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Metal effects in *Fraxinus angustifolia* and its endophytic communities

Efeitos de metais em *Fraxinus angustifolia* e sua comunidade endofítica

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à Mãe e ao Pai

o júri

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palavras-chave

fitoremediação, metais, *Fraxinus angustifolia*, bactérias endofíticas, bactérias da rizosfera, PCR-DGGE

resumo

A contaminação de solos com metais é um problema ecológico grave que requer medidas de resolução urgentes. As metodologias de remediação convencionais revelam-se muitas vezes ineficazes e muito dispendiosas. O processo de fitoremediação surge como uma alternativa promissora para a recuperação de solos, a aplicar de um modo económico e com maior potencial para recuperar os serviços do ecossistema. Os programas de fitoremediação para além de se debruçarem sobre a escolha da espécie vegetal mais adequada para o processo, devem igualmente debruçar-se sobre as comunidades microbianas associadas às raízes das plantas, nomeadamente as bactérias endofíticas e da rizosfera. No presente trabalho, é estudado o potencial da espécie Fraxinus *angustifolia* para a fitoremediação de solos contaminados com metais, recolhidos na área de exploração de uma mina de urânio abandonada, na Cunha Baixa, Mangualde, Portugal. A estratégia assumida consistiu na exposição das plantas ao solo contaminado, a um solo de referência e a um solo controlo, por um período de aproximadamente 3 meses. Com o fim de avaliar a capacidade de *F. angustifolia* de resistir ao solo contaminado, durante a exposição aos diferentes solos acompanhou-se o crescimento e o estado fisiológico das plantas medindo um conjunto de parâmetros bio- e fisiológicos (crescimento acima do solo, área foliar, conteúdo hídrico relativo, máxima eficiência e rendimento do fotosistema II e conteúdo foliar em clorofila *a*, clorofila *b*, carotenoides, prolina e malondialdeído) ao longo do período de exposição. Ademais, procedeu-se à análise genética dos perfis das comunidades bacterianas (endofíticas e rizosfera) associadas às raízes de *F. angustifolia*. Esta análise foi realizada através do método de PCR-DGGE, tendo como alvo uma região conservada 16S rDNA, antes e após a exposição aos diferentes solos. Apenas o parâmetro “crescimento acima do solo” se revelou, ao longo de toda a experiência, claramente indicativo do efeito negativo das propriedades do solo proveniente da mina nas plantas. Nos restantes parâmetros, foi observada uma resposta positiva das plantas expostas à contaminação, tendo demonstrado capacidade de manter o seu estado fisiológico ou, após oscilações, retomar ao estado normal. Comparativamente às comunidades bacterianas analisadas pré-tratamento, as alterações dos perfis das comunidades foram notáveis, principalmente aquelas referentes às plantas expostas ao solo contaminado. As comunidades referentes às plantas do solo controlo e referencia apresentaram maior similaridades entre si e entre a análise pré-tratamento. O estudo fisiológico demonstrou que, quando expostas ao solo contaminado, as plantas *F. angustifolia* apresentam capacidade de resistência e adaptativa às condições adversas, demonstrando potencial passível de ser explorado para fins de fitoremediação. Foi igualmente demonstrado que as alterações exercidas sobre as comunidades bacterianas das raízes expostas ao solo contaminado resultaram em perfis consideravelmente diferentes dos observados nas restantes comunidades. O desempenho das plantas pode estar intrinsecamente relacionado com estas alterações microbianas.

keywords phytoremediation, metals, *Fraxinus angustifolia*, endophytic bacteria, rhizosphere bacteria, PCR-DGGE

abstract

The contamination of soils with metals is a serious ecological problem requiring urgent measures to counteract the impacts. The conventional remediation techniques are, most of the times, inefficient and expensive. Phytoremediation comes as a promising alternative for the cleaning of the soils, to be applied in a more economic and eco-friendly manner, with a great potential for recovering ecosystem services. Besides looking for the best suitable plant species for the task, phytoremediation programs should also focus in the microbial communities associated with the roots of the plants, namely endophytic and rhizosphere bacteria. In this work, it was studied the potential of the species *Fraxinus angustifolia* for the phytoremediation of metal contaminated soils, collected in the Cunha Baixa uranium mine area (Mangualde, Centre of Portugal). The planned strategy consisted of the exposure of the plants to the contaminated soil, a reference soil and a control soil, for a period of about 3 months. In order to access the ability of *F. angustifolia* to tolerate the contaminated soil, growth and physiological performance of plants, a set of bio-physiologic parameters (above ground growth, leaf area, relative water content, maximum efficiency and quantum yield of photosystem II and leaf chlorophyll *a* and *b*, carotenoid, proline, malondialdehyde contents) were measured during the test. Furthermore, the analysis of the genetic profiles of the bacterial communities (endophytic and rhizosphere) associated with the roots of *F. angustifolia* was also performed. This analysis was carried out through the PCR-DGGE technique, targeted for a conserved region of 16S rDNA, pre- and post-treatment. Only the “above ground growth” parameter clearly showed to be, throughout the course of the experiment, an indicator of the negative effects of the properties of the mine soil on the plants. Concerning the remaining parameters, a good response of the exposed plants was observed, which showed the ability to maintain their physiologic status or, after some variation, return to a normal state. In comparison with the bacterial communities profiles analyzed pre-treatment, the alterations of the profiles were notable, principally those corresponding to the contaminated soil. The communities related to the plants from the control and reference soils showed more similarity between each other and the pre-treatment analysis. This physiologic study demonstrated that, when exposed to the contaminated soil, *F. angustifolia* plants had the ability to resist and adapt to the adverse conditions, revealing a potential which can be exploited for phytoremediation. It was also possible to demonstrate that the changes exerted on the bacterial communities from the roots exposed to the contaminated soil resulted in profile considerably different from those observed on the remaining communities. The performance of the plants might be related to these microbial alterations.

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Abbreviations and Acronyms

°C – Celsius Degree

Φ_{PSI} – Photosystem II Quantum Yield

ANOVA – Analysis of Variance

APS – Ammonium Peroxodisulfate

BSA - Bovine Serum Albumine

Chl *a/b* – Chlorophyll *a/b* Ratio

DGGE – Denaturing Gradient Gel Electrophoresis

dNTP – n Deoxyribonucleotide

EDTA – Ethylenediaminetetraacetic Acid

Fv/Fm – Photosystem II Maximum Efficiency

g – Relative Centrifugal Force

h – Hour

IAA – Indole-3-acetic Acid

M – Molar

MDA – Malondialdehyde

min – Minutes

MS_x – Mean Square

PBS – Phosphate Buffered Saline Solution

PCR – Polymerase Chain Reaction

PSII – Photosystem II

rDNA – Ribossomal RNA Coding DNA

RM – Repeated Measures

rpm – Revolution Per Minute

RWC – Relative Water Content

SD – Standard Deviation

TBA – Thiobarbituric Acid

TCA – Trichloroacetic Acid

TEMED – Tetramethylethylenediamine

Tris – Tris(hydroxymethyl)aminomethane

UPGMA – Unweighted Pair Group Method With Arithmetic Mean

UV – Ultra Violet Light

1. Introduction

Many human actions and activities can harm the environment in numerous different ways. Since the 70's bigger attention has been brought to this subject, in order to evaluate the dangers associated with it and find ways to fix the damaged environment (Neves and Matias 2008). The contamination of soils with metals and other pollutants is a serious problem that needs to be addressed with urgency. The soil grants shelter and food to many organisms, but in a way it also retains and concentrates chemicals, providing a natural protection of the water resources and the environment. Therefore when soils are greatly contaminated with metals, severe impacts can occur on the communities sustained by them (Abrahams 2002; O'Halloran 2006).

Arising mostly from anthropogenic activities, such as mining explorations (Figure 1.1), factory effluents, waste disposal, gas exhaust, fuel production and agricultural amendments this contamination not only has a great impact on the environment and wildlife, but might also have dire effects on human health as well (Prasad and Freitas 2006; Antunes et al. 2008; Pereira et al. 2008; Hasan et al. 2009). Soil contamination can easily lead to an uncontrolled spread of dangerous contaminants that can alter ecosystems diversity and functions, enter food chains, cause disease and invade neighbor ecosystems (Abrahams 2002; Evseeva et al. 2003). Depending on the nature of each metallic element, its legacies to the environment can be either radiological or non-radiological which themselves can end up resulting in carcinogenic and non-carcinogenic effects on human health (James 1996; WHO 2008; Nwoko 2010). For instance, metals such as uranium are of alarming concern for they can bring forth a double negative effect from acting both as a poisonous toxicant metal and as a hazardous gamma radiation emitting radioactive particle. Also, metals persist in the environment, aggravating its negative potential and empathizing the need to remove or neutralize them (Prasad and Freitas 2006). It is important to note that many of these metals actually have an important role in the physiology of some living organisms. For example, metals such as B, Cu, Mn, Mo, Ni and Zn are micronutrients needed by plant species and their deficit can cause development impairments (Phålsson 1989; Gallego et al. 1996; Mitsios and

Danalatos 2006). Thus, the real problem comes when the contamination of soils with metals reaches critical levels, especially for those with no beneficial physiological roles.

Figure 1.1 – Grounds from a surrounding area of a uranium mine in Cunha Baixa, Portugal contaminated with metals.

The dangers of these impacts turn into a much bigger issue when the main sources of pollution release are in the vicinity of dwelling places. Largely depending on the chemical and physical composition of the surrounding soil, the mobility of dangerous elements can be of great concern as they can easily reach water resources. Soil and water resources of the surrounding dwelling areas may be used either for agricultural or directly for drinking purposes, being, this way, available to humans (Pereira et al. 2008; Carvalho et al. 2009b, 2009c; Pereira et al. 2009). Additionally, some studies have already focused on the leaching and percolation of metals into private water wells and the transfer of metals to vegetables cultivated on contaminated soils or irrigated with contaminated water (Antunes et al. 2008; Neves and Matias 2008, Neves et al. 2008; Pereira et al. 2008; Neves and Abreu 2009; Pereira et al. 2009). In some cases it was found that both water

and food containing concerning levels of metals, pose a risk to consumers (Sarić et al. 1995; Hakonson-Hayes et al. 2002; Neves and Abreu 2009). In fact, some studies have also reported considerable increases in the concentrations of metals in various tissues of people living in some urbanized areas situated near the contamination (Fergusson 1991).

It is not just human life that is at stake. Wildlife can also be directly or indirectly affected by metal contamination. Organisms that contact directly with metals can suffer from their effects, while others end up being affected by means of intermediate events. For instance, when metals integrate or interact with plant tissues they can disrupt the normal growth and dissemination process of plant species (Love and Babu 2006). This, in turn, will consequently have effects on animal life and the landscape. The disappearance of some plant species due to contamination may represent a significant loss of habitat and food for animal species that may die or need to reallocate. Many plants are also linked to various soil properties and changes in the vegetation may cause soil alterations (Binkley and Giardina 1998). Animals that live and feed on the contaminated soil can also accumulate the contaminants in their tissues, which can lead to disease and their transference to other animals in the food chains (Sekhar and Prasad 2006). Several studies have shown that birds accumulate substantial amounts of metals by ingesting insects that feed on contaminated material (Sillanpää et al. 2008). In conclusion, the presence of metals in the various components of the environment can be problematic for all living organisms (Sanità di Toppi and Gabbrielli 1999).

In places where lands are highly contaminated, it becomes urgent to come up with remediation plans to secure the ecosystems that depend on them. In most places, the applied conventional methods do not always directly address the main issue of the decontamination. Among them, physical, chemical and engineering techniques, like encapsulation, “dig-and-dump” and capping are the ones mainly used which aside from being expensive, also fail to decontaminate larger areas in an eco-friendly manner (Martin and Bardos 1996; Huang et al. 1998; Pulford and Watson 2003). For example, dumping contaminated soils into a landfill only transfers the pollutants from one site to another, without neutralizing the contaminants. Furthermore, the excavation required for

the process may cause the disruption of the landscape and soil properties and structure (Jadia and Fulekar 2009). Other methods like soil washing also have unwanted effects such as soil structure and fertility issues, biological activity perturbation and high costs (Pulford and Watson 2003). Due to such factors the remediation process in large contaminated areas calls for some innovative and more appealing measures.

The remediation programs should not only be aware of the major risks intrinsically related to the soil contamination, but should also try to establish the needed conditions for the re-use of the lands for: agriculture or reforestation purposes; the (re-)habitation of the lands; the healthy development of local flora and fauna (Pereira et al. 2009). Consequently, to correctly address the problem, important criteria like the extent of contamination, the uses of water and soil resources by the local population, the geological risks and even the effects on the landscape, should always be considered (Echevarria et al. 2001; Oliveira et al. 2002; Nero et al. 2003). In summary, the remediation programs applied, should guarantee as much as possible the restoration of at least some ecosystem services previously provided.

1.1. Phytoremediation

Phytoremediation or botanical bioremediation is a class of bioremediation that relies on the use of plant species to remove or render harmless contaminants from soils, water courses and sediments (Chaney et al. 1995, 1997; Salt et al. 1995; Pradhan et al. 1998). It is indeed a technology that has grown in the last years as a promising method for metal and radionuclide remediation, and investigation towards finding the right plant species for the task is being carried all around the globe (Huseyİnova et al. 2009). Phytoremediation is a process that can take various routes and it is now commonly divided in several different classes, as follows (Huang et al. 1998):

- Phytoaccumulation or phytoextraction: this process refers to the concentration of metals in the harvestable parts of a plant, as they remove them

from the soil. Usually requires the plantation of plant species that accumulate the desired metal at high rates (Raskin et al. 1997). After a period of time, the harvesting of the aerial parts of the plant allows the removal of the metals from the site. Metal containing material can later be treated, which is often done by volume reduction, metal concentration, burning under controlled conditions and recycling the metals of economic valuable elements. If valuable metals are being extracted, its recovery is processed in a cost effective manner (Wang et al. 2006; Barceló and Poschenrieder 2003; Mukhopadhyay and Maiti 2010). In the case of radionuclide containing matter, the treatment involves the safely disposal of the treated material as waste (Dushenkov 2003). It has been proposed that, for phytoextraction to be an efficient process, plants should accumulate in their dry biomass no less than 1% of the soil total metal content (Karami and Shamsuddin 2010). Despite the lack of many evidences of the effectiveness of this technique at larger scales, improvements have been increasing and its application has been demonstrated in the effective removal of some metals and radionuclides (Huang et al. 1998, Dushenkov et al. 1999; Dushenkov 2003).

- Phytodegradation: also known as phytotransformation, this type of remediation consists in the capacity of plants to metabolize and degrade organic pollutants from contaminated soils and water resources. Depending mostly from the activity of their own enzymes, plants may be helped by the action of microorganisms, despite not being dependent on them (Salt et al. 1998; Turnau et al. 2006; Mukhopadhyay and Maiti 2010). Phytodegradation addresses mainly organic contamination and since metals cannot be degraded by this mechanism it is not adequate for their removal.

- Phytostabilization: represents the reduction of mobility and/or bioavailability of contaminants by immobilization on the substrate or on the roots, or prevention of migration (Vangronsveld et al. 1995; Salt et al. 1998). This is usually accomplished by means of complexation/precipitation, metal valence

reduction and sorption (Ghosh and Singh 2005; Prasad and Freitas 2006). Phytostabilization also refers to the use of vegetation to directly stabilize the contaminated soils and adjacent sediments, thus, big perennial trees with deep and dense root systems are good choices for this type of remediation (Chaney et al. 1997). Since pollutants remain in the soil, this technique does not accomplish the cleaning of the soil but in turn it is a very useful technique to rapidly preserve ground and surface water (Ghosh and Singh 2005; Vandenhove 2006). Studies demonstrated that chemical reduction of dangerous species of chromium and lead into safer ones, by plants, appears to be effective (Chaney et al. 1997; Cotter-Howells and Caporn 1996). A study showed that the roots of *Agrostis capillaris* plants growing in a Pb contaminated soil promoted the formation of pyromorphite, an insoluble and non-bioavailable form of Pb (Cotter-Howells and Caporn 1996).

