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PhD thesis

Exploring the *Bioremediation* potential of *Populus alba* in experimental system and in plant-rhizosphere fungi associations

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Riassunto

Il presente studio è stato organizzato in tre prove, volte a valutare il potenziale di biorimedio del *Populus alba*, in differenti condizioni sperimentali ed in associazione con funghi della rizosfera. Nella prima parte della ricerca è stata studiata la capacità di accumulo, traslocazione e tolleranza del clone "Villafranca" di *Populus alba* esposto a concentrazioni crescenti di cadmio (Cd 0, 5, 50 e 250 μM) e rame (Cu 0, 5, 50, 250 e 500 μM) in condizioni di autonomia autotrofica. L'induzione dell'autotrofia è stata garantita mediante l'applicazione di filtri che hanno consentito il miglioramento dello screening *in vitro*. I risultati hanno mostrato, dopo 15 giorni, un aumento di Cd e Cu nella parte aerea e nelle radici, all'aumentare della concentrazione dei metalli nel substrato. In "Villafranca", il Cu ha evidenziato effetti tossici sullo sviluppo delle piantine, soprattutto ad alte concentrazioni, causando una riduzione della biomassa secca.

L'indice di tolleranza (Ti) e il fattore di traslocazione (TF) denotavano una buona tolleranza e capacità di traslocazione del metallo in questo clone, quando esposto ad elevate concentrazioni. Alla luce dei risultati ottenuti, si raccomanda l'utilizzo di questo sistema di *sceening* condotto *in vitro,* come valido strumento volto a selezionare genotipi di specie vegetali resistenti ad elevate concentrazioni di metalli pesanti; permettendo di fornire rapidamente indicazioni circa le potenzialità di accumulo e di tolleranza. La seconda parte dello studio ha incluso valutazioni *in vitro* atte ad indagare i meccanismi coinvolti nell'aumento dell'assorbimento di Cd da parte di diverse specie di funghi del genere *Trichoderma*. A tale scopo, è stata inizialmente testata la capacità di tolleranza di 52 ceppi di *Trichoderma* nei confronti di diverse concentrazioni di Cd (0, 5, 50, 250 μM). Dai risultati ottenuti è stato possibile effettuare la selezione di 27 ceppi tolleranti il metallo appartenenti a 6 specie di *Trichoderma* (*T. atroviride, T. citrinoviride, T. koningii, T. hamatum, T. harzianum e T. polysporum*). Sulla base delle indagini effettuate, il meccanismo di tolleranza messo in atto dai 27 *Trichoderma* è stato attribuito ad almeno due diverse strategie: ì) esclusione del contaminante e, ii) compartimentalizzazione intracellulare del metallo e/o detossificazione enzimatica. I ceppi che hanno mostrato quest'ultima strategia potrebbero essere vantaggiosamente sfruttati nei processi di biorisanamento del suolo. A tal proposito, la terza parte dello studio si è concentrata sul possibile utilizzo di due ceppi di *Trichoderma*, tolleranti il Cd, in associazione con il clone di pioppo "Querce" nelle strategia di bonifica di terreni fortemente contaminati dal metallo. Quest'ultima parte ha inteso valutare l'effetto dell'elevata concentrazione di Cd (250 μM) sulla crescita, l''assorbimento e la traslocazione del metallo in

piante di pioppo allevate in vaso, inoculate e non inoculate con i ceppi T1 e T2. Inoltre, è stato determinato lo spettro dei composti volatili (VOC) emessi dalle piante inoculate con i due ceppi di *Trichoderma* al fine di ottenere informazioni sulla risposta fisiologica del clone dovuta all'interazione con i due microrganismi. I risultati indicavano che il trattamento con T2 induceva una maggior tolleranza al metallo in questo clone ed incrementava la traslocazione del Cd dalle radici verso le il fusto, se paragonato con il trattamento T1 ed il controllo.

In queste condizioni sperimentali, il ceppo T2 presentava delle caratteristiche interessanti riguardo il potenziamento della translocazione di Cd dalle radici al fusto nel clone "Querce". L'analisi gas-cromatografica ha permesso l'individuazione di 81 VOC. L'esaminazione di tali VOC emessi indicava che, l'inoculazione dei due ceppi di *Trichoderma* e la somministrazione di Cd inducevano una variazione significativa dei VOC emessi dalle piante stressate, rispetto alle piante di controllo. La produzione di queste sostanze, tuttavia, sembrava non essere coinvolta nell'aumento della tolleranza mediata dai ceppi T2 e T1 nelle piante di pioppo esposte al Cd;

Alla luce di tali risultati, restano ancora da accertarsi le significative variazioni osservate nell'emissione di VOC, in piante trattate con i due bio-inoculanti e cresciute in presenza o in assenza di Cd.

Abstract

The present study has been organized into three main experiments aimed to explore the bioremediation potential of *Populus alba* in experimental system and in plant-rhizosphere fungi associations. In the first part, it was investigated accumulation, translocation and tolerance of autotrophic *Populus alba* clone "Villafranca" in the response to excess concentrations of cadmium (Cd) and copper (Cu) provided to the plants. For this purpose, increasing concentrations of Cd (0, 5, 50 and 250 μM) and Cu (0, 5, 50, 250 and 500 μM) were administered to the growth medium. Filter bags, instead of the conventional in vitro screening, were applied to improve the experimental design. Results showed that metals treatment after 15 days induced Cd and Cu enhancement in shoots and roots at increasing metal concentration in the medium. The highest Cd content was found in leaves, while the highest Cu content was found in roots. In "Villafranca", Cu showed toxic effects on the development of the seedlings, especially at the highest concentrations, reducing plant dry mass. However, the tolerance index (Ti) indicated good tolerance in this clone under exposure to excess metal concentrations, whereas plants had higher translocation factor (Tf). Therefore, on the findings basis, it is recommend *in vitro* selection of tolerant genotypes, aimed at providing early indication on accumulation potentiality and tolerance capability in research on plant sensitivity to excess heavy metal concentrations. The second part of the research activity has included *in vitro* assessments aimed to investigate the mechanisms of Cd uptake-boosting by several *Trichoderma* species. Fifty-two *Trichoderma* strains were initially screened for their tolerance to a range of Cd concentrations (0, 5, 50, 250 μM). Based on fungal growth rate under metal stress, twenty-seven cadmium tolerant strains belonging to 6 different *Trichoderma* species (namely *T. atroviride, T. citrinoviride, T. koningii, T. hamatum, T. harzianum, and T. polysporum*) were selected. Based on the investigations, the mechanism of Cd-tolerance on the *Trichoderma* strains, was attributed at least two different tolerance strategies: *i*) exclusion of the toxicant and, *ii)* metal intracellular compartmentalization and/or enzymatic detoxification.

Strains that used this latter strategy may find a possible application as bioaccumulators intended for bioremediation of wastewater. Nevertheless, the behavior of these strains also need to be studied at the rhizosphere level, with regard to a possible translocation of the uptaken Cd at the interface of the plant-*Trichoderma* symbiosis. Finally, the study was focused on the phytoremediation of cadmium-contaminated soils by means of the tree species *Populus alba* L., clone "Querce", in association with two Cd-tolerant strains of *Trichoderma spp.*, rhizosphere fungi. The effect of high Cd concentration (250 μM) was evaluated on plant growth

and Cd uptake of poplar plantlets inoculated and non-inoculated with strains T1 and T2 in pot assays. Cd uptake was determined in root, leaf and stem tissues. In order to gain insights into the poplar physiological response in the interaction with the beneficial microorganisms, the spectrum of emitted volatile compounds (VOCs) of plant inoculated with *Trichoderma* strains was determined. Our results indicate that T2 treatment exhibited the highest metal tolerance and Cd translocation in stems compared with the other treatments. In these experimental conditions, T2 showed interesting trait in the enhancement of Cd translocation by roots to stems of poplar plants. Regarding VOC analyses, carried out on the 81 identificated VOCs, data indicated that, the two *Trichoderma* strains inoculation and Cd administration induced significant variation in the VOCs emitted by stressed plants, compared to control plants. However, no correlation was found between VOCs emitted and the strengthening tolerance mediated by the strains.

Therefore, further investigations are required to understand the mechanisms induced by the two bio-inoculant agents and involved in “Querce” clone tolerance under metal stress.

Thesis Outline

Poplar plants are naturally resistant to heavy metals and other contaminants in the environment exhibiting, besides that, characteristics desirable for remediation of environment (i.e fast grow, deep root system and high transpiration). For these reason several studies have been focused to test selected poplar clones for enhanced tolerance to heavy metal toxicity occurring in the soil.

Given the importance of these plants in phytoremdiation application, the following thesis was focused mainly on the evaluation of the phytoactive efficacy of white poplar clones (*Populus alba* L.), in combination or not with fungi present in the rhizosphere.

In order to test the potential use of this plant in the decontamination of soils heavily polluted by heavy metals, different experimental systems were exploited.

The choice of this topic arose from the urgent need to remedy the ever increasing pollution of the soil by human activities, which raises concerns both for human health and for the quality of the surrounding environment. The studies carried out, are in line with the recent interest of research in the use of eco-friendly and low cost reclamation techniques (e.g. Phytoremediation) recognized as valid alternatives to the more expensive and invasive conventional remediation techniques.

The experiments chosen as a thesis subject were carried out at:

- the University of Molise (Campobasso)
- the Institute of Sciences of Food Production (ISPA – CNR) of Bari (BA)
- the area of research of the Institute of Biosciences (IBBR – CNR) and Bioresources of Sesto Fiorentino (FI)

The study was performed using two main experimental models executed in a controlled and semi-controlled environment. The research activity has been divided into three central parts. The first part of the research, submitted in the Chapter II, was developed at the Agricultural, Environment and Food Department of the University of Molise (CB) and was aimed to examine the capacity of tolerance, accumulation and translocation in a clone of white poplar ("Villafranca") bred *in vitro* on substrates contaminated with cadmium (Cd) and copper (Cu) administered at different concentrations, maintaining conditions of autotrophic autonomy.

The second study (Chapter III), carried out at the ISPA, was focused on the implementation of a cadmium tolerance screening performed on different *Trichoderma* strains for their

exploitation in fungi-assisted phytodepuration techniques. Based on the results obtained, two strains were selected, showing satisfactory results, have been adopted as bioinoculants in poplar seedlings (Chapter IV).

At the list, the third part of the this work has been intended to evaluate the land restoration capacity of the white poplar ("Querce" clone) in association with rhizosphere fungi belonging to the genus *Trichoderma* , on soil contaminated with high level of cadmium (Cd). For the assay has been used micropropagated and acclimatized plantlets coming by research area of IBBR.

For the test an experimental model has been used in a semi-controlled environment. In this experimentation, in addition to the characterization of the metal inside the plant tissues, changes in the spectrum of volatile substances (VOC) emitted by the poplar have been evaluated as a result of bioinoculation with *Trichoderma* trying to correlate these data with the physiological changes of the plants in response to interaction with the beneficial microorganism (Chapter IV).

Altogether the thesis dissertion is organized into a total of five chapters. Apart from general introduction (Chapter I) and general conclusion (Chapter IV), Chapter II is publshed in reviewed journal, while Chapters III and IV are in preparation for the research publication.

The structure of the disserion above mentioned is as follows:

Beckground

Chaper I - General introduction

Chapter II - *In vitro* screening

Chapter III - *Trichoderma* experiment

Chapter IV- Semi-controlled pot experiment

Chapter V - General conclusion

Background

Large areas of land are polluted by heavy metals (HMs), as chemical residues resulting from human activities. HMs are the most important contaminants of the environment that can be found in different soil layers. HMs such as cadmium (Cd), mercuty (Hg) and lead (Pb) are known to be directly toxic to biota due to their non-biodegradable properties. All HMs are progressively accumulated along the food chain, so that chronic exposure of lower organisms to relatively low concentrations of HMs can lead to incremental exposure of predatory organisms, including humans, up to potentially harmful concentrations. They are of concern for the human health due to their toxicity and cancerogenic role even at low concentrations.

Among HMs, Cd is recognized as an extremely relevant pollutant due to its high toxicity and diffusion in plant and animal environments, and high solubility in water (Pinto et al., 2004). Other HM are common in contaminated sites, namely lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), copper (Cu), mercury (Hg), and nickel (Ni) all of which rise environmental and toxicological concerns (Raymond et al., 2011).

Chemical and physical technologies for removing HM from soil, water and sediment have been developed. Although these methods have been successful in specific situations, they have significant disadvantages, such as the high costs and the negative impact to the ecosystem. Recently, alternative and sustainable solutions to detoxify the environment have been developed through phytotechnologies. In detail, phytoremediation is a biological technique intensively used in last decades that is based on the attitude of plants to uptake, accumulate, stabilize and tolerate pollutants in the environment. These plants are defined hyper-accumulators, e.g., Brassicaceae for herbaceous species and Salicaceae for woody species (Raskin and Ensley, 2000). Among hyper-accumulator plants, poplar is an interesting plant for phytoremediation due to morphological and physiological traits. Above all, this species is characterized by easy propagation, fast-growing, high biomass production,from 22,7 to 27,5 m^3/ha/yr, (Truax et al. 204), extensive deep root system helpful for efficient metal absorption and high transpiration activity (Sebastiani et al., 2004; Di Lonardo et al., 2011). Moreover, poplar can provide multifunctional benefits added in environmental restoration contributing to pollution mitigation, bioenergy production, CO_2 storage and landscape management improvement (Tognetti et al., 2013).

The phytoremediation technique is a cost-effective, non-invasive and eco-friendly solution for the environment protection, due to easy application and low costs of management (Clements

et al, 2002; Hodson, 2012; Salt et al., 1998; Chaney et al., 1997; Cho-Rhuc et al., 2006). Phytoremediation practice is reviewed in detail in several papers shaped on the restoration of contaminated soils (Arthur et al, 2005; Cherian and Oliveira, 2005; Doty, 2008). However, novel phytoremediation strategies also involve chelating substances or rhizosphere microorganisms in order to increase the mobility of metals in soils with the consequent improvement of the plant removal potential. Despite their effectiveness, the use of chelators in field application is highly limited (Manouchehri et al., 2009) due to the potential environmental risks and disadvantages.

The addition of too high concentrations of chelating agent can compromise soil microorganisms activity and plants growth, besides can cause heavy metal leaching and thus the groundwater pollution (Kidd et al., 2015). Therefore, the practicability and feasibility of chelating agents application in the chelate-assisted phytoremediation strategy are still unclear. Conversely, a feasible tool for an improved phytoremediation process in the open field is the association of plants with microorganisms which are able to increases the efficiency of the technique. Several fungi were identified and tested in cottonwood plants for their hyphal potential of metals removal or metal tolerance (Sell et al., 2005; Bissonnette et al. 2010). Plants and associated microorganisms are able to perform processes of phytoextraction and rhizodegradation, consisting respectively in the absorption, translocation and storage of toxic contaminants from a soil matrix into plant root and shoot tissue (phytoextraction) and in the degradation of contaminants in the rhizosphere (area of soil surrounding the roots of the plants) by means of microbial activity which is enhanced by the presence of plant roots (rhyzodegradation). These proceses, therefore, can be advantageously involved in the clean-up of co-contaminated soils (Vivas et al. 2003; Khan, 2005a, b). The plant-microorganism association enhances the bioavailability of pollutant in the soil, resulting a biologically assisted strategy to overcome major constraints,which hinders the success of phytotechnologies (i.e phytotoxicity, slow degradation, limited contaminant uptake and evotranspiration of volatile contaminants) (Weyens et al 2010), thus improving the efficiency of remediation.

Concerning plant-microbe association is has been proved that rhizosphere microorganism could be effectively applied for remediating sites as they possess multiple metal detoxifying properties which coupled with other physiological attributes including an ability to degrade organic contaminants make the plant – microbe association (i.e fungi, bacteria) worthy of consideration for its bioremediation potential.

Despite metal resistant fungi have been found in the rhizosphere of various hyperaccumulator plants growing on heavy-metal contaminated soil, to date very little is known

about the associations of poplar with rhizosphere fungi which might be exploited for the improvement of soil remediation strategies.

CHAPTER I – Heavy metals pollution

1 General introduction

1.1 Soil pollution

The growing industrialization of the world over the last centuries has led a considerable soil contamination on a global scale. Environmental pollution influences both the soil system and agriculture, that are the two essential resources for life sustenance (Chauhan and Mittu, 2015). Soil can become contaminated through a variety of processes, including poor waste disposal methods, landfill sites, oil spills or nuclear pollution. In the soil, pollutants tend to have longer persistence than in water or air, rising great concerns. The soil acts as a sink or a filter, where pollutants are rapidly accumulated and slowly depleted (Lombi and Hamon, 2005). Organic and inorganic pollutants derive from anthropogenic activities (Muralikrishna et al., 2017) or naturally occur in soil (Cachada et al., 2018). The exponentially-widespread of industrialization and urbanization has led to significant levels of soil pollution of polyaromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), trinitrotoluene (TNT), HMs (HM) and a variety of dioxins. The most common soil pollutants are organic compounds (such as, pesticides, oils, tars, chlorinated hydrocarbons, PCBs and dioxins), biologically active materials (sewage sludge), combustible materials (wood, coal, oil, natural gas), and other hazardous materials (such as, asbestos).

Many of the main pollutants such as Fe, Pb, Hg, Cu, Zn, Cd, Al and cyanides, acids and alkalies derive from industrial wastes and pollute the soil through water vector, runoff or rain. The improper and continuous use of herbicides, pesticides and fungicides negatively alters the composition of the soils because they accumulate in the soil and water causing soil toxicity for plant growth (Kumar et al., 2015).

Briefly, “soil pollution” refers to the presence of a chemical or substance out of place and/ or present at a higher than normal concentration that has adverse effects on any organism (FAO and ITPS, 2015).

Soil pollution often is not directly assessed or visually perceived, making it a hidden danger. The World's Soil Resources Report (SWSR) identified soil pollution as one of the main alarming threats of soil affecting functions and the ecosystems services provided by soil (FAO, 2018; FAO and ITPS, 2015).

Soil, indeed, is a biologically active, complex mixture of weathered minerals (sand, silt and clay), organic matter, organisms, air and water that provides the foundation for life in terrestrial ecosystems. The main ecological services provided by soil reside in regulate and control water flow over

land, in addition to filtering rainfall passing through the soil to plants and groundwater. Soil influences river flows and flooding, filtration and buffering of potential pollutants and transforming and cyclesing essential nutrients such as carbon, nitrogen, phosphorus, potassium and sulphur. Furthermore, the microorganisms and minerals in soil filter, buffer, degrade, utilise, immobilise and detoxify large numbers of organic and inorganic materials, including slurries, industrial organic wastes and sewage sludge. Soil stores, transforms and cycles essential nutrients such as carbon, nitrogen, phosphorus, potassium and sulphur. Soil also takes up, stores and releases atmospheric gases. This essential function ensures good quality of groundwater and safe food production (Blum, 2005).

In spite of this, soil pollution causes negative consequences on this soil funtions and on food security, by impairing plant metabolism and crop yields and by reducing the crops safety for the animal and human consumption (Dal Corso et al., 2008) due to the propagation of the contaminants through food chain involving bioaccumulation and biomagnification process (Cristaldi et al, 2017).

Soil pollution is frequent in both developed and developing countries and the threat increases in every region (Muralikrishna et al., 2017)

Globally there are over 20 million ha of land contaminated by the HMs and or metalloids such as: As, Cd, Cr, Hg, Pb, Co, Cu, Ni, Zn, and Se; with the present soil concentrations higher than the geo-baseline or regulatory levels (Liu et al., 2018). Huge amounts of contaminated sites have been identified wideworld and require clean up.

According FAO report (FAO, 2018), in Australia, 80,000 sites are estimated to be soil contaminated. Whereas there are more than 200,000 contaminated sites in China, 320 of which were identified as serious contaminated sites covering 5.48 million hectares (Everbright Securities, 2013). There are approximately 3 million potentially polluted sites in the European Economic Area and the West Balkans. In the United States, 1,300 sites appear on that country's Superfund National Priorities list of pollution hot spots. In the Member States of European Environment Agency (EEA) the sites to be reclaimed are about 250,000, and thousands of these are located in Italy. Among the contaminated sites of Italy, 41 of these are defined as "national interest for remediation" (SNI) within the "National reclamation program" on the basis of the environmental contamination extent, health risk and social alarm (DM 471/1999). The 41 sites SNI include brownfield lands, industrial areas in reconversion, industrial areas in activity, areas subjected to release of chemical pollutants and areas with uncontrolled disposal of hazardous waste (Article 252 of Legislative Decree no. 152/06).

1.2 Soil pollution: agricultural soils

The contamination of arable soils is one of the most pressing concerns in the debate about food security and food safety in Europe (CEC, 2006a), and globally (Kong, 2014) due to the close soil influence on agriculture and natural vegetation, since soil pollution is closely related to land use. This concern is related to the direct and indirect sources of pollution in agricultural soils and their impacts in ecosystems (WHO, 2018; Mulligan et al., 2001; Rattan et al., 2005). Although the presence of pollutants in rural areas is a serious threat for human and environmental health, currently there are no standards for health risk assessment related to agricultural environment pollution (Beccaloni et al., 2010). Furthermore, agricultural sites are not explicitly indicated in the Italian Legislative Decree no. 152/06 (Environmental Protection Code), which states the key of EU-derived principles by governing environmental protection in Italy.

Modern agriculture practices accelerate soil pollution with the intensive use of fertilizer and pesticides in order to increase productivity and reduce crop losses. When pollutants reach high levels in the soil, detrimental effects on soil fertility occur and crop productivity is affected. Therefore, in addition to endangering human health and the environment, soil pollution can also cause economic losses (FAO, 2018; FAO and ITPS, 2015.

HMs in arable lands become available to crops for the absorption (Bai et al. 2010), consequently, polluted soils reduce the land suitability for agricultural production (McLaughlin et al 2000, Ling et al 2007). Moreover, HMs cause toxicological effects on soil microbes, compromising their populations and activities (Khan et al., 2010), and then the soil fertility (FAO, 2018). Poor quality of water sources for irrigation, e.g. when polluted by waste water and urban sewage, is one of the main causes of soil pollution. In agriculture the use of sludge and organic and mineral substances obtained by the purification of urban wastewater is allowed, although several limitations in the use are related to HMs content (Adriano, 1986), often these wastewater are rich e.g. in Cd, Co, Cu and Zn.

Metals contamination is differently worrisome in different counties. It's estimated that 6.24% of the of European agricultural land needs local assessment and eventual remedial action (Toth et al. 2016).In China, 19% of all arable land is polluted with HMs (Cd, Ni, As) and it is addressed to crop production for human food (Takahashi et al., 2014; CCICED, 2015). The South and Southeast Asian countries, such as Peninsular Malaysia, Vietnam, India, Thailand, Philippines, Indonesia, Bangladesh, and Pakistan have paid high attention to HMs contamination of agricultural soils and crops due to their spread and potential effects on human

health and long-term sustainability of food production in the contaminated areas (Luo Y. et al, 2009). Therefore, based on the research conducted so far, currently, HMs contamination of agricultural soils is a significant worldwide environmental problem.

1.3 Polluted sites and soils in Italy

The presence of contaminated sites is significant and well-documented in Europe and Italy. In the Member States of the European Environment Agency (EEA) there are approximately 250,000 sites awaiting remediation, thousands of which in Italy, and 41 of which are defined "remediation sites of national interest" (SNI) due to the degree of environmental contamination, the health risk and the social alarm (Ministerial Decree 134/2012). Other 18 contaminated sites, previously recognized as "SNI", are widespread throughout the country and their remediation have been assigned to the Regions competence due to Ministerial decree dated 11/01/2013 (ISPRA 2017) (Figure1).

The contaminated Sites of National Interest (SNI) have been defined by specific statutory provisions based on site characteristics, quantity and severity of hazardous materials, and by the extent of the environmental impact on health and environment. For these sites, the administrative competence in remediation procedures is a responsibility of the Ministry of the Environment, Land and Sea (MATTM) (ISPRA., 2017).

The 41 sites of the "National Remediation Programme" includes abandoned industrial areas, industrial areas in reconversion, industrial areas still being used, areas accidentally polluted and those not subjected to controlled disposal of waste. In these sites exposure to contaminating substances can occur through occupational exposure, industrial emissions and, ultimately, contaminated soil and groundwater. In details, SNI comprise Italy's most important industrial areas, e.g., petrochemical plants in Porto Marghera, Brindisi, Taranto, Priolo, Gela; urban and industrial areas of East Napoli, Trieste, Piombino, La Spezia, Brescia, Mantova; waste landfills (Ministry for the Environment, Land and Sea, 2009).

Overall, Italian SNI represent the 0,5% of the surface of the Italian territory. Contrary, at regional level, the surface area of the SNI is higher to 1% of the regional territory in only two cases (Liguria with 4.1% and Piedmont with the 3.5%). More than half (21) of the SNI reverts in Lombardy (5), Piedmont (4), Tuscany (4), Puglia (4) and Sicily (4). However, the regions with the greatest general perimeter surface (land + sea) are Piedmont (around 90.000 have), Sardinia (around 56.800 ha), Sicily (around 24.400 ha), Puglia (around 24.000 ha) and Liguria (around 22.500 ha). Currently, nearly 40% of SNI shows more than 50% of the areas with a

making safety / reclamation project approved through soil and / or groundwater decree, save only one SNI where proceeding completed exceed 50% of SNI total surface area (ISPRA, 2017).

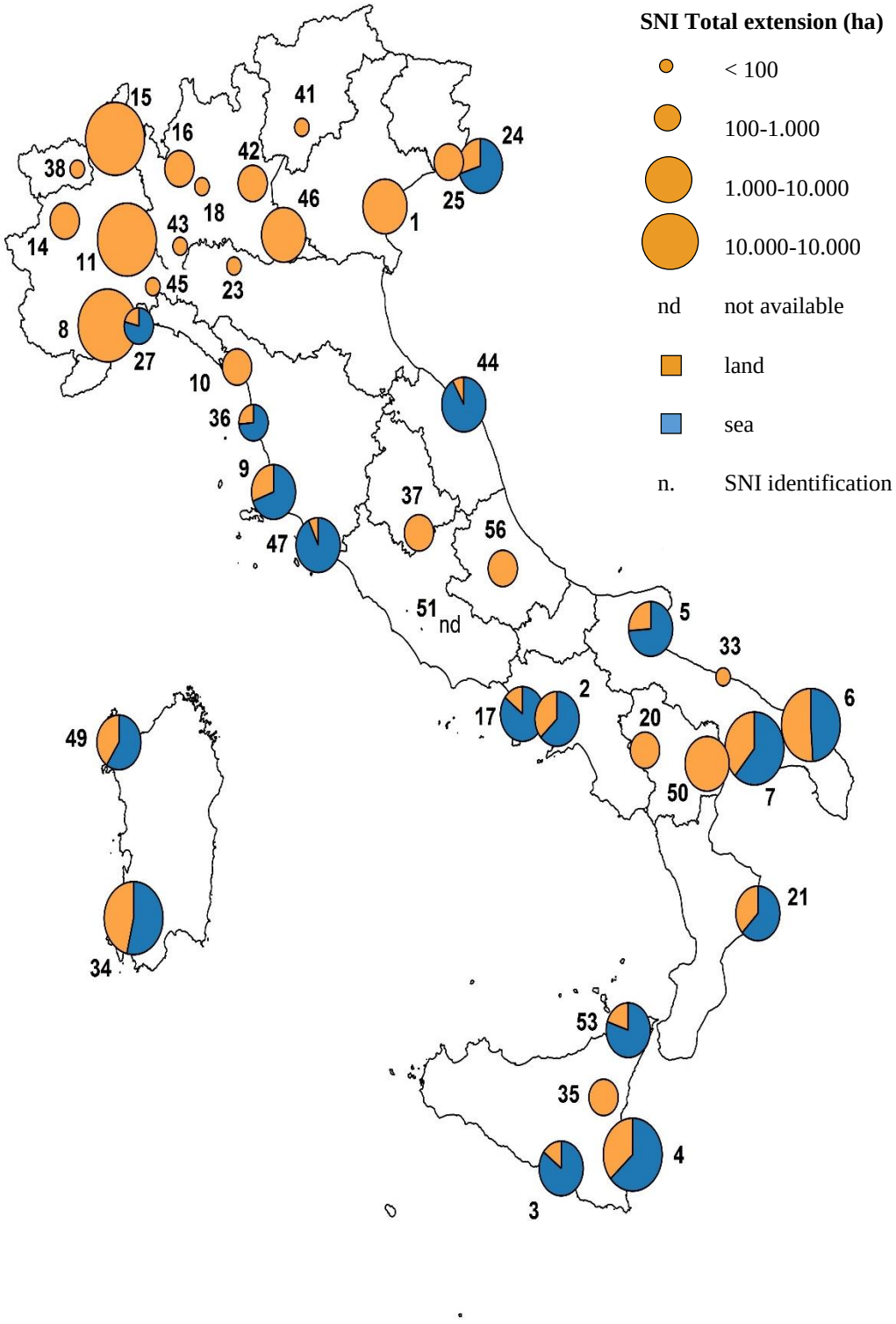


Figure 1 Location and total surface classes of the Sites of National Interest (ISPRA, 2017)

1.4 HMs pollution

The problem of HMs' pollution is becoming high serious with increasing of the industrialization and disturbance of natural biogeochemical cycles (Hazrat et al., 2013). HMs are conventionally defined as chemical elements with relatively high densities, metallic and toxic properties (Singh et al., 2011) and with an atomic number >20 (Copat et al., 2012). HMs are Ag, Ba, Cd, Co, Cr, Mn, Hg, Mo, Ni, Pb, Cu, Sn, Tl, V, Zn and some metalloids, with properties similar to those of HMs, such as As, Sb, Bi and Se. In addition to the worrying diffusion of HMs and their persistence in the soil, of major concern is their possible carcinogenic, immunological and reproductive effects, but more recently concern has also been expressed over their possible harmful effects on human development. Overall, 13 eco-toxic HMs are listed among the chemicals of major public concern by the World Health Organization (WHO., 2011) for their potential to be carcinogenic and inflict acute organ damage (Tchounwou et al., 2012).

Toxic HMs can be found naturally in the soil environment from the pedogenetic processes of weathering of parent materials at levels that are regarded as trace (< 1000 mg Kg^{-1}) and rarely toxic.

Some HMs are essential in small quantities for human, animal and plant health (life). However, HMs are non-biodegradable, therefore excessive amounts accumulate in the environment and persist for long time (Lasat et al., 2000; Adriano, 2003) becaming toxic.

The toxicity can be manifest by replacement of essential metal reaction centers of enzymes, reaction of metal ions with the sulfhydryl and phosphate groups of proteins, or displacement of key structural elements. Metal toxicity is highly dependent on the pH and redox state of the soil, as these strongly influence the oxidation state and solubility of metals and hence their bioavailability in the soil.

Currently, the spread of HMs and thereafter soil contamination is a worldwide problem widely noticed (Table 1). Metal-contaminated soil has been reported at more than 50,000 sites in the USA (Ensley, 2000), 80,000 in Germany (Liu et al., 2011), and similar areas in other countries. There were ca. 100,000 ha of land contaminated by HMs in the USA, with the losess of about 10,000 ha for agricultural land, even applying the less strict thresholds of the present soil protection regulation (Lewandowski et al., 2006). HMs, together with mineral oils, is the

main frequent contaminant affecting the solid matrix (soil, sludge, sediment) in Europe (EEA, 2011), contributing around 60% to soil contamination (Figure 2).

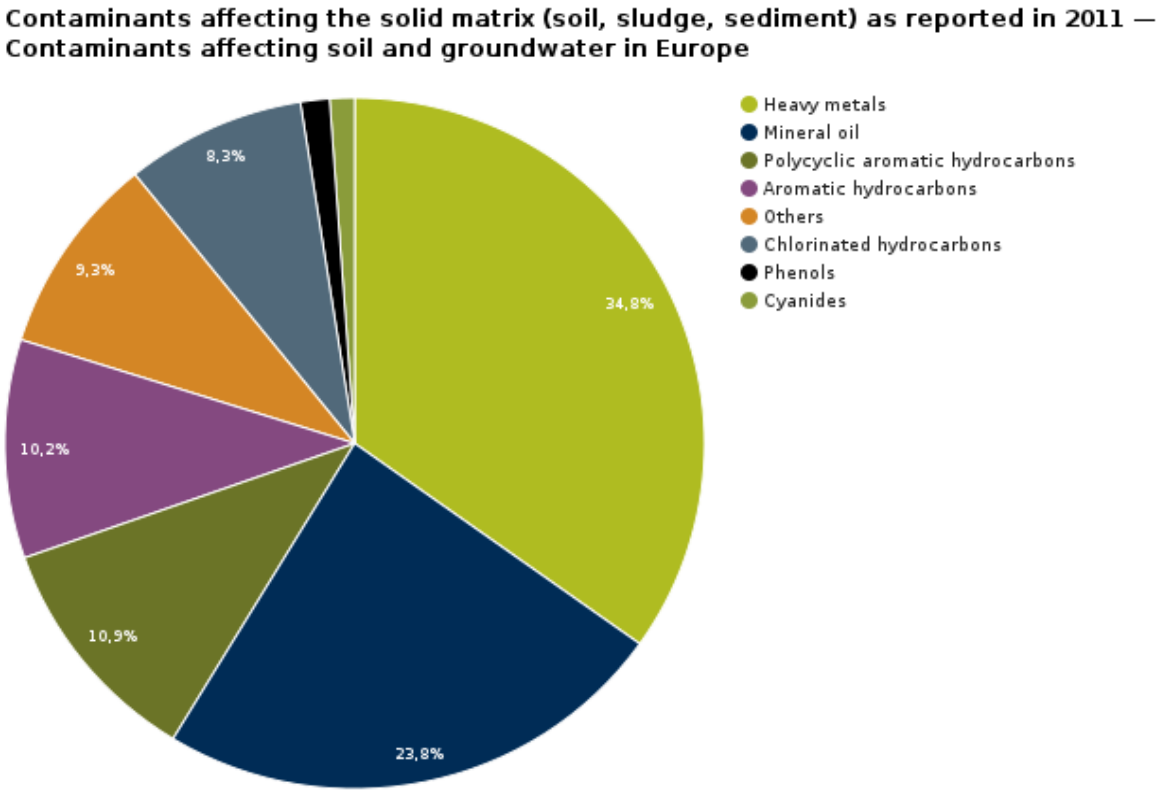


Figure 2 Contaminants affecting soil and groundwater in Europe (https://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites-3/assessment)

Pollution by HMs and many organic contaminants is practically irreversible (European Commission, 2012). In Europe, there are estimated to be around three million potentially contaminated sites, most of which are thought to be actually contaminated and in need of remediation (European Environment Agency 2010) and for this reason different studies are focused on the actual state of soil pollution.

Heavy metal	Concentration in soil (mg/Kg)	Maximum allowable limit[a]	Fold-higher than allowable limit	Study area	References
Cd	42	3	14.0	Southern Italy	Baldantoni et al., 2016
	19		604	India	Tiwari et al., 2011
	16		504	Switzerland	Quezada-Hinijosa et al., 2015
	14		407	Mexico	Torres et al., 2012
	14		4.6	China	Shi et al., 2015
Pb	4500	100	45.0	China	Luo wt al., 2011
	1988		19.9	China	Niu et al., 2015
	711		7.1	UK	Nabulo et al., 2011
	452		4.5	Uganda	Nabulo et al., 2012
	302		3.0	Brazil	Carvalho et al., 2015
As	7490	20	374.5	Spain	Breesley et al., 2014
	4357		217.9	Italy	Marabottini et al., 2013
	354		17.7	China	Wei et al., 2015
	131		6.6	Korea	Myoung Soo Ko et al., 2015
	64		3.2	Bolivia	Acosta et al., 2015
Zn	3833	300	12.8	China	Niu et al., 2015
	370		1.2	Nigeria	Obiora et al., 2016
	1168		3.9	Germany	Shaheen et al., 2014
	905		3.0	Portugal	Anjos et al., 2012
	393		1.3	-	Kwon et al., 2015
Ni	2603	50	52.1	Mexico	Torres et al., 2012
	373		7.5	Spain	Arenas-Lago et al., 2016
	201		4.0	Zimbabwe	Mapanda et al., 2007
	200		4.0	Turkey	Avci and Deveci, 2013
	153		3.1	China	Wang et al., 2015
Cu	35.582	100	355.8	Mexico	Torres et al., 2012
	19.581		195.8	Australia	Sacristán et al., 2016
	448		4.5	China	Wang et al., 2015
	235		2.4	Portugal	Anjos et al., 2011
Cr	4309	100	43.1	Spain	Arenas-Lago et al., 2016
	590		5.9	China	Xu et al., 2014
	481		4.2	Greece	Panagopoulos et al., 2015
	224		2.2	Germany	Shaheen et al., 2014

[a] World Health Organization (WHO), Food and Agricultural Organization (FAO).

