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Emerging applications of X-ray fluorescence spectroscopy to assesses the phytoremediation potential and mineral nutrition aspects: *Eucalyptus urophylla x Eucalyptus grandis* case studies

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Thesis presented to obtain the degree of Doctor in Science. Area: Forest Resources. Option in: Silviculture and Forest Management

Piracicaba
2019

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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2019

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Sant'Anna Neto, Analder

Emerging applications of X-ray fluorescence spectroscopy to assesses the phytoremediation potential and mineral nutrition aspects: *Eucalyptus urophylla* x *Eucalyptus grandis* case studies/ Analder Sant'Anna Neto. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2019.

85 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura "Luiz de Queiroz".

1. Biotecnologia 2. Fitoextração 3. Chumbo 4. Interação entre elementos 5. Cultura de tecidos 6. Fertilização 7. Seleção de materiais I. Título

RESUMO

Aplicações emergentes da espectroscopia de fluorescência de raios X na investigação do potencial para fitorremediação e aspectos nutricionais: casos de estudo com o *Eucalyptus urophylla* x *Eucalyptus grandis*

Este trabalho apresenta duas novas aplicações emergentes da espectroscopia de fluorescência de raios X (*XRF*) na área de ciências florestais. O trabalho está estruturado com um capítulo introdutório e dois capítulos técnicos. O segundo capítulo traz um método que permite acessar o potencial uso de espécies florestais em programas de fitorremediação. No terceiro capítulo, foram analisadas as diferentes tendências de uso de elementos realizada por mudas para modelar a melhor estratégia de fertilização. Finalmente, um capítulo final que apresenta as principais considerações, descobertas científicas e avanços. No capítulo 2, foi proposto um rápido método que investiga as interações entre brotações de eucalipto cultivadas *in vitro* expostas a diferentes doses de chumbo (Pb). O método explora o crescimento das brotações, tendências de tolerância ao Pb, acúmulo e mobilidade. Assim como, as interações entre o Pb e elementos essenciais em diferentes regiões das brotações. E também, possíveis distúrbios causados pelo Pb adicionado no aspecto nutricional das brotações. Assim, usando menos de 2 g de Pb foi possível analisar 256 explantes *in vitro* em um curto período, no total 100 dias. O método traz importante descobertas sobre a tolerância do material vegetal, o potencial para fitoextração e possíveis mecanismos de defesa usados pelas brotações para evitar os efeitos fitotóxicos do Pb. Permitindo assim, selecionar o melhor material vegetal disponível em escala mundial. Com amplo espectro de utilização em programas voltados a descontaminação do solo e das águas subterrâneas. O capítulo 3 apresenta um novo e rápido método que permite investigar tendências quanto às estratégias de uso dos elementos em mudas e sua relevância para o crescimento vegetativo. Mapeando regiões específicas no perfil transversal do caule das mudas, foi possível analisar as tendências de acúmulo dos elementos, transporte e afinidade. O novo método apresenta informações cruciais que permitem entender os processos internos de uso dos elementos. Característica indispensável para melhorar o entendimento sobre os aspectos nutricionais apresentados pelas mudas. Podendo ser utilizado para modelar a melhor estratégia de fertilização em viveiros florestais globalmente. A análise de *XRF* tem sido utilizada em escala mundial para revelar estes processos internos devido sua ampla aplicação. Permitindo realizar análises em diferentes tecidos vegetais com objetivo de aumentar o conhecimento sobre as habilidades das plantas antes desconhecidas. Associada com outras poderosas técnicas analíticas disponíveis, como espectroscopia que estuda a estrutura fina da absorção de raios X próxima à borda (*XANES*). É possível conseguir diversos tipos de avanços científicos nas áreas florestal, ambiental e agronômica. Neste contexto, os métodos desenvolvidos também podem ser aplicados para aumentar o conhecimento em outras áreas. Entre elas, na comparação de diferentes genótipos, a eficiência quanto ao uso de nutrientes, além da ocorrência de doenças e pragas. Possibilitando selecionar e desenvolver o melhor material vegetal disponível com maior capacidade para enfrentar efeitos ambientais adversos mundialmente. O método também permite aumentar a segurança relacionada a produção de alimentos, removendo elementos potencialmente tóxicos de áreas utilizadas na agricultura e também evitando a fertilização excessiva, respectivamente.