- Phytovolatilisation: in this mechanism plants perform the volatilization of pollutants to the atmosphere by transpiration. Initially the contaminants are taken into the roots and transported to the above ground parts. Meanwhile they are converted to modified forms, after which they are finally volatilized to the atmosphere (Salt et al. 1998; Burken and Schnoor 1999). Phytovolatilization offers the advantage of getting around the need of harvesting, as it happens with the phytoextraction, but on the downside the volatilization of these elements requires careful pondering (Salt et al. 1998). The phytovolatilization of selenium (Se) is a well known case of a metal element that is transformed to a modified form (typically to dimethylselenide) and volatilized at high rates, although mercury is also known to be eliminated by this mechanism (Neumann et al. 2003; Mukhopadhyay and Maiti 2010). Phytovolatilization has also been applied in the remediation of radionuclides. Trials with tritium (^{3}H), an hydrogen isotope, have shown that phytovolatilization can be a safer, cost-effective alternative to the remediation of radionuclides in the environment (Dushenkov 2003).

- Rhizofiltration: in this case, roots directly absorb metals from contaminated waters. Rhizofiltration applies to the treatment of surface and waste waters, industrial effluents and mine drainages, and also agricultural runoffs (Raskin et al. 1997). Acting as filters, the roots either precipitate, concentrate or absorb the pollutants in order to retain the contamination (Dushenkov 2003; Ghosh and Singh 2005; Nwoko 2010). For this task plants must have specific additional features like hypoxia tolerance and higher absorption surface, but do not necessarily need to have high accumulation rates of the contaminants (Dushenkov et al. 1995; Ghosh and Singh 2005; Mukhopadhyay and Maiti 2010). Early in the 50's, the first effort to control contamination using rhizofiltration revealed that plants like *Cladophora glomerata* and *Elodea Canadensis* were able to absorb considerable amounts of the radionuclides ^{137}Cs and ^{90}Sr (Timofeeva-Ressovskaia et al. 1962). Since then many other species have proven their utility as rhizofiltrators in the remediation process of other elements (Dushenkov et al. 1995, 1997).

Hence, by ways of immobilizing, degrading, transferring and accumulating pollutants, this mechanism seems to have the potential to become a successful "green" sustainable alternative for soil decontamination (Cunningham et al. 1995; Salt et al. 1996; Dickinson 2000). Besides, in comparison, the costs of reforesting a given land are almost insignificant comparing to the costs of soil replacement and other conventional methods. As a result, unless there is an urgent need to reuse a specific land in a short period of time, phytoremediation appears to be a more suitable replacement approach (Bollag et al. 1994; Riddell-Black 1994).

However, this process is far from perfect and some disadvantages can be pointed out. Probably the most important one is the long period of time needed to get efficient outcomes. It may take several years until plants have grown sufficient biomass and accumulated enough amounts of metals (Shah and Nongkynrih 2007). Another major drawback is the treatment and disposal of contaminated harvested material (Raskin et al. 1997). If controlled conditions cannot be ensured then contamination spread can occur.

Additionally, the requirements for particular environmental and climatic conditions, soil characteristics and nutritional *status* are also some of the challenges phytoremediation still faces (Karami and Shamsuddin 2010).

1.1.1. The process of phytoremediation and tree species

With the simple aim of cleaning up soils, phytoremediation might seem, at first, like a straightforward process. However, the process involves complex physiologic-biochemical mechanisms and interactions between the soils, plants and other organisms (Raskin et al. 1997). When it comes to choosing the plants to be applied in the phytoremediation there are certainly some preferred qualities. The investigation of this subject and its associated mechanisms has targeted plant species that vary from very small plants to big trees. The present study will focus mainly on tree based remediation.

Essentially because of their lack of mobility, trees usually face the task of adapting to environmental changes, in order to avoid death (Camejo et al. 2005). This process might involve both changes in metabolic activities and morphological characteristics. Nonetheless, given their long timed generation, trees species are not under a fast and strong natural selection for metal tolerance (Pulford and Watson 2003). Despite this low adaptation, which in turn led to few tolerant ecotypes, the trees that can still grow under contamination appear to survive for long periods of time and lack usual signs of toxicity (Dickinson et al. 1992; Kahle 1993). It is true that in most of this cases plants grow slower, but their strong persistence does point out the development of mechanisms of resistance against highly concentrated contaminants. Different research works look for many of such mechanisms, in terms of phytoremediation. Still it is important to note that facultative tolerance may also be behind the survival of some non-naturally selected trees growing in metal contaminated lands. The redistribution of the roots to less contaminated areas is an example of such tolerance (Watmough and Dickinson 1995).

Compared to smaller plants, tall trees usually have deeper root systems which allow them to reach soil and water sheets at a greater depth. Not only does this allow

them to decontaminate larger underground areas, but it also confers trees the capability to better resist drought and transpire for a longer time than short-rooted plants (Domínguez et al. 2008). Also, trees usually have higher biomass yields than smaller plant species, which enables them to accumulate more quantities of pollutants (Pulford and Dickinson 2006). The leaves may concentrate metals transferred from the roots and stems, but direct surface deposition is also a way of concentration of elements in the plant's tissues (Huseyİnova et al. 2009). If the local environment of a contaminated land presents propitious conditions for metals deposition in the leaves, taller trees may be preferable to smaller species since higher canopies reduce the concerns about herbivorous predation (Domínguez et al. 2008). Trees are also fairly accepted by the public as ecological and esthetical elements for rural and urban areas, and can have important commercial uses (Domínguez et al. 2008).

The exploitation of trees might also entail some weaknesses. When natural tree-related phenomena like litter fall, specific root/microbe interaction and soil acidification take place, increases in metal mobility and bioavailability can occur, leading to the spread of the contamination (Domínguez et al. 2008).

Some plants are able to concentrate the accumulated contaminants in their roots by root immobilization. This physiological capability should function as a protection against the soil contamination, but some plants cannot achieve this. For these plants, once the contaminants are captured at the root level they are then transferred to the above ground parts (Pulford and Watson 2003). Nowadays, most scientists believe that for the phytoremediation process to be most efficient, it is truly important that the translocation of pollutants from the root system to the above ground parts occurs at high rates (Mihalík et al. 2010; Kholodova et al. 2011). On the basis of these thoughts stands mainly the fact that by concentrating metals in the harvestable parts of the trees, they can be easily accessed and extracted from the site – phytoextraction method (*cf*. section 1.1.). Furthermore, it has been reported that high levels of some metals (like Ni) in the leaves of the plants confers them protection against insect predation and bacterial and fungal infections (Boyd et al. 1994; Raskin et al. 1997). Also, when translocated to the

upper parts, some contaminants can integrate the wood and bark tissues, which are natural sink tissues for some metals. Allied to the fact that these tissues are continuously produced every growing season, the very long decomposition time of these metabolic inactive compartments could permit the immobilization of target metals for a good amount of time, if they were to be accumulated by the plant (Lepp 1996).

Nonetheless, the movement of pollutants from the roots to the stems and leaves of the plant (or the direct absorption by the leaves) can still be of concern as it may result in several negative consequences. Situated in the base of food chains, edible plants serve as food to many organisms and every part of a plant can be eaten. Should any of these parts of a plant concentrate the target substances of the phytoremediation process, and they could easily reenter food chains (Salomons et al. 1995; Wislocka et al. 2006). In the case of deciduous tree species, the seasonal leaf fall may just accelerate the process (Bañuelos and Ajwa 1999). Therefore, in some circumstances it can be advantageous to use arboreal species that can better retain a great amount of pollutants in their root systems. This could probably minimize the need for a bigger aboveground biomass productive species and possibly assist the control of the spread and entrance of contaminants in food chains and water resources. Ultimately, the roots could be safely extracted to further treatment or elimination, and the aerial parts of the trees could be recovered for other potential uses. According to these ideas, phytostabilization (*cf*. section 1.1.) seems like a more appropriate technique since it helps to retain the metals in a restricted area preventing its mobilization to other places and into food chains.

1.1.2. Plant tolerance and accumulation mechanisms

Not every plant is able to deal with the amounts of contamination present in the soil. Even worse, not all of those that can survive under such conditions are capable of extracting or degrading the pollutants at acceptable rates (Pulford and Watson 2003). Even intra-species cultivar metal accumulation variability is observed (Yang et al. 1995). These are just some of the aspects to have in mind when choosing the appropriate plant species as a phytoremediation model. Clearly the accumulation of metals in plants

depends on various separated factors ranging from the soil properties, to total and available metal concentrations and also plant physiology related parameters (Antoniadis et al. 2006).

When the plants are able to safely accumulate metals in the roots and allow them to move up to the above ground tissues, some mechanisms must be active in order to confer them such tolerance (Figure 1.2). They might be principally associated with elevated internal requirements for such metals and highly expressed metal sequestering mechanisms (Shen et al. 1997). The "perfect" plant for phytoremediation is yet to be found, since there always seems to be a balance between some of the good and the bad properties of a species. Ideally, these plants (from the phytoextraction point of view) should:

- Be capable of tolerating the presence of the contaminants in its cells, even if at high concentrations (Punshon et al. 1996);
- Show high rates of contaminant accumulation from the soil (Prasad 2006);
- Have the ability to translocate the contaminants from the root system to the above ground parts efficiently (Mihalík et al. 2010);
- Grow in low nutrient content soils and have a deep root system (Punshon et al. 1996);
- Have high growth rates and biomass levels (Landberg and Greger 1994; Punshon et al. 1996);
- Defend itself against predation to resist more and avoid metals entry in the food chains (Prasad 2006).

Even though not directly related to the remediation itself, the plants could preferably have another economically viable use, although this refers mostly to those that are not able to accumulate in the aerial parts.

1.1.3. Hyperaccumulation

There is a rare group of plants with some attributes that makes them, indeed, specialists in phytoremediation. Named as hyperaccumulator plants, they have increasingly earned interest in the field as potential models for phytoremediation (Chaney et al. 1997). These plants can mobilize and accumulate from 10 up to 500 times more elements than normal crops, without undergoing critical yield reduction (Chaney et al. 1997; Salt et al. 1998). Additionally, they are able to accumulate greater amounts of metals from smaller metal pools than normal crops (Mitsios and Danalatos 2006). Hyperaccumulators were first defined as natural habitat growing plants with at least 1000 $\mu g.g^{-1}$ of Ni concentration in its leaves (for a Ni hyperaccumulator plant) (Brooks et al. 1977). This definition was later updated as new phytoaccumulators were studied (Chaney et al. 1997).

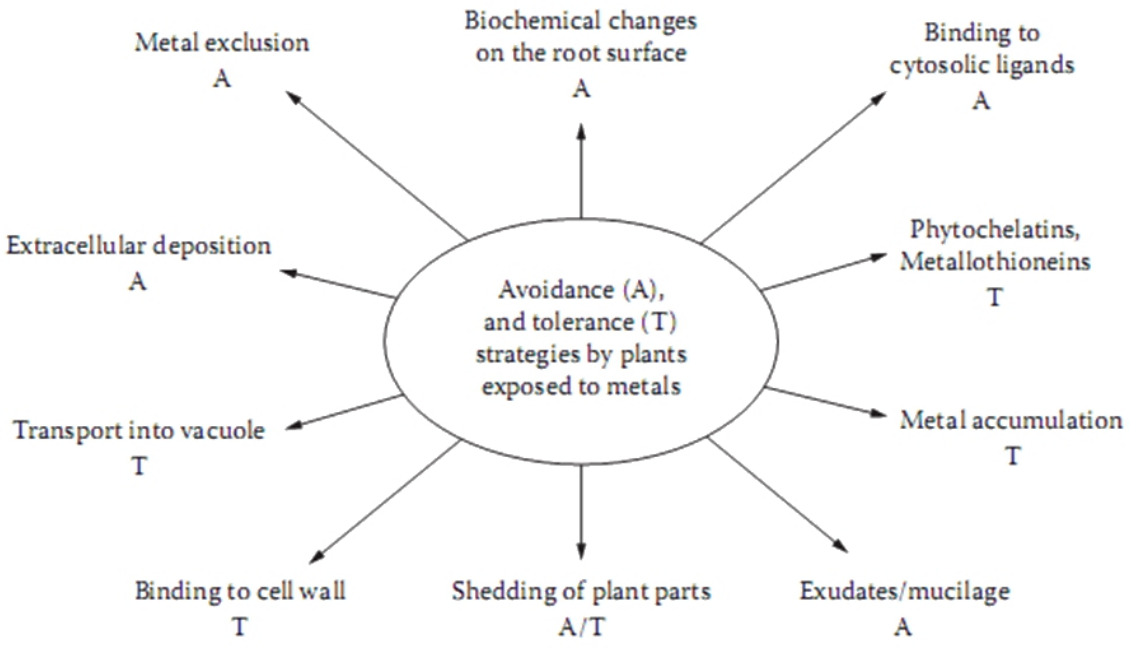

Figure 1.2 – Avoidance and tolerance mechanisms used by plants when exposed to high doses of metals (*adapted from Shaw et al. 2006*)

Despite being known as rare, not all hyperaccumulators are completely suitable for phytoremediation. It has been suggested that, for the means of soil remediation, the

hyperaccumulation ability is more important than that of having high biomass rates (Chaney et al. 1997). However, if a given hyperaccumulator shows the downside of, for instance, being extremely small, having too low biomass yield, growing very slowly or not accumulating a specific element, it may not be a good choice. The plant *Thlaspi caerulescens* comes as an example of a Zn hyperaccumulator that has no great use in the field due to its small sized and slow growing specimens (Figure 1.3) (Ebbs and Kochian 1997).

Figure 1.3 – Live specimens of the Zn hyperaccumulator *Thlaspi caerulescens* (*from http://www.hlasek.com)*

There is another class of plants often considered for phytoremediation programs. Plants from this class are called excluders due to the fact that they neither permit metal uptake in their roots (or only very small amounts), nor accumulate them in the above ground tissues (Robinson et al. 2006; Shaw et al. 2006). Since they are basically equipped with avoidance mechanisms, and do not directly interact much with the contaminants,

these are indeed, not very useful for the phytoremediation (extraction) process itself. However they are key helpers to the whole remediation process working as soils stabilizers, preventing the spread of pollutants by soil erosion and reducing metals' availability to other plants (used in phytostabilization) (Shaw et al. 2006).

1.2. Rhizosphere and endophytic bacteria in metal phytoremediation

There is yet another important factor concerning the phytoremediation process – the non-pathogenic bacterial communities associated to the plants' roots. Two major types of bacterial communities are known to have relevant effects on plants. The first is the bacteria that grow on or around normal roots' surface, called the rhizospheric bacteria (Doty 2008; Mitsios and Danalatos 2006). The rhizosphere refers to the fraction of soils that adheres to the roots of the plants, about 1 mm, and that is directly influenced by them (Gomes et al. 2001; Mitsios and Danalatos 2006). The other type is the bacteria that live inside the plant tissues, which are named endophytic bacteria (Doty 2008). Endophytic bacteria belong to a group of organisms called endophytes. These microorganisms are known for establishing an endosymbiotic relationship with the plants, in practically any of its organs from roots to seeds (Compant et al. 2010). They are not harmful to the plant in any visible way. In fact, they are typically beneficial to the plant in various manners (Weyens et al. 2011). These symbionts can be isolated either from the disinfected surface of the plants or directly from deep inside the tissues (Nimnoi et al. 2010).