Table 1 Examples of some HM (loid)s polluted soils worldwide exceeding permissible limits. (Khalid et al., 2016)

1.4.1 HMs in the soil

1.4.1.1 Lead (Pb)

Pb source

Contamination of soil with Pb occurrs on a global scale. Exposure to Pb may cause adverse effects to human health and the environment (Markus et al., 2001). Lead is found at low levels in Earth's crust, mainly as lead sulfide. However, the widespread occurrence of lead in the environment is largely the result of human activity, such as mining, smelting, refining and informal recycling of lead; use of leaded petrol (gasoline); production of lead-acid batteries and paints; jewellery making, soldering, ceramics and leaded glass manufacture in informal and cottage (home-based) industries; electronic waste; and use in water pipes and solder (WHO, 2010). When Pb enters the soil matrix, it is very difficult to remove it. The capacity of soil to adsorb Pb increases with increasing pH, cation exchange capacity, redox potential, organic

carbon content, and chelates (phosphate) levels (United States Environmental Protection Agency, 1992).

Pb effects on human health

Lead is a toxic metal whose widespread use has caused extensive environmental contamination and health problems in many parts of the world. Lead toxicity occurs at very low exposure levels and has acute and chronic effects on human health. It is a element toxicant for multiple organs as it can affect neurological, cardiovascular, renal, gastrointestinal, haematological and reproductive system. The type and severity of effects depend on the level, duration and timing of exposure. Lead is accumulated in bone and may serve as a source of exposure later in life. Organo-lead compounds, such as tri-alkyl-lead and tetra-alkyl-lead compounds, are more toxic than inorganic forms of lead (UNEP, 2010). Low Pb exposure level can cause serious and, in some cases, irreversible neurological damage in the young children who are particularly vulnerable to the neurotoxic effects of lead.

Lead causes long-term harm in adults, including increased risk of high blood pressure and kidney damage. Exposure of pregnant women to high levels of lead can cause miscarriage, stillbirth, premature birth and low birth weight, as well as minor malformations. (WHO, 2018). People can become exposed to lead through the inhalation of lead particles generated by burning materials containing lead (example, during smelting, recycling, stripping leaded paint, and using leaded gasoline or leaded aviation fuel) and ingestion of lead-contaminated dust, water (from leaded pipes), and food (from lead-glazed or lead-soldered containers) (WHO 2018).

Lead exposure is estimated to account for 0.6% of the global burden of disease, with the highest burden in developing regions. Recently, the restriction of lead use in petrol (gasoline), paint, plumbing and solder have resulted in substantial decrease in lead levels in the blood (WHO, 2010).

Pb effects on plants

Lead is a non-essential element, that can be easily absorbed and accumulated in different parts of a plant (Fazal and Tariq., 2015). Excessive lead accumulation in plant tissue can cause various toxic and deleterious symptoms impairing a range of morphological, physiological, and biochemical functions. The main effect of Pb toxicity in plants is the rapid inhibition of root growth, reduction of biomass production, probably due to the inhibition of cell division in the

root tip (Eun et al., 2000; Samardakiewicz and Wozny, 2005; Peng et al., 2005; Verma and Dubey 2003), the distortion of ultrastructure chloroplast (Elibieta and Miroslawa, 2005; Islam et al., 2007), the reduction of electron transport (Qufei et al., 2009), and the inhibition of Calvin cycle activitiy (Mishra et al. 2006; Liu et al. 2008). Moreover, Pb leads to impairment of essential elements, such as Mg and Fe (Chatterjee et al., 2004; Gopal and Rizvi, 2008), inducing deficiency of CO_2 resulting from stomatal closure (Romanowska et al. 2002, 2005, 2006) and increasing chlorophyllase activity (Liu et al., 2008). Moreover, alteration of minerals uptake, concentration and translocation in plants was found with decreasing Zn, Cu, and K contents and inhibition in Ca transporters (Sharma and Dubey, 2005; Wojas et al., 2007).

1.4.1.2 Arsenic (As)

Arsenic source

Arsenic (As) contamination is one of the major environmental problems around the world (FAO 2010). Arsenic is widely dispersed and ubiquitous in the environment and highly toxic to all forms of life, being carcinogenic (Chung et al., 2014; IARC, 1987). Arsenic pollution in soil occurs mainly from its release from parent rock into environment. Anthropogenic activities such as mining, smelting of sulphide ores, application of agricultural pesticides (e.g., fungicides, herbicides, insecticides), timber preservation, wood preservation, disposal of industrial wastes from tannery industries, disposal of chemical warfare agents, and combustion of fossil fuels can enhance arsenic concentration in soils (Miretzky and Cirelli, 2010).

Among the sources of As in the environment, drinking water probably poses the greatest threat to human health (Smedley et al., 2002).

Arsenic occurs in soil in inorganic and organic forms. Inorganic arsenic is a carcinogen and significant chemical contaminant in drinking-water, while organic arsenic compounds (such as those found in seafood) are less harmful to health (WHO, 2018). Severe arsenic contamination of soils may cause arsenic toxicity in plants, animals and human (Warren et al., 2003). Bioavailability of As in soil is powerfully influenced by the chemical and physical characteristics of soils together with the character of minerals and clay content, organic matter, texture, pH and redox potencial (Eh), cation-exchange capability (CEC), and presence and concentration of oxides and hydroxides of metals, such as Al and Mn (Hossain et al., 2010; Shrivastava et al., 2014).

Both As(III) and As(V), infact, are powerfully adsorbed to hydrous oxides of metal, Mn, and Al in acid soils. However, areas with soils of high organic carbon content are generally more susceptible to accumulating arsenic than sandy soils (Anamika et al., 2015).

Arsenic effects on human health

Arsenic and arsenic compounds are recognized as carcinogenic to humans (IARC, 1980). Long-term exposure to high levels of inorganic arsenic (for example, through drinking-water and food) impair pigmentation changes, skin lesions and hard patches on the palms and soles of the feet (hyperkeratosis) that may be a precursor of skin cancer (Uede and Furukawa, 2003). In addition to skin cancer, long-term exposure to arsenic may also cause cancers of the bladder and lungs (Mandal et al., 2001).

Other adverse health effects, associated with long-term ingestion of inorganic arsenic, include developmental effects, diabetes, pulmonary disease, and cardiovascular disease, hepatic and renal dysfunction including mortality due to chronic diseases (Chen et al., 2009). Arsenic-induced myocardial infarction, in particular, can be a significant cause of excess mortality (Manna et al., 2008).

Arsenic has been able to induce adverse pregnancy outcomes and infant mortality, with impacts on child health (Quansah et al., 2015).

Arsenic exposure in utero is also associated with increases in mortality in young adults due to multiple cancers, lung disease, heart attacks, and kidney failure (Farzan et al., 2013). Numerous studies have demonstrated negative impacts of arsenic exposure on cognitive development, intelligence, and memory (Tolins et al., 2014; WHO, 2018).

Arsenic effect on plants

Arsenic is a non-essential and generally toxic to plants. Roots exposure to As can impair root extension and proliferation. Moreover, upon translocation to the shoot, As can severely inhibit plant growth by slowing or arresting expansion and biomass accumulation, as well as compromising plant reproductive capacity through losses in fertility, yield, and fruit production (Garg and Singla, 2011). In some cases, at high concentrations, As interferes with critical metabolic processes, including cell necrosis and chlorosis, which can lead to death (Finnegan et al., 2012). One of the many interesting paradoxes related to As is that plant growth is stimulated at low As concentrations (Miteva, 2002; Garg and Singla, 2011). Furthermore, arsenic may also influence nutrient uptake by competing directly with nutrients and/or altering metabolic processes. Phosphorus is usually the nutrient whose uptake is the most affected by

As. Arsenate (As5+) and phosphate (P5+) competes for the uptake, since they are taken up via phosphate transport systems (Farnese et al., 2014).

1.4.1.3 Cadmium (Cd)

Cadmium source

Cadmium (Cd) is in the environment from natural and anthropogenic sources. Natural emissions of Cd in the environment results from volcanic eruptions, forest fires, generation of sea salt aerosols (EPA, 1985a; Morrow, 2001; Shevchenko et al., 2003). The release of Cd by anthropogenic source is defined by industrial processes, heating systems, vehicular traffic, phosphate fertilizers and mineralization of rocks (Benavides et al., 2005). The main sources of Cd directed to landfills and waste deposits are municipal waste, Cd processing, non-ferrous metal processing and cement production, and both industrial and municipal wastes are important sources of cadmium for landfilling.

The presence of cadmium in fertilisers and has been found to cause increasing atmospheric deposition of cadmium in topsoil in a number of European countries (WHO/UNECE, 2006). Once cadmium is in soil, it is persistent and cannot be broken down into less toxic substances in the environment, accumulating in soils and increasing the likelihood of human exposure via crop contamination (WHO/UNECE, 2006). Cadmium levels tend to increase moving up via the food chain (‘bioaccumulation’ and ‘biomagnification’). The Cd up-take is influenced by factors, such as soil pH, salinity, humus content, crop species and varieties and the presence of other elements (e.g. zinc).

In acidic conditions, cadmium mobility and bioavailability increase, and low adsorption of cadmium is in soil colloids, hydrous oxides, and organic matter takes place while it’s lower in chalky/lime soils. One way to control cadmium bioavailability is to lime the soil to make it less acidic.

Cd effect on human health

Cadmium is a non-essential and toxic element for humans, and has no use for plants or animals either. Cadmium produces a number of health problems and is a known carcinogen. In industry, it’s regulated by the Environmental Protection Agency (EPA) and it has a very low permissible exposure level. The negative effects of cadmium on the body are numerous and can impact nearly all systems in the body, including cardiovascular, reproductive, the kidneys, eyes,

and even the brain having a very detrimental effect on the central nervous system (Caciari et al., 2012; Pizent et al., 2012; Pacini et al., 2012). It can damage the kidneys, causing excess production of proteins in the urine, the duration and level of exposure to cadmium determines the severity of the effect. Cadmium is also carcinogenic if inhaled. Mainly stored in the liver and kidneys, excretion of cadmium is slow and it can remain in the human body for decades.

Levels of the element tend to increase in body tissues with the age. Cadmium is associated with skeletal damage, evidenced by low bone mineralisation, a high rate of fractures, increased osteoporosis and intense bone pain. These were features of itai-itai disease, first described in Japan in the 1940s among people who had eaten rice grown on fields irrigated with cadmium-polluted water. Moreover, a low calcium diet plus high cadmium exposure led to kidney disease followed by bone disease.

Cadmium effect on plants

Cd can be readily taken up by the plants roots and transported to shoots impairing plant biochemical and physiological processes (Sanita di Toppi and Gabbrielli, 1999). Roots are likely to be firstly affected by HMs since much more metal ions are accumulated in roots than shoots (Sanita di Toppi and Gabbrielli, 1999). Thus, Cd toxicity obviously led to inhibition of plant root growth (Liu et al., 2003) and alterations in their morphogenesis (Daud et al., 2009; Rascio et al., 2008). In particular, long-term exposure to Cd causes mucilaginous, browning and decomposition of roots. Cd was found to affect secondary roots formation and the occurrence of brown, rigid, and twisted main root (Krantev et al., 2008; Yadav, 2010; Rascio and Navari-Izzo, 2011).

Toxic effects of Cd in root can be related to disordered division and abnormal enlargement of epiderma and cortical cell layers in the apical region. The changes in the leaf included alterations in chloroplast ultrastructure, low contents of chlorophylls, which caused chlorosis, and restricted activity of photosynthesis (He et al., 2008; Rascio et al., 2008; Lee et al., 2010; Liu et al., 2010; Miyadate et al., 2011, Tuan et al., 2013).

Cadmium toxicity, besides, may result from disturbance in plant metabolism as a consequence of disturbance in the uptake, concentration and translocation of mineral nutrients. Plant nutrients and Cd compete for the same transporters and, therefore, Cd causes mineral nutrients deficiency or alteration (Rahat et al., 2012).

In general, Cd has been shown to interfere with the uptake, transport and use of several elements (Ca, Mg, P and K) (Wang et al., 2007; Nedjimi and Daoud, 2009) and water (Polle et al., 2013).

Excess Cd accumulation in plants can profoundly interfere with a series of physiological processes, such as Cd in plants interferes with the gas exchange (López-Climent et al., 2001; Hossain et al. 2010), the Calvin cycle (Mobin and Khan 2007; Shi et al., 2010) and the antioxidant metabolism (Khan et al., 2007). Cadmium also reduced the absorption of nitrate and its transport from root to shoot by inhibiting the nitrate reductase activity in shoots (Hernandez et al., 1996). Several researches have suggested that Cd alters the levels of metabolic enzymes and indirectly induces oxidative stress by generating reactive oxygen species (ROS) (Sandalio et al., 2001, Romero-Puertas et al., 2004). The ROS react with lipids, proteins, pigments and nucleic acids and cause oxidative damage including lipid peroxidation leading to membrane damage (López-Millàn et al., 2009).

1.4.1.4 Copper (Cu)

Copper source

Copper (Cu) is an essential trace element in both humans, plants and animals (Yruela, 2005; Bos et al., 2016). Although copper is an important sustaining life element it can become toxic when it is accumulated in the environment (Nagajyoti, 2010; Alva and Chen, 1995).

Like other metals, copper is highly persistent in the environment and biologically active long after its use has ceased (De Boer et al., 2012). Copper is a very common substance that occurs naturally in the environment and spreads through the soil via natural phenomena or wide human use. For instance, it is applied in the industries and in agricultural practices.

An important anthropogenic source of local soil enrichment in Cu is deposition of airborne dust released during metal processing; copper metallurgy is a large source of emissions containing Cu and other metals (Pb, Cd, Ni and As) (Nagajyoti, 2010). The contamination of agricultural soil with inorganic (copper) and organic pesticides is of particular concern for the state of our environment and for food safety (Komárek et al., 2010).

The excessive presence of copper in the soil is strongly correlated to the presence of organic matter and minerals which keep it without allowing it to be released into the surrounding environment, for this reason it can be accumulated in plants when it is found in soils and thus in animals. Furthermore, Copper negatively influences the activity of microorganisms and earthworms in the soil.

Health effects of copper exposure

Cu is recognized as an essential micronutrient for man, but it is toxic at high levels (Bost et al., 2016). Copper is involved in enzyme systems, and enzymes are responsible for countless metabolic processes required to sustain life. Nevertheless, high body copper burden can be responsible for several disorders. At high concentrations, copper is known to produce damage such as hypotension, heart disease, premenstrual tension, postpartum depression, paranoid and hallucinatory schizophrenias and childhood hyperactivity and autism (Pfeiffer, 1979). Copper accumulates in liver and brain altering structure and function of these organs (Wilson, 1912). Moreover, Cu toxicity is also indirectly associated with a number of neurological disorders, including Alzheimer's disease and prion diseases, including bovine.

Copper effect on plants

Cu is an essential micronutrient for growth and development of plants. Nevertheless, high Cu concentrations can become extremely toxic, causing symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth and morphology (Lombardi et al., 2005). The main plant effects of exposure to high levels of Cu are the alteration of chlorophyll concentration, of the chloroplast structure and thylakoid membrane . In particular, degradation of grana stacking and stroma lamellae, increase in the number and size of plastoglobuli, and appearance of intrathylakoidal inclusions were observed. It was proposed that high Cu concentrations directly interferes, at the cellular level, with oxidative stress caused by the increased concentration of reactive oxygen species (ROS), such as superoxide anion (O_2-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH-) (Apel and Hirt, 2004). However, ROS can damage all biomolecules, lipid peroxidation of the cell membranes is one of the most important effects observed. Furthermore, the reduction of lipid content and changes in fatty acid composition of thylakoid membranes were observed (Sandmann and Böger, 1980; Luna et al., 1994; Maksymiec et al., 1994). Because of such modifications, alteration of PSII membrane fluidity was found (Quartacci et al., 2000).

1.5 Remediation techniques for HM-contaminated soils

There is an urgent need to properly resolve the complex environmental problems related to soil pollution. In order to support ecologically and socially sustainable development, it is

necessary to coordinate the activities of the governments and markets to control and to repair the discharges of HMs. Currently, soil remediation is one of the top priorities of EU and other State environment policy. Life and living on the earth would be impossible without healthy soil. Making plan for having healthy and productive soil is essential to human survival considering that 95% of human food is derived from the earth (Khakbaz et al., 2012).

In-situ and *ex-situ* remediation techniques have been developed to clean up HMs-contaminated sites, including physical, chemical and biological methods.

Ex situ remediation method involves excavation and transportation of the contaminated top soil to somewhere else for physical-chemical and/or electrical treatments (Jankaite and Vasarevicius, 2005; Shukla et al., 2010). Contrarily, *in situ* method, involves remediating the contaminated soil on site (Wuana et al., 2010; Rahimi et al., 2012) via different method such as surface capping, encapsulation, landfilling, soil flushing, soil washing, electrokinetic extraction, stabilization, solidification, vitrification and soil (Jankaite and Vasarevicius, 2005; ICS, 2005). These remediation techniques employ containment, extraction/removal, and immobilization mechanisms to reduce the contamination effects through physical, chemical, biological, electrical, and thermal remediation processes. Some remediation methods can be carried out through the synergistic use of *in situ* and *ex- situ* methods.

Generally, *in situ* remediation technique is often preferred over *ex situ* because the former is always cheaper and less destructive to the ecosystem (Khan et al., 2000), although *ex situ* treatments are typically cost effective and can be completed in short time periods. More precisely, *ex-situ* soil reclamation techniques lead to a big reduction in soil fertility due to the soil disturbance and to the toxicity of synthetic surfactants (Fiorentino et al., 2013). The choice of remediation method depends on the site characteristics, contaminant concentration, type of contaminants or pollutants to be remediated, and the final use of the contaminated soil (Jankaite and Vasarevicius, 2005). Recently, biological techniques were developed alternatively to conventional remediation techniques (chemical and physical).

Among the available biological remediation techniques, bioremediation is recognized as a cost effective and eco-friendly technique that offers the possibility to degrade and transform environmental contaminants into harmless or less toxic forms using natural biological activity.

Bioremediation is an emerging technology that exploit the use of living organisms (fungi, bacteria, plants) to remediate polluted sites. These technique offers a potentially more effective and economical clean-up technique than conventional physicochemical method.

1.5.1 Bioremediation technology: Phytoremediation

1.5.1.1 Phytoremediation: generalities and application

Phytoremediation comprises a group of emerging biological remediation technologies shaped on the use of living plants to clean up soil, air, and water contaminated with hazardous pollutants. (Reichenauer et al., 2008). This green technique can be exploited to reduce the concentrations or toxic effects of several contaminants in the environments (Greipsson, 2011), including HMs, radionuclides and organic pollutants such as polynuclear aromatic hydrocarbons, polychlorinated biphenyls, explosives, surfactants and pesticides chlorinated (Hazrat et al., 2013; Wang et al., 2003).

Phytoremediation takes advantage of the unique and selective uptake capabilities of plant root systems, together with the translocation, bioaccumulation, and contaminant degradation abilities of the entire plant body (Hinchman et al., 1995).

Lately, it is being regarded as a highly promising technology for the remediation of polluted sites (Garbisu and Alkota al., 2003). It is considered a cost effective, non-invasive, efficient, environmentally and publicly acceptable technology to address the removal of environmental contaminants via solar-driven remediation strategy (Pilon-Smits, 2005; Meagher, et. al., 2000; Clemens,2001; Sarma, 2011; Singh and Prasad, 2011; Vithanage et al., 2012).

Compared with traditional techniques, generally, phytoremediation handles the contaminants without affecting topsoil, thus conserving its fertility (Mench et al., 2009), furthermore, it is suitable at wide range of contaminated sites were conventional practices are impracticable or extremely expensive (Kokyo et al., 2014).

Land restoration by plant relies on evolving plant strategies, which enable them to tolerate metal toxicity and then growing on metal-contaminated soils.

Although heavy metals at high concentration can negatively affect plant growth, some types of plants have indeed evolved different mechanisms to tolerance or detoxification under stressful conditions due to high heavy metal concentrations (Clemens, 2001). For, the instance, some plants are able to adopt strategies targeted to preclude or limit the entry of the metals into the plant root such us metal immobilization by mycorrhizal association, metal sequestration, or complexation by exuding organic compounds from root. Other tolerance mechanisms for metal

detoxification include metal sequestration and compartmentalization in various plant intracellular compartments (e.g., vacuole) (Hall, 2002), metal binding by cellular walls and metal chelation at root level followed by the release of several substances which have a high affinity for the absorption of heavy metals (e.g., organic acids, polysaccharides, phytochelatins and metallothioneins). Plant cells are also able to biosynthesize or accumulate osmolytes and osmoprotectants substances, for example, proline, that are typically used by plants to enhance tolerance to HMs toxicity.

According to their different adaption strategies, plants can be classified into three categories: metal excluder, indicators and accumulators/hyperaccumulators (Baker and Walker, 1990). The excluder group includes the majority of plant species that limit the translocation of HMs and maintain low levels of contaminants in their aerial tissues over an extensive range of soil concentrations. Plants that are metal indicators accumulate metals in their harvestable biomass and these levels generally are reflective of the metal concentration in soil. Metal accumulators/hyperaccumulators plants, instead, show exceptional metal-accumulating capacity in their harvestable biomass to levels that far exceed those found in the soil (Mganga et al., 2011; Cho-Rhuc et al., 2006).

Owing to hyperaccumulator plants ability to take up large amounts of metals in their shoots without showing significant signs of toxicity, this makes hyperaccumulators ideal candidates for soil metal phytoremediation (Thijs et al., 2016).

At present, plants that naturally hyper-accumulate or tolerate toxic materials (Chaney et al., 1997; Pence et al., 2000) are used for the purpose of remediation of the environment polluted by contaminates, such as Pb, Cd,Cr, As, and various radionuclides. They include, in particular, species with high biomass, like *Fragmites australis* (Massacci et al., 2001), *Cannabis sativa* (Kos and Lestan, 2004) and *Populus alba* (Di Lonardo et al., 2011) trees. A provisional list of some accumulator plants, with indication of the metal(s) accumulated, is reported in Table 2.

Vegetables	Heavy metals	Concentration in plant aerial parts (mg/kg)	Threshold level for hyper-accumulator	Type of remediation	Fold-higher than threshold level	Reference
Prosopis laevigata	Cd	8176	100	Phytoextraction	81.8	Buendía-González et al., 2010
Arabidopsis halleri		5722		Phytoextraction	57.2	Küpper et al., 2000
Thlaspi caerulescens		5000		Phytoextraction	50.0	Koptsik, 2014
Thlaspi caerulescens		3000		Phytoextraction	30.0	Sheoran et al., 2009
Viola principis		1201		Phytoextraction	12.0	Wan et al., 2016
Potentilla griffithii		852		Phytoextraction	8.5	Hu et al., 2009
Solanum nigrum		387		Phytoextraction	3.9	Sun et al., 2008
Lonicera japonica		286		Phytoextraction	2.9	Liu et al., 2009
Thlaspi caerulescens		263		Phytoextraction	2.6	Lombi et al., 2001
Eleocharis acicularis		239		Phytoextraction	2.4	Sakakibara et al., 2011
Deschampsia cespitosa		236		Phytoextraction	2.4	Kucharski et al., 2005
Solanum photeinocarpum		158		Phytoextraction	1.6	Zhang et al., 2011
Phyllanthus serpentinus	Ni	38.100	1000	Phytoextraction	38.1	Chaney et al., 2010
Alyssum murale		22.800		Phytoextraction	22.8	Chaney et al., 2008
Alyssum corsicum		18.100		Phytoextraction	18.1	Li et al., 2003
Berkheya coddii		18.000		Phytoextraction	18.0	Mesjasz-Przybyłowics et al., 2004
Thlaspi caerulescens		16.200		Phytoextraction	16.2	Koptsik, 2014
Salvinia minima		16.600		Phytoextraction	16.6	Fuentes et al., 2014
Alyssum murale		15.000		Phytoextraction	15.0	Li et al., 2003
Alyssum serpyllifolium		10.000		Phytoextraction	10.0	Prasad, 2005
Isatis pinnatiloba		1441		Phytoextraction	1.4	Altinozlu et al., 2012
Arrhenatherum elatius	Pb	24.000	1000	Phytoextraction	24.0	Deram et al., 2000
Brassica juncea		10.300		Phytoextraction	10.3	Koptsik, 2014
Brassica nigra		9400		Phytoextraction	9.4	Koptsik, 2014
Pelargonium (Atomic)		7000		Phytoextraction	7.0	Arshad et al., 2008
Helianthus annuus		5600		Phytoextraction	5.6	Koptsik, 2014
Pelargonium (Clorinda)		5000		Phytoextraction	5.0	Arshad et al., 2008
Pelargonium (Attar)		4000		Phytoextraction	4.0	Arshad et al., 2008
Viola principis		2350		Phytoextraction	2.4	Wan et al., 2016
Euphorbia cheiradenia		1138		Phytoextraction	1.1	Chehregani and Malayeri, 2007
Pteris vittate	As	23.700	1000	Phytoextraction	23.7	Ma et al., 2001
Pteris vittate		13.800		Phytoextraction	13.8	Tu et al., 2002
Pteris vittata		8331		Phytoextraction	8.3	Kalve et al., 2011
Pteris vittata		6017		Phytoextraction	6.0	Han et al., 2016
Pteris vittata		4106		Phytoextraction	4.1	Wan et al., 2016
Pteris ryukyuensis		3647		Phytoextraction	3.6	Srivastava et al., 2006
Pteris quadriaurita		2900		Phytoextraction	2.9	Srivastava et al., 2006
Corrigiola telephiifolia		2110		Microbial assisted phytoextraction	2.1	Garcia-Salgado et al., 2012
Pteris biaurita		2000		Phytoextraction	2.0	Srivastava et al., 2006
Pteris cretica		1800		Phytoextraction	1.8	Srivastava et al., 2006
Eleocharis acicularis		1470		Phytoextraction	1.5	Sakakibara et al., 2011
Arabidopsis halleri	Zn	32.000	10.000	Phytoextraction	3.2	Zhao et al., 2000
Potentilla griffithii		11.400		Phytoextraction	1.1	Hu et al., 2009
Eleocharis acicularis	Cu	20.200	1000	Phytoextraction	20.2	Sakakibara et al., 2011
Aeolanthus biformifolius		13.700		Phytoextraction	13.7	Chaney et al., 2010
Pteris vittata	Cr	20.675	1000	Phytoextraction	20.7	Kalve et al., 2011
Prosopis laevigata		5461		Phytoextraction	5.5	Buendía-González et al., 2010

Table 2 List of some hyperaccumulator plants accumulating high levels of HM(loid)s in their above ground parts. (Khalid et al., 2016)

Based on soil condition and type of contaminant and plant, phytoremediation can be used for containment (phytoimmobilization and phytostabilization) or removal (phytoextraction and phytovolatilization) purposes (Thangavel and Subhuram, 2004). This practice includes several plant-based technology such as phytoextraction (or phytoaccumulation), phytofiltration, phytostabilization, phyto-volatilization, and phytodegradation (Alkorta et al., 2004), each having a different mechanism of action for remediating metal-polluted soil, sediment, or water.

Phytostabilization

Phytostabilization involves the remediation of polluted soils (and waters) by certain plant species able to reduce the solubility and mobility of contaminants within the rhizosphere. Phytostabilization process includes absorption, adsorption onto the root surface, or by formation of insoluble compounds, which lead to neutralizing their harmful effect on the environment (Singh et al., 2015).

Phytofiltration

Phytofiltration is the use of plant roots (rhizofiltration) or seedlings (blastofiltration) to absorb or adsorb pollutants, mainly metals, from water and aqueous-waste streams (Prasad and Freitas 2003). Plant roots or seedlings grown in aerated water are able to absorb, precipitate and concentrate toxic metals from polluted effluents (Dushenkov and Kapulnik 2000; Elless et al. 2005).

Rhizofiltration

Rhizofiltration is exploited for the removal of pollutants, mainly metals from aquatic environments, such as damp soil and ground and/or surface waters by adsorption or precipitation onto roots or other submerged organs of metal-tolerant aquatic plants related to their physiological and biochemical characteristics (Kvesitadze et al., 2006; Jadia and Fulekar, 2009).

Phytovolatilization

Phytovolatilization involves a process by which plants take up contaminants from the soil and release them in a volatile form into the atmosphere through transpiration. The removal of contaminants, especially organic matters and arsenic, mercury and selenium (As, Hg, and Se),

by phytovolatilization could be achieved by genetically-modified plants capable of absorbing elemental forms of these metals from the soil, biologically converting them to gaseous species within the plant, and releasing them into the atmosphere (Kotrba, 2013).

Phytoextraction

Phytoextraction, also well known as phytoaccumulation, refers to the uptake and translocation of metal contaminants from the soil into above-ground components of the plants resulting in reduced soil metal concentrations (Pulford and Watson, 2003). This extraction process depends on the ability of selected plants, called hyper-accumulators, to grow under excessive metal stress and to accumulate greater metal concentrations in shoots than those usually found in non-accumulators, without visible symptoms (Prabha et al., 2007).

Among these phytoremediation categories, phytoextraction, can be used to remove HMs from soil using its ability to uptake and accumulate considerable amounts of metals which normally are essential for plant growth (Fe, Mn, Zn, Cu, Mg, Mo, and Ni) such as some metals with unknown biological function (Cd, Cr, Pb, Co, Ag, Se, Hg) (Cho-RuK et al.,2006).

1.5.1.2 Fungi-assisted phytoremediation

Fungi-assisted phytoremediation is a bioremediation technique based on the integration and improvement of the natural ability of plants in the removal of contaminants by rhizosphere fungi which can pay a pivotal role in bioremediation of soil rich in heavy metals. In these regard, interaction between fungi and plants in soil may led to symbiosis, allowing to combine the potential role of fungi in the eliminating the toxic effect of metal-contaminated soil and their possible role in augmenting plant growth by providing nutrient and metabolites to the plants for phytoremediation approaches.

Rhizosphere fungi, particularly associated with hyperaccumulating or non-hyperaccumulating plants, have repeatedly been demonstrated to alleviate HMs stress of plants, against abiotic stresses, stimulating plant growth and improving plant health owing to plant grow regulators production, especially phytormones such as auxin and gibberellins (Khan et al., 2015a; Deng and Cao, 2017)

Moreover, fungi associated with plant root system offer several benefits (exudates production, promotion of water and gases movement in soil, promotion of microbial activity) able to influence pollutants uptake and degradation. In details, fungi can change or reduce the

toxicity of metal contaminants or affect transformations between soluble and insoluble phases of contaminants through pH change and the combination of bioaccumulation (i.e. active metabolism-dependent processes, which may include both transport into the cell and partitioning into intracellular components) and biosorption (i.e. the binding of metals to the biomass by processes that do not require metabolic energy) process (Melgar et al., 2007).

Fungi, indeed, implement a multiplicity of physicochemical and biological mechanisms by which release intracellular /extracellular enzymes to resist the metal concentration or possess appropriate metal chelation and solubilisation and sequestration abilities that aid the removal of soil pollutant by enhancing the phytoremediation capacity of the host plant (Khan et al., 2011a; Aly et al., 2011; Mosa et al.,2016) .

Fungi-assisted phytoremediation could be an innovative technologies having a potential to advance plant-based environmental clean-up. Plant species associated with fungi and the ability of such plants to grow on metal-contaminated soils is important to phytoremediation.

Therefore, symbiotic fungi, such as, filamentous fungi and arbuscular mycorrhizal fungi, has widely been studied in relationship to phytoremediation (Entry et al., 2002; Meier et al., 2012).

In particular, the filamentous fungi of *Trichoderma* species emerged in plant-consortia clean-up system owing their high colonization ability together with their action in promoting plant growth and metal tolerance capability. Their application in phytoremediation strategy showed significant increase in heavy metals uptake in a wide range of plant species (Adams et al., 2007).

Arbuscular mycorrhizal fungi (AMF) may also have an essential contribution to phytoremediation by influencing HMs availability and enhancing plant tolerance (Gaur and Adholeya, 2004). Arbuscular mycorrhizal fungi (AMF) are ubiquitous plant symbionts that affect plant nutrient status, patterns of resource allocation and rates of plant growth (Tao et al., 2016). AMF are able to established direct relationships between soil and the roots of a host plant by symbiotic association providing macro and micro-nutrients, water and delivering a proportion to their hosts in exchange for carbon.

Similarly to the enhancing of low mobility nutrients, such as P, Zn, and Cu and the improving of plant mineral nutrition induced by AMF, HMs can taken up via the fungal hyphae and can be transported to the plant; consequently AMF could be used to further the plant phytoremediation capability stimulating phytoextraction or contribute to phytostabilization.

AMF can alleviate HMs toxicity in plant host through several mechanism including mycelium heavy metal immobilization, HMs adsorption to chitin in the cell walls.

The mechanisms exerted by AMF to alleviate HMs stress in plants may include the immobilization of HMs in the mycelium, HMs adsorption to chitin in the cell walls, the improvement of plant mineral nutrition, changes in rhizosphere pH, and the regulation of gene expression under stress conditions (Joner et al., 2000; Li and Christie, 2001; Christie et al., 2004; Wang et al., 2007b; Upadhyaya et al., 2010). However element type, plant and fungal species may influence the effectiveness of mycorrhizal symbiosis in phytoremediation (Wang et al., 2007b). The combined use of *Trichoderma* and AMF can be exploit to improve phytoremediation process and to relieve environmental stress in plants due to heavy metals, subsequently to, increase plant growth potential under these less-ideal conditions.

1.5.1.3 Poplar for the phytoremediation

Poplar plants are naturally resistant to a wide variety of HMs and other contaminants spread in the environment (Hong et al., 2001; Robinson et al., 2007; Liu et al., 2009). Several poplar species are known both for the ability to uptake (i.e. phytoextraction; Marzilli et al., 2018; Pulford and Watson, 2003; Bissonnette et al., 2010) and to stabilise HMs (i.e. phytostabilisation; Di Baccio et al., 2003; Tognetti et al., 2004) into their tissues, thus reducing the mobility of these contaminants in the soil profile (Shim et al., 2010). Compared to other plant species, poplar trees have several advantageous characteristics, such as fast-growing extensive root systems, high rates of water uptake and transpiration, high biomass production and easy propagation (Andreas et al., 2005; Guerra et al., 2011). Furthermore, poplar trees thrive in a wide range of climatic conditions and can be exploited in 'short-rotation forestry' systems for pulp and paper production or like bioenergy sources, thus providing further economic gain (Laureysens et al., 2004; Licht and Isebrands, 2005; Sebastani et al., 2014).