Palavras-chave: Biotecnologia, Fitoextração, Chumbo, Interação entre elementos, Cultura de tecidos, Fertilização, Seleção de materiais

ABSTRACT

Emerging applications of X-ray fluorescence spectroscopy to assesses the phytoremediation potential and mineral nutrition aspects: *Eucalyptus urophylla* x *Eucalyptus grandis* case studies

This work presents emerging applications of X-ray fluorescence (XRF) in the forest science area. It is structured with an introductory chapter and two technical chapters. The second chapter brings a method that allows assesses the potential use of forest species in phytoremediation programs. The thirty chapter, there were assessed the seedlings elements use trends, in order to modeling the best fertilizer strategy. Finally, a chapter with the final considerations and the most important scientific advances discovered. In the chapter 2, there was proposed a fast method to assess the interactions between eucalypt shoots cultured *in vitro* exposed to different lead (Pb) doses. The method explores the shoots growth rate aspects, Pb tolerance, accumulation and mobility trends. Such as the interactions between Pb and essential elements in different shoots regions. As possible disturbances caused by the Pb added in the shoots mineral nutrition aspects. Thus, using less than 2 g of Pb it was possible to analyze 256 explants *in vitro* in a short period, in total 100 days. Therefore, the method brings important insights about the vegetal material tolerance, phytoextraction potential and possible mechanism used by the shoots in order to avoid the Pb phytotoxic effects. That allows select the best vegetal material available worldwide, therefore, can be included in emergence programs of soil and groundwater decontamination. The chapter 3 presents a fast and new method with allows investigated the internal elements use trends and its importance to the seedlings vegetative growth. Mapping target regions in the seedlings stem cross-sections, it was possible assesses the elements accumulation, transport and affinity. This new method shows crucial insights with allows understand the internal elements use processes. Indispensable feature to provide a better understanding about the seedlings mineral nutrition aspects. That can be used to model the best fertilizer use strategy in the nurseries worldwide. The XRF analysis have been widely used to reveal important internal process due its suitable applications. Allowing to perform analysis in different vegetal tissue in order to better understand the plants ability before ignored. Associated with other powerful methods available, as the X-ray absorption near-edge structure (XANES). It is still possible to perform several scientific advances in the forest, environmental and agronomic areas. In this context, the methods built can also be applied to improve the knowledge for several purposes. Such as the comparison between different genotypes, elements use efficiency, the occurrence of diseases providing vegetal materials with higher quality against possible anthropic disturbances worldwide. The methods also can enhance the food security production, removing potential toxic elements from agricultural areas and avoiding the over fertilization, respectively.

Keywords: Biotechnology, Phytoextraction, Lead, Elements interaction, Tissue culture, Fertilizer, Vegetal material selection

1. INTRODUCTION

To understand interactions between the forest species and essential nutrients, or even, potential toxic elements can provide several benefits to the environment and also to enhance the wood productivity capacity. Regarding the increasing release of potential toxic elements to the environment and the highest requirement of forest products, as timber and pulp. Due to the globally growing anthropic activities and consumption rates, respectively, these challenges must be faced as soon as possible, avoiding potential risks in the food production security, regarding the food chain contamination possibility. Other important perspective is the approaching of the use of fertilizer in the seedling nurseries. In order to provide genotypes with best field performance avoiding the over fertilization. In these contexts, its indispensable to solve gaps about several plant internal processes, with emphasis in the elements use strategies. Such as the elements contribution to the vegetative growth, accumulation, mobility and transport trends. The knowledge will help to perform a better selection of the best material available regarding its use in phytoremediation programs. Such as, it is also possible modeling the best fertilization strategy avoiding the elements leaching to the soil and groundwater. Both aspects in order to enhance the environmental quality recovering the soil production capacity and providing a sustainable use or fertilizer during the trees initial production processes. However, new methods, approaches and techniques mostly to be developed in order to assess the plants internal processes.