Both seem to have an important role in the development of many plants species, concerning plant growth and health. Additionally, these bacteria may even be responsible for specific soil properties (Gomes et al. 2001; Compant et al. 2010). Before being considered for phytoremediation, these kinds of bacterial communities were used in the past for agricultural and forestry purposes, in order to improve plant growth and resistance to diseases (Karami and Shamsuddin 2010). However, interactions between plants and these microbial communities are rather complex and often ignored in studies

concerning metal uptake by plants. Hence, most details about such interactions are still unknown (Shtangeeva 2006).

1.2.1. Interactions between rhizosphere/endophytic bacteria with plants

The growth of a plant may be influenced by microorganisms, but in turn, the roots are also responsible for the bacterial communities' composition and structure. The benefits of bacteria on the plant's ability to grow and live healthy can depend on numerous factors, which reflect a quite complex interaction (Weyens et al. 2011). The main mechanisms utilized by bacteria to improve plant growth and productivity are: the production of a number of phytohormones that support the plants' growth (Glick et al. 1999; Lee et al. 2004; Mendes et al. 2007); the containment or elimination of injurious microorganisms (Cook et al. 1995; Walsh et al. 2001; Winding et al. 2004); indispensable nutrient solubilization (mainly phosphorus) and N_2 fixation (Christiansen-Weniger and Van Veen 1991; Höflich et al. 1994; Roesch et al. 2007); the secretion of enzymes to reduce plant ethylene levels and the sequestration of metals with specific siderophores (Glick et al. 1995; Reed and Glick 2005). This way, they not only directly improve the development process of the plants, but also grant them protection against pathogens that can cause disease and seriously decrease plant growth (Chanway 1997; Bent and Chanway 1998). Similarly to what was mentioned before for the hyperaccumulator plants, one can only hope to find the perfect bacterial population as it always seems to be a balance between some good and bad traits. Indole-3-acetic acid (IAA) is a phytohormone known for promoting plant growth by enhancing root length and distribution and that is produced by some microorganisms in favor of the plants' growth (Barazani and Friedman 1999; Kuklinsky-Sobral et al. 2004). However, what can be, for instance, a good IAA producing bacterium might not be the best nutrient solubilizer, and vice-versa (Dias et al. 2009).

On the other hand, bacterial communities do not depend solely on environmental conditions and soil properties. Root exudate patterns, allow the plants to alter soil properties, change pH values and even change substrate availability in its vicinities, which controls bacterial proliferation (Miller et al. 1989; Jaeger et al. 1999; Yang and Crowley

2000). Roots can also secrete secondary compounds capable of controlling gene expression on the bacteria (Nwoko 2010). Differences in exudates composition, root location, growth stages and even plant species are all known to selectively influence microorganism population growth and structural diversity (Miller et al. 1989; Watkinson 1998; Myers et al. 2001). It is also with its root exudates that the plants make metals more bioavailable. Being mainly organic exudates (e.g. organic acids), some of the compounds released can create strong chelates with the metal elements making them more easily absorbed by the plant (Cieśliński et al. 1998).

In fact, rhizosphere and endophytic bacterial inoculants have already been specifically used with success in the acclimatization phase of some species to promote their growth (Khalid et al. 2004; Dias et al. 2009). It is important to note, though, that the positive effects brought up to a plant, are most probably not due to a single species but instead a multiple effect achieved by a mixed community of symbiotic bacteria (Dias et al. 2009).

Protecting and promoting the growth of a plant are very important features of bacterial communities but it does not end there. Bacterial population can actually enhance the tolerance and uptake of metals by the plants species. Either by making metals more available to plants outside the roots or by absorbing and immobilizing them inside the vegetal tissues, root-associated bacteria may facilitate the phytoremediation process almost directly (Yong and Macaskie 1998). It has even been suggested that bacterial influence may actually be more relevant than root exudates alone (Jackson 1993; Zhou et al. 2004). Bacteria achieve this by means of oxidation-reduction reactions, synthesis of natural chelators and pH changes making metals, otherwise not accessible by plants, available for uptake (Wielinga et al. 1999). Bacterial specific sequestration mechanism can also significantly decrease the levels of phytotoxicity induced by metals (Lodewyckx et al. 2001).

Bacterial communities are not the only microbial populations beneficial to plants. Mycorrhiza are also known to play important roles concerning plant growth and health (Bridge and Spooner 2001; Gomes et al. 2003). Although not much emphasis will be given

to these populations in this work, it is important to stress that advantageous bacterial communities also interact with and contribute to the establishment of favorable mycorrhiza populations near the roots (Turnau et al. 2006).

1.2.2. Analysis of rhizosphere and endophytic bacterial communities

In microbiology, it is only possible to culture in the laboratory a limited small number of the total microbe communities. Coined in 1985 as the 'Great plate count anomaly', this phenomenon has been, up until recently, a huge drawback in studies concerning the analysis of rhizosphere/endophytic bacterial populations (Staley and Konopka 1985). Fortunately, molecular-based techniques came to help overcome such difficulties. Through molecular microbiology evolution, new techniques became more adequate to study the microbial population structure and dynamics, and more specifically spatial and temporal variations in rhizosphere/endophytic bacterial root communities. To replace excessive laborious and time-consuming methods, namely 16S rDNA cloning and sequencing, molecular fingerprinting methodologies have been extensively used (Muyzer et al. 1993; Gomes et al. 2001).

Denaturing gradient gel electrophoresis (DGGE) is a molecular based technique designed to identify small differences between DNA or RNA samples. It consists of an electrophoretic run, where multiple samples are run together on a denaturing gradient built gel (Muyzer et al. 1993). This method relies on the differences in mobility of partially denatured double-stranded DNA molecules. Even single nucleotide changes between samples will result in different band mobilities on the gel (Muyzer et al. 1993). Despite being originally used mainly for detection of point mutation in the medical field, with its almost 100 % of sensitivity when correctly used, DGGE is now also currently used for analyzing complex microbial community profiles (Muyzer and Smalla 1998; Aksoy and Demirezen 2006; Weyens et al. 2009). Furthermore, despite the fast evolution of the sequencing techniques this technique still proves to be a better alternative to direct sequencing by being faster, less laborious and by providing a qualitative and semiquantitative display of a microbial population's constituents (Muyzer et al. 1993).

1.3. General and Specific Objectives

Even though the overall attention over phytoremediation has clearly grown in recent years, precise information about the plant species to be recruited for the task is not enough. Moreover, the knowledge about the structure and dynamics of rhizosphere/endophytic bacterial communities, and their interactions with the plants during phytoremediation, needs more clarification. Karami and Shamsuddin (2010) have summarized some of the most recent studies on the relationship and effects of bacterial communities on plants grown in contaminated lands and the list of both plant and microbial species still appears to be short. There is a need for new investigations that would work on the unknown potential of plant species and its associated bacteria, to improve phytoremediation of metal contaminated soils, as fast as possible.

The present study aims to combine the existing knowledge about phytoremediation and rhizosphere/endophytic bacteria to investigate the potential of native plant species for the remediation of metal contaminated soil.

Belonging to the family Oleaceae the genus *Fraxinus* (commonly ash tree) comprises between 39-65 species of medium and big trees (Kostova and Iossifova 2007). There is not much knowledge about their ability to tolerate and accumulate metals from soils. Also, the research work available focused principally on a main species (*Fraxinus excelsior*) and lacks data regarding quantitative and spatial metal and radionuclides accumulation, physiological data and microbial communities analysis (Haro 2000; Rosseli 2003; Mertens 2004; Pulford and Dickinson 2006; Tlustoš et al. 2006). In this work it will be investigated the potential of a native deciduous ash species, *Fraxinus angustifolia,* for the purpose of phytoremediation.

The detailed goals of the present study are:

i) to evaluate the growth and physiological performance of 1 native tree species, *Fraxinus angustifolia*, planted in a metal and radionuclide contaminated soil from an

abandoned uranium mine in the area of Cunha Baixa, Mangualde, Center of Portugal, under controlled laboratorial conditions;

ii) to analyze several biologic and physiologic parameters (above ground growth, leaf area, leaf chlorophyll a and b, carotenoid, proline, malondialdehyde (MDA) contents, maximum efficiency and quantum yield of photosystem II and relative water content) which should reflect the health state and stress conditions induced in the plants;

iii) to evaluate if the species could be an adequate candidate for the phytoremediation of this mining area;

iv) to study spatial and temporal variations on the rhizosphere and endophytic bacterial communities isolated from the roots of plants grown in contaminated and non-contaminated soils, by DGGE methodologies.

Materials and methods

2.1. Study site and soil substrates sampling

The soils used for the experiment were obtained from a uranium mine located at the region of Cunha Baixa, Viseu (Centre of Portugal). Despite not being active at the moment, this mine remained active for many years until complete closure in 1993 (Pereira et al. 2009). The surrounding area was affected by its activity since both underground and open pit extractions were performed, which resulted in severe impacts in the area. Regarding soil contamination, metal and radionuclides and radiation are probably the most worrying problems. Several studies have already been made in order to evaluate the local ecological risks at the soil level and the area is now under close attention to minimize the impacts (Pereira et al. 2008, 2009; Carvalho et al. 2009a).

For this work two natural soils and an artificial substrate were used. One of the natural soils, the contaminated soil (soil B according to Pereira et al. 2008), was collected in the Cunha Baixa mine area, within the exploitation zone (Figure 2.1). The general physical and chemical characterization of this soil was already made by Pereira et al. (2008, 2009) and is described in Table 2.1 (A and B). This soil was one of the most contaminated soils found in the area, with high total concentrations of Al (25628.5 mg/Kg), Fe (8570.07 mg/Kg), Mn (3321.36 mg/Kg) and U (224.16 mg/Kg). The other natural soil, the reference soil, was collected from a site about 60 Km away from the mine, near Guarda city (Centre of Portugal) and it was characterized as a reference soil by Caetano et al. (unpublished data) (Table 2.1 A and B). Lastly, the artificial substrate, used as a control in the experiments, consisted of a moisturized mixture of non-acid vermiculite and humus substrate/turf at a proportion of 3:2.

2.2. Experimental design

All the plant specimens, belonging to the species *F. angustifolia*, were obtained directly from a nursery. A group of 54 rooted (grown from seed) plantlets were chosen according to their size and apparent good physiological conditions, in order to preserve group

homogeneity. The plantlets still in the soil from the nursery were initially kept in an acclimatized chamber, for 2 weeks. The local conditions of the chamber were as follows: photoperiod of $16h^{L}:8h^{D}$; temperature of 23°C; light irradiance of 160 $\mu mol.m^{-2}.s^{-1}$.

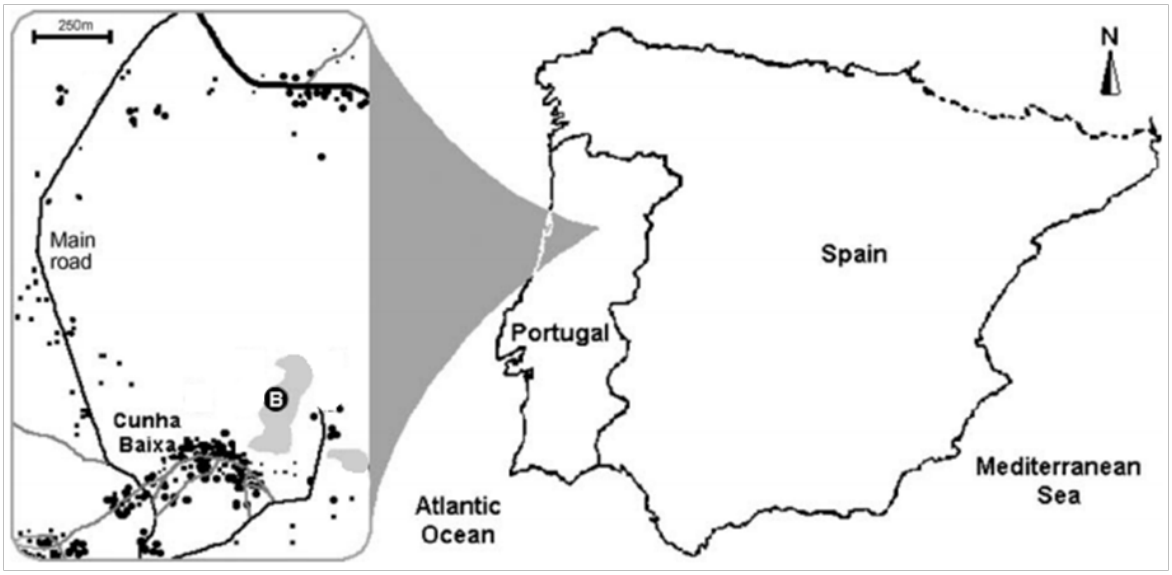

Figure 2.1 – Geographical representation of the Cunha Baixa region where the Uranium mine is situated. B – represents de location from where the "Contaminated Soil" was collected (Pereira et al. 2008).

Table 2.1 A – Summarized physical and chemical characterization of the soil "B" from the Cunha Baixa mine and the "reference soil" from Guarda. Element concentration measured in mg/Kg (± standard deviation); N/A: Not available.

	Soil "reference"	Soil B		Soil "reference"	Soil B
Ag	0.1 ±0.0	N/A	Mn	386.8 ± 77.9	3321.36 ± 42.14
Al	25628.5 ± 5130.0	26023.29 ± 160.04	Mo	0.9 ± 0.2	N/A
B	2.2 ± 0.8	N/A	Na	78.1 ± 14.9	N/A
Ba	45.8 ± 8.0	N/A	Ni	4.6 ± 0.9	53.08 ± 0.40
Be	1.2 ± 0.2	40.40 ± 0.37	Pb	12.5 ± 2.2	6.68 ± 0.87
Cd	0.1 ± 0.1	1.90 ± 0.03	Sb	0.2 ± 0.0	N/A
Co	5.6 ± 1.1	37.34 ± 0.43	Sn	10.4 ± 1.9	N/A
Cr	10.8 ± 2.1	N/A	Sr	N/A	1.76 ± 0.03
Cu	9 ± 1.8	53.38 ± 0.12	U	7.8 ± 1.7	224.16 ± 5.07
Fe	24921.4 ± 4534.4	8570.07 ± 192.84	V	37.8 ± 14.1	N/A
Li	124.4 ± 22.9	N/A	Zn	57.1 ± 8.9	219.67 ± 2.12
Hg	5253.5 ± 1025.5	N/A			

Table 2.1 B – Summarized physical and chemical characterization of the soil "B" from the Cunha Baixa mine and the "reference soil" from Guarda. N/A: Not available; SD: Standard deviation.

	Soil B	Soil "reference"
pH	7.79 ± 0.01	5.91 ± 0.1
Conductivity (mS cm-1 ± SD)	2263 ± 11.55	4860 ± 230
Organic matter content (% ± SD)	7.71 ± 0.06	6.5 ± 0.004
Soil texture class	Sandy clay	N/A
Radioactivity (cps ± SD)	850 ± 349	N/A

Following the acclimatizing period, plantlets were randomly distributed and transplanted for the 2 different natural soils and the artificial substrate. In order to try to maintain their natural structure the natural soils were not sieved nor treated in any way. A total of 20 plantlets were transplanted to the reference and contaminated soils and lastly 10 plants were transplanted to the artificial substrate. Four plantlets were immediately used for the isolation of endophytic and rhizosphere bacteria from their roots (*cf.* section 2.4.1.). Each individual plantlet was planted in a small plastic pot (of about 760 cm^3 of volume) which was filled with the corresponding soil/substrate. The 3 groups of plantlets were then kept in the chamber until the end of the test, for approximately 3 months. Throughout the test, plant maintenance included daily care procedures like regular watering, weed removal and checking for parasites and any other anomalies. Also, every 15 days a nutrient solution (Nutriquisa 5-8-10® - Agroquisa® - 5 % (w/v) of nitrogen (N), 8 % (w/v) phosphorus (P) (P2O5) and 10 % (w/v) potassium (K) (K2O), chlorine-free, 0.3 % (w/v) magnesium (Mg), 0.4 % (w/v) of sulfur (S) and the following chemical elements: boron (B), copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) and molybdenum (Mo)) was poured into the soil surface of each pot (about 50 mL per 450 g of soil).

