THE PHYSIOLOGY OF MYCORRHIZAL *Lolium multiflorum* IN THE PHYTOREMEDIATION OF PETROLEUM HYDROCARBON-CONTAMINATED SOIL

A Dissertation

by

ALEJANDRO ALARCÓN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Horticulture

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Approved by:

Chair of Committee, Frederick T. Davies Jr.
Committee Members, Robin L. Autenrieth
David Wm. Reed
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ABSTRACT

The Physiology of Mycorrhizal *Lolium multiflorum* in the Phytoremediation of Petroleum Hydrocarbon-Contaminated Soil. (August 2006)

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Arbuscular mycorrhizal fungi (AMF) can play an important role in the phytoremediation of petroleum hydrocarbon (PH)-contaminated soil. However, little is known about the effects of AMF in combination with biostimulation via fertilization or bioaugmentation with hydrocarbonoclastic microorganisms, during phytoremediation of PH in soils.

This research evaluated the influence of the AMF *Glomus intraradices* and inorganic fertilization on growth and physiological responses of *Lolium multiflorum* Lam. cv. Passarel Plus during phytoremediation of soil contaminated with Arabian medium crude oil (ACO). Also determined was the interaction of AMF with the hydrocarbonoclastic bacterium, *Sphingomonas paucimobilis* EPA505 (Sp), and the filamentous fungus, *Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce), on growth and selected physiological responses of *L. multiflorum* during phytoremediation of soil contaminated with benzo[*a*]pyrene (BaP) or ACO.

This research provides evidence that AMF enhance the phytoremediation of petroleum hydrocarbons in soils when inoculated with *L. multiflorum*. The concentration of petroleum hydrocarbons in soil was a determining factor of potential benefits of AMF

on *L. multiflorum*. Low (3000 $mg \cdot kg^{-1}$) or high (15000 $mg \cdot kg^{-1}$) concentrations of ACO resulted in limited benefits of AMF on plant growth, physiology, and degradation of ACO in soil. However, when plants were exposed to an intermediate ACO concentration in soil (6000 $mg \cdot kg^{-1}$), AMF plants had enhanced growth, physiological responses, and greater ACO-degradation than non-AMF plants. The AMF symbiosis in roots of plants was observed at all concentrations of ACO-contaminated soil.

This research is one of the first reports demonstrating the benefits of AMF on the degradation of benzo[*a*]pyrene or ACO, alone or in combination, with the hydrocarbonoclastic microorganisms. Thus, AMF resulted in a beneficial synergism with the hydrocarbonoclastic microorganisms, particularly during ACO-degradation in the rhizosphere of *L. multiflorum*. Hydrocarbonoclastic microorganisms had no negative effects on AMF colonization.

DEDICATION

In memoriam:

To my grandparents (Dolores y Josafat), my aunt (Victoria), and my cousin (Daniel).

To my familiy:

Josafat (Uncle), Eva (Mother), Esther (Aunt), Josafat (Cousin), Adan and Arturo (Brothers), Angeles, Ericka, and Rosa (Sisters), and all my nephews and nieces.

To my professional mentor and friend:

Dr. Ronald Ferrera-Cerrato

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CHAPTER I

INTRODUCTION

Contamination of soil with toxic compounds is an important environmental issue, which has attracted public attention and led to the creation of methodologies to detoxify such compounds and avoid their detrimental effects. Bioremediation is an alternative to expensive and destructive physical and chemical processes of soil remediation, in part because it is more cost-effective and environmentally sound (Juhasz and Naidu, 2000). Phytoremediation is based on the utilization of plants to detoxify and eliminate contaminants from soil (Dietz and Schnoor, 2001). The establishment of plants contributes significant changes to the soil chemical, physical and biological properties in the rhizosphere, which in turn favor the dissipation and/or degradation of contaminants in soil.

Plants can avoid toxic effects of soil contaminants via specific root physiological processes, which are stimulated by the presence of contaminants (Binet et al., 2000a; Dietz and Schnoor, 2001; Dzantor et al., 2000; Neduhuri et al., 2000). While phytoremediation research largely deals with heavy metal-contaminated soils, research on phytoremediation of organic contaminants is limited (Alkorta and Garbisu, 2001; Cunningham et al., 1997).

Although plants utilized in phytoremediation characteristically have higher tolerance to organic contaminants (Merkl et al., 2005a) such as petroleum hydrocarbons (PH), they are subjected to a stressful soil environment that limits their growth and

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This dissertation follows the style of the Journal of the American Society for Horticultural Science.

development. The exposure of plants to PH in soils limits growth by affecting water and nutrient uptake (De Jong, 1980; Merkl et al., 2005b), which can alter physiological responses and therefore reduce plant tolerance and survival. Plants adapt to abiotic stress through selected physiological responses, which can include improvement of water absorption, enhanced nutrient uptake, and the induction of free-radical scavenging systems to avoid cellular damage (Grace and Logan, 2000; Mahayan and Tuteja, 2005; Misra and Gupta, 2006; Nayyar and Gupta, 2006; Qadir et al., 2004). However, physiological response of plants utilized in phytoremediation of petroleum contaminated soils is not well studied.

Roots play an important role not only in mining the soil for water and nutrients, but also as a primary factor for dissipating soil contaminants, thus avoiding their toxic effects on plants (Heinonsalo et al., 2000; Meharg and Cairney, 2000b). Plant alleviation of soil contaminants is often increased by native microflora inhabiting the rhizosphere. Selected microorganisms can degrade soil organic contaminants and complete contaminant degradation is favored by microbial cooxidation and cometabolism (Alexander, 1999; Rivera-Cruz et al., 2002b; Trejo and Quintero, 2000). The inoculation of plants with free-living microorganisms, such as bacteria and filamentous fungi, has significantly improved the phytoremediation of organic and inorganic contaminants (Alexander, 1999; Anderson et al., 1993; Atlas, 1995; Ferrera-Cerrato, 2000; Jasper, 1994; Pérez-Vargas et al., 2000).

Arbuscular mycorrhizal fungi (AMF) are an important component of the rhizosphere, and have been well documented to enhance phytoremediation of heavy metal-contaminated soils (Cairney and Meharg, 1999; Davies et al., 2001; Gonzalez-

Chavez, 2000; Griffioen and Ernst, 1989; Meharg and Cairney, 2000a; Van Diun et al., 1991). In some of these studies, physiological mechanisms for AMF have been identified such as avoidance and tolerance (Perotto and Martino, 2001). However, few studies have reported the role of AMF on phytoremediation of soils contaminated with PH and polycyclic aromatic hydrocarbons (PAH). The establishment of this symbiosis may potentially contribute to stress alleviaton by enhancing plant nutrient and water uptake, and subsequent growth and adaptation (Smith and Read, 1997).

AMF may be an indicator of soil decontamination, since AMF-colonization in the root system increases as the contaminant is degraded or dissipated from the rhizosphere, which is attributed to the proliferation of petroleum-degrading (hydrocarbonoclastic) rhizobacteria and free-living N_2-fixing bacteria in the mycorhizosphere (Hernandez-Acosta et al., 2000). Studies have shown that AMF play a significant role on plant survival and tolerance to PH and PAH, such as anthracene, chrysene and dibenz(*a,h*)anthracene (Binet et al., 2000b; Joner and Leyval, 2001; Leyval and Binet, 1998).

Nevertheless, the physiological impact of AMF on plants during the phytoremediation of PAH-contaminated soils has not been well studied. There is evidence that AMF-plants are more tolerant of PAH-contaminated soil, which is likely related to the greater adaptation, survival, establishment and fitness of plants (Cabello, 2001; Joner and Leyval, 2003b). Some proposed mechanisms by which AMF may contribute to the dissipation/degradation of organic contaminants (Joner et al., 2001; Joner and Leyval, 2003a) include: 1) mycorrhizal modification of plant and microbial metabolism, 2) enhanced root peroxidase activity (Criquet et al., 2000), 3) modification

of rhizosphere microbial populations due to alteration of root biomass and exudation of specific compounds that induce the degradation of organic contaminants, and 4) formation of an abundant extraradical mycelium with exudates, including glomalin and extracellular enzymes, which may enhance specific bacterial activity and drive cometabolic degradation of organic contaminants.

The general objectives and hypotheses for this research were as follows:

1. To study the influence of AMF inoculation and inorganic fertilization (biostumulation) on plant growth and selected physiological responses of *Lolium multiflorum* during phytoremediation of Arabian medium crude oil (ACO)-contaminated soil.

   Hypothesis: AMF inoculation and application of inorganic fertilization enhance growth, selected physiological responses, and phytoremediation of ACO in the rhizosphere of *Lolium multiflorum*.

2. To evaluate the effect of the inoculation (bioaugmentation) of petroleum degrading (hydrocarbonoclastic) bacterium, filamentous fungus, and AMF on plant growth, selected plant physiological responses of *Lolium multiflorum* during phytoremediation of benzo[*a*]pyrene (BaP) in sand.

   Hypothesis: Plant growth, selected physiological responses, and phytoremediation of BaP in the rhizosphere of *Lolium multiflorum*, are enhanced through the inoculation of AMF and hydrocarbonoclastic microorganisms (bacteria and filamentous fungi).

3. To evaluate the influence of the inoculation of AMF and bioaugmentation with petroleum-degrading bacteria and filamentous fungi on growth and selected physiological responses of *Lolium multiflorum* during phytoremediation of ACO-contaminated soil.

   Hypothesis: The interaction of AMF inoculation with bioaugmentation of hydrocarbonoclastic microorganisms enhances growth, selected physiological responses, and phytoremediation of ACO in the *Lolium multiflorum* rhizosphere.

CHAPTER II

LITERATURE REVIEW

Roles of Arbuscular Mycorrhizal Fungi in Plants Growing in Petroleum Hydrocarbon (PH)-Contaminated Soils and Their Influence on Phytoremediation Performance

The worldwide petroleum industry produces, distributes, and consumes large quantities of oil and oil-derivatives which are the major energy source for factories and transportation. As an example, the U.S. consumes over 250 billion gallons of oil and petroleum products every year, and produces and imports 125 and 114 billion gallons per year, respectively, of crude oil and petroleum-based products (API, 2006; USEPA, 2004). The production, refining, processing, distribution and storage of petroleum products are an environmental pollution threat (API, 2006). For developed countries, successful oil programs have been designed to minimize oil spills to less than 1% of the total volume handled each year. In contrast, countries such as Mexico with less stringent environmental regulations, have had significant oil spills that have contributed to the release of approximately 1.5 million tons of contaminants per year to soil, air, and water systems (SEMARNAT, 2004). In Tabasco, Mexico which is one of the most important Mexican States for petroleum extraction and distribution, the extent of contaminated soil surface by oil spills is approximately 0.07% of the total area of the state (Rivera-Cruz et al., 2004; Zavala-Cruz et al., 2005).

As a response to the release of hazardous substances to the environment, several strategies have been designed to cleanup contaminated sites. These remedial actions are directed to protect human health and the environment, as well as to maintain protection over time and maximize waste treatment. Remedial actions are related to the containment of contaminants (e.g. physical and hydraulic barriers), contaminant removal (e.g. excavation, pumping and treatment, enhanced removal and soil vapor extraction), and treatment to allow the transformation of contaminants to less hazardous materials, e.g., bioremediation, oxygen augmentation, nutrient application, bioaugmentation, etc. (Brusseau and Miller, 1996). See Table 1.1 for definitions of concepts mentioned in this chapter.

Of the remedial techniques, bioremediation consists of the optimization of naturally occurring biodegradative processes. It has a good public acceptance and support, largely because of its success and low cost of application, compared to physical and chemical remediation techniques. Bioremediation utilizes free-living microorganisms which can grow in contaminated environments and start the oxidation, degradation and/or complete mineralization of organic contaminants by using them as carbon and energy sources. Either aerobic or anaerobic microorganisms drive biotransformations of organic contaminants such as alkanes, polycyclic aromatic hydrocarbons, volatile compounds and/or xenobiotic compounds, including polychlorinated biphenyls, etc. (Donnelly et al., 1994; Alexander, 1999; Van Hamme et al., 2003).

As an alternative to bioremediation, phytoremediation with the incorporation of green plants to remove, stabilize, volatilize, accumulate, and/ or degrade organic contaminants, is being utilized to clean up contaminated soils (Alkorta and Garbisu,

2001; Davis et al., 2002; Dietz and Schnoor, 2001). Microbial activity associated with plant roots is often directly related to enhanced ability to dissipate or degrade organic contaminants, also called rhizodegradation (Newman and Reynolds, 2004; Pilon-Smits, 2005).

Table 1.1. Definitions for common terminology utilized for decontaminating soil and water systems.

Term	Definition
Arbuscular mycorrhizal fungi (AMF)	Ubiquitous, asexual, and obligate symbiotic fungi that belong to the Phyllum Glomeromycota.
Bioaugmentation	Inoculation with specialized microorganisms to stimulate natural microbial populations and enhance contaminant degradation.
Bioremediation	Application/Management of natural free-living microorganisms.
Biostimulation	Application of mineral nutrients/surfactants, aeration, etc., to stimulate or supplement natural microbial populations.
Cometabolism	The metabolic transformation of a an organic compound by a microorganism, incapable of using the substance as a primary energy or carbon source or one of its constituent elements.
Filamentous fungi	The term filamentous fungi is intended to include fungi belonging to the Phyla Zygomycota, Ascomycota, Basidiomycota or fungi imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium* or *Humicola*.
Hydrocarbonoclastic microorganism	Microorganism with the ability to utilize petroleum hydrocarbons as sole sources of energy and carbon.
Phytoextraction	Removing contaminants from soil through accumulation in plant tissues.
Phytoremediation	Utilization of plants and associated rhizosphere microorganisms to degrade contaminants.
Phytostabilization	Immobilization of a contaminant in soil through absorption and accumulation by roots, absortion into roots or precipitation within the rhizosphere
Phytovolatilization	Uptake, metabolism and/or volatilization of a solid or liquid contaminant by a plant, i.e. metabolism of selenium and mercury.
Rhizodegradation	Also called enhanced rhizosphere biodegradation, phytostimulation, and plant assisted bioremediation; the breakdown of organic contaminants in soil by soil-dwelling microbes that is enhanced by the rhizosphere.

Alkorta and Garbisu (2001), Dietz and Schnoor (2001), Newman and Reynolds (2004), Pilon-Smits (2005), Susarla et al. (2002), and van Hamme et al. (2003).

Bioremediation or phytoremediation of petroleum-contaminated soils can be improved by introducing specific microbial species (bioaugmentation) with ideal physiological characteristics for the oxidation or degradation of organic contaminants. The inoculation with these microorganisms is often recommended when native microorganisms show low effectiveness in degrading contaminants. In addition, petroleum-contaminated soils are typically limited by available nutrients, particularly nitrogen and phosphorus due to increased C/N and C/P ratios that results in immobilization of N and P by soil microbes, depleting the availability of these elements. Thus, supplementing with organic or inorganic nutrients (biostimulation) enhances and optimizes the C:N:P ratio to improve the remediation of soils contaminated with PH.

Although bioaugmentation and biostimulation have been well documented as effective ways to improve bioremediation or phytoremediation processes, the interaction of all these remedial techniques are not well understood. The success of bioremediation or phytoremediation is a function of not only the type of organic contaminant, but also the soil physical and chemical properties. In the same manner, the physiological activity and genetic diversity of rhizosphere microorganisms influence the aerobic or anaerobic degradation of PH. The interaction of well acclimated hydrocarbonoclastic microorganisms in the rhizosphere is crucial for the detoxification and cleanup of soil contaminated with organic compounds (Alexander, 1999; Suresh and Ravishankar, 2004).

Phytoremediation of Organic Contaminants in Soils

Soils are frequently the ultimate repositories of waste products and chemicals which are utilized by society. However, such deposition can have a negative impact on all biological cycles occuring in soil. Among organic contaminants deposited in soils are pesticides, plastics, plasticizers, lubricants, refrigerants, fuels, solvents, preservatives, petroleum hydrocarbons (PH), and polycyclic aromatic hydrocarbons (PAH). Other contaminants include synthetic organic chemicals (xenobiotics) which have inserted halogen atoms (Cl, F, Br) or multivalent nonmetal atoms (S and N) in their molecular structure (Brady and Weil, 2002; Reynolds and Skipper, 2005). Some of these organic compounds are extremely toxic to humans and other living organisms, including soil microorganisms and plants.

Once organic contaminants such as PH reach the soil they are subjected to several biotic and abiotic processes including: 1) evaporation to the atmosphere without chemical change, 2) adsorption by soil components, 3) downward movement in soil profiles and loss via leaching, 4) undergoing oxidation-reduction chemical reactions, 5) degradation by microbial activity, 6) surface runoff by which contaminants move into streams and rivers, 7) absorption by plants and ultimately animals in the food chain (Brady and Weil, 2002) (Fig. 2.1).

Some plants colonize sites contaminated with inorganic or organic compounds, and have the ability not only to tolerate but also to enhance dissipation/degradation of recalcitrant contaminants. Davis et al. (2002) emphasized the significant benefits of plants in phytoremediation of complex organic molecules derived from different sources. Plants posses physiological mechanisms to enhance the remediation processes, such as

phytoextraction/phytoaccumulation (phytomining), phytopumping, water balance control, phytostabilization, phytotransformation/phytodegradation, phytovolatilization and rhizodegradation (Susarla et al., 2002). Rhizodegradation of organic contaminants is significantly enhanced by root exudation (rhizosphere effect) that stimulates microbial activity and the presence of symbiotic and facultative plant-microbe associations. Plant establishment creates favorable conditions such as accumulation of available nutrients and litter, secretion of root exudates, and enhanced oxygenation, that favor microbial activity which is responsible for initiating the degradation of organic contaminants.

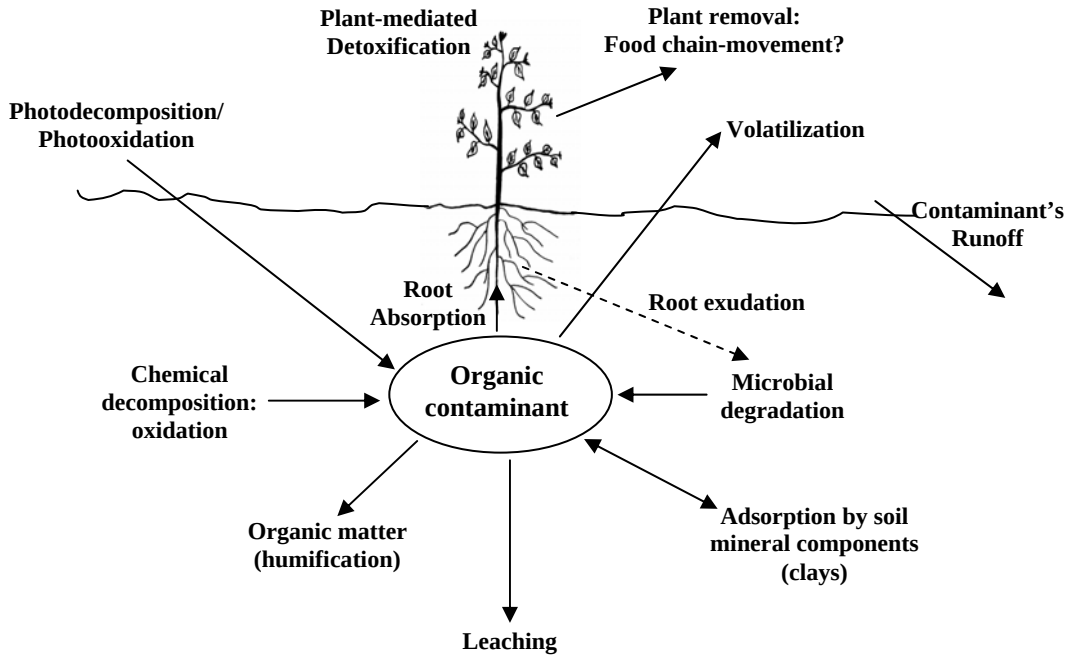

Fig. 2.1. Biotic and abiotic processes influencing the dissipation/degradation of organic contaminants in soils (Modified from Brady and Weil, 2002).

Plant Responses to Petroleum-Contaminated Soils

Petroleum hydrocarbons (PH) in soil create a stressful environment for plants. The negative effects of PH to plants have aboveground and belowground impacts.

Aboveground effects include the deposition of PH on leaves causing physiological disorders that ultimately provoke plant death. PH on the surface of leaves may reduce gas exchange (photosynthesis and transpiration) by closing or blocking stomata, disrupting chloroplast membranes, and induced-inhibition by accumulation of toxic intermediates (Baker, 1970; Daly et al., 1988; Macinnis-Ng and Ralph, 2003). In addition, respiration increases due to mitochondrial damage, which can potentially cause oxidative stress (Baker, 1970; Mittler, 2002; Torres and Dangl, 2005). Depending on the crude oil composition of alkanes, cycloalkanes, aromatics, alkenes, naphthenic acids, sulphur, nitrogen compounds and trace metals such as vanadium and nickel, phytotoxic effects can be classified from acute to severe (Van Hamme et al., 2003).

The deleterious belowground effects of PH have been little studied. Most of the negative impacts of PH in soils are related to their hydrophobic and lipophilic properties. In soils, PH may reduce water infiltration, water availability, oxygen diffusion and adversely alter physicochemical properties (Obire and Nwaubeta, 2002), which limit or inhibit either seed germination or root growth.

Phytotoxicity of PH during seed germination and seedling emergence are related to their hydrophobic properties, which restrict and reduce water availability and gas exchange, including oxygen that is depleted by microbial activity (Miller, 1996). The presence pf PH alters metabolic reactions of seeds during germination and potentially kills the embryo (Amadi et al., 1993; Udo and Fayemi, 1975). Although seed germination can be inhibited or delayed by the PH, there are reports about the beneficial effects of low and medium concentrations of PH in soils on seed germination of selected plant species (Adam and Duncan, 2003; Kirk et al., 2002; Quiñones-Aguilar et al., 2003; Siddiqui et

al., 2001). Stimulation of seed germination by PH may be due to increased soil temperature, since PH-contaminated soils have higher solar radiation absorbing properties which can increase soil temperature (Merkl et al., 2005b). Even though seed germination may be stimulated by the presence of PH, the long-term exposure to these organic compounds typically impairs seedling emergence or plant growth and development (Adam and Duncan, 2003; De Jong, 1980; Ilangovan and Vivekanandan, 1992; Kirk et al., 2002; Malallah et al., 1996; Quiñones-Aguilar et al., 2003).

Roots are an important component for plant adaptation to PH-contaminated soils, and crucial in phytoremediation. A pioneer study on root morphology under PH-contaminated soils demonstrated that root growth of sensitive species is drastically impaired. In contrast, PH-tolerant species have a modified root morphology characterized by coarser, shorter and thicker roots, characteristics that are related to higher PH-degradation (Merkl et al., 2005b) and the improvement of water and nutrient acquisition to support plant growth under soil contamination.

The negative effects of PH on plant physiological responses and root morphology varies among plant species, soil type and properties, microbial composition, and petroleum type, concentration, and composition (Salanitro et al., 1997; Siciliano et al., 2001; Wiltse et al., 1998).

Grass species are considered to be less susceptible to PH in soils than legume species (Kirk et al., 2002; Merkl et al., 2005a), and some legumes are potential indicators of phytotoxicity-induced by PH in soils (Malallah et al., 1996). The screening and selection of suitable plant species to be utilized in phytoremediation entails their ability to tolerate and grow in contaminated soils, as well as enhancing PH-degradation by directly

secreting or exuding specific oxidative enzymes, or indirectly stimulating petroleum-degrading microbial populations in the rhizosphere (Pilon-Smits, 2005; Siciliano et al., 2001; Susarla et al., 2002).

Arbuscular Mycorrhizal Fungi: Effects on Plant Stress Conditions

Among the diverse microbial populations in the rhizosphere, mycorrhizal fungi are considered as an important microbial component on plant adaptation and nutrition (Bago et al., 2001; Hodge et al., 2001; Johansson et al., 2004; Smith and Read, 1997). Arbuscular mycorrhizal fungi (AMF) are obligate symbionts in a living root system. AMF belong to the phylum Glomeromycota (Schüβler et al., 2001). In this symbiosis, the root system provides simple carbon sources for the metabolism of AMF, which allow their proliferation and life cycle (Bago et al., 2000; Bago et al., 2002; Lammers et al., 2001). In return, AMF enhance the nutrient and water uptake of plants via their external and internal mycelium (Bago et al., 1998; Drew et al., 2003; George et al., 1992; Smith and Read, 1997). AMF are an important component of the soil biota, and can account for from 5 to 50 % of total microbial biomass in agricultural soils (Olsson et al., 1999). Survival and dispersion of AMF in soils are mainly dependent on their establishment with plant root systems.

The AMF symbiosis is based on the presence of specific fungal structures, called arbuscules, which colonize the cortical cells of roots. AMF are present in more than 80% of all known-terrestrial plants (~250,000 plant species worldwide) (Smith and Read, 1997). The importance of AMF is not only their beneficial effect on plant growth and nutrition, but also their relevant role in plant evolution and adaptation to terrestrial

ecosystems (Malloch et al., 1980; Taylor et al., 1995). According to fossil evidence, AMF evolved with the first land plants, around 460 million years ago (Berbee and Taylor, 1993; Brundrett, 2002; Redecker et al., 2000).

Plant adaptation and survival under stressful environments can be enhanced by their symbiosis with AMF. Environmental stress conditions such as nutrient limitation, salinity, drought, disturbance during mining, accumulation of heavy metals, pesticides, and petroleum hydrocarbons typically impose serious difficulties to plant survival and growth. Nevertheless, the establishment of AMF symbiosis in the root system alleviates some of these abiotic stresses by improving growth as a result of enhanced nutrient and water uptake (Amaya-Carpio et al., 2005; Davies et al., 1992; Davies et al., 1993; Davies et al., 2002; Estrada-Luna et al., 2000; Smith and Read, 1997) as well as by stimulating or modifying specific physiological mechanisms related with adaptation to stressful environments (Augé, 2001, Augé et al., 2004; Azcón et al., 1996; Hause et al., 2002; Porcel et al., 2003; Querejeta et al., 2003).

As phytoremediation has emerged as an environmentally sound alternative to detoxify and remove toxic contaminants from soils, the potential utilization of AMF to improve plant adaptation and performance during phytoremediation has enormous significance. More attention has been directed to the role of AMF on phytoremediation of heavy metal-contaminated soils. AMF are a critical biological component in the rhizosphere via avoiding toxicity of potentially toxic elements to the plants (Perotto and Martino, 2001) or enabling plants to phytostabilize, as well as to improve their capability to accumulate or phytoextract heavy metals in harvestable plant tissues (Cairney and

Meharg, 1999; Davies et al., 2001; Gonzalez-Chavez, 2000; Griffioen and Ernst, 1989; Meharg and Cairney, 2000a; Van Diun et al., 1991).

Some AMF species have the ability to enhance phytostabilization of heavy metals in soil by secreting organic compounds and sequestering these elements in the external hyphae, which reduces their availability to susceptible plant species. In addition, some AMF species can potentially enhance the uptake of heavy metals (for instance, Cd, Cr, Pb, As, Ni) in plants that have been characterized as hyperaccumulators including species in the Compositae and Gramineae (Davies et al., 2001; Ernst, 2005; Leung et al., 2006; Zhou and Qiu, 2005). Effects of AMF may include either reducing or enhancing human health risks as a result of the consumption of vegetables that are cultivated at heavy metal contaminated areas (Jeffries et al., 2003). The utilization of both selected AMF isolates and tolerant-plant species or genetically-engineered plants can potentially improve the performance of phytoremediation of heavy metal-contaminated soils by establishing a more functional and efficient microbial co-operation/interaction in the rhizosphere or mycorrhizosphere (Barea et al., 2005; Jeffries et al., 2003; Johansson et al., 2004).

Arbuscular Mycorrhizal Fungi in Plant Tolerance and Phytoremediation of Petroleum Hydrocarbon (PH)-Contaminated Soils

PH drastically affect physical, chemical and biological properties of soils. As previously reported, these changes are related to altered nutrient availability, reduced oxygen diffusion and water movement through soil pore space, which impairs plant growth and thus, cause alterations on rhizosphere microbial populations (Kirk et al., 2005; Merkl et al., 2005a; Wyszkowska and Kucharski, 2001).

Microorganisms whose physiological activity permits them to grow and to utilize fractions of PH as energy sources, can significantly contribute to the dissipation, degradation, and mineralization of these organic contaminants. In particular, the proliferation of aerobic, petroleum hydrocarbon-degrading (hydrocarbonoclastic) bacteria and filamentous fungi plays an important role in both soil and rhizosphere detoxification (Atlas, 1995; Banks et al., 2003; Van Hamme et al., 2003). However, enhanced microbial activity may reduce the quantities of available nutrients and oxygen during the oxidation of the organic contaminants. Since nutrients and oxygen become limiting factors during bioremediation, the extent of degradation of organic contaminants is partially or completely inhibited (Miller, 1996). In addition, the by-products resulting from the initial and incomplete degradation of PH may result in greater toxicity, negatively affecting roots and rhizosphere microorganisms (Swoboda-Colberg, 1995).

In contrast to the activity of bacteria and filamentous fungi on PH-degradation, few studies have determined the role and benefits of AMF in the rhizosphere of plants established in PH-contaminated soils. Since PH in soils limit plant growth by reducing nutrient availability and altering water uptake, AMF could potentially alleviate abiotic stress of plants, and enhance plant adaptation, tolerance and growth (Cabello, 2001; Meharg, 2001).

AMF-communities also play an important role in phytoremediation of PH and polycyclic aromatic hydrocarbons (PAH)-contaminated soils, which is a serious worldwide problem. Increased mycorrhizal root colonization occurred when kerosene levels were reduced in the rhizosphere through the proliferation and activity of free-living N_2-fixing bacteria (García et al., 2000; Hernández-Acosta et al., 1998, Hernández-Acosta

et al., 2000). This microbial group contributes to the cometabolism of PH with indigenous hydrocarbonoclastic bacteria (Onwurah, 1998; Onwurah, 1999; Pérez-Vargas et al., 2000).

AMF can increase the survival and tolerance of plants when PAH are present in the rhizosphere (Binet et al., 2000a, Binet et al., 2000b; Leyval and Binet, 1998), but in other cases AMF-colonization can be negatively affected by aromatic compounds (Joner and Leyval, 2001). Root and microbial oxidative enzymes in soil have the greatest impact on initiating the degradation and ring fission of PAH. In this case, AMF can increase the release of root peroxidases which contribute to the degradation of anthracene (Criquet et al., 2000).

In tropical areas of Mexico, studies have been conducted to understand the role of AMF on plants established in PH-contaminated sites. An AMF strain of *Gigaspora margarita*, isolated from petroleum-contaminated soil from Veracruz, Mexico, was able to germinate with 100 $\mu g \cdot g^{-1}$ of benzo[*a*]pyrene (Alarcón et al., 2003a). Furthermore, symbiosis of this AMF with *Echinochloa polystachya* was not adversely affected at the benzo[*a*]pyrene (BaP) concentrations tested. AMF enhanced rhizosphere dehydrogenase activity of *E. polystachya*, but root polyphenol oxidase activity and dissipation of BaP from the rhizosphere were not enhanced (Alarcón et al., 2003b). Recently, Liu et al. (2004) reported higher phytoremediation of BaP with *Medicago sativa* colonized by *Glomus caledomiun*.

Inoculation with AMF species isolated from PH-contaminated soils resulted in better growth and nutrient status than plants inoculated with introduced AMF species (Cabello, 1999). Although PH contamination generally decreases AMF propagules, there

are some species, such as isolates of *Glomus aggregatum* and *Glomus mosseae*, that have better adaptation to PH-contaminated soil, and have high colonization capacity (Cabello, 1997).

Similarly, the interactions between AMF and hydrocarbonoclastic microorganisms (bacteria or filamentous fungi) in the phytoremediation of PH or PAH have received little attention. One of the few reports about this microbial interactions was published by Gaspar et al. (2002). In this study, the presence of phenanthrene reduced the intraradical colonization of *Glomus geosporum*, but was not affected by the inoculation of the hydrocarbonoclastic-yeast, *Rhodotorula glutinis*. The combined inoculation of these two microorganisms resulted in reduced accumulation of phenanthrene in maize roots. However, neither plant physiological responses nor degradation rate of phenanthrene were described.

Some of the proposed mechanisms by which AMF may contribute to the dissipation/degradation of organic contaminants are: i) mycorrhizal modification of plant and microbial metabolism, ii) enhanced root peroxidase activity, and iii) modification of the microbial composition of the rhizosphere as a consequence of the establishment of AMF-symbiosis (Joner et al., 2001). The latter mechanism is related to the modification of root biomass and exudation patterns, both qualitatively and quantitatively. For instance, AMF may induce the synthesis and release of simple phenolic compounds by roots, which may act as microbial inducers for PAH-degradation. In addition, the formation of abundant AMF-extraradical mycelium contribute to the exudation of organic compounds such as glomalin and other non-characterized compounds (Wright and

Upadhyaya, 1999), which may possibly enhance specific bacterial activity involved in the cometabolic degradation of persistent and recalcitrant organic contaminants.

Summary

The success of phytoremediation of organic contaminants in soil depends on the type and concentration of the organic compound, soil physical and chemical properties, environmental characteristics, and biological activity in the rhizosphere. The interaction of all these factors governs the rate and the extent of the degradation as well as the fate of PH in soils. The proliferation of microbial groups that oxidize, transform, mineralize, and/or utilize organic contaminants as source of carbon and energy, is paramount to phytoremediation. Free-living microorganisms as well as facultative-associated and symbiotic bacteria and fungi can contribute to PH-degradation and enhance the adaptation, growth and development of plants established in contaminated soils.

Although the benefit of AMF on plants under stressful abiotic and biotic environments has been well documented, their adaptation, functionality, and benefits on plant physiological responses in the phytoremediation of PH contaminants is not well known. There is evidence that selected AMF have tolerance to PH and PAH-contaminated soil, which enhances a plant's adaptation, survival, establishment and fitness. While some isolates of AMF species are adversely affected by the presence of PH in the rhizosphere, other AMF species can significantly enhance plant adaptation, growth and development in contaminated soils.

Questions remain about the role of AMF species in a PH-contaminated rhizosphere in regards to their capability of sequestering or stabilizing organic

contaminants in their fungal biomass, as reported by Gaspar et al. (2002). Secondly, research is needed to evaluate the influence of specific AMF ecotypes which may have higher potential for either inducing plant tolerance and adaptation in contaminated soils or stimulating the phytoremediation processes (Cabello, 1999; Joner and Leyval, 2003a). Thirdly, neither biochemical nor physiological mechanisms of AMF have been sufficiently reported to fully understand the AMF symbiosis responses during phytoremediation of organic contaminants, such as PH and PAH. The benefits of AMF on physiological responses of plants utilized in phytoremediation of PH -contaminated soil are not well known.

This research is intended to study selected physiological responses of a grass species inoculated with the AMF *Glomus intraradices*, in a PH-contaminated soil as well as to study the contribution of AMF-plants on phytoremediation of PH and BaP in the rhizosphere by utilizing biostimulation with inorganic fertilizer, and bioaugmentation with selected hydrocarbonoclastic microorganisms.

CHAPTER III

ASSESSMENT AND SELECTION OF A PETROLEUM-TOLERANT GRASS SPECIES FOR UTILIZATION IN PHYTOREMEDIATION OF PETROLEUM HYDROCARBON-CONTAMINATED SOIL

Introduction

Soil contamination with petroleum hydrocarbons (PH) due to accidental spills during extraction, refinement, distribution, storage of oil and oil-derivatives is an international environmental problem. PH drastically modifies physical, chemical and biological properties of soil. Depending on the amount, type of contaminants and environmental conditions, it may take months to many years to decontaminate soil (USEPA, 2004).

The incorporation of higher plants as a biological alternative to remove, accumulate or degrade organic contaminants (phytoremediation) is being utilized to cleanup contaminated soils. Plant establishment in contaminated soils also allows biological transformations of organic contaminants by stimulating the microbial activity via root exudation (Susarla et al., 2002). Phytoremediation of PH-contaminated soils is dependent plant species that are highly tolerant and can establish and thrive under these stressful site conditions.

Plant species utilized in phytoremediation include graminaceous plants, legumes and selected dicots that enhance the degradation of PH in soils, due in part to their influence on plant-associated microorganism populations (Rivera-Cruz et al., 2002a;

Siciliano and Germida, 1998). Grass species are important in phytoremediation of organic contaminants in soils, due to their adaptability, root growth characteristics, and microbial stimulation through root exudation (Merkl et al., 2005a; Merkl et al., 2005b; Siciliano and Germida, 1998). One of the primary characteristics of grass species for phytoremediation is their root system, which is usually fibrous and extensively branched, allowing more soil volume to be explored for nutrient absorption and uptake (Christians, 1998; Carrow et al., 2001). Plant roots exude organic compounds that serve as carbon and energy sources for the microorganisms that carry out biogeochemical transformations of organic contaminants in soils.

PH exert toxic effects on plants due to their complex chemical characteristics. Seed germination and seedling emergence are inhibited by the toxic effects of *n*-alkanes, small carbon chain hydrocarbons (*n*-C_{10} and *n*-C_{11}) in soils; once these small compounds undergo biodegradation, germination is less inhibited (Siddiqui et al., 2001). In addition, light aromatic PH, including gasoline, have phytotoxic effects on seed germination of plant species (Chaîneau et al., 1997). Typically, seedling growth and development are significantly impaired with increased concentrations of PH in soil (Chaîneau et al., 1997; Kirk et al., 2002; Quiñones-Aguilar et al., 2003; Siddiqui et al., 2001). While small PH concentrations in soils can stimulate seed germination, seedling growth, and yield (Plice, 1948; Quiñones-Aguilar et al., 2003).

