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The performance of *Fraxinus angustifolia* as a helper for metal phytoremediation programs and its relation to the endophytic bacterial communities

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ABSTRACT

Soil contamination with metals is a serious problem requiring urgent measures to counteract their impacts. The conventional soil remediation techniques are often inefficient and expensive. In this work, we studied the potential of the species *Fraxinus angustifolia* for the phytoremediation of metal contaminated soils from the Cunha Baixa uranium mine (Mangualde, Portugal). The plants were exposed to a contaminated, a reference and a control soil, for a period of about 3 months, during which metal accumulation and a set of physiologic parameters (shoot height, potential maximum efficiency of PSII (Fv/Fm) and quantum yield of PSII (Φ_{PSII}), leaf area, specific leaf area, leaf relative water content, leaf chlorophyll *a*, chlorophyll *b* and carotenoid content, leaf malondialdehyde (MDA) content and leaf proline content) were measured. The genetic profiles of the endophytic communities from the roots of the plants were also analyzed, via PCR-Denaturing Gradient Gel Electrophoresis (DGGE) targeted for a conserved region of 16S rDNA, pre- and post-exposure. Only the shoot height was a suitable indicator of the negative effects of the contamination with metals on the plants. The remaining parameters indicated good physiologic fitness and recovery. The trees did not hyperaccumulate metals but extracted Sr at a higher rate. The bacterial profiles from the control and reference soils showed more similarity with each other and with the pre-exposure profiles than with those from the contaminated soil. We showed that *F. angustifolia* has the ability to resist and adapt to the adverse conditions of contamination, revealing a potential which can be exploited for phytoremediation, specifically phytostabilization. It also revealed that changes exerted on the bacterial root communities exposed to contamination, resulted in profiles considerably different from those of the remaining communities.

1. Introduction

The contamination of soils with metals is a serious worldwide problem that needs to be urgently addressed. Such contamination can lead to severe impacts on natural ecosystems and on the communities sustained by them (Antunes et al., 2008; Hasan et al., 2009; Pereira et al., 2008; Prasad and Freitas, 2006). In places where lands are highly contaminated, it becomes urgent to come up with remediation plans to protect the ecosystems that depend on them. In most cases, the applied conventional methods (mostly physical, chemical and engineering techniques) are too expensive and fail to efficiently decontaminate larger areas in an eco-friendly manner (Pulford and Watson, 2003).

Phytoremediation consists of the use of plants to remove or render harmless contaminants from the soil and is a technology that has

Abbreviations: Φ_{PSII}, Photosystem II Quantum Yield; APS, Ammonium Peroxodisulfate; BSA, Bovine Serum Albumin; DGGE, Denaturing Gradient Gel Electrophoresis; Fv/Fm, Photosystem II Maximum Efficiency; IAA, Indole-3-Acetic Acid; MDA, Malondialdehyde; PSII, Photosystem II; RWC, Relative Water Content; TBA, Thiobarbituric Acid; TCA, Trichloroacetic Acid; TEMED, Tetramethylethylenediamine; Tris, Tris(hydroxymethyl)aminomethane.

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grown in the last years as a promising method for metal and radionuclide remediation (Bhargava et al., 2012; Huseyİnova et al., 2009; Rascio and Navari-Izzo, 2011). Phytoremediation is a process that can take various routes and is commonly divided in the following different classes (Salt et al., 1998): i) phytoaccumulation and phytoextraction; ii) phytodegradation or phytotransformation; iii) phytostabilization; iv) phytovolatilization and v) rhizofiltration.

By ways of immobilizing, degrading, transferring and accumulating pollutants, phytoremediation began to gain the potential to become a successful "green" sustainable alternative for soil decontamination. However, to this day, phytoremediation still presents some disadvantages. The very slow nature of this treatment stands as one of the most important factors, among others like the dependence on various environmental factors, variable bio-availability of the contaminants for the plants, low range of plant tolerance (some plants may tolerate well one metal but not others also present), the need of plants to accumulate contaminants in the harvestable parts for phytoextraction, and low biomass producing plants not having the capacity to support large efficient remediation programs (Glick, 2010; Sarma, 2011).

