UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

DISSIPATION OF POLYCYCLIC AROMATIC HYDROCARBONS USING PLANTS INOCULATED WITH ARBUSCULAR MYCORRHIZAL FUNGI AND SAPROTROPHIC FUNGI

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR OF SCIENCES IN NATURAL RESOURCES

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TEMUCO-CHILE

2018

DISSIPATION OF POLYCYCLIC AROMATIC HYDROCARBONS USING PLANTS INOCULATED WITH ARBUSCULAR MYCORRHIZAL FUNGI AND SAPROTROPHIC FUNGI

Esta tesis fue realizada bajo la supervisión del Dr. César Arriagada Escamilla perteneciente a la Facultad de Ciencias Agropecuarias y Forestales de la Universidad de la Frontera y ha sido aprobada por los miembros de la comisión examinadora.

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ACKNOWLEDGEMENTS

First, I would like to thank to Dr. Cesar Arriagada for his patience and invaluable support both at each stage of this doctorate and on my academic development during the last 11 years.

I would like to thank to Dr. Fernando Borie, Dr. Pablo Cornejo, Dr. Götz Palfner, Dr. Francisca Acevedo and Dr. Leandro Paulino for their contribution on this doctoral thesis.

I would especially would thank to Dr. John Larsen for allowing me made the international internship at the Laboratorio de Agroecología (UNAM, Morelia, México) and his contribution on research manuscripts. In the same context, I would like to thank to Estefanía Saucedo, Dr. Marcela Sarabia, Dr. Pablo Jaramillo and Andrea Raya for all good moments lived in Morelia.

Additionally, I would like to thank my Lab matters Dr. Alejandra Fuentes, Dr. Leonardo Almonacid, Dr. Hector Herrera, Javiera Soto, Javier Ortiz, Tedy Sanhueza, Yerko Cerda and Roxana Alvarado for their support and friendship.

One of the most special acknowledgements is for my brothers, Leonardo Lagos, David Lagos, Victor Lagos and Luciano Lagos, who I met during this doctorate almost 6 years ago. I love every one of you dear brothers.

Before put an end to these acknowledgements, I would like to thank to my friends. My best friend Luis García, Rodrigo Manriquez, Ana Lopez, Jaime Garcés, Victor Rocco, Consuelo Saldivia, Mario Bello, Fabian Silva and Monica Ñanco for all these years of friendship and

trust, for each conversation, for each smile, for each reflection about life and their difficulties, for each shared moment.

Finally, the most important gratitude to my mother Isabel Etchegaray, my grandmother Silvia Villalobos (R.I.P), my grandfather Carlos Etchegaray (R.I.P) and my uncle Julio Etchegaray for their support during all my life under any complicated event. Without their presences this achievement would have been impossible. All I am is thank to you.

This study was possible thanks to support by FONDECYT 1130662 (Cesar Arriagada), from Comisión Nacional de Investigación Científica y Tecnológica, Chile. I want also thanks the financial support of CONICYT through Doctoral Fellowship Program (21120476).

THESIS OUTLINE

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants generated by the incomplete combustion of organic materials derived by natural or anthropogenic events. PAHs have been reported as toxic compounds on living organisms related to their carcinogenic, teratogenic and embryotoxic properties. PAHs are organic compounds with two or more fused benzene rings and are widely distributed in all ecosystems, however, soil as the main reservoir hosting 90% of PAHs that are emitted into the atmosphere. Therefore, techniques to reduce or eliminate PAHs concentration in soil are required. In this sense, bioremediation represent an alternative of low cost and environmentally friendly due to the use of biological agents and non-input of chemicals for solving the soil pollution. A diverse group of organisms that includes plants, saprotrophic fungi (SF) and arbuscular mycorrhizal fungi (AMF) have been related to the degradation or removal of PAHs from soil.

Studies with plants in order to degrade PAHs have been conducted, however, depending on the PAHs and their concentrations, negative impacts of these compounds have been described and mainly related to the oxidative stress. For coping with this harmful effect, AMF are known for helping plants to establish on soils polluted by different compounds including PAHs. The AMF are directly associated to plant roots forming a mutualistic symbiosis, helping to plants to increase the uptake of water and nutrients, mainly Phosphorus. On the other hand, the soil SF are important since they take part in the mobilisation of nutrients and degradation of phytotoxic substances; produce substances that promote or inhibit the growth of other rhizosphere microorganisms; add great amounts of

microbial biomass to the soil and also contribute to the optimum use of nutrients by the plant.

Therefore, considering the beneficial effects of AMF and SF in order to degrade and help plants to establish plants on PAH-polluted soils, the use of this fungal interaction and plants could an interesting bioremediation strategy for coping with this environmental concern.

In **Chapter I,** general introduction, hypotheses, general, and specific objectives are presented. The general objective of this Doctoral Thesis was **to study the contribution of the saprotrophic fungi and arbuscular mycorrhizal fungi on plant tolerance against PAHs in soil and their further dissipation.**

Chapter II corresponds to a review entitled "**Bioremediation of polycyclic aromatic hydrocarbons (PAHs) through plants and their growth promoter fungi**". In this review, a background related to the risks associated to PAHs in different ecosystems, mainly in soil and their remediation techniques were presented, highlighting bioremediation as suitable technique in order to achieve PAHs degradation in soil. Researches that have been done related to degradation of PAHs by plants, saprotrophic and mycorrhizal fungi were presented.

Chapter III entitled "**Selection of suitable plant and rhizospheric fungi as an alternative to tolerance high concentration of polycyclic aromatic hydrocarbons".** In this chapter a fungal isolation was conducted from a Polycontaminated soil from the Puchuncaví Valley in central Chile placed 1.5 km southeast of the Ventanas copper smelter. The strains isolated were molecularly classified and corresponded to *Trichoderma viride*,

Penicillium canescens, *Penicillium glabrum*, *Alternaria consortialis*, *Alternaria dauci*, *Fimetariella rabenhorstii* and *Hormonema viticola*. Furthermore, removal of PAHs (Anthracene and Phenanthrene) under *in vitro* conditions by these fungal isolates was conducted. Parallel, a germination test was assessed using *Eucalyptus globulus*, *Solanum lycopersicum*, *Medicago sativa* and *Triticum aestivum*, in order to determine a suitable plant for phytoremediation purposes based on growth parameters. The main results suggested that *T. viride* reached the highest anthracene and phenanthrene removal *in vitro*. In the case of germination assays and plant growth parameters, *T. aestivum* seedlings showed a higher tolerance to phenanthrene and anthracene compared to the other species. In addition, phenanthrene showed a higher toxicity, therefore was selected as the model PAH for later studies involved in this research.

Chapter IV corresponds to a manuscript entitled "**Association of wheat with plant growth promoting fungi alleviates plant oxidative stress and enhances phenanthrene dissipation in soil".** In this chapter, the effects of the dual inoculation of the saprotrophic fungus *T. viride* and the arbuscular mycorrhizal fungus *Funneliformis mosseae* on tolerance of *T. aestivum* growing in a soil spiked with phenanthrene and the influence of this interaction on pollutant dissipation were evaluated. The main results showed that the dual inoculation associated to *T. aestivum* plants reached the highest phenanthrene dissipation from soil. Also, dry biomass, soil enzymes, antioxidant response, organic acid exudation and phenanthrene content in roots were stimulated by dual inoculation in phenanthrene-spiked soil, whereas lipid peroxidation and phenanthrene content in shoots were reduced. Finally, according to the undetectable phenanthrene, it was suggested that the main

dissipation way of phenanthrene in soil could be by degradation. Our results show that dual inoculation in wheat plants significantly promotes phenanthrene dissipation in soil and contributes to alleviating oxidative damage as a consequence of improved plant fitness.

Chapter V corresponds to an accepted manuscript entitled **"Dual inoculation with mycorrhizal and saprotrophic fungi suppress the maize growth and development under phenanthrene exposure".** In this chapter the interaction of AM fungus *Rhizophagus irregularis* and the saprotrophic fungus *T. viride* and their single and dual effects on maize plant growth performance and communities of rhizosphere microorganisms in a phenanthrene spiked soil. In this study, plant growth parameters, AM colonization, *Trichoderma* colony forming units (CFU) and a whole cell fatty acid profile were conducted. Phenanthrene decreased maize plant growth independent of *T. viride* and *R. irregularis* inoculation, which were also both inhibited by phenanthrene exposure. Furthermore, mutual inhibition between *R. irregularis* and *T.viride* was evidenced, which mitigated their plant growth promoting effects independent of phenanthrene exposure. Therefore, the observed mutual inhibition between *R. irregularis* and *T. viride* underline the importance of testing for compatibility between fungal inoculants when these are used in combination in order to achieve a successful bioremediation strategy on PAH-polluted soil.

Finally, **Chapter VI** corresponds to general discussion, conclusions, and future directions that must be taken in bioremediation of PAH-polluted soil by the interaction of saprotrophic fungi and arbuscular mycorrhizal fungi associated to vegetal species.

In summary it was concluded that inoculation with a selected saprotrophic fungus as *T. viride* and the AM fungus *F. mosseae* can be considered an important biotechnological

approach for increase dissipation of PAHs and improving the plant tolerance as an alternative to remediate soils polluted with PAHs. However, the selection of compatible fungi and plants is a key previous step in order to achieve remediation of PAHs in soil by this interaction. In order to get a better understanding of dissipation of PAHs in soil mainly by AMF in future researches, the study of the role of glomalin in PAHs accumulation could be an important issue for explaining the positive effects of AMF on plants under PAHs exposure.

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CHAPTER I

General Introduction, hypothesis and objectives

1.1 GENERAL INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed organic pollutants in different ecosystems, highly persistent (Maliszewska-Kordybach, 1999), carcinogenic, mutagenic and teratogenic (Wassenberg and Di Giulio, 2004). The main PAHs sink corresponds to soil, which host almost 90% of produced PAHs, being atmospheric deposition the pathway into the soils. Once in soil, PAHs are strongly sorbed depending on organic matter content in soil (Vácha et al., 2010). Also, they can be dissipated by photo-oxidation (Park et al., 1990), bioaccumulation in vegetal tissues (Meudec et al., 2006), leaching (de Jonge et al., 2008), biosorption and biodegradation by soil microorganisms (Chen and Ding, 2012).

In the case of biodegradation of PAHs in soil, it can be stimulated by different ways that could help to the effects of these microorganisms. Among them, the nutrient input such as inorganic N and P is an important aspect for being considered in bioremediation of PAHs. Due to the poor nutritional status of polluted soil, the nutrient input can be a necessary step for *in situ* biodegradation of PAHs which generally present low rates compared to studies conducted at laboratory scale (Luthy et al., 1994). The incorporation N and P is needed for increasing microbial biomass in soil and their activity, stimulating the biodegradation of these compounds in soil (Atagana et al., 2003; Leys et al., 2005).

Another limiting factor involved on PAHs biodegradation is their bioavailability due to their low aqueous solubility and strong sorption to soil (Zhu and Aitken, 2010). In this case, the use of surfactants have been demonstrated successful results for improving biodegradation of PAHs by increasing their solubility and as a consequence increase the

available fraction of PAHs (Lamichhane et al., 2017). Surfactants correspond to amphiphilic molecules (composed by hydrophobic and hydrophilic parts) and are assembled into micelles whose interior provides a hydrophobic environment to nonpolar compounds such as PAHs enhancing their solubility (Mesbaiah et al., 2014). The surfactant incorporation into polluted soil decrease the interfacial tension thus increasing the mass transfer of the pollutants (Gao et al., 2007). Surfactants can be obtained by synthetic origin and natural production. The later, is very important in terms of bioremediation of PAHs due to be produced extracellularly or as part of the cell membrane by different microorganisms such as bacteria, fungi, and yeast being called biosurfactants (Bustamante et al., 2012). Biosurfactants improve microorganisms growth on hydrophobic surfaces by overcoming the reduced bioavailability of PAHs and increasing the nutrient uptake rates (Tecon and van der Meer, 2010). Therefore, it is another advantage in the use of microorganisms for enhancing PAHs biodegradation in soil and has been tested successfully in several studies (Gao et al., 2007;Bezza and Chirwa, 2016)

In the last decade, the use of environmentally friendly technologies such as microorganisms or plants has contributed to PAHs dissipation from soil in the root zone (Cheema et al. 2010). The use of plants to remove PAHs from soils (phytoremediation) is emerging as a potential strategy for cost-effective and environmentally sound remediation of contaminated soils. Phytoremediation is the use of plants to remediate contamination of soil with xenobiotic compounds like PAHs (Cunningham and Berti, 2000). In fact, plants have mechanisms for PAHs dissipation such as degradation, accumulation, as well as mechanisms for tolerating high levels of PAHs in soil (Khan et al., 2008). Furthermore, some microorganisms are able to promote PAHs degradation in soil and help plants to

establish under adverse conditions. Among these organisms, arbuscular mycorrhizal fungi (AMF) are directly related to plants due to their dependence for completing their life cycle that ends with spore formation. Generally, these fungi promote plant growth mainly by higher acquisition of P and other elements such as N from soil through their extraradical mycelium that explores the soil in a greater extension than non-mycorrhizal roots (Smith and Read, 2008). The AMF help plants for coping against biotic and abiotic stresses. Their contribution against several pollutants such as heavy metals (Carvalho et al., 2006; Cicatelli et al., 2010; Hristozkova et al., 2016) and persistent organic pollutants (Qin et al., 2016; Wu et al., 2008) has been reported. The AMF have been reported for increasing PAHs dissipation in soil, by an enhancement on soil microbial population and activity, which lead to a PAHs biodegradation. In addition, plants inoculated with AMF show an increase of PAHs concentration in roots. Therefore, this pollutant compartmentalization enhances the dissipation process (Gao et al., 2009; Gao et al., 2011).

On the other hand, saprotrophic fungi (SF) are key organisms that contribute in important processes in soil such as organic matter decomposition and are divided mainly in two groups consisted of ligninolytic and non-ligninolytic fungi (Eriksson et al., 1990; Ryan et al., 2007). Fungal strains belonging to different phylum such as Ascomycetes, Basidiomycetes and Zygomycetes have been tested in order to degrade PAHs (Batista et al., 2017; Ravelet et al., 2000). In these sense, is important indicate that these fungi present different functional diversity between them but due to their wide-broad enzymatic machinery SF are able to degrade a large variety of organic pollutants including PAHs (Acevedo et al., 2011; Mineki et al., 2014; Zafra et al., 2015).

Zygomycetes are composed of a group of aseptate fungi (coenocytic) that produce zygospores after fusion of isogamic sex organs and are referred commonly as “molds” and they are found in soil living on soil and decomposed plant residues, dead animals, dung and are considered as primary or secondary colonizers of substrates rich in simple carbohydrates (Richardson, 2009). In relation to PAHs degradation Cytochrome P450 Monooxygenase/ Epoxide hydrolase have been identified as the main enzymes involved in this process (Mitra et al., 2013). Nevertheless, these fungi present deleterious effects directly on humans by different diseases (Muszewska et al., 2014)

Ascomycetes are ubiquitous fungi in different ecosystems (air, soil and water) and have been reported for prevailing under different pollution events being suitable candidates for remediation strategies (Aranda, 2016) and produce their sexual spores (ascospores) in tubular sacs called asci (Trail, 2017). They participate in the decay of organic substrates (wood, leaf litter, and dung) and act as mutualists, parasites, and pathogens of animals, plants and other fungi (Schoch et al., 2009). This fungal division present different properties related to positive and negative effects on mankind and their environment, especially in terms of their effects on plants and economical crops. In this sense, fungal genera such as *Aspergillus*, *Penicillium* and *Trichoderma* have been described as plant growth promotion fungi mainly related to their biocontrol over plant pathogens (Boughalleb-M’Hamdi et al., 2019), whereas, fungi genera such as *Alternaria* and *Fusarium* represent the most importants fungal pathogens for plants (Schiro et al., 2018). Furthermore, Ascomycetes have been well described such as P solubilizers in soil favoring to plants and microorganisms (Elias et al., 2016). In terms of PAHs dissipation, this fungal division presents different enzymatic ways for degrading PAHs including some ligninolytic

enzymes and Cytochrome p450 monooxygenase (Durairaj et al., 2016; Viswanath et al., 2014).

Finally, Basidiomycetes are the most complex and evolved members of the fungal kingdom and correspond to filamentous fungi that present complex lifecycles, sexual reproduction and produce basidiospores on speciallised cells called basidia (de Mattos-Shipley et al., 2016). These fungi play a vital role in terms of recycling by the descomposition of many substrates such as lignocelluloses in wood that can not be degraded by other microorganisms due to their ligninolytic enzymes that is an exclusive system of these fungi (Riley et al., 2014). This enzymatic system composed by laccase, manganese peroxidase and lignin peroxidase make of these fungi the most efficient degrader of lignin that is the most recalcitrant polymer of lignocellulosic residues (Gomes et al., 2009). These enzymes not only degrade lignin, are able to degrade a large variety of substances including organic pollutants such as PAHs (Acevedo et al., 2011; Schützendübel et al, 1999).

Therefore, based on the above statements, the inoculation with SF could increase both plant growth and PAHs dissipation in soil. In order to enhance plant growth against pollutants, microorganisms combination contributes to a diminishing of their detrimental effects by sequestration, accumulation or degradation depending on the pollutant nature. Interaction between AMF and SF has been reported for increasing plant growth and yield (Bhuvaneswari et al., 2014; Martínez- Medina et al., 2009; Yadav et al., 2015). In relation to pollutants, fungal interaction between AMF and SF has been tested mainly for heavy metals and metalloids (Arriagada et al., 2007; Arriagada et al., 2009; Fuentes et al., 2016). However, literature presents scarce or null attempts using this interaction order to mitigate the negative PAHs impacts on plant or to dissipate PAHs in soil. Therefore, the present

study aims to evaluate the plant response against PAHs by the dual inoculation and their contribution on PAHs dissipation in soil.

1.2 HYPOTHESIS:

The inoculation with saprotrophic and arbuscular mycorrhizal fungi:

1) Improves plant tolerance in soils contaminated with polycyclic aromatic hydrocarbons

2) Promotes the dissipation of polycyclic aromatic hydrocarbons in soils.

1.3 GENERAL OBJECTIVE

To study the contribution of the saprotrophic and arbuscular mycorrhizal fungi on plant tolerance against polycyclic aromatic hydrocarbons (PAHs) in soil and their further dissipation.

1.4 SPECIFIC OBJECTIVES

1. To evaluate the effect of saprotrophic fungi on PAHs removal growing under *in vitro* conditions.

2. To evaluate the effects of the inoculation with saprotrophic and arbuscular mycorrhizal fungi on PAHs dissipation in soil and plant tolerance.

3. To analyze possible mechanisms involved in PAHs dissipation and plant tolerance through the inoculation with saprotrophic fungi and the inoculation with arbuscular mycorrhizal fungi.

CHAPTER II

Bioremediation of polycyclic aromatic hydrocarbons (PAHs) through plants and their growth promoter fungi: A review.

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Review to be submitted to Critical Reviews in Plant Sciences

2.1 ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) have been declared priority pollutants due to their detrimental effects on living organisms. PAHs are recalcitrant and ubiquitous pollutants present in different ecosystems. Bioremediation of PAH-polluted soils through phytoremediation have been proven to enhance the ways these pollutants dissipate, mainly by stimulating soil microorganisms by root exudates and enhancing bioavailability of PAHs in soil. On the other hand, rhizospheric microorganisms such as saprotrophic fungi can dissipate PAHs in soil by degradation due to different enzymatic systems. Likewise, plant inoculation with arbuscular mycorrhizal fungi have also been involved in the PAHs dissipation in soil, with the main mechanisms being improved plant nutrition and a higher exudation of specific enzymes involved in PAHs removal. In order to improve PAHs dissipation in soil, a combination of these microorganisms could be an interesting alternative to achieve the reclamation of soil contaminated by these pollutants.

The aim of this review is to discuss the alternative of bioremediation through dissipation of PAHs by rizospheric and free-living microorganism. The principals mechanism involved on their bioremediation are presented.

Key Words: Recalcitrant pollutants, phytoremediation, plant-growth-promoting fungi, degradation.

2.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different compounds that have two or more fused benzene rings (Gan et al., 2009; Martorell et al., 2010). PAHs are are the most common environmental contaminants (Maliszewska-Kordybach, 1999). The difficulties in their degradation are related mainly to low water solubility that promotes their accumulation in different ecosystems (Leneva et al., 2009). Their wide distribution in the environment is due to their lipophilic properties, which allow their adsorption by atmospheric particles and direct deposition in sediments, soils and plants (Danyi et al., 2009). PAHs are considered priority organic pollutants by the US Environmental Protection Agency (EPA) due to their detrimental properties as teratogenic, mutagenic and carcinogenic (U.S. EPA, 2008). According to the U.S. EPA, 16 PAHs have been described as priority pollutants (Figure 2.1), and the following compounds are considered possible human or animal carcinogens: benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)anthracene, indeno(l,2,3-cd) pyrene. This harmful property is related to the molecular weight of PAHs; thus, the higher the molecular weight, the greater the carcinogenicity of PAHs (Ravindra et al., 2008). The main population exposures to these pollutants are through inhalation from polluted air, ingestion of contaminated food and direct contact with PAHs; moreover, exposure increases in areas with significant industrial development (Wcislo, 1998).

In order to alleviate these negative impacts on the environment, mainly at soil scale, different remediation techniques have been implemented for addressing pollution by PAHs. Biological alternatives such as bioremediation present advantages such as being

economical, environmentally friendly and useful at a larger scale in field compared to the other treatments.

Bioremediation of PAH-contaminated soil through plants and their growth-promoting fungi (saprotrophic and mycorrhizal fungi) has been demonstrated in different studies where these biological agents via different mechanisms dissipated these compounds from soil. Therefore, the aim of this review is to summarize the main mechanisms involved in PAHs remediation by plants and fungi and to establish future perspectives for their use PAH-contaminated soil.

2.3 POLLUTION BY PAHs IN DIFFERENT ECOSYSTEMS

Pollution by PAHs in several ecosystems has different origins depending on their anthropogenic or naturals causes. There are two kinds of anthropogenic causes of PAHs sources: The first is derived from the incomplete combustion of fossil fuels and organic matter, municipal incineration, vehicle emissions as well as from industrial processes, cigarette smoking and agricultural waste burning. The second is a result of accidental spillage and intentional dumping of materials such as creosote, coal tar and petroleum products (Criquet et al, 2000; Kuo et al., 2008; Singh and Tripathi, 2007), forest fires and volcanic eruptions (Liu, et al., 2006).

At atmospheric level, PAHs are widely distributed. The main anthropogenic causes of atmospheric emissions are biomass burning, coal and petroleum combustion, coke, metal production, motor vehicle and power generation (Lu et al., 2011; Zhang and Tao, 2008). Natural sources that contribute to PAHs levels in the atmosphere are volcanic eruptions and forest fires (Liu et al., 2006). The amount of PAHs in the atmosphere is affected by

meteorological conditions, such as wind, temperature and humidity, as well as fuel characteristics and types, such as moisture content, green wood and seasonal wood (Lee, 2010). The vapors from PAHs released into the atmosphere are condensed at ambient temperatures; therefore, they can be transported long distances (Levinson et al., 2005). The distribution of atmospheric PAHs depends on their physicochemical properties, so they can be associated with gas and particulate phases, mainly associated with small particles (<1µm). This facilitates atmospheric transport towards other ecosystems located far away from the original source (Fernández et al., 1999), with the transportation level being described as regional and intercontinental (Zhang and Tao, 2009).

Aquatic ecosystems represent one of the major PAHs sinks. PAHs tend to adsorb onto suspended particulate matter due to their lipophilic characteristics and to settle on the sediments (Shi et al., 2007). The main inputs of PAHs in aquatic systems are atmospheric deposition; discharge of industrial and residential wastewater; petroleum spillage by ships or urban and surface runoff (Heemken et al., 2000). In these systems, PAHs are distributed globally from inland lakes and urban rivers to the open ocean with a wide range of concentrations in water and sediments. Almost all of these polluted areas present PAHs concentrations ranging from 10 to 1000 ng L^{-1} in water (Chen et al., 2007). Given the very low water solubility of PAHs, most of their mass in the aquatic environment is found mainly in solid phases corresponding to sediments (Fernández et al., 1999; Levinson et al., 2005).

Soil is considered the major reservoir and sink for environmental pollutants due to its holding capacity and the quantity of pollutants that reach the soil (Ockenden et al., 2003). Soil pollution by PAHs begins with atmospheric emissions of these pollutants and their

further precipitation (Rong et al., 2007). In light of their hydrophobicity related to their low water solubility, high octanol-water partition coefficient and electrochemical stability, they can remain and accumulate on soils for ages (Alcantara et al., 2008). As PAHs solubility in water is low (less than 10^4), these compounds are strongly sorbed in soil, limiting the efficiency of remediation techniques (Chi, 2010). Soil pollution by PAHs is recognized as a suitable indicator of the level of environmental contamination derived from human activities (Chung et al., 2007). According to Abrahams (2002), the greatest amounts of PAHs are found in urban soils, and the close proximity of these soils to humans may increase the probability of human exposure to PAHs through inhalation, ingestion or skin contact. Once in soils, the possible fates of PAHs correspond to volatilization, photo-oxidation, chemical oxidation, adsorption, adhesion, leaching and bioaccumulation (de Jonge et al., 2008; Park et al., 1990; Singh, 2006).

2.4 PAH-REMEDIATION TECHNIQUES IN SOILS.

The main alternative treatments for remediating PAH-contaminated soil are physicochemical, chemical, thermal and biological.

a) Physicochemical. Solvent extraction is a technology for soil remediation based on a concentration process, where a non-aqueous liquid is used to remove a variety of organic pollutants (Silva et al., 2005). The negative feature is related to the *ex situ* condition where this is carried out, limiting its application in polluted soil.

b) *Chemical oxidation*. This consists of introducing strong oxidants to contaminated soil and/or groundwater. It is considered an effective method against the limitations of

bioremediation in the degradation process of toxic and organic compounds such as PAHs (Kulik et al., 2006). The most commonly used oxidants in this method correspond to ozone, hydrogen peroxide, persulfate and permanganate. Once these agents transform organic compounds, oxygenated intermediates are generated, which present higher solubility, increasing the susceptibility of these intermediates to biodegradation (Lu et al., 2010). Conversely, in the case of incomplete oxidation, the oxygenated intermediates may become toxic, mutagenic and carcinogenic (Russo et al., 2010; Xie et al., 2006). Therefore, this technique is a previous step to facilitate PAHs degradation by soil microorganisms. Hence, other limitations of this technique seem to be associated with the presence of microorganisms able to degrade these pollutants. In this sense, negative impacts of chemical oxidation agents on soil microorganisms have been reported (Kakosová et al., 2017). Although chemical oxidation is more effective in terms of time than other remediation techniques, its dependence on soil microorganisms and the negative impact on soil biology seem to limit the use of this technology.

c) Thermal treatments. Several thermal treatments have been implemented to remediate soil polluted with hydrocarbons. From low to high temperatures, thermal desorption is classified according to its temperature. Temperature between 100 and 350°C (low) and temperature between 350 and 600°C (high) both consisting of a physical separation of pollutants from the soil, whereas thermal destruction corresponding to a chemical modification of the contaminants uses temperatures between 650 and 1000°C (Falciglia et al., 2011). Other thermal treatments are incineration and pyrolysis. Incineration is a process in which the soil is incinerated at high temperatures between 870 and 1200°C, destroying organic contaminants such as PAHs (Gan et al., 2009). However, negative plant growth

parameters in incinerated soils even in comparison with the original polluted soil have been reported (Vidonish et al., 2015). Similarly, pyrolysis consists of heating the soil (400–1200 °C) for a variety of hazardous wastes (< 550 °C for hydrocarbons) under anoxic conditions (Vidonish et al., 2015). The negative aspects of thermal treatments are related to the limitations for reusing incinerated soils for agricultural purposes (Exner, 1995).

d) *Electrokinetic remediation.* This is a technology that uses the conductive properties of the soil to separate and remove organic and inorganic pollutants (metals) from soils, sludge and sediments with the use of an electric field to remove charged species (ions). It involves the application of a low intensity direct current between a positive and a negative electrode (Alcántara et al., 2012; Paillat et al., 2000). The disadvantages of this treatment are related to its practical application in terms of its low removal efficiency for non-polar organic pollutants such as PAHs (Huang et al., 2012)

e) *Photocatalytic degradation***.** This is a process based on the use of photocatalysts to stimulate oxidizing reactions, which destroy organic pollutants in the presence of light radiation (Gan et al., 2009). The most common photocatalyst agent corresponds to TiO_2, which has resulted in an efficient degradation of hydrocarbons in the presence of UV light in several studies (Dong et al., 2010; Gu et al., 2012). However, in order to achieve an effective degradation, this technique requires bioremediation process (Yang et al., 2017).

f) Bioremediation. Defined as a managed or spontaneous process, in which a biological catalysis acts on different pollutants, remedying or eliminating environmental pollution (Madsen, 1991). It presents a vast potential as a practical and cost-effective approach for solving a wide variety of pollution events (Alvarez and Illman, 2005). To carry out an

effective bioremediation process related to organic pollutants, microorganisms must attack the pollutants through enzymatic systems and convert them to harmless products (Sharma, 2012). Leneva et al. (2009) suggested that the bioremediation of PAH-contaminated soils might become an important alternative, as it is more efficient and economical than other methods due to the complete destruction of the compounds or pollutants, cheaper treatment, greater safety and fewer environmental impacts.