Besides PH, soil properties may also represent adverse conditions that result in nutritional constraints, limiting plant adaptation and growth (Dec et al., 2002). Thus, soil chemical, physical and biological properties play a significant role in biodegradation of PH in the rhizosphere. Hence, screening and selection of tolerant plants is crucial for the

success of phytoremediation of PH-contaminated soils. The hypothesis tested in this experimental stage was that grass species from the same botanical family differ in their tolerance and adaptation to PH-contaminated soil. The objectives of this research were to: 1) select plant species with high tolerance to PH in soil, 2) select the most suitable plant species to be utilized in a plant model system for subsequent experiments, and 3) determine critical PH concentrations for subsequent future experiments.

Materials and Methods

Seedling Bioassay of Five Grass Species (Poaceae) for Tolerance of Soil Contaminated with Arabian Medium Crude Oil (ACO)

Five grass species were screened with Arabian medium crude oil (ACO) contaminated soil under glasshouse conditions. The species were: bahia grass (*Paspalum notatum* Flugge var. Pensacola), Bermuda grass (*Cynodon dactylon* (L.) Pers. var. Yuma), blue grass (*Poa pratensis* L. var. Kentucky), annual ryegrass (*Lolium multiflorum* Lam. var. Passerel Plus), and tall fescue (*Festuca arundinacea* Schreb. var. Kentucky 31). Seeds were purchased from Pennington Seed Inc®, Lebanon, Oreg.

This study was conducted under glasshouse conditions at Texas A&M University, College Station, Tex. for 30 days, from 8 July to 7 August 2003. Temperature, relative humidity, and photosynthetic photon flux density (PPFD) were monitored with a watch dog data logger Model 150 (Spectrum technologies, Inc., Planfield, Ill.), and by a LI-190SA Quantum/Radiometer/Photometer and sensor (LI-COR® Biosciences, Lincoln, Nebr.), respectively. Average day/night temperature and relative humidity were 32/24°C,

and 80/75%, respectively, and average maximum PPFD determined at solar noon, was 684.7 μmoles·m^{-1}·s^{-2}.

A mixture of sand and sandy loam soil (1:1 v/v) was utilized as a substrate, with chemical properties as follows: (μg·g^{-1}) 0.9 NO_3-N, 2.1 NH_4-N, 1.5 P, 17 K, 9468 Ca, 72 Mg, 161 Na, and 53 S. The electrical conductivity was 0.17 dS·m^{-1}, pH of 7.7, and textural analysis of 85% sand, 10% clay, and 5% silt. The substrate was steam-pasteurized at 70°C for eight hours on two consecutive days. Afterward, substrate was treated with ACO at 0, 100, 1000, 50000 or 150000 mg·kg^{-1}. The viscosity of ACO contaminant was reduced through the application of dichloromethane solvent (Sigma-Aldrich®, Steinheim, Germany, <0.002 % of residue after evaporation).

Each petroleum concentration had five replicates (n=5), in which 20 seeds of the respective grass species were sown (100 seeds per treatment). Pots were watered with deionized water as needed. Seedling emergence was recorded after 30 days to identify the susceptibility or tolerance of the plant species to ACO. The 5 (grass species) x 5 (ACO concentrations) factorial experiment was in a completely randomized design with 25 treatments and five replications (n=5). Data were analyzed using analysis of variance (ANOVA) and mean separation was by standard error (± SE) (SAS Institute Inc., 2002).

Seed Germination and Growth of *Lolium multiflorum* and *Festuca arundinacea* in Soil Contaminated with Arabian Medium Crude Oil (ACO)

Annual ryegrass (*Lolium multiflorum* Lam. var. Passerel Plus) and tall fescue (*Festuca arundinacea* Schreb. var. Kentucky 31) purchased from Pennington Seed Inc®, Lebanon, Oreg., were screened for tolerance to soil contaminated with ACO. This study

was conducted under glasshouse conditions at Texas A&M University, College Station, Tex. for 20 days, from 20 August to 6 September 2003. Temperature and relative humidity were monitored with a watch dog data logger Model 150 (Spectrum technologies, Inc., Planfield, Ill.), and photosynthetic photon flux density (PPFD) was determined with a LI-190SA Quantum/Radiometer/Photometer and sensor (LI-COR® Biosciences, Lincoln, Nebr.). Average day/night temperature and relative humidity were 33/25°C, and 80/75%, respectively, and average maximum PPFD determined at solar noon, was 846.5 $\mu moles \cdot m^{-1} \cdot s^{-2}$.

A mixture of coarse sand and sandy loam soil (1:1 v/v) was prepared and steam-pasteurized as previously described. The substrate was treated with ACO at 0, 150, 300, 1000, 3000, 5000, 10000, 15000, 30000, 45000, 60000 and 120000 $mg \cdot kg^{-1}$ of substrate. The viscosity of ACO was reduced with dichloromethane solvent (Sigma-Aldrich®, Steinheim, Germany, <0.002 % of residue after evaporation), as previously described. Two controls (with and without dichloromethane) were used to determine the influence of this solvent on seed germination and seedling growth. Contaminated and non-contaminated soil was placed in 15 mm plastic petri dishes. Each petroleum concentration had five replicates in which 20 seeds of the respective grass species were sown (100 seeds per treatment). Petri dishes were sprayed daily with deionized water. Seedling emergence was monitored and recorded for 30 days in order to identify the susceptibility or tolerance of these two plant species to ACO concentrations.

The 2 grass species x 13 (ACO concentrations) factorial was in a completely randomized design, with 26 treatments and five replications (n=5). Data were analyzed using analysis of variance (ANOVA). Treatment means for each plant species exposed to

ACO were compared using least significant difference (LSD, α=0.05) or standard error (± SE). Pearson correlation coefficients between seed germination and total plant dry weight were also determined (SAS Institute Inc, 2002).

Results

Seedling Bioassay of Five Grass Species (Poaceae) for Tolerance of Soil Contaminated with ACO

Seed germination was significantly ($P \leq 0.001$) affected by ACO, grass species, and the interaction ACO x grass species. Seed germination of control plants (0 mg ACO kg^{-1} soil) varied among the plant species. *Lolium multiflorum* and *Festuca arundinacea* had the highest germination percentages, 87 and 79 %, respectively (Fig. 3.1). In contrast, very low seed germination occurred with *Paspalum notatum*, *Cynodon dactylon*, and *Poa pratensis* with 9, 30, and 15 %, respectively (Fig. 3.1).

Increased concentrations of ACO resulted in significantly ($P \leq 0.001$) reduced germination in all five species. *Lolium multiflorum* had greater seed germination and tolerance to ACO-concentrations up to 50000 $mg \cdot kg^{-1}$, followed by *F. arundinacea* with significantly reduced seed germination at ACO-concentrations up to 50000 $mg \cdot kg^{-1}$ (Fig. 3.1). Although, seed germination of *P. notatum*, *C. dactylon*, and *P. pratensis* was low, *P. pratensis* failed to germinate at ACO concentrations of 1000 $mg \cdot kg^{-1}$ or higher, while *P. notatum* and *C. dactylon* failed to germinate at 150000 $mg \cdot kg^{-1}$ (Fig. 3.1).

Due to their higher germination, *L. multiflorum* and *F. arundinacea* were selected for further evaluation of their growth tolerance to increasing concentrations of ACO, with

the goal of selecting one of the remaining two species as part of the plant model system for subsequent experiments.

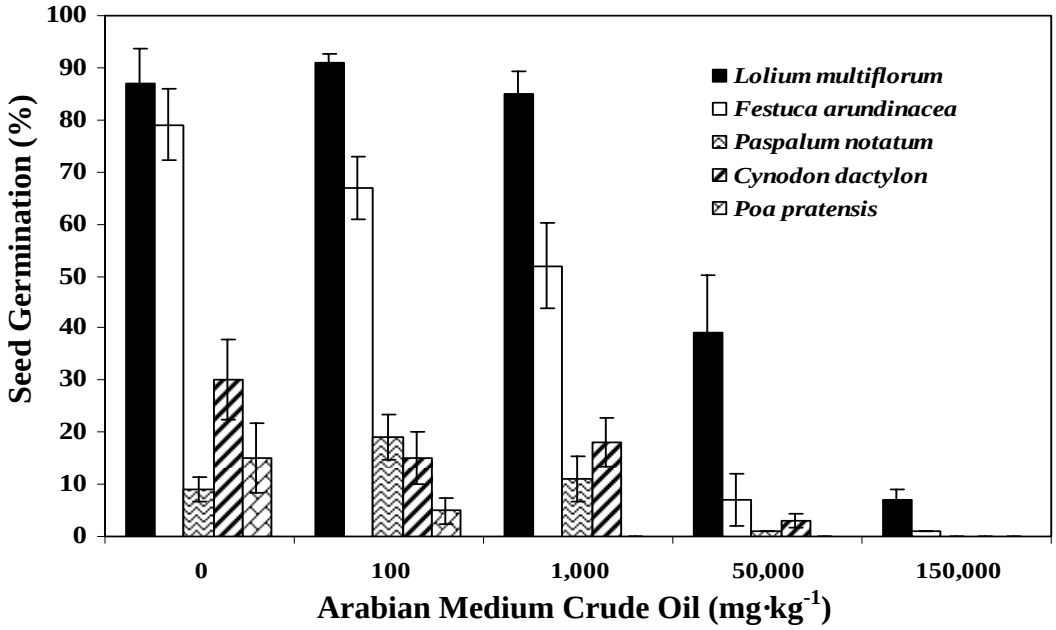

Fig. 3.1. Seed germination percentage of five grass species exposed to soil contaminated with five levels of Arabian medium crude oil (ACO) after 30 days. Main effects of ACO, grass species and the interaction of ACO x grass species were significant at $P \leq 0.001$; Bars are ± SE, n=5.

Seed Germination and Growth of *Lolium multiflorum* and *Festuca arundinacea* in Soil Contaminated with ACO

Seed germination of each plant species was significantly ($P \leq 0.001$) affected by ACO concentration, grass species and the interaction ACO x grass species. Increased concentrations of ACO resulted in reduced germination in both species (Fig. 3.2; see data in Appendix I, Table AI-3.1). However, *L. multiflorum* had significantly ($P \leq 0.001$) higher germination, tolerance, and growth in ACO contaminated soil than *F. arundinacea*. Seed germination in *L. multiflorum* was not significantly reduced in soil

contaminated with ACO concentrations from 150 to 10000 mg·kg^{-1}, but at ACO concentrations greater than 10000 mg·kg^{-1}, germination was significantly reduced (Fig. 3.2). In contrast, although germination was 70% in the control (0 mg ACO kg^{-1}), *F. arundinacea* had significant reduced germination when ACO concentrations increased up to 5000 mg·kg^{-1}. Seedling root, shoot and total plant dry weight of *L. multiflorum* was significantly ($P \leq 0.001$) reduced by increasing ACO concentrations as a treatment effect, while the root:shoot ratio was not significantly different (Table 3.1). In the case of *F. arundinacea*, seedling root, shoot and total plant dry weight was also significantly ($P \leq 0.001$) reduced by increasing ACO concentrations, while for the root:shoot ratio, the effects of ACO concentration effects were significant at $P \leq 0.05$ (Table 3.2).

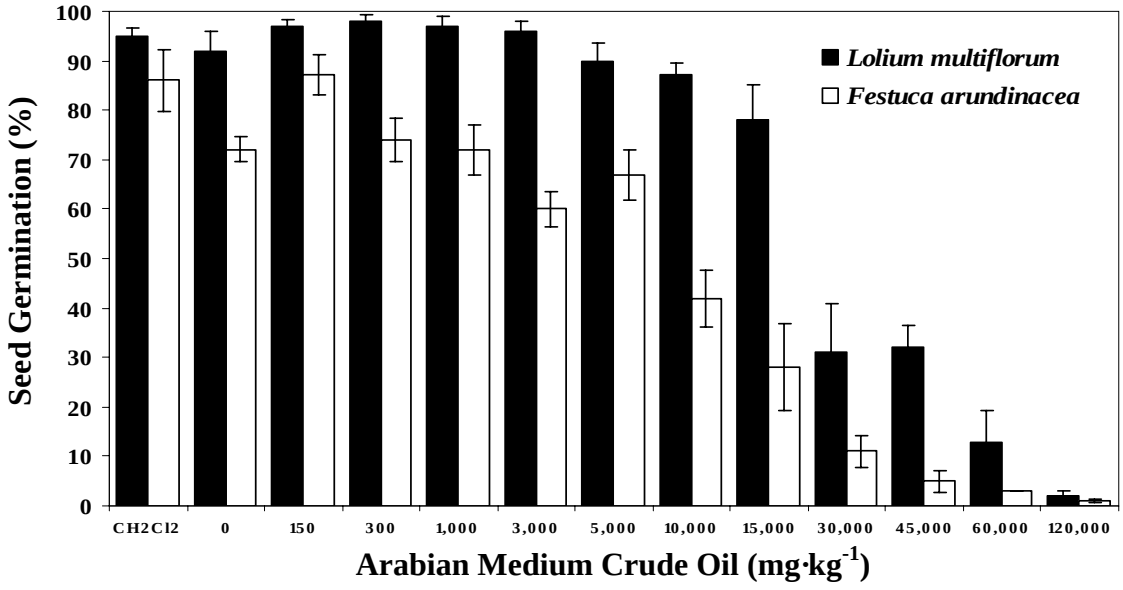

Fig. 3.2. Influence of Arabian medium crude oil (ACO) and dichloromethane solvent (CH_2Cl_2) on seed germination of *Lolium multiflorum* cv. Passerel Plus and *Festuca arundinacea* after 20 days. Main effects of ACO, grass species and the interaction of ACO x grass species were significant at $P \leq 0.001$; Bars are ± SE, n=5.

Table 3.1. Seed germination, plant dry weight (DW), and root:shoot ratio of *Lolium multiflorum* cv. Passerel Plus exposed to several concentrations of Arabian medium crude oil (ACO) in a sandy soil, after 20 days.

ACO-Treatment ($mg \cdot kg^{-1}$)	Root DW (mg)	Shoot DW (mg)	Total plant DW (mg)	Root:Shoot ratio ($g \cdot g^{-1}$)
Control	194.0 b[z]	67.6 c	261.6 b	2.9
CH_2Cl_2[y]	307.2 a	84.6 ab	391.8 a	3.6
150	265.2 a	91.0 a	356.2 a	2.9
300	164.2 b	78.4 abc	242.6 b	4.6
1,000	98.4 c	72.4 bc	170.8 c	1.4
3,000	87.0 cd	47.6 d	134.6 cd	1.8
5,000	68.6 cd	41.0 d	109.6 de	1.7
10,000	59.0 cde	33.8 de	92.8 de	1.8
15,000	49.6 def	24.4 e	74.0 ef	2.1
30,000	15.2 ef	8.2 f	25.6 fg	2.5
45,000	18.2 ef	7.4 f	23.4 fg	2.5
60,000	10.0 f	3.2 f	13.2 g	3.6
120,000	1.4 f	0.4 f	1.8 g	3.5
Significance	0.001	0.001	0.001	NS

[y]Solvent (dichloromethane) applied to reduce the oil viscosity and allow ACO homogenization in the soil.

[z]Means followed by the same letter in the same column are not significantly different (LSD, α=0.05); NS= Nonsignificant, n=5 with 5 petri dishes containing 20 seeds per petri dish.

Table 3.2. Seed germination, plant dry weight (DW), and root:shoot ratio of *Festuca arundinacea* exposed to several concentrations of Arabian medium crude oil (ACO) in a sandy soil, after 20 days.

ACO-Treatment ($mg \cdot kg^{-1}$)	Root DW (mg)	Shoot DW (mg)	Total plant DW (mg)	Root:Shoot ratio ($g \cdot g^{-1}$)
Control	96.2 ab[z]	36.0 b	132.2 bc	2.7 b
CH_2Cl_2[y]	110.8 a	52.8 a	163.6 a	2.2 b
150	100.2 ab	50.8 a	151.0 ab	2.1 b
300	97.2 ab	35.6 b	132.8 bc	2.8 b
1,000	90.0 b	32.2 b	122.2 c	2.7 b
3,000	41.4 c	21.0 c	62.4 d	2.1 b
5,000	39.2 c	17.4 cd	56.6 d	2.4 b
10,000	13.8 d	10.0 de	23.8 e	2.3 b
15,000	10.6 d	5.3 ef	15.9 ef	1.9 b
30,000	3.4 d	2.0 ef	5.4 ef	1.9 b
45,000	2.4 d	0.8 f	3.2 ef	3.3 b
60,000	1.4 d	0.2 f	1.6 ef	7.0 a
120,000	0.4 d	0.2 f	0.6 f	2.0 b
Significance	0.001	0.001	0.001	0.05

[y]Solvent (Dichloromethane) applied to reduce the oil viscosity and allow ACO homogenization in the soil.

[z]Means followed by the same letter in the same column are not significantly different (LSD, α=0.05); n=5 with 5 petri dishes containing 20 seeds per petri dish.

The application of CH_2Cl_2 and 150 mg ACO kg^{-1} stimulated seedling growth in both plant species; however, reduction in total plant DW was obtained when plants were exposed to ACO at concentrations greater than 1000 $mg \cdot kg^{-1}$ (Fig. 3.3).

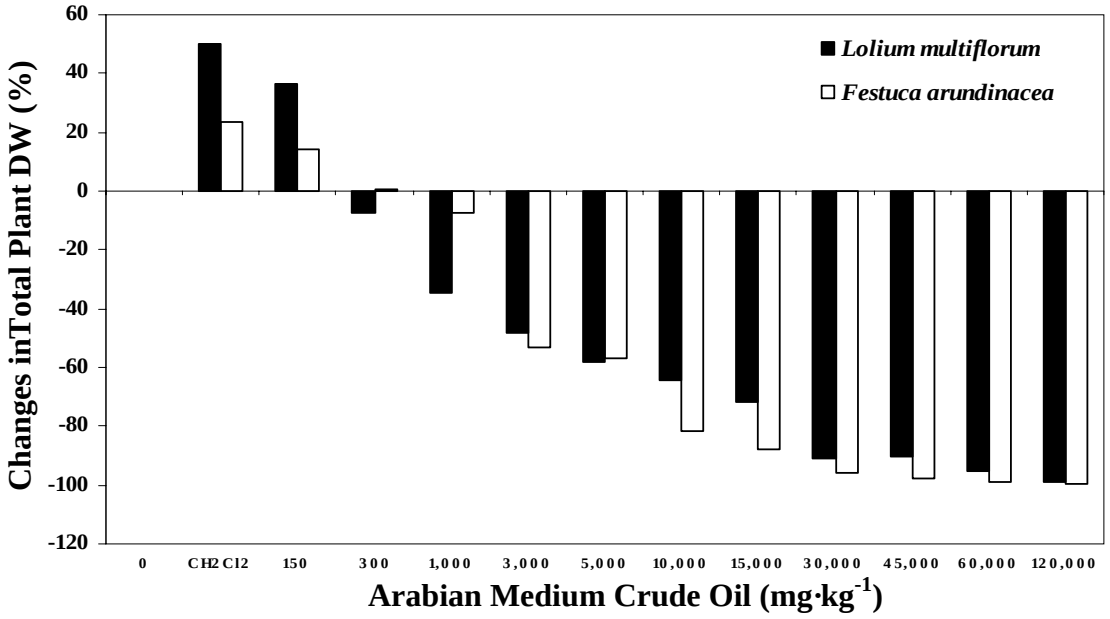

Fig. 3.3. Changes in percentage of total plant dry weight (DW) compared to the control of *Lolium multiflorum* cv. Passerel Plus and *Festuca arundinacea*, as affected by Arabian medium crude oil concentrations and dichloromethane solvent (CH_2Cl_2) application in soil, after 20 days. Values estimated from the difference expressed in percentage, of the total plant DW of controls (0 mg ACO kg^{-1}) for each grass species and the total plant DW of ACO or solvent treated plants.

Discussion

Increasing ACO concentrations reduced seed germination among all the plant species; however, *P notatum*, *C. dactylon*, and *P. pratensis* had poor germination despite ACO concentration. In constrast, *L. multiflorum* and *F. arundinacea* had greater tolerance to ACO. Thus, indicating differential responses among members of the same botanical family (Poaceae). While selected grass species are commonly used for phytoremediation (Chaîneau et al., 1997; Merkl et al., 2005a; Siddiqui et al., 2001), selection of plants that thrive under specific environmental conditions such as soil type, climate, type and

concentration of contaminants in soil, is critical for the success of the phytoremediation. Under our experimental conditions, seedling germination of the controls (0 mg ACO kg^{-1}) were: *L. multiflorum* (>85 %), *F. arundinacea* (~80 %), *P. notatum* (~9%), *C. dactylon* (~30 %), and *P. pratensis* (~ 15 %). Seed germination percentages for the latter three grass species were low, suggesting problems with seed sources, seed viability, or environmental conditions during the experiment. The expected germination percentages for *P. notatum*, *C. dactylon*, and *P. pratensis* are ~70 %, ~85 %, and ~85 %, respectively.

Although environmental conditions such as temperature and relative humidity during the experiment were considered no detrimental for grass species (Clarke and French, 2005; Larsen and Bibby, 2005), differences in seed germination observed among the species may be related to their specific temperature requirements and physiological characteristics. While *P. notatum* and *C. dactylon* are classified as warm-season grasses (C_4 photosynthetic system plants); *L. multiflorum, F. arundinacea* and *P. pratensis* are classified as cool-season grasses (C_3 photosynthetic system plants) (Christians, 1998). According to the recommended specifications given by the commercial provider, *P. notatum*, *F. arundinacea*, are tolerant to heat and drought stress, while *P. pratensis* and *L. multiflorum* are more suitable for cold climates (Carrow et al., 2001). However, in this study, factors that may have caused a direct effect on seed germination and seedling growth besides ACO in soil, were related with low seed viability, particularly for *P. notatum*, *C. dactylon*, and *P. pratensis*, or the temperature effects during the experiment (average day/night 32/24°C).

Increasing concentrations of ACO resulted in significant reductions of tolerance of *L. multiflorum* and *F. arundinacea*. Root DW was greater than shoot DW in both plant

species (Tables 3.1 and 3.2). However, differences on root sensitivity were observed between *L. multiflorum* and *F. arundinacea*. When *F. arundinacea* seedlings were exposed to low ACO concentrations, such as 300 and 1000 $mg \cdot kg^{-1}$, root DW reductions were negligible (from 0 to -0.06 %, respectively) with respect to the control (0 mg ACO kg^{-1}); in constrast, the reduction in root DW for *L. multiflorum* was -15% and -49%, respectively. Thus, *L. multiflorum* was more sensitive to low ACO concentrations in comparison to *F. arundinacea*. In contrast, when ACO concentrations in soil increased up to 10000 $mg \cdot kg^{-1}$, *L. multiflorum* was less sentive than *F. arundinacea*. For instance, the exposure of *L. multiflorum* seedlings to ACO concentrations of 10000 and 15000 $mg \cdot kg^{-1}$ resulted in a reduction of root DW of -70%, and -74%, respectively; while for *F. arundinacea* this reduction was -86% and -90%, respectively.

Seed germination of *L. multiflorum* correlated with the total plant dry weight in 70.6% (Pearson Correlation Coefficient, $P \leq 0.001$), while for *F. arundinacea* the correlation was 82.7%. By comparing total plant DW of the control seedlings with the seedlings exposed to ACO, it was observed that the reduction of total plant growth in *L. multiflorum* exposed to ACO concentration from 300 to 1000 $mg \cdot kg^{-1}$ was -0.003% and -48%, respectively. In contrast, the reduction observed in *F. arundinacea* at the same ACO concentrations was 0.0% and -0.08%, respectively (Fig 3.3). This suggests that *F. arundinacea* is less sensitive to ACO concentrations ≤ 1000 $mg \cdot kg^{-1}$ in comparison to *L. multiflorum*. However, when ACO concentrations were higher than 3000 the extent of reduction in total DW for *F. arundinacea* was significantly greater than in *L. multiflorum*. The reductions observed for *F. arundinacea* were -53%, -57%, -82%, and -87% when exposed to ACO concentrations of 3000, 5000, 10000, and 15000 $mg \cdot kg^{-1}$, respectively;

while in *L. multiflorum* the reductions were -48%, -58%, -64%, and -72%, respectively (Fig 3.3). Hence, *L. multiflorum* was considered as a tolerant grass species when exposed to high concentrations of ACO in soil since this species had greater plant biomass than *F. arundinacea* (Tables 3.1 and 3.2). However, ACO concentrations greater than 15000 $mg \cdot kg^{-1}$ resulted in plant growth inhibitions for both grass species.

The beneficial effect of the solvent dichloromethane (CH_2Cl_2) on total plant DW of both grass species (Fig. 3.3) may be explained in part due to its influence on enhancing release of nutrients that were adsorbed on the surface of both clay and silt fractions of the substrate (Brady and Weil 2001). As a consequence of this nutrient desorption, nutrients may have been more available to the seedlings, resulting in enhanced dry weight of roots, shoots and total biomass in comparison to seedling growth in control petri dishes. There was no supplementary fertilization added during the experimental bioassay.

In addition, some benefits on total plant DW were observed in plants of both species when exposed to low concentrations of ACO such as 150 $mg \cdot kg^{-1}$. For instance, the increase in total DW with respect to the corresponding control plants was +36% for *L. multiflorum*, and +14% for *F. arundinacea* (Fig. 3.3). Benefits of small concentrations of PH in soils on germination and plant growth have been previously reported (Plice, 1948; Quiñones-Aguilar et al., 2003).

Selected grass species have promise for phytoremediation of organic contaminants in soils due to their adaptability and root growth characteristics (Merkl et al., 2005b). Grass root systems are usually fibrous and highly branched, allowing more soil volume to be explored for nutrient absorption and uptake (Christians, 1998; Carrow et al., 2001). Thus, phytoremediation of PH in soils requires the utilization of plant

species with higher tolerance and ablity to establish and develop under stressful conditions. In this study, grass species from the same botanical family differed in their tolerance and adaptation to contaminated soil with ACO. However, for *P. notatum*, *C. dactylon*, and *P. pratensis*, the results may have been confounded in their response to ACO, due to their initial low seed viability and temperature conditions of the experiment.

Furthermore, *Lolium multiflorum* was selected for future experiments based on its growth rate and greater tolerance to ACO in the sandy soil with limited nutrient availablility system utilized in these experiments.

Summary

Phytoremediation can be used to cleanup soils containing organic or inorganic contaminants, and consists of utilizing plants which improve soil aeration and stimulate rhizosphere microbial activity via root exudation. Success of phytoremediation of petroleum hydrocarbons (PH) depends on the utilization of plant species with high tolerance and ablity to establish and develop in contaminated soils. Two glasshouse biossays were established to select grass species with high tolerance to Arabian crude oil (ACO) and to determine critical petroleum concentrations for future experiments.

The first biossay consisted of screening the germination of five grass species (Poaceae) to increased ACO concentrations (0, 1000, 50000, and 150000 $mg \cdot kg^{-1}$) in a sand-sandy loam soil mixture for 30 days. Screened grass species were: *Lolium multiflorum*, *Festuca arundinacea*, *Paspalum notatum*, *Cynodon dactylon*, and *Poa pratensis*. Significant ($P \leq 0.001$) effects on seed germination were observed by ACO, grass species and the interaction of ACO x grass species. Increased ACO concentrations

resulted in reduced seed germination, but higher germination and tolerance to ACO was observed for *Lolium multiflorum* and *Festuca arundinacea*. However, the reduced germination observed for *P. notatum*, *C. dactylon*, and *Poa pratensis*, suggested viability or higher temperature problems during germination of seeds of these species.

The second biossay consisted on exposing *L. multiflorum* and *F. arundinacea* to increased ACO concentrations (0, 150, 300, 1000, 3000, 5000, 10000, 15000, 30000, 45000, 60000, and 120000 $mg \cdot kg^{-1}$) in sand-sandy loam soil mixture for 20 days. Germination and growth parameters were significantly ($P \leq 0.001$) affected by ACO, grass species, and the interaction ACO x grass species. Increased concentrations of ACO resulted in reduced germination, root, shoot, and total dry weight in both species. Solvent dichloromethane and ACO concentrations of 150 $mg \cdot kg^{-1}$, enhanced seedling growth.

Lolium multiflorum was selected as part of the plant system for future experiments based on its growth rate and greater tolerance to ACO. The evaluation of PH-degradation in the rhizosphere of *L. multiflorum* inoculated with arbuscular mycorrhizal fungi (AMF) in combination with different treatments of biostimulation or biougmentation are considered in the following chapters.

CHAPTER IV

MYCORRHIZA AND BIOSTIMULATION AFFECT GROWTH, AND PHYSIOLOGICAL RESPONSES OF *Lolium multiflorum* IN THE PHYTOREMEDIATION OF SOIL CONTAMINATED WITH ARABIAN MEDIUM CRUDE OIL

Introduction

Phytoremediation utilizes plants to detoxify and eliminate contaminants from the soil (Dietz and Schnoor, 2001). Plant establishment contributes to significant changes in chemical, physical and biological properties in the rhizosphere, which favor the dissipation and/or degradation of organic contaminants such as petroleum hydrocarbons (PH) and polycyclic aromatic hydrocarbons (PAH) in the soil. While phytoremediation of heavy metal-contaminated soils is more commonly reported, research on phytoremediation of organic contaminants is limited (Alkorta and Garbisu, 2001).

Plants utilized in phytoremediation characteristically have higher tolerance to organic contaminants such as PH, however growth can be limited, in part due to reduced water and nutrient uptake (De Jong, 1980; Merkl et al., 2005a, Merkl et al., 2005b). Plants adapt to abiotic stress by adjusting selected physiological responses, which include improvement of water absorption, enhanced nutrient uptake, and induction of free-radical scavenging systems to avoid cellular damage (Grace and Logan, 2000; Mahayan and Tuteja, 2005; Misra and Gupta, 2006; Nayyar and Gupta, 2006; Qadir et al., 2004).

However, physiological responses of plants utilized in phytoremediation of PH-contaminated soils are not well understood.

Alleviation of soil contaminants can be increased by bioremediation with microflora inhabiting the rhizosphere. Some microorganisms degrade soil organic contaminants more efficiently under natural conditions, and contaminant degradation is favored by microbial cometabolism (Trejo and Quintero, 2000: Rivera-Cruz et al., 2002b). Bioaugmentation via inoculation of plants with free-living microorganisms, such as bacteria and filamentous fungi, may significantly improve the phytoremediation performance in detoxifying or degrading organic and inorganic contaminants (Binet et al., 2000b; Pérez-Vargas et al., 2000).

As an important component of the rhizosphere, arbuscular mycorrhizal fungi (AMF) can enhance phytoremediation of soils contaminated with heavy metals (Davies et al., 2001). In some of these studies, some physiological mechanisms for AMF have been identified such as avoidance or tolerance (Perotto and Martino, 2001). However, few studies have been conduced to determine the role of AMF on phytoremediation of soils contaminated with petroleum and polycyclic aromatic hydrocarbons (PAH).

Although AMF can be affected by the presence of organic contaminants in soils, the establishment of this symbiosis may confer some benefits to the plants. Presence of AMF may be an indicator of soil decontamination, since root colonization increased as the levels of contaminants decreased in soils (Hernandez-Acosta et al., 1998, Hernandez-Acosta, 2000). AMF play a significant role in plant survival and tolerance to petroleum hydrocarbons and PAH (Binet et al., 2000a; Joner and Leyval, 2001; Leyval and Binet, 1998). Physiologically, the effect of AMF on *Medicago sativa* has been reported to

induce higher release of oxidoreductases by roots in soil contaminated with anthracene (Criquet et al., 2000). There is evidence that AMF have tolerance in petroleum-contaminated soil (Cabello, 2001), likely related to adaptation, survival, establishment and fitness of plants under these environmental conditions.

The present research was conducted to determine the role of an AMF on selected physiological responses of plants grown in soil contaminated with Arabian medium crude oil (ACO), and to evaluate its performance on phytoremediation of PH. The hypotheses were that: 1) AMF increase plant production of antioxidants in the phytoremediation of ACO-contaminated soil, 2) inorganic fertilization increases growth, gas exchange and selected physiological responses of plants during phytoremediation, 3) microbial population and soil respiration are increased via biostimulation with inorganic fertilization using *Lolium multiflorum* in the phytoremediation of ACO-contaminated soil, and 4) AMF-inoculation and fertilization increase phytoremediation of ACO in soil measured as the reduction in ACO levels.

The objectives of this research were to: 1) determine the antioxidant production of AMF-plants grown during phytoremediation of ACO-contaminated soil, 2) determine the effect of inorganic fertilization on growth, gas exchange and selected physiological responses of *L. multiflorum* during phytoremediation, 3) determine the effect of the fertilization and AMF on microbial populations and soil respiration in the rhizosphere of *L. multiflorum* grown in an ACO-contaminated soil, and 4) determine the phytoremediation of Arabian medium crude oil (ACO) as a response of AMF-inoculation and the biostimulation with inorganic fertilization.

Materials and Methods

Cultural Conditions, Soil Contamination, Transplant and Mycorrhizal Inoculation

The study was conducted for 80 days under glasshouse conditions at Texas A&M University, College Station, TX. The experiment started on 28 August and terminated on 17 November 2004. Temperature and relative humidity were monitored with a watch dog data logger Model 150 (Spectrum technologies, Inc., Planfield, Ill.), and photosynthetic photon flux density (PPFD) was determined with a LI-190SA Quantum/Radiometer/Photometer and sensor (LI-COR® Biosciences, Lincoln, Nebr.). Average day/night temperature and relative humidity were 24.8/23.1°C, and 75.3/78.4%, respectively; and average maximum PPFD determined at solar noon, was 758.5 $\mu moles \cdot m^{-1} \cdot s^{-2}$. A 14 h of photoperiod was maintained by artificially lighting plants from 18:00 to 22:00 during October and November.

The container substrate was a mixture of sand and sandy loam soil (1:1, v/v) with chemical properties of: ($\mu g \cdot g^{-1}$) 0.9 NO_3-N, 2.1 NH_4-N, 1.5 P, 17 K, 9468 Ca, 72 Mg, 161 Na, and 53 S. The electrical conductivity (EC) was 0.17 $dS \cdot cm^{-1}$, pH of 7.7, and textural analysis of 85% sand, 10% clay, and 5% silt. Substrate was steam-pasteurized at 70°C for eight hours on two consecutive days. The substrate was treated with Arabian medium crude oil (ACO) at 0, 3000, or 15000 $mg \cdot kg^{-1}$. The viscosity of ACO contaminant was reduced through the application of dichloromethane solvent (Sigma-Aldrich®, Steinheim, Germany, <0.002 % of residue after evaporation), as previously described. Dichloromethane was allowed to evaporate from the substrate for a week prior to transplanting seedlings. Two week-old seedlings of *Lolium multiflorum* Lam. cv. Passerel

Plus were transplanted to two-kilograms of either non-contaminated or ACO-contaminated substrates. Half of the seedlings were non-inoculated (Non-AMF), and the remaining seedlings were inoculated (AMF) with 500 spores of *Glomus intraradices* (Mycorise® ASP, PremierTech Biotechnologies, Canada) per pot. Plants were weekly fertilized with 100 mL of Long Ashton Nutrient Solution (LANS; Hewitt, 1966; see Appendix I, Table AI-4.1) at 0.5X, 1.0X and 2.0X strength, modified to supply 30 μg P mL^{-1} in all the treatments to maximize establishment of mycorrhizal symbiosis and encourage comparable growth between AMF and Non-AMF plants.

Plant Growth Evaluation

After 80 days, plants were harvested to determine: leaf area (cm^2), leaf dry weight (DW), number and DW of pseudostems, aerial parts, roots, and total plant DW (g). In addition, leaf area ratio [(LAR): leaf area/total DW, $cm^2 \cdot g^{-1}$], specific leaf area [(SLA): leaf area/leaf DW, $cm^2 \cdot g^{-1}$], and root to shoot ratio [(RSR): root DW/aerial part DW, $g \cdot g^{-1}$] were determined. Leaf area was determined with a portable area meter LI-COR Model LI-3000 (LI-COR Biosciences, Lincoln, Nebr). Detached plant organs were placed in an oven at 70°C for two days, and plant samples were weighed.

Selected Plant Physiological Responses

Gas exchange measurements (photosynthesis and stomatal conductance) were taken at the end of the experiment with a portable photosynthesis system model LI-6400 (LI-COR Inc., Lincoln, Nebr.) with red/blue LED light source (LI6400-02B) at photosynthetically active radiation (PAR) levels of 500 μmol $m^{-2} \cdot s^{-1}$, and CO_2

concentration of 360 μmol·s^{-1}. This determination was performed on individual mature leaf blades from three randomly chosen plants per treatment (n=3).

Leaf chlorophyll content was determined with 80% acetone extraction using the procedure of Harborne (1998). Absorbance readings were taken at 645 and 663 nm wavelengths with a Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Chlorophyll content (total, *a* and *b*) were estimated with the following equations:

$$\text{ChlTotal } (mg \cdot L^{-1}) = 17.3(\text{Absorbance}_{646}) + 7.18(\text{Absorbance}_{663})$$

$$\text{Chl}a \ (mg \cdot L^{-1}) = 12.21(\text{Absorbance}_{663}) - 2.81(\text{Absorbance}_{646})$$

$$\text{Chl}b \ (mg \cdot L^{-1}) = 20.13(\text{Absorbance}_{646}) - 5.03(\text{Absorbance}_{663})$$

Leaf samples were taken to measure the content of total phenolic compounds (Singleton and Rossi, 1965; Soong and Barlow, 2004), antioxidant activity (Re et al., 1999) and ascorbic acid (Hernandez et al., 1999; Jimenez et al., 1997b). Proline content in leaves was determined as described by Bates et al. (1973) and Gzik (1996).

