Universidad de La Frontera

Facultad de Ingeniería Ciencias y Administración

Programa de Doctorado en Ciencias en Recursos Naturales

Study of the native saprophytic fungi incubated on organic residues and their effect on the growth of agroforestry species colonized by arbuscular mycorrhizal fungi

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

LEONARDO JORGE ALMONACID MUÑOZ

TEMUCO-CHILE 2014

Study of native saprophytic fungi incubated on organic residues and their effect on agroforestry species colonized by arbuscular mycorrhizal fungi

Esta tesis fue realizada bajo la supervisión del Dr. Cesar Arriagada Escamilla, perteneciente a la Facultad de Ciencias Agropecuarias y Forestales de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

LEONARDO JORGE ALMONACID MUÑOZ

……
DIRECTOR PROGRAMA DOCTORADO
EN CIENCIAS DE RECURSOS NATURALES

…..……
Dr. Cesar Arriagada Escamilla

……..
DIRECTOR DE POSTGRADO
UNIVERSIDAD DE LA FRONTERA

…..……
Dr. Fernando Borie Borie

…..……
Dr. Pablo Cornejo Rivas

…..……
Dra. Mara Cea Lemus

..……
Dra. Inmaculada García-Romera

..……
Dr. Jorge Mendoza Crisosto

Acknowledgments

Acknowledgments

I would like to thank to my thesis evaluation committee Dr. Fernando Borie, Dr. Pablo Cornejo, Dra. Mara Cea and Dr. Jorge Mendoza for their comments and support in this thesis.

I would like to thanks to research group of Laboratorio de Biorremediación, Claudio Lagos, Alejandra Fuentes, Javiera Soto, Hector Herrera, Javier Ortiz and Sebastián Gutiérrez for their help and friendship.

I would like to express my special thanks to my thesis advisor Dr. Cesar Arriagada for their support, help and advice during the development of this thesis and in general over the years as a doctoral student.

I would like to thanks to research group of Biofertilización y biorremediación por Hongos Rizosféricos of Estación Experimental del Zaídin, Granada, España. Especially to Dr. Juan Antonio Ocampo and Dra. Inmaculada García-Romera for their valuable assistance collaboration and assistance during my doctoral internship and by their contribution in the improving this thesis.

Finally I would like to thanks to my family especially my mother for their unconditional support.

This research was financially supported by FONDECYT N° 1130662, 1100752 and CONICYT scholarship 24121357, 75120067 and 24080106.

Summary

The increase in food demand and progressive enhance in the use of pesticides in agriculture have led to a constant loss of soil fertility, generating an excessive use of chemical fertilizers. Thus, the main aim of agriculture is to increase crop production and the addition of organic residues and microorganisms is a friendly environment alternative and lower cost compared with traditional methods of fertilization. Wheat straw and sewage sludge are two types of organic residues containing high amounts of organic matter and essential nutrients to growth and development of plants. Similarly, the increase in crop yield may be generated by the introduction to soil, either free or symbiotic microorganisms such as arbuscular mycorrhizal fungi, which improve growth through several strategies as: organic matter degradation, phosphorus solubilization, protection against disease, nutrient translocation, among others. The objective of this study was to generate an organic amendment from the combination of wheat residue with sewage sludge, and to evaluate its effect on arbuscular mycorrhizal symbiosis and agroforestry plant growth. Two kinds of organic residues (wheat straw and sewage sludge) single and in combination (1:3 w:w) were used. These residues were inoculated (added at the same time) and incubated for 2 and 4 weeks with saprophytic fungi. Three species of saprophytic fungi *Coriolopsis rigida*, *Phanerochaete crysosporium* and *Trichoderma harzianum* and one arbuscular mycorrhizal fungi *Rhizophagus irregularis* were used. As test plants *Solanum lycopersicum* and *Eucalyptus globulus* were used. In order to evaluate the degradation process of this organic residue by saprophytic fungi, ligninolytic enzymes, hydrolytic enzymes, nutrient content and pH were determined during 4 weeks under *in vitro* condition. In relation to the organic residue degradation *C.rigida* showed higher values of ligninolytic enzymes and FDA activity associated with an increased in the degradation of these residues. Similarly, dry matter, cellulose, carbon, nitrogen and pH values decreased after 4 weeks of incubation with both saprophytic fungi can be attributed to an

increased in the degradation process. After, were conducted experiments with addition of these organic residues.Both plants determination (shoot and root biomass, mycorrhizal colonization, alkaline phosphatase activity, relative chlorophyll content and nutrient concentration) as biochemical and biological determinations of the rhizospheric soil (enzymatic activities, nutrient content, rhizospheric microorganisms) were performed. In relation to evaluate the influence of organic residues, saprophytic and mycorrhizal fungi on soil quality and growth of *E. globulus* greenhouse experiments were performed. Sewage sludge addition not increased biomass of *E. globulus* plants. However, the co-inoculation of *C. rigida* and *P. chrysosporium* increased the biomass, mycorrhizal colonization and SDH activity of *E. globulus* plants inoculated with *R.irregularis* in the presence of mixed residues. Similarly, the treatments with mixed residues enhance the nutrient translocation and increased FDA and β-glucosidase activities of the rhizospheric soil. On the other hand, the effect of organic residues addition on tomato dry weight and on chemical and biochemical properties of rhizospheric soil was evaluated. Organic residue increased the biomass of *S.lycopersicum* especially in arbuscular mycorrhizal plants. This increase was greater when the mixture of residues was inoculated with *T.harzianum.* The co-inoculation also increased nutrients translocation and relative chlorophyll content. Similarly, the presence of saprophytic fungi increased pH and nutrients content of rhizospheric soil especially when the mixture of residue was inoculated with saprophytic fungi. Finally, the effect of organic residues addition incubated with *T.harzianum* on bacterial communities was evaluated. The addition of residues increases the number of cultivable bacteria especially after 45 days from organic residues addition. These results show the potential use of these organic amendments applied in combination with free-living and symbionts microorganisms for improving plant growth and soil fertility increasing nutrients concentration and stimulating the proliferation of microorganisms being both essential for enhance crops yield.

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CHAPTER I

GENERAL INTRODUCTION

2.1 General introduction

The constant addition of mineral fertilizers for improving crop yield can generate a negative effect on environment especially for the large inputs of nitrogen. Furthermore, these fertilizers in high concentrations affect negatively the development of soil microorganisms (Oehl et al., 2004). In opposite, organic residues used as biofertilizers enhance the development of soil microorganisms increasing the biological activity of agricultural soils (Gryndler et al., 2006).Therefore, new alternatives to sustain agricultural productivity are required, where the optimization processes (biostimulation and bioaugmentation). In this case the microorganisms promoters of plant growth may play a key role. Both strategies can be used as alternatives to current methods of fertilization, considering some advantages such as lower cost and minimal environmental impact.

The process of biostimulation involves the addition of nutrients in order to stimulate native microorganisms. Several types of organic residues are used in agroforestry systems, including crop residues and others as sewage sludge. Crop residues such as wheat straw can be used as source of nutrients, e.g. nitrogen or phosphorus, for the development of crops (Cassman et al., 1996). According to the office of Agricultural Studies and Policies (ODEPA) in Chile there are 264.305 ha of wheat, of which 115.625 correspond to the Araucanía Region. According to Bamaga et al., (2003), each kilogram grain produces 1.1 kilogram residues, Therefore, it is estimated that 1.676.313 tons of crop residues are annually produced in Chile. On the other hand, sewage sludge from wastewater treatment plants contains large amounts of organic matter and nutrients such as nitrogen, phosphorus and micronutrients (Chodak et al., 2001, Mantovi et al., 2005) that can be used for crop nutrition. Currently in Chile 266 wastewater treatment plants are in operation and 34 of them are located in the Araucania Region (Ramirez et al., 2002).

The use of sewage sludge in combination with wheat straw as bulking agent improve some soil characteristics (Petric et al., 2009). The input of carbon provided by wheat straw stabilizes C:N ratio, decreases moisture and increases particle size of the substrate improving aeration and stabilizing pH improving the development of microorganisms (Barrington et al., 2002).

The addition of organic matter and nutrients into the soil can affect processes such as plant exudation having a strong influence on rhizospheric microorganisms with diverse functions. Organic anions, such as citrate, malate and oxalate, may be complexed with iron and aluminum preventing formation of insoluble compounds between these elements and phosphorus, increasing their bioavailability (Dakora & Phillips, 2002). Furthermore, the addition of organic waste has an effect on soil enzyme activities, which are directly related to the organic matter degradation. For instance, phosphatase is responsible for the mineralization of organic phosphorus (Amador et al., 1997, Roldán et al., 1996). Similarly, β-glucosidase is directly involved in the carbon cycle and degradation of organic matter (Turner et al., 2002). On the other hand, other enzyme activities are used as indexes of biological activity. Dehydrogenase is considered to be a general index of biological activity on account of its role in the respiratory metabolism of microorganisms (Delgado et al., 2004). Similarly, hydrolysis of fluorescein diacetate (FDA) is used for estimating total soil microbiological activities (Adam & Duncan, 2001) reflecting the activity of hydrolases involved in organic matter degradation (Sánchez-Monedero et al., 2008).

Saprophytic fungi are natural inhabitants of the soil, especially in areas rich in organic matter, degraded metabolic and structural components of dead plants and animals (Dix & Webster, 1995), being crucial in the recycling and availability of nutrients in ecosystems. White rot fungi, are capable of degrading several compounds usually resistant to microbial action

mainly due to the production of broad-spectrum enzymes (Matsubara et al., 2006).The lignin degrading enzyme systems are composed of a variety of enzymes such as laccase, Manganese peroxidase and manganese-independent peroxidase as well as hydrolytic enzymes (Pelaez, et al., 1995, Dinis et al., 2009). The biotransformation process transforming complex organic molecules in assimilable compounds o as well as occurs organic matter stabilization and decreasing the amount of toxic compounds. Likewise, these microorganisms can be used in bioaugmentation processes, which consist of the introduction of microorganisms either exogenous or endogenous to a system, which, when applied to the soil, may facilitate processes such as, improving soil structure, increasing biodiversity of microorganisms and promoting plant growth (Van Veen et al., 1997). Plant growth promotion by these microorganisms is carried out by strategies such as phytohormone production, phosphate solubilization, or by induction of systemic resistance (Meera et al., 1995, Azcon & Barea, 1996, Shivanna et al., 1996).

Mycorrhizal fungi are other important components of the soil, improving plant growth primarily by nutrient and water uptake (Smith & Read, 2008). Mycorrhizal fungi are very important microorganisms especially in relation to phosphorus absorption in soils with a high adsorption capacity such as Andisols increasing the sustainability and productivity of crops (Borie et al., 1998, Borie et al., 2010). Furthermore, these fungi are able to alleviate different kind of stresses for instance phytotoxicity of heavy metals in polluted soils. Mycorrhizal fungi are able to help to their removal, through various mechanisms including precipitation, active uptake, complexation, biosorption to cell wall among others (Gadd, 1990, Gadd, 1993).

Several studies have shown the synergistic effect between arbuscular mycorrhizal fungi and saprophytic fungi. However, this effect are not always positive; in some cases, it may be neutral or even negative (McAllister et al., 1997, Fracchia et al., 1998, García-Romera et al.,

1998, Gryndler et al., 2000), depending on factors such as microorganisms, plants and growth substrate (Fracchia et al., 2000).

Another important factor to be considered is the effect the use of residues and microorganisms may have on soil microbial communities, being bacteria key microorganisms in recycling and the availability of nutrients in ecosystems. The structure and composition of microorganisms can be influenced by anthropogenic factors. The application of the organic amendments generally increases the activity of microorganisms (Stark et al., 2007). This may be due to the increased metabolic activity, cell proliferation or changes in the structure and composition of soil microorganisms (Cytryn et al., 2011).

Considering that: (1) the addition of organic residues improve chemical, physical and biological soil properties increasing crop yields, (2) microorganisms such as saprophytic fungi are highly efficient in the degradation of organic matter. Thus, it is expected that the process of biostimulation with organic residues and bioaugmentation with arbuscular mycorrhizal and saprophytic fungi will improve the plant growth including agroforestry species.

2.2 Hypotheses

This Thesis proposes the following hypotheses:

- The combination of wheat residues with sewage sludge and their inoculation and incubation with native saprophytic fungi will enhance quality of this organic residue (amendment).

- The use of this organic amendment will promote the growth of agroforestry species through the synergistic effect between mycorrhizal and native fungal strains.

2.3 General objectives:

To generate an organic amendment from the combination of wheat residue with sewage sludge, and to evaluate its effect on arbuscular mycorrhizal symbiosis and agroforestry plant growth.

Specific objectives

1. To study some ligninolytic and hydrolytic enzymes involved on the degradation process of organic residues (incubation) with native saprophytic fungi.

2. To evaluate the effect of addition of organic residue (amendment) on the growth and nutrient translocation on *Eucalyptus globulus* and *Solanum lycopersicum* plants inoculated with arbuscular mycorrhizal fungi.

3. To analyze changes on the microbial rhizosphere communities and biochemical soil parameters by soil application of organic residue.

CHAPTER II

Soil fungi as promoters of the plant growth and their interaction with organic residues on agricultural soils. A REVIEW

Soil fungi as promoters of the plant growth and their interaction with organic residues on agricultural soils.

Leonardo Almonacid and Cesar Arriagada

Center of Amelioration and Sustainability of Volcanic Soils.BIOREN. Bioremediation Laboratory. Universidad de La Frontera. P.O. Box 54-D. Temuco-Chile.

Abstract

Several fungi are capable to promote plant growth through several mechanisms associated with better conditions for plant development such as organic acids production, enzymes production, phosphate solubilization, induction of defense, among others. Similarly, the use of these microorganisms as biotechnological tools are widely practiced in several areas, such as industry, bioremediation, agriculture and forestry production. In the latter case, increasing the growth, productivity and health of plant species. Another application area is organic residues management which seeks to reduce the harmful effects they may generate due to the presence of elements such as phenols or heavy metals. The use the plant growth promoting fungi and organic residues as amendments increases plant growth due to the high amount of organic matter and nutrients promoting physical, biological and chemical properties of soil enhancing plants nutrition. On the other hand the addition of both organic residues and fungi has a lower cost and are friendly environmental alternative compared with traditional methods of fertilization.

Keywords: Bioaugmentation, Lignocellulosic residues, Mycorrhizal fungi, Organic amendment, Plant nutrition, Saprophytic fungi.

3.1 Introduction

Plant growth promoting fungi (PGPF), are capable to improve plant growth through different strategies such as, mineral solubilization, organic acid production, hormones stimulation, abiotic and abiotic stress tolerance and protection against pathogens (Chandanie et al., 2006, Van der Ent et al., 2009). Within this classification, saprophytic fungi and mycorrhizal fungi are found, which are of vital importance in the uptake of nutrients and water and other processes associated with the promotion of growth.

On the other hand, crop development demands the generation of new alternatives to sustain productivity, where generation and optimization of processes, such as organic fertilizers, can play an important role in terms of being an alternative for current fertilization methods, considering some advantages such as lower cost and lower environmental impact.

Several production processes, such as wastewater treatment, olive oil production or crop establishment, generate large amounts of residues, which are applied to the soil as common practice in some countries, improving physical, chemical and biological properties (Tsadilas et al., 1995) due to large amounts of organic matter and nutrients, such as nitrogen and phosphorus. These residues, especially those related to industrial processes, can also contain high amounts of toxic elements, such as heavy metals or phenols, which limit their application to soil (Saadi et al., 2007, Sampedro et al., 2008). Direct application of residues to the soil can cause detrimental effects on crops. Therefore, is necessary to handle the residues previous to application for reducing the negative impact they may have on the soil or directly on crops, decreasing the amounts of toxic elements (Sampedro et al., 2004, Aranda et al., 2009).

There are several studies about the management of organic residues using microorganisms, particularly white rot fungi due to characteristics such as high efficiency in the degradation of

lignin, phenolic compounds and organic pollutants, among others. Through their broad-spectrum enzyme system that allows them to act on several substrates. The use of this fungi reduce the amount of harmful elements for plant transforming these residues in amendments that enhance plant growth, especially if these are associated with mycorrhizal fungi due to their synergy effects between saprophytic fungi and mycorrhizal fungi and the optimization of nutrient and water uptake. The aim of this review is to explore current understanding of the main mechanisms used by saprophytic and mycorrhizal fungi to promote plant growth and their role in managing organic residues for subsequent use as amendments.

3.2 Beneficial rhizospheric fungi

3.2.1 Saprophytic fungi

Saprophytic fungi are natural inhabitants of soil able to degraded metabolic and structural constituents of plant debris and dead animals (Dix & Webster, 1995), key factors in the recycling and nutrient availability in ecosystems. Some of these fungi are capable of degrading several compounds resistant to microbial action, such as lignin, polycyclic aromatic hydrocarbons (Baldrian, 2008) or humic substances (Hofrichter & Fritsche, 1996) mainly due to the production of broad-spectrum enzymes (Matsubara et al., 2006). Thus this fungi are very important in industrial and bioremediation processes, for instance, in the degradation of synthetic dyes, biobleaching, wastewater treatment or in pollutant removal. Furthermore, these fungi are able to promote the plant growth through several processes such as phosphate solubilization, organic acids production or induction of defense. This capacity to promote the plant growth is mainly by the excretion of enzymes that degrade and mineralize organic matter, the main carbon sink of the soil (Grinhut et al., 2007) and therefore increasing the amount of nutrients such as, carbon, nitrogen and phosphorus available to plants. Lignin degrading enzyme system is composed by enzymes as manganese-dependent lignin

peroxidase, versatile peroxidase, lignin peroxidase, manganese peroxidase and laccase, as well as, cellulases, hemicellulases and esterase (Pelaez et al., 1995, Dinis et al., 2009).Several fungi, especially white rot fungi, are capable of producing these enzymes conferring different capacities for degradation of compounds. Many studies report the production of enzymes by some fungi thus *Trichoderma viride*, *Paecilomyces farinosus*, *Wardomyces inflatus* and *Pleurotus ostreatus* show xyloglucanase activity (Tribak et al., 2002), *Irpex lacteus* shows production of laccase, manganese peroxidaseand lignin peroxidase (Rothschild et al., 2002), even Ascomycota fungi as *Coniochaeta ligniaria* presents production of cellulase, xylanase, manganese peroxidaseand lignin peroxidase (Lopez et al., 2007) or the litter decomposition fungi *Mycena inclinata*, *Collybia dryophila* produce manganese peroxidase(Baldrian & Šnajdr, 2006).

3.2.2 Arbuscular mycorrhizal fungi

Mycorrhizae are fungi that have a symbiotic association with plant roots. There are seven types of mycorrhizae, being the most common arbuscular mycorrhiza (Phylum Glomeromycota) and ectomycorrhizae (Phylum basidiomycota and ascomycota). The latter are obligate symbionts of about 85% of plants (Smith & Read, 2008, Brundrett, 2002). These are important components of soil microorganism, promoting plant growth, mainly by water and nutrients uptake (Smith & Read, 2008) being very important in phosphorus uptake in soil with high adsorption capacity such as Andisol, increasing the sustainability and productivity of crops (Borie et al., 1998). On the other hand, mycorrhizal fungi are able to promote plant growth through other strategies such as metals detoxification (Göhre & Paszkowaski, 2006), or diseases and abiotic stress protection (Ryan & Graham, 2002).

Mycorrhizal fungi can be used in bioremediation processes due to their ability to persist in places with high levels of heavy metals, helping to their detoxification through several

processes such as extra and intra cellular chelation, adsorption to the wall and membrane incorporation into the cytosol and vacuoles and transport through the hyphae (Göhre & Paszkowski, 2006, Cornejo et al., 2008). In relation to the acquisition of elements by mycorrhizal plants in soils, the results are varied because this fungi may increase or decrease nutrients uptake, depending of mycorrhizal fungi and plant species (Ryan & Angus, 2003, Jankong & Visoottiviseth, 2008). However, many studies show an increase in yield and production of crops inoculated with arbuscular mycorrhizal fungi (Karagiannidis & Hadjisavva-Zinoviadi, 1998).

3.3 Synergistic effect between saprophytic and mycorrhizal fungi

It has been develop a series of relationships develop among organisms that inhabit the soil as in the case of mycorrhizal and saprophytic fungi. Studies show that exudate of saprophytic fungi have a positive influence on mycorrhizal fungi in terms of spore germination and growth mycelium, thus promoting plant growth. However, this answer is not widespread, in some cases the effect can be negative or neutral (McAllister et al., 1997, Fracchia et al., 1998, García-Romera et al., 1998, Gryndler et al., 2000), depending on factors, such as, microorganisms, plants and substrates (Fracchia et al., 2000).

Several studies show the synergistic effect between arbuscular mycorrhizal and saprophytic fungi, such as, *Trichoderma sp* stimulates germination and mycelial growth of *Glomus mosseae* under *in vitro* conditions attributed to the presence of volatile compounds produced by saprophytic fungi (Calvet et al., 1992). Similarly, the combined inoculation of *Trichoderma aureoviride* and *G. mosseae* stimulates the growth of *Tagetes erecta*, due to the effect of the fungi on mycorrhizal colonization (Calvet et al., 1993).

Arriagada et al., (2007) showed that *Fusarium concolor* and *Trichoderma koningii* enhance the *G. mosseae* percentage of colonization, increased the dry weight and N, P and K content of plant. This influence could be the direct effect of these AM fungi *G. mosseae* or an indirect effect due to role that have the mycorrhizal fungi in the modification of plant exudates. Similarly, the inoculation with *G. deserticola* and *Coriolopsis rigida* have synergistic effect on growth of *Eucalyptus globulus* plants as increase their tolerance to Cu, decrease their concentration and improve the colonization of mycorrhizal fungi (Arriagada et al., 2009a).On the other hand, results presented by McAllister et al., (1997) showed an inhibitory effect on *G. mosseae* when *F. equiseti* and *Alternaria alternata* were inoculated before or at the same time as mycorrhizal fungi suggesting a direct effect on the extramatrical phase. The synergistic effects of some saprophytic fungi and mycorrhizal fungi on plant growth are shown in table 2.

3.3.1 Organic acid production

Organic acids are produced by a several fungi: Brown rot fungi, Basidiomycetes, Mucorales, Ascomycetes, Deuteromycetes and white rot fungi, even mycorrhizal fungi have the ability to secrete organic acids. Johansson et al., (2008) showed production of acetic, propionic and butyric acids by ectomycorrhiza growing in a liquid medium with different concentrations of metals. Along with the action of enzyme, compounds of low molecular weight are considered as mediators in fungal degradation processes, for instance: preparing cell wall to enzymatic action is the case of oxalic acids that can chelates and stabilizes Mn (III), providing H_2O_2 and decreasing the pH of medium and improving peroxidases action. Mäkela et al., (2002) shows a direct relationship between manganese peroxidase activity and oxalate concentration, present in spruce chip cultures of *Trametes ochracea* and *Trametes versicolor*.

Oxalic acid is the most common organic acid involved in lignocellulosic residue degradation, metal detoxification and other processes as biodeterioration or breakdown pollutants (Dutton & Evans, 1996, Gadd, 1999, Shimada et al.,1997) allowing an increase in the amount of nutrients available to plants and microorganisms (Jones, 1998). The production of organic acids as oxalic acid by complex formation decreases the amount of metal conferring tolerance to plant (Jarosz-Wilkolazka & Gadd, 2003). On the other hand, organic acids production is an important aspect in phosphate solubilization into usable forms by plants, being fungi more efficient than bacteria in this process (Nenwani et al., 2010). This process would be mainly due to the reduction of pH and cations chelation (Bar-Yosef, 1991, Whitelaw et al., 1999). Altomare et al., (1999) shows the solubilization of different elements by a strain of *Trichoderma harzianum.* The Fe chelation occurs in the absence of organic acids, suggesting that this could have been caused by constituents of fungi that may have chelating capacity such as, protein, chitin or phenols. Studies by Whitelaw et al., (1999) with *Penicillium radicum* report that phosphate solubilization is mainly due to the reduction of pH by H^+ and gluconic acid production that would act as a chelator.

3.3.2 Hormones production

Production of hormones such as ethylene, auxins, gibberellins, cytokinins and abscisic acid (Tsavkelova et al., 2006, Van der Ent et al., 2009) by microorganisms is a mechanisms capable to stimulate plant growth. Fungi as *Trichoderma* are widely used as biological control agents against fungal pathogens such as *Pythium sp*, *Verticillium sp* or *Rizoctonia sp* for their ability to promote mechanisms of plant defense (Benitez et al., 2004) similar to other fungi such as *Fusarium sp*, *Penicillium sp*, *Pythium sp*. Beside, phytohormones such as jasmonic acid are related to signaling between plants and mycorrhizae for the formation of the symbiosis and the arbuscule formation (Isayenkov et al., 2005,Herrera-Medina et al., 2008).

The abscisic acid is known to be key in the penetration spread of the fungus in the root and the development of arbuscule (Herrera-Medina et al., 2007) and its effect could be related to salicylic acid, jasmonate or ethylene. Auxin and salicylic acid facilitate mycorrhizal colonization (Hause et al., 2007). Similarly, salicylic has related with the systemic acquired resistance, while gibberellins would have no clear effect on mycorrhizal colonization and ethylene generally inhibits the colonization process.

3.3.3 Plant growth promotion in contaminated soils

Degraded or contaminated soils produce a reduction in plant growth thus the establishment and development of good plants production can be help by the presence of indigenous microorganisms acclimated to local conditions and capable of promoting plant growth (Carrasco et al., 2011). For instance, saprophytic fungi are able to tolerate and develop in places polluted with heavy metals being able to help their removal by several mechanisms including precipitation, active uptake, complexation and biosorption to cell wall (Gadd, 1993). The removal of this compound can be either intra or extra cellularly. At extracellular level, it can form complexes with carboxyl, amide, thiol, phosphate and hydroxyl groups (Săg, 2001). Similarly, mycorrhizal fungi can be used in bioremediation processes due to their ability to persist in places with high levels of heavy metals, helping their detoxification through various processes such as extra and intra cellular chelation, adsorption to the wall and membrane, incorporation into the cytosol and vacuoles and transport through hyphae, among others (González-Chávez et al., 2004, González-Guerrero 2005, Cornejo et al., 2008).