2.5 EFFECTS OF PLANTS AND RHIZOSPERIC FUNGI ON PAHs DISSIPATION.

2.5.1 Phytoremediation.

Phytoremediation is the use of plants to reduce or remove different types of contaminants from soil and water. In plants this process can occur through the direct uptake of the pollutant into vegetative tissues, causing a transformation by plant enzymes, sequestration within the plant or transpiration through leaves (Schnoor et al., 1995). Furthermore, the phytoremediation is related to the stimulation of microbial activity in the rhizosphere creating optimal conditions for organic pollutant degradation and their further mineralization (Muratova et al., 2003). In this order, plant roots are actively or passively releasing a range of organic compounds (known as root exudates) that include carbohydrates, organic acids, proteins and amino acids (Zhang and Dong, 2008). As a carbon and nutrient source, root exudates represent an important resource for the rapid growth of soil microbes and can alter the rhizospheric species composition, influencing nutrient transformation, decomposition and the mineralization of organic substances (Marschner et al., 2004). Plant roots can improve the dissipation of organic pollutants such as PAHs presents in soils (Table 2.1), and this can be mainly attributed to supply of root

exudates for co-metabolic processes (Yoshitomi and Shann, 2001). The root exudates play important roles in PAHs bioavailability in soils, which is limited due to the strong sorption between soil components and these compounds depending on the organic matter content in soil (Vácha et al., 2010). According to Gao et al. (2010a), the sorption–desorption process is considered one of the main factors in the phytoremediation process. In this sense Low-molecular-weight organic anions (LMWOAs) have been described as playing an important role in PAHs desorption processes in soil affecting their bioavailability; therefore, degradation process are affected subsequently. LMWOAs are the primary components of root exudates and have been involved in different soil processes that include the mobilization and uptake of nutrients by plants and microorganisms, detoxification of metals by plants, microbial proliferation in the rhizosphere and dissolution of soil minerals (Marschner, 1995a). Gao et al. (2010a) found that PAHs such as phenanthrene and pyrene desorption was higher with the addition of citric and oxalic acid. The availability of phenanthrene in soil with various treatments decreased in the following order: organic acids (citric acid > oxalic acid > malic acid) > amino acids (alanine > serine) > fructose treatments (Sun et al., 2013). Improved bioavailability of PAHs due to LMWOAs is related to the increase of dissolved organic matter in solution and the decrease of soil organic matter (solid) and given by the affinity of PAHs to dissolved organic matter (Gao et al., 2010a; Sun et al., 2013).

Related to PAH dissipation, plants can bioaccumulate them into their vegetative tissues (Meudec et al., 2006; Yakovleva et al., 2012), transform PAHs within theirs tissues (Chroma et al., 2002), stimulate their biodegradation enhancing the microbial activity in soil (Muratova et al., 2003), as well as improving the enzyme production involved in PAH

degradation (Liu et al., 2015). Several enzymes released by plants into the rhizosphere or stimulated in soil by plants have been related to PAHs degradation, for example polyphenol oxidase, peroxidase, dehydrogenase, urease, catalase, laccase, nitroreductase, dehalogenase (Alagic et al., 2015; Liu et al., 2015).

2.5.2 Saprotrophic fungi.

The main function of saprotrophic fungi (SF) is the decomposition and mineralization of organic residues in the soil, obtaining their energy and food source (Penttilä and Saloheimo, 1999). These fungi release many enzymes through their metabolism that can degrade compounds of complex organic structures and promote plant growth. Extracellular enzymes released into the soil include proteases, amylases, pectinases, cellulases and ligninases, xylanases, chitinases, cutinases, phytases and phosphatases (Allison and Vitousek, 2005; Geisseler and Horwath, 2009; Moore et al., 2005). These fungi use organic matter, mainly polysaccharides (components of cell-wall such as cellulose, hemicellulose, lignin and pectin) and other biopolymers such as carbonated sources (Dashtban et al., 2009). In addition, nitrogen compounds such as ammonium or nitrates, proteins, nucleic acids, among others, which are degraded by enzymes secreted to release nutrients that can be taken up and used by SF (Adney et al., 2008). These fungi can also mobilize nutrients, degrade phytotoxic substances and promote an efficient use of nutrients by plants (Fracchia et al., 2000). The mechanisms commonly shown by SF in promoting plant growth are related to their ability to suppress harmful soil microorganisms through antagonism activities such as hyperparasitism, antibiosis and compensation (Hyakumachi and Kubota, 2004) as well as involving plant growth-promoting substances (Chanclud and Morel, 2016)

It is important consider use these fungi, mainly for their ability to degrade pollutant load in places with complex degradation (Van Beek et al., 2007). The SF involved in PAHs degradation are mainly basidiomycetes and includes non-ligninolytic fungi and ligninolytic fungi either in liquid cultures and soil (Table 2.2; Table 2.3)

2.5.2.1 Ligninolytic fungi.

Ligninolytic fungi or white rot fungi are among the few groups of microorganisms capable of degrading phenolic polymer sources entirely, including lignin (Ryan et al., 2007).The group of white rot fungi in wood is the most efficient lignin degraders due to the extracellular oxidative enzymes involved in this process, including a variety of oxidases and peroxidases (Wymelenberg et al., 2006). These microorganisms use cellulose as the carbon source and have the unique ability to degrade lignin into carbon dioxide completely by accessing the cellulose molecule (Aust and Benson, 1993). In particular, white rot fungi are a highly eco-physiologically diverse group belonging mostly to basidiomycetes and to a lesser extent to ascomycetes. These fungi are efficient lignin decomposers because they produce various non-specific oxidizing enzymes: manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Grinhut et al., 2007; Hao et al., 2006; Hatakka, 2001), the so-called "ligninolytic system"(Kluczek-Turpeinen, 2007).

The degradation of different organic pollutants by ligninolytic fungi has been studied intensively during the past few years. Due to the irregular structure of lignin, fungi produce extracellular enzymes with very low substrate specificity, making them suitable organisms for the degradation of different compounds that impact on the environment, mainly soil

(Haritash and Kaushik, 2009). The group of white rot fungi produces four major groups of enzymes for the degradation of lignin: manganese-dependent peroxidase (manganese peroxidase, MnP; EC 1.11.1.13), laccase (LAC; EC 1.10.3.2), versatile peroxidase (VP; EC 1.11.1.16) and lignin peroxidase (LiP; EC 1.11.1.14) (Wong, 2009). Manganese peroxidase consists of a heme-containing glycoprotein that requires hydrogen peroxide (H_2O_2) as an oxidant. The MnP catalyzes the oxidation of Mn^{2+} to Mn^{3+}, then oxidizing phenolic rings to phenoxy radicals, and finally leading to the decomposition of compounds (Pozdnyakova, 2012). Laccase belongs to a group of polyphenol oxidases containing copper atoms in their catalytic center. This enzyme catalyzes the reduction of oxygen to water in addition to generating the oxidation of a substrate, typically a p-dihydroxy phenol or other phenolic compound (Pozdnyakova, 2012). Versatile peroxidase consists of a group of enzymes that is not only specific to Mn^{2+} but also oxidizes phenolic and non-phenolic substrates that are typical for LiP, including veratryl alcohol, methoxybenzenes and lignin model compounds, in the absence of manganese (Wong, 2009). LiP is a heme-containing glycoprotein and plays a central role in the biodegradation of the cell wall constituent, lignin (Piontek et al., 2001). LiP catalyzes the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds. LiP oxidizes the substrates in multi-step electron transfers and forms intermediate radicals, such as phenoxy radicals and veratryl alcohol radical cations (Dashtban et al., 2010).

Depending on the species and cultural conditions, these fungi may produce LiP, MnP and laccase, showing that they have multiple enzymatic mechanisms to degrade PAHs (Cerniglia and Sutherland, 2010).The catalytic action of these enzymes generates more polar and water-soluble metabolites, such as quinones, which are more susceptible to

further degradation by indigenous bacteria present in soils and sediments (Brodkorb and Legge, 1992). In soil, these metabolites are used as substrates for microbial populations and are mineralized to carbon dioxide and may undergo polymerization and become part of the humus pool (May et al, 1997).

According to Pozdnyakova (2012), knowledge of the metabolites formed during PAHs degradation by fungi is a key requirement to validate bioremediation on different scales. In general, the first step to choose a suitable fungal strain for PAHs degradation is through submerged cultures prior to its application in the soil. In various studies using submerged cultures, the main metabolites identified derived from fungal degradation of PAHs are quinones. In the case of anthracene, one of the most common PAHs, the appearance of 9, 10-anthraquinone coincides with anthracene degradation, and depending on the fungi used, 9,10-anthraquinone can behave as a dead-end metabolite for the genera *Bjerkandera* and *Phanerochaete* (Field et al, 1992) . The 9,10-anthraquinone metabolite has been reported in numerous studies with white rot fungi; i.e., *Pleurotus ostreatus* and *Trametes versicolor*have been described as generating this metabolite with the action of MnP and laccase enzymes in*in vitro* experiments (Schützendübel et al., 1999; Johaness et al., 1996, Collins et al., 1996). In contrast, *Phanerochaete chrysosporium* LiP and MnP have been described as being involved in the formation of 9,10-anthraquinone during anthracene degradation, as well as in the formation of a successive product such as phthalic acid (Hammel et al., 1991; Vyas et al., 1994). Manganese peroxidase derived from ligninolytic fungi has been reported to catalyze anthracene conversion into anthrone in media containing organic solvents (Eibes et al., 2006).

In the case of phenanthrene, Sutherland et al. (1991) found that metabolites produced by phenanthrene degradation in submerged cultures using *P. chrysosporium* were phenanthrene-*trans*- 9,10-dihydrodiol, phenanthrene-*trans*-3,4-dihydrodiol, 9- phenanthrol, 3-phenanthrol, 4-phenanthrol and the conjugate 9-phenanthryl-*D*-glucopyranoside. These authors did not observe Lip activity; therefore, they suggested that monooxygenase and epoxide hydrolase activity started the oxidation and hydration of phenanthrene by the fungus. In addition, Tatarko and Bumpus (1993) observed that LiP from this fungus was able to catalyze the oxidation of an intermediate product of phenanthrene degradation such as 9-phenanthrol to phenanthrene 9,10-quinone. Likewise, Ning et al. (2010) observed that the MnP in *P.chrysosporium* was able to transform phenanthrene into 9,10-phenanthrenequinone and 2,2′ -diphenic acid. Similarly, phenanthrene degradation by white rot fungi *Polyporus sp.* S133 produced two metabolites corresponding to 9,10-phenanthrenequinone, 2,2′ -diphenic acid, phthalic acid and protocatechuic acid (Hadibarata and Tachibana, 2009: Hadibarata and Tachibana, 2010). According to Bezalel et al. (1997), *P. ostreatus* initially oxidizes phenanthrene stereoselectively by a cytochrome P-450 monooxygenase and this is followed by epoxide hydrolase-catalyzed hydration reactions. PAHs degradation by white rot fungi may be performed by a combination of ligninolytic enzymatic systems consisting of cytochrome p450 and epoxide hydrolases, which can result in a complete mineralization of these compounds. Involvement of both cytochrome p450 and MnP in phenanthrene metabolism by *P. chrysosporium* has been reported (Ning et al., 2010).

Studies about metabolites produced in soil by fungal PAHs degradation are scarce compared to metabolites produced in submerged cultures. Anthracene degradation in soils

by *P. ostreatus* mainly produces anthraquinone, with wheat bran and compost additives being used as fungal substrate accelerating the removal of anthracene from the spiked soil used in the study (Vargas et al., 2001). Using native white-rot fungi *Anthracophyllum discolor*, Acevedo et al. (2011) found that the metabolites derived by anthracene degradation in soil were anthraquinone and phthalic acid.

2.5.2.2 Non-ligninolytic fungi.

According to type of decay produced by these fungi, they are called either brown rot fungi or soft rot fungi. The former degrade cellulose and hemicellulose leaving a brown residue formed by lignin (Eriksson et al., 1990). The latter are less destructive than brown rot and degrade the secondary wall of cellulose, causing the soft final appearance of the wood similar to brown rot decay (Goodell et al., 2008; Nilsson et al., 1989). In non-ligninolytic fungi, PAHs metabolism is performed primarily by the cytochrome P450s monooxygenase enzyme that oxides the aromatic ring to produce an arene oxide by incorporating one oxygen atom onto the ring (Bamforth and Singleton, 2005). The metabolic pattern of PAHs degradation through fungal cytochrome P450 enzyme systems are related to the metabolic pattern observed in mammals (Bamforth and Singleton, 2005; van den Brink et al., 1998). Cytochrome P450s belongs to a superfamily of heme-containing enzymes that are often involved in the initial oxidative metabolism of a wide variety of xenobiotic compounds (Gonzalez, 1998; van den Brink et al., 1998). Cytochrome P450s are produced not only by non-ligninolytic fungi but also produced by ligninolytic fungi (Chigu et al., 2010; Ning et al., 2010). However, this is not the only enzymatic mechanism by non-ligninolytic fungi to dissipate PAHs through degradation. Zafra et al. (2015) found that PAHs degradation by

Trichoderma asperellum was promoted by enzymes such as catechol 1, 2 dioxygenase, peroxidase and laccase. In this sense, reports have described fungi from the genus *Trichoderma* as producing a wide range of enzymes that also include those produced by ligninolytic fungi in order to degrade organic compounds (Sowmya et al., 2014)

2.5.3 Mycorrhizal fungi.

A mycorrhiza is defined as the association between the root system of plants and soil fungi capable of forming a mutualistic association (Brundrett et al., 1996), and it is currently estimated that over 90% of plant species form this symbiotic relationship (Arriagada et al., 2007). These fungi are recognized as growth-promoting plant species, mainly because of the involvement of the extraradical mycelium in the acquisition of plant nutrients, particularly phosphorus (P) (Smith and Read, 1997). Among the different types of mycorrhizae, arbuscular mycorrhizal fungi (AMF) are the most common that form symbiosis with plants, and are so called because they form a structure in the root cells known as arbuscules, which are a dichotomous branching of the intracellular hyphae involved in transport of nutrients (Smith and Gianinazzi-Pearson, 1988). Therefore, this association with AMF improves mineral nutrition by inducing changes in the root system, such as increasing root length and branching, thereby enhancing nutrient uptake (Padilla and Encina, 2005).

The extraradical hyphae of fungi can absorb nutrients from those areas beyond the radical depletion area of non-mycorrhizal roots and then be translocated to the host (Marschner, 1995b); Due to their small diameter (1-12μm), they are highly effective at nutrient uptake

from microsites that contain high concentrations of nutrients such as P, N, Fe, Cu and Zn. Simultaneously, the total length of extraradical hyphae for AMF species may be in the range of 1-10 m cm^{-1} of colonized root length, with a high area available to capture the nutrient which is then translocated to the colonized root and hydrolyzed in the arbuscules (Marschner, 1995b; Nichols, 2008).

According to Leyval et al. (2002), AMF help establish plants in PAH-contaminated soils and promote the dissipation of these compounds. One of the firsts approaches related to the effect of PAHs on plant survival and growth was evaluated by Leyval and Binet (1998), by the inoculation of AM fungus *Funneliformis mosseae* in *Allium porrum, Zea mays, Lolium perenne and Trifolium subterraneum*. At 5 g of PAHs kg^{-1}, only mycorrhizal plants survived; the authors suggested that these effects depend on the plant-fungus interaction.

Studies that help to understand the plant survival on PAH-polluted soil due to AMF have been conducted. Gao et al. (2010c) found that hyphae of AMF such as *Glomus etunicatum* and *F. mosseae* are able to uptake PAHs from soil. Complementary, Gao et al. (2010a) that found that the AM fungus *F. mosseae* reduce the pyrene and phenanthtrene translocation into the shoot and increase the PAHs content in roots in *Medicago sativa* and was well correlated to the rate of AM colonization . In addition, the results associated to the highest degradation were directly related to the plant survival in soil.

Studies using plants inoculated with AMF to dissipate PAHs from soil have been widely reported (Table 2.4) One of the first successful studies was performed using two industrial soils contaminated with different PAHs, where *Lolium perenne* and *Trifolium repens* plants

were used and inoculated with the AM fungi *F. mosseae*, which resulted in both an enhanced dissipation of PAHs with the use of colonized plants and improved growth in both plants (Joner and Leyval, 2003). In the same study, the relation between PAHs dissipation and the proximity to the roots was evaluated; it was found that the shorter the distance from the roots, the higher the dissipation of the different PAHs. These authors suggested that a major dissipation of PAHs in soil by the presence of plants colonized by AMF can be attributed to two possible explanations: (1) The mycorrhizal symbiosis modifies root physiology (enzyme activity, exudation, longevity), thereby stimulating PAHs degradation, either by root-derived enzymes or by rhizosphere organisms, or (2) Mycorrhizal colonization affects root surface properties as well as rhizosphere soil properties that can affect PAHs availability through adsorption. In addition, Gao et al. (2011) suggested that the mechanism underlying PAHs dissipation by AMF in soil is the improvement of microbial activity that promotes the degradation of these pollutants.

Enhancement of enzymatic and microbial activities involved in PAHs degradation in plants inoculated with AMF has been observed in several studies. Catalase and polyphenol oxidase activities in soil vegetated with *Trifolium repens* were improved by the AMF *F. mosseae* and *Glomus etunicatum,* and higher microbial activities mainly in the hyphosphere (soil area close to the hyphae of AMF) were also noted. Moreover, a change in soil microflora was observed because of AMF fungi inoculation (Xiao et al., 2009).

Rabie (2005) observed that inoculation with the AM fungus *F. mosseae* contributed to enhancing the dissipation of a PAHs mixture in soil composed of anthracene, pyrene, chrysene and benzo(a, h) anthracene using *Triticum aestivum, Solanium melongena* and

Vigna radiate as host plants. In this study, *V. radiate* presented the highest colonization of AMF in its roots and highest polyphenol oxidase, dehydrogenase and peroxidase activity in the soil, as well as, the highest number of bacteria and fungi in the soil. All these results were directly related to a higher dissipation of PAHs in soil. Liu et al. (2004) also observed that inoculation with the AM fungus *Glomus caledoniun* in *Medicago sativa* plants increased the degradation of benzo(a)pyrene added at 1, 10 and 100 mg kg^{-1} without *G. caledonium* were 76, 78 and 53%, and with mycorrhizal inoculation were 86, 87 and 57%. A higher dehydrogenase activity was found in mycorrhizal plants under benzo(a)pyrene.

The AMF effects on the reduction of anthracene and phenanthrene content in soil were evaluated by Liu and Dalpé (2009) with *Allium porrum* plants inoculated with *Rhizophagus irregularis* or *Glomus versiforme*. Results obtained suggested that the AMF reduced the amount of PAHs in the polluted soil. This result was related to the enhanced nutrient uptake by the AMF, leading to improved plant growth, which in turn may stimulate soil microbial activity.

Wu et al. (2009) observed mechanism that can affect PAHs dissipation in soil using *Medicago sativa* plants were grown in phenanthrene-contaminated soil and inoculated with the AM fungus *Glomus etunicatum*. Direct visualization using two-photon excitation microscopy identified a higher phenanthrene accumulation in epidermal cells of roots and lower transport into the root interior and stem in mycorrhizal plants than in non-mycorrhizal controls.In this sense, a higher accumulation of PAHs in mycorrhizal plant roots than in non-mycorrhizal plants has been reported. Gao et al. (2010c) found that

phenanthrene and fluorene were accumulated mainly in *Lolium multiflorum* roots inoculated with the AMF *Funneliformis mosseae* and *Glomus etunicatum*, whereas the concentration of these two PAHs was lower in colonized plants than in non-mycorrhyzal plants. Moreover, the same authors suggested that the hyphae of AMF contribute to the uptake of PAHs from soil into roots based on a three- compartment system study.

2.6. CONCLUSIONS AND FUTURE PERSPECTIVES.

Plants and fungi involved in the bioremediation of PAHs have been described for the degradation or dissipation of these pollutants present in the soil. Plants contribute to this process by improving soil microbial activity by roots exudates, increasing the degradation and bioavailability of this pollutant, with the latter being an important factor in an effective remediation process. According to the statements above, plants interacting with mycorrhizal fungi can enhance PAHs dissipation in soil depending on fungus-plant compatibility. This symbiosis is able to dissipate PAHs from soil by enhancing the nutritional status of the plant, with a higher exudation of enzymes from the plant increasing microbial activity, while the mechanism of the fungi could be a higher accumulation of PAHs in roots than in non-mycorrhizal plants. On the other hand, the mostly frequently studied fungi such as SF are directly involved in PAHs degradation by ligninolytic and non-ligninolytic enzymes.

Several studies have reported the synergistic effects of SF and AMF mainly in heavy metal-contaminated soil (Arriagada et al., 2004; Arriagada et al., 2007; Arriagada et al., 2009) as well as in soils amended with toxic organic wastes (Sampedro et al., 2008). Therefore, a combination of these fungi could be an interesting alternative for PAHs dissipation in soil

by degradation, which could be stimulated by the enzymes produced by SF and the root exudates (including enzymes) generated by mycorrhizal plants, thus improving microbial activity in soil and bioavailability of PAHs as well as increasing plant tolerance and growth in contaminated soils (Figure 2.2). Therefore, the study of these interactions is relevant to improving the bioremediation process in PAH-contaminated soil and the establishment of plants.

Table 2.1 Removal of PAHs by plants influence in soil.

Plants	PAHs (concentration)	Removal (%)	Time (days)	Author
Mimosa monancistra	Benzo[a]pyrene (50 mg kg^{-1})	96	90	Alvarez-Bernal et al., 2007
Zea mays	Benzo[a]Pyrene (10 mg kg^{-1})	82	60	Chigbo, 2015
Phragmites australis	PAHs (2-3 rings, 2.7 mg kg^{-1})	66	25	Cui et al., 2017
	PAHs (4 rings, 1.9 mg kg^{-1})	57		
	PAHs (5-6 rings, 1.1 mg kg^{-1})	32		
Medicago sativa	Pyrene(100 mg kg^{-1})	32	90	D´Órazio et al., 2013
Brassica napus		30		
Lolium perenne		28		
Lolium multiflorum	Phenanthrene (200 mg kg^{-1})	85	90	Liu et al., 2013
Echinacea purpurea	8 PAHs (34 to 122 mg kg^{-1})	93	150	Liu et al., 2015a
Festuca arundinacea		98		
Medicago sativa		98		
Callistephus chinensis		85		
Sorghum bicolor	Pyrene (300 mg kg^{-1})	53	30	Rostami et al., 2016
Zea mays	Phenanthrene (53 mg kg^{-1})	98	60	Xu et al ., 2006
	Pyrene (58 mg kg^{-1})	95		
Juncus subsecundus	Phenanthrene (50 mg kg^{-1})	97	70	Zhang et al., 2012
	Pyrene (50 mg kg^{-1})	43		

Table 2.2. Removal of PAHs by SF in liquid cultures.

Fungi	PAHs (Concentration)	Removal (%)	Liquid medium	Time (days)	Author
Anthracophyllum discolor	Phenanthrene (50 mg L^{-1})	23	Kirk medium	28	Acevedo et al., 2011
	Anthracene (50 mg L^{-1})	7			
	Fluoranthene (50 mg L^{-1})	20			
	Pyrene (50 mg L^{-1})	9			
Penicillium janthinellum	Pyrene (250 mg L^{-1})	94	Malt yeast potato dextrose	28	Boonchan et al., 2000
	Chrysene (50 mg L^{-1})	32			
	Benzo[a]anthracene (50 mg L^{-1})	23			
	Benzo[a]pyrene (50 mg L^{-1})	72			
	Dibenzo[a,h]anthracene (50 mg L^{-1})	36			
Irpex lacteous	Anthracene (20 mg L^{-1})	60	Malt extract	25	Drevinskas et al., 2016
Pleurotus ostreatus		40			
Phanerochaete chrysosporium	Phenanthrene (50 mg L^{-1})	93	Basal III medium	5	Dhawale et al., 1992
Pseudotrametes gibbosa	Anthracene (5 mg L^{-1})	43	Liquid medium I	21	Gao et al., 2010b
	Pyrene (5 mg L^{-1})	24			
Trametes versicolor	Phenanthrene (100 mg L^{-1})	77	YMG broth (yeast, malt extract and glucose)	2	Han et al., 2004
Aspergillus terricola	Benzo[a]pyrene (30 mg L^{-1})	56	Mineral salt	7	Guntupalli et al., 2016
	Benzo[a]fluoranthene (30 mg L^{-1})	42			
Armillaria sp. F022	Pyrene (5 mg L^{-1})	81	Mineral salt broth	30	Hadibarata and Kristanti, 2013
Pestialopsis sp	Fluorene (5 mg L^{-1})	100	Mineral salt	23	Kristanti and Hadibarata., 2015

Fungi	PAHs (Concentration)	Removal (%)	Liquid medium	Time (days)	Author
Trichoderma Tormentosum	Anthracene (50 mg L^{-1})	64	Bushnell-Haas	49	Marchand et al., 2017
	Phenanthrene (50 mg L^{-1})	77			
	Fluorene (50 mg L^{-1})	90			
	Pyrene (50 mg L^{-1})	64			
Fusarium Oxysporum	Anthracene (50 mg L^{-1})	63			
	Phenanthrene (50 mg L^{-1})	85			
	Fluorene (50 mg L^{-1})	99			
	Pyrene (50 mg L^{-1})	55			
Trichoderma/Hypocrea sp	Anthracene (10 mg L^{-1})	36	Czapek-Dox	7	Mineki et al., 2014
	Pyrene (10 mg L^{-1})	37			
	Fluoranthene (10 mg L^{-1})	18			
Bjerkandera adusta	Anthracene (500 mg L^{-1})	38	Basic medium	3	Schützendübel et al., 1999
	Fluorene (500 mg L^{-1})	56			
Pleurotus ostreatus	Anthracene (500 mg L^{-1})	60			
	Fluorene (500 mg L^{-1})	43			
Tramates polyzona	Phenanthrene (100 mg L^{-1})	100	Mineral salt glucose medium	32	Teerapatsakul et al., 2016
	Fluorene (100 mg L^{-1})	100			
	Pyrene (100 mg L^{-1})	85			

Table 2.3 Removal of PAHs by SF in soil. * Non Sterilized soil ** Sterilized soil

Fungi	PAHs (Concentration)	Removal (%)	Inoculation substrate	Time (days)	Author
Pleurotus ostreatus	Phenanthrene (10 mg kg^{-1})	40	Birch wood	70 **	Andersson et al., 2000
	Anthracene (5 mg kg-^{1})	50			
	Pyrene (31 mg kg^{-1})	26			
	Indeno [1,2,3,cd]pyrene (15 mg kg^{-1})	20			
Phanerochaete chrysosporium	Phenanthrene (10 mg kg^{-1})	45			
	Anthracene (5 mg kg^{-1})	52			
	Pyrene (27 mg kg^{-1})	37			
	Indeno [1,2,3,cd]pyrene (13 mg kg^{-1})	46			
Penicillium frequentans	Phenanthrene (200 mg kg^{-1})	67	Bagasse	30 *	Amezcua-Allieri et al., 2005
Trichoderma sp	Phenanthrene (250 mg kg^{-1})	89	Barley grains	70**	Atagana, 2009
	Chrysene (150 mg kg^{-1})	83			
	Benzo[a]anthracene (220 mg kg^{-1})	90			
	Benzo[a]fluoranthene (100 mg kg^{-1})	75			
	Benzo[a]pyrene (200 mg kg^{-1})	85			
Trametes versicolor	Phenanthrene (250 mg kg^{-1})	94			
	Chrysene (150 mg kg^{-1})	91			
	Benzo[a]anthracene (220 mg kg^{-1})	93			
	Benzo[a]fluoranthene (100 mg kg^{-1})	86			
	Benzo[a]pyrene (200 mg kg^{-1})	92			

Fungi	PAHs (Concentration)	Removal (%)	Inoculation substrate	Time (days)	Author
Agaricus bisporum	Acenaphthene (50 mg kg^{-1})	24	Spent mushroom	63*	García-Delgado et al., 2015
	Anthracene (7.4 mg kg^{-1})	100			
	Phenanthrene (29 mg kg^{-1})	35			
	Fluorene (0.8 mg kg^{-1})	32			
	Fluoranthene (71 mg kg^{-1})	31			
	Pyrene (95.8 mg kg^{-1})	30			
	Benzo(a)anthracene (56.8 mg kg^{-1})	28			
	Chrysene (76.4 mg kg^{-1})	27			
	Benzo[b]fluoranthene (112 mg kg^{-1})	28			
	Benzo[k]fluoranthene (32.3 mg kg^{-1})	22			
	Benzo[a]pyrene (93.1 mg kg^{-1})	39			
	Indeno[1,2,3,cd]pyrene (49.5 mg kg^{-1})	30			
Fusarium sp	Phenanthrene (100 mg kg^{-1})	84	Lotus roots	14**	Li et al., 2005
	Pyrene (100 mg kg^{-1})	75			
Talaromyces spectabilis	PAHs Mixture (2 g kg^{-1})	23	Sugarcane bagasse	14**	Reyes-César et al., 2014
Fusarium sp		25			
Aspergillus terreus		25			
Trichoderma reesei	PAHs (2-3 rings) (0.45 mg kg^{-1})	36	Wheat bran–orange peel	28*	Yao et al., 2015
	PAHs (4 rings) (3.8 mg kg^{-1})	35			
	PAHs (5-6 rings) (5.4 mg kg^{-1})	25			
Trichoderma asperellum	Phenanthrene (333 mg kg^{-1})	74	Sugarcane bagasse	18**	Zafra et al., 2015
	Pyrene (333 mg kg^{-1})	63			
	Benzo[a]pyrene (333 mg kg^{-1})	81			

Table 2.4 Removal of PAHs by plants inoculated with mycorrhizal fungi.

