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Study of arbuscular mycorrhizal fungi under hydrocarbon-polluted conditions and diversity associated to plants communities naturally recolonizing oil ponds in the Amazonian areas.

Thèse de doctorat présentée en vue de l'obtention du grade de Docteur en Sciences agronomiques et ingénierie biologique

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List of abbreviations

AC	Arbuscular Colonization
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	ANalysis Of VAriance
B[a]P	benzo[a]pyrene
BCCM	Belgian Coordinated Collections of Microorganisms
bp	base pair
BSA	Bovine Serum Albumin
CAT	Catalase
CESAMM	Center of Study on Arbuscular Mycorrhizal Monoxenics
CIPF	Centre Indépendant de Promotion Fourragère
CMNs	Common Mycorrhizal Networks
DBA	dibenzo(a,h)anthracene
ECM	Ectomycorrhizae
ELI-A	Earth and Life Institute - Agronomy
ELIM	Earth and Life Institute - Microbiology
EPA	Evolutionary Placement Algorithm
ERM	Extraradical Mycelium
FAO	Food and Agriculture Organization
GC-MS	Gas Chromatography-Mass Spectrometry
GHT	Growing Hyphal Tip
GINCO	Glomeromycota *IN vitro* COllection
GRPV	Groupe de Recherche en Physiologie Végétale
GTR	General Time-Reversible
HCl	Hydrochloric acid
HHM	Hyphal Healing Mechanism
Hinf I	*Haemophilus influenzae* (Restriction enzyme)
HPLC	High Performance Liquid Chromatography
HTS	High-Throughput Sequencing
ICP-AES	Inductive Coupled Plasma Atomic Emission Spectrometer
ITS	Internal Transcribed Spacers
KOH	Potassium hydroxide
kp	kilo-base pair
LHS	Life-History Strategies
LSU	Large SubUnit (gene)

MAFFT	Multiple Alignment using Fast Fourier Transform
MDA	Malondialdehyde
MID	Multiplex Identifier
MSR	Modified Strullu-Romand (medium)
MUCL	Mycothèque de l'Université catholique de Louvain
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NM	Non- Mycorhized
NM+D	Non Mycorrhized with Diesel (treatment)
NM-D	Non Mycorrhized without Diesel (treatment)
nrDNA	nuclear ribosomal DeoxyriboNucleic Acid
OTU	Operational Taxonomic Unit
8-OHdG	8-hydroxy-2' –deoxyguanosine
PAH	Polycyclic Aromatic Hydrocarbon
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PHE	Phenanthrene
PhyDE	Phylogenetic Data Editor
Pi	Inorganic Phosphorus
PM	Pre Mycorrhized
PM+D	Pre-Mycorrhized with Diesel (treatment)
PM-D	Pre-Mycorrhized without Diesel (treatment)
POD	Peroxidase
poly-P	Polyphosphate
PPF	Photosynthetic Photon Flux
PUCE	Pontificia Universidad Catolica del Ecuador
PYR	Pyrene
QIIME	Quantitative Insights into Microbial Ecology
RAxML	Randomized Axelerated Maximum Likelihood
rDNA	ribosomal DeoxyriboNucleic Acid
RDW	Roots Dry Weight
RFLP	Restriction Fragment Length Polymorphism
RFW	Roots Fresh Weight
RH	Relative Humidity
ROC	Root Organ Culture
rRNA	ribosomal RiboNucleic Acid

RS	Representative Sequence
RsaI	Rhodopseudomonas sphaeroides (Restriction enzyme)
SD	Standard Deviation
SDW	Shoot Dry Weight
SFW	Shoot Fresh Weight
SM	Standard Methods
SOC	Super Optimal broth with Catabolite repression (medium)
SOD	Superoxide dismutase
SPSS	Statistical Package For The Social Sciences
SSU	Small SubUnit (gene)
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
TE	Trace Elements
TC	Total Colonization
TDW	Total Dry Weight
TPH	Total Petroleum Hydrocarbon
U	Unit (of enzyme activity)
US.EPA	United States - Environmental Protection Agency
VC	Vesicular Colonization
XSEDE	Extreme Science and Engineering Discovery Environment

Summary

Oil exploitation and its mishandling have left numerous polluted environments all over the world. The impact of oil on flora and fauna is perceptible in natural as well as anthropogenic environments and has resulted in the emergence of human diseases. In the Amazon basin of Ecuador, around 2550 oil-polluted sites have been identified and the government, supported by the public enterprise PetroAmazonas EP, has become responsible for the recovery of the hydrocarbon-polluted soils. Physical and chemical approaches are the more common methods used to remediate polluted soils, while the use of biological methods has increasingly attracted the attention of scientists, industrials and policy makers. Among the most promising methods is the phytoremediation assisted by arbuscular mycorrhizal fungi (AMF). AMF are obligate root symbionts that improve the nutrition of plants (particularly P) and increase their resistance/tolerance to abiotic (e.g. hydrocarbon-pollutants) stresses. Nowadays, their community composition in hydrocarbon-polluted soils as well as the impacts of hydrocarbon-pollutants on AMF physiology is poorly known. In this context, the first objective of the thesis was to study the AMF community composition in weathered crude oil ponds and surrounding soils naturally recolonized by native plants. High levels of root colonization were measured and the presence of four AMF families (i.e. Acaulosporaceae, Glomeraceae Archaeosporacea and Paraglomeraceae) and several species were detected by the Sanger sequencing and NGS pyrosequencing methods. This suggested the tolerance of AMF to hydrocarbon pollutants and their potential to help plants establishing and remaining in the polluted soils. These observations opened the door to our second objective which was to determine the role of the standard AMF *R. irregularis* MUCL 41833 on the uptake and transport of Pi to maize plantlets in presence of diesel. No significant effect of AMF was observed on Pi uptake and growth of the maize plantlets. Conversely, a detrimental effect of diesel was noticed both on plant growth

and on fungus root colonization. Thus, it was assumed that this specific fungus was not adequate for phytoremediation, which does not exclude that other AMF (e.g. those from the oil-polluted soils) could improve plant tolerance to diesel and improve Pi uptake. Finally, in a third objective, we investigated the impact of diesel on an essential attribute of AMF to recover integrity following physical injury; i.e. the hyphae healing mechanism (HHM). This mechanism was monitored in two AMF species differing in their life history strategies, *R. irregularis* MUCL 41833 (an r- strategist) and *Gigaspora* sp. MUCL 52331 (a K-strategist). Both fungi differed in their ability to heal under increasing concentrations of diesel. In *Gigaspora* sp., the HHM was the most efficient way to maintain the viability of hyphae under adverse conditions, while in *R. irregularis*, this mechanism was of more limited importance, the fungus being able to reconnect injured hyphae only at low frequencies, probably investing more energy in connecting neighboring hyphae or roots as reported previously in absence of pollutants.

Outline of the thesis

Oil exploitation is a major activity conducted worldwide by oil companies. It includes the exploration, extraction, transport, refining, storage and sale. Accidents (e.g. pipeline damage and/or oil spills) or inadequate waste management have resulted in pollution of soils requiring physical, chemical or biological cleaning technologies.

The increasing interest for plant remediation techniques, so-named phytoremediation, and their association to microbes such as fungi, so-named mycoremediation, has gained interest in the last decade over the conventional physicochemical methods. Physicochemical methods are most of the time highly efficient but expensive, changing soil structure, decreasing soil microbial activity and depleting available nutrients to plants due to the use of aggressive solvents and higher temperatures (Gan *et al.*, 2009).

Among the microorganisms more frequently reported in the rhizosphere are the arbuscular mycorrhiza fungi (AMF). These belowground fungi improve plant nutrition and increase their resistance/tolerance to biotic and abiotic stresses. They could thus represent an interesting option for bioremediation of soils polluted by oil. Their presence in oil-polluted soils has been observed but studies on community composition are rare. Their roles on the uptake of inorganic phosphorus (Pi) by plants in presence of hydrocarbons are inexistent as well as the impact of the pollutants on the hyphal healing mechanism (HHM), an essential process for AMF to survive under stress conditions.

In the present thesis, two main objectives were pursued. The first one aimed to assess, via molecular tools, the AMF community composition in roots of native plants colonizing petroleum-polluted soils. The second one aimed to investigate the role of AMF on plant development and physiology

(e.g. Pi uptake by extraradical hyphae and accumulation in plants) in presence of diesel and conversely to study the impact of diesel on the HHM of two AMF having contrasting life history strategies (LHS). The outline of the thesis is presented in Fig. 1.

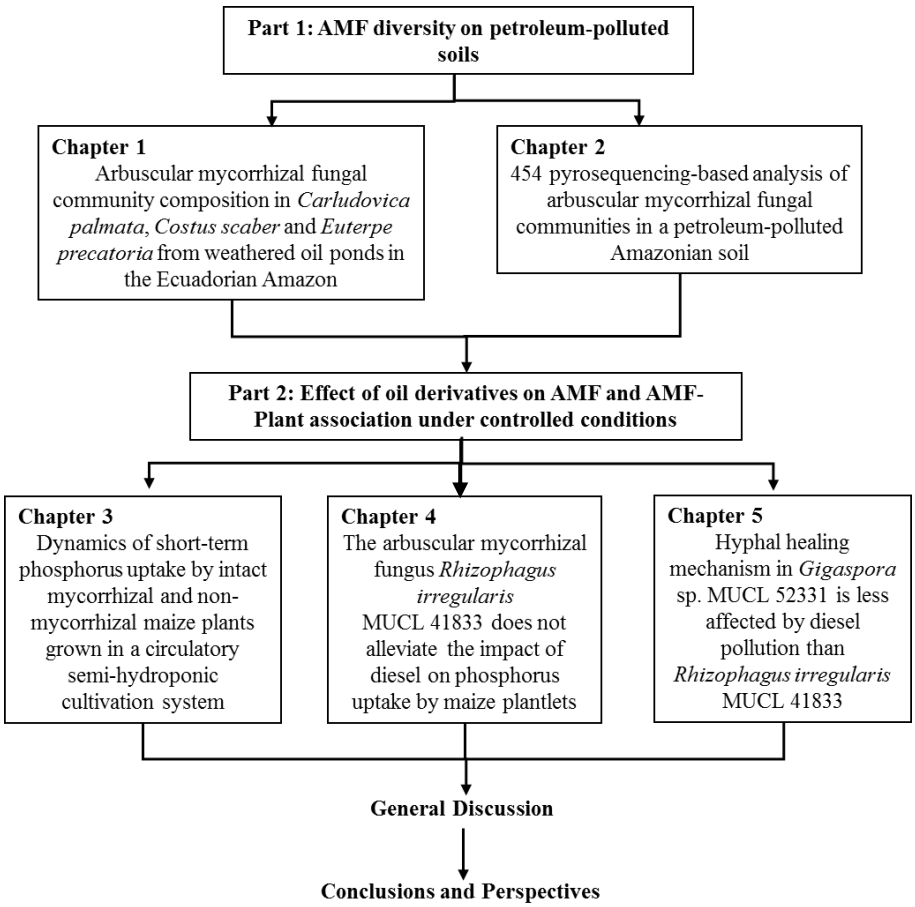

Fig. 1 Outline of the thesis.

In the **Introduction** section, the context and the general objectives of the thesis were presented. In the **State of the Art** section, an exhaustive bibliography review was made on AMF, their diversity and ecological importance in hydrocarbon-polluted ecosystems. The review also included a

description of petroleum hydrocarbons, their classification and detrimental effects on soil microorganisms.

In the **Materials and Methods** section, we detailed the biological material and provided a brief description of the sampling sites and of the most important techniques used throughout the thesis.

The **research results** section was divided into two parts (Fig. 1).

The first part was divided in two chapters and aimed to assess the AMF community composition in crude oil ponds in the Charapa field of the province Sucumbíos (Amazon region of Ecuador). Two ponds of weathered crude oil as well as their surrounding soil were considered. These polluted soils were abandoned for more than 30 years and were naturally recolonized by several plant species.

The objectives of **Chapter 1**, were to (1) evaluate the AMF root colonization of plant species growing in Pond 1 and 2 and the surrounding soil and (2) describe the AMF community composition in three plant species (*Carludovica palmata*, *Costus scaber* and *Euterpe precatoria*) present in the three locations (Pond 1, 2 and surrounding soil) by the Sanger method. Regardless of the sites, all the plants under study were colonized by AMF with values for total root colonization exceeding 56%. Four genera (i.e. *Glomus*, *Rhizophagus*, *Acaulospora* and *Archaeospora*) from the Glomeromycota Phylum were detected in the roots of the three selected plant species. However, 74% of the OTUs obtained could not be attributed to identified AMF species, suggesting the probable existence of new taxa.

The results of chapter 1 were published in Frontiers in Microbiology (Garcés-Ruiz *et al.*, 2017b).

The objective of **Chapter 2** was to investigate in more details the AMF communities in Pond 2. Community composition was assessed 3 m outside and inside the border of the Pond and in the center of the Pond. The Next Generation Sequencing (NGS) technique was used and a large number of sequences (fragments size of approx. 800 bp) were obtained and analyzed. The presence of three families (*Glomeraceae*, *Acaulosporaceae* and *Archaeosporaceae*) containing the four genera reported above was confirmed. Other genera (i.e. *Sclerocystis, Dominikia* and *Kamienskia*) were also detected. As in the previous study, 75% of representative sequences belonged to undescribed species.

The results of chapter 2 were submitted in MicrobiologyOpen.

Among the various methods used to remediate hydrocarbon-polluted soils is the use of plants (or phytoremediation). This method has received an increasing interest in the last decade. However, plants may be susceptible to pollutants, limiting their use in phytoremediation. It is thus important to improve their resistance/tolerance (where resistance is the ability of the plant to limit the stress itself, while tolerance is the ability to limit the harm or damage caused by a given stress (Kover & Schaal, 2002; Schneider & Ayres, 2008)) to pollutants or increase their biomass to stimulate their use in remediation. One way to improve plant resistance/tolerance of plants is their association with AMF. These soil fungi have been repeatedly reported to improve plant nutrition (especially phosphorus). However, this effect remains poorly understood in presence of hydrocarbon pollutants (Hernández-Ortega *et al.*, 2012). Therefore, **the second part of the thesis**, was focused on the effects of diesel, a crude oil derivative from complex composition (alcanes, PAHs…) (Rollin *et al.*, 2005), on (1) plant growth and physiology (especially Pi uptake and transport) in presence/absence of AMF, and (2) a crucial

mechanism for AMF to survive under physical disturbance (i.e. the hyphae healing mechanism).

In **Chapter 3,** we described a circulatory semi hydroponic cultivation system developed in our laboratory for investigating the Pi uptake dynamics by AMF-colonized or non-colonized maize plantlets grown in controlled conditions.

The results of chapter 3 were published in Frontiers in Plant Science (Garcés-Ruiz *et al.*, 2017a).

In **Chapter 4**, the role of *Rhizophagus irregularis* MUCL 41833 on maize resistance/tolerance to diesel was investigated in the circulating semi-hydroponic cultivation system (**chapter 3**). This non-destructive system allowed measuring the dynamics of Pi uptake from a circulating nutrient solution. Pi uptake was quantified at 9, 21 and 42 hours. At the end of the experiment, fresh and dry weights, P concentration and content in shoots and roots and the oxidative stress marker malondialdehyde (MDA) were analyzed. Root colonization by the AMF in presence/absence of diesel was also evaluated. The presence of diesel drastically impacted root colonization by *R. irregularis.* Pi uptake was seriously decreased in mycorrhized and no-mycorrhized plants and no beneficial effects were noticed on the maize plantlets, questioning the option of using AMF species isolated from polluted soils.

The results of chapter 4 are ready for submission to the Journal of Environmental Science and Pollution Research.

In **chapter 5**, we investigated the role of diesel on an essential attribute of the AMF to survive under physically-perturbed conditions: the HHM. This mechanism is known to differ between r and K-strategists (de la Providencia *et al.*, 2005), but the impact of diesel on this mechanism is totally unknown.

The HHM was investigated under *in vitro* culture conditions with two AMF species differing in their LHS; *R. irregularis* MUCL 41833 (r- strategy) and *Gigaspora* sp. MCL 52331 (K-strategy) (IJdo *et al.*, 2010). Increasing concentrations of diesel were used and hyphal recovery was monitored during 48 hours. The results revealed that both strains differed in their ability to heal. *R. irregularis* was severely affected in the presence of diesel comparing to *Gigaspora* sp. Moreover, we demonstrated that diesel delayed the steps concurring to a total healing in both species.

The results of chapter 5 are ready for submission to Environmental microbiology.

In the **General Discussion** section, the overall results of the thesis were summarized and discussed. In the **Conclusions and Perspectives** section, the most important findings and future prospects for the potential applications of AMF in phytoremediation of hydrocarbon-polluted soils were presented.

I. Introduction

Oil, gas and coal are byproducts from fossil fuels that are used as energy sources all over the world (UNCTAD, 2009). Crude oil is also known as the "black gold" due to its global economic and political importance (Darkwah, 2010). The oil industry involves several processes from exploration to extraction, transport, refining, storage and consumption. The extraction and exploitation of oil represent a threat for human health as well as for the environment due, among others, to accidental pollutions such as the formation of crude oil ponds, pipelines spills and waste generation (Kvenvolden & Cooper, 2003; Darkwah, 2010). The consequence of such pollution may be dramatic, resulting in the leaching of organic pollutants contaminating soils and underground water.

The Amazon basin of Ecuador covers an area of 100 000 Km^2 of tropical rain forest. This region, especially the Yasuni Park, has been acclaimed biosphere reserve by the UNESCO in 1989. It harbors one of the most diverse collection of plants, animals, insects, and microorganisms. However, it is also a major reservoir of fossil fuels.

Oil exploitation has become a major economic resource for Ecuador since the early seventies (San Sebastián & Hurtig, 2004). However, the undiscerned development of oil exploration and exploitation have seriously damaged and degraded great part of the environment in most provinces of the Amazon region. The lack of environmental legislation until the early nineties (Armstrong & Vallejo, 1992) left around 2550 environmental liabilities (MAE, 2015) and also generated water and soil pollution with various side effects impacting the environment and the overall biodiversity.

In 2007, the Vice President for environmental Protection office made a diagnostic assessment of the environmental damage in the area exploited by foreign and national oil companies (MAE, 2016).

Since then, remediation of crude oil-polluted soils via chemical, physical and biological techniques has become a priority for the country (MAE, 2015). Several physical and chemical methods exist to remediate oil-polluted soils (Chibuike, 2013). These methods are most often too expensive and may have limited effectiveness in the removal of the pollutants (Das & Chandran, 2011). An interesting alternative is phytoremediation with resistant/tolerant plants. This technique is cost-effective and adequate in an integrated environmental restoration effort. The association of plants with microbes such as bacteria or fungi (so-named mycoremediation in the last case) further represent a serious option for phytoextraction, rhizofiltration, and/or phytostabilization (Atlas & Philp, 2005; Garg & Chandel, 2010).

Nowadays, microbial-assisted phytoremediation with arbuscular mycorrhizal fungi (AMF) is a strategy increasingly considered, due to the widespread distribution of these fungi and their capacity to colonize the vast majority of terrestrial plants (Smith & Read, 2008). AMF are considered as key players for the survival of plants, particularly for ecosystems restoration (Hafidi *et al.*, 2015). They improve plant mineral nutrition and water absorption increasing the resistance/tolerance of plants against various abiotic (see review by Plouznikoff *et al.*, 2016) and biotic (Pozo *et al.*, 2013) stresses. Phytoremediation assisted by AMF has been considered for several oil-pollutants and a number of results clearly demonstrated a better plant establishment, a reduction of toxic effects and a dissipation of hydrocarbon pollutants (Leyval & Binet, 1998; Joner & Leyval, 2001; Gaur & Adholeya, 2004; Alarcón *et al.*, 2006, 2008; Verdin *et al.*, 2006; Liang *et al.*, 2009; Salami *et al.*, 2010; Hernández-Ortega *et al.*, 2012).

Interestingly, a number of studies have reported the presence of AMF in the roots and rhizosphere of plants growing in oil-contaminated soils (Cabello, 1997; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016). However, their presence and community composition in oil-polluted sites of the Amazonian region is almost unknown. Since the environmental liabilities

in the Charapa field, in the province of Sucumbíos are naturally recolonized by plants, it is highly probable that AMF may also be present and associated with these plants.

In the **first part of the study**, it was our objective to investigate the AMF communities composition associated with the roots of plants that naturally recolonized the crude oil weathered ponds and the surrounding soils in the Charapa field. Two different molecular techniques (i.e. Sanger sequencing and pyrosequencing by 454 ROCHE) were used to assess AMF community composition (**chapter 1** and **2**).

The efficiency of phytoremediation depends on the resistance/tolerance of the plants used as well as their associated rhizosphere microorganisms. Improving plant nutrition may increase plant resistance/tolerance against the pollutants. AMF have been repeatedly reported to improve plant nutrients uptake (especially phosphorus) under normal soil conditions (Smith & Smith, 2010; Smith *et al.*, 2011), but only few studies reported on this role under oil-polluted conditions (Hernández-Ortega *et al.*, 2012).

In the **second part of the study,** it was our objective to evaluate the effects of an oil derivative (i.e. diesel) on the phosphorus nutrition of AMF-colonized plant. A non-destructive circulatory semi-hydroponic cultivation system was developed to study the dynamics of Pi uptake and accumulation of AMF-colonized and non-colonized plants growing under controlled conditions (**chapter 3**). The system was based on a method developed by Colpaert *et al.* (1999) for ECM where a circulating nutrient solution impoverished in Pi was sampled from each plantlet at defined intervals. The effect of diesel on the Pi absorption of maize plantelts was subsequently evaluated with this system (**chapter 4**). It is well known that the uptake and transport of Pi is an essential attribute of the AMF for which the extraradical mycelium plays a key role. This network may be impacted by physical injuries reducing its capacity to transport Pi to the plant but also, more largely, affecting the viability of the fungi. AMF have developed a mechanism of repair named the hyphae healing

mechanism (HHM). This mechanism helps the fungus to survive under adverse conditions (de la Providencia *et al.*, 2005, 2007). Nowadays, it is totally unknown whether hydrocarbons may impact this essential mechanism. Therefore, the impact of diesel on the HHM was investigated with two AMF strains having contrasting life history strategies; *Rhizophagus irregularis* MUCL 41833, an r-strategist, and *Gigaspora* sp. MUCL 52331, a K-strategist (IJdo *et al.*, 2010). The recovery of injured extraradical hyphae was evaluated in presence of increasing concentrations of diesel (**chapter 5**) and strategies of both contrasting AMF to survive under stress conditions discussed.

II. State of the art

II.1 Total Petroleum Hydrocarbon (TPH)

Petroleum is a complex mixture of hydrocarbons (molecules are formed only by carbon and hydrogen) basically composed of alkanes, iso- or branched-chain alkanes, cycloalkanes, aliphatic, monoaromatic, polyaromatics and asphaltene fractions (Varma & Ward, 2004) and non-hydrocarbons compounds such as sulfur nitrogen and oxygen (Overton *et al.*, 1994). This composition might highly differ according to the petroleum source (Salanitro, 2001).

Petroleum has been formed naturally during geological era through the burial of microorganisms, animals and plants as a complex fossilized organic matter (Salanitro, 2001), accumulating majoritarily on the ocean floor (Kvenvolden & Cooper, 2003). Thus, natural seepage of crude oil and tar are geographically common and have likely been active through much of geologic time (Kvenvolden & Cooper, 2003), creating ecosystems with adaptive microorganism that use petroleum as carbon source (Darkwah, 2010).

Petroleum and its derivatives are used all over the world. It has been baptized "the black gold" for being a financial income source for many countries, especially in the developing regions of the world (Kharaka & Dorsey, 2005; UNCTAD, 2009). Due to the steadily increasing world population, petroleum is expected to remain the main source of primary energy in the next decades (Salanitro, 2001).

II.1.1 Petroleum derivatives

Following extraction, crude oil is transformed in a variety of fuels (e.g., gasoline, diesel, jet fuel, heating oil, marine diesel), lubricants oil and

chemical feedstocks (e.g., ethylene, propylene, and aromatics) for transportation and energy application (Overton *et al.*, 1994). Therefore, a refining process is required, where distillation techniques with different boiling point ranges and catalytic cracking processes are used to separate crude oil in useful petroleum products (Varma & Ward, 2004) characterized by the hydrocarbon class composition. Generally, light crude oils contain more monoaromatics and less heterocyclics compounds than heavy viscous crude oils (Overton *et al.*, 1994). It is thus important to take into consideration the composition of the different derivatives to understand their potential environmental impacts following oil spill or release from soil or water pollution (Kharaka & Dorsey, 2005).

The toxicity of oil spills is of special concern owing to the complex and diverse range of petroleum compounds released such as aliphatic (e.g. alkanes and alkenes) aromatics (e.g. benzene, toluene, ethylbenzene and xylenes (BTEX)) and polycyclic aromatic hydrocarbons (PAHs), which are recalcitrant in natural environments with potential toxicity, carcinogenicity and mutagenicity (Wilson & Jones, 1993; Atlas & Philp, 2005) effects.

Aliphatic hydrocarbons

Aliphatic hydrocarbons are composed of alkanes (saturated) alkenes and alkynes (unsaturated) (Watkinson & Morgan, 1990). They may be straight-chain compounds, simple branched or highly branched compounds (Watkinson & Morgan, 1990; Margesin & Schinner, 2001). They range from gases, such as methane and ethane, through liquids to long-chain molecules of 40 or more C atoms, which change to solid at physiological temperatures. These hydrophobic molecules are composed entirely of C-C and C-H linkages (Watkinson & Morgan, 1990). Additionally, n-alkanes are the major constituents of petroleum hydrocarbons (Margesin & Schinner, 2001) but the most rapidly degraded components (Watkinson & Morgan, 1990).

Aromatic hydrocarbons

This group is formed by benzene, toluene, ethylbenzene, xylenes (BTEX). They are non-oxygenated monoaromatic hydrocarbons often found together (Margesin & Schinner, 2001; Margesin *et al.*, 2003; Atlas & Philp, 2005). They have a high water solubility compared to other hydrocarbon components. Their mobility is thus non-negligible, facilitating migration in the soil surface and contaminating drinking water supplies. However, the permanence of BTEX compounds in soil before leaching or evaporation depends on their sorption to soil components (i.e. physico-chemical soil properties) as well as degradation activity by soil microorganisms (Margesin *et al.*, 2003).

Polycyclic aromatic hydrocarbons (PAH)

These compounds are found in higher concentrations associated with the petroleum, gas-production and wood-preserving industries. PAHs are relatively stable and recalcitrant in the environment, therefore it is more difficult to degrade than other organic compounds present in the soil (Wilson & Jones, 1993).

The PAHs consist of two or more fused benzene rings in linear, angular or cluster arrangements formed by C and H atoms. However N, S and O atoms may substitute in the ring to form heterocyclic aromatic compounds, also grouped with the PAHs. The linear benzene rings are the most unstable while the angular rings the most stable (Wilson & Jones, 1993). PAHs are relatively insoluble in water (hydrophobic molecules). However their solubility depends on the molecular weight (i.e. number of rings). PAHs with 2-3 rings have a low molecular weight (LMW) while ≥ 4 rings have a high molecular weight (HMW) (Wilson & Jones, 1993; Dumas, 2013), thereby LMW PAHs are more solubles than HMW PAHs.

Diesel oil

Diesel is an oil refined complex mixture of petroleum hydrocarbons containing low molecular weight volatile alkanes, aromatic compounds and PAHs (Adam & Duncan, 1999). However, this composition may vary with the origin of the oil and with the current legislation (Driai *et al.*, 2015). According to the international Agency for Research on Cancer (IARC, 1990), the number of carbons in diesel varies from 9 to 28 and boiling temperature range is approx. 163-357°C. Additives may also be present in diesel fuels, improving and preserving the quality of diesel (IARC, 1990). Diesel is harmful to humans. It has toxic effects such as anuria, renal failure, gastro-intestinal symptons and cutaneous hyperkeratosis. An increasing risk for squamous cell carcinoma of lungs has also been evidenced (IARC, 1990). In mice, skin application resulted in few squamous-cell carcinomas and papillomas (IARC, 1990).

II.1.2 Environmental contamination by Petroleum and its derivatives

Consumption of petroleum may have major environmental impacts at regional and/or global scale (Kharaka & Dorsey, 2005). Soil, air, surface and groundwater pollution as well as global climate change are the principal consequences of oil exploration, production, exploitation, unsafe disposal, leaking or spills (Varma & Ward, 2004) resulting in a serious deterioration in the quality of environments (Debiane *et al.*, 2012; Oludele & Ogundele, 2013; Gao *et al.*, 2014). Several hydrocarbons such as diesel are highly hydrophobic (Rajtor & Piotrowska-Seget, 2016) favoring their accumulation and sequestration in the soil (Cruz *et al.*, 2013) and also influencing the uptake of water and nutrients by plants (Varma & Ward, 2004).

Petroleum contaminations may have different origin, leakage of underground storage, tanks and pipelines, spills at production wells and distribution

terminals and seepage of gasoline from underground storage tanks. This pollutions have caused major soil and water contaminations (Atlas & Philp, 2005). A review of the contaminated sites in Europe (estimated to 342 000) demonstrated that the main contaminants are heavy metals and mineral oil, contributing together to around 60% in soil contamination and 53% in groundwater contamination. Moreover BTEX, PAH and other contaminants contribute to soil contamination varying between 8 and 11% (Panagos *et al.*, 2013). According to the study of Panagos *et al.*, (2013) the management of contaminated soils (including as well production sectors such as chemical, metal, textil, electronic industry, mining sites and service sectors such as gasoline and car service station, dry cleaning…) in Europe may be estimated to be 6.526 million € per year. Besides the calculated budget per remediated site (RS)varies from 7.5000 to 232 000 €. However the real budget reported in projects remediation is between 50 000 to 500 000 € and in some cases even less 5 000 to 50 000 € per RS (Panagos *et al.*, 2013).

In addition, during long transportation of crude oil and its derivatives, spills have caused devastating accidents in different environments (Atlas & Hazen, 2011). Million of oil gallons have been released, leaving long-term contamination in shorelines and seas (Table 1) due to the failure in oil removal (Lim *et al.*, 2016).

Thus, Physical, chemical and biological methods are necessary to remediate contaminated ecosystems (Singh & Seneviratne, 2017). Biological processes (i.e. bioremediation and phytoremediation) are less expensive compared to physical-chemical processes (Debiane *et al.*, 2009) and have been used on large scale to clean up hydrocarbon contaminated soils (Varma & Ward, 2004).

Table 1 Top 10 largest marine oil spills and the corresponding clean-up costs, ranging from controversial oil spills to accidental oil spills in history. Adapted from Lim *et al.* (2016).

No	Name of oil spill	Year	Place of oil spill	Type of fuel	Amount of oil spilled(million gallons)	Clean-up cost estimated in 2010 (US dollars)
1	Gulf War	January, 1991	Persian Gulf, Kuwait	Crude oil	240–336	540 million
2	Deepwater Horizon	April, 2010	Mexican Gulf, Mexico	Crude oil	210	10 billion
3	Ixtoc 1 oil well	June, 1979	Mexican Gulf, Bay of Champeche	Crude oil	140	283.9 million
4	Atlantic Empress oil spill	July, 1979	Carribean Sea, off the coast of Trinidad and Tobago	Light crude oil	9	187 million
5	Nowruz oil fiel	February, 1983	Persian Gulf, Nowruz Field Platform	Oil	80	161.5 million
6	ABT Summer	May, 1991	Off coast of Angola, Africa	Iranian crude oil	80	163.2 million
7	Castillo de Bellver oil spill	August, 1983	Table Bay; Saldanha Bay, South Africa	Light crude oil	78.5	153 million
8	Amoco Cadiz	March, 1978	Brittany coast, up to Channel Islands; Portsall, France	Light Iranian and Arabian crude oil and bunker fuel	68.7	136 million
9	M/T Haven Tanker oil spil	April, 1991	Mediterranean Sea; Genoa, Italy	Crude oil	45	85 million
10	Odyssey oil spill	November, 1988	North Atlantic, off the coast of Nova Scotia	North Sea crude oil	43	86.7 million

II.2 Remediation methods for hydrocarbon-polluted soil

Several techniques have been developed to remediate hydrocarbon-contaminated environments (Table 2).

Table 2 Remediation technologies for hydrocarbon-polluted soil. Adapted from Lim *et al.* (2016).

Chemical remediation	Thermal remediation	Physical-chemical remediation	Bioremediation	Phytoremediation
Chemical oxidation	Incineration	solvent extraction	Bioaugmentation	Phytostabilization
Electrokinetic remediation	Thermal desorption	Soil vapour extraction	Biostimulation	Phytodegradation
	Microwave frequency heating	Flotation	Bioventilation	Phytovolatization
		Ultrasonication		Rhizodegradation

II.2.1 Chemical remediation

Different chemicals have been used to remediate oil-contaminated soils via oxidation reactions. Reangents such as hydrogen peroxide (H_2O_2), ozone, peroxymonosulfate, permanganate and persulfate were used. They were delivered via injection of liquid/gaseous chemicals to the ground (Lim *et al.*, 2016). The most studied reagent, H_2O_2 generates highly reactive free radicals capable to oxidize unsaturated organic contaminants forming free radicals, which may be transformed into an organic product through electrophilic addition, or saturated organic compounds. Finally, free radical from the organic compounds and water via hydrogen substraction are produced (Villa *et al.*, 2010). In the case of ozone, oil molecules were degraded by splitting C-H bond and transformed into decomposition products (Yu *et al.*, 2007) which may be used by microbial community due to the supply of oxygen (Gan *et al.*, 2009). Persulfate is a strong oxidant which have less affinity for natural soil organics, resulting in a higher remediation efficiency (Usman *et al.*, 2012; Usman, 2016).

Chemical oxidation method is a non-selective remediation technology useful for oil-contaminated soils that are not affected by the toxicity of the

contaminant. The simplicity of the technique could provide fast results with low operational costs. Moreover, the degradation of oil contaminants result in the generation of more biodegradable compounds without the formation of toxic by-products (Lim *et al.*, 2016). However, the dosage of chemical oxidants are important to avoid adverse impacts on the environment (Goi *et al.*, 2006). It has been demonstrated that the use of these chemicals decrease the organic matter content in the soil causing soil infertility due to reduction in water and nutrients retention with a probable impact on microbial population (Lim *et al.*, 2016).

Electrokinetic remediation is an *in situ* remediation method which employs low levels of direct electric power between electrodes (anodes and cathodes) embedded at each side of the oil-contaminated soil mass, forming an electric field across the area (Lim *et al.*, 2016). This forms a voltage potential gradient which causes the flow towards the cathode while dragging the contaminant together with the bulk flow (Sri Ranjan *et al.*, 2006). This method combines several mechanisms such as electroosmosis, electromigration, and electrophoresis. It contributes to the effectiveness of fluid flow, especially electroosmosis that move the soil moisture and groundwater from the positive cathode to the negative anode. The excess of cations in the electrolyte system to neutralize negatively charged soil particles impart a viscous drag into the aqueous phase (Elektorowicz & Boeva, 1996). Then the contaminant is mobilized in the fluid and transported towards the cathode. The efficiency of this mechanism thus depends on the flow rate, if flow increase migration of contaminants increase along the fluid. The advantage of this method is its performance *in situ,* which provides a faster response time and lower operating cost. Further, it has more control on the movement of the aqueous phase and contaminants through heterogeneous soils; also, it could provide a uniform flow distribution, particulary in low permeability soils sand or sludges (Sri Ranjan *et al.*, 2006). However, the electrolysis process could create thermal

hot spots and alter the pH of the soil after long periods. Besides, electrokinetic is not effective at low concentrations of contaminants (Mosavat *et al.*, 2012). Hence, work is still required to understand the interactions between electrokinetic parameters, contaminant and soil microbial communities (Lim *et al.*, 2016).

II.2.2 Thermal remediation

Currently, three different methods (i.e. incineration, thermal desorption and microwave frequency heating) use heat to remove oil from the soil (Lim *et al.*, 2016).

Incineration: this method use temperatures up to 1000°C to destroy via burning the oil contaminants from the soil. It has been demonstrated that almost 100% of the contaminant is remove under rapid heating conditions (Bucala *et al.*, 1994; Anthony & Wang, 2006; Rushton *et al.*, 2007).

Thermal desorption: this method manipulates the temperatures (100-900°C) to increase the vapor pressure of the organic contaminants. Thus, volatiles are produced and subsequent desorption from contaminated soil is obtained. After this process, a sweep gas pass through to take the volatile contaminants and have a further treatment or disposal (Rushton *et al.*, 2007). Studies demonstrated high efficiency of removal. However, this efficiency depends on the chemical composition of the contaminated soil since this strongly influence the amount and composition of the combustion gases produced (Piña *et al.*, 2002).

Microwave frequency heating: this method removes contaminants via heating and volatilization through microwave energy. However, several organic compounds and soil particles are resistant to microwaves, thereby microwave absorbers (i.e. granulated activated carbon, graphite fibre, activated carbon powder, MnO_2 and Cu_2O) are used and mixed together with the contaminated soil giving a highly removal efficiency of oil from soil (Rushton *et al.*, 2007; Li *et al.*, 2009).

II.3 Physico-chemical remediation

Different physico-chemical technologies have been developed for soil remediation.

Solvent extraction removes soil contaminants using a single solvent or a mixture of solvents (i.e. organic solvents, surfactant-aided aqueous solutions, supercritical and subcritical fluids) (Gan *et al.*, 2009; Wu *et al.*, 2011a). They are used to remove organic contaminants by an *ex situ* separation and concentration process (Silva *et al.*, 2005). The use of ethyl-acetate/acetone/water, hexane/acetone and petroleum ether solvents showed interesting results in petroleum removal.

Surfactant-aided extraction reduces the interfacial tension between oil/soil and oil/water interfaces. Surfactants are amphiphilic molecules (i.e. hydrophobic tail, hydrophilic head) capable to aggregate the contaminant molecules by the micelles formation. The use of high temperatures (80°C) and agitations are efficient for removing oil from soil (Han *et al.*, 2009).

Subcritical fluid extraction uses high temperature and pressure fluids. Thus, at hight temperatures H bonding forces between water molecules weaken, decreasing the dielectric constant and polarity. Thereby water adopts a hydrophobic nature behaving as an alternative organic solvent (Gan *et al.*, 2009).

Although the use of solvents are efficient for remediation, it presents a major disadvantage, which is the introduction of a secondary pollutant, from the solvent used (Lim *et al.*, 2016). Nevertheless, the use of biosurfactants (green solvents) from bacterial consortia has been used to increase solubility of the petroleum oil in soil to enhance bioavailability to the microorganisms (Grace Liu *et al.*, 2011; Agnello *et al.*, 2016).

Although several physical and chemical methods are efficient in remediation of hydrocarbon pollutants from soil, they impact the microbial diversity.

Therefore, the promotion and development of biological methods increased in the last years.

II.3.1 Bioremediation

Bioremdiation is the use of biological processes to detoxify or remove different environmental pollutants (Gao *et al.*, 2014). It was developed in 1940 and its effectiveness was demonstrated in the removal of weathered crude oil from the Exxon Valdez oil spill in the coast of Alaska in 1994 (Varma & Ward, 2004; Atlas & Philp, 2005; Atlas & Hazen, 2011). This technology involves several disciplines such as microbiology, engineering, geology, ecology, chemistry, … (Varma & Ward, 2004). Biodegradation is the metabolic ability of microorganisms to transform or mineralize organic contaminants into less harmful, non-hazardous substances further integrated into natural biogeochemical cycles (Margesin & Schinner, 2001). Several factors influence this process such as nutrients, oxygen, pH, temperature, concentration and bioavailability of the contaminants, and chemical-physical characteristics of the contaminated environment (Margesin & Schinner, 2001; Lim *et al.*, 2016).

Three mechanisms (i.e. bioaugmentation, biostimulation and bioventilation) have been successfully described in bioremediation.

Bioaugmentation is the addition of selected and acclimated cultured microbial strains (bacteria or fungi), or genetically engineered bacteria with specific catabolic activities. Further, the addition of biodegradation relevant genes packaged in a vector to be transferred by conjugation into native microorganism to enhance and increase the rate of degradation has been studied (Tyagi *et al.*, 2011; Lim *et al.*, 2016). The selection of microorganisms is based on their metabolic potential and capabilities to be efficient and persistent under specific environmental conditions. Hence a prior knowledge

of the microbial communities inhabiting in the target site is highly desired (Tyagi *et al.*, 2011).

Biostimulation is the addition of nutrients (i.e. N and P), biosurfactants and other co-substrates in the contaminated zone where degrading microorganism exist, but nutrients and other factors are limited to promote microbial activity. Moreover providing the appropiate pH and humidity may promote cells growth, especially if the contaminant is degraded by co-metabolism (Varma & Ward, 2004; Tyagi *et al.*, 2011).

Bioventilation is an *in situ* process, based on vacuum-enhanced soil vapour extraction, which stimulates the growth of microorganisms by the supply of air (oxygen). Thus, the aerobic conditions may enhance the metabolisation of organic matter and generate more energy (Satyanarayana *et al.*, 2012).

Some limitations for bioaugmentation could be the adaptation of the inoculated consortia to the environmental conditions and the competition between introduced and indigenous microbes (Tyagi *et al.*, 2011).

A limited step in biodegradation is the temperature, thus, bioavailability and solubility of less soluble hydrophobic substances such as aliphatic and polyaromatic hydrocarbons are affected. If the temperature increases, oil viscosity decreases affecting the degree of distribution and an increase in the difussion rates of organic compounds. Thereby, smaller boundery layers are expected at elevated temperature performing higher reaction rates (Margesin & Schinner, 2001).

Bioremediation of oil-contaminated soils was successfully used under *in situ* as well as *ex situ* conditions, showing the capacity to degrade the oil contaminants permanently with no adverse long-term toxic effects on the environment. Aditionally, this technology eliminates wastes permanently, does not disrupt the environment and is low-cost (Lim *et al.*, 2016).

As drawbacks, bioremediation requires long treatment periods up to several years. High concentrations of oil contaminants may result in low removal

efficiency by the microbial communities. Moreover, inconsistent results efficiency have been revealed do to soil parameters, such as temperature, pH, salinity, contaminant compositition and concentration and weathering effect (Margesin & Schinner, 2001; Lim *et al.*, 2016).

II.3.2 Phytoremediation

Phytoremediation, also known as plant-assisted bioremediation is a method that seeks to remove toxic contaminants using plants, their roots and the microorganism from the rhizosphere (Merkl *et al.*, 2005a).

Different phytoremediation methods have been described: phytostabilization phytodegradation, phytovolatilization, and rhizodegradation (Conesa *et al.*, 2012) (Fig 2). The presence of the plants in the oil-polluted soil help in the degradation of the pollutants by the production of root enzymes that may alter or degrade them. In addition, the production of root exudates (enzymes) and mucilage secretion could alter and degrade the oil contaminants (Lim *et al.*, 2016).

The addition of fertilizer as an extra source of nutrients for plants microorganisms may improve phytoremediation success (Lim *et al.*, 2016).

Phytostabilization is the inmobilization of contaminants in the root zone. The roots inmobilize the contaminants preventing soil leaching or dispersion. Root cells and roots membrane absorb and accumulate the hydrocarbons, necessitating a large root system (Conesa *et al.*, 2012; Lim *et al.*, 2016). Plants can act as organic pumps, transpiring water to keep the contaminants in the root zone and preventing its migration off-site. The phytostabilization of petroleum hydrocarbons may involve the incorporation of contaminant molecules into humic materials in the rhizosphere where plant roots may supply enzymes that bind the pollutant to soil organic matter increasing the organic content of the soil (Germida *et al.*, 2002).

Phytodegradation or phytotransformation, involves the breakdown of the hydrocarbon molecules, through metabolic process or through enzymes release (i.e. dehalogenase, nitroreductase, peroxidase, nitrilase and laccase), acting as catalysts to chemical reaction, capable to accelerate the degradation processes (Germida *et al.*, 2002; Lim *et al.*, 2016) without the intervention of microorganisms (Conesa *et al.*, 2012).

Phytovolatilization is the uptake and transpiration of the pollutant by a plant. The molecule may be metabolized or not and released into the atmosphere. It is suggested that volatilization may be through the stems and leaves. The removal of pollutants by this mechanism may have implication on the quality of the air (Germida *et al.*, 2002).

Rhizodegradation is the intervention of microorganisms degrading the organic compound present in the rhizosphere (Conesa *et al.*, 2012). Microorganisms are involved in direct and indirect degradation or transformation of the petroleum hydrocarbons. Plants and microorganisms in the rhizosphere are considered essential actors in the degradation of petroleum hydrocarbon in soils (Germida *et al.*, 2002).

The advantage of phytoremediation is the facility to implement and the minimal maintenance costs. This method is not limited to size of the site and can be easily used in any area that supports plant growth. The addition of fertilizers provides sufficient nutrient and organic materials to help the improvement of remediation. Moreover plants help to stabilize soil with the development of an extensive root system (Lim *et al.*, 2016).

Phytoremediation is a slow process, which may be considered as a long-term solution. Several factors must be taken into account such as type and concentration of the pollutant, soil parameters, water content, nutrient concentration, plant species (i.e. type, characteristics and resistance) (Öztürk *et al.*, 2015; Lim *et al.*, 2016).

Phytoremedation is a friendly method, which could be used in situ to promote ecological recovery. Moreover, several studies have demonstrated that the use

of phytoremediation combined with bioaugmentation have better results in remediation of hydrocabons-polluted soils that each one separately (Vosátka *et al.*, 2006; Dong *et al.*, 2014; Lim *et al.*, 2016). Thus, the use of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) assisting phytoremediation strategies may help the plants resist/tolerate the stress conditions induced by hydrocarbon pollutants (Gao *et al.*, 2014).

Table 3 present an exhaustive list of remedation methods with their advantages and limitations.

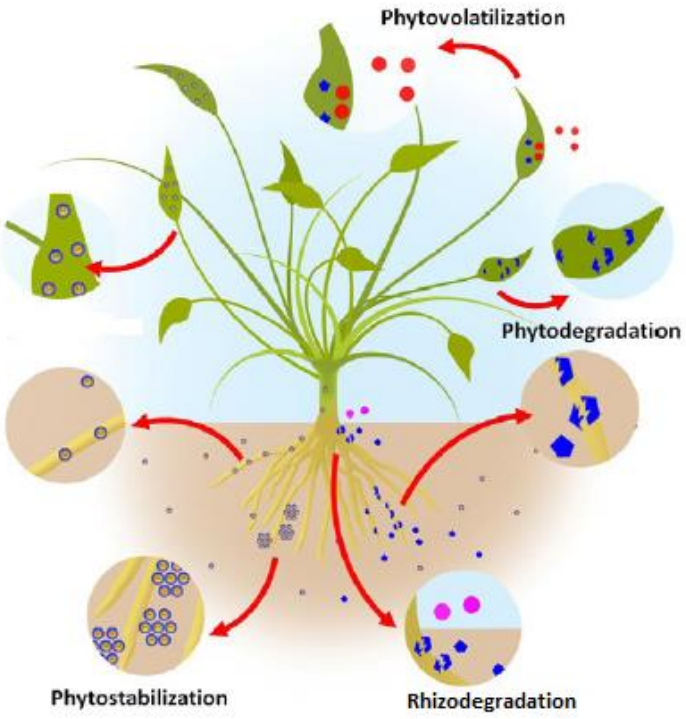

Fig. 2 Schematic representation of phytotechnologies (adapted from Parmar & Singh, 2015).

Table 3 Remediation technologies of oil contaminated soil. Advantages and limitations adapted from Lim *et al.* (2016).

Classification	Remediation technology	Current application for on-site remediation	Advantages	Limitations	Maximum contaminant level	Efficiencies (%)	Duration
Biological	Bioremediation	Yes	Low cost, low environmental impact, high potential for on-site treatments, no waste treatment required	Long treatment durations, inconsistent results and highly dependent on the contaminated site, limited to biodegradable contaminants	2–10%	≈50–90+	Several months/years
	Phytoremediation	Yes	Aesthetically pleasing, low operational cost, roots of plants helps to stabilize soil which can prevent erosion, capable of remediating large contaminated areas	Time-consuming, difficult to implement a specific plant type for every oil spill, dependent on many environmental factors, could only tolerate low concentrations of toxins	1–8%	≈50–90	Several months/years
Chemical	Chemical oxidation	No	High efficiency, low operational costs, ease of operation, formation of environmental friendly by-products	Limited by low soil permeability, highly dependent on pH, destroys natural microorganisms	5–15%	N80	Up to 72 h
	Electrokinetic remediation	Yes[a]	Uniform flow distribution, precise control of movement, low operating cost, low power consumption	Dependent on desorption of contaminant, unenvironmental friendly, time consuming, may affect microbial activity due to mobilization of soil nutrients	10–20%	≈40–70	14–45 Days
Thermal	Incineration, thermal desorption, microwave heating	Yes	High efficiency, quick, reliable, able to treat large volumes of contaminated soil	High costs, formation of greenhouse gases, affected by high moisture content	2–10%	N95	Several seconds–2 h

Physical–chemical	Solvent extraction	No	Easily implemented, high efficiency, quick	High cost, consumes large amount of solvent, unenvironmental friendly	0.5–30%	60–98	Several hours–10 months
	Soil vapour extraction	Yes	High efficiency for large quantities of volatile content, quick, low cost, enhances growth of microorganisms	Ineffective for low volatility contaminants and soils with low air permeability	3–15%	65–95	Several days–months
	Flotation technology	No	Easy application, rapid operation time, low operational cost, low space requirements	Requires large amounts of water, unable to treat soil contaminated with non-buoyant contaminants	7.5–35%	65–97	Several hours
	Sonication	No	Environmental friendly, low energy consumption, eliminates the need for chemical addition	High equipment cost, limited to small area of treatment per run	1–15%	20–100	Several seconds–45 min

II.4 Arbuscular Mycorrhiza Fungi (AMF)

Numerous groups of microorganisms (e.g. bacteria, fungi) are present in the soil, many of which, establishing mutualistic associations with plants (Chapin *et al.*, 2000). Among these are the arbuscular mycorrhizal fungi (AMF), the most ancient and important plant symbiont (Souza, 2015). These fungi develop a massive hyphal network in the soil structuring and modifying profoundly the microflora composition (e.g. bacteria) in an environment known as the mycorrhizosphere (van der Heijden & Sanders, 2003). The hyphae are used by bacteria as "fungal highways" to move within the soil, to acquire nutrients and/or to colonize neighbor microcosms (de Menezes *et al.*, 2017). The network of hyphae also strongly contribute to the formation of micro-aggregates via the secretion of glomalin (i.e. a glycoprotein (Rillig, 2004)) and thus to the stabilization of soils (Wu, 2017).

The term mycorrhiza comes from the contraction of two words, myco from a Greek word meaning fungus and rhiza from a Latin word meaning root. The AMF colonize roots from an estimate of 74% of vascular land plants (Brundrett 2009) as well as the thalli of gametophytes of liverworts, hornworts, and lycophytes (Kenrick & Strullu-Derrien, 2014; Smith & Read, 2008). It is hypothesized that the origin of AMF dates back 400 million years ago and that they were present in several ecosystems forming symbiosis with ancient land plants (Schwarzott & Schüßler, 2001; Wu, 2017). A review by Berbee *et al.* (2017) documented the evidence of spore and arbuscules of AMF associated with Rhynie Chert fossils dated 407 million years ago.

Arbuscular mycorrhizal fungi provide mineral nutrients to the plant in exchange for C acquired by the intraradical mycelium (IRM) and transferred to the extraradical mycelium (ERM) (Giovannetti *et al.*, 2015; Smith & Read, 2008). This association thus improves the growth of plants but also their resistance/tolerance to abiotic (e.g. drought, salinity – Plouznikoff *et al.*, 2016) and biotic (pests and diseases – Pozo *et al.*, 2013) stresses.

II.4.1 AMF life cycle

Arbuscular mycorrhizal fungi are obligate biotrophs. Their life cycle thus fully rely on the colonization of living host plants (Berruti *et al.*, 2014). This interaction starts with the germination of a propagule (i.e. a spore, a vesicle or an AMF-colonized root piece). In numerous cases, root exudates will orient hyphal growth and induce branching until contact with the epidermis. A signal exchange between both organisms takes place to allow the formation of an appressorium (or hyphopodium) on the epidermal cells of the root, before hyphal penetration inside the root (David-Schwartz *et al.*, 2003) suppressing transiently plant defenses (Helber *et al.*, 2011). After penetration, the hyphae branches dichotomously within each cells of the cortex forming arbuscules (Varma, 2008) without penetrating the plasma membrane. These structures, which shape reminds like a tree are the preferential site for the exchange of nutrients for carbon (Berruti *et al.*, 2014). Vesicles are also formed by most of the genera (except for *Archaeospora*, *Gigaspora*, *Paraglomus*, *Racocetra* and *Scutellospora*) intra or intercellularly (Varma, 2008; Berruti *et al.*, 2014). Their function is storage and propagation (Declerck *et al.*, 2004). The formation of these structures (ie. arbuscules, vesicles and intraradical hyphae) is known as the intraradical phase of the fungus. Starting from the intraradical mycelium, the fungus develops and extraradical mycelium with spores, auxiliary cells and branched absorbing structures (BAS). This extraradical mycelium develops and spreads over long distances to explore the soil for water and nutrients (Horton, 2015). Spores formation is the response to particular environmental conditions (e.g. stress conditions) (Berruti *et al.*, 2014; Souza, 2015) (Fig. 3). Some studies observed, during the pre-symbiotic phase, a molecular dialogue where plants exude strigolactones (phytohormone) which activates and up-regulates the mycorrhizal factor (myc factor) genes of AMF (Bonfante & Genre, 2010) to stimulate spore germination and hyphal branching (Chang *et al.*, 2017). The myc factor is a

mixture of sulfated and non-sulfated oligosaccharides (LCOs) which stimulates mycorrhization at early stages. This signaling pathway is known as the common symbiotic pathway (CSP) (Bonfante & Genre, 2010; Chang *et al.*, 2017).

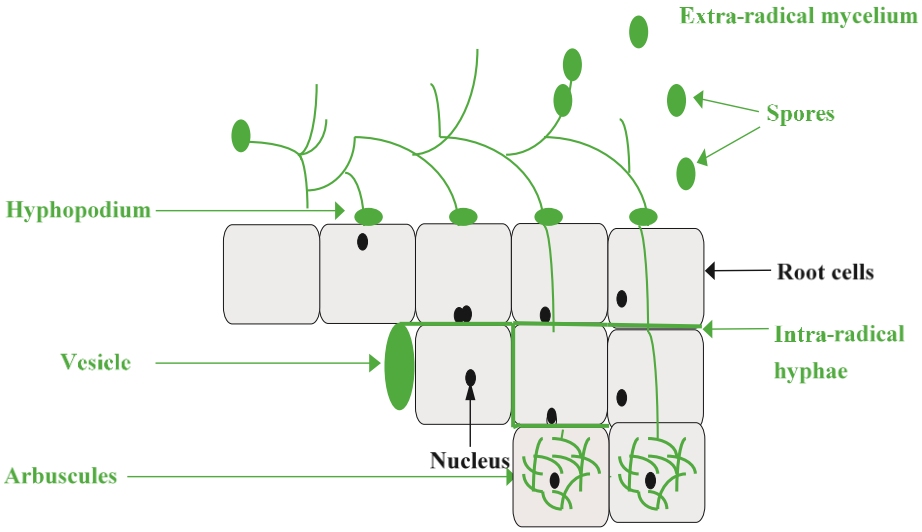

Fig. 3 Development of the different AMF structures in the intra-and extraradical phases (adapted from Recorbet *et al.*, 2013).

To understand AMF community dynamics in environments (i.e. natural or anthropogenic ecosystems), AMF have been classified on the basis of their life-history strategies (LHS) (IJdo *et al.*, 2010) or into different functional groups (van der Heijden & Scheublin, 2007).

In the classification on LHS, two strategies have been defined, i.e. the r-K strategies, where r-endpoint represents a quantitative and K-endpoint a qualitative extreme.