Several studies have compared the potentiality to tolerate and accumulate wide variety of HMs in many poplar species through *in vivo* (Dos Santos et al., 2007; Legault et al., 2017) and *in vitro* observations (Di Lonardo et al., 2011, Katanić et al., 2014). In this regard, the occurrence of different detoxification strategies and response to HMs stress between poplar species (included clones, cultivars and hybrids) exposed to HMs were discovered. Many studies have thus been focused on the use of poplar in phytoremediation strategy. For the instance, Sebastiani et al., (2004) investigation on the effects of HMs-enriched organic waste on biomass partitioning and HMs accumulation into plant organs for two poplar clones (“I-214” and “Eridano”) displayed both phytoextraction and phytostabilisation strategies in the two clones

studied among the four HMs (Zn, Cu, Cr and Cd) contained in the industrial organic waste. The ability of the same two poplar clones, commonly used in Italian poplar plantations, to remediate and achieve substantial reductions in the concentration of Cd in contaminated soil have been reported by Di Baccio et al. (2014). Moreover, variability of cadmium tolerance and distribution in plant organs was set up in ten selected poplar clones from different species, hybrids and genotypes via hydroponic system (Pietrini et al., 2009).

Metals accumulation capacity of two Cu and Zn tolerant poplar clones, namely "AL22" (*Populus alba* L.) and "N12" (*Populus nigra* L.), to phytostabilize or phytoextract Cd, Cu, Fe, Pb and Zn from an artificially Fe polluted soil, was investigated and compared in a pot experiment (Baldantoni et al., 2014). Nikolić et al. (2017), indeed, evaluated the potential of *Populus deltoides* (clone B-81) and *Populus* × *euramericana* (clone "Pannonia") for phytoremediation of Cd contamination in soil suggesting *P. deltoides* for its best phytoextraction performance under Cd exposure. Recently, genetically engineering poplar has been proposed as a promising HMs captor. The transgenic poplar plants, overexpressing genes involved in HMs resistance, have exhibited remarkable removal rates of these pollutants in a wide number of studies (Shim et al., 2012; Doty et al 2007).

Concerning transgenic poplar phytoremediation ability, Doty et al. (2007) developed transgenic poplar (*Populus tremula* × *Populus alba*) plants through the overexpression of cytochrome P450 2E1, showing increased removal rates of some common environmental pollutants (volatile hydrocarbons, including trichloroethylene, vinyl chloride, carbon tetrachloride, benzene, and chloroform) from hydroponic solution reporting a 100-fold enhancement of phytoremediation capacity. In addition, the poplar hybrids (*Populus alba* ×*Populus tremula*), "INRA" clone "717 1-B4", over-expressing γ-glutamylcysteine synthetase (γ-ECS) exhibited significantly more Cd accumulation in root tissue than wild type (Koprivova et al., 2002).

Other studies have mentioned the synergic use of poplar and rhizosphere microbial organisms for enhancing the effectiveness of phytoremediation (Cocozza et al., 2014, 2015). Based on their potential for remediation and/or their potential benefits on plants, symbiotic and ectomycorrhizal fungi have been payed remarkable attention in fungi-assisted phytoremediation program.

Symbiotic fungi like *Trichoderma* spp., highly resistant to a range of toxicants (Harman et al., 2004b; Ezzi and Lynch., 2005), inoculated in poplar seedlings have led to the poplar increasing resistance against pathogenic fungi infection under saline or alkaline stress (Guo et al., 2018). The resistance induced in *Trichoderma*-plants interaction could be positively

exploited for the enhancement of HMs removal efficiency and of poplar tolerance to HMs. Although *Trichoderma* fungi can effectively reduce the physiological damage caused by HMs stress, there are no studies regarding the application of *Trichoderma* tolerant strains in association with poplar for phytoremediation purpose.

Indeed, *Trichoderma* -assisted phytoremediation, has been only examined in combination with other plant species, showing promising results (Lynch and Moffat, 2005; Teng et al., 2015). In the same way, the responses of poplar clones inoculated with arbuscular mycorrhizal fungi were investigated under high Cu (Todeschini et al., 2007) and Zn (Lingua et al., 2008) concentration with the aim to exploit the fungi capacity to protect their host plant from metal contamination. In addition, they can be propagated by *in vitro* techniques and are conveniently exploited in genetic engineering (Confalonieri et al., 2003).

1.6 Aim of the study

The present research was aimed to the study of the potential use of two *Populus alba* clones in phytoremediation system utilizing different screening models.

The study was performed through two main experimental models: controlled and semi-controlled environments. Briefly, the research activity was constituted by three parts.

The first part of the research examined the use of white poplar clone ("Villafranca") to assess the potential to absorb metals in autotrophic autonomy under controlled conditions. *P. alba* clone responses were evaluated under several metals concentrations of Cd and Cu through the *in vitro* approach. Results highlight an efficient system for the plants screening in bioremediation and plant response assessment to environmental contaminants.

In the second part of the work, the study defined fungal strains tolerant to Cd for their exploitation in fungi-assisted phytoremediation techniques.

The third part of the research activity considered the study of Cd phytoremediation ability of white poplar clone ("Querce") in association with fungi of the genus *Trichoderma*. The experiment was performed using micropropagated plants acclimatized and grown in pots under semi-controlled conditions in order to minimize the impact of other environmental stress factors (such as drought, pests), but under conditions as similar as possible to those of the field.

Summarising, the different approaches used in the research activity were aimed to: i) value metal tolerance, accumulation and translocation of poplar clone ("Villafranca") established on Cd and Cu- polluted medium by means of *in vitro* screening; ii) select fungi belonging to the

genus *Trichoderma* based on the Cd-tolerance and investigation of the mechanisms of Cd-tolerance in *Trichoderma* ; iii) investigate the effect of *Trichoderma* selected strains on a Cd-tolerant poplar clone (“Querce”) via semi-controlled screening.

CHAPTER II – *In vitro* screening

2. Cd and Cu accumulation, translocation and tolerance in *Populus alba* clone (Villafranca) in autotrophic *in vitro* screening

2.1 Abstract

The present study investigated accumulation, translocation and tolerance of autotrophic *Populus alba* clone "Villafranca" in response to excess concentrations of cadmium (Cd) and copper (Cu) provided to the plants. For this purpose, increasing concentrations of Cd (0, 5, 50 and 250 μM) and Cu (0, 5, 50, 250 and 500 μM) were administered to the growth medium in which micropropagated poplar plantlets were exposed to metal treatments for 15 days. Filter bags, instead of the conventional *in vitro* screening, were applied to improve the experimental design.

Results showed that Cd and Cu increased in shoots and roots at increasing metal concentration in the medium. The highest Cd content was found in leaves, while the highest Cu content was found in roots. In "Villafranca", Cu showed toxic effects on the development of the seedlings, especially at the highest concentrations, reducing plant dry mass. However, the tolerance index (Ti) indicated good tolerance in this clone under exposure to excess metal concentrations, whereas plants had higher translocation factor (Tf).

We recommend *in vitro* selection of tolerant genotypes, aimed at providing early indication on accumulation potentiality and tolerance capability in research on plant sensitivity to excess heavy metal concentrations.

Keywords: Pollution, cadmium, copper, phytoremediation, white poplar, micropropagation, autotrophy

2.2 Introduction

Urbanization, industrialization and agricultural practices cause major disturbances on terrestrial ecosystems, releasing pollutants in the environment, altering the biogeochemical cycles and, consequently, ecological global processes (Kabata-Pendias and Pendias, 1989; Zacchini et al., 2009; Moss 2008; Xu et al., 2016). Pollutant toxicity is due to their persistence in the environment, soil, water and organisms for long time (Lone et al., 2008). In particular, soil contamination by heavy metals and organic chlorinated compounds is of concern in

industrialized countries, and the former may inhibit biodegradation of the latter contaminants (Alloway, 2013; Ahsan et al., 2009). High levels of pollutants in the soil alter its characteristics, potentially causing problems in groundwater and surface water, atmosphere and damaging microbial, human and plant health (Nazar et al., 2012; Olaniran et al., 2013). Cadmium (Cd) is recognized as an extremely relevant pollutant due to high toxicity, diffusion in plants and animals and high solubility in water (Pinto et al., 2004; WHO, 2010). Cadmium is released in the environment by industrial processes, heating systems, vehicular traffic, phosphate fertilizers and mineralization of rocks (Benavides et al., 2005). The concentration of Cd is usually below 0.5 mg kg^{-1} in uncontaminated soil, but it can reach 3.0 mg kg^{-1}, depending on soil conditions (Vahter et al., 1991). In highly contaminated soil, Cd concentration may exceed regulatory limits (5 mg kg^{-1} for public or private green areas or residential areas and 12 mg kg^{-1} for industrial sites), as established by the Italian legislation (no. 152/06, Italian Regulation 2006). The accumulation of Cd in plants impairs physiological and biochemical processes and alters structural traits (Khan et al., 2007; Feng et al., 2010). Cadmium in plants interferes with: (i) the uptake, concentration and translocation of nutrients, (ii) the gas exchange (Becerril et al., 1989; Hossain et al. 2010; Hatch et al. 1988), (iii) the Calvin cycle (Mobin and Khan, 2007; Shi et al. 2010) and (iv) the antioxidant metabolism (Khan et al. 2007), thus inducing significant losses of plant productivity (Manciulea and Ramsey, 2006).

Copper (Cu) is another important pollutant, because, despite being an essential element (required in the growth of plants, in seed production, disease resistance and regulation of water), excessive amounts of this metal become toxic to biota and humans (Adrees et al., 2015). Copper contamination derives by agricultural practices and industrial or municipal wastes (Ali et al., 2004). Contaminated sites show metal levels up to 990 mg Cu kg^{-1} (Yoon et al., 2006). Excess Cu concentration affects negatively plant growth, largely damaging root growth and morphology (Sheldon and Menzies, 2004; Borghi et al., 2007; Mallick et al., 2010). Conventional technologies (chemical and physical) for environment cleaning by pollutants are quite expensive and invasive (Vassilev et al., 2004; Van Nevel et al., 2007). Phytoremediation can be considered an alternative solution for decontamination purposes at relatively low costs (Saier and Trevors, 2010; Kalve et al., 2011; Sarma, 2011; Singh and Prasad, 2011; Vithanage et al., 2012). Phytoremediation technologies use herbaceous (e.g. Brassicaceae) and woody (e.g. Salicaceae) plant species able to tolerate, uptake and accumulate pollutants (Clements et al., 2002; Hodson 2012, Salt et al., 1998, Chaney et al., 1997; Raskin and Ensley, 2000; Cho-Ruk et al., 2006). In particular, poplars are of specific interest in environmental restoration programmes (Di Baccio et al., 2014; Fuzhong et al., 2010); for the multiple services, they

provide such as bioenergy, windbreaks, lignocellulosic feedstock for pulp, paper production, and other ecosystem services, including landscape enrichment by increasing structural and biological diversity and carbon sink (Tognetti et al., 2013). Recently, *in vitro* cultures, namely micropropagation, have promoted plant-based experimental system to analyze the plant responses to environmental contaminants (Di Lonardo et al., 2011; Di Santo et al., 2017). Plant production through *in vitro* culture remains necessary for genetic manipulation of plants and subsequent benefits for plant breeding. Moreover, an advantage of micropropagation is defined by the high number of homogeneous plants that can be screened in a short time. The present study aimed to assess the efficiency of *in vitro* system for screening plants intended for bioremediation programmes. The phytoremediation capacity of candidate *Populus alba* L. clone "Villafranca" in *in vitro* screening was previously tested in heterotrophic conditions (Borghi et al., 2008; Di Lonardo et al., 2011). In this study, we focused for the first time on metal accumulation, translocation and tolerance of the fast-growing commercial clone "Villafranca" exposed to excess Cd and Cu concentrations in autotrophic conditions. Cadmium is considered one of the most pressing concerns in the debate on food security and food safety in Europe (CEC, Commission of the European Communities, 2006) and globally (Kong, 2014), due to the diffusion of this metal in the food chain, whereas Cu is the third most used metal in the world (VCI, 2011).

In this experimental setting, increasing concentrations of Cd (0, 5, 50 and 250 μM; concentrations higher than 250 μM were not considered in this trial because they were toxic in preliminary tests; data not shown) and Cu (0, 5, 50, 250 and 500 μM) were used to (i) test whether clone "Villafranca" tolerates and accumulates excess metal concentrations in the growing medium already in the early stages of development, (ii) investigate metal translocation from roots to shoots and anatomical traits in root and leaf tissues and (iii) assess the validity of autotrophic micropropagation in screening poplar responses to excess Cd and Cu concentrations. Tissue culture was hypothesized to be useful for studying the potential of poplar clones to withstand heavy metal excess in the cultivation media. Cell and organ culture allows very fast plant testing in comparison with plant experiments, as well as with field trials (Golan-Goldhirsh et al., 2004), and provides the advantage of separating single effects of ready available experimental factors in controlled conditions (Harms, 1992).

In the current study, the *in vitro* system was improved through the use of filter bags that facilitate gas exchange, allowing to develop a close-tonature *in vitro* screening system, unlike other works carried out in heterotrophy (e.g. Di Lonardo et al., 2011).

2.3 Materials and methods

2.3.1 Plant culture and metals treatment

Populus alba L. clone "Villafranca" was micropropagated through microshoot cultures, according to Confalonieri et al. (2000). The growth substrate was defined by Woody Plant Medium (WPM) (Lloyd and McCown, 1980) at pH 5.5, supplied with sucrose (30 g L^{-1}), agar (6 g L^{-1}) and benzyl adenine (0.5 mg L^{-1}), in Magenta GA-7 plastic vessels. Cultures were kept in a growth chamber for 20 days at 23 ± 1 °C with 8-h photoperiod and 3000 lx of light intensity (T5 fluorescent lamps, 28 watts) and routinely subcultured. Then, aseptic cultures were transferred on BA (benzyl adenine) devoid medium for 3 weeks to prevent its carry-over effect and to promote rhizogenesis. The seedling autotrophy was induced removing sucrose from the substrate, and then jars have been provided by filter bags. The filter bags (25 × 38 cm) are made of polypropylene and are equipped with filtration system based on membranes with a fluxing surface that allows efficient gas exchange. They are composed of randomly arranged fibres in which microorganisms get trapped. This allows a fluent gas exchange without a drying-out zone below the filters, while still forming a firm barrier against microorganisms. The filtration system was assembled with Schott GLS 80 jars containing the seedlings. This innovative filtration technology results in a barrier against contaminations, high gas exchange, no dehydration and sterile conditions allowing ideal conditions for consequent healthy *in vitro* plant development. Treatments were carried out preparing a solid medium with half-strength WPM basal solution. The solid medium was contaminated with $CdSO_4$ (Sigma-Aldrich, Germany) and $CuSO_4$ (Sigma-Aldrich, Germany) showing increasing metal concentrations, respectively, 0 (control), 5, 50 and 250 μM Cd and 0 (control), 5, 50, 250 and 500 μM Cu, namely experimental treatments. The plants were then transferred into jars containing 200 ml Schott GLS 80 of contaminated substrate. Trial was set up in completely randomized design with four replicates (n = 4) for each experimental treatment (n = 4). The seedling exposure to Cd and Cu treatment was 15 days long.

2.3.2 Plant traits and element analysis

At the end of treatment with excess metal concentrations, seedlings were harvested collecting separately shoots and
roots. Leaf number and root and shoot length were measured (n = 4). Leaf area was measured using a leaf area meter (LICOR LI-3100; LI-COR, Lincoln, NE). Element contents were determined in roots and shoots. Roots were washed in distilled water (50 °C) to eliminate the agar and then placed for 10 min in 10 mM $CaCl_2$ solution to remove metals adhered to the root surface. Roots and shoots were then dried in a ventilated stove at 70 °C for 24 h until constant weight for the determination of dry mass. Dried tissues were digested with a concentrated acid mixture of $HNO_3/HClO_4$ (3:1, v/v) in DK6 Heating Digester. The Ca, Cd, Cu, Fe, K, Mg, Mn, Na, P, S and Zn concentrations in plant tissues were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 2000 DV, PerkinElmer Instruments). Element content was defined as the mean value of the element concentration in relation to the dry mass of plant tissue. Tolerance index and translocation factor were determined to define the effects of metal treatment. The tolerance index (Ti) was calculated to measure the plant ability to grow on metal-polluted substrate using the following formula according to Wilkins (1978):

$$Ti = \frac{\text{Dry mass of the plants growing in polluted medium}}{\text{Dry weight of the plants growing in control conditions}}$$

The translocation factor (Tf) was used to quantify the plant ability to translocate Cd and Cu from roots to shoots using the following formula (Liu et al., 2009):

$$Tf = \frac{\text{Metal concentration in shoots}}{\text{Metal concentration in roots}}$$

2.3.3 Scanning electron microscopy and energy-dispersive X-ray analysis

Scanning electron microscopy (SEM-EDX, DSM940, ZEISS) equipped with X-ray detector (INCA, Oxford, Great Britain) was used to observe leaf morphology and to measure the weight percentage of Cd and Cu in leaf and root tissues in control and metal-treated plants through microanalysis. Moreover, microanalysis allowed to define the distribution of metals

and nutrients (Ca, Fe K, Mg, Mn, Na, S, P and Zn) within portions of leaf and root tissues. Roots and leaves of poplar seedlings were fixed in 2.5% glutaraldehyde in 0.1 mmol L^{-1} phosphate buffer (pH 7.2) overnight. The samples were cut in cross sections, subsequently rinsed several times with 0.1 mmol L^{-1} phosphate buffer (pH 7.2) and dehydrated by immersion in ethanol at increasing concentrations (20, 30, 40, 60, 80, 95, 100%) with a final wash in acetone to exchange ethanol. Afterwards, the samples were dried by critical point drying (EMITECH K850) and coated with colloidal graphite to EDX analysis, and with a thin layer of gold (SEM) to morphological observation by a sputter coater (EMITECH K550). The samples were examined in EDX environment with working conditions: accelerating voltage, 25 kV; distance, 20 mm; and emission current, 100 μA. For the quantitative analysis, X-ray analysis data were processed by a standard quantitative analysis program for every element to compare relative concentrations.

The results were normalized to 100% excluding C and O and were expressed as % weight of element. Stomatal density (number of stomata per mm^2 of leaf area), the polar (length) and equatorial (width) of stomatal size and the distance between guard cells were determined at 10 kV.

2.3.4 Statistical analysis

Statistical analysis was performed in quadruplicate (repeated at least four times) independent experiments. The effects of Cd and Cu treatment on growth parameters, element contents, tolerance index and translocation factor were defined through one-way analysis of variance (ANOVA), while metal effects on nutrient distribution and localization within cross sections were tested via two-way analysis of variance (ANOVA). Differences of measured parameters between treatments were assessed through a post hoc comparison of means using the least significant difference (LSD) at the 0.05 and 0.001 significance levels. Statistical analysis was performed using the statistical program Statistica (StafSoft Inc., Tulsa, OK, USA) and SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA).

2.4 Results

2.4.1 Plant traits

Micropropagated poplar seedlings showed differences in growth parameters, leaf number, leaf area, root and shoot dry biomass and root and shoot length (Table 3). Leaf chlorosis symptoms wereobserved in treated plants. Leaf number was significantly reduced by 250 μM Cd treatment ($p < 0.0001$); 50 and 250 μM Cd treatments reduced leaf area of seedlings ($p < 0.0001$) (Table 3). Root length and shoot biomass were clearly affected by Cd treatment, reaching higher values in 5 μM than in other treatments. Leaf number was significantly reduced by 250 and 500 μM Cu treatments. Positive effects of Cu were observed on leaf area at metal concentrations of 5 and 50 μM and on root length at 5, 50 and 250 μM. The dry mass of shoots was remarkably reduced in 250 and 500 μM Cu treatments (Table 3). Stomatal density was reduced in 5 μM Cd and Cu treatments, whereas increasing in 250 and 500 μM Cu treatments.

The length and width of the stomata were reduced at high metal concentrations (250 μM Cd and 250 and 500 μM Cu) (Table 4). The distance between guard cells increased in response to metal treatments (both Cd and Cu) in comparison with control plants.

Treatment	Leaf number (n)	Leaf area index (cm^2)	Root length (cm)	Shoot length (cm)	Root dry mass (g)	Shoot dry mass (g)
Cd						
control	11.29 ± 1.10 a	2.44± 1.41 a	2.66 ± 0.39 a	6.31 ± 0.51 a	0.0025 ± 0.0008 a	0.0093 ± 0.0017 a
5 μM	12.7 ± 0.47 a	6.64± 3.59 b	5.18 ± 0.63 b	7.67 ± 0.41 a	0.0029 ± 0.0005 a	0.0167 ± 0.0017 b
50 μM	11.08 ± 0.95 a	1.24± 0.25 c	3.55 ± 0.41 a	7.38 ± 0.57 a	0.0024 ± 0.0002 a	0.0095 ± 0.0014 a
250 μM	8 ± 0.80 b	0.62± 0.12 c	3.13 ± 0.43 a	6.46 ± 0.49 a	0.0019 ± 0.0004 a	0.0063 ± 0.0011 a
p level	*0.0001*	*0.0001*	*0.004*	0.171	0.370	*0.0001*
Cu						
control	11.29 ± 1.10ab	2.24 ± 0.93a	2.66 ± 0.39a	6.31 ± 0.51ab	0.003 ± 0.001a	0.009 ± 0.002ab
5 μM	13.00 ± 0.92a	5.22 ± 0.27b	5.08 ± 0.27b	7.54 ± 0.37a	0.005 ± 0.002a	0.013 ± 0.002a
50 μM	11.42 ± 1.09ab	5.77 ± 0.95b	4.29 ± 0.38bc	8.00 ± 0.87a	0.003 ± 0.001a	0.010 ± 0.002a
250 μM	9.25 ± 0.99b	0.41 ± 0.11c	3.25 ± 0.48ac	5.46 ± 0.44b	0.002 ± 0.001a	0.006 ± 0.001b
500 μM	4.67 ± 0.57c	1.06 ± 0.05ac	3.96 ± 0.50c	4.4 ± 0.67b	0.002 ± 0.001a	0.006 ± 0.001b
p level	*0.0001*	*0.0001*	*0.002*	*0.0001*	0.207	*0.001*

Table 3 Plant traits (leaf number, root and shoot length, root and shoot dry biomass) of clone "Villafranca" subjected to Cd (control, 5, 50 and 250 μM Cd concentration) and Cu (control, 5, 50, 250 and 500 μM Cu concentration) treatments. Values are means ± standard errors; significant differences between the means ($p < 0.05$, according to ANOVA and LSD test) appear with different letters.
(Italic numbers report significant difference between treatments for each parameter.)

Treatment	Stomatal density (n mm^{-2})	Polar size (µm)	Equatorial size (µm)	Distance between guard cell (µm)
Cd				
control	8.33±0.43a	11.43±0.77b	2.68±0.36b	6.37±0.34b
5	6.07±0.21b	12.10±0.70a	3.92±0.38a	10.27±0.30a
50	8.07±0.21a	11.92±0.75a	1.22±0.26c	10.69±0.65a
250	8.53±0.34a	7.04±0.51c	0.60±0.18d	8.66±0.42c
P level	*0.000*	*0.000*	*0.000*	*0.000*
Cu				
control	8.33±0.43b	2.68±0.36ab	2.68±0.36b	6.37±0.34c
5	5.53±0.30d	3.78±0.31ac	3.78±0.31a	11.55±0.42b
50	10.07±0.53c	3.13±0.53b	3.13±0.53ab	13.70±0.42a
250	7.47±0.39b	2.38±0.30b	2.38±0.30b	10.63±0.31b
500	12.8±0.52a	2.31±0.43b	2.31±0.43b	14.48±0.60a
P level	*0.000*	*0.003*	*0.053*	*0.000*

Table 4 Leaf traits (stomatal density, polar size, equatorial size and distance between guard cells) of clone "Villafranca" subjected to Cd (control, 5, 50 and 250 µM Cd concentration) and Cu (control, 5, 50, 250 and 500 µM Cu concentration) treatments. Values are means ± standard errors. Significant differences between the means ($p < 0.05$, according to ANOVA and LSD test) appear with different letters.
(Italic numbers report significant difference between treatments for each parameter.)

2.4.2 Metal accumulation, tolerance index and translocation factor

Cadmium and Cu contents significantly increased in roots and shoots with increasing Cd and Cu contamination (50 and 250 µM Cd treatment; 250 and 500 µM Cu treatment), showing higher Cd values in leaves than in roots ($p < 0.05$) and higher Cu values in roots than in leaves ($p < 0.001$) (Figure 3). The tolerance index of shoots decreased in 50 and 250 µM Cd treatments, whereas the translocation factor was low at 250 Mm Cd (Table 5). The tolerance index of shoots decreased in 250 and 500 µM Cu treatments, while the metal did not affect root tolerance (Table 5). The translocation factor significantly declined in 250 µM Cd treatment ($p < 0.001$), whereas it was not affected by Cu treatment (Table 5).

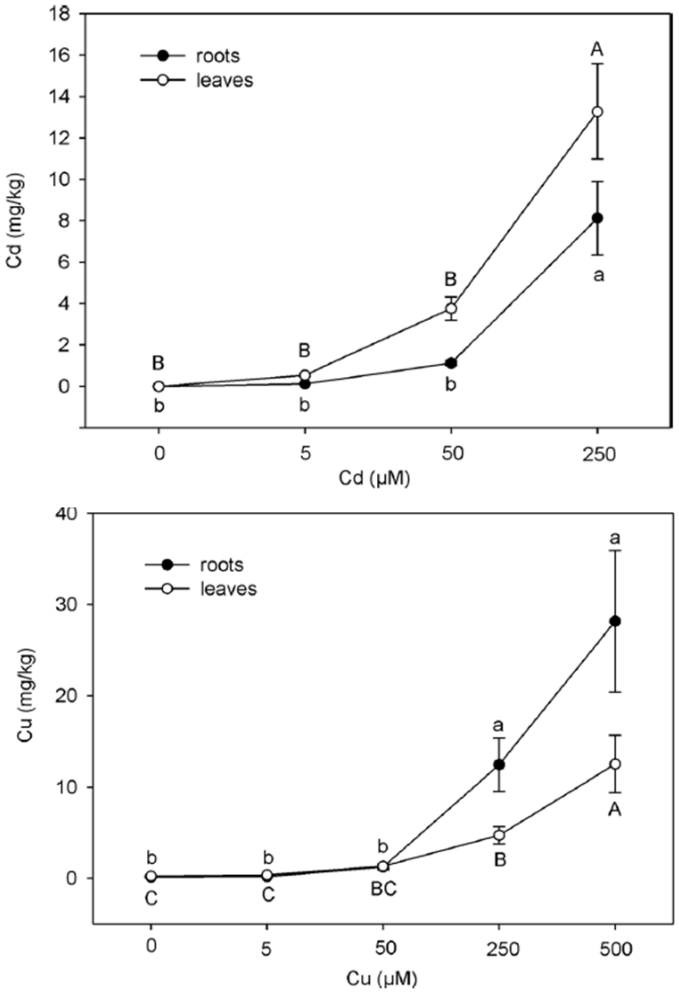


Figure 3 Cadmium (Cd) and Copper (Cu) contents in roots (black circles) and leaves (white circles) of clone "*Villafranca*" subjected to Cd (control, 5, 50 and 250 µM Cd concentration) and Cu (control, 5, 50, 250 and 500 µM Cu concentration) treatments. Values are means ± standard errors (n = 12 seedlings); values marked with the same letter are not statistically different (lowercase letters for the comparison of Cd and Cu treatment in roots; capital letters for the comparison of Cd and Cu treatment in leaves) (LSD test, $p \leq 005$). One-way ANOVA was applied to weigh the effects of metal treatment in roots and leaves: Cd in roots (p level > 0.0001), Cd in leaves (p level > 0.004), Cu in roots (p level > 0.0001), and Cu in leaves (p level > 0.0001)

Treatment	Ti root	Ti shoot	Tf
Cd			
5 µM	116.80 ± 19.68 a	179.68 ± 18.77 a	4.66 ± 0.59 b
50 µM	96.00 ± 9.93 a	102.42 ± 14.96 b	3.51 ± 0.53 b
250 µM	75.00 ± 16.53 a	67.65 ± 11.59 b	1.88 ± 0.25 a
p level	*0.161*	*0.0001*	*0.0001*
Cu			
control			3.13 ± 1.07a
5 µM	189.67 ± 75.72a	143.10 ± 19.66a	4.16 ± 1.52a
50 µM	126.33 ± 26.18a	110.30 ± 18.86a	1.77 ± 0.42a
250 µM	71.33 ± 20.89b	63.53 ± 10.74b	1.08 ± 0.37a
500 µM	74.33 ± 20.98b	59.50 ± 11.91b	1.04 ± 0.31a
p level	*0.153*	*0.0001*	*0.456*

Table 5 Tolerance index (Ti) in roots and shoots and translocation factor (Tf) in clone "Villafranca" subjected to Cd (control, 5, 50 and 250 µM Cd concentration) and Cu (control, 5, 50, 250 and 500 µM Cu concentration) treatments. Values are means ± standard errors significant differences between the means ($p < 0.05$, according to ANOVA and LSD test) appear with different letters.
(Italic numbers report significant difference between treatments for each parameter.)

2.4.3 Element concentrations

Element concentrations (Ca, Fe K, Mg, Mn, Na, S, P and Zn) varied in roots and shoots in relation to Cd and Cu treatments. In roots exposed to excess Cd, nutrients (Ca, Mg, Mn, Na, P, Sand Zn) were not affected by the treatment, though they changed in the shoot. Moreover, Fe and K concentrations were significantly affected in shoots and roots by Cd treatment (Table 6). Concentrations of Fe and K were significantly reduced in 250 μM Cd treatment (Table 6). At shoot level, Ca, Fe, K, Mg, Mn, P, S and Zn were significantly reduced (Table 6). The 500 μM Cu treatment induced low contents of K and Mg in root tissues (Table 6). Contents of Ca, K, Mg, Mn, Na, P, S and Zn were lower in 500 μM Cu than in other treatments (Table 6).

	mg kg^{-1}										
Cd treatment	**Ca**	**Cd**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**S**	**Zn**
Roots											
control	25.278±4.135a	0.000±0.000 b	0.109±0.034b	6.714±2.091a	235.935±53.543 b	5.398±0.883a	0.404±0.126a	40.286±5.516a	0.001±0.000a	0.504±0.157a	1.018±0.317a
5 µM	37.630±3.914a	0.128±0.023 b	0.233±0.036b	21.662±2.535b	405.099±45.125 a	6.600±1.041a	0.520±0.089a	43.738±6.912a	0.002±0.000a	0.683±0.123a	1.358±0.221a
50 µM	27.775±2.274a	1.121±0.116 b	0.173±0.021b	13.271±1.954c	236.356±28.507 b	5.104±0.508a	0.349±0.047a	28.700±2.618a	0.002±0.000a	0.458±0.054a	1.292±0.128a
250 µM	23.145±4.655a	8.125±1,765 a	0.730±0.276a	8.000±1.788a	133.393±16.326 c	5.104±1.126a	0.521±0.114a	27.815±6.358a	0.002±0.000a	0.371±0.080a	1.413±0.307a
p level	0.591	*0.000*	*0.026*	*0.000*	*0.008*	0.421	0.398	0.314	0.684	0.115	0.731
Shoots											
control	56.134±12.739 b	0.000±0.000 b	0.225±0,042a	4.571±0.848 c	39.143±5.360b	25.249±5.351 a	3.568±0.662 b	48.214±8.947 a	0.073±0.014b	2.429±0.451a	2.386±0.443b
5 µM	95.650±11.944 a	0.542±0.063 b	0.308±0.032a	9.275±1.088 b	56.083±13.950a	37.666±4.269 b	5.450±0.587 a	35.550±5.290 a	0.137±0.014a	4.160±0.441b	4.570±0.520a
50 µM	70.708±9.066 b	3.763±0.565 b	0.271±0.049a	9.876±2.335 a	51.250±5.307b	21.204±2.924 a	3.323±0.517 b	31.189±5.749 a	0.065±0.010b	2.168±0.332a	3.308±0.610ab
250 µM	46.124±7.828 b	13.271±2.291 a	0.180±0.031a	3.965±0.737 c	21.334±5.055c	12.484±1.876 c	1.954±0.335 c	33.084±5.261 a	0.023±0.005c	1.282±0.224c	2.425±0.428b
p level	*0.011*	*0.004*	0.089	0.012	*0.000*	*0.000*	*0.000*	0.590	*0.000*	*0.000*	*0.014*
Cu treatment	**Ca**	**Cd**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**S**	**Zn**
Roots											
control	34.786±10.833a	nd	0.109±0.034b	6.714±2.091a	48.929±15.237ab	7.429±2.313a	0.404±0.126a	50.357±15.682a	0.001±0.000b	0.109±0.157b	1.018±0.317a
5 µM	49.223±14.838a	nd	0.174±0.052b	13.945±3.278a	66.892±17.149a	6.548±2.020a	0.315±0.075a	49.092±16.105a	0.004±0.001ab	0.726±0.178b	2.284±0.818a
50 µM	74.072±19.808a	nd	1.328±0.290b	25.944±6.330a	44.535±9.819ab	8.274±2.424a	0.421±0.111a	56.802±14.867a	0.005±0.001a	2.086±0.995a	2.551±0.708a
250 µM	41.014±11.322a	nd	14.646±4.101a	14.958±4.997a	64.125±19.634ab	7.563±2.086a	0.983±0.295a	39.000±14.806a	0.002±0.000b	0.402±0.129b	1.965±0.623a
500 µM	32.184±10.218a	nd	26.665±8.254a	35.106±13.886a	8.998±3.008b	3.070±1.058a	0.628±0.295a	22.208±7.568a	0.001±0.000ab	0.221±0.071b	14.425±7.644a
p level	0.534	nd	*0.000*	*0.114*	*0.023*	0.321	0.080	0.388	0.124	0.470	*0.055*
Shoots		nd									
control	70.357±13.056a	nd	0.225±0.042bc	4.571±0.848a	295.714±54.874ab	27.571±5.116a	3.568±0.662a	48.214±8.947a	0.073±0.014a	0.225±0.451b	2.386±0.443a
5 µM	121.542±22.386b	nd	0.346±0.060c	8.021±1.932a	408.451±69.387a	42.945±6.993b	5.763±0.854b	54.644±15.398a	0.104±0.015a	4.491±0.639a	5.523±0.939b
50 µM	68.342±14.928a	nd	1.317±0.256bc	6.402±1.327a	235.349±52.745b	23.312±5.052a	3.216±0.651a	41.990±10.099a	0.113±0.038 a	2.437±0.510b	3.273±0.671a
250 µM	50.969±8.366a	nd	4.707±0.950b	10.896±2.461ab	119.167±21.509bc	10.753±2.307c	1.573±0.266c	51.875±8.693a	0.010±0.003b	1.093±0.200c	2.210±0.430a
500 µM	44.694±10.799a	nd	13.273±3.126a	16.704±4.021b	79.724±19.602c	7.290±1.829c	1.115±0.268c	30.317±9.354a	0.008±0.002b	0.850±0.205c	1.712±0.469a
p level	*0.002*	nd	*0.000*	*0.012*	*0.000*	*0.000*	*0.000*	0.486	*0.000*	*0.000*	*0.000*

Table 6 Concentrations of Ca, Fe, K, Mg, Mn, Na, P, S and Zn (mg kg−1) in roots and shoots of clone "Villafranca" subjected to Cd (control, 5, 50 and 250 µM Cd) and Cu (control, 5, 50, 250 and 500 µM Cu) treatments. Values are the result of ICP analysis. Values are means ± standard errors. Significant differences between the means ($p < 0.05$, according to ANOVA and LSD test) appear with different letters. (Italic numbers report significant difference between treatments for each parameter.)
*nd = not detected

2.4.4 Distribution of elements in leaf and root cross-sections

Elements were differently distributed in cross-sections of leaf and root tissues of treated plants (SEM-EDX analysis) (Table S1). In leaves of Cd-treated plants, Ca, P and Zn showed significant differences between portions of the cross-sections, highlighting higher values in the mesophyll than in the epithelium. For other elements, Cd treatment determined high element allocation in the epithelium, without affecting element allocation in the mesophyll. In roots, only Na content was significantly different among the epithelium, cortex and central cylinder, with high values in the cortex and central cylinder. In addition, Cd threatened the allocation of most of the elements (Ca, Cu, Fe, K, Mn, Na, S), especially in the epithelium and central cylinder. The mapping of elements through microanalysis showed that the localization of Cd in root and leaf tissues was associated with that of P (Figure 4). In roots, Cd and P were concentrated in the central cylinder, while in leaves, in the epithelium and mesophyll.