Fungi	Host Plants	PAHs (Concentration)	Removal (%)	Time (days)	Author
Funneliformis mosseae	*Lolium perenne*	Phenanthrene (500 mg kg^{-1})	92		Corgié et al., 2006
Funneliformis mosseae+	*Medicago sativa*	Pyrene (74 mg kg^{-1})	98	70	Gao et al., 2011
Glomus etunicatum		Phenanthrene (103 mg kg^{-1})	88		
Funneliformis mosseae	*Lolium perenne* + *Trifolium repens*	12 PAHs (2g kg^{-1})	25	90	Joner and Leyval, 2003
Suillus bovinus	*Pinus sylvestris*	Anthracene (1 g Kg^{-1})	95	112	Joner et al., 2006
		Chrysene (1 g Kg^{-1})	66		
		Dibenzo[a,h]anthracene (50 mg Kg^{-1})	42		
Rhizophagus irregularis	*Triticum aestivum*	Naphthalene (24.8 mg kg^{-1})	10	112	Lenoir et al., 2016
		Acenaphthylene (2 mg kg^{-1})	20		
		Acenaphthene (1.1 mg kg^{-1})	9		
		Fluorene (2 mg kg^{-1})	15		
		Phenanthrene (16.1 mg kg^{-1})	29		
		Anthracene (20.4 mg kg^{-1})	19		
		Pyrene (25.3 mg kg^{-1})	40		
		Benzo[a]anthracene (13.1 mg kg^{-1})	41		
		Chrysene (18.4 mg kg^{-1})	41		
		Benzo[b]fluoranthene (18.5 mg kg^{-1})	29		
		Benzo[k]fluoranthene (17.2 mg kg^{-1})	37		
		Benzo[a]pyrene (19.4 mg kg^{-1})	37		
		Benzo[g,h,i]perylene (29.2mg kg^{-1})	31		

Fungi	Host Plants	PAHs (Concentration)	Removal (%)	Time (days)	Author
Rhizophagus irregularis +	*Allium porrum*	Anthracene (7 g kg^{-1})	30	84	Liu and Dalpé., 2009
Glomus versiforme		Phenanthrene (7 g kg^{-1})	77		
Glomus caledoniun	*Medicago sativa*	Benzo[a]pyrene (100 mg kg^{-1})	57	90	Liu et al., 2004
Glomus caledonium	*Festuca arundinacea*	2-ring PAHs (620 mg kg^{-1})	85	120	Lu and Lu, 2015
Glomus etunicatum	*Medicago sativa*	Phenanthrene (10 mg kg^{-1})	70	60	Wu et al., 2008
Funneliformis mosseae	*Lolium multiflorum*	Phenanthrene (50 mg kg^{-1})	100	60	Yu et al., 2011
		Pyrene (50 mg kg^{-1})	93		
Rhizophagus irregularis	*Festuca arundinacea*	Phenanthrene (500 mg kg^{-1})	98	42	Zhou et al., 2009
	Medicago sativa	Pyrene (500 mg kg^{-1})	60		
		Dibenzo[a]anthracene (65mg kg^{-1})	72		
Rhizophagus irregularis	*Medicago sativa*	Phenanthrene (500 mg kg^{-1})	95	42	Zhou et al., 2013
		Pyrene (500 mg kg^{-1})	64		
		Dibenzo[a,h]anthracene (50 mg kg^{-1})	20		
	Festuca arundinacea	Phenanthrene (500 mg kg^{-1})	95		
		Pyrene (500 mg kg^{-1})	62		
		Dibenzo[a,h]anthracene (50 mg kg^{-1})	10		
	Lolium multiflorum	Phenanthrene (500 mg kg^{-1})	93		
		Pyrene (500 mg kg^{-1})	50		
		Dibenzo[a,h]anthracene (50 mg kg^{-1})	20		
	Apium graveolen	Phenanthrene (500 mg kg^{-1})	94		
		Pyrene (500 mg kg^{-1})	49		
		Dibenzo[a,h]anthracene (50 mg kg^{-1})	1		

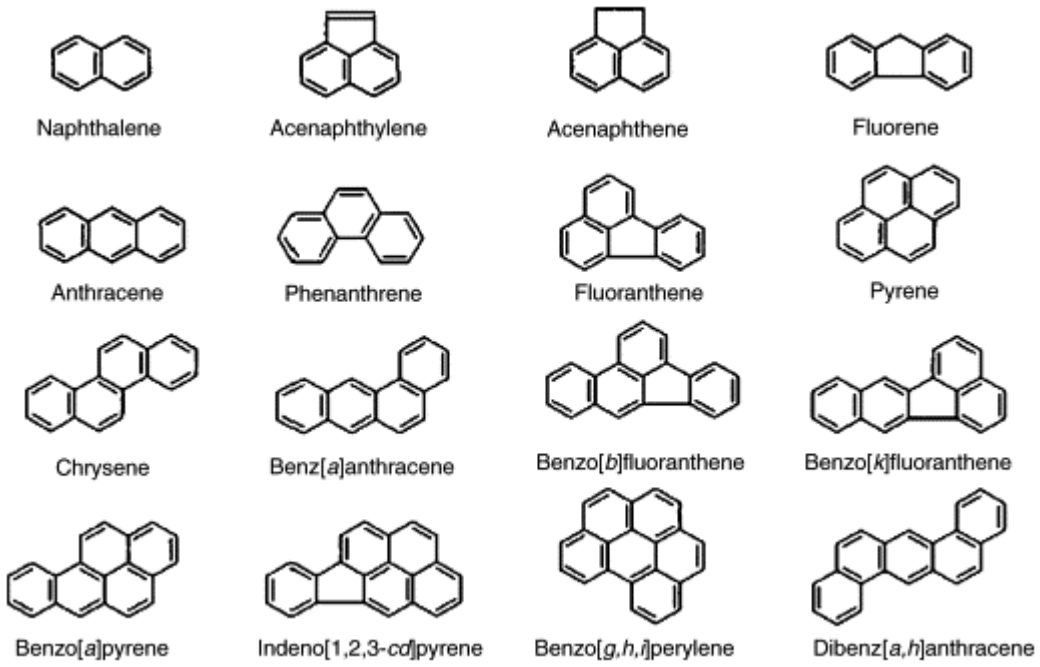

Figure 2.1 The 16 priority PAHs according to USEPA.

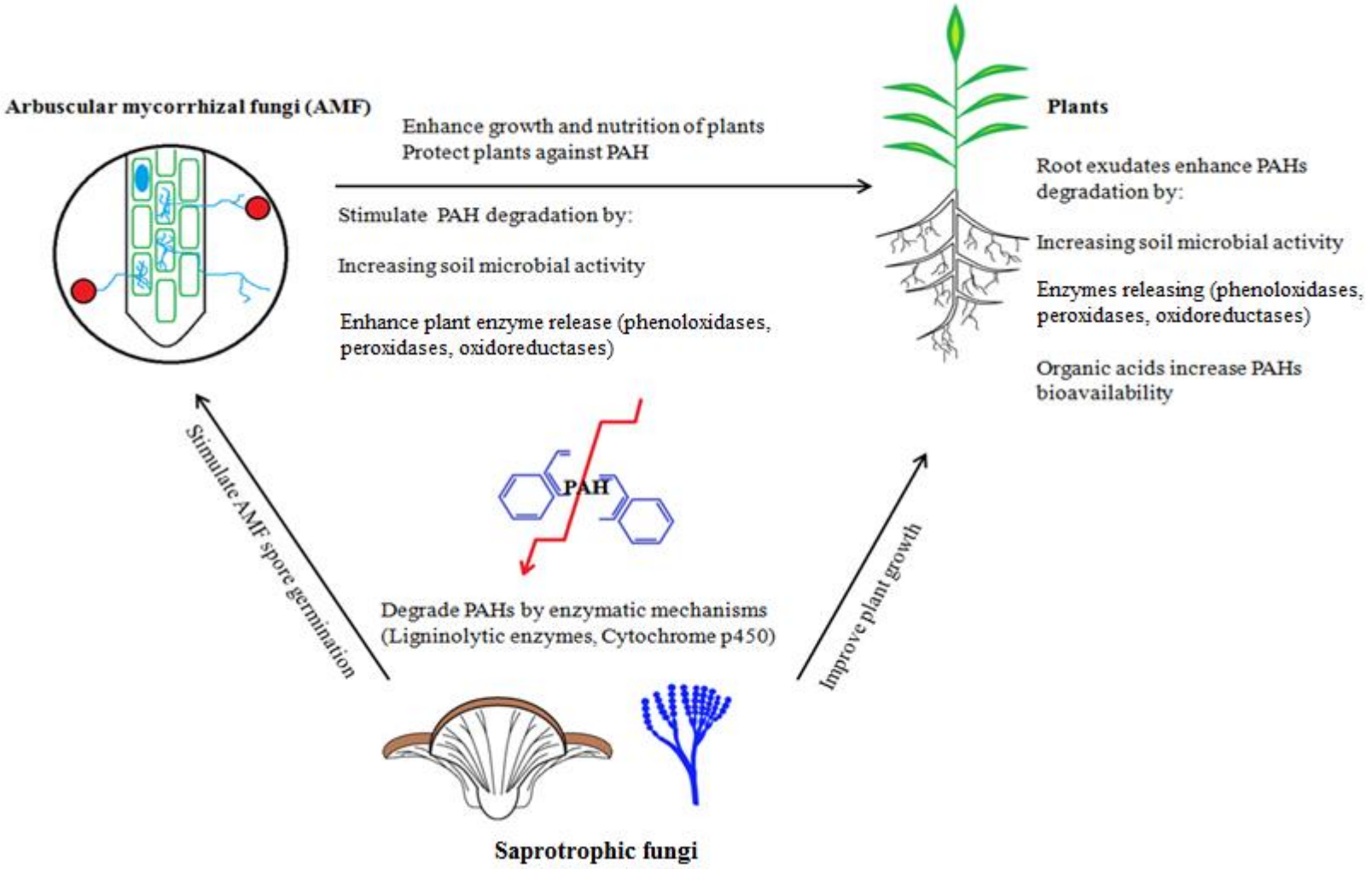

Figure 2.1 Scheme of the interaction of arbuscular mycorrhizal fungi, saprotrophic fungi and plants in order to enhance PAHs degradation.

CHAPTER III

Selection of suitable plant and rhizospheric fungi as an alternative to tolerance high concentration of polycyclic aromatic hydrocarbons

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Manuscript to be submitted to Plant Soil and Environment

3.1 ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are detrimental organic contaminants in soils. Our study analyzed the potential of fungi isolated from heavy metal polluted soil to remove PAHs under in vitro conditions and seedlings able to tolerate and growth in PAH-spiked substrate. In vitro removal was done using the fungal strains in liquid culture polluted individually with 100 mg L^{-1} of anthracene and phenanthrene and their mixture (50 mg L^{-1} for each one) for 14 days. PAHs removal, ligninolytic enzymes, fungal dry biomass and final pH of the liquid medium were determined. Seven fungal strains were identified through molecular methods: *Trichoderma viride*, *Penicillium canescens*, *Penicillium glabrum*, *Fimetariella rabenhorstii*, *Alternaria consortialis*, *Alternaria duci* and *Hormonema viticola*. All strains removed PAHs in the liquid culture. *T. viride* reached the highest PAHs removal, ligninolytic enzymatic activity and fungal dry biomass. Among the tested seedlings, *Triticum aestivum* was able to reach the highest germination rates and growth parameters. Our results provide evidence that fungi isolated from a heavy metal polluted soil can be regarded as a strategy for remediating PAH-contaminated soil, as well as *T. aestivum* plants represent a suitable plant for achieving these purposes.

Keywords: Bioremediation; PAHs removal; Recalcitrant pollutants; Soil fungi.

3.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of over 100 different compounds formed by carbon and hydrogen atoms consisting of two or more fused aromatic rings in a linear or clustered arrangement (Martorell et al., 2010). Given their carcinogenic, mutagenic, embryotoxic and teratogenic properties, PAHs are persistent organic

compounds that have a negative impact on all broad living organisms in different ecosystems (Sogbanmu et al., 2016). Their wide distribution in the environment is due to their lipophilic properties, which allow their adsorption onto atmospheric particles and direct deposition in sediments, soils and plants (Danyi et al., 2009). In addition, their capacity for remaining in the environment for extended periods is associated with their degradation difficulties related to low water solubility, which increases at a higher fused ring number of their chemical structure (Malachowska-Jutsz and Niesler, 2015). Once in soil, PAHs are strongly sorbed depending on the organic matter content in soil (Vácha et al., 2010). In addition, they can be dissipated by photo-oxidation (Park et al., 1990), bioaccumulation in plant tissues (Meudec et al., 2006), leaching (de Jonge et al., 2008), biosorption and biodegradation by soil microorganisms (Chen and Ding, 2012).

In order to alleviate these environmental concerns, bioremediation is considered an economical and environmentally friendly alternative to remediate PAH-contaminated soil by using different microbes (Guntupalli et al., 2016). Among the organisms involved in this remediation option, saprotrophic fungi (SF) have yielded successful results related to PAHs degradation as a dissipation method in soil or indifferent growing conditions or substrate (Aranda et al., 2013). PAHs degradation by these fungi has been associated with their enzymatic machinery comprised of ligninolytic and non-ligninolytic systems able to transform these compounds into oxidized intermediates and CO_2 (Bezalel et al., 1996; Pozdnyakova, 2012). In order to degrade PAHs at different scales, the use of SF isolated from PAH-contaminated soil has important advantages since most of them are adapted to this condition and have been reported to be a highly effective in PAHs removal in soil and

liquid cultures (Marchand et al., 2017). In this regard, most research about fungal strains degrading PAHs has examined using fungi isolated from PAH-contaminated soils.

Heavy metal pollution is one of the main issues affecting soil and is usually found with PAHs derived from a common pollution origin related to anthropogenic activities (Bradley et al., 1994). In addition, fungal strains isolated from PAHs combined with heavy metal-contaminated soil have been tested successfully for degrading PAHs (D'Annibale et al., 2006). However, few studies have used fungi isolated from only heavy metal-contaminated soils for this purpose. Therefore, the aims of this study were to isolate and classify SF strains from a heavy metal-contaminated soil and assess the removal of PAHs by these isolated fungi in liquid cultures.

The use of plants for remediation proposal is related to the stimulation of microbial activity in the rizhosphere due to the presence of vegetal organisms increasing the microbial population and enhancing its activity for creating optimal degradation conditions for organic pollutant mineralization (Muratova et al., 2003). Nevertheless, the sensitivity of many plant species to contaminants including PAHs is one of the most important limitations about phytoremediation and has been demonstrated in several studies (Berteigne et al., 1989; Kummerová et al., 2006; Liu et al., 2009; Pasková et al., 2006). Huang et al. (2004) suggested that due to sensitivity of many plants to PAHs their growth is slow; therefore, it is difficult to establish enough biomass to carry out phytoremediation of PAHs-polluted soils, even more when the number of microorganisms present in these soils is limited compared with an uncontaminated soil, which does not facilitate the establishment of plants. Therefore the selection of

suitable plants and fungi is a key step for achieving a successful bioremediation of PAH-polluted soil.

3.3 Materials and methods

3.3.1 Soil

The soil was collected from the Puchuncaví Valley in central Chile placed 1.5 km southeast of the Ventanas copper smelter (32°46'30''S; 71°28'17''W). The soil has been classified as an Entisol of the Chilicauquén series, with a pH of 5.54 (1:10, H_2O), 2.41% organic matter and 29 mg N kg^{-1}, 40 mg P kg^{-1} and 210 mg K kg^{-1} content (Fuentes et al., 2016). PAHs where found in negligible quantities due to rains and further soil washing associated to the low organic matter content that favor the erosion process and soil drag by rains. However, PAHs at atmospheric level should be important in this area, given by the high industrial activity that copper smelter and thermoelectrics which produces PAHs in their process.

3.3.2 Isolation of fungal strains from a heavy metal contaminated soil

Fungi were isolated from soil by the particle washing method using a multichamber washing apparatus (Bisset and Widden, 1972). The SF were transferred to tubes of potato dextrose agar (PDA, DIFCO) plus streptomycin (100 mg L^{-1}) and incubated in the dark for seven days at 28°C. The strains were stored on PDA plates at 4 °C and periodically sub-cultured. The SF were molecularly characterized and classified as described by White et al. (1990).

3.3.3 Molecular fungal characterization

Isolated pure colonies were grown potato dextrose broth (PDB) medium for 10 days. Genomic DNA extraction was performed using the DNeasy Plant Mini Kit (QIAGEN) for fungi. Then, a polymerase chain reaction (PCR) was carried out with primers ITS1 - ITS4 (fungi). The PCR amplification was conducted in 25 µl in a MultiGene Labnet thermocycler (International Inc., USA) with the following profile: 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 59.5 °C for 30 s, and 72 °C for 30 s, followed by a final extension step of 72 °C for 5 min. Negative controls (without DNA sample) were prepared for each set of amplifications. The PCR products with the transformation of competent cells (*E. coli* DH5) were prepared with the pGEM T-Easy (Promega) Vector for subsequent extraction and plasmid DNA sequencing. The sequences were compared with others in GenBank using BLASTn (Altschul et al., 1990) to find the best match.

3.3.4 Experimental Set-up

The 3-ring PAHs anthracene (ANT) and phenanthrene (PHE) were diluted in acetone and then introduced into sterile 100 mL flasks under aseptic conditions using concentrations of ANT (100 mg L^{-1}), PHE (100 mg L^{-1}) and their mixture (50 mg L^{-1} for each one). Acetone was left for evaporation and then 40 mL of sterile asparagine liquid medium was introduced into the flasks. Then, a 1 cm^2 plug of each fungal strain previously subcultured in PDA was incorporated into the flasks and kept in the dark under constant agitation at 150 rpm at 28°C for 14 days. Control treatments were inoculated cultures without PAHs. The experimental design consisted of 8 strains, 4 treatments with PAHs (control, ANT, PHE

and their mixture) and 3 replicates for each treatment resulting in 96 experimental units. ANT and PHE were purchased from Sigma-Aldrich Corporation. Determinations were made at 14 days after fungal inoculation.

3.3.5. PAHs extraction and fungal biomass

Prior to PAHs extraction, the final pH of each flask was determined. The PAHs extraction from liquid culture was determined according to the method described by Cajthaml et al. (2008). The entire flask content (mycelium with the liquid) of each replicate was homogenized. Then, it was acidified at pH 2 and extracted with ethyl acetate. The organic phase (ethyl acetate) was recovered and dried with disodium sulfate in order to clean the extracted liquid. Afterward, the extract was concentrated with a rotary evaporator for measuring PAHs concentration. On the other hand, the removed flask content was filtered to determine the dry fungal biomass.

3.3.6 PAHs determination

PAHs concentration in liquid culture was determined as described by Cajthaml et al. (2008). To perform the high performance liquid chromatography (HPLC) analysis, an aliquot of the extract was mixed with acetonitrile 1 : 10 (V/V) and the mixture was used for injection. The remaining PAHs were measured by a HPLC system equipped with a diode-array detector. A SUPELCOSIL™LC-18 HPLC column was used to determine PAHs concentration to express their removal from liquid culture.

3.3.7 Ligninolytic enzymes

Laccase activity was measured by spectrophotometry at 405 nm. The reaction mixture consisted of 0.5 ml of culture of the sample, 0.25 ml 100 mM glycine buffer (pH 3.0) and 0.25 ml of 4 mM of ABTS. Manganese peroxidase (MnP) activity was measured by spectrophotometry at 610 nm. The reaction mixture consisted of 0.5 ml of culture sample, 0.2 ml of buffer 0.5 M sodium tartrate (pH 5), 0.1 ml of 10 mM guaiacol, 0.1 ml 1 mM $MnSO_4 * H_2O$ and 0.1 ml of 1 mM H_2O_2 prepared in 20 mM sodium succinate buffer (pH 4.0). All enzyme reaction mixtures were incubated at 25 °C for 5 min before the measurement. All the methodologies were determined as described Ting et al. (2011).

3.3.8 Germination Test

Germination assay was performed in petri dishes using seeds of *Solanum lycopersicum* (tomato), *Medicago sativa* (alfalfa), *Triticum aestivum* (wheat) and *Eucalyptus globulus* (eucalyptus). These species were chosen by their high compability with saprotrophic and arbuscular mycorrhizal fungi previously tested in assays using fungal consortia. In addition, *T. aestivum* and *M. sativa* has been reported for their ability to enhance PAHs dissipation (Dubrovskaya et al., 2017; Shtangeeva et al., 2018) *The* seeds were put in petri dishes, which were spiked with 250, 500 and 1000 mg L^{-1} of ANT, PHE and their mixture. For each treatment, three petri dishes were used, containing 10 seeds of each plant. The control for this assay corresponded to seed without PAHs. Relative germination percentage (RGP) and relative radicle growth percentage (RRGP) were evaluated at 24, 48 and 96 hours. At the end of this experiment shoot and root dry weight, shoot and root height was measured.

3.3.9 Statistical Analysis

Statistical procedures were carried out with the SPSS software, version 11.0 (SPSS Inc., 1989–2001). Statistical significance was determined at $p< 0.05$. All variables were tested for normality and variance homogeneity (Kolmogorov-Smirnov and Cochran's C tests, respectively) and data was log transformed when needed. Percentage values were arcsine transformed for their analyses.

3.4 RESULTS

3.4.1 Fungal isolates

Seven strains were isolated from the heavy metal-contaminated soil and were classified according to Table 3.1: *Trichoderma viride*, *Penicillium canescens*, *Penicillium glabrum*, *Alternaria consortialis*, *Alternaria dauci*, *Fimetariella rabenhorstii* and *Hormonema viticola*

3.4.2 PAHs removal from liquid culture

After 14 days the highest removal in all PAHs treatments was reached significantly by *T. viride*. In the individual PAH incorporation (Figure 3.1a), *T. viride* removed 84.9% of ANT and 91.8% of PHE, followed by *Penicillium canescens* (ANT: 70.8%; PHE: 81.7%), *Penicillium glabrum* (ANT: 70%; PHE: 80.2%) , *Hormonema viticola* (ANT:69%; PHE: 74.3%), *Alternaria consortialis* (ANT: 59.4%; PHE: 69.4%), *Alternaria dauci* (ANT: 58.8%; PHE: 71.3%) and *Fimetariella rabenhorstii* (ANT: 49.9%; PHE: 58.7).

In the PAH mixture (Figure 3.1b), *T. viride* removed 74% of ANT and 82% of PHE, followed by *Penicillium canescens* (ANT: 60.1%; PHE: 73.5%), *Penicillium glabrum* (ANT: 59.1%; PHE: 72.3%), *Hormonema viticola* (ANT:59.3%; PHE: 69.12%), *Alternaria dauci* (ANT: 56.2%; PHE: 64%), *Alternaria consortialis* (ANT: 57.7%; PHE: 60.3%) and *Fimetariella rabenhorstii* (ANT: 38%; PHE: 56.2).

3.4.3 Ligninolytic enzymes

In general, ligninolytic enzymes were stimulated significantly in the presence of PAHs, mainly when PHE was incorporated. *T. viride* showed the highest laccase and manganese peroxidase activities either in the presence of PAHs or not. Significant differences between *T. viride* and all other fungal treatments were observed (Table 3.2). Fungi from *Penicillium* strains assessed in our study produced considerable laccase and manganese peroxidase mainly in the presence of PHE, whereas *Alternaria* strains produced only laccase.

3.4.4. Dry fungal matter and pH

T. viride reached significantly the highest dry fungal biomass compared to all other fungal treatments under either PAHs presence or absence. In general, ANT, PHE and their mixture stimulated the fungal growth in all treatments significantly (Table 3.3). With respect to the final pH after 14 days, in all cases it decreased significantly once the PAHs were incorporated. The highest pH decrease was observed with *T. viride* in the presence of PHE, being statistically significant either under PAH treatments or fungal treatments (Table 3.3).

3.4.5 Relative germination percentage

In general, the relative germination percentage **(**RGP) of all seeds decreased at each PAHs concentration including their mixture at different germination times. The only exception was *T. aestivum* seeds that reached the 100% of RGP under 250 mg L^{-1} of ANT and 250 mg L^{-1} of PAHs mixture after 96 and 144 hours (Table 3.4)

The increasing PAHs concentration decreased the RGP. In the cases of *M. sativa* and *S. lycopersicum* at 500 mg L^{-1} of PHE, 1000 mg L^{-1} of ANT, PHE and their mixture, the germination was completely inhibited at the end of the experiment. However, after 144 hours *T. aestivum* seeds reached 77%, 73% and 73% of RGP under 1000 mg L^{-1} of ANT, PHE and their mixture respectively. At the same germination time *E. globulus* seeds reached 36% of RGP only under 1000 mg L^{-1} of ANT, whereas, under PHE and the mixture, seeds did not germinate. On the other hand, *S. lycopersicum* presented the lowest RGP in all treatments after 144 hours. Respect to the negative effect of each PAH, PHE clearly decreased the RGP at all concentrations when compared to ANT and the mixture at each germination time.

3.4.6. Relative radicle growth percentage

The relative radicle growth percentage **(**RRGP) was decreased by all PAHs treatments tested at different concentrations and germination times. In no one case the RRGP was equal of higher than 100%. However, among all tested seeds, *T. aestivum* was less negatively affected by PAHs concentrations compared to the other seeds, especially at 500

and 1000 mg L^{-1} of ANT, PHE and their mixture, due that in these concentration the other seeds clearly decreased their RRGP (Table 3.5)

On the other hand, at 1000 mg L^{-1} of PAHs treatments only in seeds of *T. aestivum* and *E. globulus* RRGP was assessed due to *M. sativa* and *S. lycopersicum* did not germinate at this concentration. After 144 hours *T. aestivum* seeds reached 46%, 38% and 40% of RRGP under 1000 mg L^{-1} of ANT, PHE and their mixture, whereas, *E. globulus* only reached a 17% of RRGP under 1000 mg L^{-1} of ANT.