The AMF K-strategists are more adapted to survive in stable and predictable environments where competition is high. The resources are mostly used for vegetative growth and enhanced survival. Conversely, the AMF r-strategists invest their energy mostly in the production of many offspring, favoring their development in unstable environments (IJdo *et al.*, 2010). Species belonging

to the family Gigasporaceae are often considered as K-strategists due to their slow root colonization and production of few large offprings, while most Glomeraceae are considered as r-strategist. Gigasporaceae may produce few and large spores long after roots had ceased growth (de Souza *et al.*, 2005), while Glomeraceae produce small and high numbers of spores in parallel to root growth (Declerck *et al.*, 2001) within a short period of time. Anastomoses and hyphae healing mechanisms (HHM) also differ between K and r strategists. For instance, *Glomus intraradices* (a r-strategist) was suggested to be more adapted to perturbed environments than *Scutellospora reticulata* (a K-strategist) in the HHM and anastomosis process (de la Providencia *et al.*, 2005; Voets *et al.*, 2006). In the HHM *Glomus* developed several new branches while *Scutellospora* invested its energy to reconnect the hyphae after injury.

In the classification of functional groups, the Grime´s C-S-R (competitor, stress tolerator, ruderal) framework (Fig. 4) (Chagnon *et al.*, 2013) is used. The competitive AMF have the capacity to acquire growth-limiting resources, which is the plant-derived carbon. Thus competitors may increase C aquisition from their host plant by producing high soil mycelial biomass for exploration and P uptake, low investment in carbon storage and late production of spores (asexual reproduction) (Chagnon *et al.*, 2013). This characteristics are typical for the Gigasporaceae family that invest more energy in the production of extraradical biomass than in intraradical fungal structures as compared to others AMF families (Hart *et al.*, 2002; Maherali & Klironomos, 2007). The stress-tolerant AMF are characterized by their limited requirements in C supplied by the host plant and its use in an efficiently way. These AMF are capable to complete their life cycle producing low biomass, which may reduce metabolic maintenance costs, further the production of little extraradical biomass may reduce the exposure to abiotic factors (i.e. salinity, heavy metals, acidity) (Chagnon *et al.*, 2013). A representative of this strategy is the family Acaulosporaceae which produce less extraradical hyphae and intraradical root

structures as compared to Glomeraceae and Gigasporaceae (Hart *et al.*, 2002; Maherali & Klironomos, 2007; Chagnon *et al.*, 2013). The ruderal AMF are characterized by a high growth rate and an efficient mechanism of anastomosis. These AMF invest their energy in the production of spores suggesting a strategy by wich ruderal AMF cope with disturbances. Most Glomeraceae belongs to this ruderal strategy. They grow fast (de Souza *et al.*, 2005), fuse hyphae more readily (de la Providencia *et al.*, 2005), invest earlier and more abundantly in spore formation (de Souza *et al.*, 2005) and high root colonization (Declerck *et al.*, 2001) as well as larger extraradical hyphal. Ruderal AMF with high growth rates and short life cycles produce low cost nonenduring biomass.

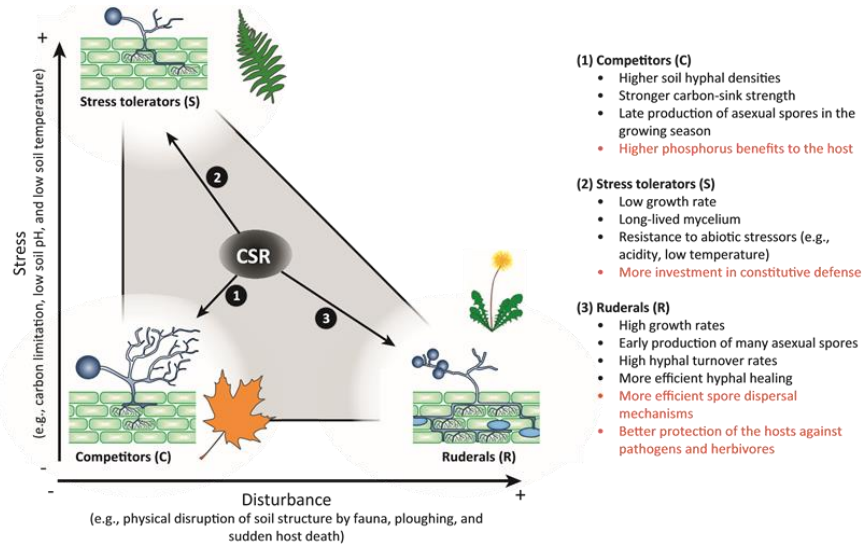

Fig. 4 A C-S-R triangle identifying stress and disturbance factors as well as phenotypic traits of AMF classified as competitors, stress tolerators, or ruderals. Empirical evidence is lacking for the suggested traits highlighted in red. On the triangle, is illustrated the corresponding plant life history strategy that may match each fungal strategy given that preferential associations are likely between plants and fungi with similar C, S, or R strategies (adapted from Chagnon *et al.*, 2013).

II.4.2 Phylogeny of AMF

Arbuscular mycorrhizal fungi is a monophyletic group (Schwarzott & Schüßler, 2001) placed in the phylum Glomeromycota (Redecker *et al.*, 2013; Schüßler & Walker, 2010; Schüβler *et al.*, 2001). Identification of species in this phylum was most often based on spores. Thus concepts of spore wall characteristics and murographs as proposed by Walker (1983) were used until today (Redecker *et al.*, 2013). However, this method was not powerful enough to clarify many doubts about undescribed new species, requiring novel tools to improve AMF identification (Souza, 2015). DNA extraction techniques from one to several spores (Schwarzott & Schüßler, 2001) as well as several molecular markers (i.e. COX1 region, β-tubulin, elongation factor 1-α, the mitochondrial LSU (mtLSU) rRNA gene and SSU-ITS-LSU gene rDNA barcode) (Krüger *et al.*, 2009; Schüßler & Walker, 2010; Senés-Guerrero, 2014) were developed. DNA-methods thus gained in importance through the years facilitating the identification of conspecific (i.e. species with spores having the same morph within distinct families (Stockinger *et al.*, 2010)) organisms. Furthermore, many AMF are non-culturable and their identification from environmental samples can be achieved via these molecular methods (Davison *et al.*, 2015). However, the sequencing of DNA from AMF has left a the large number of publication with taxon names at all levels, generating confusion (Redecker *et al.*, 2013). Thereby, a current classification mainly based on the studies of Schüßler & Walker (2010); Krüger *et al.* (2012) and Redecker *et al.* (2013) is proposed, encompassing the identification of AMF based on DNA sequencing and classical taxonomy (see -http://amf-phylogeny.com/). The Glomeromycota thus has been divided in four orders Glomerales, Diversisporales, Paraglomerales and Archaeosporales (Schüβler *et al.*, 2001; Schüßler & Walker, 2010; Redecker *et al.*, 2013) with eleven families (Acaulosporaceae, Ambisporaceae, Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae, Geosiphonaceae, Gigasporaceae,

Glomeraceae, Pacisporaceae, Paraglomeraceae, and Sacculosporaceae), twenty-five genera (*Acaulospora, Ambispora, Archaeospora, Cetraspora, Claroideoglomus, Corymbiglomus, Dentiscutata, Diversispora , Funneliformis, Geosiphon, Gigaspora, Glomus, Intraornatospora, Otospora, Pacispora, Paradentiscutata, Paraglomus, Racocetra, Redeckera, Rhizophagus, Sacculospora, Sclerocystis, Scutellospora, Septoglomus, and Tricispora*), and more than 200 species (Redecker *et al.*, 2013; Souza, 2015) (Fig. 5).

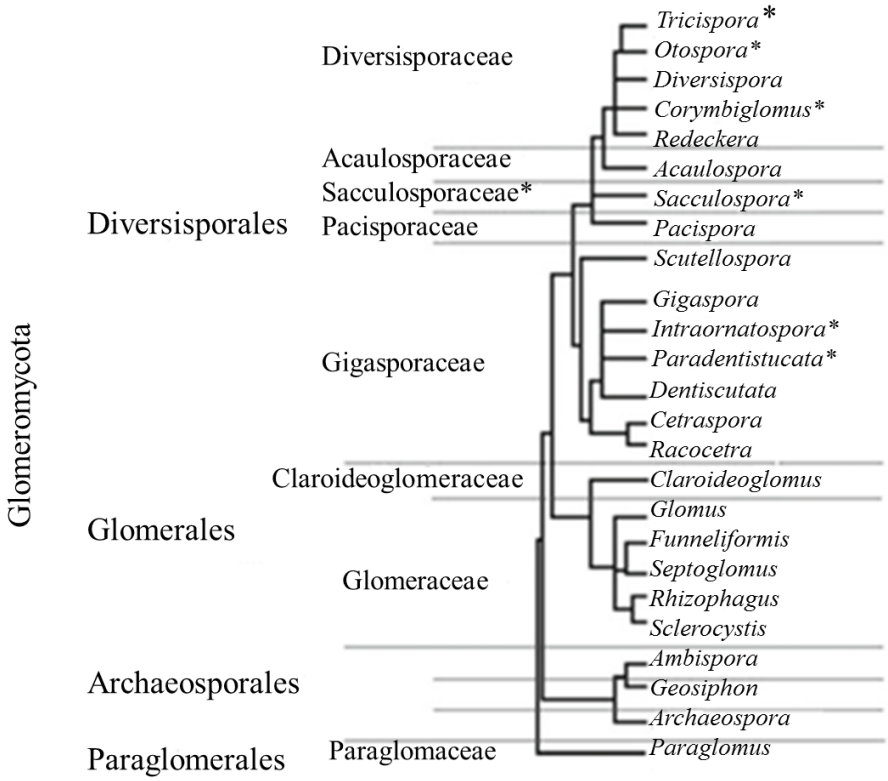

Fig. 5 Classification of Glomeromycota based on Redecker *et al.* (2013). Phylogenetic tree adapted from Souza (2015). Asterisks indicate insufficient evidence, but no formal action taken.

II.4.3 AMF diversity in hydrocarbon polluted soils

In the last years, several studies have been conducted in hydrocarbon-polluted soils where different plant species were observed to growth, suspecting that AMF may play a role in plant adaptation to hydrocarbon pollutants (Hassan *et al.*, 2014). A number of studies have reported the presence of AMF associated with several plant species growing in hydrocarbon-polluted soils (Cabello, 1997; Villacrés *et al.*, 2014; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016; Garcés-Ruiz *et al.*, 2017b). These findings suggest their tolerance/resistance to petroleum hydrocarbons pollutants (Cabello, 1997; Franco-Ramírez *et al.*, 2007). Their presence has been demonstrated and identification conducted through the isolation of spores as well as the use of molecular tools. Table 4, summarizes the AMF taxa found in different hydrocarbon-contaminated sites.

II.4.4 Impact of hydrocarbon pollutants on the AMF physiology

A number of studies have been conducted on the effects of hydrocarbon pollutants on the AMF physiology (i.e. germination, colonization, extradical elongation, sporulation). Table 5 shows the impact of hydrocarbon-pollutants on the physiology of different AMF species.

Table 4 AMF species indentified by different methodologies under hydrocarbon polluted soils associated with several plant species.

Site	Sample/Host plant	Hydrocarbon pollutant	AMF	Identification metodology	Reference
Ensenada, Argentina	soil and root system from *Cynodon dactylon*	Crude oil	*Glomus aggregatum, Gigaspora* sp.	Spore extracted from trap cultures	(Cabello, 1997)
Rositz, Germany	soil and root system from *Solidago* sp. and *Dactylis glomerata*	Polycyclic aromatic hydrocarbons	*Glomus mosseae*		
Joya de los Sachas, Ecuador	Soil	Total hydrocarbon petroleum (TPH)	Predominant Genera *Glomus* and *Acaulospora*. *Entrophospora*	Spore extracted from trap cultures	(Villacrés *et al.*, 2014)
Moxi oil field, China	Soil	Petroleum	*Glomus* (*G. constrictum* and *G. mosseae*), *Acaulospora and Archaeospora*	spores isolated from contaminated soil	(Huang *et al.*, 2007b)
Varennes, Canada	Rhizosphere soil of introduced willow cultivars	Petroleum hydrocarbons from petrochemical refining activities	Archaeosporaceae, Claroideoglomeraceae, Diversisporaceae, Gigasporaceae, Glomeraceae (*Funneliformis, Septoglomus, Glomus and Rhizophagus*), Paraglomeraceae	454-pyrosequencing 18S rDNA region	(Hassan *et al.*, 2014)
Varennes, Canada	Rhizosphere and roots from *Eleocharis obtusa* and *Panicum capillare*	Petroleum hydrocarbons contaminated sediments Sedimentation basin	*Claroideoglomus, Diversispora, Rhizophagus Paraglomus* (more abundant)	high-throughput PCR, cloning and sequencing of 18S rDNA	(de la Providencia *et al.*, 2015)
St-Lawrence River, Canada	Roots *Solidago rugosa*	Former petrochemical plant aliphatic and aromatic hydrocarbons	Claroideoglomeraceae, Diversisporaceae, Glomeraceae and Archaeosporaceae	454-pyrosequencing 18S rDNA region	(Iffis *et al.*, 2014)
St-Lawrence River, Canada	Solidago canadensis, Populus balsamifera and Lycopus europaeus	Petroleum hydrocarbon waste in three decantation basins	Mainly *Diversispora , Glomus, Acaulospora, Claroideoglomus Rhizophagus, Entrophospora, Funneliformis, Acaulospora Claroideoglomus* unclassified Archaeosporaceae	454-pyrosequencing 18S rDNA region 454-pyrosequencing ITS dataset	(Iffis *et al.*, 2016)
Activo Cinco Presidentes, Mexico	Rhizosphere and roots from *Echinochloa polystachya, Citrus aurantifolia, C. aurantium*	Chronically petroleum-contaminated	From *E. polystachya*: *Glomus ambisporum, G. sinuosum, Acaulospora laevis, Ambispora gerdermanni*. From citrus trees: *Scutellospora heterogama, G. ambisporum, Acaulospora scrobiculata*, and *G. citricola*.	Spore extracted from trap cultures	(Franco-Ramírez *et al.*, 2007)

Charapa field, Ecuadorian Amazon	Roots from *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria*	Weathered Oil Ponds	*Rhizophagus proliferus*, *Rhizophagus* sp. *Acaulospora* sp. *Acaulospora longula*, *Acaulospora kentinensis*, *Glomus* sp. *Archaeospora* sp.	high-throughput PCR, cloning, RFLP from partial SSU, the whole ITS and partial LSU rDNA region	(Garcés-Ruiz *et al.*, 2017b)

Table 5 Impact of hydrocarbon-pollutants on the physiology of AMF. Adapted from Lenoir *et al.* (2016).

Stress type	AMF	Culture	AMF physiological step affected					Reference
			Spore germination	Germination hyphae elongation	Root colonization	Extraradical hyphal elongation	Sporulation	
Crude oil	*Funneliformis mosseae, Glomus ambisporum, Scutellospora heterogama*	water-agar	x	x		x		(Franco-Ramírez *et al.*, 2007)
	Rhizophagus irregularis	in vitro *Daucus carota*		x	x	x		(Kirk *et al.*, 2005)
	Funneliformis constricum				x			(Tang *et al.*, 2009)
Diesel	*Glomus* Zac-19	Pot (artificial contamination)			x			(Hernández-Ortega *et al.*, 2012)
	Rhizophagus irregularis	M medium associated to transformed chicory (*C. intybus* L.)	x	x	x	x	x	(Driai *et al.*, 2015)
	Gigaspora margarita	*Echynochloa polystachya* water-agar	x	x	x	x	x	(Alarcón *et al.*, 2006)
PAH	*Funneliformis caledonium, Rhizophagus irregularis, Claroideoglomus lamellosum, Rhizophagus proliferus, Funneliformis mosseae, Glomus ambisporum, Scutellospora heterogama, Rhizophagus custos*	M medium associated to transformed chicory (*C. intybus* L.)	x	x	x	x	x	(Liu *et al.*, 2004; Verdin *et al.*, 2006; Debiane *et al.*, 2008, 2009, 2011; Aranda *et al.*, 2013)
	Funneliformis geosporum, Funneliformis mosseae, Claroideoglomus etunicatum	Pot (artificial contamination)	x	x	x	x	x	(Gaspar *et al.*, 2002; Rabie, 2004, 2005; Wu *et al.*, 2009)
	Funneliformis mosseae	Pot (artificial contamination)			x			(Leyval & Binet, 1998)

II.4.5 AMF protection mechanism to mitigate plant stress under hydrocarbon pollutants

Several reponse of AMF to mitigate plant stress in the presence of pollutants have been observed: 1) Improved plant mineral nutrition, 2) qualitative and quantitavie changes of sugars, polyamines and lipids, 3) increased tolerance to oxidative stress, 4) modifications in plant physiology (photosynthetic activity, osmotic balance) and 5) Root and fungal chelation and inactivation/exclusion of pollutants (Plouznikoff *et al.*, 2016).

Table 6 shows the parameters which are improved in plant species associated with different AMF under hydrocarbon conditions.

Table 6 Impact of AMF on plant parameters under under hydrocarbon-polluted conditions. Adapted from Plouznikoff *et al.* (2016).

				Improved parameters						
Stress	**AMF**	**Host plant**	**Growth conditions**	**Growth promotion**	**Nutrition**	**Antioxidant system**	**Osmotic balance**	**Photosynthesis**	**Remarks**	**References**
Petroleum (5, 10 g/kg soil)	*R. intraradices/ irregularis*	*Avena sativa* cv. Baiyan No. 7	Pot culture greenhouse	+		+	+		- MDA, proline +SOD, CAT, POD +Ureases, sucrases, dehydrogenases	(Xun *et al.*, 2015)
Petroleum (3.5 g/kg soil)				+		+			+SOD, urease, dehydrogenase = CAT, POD	(Dong *et al.*, 2014)
Crude oil (2, 4, 8% soil)	*F. mosseae*	*Phaseolus vulgaris* L.	Pot culture greenhouse	+						(Nwoko, 2014)
Crude oil (5%)	*Glomus desertícola, G. fasciculata; G; geosporum; G. intraradices, G. mosseae*	*Medicago sativa*	Pot culture	+	+ P and Zn					(Cabello, 1999)
Diesel (0.05, 0.1, 0.25, 0.5, 1% of M medium)	*R. irregularis* (MUCL43194)	Transformed *Cichorium intybus* roots	In vitro	+(In low diesel conc.: 0.05, 0.1, and 0.25 %)		+			= MDA - SOD + POD	(Driai *et al.*, 2015)
Diesel (2, 6, 10, 15 g/kg soil)	*G. constrictum*	*Zea mays* cultivars Yuyu 22	Pot culture	+		+	+	+	*G. constrictum* was isolated in a petroleum polluted soil Proline, MDA (10 and 15 g/kg) +SOD, CAT, POD	(Tang *et al.*, 2009)

Diesel (7500 mg /kg^{-1})	*Glomus Zac-19*	*Melilotus albus* Medik	Pot culture	+		+N,P,K, Ca	+		MP had higher degradation of TPH tan NM	(Hernández-Ortega *et al.*, 2012)
Diesel /Biodiesel (7%)	Mix of 12 AMF *Acaulospora morrowiae, A. spinosa, A. scrobiculata, Funneliformis mosseae, F. geosporus, Gigaspora rosea, Gi. decipiens, Glomus macrocarpum, G. aggregatum, R. intraradices, Scutellospora pellucida Claroideoglomus etunicatum*	*Brachiaria decumbens*	Pot culture		+				Biodiesel more toxic than diesel Both were degraded by MP	(Trejo *et al.*, 2013)
Anthracene (6.25, 12.5, 25, and 50 mg/L)					=		+		-MDA, =SOD, - 8-OHdG (50 mg/L) No DNA fragmentation in M and NM roots	(Debiane *et al.*, 2008)
Anthracene (30 and 140 mg/L)	*R. irregularis* (MUCL43194)	Transformed *Cichorium intybus* roots	In vitro		+				+ POD	(Verdin *et al.*, 2006)
B[a]P (35, 70,140, 280 µM)					+		+		-MDA, 8-OHdG, +SOD -POD (35 and 140 µM)	(Debiane *et al.*, 2009)

Anthracene, Phenantrene	*R. irregularis*, *G. versiforme*	*Allium porrum* L. cv. Musselburgh	Pot culture greenhouse	+	+ (N and P)		(Liu & Dalpé, 2009)
Anthracene, Phenantrene, dBT (60, 120, 240 µM)	*R. custos*	Transformed *Daucus carota* L.	In vitro	+ Anthacene Phenantrene = (dBT)		ANT did not impacted NM and M root dry weight, whereas PHE and dBT did	(Aranda *et al.*, 2013)
PAH phenanthrene (PHE), pyrene (PYR), dibenzo(a,h)anthracene (DBA)	*G. intraradices*	*Medicago sativa* cv. Europe, *Festuca arundinacea* cv. Bariane, *Lolium multi-florum* cv. Barclay Apium graveolen)	Pot culture Growth chamber	+	+ P	PHE dissipation but not PYR and DBA	(Zhou *et al.*, 2013)
Coal mine	*G. aggregatum* (BGC HK02D), *R. intraradices* (BGC BJ09), *F. mosseae* (BGC XJ01)	*Zea mays* L	Pot culture	+	+ N,P,K,C		(Guo *et al.*, 2014)

Each sign +, =, and - indicates an increase, an equal, or a decrease effect of AMF on host plant (MP) as compared to non-mycorrhized (NM) controls, respectively. Abbreviation meaning SOD = Superoxide dismutase, POD = peroxidase, CAT=catalase, MDA= malondialdehyde, 8-OHdG = 8-hydroxy-2' –deoxyguanosine.

II.5 Plant phosphorus nutrition in presence of AMF under normal and stress conditions

Phosphorus is a major nutrient for plant growth. Despite its relatively large abundance in soil, P acquisition by plants is difficult because this mineral is poorly available in soil due to its low solubility and slow movement (Smith *et al.*, 2011). Arbuscular mycorrhizal fungi are known for their capacity to take up nutrients (i.e. P, N, Cu, Fe, K, Zn) from the soil. Hence, numerous studies have reported on the role of these fungi in plant nutrition and several mechanisms were proposed, especially for the uptake and transport of Pi to plants (Smith *et al.*, 2004; Javot *et al.*, 2007; Smith *et al.*, 2011; Zhang *et al.*, 2016; Garcés-Ruiz *et al.*, 2017). Two pathways have been identified for P transport to plants in presence of AMF. The direct pathway where root cells take up P from the rhizosphere (Smith & Smith, 2012) and the indirect or mycorrhizal pathway that correspond to the uptake and transport of Pi by the extraradical mycelium often far beyond the root exploration area (Ferrol *et al.*, 2016) (Fig. 6). Furthermore, P deficiency in soil increases the flow of plant C to the AMF, revealing that the flow of C to the fungus is proportional to the quantity of Pi received by the plant (Chagnon *et al.*, 2013). The exchange of this nutrient for C depends of the extraradical mycelium development rather than on the intensity of root colonization (Chagnon *et al.*, 2013). The Pi uptake is achieved by the high affinity Pi transporters (i.e. H^+- and Na^+ -membrane dependent transporters). The Pi is transferred into the vacuole of the extraradical mycelium and synthesized into polyP, which is a polyanionic compound (Zhang *et al.*, 2016). The polyP is then broken to orthophosphate by phosphatases and translocated to the intraradical mycelium, where it is transferred from the fungal cells to the root cells (Smith & Read, 2008).

The Pi absorption in presence of AMF has been widely studied under greenhouse (Nouri *et al.*, 2014; Walder *et al.*, 2015; Garcés-Ruiz *et al.*, 2017a) in the field (Yang *et al.*, 2014) and under *in vitro* culture conditions (De Jaeger

et al., 2011; Zocco *et al.*, 2011; Calonne *et al.*, 2014b) using isotopes (i.e., ^{33}P and ^{32}P). However, the role of AMF on Pi uptake and transport has been less studied under stress conditions (e.g. hydrocarbon pollutants). Pi transport from the extraradical mycelium of *R. irregularis* to the host transformed chicory roots was monitored in presence of anthracene and benzo[a]pyrene (B[a]P) by Calonne *et al.* (2014). These authors demonstrated a detrimental effect of these hydrocarbons on the quantity of Pi transported to the roots. Others studies revealed the efficiency of mycorrhizal root colonization, increasing P and N uptake as well as plant growth in presence of polyaromatic hydrocarbons (PAHs) (Liu & Dalpé, 2009; Zhou *et al.*, 2013)diesel (Hernández-Ortega *et al.*, 2012) crude oil (Cabello, 1999) and in coal mine spoils (Guo *et al.*, 2014).

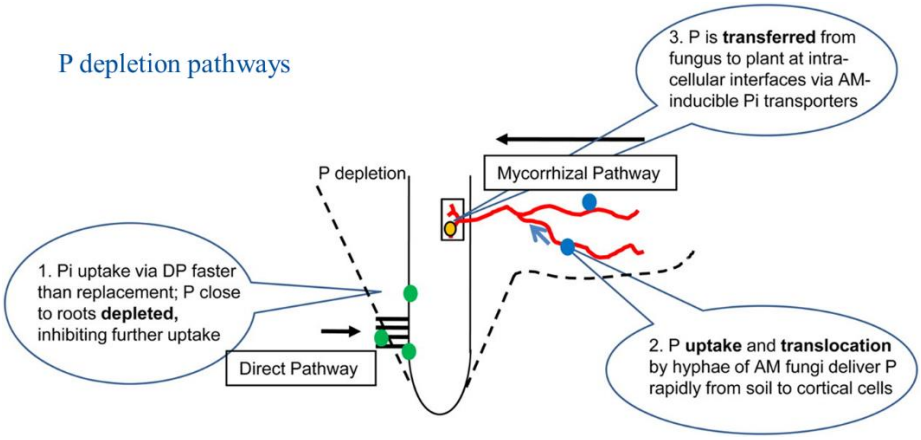

Fig. 6 Two pathways of P absorption in AMF-colonized roots. The direct pathway (DP) and mycorrhizal pathway, each of them involves different regions of the roots, different cell types and different Pi transporters. In the DP, Pi is absorbed from the rhizosphere by plant Pi transporters in epidermis and root hairs (green circles) close to the root surface. Uptake is normally faster, resulting in reduced Pi concentrations (depletion) close to the roots (callout 1). In the mycorrhizal pathway, Pi is taken up into AMF hyphae by fungal Pi transporters (blue circles) several centimeters away from the root and translocated to intracellular fungal structures (arbuscules and hyphal coils) in root cortical cells (callout 2). Plant Pi transporters, induced in colonized cells (yellow circle), transfer Pi from the interfacial apoplast to plant cortical cells (callout 3). Adapted from Smith *et al.* (2011).

II.6 Anastomosis and Hyphae Healing Mechanism (HHM) of AMF

Anastomosis, which is the ability of compatible hyphae to fuse or reconnect sectors that grow apart or were disrupted by physical injury (de la Providencia *et al.*, 2005; Purin & Morton, 2011) is a fundamental mechanism for fungi to create large networks (Novais *et al.*, 2017), to survive (Giovannetti *et al.*, 2015), to favor nutrient flow and to maintain genetic diversity (Chagnon, 2014; Novais *et al.*, 2017). Anastomosis in injured hyphae is also known as hyphae healing mechanism (HHM) (de la Providencia *et al.*, 2005), defined as damaged hyphae capable to reconnect or overcoming obstructed areas within a hyphae (de Souza & Declerck, 2003; Gerdemann, 1955). The hyphal bridges formed after injury have been mostly observed in Gigasporaceae, while it is less frequent in Glomeraceae (de la Providencia *et al.*, 2005, 2007; de Souza & Declerck, 2003; Gerdemann, 1955). de la Providencia *et al.*, (2005) studied under *in vitro* culture conditions the HHM in several AMF species belonging to Gigasporaceae and Glomeraceae. These authors described four-steps in the HHM: (1) septum formation, (2) initiation of growing hyphal tips (GHTs), (3) GHT elongation, orientation and contact, and (4) GHT fusion and cytoplasmic/protoplasmic flow re-establishment (Fig.7). They further noticed that the HHM differed considerably between representatives of these two families that diverge in life history strategies (i.e. r and K strategies). In *Gigaspora margarita, Gigaspora rosea* and *Scutellospora reticulata* (K-strategy representatives) a complete recovery (i.e. flow re-establishment) between growing hyphal tips (GHTs) formed at both sides of the cut hyphae was observed. Conversely, in *Glomus intraradices*, *Glomus proliferum* and *Glomus hoi* (r-strategy representatives), the production of GHTs at both sides were abundant and disorganized. The fungus was able to reconnect but also to connect neighbor hyphae or even roots.

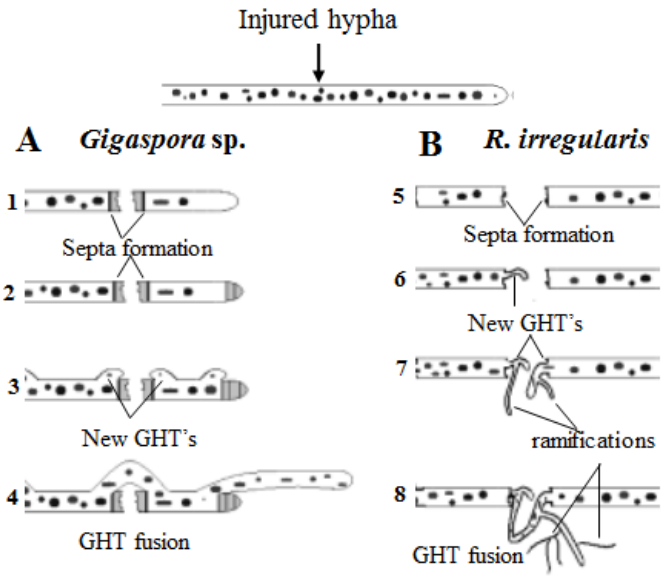

Fig. 7 Diagram showing the HHM in injured hyphae of *Gigaspora* sp. and *Rhizopagus irregularis*. (A) In *Gigaspora* sp., (1-2) septa produced at the extremities of each cut section. (3) GHTs formation behind the septa. (4) GHTs fusion, complete hyphal recovery (B) In *R. irregularis*, (5) Septa produced at the cut-extremities of each section. (6) The first GHT initiated its elongation from the cut-extremities. (7) GHTs with ramifications. (8) GHT fusion. Adapted from de la Providencia *et al.* (2007).

Several authors have studied anastomosis of AMF under "non-perturbed conditions" (de Souza & Declerck, 2003; Giovannetti *et al.*, 1999; Pepe *et al.*, 2016; Sbrana *et al.*, 2011; Voets *et al.*, 2006), while only a few studies were conducted under stress conditions, such as injured hyphae (de la Providencia *et al.*, 2005; 2007). Moreover, no information is available on the impact of pollutants (e.g. hydrocarbons) on this essential attribute for the fungus to survive, interconnect mycelia and plants and transport nutrients and genetic information. It is thus of prime importance to know these impacts for remediation strategies involving the introduction of species in hydrocarbon-contaminated soils.

III. Materials and methods

a. Sampling site description

The sampling location was situated in the Amazonian region in the Province of Sucumbíos, Ecuador, in the environmental liability known as Charapa field at 309 masl (Fig. 8). This field is handled by Petroamazonas EP, Ecuadorian public enterprise. The field has a surface area of 244 Km^2 and contains weathered crude oil polluted ponds. This field was abandoned ~30 years ago and was naturally recolonized by native plants. The flora ecosystem was characterized by the botanist A. Pérez (2014) who works at PUCE, Facultad de Ciencia Exactas y Naturales. The plant community was described as a Lowland Evergreen Forest of Napo-Curaray (Guevara, 2013)(Fig. 9). Two ponds and the surrounding soil were considered for the AMF diversity study. Pond 1 of 330 m^2 (76°48'57" W, 00° 11'49" S**)** and Pond 2 of 450 m^2 (76°48'54" W, 00°11'46" S).

Physico-chemical properties of the soil inside the pond 2 and surrounding soil were analyzed (Table 7). A layer of ~ 10 cm of organic matter was above the weathered crude oil (Fig 10 A). Conversely in the surrounding site, the soil consisted of clay (Fig. 10 B).

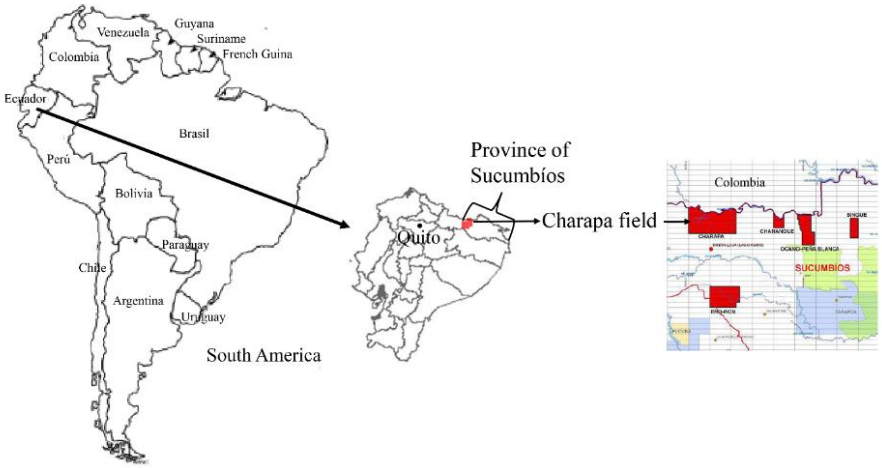

Fig. 8 Charapa field location. Situated in the Province of Sucumbíos, approximately 15 km to the NE of Lago Agrio (currently Nueva Loja, Capital), and limited to the North with the Republic of Colombia. As Charapa, Chanangue, Oceano-Peña Blanca, Singue and Eno-Ron (red sites) represent oil wells. Source: http://rondaspetroleras.yage.ec/portal/web/ronda-petrolera/charapa.

Table 7 Physical and chemical analysis from soil collected in Pond 2 and in the surrounding soil (i.e. control) from Charapa field.

Analysis	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Analytical method
pH	8	6	5	5	8.1	CP-PEE-S004
Organic matter %	> 95	> 95	> 95	>95	44.55	Gravimetric
P mg Kg^{-1}	> 450	404.8	> 450	321.8	49	SM 4500 P B-C
N mg Kg^{-1}	> 1500	666.6	> 1500	1138.6	> 1500	SM 4500-N C
K mg Kg^{-1}	133.7	42.3	246.1	26.8	82.1	EPA 3051/7000A
TPH mg Kg^{-1}	> 5000	> 5000	> 5000	>5000	1188.8	CP-PEE-S003

Soil samples description: three independent samples and two composite samples were analyzed. Samples: (1) a surface sample and (2) a sample collected at 30 cm depth, close to the center of the pond, (3) a composite surface sample and (4) a composite sample collected at 30 cm depth, made of soil cores collected in the 4 corners inside the pond, and (5) a sample at 30 cm depth collected 70 m outside the Pond (i.e. control).

Fig. 9 Charapa field, sampling site showing, (A) the flora of the surrounding soil of the ponds, (B) species plants present in the pond 2, (C) setting up the transect point for collection and (D) Old trees growing inside the hydrocarbon polluted soil.

Fig. 10 (A) Soil inside the ponds 1and 2, (B) surrounding soil.

b. Biological Material

For the experiments in the greenhouse and *in vitro* culture, AMF MUCL strains were used due to their relative fast growth under the conditions required.

i. Microbial Material for Greenhouse and *in vitro* experiments

***Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as ['irregulare'] MUCL 41833:** formerly named *Glomus intraradices*/*irregulare* (Redecker *et al.*, 2013) was isolated from the Canary Islands (Spain). The strain was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). It was grown *in vitro* in association with Ri T-DNA transformed chicory (*Cichorium intybus* L.) roots clone A4NH (Fontaine *et al.*, 2004) in Petri dishes (135 mm diam.) containing 90 mL MSR (Declerck *et al.*, 1998) medium solidified with 3 g L^{-1} GelRite® (Carl Roth, Germany). Petri dishes were incubated in the dark at 27°C for one month until proliferation of runner hyphae were observed.

R. irregularis MUCL 41833 is a model fungus used for *in vivo* (Rufyikiri *et al.*, 2000; Anene & Declerck, 2016; Buysens *et al.*, 2016) as well as for *in vitro* studies (Anene *et al.*, 2013; Koffi *et al.*, 2013; Buysens *et al.*, 2015; Kothamasi *et al.*, 2015; Loján *et al.*, 2017). *R. irregularis* is widely distributed all over the world. It is known as a generalist symbiont and has been classified as an r-strategist or ruderal basis on the AMF life-history strategy (LHS) (IJdo *et al.*, 2010; Chagnon *et al.*, 2013).

***Gigaspora* sp. (Gerdemann and Trappe) MUCL 52331:** was isolated from an agro-ecosystem from winter crop of Secale cereal in the center for Environmental Farming Systems in North Carolina (USA) and supplied by the Glomeromycota *in vitro* collection (GINCO - http://www.mycorrhiza.be/ginco-bel). The strain was grown in the same conditions as *R. irregularis* (see above) during one month.

Gigaspora is considered an inhabitant of undisturbed environments (de Souza *et al.*, 2005). According to the LHS, this genus is classified as a K-strategist or competitor (IJdo *et al.*, 2010; Chagnon *et al.*, 2013).

c. Plant Material

i. Plant material used for AMF root colonization evaluation, diversity assessment and trap cultivation

Different plant species recolonizing the weathered crude oil ponds and the surrounding soil of the Charapa field in the Amazonian region were harvested for assessing AMF root colonization by the technique of McGonigle *et al.* (1990) (see section e) and community composition by molecular tools (see section d). Three samplings campaigns were conducted in the polluted area (Table 8). The first and the second one were at random. Several plant species were collected inside Pond 1, Pond 2 and the surrounding soil (Table 9). The third one was done only in Pond 2. Transects were traced from the contour size of the pond and every ~7.3 m a sample was taken 3 meters outside and 3 meters inside de pond. Several plants species were collected. Moreover, some plants were sampled in the center of the pond (Table10, Fig. 11). The leaves from the collected plants of the second and third sampling were used for species identification by the herbarium at PUCE.

Table 8 Information of sampling performed in the Charapa field in the Amazonian forest.

Sampling	#_Samples	Collection date	Side	Aim
1	26	October 2012	Pond 1, 2 and surrounding soil	Conditions establishment for ink staining, pot culture and DNA extraction
2	54	December 2012	Pond 1, 2 and surrounding soil	Chapter 1
3	40	December 2013	Pond 2	Chapter 2

Table 9 Plant species and number of plants shared by site (Pond 1, Pond 2 and surrounding soil) within Charapa field (Province of Sucumbíos, Amazonian forest Ecuador**).** Sampling December 2012.

Plant name	Pond 1	Pond 2	Surrounding soil
Monotagma sp. 1	4	4	
Polybotrya sp. 2		1	1
Geonoma cf. deversa (Poit.) Kunth	1	2	
Euterpe precatoria Mart.	7	4	2
Costus scaber Ruiz & Pav.	2	1	1
Costus pulverulentus C. Presl	1	2	
Costus lima var. scabremarginatus Maas	2	3	1
Carludovica palmata Ruiz & Pav	2	3	1
Polybotrya sp. 1	2	1	
Asplenium sp. 2 *	1		
Heliconia chartacea Lane ex Barreiros *			1
Heliconia sp. 1 *			1
Iriartea deltoidea Ruiz & Pav. *	1		
Renealmia cf. thyrsoidea Ruiz & Pav. *		1	
Tococa guianensis Aubl. *	1		

Plants with (*) had only one representative and were not used in the statistic of AMF colonization percentage. Plants in grey were used for AMF identification by Sanger sequencing.

Table 10 Plant species and number of plants sampled 3 meters outside and inside the Pond 2 and in the center. From December 2013.

Plant name	3 meters outside	3 meters inside	Center
Piper sp.	1	2	
Miconia sp.	1		
Inga auristellae	2		
Costus sp.	2	2	
Calathea	1		
Anthurium sp	1	2	
Heliconia sp.	1		
Euterpe precatoria	1	2	
Urera baccifera	1	1	
Urera caracasana		1	
Stigmatopteris		1	
Acalypha	1		
Geonoma macrostachys		1	
Poaceae	2		2
Pouruma sp.	1		
Leandra sp.	1		
Cyathea sp.	1	1	
Dendropanax sp.	1		
Caladium sp.		1	
Cordia		1	
Acacia	1		
Renealmia sp.	1		
Cyclanthus bipartitus		1	
Faramea sp.			1
Paullinia sp.			1

Plants were used (1) to determine the AMF root colonization percentage, (2) to assess AMF community composition by DNA extraction and new generation sequencing by 454 GS-FLX.

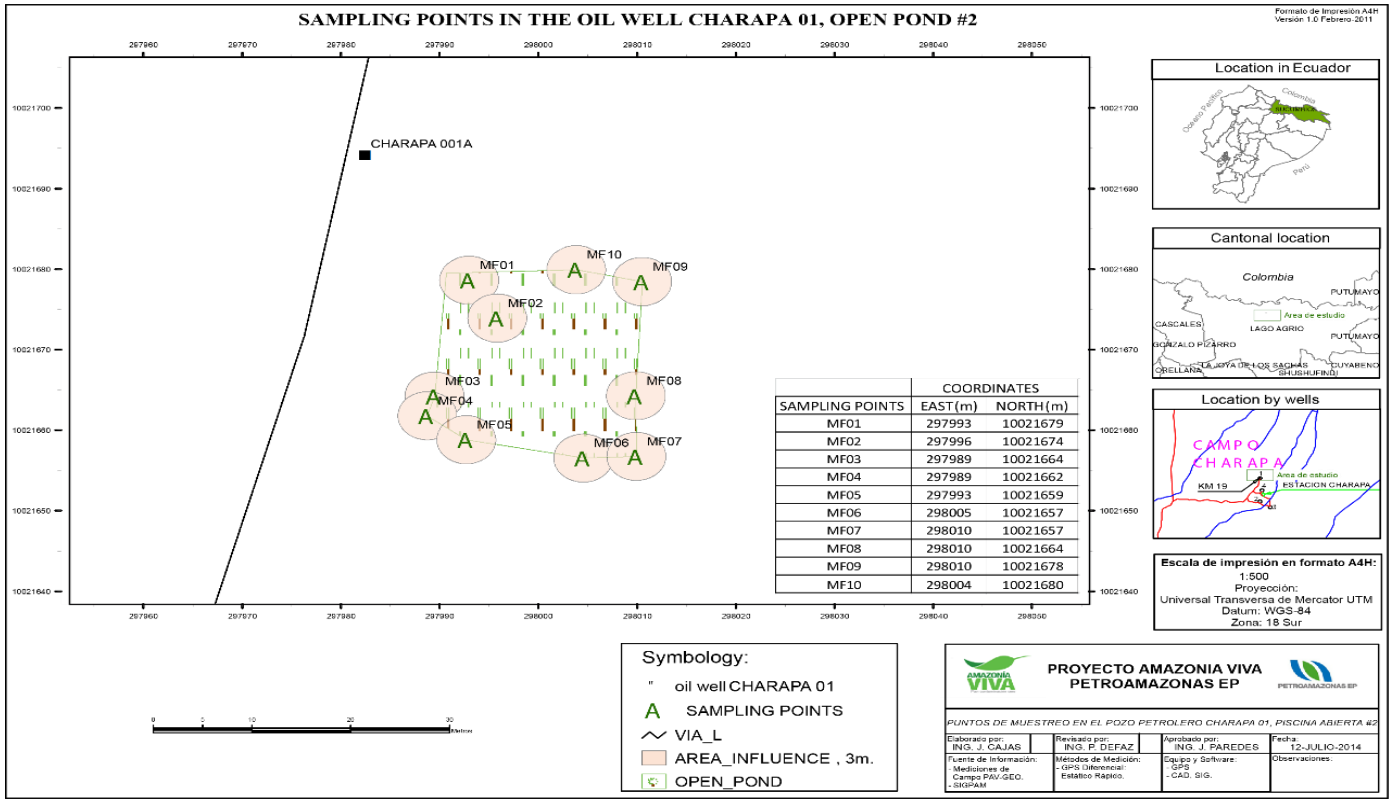

Fig. 11 Sampling points of plants (i.e. circles with A) according to the geographical coordinates in the Pond 2 in Charapa field, province of Sucumbíos. Carried out by the staff of PetroEcuador.

ii. Plant material used for Greenhouse experiment

Maize (Zea Mays L.) cv. ES Ballade was supplied by the Centre Indépendant de Promotion Fourragère (CIPF – http://www.cipf.be/). Before use, seeds were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 15 minutes and rinsed three times with sterilized (121°C for 15 min) deionized water during 10 minutes. The seeds were then placed on wet paper and germinated in plastic boxes in the dark at room temperature (~20°C). This crop plant originated from America, specifically from Mexico. Nowadays maize has been established as an important food crop all over the world (FAO, 2015). Its principles characteristics are: (1) to develop in a range of temperatures from temperate to tropical, (2) a varieties selection and (3) very fast growth (i.e. 6 months) making it a successful crop for cultivation (FAO, 2015). These characteristics have promoted its use in several experiments confronted to hydrocarbon pollutions (Agbogidi *et al.*, 2007a,b; Njoku *et al.*, 2009a; Oludele & Ogundele, 2013).

iii. Plant material for *in vitro* trial

Chicory (*Cichorium intybus* L.): Ri T-DNA transformed roots clone A4NH (Fontaine *et al.*, 2004) was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). The transformed roots was grown on MSR medium and incubated in an inverted position in the dark at 27°C. Chicory was sub-cultured in Petri plates (135 mm diam.) and associated with the AMF *R. irregularis* MUCL 41833 or *Gigaspora* sp. MUCL 52331 on 90 mL MSR medium.

d. Molecular Methods

For the characterization and identification of AMF communities, DNA from several roots of different plant species was obtained. Root pieces were

completely ground with liquid N_2 in a mortar to get fine powder. DNA from roots powder was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) following manufacturer's instructions.

Two sequencing methods (with nested PCR) were used: 1) Sanger sequencing of 1.5 kb extended rDNA-barcode from clone libraries and 2) high throughput 454 GS-FLX+ pyrosequencing of a 800 bp rDNA fragment (Fig. 12) (Senés-Guerrero & Schüßler, 2016a).

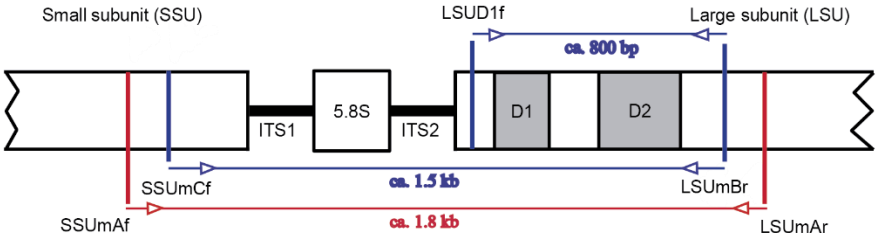

Fig. 12 Schematic representation of the nuclear ribosomal DNA regions targeted by PCR and nested primers in Sanger and/ or pyrosequencing method. Arrows in red represented the primers used in the first PCR for both techniques. Blue arrows represent the primers used in the nested PCR. The blue arrows below the target region represent the primers used in the Sanger method. The blue arrows upper the regios represent the primers used in the pyrosequencing method. (Not at scale). Adapted from Senés-Guerrero & Schüßler (2016a).

AMF primers

Specific primers were used for the two methods

1. **Sanger sequencing:** For the first PCR, the forward primer SSUmAf is a mix of 10 µL of each primer SSUmAf1 - 2 with 80 µL of water. For the reverse primer, LSUmAr is a mix of 10 µL of each primer LSUmAr1 - 4 with 60 µL of water. For the nested PCR, the forward primer SSUmCf is a mix of each primer SSUmCf 1-3 with 70 µL of water. For the reverse primer LSUmBr is a mix 10 µL of each primer LSUmBr1 - 5 with 50 µL of water. (Table 11).

Table 11 Primers for the first and nested PCR used in Sanger sequencing of a 1.5 kb rDNA fragment. Adapted from Senés-Guerrero & Schüßler (2016a).

Step	Primer	Nucleotide sequence (5´-3´)	Primer mixtures
First PCR (1.8 kb)	SSUmAf1	TGGGTAATCTTTTGAAACTTYA	SSUmAf: mix SSUmAf1-2 (equimolar)
	SSUmAf2	TGGGTAATCTTRTGAAACTTCA	
	LSUmAr1	GCTCACACTCAAATCTATCAAA	LSUmAr: mix LSUmAr1-4 (equimolar)
	LSUmAr2	GCTCTAACTCAATTCTATCGAT	
	LSUmAr3	TGCTCTTACTCAAATCTATCAAA	
	LSUmAr4	GCTCTTACTCAAACCTATCGA	
Nested PCR (1.5 kb)	SSUmCf1	TCGCTCTTCAACGAGGAATC	SSUmCf: mix SSUmCf1-3 (equimolar)
	SSUmCf2	TATTGTTCTTCAACGAGGAATC	
	SSUmCf3	TATTGCTCTTNAACGAGGAATC	
	LSUmBr1	DAACACTCGCATATATGTTAGA	LSUmBr: mix LSUmBr1-5 (equimolar)
	LSUmBr2	AACACTCGCACACATGTTAGA	
	LSUmBr3	AACACTCGCATACATGTTAGA	
	LSUmBr4	AAACACTCGCACATATGTTAGA	
	LSUmBr5	AACACTCGCATATATGCTAGA	

2. **New generation sequencing:** For the first PCR, the primers SSUmAf – LSUmAr or SSUmCf – LSUmBr (Table 3) were used. For the nested PCR the primers with an adapter and 3 molecular identifiers (MIDs) were used to obtain sequences from 3 different sites LSUDf + MID+ D1f A – LSUBr (Table 12).

Table 12 Composition of primers for nested PCR used in high throughput 454 GS-FLX + pyrosequencing of a 800 bp rDNA fragment. Designed primers by ROCHE.

Forward primer name	Adapter A	MID	LSU-D1f
LSU-D1f-A-MID1	CGTATCGCCTCCCTCGCGCCATCAG	ACGAGTGCGT	TAAGCGGAGGAAAAGAAAMTAAC
LSU-D1f-A-MID2	CGTATCGCCTCCCTCGCGCCATCAG	ACGCTCGACA	TAAGCGGAGGAAAAGAAAMTAAC
LSU-D1f-A-MID3	CGTATCGCCTCCCTCGCGCCATCAG	AGACGCACTC	TAAGCGGAGGAAAAGAAAMTAAC
Reverse primer name	**Adapter B**		**LSUmBr**
LSUmBr1-B	CTATGCGCCTTGCCAGCCCGCTCAG	N/A	DAACACTCGCATATATGTTAGA
LSUmBr2-B	CTATGCGCCTTGCCAGCCCGCTCAG	N/A	AACACTCGCACACATGTTAGA
LSUmBr3-B	CTATGCGCCTTGCCAGCCCGCTCAG	N/A	AACACTCGCATACATGTTAGA
LSUmBr4-B	CTATGCGCCTTGCCAGCCCGCTCAG	N/A	AAACACTCGCACATATGTTAGA
LSUmBr5-B	CTATGCGCCTTGCCAGCCCGCTCAG	N/A	AACACTCGCATATATGCTAGA

The 3 forward primers are a fusion of adapter A with MID (1, 2 or 3) plus LSU D1f. The reverse primer is a fusion of adapter B with the mix of 10 µL of each primer LSUmBr1 – 5.

PCR conditions

Sanger methods

The Sanger sequencing is a suitable method to amplify a 1.5 kb fragment covering the SSU-ITS-LSU rDNA region for members of the Glomeromycota as an extended DNA barcode providing species-level resolution. It is used to identify AMF from trap cultures (i.e. single spores or mycorrhizal roots) and also in field studies (Krüger *et al.*, 2009; Stockinger *et al.*, 2010; Senés-Guerrero *et al.*, 2014). The amplification of the SSU-ITS-LSU region was made according to Krüger *et al.* (2009). Briefly, the reaction mix contained 0.02 U μL^{-1} Phusion polymerase (Thermo Scientific, Lithuania), 1 x Phusion HF Buffer with 1.5 mm $MgCl_2$, (Thermo Scientific, USA) 200 µM of each dNTP (Promega, USA), 0.2 ug mL^{-1} BSA (Albumin Bovine, AMRESCO, USA) and 0.5 µM of each primer (Sigma, Germany) with 5 µL of template DNA (undiluted and 20 x DNA diluted) in 20 µL of final reaction. Thermal cycling was done in an Eppendorf Mastercycler Gradient (Eppendorf, Germany) with the following conditions for the first PCR: Five min initial denaturation at 99 °C; 40 cycles of 10 s. denaturation at 99 °C, 30 s. annealing at 60 °C and 1 min elongation at 72 °C; and a 10 min final elongation.

In the nested PCR, 1 µL of the first PCR product was used in the same final reaction (20 µL), the thermal cycling conditions were the same as for the first PCR, except that the annealing temperature was 63 °C and only 30 cycles were done. The PCR products were loaded on 1% agarose gel. Five µL of DNA with 5 µL of blue buffer 1 x (Sambrook, Fritsch, and Maniatis, 1989) were visualized on the electrophoresis Gel (FastGene® Agarose Tablets, NIPPON GENETICS EUROPE, Germany) with 100 x GelRed™ (Nucleic Acid Gel Stain, Biotium, Belgium) in 0.5 x TAE buffer (50 x, Ultra-Pure Grade,

AMRESCO®, USA). Migration parameters were: 100 V, 200 mA for 18 min and visualized in camera UV light (Vilber Lourmat, Belgium).

It is known that AMF spores contain heterogeneous nuclei (i.e. genetic variation intra- and inter - species) (Redecker *et al.*, 2003; Reddy *et al.*, 2005). Moreover, several AMF may colonize the same root (Redecker *et al.*, 2003) thus, cloning the PCR products help to identify the possible different patterns present in a multi-spores trap culture or in environmental samples (i.e. colonized roots or soil). Cloning, selection and sequencing were conducted as described in Krüger *et al.* (2009). In brief, PCR products were cloned with the Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific, USA) following the manufacturer's protocol, with only one-third of the specified volume of all components used. The ligation reaction between the PCR product and the PCR vector lasted one hour at room temperature. Cells of *E. coli* genetically modified (DH5α/ chemically competent) were transformed with the ligation product by thermal shock (42°C/ 1 min) and immediately stocked in ice to finish the process. Super Optimal broth with Catabolite repression (SOC, Thermo Fisher Scientific, USA) medium was used for initial bacterial growth after transformation. After 2 hours, 100µL of the bacterial inoculum was spread in the Lysogeny Broth (LB) (Fluka Sigma-Aldrich, India) medium with kanamycin antibiotic (Alfa Aesa, Germany) (50µg mL^{-1}) and incubated in the dark at 37°C for 24 hours.

Forty-eight clones from each sample were analyzed for correct length of plasmid inserts and clone selection (RFLP).

Colony-PCR was performed with 1x GoTaq DNA Polymerase (Promega, USA) and M13F and M13R primers. To roughly detect variable sequences between the clones, RFLP with two different enzymes was performed in 10 µL reaction volume, containing 5 µL colony-PCR product, with one of the restriction enzymes Hinf I (1 U), or RsaI (1 U), and the specific buffer (Roche, Germany). During 1 hour the samples were at 37°C. The restriction products were loaded on 1.5% agarose (Nippon Genetics Europe, Germany) gel. The

10 µL of product with 2 µL of blue buffer 6 x were visualized on electrophoresis Gel with 100 x GelRed™ (Biotium, USA) in 0.5 x TAE buffer (ROTH, Germany), migration parameters were: 100 V, 200 mA for 1 hour and visualized in camera UV light. Photos of each restriction pattern were analyzed to select the different clones.

Clones (e.g. 12 to 29 per cloning reaction) were purified using the Invisorb® Spin Plasmid Mini Two kit (STRACTEC, Germany) according to the manufacturer's protocol. Before purification tubes with 5 mL of LB with kanamycin antibiotic were inoculated with 50 µL of cloning mix and incubated overnight. After extraction, a PCR using M13 primers was performed and sequenced at Macrogen Inc. (Korea).

All the steps followed to AMF identification are presented in the Fig. 13.