Total phenolics content of shoots was evaluated by the Folin-Ciocalteu reagent assay utilizing chlorogenic acid for a standard curve (Singleton and Rossi, 1965; Soong and Barlow, 2004). In brief, 0.150 g of leaf fresh tissue was macerated in a chilled mortar with 3 mL of 80% methanol. Extracts were centrifuged for 15 min at 15,000 rpm. Reaction mixture consisted of mixing 30 μL of the extract added with 90 μL of Na_2CO_3 and 150 μL of Folin-Ciocalteau reagent in a 96-well microplate. After 30 min the absorbance was measured at 725 nm using a KC-4 spectrophotometer (Biotek® Instruments, Inc. Winooski, Vt.). Results were expressed as micrograms of chlorogenic acid equivalents per gram of fresh weight tissue.

Shoot total antioxidant activity was determined by the 1,1-diphenyl-2-picryldrazyl (DPPH) radical decoloration assay (Matthäus, 2002; Re et al., 1999). Briefly, leaf extracts (0.150 g in 3 mL of 80 % methanol) were obtained and immediately centrifuged at 15,000 rpm for 15 min. The reaction mixture consisted of mixing 75 µL of the extract added with 250 µL of DPPH-solution in 96-well microplates. Initial absorbance readings at 515 nm were taken and then, microplates were incubated for 15 min to take a final absorbance reading using a KC-4 spectrophotometer (Biotek® Instruments, Inc. Winooski, Vt.). Antioxidant activity was calculated by applying known aliquots of Trolox (antioxidant compound) to known concentrations of DPPH solution. Results were expressed as micromoles Trolox equivalents per gram of fresh tissue.

Proline content in leaves was determined with the procedures of Bates et al. (1973) and Gzik (1996). Briefly, 0.100 g of leaf fresh tissue was macerated in an iced-mortar with 3 mL of 3 % sulfosalicylic acid. After centrifugation at 10,000 g for 30 minutes, an 200 µL aliquot of the extract was reacted with 200 µL ninhydrin reagent and 200 µL glacial acetic acid, and incubated at 100 °C for one hour. Reaction mixture was stopped with an ice bath, and proline was extracted with toluene. Absorbance readings were taken at 520 nm (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Proline concentration was determined from a standard curve of D,L-proline, and the results were expressed as micromoles of proline per gram of fresh tissue.

Ascorbic acid was extracted from leaves with 2 mL of 2 % metaphosphoric acid, and determined by HPLC (Jimenez et al., 1997a; Jimenez et al., 1997b; Hernandez et al.,

1999). Ascorbic acid was used as standard and results were expressed as micrograms of ascorbic acid per gram of fresh tissue.

Plant Nutrient Analysis

Dried tissue samples from shoots (leaves and pseudostems) from three plants per treatment were ground (Wiley Mill, Arthur H. Thomas Co. Scientific Apparatus, Philadelphia, Pa.) to pass a 40-mesh screen. Complete tissue analysis (N, P, K, Mg, Ca, S, Na, Fe, Mn, Zn, Cu, Al, B, and Mo) was conducted (MDS Harris Laboratory Services, Lincoln, Nebr.).

Microbial Populations, Microbial Respiration, and Mycorrhizal Colonization

Naturally occurring populations of bacteria and filamentous fungi were estimated by performing the dilution plate count method (Alexander, 2005). Soil samples were prepared in serial dilutions (10^{-1} to 10^{-6}) with sterile distilled water. Briefly, 10 g of rhizosphere soil were mixed in 95 mL of sterile water (10^{-1} dilution). The suspension was agitated vigorously for 10 min to suspend either bacterial or fungal cells in the liquid, and then allowed to settle. Subsequent dilutions were prepared by transferring 1 mL of the cell suspension to test tubes containing 9 mL of sterile water. Aliquot transfers were made sequentially from tube to tube to obtain increasingly dilute cell suspensions. From dilution 10^{-5} to 10^{-6}, 100 µL aliquots were taken to estimate bacterial colony forming units (CFU), and from dilutions 10^{-2} to 10^{-3}, aliquots were taken to estimate fungal CFU. Aliquots of 100 µL were transferred and spread out on nutrient agar (total bacteria) or

potato dextrose agar (filamentous fungi) plates. For free-living N_2-fixing bacteria (bacteria able to grow on N-free medium), aliquots were taken from dilutions 10^{-4} to 10^{-5} and spread out on Petri dishes containing Rennie's medium (Rennie, 1981). Plates were inverted, incubated at 26°C for 2-5 days, and bacterial CFU were counted from plates that yield between 30 and 300 CFU.

Rhizosphere soil respiration (CO_2) was determined at the end of the experiment as described by Anderson (1982) and Zuberer (1996). Briefly, 30 g of rhizosphere soil was weighed and put in 150 mL-glass jars and 3.5 mL of nanopure deionized water were added to bring the soil sample to approximately 60% of field capacity. Simultaneously, 4 mL of 1.0 N NaOH were put into 4-mL vials. Vials containing the alkali solution were carefully placed on the surface of the soil in the jars, including in three empty jars without soil as controls. Immediately, jars were tightly sealed and incubated in an incubator at 30 °C for 24 h. The evolution of CO_2 from soil samples and controls was determined when NaOH-vials were lifted out from the jars. Vials received with 2 or 3 drops of phenolphthalein as indicator and 1.0 mL of 50% $BaCl_2$, to precipitate the carbonate as an insoluble barium carbonate. The resulting solution was titrated with 1.0 N HCl, which was slowly added to the vials by means of a buret. Vials were gently stirred until the pink color disappeared. The amount of acid solution required to get the endpoint of the titration of each sample was recorded. The amount of CO_2 evolved from each sample was estimated by the following formula:

$$\text{mg } CO_2 = (B\text{-}V)\ NE,$$

where B is the volume of acid (mL) to titrate the alkali in the vials from controls, V is the volume of acid to titrate the alakali from soil samples, N is the normality of HCl, and E is

the equivalent weight for CO_2 which is equals to 22. Results were expressed as milligrams of CO_2 per kilogram per hour.

Three plants per treatment were randomly taken and assayed for AMF-colonization (Phillips and Hayman, 1970). Roots were placed in plastic capsules and cleared with 10% KOH exposed to 121°C for 10 min. After rinsing with tap water, roots were exposed to a commercial hydrogen peroxide (~3%) for 15 min and rinsed with tap water. Immediately, a 10% hydrochloric acid solution was added to the roots for 15 min. Roots were stained with 0.05% trypan blue in a lactoglycerol solution (glycerol-lactic acid-distilled water, 1:1:1, v/v) at 121°C for 10 min. Finally, 1-cm root segments (20 per slide) were placed on slides, covered with cover slip, and observed under a compound microscope at 100X magnification. The frequency of arbuscules, vesicles, and hyphae (total colonization) was determined, and results were expressed as a percentage of each AM-fungal structure (Biermann and Linderman, 1981).

Total Petroleum Hydrocarbon (TPH) Degradation

Analysis of TPH was performed using a modified EPA SW-846 Method 8270B (Louchouarn et al., 2000; USEPA, 1986). The extraction of TPH from pre-dried samples (15 g) was performed with an automated accelerated solvent extractor (Dionex ASE-200, Dionex Corp., Sunnyvale, Calif.) (Berset et al., 1999; Popp et al., 1997; Richter et al., 1997). The extractions were performed using 100% dichloromethane, with stainless-steel extraction cells held at elevated temperature (100°C) and solvent pressure (1200 psi). The extracted TPH dissolved in the hot solvent were collected in 60 mL glass vials, and immediately concentrated to a volume of 1 mL, using an evaporative solvent reduction

apparatus (Zymark TurboVap II, Zymark Corp. Hopkinton, Mass.). Final extracts were used in the quantitative determination of TPH by gas chromatographic mass spectrometry (HP 5890 Series II Gas Chromatograph Hewlett-Packard Co., Wilmington, Del.).

Rhizosphere Soil pH and Electrical Conductivity Changes

Rhizosphere soil pH and electrical conductivity (EC) were determined at the end of the experiment to evaluate the influence of LANS, AMF, and ACO on changes in pH and salinity build-up. Rhizosphere soil samples were taken and dried at room temperature, and three grams of sample were separately weighed and added with 6 mL of nanopure water. Soil-water suspensions were agitated for five minutes and soil particles allowed to settle to the bottom. Once the supernatant was clear, aliquots were taken and measured by means of a pH meter (Model B-213 HORBIA Ltd. Kyoto, Japan) and EC-meter (Model B-173 HORBIA Ltd. Kyoto, Japan).

Experimental Design

The experiment was a 3x2x3 factorial in a completely randomized design including three levels of fertilization (0.5X, 1X, and 2X strength LANS), two AMF levels (Non-AMF and AMF), and three levels of ACO (0, 3,000 or 15,000 $mg \cdot kg^{-1}$). Data were analyzed by using analysis of variance (ANOVA), except for N content and concentration in shoots, which was analyzed by using the General Linear Model (GLM). LSD (LSD, $\alpha=0.05$) or mean standard error (± SE) was also utilized for means comparison tests (SAS Institute Inc, 2002). Numbers of replications were: for plant DW, n=7; for gas exchange, phenolic content, antioxidant activity, and ascorbate content, n=3;

for microbial population, n=5; and for microbial respiration, AMF-colonization, leaf elemental analysis, TPH-degradation, rhizosphere soil pH, and EC, n=3.

Results

Plant Growth Responses

After 80 days, plant growth measured as the number of pseudostems, and the dry weight (DW) of pseudostems, leaves, aerial part, roots, and total plant was significantly ($P \leq 0.01$) reduced by increasing ACO in soil (Table 4.1; see Appendix I, Fig. AI-4.1 for visual responses of plants). Increasing LANS enhanced ($P \leq 0.01$) all growth parameters at 0 mg ACO kg^{-1} (except root DW) and at 3000 mg ACO kg^{-1} (except root and total plant DW). LANS had no growth effects at 15000 mg ACO kg^{-1} (Table 4.1). Significant effects ($P \leq 0.05$) were observed for AMF for pseudostems, leaf, and aerial DW of plants at 0 mg ACO kg^{-1} (Table 4.1). However, no AMF enhancement occurred at 3000 or 15000 mg ACO kg^{-1}. Interactions of LANS x ACO and LANS x AMF x ACO resulted in significant effects ($P \leq 0.01$) on plant growth, whereas the effects of the interactions LANS x AMF and AMF x ACO were nonsignificant (Table 4.1). Total plant DW was significantly affected by LANS ($P \leq 0.05$), ACO ($P \leq 0.01$), and the interaction LANS x AMF x ACO ($P \leq 0.01$) (Table 4.1, Fig. 4.1).

Independent factors LANS, AMF, and ACO as well as the interactions LANS x ACO and LANS x AMF x ACO significantly ($P \leq 0.01$) affected leaf area (Table 4.2). Increasing ACO significantly ($P < 0.01$) decreased leaf area (Table 4.2; Fig. 4.2). The RSR was also reduced by ACO ($P < 0.01$), and increased by increasing LANS at 0, but not at 3000 or 15000 $mg \cdot kg^{-1}$ ACO. In general, SLA, LAR and RSR were significantly affected

($P \leq 0.01$) by ACO, and the interactions LANS x ACO and LANS x AMF x ACO (Table 4.2). SLA and RSR were significantly affected by LANS application ($P \leq 0.01$ and $P \leq 0.05$, respectively). The main effects of AMF, and the interactions LANS x AMF and AMF x ACO were nonsignificant for SLA, LAR, and RSR (Table 4.2).

Table 4.1. Growth response of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) plants of *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	No. of pseudo-stems	Pseudo-stems DW (g)	Leaf DW (g)	Aerial DW (g)	Root DW (g)	Total plant DW (g)
0	0.5	No	21.1 def [y]	2.4 cde	2.9 c	5.3 d	18.4 ab	23.9 c
		Yes	21.4 def	2.6 cd	2.9 c	5.5 d	23.9 a	29.3 bc
	1	No	32.4 c	4.7 b	5.4 b	10.0 c	25.9 a	35.9 ab
		Yes	33.7 bc	4.9 b	5.0 b	10.0 c	25.3 a	34.8 ab
	2	No	48.7 a	5.1 b	7.5 a	12.7 b	10.6 bc	22.1 c
		Yes	46.1 a	6.4 a	8.5 a	14.9 a	23.4 a	38.9 a
3,000	0.5	No	12.6 fgh	0.6 f	1.0 de	1.6 ef	2.3 d	3.9 de
		Yes	15.0 efg	0.7 f	1.3 d	2.1 e	1.8 d	3.7 de
	1	No	26.2 cd	1.7 de	2.8 c	4.6 d	3.4 cd	7.6 de
		Yes	22.4 de	1.7 e	2.6 c	4.3 d	3.6 cd	7.7 cde
	2	No	41.6 ab	3.1 c	5.2 b	8.3 c	3.3 cd	10.8 d
		Yes	43.5 a	3.2 c	5.2 b	8.4 c	3.2 cd	10.8 d
15,000	0.5	No	4.0 h	0.1 f	0.1 e	0.2 f	0.2 d	0.5 e
		Yes	5.7 h	0.1 f	0.2 e	0.3 ef	0.3 d	0.7 e
	1	No	5.1 h	0.1 f	0.2 e	0.4 ef	0.4 d	0.8 e
		Yes	6.6 gh	0.3 ef	0.5 de	0.8 ef	0.9 d	2.0 de
	2	No	6.3 gh	0.3 f	0.4 de	0.6 ef	1.2 d	2.0 de
		Yes	4.5 h	0.1 f	0.2 e	0.3 ef	0.1 d	0.4 e
	Significance							
		LANS	0.01	0.01	0.01	0.01	NS	0.05
		AMF	NS	0.01	0.05	0.05	NS	NS
		ACO	0.01	0.01	0.01	0.01	0.01	0.01
		LANS x AMF	NS	NS	NS	NS	NS	NS
		LANS x ACO	0.01	0.01	0.01	0.01	NS	NS
		AMF x ACO	NS	NS	NS	NS	NS	NS
		LANS x AMF x ACO	0.01	0.01	0.01	0.01	0.01	0.01

[y] Means followed by same letter in the same column are not significantly different (LSD, $\alpha=0.05$); NS=Nonsignificant, n=7.

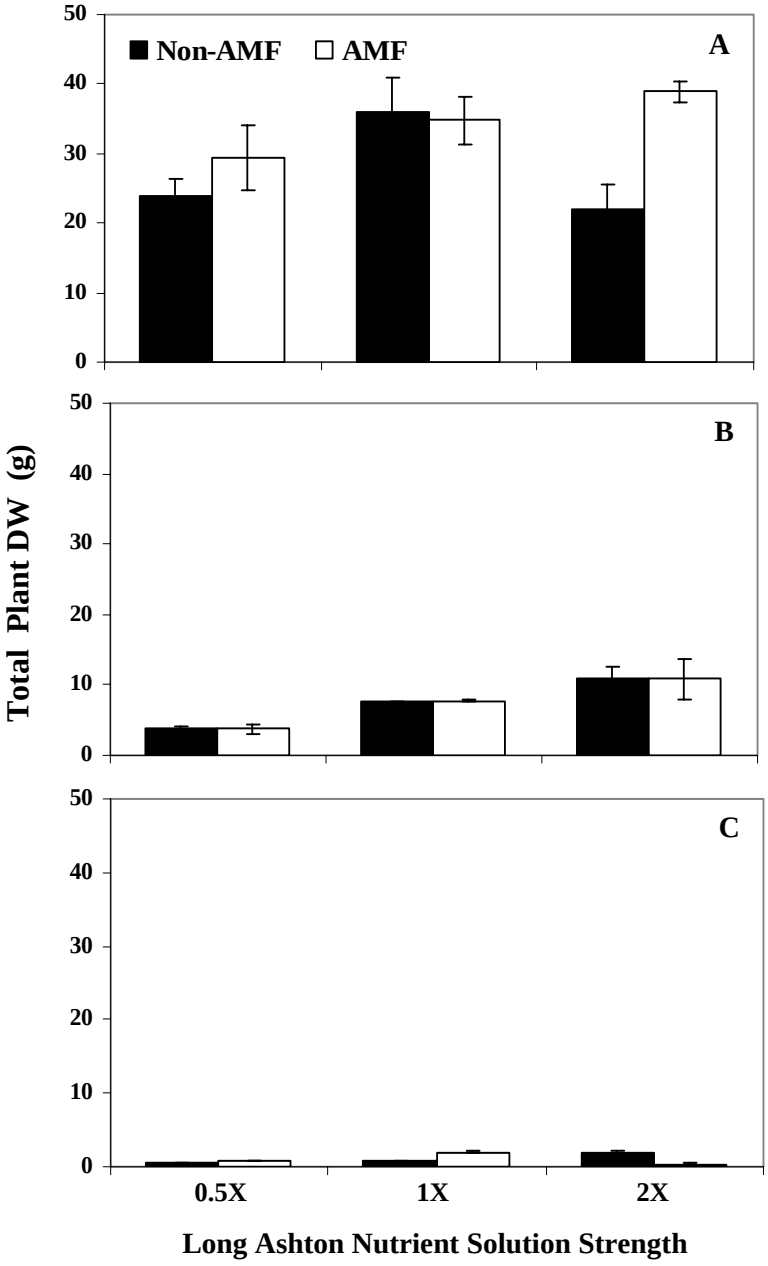

Fig. 4.1. Total plant dry weight of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) 0 $mg \cdot kg^{-1}$, B) 3,000 $mg \cdot kg^{-1}$, C) 15,000 $mg \cdot kg^{-1}$. Main effects of LANS, ACO, and the interaction LANS x AMF x ACO were significant at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.01$, respectively. Main effects of AMF, and the interaction of LANS x AMF, LANS x ACO, and AMF x ACO were nonsignificant. Bars ± SE; n=7.

Table 4.2. Leaf area, specific leaf area (SLA), leaf area ratio (LAR), and root to shoot ratio (RSR) of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) plants of *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS Strength (X)	AMF	Leaf area (cm^2)	SLA ($cm^2 \cdot g^{-1}$)	LAR ($cm^2 \cdot g^{-1}$)	RSR ($g \cdot g^{-1}$)
0	0.5	No	721.9 g	246.3 efgh[y]	31.2 jk	3.4 ab
		Yes	736.9 fg	241.5 fgh	23.9 k	4.5 a
	1	No	1570.0 d	314.6 cdef	47.9 ijk	2.6 bcd
		Yes	1705.4 cd	366.6 abcd	62.7 ghijk	2.5 bcd
	2	No	2482.3 b	335.5 cde	106.5 cdefgh	0.9 e
		Yes	2936.4 a	342.4 bcd	80.2 efghij	1.5 cde
3,000	0.5	No	403.5 ghi	393.4 abc	131.4 bcde	1.2 de
		Yes	491.2 gh	383.6 abc	142.3 abc	1.0 e
	1	No	1127.7 e	433.9 ab	155.4 abc	0.9 e
		Yes	1082.6 ef	451.2 a	166.3 ab	0.8 e
	2	No	2022.2 c	406.2 abc	178.8 ab	0.4 e
		Yes	1963.8 c	395.9 abc	184.9 a	0.4 e
15,000	0.5	No	53.8 ij	355.2 bcd	112.8 cdefg	0.9 e
		Yes	78.3 ij	321.9 cdef	136.0 abcd	0.6 e
	1	No	60.5 ij	277.7defg	89.4 defghi	0.8 e
		Yes	184.1 hij	381.8 abc	127.8 bcdef	0.9 e
	2	No	104.4 ij	212.2 gh	55.5 hijk	2.0 bc
		Yes	32.0 j	183.5 h	77.8 fghij	0.4 e
Significance						
		LANS	0.01	0.01	NS	0.05
		AMF	0.01	NS	NS	NS
		ACO	0.01	0.01	0.01	0.01
		LANS x AMF	NS	NS	NS	NS
		LANS x ACO	0.01	0.01	0.01	0.01
		AMF x ACO	NS	NS	NS	NS
		LANS x AMF x ACO	0.01	0.01	0.01	0.01

[y] Means followed by same letter in the same column are not significantly different (LSD, α=0.05); NS=Nonsignificant, n=7.

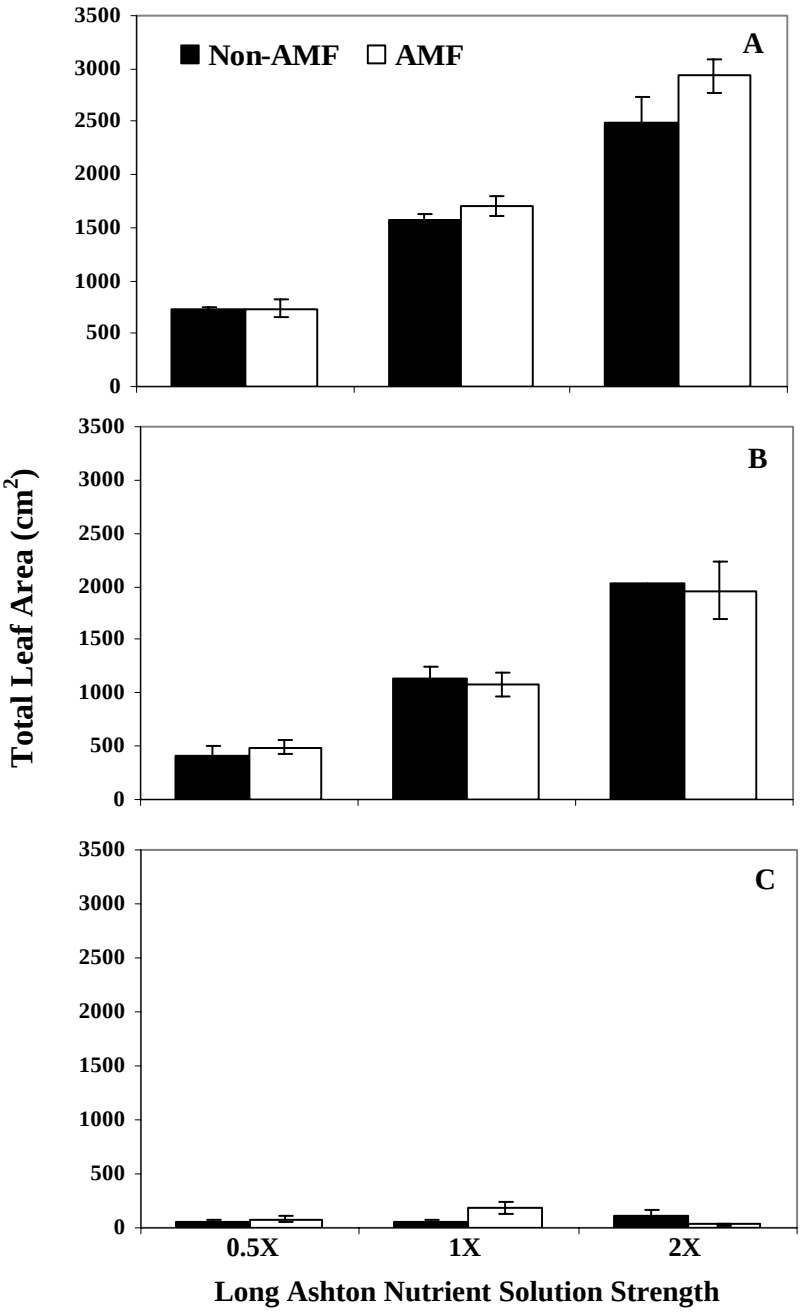

Fig. 4.2. Total leaf area of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) 0 mg·kg^{-1}, B) 3,000 mg·kg^{-1}, C) 15,000 mg·kg^{-1}. Main effects of LANS, AMF, ACO, and the interactions LANS x ACO, and LANS x AMF x ACO were significant at $P \leq 0.01$, respectively. The interactions LANS x AMF, and AMF x ACO were nonsignificant. Bars ± SE, n=7.

Selected Physiological Responses

Total chlorophyll, chlorophyll *a* and *b* were not significantly affected by LANS, AMF or the interactions LANS x AMF, LANS x ACO, and AMF x ACO, but significant effects ($P \leq 0.05$) were observed for the interaction LANS x AMF x ACO. ACO significantly affect ($P \leq 0.05$) chlorophyll *a* and *b*, but had no effect on total chlorophyll (Table 4.3). With increasing ACO there were trends in increased chlrophyll *a* and decreased chlorophyll *b*.

Photosynthesis was significantly ($P \leq 0.01$) reduced with increasing ACO, but no significant effects occurred with LANS and AMF (Table 4.4). The only significant ($P \leq 0.01$) interaction on photosynthesis was LANS x AMF x ACO (Table 4.4).

Stomatal conductance was significantly affected by ACO, LANS, AMF, and the interactions LANS x ACO, and LANS x AMF x ACO (Table 4.4). Generally, there was a trend of lowest stomatal conductance at highest ACO. AMF had no effect on stomatal conductance at 3000 or 15000 mg ACO kg^{-1}. In constrast, ACO was the only factor that significantly ($P \leq 0.05$) affected *WUE* of plants, however, the response was not consistent (Table 4.4.)

The total soluble phenolics content of leaves were significantly affected by ACO ($P \leq 0.05$), LANS ($P \leq 0.01$), and the interaction LANS x AMF xACO ($P \leq 0.01$). Increasing ACO caused a significant increase in phenolic compounds at 2X LANS, but had no effect at 0.5X or 1.0X LANS (Fig. 4.3). Phenolics content was generally higher at 0.5X than 2X LANS regardless of ACO levels (Fig. 4.3). AMF had no significant effects on phenolics (Fig. 4.3 C).

Table 4.3. Chlorophyll concentration (total, *a*, and *b*) of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) plants of *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	Chlorophyll Total	*a*	*b*
				($\mu g \cdot g^{-1}$)	
0	0.5	No	1171.5 bc[y]	570.2 c	602.1 abcd
		Yes	1128.9 c	601.8 c	582.0 bcd
	1	No	2070.3 ab	1202.8 abc	868.9 a
		Yes	1392.0 abc	710.2 bc	682.9 abc
	2	No	1138.1 c	527.7 c	611.3 abcd
		Yes	1589.2 abc	851.0 bc	739.3 ab
3,000	0.5	No	1619.5 abc	898.9 bc	721.8 ab
		Yes	1401.1 abc	763.0 bc	639.1 abcd
	1	No	1323.1 abc	648.8 bc	675.3 abcd
		Yes	1589.4 abc	833.0 bc	757.6 ab
	2	No	1576.4 abc	695.5 bc	882.1 a
		Yes	1825.2 abc	937.2 bc	889.3 a
15,000	0	No	2155.8 a	1667.7 a	489.3 bcde
		Yes	1414.5 abc	1041.9 abc	373.5 de
	1	No	1551.3 abc	1156.1 abc	396.1 cde
		Yes	1343.8 abc	861.9 bc	482.9 bcde
	2	No	1015.2 c	794.2 bc	221.5 e
		Yes	1776.7 abc	1319.5 ab	458.2 bcde
	Significance				
		LANS	NS	NS	NS
		AMF	NS	NS	NS
		ACO	NS	0.05	0.01
		LANS x AMF	NS	NS	NS
		LANS x ACO	NS	NS	NS
		AMF x ACO	NS	NS	NS
		LANS x AMF x ACO	0.05	0.01	0.01

[y] Means followed by same letter in the same column are not significantly different (LSD, α=0.05); NS=Nonsignificant, n=3.

Total antioxidant (AOX) activity in leaves was significantly affected by LANS ($P \leq 0.01$), and the interaction LANS x AMF x ACO ($P \leq 0.05$). Neither ACO nor AMF significantly affected AOX activity (Fig. 4.4). Increasing ACO caused a significant increase in antioxidant activity at 2X LANS (Fig. 4.4). Antioxidant activity was generally higher at 0.5X than 2X LANS regardless of ACO levels (Fig. 4.4). AMF had no significant effects on activity (Fig. 4.4 C).

Table 4.4. Photosynthesis (*Pn*), stomatal conductance (g_s) and water use efficiency (*WUE*) of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) plants of *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	Photosynthesis ($\mu moles\ CO_2\ m^{-2} \cdot s^{-1}$)	Stomatal conductance ($moles\ m^{-2} \cdot s^{-1}$)	*WUE* (Pn/g_s)
0	0.5	No	6.1 abcd [y]	0.04 cde	169.7 bcd
		Yes	4.7 bcdef	0.04 cde	120.9 bcd
	1	No	3.8 cdefg	0.02 cde	148.3 bcd
		Yes	8.0 a	0.05 c	171.3 bcd
	2	No	7.7 ab	0.03 cde	558.7 abc
		Yes	5.1 abcde	0.09 b	55.5 cd
3,000	0.5	No	3.6 defg	0.02 cde	141.6 bcd
		Yes	3.3 defg	0.01 cde	624.9 ab
	1	No	3.7 cdefg	0.02 cde	164.2 bcd
		Yes	6.8 abc	0.04 cd	158.8 bcd
	2	No	3.2 defg	0.11 ab	24.0 d
		Yes	7.8 ab	0.13 a	62.0 cd
15,000	0.5	No	1.6 fg	0.00 e	414.6 abcd
		Yes	2.6 efg	0.01 cde	214.3 bcd
	1	No	2.6 efg	0.01 de	472.6 abcd
		Yes	2.0 fg	0.02 cde	159.4 bcd
	2	No	1.4 g	0.01 de	483.0 abcd
		Yes	2.6 fg	0.01 de	890.6 a
	Significance				
		LANS	NS	0.01	NS
		AMF	NS	0.05	NS
		ACO	0.01	0.01	0.05
		LANS x AMF	NS	NS	NS
		LANS x ACO	NS	0.01	NS
		AMF x ACO	NS	NS	NS
		LANS x AMF x ACO	0.01	0.01	NS

[y] Means followed by same letter in the same column are not significantly different (LSD, α=0.05); NS=Nonsignificant. n=3.

Ascorbate content was significantly ($P \leq 0.01$) affected by ACO, while nonsignificant effects occurred for LANS, AMF, or the interactions LANS x ACO, LANS x AMF, and LANS x AMF x ACO (Fig. 4.5). At 0.5X and 1X LANS, ascorbate decreased from 0 to 15000 mg ACO kg^{-1}. Conversely, ascorbate increased at 2X LANS from 0 to 15000 mg ACO kg^{-1} (Fig. 4.5).

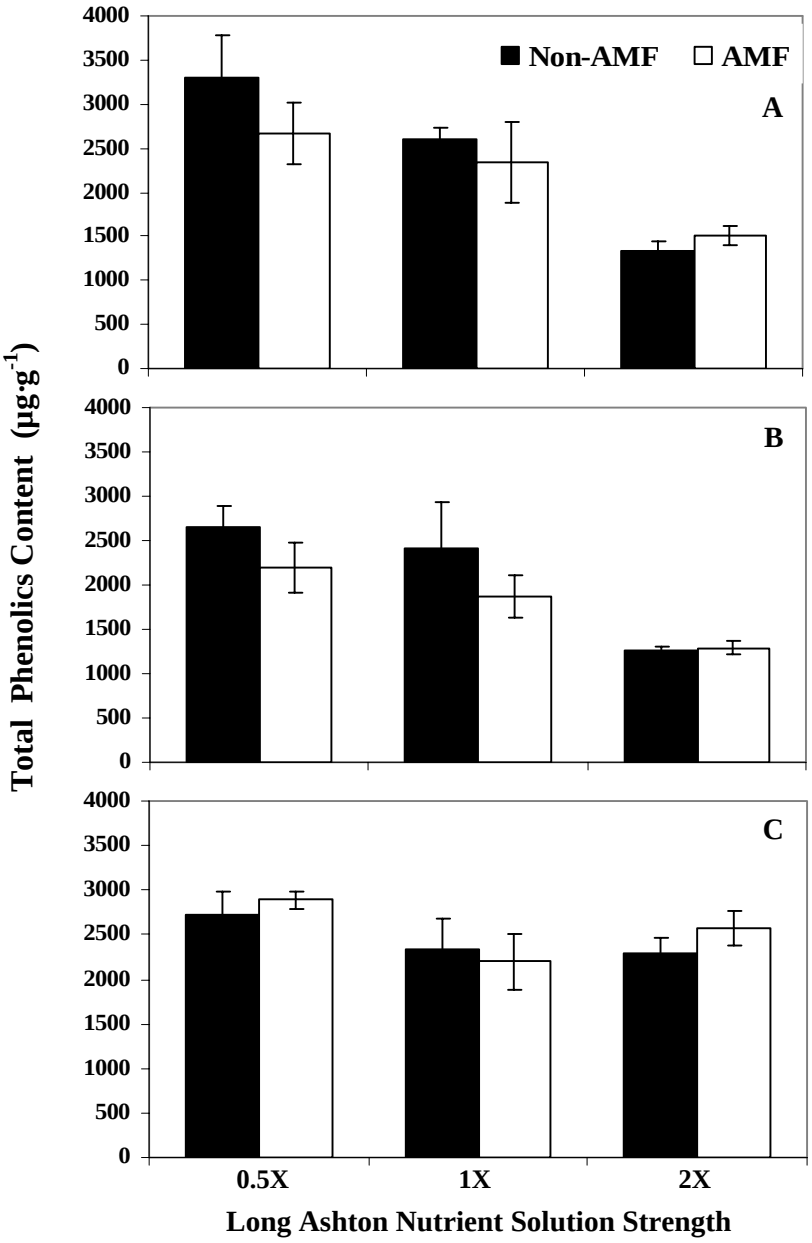

Fig. 4.3. Total soluble phenolics in leaf blades of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) 0 mg·kg^{-1}, B) 3,000 mg·kg^{-1}, C) 15,000 mg·kg^{-1}. Main effects of LANS, ACO, and the interaction LANS x AMF x ACO were significant at $P \leq 0.01$, $P \leq 0.05$, and $P \leq 0.01$, respectively. Main effects of AMF, and the interactions LANS x AMF, LANS x ACO, and AMF x ACO were nonsignificant. Bars ± SE, n=3.

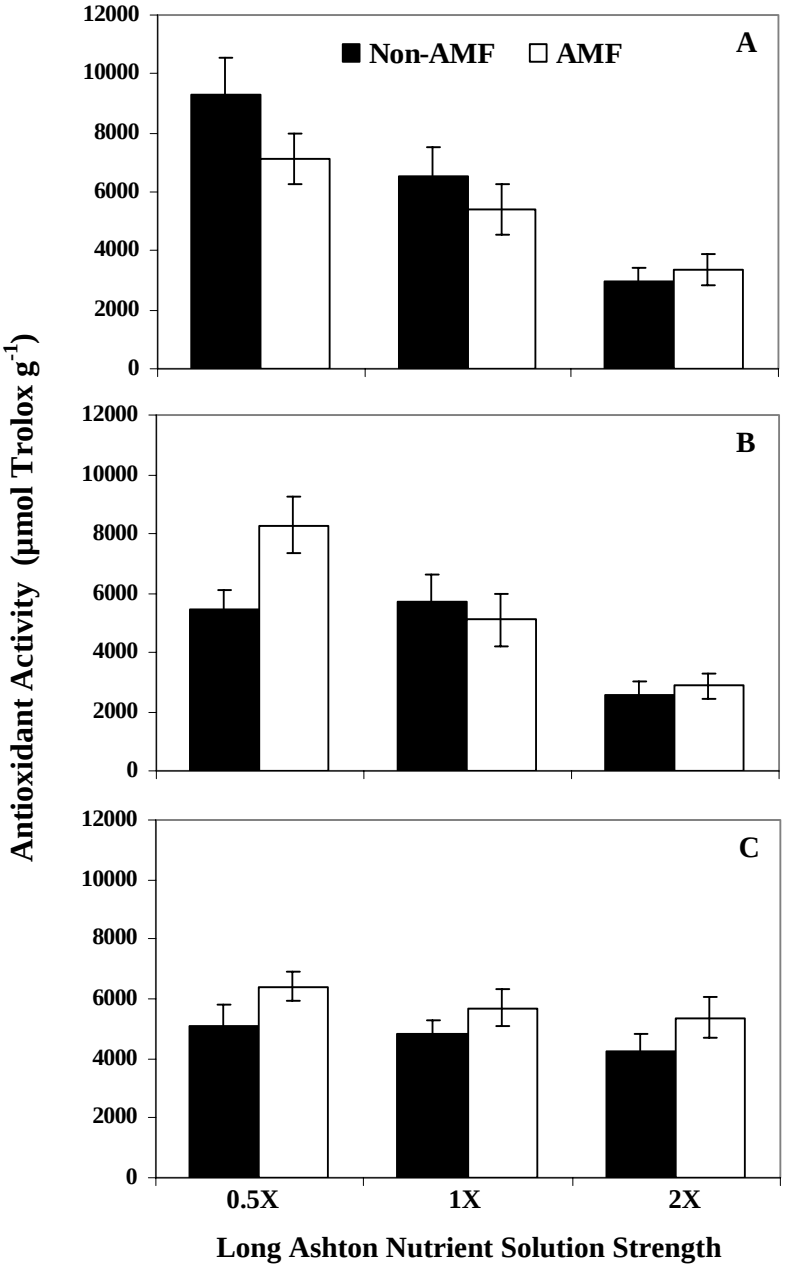

Fig. 4.4. Antioxidant activity in leaf blades of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) 0 mg·kg^{-1}, B) 3,000 mg·kg^{-1}, C) 15,000 mg·kg^{-1}. Main effects of LANS and the interaction LANS x AMF x ACO were significant at $P \leq 0.001$, and $P \leq 0.05$, respectively. Main effects of LANS, AMF, and the interactions LANS x AMF, LANS x ACO, AMF x ACO were nonsignificant. Bars ± SE, n=3.