2.3. Plant measurements endpoints

2.3.1. Fortnightly measurements

A couple of non-destructive parameter measurements, namely above ground growth and maximal efficiency (Fv/Fm) of photosystem II (PSII) and quantum yield of PSII (Φ_{PSI}), were performed at intervals of 2 weeks.

Above ground growth

The growth of the plantlets was quantified for all the specimens with a simple measurement of the height of each plant, from the lowest visible stem point above the soil level to its highest point.

Photosystem II efficiency

To study the efficiency of the PSII, the quantification of the maximum efficiency as the ratio between the variable and the maximum fluorescence (Fv/Fm) and quantum yield (Φ_{PSI}) of PSII was carried out. This fluorescence analysis allows the calculation of the *in vivo* photosynthetic capacity and so it gives an indication of global photosynthesis. These measurements were made with a portable fluorometer (Walz® MINI-PAM Photosynthesis Yield Analyzer) which allowed the reading of fluorescence values directly from the leaves in the plants without their removal. For each point of analysis, one leaf was chosen from ten randomly selected plantlets from each of the three treatments. For light-adapted leaves, the fluorometer was connected to one fully developed leaf of each plant and both the basal (Ft) and maximum (F'_m) levels of fluorescence were recorded. According to the photoperiod of the chamber, the plants must had been under light emission for at least 30 min. To read the values from dark-adapted leaves, specialized dark adapted clips were placed in the leaves for at least 30 min, allowing a small area of the leaf to be completely devoid of light, putting the leaves' photosystems in a steady state. The fluorometer was then adapted to the clips and the basal (F_0) and maximum (F_m) fluorescence values were read. The raw basal and maximum fluorescence values required conversion into photosystem efficiency values. The efficiency values were obtained according to Maxwell and Johnson (2000):

$$Fv/Fm = \frac{(F_m - F_0)}{F_m}$$

$$\Phi_{PSI} = \frac{(F'_m - F_t)}{F'_m}$$

Where,

Φ_{PSI} – Quantum yield of photosystem II

F'_m – Maximum fluorescence in light-adapted state

F_0 – Basal fluorescence in dark-adapted state

Fm – Maximum fluorescence in dark-adapted state

Ft – Steady state fluorescence value

Fv/Fm – Maximum photochemical efficiency of photosystem II

2.3.2. Monthly measurements

A different set of parameters were also analyzed every month (starting at day 15). These parameters were the measurement of leaf area, leaf relative water content, leaf chlorophyll *a*, chlorophyll *b*, carotenoid, MDA and proline content. For future utilization, every month one or more leaves were cautiously removed from each plant and immediately preserved at -80°C, until further use, to avoid any unwanted physiological alterations.

Leaf area

The calculation of the leaf area was achieved by computer analysis of leaf photographs with the aid of the image editing software ImageTool® (The University of Texas Health Science Center in San Antonio, Ver. 3.00). From ten randomly chosen plants of each group, one leaf was carefully removed and photographed with a metric scale.

Leaf relative water content

To calculate relative water content (RWC) values, which give an index of plant water status, the protocol originally described by Weatherley (1950) was followed with a few minor changes. One leaf from ten plants was carefully removed and its fresh weight was recorded immediately to prevent drying. Next, each leaf was immersed in distilled water

for 24 h, after which their turgid weight was recorded. Excess water was removed from the leaves with absorbent paper before weighting, to ensure true turgid weights. Turgid leaves were then left to dry for at least 72 h on an incubator, at 60°C. After that, the dry weight of the leaves was quickly recorded and the RWC was calculated according to the following formula:

$$RWC\ (\%) = \left(\frac{FW\ \times DW}{TW\ \times DW}\right) \times 100$$

Where:

DW – Dry Weight (g)

FW – Fresh Weight (g)

RWC – Relative Water Content (%)

TW – Turgid Weight (g)

Leaf chlorophyll a, chlorophyll b and carotenoid content

To quantify chlorophyll *a*, chlorophyll *b* and carotenoid leaf content, a spectrophotometry approach based on the methodology of Sims and Gamon (2002) with a few modifications was followed. Every spectrophotometric analysis was performed in a Thermo Scientific™ 10S Vis spectrophotometer.

Tissue samples with fresh weights ranging between 0.02 g and 0.072 g were cut from the previously frozen leaves and were homogenized with a mortar and pestle in 1.5 mL of an acetone/Tris buffer solution [50 mM] (LabSolve – 99%; Applichem – pH 8.8) (80:20 v/v). For each sample the resulting suspension was then mixed in a vortex for 30 s and subsequently centrifuged for 5 min at 2800 g, in order to remove floating particles.

The supernatant was then transferred to falcon tubes covered with aluminum foil, to prevent contact with UV radiation, and 1.5 mL of the acetone/Tris buffer solution was added to the pellet. The pellet was briefly remixed with the solution in a vortex and the centrifugation step was repeated. The resulting supernatant was added to the previous extraction and acetone/Tris buffer solution was added until a final volume of 3 mL was reached. All samples were stored in ice and in the dark. Lastly, the absorbance of the samples was read at the wavelengths of 470 nm, 537 nm, 647 nm and 663 nm with the blank reading being set with the acetone/Tris buffer solution. Whenever needed, due to high absorbance values, samples were diluted until values fitted an acceptable range.

The leaf chlorophyll *a* and *b* and carotenoid content values were obtained from the following calculations:

$$Chl_a = ((0.01373 \times A_{663}) - (0.000897 \times A_{537}) - (0.003046 \times A_{647})) \times Df \div W$$

$$Chl_b = ((0.02405 \times A_{647}) - (0.004305 \times A_{537}) - (0.005507 \times A_{663})) \times Df \div W$$

$$Crt = ((A_{470} - (17.1 \times (Chl_a + Chl_b) - 9.479 \times ant)) \div 119.26) \times Df \div W$$

Where,

ant – Anthocyanins, calculated as:

$$ant = (0.08173 \times A_{537}) - (0.00697 \times A_{647}) - (0.002228 \times A_{663})$$

A_x – Absorbance (x = wavelength [nm])

Chl_a – Chlorophyll a content (μmol/gFW)

Chl_b – Chlorophyll b content (μmol/gFW)

Crt – Carotenoids content (μmol/gFW)

Df – Dilution Factor (mL)

W – Leaf Weight (g)

Note that the "ant" (anthocyanins) value calculated above is an intermediate calculation and does not represent the true anthocyanins content on the leaves. It only serves the purpose of calculating the carotenoid values (see Simon and Gamon 2002). After the determination of the chlorophyll *a* and *b* content the ratio chlorophyll *a*/chlorophyll *b* (Chl *a/b*) was also calculated from the mean values of each measurement.

Leaf MDA content

Lipid peroxidation was analyzed through the quantification of leaf MDA content, which is a known product of lipid peroxidation at tissue levels (Cai et al. 2011). MDA is a natural marker of oxidative stress and its quantification is an essential parameter to assess membrane damage and serves as another way of determining the plant stress state (Gallego et al. 1996; Shah et al. 2001). Small samples of leaf tissue were obtained from frozen leaves as described previously. Tissue samples with a fresh biomass ranging from 0.033 g and 0.103 g were homogenized in 5 mL of trichloroacetic acid (TCA) 0.1 % (w/v) (Acros Organics – Extra Pure) with a mortar and pestle. The resulting solutions were then centrifuged for 5 min at 10000 g. Then 4 mL of a TCA solution 20 % (w/v) containing 0.5 % (w/v) of thiobarbituric acid (TBA) (Sigma-Aldrich – 98 %) were added to 1 mL of the resulting supernatant and kept in a hot bath (95°C), for 30 min. After the hot bath, the samples were rapidly put on ice. When cooled, the absorbance of the samples was measured for 532 nm and 600 nm wavelengths, using a mixture of TCA/TBA (same as above) and H_2O (4:1) to set the blank reading.

The next formulas were used to determine the MDA content:

$$Abs(MDA) = A_{532\,nm} - A_{600\,nm}$$

$$MDA = (Abs \div \varepsilon \times CW \times Df \div W) \times 1000$$

Where,

ε – Molar extinction coefficient (155 $mM^{-1}.cm^{-1}$)

Abs(MDA) – Corrected absorbance value

CW – Cuvette width

Df – Dilution Factor (mL)

MDA – Malondialdehyde content (μmol/gFW)

W – Leaf Weight (g)

Leaf proline quantification

Leaf proline content was also quantified through a spectrophotometric approach. Small samples of leaf tissue were obtained from frozen leaves. For each sample fresh tissue that ranged from 0.029 g and 0.082 g was homogenized in 1 mL of sulphosalicylic acid 3 % (Sigma-Aldrich - ≥99 %) (v/v). The resulting solution was centrifuged for 10 min at 10000 g and 100 μL of the supernatant was mixed with 2 mL of glacial acetic acid (Panreac – 99 %) and 2 mL of ninhydrin acid (1.25 g of ninhydrin (Riedel-de Haën – Pro Analysis) dissolved with heating in 30 mL of glacial acetic acid and 20 mL phosphoric acid (6M) (Merck – Pro Analysis). Samples were then incubated at 100°C, for 60 min, and kept in an ice bath afterwards. Then, samples were warmed up to room temperature and 1 mL of toluene (Merck – 99 %) was added to the mixture. As the chromophore phase formed at the surface it was aspirated and its absorbance was recorded for the wavelength of 520 nm. The proline content was calculated from a calibration curve and its respective linear regression equation (Table 2.2).

2.4. Microbiological analysis

2.4.1. Endophytic and rhizosphere bacteria isolation

At the beginning and at the end of the experiment, rhizosphere and endophytic bacteria were isolated from the roots of *F. angustifolia* specimens, to later proceed with the analysis of these communities' 16S rDNA profiles. The protocol followed in this study was based on the works of Gomes et al. (2001), Forcheti et al. (2007), Aravind et al. (2009), Dias et al. (2009). During the isolation process all steps required sterile conditions and the procedure was mostly held in a laminar flow chamber using only sterilized equipment.

Table 2.2 – Proline calibration curve values and linear regression equation

Proline concentration (mg/mL)	Abs (520 nm)	Linear regression equation
0.06	0.5645	
0.03	0.2725	
0.02	0.14	
0.01	0.0695	$y = 8.9192 x + 0.0023$
0.004	0.0385	$R^2 = 0.9995$
0.002	0.024	
0.001	0.013	
0.0005	0.007	

The plants were firstly taken out from the pots and the soil was gently desegregated and shaken off. The roots of the plants in a total of 16 (4 random plants chosen before the transplanting procedure and 4 plants from each soil test at the end of the experiment) were cut with a pair of scissors and up to 5 g of each root was placed on a plastic sampling cup. Roots were then rinsed 4 times in a phosphate buffered saline solution (PBS) (Fisher – BioReagents 10x) to remove adherent soil particles and loose microorganisms. Excess PBS on the roots was removed with absorbent paper and, at that point, visible soil particles were removed with a pair of tweezers. Roots where put inside Erlenmeyer flasks together with glass beads of approximately 4 mm of diameter (the amount of beads on each flask was approximately equal to the weight of the corresponding roots) and immersed in a solution of sodium pyrophosphate 0.1 % (Acros Organics – Pro Analysis) containing 0.1 % Tween20 (Sigma-Aldrich) (the volume of the added solution was 45 mL per 5 g of roots). Next, the flasks were placed on an orbital shaker at 200 rpm for 30 min. This procedure allowed rhizosphere bacteria to concentrate in the liquid solution. Lastly, 1 mL of this root solution was transferred to eppendorf tubes and centrifuged for 20 min at 5000 g. To conserve the isolated rhizosphere bacteria, the supernatant was discarded and the pellet was resuspended with 1 mL of ethanol 96 % (Merck - Pro Analysis) and preserved at -20°C.

To proceed with the extraction of the endophytic bacteria, the same roots were again washed with distilled water and then cut into small 2-3 cm long segments with a pair of scissors. Root segments were immersed in PBS and let to soak for 10 min. In order to sterilize the root surfaces, root segments were sequentially immersed in ethanol 70 % for 1 min, in sodium hypochlorite 2.5 % (Sigma-Aldrich – 6-14 % Cl active) containing 0.1 % Tween20 for 20 min and lastly again in ethanol 70 % for 30 s. After the immersions, root segments were once more rinsed in distilled water 5 times. From the resulting solution of the last washing, 100 μL were spread into tryptone soya agar (Himedia – IVD) plates containing cyclohexamide (Sigma-Aldrich – ≥94 %), to prevent fungi growth, in duplicate for each sample. Plates were incubated at 28°C, for 48 h, and were checked for the presence of fast growing bacteria. The absence of bacterial growth indicates the success of the sterilizing process. Using a mortar and pestle the roots were grounded in PBS buffer (approximately enough volume to immerse the roots) which released the endophytic bacteria into the solution. From the resulting root solution 1000 μL were centrifuged for 7 min at 13000 g and, after discarding the supernatant, 1 mL of ethanol 96% was added to the pellet and the samples were preserved at -20°C.

2.4.2. Bacterial DNA extraction

The extraction of DNA from rhizosphere and endophytic bacteria was performed with the Ultra Clean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc.). The procedure was performed according to the manufacturer protocol manual. Due to the high ethanol content of the samples, the protocol's first step was replaced by an alternative pre-treatment. Samples previously stored at -20°C were warmed up to room temperature and centrifuged for 5 min at 10000 g. Sample tubes were then left open at room temperature to allow the ethanol phase to evaporate. The buffer solution from the specialized bead tube (provided in the kit) was added to the sample pellet, briefly mixed in a vortex and transferred back to the kit bead tube. Samples were then ready to start the step 2 of the manufacturer protocol.

2.4.3. PCR-DGGE Fingerprinting

DNA extracted from rhizosphere and endophytic bacteria was amplified and set ready for DGGE by nested polymerase chain reaction (PCR). Amplification was achieved with the following primers: 1st reaction, F27 5'-AGA GTT TGA TCM TGG CTC AG-3', R1512 5'-ACG GCT ACC TTG TTA CGA CTT-3'; 2nd reaction, F984-GC 5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3', R1378 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The underlined sequence represents a GC-clamp. This introduces a Guanine (G)/Cytosine (C) rich high melting domain in the double stranded DNA sample preventing its complete dissociation into single strands, increasing the sensitivity of the DGGE output (Muyzer and Smalla 1998). For the first nested PCR run was used the following 25 μL PCR cocktail: Bovine Serum Albumine (BSA) (0.4 μg/μL), 2.5 μL 10X DramTaq™ Buffer, 2.5 mM $MgCl_2$, 200 μM of dNTPs, 100 nM of each primer, 1U DramTaq™, 1 μL DNA sample. The PCR reactions were carried on a thermal cycler (BIO RAD C1000™ Thermal Cycler), and the cycling regime used was of 94°C for 5 min (1 cycle); 94°C for 45 s, 56°C for 45 s, 1,5 min for 72°C (30 cycles); 72°C for 10 min (1 cycle). For the second nested PCR run the same PCR cocktail was used, with a change of acetamide instead of the BSA. The cycling of the second PCR was achieved as follows: 94°C for 4 min (1 cycle); 95°C for 1 min, 53°C for 1 min, 1,5 min for 72°C (34 cycles); 72°C for 7 min (1 cycle). For both runs was included a negative control containing only the cocktail solution without any DNA sample.