Similar to RGP, at all concentrations PHE produced the highest negative effect on RRGP of all tested seeds when were compared at each germination time.

3.4.7 Seedling growth parameters

According to the Table 3.6, after 144 hours all seedling growth parameters decreased under the different PAHs and their concentrations compared to the control treatment. Similarly to the observed results of RGP and RRGP, PHE produced the most negative effect on the growth parameters, mainly at 1000 mg L^{-1}. Under this concentration only *T. aestivum* seeds were able growth. Indeed, *T. aestivum* was not critically affected compared to the other seedlings under PAHs treatments. On the other hand, *M sativa* and S. *lycopersicum* were dramatically affected from 500 mg L^{-1} of each PAH and their mixture

At overall, *T. aestivum* was the most tolerant seed to each PAH and their mixture based on RGP, RRGP and seedling growth parameters. On the contrary, *S. lycopersicum* was the most sensitive seed. Finally, increasing concentrations of each PAH decreased all assessed parameters mainly by the single incorporation of PHE.

3.5. DISCUSSION

The genus *Trichoderma* corresponds to a cosmopolitan kind of fungus that can be present in different ecosystems (Das and Gupta-Bhattacharya, 2009) and under extreme environmental conditions (Harman et al., 2004). The presence of *Trichoderma* species isolated from heavy metal-contaminated soil has been reported in several studies (Nongmaithem et al., 2016). In addition, *T. viride* has been isolated from sewage, sludge and industrial effluents containing heavy metals (Joshi et al., 2011); thus, the occurrence of *T. viride* in the heavy metal-contaminated soil studied here is a common trait of these soils.

According to classified fungi, two *Penicillium* species were identified. *P. canescens and P. glabrum*, both ubiquitous fungal species present in different ecosystems able to adapt to extreme environments. (Nicoletti and De Stefano, 2012).

*Alternaria*strains seem not to be a typical fungal genus present in heavy metal-contaminated soils due to the paucity of literature describing this fungal trait compared to *Trichoderma* and *Penicillium*. However, several studies have demonstrated that fungi from *Alternaria* genus are able to tolerate heavy metals in soil (Mohammadian et al., 2017; Zafar et al., 2007), in solid waste at copper smelting industry (Saba et al., 2017) and under *in vitro* conditions (Shoaib et al., 2015). Information about isolation of the genus *Fimetariella* and *Hormonema* is lacking on the literature, therefore, there is new information in relation to these fungi and their ability to prevail in heavy metal polluted soil.

In all cases PHE presented higher removal than ANT. These PAHs are composed of the same ring number; however, PHE solubility is higher than ANT, which may increase its susceptibility to degradation (Bossert and Bartha, 1986). Zhu et al. (2016) found that *in*

vitro degradation of single PAHs such as PHE, naphthalene, fluorene, pyrene and benzo(a)pyrene by endophytic bacteria was higher than in a mixture of these PAHs. A competitive inhibition on single PAH may be caused by other PAHs, reducing their removal (Ma et al., 2013). Fungi belonging to the genus *Trichoderma* have been reported as degrading a wide range of organic material pollutants including PAHs at different scales (Sowmya et al., 2014). Many PAHs have been removed using *Trichoderma* strains isolated from PAH-contaminated soil (Zafra et al., 2015). Degradation of benzo(a)pyrene (5-ring PAH) by *T. viride* isolated from polluted soil (without specification) was assessed by Verdin et al. (2004), obtaining 39% degradation for 10 mg L^{-1} after 30 days of incubation. Due to its number of rings, benzo(a)pyrene is more recalcitrant than ANT and PHE; therefore, these findings demonstrate the potential of *T. viride* to degrade PAHs. In addition, *T. viride* has been isolated from extra-heavy crude oil-contaminated soil that includes PAHs (Naranjo et al., 2007), suggesting both its tolerance to these compounds and its potential as a PAH-biodegrader.

Govarthanan et al (2017) showed that a *Penicillium* sp. CHY-2 strain isolated from Antarctic soil was able to degrade naphthalene, acenaphthene and benzo(a) pyrene in liquid medium at 100 and 500 mg L^{-1}. However, in no case did this degradation pass 20 %. By contrast, PAHs degradation by *Penicillium* strains isolated from PAH-contaminated soil has been reported. Boonchan et al. (2000) found that *Penicillium janthinellum* isolated from creosote-contaminated soil was able to degrade 250 mg L^{-1} of pyrene (94%) and 50 mg L^{-1} of chrysene (32%), benzo(a) anthracene (23%) benzo(a) pyrene (72%) and dibenzo(a,h) anthracene (36%) in liquid medium (MYPD) after 28 days. With respect to *Penicillium glabrum*, Wunder et al. (1997) isolated a strain of fungus from a highly PAH-contaminated

soil. This strain was able to degrade pyrene in liquid culture determined by the appearance of two novel metabolites derived from fungal metabolism identified as 1-methoxypyrene and 1,6-dimethoxypyrene. On the other hand, *in vitro* removal of PAHs by *Penicillium canescens* has not been described yet, therefore our results provide new achieves oh this fungus in the field of bioremediation of organic pollutants.

Gerginova et al. (2013) showed that *Alternaria maritima* was able to degrade phenanthrene (300 mg L^{-1}) under *in vitro* conditions. In addition, Verma et al. (2016) found that *Alternaria alterna* was able to degrade among 30 - 40% of anthracene, phenanthrene and naphthalene (200 mg L^{-1}) under *in vitro* studies. Nevertheless, there is no information about PAHs removal by *Alternaria consortialis* and *Alternaria dauci* was assessed in our study. Furthermore, PAHs removal by fungal strains from the genus *Fimetariella* and *Hormonema* is lacking on the literature. Therefore, our results provide new evidence of PAHs removal by these fungal strains.

The highest production of ligninolytic activities by *T. viride* is related to the greatest removal of PAHs in all treatments. Fungi from the genus *Trichoderma* possess a versatile and powerful enzymatic machinery that allows them degrade a wide range of substrates and compounds (Jaklitsch, 2009). Despite the genus *Trichoderma* belonging to non-ligninolytic fungi, ligninolytic activities from *Trichoderma* have been reported in several studies. Zafra et al. (2015) found that *Trichoderma asperellum* was able to produce laccase and other oxidizing enzymes such as peroxidases and catechol 1,2 dioxygenases under PHE and pyrene exposure in liquid culture. *Penicillium* and *Alternaria* strains showed fewer ligninolytic activities compared to *T. viride*, but with a more effective PAHs removal In this case, it is possible that other enzymatic systems are operating to remove PAHs in liquid

medium that could include cytochrome p450, commonly recognized as the main enzymatic mechanism involved in PAHs oxidation and their further degradation by non-ligninolytic fungi (Črešnar and Petrič, 2011). However, in the case of *Penicillium*, the production of ligninolytic activities seems to be common for this genus (Govarthanan et al., 2017; Omaka and Kalu, 2016). In general, a combination of enzymatic systems has been suggested for increasing PAHs degradation and mineralization compared to a unique enzymatic system.

Saraswathy and Hallberg (2002) suggested that a higher fungal biomass increases the PAHs oxidation. This suggestion is related to the results shown by *T. viride* due to it reaching the highest biomass, ligninolytic activity and PAHs removal. The direct relationship between fungal growth and enzymatic activities has been reported previously (Anusha et al., 2012; Costa and Nahas, 2012). Therefore, all strains showed tolerance against ANT and PHE. Tolerance to PAHs is considered the primary factor in identifying suitable fungi for degrading PAHs for biotechnological purposes (Carrera, 2010).

In terms of the pH decrease in liquid medium by fungal strains, Srivastava et al. (2011) suggested that fungi decreased the pH of their environment possibly due to the end products of metabolism secreted in the medium, mainly carbon dioxide. This suggestion is also in line with the removal percentages of our study due to a possible mineralization of the PAHs tested by the isolated strains. Moreover, according to Srivastava et al. (2011) and Odeyini et al. (2009), an increased fungal growth tended to decrease pH in the medium. In our study, all strains decreased pH when PAHs were incorporated and also increased the fungal dry biomass.

For instance, in the case of *T. viride*, this fungus reached the highest biomass, produced the lowest pH and reached both the highest number of ligninolytic enzymes and greatest PAHs removal in our study. Therefore, the sum of all these results can explain the findings of our research regarding PAHs removal from liquid culture by this fungus.

The toxic effect of PAHs on seed germination has been showed previously (Hong et al., 2009; Wei et al, 2014). An eventual destruction of seed embryo by hydrocarbons has been implied on the reduction of seed germination (Reynoso-Cuevas et al., 2008) and have been shown to have indirect secondary effects including disruption of plant–water–air relationships (Renault et al., 2000)

On the other hand, the toxicity of PAHs on seeds of plants is related to their solubility in water. PAHs with higher water solubility; it means less aromatic rings; in water tend to produce more negative effects on seed germination than PAHs with lower water solubility (Henner et al., 1999; Somtrakoon and Chouychai, 2013). In this sense, anthracene and phenanthrene present the same ring number. Nevertheless, phenanthrene higher water solubility (1.6 mg L^{-1}) compared to anthracene (0.045 mg L^{-1}), being almost thirty-six folds more soluble and more phytotoxic to the plants. In addition, more soluble PAHs tend to present higher mobility than less soluble PAHs (Somtrakoon and Chouychai, 2013).

A higher germination rate of wheat seeds compared with other plants under PAHs exposure has been reported in literature. Salehi-Lisar and Deljoo (2015) found that different concentrations of Fluorene (50 and 100 mg kg^{-1}) did not affect the germination rate of wheat seeds compared to alfalfa (*Medicago sativa* L.), and sunflower (*Helianthus annus* L.) seeds which were negatively affected. Another explanation is related to the seeds size due

to a smaller seed is more susceptible for being covered by these hydrophobic compounds that impede the passage of water and possibly gases to the interior of the seed, reducing and delaying the seed germination (Bona et al, 2011). In this sense, wheat seeds were the largest used in our experiment and can be considered an advantage compared to the other seeds under the experimental conditions carried out. These results agree with Bouranis et al. (2012) due that Poaceae have been considered one of the most important plant family used in phytoremediation due to their tolerance to a wide range of pollutants such as trace elements and petroleum.

The inhibition of physiological processes, detected by chlorosis, yellowing, and decreased growth parameters, is one of the most common effects of PAHs pollution on plants (Meudec et al., 2006). In addition, the biomasses of root and shoot decrease as the plant spend most of its energy in order to adjust to the polluted conditions and absorbing water and nutrients from the substrate (Besalatpour et al., 2008).

Literature have described that oxidative damage is the main detrimental effect of PAHs on plants (Paskova et al., 2006). Wei et al (2014) found that phenanthrene at increasing doses (0 up to 250 mg L^{-1}) decrease the root and stem growth of wheat seedlings, reduced the chlorophyll content, decrease the antioxidant response and increase the lipid peroxidation. Their results showed that antioxidant enzymes Superoxide dismutase, Catalase and Guaiacol peroxidase decreased by effect of phenantrene, whereas, an increase in lipid peroxidation was well correlated to the reduced seedling growth parameters. Therefore the oxidative damage even at seedling stage is one of the main detrimental effects of PAHs on plants. This kind of damage is due to the formation of reactive oxygen species (ROS), which exceed the plant antioxidant systems within the vegetal tissues. These accumulations

of ROS produce the reduction in seed germination (Liu et al., 2009) and contribute to increase the oxidative damage of these compounds on plants at their earlier stages (Kummerova et al., 2012).

Root elongation and seed germination are suitable parameters involved on plant development due to their sensitivity to environmental phytotoxicity (Paquin et al., 2012). In our study, both parameters evidenced that wheat is a suitable plant for developing future strategies in order to assess their impact on PAHs dissipation in soil.

3.6 CONCLUSIONS

All fungal strains isolated from heavy metal-contaminated soil were able to remove ANT and PHE and their respective mixture after 14 days of incubation. *Trichoderma viride* reached the highest removal of ANT and PHE both individually and in the PAHs mixture. In addition, new information of PAHs removal by fungi such as *Penicillium canescens*, *Alternariaconsortialis*, *Alternaria dauci*, *Fimetariella rabenhorstii* and *Hormonema viticola* is provided for fisrt time in our study. These results are one of the first approaches related to PAHs removal using fungi isolated from heavy metal-contaminated soil. Therefore, their use in PAH-polluted soil remediation could be useful in cleanup strategies. On the other hand, wheat plants were showed the great potential to tolerate and grow in substrate piked with ANT and PHE and its further use in remediation in PAH-polluted soil.

Table 3.1 Molecular classification of fungi isolated from heavy metal polluted soil.

STRAIN	MICROORGANISMS	IDENTITY	ACCESSIONS NUMBER
V1	*Trichoderma viride*	99%	HM037962.1
V2	*Penicillium canescens*	100%	KX359603.1
V3	*Penicillium glabrum*	99%	HM77643
V4	*Alternaria consortialis*	99%	LC228637.1
V5	*Alternaria dauci*	100%	HE796759.1
V6	*Fimetariella rabenhorstii*	94%	HQ406808
V7	*Hormonema viticola*	100%	NR137620.1

Table 3.2 Ligninolytic activities produced by the isolated fungal strains after 14 days. ANT: anthracene; PHE: phenanthrene; ANT+PHE: mixture of anthracene and phenanthrene.

	Laccase (U L^{-1})				Manganese peroxidase (U L^{-1})			
Fungi	Control	ANT	PHE	ANT+PHE	Control	ANT	PHE	ANT+PHE
Trichoderma viride	54.2 Ca	155.9 Db	181.4 Dc	172.6 Dbc	21.5 Ba	65.4 Cb	76.0 Cb	68.8 Cb
Penicillium canescens	34.3 Ba	76.6 Cb	85.7 Cb	78.9 Cb	4.5 Aa	31.8 Bb	35.8 Bb	33.6 Bb
Penicillium glabrum	35.9 Ba	78.8 Cb	88.6 Cb	82.4 Cb	4.2 Aa	32.5 Bb	38.6 Bb	34.7 Bb
Alternaria consortialis	23.7 ABa	65.4 Bb	75.3 BCb	66.5 Bb	ND	ND	ND	ND
Alternaria dauci	17.5 Aa	53.1 Bb	67.7 Bb	66.4 Bb	ND	ND	ND	ND
Fimetariella rabenhorstii	11.4 Aa	22.2 Ab	22.1 Ab	17.1 Aab	3.1 Aa	4.1 Aab	4.9 Ab	4.8 Ab
Hormonema viticola	38.4 Ba	53.5 Bab	61.5 Bb	57.8 Bb	ND	ND	ND	ND

Different capital letter s within the same column indicated significant (p>0.05) difference between different fungal treatments, while different small letters within the same row indicated significant (p>0.05) difference between different PAHs treatments.

Table 3.3 Fungal dry biomass and final pH of the fungal growing medium after 14 days. ANT: anthracene; PHE: phenanthrene; ANT+PHE: mixture of anthracene and phenanthrene.

	Fungal dry biomass (g)				Final pH			
Fungi	Control	ANT	PHE	ANT+PHE	Control	ANT	PHE	ANT+PHE
Trichoderma viride	0.146 Ca	0.177 Cb	0.191 Dc	0.169 Db	4.79 Ac	4.11 Ab	3.82 Aa	4.19 Ab
Penicillium canescens	0.129 Ba	0.149 Bb	0.158 Cb	0.145 Cb	5.09 Bc	4.35 Bb	4.16 Ba	4.31 ABb
Penicillium glabrum	0.124 Ba	0.140 Bab	0.154 Cb	0.131 BCab	5.06 Bc	4.39 Bb	4.12 Ba	4.29 ABab
Alternaria consortialis	0.116 ABa	0.131 Bab	0.143 BCb	0.115 Ba	5.11 Bb	4.66 Cab	4.40 Ca	4.62 Bab
Alternaria dauci	0.111 Aa	0.127 ABab	0.141 BCb	0.119 Ba	5.27 Cc	4.57 Cab	4.41 Ca	4.65 Bb
Fimetariella rabenhorstii	0.102 Aab	0.102 Aab	0.108 Ab	0.093 Aa	5.03 Bb	4.53 Cab	4.39 Ca	4.58 Bab
Hormonema viticola	0.132 Ba	0.141 Bab	0.151 Cb	0.144 Cab	5.18 BCb	4.55 Cab	4.35 Ca	4.39 ABa

Different capital letter s within the same column indicated significant (p>0.05) difference between different fungal treatments, while different small letters within the same row indicated significant (p>0.05) difference between different PAHs treatments.

Table 3.4 Relative germination percentage at 24, 48, 96 and 144 hours. ANT: anthracene; PHE: phenanthrene; ANT+PHE: mixture of anthracene and phenanthrene.

		PAHs Concentration											
		250 mg L^{-1}				500 mg L^{-1}				1000 mg L^{-1}			
Plant	**Time (Hours)**	**24**	**48**	**96**	**144**	**24**	**48**	**96**	**144**	**24**	**48**	**96**	**144**
	PAHs												
	ANT	0%	78%	100%	100%	0%	44%	90%	90%	0%	0%	77%	77%
T. aestivum	PHE	0%	44%	93%	93%	0%	33%	80%	87%	0%	11%	63%	73%
	ANT+PHE	0%	67%	100%	100%	0%	56%	83%	87%	0%	22%	70%	73%
	ANT	59.1%	80.0%	80.0%	80.0%	45.5%	50.0%	56.7%	60.0%	0.0%	0.0%	0.0%	0.0%
M. sativa	PHE	54.5%	46.7%	60.0%	63.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	ANT+PHE	59.1%	70.0%	73.3%	73.3%	54.5%	50.0%	63.3%	66.7%	0.0%	0.0%	0.0%	0.0%
	ANT	61.9%	50.0%	53.3%	53.3%	28.6%	26.7%	30.0%	30.0%	0.0%	0.0%	0.0%	0.0%
S. lycopersicum	PHE	52.4%	40.0%	43.3%	43.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	ANT+PHE	57.1%	46.7%	46.7%	46.7%	23.8%	20.0%	20.0%	26.7%	0.0%	0.0%	0.0%	0.0%
	ANT	0.0%	29.4%	63.3%	73.3%	0.0%	17.6%	40.0%	56.7%	0.0%	5.9%	23.3%	36.7%
E. globulus	PHE	0.0%	17.6%	53.3%	63.3%	0.0%	5.9%	36.7%	43.3%	0.0%	0.0%	0.0%	0.0%
	ANT+PHE	0.0%	23.5%	60.0%	73.3%	0.0%	11.8%	33.3%	43.3%	0.0%	0.0%	0.0%	0.0%

Table 3.5 Relative radicle growth percentage at 24, 48, 96 and 144 hours. ANT: anthracene; PHE: phenanthrene; ANT+PHE: mixture of anthracene and phenanthrene.

		PAHs Concentration											
		250 mg L^{-1}				500 mg L^{-1}				1000 mg L^{-1}			
Plant	**Time (Hours)**	**24**	**48**	**96**	**144**	**24**	**48**	**96**	**144**	**24**	**48**	**96**	**144**
	PAHs												
	ANT	0%	56%	83%	79%	0%	28%	40%	61%	0%	0%	22%	46%
T. aestivum	PHE	0%	21%	67%	77%	0%	14%	21%	55%	0%	0%	16%	38%
	ANT+PHE	0%	47%	78%	78%	0%	33%	37%	51%	0%	0%	20%	40%
	ANT	62%	60%	48%	53%	38%	46%	22%	20%	0%	0%	0%	0%
M. sativa	PHE	39%	40%	41%	43%	0%	0%	0%	0%	0%	0%	0%	0%
	ANT+PHE	47%	44%	45%	44%	38%	35%	20%	16%	0%	0%	0%	0%
	ANT	59%	75%	75%	75%	33%	34%	24%	30%	0%	0%	0%	0%
S. lycopersicum	PHE	52%	64%	53%	66%	0%	0%	0%	0%	0%	0%	0%	0%
	ANT+PHE	57%	71%	69%	69%	22%	22%	19%	21%	0%	0%	0%	0%
	ANT	0%	75%	82%	74%	0%	47%	41%	43%	0%	20%	15%	17%
E. globulus	PHE	0%	12%	32%	60%	0%	7%	13%	20%	0%	0%	0%	0%
	ANT+PHE	0%	42%	37%	61%	0%	24%	29%	33%	0%	0%	0%	0%

Table 3.6 Seedling growth parameters after 144 hours. ANT: anthracene; PHE: phenanthrene; ANT+PHE: mixture of anthracene and phenanthrene.

		Treatments									
		Control	ANT (mg L^{-1})			PHE (mg L^{-1})			ANT + PHE (mg L^{-1})		
Plants			250	500	1000	250	500	1000	250	500	1000
T. aestivum	Shoot lenght (cm)	8,3	7,1	5,7	4,3	6,6	4,7	3,4	6,9	5,4	3,9
	Rooth lenght (cm)	8,4	6,6	5,1	3,7	5,7	4,4	3,1	6,2	4,2	3,4
	Shoot weight (g)	0,351	0,261	0,193	0,108	0,233	0,154	0,091	0,251	0,181	0,102
	Root weight (g)	0,151	0,099	0,055	0,031	0,076	0,043	0,027	0,085	0,048	0,030
M. sativa	Shoot lenght (cm)	3,4	1,5	0,5	ND	1,2	ND	ND	1,3	0,4	ND
	Rooth lenght (cm)	3,1	1,6	0,6	ND	1,3	ND	ND	1,1	0,5	ND
	Shoot weight (g)	0,048	0,024	0,015	ND	0,016	ND	ND	0,021	0,011	ND
	Root weight (g)	0,026	0,017	0,011	ND	0,011	ND	ND	0,014	0,009	ND
S. lycopersicum	Shoot lenght (cm)	6,4	3,1	1,1	ND	2,7	ND	ND	2,9	0,8	ND
	Rooth lenght (cm)	5,7	4,0	1,6	ND	3,5	ND	ND	3,7	1,1	ND
	Shoot weight (g)	0,040	0,025	0,011	ND	0,016	ND	ND	0,021	0,009	ND
	Root weight (g)	0,028	0,017	0,010	ND	0,012	ND	ND	0,015	0,008	ND
E. globulus	Shoot lenght (cm)	2,4	1,8	1,1	0,5	1,4	0,7	ND	1,6	0,9	ND
	Rooth lenght (cm)	3,0	2,2	1,3	0,5	1,8	0,6	ND	1,8	1,0	ND
	Shoot weight (g)	0,021	0,016	0,011	0,007	0,012	0,009	ND	0,014	0,010	ND
	Root weight (g)	0,018	0,012	0,009	0,006	0,010	0,005	ND	0,110	0,006	ND

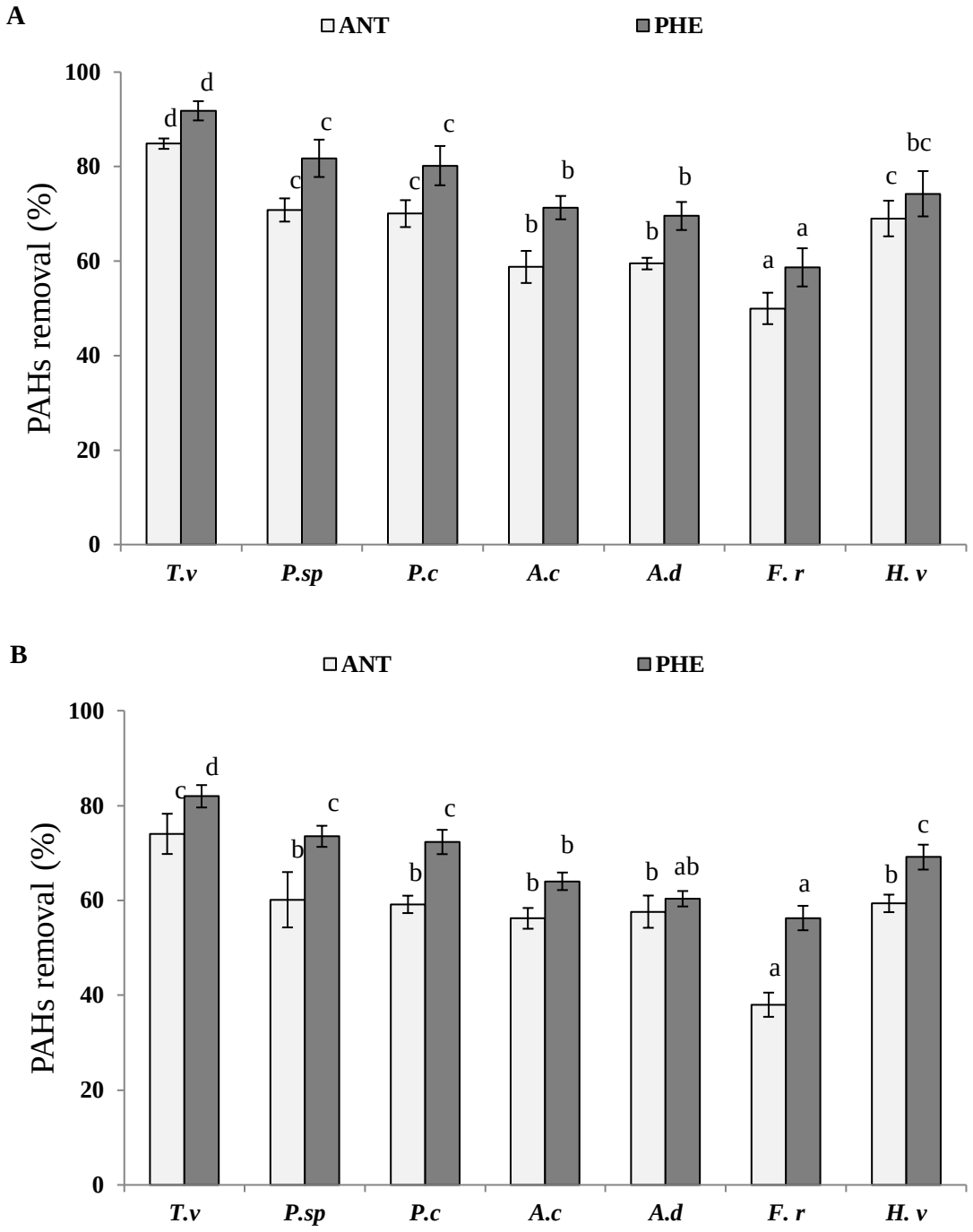

Figure 3.1 Percentage of individual PAHs removal in liquid cultures by fungi isolated from heavy metal polluted soil. A) Removal of individual PAHs. B) Removal of individual PAHs after mixed incorporation. *T.v*: *T. viride*; *P.c*: *Penicillium canescens*; *P.g*: *Penicillium glabrum*; *A. c*: *Alternaria consorcialis*; *A.d*: *Alternaria dauci*; *F. r*: *Fimetariella rabenhorstii* ; *H.v*: *Hormonema viticola*. Different letters on bars means significant differences among fungi for each PAHs.

CHAPTER IV

Association of wheat with plant growth promoting fungi alleviates plant oxidative stress and enhances phenanthrene dissipation in soil

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Manuscript Submitted to Bioremediation Journal

4.1 ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are environmentally hazardous due to their strong toxic effect on living organisms, being the soil the main reservoir. Microbiological methods provide an economic and efficient alternative for such soil decontamination. Here, we examined the effects of the saprotrophic fungus *Trichoderma viride* and the arbuscular mycorrhizal (AM) fungus *Funneliformis mosseae* on PAH dissipation in soil and alleviation of oxidative stress in *Triticum aestivum* plants growing in soil spiked with two doses of phenanthrene (500 and 1000 mg kg^{-1} soil). Phenanthrene dissipation, soil enzymatic activities, dry biomass, antioxidant enzymes, lipid peroxidation and organic acid exudation of plants were determined. The dual inoculation with *T. viride* and *F. mosseae* increased phenanthrene dissipation in soil Also, dry biomass, soil enzymes, antioxidant response, organic acid exudation and phenanthrene content in roots were increased by dual inoculation in phenanthrene-spiked soil, whereas lipid peroxidation and phenanthrene content in shoots were reduced. Dissipation of phenanthrene by degradation could be the main way to remove it from soil as undetectable phenanthrene was higher under dual inoculation. Our results show that dual inoculation in wheat plants significantly promotes phenanthrene dissipation in soil and contributes to alleviating oxidative damage as a consequence of improved plant fitness.