Considering Cu treatment, Ca, P, S and Zn contents were significantly different between portions of leaves, generally high values being detected in the mesophyll. Nutrient distribution, whereas, was significantly variable in leaf cross sections, at increasing Cu levels, with relatively higher allocation in the mesophyll than in the epithelium. In roots, Fe and Zn were localized principally in the epithelium, where high Cu levels caused increasing element allocation, while Na, Mg and P were mainly distributed in the cortex and central cylinder.

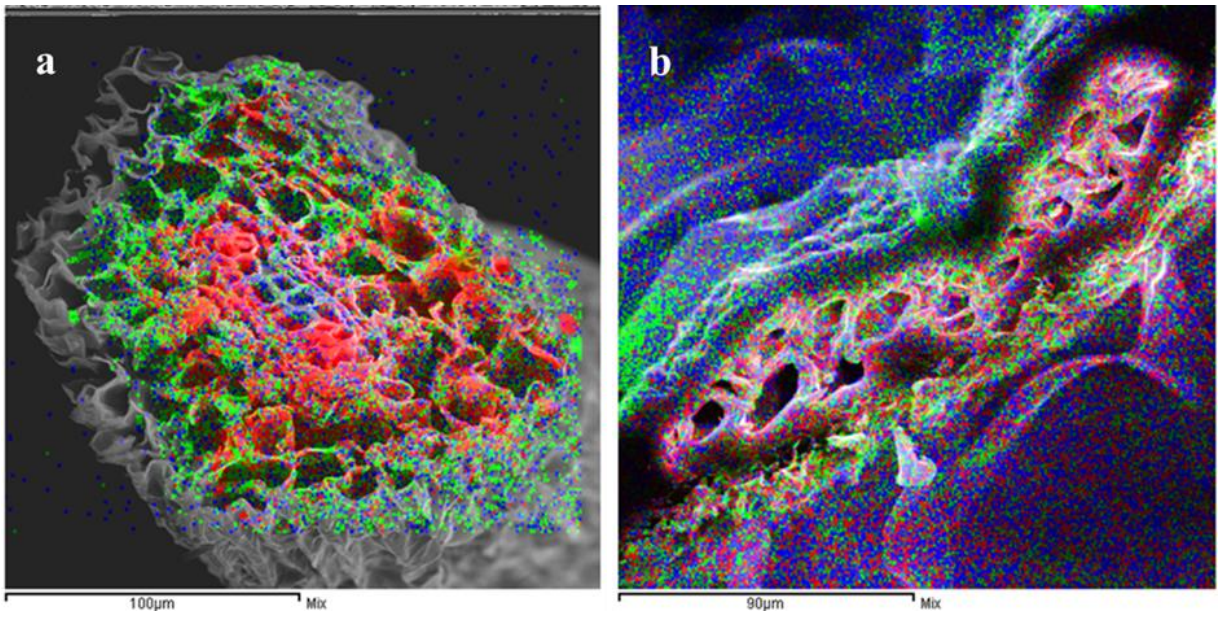


Figure 4 Localization of Cd (blue), P (red) and Ca (green) in the central cylinder of roots (a) and in the mesophyll (b) of leaves exposed to 250 µM Cd (SEM-EDX analysis).

2.5 Discussion

2.5.1 Effects of Cd and Cu on plant growth

Clear symptoms of toxicity to excess Cd and Cu were observed in plant growth (shoot and root length) and allocation of essential nutrients among plant tissues. Plants used in phytoremediation must, obviously, tolerate the contaminants at concentrations present in the contaminated environment in order to maintain physiological and metabolic processes, e.g., under excess heavy metal concentrations (Pietrini et al., 2003), through the combination of metal exclusion, damage reduction and metal absorption (Zacchini et al., 2009). The autotrophic micropropagation of clone "Villafranca", improved through the use of filter bags to facilitate gas exchange, was proved useful in screening early responses to excess Cd and Cu concentrations in poplar, thus providing information on the suitability of this clone for being used as an indicator of soil pollution in phytoremediation programmes (Borghi et al., 2008).

High Cd concentrations (50 and 250 μM) induced a reduction of leaf development (leaf area and leaf number) and visible symptoms of toxicity on various organs and tissues of seedling shoots (chlorosis, necrosis and curling of leaves reddish on the stem). Damages of leaf tissue are indicative of Cd effects, considering the importance of an efficient photosynthetic apparatus in order to maintain an effective transpiration flux that drives metals from belowground structures to the aerial parts (Zacchini et al., 2009). Di Baccio et al. (2014) found similar sensitivity to excess Cd in two poplar clones in terms of biomass production, photosynthetic activity and metal accumulation, though physiological and biochemical traits revealed different response strategies between clones; in particular, clone "Eridano" maintained the number of leaves, while the number of leaves was reduced in clone "I-214". In the same clones, excess Cd induced a reduction in stomatal conductance and photosynthetic rates, as well as changes in antioxidant capacity and ethylene evolution (Castagna et al., 2013).

Although recent studies have compared the potentiality to tolerate and accumulate heavy metals in various poplar (and salix) species, proving their good potential for phytoremediation (e.g. Dos Santos Utmazian et al., 2007), clonal comparison and genetic analysis are needed to screen for metal uptake to determine clone-specific suitability for phytoremediation (Di Baccio et al., 2011). On the contrary, at low Cd concentration (5 μM), the development of leaf area was positively affected; as well, the leaf number and shoot dry biomass exhibited a positive

correlation, highlighting positive effects of low metal concentration on plant growth and development (Wu et al., 2003).

Wu and Zhang (2002) carried out a hydroponic experiment in greenhouse to study the effect of four Cd levels in barley, during plant ontogenesis, proving that there is some potentially positive impact of Cd on plant growth at low metal concentration. Guo et al. (2004), in a hydroponic experiment using winter barley genotypes, also noticed that low Cd level had positive impact on plant growth.

Stimulation of growth found at low cadmium concentrations (5 and 50 μM) on poplar seedling could be attributed to the effect of the sulphate ion (SO_4) that accompanies the used metal ($CdSO_4$). SO4 is indeed recognized as an important ion for plant growth and vigour (Rouached, 2011) and hence could be involved in boosting growth of Villafranca clone under lower Cd stress.

Stomatal density decreased with increasing leaf area under low Cu and Cd concentrations (5 μM). Stomatal density was found to increase in leaves exposed to excess heavy metals, in other studies, including Cd and Cu (Baryla et al., 2001). Di Baccio et al. (2009) observed that, in young leaves of clone "I-214", the increase in stomatal density on adaxial and abaxial surfaces at 5 and 10 mM Zn corresponded to a decrease in leaf area, whereas in old leaves, the stomatal density on abaxial surface increased without changes in leaf area.

The increased stomatal density in conjunction with a small stomatal size (as observed in the current study) may compensate for a reduced transpiring surface area, while maintaining gas exchange (Mazid et al., 2011; Melo et al., 2007). This adaptation to toxic levels of heavy metals may preserve leaf functions under stress conditions (Di Baccio et al., 2009). Leaf transpiration is closely related to rooting absorption, and an increase in water loss through leaf transpiration can, thus, be related to an increase in the uptake of metal ions (Gomes et al., 2011). Indeed, a positive effect of treatments with excess Cd and Cu was also observed in root elongation, as evidenced by Liu et al. (2009) and Wang et al. (2007).

The length of shoots and roots decreased in 250 and 500 μM Cu treatments, which caused strong inhibition of leaf number and leaf area, as well as a reduction in shoot and root dry mass, though to a smaller extent. Leaf growth was strongly inhibited above 50 μMCd and Cu levels (e.g. Kovacs et al., 2005).

Nevertheless, root length and leaf area were significantly higher at 5 μM Cd and 5 and 50 μM Cu levels than in other treatments. In 5 and 50 μM Cu treatments, plant development was relatively stimulated at root and shoot level (Bojarczuk, 2004), with similar effects in both plant compartments at low Cd concentrations. Indeed, positive or negative impacts on shoot and root

growth, as well as morphological disorders and physiological traits, can be used to determine thresholds of metal toxicity in screening experiments. The potential of autotrophic micropropagated poplars to grow in conditions of limiting heavy metal concentrations was further validated by Ti of shoots and roots, which decreased at increasing metal concentrations.

Values of Ti were higher than 100% at low Cd and Cu levels (5 and 50 μM) (Di Lonardo et al., 2011). Generally, shoots and roots showed marked sensitivity to both metals at high concentrations.

Moreover, clone "Villafranca" showed the specific ability to accumulate Cd in shoots and Cu in roots, which points to the use of this poplar genotype as candidate indicator for Cd and as soil stabilizer for Cu (Borghi et al., 2008). It is emphasized that clone "Villafranca" had high Ti values (> 60) (e.g. Lux et al., 2004).

2.5.2 Contents of Cd, Cu and Tf

As expected, Cd and Cu contents in roots and shoots significantly increased with the increasing of metals supplied to the substrate. The concentration of Cd in shoots was higher than in roots, indicating root-to-shoot translocation in this autotrophic micropropagated poplar clone, as occurred in the same genotype but heterotrophic micropropagated (Di Lonardo et al., 2011), whereas Cu-treated seedlings showed higher concentration of Cu in roots than in shoots. Plants exhibited foliar damages (chlorosis and necrosis) at 250 and 500 μM Cu, although they did not store higher Cu contents in leaves than roots, indicating symptoms of toxicity to Cu due to decreases in chlorophyll content (Taylor and Foy, 1985).

High Cd accumulation in shoots and Cu accumulation in roots were in accordance with findings of Kacálková et al. (2009). Accordingly, although Tf declined with the increasing of Cd concentrations, values were greater than one. Generally, plant species can be labelled as accumulators of pollutants when Tf > 1 (Fitz and Wenzel, 2002; Rizzi et al., 2004).

In contrast, most of Cu was immobilized in the roots, without significant variation in Tf. While Cu can be bound to the root surface or adsorbed in the apoplast, where its effects are less detrimental, increasing levels of this metal in root tissues of poplar seedlings were found to reduce root biomass and change root morphology (Arduini et al., 1995; Borghi et al., 2008).

Two basic strategies of plant responses to heavy metals were proposed by Baker (1981). Plants may act as "excluders" detoxifying metals in the roots, whereas "accumulators" transport

metal ions to the shoot, where they can be stored in the vacuoles of leaf cells. Our results showed that autotrophic micropropagated poplars showed translocation capacity for Cd, while this was not the case for Cu (Table 5), suggesting that clone "Villafranca" can be considered Cd accumulator (cf., Jiali et al., 2013) and Cu excluder (cf., Borghi et al., 2008).

Nevertheless, selective substrate-specific uptake and defined metal-induced responses may occur because of interference with other ions (e.g. forming precipitates with P), which warrants the application of multiple investigation methods (Cocozza et al., 2008).

2.5.3 Essential elements

Nutrient contents were significantly affected by metal treatments (Pulford and Watson, 2003), Cd and Cu interfering with the uptake of, especially, Ca, Fe, K, Mg, Mn, P, S and Zn. Indeed, the absorption process of Cd may compete with transmembrane carriage of nutrients, such as K, Ca, Mg, Fe, Mn, Cu, Zn and Ni (Clarkson and Lüttge, 1989; Rivetta et al., 1997), affecting physiological and biochemical processes (Nazar et al., 2012). The absorption of Ca, K, Mg, Mn, P, S and Zn, relatively high in shoots at 5 μM Cd, was inhibited at greater Cd concentrations, except for Ca, Fe and Zn. These correlations may underline a mechanism involved in maintaining ion homeostasis under Cd stress, rather than being an effect of the competition between elements (Przedpełska Wąsowicz et al., 2012).

Results of distribution of elements within portions of leaf tissues, obtained by microanalysis, showed that P and Cd were mainly located in the central cylinder of roots and in the mesophyll of leaves, suggesting phosphate sequestration, as previously found by Van Belleghem et al. (2007).

Furthermore, Cocozza et al. (2011) observed a relationship between Cd and Ca distribution, showing that the accumulation of Cd did not interfere with the absorption of this macronutrient. Indeed, the uptake of Cd ions occurs through the transmembrane carriers used for the uptake of Ca divalent cations (Dal Corso et al., 2008). Correlations between element concentration in leaves and metal availability in soils can be used to monitor trace elements in contaminated areas (Madejón et al., 2004). The excess of Cu in the nutrient solution caused a deficiency of essential ions (i.e. K, Mg, Mn and P) at root level, with visible symptoms of toxicity (Table 6).

The inhibition of Mg uptake may explain the chlorosis symptoms, considering the role of this element as an essential cofactor of the polypeptide enzymes in PS-I and PS-II (Farhat et al. 2015). Interestingly, the values of Mg and Mn were higher at low Cd level (5 μM) than in

control plants. Additionally, the tissue concentrations of Ca, Fe, S and Zn were generally enhanced by high Cu concentration in the culture solution. In other experiments, poplar clones were found rather tolerant to Cu excess in hydroponics (Borghi et al., 2007), although the metal is readily available to plants.

2.6 Conclusions

In vitro screening of the poplar clone "Villafranca" was proved a suitable preliminary tool to quickly test for Cd and Cu tolerance, translocation and accumulation, in axenic conditions, as metals are readily available to the plant, reducing the growth and the treatment intervals, as well as the space required for trials. The present study highlighted the suitability of "Villafranca" to be micropropagated in autotrophic culture system, allowing to set the threshold values for Cd and Cu toxicity and to investigate its candidacy for bioremediation for sites contaminated with heavy metals. Clone "Villafranca" showed a remarkable ability to translocate Cd in shoots and to accumulate Cu in roots, which was associated with compartment-specific metal tolerance. Clone "Villafranca" proved to be promising in the phytostabilization of Cu contaminated sites and in being used as early bioindicator of exposure to Cd pollution.

The present study reports, for the first time, on plant tissue cultures under autotrophic conditions in filter bags. Filter bags, in contrast to conventional *in vitro* screening, have exceptionally efficient gas exchange capacity, complete barrier against pests and contaminations and enhance the survival capacity of the micropropagation culture, which may explain the different outcomes of this study in comparison with experiments using other media, in terms of Cd and Cu allocation in plant tissues.

Results obtained with *in vitro* autotrophy need to be confirmed in field conditions, though the high potential for testing plant adaptation to stress conditions in short time and to focus investigation on specific plant response mechanisms makes this screening system promising.

Appendix

Supplementary Table

Table S1.

Cd and Cu determined by SEM-EDX in leaf (S1a, S1b) and root (S1c, S1d, S1e) tissues of control and treated plants of clone "Villafranca" (values are reported in % weight, means ± standard errors). Mean values (± standard error) marked with the same letter are not statistically different (LSD test, $p \leq 0.05$). Lowercase letters refer to the comparison between portions of cross-section within each metal treatment (S1a vs S1b; S1c vs S1d vs S1e); capital letters refer to the comparison between treatments within each portion of cross-section (S1a, S1b, S1c, S1d, S1e). Two-way ANOVA was performed for heavy metals and nutrients between cross-sections; one-way ANOVA was applied for heavy metals and nutrients in each cross section (S1f).

Leaf									
	MESOPHYLL								
elements (mg kg $^{-1}$)	Cd treatment				Cu treatment				
	Control	5 µM	50 µM	250 µM	Control	5 µM	50 µM	250 µM	500µM
Ca	13.31±9.80^{aA}	6.88±0.97^{aA}	7.16±1.01^{aA}	4.65±1.22^{aA}	13.31±9.80^{aA}	-	4.96±0.80^{aA}	0.98±0.33^{aA}	3.77±0.41^{aA}
Cu	1.33±0.93^{aA}	0.81±0.67^{aA}	0.58±0.10^{aA}	0.94±0.18^{aA}	1.33±0.93^{aA}	-	1.18±0.21^{aA}	0.35±0.49^{aAB}	0.35±0.10^{aB}
Fe	0.25±0.04^{aA}	0.27±0.16^{aA}	0.20±0.06^{aA}	0.30±0.12^{aA}	0.25±0.04^{aA}	-	0.36±0.12^{aA}	0.14±0.20^{aA}	0.36±0.20^{aA}
K	0.17±0.00^{aA}	0.26±0.05^{aA}	0.22±0.07^{aA}	0.21±0.11^{aA}	0.17±0.00^{aA}	-	0.27±0.13^{aA}	0.43±0.28^{aA}	0.40±0.04^{aA}
Mg	0.00±0.00^{aA}	1.10±0.17^{aA}	1.00±0.28^{aA}	0.83±0.26^{aA}	0.00±0.00^{aA}	-	0.26±0.12^{aA}	0.13±0.18^{aA}	0.54±0.07^{aB}
Mn	0.08±0.11^{aA}	0.00±0.00^{aA}	0.10±0.03^{aA}	0.18±0.08^{aA}	0.08±0.11^{aA}	-	0.14±0.10^{aA}	-	0.10±0.05^{aA}
Na	0.14±0.20^{aA}	9.50±0.98^{aB}	11.76±1.33^{aB}	9.67±1.81^{aB}	0.14±0.20^{bA}	-	8.85±0.90^{bB}	18.71±0.83^{bC}	14.06±0.46^{bD}
P	0.77±0.66^{aA}	3.35±0.44^{aA}	4.76±1.31^{aA}	5.27±0.91^{aA}	0.77±0.66^{aA}	-	3.32±0.76^{aB}	7.22±0.58^{aC}	9.55±0.80^{aC}
S	1.11±0.97^{aA}	2.52±0.25^{bA}	2.09±0.34^{bA}	2.07±0.28^{bA}	1.11±0.97^{aA}	-	6.11±1.64^{aB}	3.08±0.03^{aAB}	0.46±0.07^{aA}
Zn	0.08±0.11^{aA}	0.46±0.29^{aA}	0.14±0.09^{bA}	0.38±0.18^{aA}	0.08±0.11^{aA}	-	0.39±0.15^{aA}	0.15±0.00^{aA}	0.05±0.06^{aA}
Cd	0.00±0.00^{aA}	0.10±0.12^{aA}	0.14±0.07^{aA}	0.76±0.44^{aA}	-	-	-	-	-

Table S1a Cd and Cu determined by SEM-EDX in leaf at mesophyll level

Leaf

elements (mg kg $^{-1}$)	EPITHELIUM								
	Cd treatment				Cu treatment				
	Control	5 µM	50 µM	250 µM	Control	5 µM	50 µM	250 µM	500µM
Ca	0.87±0.32^{bA}	4.33±0.28^{bB}	3.35±0.28^{bC}	2.71±0.24^{bC}	0.87±0.32^{bA}	3.69±0.35^{bB}	4.61±0.35^{bB}	1.10±0.09^{bA}	2.21±0.71^{bA}
Cu	0.20±0.06^{aA}	0.65±0.07^{aB}	0.79±0.08^{aBC}	0.95±0.11^{aC}	0.20±0.06^{aA}	1.05±0.12^{aB}	0.73±0.08^{aC}	0.84±0.09^{aBC}	1.14±0.16^{aB}
Fe	0.05±0.02^{aA}	0.31±0.04^{aB}	0.16±0.03^{aA}	0.17±0.04^{aA}	0.05±0.02^{aA}	0.31±0.07^{aB}	0.20±0.05^{aA}	0.20±0.03^{aA}	0.38±0.09^{aB}
K	0.05±0.01^{aA}	0.23±0.03^{aB}	0.14±0.02^{aA}	0.33±0.03^{aC}	0.05±0.01^{aA}	0.51±0.06^{aB}	0.26±0.03^{aC}	0.56±0.06^{aD}	0.44±0.10^{aE}
Mg	0.06±0.02^{aA}	0.94±0.08^{aB}	0.93±0.08^{aC}	0.48±0.04^{aD}	0.06±0.02^{aA}	0.95±0.08^{aB}	0.48±0.06^{aC}	0.32±0.04^{aD}	0.37±0.06^{aC}
Mn	0.03±0.01^{aA}	0.14±0.04^{aB}	0.05±0.01^{aA}	0.15±0.03^{aB}	0.03±0.01^{aA}	0.19±0.04^{aA}	0.15±0.03^{aA}	-	0.03±0.02^{aA}
Na	0.71±0.12^{aA}	10.28±0.46^{aB}	9.62±0.86^{aBC}	12.32±0.44^{aD}	0.71±0.12^{aA}	11.11±0.66^{aB}	9.92±0.57^{aB}	16.13±0.43^{aC}	15.48±1.06^{aC}
P	0.22±0.03^{aA}	1.66±0.14^{aBC}	2.47±0.19^{aC}	6.63±0.44^{aD}	0.22±0.03^{bA}	3.82±0.21^{bB}	2.46±0.16^{bC}	7.33±0.33^{bD}	5.26±0.66^{bE}
S	0.40±0.03^{bA}	3.19±0.23^{aB}	3.26±0.31^{aB}	3.15±0.29^{aB}	0.40±0.03^{bA}	4.34±0.31^{bB}	3.26±0.23^{bC}	2.59±0.14^{bD}	1.81±0.26^{bC}
Zn	0.02±0.01^{bA}	0.16±0.04^{bA}	0.16±0.04^{aA}	0.17±0.05^{bA}	0.02±0.01^{aA}	0.21±0.08^{aB}	0.21±0.05^{aB}	0.15±0.03^{aAB}	0.00±0.00^{aA}
Cd	0.00±0.00^{aA}	0.14±0.05^{aA}	0.12±0.04^{aA}	0.34±0.08^{aB}	-	-	-	-	-

Table S1b Cd and Cu determined by SEM-EDX in leaf at epithelium level

Root									
					EPITHELIUM				
elements mg kg^{-1}	Cd treatment				Cu treatment				
	Control	5 µM	50 µM	250 µM	Control	5 µM	50 µM	250 µM	500µM
Ca	2.59±0.54^{aA}	2.68±0.28^{aA}	2.19±0.21^{aA}	2.73±0.16^{aA}	2.59±0.54a^{AB}	2.44±0.17^{aA}	2.52±0.30^{aA}	3.53±0.45^{aB}	1.98±0.23^{aA}
Cu	1.09±0.19^{aA}	0.77±0.10^{aB}	0.65±0.12^{aB}	0.65±0.05^{aB}	1.09±0.19^{aA}	0.72±0.07^{aA}	9.58±0.05^{aA}	6.34±1.46^{aB}	2.24±0.58^{aA}
Fe	0.33±0.10^{aA}	0.60±0.16^{aA}	1.86±0.29^{aB}	3.03±0.32^{aC}	0.33±0.10^{bA}	4.27±0.38^{bB}	3.90±0.80^{bB}	1.58±0.30^{bA}	2.63±1.03^{bB}
K	0.31±0.07^{aA}	0.50±0.07^{aA}	0.35±0.09^{aA}	0.34±0.03^{aA}	0.31±0.07^{aA}	0.75±0.05^{aB}	0.89±0.17^{aB}	0.48±0.06^{aA}	1.73±0.28^{aC}
Mg	0.54±0.16^{aA}	0.57±0.08^{aA}	0.56±0.10^{aA}	0.78±0.06^{aA}	0.54±0.16^{aA}	0.80±0.07^{aAB}	0.71±0.08^{aA}	0.99±0.09^{aB}	0.77±0.06^{aAB}
Mn	0.16±0.06^{aA}	0.00±0.00^{aA}	0.19±0.18^{aA}	0.01±0.01^{aA}	0.16±0.06^{aA}	0.15±0.02^{aA}	0.16±0.02^{aA}	1.28±0.57^{aA}	0.15±0.00^{aA}
Na	7.69±0.36^{aA}	9.62±0.95^{aA}	8.47±0.93^{aA}	8.72±0.74^{aA}	7.69±0.36^{aAB}	7.77±0.20^{aAB}	8.40±0.91^{aA}	6.22±0.36^{aB}	7.57±0.29^{aA}
P	1.68±0.14^{aA}	5.63±0.70^{aB}	3.54±0.21^{aC}	6.60±0.40^{aB}	1.68±0.14^{bA}	6.60±0.40^{bB}	6.42±0.44^{bB}	3.79±0.37^{bC}	4.90±1.04^{bC}
S	1.71±0.32^{aA}	1.28±0.13^{aA}	1.61±0.12^{aA}	1.49±0.10^{aA}	1.71±0.32^{aA}	1.22±0.11^{aA}	1.34±0.12^{aA}	2.78±0.34^{aB}	1.64±0.16^{aA}
Zn	0.22±0.09^{aA}	0.06±0.03^{aA}	0.05±0.06^{aA}	0.17±0.04^{aA}	0.22±0.09^{aA}	0.17±0.05^{aA}	0.21±0.04^{aA}	0.24±0.05^{aA}	0.37±0.09^{aA}
Cd	0.00±0.00^{aA}	0.05±0.04^{aA}	0.22±0.07^{aA}	2.38±0.19^{aB}	-	-	-	-	-

Table S1c Cd and Cu determined by SEM-EDX in root at epithelium level

Root									
	CORTEX								
elements (mg kg $^{-1}$)	Cd treatment				Cu treatment				
	Control	5 µM	50 µM	250 µM	Control	5 µM	50 µM	250 µM	500µM
Ca	2.18±0.19^{aA}	1.78±0.35^{aA}	2.40±0.15^{aA}	3.28±0.41^{aB}	2.18±0.19^{aA}	8.75±4.79^{aB}	1.74±0.30^{aA}	2.16±1.00^{aA}	4.09±0.68^{aA}
Cu	0.84±0.32^{aA}	0.53±0.11^{aA}	0.60±0.21^{aA}	0.57±0.13^{aA}	0.84±0.32^{aA}	1.00±0.50^{aA}	0.55±0.15^{aA}	6.75±3.99^{aB}	1.74±0.66^{aAB}
Fe	0.89±0.37^{aA}	0.68±0.27^{aA}	1.54±0.56^{aA}	2.17±0.52^{aA}	0.89±0.37^{aA}	0.34±0.15^{aA}	1.79±0.95^{aA}	0.97±0.66^{aA}	0.96±0.40^{aA}
K	0.28±0.09^{aA}	0.18±0.04^{aA}	0.45±0.07^{aA}	0.65±0.13^{aB}	0.28±0.09^{abA}	0.58±0.18^{abA}	0.45±0.11^{abA}	0.50±0.21^{abA}	0.37±0.18^{abA}
Mg	1.05±0.21^{aA}	0.45±0.16^{aA}	0.63±0.15^{aA}	1.56±0.83^{aA}	1.05±0.21^{abA}	0.62±0.08^{abA}	0.40±0.13^{abA}	0.94±0.30^{abAB}	1.64±0.70^{abB}
Mn	0.02±0.02^{aA}	0.00±0.00^{aA}	0.86±0.45^{aB}	0.00±0.00^{aA}	0.02±0.02^{aA}	0.15±0.00^{aB}	0.15±0.00^{aB}	0.15±0.00^{aB}	0.14±0.06^{aB}
Na	9.28±0.99^{bAB}	7.79±0.82^{bA}	13.16±1.66^{bB}	12.05±1.20^{bB}	9.28±0.99^{abA}	9.07±2.13^{abA}	10.18±1.42^{abA}	8.41±0.20^{abA}	7.07±0.87^{abA}
P	2.33±0.27^{aA}	3.38±0.43^{aAB}	4.67±0.44^{aB}	7.22±0.94^{aC}	2.33±0.27^{aA}	2.51±0.10^{aA}	4.69±0.32^{aB}	3.59±1.40^{aAB}	4.74±0.70^{aB}
S	1.60±0.17^{aA}	0.81±0.13^{aB}	1.76±0.20^{aA}	1.67±0.27^{aA}	1.60±0.17^{aA}	0.78±0.11^{aB}	1.35±0.18^{aA}	3.64±0.75^{aC}	1.88±0.32^{aA}
Zn	0.09±0.05^{aA}	0.10±0.06^{aA}	0.00±0.00^{aA}	0.15±0.08^{aA}	0.09±0.05bA	0.15±0.00^{bA}	0.15±0.00^{bA}	0.15±0.00^{bA}	0.19±0.09^{bA}
Cd	0.00±0.00^{aA}	0.14±0.11^{aA}	0.30±0.10aA	2.55±0.34aB	-	-	-	-	-

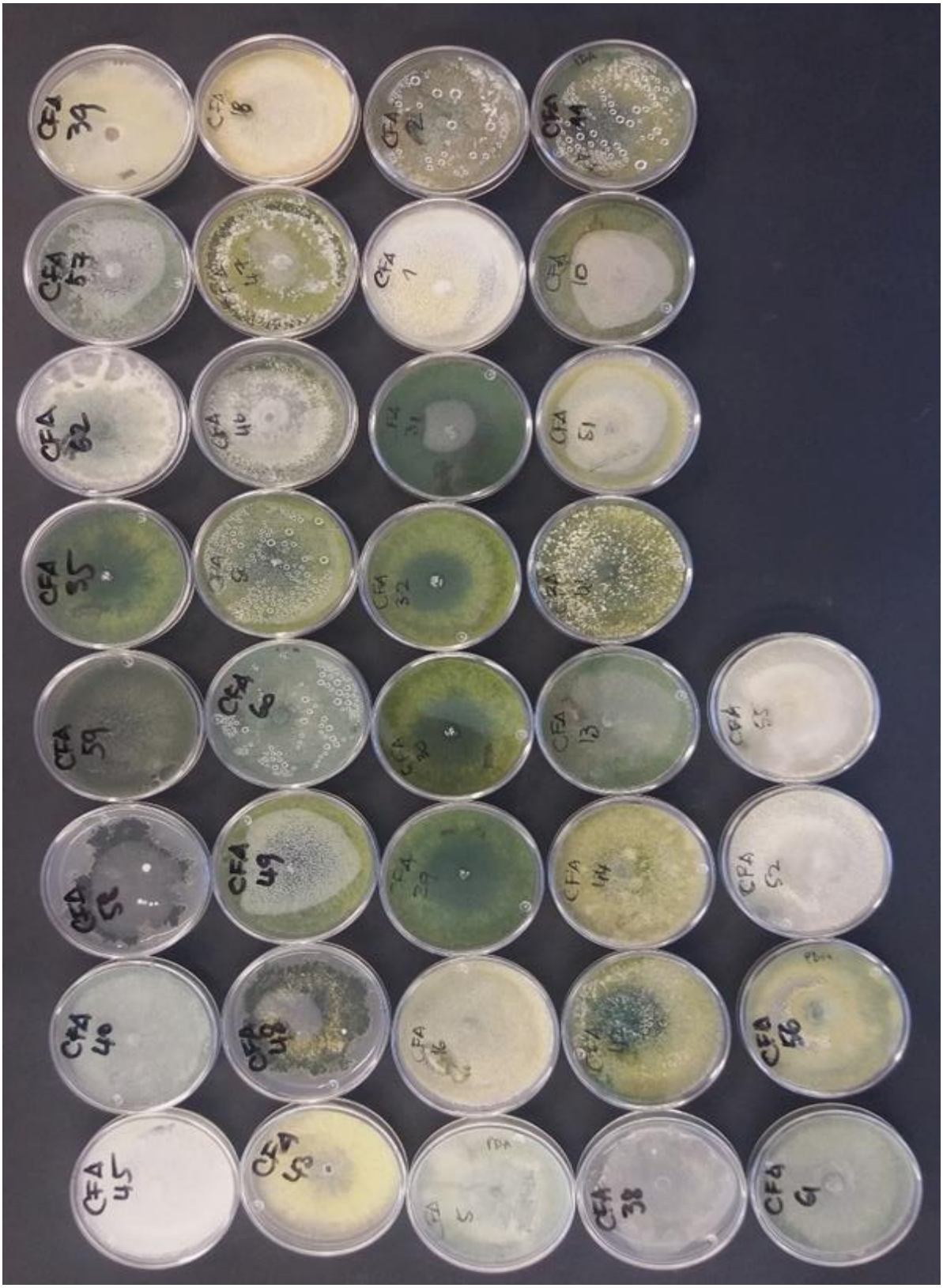
Table S1d Cd and Cu determined by SEM-EDX in root at cortex level

Root

elements (mg kg $^{-1}$)	CENTRAL CYLINDER								
	Cd treatment				Cu treatment				
	Control	5 µM	50 µM	250 µM	Control	5 µM	50 µM	250 µM	500µM
Ca	2.73±0.35^{aA}	2.96±1.53^{aA}	2.02±0.20^{aA}	2.68±0.35^{aA}	2.73±0.35^{aAB}	4.23±0.34^{aA}	2.22±0.75^{aB}	1.81±0.13^{aB}	3.56±0.88^{aA}
Cu	0.60±0.11^{aA}	1.16±0.84^{aA}	0.60±0.18^{aA}	0.58±0.06^{aA}	0.60±0.11^{aA}	0.85±0.16^{aA}	0.87±0.37^{aA}	6.48±0.42^{aB}	2.55±0.49^{aC}
Fe	0.66±0.14^{aA}	0.38±0.53^{aA}	1.19±0.27^{aA}	1.85±0.90^{aA}	0.66±0.14^{aA}	3.11±3.71^{aA}	0.38±0.25^{aA}	0.88±0.16^{aA}	0.61±0.15^{aA}
K	0.30±0.09^{aA}	0.38±0.18^{aA}	0.30±0.05^{aA}	0.41±0.03^{aA}	0.30±0.09^{bA}	0.72±0.35^{bA}	0.68±0.18^{bA}	0.49±0.09^{bA}	0.43±0.10^{bA}
Mg	0.90±0.31^{aA}	0.24±0.34^{aA}	0.46±0.15^{aA}	0.55±0.17^{aA}	0.90±0.31^{bA}	0.79±0.25^{bA}	0.16±0.0^{bA}	1.10±0.11^{bB}	3.15±0.60^{bC}
Mn	0.15±0.08^{aAB}	0.00±0.00^{aAB}	0.30±0.10^{aA}	0.00±0.00^{aB}	0.15±0.08^{aA}	0.15±0.00^{aA}	0.15±0.00^{aA}	0.15±0.00^{aA}	0.25±0.05^{aA}
Na	9.07±0.70^{bA}	8.99±0.40^{bA}	11.63±1.09^{bA}	12.14±2.10^{bA}	9.07±0.70^{bA}	8.02±1.12^{bA}	12.36±1.26^{bB}	9.51±0.69^{bA}	9.70±0.61^{bA}
P	3.21±0.74^{aA}	2.99±0.77^{aA}	3.63±0.22^{aA}	7.15±1.00^{aB}	3.21±0.74^{aA}	5.83±3.04^{aA}	6.67±1.18^{aA}	4.03±0.28^{aA}	5.70±0.57^{aA}
S	1.31±0.19^{aA}	1.09±0.51^{aA}	2.05±0.20^{aB}	1.77±0.16^{aAB}	1.31±0.19^{aAB}	0.89±0.19^{aA}	1.44±0.16^{aAB}	2.52±0.36^{aB}	1.91±0.14^{aB}
Zn	0.05±0.05^{aA}	0.22±0.31^{aA}	0.06±0.06^{aA}	0.09±0.05^{aA}	0.05±0.05^{abA}	0.10±0.06^{abA}	0.10±0.06^{abA}	0.15±0.00^{abA}	0.31±0.20^{abA}
Cd	0.00±0.00^{aA}	0.00±0.00^{aA}	0.43±0.16^{aA}	3.22±0.43^{aB}	-	-	-	-	-

Table S1e Cd and Cu determined by SEM-EDX in central cylinder level

CHAPTER III – *Trichoderma* experiment



3. *Trichoderma* strains tolerance, accumulation, chelation, phosphorous solubilisation activity and rhizosphere competence under Cd stress

3.1 Abstract

The present research activity has included in vitro assessments aimed to investigating the mechanisms of Cd uptake-boosting by several *Trichoderma* species. Fifty-two *Trichoderma* strains were initially screened for their tolerance to a range of Cd concentrations (0, 5, 50, 250 µM). Based on fungal growth rate under metal stress, twenty-seven cadmium tolerant strains were selected. Fungi belonged to at least 6 different *Trichoderma* species (namely *T. atroviride, T. citrinoviride, T. koningii, T. hamatum, T. harzianum, and T. polysporum*)

Further studies were focused on the selected strains with regard to their capability of Cd compartimentalization inside fungal hyphae and the capability to produce metal binding compounds (siderophores) and thus solubilize sparingly soluble complexes that Cd may form with phosphates. The screening of Cd tolerance of the *Trichoderma* strains was based on the evaluation of hyphal growth rate in Petri dishes containing an agar medium (PDA) contaminated with three doses of Cd for identification of tolerant strains and one further test at a single high dose of Cd (250 µM) as confirmatory test on the candidate tolerant strains previously selected. For this purpose, the growth of each fungal colony was recorded daily and compared with control plates not containing Cd (0 µM Cd). The chelating activity of *Trichoderma* culture filtrates was determined by a method based on measurement of the equilibrium concentration of the chrome azurol S complex in the presence of other chelating substances while the ability of isolates to solubilize phosphate under Cd stress was estimated using Fiske and Subbarow method (1925).