The acrilamide gel for the DGGE electrophoretic run was prepared with the low and high gradient solutions as described in Table 2.3. The Tris acetate/Na_2EDTA (2M) (National Diagnostics – Ultra Pure), bisacrilaminde (Sigma-Aldrich – 30 %) , formamide (Acros Organics – Extra Pure), urea (Panreac – Pro Analysis) and distilled water were previously mixed in a falcon tube and the ammonium peroxodisulfate (APS) 10% (Panreac – Pro Analysis) and Tetramethylethylenediamine (TEMED) (Sigma-Aldrich – 99 %) were only added immediately before the solutions were ready to be poured in the gel support, due to the fast polymerization of the solutions. When the gel was completely polymerized 8 μL of each PCR (2nd run) product, mixed with 5 μL of DNA Loading dye buffer

(Fermentas - 6x) was loaded into the wells and the electrophoretic run was performed in a DCode™ System for DGGE (Bio-Rad Laboratories, Inc.) for 5 h at 200 V and 60°C. After the DGGE run was completed the gel was stained for 5 minutes in a ethidium bromide 5 % solution and digitally scanned with a Molecular Imager® FX (Bio-Rad Laboratories, Inc.). DNA profiles were then analyzed with the software GelCompar II® (Applied Maths NV, Ver. 6.4). For the analysis, the similarity between the bacterial communities was measured with the calculation of the Pearson correlation coefficient for each pair of lanes in the gel (similarity matrix). Dendrograms were built by applying the unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering to the similarity matrix obtained before.

Table 2.3 – Acrilamide gel low and high gradient solutions (Muyzer et al. 1993)

Reagent	35 % (low)	65 % (high)
TAE 50x	320 μL	320 μL
40 % Bis acrilamide	3.2 mL	3.2 mL
Formamide	2.24 g	4.16 g
Urea	4.36 g	2.35 g
dH_2O	Adjust to 16 mL	Adjust to 16 mL
APS 10 %	140 μL	140 μL
TEMED	14 μL	14 μL

2.5. Statistical analysis

To test the responses of the plants to the conducted measurements, the data was subjected to a univariate two-way analysis of variances (Two-Way ANOVA). When no significant interaction was found between the “soil” and “time” factors an independent one-way analysis of variances (One-Way ANOVA) was performed for the levels of each factor to search for significant differences. The established significant level was of $\alpha = 0.05$. When there was significant interaction between factors (chlorophyll *b*, carotenoid and Fv/Fm) simple main effect tests were done for each factor, using the $MS_{residual}$ of the

previous Two-way ANOVA as the denominator for the calculation of the F statistics (Quinn and Keough 2003). When significant differences were found, the previous $MS_{residual}$ was used as well for the calculation of q statistics in the Tukey multiple comparison tests to reduce the pairwise type I error. Both Tukey multiple comparison tests for equal sample sizes and Tukey-Kramer tests for unequal sample sizes, were performed to test for significant differences among the levels of the factor "time" for each soil, and among levels of the factor "soil" for each time, respectively.

In the case of the "above ground growth" since every single plant was repeatedly measured over the course of time, a repeated measures (RM) ANOVA was performed to test for significant effect of soil and time on this parameter. Plants were the subjects, and soil and time were the between subjects and within subject factors respectively. Since the sphericity assumption was not met, the Greenhouse-Geisser estimate of ε was applied for the adjustment of the degrees of freedom of the repeated measures ANOVA (Quinn and Keough 2003). Since a significant interaction between factors was recorded, simple main effect tests were performed as described previously, using the $MS_{residual}$ of the RM-ANOVA. All the statistical analysis was performed with the SPSS© Statistics for windows (IBM© Ver. 19) software.

3. Results

3.1. Plant measurements endpoints

Above ground growth

At the end of the test not all plants showed size increase over time, even though none of them died. However, the mean above ground growth values for all the groups increased after each measurement (Figure 3.1). Both soil and time factors had a significant effect on this parameter (Soil: F=5.521, d.f.=2, p=0.007; Time: F=38.457, d.f.=1.538, p=0.000). Further a significant interaction between both factors was recorded (F=11.165, d.f.=3.007, p=0.000). With respect to soil, the control plants showed the most pronounced growth, and significant differences in terms of this parameter between exposure times to the soil were recorded by the simple main effect test (F=12.521; d.f.=5.54; p<0.001). In the control soil, and after the second measurement (including T30), the average height of the plants started to be significantly different (p<0.05) from the initial measurements (T0 and T15), according to the Tukey's multiple comparison test. After T30, significant differences in the growth of plants were recorded only between the reference and the control soil (p<0.05), but after T45, the plants from the control soil were significantly higher than those exposed to both the contaminated and the reference soil. No significant differences were recorded between plants from the reference and the contaminated soil, at any exposure period. Nevertheless, at T75 it was also clearly visible that in the reference and especially in the contaminated group, most of the plants were beginning to develop new shoots and leaves.

Leaf Area

For the 3 groups of plants, the same variation pattern was observed, concerning the average leaf area values (Figure 3.2) and no significant differences were recorded for plants exposed to the different soils (F=1.238; d.f.=2; p=0.296). The interaction between both factors was also non-significant (F=2.046; d.f.=4; p=0.096). However, the exposure time had a significant effect on this parameter (F=14.449; d.f.=2, p=0.000). Plants from

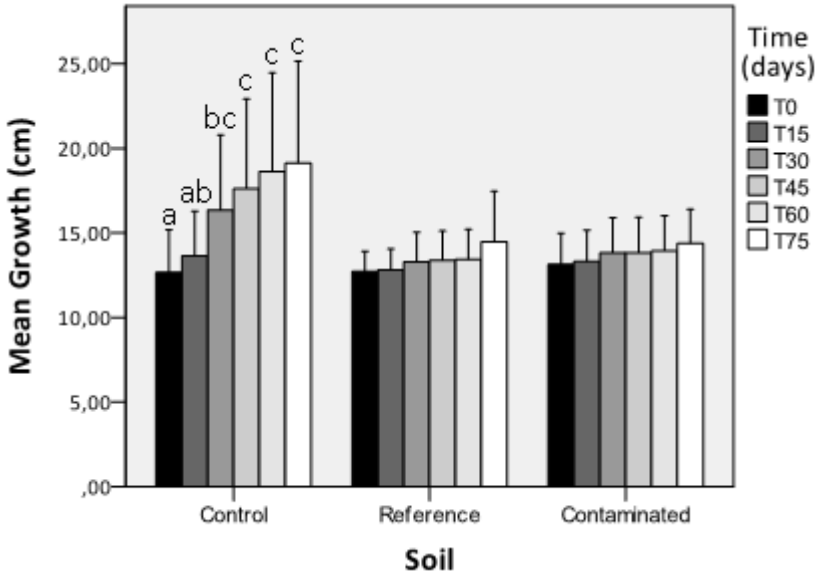

Figure 3.1 – Mean values of the above ground growth (cm) of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 0 (T0), 15 (T15), 30 (T30), 45 (T45), 60 (T60) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a, b or c) are significantly different to each other.

the control soil showed a significant higher leaf area after 45 days of exposure, while for the reference soil it occurred after 75 days ($p<0.05$). However, no significant differences were recorded among exposure periods for the plants exposed to the contaminated soil (One-way ANOVA: F=2.776, d.f. 26,29; p=0.081).

Leaf chlorophyll a, chlorophyll b and carotenoid quantification

The reference and contaminated groups showed, each, a particular pattern in the content of the 3 measured pigments. For the chlorophyll *a* content assay the two-way ANOVA revealed significant differences among soils (F=6.281, d.f.=2, p=0.004) and exposure times (F=13.529, d.f.=2, p=0.000). No significant interaction between both factors was recorded (F=1.988, d.f.=4, p=0.112). The control soil was significantly different from the contaminated soil ($p<0.05$). Considering each soil individually, significant differences among exposure times were recorded only for the reference (F=7.428, d.f.=15.18, p=0.006) and contaminated soils (F=8.931, d.f.=15.18, p=0.003). The chlorophyll *a* content

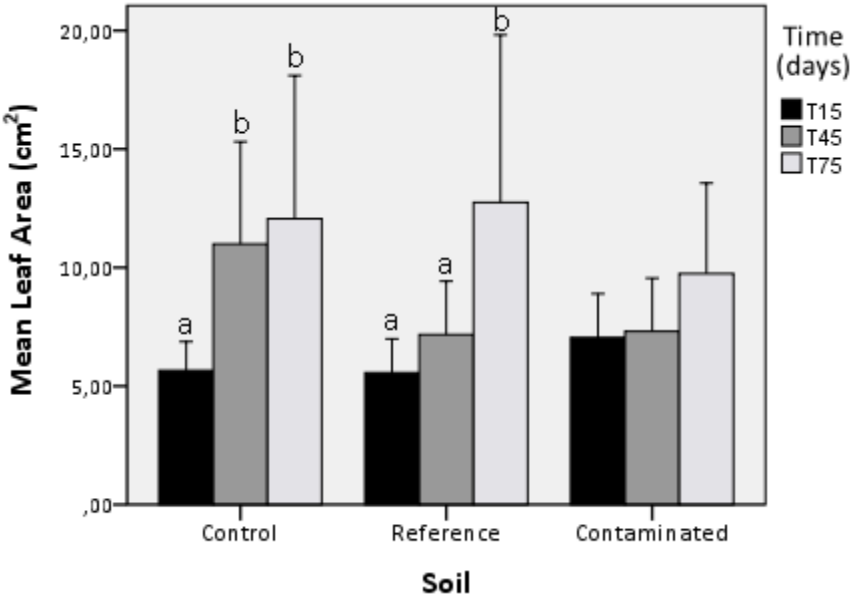

Figure 3.2 – Mean values of the area (cm^2) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a or b) are significantly different to each other.

on the leaves of plants growing on both soils was significantly higher after 75 days of exposure ($p<0.05$). The control group showed successive increases of chlorophyll *a* content over time, although without any significant differences found between measurements (Figure 3.3). As far as chlorophyll *b* content was considered no significant differences among soils were recorded ($F=2.104$, d.f=2, $p=0.134$), despite the significant effect of the exposure time ($F=6.468$, d.f.=2, $p=0.003$) and the significant interaction between both factors ($F=4.497$, d.f.=4, $p=0.004$) (Figure 3.4). Considering each soil individually significant differences in chlorophyll *b* content, throughout the exposure, was recorded only for plants exposed to the contaminated soil ($F=12.704$, d.f.=2.15, $p=0.0006$). This parameter displayed significantly higher values after 75 days of exposure ($p<0.05$). The Chl *a/b* ratio calculated for the 3 groups were the following: control 2.51 (T15), 2.26 (T45), 2.98 (T75); reference 2.86 (T15), 2.56 (T45), 2.76 (T75); contaminated 2.46 (T15), 2.52 (T45), 2.13 (T75).

No significant differences were recorded in the carotenoid content on plants exposed to different soils (F=2.324, d.f.=2, p=0.109) and for the different exposure times (F=2.109, d.f.=2, p=0.133) (Figure 3.5).

Leaf relative water content

The 3 different groups showed significant differences among exposure times (F=19.132; d.f.=2; p=0.000) and soils (F=4.586; d.f.=2; p=0.013) for the RWC (%) values (Figure 3.6). However, no significant interaction was recorded for both factors, regarding this parameter (F=1.061; d.f.=4; p=0.381). The average RWC percentage was significantly higher in plants from the control soil, when compared with those from the reference soil (p=0.005). One-way ANOVAS performed for each soil have shown significant differences among exposure times for each soil (control soil: F=11.633; d.f. 25,28, p=0.000; reference soil: F=3.914; d.f. 26,29; p=0.033; contaminated soil: F=7.920; d.f. 26,29, p=0.002). A significant decrease in this parameter was recorded for the plants exposed to the three different soils, between the measurements of 45 and 75 days of exposure (T75: Control – 69.4 %; Reference – 65.9 % ; Contaminated – 69.6 %).

Leaf proline quantification

No significant effect of both exposure time (F=0.596, d.f.=2, p=0.555) and soil (F=2.918; d.f.=2; p=0.065) were detected in the proline content of leaves. Furthermore, no significant interaction was recorded between both factors (F=0.379, d.f.=4, p=0.823) as well (Figure 3.7).

Leaf MDA content

As far as the MDA content on the leaves was concerned, only significant differences among times of exposure were recorded (F=6.386, d.f.=2, p=0.004) without a significant interaction between this factor (F=0.467, d.f.=4, p=0.759) and the type of soil tested

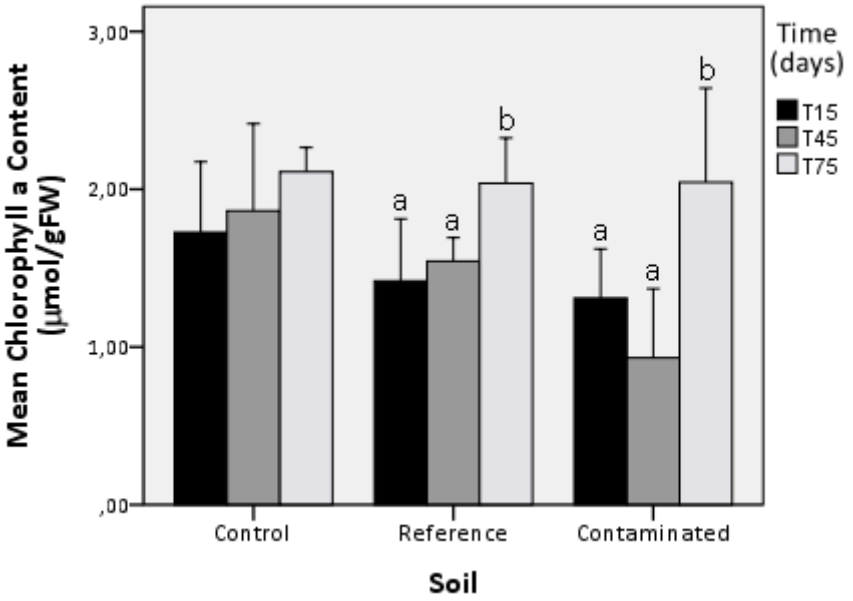

Figure 3.3 – Mean values of the relative chlorophyll *a* content (µmol/gFW) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a or b) are significantly different to each other.

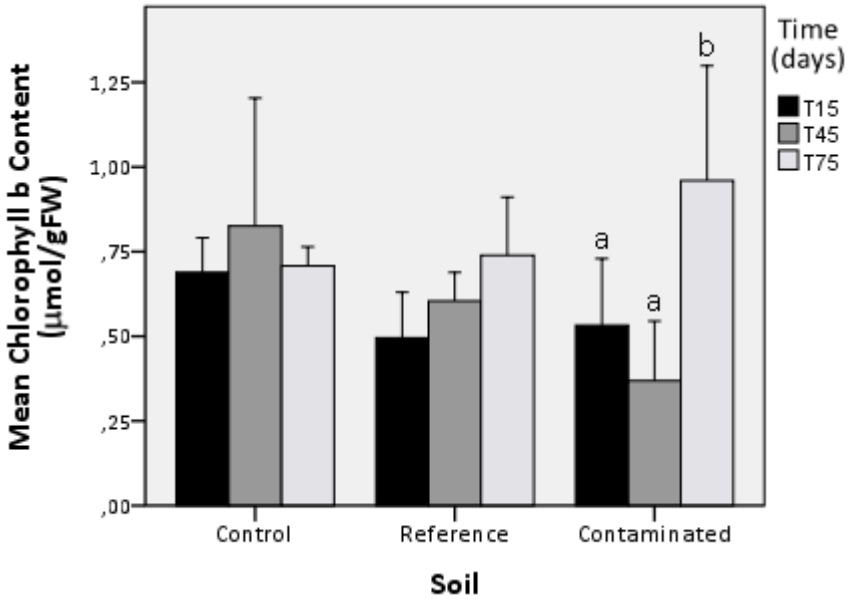

Figure 3.4 – Mean values of the relative chlorophyll *b* content (µmol/gFW) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a or b) are significantly different to each other.