Keywords: Antioxidant enzymes, arbuscular mycorrhizal fungi, polycyclic aromatic hydrocarbons; saprotrophic fungi, soil bioremediation.

4.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic compounds that adversely affect the environment with negative impacts on all broad living organisms related to the carcinogenic, mutagenic, embryotoxic and teratogenic properties of these compounds (Rajendran et al., 2013; Sogbanmu et al., 2016). In the last decade, the use of environmentally friendly technologies such as microorganisms or plants has contributed to PAHs dissipation from soil in the root zone (Cheema et al., 2010), but may also have resulted in the bioaccumulation of PAHs in plants, leading to food chain contamination (Li et al., 2008; Wild et al., 2005). Therefore, it is necessary to understand the process of PAHs dissipation in the rhizosphere (plant roots and their associated microbiota) to develop efficient and environmentally friendly bioremediation strategies.

Soil microorganisms are important in the recovery of contaminated soils because they can produce plant-growing substances, sequester toxic substances and contribute to nutrient availability for plants (Arriagada et al., 2009). Previous studies have determined that microorganisms such as fungi are able to improve PAHs degradation as a dissipation way in soil under different growing conditions or substrates (Aranda et al., 2013; Atagana, 2009; Yu et al., 2013). In addition, some plants species enhance PAHs dissipation (Yi and Crowley, 2007) by favoring the proliferation of soil microorganism involved in the degradation of recalcitrant compounds. However, plant establishment in polluted soils depends on their sensitivity to PAHs. The negative effects of PAHs on plants are related to oxidative stress through reactive oxygen species (ROS) that cause oxidative damage, affecting normal plant growth (Liu et al., 2015b; Pasková et al., 2006); therefore, alternatives are needed to manage these detrimental effects of PAHs on plants.

Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs from Glomeromycota forming mutualistic associations with plant roots, which through colonization of the soil with external mycelia extend the nutrient and water absorption area of their host plant, resulting in plant growth promotion. Alleviating plant stress caused by biotic and abiotic factors is another important trait of AMF (Smith and Read, 2008). These symbionts are also known to play an important role in the phytoremediation of contaminated soils (Fuentes et al., 2016). In terms of PAHs bioremediation, AMF have been found to promote PAHs dissipation from soil through strategies such as the accumulation of PAHs in roots and reducing the PAHs content in leaves (Gao et al., 2010a). Furthermore, indirect effects of AM fungi in relation to PAHs dissipation through degradation have been suggested via proliferation of mycorrhizal-associated microorganisms (Gao et al., 2011). Saprotrophic fungi (SF) such as *Trichoderma* spp. associated with roots are also able to alleviate plant stress and improve plant growth (Contreras-Cornejo et al., 2016; Harman, 2006), and given their broad enzyme profile, these fungi also exhibit traits for the bioremediation of recalcitrant contaminants like PAHs (Tripathi et al., 2013). The joint inoculation AMF and SF have been reported as promising for the amelioration of several pollution events occurring in soil (Arriagada, et al., 2007; Arriagada et al., 2010). However, information on combination effects of AM fungi and *Trichoderma* spp. in relation to PAHs soil bioremediation is still limited. Thus, the aim of this study was to evaluate the dissipation of phenanthrene (three-ring PAH) and stress alleviation in wheat from dual inoculation with the AM fungus *Funneliformis mosseae* and saprotrophic fungus *Trichoderma viride*. In order to achieve these purposes, *Triticum aestivum* (wheat) belonging to Poaceae have been considered one of the most important plant families used in phytoremediation due to their

tolerance to a wide range of pollutants such as trace elements and PAHs (Bouranis et al., 2012; Kuang et al., 2016; Yu et al., 2013).

4.3 MATERIALS AND METHODS

4.3.1 Soil

The soil was collected from Puchuncaví Valley in central Chile placed 1.5 km southeast of the Ventanas copper smelter (32°46'30''S; 71°28'17''W). The soil has been classified as an Entisol Chilicauquén series and has a pH of 5.54 (1:10, H_2O), 2.41% organic matter and 29 mg N kg^{-1}, 40 mg P kg^{-1} and 210 mg K kg^{-1} content (Fuentes et al., 2016) .The soil samples were collected at a depth up to 20 cm.

4.3.2 Microorganisms

The saprotrophic fungus *Trichoderma viride* (Accession number: HM037962.1) was previously isolated and molecularly classified. Isolation occurred in soil from the Puchuncaví valley in central Chile, located 1.5 km from the Ventanas copper smelter and was used because it had shown the best phenanthrene degradation in liquid culture in previous tests. *T. viride* was stored in potato dextrose agar (PDA) dishes at 4 °C and periodically subcultured to ensure its viability.

The inoculum of the AM fungus *Funneliformis mosseae* (Accession number: JN847444.1) was obtained from culture pots performed at the Bioremediation Laboratory at the Universidad de La Frontera, Temuco, Chile. This inoculum was a root and soil mixture consisting of rhizosphere soil containing spores and colonized root fragments of *Sorghum bicolor* with high levels of root colonization.

4.3.3 Greenhouse experiment

A greenhouse pot experiment was performed with a fully factorial design with three factors: phenanthrene (three levels, 0, 500 and 1000 mg kg^{-1} soil), *F. mosseae* (two levels, without and with) and *T. viride* (two levels, without and with). Each of the resulting 12 treatments had twelve replicates yielding a total of 144 experimental units.

Prior to the experiment, the soil was spiked with phenanthrene by first diluting with acetone as an organic solvent and then a portion of the soil (10%) was contaminated and mixed with the remaining soil (Northcott and Jones, 2000). Treatments without phenanthrene were also supplied with acetone in order to discard a negative effect of this organic solvent on the model plant used in this research. Then, acetone was left to evaporate for 48 hours. The phenanthrene concentrations were based on the growth parameters and tolerance of the model plant used in this study, *Triticum aestivum* L. (wheat), due to its ability to tolerate high phenanthrene concentrations compared to other plants, whether in germination or potting trials, which had been previously conducted. Wheat seeds were sterilized with NaClO for 15 min and washed with distilled water. Then, seeds were sown in sterile conditions in vermiculite. One week after germination, plants were transplanted into 250 cm^3 pots with 250 g of soil. *T. viride* was previously grown on sterile wheat seeds and then incorporated into the soil (5g per pot). In the same way, each pot was inoculated with 8g of *F. mosseae* inoculum, which was predetermined to have achieved high levels of root colonization. This assay was performed in a greenhouse with supplementary light through Sylvania incandescent and cool white lamps (400 $E/m^2/s$, 400–700 nm) with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity. Each pot was watered with 25 mL of distilled water every 48 hours. Plants were watered from below and fed every two weeks

with 10 mL of a nutrient solution plus 50 mg L^{-1} of P (Hewitt, 1966). The nutrient solution used was composed as follows (L^{-1} of solution): NO_3K (3.03 mg), $(NO_3)_2Ca$ $4H_2O$ (28.32 mg), SO_4Mg $7H_2O$ (7.36 mg), EDTA Fe (II) (0.025 mg), SO_4Mn $4H_2O$ (0.22µg), SO_4Cu $5H_2O$ (0.024µg), SO_4Zn $2H_2O$ (0.029 µg), BO_3H_3 (0.186 µg), Na_2MoO_4 $2H_2O$ (0.003µg), PO_4H_2Na $2H_2O$ (2.08 mg).

After eight weeks plants were harvested and the dry biomass of shoots and roots were determined. The shoots and roots were separated and stored at -80 °C to determine antioxidant enzyme activity and the phenanthrene content in the plant tissues.

4.3.4 Mycorrhizal colonization parameters

After the harvest, samples of fresh roots were taken at random, cleared in KOH and stained with Trypan blue in lactic acid (Phillips and Hayman, 1970). The percentage of mycorrhizal root colonization was evaluated microscopically using 30 root fragments of 1cm on each slide (3 per treatment). Mycorrhizal frequency (F%), mycorrhizal intensity (M%) and arbuscules abundance (A%) were performed using the Mycocalc software(https://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html). Number of vesicles was counted for each root placed on the respective slides.

4.3.5 Phenanthrene in soil

According to the method used by Acevedo et al. (2011), phenanthrene in soil was extracted by weighing 10 g from each treatment placed in 50 mL tubes, adding 10 mL toluene and 10 mL sodium pyrophosphate (0.05M) to each tube and then shaking for 16 hours. Toluene extract was evaporated to dryness under N_2. Finally, the residue was re-suspended in acetonitrile and analyzed by high performance liquid chromatography (HPLC) Shimadzu

Prominence (Kyoto, Japan). A SUPELCOSIL™LC-18 HPLC column was used to determine phenanthrene concentration by preparing different concentrations of phenanthrene standard (99.2% Purity) at 0, 0.5, 1, 5, 25, 50, 100, 250, 500 and 1000 ppm, which were dissolved in acetonitrile. Then, a calibration curve was performed for establishing phenanthrene detection limits. As complementary information, PAHs were not detected in the soil studied. In addition, a phenanthrene recovery percentage in non-sterilized soil spiked with phenanthrene (500 and 1000 mg kg^{-1} soil) without wheat plants was measured at the beginning and at the end of the experiment to understand the impact of native microorganisms and physicochemical processes of phenanthrene dissipation on soil. These percentages were 94.3% and 96.7% for 500 and 1000 mg kg^{-1} respectively at the beginning of the experiment and 89.5% and 87.9 % at the end of the experiment (data previously obtained).

Phenanthrene dissipation percentages in soil were calculated by dividing the residual phenanthrene concentration by initial phenanthrene concentration in soil. Then, in order to explain this dissipation, phenanthrene accumulation in plant tissues (shoot and root) and undetectable phenanthrene were calculated and expressed in percentages. The percentages of roots and shoots on phenanthrene accumulation was calculated by the concentration obtained from HPLC (transformed into mg) and multiplied by the fresh weight of the respective samples. An undetectable percentage of phenanthrene was calculated by the difference of initial concentration of both phenanthrene doses (100%) and the sum of percentages of root and shoot accumulation and residual phenanthrene in the soil. Therefore, undetectable phenanthrene was considered to be the quantity of phenanthrene dissipated in soil by means other than accumulation in plant tissues.

4.3.6 Phenanthrene determination in plant tissues and translocation factor (TF)

Determination of phenanthrene in plant tissues was performed according to the method described by Zitka et al. (2012). Plant tissue samples (0.1 g) of separated roots and shoots were homogenized in a mortar using a 2 mL mixture of benzene and ethanol in a 3:1 (v: v) ratio. Then this mixture of solvents was evaporated, and 1mL of the solvent mixture was transferred to 1.5 mL Eppendorf tubes and shaken for 30 min at 100 rpm. Next, samples were sonicated for 30 min at 4 ℃ and 40 W, and the tubes were centrifuged at 15,000 g for 30 min. Finally, the supernatant was completely evaporated using a vacuum rotary evaporator and dissolved in 1000 μL of acetonitrile. The samples were stored at -20 ℃ in microtubes for analysis. The determination was made using a HPLC as described before. The translocation factor was determined by dividing the phenanthrene concentration in shoots by the phenanthrene concentration in roots using the data obtained from the determination of phenanthrene in plant tissues.

4.3.7 Soil enzymatic activities

Dehydrogenase activity (DHA) was determined according to the method described by Casida et al. (1964), and fluorescein diacetate hydrolysis (FDA) was determined according to the method described by Adam and Duncan (2001) for measuring total soil biological activity. Absorbance was measured by spectrophotometry at 490 nm. Ligninolytic enzymes were extracted according to Elgueta et al. (2016). Laccase (EC 1.10.3.2) was determined as described by Castillo and Torstensson (2007), whereas Manganese peroxidase (MnP, EC 1.11.1.13) was determined according to a method developed by Moreira et al. (1997).

4.3.8 Organic acid extraction and determination

Organic acid derived from root exudates were extracted according to Rosas et al. (2007). Roots were carefully washed with deionized water and then placed in 50 mL flasks with 25 mL of deionized water for 1 hour in order to collect root exudates. After this, the collected samples were sonicated for 30 min, and filtered through 0.2 µm. Collected root exudates samples were freeze-dried and suspended in ultrapure water for organic acid determination by HPLC.

4.3.9 Antioxidant activities

The plant samples were macerated with liquid nitrogen using a ceramic mortar. The 0.1 g plant tissue was weighed and 2 mL of 0.1 M potassium phosphate buffer (pH 7.0) was added. The macerated tissues were transferred to 1.5 mL Eppendorf tubes and centrifuged at 17,000 g for 15 min at 4°C. The supernatant was used to measure the enzymatic activities of superoxide dismutase (SOD) and guaiacol peroxidase (GPX).

Total SOD activity (EC 1.15.1.1) was determined by measuring the inhibition of photochemical reduction of nitrobluetetrazolium (NBT) based on the method described by Donahue et al. (1997). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the NBT photochemical reduction rate expressed as U mg^{-1} of dry mass.GPX activity (EC 1.11.1.11) was determined according to Pinhero et al. (2007) following the change in absorbance at 470 nm as a consequence of guaiacol oxidation. A molar extinction coefficient (26.6 nM^{-1} cm^{-1}) was used to calculate the enzymatic activity.

4.3.10 Lipid peroxidation

Lipid peroxidation was determined as described by Health and Packer (1991) and modified by Du and Bramlage (1992) based on the doses of malondialdehyde (MDA), which are determined by the reaction given by thiobarbituric acid (TBA). A molar extinction coefficient (155 mM^{-1} cm^{-1}) was used to calculate the MDA concentration.

4.3.11 Statistical analysis

The data were analyzed using factorial design analysis of variance with *F. mosseae*, *T. viride* and phenanthrene as the main factors. Statistical procedures were carried out with the software SPSS v.11.0 (SPSS Inc., 1989–2001). Statistical significance was determined at $p< 0.05$. All variables were tested for normality and variance homogeneity (Kolmogorov-Smirnov test and Cochran's C test, respectively), and data were log transformed when needed. Percentage values were arcsine transformed for their analyses. Student

4.4 RESULTS

4.4.1 Dry biomass

Figure 4.1 shows that the shoot and root biomass of wheat plants decreased at the different doses of phenanthrene tested, mainly at 1000 mg kg^{-1}. The shoot and root dry weights of wheat plants were increased by *T. viride*, *F. mosseae* and their interaction. Additionally, at all phenanthrene concentrations (500 and 1000 mg kg^{-1}) *F. mosseae* and its interaction with *T. viride* resulted in a higher biomass than in non-inoculated plants (control) or plants inoculated only with *T. viride* or *F. mosseae*.

4.4.2 Arbuscular mycorrhizal colonization parameters

The phenanthrene doses significantly reduced the parameters of AM colonization in roots (frequency, intensity and arbuscule abundance) (Table 4.2). However, *T. viride* significantly increased the AM fungus root colonization parameters, reaching the highest colonization level in combination with phenanthrene. Vesicles were increased by both phenanthrene doses and reached the highest number at 1000 mg kg^{-1} of phenanthrene combined with *T. viride* or not (Table 4.4)

4.4.3 Phenanthrene content

The residual phenanthrene content in the soil was significantly decreased by the inoculation with *T. viride* and *F. mosseae* (Table 4.3a). Particularly, when these were inoculated in combination, resulting in the lowest residual phenanthrene in soil in both treatments (500 and 1000 mg kg^{-1}) (Table 4.5).

For both phenanthrene doses applied the subsequent content of phenanthrene in shoots was significantly reduced by *F. mosseae,* whereas the content in roots was significantly increased also in combination with *T. viride* (Table 4.5).

In shoots, the highest phenanthrene content was observed in plants without fungal inoculation (control) mainly at 1000 mg kg^{-1}, whereas the lowest phenanthrene content in shoots was observed after dual inoculation with *F. mosseae* and *T. viride.* By contrast, the highest phenanthrene content in roots was obtained with dual fungal inoculation (Table 4.5).These results were also reaffirmed by the reduced translocation factor (TF) of phenanthrene after inoculation with *F. mosseae*. The lowest TF was observed after dual fungal inoculation (Table 4.5).

4.4.4 Phenanthrene dissipation

Phenanthere dissipation was significantly increased by dual inoculation (Table 4.3b). The highest phenanthrene dissipation in soil was reached by the dual inoculation for the 500 and 1000 mg kg^{-1} doses (74.5% and 66% respectively) (Table 4.6). Accumulation in roots or shoots only accounts for a small portion of phenanthrene dissipation, whereas undetectable phenanthrene accounted for the highest amount in treatments with dual fungal inoculation for the 500 and 1000 mg kg^{-1} doses (68.3% and 61.6%, respectively).

4.4.5 Soil enzymatic activities

The highest DHA and FDA activities for each phenanthrene level were observed after dual inoculation with *F. mosseae* and *T. viride* (Figure 4.2a and 2b). In general, DHA and FDA increased in all treatments with 500 mg kg^{-1} of phenanthrene, but decreased at 1000 mg kg^{1}, mainly in treatments without *F. mosseae* inoculation. However, both single and dual fungal inoculation enhanced these activities in combination with 1000 mg kg^{-1} phenanthrene compared with the non-inoculated control treatment (Figure 4.2a and 4.2b). Enzymes related to PAHs degradation such as Laccase and Manganese peroxidase **(**MnP) also reached their highest levels by this interaction. In the case of MnP, the highest level was obtained at 1000 mg kg^{-1} of phenanthrene, whereas Laccase reached its highest activity by dual inoculation without phenanthrene and decreased under increasing phenanthrene doses (Figure 4.2c and 4.2d)

4.4.6 Organic acids

The main organic acids determined in root exudates were citric and malic acid. The interaction of the three factors significantly increased malic and organic acid exudation

(Table 4.1). The highest malic and citric acid exudation was observed by the interaction of *T. viride*, *F. mosseae* and phenanthrene at 1000 mg kg $^{-1}$ (Figure 4.3a and 4.3 b).

4.4.7 Antioxidant enzymes and lipid peroxidation

Antioxidant activities in wheat plants increased along the phenanthrene doses applied as well as by *F. mosseae* inoculation (Figure 4.4a and 4.4b). SOD and GPX activities increased significantly with dual fungal inoculation in combination with phenanthrene, although the main factor in this response was ascribed to phenanthrene (Table 4.1). In the case of the significant increase in GPX activity, *F. mosseae* was the main factor involved in this response, followed by phenanthrene and *T. viride*. In terms of the overall antioxidant response, the results for SOD and GPX show that the highest activities were produced by dual fungal inoculation at 1000 mg kg^{-1} of phenanthrene applied to the soil (Figure 4.4a and 4.4b). Lipid peroxidation in wheat plants in terms of MDA production increased significantly after phenanthrene application, whereas single inoculation of *F. mosseae* and *T. viride* significantly reduced lipid peroxidation. MDA production was highest in non-inoculated plants at 1000 mg kg^{-1} of phenanthrene in soil, whereas dual fungal inoculation resulted in the lowest MDA production at this phenanthrene concentration (Figure 4.4c).

4.5 DISCUSSION

The adverse effects of PAHs on plants have been widely reported in the literature (Jajoo et al., 2014; Weisman et al., 2010). Results from the present study show that plant stress response to the PAH phenanthrene were alleviated by single and dual inoculation of the root-associated fungi *F. mosseae* and *T. viride*.

Plant growth promotion in wheat was observed after inoculation with *F. mosseae* even in phenanthrene-spiked soil. In general AMF are known for improving plant growth by increasing P acquisition and protecting their host plant against biotic and abiotic stress (Smith and Read, 2008). On the other hand, the observed plant growth promotion by *T. viride* seems to be related to phytohormone production (Contreras-Cornejo et al., 2009; Korolev et al., 2008), and plant stress alleviation may be related to the enzymatic capacity of *T. viride* to degrade phenanthrene (Zafra et al., 2015).

Plants inoculated with *F. mosseae* presented the highest colonization parameter when co-inoculated with *T. viride*, which also resulted in improved plant growth. To the best of our knowledge, this is the first report on plant stress alleviation with AMF and SF in PAH-polluted soils, which should be further explored when developing phytoremediation strategies to clean up soils contaminated with PAHs.

The decrease in AM colonization parameters by PAHs has been previously reported (Nwoko, 2014). The negative impacts of PAHs on AM fungus root colonization have been linked with the inhibition of spore germination and hyphal growth in soil (Aranda et al., 2013; Franco-Ramírez et al., 2007). Lipid peroxidation of the extraradical mycelium of the AM fungus *Rhizophagus irregularis* from PAH contamination coincided with reduced AM fungus root colonization (Calonne et al., 2014). In addition, Gaspar et al. (2002) reported that phenanthrene reduced the hyphal metabolic activity of the AM fungus *Glomus geosporum* in symbiosis with maize, which negatively affected formation of the mycorrhizal association. On the other hand, inoculation with *T. viride* enhanced AM fungus root colonization at both phenanthrene doses. SF have been found to promote or stimulate the spore germination of AM fungi (Fracchia et al. 2000). Indeed, Fracchia et al. (1998)

suggested that the volatile compounds of *Trichoderma harzianum* promote the formation of auxiliary AM fungal cells. Similar mode of interaction may occur between *T. viride* and *F. mosseae* in our study.

In order to examine the physiological response of *F. mosseae* to phenanthrene, we counted vesicles, which may be produced by AM fungi as a stress response. Indeed, the highest number of vesicles in roots was obtained at 1000 mg kg^{-1} of phenanthrene in combination with *T. viride*. However, the abundance of arbuscules decreased at this phenanthrene dose. Similarly, Cabello (1998) found that hydrocarbon-contaminated soil (crude oil containing phenanthrene at 857.46 mg kg^{-1} and other PAHs) respectively decreased and increased the abundance of arbuscules and vesicles in roots, which was suggested to be a stress response of AMF to contamination by hydrocarbons. Indeed, when exposed to biotic or abiotic stress conditions, AMF can reallocate the carbon resources obtained from their host plant, reducing arbuscule formation and increasing intraradical hyphae and vesicles (Hawkes et al., 2008; Johnson et al., 1997).

Phenanthrene dissipation from the soil was at least twofold higher in plants inoculated with *F. mosseae* than in non-inoculated plants, whereas a significant enhancement on the dissipation was observed with the interaction of *F. mosseae* and *T. viride* (maximum phenanthrene removal at both doses). Also, improved PAHs dissipation from soil by AMF has been shown in other studies (Gao et al., 2011; Nwoko, 2014). The improvement in microbial activity has been proposed as the main mechanism through which AMF increase PAHs dissipation in the soil due to an enhancement in enzymatic activities brought about by microbial exudation (Gao et al., 2011). Enzymes such as peroxidases and polyphenol oxidase have been well documented as being enriched by AMF in PAH-polluted soil and

contributing to the biodegradation of these pollutants (Lu and Lu, 2015; Yu et al., 2011). In relation to *T. viride*, PAHs dissipation by the genus *Trichoderma* in soil has also been reported for degrading PAHs in soil and ascribed to enzymes such as laccase, catechol 1, 2 dioxygenase and peroxidase produced by *Trichoderma asperellum* (Zafra et al. 2015). Hence, enhanced phenanthrene removal from soil by combined inoculation with *F. mosseae* and *T. viride* could be linked to the combined effect on phenanthrene degradation by enzymatic activities that both fungi stimulate in PAH-contaminated soil.

Another mechanism that has been suggested in PAHs dissipation from the soil is the accumulation in plant tissues (Wu et al., 2011). The phenanthrene content in shoots was higher in non-inoculated plants, whereas plants with dual inoculation exhibited lower phenanthrene content. Conversely, phenanthrene accumulation was higher in roots with the dual inoculation. Additionally, the TF showed that dual inoculation reduced this parameter significantly, thus reflecting the higher accumulation in roots and lower translocation into the shoots, mainly at 1000 mg kg^{-1}.This response was produced mainly by inoculation with *F. mosseae*. It has been reported that PAHs are mainly accumulated in the roots of plants colonized by AM fungi, which restrict PAHs translocation into the shoot (Gao et al., 2010a; Gao et al., 2011). A relation between a higher root biomass and accumulation can be established according to our results given that the treatment that produced the highest root biomass under phenanthrene exposure was the dual fungal inoculation, but mainly provided by *F. mosseae*. PAHs accumulation in roots depends on the lipid content, including protein, fats, nucleic acids, cellulose tissues and other components all containing lipophilic components (Gao and Zhu, 2004). PAHs are lipophilic compounds (Sverdrup et al., 2002) that accumulate in lipids. In this sense, AM fungal structures like vesicles and mycelia have

been described as lipid structures (Bago et al., 2002; Jabaji-Hare et al., 1984). In particular, the number of vesicles in our study was higher under phenanthrene exposure, which coincided with phenanthrene accumulation in roots, thereby suggesting that phenanthrene may be accumulated in vesicles. Accumulation of PAHs from soil has also been reported for AM fungal spores, which are rich in lipids (Verdin et al., 2006).

The enhancement by the dual inoculation on soil enzymes under phenanthrene exposure reflects the positive effect of dual inoculation both by the phenanthrene degradation processes and microbial activity. Laccase and MnP have been reported as a part of the main enzymes responsible for phenanthrene degradation (Acevedo et al., 2011; Wang et al., 2009). In our results, Laccase and MnP enzymes were stimulated mainly by *T. viride*, which catalyze the oxidation of various hydrocarbons into intermediate products like phenanthrene (Gerhardt et al., 2009).

Increased soil biological activities in terms of DHA and FDA in phenanthrene-spiked soil in combination with dual fungal inoculation coincided with the highest phenanthrene dissipation, which may be due to a corresponding higher microbial biomass, but this needs to be further examined in future studies**.** However, FDA activity has been used as an indicator of microbial activity to evaluate the sensitivity of microorganisms in a broad range of stress conditions in soil (Smith and Paul, 1990). Similarly, DHA activity has been suggested as an important parameter involved in organic matter oxidation as a result of biological degradation by soil microorganisms (Zhang et al., 2010) as well as an indicator of their activity and biomass (Kumar et al., 2013). Hence, a higher DHA in soil with both fungi could be related to a higher microbial biomass in the soil, stimulating the phenanthrene dissipation from soil spiked by biodegradation. In our results, the root and

shoot dry weight and their improved fitness as a result of dual fungal inoculation could be increasing root exudates into the rhizosphere, thereby stimulating microbial activity, which in turn helps to dissipate PAHs from soil. Indeed, increased DHA has previously been linked to PAHs dissipation in soil grown with *Triticum aestivum*, *Solanum melongena* and *Vigna radiate* associated with *F. mosseae* (Rabie, 2005).

Organic acid release from roots is often related to biotic or abiotic stresses. Malic and citric acid have been described as the main root exudates from plants under stress (Ma and Furuwaka, 2003; Patel et al., 2015). In our results, these organic acids decreased in non-inoculated plants exposed to phenanthrene, which is attributed to the drastic negative impacts of phenanthrene on plant growth observed in our study. Thus, we suggest that these plants were not able to produce higher root exudates due to poor biomass. Conversely, when plants presented single or dual inoculation under phenanthrene exposure, malic and citric acid production increased. It was more evident by fungal interaction, which was related to a better plant performance by reaching the highest biomass (shoots and roots). Moreover, by increasing plant growth, the carbon cost associated with root exudation into the rhizosphere should be lower than plants critically damaged by phenanthrene (without fungal inoculation). On this point, plants release up to 40% of their photosynthetically fixed carbon into the rhizosphere by root exudates (Bais et al., 2006). Thus, plants with a better performance can increase organic acid secretion compared with damaged plants. Furthermore, malic and citric acid mobilize inorganic P in soil, promoting plant growth (Wei et al., 2010). Therefore, under phenanthrene exposure, the increase of organic acid exudation can be considered a mechanism by AM fungi and *T. viride* to enhance plant nutrition to cope with adverse conditions. Enhancement in organic acid

exudation by AM fungi has been reported by Klugh-Stewart and Cumming (2009) under aluminum stress in *Andropogon virginicus*. Organic acids, as components of root exudates, act as a carbon source and energy for microorganisms, and also improve the hydrocarbon degradation in the rhizosphere by stimulating the population able to degrade PAHs (Gao et al., 2011). In addition, a higher organic acid exudation by plants promotes the bioavailability of PAHs in soils, increasing their desorption in soil depending on organic matter content. Thus, their degradation susceptibility by soil microorganisms increases (Ling et al., 2015).