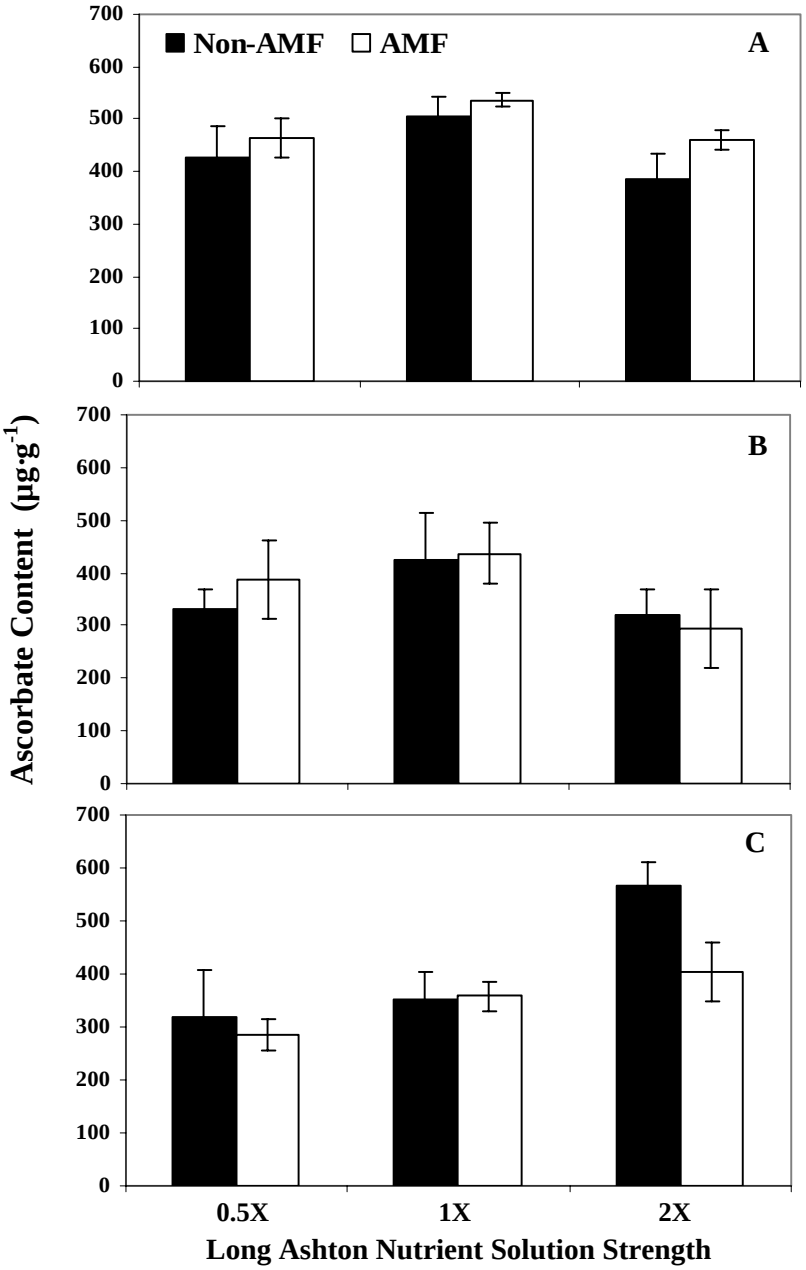

Fig. 4.5. Ascorbate content in leaf blades of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) 0 $mg \cdot kg^{-1}$, B) 3,000 $mg \cdot kg^{-1}$, C) 15,000 $mg \cdot kg^{-1}$. Main effects of ACO were significant at $P \leq 0.05$. Main effects of LANS, AMF, and the interactions LANS x AMF, LANS x ACO, AMF x ACO, and LANS x AMF x ACO were nonsignificant. Bars ± SE; n=3.

Plant Nutritional Responses

Increased levels of LANS application resulted in higher total macroelement (N, P, K, Mg, Ca, S, and Na) content in plant shoots (leaves and pseudostems), while ACO-contamination caused a significant reduction, particularly at 15000 mg·kg^{-1} (Table 4.5). LANS, ACO, and the interaction LANS x ACO had significant ($P \leq 0.001$) effects on N, P, K, Mg, Ca, S, and Na. AMF had no significant effects on total content of macronutrients, except for N and Ca ($P \leq 0.05$), which were highest at 2X LANS at 0 mg ACO kg^{-1} (Table 4.5).

Increasing ACO significantly ($P \leq 0.001$) reduced total content of all microelements (Zn, Mn, Cu, Fe, B, Al, and Mo) in shoots (Table 4.6). Increasing LANS strength enhanced ($P \leq 0.001$) all microelemental content, except Mn (Table 4.6). AMF only enhanced ($P \leq 0.05$) Zn and Fe content at 0 ACO mg·kg^{-1} at 2X LANS (Table 4.6). The interaction LANS x AMF x ACO was only significant ($P \leq 0.01$) only for Mn content, while LANS x ACO effects were significant for Zn ($P \leq 0.05$), Cu, Fe, B, and Al ($P \leq 0.001$). There were no significant interactions of LANS x AMF or AMF x ACO on microelement content (Table 4.6).

In regard to macronutrient concentrations, increasing LANS strength enhanced ($P \leq 0.001$) N, K and Mg concentration, but had no effect on P, S, or Na; Ca decreased (Table 4.7). ACO had significant ($P \leq 0.001$) effects on macronutrient concentration. Increasing ACO enhanced N and P (up to 3000 mg ACO kg^{-1}), K, Mg, Ca, and S; Na was not affected (Table 4.7). There were significant effects of AMF ($P \leq 0.001$) on Mg, Ca, and S, however, AMF only enhanced concentration of Mg, Ca, and S, at 15,000 mg ACO kg^{-1} at 2X LANS.

Table 4.5. Total macroelement content in shoots of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	Macroelements						
			N	P	K	Mg ($mg \cdot plant^{-1}$)	Ca	S	Na
0	0.5	No	70.4 fgh [y]	17.5 efg	128.1 efgh	17.1 cde	25.0 cd	17.2 def	19.8 bcde
		Yes	71.2 efgh	15.5 efg	129.6 efgh	15.1 de	23.6 cd	17.3 def	20.7 bcde
	1	No	143.9 def	26.2 bcde	228.5 de	33.0 bc	35.7 bc	31.2 bcd	37.9 bc
		Yes	146.8 de	29.3 bc	228.9 de	36.3 b	42.5 b	32.7 bc	68.9 a
	2	No	263.9 b	31.9 b	422.3 ab	45.7 b	35.2 bc	39.0 b	32.3 bcd
		Yes	350.4 a	45.0 a	520.6 a	71.0 a	59.7 a	56.4 a	41.6 b
3,000	0.5	No	29.3 gh	7.7 gh	50.0 fgh	4.8 de	6.9 ef	6.4 ef	3.9 e
		Yes	50.1 gh	14.2 fg	92.9 fgh	8.9 de	15.2 def	10.2 ef	4.3 e
	1	No	85.1 defg	18.4 defg	165.8 def	17.9 cde	23.1 cd	18.1 cde	6.4 e
		Yes	95.4 defg	17.4 efg	159.7 defg	18.9 cd	21.0 cde	19.0 cde	10.6 de
	2	No	159.1 cd	19.6 cdef	261.9 cd	32.6 bc	32.3 bc	29.3 bcd	6.8 e
		Yes	231.4 bc	28.8 bcd	360.3 bc	42.7 b	42.9 b	40.4 b	17.9 cde
15,000	0.5	No	7.2 h	1.9 h	15.1 h	2.2 e	2.7 f	3.1 f	1.8 e
		Yes	8.4 h	2.1 h	18.3 h	2.1 e	3.5 f	3.3 f	1.5 e
	1	No	6.5 h	1.0 h	16.5 h	2.2 e	2.6 f	3.0 f	1.1 e
		Yes	23.1 gh	2.7 h	42.0 gh	5.7 de	5.5 f	6.6 ef	2.8 e
	2	No	22.5 gh	2.2 h	41.4 gh	5.1 de	3.7 f	6.0 ef	0.9 e
		Yes	md	0.9 h	15.7 h	3.9 de	2.5 f	5.2 ef	0.8 e
	Significance								
		LANS	0.001 [§]	0.001	0.001	0.001	0.001	0.001	0.05
		AMF	0.01	NS	NS	NS	0.05	NS	NS
		ACO	0.001	0.001	0.001	0.001	0.001	0.001	0.001
		LANS x AMF	NS	NS	NS	NS	NS	NS	NS
		LANS x ACO	0.01	0.01	0.001	0.001	0.01	0.01	0.05
		AMF x ACO	NS	NS	NS	NS	NS	NS	NS
		LANS x AMF x ACO	NS	NS	NS	NS	NS	NS	NS

[y] Means followed by same letter in the same column are not significantly different (LSD, α=0.05); [§]N significance was estimated via GLM procedure due to missing data (md).; NS=Nonsignificant, n=3.

Table 4.6. Total microelement content in shoots of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	Microelements						
			Zn	Mn	Cu	Fe ($\mu g \cdot plant^{-1}$)	B	Al	Mo
0	0.5	No	244.9 cdef[y]	1404.4 b	39.5 ef	280.6 efgh	298.5 de	117.4 cde	39.7 cdef
		Yes	238.3 def	1173.1 bc	36.9 efg	250.3 fghi	263.3 def	88.8 de	46.3 bcde
	1	No	324.8 bcde	1134.7 bc	75.5 bcd	463.7 def	497.6 bc	163.7 bcd	49.1 bcd
		Yes	395.1 bc	1017.5 bc	87.9 bc	607.5 bcd	529.8 bc	269.8 ab	62.8 abc
	2	No	405.6 b	628.3 defgh	105.8 b	752.7 ab	693.4 b	198.3 abc	82.5 a
		Yes	686.4 a	2043.7 a	143.8 a	936.0 a	913.4 a	256.7 ab	72.7 ab
3,000	0.5	No	66.0 gh	250.8 defgh	12.1 fgh	94.5 ghi	80.1 fgh	43.1 e	4.53 g
		Yes	136.2 fgh	769.0 cdef	26.2 efgh	182.6 ghi	121.6 efgh	79.6 de	9.0 fg
	1	No	217.9 efg	798.8 bcde	49.8 de	269.5 efgh	228.9 defg	81.3 de	15.6 efg
		Yes	218.3 efg	668.0 cdefg	39.5 ef	314.2 efg	266.9 def	69.6 de	18.4 defg
	2	No	346.2 bcde	877.2 bcd	57.1 cde	499.2 cde	415.0 cd	239.2 ab	27.6 defg
		Yes	387.5 bcd	996.8 bc	75.0 bcd	709.3 abc	618.2 b	299.4 a	10.5 fg
15,000	0.5	No	20.4 h	123.9 gh	3.1 h	54.6 hi	21.7 h	20.2 e	0.93 g
		Yes	18.9 h	161.4 fgh	3.8 h	81.8 ghi	27.0 h	81.5 de	1.0 g
	1	No	13.9 h	119.2 gh	3.1 h	26.3 i	24.2 h	9.2 e	2.3 g
		Yes	37.0 h	203.6 efgh	6.6 fgh	64.5 hi	60.5 gh	16.9 e	3.7 g
	2	No	27.4 h	75.5 gh	5.4 gh	57.5 hi	65.3 gh	29.9 e	5.3 g
		Yes	9.8 h	29.0 h	5.1 gh	48.3 hi	42.5 gh	41.4 e	1.8 g
	Significance								
		LANS	0.001	NS	0.001	0.001	0.001	0.001	0.05
		AMF	0.05	NS	NS	0.05	NS	NS	NS
		ACO	0.001	0.001	0.001	0.001	0.001	0.001	0.001
		LANS x AMF	NS	NS	NS	NS	NS	NS	NS
		LANS x ACO	0.01	NS	0.001	0.001	0.001	0.001	NS
		AMF x ACO	NS	NS	NS	NS	NS	NS	NS
		LANS x AMF x ACO	NS	0.01	NS	NS	NS	NS	NS

[y] Means followed by same letter in the same column are not significantly different (LSD, α=0.05); NS=Nonsignificant, n=3.

Table 4.7. Macronutrient concentration (%) in shoots of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	Macronutrients						
			N	P	K	Mg (%)	Ca	S	Na
0	0.5	No	1.3 g [y]	0.32 de	2.37 g	0.33 fg	0.46 hi	0.32 g	0.36 bcd
		Yes	1.3 g	0.29 defg	2.43 g	0.28 g	0.44 i	0.33 g	0.38 bcd
	1	No	1.6 fg	0.28 defg	2.47 g	0.35 f	0.38 jk	0.33 g	0.43 bc
		Yes	1.6 fg	0.31 def	2.42 g	0.37 f	0.43 ij	0.34 fg	0.73 a
	2	No	2.6 abc	0.30 defg	3.98 bcde	0.45 e	0.35 k	0.38 efg	0.35 bcd
		Yes	2.2 bcd	0.29 defg	3.34 f	0.45 e	0.38 jk	0.36 fg	0.28 bcdef
3,000	0.5	No	2.1 de	0.50 ab	3.87 cde	0.35 fg	0.52 g	0.45 de	0.26 bcdef
		Yes	2.0 de	0.58 a	3.77 def	0.36 f	0.62 cd	0.42 def	0.18def
	1	No	2.2 cd	0.48 b	4.25 bc	0.47 de	0.60 de	0.48 d	0.17 def
		Yes	2.4 abcd	0.45 b	4.16 bcd	0.49 de	0.54 fg	0.49 d	0.22 cdef
	2	No	2.6 ab	0.33 de	4.30 bc	0.52 cd	0.52 gh	0.47 d	0.12 ef
		Yes	2.8 a	0.36 cd	4.37 ab	0.50 de	0.52 gh	0.48 d	0.18 def
15,000	0.5	No	1.8 ef	0.47 b	3.70 def	0.53 cd	0.67 bc	0.80 b	0.45 b
		Yes	1.8 ef	0.44 bc	3.87 cde	0.45 e	0.74 a	0.69 c	0.32 bcde
	1	No	1.4 fg	0.22 g	3.69 ef	0.49 de	0.58 def	0.68 c	0.26 bcdef
		Yes	2.3 bcd	0.28 defg	4.29 bc	0.58 bc	0.56 efg	0.68 c	0.28 bcdef
	2	No	2.5 abc	0.26 fg	4.83 a	0.60 b	0.43 ij	0.70 c	0.11 f
		Yes	md	0.24 fg	4.32 bc	1.10 a	0.68 b	1.42 a	0.22 cdef
	Sufficiency range [z]		3.3-5.1	0.35-0.55	2.0-3.4	0.16-0.32	0.25-0.51	0.27-0.56	NR
Significance		LANS	0.001 [§]	0.001	0.001	0.001	0.001	0.001	0.01
		AMF	NS	NS	NS	0.001	0.001	0.001	NS
		ACO	0.001	0.001	0.001	0.001	0.001	0.001	0.001
		LANS x AMF	0.05	NS	0.05	0.001	0.001	0.001	NS
		LANS x ACO	NS	0.001	0.001	0.001	0.001	0.001	0.05
		AMF x ACO	0.05	NS	NS	0.001	0.001	0.001	NS
		LANS x AMF x ACO	NS	NS	NS	0.001	0.001	0.001	NS

[y]Means followed by same letter in the same column are not significantly different (LSD, α=0.05); [z]Carrow et al. (2001); NR=No reported. [§]N significance was estimated via GLM procedure due to missing data (md); NS=Nonsignificant, n=3.

Table 4.8. Micronutrient concentration in shoots of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF) *Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	AMF	Micronutrients						
			Zn	Mn	Cu	Fe ($mg \cdot g^{-1}$)	B	Al	Mo
0	0.5	No	45.3 cde[y]	260.7 cde	7.33 de	52.0 fg	55.3 fg	22.0 def	7.3 abc
		Yes	44.7 cde	220.7 def	7.00 de	46.7 g	50.0 g	16.0 f	8.7 a
	1.0	No	35.3 fg	113.7 ijk	8.33 bcde	51.3 fg	54.0 fg	19.0 ef	5.7 abcde
		Yes	40.3 def	100.0 ijkl	9.00 bcd	65.7 def	55.3 fg	33.3 cdef	6.0 abcd
	2.0	No	40.0 def	63.0 l	10.00 bc	70.7 cde	71.0 bcd	19.0 ef	8.3 ab
		Yes	43.7 cde	128.7 hij	9.33 bcd	60.3 efg	59.0 efg	16.7 f	4.7 bcdef
3,000	0.5	No	55.7 a	207.3 fg	10.33 bc	72.7 cde	57.3 efg	37.7 cd	3.3 def
		Yes	55.3 a	313.0 ab	10.67 b	74.3 cde	49.3 g	32.0 cdef	3.7 cdef
	1.0	No	56.3 a	214.7 efg	13.33 a	70.0 cde	60.7 defg	22.0 def	4.7 bcdef
		Yes	56.0 a	167.7 gh	10.67 b	78.0 cd	68.0 bcde	20.3 def	3.7 cdef
	2.0	No	54.7 ab	137.7 hi	9.33 bcd	80.0 cd	68.3 bcde	38.3 cd	5.0 abcdef
		Yes	46.7 bcd	120.3 hijk	9.33 bcd	87.0 c	73.7 bc	37.3 cd	1.3 f
15,000	0.5	No	50.0 abc	305.0 bc	8.00 cde	134.0 b	53.0 fg	50.0 c	2.0 ef
		Yes	40.0 def	341.0 a	8.00 cde	173.0 a	57.0 efg	172.0 a	2.0 ef
	1.0	No	31.0 gh	267.0 bcd	7.00 de	59.0 efg	54.0 fg	20.7 def	5.0 abcdef
		Yes	38.0 efg	208.0 fg	7.00 de	66.0 def	62.0 cdef	17.0 f	4.0 cdef
	2.0	No	31.7 gh	87.7 jkl	6.33 e	70.3 cde	79.3 b	35.7 cde	6.0 abcd
		Yes	27.0 h	80.0 kl	14.00 a	133.0 b	117.0 a	114.0 b	5.0 abcdef
	Sufficiency range[z]		14-64	30-73	6-38	97-934	5-17	NR	0.5-1.0
Significance		LANS	0.001	0.001	NS	0.001	0.001	0.001	NS
		AMF	NS	NS	NS	0.001	0.05	0.001	NS
		ACO	0.001	0.001	0.001	0.001	0.001	0.001	0.001
		LANS x AMF	NS	0.01	0.01	NS	0.05	0.001	NS
		LANS x ACO	0.01	0.001	0.001	0.001	0.001	0.001	0.05
		AMF x ACO	NS	NS	0.01	0.001	0.001	0.001	NS
		LANS x AMF x ACO	NS	0.001	0.01	0.001	0.01	0.001	NS

[y] Means followed by same letter in the same column are not significantly different (LSD, α=0.05); [z]Carrow et al. (2001); NS=Nonsignificant; NR=No reported, n=3.

For micronutrient concentrations, LANS had significant effects ($P \leq 0.001$) on Zn, Mn, Fe, B, and Al (Table 4.8). While ACO had significant ($P \leq 0.001$) effects on micronutrient concentration, no consistent effects were observed. The interaction LANS x ACO had significant ($P \leq 0.05$) effects in all micronutrients, at 2X LANS at 15,000 mg ACO kg^{-1} the concentration of Zn and Mn was significantly lower (Table 4.8). There were significant effects of AMF on Fe ($P \leq 0.001$), B ($P \leq 0.05$), and Al ($P \leq 0.001$). AMF at 15,000 mg ACO kg^{-1} enhanced the concentration of Fe and Al at 0.5X LANS, and B at 2X (Table 4.8).

Microbial Populations, Microbial Respiration, and Mycorrhizal Colonization

The main effects of LANS, ACO, and AMF significantly ($P \leq 0.01$) affected the total rhizosphere bacteria population analyzed as logarithmic units (Table 4.9; see Appendix I, Table AI-4.2 for data analyzed by the actual microbial counts). The total bacterial numbers increased significantly in the presence of 15,000 mg ACO kg^{-1} with the combination of 0.5X and 2X LANS. However, AMF plants had higher bacterial populations at 0.5X LANS, but lower at 2X (Table 4.9).

The population of bacteria able to grow on N-free medium (NFB) were significantly ($P \leq 0.01$) affected by LANS, ACO, and the interactions of LANS x AMF, LANS x ACO, and LANS x AMF x ACO (Table 4.9). Higher concentration of ACO tended to decrease NFB; and the combination of LANS and ACO concentrations stimulated or diminished the colony forming units of NFB (Table 4.9). Neither AMF or AMF x ACO interaction effects were statistically significant (Table 4.9). Non-AMF

plants with the combination of 0.5 X LANS and either 0 or 15000 mg ACO kg^{-1} had a significantly (LSD, α=0.05) higher population of NFB than AMF plants. In contrast, AMF plants at 1 X LANS with the combination of either 0 or 15000 mg ACO kg^{-1} had significantly ($P \leq 0.01$) greater NFB population than Non-AMF plants (Table 4.9).

Filamentous fungi were significantly affected ($P \leq 0.01$) by LANS, ACO, and the interactions of LANS x ACO, and LANS x AMF x ACO (Table 4.9). ACO stimulated the fungal population, particularly when applied at 15000 $mg \cdot kg^{-1}$ in combination with 0.5 and 1X LANS (Table 4.9). Non-AMF plants with the combination of 0.5 X LANS and 15000 mg ACO kg^{-1} had a significantly ($P \leq 0.01$) greater fungal population than AMF-plants (Table 4.9).

CO_2-evolution from the activity of rhizosphere microorganisms (microbial respiration) was significantly affected by LANS ($P \leq 0.01$), and ACO, LANS x ACO and LANS x AMF x ACO ($P \leq 0.001$). Increasing LANS and ACO concentration resulted in higher microbial respiration (Fig. 4.6C). AMF had no significant effects on microbial respiration at any level of ACO or LANS.

Total mycorrhizal colonization and arbuscule formation were significantly affected ($P \leq 0.01$) by ACO and LANS. ACO at 15000 $mg \cdot kg^{-1}$ generally resulted in greater total colonization and arbuscule formation at 1X and 2X LANS (Figure 4.7). The lowest values of total colonization and arbuscule formation were observed at plants with 0.5X LANS (Fig. 4.7). No significant effects of LANS, ACO and their interaction were observed for vesicle formation. No mycorrhizal fungal structures were found in root cortical cells of Non-AMF plants.

Table 4.9. Colony forming units ($Log_{10}CFU$) of rhizosphere microorganisms of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF)-*Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days.

ACO ($mg \cdot kg^{-1}$)	LANS Strength (X)	Mycorrhiza	Total bacteria ($Log_{10}CFU$)	Bacteria growing at N-free medium ($Log_{10}CFU$)	Filamentous fungi ($Log_{10}CFU$)
0	0.5	No	7.30 def [y]	6.92 bc	3.68 def
		Yes	7.30 def	6.78 de	3.48 fgh
	1.0	No	7.20 efg	6.86 cd	3.76 bcde
		Yes	7.37 de	7.00 ba	3.60 efg
	2.0	No	7.42 de	7.04 a	3.88 abcd
		Yes	7.27 def	7.10 a	3.70 cdef
3,000	0.5	No	7.42 de	6.56 gh	3.50 fgh
		Yes	7.52 cd	6.60 gh	3.34 hi
	1.0	No	7.00 ghi	6.54 gh	3.27 hi
		Yes	6.77 i	6.46 h	3.37 ghi
	2.0	No	7.02 gh	6.48 h	3.27 hi
		Yes	7.10 fgh	6.72 e	3.50 fgh
15,000	0	No	8.06 b	7.02 ab	3.94 abc
		Yes	8.12 b	6.74 e	3.78 bcde
	1.0	No	6.20 j	6.20 i	3.98 ab
		Yes	6.92 hi	6.70 ef	4.04 a
	2.0	No	8.57 a	6.80 de	3.20 i
		Yes	7.70 c	6.78 de	3.40 ghi
	Significance:				
		LANS	0.001	0.001	0.01
		AMF	0.01	0.01	NS
		ACO	0.001	0.001	0.001
		LANS x AMF	0.001	0.001	0.05
		LANS x ACO	0.001	0.001	0.001
		AMF x ACO	NS	NS	0.05
		LANS x AMF x ACO	0.001	0.001	NS

[y]Means followed by same letter in the same column are not significantly different (LSD, α=0.05); NS=Nonsignificant, n=5. (see Appendix I, Table AI-4.2 for data analyzed by the actual microbial counts).

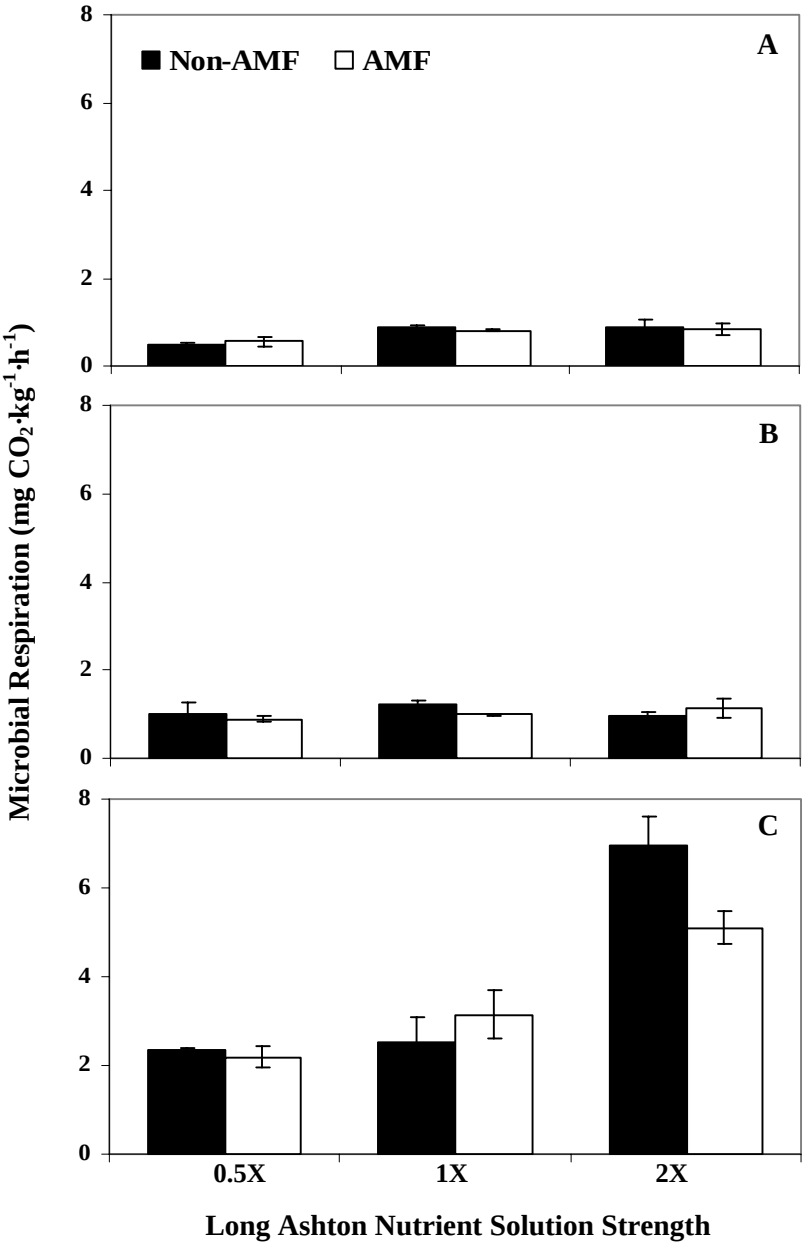

Fig. 4.6. Microbial respiration in the rhizosphere soil of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) 0 mg·kg^{-1}, B) 3,000 mg·kg^{-1}, C) 15,000 mg·kg^{-1}. Main effects of LANS were significant at $P \leq 0.01$; main effects of ACO, and the interactions LANS x ACO and LANS x AMF x ACO were significant at $P \leq 0.001$; main effects of AMF, and the interactions LANS x AMF, and AMF x ACO were nonsignificant. Bars ± SE, n=3.

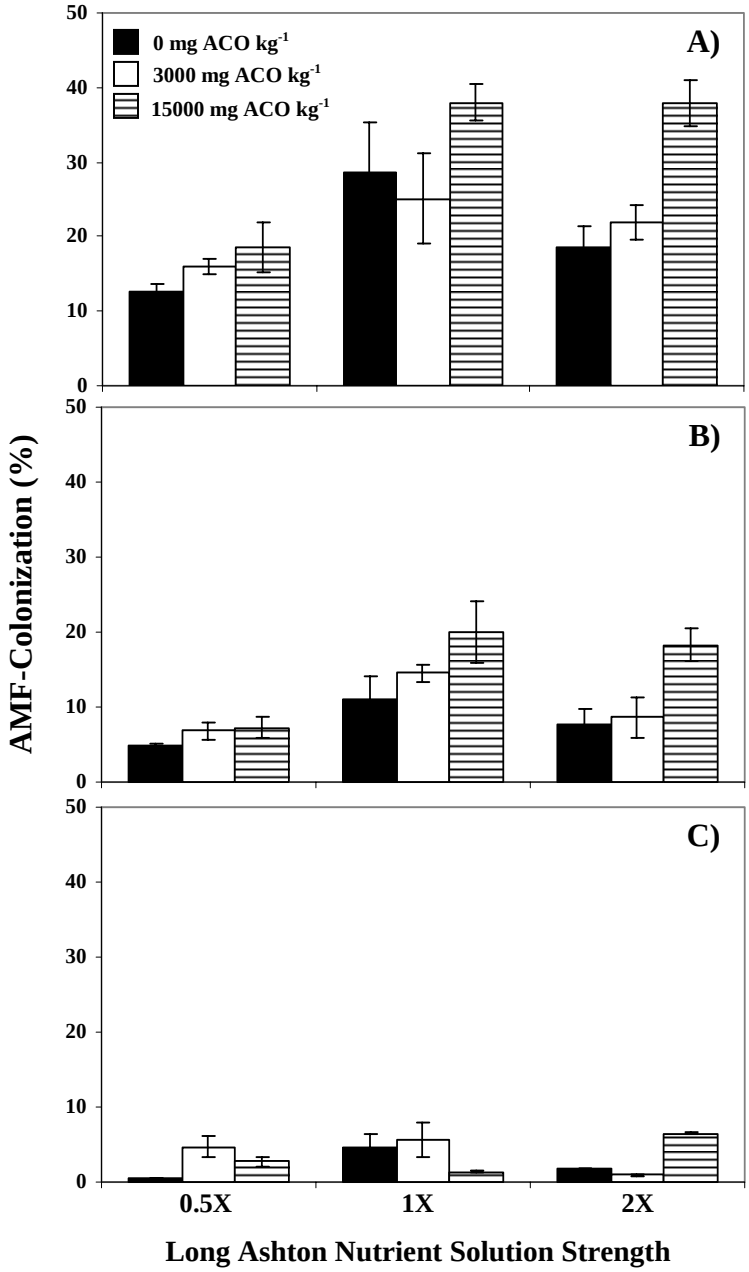

Fig. 4.7. Root mycorrhizal colonization in *Lolium multiflorum* after 80 days, treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A) total colonization, B) arbuscules and C) vesicles. For total colonization and arbuscules, main effects of LANS and ACO were significant at $P \leq 0.01$ and $P \leq 0.001$, respectively, while effects of LANS x ACO interaction were nonsignificant; for vesicles, effects of LANS x ACO interaction were significant at $P \leq 0.05$, while main effects of LANS and ACO were nonsignificant. Bars ± SE; n=3.

Total Petroleum Hydrocarbon Degradation

Degradation of TPH was significantly affected ($P \leq 0.01$) by ACO, LANS, and the interactions of LANS x ACO and LANS x AMF x ACO (Fig. 4.8). The interaction of LANS x AMF and AMF x ACO were also significant ($P \leq 0.05$), but the main effect of AMF did not significantly affect TPH-degradation. Greater percentages of TPH-degradation occurred at 3000 than 15000 mg ACO kg^{-1}, however, the former also had initial 5-fold higher ACO levels (Fig. 4.8). At 0.5X and 1X LANS, TPH-degradation was generally higher for Non-AMF plants at 3,000 and 15,000 mg ACO kg^{-1}. However, fertilization with 1X or 2X LANS did not enhance the degradation of ACO at 3000 $mg \cdot kg^{-1}$ (Fig. 4.8A). AMF-plants at 3,000 mg ACO kg^{-1} and 1X LANS had lower (60.3%) TPH-degradation than Non-AMF (84.6%) plants (Fig. 4.8A). Lower TPH-degradation occurred at 15000 than 3000 mg ACO kg^{-1}. The lowest TPH-degradation occurred with 1.0X LANS at 15000 mg ACO kg^{-1} (Fig. 4.8B). At 15000 mg ACO kg^{-1}, no treatment had greater TPH-degradation than 0.5X LANS, regardless of AMF inoculation (Fig. 4.8B).

Rhizosphere Soil pH and Electrical Conductivity Changes

Rhizosphere soil pH was significantly ($P \leq 0.001$) affected by the application of LANS and ACO while AMF had no significant effects (Fig. 4.9A,B,C). Significant interactions occurred with LANS x AMF ($P \leq 0.05$), LANS x ACO and LANS x AMF x ACO ($P \leq 0.001$).

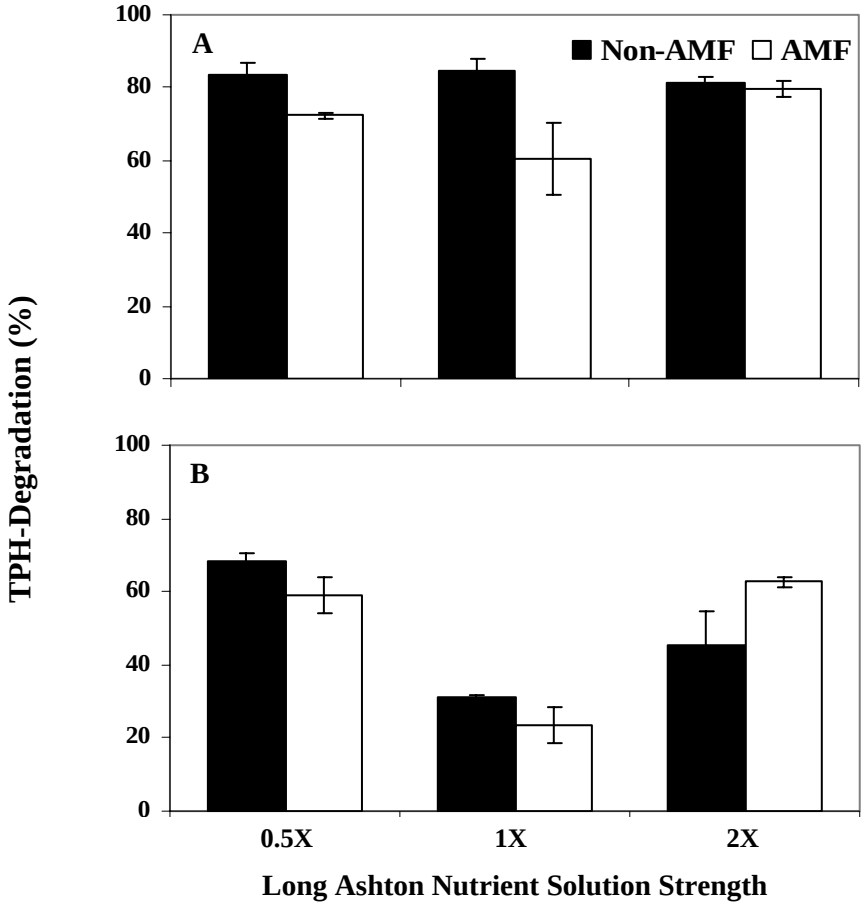

Fig. 4.8. Total petroleum hydrocarbons (TPH)-degradation in rhizosphere soil of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and two concentrations of Arabian medium crude oil (ACO). A) 3,000 $mg \cdot kg^{-1}$, B) 15,000 $mg \cdot kg^{-1}$. Main effects of LANS, ACO, and the interactions LANS x ACO and LANS x AMF x ACO were significant at $P \leq 0.01$. Effects of LANS x AMF, and AMF x ACO interactions were significant at $P \leq 0.05$. Main effects of AMF were nonsignificant. Bars ± SE; n=3.