As not every plant is able to deal with the amounts of contamination present in the soil, also not all of those that can survive under such conditions are capable of extracting or degrading the pollutants at acceptable rates (Bhargava et al., 2012; Pulford and Watson, 2003). The accumulation of metals in plants depends on various separated factors ranging from soil properties, to total and available metal concentrations and also plant physiology related parameters (Antoniadis et al., 2006). Named as hyperaccumulator plants, they have increasingly earned interest in the field as potential models for phytoremediation. 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). 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 a significant metal uptake in their roots, nor accumulate them in the above ground tissues (Robinson et al., 2006; Shaw et al., 2006). Since they are basically equipped with avoidance/resistance mechanisms, while they are not applicable for the metal extraction process they are, however, key helpers to the whole remediation process working as soil stabilizers, preventing the spread of pollutants by soil erosion and reducing metal availability to other plants (Shaw et al., 2006). Even though it is impressive how 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. Recent reviews show that the list of potential candidates to accumulate metals comprises already a few hundred species (Sarma, 2011; Wuana and Okieimen, 2010). Despite this, we believe that considering the complexity of phytoremediation itself and whole phytoremediation programs there is still the need for more detailed information not only on some of the already listed species (regarding the extent of extraction, their real application in large scale remediation programs, physiology analysis), but also studies featuring less known and studied species. In this study we attempt to help filling that gap.

As most of the studies that approach the theme of phytoremediation focus primarily on the plant species themselves, they tend to overlook what seems to be a factor much more relevant than was previously thought. This important factor consists of the non-pathogenic bacterial communities associated to the plants' roots and their interactions. The rhizospheric and endophytic bacteria are the two major types of root-bacteria known to have relevant effects on plants (Doty, 2008; Gomes et al., 2001). These are known for establishing a close relationship with the plants and for being beneficial to them, not only by directly aiding the plants' development, but also by grating protection against pathogens (Gomes et al., 2001). These bacteria can also enhance the tolerance and uptake of metals by the plant species either by making metals more or less available to plants outside the roots or by absorbing and immobilizing them inside the vegetal tissues. Hence, root-associated bacteria may facilitate the phytoremediation process almost directly, possibly during either phytoextraction or phytostabilization (Glick, 2010; Sheng et al., 2008). The interactions between plants and these microbial communities are rather complex and often ignored in studies concerning metal uptake by plants, therefore needing further characterization. Moreover, the impacts of the contaminated soil on these communities need more clarification. We believe that this sub-field of phytoremediation still stands as a new big challenge on the list of unresolved questions of phytoremediation.

Compared to smaller plants, tall trees have deeper root systems allowing them to reach soil and water sheets at a greater depth (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). Trees are also fairly accepted by the public as ecological and esthetical elements for rural and urban areas (Domínguez et al., 2008). Belonging to the family Oleaceae the genus *Fraxinus* (commonly ash tree) comprises between 39 and 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 accumulation, physiological data and microbial community analysis (Haro et al., 2000; Mertens et al., 2004; Pulford and Dickinson, 2006; Rosseli et al., 2003; Tlustoš et al., 2006).

The main objective of this work was to investigate the potential of a native deciduous ash species, *Fraxinus angustifolia*, for the purpose of metal phytoremediation. In an attempt to enrich our characterization we performed simultaneously an evaluation of a set of physiologic parameters, an assessment of metal accumulation in the plants and, since most studies are based on controlled laboratory conditions only (Glick, 2010), a study of the possible roles of the species in an eventual field phytoremediation program was performed. It was also our goal to help fill the void on the emerging branch of phytoremediation that concerns microbial communities, trying to engage a more novel and complete approach. Hence, we did so by applying the molecular based technique of denaturing gradient gel electrophoresis (DGGE) in order to check for spatial and temporal variations on the endophytic bacterial communities isolated from the roots of plants grown in non-contaminated soils and contaminated soils from a uranium mine in Cunha Baixa (Mangualde), center of Portugal.

2. Materials and methods

2.1. Test soils and artificial substrate

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 uranium mine area (central Portugal). The general physical and chemical characterization of this soil was already made by Pereira et al. (2008, 2009) and is summarized in Tables (1A and 1B). This soil was one of the most contaminated soils found in the area, with high total concentrations of Al (25,628.5 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 (center of Portugal). It was characterized as a reference soil by Caetano et al. (2012) (Tables 1A and 1B). 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

A group of 54 rooted (grown from seed) plantlets, belonging to the species *F. angustifolia*, was obtained directly from a nursery. They were chosen according to their size and apparent good physiological conditions, in order to preserve group homogeneity. While still in the soil from the nursery, all the plantlets were initially kept in an acclimatized