New Generation Sequence 454 Roche method

The 454 GS-FLX+ is a high throughput method that performs sequencing of millions of small fragments of DNA at the same time. While bioinformatics analyses are on charge to piece together these fragments by mapping the individual reads (Behjati & Tarpey, 2013) offering the best phylogenetic resolution power to monitor AMF in the field and in the plant roots, providing read lengths of up to approx. 1kb (Senés-Guerrero & Schüßler, 2016b). This technology overcomes the cloning step and provides a more extensive data of AMF communities while saving time, labor and financial costs (Kohout *et al.*, 2014). Thus, AMF-communities associated to different plant species growing in a hydrocarbon polluted Pond were evaluated.

Two PCR were performed as in the Sanger sequencing. For the first PCR the primers to obtain long sequence (i.e. 1.8 or 1.5 kb) were used (see Table 11). The nested PCR was performed with the primers of the Table 12, which have adapter named multiplex identifiers (MIDs). These MIDs were used according to the place where the roots plants were sampled (outside, inside and center of the pond). The second PCR was replicated 3-fold per sample of the first PCR.

Conditions of the first PCR was the same as in Sanger method as well as for the nested PCR, but instead of 40 cycles 25 cycles were used. After nested PCR, the 3 products for each sample were mixed. The pooled products were loaded on 1% agarose to purify with the QIAquick® Gel Extraction Kit (Qiagen, Germany). DNA quantification was performed with the Quant-iT^{TM} PicoGreen® dsDNA Assay Kit (Life technologies, USA) according to the manufacturer's protocol. The samples were quantified in a fluorimeter (Fluoroskan Ascent FL, Labsystem, USA) with the Ascent Software (Louisc, nsku91). According to the results, the samples were diluted until they reached 10^9 molecules μL^{-1}. The samples were mixed to equimolar concentration according to the labeled MID (i.e. 1, 2 and 3). Finally, diluted PCR products were pooled in an equimolar concentration to obtain only one sample. Sequencing was done by using the 2 XLR GS Junior Sequencing (Nucleomics Core, Leuven Belgium, http://www.nucleomics.be/).

The bioinformatics pipeline for analyzing 454 sequence reads follow several steps (Fig. 13) and analysis were done following Senés-Guerrero & Schüßler (2016a). Downstream analyses were done with QIIME pipeline (Caporaso *et al.*, 2010). The criteria to select reads were: (1) no more than 15 ambiguous bases, (2) maximum length of homopolymer run of 15, (3) a maximum number of 5 primer mismatches and (4) sequences with a minimum length of 500 bp including the primers. Sequences were divided into clusters using UCLUST (Edgar, 2010) at a similarity threshold of 98%. For downstream analyses, representative sequences (RS) were used as input sequences for evolutionary placement algorithm (EPA) based species affiliation. Representative sequences were sorted into different clusters, by analyzing the approx. 800 bp target region sequences from a reference tree well-defined AMF reference sequences. After clustering, singletons were removed and the remaining RS were blasted against the NCBI database to identify and remove non-AMF sequences.

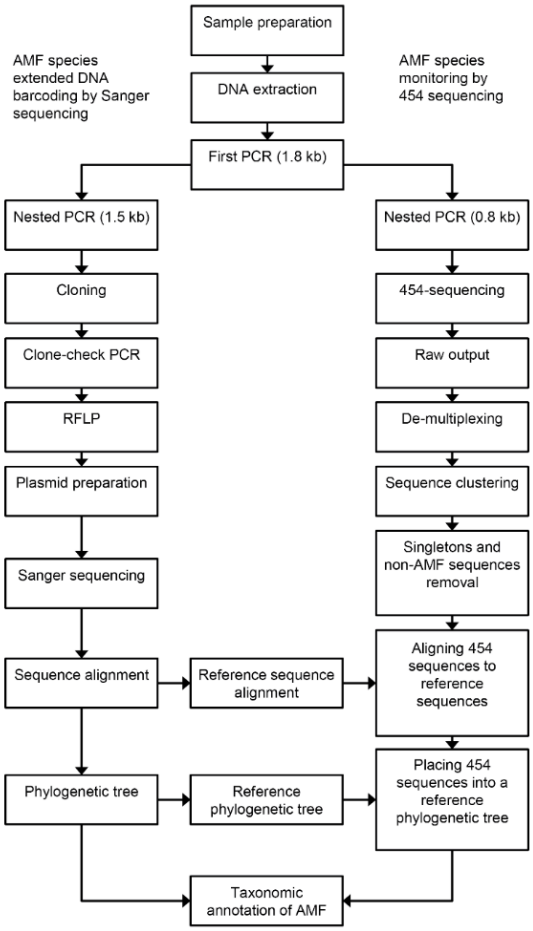

Fig. 13 Scheme of every step followed in the AMF identification by molecular biology with Sanger method and NGS-454 ROCHE method. Adapted from (Senés-Guerrero & Schüßler, 2016a).

e. Evaluation of root colonization by AMF

Root colonization was estimated by the method of McGonigle *et al.* (1990). Roots were first stained with ink (Parker, France) to reduce the use of noxious and dangerous chemicals (Walker, 2005). The roots were then cut into small pieces and placed in Falcon tubes. KOH 10% was added to the roots and incubated at 70°C in a water bath for 1 h for clearing the cells. The KOH was then removed and roots washed with HCl 1% to neutralize the pH and increase

the affinity with the ink solution. The staining step consisted in adding blue ink 2% in HCl 1%. The tubes were placed again in a water bath at 70°C for 1 h. The roots were rinsed and stored in deionized water or for long periods in acidified glycerol before observation (Walker, 2005). Total, arbuscular and vesicular/spore colonization were estimated under a dissecting microscope (Olympus BH2–RFCA, Japan) at 10 x magnification. Around 200 intersections were observed per plantlet. At each intersection, the presence or absence of the fungus was counted. However, if the vertical crosshair crosses more than one arbuscule or vesicle/spore per intersection, the number of each is recorded but the total number of interaction is only one (McGonigle *et al.*, 1990).

f. Root Organ Culture (ROC) to investigate the hyphae healing mechanism

The two AMF strains (*R. irregularis* MUCL 41833 and *Gigaspora sp.* MUCL 52331) were grown in 135 mm diameter Petri dishes in association with transformed chicory roots. The Petri dishes contained 90 mL MSR medium solidified with 3 g L^{-1} GelRite®. The Petri dishes were inclined at 5° during filling with the MSR medium to obtain a slope from one to the other end of the Petri dishes on solidification of the medium. One Petri dish from each strain containing well developed inoculum was cut into squares. Each square of medium with several spores and colonized root fragments were associated to chicory roots in the thicker part of the MSR medium. Twenty Petri dishes per strain were considered. The Petri dishes were incubated in an inverted position at 27°C in the dark. During one month, the association was observed using a binocular microscope (Olympus SZ2-ILST, Japan) to determine the presence of well-developed runner hyphae showing a dense cytoplasmic/protoplasmic flow.

g. Hyphal healing mechanism in presence of diesel

The AMF mycelial networks has been carefully studied by several researchers (de la Providencia *et al.*, 2005; de Souza & Declerck, 2003; Giovannetti *et al.*, 1999; Giovannetti *et al.*, 2001; Giovannetti *et al.*, 2004; Voets et al., 2006) showing its capacity to link plants together and /or AM hyphae via anastomosis or hyphal healing mechanism (HHM) (de la Providencia *et al.*, 2005, 2007). The ability of fusion between hyphae may represent an important mechanism to increase the chances to survive (Giovannetti *et al.*, 2015) in a/biotic stress conditions.

For the experiment, four concentrations of diesel (Q8, Belgium) (i.e. 0%, 1%, 2% and 5%) were chosen and added (previously filtered 0.2 µm- sterilized) to the bottles with autoclaved MSR medium maintained at 40°C. Runner hyphae growing close to the surface of the medium were selected. The hyphae were gently cut with a sterile scalpel under binocular microscope at 6.7 x or 40 x magnification. After each cut, 20 µL of MSR supplemented or not with diesel was added at the place of cutting. During the first 60 min following cutting, the hyphae in each Petri dish was observed following the order of cutting, then each hour until 16 h and finally at 24, 30, 36 and 48 h (Fig. 14).

The cut hyphae were monitored cautiously by images captured with a digital camera (Canon EOS 60D) coupled to a compound bright-field light microscope (Olympus BH2–RFCA, Japan) at 100 x or 200 x magnification and displayed on a 15-inch of HP Compaq nc 6320 screen to reconstitute the sequence of hyphal healing mechanism (HHM) using the image manager software: EOS Utility – Canon USA Inc.at regular intervals under a dissecting bright-field light microscope at 100 x and 200 x.

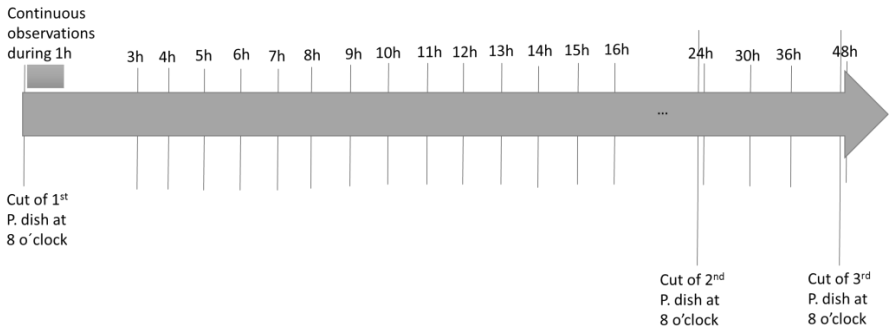

Fig. 14 Estimated monitoring time for hyphal healing mechanism per cut.

IV. Research questions

Three research questions were addressed:

1. **How diverse are the AMF communities associated to plant species naturally recolonizing weathered crude oil ponds?**
2. **Does *Rhizophagus irregularis* MUCL 41833 protect maize plantlets and facilitate its phosphorus uptake in presence of diesel?**
3. **Does diesel impact the AMF hyphal healing mechanism of species belonging to different life history strategies?**

V. RESEARCH RESULTS

Chapter 1

Arbuscular mycorrhizal fungal community composition in *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria* from weathered oil ponds in the Ecuadorian Amazon

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Sylvie Cranenbrouck

My contribution to this chapter was approximately 75% and involved experimental design, sampling, analyses of the samples, data analysis and the writing of the manuscript. Co-authors of the chapter have been involved in statistical analyses and revision of the manuscript.

Preface

Arbuscular mycorrhizal fungi (AMF) are obligate root symbionts, which have been isolated from natural as well as anthropogenic environments. Their identification most often is determined by morpho-taxonomy of the spores present in the soil thus, leaving AMF inside the roots undiscovered. However, in the last years, molecular tools have been developed allowing the identification of AMF from environmental samples such as soils or roots.

In the recent years, several studies have reported the presence of AMF in oil-polluted environments, suggesting their potential resistance/tolerance to hydrocarbons as well as their possible role in increasing the resistance/tolerance of their plant associates to these pollutants. However, the diversity or communities composition of AMF in oil-polluted soils/plants are poorly known. Thus, in-deep studies are necessary owing to (1) the key roles of these microorganisms in the ecology of plants in polluted soils and (2) their potential use in bioremediation.

Molecular tools such as Sanger and/or Next Generation Sequencing (NGS) are useful to investigate the AMF community structure of environments in which isolation of AMF spores is particularly difficult.

In **chapter 1**, we studied the root colonization as well as the community composition of AMF associated to different native plants, which have naturally re-colonized hydrocarbon-polluted ponds. The study was conducted in the Charapa field in the Amazon region of Ecuador. The molecular characterization and identification of the AMF community was performed by Sanger sequencing. Roots of three different plants (*Carludovica palmata, Costus scaber* and *Euterpe precatoria*) present in the three different sites of study (i.e. Pond 1, Pond 2 and the surrounding soil) were analyzed.

Abstract

Arbuscular mycorrhizal fungi (AMF) are ubiquitous to most natural and anthropized ecosystems, and are often found in polluted environments. However, their occurrence and diversity in highly weathered petroleum-polluted soils has been infrequently reported. In the present study, two ponds of weathered crude oil and their surrounding soil from the Charapa field in the Amazon region of Ecuador were selected and root colonization by AMF of their native plants investigated. The AMF community was further analyzed in three selected plant species (i.e. *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria*) present in the two ponds and the surrounding soil. A fragment covering partial SSU, the whole ITS and partial LSU rDNA region was amplified (i.e.1.5 kb), cloned and sequenced from the roots of each host species. AMF root colonization exceeded 56% in all plant species examined and no significant difference was observed between sites or plants. For AMF community diversity analysis, a total of 138 AMF sequences were obtained and sorted into 32 OTUs based on clustering (threshold ≥ 97%) by OPTSIL. The found OTUs belonged to the genera *Rhizophagus* (22%), *Glomus* (31%), *Acaulospora* (25%) and *Archaeospora* (22%). *Glomus* and *Archaeospora* were always present regardless of the plant species or the site. *Acaulospora* was found in the three plant species and in the two ponds while *Rhizophagus* was revealed only in the surrounding soil in one plant species (*E. precatoria*). Our study contributed to the molecular community composition of AMF and revealed an unexpected presence of four AMF genera which have established a symbiosis with roots of native plants from the Amazon forest under high polluted soil conditions.

Keywords: Arbuscular mycorrhizal fungi; weathered crude oil pond; symbiosis; Amazon forest, Ecuador, molecular diversity

Introduction

Ecuador is considered as a biodiversity hot spot, especially the Amazonian region which is emblematic for its rich and diverse flora and fauna. Despite of its ecological importance, the seventies marked the initiation of oil exploration and exploitation of the Amazonian forest. This led to a series of environmental disasters such as the contamination of rivers and groundwater, deforestation, precarious waste storage or weathered crude oil ponds (Armstrong & Vallejo, 1992).

Crude oil is a mixture of many compounds, such as alkanes, aromatics, asphaltenes and resins (Robertson *et al.*, 2007). The alkanes and the LMW polyaromatic hydrocarbons are the most easily biodegradable, by contrast to the asphaltenes and resins which are more resistant (Singh, 2006). The physicochemical composition affected by temperature, sun radiation, humidity and biological action left weathered crude oil accrued, eventually turning them in environmental liabilities (Singh, 2006).

The lack of environmental laws in Ecuador until 1990 (Armstrong & Vallejo, 1992) left around 2550 environmental liabilities caused by the oil industry reported by the Ministerio del Ambiente de Ecuador (MAE), (2015). Petroamazonas EP, an Ecuadorian public enterprise engaged in the exploration and extraction of oil reserves has concentrated its activities in production fields in the Amazon basin. Moreover, it is committed to the recovery of some environmental liabilities. Some of the weathered crude oil ponds are between 30 to 40 years old, despite of the intensive soil contamination, gradually these have been naturally recolonized by plants from the area.

The environmental consequences of weathered crude oil have mostly been measured in terms of flora and fauna, while microbial diversity has been most often ignored, even though microbes provide many ecological services.

Arbuscular mycorrhizal fungi (AMF) are soil inhabitants forming associations with the vast majority of plant species. They play key roles in soil processes

(e.g. soil structure, biogeochemical cycles) (Rillig, 2004; Simard & Austin, 2010), help plants to acquire nutrients in exchange for carbohydrates (Solaiman *et al.*, 2014) and protect them from biotic (Smith and Read, 2008) and abiotic (Plouznikoff *et al.*, 2016) stresses. For instance, Cabello (1999) observed an increase in plant height and shoot biomass as well as P and Zn content in AMF-colonized *Medicago sativa* species grown in a hydrocarbon-polluted substrate as compared to non-colonized plants. Rajtor & Piotrowska-Seget (2016) argued that phytoremediation with AMF and/or hydrocarbon-degrading microorganisms are an effective strategy for dissipation of organic pollutants.

Arbuscular mycorrhizal fungi have been described in various biomes from temperate (Dodd, 2000) to tropical (Declerck *et al.*, 1998; Homeier *et al.*, 2013) and polar/boreal ecosystems (Öpik *et al.*, 2013), under natural forests (Schüßler *et al.*, 2015) as well as under crop cultivation systems (Senés-Guerrero *et al.*, 2014; Buysens *et al.*, 2016). A number of studies also reported their presence in environments polluted by heavy metals (Vallino *et al.*, 2006; Liang *et al.*, 2009; Gil-Cardeza *et al.*, 2014; Wei *et al.*, 2015; Ferrol *et al.*, 2016) or petroleum hydrocarbons (Cabello, 1997; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015). Although, their diversity in Ecuadorian petroleum hydrocarbons polluted soils is mostly unknown. Only one study (Villacrés *et al.*, 2014) reported the presence of spores belonging to *Glomus* and *Acaulospora* in hydrocarbon-polluted soils from the Orellana province in the city La Joya de los Sachas (Amazon region). Species identification was done via morphological examination of spores which could highly change with age and due to the influence of different environmental stresses (Clapp *et al.*, 1995; Bever *et al.*, 1996; Declerck *et al.*, 2000; Rillig *et al.*, 2013).

Nowadays, molecular characterization is the current methodology that has reclassified a high number of AMF species (Souza, 2015). Krüger *et al.* (2012) developed a phylogenetic reference data for systematics and phylotaxonomy

of AMF, that has been used as DNA barcode for AMF (Senés-Guerrero & Schüßler, 2016a; Wang *et al.*, 2016). This reference dataset is an important tool to identify AMF from environmental samples. Interestingly, various surveys have been conducted on AMF diversity in industrial soils polluted by oil, using morpho-taxonomy (Cabello, 1997; Villacrés *et al.*, 2014), and more importantly molecular tools (Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016) thus increasing the accuracy of species identification. It is therefore interesting to study microbial communities associated with these plants and polluted sites, focusing in particular on AMF. Among these environmental liabilities is the Charapa field, in the province of Sucumbíos. The site is heavily contaminated with petroleum hydrocarbons. However, through the years many plants have recolonized these sites naturally.

The aim of this study was to analyze AMF root colonization in different plants species composing the ponds and surrounding soil and to determine the AMF community from three specific plants (*Carludovica palmata*, *Costus scaber* and *Euterpe precatoria*) present across the sites.

Material and Methods

Sampling location

The sampling site was located in the Amazonian region in the Province of Sucumbíos, Ecuador, in the environmental liability known as Charapa field at 309 masl. This area corresponds to a plant community known as Lowland Evergreen Forest (Palacios *et al.*, 1999; Pérez, 2014).

The site has a surface of 244 km^2 and contains weathered crude oil polluted ponds.

Two ponds were considered for this study. Pond 1 (76°48’57” W, 00° 11’49” S) has a surface of 330 m^2 and Pond 2 (76°48’54” W, 00°11’46” S) a surface of 450 m^2. The flora ecosystem was characterized by the botanist A. Pérez

(2014). Both ponds are covered with a 10 cm layer of decomposed organic matter and are colonized by native plants from a secondary forest dominated by herbaceous plants and trees (Fig. 15). In Pond 1, the predominant tree species are *Ficus insipida* (wild fig), *Ficus* cf. *americana* (West Indian laurel fig or Jamaican cherry fig), *Hieronyma alchorneoides* (*mascarey*) and *Croton lechleri* (dragon's blood); while the more abundant herbaceous species are *Dimerocostus strobilaceus* (sour cane), *Carludovica palmata* (Panama hat plant –palm like), *Heliconia cf. chartacea* and several species of Araceae (i.e. *Euterpe precatoria* Mart. - palm like). In Pond 2, the predominant tree species are *Ficus insipida*, *Ficus* cf. *americana*, and several species of *Miconia*; while the dominant herbaceous species are *D. strobilaceus* (sour cane) and several species of *Costus* (sour cane - ginger like), *C. palmata*, *H.* cf. *chartacea* and several species of Marantaceae. In the surrounding soil the predominant tree species are *Ficus*, *Croton lechleri* and *Sapium glandulosum* while the dominant herbaceous species are *Costus scaber*, *Carludovica palmata*, *Heliconia* cf. *chartacea* several species of Araceae, besides pasture and crop species (i.e. cassava, banana cacao) were identified.

Fig. 15 Charapa field. Vegetation present in the ponds and surrounding soil. (A) Herbaceous plants outside the ponds. (B) Trees present inside the ponds. (C) Herbaceous plants inside the ponds. (D) Contaminated soil where plants grow.

Experimental site

Herbaceous plants were randomly harvested from the two ponds and the surrounding soil in December 2012. Forty eight plants representing 9 different species were sampled (i.e. 21 in Ponds 1 and 2 and 6 in the surroundings) (Table 13) for root colonization evaluation. Due to the complexity of the sampling environment and the difficulties in the plant identification, three plant species (*Carludovica palmata*, *Costus scaber*, and *Euterpe precatoria*) found in the two ponds and surrounding soil, were further considered for AMF diversity assessment via molecular tools. For both analyses, the root samples were kept within the rhizosphere soil and dried at room temperature (28-30ºC, standard temperature in the Amazon region) for four days and stored at 4°C. Before processing, roots were cleared from mineral and organic debris.

Table 13 Number of plant species sampled by site.

Plant species	Pond 1	Pond 2	Surrounding soil
Monotagma sp. 1	4	4	
Polybotrya sp. 2		1	1
Geonoma cf. *deversa* (Poit.) Kunth	1	2	
Euterpe precatoria Mart.	7	4	2
Costus scaber Ruiz & Pav.	2	1	1
Costus pulverulentus C. Presl	1	2	
Costus lima var. scabremarginatus Maas	2	3	1
Carludovica palmata Ruiz & Pav	2	3	1
Polybotrya sp. 1	2	1	

AMF Root colonization

Roots were cut into pieces of 2-3 cm and stained into acidic-blue ink as described by Walker (2005). The percentage of total colonization (%TC), arbuscular (%AC) and spores/vesicles (%VC) colonization were estimated under a dissecting microscope (Olympus BH2–RFCA, Japan) at 10x

magnification according to McGonigle *et al.* (1990). An approximate of 100 intersections were observed per plant.

DNA extraction

Roots from *C. palmata*, *C. scaber* and *E. precatoria* were selected for AMF community composition. One plant per species was selected in each site and thus a total of 9 plants were analyzed. Between 55 – 100 mg of roots were grinded in liquid N_2 with a mortar pestle to obtain a complete root disruption. The grounded material was transferred into the Lysing Matrix E tube from the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) and DNA was extracted following the manufacturer's protocol. DNA integrity was analyzed by electrophoresis using 5 µl of DNA stained with 100x GelRed™ (Nucleic Acid Gel Stain, Biotium, Belgium). Samples were run at 100 V for 18 min in 0.5 x TAE buffer and stored at -20°C until further use.

PCR, cloning and restriction fragment length polymorphism (RFLP)

For AMF species delimitation, an extended DNA barcode region which comprises a part of the SSU rRNA gene, the complete ITS region (including the 5.8S rRNA gene) and approx. 800 bp of the LSU rRNA gene was amplified according to Krüger *et al.* (2009). The amplification requires a two-step PCR using AMF specific primers. For the first and nested PCR, the SSUmAf – LSUmAr and the SSUmCf – LSUmBr primer pairs were used, respectively. These primers have the widest taxon coverage when compared to other commonly used primers targeting a single nuclear rDNA marker (Kohout *et al.*, 2014). Briefly, the reaction mix for both PCRs was done using the Phusion High Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Lithuania) with 0.5 µM as a final primer concentration for each primer (Sigma, Germany) and 0.2 µg mL^{-1} BSA (Albumin Bovine, AMRESCO, USA). Five µL of template DNA were used in 20 µL of final

reaction. Thermal cycling was done in an Eppendorf Master-cycler Gradient (Eppendorf Nexus X2, Germany) with the following conditions for the first PCR: Five min. initial denaturation at 99 °C; 40 cycles of 10 s. denaturation at 99 °C, 30 s. annealing at 60 °C and 1 min elongation at 72 °C; and a 10 min. final elongation. In the nested PCR, 1 μL of the first PCR product was used as template in the same final reaction volume (20 μL), the thermal cycling conditions were the same as for the first PCR, except that the annealing temperature was 63 °C and only 30 cycles were done. The amplification results in a single 1.5 kb fragment with species resolution power (Stockinger *et al.*, 2010).

Cloning as well as RFLPs were as described by Krüger *et al.* (2009). In brief, after a nested PCR amplification, PCR products were loaded on 1% agarose gel to determine positive amplification (see above). The 1.5 kb fragments were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, USA) following the manufacturer's protocol, with only one-third of the specified volume of all components used.

Forty eight clones from each cloning reaction were analyzed for correct length of plasmid inserts by colony-PCR using 1x GoTaq DNA Polymerase (Promega, USA) and M13F-M13R primers.

To detect variable sequences between the clones, RFLPs with two different enzymes were performed in 10 μL reaction volume, containing 5 μL colony-PCR product, with one of the restriction enzymes Hinf I (1 U), or RsaI (1 U) and the specific buffer (Roche, Germany).

Selected clones (e.g. 12 to 29 per cloning reaction) were purified using the Invisorb® Spin Plasmid Mini Two kit (STRACTEC, Germany) according to the manufacturer's protocol and sequenced using M13F-M13R primers at Macrogen Inc. (Korea).

Sequence assembly and phylogenetic analysis

Sequences were assembled and edited with SeqMan (Lasergene, Madison, Wisconsin). The search for homologous sequences was conducted by blastn at the National Center for Biotechnology information (NCBI) website and Blast2GO (https://www. blast2go.com/b2ghome) (Conesa *et al.*, 2005). Although a high-fidelity enzyme such as the Phusion DNA polymerase prevents chimera formation, sequences were checked manually to remove chimeric sequences. The AMF sequences were subsequently grouped into operational taxonomic units (OTUs) at a threshold of ≥97% sequence similarity, using the optimization of threshold-based linkage clustering runs from OPTSIL program (Goeker *et al.*, 2009).

Nucleotide sequence alignments were performed with MAFFT version 7 (http://mafft.cbrc.jp/alignment/software) (Katoh & Standley, 2013), followed by manual adjustments using an AMF freely-available reference alignment (Krüger *et al.*, 2012; http://www.amf-phylogeny.com) and environmental sequences from the NCBI platform at the Phylogenetic Data Editor (PhyDE) (http://www.phyde.de/).

A maximum-likelihood phylogenetic tree was assembled with 256 reference and environmental sequences and 32 single representative sequences from each OTU using RAxML-HPC2 (Stamatakis *et al.*, 2008) on XSEDE ver. 8.2.9. on the CIPRES Science Gateway (http://www.phylo.org) with 1000 bootstrap and the GTRGAMMA model (Krüger *et al.*, 2012). Taxonomic annotations follow the classification of Schüßler & Walker (2010). All variant sequences obtained from the clone library were deposited at NCBI with accession numbers MF589988-MF590019.

Diversity analysis

Analysis of the community composition of AMF, in roots, was based on the number of clones (determined by analyzing the RFLP patterns) representing

each AMF species (determined by phylogenetic analysis) present in the site and plant species. The Shannon diversity index (H′), was calculated using the vegan package (Oksanen *et al.,* 2017) in R Core Team (2016). The Shannon diversity index (H′) was calculated using the diversity function by the formula H′=−∑pi log(b) pi, where pi is the proportional abundance of AMF species or genera *i* and *b* is the base of the logarithm (Oksanen *et al.,* 2017).
Principal component analysis (PCA) was used to analyze the similarity of the AMF community composition present in the plant species and the sites. Data were square root normalized and analyzed using the vegan package of R adjusting the total inertia to the number of variables.

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistic 22 software. Data for AMF root colonization percentage were analyzed by one way ANOVA. Normal distribution was checked and non-normal data were normalized by arcsine transformation before analysis. One way ANOVA was used to determine significant difference between plant species and between sites AMF root colonization.

Results

AMF Root colonization

Regardless of the origin of the sample, root colonization was observed on all nine plant species (Pond 1 and 2 and surrounding soil) (Fig. 16). All the plants surveyed had a total colonization percentage (%TC) above 56%± 12.7 and a total vesicle colonization percentage (%VC) between 4% ± 1.5 and 20% ± 18 (Fig. 17). Arbuscules were observed only in six plants out of the 48 observed (i.e. 1 (*Costus pulverulentus*), 2 (*Costus scaber*), 1 (*Costus lima* var. *scabremarginatus*), 1 (*Carludovica palmata*) and 1 (*Monotagma* sp. 1)). The

%AC was between 1% and 12% (data not shown). Due to the presence of few representatives of some plant species and to preserve the plant diversity in the ponds, it was not always possible to sample the same plant species with the same number of replicate in the three sites (Table 13). However no significant difference were observe in the %TC or %VC between plant species (P = 0.216 and P = 0.382, respectively) (Fig. 17) and between sites (P = 0.495 and P = 0.284, respectively – data not shown).

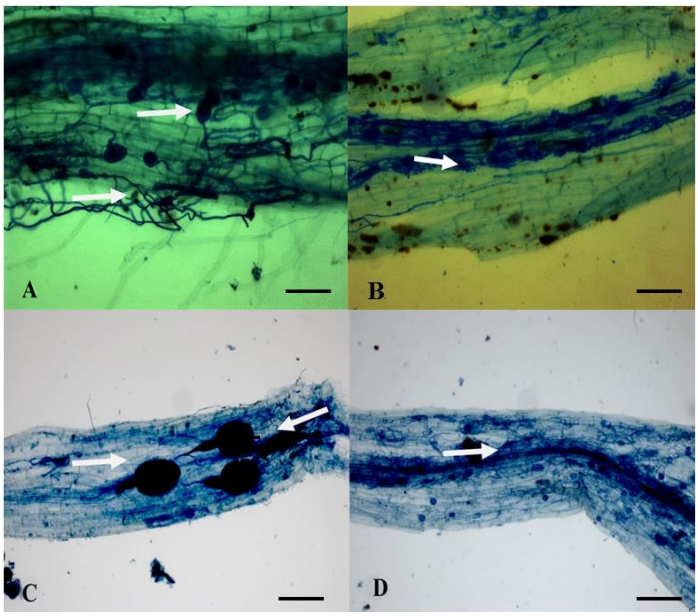


Fig. 16 Ink staining of AMF root colonization (Walker, 2005). (A) Spores/vesicles (white arrow above), and hyphae (arrow below) in *Carludovica palmata*. (B) Arbuscules (white arrow) in *Costus scaber*. (C) Big spores/vesicles presenting a well -defined subtending hypha (white arrows) in *Polybotrya* sp. 2 (D) Hyphae (white arrow) in *Euterpe precatoria*. Scale bar (A-B) 300 µm, (C-D) 100 µm.

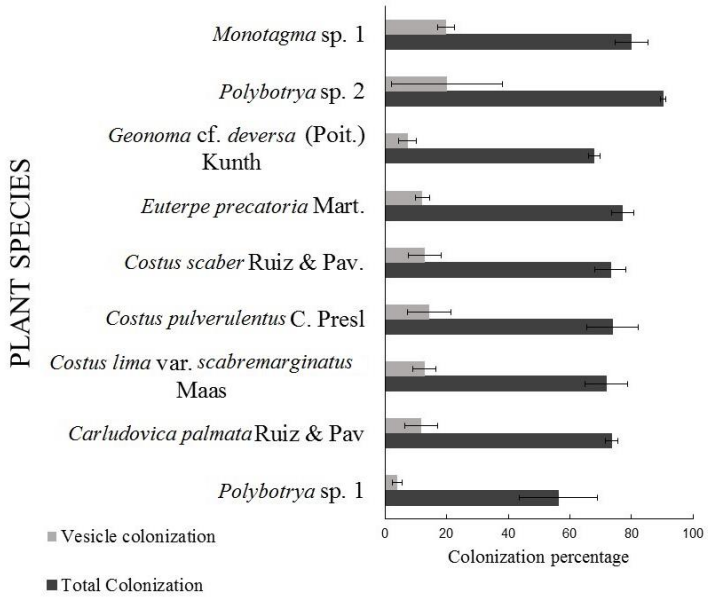


Fig. 17 AMF total and intraradical spores/vesicles colonization (McGonigle *et al*. 1990) of plant species present in Ponds 1, 2 and surrounding soil. Data represent mean values and standard errors (SE) of 8 (*Monotagma* sp. 1), 2 (*Polybotrya* sp. 2), 3 (*Geonoma* cf. *deversa*), 13 (*Euterpe precatoria.*), 4 (*Costus scaber*), 3 (*Costus pulverulentus*), 6 (*Costus lima*), 6 (*Carludovica palmata*), 2 (*Polybotrya* sp. 1) replicates. No significant differences were observed on %VC or %TC between plant species ($P \leq 0.05$, Tukey HSD test).

AMF root diversity

The nested PCR amplified the SSU-ITS-LSU (1.5 kb) rDNA region from all selected root samples. The number of sequences amplified was 138. According to the clustering (threshold ≥ 97%) by OPTSIL the sequences were grouped in 32 OTUs (Table 14- Fig. 18). Four AMF genera and seven species were detected in the roots of the three plant species examined by maximum-likelihood phylogeny. Based on homology in GenBank and the phylogenetic tree (Fig. 18), the percentage of OTUs per AMF genus was 22% (7 OTUs) of *Rhizophagus*, 31% (10) of *Glomus*, 25% (8) of *Acaulospora* and 22% (7) of *Archaeospora* (Fig. 19 A). The genus *Acaulospora* was found in the three plant species in the two ponds but not in the surrounding soil. On the contrary,

Rhizophagus was only observed in the surrounding soil specifically in *E. precatoria*. *Glomus* and *Archaeospora* were detected in all plant species in the three sites (Table 14, Fig. 20 A-C). According to AMF species, 5 OTUs (16%) belonged to *Rhizophagus proliferus* and *Acaulospora* sp. (minuta-scrobiculata-like), 2 (6%) belonged to *Rhizophagus sp.* and *Acaulospora longula*, 1 (3%) to *Acaulospora kentinensis*, 10 (31%) to *Glomus* sp., and 7 (22%) to *Archaeospora* sp. (Fig. 18 - Fig. 19 B). The distribution was similar at the genus-level.

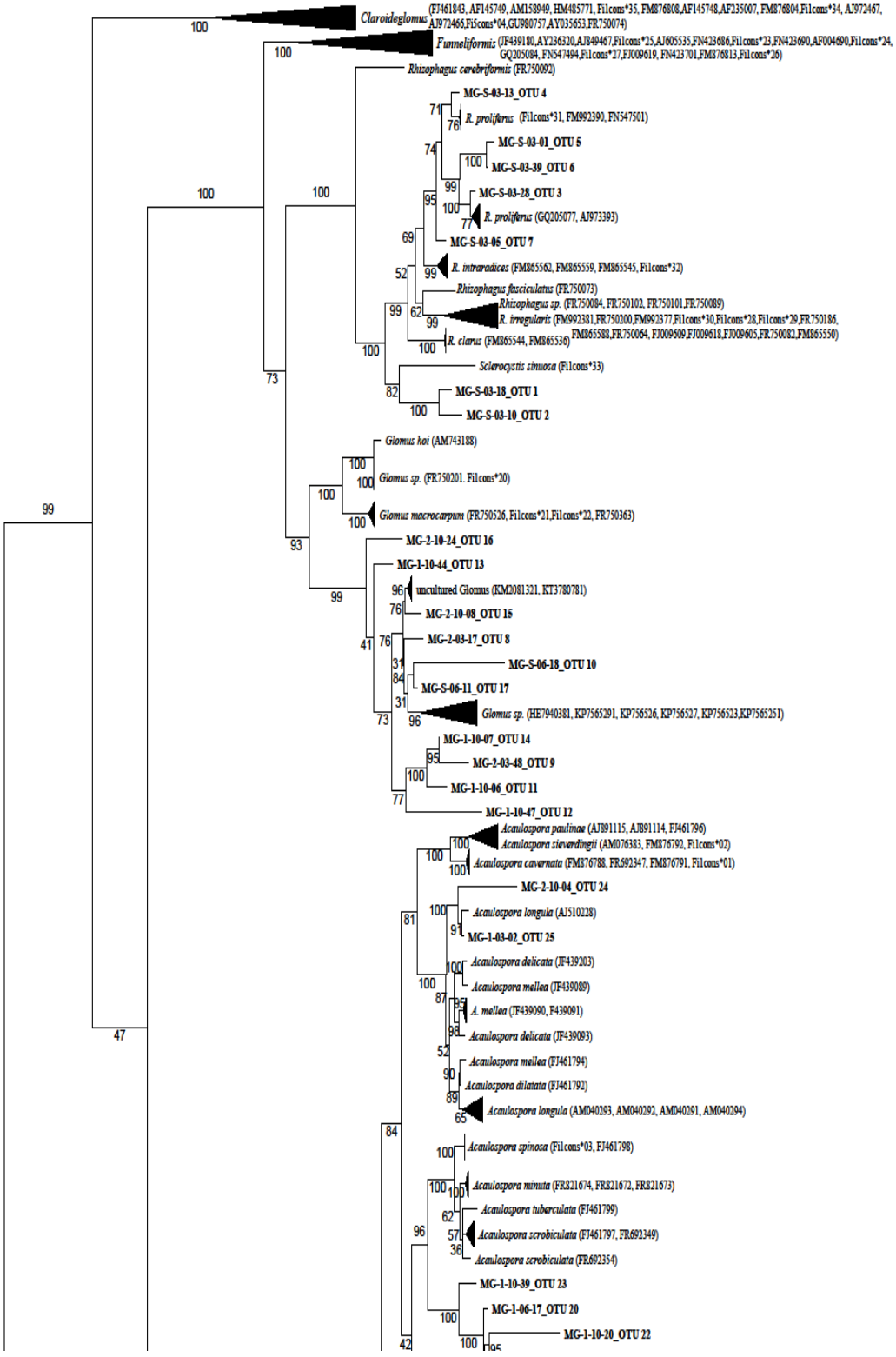
Table 14 OTUs identification according to the site and plant species.

Family	Genus	# OTU	OTU species ID	Site	Plant species
Glomeraceae	***Rhizophagus***	**1**	*Rhizophagus* sp. 1	Surrounding soil	*E. precatoria*
		2	*Rhizophagus* sp. 1	Surrounding soil	*E. precatoria*
		3	*R. proliferus*	Surrounding soil	*E. precatoria*
		4	*R. proliferus*	Surrounding soil	*E. precatoria*
		5	*R. proliferus*	Surrounding soil	*E. precatoria*
		6	*R. proliferus*	Surrounding soil	*E. precatoria*
		7	*R. proliferus*	Surrounding soil	*E. precatoria*
	Glomus	**8**	*Glomus* sp. 1	Pond 2	*E. precatoria*
		9	*Glomus* sp. 1	Pond 2	*E. precatoria*
		10	*Glomus* sp. 1	Surrounding soil	*C. scaber*
		11	*Glomus* sp. 1	Pond 1	*C. palmata*
		12	*Glomus* sp. 1	Pond 1	*C. palmata*
		13	*Glomus* sp. 1	Pond 1	*C. palmata*
		14	*Glomus* sp. 1	Pond 1	*C. palmata*
				Pond 2	*E. precatoria*
		15	*Glomus* sp. 1	Pond 2	*C. palmata*
		16	*Glomus* sp. 1	Pond 2	*C. palmata*
		17	*Glomus* sp. 1	Pond 2	*E. precatoria* *C. palmata*
				Surrounding soil	*C. scaber*
Acaulosporaceae	***Acaulospora***	**18**	*A. kentinensis*	Pond 1	*C. palmata*
		19	*Acaulospora* sp. 1	Pond 2	*C. scaber*
		20	*Acaulospora* sp. 1	Pon1, Pond 2	*C. scaber*

		21	*Acaulospora* sp. 1	Pond 1	*C. palmata*
		22	*Acaulospora* sp. 1	Pond 1	*C. palmata*
		23	*Acaulospora* sp. 1	Pond 1	*C. palmata*
		24	*A. longula*	Pond 2	*C. palmata*
		25	*A. longula*	Pond 1	*E. precaria*, *C. scaber*
				Pond 2	*C. palmata*
Archaeosporacea	***Archaeospora***	**26**	*Archaeospora* sp. 1	Pond 2	*C. scaber*
		27	*Archaeospora* sp. 1	Pond 2	*C. scaber*
		28	*Archaeospora* sp. 1	Pond 2	*C. scaber*
		29	*Archaeospora* sp. 1	Surrounding soil	*C. palmata*
		30	*Archaeospora* sp. 1	Pond 1 and 2	*E. precaria*, *C. scaber*
				Pond 1	*C. palmata*
		31	*Archaeospora* sp. 1	Surrounding soil, Pond 1	*C. palmata*
		32	*Archaeospora* sp. 1	Pond 1	*C. palmata*

Regardless of the site, the genera *Rhizophagus* sp. and *R. proliferus* were colonizing only *E. precatoria*. *A. kentinensis* was recorded only in *C. palmata*. *Acaulospora* sp. was shared between *C. palmata* and *C. scaber*. Finally *Glomus* sp., *A. longula* and *Archaeospora* sp. were revealed in the 3 plant species (Fig. 21 A-C Table 14).

The PCA from the AMF community composition analyzed at the genus-level, demonstrated a similarity in the AMF community between *C. palmata*, *C. scaber* and Pond 2. Also similar are the communities present in *E. precatoria* and surrounding soil whereas Pond 1 was the most dissimilar (Fig. 20 D). A very similar pattern was observed when the AMF community composition was analyzed at the species-level (Fig. 21 D). Shannon diversity from AMF presented similar indices regardless of the site or plant species (Table 15).


Claroideglomus (FJ461843, AF145749, AM158949, HM485771, Filcons*35, FM876808,AF145748,AF235007, FM876804,Filcons*34, AJ972467, AJ972466,Fi5cons*04,GU980757,AY035653,FR750074)
Funneliformis (JF439180,AY236320,AJ849467,Filcons*25,AJ605535,FN423686,Filcons*23,FN423690,AF004690,Filcons*24, GQ205084, FN547494,Filcons*27,FJ009619, FN423701,FM876813,Filcons*26)
Rhizophagus cerebriformis (FR750092)
MG-S-03-13_OTU 4
R. proliferus (Filcons*31, FM992390, FN547501)
MG-S-03-01_OTU 5
MG-S-03-39_OTU 6
MG-S-03-28_OTU 3
R. proliferus (GQ205077, AJ973393)
MG-S-03-05_OTU 7
R. intraradices (FM865562, FM865559, FM865545, Filcons*32)
Rhizophagus fasciculatus (FR750073)
Rhizophagus sp. (FR750084, FR750102, FR750101,FR750089)
R. irregularis (FM992381,FR750200,FM992377,Filcons*30,Filcons*28,Filcons*29,FR750186, FM865588,FR750064, FJ009609,FJ009618,FJ009605,FR750082,FM865550)
R. clarus (FM865544, FM865536)
Sclerocystis sinuosa (Filcons*33)
MG-S-03-18_OTU 1
MG-S-03-10_OTU 2
Glomus hoi (AM743188)
Glomus sp. (FR750201. Filcons*20)
Glomus macrocarpum (FR750526, Filcons*21,Filcons*22, FR750363)
MG-2-10-24_OTU 16
MG-1-10-44_OTU 13
uncultured Glomus (KM2081321, KT3780781)
MG-2-10-08_OTU 15
MG-2-03-17_OTU 8
MG-S-06-18_OTU 10
MG-S-06-11_OTU 17
Glomus sp. (HE7940381, KP7565291, KP756526, KP756527, KP756523,KP7565251)
MG-1-10-07_OTU 14
MG-2-03-48_OTU 9
MG-1-10-06_OTU 11
MG-1-10-47_OTU 12
Acaulospora paulinae (AJ891115, AJ891114, FJ461796)
Acaulospora sieverdingii (AM076383, FM876792, Filcons*02)
Acaulospora cavernata (FM876788, FR692347, FM876791, Filcons*01)
MG-2-10-04_OTU 24
Acaulospora longula (AJ510228)
MG-1-03-02_OTU 25
Acaulospora delicata (JF439203)
Acaulospora mellea (JF439089)
A. mellea (JF439090, F439091)
Acaulospora delicata (JF439093)
Acaulospora mellea (FJ461794)
Acaulospora dilatata (FJ461792)
Acaulospora longula (AM040293, AM040292, AM040291, AM040294)
Acaulospora spinosa (Filcons*03, FJ461798)
Acaulospora minuta (FR821674, FR821672, FR821673)
Acaulospora tuberculata (FJ461799)
Acaulospora scrobiculata (FJ461797, FR692349)
Acaulospora scrobiculata (FR692354)
MG-1-10-39_OTU 23
MG-1-06-17_OTU 20
MG-1-10-20_OTU 22

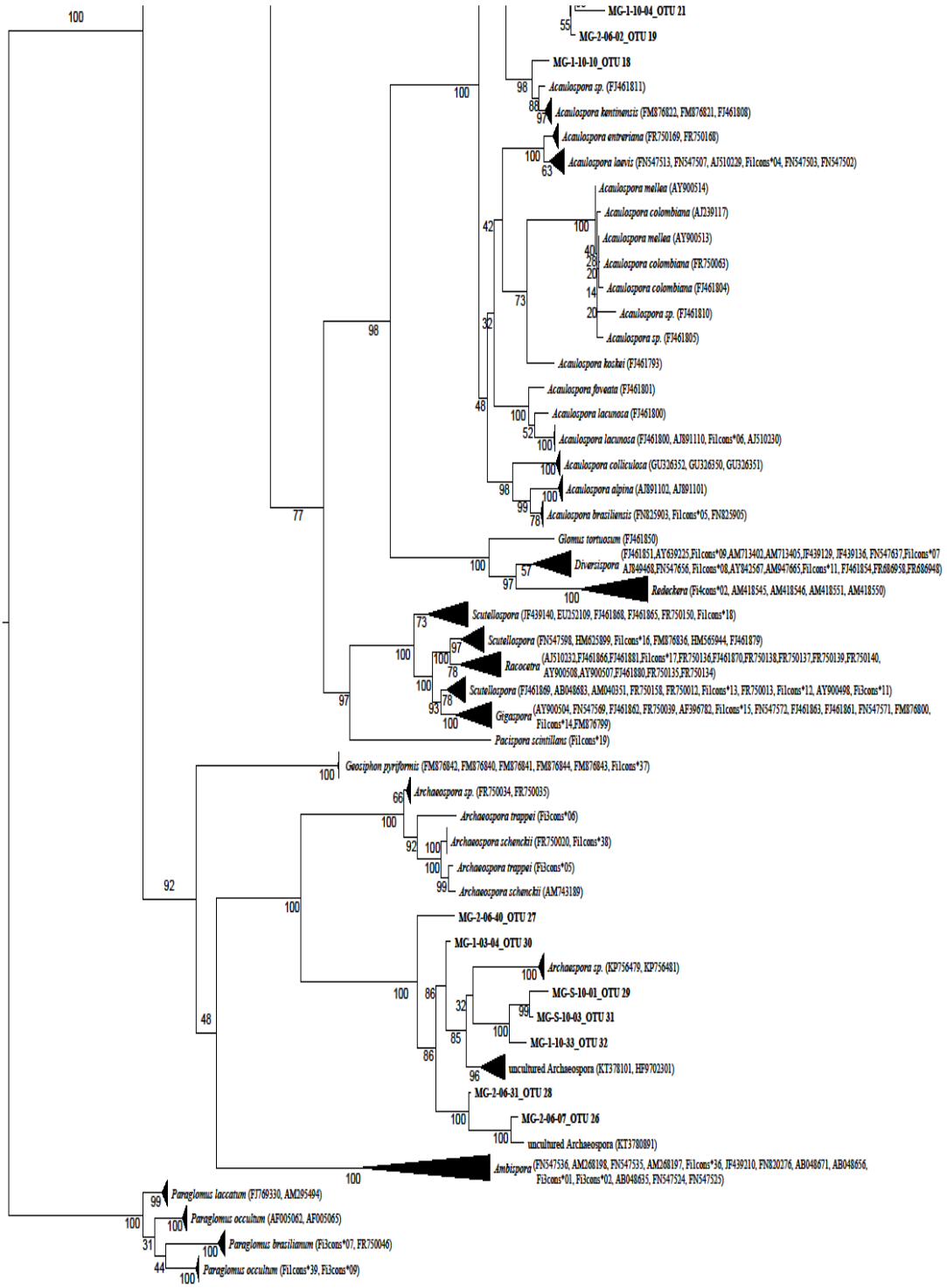

MG-1-10-04_OTU 21
MG-2-06-02_OTU 19
MG-1-10-10_OTU 18
Acaulospora sp. (FJ461811)
Acaulospora kentinensis (FM876822, FM876821, FJ461808)
Acaulospora entreriana (FR750169, FR750168)
Acaulospora laevis (FN547513, FN547507, AJ510229, Fi1cons*04, FN547503, FN547502)
Acaulospora mellea (AY900514)
Acaulospora colombiana (AJ239117)
Acaulospora mellea (AY900513)
Acaulospora colombiana (FR750063)
Acaulospora colombiana (FJ461804)
Acaulospora sp. (FJ461810)
Acaulospora sp. (FJ461805)
Acaulospora koskei (FJ461793)
Acaulospora foveata (FJ461801)
Acaulospora lacunosa (FJ461800)
Acaulospora lacunosa (FJ461800, AJ891110, Fi1cons*06, AJ510230)
Acaulospora colliculosa (GU326352, GU326350, GU326351)
Acaulospora alpina (AJ891102, AJ891101)
Acaulospora brasiliensis (FN825903, Fi1cons*05, FN825905)
Glomus tortuosum (FJ461850)
Diversispora (FJ461851,AY639225,Fi1cons*09,AM713402,AM713405,JF439139, JF439136, FN547637,Fi1cons*07 AJ849468,FN547656, Fi1cons*08,AY842567,AM947665,Fi1cons*11, FJ461854,FR686958,FR686948)
Redeckera (Fi4cons*02, AM418545, AM418546, AM418551, AM418550)
Scutellospora (JF439140, EU252109, FJ461868, FJ461865, FR750150, Fi1cons*18)
Scutellospora (FN547598, HM625899, Fi1cons*16, FM876836, HM565944, FJ461879)
Racocetra (AJ510232,FJ461866,FJ461881,Fi1cons*17,FR750136,FJ461870,FR750138,FR750137,FR750139,FR750140, AY900508,AY900507,FJ461880,FR750135,FR750134)
Scutellospora (FJ461869, AB048683, AM040351, FR750158, FR750012, Fi1cons*13, FR750013, Fi1cons*12, AY900498, Fi3cons*11)
Gigaspora (AY900504, FN547569, FJ461862, FR750039, AF396782, Fi1cons*15, FN547572, FJ461863, FJ461861, FN547571, FM876800, Fi1cons*14,FM876799)
Pacispora scintillans (Fi1cons*19)
Geosiphon pyriformis (FM876842, FM876840, FM876841, FM876844, FM876843, Fi1cons*37)
Archaeospora sp. (FR750034, FR750035)
Archaeospora trappei (Fi3cons*06)
Archaeospora schenckii (FR750020, Fi1cons*38)
Archaeospora trappei (Fi3cons*05)
Archaeospora schenckii (AM743189)
MG-2-06-40_OTU 27
MG-1-03-04_OTU 30
Archaeospora sp. (KP756479, KP756481)
MG-S-10-01_OTU 29
MG-S-10-03_OTU 31
MG-1-10-33_OTU 32
uncultured Archaeospora (KT378101, HF970201)
MG-2-06-31_OTU 28
MG-2-06-07_OTU 26
uncultured Archaeospora (KT378089)
Ambispora (FN547536, AM268198, FN547535, AM268197, Fi1cons*36, JF439210, FN820276, AB048671, AB048656, Fi3cons*01, Fi3cons*02, AB048635, FN547524, FN547525)
Paraglomus laccatum (FJ769330, AM295494)
Paraglomus occultum (AF005062, AF005065)
Paraglomus brasilianum (Fi3cons*07, FR750046)
Paraglomus occultum (Fi1cons*39, Fi3cons*09)

Fig. 18 Maximum-likelihood phylogenetic tree based on the SSU-ITS-LSU rDNA region showing the distribution of the 19 OTUs obtained from the three sites (Pond 1, Pond 2 and surrounding soil) associated with the roots of 3 different plant species (*E. precatoria*, *C. scaber* and *C. palmata*). Sequence data were analyzed in combination with the reference sequences from Krüger *et al.* (2012) and GenBank sequences. Bootstrap values (1000 replicates) are shown above the branches.

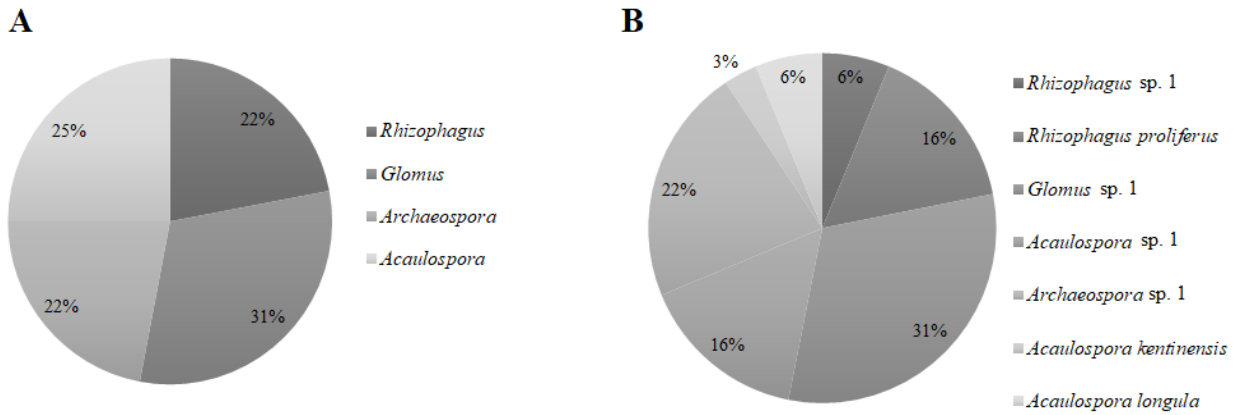


Fig. 19 Occurrence of AMF Operational Taxonomic Units (OTUs) according to the (A) percentage of OTUs representing Glomeromycota genera, (B) percentage of OTUs representing Glomeromycota species independently of the site or plant species.

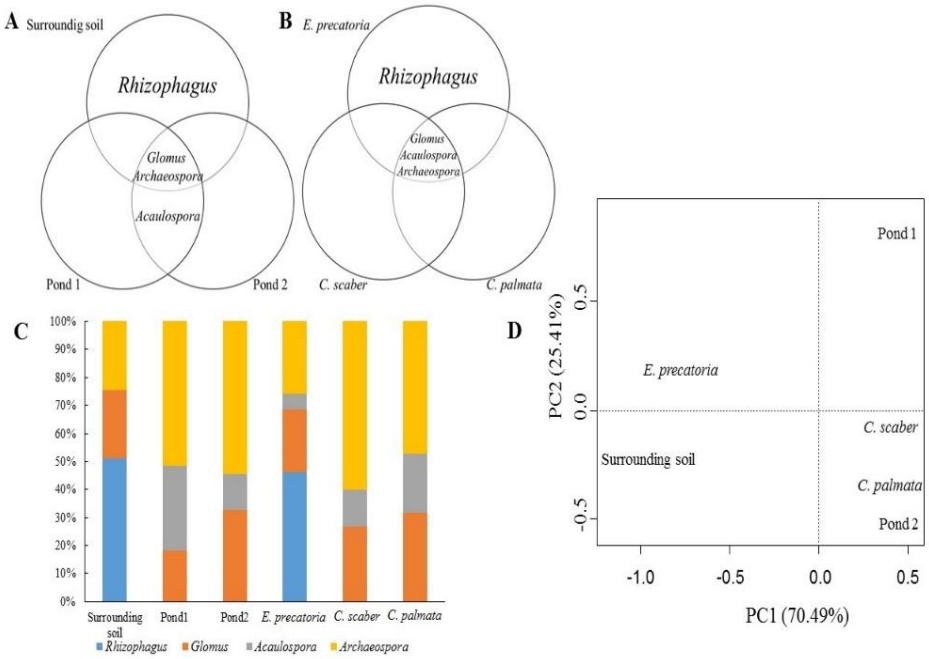


Fig. 20 Distribution of the AMF genera according to the sites (A) and the three plant species (B). Relative abundance distribution (C) and principal component analysis (PCA) of AMF genera (D). Sites: oil ponds 1, 2 and surrounding soil. Plant species: *E. precatoria*, *C. scaber* and *C. palmata.*

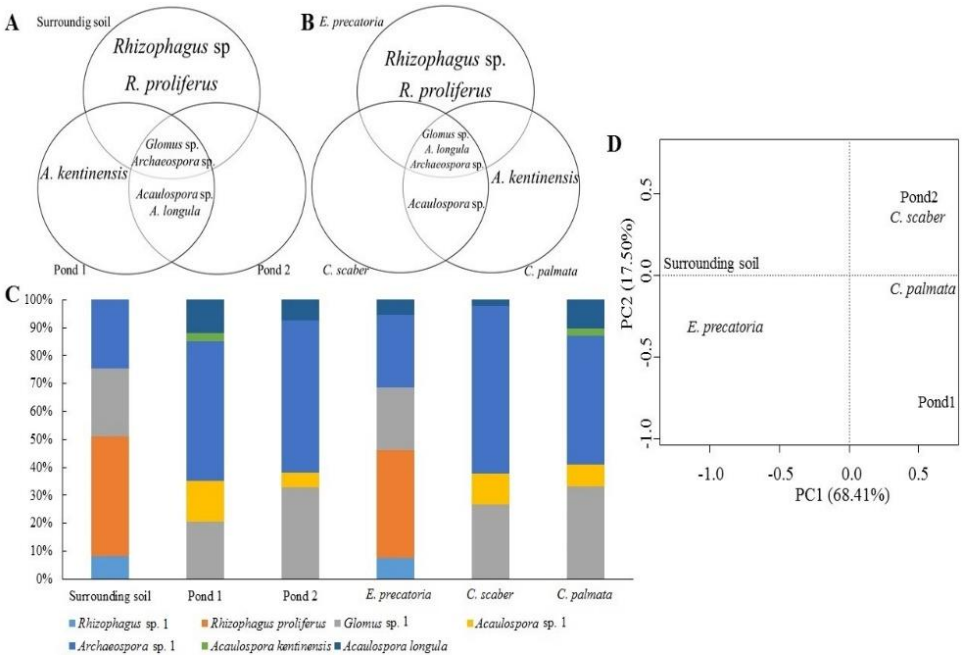


Fig. 21 Distribution of the AMF species according to the sites (A) and the three plant species (B). Relative abundance distribution (C) and principal component analysis (PCA) of AMF species (D). Sites: oil ponds 1, 2 and surrounding soil. Plant species: *E. precatoria, C. scaber* and *C. palmata.*

Table 15 Values of AMF diversity (species and genera) by site and plant species according to Shannon's index.