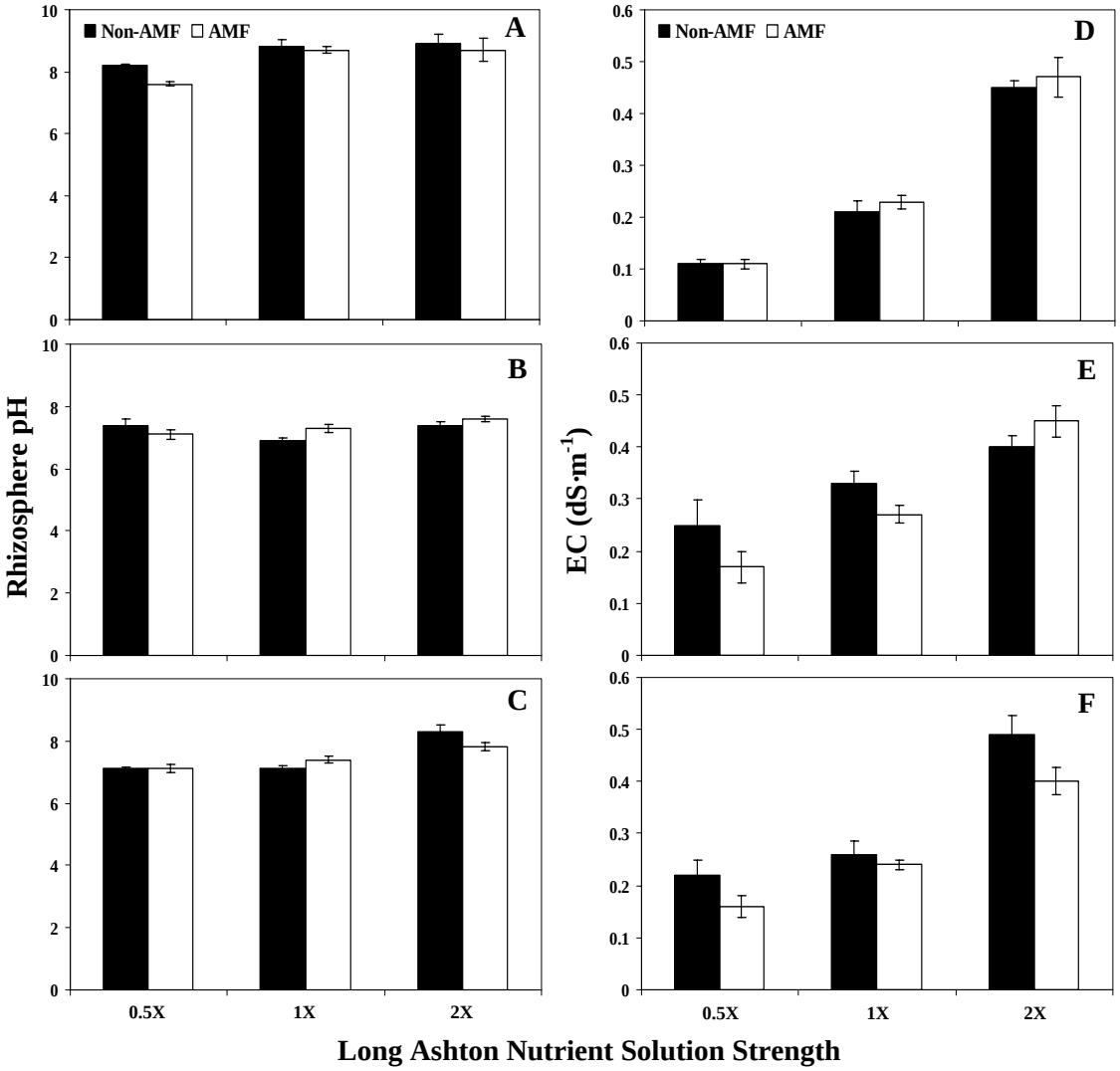

Fig. 4.9. Changes in the pH (A, B and C) and electrical conductivity (D, E, and F) of the rhizosphere soil of *Lolium multiflorum* after 80 days, inoculated with *Glomus intraradices* (AMF) or without (Non-AMF), treated with three levels of Long Ashton Nutrient Solution (LANS) and three concentrations of Arabian medium crude oil (ACO). A and D) 0 mg·kg^{-1}, B and E) 3,000 mg·kg^{-1}, C and F) 15,000 mg·kg^{-1}; For pH, main effects of LANS, ACO, and the interactions LANS x ACO and LANS x AMF x ACO were significant at $P \leq 0.001$. Main effects of AMF, LANS x AMF, and AMF x ACO were nonsignificant. For EC, main effects of LANS and the interaction LANS x AMF x ACO were significant at $P \leq 0.001$. Main effects of AMF, ACO, and the interactions LANS x AMF, LANS x ACO and AMF x ACO were nonsignificant. Bars ± SE, n=3.

In general, rhizosphere soil pH varied across 2 units (from 6.9 to 8.9). Increasing LANS at 0 mg ACO kg^{-1} increased (>8.5) pH (Fig. 4.9A); while increasing ACO resulted in a pH decreasing (~7.5); however, at 15,000 mg ACO kg^{-1} with 2X LANS had greater pH (~8.0) than with the application 0.5X and 1X LANS (Fig. 4.9C).

Rhizosphere soil electrical conductivity (EC) was significantly ($P \leq 0.001$) affected by LANS. ACO, AMF, and interactions LANS x AMF, LANS x ACO, and AMF x ACO were not significant (Fig. 4.9 D,E,F). Increasing LANS resulted in higher EC (Fig. 4.9D-F). The interaction LANS x AMF xACO also resulted in significant ($P \leq 0.01$) effects on EC. In general, the EC-values ranged from 0.11 to 0.49 $dS \cdot m^{-1}$ (Fig. 4.9 D and F).

Discussion

Plant Growth Responses

Growth of *L. multiflorum* was impaired by ACO in the soil as has been observed with other plant species (Adam and Duncan, 2003; Malallah et al., 1996; Quiñones-Aguilar et al., 2003). The negative impact of ACO on plant biomass, pseudostem number and leaf area were partially compensated by higher levels of LANS. SLA, LAR, and RSR can be useful to understand plant growth performance under stressful cultural conditions (Reich et al., 1997; Westoby, 1998; Wright and Westoby, 2001). Higher SLA (thinner leaves) values were found in plants grown in soil contaminated with 3000 mg ACO kg^{-1}, regardless of LANS concentration (Table 4.2). In contrast, lower SLA (thicker leaves) was obtained with plants exposed at 15000 mg ACO kg^{-1} at 2X LANS (Table 4.2). While growth was depressed by 3000 mg ACO kg^{-1}, plants were less stressed with greater growth than those exposed to 15000 mg ACO kg^{-1}. The application of 1X and 2X LANS to plants exposed to 3000 mg kg^{-1} enhanced growth and development, except root and

total plant DW. Thus, 0.5X LANS was nutritionally limiting growth of plants at 3000 mg ACO kg^{-1}.

A similar tendency was observed for LAR which is an indication of the efficiency of a given leaf area to overall plant biomass (Hunt, 1982; Lafarge and Hammer, 2002). Plants at 3000 mg ACO kg^{-1} had a higher LAR than those at 0 mg ACO kg^{-1}, regardless of LANS or AMF treatment (Table 4.2). These results suggest that plants under ACO-stress require a greater leaf area to produce higher biomass, enhancing survivability and adaptation to contaminated soil. However, at the more toxic levels of ACO (15,000 mg ACO kg^{-1}), LAR was greater at 0.5X and 1X LANS (but not 2X LANS) compared to plants without ACO. Stressfull environmental conditions typically result in significant reduction of leaf area expansion (i.e. leaf area was reduced) (Bayuelo-Jimenez et al., 2003; Bruggink and Heuvelink, 1987; Estrada-Luna and Davies, 2003; Liu and Stützel, 2004), which may in part explain the negative effects of ACO on inhibiting growth of *L. multiflorum*. The RSR was also reduced under increasing ACO levels, indicating reduced carbon partitioning to the root system.

AMF can enhance plant growth under biotic and abiotic stress (Smith and Read, 1997). The effect of AMF on plant growth under PH-contaminated soil has received little attention (Binet et al., 2000a; Cabello, 1999; Joner and Leyval, 2001; Leyval and Binet, 1998). However, in this study, AMF did not significantly enhance plant growth at any level of ACO. This null effect of AMF on overcoming the abiotic stress of ACO may in part be attributed to the characteristic low dependency of monocots including grass species to AMF-symbiosis (Smith and Read, 1997), or to the utilization of an ineffective AMF strain (Cabello, 2001). The nonsignificant reduction in total plant DW (-80%) of

AMF-plants at 15000 mg ACO kg^{-1} at 2X LANS may in part be explained to increased carbon drain from the plant to the AMF (Smith and Read, 1997; Sylvia, 2005). Oil spills inhibit photosynthesis, which alters carbon translocation and repartitioning towards plant sink regions, i.e., new shoot and root growth (Daly et al., 1988), and AMF-symbiosis (Smith and Read, 1997). Growth suppression in AMF-plants may occurr when photosynthesis is limited, or due to incompatibilities between AMF and plant (Hart et al., 2003; Klironomos, 2003; Sylvia, 2005).

In the current experiment, even though plants were supplied with different levels of LANS, the P-level in nutrient solution was the same for all treatments (30 $\mu g \cdot mL^{-1}$). The rationale of this was to avoid P-defficiency in Non-AMF and to have comparable growth between AMF and Non-AMF plants. AMF-species may differ in their ability to tolerate and enhance plant growth under PH-contaminated soil; hence, AMF isolated from contaminated soils may be more effective in improving plant growth than introduced AMF species (Cabello, 1999; Cabello, 2001). Although *Glomus intraradices* has been reported as an effective fungal isolate for plant growth under petroleum contaminated soil (Cabello, 1999), the fungal strain utilized in this study was from a commercial pure liquid inoculum originally isolated from a non PH-contaminated site in Canada. While the *G. intraradices* isolate was able to colonize the root system, its effects on plant growth under the tested ACO conditions were limited.

Plant Nutritional Responses

Macro- and microelement content and concentration were enhanced with increasing LANS concentration. Greater total nutrient content and concentration was

generally observed in plants growing in non-contaminated soil with 2X LANS. Increasing ACO concentrations reduced macro- and microelement content in plants, but that reduction was significantly alleviated by biostimulation with LANS (Tables 4.5, 4.6). The main effect of AMF was not significant, except for Ca, Zn, and Fe ($P \leq 0.05$); however, there was observed a trend of generally higher content of macro- and microelement content in AMF than non-AMF plants at 1X and 2X LANS (Tables 4.5, 4.6). The highest contents of N, P, K, Mg, Ca, S, Zn, Mn, Cu, and B were in AMF-plants at 2X LANS at 0 mg ACO kg^{-1}, which were significantly different (LSD, $\alpha=0.05$) than those at Non-AMF plants with the same combination of LANS and ACO (Tables 4.5, 4.6). At 3000 mg ACO kg^{-1}, there was a nonsignificant trend of greater total content of macro- and microelements with AMF than Non-AMF plants, particularly at 0.5X and 2X LANS. Beneficial effects of AMF on plant nutrition has been well documented in the literature (Amaya-Carpio et al., 2005; Smith and Read, 1997; Sylvia, 2005). However, in this study, the benefits of AMF on plant nutrient uptake were significantly diminished, particularly at high levels of ACO. For instance, at 15000 mg ACO kg^{-1}, nutrient uptake was drastically reduced for AMF- and Non-AMF plants (Tables 4.5, 4.6).

LANS and ACO had significant effects on macro- and microelement concentration, while the main effect of AMF was significant for only 50% of elemental concentrations (Tables 4.7, 4.8). Regardless of ACO, LANS or AMF, plants were sufficient in K, Mg, Ca, S, Na, Zn, Mn, Cu, Fe, B, and Mo leaf tissue concentrations (Carrow et al., 2001). All treatments were deficient in leaf N concentration, which may be in part due to the high N-requirement for this grass species (120 to 300 kg N ha^{-1}). However, *Lolium* species are characterized by their high degree of adaptation to N

availability (Gilsum et al., 2003). A decline in N concentration in leaf tissue of *Lolium* genotypes during N deficiency may be related to greater efficiency of N utilization by which those plants can sustain biomass production and growth, from the utilization of amino acids derived from protein turnover, and from stored NO_3^- (Maduff et al., 1989; Marino et al., 2004).

In this study, plants were differentially fed with 30 μg P mL^{-1} at all LANS levels to minimize greater P-efficiency of AMF, so that comparable P concentration and subsequent equal plant size would be maintained between AMF and Non-AMF plants. At 0 and 15000 mg ACO kg^{-1} at 1X and 2X, plants were deficient in P, whereas P sufficiency occurred at all treatments at 3000 and 15000 mg ACO kg^{-1} with 0.5X LANS. Growth was greater at 0 mg ACO kg^{-1}, which may have depleted tissue P, whereas higher LANS (1X and 2X) under the more toxic levels of 15,000 mg ACO kg^{-1} led to deficiency. Sufficiency levels of Na and Al have not been reported for *L. multiflorum*. For graminaceous plants, Al should be less than 200 $\mu g \cdot g^{-1}$, hence, the concentration of Al in *L. multiflorum* was well below toxicity level (Jones, 1998).

There are no previous reports on the effects of PH on nutrient uptake of plants utilized during phytoremediation. Thus, this study is one of the first reports describing the effects of ACO and biostimulation on the nutritional status of *L. multiflorum* during phytoremediation.

Selected Physiological Responses

This is one of the first studies to report the physiological responses of AMF plants in ACO-contaminated soil, in combination with the biostimulation of inorganic

fertilization (LANS) to enhance TPH degradation/dissipation. Little has been reported on the physiological responses of plants to PH in soils (Malallah et al., 1996).

Regardless of ACO concentrations, chlorophyll (total, *a*, and *b*) content in leaves was similar in all treatments (Table 4.3). The highest content of total and *a* chlorophyll was at Non-AMF with 15000 mg ACO kg^{-1} and 0.5X LANS; while for chlorophyll *b*, the lowest content was at Non-AMF plants with 15000 mg ACO kg^{-1} and 2X LANS.

Photosynthesis of *L. multiflorum* plants was significantly impaired at 15000 mg ACO kg^{-1}, and consequently plant growth was also affected. There was a nonsignificant trend of reduced stomatal conductance in plants exposed to ACO, particularly at 15000 $mg \cdot kg^{-1}$. Consequently, the *WUE* of these plants was nonsignificantly higher than that from plants at 0 mg ACO kg^{-1}. The negative effects of ACO on chlorophyll and photosynthesis are in aggrement with those previously reported for terrestrial plants (Baker, 1970; Daly et al., 1988; Ilangovan and Vivekanandan, 1992; Malallah et al., 1996) and seagrasses (Durako et al., 1993; Macinnis-Ng and Ralph, 2003; Ralph and Burchett, 1998).

Some legume species growing in soil contaminated with PH have higher phenolics when compared to plants growing in non-contaminated soil (Ilangovan and Vivenkanandan, 1992; Malallah et al., 1996). In contrast, *L. multiflorum* had variable phenolic content, which was dependent on LANS and ACO concentrations. There was a nonsignificant trend of AMF-plants generally having lower phenolics when fertilized with 0.5X and 1X LANS in combination with 0, 3000, or 15000 mg ACO kg^{-1}, which may indicate that AMF-plants were less stressed by the presence of ACO (Fig. 4.3). Total phenolics and antioxidants (Fig. 4.4) decreased with increasing LANS concentration at 0

and 3000 mg ACO kg^{-1}. In contrast, when plants were exposed to 15000 mg ACO kg^{-1} there was no consistent trend of LANS or AMF on phenolics and antioxidants. ACO affected leaf ascorbate content (Fig. 4.5). In general, increased ACO concentrations resulted in nonsignificant lower ascorbate content in plants, except for the combination of 2X LANS with 15000 $mg \cdot kg^{-1}$ in which ascorbate content increased, indicating higher stress for plants, as reported with plants under abiotic and biotic stress (Sharma and Dubey, 2005; Song et al., 2005; Wolucka et al., 2005).

Plant response to stressful environmental conditions may be evaluated by the synthesis of specific compounds such as phenolics and ascorbate content that confer higher detoxification of reactive oxygen species (ROS) in the internal tissues (Mittler, 2002; Singer et al., 2003; Noctor, 2006). Phenolic compounds play an important ecological role. Their synthesis and storage are considered good indicators of biotic and abiotic stress to plants. In general, phenolics and ascorbate, reflect the antioxidant activity in stressed plants, and may contribute to protecting cells from oxidative damage of free radicals by reducing their toxicity on cytoplasmic structures (Ferrat et al., 2003; Misra and Gupta, 2006; Mittler, 2002; Wahid and Ghazanfar, 2006). Phenolics and antioxidant activity were particularly higher in plants with 0.5X than plants with 2X LANS. This observation is in agreement with reports describing the increase of phenolics at low fertility, providing greater host plant resistance to insect herbivory and pathogen interactions (Dudt and Shure, 1994; Hakulinen, 1988; Witzell and Shevtsova, 2004).

The contribution of AMF to phenolic compounds, antioxidant activity, and ascorbate content in plants exposed to ACO-contaminated soil had not been previously reported. However, studies under drought stress postulated that AMF protect plants

against oxidative damage by increasing levels of enzymatic or non-enzymatic antioxidants (Alguacil et al., 2003; Porcel et al., 2003; Ruiz-Lozano, 2003; Wu et al., 2006). Perhaps with the inoculation of AMF (single or multiple isolates) more tolerant to ACO, there may have been more significant responses.

Microbial Populations, Microbial Respiration, and Mycorrhizal Colonization

Microbial populations were significantly affected by the application of ACO and LANS. Total bacteria were stimulated by 15000 mg ACO kg^{-1}, when fertilized with 0.5X and 1.0X LANS. Population of bacteria able to grow on N-free medium generally decreased as ACO levels increased, particularly at 1X and 2X LANS. Conversely, filamentous fungi were generally higher at 15000 mg ACO kg^{-1} at 0.5X and 1X LANS. Microbial respiration was greater in the rhizosphere soil at 15000 mg ACO kg^{-1}, and significantly higher with 2X LANS. AMF-colonization was detected in all plants inoculated with *Glomus intraradices*, and higher total colonization and arbuscule formation was found at plants exposed to 15000 mg ACO kg^{-1} with 1X or 2X LANS.

Plant adaptation to PH-contaminated environments is a result of microbial interactions in the rhizosphere that facilitate mineralization of organic contaminants by cooxidation and cometabolism processes (Barea et al., 2005; Dec et al., 2002; Jeffries et al., 2003; Rillig and Steinberg, 2002; Robson et al., 2004; Siciliano and Germida, 1998). The dynamic, diversity and significance of microbial populations in the rhizosphere are critical in phytoremediation, but they are not well understood. Microbial interactions are dependent on soil properties, type and concentration of contaminants, nutrient availability, plant genotype, and root exudation pattern. The carbon partitioning, below-

ground translocation, and transfer of soluble and insolube material from roots to soil (rhizodeposition) also stimulate specific microbial populations involved in degradation of PH in soils (Bundy et al., 2002; Cohen et al., 2004; Kim et al., 2005; Kirk et al., 2005; Kuzyakov et al., 2001; Onwurah, 1999; Siciliano et al., 2001; Wyszkowska and Kucharski, 2001). Rhizosphere microorganisms use substances from the roots for their nutrition and degrade organic materials into inorganic forms e.g., mineralization of carbon and nitrogen. The metabolic products of microbial organisms can subsequently influence plant growth (Hodge et al., 2000).

Total Petroleum Hydrocarbon Degradation

Degradation of PH is a result of combined factors such as soil type, microbial activity in the soil/rhizosphere, type, bioavailability, and concentration of the contaminant, and available nutrients for plants and microorganisms (Anderson et al., 2002; Dec et al., 2002; Kuzyakov et al., 2001; Newman and Reynolds, 2004). Phytoremediation performance was higher at low ACO concentration, and the application of 0.5X LANS showed similar TPH-degradation than that obtained with either 1X or 2X. In general, there was no consistent AMF treatment response. TPH-degradation of AMF was lower at 1X LANS at 3000 mg ACO kg^{-1}, and higher at 2X LANS at 15,000 mg ACO kg^{-1}. AMF may alleviate the toxic effects produced by PH (Cabello, 1999; Joner and Leyval, 2003a; Leyval and Binet, 1998). However, mechanisms of AMF benefit on phytoremediation of organic contaminants in soils is not well documented. AMF enhancement in plants during phytoremediation may be related to release of oxidative enzymes by roots, improved plant nutrition and growth, creation of favorable

microenvironments in the mycorrhizospehere, including changes on microbial populations that may degrade organic contaminants (Criquet et al., 2000; Joner and Leyval, 2003a; Rillig, 2004; Sylvia, 2005).

With 15000 mg ACO kg^{-1}, 0.5X LANS had significantly higher TPH-degradation (~60%) than 1X LANS (~25%). The application of 0.5X seemed to stimulate microbial population and root activity, which may have contributed on higher TPH-degradation, as previously mentioned (Günther et al., 1996). In contrast, TPH-degradation significantly decreased with the application of 1X LANS. This effect is contrary to that reported by Hutchinson et al. (2001) who obtained higher degradation of aged petroleum sludge via biostimulation with higher rates of fertility. This effect may be in part explained by the expression of nutrient immobilization and competition, between microorganisms and plants. In this study, the results denote the complex interaction among PH, plant, rhizosphere microbial activity, AMF inoculation, and inorganic fertilization. The ultimate benefit of plants during phytoremediation is evaluated by their contribution on the dissipation/degradation of petroleum hydrocarbons (Banks et al., 2003). In general, the extent of TPH-degradation was dependent on ACO-concentration in the soil, as well as bioremediation with LANS. However, further research is needed to understand the interaction of AMF and hydrocarbonoclastic microorganisms in PH-contaminated soil.

Rhizosphere Soil pH and Electrical Conductivity Changes

Soil pH is a chemical variable that significantly influences physical, chemical and biological soil properties. In addition, soil pH is a critical factor that not only affects the mobility of organic contaminants, but also influences their biochemical breakdown,

solubility, adsorption to colloids, and transport and distribution along soil profile (Brady and Weil, 2002; Pepper, 1996). In this study, rhizosphere soil pH showed variations among treatments of 2.0 units, ranging from 6.9 to 8.9, depending on the interaction of all the tested factors (LANS, AMF, or ACO). These variations resulted in increased alkalinity (>8.0 pH) for some treatments as a direct effect of the application of 1Xor 2X LANS and its combination with 0 or 15000 mg ACO kg^{-1}. Although AMF-inoculation did not have significant effects on the rhizosphere soil pH, AMF-symbiosis can change the mycorrhizosphere pH, depending on the N-form ($N\text{-}NO_3^-$ or $N\text{-}NH_4^+$) (Bago et al., 1996; Ortas et al., 2004). Furthermore, AMF-response to soil pH is differentially affected, thus, some species are more tolerant to acidic or alkaline soil conditions in soil (Mohammad et al., 2005; Rohyadi et al., 2004; van Aarle et al., 2002).

Salinity build-up was observed as a consequence of the application of LANS to the soil. Salinity may stress susceptible plants and impair their physiology and growth (Taiz and Zeiger, 2002). However, salt accumulation was minimal (low EC) and not a factor with growth of *L. multiflorum*. Hence, the main abiotic factor that limited physiological responses and plant growth was ACO. Conversely, LANS application minimized the adverse effects of ACO to plants, particularly at 3000 $mg \cdot kg^{-1}$.

Summary

While arbuscular mycorrhizal fungi (AMF) are an important component in the phytoremediation of petroleum hydrocarbons (PH), the physiological responses and growth of AMF-plants during phytoremediation of PH, in combination with the biostimulation through inorganic fertilization, are not well understood. The present study

evaluated the effects of AMF and biostimulation with fertilization on selected physiological responses and growth of *Lolium multiflorum* in the phytoremediation of soil contaminated with Arabian medium crude oil (ACO). A 2x3x3 factorial experiment was conducted in glasshouse conditions for 80 days with *L. multiflorum* seedlings inoculated (AMF) or not (Non-AMF) with *Glomus intraradices*, established in a sand:sandy loam soil mixture (1:1 v/v), contaminated with three levels of ACO (at 0, 3000, or 15000 $mg \cdot kg^{-1}$), and fertilized with three levels of Long Ashton Nutrient Solution [(LANS) at 0.5X, 1X, or 2X].

Plant growth, photosynthesis, and nutrient content in shoots were significantly reduced by increased ACO. Growth, photosynthesis and nutrient uptake of plants in ACO-contaminated soil were enhanced by LANS. Microbial populations and soil respiration were stimulated by ACO and LANS. Regardless of ACO concentration, total phenolics and antioxidant activity in leaves decreased with increasing LANS. Increasing ACO concentrations resulted in decreased ascorbate content at 0.5X and 1X LANS, but increased at 2X LANS.

AMF had minimal effects on plant growth, photosynthesis, and nutrient content, depending on ACO and LANS combinations. Mycorrhizal colonization and arbuscule formation was observed at all ACO levels. At 3000 mg ACO kg^{-1}, total petroleum hydrocarbon (TPH)-degradation was >60% in average in all treatments, while at 15000 mg ACO kg^{-1} the higher TPH-degradation occurred at 0.5X LANS. Neither LANS nor AMF consistently increased TPH-degradation. LANS and ACO significantly affected soil pH, which ranged from 6.9 to 8.9. While LANS significantly enhanced electrical conductivity (EC) in soil, no toxic levels of salt build-up occurred.

CHAPTER V

BIOREMEDIATION AND PHYTOREMEDIATION OF BENZO[*a*]PYRENE CONTAMINATED SAND

Introduction

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous and can have negative impacts on human health as carcinogenic compounds (Cunningham et al., 1997). PAH are organic compounds of low or high-molecular-weight, such as flouranthene, pyrene, benzo[*a*]antrhacene, chrysene, benzo[*a*]pyrene and dibenzo[*a*,*h*]anthracene (Kanaly and Harayama, 2000). The mechanisms of PAH formation is complex, but they originate from incomplete combustion of fossil fuels, organic material, and wood, and typically have 2-6 aromatic rings and less alkylated substitution (Hwang et al., 2003; Masclet et al., 1987; NCR, 1983). The U.S. Environmental Protection Agency (EPA) is developing strategies for controlling source, persistence and toxicity effects of PAH benzo[*a*]pyrene (BaP) (Renner, 1999, Kanaly and Harayama, 2000). BaP in soil ranges between 26 and 12,600 $\mu g \cdot kg^{-1}$ (Coleman and Mauro, 2002) and this compound is found as a part of a complex mixture of PAH in industrially contaminated soils, ranging from 3.1 to 8.1 $g \cdot kg^{-1}$ (Joner et al., 2002; Leyval and Binet, 1998).

Bioremediation with free-living microorganisms is utilized to detoxify PAH-contaminated soils. Bacteria, such as *Sphingomonas* spp. or *Mycobacterium* spp., with hydrophobic cell surfaces have a high mineralization rate of flouranthene, as well as oxidizing other PAH (Cerniglia, 1992; Juhasz and Naidu, 2000; Willumsen and Karlson,

2001). In particular, the bacterial genus *Sphingomonas* is effective in oxidizing two- or three-ring PAH, thus contributing to their initial degradation (Dutta et al., 1998; Kästner et al., 1998; van Hamme et al., 2003).

On the other hand, filamentous fungi have physiological mechanisms that allow the mineralization of PAH and are important component in bioremediation of persistant organic compounds in soil. Fungal species such as *Phanerochaete chysosporium*, *Candida utilis*, *Penicillium chrysogenum*, *Aspergillus niger*, *Cunninghamella elegans*, and *Cunninghamella echinulata* are characterized as PAH-oxidizers (Cerniglia, 1992). *Cunninghamella* is one of the most studied fungal genera for degradation of PAH in liquid media. Some species of this genus, such as *C. elegans* or *C. echinulata* var. *elegans* have a particular enzymatic system (cytochrome P450 oxidoreductase), which is involved in the metabolism of PAH and aliphatic contaminants (Cutright, 1995; Garon et al., 2000; Yadav and Loper, 2000). The effect of sphingomonads bacteria and *Cunninghamella* fungal strains (bioremediation) has not been tested during phytoremediation (with plants) of PAH-contaminated soils.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous rhizosphere microorganisms that form a mutually beneficial symbiosis with the root system of approximately 80% of all terrestrial plants (Smith and Read, 1997). The symbiosis between AMF and plants can be an important component during phytoremediation of soils contaminated with both inorganic and organic compounds (Cabello, 2001; Meharg, 2001; Joner and Leyval, 2003b). Phytoremediation of petroleum hydrocarbons (PH) in soil can be successful (Günther et al., 1996; Newman and Reynolds, 2004), but the contribution of AMF during phytoremediation of PAH is not well documented (Joner and Leyval, 2003b).

Direct benefits of AMF in phytoremediation of organic contaminants in soils are related to enhanced plant adaptation and tolerance as well as improved nutritional and physiological status. Indirect benefits of AMF are associated with the modification of microbial groups in the mycorrhizosphere, including the proliferation of PH-degrading (hydrocarbonoclastic) microorganisms via hyphae exudation (Joner and Leyval, 2003a). Nevertheless, few studies have been conducted to understand the role of AMF on plant physiological responses, rhizosphere microbial interactions, and phytoremediation of PAH. In addition, the interaction of AMF and hydrocarbonoclastic microorganisms (bacteria and filamentous fungi) is not well understood. Gaspar et al. (2002) reported that *Zea mays* inoculated with the AMF *Glomus geosporum* and the yeast, *Rhodotorula glutinis*, had reduced accumulation of the PAH, phenanthrene, in roots.

The present study tested the following hypotheses: 1) inoculation with hydrocarbonoclastic, free-living microorganisms does not impede the establishment of AMF, 2) gas exchange and selected physiological responses of *Lolium multiflorum* plants are enhanced by bioaugmentation of AMF (*Glomus intraradices* [AMF]), bacteria (*Sphingomonas paucimobilis* [Sp]), and filamentous fungus (*Cunninghamella echinulata* var. *elegans* [Ce]), 3) dissipation/degradation of the PAH benzo[*a*]pyrene (BaP), from the rhizosphere of *L. multiflorum* is enhanced by bioaugmentation of AMF, Sp, and Ce, and 4) phytoremediation (with *L. multiflorum* inoculated with Sp and Ce) is more effective in the degradation of BaP than bioremediation with the two hydrocarbonoclastic microorganisms (Sp and Ce) inoculated separately.

The objectives of this research were to: 1) evaluate the interaction of BaP-degrading microorganisms (Sp and Ce) and the AMF, *G. intraradices* (AMF), inoculated

in the rhizosphere of *L. multiflorum* during phytoremediation of BaP, 2) evaluate the effect of Sp, Ce, and AMF, on gas exchange, proline content, phenolic content, antioxidant activity, and nitrate reductase activity of *L. multiflorum*, 3) determine if phytoremediation was enhanced by bioaugmentation with Sp, Ce, and AMF, on the dissipation/degradation of BaP from the rhizosphere of the *L. multiflorum* phytoremediation system, and 4) compare the phytoremediation of *L. multiflorum* and bioaugmentation of Sp, and Ce in the BaP-degradation with the bioremediation of the two hydrocarbonoclastic microorganisms applied separately.

Materials and Methods

The study was conducted under greenhouse conditions at Texas A&M University, College Station, TX for 60 days from 11 April to 9 June 2005. Temperature and relative humidity were monitored with a watch dog data logger Model 150 (Spectrum technologies, Inc., Planfield, Ill.), and photosynthetic photon flux density (PPFD) was determined with a LI-190SA Quantum/Radiometer/Photometer and sensor (LI-COR® Biosciences, Lincoln, Nebr.). Average max/min temperature and relative humidity were 34.6/18.9°C, and 71.7/78.4%, respectively, and average maximum PPFD determined at solar noon, was 701.5 $\mu moles \cdot m^{-1} \cdot s^{-2}$. Autoclaved sand was utilized as substrate, and treated with BaP (Sigma-Aldrich®) at 100 $mg \cdot kg^{-1}$ dissolved in 50 mL of dichloromethane solvent (Sigma®, <0.002% of residue after evaporation) to contaminate the sand as previously described.

Growth and Physiological Responses of *Lolium multiflorum* in the Phytoremediation of BaP – Effect of Rhizosphere Microorganisms

One week old-*Lolium multiflorum* Lam. cv. Passerel Plus seedlings were transplanted to BaP contaminated sand, and immediately inoculated with selected microorganisms. Bacterial cells on culture medium were washed with sterile water, and two milliliters of bacterial liquid inoculum ($4.0x10^8$ CFU mL^{-1}) [flouranthene-preadapted *S. paucimobilis* (Sp), strain EPA505; Mueller et al., 1990] were applied directly to the root system of the seedlings. The filamentous fungus *C. echinulata* var. *elegans* [(Ce), ATCC-36112] was cultured for five days at 26°C, in Petri dishes containing PDA medium. Spores were collected in a suspension with sterile water, and two milliliters ($1.2x10^4$ CFU mL^{-1}) were applied to the root system of the respective seedlings. For AMF treatments, seedlings were inoculated with 500 spores of *G. intraradices* (AMF) [Mycorise® ASP, PremierTech Biotechnologies, Quebec, Canada]. Non-inoculated seedlings were utilized as the control.

Plants were fertilized weekly with 30 mL of 1X strength Long Ashton Nutrient Solution (Hewitt, 1966; see Appendix I, Table AI-4.1) modified to supply 22 μg P mL^{-1} to maximize the AMF-colonization.

Plant growth responses were evaluated at the end of the experiment (60 days), including leaf area (cm^2), and dry weight (DW) of leaves, pseudostems, roots and total plants. Leaf area ratio (LAR=leaf area/total plant DW), specific leaf area (SLA=leaf area/leaf DW), and root to shoot ratio were also determined.

Net photosynthesis (*Pn*), stomatal conductance (g_s), and water use efficiency (*WUE*, *Pn*/*gs*) were evaluated at 30 and 60 days, with a portable photosynthesis system

model LI-6400 (LI-COR Inc., Lincoln, Nebr.) with red/blue LED light source (LI6400-02B) at photosyntheticaly active radiation (PAR) levels of 500 μmol $m^{-2} \cdot s^{-1}$, and CO_2 concentration of 360 μmol$\cdot s^{-1}$.

Leaf samples were taken at 30 and 60 days for proline, total phenolics and antioxidant activity. Proline content in leaves was determined as described by Bates et al. (1973) and Gzik (1996). Briefly, 0.100 g of leaf fresh tissue was macerated in a chilled mortar with 3 mL of 3% sulfosalicylic acid. After centrifugation at 10,000 rpm for 15 min, 200 μL aliquot of the extract was reacted with 200 μL of ninhydrin reagent and 200 μL of glacial acetic acid, and incubated at 100°C for one hour. Reaction mixture was stopped with an ice bath, and proline was immediately extracted with toluene. Absorbance readings were taken at 520 nm by means of a Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Proline concentration was determined from a standard curve of D,L-proline.

Total phenolics were evaluated by the Folin-Ciocalteu reagent assay utilizing chlorogenic acid as a standard curve (Singleton and Rossi, 1965; Soong and Barlow, 2004). In brief, 0.150 g of leaf fresh tissue was macerated in a chilled mortar with 3 mL of 80% methanol. Extracts were centrifuged for 15 min at 15,000 rpm. Reaction mixture consisted on mixing 30 μL of the extract added with 90 μL of Na_2CO_3 and 150 μL of Folin-Ciocalteau reagent in a 96-well microplate. After 30 min the absorbance was measured at 725 nm using a KC-4 spectrophotometer (Biotek® Instruments, Inc. Winooski, Vt.). Results were expressed as micrograms of chlorogenic acid equivalents per gram of fresh weight tissue.

Total antioxidant activity was determined by the 1,1-diphenyl-2-picryldrazyl (DPPH) radical decoloration assay (Matthäus, 2002; Re et al., 1999). Briefly, leaf extracts (0.150 g in 3 mL of 80 % methanol) were obtained and immediately centrifuged at 15,000 rpm for 15 min. The reaction mixture consisted of mixing 75 µL of the extract added with 250 µL of DPPH-solution in 96-well microplates. Initial absorbance readings at 515 nm were taken and then, microplates were incubated for 15 min to take a final absorbance reading using a KC-4 spectrophotometer (Biotek® Instruments, Inc. Winooski, Vt.). Antioxidant activity was calculated by applying known aliquots of Trolox (antioxidant compound) to known concentrations of DPPH solution. Results were expressed as micromoles Trolox equivalents per gram of fresh tissue.

Leaf chlorophyll was determined at the end of the experiment with 80% acetone extraction using the procedure of Harborne (1998). Absorbance readings were taken at 645 and 663 nm with a Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Chlorophyll content (total, *a* and *b*) were estimated with the following equations:

$$\text{ChlTotal } (\text{mg L}^{-1}) = 17.3(\text{Absorbance}_{646}) + 7.18(\text{Absorbance}_{663})$$

$$\text{Chl}a \ (\text{mg L}^{-1}) = 12.21(\text{Absorbance}_{663}) - 2.81(\text{Absorbance}_{646})$$

$$\text{Chl}b \ (\text{mg L}^{-1}) = 20.13(\text{Absorbance}_{646}) - 5.03(\text{Absorbance}_{663})$$

Nitrate reductase activity was performed with the procedure of Foyer et al. (1998). Briefly, leaf samples were ground with 3 mL of extraction buffer solution, consisting of 50 mM Mops-KOH, pH 7.8, 5 mM NaF, 1 µM Na_2MoO_4, 10 µM FAD, 1 µM leupeptin, 1 µM microcystin, 0.2 g^{-1} fresh weight PVP, 2 mM ß-mercaptoethanol, and 5 mM EDTA. The homogenate was centrifuged at 4°C for 15 min at 12,000 rpm. An

aliquot of 200 µL was taken and then reacted to 200 µL of reaction mixture solution consisting of 50 mM Mops-KOH buffer, pH 7.5, supplemented with 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH, and 5 mM EDTA. Reaction was terminated after 15 min with the addition of 200 µL of sulfanilamide (1% [w/v] in 3 N HCl) and 200 µL of naphthylethylene-diamine dihydrochloride (0.02% [w/v]) to the reaction mixture, and the absorbance at 540 nm was measured by means of a Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640, Beckman Coulter, Inc. Fullerton, Calif.). Nitrate reductase activity was determined from a standard curve of NO_2Cl.