Inconsistent results have been reported regarding the activities of antioxidant enzymes in plants exposed to PAHs. Song et al. (2011) observed that SOD and other antioxidant enzymes were increased by PAHs in *Kandelia candel* leaves and roots. By contrast, Wei et al. (2014) found that phenanthrene tended to decrease antioxidant activities. In our study, increasing phenanthrene doses increased the antioxidant response in leaves of wheat plants due to the ROS generated by this pollutant. Dual inoculation with *F. mosseae* and *T. viride* yielded the highest production of antioxidant enzymes as well as the lowest MDA production at the highest phenanthrene level in the soil. These results suggest that wheat plants dually inoculated with *F. mosseae* and *T. viride* benefit from improved growth and fitness to mitigate the negative effects of phenanthrene by increasing their production of antioxidant enzymes to alleviate stress. Moreover, reduced translocation of phenanthrene from the soil to the shoot in mycorrhizal wheat plants may allow a higher production of antioxidant enzymes against PAHs.

4.6 CONCLUSIONS

Our study shows that the association of wheat roots with the plant growth-promoting fungi *F. mosseae* and *T. viride* improved phenanthrene soil dissipation and alleviated oxidative stress in wheat. The enhancement in soil biological activities and enzymes associated with PAHs removal, organic acid exudation were related to the improved plant fitness under phenanthrene exposure when dual inoculation was used. Additionally, according to the undetectable phenanthrene results, the main mode of phenanthrene dissipation in soil could be attributed to its degradation.

ACKNOWLEDGEMENTS

Financial support of this study was provided by the Fondo Nacional de Desarrollo Científico y Tecnológico, FONDECYT 1130662; CONICYT Doctoral Fellowship 21120476 and the Universidad de La Frontera-MECE Program 2 FRO 1204 Ministerio de Educación, Chile. We would like to also thank Javier Ortiz, Javiera Soto and Hector Herrera for their technical assistance.

Table 4.1 Significance of the main treatment effects based on factorial ANOVA.

	F **- values**						
	Phe	T.v	F.m	T.v x F.m	Phe x T.v	Phe x F.m	Phe x T.v x F.m
Shoot dry weight	116.23**	105.25***	195.24***	9.18**	1.44 n.s	15.99**	5.07 *
Root dry weight	47.47***	13.28***	74.26***	8.61*	1.86 n.s	6.37*	6.62 *
Superoxide dismutase	112.64***	7.03*	50.81***	7.66*	2.78 n.s	23.12****	3.7*
Guaiacol peroxidase	75.22***	54.66***	271.4***	22.26***	3.18 n.s	8.59**	5.32*
Malondialdehyde	78.79***	6.14*	18.05***	6.82*	2.16 n.s	6.99 **	6.44*
Fuorescein diaceteate	26.1***	36.2****	40.4***	60.4*	9.8*	49 ***	22.1***
Dehydrogenase	33.6***	23.7***	39.7***	16.3**	6.9 *	20.9***	12.4 **
Laccase	105.3***	375.3 ***	39.9***	38.2 ***	21.1 **	31.8 ***	21.6 ***
Manganese peroxidase	24.9***	148.9***	5.7 *	75.3***	11.7 **	0.4 n.s	18.9***
Malic acid	41.6***	33.6***	69.3***	28.3***	37.9***	6.5*	40.77***
Citric acid	78.7***	44.4***	76.9***	27.8***	54.1***	12.5**	35.7***

Phe: Phenanthrene; T.v: *Trichoderma viride* ; F.m: *Funneliformis mosseae*. *p<0.05; **p<0.01; ***p<0.001

Table 4.2 Significance of the main treatment effects based on factorial ANOVA on arbuscular mycorrhizal colonization parameters.

	F - values		
	Phe	T.v	Phe x T.v
Frequency	65.2**	105.25***	195.24***
Intensity	47.47***	131.28***	174.26***
Arbuscule abundance	65.64***	97.03*	117.81***
Vesicles	197.22***	54.66***	71.4***

Phe: Phenanthrene; T.v: *Trichoderma viride* . *p<0.05; **p<0.01; ***p<0.001

Table 4.3 Significance of the main treatment effects based on factorial ANOVA on phenanthrene quantity in soil and vegetal tissues (A) and phenanthrene dissipation (B).

A

	F-values		
	T.v	F.m	T.v x F.m
Phe Residual in soil	12.5**	181.***	22.5***
Phe Content in shoot	42.5***	137.8***	31.4***
Phe Content in roots	17.2 **	85.1 ***	22.1***
Translocation factor	17.9**	95.3***	44.5***

B

	F-values		
	T.v	F.m	T.v x F.m
Phe Soil	13.77**	104.7***	24.88***
Phe Shoots	32.7***	107.8***	37.8 ***
Phe Roots	21.2 **	73.1 ***	19.9***
ND	18.9**	85.9***	35.5***

Phe: Phenanthrene; T.v: *Trichoderma viride* ; F.m: *Funneliformis mosseae*. *p<0.05; **p<0.01; ***p<0.001

Table 4.4 Frequency (F%) and intensity (M%) of arbuscular mycorrhizal colonization, abundance of arbuscules (A%) and vesicles in root system of *T. aestivum* grown in soil spiked with phenanthrene (0, 500 and 1000 mg Kg $^{-1}$).

Treatments	F%			I%			A%			Vesicles per 100 cm root		
	Phenanthrene mg Kg $^{-1}$			Phenanthrene mg Kg $^{-1}$			Phenanthrene mg Kg $^{-1}$			Phenanthrene mg Kg $^{-1}$		
	0	500	1000	0	500	1000	0	500	1000	0	500	1000
F. mosseae	73.1 Ac	51.3 Ab	39.8 Aa	46.1 Ab	36.1 Aab	28.5 Aa	53.3 Ac	36.8 Ab	27.0 Aa	18.0 Aa	52.0 Ab	165.2 Ac
F. mosseae + *T. viride*	80.3 Ac	60.5 Bb	49.4 Ba	51.2 Ac	42.8 Bb	34.9 Ba	56.5 Ac	44.1 Ab	32.6 Ba	28.4 Ba	69.2 Bb	178.6 Ac

Different capital letters within the same column indicate significant (p>0.05) differences between different inoculation treatments, whereas different small letters within the same row indicate significant (p>0.05) differences between different phenanthrene doses.

Table 4.5 Residual phenanthrene in soil and phenanthrene content in plant tissues.

Treatments	**Phe residual in soil (ppm)**		**Phe content in shoots (ppm)**		**Phe content in roots (ppm)**		**Translocation Factor**	
	Phenanthrene mg Kg $^{-1}$		**Phenanthrene mg Kg $^{-1}$**		**Phenanthrene mg Kg $^{-1}$**		**Phenanthrene mg Kg $^{-1}$**	
	500	**1000**	**500**	**1000**	**500**	**1000**	**500**	**1000**
Control	422.8 Da	753.1 Cb	11.1 Ba	19.1Bb	18.8 ABa	25.2 Ab	0.81 Ca	0.76 Da
Trichoderma viride	351.7 Ca	691.3 Cb	10.4 Ba	17.4 Bb	12.8 Aa	28.2 Ab	0.49 Ba	0.61 Cb
Funneliformis mosseae	211.8 Ba	426.6 Bb	5.0 Aa	9.3 Bb	23.1 Ba	34.0 Bb	0.21 Aa	0.27 Bb
F. mosseae + *T. viride*	164.3 Aa	339.6 Ab	4.7 Aa	3.7 Aa	26.4 Ba	40.4 Cb	0.18 Ab	0.09 Aa

Different capital letters within the same column indicate significant (p>0.05) differences between different inoculation treatments, whereas different small letters within the same row indicate significant (p>0.05) differences between different phenanthrene doses.

Table 4.6 Phenanthrene dissipation from soil, dissipation by accumulation in plant tissues of *T. aestivum* (roots % and shoots %); and phenanthrene not detected (ND %).

Treatments	Soil (%)		Shoots (%)		Roots (%)		ND (%)	
	Phenanthrene mg Kg $^{-1}$		Phenanthrene mg Kg $^{-1}$		Phenanthrene mg Kg $^{-1}$		Phenanthrene mg Kg $^{-1}$	
	500	1000	500	1000	500	1000	500	1000
Control	15.4 Da	24.7 Ca	2.2 Ba	1.9 Ba	4.6 Bb	2.5 Aa	8.6 Aa	20.3 Ab
Trichoderma viride	29.7 Ca	30.3 Ca	2.1 Ba	1.7 Ba	2.6 Aa	2.8 Aa	25.0 Ba	25.8 Aa
Funneliformis mosseae	57.6 Ba	56.3 Ba	1.0 Aa	0.9 Aa	4.6 Bb	3.4 Aba	52.0 Ca	53.0 Ba
F. mosseae + *T. viride*	74.5 Aa	66.0 Ab	0.9 Ab	0.4 Aa	5.3 Ba	4.0 Ba	68.3 Da	61.6 Ba

Different capital letters within the same column indicate significant (p>0.05) differences between different inoculation treatments, whereas different small letters within the same row indicate significant (p>0.05) differences between different phenanthrene doses.

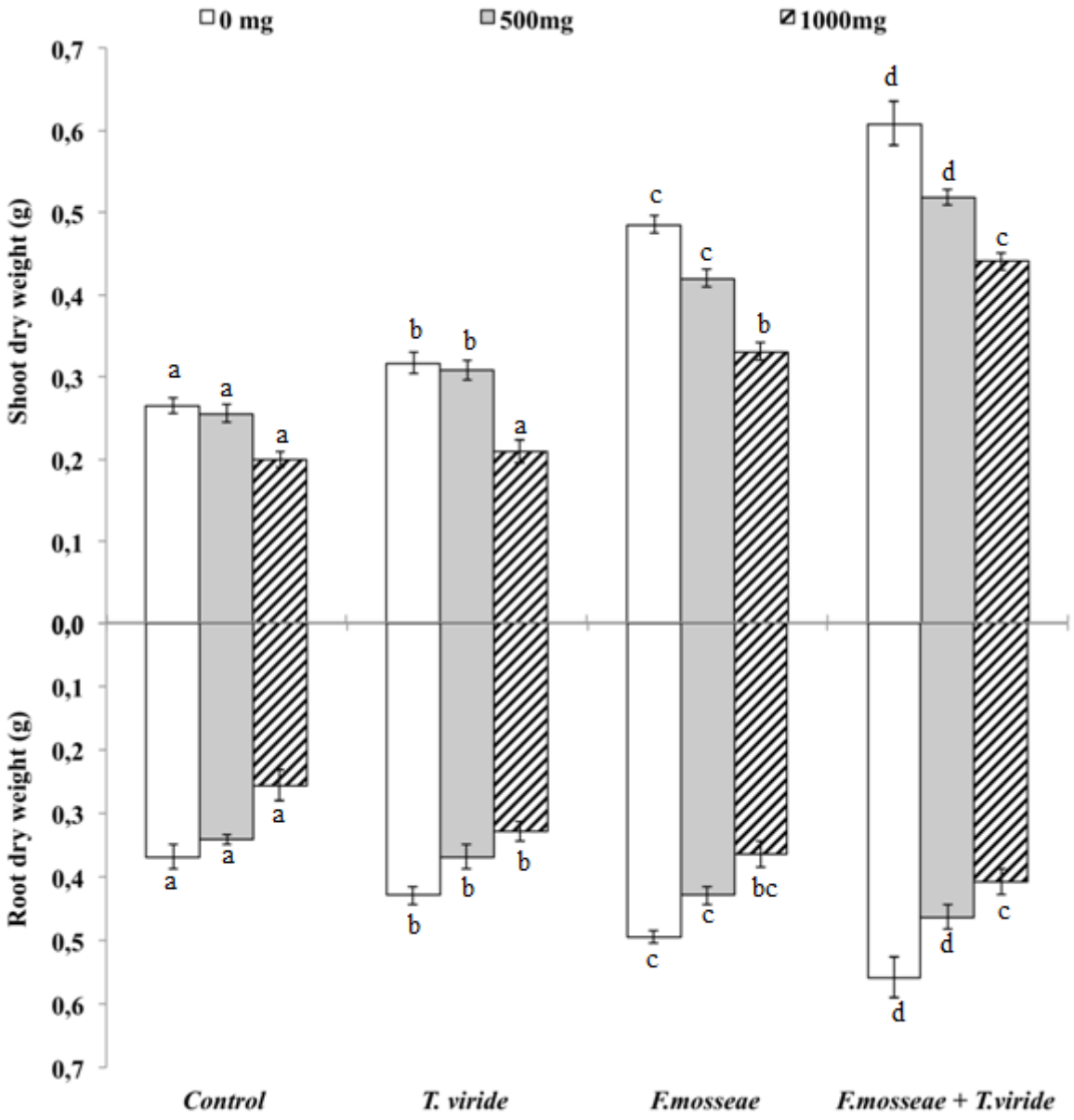
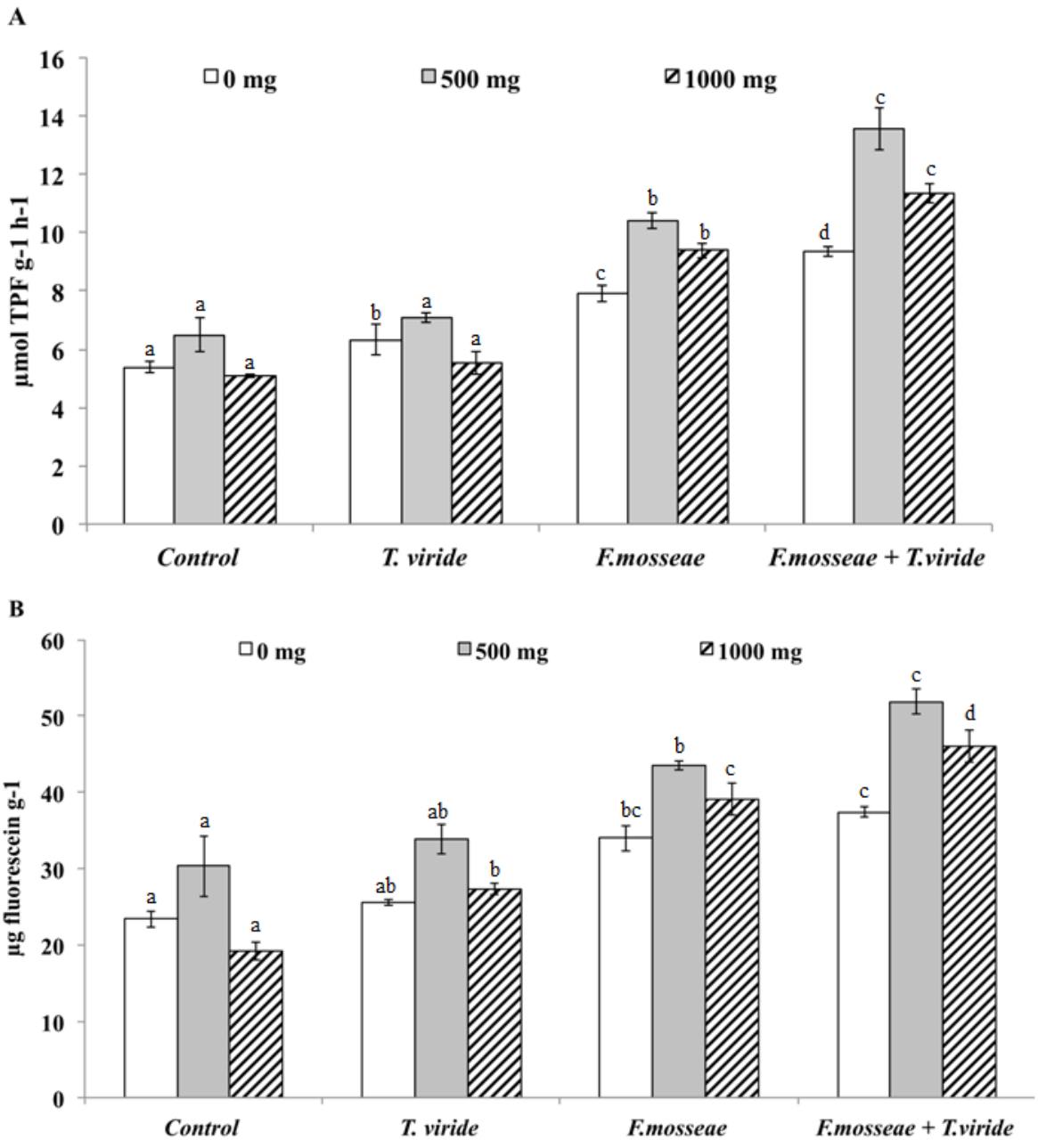


Figure 4.1 Shoot (A) and root (B) dry weight of wheat plants inoculated with *F. mosseae* and *T. viride* grown in soil spiked with phenanthrene(0, 500 and 1000 mg Kg^{-1}). The data are the means ± standard errors. Different letters indicate significant differences between different inoculation treatments and their respective doses (p>0.05).


A
0 mg
500 mg
1000 mg
µmol TPF g-1 h-1
16
14
12
10
8
6
4
2
0
a
a
a
b
a
a
c
b
b
d
c
c
Control
T. viride
F.mosseae
F.mosseae + T.viride
B
0 mg
500 mg
1000 mg
µg fluorescein g-1
60
50
40
30
20
10
0
a
a
a
ab
ab
b
bc
b
c
c
c
d
Control
T. viride
F.mosseae
F.mosseae + T.viride

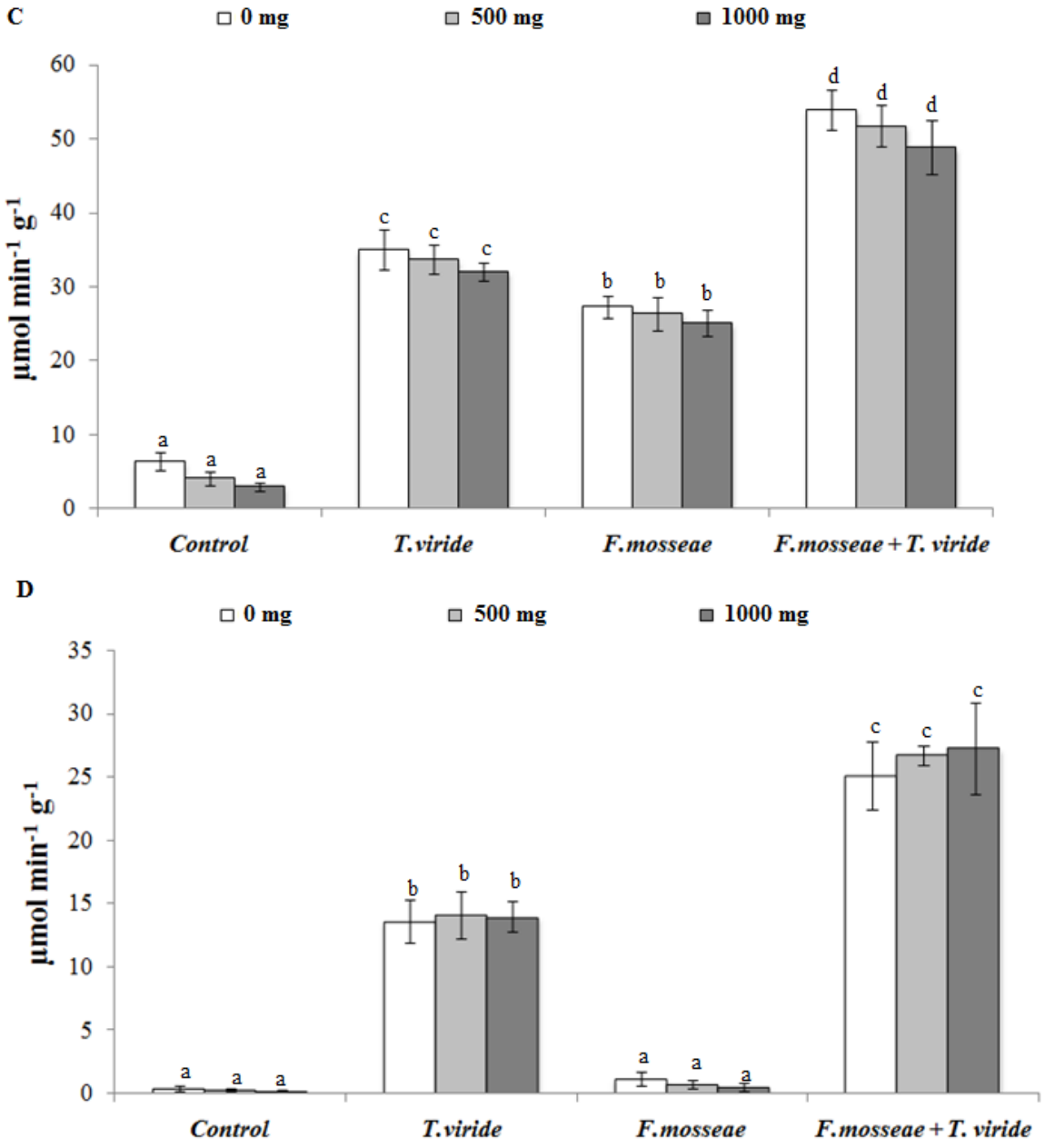


Figure 4.2. Biological activities in soil of wheat plants inoculated with *F. mosseae* and *T. viride* grown in soil spiked with phenanthrene (0, 500 and 1000 mg Kg^{-1}). A) Dehydrogenase activity (µmol TPF g^{-1} h^{-1}). B) Fluorescein diacetate activity (µg fluorescein g^{-1}). C) Laccase activity (µmol min^{-1} g^{-1}). D) Manganese peroxidase activity (µmol min^{-1} g^{-1}).The data are the means ± standard errors. Different letters indicate significant differences between different inoculation treatments and their respective doses (p>0.05).

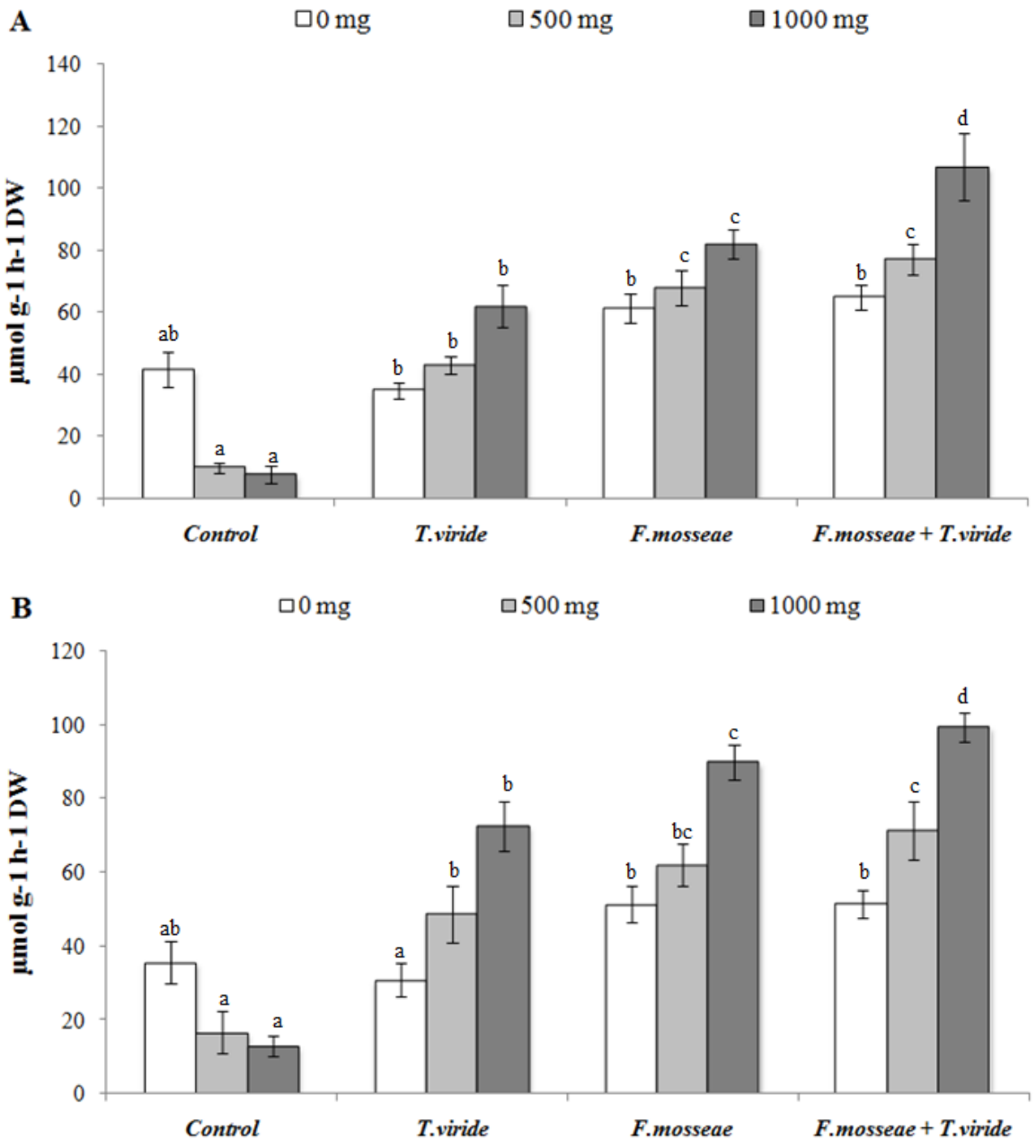
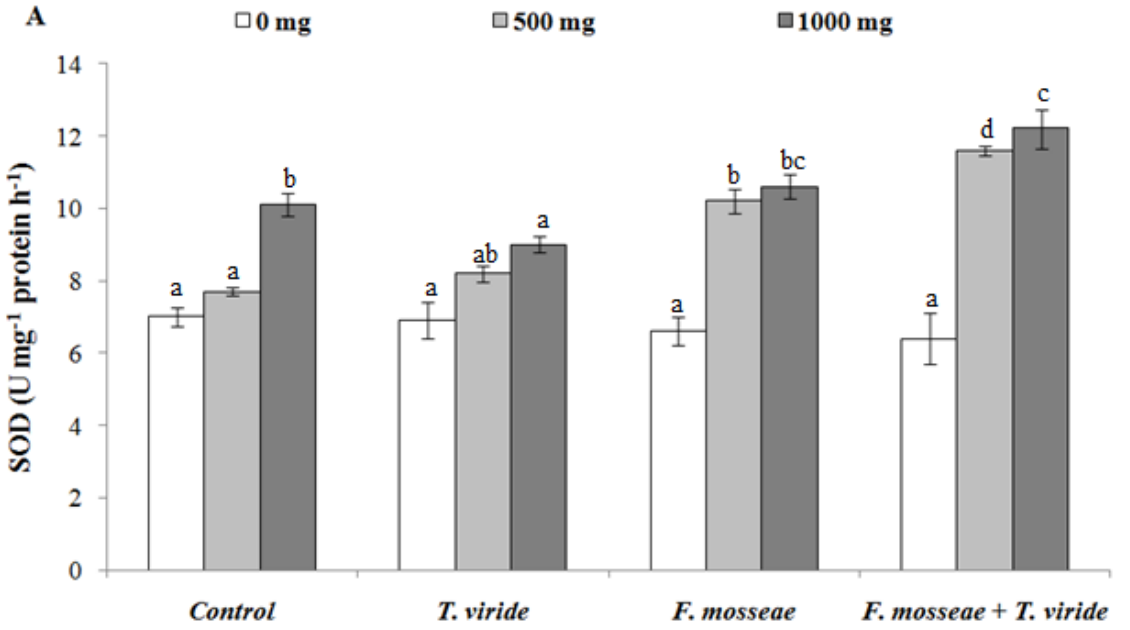
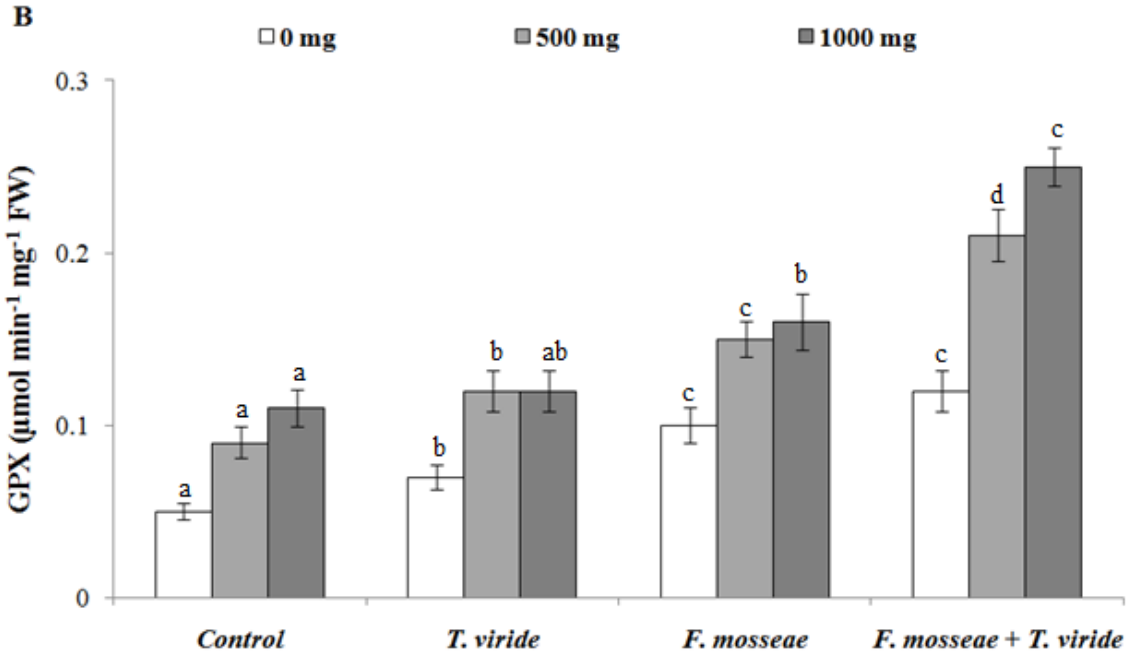


Figure 4.3 Organic acids collected from root exudates of wheat plants inoculated with *F. mosseae* and *T. viride* grown in soil spiked with phenanthrene (0, 500 and 1000 mg Kg^{-1}). A) Malic acid (µmol g^{-1} h^{-1} DW). B) Citric acid (µmol g^{-1} h^{-1} DW). The data are the means ± standard errors. Different letters indicate significant differences between different inoculation treatments and their respective doses (p>0.05).