3.4 Saprophytic and mycorrhizal fungi and their interaction on the rhizosphere with organic amendments

The addition of organic amendments improves chemical, physical and biological properties of soils (Roldán et al., 1996) in addition to increasing fertility, resulting in higher production. There are several types of residues used as organic amendments in agroforestry systems, including crop residues, sewage sludge, sugar beet and olive mill, generated in different production processes, but they differ in their composition and their effect on soil and crops. Crop residues reached about 149 millions ton annually (FAO, 2004) only in Europe, they are usually burnet (Bierke et al., 2008) causing a negative impact on environment. These residue are lignocellulosic which contain lignin, cellulose and hemicellulose, the most abundant natural polymers in nature (Buranov & Mazza, 2008) and an important source of organic matter (Howard et al., 2003), can be used as a source of nitrogen or phosphorus (Cassman et al., 1996). These crop residues may promote physical, chemical and biological soil properties. Another important residue is sewage sludge, which generates as wastewater treatment product which contain large amounts of organic matter and macronutrients such as N and P (Singh & Pandey, 1998, Gonzalez et al., 1992, Sikora & Enkiri, 1999, He et al., 2000, Mantovi et al., 2005). The presence of this element is variable in composition and concentration, depending on factors such as methods of waste treatment and storage, among others (Sommers, 1977). Furthermore, the residue of the olive oil production is one of the main residue generated in the Mediterranean regions (Taccari et al., 2009), producing annually more than 4 tons of waste. They contain organic matter, cellulose, hemicellulose, carbohydrates and lignin. Another organic residue is the sugar beet that is generated in sugar production, which is used mainly for animal feed (Medina et al., 2006). However some of these organic residues apart from containing significant amounts of nutrients contain also high amounts of toxic elements which

restrict the use of them as amendment. Sewage sludge may contain significant amounts of heavy metals, pathogens and organic contaminants such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls (Dean & Suesst, 1985, Xin Hu et al., 2005) as well as olive mill waste presents large amounts of phenolic compounds (Alburquerque et al., 2004, Aranda et al., 2006).

The addition of amendments to soil has the positive effect of improving soil fertility, but it also contains elements that can cause toxic effects on crops. The application of sewage sludge to land is a usual practice for over 40 years in U.S.A., Canada and Europe (Warman &Termeer, 2005), they provide the new physical, chemical and biological soil properties (Tsadilas et al., 1995). The sewage sludge application can increase short term macro porosity, bulk density, electrical conductivity or soil stability due to the amount of organic matter, helping to the erosion processes (Cuevas et al., 2006). Many studies show that application of olive mill waste increases organic carbon, nitrogen, phosphorus, potassium ,electrical conductivity and pH and in soil (Brunetti et al., 2007, Lopez-Piñeiro et al., 2008, Altieri & Esposito, 2010, Karpouzas et al., 2010). Mahmoud et al., (2010) show that organic carbon increased from 2.3 g kg^{-1} to 39.1 g kg^{-1} and C/N ratio of 2.9 to 10 with the application of olive mill wastewater after 15 years. While the implementation of these residues promotes parameters such as soil nutrient status and biological activity, it also can have negative effects. Mahmoud et al., (2010) observed that after 15 years of application of olive mill waste, conductivity water and soil infiltration decreased, which could cause problems such as water pollution. Similarly, the continuous application of sewage sludge can increase the amounts of heavy metals (Yongjie &Yangsheng, 2005), whose toxic effect depends not only on the concentration, but also on bioavailability (Renoux et al., 2001). The toxicity of this sewage sludge is regulated by biological processes and variables as: Physical and chemical properties

of soil, pH, organic matter, oxides of Fe, Mn and Al, clay content and cation exchange capacity (Jones & Jarvis, 1981, Katanda et al., 2007). The metals of this residue can persist for long periods in the environment can be captured by plants and may even be accumulate in the tissues of grazing animals (Wilkinson et al., 2001) or be leaching into bodies of water, causing a deterioration of environmental health (McBride et al., 1997, Bhogal et al., 2003). Changes in physical and chemical parameters of soil due to the application of different organic residues are shown in Table 3. Brunetti et al., (2007) reported that an increase in the productivity of wheat plants resulted in a rise in grain number, weight and density of the spikes by adding catalytically digested wastewater. Altieri & Esposito (2010) observed increases in *L. sativum* and *L. esculentum* plants amended with olive mill residue similar to those promoted by an inorganic fertilizer. Lopez-Piñeiro et al., (2008) reported that in mediterranean soils amended with residues from the processing of two-phase olive mill waste and d-oiled two-phase olive mill waste, an increase in nitrogen, potassium and phosphorus content in olive trees was observed, reaching the highest level in 5 years. However, the direct application of these residues without a proper management leading to the reduction of toxic elements can cause a negative effect on crops. Saadi et al., (2007) showed that short-term application of high doses of olive mill wastes was toxic to cress seeds, which only reached 20% germination and root growth. This fact was attributed mainly to low pH of waste, high amount of phenols (2500 mg L^{-1}) and high salinity.

3.4.1. Effects on the soil biochemical and biological parameters.

The application of organic amendments causes an effect on soil microorganisms and their activities. Generally addition of organic residues improves soil enzymes activities such as β-glucosidase, phosphatase and dehydrogenase (Medina et al., 2005). Saadi et al., (2007) reported an increase in Fluorescein diacetate (FDA) activity and respiration rate in short-term,

after the application of olive mill waste. Similarly, Karpouzas et al., (2010) observed changes in the structure of actinomycetes and aerobic ammonia-oxidizing bacteria. Piotrowska et al., (2011) showed an increase in dehydrogenase activity of 8.72 and 4.25 $TPFg^{-1}$ after application of crude olive mill waste and dephenolized olive mill waste. Studies performed by Sampedro et al., (2009) show that application of dry olive mill residue does not decrease bacterial or fungal activity, it even encourages fungal activity. In this study an increase in dehydrogenase and FDA activities with respect to control was observed corresponding to soil without amendment, attributable to the increase in microorganisms due to the addition of substrate. Lakhdar et al., (2010) applied two doses of municipal solid waste compost and two doses of sewage sludge and observed a differential effect with respect to different types and doses of amendment. It was observed in this case an increase in enzyme activities as β-glucosidase, urease and especially by dehydrogenase and catalase.

On the other hand, it is necessary to handle the residues previous to application for reducing the negative impact that they may produce on soil or directly on crops, decreasing the amounts of heavy metals, phenols and other contaminants in order to improve their nutritional properties that have a direct effect on increasing crop productivity. Several saprophytic fungi are used for the previous treatment of residue application in order to reduce possible toxic effects of these. *Phanerochaete chrysosporium* is capable of reducing significantly phenol content of olive mill waste combined with lignocellulosic residues, reducing the phytotoxic effects on plants (Taccari et al., 2009). *Pycnoporus cinnabarinus* and *C. rigida* reduce the amount of tyrosol and hydroxytyrosol of olive mill waste after 5 and 15 days of incubation respectively, associated with the action of ligninolytic enzymes that participate in the removal of these phenolic compounds (Sampedro et al., 2004, Aranda et al., 2006). Similarly, *F. lateritium* reduced completely hydroxytyrosol, catechol, vanillic and

dimethoxy ciannamic acid contents after 20-week incubation (Sampedro et al., 2005). The direct application of dry olive mill residue reduces shoot and root dry weight and mycorrhizal colonization, it decreases mainly due to the presence of phenols. However, after the treatment of residue for 20 weeks it reduces significantly the amount of phenols from 27 g kg^{-1} to 4.5, 5.2, 5.7 g kg^{-1} for *Paecilomyces farinosus*, *C. rigida* and *Poria subvermispora*, respectively. Finally, addition of dry olive mill residue treated with these fungi increased growth of *Solanum lycopersicum* and *Medicago sativa*, especially when plants were inoculated with *G.deserticola* (Sampedro et al., 2008). Similarly, Medina et al., (2010) showed that the application of dry olive cake mixed with rock phosphate, handled with *Aspergillus niger* in semi-arid soils, promoted the growth of *Trifolium repens* plants inoculated with arbuscular mycorrhizal. On the other hand, this residues handled with *P. chrysosporium* added to *Dorycnium pentaphyllum* plants inoculated with AM fungi increased the phosphorous content in shoot ina 933% respect to the control, without residue and AM fungi. The application of sugar beet biotransformed by *A. niger* increased in 1089% growth of mycorrhizal *T. repens* plants in soils contaminated with cadmium, showing strong root development also when applying sugar beet combined with phosphate rock. It increased significantly the amount of phosphorus in the shoot and cadmium was reduced, decreasing the toxic effect of this factor (Medina et al., 2005), whereas without the application of the amendment and the presence of mycorrhizal fungi, there was not establishment. Similarly, an amendment promotes plant growth in soils contaminated with Zn (Medina et al., 2006).

3.6.Conclusions and future research trends

Several studies show the benefits and mechanisms by which plant growth promoting fungi both free living fungi and arbuscular mycorrhizal fungi are able to promote plant growth. It has been described that several fungi are efficient in the biotransformation process of different organic residues avoiding the toxic effects of elements such as heavy metals or phenols generating biotransformed residues that can improve physical, chemical and biological soil properties, thus improving plant growth. However, it is still necessary to understand which are the main mechanisms and how they interact in the transformation of organic residues in order to optimize the process and so be able to generate these amendments in a larger scale.

Table 1 Effects of saprophytic fungi on plant growth

Saprophytic fungi	Plants	Effect (%)	Experimental conditions	References
		Shoot and root dry weight		
	S. vulgare	41/55		Fracchia et al., (2000)
F. oxysporum	*L. esculentum*	128/x		
	P. sativum	91/5		
		Shoot N/P/Kcontent		
T. harzianum	*C. melo*	27/137/27	Greenhouse condition with reduced fertilizer dose 1/3 (0.51 gL^{-1} NH_4NO_3, 0.51 gL^{-1} $NH_4H_2PO_4$.)	Martínez-Medina et al., (2011)
	C. melo	19/7/9	Conventional fertilizer dose 0.51 gL^{-1} NH_4NO_3 , 0.51 gL^{-1} $NH_4H_2PO_4$.	
		Shoot and root dry weight.		
A.niger		26/34	Growth chamber	Yadav et al., (2011)
P.citinum	*C. arietinum*	47/58		
T. harzianum		39/58		
		Reducing area of anthracnose lesion by *C. orbiculare*		
Phoma GS8-2		82	Incubated in growth chamber	Chandanie et al., (2006)
Phoma GS8-3	*C. sativus*	70		
P. simplicissimum		78		
		Increasing shoot and root dry weight		
W.inflatus		13/13	Unsterilised soil with sterilised quartz sand in proportions of 1/5	
G.roseum	*G. max*	3/43		Godeas et al.,(1999)
T.harzianum		7/84		
P.farinosus		22/28		
T.pseudokoningii		28/56		
		Increasing shoot and root dry weight		
A.niger		1/-9		
P.restrictum	*G. max*	14/-2	Greenhouse condition with sterilized soil	Fracchia et al.,(2004)
T.harzianum		17/-13		
		Increasing shoot and root dry weight		
F.concolor	*E. globulus*	1/6		
T.koningii		1/12	Heavy metal contaminated soil	Arriagada et al.,(2007)
F.concolor	*E. globulus* + *G. max*	1/2		
T.koningii		2		

Table 1 (Continued)

Saprophytic fungi	Plants	Effect (%)	Experimental conditions	References
		Increasing shoot and decreasing root dry weight	Incubated in greenhouse and inoculated at diferent times.	
A. alternata	*Z. mays*	1/4	Two weeks before *G.mosseae*	
		1/3	The same time *G.mosseae*	
		8/-2	Two weeks after *G.mosseae*	
F.equiseti	*Z. mays*	-6/9	Two weeks before *G.mosseae*	McAllister et al.,(1997)
		3/12	The same time that *G.mosseae*	
		11/8	Two weeks after *G.mosseae*	
A. alternata	*L. sativa*	27/-1	Two weeks before *G.mosseae*	
		12/36	The same time that *G.mosseae*	
		-9/-9	Two weeks after *G.mosseae*	
		Protection from damping-off disease caused by *R. solani.*	Incubated in growth chamber Inoculation at diferent times	
P.simplicissimum	*C. sativus*	38	Simultaneously with the pathogen	
		54	7 days prior to the pathogen infection.	Chandanie et al.,2009
		53	12 days prior to the pathogen infection.	
T.harzianum	*C. sativus*	22	simultaneously with the pathogen	
		48	7 days prior to the pathogen infection.	
		56	12 days prior to the pathogen infection.	
		Protection of disease caused by *C. orbiculare*	Plants growing in potting medium.	
Trichoderma GT3-2	*C. sativus*	14	Amended with barley grain inocula.	
Fusarium GF19-2		65		Koike et al., (2001)
Penicillium GP17-2		66		
Phoma GS8-2		73		
Sterile GU23-3		76		
Trichoderma GT3-2	*C. sativus*	59	Unamended potting medium, roots treated with mycelial inocula	
Fusarium GF19-2		51		
Penicillium GP17-2		85		
Phoma GS8-2		83		
Sterile GU23-3		79		

Table 1 (Continued)

Saprophytic fungi	Plants	Effect (%)	Experimental conditions	References
		Protection of disease caused by *P. syringae* pv. *lachrymans*		
Trichoderma GT3-2	*C. sativus*	52	Unamended potting medium, roots treated with culture filtrates	Koike et al., (2001)
Fusarium GF19-2		76		
Penicillium GP17-2		82		
Phoma GS8-2		76		
Sterile GU23-3		80		
Trichoderma GT3-2	*C. sativus*	10	Amended with barley grain	Koike et al., (2001)
Fusarium GF19-2		19		
Penicillium GP17-2		52		
Phoma GS8-2		32		
Sterile GU23-3		59		
		Increasing shoot dry weight.	Incubated greenhouse in sterilized soil and sand (1:1) with arsenic.	
T.versicolor	*E. globulus*	8	50 mg As kg^{-1}.	Arriagada et al.,(2009a)
T.harzianum		8	100 mg As kg^{-1}.	
		Increasing shoot and root dry weight.	Incubated greenhouse in sterilized soil and sand (1:1) with Cu.	
T.versicolor	*E. globulus*	5/5	0 mg Cu kg^{-1}.	
		4/5	10 mg Cu kg^{-1}.	
		11/4	100 mg Cu kg^{-1}.	
		3/8	1000 mg Cu kg^{-1}.	
		3/15	2000 mg Cu kg^{-1}.	
C.rigida	*E. globulus*	-5/-3	0 mg Cu kg^{-1}.	Arriagada et al., (2009b)
		-8/4	10 mg Cu kg^{-1}.	
		9/4	100 mg Cu kg^{-1}.	
		15/-3	1000 mg Cu kg^{-1}.	
		52/5	2000 mg Cu kg^{-1}.	
		Shoot and root dry weight.	Incubated greenhouse in sterilized soil and sand (1:1) with	
F.concolor	*G. max*	23	0 mg Cd kg^{-1}.	
		-8	25 mg Cd kg^{-1}.	
		17	50 mg Cd kg^{-1}.	Arriagada et al., (2004)
T.koningii	*G. max*	10	0 mg Cd kg^{-1}.	
		12	25 mg Cd kg^{-1}.	
		-8	50 mg Cd kg^{-1}.	
F.concolor	*E. globulus*	-6	0 mg Cd kg^{-1}.	
		4	25 mg Cd kg^{-1}.	
		-6	50 mg Cd kg^{-1}.	
T.koningii	*E. globulus*	-11	0 mg Cd kg^{-1}.	
		-1	25 mg Cd kg^{-1}.	
		3	50 mg Cd kg^{-1}.	

Table 2 Synergistic effects of mycorrhizal fungi and saprophytic fungi on plant growth

Saprophytic fungi	Mycorrhizal fungi	Host Plants	Effect (%)	Experimental conditions	References
			shoot/ root dry weight		
F. oxysporum	*G. mosseae*		93	Sterilized soil	
	G. deserticola		108		Fracchia et al., (2000)
F. oxysporum	*G. mosseae*		58/33	Unsterilized soil	
	G. deserticola		89/14		
			Reducing area of anthracnose lesion by *Colletotrichum orbiculare*	Incubated in growth chamber	
Phoma GS8-2	*G. mosseae*	*C.* sativus	63		Chandanie et al., (2006)
Phoma GS8-3	*G. mosseae*		57		
P. simplicissimum	*G. mosseae*		77		
			Increasing shoot and root dry weight.	Incubated in greenhouse and inoculated at diferent times	
A. alternata	*G. mosseae*	*Z. mayz*	16/11	Two weeks before *G.mosseae*	
			26/2	The same time *G. mosseae*	McAllister et al., (1997)
			50/5	Two weeks after *G.mosseae*	
F.equiseti	*G. mosseae*	*Z. mays*	4/4	Two weeks before *G.mosseae*	
			29/9	The same time *G.mosseae*	
			53/25	Two weeks after *G.mosseae*	
A.alternata	*G.mosseae*	*L. sativa*	22/-2	Two weeks before *G.mosseae*	
			33/-2	The same time *G.mosseae*	
			61/20	Two weeks after *G.mosseae*	
F.equiseti	*G.mosseae*	*L. sativa*	-10/26	Two weeks before *G.mosseae*	
			26/49	The same time *G.mosseae*	
			47/33	Two weeks after *G.mosseae*	

Table 2 (Continued)

Saprophytic fungi	Mycorrhizal fungi	Host Plants	Effect (%)	Experimental conditions	References
			Increasing shoot and root dry weight.	Heavy metal contaminated soil	
F.concolor	*G. mosseae*		21/-17		
F.concolor	*G. deserticola*	*E.globulus*	40/-13		
T.koningii	*G. mosseae*		22/-18		
T.koningii	*G. deserticola*		40/-16		
F.concolor	*G. mosseae*		38/-25		Arriagada et al., (2007)
F.concolor	*G. deserticola*	*E. globulus* + *G. max*	84/-25		
T.koningii	*G. mosseae*		14/-24		
T.koningii	*G. deserticola*		87/-21		
			Increasing fresh shoot weight	Greenhouse condition	
T.harzianum	*G. constrictum*	*C. melo*	15	Reduced fertilizer dose 1/3 (0.51 gL-1 NH_4NO_3, 0.51 gL^{-1} $NH_4H_2PO_4$.)	
	G. mosseae		27		
	G. claroideum		6		
	G. intraradices		18		Martinez-Medina et al., (2011)
T.harzianum	*G. constrictum*	*C. melo*	1	Conventional fertilizer dose 0.51 gL^{-1} NH_4NO_3 , 0.51 gL^{-1} $NH_4H_2PO_4$.	
	G. mosseae		13		
	G. claroideum		7		
	G. intraradices		11		
			Increasing shoot and root dry weight.		
A.niger	*G.mosseae*	*G. max*	48/87		
	Gi.rosea		37/46		
P.restrictum	*G.mosseae*	*G. max*	1/8	Greenhouse condition with sterilized soil	Fracchia et al.,(2004)
	Gi.rosea		-47/-45		
T.harzianum	*G.mosseae*	*G. max*	10/34		
	Gi.rosea		-4/19		

Table 2 (Continued)

Saprophytic fungi	Mycorrhizal fungi	Host Plants	Effect (%)	Experimental conditions	References
			Increasing shoot and root dry weight.	Unsterilised soil with sterilised quartz sand in proportions of 2/3	
W.inflatus	*G.mosseae*	*G. max*	-20/3		
G.roseum			-11/20		
T.harzianum			7/-8		
P.farinosus			6/19		Godeas et al.,(1999)
T.pseudokoningii			5/-6		
W.inflatus			-31/4	Unsterilised soil with sterilised quartz sand in proportions of 1/5	
G.roseum			-41/32		
T.harzianum			34/42		
P.farinosus			93/79		
T.pseudokoningii			86/58		
			Increasing shoot and root dry weight.	Incubated in greenhouse and inoculated at diferent times	
A.niger	*G.mosseae*	*Z. mays*	30/2	The same time that *G.mosseae*	
			55/-9	Two weeks after *G.mosseae*	
			17/-6	Two weeks before *G.mosseae*	McAllister et al., (1995)
A.niger	*G.mosseae*	*L. sativa*	-11/3	The same time *G.mosseae*	
			78/34	Two weeks after *G.mosseae*	
			21	Two weeks before *G.mosseae*	
			Protection from damping-off disease caused by *R. solani*.	Incubated in growth chamber Inoculation at diferent times	
			40	Simultaneously with the pathogen	Chandiane et al., (2009)
			71	7 days prior to the pathogen infection.	
P.simplicissimum	*G.mosseae*	*C. sativu*	72	12 days prior to the pathogen infection.	
			24	Simultaneously with the pathogen	
			61	7 days prior to the pathogen infection.	
T.harzianum	*G.mosseae*	*C. sativu*	68	12 days prior to the pathogen infection.	
			Root length colonized by mycorrhizal fungi.	Growing under axenic condition	
			75	Inoculation at the same time of mycorrhizal fungi	McAllister et al.,1994
T.koningii	*G.mosseae*	*Z.mays*	46	Inoculation two weeks after of mycorrhizal fungi.	
			63	The same time of mycorrhizal fungi	
F.solani	*G.mosseae*	*Z.mays*	56	Two weeks after of mycorrhizal fungi	

Table 2 (Continued)

Saprophytic fungi	Mycorrhizal fungi	Host Plants	Effect (%)	Experimental conditions	References
			Root length colonized by mycorrhizal fungi.		
R. mucilaginosa	*G.mosseae*	*G. max*	43/43	Two weeks before *G.mosseae*	Sampedro et al., (2004)
			6/6	Two weeks after *G.mosseae*	
			-5/-2	The same time *G.mosseae*	
C. laurentii	*G.mosseae*	*G. max*	19/20	Two weeks before *G.mosseae*	
			41672	Two weeks after *G.mosseae*	
			2	The same time *G.mosseae*	
S. kunashirensis	*G.mosseae*	*G. max*	46/40	Two weeks before *G.mosseae*	
			10/10	Two weeks after *G.mosseae*	
			-2/-3	The same time *G.mosseae*	
			Increasing shoot dry weight.	Incubated greenhouse in sterilized soil and sand (1:1) with As.	
T.versicolor	*G.mosseae*	*E. globulus*	33	0 mg As kg^{-1}.	
			46	25 mg As kg^{-1}.	
			58	50 mg As kg^{-1}.	
			58	75 mg As kg^{-1}.	
			58	100 mg As kg^{-1}.	
T.harzianum	*G.mosseae*	*E. globulus*	40	0 mg As kg^{-1}.	
			46	25 mg As kg^{-1}.	
			58	50 mg As kg^{-1}.	
			58	75 mg As kg^{-1}.	
			67	100 mg As kg^{-1}.	
T.versicolor	*G.claroideum*	*E. globulus*	40	0 mg As kg^{-1}.	
			54	25 mg As kg^{-1}.	
			67	50 mg As kg^{-1}.	Arriagada et al.,(2009a)
			58	75 mg As kg^{-1}.	
			67	100 mg As kg^{-1}.	
T.harzianum	*G.claroideum*	*E. globulus*	73	0 mg As kg^{-1}.	
			100	25 mg As kg^{-1}.	
			125	50 mg As kg^{-1}.	
			125	75 mg As kg^{-1}.	
			133	100 mg As kg^{-1}.	

Table 2 (Continued)

Saprophytic fungi	Mycorrhizal fungi	Host Plants	Effect (%)	Experimental conditions	References
			Increasing shoot and root dry weight.	Incubated greenhouse in sterilized soil and sand (1:1) with Cu.	
T.versicolor	*G.deserticola*	*E. globulus*	29/40	0 mg Cu kg^{-1}.	
			23/48	10 mg Cu kg^{-1}.	Arriagada et al.,(2009b)
			75/115	100 mg Cu kg^{-1}.	
			121/132	1000 mg Cu kg^{-1}.	
			152/120	2000 mg Cu kg^{-1}.	
C.rigida	*G.deserticola*	*E. globulus*	46/36	0 mg Cu kg^{-1}	
			39/53	10 mg Cu kg^{-1}.	
			111/112	100 mg Cu kg^{-1}.	
			169/142	1000 mg Cu kg^{-1}.	
			386/30	2000 mg Cu kg^{-1}.	
			Shoot and root dry weight.	Incubated greenhouse in sterilized soil and sand (1:1) with Cd.	
F.concolor	*G.deserticola*	*G. max*	51	0 mg Cd kg^{-1}.	
			20	25 mg Cd kg^{-1}.	
			58	50 mg Cd kg^{-1}.	
T.koningii	*G.mosseae*		33	0 mg Cd kg^{-1}.	
			16	25 mg Cd kg^{-1}.	
			-17	50 mg Cd kg^{-1}.	Arriagada et al.,(2004)
T.koningii	*G.deserticola*		67	0 mg Cd kg^{-1}.	
			32	25 mg Cd kg^{-1}.	
			67	50 mg Cd kg^{-1}.	
F.concolor	*G.mosseae*	*E. globulus*	3	0 mg Cd kg^{-1}.	
			6	25 mg Cd kg^{-1}.	
			6	50 mg Cd kg^{-1}.	
F.concolor	*G.deserticola*		28	0 mg Cd kg^{-1}.	
			50	25 mg Cd kg^{-1}.	
			31	50 mg Cd kg^{-1}.	
T.koningii	*G.mosseae*		-3	0 mg Cd kg^{-1}.	
			12	25 mg Cd kg^{-1}.	
			7	50 mg Cd kg^{-1}.	
			Percentage of internal colonization	Sterilized peat-perlite (1:1) in growth chamber	
T.aureoviride	*G.mosseae*		35	Inoculation with *T. aureoviride prior* to introduction of *G.mosseae.*	Calvet et al.,1993
T.aureoviride	*G.mosseae*	*T. erecta*	14	Medium infested with *P. ultimum var. Ultimum.*	

Table 3 Properties of soil amended with different organic residues

	OC (g kg^{-1})	N (g kg^{-1})	EC (µS cm^{-1})	pH	P (mg kg^{-1})	CEC (cmol kg^{-1})	Ca (cmol kg^{-1})	Mg (cmol kg^{-1})	K (cmol kg^{-1})	C/N	
Control		0.68	226	6.96	3.5	26.71	15.96	3.72	0.15		Altieri et al., 2010
SP		0.91	171	6.84	6.6	26.68	14.68	3.43	0.36		
C		0.89	326	7.11	6.5	28.19	13.05	5.12	0.40		
SPF		0.86	266	6.95	6.8	27.96	14.79	3.58	0.42		
CF		0.76	217	6.95	6.0	24.33	12.98	3.06	0.29		
Control	23	0.8		7.93						2.86	Mahmoud et al., 2010
OMW5	25	2.3		7.73						10.87	
OMW15	39.1	3.9		7.81						10.03	
									(mg kg^{-1})		López-Piñeiro et al., 2008
Control	11.6	1.26	427	8.14	10				235		
TPOMW1	14.5	1.62	620	8.06	19.8				491		
TPOMW2	18.8	1.66	592	7.97	30.4				467		
DTPOMW1	14.3	1.64	475	8.12	15.1				325		
DTPOMW2	16	1.73	549	8.02	23.8				370		
								mg g^{-1}			
Control	2	0.23		7.9	20		14.70	0.25	0.14	8.7	Mekki et al., 2006
P1	8	0.56		7.9	30		19.80	0.35	1.05	14.29	
P2	15	0.95		7.6	80		16.20	0.40	1.60	16.32	
P3	16	0.91		7.4	80		15.80	0.37	1.80	18.68	
									(mg kg^{-1})		Brunetti et al., 2007
Control	10.3	1.0		8.0	33				186	10	
LW300	11.8	1.0		7.8	48				321	11.5	
LW600	13.8	1.1		7.9	70				476	13.1	
CW300	12.4	1.1		7.9	52				326	11.1	
CW600	14.5	1.1		7.8	68				460	12.6	
Control		0.09		4.1		5	1.01	0.41	0.24		Katanda et al., 2007
SI		0.18		5.5		9.7	5.34	2.97	0.15		
Control	2.5	1.2	647	8.27			0.16		0.25	2.1	Lakhdar et al., 2010
C1	10.9	1.8	741	8.28			0.17		0.27	6.1	
C2	12.7	1.9	843	8.31			0.17		0.24	6.7	
S1	10.1	1.8	731	8.26			0.22		0.23	5.6	
S 2	13.7	2.1	863	8.22			0.34		0.26	6.5	
Control	8.2	0.05			10.9		3.0	0.4	0.3	17.1	Kidd et al., 2007
	5.9	0.06			131.2		42	0.7	0.9	10	

Control: unamended, SP: stack pile, C: compost, SPF: stack pile + mineral fertilization, CF: compost + mineral fertilization, OMW5: olive mill waste irrigated during 5 years, OMW10: olive mill waste irrigated during 10 years, TPOMW1: 30 Mg ha^{-1} two- phase olive mill waste, TPOMW2: 60 Mg ha^{-1} two- phase olive mill waste, DTPOMW1: 27 Mg ha^{-1} de-oiled-two phase olive mill waste, DTPOMW2: 54 Mg ha^{-1} d-oiled-two phase olive mill waste P1,P2, and P3: Soils amended with 50, 100, and 200 m^3 h^{-1} of untreated OMW respectively,LW 300:lagooned digested olive at rates of 300 m^3 ha^{-1}, LW 600: lagooned digested olive at rates of 600 m^3 ha^{-1}, CW300: catalytically oil mill wastewater rates of 300 m^3 ha^{-1} CW600: : catalytically oil mill wastewater rates of 600 m^3 ha^{-1},SI: Sewage irrigated, C1: soil amended with 13.3 g kg-1 of MSW compost; C2: soil amended with 26.6 g kg-1 of MSW compost; S1: soil amended with 13.3 g kg^{-1} of sewage sludge; S2: soil amended with 26.6 g kg^{-1} of sewage sludge.