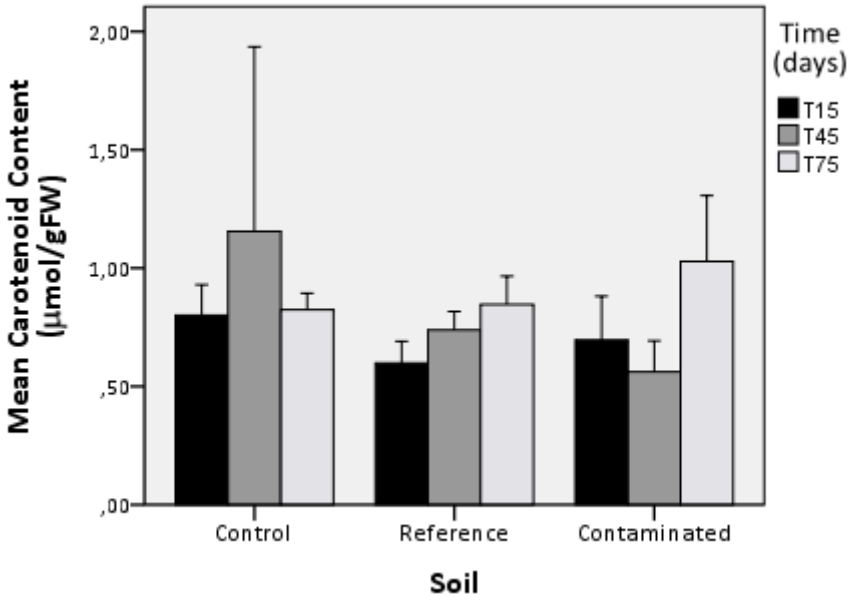

Figure 3.5 – Mean values of the relative carotenoid content (µmol/gFW) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation.

(F=1.564, d.f.=2, p=0.221) (Figure 3.8). The MDA content was significantly different among exposure times only for plants exposed to the reference soil (F=6.890, d.f.=14.17, p=0.008). In these plants, the MDA content was significantly higher after 45 days of exposure ($p<0.05$), decreasing again for values similar to those recorded in the beginning of the experiment.

Photosystem II efficiency

Concerning the PSII efficiency, the Fv/Fm values obtained for the 3 groups indicated significant differences among soils (F=6.377; d.f.=2; p=0.002), exposure times (F=18.461; d.f.=4, p=0.000), as well as a significant interaction between both factors (F=3.210; d.f.=8; p=0.002) (Figure 3.9). In the 3 groups, the variation pattern was very similar, with the highest mean values detected at T15 and T60. However, significant differences among soils were recorded by a simple main effect test only after 60 days of exposure (T60) (F=15.0; d.f.=2.27, $p<0.0001$). However in the control soil, after such increase, a decrease

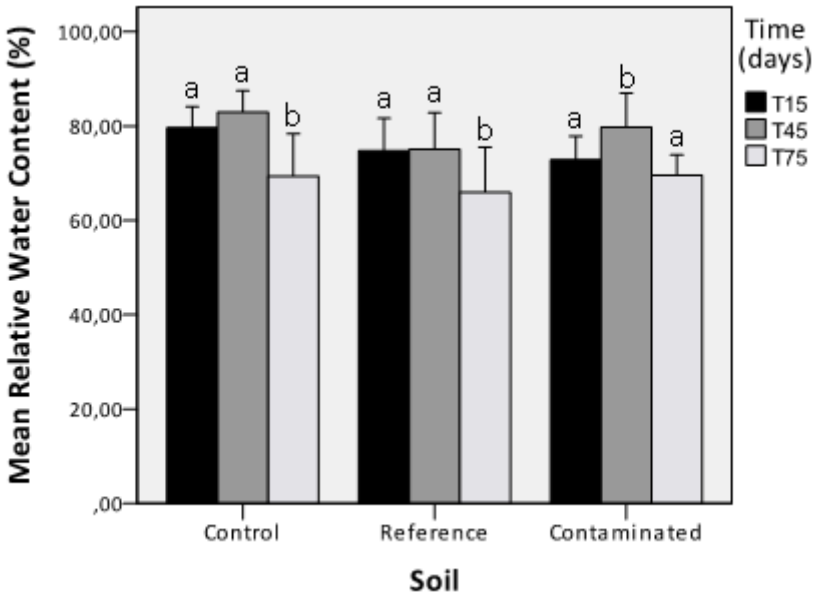

Figure 3.6 – Mean values of the relative water content (%) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a or b) are significantly different to each other.

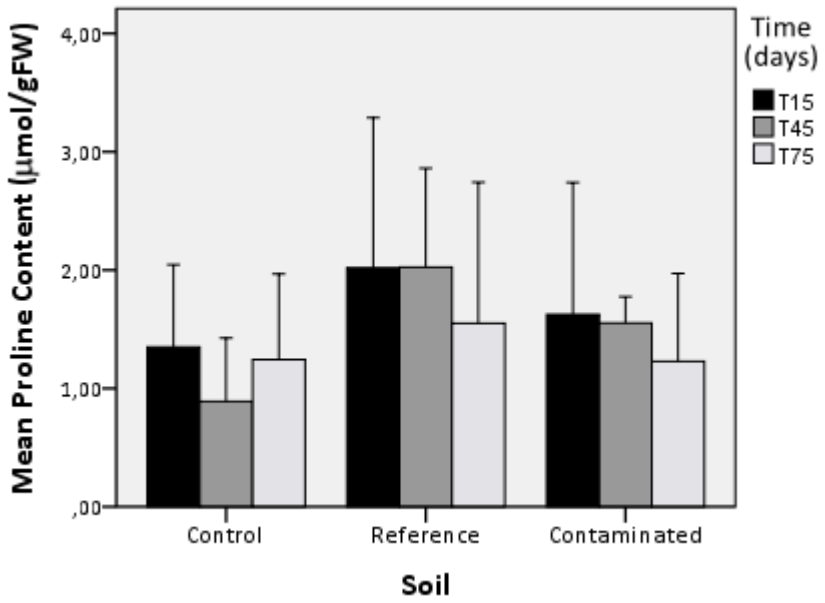

Figure 3.7 – Mean values of the proline content (µmol/gFW) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation.

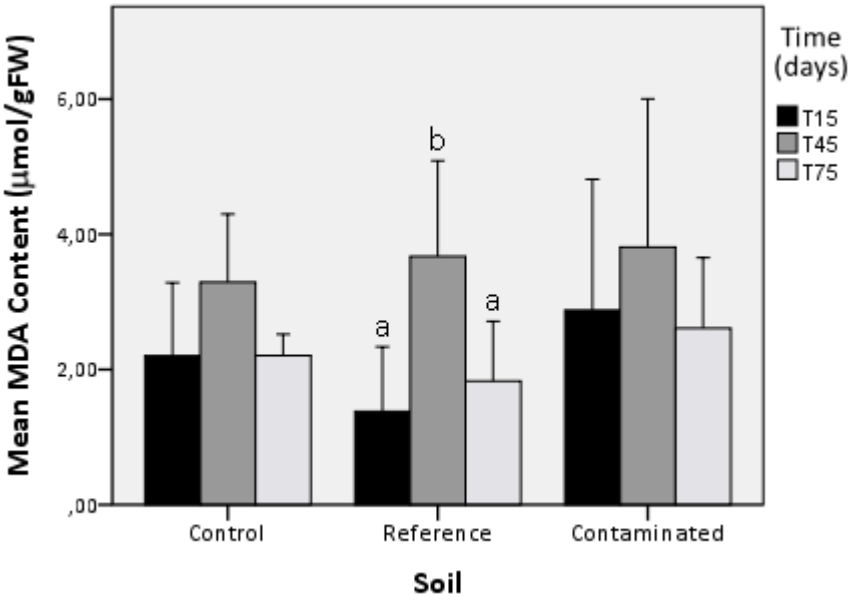

Figure 3.8 – Mean values of the MDA content (µmol/gFW) of the leaves of *F. angustifolia* plants from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 45 (T45) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a or b) are significantly different to each other.

of similar order of magnitude was registered. Considering each soil individually, significant differences in the Fv/Fm values obtained on plants among exposure times were recorded only for the control and contaminated soils ($F=15$, d.f.=4.42, $p<0.0001$ and $F=12$, d.f.=4.43, $p<0.0001$, respectively). The contaminated group of plants showed the most considerable decrease after at T30 and T45 but this was also later on followed by a recovery which led to a significant higher value at T60.

For the Φ_{PSI} measurements both time of exposure ($F=1.143$; d.f.=4; $p=0.233$) and soil ($F=2.199$; d.f.=2; $p=0.124$) did not have a significant effect on this parameter (Figure 3.10). The interaction between both factors was also non-significant ($F=1.846$; d.f.=8; $p=0.074$).

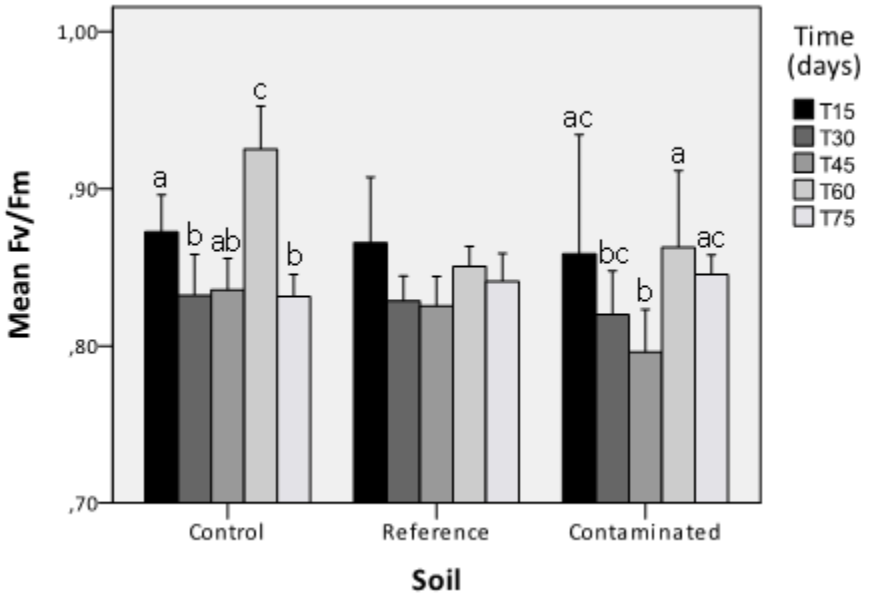

Figure 3.9 – Mean values of the Fv/Fm ratio of *F. angustifolia* leaves from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 30 (T30), 45 (T45), 60 (T60) and 75 days (T75) of testing. Error bars represent standard deviation. Bars not sharing any common letter (a, b or c) are significantly different to each other.

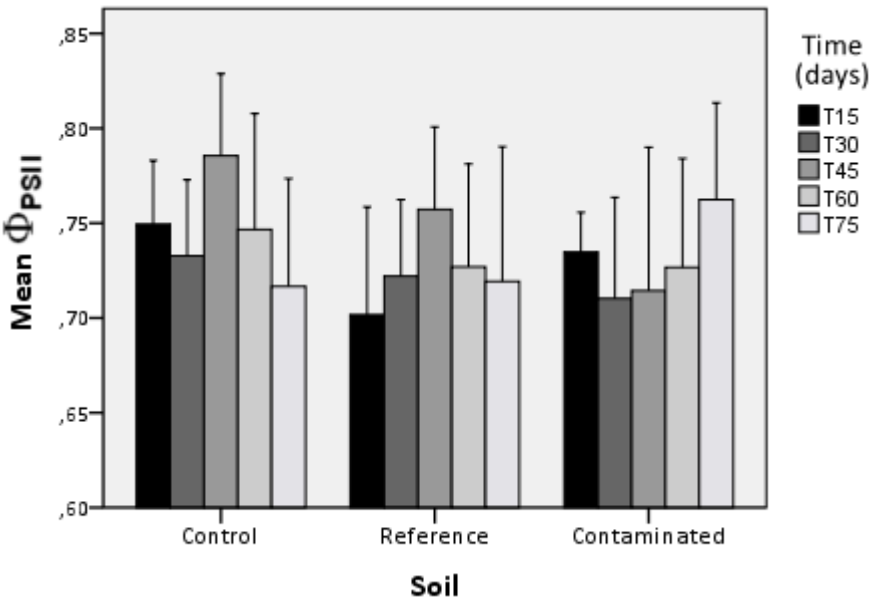

Figure 3.10 – Mean values of the Φ_{PSII} ratio of *F. angustifolia* leaves from the 3 groups (Control; Reference; Contaminated) after 15 (T15), 30 (T30), 45 (T45), 60 (T60) and 75 days (T75) of testing. Error bars represent standard deviation.

3.2. Microbiological data

Agar plates

The plates where the washing solutions from the bacterial extraction process were incubated showed none or very little bacterial growth after 48 h (data not shown).

PCR-DGGE band profile analysis

In Figure 3.11 (A and B) are depicted the PCR-DGGE 16S rDNA band profiles of the extracted endophytic bacteria, in the beginning and at the end of the experiment, and the dendrogram cluster analysis of the same samples. The same information is present in Figure 3.12 (A and B) for the rhizosphere communities.

Both fingerprints provided clear rDNA profiles where it was possible to detect distinct bands for each groups of samples analyzed but there were also bands with similar melting behavior common for all the analyzed lanes (shown in the rectangle boxes in both figures). There was more variety on the rhizospheric fingerprinting, as the band count was higher.

In the endophytic bacteria gel the 4 lanes corresponding to the contaminated soil, although being similar among them, they appeared to show the most different profile comparing to the other lanes. An exception was lane nº 8 (control soil - sample 4) where the bands also showed a particular melting profile, in fact, quite similar to the "contaminated soil" lanes. As it is shown in the dendrogram (Figure 3.11 B), the 4 "contaminated soil" lanes and lane nº 8 form a cluster very distinct to the other one. Despite such clustering, lane nº 8 still showed the smallest similarity index within the cluster. The other 3 groups of samples appear to be more similar in the gel, where 3 bands appear with the same melting behavior in all samples, except lane 8 (indicated by the arrows in Figure 3.11 A). The cluster for these samples shows that the similarity between the "initial" samples and the control samples is greater than with the reference

ones. An intense band present in the “initial”, reference and in 3 of the control lanes is clearly absent in the “contaminated soil” lanes (lower arrow in Figure 3.11 A).

In the gel of the rhizosphere samples the bands from the “contaminated soil” group of lanes once again showed a particular profile distinct from the rest (Figure 3.12 A). In turn, the profiles from the other 3 groups are more similar between them, although some distinct melting behavior could be found for some bands between the groups. The data from the dendrogram supports the visual analysis and again 2 big individual clusters were formed (Figure 3.12 B). On the smaller cluster were the group of lanes from the “contaminated soil” samples and lane nº 8 (control soil - sample 4) that, in line to what happened in the other gel, had a profile more similar to that of the “contaminated soil” lanes comparatively to all the others.

Concerning the bigger cluster, the grouping of the data from rhizosphere communities seems to differ from what was observed in the endophytic samples. This time the 3 groups appear to be more similar between each other than before. It is notable that an intense band (arrow in Figure 3.12 A) appeared in all the lanes of the contaminated soil samples although it did not appear in the other samples.