		Pond1 # of AMF		**Pond2 # of AMF**		**Surrounding soil # of AMF**		***Shannon´s diversity index (plant species)***	
Plant species	**# of plant**	species	genera	species	genera	species	genera	species	genera
E. precatoria	3	2	2	2	2	2	1	1.40	1.20
C. scaber	3	3	2	2	2	1	1	0.99	0.93
C. palmata	3	4	3	2	2	1	1	1.25	1.05
Shannon´s diversity index (sites)		1.31	1.01	1.05	0.96	1.26	1.03		

Discussion

Soils heavily contaminated by hydrocarbons are generally poor in plant and microbial diversity (Chibuike, 2013). Exploring AMF in hydrocarbon-polluted soils from natural environments (e.g. tropical rain forest) may provide unique information about the diversity and resilience of these root symbionts and their potentialities to protect plants from such stresses.

The presence of AMF in natural or polluted soils in the Amazonian basin of Ecuador has been poorly studied. The first report, to our knowledge, was made by Lunt and Hedger (1996) on the mycorrhizal colonization of trees in *Terra Firme* Cuyabeno, a wildlife reserve in the rainforest. They found that AMF colonization was the dominant mycorrhizal type but they did not study their diversity. Still today there are limited reports on AMF community composition in the Amazon region of Ecuador, contrary to the Amazon regions from Brazil, Colombia, Venezuela and Peru which have been much more explored (Janos and Sahley, 1995; Salamnaca and Silva, 1998; Lopes

Leal *et al.*, 2009; Stürmer and Siqueira, 2011; Kalinhoff *et al.*, 2009; Peña-Venegas, 2011; Peña-Venegas *et al.*, 2006).

Forty years ago, during the exploration of oil in the Charapa field, crude oil was spilled and accumulated in artificial ponds. Since then, a layer of organic matter consistently amassed allowing a variety of different trees and herbaceous plants to colonize and establish naturally. Total petroleum hydrocarbon (TPH) concentration inside the ponds and surrounding soil was high (i.e. above 5000 mg Kg^{-1} in Pond 1 and 2 and ~1200 mg Kg^{-1} in the surrounding soil) (Personal communication by Centro de Servicios Ambientales y Químicos – PUCE). The presence of TPH in the surrounding soil suggested lixiviation from the ponds. Pollution was thus observed in the three sites, although four times higher in the two ponds as compared to the surrounding soil.

The thorough analysis of plant species in the ponds as well in the surrounding soil, demonstrated the systematic presence of AMF in roots with levels of colonization above 50% in each species analyzed. Root colonization was observed for the first time in *E. precatoria, C. scaber, C. palmata, Monotagma* sp.1, *Polybotrya* sp.2, *Geonoma* cf. *deversa*, *Costus pulverulentus*, *Costus lima*, and *Polybotrya* sp.1 isolated from oil polluted soils. Nonetheless AMF association in some of these genera has been already revealed, due to its presence in the rhizosphere on natural, agriculture or greenhouse conditions (Trufem, 1990; Santos *et al.*, 2000; Wang and Qiu, 2006; Reyes-Jaramillo *et al.*, 2008).

Numerous hyphae and vesicles were observed but curiously few or no arbuscules in agreement with the findings of Iffis *et al.* (2016). These authors recorded the presence of vesicles and hyphae but no arbuscules in the roots of three plants species (i.e. *Solidago canadensis, Populus balsamifera* and *Lycopus europaeus*) growing in petroleum hydrocarbon wastes of three

decantation basins from a former petrochemical plant, located on the south shore of the St-Lawrence River near Montreal (Canada). This very low or erratic occurrence of arbuscules could be attributed to several factors among which is the presence of pollutants as suggested by Cabello (1997). Interestingly, in soils contaminated by heavy metals, both Sonjak *et al.* (2009) and Maček *et al.* (2016) reported a low frequency or absence of arbuscules in the roots of plants naturally colonizing these soils.

An in-deep diversity analysis was conducted on *C. scaber, E. precatoria and C. palmata* because these plants are known to form associations with AMF (Wang and Qiu, 2006; Janos, 2007; Stürmer and Siqueira, 2011; Janos, 1980; Santos *et al.*, 2000; Lopes Leal *et al.*, 2009) and they were present in the two contiguous oil ponds and surrounding soil of the Charapa field study. To our knowledge, no information on AMF communities associated with these species exist. A number of AMF species belonging to *Acaulospora, Entrophospora* and *Glomus* were detected in an agroforestry system at the Upper Solimões River, at the heart of the Amazon region in Brazil, in which *E. precatoria* was present along with other plants known as regenerating species from secondary forest. Though no root colonization or AMF species diversity within roots was evaluated (Stürmer and Siqueira, 2011; Lopes Leal *et al.*, 2009). Similarly, *C. palmata* (Janos, 1980, 2007) and *C. scaber* (Santos *et al.*, 2000) are highly mycotrophic plants but the AMF communities in their roots remain undescribed.

Interestingly, from the DNA extraction of roots of the three plant species, four AMF genera (*Rhizophagus, Glomus, Acaulospora* and *Archaeospora*) were detected. The taxonomic diversity at the genera level was low, although seven species within 138 AMF sequences were obtained for a total of 10, 8, 7 and 7 different OTUs within *Glomus, Acaulospora, Rhizophagus* and *Archaeospora,* respectively. Hassan *et al.* (2014) and de la Providencia *et al.*

(2015) revealed lower number of OTUs (27 and 21, respectively) in different hydrocarbon-polluted soils, although they analyzed a much larger number of sequences with a different molecular marker (69282 AMF 18S rDNA sequences from 454-pyrosequencing and 824 AMF 18S rDNA Sanger sequences).

The presence of *Archaeospora* was detected in 22% of the total number of OTUs. This family was present in the three sites and associated with all plant species analyzed. However all the sequences were represented by unknown *Archaeospora* spp. (OTUs 24 to 32) in several well-resolved phylogenetic clades (Fig. 18), suggesting the presence of new species. This genus was not reported when the 18S rDNA was sequenced in the studies of de la Providencia *et al.* (2015) and Iffis *et al.* (2016) on hydrocarbon-polluted soils. However Iffis *et al.*, (2016) reported, in the same study, low abundance of unclassified Archaeosporaceae using the AMF ITS dataset. Similarly, Cabello (1997) and Villacrés *et al.* (2014) using classical spores taxonomy never described this genus in hydrocarbon polluted soils. Hassan *et al.* (2014) and Iffis *et al.* (2014, 2016) recorded virtual taxa belonging to Archaeosporacea with a molecular approach in soils from a phytoremediation field trial at the site of a former petrochemical plant in Varennes and St-Lawrence River, Canada, respectively. Interestingly, Peña-Venegas (2011) have documented two species of *Archaeospora* (*A. leptoticha and A. trappei*) in the Amazonian region of Brazil and one in Colombia (*A. leptoticha*) demonstrating the presence of these genera in the Amazonian region. Further one phylotype belonged to this genus was recorded in roots of *Prunus persica* under two types of fertilization (inorganic, with or without manure) in a tropical agro-ecosystem of Venezuela (Alguacil *et al.*, 2014).

The genus *Acaulospora* was found in 25% of the total number of OTUs. This genus was present in both ponds but not in the surrounding soil. Two OTUs

belonged to *A. longula* (OTU 24 and 25) and 1 was related to *Acaulospora kentinensis* (OTU 18) and putatively unknown *Acaulospora* spp. (OTU 19 to 23), suggesting undescribed new species. The Acaulosporacea family has been defined as a stress-tolerant AMF due to its capacity to complete its life cycle with low biomass production that would thus reduce exposure to abiotic stress agents (Chagnon *et al.*, 2013). This family was recorded in hydrocarbon-polluted soils with a predominance of 16% of the total number of species detected (Iffis *et al.*, 2016). Villacrés *et al.* (2014) also noticed, by spores morpho-analysis, a predominance of *Acaulospora* as well as *Glomus* in hydrocarbon-polluted soils from the Orellana province in the Amazon region of Ecuador. Conversely, de la Providencia *et al.* (2015) obtained only a single OTU belonging to *Acaulospora*, associated with *Eleocharis obtusa* and *Panicum capillare*. Hassan *et al.* (2014) did not detect any *Acaulospora* spp. in Willow (*Salix* spp. L.) under hydrocarbon-polluted conditions. These suggest that the presence of *Acaulospora* spp. is not related to hydrocarbon contamination, but can be associated with the Ecuadorian Amazon region.

Finally, the predominance of *Glomus* (31%) with 10 different OTUs detected in the roots of the three plants species from the ponds and surrounding soil was in agreement with several studies that revealed the predominance of Glomeraceae in hydrocarbon-polluted soils (Cabello 1997; Hassan *et al.*, 2014; Villacrés *et al.*, 2014; de la Providencia *et al.*, 2015). The *Rhizophagus* genus was represented by 5 OTUs of *R. proliferus* (OTUs 3 to 7; synonymous of *Glomus proliferum*), 2 OTUs of *Rhizophagus* sp. closely related to *Sclerocystis sinuosa* (OTUs 1 and 2), revealing again the possibility of undescribed new species. Chagnon *et al.* (2013) determined that the Glomeraceae family has the capacity to colonize roots faster and that their extraradical hyphae abundance is higher than other AMF families (i.e. Acaulosporaceae, Gigasporaceae). This family is considered to have a ruderal life history, in agreement with its dominance in polluted environments.

However, the OTUs belonging to the genus *Rhizophagus* were observed only in the surrounding soil in one plant species (*E. precatoria*). Conversely, *Glomus* spp. were observed in all sites in all plant roots. Thus, *E. precatoria* presented the most elevated percentage (58%) of OTUs compared to *C. scaber* (47%) and *C. palmata* (37%). *Rhizophagus* was dominant with 5 different OTUs according to the clustering (threshold ≥ 97%) by OPTSIL. Hassan *et al.* (2014) and de la Providencia *et al.* (2015) showed an elevated occurrence of *Rhizophagus* in hydrocarbon-polluted rhizosphere. Conversely de la Providencia *et al.* (2015) demonstrated that the AMF community structure in roots was totally different compared to the rhizosphere in the sediments associated with *E. obtusa* and *P. capillare*, where just one OTU belonging to the *Rhizophagus* genus was recorded.

These three AMF families (i.e. Glomeraceae, Acaulosporaceae, and Archaeosporaceae) have also been recorded associated with plants in the tropical mountain rain forest in the San Francisco River, Cordillera Real, in the south of Ecuador after reforestation experiments with local trees species (*Cedrela montana* Moritz ex Turcz., *Heliocarpus americanus* L., *Juglans neotropica* Diels and *Tabebuia chrysantha* (Jacq.) G. Nicholson) where 40 year old abandoned pastures were compared with old trees from the nearest pristine forest (Haug *et al.*, 2010). Moreover, in potato crops in the Andean region of Ecuador, the same genera as the ones found in our study were detected and several *Acaulospora* spp. were recorded as the main root colonizers (Senés-Guerrero and Schüßler, 2016). These results suggest a prevalence of these families in different environmental conditions in Ecuador. Thus, these AMF families could be considered as widely distributed and adapted to an extensive range of host plants and soil conditions in Ecuador. Indeed, a global assessment of AMF diversity revealed a high number of AMF taxa shared between continents (Davison *et al.*, 2015), suggesting that

dispersal mechanism such as wind, animals and human activities are responsible of their distribution in all kind of vegetative communities.

Additionally, results from de la Providencia *et al.* (2015) and our results showed the presence of almost all families of Glomeromycota with exception of Ambisporaceae, Geosiphonaceae and Pacisporaceae in oil-polluted soils with abundance varying depending on environmental conditions (rainforest) as well as on the levels of pollution and native flora. Suggesting, one again, the world-wide distribution of this three families.

For the first time, AMF community compositions was studied in crude oil ponds in a natural environment from the Amazon basin of Ecuador via molecular tools. Roots from several native plants species were highly colonized and diverse communities of AMF belonging to *Glomus*, *Rhizophagus*, *Archaeospora* and *Acaulospora* were associated with *C. scaber, E. precaria* and *C. palmata.*

Seventy four percent of OTUs could not be ascribed to an existing AMF species suggesting the presence of a high number of potential new species. This is in agreement with global AMF molecular surveys where many unknown species are living in unstudied areas (Kivlin *et al.*, 2011; Öpik *et al.*, 2013) such as the Tibetan Plateau (Li *et al.*, 2015) and the Andean region (Senés-Guerrero *et al.*, 2014), suggesting that the diversity within this group of fungi is still underestimated. The unexpectedly high percentage of AMF colonization of the roots from plants growing in the hydrocarbon-polluted ponds of the Charapa field, further suggested that the AMF taxa found were able to adapt to these harsh conditions, representing potentially interesting strains for plant establishment and remediation of polluted soils.

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Author contributions

MG-R: sampling, development of samples analysis, data collection, data analysis, interpretation of data. Drafting the work, commentaries corrections, final approval and agreement with all aspects of the work. CS-G: contribution to the analysis and interpretation of the data, draft correction and final approval and agreement with all aspects of the work. SD: contributions to analysis of the results, draft corrections final approval and agreement with all aspects of the work. SC: substantial contributions from conception to data analysis, draft correction and final approval and agreement with all aspects of the work.

Chapter 2

454 pyrosequencing-based analysis of arbuscular mycorrhizal fungal communities in a petroleum-polluted Amazonian soil

Adapted from the research article submitted in MicrobiologyOpen

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My contribution to this chapter was approximately 70% and involved experimental design, sampling, analyses of the samples, statistical analyses and the writing of the manuscript. Co-authors of the chapter have been involved in bioinformatics analyses, experimental design and revision of the manuscript.

Preface

In **chapter 1** we evaluated the AMF colonization of several plant species as well as the AMF community composition of three selected plant species (*Carludovica palmata, Costus scaber* and *Euterpe precatoria*) present in the three environments under study (i.e. Pond 1, Pond 2 and surrounding soil). Results revealed the occurrence of four AMF genera associated to the three native plants and a high occurrence of unidentified taxa possibly associated to novel species. However, the Sanger sequencing methodology presents some drawbacks which are the time needed for cloning, RLFP set up and plasmid extraction as well as the low number of sequences obtained. It is thus not excluded that more AMF genera or species might be present in the petroleum-polluted sites.

In order to improve the AMF molecular identification in the oil ponds, we used the new generation sequencing (NGS) 454 GS-Junior technology in **chapter 2.** We focused on Pond 2 from the Charapa field and investigated in depth the AMF community composition as well as the root colonization of plants. Transects were established from the border of the Pond to 3 m inside and 3 m outside the Pond. A sampling was also done in the middle of the Pond. Forty samples belonging to different plant species were collected. We characterized and identified by NGS the AMF species present. In addition, we evaluated the frequency of occurrence and relative abundance of the AMF community according to the site of sampling (i.e. 3 m inside, 3 m outside and in the center of the Pond).

Abstract

Arbuscular mycorrhizal fungi (AMF) are mutualistic plant symbionts distributed worldwide. Their occurrence in hydrocarbon-polluted environments is less investigated, although specific communities may be present with possible interest for remediation strategies. Here, we investigated the AMF species community composition associated with the roots of diverse plants species naturally recolonizing a weathered crude oil pond in the Amazon region of Ecuador. Next generation 454 GS-Junior sequencing of an 800 bp LSU rRNA gene PCR amplicon was used. PCR amplicons were affiliated to a maximum-likelihood phylogenetic tree computed from 1.5 kb AMF reference sequences. A high throughput phylogenetic annotation approach using an evolutionary placement algorithm (EPA) allowed the characterization of sequences to the species level. Fifteen species were detected. *Acaulospora* species were identified as dominant colonizers, with 73% of relative read abundance (i.e. six representative species) co-occurring with *Archaeospora* (19.6%) (i.e. two unidentified species) and several genera from the Glomeraceae (*Rhizophagus, Glomus macrocarpum*-like, *Sclerocystis, Dominikia* and *Kamienskia*). Although, a diverse community belonging to Glomeraceae was observed, they represented less than 10% of the relative abundance in the Pond. The presence of 75% unidentified species suggests possible new species associated with roots of plants co-occurring under highly hydrocarbon-polluted conditions.

Keywords: Arbuscular mycorrhizal fungi; weathered crude oil pond; community composition; Amazon forest, Ecuador, 454-pyrosequencing.

Introduction

Oil pollution is an actual problem whose effects on fauna and flora are perceptible in natural as well as anthropized environments (Atlas & Philp, 2005; Labana *et al.*, 2007). It is often reported that contamination by hydrocarbons change the microbial community structure and decrease the microbial diversity (Satyanarayana *et al.*, 2012). However some microbial populations have demonstrated their capacity to adapt to the pollutants, resulting in the development of microbial consortia able to degrade a variety of petroleum molecules (Sahoo *et al.*, 2012). Due to the rapidly growing demand for hydrocarbon derivatives all over the world (Lee, 2015), it is expected that environmental pollution will increase in the coming years (Öztürk *et al.*, 2015). It is thus of the highest priority either to apply physico-chemical or to develop biological-friendly remediation strategies. The identification of native microbial communities well-adapted to polluted conditions and their further isolation, mass-production and application to polluted soils are part of this strategy and may represent an interesting approach for handling oil-contaminated sites.

The Amazonian region of Ecuador is a hotspot of biodiversity, which unfortunately is also a major reservoir of hydrocarbons (MAE, 2016). Therefore, the effects on fauna and flora regularly reported in literature (see above) are also significant here. Nevertheless, in hydrocarbon-polluted sites of the Charapa field in the Amazonian region, a natural re-colonization of the abandoned weathered oil ponds was observed through the years (Garcés-Ruiz *et al.*, 2017b). This suggested that plant roots and microbial communities associated with the rhizosphere were able to establish, probably enhancing the degradation of petroleum compounds, and thus representing a potentially important approach for the *in situ* treatment of hydrocarbon-polluted soils (Öztürk *et al.*, 2015).

Among the rhizosphere microbial communities, one of the most important is the arbuscular mycorrhizal fungi (AMF). These obligate root symbionts contribute to the formation and stability of soil aggregates, and to the transport of nutrients and water to the plant (Smith & Read, 2008). Thus, phytoremediation assisted by AMF has been suggested for hydrocarbon-polluted environments (Lenoir *et al.*, 2016b). The application of AMF may enhance plant growth (several studies, in controlled conditions, demonstrated increased plant biomass, root and shoot length, P and N uptake and chlorophyll content), increased biodegradative activity of roots and rhizosphere microorganism, improve adsorption and bioaccumulation of hydrocarbons by roots (see review Rajtor & Piotrowska-Seget, 2016).

In a previous study, conducted in a hydrocarbon-polluted soil from a natural environment in the Amazonian region of Ecuador (i.e. the Charapa field), Garcés-Ruiz *et al.* (2017) observed the presence of a relatively diverse AMF community associated with various herbaceous plants. A high root colonization was noticed in all the plants sampled and a molecular diversity analysis using a clone library and Sanger sequencing approach of a 1.5 kb fragment defined as the DNA barcode for AMF (Stockinger *et al.*, 2010) identified four AMF genera (i.e. *Glomus*, *Rhizophagus*, *Acaulospora* and *Archaeospora*) associated with three different plant species (*Euterpe precatoria*, *Carludovica palmata* and *Costus scaber*) (Garcés-Ruiz *et al.*, 2017b). However, more than 74% of the species could not be ascribed to an identified AMF, suggesting the presence of numerous unidentified taxa.

Sanger sequencing suffers from a number of drawbacks such as a tedious cloning step and the difficulty to collect and sequence all the clones, requiring their selection via restriction fragment length polymorphism (RFLP) patterns, which may create biases depending of the selection criteria. This method is time-consuming, costly and the number of samples that can be processed and sequences generated is often limited. Consequently, novel technologies such as high throughput sequencing (HTS) using different platforms such

as Illumina (Illumina, Inc.; Hafidi *et al.* 2015) or 454-pyrosequencing (Roche Applied Science) are increasingly used (Öpik *et al.*, 2009; Stockinger *et al.*, 2014; Senés-Guerrero & Schüßler, 2016b; Bouffaud *et al.*, 2017). For example the major advantages of the 454-pyrosequencing technology are: (1) the provision of unprocessed sequence data of sufficient length (~1kb) for robust phylogenetic analysis, (2) the elimination of the cloning step, (3) the obtainment of a large number of sequences per sample and (4) the sequencing of multiple samples in a single run through the use of barcoded primers (multiplex identifiers – MIDs) (Öpik *et al.*, 2009). The use of this high-throughput sequencing method provides the best phylogenetic resolution power to monitor AMF in the field (Senés-Guerrero & Schüßler, 2016b). This resolution has been improved by the use of a "phylogenetic backbone tree" constructed from reference AMF sequences of approx. 1.5 kb amplifying the SSU-ITS-LSU rRNA gene region which was defined as an extended DNA barcode resolving closely related AMF species (Stockinger *et al.*, 2010; Schoch *et al.*, 2012; Senés-Guerrero & Schüßler, 2016b). Moreover the AMF specific primers (Krüger *et al.*, 2009) used to amplify this gene region were confirmed to have the widest taxonomic coverage for AMF detection in environmental samples (Kohout *et al.*, 2014).

Thereby, the aim of the present study was to explore in-deep the community composition of AMF associated with roots of a diverse assemblage of plants present in a weathered crude oil Pond, from the Charapa field (see Garcés-Ruiz *et al.*, 2017). High-throughput 454-pyrosequencing of an ~800 bp rDNA fragment was conducted and analyzed using a reference "phylogenetic backbone" based on long AMF sequences (i.e. SSU-ITS-LSU 1.5 kb fragment) (Stockinger *et al.*, 2010; Krüger *et al.*, 2012) and an evolutionary placement algorithm (EPA) which allows the phylogenetic annotation of sequences to the species level (Senés-Guerrero & Schüßler, 2016b). The AMF community composition in plant roots was evaluated according to the site of

collection within the Pond by comparing the relative read abundance (RA) of AMF species (Loján *et al.*, 2016; Senés-Guerrero & Schüßler, 2016b).

Experimental procedures

Sampling location and experimental design

The sampling was done on December 2013 in a weathered crude oil Pond of 450 m^2 (76°48'54" W, 00°11'46" S) in the Charapa field located in the province of Sucumbíos in the Amazonian region of Ecuador. More details and description of this site can be found in Garcés-Ruiz *et al.* (2017). The Pond has an irregular shape; the west, north and south sides measured around ~23 m while the east side was only ~15 m. The perimeter of the Pond was marked every ~ 7.3 m (11 points in total). From each point, a straight line to the center of the Pond was traced and plants sampled 3 m inside and 3 m outside the Pond. Due to the complexity of the sampling environment, only an extra point was delimited close to the center of the Pond. In total 40 plants were collected (18 inside and outside and 4 in the middle) (Table 15). The number of plants sampled at each point varied from 1 to 3 according to their abundance and in a few cases, no plant was present.

Table 15 Plant species and number of individuals collected outside, inside and in the center of the pond.

Plant species	3 m outside	3 m inside	Center
Piper sp.	1	2	
Miconia sp.	1		
Inga auristellae	2		
Costus sp.	1	3	
Calathea		1	
Anthurium sp.	1	2	
Heliconia sp.	1		
Euterpe precatoria	1	2	
Urera baccifera	1	1	

Urera caracasana		1	
Stigmatopteris		1	
Acalypha	1		
Geonoma macrostachys		1	
Poaceae	2		2
Pouruma sp.	1		
Leandra sp.	1		
Cyathea sp.	1	1	
Dendropanax sp.	1		
Caladium sp.		1	
Cordia		1	
Acacia	1		
Renealmia sp.	1		
Cyclanthus bipartitus		1	
Faramea sp.			1
Paullinia sp.			1
Total	**18**	**18**	**4**

Root processing

After sampling, the shoots were used for plant identification and separated from roots that were kept within the soil at 4°C. Roots were cleaned from the soil particles with tap water. They were further washed for 10 min with Tween ® 80 (Panreac, Spain) diluted in sterilized distilled water (3%), to detach the crude oil (from Gordillo and Decock, personal communication). Roots were finally rinsed with sterilized distilled water. The cleaned roots were divided for evaluation of AMF colonization and DNA extraction.

Physico-chemical soil analysis

The Pond consisted of a layer of organic matter and soil above the weathered crude oil known as “tar”. Soil was sampled below the organic matter and until 30 cm depth, at different places inside the Pond. Three independent samples and two composite samples, each of 500 g, were analyzed: (1) a surface

sample and (2) a sample collected at 30 cm depth, close to the center of the pond, (3) a composite surface sample and (4) a composite sample collected at 30 cm depth, made of soil cores collected in the 4 corners inside the pond, and (5) a sample at 30 cm depth collected 70 m outside the Pond (i.e. non polluted soil – as control). The physico-chemical analyses were conducted by the CESAQ-PUCE laboratory (Table 16). The reference methods used were from the Environmental Protection Agency (US.EPA) and the Standard Methods for the Examination of Water and Wastewater (SM). The analysis performed were pH (US.EPA 9045 D, 2004), organic matter percentage (gravimetric), P mg Kg^{-1} (SM 4500 P, 1999), N mg Kg^{-1} (SM 4500-N, 1997), K mg Kg^{-1} (EPA 3051/7000A) and total petroleum hydrocarbon (TPH) mg Kg^{-1}(SM 5520 E; US.EPA 3550 B, 2012).

AMF Root colonization

After cleaning, the roots were stained in acidic-blue ink (Walker, 2005) and percentage of total colonization (%TC), arbuscular (%AC) and spores/vesicles (%VC) colonization were estimated under a dissecting microscope (Olympus BH2–RFCA, Japan) at 10 x magnification following the method of McGonigle *et al.* (1990). An approximate of 100 intersections were observed per sample.

DNA extraction

DNA was extracted from the 40 root samples according to Garcés-Ruiz *et al.* (2017). In brief, ~70 mg of dried roots from each sample was ground and material was transferred into the Lysing Matrix E tube from the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA). DNA was extracted following the manufacturer's protocol. The DNA integrity was visualized in 1% electrophoresis gel. Five µl of the product were stained with 100 x GelRed™

(Nucleic Acid Gel Stain, Biotium, Belgium). Samples were ran at 100 V for 18 min in 0.5 x TAE buffer and stored at -20°C until further use.

PCR conditions and 454-pyrosequencing

Two PCR were performed. The first PCR was developed according to Krüger *et al.* (2009). The amplification of the partial SSU, the complete ITS region and partial LSU rRNA gene using the SSUmAf–LSUmAr or SSUmCf-LSUmBr primers pairs was done. The primers targeted a 1.8 or 1.5 kb region, respectively. The nested PCR was performed as described by Senés-Guerrero & Schüßler (2016b), the product of the first one served as template. Nested PCR primer pairs amplified a fragment of around 800 bp from the LSU rRNA gene region. A fusion- primers amplicon was used. The forward primer LSU-D1f (5'-TAAGCGGAGGAAAAGAAAMTAAC-3') was synthesized together with the 454 adaptor A (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') and different multiplex identifiers (MIDs). Three different MIDs were used according to the site where the plants were sampled (i.e. MID 1: 3 m outside, MID 2: 3 m inside and MID 3: center of the Pond). The reverse primer LSUmBr (Krüger *et al.*, 2009) was synthesized with the 454 adaptor B (5'-CTATGCGCCTTGCCAGCCCGCTCAG-3') (Sigma, Germany). The reaction mix contained 0.02 U μL^{-1} Phusion polymerase (Thermo Scientific, Lithuania), 1 x Phusion HF Buffer with 1.5 mm $MgCl_2$, (Thermo Scientific, Lithuania) 200 µM of each dNTP (Promega, USA), 0.2 ug mL^{-1} BSA (Albumin Bovine, AMRESCO, USA) and 0.5 µM of each primer (Sigma, Germany) with 5 µL of template DNA in 20 µL of final reaction. Thermal cycling was done in an Eppendorf Mastercycler Gradient (Eppendorf, Germany) with the following conditions for the first PCR: Five min. initial denaturation at 99 °C; 40 cycles of 10 s. denaturation at 99 °C, 30 s. annealing at 60 °C and 1 min elongation at 72 °C; and a 10 min. final elongation.

In the nested PCR, 1 µL of the first PCR product was used in the final reaction (20 µL). The thermal cycling conditions were the same as for the first PCR, except that only 25 cycles were done (Senés-Guerrero & Schüßler, 2016b). For each sample, three separate PCRs were performed and the products were loaded on 1% agarose gel electrophoresis as above. Then, PCR replicates for each sample were pooled after confirming a visible band. The pooled products were loaded on 1% agarose to purify with the QIAquick® Gel Extraction Kit (Qiagen, Germany). DNA quantification was performed with the Quant-iTTM PicoGreen® dsDNA Assay Kit (Life technologies, USA) following the manufacturer's protocol. The samples were quantified in a fluorimeter (Fluoroskan Ascent FL, Labsystem, USA) with the Ascent Software (Louisc, nsku91). According to the results, the samples were diluted until they reached 10^9 molecules μL^{-1}. The samples were mixed to equimolar concentration according to the labeled MID (i.e. 1, 2 and 3). Finally, diluted PCR products were pooled in an equimolar concentration to obtain only one sample. 454 pyrosequencing was done by using the 2 XLR GS Junior Sequencing (Nucleomics Core, Leuven Belgium, http://www.nucleomics.be/).

Bioinformatic analyses

Analyses were performed according to Senés-Guerrero & Schüßler (2016a,b). In an initial step, sequences were quality-filtered and clustered to obtain representative sequences (RS). The next step involved the phylogenetic placement of the RS into a reference phylogenetic tree. QIIME pipeline (Caporaso *et al.*, 2010) was used for the initial analysis. Selection parameters to obtain reads for downstream analyses consisted on reads with no more than 15 ambiguous bases, a maximum length of homopolymer run of 15, a maximum number of 5 primer mismatches and sequences with a minimum length of 500 bp including the primers. The remaining sequences were clustered at a 98% similarity threshold to obtain RS and to avoid merging of

different species in the same cluster (Senés-Guerrero & Schüßler, 2016b). After clustering, singletons were removed and the remaining RS were blasted against the NCBI nucleotide database using Blast2GO (Conesa *et al.*, 2005) to identify and remove non-AMF sequences.

The remaining RS (with no non-AMF sequences and no singletons) were taken for species delimitation by means of the RAxML Evolutionary Placement Algorithm (EPA) with the GTRGAMMA model performed through a web interface (Berger & Stamatakis, 2011; Berger *et al.*, 2011) using a "phylogenetic backbone tree" based on 1.5 kb reference sequences (Krüger *et al.*, 2012) for sequence placement. To allow comparisons, species were annotated with the same species numbers as used in previous studies (Senés-Guerrero *et al.*, 2014; Loján *et al.*, 2016; Senés-Guerrero & Schüßler, 2016b).

The data of the sequencing run were deposited in the Sequence Read Archive at NCBI under BioProject ID PRJNA419893.

Statistical and data analysis

Data for AMF root colonization percentage were analyzed by one way ANOVA. Normal distribution was checked and non-normal data were normalized by arcsine transformation before analysis. One way ANOVA was used to determine significant difference between sites AMF root colonization. Statistical analyses were performed using the IBM SPSS statistic 25 software. To compare between sites the AMF community composition, 454-read relative abundance and principal component analysis (PCA) were the criteria (Senés-Guerrero & Schüßler, 2016b). Data from PCA were square root normalized and analyzed using the vegan package (Oksanen *et al.*, 2017) in R version 3.3.3 (R Development Core Team 2016).

The Shannon diversity index (H′) was calculated using the formula:

$H' = -\sum pi \log (pi)$,

where pi is the proportional abundance of AMF species according to the site of sampling.

Results

Physic-chemical soil properties

The level of TPH in the Pond was > 5000 mg Kg^{-1} (limit of quantification LOQ), while in the control (sample 5) it was 1188.8 mg Kg^{-1} (Table 16). The pH was alkaline in the control and the superficial sample from the middle of the pond (sample 1) (Table 16). The pH was neutral in samples 2, 3 and 4 (Table 16). The mineral nutrients (P, N and K) were higher in the superficial samples (1 and 3), although the sampling was performed below the organic matter layer. Conversely, sample 5 (control) had a low amount of P compared with the other samples (Table 16) while N was similar or higher compared to the others as well as K (Table 16). The analysis of organic matter was higher than the LOQ in all the samples with the exception of the control soil (Table 16).

Table 16 Chemical and physical analysis from soil collected in Pond in the Charapa field.

Analysis	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Analytical method
pH	8	6	5	5	8.1	CP-PEE-S004
Organic matter %	> 95	> 95	> 95	>95	44.55	Gravimetric
P mg Kg^{-1}	> 450	404.8	> 450	321.8	49	SM 4500 P B-C
N mg Kg^{-1}	> 1500	666.6	> 1500	1138.6	> 1500	SM 4500-N C
K mg Kg^{-1}	133.7	42.3	246.1	26.8	82.1	EPA 3051/7000A
TPH mg Kg^{-1}	> 5000	> 5000	> 5000	>5000	1188.8	CP-PEE-S003

Chemical and physical analysis from three independent samples and two composite samples: (1) a surface sample and (2) a sample collected at 30 cm depth, close to the center of the pond, (3) a composite surface sample and (4) a composite sample collected at 30 cm depth, made of soil cores collected in the 4 corners inside the pond, and (5) a sample at 30 cm depth collected 70 m outside the Pond (i.e. non polluted soil – as control).

AMF root colonization

The roots of all the plants sampled contained AMF structures. Colonization percentages were analyzed according to the sampling place (outside, inside and in the center of the Pond). The %TC was 62.5% ± 4.3, 51.2% ± 5.1 and 43.8% ± 13.3, outside, inside and in the center of the Pond, respectively, without any significant difference (P = 0.138). The %VC outside the Pond was 4.7% ± 0.9 while it was higher inside and in the center of the Pond (i.e. 10.2% ± 1.9 and 11.5% ± 6.2, respectively). However, no significant difference was noticed (P = 0. 058). The %AC did not differ between the sampling places (P= 0.054). The values were low and varied from 1.05% ± 0.46 to 2.3 % ±1.2, outside and inside the Pond, respectively. No arbuscules were observed in plants from the center of the Pond.

AMF community composition of roots

From a total of 40 root samples collected in the Pond (Table 15), 17085 raw input sequences resulted from 454-pyrosequencing analysis, while 5596 sequences fulfilled the parameters of selection. A full reference phylogenetic tree was used as "backbone" for the EPA approach and the placement of the query sequences, revealing 150 well-defined AMF representative sequences from 2557 reads (i.e. from approx. 800 bp). The 150 AMF representative sequences were annotated as 15 species belonging to 7 genera (Figure 22A-B). Some sequences could not be classified at family or genus level. For instance, 5 OTUs were related either to *Rhizophagus* sp. or *Dominikia* sp. but could not be defined at the genus level and 1 OTU was in an undefined branch from the reference phylogenetic tree, in-between Paraglomerales and Archaeosporales (Figure 22A-B).

The genus *Archaeospora* was represented by two undefined species, one of them, *Archaeospora* sp. 1 was recorded only inside the Pond while *Archaeospora* sp. 2 was found in the three sampling places, with a lower

abundance in the center of the Pond (Figure 22A). The genus *Acaulospora*, was represented by 6 species, *Acaulospora kentinensis*, *Acaulospora scrobiculata* and 4 *Acaulospora* spp. *Acaulospora kentinensis* and 2 *Acaulospora* spp. (i.e. 2 and 4) were recorded in all the sites. *Acaulospora* sp. 3 was registered inside and in the center of the Pond, while *A. scrobiculata* and *Acaulospora* sp. 1 were detected only inside the Pond (Figure 22A). The genus *Rhizophagus* was represented by *R. proliferus* detected in the three sites and *Rhizophagus* sp. detected only inside the Pond. Additionally, 64 sequences detected in the center of the Pond were classified as either *Rhizophagus* sp. or *Dominikia* sp. *Glomus macrocarpum* was present inside and in the center of the Pond. The genus *Kamienskia* and *Sclerocystis* were represented by one undefined species and were detected only inside the Pond (Figure 22A). In contrast, only one OTU represented by 3 reads could not be classified at family level, thus it was associated with Paraglomerales/Archaeosporales, representing less than 1% of the species identified (Figure 22B) detected only inside the pond**.**

The PCA analysis showed differences in the AMF community composition presented in the three sampling sites (Figure 22C). While the Shannon diversity index presented similar indices regardless of the sampling place (i.e. inside, outside or center of the Pond with values of 1.60, 1.09 and 1.38, respectively).

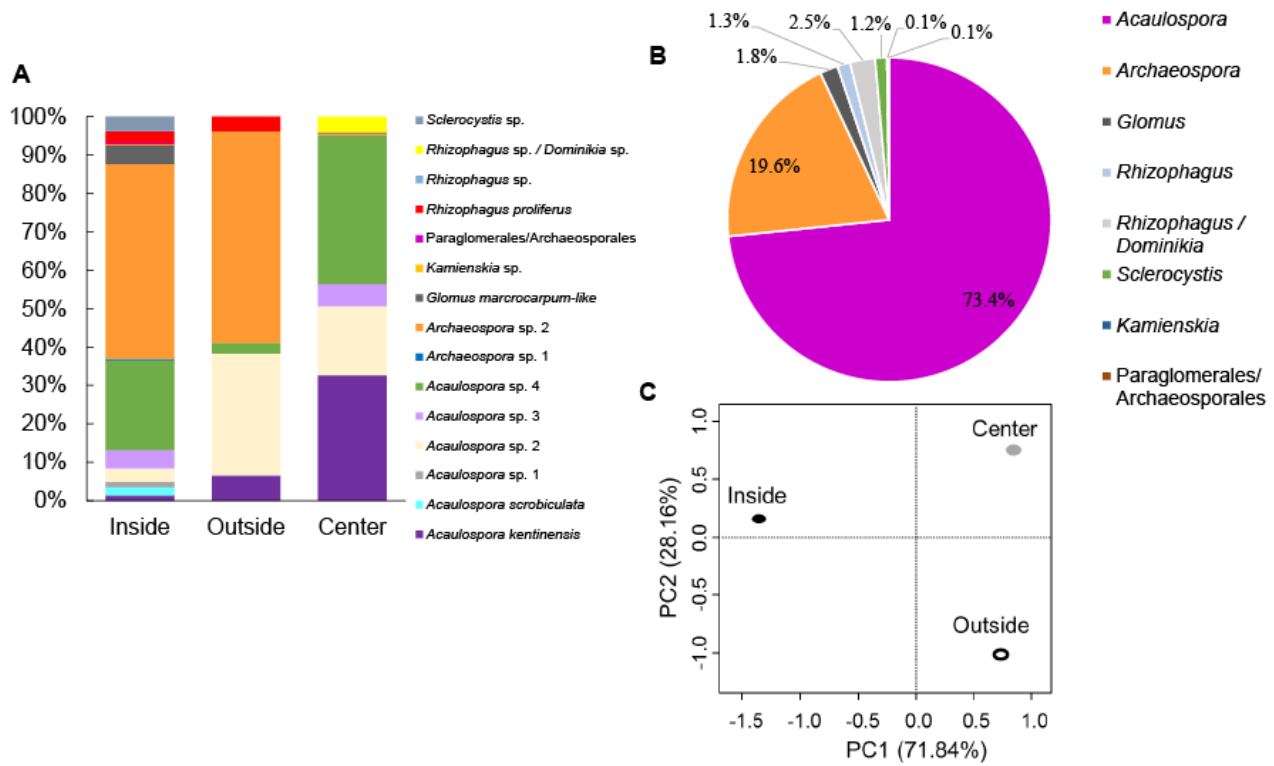

Fig. 22 (A) Relative read abundance (%) of AMF species present in pooled root samples from three different sides of the hydrocarbon polluted pond. (B) Pie chart shows the relative abundance (%) of each genus found in the hydrocarbon polluted pond. (C) Principal component analyses (PCA) of AMF community composition by place of sampling.

Discussion

Arbuscular mycorrhizal fungi are obligate roots symbionts associated with an approximate of 74% of angiosperms (Brundrett, 2009) and are occurring in almost every ecosystem (Cabello, 2001). However, their presence in hydrocarbon-polluted soils is poorly reported, even when they may be of interest for remediation strategies (Lenoir *et al.*, 2016a; Rajtor & Piotrowska-Seget, 2016). Twenty-five plant species, represented in 40 samples, were collected within the weathered oil crude Pond located in the Charapa field from the Amazon Basin of Ecuador. Root colonization by AMF was observed in all the samples analyzed. Total root colonization was above 40% and was very similar to a previous study using a clone library and Sanger sequencing approach in the same location (Garcés-Ruiz *et al.* 2017). High colonization was also reported by Huang *et al.* (2007) in 13 plant species growing in a petroleum-contaminated soil at the Sichuan Province in China. Both results suggested that plants are strongly dependent of AMF under highly hydrocarbon-polluted conditions.

The identification of AMF in roots can only be accurately established by molecular markers (Senés-Guerrero & Schüßler, 2016b). Thus, it is important to use molecular approaches capable of providing the best phylogenetic resolution for environmental samples. The 454-pyrosequencing of an ~800 bp LSU rDNA fragment and phylogenetic annotation method were used in the present study due to their robustness in short sequence characterization to species (Senés-Guerrero and Schüßler, 2016a). The 454-pyrosequencing and EPA-based approach revealed a community composition of 15 AMF species composed of 1 *Glomus macrocarpum*–like species, 2 *Rhizophagus* spp., 6 *Acaulospora* spp., 2 *Archaeospora* spp. 1 *Sclerocystis* sp., 1 *Kamienskia* sp. One species was not attributed to a specific genus thus, leaving it in-between *Dominikia* sp. and *Rhizophagus* sp. and one last RS was attributed in-between

the order Archaeosporales/Paraglomerales. Interestingly, only the four first genera (*Glomus, Rhizophagus, Acaulospora* and *Archaeospora*) were detected by Sanger sequencing in Garcés-Ruiz *et al.* (2017) in the same study site. However, in the study of Garcés-Ruiz *et al.* (2017), only three plants species (representing 9 samples) were analyzed. The higher number of genera/species detected in the present study could thus be attributed, at least partly, to the greater number of sequences generated from the 25 plants species (40 samples) analyzed.

Acaulospora was the more frequent genera (i.e. 73%) observed in the Pond. This prevalence was confirmed by the presence of *A. ketinensis*, *A. scrobiculata* and four unidentified species. Conversely, in the study of Garcés-Ruiz *et al.* (2017), this genus was less abundant (13%) with the presence of *A. longula* associated with *Carludovica palmata* and one unindentified species associated with *Costus scaber* inside the Pond, while *A. ketinensis* was observed outside the Pond colonizing *C. palmata* (Garcés-Ruiz *et al.*, 2017b). The study performed by Iffis *et al.* (2016) also identified *Acaulospora* as a dominant genus in hydrocarbon-polluted soils. Our study as well as the previous one (Garcés-Ruiz *et al.*, 2017b) demonstrated thus the prevalence of this genus at different abundances. Moreover, the high occurrence of *Acaulospora* could be related to the life history strategy (LHS) from the Grime C-S-R (competitor, stress-tolerator, ruderal) framework (Chagnon *et al.*, 2013). These authors classified this genus as stress-tolerant. Indeed, it was frequently reported under harsh climatic condtions, in acidic soils (i.e. pH 3.6-4.20 (Morton, 2017)) as well as under high elevation sites (such as the Andes region 2500 m asl (Loján *et al.*, 2016). In our study, this genus was observed in alcaline soils (pH 8) as well at lower pH (5-6) at 300 m asl, in highly hydrocarbon-polluted conditions, demonstrating its ability to develop under highly contrasting conditions.

Archaeospora was represented by two unidentified species. The relative abundance of this genus was 19.6%. *Archaeospora* sp.1 had a low occurrence

and was observed only inside the Pond. Conversely, the occurrence of *Archaeospora* sp. 2 was higher inside as well as outside the Pond with a low frequency in the center of the Pond. The abundance of this genus was different to the results obtained by Garcés-Ruiz *et al.* (2017). *Archaeospora* was revealed in the same Pond associated only with *C. scaber* with an abundance of ~ 50% and represented by one unidentified species. A Blast analysis between *Archaeospora* sp. sequences previously obtained from Sanger sequencing (Garcés-Ruiz *et al.*, 2017b) and the RS from 454-sequencing revealed a 95-97% of shared identity (data not shown). Thus we may hypothesize that the same unidentified species was detected in both studies.

Finally, the family Glomeraceae was represented by several species, although their relative abundance only represented 7% of the total AMF community. This could possibly be attributed to a lower affinity with the plant species sampled from the Pond or to an AMF species competition within the hydrocarbon-polluted soil. However, it is not excluded that the low amount of plants from the center of the Pond could be also related with this low percentage although the low number of AMF species was observed outside of the Pond (5 species) compared to inside (14 species) and the center (8 species). The AMF species that were not detected by Sanger but by pyrosequencing were *Glomus macrocarpum-like*, *Sclerocystis* sp., *Kamienskia* sp. and *Dominikia* sp. The last two genera were only recently described (Błaszkowski *et al.*, 2015). *Kamienskia* sp. was observed only inside the Pond, while *Dominikia* sp. was discovered only in the center of the Pond. Two species belonging to *Kamienskia* (i.e. *K. bistrata* and *K. perpusila*) were also recently erected by Błaszkowski *et al.* (2015). Their spores were isolated from *Triticum aestivum* L. in Iran, while in *Dominikia*, four species (i.e. *D. iranica*, *D. disticha*, *D. minuta* and *D. achra*) were described associated with roots of *Thinopyrum distichum* and other plants colonizing the maritime sand dunes of the Reserve Rooiels in South Africa. Until today, these are the only sites of their occurrence from around 3000 field-collected soils representing other

regions of Africa, Asia, Europe and the U.S.A (Błaszkowski *et al.*, 2015). It is suggested that their low occurrence may be attributed to a rare or seasonally sporulation or to their delicate spores easily decomposed by other organisms (Stutz & Morton, 1996; Błaszkowski *et al.*, 2000). None of these new erected species were identified in the hydrocarbon-polluted Pond, suggesting the presence of possible new taxa from these two genera.

Sclerocystis sp. was found only inside the pond, while *Glomus macrocarpum*-like was present inside and in the center of the Pond, both at low occurrence. Conversely, in Garcés-Ruiz *et al.* (2017), *Glomus* sp. was detected in *E. precatoria* and *C. palmata*. Its abundance was around 30%, while our results showed a relative abundance of 1.8%. Moreover, two OTUs of *Rhizophagus* sp. were closely related to *Sclerocystis sinuosa* (Garcés-Ruiz *et al.*, 2017b), thereby our results by pyrosequencing confirmed its presence in the hydrocarbon-polluted Pond.

Rhizophagus proliferus was found outside, inside and in the center of the pond, while a number of other undescribed *Rhizophagus* species were detected only inside the pond. These results demonstrated the presence of this genus in soil containing high levels of TPH. This was not observed by Garcés-Ruiz *et al.* (2017) with the Sanger sequencing method. In their study, this genus was only detected in the low-hydrocarbon contaminated surrounding soil associated with *Euterpe precatoria.* Although we could not precise to which plant species it was associated, it suggested the adaptability of this genus to highly oil-polluted soil as already observed by Hassan *et al.* (2014), de la Providencia *et al.* (2015) and Iffis *et al.* (2016). According to Hassan *et al.* (2014) and de la Providencia *et al.* (2015), *Rhizophagus* was the dominant genus in the rhizosphere of plants developing in hydrocarbon-polluted soils.

As in Garcés-Ruiz *et al.* (2017), three representing families (i.e. Glomeraceae, Acaulosporaceae and Archaeosporaceae) were detected in the Pond. However, the AMF distribution revealed the genus *Archaeospora* (55%) as the dominant followed by *Glomus* (33%) and *Acaulospora* (13%), while in the

present study *Acaulospora* was the dominant genus (73%) followed by *Archaeospora* (20%) and Glomeraceae (7%). The difference observed in the relative abundance could be ascribed to the difference of the analyses, such as the number of plants sampled, the larger plant diversity collected in the present study as well as the higher number of sequences generated. Thereby, our study revealed a large AMF community composition associated with a variety of plant species. These results improved the characterization of the AMF diversity capable to inhabit under highly polluted conditions.

Unidentified AMF species accounted for 75% the RS detected in our study. This corroborates the study of Garcés-Ruiz *et al.* (2017) conducted in the same location but using Sanger sequencing. Thereby we may assume, whatever the molecular technology applied, in this case Sanger or HTS-454 sequencing, the percentage of unknown species remains the same. As Błaszkowski *et al.* (2015) suggested, only 5% of AMF species are identified to date based on phylogenetic analyses of sequences of nrDNA extracted from plant roots. Our results confirmed this hypothesis. Hence, it is not excluded that under highly perturbed and unexplored environments the number of undescribed species may be even higher.

Within this study, a larger number of different AMF species were detected thanks to a wider number of plants collected and sequences generated as compared to the previous study of Garcés-Ruiz *et al.* (2017). *Acaulospora* was the dominant genus, supporting the results of Villacrés *et al.* (2014) which demonstrated its dominance by classical spores taxonomy in environmental liabilities in the Amazon region of Ecuador. Moreover, *Acaulospora* was also observed as dominant in potato crop culture in the Andean region (Loján *et al.*, 2016; Senés-Guerrero & Schüßler, 2016b) co-occurring with other AMF species. Further, the dominance of *Glomus* and *Acaulospora* representing more than 50% of the total AMF community has been observed in natural and disturbed Amazonian soils (Peña-Venegas, 2011). Recently, in a comparative study between a temperate (the Colorado Plateau in the south- western USA)

and a tropical (La Gran Sabana in south- eastern Venezuela) ecosystem, the presence of several *Acaulospora* spp. was demonstrated in agricultural sites, forest and shrub lands, however the family Glomeraeae was found in higher abundance (Chaudhary *et al.*, 2017). These results confirm *Acaulospora* as a worldwide-distributed stress-tolerant genus, especially in the tropical regions. In other studies on AMF community composition in hydrocarbon-polluted environment, Claroideoglomeraceae, Diversisporaceae, Gigasporaceae, Glomeraceae Paraglomeraceae, Archaeosporaceae were detected (Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016). Therefore, our findings suggest a moderate AMF diversity, which may be related for instance to plant species, type and level of hydrocarbon-pollution, soil characteristics or environmental variables.

Finally, the identification and characterization of undescribed species adapted to hydrocarbon-pollutants could help in the selection of AMF species, production of inoculum and its possible application for phytoremediation strategies.

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Chapter 3

Dynamics of short-term phosphorus uptake by intact mycorrhizal and non-mycorrhizal maize plants grown in a circulatory semi-hydroponic cultivation system

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My contribution to this chapter was approximately 50% and involved experimental data analysis, statistical analyses and the writing of the manuscript. Co-authors of the chapter have been involved in development of the system, experimental work, statistical analyses and revision of the manuscript.

Preface

In **chapters 1** and **2**, we evaluated the presence of AMF in roots of native plants growing in petroleum-polluted soils (crude oil Ponds and surrounding soil) and used two sequencing methods (Sangers and NGS) to identify the AMF community composition in the roots of these plants. Several families, genera and species were detected, but 75% remained undescribed, suggesting novel taxa. One of the genus observed with the Sanger and NGS sequencing methods was *Rhizophagus*. This genus contains ruderal species found in almost all environments. Therefore, it is used as a model AMF species for physiological studies.

The uptake and transport of inorganic phosphorus (Pi) is one of the most important attributes of AMF resulting in plant growth promotion. In **chapter 3,** we aimed to develop, a non-destructive circulatory semi-hydroponic cultivation system adapted from the method of Colpaert *et al.*, (1999) used to determine the Pi uptake dynamics from plants associated with ectomycorrhizal (ECM) fungi. Maize plantlets associated or not to the AMF *Rhizophagus irregularis* MUCL 41833 were evaluated during ~ one month. A nutrient solution empoverished in Pi circulated in the system during 42h, thereby depletion of Pi was evaluated at fixed intervals. The accumulation of P in maize plantlets was also assessed.

Abstract

A non-destructive cultivation system was developed to study the dynamics of phosphorus (Pi) uptake by mycorrhizal and non-mycorrhizal maize plantlets. The system consisted of a plant container connected via silicon tubes to a glass bottle containing a nutrient solution supplemented with Pi. The nutrient solution is pumped with a peristaltic pump to the upper part of the container via the silicon tubes and the solution percolate through the plantlet container back into the glass bottle. Pi is sampled from the glass bottle at regular intervals and concentration evaluated. Maize plantlets were colonized by the AMF *Rhizophagus irregularis* MUCL 41833 and Pi uptake quantified at fixed intervals (9, 21 and 42 h) from the depletion of the Pi in the nutrient solution flowing through the plantlets containers. Plants and fungus grew well in the perlite substrate. The concentration of Pi in the bottles followed an almost linear decrease over time, demonstrating a depletion of Pi in the circulating solution and a concomitant uptake/immobilization by the plantlet-AMF associates in the containers. The Pi uptake rate was significantly increased in the AMF-colonized plantlets (at 9 and 21 h) as compared to non-colonized plantlets, although no correlation was noticed with plant growth or P accumulation in shoots.

The circulatory semi-hydroponic cultivation system developed was adequate for measuring Pi depletion in a nutrient solution and by corollary Pi uptake/immobilization by the plant-AMF associates. The measurements were non-destructive so that the time course of Pi uptake could be monitored without disturbing the growth of the plant and its fungal associate. The system further opens the door to study the dynamics of other micro and macro-nutrients as well as their uptake under stressed growth conditions such as salinity, pollution by hydrocarbon contaminants or potential toxic elements.

Keywords *Rhizophagus irregularis*, maize (*Zea mays* L.), short term

phosphorus depletion, phosphorus uptake, Hoagland modified solution, non-destructive, circulatory semi-hydroponic cultivation system.

Introduction

The arbuscular mycorrhizal fungi (AMF) are normal and ubiquitous biota belonging to the soil (Smith & Smith, 2010). They occupy a singular place at the interface between soil and roots, colonizing a wide variety of plant species (Smith & Read, 2008), providing minerals to their hosts (mainly phosphorus (P)) in exchange for carbon (Smith & Read 2008; Smith *et al.,* 2011). These fungi also increase the resistance/tolerance of plants to a/biotic stresses (Smith et al., 2011).