CHAPTER III

Optimization of organic residues by their inoculation and incubation with native saprophytic fungi

Manuscript in preparation

Optimization of organic residues by their inoculation and incubation with native saprophytic fungi

Leonardo Almonacid and Cesar Arriagada

Center of Amelioration and Sustainability of Volcanic Soils. BIOREN. Bioremediation Laboratory. Universidad de La Frontera. P.O. Box 54-D. Temuco-Chile.

Abstract

Several industrial processes and agricultural practices generate large amounts of residues which are considered beneficial to their land application. The sewage sludge and crop residues can be used as an soil amendment to improve chemical, physical and biological soil parameters improving soil fertility. These residues contain high levels of organic matter and nutrients, which previously handled could be used as amendment in agroforestry systems. The saprophytic fungi are natural inhabitants of soil, degrade metabolic and structural constituents of plant debris and dead animals by their enzymes production capacity. In this context, the aim of this work was to evaluate the effect of some ligninolytic and hydrolytic enzymes in the process of degradation and optimization of the organic residues. These residues consist in wheat straw combined with sewage sludge. In the *in vitro* experiments the highest activity was presented for laccase and manganese independent peroxidase mainly in the organic residues inoculated with *Coriolopsis rigida.* Similarly, this fungus showed high values of Fluorescein diacetate activity compared with *Trichoderma harzianum.* The dry matter, cellulose, carbon and nitrogen content decreased after 4 weeks of incubation with both saprophytic fungi. Besides, the pH decreased when the time of incubation increased. Thus, our results suggest the utility potential of these microorganisms in the optimization process of organic residues and the possibility of development of these combined residues as a alternative of organic soil amendment.

Key words: Organic amendment, *Coriolopsis rigida, Trichoderma harzianum,* ligninolytic enzymes.

3.1 Introduction

The organic soil amendments are widely used to improve chemical, physical and biological soil properties increasing their fertility as well as plant nutrition. There are several types of organic residues used as amendments in agroforestry systems, including crop residues, sugar beet, olive mill residue or sewage sludge from wastewater treatment plant. Crop residues can be used as a source of nitrogen or phosphorus (Cassman et al., 1996) increasing crop yield of agricultural soils. This residue is composed of lignin, cellulose and hemicellulose being and an important source of organic matter (Howard et al., 2003). On the other hand, sewage sludge generated in wastewater treatment plants contains large amounts of organic matter, nitrogen and phosphorus and micronutrients as copper, zinc, molybdenum, boron, iron, magnesium (He et al., 2000, Mantovi et al., 2005).

The possibility of combination of these residues can improve the properties of both, and generate a better quality substrate. Some characteristics of sewage sludge, such as moisture, C/N ratio and pH are not optimal for the degradation process (Petric et al., 2009). Wheat straw is an essential nutrient and carbon source that promotes the development and increases the metabolic activity of microorganisms. However, being a lignocellulose residue their decomposition is very slow due to the high recalcitrance of lignin. The combination of sewage sludge with wheat straw as bulking agent may optimizing the substrate properties. The input of carbon provided by the wheat straw stabilizes the C/N ratio which is an important parameter in the biodegradation process of estimating an optimal of 25 to 30 C/N ratio for composting is proposed (Huang et al., 2004). On the other hand, the wheat crop residues combined with sewage sludge decreases moisture and increases the particle size of substrate which improve aeration, avoiding nitrogen losses and also improving fungi colonization (Bhamidimarri & Pandey et al., 1996, Barrington et al., 2002). Another

important aspect for the viability and metabolism of fungi is the pH (Yang & Liau, 1998). The sewage sludge before leaving from wastewater treatment plant is stabilized usually with $CaCO_3$ so that the pH is basic, with the combination of residues we can reduce pH values thus improving the development of microorganisms.

On the other hand, some microorganisms, especially white rot fungi, are capable of degrading several compounds usually resistant to microbial action, mainly due to the production of broad-spectrum enzymes (Matsubara et al., 2006). The production of these enzymes has been determined in different species of saprophytic fungi in the biotransformation of residues (Pelaez, et al., 1995, Dinis et al., 2009). The enzyme activity is a efficient indicators of substrate quality (Avidano et al., 2005). Hydrolysis Fluorescein diacetate is used to estimate soil microbiological activities (Adam & Duncan, 2001) reflecting the activity of hydrolases involved in organic matter degradation (Sánchez-Monedero et al., 2008). Phosphatase is an enzyme involved in the phosphorus cycling, responsible for the mineralization of organic phosphorous in available inorganic phosphorous (Amador et al., 1997) increasing their availability by plants uptake.

The aim of this work was to evaluate ligninolytic and hydrolytic enzymes produced by saprophytic fungi involved in the process of degradation of wheat straw combined with sewage sludge.

3.2 Materials and methods

3.2.1 Saprophytic and mycorrhizal fungi

Two native saprophytic fungi from Rucamanque field were used: *Coriolopsis rigida*, and *Trichoderma harzianum*. These fungi were grown on potato dextrose agar (PDA) slants at 25ºC for 7 days. Stock cultures of the fungi were stored in PDA slants at 4º C. Strains were

provided by the fungal culture collection of the Bioremediation Laboratory, Universidad de La Frontera, Temuco, Chile.

3.2.2 Organic residues

Sewage sludge was collected from a wastewater plant (Vilcún, Chile) and stored at 4°C until use. Wheat straw was obtained from crop residues from the Araucanía Region. Wheat straw was mixed with sewage sludge (1:3 w:w) and called organic residue.

3.2.3 *In vitro* experiments

The effect of ligninolytic and hydrolytic enzymes of saprophytic fungi involved in the degradation and optimization process of combined residues was tested in a 250 mL Erlenmeyer flask autoclaved at 121 °C for 20 min.

For *C. rigida* inoculum, one slant of active mycelia from stock culture was diluted in 40 mL of sterile distilled water, 10 mL of this suspension, equivalent to 70 mg of dry mycelium, was added to the organic residues. *T. harzianum* was grown on a slant and spores were scraped in sterile distilled water; 3 mL of spore suspension, equivalent to 1.8 $x10^6$ spores, was spread over the surface of the organic residues. Each fungus was added to flasks with 40 g of sterilized organic residues (10 g wheat straw + 30 g sewage sludge). After the inoculation, the substrate was incubated 2 and 4 weeks at 23 ± 2 °C in darkness.

To analyzed the ligninolytic enzyme 10 g of combined residues was placed into a 100 mL flask and 25 mL of succinate lactate buffer was added, the flask was placed in orbital shaker for 1 h to 150 rpm. Laccase (Lac), manganese peroxidase (MnP), and manganese-independent peroxidase (MIP) activities were determined according to the method described by Castillo et al., (1997) by the oxidation of 2.6-dimethoxyphenol (DMP). Lac activity was performed in the reaction mixture with 200 µL of sodium malonate (250 mM, pH 4.5), 50 µL of 2,6-DMP

(20 mM) and 600 µL of sample. The MnP was determined from the same reaction mixture, which was added 50 µL of Mn^{2+} (20 mM), initiated by adding 100 µL of H_2O_2. Finally, MIP activity was determined in the reaction mixture with 200 µL sodium malonate (250 mM, pH 4.5), 50 µL of 2,6- DMP (20 mM), 1 mM EDTA and 550 µL of sample. This reaction was determined at 468 nm. The activity unit (U) was defined as the amount of enzyme needed to transform 1 mole of substrate per minute.

To evaluate hydrolytic enzymes were extracted with 50mM sodium-phosphate buffer at pH 6.5. The mixture was placed in shaker at 150 rpm for 1 h. Acid phosphatase was measuring using the buffer TRIS-HCl. TRIS is prepared at 0.1 M in distilled water and then with 20% HCl to adjust pH to 5, and FDA hydrolysis was performed according to Adam & Duncan (2001), 60 mM potassium phosphate buffer was used, pH 7.6. The enzymatic hydrolysis started by adding 0.4 ml FDA solution (1 mg ml^{-1}). The absorbance of filtrates was measured at 490 nm. The all enzymatic activities were measured after 0, 2 and 4 weeks. Three replicates were used within all experiments with the respective controls not incubated (0 weeks).

After of 4 weeks of incubation chemical characterization of organic residues was performed. Total N was measuring using a modified Kjeldahl method. The pH was determined in a 1:2.5 (v/v) suspension of substrate in H_2O by each sample. Cellulose was determined according to Goering et al., (1970), carbon and nitrogen content were determined using Flash EA 1112 Series LECO-TRUSPEC elemental analyser.

3.2.4 Statistical analysis

Statistical analyses were conducted in Statistica software. The percentage values were arcsine transformed for statistical analyses. The interaction among the main factors data were analyzed using a two way ANOVA followed by Tukey's multiple range test. Statistical significance was determined at $P< 0.05$.

3.3 Results and discussion

The most important mechanisms involved in the phenol compounds degradation is the production of ligninolytic and hydrolytic enzymes (Pérez et al., 1998, Hamman et al., 1999). The white-rot fungi as *C.rigida* are considered the most efficient degraders of lignin by the action of enzymes such as laccase, manganese peroxidase and manganese-independent peroxidase. After 4 weeks of incubation the main ligninolytic enzymes produced by *C.rigida* were Lac and MIP after one day of inoculation, decreasing when the time of incubation increased. Whereas, MNP was produced in lesser amount (Fig 1A). *C.rigida* is a white-rot fungus highly efficient in the degradation of organic matter especially by the production ligninolytic enzymes such as laccase that is a phenol oxidase capable of degraded a wide range of phenolic compounds. The high Lac and MIP activity after one day of incubation could be due to faster stimulation of enzymatic activity of microorganisms in presence of high organic matter content in the combined residues. Studies performed by Rodriguez et al., (2004) showed a high Lac and MIP activities in solid state fermentation in the first week of incubation. On the other hand, studies performed by Calvo et al., (1998) showed that theses enzymes activities are mainly expressed in cultured with high concentration of nitrogen. *T. harzianum* showed lower ligninolytic enzymes activity in inoculated and incubated organic residues (Figure 1B).

Organic residues inoculated and incubated with *C. rigida* and *T. harzianum* showed high FDA activity. The FDA activity reflects the activity of esterases and proteases enzymes involved in the microbial decomposition of organic matter (Schnürer & Rosswall, 1982).Therefore, both fungi would be promoting the degradation of organic residues (Figure 2). In relation to acid phosphatase activity organic residues inoculated and incubated with saprophytic fungi showed similar values compared with organic residues without fungi. This can be attributable to the

high amount of phosphorus present in the residue that inhibits the activity of this enzyme (Azcon & Barea, 1997).

Table 1 show a significant decreasing of dry matter, cellulose, nitrogen and carbon content after 4 weeks of incubation with both saprophytic fungi. The organic residues incubation with *C. rigida* and *T. harzianum* decreased dry matter content in a 35 and 34%, respectively. The cellulose content decreased in a 33 and 24% with *C.rigida* and *T.harzianum*, respectively. Similarly, carbon content decreased in a 4 and 19% by *C. rigida* and *T. harzianum*, whereas nitrogen content was decreased in a 39 and 26% by *C. rigida* and *T. harzianum*, respectively. The decrease in dry matter and cellulose percentage demonstrates the ability of both fungi to degrade organic matter. Similarly, the reduction of carbon content after the incubation time can be attributed to lose as CO_2 or CH_4 due to organic matter degradation (Tiquia et al., 2002). Besides, the reduction in nitrogen content may be due to the loss of nitrogen through gaseous emission such as NH_3 (Sommer, 2001).

One important aspect for the viability and metabolism of fungi is the pH. Wheat straw addition stabilizes the pH values of sewage sludge which before leaving from waste water treatment plant is stabilized with $CaCO_3$. In relation to pH in the degradation process of organic residues this present values that close to 8 in the treatments inoculated with saprophytic fungi compared with organic residues without fungi. However, organic residues incubated for 4 weeks with *C. rigida* or *T. harzianum* presented lower values compared with the organic residues without fungi (Figure 3).The pH values decreased when the residues were incubated for 2 to 4 weeks. This can be attributable to the degradation process by these fungi, where microorganisms through their metabolic activities decreased the pH of the medium (Kishan et al., 1993). Being these fungi described as highly efficient in by degrade compounds as lignin, being mainly used in the treatment and bioconversion of lignocellulosic residues (Bosco et al., 1999, Ruggeri & Sassi, 2003).

3.4 Conclusions

The production of ligninolytic enzymes such as, laccase and manganese independent peroxidase mainly produced by *C. rigida* indicates the ecological importance of this fungus in the processes of lignin degradation and transform organic residues degradation. Similarly, *C. rigida* showed higher values of FDA activity compared with *T. harzianum.* The incubation with both saprophytic fungi generates a degradation of the organic residue which can be attributed to the action of enzymes produced by these fungi. These results shows the potential of these microorganisms in the optimization process of organic residues and the possibility of development of these combined residues as a alternative of organic soil amendment the handled of organic residues.

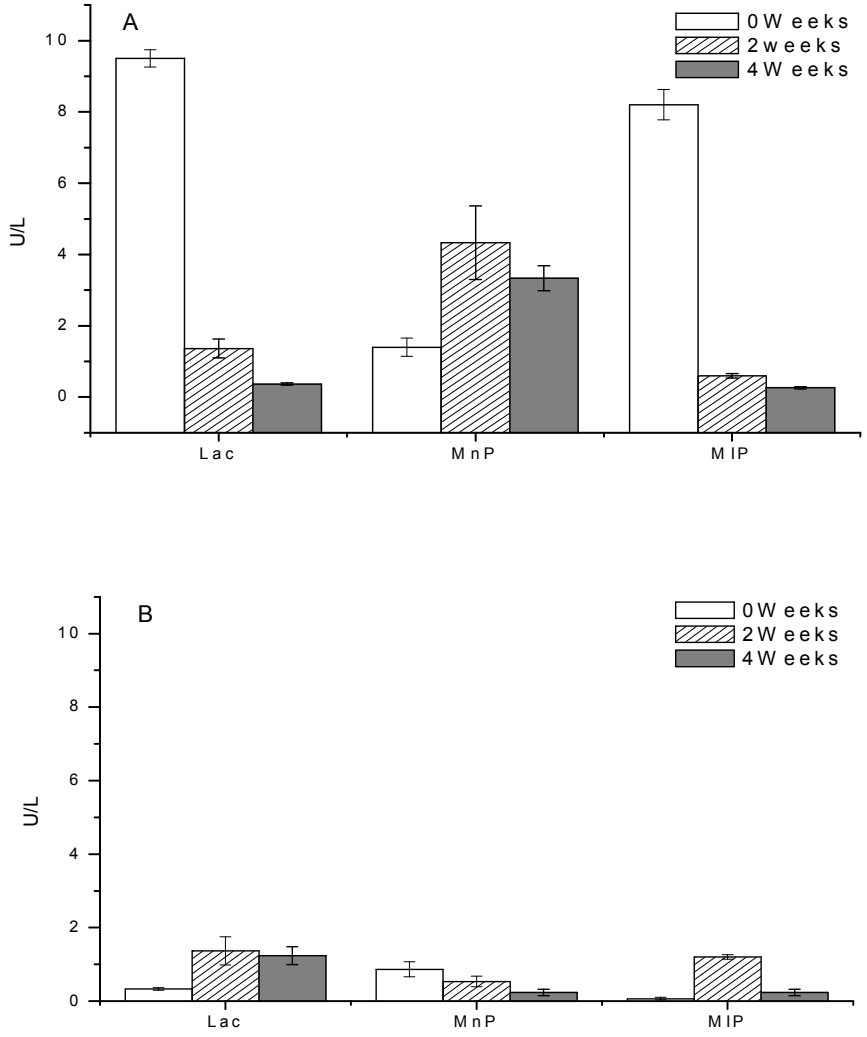

Figure 1. Ligninolytic enzymes activities Laccase (Lac), Mn-peroxidase (MnP) and Manganese-independent (MIP) peroxidase on organic residues inoculated and incubated with A) *Coriolopsis rigida* and B) *Trichoderma harzianum*. Data are the means ± standard error.

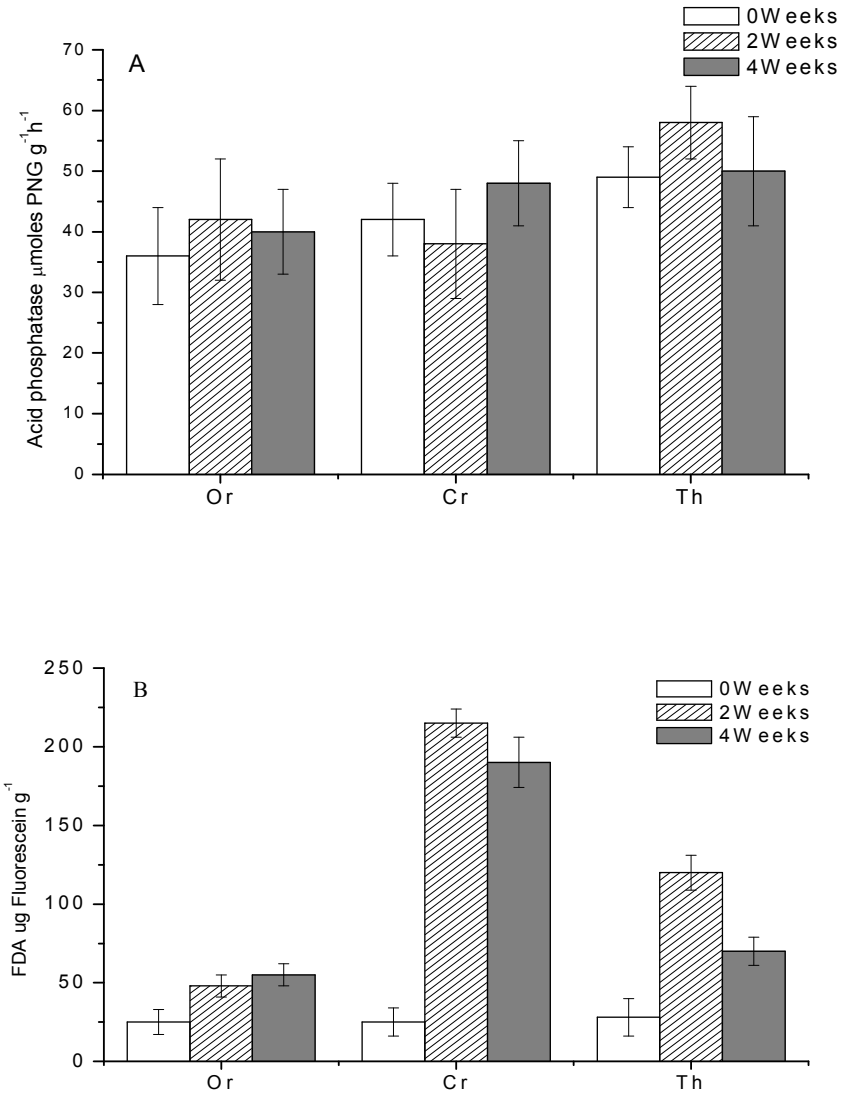

Figure 2. Biochemical parameters of organic residues inoculated and incubated with saprophytic fungi. A) Fluorescein diacetate and B) Acid phosphatase.Or: Organic residues Cr: *Coriolopsis rigida*, Th: *Trichoderma harzianum*. Data are the means ± standard error

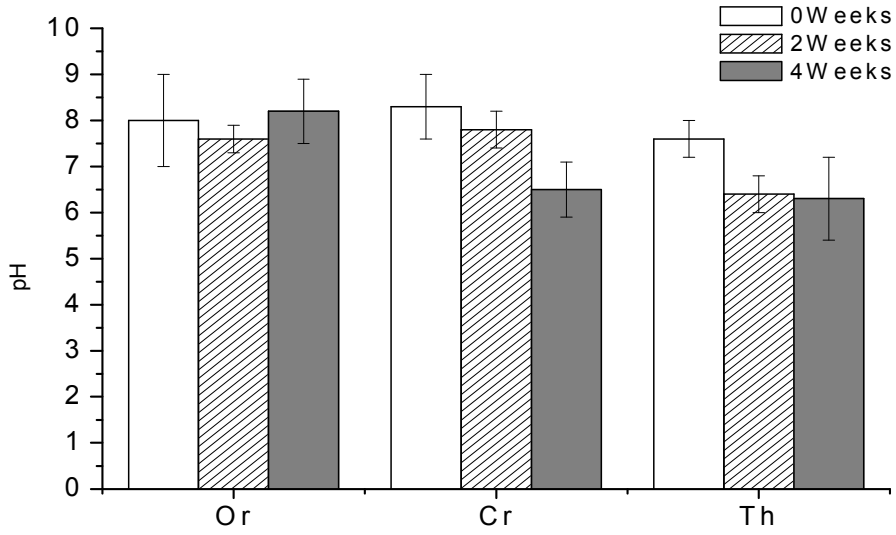

Figure 3.pH of organic residues inoculated and incubated with *Coriolopsis rigida* and *Trichoderma harzianum* for 2 and 4 weeks. Or: Organic residues, Cr: *Coriolopsis rigida*, Th: *Trichoderma harzianum*. The data are the means ± standard error.

Table 1: Chemical analysis of organic residues inoculated and incubated with *Coriolopsis rigida* and *Trichoderma harzianum*.

	Dry matter (%)	Cellulose	C (ppm)	N (ppm)
Control	67 b	28 b	255800 b	21540 b
***C. rigida* 0**	67 b	28 b	255000 b	20590 b
***C. rigida* 4**	43 a	19 a	244900 a	12500 a
***T. harzianum* 0**	69 b	26 b	255600 b	21130 b
***T. harzianum* 4**	45 a	19 a	208100 a	15700 a

Control:Organic residues, *C.rigida* 0: Organic residues inoculated with *C. rigida, C.rigida* 4: Organic residues incubated for 4 weeks with *C. rigida; T.harzianum* 0: Organic residues inoculated with *T.harzianum*; *T.harzianum*4: Organic residues incubated for 4 weeks with *T.harzianum.* Values followed by the same letter are not significantly different as determined by Tukey's multiple range test ($p<0.05$).

CHAPTER IV

Article published inArchives of Agronomy and Soil Science, 2014

Vol. 60, No. 9, 1229–1248, http://dx.doi.org/10.1080/03650340.2013.878455

Influence of an organic amendment comprising saprophytic and mycorrhizal fungi on soil quality and growth of *Eucalyptus globulus* in the presence of sewage sludge

Cesar Arriagada[a], Leonardo Almonacid[a], Pablo Cornejo[a], Inmaculada García-Romera[b]& Juan Ocampo[b]

[a]Laboratorio de Biorremediacion, Universidad de La Frontera, Temuco, Chile; [b]Estación Experimental del Zaidín, CSIC, Granada, España

Influence of an organic amendment comprising saprophytic and mycorrhizal fungi on soil quality and growth of *Eucalyptus globulus* in the presence of sewage sludge

Abstract

The single application of either sewage sludge with high aluminium concentration, wheat straw or the mixture of both residues to soil did not increase the growth of *Eucalyptus globulus* Labill. However, inoculation with either the arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis*, the saprophytic fungi *Coriolopsis rigida* (Berk.Et Mont.) Murrill and *Phanerochaete chrysosporium* Burds or the combination of each saprophytic with the AM fungus increased both the P concentration and dry weight of *E. globulus* shoots. These effects were greater in the presence of wheat straw or sewage sludge, but were greatest in the presence of the mixed residue. *P. chrysosporium* had the greatest effect on plant dry weight when co-inoculated with *R. irregularis* in the treatment with mixed residue. The co-inoculation of AM and saprophytic fungi increased fluorescein diacetate and β-glucosidase activities in the bulk soil of *E. globulus* grown in the treatment with mixed residue. However, only the AM fungus increased dehydrogenase activity, and phosphatase activity was similar in all treatments tested. Our results showed that sewage sludge with high aluminium concentration could be used as a soil amendment to improve the growth of *E. globulus* when mixed with wheat straw and co-inoculated with saprophytic and arbuscular fungi.

Keywords: arbuscular mycorrhizae; biological fertilizers; saprophytic fungi; sewage sludge, soil biochemical parameters, wheat straw.

4.1 Introduction

Agricultural soils are often subject to a decrease in organic matter due to agricultural practices that can alter the soils' physical, chemical and biological properties, and this can lead to a decrease in soil fertility. The use of sewage sludge as an organic soil amendment in agriculture is a widespread practice because the sewage sludge provides high levels of organic matter and plant nutrients, which can increase soil fertility (Hargreaves et al., 2008, Fagnano et al., 2011, Gonzalez-Ubierna et al., 2012). However, the potential presence of heavy metals in sewage sludge and the effects of these contaminants on plant and soil fertility are well known (Tella et al., 2013). High aluminium (Al) concentration has been found in sewage sludge (Busetti et al., 2005, Üstün 2009). In acidic conditions, Al phytotoxicity is one of the major factors limiting crop production (Kochian 1995, Von Uexküll & Mutert 1995).