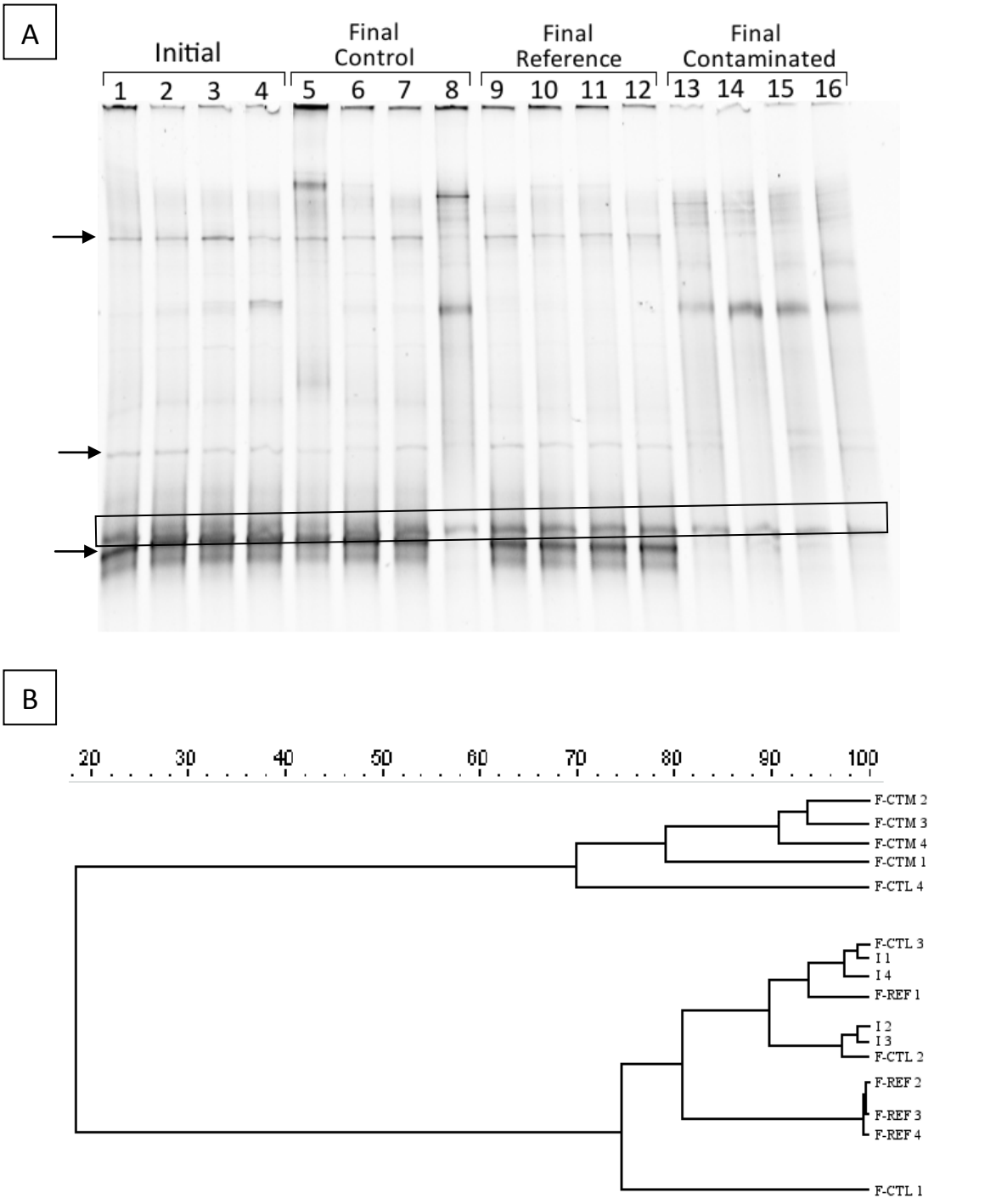

Figure 3.11 – A) PCR-DGGE fingerprinting of the 16S rDNA fragments from the endophytic bacteria extracted from the roots of 4 plants in the beginning of the test (Initial-I) and of 4 plants of each group in the end of the experiment [Final – Control (F-CTL); Final-Reference (F-REF); Final-Contaminated (F-CTM)]. Black arrows point to a common bands to all groups except the "Contaminated"; box represents a common band for all the lanes. B) Similarity cluster based dendrogram of the endophytic bacteria rDNA profiles. Built with the Pearson correlation coefficient and unweighted pair group method with arithmetic mean; genetic profiles differences shown as similarity percentage.

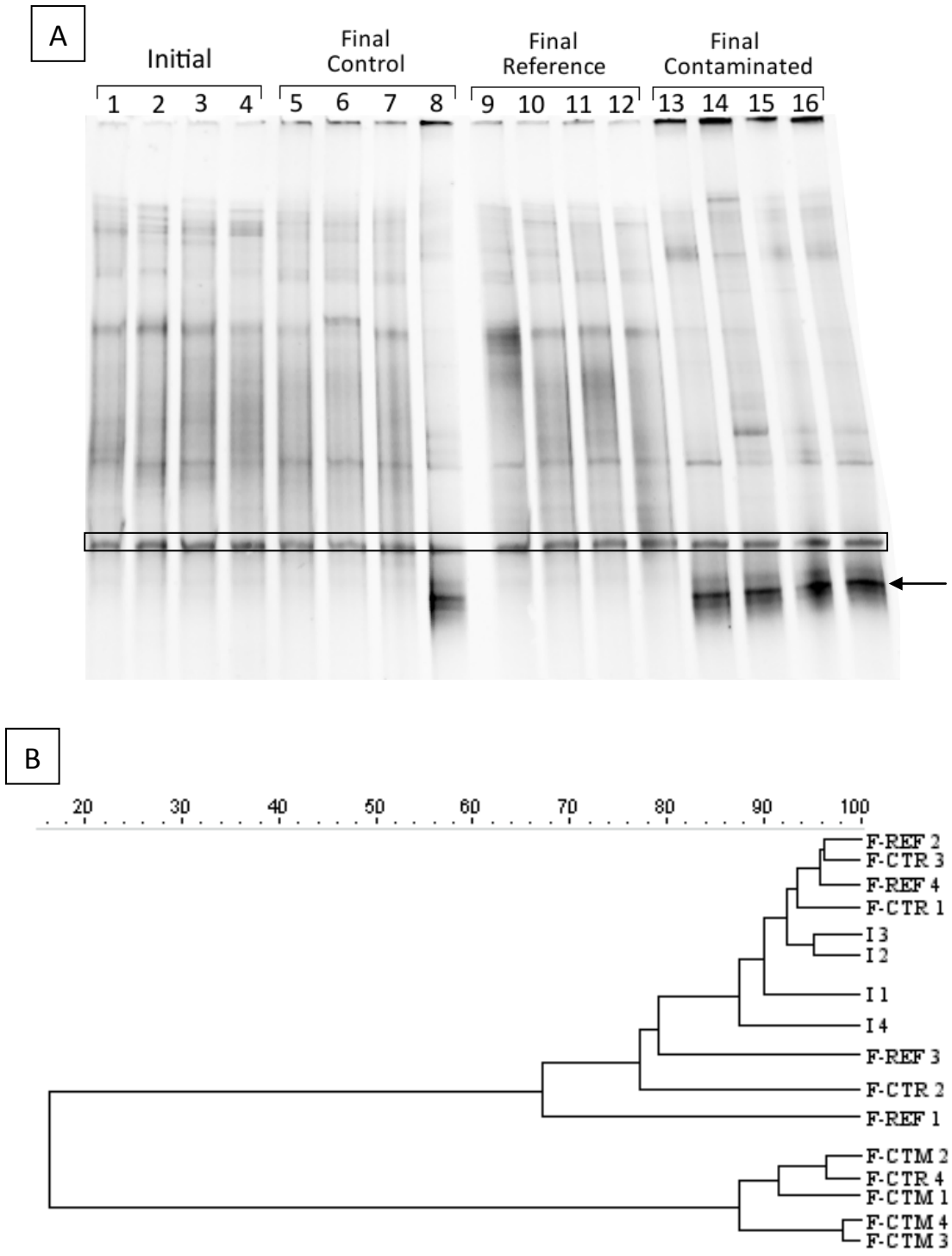

Figure 3.12 – A) PCR-DGGE fingerprinting of the 16S rDNA fragments from the rhizosphere bacteria extracted from the roots of 4 plants in the beginning of the test (Initial) and of 4 plants of each group in the end of the experiment [Final – Control (F-CTL); Final-Reference (F-REF); Final-Contaminated (F-CTM)]. Black arrow point to an intense band present only in the "Contaminated" samples; box represents a common band for all the lanes. B) Similarity cluster based dendrogram of the rhizospheric bacteria rDNA profiles. Built with the Pearson correlation coefficient and unweighted pair group method with arithmetic mean; genetic profiles differences shown as similarity percentage.

4. Discussion

To evaluate the phytoremediation potential of the *F. angustifolia* trees, a set of parameters were analyzed to study the effects of contamination on the plants. Contamination of soils with metals is known to cause several effects in plants, ranging from oxidative stress to growth inhibition, reduction of photosynthesis, degradation of pigments, damage to chloroplast and mitochondria, inhibition of enzyme activities and changes in their ultrastructure and water status, among others (Barceló and Poschenrieder 1990; Van Assche and Clijsters 1990; Shanker et al. 2005; Hu et al. 2007; Maksymiec 2007; Kholodova et al. 2011). Some of these effects were targeted for evaluation in this study.

The results from the two parameters most related to biomass, the above ground growth and the leaf area tests, suggest that the development of the plants was affected by the factor soil only for the former one, since plants from the control soil displayed a significantly higher values than the others. The leaf area has significantly increased for the control and reference plants throughout the experiment, however at the end no significant differences among the 3 soils were recorded. This happened because in the last 3 weeks of the test a rapid burst growth appeared to have began in the contaminated plants group. Such observation suggests that despite an evident growth inhibition of the contaminated group of plants has occurred, after such amount of time, should the test had continued and these plants would start to develop significantly. Several studies have demonstrated that the growth rates and biomass production of plants is inhibited by the contamination with metals (Shah et al. 2001; Manios et al. 2003). Previous studies that focused on the effects of cadmium also showed that metals can decrease plant cells and intercellular space sizes and have irreversible effects on proton pumps that intervene in the elongation of the cells and, thus, interfere with the plants' growth (Barceló et al. 1988; Aidid and Okamoto 1993). Metal contamination has also been implicated in the inhibition of leaf growth (Clijsters and Van Assche 1985). Since the over time growth of the reference and contaminated groups was very similar, it seems that the delay in the

growth of the plants from these groups was related to some other factors, like the soil structure, instead of being essentially related to the contamination levels.

The quantification of chlorophyll *a* and *b* showed significant differences between the control and the contaminated group of plants. In fact, plants exposed to the contaminated soil displayed significantly lower contents of chlorophyll *a* after 45 days, only showing a significant recover in both chlorophyll *a* and *b* parameters after 75 days. The same trend was observed for plants exposed to the reference soil for chlorophyll *a* content. Nevertheless, Chl *a/b* ratios had little variations and always remained above the value of 2 for all groups. Chlorophyll content is considered to be one of the most important parameters in the evaluation of plant stress (Zarco-Tajeda et al. 2000). The stress caused by metal contamination is known to result in the reduction of total chlorophyll *a* and *b* and carotenoid contents (Van Assche and Clijsters 1990; Wonzy and Krzeslowska 1993; Krupa et al. 1996; Kastori et al. 1998; Fargašová 2001; Macfarlane and Burchett 2001; Pandey and Sharma 2002). This decrease in chlorophyll is usually explained due to impacts that metals have on the photosystem II and negative interferences in the chlorophyll synthesis process and associated enzymes (Prasad and Prasad 1987; Van Assche and Clijsters 1990; Ouzounidou 1993; De Filippis and Pallaghy 1994; Moustakas et al. 1994; Sánchez-Viveros et al. 2010). This way, the analysis of the chlorophyll content relates to the physiological status and the productivity of a plant. This might give information related not only to the damage on the PSII but can also be related to growth and survival limitations (Vangronsveld and Clijsters 1994; Blackburn 1998). The somehow similar pattern observed in chlorophyll content on plants exposed to both natural soils (the contaminated and the reference soil) with a recovery (sometimes significant) near the end of the experiment, suggests, that not only the contamination with metals but other soil properties, as well, may interfere with plant performance. However, as far as the contaminated soil is considered *F. angustifolia* plants seemed to have surpassed the effects of such levels of contamination and were able to restore the pigments levels.

It is clear that metal toxicity has a tendency to reduce the chlorophyll content in plants but weather it reduces or increases the Chl *a/b* ratio is still debatable. The Chl *a/b* ratio is important because it can reveal some insights about the changes and adaptations of the plants under stress. Some studies reported that plants suffering from metal-induced stress showed a reduction in the ratio (Ouzounidou 1993; Moustakas et al. 1994; Ewais 1997; Pandey and Sharma 2002) while others stated the opposite (Loggini et al. 1999; Li et al. 2011). On the contaminated group of plants the Chl *a/b* ratios were of 2.46 (T15), 2.52 (T45) and 2.13 (T75), while the ratios for the control soil were 2.51 (T15), 2.26 (T45), 2.98 (T75) and for the reference soil were 2.86 (T15), 2.56 (T45), 2.76 (T75). Despite the very slight changes, the ratio only increased when the contamination effects were most notable. This contradicts the supposable reported greater sensitivity of chlorophyll *a* to environmental pollutants (Wong and Chang 1991; Somashekaraiah et al. 1992) but it may actually represent an adaptive mechanism against metal contamination based on beneficial pigment changes in the photosynthetic centers (Loggini et al. 1999).

Proceeding with pigments evaluation, the analysis of the carotenoid content data revealed that the pattern of variation of the mean values was very similar to those observed for the chlorophyll *b* content. It has been suggested that the increase or decrease in the carotenoid content of the plants growing under metal contamination, can be either the result of tolerance or susceptibility to the contamination (Sánchez-Viveros et al. 2011). While a decrease in the carotenoid content is probably related to the toxic effects of the metals, an increase can be related to protective effects of carotenoids against the metal-induced stresses (Vajpayee et al. 2001; Mascher et al. 2002). Due to the lack of statistical proof, it is risky to draw many conclusions, but 2 important observations can be pointed out: on the one hand, since there were no significant differences at any time between the contaminated group and the others, carotenoid content seem to have not been affected by the contamination; on the other hand, the increase recorded for the contaminated group at T75, following the low value at T45, could possibly be the beginning of a late adaptive response to the metal stress, i.e. metal-induced oxidative stress (Foyer and Harbinson 1994). Carotenoids seem to have beneficial effects against stress by helping maintaining the integrity of photosynthetic membranes, and although in

small amounts, this might have been true in the contaminated soil group of plants (Havaux 1998; Havaux et al. 1998).

The term “relative water content” was firstly coined as the water content of the leaves (in per cent) correlated with their water content at a maximum turgor state. By expressing the amount of water that a plant needs in order to achieve a state of artificial full saturation, it is an indicator of the plant’s water balance and still serves today as another useful tool among plant stress related parameters (Weatherley 1950; Roger 2003). Normal RWC values vary among species and may also be under influence of considerable genotypic diversity (Babu et al. 1999). However a range of RWC values is actually accepted as a rule for most species: RWC of 100 - 90 % represent normal leaf stomata pores closing and decreasing cell growth and expansion; values of 90 - 80 % are related to changes in photosynthesis and respiration rates and tissue composition, but plants are still healthy; <80 % values represent low water potentials associated with metabolism alterations and proline and abscisic acid accumulation (Roger 2003). The initial (T15) RWC values registered for the 3 groups of plants were not too low but were still below 80 %. These results might indicate some physiological changes due to the adaptation to the new conditions, reaction to the new soil (specially the contaminated plants since they had the lowest mean value – 72.8 %) or both. At T15, contaminated plants’ RWC mean value was significantly lower than the control value but not comparing to the reference plants and after 30 days (T45) the RWC values increased in all 3 groups of plants. Such reaction might mean a positive over time response of the plants, as an adaptation to the transplanting and an initial physiological shock to any adverse conditions of the soil. It is also possible that mechanisms of osmotic adjustment might have been behind such RWC values. Such mechanisms usually include the cellular accumulation of compatible solutes (or compatible osmolytes). Compatible solutes are soluble compounds, generally nontoxic to the plant cells even at high concentrations, which protect the cells in various ways. These include not only the lowering of the cell osmotic potential and consequently the rise of RWC, but also the detoxification of reactive oxygen species, buffering cellular redox potential, stabilization of proteins/enzymes and maintenance of membrane integrity (Yancey et al. 1982; Babu et

al. 1999; Bohnert and Jensen 1996). It is very typical for plants under several stresses, including metal contamination, to accumulate such molecules and would not be unexpected if it was found that such phenomenon took place in this case (Bassi and Sharma 1993; Kuznetsov and Shevyakova 1999).