Dehydrogenase activity of the rhizosphere soil was performed following the procedures of Casida et al. (1964). Briefly, 1.5 g of rhizosphere sand were added in a test tube with 0.001 g $CaCO_3$, 250 µL of 3% triphenyl tetrazolium chloride, and 2.5 mL of nanopure water. Tubes were sealed with a rubber stopper and incubated for 24 h at 37°C. Immediately after incubation, 2.5 mL of methanol were added to each tube and mixed with vortex for a few seconds. Suspensions were filtrated using Whatman paper filter # 1. Aliquots of 500 µL were taken and transferred to 1.5 mL microtubes and added with 1 mL methanol. Absorbance readings were taken at 485 nm with a Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Dehydrogenase activity was determined from a standard curve prepared with known quantities of triphenyl formazan.

Mycorrhizal colonization was determined by the alkaline phosphatase vital stain procedure (Pearse, 1968; Tisserant et al., 1993). Briefly, roots were incubated for two hours at room temperature in a digestion solution consisting of 0.05 M Tris/Citric acid buffer (pH 9.2), 0.05% sorbitol, 15 units cellulase mL^{-1} and 15 units pectinase mL^{-1}.

Roots were exposed to a sodium chloride solution (1% active chlorine) for five minutes. Staining procedure consisted on overnight exposure of roots to reaction medium consisted on 0.05 Tris/citric acid buffer (pH 9.2), 1 $mg \cdot mL^{-1}$ fast blue RR salt , 1 $mg \cdot mL^{-1}$ α-naphtyl acid phosphate, 0.5 $mg \cdot mL^{-1}$ $MgCl_2$, and 0.8 $mg \cdot mL^{-1}$ $MnCl_2 \cdot 4H_2O$. Fractional colonization was estimated microscopically as the intensity of AMF-colonization of the root cortex, expressed as a percentage.

Analysis of BaP was performed by a modified EPA SW-846 Method 8270B (Louchouarn et al., 2000; USEPA, 1986). Extraction and concentration of BaP from pre-dried samples (15 g) was done with an automated accelerated solvent extractor (Dionex ASE-200, Dionex Corp., Sunnyvale, Calif.) following the procedures of Berset et al. (1999), Popp et al. (1997), and Richter et al. (1997). The extractions were done using 100% dichloromethane, and stainless-steel extraction cells held at elevated temperature (100°C) and solvent pressure (1200 psi). The extracted BaP dissolved in the hot solvent was collected in 60 mL glass vials, and immediately concentrated to a volume of 1 mL, using an evaporative solvent reduction apparatus (Zymark TurboVap II, Zymark Corp. Hopkinton, Mass.). Final extracts were utilized in the quantitative determination of BaP by gas chromatographic mass spectrometry (HP 5890 Series II Gas Chromatograph Hewlett-Packard Co., Wilmington, Del.).

The experiment was a completely randomized experimental design with six treatments (Control, Ce, Sp, AMF, Ce+SP, Ce+Sp+AMF) and eight replications per treatment. Data were analyzed with analysis of variance (ANOVA), and LSD test for mean comparison (LSD, $\alpha=0.05$) or mean standard error (± SE) (SAS Institute Inc, 2002). The number of replications was as follows: plant growth responses, n=5; and for gas

exchange, chlorophyll, proline, total phenolics, antioxidant activity, nitrate reductase activity, mycorrhizal colonization, dehydrogenase activity, and BaP-degradation, n=3.

Bioremediation and Phytoremediation of BaP with Free-Living Hydrocarbonoclastic Microorganisms

This experiment was conducted to determine the effect of bioagumentation with hydrocarbonoclastic microorganisms on bioremediation and phytoremediation of sand contaminated with 100 mg BaP kg^{-1}. It was run concurrently under the same glasshouse and cultural conditions of the previously described experiment. Treatments consisted of bioremediation (without plants) or phytoremediation with *L. multiflorum*. Plants and plantless containers were immediately inoculated, as previously described, with *S. paucimobilis* (Sp), *C. echinulata* (Ce), or with the combination of both microorganisms, including a non-inoculated as control.

Soil dehydrogenase activity and BaP-degradation were determined as previously described. The experiment was a 2x4 factorial, with two remediation levels (phytoremediation and bioremediation), and four microbial levels (control, Sp, Ce, and Ce+Sp), n=3. AMF-treatments were not considered for bioremediation since AMF is an obligate symbiont in the rhizosphere, requiring a host plant. Soil dehydrogenase activity (n=3) and BaP-degradation (n=3) were statistically analyzed with analysis of variance (ANOVA), and LSD test for mean comparison (LSD, α=0.05) or mean standard error (± SE) (SAS Institute Inc, 2002).

Results

Growth and Physiological Responses of *Lolium multiflorum* in the Phytoremediation of BaP – Effect of Rhizosphere Microorganisms

Rhizosphere inoculation with Ce, Sp, AMF, singly or in combination had no significant effects on leaf area, DW of leaves and pseudostems, SLA, and shoot number (Table 5.1). Microbial treatments had significant effects on root DW ($P \leq 0.01$), and total plant DW, RSR, and leaf area ratio LAR ($P \leq 0.05$) (Table 5.1). However, inoculation with Ce, AMF, and Ce+Sp+AMF had significantly reduced total plant DW than control. AMF-plants had significantly higher LAR than control and plants with Sp or Ce+Sp+AMF (Table 5.1; see Appendix I, Fig. AI-5.1 for visual responses of plants).

Gas exchange [net photosynthesis (*Pn*), stomatal conductance (g_s), and water use efficiency (*WUE*)] of *L. multiflorum* at 30 and 60 days, was not significantly different due to microbial inoculation (Table 5.2). Level of *Pn*, g_s, and *WUE* were high among treatments, and not adversely affected by BaP (Table 5.2). Chlorophyll content (total, *a* and *b*) was not significantly different among microbial treatments (Table 5.3). Effects of microbial inoculation on leaf proline, total phenolics, and antioxidant activity at 30 and 60 days, were nonsignificant (Tables 5.4, 5.5). Neither leaf nitrate reductase nor soil dehydrogenase activity were significantly enhanced by microbial inoculation (Table 5.6).

Total arbuscular mycorrhizal colonization and arbuscule formation were not adversely affected by the co-inoculation with hydrocarbonoclastic microorganisms (Fig. 5.1). While vesicle formation was statistically ($P \leq 0.05$) higher in AMF treatment alone, the AMF isolate of *G. intraradices* had very low vesicle formation of less than 5% (Fig. 5.1).

Table 5.1. Effect of *Glomus intraradices* (AMF) and two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on plant growth of *Lolium multiflorum* var. Passerel Plus during the phytoremediation of benzo[*a*]pyrene-contaminated sand, after 60 days.

Treatment	Leaf Area (cm^2)	Leaves DW (g)	Pseudostems DW (g)	Root DW (g)	Total plant DW (g)	RSR ($g \cdot g^{-1}$)	SLA ($cm^2 \cdot g^{-1}$)	LAR ($cm^2 \cdot g^{-1}$)	Shoot number
Control	306.0	1.8	1.8	4.6 a [z]	8.2 a	1.3 a	170.9	38.3 b	25.8
Ce	267.3	1.6	1.7	2.9 bc	6.3 b	0.9 bc	167.4	42.4 ab	30.5
Sp	232.8	1.7	1.7	4.6 a	8.0 a	1.3 a	138.5	29.8 b	23.8
AMF	304.7	1.8	1.7	2.5 c	6.0 b	0.7 c	172.7	51.7 a	30.8
Ce+Sp	298.5	1.7	1.7	4.0 ab	7.4 ab	1.2 ab	182.2	42.1 ab	27.7
Ce+Sp+AMF	208.8	1.5	1.8	3.0 bc	6.4 b	0.9 abc	132.6	33.3 b	30.1
Significance Treatment	NS	NS	NS	0.01	0.05	0.05	NS	0.05	NS

[z]Means in the same column followed by the same letter are not significantly different (LSD, α=0.05); NS=Nonsignificant, n=5.

Table 5.2. Effect of *Glomus intraradices* (AMF) and two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on gas exchange of *Lolium multiflorum* var. Passerel Plus during the phytoremediation of benzo[*a*]pyrene-contaminated sand.

Treatment	30 days			60 days		
	Pn (μmoles CO_2 $m^{-2} \cdot s^{-1}$)	g_s (moles $m^{-2} \cdot s^{-1}$)	*WUE* [y] (*Pn*/ g_s)	*Pn* (μmoles CO_2 $m^{-2} \cdot s^{-1}$)	g_s (moles $m^{-2} \cdot s^{-1}$)	*WUE* (*Pn*/ g_s)
Control	22.1	0.05	398.5	16.4	0.05	507.2
Ce	20.3	0.06	309.5	21.3	0.04	794.3
Sp	19.3	0.06	296.8	22.7	0.04	600.7
AMF	24.1	0.08	308.3	14.5	0.03	481.2
Ce+Sp	21.5	0.08	275.0	23.6	0.04	578.3
Ce+Sp+AMF	21.2	0.08	282.2	18.1	0.03	556.6
Significance Treatment	NS	NS	NS	NS	NS	NS

[y] *WUE*= Water Use Efficiency, μmoles $C0_2$/moles H_20 $m^{-2} \cdot s^{-1}$; NS=Nonsignificant, n=3.

Table 5.3. Effect of *Glomus intraradices* (AMF) and two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on chlorophyll content in leaves of *Lolium multiflorum* var. Passerel Plus during the phytoremediation of benzo[*a*]pyrene-contaminated sand, after 60 days.

Treatment	Chlorophyll content ($\mu g \cdot g^{-1}$ FW)		
	Total	*a*	*b*
Control	1032.4	723.6	309.4
Ce	1521.5	999.4	523.1
Sp	1333.2	900.8	433.3
AMF	1385.2	913.6	472.5
Ce+Sp	1531.9	1091.4	441.4
Ce+Sp+AMF	1730.1	1124.3	606.9
Significance			
Treatment	NS	NS	NS

FW=Fresh weight; NS=Nonsignificant, n=3.

Table 5.4. Effect of *Glomus intraradices* (AMF) and two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on proline content in leaves of *Lolium multiflorum* var. Passerel Plus during the phytoremediation of benzo[*a*]pyrene-contaminated sand.

Treatment	Proline (μmoles g^{-1} FW)	
	30 days	60 days
Control	1.8	1.5
Ce	2.8	2.0
Sp	3.0	1.5
AMF	2.3	1.3
Ce+Sp	1.9	1.8
Ce+Sp+AMF	1.8	2.3
Significance		
Treatment	NS	NS

FW=Fresh weight; NS=Nonsignificant, n=3.

Table 5.5. Effect of *Glomus intraradices* (AMF) and two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on total phenolics and total antioxidant activity in leaves of *Lolium multiflorum* var. Passerel Plus during the phytoremediation of benzo[*a*]pyrene-contaminated sand.

Treatment	Total phenolics (µg Chlorogenic acid g^{-1} FW)		Total antioxidant activity (µmoles Trolox g^{-1} FW)	
	30 days	60 days	30 days	60 days
Control	3437.5	4381	12.5	13.1
Ce	4224.9	5033	15.0	16.2
Sp	4107.5	4696	15.4	17.0
AMF	4158.4	5533	13.3	18.5
Ce+Sp	3558.5	3900	12.9	13.5
Ce+Sp+AMF	4785.7	6791	18.2	21.8
Significance				
Treatment	NS	NS	NS	NS

FW=Fresh weight; NS=Nonsignificant, n=3.

Table 5.6. Effect of *Glomus intraradices* (AMF) and two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on leaf nitrate reductase activity, and dehydrogenase activity in the rhizosphere of *Lolium multiflorum* var. Passerel Plus during the phytoremediation of benzo[*a*]pyrene-contaminated sand, after 60 days.

Treatment	Leaf Nitrate Reductase (µmoles NO_2 g^{-1} FW)	Rhizosphere soil dehydrogenase activity (µg Formazan g^{-1} soil)
Control	67.9	2.2
Ce	71.9	1.7
Sp	74.9	3.3
AMF	62.3	1.7
Ce+Sp	70.4	2.7
Ce+Sp+AMF	92.8	1.9
Significance		
Treatment	NS	NS

FW=Fresh weight; NS=Nonsignificant, n=3.

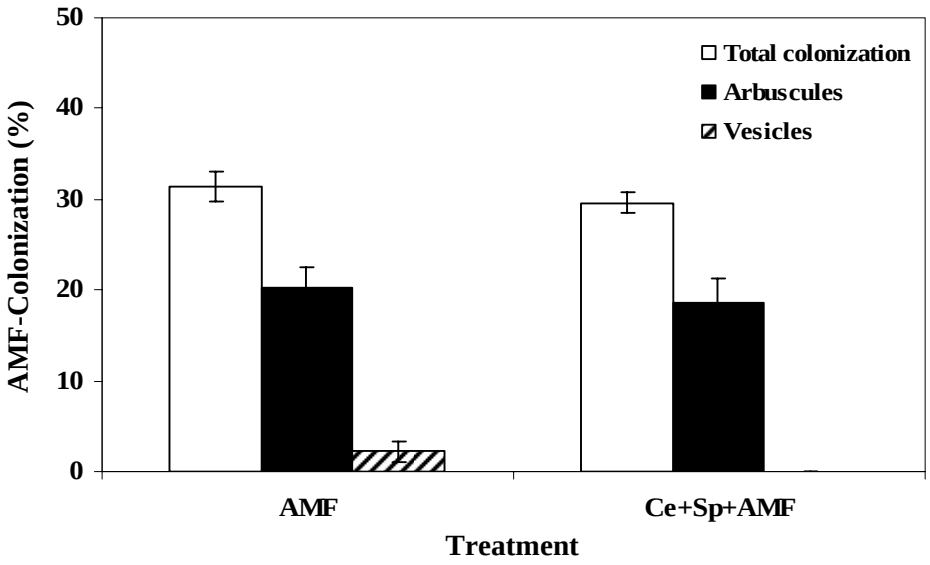

Fig. 5.1. Influence of the inoculation of hydrocarbonoclastic-microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA505 (Sp)] on *Glomus intraradices* (AMF)-colonization with positive reaction of fungal alkaline phosphatase activity in roots of *Lolium multiflorum* var. Passerel Plus during phytoremediation of benzo[*a*]pyrene-contaminated sand, after 60 days. Main effects of microbial inoculation were nonsignificant for total colonization and arbuscules, but significant ($P \leq 0.05$) for vesicles. Bars ± SE, n=3.

In regards to phytoremediation, degradation of BaP in the rhizosphere of *L. multiflorum* was significantly ($P \leq 0.05$) enhanced by the microbial inoculation (Fig. 5.2A). The single inoculation of Ce and the combination Ce+Sp+AMF, resulted in increased degradation of BaP, compared to control plants (Fig. 5.2). The inoculation of AMF stimulated BaP-degradation in the rhizosphere of *L. multiflorum*, which was comparable to the single inoculation of Sp, but significantly different than control plants (Fig. 5.2A). Thus, phytoremediation of BaP was more efficient when plants where inoculated with either hydrocarbonoclastic microorganisms (Ce or Sp) or with AMF (Fig. 5.2B).

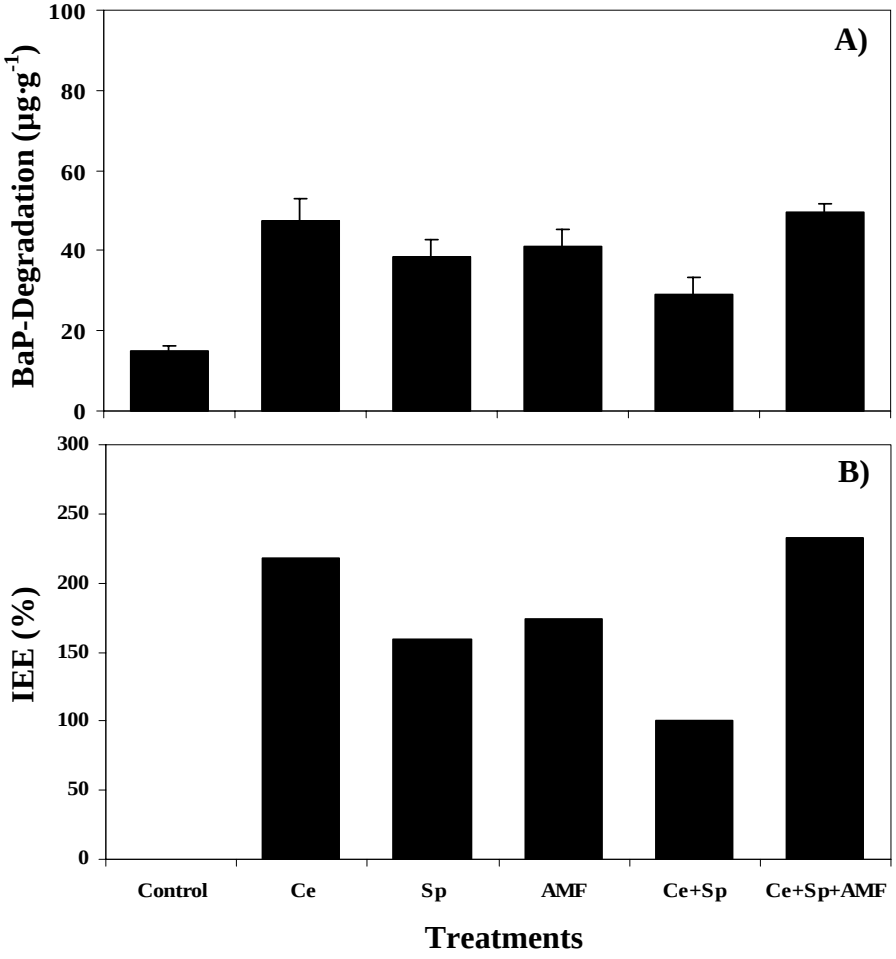

Fig. 5.2. Phytoremediation of benzo[*a*]pyrene (BaP) (100 mg·kg^{-1}), after 80 days, with *Lolium mutiflorum* inoculated with *Glomus intraradices* (AMF), *Cunninghamella echinulata* var. *elegans* (Ce), and *Sphingomonas paucimobilis* (Sp). A) Degradation of BaP. Main effects of microbial inoculation were significant ($P \leq 0.01$), Bars ± SE, n=3. B) Inoculation Efficiency Effect (IEE) on BaP-degradation: IEE (%)= [(BaP degradation (µg·g^{-1}) of inoculated plants–BaP degradation (µg·g^{-1}) of control plants)/(BaP degradation (µg·g^{-1}) of control plants)] x 100.

Bioremediation and Phytoremediation of BaP with Free-Living Hydrocarbonoclastic Microorganisms

Main effects of remediation (bio- or phytoremediation), microbial inoculation, and their interaction had no significant effect on dehydrogenase activity (Table 5.7).

BaP-degradation was significantly affected by remediation, microbial inoculation, and their interaction ($P \leq 0.001$). BaP-degradation was significantly higher with bioaugmentation via bioremediation than phytoremediation (Fig. 5.3A). Thus, the single or combined inoculation of Ce and Sp during bioremediation (no plants) was more efficient on BaP-degradation than phytoremediation (Fig. 5.3B).

Table 5.7. Effect of the inoculation of two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce), and *Sphingomonas paucimobilis* EPA505 (Sp)] on soil dehydrogenase activity during bioremediation (no plant) or phytoremediation (with *Lolium multiflorum* var. Passerel Plus) of benzo[*a*]pyrene (100 $mg \cdot kg^{-1}$), after 60 days.

Remediation	Microbial Inoculation	Rhizosphere soil dehydrogenase activity (μg Formazan g^{-1})
Bioremediation	Control	1.64
	Ce	2.81
	Sp	1.80
	Ce+Sp	2.28
Phytoremediation	Control	2.16
	Ce	1.71
	Sp	3.34
	Ce+Sp	2.72
Significance		
	Remediation	NS
	Microbial inoculation	NS
	Remediation x Microbial inoculation	NS

NS=Nonsignificant, n=3.

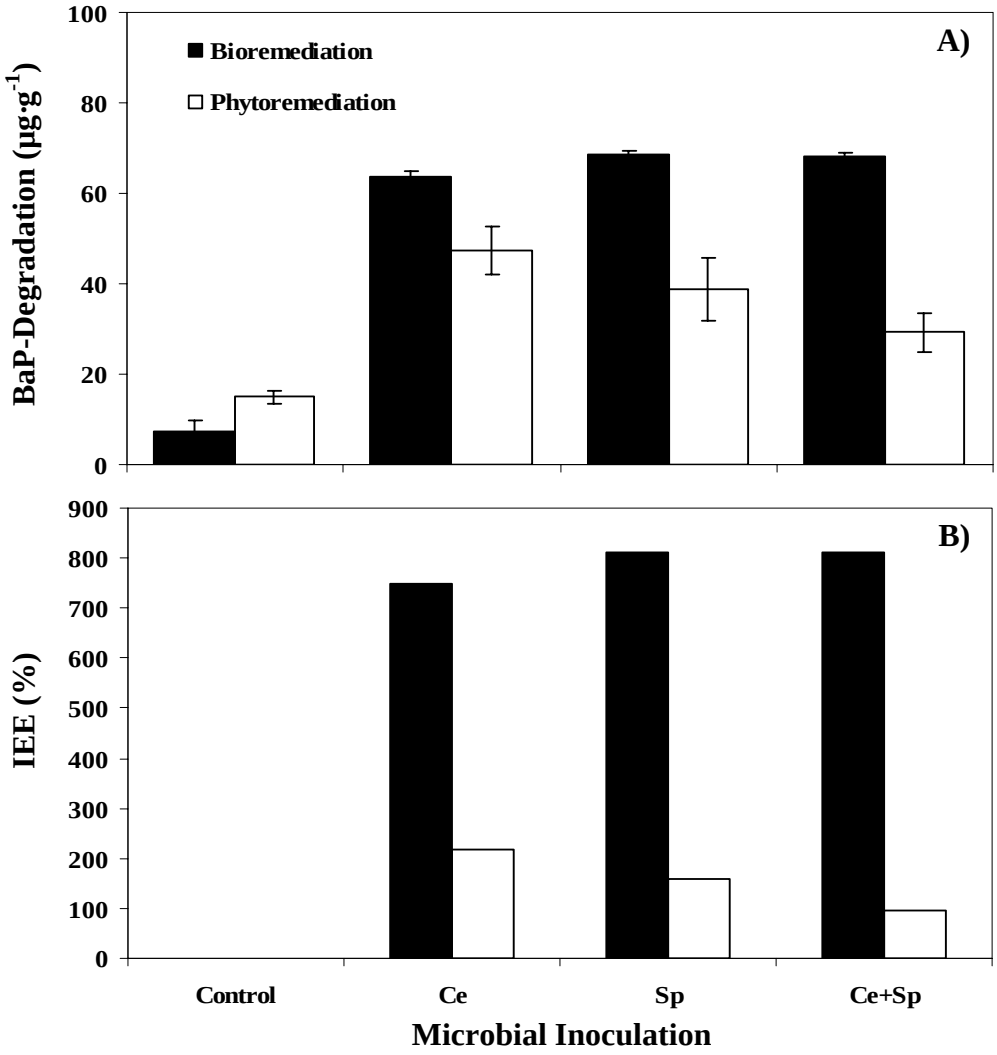

Fig. 5.3. A) Effect of bioaugmentation with two hydrocarbonoclastic microorganisms [*Cunninghamella echinulata* var. *elegans* (Ce), and *Sphingomonas paucimobilis* (Sp)] on the bioremediation (no plants) and phytoremediation (with *Lolium multiflorum*) of benzo[*a*]pyrene (BaP) (100 mg·kg^{-1})-contaminated sand, after 60 days. Main effects of remediation, microbial inoculation, and their interaction were significant at $P \leq 0.001$, Bars ± SE, n=3. B) Inoculation Efficiency Effect (IEE) on BaP degradation. IEE (%)= [(Inoculated treatments of bioremediation or phytoremediation–Non-inoculated treatments of bioremediation or phytoremediation)/(Non-inoculated treatments of bioremediation or phytoremediation)] x 100.

Discussion

This research demonstrates the benefit of incorporating AMF with a hydrocarbonoclastic bacterium and a filamentous fungus for phytoremediation of benzo[*a*]pyrene (BaP) with a *Lolium multiflorum* system. In addition, this research is one of the first reports that shows that the efficiency of BaP-degradation by hydrocarbonoclastic microorganisms is higher with the bioremediation, solely using free-living microorganisms than phytoremediation with the *L. multiflorum* system.

Bioaugmentation with Ce, Sp, AMF or their combination during phytoremediation of BaP, had no significant effect on *L. multiflorum* growth. In general, growth responses of inoculated plants were similar or lower than control plants. Thus, the hypothesis that bioaugmentation improves plant growth was rejected. AMF-plants had significantly higher LAR but significantly lower root DW and RSR, compared to control plants. Similar trends were reported in non-phytoremediation studies of abiotic stress of P and drought, respectively (Aguilera-Gomez et al., 1999; Davies et al., 1999). The LAR suggests that AMF-inoculated plants required a greater leaf area to produce a unit of plant DW, but less dry matter was partitioned to the root system.

The inoculation with Ce, AMF, and Ce+Sp+AMF resulted in significant reduction of total plant DW (~24.4% less), compared to control plants. In the case of AMF, this effect may be related with the carbon drain from the plant to the roots to satisfy the greater carbon requirements to support growth and metabolism of AMF (Bago et al., 2000; Bago et al., 2003). This metabolic cost to the plants may explain, in part, the reduced total plant biomass and the enhanced LAR. Nonsignificant effects of AMF-inoculation on plant growth response during phytoremediation of anthracene were

previously reported (Binet et al., 2000a). However, this is one of the first reports of the inoculation of Ce and Sp on growth of *L. multiflorum* during BaP-phytoremediation.

Physiological responses of plants utilized in phytoremediation of PAH have received little attention, especially when inoculated with either hydrocarbonoclastic bacteria or filamentous fungi with the ability to degrade these persistent organic contaminants. In this study, microbial inoculation did not significantly enhance gas exchange, chlorophyll content, proline content, antioxidant activity, nitrate reductase, and soil rhizosphere dehydrogenase activity. Thus, the tested hypothesis that bioaugmentation enhanced physiological plant response was rejected. However, there was a nonsignificant trend of enhanced chlorophyll content, total phenolics, and antioxidant activity with the inoculation of Ce+Sp+AMF, compared to control plants. These apparent benefits provided by the inoculation of the three microorganisms may represent a physiological advantage to plants during phytoremediation of BaP, which may minimize toxic effects to plants (Binet et al., 2000b; Qiu et al., 1994; Schwab and Banks, 1994). Some of the physiological evaluations of plants exposed to PAH are related with the modification on the synthesis of specific enzymes (i.e., laccases, dehalogenases, nitroreductases, nitrilases, and peroxidases) that contribute to the initial oxidation and degradation of PAH in the rhizosphere (Alkorta and Garbisu, 2001; Criquet et al., 2000; Criquet et al., 2001; Schnoor et al., 1995; Susarla et al., 2002).

Total mycorrhizal root colonization and arbuscule formation, measured as the positive reaction of the alkaline phosphatase (ALP) activity in the intraradical hyphae (Tisserant et al., 1993; Smith and Gianinazzi-Pearson, 1990), was not affected by the interaction with the two hydrocarbonoclastic microorganisms. Previous results showed

that ALP of AMF-symbiosis in *Echinochloa polystachia* was not reduced by the presence of several concentrations of BaP in soil (Alarcón et al., 2006). Nevertheless, negative effects of either petroleum hydrocarbons and mixtures or single PAH on AMF colonization have been reported (Cabello, 1997; Gaspar et al., 2002; Leyval and Binet, 1998; Liu et al., 2004). In one of the few reports on the interaction of AMF with hydrocarbonoclastic-microorganisms, Gaspar et al. (2002) reported that the inoculation of maize plants with the yeast *Rhodotorula glutinis* caused significant reduction of mycorrhizal colonization measured as succinate-dehydrogenase activity of *Glomus geosporum* hyphae, exposed to the PAH, phenanthrene.

This is one of the first reports describing the microbial interaction of *G. intraradices* with the bacteria, *S. paucimobilis*, and the filamentous fungus, *C. echinulata* var. *elegans*, during phytoremediation of BaP. The single or combined microbial inoculation resulted in significantly enhanced efficiency of BaP-degradation in the rhizosphere of *L. multiflorum* when compared to the control (Fig. 5.2B). The beneficial effects of Ce and Sp on the degradation of PAH has been well demonstrated, particularly in liquid cultures and soils without plants (Casillas et al., 1996; Cerniglia et al., 1980; Daugulis and McCracken, 2003; Kanaly and Harayama, 2000; Story et al., 2001; Sutherland, 1992), but effects of hydrocarbonoclastic microorganisms during phytoremediation has received little attention (Kelley et al., 2001). In this study, the combined inoculum of Ce+Sp+AMF showed similar BaP-degradation to plants inoculated with Ce alone, but significantly higher than the inoculation with Ce+Sp (Fig. 5.2A). In this particular case, the reduced capability of both hydrocarbonoclastic microorganisms when coinoculated may be in part due to microbial competition for

carbon compounds, rather than antagonism between both microorganisms. The possible antibiosis of Sp to Ce was tested *in vitro* but results were negative, indicating that the presence of Sp did not inhibit the growth of Ce (see Appendix I, Fig. AI-5.2). Thus, the reduced BaP-degradation observed in the rhizosphere inoculated with Sp+Ce was not attributed to antibiotic effects of Sp to Ce. Furthermore, microbial competition for carbon sources derived from root exudation may result in greatly reduced or minimal degradation of organic contaminants (Siciliano and Germida, 1998).

AMF can contribute directly or indirectly to the phytoremediation of PAH in the rhizosphere (Joner and Leyval, 2003a). The BaP-degradation observed in AMF-inoculated plants in this study is in agreement with Liu et al. (2004). More recently, Volante et al. (2005) published the first report that AMF dissipate aromatic compounds in the rhizosphere of *Allium porrum*; however, the mechanisms remain unknown. Since this experiment was not in a completely closed system, it is possible that the mycorrhizal symbiosis may have stimulated either naturally-occurring microorganisms or root activity (Joner and Leyval, 2003a), thus contributing on the BaP-degradation in the rhizosphere. However, in our study neither microbial populations nor root physiology was determined. Furthermore, the identification of those functional PAH-degrading microorganisms stimulated by the presence of AMF in the root systems merits further investigation. To our knowledge, no studies have reported on the bioaugmentation of AMF-plants for enhancing BaP-degradation in the rhizosphere. Thus, this study showed the beneficial effect of *G. intraradices* on the degradation of BaP in the rhizosphere of *L. multiflorum*, although *C. echinulata* var. *elegans* was the most efficient microorganism.

On the other hand, bioaugmentation with Ce, Sp, and their combination during bioremediation (no plants) or phytoremediation (with *L. multiflorum*) did not significantly enhance soil dehydrogenase activity. Most importantly, by comparing the effect of bioaugmentation on bioremediation or phytoremediation of BaP, it was observed that the extent of BaP-degradation was significantly higher during bioremediation than phytoremediation (Fig. 5.3B). In phytoremediation, the inoculation with Ce resulted in higher BaP-degradation than in the combination of Ce+Sp, while in bioremediation, the single or combined inoculation of Ce and Sp had similar BaP-degradation.

Conversely to the expected outcome of one hypothesis proposed in this work, phytoremediation was less efficient for BaP-degradation than bioremediation, when bioaugmentation was applied. This effect may be attributed to competition for carbon sources in the rhizosphere as previously discussed. Root exudates could have delayed BaP-degradation since they may represent rapidly assimilable carbon to hydrocarbonoclastic microorganisms (Siciliano and Germida, 1998), which resulted in the inhibition of their expression of PAH-degrading behavior. Furthermore, Kelley et al. (2001) reported that degradation of recalcitrant contaminants was higher in soil without plants, suggesting that plants may impede the metabolism of the contaminant via soil microorganisms.

Summary

While bioaugmentation with hydrocarbonoclastic microorganisms (bacteria and filamentous fungi) can improve bioremediation of polycyclic aromatic hydrocarbons (PAH), their interaction with arbuscular mycorrhizal fungi (AMF) during

phytoremediation of PAH is not well known. Glasshouse experiments were conducted to evaluate the effect of bioaugmentation on phytoremediation and bioremediation of benzo[*a*]pyrene (BaP). Phytoremediation of BaP (100 $mg \cdot kg^{-1}$) was done with a *Lolium multiflorum* plant system inoculated with a hydrocarbonoclastic bacterium [*Sphinghomonas paucimobilis* EPA505 (Sp)], a filamentous fungus [*Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce)], and an arbuscular mycorrhizal fungus [*Glomus intraradices* (AMF)], or combinations of the three microorganisms. Microbial inoculation did not significantly enhance plant growth during phytoremediation of BaP, and the inoculation with Ce, AMF, and Ce+Sp+AMF significantly reduced total plant dry weight (~24.4% less) and root-to-shoot ratio when compared to the control. In addition, microbial inoculation did not significantly enhance net photosynthesis, stomatal conductance, chlorophyll content, proline, nitrate reductase, total phenolics, antioxidant activity, and soil rhizosphere dehydrogenase activity. The inoculation of Ce+Sp did not impede root colonization or arbuscule formation of AMF. Most importantly, single or combined microbial inoculation enhanced BaP-degradation in the rhizosphere of *L. multiflorum*, and inoculation with Ce or Ce+Sp+AMF caused the greatest BaP-degradation. Bioaugmentation with Ce, Sp, or Ce+SP enhanced both bioremediation and phytoremediation of BaP. Bioremediation was more efficient than phytoremediation for BaP-degradation.

CHAPTER VI

PHYTOREMEDIATION OF PETROLEUM-CONTAMINATED SOIL VIA BIOAUGMENTATION WITH ARBUSCULAR MYCORRHIZA AND HYDROCARBONOCLASTIC MICROORGANISMS IN A *Lolium multiflorum* RHIZOSPHERE SYSTEM

Introduction

Enhanced degradation of petroleum hydrocarbons (PH) via phytoremediation is dependent on abiotic and biotic conditions of: 1) soil type, nutrient and water availability, 2) type and concentration of PH in soil, and 3) proliferation of hydrocarbonoclastic microorganisms that degrade available contaminants in the soil and rhizosphere (Schnoor et al., 1995; Cunningham et al., 1997; Siciliano y Germida, 1998). Plant sensitivity to contaminants, low soil fertility, slow plant growth rates, and reduced microbial populations in soils due to chronic exposure to contaminants are factors limiting phytoremediation of PH-contaminated soils (Alkorta and Garbisu, 2001; Susarla et al., 2002; Pilon-Smits, 2005).

During phytoremediation, plants may improve aeration via their root system, which enhances rhizosphere microbial activity and contaminant degradation. Microbial activity is stimulated by root exudates that serve as alternate sources of carbon and energy for microorganisms that oxidize and/or degrade organic contaminants. However, in spite of the apparent benefit of plants in the phytoremediation of PH-contaminated soils, there is little information about the effect of combining phytoremediation with

bioaugmentation utilizing hydrocarbonoclastic microorganisms (Huang et al., 2004). Microorganisms with the ability to degrade PH are ubiquitous, however when their populations are depleted, bioaugmentation via inoculation with specific microbes to enhance bioremediation and/or phytoremediation is usually recommended. Free-living bacteria such as *Sphingomonas paucimobilis*, as well as filamentous fungi, such as *Cunninghamella* spp., are ideal microorganisms that oxidize and/or degrade PH, including polycyclic aromatic hydrocarbons (PAH) in soil (Cerniglia, 1992; van Hamme et al., 2003). Although the physiological and biochemical mechanisms by which these microorganisms degrade PH are known, there are few reports about their effects on plants utilized in phytoremediation.

Arbuscular mycorrhizal fungi (AMF) are ubiquitous rhizosphere microorganisms that form mutually beneficial symbiosis with the root system of approximately 80% of the terrestrial plants (Smith and Read, 1997). This symbiosis can have important effects on phytoremediation of soils contaminated with inorganic and organic compounds (Cabello, 2001; Meharg, 2001; Joner and Leyval, 2003a; Joner and Leyval, 2003b). Direct benefits are related with enhanced plant adaptation and growth, including enhanced nutrition, and abiotic and biotic stress resistance. Indirect benefits include modification of microbial groups in the mycorrhizosphere, and potential proliferation of PH-degrading (hydrocarbonoclastic) microorganisms via extraradical hyphae exudation (hyphosphere effect). The interaction of AMF and hydrocarbonoclastic microorganisms has received little attention (Gaspar et al., 2002). In addition, little is known about the role of AMF and their interaction with hydrocarbonoclastic microorganisms on plant growth, physiological responses, and rhizosphere microbial populations in the

phytoremediation of PH in soils. The hypotheses in this research were that: 1) bioaugmentation with a hydrocarbonoclastic bacterium and a filamentous fungus improves the physiological response of AMF-colonized plants in the phytoremediation of PH-contaminated soil, and 2) degradation of PH in the rhizosphere of *Lolium multiflorum* colonized with AMF is enhanced by inoculation with hydrocarbonoclastic microorganisms. Thus, objectives of this research were to: 1) determine selected physiological responses (gas exchange, total antioxidant activity, nitrate reductase, and proline) of AMF-colonized plants of *Lolium multiflorum* utilizing bioaugmentation with *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans* in Arabian medium crude oil (ACO)- contaminated soil, and 2) determine the phytoremediation of PH via bioaugmentation of hydrocarbonoclastic microorganisms in soil contaminated with ACO.