The use of soil microorganisms is a suitable method to improve soil fertility and plant protection against the deleterious effect of soil contaminants such as Al (Batty & Dolan, 2013). Arbuscular mycorrhizal (AM) fungi are the most extended symbiosis among plant roots and many studies have demonstrated the efficiency of AM fungi in improving plant growth mainly by higher P uptake from soil through their external mycelium (Smith & Read 2008). AM fungi play a crucial role in plant tolerance against environmental stress as metal contamination, as Al resistance in plants has been ascribed to mycorrhizal symbiosis (Kanu et al., 2013, Seguel et al., 2013). Studies have shown that the addition of organic fertilizer can either reduce or increase the growth of AM fungi (Gryndler et al., 2009, Hammer et al., 2011). Most studies have found that sewage sludge reduces both the pre-symbiotic and in plant stages of the development of mycorrhizal fungus (Jacquot-Plumey et al., 2003, Ghanavati et al., 2012). In addition to AM fungi, saprophytic fungi are another important group of microorganisms that can provide energy for other microorganisms, including AM fungi, by breaking down cellulosic materials into simple sugars (Radford et al., 1996). Some

saprophytic fungi increase the effectiveness of AM fungi in root colonization and in the promotion of both plant growth and resistance against Al phytotoxicity (Arriagada et al., 2007,Vaz et al., 2012). Saprophytic fungi can also increase the metabolic activity of AM fungi inside the root in the presence of sewage sludge (Arriagada et al., 2009c).

The effects of sewage sludge as a fertilizer can be improved by applying different doses of wheat straw. In fact, the application of wheat straw to sludge increases N mineralisation and nutrient availability to plants and helps to reduce the toxicity of heavy metals (Jia et al., 2008, Juwarkar & Jambhulkar, 2008). Additionally, application of sewage sludge increases the biomass content and the enzymatic activities of microbes in the soil. Soil enzymes such as fluorescein diacetate hydrolase (FDA), β-glucosidase, phosphatase and dehydrogenase can be used as indicators of soil quality and microbial activity, because these enzymes are highly sensitive to alterations in soil management (Paz-Ferreiro et al., 2012).

Because of the positive impacts of mycorrhizal and saprophytic fungi on soil fertility and plant growth, the AM fungus *Rhizophagus irregularis* and the saprophytic fungi *C. rigida* and *P.chrysosporium* were examined for beneficial effects on FDA, β-glucosidase, dehydrogenase and phosphatase activities in the soil and on the growth and nutrient concentrations of *Eucalyptus globulus* shoots. The effects of these fungi were also analysed together with the application of sewage sludge with high Al concentration, wheat straw or a combination of sewage sludge and wheat straw, because these components have also been shown to have positive impacts on soil fertility and plant growth.

4.2 Materials and methods

4.2.1 Soil characteristics and measurements

Soil samples were collected at 0–20 cm depth from the Maquehue Experimental Station (38° 50′ S, 72° 41′ W) of La Frontera University, Araucania Region, Southern Chile. The soil is classified as Andisol (Acrudoxic Hapludands) from an agricultural area with pH 5.35. Soil pH was analysed in H_2O suspensions (1:2.5 w:v) at the beginning and end of experiment. Organic matter was estimated by wet digestion using the Walkley & Black (1934) method. The total organic C concentration was determined using dichromate oxidation followed by titration with ferrous ammonium sulphate. Olsen P was measured using the Olsen P test, in which inorganic P was extracted from soil with 0.5 $NaHCO_3$ pH 8.5 (Olsen & Sommers 1982); total P was determined using the alkaline oxidation method of Dick & Tabatabai (1977). Total N concentration was obtained using the Kjeldahl method. Exchangeable cations (Ca^{2+}, Na^{+}, Mg^{2+} and K^{+}) were extracted with 1 M ammonium acetate at pH 7.0 (Hendershot et al., 2007) and exchangeable Al was extracted with 1 M KCl analysed by atomic absorption spectroscopy using a Perkin Elmer 3110 atomic absorption spectrometer (Perkin Elmer, Norwalk, CT, USA). Available K and total concentrations of heavy metals were determined as described by Mingorance (2002). The mean values of chemical characteristics for soil samples are listed in Table 1.

4.2.2 Organic residues

Sewage sludge was collected from a wastewater plant (Vilcún, Chile) and stored at 4°C until use. The wastewater plant produces sewage sludge that has been aerobically digested and dried to 19% dry matter. Wheat straw was obtained from crop residues from the Araucanía Region. Wheat straw was mixed with sewage sludge (1:3 w:w) and called mixed residue. Standard methods (Dane & Topp, 2002) were used to determine the principal properties of

wheat straw, sewage sludge and mixed residue samples; each sample was analysed in triplicate.

4.2.3 Plant species

After surface sterilization and a thorough rinse with sterilized water, *E. globulus* seeds were sown in moistened sand. After germination, uniform seedlings were planted in 0.3 L pots (measuring 10 cm of height × 8 cm of diameter) filled with a 1:4 (v:v) mixture of sterilized sand and soil (substrate density 0.8 g cm^{-3}). Plants were grown in growth chambers with supplementary light provided by Sylvania incandescent and cool white lamps (400 E m^{-2} s^{-1}; 400–700 nm) with a 16/8 h day/night cycle at 25/19°C and 50% relative humidity.

Table 1. Chemical characteristics of soil.

Parameter	Amount
Soil organic matter (%)	12
pH (H_2O)	5.35
C (g kg^{-1})	60
Total P (mg kg^{-1})	1270
Olsen-P (mg kg^{-1})	8.01
Available-K (mg kg^{-1})	414
Total N (g kg^{-1})	2.3
Exchangeable Ca (cmol kg^{-1})	30.87
Exchangeable Na (cmol kg^{-1})	0.24
Exchangeable Mg (cmol kg^{-1})	5.97
Exchangeable K (cmol kg^{-1})	1.12
Exchangeable Al (cmol kg^{-1})	0.03
CEC* (cmol kg^{-1})	38.23
Al-saturation (%)	0.06
Extractable Al (mg kg^{-1})	1891
Total Fe (mg kg^{-1})	48.1
Total Cu (mg kg^{-1})	3.4
Total Zn (mg kg^{-1})	2.9

Note: *Cationic exchange capacity = Σ(K, Ca, Mg, Na and Al).

4.2.4 AM inoculation

The mycorrhizal fungus *R. irregularis* was used for inoculation (Krüger et al., 2012). The mycorrhizal fungal inoculum used in these experiments was a mixture of soil spores and root fragments of *Medicago sativa* L. grown in pots. The inoculum was applied in the amount of 2.7 g per 100 g of soil, which has been previously determined to achieve high levels of root colonization (approximately 1000 spores per 100 g). Non-AM-fungus-inoculated pots received a water filtrate (Whatman no. 1 paper) of the AM inoculum which contained common soil microflora but were free of AM fungal propagules.

4.2.5 Saprophytic fungi

Two saprophytic fungi were used, *C. rigida* (Berk. Et Mont.) Murrill (obtained from the Culture Collection of the Centro de Investigaciones Biológicas (CIB) in Madrid) and *P. chrysosporium* (obtained from the fungal collection of the Laboratorio de Biorremediación, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Chile). The strains were stored on malt extract agar (MEA) plates at 4°C and periodically subcultured. To prepare inoculum, barley seeds (as substrate to form fungal mycelium) were inoculated with MEA (disks of 1 cm^2), withdrawn from 14-day-old fungal cultures grown at 28°C. Once a dense culture of fungal mycelium was generated, 10 barley seeds completely colonized by the fungal mycelium were added per pot to inoculate the soil.

4.2.6 Experimental design

The treatments consisted of

(1) uninoculated controls,

(2) soil inoculated with *C. rigida* or *P. chrysosporium* with or without sewage sludge or wheat straw or mixed residue,

(3) soil inoculated with *R. irregularis* with or without sewage sludge or wheat straw or mixed residue and

(4) soil inoculated with *C. rigida* or *P. chrysosporium* with or without *R. irregularis* and with or without sewage sludge or wheat straw or mixed residue.

The plants were inoculated with AM (inoculum obtained from *M. sativa*) and saprophytic fungi (inoculated on barley seeds) when they were transplanted (after 4 weeks of growth). One seedling was planted per pot and six replicates were performed per treatment. Sewage sludge was applied to Eucalyptus pots at a concentration of 4 g per 100 g of soil (dry matter basis). Wheat straw was chopped into 1–2 cm pieces and applied at a concentration of 1.5 g per 100 g of soil. The mixed residue was applied at a concentration of 1.5 g wheat straw and 4 g of sewage sludge per 100 g of soil.

4.2.7 Plant analysis

The Eucalyptus plants were harvested after 16 weeks and dry biomass (root and shoot) was determined. For determination of nutrient concentration, shoot was ground to pass through a 0.5 mm sieve and digested in a $H_2SO4–H_2O_2$ mixture. Phosphorus and N concentrations in the digest were determined as described by Jackson (1973). Potassium (K), magnesium (Mg), calcium (Ca) and iron (Fe) concentrations were determined as described by Mingorance (2002).

4.2.8 Fungi analysis

After harvest, two fresh root samples were randomly taken from the entire root system. One of the two root samples was used to determine the percentage of root length colonized by AM fungi using the gridline intersect method (Giovannetti & Mosse 1980) after the sample had been cleared and stained with trypan blue (Phillips & Hayman 1970). The second root sample was used to measure the succinate dehydrogenase (SDH) activity (EC 1.3.99.1) in fungal mycelia through the reduction of tetrazolium salts at the expense of added succinate (MacDonald & Lewis 1978);

the percentage of AM fungal mycelia with SDH activity was determined under a compound microscope Nikon Eclipse E200 (Nikon, Tokyo, Japan) (Ocampo & Barea 1985).

4.2.9 Biochemical determinations

In each pot six samples were randomly collected for biochemical analyses, approximately10 g of soil per pot was used. FDA was assessed as described by Adam & Duncan (2001) and expressed as μg fluorescein g^{-1}soil. β-glucosidase activity was determined by measuring p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside (PNG) according to the method of Eivazi & Tabatabai (1990) and expressed as μmol p-nitrophenol g^{-1} dry soil h^{-1}. Acid phosphatase was measured using the same procedure as β-glucosidase, but using p-nitrophenyl phosphate (PNPP) instead of PNG (Sannino & Gianfreda, 2001). Dehydrogenase activity was determined according to the method described by Casida et al., (1964) and expressed as μg TPF (triphenyl formazan) g^{-1}dry soil h^{-1}. The average and standard deviations of the enzymatic activities from triplicate cultures are reported.

4.2.10 Statistical analysis

All data expressed as a percentage were arcsine-square root transformed before statistical analysis. The data were analysed using factorial design analysis of variance with AM fungi treatment (control, *R.irregularis*), saprophytic fungi treatment (control, *C.rigida* and *P. chrysosporium*), organic residue treatment (control, sewage sludge, wheat straw and mixed residue) and their interactions as sources of variation. Statistical procedures were carried out with the software package SPSS 11.0 for Windows (Aranaz, 1996). Means were compared using Tukey's multiple range test. Statistical significance was determined at $p < 0.05$. Prior to statistical analysis, data were tested for normality and homogeneous variances.

4.3 Results

The nutritional values of the organic residues applied to the soil are summarized in Table 2. After 16 weeks of application of wheat straw, sewage sludge or mixed residue to the soil, the pH of the soil changed from 5.35 (without amendment) to 5.43, 6.16 and 6.63, respectively. The statistical results of the factorial analyses can be found in Table 3. Application of wheat straw, sewage sludge or mixed residue to the soil without fungal inoculation did not increase the dry weight of *E. globulus* shoots (Figure 1). Inoculation of *R. irregularis* increased the dry weight of *E. globulus* shoots in all treatments, with the highest increase obtained from the addition of mixed residue. Inoculation with the saprophytic fungi *C. rigida* or *P. chrysosporium* increased the dry weight of plants when inoculated in the presence of sewage sludge or mixed residue. Inoculation with *C. rigida* or *P. chrysosporium* also increased the dry weight of *E. globulus* shoots when colonized together with *R. irregularis*. The effects of each saprophytic fungus on the dry weight of *E. globulus* shoots colonized with *R. irregularis* were increased in the presence of wheat straw, sewage sludge or mixed residue. The most significant effect on the dry weight of *E. globulus* shoots was observed when *P. chrysosporium* was co-inoculated with *R. irregularis* in the presence of mixed residue (Figure 1).

The dry weight of Eucalyptus roots increased when grown in soil amended with sewage sludge, whether or not it was inoculated with *R. irregularis* (Figure 2). *Coriolopsis rigida* had no effect on the dry weight of roots in any of the treatments; however, dual treatment of *C. rigida* together with *R. irregularis* increased the dry weight of *E. globulus* roots when plants were grown in the presence of sewage sludge or mixed residue. In contrast to *C.rigida*, inoculation with *P. chrysosporium* alone had a stimulating effect on the growth of *E. globulus* roots when grown in the presence of sewage sludge. Dual treatment of *P. chrysosporium* together with *R. irregularis* also increased the dry weight of *E. globulus* roots when plants were grown in the presence of sewage sludge or mixed residue.

The percentage of root length colonization of *E. globulus* by AM decreased when plants were grown in the presence of sewage sludge (Figure 3). Inoculation with either saprophytic fungi did not have a significant effect on the percentage of root length colonization of *E. globulus* by AM. The co-inoculation of both arbuscular and saprophytic fungi increased the percentage of AM root length colonization in the presence of the mixed residue. Similar levels of SDH activity in E. globulus roots were observed whether or not residues were applied (Figure 3). However, internal AM mycelium in the root of *E. globulus* had higher SDH activity when co-inoculated with saprophytic fungi in the presence of sewage sludge or mixed residue (Figure 3).

Table 2. Chemical characteristics of mixed residue.

Parameter	Amount
pH	8.1
C (%)	36.5
Total N (%)	2.15
Total P (mg kg^{-1})	1124
Total K (mg kg^{-1})	2780
Extractable Al (mg kg^{-1})	9679
Total Zn (mg kg^{-1})	733
Total Cu (mg kg^{-1})	750
Total Mn (mg kg^{-1})	187
Total As (mg kg^{-1})	2.5
Total Pb (mg kg^{-1})	59.7

Table 3. Significance of the main treatment effects and their interaction based on factor ANOVA.

	F-values			
	AM	OR	OR × SF	AM × OR × SF
Shoot dry weight	73.24**	221.93**	1.08*	23.19**
Root dry weight	20.35 (ns)	152.7*	0.53 (ns)	8.61*
β-glucosidase	35.19 (ns)	116.1 (ns)	0.82*	9.27*
Fluorescein diacetate	83.26**	97.18 (ns)	0.49 (ns)	8.63*
Dehydrogenase	110.1*	104.82 (ns)	0.37 (ns)	6.24*
Phosphatase	19.33 (ns)	86.69 (ns)	0.33 (ns)	5.99*

Notes: AM, arbuscular mycorrhiza; OR, organic residue; SF, saprobe fungi.
(ns), not significant; *significant at $p < 0.05$; **significant at $p < 0.01$.

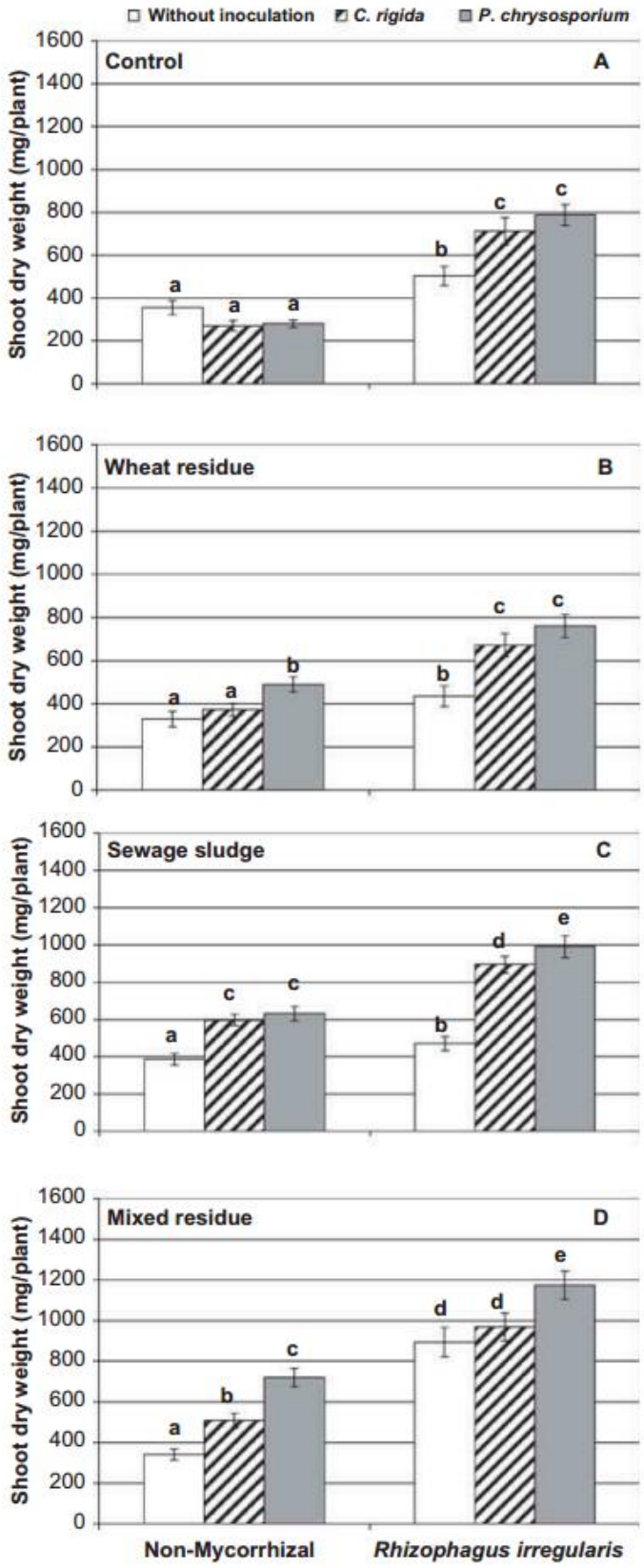

Figure 1. Shoot dry weight of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means (n = 6). Bars with the same letter are not significantly different ($p < 0.05$).

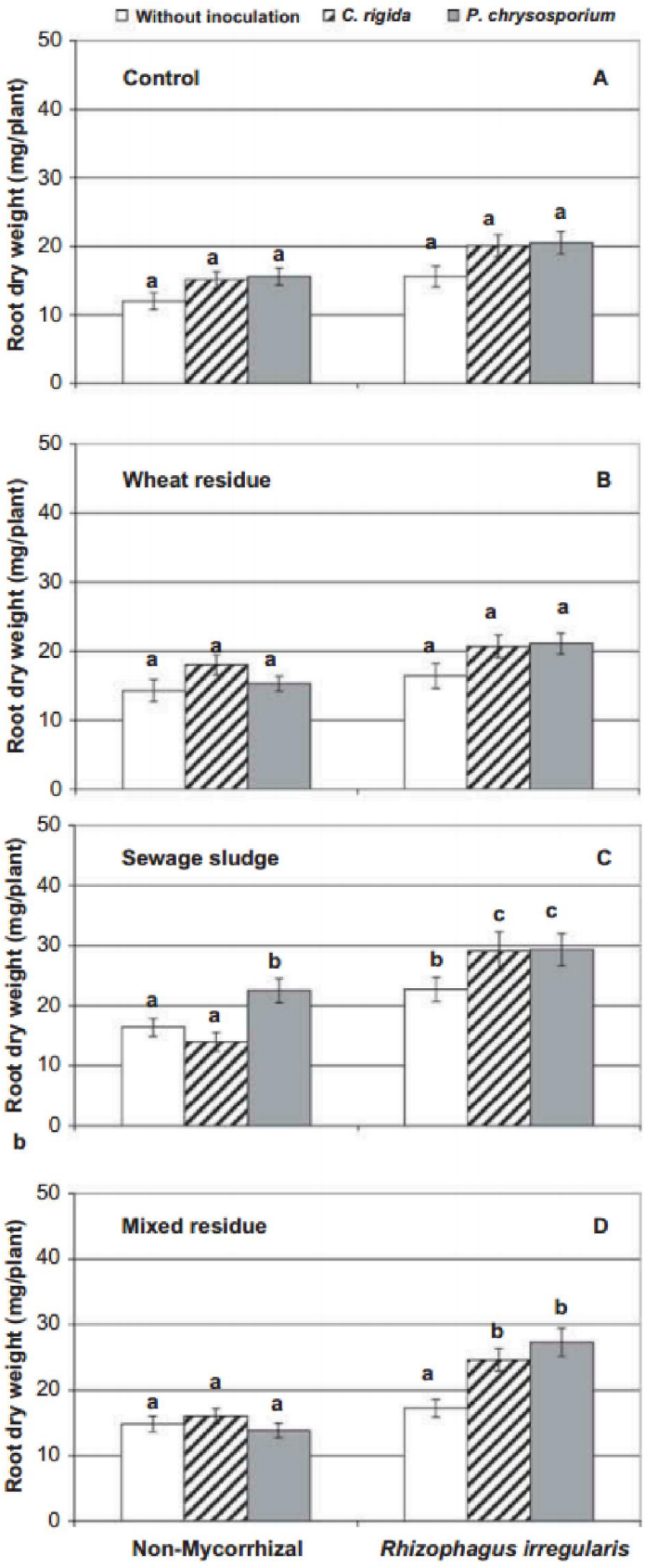

Figure 2. Root dry weight of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means (n = 6). Bars with the same letter are not significantly different ($p < 0.05$).

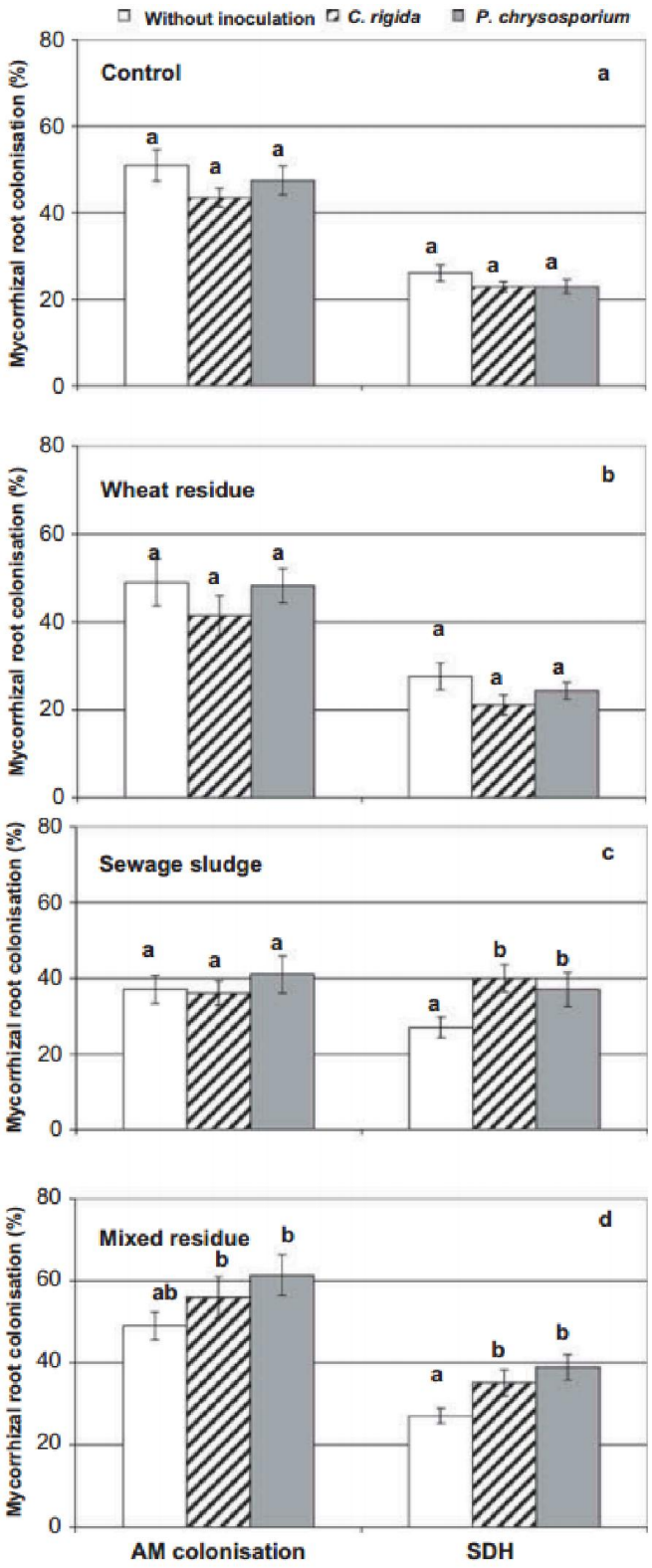

Figure 3. Arbuscular mycorrhizal root length colonisation and arbuscular mycorrhizal mycelium with succinate dehydrogenase (SDH) activity of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means (n = 6). Bars with the same letter are not significantly different ($p < 0.05$).

The application of wheat straw, sewage sludge or mixed residue to the soil did not increase the FDA (Figure 4). Inoculation with *R. irregularis* increased the FDA activity in the bulk soil of *E. globulus* in the presence of all organic residues, but no significant differences between them were observed. The co-inoculation of *C. rigida* or *P. chrysosporium* together with *R. irregularis* in the presence of mixed residue was the most effective in increasing the FDA.

Increased levels of β-glucosidase activity in the bulk soil of *E. globulus* were observed with the addition of wheat straw, sewage sludge or mixed residue. Inoculation of the soil with *R. irregularis* alone did not increase the β-glucosidase activity in the presence of any of the three residues in soil without saprophytic fungi. Inoculation of the soil with AM fungi combined with *C. rigida* or *P. chrysosporium* increased the β-glucosidase activity in the soil samples of plants grown in the presence of wheat straw or mixed residue. Inoculation with either *C. rigida* or *P. chrysosporium* saprophytic fungi together with *R. irregularis* in the presence of the mixed residue induced the greatest increase in the β-glucosidase activity of soil samples (Figure 5).

The addition of the different organic residues to the soil had a negligible effect on phosphatase activity. Similarly, inoculation of the soil with the AM fungi *R. irregularis* or the saprophytic *C. rigida* and *P. chrysosporium* did not induce any change in phosphatase activity (Figure 6).

The addition of the different organic residues to the soil did not increase the dehydrogenase activity. Inoculation of the soil with *R. irregularis* increased dehydrogenase activity compared to the non-AM-fungus-inoculated control, but the saprophytic fungi had no significant effect in any of the treatments tested (Figure 7).