A
0 mg
500 mg
1000 mg
SOD (U mg-1 protein h-1)
14
12
10
8
6
4
2
0
a
a
b
a
ab
a
a
b
bc
a
d
c
Control
T. viride
F. mosseae
F. mosseae + T. viride


B
0 mg
500 mg
1000 mg
GPX (µmol min-1 mg-1 FW)
0.3
0.2
0.1
0
a
a
a
b
b
ab
c
c
b
c
d
c
Control
T. viride
F. mosseae
F. mosseae + T. viride

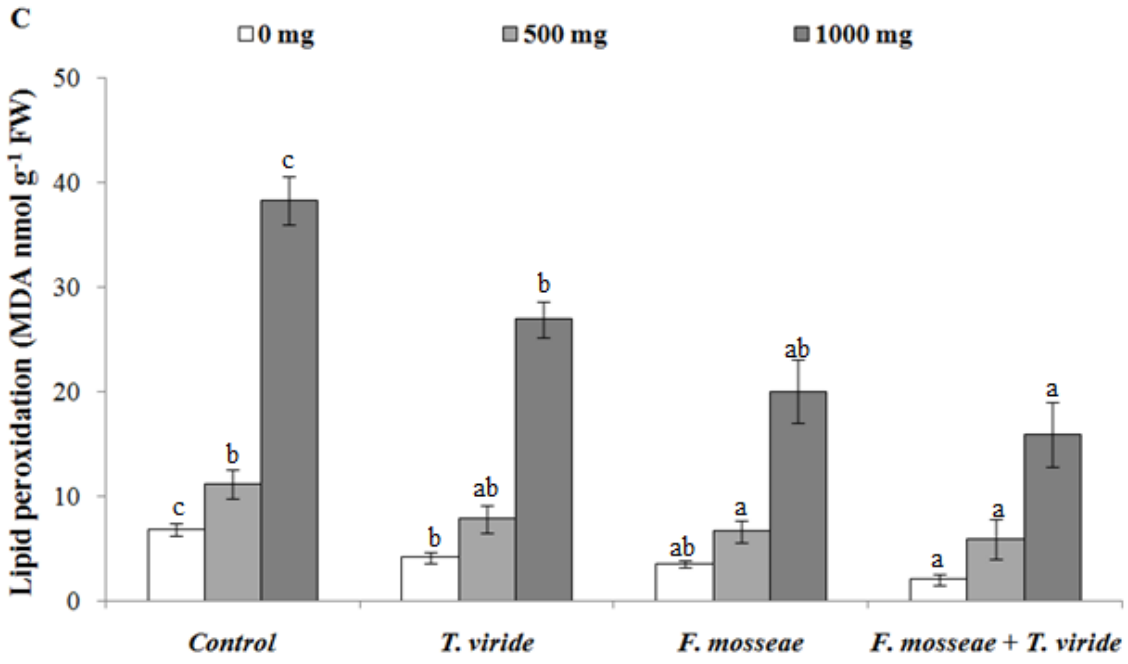


Figure 4.4 Antioxidant enzymes activities and lipid peroxidation in leaves of *T. aestivum* inoculated with *F. mosseae* and *T. viride* grown in soil spiked with phenanthrene (0, 500 and 1000 mg Kg $^{-1}$). A) Superoxide dismutase activity (U mg^{-1} protein h^{-1}). B) Guaiacol peroxidase activity (µmol min^{-1} mg^{-1} FW). C) Lipid peroxidation (MDA nmol g^{-1} FW). The data are the means ± standard errors. Different letters indicate significant differences between different inoculation treatments and their respective doses (p>0.05).

CHAPTER V

Dual inoculation with mycorrhizal and saprotrophic fungi suppress the maize growth and development under phenanthrene exposure

Journal of Soil Science and Plant Nutrition
doi: http://dx.doi.org/10.4067/S0718-95162018005002102

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5.1ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) belong to the group of persistent organic pollutants with detrimental impact on the environment and its living organisms. Soil acts as the main PAHs sink where strategies to remediate PAHs contaminated soil are needed. Inoculation with plant growth promoting and stress alleviating fungi such as arbuscular mycorrhizal fungi (AMF) and saprotrophic fungi (SF) could be an efficient bioremediation strategy for PAHs contaminated soils, but information about their compatibility is missing. In a greenhouse pot experiment we examined interactions between the AM fungus *Rhizophagus irregularis* and the saprotrophic fungus *Trichoderma viride* and their single and dual effects on maize plant growth performance and communities of rhizosphere microorganisms in a phenanthrene spiked soil. Phenanthrene reduced maize plant growth independent of both fungal inoculants, which were also both inhibited by phenanthrene. On the other hand strong mutual inhibition between *R. irregularis* and *T.viride* was observed, which mitigated their plant growth promoting effects independent of phenanthrene. In conclusion, the observed mutual inhibition between *R. irregularis* and *T. viride* underline the importance of testing for compatibility between microbial inoculants when these are used in combination.

Keywords: Polycyclic aromatic hydrocarbons; phenanthrene; maize; fungal interactions, rhizozphere

5.2 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are organic pollutants widely distributed in different ecosystems, highly persistent and toxic for living organisms because of their carcinogenic, mutagenic and teratogenic properties (Maliszewska-Kordybach, 1999).Soil is the main sink of PAHs hosting almost 90% of PAHs produced, which are deposited into the soil from the atmosphere, and once in the soil environment, PAHs are strongly sorbed depending on the content of organic matter in soil. Also, PAHs can be dissipated from the soil by photo-oxidation, bioaccumulation in plant tissues, leaching, biosorption and biodegradation by soil microorganisms (Vácha et al., 2010).

Plants play a key role in soil PAHs dissipation via their enhancement on PAHs degrading rhizosphere microorganisms leading to an increase in soil enzymatic activity such as polyphenol oxidase, dehydrogenase, urease, peroxidase and tyrotinase that are involved in the degradation of PAHs (Liu et al., 2015). Also bioaccumulation of PAHs in plant tissue contributes to their dissipation from soil. Nevertheless, successful phytoremediation depends on how the plants and their root associated microorganisms respond to the pollutant (Parrish et al., 2006).

Among these root associated microorganisms, arbuscular mycorrhizal fungi (AMF) form biotrophic symbiosis with roots, which is characterized by a reciprocal exchange of nutrients, where the fungus receive carbohydrates from the host and the AM fungus provide the host with mineral nutrients mainly P via the extraradical mycelium. Besides improving host nutrition AM fungi are also known to alleviate the plant host from biotic and abiotic stress (Smith and Read, 2008). Plant stress alleviation by AM fungi has been shown for

many soil contaminants, including heavy metals and persistent organic pollutants (Fuentes et al., 2016). AM fungi have been shown to increase soil PAHs dissipation both by promoting the soil microbial population leading to a PAHs biodegradation and via accumulation of PAHs in fungal tissue in roots (Gao et al., 2011).

On the other hand, SF are key drivers of soil organic matter decomposition due to their ample enzymatic machinery also allowing degradation of recalcitrant organic compounds including soil pollutants such as PAHs (Mineki et al., 2014). Several species such as *Trichoderma* are widely known as plant health and growth promoters mainly linked to their production of biological active secondary metabolites including phytohormones and antibiotics against plant pathogens (Contreras-Cornejo et al., 2016).

Combination of AMF and SF such as *Trichoderma* spp has been reported to increase plant growth and yield (Yadav et al., 2015). Regarding soil pollution combination of AMF and SF has been tested mainly for heavy metals and metalloids (Arriagada et al., 2009; Fuentes et al., 2016), while information on combined inoculation with AMF and SF to mitigate the adverse impacts of PAHs on plant growth and possible bioremediation is limited.

The objective of this study was to examine interactions between the AM fungus *Rhizophagus irregularis* and the saprotrophic fungus *Trichoderma viride* and their single and combined effects on maize plant growth and soil microbial communities in soil with and without the PAH phenanthrene. The main hypothesis was that dual inoculation with *R. irregularis* and *T. viride* would improve plant growth and mitigate plant stress from phenanthrene application to a higher extent than from single fungal inoculation.

5.3 MATERIAL AND METHODS

5.3.1 Experimental design

A completely randomized factorial greenhouse pot experiment was performed with the three factors: 1) AM fungus (with or without *Rhizophagus irregularis*); 2) Saprotrophic fungus (with or without *Trichoderma viride*) and 3) phenanthrene (0 and 250 mg kg^{-1}). Each of the eight treatments had five replicates resulting in a total of 40 experimental units.

5.3.2 Biological materials

Soil was obtained from the experimental field station of the National Agricultural University of Mexico, Campus Morelia, Michoacán, Mexico. Soil texture was clayish (53.2% clay, 27.3% silt and 19.5% sand) and with the following chemical characteristics: 2.7% organic matter, 23.2 mg kg^{-1} inorganic nitrogen, 5.8 mg kg^{-1} available phosphorus (Olsen P) and pH (H_2O) 7.3. Soil was mixed with quartz sand (1:1, w:w) and disinfected in an autoclave in two consecutive days. Mineral fertilization except P was applied to the soil so that P was the only nutrient limiting plant growth (mg kg^{-1} dry soil): K_2SO_4(370.3), $CaCl_2$ x $2H_2O$(75.0), $CuSO_4$ x $5H_2O$ (2.1), $ZnSO_4$ x $7H_2O$ (5.4), $MnSO_4$ x H_2O (10.5), $MgSO_4$ x $7H_2O$ (405.4), Na_2MoO_4 x $2H_2O$(0.18), NH_4NO_3(285.71).

The *Zea mays* L. hybrid DK2061 was used in the present study as a model plant due to its important as the main Mexican economic crop and for its ability to promote PAHs dissipation from soil (Guo et al., 2017). In each pot three seeds were sown, but after seedling emergence, one week after sowing, pots were unified so that only one seedling was left in each pot.

The AM fungus *Rhizophagus irregularis* (BEG87) and the saprotrophic fungus *Trichoderma viride* (BAFC8850) were obtained from the culture collection at the Agroecology Laboratory at the Universidad Nacional Autonoma de México, Campus Morelia, México. Inoculum of *R. irregularis* was obtained from a maize trap culture consisting of soil with root segments, mycelium and spores.

Inoculum of *T. viride* consisted of conidia obtained from two week old cultures with potato dextrose agar. Conidia were suspended in Milli-Q water and the counted by microscopy using a hemocytometer.

5.3.3 Experimental setup

The experiment was carried out using the bi-compartmented system, hyphal compartments (HCs) and root + hyphal compartments (RHC). Pots were filled with 1 kg of soil:sand mix and AM fungal inoculum (10%, w/w) was mixed into the soil in the treatments with *R. irregularis*. Then two empty tubes (200 ml) were inserted in the soil in each pot oppositely to the center, where the seeds were sown. Two weeks after seedling emergence plants were thinned to one seedling per pot. Three weeks after sowing, when the mycorrhizal association was supposed to be established, tubes were removed and replaced by mesh bags made from nylon mesh of the same shape. One mesh bag with 20 µm mesh diameter, allowing entrance of hyphae but not roots (HCs) and another mesh bag with 400 µm mesh diameter allowing entrance of roots and hyphae (RHC). Each set of mesh bags were filled with 100 g soil:sand substrate spiked with phenanthrene (250 mg kg^{-1} soil) in the corresponding phenanthrene treatments. phenanthrene dissolved in acetone (25 mg phenanthrene in 5 ml acetone) was thoroughly mixed in to the soil (100 g) and left to evaporate for 48 hours in a fume hood. The same amount of acetone was applied to the soil

in the treatments without phenanthrene. Hereafter inoculum of *T. viride* (1 x 10^6 conidian g^{-1} soil) was also mixed into the soil:sand substrate. Finally, the filled mesh bags were inserted into pots according to the experimental design.

5.3.4 Plant growth conditions

Plants were grown under greenhouse conditions with minimum and maximum temperature of 15°C and 30°C, respectively. Watering was made daily by weight to 70% of the water holding capacity throughout the experiment. Plants were harvested 10 weeks after sowing. Mesh bags were carefully removed from the pots and the soil within the mesh bags was collected and stored according to the respective measurements. Roots were gently washed free of growth substrate and separated from the shoot. Dry weights of shoots and roots were obtained after drying at 80°C for 48 hr. Prior drying of roots, they were cut into 5-to 10-mm segments and a representative two-gram root subsample was taken for measurement of AM colonization.

5.3.5 Population density of *T. viride*

From both mesh bags and the pot one gram of soil was individually applied to a 15 ml Falcon tube with 9 ml sterile Milli-Q water and Triton (0.01%) and hereafter serial dilutions were made up to 10^{-3} from which 0.1 ml was placed on *Trichoderma* selective medium according to Elad et al. (1981, with modifications). Plates were incubated in darkness at 27°C and observed on a daily basis. Population density of *T. viride* was calculated according to the amount of soil in the respective dilutions and expressed as colony forming units (CFU) g^{-1} dry soil.

5.3.6 Arbuscular mycorrhizal colonization

Two gram of fresh roots from all experimental units was stained with trypan blue according to Phillips and Hayman (1970) and AM colonization was measured by microscopy according to Giovanetti and Mosse (1980).

5.3.7 Biomarker fatty acids

Ten gram of soil samples were freeze dried and powderized in a mortar of which one-gram subsamples were used for extracting of whole cell fatty acids according to the method of Sasser (1990). To enable quantification of the extracted fatty acid methyl esters, a known amount of an internal standard, nonadecanoate fatty acid methyl ester 19:0 was added to each sample. Analyses of fatty acid methyl esters were performed using the software package Sherlock Version 6.0 (MIDI Inc.). Hydroxylic and cyclic fatty acids were used as biomarkers for Gram-negative bacteria, fatty acids with branched chains localized on positions iso and anteiso were used as Gram-positive bacteria biomarkers and methylated fatty acids were used a biomarker for Actinobacteria (Ratledge and Wilkinson,1988). The fatty acid 16:1ω5 and 18:2ω6,9 were used as biomarkers for AM (Olsson, 1999) and SF (Frostegård and Bååth, 1996), respectively. Fatty acid analysis was performed with the Agilent gas chromatograph 7890B.

5.3.8 Statistical analysis

Data were analyzed according to the factorial design with three way analyses of variance. Statistical significance was determined at $p<0.05$. Data sets were tested for normality and variance homogeneity with Kolmogorov Smirnov and Cochran's C tests respectively and log transformation was applied when necessary. The variable AM colonization was arcsine

transformed before statistical analysis. All statistical analyses were performed with the SPSS software, version 11.0 (SPSS Inc., 1989–2001).

5.4 RESULTS

5.4.1 Shoot and root dry weight

Significant "AM x saprotrophic fungus" interactions and single factor phenanthrene effects were obtained for both shoot and root dry weight (Table 5.1). Application of phenanthrene reduced shoot and root dry weight independent of fungal inoculation *F* values: 5.95 and 32.62 respectively). Highest shoot dry weight was found in plants inoculated with *R. irregularis*, which was however reduced after dual inoculation with *T. viride* (*F* value: 4.85), independent of phenanthrene application Individual inoculation with *R. irregularis* and *T. viride* caused improved root dry weight, which was mitigated by dual fungal inoculation (*F* value: 51.6), again independent of phenanthrene application.

5.4.2 Population density of *T. viride*

In the RHC a significant "AM x phenanthrene" interaction was obtained for the population density of *T. viride* (Table 5.2). Inoculation with *R. irregularis* reduced the population density of *T. viride*, which was further suppressed in combination with phenanthrene application, though phenanthrene alone had no effect on *T. viride* population density (Figure 5.1). In the HC significant single factor effects where observed for both factors AM fungi and phenanthrene, which also both reduced the population density of *T. viride* (Figure 5.1). In the NCS, where *T. viride* was not inoculated a significant reduction from phenanthrene application was found independent of inoculation with *R. irregularis* (Table 5.1, Figure 5.1).

5.4.3 AM colonization

A significant “SF x phenanthrene” was observed for AM colonization in roots from the RHC (Table 5.2). Single inoculation with *T. viride* and individual application of phenanthrene had no effect on AM colonization, whereas combination of phenanthrene and *T. viride* reduced AM colonization (Figure 5.2). In the NCS single factor effects where observed for both factors SF and phenanthrene, which both reduced the AM root colonization (Figure 5.2).

5.4.4 Biomarker fatty acids

In the RHC significant SF single factor effects were observed for Gram negative bacteria, Gram positive bacteria and actinobacteria, independent of the two other factors AM fungus and phenanthrene (Table 5.3),whereas in the HCs the abundance of bacteria was unaffected by all three factors examined (Table 5.3). The abundance of Gram positive bacteria, Gram negative bacteria and actinobacteria in the RHC was higher in the factor treatment mean with *T. viride* compared to the factor treatment mean without *T. viride* (Figure 5.3).

A significant single factor effect of phenanthrene was observed for the abundance of SF in the HCs (Table 5.3), where application of phenanthrene decreased the abundance of SF (Figure 5.3).

For the treatments with *R. irregularis* inoculation significant single factor effects with SF and phenanthrene were observed for the abundance of AM fungus in both RHC and HCs (Table 5.4). In both RHC and HCs inoculation with *T. viride* and application of phenanthrene reduced the abundance of AM fungus (Figure 5.4). The background values of

the AMF biomarker fatty acid 16:1ω5 in the RHC and the HCs were 1.07 and 0.73 mol g^{-1} dry soil, respectively.

5.5 DISCUSSION

Here we show that soil inoculation with the AM fungus *R. irregularis* and the saprotrophic fungus *T. viride* did not mitigate phenanthrene toxicity in maize as expected. On the contrary dual inoculation with *R. irregularis* and *T. viride* resulted in maize plant growth suppression independent of phenanthrene application. These results reject our main hypothesis that dual inoculation with *R. irregularis* and *T. viride* improve plant growth and mitigate plant stress from phenanthrene application to a higher extent than from single fungal inoculation.

The observed maize shoot and root growth suppression when grown in phenanthrene spiked soil is in accordance with other reports on this matter, which has been suggested to be linked with oxidative damage through reactive oxygen species (Salehi-Lisar and Deljoo, 2015). Soil inoculation with AM fungus and *Trichoderma* spp has been found to mitigate toxic effects of phenanthrene on plants linked to dissipation in terms of bioaccumulation and/or degradation (Wu et al., 2011). In the present study single or dual inoculation with *R. irregularis* and *T. viride* had no effect on the phenanthrene toxicity in maize, which may be due to differences in environmental conditions and fungal species employed in these studies. On the contrary phenanthrene application decreased both AMF root and soil colonization and *T. viride* population density, which maybe caused from a direct toxic effect. Another explanation is the possible reduction in root exudation after phenanthrene application as reported by Muratova et al. (2009) who found that 100 mg phenanthrene kg^{-1}

soil decreased root exudation of carbohydrates, amino acids and carboxylic acids in *Sorghum bicolor*. Phenanthrene had no effects on the abundance of soil bacteria, which is in contrast to the observed effects on AMF and *T. viride*. Phenanthrene also reduced the abundance of SF in terms of biomarker fatty acids though only significantly in the HCs. These results suggest a differential response of bacteria and fungi to phenanthrene, which however needs to be further addressed.

The observed maize growth promotion from inoculation with *R. irregularis* is a common mycorrhiza response in maize though depending on maize genotype and phosphorus fertilization (Sawers et al., 2017). In the present study dual inoculation with *R. irregularis* and *T. viride* resulted in plant growth suppression compared to single inoculation with *R. irregularis*. *Trichoderma spp*. are also known plant growth promoters, though in the present study *T. viride* only promoted root growth and not shoot growth. Improved plant growth promotion from dual inoculation with AMF and *Trichoderma* spp has been reported in other plants such as pepper (Bhuvaneswari et al., 2014) and cucumber (Chandanie et al., 2009), but to our best knowledge plant growth suppression from dual inoculation with AMF and *Trichoderma* spp. as observed in the present study has not been reported before. However, plant growth suppression by the interaction between AMF and other rizhosphere microorganisms such as bacteria was found by Larsen et al (2009) using *Cucumis sativa* plants inoculated with *R. irregularis* in combination with *Paenibacillus polymyxa* and *Paenibacillus macerans*. The reduction on plant growth parameters where associated to damaged roots and their abnormal growth.

The observed maize plant growth suppression after dual fungal inoculation coincided with mutual inhibition between *R. irregularis* and *T. viride* independent of phenanthrene

application. Inoculation with *T. viride* especially reduced the growth of *R. irregularis* in the soil, which may impaired the AM hyphal P uptake capacity of *R. irregularis* and consequently resulting in plant growth suppression since the plant benefits from hosting the fungal partner was lower than the costs in terms of photosynthates. The underlying mechanism involved in the inhibition of *R. irregularis* soil colonization by *T. viride* needs to be further addressed, but may be linked to competition for soil nutrients as also suggested by Green et al. (1999). Other possible modes of interactions include mycoparasitism and/or production of antagonistic secondary compounds or changes in soil microbial community. In the present study inoculation with *T. viride* increased the abundance of soil bacteria, which may have led to escape from competition between AM fungi and soil bacteria, but this needs to be further addressed. Several studies have demonstrated that bacteria are able to survive or recover after autoclaving (Nowak and Wronkowska, 1991; O'Sullivan et al., 2015). In other studies of AM fungi-*Trichoderma* interactions both inhibition and promotion of AM colonization from inoculation with different *Trichoderma* species has been observed (Contreras-Cornejo et al., 2016; Dehariya et al., 2015a, 2015b). Our results showed that *R. irregularis* reduced the population density of *T. viride* in the RHC, but had no effect in the HCs, indicating that the effects of *R. irregularis* maybe plant mediate perhaps from reduced root exudation, which is a known plant mycorrhiza response (Graham et al., 1981). However, this needs to be further examined. On the contrary mycelium of *R. irregularis* has been found to reduce the population density of *T. harzianum* in root-free soil (Green et al., 1999), showing that direct effects of *R. irregularis* on the population density of *T. harzianum* occurred. In the case of soil growth of *R. irregularis* similar inhibition from *T. viride* inoculation was observed independent of the absence or presence of roots, which suggest a direct effect of *T. viride*

on *R. irregularis*, which was also the case in the study of Green et al. (1999), who suggested competition for nutrients as the main mode of interaction.

In general, the outcome of AMF-*Trichoderma* interactions seems to be complex depending on fungal species and genotypes as well as the host plant and environmental conditions. When developing fungal consortia for different purposes such as pest biocontrol, biofertilization and/or bioremediation it is important to consider this complexity and ensure compatibility between the fungi in question. However, it is also important to consider that mutual inhibition between AMF and SF may also result in plant growth promotion as was shown when dually inoculating the AMF *R. irregularis* and the SF *Clonostachysrosea* associated with tomato grown in soil amended with organic matter (Ravnskov *et al*., 2006).

5. 6. CONCLUSION

In conclusion our results show that mutual inhibition between the plant growth promoting fungi *R. irregularis* and *T. viride* can result in plant growth suppression emphasizing the importance to test for fungal compatibility when AM fungi and *Trichoderma* spp. are dually inoculated in order to achieve suitable strategies for PAHs bioremediation.

ACKNOWLEDGEMENTS

Financial support of this study was provided by the Fondo Nacional de Desarrollo, Científico y Tecnológico, FONDECYT 1130662, and CONICYT Doctoral Fellowship 21120476.

Table 5.1 Significance of the main treatments on plant biomass based on factorial ANOVA.

	R.i	T.v	PHE	R.i x T.v	R.i x PHE	T.v x PHE	R.i x T.v x PHE
F-values							
Shoot dry weight	0.49 n.s	5.72*	5.95*	4.85*	0.57 n.s	0.28 n.s	0.2 n.s
Root dry weight	0.24 n.s	6.2*	32.62***	51.6***	3.31 n.s	3.35 n.s	0.96 n.s

R.i: *R. irregularis*; T.v: *T. viride*; PHE: phenanthrene. n.s: Not significant. *P<0.05; **P<0.01, ***P<0.001

Table 5.2 Significance of the main treatments on *Trichoderma* CFU and AM colonization based on factorial ANOVA.

Trichoderma CFU				AM colonization			
	R.i	PHE	R.i x PHE		T.v	PHE	T.v x PHE
F-values				*F-values*			
NCS	15.92***	9.42**	0.28 n.s	NCS	13.2**	50.61***	0.10 n.s
RHC	76.54***	26.04***	7.30*	RHC	2.47 n.s	43.11***	25.07***
HC	2.45 n.s	39.72***	3.61 n.s	HC	-	-	-

R.i: *R. irregularis*; PHE: phenanthrene; T.v: *T. viride*. NCS: Not compartmented soil; RHC: Root and hyphal compartment; HC: Hyphal compartment. n.s: Not significant. *P<0.05; **P<0.01, ***P<0.001

Table 5.3 Significance of the main treatments on whole cell fatty acids of different microorganisms in compartmented soil. A) Root and hyphal compartment. B) Hyphal compartment.

A

	R.i	T.v	PHE	R.i x T.v	R.i x PHE	T.v x PHE	R.i x T.v x PHE
F-values							
AMF	20.43***	5.85*	11.31**	6.16*	11.17**	2.61 n.s	3.27 n.s
SF	0.05 n.s	0.37 n.s	1.54 n.s	0.86 n.s	0.43 n.s	0.13 n.s	0.02 n.s
Gram negative	0.07 n.s	6.80*	0.02 n.s	1.13 n.s	0.12 n.s	0.39 n.s	3.22 n.s
Gram possitive	0.01 n.s	12.88**	1.23 n.s	1.17 n.s	0.05 n.s	0.68 n.s	3.89 n.s
Actinomycetes	0.05 n.s	11.08**	0.27 n.s	2.69 n.s	2.16 n.s	1.87 n.s	2.33 n.s

B

	R.i	T.v	PHE	R.i x T.v	R.i x PHE	T.v x PHE	R.i x T.v x PHE
F-values							
AMF	20.94***	2.93 n.s	5.08 *	3.20 n.s	5.28*	0.46 n.s	0.49 n.s
SF	0.71 n.s	0.55 n.s	5.26*	1.52 n.s	1.00 n.s	1.91 n.s	1.60 n.s
Gram negative	0.00 n.s	0.00 n.s	0.16 n.s	1.94 n.s	0.00 n.s	0.76 n.s	0.02 n.s
Gram possitive	0.54 n.s	0.27 n.s	0.02 n.s	2.49 n.s	0.62 n.s	2.65 n.s	0.99 n.s
Actinomycetes	0.36 n.s	0.52 n.s	0.16 n.s	0.22 n.s	0.14 n.s	0.25 n.s	0.05 n.s

R.i: *R. irregularis*; T.v: *T. viride*; PHE: phenanthrene. n.s: Not significant. *P<0.05; **P<0.01, ***P<0.001

Table 5.4 Microbial biomass by whole cell fatty acids analysis (nmol g^{-1} dry soil).