Investigations on the mechanism of Cd-tolerance, showed that this was not univocal, but at least two different tolerance strategies could be distinguished as following: *i*) exclusion of the toxicant and, *ii)* metal intracellular compartmentalization and/or enzymatic detoxification.

Strains that used this latter strategy may find a possible application as bioaccumulators intended for bioremediation of wastewater. Nevertheless, the behavior of these strains also need to be studied at the rhizosphere level, with regard to a possible translocation of the uptaken Cd at the interface of the plant-*Trichoderma* symbiosis.

3.2 Introduction

The global environmental pollution is one of the most concerning issues in the public debate about human health and sustainable development (UNEP, 2017). Soil pollution is a growing problem, mostly due to human activities such as mining, agriculture and industrial activities or to inadequate management of waste and sewage materials that result in the accumulation of toxicants in agricultural soils and diffusion to fresh and sea waters (FAO, 2017). Trace (heavy) metals (HMs), such as arsenic, cadmium (Cd), lead and mercury, are among the most diffused soil toxicants worldwide. In biological systems, metal cations play an important role as co-factors in biochemical reactions.

However, at high concentrations HMs ions form unspecific complex compounds that have strong cytotoxic effects. Excess levels of trace metals in agricultural soils can impair plant metabolism and severely affect crop productivity, ultimately putting pressure on arable land (UNEP, 2017). HMs that enter the food chain pose risks to consumers, water resources and rural livelihoods, with serious consequences for human health (Järup, 2003).

Cd is the best-known among the toxic HMs because of its implication in the bone softening and kidney impairing “Itai-Itai disease” (Yukimasa, 1975). It is more soluble, and thus more bioavailable and subject to bioaccumulation than zinc (Zn) and most of the other trace metals (Pinto et al., 2004). The cytotoxicity of Cd is due to thiol-binding and protein denaturation properties; it also interferes with calcium and Zn cell metabolism. According to the United States Environmental Protection Agency (U.S. EPA), Cd is a probable human carcinogen. The chronic exposure to Cd results in kidney dysfunction and at high levels it may cause death of exposed subjects (Fu and Wang, 2011). The human maximum intake of Cd recommended by the World Health Organisation (WHO) is 0.4–0.5 mg per week, while U.S. EPA set the maximum admissible concentration of Cd in drinking water at 0.005 mg/L (Huang et al., 2013).

In most cases, a Cd content over 0.5 mg/Kg (or ppm) in agricultural soils is of anthropogenic origin. It is poorly degradable in nature and has a half-life of ten to thirty years (Jan et al., 1999). In plants, it is taken up by the calcium uptake system (Clemens et al., 1998). Excess Cd in plants causes necrosis, leaf chlorosis, leaf roll, reduction in plant growth, damage of photosynthetic machinery specially PS-I and PS-II (photosystem) and reduction in chlorophyll synthesis. The plant uptake and transport of mineral nutrients is also affected (Foy et al., 1978).

The need to remediate polluted soils has led to the development of biology-based technologies, which are the most durable options for amendment of altered soils and restoration of natural aedaphic conditions (Shukla et al., 2010). These technologies are collectively known

as bioremediation and include the use of plants (phytoremediation), bacteria (bacterial bioremediation) and fungi (mycoremediation). The interest in mycoremediation is increasing, due to recent successful applications in detoxification of diverse environments and remediation of soils contaminated with organic and inorganic pollutants (Bhattacharya et al., 2014; Babu et al., 2014a). Fungi are able to tolerate and remove a wide variety of metals from the environment by several biological mechanisms, including biodegradation, biosorption, bioaccumulation and bioconversion (Kulshreshtha et al., 2014, Akinyele et al., 2012). These biological mechanisms allow fungi to adsorb pollutant in mycelium (biosorbition and bioaccumulation processes), either by transformation into less toxic forms (bioconversion) that are utilizable for their metabolic processes, or to degrade (biodegradation) complex and recalcitrant organic compounds by the release of highly efficient enzymes (Kapahi and Sachdeva, 2017). In the case of HMs, fungal species adopt one or more metal tolerance strategies that include extracellular metal sequestration and precipitation, suppressed influx, enhanced metal efflux, production of intracellular/extracellular enzymes, metal binding to cell walls, intracellular sequestration and complexation (Oladipo et al., 2017).

Trichoderma spp. are opportunistic, ubiquitous, antagonistic and avirulent plant symbionts which exhibit remarkable metabolic flexibility and colonization ability, fast grow and high resistance to several xenobiotic compounds such as antibiotics, fungicides and pollutants (Harman et al., 2004a, 2004b; Lynch and Moffat, 2005). Although tolerance of these filamentous fungi to different heavy metals, including Cd, has been reported (Anad et al., 2006; Sahu et al., 2012; Prasad et al., 2013; Feng et al., 2015; Oladipo et al., 2017), the mechanism of Cd tolerance of *Trichoderma* sp. has been sparingly investigated at biochemical and molecular levels. In general, the tolerance of *Trichoderma* to Cd has been associated to the capability of these fungi to reduce the bioavailability of metals in the surrounding environment by an adsorption process based on binding of metal cations to components of the fungus cell walls in a temperature-, pH- and concentration-dependant manner (Mohsenzadeh and Shahrokhi, 2014; Hlihor et al., 2015). López Errasquín and Vázquez (2003) isolated from wastewater sludge a strain of *T. atroviride* tolerant to high concentrations of copper (Cu), zinc (Zn) and Cd. These authors observed that the highest efficiency of heavy metals removal occurred in a poor growth medium (saline solution), in which the biomass was greatly reduced and the mycelium was subjected to partial autolysis. They concluded that the removal was due to physical binding to cell wall surfaces, enhanced in the lysized cells by the increase of wall surface exposition and also by the exposure of intercellular binding sites, rather than to an active process. Biosorption was also assumed by Hlihor et al. (2015) to be the mechanism of removal of Cd from a batch solution by dead and living biomass of *T. viride*. Nongmaithem et al. (2016)

carried out a screening of fourteen isolates of *Trichoderma* spp. and identified three isolates that showed medium to high levels of tolerance to Cd (MIC_{50} for biomass production ranging from 57.41 to 227.92 ppm).

The uptake of Cd by *Trichoderma* mycelia was measured by the depletion of Cd from the culture broth and no further experiment was carried out to investigate the mechanism of removal, but biosorption was postulated to be the mechanism involved (Nongmaithem et al., 2016). However, evidences have been reported that support the existence of mechanisms of Cd-tolerance in *Trichoderma* other than adsorbtion on the cell wall. For instance, exposure to Cd and other HM is known to induce increased production of reactive oxygen species (ROS) (Miller et al., 2010). *Trichoderma* spp. have been shown to be able to mitigate the cell oxidative damage by means of an array of highly efficient enzymes such glutathione transferases (GSTs) which scavenge ROS (Mastouri et al., 2010). A GTS gene from *T. virens* was heterougously expressed in tobacco plants by Dixit and co-workers (2011). The genetically modified plants showed higher levels of GTS and enhanced Cd tolerance, along with lower lipid peroxidation and higher levels of antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, guaiacol peroxidase and catalase.

Trichoderma species are utilized world-wide as biocontrol agents of plant pathogens and as plant biostimulants (Woo et al., 2014). They have been also utilized in association with plants to enhance phytoremediation of Cd- polluted soils (Teng et al., 2015).

While in the latter case the capability of *Trichoderma* to enhance the Cd uptake by plants is a desirable feature, it is apparent that this is not the case if *Trichoderma* are used as biocontrol agents for agricultural crops in soils with high levels of Cd, where the plant uptake should be limited as much as possible. It is, therefore, important to gain more knowledge about the physical and the biochemical processes that drive Cd tolerance and sequestration / compartimentalization of Cd in *Trichoderma*. In the research work herein presented we investigated the occurrence of Cd tolerance in *Trichoderma* spp. and the relevant mechanisms. The Cd tolerance of different species and strains, including *T. atroviride*, *T. citrinoviride*, *T. hamatum*, *T. harzianum* and *T. polysporum*, was studied under high Cd concentration pressure to assess the capability of strains to grow and detoxify the surrounding environment by reducing Cd bioavailability. This experimental activity was intended as a preparatory step in the study of the *Trichoderma* /plant association for phytoremediation of Cd-polluted soils. The research work about *Trichoderma* /plant association is indeed presented in chapter 4 of this thesis.

3.3 Materials and Methods

3.3.1 Selection of cadmium-tolerant strains of *Trichoderma* spp.

3.3.1.1 *Trichoderma* isolates.

Fifty-two *Trichoderma* strains belonging to different species and of various origins were used in this study (Table 7). All the isolates derived from single-spore isolations and were morphologically identified according to Gams and Bisset (1998). The fungal isolates were stored at + 4 °C on potato-dextrose-agar (PDA) slants and used as inocula for sub-cultures throughout the work.

3.3.1.2 Selection of cadmium-tolerant strains of *Trichoderma* spp. by fast Cd screening

Fifty-two *Trichoderma* spp. isolates strains were evaluated for their *in vitro* tolerance to cadmium (Cd) stress at different concentrations. The fungal strains were obtained from the culture collection of the Institute of Sciences of Food Production, National Research Council, Bari (ISPA). Cadmium tolerance of the strains was determined by the radial growth-rate of colonies exposed to Cd, in comparison with Control colonies. Appropriate volumes of a 5 mM $CdSO_4$ stock solutions were added to PDA to get the required Cd concentrations (0, 5, 50 and 250 µM). The plates were inoculated with 6-mm mycelial disks from 3-day-old cultures of *Trichoderma* and incubated at 25 ± 1°C for 3 days.

The colony growth was assessed every 24 h for 3 subsequent days by the average value of two orthogonal diameters and the percent inhibition (I %) with respect to Control was calculated (Table. 13).

The percent growth inhibition (I%) of fungal colonies due to the Cd exposure was calculated utilizing the following equation:

$$I\ (\%) = \frac{(Dc-Dt)}{Dc} * 100$$

where, I(%) = fungal growth inhibition , Dc = diameter of fungal colony in control plates ,Dt = diameter of the fungal colony exposed to Cd,

Based on values of I (%), several tolerant *Trichoderma* strains whose growth was not significantly inhibited by high levels of Cd were identified. The selection of the colonies

showing good tolerance to Cd was carried out based on the inhibition rate (I%) after 48 h of growth.Thus, twenty-seven isolates were selected for further studies (Table 8).

ITEM/ CFA	GENUS	SPECIES	ITEM/ CFA	GENUS	SPECIES
29	*Trichoderma*	*atroviride*	**55**	*Trichoderma*	*harzianum*
30	*Trichoderma*	*atroviride*	**56**	*Trichoderma*	*harzianum*
31	*Trichoderma*	*atroviride*	**61**	*Trichoderma*	*harzianum*
35	*Trichoderma*	*atroviride*	**2683**	*Trichoderma*	*harzianum*
32	*Trichoderma*	*atroviride*	**1412**	*Trichoderma*	*harzianum*
38	*Trichoderma*	*atroviride*	**7049**	*Trichoderma*	*harzianum*
39	*Trichoderma*	*atroviride*	**1323**	*Trichoderma*	*harzianum*
43	*Trichoderma*	*atroviride*	**1411**	*Trichoderma*	*harzianum*
48	*Trichoderma*	*atroviride*	**4482**	*Trichoderma*	*harzianum*
49	*Trichoderma*	*atroviride*	**7054**	*Trichoderma*	*harzianum*
50	*Trichoderma*	*atroviride*	**4483**	*Trichoderma*	*harzianum*
33	*Trichoderma*	*citrinoviride*	**7050**	*Trichoderma*	*harzianum*
53	*Trichoderma*	*citrinoviride*	**908-5**	*Trichoderma*	*harzianum*
54	*Trichoderma*	*citrinoviride*	**1324**	*Trichoderma*	*harzianum*
13	*Trichoderma*	*hamatum*	**7053**	*Trichoderma*	*harzianum*
40	*Trichoderma*	*hamatum*	**908-WT**	*Trichoderma*	*harzianum*
1	*Trichoderma*	*harzianum*	**5**	*Trichoderma*	*koningii*
2	*Trichoderma*	*harzianum*	**57**	*Trichoderma*	*koningii*
10	*Trichoderma*	*harzianum*	**60**	*Trichoderma*	*polysporum*
11	*Trichoderma*	*harzianum*	**18**	*Trichoderma*	*spp.*
16	*Trichoderma*	*harzianum*	**46**	*Trichoderma*	*spp.*
41	*Trichoderma*	*harzianum*	**47**	*Trichoderma*	*spp.*
42	*Trichoderma*	*harzianum*	**45**	*Trichoderma*	*viride*
44	*Trichoderma*	*harzianum*	**62**	*Trichoderma*	*viride*
51	*Trichoderma*	*harzianum*	**59**	*Trichoderma*	*virens*
52	*Trichoderma*	*harzianum*	**58**	*Trichoderma*	*asperellum*

Table 7 *Trichoderma* species/isolates utilized in the study

3.3.1.3 Assessment of Cd -tolerance of the twenty-seven selected *Trichoderma* isolates

In order to ascertain the metal tolerance of the twenty-seven *Trichoderma* strains previously selected by the screening test, a confirmatory test was carried out by repeating the procedure in triplicates under the same conditions described above, but only at the highest Cd concentration (250 µM). The colony growth was assessed by the average value of two orthogonal diameters and the fungi response to metal stress was evaluated by Tolerance index (Ti) (growth of the strain exposed to the metals divided by growth of the same strain in the control plates).

Species and strain (*)	Origin	Notes, References
T. atroviride (Ta)	NS**	NS**
T29	NS**	NS**
T31	NS**	NS**
T32	NS**	NS**
T39	NS**	NS**
T49	NS**	NS**
T. citrinoviride (Tc)	NS**	NS**
T33	NS**	NS**
T53	NS**	NS**
T. hamatum (Th)	NS**	NS**
T13	NS**	NS**
T. harzianum (TH)	NS**	NS**
ITEM 908	Italy, ex soil	Biocontrol agent, Leonetti et al., 2017.
ITEM 908-5	UV-mutant of ITEM 908	Tolerant to fusaric acid, Marzano et al., 2013.
ITEM 1323	USA, ex Sclerotium solfsii on Lupinus angustifolius	Gallo et al., 2004
ITEM 1324	USA, ex soil	NS**
ITEM 1412	The Netherlands, ex Polyporus badius	NS**
ITEM 4482	Italy, ex Asparagus officinalis	Gallo et al., 2004
ITEM 4483	Italy, ex Asparagus officinalis	NS**
ITEM 7049	Italy, ex Mushroom substrate	Gallo et al., 2004
ITEM 7050	Italy, ex Zea mays kernel	NS**
ITEM 7053	Italy, ex Seedling substrate	Gallo et al., 2004
ITEM 7054	Italy, ex Mushroom substrate	Gallo et al., 2004
T01	NS**	NS**
T11	NS**	NS**
T16	NS**	NS**
T56	NS**	NS**
T. koningii (Tk)	NS**	NS**
T5	NS**	NS**
T. polysporum (Tp)	NS**	NS**
T60	NS**	NS**
T. spp (Ts)	NS**	NS**
T18	NS**	NS**
T46	NS**	NS**

Table 8 *Trichoderma* isolates selected by Cd tolerance
(*) The isolates with a ITEM code were obtained from the culture collection of the Institute of Sciences of Food Production, National Research Council, Bari (www.ispa.cnr.it/Collection) **(**)** NS: Not Specified

3.3.3 Fungal biomass estimation in liquid culture

3.3.3.1 Liquid culture preparation

Selected *Trichoderma* isolates were inoculated on PDA plates and incubated at room temperature (25 ± 1°C) for three days. After three days, ten mycelial disks (6 mm-diameter) for each *Trichoderma* strain were put into a 250 ml conical flask containing 100 ml of Potato Dextrose Broth (PDB) supplemented with 250 µM Cd (Figure 5 a,b).

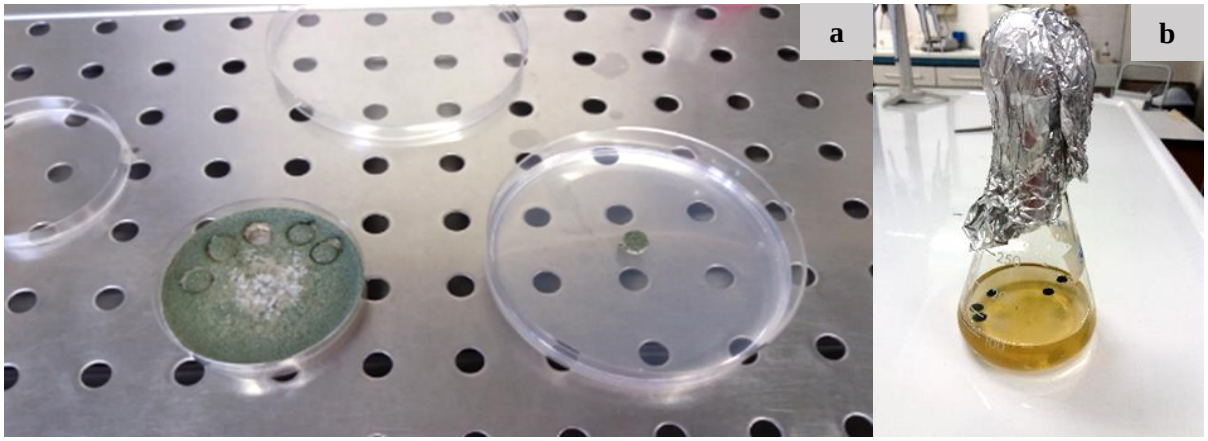


Figure 5 **a)** Plates inoculation**, b)** PDB inoculated with 6-mm mycelial disks

Unsupplemented PDB containing mycelial disks represented the control, while an uncontaminated and uninoculated conical flask represented the blank. Mycelial cultures were incubated at 25 ± 1°C for five day in a rotary shaker incubator at 160 rpm for five days.

3.3.1.2 Biomass production by *Trichoderma* isolates

After five day, fungal biomass was harvested by filtering through filter paper (Whatman No. 1) and then washed thoroughly three times with 50 ml deionized water to remove the growth medium and Cd on the mycelium surface.

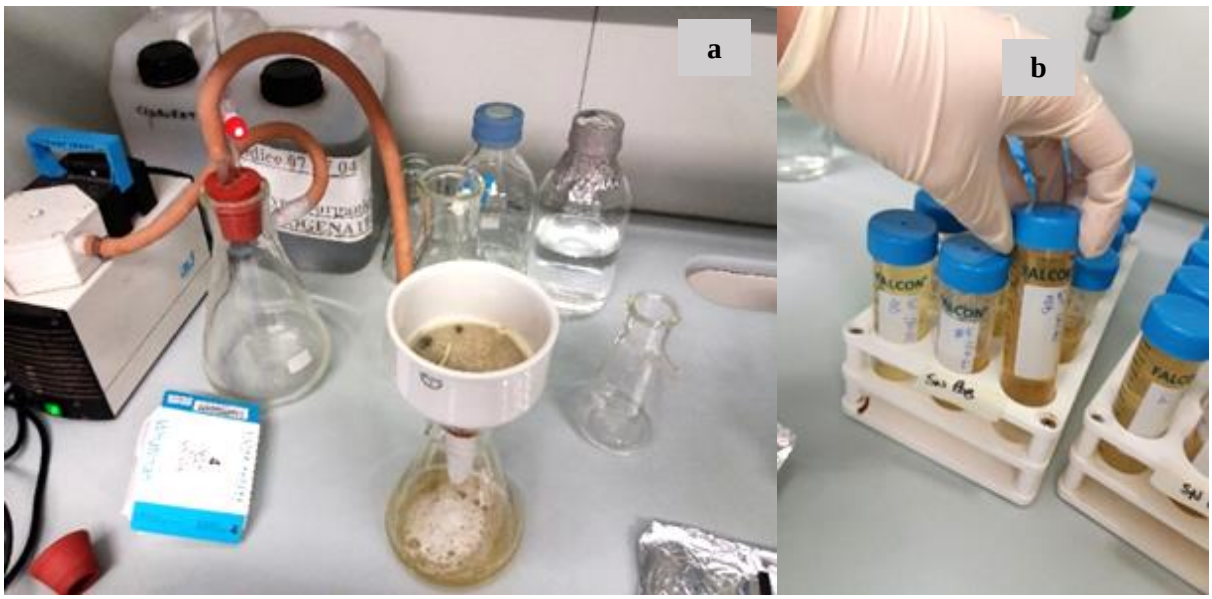


Figure 6 ***a)*** *Collected and filtered mycelia and* ***b)*** *culture supernatant*

Fresh harvested mycelium was weighed, freeze-dried and stored at −20° while the supernatant was separated, measured and frozen until metal content evaluation. The freeze-dried fungal biomass was weighed to determine the biomass dry weight (g)(Figure 6 a, b).

The inhibition of biomass production was calculated based on the dry weight using the following formula:

$$PI\ (\%) = \frac{(W_0 - W_{cd})}{W_0} \times 100$$

Where PI(%) is the percentage of inhibition; W_0 is biomass in the control (0 µM) broth; and W_{cd} is biomass in the metal-containing broth.

3.3.4 Determination of Cd-uptaking by *Trichoderma* mycelia and Cd supernatant content

Dry mycelia produced after 5 days in PDB medium were ground in a mortar, dried again overnight in an oven at 65 °C and mineralized. For the mineralization process of fungal material, accurate amounts of dray mycelia (0.25 g) were weight in microwave digestion vessels followed by addition of 6 ml of 65% HNO_3 (VWR Chemicals) and digested in a closed-vessel microwave assisted digestion system (MARS 6, CEM Corporation, Matthews, NC, USA) (Figure 7)

The digestion procedure were performed in two steps: 15 min to reach 200 °C and 10 min maintained at 200 °C (power set at 900–1050 W; 800 psi). Blanks, HNO_3 without sample, were

also prepared and digested in the same conditions. The digested solutions were cooled and quantitatively transferred to 50 ml volumetric flask. Supernatants and mycelia mineralized solution were diluted to volume with ultrapure H_2O (Milli-Q Millipore 18MΩ cm^{-1}); thus, were filtered by syringe tip polyethersulfone filters (0.2 μm and 0.45 μm rispectively). Samples were analyzed using an inductively coupled plasma optical emission spectroscopy (ICP-OES; 5100 VDV, Agilent Technologies, Santa Clara, CA, USA) to measure the concentration of Cd in the supernatants and mineralized mycelia (Figure 7).

The amount of the heavy metal uptake was estimated by the difference between the metal content before and after adsorption and expressed as a percentage of the initial metal content.

Figure 7 Filtered supernatant and mineralized mycelia

3.3.5 Chemical assay for detection of chelators in culture filtrates

The chelating activity of twenty-seven *Trichoderma* spp. culture filtrates was assayed by the method described by Shenker et al. (1995). This method is based on the measurement of the equilibrium concentration of the Cu-CAS (chrome azurol S) complex in the presence of other chelating substances. In the presence of complexing substances in the filtrates, the Cu is competitively lost from the Cu-CAS complex and a decrease in absorbance occurs.

The method was adapted to use 96-well microtitration plates for spectrophotometric measurements. *Trichoderma* strains were grown both in PDB and in a modified Richard's solution (RMT, Table 9), supplemented with 250 μM Cd, adjusted to pH 7.0 with NaOH.

Cultures were grown for five days and then the cultural filtrates were harvested. No inoculated culture broth, either supplemented with Cd or not, were used as a control.

Components	Amounts (g l^{-1})
KNO_3	10
KH_2PO_4	5
$MgSO_4$	1.3
$FeCl_3$	0.02
Sucrose ($C_{12}H_{22}O_{11}$)	20
$ZnSO_4 \cdot 7\ H_2O$	0.0035
$CuSO_4 \cdot 5\ H_2O$	0.0004
$MnSO_4$	0.00031
$(NH_4)\ 6\ Mo_7O_{24} \cdot 4\ H_2O$	0.00013

Table 9 Composition of modified Richard's solution (RMT) for chelator assay.

A stock solution of the Cu-CAS reagent was prepared with 200 µM $CuCl_2$, 210 µM CAS (Sigma), and 40 mM (N-morpholino ethanesulfonic acid (MES) (Sigma) with pH adjusted to 5.7 with NaOH. The culture filtrates, with and without Cd, were centrifuged (4000 rpm for 10 min at 4°C), filter sterilized (0.22 µm filters). The reagent solution and the culture filtrate were mixed in a 1:1 ratio in microtitration plate wells to a total volume of 100 µl and incubated at room temperature for 5 min, than absorbance at 540 nm was measured by Varioskan. (Figure 8)

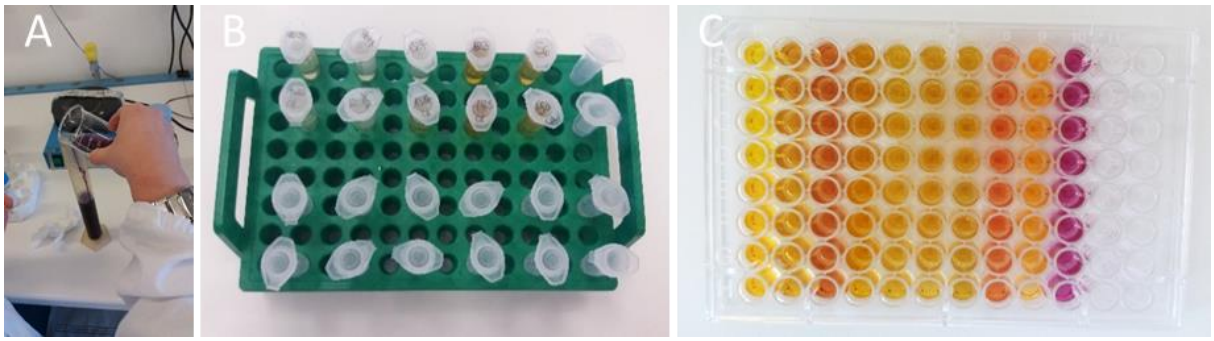


Figure 8 *a) Reagent solution preparation; b) Reagent solution and culture filtrate mix; c) 96-well microtitration plates setting*

3.3.6 Screening of *Trichoderma* isolates for phosphate solubilization

Twenty-seven *Trichoderma spp.* isolates were screened for their *in vitro* phosphate solubilizing potential. In order to study heavy metal (cadmium) influence on P solubilizing capacity the fungal culture were grown in National Botanical Research Institute Phosphate

(NBRIP) broth medium (Nautiyal, 1999) containing (g l^{-1}): glucose (10), tricalcium phosphate (10), $MgCl_2 \cdot 6H_2O$ (5.0), $MgSO_4 \cdot 7H_2O$ (0.25), KCl (0.2) and $(NH_4)_2SO_4$ (0.1). Tricalcium phosphate represented an insoluble phosphorous source.

Qantitative estimation of phosphate solubilisation was carried in Erlenmeyer flasks (250 ml) containing 50 ml of NBRIP broth medium supplemented with 250 µM of cadmium and inoculated with five 6 mm-diameter mycelial discs from active cultures of *Trichoderma* .

Untreated fungal culture represented the control samples. Culture flask were incubated at 28±1 °C in an orbital shaker at 150 rpm for 5 days. The samples were filtered by syringe tip polyethersulfone filters (0.45 um) and the pH of the supernatants were measured by a pH meter (pH 315i/SET, Wissenschaftlich Technische Werkstatten, Germany). Phosphate concentration in the supernatant was also estimated using Fiske and Subbarow method (1925).

Culture supernatant was mixed, in microtitration plate wells to a total volume of 100 µl, with color reagent (1:1 ratio) containing ammonium molybdate 1.5% (w/v), sulfuric acid solution 5.5% (v/v) and ferrous sulfate solution 2.7% (w/v) and then measured spectrophotometrically at 600 nm. The level of phosphate concentration was determined by using a standard graph of KH_2PO_4 and is expressed as equivalent phosphate (µg ml^{-1}).

3.3.7 Statistical analysis

The effects of Cd treatment on tolerance index parameters, Cd uptake, chelation and solubilization ability were defined through one-way analysis of variance (ANOVA). The fungal assay to Cd was set as a completely randomized experimental design by 27 x 1 x 1 factorial experiment (twenty seven *Trichoderma* strains, one metal, and one dose).

Data were subjected to ANOVA using the statistical program Statistica (StafSoft Inc., Tulsa, OK, USA) and the SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA).

3.4 Results

3.4.1 Screening for selection of candidate Cd- tolerant *Trichoderma* strains

The 52 *Trichoderma* strains subjected to the preliminary screening for tolerance to Cadmium exhibited different rates of growth inhibition, depending on the metal concentration in the medium (0, 5, 50 and 250 µM), time of exposure (24, 48 and 72 h), and fungal strain.

The presence of the metal affected the colony growth of *Trichoderma* strains, mostly resulting in moderately or strongly reduced growth, but in some cases also in increased growth

rate (negative values of I, Table 10). All the colonies were already visible after 24 hours of incubation, except for the strain T58, which started to grow after 48 h of incubation. In general, the I values did not change much by increasing the dose of Cadmium from 5 µM to 50 µM, while a significant increase in I values was observed in colonies grown in the presence of 250 µM of $CdSO_4$). Apart from growth inhibition, some Cd-toxicity effects were observed on the mycelium abundance, and sporulation patterns of *Trichoderma* strains (Figure 9).

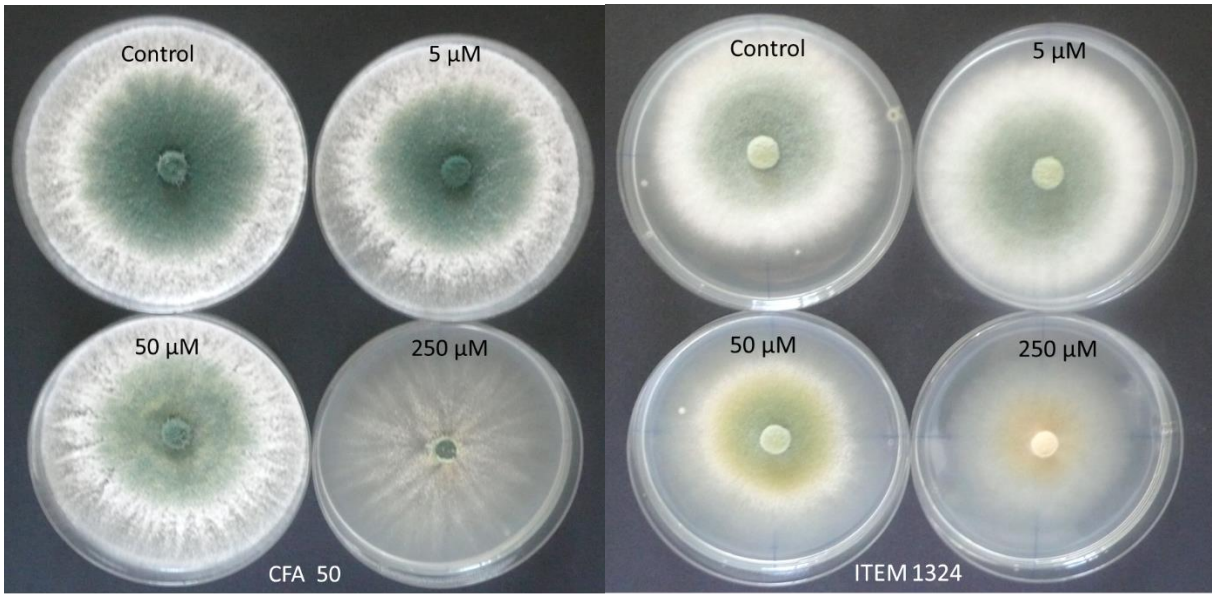


Figure 9 Cd-toxicity effects were observed on the mycelium abundance, and sporulation patterns of *Trichoderma* strains

Among the 52 strains used in the preliminary screening, 27 (highlighted in blue in Table 10) were selected for further studies. The 27 strains, representatives of each one of the *Trichoderma* species examined were chosen based on the values and consistency of 24 h and 48 h responses

ITEM/ CFA	I (%) [a] 24 h 5µM	24 h 50µM	24 h 250µM	48 h 5µM	48 h 50µM	48 h 250µM	72 h 5µM	72 h 50µM	72 h 250µM
Trichoderma atroviride									
29	-9,52	-9,52	19,05	0,00	-4,65	15,50	n.d. [b]	n.d.	n.d.
30	-4,08	2,04	26,53	-0,87	4,35	25,22	n.d.	n.d.	8,82
31	0,00	-4,88	29,27	0,90	-1,80	19,82	n.d.	n.d.	n.d.
32	-1,72	5,17	22,41	-4,76	-3,17	19,84	n.d.	n.d.	2,94
35	-50,00	-11,76	-14,71	-16,67	5,00	6,67	-53,85	-3,85	6,41
38	8,33	36,36	33,33	14,46	32,53	38,55	10,53	26,32	43,86
39	-15,79	2,27	23,68	-15,05	-2,15	13,98	-1,80	-1,80	3,59
43	-29,41	13,64	41,18	0,00	29,46	65,18	n.d.	5,29	62,35
48	-9,09	91,67	100,00	8,91	9,90	27,72	8,04	33,93	33,04
49	-9,62	-9,62	19,23	-0,76	-0,76	21,97	n.d.	n.d.	n.d.
50	10,71	12,50	37,50	4,72	4,72	29,92	n.d.	n.d.	n.d.
Trichoderma citrinoviride									
33	-3,12	-3,12	12,50	-5,48	-12,33	-1,37	n.d.	-11,11	-5,93
54	20,00	17,14	37,14	1,53	5,34	45,80	0,60	16,87	36,14
53	-11,54	-15,38	11,54	-4,92	-21,31	9,84	-7,41	1,85	20,37
Trichoderma koningii									
5	-9,30	2,33	100,00	-7,62	-3,81	29,52	n.d.	n.d.	35,29
57	-9,43	-10,34	22,64	0,00	1,44	25,18	n.d.	n.d.	n.d.
Trichoderma hamatum							n.d.	n.d.	n.d.
13	-1,67	-1,64	28,33	-4,51	-3,76	19,55	n.d.	n.d.	n.d.
40	18,52	6,82	31,48	-10,00	3,00	8,00	n.d.	n.d.	n.d.
Trichoderma harzianum									
1	2,22	31,11	40,00	13,48	-11,24	23,60	1,39	3,47	29,17
2	10,00	13,75	72,50	28,75	23,75	54,38	n.d.	n.d.	22,35
10	-5,36	23,21	60,71	-6,06	6,82	31,06	n.d.	n.d.	n.d.
11	-19,35	-14,52	38,71	-6,38	-8,51	25,53	n.d.	n.d.	n.d.
16	-21,88	-37,50	12,50	-7,02	-11,40	6,14	n.d.	n.d.	n.d.
41	25,00	21,59	39,77	3,31	0,66	24,50	n.d.	n.d.	n.d.
42	1,25	12,50	48,75	0,00	1,23	20,99	n.d.	n.d.	n.d.
44	-6,76	17,57	40,54	-3,36	6,04	23,49	n.d.	n.d.	n.d.
51	-23,91	-6,52	23,91	3,13	6,25	26,56	n.d.	n.d.	n.d.
52	-9,68	9,68	16,13	-6,67	6,67	20,00	-4,43	12,66	19,62
56	-2,13	-21,28	-2,13	-3,10	-10,85	-6,20	n.d.	n.d.	n.d.
55	31,25	28,13	34,38	10,00	16,67	28,89	0,60	16,87	36,14
59	-40,48	11,86	11,43	-9,52	20,95	18,10	0,00	16,47	8,82
61	2,27	2,27	0,00	0,68	11,56	19,73	n.d.	n.d.	1,18
908-5	-10,20	-14,29	-6,12	-3,42	-5,13	11,11	n.d.	n.d.	n.d.
908 WT	-7,69	-15,38	-5,13	0,00	1,08	19,35	n.d.	n.d.	9,41
1323	0,00	-4,76	42,86	-4,46	-4,46	16,07	n.d.	n.d.	n.d.
1324	-56,00	4,00	0,00	-27,03	-8,11	6,76	-11,26	0,66	17,88
1411	4,35	-8,70	-4,35	-27,45	11,76	21,57	-11,93	-13,76	33,94
1412	-4,08	-8,16	20,41	-4,63	-10,19	12,96	n.d.	n.d.	4,71
2683	-11,36	22,73	45,45	-3,31	13,22	29,75	n.d.	n.d.	n.d.
4482	0,00	9,52	42,86	-28,40	-22,22	-1,23	-6,25	-6,25	8,75
4483	-18,18	-16,36	29,09	-9,68	-3,87	24,52	n.d.	n.d.	n.d.
7050	-6,78	-3,39	57,63	-6,25	-6,25	33,75	n.d.	n.d.	n.d.
7049	-4,44	-2,22	8,89	-3,12	-6,25	15,63	n.d.	n.d.	n.d.
7053	-14,63	-17,07	26,83	0,00	-2,50	22,50	n.d.	n.d.	n.d.
7054	6,52	-2,17	21,74	-1,67	-1,67	13,33	n.d.	n.d.	1,76
Trichoderma polysporum									
60	-11,43	0,00	0,00	-9,00	-1,00	13,00	n.d.	n.d.	7,06
Trichoderma spp.									
18	-9,68	-25,81	-22,58	-9,09	-14,14	-5,05	n.d.	n.d.	n.d.
45	1,59	6,45	52,38	2,58	9,03	42,58	n.d.	n.d.	5,88
46	**-3,33**	**0,00**	**10,00**	**2,78**	**-4,17**	**6,94**	**-5,31**	**-5,31**	**15,04**
47	-22,41	-17,24	29,31	1,16	2,91	48,26	n.d.	n.d.	n.d.
58	n.g. [c]	n.g.	n.g.	-25,00	20,00	15,00	-25,00	20,00	15,00
62	-7,69	7,14	30,77	-7,69	7,69	30,77	2,68	16,11	18,12

Table 10 Rates of growth inhibition (I%) of *Trichoderma* strains used in the preliminary screening

[a] Negative values indicate increased growth of the colonies compared to control.