A major benefit of AMF to plants is the increased/facilitated acquisition of Pi via the so-named AMF pathway, thus bypassing direct uptake by roots (Javot *et al.,* 2007), as evidenced in maize (Wright *et al.,* 2005; Tian *et al.,* 2013). The contribution of AMF to Pi uptake and plant nutrition has been investigated extensively (see Smith *et al.,* 2011) under greenhouse (Nouri *et al.,* 2014; Walder *et al.,* 2015), in the field (Yang *et al.,* 2014) and for some under *in vitro* culture conditions (Dupré de Boulois *et al.,* 2006; Calonne *et al.,* 2014) using various isotopes (i.e. ^{33}P and ^{32}P). Studies were almost all destructive (Castillo *et al.,* 2013; Nazeri *et al.,* 2014; Xie *et al.,* 2014; Yang *et al.,* 2014; Gonçalves de Oliveira *et al.,* 2017) thus preventing continuous measurements of Pi uptake by intact non-perturbed plant-AMF associates. Studies conducted by Colpaert *et al.* (1999) and Van Tichelen *et al.,* (1999) with ectomycorrhizal (ECM) fungi implemented a short-term Pi uptake dynamics on intact ectomycorrhizal and non-mycorrhizal *Pinus sylvestris* seedlings using a non-destructive semi-hydroponic cultivation system (Colpaert *et al.,* 1999). The depletion of Pi was monitored in a nutrient solution percolating through plant containers. The authors concluded that the extraradical mycelium of *Paxillus involutus*, *Suillus luteus*, *Suillus bovinus* and *Thelephora terrestris* strongly

influence the Pi-uptake capacity of the pine seedlings. Van Tichelen *et al.* (1999) used this system to monitor nutrients uptake by the ECM-colonized *P. sylvestris* plantlets under copper toxicity and demonstrated that mycorrhizal plants consistently had higher Pi uptake capacities than non-mycorrhizal plants.

In the present study a circulatory semi-hydroponic cultivation system, derived from the system of Colpaert *et al.* (1999) for ECM, was used to analyze the short-term Pi uptake dynamics in non-mycorrhizal and AMF-colonized maize plantlets. For the first time, short-term Pi uptake was studied on intact plant–AMF association. The measurements were non-destructive so that the time course of the Pi uptake capacity of individual plants could be followed. Pi depletion in the nutrient solution was monitored during 42 h and converted into Pi uptake rates by the plant/fungus associates and in shoots and roots concentration of P at the end of the experiment.

Materials and methods

Biological material

The arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as [‘irregulare'] MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel). The fungus was proliferated on *Zea mays* L. cv. ES Ballade (Euralis, France) in a plastic box containing a sterilized (121°C for 15 min) substrate of vermiculite and sand (w:w, 1:1) supplemented with 20 beads of Osmocote® Pro 5-6M (NPK 17-11-10+2MgO+TE (trace elements)). In parallel, *Zea mays* L. seeds were inoculated in a similar substrate without *R. irregularis* (i.e. the non-mycorrhized – NM – control). The AMF and NM control plastic boxes were maintained under greenhouse conditions at 25°C/18°C (day/night), a relative humidity (RH) of 38%, a photoperiod of 16 h day^{-1} and a photosynthetic

photon flux (PPF) of 120 µmol m^{-2} s^{-1}. Root colonization (see section *AMF root colonization*) was estimated after two months on three randomly selected maize plantlets. The total root colonization reached 87% ± 0.2, while no colonization was noticed for the NM control plantlets. Chopped roots and substrate from both inocula were used in the experiments below.

Maize (cv. ES Ballade) was supplied by the Centre Indépendant de Promotion Fourragère (CIPF – http://www.cipf.be/). The seeds were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 15 minutes and rinsed three times with sterilized (121°C for 15 min) deionized water during 10 minutes. The seeds were then placed on wet paper and germinated in plastic boxes in the dark at room temperature (~20°C). Eighty percent of the seeds germinated within three days.

Short term Pi depletion in a nutrient solution circulating through containers with pre-mycorrhized and non-mycorrhized plantlets

Pre-mycorrhization of maize plantlets

For the pre-mycorrhization of maize plantlets, two plastic nursery boxes (Modiform, Netherlands) with compartments of 0.08 L (4x4x5 cm size) were used. Thirty grams of lava stone substrate (DCM, Belgium) mixed with 1 g of AMF inoculum was added in each compartment and covered with 33 g of substrate. A control was similarly set up with the NM inoculum. Three-days-old maize seedlings were planted in each box. The seedlings were kept under greenhouse conditions set at 25/20°C (day/night), a RH of 50%, a photoperiod of 16 h day^{-1} and a PPF of 150 µmol m^{-2} s^{-1}.

Just before transfer in containers, three randomly selected pre-mycorrhized (PM) plantlets and NM plantlets were harvested and analyzed for root colonization. The total root colonization was 80.7% ± 1.7 for the PM plantlets, while no colonization was noticed for the NM plantlets.

Experimental set up

After 17 days of pre-mycorrhization, 9 PM maize plantlets and the same number of NM plantlets were transferred into the containers (Figure 23) containing dry perlite. The roots of the PM and NM plantlets were rinsed with deionized water to eliminate the lava stone substrate and seed debris prior to transfer into the containers.

Containers consisted of inverted 75 mm diam. wash bottles (500 mL - VWR, USA) cut at the base. A mesh of 100 µm size pore (Prosep B.V.B.A., Belgium) was glued on the top of the bottle to avoid losses of the growing substrate and to prevent roots to grow outside the bottle. Each bottle was filled with 32 g perlite (KNOUF- GMBH, Germany), beforehand sieved at 1 mm diam., rinsed with deionized water and dried in an oven for 48 h at 50°C. Maize plantlets were kept in the same conditions as above.

According to the equivalence test (Robinson et al., 2005), the height of plantlets presented a difference ($P = 0.74$) between the PM (36.7 ± 1.2 cm) and NM (32.4 ± 3.3 cm) treatments.

The containers with plantlets were randomly disposed in holes made in flex foam supports on 3 separate tables. A control without plantlet was also included (see Fig 23B). Each container was wrapped with black plastic foil to prevent algae development. The surface of the perlite was covered with the same black plastic foil (see Fig 23B).

Before set up of the system, the PM and NM maize plantlets were acclimatized during 25 days in the perlite. The plantlets received 200 mL of Hoagland^{-P} solution every 48 h (containers were closed and old solution was discarded before fresh was introduced). The modified Hoagland (Hoagland & Arnon, 1950) solution (i.e. 90% Pi-impoverished solution – Phosphorus = 6.245 mg L^{-1}, referred as Hoagland^{-P} throughout the text) contained in mg L^{-1} deionized water: NH_4NO_3, 80; $Ca(NO_3)_2 \cdot 4H_2O$, 826; KNO_3, 357; KCl, 45.1; K_2SO_4 105.4; KNO_3, 50; KH_2PO_4, 27.4; $MgSO_4$, 120.4; $MnSO_4 \cdot H_2O$, 0.5; H_3BO_3,

1.4; $CuSO_4 \cdot 5H_2O$, 0.2; $(NH_4)_6Mo7O_2 \cdot 4H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.6 and Fe-EDTA, 19. The pH of the solution was adjusted to 5.6 ± 0.2 before use.

After acclimatization, Pi depletion in a fresh circulating Hoagland^{-P} solution was monitored in the PM and NM maize plantlets.

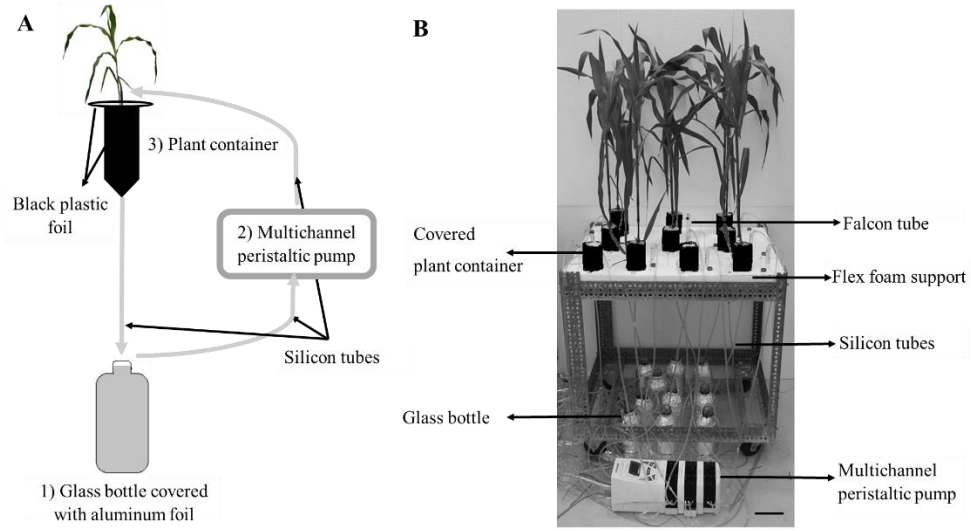

Fig. 23 The circulatory semi-hydroponic cultivation system**. (A)** Schematic representation of the experimental set-up to monitor Pi depletion in Hoagland^{-P} solution circulating through containers with pre-mycorrhized (PM) or non-mycorrhized (NM) maize plantlets. The nutrient solution in the glass bottle (1) is pumped with a peristaltic pump (2) to the upper part of the container (3) via silicon tubes. The solution percolate through the plantlet container back into the glass bottle. Grey arrows indicate the direction of flow of the nutrient solution in the tubing. **(B)** Experimental set up under greenhouse conditions. One table contained randomly disposed containers. Scale bar 10 cm.

Monitoring Pi depletion in Hoagland^{-P} solution

A circulatory semi-hydroponic cultivation system was set up to monitor the short term depletion of Pi in the modified Hoagland solution. The Hoagland^{-P} solution circulated via a peristaltic pump through the containers with PM or NM maize plantlets as described by Colpaert *et al.* (1999), Van Tichelen *et al.* (1999) and Crahay *et al.* (2013) for ectomycorrhizal fungi (see detailed

description Fig 23A-B). From the Pi depletion in the circulating solution, P uptake rates were deduced for the PM and NM maize plantlets.

The system consisted of 1L glass bottles covered with aluminum foil and containing the Hoagland^{-P} solution. Sterilized (sodium hypochlorite solution 50% overnight) 3 mm inner diam. silicon tubes (VWR, USA) linked the maize plantlets containers to the glass bottles *via* a multichannel peristaltic pump (Gilson's Minipuls Evolution, France). Each plantlet was connected to a single bottle. The tubes plunged in the bottles and the solution was pumped to the containers. The bottom of the containers was similarly connected to another silicon tube and the solution percolated through the plantlets and returned to the individual bottles *via* a silicon tube (Figure 23A and B).
Prior to the Pi-depletion short term experiment, a flushing was performed to establish an equal nutrient concentration in all containers (Colpaert *et al.*, 1999). Each plantlet received 200 mL (4 aliquots of 50 mL) of Hoagland^{-P} solution. The flow rate in the multichannel peristaltic pumps was 44 mL min^{-1} (see Figure 24). One L of Hoagland^{-P} solution per plantlet and control containers was prepared. Twenty mL of the solution was sampled from each bottle before the start of the circulatory system (considered as Time 0 (T_0)) to determine initial Pi concentration in the glass bottles. The circulating system was then initiated at a speed of 7.4 mL min^{-1}. At defined intervals, the nutrient solution was sampled in Falcon tubes of 50 mL. At each sampling time, the pumps were stopped, the end of the silicon tubes connecting the pump to the plantlets were placed over Falcon tubes and the pumps set on again. Similarly, after sampling, the pumps were stopped, the silicon tubes placed over the containers, and pumps set on again. Twenty mL of nutrient solution was collected at 9, 21 and 42 h following the start of the nutrient flow. The samples were then stored at 4°C in the dark before Pi analysis by inductive coupled plasma atomic emission spectrometer (ICP-AES).

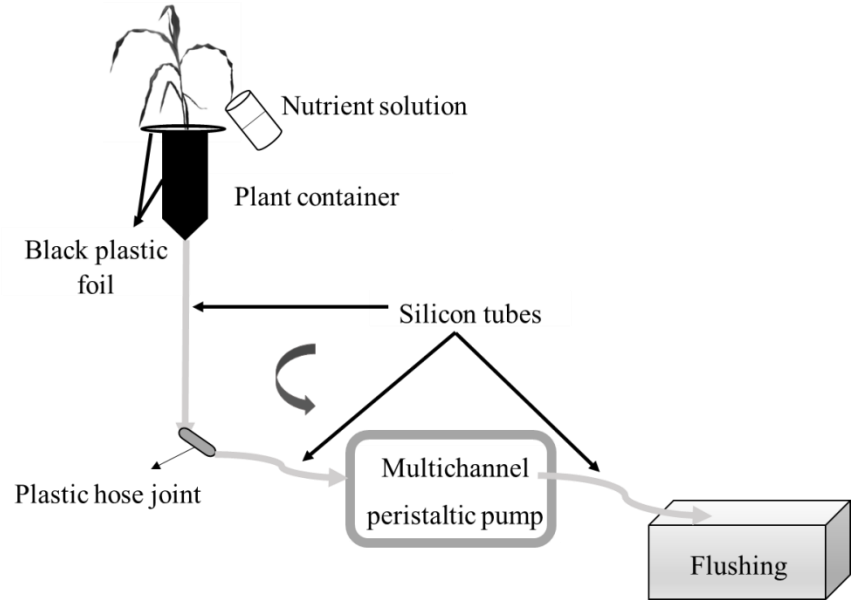

Fig. 24 Schematic representation of nutrient solution flushing before Pi short term monitoring. Hoagland^{-P} solution was added to the plant (4X50 mL). Silicon tubes were unified with a plastic hose joint. The multichannel peristaltic pump was turned on. Grey arrows indicate the direction of flow of the nutrient solution in the tubing.

Maize harvest and analysis

At the end of the experiment, i.e. 25 days after transfer into perlite, the plantlets were harvested. The shoot and roots fresh weights (SFW and RFW, respectively) were estimated before drying in an oven at 50°C for 48 hours and evaluation of shoot, root and total dry weights (SDW, RDW and TDW, respectively). The root systems were subsequently separated in two parts to evaluate AMF root colonization and P concentration.

AMF root colonization determination

Root colonization was estimated on three replicates from the PM and NM plantlets before experiments set up and all the PM replicates at the end of the experiment (i.e. at 17 and 46 days, respectively). Roots were sampled and

analyzed *via* ink staining (Walker, 2005). The roots were cut into small pieces and placed in Falcon tubes (Sarstedt, Germany). Twenty-five mL of KOH 10% was added to the roots before incubation at 70°C in a water bath for 1 h. The KOH was then removed and roots washed with HCl 1%. The staining step consisted in adding 25 mL of ink 2% (Parker blue ink, USA) in HCl 1%. The tubes were then placed at 70°C in a water bath for 1 h. The roots were rinsed and stored in deionized water before observation (Walker, 2005). Total (TC), arbuscular (AC) and vesicular (VC) colonization were estimated under a dissecting microscope (Olympus BH2–RFCA, Japan) at 10x magnification (McGonigle *et al.*, 1990). Around 170 intersections were observed per plantlet. At each intersection the presence or absence of the fungus was noted.

Phosphorus quantification

Phosphorus quantification in Hoagland-P solution

The Hoagland^{-P} solution was sampled at 0, 9, 21 and 42 hours and Pi concentration assessed. Two mL of each sample was diluted 5 times with ultrapure water (Millipore, France). The solution was then acidified with 20 µL of HNO_3 at 65% (Merck, Germany) and subsequently analyzed by ICP-AES (ICAP 6500, Thermo-Scientific, USA). The Pi content was determined with axial viewing of the emitted radiation. A peristaltic pump was used to introduce the solutions into the ICP-AES at a flow rate of 1.5 mL min^{-1}. Operating parameters for the instrument included forward power 1150 W, coolant gas flow rate 12 L min^{-1}, auxiliary gas flow rate 1 L min^{-1} and nebulizer gas flow rate 0.6 L min^{-1}. Pi quantification was analyzed under a wavelength of 177.495 λ nm^{-1}. The limit of detection was < 100 ppb. Data obtained (in ppm) were converted in mg L^{-1}.

The Pi depletion values obtained from the medium were standardized according to those obtained by their respective blanks and Pi concentration at

time 0 h, following the formula below:

$$\mathbf{[P]_X =} [P]_X \text{ quantified with ICP-AES at time T}$$

$$+ ([P]_{blank} \text{ at } T_0 - [P]_{blank} \text{ at T})$$

where:
[P] = Pi concentration in the solution
X = sample
blank = blank (i.e. perlite control) respective to the sample analyzed
T = time considered (9, 21 or 42 h after the start of the circulatory system)
T_0 = 0 h before the start of the circulatory system
Net Pi uptake was determined from the depletion of Pi in a Hoagland^{-P} solution circulating through the plantlet containers.

Phosphorus quantification in maize plantlets

After evaluation of dry weight, 300 mg of shoot and 100 mg of roots were ground separately in a grinder for P analysis. The ground material was then placed at 50°C overnight. Each sample was transferred into an Erlenmeyer (Pyrex, USA) and 4 mL of HNO_3 65% was added for the digestion process. Erlenmeyers were covered with a watch-glass and placed on a heating plate at 60°C overnight. After concentration at 120°C, samples were diluted with 3 mL HCl 38% (Merck, Germany), 1 mL HNO_3 65% and 10 mL of ultrapure water (Millipore, France). The solution was filtered with filter paper N°1 (Whatman, UK) in a 25 mL volumetric flask, before analysis. P concentration was converted from ppm to mg Kg^{-1} and content of P was determined according to the dry weight from shoot part and root system.

Statistical analysis

Equivalence test (Robinson *et al.*, 2005) was used to determine differences in height of maize plantlets before transfer into the containers.

Plant dry weight, P concentration and root colonization were analyzed by a student-t test and the homogeneity of variance was tested (Levene).
Phosphorus depletion in the Hoagland^{-P} solution was firstly subjected to a repeated-measures ANOVA with REML estimation where "Time" (of sampling, i.e.: 0, 9, 21, 42 h) and "table" (3 different flex foam supports in the greenhouse) were regarded as random factors. Each time was secondly analyzed independently using a mixed model, with "AMF" as fixed factor and "table" as random factor.
Normal distribution of residuals was checked and non-normal data were normalized by log transformation before analysis. Results are followed by the standard error (SE). All the tests were performed using the IBM SPSS statistic 23 software and JMP Pro 12 statistical software (SAS Institute Inc., USA).

Results

Short term Pi depletion

The concentration of Pi in the bottles of the PM and NM treatments followed the same time-course with an almost linear decrease over time (Figure 25), demonstrating a depletion of Pi in the circulating solution and a concomitant uptake/immobilization by the plantlet-AMF associates in the containers. However, differences were noticed between both treatments, as noticed by the repeated-measures ANOVA ($P = 0.0103$, results not shown). A mixed model was thus used to determine the difference in Pi absorption by PM and NM plantlets for each time. At 9 h, the concentration of Pi in the bottles (i.e. the nutrient circulating solution) was significantly higher in the NM treatment as

compared to the PM treatment (P = 0.037). Consequently, the relative Pi uptake/immobilization in the PM treatment was significantly higher than in the NM treatments (i.e. 16.4% versus 11.3% of the initial Pi concentration in the PM and NM treatments, respectively). A similar observation was made at 21 h. The Pi concentration in the bottles was significantly higher in the NM treatment as compared to the PM treatment (P = 0.002) and as a consequence the relative Pi uptake/immobilization in the PM treatment was significantly higher as compared to the NM treatment (i.e. 53% and 38.5% of the initial concentration of Pi in the PM and NM treatments, respectively). Conversely, at 42 h, no significant differences (P = 0.592) in Pi concentration was noticed between the PM and NM treatments (Figure 3). In both treatments, the relative Pi uptake/immobilization was 65% of the initial concentration of Pi in the bottles.

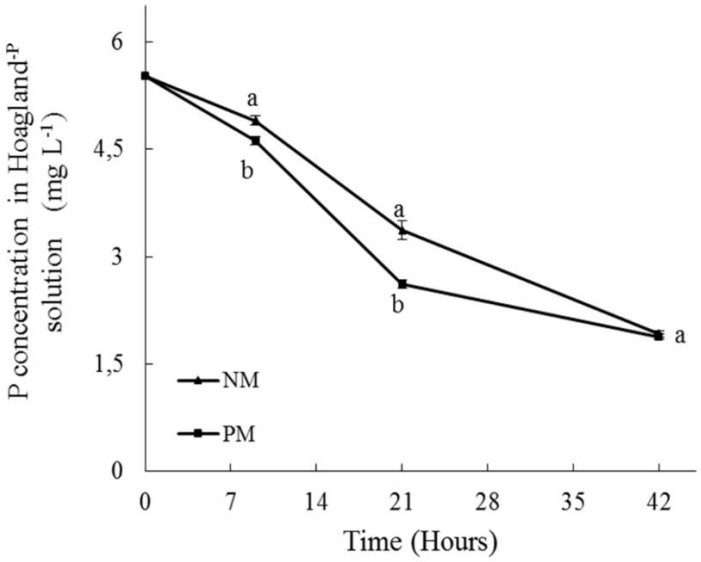

Fig. 25 Short term time course of phosphorus (Pi) depletion (mg L^{-1}) from a 90% P-impoverished Hoagland solution (i.e. Pi = 6.245 mg L^{-1}) circulating through plant containers in presence of pre-mycorrhized (PM) and non-mycorrhized (NM) maize plantlets. Pi was sampled at 0, 9, 21 and 42 hours of circulating solution. Data are mean values and standard errors (SE) of 8 (NM) and 9 (PM) replicates. Different letters at 9, 21 and 42 h indicate significant differences between treatments.

Plant growth parameters and AMF-root colonization

At the end of the experiment, no significant differences were observed in SDW and RDW between the plantlets in the PM and NM treatments (Table 17). The height of plantlets in the PM and NM treatments were similar at the end of the experiment (i.e. 25 days – P = 0.14). For both treatments, a relative increase of 60% was noticed as compared to the initial height (PM plantlets 36.7 ± 1.2 cm and NM plantlets 32.4 ± 3.3 cm). Similarly, no significant differences were noticed in shoot P concentration and total P content of plantlets between the PM and NM treatment (Table 17). Conversely, the plantlets in the PM treatment had a significantly higher root P concentration and total P content as compared to the plantlets in the NM treatment (Table 17).

At the end of the experiment, the %TC, %AC and %VC of the maize plantlets in the PM treatment was 90% ± 0.5, 64.5% ± 1.8 and 24.8% ± 1.4, respectively. Colonization before transfer to the containers was 80.7% ± 1.7, 35.9% ± 3.3 and 14.8% ± 1.5 for %TC, %AC and %VC, respectively. The increase in colonization percentages was significant for %TC and %AC ($P< 0.0001$) and %VC ($P< 0.005$).

Table 1 Shoot and root dry weight (g), P concentration (mg g^{-1} of dry weight) and total P content (mg $plant^{-1}$) of pre-mycorrhized (PM) and non-mycorrhized (NM) maize plantlets after 25 days of growth in perlite and after circulation through containers during 42h of an 90% P-impoverished (i.e. Pi = 6.245 mg L^{-1}) Hoagland solution.

	Dry weight (g)		**P concentration (mg g^{-1})**		**P content (mg $plant^{-1}$)**	
	Shoot	**Root**	**Shoot**	**Root**	**Shoot**	**Root**
PM	3.56 ± 0.08 a	1.55 ± 0.05 a	2.68 ± 0.07 a	1.8 ± 0.11 a	9.51 ± 0.19 a	2.72 ± 0.13 a
NM	3.47 ± 0.08 a	1.54 ± 0.06 a	2.85 ± 0.13 a	1.35 ± 0.04 b	9.86 ± 0.45 a	2.10 ± 0.11 b

Data are mean values and standard errors (SE) of 9 (PM) and 8 (NM) replicates. Values within a column followed by different letters are significantly different ($P \leq 0.05$, Student-t test).

Discussion

A non-destructive circulatory semi-hydroponic cultivation system with perlite as substrate was developed to investigate the short-term Pi depletion in a nutrient solution and by corollary Pi uptake/immobilization by non-mycorrhized and AMF-colonized maize plantlets. The system was adequate either for the maize plantlet and its fungal associate *R. irregularis*. Indeed, maize growth increased by 60% in a period of 25 days. Similarly, *R. irregularis* developed profusely with values of total root colonization reaching 90% in the same period. This suggested that the Hoagland^{-P} nutrient solution was adequate either to support plant growth and AMF development within the roots. These results are in agreement with Wright *et al.* (2005) and Tian *et al.* (2013), who demonstrated the ability of maize cultivars to be extensively colonized by the AMF *R. irregularis* at Pi concentrations below 1 mM.

Short-Term Pi uptake dynamics was first investigated by Colpaert *et al.* (1999) in roots of intact *P. sylvestris* seedlings colonized by four different ectomycorrhizal fungi and further extended to the same ECM-plant couples by Van Tichelen *et al.* (1999) to study Pi uptake under copper toxicity. For AMF, P acquisition by plants was mostly investigated using ^{33}P or ^{32}P (Smith *et al.*, 2011; Walder *et al.*, 2015; Kavka & Polle, 2016) or by destructive harvest of plants and analysis of P concentration in shoots and roots (Castillo *et al.*, 2013; Nazeri *et al.*, 2014; Xie *et al.*, 2014; Gonçalves de Oliveira *et al.*, 2017). For instance, Poss *et al.* (1985) analyzed P concentration from harvested plants as well as from the leachate volume collected during irrigation, to establish the P loss from mixed substrate. Hydroponic cultivation systems have also been used to determine P content in shoots and roots using different nutrient solutions (i.e. modified Long Ashton nutrient solution, modified Clark nutrient solution, half-strength Hoagland's solution) (Ojala & Jarrell, 1980; Medeiros *et al.*, 1994; Hawkins & George, 1997; White & Charvat, 1999). For instance, Ojala & Jarrell (1980) used a recirculating

hydroponic sand culture system and demonstrated that at low concentration of Pi (i.e. 0.1mg L^{-1}) colonization by the AMF was higher, with numerous arbuscules and vesicles, as compared to a concentration of 0.3 mg P L^{-1}. However, none of these studies considered depletion curves of Pi in a nutrient solution and by corollary Pi uptake/immobilization by intact plant-AMF associates. Thus, it seems that the circulatory semi-hydroponic system developed here could ease the determination of P dynamics in plant-AMF associations by the indirect analysis of Pi in the nutrient solution, avoiding disturbing the extraradical mycelium development, Pi uptake, translocation and transfer function and preventing to harvest plants.

Colonization of roots by *R. irregularis* resulted in a significant increase of Pi uptake in the PM maize plantlets as compared to the NM plantlets at 9 and 21 h, while no difference was noticed at 42 h in the experiments. The difference of Pi uptake at 9 and 21 h was probably not ascribed only to the higher volume of perlite explored by the AMF-colonized roots. Indeed, as suggested by Colpaert *et al.* (1999) using the same system with ECM fungi, the constant percolation of the nutrient solution during the uptake analyses counteracts the development of depletion zones around roots and hyphae. Therefore the high Pi-uptake rates of the mycorrhizal root systems cannot be ascribed uniquely to an improved nutrient scavenging of the substrate by the fungus but possibly also to a higher number of Pi transporters operating in the mycorrhizal root systems than in the NM roots (Colpaert *et al.,* 1999). It is not excluded that these Pi transporters (on root and hyphae surface) were more abundant in the PM maize plantlets. Nevertheless, as reported by Tian *et al.* (2013), little is known about the relationship between the expression of Pi transporters in maize roots colonized by AMF and the growth or Pi uptake in maize. Indeed, even if short term Pi uptake rates increased in the PM plantlets as compared to the NM ones, no difference in biomass was observed. This results corroborates the findings of Smith *et al.* (2004) in tomato. These authors suggested that there is no clear relationship between total root colonization

and plant growth or P response.

The absence of differences in biomass between the AMF-colonized and control plantlets, could be attributed to the fact that each plant received strictly the same amount of nutrients flowing on the root system during several weeks and uptake rates is only established for short term at fixed intervals and with refreshed nutrient solutions. In presence of AMF, the nutrient solution is much faster depleted, which would find its advantages in a non-resource restricted environment, thus impacting growth, while in the semi-hydroponic system, the same amount of nutrients would at the end be taken up in presence as well as in absence of AMF, resulting in the absence of visible impact on plant growth. This could possibly explain the absence of difference at 42 h for the experiments.

The higher P concentration and content in the root system of the PM plantlets, suggested a better P nutrition of the PM plantlets. Curiously, these differences were not reflected in the shoot parts. This could indicate that P was accumulated in the intraradical hyphae of the AMF after synthesis into polyphosphate granules (poly-P) in the vacuole of the extraradical/intraradical hyphae (Ezawa *et al.,* 2001; Zhang *et al.,* 2016). Fiorilli *et al.* (2013) suggested that, once the AMF satisfied their Pi requirements, most of the Pi flux is oriented towards the host. Because of the high presence of the fungus inside the roots (90% of colonization rate), this stored poly-P could represent a reservoir of P available for the fungus metabolism or for plant growth. Nazeri *et al.* (2014) also observed that after the P pulse, shoot P concentration increased by 143% in uncolonized plants, while in colonized plants the increase was only 53% resulting in shoot P being highest in uncolonized plants. They suggest that AMF affects shoot P concentration at low and high availability, restricting its transfer when P becomes excessive to plant requirements.

The major advantage offered by the semi-hydroponic cultivation system developed in the present study is the capacity to monitor and compare non-destructively the Pi uptake dynamics of mycorrhizal and non-mycorrhizal plants via the depletion of Pi in the nutrient solution flowing through the plantlets containers. The system could be extended to analyze the uptake dynamics of other macro and micro-nutrients from their depletion in a nutrient solution, as well as to the dynamics of uptake of various plant species at different phenological development steps (e.g. at vegetative growth, flowering, production of seeds, senescence) associated to AMF having different life history strategies and under non-stress as well as abiotic (e.g. salinity, pollution by hydrocarbons pollutants or potential toxic elements) stress conditions. However, complementary studies and improvement of the circulatory semi-hydroponic cultivation system are necessary to separate the contribution of roots and fungi to the uptake, and thus to determine the relative contribution of each in the phosphorus uptake dynamics and accumulation within plants.

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Chapter 4

The arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 does not alleviate the impact of diesel on phosphorus uptake by maize plantlets

Research paper in preparation for Journal of Environmental Science and Pollution Research

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My contribution to this chapter was approximately 70% and involved experimental design and work, data analysis and the writing of the manuscript. Co-authors of the chapter have been involved in experimental work, statistical analyses and revision of the manuscript.

Preface

In **chapter 3** a non-destructive cultivation system was developed to study the dynamics of phosphorus (Pi) uptake by mycorrhizal and non-mycorrhizal maize plantlets. Maize plantlets were colonized by the AMF *Rhizophagus irregularis* MUCL 41833 and Pi uptake quantified at fixed intervals from the depletion of the Pi in the nutrient solution flowing through the plantlets containers. We demonstrated a significant depletion of Pi at 9 and 21 h by mycorrhizal plantlets compared to the non-mycorrhizal ones. The circulatory semi-hydroponic cultivation system developed was adequate for measuring Pi depletion in a nutrient solution and by corollary Pi uptake/immobilization by the plant-AMF associates. Since sampling was non-destructive, the time course of Pi uptake could be monitored without disturbing the growth of the plant and its fungal associate. Moreover the use of different stress may be applied to study Pi uptake mechanism. Thus, in **chapter 4,** we aimed to evaluate, via the non-destructive circulatory semi-hydroponic cultivation system, the Pi uptake dynamics under hydrocarbon-polluted conditions (with diesel as the pollutant) of maize plantlets associated or not with the AMF *Rhizophagus irregularis* MUCL 41833. The accumulation of P in maize plantlets was also assessed.

Abstract

Soil contamination by petroleum byproducts is an important environmental problem that affects plants as well as rhizosphere microorganisms. Here, the impact of diesel on short-term phosphorus (Pi) uptake/immobilization by maize plantlets colonized by the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* MUCL 41833 was evaluated in a non-destructive circulatory semi-hydroponic cultivation system. Pi uptake/immobilization was quantified at regular intervals (9, 21 and 42 h) from the % of Pi depletion in a nutrient solution flowing through the plantlets containers. Plants and fungus grew well in the perlite substrate in absence of diesel. In presence of diesel, a detrimental effect was noticed on the growth of the maize plantlets, their Pi uptake/immobilization and induction of oxidative stress in presence as well as in absence of AMF. The combination of maize plantlets with the AMF *R. irregularis* MUCL 41833 thus seems not appropriate for phytoremediation and plead for the selection, testing and utilization of AMF species isolated from petroleum-polluted soils for bioremediation strategies.

Keywords: *Rhizophagus irregularis*, maize (*Zea mays* L.), short-term phosphorus depletion, inorganic phosphorus uptake/immobilization, diesel, Hoagland modified solution

Introduction

Diesel is a type of fuel used for machinery like power generators and vehicles. It is a distilled mixture from crude oil containing a combination of 65–85% saturates, 5–30% aromatics and 0–5% olefins, although this percentage is variable according to the petroleum source, the refining process and the sulfur content (Liang *et al.*, 2005). Diesel is toxic to the environment, owing to the presence of low molecular weight compounds with high volatility, solubility

and bioavailability, as polyaromatic hydrocarbons (PAHs) and alkanes (Dorn & Salanitro, 2000). Its accumulation in soil is particularly harmful to plants and their microbial root-associates as well as a source of contamination of ground water (Adam & Duncan, 1999; Driai *et al.*, 2015).

Several studies have reported a detrimental effect of petroleum hydrocarbons (crude oil, diesel, PAHs…) on plant development (Verdin *et al.*, 2006; Debiane *et al.*, 2008, 2009; Tang *et al.*, 2009; Hernández-Ortega *et al.*, 2012; Cruz *et al.*, 2013; Oludele & Ogundele, 2013; Shukry *et al.*, 2013; Nwoko, 2014). For instance, petroleum derivatives like lubricants and diesel fuel have been reported to delay or inhibit roots and hypocotyl growth in *Cucumis sativus* (Cruz *et al.*, 2013) as well as to decrease the capacity of roots to take up minerals and water (Leyval & Binet, 1998; Tang *et al.*, 2009; Oludele & Ogundele, 2013). A decrease in biomass of chicory transformed roots was also noticed by Driai *et al.* (2015) and these authors suggested that it was the result of a deficiency in the water and mineral absorption. The induction of oxidative stress as malondialdehyde (MDA) production and lipid changes in plant cells altering membranes, have been reported in presence of PAHs (Debiane *et al.*, 2008, 2009, 2012). Moreover, total antioxidant activity (AOX) was significantly increased in *Melilotus albus* in presence of diesel suggesting the increase of reactive oxygen species (ROS) (Hernández-Ortega *et al.*, 2012). Interestingly, a variation in tolerance to petroleum products has been observed with different plant species (Hernández-Ortega *et al.*, 2012; Hernández-Ortega, 2014). For instance, *Zea mays* showed higher survival than *Arachis hypogaea, Vigna unguiculata* and *Sorghum bicolor* (Ogbo, 2009), and superior seedlings growth than *Abelmoschus esculentus* and *Amaranthus hybridus* in presence of diesel, even if it was impacted by the fuel (Oludele & Ogundele, 2013). Therefore, maize is nowadays frequently used in phytoremediation studies because of its higher tolerance to many pollutants such as petroleum (Wuana & Okieimen, 2010). The variation in tolerance could be related to various factors, among which (a) the development of a

profuse root system (Surridge *et al.*, 2009) (b) a rapid growth with development of high biomass (Wuana & Okieimen, 2010), (c) the production of enzymes capable to metabolize/degrade many organic pollutants (Merkl *et al.*, 2005b; Macek *et al.*, 2009) and (d) an elevated bioaccumulation potential (Macek *et al.*, 2009; Wuana & Okieimen, 2010; Agamuthu & Dadrasnia, 2014).

Interestingly, the capacity of plants to associate with rhizosphere microorganisms involved in the degradation, transformation, mineralization and/or sequestration of petroleum contaminants has also been mentioned in several studies (Atlas & Philp, 2005; Macek *et al.*, 2009; Das & Chandran, 2011). Their presence within or close to the roots thus represent a serious option to consider for increasing the resistance/tolerance of plants against hydrocarbon pollutants (Ferrera-Cerrato *et al.*, 2006; Li *et al.*, 2007; Alarcón *et al.*, 2008; Hernández-Ortega *et al.*, 2012).

Among the soil microorganisms, arbuscular mycorrhizal fungi (AMF) occupy a singular place at the interface between soil and roots. They colonize a large variety of plant species (Smith & Read, 2008), improving their mineral nutrition (mainly inorganic phosphorus (Pi)) and resistance/tolerance to several biotic (Pozo *et al.*, 2013) and abiotic (Plouznikoff *et al.*, 2016) stresses. Diesel was reported to affect AMF (Kirk *et al.*, 2005; Tang *et al.*, 2009; Trejo *et al.*, 2013; Driai *et al.*, 2015). However, the role of these fungi in increasing plant resistance/tolerance to this pollutant (Alarcón *et al.*, 2008; Trejo *et al.*, 2013; Driai *et al.*, 2015) or its components (Verdin *et al.*, 2006; Debiane *et al.*, 2008, 2009, 2012) have also been documented. Several mechanisms such as the (a) accumulation of pollutants in colonized roots and fungal structures (Verdin *et al.*, 2006), (b) improved plant mineral nutrition (Liu & Dalpé, 2009), (c) qualitative and quantitative changes of lipid content (Debiane *et al.*, 2012) and (d) increased tolerance to oxidative stress (Debiane *et al.*, 2008, 2009; Hernández-Ortega, 2014; Driai *et al.*, 2015) were attributed to AMF in colonized plants.

A major benefit of AMF for plants is the increased/facilitated acquisition of Pi via the so-named AMF pathway, thus bypassing direct uptake by roots, as evidenced in maize (Wright *et al.*, 2005; Tian *et al.*, 2013; Garcés-Ruiz *et al.*, 2017a). In presence of anthracene and phenanthrene, two low molecular weight PAHs often reported in diesel (Tong & Karasek, 1984; Rollin *et al.*, 2005), Liu & Dalpé (2009) observed an increase in N and P uptake in pre-mycorrhized plants as compared to non-mycorrhized plants. Conversely, Hernández-Ortega *et al.* (2012) reported no differences in P content in *M. albus* associated or not to AMF after 60 days of growth in presence of diesel. However, these studies were conducted under soil-substrate (with P-buffer capacity) conditions and were destructive. Therefore, Pi uptake/immobilization kinetics in presence/absence of the pollutant could not be investigated. To avoid these limitations, a circulatory semi-hydroponic cultivation system was developed to monitor the short-term Pi uptake/immobilization in intact mycorrhizal and non-mycorrhizal maize plantlets (Garcés-Ruiz *et al.*, 2017a). This system was adequate for growing AMF-colonized maize plantlets and to investigate the short-term dynamics of Pi uptake/immobilization. This system was used in the present study to determine the impact of diesel on Pi uptake by maize plantlets associated or not to the AMF *R. irregularis* MUCL 41833. The % of Pi depletion in the nutrient solution was monitored during 42 h and converted into Pi uptake/immobilization by the plant/fungus associates. In parallel, the concentration of P and MDA in roots and shoots were evaluated at the end of the experiment in order to assess the impact of diesel on lipid membranes.

Materials and methods

Biological material

The arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as [‘irregulare']

MUCL 41833 was supplied by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel) and the maize plantlets (cv. ES Ballade) by the Centre Indépendant de Promotion Fourragère (CIPF – http://www.cipf.be/).

Mass-production of the fungus as well as the protocols for maize seed disinfection and growth are detailed in Garcés-Ruiz *et al.* (2017a)

Experimental design

Preliminary experiment: Diesel toxicity to maize plantlets

Six one-month old non-mycorrhized (NM) maize plantlets were used to determine the impact of increasing concentration of diesel on plant survival. The plantlets were transferred into containers, which consisted of inverted 75 mm diam. wash bottles (500 mL - VWR, USA) cut at the base. A mesh of 100 µm size pore (Prosep B.V.B.A., Belgium) was glued on the top of the bottles to avoid losses of the growing substrate and to prevent roots to grow outside the containers. Each container was filled with 32 g perlite (KNOUF- GMBH, Germany), beforehand sieved at 1 mm diam., rinsed with deionized water and dried in an oven for 48 h at 50°C (Garcés-Ruiz *et al.*, 2017a). The plantlets were grown under growth chamber conditions (22/18°C day/night, relative humidity (RH) of 80%, photoperiod of 16 h day^{-1} and photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}).

Two hundreds mL of modified Hoagland solution (i.e. 90% P-impoverished solution, referred as Hoagland^{-P} throughout the text) was added every 48 hours (see Garcés-Ruiz *et al.* (2017a) for detailed composition). The plantlets were acclimatized for 7 days in the containers. Afterwards (from day 8 to 11), increasing amounts (i.e. 0.8, 1.6, 2.4, 3.2 or 4 mL) of filtered diesel from gas station (Q8, Louvain la Neuve, Belgium) was spiked on the perlite surface. At day 12, 0.8 mL of diesel was added in each container and finally at day 15,

0.8 mL was spiked again to the top of the perlite to reach a final concentration of 2.4, 3.2, 4, 4.8 and 5.6 mL of diesel per container. Survival of the plantlets and symptoms were estimated at day 18.

The plantlets grown in presence of 4 mL or less diesel, including the control plantlets (i.e. no diesel added to the substrate) survived, even if an impact on plant development was observed as compared to the control. According to this result, the experiment below was conducted with 4 mL diesel (i.e. 125 mL Kg^{-1} of perlite) per container.

Pre-mycorrhization of maize plantlets and estimation of root colonization prior to experimental set up

Pre-mycorrhization of maize plantlets was performed according to Garcés-Ruiz *et al.* (2017a) except that sand/vermiculite (Euroquartz/Agra-Vermiculite Pull Rhenen) substrate was used instead of lava stone.

Before transfer into the containers, three randomly selected pre-mycorrhized (PM) plantlets and three non-mycorrhized (NM) plantlets were harvested and analyzed for root colonization. The total root colonization at day 17 following transfer of the plantlets into the containers was 60 ± 2% for the PM plantlets. No root colonization was noticed in the NM plantlets.

Experimental set up

After 21 days of pre-mycorrhization, 12 PM and NM plantlets were harvested and their roots cleaned with deionized water to eliminate the sand/vermiculite substrate. The plantlets were then transferred into the containers containing dry perlite (32 g) prepared as explained for the preliminary experiment. At the start of the experiment, the height of the plantlets in the PM and NM treatments was similar according to the equivalence test (Robinson *et al.*, 2005), and reached 50.4 ± 0.9 and 49.5 ± 0.8 cm for the PM and NM treatments, respectively ($P = 0.046$).

Four treatments with six plantlets (i.e. replicates) were considered: PM plantlets grown in presence (PM+D) or absence (PM-D) of diesel and NM plantlets grown in presence (NM+D) or absence (NM-D) of diesel. Two additional controls without plantlets were considered: a blank with diesel ($blank^{+D}$) and a blank without diesel ($blank^{-D}$). Three containers (i.e. replicates) were considered for both controls. The plantlets and controls were randomly disposed in flex foam supports. The PM and NM maize plantlets were first acclimatized four days in absence of diesel. During the next days, diesel was gradually applied to the substrate in half of the containers and $blank^{+D}$ containers: 0.8 mL added on the top of perlite substrate after 5 days, 1.6 mL added after 7 days and finally 1.6 added after 9 days of acclimatization. A final concentration of 4 mL of diesel per container (125 mL Kg^{-1} of perlite) was reached at the start of Pi depletion monitoring. During the 21 days of acclimatization, 200 mL of fresh $Hoagland^{-P}$ solution was added to all treatments every 48 hours. The outlet of the containers were sealed during the acclimatization period to avoid lixiviation of diesel.

Short-term Pi depletion in Hoagland-P solution circulating through containers with pre-mycorrhized and non-mycorrhized plantlets in presence of diesel

After the acclimatization period, where plantlets were in contact with 4 mL diesel per container during 12 days, a flushing step was performed to establish an equal nutrient concentration in all containers (Garcés-Ruiz *et al.*, 2017a). A minimal loss of diesel was expected, even though it was visibly adsorbed on perlite and solution was not yellow colored and/or no oil on solution surface was observed after flushing.

Briefly, time-course of Pi depletion was monitored in the four treatments and blanks, with 1 L of $Hoagland^{-P}$ solution per container. Twenty mL of the solution was sampled from each glass bottle before the start of the circulatory

system (T_0) to assess the initial Pi concentration. After 9, 21 and 42 h, the nutrient solution was sampled, and stored at 4°C in the dark prior to ICP-AES analysis.

Maize harvest and analysis

At the end of the experiment, i.e. 25 days after transfer into containers and 20 days after the first contact with diesel, the plantlets were harvested. The shoots and roots fresh weights (SFW and RFW, respectively) were estimated before drying in an oven at 50°C for 48 hours and evaluation of shoot, root and total dry weights (SDW, RDW and TDW, respectively). The root systems were subsequently separated in two parts to evaluate AMF root colonization and P concentration. Finally, one fresh part of shoot and root samples (between 1.5 and 13.5% of the fresh weight) were stored at -80°C for malondialdehyde (MDA) analysis.

AMF root colonization

Root colonization was estimated on three replicates from the PM and NM plantlets before experiment set up and on all the plantlets at the end of the experiment (i.e. at 17 and 46 days, respectively). Roots were sampled, stained with ink and analyzed as in Garcés-Ruiz *et al.* (2017a). Total (TC), arbuscular (AC) and vesicular (VC) colonization were estimated under a dissecting microscope (Olympus BH2–RFCA, Japan) at 10 x magnification (McGonigle *et al.*, 1990a).

Phosphorus quantification in Hoagland-P solution and maize plantlets

The Hoagland^{-P} solution was sampled at 0, 9, 21 and 42 hours and Pi depletion assessed. Samples were prepared and analyzed by ICP-AES (ICAP 6500, Thermo-Scientific, USA). Data obtained (in ppm) were converted in mg L^{-1}.

Pi depletion values obtained from the medium were standardized according to those obtained by their respective blank containers and Pi concentration at time 0 h. Data were then converted as % of Pi depletion which is the percentage of the Pi concentration remaining in the bottles at time 9, 21 and 42 h relative to the initial Pi concentration in the bottles at time T0 = 100%.

The P concentration in maize plantlets was determined as follows: One hundred mg of shoot or roots were ground separately in a grinder and then placed at 50°C overnight. Each sample was transferred into an Erlenmeyer (Pyrex, USA) and 4 mL of HNO_3 65% was added for the digestion process. Erlenmeyers were covered with a watch-glass and placed on a heating plate at 60°C overnight. After concentration at 120°C, samples were diluted with 3 mL HCl 38% (Merck, Germany), 1 mL HNO_3 65% and 10 mL of ultrapure water (Millipore, France). The solution was filtered with filter paper N°1 (Whatman, UK) in a 25 mL volumetric flask, before analysis. Phosphorus concentration was converted from ppm to mg g^{-1} and content of P was determined according to the dry weight of shoots and roots.

Analysis of malondialdehyde

The level of lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances (mainly MDA). Frozen samples (0.25 g of FW) were homogenized with a pre-chilled mortar and pestle with 5 mL of ice-cold 5% (w/v) trichloroacetic acid and centrifuged at 12000 g for 15 min and at 4 °C. The assay was performed according to Heath & Packer, (1968) and MDA concentration was calculated using a molar extinction coefficient of 155 mM^{-1} cm^{-1} (Ghanem *et al.*, 2008).

Statistical analysis

To determine differences in height of maize plantlets before transfer into the containers equivalence test (Robinson *et al.*, 2005) was used.

Root colonization in presence and absence of diesel was analyzed by a student-t test and the homogeneity of variance was tested (Levene) after arcsine transformation.

Arcsin-transformed percentages of Pi remaining in the Hoagland^{-P} solution were firstly subjected to a repeated-measures ANOVA with REML estimation, where "AMF" and "Diesel" were regarded as fixed factors. "Time" (of sampling, i.e. 0, 9, 21, 42 h) and "Table" (3 different supports in the greenhouse) were regarded as random factors. Each time was secondly analyzed independently using a mixed model, with "AMF" and "Diesel" as fixed factors and "Table" as random factor. The same model was used to analyze P concentration and content in root and shoot, SDW, RDW and TDW and MDA concentration in root and shoot.

Normal distribution of residuals was checked and non-normal data were normalized by logarithm transformation before analysis. After transformation, data remaining non-normal were analyzed by a non-parametric test: the Wilcoxon Mann-Whitney U test, without interaction analysis. Means are followed by the standard error (SE). All the tests were performed using the IBM SPSS statistic 23 software and JMP Pro 12 statistical software (SAS Institute Inc., USA).

Results

Maize plant growth and development

The height of the plantlets was drastically reduced in presence of diesel (Fig. 26A, B). The leaves had reduced surface and were most often yellow and flabby (Figs. 26B). They tended to detach from the stem (Figs 26C, D, E, and F) and were generally tapered at their base (Fig. 26E). The stems were thin, usually wilted and in most case were unable to ensure the upright growth of plants.

No significant difference was noticed in the initial size of the plantlets in the PM and NM treatments with/without diesel before transfer to the perlite substrate (i.e. before addition of diesel), according to the equivalence test ($P = 0.046$). At the end of the experiment (i.e. 25 days), the height of the plantlets in the PM and NM treatments growing in presence of diesel remained almost similar (51.9 ± 1.7 and 51.9 ± 1 cm) comparing to the initial height (50.4 ± 0.9 cm and 49.5 ± 0.8 cm for the PM and NM treatments), while in absence of diesel, the height of the plantlets increased markedly and reached 92.6 ± 0.9 and 87.5 ± 1.3 cm in the PM and NM treatments, respectively. Whatever the treatment, the height of the plantlets growing in presence of diesel was significantly smaller as compared to the plantlets grown in absence of diesel ($P < 0.001$). The presence of AMF did not improve the growth of plantlets as compared to the treatment without AMF association ($P = 0.237$).

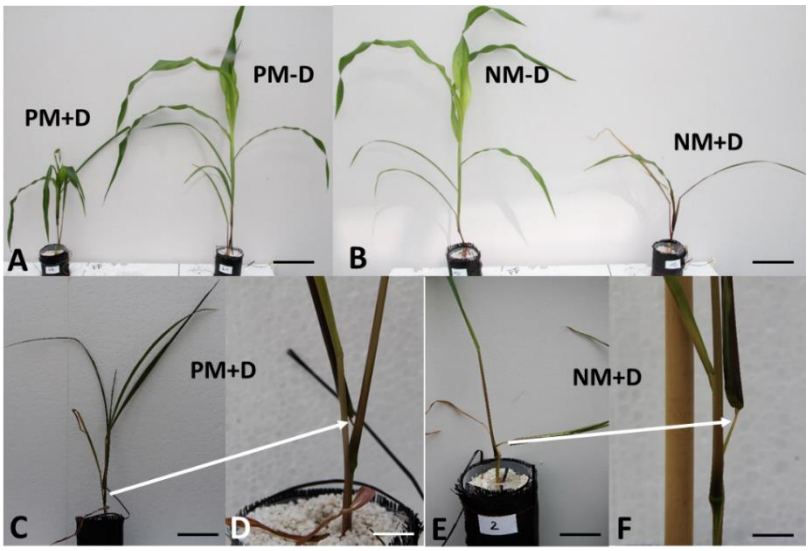
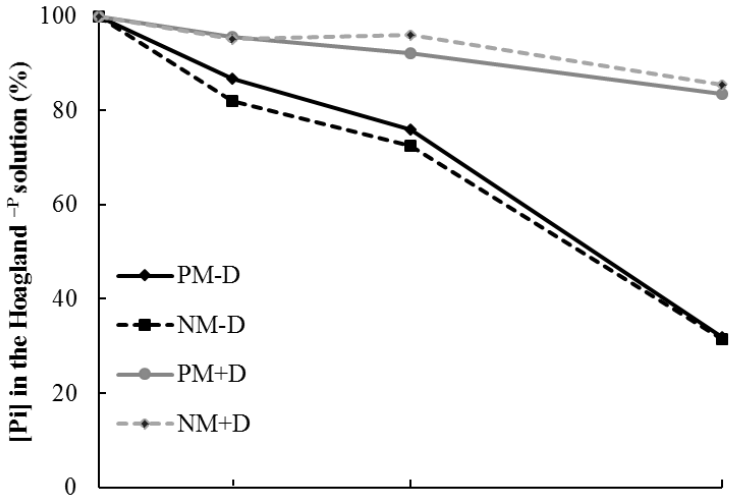


Fig. 26 Impact of diesel on maize plantlets associated (PM) or not (NM) to *R. irregularis* MUCL 41833. (**A)** PM maize leaves show differences in size when grown in presence (PM+D) or in absence (PM-D) of diesel. (**B)** NM maize leaves show differences in size when grown in presence (NM+D) or in absence (NM-D) of diesel. Scale bar: 10 cm (**A, B**). **(C - F)** PM and NM maize plantlets grown in presence of diesel present thinner stem (white arrows) with weakened rigidity, no longer ensuring the upright growth of the plants. Scale bar 5 cm for **C** and **E**. For **D** and **F** closed view of stem. Scale bar: 1 cm.

Percentages of Pi depletion

The % of Pi remaining in the bottles (expressed throughout the text as the % of Pi depletion – see definition in materials and method section) decreased over time for the treatments without diesel demonstrating a depletion of Pi in the circulating solution (Fig. 27) and suggesting a concomitant uptake/immobilization by the plantlet-AMF associates or adsorption on the substrate of the containers. In presence of diesel, the curves of % of Pi depletion were drastically different. The plantlets took up/immobilized significantly lower amounts of Pi as compared to the plantlets grown in absence of diesel (PM-D and NM-D treatments), as demonstrated by the repeated-measures ANOVA ($P < 0.001$, result not shown). Difference in Pi uptake/immobilization by plantlets grown in absence or presence of diesel was thus studied at each time of sampling. At 9 h, a significant effect ($P < 0.001$) of the factor "Diesel" was observed on the % of Pi depletion. Conversely, the factor "AMF" had no significant effect ($P = 0.561$) and no interaction was observed between both factors (Fig. 27). The relative Pi uptake/immobilization in absence of diesel was significantly higher than in presence of diesel (i.e. NM-D and PM-D, 18% and 13.1% respectively; and PM+D and NM+D 4.4% and 4.8% respectively of the initial Pi concentration in the bottle). At 21 h, a significant effect of the factors "Diesel" ($P < 0.001$) was observed on the % of Pi depletion, while no significantly effect was detected on the factor "AMF" and on the interaction between both factors (Fig. 27). As a result, the relative Pi uptake/immobilization in the treatments without diesel was significantly higher as compared to the treatments in presence of diesel (i.e. NM-D: 27.5%; PM-D: 24%; PM+D: 7.8% and NM+D: 4% of the initial % of Pi concentration in the bottle). At 42 h, the % of Pi in the bottles was significantly affected by the factor "Diesel" ($P < 0.001$), while no significant effect was detected for the factor "AMF" or the interaction between both factors (Fig. 27). At the end of the experiment, the relative Pi

uptake/immobilization was significantly larger in absence of diesel (i.e. 68.4% and 68.2% for NM-D and PM-D treatments, respectively) than in presence of diesel (i.e. 14.5% and 16.4% for NM+D and PM+D treatments, respectively).



Effects (p-value)	9 hours	21 hours	42 hours
AMF	0.561	0.296	0.506
Diesel	**<0.001**	**< 0.001**	**< 0.001**
AMF*Diesel	0.453	0.093	0.509

Fig. 27 Short-term Pi depletion analysis (expressed as the percentage of Pi concentration remaining in the bottles at time 9, 21 and 42 h relative to the initial Pi concentration in the bottles at T0) in *Rhizophagus irregularis* MUCL 41833 pre-mycorrhized (PM) or non mycorrhized (NM) maize plantlets grown in presence (+D) or absence (–D) of diesel. Data are presented as means (N=5 for the NM+D; N=6 for the other treatments). For each time, data were analyzed with a linear mixed model with "Table" as random factor ($p \leq 0.05$). Main effects and interaction between the factors: "AMF" and "Diesel" are presented.