Compartments		Control	R. i	T. v	R.i + T.v	PHE	R.i + PHE	T.v + PHE	R.i + T.v + PHE
RHC	AMF	0.82 ± 0.19	26.36 ± 6.83	1.36 ± 0.05	8.87 ± 3.8	1.18 ± 0.23	5.08 ± 3.07	0.91 ± 0.14	1.97 ± 0.16
	SF	5.09 ± 1.11	9.9 ± 3.94	7.17 ± 0.44	5.81 ± 0.16	13.48 ± 6.46	14.28 ± 11.20	13.96 ± 8.5	6.11 ± 0.68
	Gram (-)	4.51 ± 1.41	4.82 ± 0.35	6.43 ± 0.23	6.03 ± 0.35	5.56 ± 0.33	4.48 ± 1.1	5.14 ± 0.37	6.82 ± 0.08
	Gram (+)	7.93 ± 2.07	8.44 ± 0.3	11.66 ± 2.27	10.92 ± 2.27	10.51 ± 0.16	8.58 ± 1.73	10.31 ± 0.49	12.67 ± 015
	Actinomycetes	1.71 ± 0.43	1.33 ± 0.22	2.66 ± 0.13	2.33 ± 0.15	2.11 ± 0.29	1.71 ± 0.53	1.86 ± 0.22	2.78 ± 0.06
HC	AMF	0.62 ± 0.05	17.44 ± 7.12	0.74 ± 0.08	12.95 ± 1.95	0.67 ±0.03	10.74 ± 4.08	0.88 ± 0.16	0.43 ± 0.22
	SF	1.19 ± 0.4	1.31 ± 0.14	1.42 ± 0.37	2.14 ± 1.34	1.30 ± 0.27	1.29 ± 0.31	1.29 ± 0.24	1.48 ± 0.82
	Gram (-)	3.03 ± 0.29	3.5 ± 0.13	3.58 ±0.2	4.08 ± 0.13	2.71 ± 0.25	3.33 ± 0.35	3.20 ± 0.53	2.49 ± 0.72
	Gram (+)	7.76 ± 0.34	8.25 ± 0.31	8.51 ± 2.02	8.06 ± 0.42	7.96 ± 0.27	8.34 ± 0.63	8.09 ± 0.71	7.70 ± 0.38
	Actinomycetes	1.37 ± 0.09	1.48 ± 0.09	1.66 ± 0.15	1.57 ± 0.29	1.59 ± 0.13	1.77 ± 0.07	1.66 ± 0.3	0.96 ± 0.48

R.i: *R. irregularis*; T.v: *T. viride*; PHE: phenanthrene; RHC: Root and hyphal compartment; HC: Hyphal compartment. AMF : Fatty acid for arbuscular mycorrhizal fungi; S.F: Fatty acid for saprotrophic fungi ; Gram (-): Fatty acid for gram negative bacteria; Gram (+): Fatty acid for gram positive bacteria; Actinomycete: Fatty acid for Actynomycetes;. The values shown are the mean ± standard deviation of triplicates.

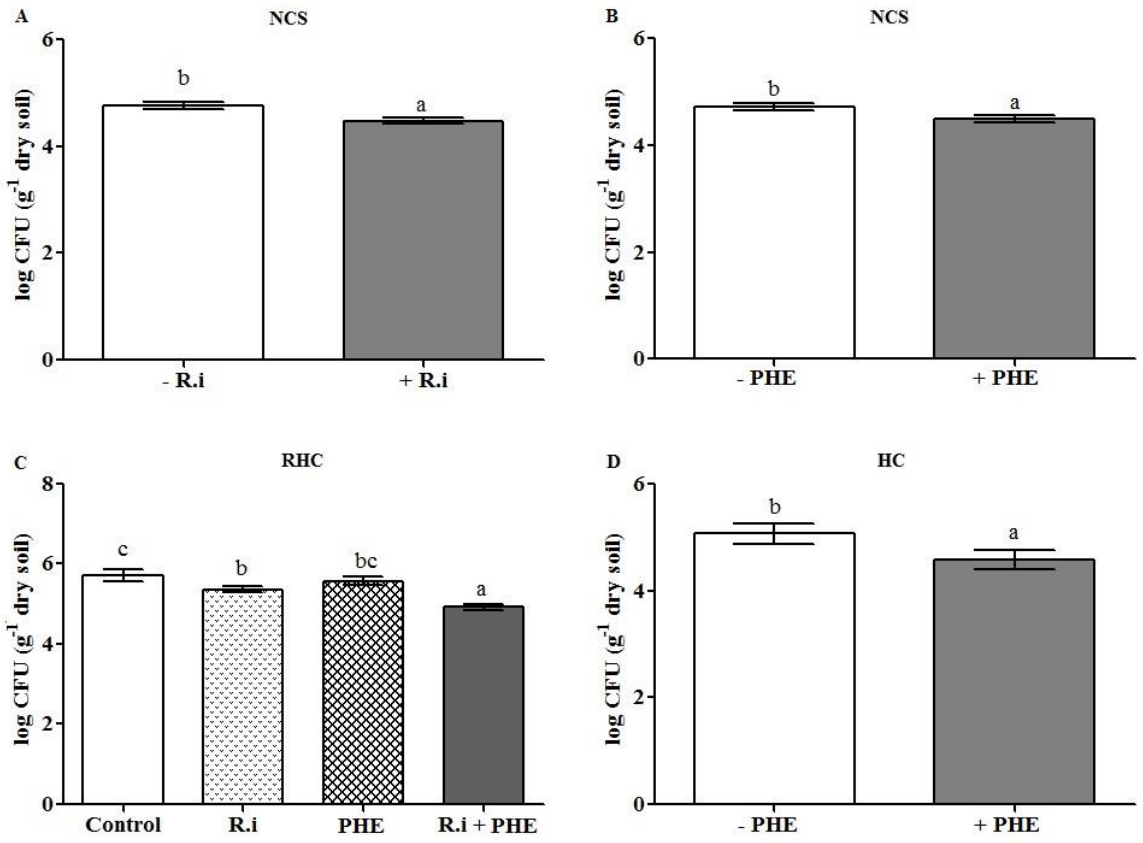

Figure 5.1 Factor treatments means for population of *T. viride* (CFU g^{-1} dry soil) in the different compartments. A) Effect of *R. irregularis* in NCS. B) Effect of phenanthrene in NCS. C) Effect of factor interaction comprised by *R. irregularis* and phenanthrene in RHC. D) Effect of phenanthrene in HC. NCS: Non-compartmented soil; RHC: Root and hyphal compartments; HC: Hyphal compartments; R.i: *R. irregularis*; PHE: phenanthrene. Different letters indicate significant factor effects and error bars represent standard error of the mean.

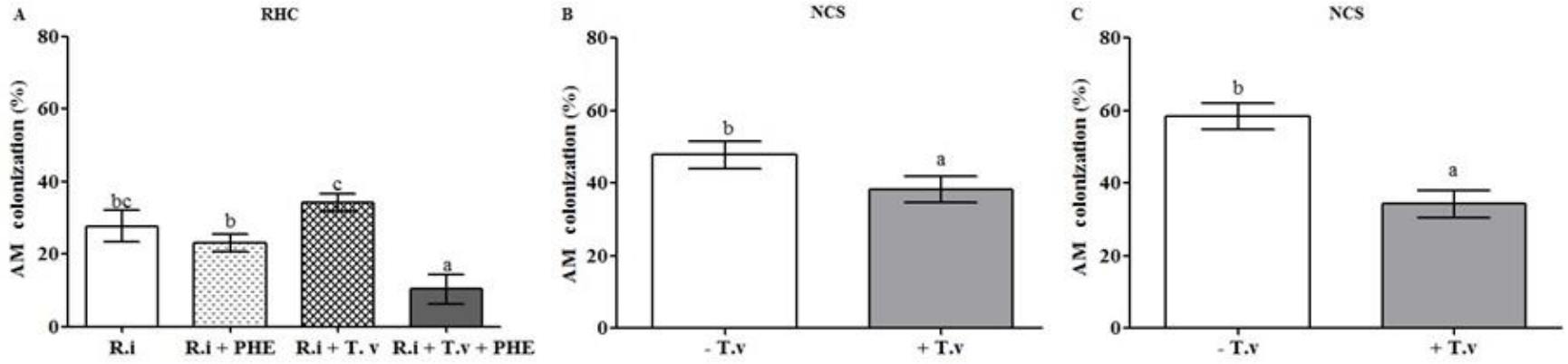

Figure 5.2 Factor treatments means for AM colonization in the different compartments. A) Effect of factor interaction comprised by phenanthrene and *T. viride* on AM colonization in RHC B) Effect of *T. viride* in NCS. C) Effect of phenanthrene in NCS. NCS: Non-compartmented soil; RHC: Root and hyphal compartments; R.i: *R. irregularis*; PHE: phenanthrene; T.v: *T. viride*. Different letters indicate significant factor effects and error bars represent standard error of the mean.

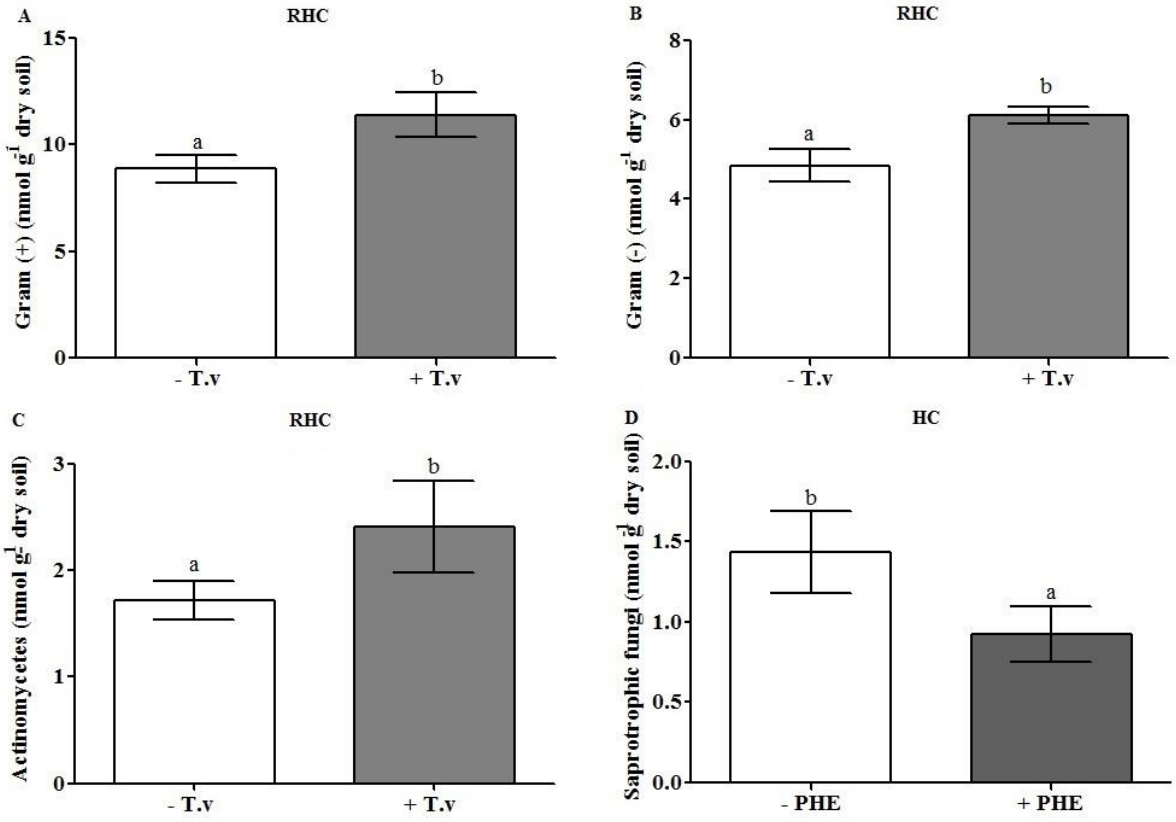

Figure 5.3 Factor treatments means for fatty acids profile of different organisms in the different compartments. A) Effect of *T. viride* on gram negative fatty acid profile in RHC. B) Effect of *T. viride* on gram positive fatty acid profile in RHC. C) Effect of *T. viride* on actinomycetes fatty acid profile in RHC. D) Effect of phenanthrene on saprotrophic fungi in HC. RHC: Root and hyphal compartments; HC: Hyphal compartments; T.v: *T. viride*; PHE: phenanthrene. Different letters indicate significant factor effects and error bars represent standard error of the mean.

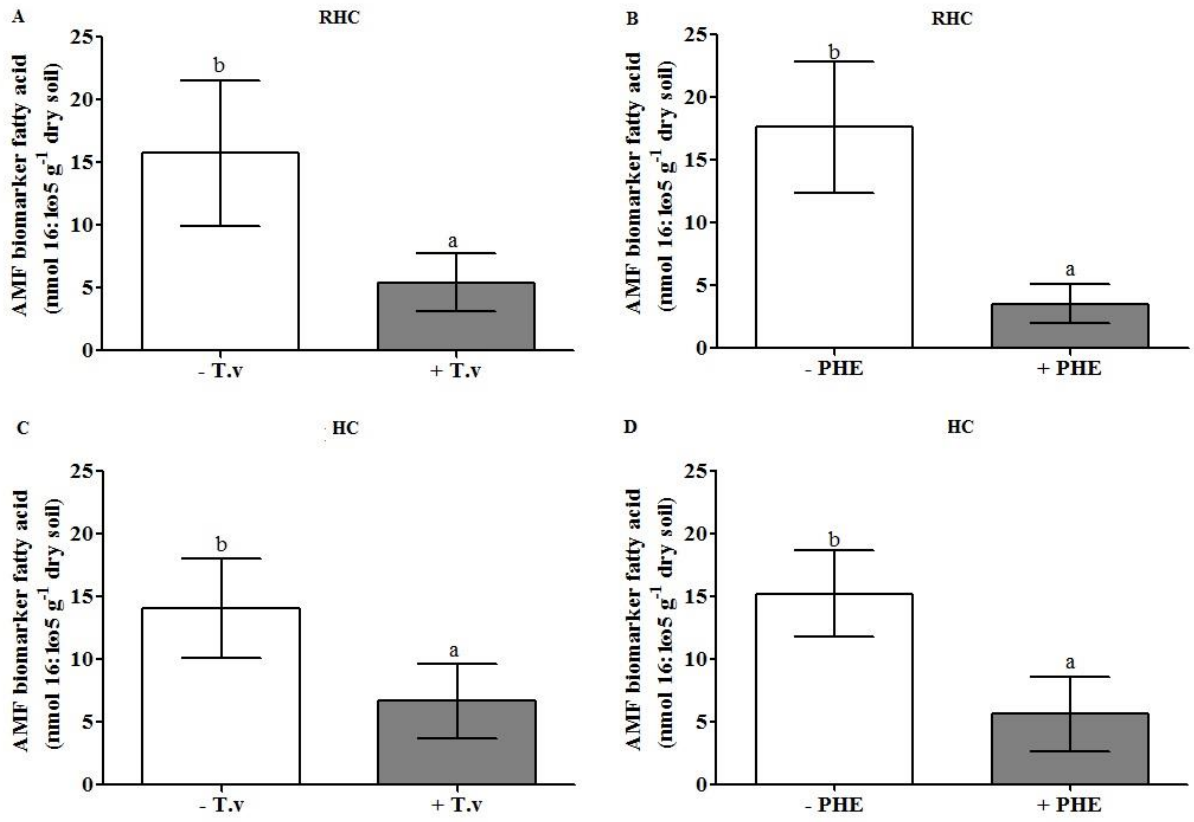

Figure 5.4 Factor treatments means for 16:1ω5 (AMF fatty acid) in the different compartments. A) Effect of *T.viride* in RHC. B) Effect of phenanthrene in RHC. C) Effect of *T.viride* in HC. D) Effect of phenanthrene in HC; RHC: Root and hyphal compartments; HC: Hyphal compartments; T.v: *T. viride*; PHE: phenanthrene. Different letters indicate significant factor effects and error bars represent standard error of the mean.

CHAPTER VI

General discussion, general conclusions and future directions.

6.1 GENERAL DISCUSSION

Pollution by polycyclic aromatic hydrocarbons (PAHs) in soil has become of one of the most important environmental challenges where bioremediation through living organisms such as plants and fungi area useful strategy for decline or remove PAHs from soil (Acevedo et al., 2011; Gao et al., 2009; Liu et al., 2015a). In the case of plants, they are able to dissipate PAHs by bioaccumulation within their tissues and through degradation due to their beneficial effect on soil microorganism that promote PAHs degradation (Denys et al., 2006; Parrish et al., 2006; Sun et al., 2013). However, plants are sensitive to PAHs; therefore the selection of plants able to tolerate high PAHs levels is desirable. In this study, performed in **Chapter III,** among tested plants, *T. aestivum* (wheat) presented the higher tolerance parameters at 1000 mg L^{-1} based on germination and plant growth parameters. The Poaceae have been considered one of the most important plant family used in phytoremediation due to their tolerance to a wide range of pollutants such as trace elements and petroleum (Bouranis et al., 2012).

The use of microorganism as a bioremediation alternative for PAH-polluted soils has gained importance due to the advantages related to their environmentally friendly features (Hwang et al., 2007). In this regard, fungi and bacteria have proved to be excellent PAHs degraders by their enzymatic activities (Morelli et al., 2013; Vila et al., 2015). In order to optimize this degradation process, the suitable microorganisms used have been isolated from PAH-polluted soil due to its abilities related to prevail and tolerance for coping these compounds (Marchand et al., 2017). In soil, bacteria degrade PAHs via oxygenase-mediated metabolism involving either monooxygenase/dioxygenase enzymes and cytochrome P450 (Ghosal et al., 2016; Moody et al., 2004). In the case of SF, they can

degrade PAHs by ligninolytic enzymes such as manganese peroxidase, lignin peroxidase and laccase, as well as, by non-ligninolytic enzymes such as cytochrome P450 (Bamforth and Singleton, 2005; Cerniglia and Sutherland, 2010). Generally fungal rates of PAHs in terms of degradation are slow and inefficient compared to bacteria, nevertheless, fungi can degrade PAHs of high molecular weight by their wide broad enzymatic system (Leitão et al., 2009). Furthermore, fungal traits such as their physiological versatility, ability for growing in environments with low nutrient concentrations, low humidity and acidic pH, their filamentous way of growing that allows an efficient colonization and exploration of polluted soil and the large fraction of the soil biomass represented by fungal mycelia, provide to the fungi an important ecological role, contributing significantly to the transformation of PAHs in soil (Morelli et al., 2013).

In the **Chapter III,** The selection of SF was evaluated under *in vitro* conditions where *T. viride* showed the greatest PAHs remotion after 14 days. It is explained due to *T. viride* presented the highest values on different parameters such as ligninolytic activities and fungal dry biomass, as well as, the lowest pH that can be attributed to a CO_2 production (Srivastava et al., 2011). *T. viride* and all fungi tested in this chapter corresponded to Ascomycota phylum whose species have been described successfully in order to degrade PAHs and prevail under PAHs pollution in soil and are able to produce ligninolytic enzymes even being considered non-ligninolytic fungi compared to Basidiomycota phylum that generally produce different ligninolytic enzymes (Viswanath et al., 2014). Ascomycetes possess some advantages compared to ligninolytic fungi such as their fast growing at neutral pH, they do not require lignocelullosic substrates for enzyme production, since the degradation of these compounds is linked to the intracellular metabolism of

xenobiotics, and they are commonly found in extremely polluted areas (Kües, 2015; Marco-Urrea et al., 2015). In terms of performance of PAHs degradation by different fungal phylum several studies have been conducted showing different results. Batista et al. (2017) observed that among Zygomycetes, Ascomycetes and Basidiomycetes, the two later presented performances in order to degrade PAHs *in vitro*. However, all strain where indicates as potential PAHs degraders. Juckpech et al. (2012) observed that Basidiomycetes produce a significant degradation of PAHs compared to Ascomycetes. Conversely, Olivella et al. (2016) found that Ascomycetes degraded a higher PAHs quantity compared to Basidiomycetes. It seems that this opposite results depends on the fungi and their respective origin.

In the **Chapter IV**, we studied the interaction of wheat plants, saprotrophic fungus *T. viride* and the third component of this interaction corresponding to the AM fungus *F. mosseae* in soil spiked with phenanthrene. We observed that the dual interaction of *T. viride* and *F. mosseae* produced beneficial effects on plant growth under phenanthrene presence compared to single inoculation and the control treatment. These results are related to an enhancement on antioxidant enzymes by dual inoculation that in turn reduced the oxidative damage in wheat leaves measured through lipid peroxidation. In addition, AMF have been reported for protecting plants when exposed to PAHs mainly by increasing accumulation in roots reducing their transference into shoot and leaves (Gao et al., 2010a), whereas, *T. viride* have been reported for increasing plant growth by the secretion of phytohormones (Contreras-Cornejo et al., 2016; Harman, 2006), as well as, for the enzymatic degradation of PAHs and other organic pollutants (Sowmya et al., 2014; Tripathi et al., 2013). Moreover, a higher AM colonization parameters were found when *T. viride* was co-

inoculated improving growth of wheat plants with or without phenanthrene. Enhancement on AM colonization by SF followed by an increase in biomass has been reported in several researches.

Dual inoculation reached the highest phenanthrene dissipation in the contaminated soil according to observed in the **Chapter IV.** These results are explained in different ways. First, dual inoculation increased soil biological activities such FDA and Dehydrogenase activities being the later related to PAHs degradation (Rabie, 2005). An increase in soil biological activities are related to the highest phenanthrene dissipation. In this sense, AMF have been reported for improving microbial activity as a way to dissipate PAHs by degradation in soil (Gao et al., 2011). Moreover, in **Chapter IV**, our study showed that dual inoculation reached the highest organic acids exudation which are related with the proliferation of soil microorganism (Marschner, 1995), as well as, have been suggested for increasing PAHs bioavailability in soil (Ling et al., 2015)

Second, the highest levels of enzymatic activities involved in PAHs degradation such as Laccase and manganese peroxidase were found when dual inoculation was performed. These enzymes have been related mainly to SF including *Trichoderma* species (Zafra et al., 2015), therefore the role of *T. viride* in this study is vital for increase PAHs dissipation in soil by degradation. Several enzymes not determined in this study have been described in order to degrade PAH (Zafra et al., 2015).

Third, *F. mosseae*, increased the phenanthrene content in roots compared with non-inoculated plants and reducing content in aerial biomass. This effect was more pronounced

under *T. viride* co-inoculation. Nevertheless, accumulation in vegetal tissues contributes with small percentages of phenanthrene dissipation. Therefore, in general terms, the main dissipation way of phenanthrene by the dual inoculation seemed to be degradation. In **Chapter IV** not detectable phenanthrene was at least tenfold greater than phenanthrene accumulated in vegetal tissues.

Related to a greater accumulation of phenanthrene in roots by *F. mosseae* inoculation and enhanced by *T. viride*, a protective effect is provided by this fungal interaction and is related to the decrease in lipid peroxidation besides an enhancement on antioxidant activities. In addition, lipid structures of AMF such as vesicles were higher in plants dual inoculated under phenanthrene presence and may explain the increase of phenanthrene in roots due to the lipophilic nature of PAHs (Abdel-Shafy, 2016). In addition, this protection is related to the higher dissipation provided by this dual inoculation and the plant growth promotion by these fungi under phenanthrene presence.

The sum of all results suggest that plant growth promotion given by dual inoculation is the driving force in order to achieved the objectives purposed in this study related to improve PAHs dissipation and plant protection for coping to these compounds. The highest values related either the highest phenanthrene dissipation such as biological activities, ligninolytic enzymes, organic acid exudation, and plant protection such as AM colonization, antioxidant activities, were observed when plants under phenanthrene treatments reached the highest biomass. Nevertheless, compatibility among plants and fungi is an important factor for achieving the goals of this study. **In Chapter V,** a root compartment experiment the interaction of the AM fungus *R. irregularis* and *T. viride* showed the lowest biomass of

maize plants under phenanthrene presence in the root and hyphal compartments. Mutual fungal inhibition based on *Trichoderma* CFU and WCFA was observed.

The results achieved by this doctoral thesis show that dual inoculation with *F. mosseae* and *T. viride* using wheat plants, increased significantly the phenanthrene dissipation in soil as a consequence of better plant growth produced by this interaction derived by the protection provided by them, unleashing an enhancement in enzymatic and biological activities in soil that promote PAHs dissipation in soil. Therefore, the implementation of this biotechnological seems an interesting option alternative to remediate PAHs polluted as a bioremediation strategy.

6.2 GENERAL CONCLUSIONS

- In our study, we observed that wheat could be use as a model plant to develop an alternative to remediation of soil polluted with PAHs due to their capacity to tolerate high phenanthrene concentrations, as well as, presented a suitable compatibility when dual inoculation was used in order to promote phenanthrene dissipation.

- The fungal dual inoculation between AM fungus *Funneliformis mosseae* and *T. viride* increased plant growth compared to single inoculation or control treatment with or without phenanthrene presence.

- Dual inoculation reached the highest phenanthrene dissipation in soil directly related to an enhancement on soil biological activities such as FDA, dehydrogenase, ligninolytic activities such as Laccase and MnP, and exudation of organic acids.

- Degradation seems to be the main dissipation way of phenanthrene in soil by dual inoculation according to not detectable phenanthrene. The accumulation in vegetal tissues accounts with small percentages of phenanthrene dissipation and did not have a significant impact on phenanthrene dissipation in soil.

- A higher accumulation of phenanthrene in roots and its further decrease in shoot is one of the most important mechanism that *F. mosseae* provided to wheat plants in

order to alleviate them from phenanthrene through a better plants antioxidant response and a decrease in lipid peroxidation. This is related to the increase of AM fungus lipid structures (such as vesicles) important on the accumulation of PAHs due to their lipophilic nature.

6.3 FUTURE DIRECTIONS

The dual inoculation composed by SF and AMF has showed to be an interesting alternative in order to increase the dissipation of polycyclic aromatic hydrocarbons in soil. Nevertheless, practical application of this biotechnological into a greater scale than a laboratory condition is a future challenge for achieving this remediation purposes. In addition, the selection of the suitable fungi and plants is the key step for using their beneficial effects. On the other hand, mineralization studies are required in order to ensure an appropriate dead end product derived from polycyclic aromatic hydrocarbons degradation by this interaction. On the other hand, the detailed study related to the vesicles and their role on polycyclic aromatic hydrocarbons accumulation or sequestration could be important findings in order to elucidate AMF mechanisms that protect plants against polycyclic aromatic hydrocarbons. Similarly, determinations related to the trends of extraradical mycelium of AMF and their spores under PAHs pollution will help to achieve this purpose. In addition, the study of the effects of glomalin on polycyclic aromatic hydrocarbons behavior in soils is an interesting research for reaching a greater understanding of the mechanism underlying on the AM symbiosis as an alternative for coping polycyclic aromatic hydrocarbons.

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GLOSARY AND CONCEPTS

ANT: anthracene

AMF: arbuscular mycorrhizal fungi

CFU: colony forming units

HC: hyphal compartment

NCS: non-compartmented soil

SF: saprotrophic fungi

PAHs: polycyclic aromatic hydrocarbons

PHE: phenanthrene

RHC: root and hyphal compartment

TF: translocation factor

WCFA: whole cell phatty acids

Dissipation: In terms of environmental pollution, dissipation comprises all ways that reduce the final concentration of a compound in a determined solution by the effect of external factor. The dissipation of PAHs by plant-fungi interaction involves degradation, bioaccumulation, adsorption and transference (Lenoir et al., 2016).