Nutrient analysis of *E. globulus* showed that the inoculation of the AM fungus alone or together with the saprophytic fungi *C. rigida* or *P. chrysosporium* increased the shoot concentrations of N, P, K, Ca, Mg and Fe in comparison to non-inoculated controls. In addition, shoots of *E. globulus* had the highest concentration of P when co-inoculated with AM and saprophytic fungi in the presence of the mixed residue (Table 4).

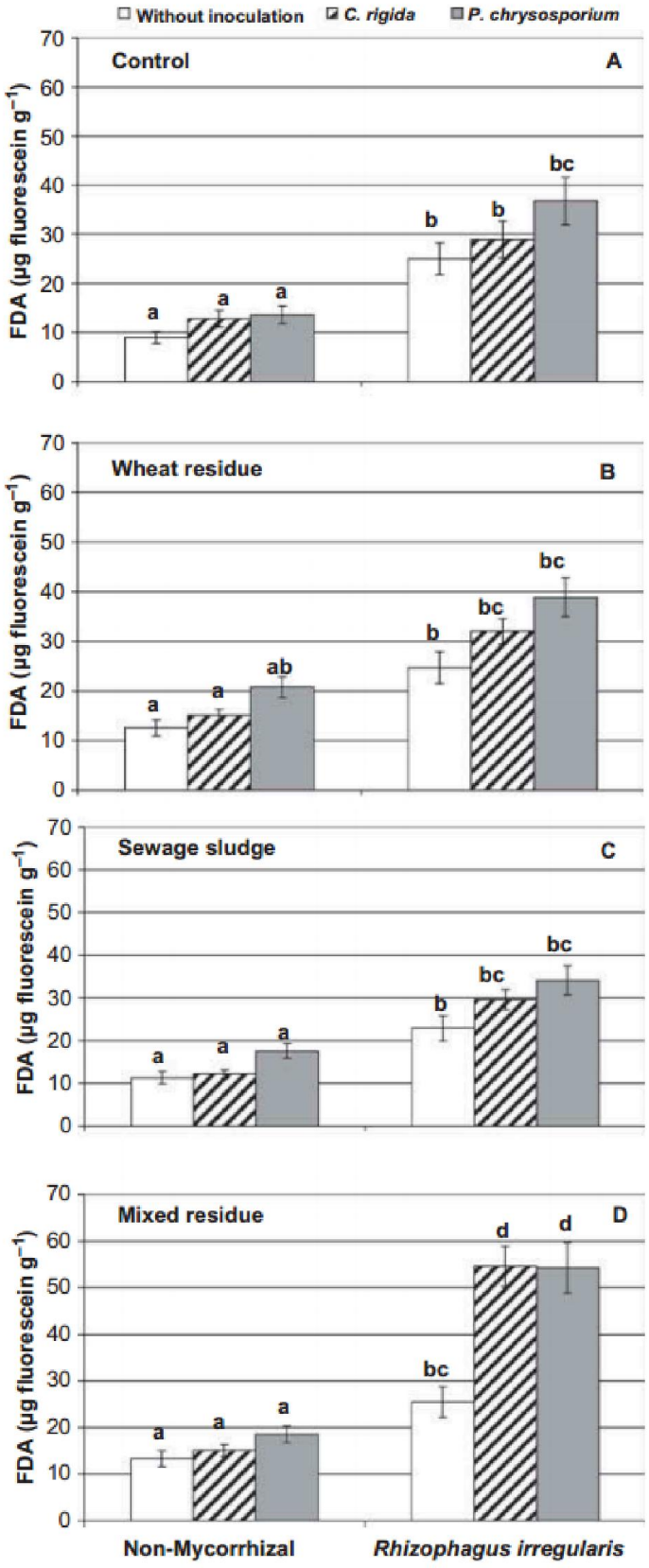

Figure 4. Fluorescein diacetate hydrolase (FDA) activity in the bulk soil of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means (n = 6). Bars with the same letter are not significantly different ($p < 0.05$).

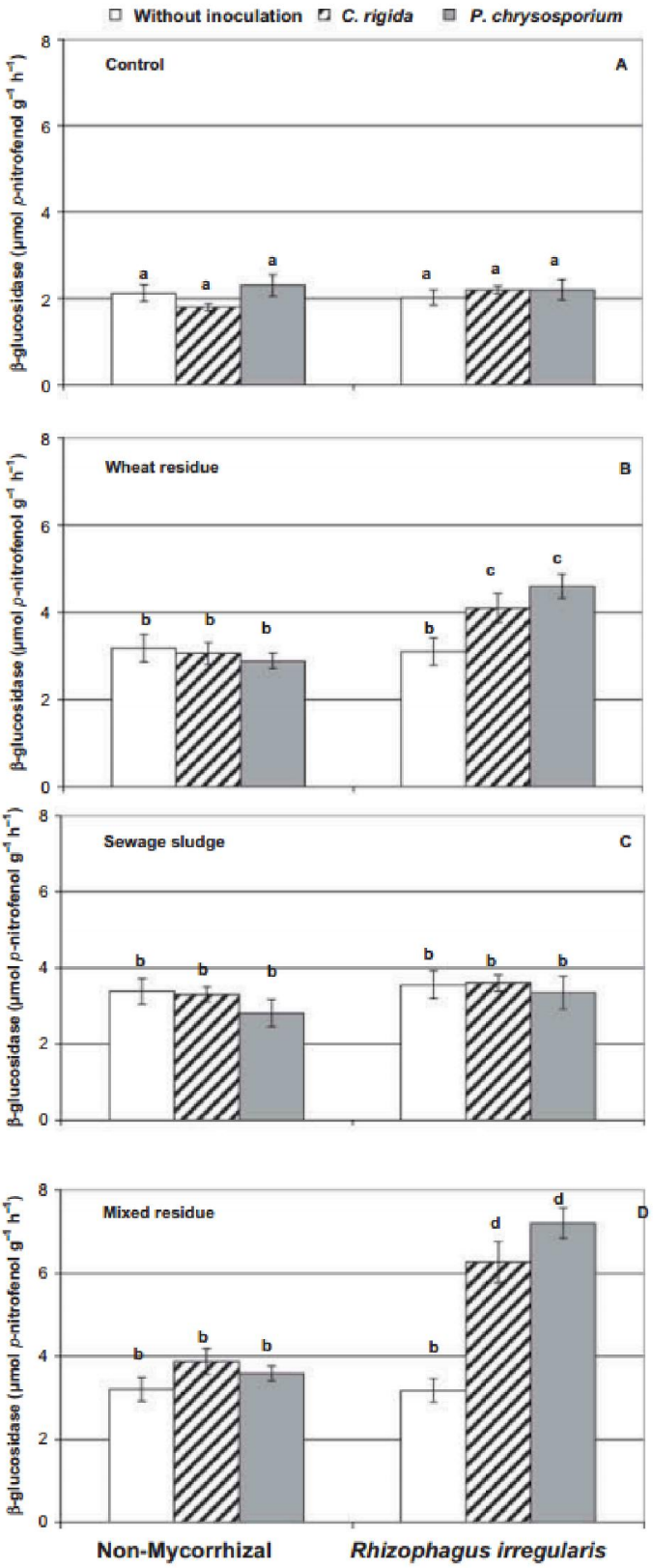

Figure 5. β-glucosidase activity in the bulk soil of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means ($n = 6$). Bars with the same letter are not significantly different ($p < 0.05$).

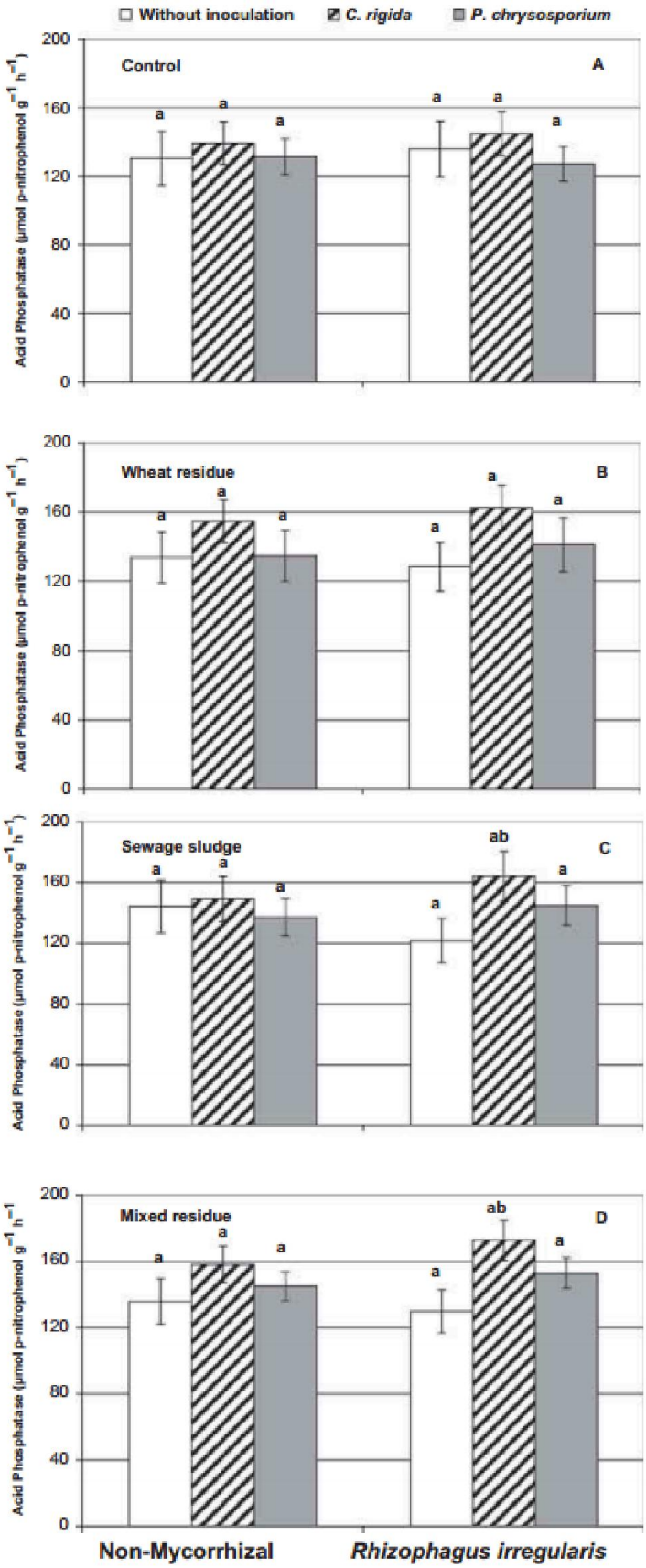

Figure 6. Phosphatase activity in the bulk soil of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means ($n = 6$). Bars with the same letter are not significantly different ($p < 0.05$).

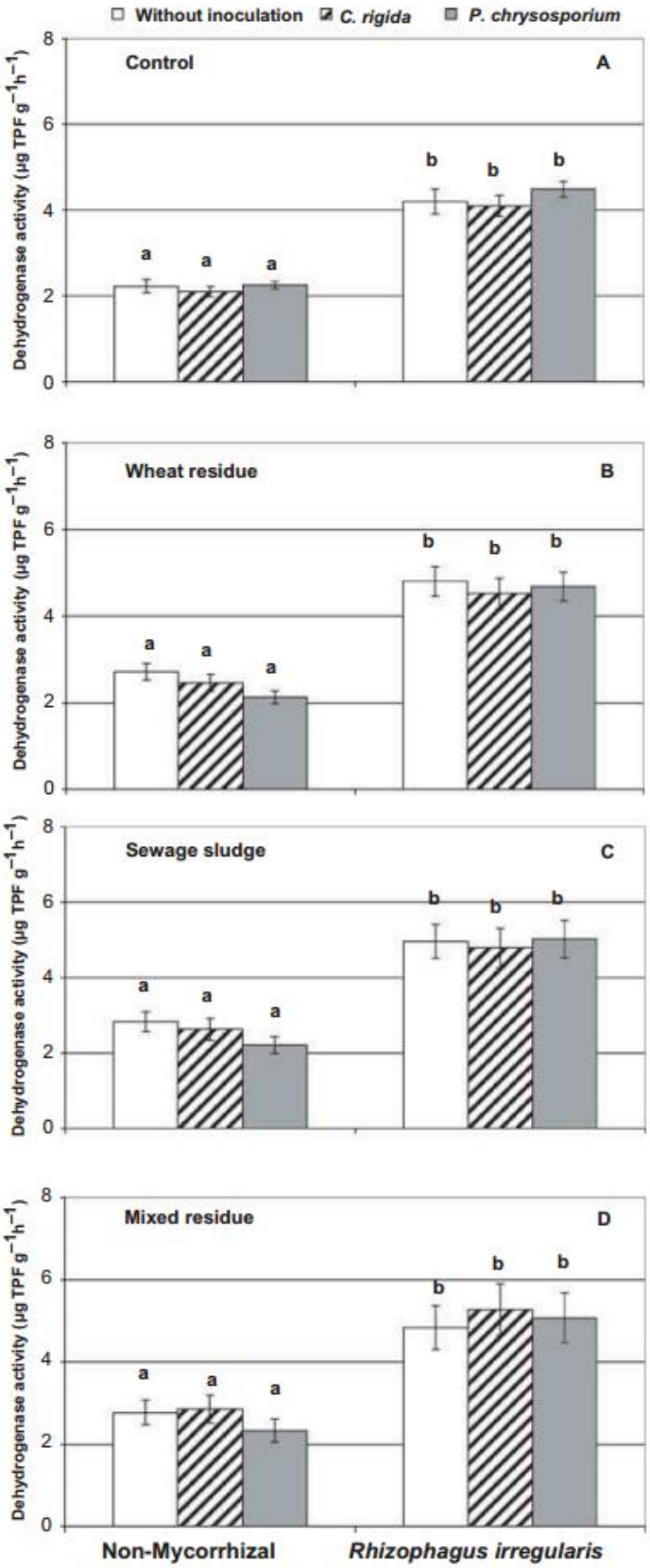

Figure 7. Dehydrogenase activity in the bulk soil of *Eucalyptus globulus* inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with wheat straw, sewage sludge or mixed residue. Residue type: A = Without residue; B = Wheat straw; C = Sewage sludge; and D = Mixed residue. The data are the means ± standard errors of means ($n = 6$). Bars with the same letter are not significantly different ($p < 0.05$).

Table 4. Mineral concentration (on dry matter basis) in shoots from *Eucalyptus globulus* plants (%) inoculated with the AM fungus *Rhizophagus irregularis* and the saprobe fungi *Coriolopsis rigida* and *Phanerochaete chrysosporium* in soil amended with organic residue. The plants were harvested at 20 weeks after transplanting. Values followed by the same letter are not significantly different as determined by Tukey's multiple range test ($p < 0.05$).

	N%	P	K	Ca	Mg	Fe
Control						
Without inoculation	1.04 ba	0.04 a	1.14 a	0.61 a	0.17 a	183 a
Rhizophagus irregularis	1.37 b	0.13 b	2.02 b	1.14 b	0.35 b	218 b
Coriolopsis rigida	1.05 a	0.04 a	1.13 a	0.72 a	0.22 a	147 a
Phanerochaete chrysosporium	1.01	0.04	1.16	0.70	0.21	153
R. irregularis + *C. rigida*	1.78 c	0.14 b	2.34 b	1.16 b	0.31 b	315 c
R. irregularis + *P. chrysosporium*	1.77 c	0.14 b	2.37 b	1.11 b	0.30 b	308 c
Sewage sludge						
Without inoculation	1.03 a	0.05 a	1.16 a	0.73 a	0.21 a	179 a
Rhizophagus irregularis	1.35 b	0.17 b	2.21 b	1.15 b	0.36 b	235 b
Coriolopsis rigida	1.18 a	0.07 a	1.39 a	0.79 a	0.26 a	194 a
Phanerochaete chrysosporium	1.17 a	0.06 a	1.45 a	0.81 a	0.27 a	206 ab
R. irregularis + *C. rigida*	1.49 b	0.17 b	2.24 b	1.12 b	0.36 b	374 c
R. irregularis + *P. chrysosporium*	1.54 b	0.18 b	2.21 b	1.15 b	0.37 b	359 c
Wheat straw						
Without inoculation	1.02 a	0.04 a	1.15 a	0.65 a	0.18 a	172 a
Rhizophagus irregularis	1.35 b	0.14 b	2.20 b	1.16 b	0.32 b	224 b
Coriolopsis rigida	1.20 ab	0.11 ab	1.31 a	0.75 a	0.25 ab	213 ab
Phanerochaete chrysosporium	1.12 a	0.09 ab	1.18 a	0.83 a	0.25 ab	189 a
R. irregularis + *C. rigida*	1.50 b	0.18 b	2.20 b	1.18 b	0.36 b	326 c
R. irregularis + *P. chrysosporium*	1.75 b	0.17 b	2.28 b	1.18 b	0.35 b	328 c
Mixed residue						
Without inoculation	0.98 a	0.04 a	1.17 a	0.67 a	0.16 a	164 a
Rhizophagus irregularis	1.36 b	0.15 b	2.13 b	1.17 b	0.30 b	237 b
Coriolopsis rigida	1.21 ab	0.10 ab	1.45 a	0.82 a	0.27 ab	216 ab
Phanerochaete chrysosporium	1.20 ab	0.08 a	1.48 a	0.85 a	0.31 b	229 ab
R. irregularis + *C. rigida*	1.60 c	0.21 c	2.99 c	1.90 c	0.39 bc	427 c
R. irregularis + *P. chrysosporium*	1.76 c	0.25 c	3.23 c	2.23 d	0.45 d	493 c

4.4 Discussion

Studies about the use of sewage sludge as an organic fertilizer have been shown to benefit plant growth (Khan et al., 2013). However, in the present study, no beneficial effects of sewage sludge were observed on either the dry plant matter or the nutrient concentration in shoots of *E. globulus*. Sewage sludge from different backgrounds may have different compositions. The sewage sludge used in these experiments contained high levels of Al. It is known that the application of 600 mg kg^{-1} of Al in soil decreases the weight of *E. globulus* (Arriagada et al., 2007). When sewage sludge was applied to the soil pots, the amount of Al reached 290 mg kg^{-1} of soil. Therefore, the high concentration of Al in the sewage sludge may be responsible for the lack of increases in the dry weight and nutrient concentrations of *E. globulus*. In addition, while the application of wheat straw and sewage sludge has been shown to be beneficial to plant growth by increasing the total N supply of the mixture (Jia et al., 2008), the application of wheat straw mixed with sewage sludge had no beneficial effects on either the dry weight or nutrient concentration in the shoots of *E. globulus* in this study.

Several authors have described an inhibition of the effectiveness of AM fungi in improving plant growth from constant application of sewage sludge, most likely due to the accumulation of Al in the soil (Kanu et al., 2013, Seguel et al., 2013). The high Al concentration in the sewage sludge in these studies may explain the observed decrease in the percentage of AM root length colonization and the unchanged dry weight of the plant. However, the negative effect of sewage sludge on AM colonization and its lack of a beneficial effect on plant dry weight were overcome by the application of wheat straw to the residue. It is well known that increasing organic matter in the soil after sludge application contributes to the beneficial effects of AM fungus on plant growth by reducing the toxicity of metals and heavy metals in AM-colonized plants through their immobilisation. Likewise, organic matter and AM fungi both prevent the translocation of heavy metals to the plant (Juwarkar & Jambhulkar, 2008). Additionally, in the presence of Al in the soil, mycorrhizal plants have been shown to have increased growth and P uptake when compared to

non-mycorrhizal plants (Seguel et al., 2013). These symbionts play a role critical in the protection of roots by avoiding aluminium stress by altering their bioavailability. In addition, in the soil rhizosphere, the mycorrhizal fungi may extract phosphorus from $AlPO_4$ (Cumming & Weinstein 1990) due to changes in soil pH as an aluminium detoxification mechanism. In our research, changes in soil pH from the application of organic residues may be an important factor in Al tolerance for agricultural plants cropped in acidic Chilean soils.

The synergistic effects of AM fungi and *C. rigida* and *P. chrysosporium* saprophytic fungi on both the AM root colonization and the dry weight of plants have already been observed (Arriagada et al., 2009c, Medina et al., 2010). In the present study, the saprophytic fungi *C. rigida* and *P. chrysosporium* decreased the toxicity of sewage sludge on AM root length colonization of *E. globulus*, increased the metabolic activity of AM fungi inside *E. globulus* roots and improved the dry weight of the plants. Addition of wheat straw to the sewage sludge increased the benefits of saprophytic fungi even more, as this addition further increased the AM root length colonization, the SDH activity of AM fungi inside roots and the P concentration and growth of shoots of *E. globulus*. Saprophytic fungi are able to produce hydrolytic enzymes which degrade organic matter into compounds accessible for plant uptake (polysaccharidases as endoglucanase, endopolymethylgalacturonase and endoxyloglucanase). In addition, the extraradical hyphae of AM fungi are highly efficient in the acquisition and translocation of inorganic compounds (capture of inorganic N and release of inorganic N as NH_4^+) from organic matter to host plants (Hodge et al., 2001, Tribak et al., 2002). Therefore, the increased availability of organic nutrient compounds (polysaccharidases) from the production of hydrolytic enzymes of saprophytic fungi is one reason why saprophytic fungi may complement the benefits of AM fungi for plant growth (Aranda et al., 2004). However, in the presence of the mixed residue, the highest plant growth took place when *E. globulus* was co-inoculated with *P. chrysosporium* and *R. irregularis*. Differential synergistic effects of various saprophytic fungi on the growth of AM plants have already been documented (Fracchia et al., 1998, Medina et al., 2010).

The enzymatic machinery of the saprophytic and AM fungi increased the FDA, β- glucosidase and dehydrogenase activities in the soil where the mixed residue was applied. It is known that production of these enzymes are an indication not only of the ongoing metabolic and biological activities of soil microorganisms but also of the microbial hydrolytic processes involved in the breakdown of organic matter, which is important for nutrient availability (Bandick & Dick 1999,Turner et al., 2002).

4.5 Conclusions

The application of sewage sludge to soil as an organic fertilizer does not always improve plant growth, because sewage sludge can have high levels of elements such as Al that is not beneficial for plant growth. The application of wheat straw alone did not increase the benefits of the sewage sludge. However, the co-inoculation of AM and saprophytic fungi with a mixture of sewage sludge and wheat straw increased the P concentration and growth of *E. globulus* shoots and FDA and β-glucosidase activities in the soil. The combination of saprophytic and arbuscular fungi together with wheat straw may allow the use of sewage sludge, even with high aluminium concentration (12,620 mg kg^{-1} of extractable Al), as a biological fertilizer to improve the growth of plants such as *E. globulus*.

CHAPTER V

Effect of mixing soil saprophytic fungi with organic residues on the response of *Solanum lycopersicum* to arbuscular mycorrhizal fungi

Almonacid, L.[1,2], Fuentez, A. [1], Ortiz, J. [1],Salas, C. [1], García-Romera, I. [3], Ocampo, J.[3] and Arriagada, C.[1]

(1) Laboratorio de Biorremediacion, Universidad de La Frontera, Casilla 54-D.Temuco-Chile.

(2) Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de la Frontera. Temuco-Chile

(3) Estación experimental del Zaidín, CSIC. Profesor Albareda 1,18008.Granada-España.

Soil Use and Management

SUM 2014-016.R3

Effect of mixing soil saprophytic fungi with organic residues on the response of *Solanum lycopersicum* to arbuscular mycorrhizal fungi

Abstract

The effect of the dual inoculation with AM and saprobe fungi and a combination of wheat straw and sewage sludge residues was studied by determining their effect on dry weight of tomato and on chemical and biochemical properties of soil. Soil application of sewage sludge combined with wheat straw (organic residues), increased the dry weight of tomato inoculated with *Rhizophagus irregularis*. The greatest shoot dry mass was obtained when the organic residues were incubated with *Trichoderma harzianum* and applied to arbuscular mycorrhizal (AM) plants. However, the greatest percentage of root length colonized with AM in presence of the organic residues was obtained with inoculation with *Coriolopsis rigida*. The relative chlorophyll was greatest in mycorrhizal plants regardless of the presence of both saprobe fungi. The presence of the saprobe fungi increased soil pH as the incubation time of residue with both saprobes increased. Soil nitrogen and phosphorus contents and acid phosphatase were stimulated by the addition of organic residues, and contents of N and P. Total N and P content in soil increased when the organic residue was incubated with saprobe fungi but this effect decreased as the incubation period of the residue with saprobe fungi increased. The same trend was observed for soil β-glucosidase and FDA activities. The application of organic residues in presence of AM and saprobe fungi seems to be an interesting option as a biofertilizer to improve plant growth and biochemical parameters of soils.

Key words: *Coriolopsis rigida*, Mycorrhizal fungi, Sewage sludge, *Trichoderma harzianum,* Wheat straw.

5.1 Introduction

The application of organic amendments improves chemical, physical and biological soil properties increasing soil fertility and crop production (Cuevas et al., 2006). Among the different types of organic residues used as amendments in agroforestry systems, crop residues and sewage sludge from wastewater treatment plants are widely used. The effect of sewage sludge as fertilizer can be improved by applying wheat straw. The input of carbon provided by wheat straw stabilizes the C:N ratio which is an important parameter in the biodegradation process, avoiding nitrogen losses and also improving fungal colonization and stabilizing pH, improving the development of microorganisms (Barrington et al., 2002; Huang et al., 2004).

Arbuscular mycorrhizal (AM) fungi are an important group of microorganisms that can improve plant growth by enhancing nutrient and water uptake, especially for phosphorus uptake from soils with high adsorption capacity such as Andisols (Smith & Read, 2008, Borie et al., 2010). Saprobe fungi are another important group of microorganisms which, by breaking down cellulosic materials to simple sugars, can provide energy sources for other microorganisms, including AM fungi (Radford et al., 1996). Some saprobe fungi such as *Coriolopsis rigida* and *Trichoderma harzianum* have been shown to increase the effectiveness of root colonization by AM fungi and plant growth (Arriagada et al., 2009a). Among saprobe fungi, *C. rigida*, is capable of degrading a variety of compounds usually resistant to microbial action, such as lignin and other phenolic substances mainly due to the production of broad-spectrum enzymes (Baldrian, 2008).

The measurement of enzyme activity is an efficient indicator of soil quality. Hydrolysis of fluorescein diacetate (FDA) is used to estimate microbiological activities of soil reflecting the activity of hydrolases involved in organic matter degradation (Sánchez-Monedero et al., 2008). Phosphatase is responsible for the mineralization of organic phosphorus increasing its availability to plants (Amador et al., 1997). β-glucosidase is involved directly in the carbon cycle and degradation of organic matter (Turner et al., 2002). Dehydrogenase is considered to be a general

index of biological activity on account of its role in the respiratory metabolism of microorganisms (Delgado et al., 2004).