Materials and Methods

Cultural Conditions, Soil Contamination, Transplant, Microbial and Mycorrhizal Inoculation

The study was conducted under glasshouse conditions at Texas A&M University, College Station, TX for 80 days. The experiment was initiated on 6 September and terminated on 24 November, 2005. Temperature and relative humidity were monitored with a watch dog data logger Model 150 (Spectrum technologies, Inc., Planfield, Ill.), and photosynthetic photon flux density (PPFD) was determined with a LI-190SA Quantum/Radiometer/Photometer and sensor (LI-COR® Biosciences, Lincoln, Nebr.). Average day/night temperature and relative humidity was 25.7/22.3°C, and 64.9/72.4%,

respectively, average maximum PPFD determined at solar noon, was 583.5 $\mu moles \cdot m^{-1} \cdot s^{-2}$. A 14 h of photoperiod were maintained by artificially lighting plants from 18:00 to 22:00 during October and November.

A mixture of sand and sandy loam soil (1:1 v/v) was utilized as a substrate, with chemical properties of: ($\mu g \cdot g^{-1}$) 0.9 NO_3-N, 2.1 NH_4-N, 1.5 P, 17 K, 9468 Ca, 72 Mg, 161 Na, and 53 S. The electrical conductivity was 0.17 $dS \cdot m^{-1}$, pH of 7.7, and textural analysis of sand 85 %, clay 10 % and silt 5%.

Substrate was steam-pasteurized at 70°C for eight hours on two consecutive days. Arabian medium crude oil (ACO) concentrations were 0 and 6000 $mg \cdot kg^{-1}$. The viscosity of ACO contaminant was reduced through the application of dichloromethane solvent (Sigma®, <0.002% of residue after evaporation), as previously described.

One week-old seedlings of *Lolium multiflorum* Lam. cv. Passerel Plus were transplanted, with one seedling per container, and selected treatments were inoculated with 500 spores of *Glomus intraradices* (AMF) [Mycorise® ASP, PremierTech Biotechnologies, Quebec, Canada] and/or with two milliliters of liquid inoculum ($8.8x10^8$ CFU mL^{-1}) of the flouranthene-preadapted bacterium, *Sphingomonas paucimobilis* (Sp, EPA505; Mueller et al., 1990) and two milliliters of liquid inoculum ($5.5x10^4$ CFU mL^{-1}) of the filamentous fungus, *Cunninghamella echinulata* var. *elegans* (Ce, ATCC-36112). Microbial inoculation was applied directly to the root system of the seedlings. Non-inoculated seedlings in ACO-contaminated or non-contaminated soil were utilized as controls.

Plants were fertilized weekly with Long Ashton Nutrient Solution (LANS) at 1X (Hewitt, 1966; see Appendix I, Table AI-4.1) modified to supply 30 µg P mL^{-1}. Plants

were watered as needed with deionized water. An additional control treatment with LANS modified to supply 44 μg P mL^{-1} was included to determine if high P substituted or equaled AMF effects on plant physiology and phytoremediation.

Plant Growth Evaluation

At the termination of the experiment, plants were harvested to determine the following parameters: leaf area (cm^2), and dry weights (DW) of leaves, pseudostems, roots, and total plants. The leaf area ratio [(LAR): leaf area/total plant DW, $cm^2 \cdot g^{-1}$], specific leaf area [(SLA): leaf area/leaf DW, $cm^2 \cdot g^{-1}$], and root to shoot ratio [(RSR): root DW/leaf + pseudostems DW, $g \cdot g^{-1}$] were also estimated. Leaf area was determined using a portable area meter LI-COR Model LI-3000 (LI-COR Biosciences, Lincoln, Nebr.). Detached plant organs were placed in an oven at 70°C for two days, then plant samples were subsequently weighed.

Plant Gas Exchange, Chlorophyll Content and Selected Physiological Responses

Gas exchange measurements (photosynthesis and stomatal conductance) were taken at the end of the experiment at 80 days. Measurements were done on individual mature leaf blades from three random plants per treatment (n=3), with a portable photosynthesis system model LI-6400 (LI-COR Inc., Lincoln, Nebr.) with red/blue LED light source (LI6400-02B) at photosyntheticaly active radiation (PAR) levels of 500 μmol $m^{-2} \cdot s^{-1}$, and CO_2 concentration of 360 $\mu mol \cdot s^{-1}$.

Leaf chlorophyll content was determined with 80% acetone extraction using the procedure of Harborne (1998). Absorbance readings were taken at 645 and 663 nm with a

Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Chlorophyll content (total, *a* and *b*) were estimated by the following equations:

$$\text{ChlTotal } (\text{mg L}^{-1}) = 17.3(\text{Absorbance}_{646}) + 7.18(\text{Absorbance}_{663})$$

$$\text{Chl}a \ (\text{mg L}^{-1}) = 12.21(\text{Absorbance}_{663}) - 2.81(\text{Absorbance}_{646})$$

$$\text{Chl}b \ (\text{mg L}^{-1}) = 20.13(\text{Absorbance}_{646}) - 5.03(\text{Absorbance}_{663})$$

Nitrate reductase activity in leaves was performed by the procedures described by Foyer et al. (1998). Briefly, leaf samples were ground with 3 mL of extraction buffer solution consisted on 50 mM Mops-KOH, pH 7.8, 5 mM NaF, 1 µM Na_2MoO_4, 10 µM FAD, 1 µM leupeptin, 1 µM microcystin, 0.2 g PVP g^{-1} fresh weight, 2 mM ß-mercaptoethanol, and 5 mM EDTA. The homogenate was centrifuged at 4°C for 15 min at 12000 rpm. An aliquot of 200 µL was taken and then reacted to 200 µL of reaction mixture solution consisted on 50 mM Mops-KOH buffer, pH 7.5, supplemented with 1 mM NaF, 10 mM KNO_3, 0.17 mM NADH, and 5 mM EDTA. The reaction was terminated after 15 min by the addition of 200 µL of sulfanilamide (1% [w/v] in 3 N HCl) and 200 µL of naphthylethylene-diamine dihydrochloride (0.02% [w/v]) to the reaction mixture, and the absorbance at 540 nm was measured by means of a Beckman UV-Vis spectrophotometer (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Nitrate reductase activity was determined from a standard curve of NO_2Cl.

Proline in leaves was determined with the procedures of Bates et al. (1973) and Gzik (1996). Briefly, 0.100 g of leaf fresh tissue was macerated in an iced-mortar with 3 mL of 3 % sulfosalicylic acid. After centrifugation at 10000 g for 30 minutes, an aliquot

the extract was reacted with ninhydrin reagent and glacial acetic acid, and incubated with at 100 °C for one hour. Reaction mixture was stopped with an ice bath, and proline was extracted with toluene. Absorbance readings were taken at 520 nm (Beckman Coulter™ Du® Series 640 UV/Vis Spectrophotometer, Beckman Coulter, Inc. Fullerton, Calif.). Proline concentration was determined from a standard curve of D,L-proline, and the results were expressed as micromoles of proline per gram of fresh tissue.

Leaf total antioxidant activity was determined by the 1,1-diphenyl-2-picryldrazyl (DPPH) radical decoloration assay (Matthäus, 2002; Re et al., 1999). Briefly, leaf extracts (0.150 g in 3 mL of 80 % methanol) were obtained and immediately centrifuged at 15,000 rpm for 15 min. The reaction mixture consisted of mixing 75 µL of the extract added with 250 µL of DPPH-solution in 96-well microplates. Initial absorbance readings at 515 nm were taken and then, microplates were incubated for 15 min to take a final absorbance reading using a KC-4 spectrophotometer (Biotek® Instruments, Inc. Winooski, Vt.). Antioxidant activity was calculated by applying known aliquots of Trolox (antioxidant compound) to known concentrations of DPPH solution. Results were expressed as micromoles Trolox equivalents per gram of fresh tissue.

Total phenolics in leaves were evaluated by the Folin-Ciocalteu reagent assay utilizing chlorogenic acid as a standard curve (Singleton and Rossi, 1965; Soong and Barlow, 2004). In brief, 0.150 g of leaf fresh tissue was macerated in a chilled mortar with 3 mL of 80% methanol. Extracts were centrifuged for 15 min at 15,000 rpm. Reaction mixture consisted on mixing 30 µL of the extract added with 90 µL of Na_2CO_3 and 150 µL of Folin-Ciocalteau reagent in a 96-well microplate. After 30 min the absorbance was measured at 725 nm using a KC-4 spectrophotometer (Biotek®

Instruments, Inc. Winooski, Vt.). Results were expressed as micrograms of chlorogenic acid equivalents per gram of fresh weight tissue.

Total Microbial Populations and Mycorrhizal Colonization

Populations of bacteria and filamentous fungi were estimated by performing the dilution plate count method (Alexander, 2005). Soil samples were prepared in serial dilutions (10^{-1} to 10^{-6}) with sterile distilled water. Briefly, 10 g of rhizosphere soil were mixed in 95 mL of sterile water (10^{-1} dilution). The suspension was agitated vigorously for 10 minutes to suspend either bacterial or fungal cells in the liquid, and then allowed to settle. Subsequent dilutions were prepared by transferring 1 mL of the cell suspension to culture tubes containing 9 mL of sterile water. Aliquots transfers were made sequentially from tube to tube to obtain increasingly dilute cell suspensions. From dilution 10^{-5} to 10^{-6}, 100 µL aliquots were plated to estimate bacterial colony forming units (CFU), and from dilutions 10^{-2} to 10^{-3}, aliquots were plated to estimate fungal CFU. Aliquots of 100 µL were transferred and spread out on nutrient agar (total recoverable bacteria) or potato dextrose agar (filamentous fungi) plates. For free-living N_2-fixing bacteria (bacteria able to grow on N-free medium), aliquots were taken from dilutions 10^{-4} to 10^{-5} and spread out on Petri dishes containing Rennie's medium (Rennie, 1981). Plates were inverted and incubated at 26°C for 2 to 5 days. Bacterial CFU were counted from plates that yield between 30 and 300 CFU.

After 80 days, three plants per treatment were randomly taken and assayed for AMF-colonization (Phillips and Hayman, 1970). Briefly, roots were placed in plastic capsules and cleared with 10% KOH exposed to 121°C for 10 min. After being rinsed

with tap water, roots were exposed to a commercial hydrogen peroxide for 15 min and rinsed with tap water. Immediately, a 10% hydrochloric acid solution was added to the roots for 15 min. Roots were stained with 0.05% tripan blue colorant in a lactoglycerol solution (glycerol-lactic acid-distilled water, 1:1:1 v/v) at 121°C for 10 min. Root segments (20 per slide) were placed on slides, covered with cover slip, and observed under light microscope at 100X magnification. The frequency of arbuscules, vesicles, and hyphae (total colonization) was determined, and results were expressed as a percentage of each AM-fungal structure (Biermann and Linderman, 1981).

Total Petroleum Hydrocarbon Degradation

Analysis of total petroleum hydrocarbons (TPH) was performed by a modified EPA SW-846 Method 8270B (Louchouarn et al., 2000; USEPA, 1986). Extraction of TPH from pre-dried samples (15 g) was done with an automated accelerated solvent extractor (Dionex ASE-200, Dionex Corp., Sunnyvale, Calif.) following the procedures of Berset et al. (1999); Popp et al. (1997), and Richter et al. (1997). Extractions were performed using 100% dichloromethane, and stainless-steel extraction cells held at elevated temperature (100°C) and solvent pressure (1200 psi). The extracted TPH dissolved in the hot solvent were collected in 60 mL glass vials, and immediately concentrated to a volume of 1 mL, using an evaporative solvent reduction apparatus (Zymark TurboVap II, Zymark Corp. Hopkinton, Mass.). Final extracts were used in the quantitative determination of TPH by gas chromatographic mass spectrometry (HP 5890 Series II Gas Chromatograph Hewlett-Packard Co., Wilmington, Del.).

Experimental Design

The experiment was a 5 x 2 factorial in a completely randomized design with five levels of rhizosphere management (Control-44 P μg·mL^{-1}; Control, AMF, SpCe, and AMF+SpCe at 30 μg P mL^{-1} of modified LANS), and two levels of ACO-contamination (contaminated and non-contaminated soil). Each pot containing one plant was one replicate, (n=15). Data were analyzed by using analysis of variance (ANOVA) and LSD test for mean comparison (LSD, α=0.05) or mean standard error (± SE) (SAS Institute Inc, 2002). The number of replications was as follows: plant DW, n=7; gas exchange, chlorophyll, proline, total phenolics, antioxidant and nitrate reductase activities, n=3; microbial population, n=5; mycorrhizal colonization, n=3; and TPH-degradation, n=3.

Results

Plant Growth Responses

Plant growth was significantly ($P \leq 0.001$) reduced by ACO, and enhanced by rhizosphere management (RM). Treatments of AMF, SpCe, or AMF+SpCe increased leaf area, leaf ($P \leq 0.001$) and pseudostem DW ($P \leq 0.05$) at non-contaminated soil compared to control with 30 μg P mL^{-1} (Table 6.1). The interaction of ACO x RM had no effect on plant growth (Table 6.1). However, none of the RM treatments of AMF, SpCe or their combination overcame reduced plant growth in ACO-contaminated soil (Table 6.1; see Appendix I, Fig. AI-6.1 for visual responses of plants).

ACO ($P \leq 0.001$) and ACO x RM interaction ($P \leq 0.05$) resulted in higher specific leaf area (SLA), leaf area ratio (LAR), and lower root:shoot ratio (RSR) compared to plants in non-contaminated soil (Table 6.2). RM treatment had no effects on SLA, LAR

or RSR (Table 6.2). Highest SLA was obtained in plants inoculated with AMF+Sp and in control-44 µg P mL^{-1} plants at 6,000 mg ACO kg^{-1} (Table 6.2). Control plants and plants inoculated with either SpCe or AMF+SpCe in non-contaminated soil had the highest RSR (Table 6.2). RM had no effect on LAR or RSR of plants exposed to ACO.

Plant Gas Exchange, Chlorophyll Content and Selected Physiological Responses

ACO significantly reduced ($P \leq 0.001$) net photosynthesis (*Pn*), stomatal conductance (g_s), and transpiration (*E*), and increased water use efficiency (*WUE*), while nonsignificant effects for RM and the interaction of ACO x RM interaction were observed (Table 6.3).

Table 6.1. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on plant growth of *Lolium multiflorum* cv. Passerel Plus, after 80 days.

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	Leaf Area (cm^2)	Leaf dry weight (g)	Pseudostem dry weight (g)	Root dry weight (g)	Total plant dry weight (g)
Control-44 P	0	887.6 a [w]	2.9 bc	2.9 bc	6.0 c	11.7 b
Control [x]		710.6 b	2.7 c	2.5 c	12.4 ab	17.6 a
AMF [xy]		921.4 a	3.4 ab	3.8 a	9.1 bc	16.3 ab
SpCe [xz]		867.3 ab	3.3 abc	2.8 bc	12.8 ab	18.9 a
AMF + SpCe[x]		809.7 ab	3.6 a	3.5 ab	14.2 a	21.3 a
Control-44 P	6,000	121.9 c	0.3 d	0.1 d	0.6 d	1.0 c
Control [x]		66.6 c	0.2 d	0.1 d	0.3 d	0.6 c
AMF [x]		148.9 c	0.4 d	0.2 d	0.5 d	1.1 c
SpCe [x]		152.6 c	0.5 d	0.2 d	0.6 d	1.3 c
AMF + SpCe [x]		163.8 c	0.4 d	0.2 d	0.6 d	1.2 c
Significance						
	ACO	0.001	0.001	0.001	0.001	0.001
	RM	0.01	0.01	0.05	NS	NS
	ACO x RM	NS	NS	NS	NS	NS

[w]Means in the same column followed by the same letter are not significantly different (LSD, α= 0.05). NS= Nonsignificant, n=7. [x]Treatments fertilized with Long Ashton Nutrient Solution (LANS), modified to supply 30 µg P mL^{-1}; [y]AMF= *Glomus intraradices*; [z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

Table 6.2. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on plant growth parameters of *Lolium multiflorum* cv. Passerel Plus, after 80 days.

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	Specific Leaf Area (SLA) ($cm^2 \cdot g^{-1}$)	Leaf Area Ratio (LAR) ($cm^2 \cdot g^{-1}$)	Root:Shoot Ratio (RSR) ($g \cdot g^{-1}$)
Control 44 P	0	312.3 bcd [w]	82.1 dc	1.0 c
Control [x]		289.7 cd	71.7 de	2.0 a
AMF [xy]		272.3 cd	57.9 de	1.3 bc
SpCe [xz]		263.8 de	48.9 de	2.2 a
AMF + SpCe [x]		227.8 e	39.5 e	2.1 a
Control 44 P	6,000	392.7 a	119.8 ab	1.2 c
Control [x]		321.3 bcd	114.3 bc	0.9 c
AMF [x]		363.0 ab	130.8 ab	0.9 c
SpCe [x]		327.1 bc	118.8 ab	0.9 c
AMF + SpCe [x]		398.4 a	151.2 a	0.8 c
Significance				
	ACO	0.001	0.001	0.001
	RM	NS	NS	NS
	ACO x RM	0.01	0.05	0.05

[w]Means in the same column followed by the same letter are not significantly different (LSD, α= 0.05). NS= Nonsignificant, n=7. [x]Treatments fertilized with Long Ashton Nutrient Solution (LANS), modified to supply 30 µg P mL^{-1}; [y]AMF= *Glomus intraradices*; [z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

Table 6.3. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on plant gas exchange of *Lolium multiflorum* cv. Passerel Plus, after 80 days.

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	Photosynthesis (*Pn*) (µmoles CO_2 $m^{-2} \cdot s^{-1}$)	Stomatal conductance (g_s) (moles $m^{-2} \cdot s^{-1}$)	Transpiration (*E*) (moles $m^{-2} \cdot s^{-1}$)	Water use efficiency (*WUE*) (Pn/g_s)
Control 44 P	0	8.4 a [w]	0.05 a	2.7 a	158.1 c
Control [x]		6.5 abc	0.03 bc	1.5 bc	229.4 c
AMF [xy]		5.9 bcd	0.03 bcd	1.3 bcd	229.8 c
SpCe [xz]		6.9 ab	0.04 b	1.9 ab	212.3 c
AMF+SpCe [x]		7.3 ab	0.04 ab	1.9 ab	189.7 c
Control 44 P	6,000	4.3 de	0.01 cd	0.8 cd	298.9 bc
Control [x]		3.9 e	0.01 d	0.5 d	551.9 a
AMF [x]		4.8 cde	0.02 cd	0.9 cd	297.0 bc
SpCe [x]		5.7 bcde	0.02 cd	0.9 cd	319.2 abc
AMF+SpCe [x]		4.8 cde	0.01 cd	0.7 cd	484.2 ab
Significance					
	ACO	0.001	0.001	0.001	0.001
	RM	NS	NS	NS	NS
	ACO x RM	NS	NS	NS	NS

[w]Means in the same column followed by the same letter are not significantly different (LSD, α= 0.05). NS= Nonsignificant, n=3. [x]Treatments fertilized with Long Ashton Nutrient Solution (LANS), modified to supply 30 µg P mL^{-1}; [y]AMF= *Glomus intraradices*; [z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

Total chlorophyll content was significantly ($P \leq 0.05$) increased by ACO, RM, and the ACO x RM interaction (Table 6.4). Plants exposed to ACO had higher total chlorophyll ($P \leq 0.05$), chlorophyll *a* ($P \leq 0.01$), and chlorophyll *a*/*b* ratio ($P \leq 0.05$) than plants in non-contaminated soil (Table 6.4). RM significantly affected ($P \leq 0.001$) chlorophyll *b* and the chlorophyll *a*/*b* ratio. The interaction of ACO x RM had no significant effect on chlorophyll *a*, *b* and *a*/*b* ratio (Table 6.4).

Leaf nitrate reductase and proline of *L. multiflorum* were significantly ($P \leq 0.01$) affected by ACO, RM and the interaction of ACO x RM; however, RM had no significant effect on leaf nitrate reductase (Table 6.5). Nitrate reductase activity and proline were significantly increased ($P \leq 0.001$) in plants exposed to ACO (Table 6.5).

Table 6.4. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on leaf chlorophyll of *Lolium multiflorum* cv. Passerel Plus, after 80 days.

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	Chlorophyll content Total	*a*	*b*	Chlorophyll ratio *a*/*b*
		($\mu g \cdot g^{-1}$)			
Control 44 P	0	804.6 cd [w]	620.1 bc	184.9 cd	3.4 ab
Control [x]		627.6 d	453.4 c	174.5 d	2.6 abc
AMF [xy]		871.8 bcd	617.2 bc	255.1 bcd	2.6 abc
SpCe [xz]		1101.4 abc	647.7 bc	454.4 a	1.4 d
AMF + SpCe [x]		1094.8 abc	614.6 bc	480.9 a	1.3 d
Control 44 P	6,000	792.5 cd	590.7 bc	202.4 cd	3.1 abc
Control [x]		1063.4 abc	829.5 ab	234.6 cd	3.5 a
AMF [x]		1296.3 a	952.9 a	317.1 bc	3.3 abc
SpCe [x]		898.3 bcd	624.2 bc	274.7 bcd	2.2 bcd
AMF + SpCe [x]		1208.8 ab	827.0 ab	382.5 ab	2.2 cd
Significance					
	ACO	0.05	0.01	NS	0.05
	RM	0.05	NS	0.001	0.001
	ACO x RM	0.05	NS	NS	NS

[w]Means in the same column followed by the same letter are not significantly different (LSD, $\alpha = 0.05$). NS= Nonsignificant, n=3. [x]Treatments fertilized with Long Ashton Nutrient Solution (LANS), modified to supply 30 µg P mL^{-1}; [y]AMF= *Glomus intraradices*; [z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

Table 6.5. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on leaf nitrate reductase activity, proline, antioxidant activity, and total phenolics of *Lolium multiflorum* cv. Passerel Plus, after 80 days.

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	NO_3-reductase ($\mu M\ NO_2\ g^{-1}$)	Proline ($\mu g \cdot g^{-1}$)	Antioxidant activity (μM Trolox g^{-1})	Total phenolics (μg chlorogenic acid g^{-1})
Control 44 P	0	174.6 cde [w]	1.8 cd	7821.3 a	4502.9 b
Control [x]		186.7 bcd	0.8 de	1377.5 d	4483.8 b
AMF [xy]		136.1 de	0.5 e	1544.2 cd	3647.6 bcd
SpCe [xz]		172.7 cde	0.9 de	1513.0 cd	6274.5 a
AMF + SpCe [x]		123.7 e	1.4 de	1448.4 cd	4187.8 bc
Control 44 P	6,000	152.7 de	0.9 de	6534.5 b	1920.2 f
Control		233.3 ab	3.8 ab	1685.6 cd	3406.8 cde
AMF		214.6 abc	0.9 de	1807.9 c	2962.4 de
SpCe		251.1 a	5.0 a	1561.4 cd	2629.6 ef
AMF + SpCe		229.1 abc	3.1 bc	1624.8 cd	3171.6 de
Significance					
	ACO	0.001	0.001	0.001	0.001
	RM	NS	0.001	0.001	0.001
	ACO x RM	0.05	0.001	0.001	0.001

[w]Means in the same column followed by the same letter are not significantly different (LSD, α= 0.05). NS= Nonsignificant, n=3.[x]Treatments fertilized with Long Ashton Nutrient Solution (LANS), modified to supply 30 µg P mL^{-1}; [y]AMF= *Glomus intraradices*; [z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

Antioxidant activity and total phenolics were significantly ($P \leq 0.001$) affected by ACO, RM, and their interaction (Table 6.5). Control-44 µg P mL^{-1} plants in non-contaminated or ACO-contaminated soil had significantly higher antioxidant activity than other RM treatments at 30 µg P mL^{-1}. For total phenolics, in non-contaminated soil, plants inoculated with SpCe had significantly higher phenolic content (Table 6.5); while control-44 µg P mL^{-1} plants in ACO-contaminated soil had significantly lower total phenolics than the other RM treatments with 30 µg P mL^{-1} (Table 6.5).

Microbial Populations in the Rhizosphere and Mycorrhizal Colonization

Populations of total bacteria, bacteria able to grow on N-free medium, and filamentous fungi populations analyzed as logarithmic units, were significantly ($P \leq 0.001$) affected by the ACO, RM, and the interaction ACO x RM (Table 6.6; see Appendix I, Table AI-6.1 for data analyzed by the actual microbial counts). Total bacteria and filamentous fungi were generally stimulated when the rhizosphere was contaminated with ACO, compared to non-contaminated soil. However, the lowest bacterial and fungal populations in ACO-contaminated soil, were with control-44 μg P mL^{-1} treated plants (Table 6.6).

Table 6.6. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on logarithmic colony forming units ($Log_{10}CFU$) of total bacteria, filamentous fungi, and bacteria able to grow on N-free medium, in the rhizosphere of *Lolium multiflorum* cv. Passerel Plus after 80 days.

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	Total bacteria ($Log_{10}CFU$)	Filamentous fungi ($Log_{10}CFU$)	Bacteria able to grow on N-free medium ($Log_{10}CFU$)
Control 44 P	0	6.77 f [w]	3.60 e	5.90 ab
Control [x]		6.87 de	3.77 e	5.07 f
AMF [xy]		6.73 f	4.47 b	5.88 abc
SpCe [xz]		6.90 d	3.60 e	5.76 cd
AMF + SpCe [x]		6.80 ef	4.03 d	5.97 a
Control 44 P	6,000	7.10 c	3.10 f	5.80 bcd
Control		7.50 a	4.30 bc	5.18 f
AMF		7.47 a	4.10 cd	5.99 a
SpCe		7.23 b	5.33 a	5.51 e
AMF + SpCe		7.50 a	5.40 a	5.71 d
Significance:				
	ACO	0.001	0.001	0.05
	RM	0.001	0.001	0.001
	ACO x RM	0.001	0.001	0.001

[w]Means in the same column followed by the same letter are not significantly different (LSD, α= 0.05). n=5

[x]Treatments fertilized with Long Ashton Nutrient Solution, modified to supply 30 μg P mL^{-1}

[y]AMF= *Glomus intraradices*

[z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

See Appendix I, Table AI-6.1 for data analyzed by the actual microbial counts.

For bacteria able to grow on N-free medium, ACO generally resulted in significantly lower populations (Table 6.6). With non-contaminated soil, the inoculation of AMF, AMF+SpCe, or control-44 μg P mL^{-1} plants significantly (LSD, α=0.05) enhanced the proliferation of this bacterial group compared to control plants (Table 6.6). In ACO-contaminated soil, the highest population of these bacteria occurred with AMF-plants, while the control at 30 μg P mL^{-1} had the lowest population (Table 6.6).

The main effect of ACO significantly ($P \leq 0.001$) reduced total mycorrhizal colonization, but had no effect on arbuscules and vesicles formation (Fig. 6.1A-C). The main effects of RM (AMF or AMF+ScCe), and the interaction of ACO x RM had no effect on total colonization, arbuscule, and vesicle formation. AMF-structures were not found in plants without AMF-inoculation.

Total Petroleum Hydrocarbon Degradation

Rhizosphere management (RM) resulted in significant ($P \leq 0.001$) effects on TPH-degradation in the rhizosphere of *Lolium multiflorum* (Fig. 6.2A). The lowest TPH-degradation was with control plants, while the highest degradation occurred with plants inoculated with AMF+SpCe. In general, all inoculated plants and control-44 μg mL^{-1} had significantly greater TPH-degradation than the control at 30 μg P mL^{-1} (Fig. 6.2A).

The treatment effect efficiency (TEE) on TPH-degradation mirrored that of the TPH-degradation. Plants inoculated with AMF+SpCe had 60% more TPH-degradation than the 30 μg P mL^{-1} control plants, while control-44 μg mL^{-1}, SpCe or AMF plants, had 34%, 26%, and 24% more TEE of TPH-degradation, respectively, than the 30 μg P mL^{-1} control plants (Fig. 6.2B).

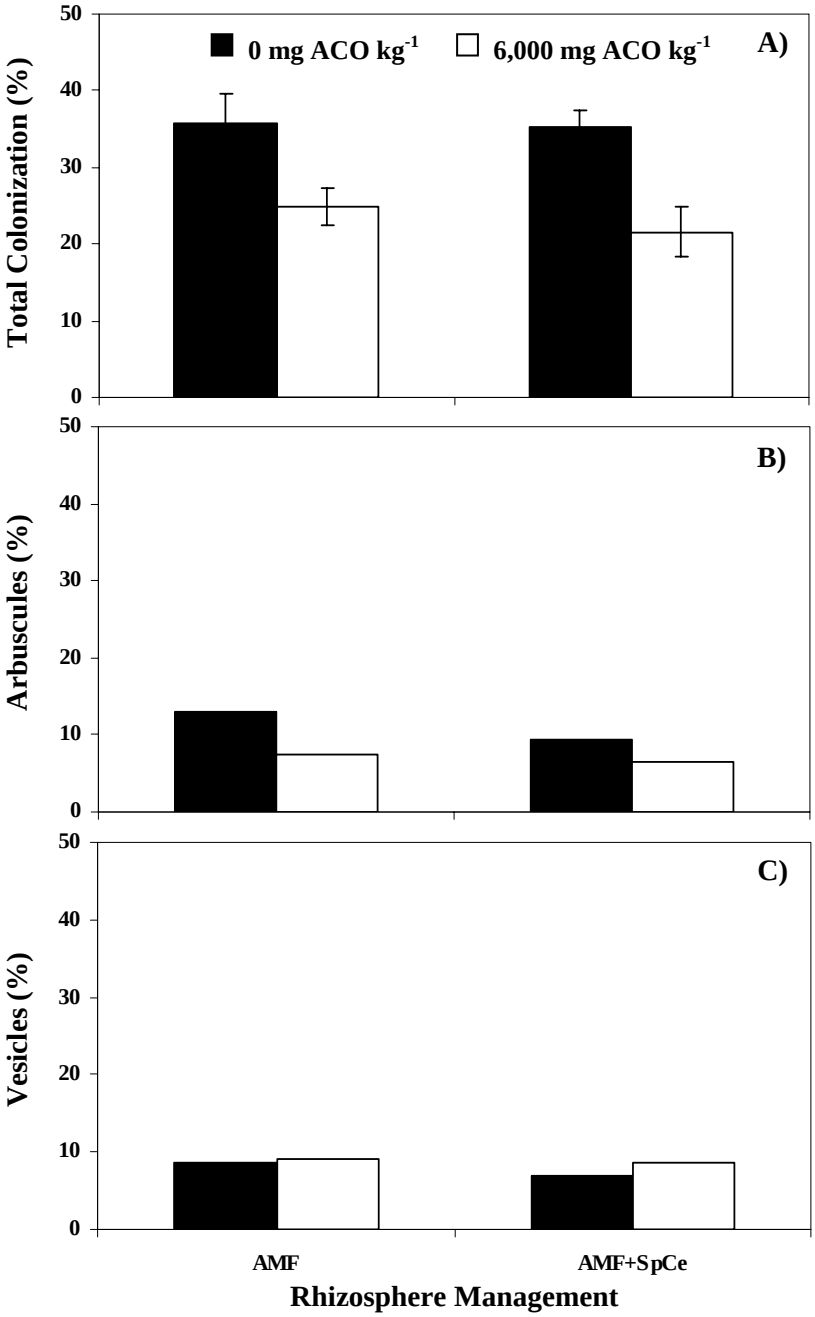

Fig. 6.1. Effect of hydrocarbonoclastic microorganisms and ACO contamination on the colonization of *Glomus intraradices* (AMF) in roots of *Lolium multiflorum* cv. Passerel Plus after 80 days. SpCe=*Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*. Main effects of Arabian medium crude oil (ACO) were significant only for total colonization ($P \leq 0.001$). Main effects of Rhizosphere management (RM) and ACO x RM interaction were not significant for total colonization, arbuscules or vesicles. Bars ± SE, n=3.

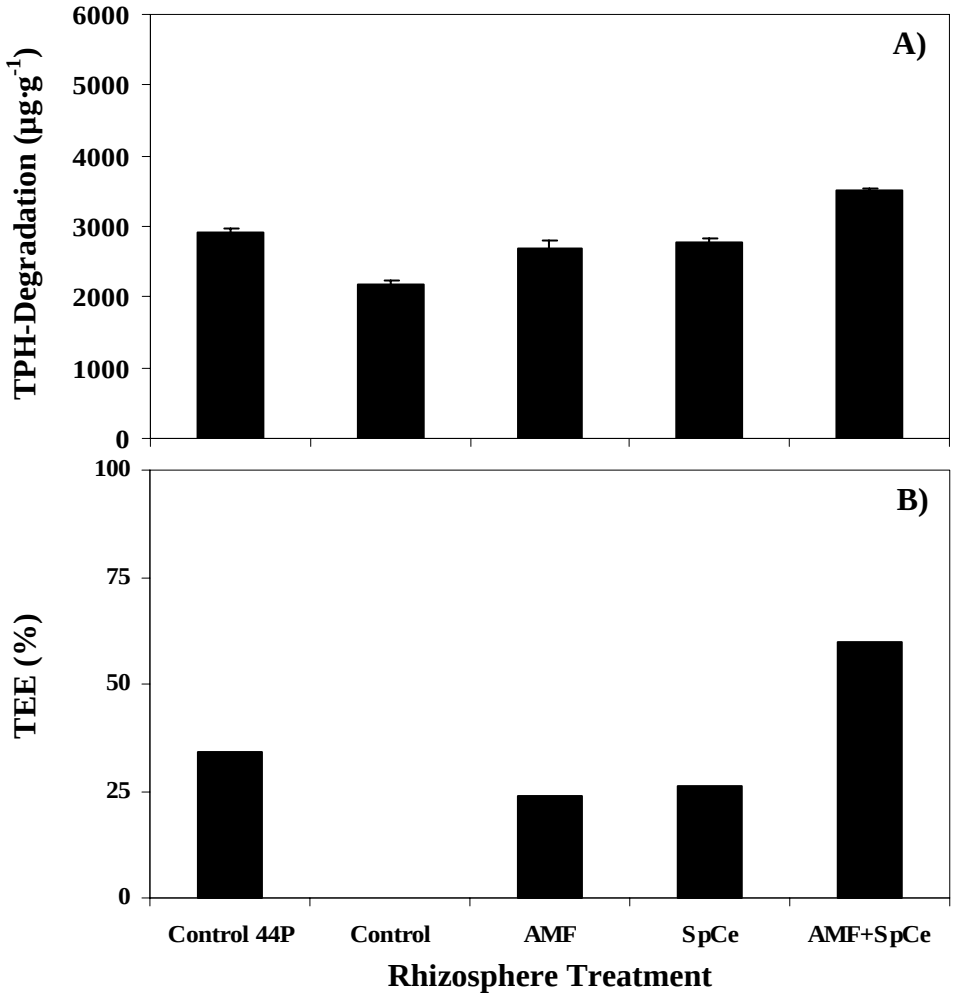

Fig. 6.2. A) Effect of hydrocarbonoclastic microorganisms [*Sphingomonas paucimobilis* (Sp) and *Cunninghamella echinulata* var. *elegans* (Ce)], *Glomus intraradices* (AMF), and higher fertility [control with modified Long Ashton Nutrient Solution (LANS) to supply 44 µg P mL^{-1} (Control 44P)] on total petroleum hydrocarbon (TPH) degradation in the rhizosphere of *Lolium multiflorum* contaminated with Arabian medium crude oil (ACO) at 6,000 $mg{\cdot}kg^{-1}$)], after 80 days. All treatments supplied with 30 µg P mL^{-1} LANS, except Control 44P. Main effect of treatment was significant at $P \leq 0.001$. n=3. B) Treatment Efficiency Effect (TEE) of TPH degradation. TEE (%)= [(TPH degradation of treated plants – control plants)/(control plants)] x 100.

Discussion

This research is one of the first studies that demonstrates the importance of bioaugmentation utilizing a mixture of hydrocarbonoclastic microorganisms with AMF during phytoremediation of soil contaminated with Arabian medium crude oil (ACO) with a *L. mutiflorum* system.

Plant Growth Responses

ACO in soil caused a significant reduction in plant growth (leaf area, leaf, pseudostem, root and total plant DW). ACO reduced leaf area and total plant DW by -91% and -96%, respectively. Inoculation with AMF or application of 44 µg P mL^{-1} resulted in significantly greater plant growth than control plants only in non-contaminated soil. Although no significant effects were observed, bioaugmentation with microorganisms and biostimulation with 44 µg P mL^{-1}, resulted in two-fold increased leaf area and total plant DW of ACO-treated plants. Results on impaired plant growth of *L. multiflorum* in ACO-contaminated soil were in agreement with reports for other plant species (Adam and Duncan, 2003; Malallah et al., 1996; Quiñones-Aguilar et al., 2003).

Bioaugmentation with AMF+SpCe in ACO-contaminated soil resulted in significantly higher SLA and LAR than control plants. ACO also increased SLA and LAR, and decreased RSR. These plant growth ratios can be useful in determining how plants cope with stressful environmental conditions (Wright and Westoby, 2001). Increased SLA (thinner leaves) reflects more leaf area that may contribute to enhanced light capture per unit of leaf mass (Westoby, 1998; Reich et al., 1997), while LAR indicates the efficiency of a given leaf area to produce a given plant size (Hunt, 1982;

Lafarge and Hammer, 2002). Since SLA and LAR were significantly increased by AMF+SpCe plants exposed to ACO-contaminated soil, plants in this treatment may have better adaptation.

Plant Gas Exchange, Chlorophyll Content and Selected Physiological Responses

Photosynthesis, stomatal conductance, and transpiration of *L. multiflorum* plants were significantly diminished by ACO. Furthermore, neither bioaugmentation with the microorganisms nor biostimulation with 44 μg P mL^{-1} overcame the deleterious effect of ACO. The negative effects of PH on photosynthesis of marine and terrestrial plants have been previously described (Baker, 1970; Daly et al., 1988; Durako et al., 1993; Macinnis-Ng and Ralph, 2003).