Environmental pollution and the risk to the all living beings is not a recent concern. Since the Second World War the anthropogenic release of potential toxic elements to the environment have been increasing worldwide. The reduction of the environmental quality brings directly and indirectly effects that can affect negatively the biosphere. There are many pathways of potential toxic elements inputs in the ecosystem. An important source of trace metals pollution is the industry activity, a recently research presents the status of trace elements release to the environment. The smelting, metal production and leather industries, lead the was the most significant metal that contributed to the environmental pollution (Kabir et al., 2012). The atmosphere emissions are also an important pathway of trace elements input in the environmental. In the 1970's, this issue it was a global concern due the drastically increase of potential toxic elements in the atmosphere (NRIAGU, 1979). The author also highlights the massive redistribution of trace metals, was related mostly with the anthropogenic activities. Due the several soil pollutions sources, different sources act distinct way regarding the negative impacts, such as its main interaction between these sources and the environment (Figure 1).

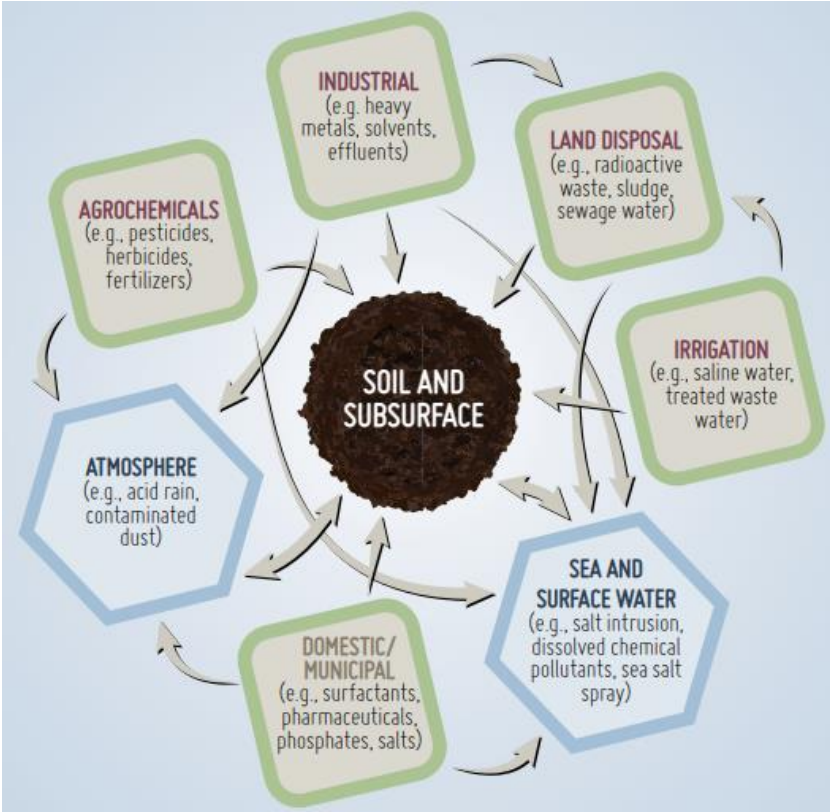

Figure 1. Main sources of soil pollution and its interactions and pollutants (Yaron et al., 2012).

Between the potentially hazardous trace metals, the lead (Pb) are listed as a great risk to the environmental health. The bulletin of the World Health Organization classified the Pb as the major public health hazard in a global scale (Tong et al., 2000). Still about the latest work, the author emphasized the developing countries as the group with highest risk, due the fast industrialization and the reduced public polices implement capacity. The total man-induced mobilization of heavy metals in the 1980's was in thousand tons per year 1,160 for Pb, with a considerable risk for the human beings due its food chain contamination possibility (Nriagu and Pacyna, 1988). In 2011, an article showed that the Pb levels in the children's blood mainly in developing countries can be over than 20 μg dl-1 (Clune et al., 2011). With encompass a great risk to the children's development due the several diseases associated with high amounts of Pb in the blood. One of the highest damages is the Pb exposure to low concentrations, causing deficiencies in the intellectual children's development.