The aim of this work is to evaluate the effect of the application of wheat straw and sewage sludge on tomato dry weight and on chemical and biochemical properties of rhizosphere soil. The use of AM and saprobe fungi in combination of wheat straw and sewage sludge residues to improve their effect as soil amendment was expected.

5.2 Materials and Methods

5.2.1 Microorganisms

Native strains of *Coriolopsis rigida* (CECT 20449) and *Trichoderma harzianum* were obtained from the fungal culture collection of the Bioremediation Laboratory, Universidad de La Frontera, Temuco, Chile. Stock cultures of the fungi were stored in PDA slants at 4 ºC and periodically subcultured.

The arbuscular mycorrhizal fungi *Rhizophagus irregularis*(Krüger et al., 2012), was obtained from the collection of Bioremediation Laboratory, Facultad de Ciencias Agropecuarias y Forestales, Universidad de La Frontera, Temuco, Chile.

5.2.2 Soil and residues

The experiments were carried out in a soil classified as an Andisol (Acrudoxic Hapludands), moderately acidic that was obtained from Rucamanque field, Temuco (Chile). Wheat straw corresponded to crop residues of Araucanía Region and the stabilized sewage sludge was collected from a municipal wastewater plant ESSAL S.A., Osorno, Chile. Cellulose, hemicellulose and lignin were determined according to Goering et al. (1970). Carbon and nitrogen content were determined using a Flash EA 1112 Series LECO-TRUSPEC elemental analyzer. Sodium, Cu, Zn, Cd and P concentrations were analyzed by plasma emission spectroscopy (ICP). Each sample was analysed in triplicate (Table 1).

Table 1. Chemical characteristics of wheat straw, sewage sludge organic residues and soil.

	Wheat straw	Sewage sludge	Organic residue	Soil
Cellulose (%)	43	1.96	18	-
Hemicellulose (%)	30	11	28	-
Lignin (%)	9	1.3	5.4	-
C:N ratio	87	8.5	11.9	-
pH	5.5	12	8.3	5.7
Organic matter (%)	--	81	--	12.3
Carbon (%)	46	34	26	10.2
Total N (%)	0.5	0.6	1.8	0.002
Total P (mg kg^{-1})	1.5	19500	5000	8.0*

*Olsen

Table 2. Significance of the main treatment effects and their interaction based on factorial ANOVA.

	SF	IT	AM	SFxIT	SFxAM	ITxAM	SFxITxAM
F-values							
Shoot dry weight	31.421**	0.813n.s	387.6**	0.773n.s	25.82**	0.043n.s	0.118n.s
Root dry weight	11.198**	1.021n.s	89.247**	2.78 n.s	10.911**	0.090n.s	1.642n.s
R:SRatio	1.135n.s	2.890**	44.026**	1.711n.s	0.722n.s	3.469*	1.363n.s
Nitrogen	0.010n.s	30.23**	2.617 n.s	0.040n.s	0.028n.s	0.433n.s	0.045n.s
P-Olsen	1.747n.s	20.65**	0.313 n.s	0.275n.s	0.786n.s	2.017n.s	0.101n.s
pH	107.88**	173.37**	3.391 n.s	31.295**	2.915*	0.760n.s	9.939**
β-glucosidase	28.15**	17.703**	0.741 n.s	6.239**	2.744*	1.266n.s	0.686n.s
Dehydrogenase	30.093**	1.528n.s	0.558 n.s	1.382n.s	23.835**	0.492n.s	0.979n.s
Acid phosphatase	9.918**	2.403n.s	9.255*	2.005n.s	3.813*	0.126n.s	0.113n.s
Fluorescein diacetate	30.089**	130.44**	2.936 n.s	44.674**	3.215*	1.856n.s	0.752n.s
Chlorophyll	4589.ns	1.073ns	319.196**	0.422ns	3.261*	1.101ns	0.533ns

SF: Saprophytic fungi, IT: Incubation Time, AM: Arbuscular Mycorrhiza, ns: Not significant, *p <0.95, **p <0.99

5.2.3 Substrate inoculation

For *C. rigida* inoculum, one slant of active mycelia from stock culture was diluted in 40 mL of sterile distilled water that was homogenized and strongly agitated; 10 mL of this suspension, equivalent to 70 mg of dry mycelium, were added to the organic residues. *T. harzianum* was grown on a slant and spores were scraped in sterile distilled water; 3 mL of spore suspension, equivalent to 1.8 $x10^6$ spores, was spread over the surface of the organic residues. Sterilized slants were added to non-inoculated controls.

Each fungus was added to flasks with 40 g of sterilized organic residues (10 g wheat straw + 30 g sewage sludge), in a 250 mL Erlenmeyer flask autoclaved at 121 °C for 20 min. After the inoculation, the substrate was incubated for 2 and 4 weeks at 23 °C in darkness.

5.2.4 Greenhouse experiments

The experiments were carried out using tomato (*Solanum lycopersicum* L) as test plant. Seeds were surface-sterilised with NaClO at 0.5 % for 15 min, thoroughly rinsed with sterilised water, and then sown in a seedling bed. After 15 days, seedlings were inoculated with 8 g of inoculum of *R. irregularis* 10 days before transplanting to 0.3 L pots. The AM fungal inoculum consisted of substrate containing spores and colonized root fragments of *Medicago sativa* L with high levels of root colonization. Plants non-inoculated with AM fungi were given a filtrate (Whatman no. 1 paper) of the inoculum containing the common soil microflora, but free of AM propagules.The plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 E m^{-2} s^{-1},400-700 nm, with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity.

Eight grams of incubated residues (equivalent to a field application of 80000 kg per ha) (inoculated with the saprobe fungi as described before) were mixed with the soil in each pot. The treatments were: (1) non-inoculated soil or plants - control, (2) plants inoculated with *R. irregularis* in non-inoculated soil(3) non-inoculated plants and soil plus organic residues, (4) soil plus organic residue and plants inoculated with *R. irregularis*, (5) non-inoculated plants in soil with organic residues incubated with *C. rigida*, (6) inoculated plants in soil with organic residues incubated with *C. rigida*, (7) non-inoculated plants in soil with organic residue incubated with *T. harzianum* and (8) inoculated plants in soil with organic residues incubated with *T. harzianum*. Each plant was amended with the organic residues inoculated with *T.harzianum* (incorporated at the same time that organic residues) and incubated with *T.harzianum* for 2 and 4 weeks.(Toole 1971).Four replicates per each treatment were used. The relative chlorophyll content was measured with the Minolta SPAD-502 chlorophyll meter. Shoots and roots were harvested 30 days after transplant and dry mass was determined. Small amounts of roots were taken from the entire root system at random, cleared in KOH, stained with trypan blue in lactic acid according to Phillips & Hayman (1970) and examined microscopically to determine AM root length colonization by the grid intersections plate using a stereo microscope (Giovanetti & Mosse, 1980).

5.2.5 Chemical and biochemical postharvest soil analysis

After harvest, soil N and P were analyzed as described before. β-glucosidase activity was determined by measuring p-nitrophenol released from *p*-nitrophenyl-β-D-glucopyranoside (PNG) according to the method of Tabatabai & Bremmer (1969) and expressed as μmol p-nitrophenol g^{-1} dry soil h^{-1}. Acid phosphatase was measured using the same procedure as β-glucosidase, but using p-nitrophenyl phosphate instead of PNP (Tisserant et al. 1993). Dehydrogenase activity was determined according to the method described by Casida et al. (1964) and expressed as μg TPF (triphenyl formazan) g^{-1} dry soil h^{-1} with modifications. The activity of fluorescein diacetate (FDA) was assessed as described by Adam & Duncan (2001) and expressed as μg fluorescein g^{-1} soil.

5.2.6 Statistical analysis

The percentage values were arcsine transformed for statistical analyses. We studied the following three main factors and their respective levels as follows: AM fungal (control and inoculation with *R. irregularis*), Saprobe fungi (control and organic residues incubated with *C. Rigida* or *T. harzianum*), Incubation Time (amended organic residues incubated during 0, 2, and 4 weeks). We also analyzed the interaction among the main factors using a factorial analysis of variance (Sokal & Rohlf, 1981). Statistical significance was determined at $P< 0.05$. Statistical analyses were conducted using SPSS software, version 11.0 (SPSS Inc., 1989–2001).

5.3 Results

5.3.1 Plant Biomass

There were significant differences in the response of plant and soil variables to the main factors and their interactions (Table 2). Organic residues and inoculation with *C. rigida* or *T.harzianum* did not increase shoot or root dry weight. *R. irregularis* inoculation increased shoot dry weight and the effect of the AM fungus was greater in plants grown in the presence of the organic residues inoculated with *T. harzianum* (Figure 1). Root biomass and SPAD values were greater in mycorrhizal than in non-inoculated plants (Figures 1 and 2). The addition of organic residues further increased the root dry weight of mycorrhizal plants (Figure 1), but not SPAD values (Figure 2). The shoot concentration of N increased with the application of organic residues, and in inoculated plants, while P increased in all treatments compared with control (Table 3).

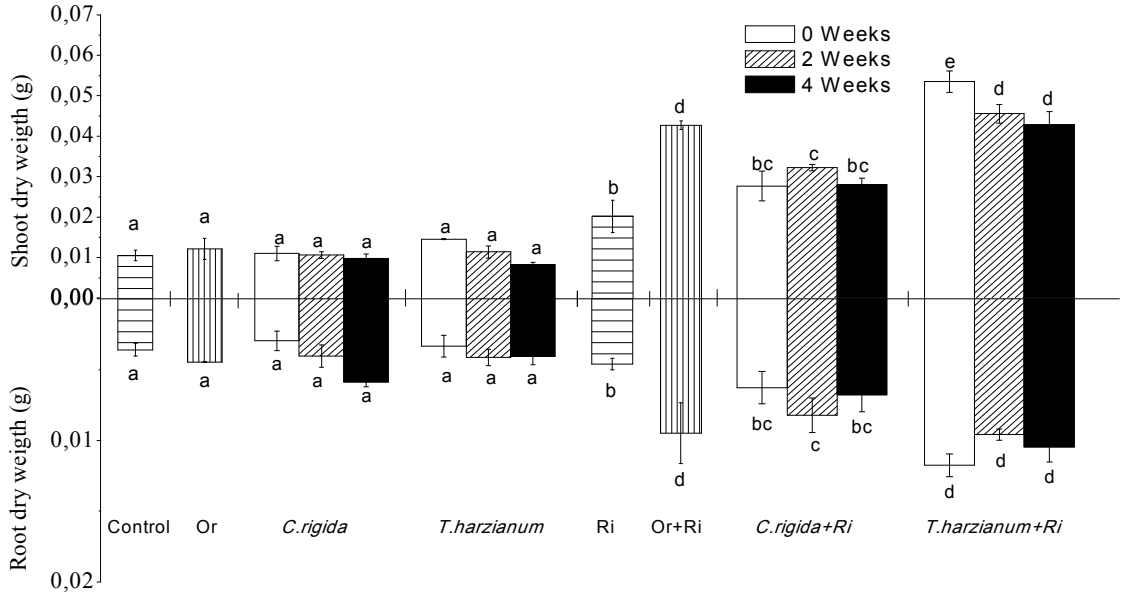

Figure 1. Shoot and root dry weight (g) of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* grown in presence of organic residues inoculated and incubated with the saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residue without fungi, *C. rigida*: Organic residue incubated with *C. rigida, T. harzianum*: Organic residue incubated with *T. harzianum,* Ri: *R. irregularis,* Or+Ri: Organic residue + *R. irregularis, C. rigida*+Ri: Organic residue incubated with *C. rigida* + *R. irregularis*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different ($P< 0.05$).

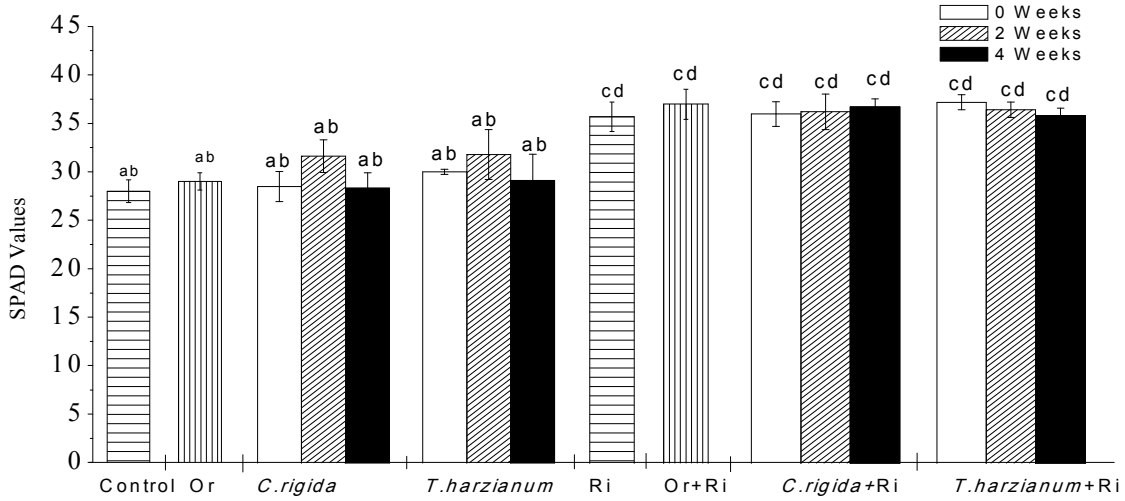

Figure 2: SPAD relative chlorophyll values (a unit less index from 0 to 100) of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* grown in presence of organic residues inoculated and incubated with the saprophytic fungi for 0, 2 and 4 weeks. C: Control, Or: Organic residue without fungi, *C. rigida*: Organic residue incubated with *C. rigida, T. harzianum*: Organic residue incubated with *T. harzianum,* Ri: *R. irregularis,* Or+Ri: Organic residue + *R. irregularis, C. rigida*+Ri: Organic residue incubated with *C. rigida* + *R. irregularis*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different ($P< 0.05$).

Table 3. Mineral concentration in *Solanum lycopersicum* plants (mg kg^{-1}) inoculated with *Rhizophagus irregularis* and organic residues inoculated (0) and incubated with saprophytic fungi for 2 and 4 weeks.

	N	SE		P	SE		K	SE		Ca	SE		Mg	SE	
Control	9600	956	a	900	85	a	13400	1320	a	7900	679	a	7500	633	a
Rhizophagus irregularis	15600	1589	b	1800	179	b	25700	2500	b	11500	1150	a	8300	800	a
Or	17000	1500	b	1857	166	b	32444	3144	d	30646	2990	b	13119	1213	ab
Or+Ri	18500	1790	b	2530	246	c	35900	3590	d	35650	3479	b	17900	1790	b
***C. rigida* 0 weeks**	11600	1150	a	1600	156	b	14200	1520	a	23100	2211	b	11400	1144	a
***C. rigida* 2 weeks**	11000	1100	a	1550	144	b	13800	1380	a	23550	2433	b	11090	1000	a
***C. rigida* 4 weeks**	9440	933	a	1500	149	b	12470	1222	a	22530	2359	b	10870	9870	a
***C. rigida*+ Ri 0 weeks**	19000	1870	bc	1700	166	b	24800	2380	b	11300	1130	a	12400	1140	ab
***C. rigida*+Ri 2 weeks**	22800	2200	c	1799	175	b	25200	2520	b	12800	3234	a	12980	1000	ab
***C. rigida*+ Ri 4 weeks**	23540	2110	c	1840	175	b	25900	2490	b	13900	1390	a	14500	1333	ab
***T. harzianum* 0 weeks**	11600	1230	a	1574	157	b	30945	3000	bc	32007	3103	b	16255	1598	b
***T. harzianum* 2 weeks**	11200	1321	a	1402	140	b	28940	2789	bc	32112	3311	b	15990	1599	b
***T. harzianum* 4 weeks**	10100	9809	a	1385	128	b	28669	2867	bc	31000	3600	b	14700	1320	b
***T. harzianum*+Ri 0 weeks**	25200	1460	d	1414	141	b	34314	3531	d	29444	2644	b	15192	1467	b
***T. harzianum*+Ri 2 weeks**	27900	1690	d	1547	143	b	32193	3319	d	29527	2953	b	15889	1200	b
***T. harzianum*+Ri 4 weeks**	28300	1730	d	1684	166	b	31235	3224	d	30935	3033	b	16143	1437	b

Or: Organic residue without fungi, Or+Ri: Organic residue + *R. irregularis*, *C. rigida*: Organic residue incubated with *C. rigida*, *C. rigida*+Ri: Organic residue incubated with *C. rigida*+ *R. irregularis*, *T. harzianum*: Organic residue incubated with *T. harzianum*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error (SE).Columns with the same letter are not significantly different ($P< 0.05$).

5.3.2 *Chemical parameters of rhizospheric soil*

Total N content in soil was greater in all treatments, with the exception of soil inoculated with *C. rigida* after four weeks of incubation (Figure 3). The greatest total N content was observed when the organic residues were inoculated with *T. harzianum* or *C. rigida* in the presence of mycorrhizal plants (Figure 3). Addition of organic residues also increased available soil P content; the greatest value was obtained when the mixture without fungi was used. The addition of *T. harzianum* or *C. rigida* to the residues led to a decrease of soil P, especially when the incubation period was longer (Figure 4).

Soil pH increased in all treatments compared with control. Organic residues incubated for 4 weeks with *T. harzianum* or *C. rigida* and plants inoculated with *R. irregularis* led to a greater soil pH than when residues were inoculated (Figure 5).

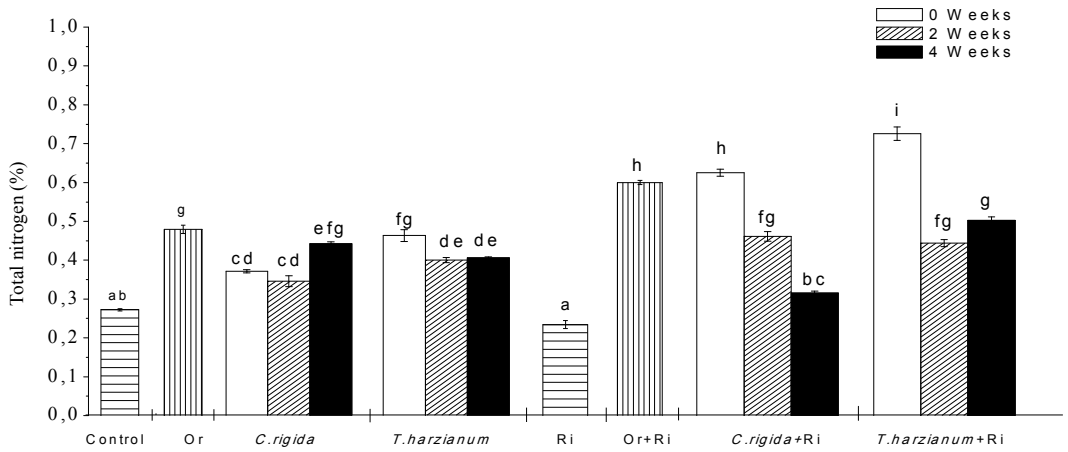

Figure 3. Total nitrogen in soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* grown in presence of organic residues inoculated and incubated with the saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residue without fungi, *C. rigida*: Organic residue incubated with *C. rigida, T. harzianum*: Organic residue incubated with *T. harzianum,* Ri: *R. irregularis,* Or+Ri: Organic residue + *R. irregularis, C. rigida*+Ri: Organic residue incubated with *C. rigida* + *R. irregularis*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different ($P< 0.05$).

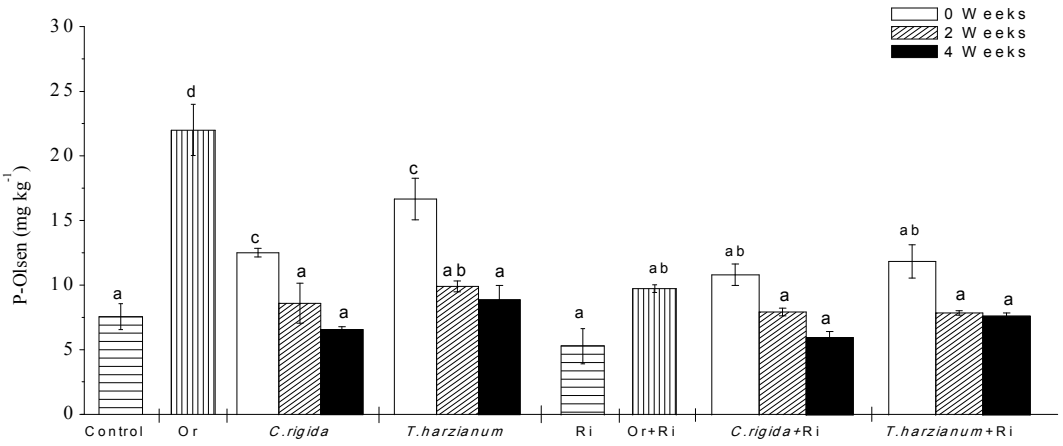

Figure 4. P-Olsen in soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* and grown in presence of organic residues inoculated (0) and incubated with the saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residue without fungi, *C. rigida*: Organic residue incubated with *C. rigida, T. harzianum*: Organic residue incubated with *T. harzianum,* Ri: *R. irregularis,* Or+Ri: Organic residue + *R. irregularis ,C. rigida*+Ri: Organic residue incubated with *C. rigida* + *R. irregularis*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different ($P< 0.05$).

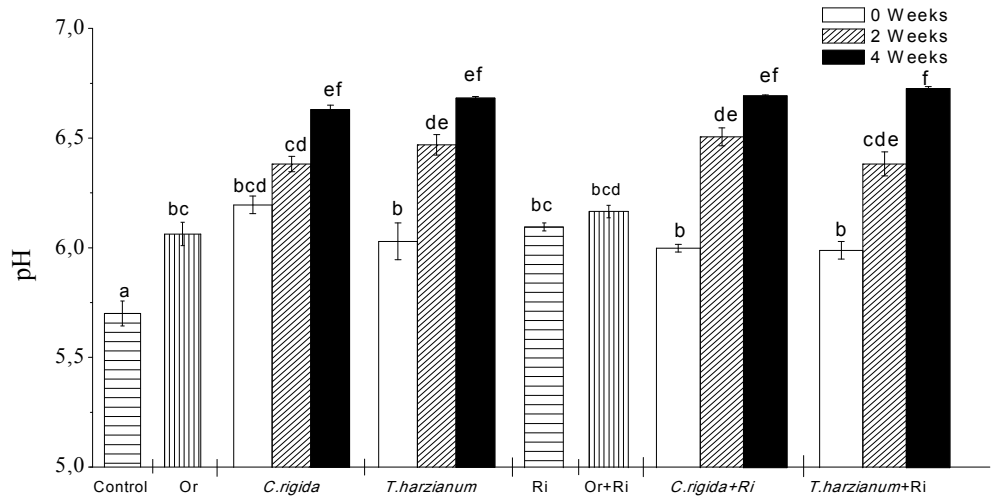

Figure 5.pH of soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* and grown in presence of organic residues inoculated (0) and incubated with the saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residue without fungi, *C. rigida*: Organic residue incubated with *C. rigida, T. harzianum*: Organic residue incubated with *T. harzianum,* Ri: *R. irregularis,* Or+Ri: Organic residue + *R. irregularis, C. rigida*+Ri: Organic residue incubated with *C. rigida* + *R. irregularis*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different ($P< 0.05$).

5.3.3 *Biochemical parameters of rhizosphere*

Acid phosphatase and dehydrogenase activities were stimulated in rhizosphere soil when organic residues were added, but the presence of saprobe and arbuscular fungi did not influence these enzymatic activities (Table 4). β-glucosidase activity was only stimulated by the addition of organic residues inoculated with *C. rigida* or *T. harzianum* compared with treatments without saprobe fungi. Fluorescein diacetate activity (FDA) was increased by the addition of organic residues inoculated with *T. harzianum* or *C. rigida.* However, this enzymatic activity decreased with the addition of organic residues incubated with both saprobe fungi for 2 and 4 weeks (Table 4).

Table 4. Biochemical parameters of rhizospheric soil. Activities of β-glucosidase, Dehydrogenase, Acid phosphatase and Fluorescein diacetate in the rhizospheric soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* and the addition of organic residues inoculated (0) and incubated with saprophytic fungi for 2 and 4 weeks.

Treatments	β-glucosidase (µmol PNG g^{-1} h^{-1})			Dehydrogenase (µg TPF g^{-1} h^{-1})			Acid phosphatase (µmol PNP g^{-1} h^{-1})			Fluorescein diacetate (µg fluorescein g^{-1})		
Control	99.8	(8.0)	a	3.60	(0.73)	ab	178.0	(14.00)	ab	52.0	(2.60)	a
Rhizophagus irregularis	104.5	(9.0)	a	2.85	(0.26)	a	170.0	(12.03)	ab	56.5	(4.13)	a
Or	120.7	(7.8)	ab	5.50	(0.40)	b	217.0	(24.88)	c	64.8	(1.68)	b
Or+Ri	122.5	(6.3)	ab	4.08	(0.44)	ab	167.0	(18.63)	ab	64.1	(3.93)	b
***C. rigida* 0 weeks**	156.6	(20.0)	cd	3.10	(0.12)	ab	170.0	(19.14)	ab	79.0	(2.56)	c
***C. rigida* 2 weeks**	139.0	(17.0)	cd	3.30	(0.52)	ab	157.7	(5.60)	ab	30.1	(2.60)	b
***C. rigida* 4 weeks**	123.6	(7.5)	abc	3.29	(0.28)	ab	164.4	(11.97)	ab	45.6	(4.93)	bc
***C. rigida*+Ri 0 weeks**	148.2	(12.0)	cd	4.40	(0.41)	ab	157.0	(13.18)	ab	81.0	(4.62)	c
***C. rigida*+Ri 2 weeks**	108.9	(13.0)	ab	4.63	(0.50)	ab	169.0	(8.17)	ab	18.3	(0.90)	a
***C. rigida*+Ri 4 weeks**	118.2	(19.0)	abc	3.50	(0.20)	ab	154.5	(7.17)	ab	34.9	(2.56)	a
***T. harzianum* 0 weeks**	169.0	(15.0)	cd	3.20	(0.41)	ab	183.0	(9.93)	ab	81.0	(1.16)	c
***T. harzianum* 2 weeks**	132.0	(8.1)	cd	3.10	(0.25)	ab	154.0	(17.86)	ab	36.8	(2.43)	bc
***T. harzianum* 4 weeks**	108.4	(15.0)	ab	3.27	(0.32)	ab	126.3	(5.63)	a	35.5	(3.69)	a
***T .harzianum*+Ri 0 weeks**	157.0	(16.0)	cd	4.62	(0.38)	ab	175.0	(17.51)	ab	80.0	(1.40)	c
***T. harzianum*+Ri 2 weeks**	127.8	(9.2)	cd	4.30	(0.26)	ab	148.5	(7.80)	ab	23.4	(1.65)	ab
***T .harzianum*+Ri 4 weeks**	122.3	(12.1)	abc	4.30	(0.60)	ab	121.2	(7.78)	a	30.4	(3.27)	a

Or: Organic residue without fungi, Or+Ri: Organic residue + *R. irregularis*, *C. rigida*: Organic residue incubated with *C. rigida*, *C. rigida*+Ri: Organic residue incubated with *C. rigida* + *R. irregularis*, *T. harzianum*: Organic residue incubated with *T. harzianum*, *T. harzianum*+Ri: Organic residue incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Columns with the same letter are not significantly different ($P< 0.05$).