[b] n.d. = Not determined. The colonies were grown up to cover the entire plate both in the treatment and control plates.

[c] n.g. = Not grown

To confirm the results of the preliminary screening test, a confirmatory test was carried out with the 27 selected strains, in triplicate and using only the highest Cd dosage (250 µM). The results are shown in Figure 12. While the experiment was carried out over 3 days, and the whole dataset records included the TI values at 24, 48 and 72 h, only the 48 h TI values are shown in figure 10.

The 48 h values were judged to be the most representative of the strain tolerance, since the 24 h values reflected an initial adaptative hurdle and some of the 72 h value were not determined because the colonies grew up to cover the entire plate both in the treatment and control plates.

Based on the TI values, the *Trichoderma* strains were classified into 3 classes of tolerance: highly tolerant strains (negative TI values: strains 33 and 39), tolerant strains (TI values > 0.8: strains 29, 53, 11, 16, 908-5, 908WT, 1323, 1412, 4482, 7049, 7054, 60, 18) and moderately tolerant strains (TI values ≤ 0.8: strains 31, 12, 49, 5, 13, 1, 56, 1324, 4483, 7050, 7053, 46).

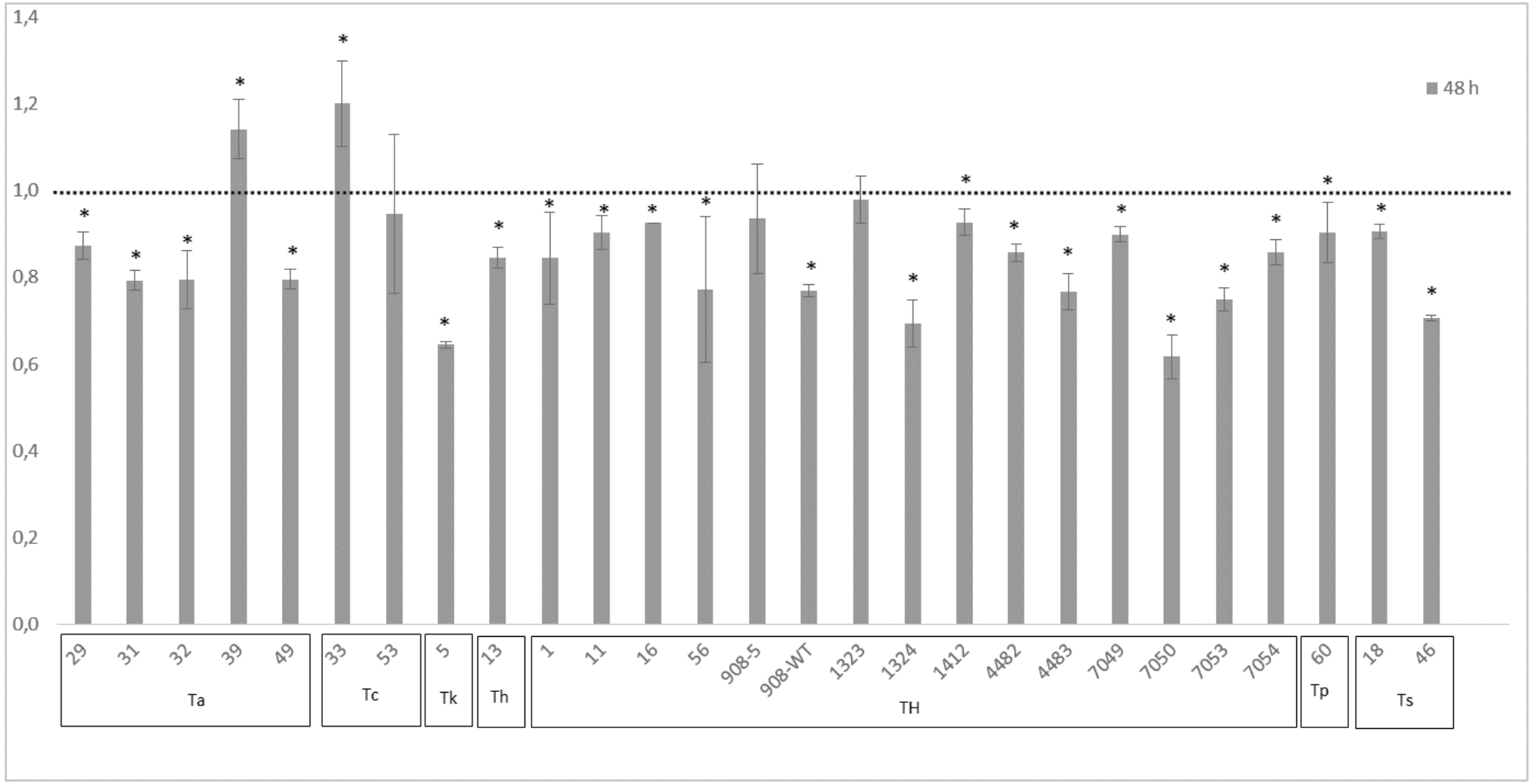


Figure 10 Tolerance index of *Trichoderma* strains subjected to treatment with 250 µM Cd at 48, h. Values are means ± standard error. Values significantly different by the Control value (Black dashed line) are identified by an asterisk above the bar ($P < 0.05$, one-way ANOVA). Bars represent the standard deviation (n=3)

In this study Twenty-seven isolates, were screened due to their low growth inhibitory rate under Cd exposure. Screening of the *Trichoderma* isolates indicated that several doses of cadmium had significant effects on the fungal growth. These significant effects were observed from 24 h to 72 h after Cd exposure and the growth fungal rate was expressed by the tolerance index. Generally, in most of the tested strains, Cd significantly slowed fungal colony growth than controls; nevertheless all fungal strains were able to grow at 250 µM Cd. Moreover, the fungal growth for controls (0 µM Cd) and for the all treated filamentous fungi was observed in average already after 24 h; despite some Cd effects were observed on the mycelium abundance, and sporulation patterns of treated *Trichoderma* strains (Figure 11).

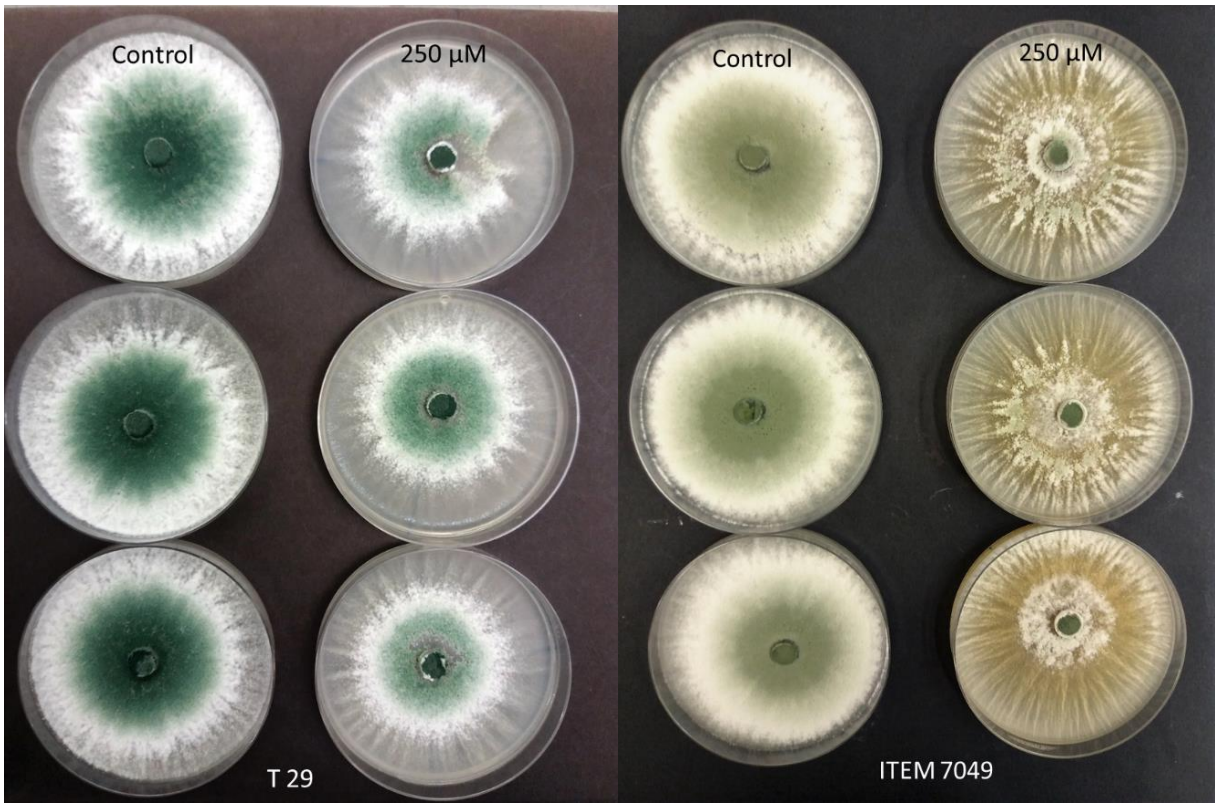


Figure 11 Cd effects observed on the mycelium abundance, and sporulation patterns of 27 treated *Trichoderma* strains

Irrespective of the evaluation period of fungi proliferation, T33 showed the highest resistance to the metal effect followed by T39. From 24 h to 48 h, indeed, the strains with higher tolerance to Cd were T1323 followed byT1412, T7049, T16, T908-5 while the strains T7050, T5, T1324, T1, T7053 and T4483 showed the lowest tolerance (Figure 12). However, the remaining *Trichoderma* strains showed a moderate tolerance to cadmium evidencing a slight inhibition of growth compared to control. Contrary, more than half of the tested strains completely covered the Petri dish at 72 h.

After all, the average fungal adaptation period to Cd was shorter (24 h); in this period the strains T33, T39, T1412 and T16 showed growth stimulation under metal stress; this stimulatory effect was observed still at 48h and 72 h for T33 and T39 than the rest of the strains mentioned above. The strain T7050 was the only fungus to highlight remarkable significant reduction in the degree tolerance (low tolerance) comparing to the control showing minimum resistance to the metal from 24 h to 72 h.

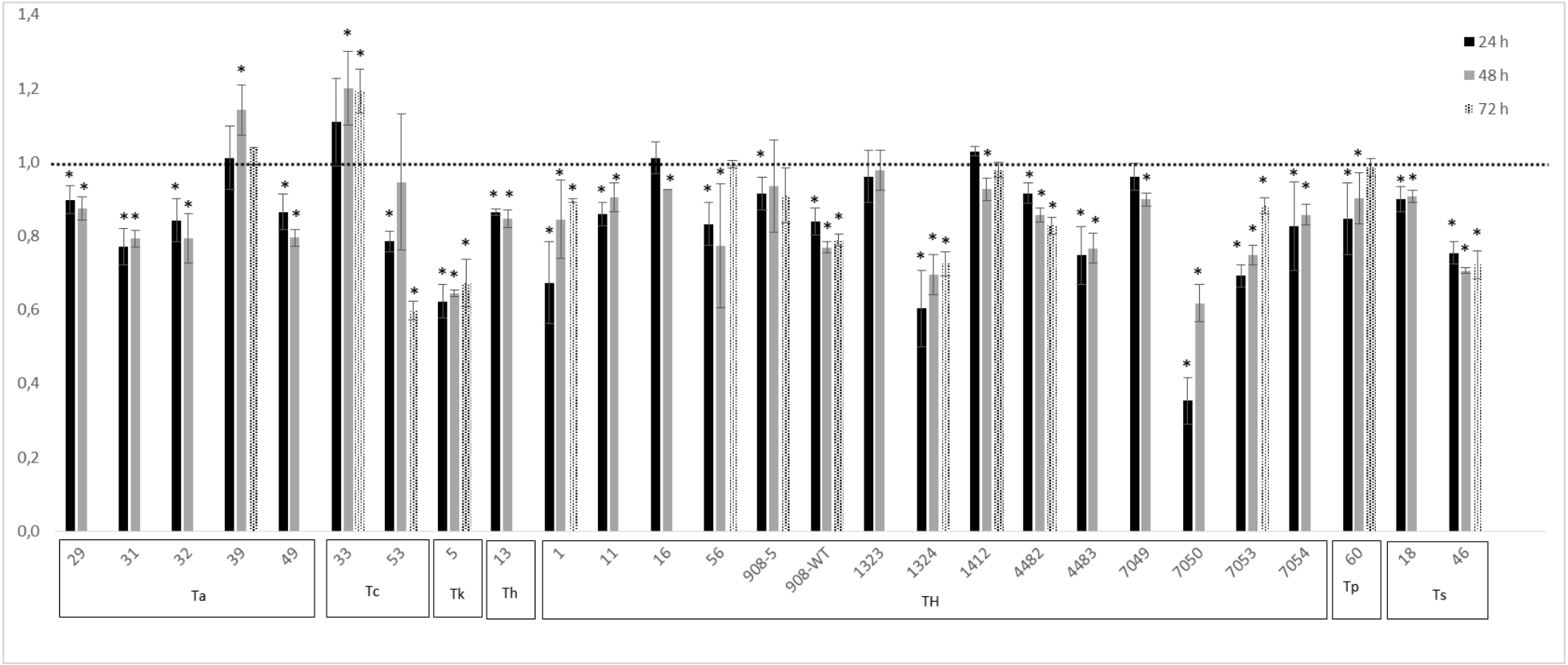


Figure 12 Tolerance index of *Trichoderma* strains subjected to treatment with 250 µM Cd at 24, 48, 72 h. Values are means ± standard error. Values significantly different by the Control value (Black dashed line) are identified by an asterisk above the bar ($P < 0.05$, one-way ANOVA). Bars represent the standard deviation (n=3).

3.4.3 Metal chelating activity of *Trichoderma* spp. isolates

Production of metabolites with chelating activity by the 27 *Trichoderma* strains was initially investigated using PDB, a complex medium containing undefined plant components, for fungal growth.

However, a significant chelating activity was observed in the medium itself (data not shown), which made the interpretation of the results difficult due to complexing activity from the medium overwhelming the activity from *Trichoderma* metabolites. Therefore, to avoid any interference from the culture medium, the assay was subsequently repeated using RMT, a chemically-defined (synthetic) medium. The results of the Chrome-Azurol S (Cu-CAS) assay for chelating activity of *Trichoderma* culture filtrates obtained either in the absence or in the presence of Cadmium are shown in (Figure 13).

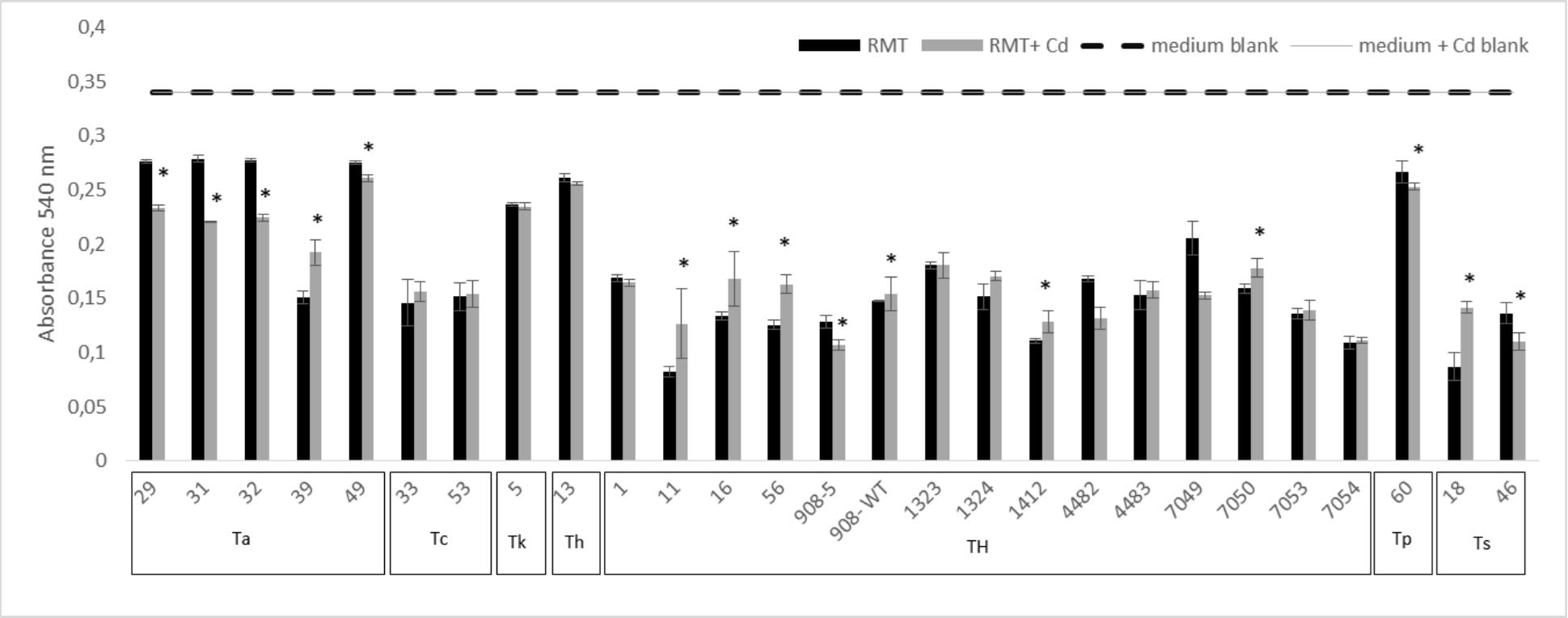


Figure 13 Chelation activity of *Trichoderma* strains grown in RMT in the absence (back bars) or in the presence (grey bars) of Cd. Decrease of the absorbance at 540 nm indicates the production of chelating metabolites. Values are means ± standard deviation. Values of absorbance of cultures with Cd (grey bars) that are significantly different by their respective control values (cultures without Cd, black bars) are identified by an asterisk ($P < 0.05$, one-way ANOVA). Error bars represent the standard deviation (n=3). Black dashed line and grey line represent the absorbance of "RMT" and "RMT + Cd" (medium blanks) , respectively.

Based on the results of the Cu-CAS assay on the culture filtrates without Cd (Figure 13), black bars), the strains can be classified into 2 arbitrary classes of chelating capability, that is strong producers of chelating metabolites (absorbance reduced by more than 50% in respect to the blank, strains 39, 33, 53, 1, 11, 16, 56, 908-5, 908WT, 1324, 1412, 4482, 4483, 7050, 7053, 7054, 18, 46) and moderate producers (absorbance reduced by less than 50% in respect to the blank, strains 29, 31, 32, 49, 5, 13, 1323, 7049, 60). The presence of Cd in the culture medium (grey bars) significantly altered, either by increasing or by decreasing, the production of chelating metabolites by most of the strains. In 9 strains (*viz.* strains 29, 31, 32, 49, 908-5, 4482, 7049, 60, 46) the production of chelating metabolites was increased, in 9 strains (*viz.* strains 39, 11, 16, 56, 908WT, 1324, 1412, 7050, 18) it was decreased, and in the remaining 9 strains (*viz.* strains 33, 53, 5, 13, 1, 1323, 4483, 7053, 7054) it was not significantly affected.

3.4.5 Fungal biomass inhibition

Effects of high cadmium concentration in broth culture on fungal biomass production were studied using the twenty-seven most tolerant *Trichoderma* isolates. The maximum biomass inhibition was recorded for T33 (61.43%), followed by T53 (52.13%), T46 (46.51%) and so on (Figure 14).

Isolate T39 (- 42,86 %), instead, exhibited biomass stimulation upon exposure to cadmium toxicity whose biomass production was higher than the values obtain for T16 (-33.87%), T7053 (-33.87%), T1 (-30.91%), T18 (-29.09%), T7054 (-6.67) and T908-WT (-2.99%) also characterized by a stimulatory effect in the presence of metal.

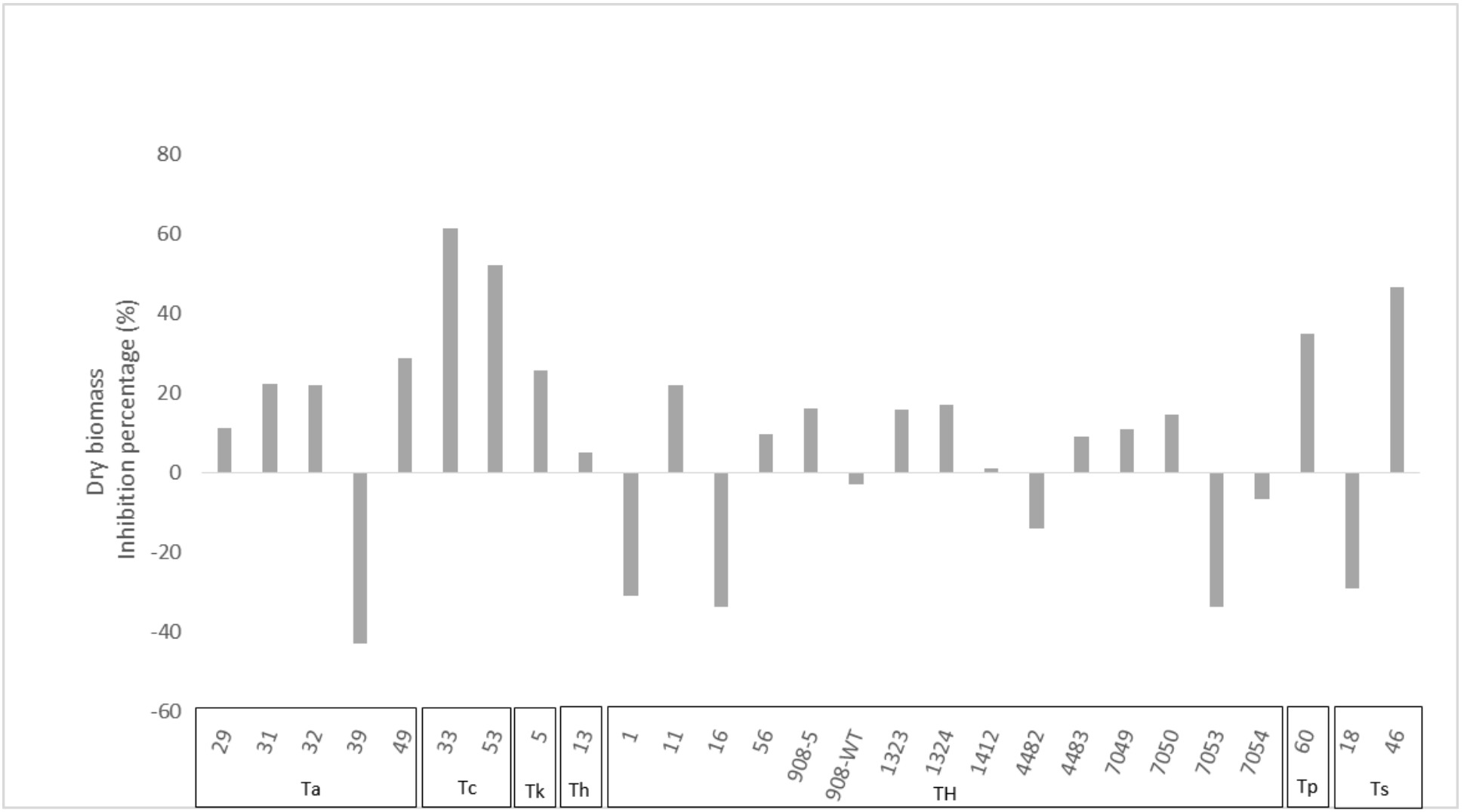


Figure 14 Fungal biomass inhibition in broth culture

3.4.6 Cd-uptake by *Trichoderma* mycelium

The capability of the *Trichoderma* strains to take up Cadmium from the surrounding environment was studied using cultures grown in a liquid medium supplemented with 250 µM Cd. After five days of growth the ratio of the up-taken Cd versus the Cd that was still in solution was determined by spectroscopical analysis (ICP-OES) of both mycelium and culture broth.

The results are shown in figure 15. The data show a large variation in respect to the Cd-uptake capability of *Trichoderma* strains, which varied from 20% (strain 1412) to higher than 97.54% (strain 5). Variation in Cd-uptake capability was found both interspecifically and intraspecifically. The best strains, in terms of Cd-uptake capability were strain 5 > 7050 > 11 > 4483 > 39 > 56 > 13 > 908WT > 1323 > 53 >32 >7049 > 31 > 46.

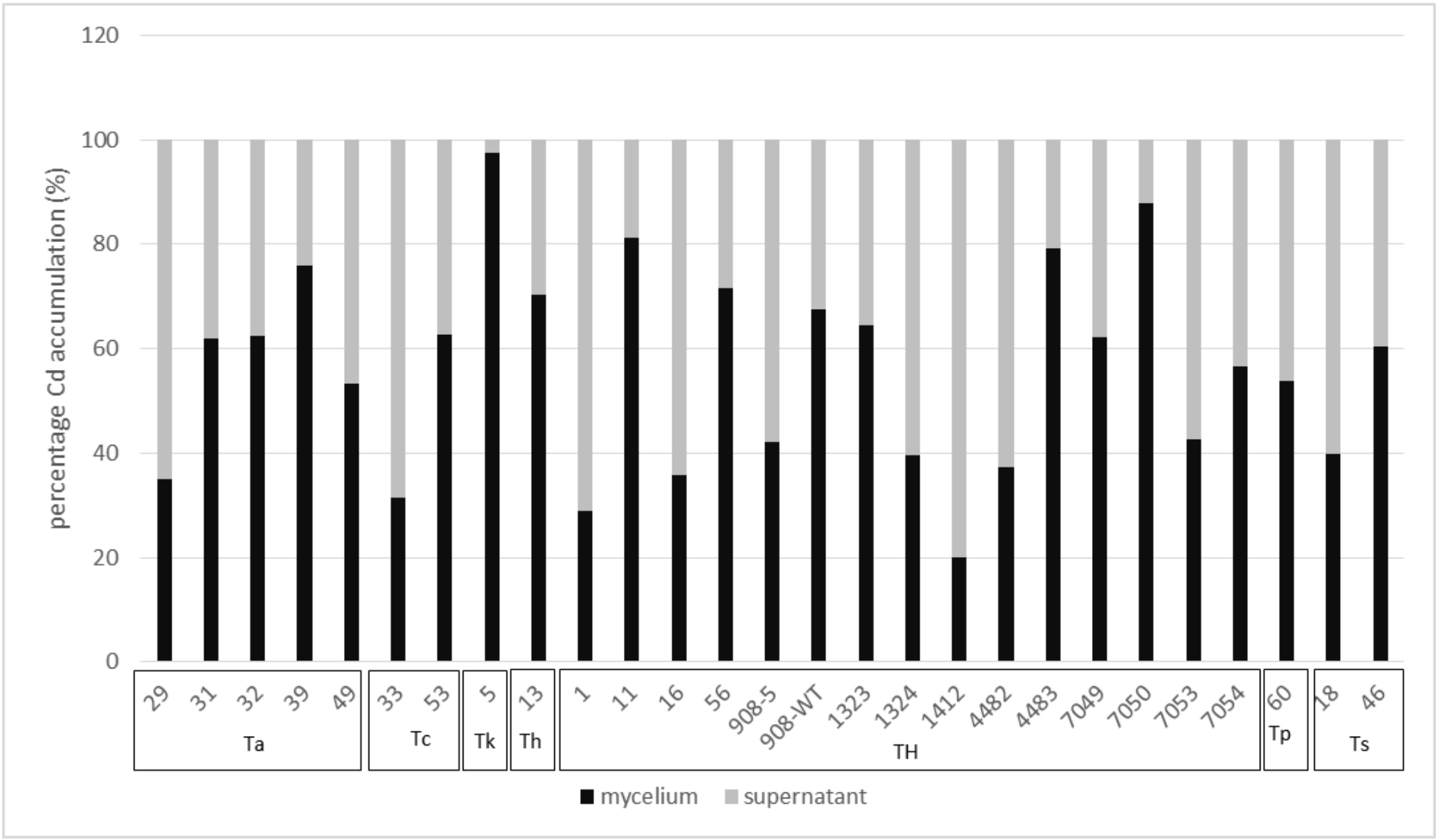


Figure 15 Normalized Cd content in mycelia (black bars) and culture filtrates (grey bars) of *Trichoderma* strains grown in PDB supplemented with 250 µM $CdSO_4$. Cd content of each fraction (mycelium or filtrate) is expressed as percentage (w/w) of total Cd in the culture (mycelium + filtrate)

In addition, in order to evaluate the efficiency of different strains as bioaccumulators of Cadmium, the quantity (μg) of Cadmium fixed by the mycelium of different strains was determined in respect to both fresh weight (Figure 16) and dry weight (Figure 17) of fungal biomass. In terms of bioaccumulation capability, the strains 7050, 5, 53 and 11 were the most efficient ones.