It was one of the aims of this study to analyze the accumulation of one of these compatible solute molecules, proline. Proline is an amino acid that is known to occur and accumulate in large quantities in higher plants, when they are under stressful conditions (Hare and Cress 1997; Ashraf and Foolad 2007). This usually allows plants to overcome stress situations for a period of time. The analysis of the proline content values did not provide much information regarding its relation with the RWC values. Since not many considerable variations and differences were observed it is difficult to establish a solid relation between both parameters.

Nonetheless, at T75 the measurements of RWC showed a decrease in the 3 groups, that were significant for the 3 groups. At this point the contaminated group's mean value (69.6 %) was very close to the control mean value (69.4 %) and no significant difference existed between the 3 groups of plants (65.9 % for the reference soil) which were all notably below the 70 % mark. Contamination by metals has already been reported to be related to impairments in the water status of the plants and should allow the detection of metal effects on them (Barceló and Poschenrieder 1990). However, in this case it is uncertain if such a late decrease could have been due to a failure to overcome the contamination and adverse soil properties by the plants of the reference and contaminated lots. Still, that would not explain the similar drop of RWC for the control plants. Therefore another explanation to such reaction could be that the fact that the plants' roots were already overgrowing the pots at that point of the experiment, were also responsible for changes in the plants' water balance and the consequent decrease of the RWC values. Additionally, at the end (T75) there were no significant differences between the mean RWC values of the 3 groups, although surprisingly the RWC of the contaminated lot was actually higher than the value of the control lot. This set of data also suggests a good response of the plants from the contaminated group.

The potential danger of metals is believed to be closely related to their oxidation-reduction biological activity, which leads to the formation of harmful reactive oxygen species in plant cells (Gallego et al. 1996; Shah et al. 2001; Pandey and Sharma 2002). Since this can easily lead to dangerous levels of oxidative stress and damage to the membranes, this study also tried to evaluate the extent of that kind of cellular damage related to metal exposure, by means of MDA quantification. Increased rates of lipid peroxidation was already observed in plants growing on medium supplied with various metals (Gallego et al. 1996). However, metals can have different ways of action like the direct induction of radicals production or the decrease of enzymatic and non-enzymatic antioxidants (Gallego et al. 1996; Romero-Puertas et al. 2002). The fact that in this study the mean MDA content values for any of the measuring times were not significantly different between the 3 plant groups, seems to mean that lipid peroxidation was not significantly pronounced in a specific group of plants. Although not significant, the values for the contaminated lot were always slightly higher than the values obtained for the other groups so the contamination on soil probably had induced some oxidative stress, even if at a low extent. It is also curious to note that the mean values for all the lots increased from T15 to T45 and decreased again at T75. Like before, these results suggest that the existing stressful conditions affecting the plants triggered MDA production in the leaves, at first, but after some time the plants recovered from or adapted to such conditions and returned to a normal state. Once again the control lot followed the same pattern as the other groups, which makes it difficult to establish any direct relation between MDA values and soil contamination. Xu and colleagues (2009) also suggested that there could be a negative correlation between MDA levels and photosynthesis efficiency. The analysis of the results of the MDA content and PSII efficiency assays for *F. angustifolia* shows that there is some relation between increases of MDA in the leafs and decreases in PSII efficiency, especially in the contaminated group. It is, however, not possible to assure that there is a direct physiological connection. Firstly, many of the differences observed did not reach statistical significance and secondly, in this case the decreases of photosynthetic efficiency are probably related to multiple factors instead of just the MDA content and lipid peroxidation, which itself may be dependable on several

other factors (i.e. MDA may accumulate in order to help restore cellular osmotic homeostasis) (Cao et al. 2011).

The absence of significant differences in the proline mean values seems like another indicator of the positive response of the *F. angustifolia* trees to the contaminated soil. Proline is known to have multiple roles in plant physiology, aside from the compatible solute function, some of them closely related to stress response (Pandey and Sharma 2002; Szabados and Savouré 2009). While in many cases it still remains unclear how precisely proline acts as a protective molecule and how it contributes to cellular homeostasis under stressful conditions, it has already been reported to be associated with many kinds of plant stresses including metal contamination (Schat et al. 1997; Rai 2002). It is even considered one of the most common adaptive responses of plants to the contamination with metals (Zhao et al. 2008). It has been suggested that, in a situation of high concentration of metals, proline can act as a protector of membranes and proteins (i.e. protection of nitrate reductase), help maintain the $NADP^+$/NADPH ratios, regulate the osmotic pressure, act as a free radical scavenger, regulate metal-induced cellular acidification, be a source of nitrogen and carbon and act as a component of stress signal transduction pathways (Smirnoff and Cumbes 1989; Saradhi 1993; Hare and Cress 1997; Sharma and Dubey 2005; Saygideger and Deniz 2008; Shevyakova et al. 2009). However, it has also been suggested that in some cases proline accumulation can mean nothing but a consequence of stress-induced damage in the plants' cells (Matysik et al. 2002).

A study with the plant *Silene vulgaris* demonstrated interesting results concerning the relation between proline and metal tolerance. When exposed to metal contamination, an ecotype of *S. vulgaris* non-tolerant to metals showed increments in the proline content while a metal tolerant ecotype of the same species already had higher basal constitutive proline levels before exposure (Schat et al. 1997).

It is possible that the T15 proline value for the contaminated group, already higher than control, represented a physiological response to the contamination. It is unclear, though, if the following decreases after 30 and 60 days could represent an after-response stabilization of the levels. However, according to the proline contents of the reference

plants, these appeared to be the most stressed ones, having showed the highest values in all the measurements. It is suspected that in the reference soil, plants were probably under other kind of stress to which the leaves responded by producing higher amounts of proline. Another explanation to the high values in the reference lot and the increase in T75 for the control lot, could be that some water related stress (probably linked to the decrease in the RWC values) was responsible for the proline production. It would be also interesting to evaluate if compared to different ecotypes or similar species, the basal constitutive proline accumulation was higher for these trees.

According to the Fv/Fm results, the plants from the contaminated groups did not differ much from the reference group of plants, only from those in the control soil. The Fv/Fm ratio is a physiologic non-invasive measure that is directly correlated with the photosynthetic efficiency (Lafabrie et al. 2011). It has an inverse correlation with the damage inflicted to the PSII reaction centers and is currently the most used fluorescence parameter in plant physiology (Björkman and Demmig 1987; Farquhar et al. 1989). When plants are under environmental stresses, the decreases in the Fv/Fm ratio are related to the reduction of photosynthetic capacity, by means of photoprotective mechanisms that are triggered to dissipate excess energy in a situation of a dynamic/reversible photoinhibition. Still, chronic photoinhibition can occur and in this case it might be related to permanent damage to the PSII reaction center complexes (Peñuelas and Llusià 2002; Oliveira and Pañuelas 2004; Prieto et al. 2009). The inhibition of PSII activity has been related to metal exposure many times (Clijsters and Van Assche 1985). It has also been suggested that exposure to metals can cause several damage at the cell and ultrastructure level, like the destruction of sub-cellular structures and notably the disorganization in the chloroplasts, including the disappearance of grana (Clijsters and Van Assche 1985; Gallego et al. 1996; Huseyİnova et al. 2009).

Significant differences in these parameters, among times of exposure, were recorded both for the control and contaminated groups of plants. Nevertheless, all the mean values recorded for all the groups of plants were above 0.8, with the exception of the T45 contaminated group value of 0.796 which is still very close to 0.8. Such values

usually show that the plants are healthy. Despite some inter-species variability, for most species it has been established that values of Fv/Fm above 0.8 are characteristic of healthy and vigorous plants and that values below 0.8 are usually related to some kind of stressful conditions (Schulze and Caldwell 1997; Zarco-Tejada et al. 2000). A noteworthy fact is that for the 3 groups an initial drop in the Fv/Fm mean values was recorded in the first 45 days of exposure, but it was followed by an increase until T60. This variation was especially significant for the control and contaminated plants. It appears that after an initial shock, especially in the contaminated group where the drop was the highest (probably due to metal contamination), the plants were able to adapt and restore their photosynthetic rates; even those exposed to metal contamination conditions.

Being related with the reduction of photosynthesis, the low values of Fv/Fm are usually also associated with reduction of growth and biomass production (Clijsters and Van Assche 1985; Farquhar et al. 1989; Oliveira and Peñuelas 2004; Hermle et al. 2006). The outcome of the analysis of the Fv/Fm data actually reveals similarities with those of the growth test. Just like what was recorded for the growth test, in this case the reference and contaminated groups were also significantly different from the control group, but not between each other. This could mean that the higher Fv/Fm values in intermediate measurements for the control group could be related to the greater growth of the plants belonging to the group. Despite this, in the last measurement there were again no differences between the groups and the contaminated group showed, in fact, the highest value. This could also be related to the emergence of new shoots and leaves on the plants of this group. Still, given the similarity between the reference and the contaminated group one can believe that the growth limitation observed for both groups was probably not only related to metal-induced photoinhibition. It is likely that growth inhibition was also due to other factors like damage to the roots, inhibition of water uptake or reduced microbial activity, even though these could still be related both to soil characteristics and the presence of metals in the soil, or even the lack of soil volume to the evident outgrowing roots (Sanità di Toppi and Gabbrielli 1999). The data corresponding to the Φ_{PSI} ratio did not show any significant difference between any type of soil or time of exposure. This shows that despite the differences observed for the Fv/Fm ratio, the

quantum yield of the photosystems II from the leaves of the plants were not affected by the imposed conditions.

From the analyzed data, few parameters point to a negative effect of the contamination on the plants' health status. Furthermore, at the end of almost 3 months of exposure, the plants growing on the contaminated soil even appear to be more healthy than the other plants. The evident differences found for the growth parameter may possibly be related to the soil properties or for instance to the metal-induced inhibition of nutrient uptake, which could be determinant to the plants' growth rates (Pandey and Sharma 2002). Abundant metals in the soil can compete for root uptake, leading to the deficiency of other important elements that might influence the plant's growth (Jarvis et al. 1976; Clijsters and Van Assche 1985). Plus, many of the existing data are based on the study of 1 or 2 metals under controlled conditions while little is known about the effects and responses to mixed contamination (Hermle et al. 2006). The possible synergistic effects of 2 or more metals, like those found in the contaminated soil, should not be ignored and could be a target of a new study.

The metal quantification analysis for the various parts of the plants is a key point in a study based on the potential of a plant to be applied in phytoremediation. Being able to specify whether the plant is accumulating any metals and to what extent it does so, is crucial to clarify some of the observed physiological behavior patterns, to determine which phytoremediation class might be more suitable and also to reveal if the plant can be classified as a hyperaccumulator or even as an excluder. Unfortunately, due to the long duration of the quantification process and the need to be done outside the country, this analysis is still ongoing and the corresponding results will only be reported in a future opportunity.

Lastly, it is also worth mentioning that when plant assays are done in the laboratory under controlled conditions, some of the natural conditions are difficult to replicate. The light intensity from the lamps in the experimental chamber is constant and hundreds of times far below the intensity levels of solar light in the field. This does not

mean the output from the measurements would had greatly changed but maybe the global growth rates would be different, for instance.

The band profiling obtained through the technique of PCR-DGGE provided some insight on the variations in the composition of the endophytic and rhizospheric bacterial communities in the roots of the tested plants. The profiles indicated that for both types of bacteria, those associated with the roots of the plants from the “contaminated soil” were the ones which varied the most, over the course of the experiment. Naturally, this was most probably caused by the metal contamination present in the respective soil. Overall, the control and reference samples showed much less differences between each other and the “initial” extraction samples. Also, since these 3 groups showed more similarity concerning the rhizosphere communities, it may be a sign that the endophytic ones are more susceptible to change in response to changes in the environmental conditions. This is not in line with the concept that the communities of the rhizosphere are more prone to compete with each other and more vulnerable to soil-related conditions.

Although root exudates interfere with the bacterial composition on the rhizosphere, endophytic communities are under a more direct selection and control by the plant’s roots, which might explain why there is an overall smaller variety in the endophytic communities (Doty 2008).

In the rhizosphere fingerprinting the most intense bands, in the low end of the gel, were clearly present in the “contaminated” lanes but were nowhere visible on the remaining lanes (except lane 8). It seems that initially, and later on the control and reference soil samples, the bacterial communities showed more variety probably due to a more competitive balanced environment. However, the presence of the contaminants probably induced a more restrict environment, which favored only the most adapted species. Maybe the competitive tension was reduced and maybe the intense bands reflect the presence of some species that were able to thrive better in that environment.

By contrast, in the endophytes profiles, the intense bands suggest bacterial abundance for some species that were present in the “initial” samples and that remained in the others groups except on the “contaminated” one (except lane 8). It appears that

what was initially an abundant bacterial community inside the roots, was later, upon contamination exposure, almost completely eliminated in detriment of other ones. This is understandable since the root tissue consists of a more controlled environment. Although this study does not clarify it, the bacteria that persisted in the tissues throughout the entire experimental period could possibly be determinant to the plants' health and their capability to survive and develop under such conditions. Both analysis indicate that lane nº 8 should be classified as an experimental mistake and not be considered as representative of the "control group". These results indicate that the metal contamination had strong effects in the structure of the bacterial communities associated with the roots. This strongly corroborates the documented roles of the root bacteria as helper simbionts under adverse conditions.

It would be interesting to further study these bacterial communities in order to analyze their specific composition and discriminate potential useful bacterial species. These could be directly applied as new inoculants to promote the growth and provide resistance to other trees species.

5. Conclusions

Through the measurements of a selected set of parameters, the results allowed to demonstrate that the *F. angustifolia* species is indeed a good candidate for field pilot tests. The only pronounced stress factor detected in the trees growing in the contaminated soil was some growth limitation. The analysis of the growth and leaf area appears to be the most sensitive indicators of the effects of contamination. However, the similar growth between the reference and contaminated groups indicates that the inhibition of growth was probably not exclusively related to the contamination. Among the remaining parameters, some revealed supposable mid-term metal-induced effects soon after the transplantation, but all of them indicated that once the experimental period was completed no significant differences existed between the plants growing in the contaminated soil and the control and reference groups.

In conclusion, the tested species appear to show tolerance and resistance mechanisms against the tested contamination but that there is a need for a certain period of time for the plants to adapt to the challenging conditions and resume normal growth and physiological condition. Still, it would be interesting to develop further studies in a larger scale and with longer periods, in order to investigate which factors besides the metal contamination could significantly hold back the growth of the plants.

The PCR-DGGE analysis of the bacterial communities' profiles proved to be a good indicator of the variations of these communities in the vicinities and inside the roots of the tested plants. It could also be concluded that the presence of contamination in the soil had strong effects in the communities growing in the roots of the respective plants. It is very likely that these changes had a preponderant influence in the adaptation and development of the plants. Further studies should be conducted in order to evaluate the use of these communities in the improvement of the establishment of this and other plant species for the end of phytoremediation.

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