Global food security is a constant concern regarding the increase of food demand worldwide. Recently, the Food and Agriculture Organization of the United Nations (FAO) published recently a report that showed the three mainly challenges in the global agriculture. Employing a safe production of nutritious food using sustainable techniques that also act against the global climate changes and in the resources conservation ((FAO) and (OECD), 2018). The environmental contamination can expose the humankind and all biosphere to a high risk. One of the major global concern it is the soil and water pollution due the excessive nutrient application to supply the increasing worldwide food demand (Rodríguez et al., 2018). The authors also highlight from the 20th century until now, the use of fertilizers in intensified crops systems for food production increase as the environmental discharge of nutrients to the water. Besides that, agriculture represents the major role in water pollution, farms activities also discharge large amounts of agrochemicals and livestock manure (Mateo-Sagasta et al., 2018).

In this context, the contamination of agricultural soils has been attracted global attention due the negative effects decreasing the food production capacity. Or even, in extreme cases, cause the total loss of soil use capacity. Negative effects are also related in the living beings community exposed to high concentrations of potential toxic elements. Both cases, due the directly contact or in an indirectly way by the ingestion of contaminated food or drink polluted water. A global strategy was proposed in order to brings the culture production capacity of agricultural contaminated soils. Enhancing the food production capacity and consequently, increase the security regarding the food production. The lasted FAO report about the soil pollution the authors showed the main sources of potential toxic elements in these areas (Rodríguez et al., 2018). The soil and groundwater pollution process can be considered a high risk regarding the food production and the increasing global population and food demand. The over fertilization, irrigation with poor quality water plus other important agricultural practices are the mostly important pathways of pollutants input in the agricultural soils (Figure 2). Other important agent that contribute to the release of mixed potential toxic elements are the pesticides, due the intensification uses in the last few decades (Silva et al., 2019).

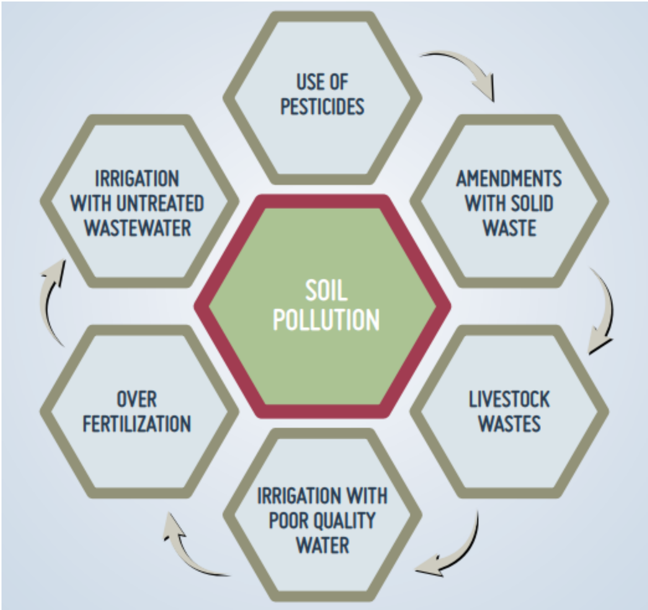

Figure 2. Agricultural sources of soil pollution (Rodríguez et al., 2018).

One of the highest challenges to restore these contaminated areas it is the heterogeneous conditions. Regarding the pollutants behavior in the soil, several techniques have been employed to remove, immobilize or even volatilize potential toxic elements. However, these techniques employ high investments, sometimes, can also cause a negative impacts in the environment (Wan et al., 2016a). Causing disturbances in the ecosystem balance affecting mostly in the biologic aspects from the soil (Kibria, 2014). The behavior of potential toxic elements in the environment and its ecotoxicology effects are also important factors (Ali et al., 2019). Between the techniques available, the phytoremediation of contaminated soils and groundwater have been widely explored. With encompass in the use of plants to remove potential toxic elements from the soil.