5.3.4 *Root colonization*

The AM root length colonization was increased by the application of the organic residues. The percentage of AM root colonization was significantly greater when the organic residues were inoculated and incubated for 4 weeks with *C. rigida*, but not when was incubated for 2 weeks. The incubation of the organic residue with *T. harzianum* did not increase the percentage of AM colonization (Figure 6).

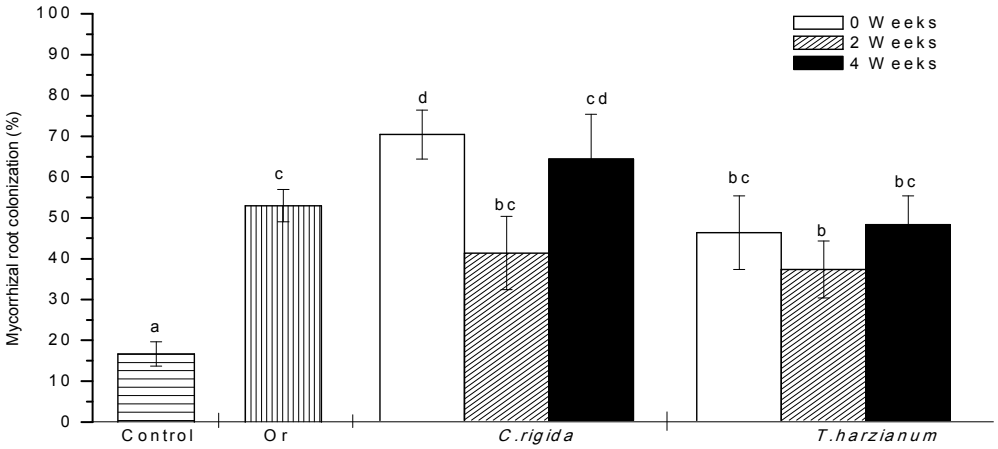

Figure 6. Percentage of root length colonization of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* grown in presence of organic residues inoculated (0) and incubated with the saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residue, *C .rigida*: Organic residue incubated with *C. rigida*, *T. harzianum*: Organic residue incubated with *T. harzianum*. Data are means ± standard error. Bars with the same letter are not significantly different ($P< 0.05$).

5.4 Discussion

Mycorrhizal plants had greater biomass than non-mycorrhizal plants, and these parameters were increased by the presence of the organic residues. The combination of wheat straw with sewage sludge seems to avoid the negative effect of the sewage sludge observed on AM colonization of some plants (Arriagada et al., 2009a). The effect of *T. harzianum* on tomato growth was superior to that of *C. rigida* and this can stimulate growth in a broad range of AM colonized plants including tomato, through several mechanisms, including production or stimulation of hormones or P solubilization (Altomare et al., 1999; Hoyos-Carvajal et al., 2008). However, *T. harzianum* did not increase the percentage of AM root length colonization of tomato in the presence of the organic residues, while *C. rigida* increased the percentage of AM root length colonization but decreased the dry weight of the plants compared with organic residues on their own. It is known that saprobe fungi can increase the infectiveness of AM fungi although they do not always increase their effects on plant growth (Aranda et al., 2007). Moreover, the absence of a close relationship between AM root length colonization and AM effect on plant growth has been observed before (Treseder, 2013). On the other hand, root biomass was not increased by the AM fungus. It is known that mycorrhizal plants do not need to increase root biomass due to the increase in surface which allows a greater area of absorption to be achieved, provided by the mycelium of mycorrhizal fungi (Gonzalez-Monterrubio et al., 2005).

The increased levels of chlorophyll in leaves of AM colonized plants (SPAD values) presumably contributed to higher photosynthetic rates in tomato, thus benefiting the development and functionality of the symbiosis. However, the enhancement of the relative chlorophyll in leaves of plants can be independent on environmental and nutritional factors (Baslam & Goicoechea, 2012). In fact, we found that *R. irregularis* increased relative chlorophyll (SPAD values) regardless of the different level of mycorrhization reached in the presence of either saprophytic fungi.

Soil N and P contents in soil were stimulated by the addition of the organic residues. This could be due to the large amounts of organic matter and nutrients that the organic residues contain mainly because of the input of sewage sludge (Montemurro & Maiorana, 2008). Both soil N and P were decreased by the saprobe fungi, especially as the time of incubation of the residue increased. This would suggest increased degradation of organic matter and mineralization of N and P (Barbarick & Ippolito, 2007) increasing the bioavailability of these nutrients for plant uptake, therefore reduced the amounts N and P in the soil (Diacono & Montemurro, 2010), but if so, the effect was not enough to significantly increase plant growth or the concentrations of N and P in tomato.

The limitation in crop production in acid soils is mainly due to high concentrations of toxic elements, such as aluminum (Al) or manganese (Mn) and by phosphorus deficiency (Kochian et al., 2004). The increase in pH values in the treatments with organic residues may be due to the contribution of these residues in the exchangeable bases and their ability to form complexes with Al^{+3} (Bulluck et al., 2002, Valarini et al., 2009). The increase in pH could enhance mycorrhizal colonization (Coughlan et al., 2000) and crop nutrition in acid soils and may have contributed to the beneficial effect of *T. harzianum* on the dry weight of tomato (Tang et al., 1999). However, although both saprobe fungi increased soil pH there was no beneficial effect of *C. rigida* or *T. harzianum* on plant growth. It is known that the metabolical capacity of both saprobe fungi are different and the role of these fungi on degradation of organic residues are carried out by different metabolical system in which the production of lacasse by *C. rigida* is the main enzymatic way of organic residues degradation (Saparrat et al., 2014), whereas, the production cell wall degrading enzymes, such as cellulase and hemicellulase by *T. harzianum* are the main enzymes implicates in the degradation of biomass (Horta et al., 2014). This may explain their different effects of these fungi on the stimulation of biomass accumulation of mycorrhizal plants in the presence of organic residues.

The enhancement of acid phosphatase, FDA and dehydrogenase activities by organic residues indicates beneficial effects of organic matter, carbon, nitrogen, phosphorus and other physicochemical characteristics of the amendment (Ros et al., 2006). However, organic residues only increased soil β-glucosidase when it was incubated with the saprobe fungi *C. rigida* or *T. harzianum*, but the enhancement of these soil enzymatic activities decreased when the time of incubation of the organic residue with the saprobe fungi increased. Fresh organic amendments provide easily degradable and metabolizable materials via the enzymatic systems of soil microorganisms, resulting in greater soil enzyme activity whereas incubation of organic amendments stabilizes the residues generating more resistant compounds (Bastida et al., 2008). Thus, during the prolonged period of incubation with the saprobe fungi, the nutrients in the substrate may have been used by these fungi in the growth of their mycelium resulting in the decrease of available nutrients for native soil microorganisms.

5.5 Conclusions

The combination of sewage sludge with wheat straw applied to the soil and the inoculation with *S. lycopersicum* with *R. irregularis* improved plant biomass. The highest shoot dry mass was achieved when the organic residue was incubated for 24h with *T. harzianum* and added to mycorrhizal plants. However, *C. rigida* inhibited the positive interaction between the AM fungus and the organic residue on plant growth. N and P contents of soil as well as several soil enzymatic activities were increased with addition of the organic residues, contributing positively to promote plant growth when it was inoculated with AM fungi. These results show the potential of these residues and microorganisms to improve chemical and biochemical properties of soils and enhance the growth of tomato.

CHAPTER VI

Effects of organic residues optimized by saprophytic fungi on the Bacterial soil community

Almonacid, L.[1,2], Fuentez, A.[1], García-Romera, I.[3], Ocampo, J.[3] and Arriagada, C.[1]

(1) Laboratorio de Biorremediación, Universidad de La Frontera. Casilla 54-D. Temuco - Chile.

(2) Programa de Doctorado en Ciencias de Recursos Naturales, Universidad de La Frontera. Temuco - Chile.

(3) Estación Experimental del Zaidín, CSIC. Profesor Albareda 1, 18008. Granada-España.

In preparation

Agricultural and Biological Science

Effects of organic residues optimized by saprophytic fungi on the Bacterial soil community

Abstract

Addition of organic residues and plant growth promoting microorganisms can be used as a strategy to promote plant growth. However, the effect of both depends on several factors, such as soil, quality of residue and microorganism and plant species, among others. The effects of two kinds of residues in combination, wheat straw and sewage sludge as amendment previously inoculated (added at the same time that organic residues) and incubated with *Trichoderma harzianum* for 2 and 4 weeks on rhizospheric soil bacterial communities of *Solanum lycopersicum* plant were evaluated. The addition of both residues as amendment previously inoculated and incubated with *T.harzianum* increase the number of cultivable bacteria especially in the last sampling period (45nd day). Similarly, denaturing gradient gel electrophoresis (DGGE) analysis presented the same trend that cultivable method, showed high species richness values with amendment addition mainly in the last sampling period. The addition of organic residues with *T.harzianum* as amendment and the stimulation of native microorganisms is an essential strategy directly related with plant growth. In this study the addition of this amendment increased growth of *S. lycopersicum* plants inoculated with *Rhizophagus irregularis*, frequency and intensity of mycorrhization and alkaline phosphatase activity of mycorrhizal fungi. These results show that the addition of organic residues especially inoculated with *T.harzianum* improve the growth and mycorrhization of *S.lycopersicum* plants as well as the proliferation of rhizospheric soil bacterias.

Key words: Organic amendments, arbuscular mycorrhizal fungi, cultivable microorganisms.

6.1 Introduction

Addition of organic residues as biostimulation agents and microorganisms as bioaugmentation agents is a friendly environment alternative with lower costs compared with traditional methods (Altieri & Esposito, 2010, Karpouzas et al., 2010, Mahmoud et al., 2010) to maintain the fertility and productivity of soils. However, depending on the nature or treatment of the residues, these may exert different effects on soil properties and soil microorganisms (Abbasi et al., 2002).Both amendments as microorganism addition have an effect on physical chemical and biological parameters of soil. Organic residues are an important source of usable nutrients by microorganisms and this can change the composition and activities of soil microorganisms (Debosz et al., 2002, Karpouzas et al., 2010) depending on factors such as soil or amendment (Perez-Piqueres et al., 2006). On the other hand, the addition of free living or symbiont microorganisms can improve plant growth through processes such as, degradation of organic matter, phosphorus solubilization, protection against disease or nutrients translocation, among others. Soil native microorganisms are essential factors in soil sustainability being directly related to processes such as biogeochemical cycles and degradation of organic matter improving the availability of nutrients by plants. Therefore, is crucial to take into account the possible effect of the amendments addition on the structure and function of soil microorganisms. Several techniques can be used for determining soil microorganisms and evaluate the effect that organic residues may have on soil microorganisms. DNA-based techniques do not give us wide information about the activity of microorganisms (Sessitsch et al., 2013). Whereas the culturable-based methods have the disadvantage that most of microorganisms are uncultivable. Therefore it is desirable to explore both methods. One DNA-based method is DGGE which is used for determining microbial community structure of bacteria, fungi and mycorrhizal fungi (Muyzer et al., 1993, Kowalchuket al., 1997, Ma et al*.,* 2005). Considering that organic residues and fungi addition as amendment can be a direct effect on the stimulation of native microorganisms that are essential for improve plant

growth. In this study sewage sludge and wheat straw in combination with the saprophytic fungi (*Trichoderma harzianum*) and arbuscular mycorrhizal fungi (*Rhizophagus irregularis*), were used.

The aim of this work was to evaluate the effect of mixed organic residues inoculated and incubated with *T.harzianum* on bacterial communities and plant growth of *Solanum lycopersicum* plants inoculated with *R.irregularis*

6.2 Materials and methods

6.2.1 *In vitro* experiments

Saprophytic fungus *Trichoderma harzianum* and arbuscular mycorrhizal fungus *Rhizophagus irregularis* (Krüger et al., 2012), were obtained by fungal culture collection of the Bioremediation Laboratory, Universidad de La Frontera, Temuco, Chile. Sewage sludge was collected from a wastewater plant (Vilcún, Chile) and stored at 4°C until use. Wheat straw was obtained from crop residues from the Araucanía Region. Sewage sludge and wheat straw were mixing (1:3 w:w) and autoclaved at 121°C for 20 min. Subsequently 3 mL of spore suspension equivalent to 1.8 $x10^6$ spores was spread over the mixture and this was incubated for 2 and 4 weeks at 23 °C in darkness.

6.2.2 Rhizospheric soil determinations

Rhizospheric soil samples were taken in order to determine cultivable microorganisms and non cultivable microorganisms by DNA extraction and determination of microorganism diversity by DGGE. For determining number of cultivable microorganisms, 1g of homogeneous soil samples of each pot of *S.lycopersicum* plants previously amended with 8 g of combined residues inoculated with *T.harzianum* (incorporated at the same time that organic residues) and incubated with *T.harzianum* for 2 and 4 weeks was taken and dissolved in 10 mL of sterilized distilled water. Aqueous dilution from 10^{-1} to 10^{-5} was performed. The colony forming units (CFUs) was determined in R2A Agar media. Five replicates were performed by each dilution; CFU_S was expressed per gram of dry rhizospheric soil (Godeas et al., 1999).

DNA samples were extracted using "Power Soil TM DNA isolation Kit; MOBIO", DNA samples were checked using the NanoDrop®ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Selective amplification of DNA was performed using specific primers for bacteria V3f (5'-CCT ACG GGA GGCAGCAG-3') and V3r (5'-ATTACCGCG GCTGCT GG-3'). To the forward primer a GC-clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G) was added according to Muyzer et al., (1993). The reactions were carried out with 25 µL volume, which contained 5 µL Buffer (160 mM $(NH4)_2SO_4$, 670 mM Tris HCl), 2,5 µL Mg Cl_2, 2.5 µL of dNTPs (25 mM), 0,5 µL of each primer, 0.5 µL of Taq DNA Polymerase, and 1 µL DNA (20 ng) and sterile Mili-Q water. The 20 amplification cycles were performed decreasing 0.5 °C per cycle (Touchdown PCR) consisting of an initial denaturation for 5 min at 94 °C, 1 minute at 94 °C, annealing for 1 minute at 65 °C and extension for 1 min at 72 °C, followed by 12 amplification cycle, with an initial denaturation for 5 min at 94 °C, 1 min at 94 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C and final extension for 10 min at 72 °C. The PCR products were checked in a gel 1.5% agarose and frozen at -20 ° C until analysis. The products generated in PCR reaction were analyzed by DGGE on DCode system (BioRad Laboratories, Inc.). 15uL PCR sample was applied and the electrophoresis was carried out at 85 V for 16 hours. A gradient from 45% to 65% denaturant was used. The gels were placed in a fixing solution (10% v/v ethanol and 0.5% v/v acetic acid) for 15 min in staining solution (0.2% w/v $AgNO_3$) for 20 min and in developing solution for 10 min (1.5% w/v sodium hydroxide and 0.8% v/v formaldehyde). Finally they were washed in fixing solution for 5 min (Abreu-Tarazi et al., 2010). The gels were visualized under UV light.

The DGGE banding patterns were digitized and processed using Phoretix 1D analysis software (TotalLab Ltd.). Species richness (R), Shannon-Weaver (H´) and Evenness (E) indexes. The similarity of bacterial profiles was evaluated using the unweighted pair-group method with arithmetical averages (UPGMA) and differences in the abundance of bacterial groups were

measured using non-metric multidimensional scaling (MDS) using freeware PAST (http://folk.uio.no/ ohammer / past /) With the Bray-Curtis similarity index (Jorquera et al., 2014)

6.2.3 Greenhouse experiments

The treatments used in this work consisted of: (1) non-inoculated plants, (2) plants inoculated with *R. irregularis* (3) non-inoculated plants and soil plus organic residues, (4) plants inoculated with *R. irregularis* and soil plus organic residue, (5) non-inoculated plants in soil with organic residues inoculated and incubated with *T. harzianum*, (6) inoculated plants in soil with organic residues inoculated and incubated with *T. harzianum*.

Seeds of *S. lycopersicum* were sterilized with NaClO at 0.5 % for 15 min rinsed and sown in vermiculite. Seedlings were inoculated with a piece of monoxenic culture in a Gel-Gro (ICN Biochemical, Aurora, OH, USA) medium containing *R. irregularis* spores and infected carrot roots. Monoxenic culture (*R. irregularis* and carrot roots) was produced according to the method described by Chabot et al., (1992). Plants were grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, 400 E m^{-2} s^{-1},400-700 nm, with a 16/8 h day/night cycle at 25/19° C and 50% relative humidity.

6.2.4 Plants determination

Plants were harvested 45 days after organic residue addition. Shoot and root were dried in a forced air oven and dry weight was determined with a digital scale. Mycorrhizal colonization was determining according to Trouvelot et al., (1986). Frequency of colonization (%F), intensity of colonization (%M) and arbuscular abundance (%A) with MYCOCALC software http://www.dijon.inra.fr/mychintec/ Mycocal prg/download.html) were determined. Alkaline phosphatase (ALP) activity of mycorrhizal fungi reported as a marker for the efficiency of symbiosis was determined according to Tisserant et al., (1993).

6.2.5 Statistical analysis

The percentage values were arcsine transformed before statistical analyses. Data were The interactions among the main factors data were analyzed using a Factorial analysis of variance (ANOVA)and Fisher's protected least significant differences (LSD) when appropriate (Sokal & Rohlf, 1981). Statistical procedures were carried out with the SPSS software, version 11.0 (SPSS Inc., 1989–2001). Statistical significance was determined at $P< 0.05$.Data sets were tested for normality and equal variance (Kolmogorov Smirnov and Cochran's C test, respectively) and a log transformation was applied when significant departures from normality were found.

6.3 Results and discussion

6.3.1 Rhizospheric soils microorganism determination

Amendment application not only improves soil characteristics but also has a strong influence on soil microorganisms (Pérez- Piqueres et al., 2006, Bastida et al., 2008). The biodiversity of microorganisms is essential factor for the maintenance of soil fertility mainly because of their role in biogeochemical cycles. Organic residue addition increase considerably the number of cultivable bacteria in all treatments compared with treatments without addition of organic residues increased about three-fold bacterial CFU (Table 1). This may be due to stimulation of soil bacteria by the abundance of nutrients, organic matter and easily degradable compounds provided by the amendments which are used by them (Marschner et al., 2003). Besides, soil pH is higher in all treatments with organic residues application (not shown data), increasing to nearly neutrality values, which improve the presence of bacteria.On the other hand,to compare the different sampling period a greater quantity of bacterial CFU was determinated after 45 days of organic residues addition in relation to the first sampling period (Table1).

Regarding the DGGE analysis Shannon-weaver index that correspond to the proportional abundance of species in a community showed values around 3 which is considered as a high level

of biodiversity. On the other hand, similar results that cultivable method was observed. In the sampling one day after the organic residue addition all treatments showed a greater species richness that the control (Table2). Similarly this index also showed the same trend that results obtained by cultivable methods increasing in the last sampling period (Table2). In order to determine whether there is any grouping of results among treatments an unweighted pair-group method cluster analysis and multidimensional scaling analysis of DGGE profiles was performed for the three sampling periods. The cluster analysis showed a similar trend among the treatments not showing a clear grouping among treatments with to the exceptions of Th4+Ri (Figure1). However, when analyzing the samples in set, 3 different groups were observed showing that the greater differences were observed among the different sampling times and not among the treatments (Figure 2).

Microbial biomass changes are associated with changes in the availability of mineralized nutrients (Spedding et al., 2004).The addition of these residues improves rapid proliferation of bacteria in the short term attributable to the high amount of nutrients that are readily usable by these microorganisms. On the other hand, changes in the composition of organic matter due to decomposition processes produced changes in the amount and even in the community and structure of soil microorganisms (Marschner et al., 2003). This could explain higher number of bacteria in the last sampling period. Similarly, the phenological stage of plants can modify the community of soil microorganisms producing changes in the time (Buyer et al., 2002). Even these differences could be associated to natural variations mainly due to the high value of species richness found at 45 days in the control. At 45 days the organic residues could be being degraded by other microorganisms such as fungi acting on the more recalcitrant fraction of organic matter which could improve the presence of this microorganism in relation to bacterias phenomenon that would not be observed in the control.

6.3.2 Plant determinations

The addition of organic residues incubated with *T.harzianum* also improves growth of *S.lycopersicum* plants attributable to the nutrient input and the stimulation of the native soil microorganisms. High biomass production was observed in plants inoculated with *R. irregularis* and amended with residues inoculated with *T. harzianum*. Organic residues addition improves the growth of *S. lycopersicum* plants in average ten-fold compared with the control. While, in treatments inoculated with *R.irregularis* the plants were in average 20-fold higher than controls presenting the highest growth in the treatment inoculated with *T.harzianum* (Figure 3).Plant growth may be associated with a greater amount of nutrients and organic matter provided by the amendment inoculated with *T. harzianum* and unincubated, being both sources of fresh organic matter compared with the residue incubated for 2 or 4 weeks. Organic residues inoculated with *T.harzianum* compared with longer incubation (Th4) shows a greater amount of P (7301-3124 mg kg^{-1}) and Mg (4388-2311 $mgkg^{-1}$) (unpublished data). *T. harzianum* can enhance the solubilization of phosphorus, which would explain the higher growth in the treatment inoculated with *T.harzianum*, increasing the availability of this element to plant growth, whose transport to the plant can be improved by the mycorrhizal colonization

Similarly, the addition of organic residues improves the frequency and intensity of mycorrhization, especially in the treatments with amendment addition inoculated with *T.harzianum* and in the treatments with addition of organic residues (Figure 4 A, B, C). Organic residues addition with or without *T.harzianum* increased alkaline phosphatase activity of mycorrhizal fungi concerning the inoculated treatments without residues addition (Ri) (Figure 5).Mycorrhizal colonization may be associated with a greater amount of nutrients and organic matter provided by the amendment. Organic matter of this can have an effect on the development of mycorrhizal fungi and on the interaction of these with other soil microorganisms (Larsen et al., 2009) and even accelerate organic matter decomposition (Hodge et al., 2001). On the other hand, plants grown in soil with a

higher nutritional quality also contain native microorganisms, which could interact with the mycorrhizal fungus and be responsible for higher values of mycorrhization.

6.4 Conclusions

Organic residues addition increased the number of bacterial CFU especially in the last sampling period. Furthermore, UPGMA cluster analysis and Multidimensional scaling showed that the main differences were obtained in the sampling periods and not among the treatments. Both cultivable method (plate counting) and uncultivable method (DGGE) show the same results confirming that both methods are complementary and can be used in this kind of studies.

On the other hand, organic residues inoculated with *T.harzianum* presented the best results concerning biomass production increasing the growth over twenty-fold compared with the treatment inoculated with *R.irregularis* without organic residues. Similarly, the same treatment presented the best frequency of mycorrhization and alkaline phosphatase mainly attributed to the major amount of nutrients present in this treatment, as well as, the presence of native microorganisms.

Table 1. Number of Bacteria CFUs sampled after 1, 22 and 45 days from organic residues addition in the rhizospheric soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* and the addition of organic residues inoculated and incubated with saprophytic fungi for 2 and 4 weeks.

(A) 10^5	**1**			**22**			**45**		
Control	0.25	±0.08	b	0.28	±0.008	a	0.8	±0.50	a
Rhizophagus irregularis	0.15	±0.04	a	0.11	±0.005	a	3.45	±0.15	b
Or	0.72	±0.30	c	0.30	±0.030	ab	3.67	±0.072	b
Or+Ri	0.32	±0.06	b	0.56	±0.020	abc	4.45	±0.284	abc
***T.harzianum* 0 Weeks**	0.36	±0.14	b	0.47	±0.044	abc	6.65	±0.548	cd
***T.harzianum* 2 Weeks**	0.87	±0.29	c	0.44	±0.034	abc	4.63	±0.578	abc
***T.harzianum* 4 Weeks**	1.22	±0.49	c	0.48	±0.028	abc	4.15	±0.321	abc
***T.harzianum* +Ri 0 Weeks**	0.84	±0.32	c	0.64	±0.064	abc	9.85	±1.44	d
***T.harzianum* +Ri 2 Weeks**	0.92	±0.49	c	0.29	±0.030	ab	5.90	±0.316	bc
***T.harzianum* +Ri 4 Weeks**	0.93	±0.25	c	0.49	±0.031	abc	4.88	±0.116	abc

Or: Organic residue without fungi, Or+Ri: Organic residue + *R. irregularis*, *T. harzianum*: Organic residue inoculated or incubated with *T. harzianum*, *T. harzianum*+Ri: Organic residue inoculated or incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error. Columns with the same letter are not significantly different according to Fisher's LSD test ($P< 0.05$).

Table 2. Species richness (R), Shannon (H') and evenness (E) diversity indexes of bacterial diversity sampled after 1, 22 and 45 days from organic residues addition in the rhizospheric soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* and the addition of organic residues inoculated and incubated with saprophytic fungi for 2 and 4 weeks.

	1 days			**22 days**			**45 days**		
Treatments	**R**	**H'**	**E**	**R**	**H'**	**E**	**R**	**H'**	**E**
Control	19	2.7	0.75	36	3.19	0.67	36	3.27	0.73
Or	21	2.7	0.73	28	2.98	0.70	33	3.11	0.68
Rhizophagus irregularis	23	2.7	0.67	29	3.11	0.77	38	3.33	0.74
Or+Ri	22	2.8	0.75	32	3.25	0.80	38	3.34	0.74
***T.harzianum* 0 Weeks**	23	2.8	0.74	30	3.14	0.77	35	3.23	0.72
***T.harzianum* 2 Weeks**	23	2.9	0.79	29	3.07	0.74	31	3.05	0.68
***T.harzianum* 4 Weeks**	21	2.8	0.76	38	3.36	0.76	23	2.74	0.67
***T.harzianum* +Ri 0 Weeks**	21	2.8	0.77	31	3.12	0.73	33	3.17	0.72
***T.harzianum* +Ri 2 Weeks**	23	2.9	0.77	24	2.96	0.80	23	2.83	0.73
***T.harzianum* +Ri 4 Weeks**	28	3.1	0.82	35	3.28	0.76	23	2.74	0.74

Or: Organic residue without fungi, Or+Ri: Organic residue + *R. irregularis*, *T. harzianum*: Organic residue inoculated or incubated with *T. harzianum*, *T. harzianum*+Ri: Organic residue inoculated or incubated with *T. harzianum* + *R. irregularis*. Data are means ± standard error.

