UNIVERSITA' DEGLI STUDI DI PISA

Dottorato in Scienze delle Produzioni vegetali eco-compatibili

OUTLINE OF THE THESIS. PHYTOEXTRACTION TECHINIQUES OF HEAVY METALS

Metals are present naturally in the Earth's crust at various levels. Mining, industry and agriculture accelerate their release into ecosystems, causing serious environmental problems and posing a threat to human health. Unlike organic contaminants, metals cannot be degraded.

The use of plants for remediation of metals offers an attractive alternative to conventional remediation methodologies. It doesn't need use of energy, being solar driven, and can be carried out *in situ*, minimizing cost and human exposure. However, as a technology, phytoremediation, and particularly phytoextraction, is still in its developmental stage. Before phytoextraction becomes a widely accepted technique, several questions must be resolved starting from the biochemical, biological and molecular mechanisms which determine metal accumulation and tolerance in plants.

The principal aim of this thesis is, therefore, to underline some of the approaches that can be used to further enhance the efficiency of metal phytoextraction, studying and improving well-known techniques and developing new approaches.

Several studies concerning phytoextraction of metals have been performed in hydroponic culture or in artificially contaminated substrates, while only few investigations have been performed using soils naturally contaminated by more than one metal, as done in the present work. The soil of interest was taken from a site, identified in collaboration with the Agency for Environment Protection of Friuli-Venezia Giulia, located within the perimeter of a chemical factory; it is represented by a dump of pyrite ashes deriving from the burning process of the mineral for sulphur extraction and showed a multiple contamination from As, Cd, Cu, Pb and Zn. At the present the site is included within the perimeter of the polluted area named "Grado and Marano lagoon and neighbouring water-courses" identified as a site of national interest by the Cabinet Decree 468/2001 "National Programme for Environmental Restoration of Polluted Sites".

The work has been developed as follow.

Firstly, a screening study in hydroponic culture was performed to identify the most suitable plant species for the remediation of the site. Secondly, once *B. carinata* cv. 079444 was selected, studies directed to maximize its phytoextraction capacity (for instance enhancing metal accumulation without decreasing biomass) were conducted.

Considering that plant uptake is generally related to the availability of metals in soil, the addition of synthetic chelators must be considered a suitable approach to increase the accumulation of metals. Such approach has been extensively used in the last years, but not without problems. The use of such synthetic chelators could have, in fact, toxic effects on the environment enhancing both leaching of metals to groundwater and promotion of off-site migration. Due to the above-mentioned potential risks, chelators that combine high biodegradability, low phytotoxicity and chelating strength, such as nitrilotriacetic acid (NTA) and [S,S]-ethylenediamine disuccinic acid (EDDS), were the only proposed in this chemically-assisted phytoextraction program. The aim of this part of the work was improving the well-known chemically-assisted phytoextraction technique using chelators that are promising alternatives to the most common benchmark chelators (EDTA or its derivatives).

However, the development a technique totally environmental safe is still a goal for phytoremediation.

The awareness that some compounds commonly exuded from plant roots are implicated in several mechanisms including mobilisation, uptake and detoxification of metals by plants and that the non-accumulator plants generally exude more organic compounds than hyperaccumulator ones do, have suggested the idea to combine, in succession, different plant species in order to reach a maximal phytoextraction efficiency. Three excluder plant species, endemic of metalliferous soils, (*Pinus pinaster*, *Plantago lanceolata* and *Silene paradoxa*) were used for producing natural chelators for assisted phytoextraction by *B. carinata*. The results, in terms of metal mobilisation and accumulation, encouraged further researches about this new promising totally environmentally safe approach.

Since the risk associated to the transgenic plants or their gene "escaping" is not considered a significant problem, the genetic engineering approach was also taken into consideration. This approach allows to insert a desired property in a plant with a natural good phytoextraction capacity in order to strengthen its potentiality.

The MT2b gene from *Arabidopsis thaliana* was chosen since it is well known that MTs proteins are involved in metal homeostasis and tolerance. Sometimes, as an indirect effect, they are also involved in enhanced metal accumulation.

In the genetic engineering approach, the obtained transformed plants are initially tested with one metal at a time. Once the phenotype is well characterised, the simultaneous effects of more metals can be evaluated. Since the effects of MTs genes on Cu and Cd tolerance are widely investigated, As(III) was chosen as the most interesting metal, among those present in the soil, to start with.

The AtMT2b gene was initially tested in the model plant *Nicotiana tabacum* following a pathway generally used by geneticists. Tobacco is considered a model plant because it is a high biomass plant species, it is sufficiently hardy and competitive and it has a good phytoremediation capacity to start with. In addition, a transformation protocol for the species has been already developed.

Unfortunately, the insertion of the AtMT2b gene into the tobacco genome didn't show any promising results in terms of enhanced As(III) accumulation and tolerance. However, if the insertion of AtMT2b leads to transformed tobacco plants with enhanced capacity of phytoextraction of the other metals present in the multiple-contaminated soil (Cd, Cu, Pb and Zn), the development of a transformation protocol for *B. carinata* will be taken into consideration.

Investigating some of the uptake and translocation mechanisms of *Brassica carinata* is another way for improving its phytoextraction capacity since accumulation and transport of metals in the harvestable part of plants are two of the most important features in phytoextraction technique.

In order to reach this aim, arsenic and copper, among the metals present in the dump area, were studied. These two metals were chosen because of their similarities (both redox metals that could generate active oxygen species) and their differences in chemical properties (arsenic is present in the soil as anion while copper as cation) and toxicity (copper is a micronutrient while arsenic is not).

Studying the uptake kinetics it was possible to evaluate the differences and the similarities in the absorption of a toxic anion and an essential cation by *B. carinata* roots. As regard copper, the involvement of amino acids in its xylematic transport was evaluated as well, in order to understand and potentially improve the translocation of this cation towards the harvestable part of *B. carinata*.