Maize harvest

No significant effect of the factor "AMF" was noticed on SDW, RDW and TDW, while a significant effect of the factor "Diesel" was noticed on these three parameters (Table 18). The dry weights were higher for maize plantlets grown in absence of diesel (PM-D and NM-D treatments) as compared to

those grown in presence of diesel (PM+D and NM+D, $P < 0.001$ for SDW, RDW and TDW). Similarly, no significant effect of the factor "AMF" was observed on the shoot and root P concentration and shoot, root and total P content of plants, whereas the factor "Diesel" had a significant effect on shoot ($P = 0.001$) and root ($P < 0.001$) P concentration and on shoot ($P < 0.001$) and total ($P < 0.001$) P content of the plantlets (Table 18). No significant effect of the factor "Diesel" was observed on root P content. No significant effect of the interaction between "AMF" and "Diesel" factors was noticed for any of the parameters evaluated (Table 18).

At the end of experiment, the %TC was significantly higher ($P < 0.05$) in the PM-D plantlets (i.e. 64.6 ± 10.9%) as compared to the PM+D plantlets (i.e. 29.2 ± 6.7%). Interestingly, the relative %TC increased by 6.6% in the plantlets grown in absence of diesel (PM-D) as compared to the initial %TC (i.e. 60 ± 2% determined after 17 days of pre-mycorrhization), although it was not significant ($P = 0.767$). Conversely, the relative %TC significantly decreased ($P < 0.001$) by 48.3% in the plantlets grown in presence of diesel (PM+D). No colonization was observed in NM plantlets.

Whatever the treatment no differences in MDA was observed in shoots (Table 19). Similarly, no effect of the factors "AMF" and of the interaction "AMF x Diesel" was observed on MDA content in roots. To the contrary, a significant effect of the factor "Diesel" was noticed, with higher contents in the roots of the plants in the PM+D and NM+D treatments (Table 19).

Table 18 Shoot dry weight (SDW), root dry weight (RDW) and total dry weight (TDW) dry weights, P concentration (mg g^{-1} of dry weight) and total P content (mg $plantlet^{-1}$) of maize plantlets pre-mycorrhized with *Rhizophagus irregularis* MUCL 41833 and grown in presence (PM+D) or absence (PM–D) of diesel and non mycorrhized (NM) maize plantlets grown in presence (NM+D) or absence (NM–D) of diesel, after 23 days of growth in perlite amended or not with diesel.

	Dry weight (g)			**P concentration (mg g^{-1} of dry weight)**		**P content (mg $plantlet^{-1}$)**		
	Shoot	Root	Total	Shoot	Root	Shoot	Root	Total
PM+D	0.98 ± 0.12	0.35 ± 0.03	1.33 ± 0.14	2.89 ± 0.11	1.93 ± 0.29	2.86 ± 0.45	0.68 ± 0.06	3.54 ± 0.44
NM+D	0.87 ± 0.11	0.33 ± 0.05	1.2 ± 0.15	2.57 ± 0.31	1.9 ± 0.11	2.36 ± 0.6	0.63 ± 0.1	2.99 ± 0.66
PM-D	2.41 ± 0.10	0.6 ± 0.08	3.01 ± 0.17	3.4 ± 0.13	1.38 ± 0.06	8.16 ± 0.24	0.85 ± 0.14	9.01 ± 0.33
NM-D	2.12 ± 0.13	0.53 ± 0.04	2.66 ± 0.15	3.35 ± 0.11	1.43 ± 0.12	7.12 ± 0.49	0.77 ± 0.09	7.89 ± 0.55
Effects (P- values)								
AMF	0.216	0.346	0.174	0.275	0.887	0.442	0.437	0.085
Diesel	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	0.099	**<0.001**
AMF*Diesel	0.86	0.705	0.914	0.424	0.698		0.944	0.573

Data are presented as means ± SE (N=5 for the NM+D; N=6 for the other treatments). Main effects and interactions between the factors "Inoculation" and "Diesel" are presented with significant effect (in bold) and significance values set at $P \leq 0.05$ (mixed model). The values from shoot P content were non-normal after transformations and were thus subjected to a Wilcoxon-Mann Whitney U test, without interaction analysis.

Table 19 Malondialdehyde (MDA) concentration (µmol g^{-1} of fresh weight) in shoot and root system, measured in the pre-mycorrhized (PM) or non mycorrhized (NM) maize plantlets grown in presence (+D) or absence (–D) of diesel after 23 days of growth in an 90% P-impoverished (i.e. Pi = 6.245 mg L^{-1}) Hoagland circulating solution.

	MDA µmol/ g	
	Shoot	Roots
PM+D	18.9±4.043	0.42± 0.031
NM+D	16.1±4.043	0.35±0.035
PM-D	23.8±4.043	0.33±0.035
NM-D	20.4±2.859	0.31±0.027
Effects (P- values)		
AMF	0.2979	0,0951
Diesel	0.4771	**0,0249**
AMF * Diesel	0.9442	0,3029

Data are presented as means ± SE of 3 (PM-D, PM+D, NM+D treatments) and 6 (NM-D treatment) replicates for shoot analysis and 4 (PM+D treatment), 5 (NM-D treatment) and 3 (PM-D, NM+D treatments) replicates for root analysis. Data were analyzed with a mixed model with "Table" as random factor.

Discussion

Plants can acquire their P via root epidermis including root apexes and root hairs when present (i.e. the direct pathway) and via the AMF (i.e. indirect pathway) (Javot *et al.*, 2007). Numerous studies have reported on the role of AMF and mechanisms involved in Pi uptake/immobilization and transport to plants (Smith *et al.*, 2004, 2011; Javot *et al.*, 2007; Zhang *et al.*, 2016; Garcés-Ruiz *et al.*, 2017a), but only a few were conducted in presence of organic pollutants such as diesel. Maize was selected because of its relative tolerance to hydrocarbons (Agbogidi *et al.*, 2007a,b; Njoku *et al.*, 2009b; Oludele & Ogundele, 2013). Similarly, the AMF *R. irregularis*, formerly named *Glomus intraradices*/*irregulare* (Redecker *et al.*, 2013), was chosen for its capacity to colonize extensively maize roots and improve its growth under low Pi

concentration (Mickelson & Kaeppler, 2005; Wright *et al.*, 2005; Tian *et al.*, 2013).

The circulatory semi-hydroponic system with perlite was adequate for growing the non-mycorrhized and AMF-colonized maize plantlets, as well as for measuring short-term % of Pi depletion in the nutrient solution and by corollary Pi uptake/immobilization in the plant systems under diesel stress conditions. Indeed, the plantlets as well as the fungus developed well in the perlite in absence of diesel, under low Pi concentration as already demonstrated by Garcés-Ruiz *et al.* (2017a) with the same system. Maize growth increased by 45% in a period of 25 days. Similarly, *R. irregularis* developed profusely with value of total root colonization reaching 65% at 25 days in absence of diesel.

Nevertheless, the colonization of roots by *R. irregularis* did not result in a significant increase of Pi uptake/immobilization by the PM maize plantlets in absence as well as presence of diesel, contrarily to results previously obtained (Garcés-Ruiz *et al.*, 2017a). It is not excluded that the lower percentage of root colonization (i.e. 65%) obtained in our study as compared to the 90% obtained by Garcés-Ruiz *et al.* (2017a) was the reason, suggesting the necessity for higher colonization percentages for increased Pi uptake via the AMF pathway. Diesel could interfere with the uptake/immobilization of nutrients, especially Pi, due to the osmotic stress caused to the roots by its hydrophobic and lipophilic properties (Ko & Day, 2004; Merkl *et al.*, 2005a; Robertson *et al.*, 2007; Hernández-Ortega *et al.*, 2012). This could explain why a significant decrease in Pi uptake/immobilization was observed in the NM+D and PM+D treatments as compared to the plantlets from the NM-D and PM-D treatments. The absence of difference in P content in roots of AMF plantlets from the PM-D and PM+D treatments could be explained by a lower colonization rate (65% and 24% PM-D and PM+D plantlets), thus resulting in a similar P accumulation in intraradical structures, satisfying first the AMF requirements (Fiorilli *et al.*, 2013), regardless the presence of diesel.

Diesel also impacted root and shoot P concentration in the PM and NM plantlets. Indeed, whereas diesel-stressed roots accumulated more P than non-stressed ones, the contrary was observed in shoot where P concentration was higher in non-stressed treatments. Changes in plant morphology, structure and hydraulic parameters was already observed in plants confronted to the presence of diesel or fluoranthene (PAH) (Hernández-Ortega *et al.*, 2012; Kummerová *et al.*, 2013), which could impact P mobilization inside plant. Phosphorus transporters involved in P translocation from roots to shoots were also possibly inhibited by diesel, resulting in P accumulation in roots.

During the three weeks of exposure to diesel, one maize plantlet died (results not shown) and the height of the other plantlets remained almost the same. Their stems strongly suffered from a lack of turgor and leaves were smaller and almost all discolored. Tang *et al.* (2009) showed that chlorophyll content of maize plantlets was reduced in presence of diesel. They suggested that the fuel might affect the synthesis of chlorophyll enzyme, therefore reducing plants photosynthesis and inhibiting plant growth. In our study, concentration of chlorophyll A, B and carotenoids per mg of shoot remained similar (data not shown). It could thus be expected that the photosynthetic system was not impacted by diesel. On the other hand, diesel induced an oxidative stress in maize roots. Previous studies reported that abiotic stresses, such as metal pollution, salinity, drought and PAHs induced MDA production by plant cells (Mukherjee & Choudhuri, 1983; Sinha *et al.*, 2005; Abad & Jalil, 2007; Debiane *et al.*, 2008, 2009; Bidar *et al.*, 2009). MDA concentration was higher in the roots of the PM+D and NM+D plantlets, whereas no differences were observed in shoot concentration whatever the presence or the absence of diesel. This result reflects the ability of diesel to cause cell membrane damage in root cells in presence as well as in absence of *R. irregularis*. In addition, a membrane alteration could impair cell functioning, and thus explain the disruption in Pi uptake/immobilization as well as P accumulation in diesel-stressed roots. Furthermore, a direct impact of diesel inhibiting nutrients

absorption as mentioned by Ogbo, (2009) and Oludele & Ogundele (2013) could be related to the decrease of AMF colonization in presence of diesel, thus affecting Pi transport from the extraradical AMF to the root cells. Phosphorus transport from the extraradical mycelium to the host plant was previously demonstrated to be affected by various molecules, including fungicides and PAHs (Zocco *et al.*, 2011; Calonne *et al.*, 2014a) and thus probably diesel.

In conclusion, in presence of diesel, plant growth, root colonization and Pi uptake/immobilization rates were drastically decreased. Curiously P concentration in roots was higher in presence of diesel suggesting that translocation from roots to shoot was inhibited/disrupted. The direct exposure of roots to increasing concentrations of diesel induced a reduction in shoot length and shoot or root dry weights, besides a detrimental effect on AMF root colonization. Tong & Karasek (1984) and Rollin *et al.* (2005) concluded in their studies that *Z. mays* is the best plant choice for phytoremediation of petroleum hydrocarbons and diesel. Nevertheless, the authors used lower concentrations (i.e. between 3 and 5%) as compared to our experiment and no AMF were considered. At high concentration of diesel (12.5% in our study), no resistance was observed for maize with or without the AMF. The fungus originating from non-contaminated conditions appeared inappropriate to increase the tolerance/resistance of maize to petroleum hydrocarbons. Probably the couple *Zea mays*/*R. irregularis* MUCL 41833 is not suitable for phytoremediation of diesel. It is not excluded that AMF isolated from polluted soils may be more efficient in plant nutrition and on increasing the resistance/tolerance of the plantlets to the pollutant and thus in phytoremediation strategies. Indeed, AMF species have been detected in oil-polluted soils (Cabello, 1997; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016; Garcés-Ruiz *et al.*, 2017b) that may potentially be efficient in protecting the plants from polluted conditions.

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Chapter 5

Hyphae healing mechanism in *Gigaspora* sp. MUCL 52331 is less affected by diesel pollution than *Rhizophagus irregularis* MUCL 41833

Research paper in preparation for Environmental microbiology

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My contribution to this chapter was approximately 75% and involved experimental design, data collection, statistical analysis of the data and the writing of the manuscript. Co-authors of the chapter have been involved in collection and analysis of the data and revision of the manuscript.

Preface

In **chapter 4**, we evaluated the Pi uptake dynamics of AMF-colonized and non-colonized maize plantlets grown in presence of diesel. A non-destructive circulatory semi-hydroponic cultivation system was set up to determine the Pi depletion from a modified Hoagland solution impoverished in Pi. We observed a detrimental effect of diesel on plant growth and P concentration in shoots, as well as an oxidative stress in cell roots. No significant difference was further observed on Pi uptake or growth development between AMF-colonized or non-colonized plantlets. Moreover, a decrease in root colonization by the AMF (*Rhizophagus irregularis* MUCL 41833) was observed. These results suggested that the AMF used was probably inappropriate (isolated from a non-polluted soil) to improve Pi uptake and transport to plant. Thus, AMF isolated from oil-polluted soils (i.e. as those found in **chapter 1** and **2**) might be more efficient in Pi uptake and transport under oil-polluted conditions as compared to species isolated from natural or non-contaminated environments. This needs to be assessed in the future.

Another essential function of AMF is the capacity of the fungus to repair hyphae after physical injury. This mechanism (i.e. the hyphae healing mechanism – HHM) may differ between AMF species having different life history strategies (LHS). Indeed, most of the species within the *Glomeraceae* and *Gigasporaceae* families belongs to r- or K-strategists, respectively. However, it is totally unknown how the HHM might be influenced by hydrocarbons. Therefore **chapter 5,** aimed to investigate the effects of increasing concentrations of diesel on the HHM in *Rhizophagus irregularis* MUCL 41833 (an r-strategist) and *Gigaspora* sp. MUCL 52331 (a K-strategist) and on the successive steps that contribute to a complete healing, using image analysis.

Abstract

Hydrocarbon pollution is an increasing problem affecting ecosystems. The impact of such pollutants on arbuscular mycorrhizal fungi (AMF) has been shown on several fungal traits (i.e. spore germination…) but never on the capacity of hyphae to heal following injury. However, the hyphae healing mechanism (HHM) is a major process allowing the fungus to maintain its integrity following injury, which may further vary according to the life history strategy (LHS) of the organism. Here, the HHM was monitored *in vitro* on two AMF with contrasting LHS (a r-strategist, *Rhizophagus irregularis* MUCL 41833 and a K-strategist, *Gigaspora* sp. MUCL 52331) under increasing concentrations of diesel (0, 1, 2 and 5%). In both AMF, all the steps concurring to a total healing were slowed-down in presence of diesel suggesting an impact on the process of signal-response between growing hyphal tips (GHTs) at both sides of the injured hyphae. However, in *Gigaspora* sp., the impact was limited. Most hyphae were able to heal as in the controls without diesel. In *R. irregularis*, the HHM was more severely affected. Healing of injured sections was infrequent, as in the control, but the number of GHTs was strongly decreased with increasing concentration of diesel. As a conclusion, in *Gigaspora* sp., the HHM was the most efficient way of maintaining the viability of hyphae under adverse conditions, while in *R. irregularis*, this mechanism was of more limited importance, the fungus being able to reconnect injured hyphae only at low frequencies.

Importance

The impact of hydrocarbon pollutants on arbuscular mycorrhizal fungi (AMF) has been investigated on several traits, but never on the hyphae healing mechanism (HHM). This mechanism is a major process allowing the fungus to maintain its integrity following injury. This mechanism differs according

to the life history strategy (LHS) of the AMF. In K-strategists, it is most often oriented towards the complete recovery of hyphae integrity, while in r-strategists hyphae recovery is less frequent but more often oriented towards disorganized hyphae regrowth. How hydrocarbon-pollutants (i.e. diesel) affect this essential mechanism is totally unknown. Therefore, this study will increase our understanding on the mechanisms developed by AMF to maintain their integrity, following hyphae injury, under hydrocarbon-polluted conditions. This may be of importance to understand the ecology of these microorganisms in polluted soils and more pragmatically for the development and application of AMF inoculants to be applied on polluted soils for phytoremediation.

Introduction

Crude oil pollution deriving from exploration and processing of petroleum is a widespread environmental problem (Vwioko & Omamogho, 2012), whose impacts on fauna and flora is particularly marked and most often results from oil spillages in water and/or soil (Jernelöv & Lindén, 1981; Jernelöv, 2010; Kennedy, 2010). Some emblematic examples are the Exxon Valdez (in 1989) and Deepwater spills (in 2010) known as the two worst accidents in the US, with devastating environmental impacts (Atlas & Hazen, 2011). Oil pollution also influence and alter the water and soil microbial communities (Amadi *et al.*, 1996; Nicolotti & Egli, 1998; Gan *et al.*, 2009; Abha & Singh, 2012; Joye *et al.*, 2014) and may thus affect ecosystem functions and sustainable soil fertility as well as environmental quality (Peng *et al.*, 2015). The toxicity of petroleum molecules is depending on their chemical and physical nature as well as on the time of exposure, way of contamination and susceptibility of the organisms (Abha & Singh, 2012). Interestingly, under oil pollution, a shift of bacterial communities towards the selection for many potentially petroleum-degrading bacteria has been reported (Peng *et al.*, 2015). This result

may provide useful information for bioremediation of petroleum-contaminated soils since the ability of microbial communities to degrade hydrocarbons compounds has been reported in several studies (Kauppi *et al.*, 2011; Faure *et al.*, 2015; Stefani *et al.*, 2015). For instance, the production of natural surfactants by microbial oil degraders that emulsify oil, increasing its bioavailability to the general microbial community has been demonstrated (Joye *et al.*, 2014). More generally, phytoremediation associated to rhizosphere microorganisms has revealed the improvement of degradation of hydrocarbon pollutants (Joner & Leyval, 2001; Merkl *et al.*, 2005b; Gan *et al.*, 2009).

One major key player in the plant rhizosphere is the arbuscular mycorrhizal fungi (AMF). These soil inhabitants form symbiotic associations with an estimate of 80% of plant species (Smith & Read, 2008). They are considered nowadays a crucial, although still enigmatic, component of the plant microbiome (Lanfranco & Young, 2012). These fungi provide the plants with nutrients, mainly phosphorus (P) and nitrogen (N), in exchange for carbon (C). Besides promoting plant growth, AMF sustain other ecological and economic important functions such as improved plant resistance/tolerance to salinity and drought (Plouznikoff *et al.*, 2016) but also to organic pollutants such as polyaromatic hydrocarbons (PAHs) (Leyval & Binet, 1998; Verdin *et al.*, 2006; Debiane *et al.*, 2008, 2009; Lenoir *et al.*, 2017), diesel (Kirk *et al.*, 2005; Tang *et al.*, 2009) and crude oil (Cabello, 1999; Salami *et al.*, 2010; Nwoko, 2014). These microorganisms are thus considered as potential actors in remediation strategies (Lenoir *et al.*, 2016b; Rajtor & Piotrowska-Seget, 2016). Conversely, a number of studies have also reported the impact of oil pollutants (i.e. benzo[a]pyrene, anthracene, phenanthrene and diesel) on the AMF development and physiology (Kirk *et al.*, 2005; Franco-Ramírez *et al.*, 2007; Debiane *et al.*, 2008, 2009; Driai *et al.*, 2015). These authors generally observed a significant decrease in spore germination, hyphal length and root colonization of different *Glomus* species in presence of these pollutants.

A fascinating feature of AMF is the ability of their extraradical mycelium (ERM) to develop through the soil and interconnect individual plants of the same or different species to form 'common mycelial networks' (CMNs) (Simard *et al.*, 2012). These "mycorrhizal webs" are thought to play key roles in ecosystems (Giovannetti *et al.*, 2015). These networks may also result from the interconnection of hyphae belonging to the same or different AMF germlings from the same strains via a mechanism called anastomosis (Giovannetti *et al.*, 1999, 2001, 2004; de Souza & Declerck, 2003; de la Providencia *et al.*, 2005; Voets *et al.*, 2006, 2009; Purin & Morton, 2011) and their survival to environmental stresses (e.g. physical injury) (de la Providencia *et al.*, 2005, 2007) may result from a mechanism known as hyphae healing mechanism (HHM – (de la Providencia *et al.*, 2005)). The HHM is a major process allowing the fungus to maintain its integrity following physical injury. In some Gigasporaceae (e.g. *Scutellospora reticulata*, *Gigaspora margarita*, *Gigaspora rosea*), reconnection of cut hyphae was the principal mechanism of repair, suggesting the presence of specific signals to maintain the viability of the hyphae under adverse conditions (de Souza & Declerck, 2003; de la Providencia *et al.*, 2005). Conversely, in species belonging to the Glomeraceae (e.g. *Glomus intraradices*, *Glomus proliferum*, *Glomus hoi*, *Glomus clarum*), high numbers of new hyphae were produced at both sides of the injured hyphae, capable to reconnect the affected hyphae, other hyphae in the network or to connect neighbor roots (de la Providencia *et al.*, 2005, 2007).

The HHM has mostly been investigated *in vitro* on species belonging to the two AMF phylogenetic groups mentioned above (i.e. Glomeraceae and Gigasporaceae) (de la Providencia *et al.*, 2005, 2007), which were reported to differ in their life-history strategies (LHS) (i.e. r and K strategies or "competitors" and "ruderals" from the Grimes'C-S-R framework (IJdo *et al.*, 2010; Chagnon *et al.*, 2013)). Ecological differences in functional traits between different AMF are of practical importance for plant growth and

ecosystem functioning (van der Heijden & Scheublin, 2007). It is considered that r-strategists or ruderals are more adapted to develop in disturbed environments, in contrast to K-strategists or competitors which are more prone to develop in stable environments (de la Providencia *et al.*, 2005, 2007)

Four-steps, from physical injury to complete fusion with cytoplasmic/protoplasmic flow re-establishment, have been described for the HHM: (a) septum formation, (b) initiation of growing hyphal tips (GHTs), (c) GHT elongation, orientation and contact and (d) GHT fusion and cytoplasmic/protoplasmic flow re-establishment. These four steps differentiated the fungi under study in the Glomeraceae and Gigasporaceae (de la Providencia *et al.*, 2005) suggesting that they have developed different strategies (i.e. r and K strategies) for colony growth to survive under adverse conditions (de la Providencia *et al.*, 2007). However, the full range of functions undertaken by CMNs as well as the environmental factors that may influence/impact their functioning is still poorly understood. For instance, it is totally unknown how hydrocarbon pollutants may affect the HHM of AMF belonging to r and K strategists and thus affect their integrity and functions.

In the present study, *R. irregularis* MUCL 41833 (a r-strategist or ruderal) and *Gigaspora* sp. MUCL 52331 (a K-strategist or competitor) (IJdo *et al.*, 2010; Chagnon *et al.*, 2013) were selected and grown *in vitro* on transformed root organs of chicory. Increasing concentrations of diesel, a hydrocarbon derivate, was applied on injured hyphae. The healing mechanism was monitored and analyzed by video-camera imaging.

Materials and Methods

Biological material

The AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker and Buscot) C. Walker and A. Schüßler as [‘irregulare'] MUCL 41833 and *Gigaspora* sp.

(Gerdemann and Trappe) MUCL 52331 were supplied by the Glomeromycota *in vitro* collection (GINCO - http://www.mycorrhiza.be/ginco-bel) and maintained on Ri T-DNA transformed chicory (*Cichorium intybus* L.) roots clone A4NH (Fontaine *et al.*, 2004) as described by Cranenbrouck *et al.* (2005).

Diesel medium preparation

Four 100 ml bottles (VWR, USA) containing 50 ml of modified Strullu–Romand (MSR) medium (Declerck *et al.*, 1998) supplemented with 3 g L^{-1} GelRite® (Carl Roth, Germany) were sterilized (121°C for 15 min) and maintained at 50°C before use. The bottles contained a stirring magnet. Filtered (0.45 µm Millipore-Merck, USA) diesel from gas station (Q8, Louvain la Neuve, Belgium) was added to the bottles at 1%, 2% or 5% (v:v of MSR medium) concentrations. One bottle without diesel was used as control.

Experimental design

The two AMF strains were grown in Petri dishes (135 mm diam.) in association with transformed chicory roots. The Petri dishes contained 90 mL MSR medium solidified with 3 g L^{-1} GelRite®. Briefly, the Petri dishes were inclined at 5° during filling with the MSR medium so as to ensure a slight slope from one to the other end of the Petri dishes on solidification of the medium. The AMF were associated to chicory roots in the thicker part of the MSR medium. Roots tended to grow preferentially in this part, while the hyphae developed more easily in the thinner part of the MSR medium facilitating the microscopic observations. The Petri dishes were incubated in an inverted position at 27°C in the dark.

After one-month of incubation, four Petri dishes of each AMF were selected. In each of them, between 6 and 12 runner hyphae showing a dense

cytoplasmic/protoplasmic flow under binocular microscope (Olympus SZ2-ILST, Japan) and growing close to the surface of the medium were carefully chosen and labeled by numbers. The selected hyphae were sufficiently distant from each other to avoid as much as possible interferences. The hyphae were gently cut with a sterile scalpel under binocular microscope at 6.7x or 40x magnification. Twenty µL of MSR medium maintained at 40°C without diesel (i.e. the Control) or containing 1%, 2% or 5% diesel was added at the place of cutting. For each plate (i.e. one per treatment), all cuttings were done at the same time. One cutting and addition of diesel in a single plate took approx. one minute. The experiment was repeated threefold independently.

Monitoring of hyphae healing mechanism

The cut hyphae were observed cautiously at regular intervals under a dissecting bright-field light microscope (Olympus BH2–RFCA, Japan) at 100x or 200x magnification. During the first 60 min following cutting, the hyphae in each Petri dish were observed following the order of cutting, then each hour until 16 h and finally at 24, 30, 36 and 48 h.

Images of growing hyphal tips (GHTs) were captured with a digital camera (Canon EOS 60D) coupled to a compound bright-field light microscope and displayed on a 15-inch of HP Compaq nc6320 screen to reconstitute the sequence of hyphal healing mechanism (HHM) using the image manager software EOS Utility – Canon USA Inc.

Data collection

Following cutting, the time to septum formation and GHT initiation at one or both extremities of the cut hyphae, contact and fusion between GHTs and subsequent cytoplasmic/protoplasmic flow were measured (de la Providencia *et al.*, 2005). In addition, the number of new GHTs from cut hyphae as well as the number of ramifications (i.e. branches that were produced from the

GHTs) was enumerated. Cut hyphae which became empty and showed multiple retraction septa were also recorded. Finally, the timing for hyphae to heal was measured and compared between the two strains.

Statistical analysis

The impact of diesel on the percentage of hypha with septum formation, GHTs initiation and contact with or without cytoplasm/protoplasm flow re-establishment, ramification and cut hyphae showing multiple retraction septa after cutting was analyzed for each experiment independently or pooled over the three experiments. Data were subjected to Chi-Square test. Normal distribution was checked and non-normal data were normalized by logarithm transformation before analysis. After transformation, data remaining non-normal were analyzed by a non-parametric test. The Kruskal-Wallis test was used to compare the timing of the different steps in the healing process. Results are presented as mean values followed by the standard errors. All the tests were performed using the IBM SPSS statistic 24 software.

Results

Hyphae healing mechanism in *Gigaspora* sp. MUCL 52331 under increasing concentrations of diesel

The experiment was conducted three times independently. However, no significant differences ($P > 0.05$) were noticed for any parameter between the Controls (data not shown). It was thus decided to combine all the data from the three independent experiments for analysis.

Whatever the treatment, cytoplasmic/protoplasmic loss was noticed at both extremities of the cut hyphae with the formation of a plug at one or both cut ends (Fig. 28A). This was followed by the rapid formation of a septum in above 70% of the cut hyphae, without significant difference between the

treatments (Table 20). The damaged section ahead the septum changed in color turning darker (Fig. 28A). The emergence of GHTs was always initiated behind the septum (Fig. 28A) or distant from the cut end when septum was not observed. Whatever the treatment, no significant difference was noticed in the % of hyphae showing at least one GHT emergence, which in all cases exceeded 80% (Table 20). Conversely, the % of GHT formed at both sides of the cut ends significantly differed between the treatments. It was significantly lower in the diesel 5% treatment as compared to the three other treatments (Table 20). No significant difference was further noticed between the Control, diesel 1% and diesel 2% treatments (Table 20). The number of GHTs per cut hyphae varied from 2.71 ± 0.33 in absence of diesel to 2.59 ± 0.38, 3.28 ± 0.41 and 2.06 ± 0.51 GHT in presence of 1%, 2% and 5% diesel, respectively. The % of cut hyphae showing GHTs contact (Fig. 28 A) as well as the % of GHTs contact without visible fusion did not differ between treatments (Table 20). Similarly, no difference between treatments was observed in the % of fused GHTs showing cytoplasmic/protoplasmic flow (Table 13, Fig. 28B). The % of ramifications on GHTs (Fig. 28C) and the % of non-fused GHTs with retraction septa were also enumerated at 48 hours (Table 20). For both parameters, no significant differences were noticed between treatments (Table 20). In all treatments, the shape of the GHTs and ramifications were wavy or straight. The number of ramifications on GHTs were 3.67 ± 1.92, 2.09 ± 1.13, 2.28 ± 0.83 and 2.33 ± 1.16 per cut hyphae in the Control, diesel 1% 2% and 5% treatments, respectively. However, in some rare cases high numbers of short ramifications (up to 33 in one single case) were noticed. In one single case of the Control and diesel 5% treatments, a ramification of a GHT fused with a non-injured hypha from the hyphal network and cytoplasmic/protoplasmic flow was observed.

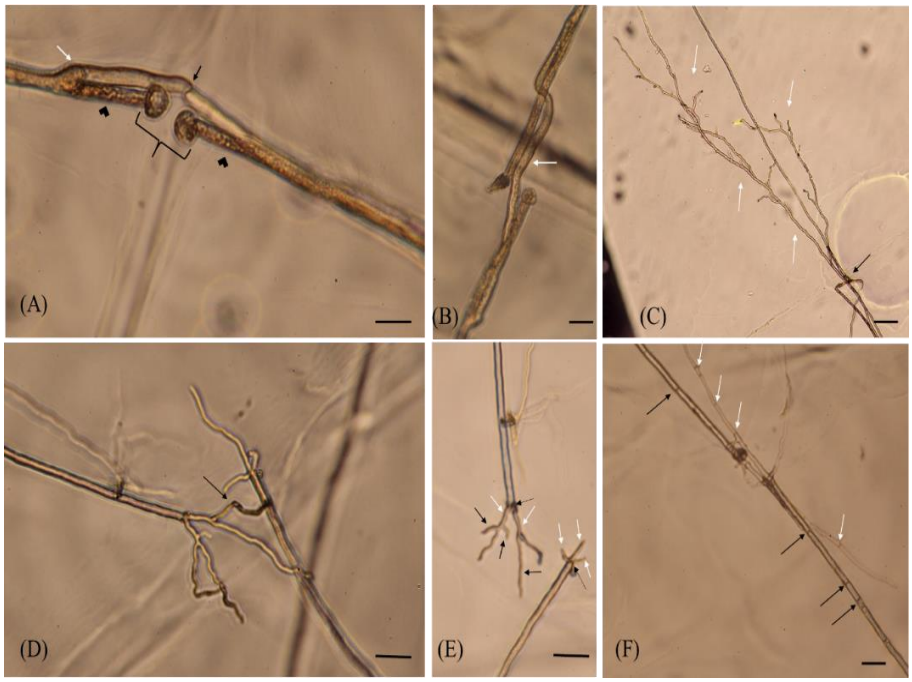

Fig. 28 Impact of diesel on the hyphae healing mechanism in *Gigaspora* sp. MUCL 52331 or *Rhizophagus irregularis* MUCL 41833. From A – C *Gigaspora* sp. **(A)** At 5% diesel concentration. Plugs were formed at both extremities in front of the cut ends (brace). The cut ends behind the plug changed color turning darker (arrowheads). GHTs emergd at both extremities of cut ends (white arrow) and contacted (black arrow) (scale bar 50 µm). **(B)** At 1% diesel concentration. GHT contact and fusion (white arrow) (scale bar 50 µm). **(C)** Control treatment. Development of different size of ramifications (white arrows) from one side of cut hyphae (black arrow) at the end of the experiment (scale bar 100 µm). From D – F *Rhizophagus irregularis*. **(D)** Control treatment. GHTs contact and fusion following cutting. **(E)** Septa formation after cutting with no plug formation (discontinued arrow). GHTs emerging (white arrows) through the cut end and ramification (black arrows) development from GHTs. **(F)** Control treatment showing hyphae (black arrows) and GHTs (white arrows) with septa formation (Scale bar 50 µm).

Table 20 Hyphal healing in *Gigaspora* sp. MUCL 52331 in absence (Control) or presence of 1% (Diesel 1%) 2% (Diesel 2%) or 5% (Diesel 5%) diesel added at the place of cutting.

	***Gigaspora* sp. MUCL 52331**					
	Parameter	**Control**	**Diesel 1%**	**Diesel 2%**	**Diesel 5%**	**Chi^2**
1	% of hyphae showing septum formation at both sides of cut ends	95.8%±4.3	86%±1.7	87.5%±7.2	72.7%±3.8	P= 0.215
2	% of hyphae showing at least one GHT emergence at one side of cut ends	90.5%±9.7	84.9%±8.4	100%±0	83%±1.7	P = 0.365
3	% of hyphae showing at least one GHT at both sides of cut ends	90.5%±9.7 **a**	80.2%±12.5 **a**	72.2%±16.0 **a**	51.3%±15.1 **b**	**P < 0.05**
4	% of GHT contact**s**	96%±4	66.7%±33.0	87.7%±7.2	59%±4.9	P = 0.051
5	% of GHT contact**s** without fusion	100%	16.7%	22%±11	22%±11	P = 0.212
6	% of GHT contacts with fusion and cytoplasm/protoplasm flow	100% ±0	90%±10	78.6%±14.9	80.6%±9.9	P = 0.107
7	% of GHT showing ramifications	44%±12.7	30% ± 15	55.6 %± 15.5	25% ±14	P = 0.293
8	% of non-fused GHT**s** showing retraction septa	0%	14.5%±14.5	33.3%±33.3	8.3%±8.3	P = 0.738

Data for parameter 1 and 2, are percentage of 21 (Control treatment), 22 (Diesel 1% treatment) and 18 (Diesel 2% and 5% treatments) replicates. Data for parameters 3, 4 and 7 are percentage of 19 (Control and Diesel 1% treatments), 18 (Diesel 2% treatment) and 15 (Diesel 5% treatment) replicates. Data for parameter 5, are percentage of 1 (Control treatment), 6 (Diesel 1% and Diesel 2% treatments) and 8 (Diesel 5% treatment) replicates. Data for parameter 6, are percentage of 18 (Control treatment), 14 (Diesel 1% treatment) 16 (Diesel 2% treatment) and 9 (Diesel 5% treatment) replicates. Data for parameter 8, are percentage of 3 (Control treatment), 9 (Diesel 1% treatment) 6 (Diesel 2% treatment) and 11 (Diesel 5% treatment) replicates. Data are mean values and ± standard error. Values in a same row followed by different letter differed significantly (P <0.05, chi square test).

The HHM started with the formation of septa behind the cut ends and emergence of GHTs. The time required for septum formation was significantly shorter in the Control treatment as compared to the diesel 1% and 5% treatments ($P < 0.05$), while no difference was noticed with the diesel 2% treatment ($P = 0.052$) (Fig. 29A). No significant differences were further noticed between the diesel 1%, 2% and 5 % treatments (Fig. 29A). Similarly, the time needed for GHT emergence was significantly shorter in the Control treatment as compared to the other treatments, that did not differ among them ($P < 0.05$) (Fig. 29B). Following the first GHT emergence, a second GHT was generally formed on the other end of the cut hyphae. The time needed for GHTs to contact was significantly shorter in the Control treatment as compared to the diesel 2% and 5% treatments ($P < 0.001$) (Fig. 29C), while no significant difference was noticed with the diesel 1% treatment ($P = 0.056$). No significant differences were further observed between the three diesel treatments (Fig. 29C). Whatever the treatment, a complete healing with cytoplasmic/protoplasmic flow was observed between two GHTs or very rarely between one GHT and a PEG (i.e. a very short ramification that did not develop further). Despite a high percentage of GHTs contact in all treatment, the timing of cytoplasmic/protoplasmic flow in the fused hyphae differed between treatments. It was significantly shorter in the Control treatment as compared to the diesel 2% and 5% treatments ($P<0.05$), while no difference was noticed with the diesel 1% treatment ($P = 0.259$) (Fig. 29D). No significant differences were further observed between the diesel 1%, 2% and 5 % treatments. Arrest of cytoplasmic/protoplasmic flow was observed a few times following complete healing. The time for observing empty hyphae with multiple septa after cutting was not significantly different ($P=0.34$) between the treatments (Fig. 29E).

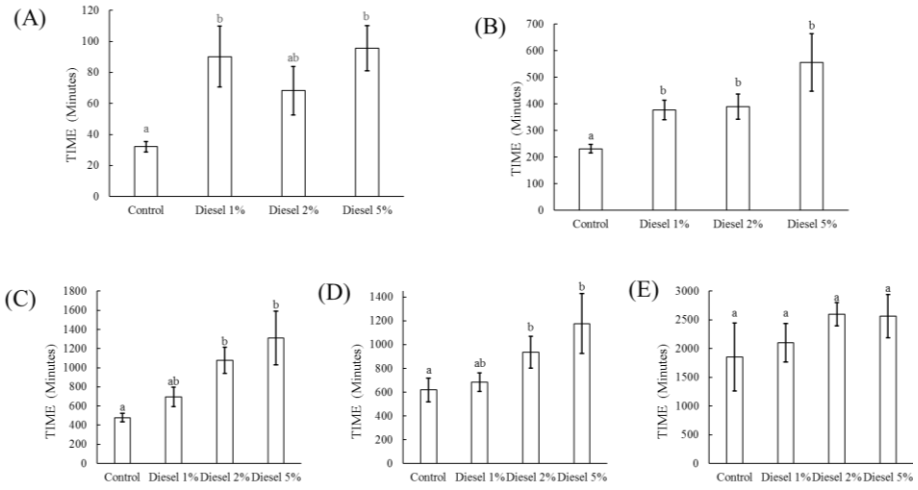

Fig. 29 Time to septum formation **(A)**, emergence of first GHT **(B)**, time to GHTs contact **(C)**, time to cytoplasmic/protoplasmic flow in fused GHT **(D)** and time to retraction septa in cut hyphae **(E)** of *Gigaspora* sp. MUCL 52331 in absence (Control) or presence of 1% (Diesel 1%), 2% (Diesel 2%) or 5% (Diesel 5%) diesel added at the place of cutting**.** Data are mean values and SE of 21 (Control and Diesel 1% treatments), 18 (Diesel 2% treatment) and 16 (Diesel 5% treatment) replicates. Zero values from data were excluded. Data were analyzed with non-parametric test Kruskal Wallis and all pairwise multiple comparisons. Different letters indicate significant differences between treatments ($P < 0.05$).

Hyphae healing mechanism in *R. irregularis* MUCL 41833 under increasing concentrations of diesel

Similar to *Gigaspora* sp., the experiment with *R. irregularis* was conducted three times independently. No significant differences were noticed for any parameter between the Control of the three experiments ($P > 0.05$) (data not shown) and data were thus combined.

Whatever the treatment, cytoplasmic/protoplasmic loss was noticed at both extremities of the cut hyphae. The formation of a plug was rarely observed (Fig. 28E). The percentage of septa formation in the cut hyphae was above 33% with a significant difference between the treatments ($P < 0.001$) (Table 21). It was significantly higher in the Control treatment as compared to the

three other treatments. No difference was further observed between the three diesel treatments. The GHT most often emerged through the septum (Fig. 28D, E) or through the lumen of the cut hyphae when septum was not apparent or present. The percentage of GHTs formed at one ($P < 0.05$) or both extremities ($P < 0.001$) of the cut ends significantly differed between the treatments (Table 21). In both cases, the % GHT was significantly higher in the Control treatment as compared to the three other treatments. No difference was observed between the diesel treatments in the % GHT formed at one or both sides of the cut hyphae (Table 21). The number of GHTs developed in the Control treatment was 3.33 ± 0.45 and was 1.5 ± 0.33, 2 ± 0.49 and 1.55 ± 0.21 in the diesel 1%, 2% and 5% treatments, respectively. The % of hyphae showing GHTs contact and GHTs contact without fusion significantly differed between the treatments (Table 21). It was significantly higher in the Control treatment as compared to the diesel 1%, 2% and 5% treatments. No significant differences were observed between the three diesel treatments (Table 21). The % of fused GHTs showing cytoplasmic/protoplasmic flow did not differ between treatments (Table 21). The % of ramification from GHT as well as the % of non-fused GHT showing retraction septa (Fig. 28F) was enumerated at 48 h. For both parameters, significant differences were noticed between treatments. The Control treatment presented a higher % of ramifications as compared to the diesel 1% and 5% treatments, while no difference was observed with the diesel 2% treatment (Table 21). No difference was observed for hyphae showing retraction septa between the diesel treatments (Table 21). In all treatments, GHTs and ramification shapes were wavy or straight. The number of ramifications on GHTs per cut hyphae was 2.13 ± 0.53 in the Control treatment and 1.39 ± 0.77, 0.47 ± 0.24 and 0.48 ± 0.45 for the diesel 1%, 2% and 5% treatments, respectively. However, in some rare cases, up to 18 ramifications per cut hyphae were noticed. During the 48 h of observation, no contact or fusion was noticed between the ramifications or between a ramification and a GHT or another non-injured hypha.

The HHM started with the formation of septa at one or both cut ends followed by the emergence of GHTs. No difference in time needed for septum formation was found between treatments ($P = 0.156$) (Fig. 30A). To the contrary, the time needed for GHT emergence was significantly shorter in the Control treatment as compared to the other treatments, that did not differ among them ($P < 0.01$) (Fig. 30B). After the first GHT emergence, a second GHT was generally formed at the other end of the cut hyphae. The time needed for GHT contact was significantly shorter in the Control treatment as compared to the diesel 2% and 5% treatments ($P < 0.001$) (Fig. 30C), while no significant difference was noticed with the diesel 1% treatment ($P = 0.056$). No significant differences were further observed between the three diesel treatments. Healing with cytoplasmic/protoplasmic flow was observed with no significant difference between treatments ($P = 0.162$). Nevertheless, the number of cut hyphae showing complete healing was very few per treatment (Fig. 30D). Empty hyphae showing multiple septa after cutting were observed without significant difference ($P = 0.143$) between treatments (Fig. 30E).

Table 21 Hyphal healing in *Rhizophagus irregularis* MUCL 41833 in absence (Control) or presence of 1% (Diesel 1%) 2% (Diesel 2%) or 5% (Diesel 5%) diesel added at the place of cutting.

	***R. irregularis* MUCL 41833**					
	Parameter	**Control**	**Diesel 1%**	**Diesel 2%**	**Diesel 5%**	**Chi^2**
1	% of hyphae showing septum formation at both sides of cut ends	85.7%±8.3 **a**	33.8%±9.2 b	43.3%±23.3 b	35.2%±11.5 b	**P < 0.001**
2	% of hyphae showing at least one GHT emergence at one side of cut ends	100%±0 **a**	75.1%±19.7 b	68.3%±22.4 b	82.4%±12.5 b	**P < 0.05**
3	% of hyphae showing at least one GHT at both sides of cut ends	82.4%±5.78 **a**	27.1%±12.1 b	47.8%±24.74 b	51.9%±14.57 b	**P < 0.001**
4	% of GHT contact**s**	55%±16.6 **a**	29.3%±10.9 b	5.7% b	12.7%±12.7 b	**P < 0.05**
5	% of GHT contact**s** without fusion	51%±15.1 **a**	20%±6.5 b	0% b	9.7%±9.7 b	**P < 0.001**
6	% of GHT contacts with fusion and cytoplasm/protoplasm flow	17.7%±9.5	33%±16.7	100%	33%	P = 0.430
7	% of GHT showing ramifications	55%±16.6 **a**	29.3%±15.1 b	30.7%±22.7 **ab**	8.3%±8.3 b	**P < 0.05**
8	% of non-fused GHT**s** showing retraction septa	89%±11 **a**	31%±10 b	36.7%±8.8 b	21%±21 b	**P < 0.05**

Data for parameter 1 and 2, are percentage of 24 (Control treatment), 28 (Diesel 1% treatment), 15 (Diesel 2% treatment) and 29 (Diesel 5% treatments) replicates. Data for parameters 3, 4 and 7 are percentage of 24 (Control treatment), 20 (Diesel 1% treatment), 11 (Diesel 2% treatment) and 23 (Diesel 5% treatments) replicates. Data for parameter 5, are percentage of 21 (Control treatment), 18 (Diesel 1% treatment), 10 (Diesel 2% treatment) and 22 (Diesel 5% treatments) replicates. Data for parameter 6, are percentage of 13 (Control treatment), 5 (Diesel 1% treatment), 1 (Diesel 2% treatment) and 3 (Diesel 5% treatments) replicates. Data for parameter 8, are percentage of 21 (Control treatment), 26 (Diesel 1% treatment) 14 (Diesel 2% treatment) and 28 (Diesel 5% treatment) replicates. Data are mean values ± standard error. Values in a same row followed by different letter differed significantly (P <0.05, chi square test).

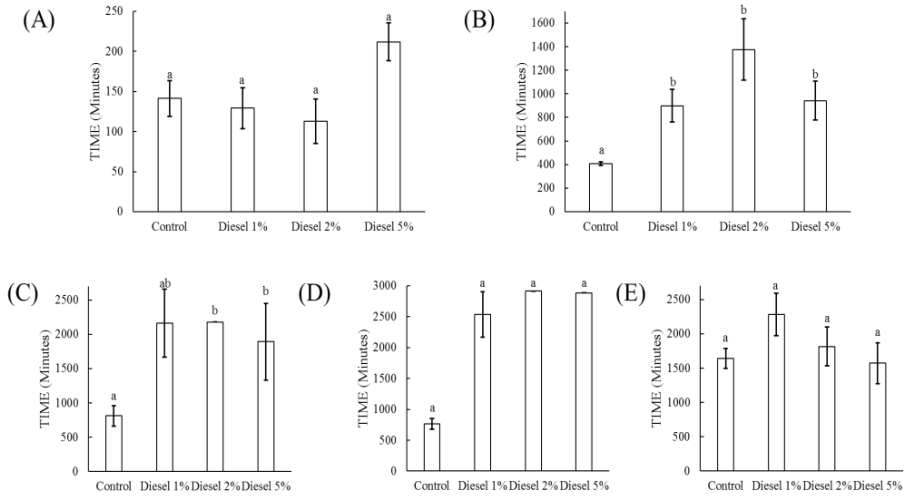

Fig. 30 Time to septum formation **(A)**, emergence of first GHT **(B)**, time to GHTs contact **(C**) time to cytoplasmic/protoplasmic flow in fused GHT **(D)** and time to retraction septa in cut hyphae **(E)** of *R. irregularis* MUCL 41833 in absence (Control) or presence of 1% (Diesel 1%), 2% (Diesel 2%) or 5% (Diesel 5%) diesel added at the place of cutting. Data are mean values and standard errors of 24 (Control treatment), 21 (Diesel 1% treatment), 13 (Diesel 2% treatment) and 23 (Diesel 5% treatment) replicates. Zero values from data were excluded. Data were analyzed with non-parametric test Kruskal-Wallis and all pairwise multiple comparisons. Different letters indicate significant differences between treatments ($P < 0.05$)

Comparison of hyphae healing mechanism between *Gigaspora* sp. MUCL 52331 and Rhizophagus irregularis MUCL 41833 in presence of increasing concentrations of diesel

Following hyphae cutting, *Gigaspora* sp. (whatever the concentration of diesel) generally formed a plug at cut ends to circumvent cytoplasmic/protoplasmic loss, while *R. irregularis* rarely formed plugs. Both strains developed GHTs bearing sometimes short hyphae ramifications (Fig. 28A-F). A higher number of GHTs and ramifications formation was noticed

in *Gigaspora* sp. compared to *R. irregularis* in presence of diesel. Septum formation in the Control, diesel 2% and diesel 5% treatments was significantly ($P < 0.001$, $P < 0.05$ and $P < 0.05$, respectively) faster in *Gigaspora* sp. than in *R. irregularis*, while no significant difference was noticed for the diesel 1% treatment ($P = 0.232$) (Fig. 31A). The emergence of GHTs also differed between the two strains. Whatever the treatment, the emergence of GHTs was significantly faster ($P < 0.001$) in *Gigaspora* sp. as compared to *R. irregularis* (Fig. 31B). The GHT contact in the Control as well as in diesel 1% treatments of *Gigaspora* sp. was significantly faster ($P < 0.05$) than in *R. irregularis*, while no differences were noticed between both strains in the diesel 2% and 5% treatments (Fig. 31C). Time to total healing did not differ between the Control, 2% and 5% diesel treatments ($P = 0.120$), while in the diesel 1% treatment the time to total healing was significantly ($P < 0.05$) faster in *Gigaspora* sp. than in *R. irregularis* (Fig. 31D). Cytoplasmic/protoplasmic retraction with septa formation did not significantly differ between the two strains, whatever the treatment ($P = 0.063$) (Fig. 31E).

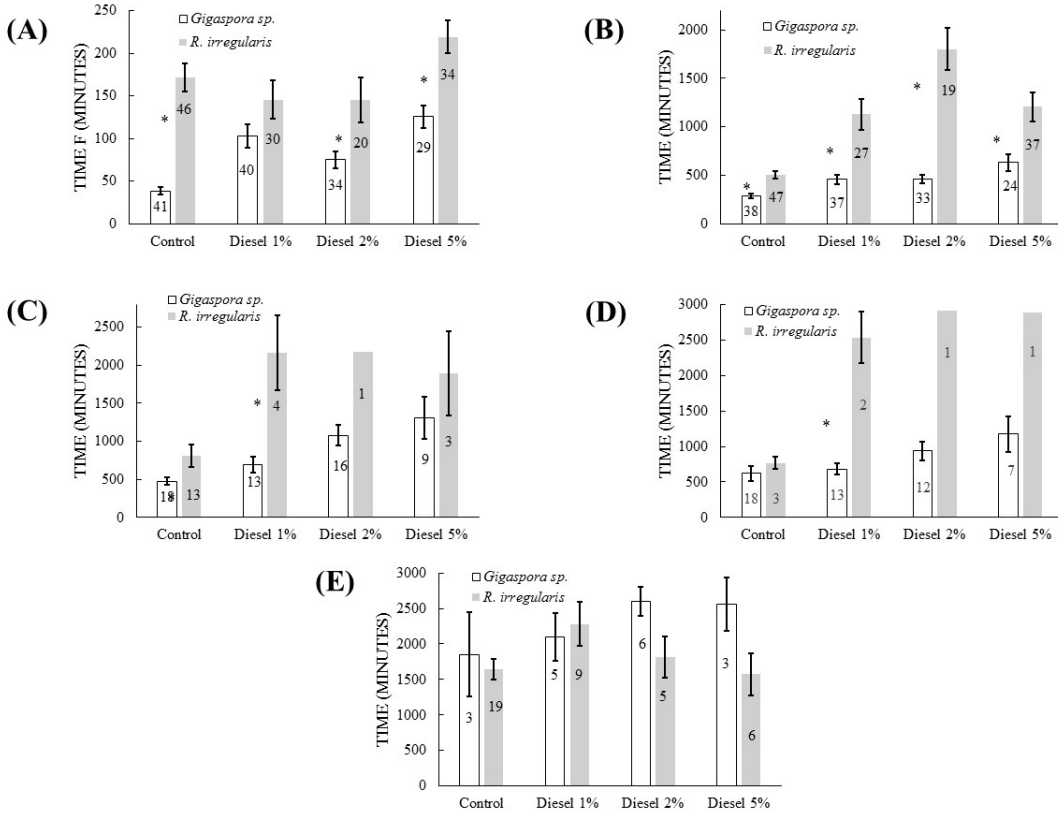

Fig. 31 Comparison of hyphal healing mechanism, time to septum formation **(A)**, emergence of first GHT **(B)**, time to GHTs contact **(C)** time to cytoplasmic/protoplasmic flow in fused GHT **(D)** and time to retraction septa in cut hyphae **(E)** between *Gigaspora* sp. MUCL 52331 and *R. irregularis* MUCL 41833 in presence of different concentrations of Diesel (0%, 1%, 2% and 5%). Data are mean values and standard errors of different number of replicates (see histograms) for Control treatment, Diesel 1% treatment, Diesel 2% treatment and Diesel 5% treatment. Zero values from data were excluded. Data were analyzed with non-parametric test Kruskal Wallis and all pairwise multiple comparisons. Asterisk (*) indicate significant differences between AMF within treatments ($P < 0.05$).

Discussion

Fungal mycelium can experience various forms of disturbance (e.g. loss of functional biomass due to physical injury of the mycelium), either caused by soil fauna, soil freezing, wetting-drying cycles or anthropogenic factors (Chagnon, 2014). To survive under adverse conditions, AMF have developed several mechanisms, among which the HHM allowing the fungus to repair hyphae damaged by physical injuries. The impact of anthropogenic pollution (e.g. the spillage of diesel) on this mechanism in AMF differing in LHS is totally unknown. Although it is well established that the release of petroleum pollutants in soils affect the AMF network (Kirk *et al.*, 2005; Tang *et al.*, 2009; Hernández-Ortega *et al.*, 2012; Trejo *et al.*, 2013; Driai *et al.*, 2015), endangering the integrity and development of the colony and thus its potential benefits to plants. In the present study, the effects of diesel on two AMF with different LHS, *Gigaspora* sp. MUCL 52331 (a K-strategist or competitor) and *R. irregularis* MUCL 41833 (a r-strategist or ruderal), were evaluated under *in vitro* culture conditions. Increasing concentrations of diesel (i.e. 0%, 1%, 2% and 5%) were applied on cut hyphae and HHM monitored by camera imaging.

Rhizophagus irregularis and *Gigaspora* sp. strongly differed in their response to diesel application. *Gigaspora* sp. seemed to be more tolerant with no major impact observed on the different steps of the HHM, unlike *R. irregularis* for which all the steps involved in the mechanism of repair were affected. However, for both fungi, the time needed for complete healing with cytoplasmic/protoplasmic flow re-establishment was strongly delayed in presence of diesel, notably at the two highest concentrations (i.e. 2% and 5%).

Septum formation was the first step following hyphae cut. For both AMF strains, septum formation was observed a few minutes after injury, at the cut ends for *R. irregularis* and some µm behind the cut ends for *Gigaspora* sp., as

previously observed by de la Providencia *et al.* (2005). Following cutting, a plug of cytoplasm/protoplasm material was nearly always observed at the cut ends in *Gigaspora* sp., while this was not the rule in *R. irregularis.* It is supposed that this plug prevented further loss of cytoplasmic/protoplasmic material. In presence of diesel, septum formation was not affected in *Gigaspora* sp. Indeed, the % of hyphae with septum formation was above 72% whatever the concentration of diesel and did not differ significantly from the Control treatment. Conversely, it was significantly impacted in *R. irregularis*. The % of hyphae with septum formation never exceeded 44% whatever the concentration of diesel and was significantly lower as compared to the Control treatment. For both AMF strains, the time to septum formation was delayed in presence of the pollutant, at the concentrations of 1% and 5% of diesel for *Gigaspora* sp. and at all concentrations for *R. irregularis.* Overall, the septum formation was faster for *Gigaspora* sp. than for *R. irregularis*.

Following septum formation, GHTs emerged at one or both cut ends of the injured hyphae, behind the septum for *Gigaspora* sp. and most often through the septum or lumen of the cut hyphae for *R. irregularis* as earlier observed by de la Providencia *et al.* (2005). In *Gigaspora* sp., all cut hyphae showing one or more GHT emergence at one cut ends presented also at least one GHT at the other cut ends, even if the second GHT most often appeared later. This was not the case for *R. irregularis* for which the emergence of a GHT at one cut end was not necessarily accompanied by the emergence of a GHT at the other cut end. In presence of diesel, the capacity to produce one GHT at one side of the cut ends was not affected in *Gigaspora* sp. Indeed, it was above 83% whatever the concentration of diesel and did not differ from the Control treatment. Conversely, the % of GHT at one cut end was drastically impacted in *R. irregularis*. With this fungus, the production of at least one GHT per cut end decreased by ~ 25%, 32% and 18% as compared to the Control treatment in presence of 1%, 2% and 5% of diesel, respectively. The production of at least one GHT at both side of the cut end was affected for both AMF strains.