Conversely, water use efficiency (*WUE*) was significantly higher (+48%) with plants exposed to ACO-contamination than plants in non-contaminated soil. *WUE* can be used to determine the plant performance under stressful conditions, since it relates carbon gain and biomass accumulation to transpiration and water loss (Kramer and Boyer, 1995); this is in agreement with the observed decreased growth, *Pn* and g_s of *L. multiflorum* plants exposed to ACO-contamination. *WUE* is the ratio of carbon fixed into dry weight to the total amount of water lost by evapotranspiration (Klingeman et al., 2005) or Pn/g_s, and indicates the biomass accumulated per total water consumed (Davies et al., 2002). Thus, high *WUE* under ACO-contamination suggests that plants are more efficiently accumulating biomass (μmoles CO_2 $m^{-2} \cdot s^{-1}$) per mole of water utilized or lost, albeit at a much reduced rate.

Plants exposed to ACO had an increased total chlorophyll and chlorophyll *a* as well as a higher chlorophyll *a*/*b* ratio, though nonsignificant effects of ACO were observed for chlorophyll *b*. Neither microbial inoculation nor application of 44 μg P mL^{-1} resulted in increased chlorophyll content compared to control plants. Similar responses on increased chlorophyll and chlorophyll *a*/*b* ratio have been reported for Non-AMF plants grown in soil contaminated with organic compounds (Huang et al., 2004). The chlorophyll *a*/*b* ratio generally increases when plants are nutrient deficient (Kitajama and Hogan, 2003) and consequently under stress. Furthermore, increased chlorophyll *a*/*b* ratio can be used as an indicator of stress in plants so that it may represent higher sensitivity to light by which electron transport from PSII to PSI is impeded. Then, photochemical oxidation of light harvesting complexes that bind chlorophyll *b* can occur (Huang et al., 1997). However, further research is needed to understand the benefits of free-living and symbiotic rhizosphere microorganisms on the alleviation of the stress induced by ACO-contaminated soil on the photosynthetic apparatus of *L. multiflorum*.

Enzymatic and biochemical responses of plants during phytoremediation of PH in soils have been little studied (Malallah et al., 1996). In our study, leaf nitrate reductase activity was significantly higher in plants growing at ACO-contaminated soil. Nitrate reductase in control plants in ACO-soil increased ~25%, while plants inoculated with AMF, SpCe, and AMF+SpCe showed an increase of ~58%, ~45%, and 85%, respectively, when compared to their corresponding treatment at non-contaminated soil. This effect indicates that microbial inoculation stimulated N-assimilation in plants under ACO-contaminated soil through enhanced nitrate reductase. In this respect, the nitrate

reductase is an important enzymatic activity in plants exposed to different environmental stresses (Foyer et al., 1998; Sinha and Nicholas, 1981; Taiz and Zeiger, 2002).

The presence of ACO in soil significantly increased the proline in plants, except for control-44 μg P mL^{-1} and for AMF-inoculated plants, compared to control-30 μg P mL^{-1} plants. Proline in plants can be a water stress indicator (Aspinall and Paleg, 1981) induced by PH in soils due to their hydrophobicity properties (Binet et al., 2000a; Qiu et al., 1994; Schwab and Banks, 1994). The low proline content in either AMF plants or control-44 μg P mL^{-1} may indicate that these plants are less affected by the ACO-induced stress. This suggests that either AMF-inoculation or improved P-nutrition may contribute towards PH stress alleviation, as also indicated for other non-PH stress conditions (Diouf et al., 2005; Ramakrishnan et al., 1988; Ruiz-Lozano et al., 1995; Ruiz-Lozano et al., 1996; Wu and Xia, 2005). However, more research is required to understand these benefits on plants exposed to PH-contaminated soils.

Antioxidant activity in control-44 μg P mL^{-1} was significantly reduced ($P \leq 0.001$) by ACO-contamination, compared to the same treatment at non-contaminated soil. Regardless of ACO-contamination, the antioxidant activity of control-44 μg P mL^{-1} plants was significantly higher than control-30 μg P mL^{-1} and plants bioaugmented with the microorganisms, which did not show a consistent or significant trend in antioxidant activity. In contrast, both controls (at 30 and 44 μg P mL^{-1}) or inoculated plants in ACO-contaminated soil had significantly reduced phenolics (-39%) than plants at non-contaminated soil. Generally, in ACO-contaminated soil control-30 μg P mL^{-1} and inoculated plants in ACO-contaminated soil, except SpCe plants, had significantly greater phenolics than control-44 μg P mL^{-1}.

The reduction in phenolic compounds by ACO is in agreement with reports of other researchers studying non-inoculated plant species exposed to PH-contaminated soil (Ilangovan and Vivenkanandan, 1992; Malallah et al., 1996). While the inoculation of plants under abiotic stress with AMF seems to confer more plant protection to oxidative damage (Alguacil et al., 2003; Porcel et al., 2003; Ruiz-Lozano, 2003; Wu et al., 2006), neither AMF nor SpCe enhanced plant antioxidant and phenolics in ACO-contaminated soil. However, the effects of AMF and hydrocarbonoclastic microorganisms on plant protection against oxidative damage had not been previously reported during phytoremediation of PH-contaminated soil.

Microbial Populations in the Rhizosphere and Mycorrhizal Colonization

ACO increased the populations of total bacteria and filamentous fungi, but reduced population of bacteria able to grow on N-free medium. However, the highest population of N_2-fixing free bacteria occurred with AMF in ACO-contaminated soil, and with AMF+SpCe in non-contaminated soil. The rhizosphere microbial populations may enhance plant adaptation to PH-contaminated soils by detoxifying soils through mineralization of organic contaminants directly or as a result of cooxidation and cometabolism processes (Barea et al., 2005; Dec et al., 2002; Jeffries et al., 2003; Robson et al., 2004; Siciliano and Germida, 1998).

Total colonization by AMF was significantly decreased from 35% in non-contaminated to 23% in plants exposed to ACO. Arbuscules, which are the exchange site between host plant and symbiont, were also reduced by ACO, whereas vesicles, which are the main fungal structure for storage in the root system, were not affected. Negative

effects of crude oil and other fractions of petroleum on AMF-colonization have been previously reported (Cabello, 1997; Gaspar et al., 2002; Leyval y Binet, 1998). Importantly, bioaugmentation with hydrocarbonoclastic microorganisms (SpCe) did not negatively affect AMF-colonization in roots.

Total Petroleum Hydrocarbon Degradation

The TPH-degradation in the rhizosphere of *L. multiflorum* was significantly enhanced by either the application of 44 μg P mL^{-1} or bioaugmentation, particularly with AMF+SpCe. TPH-degradation at control-44 μg P mL^{-1} was 34% more than control plants at 30 μg P mL^{-1}. For inoculated plants, all of which were treated with LANS at 30 μg P mL^{-1}, the extent of TPH-degradation was 23%, 26%, and 60% greater with AMF, SpCe, and AMF+SpCe, respectively, when compared to the non-inoculated control with 30 μg P mL^{-1}. Plants inoculated with AMF+SpCe had 26% more TPH-degradation than control-44 μg P mL^{-1} plants.

The biostimulation with 44 μg P mL^{-1} in control plants, enhanced TPH-degradation. In this case, P is a critical nutrient during bioremediation of PH (Chang et al., 1996), and in this study, P not only enhanced adaptation and growth of *L. multiflorum* but also improved phytoremediation of ACO, which may be due in part, to supporting rhizosphere bacterial activity and plant growth.

On the other hand, the inoculation with a mixture of beneficial bacteria such as *Pseudomonas putida*, *Azospirillum brasilense*, and *Enterobacter cloacae* to grass species enhances the effective remediation of organic contaminants (Huang et al., 2004). In addition, AMF are an important rhizosphere component that contributes to the alleviation

of toxic effects induced by PH, as well as to enhanced growth, tolerance and dissipation of contaminants in the rhizosphere (Cabello, 1999; Joner and Leyval, 2003a, Joner and Leyval, 2003b; Leyval and Binet, 1998; Volante et al., 2005).

This study is one of the first reports of the interaction of hydrocarbonoclastic microorganisms (bacteria and filamentous fungi) with AMF on growth and physiological responses of plants during phytoremediation of PH. Furthermore, this is one of the first reports on the microbial synergism between the AMF and two hydrocarbonoclastic microorganisms in stimulating higher TPH-degradation in the rhizosphere of the *L. multiflorum* system. The results suggest that AMF can indirectly enhance phytoremediation of PH in soils by enhancing the activity of hydrocarbonoclastic bacteria and filamentous fungi. Although the mechanisms are not well understood, this study supports the hypothesis that AMF may induce changes in plant physiology, and create favorable microenvironments (mycorrhizosphere/hyphosphere effect) that allow the proliferation and activity of hydrocarbonoclastic microorganisms during phytoremediation of PH (Criquet et al., 2000; Joner and Leyval, 2003b; Rillig, 2004; Sylvia, 2005). The indirect effects of AMF are a significant benefit for plants used in the phytoremediation of a complex mixture of PH such as ACO.

Summary

Plants can phytoremediate soils contaminated with petroleum hydrocarbons (PH), however, bioaugmentation with hydrocarbonaclastic microorganisms and arbuscular mycorrhizal fungi (AMF) is not well understood. Phytoremediation of Arabian medium crude oil (ACO) was done with a *Lolium multiflorum* plant system inoculated with an

AMF [*Glomus intraradices*], a mixture of hydrocarbonoclastic microorganisms [the bacteria, *Sphingomonas paucimobilis* EPA-505 (Sp) and the filamentous fungus, *Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce)], or with a combination of microorganisms (AMF+SpCe). A glasshouse experiment was conducted with *L. multiflorum* plants exposed to ACO-contaminated soil (6000 $mg \cdot kg^{-1}$). A modified Long Ashton Nutrient Solution (LANS) was supplied to all treatments at 30 μg P mL^{-1}, except for a second control treatment at 44 μg P mL^{-1}. After 80 days, ACO-contamination reduced plant growth, photosynthesis, stomatal conductance, transpiration, leaf phenolics, but increased water use efficiency, total chlorophyll, nitrate reductase, and proline. Plant growth and physiological responses were not significantly enhanced by 44 μg P mL^{-1} or bioaugmentation. ACO generally increased total bacteria and filamentous fungi, while bacteria able to grow on N-free medium decreased (except for an increase with AMF plants). While total colonization and arbuscule formation were reduced by ACO-contamination, average colonization was ~20% and ~8%, respectively. There were no adverse effects of SpCe on the AMF symbiosis in roots. Most importantly, TPH-degradation was significantly enhanced by 44 μg P mL^{-1} and the microbial inoculation. Highest TPH-degradation and efficiency on TPH-degradation was observed with AMF+SpCe (60%), followed by control plants at 44 μg P mL^{-1} (34%). Hence, there was a beneficial synergism between hydrocarbonoclastic microorganisms and AMF on TPH-degradation. Phytoremediation of ACO was also improved by the single inoculation of AMF or SpCe, compared to controls.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Summary

Phytoremediation utilizes plants to cleanup soils contaminated with organic or inorganic contaminants, in part, through improvement of soil aeration and stimulation of rhizosphere microbial activity via root exudation. Bioaugmentation with hydrocarbonoclastic bacteria, filamentous fungi, and arbuscular mycorrhizal fungi (AMF) may enhance phytoremediation. However, the effect of inorganic nutrients (biostimulation) and bioaugmentation with microorganisms beneficial to plants during phytoremediation is not well understood.

The objective of this dissertation was to evaluate the effects of AMF on plant growth, selected physiological responses, and degradation of PH in soil in combination with biostimulation and/or bioaugmenation with hydrocarbonoclastic bacteria and a filamentous fungus during phytoremediation.

The first study (Chapter III) was conducted to screen and select a grass species (Poaceae) tolerant to increased levels of Arabian medium crude oil (ACO) to determine the most suitable grass species and critical petroleum concentrations for subsequent phytoremediation experiments. Among five grass species tested, *Lolium multiflorum* Lam. cv. Passerel Plus was selected as the most tolerant, based on seed germination and growth responses to ACO in a sand-sandy loam soil mixture (1:1 v/v). ACO concentrations selected for a subsequent experiment, were 3000 and 15000 $mg \cdot kg^{-1}$.

The second study (Chapter IV) evaluated the effects of inoculation with *Glomus intraradices* (AMF) and fertilization with three levels of Long Ashton Nutrient Solution [(LANS) 0.5X, 1X, and 2X] on plant growth, selected physiological responses, and phytoremediation of total petroleum hydrocarbons (TPH) in a soil contaminated with ACO, at three levels: 0, 3000 and 15000 $mg \cdot kg^{-1}$. Plant growth, photosynthesis, and nutrient uptake in shoots were significantly reduced by increased ACO. Growth, photosynthesis and nutrient uptake of plants in ACO-contaminated soil were enhanced by LANS. Microbial populations and soil respiration were stimulated by ACO and LANS. Regardless of ACO concentration, total phenolics and antioxidant activity in leaves decreased with increasing LANS. Increasing ACO concentrations resulted in decreased ascorbate content at 0.5X and 1X LANS, but increased at 2X LANS. AMF had minimal effects on plant growth, photosynthesis, and nutrient content, depending on ACO and LANS combinations. Mycorrhizal colonization and arbuscule formation was observed at all ACO levels. At 3000 mg ACO kg^{-1}, TPH-degradation was >60% in all rhizosphere treatments, while at 15000 mg ACO kg^{-1} higher TPH-degradation occurred at 0.5X LANS. Neither LANS nor AMF consistently increased TPH-degradation. LANS and ACO significantly affected soil pH, which ranged from 6.9 to 8.9; while LANS increased EC. No toxic effects of salt accumulation occurred.

The third study (Chapter V) evaluated inoculation with the hydrocarbonoclastic bacterium, *Sphinghomonas paucimobilis* EPA505 (Sp), and a filamentous fungus, *Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce), and AMF, *Glomus intraradices*, in a *L. multiflorum* system during phytoremediation of sand contaminated with benzo[*a*]pyrene (BaP) at 100 $mg \cdot kg^{-1}$. The effectiveness of bioaugmentation with

Ce, Sp, or Sp+Ce on phytoremediation and bioremediation of BaP was also determined. Microbial inoculation did not significantly enhance plant growth during phytoremediation of BaP, although Ce, AMF, and Ce+Sp+AMF significantly reduced total plant dry weight and the root-to-shoot ratio, compared to control plants. Microbial inoculation did not significantly enhance photosynthesis, stomatal conductance, chlorophyll, proline, nitrate reductase, total phenolics, antioxidant activity, and rhizosphere dehydrogenase activity. The inoculation of Ce+Sp did not adversely affect total root colonization and arbuscule formation of AMF. Single or combined microbial inoculation significantly enhanced BaP-degradation in the rhizosphere of *L. multiflorum* when compared to the control, but inoculation with Ce or Ce+Sp+AMF had the highest BaP-degradation. Bioaugmentation with Ce, Sp, and Ce+Sp enhanced bioremediation and phytoremediation of BaP; however, bioremediation was more efficient than phytoremediation.

The fourth study (Chapter VI) evaluated phytoremediation of ACO utilizing a microbial mixture of Sp and Ce (SpCe), AMF, and their combination on plant growth, selected physiological responses, and TPH-degradation with *L. multiflorum*. Inoculated plants and a control were irrigated with LANS modified to supply 30 μg P mL^{-1}, and an additional control with 44 μg P mL^{-1} was included. After 80 days, 6,000 mg ACO kg^{-1} reduced plant growth, photosynthesis, stomatal conductance, transpiration, leaf phenolics, but increased water use efficiency, total chlorophyll, nitrate reductase, and proline. Plant growth and physiological responses were not significantly enhanced by 44 μg P mL^{-1} or bioaugmentation. ACO generally increased total bacteria and filamentous fungi, while bacteria able to grow on N-free medium decreased (except for an increase in AMF

plants). While total colonization and arbuscule formation were reduced by ACO-contamination, there were no adverse effects of SpCe on AMF symbiosis. TPH-degradation was significantly enhanced by 44 μg P mL^{-1} and the microbial inoculation. Highest TPH-degradation was observed with AMF+SpCe (60%), followed by control plants at 44 μg P mL^{-1} (34%). A beneficial synergism occurred among the hydrocarbonoclastic microorganisms and AMF in TPH-degradation during phytoremediation, which was also enhanced by the single inoculation of AMF and SpCe when compared to controls.

Conclusions

The present research demonstrated variations among grass species from the same botanical family in their tolerance to petroleum contaminated soil. *Festuca arundinacea* and particularly *Lolium multiflorum*, showed greater seed germination than *Paspalum notatum*, *Cynodon dactylon*, and *Poa pratensis*.

Most importantly, the results of this research provided further evidence about the effects of AMF on improving the phytoremediation of petroleum hydrocarbons in soils when inoculated with *Lolium multiflorum* Lam. cv. Passarel Plus.

In addition, the concentration of petroleum hydrocarbons in soil was determining factor of potemtial benefits of AMF on *L. multiflorum*. Low (3000 $mg \cdot kg^{-1}$) or high (15000 $mg \cdot kg^{-1}$) concentrations of Arabian medium crude oil (ACO) resulted in limited benefits of AMF, *Glomus intraradices*, on plant growth, physiology, and degradation of ACO in soil. However, when plants were exposed to an intermediate ACO concentration in soil (6000 $mg \cdot kg^{-1}$), AMF plants had enhanced growth, physiological responses and

greater ACO-degradation in comparison with Non-AMF plants. AMF symbiosis was observed at all concentrations of ACO-contaminated soil.

This research is one of the first reports demonstrating the benefits of AMF on the degradation of benzo[*a*]pyrene or ACO in combination with hydrocarbonoclastic bacteria (*Sphingomonas paucimobilis* EPA-505) or filamentous fungi (*Cunninghamella echinulata* var. *elegans* ATCC-36112). Thus, AMF resulted in a beneficial synergism with the hydrocarbonoclastic microorganisms, particularly during ACO-degradation in the rhizosphere of *L. multiflorum*.

Thus, further studies must be conducted to understand the effects of AMF on plants exposed to different concentrations of PH and to single or mixtures of specific petroleum fractions such as polycyclici aromatic hydrocarbons. In the same manner, the physiological responses of AMF, and their interaction with different hydrocarbonoclastic microorganisms during phytoremediation merits future research.

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APPENDIX I

Table AI-3.1. Seed germination of *Lolium multiflorum* and *Festuca arundinacea* exposed to several concentrations of Arabian medium crude oil (ACO) in a sandy soil, after 20 days (Chapter III).

ACO-Treatment ($mg \cdot kg^{-1}$)	Germination (%)	
	Lolium multiflorum	*Festuca arundinacea*
Control	92.0 a[z]	72.0 de
CH_2Cl_2[y]	95.0 a	86.0 abc
150	97.0 a	87.0 ab
300	98.0 a	74.0 cd
1,000	97.0 a	72.0 de
3,000	96.0 a	60.0 e
5,000	90.0 a	67.0 de
10,000	87.0 ab	42.0 f
15,000	76.0 bcd	28.0 g
30,000	31.0 fg	11.0 h
45,000	32.0 fg	5.0 h
60,000	13.0 h	3.0 h
120,000	2.0 h	1.0 h
Significance:		
ACO	0.001	
Grass species	0.001	
ACO x Grass species	0.001	

[y]Solvent (Dichloromethane) applied to reduce the oil viscosity and allow ACO homogenization in the soil.

[z]Means followed by the same letter between columns are not significantly different (LSD, α=0.05); NS= Nonsignificant, n=5 with 5 petri dishes containing 20 seeds per petri dish.

Table AI-4.1. Modified Long Ashton Nutrient Solution Composition, full strength (1X). (Chapter IV).

Stock Solution	Amount of reagent to weight ($g \cdot L^{-1}$)	Amount of stock to use to make 1 L (mL)
KNO_3	80.8	5.0
$MgSO_4 \cdot 7H_2O$	73.6	5.0
$Ca(NO_3)_2 \cdot 4H_2O$	188.8	5.0
$NaH_2PO_4 \cdot H_2O$	36.8	1.25 for 10.25 $\mu g \cdot mL^{-1}$ 2.50 for 20.5 $\mu g \cdot mL^{-1}$ 5.0 for 41 $\mu g \cdot mL^{-1}$
Trace elements		1.0
Citrate solution (Add just before fertilizing)		5.0
Trace Element Stock Solution: • Make up to 1000 mL with distilled water		
$MnSO_4 \cdot H_2O$	1.69	
$CuSO_4 \cdot 5H_2O$	0.25	
$ZnSO_4 \cdot 7H_2O$	0.29	
H_3BO_3	3.10	
NaCl	5.90	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.088	
Citrate Stock Solution: • Make up to 1000 mL with distilled dionized water • Stir for a few minutes and autoclaved for complete dissolution • Store in cooler or refrigerator		
Ferric citrate ($FeC_6H_5O_7$)	4.9	
Citric acid ($H_3C_6H_5O_7 \cdot H_2O$)	4.9	

Hewitt (1966).

Table AI-4.2. Microbial population in the rhizosphere of non-mycorrhizal (Non-AMF) and mycorrhizal (AMF)-*Lolium multiflorum* treated with Long Ashton Nutrient Solution (LANS) in soil contaminated with Arabian medium crude oil (ACO), after 80 days. (Chapter IV).

ACO ($mg \cdot kg^{-1}$)	LANS strength (X)	Mycorrhizal	Microbial colony forming units (CFU)		
			Total bacteria ($CFUx10^6\ g^{-1}$ soil)	Bacteria growing at N-free medium ($CFUx10^6\ g^{-1}$ soil)	Filamentous fungi ($CFUx10^3\ g^{-1}$ soil)
0	0.5	No	20.8 efgh [y]	8.4 cd	5.4 cde
		Yes	20.4 efgh	5.8 ef	3.4 efg
	1	No	15.7 efgh	7.4 de	5.6 cde
		Yes	26.2 ef	9.9 bc	4.2 def
	2	No	22.4 efg	11.5 ab	7.6 bc
		Yes	20.0 efgh	12.7 a	5.2 cde
3,000	0.5	No	27.4 ef	3.7 ghi	3.6 efg
		Yes	32.6 e	4.0 ghi	2.4 fg
	1	No	11.2 fgh	3.5 hi	2.0 fg
		Yes	7.2 hg	2.9 ij	2.5 fg
	2	No	10.2 fgh	3.2 ij	2.0 fg
		Yes	12.8 fgh	5.2 fgh	3.6 efg
15,000	0.5	No	114.4 c	10.5 b	9.0 ab
		Yes	143.6 b	5.4 fg	6.4 cd
	1	No	2.0 h	1.7 j	10.2 a
		Yes	11.7 fgh	5.3 fgh	11.4 a
	2	No	377.5 a	6.3 ef	1.7 g
		Yes	51.4 d	6.3 ef	2.5 fg
	Significance				
		LANS	0.01	0.01	0.01
		AMF	0.01	NS	NS
		ACO	0.01	0.01	0.01
		LANS x AMF	0.01	0.01	NS
		LANS x ACO	0.01	0.01	0.01
		AMF x ACO	0.01	NS	NS
		LANS x AMF x ACO	0.01	0.01	0.01

[y]Means followed by same letter in the same column are not significantly different (LSD, α=0.05); NS=Nonsignificant, n=5. (see Appendix I, Table AI-4.2 for data analyzed as logarithms).

Table AI-6.1. Effect of rhizosphere management (RM) and Arabian medium crude oil (ACO) on colony forming units of total bacterial, filamentous fungi, and nitrogen-fixing free bacteria population (bacteria able to grow on N-free medium), in the rhizosphere of *Lolium multiflorum* cv. Passerel Plus, after 80 days. (Chapter VI).

Rhizosphere management (RM)	ACO ($mg \cdot kg^{-1}$)	Total bacteria (CFU x 10^6 g^{-1} soil)	Filamentous fungi (CFU x 10^4 g^{-1} soil)	Bacteria growing at N-free medium (CFU x 10^4 g^{-1} soil)
Control 44 P	0	6.0 gf [w]	0.4 f	79.7 b
Control [x]		7.4 ef	0.6 ef	12.0 f
AMF [xy]		5.2 g	2.9 c	76.3 bc
SpCe [xz]		7.7 e	0.4 f	58.3 d
AMF + SpCe [x]		6.5 efg	1.2 e	93.7 a
Control 44 P	6,000	12.3 d	0.1 f	65.0 cd
Control		34.0 a	2.0 d	15.7 f
AMF		28.9 b	1.3 de	98.0 a
SpCe		17.2 c	22.3 a	32.7 e
AMF + SpCe		28.7 b	26.8 a	51.7 d
Significance:				
	ACO	0.001	0.001	0.001
	RM	0.001	0.001	0.001
	ACO x RM	0.001	0.001	0.001

[w]Means in the same column followed by the same letter are not significantly different (LSD, $\alpha = 0.05$). n=5. [x]Treatments fertilized with Long Ashton Nutrient Solution (LANS), modified to supply 30 µg P mL^{-1}; [y]AMF= *Glomus intraradices*; [z]SpCe= *Sphingomonas paucimobilis* and *Cunninghamella echinulata* var. *elegans*.

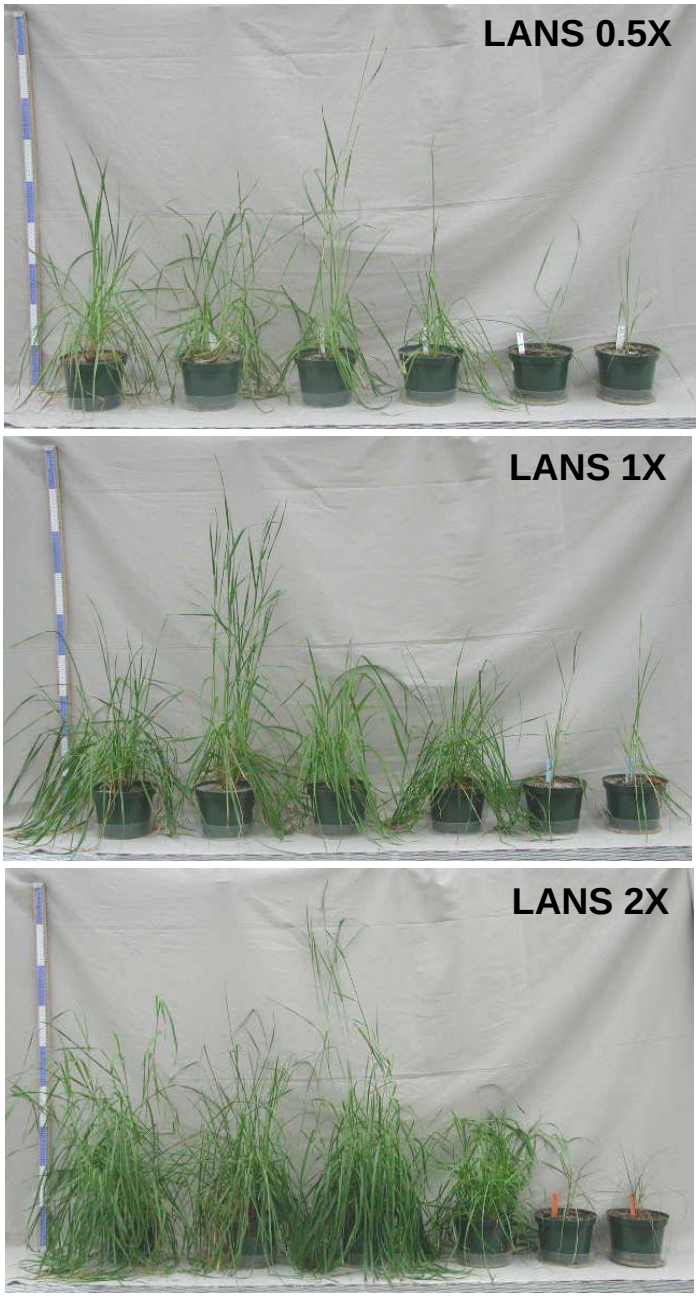


Fig. AI-4.1. Growth responses of *Lolium multiflorum* Lam. cv. Passerel Plus inoculated with *Glomus intraradices* (AMF) or not (C), and treated with three levels of Long Ashton Nutrient Solution (LANS) in soil contaminated with three levels of Arabian medium crude oil (ACO), after 80 days (Chapter IV).

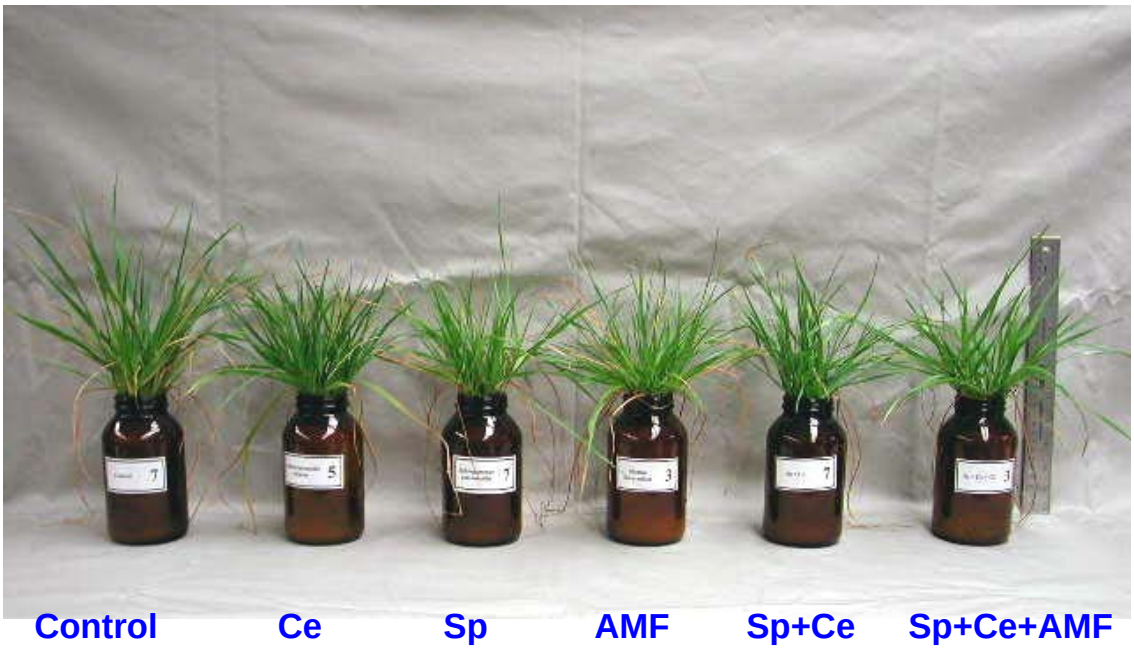
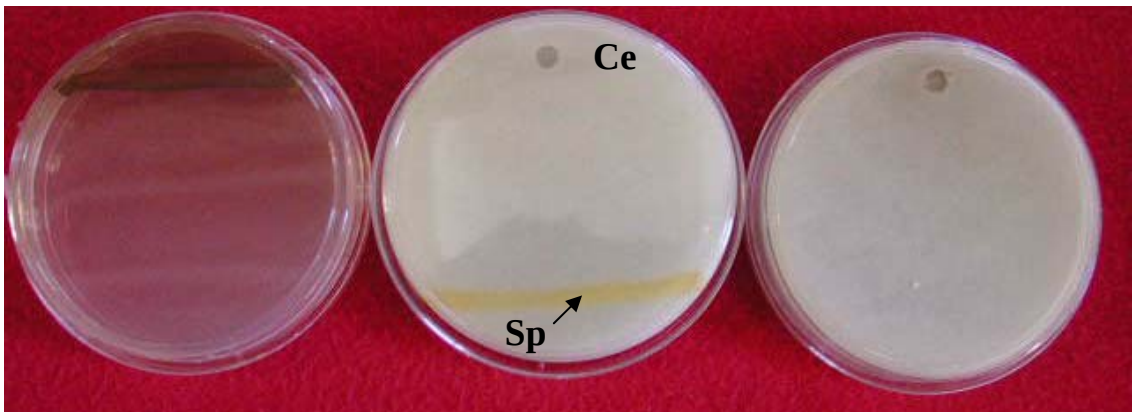


Fig. AI-5.1. Growth responses of *Lolium multiflorum* Lam. cv. Passerel Plus inoculated with *Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce), *Sphingomonas paucimobilis* EPA-505 (Sp), *Glomus intraradices* (AMF), and their combination, in sand contaminated with benzo[*a*]pyrene (100 $mg \cdot kg^{-1}$), after 60 days (Chapter V).

PDA-culture medium, growth after 10 days



Sphinghomonas paucimobilis **(Sp) EPA505**

Cunninghamella echinulata var. elegans **(Ce) ATCC-36112**

Fig. AI-5.2. *In vitro* evaluation of the antiobiosis of *Sphingomonas paucimobilis* to *Cunninghamella echinulata* var. *elegans* Note the overgrowth of *Sphingomonas* by *Cunninghamella* in the center Petri plate demonstrating lack of antagonism of the fungus by the bacterium. (Chapter V).

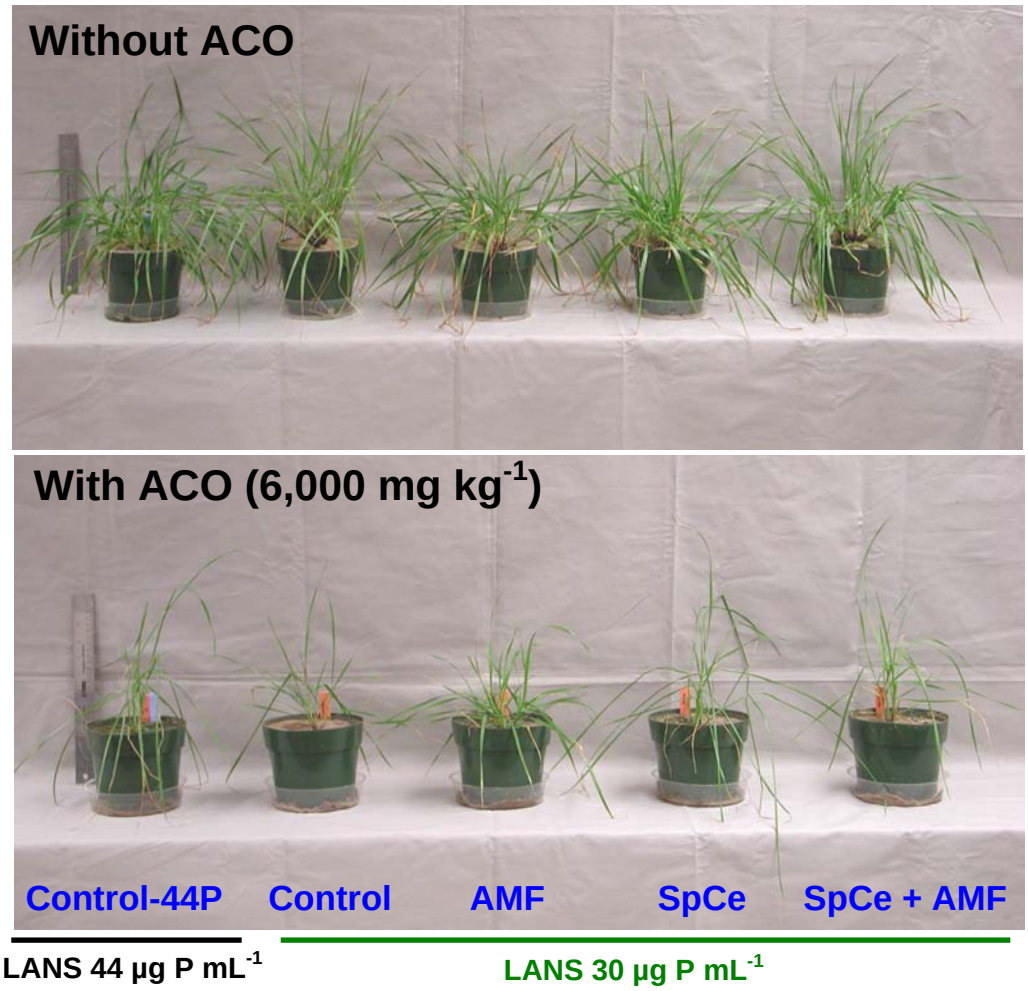


Fig. AI-6.1. Growth responses of *Lolium multiflorum* Lam. cv. Passerel Plus biaugmented with a microbial mixture constituted by *Cunninghamella echinulata* var. *elegans* ATCC-36112 (Ce) and *Sphingomonas paucimobilis* EPA-505 (Sp), and *Glomus intraradices* (AMF), and biostimulated with phosphorus supplied in Long Ashton Nutrient Solution (LANS), in soil contaminated with Arabian medium crude oil (ACO) (6000 mg·kg^{-1}), after 80 days (Chapter VI).

VITA

Alejandro Alarcón was born in Xalapa, Veracruz, México. He received his Bachelor of Science in agronomy from the Universidad Veracruzana, México in 1993. He also received a Master of Science degree in soil science from Colegio de Postgraduados, Montecillo, estado de México, México in 1997. He entered the Department of Horticultural Sciences at Texas A&M University in September 2002, and he received his Doctor of Philosophy degree in August 2006. He become a researcher and professor in soil microbiology, specializing in applications of mycorrhiza in horticultural and forestry plants and symbiotic nitrogen fixation in legumes at the Colegio de Postgraduados, where he has been working since 1992. His areas of research interest are related to the ecology, propagation, and application of arbuscular mycorrhizal fungi, physiology of mycorrhizal plants, and utilization of mycorrhizal fungi in the phytoremediation of organic contaminants in soils.

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