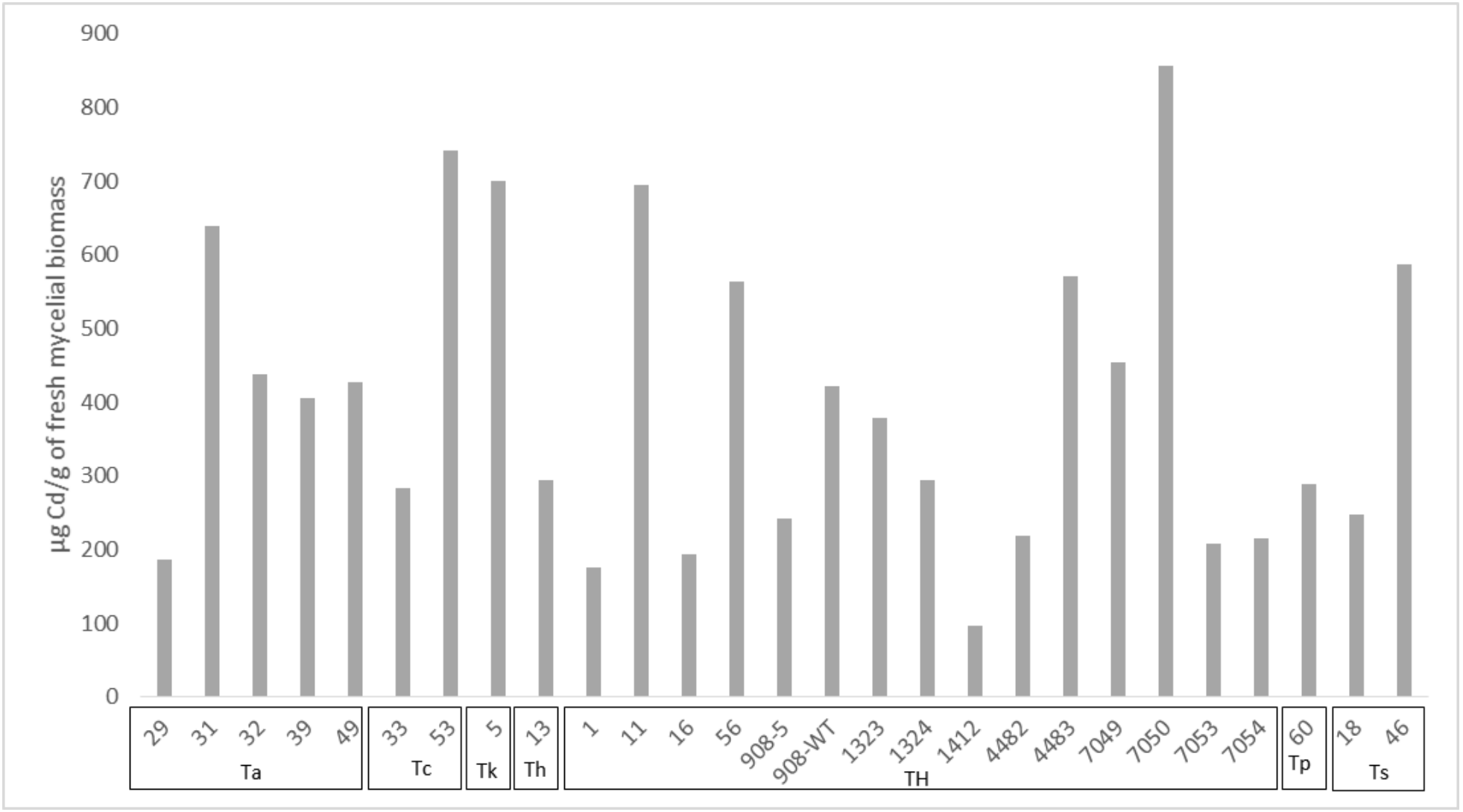


Figure 16 Evaluation of *Trichoderma* spp. mycelia as bioaccumulators of Cadmium. Quantity of Cadmium fixed per unit of fresh

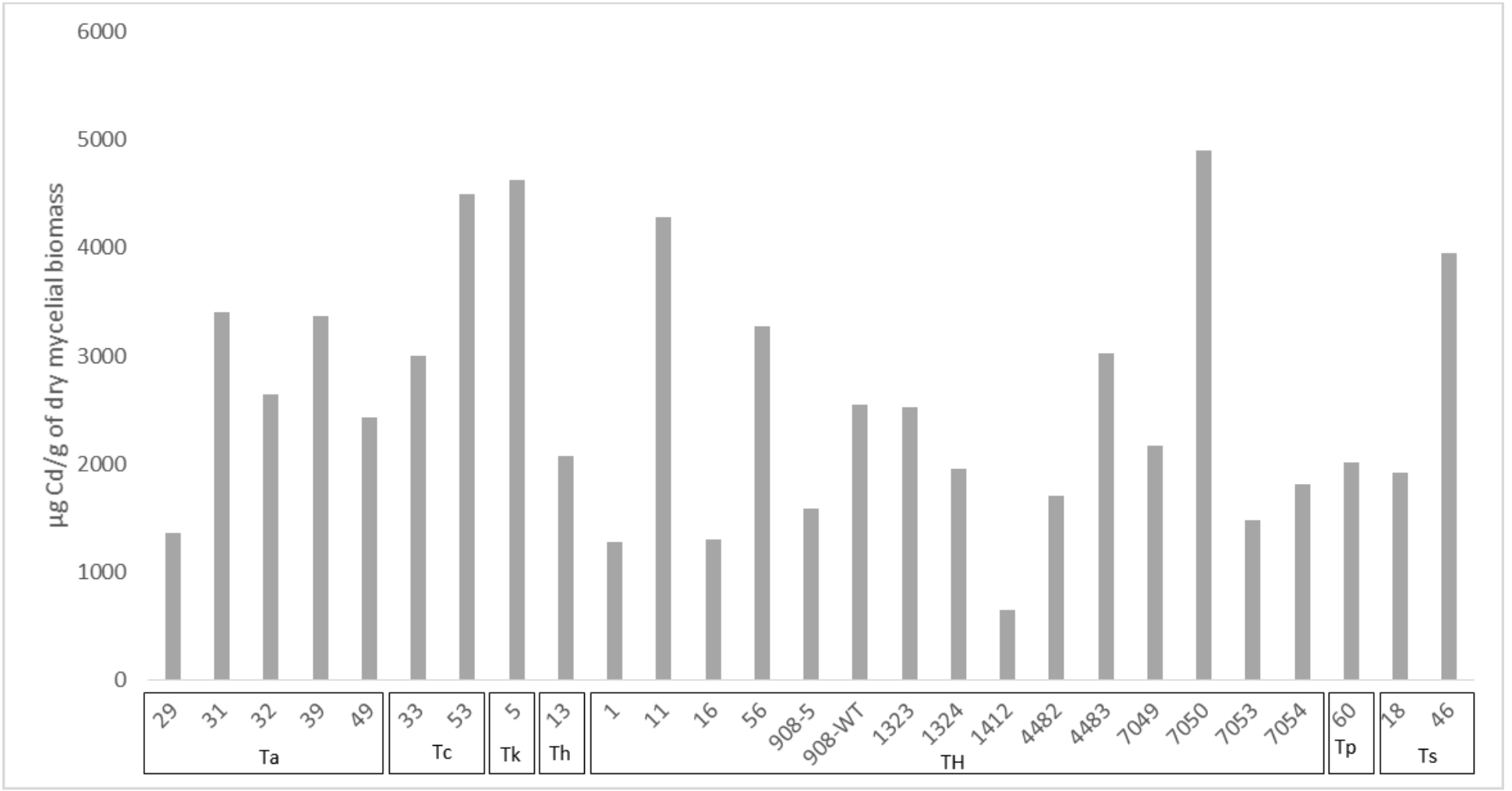


Figure 17 Evaluation of *Trichoderma* spp. mycelia as bioaccumulators of Cadmium. Quantity of Cadmium fixed per unit of dry biomass.

3.4.7 Phosphate solubilisation capability of *Trichoderma* spp. strains

The capability of *Trichoderma* strains to solubilize inorganic phosphates (tricalcium phosphate) is shown in figure 18. In general, all the strains were able to solubilize tricalcium phosphate, with a distinctly higher capability exhibited by the strain 5. In most cases the addition of $CaSO_4$ to the growth medium did not affect (strains 31, 33, 1, 11, 16, 56, 908-5, 1323, 1324, 4483, 7053) or only slightly affected the P-solubilizing capability of *Trichoderma*

In few cases (strains 39, 49, 53, 4482 and 7050) the presence of Cd in the medium increased the P-solubilizing capability of *Trichoderma* strains.

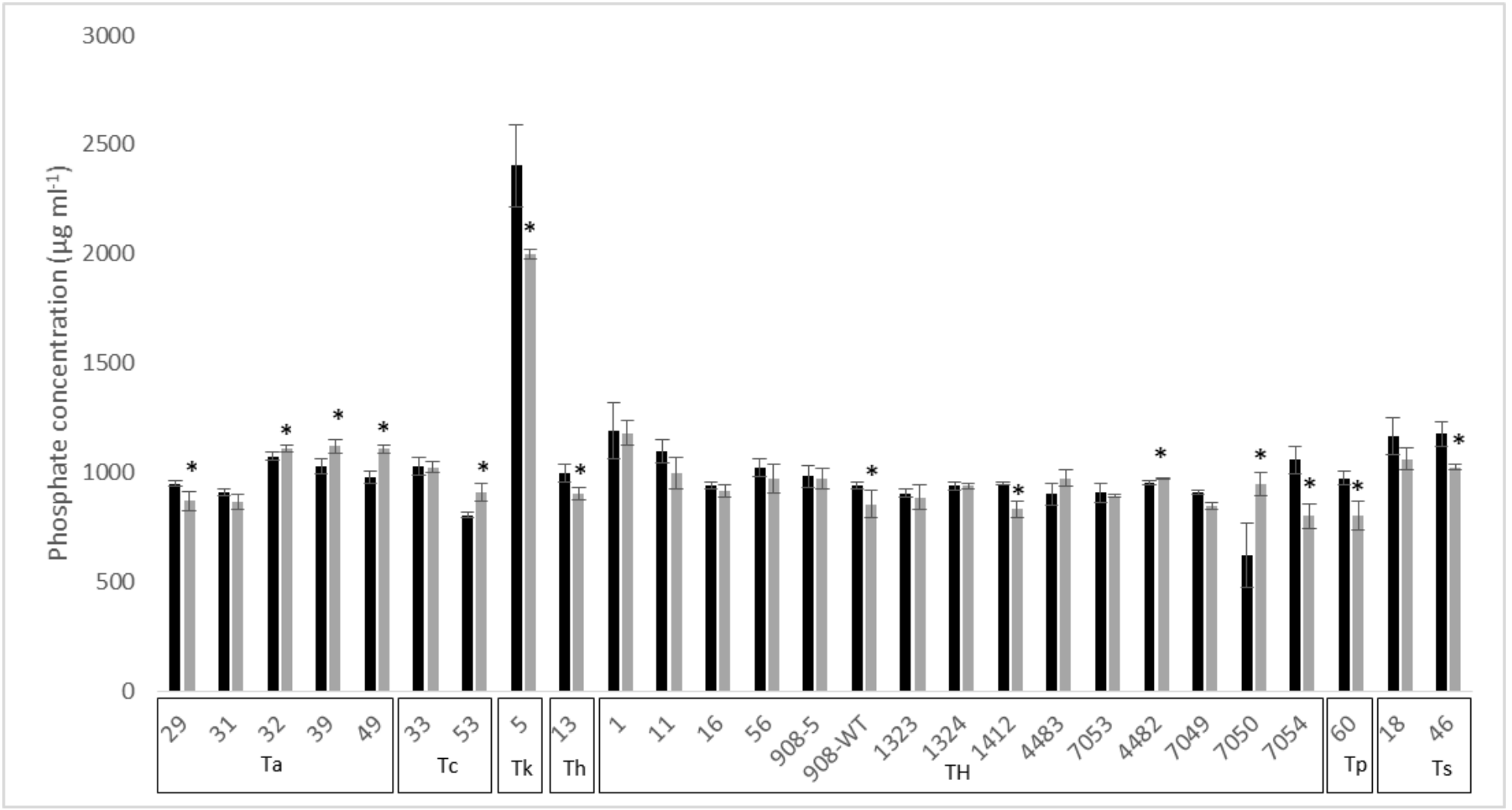


Figure 18 Concentration of phosphate released upon solubilization of tricalcium phosphate (TCP) by *Trichoderma* strains under high cadmium concentration (250 µM)

3.5 Discussion

In soil, Cd likewise other metals such as Fe, Cu, Mn and Zn undergoes a complex dynamic equilibrium of solubilization and insolubilization that ultimately affect its bioavailability, that is availability to living receptors (e.g., plant roots) through direct contact or uptake. Metal bioavailability in soil, rather than total metal concentration, is of main importance, because it is assumed that the available concentration is an indication of the amount available for plant uptake. Soil pH, activity of soil microflora and organic matter content are the most important factors that influence Cd bioavailability in soil (Kirkham, 2006; Pinto et al., 2004; Vig et al., 2003).

Biovailability of Cd should be increased as much as possible by appropriate management of the factors that control its solubility, particularly activity of soil microflora.

Trichoderma spp. are ubiquitous soil fungi able to establish a symbiotic relationship with plants that results in increased plant growth and resistance to both biotic and abiotic stresses. For this reason, these fungi have long been used world-wide for biological control of plant diseases and as plant biostimulants. More recently, a potential for use of *Trichoderma* spp. in phytoremediation as enhancers of HM-phytoextractive capability of plants has been highlighted (Adams et al., 2007; Teng et al., 2015). Boosting the capability of plants to hyperaccumulate heavy metals might be useful for phytoremediation and removal of toxic materials from soils and water. On the other hand, the uptake of heavy metal ions, including Cd, by agricultural plants may pose risks to human health. In this respect, understanding the mechanism of enhancement of Cd solubility/bioavailability appears of utmost importance in order to allow to select the best *Trichoderma* strain for one particular use.

The first set of experiments that we carried out were aimed to evaluate the tolerance level of different species and strains of *Trichoderma* to cadmium, as well as to assess their bioaccumulation capability. A preliminary rapid screening of fifty-two *Trichoderma* strains showed a large variability within the genus in respect to Cd-tolerance. The result of the screening allowed to select twenty-seven isolates that exhibited moderate to high tolerance to high concentration (250 µM) of Cd. Tolerance of fungi of the genus *Trichoderma* to HMs has been reported in others studies (Harman et al 2004b;.Zafar et al 2007; Errasquın et al.,2003) as well as the multiple tolerance to Ni, As, and Zn (Pratibha et al., 2013). In our study, Cd-tolerant strains were found in all of the taxonomic groups examined. The growth of strains *T. citrinoviride* 33 and *T. atroviride* 39 was stimulated by Cd-exposure. (negative values of TI), while the growth of 13 isolates was only slightly (less than 20%) inhibited.

The exceptional tolerance of *Trichoderma* spp. may be due to several mechanisms, which include strategies of exclusion, such as metal binding to cell walls, extracellular metal sequestration and suppressed influx, as well as detoxification strategies, such as production of ROS scavenging intracellular enzymes, intracellular compartmentalization and enhanced metal efflux.

Cultures of the tolerant strains grown in PDB amended with 250 μM of Cd displayed a decrease in biomass production, particularly *T. citrinoviride* 33, the same strain that exhibited the highest TI value. This discrepancy can be explained by the fact that Cd detrimental effect on production of fungal biomass not necessarily correspond to a limitation of the radial expansion of fungal colonies. Based in this finding, the TI value alone appears not to be a reliable measure of fungal tolerance, unlike assumed in a number of previous research works. On the contrary, isolates 39, 1, 16, 908WT, 4482, 7053, 7054 and 18 exhibited a stimulation of biomass production under metal stress. In general, the lower inhibition of *Trichoderma* biomass formation, the higher residual Cd was found in culture filtrates, suggesting a cell exclusion mechanism of Cd-tolerance for these strains. On the contrary, some strains (viz. 5, 11, 4483, 7050) exhibited only a moderate inhibition of growth as evaluated by TI and dry biomass formation, despite of a high Cd-uptake (accumulation ranging from 97,54% to 75,94 % of 250 μM of Cd). For these strains, a mechanism of Cd-tolerance based on intracellular compartmentalization and/or detoxification can be hypothesized. Therefore, these strains appear to be good candidates for an use as bioaccumulators for bioremediation applications (Nongmaithem et al., 2016).

The application of chelating agents has shown positive effects in increasing the solubility of heavy metals in soil and therefore in enhancing land restoration practices. Chelators extract HMs adsorbed by soil particles or immobilized as insoluble phosphates and increase their bioavailability for plant uptake. Both synthetic (EDTA) and natural chelating agent have been used in phytoremediation research (Evangelou et al., 2007). However, this technology has shown major drawbacks, since leaching of HMs may produce undesirable environmental risks that include pollution of groundwater used as a source of drinking water. Strategies to overcome the issue have been suggested, such as use of biodegradable and less-toxic natural chelators, like humic acids and microbial metabolites produced by bacteria and yeast (Wang and Mulligan, 2004). *Trichoderma* spp. are known to produce different classes of siderophores (namely coprogen, coprogen B and ferricrocin) and other chelating secondary metabolites, such as harzianic acid (Vinale et al., 2013). In addition, other compounds, such as low molecular weight organic acids, excreted by *Trichoderma* have chelating properties (Evangelou et al., 2007) and also facilitate metal solubilization by lowering the soil pH. All the

Trichoderma strains tested in this work were able to produce metal binding compounds, although with significant differences among strains in chelating activity. Some of the most efficient chelator producers, viz. strains 39, 1, 16, 908WT, 4482, 7053, 7054 and 18, were also the strains whose biomass production was not inhibited, but conversely stimulated, by Cd. This suggests for these isolates a strategy of Cd-tolerance at least partly based on extracellular metal sequestration.

One of the most common amendments added to soil to tie up heavy metals and prevent them from leaching is phosphate. Addition of phosphorus to soil is the basis of a patented process to reduce bioavailability of metals (Pierzynski and Hettiarachchi, 2002). Also, a study by Brown et al. (2004) showed that phosphate reduces Cd availability. Since metal cations are fixed by phosphorus in the form of insoluble phosphates, the proven capability of some *Trichoderma* strains to solubilize insoluble phosphates (Altomare et al., 1999) is of great importance for bioavailability of Cd in soil and enhancement of phytoextraction. Our results show that all the *Trichoderma* strains examined have the capability to solubilize phosphorous from and an insoluble mineral source (tricalcium phosphate), but strong variation with regard to this capability can be found within the genus. In fact, the strain 5 was able to solubilize approximately twice the amount of phosphorous solubilized in average by the other strains. Also it is important to be noted that the presence of Cd at high concentration did not impair the P-solubilizing capability of *Trichoderma* strains to a great extent, although in some instances it did at a statistically significant level.

3.6 Conclusion

In conclusion, within a group of 52 strains of *Trichoderma* spp. screened for Cd-tolerance, 27 isolates were selected which displayed good to high Cd-tolerance. The tolerant strains belonged to at least 6 different *Trichoderma* species (namely *T. atroviride*, *T. citrinoviride*, *T. koningii*, *T. hamatum*, *T. harzianum*, and *T. polysporum*). Investigations on the mechanism of Cd-tolerance, showed that this was not univocal, but at least two different tolerance strategies could be distinguished. The first is based on cell exclusion of the toxicant. Strains that used this strategy are efficient producers of chelating compounds and solubilizer of insoluble phosphates. They are good candidates for increasing bioavailability of Cd in soils and thus enhance phytoextraction. Other strains appear to function via a highly efficient Cd-uptake system coupled with intracellular compartmentalization and/or enzymatic detoxification of Cd.

These strains may find a possible application as bioaccumulators intended for bioremediation of wastewater. Nevertheless, the behavior of these strains also need to be studied at the rhizosphere level, with regard to a possible translocation of the uptaken Cd at the interface of the plant-*Trichoderma* symbiosis.

CHAPTER IV– Semi-controlled pot experiment

4. Exploring *Trichoderma* -assisted phytoremediation potential of *Populus alba* clone (Querce) in semi-controlled Cadmium-contaminated soil

4.1 Abstract

The present study is focused on the phytoremediation of cadmium-contaminated soils by means of the tree species *Populus alba* L., clone "Querce", in association with two Cd-tolerant strains of *Trichoderma spp.*, rhizosphere fungi which had been previously reported to increase plant uptake of soil minerals. The tolerance of *Trichoderma polysporum* T60 (T1) and *Trichoderma harzianum* ITEM 908 (T2) to Cd was preliminarily assessed. Then, the effect of high Cd concentration (250 µM) was evaluated on plant growth and Cd uptake of poplar plantlets inoculated and non-inoculated with the selected strains T1 and T2 in pot assays. The Cd uptake was determined in root, leaf and stem tissues through the inductively coupled plasma optical emission spectrometry (ICP-OES). In order to gain insights into the poplar physiological response in the interaction with the beneficial microorganisms, the spectrum of emitted volatile compounds (VOCs) of plant inoculated with *Trichoderma* strains was determined by gas chromatography-mass spectrometry (GC-MS). Our results indicate that T2 treatment enhanced fresh root biomass by 44.3 % as compared to the Cd-treated plants and by 54.6% as compared to plants inoculated with T1. Although the "Querce" clone inoculated with T1 and T2 did not show significant increase in Cd-uptake compared to control plants, plants inoculated with the strain T2 exhibited the compartmentalization of metal mainly in stems, indicating that this strain was effective in enhancing Cd translocation by root to shoot. The tolerance to excess metal concentrations of clone was high when inoculated with *Trichoderma* T2, in comparison to T1 inoculation. Moreover, the plants treated with T2 exhibited a higher translocation factor (Tf) indicating a remarkable potential for a phytoremediation strategy. In these experimental conditions, the *T. harzianum* ITEM 908 (T2) resulted a fungal strain as a promising agent for the enhancement of Cd-translocation by poplar plantlets, to be used in phytoremediation of highly Cd-contaminated soils. Moreover, preliminary list of VOCs emitted by the "Querce" clone was obtained, resulting in the detection of 81 VOCs as a starting VOC pool necessary to further investigations.

VOC analyses indicated that heavy dose of Cd induced significant variation in the VOCs emitted by stressed plants, compared to control plants. Reduction ($p < 0.05$) of the contents of five VOCs (i.e. α-pinene, camphene, benzaldehyde, benzyl alcohol and nonanoic acid) was

observed with respect to poplar plants in pristine soil. Likewise, two *Trichoderma* strains inoculation induced alteration in the profiles of leaf VOCs, either by up-regulating or down-regulating biosynthesis of some compounds. *Trichoderma harzianum* T2 (ITEM 908) inoculation led to an increase in the emission of the isoprenoids β-myrcene and α-citral in uncontaminated plants; similar result was not observed in treated plants subjected to Cd-stress. It is possible to concludde that elicitation of isoprenoid production does not seem to be involved in the Cd-tolerance strengthening mediated by the strains *T. harzianum* ITEM 908 and *T. polysporum* T60 in poplar plants, under the experimental conditions used.

Therefore, further investigations are required to understand the mechanisms induced by the two bioinoculants and involed in "Querce" clone improved tolerance under metal stress.

4.2 Introduction

The remediation of land polluted sites has recently led to exploration of appropriate phytoremediation strategies based on bio-reclamation. One *in situ* soil bioremediation strategy that has recently gained popularity is fungi-assisted phytoremediation.

In the recent years, indeed, considerable efforts have been dedicated to the study of the capability of fungi to enhance the efficiency of phytoremediation (Rajkumar et al., 2012).

Among the fungi suitable for phytoremediation, there are species belonging to the genus *Trichoderma*, which are extremely common in nature and are characterized by high colonisation potential of very different habitats (Kredics et al., 2001; Ezzy and Lynch., 2005; Harman et al 2004b).

Trichoderma species are able to support plant growth and stimulate the plant development owing to their remarkable rhizosphere competence (Rigot and Matsumura 2002), the capability to produce phytohormones and hormone-like compounds (Harman et al.2004a; Contreras-Cornejo et al. 2009; Lorito et al. 2010), solubilize and increase the avalaibility of soil nutrients for plant uptake (Altomare et al. 1999, Harman et al. 2004 a,b), and enhance the root system architecture (Contreras-Cornejo et al. 2009; Harman et al. 2004 b).

Moreover, beneficial fungi are considered the most resistant microorganisms to natural or synthetic chemicals and toxins, including heavy metals (Kacprzak et al. 2014; Pratibha et al. 2013, Harms et al., 2011).

Filamentous fungi of *Trichoderma* species are also prolific producers of some volatile metabolites, such us VOC, which have been shown to be involved in the defence responses to

plant biotic and abiotic stress leading to improved plant health (Shoresh et al., 2010; Lee et al., 2010).

Despite the high ability to decontaminate and to tolerate hazardous heavy metals and the remarkable capability to establish beneficial relationships with plants, the potential of *Trichoderma* strains for *in situ* phytotechnology has been scarcely investigated and remains to be proved.

Therefore, the aim of the present study was to explore the effects of two *Trichoderma* strains (previously isolated and preserved in ISPA laboratory) on Cd-phytoremediation operated by means of *Populus alba* "Querce" clone, with the aim to evaluate the possibility of increasing the metal uptake of the plants and to assess the capability of *Trichoderma* to colonize the roots of the "Querce" clone. The two fungi tested for this experiment were selected among twenty-seven *Trichoderma* strains, previously characterized for their Cd tolerance (chapter III), based on their remarkable abilities proved under Cd exposure.

Given the absence of any previous data focused on the *Trichoderma*-poplar interaction, poplar VOCs emission was also evaluated by GC-MS, and the changes in the spectrum of volatiles emitted by poplar as a result of bioinoculation with *Trichoderma* were determined. The results allowed to proof some physiological modifications of poplar plants with respect to volatiles compouns emitted in response to Cd exposure and within the plant-microbe association.

4.3 Material and Method

4.3.1 Experimental design

A pot experiment was set up under a completely randomized experimental block design included three bioinoculant treatments in pristine soil (blank samples): i) uninoculated negative control (C-); ii) *Trichoderma polysporum* T60 inoculum (T1 -); iii) *Trichoderma harzianum* 908 WT inoculum (T2-), as well as three bioinoculants treatments in Cd-contaminated soil: iv) uninoculated positive control (C+); v) *Trichoderma polysporum* T60 inoculum (T1+); vi) *Trichoderma harzianum* 908 WT inoculum (T2 +).

The treatments identified as C - , T1 - and T2-, carried out in absence of cadmium, were used as controls. Each treatment had ten replicates and each experimental unit consisted in one individual plant, giving a total of 60 plants.

4.3.2 Plant Material

Populus alba L. clone "Querce" was micropropagated through microshoot cultures, according to Confalonieri et al. (2000). The "Querce" clone, selected in Greve in Chianti (FI), was routinely subcultured on Woody Plant Medium substrate (WPM; Lloyd and McCown 1980) at pH 5.5. Cultures were incubated in a growth chamber for four week at 23 ± 1 °C with 8-h photoperiod and 3000 lx of light intensity (T5 fluorescent lamps, 28 watts). Then, aseptic cultures were transferred on BA (benzyl adenine) devoid medium for 3 weeks to prevent its carry-over effect and to promote rhizogenesis. After four proliferation weeks, the deeply-rooted seedlings were transferred in 120 ml- plastic pots filled with perlite substrate.

Plants were acclimatized into Microbox (model type: OS114+ODS114), provided by depth-filtration system, and thus irrigated alternately with sterile water and Hoagland nutrient solution (pH 5.8). After 15 days of acclimation the treatment was started.

4.3.3 *Trichoderma* strains

The fungal isolates, *Trichoderma polysporum* T60 (T1) and *Trichoderma harzianum* T908 WT (T2), derived from single-spore isolations and were identified on the basis of morphological characteristics according to Gams and Bisset (1998). Bioinoculants were stored at + 4 °C on Potato-Dextrose-Agar (PDA) slants and used as inocula for sub-cultures throughout the work.

4.3.4 Inoculation and pot experiment

Trichoderma strains were activated in PDA medium and then prepared as a conidial suspension (10^5 conidia/ml). The inoculation was performed by keeping the conidial suspension onto a liquid matrix (sterile water) that was used to fill the pots. Further, Cd was supplied as $CdSO_4$ totally dissolved in the inoculated liquid matrix and distributed in each pot, never exceeding the field capacity; for blank treatments, no cadmium was added.

The experiment was conducted in a growth chamber at room temperature with a 8-h photoperiod and 3000 lx of light intensity (T5 fluorescent lamps, 28 watts). During the experiment the plants, were grown in pots, amending them with half strength Hoagland solution that was renewed in each treatment every two days to ensure the steady rhizosphere environment.

4.3.5 Plant biomass and Cd content

Plants (n= 10) were harvested after 20 days growth, splitting leaf, stem and root. The roots were carefully washed with deionized water and then placed for 10 min in 10 mM $CaCl_2$ to remove all metal adherent to the root surface. Plant materials were collected and then dried in a ventilated stove at 65 °C for 24 h until constant weight for the determination of dry mass.

4.3.6 Cadmium content

4.3.6.1 Chemical tissue analysis

For determination of inorganic elements, 0.25 g of dried material, sampled at the moment of the harvest, were accurately weighted in microwave digestion vessels followed by addition of 6 ml of 65% HNO_3 (VWR Chemicals) and digested in a closed-vessel microwave assisted digestion system (MARS 6, CEM Corporation, Matthews, NC, USA). The digestion procedure was performed in two steps: 15 min to reach 200 °C and 10 min maintained at 200 °C (power set at 900–1050 W; 800 psi). Blanks, HNO_3 without sample, were also prepared and digested in the same conditions. The digested solutions were cooled and quantitatively transferred to 50 ml volumetric flask. Each solution was diluted to volume with ultrapure H_2O (Milli-Q Millipore 18MΩ cm^{-1}) and filtered using a 0.45 μm filter. Samples were analyzed with ICP-OES (5100 VDV, Agilent Technologies, Santa Clara, CA, USA) to measure both major (Ca, Mg, Na and K) and minor elements (Cu, Fe, Mn, Al and Zn). The first group was measured in radial mode, the second one in axial mode.

4.3.7 Tolerance index and Translocation factor

Phytoremediation parameters such as tolerance index (Ti) and translocation factor (TF) were examined (Wilkins 1978; Liu et al. 2009) to value the metal toxicity effect on poplar growth and the metal translocation capability of “Querce” clone. In the current study, the Ti was expressed on the basis of plant growth parameters including green biomass of the seedlings, while the TF provided disclosure about Cd uptake from the roots to shoots. Ti and TF values for cadmium were calculated as follows (eqn. 1 and eqn. 2):

$$Ti = FW_t / FWc \quad (1)$$

$$TF = shoot_t / root_t \quad (2)$$

where FW_t and FW_c are the fresh weights of the plants grown in contaminated soil and control, respectively, and $shoot_t$ and $root_t$ are the concentrations of cadmium in shoots and roots, respectively.

Furthermore, basing on the concentrations of total cadmium in the plants and in the shoots, metal translocation efficiency (ratio between Cd shoot content and total Cd content in pants) was calculated; then the capacity of poplar clone alone and in combination with the two bioinoculants in translocating the metal in the aerial part was compared. Data was expressed as percentage (%).

4.3.8 Chlorophyll Content

Photosynthetic efficiency of heavy metal-stressed poplars (n = 3) was assessed by measuring the chlorophyll fluorescence of primary leaves. Chlorophylls (Chl) content was determined spectrophotometrically using the extraction procedure reported by Bonasia et al. (2013). Lyophilized sample were homogenized in 80% acetone for 24 hrs at room temperature; after extraction, the absorbance was measured at 662, 645 and 470 nm, using a UV-1800 spectrophotometer (Perkin- Elmer Lambda 25 spectrophotometer, Boston, MA, USA).

4.3.9 Plant-root colonization ability of *Trichoderma* strains

After plant harvesting procedure, root samples (n=3) from each treatment plot were collected cleaning up gentled by the surrounding soil. Detection and quantification of fungal colony forming units (CFU) was conducted using standard soil microbial plating techniques by serial dilutions using a protocol exploited by Rosa and Herrera (2009). Fungal colony forming units (CFUs) was estimated on fresh *Trichoderma* Selective Medium (TSM) (Smith et al., 1990) supplemented with 0.05% (v/v) Igepal (Sigma-Aldrich, Milan, Italy).

Root, was cut into pieces and transferred to Erlenmeyer flask containing 100 ml sterile distilled water, then 5-fold serial dilutions of the suspension were performed. A 0.1 ml aliquot of each dilution was spread on the surface of the solid media and plated in two replicates.

Therefore, plates were incubated for 5 days at 25ºC; after this time, the population *Trichoderma* colonies thrived on each Petri dish were recorded expressing data as Log_{10} of colony forming units (CFU) per gram dry root weight (dw) (Log_{10} CFU g^{-1} dw). *Trichoderma* strains abundance on plant Cd-treated root was compared with fungal abundance at root level

in plants grown in pristine soil in order to value *Trichoderma* root-colonization ability after 20 days of metal exposition.

4.3.10 Emission of VOCs and SPME-GC/MS analyses

4.3.10.1Chemicals and reagents

Methanol (HPLC grade) was purchased from SigmaAldrich (Milan, Italy). Ten milliliters headspace vials with magnetic screw cap containing a pierceable PTFE/silicon septa were purchased from Agilent Technologies (Palo Alto, CA, USA). The SPME fiber holder was obtained from Gerstel (Mulheim an der Ruhr, Germany) and SPME-Fast Fit Fiber Assembly (FFA) divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm film thickness, 1 cm fiber length) was purchased from Supelco (Bellafonte, PA, USA). Helium at a purity of 99.9995% was obtained by Sapio s.r.l. (Bari, Italy). SPME liner, 0.75 mm was purchased from Agilent Technologies (Palo Alto, CA, USA). The 4-Methyl-2-pentanol (≥ 98%) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). A mixture of normal alkanes (C5-C29) was from o2si smart solutions (Charleston, SC, USA).

4.3.10.2 Sample Preparation for VOCs analysis

Three plants selected randomly from each treatments were used for evaluating by GC-MS the changes in the spectrum of volatiles emitted by poplar under Cd stress as a result of bioinoculation with *Trichoderema*. VOCs from poplar leaf were extracted under optimized experimental conditions in the four youngest apical leaves (collected for each biological replicate) from 60 days old poplar plants. Thus, VOCs extraction was totally performed on 18 leaves samples (4 leaves from the apex were collected for each biological replicate). The freshly picked leaves were immediately placed into vials and processed for VOC extraction.

4.3.10.3 HS-SPME/GC–MS analysis

A Headspace Solid Phase Microextraction (HS-SPME) procedure for VOC extraction and desorption was automatically performed using a multi-purpose sampler MPS 2 (Gerstel, Mulheim an der Ruhr, Germany), equipped with headspace incubation chamber and SPME sampling unit. In particular, 0.45 g of sample were placed in a 10 mL headspace vial and kept at temperature of 40 °C for 10 min in incubator-agitator of the MPS 2 autosampler to generate the headspace. The extraction from the headspace was performed exposing a

divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/ PDMS, 1 cm fiber length) fiber at 40 °C for 30 min.

After extraction, compounds were thermally desorbed in the cis-4 programmed temperature vaporization (PTV) injector (Gerstel) of the gas chromatograph at 250 °C for 5 min. The analyses were carried out by an Agilent 7890A GC System (Agilent Technologies, Palo Alto, CA, USA) with an Agilent 5975C inert MSD mass spectrometer equipped with MPS 2 autosampler and using a VF-WAXms (60 m ×0.25 mm i.d., 0.25 μm film tickness, Agilent Technologies) fused-silica capillary column. The injection port fitted with a 0.75 mm i.d. Ultra Inert liner Straight was maintained at 250 °C in splitless mode. The analyses were performed with programmed temperature mode: the temperature program started at 40 °C and held for 5 min, then raised at the rate of 2 °C min^{-1} to 140 °C, then raised at the rate of 5 °C min^{-1} to 210 °C, then raised at the rate of 20 °C min^{-1} to 230 °C and held for 10 min. The total chromatographic run time was 80 min. Each sample was analyzed in triplicate. The helium flow rate was held constant at 1 mL min^{-1}. The transfer line, ion source and quadrupole temperatures were 280, 230 and 150 °C, respectively. Electron impact Ionization (EI +) mode with an electron energy of 70 eV was used. The mass spectrometer acquired data in full scan mode (scan range: 40–300 μ). The compounds were identified by comparison of experimental mass spectra with spectra in the NIST/EPA/NIH Mass Spectral Database (National Institute of Standards and Technology, Version 2.0 f, 2008, USA) using a match quality higher than 80.

The identification of volatile compounds was also verified by comparison of their linear retention indices (LRI) (as Kovats indices, Zellner et al., 2008) determined in relation to the retention times of C5–C29 n-alkanes series, and compared with those reported in literature (Zellner et al., 2008; www.chemspider.com; www.flavornet.org/f_kovats.html; www.nist.gov; www.pherobase.com).

Quantification of compounds was performed with internal standardization by adding 2.5 μL of 4-Methyl-2-pentanol 200 mg L^{-1}. The quantitative evaluation of the compounds was determined as ratio between their peak areas and the 4-Methyl-2-pentanol peak area. All acquired data were combined to obtain one data matrix containing 18 objects (samples) and 81 variables (identified volatile molecules) including all samples and two data matrices, containing 9 objects and 81 variables, relevant to the single case studies (poplar plants growth in presence or absence of cadmium), that were submitted to statistical analyses. The SPME-GC/MS data matrices were analysed by Pirouette software ver. 4.0 (Infometrix, Inc., Bothell, WA, USA) and using Principal Component Analysis (PCA) (unsupervised statistical approach) as an exploratory tool of data structure (Vandeginste et al., 1997) with the aim to visualize if sample clustering was present. In addition, one-way ANOVA was performed by Statistica 6.0 (StatSoft,

Tulsa) on the HS-SPME/GC–MS data matrices to investigate the effect of presence of cadmium and fungal treatments with respect to the control samples (Figure 19).

Figure 19 HS-SPME/GC–MS analysis on poplar leaves

4.4 Statistical analysis

Statistical analysis was performed in triplicate (repeated at least four times) independent experiments. Data were averaged on plant and fungal basis. The effects of Cd treatment on growth parameters, elements contents, tolerance index, translocation factor and chlorophyll content were defined through one-way analysis of variance (ANOVA) while HMs influence on nutrients content within plant portions (root, stem and leaf) were tested via Two-way analysis of variance (ANOVA). Differences of measured parameters between treatments were assessed through a post hoc comparison of means using the least significant difference (LSD) at the 0.05 and 0.001 significance level. Statistical analysis was performed using the statistical program Statistica (StafSoft Inc., Tulsa, OK, USA) and SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA).

The SPME-GC/MS data matrices were analysed by Pirouette software ver. 4.0 (Infometrix, Inc., Bothell, WA, USA) and using Principal Component Analysis (PCA) (unsupervised statistical approach) as an exploratory tool of data structure (Vandeginste et al., 1997) with the aim to visualize if sample clustering was present. In addition, one-way ANOVA was performed by Statistica 6.0 (StatSoft, Tulsa) on the two HS-SPME/GC–MS data matrices (poplar plants

growth in presence or absence of cadmium) to investigate the effect fungal treatments with respect to the control samples.