In *Gigaspora* sp., it was decreased by 44% at the highest diesel concentration as compared to the Control treatment, while for *R. irregularis*, it was decreased by ~ 67%, 42% and 37% as compared to the Control treatment in presence of 1%, 2% and 5% of diesel, respectively. Similarly, the % of ramification per GHT decreased in *R. irregularis* whatever the diesel concentration as compared to the Control treatment, thus decreasing the chance to meet ramification of a neighbor GHT or other hypha from the network. Conversely, this % remained similar whatever the treatment for *Gigaspora* sp.

For both AMF strains, the emission of GHT was delayed in presence of diesel at the three concentrations tested. Even though, it was faster in *Gigaspora* sp. than in *R. irregularis*, in absence as well as in presence of diesel.

The majority of GHTs at both cut ends in *Gigaspora* sp. grew toward each other as previously described by de la Providencia *et al.* (2005) and in most of the cases (i.e. 96%, 67%, 88% and 59% for the control, diesel 1%, 2% and 5%, respectively) entered into contact. This process seemed not affected by diesel with this fungus. To the contrary, only 55% of the GHTs in *R. irregularis* entered into contact in absence of diesel and this percentage decreased by ~ 46%, 90% and 77% as compared to the Control treatment in presence of 1%, 2% and 5% of diesel. Although there was no significant difference between the treatments within *Gigaspora* sp., the decrease observed at the highest concentration with this fungus, and at all concentrations for *R. irregularis*, could be related to the impact of diesel on signal molecules emitted by GHTs in a sequence of dose-response. Indeed, de la Providencia *et al.*(2007) observed that long distances (>5000 µm) separating extremities of cut hyphae drastically decreased the capacity of GHTs to reconnect in *Scutellospora reticulata* (a Gigasporaceae), while it was even absent in *Glomus clarum* (a Glomeraceae), and it was maximum at short distances for *S. reticulata* (de la Providencia *et al.*, 2007). This suggested that

the growth of GHTs towards each other was due to the elicitation of specific diffusible substances in a sequence of signal response (de la Providencia *et al.*, 2005), which may be diluted at too long distances to initiate the mechanism. In our experiment, the distance between cut sections was short but it is not excluded that the emission of these signal molecules may have been impacted/inhibited by diesel or possibly masked in the medium by the pollutant, reducing the capacity of GHT to grow toward each other at the higher diesel concentrations for *Gigaspora* sp. and at all concentrations for *R. irregularis*. The presence of diesel thus seemed to decrease the chance of perception of signals emitted by GHT, particularly with *R. irregularis*. This was further supported by the time needed for GHT contact in both strains, which was strongly delayed in presence of diesel 2% and 5%. Interestingly, whereas a significant difference between both AMF strains was observed for the time to contact in absence of diesel, this difference disappeared in presence of the two highest concentrations of diesel further supporting the impact of the pollutant on GHT orientation and contact.

Contact of GHTs was always followed by GHT/GHT or GHT/PEG fusion and cytoplasmic/protoplasmic flow re-establishment in *Gigaspora* sp. In *R. irregularis*, GHTs contacts were also observed, which in 18% of the cases (i.e. 3 cuts out of 13 which showed GHT contact) resulted in cytoplasmic/protoplasmic flow re-establishment in absence of diesel. In presence of diesel only 2 cuts out of 5 and only 1 out of 3 resulted in cytoplasmic/protoplasmic flow re-establishment for diesel 1% and 5%, respectively. For diesel 2% only one cut showed GHT contact and resulted in cytoplasmic/protoplasmic flow re-establishment. With this fungus, a pre-contact interaction between the GHT hyphal tips, especially in the Control treatment (51%) was observed, probably via a remote signaling process (Novais *et al.*, 2017), but ended by overlapped GHTs without further fusion. Anastomosis is a complex process for which incompatibility interactions have been described (Novais *et al.*, 2017). Sbrana *et al.*(2011) classified hyphal

contacts into four categories: (1) Perfect fusion (i.e. hyphae anastomosed with cytoplasmic/protoplasmic flow verified), (2) Pre-fusion incompatibility (i.e. morphological changes in the hyphae tips with cytoplasmic/protoplasmic retraction and septa formation); (3) Post-fusion incompatibility (i.e. protoplasm withdrawal occurred after anastomosis) and (4) Hyphal contacts (i.e. close contact with no fusion and no morphological changes). This last category was often observed in our experiment with *R. irregularis* when GHTs from both sides of the cut hyphae contacted without fusion (e.g. Control, diesel 1% and 5% treatments).

The time for cytoplasmic/protoplasmic flow re-establishment in fused GHT was delayed for *Gigaspora* sp. at the concentrations of 2% and 5% of diesel as compared to the Control treatment. This was not evidenced for *R. irregularis*, even if the capacity to restore flow after fusion was faster for *Gigaspora* sp. than *R. irregularis* with increasing concentrations of diesel.

Curiously, in *Gigaspora* sp., the ramifications on the fused GHTs as well as the GHTs that did not fuse stopped growth and septa formation were observed. This suggested a very specific role of GHT to fuse with a neighbor GHT and re-establish cytoplasmic/protoplasmic flow in injured hyphae. When the process is completed, the other GHTs and their ramifications became obsolete and cytoplasmic/protoplasmic material was reallocated inside the colony to save energy. In several AMF species (*G. mosseae*, *Gigaspora margarita*, and *Glomus etunicatum*) arrest of hyphal growth was observed in absence of roots followed by the formation of septa with cytoplasmic/protoplasmic retraction. This mechanism is thus not only associated to mycelial senescence and aging but possibly also to control the energy resources in the fungus (i.e. spores) (Logi *et al.*, 1998). Surprisingly, this mechanism was also noticed in *R. irregularis* in our study, but at very low frequency (only two cases, one in the control and one in the diesel 5%).

Interestingly, in one cut hypha from Control and another one from diesel 1% treatment in the *Gigaspora* strain, arrest of cytoplasmic/protoplasmic flow was observed after total fusion. This process is known as post-fusion incompatibility and was reported in AMF hyphal anastomosis only in Glomeraceae species between combinations of genetically different isolates (i.e. several isolates of *R. intraradices, R. irregularis, F. mosseae* and *F. coronatus*) (Croll *et al.*, 2009; Sbrana *et al.*, 2011; de la Providencia *et al.*, 2013; Giovannetti *et al.*, 2015; Pepe *et al.*, 2016).

Conclusion

The mechanism to repair damage within hyphae highly differed between *Gigaspora* sp. and *R. irregularis* in absence as well as in presence of diesel. For both fungi, the steps leading to a complete healing were slowed down in presence of diesel. Although, in *Gigaspora* sp., the impact of this pollutant was limited and hyphae in most cases were able to heal following injury as observed in absence of diesel. The healing process is thus the most probable way to maintain the viability of the hyphae in adverse conditions, and represent a mechanism for hyphae to survive. Conversely, in *R. irregularis*, the HHM was more severely affected. However, the GHTs of this fungus were able to reconnect the affected hyphae (although at low frequency) as in the control, and probably neighboring hyphae or roots as reported by de la Providencia *et al.* (2005) in absence of pollutants, being another strategy to survive under stress conditions. The significance of the two strategies for the fungi to survive under oil-polluted soils remains to be elucidated. It necessary involve understanding of the signal molecules implicated in the process of signal-response between injured sections of hyphae to re-establish a functional and effective mycelial network under polluted conditions. In *Gigaspora* sp., the strategy to reconnect injured hyphae is probably less costly and allows the existing mycelium network to keep functioning with the connected plants. This mechanism is probably the most efficient and cost-effective under stable

(i.e. physically-limited disturbance) environments in presence/absence of the pollutant. Conversely, in *R. irregularis*, the limited healing within the injured hyphae, but capacity to connect neighboring hyphae or even roots (de la Providencia *et al.*, 2005, 2007) may result in the loss of sections of the network, which is probably more costly, but may as well increase the chances of any part of injured hyphae to interconnect neighboring hyphae and roots. These hypotheses are fundamental to understand the AMF ecology in polluted as well as non-polluted soils and to investigate genetics and evolution of these obligate symbionts (de la Providencia *et al.*, 2005; Novais *et al.*, 2017).

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Author Contributions

Mónica Garcés Ruiz: design and performance of the experiment. Collection, analysis and interpretation of data. Drafting of the manuscript, commentaries corrections, final approval and agreement with all aspects of the work.

Maryline Calonne-Salmon: design and performance of the experiment. Analysis and interpretation of data. Correction of the manuscript, final approval and agreement with all aspects of the work.

Stéphane Declerck: Substantial contributions to the conception and design of the experiments, interpretation of the data. Correction of the manuscript, final approval and agreement with all aspects of the work.

VI. General discussion

Petroleum exploitation is the first income source for Ecuador and for most of the developing countries (e.g. Venezuela, Nigeria, Iran, Iraq...) that have crude oil resources underground. However, oil extraction and its mishandling have left numerous polluted environments around the world. Particularly in Ecuador, several sites have been abandoned after petroleum exploration and extraction (see **chapter 1**) and the way to manage these petroleum-contaminated sites has become a topical issue since the early nineties.

Different methods to remediate contaminated sites and dissipate hydrocarbon pollutants from water and soils have been explored and applied. Solvent extraction and oxidation (Chibuike, 2013), incineration, burials or removal of polluted soils (Das & Chandran, 2011) are chemical and physical techniques often considered. Biological approaches such as the use of bacteria (Atlas & Hazen, 2011), fungi (Stefani *et al.*, 2015) or plants, termed phytoremediation/rhizoremediation/mycoremediation (Mackova *et al.*, 2006) have also been assayed. Indeed, below-ground microorganisms are known to play key roles in the growth and increased resistance/tolerance of plants to difference a/biotic stresses (Wu, 2017). Among these microorganisms are the arbuscular mycorrhizal fungi (AMF). These fungi are obligate root symbionts. Their effects on plant growth and resistance/tolerance to biotic and abiotic stresses have been repeatedly reported (Smith & Read, 2008; Pozo *et al.*, 2013; Vosátka *et al.*, 2013). Some of these AMF have also been isolated from polluted soils and are thus potential candidates to help plants establishing under a variety of polluted environments (Garg & Chandel, 2010; Plouznikoff *et al.*, 2016; Wu, 2017).

Organic pollutants may have contrasting effects on AMF. Some studies have demonstrated a detrimental effect of these pollutants on AMF (e.g. inhibition

of spore germination or germ tube elongation, decrease of root colonization and production of spores) (Alarcón, 2006; Franco-Ramírez *et al.*, 2007; Hernández-Ortega *et al.*, 2012; Driai *et al.*, 2015), while others have observed a tolerance of these fungi against pollutants (see review Lenoir *et al.*, 2016a). The mechanisms most often suggested to explain the improved tolerance/resistance are: (1) morphological adaptation such as the modification of membrane lipids and (2) production of enzymes (i.e. glutaredoxins and thioredoxins) that act as redox regulators (antioxidants) and/or glomalin, a glycoprotein, capable to sequester pollutants (Verdin *et al.*, 2006; Lenoir *et al.*, 2016a).

A number of studies have also reported the beneficial effects of AMF on their host plants under hydrocarbon-polluted conditions, such as growth promotion (as compared to the non-colonized plants), improved P and N nutrition, increased tolerance to oxidative stress and/or facilitation of water uptake adjusting the osmotic balance (Cabello, 1999; Liu & Dalpé, 2009; Tang *et al.*, 2009; Dong *et al.*, 2014; Nwoko, 2014; Driai *et al.*, 2015; Xun *et al.*, 2015; Plouznikoff *et al.*, 2016).

In the last twenty years, some studies have reported the presence of AMF in petroleum-polluted soils (Cabello, 1997, 2001; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016).This suggested their tolerance to these pollutants and their potential for helping the plants to establish under these harsh environments.

Within this thesis, we first evaluated the AMF diversity and communities' composition in the roots of plants naturally re-colonizing weathered oil Ponds abandoned for more than 30 years in the Amazonian region of Ecuador (**chapter 1 and 2**). Whatever the plant species and site of sampling, root colonization by AMF was above 40%. This corroborated earlier studies by Cabello (1997), Huang *et al.* (2007a) and de la Providencia *et al.* (2015) that noticed the presence of AMF in hydrocarbon-polluted soils, even if root

colonization varied from low (i.e. de la Providencia *et al.*, 2015) to high (Cabello, 1997). Our findings supported a well-established AMF community in our experimental sites and a general adaptability of these obligate symbionts to hydrocarbon-polluted soils. As suggested by Huang *et al.* (2007), an intensive root colonization may indicate that plants in petroleum-contaminated soil might be highly dependent on AMF to establish and remain in place.

We further revealed a relatively diverse AMF community composition associated with native plants. This was demonstrated using two different molecular techniques (e.g. Sanger and NGS-454 sequencing). In **chapter 1** three plant species (i.e. *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria*) present in three different polluted environments (Pond 1, Pond 2 and the surrounding soil) were colonized by seven different AMF species (Sanger method). In **chapter 2**, root samples from Pond 2 revealed the presence of 15 AMF species associated with root of several plant species (454-method). However, in both studies, most of the AMF species could not be ascribed to an identified fungus, suggesting the presence of new species inhabiting these polluted soils. It is generally admitted that less than 5% of the existing AMF species are identified according to phylogenetic results from nrDNA extracted from plant roots (Błaszkowski *et al.*, 2015) and it is not excluded that under highly perturbed and unexplored environments the number of undescribed species may be even higher.

Acaulospora, *Archaeospora Glomus* and *Rhizophagus* genera were detected by the two methods supporting the reproducibility of results between both studies. This reproducibility could be ascribed to the use of specific primers that were designed from a diverse phylogenetic AMF lineages targenting one binding site in the small subunit (SSU) or the large subunit (LSU) rDNA (Krüger *et al.*, 2009). Thus, four mixed primers including a fragment covering partial SSU, complete internal transcribed spacer (ITS) and partial LSU rDNA

region, allow species resolution (Krüger *et al.*, 2009). Interestingly, a comparison between different primers sets targeting only AMF was perfomed on roots of six plant species differing in AMF community composition (Kohout *et al.*, 2014). The results demonstrated that the primers set used in our study ("Krügers primer" targeting partial SSU-ITS- partial LSU rDNA region) were the most efficient to trap the AMF diversity as compared to primers that only focused on the LSU or SSU rDNA region (Kohout *et al.*, 2014).

Sanger and 454-pyrosequencing methods revealed a relatively high number of undescribed taxa (74-75%, respectively). Sanger method detected less AMF species as well as less OTUs as compared to the 454-method. This might be due to the lower number of root samples as compared to the 454 experiment and the difficulty to obtain a high number of ~1.5 kb (SSU-ITS-LSU) sequences to analyze. Contrary, the use of short sequences (~ 800 bp (LSU)) with the evolutionary placement algorithm (EPA) - based approach used in the 454-pyrosequencing (**chapter 2**) has been considered precise and robust to unveil the AMF species community composition (Senés-Guerrero & Schüßler, 2016b) from environmental samples. Although, this method demonstrated the presence of sequences of AMF that could not be classified at the level of genera (i.e. Paraglomeraceae/Archaeosporaceae) or species (i.e. *Rhizophagus* sp. /*Dominikia* sp.) maybe due to the huge unknown diversity and lack of information (i.e. good quality and reliable reference sequences in online databases to which sequences may be compared). Additionally, the similarity threshold used with this method was 98% as compared to the 97% in the Sanger method. The use of 98% as similarity threshold, avoid the clustering of the short sequences of different AMF species as previously observed by Senés-Guerrero & Schüßler, (2016b). In addition, other species like *Sclerocystis* sp. and *Kamienskia* sp. belonging to the *Glomeraceae* family were detected with the 454-method.

Even though 454-method was the more robust and precise as compared to the

Sanger method, it will be soon replaced by new technologies that improve the analysis of long sequencing (i.e. 1.5kb) such as PacBio (http://www.pacb.com/). This method provides length sequence reads up to 60 kb. This technology has already been used in the study of the genome of *R. irregularis* DAOM-197198 (Tisserant *et al.*, 2014). Moreover one investigation has used the PacBio method with the Krüger *et al.* (2009) primers, and yielded strong data for AMF community analysis (Schlaeppi *et al.*, 2016).

The diverse AMF-community associated with plants grown in the hydrocarbon-polluted soils, suggested the potential occurrence of unknown "specialized/adapted" species belonging to three worldwide-distributed families (*Archaeosporaceae, Glomeraceae* and *Acaulosporacea*) capable to persist under high concentrations of hydrocarbons pollutants. Previous studies conducted in hydrocarbons-contaminated areas demonstrated the presence of species belonging to Glomeraceae as well to Acaulosporaceae, while the presence of Archaeosporaceae was rarely reported (Cabello, 1997; Huang *et al.*, 2007b; Villacrés *et al.*, 2014; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016). These families have also been observed in natural and disturbed soils of the Amazon region from other countries (i.e. Brazil, Venezuela, Colombia, Peru) where the dominance of *Glomus* and *Acaulospora* in the AMF community composition was reported (Peña-Venegas, 2011). Moreover, these three AMF families have been identified in different environments from Ecuador such as potato crops in the Andean region (Loján *et al.*, 2016; Senés-Guerrero & Schüßler, 2016b) as well as in the tropical rain forest in the south of the country (Haug *et al.*, 2010). Nevertheless, these anthropogenic and natural environments were also colonized by other families (i.e. Gigasporaceae, Claroideoglomeraceae, Diversisporaceae, Pacisporaceae, Paraglomeraceae), demonstrating a larger diversity in their community composition compared to the highly petroleum-polluted Ponds and surrounding soil from the Charapa field.

Several species are shared between different ecosystems/environments in Ecuador, thereby, we might assume that some of them are more ruderal and stress-tolerant resulting from a long adaptation to complicated environments. It is also postulated that the layer of organic matter developing over the weathered crude oil in our study sites allowed the establishment of different plants species. Their further growth in the soil would have been facilitated by their association to AMF increasing the accessibility to water and nutrients of roots growing inside the crude oil. Furthermore, the mycorrhizal networks can simultaneously colonize the roots of different/diverse plants from the same or different species, genera and families (Giovannetti *et al.*, 2015) as we already observed in **chapter 1**. This may significantly increase the root absorptive area of the plants and consequently enhance their supply with mineral nutrients (Błaszkowski *et al.*, 2015). These results suggest the importance of AMF in harsh conditions. Indeed, these characteristics make AMF widely appreciated either in agriculture as an alternative or complementary approach to decrease the amount and frequency of applications of agrochemicals or in phytoremediation strategies by enhancing pollutant dissipation as well as increasing the resistance/tolerance of plants (Vosátka *et al.*, 2013).

Nutrients uptake and transport (especially phosphorus), is an attribute of AMF which has never been investigated in presence of hydrocarbon pollutants. Different techniques have been developed to determine the dynamics of inorganic Phosphorus (Pi) uptake in the AMF-plant association. Most are destructive and Pi uptake/immobilization was measured in the shoot and root system (i.e. P concentration/content) or with the use of radioactive isotopes ^{32}P, ^{33}P, (Smith *et al.*, 2011; Hernández-Ortega *et al.*, 2012; Walder *et al.*, 2015; Kavka & Polle, 2016; Gonçalves de Oliveira *et al.*, 2017) which are more difficult to manage. Therefore, a new method borrowed from the system developed by Colpaert *et al.* (1999) for ECM, was developed during this thesis to study the uptake dynamics of Pi. The system consisted in a non-destructive

circulatory semi-hydroponic cultivation device in which plants were growing on perlite in containers irrigated by a nutrient solution impoverished in Pi (**chapter 3**). With this system, it was possible to evaluate the short-term Pi uptake/immobilization of maize plantlets associated or not to the AMF *R. irregularis* MUCL 41833. The system demonstrated that the AMF-colonized plantlets absorbed more Pi at 9 and 21 h as compared to non-colonized controls but not at 42 h. This system was further applied to study the dynamics of Pi depletion by maize plantlets associated or not to *R. irregularis* in presence of diesel (**chapter 4**).

Several authors have demonstrated that maize is moderately tolerant to hydrocarbons (Agbogidi *et al.*, 2007a; Njoku *et al.*, 2009a; Wu *et al.*, 2011b). Moreover, *R. irregularis* is a ruderal fungus (Chagnon *et al.*, 2013) capable of colonizing extensively maize roots (Wright *et al.*, 2005; Tian *et al.*, 2013; Garcés-Ruiz *et al.*, 2017) and improving plant growth at low Pi concentrations (Mickelson & Kaeppler, 2005; Tian *et al.*, 2013). Therefore, we combined maize to *R. irregularis* MUCL 41833 to investigate the dynamics of Pi uptake in presence of diesel. A significant and detrimental effect of diesel was noticed on AMF root colonization as well as on plant development, and no increase in Pi uptake was observed. This suggested that the hydrophobic and lipophilic properties of diesel (Ko & Day, 2004; Robertson *et al.*, 2007; Hernández-Ortega *et al.*, 2012) decreased or inhibited the uptake/immobilization of Pi by the AMF and by the roots, therefore impacting the direct and indirect uptake pathways (**chapter 4**). Interestingly, roots spiked with diesel accumulated more P than non-stressed ones suggesting that the Pi transporters in the roots were perturbed or possibly not synthesized.

R. irregularis has been extensively used as a model AMF for *in vitro* (Gallou *et al.*, 2010; Anene *et al.*, 2013; Koffi *et al.*, 2013; Buysens *et al.*, 2015) as well as *in vivo* (Anene & Declerck, 2016; Buysens *et al.*, 2016; Loján *et al.*, 2016; Garcés-Ruiz *et al.*, 2017a) experiments due, among others, to its easy

manipulation. However, our findings demonstrated that the specific isolate MUCL 41833 was not appropriate to increase maize resistance/tolerance or Pi uptake and transport under high concentration of diesel (12.5%). Therefore, other AMF either belonging to *Rhizophagus*, other genera (already identified from other environments) or isolated from hydrocarbon-polluted soils (e.g. see **chapter 1** and **2**) should be considered.

Phosphorus is one of the most important nutrients for plant development. Its uptake from beyond the roots depletion zone and further transport is attributed to the AMF, and more precisely to the extensive extraradical mycelium developing into the soil. However, this extraradical hyphal network may be damaged by anthropogenic activities such as ploughing or burning agricultural practices (Guadarrama *et al.*, 2014), grazing by soil fauna, soil freezing or wetting-drying cycles (Chagnon, 2014) requiring adaptations strategies to repair injured hyphae and restore fungal integrity. This process is named hyphae healing mechanism (HHM) and was thoroughly investigated by de la Providencia *et al.* (2005, 2007). These authors showed differences between AMF having contrasting life history strategies (LHS). However, until today, no study has been conducted on the effects of hydrocarbons (i.e. diesel) on the HHM and thus impact on the survival of the fungi and their subsequent functions in the uptake and transport of nutrients such as Pi to the plants.

The *in vitro* cultivation system was used to investigate this mechanism. This system avoids contaminations by undesirable microbes and facilitates the non-destructive time-course observation and imaging of the HHM. In **chapter 5**, the impact of increasing concentrations of diesel was investigated on the HHM. Two AMF strains with different LHS (i.e. *R. irregularis* MUCL 41833 – an r-strategist, and *Gigaspora* sp. MUCL 52331 – a K-strategist) were evaluated in absence of diesel or in presence of three concentrations of diesel (i.e. 1, 2 and 5%). The same imaging approach as described in de la Providencia *et al.* (2005, 2007) was followed. These authors identified four

successive steps for hyphae to repair and two contrasting strategies between four *Glomus* strains (r-strategists-ruderals) on one side and one *Scutellospora* and two *Gigaspora* strains (K-strategists-competitors) on the other side. The HHM in *Scutellospora* and *Gigaspora* was oriented toward a complete recovery of injured hyphae, while *Glomus* strains developed abundant growing hyphal tips (GHTs) and disorganized hyphal re-growth. Thus, the positive tropism observed in the two K-strategist strains was not demonstrated with the *Glomus* strains. Similar results were observed in our experiment, particularly in absence of diesel (i.e. the control treatment). In *Gigaspora* sp., 100% of the cut hyphae which developed GHTs at both sides and contacted each other re-established the cytoplasmic/protoplasmic flow. Conversely, in *R. irregularis*, only 18% of the developed GHTs at both sides of the cut hyphae completely recovered. In presence of diesel, the four steps leading to a total hyphae healing were similarly observed but each step took much more time as compared to the control. In *Gigaspora* sp. a complete hyphae recovery was observed at all concentrations of diesel and no significant differences were observed between the treatments (i.e. including the control). Conversely, *R. irregularis* was more severely affected and the percentages of hyphae with complete recovery were particularly low whatever the diesel treatment. However, no significant difference was observed as compared to the control. In absence of diesel, *R. irregularis* showed a HHM with production of abundant GHTs and ramifications rather than a complete recovery as suggested by de la Providencia *et al.* (2005, 2007). In presence of diesel, a similar strategy was observed suggesting that *R. irregularis* expend its energy in abundant production of new GHTs and ramifications from the injured hyphae rather than in complete recovery of the cut hyphae in presence or absence of diesel. Nevertheless, the production of abundant GHTs/ramifications almost disappeared under diesel-polluted conditions, especially at 5%. In contrast, the strategy of *Gigaspora* sp. was to use its energy to reconnect the cut extremities rather than to develop abundant

ramifications in absence as well as in presence of diesel. Thus, the K-strategy (competitor) may be considered as a qualitative survival while the r-strategy (ruderal) is considered as a quantitative strategy. Additionally, we hypothesized that signal response to the molecules generated by the AMF and/or their host to form/maintain the hyphal network as well as to recover might be inhibited/masked by diesel.

Although in our results from **chapter 1** and **2**, the Gigasporaceae family was not detected in the oil-polluted soils, others studies in hydrocarbon-polluted conditions have reported its presence (Cabello, 1997; de la Providencia *et al.*, 2005; Hassan *et al.*, 2014). Therefore, we may assume that AMF species within this family have also the ability to growth and colonize plants in oil-polluted soils. It is, however, important to mention that a high species richness of Gigasporaceae are more often reported in stable plant ecosystems than in agricultural soils (de Souza *et al.*, 2005) or in anthropogenic-perturbed ecosystems. To the contrary, the Glomeraceae family has been found in all types of ecosystems (Guadarrama *et al.*, 2014), including hydrocarbon polluted soils. Although *R. irregularis* MUCL 41833, seems to be sensitive to diesel (**chapter 4 chapter 5**) it is not excluded that others *Rhizophagus* spp. isolated from polluted hydrocarbon environments would be more tolerance and efficient in presence of oil derivatives.

Finally, according to our previous results (**chapter 1** and **2**) where several AMF species could be identified, we propose further investigations with native AMF species/strains originating from highly hydrocarbon-polluted soils. Their beneficial effect on Pi uptake/immobilization could be investigated as in **chapter 4**. To do so, AMF should first be isolated by trap-cultures and their identity assessed by the appropriate techniques (i.e. spore-morphotype identification and high throughput sequencing). An in-deep study of plant resistance/tolerance to hydrocarbon-polluted soils via their association with AMF isolated from such soils could then be investigated to truly assess their potential for phytoremediation.

VII. Conclusion and Perspectives

Soil pollution is a worldwide problem that has increased steadily since the industrial revolution. In Ecuador, the oil industry is the major economical resource since the early seventies and it is more than likely that petroleum extraction will continue in future years to support the economy of the country. It is thus essential to develop efficient tools to remediate current and possible future spills.

Among the several techniques that exist, biological approaches (i.e. phytoremediation, bioremediation) have attracted the attention of scientists, industrials as well as policy makers in the recent years. For instance, the application of microbial inoculants such as arbuscular mycorrhizal fungi (AMF) isolated from oil-polluted soils could represent a promising alternative to be combined with phytoremediation strategies, even if it is more than probable that this kind of remediation could take more time than the classical technologies (i.e. physical and chemical remediation). Interestingly, biological remediation is considered less aggressive than physical or chemical approaches and could be applied *in situ*. They could be used in combination or following the more phyisco-chemical methods, where physical-biological or chemical-biological methods (Lim *et al.*, 2016). The authors reviewed that the use of physical techniques such as the use of solvents (hexane, petane, surfactants, Triton x-100) prior the use of microorganism consortia provide a favourable environment, obtaining a highly percentage of hydrocarbon removal, especially in weathered crude oil. The use of chemical oxidation methods (H_2O_2) as pre-treament, may not destroy the oil compounds but it is possible to reduce their toxicity for the bioremediation or phytoremediation processes (Lim *et al.*, 2016).

In the present study, our attention was focused on two aspects. Firstly, we evaluated the AMF community composition in the roots of plants naturally recolonizing weathered crude oil ponds, which were abandoned in the Amazon region of Ecuador. The obtained information revealed the presence of different AMF species and probably new taxa adapted to inhabit such harsh conditions. Hence, the isolation of these indigenous AMF will increase the knowledge of the phylum Glomeromyctota. Secondly, we investigated the effect of diesel (a derivative from petroleum) on two principal attributes of AMF: (i) their capacity to take up and transport phosphorus to the plant and (ii) their capacity to heal following physical injury of hyphae.
Three research questions were addressed:

1. **How diverse are the AMF communities associated to plant species naturally recolonizing weathered crude oil ponds?**

The study on AMF community composition was conducted in oil ponds abandoned since 30 years in the Charapa field situated in the Amazon rain forest of Ecuador and naturally recolonized by a diverse community of plant species. Two molecular techniques were applied (i.e. the Sanger sequencing and NGS-454 pyrosequencing). All the plants sampled were associated to AMF with high colonization percentage generally observed. The more abundant genera were *Acaulospora, Glomus, Rhizophagus* and *Archaeospora,* with an approximate of 70% of sequences attributed to undescribed species.
We concluded that some AMF species/genera were more prevalent in crude oil-polluted soils and were able to colonize plants under these harsh conditions. Moreover, a high number of sequences could not be attributed to identified species, suggesting that these polluted soils are a reservoir of novel organisms with potential interests for bioremediation strategies. The isolation, testing and selection of the more efficient strains to help plant resist/tolerate oil-pollution and their further mass-production would be an option for the

development of efficient phytoremediation strategies for the several environmental liabilities present in the Ecuadorian Amazon and other polluted environments.

AMF live in close relation with other rhizosphere microbial inhabitants such as bacteria (i.e. *Rhizobium*-N fixation for the plants). It is not excluded, that the interaction between AMF and bacteria in the rhizosphere improve the remediation of polluted soils (Lenoir *et al.*, 2016b; Rajtor & Piotrowska-Seget, 2016), due to the ability of several bacteria to degrade and use hydrocarbon as source of energy or as an essential nutrient (Robertson *et al.*, 2007). Thus, a better understanding of these rhizo-communities may help improving hydrocarbon-degradation and efficient phyto- management.

2. Does *Rhizophagus irregularis* MUCL 41833 protect maize plantlets and facilitate its phosphorus uptake in presence of diesel?

In the study conducted on the impact of diesel on the short-term uptake of inorganic phosphorus (Pi) by maize plantlets colonized by *R. irregularis,* we demonstrated that diesel drastically impacted plant development as well as root colonization by the fungus. The AMF selected (i.e. a model fungus isolated from an agricultural soil without oil-pollution) did not improve Pi uptake rates of maize in presence of diesel. This observation demonstrated that the AMF selected was not adapted to diesel pollution and plead for the utilization of strains isolated from oil-polluted soils such as those associated to the plants in the Charapa field. Interestingly, P concentration in roots was higher in presence of diesel, which suggested inhibition/disruption of P translocation to the shoot. Thereby, a comprehensive understanding of the expression of the AM inducible Pi transporters (Walder *et al.*, 2015; Volpe *et al.*, 2016) under hydrocarbon stress condition could provide new findings to realize at what level and how this important mechanism is affected.

3. Does diesel impact the AMF hyphal healing mechanism of species belonging to different life history strategies?

In the study conducted on the hyphal healing mechanism (HHM) in *R. irregularis* MUCL 41833 and *Gigaspora* sp. MUCL 52331, we demonstrated that increasing concentrations of diesel delayed or/and inhibited total recovery in both genera. Nevertheless, *Gigaspora* sp. seemed more prone to repair than *R. irregularis.*

Several signaling molecules at fungal and plant level have been detected playing a key role in the association between AMF and plants. For instance, "Myc factors" (i.e. a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs)) play key roles in the colonization of roots (Maillet *et al.*, 2011), while strigolactones released by roots are responsible for the induction of hyphae branching (Parniske, 2008). The signal-response mechanism noticed in the healing of injured hyphae, particularly in *Gigaspora* sp. suggest the existence of specific molecules involved in the HHM. However, the nature of these molecules is unknown and probably difficult to assess due to the specific localized site of emission and low levels of production. Chromatography methods such as GC-MS or HPLC would help to unravel the molecules involved in the HHM and the impact of pollutants on the mechanisms involved in their production.

It would also be of great interest to study the HHM in species belonging to the r-strategists or ruderals such as other *Rhizophagus* or *Glomus* as well as stress-tolerant strains belonging to Acaulosporaceae as suggested by Chagnon *et al.* (2013) since this families were the more abundant in the hydrocarbon polluted soil.

In conclusion, we discovered a relatively diverse community of AMF in hydrocarbon-polluted soils. However, no effect was noticed on the Pi nutrition of maize plantlets using a standard strain, and the HHM mechanism was more

severely affected in *R. irregularis* than in *Gigaspora* sp. These results demonstrated that different strains might have different abilities to develop and fulfil their functions under diverse soils conditions. It is thus highly recommended to isolate, mass-produce and study strains from the polluted soils for their potential in plant nutrition and growth promotion as well as for their utilization under high hydrocarbon-contaminated soils (Cabello, 1997; Hassan *et al.*, 2014; de la Providencia *et al.*, 2015; Iffis *et al.*, 2016; Garcés-Ruiz *et al.*, 2017b). Their use to assist plants in phytoremediation as well as their combination with hydrocarbon-degrading microbial communities is expected to decrease the detrimental effects of hydrocarbon-pollutants on plants and to shift from chemical or physical remediation technologies to more environmental-friendly strategies of remediation.

VIII. Overview of the scientific achievements

Scientific publications

Articles published

Garcés-Ruiz, M., Calonne-Salmon, M., Plouznikoff, K., Misson, C., Navarrete-Mier, M., Cranenbrouck, S., & Declerck, S. (2017). Dynamics of short-term phosphorus uptake by intact mycorrhizal and non-mycorrhizal maize plants grown in a circulatory semi-hydroponic cultivation system. *Journal Frontiers in Plant Science*, *8*, 1–8. http://doi.org/10.3389/fpls.2017.01471

Garcés-Ruiz, M., Senés-Guerrero C., Declerck, S., &. Cranenbrouck, S. (2017). Arbuscular Mycorrhizal Fungal Community Composition in *Carludovica palmata*, *Costus scaber* and *Euterpe precatoria* from Weathered Oil Ponds in the Ecuadorian Amazon. *Journal Frontiers in Microbiology*, 8, 1-13. doi: 10.3389/fmicb.2017.02134

Article submitted

Garcés-Ruiz, M., Senés-Guerrero C., Declerck, S., &. Cranenbrouck, S. 454 pyrosequencing-based analysis of arbuscular mycorrhizal fungal communities in a petroleum-polluted Amazonian soil.

Articles in preparation

Garcés-Ruiz, M., Plouznikoff K., Cranenbrouck S., Declerck S. & Calonne-Salmon, M. The arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833 does not alleviate the impact of diesel on phosphorus uptake by maize plantlets.

Garcés-Ruiz, M., Calonne-Salmon, M., & Declerck, S. (2017). Hyphal healing mechanism in *Gigaspora* sp. MUCL 52331 is less affected by diesel pollution than *Rhizophagus irregularis* MUCL 41833.

Conference participation

Garcés-Ruiz, M., Calonne-Salmon, M., & Declerck, S. (2016) *Presentation:* Phosphorus absorption by *Zea mays* (L.) colonized by the arbuscular mycorrhizal fungus *Rhizophagus irregularis* in presence of diesel. XL Jornadas Nacionales de Biología

Garcés-Ruiz, M. (2016) *Presentation:* Estudio de hongos micorrízicos arbusculares en condiciones de contaminación por hidrocarburos y su diversidad asociada a plantas nativas en piscinas de petróleo en áreas amazónicas de Ecuador. Simposio Diversidad fúngica en ecosistemas naturales y contaminados y colecciones de cultivos de microorganismos.

Gordillo, A., **Garcés-Ruiz, M.,** Cevallos S., & Decock C. (2015) *Poster:* Biodiversity and Development, a global heritage. Royal Belgian Institute of Natural Sciences

Garcés-Ruiz, M., Calonne-Salmon, M., & Declerck, S. (2015) *Poster:* Impact of diesel on the symbiotic partners Medicago truncatula/Rhizophagus irregularis under *in vitro* conditions. Rhizosphere 4 / Journée des bioingénieurs à l'UCL.

Garcés-Ruiz, M. (2014) *Presentation:* Diversidad de hongos micorrizicos arbusculares asociados a comunidades de plantas que recolonizan pasivos ambientales en la amazonia ecuatoriana. Jornadas de Microbiología.

Teaching and **Student supervision**

1. International Training on in vitro Culture of Arbuscular Mycorrhizal Fungi. (May 2014, 2015 and 2016): Root disinfection session/ Roots and spore association session. Louvain-la-Neuve, Belgium

2. Professor of environmental microbiology and mycological techniques updated at PUCE (1 semester, 2014-2015). Quito, Ecuador.

3. Professor of Unidad de titulación in environmental microbiology and Biosafty at PUCE (1 semester, 2015-2016). Quito, Ecuador.

4. Professor of mycological techniques updated at PUCE (1 semester, 2016-2017). Quito, Ecuador.

5. Student supervision of Cédric Wilhelmi, Marine Plichon from the Faculty of Pharmacy (ULG), (September to December 2016). Performance of their final work in the mycology field (Arbuscular mycorrhizal fungi). Prof. Gabriel Castillo. Quito, Ecuador.

IX. References

Abad AK, Jalil K. **2007**. Effect of Cadmium Toxicity on the Level of Lipid Peroxidation and Antioxidative Ezymes Activity in Wheat Plants Colonized by Arbuscular Mycorrhizal Fungi. *Pakistan Journal of Biological Sciences* **10**: 2413–2417.

Abha S, Singh CS. **2012**. Hydrocarbon Pollution: Effects on Living Organisms, Remediation of Contaminated Environments, and Effects of Heavy Metals Co-Contamination on Bioremediation. *Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil-Contaminated Sites*: 185–206.

Adam G, Duncan H. **1999**. Effect of diesel fuel on growth of selected plant species. *Environmental Geochemistry and Health* **21**: 353–357.

Agamuthu P, Dadrasnia A. **2014**. Dynamics Phytoremediation of Zn and Diesel Fuel in Co-contaminated Soil using Biowastes. *Bioremediation & Biodegradation* **S4**.

Agbogidi O, Eruotor P, Akparobi S. **2007a**. Effects of crude oil levels on the growth of maize (Zea mays L.). *American Journal of Food Technology* **2**: 529–535.

Agbogidi OM, Eruotor PG, Akparobi SO, Nnaj GU. **2007b**. Evaluation of Crude Oil Contaminated Soil on the Mineral Nutrient Elements of Maize (Zea mays L.). *Journal of Agronomy*: 188–193.

Agnello AC, Bagard M, van Hullebusch ED, Esposito G, Huguenot D. **2016**. Comparative bioremediation of heavy metals and petroleum hydrocarbons co-contaminated soil by natural attenuation, phytoremediation, bioaugmentation and bioaugmentation-assisted phytoremediation. *Science of the Total Environment* **563**–**564**: 693–703.

Alarcón A. **2006**. The physiology of mycorrhizal Lolium multiflorum in the phytoremediation of petroleum hydrocarbons contaminated soil. *Office of Graduate Studies of Texas A&M University*.

Alarcón A, Davies FT, Autenrieth RL, Zuberer DA. **2008**. Arbuscular mycorrhiza and petroleum-degrading microorganisms enhance phytoremediation of petroleum-contaminated soil. *International journal of phytoremediation* **10**: 251–63.

Alarcón A, Delgadillo-Martínez J, Franco-Ramírez A, Davies FT, Ferrera-Cerrato R. **2006**. Influence of two polycyclic aromatic hydrocarbons on spore germination, and phytoremediation potential of Gigaspora margarita-Echynochloa polystachya symbiosis in benzo[a]pyrene-

polluted substrate. *Revista Internacional de Contaminacion Ambiental* **22**: 39–47.

Alguacil M del M, Torrecillas E, Lozano Z, Torres MP, Roldán A. **2014**. Prunus persica crop management differentially promotes arbuscular mycorrhizal fungi diversity in a tropical agro-ecosystem. *PLoS ONE* **9**.

Amadi A, Abbey SD, Nma A. **1996**. Chronic effects of oil spill on soil properties and microflora of a rainforest ecosystem in Nigeria. *Water, Air, and Soil Pollution* **86**: 1–11.

Anene A, Declerck S. **2016**. Combination of Crotalaria spectabilis with Rhizophagus irregularis MUCL41833 decreases the impact of Radopholus similis in banana. *Applied Soil Ecology* **106**: 11–17.

Anene A, Koffi MC, Vos C, Draye X, Declerck S. **2013**. Rhizophagus irregularis MUCL 41833 decreases the reproduction ratio of Radopholus similis in the banana cultivar Yangambi km5. *Nematology*: 629–632.

Anthony EJ, Wang J. **2006**. Pilot plant investigations of thermal remediation of tar-contaminated soil and oil-contaminated gravel. *Fuel 85*: 443–450.

Aranda E, Scervino JM, Godoy P, Reina R, Ocampo JA, Wittich RM, García-Romera I. **2013**. Role of arbuscular mycorrhizal fungus Rhizophagus custos in the dissipation of PAHs under root-organ culture conditions. *Environmental Pollution* **181**: 182–189.

Armstrong AG, Vallejo M. **1992**. Ecuador Oil Export Dispute. *Database Trade and Environment TED*.

Atlas RM, Hazen TC. **2011**. Oil biodegradation and bioremediation: A tale of the two worst spills in U.S. history. *Environmental Science and Technology* **45**: 6709–6715.

Atlas RM, Philp J (Eds.). **2005**. *Bioremediation Applied Microbial Solutions for Real-World Environmental Cleanup*. American Society for Microbiology.

Behjati S, Tarpey PS. **2013**. What is next generation sequencing? *Archives of disease in childhood - Education & practice edition* **98**: 236–238.

Berbee ML, James TY, Strullu-Derrien C. **2017**. Early Diverging Fungi: Diversity and Impact at the Dawn of Terrestrial Life. *Annual Review of Microbiology* **71**: 41–60.

Berger SA, Krompass D, Stamatakis A. **2011**. Performance, accuracy, and web server for evolutionary placement of short sequence reads under maximum likelihood. *Systematic Biology* **60**: 291–302.

Berger SA, Stamatakis A. **2011**. Aligning short reads to reference alignments and trees. *Bioinformatics* **27**: 2068–2075.

Berruti A, Borriello R, Orgiazzi A, Barbera AC, Lumini E, Bianciotto V. **2014**. Arbuscular Mycorrhizal Fungi and their Value for Ecosystem Management. In: Grillo O, ed. Biodiversity - The Dynamic Balance of the Planet. INTECH, 159–191.

Bever JD, Morton JB, Antonovics J, Schultz P a. **1996**. Host-dependent sporulation and species diversity of arbuscular mycorrhizal fungi in a mown grassland. *The Journal of Ecology* **84**: 71–82.

Bidar G, Pruvot C, Garçon G, Verdin A, Shirali P, Douay F. **2009**. Seasonal and annual variations of metal uptake, bioaccumulation, and toxicity in Trifolium repens and Lolium perenne growing in a heavy metal-contaminated field. *Environmental Science and Pollution Research* **16**: 42–53.

Błaszkowski J, Chwat G, Góralska A, Ryszka P, Kovács GM. **2015**. Two new genera, Dominikia and Kamienskia, and D. disticha sp. nov. in Glomeromycota. *Nova Hedwigia* **100**: 225–238.

Błaszkowski J, Tadych M, Madej T. **2000**. Glomus minutum, a new species in Glomales (Zygomycetes) from Poland. *Mycotaxon* **76**: 187–195.

Bonfante P, Genre A. **2010**. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nature Communications* **1**: 1–11.

Bouffaud ML, Bragalini C, Berruti A, Peyret-Guzzon M, Voyron S, Stockinger H, van Tuinen D, Lumini E, Wipf D, Plassart P, *et al.* 2017. Arbuscular mycorrhizal fungal community differences among European long-term observatories. *Mycorrhiza* **27**: 331–343.

Brundrett MC. **2009**. Mycorrhizal associations and other means of nutrition of vascular plants: Understanding the global diversity of host plants by resolving conflicting information and developing reliable means of diagnosis. *Plant and Soil* **320**: 37–77.

Bucala V, Saito H, Howard JB, Peters WA. **1994**. Thermal treatment of fuel oil- contaminated soils under rapid heating conditions. *Environ. Sci. Technol.*: 1801–1807.

Buysens C, César V, Ferrais F, Dupré de Boulois H, Declerck S. **2016**. Inoculation of Medicago sativa cover crop with Rhizophagus irregularis and Trichoderma harzianum increases the yield of subsequently-grown potato under low nutrient conditions. *Applied Soil Ecology* **105**: 137–143.

Buysens C, Dupré de Boulois H, Declerck S. **2015**. Do fungicides used to

control Rhizoctonia solani impact the non-target arbuscular mycorrhizal fungus Rhizophagus irregularis? *Mycorrhiza* **25**: 277–288.

Cabello M. **1997**. Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). *FEMS Microbiology Ecology* **22**: 233–236.

Cabello MN. **1999**. Effectiveness of indigeneous arbuscular mycorrhizal fungi (AMF) isolated from hydrocarbon polluted soils. *Basic Microbiol* **39**: 89–95.

Cabello MN. **2001**. Glomus tortuosum (Glomales), an arbuscular-mycorrhizal fungus (AMF) isolated from hydrocarbon-polluted soils. *Nova Hedwigia* **73**: 513–520.

Calonne M, Fontaine J, Debiane D, Laruelle F, Grandmougin-Ferjani A, Lounès-Hadj Sahraoui A. **2014a**. The arbuscular mycorrhizal Rhizophagus irregularis activates storage lipid biosynthesis to cope with the benzo[a]pyrene oxidative stress. *Phytochemistry* **97**: 30–37.

Calonne M, Fontaine J, Tisserant B, Dupré de Boulois H, Grandmougin-Ferjani A, Declerck S, Lounès-Hadj Sahraoui A. **2014b**. Polyaromatic hydrocarbons impair phosphorus transport by the arbuscular mycorrhizal fungus Rhizophagus irregularis. *Chemosphere* **104**: 97–104.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, *et al.* 2010. correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nature Publishing Group* **7**: 335–336.

Castillo C, Acuña H, Zagal E, Inostroza L. **2013**. Phosphorus absorption and use efficiency by Lotus spp. under water stress conditions in two soils: A pot experiment. *Chilean journal of …* **73**: 31–40.

Chagnon P-L. **2014**. Ecological and evolutionary implications of hyphal anastomosis in arbuscular mycorrhizal fungi. *FEMS microbiology ecology* **88**: 437–44.

Chagnon PL, Bradley RL, Maherali H, Klironomos JN. **2013**. A trait-based framework to understand life history of mycorrhizal fungi. *Trends in Plant Science* **18**: 484–491.

Chang C, Nasir F, Ma L, C.Tian. **2017**. Molecular Communication and Nutrient Transfer of Arbuscular Mycorrhizal Fungi, Symbiotic Nitrogen-Fixing Bacteria, and Host Plant in Tripartite Symbiosis. In: Sulieman S. TL, ed. egume Nitrogen Fixation in Soils with Low Phosphorus Availability. Cham: Springer.

Chapin FS, Zavaleta ES, Eviner VT, Naylor RL, Vitousek PM,

Reynolds HL, Hooper DU, Lavorel S, Sala OE, Hobbie SE, *et al.* 2000. Consequences of changing biodiversity. *Nature* **405**: 234–242.

Chaudhary VB, Cuenca G, Johnson NC. 2017. Tropical-temperate comparison of landscape-scale arbuscular mycorrhizal fungal species distributions. *Diversity and Distributions*: 1–13.

Chibuike GU. 2013. Use of mycorrhiza in soil remediation: A review. *Scientific Research and Essays* **8**: 1679–1687.

Cicatelli A, Torrigiani P, Todeschini V, Biondi S, Castiglione S, Lingua G. 2014. Arbuscular mycorrhizal fungi as a tool to ameliorate the phytoremediation potential of poplar: Biochemical and molecular aspects. *IForest* **7**: 333–341.

Clapp JP, Young JPW, Merryweather JW, Fitter AH. 1995. Diversity of fungal symbionts in arbuscular mycorrhizas from a natural community. *New Phytologist* **130**: 259–265.

Colpaert J V., Van Tichelen KK, Van Assche JA, Van Laere A. 1999. Short-term phosphorus uptake rates in mycorrhizal and non-mycorrhizal roots of intact Pinus sylvestris seedlings. *New Phytologist* **143**: 589–597.

Conesa HM, Evangelou MWH, Robinson BH, Schulin R. 2012. A Critical View of Current State of Phytotechnologies to Remediate Soils: Still a Promising Tool? *The Scientific World Journal* **2012**: 1–10.

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. 2005. Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**: 3674–3676.

Crahay C, Wevers J, Munaut F, Colpaert J V., Declerck S. 2013. Cryopreservation of ectomycorrhizal fungi has minor effects on root colonization of Pinus sylvestris plantlets and their subsequent nutrient uptake capacity. *Mycorrhiza* **23**: 463–471.

Cranenbrouck S, Voets L, Bivort C, Renard L, Strullu D-G, Declerck S. 2005. Methodologies for in vitro cultivation of arbuscular mycorrhizal fungi with root organs. In: Declerck S, Strullu D, Fortin J, eds. In Vitro Culture of Mycorrhizas. Berlin: Springer Berlin Heidelberg, 341–348.

Croll D, Giovannetti M, Koch AM, Sbrana C, Ehinger M, Lammers PJ, Sanders IR. 2009. Nonself vegetative fusion and genetic exchange in the arbuscular mycorrhizal fungus Glomus intraradices. *New phytologist* **181**: 924–937.

Cruz JM, Lopes PRM, Montagnolli RN, Tamada IS, Silva NMMG, Bidoia ED. 2013. Phytotoxicity of Soil Contaminated with Petroleum Derivatives and Biodiesel. *Journal of the Brazilian Society of Ecotoxicology*

8: 49–54.

Darkwah AK. **2010**. *The impact of oil and gas discovery and exploration on communities with emphasis on women.* Ghana.

Das N, Chandran P. **2011**. Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnology research international*: 941810.

David-Schwartz R, Gadkar V, Wininger S, Bendov R, Galili G, Levy A a, Kapulnik Y. **2003**. Isolation of a premycorrhizal infection (pmi2) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonization. *Molecular plant-microbe interactions : MPMI* **16**: 382–388.

Davison J, Moora M, Öpik M, Adholeya A, Ainsaar L, Bâ A, Burla S, Diedhiou a G, Hiiesalu I, Jairus T, *et al.* 2015. Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. *Science* **127**: 970–973.

Debiane D, Calonne M, Fontaine J, Laruelle F, Grandmougin-Ferjani A, Lounes-Hadj Sahraoui A. **2011**. Lipid content disturbance in the arbuscular mycorrhizal, Glomus irregulare grown in monoxenic conditions under PAHs pollution. *Fungal Biology* **115**: 782–792.

Debiane D, Calonne M, Fontaine J, Laruelle F, Grandmougin-Ferjani A, Lounès-Hadj Sahraoui A. **2012**. Benzo[a]pyrene induced lipid changes in the monoxenic arbuscular mycorrhizal chicory roots. *Journal of Hazardous Materials* **209**–**210**: 18–26.

Debiane D, Garçon G, Verdin A, Fontaine J, Durand R, Grandmougin-Ferjani A, Shirali P, Sahraoui ALH. **2008**. In vitro evaluation of the oxidative stress and genotoxic potentials of anthracene on mycorrhizal chicory roots. *Environmental and Experimental Botany* **64**: 120–127.

Debiane D, Garçon G, Verdin A, Fontaine J, Durand R, Shirali P, Grandmougin-Ferjani A, Lounès-Hadj Sahraoui A. **2009**. Mycorrhization alleviates benzo[a]pyrene-induced oxidative stress in an in vitro chicory root model. *Phytochemistry* **70**: 1421–1427.

Declerck S, Cranenbrouck S, Dalpé Y, Séguin S, Grandmougin-Ferjani A, Fontaine J, Sancholle M. **2000**. Glomus proliferum sp. nov.: A Description Based on Morphological, Biochemical, Molecular and Monoxenic Cultivation Data. *Mycologia* **92**: 1178–1187.

Declerck S, D'or D, Bivort C, de Souza FA. **2004**. Development of extraradical mycelium of Scutellospora reticulata under root-organ culture: spore production and function of auxiliary cells. *Mycological research* **108**: 84–92.

Declerck S, D'or D, Cranenbrouck S, Boulengé LE. **2001**. Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. *Mycorrhiza* **11**: 225–230.

Declerck S, Strullu DG, Plenchette C. **1998**. Monoxenic culture of the intraradical forms of Glomus sp. isolated from a tropical ecosystem: a proposed methodology for germplasm collection. *Mycologia* **90**: 579–585.

Dodd JC. **2000**. The role of arbuscular mycorrhizal fungi in agro-and natural ecosystems. *Outlook on Agriculture* **29**: 55–62.

Dong R, Gu L, Guo C, Xun F, Liu J. **2014**. Effect of PGPR Serratia marcescens BC-3 and AMF Glomus intraradices on phytoremediation of petroleum contaminated soil. *Ecotoxicology* **23**: 674–680.

Dorn PB, Salanitro JP. **2000**. Temporal ecological assessment of oil contaminated soils before and after bioremediation. *Chemosphere* **40**: 419–426.

Driai S, Verdin A, Laruelle F, Beddiar A, Lounès-Hadj Sahraoui A. **2015**. Is the arbuscular mycorrhizal fungus Rhizophagus irregularis able to fulfil its life cycle in the presence of diesel pollution? *International Biodeterioration and Biodegradation* **105**: 58–65.

Dumas A. **2013**. Mécanismes moléculaires de tolérance des plantes aux xénobiotiques : Application à la phytoremédiation des Hydrocarbures Aromatiques Polycycliques.

Dupré de Boulois H, Voets L, Delvaux B, Jakobsen I, Declerck S. **2006**. Transport of radiocaesium by arbuscular mycorrhizal fungi to Medicago truncatula under in vitro conditions. *Environmental Microbiology* **8**: 1926–1934.

Edgar RC. **2010**. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**: 2460–2461.

Elektorowicz M, Boeva V. **1996**. Electrokinetic supply of nutrients in soil bioremedia- tion. *Environ. Technol.*: 1339–1349.

Environmental Protection Agency (US-EPA) Microwave assisted acid digestion of Sediments, sludges, soils, and oils. EPA 3051, 2007 [WWW document] URL https://www.epa.gov/sites/production/files/2015-12/documents/3051a.pdf (Accessed 04/10/2017)

Environmental Protection Agency (US-EPA) Ultrasonic extraction EPA 3550 B, 1996 [WWW document] URL http://www.gtanalytical.com/media/pdf/3550b.pdf (Accessed 04/10/2017)

Environmental Protection Agency (US-EPA) Atomic absorption methods EPA 7000A, 1990 [WWW document] URL https://archive.epa.gov/epawaste/hazard/testmethods/web/pdf/method%207000a%2c%20revision%201%20-%201990.pdf (Accessed 04/10/2017)

Environmental Protection Agency (US-EPA) Soil and waste pH EPA 9045 D, 2004 [WWW document] URL https://www.epa.gov/sites/production/files/2015-12/documents/9045d.pdf (Accessed 04/10/2017)

Ezawa T, Smith SE, Smith FA. **2001**. Differentiation of Polyphosphate Metabolism between the Extra- and Intraradical Hyphae of Arbuscular Mycorrhizal Fungi. *New Phytologist* **149**: 555–563.