The phytoremediation concept was created observing the high accumulation rate of specific chemical species expressed by many plant species since the middle of last century (Brooks and Wither, 1977; R. Brooks et al., 1974). The scientists associated the potential use of plants to sink and accumulate highest amount of nickel. With the potential use of these species in remediation programs from polluted soils and groundwater (E. Salt et al., 1995). One important advantage of this technique is the lowest costs comparing with the other usual techniques. In less than 7 years, the benefits would offset the capital used to the remediation process (Wan et al., 2016b). The phytoremediation is also classified as an environmentally friendly method, due its minimal ecological disturbances (Stanley Rungwa et al., 2013). In 2008, more than 400 plant species were identified with a potential soil and groundwater decontamination (Lone et al., 2008). Regarding the technique perspective, the authors also highlight the continuous improvement of 15 hyperaccumulator genes and the gain about the increasing rates of vegetal material available with target features. Therefore, phytoremediation can be understood as the potential of a specific plant variety to restore contaminated soil for any of a variety of uses and have attracted increasing attention in the lasted 50 years (Cunningham et al., 1995; Reeves et al., 2018). The developing countries do not showed a consistency dataset about the soil sustainability conditions and potential plants species that can mitigate adverse effects in polluted areas (Reeves, 2003). In a successful phytoremediation program is essential understand the internal process in the plants. The use of suitable genotypes that can sink and tolerate great amounts of potential toxic elements and also show a viable biomass production rate its crucial.

The elements behavior in the soil is also an important factor in the phytoremediation projects. Therefore, elements bioavailability in the soil, defined as a complex dynamic process comprising the environmental availability, bioavailability and the toxicological bioavailability (Balabanova et al., 2015). To understand these complex elements behavior is a key step to the phytoremediation programs successfully such as the elements mobility and accumulation plants organs trends. Several works showed the potential use of fast-growing trees in phytoremediation programs (Couselo Bandin et al., 2012). Due to the early hyperaccumulator species mostly explored the superficial soil layers with a lower biomass production as the sunflower (Adesodun et al., 2010) and Indian mustard (E. Salt et al., 1995). Many authors cited the potential use of trees stands in phytoremediation programs, using *Dalbergia sissoo* (Kalam et al., 2019), willow (Malá et al., 2010) and *Populus* (Lonardo et al., 2011; Shi et al., 2019) genus. The trees can explore and sink greatest amounts of potential toxic elements due its highest biomass production and soil exploration capacity. Even comparing with the hyperaccumulator plants, the viability in order to use non-hyperaccumulator species have been attracted the scientist attention, mostly of the cases, using fast-growing trees (Souza et al., 2013).

As early discussed, the incorrect use of fertilizer can cause adverse effects in the environmental, loss of capital due to its higher value aggregated. Therefore, its indispensable understand the elements use trends and influences in the vegetative growth to modeling the best fertilizer strategy (Gonçalves and Benedetti, 2004). Brazilian forest-based industry has heavily invested in forest management to improve the land use efficiency and the production. *Eucalyptus* is the main planted forest species in Brazil with 72.3% of total planted area. The country also has the highest productivity in the world, in average 35.7 m^3 ha^{-1} year and the shortest cutting cycle, between 6 to 7 years (IBÁ, 2017). Many decades improving the genotypes available, performing new material introduction and investing in the mineral nutrition supplying process. These high rates of wood production can also be associated with the Brazilian climate and soil conditions, as 16 well as continuous improvements investments in forest management and genetic engineering

(IBÁ, 2017). Thus, its necessary work in new tools or even method that allows investigated the plant mineral nutrition status, as the elements use and transport trends avoiding the over fertilization and consequently, the soil and groundwater pollution (Hendricks et al., 2019).