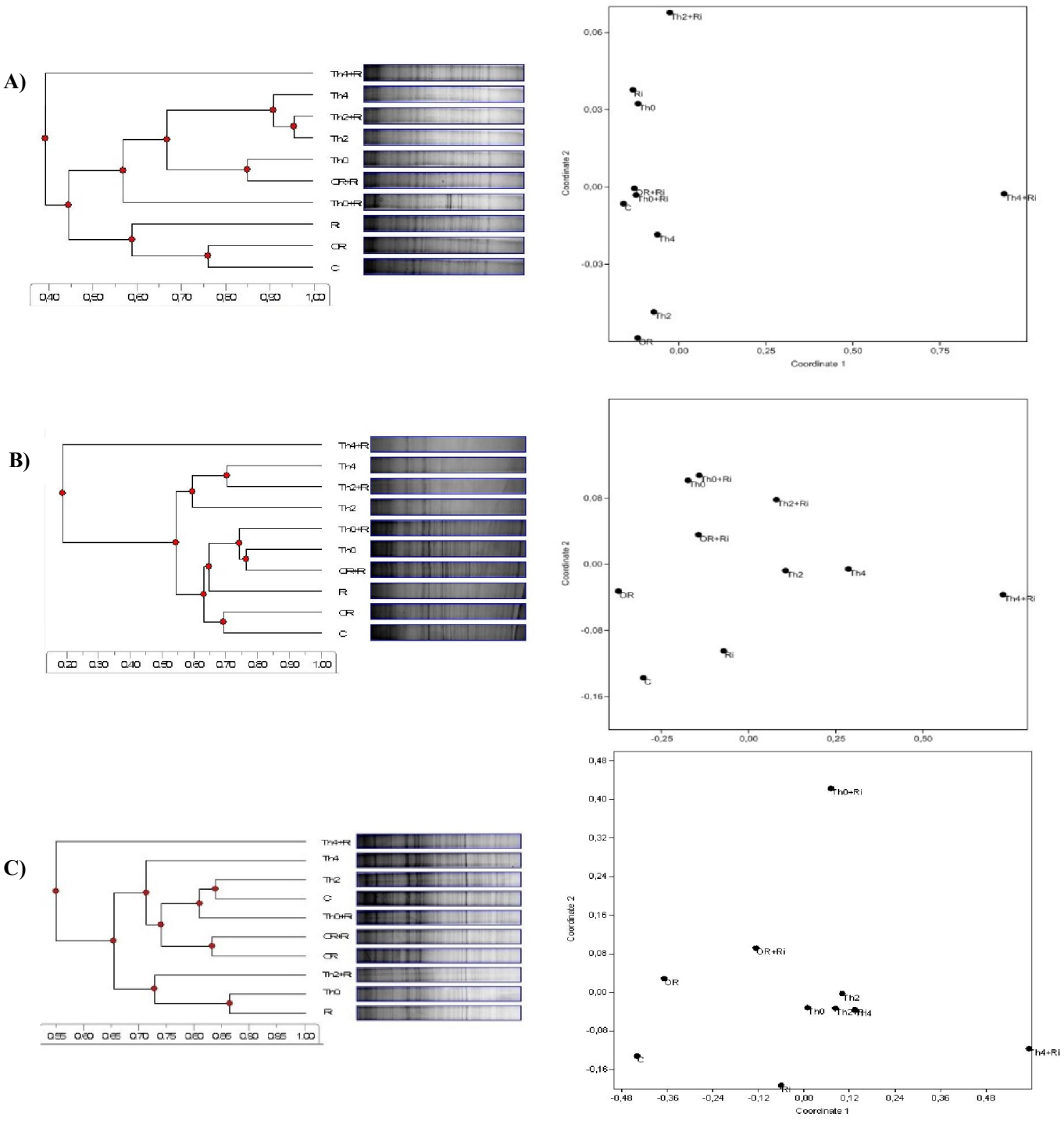

Figure 1. Unweighted pair-group method with arithmetical averages (UPGMA) cluster analysis and multidimensional scaling (MDS) analysis of DGGE profiles for bacteria communities after A) 1, B) 22 and C) 45 days from organic residues addition in the rhizospheric soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis* with addition of organic residues inoculated and incubated with saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residues, Ri: *R. irregularis*, OR+Ri: Organic residues + *R. irregularis*, Th: Organic residues + *T. harzianum*, Th+Ri: Organic residues + *T. harzianum* + *R. irregularis*.

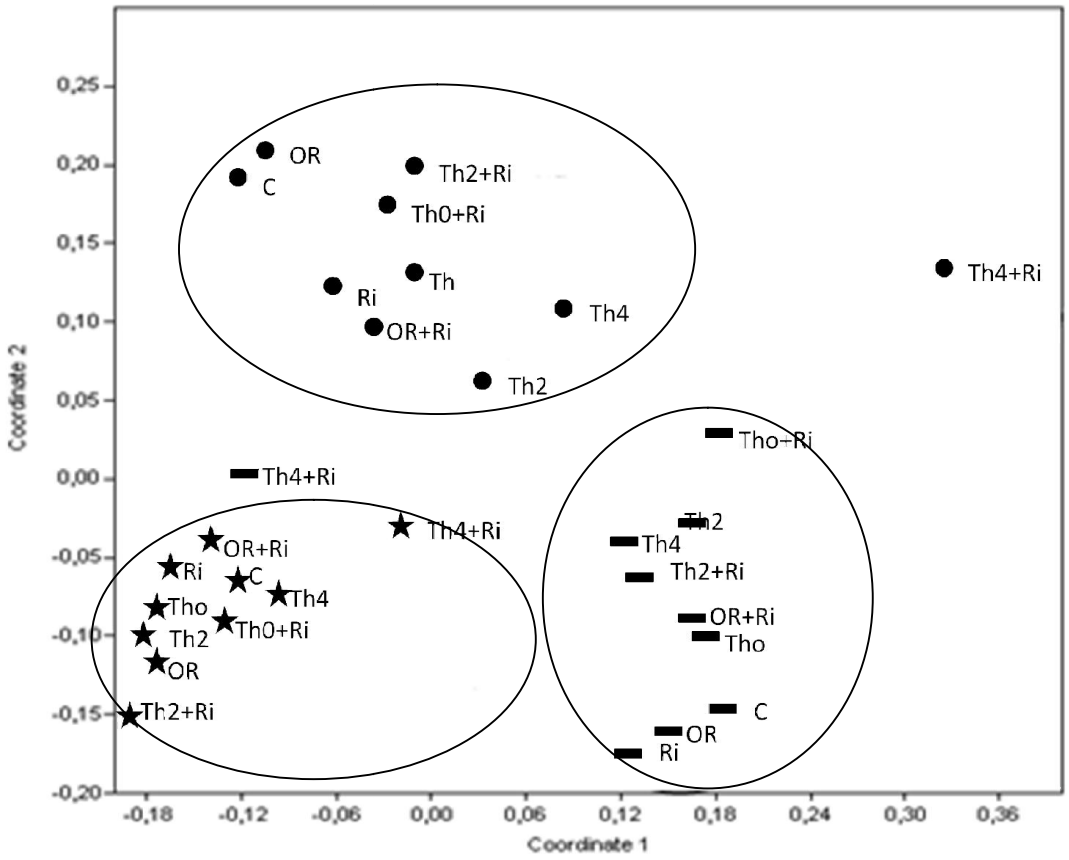

Figure 2. Multidimensional scaling (MDS) analysis of DGGE profiles for bacteria communities from rhizospheric soil of *Solanum lycopersicum* plants sampled after (1= ━ 22= ★ 45= ●) days from organic residues addition in the rhizospheric soil of *Solanum lycopersicum* inoculated with *Rhizophagus irregularis*. C: Control, Or: Organic residues, Ri: *R. irregularis*, OR+Ri: Organic residues + *R. irregularis*, Th: Organic residues + *T. harzianum*, Th+Ri: Organic residues + *T. harzianum* + *R. irregularis*.

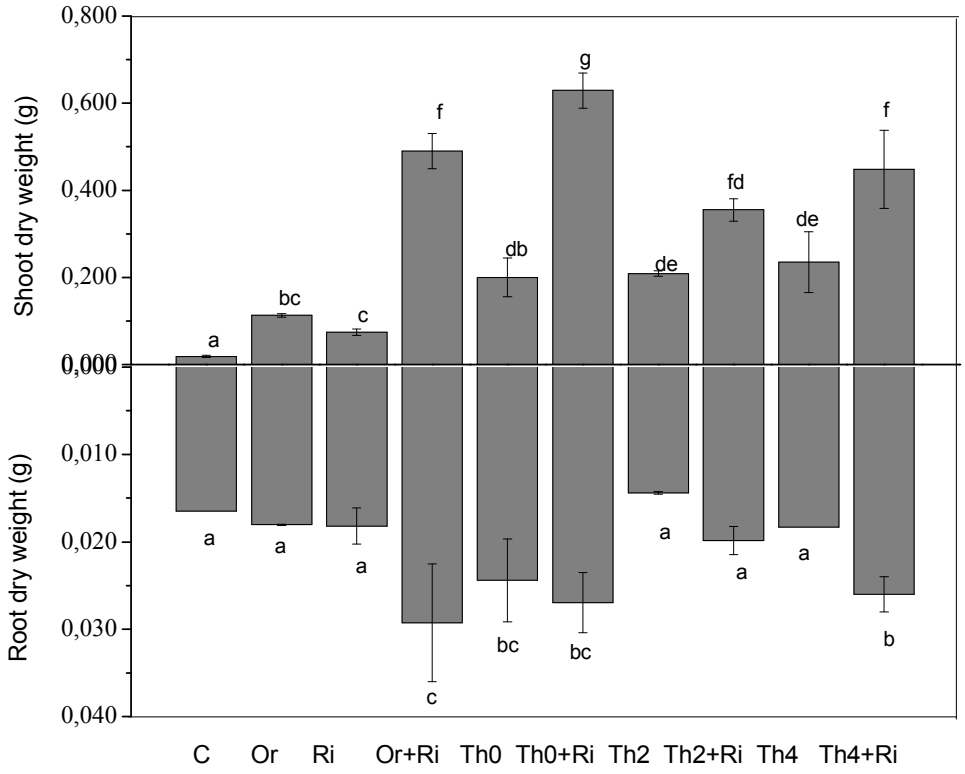

Figure 3. Shoot and root dry weight (g) of *Solanum lycopersicum* plant amendment with organic residues inoculated and incubated with saprophytic fungi for 2 and 4 weeks. C: Control, Or: Organic residues, Ri: *R. irregularis*, Or+Ri: Organic residues + *R. irregularis*, Th0: Organic residues inoculated with *T. harzianum*, Th0+Ri: Organic residues inoculated with *T. harzianum* + *R. irregularis*, Th2: Organic residues incubated with *T. harzianum* for 2 weeks, Th2+Ri: Organic residues incubated with *T. harzianum* for 2 weeks+ *R. irregularis*, Th4: Organic residues incubated with *T. harzianum* for 4 weeks, Tht4+Ri: Organic residues incubated with *T. harzianum* for 4 weeks + *R. irregularis*. Data are means ± standard error.Bars with the same letter are not significantly different according to Fisher's LSD test ($P< 0.05$).

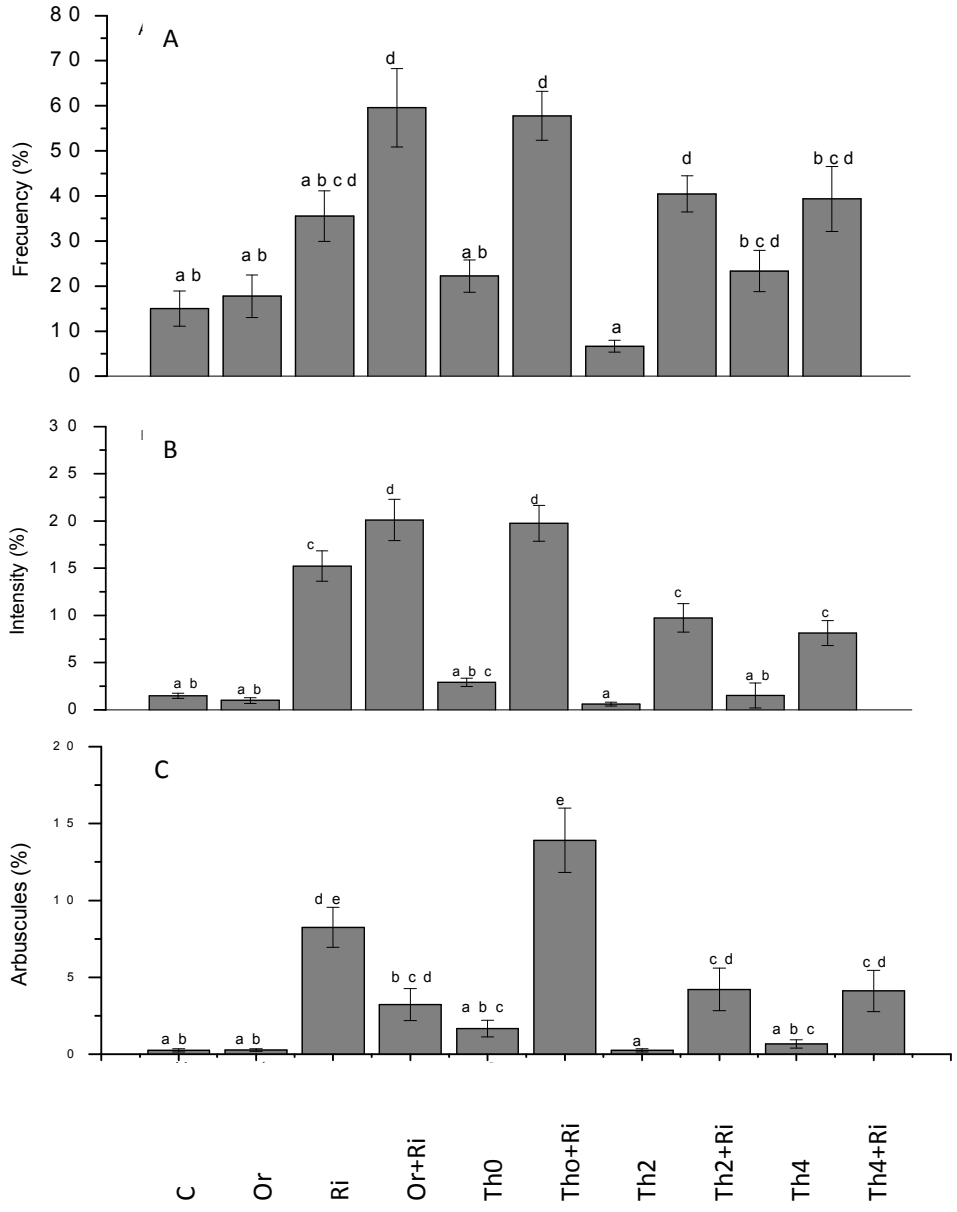

Figure 4. Root colonization (%) of *Solanum lycopersicum* plant amendment with organic residues inoculated and incubated with saprophytic fungi for 2 and 4 weeks; A).Frequency, B) Intensity, C) Arbuscules.C: Control, Or: Organic residues, Ri: *R. irregularis*, Or+Ri: Organic residues + *R. irregularis*, Th0: Organic residues inoculated with *T. harzianum*, Th0+Ri: Organic residues inoculated with *T. harzianum* + *R. irregularis*, Th2: Organic residues incubated with *T. harzianum* for 2 weeks, Th2+Ri: Organic residues incubated with *T. harzianum* for 2 weeks+ *R. irregularis*, Th4: Organic residues incubated with *T. harzianum* for 4 weeks, Tht4+Ri: Organic residues incubated with *T. harzianum* for 4 weeks + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different according to Fisher's LSD test ($P< 0.05$).

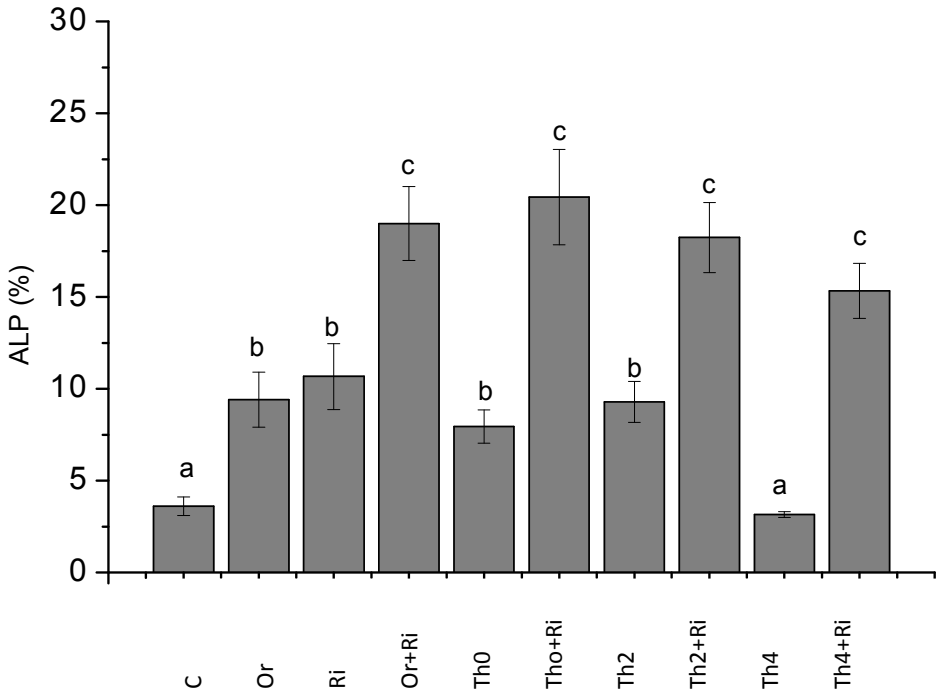

Figure 5. Fungal alkaline phosphatase activity (ALP) in *Solanum lycopersicum* plants amendment with organic residues inoculated and incubated with saprophytic fungi for 2 and 4 weeks. C: Control, OR: Organic residues, Ri: *R. irregularis*, OR+Ri: Organic residues + *R. irregularis*, Th0: Organic residues inoculated with *T. harzianum*, Th0+Ri: Organic residues inoculated with *T. harzianum* + *R. irregularis*, Th2: Organic residues incubated with *T. harzianum* for 2 weeks, Th2+Ri: Organic residues incubated with *T. harzianum* for 2 weeks+ *R. irregularis*, Th4: Organic residues incubated with *T. harzianum* for 4 weeks, Tht4+Ri: Organic residues incubated with *T. harzianum* for 4 weeks + *R. irregularis*. Data are means ± standard error. Bars with the same letter are not significantly different according to Fisher's LSD test ($P< 0.05$).

CHAPTER VII

General discussion, general conclusions and future directions

7.1 General discussion

There are several types of organic residues used as amendments in agroforestry systems, including crop residues, sugar beet, olive mill residue or sewage sludge from wastewater treatment plant which exert different effects on soil. Sewage sludge contains large amounts of organic matter and nutrients (He et al., 2000, Chodak et al., 2001, Tejada et al., 2001, Mantovi et al., 2005). This residue can improve soil fertility increasing the efficiency of agricultural production and be an alternative to chemical fertilization, but can also be a source of environmental pollution, especially when they are improperly used. In the chapter number III the optimization of organic residues by the inoculation and incubation of organic residues with *C.rigida* and *T.harzianum* was evaluated. In this experiments *C.rigida* showed greater production of ligninolytic enzymes associated with increased degradation of residues compared with *T.harzianum*. However, both saprophytic fungi produce a degradation of the organic residues which is reflected in the decrease of dry matter and cellulose content after 4 weeks of incubation. This degradation process is mediated by the action of ligninolytic and hydrolytic enzymes. Both fungi showed enzymatic activity when they were incubated with the organic residue. *C.rigida* showed high activity of ligninolytic and hydrolytic enzymes reflecting in the FDA activity (Sanchez-Monedero et al., 2008). However, the later studies showed that to despite the residue incubated for 4 weeks has a higher degradation it's not improve the growth and development of symbiosis as the residue which was added at the same time with the organic residue (inoculated).

In the study showed in Chapter number IV, no beneficial effects of sewage sludge were observed on either yield or the nutrient concentration in shoots of *E.globulus*, mainly due to the high levels of soil Al that reached 290 mg kg^{-1} soil. The high Al concentration in the sewage sludge used in this study may explain the decrease in the percentage of AM root length colonization and the unchanged dry weight of the plant. Nevertheless, the negative effect of sewage sludge on AM colonization and its lack of a beneficial effect on plant dry weight were overcome by the addition of wheat straw reducing the toxicity of metals in AM-colonized *E. globulus* plants, probably

through their immobilisation and preventing the translocation of heavy metals into the plant (Juwarkar & Jambhulkar, 2008). Therefore, the wheat straw would mitigate the toxicity of the sewage sludge, in addition to increasing the metabolic activity of the mycorrhizal fungi resulting also in a higher biomass. On the other hand, this increase in biomass production may be related to the increased amount of nutrients present in the treatment with mixture addition, mycorrhizal inoculation and especially with the application of both types of microorganisms, where saprophytic would degrade the organic matter through the production of hydrolytic enzymes and the subsequent mycorrhiza fungi transport of inorganic nutrients into the plant. On the other hand, FDA, β-glucosidase and dehydrogenase increased with the addition of mixture. This can be due to the nutrients input especially by the presence of sewage sludge that increases the metabolic activity of soil microorganisms.

Generally the addition of amendments and microorganisms inoculation increases growth of plants but, in some cases, this effect is neutral or negative depending on several factors such as plant species. In Chapter number V, we reported the effect of the addition of wheat straw and sewage sludge in combination, inoculated an incubated with *T.harzianum* on growth of *S.lycopersicum* plants and on chemical and biochemical properties of rhizospheric soil. Similar to the results observed in Chapter number IV, the combination of wheat straw with sewage sludge seems to avoid the negative effect of the sewage sludge on AM colonization (Arriagada et al., 2009c). Therefore, the addition of this organic residue and the root colonization by the AM fungi were key factors for improving the growth of plants, especially when the organic residue was incubated with *T.harzianum*. Several studies show the beneficial effects of *T.harzianum* through several mechanisms, including production or stimulation of hormones or phosphorus solubilization (Altomare et al., 1999, Hoyos-Carvajal et al., 2009). Similarly, mycorrhizal fungi enhance, the levels of chlorophyll in leaves regardless the different level of mycorrhization reached in presence of both saprophytic fungi. On the other hand, soil nitrogen and phosphorus content in soil were stimulated by the addition of organic residues. This could be due to the high amounts of organic

matter and nutrients that organic residues contain mainly because of the input of sewage sludge (Montemurro & Maiorana, 2008). Furthermore, organic residues amendment increased soil β-glucosidase and FDA activities when it was incubated with both saprophytic fungi. However, the enhancement of these soil enzymatic activities decreased when the time of incubation of the organic residue with the saprophytic fungi was increased. Thus, during the prolonged period of incubation with the saprophytic fungi, the nutrients in the substrate might have been used by these fungi in the growth of their mycelium resulting in the decrease of available nutrients for native soil microorganisms.

Amendment application not only improves soil characteristics but also has a strong influence on soil microorganisms (Pérez-Piqueres et al., 2006, Bastida et al., 2008). Therefore, in Chapter number VI the effect of *T. harzianum* inoculated (added at the same time with organic residues) and incubated for 2 and 4 weeks on growth and rhizospheric microorganisms of *S. lycopersicum* plants were evaluated.A higher amount of cultivable bacteria was observed in the treatments with inoculated and incubated amendment addition especially in the last sampling period, this may be due to stimulation by the abundance of nutrients, and organic matter (Marschner et al., 2003). The differences in the sampling periods can be explained by the development state of plants that can modify the community of soil microorganisms (Buyer et al., 2002) and by changes in the composition of organic matter due to decomposition processes (Calbrix et al., 2007). Besides, DGGE analysis showed greater species richness values than the control in the first sampling period (one day). On the other hand, were more important the differences among sampling periods than among treatments, as it was demonstrated when analyzing the three sampling periods together.

The results obtained in this doctoral thesis show that wheat straw and sewage sludge in combination and incubated with saprophytic fungi improve biomass production of plant inoculated with mycorrhizal fungi and the biochemical and biological parameters of the rhizospheric soil. Therefore the addition of this residues incubated and the AM fungi to agroforestry plants seems to

be an interesting option as fertilizer because it is able to improve plant growth and biochemical and biological parameters of soil.

7.2 General Conclusions

- The incubation of organic residues for 4 weeks with *C.rigida* and *T.harzianum* cause the degradation of substrate. However, the addition of this degraded organic residue generates lower plant growth, nutrients inputs and a lower stimulation of soil enzyme activities than the residue added at the same time with saprophytic fungi.

- The combination of saprophytic fungi, *Coriolopsis rigida* and *Phanerochaete chrysosporium* with the inoculation of arbuscular mycorrhizal fungi *Rhizophagus irregularis* and addition of wheat straw reduce the toxic effects of sewage sludge with high aluminium concentration increasing nutrients content and growth of *Eucalyptus globulus* plants as well as enzymatic activities of rhizospheric soil.

- The addition of organic residues inoculated (added at the same time that orgánico residues) with *Trichoderma harzianum* increased plant biomass production of *Solanum lycopersicum* plants inoculated with *Rhizophagus irregularis* over twenty-fold compared with the control. Similarly, the addition of this amendment improves nutrients translocation and increased nitrogen and phosphorus content as well as β-glucosidase and FDA activity of rhizospheric soil.

- Organic residues and saprophytic fungi addition increased the percentage of mycorrhization and activity of *Rhizophagus irregularis* being a key factor in biomass production of *Solanum lycopersicum* and *Eucalyptus globulus* plants.

- Amendment addition increased the number of soil bacteria being key microorganisms in the degradation of organic matter increasing the bioavailability of nutrients and promoting plant growth agroforestry species.

- These results show the potential of these residues and microorganisms in order to generate optimized organic amendments for improving chemical, biochemical and biological properties of soils apart from increasing soil fertility, thereby enhancing the growth of *Eucalyptus globulus* and *Solanum lycopersicum.*

7.3 Future directions

The positive effect of organic amendments depends on several factors and it is necessary to develop research in order to establish optimal combinations of organic amendments and microorganisms for improving plant growth. On the other hand, is essential to transfer this technology into productive activities such as horticulture or forestry production for instance through the production of inoculum generated from organic amendments and microorganisms both free living and symbiotic in order to promote plant growth. Besides, another area of application of inoculum could be in bioremediation processes through growth promotion strategies, such as phytohormone production, chelating agents or solubilizing phosphates to play a key role in the establishment and development of plants. Likewise they can use in processes of recovery degraded soils, increasing fertility and productivity of these by the input of nutrients from the incorporation of these residues and through the promotion of growth of both types of fungi allowing restoration of plant species.

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