Faure D, Bonin P, Duran R. **2015**. Environmental microbiology as a mosaic of explored ecosystems and issues. *Environmental Science and Pollution Research* **22**: 13577–13598.

Ferrera-Cerrato R, Rojas-Avelizapa NG, Poggi-Varaldo HM, Alarcón A, Cañizares-Villanueva RO. **2006**. Bioremediation processes of soil and water contaminated with petroleum hydrocarbons and other organic compounds. *Revista Latinoamericana de Microbiologia* **48**: 179–187.

Ferrol N, Tamayo E, Vargas P. **2016**. The heavy metal paradox in arbuscular mycorrhizas: from mechanisms to biotechnological applications. *Journal of Experimental Botany* **67**: 6253–6265.

Fiorilli V, Lanfranco L, Bonfante P. **2013**. The expression of GintPT, the phosphate transporter of Rhizophagus irregularis, depends on the symbiotic status and phosphate availability. *Planta* **237**: 1267–1277.

Fontaine J, Grandmougin-Ferjani A, Glorian V, Durand R. **2004**. 24-Methyl/methylene sterols increase in monoxenic roots after colonization by arbuscular mycorrhizal fungi. *New Phytologist* **163**: 159–167.

Food and Agricultrure Organization of the United Nation - FAO 2015, Water development and Management Unit Crop Water Information: Maize. [WWW document] URL http://www.fao.org/nr/water/cropinfo_maize.html (Accessed 31 July 2016)

Franco-Ramírez A, Ferrera-Cerrato R, Varela-Fregoso L, Pérez-Moreno J, Alarcón A. **2007**. Arbuscular mycorrhizal fungi in chronically petroleum-contaminated soils in Mexico and the effects of petroleum hydrocarbons on spore germination. *Journal of Basic Microbiology* **47**: 378–383.

Gallou A, De Jaeger N, Cranenbrouck S, Declerck S. **2010**. Fast track in

vitro mycorrhization of potato plantlets allow studies on gene expression dynamics. *Mycorrhiza* **20**: 201–207.

Gan S, Lau E V, Ng HK. **2009**. Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). *Journal of Hazardous Materials* **172**: 532–549.

Gao YC, Guo SH, Wang JN, Li D, Wang H, Zeng DH. **2014**. Effects of different remediation treatments on crude oil contaminated saline soil. *Chemosphere*.

Garcés-Ruiz M, Calonne-Salmon M, Plouznikoff K, Misson C, Navarrete-Mier M, Cranenbrouck S, Declerck S. **2017a**. Dynamics of short-term phosphorus uptake by intact mycorrhizal and non-mycorrhizal maize plants grown in a circulatory semi-hydroponic cultivation system. *Journal Frontiers in Plant Science* **8**: 1–8.

Garcés-Ruiz M, Senés-Guerrero C, Declerck S, Pirttilä AM, Pagano MC. **2017b**. Arbuscular Mycorrhizal Fungal Community Composition in Carludovica palmata , Costus scaber and Euterpe precatoria from Weathered Oil Ponds in the Ecuadorian Amazon. *Frontiers in Microbiology* **8**: 1–13.

Garg N, Chandel S. **2010**. Arbuscular mycorrhizal networks: process and functions. A review. *Agronomy for Sustainable Development* **30**: 581–599.

Gaspar ML, Cabello MN, Cazau MC, Pollero RJ. **2002**. Effect of phenanthrene and Rhodotorula glutinis on arbuscular mycorrhizal fungus colonization of maize roots. *Mycorrhiza* **12**: 55–59.

Gaur A, Adholeya A. **2004**. Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. *Current Science* **86**.

Germida JJ, Frick CM, Farrell RE. **2002**. Phytoremediation of oil-contaminated soils. *Developments in Soil Science* **28**: 169–186.

Ghanem ME, Albacete A, Martínez-Andújar C, Acosta M, Romero-Aranda R, Dodd IC, Lutts S, Pérez-Alfocea F. **2008**. Hormonal changes during salinity-induced leaf senescence in tomato (Solanum lycopersicum L.). *Journal of Experimental Botany* **59**: 3039–3050.

Gil-Cardeza ML, Ferri A, Cornejo P, Gomez E. **2014**. Distribution of chromium species in a Cr-polluted soil: Presence of Cr(III) in glomalin related protein fraction. *Science of the Total Environment* **493**: 828–833.

Giovannetti M, Avio L, Sbrana C. **2015**. Functional Significance of Anastomosis in Arbuscular Mycorrhizal Networks. In: Horton TR, ed. Mycorrhizal Networks, Ecological Studies. Springer Science+Business, 41–68.

Giovannetti M, Azzolini D, Citernesi AS. **1999**. Anastomosis Formation and Nuclear and Protoplasmic Exchange in Arbuscular Mycorrhizal Fungi. *Applied and Environmental Microbiology* **65**: 5571–5575.

Giovannetti M, Fortuna P, Citernesi AS, Morini S, Nuti MP. **2001**. The occurrence of anastomosis formation and nuclear exchange in intact arbuscular mycorrhizal networks. *New Phytologist* **151**: 717–724.

Giovannetti M, Sbrana C, Avio L, Strani P. **2004**. Patterns of below-ground plant interconnections established by means of arbuscular mycorrhizal networks. *New Phytologist* **164**: 175–181.

Goeker M, García-Blázquez G, Voglmayr H, Tellería MT, Martín MP. **2009**. Molecular taxonomy of phytopathogenic fungi: A case study in Peronospora. *PLoS ONE* **4**: 8–10.

Goi A, Trapido M, Kulik N, Palmroth M, Tuhkanen T. **2006**. Ozonation and Fenton treatment for remediation of diesel fuel contaminated soil. *Ozone Sci. Eng.*: 37–46.

Gonçalves de Oliveira JR, de Resende GM, de Melo NF, Yano-Melo AM. **2017**. Symbiotic compatibility between arbuscular mycorrhizal fungi (autoctone or exotic) and three native species of the Caatinga in different phosphorus levels. *Acta Scientiarum. Biological Sciences* **39**: 59.

Grace Liu PW, Chang TC, Whang LM, Kao CH, Pan PT, Cheng SS. **2011**. Bioremediation of petroleum hydrocarbon contaminated soil: Effects of strategies and microbial community shift. *International Biodeterioration and Biodegradation* **65**: 1119–1127.

Guadarrama P, Castillo S, Ramos-Zapata J a., Hernández-Cuevas LV, Camargo-Ricalde SL. **2014**. Arbuscular mycorrhizal fungal communities in changing environments: The effects of seasonality and anthropogenic disturbance in a seasonal dry forest. *Pedobiologia* **57**: 87–95.

Guo W, Zhao R, Fu R, Bi N, Wang L, Zhao W, Guo J, Zhang J. **2014**. Contribution of arbuscular mycorrhizal fungi to the development of maize (Zea mays L.) grown in three types of coal mine spoils. *Environmental Science and Pollution Research* **21**: 3592–3603.

Hafidi M, Qaddoury A, Duponnois R, Wipf D, Hijri M, Bâ A. **2015**. International Congress on Mycorrhizae: mycorrhizal symbiosis a key factor for improving plant productivity and ecosystems restoration. *Mycorrhiza*.

Han M, Ji G, Ni J. **2009**. Washing of field weathered crude oil contaminated soil with an environmentally compatible surfactant, alkyl polyglucoside. *Chemosphere* **76**: 579–586.

Hart MM, Reader RJ, Hart MM. **2002**. Taxonomic basis for variation in

the colonization strategy of arbuscular mycorrhizal fungi. *New Phytologist* **153**: 335–344.

Hassan SE-D, Bell TH, Stefani FOP, Denis D, Hijri M, St-Arnaud M. **2014**. Contrasting the community structure of arbuscular mycorrhizal fungi from hydrocarbon-contaminated and uncontaminated soils following willow (Salix spp. L.) planting. *PloS one* **9**: e102838.

Haug I, Wubet T, Weiß M, Aguirre N, Weber M, Günter S, Kottke I. **2010**. Species-rich but distinct arbuscular mycorrhizal communities in reforestation plots on degraded pastures and in neighboring pristine tropical mountain rain forest. *Tropical Ecology* **51**: 125–148.

Hawkins H-J, George E. **1997**. Hydroponic culture of the mycorrhizal fungus Glomus mosseae with Linum L., Sorghum bicolor L. and Triticum aestivum L. *Plant and Soil*: 143–149.

Heath RL, Packer L. **1968**. Photoperoxidation in Isolated Chloroplasts. *Archives of Biochemestry and Biophysics* **125**: 189–198.

van der Heijden MGA, Sanders IR (Eds.). **2003**. *Mycorrhizal Ecology*. Springer-Verlag Berlin Heidelberg GmbH.

van der Heijden MGA, Scheublin TR. **2007**. Functional traits in mycorrhizal ecology: Their use for predicting the impact of arbuscular mycorrhizal fungal communities on plant growth and ecosystem functioning [3]. *New Phytologist* **174**: 244–250.

Helber N, Wippel K, Sauer N, Schaarschmidt S, Hause B, Requena N. **2011**. A Versatile Monosaccharide Transporter That Operates in the Arbuscular Mycorrhizal Fungus Glomus sp Is Crucial for the Symbiotic Relationship with Plants. *The Plant Cell* **23**: 3812–3823.

Hernández-Ortega HA. **2014**. RESPUESTAS MORFOLÓGICAS Y FISIOLÓGICAS DE Rhizophagus intraradices Y Medicago sativa ANTE CONTAMINACIÓN POR DIÉSEL.

Hernández-Ortega HA, Alarcón A, Ferrera-Cerrato R, Zavaleta-Mancera HA, López-Delgado HA, Mendoza-López MR. **2012**. Arbuscular mycorrhizal fungi on growth, nutrient status, and total antioxidant activity of Melilotus albus during phytoremediation of a diesel-contaminated substrate. *Journal of Environmental Management* **95**.

Hoagland DR, Arnon DI. **1950**. *The water-culture method for growing plants without soil* (U of California, Ed.). Berkeley: The College of Agriculture.

Homeier J, Leuschner C, Bräuning A, Cumbicus NL, Hertel D, Martinson GO, Spannl S, Veldkamp E. **2013**. *Ecosystem Services,*

Biodiversity and Environmental Change in a Tropical Mountain Ecosystem of South Ecuador (J Bendix, E Beck, A Bräuning, F Makeschin, R Mosandl, S Scheu, and W Wilcke, Eds.). Springer-Verlag Berlin Heidelberg.

Horton TR (Ed.). **2015**. *Mycorrhizal Networks*. Springer Science+Business Media Dordrecht.

Huang J, Tang M, Niu Z, Zhang R. **2007a**. Arbuscular mycorrhizal fungi in petroleum contaminated soil in Suining area of Sichuan Province. *Chinese Journal of Ecology*.

Huang J-C, Tang M, Niu Z-C, Zhang R-Q. **2007b**. Arbuscular mycorrhizal fungi in petroleum-contaminated soil in Suining area of Sichuan Province. *Chinese Journal of Ecology* **26**: 1389–1392.

IARC. **1990**. Diesel fuels. *International Agency for Research on Cancer* **45**: 219–237.

Iffis B, St-Arnaud M, Hijri M. **2014**. Bacteria associated with arbuscular mycorrhizal fungi within roots of plants growing in a soil highly contaminated with aliphatic and aromatic petroleum hydrocarbons. *FEMS Microbiology Letters* **358**: 44–54.

Iffis B, St-Arnaud M, Hijri M. **2016**. Petroleum hydrocarbon contamination, plant identity and arbuscular mycorrhizal fungal (AMF) community determine assemblages of the AMF spore-associated microbes. *Environmental Microbiology* **18**: 2689–2704.

IJdo M, Schtickzelle N, Cranenbrouck S, Declerck S. **2010**. Do arbuscular mycorrhizal fungi with contrasting life-history strategies differ in their responses to repeated defoliation? *FEMS Microbiology Ecology* **72**: 114–122.

J. W. Gerdemann. **1955**. Wound-Healing of Hyphae in a Phycomycetous Mycorrhizal Fungus. *Mycologia* **47**: 916–918.

De Jaeger N, de la Providencia IE, Dupré de Boulois H, Declerck S. **2011**. Trichoderma harzianum might impact phosphorus transport by arbuscular mycorrhizal fungi. *FEMS Microbiology Ecology* **77**: 558–567.

Janos DP. 1980. Vesicular-Arbuscular Mycorrhizae Affect Lowland Tropical Rain Forest Plant Growth. *Ecological society of America* **61**: 151–162.

Janos DP. **2007**. Plant responsiveness to mycorrhizas differs from dependence upon mycorrhizas. *Mycorrhiza* **17**: 75–91.

Janos DP, Sahley CT. **1995**. Rodent Dispersal of Vesicular-Arbuscular Mycorrhizal Fungi in Amazonian Peru. *Ecology* **76**: 1852–1858.

Javot H, Pumplin N, Harrison MJ. **2007**. Phosphate in the arbuscular mycorrhizal symbiosis: Transport properties and regulatory roles. *Plant, Cell and Environment* **30**: 310–322.

Jernelöv A. **2010**. How to defend against future oil spills. *Nature* **466**: 182–183.

Jernelöv A, Lindén O. **1981**. Ixtoc I: A Case Study of the World's Largest Oil Spill. *Ambio* **10**: 299–306.

Joner EJ, Leyval C. **2001**. Influence of arbuscular mycorrhiza on clover and ryegrass grown together in a soil spiked with polycyclic aromatic hydrocarbons. *Mycorrhiza* **10**: 155–159.

Joye SB, Teske AP, Kostka JE. **2014**. Microbial dynamics following the macondo oil well blowout across gulf of Mexico environments. *BioScience* **64**: 766–777.

Kalinhoff C, Cáceres A, Lugo L. **2009**. Cambios en la biomasa de raíces y micorrizas arbusculares en cultivos itinerantes del Amazonas Venezolano. *Interciencia* **34**: 571–576.

Katoh K, Standley DM. **2013**. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

Kauppi S, Sinkkonen A, Romantschuk M. **2011**. Enhancing bioremediation of diesel-fuel-contaminated soil in a boreal climate: Comparison of biostimulation and bioaugmentation. *International Biodeterioration and Biodegradation* **65**: 359–368.

Kavka M, Polle A. **2016**. Phosphate uptake kinetics and tissue-specific transporter expression profiles in poplar (Populus × canescens) at different phosphorus availabilities. *BMC Plant Biology* **16**: 206.

Kennedy D. **2010**. Beyond Petroleum? *Science* **329**: 727-.

Kenrick P, Strullu-Derrien C. **2014**. The Origin and Early Evolution of Roots. *Plant Physiology* **166**: 570–580.

Kharaka YK, Dorsey NS. **2005**. Environmental issues of petroleum exploration and production: Introduction. *Environmental Geosciences* **12**: 61–63.

Kirk J, Moutoglis P, Klironomos J, Lee H, Trevors J. **2005**. Toxicity of diesel fuel to germination, growth and colonization of Glomus intraradices in soil and in vitro transformed carrot root cultures. *Plant and Soil* **270**: 23–30.

Kivlin SN, Hawkes C V., Treseder KK. **2011**. Global diversity and

distribution of arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry* **43**: 2294–2303.

Ko JY and, Day JW. **2004**. A review of ecological impacts of oil and gas development on coastal ecosystems in the Mississippi Delta. *Ocean & Coastal Management* **47**: 597–623.

Koffi MC, Vos C, Draye X, Declerck S. **2013**. Effects of Rhizophagus irregularis MUCL 41833 on the reproduction of Radopholus similis in banana plantlets grown under in vitro culture conditions. *Mycorrhiza* **23**: 279–288.

Kohout P, Sudová R, Janoušková M, Čtvrtlíková M, Hejda M, Pánková H, Slavíková R, Štajerová K, Vosátka M, Sýkorová Z. **2014**. Comparison of commonly used primer sets for evaluating arbuscular mycorrhizal fungal communities: Is there a universal solution? *Soil Biology and Biochemistry* **68**: 482–493.

Kothamasi D, Wannijn J, van Hees M, Nauts R, van Gompel A, Vanhoudt N, Cranenbrouck S, Declerck S, Vandenhove H. **2015**. Rhizophagus irregularis MUCL 41833 can colonize and improve P uptake of Plantago lanceolata after exposure to ionizing gamma radiation in root organ culture. *Mycorrhiza*.

Kover PX, Schaal BA. **2002**. Genetic variation for disease resistance and tolerance among Arabidopsis thaliana accessions. *Proceedings of the National Academy of Sciences of the United States of America* **199**: 11270–11274.

Krüger M, Krüger C, Walker C, Stockinger H, Schüßler A. **2012**. Phylogenetic reference data for systematics and phylotaxonomy of arbuscular mycorrhizal fungi from phylum to species level. *New Phytologist* **193**: 970–984.

Krüger M, Stockinger H, Krüger C, Schüßler A. **2009**. DNA-based species level detection of Glomeromycota: One PCR primer set for all arbuscular mycorrhizal fungi. *New Phytologist* **183**: 212–223.

Kummerová M, Zezulka Š, Babula P, Váňová L. **2013**. Root response in Pisum sativum and Zea mays under fluoranthene stress: Morphological and anatomical traits. *Chemosphere* **90**: 665–673.

Kvenvolden KA, Cooper CK. **2003**. Natural seepage of crude oil into the marine environment. *Geo-Marine Letters* **23**: 140–146.

de la Providencia IE, Fernández F, Declerck S. **2007**. Hyphal healing mechanism in the arbuscular mycorrhizal fungi Scutellospora reticulata and Glomus clarum differs in response to severe physical stress. *FEMS*

Microbiology Letters **268**: 120–125.

de la Providencia IE, Nadimi M, Beaudet D, Rodriguez Morales G, Hijri M. 2013. Detection of a transient mitochondrial DNA heteroplasmy in the progeny of crossed genetically divergent isolates of arbuscular mycorrhizal fungi. *New Phytologist* **200**: 211–221.

de la Providencia IE, de Souza FA, Fernández F, Séjalon Delmas N, Declerck S. 2005. Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. *New Phytologist* **165**: 261–271.

de la Providencia IE, Stefani FOP, Labridy M, St-Arnaud M, Hijri M. 2015. Arbuscular mycorrhizal fungal diversity associated with Eleocharis obtusa and Panicum capillare growing in an extreme petroleum hydrocarbon-polluted sedimentation basin. *FEMS Microbiology Letters* **362**.

Labana S, Kapur M, Malik DK, Prakash D, Jain RK. 2007. Diversity, biodegradation and bioremediation of polycyclic aromatic hydrocarbons. In: Environmental Bioremediation Technologies. chandigarh, India: Springer Berlin Heidelberg, 409–443.

Lanfranco L, Young JPW. 2012. Genetic and genomic glimpses of the elusive arbuscular mycorrhizal fungi. *Current Opinion in Plant Biology* **15**: 454–461.

Lee YT. 2015. Energy, Environment and the Future of Mankind. In: Öztürk M, Ashraf M, Aksoy A, Ahmad MSA, eds. Phytoremediation for Green Energy. New York: Springer Dordrecht, 1–10.

Lenoir I, Fontaine J, Lounès-Hadj Sahraoui A. 2016a. Arbuscular mycorrhizal fungal responses to abiotic stresses: A review. *Phytochemistry* **123**: 4–15.

Lenoir I, Fontaine J, Tisserant B, Laruelle F, Lounès-Hadj Sahraoui A. 2017. Beneficial contribution of the arbuscular mycorrhizal fungus, Rhizophagus irregularis, in the protection of Medicago truncatula roots against benzo[a]pyrene toxicity. *Mycorrhiza* **27**: 465–476.

Lenoir I, Lounes-Hadj Sahraoui A, Fontaine J. 2016b. Arbuscular mycorrhizal fungal-assisted phytoremediation of soil contaminated with persistent organic pollutants: a review. *European Journal of Soil Science* **67**: 624–640.

Leyval C, Binet P. 1998. Effect of Polyaromatic Hydrocarbons in Soil on Arbuscular Mycorrhizal Plants. *J. Environ. Qual.* **27**: 402–407.

Liang F, Lu M, Keener TC, Liu Z, Khang S-J. 2005. The organic composition of diesel particulate matter, diesel fuel and engine oil of a non-

road diesel generator. *Journal of environmental monitoring : JEM* **7**: 983–988.

Liang C-C, Tao L, Yan-Ping X, Mao-Jun L, Han-Bo Z, Zhao Z-W. **2009**. Effects of inoculation with arbuscular mycorrhizal fungi on maize grown in multi-metal contaminated soils. *International Journal of Phytoremediation* **11**: 692–703.

Li X, Zhang J, Gai J, Cai X, Christie P, Li X. **2015**. Contribution of arbuscular mycorrhizal fungi of sedges to soil aggregation along an altitudinal alpine grassland gradient on the Tibetan Plateau. *Environmental Microbiology* **17**: 2841–2857.

Li H, Zhang Y, Kravchenko I, Xu H, Zhang C. **2007**. Dynamic changes in microbial activity and community structure during biodegradation of petroleum compounds: a laboratory experiment. *Journal of environmental sciences (China)* **19**: 1003–13.

Li D, Zhang Y, Quan X, Zhao Y. **2009**. Microwave thermal remediation of crude oil contaminated soil enhanced by carbon fiber. *J. Environ. Sci.*: 1290–1295.

Lim MW, Lau E Von, Poh PE. **2016**. A comprehensive guide of remediation technologies for oil contaminated soil — Present works and future directions. *Marine Pollution Bulletin* **109**: 14–45.

Liu A, Dalpé Y. **2009**. Reduction in Soil Polycyclic Aromatic Hydrocarbons By Arbuscular Mycorrhizal Leek Plants. *International Journal of Phytoremediation* **11**: 39–52.

Liu SL, Luo YM, Cao ZH, Wu LH, Ding KQ, Christie P. **2004**. Degradation of benzo[a]pyrene in soil with arbuscular mycorrhizal alfalfa. *Environmental Geochemistry and Health* **26**: 285–293.

Logi C, Sbrana C, Giovannetti M. **1998**. Cellular events involved in survival of individual arbuscular mycorrhizal symbionts growing in the absence of the host. *Applied and Environmental Microbiology* **64**: 3473–3479.

Loján P, Demortier M, Velivelli SLS, Pfeiffer S, Suárez JP, de Vos P, Prestwich BD, Sessitsch A, Declerck S. **2017**. Impact of plant growth-promoting rhizobacteria on root colonization potential and life cycle of Rhizophagus irregularis following co-entrapment into alginate beads. *Journal of Applied Microbiology* **122**: 429–440.

Loján P, Senés-Guerrero C, Suárez JP, Kromann P, Schüssler A, Declerck S. **2016**. Potato field-inoculation in Ecuador with Rhizophagus irregularis: no impact on growth performance and associated arbuscular

mycorrhizal fungal communities. *Symbiosis*: 1–12.

Lopes Leal P, Stürmer SL, Siqueira JO. **2009**. Occurrence and diversity of arbuscular mycorrhizal fungi in trap cultures from soils under different land use systems in the amazon, Brazil. *Brazilian Journal of Microbiology* **40**: 111–121.

Lunt PH, Hedger JN. **1996**. A survey of mycorrhizal infection of trees in the Terra Firme rainforest, Cuyabeno, Ecuador. *The Mycologist* **10**: 161–165.

Maček I, Šibanc N, Kavšček M, Lestan D. **2016**. Diversity of arbuscular mycorrhizal fungi in metal polluted and EDTA washed garden soils before and after soil revitalization with commercial and indigenous fungal inoculum. *Ecological Engineering* **95**: 330–339.

Macek T, Uhlik O, Jecna K, Novakova M, Lovecka P, Rezek J, Dudkova V, Stursa P, Vrchotova B, Pavlikova D, *et al.* 2009. Advances in Phytoremediation and Rhizoremediation. In: Advances in Applied Bioremediation. In: Singh A, Kuhad CR, Ward PO, eds. Advances in Applied Bioremediation. Berlin, Heidelberg: Springer Berlin Heidelberg, 257–277.

Mackova M, Dowling D, Macek T (Eds.). **2006**. *Phytoremediation and Rhizoremediation: Theoretical Background.* Dordrecht: Springer.

Maherali H, Klironomos JN. **2007**. Influence of Phylogeny on Fungal Community Assembly and Ecosystem Functioning. *Science* **316**: 1746–1748.

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, *et al.* 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. *Nature* **469**: 58–63.

Margesin R, Schinner F. **2001**. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology* **56**: 650–663.

Margesin R, Walder G, Schinner F. **2003**. Bioremediation assessment of a BTEX-contaminated soil. *Acta Biotechnologica* **23**: 29–36.

McGonigle TP, Evans DG, Miller MH. **1990a**. Effect of degree of soil disturbance on mycorrhizal colonization and phosphorous absorption by maize in growth chamber and field experiments. *New Phytologist* **116**: 629–636.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. **1990b**. A new method which gives an objective measure of colonization of roots by

vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **115**: 495–501.

Medeiros CAB, Clark RB, Ellis JR. **1994**. Growth and nutrient uptake of sorghum cultivated with vesicular-arbuscular mycorrhiza isolates at varying pH. *Mycorrhiza* **4**: 185–191.

de Menezes AB, Richardson AE, Thrall PH. **2017**. Linking fungal – bacterial co-occurrences to soil ecosystem function. *Current Opinion in Microbiology* **37**: 135–141.

Merkl N, Schultze-Kraft R, Infante C. **2005a**. Assessment of tropical grasses and legumes for phytoremediation of petroleum-contaminated soils. *Water, Air, and Soil Pollution* **165**: 195–209.

Merkl N, Schultze-Kraft R, Infante C. **2005b**. Phytoremediation in the tropics - Influence of heavy crude oil on root morphological characteristics of graminoids. *Environmental Pollution* **138**: 86–91.

Mickelson S, Kaeppler S. **2005**. Evaluation of six mycorrhizal isolates for their ability to promote growth of maize genotypes under phosphorus deficiency. *Maydica* **50**: 137–146.

Ministerio del Ambiente de Ecuador (MAE). **2015**. Percentage of pollution sources of Hydrocarbon Industry eliminated, remedied and endorsed by the Environmental National Authority. *Ministerio del Ambiente Ecuador*.

Ministerio del Ambiente de Ecuador (MAE). **2016**. *Pasivos ambientales y reparación integral: experiencias de gestión en el Ecuador*. Quito, Ecuador.

Morton JB. **2017**. Three New Species of Acaulospora (Endogonaceae) from High Aluminum , Low pH Soils in West Virginia. *Mycologia* **78**: 641–648.

Mosavat N, Oh E, Chai G. **2012**. A review of electrokinetic treatment technique for improving the engineering characteristics of low permeable problematic soils. *Int. J. GEOMATE*: 266–272.

Mukherjee S, Choudhuri MA. **1983**. Implications of water stress-induced changes in the levels of endogenous ascorbic acid and hydrogen peroxide in Vigna seedlings. : 166–170.

Nazeri NK, Lambers H, Tibbett M, Ryan MH. **2014**. Moderating mycorrhizas: Arbuscular mycorrhizas modify rhizosphere chemistry and maintain plant phosphorus status within narrow boundaries. *Plant, Cell and Environment* **37**: 911–921.

Nicolotti G, Egli S. **1998**. Soil contamination by crude oil: Impact on the mycorrhizosphere and on the revegetation potential of forest trees.

Environmental Pollution **99**: 37–43.

Njoku KL, Akinola MO, Ige TO. **2009a**. Comparative Effects of Diesel Fuel and Spent Lubricating Oil on the Growth of Zea Mays (Maize). *American - Eurasian Journal of Sustainable Agriculture* **3**: 428–434.

Njoku KL, Akinola MO, Taiwo BG. **2009b**. Effect of gasoline diesel fuel mixture on the germination and the growth of Vigna unguiculata (Cowpea). *Science And Technology* **3**: 466–471.

Nouri E, Breuillin-Sessoms F, Feller U, Reinhardt D. **2014**. Phosphorus and nitrogen regulate arbuscular mycorrhizal symbiosis in petunia hybrida. *PLoS ONE* **9**.

Novais CB De, Pepe A, Siqueira JO, Giovannetti M, Sbrana C. **2017**. Compatibility and incompatibility in hyphal anastomosis of arbuscular mycorrhizal fungi. *Scientia Agricola* **74**: 411–416.

Nwoko CO. **2014**. Effect of Arbuscular Mycorrhizal (AM) Fungi on the Physiological Performance of Phaseolus vulgaris Grown under Crude Oil Contaminated Soil. *Journal of Geoscience and Environment Protection* **2**: 9–14.

Ogbo E. **2009**. Effects of diesel fuel contamination on seed germination of four crop plants - Arachis hypogaea, Vigna unguiculata, Sorghum bicolor and Zea mays. *African Journal of Biotechnology* **8**: 250–253.

Ojala JC, Jarrell WM. **1980**. Hydroponic sand culture systems for mycorrhizal research. *Plant and Soil* **57**: 297–303.

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, *et al.* 2017. vegan: Community ecology package. R package version 2.4-3.

Oludele E, Ogundele T. **2013**. The Impact of Diesel Contaminated Soil on Seedling Growth of Okro (Abelmoschus Esculentus), Pumpkin (Amaranthus Hybridus) Maize (Zea Mays) and. *International Journal of Scientific Research* **2**: 237–239.

Öpik M, Metsis M, Daniell TJ, Zobel M, Moora M. **2009**. Large-scale parallel 454 sequencing reveals host ecological group specificity of arbuscular mycorrhizal fungi in a boreonemoral forest. *New Phytologist*: 424–437.

Öpik M, Zobel M, Cantero JJ, Davison J, Facelli JM, Hiiesalu I, Jairus T, Kalwij JM, Koorem K, Leal ME, *et al.* 2013. Global sampling of plant roots expands the described molecular diversity of arbuscular mycorrhizal fungi. *Mycorrhiza* **23**: 411–430.

Overton E, Sharp W, Roberts P. **1994**. Toxicity of Petroleum. In: Cockerham L, Shane B, eds. Basic Environmental Toxicology. Boca Raton: CRCPress, Inc., 133–156.

Öztürk M, Ashraf M, Aksoy A, Ahmad MSA (Eds.). **2015**. *Phytoremediation for Green Energy*. New York London: Springer Dordrecht.

Palacios R, Cerón W, C. V, Sierra R. **1999**. Las Formaciones Naturales de la Amazonía del Ecuador. In: Sierra R, ed. Propuesta Preliminar de un Sistema de Clasificación de Vegetación para el Ecuador Continental. Quito, Ecuador: Proyecto INEFAN/GEF-BIRF y EcoCiencia, 114–116.

Panagos P, Hiederer R, Van Liedekerke M, Bampa F. **2013**. Review Article Contaminated Sites in Europe: Review of the Current Situation Based on Data Collected through a European Network. *Journal of Environmental and Public Health* **Artical ID**: 1–11.

Parmar S, Singh V. **2015**. Phytoremediation Approaches for Heavy Metal Pollution : A Review. *Journal of Plant Sciences & Research* **2**.

Parniske M. **2008**. Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nature Reviews Microbiology* **6**: 763–775.

Peña-Venegas CP. **2011**. Arbuscular mycorrhizal fungi in the Amazon region. In: Mycorrhiza: Occurrence in Natural and Restored Environments. 75–86.

Peña-Venegas CP, Cardona GI, Mazorra A, J.H. A, Arcos AL. **2006**. Micorrizas arbusculares de la Amazonia colombiana. Catálogo ilustrado. *Instituto Amazónico de Investigaciones Científicas SINCHI*: 90.

Peng M, Zi X, Wang Q. **2015**. Bacterial community diversity of oil-contaminated soils assessed by high throughput sequencing of 16s rRNA genes. *International Journal of Environmental Research and Public Health* **12**: 12002–12015.

Pepe A, Giovannetti M, Sbrana C. **2016**. Different levels of hyphal self-incompatibility modulate interconnectedness of mycorrhizal networks in three arbuscular mycorrhizal fungi within the Glomeraceae. *Mycorrhiza* **26**: 325–332.

Pérez A. **2014**. *Diversity , composition and floristic structure of Charapa 1 well pad, Sucumbios-Ecuador*. Quito, Ecuador.

Piña J, Merino J, Errazu AF, Bucalá V. **2002**. Thermal treatment of soils contaminated with gas oil: influence of soil composition and treatment temperature. *J. Hazard. Mater.*: 273–290.

Plouznikoff K, Declerck S, Calonne-Salmon M. **2016**. Mitigating Abiotic Stresses in Crop Plants by Arbuscular Mycorrhizal Fungi. In: Vos CMF, Kazan K, eds. Belowground Defence Strategies in Plants, Signaling and Communication in Plants. Springer International Publishing Switzerland, 341–400.

Poss JA, Pond E, Menge JA, Jarell WM. **1985**. Effect of salinity on mycorrhizal onion and tomato in soil with and without additional phosphate. **88**: 307–319.

Pozo MJ, Jung SC, Martínez-Medina A, López-Ráez JA, Azcón-Aguilar C, Barea J-M. **2013**. Root Allies: Arbuscular Mycorrhizal Fungi Help Plants to Cope with Biotic Stresses. In: Aroca R, ed. Symbiotic Endophytes. Berlin, Heidelberg: Springer Berlin Heidelberg, 289–307.

Purin S, Morton JB. **2011**. In situ analysis of anastomosis in representative genera of arbuscular mycorrhizal fungi. *Mycorrhiza* **21**: 505–514.

Rabie GH. **2004**. Using wheat-mungbean plant system and arbuscular mycorrhiza to enhance in-situ bioremediation. *International journal of food, agriculture and environment* **2**: 381–390.

Rabie GH. **2005**. Role of Arbuscular Mycorrhizal Fungi in Phytoremediation of Soil Rhizosphere Spiked with Poly Aromatic Hydrocarbons. *Mycobiology* **33**: 41.

Rajtor M, Piotrowska-Seget Z. **2016**. Prospects for arbuscular mycorrhizal fungi (AMF) to assist in phytoremediation of soil hydrocarbon contaminants. *Chemosphere* **162**: 105–116.

Recorbet G, Abdallah C, Renaut J, Wipf D, Dumas-Gaudot E. **2013**. Protein actors sustaining arbuscular mycorrhizal symbiosis: Underground artists break the silence. *New Phytologist* **199**: 26–40.

Reddy SR, Pindi PK, Reddy SM. **2005**. Molecular methods for research on arbuscular mycorrhizal fungi in India: Problems and prospects. *Current Science* **89**: 1699–1709.

Redecker D, Hijri I, Wiemken A. **2003**. Molecular identification of arbuscular mycorrhizal fungi in roots: Perspectives and problems. *Folia Geobotanica* **38**: 113–124.

Redecker D, Schüßler A, Stockinger H, Stürmer SL, Morton JB, Walker C. **2013**. An evidence-based consensus for the classification of arbuscular mycorrhizal fungi (Glomeromycota). *Mycorrhiza* **23**: 515–531.

Reyes-Jaramillo I, Camargo-Ricalde SL, Aquiahuatl-Ramos M de los A. **2008**. Mycorrhizal-like interaction between gametophytes and young sporophytes of the fern dryopteris muenchii (Filicales) and its fungal

endophyte. *Revista de Biologia Tropical* **56**: 1101–1107.

Rillig MC. **2004**. Arbuscular mycorrhizae, glomalin, and soil aggregation. *Canadian Journal of Soil Science* **84**: 355–363.

Rillig MC, Camenzind T, Gawlik J, Haug I, Maraun VKM, Scheu DSS. **2013**. Diversity in Soil Fungi, Protists, and Microarthropods. In: Bendix J, Beck E, Bräuning A, Makeschin F, Mosandl R, Scheu S, Wilcke W, eds. Ecosystem Services, Biodiversity and Environmental Change in a Tropical Mountain Ecosystem of South Ecuador. Springer-Verlag Berlin Heidelberg, 80–92.

Robertson SJ, McGill WB, Massicotte HB, Rutherford PM. **2007**. Petroleum hydrocarbon contamination in boreal forest soils: A mycorrhizal ecosystems perspective. *Biological Reviews* **82**: 213–240.

Robinson AP, Duursma RA, Marshall JD. **2005**. A Regression-based equivalence test for model validation: shifting the burden of proof. *Tree Physiology* **25**: 903–13.

Rollin C, Quiot F, Denys S, Bureau J. **2005**. *Hydrocarbures Aromatiques Polycycliques. Guide méthodologique*.

Rufyikiri G, DeClerck S, Dufey JE, Delvaux B. **2000**. Arbuscular mycorrhizal fungi might alleviate aluminum toxicity in banana plants. *New Phytologist* **148**: 343–352.

Rushton D, Ghaly AE, Martinell K. **2007**. Assessment of Canadian regulations and remediation methods for diesel oil contaminated soils. *Am. J. Appl. Sci.*: 465.

Sahoo NK, Ramesh A, Pakshirajan K. **2012**. Bacterial Degradation of Aromatic Xenobiotic Compounds: An Overview on Metabolic Pathways and Molecular Approaches. In: Satyanarayana T, Johri BN, Prakash A, eds. Microorganisms in Environmental Management. Springer, 201–221.

Salami, Olusola A, Elum A, Ejiro A. **2010**. Bioremediation of a Crude Oil Polluted Soil with Pleurotus Pulmonarius and Glomus Mosseae Using Amaranthus Hybridus as a Test Plant. *Journal of Bioremediation & Biodegradation*.

Salamnaca CR, Silva M del R. **1998**. Las Micorrizas como alternativa para el Manejo Sostenble de los Agroecosistemas Tropicales. *Boletin Tecnico Corpoica*: 26.

Salanitro JP. **2001**. Bioremediation of petroleum hydrocarbons in soil. *Advances in Agronomy* **72**: 53–105.

San Sebastián M, Hurtig AK. **2004**. Oil exploitation in the Amazon basin

of Ecuador: a public health emergency. *Pan Am Journal of Public Health* **15**: 205–211.

Santos BA, Silva GA, Maia LC, Alves M V. 2000. Mycorrhizae in monocotyledonae of Northeast Brazil: Subclasses Alismatidae, Arecidae and Zingiberidae. *Mycorrhiza* **10**: 151–153.

Satyanarayana T, Johri BN, Prakash A (Eds.). **2012**. *Microorganisms in Environmental Management*. Delhi: Springer.

Sbrana C, Fortuna P, Giovannetti M. **2011**. Plugging into the network: belowground connections between germlings and extraradical mycelium of arbuscular mycorrhizal fungi. *Mycologia* **103**: 307–316.

Schlaeppi K, Bender SF, Mascher F, Russo G, Patrignani A, Camenzind T, Hempel S, Rillig MC, van der Heijden MGA. **2016**. High-resolution community profiling of arbuscular mycorrhizal fungi. *New Phytologist*.

Schneider DS, Ayres JS. **2008**. Us About Treating Infectious Diseases. *Nature Reviews Immunology* **8**: 889–895.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, *et al.* 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences* **109**: 6241–6246.

Schüßler A, Krüger C, Urgiles N. **2015**. Phylogenetically diverse AM fungi from Ecuador strongly improve seedling growth of native potential crop trees. *Mycorrhiza*.

Schüßler A, Walker C. **2010**. *The Glomeromycota A species list with new families and new genera*. Gloucester, UK.

Schüβler A, Schwarzott D, Walker C. **2001**. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research* **105**: 1413–1421.

Schwarzott D, Schüßler a. 2001. A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AM fungal spores. *Mycorrhiza* **10**: 203–207.

Senés-Guerrero C. **2014**. DNA-based characterization of arbuscular mycorrhizal fungi associated with potato roots from the Andean region.

Senés-Guerrero C, Schüßler A. **2016a**. DNA-based characterization and identification of arbuscular mycorrhizal fungi species. In: Uroz F, Martin S, eds. Microbial Environmental Genomics. Series: Methods in Molecular Biology. New York: Springer New York, 101–123.

Senés-Guerrero C, Schüßler A. **2016b**. A conserved arbuscular mycorrhizal fungal core-species community colonizes potato roots in the Andes. *Fungal Diversity* **77**: 317–333.

Senés-Guerrero C, Torres-Cortés G, Pfeiffer S, Rojas M, Schüßler A. **2014**. Potato-associated arbuscular mycorrhizal fungal communities in the Peruvian Andes. *Mycorrhiza* **24**: 1–13.

Shukry WM, Al-Hawas GHS, Al-Moaikal RMS, M. A. El-Bendary. **2013**. Effect of Petroleum Crude Oil on Mineral Nutrient Elements, Soil Properties and Bacterial Biomass of the Rhizosphere of Jojoba. *British Journal of Environment and Climate Change* **3**: 103–118.

Silva A, Delerue-Matos C, Fiúza A. **2005**. Use of solvent extraction to remediate soils contaminated with hydrocarbons. *Journal of Hazardous Materials* **124**: 224–229.

Simard SW, Austin ME. **2010**. The Role of Mycorrhizas in Forest Soil Stability with Climate Change x The role of mycorrhizas in forest soil stability with climate change. In: Suzanne Simard, ed. Climate Change and Variability. 275–301.

Simard SW, Beiler KJ, Bingham MA, Deslippe JR, Philip LJ, Teste FP. **2012**. Mycorrhizal networks: Mechanisms, ecology and modelling. *Fungal Biology Reviews* **26**: 39–60.

Singh H. **2006**. *Mycoremediation: Fungal Bioremediation*. New Jersey: John Wiley & Sons, Inc.

Singh JS, Seneviratne G. **2017**. *Agro-Environmental Sustainability* (JS Singh and G Seneviratne, Eds.).

Sinha S, Saxena R, Singh S. **2005**. Chromium induced lipid peroxidation in the plants of Pistia stratiotes L.: Role of antioxidants and antioxidant enzymes. *Chemosphere* **58**: 595–604.

Smith S, Jakobsen I, Grønlund M, Smith FA. **2011**. Roles of Arbuscular Mycorrhizas in Plant Phosphorus Nutrition: Interactions between Pathways of Phosphorus Uptake in Arbuscular Mycorrhizal Roots Have Important Implications for Understanding and Manipulating Plant Phosphorus Acquisition. *Plant Physiology* **156**: 1050–1057.

Smith SE, Read D. **2008**. *Mycorrhizal Symbiosis*. Cambridge, UK: Academic Press.

Smith S, Smith FA. **2010**. The Role of Arbuscular Mycorrhizal Fungi (AMF) in Crop Phosphorus (P) Nutrition : A Need for Changed Ideas. *Eyre Peninsula Farming Systems 2010 Summary*: 125–127.

Smith SE, Smith FA. **2012**. Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. *Mycologia* **104**: 1–13.

Smith SE, Smith FA, Jakobsen I. **2004**. Functional diversity in arbuscular mycorrhizal (AM) symbioses: The contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. *New Phytologist* **162**: 511–524.

Solaiman ZM, Abbott LK, Varma A (Eds.). **2014**. *Mycorrhizal Fungi: Use in Sustainable Agriculture and Land Restoration*. Springer-Verlag Berlin Heidelberg.

Sonjak S, Beguiristain T, Leyval C, Regvar M. **2009**. Temporal temperature gradient gel electrophoresis (TTGE) analysis of arbuscular mycorrhizal fungi associated with selected plants from saline and metal polluted environments. *Plant Soil* **314**: 25–34.

Souza T. **2015**. *Handbook of Arbuscular Mycorrhizal Fungi*. Springer Science+Business Media.

de Souza F, Dalpé Y, Declerck S, Providencia I de la, Séjalon-Delmas N. **2005**. Life history strategies in Gigasporaceae: insight from monoxenic culture. In: Declerck S, Strullu D., Fortin JA, eds. In vitro culture of mycorrhizas. Heidelberg, Springer-Verlag, 74–94.

de Souza FA, Declerck S. **2003**. Mycelium development and architecture, and spore production of Scutellospora reticulata in monoxenic culture with Ri T-DNA transformed carrot roots. *Mycologia* **95**: 1004–1012.

Sri Ranjan R, Qian Y, Krishnapillai M. **2006**. Effects of electrokinetics and cationic sur- factant cetyltrimethylammonium bromide [CTAB] on the hydrocarbon removal and retention from contaminated soils. *Environ. Technol*: 767–776.

Stamatakis A, Hoover P, Rougemont J. **2008**. A rapid bootstrap algorithm for the RAxML Web servers. *Systematic biology* **57**: 758–71.

Standard Methods for the Examination of Water and Wastewater SM 4500-N, 1997 [WWW document] URL http://www.standardmethods.org/store/ProductView.cfm?ProductID=194 (Accessed 04/10/2017)

Standard Methods for the Examination of Water and Wastewater SM 4500 P, 1999 [WWW document] URL https://www.standardmethods.org/store/ProductView.cfm?ProductID=197 (Accessed 04/10/2017)

Standard Methods for the Examination of Water and Wastewater SM

5520 E, 2012 [WWW document] URL https://www.standardmethods.org/store/ProductView.cfm?ProductID=41 (Accessed 04/10/17)

Stefani FOP, Bell TH, Marchand C, De La Providencia IE, El Yassimi A, St-Arnaud M, Hijri M. **2015**. Culture-dependent and -independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. *PLoS ONE* **10**: e0128272.

Stockinger H, Krüger M, Schüßler A. **2010**. DNA barcoding of arbuscular mycorrhizal fungi. *New Phytologist* **187**: 461–474.

Stockinger H, Peyret-Guzzon M, Koegel S, Bouffaud M-L, Redecker D. **2014**. The largest subunit of RNA polymerase II as a new marker gene to study assemblages of arbuscular mycorrhizal fungi in the field. *PloS one* **9**: e107783.

Stürmer SL, Siqueira JO. **2011**. Species richness and spore abundance of arbuscular mycorrhizal fungi across distinct land uses in Western Brazilian Amazon. *Mycorrhiza* **21**: 255–267.

Stutz JC, Morton JB. **1996**. Successive pot cultures reveal high species richness of arbuscular endomycorrhizal fungi in arid ecosystems. *Canadian Journal of Botany* **74**: 1883–1889.

Surridge AKJ, Wehner FC, Cloete TE. **2009**. Bioremediation of Polluted Soil. In: Advances in Applied Bioremediation. London: Springer Dordrecht Heidelberg.

Tang M, Chen H, Huang JC, Tian ZQ. **2009**. AM fungi effects on the growth and physiology of Zea mays seedlings under diesel stress. *Soil Biology and Biochemistry* **41**: 936–940.

Team RDC. **2016**. R: a language and environment for statistical computing.

Tian H, Drijber RA, Li X, Miller DN, Wienhold BJ. **2013**. Arbuscular mycorrhizal fungi differ in their ability to regulate the expression of phosphate transporters in maize (Zea mays L.). *Mycorrhiza* **23**: 507–514.

Van Tichelen KK, Vanstraelen T, Colpaert J V. 1999. Nutrient uptake by intact mycorrhizal Pinus sylvestris seedlings: a diagnostic tool to detect copper toxicity. *Tree physiology* **19**: 189–196.

Tisserant E, Malbreil M, Kuo A, Kohler A, Symeonidi A, Balestrini R, Charron P, Duensing N, Frey NF dit, Vivienne Gianinazzi-Pearsoi LBG, *et al.* 2014. Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences* **111**: 562–563.

Tong HY, Karasek FW. **1984**. Quantitation of polycyclic aromatic hydrocarbons in diesel exhaust particulate matter by high-performance liquid chromatography fractionation and high-resolution gas chromatography. *Analytical chemistry* **56**: 2129–2134.

Trejo D, Moreira C, Bañuelos I, Lara L, Alafita G, Reyes A. **2013**. Effect of Diesel and Biodiesel on the Growth of Brachiaria decumbens inoculated with arbuscular mycorrhizal fungi. *Tropical and Subtropical Agroecosystems* **16**: 391–398.

Trufem SFB. **1990**. Aspectos ecológicos de fungos micorrízicos vesículo-arbusculares da mata tropical úmida da Ilha do Cardoso, SP, Brasil. *Acta Botanica Brasilica* **4**: 31–45.

Tyagi M, da Fonseca MMR, de Carvalho CCCR. **2011**. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation* **22**: 231–241.

UNCTAD. **2009**. Energy-related issues from the trade and development perspective. In: United Nations Conference on Trade and Development. 1–21.

Usman M. **2016**. Comment on 'Acomprehensive guideof remediation technologies for oil contaminated soil— Presentworks and future directions'. *Marine Pollution Bulletin* **109**: 14–45.

Usman M, Faure P, Hanna K, Abdelmoula M, Ruby C. **2012**. Application of magnetite catalyzed chemical oxidation (Fenton-like and persulfate) for the remediation of oil hydrocarbon contamination. *Fuel*: 270–276.

Vallino M, Massa N, Lumini E, Bianciotto V, Berta G, Bonfante P. **2006**. Assessment of arbuscular mycorrhizal fungal diversity in roots of Solidago gigantea growing in a polluted soil in Northern Italy. *Environmental Microbiology* **8**: 971–983.

Varma A (Ed.). **2008**. *Mycorrhiza*. Springer-Verlag Berlin Heidelberg.

Varma A, Ward OP (Eds.). **2004**. *Applied Bioremediation and Phytoremediation*. New Delhi: Springer-Verlag.

Verdin A, Lounès-Hadj Sahraoui A, Fontaine J, Grandmougin-Ferjani A, Durand R. **2006**. Effects of anthracene on development of an arbuscular mycorrhizal fungus and contribution of the symbiotic association to pollutant dissipation. *Mycorrhiza* **16**: 397–405.

Villa RD, Trovó AG, Nogueira RFP. **2010**. Diesel degradation in soil by Fenton process. *J. Braz. Chem. Soc.*: 1088–1095.

Villacrés BH, Medina ME, Cumbal L, Villarroel A. **2014**. Implementación de un banco de Hongos Micorrícicos Arbusculares, aislados de suelos del área de influencia de EP PETROCUADOR y su efecto en el desarrollo de plantas de Maíz (Zea mays) en condiciones de estrés por cadmio, en La Joya de los Sachas, Provincia (GF Olmedo C., Ed.). *Revista Digital IX Congreso de Ciencia y Tecnología*: 24–35.

Voets L, de La Providencia IE, Declerck S. **2006**. Glomeraceae and Gigasporaceae differ in their ability to form hyphal networks. *New Phytologist* **172**: 185–188.

Voets L, de La Providencia IE, Fernandez K, IJdo M, Cranenbrouck S, Declerck S. **2009**. Extraradical mycelium network of arbuscular mycorrhizal fungi allows fast colonization of seedlings under in vitro conditions. *Mycorrhiza* **19**: 347–356.

Volpe V, Giovannetti M, Sun XG, Fiorilli V, Bonfante P. **2016**. The phosphate transporters LjPT4 and MtPT4 mediate early root responses to phosphate status in non mycorrhizal roots. *Plant, Cell and Environment* **39**: 660–671.

Vosátka M, Látr A, Gianinazzi S, Albrechtová J. **2013**. Development of arbuscular mycorrhizal biotechnology and industry: Current achievements and bottlenecks. *Symbiosis* **58**: 29–37.

Vosátka M, Rydlová J, Sudová R, Vohník M. **2006**. Mycorrhizal Fungi as Helping Agents in Phytoremediation of Degraded and contaminated Soils. In: Mackova M, Dowling D, Macek T, eds. Phytoremediation Rhizoremediation. Dordrecht: Springer, 237–257.

Vwioko DE, Omamogho CE. **2012**. Assessing successive plant growth on petroleum hydrocarbon degradation in highly polluted soil augmented with wood ash. *International Journal of Applied Science and Technology* **2**: 247–267.

Walder F, Brulé D, Koegel S, Wiemken A, Boller T, Courty PE. **2015**. Plant phosphorus acquisition in a common mycorrhizal network: Regulation of phosphate transporter genes of the Pht1 family in sorghum and flax. *New Phytologist* **205**: 1632–1645.

Walker C. **1983**. Taxonomic concepts in the Endogonaceae: spore wall characteristics in species descriptions. *Mycotaxon* **18**: 443–455.

Walker C. **2005**. A Simple Blue Staining Technique for Arbuscular Mycorrhizal and Other Root-Inhabiting Fungi. *Inoculum* **56**: 68–69.

Wang Y, Li Y, Bao X, Björn LO, Li S, Olsson PA. **2016**. Response differences of arbuscular mycorrhizal fungi communities in the roots of an

aquatic and a semiaquatic species to various flooding regimes. *Plant and Soil* **403**: 361–373.

Wang B, Qiu YL. **2006**. Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* **16**: 299–363.

Watkinson RJ, Morgan P. **1990**. Physiology of aliphatic hydrocarbon-degrading microorganisms. *Biodegradation* **1**: 79–92.

Wei Y, Chen Z, Wu F, Li J, ShangGuan Y, Li F, Zeng QR, Hou H. **2015**. Diversity of Arbuscular Mycorrhizal Fungi Associated with a Sb Accumulator Plant, Ramie (Boehmeria nivea), in an Active Sb Mining. *Microbiology biotechnology* **25**: 1205–1215.

White JA, Charvat I. **1999**. The mycorrhizal status of an emergent aquatic, Lythrum salicaria L., at different levels of phosphorus availability. *Mycorrhiza* **9**: 191–197.

Wilson SC, Jones KC. **1993**. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environmental Pollution* **81**: 229–249.

Wright DP, Scholes JD, Read DJ, Rolfe SA. **2005**. European and African maize cultivars differ in their physiological and molecular responses to mycorrhizal infection. *New Phytologist* **167**: 881–896.

Wu Q-S (Ed.). **2017**. *Arbuscular Mycorrhizas and Stress Tolerance of Plants*. Singapore: Springer Nature.

Wu N, Huang H, Zhang S, Zhu Y-G, Christie P, Zhang Y. **2009**. Phenanthrene uptake by Medicago sativa L. under the influence of an arbuscular mycorrhizal fungus. *Environmental Pollution* **157**: 1613–1618.

Wu G, Li X, Coulon F, Li H, Lian J, Sui H. **2011a**. Recycling of solvent used in a solvent extraction of petroleum hydrocarbons contaminated soil. *J. Hazard. Mater.*: 533–539.

Wuana RA, Okieimen FE. **2010**. Phytoremediation Potential of Maize (Zea mays L.). A Review. *African Journal of General Agriculture* **6**: 275–287.

Wu FY, Yu XZ, Wu SC, Lin XG, Wong MH. **2011b**. Phenanthrene and pyrene uptake by arbuscular mycorrhizal maize and their dissipation in soil. *Journal of Hazardous Materials* **187**: 341–347.

Xie X, Weng B, Cai B, Dong Y, Yan C. **2014**. Effects of arbuscular mycorrhizal inoculation and phosphorus supply on the growth and nutrient uptake of Kandelia obovata (Sheue, Liu & Yong) seedlings in autoclaved soil. *Applied Soil Ecology* **75**: 162–171.

Xun F, Xie B, Liu S, Guo C. **2015**. Effect of plant growth-promoting bacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) inoculation on oats in saline-alkali soil contaminated by petroleum to enhance phytoremediation. *Environmental Science and Pollution Research* **22**: 598–608.

Yang G, Liu N, Lu W, Wang S, Kan H, Zhang Y, Xu L, Chen Y. **2014**. The interaction between arbuscular mycorrhizal fungi and soil phosphorus availability influences plant community productivity and ecosystem stability. *Journal of Ecology*: 1072–1082.

Yu D-Y, Kang N, Bae W, Banks MK. **2007**. Characteristics in oxidative degradation by ozone for saturated hydrocarbons in soil contaminated with diesel fuel. *Chemosphere*: 799–807.

Zhang L, Jiang C, Zhou J, Declerck S, Tian C, Feng G. **2016**. Increasing phosphorus concentration in the extraradical hyphae of Rhizophagus irregularis DAOM 197198 leads to a concomitant increase in metal minerals. *Mycorrhiza*.

Zhou X, Zhou J, Xiang X, Cébron A, Béguiristain T, Leyval C. **2013**. Impact of Four Plant Species and Arbuscular Mycorrhizal (AM) Fungi on Polycyclic Aromatic Hydrocarbon (PAH) Dissipation in Spiked Soil. *Pol. J. Environ. Stud:* **22**: 1239–1245.

Zocco D, Van Aarle IM, Oger E, Lanfranco L, Declerck S. **2011**. Fenpropimorph and fenhexamid impact phosphorus translocation by arbuscular mycorrhizal fungi. *Mycorrhiza* **21**: 363–374.