Several publications have been enhancing the knowledge on the mineral nutrition regarding the growth rate aspects. The elements and water use efficiency of its directly linked to the potential growth of eucalypt stands (Stape et al., 2004). However, find potential species, as the use of fast-growing trees. In order to modeling the best fertilizer application reaching the optimal growth isn't an easy task. Due the heterogeneous vegetal material available and widely used supplying the paper and pulp industries. Sometimes, the same material can present a high variation and different responses to the same factor of disturbance. The mineral nutrition it is a complex issue, therefore, several methods can describe the influences of nutrient levels and its influence in the plant development, as the law of the minimum (van der Ploeg et al., 1999). Or even the concept of the nutrient flux density models that allow assess the elements internal use strategies employed by several conifer species (Ingestad et al., 1981). Fast-growing trees shows an additional challenge, due the highest biomass production the elements uptake and utilization rate is higher. This highest elements utilization highlight the need to model the best fertilizer use strategy in order to avoid disturbances in the plant development (Marschner, 1995; Tng et al., 2014).

Besides that, mineral nutrition investigation of forest species has been performed assessing the elements concentrations in the leaves (Linder, 1995; van den Driessche, 1974). This usual technique presents limitations in order to assess the elements internal utilization. Thus, the analytical methods have been increasing such as the technology employed to perform vegetal tissue analysis for several purposes. These innovation process allows investigate different plants regions with extreme precision. And also investigate different plants aspects regarding its development under different conditions. Between the mostly used techniques, there were the inductively coupled plasma atomic emission spectroscopy. It is widely used to perform the chemistry of essential and trace metals in plants (Malá et al., 2010; Wiszniewska et al., 2016). Such as laser ablation coupled to mass spectrometry (Pedrosa Diniz et al., 2019) and the atomic emission spectrometry with inductively coupled plasma (Balabanova et al., 2015). In addition, different methods allowed perform 2D maps from different plant regions using a benchtop spectrometer by X-ray fluorescence spectroscopy (XRF) analysis, or even, using a synchrotron source (Campos et al., 2015; Kopittke et al., 2018). As the laser ablation-inductively coupled plasma-mass spectrometry have been also employed (Callahan et al., 2015). These imaging
17 methods allow to investigate many internal processes and perspectives from the plant science. Using the spatial distribution trends from specific regions to assesses the mineral nutrition, diseases

control, phytotoxic and for phytoremediation proposes (Carvalho et al., 2018; Rodrigues et al., 2018). The X-ray fluorescence is a non-destructive technique, required minimal sample preparation and can analyze several elements simultaneously. Allowing perform analysis *in vivo*, or even, in fresh vegetal tissues preserving the elements redistribution and possible changes along the investigated area (Nishida Máximo da Cruz et al., 2017).

In these contexts, we developed two novel and fast methods, the first one, allowed assess the potential use of fast-growing trees in phytoremediation programs. Using the method its possible select and promptly propagate the best vegetal material available worldwide cultivated under controlled conditions. The second method was developed in order to better understand the internal elements utilization in eucalypt seedlings. Assessing the elements spatial distribution, it was possible assessed the elements interactions and use trends. Such as modeling the best fertilizer strategy in the nursery growth stage.

2. FINAL CONSIDERATIONS

The first method developed allows perform a further investigation between the eucalypt shoots aspects facing the Pb exposition. Temporal analysis also provides possible essential elements interactions and nutritional disturbances insights. Using less than 2 g of Pb its possible analyzed 256 explants in a short period. There can be assessed the potential use of forest species in phytoremediation programs worldwide. Regarding to the elements interactions inside the shoots, the Pb showed a synergistic effect between with specific elements. On the other hand, the potential toxic element showed a strong antagonistic effect with the Ca.

The second method proposed, allowed build 2D μ-XRF maps of P, S, K, Ca, Mn and Fe simultaneously from the eucalypt stem cross-section seedlings. We solved the matrix effect due the heterogeneous superficial density using the Compton scattering as internal standard. Such as findings regarding the elements distribution and contribution trends to the vegetative growth. Elements showed a synergistic effect and sometimes a high competition in the tissue vessels reflecting an antagonistic behavior. Thus, the best fertilizer strategy can be develop using these internal processes in order to maximize the nutrients utilization.

Both methods can be used to perform the XRF analysis in several forest species and answer fundamental questions about the interactions between the vegetal material and the essential and potential toxic elements worldwide. As its application for several purposes, helping in the mineral nutrition modeling, comparing different genotypes, in the diseases and pests control.

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