4.5 Result

4.5.1 Plant fresh and dry biomass.

Poplar plants grown in Cd-contaminated soil showed reduced values of fresh weights of roots, stems and leaves (Table 11), compared to blank plants grown in absence of Cd. Only the *Trichoderma* strain T2 enhanced fresh biomass of roots by 44.3 % in comparison to the control plants (amended only with Cd) and by 54.6% as compared to plants inoculated with T1 ($P < 0.05$). Moreover, T2 associated with "Querce" plants determined a slight decrease in the dry weight at the stem and leaf level. For the rest (blank, Cd control, T1), the dry weights of stems and leaves did not significantly differ among treatments.

Treatment	Fresh weight (g)	Dry weight (g)
Root		
Blank	0.42 ± 0.04 a	0.04 ± 0.01 a
Cd control	0.21 ± 0.03 b	0.03 ± 0.00 b
T1	0.26 ± 0.03 b	0.03 ± 0.01 ab
T2	0.48 ± 0.04 a	0.02 ± 0.00 b
Stem		
Blank	0.25 ± 0.01 a	0.04 ± 0.00 a
Cd control	0.18 ± 0.02 b	0.04 ± 0.00 a
T1	0.18 ± 0.01 b	0.04 ± 0.00 a
T2	0.18 ± 0.02 b	0.03 ± 0.00 b
Leaf		
Blank	0.9 ± 0.04 a	0.14 ± 0.01 a
Cd control	0.46 ± 0.05 b	0.13 ± 0.01 a
T1	0.45 ± 0.04 b	0.14 ± 0.01 a
T2	0.49 ± 0.04 b	0.10 ± 0.01 b

Table 11 Fresh and dry biomass of root, stem and leaf of inoculated and blank plants of "Querce" clone subjected to high Cd concentration (250 μM). Values are means ± standard deviation; significant differences between the means ($p < 0.05$, according to ANOVA and LSD test) appear with different letters

4.5.2 Chlorophyll content

Plants subjected to high Cd concentration, inoculated and not with *Trichoderma*, displayed significant decrease in Chl content compared with the blank, while no differences were found among control plants and bioinoculated plants (Figure 20).

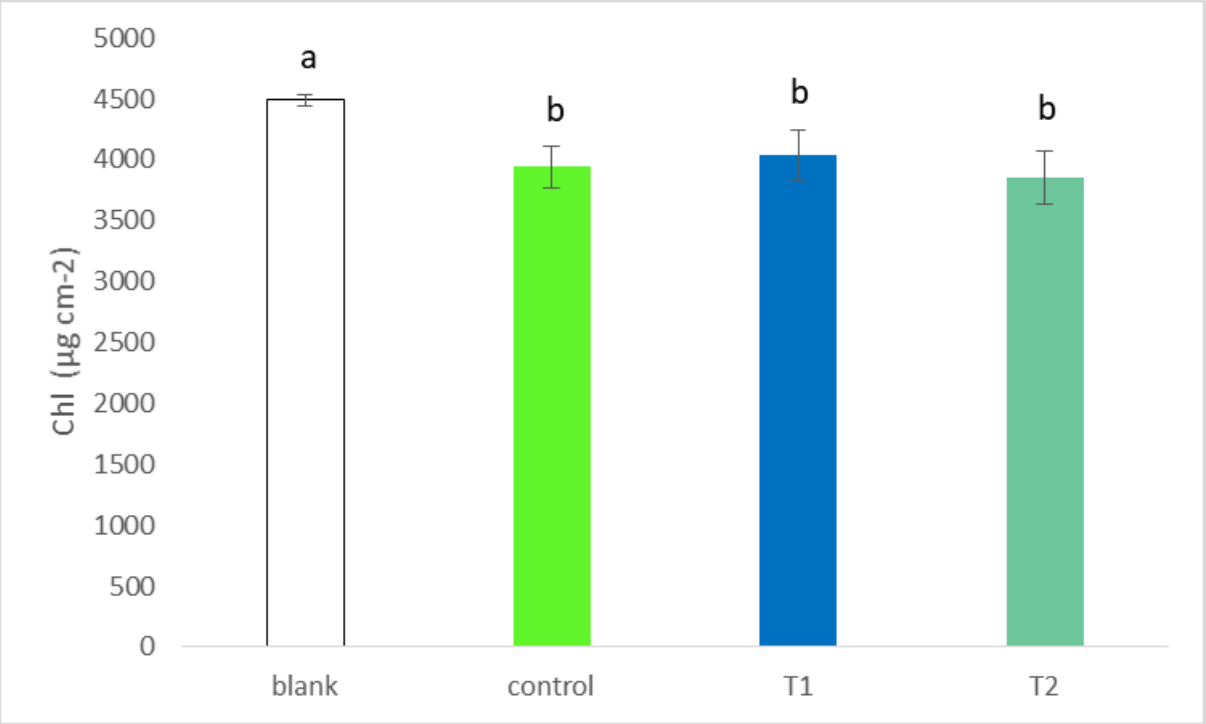


Figure 20 Chlorophyll content in inoculated and blank plants of "Querce" clone subjected to high Cd concentration (250 µM). Values are means ± standard deviation; significant differences between treatment are reported with different letters ($p < 0.05$, according to ANOVA and LSD test).

4.5.3 Cd tolerance, translocation and uptake capability

The bio-inoculation of poplar plants with *Trichoderma* strains did not affect the total metal content of both inoculated and not inoculated plants (Figure 21)

"Querce" clone showed no significant difference in Cd content in root level of T1 and T2 inoculated plants compared to control plants (Figure 22); while T2-inoculated plants exhibited the highest Cd content in stems (Figure 22). T2 inoculum improved the Cd content by 53.86 % in the stems as compared with the control. The same treatment showed a reduction of Cd content at root level by 27.33% with regard to the not inoculated plants in contrast to the reduction of metal concentration by only 7.29 % in T1-inoculated plants although the differences in the metal content at root level between the various treatments. were not significant. Comparing plants treated with T1 with the control no remarkable improvement in the Cd translocation was found (no Cd enhancement in steam and 29.47 % reduction of metal in leaves).

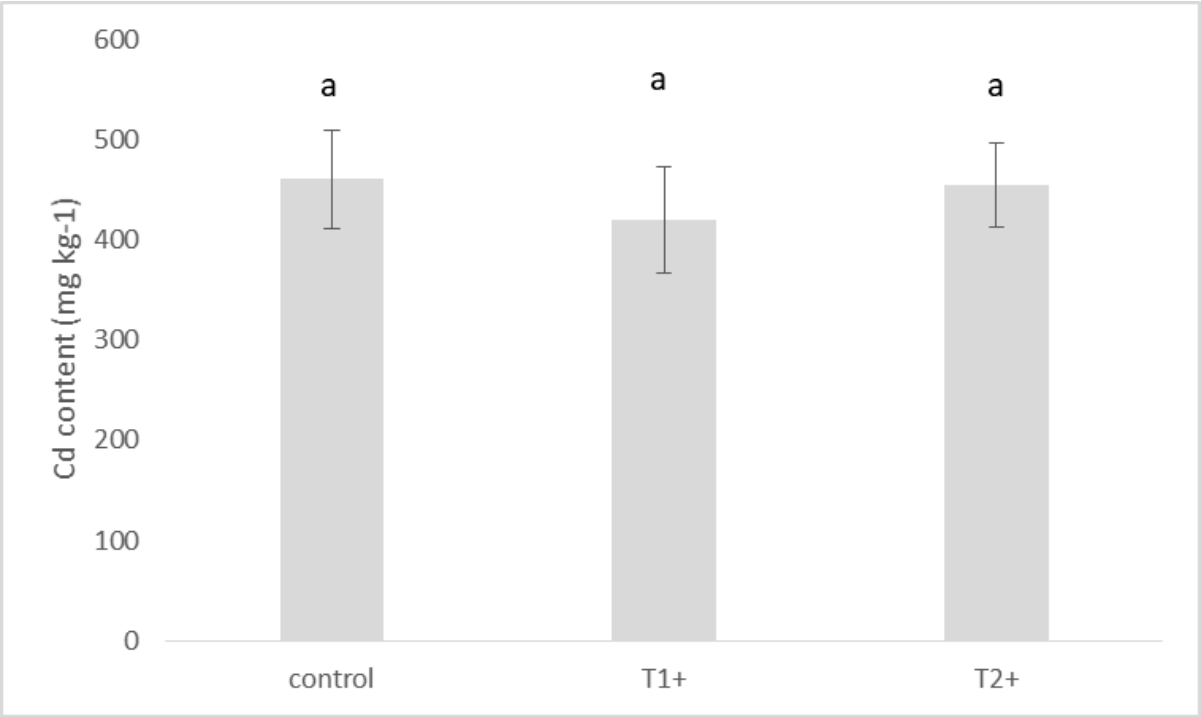


Figure 21 Cadmium content in inoculated and controls plants of "Querce" clone after 20 days of exposure to high Cd concentrations (250 µM). Values are means ± standard deviation, significant differences between treatments are reported with different letters ($p < 0.05$, according to ANOVA and LSD test).

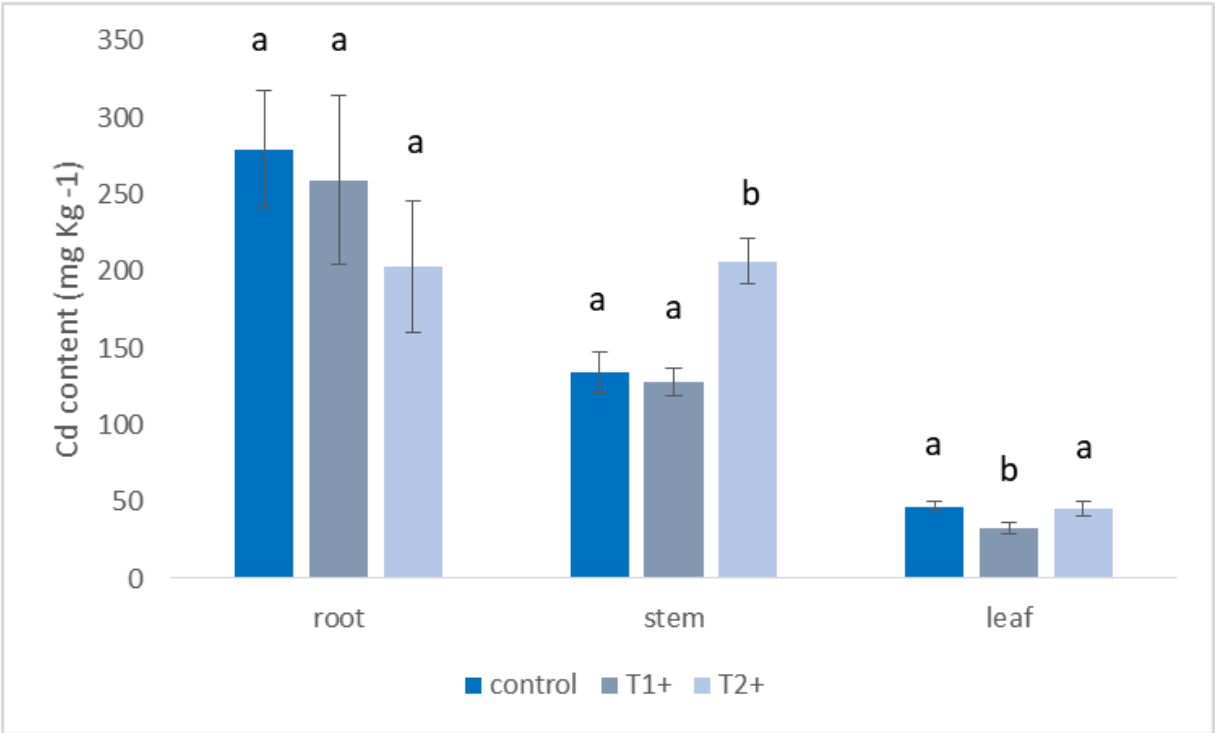


Figure 22 Cadmium content in inoculated and controls plants of "Querce" clone at root, stem and shoot level after 20 days of exposure to high Cd concentrations (250 µM). Values are means ± standard deviation, significant differences between treatments are reported with different letters ($p < 0.05$, according to ANOVA and LSD test).

The values of tolerance index indicated tolerance of this clone under exposure to excess metal concentration in presence of *Trichoderma* T2 (0.78), while T1 inoculation did not show significant tolerance (0.61) in "Querce" clone compared with the control (0.56) (Table 12, Figure 23). Moreover, the plants treated with T2 exhibited a higher translocation factor (1.24) (Table 12, Figure 24).

	Ti	**TF**
Control	0.56 ± 0,07 a	0,65 ± 0,05 a
T1+	0.61 ± 0,055 a	0,62 ± 0,03 a
T2+	0.78 ± 0,65 b	1,24 ± 0,06 b

Table 12 Tolerance index (Ti) and Translocator factor (TF) in inoculated and controls plants of "Querce" clone at after 20 days of exposure to high Cd concentrations (250 µM). Values are means ± standard deviation, significant differences between treatments are reported with different letters ($p < 0.05$, according to ANOVA and LSD test).

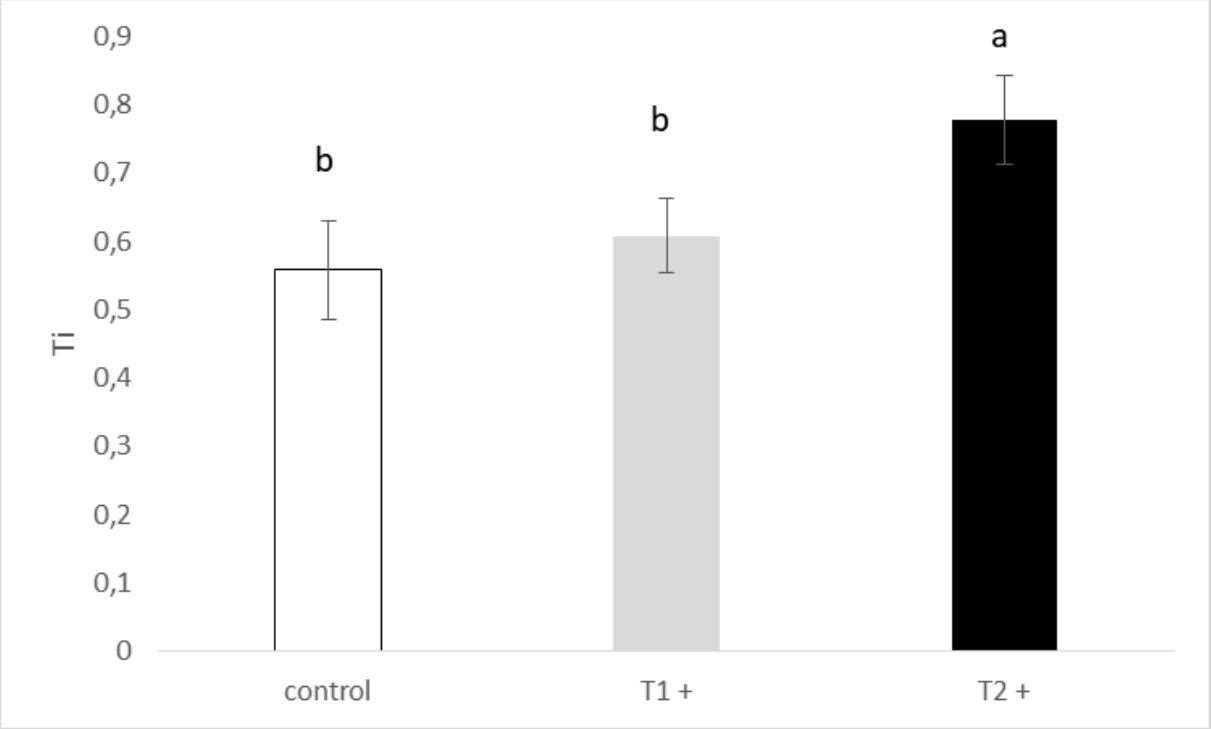


Figure 23 Tolerance index (Ti) in shoot and root of "Querce" clone after 20 days of exposure to high Cd concentrations (250 µM). Values are means ± standard deviation, significant differences between treatments are reported with different letters ($p < 0.05$, according to ANOVA and LSD test).

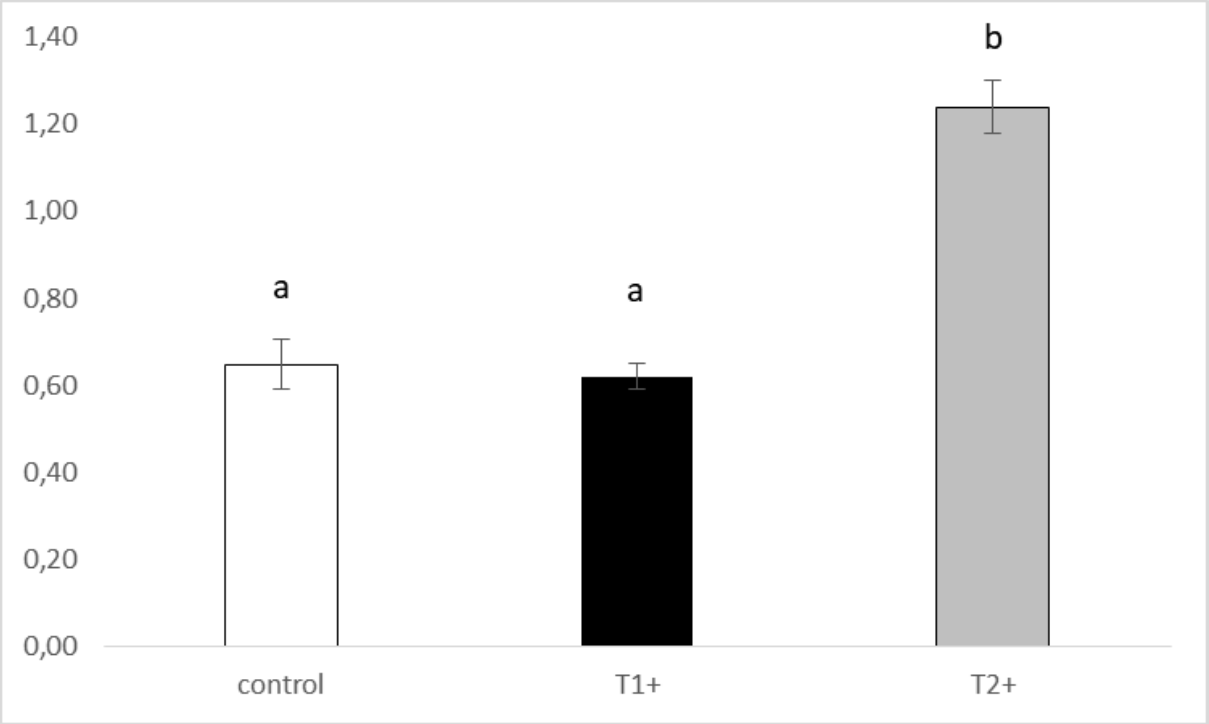


Figure 24 Translocator factor (TF) in shoot and root of "Querce" clone after 20 days of exposure to high Cd concentrations (250 µM). Values are means ± standard deviation, significant differences between treatments are reported with different letters ($p < 0.05$, according to ANOVA and LSD test).

The metal translocation efficiency of "Querce" alone (the control treatment) were 40.39 % (Table 13). After the inoculation with T1 and T2, the metal translocation efficiency of poplar was 42.17 % and 57.99 % respectively. This latter value was significantly higher than the control, unlike the metal translocation efficiency of T1.

	Metal translocation efficiency (%)
control	40.39 ± 2.68 a
T1+	42.17 ± 4.70 a
T2+	57.99 ± 4.72 b

Table 13 Metal translocation efficiency of "Querce" clone after 20 days of exposure to high Cd concentrations (250 µM).

4.5.4 Elements concentration

Plants inoculated with *Trichoderma* and exposed to Cd underwent significant changes of elements concentration (Ag, Al, B, Ba, Cr, Cu, Fe, Mg, Mn, Na, Sr, Zn) in root, shoot and leaf levels compared to blank plants (Table 14). Nutrients concentrations in root were significantly reduced in biotreated plants except for Ag, Cu, Na and Sr, which were enhanced in T1 and T2

inoculated plants in relation to the blank. At stem level, Ag, Al, Ba, Cr, Fe, Mn concentrations increased in T2 treatment under Cd stress, while Zn content was affected by the Cd in T1 and T2 treatments. Elements content was significantly reduced in leaf by the Cd in inoculated and not plants whereas, Al showed higher concentration in the control than in other treatments.

	mg kg^{-1}											
	Ag	**Al**	**B**	**Ba**	**Cr**	**Cu**	**Fe**	**Mg**	**Mn**	**Na**	**Sr**	**Zn**
Root												
blank	67.75 ± 2.01 aA	528.56 ± 1.79 aA	61.23 ± 0.40 aA	12.65 ± 0.07 aA	2.29 ± 0.08 aA	11.76 ± 0.42 aA	1103.38 ± 11.08 aA	6302.90 ± 69.32 aA	298.42 ± 1.04 aA	7336.46 ± 88.20 aA	8.65 ± 0.04 aA	64.76 ± 0.26 aA
control	137.73 ± 3.18 bA	405.36 ± 3.48 bA	47.46 ± 0.30 bA	8.37 ± 0.08 bA	1.47 ± 0.04 bA	23.11 ± 0.44 bA	971.61 ± 3.38 bA	4063.12 ± 11.06 bA	49.09 ± 0.38 bA	7847.11 ± 26.90 bA	12.75 ± 0.09 bA	53.03 ± 0.62 bA
T1+	133.54 ± 3.3 bcA	475.62 ± 2.66 cA	37.95 ± 0.05 cA	8.02 ± 0.04 cA	1.99 ± 0.06 cA	12.24 ± 0.56 bA	894.34 ± 1.03 cA	4681.67 ± 4.99 cA	43.41 ± 0.06 cA	7193.18 ± 8.86 cA	13.03 ± 0.01 cA	49.95 ± 0.13 cA
T2+	128.41 ± 5.34 cA	416.67 ± 0.36 dA	39.77 ± 0.13 dA	9.34 ± 0.02 dA	2.57 ± 0.05 dA	12.55 ± 0.65 bcA	825.51 ± 3.38 dA	4087.99 ± 22.28 dA	74.34 ± 0.18 dA	7444.01 ± 8.32 aA	12.12 ± 0.04 dA	51.45 ± 0.61 dA
Stem												
blank	108.93 ± 5.33 aB	72.15 ± 0.89 aB	39.47 ± 0.10 aB	7.31 ± 0.02 aB	0.44 ± 0.07 aB	0.00 ± 0.00 aB	81.35 ± 0.94 aB	3723.22 ± 10.07 aB	32.28 ± 0.03 aB	2227.43 ± 4.08 aB	11.31 ± 0.03 aB	60.02 ± 0.34 aB
control	97.83 ± 2.39 bB	51.22 ± 1.00 bB	36.49 ± 0.36 bB	5.87 ± 0.04 bB	0.51 ± 0.02 bB	0.00 ± 0.00 aB	47.85 ± 0.47 bB	5043.44 ± 26.72 bB	29.18 ± 0.13 bB	3285.76 ± 31.10 bB	8.95 ± 0.04 bB	52.31 ± 0.90 bA
T1+	107.26 ± 2.48 aB	45.63 ± 1.63 cB	39.88 ± 0.13 aB	6.30 ± 0.02 aB	0.37 ± 0.04 bB	0.00 ± 0.00 aB	36.14 ± 0.52 cB	5930.14 ± 45.96 cB	38.71 ± 0.10 cB	2790.48 ± 27.77 cB	12.39 ± 0.05 cB	57.67 ± 0.33 cB
T2+	129.27 ± 1.75 cA	411.9 ± 1.63 dA	39.76 ± 0.22 aA	9.18 ± 0.02 cB	2.58 ± 0.03 cA	0.00 ± 0.00 aA	823.40 ± 4.30 dA	4071.25 ± 26.51 dA	74.52 ± 0.14 bA	7249.00 ± 29.66 bB	12.03 ± 0.03 cA	53.11 ± 0.2 bB
Leaf												
blank	148.49 ± 2.56 aC	88.03 ± 0.55 aC	143.96 ± 0.92 aC	6.79 ± 0.04 aC	2.04 ± 0.13 aA	0.00 ± 0.00 aB	244.46 ± 3.78 aC	7348.08 ± 75.23 aC	138.82 ± 0.74 aC	449.90 ± 4.32 aC	14.53 ± 0.05 aC	61.65 ± 0.32 aC
control	95.23 ± 3.56 bC	107.95 ± 1.31 bC	126.92 ± 0.72 bC	3.39 ± 0.03 bC	1.26 ± 0.05 bC	0.00 ± 0.00 aB	85.67 ± 0.58 bC	4685.36 ± 32.74 bC	110.60 ± 0.31 bC	443.64 ± 1.78 bC	6.30 ± 0.01 bC	61.75 ± 0.29 aB
T1+	65.02 ± 4.82 bcC	67.78 ± 0.26 cC	96.33 ± 0.77 cC	2.90 ± 0.06 cC	0.95 ± 0.02 cC	0.00 ± 0.00 aB	50.11 ± 0.60 cC	3503.88 ± 21.79 cC	75.56 ± 0.52 cC	348.31 ± 2.46 cC	6.28 ± 0.05 cC	46.17 ± 0.40 cC
T2+	122.29 ± 4.19 cA	75.08 ± 2.66 cB	131.48 ± 0.81 dB	4.62 ± 0.04 dC	0.74 ± 0.05 dB	0.00 ± 0.00 aB	95.53 ± 0.37 dB	5676.32 ± 15.50 bB	114.00 ± 0.57 dB	427.87 ± 2.48 aC	7.65 ± 0.05 dB	48.59 ± 0.31 dC

Table 14 Concentrations of elements (mg kg^{-1}) in roots and stem and leaf of clone “Querce” subjected to Cd (250 µM Cd) treatments after 20 dayes. Values are the result of ICP analysis. Values are means ± standard errors. Significant differences between the means ($p < 0.05$, according to ANOVA and LSD test) appear with different letters

4.5.5 *Trichoderma* Colonization Ability

In general, the total fungal concentration (CFU) of the two applied *Trichoderma* bioinoculants seemed to be not influenced by high metal presence (Figure 25). Cd treatment did not negatively affected the fungal CFU in Querce root, on the contrary a significant increase (by 17.72%) of *Trichoderma* concentration was observed with the T1 inoculum comparing data with the control and T2 inoculation. The results show that the abundance of *Trichoderma* T1 under Cd treatment reached great colonization level of 2.08 x 10^6 CFU g^{-1} dw after phytoremediation, which was significantly higher ($p < 0.05$) than the other three treatments (T1: 1,95x10^5 CFU g^{-1} dw, T2: 6,7x10^5 CFU g^{-1} dw, T2+:3,08x10^5 CFU g^{-1} dw).

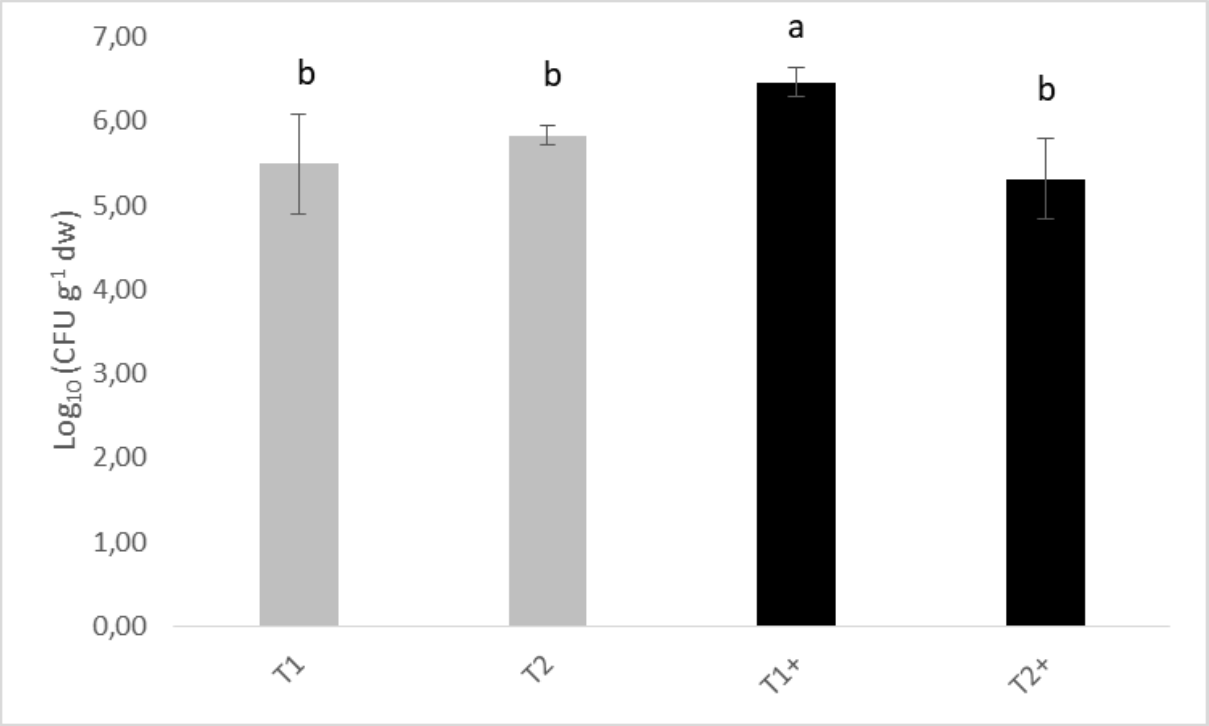


Figure 25 *Trichoderma* colonization ability at root level

4.5.6 Analysis of volatile organic compounds (VOCs) by HS-SPME/GC-MS

Volatile organic compounds were isolated from poplar leaves using headspace solid-phase microextraction (HS-SPME) technique. A HS-SPME/GC-MS method was optimized to characterize the pattern of VOCs related with the two different fungal treatments, i.e. T. atrobrunneum ITEM 908 and T. polysporum T60, in poplar plants grown in presence or absence of cadmium. In particular, SPME fibers (DVB/CAR/PDMS and PDMS/DVB, 1 cm fiber length), extraction temperatures (ranging from 30 to 60 °C), headspace equilibration times (ranging from 5 to 60 min) and extraction times (ranging from 5 to 60 min) were tested for their

efficacy in isolating VOCs. Individual parameters were changed once at a time while keeping constant the other parameters. The optimal experimental parameters, in terms of intensity and resolution of peaks, for headspace analysis were DVB/CAR/PDMS fiber, extraction temperature 40 °C, equilibration time of headspace 10 min and extraction time 30 min. Volatile compounds of poplar leaves were analysed under the optimized experimental conditions from poplar plants grown for 60 days. A total of 81 volatile compounds was identified by HS-SPME/GC-MS analysis of 18 poplar leave samples belonging to a wide range of chemical classes including aldehydes (7), ketones (8), esters (26), acids (4), alcohols (18), terpenes (14), saturated, unsaturated and aromatic hydrocarbons (3) and sulfide (1). The identified volatile compounds for all poplar leave samples and the retention times are listed in Table 15.

PCA was applied to the entire data set accounting only for 61.0% of the total variance, with factors (PC) 1 and 2 accounting for 34.4% and 23.8%, respectively. However, the scatter plot of scores of the first two components PC1 *vs* PC2 (Figure 26) showed a partial separation between poplar samples growth in presence of cadmium with respect to those growth in absence of cadmium, independently to their fungal treatments.

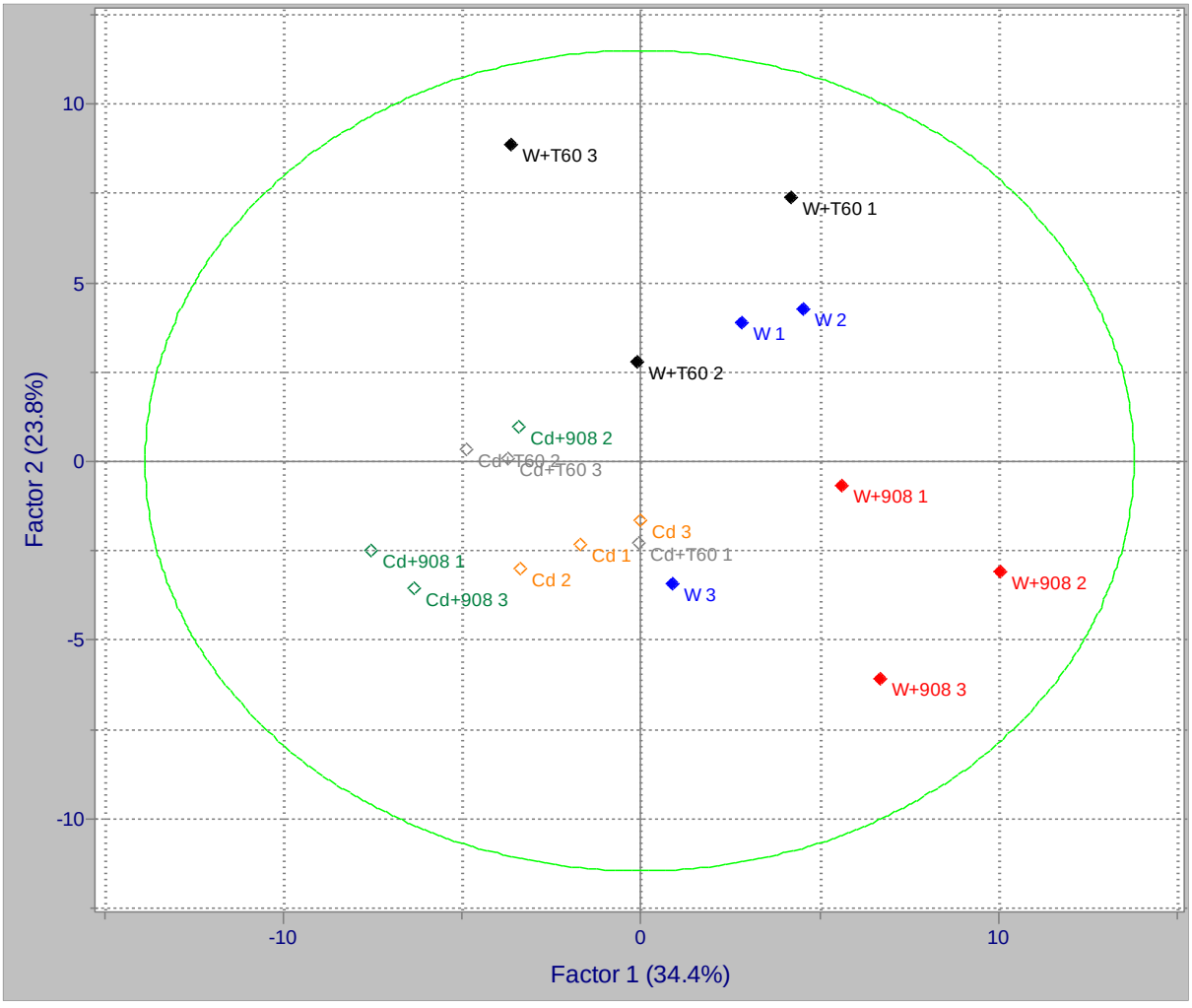


Figure 26 PC1 vs. PC2 scatter plot for the entire data set.

Figures 27 and 28 showed the scatter plots PC1 vs PC2 obtained for poplar samples not inoculated (controls) and inoculated with *Trichoderma* strains ITEM 908 and T60 and grown in absence or presence of cadmium, respectively. Although no separation was observed among poplar samples grown in presence of cadmium (Figure 28) a partial separation on the PC1 was obtained between samples not inoculated (W 1-3) and inoculated with *Trichoderma* strains ITEM 908 (W+908 1-3) or T60 (W+T60 1-3) and grown in absence of cadmium. (Figure 27).

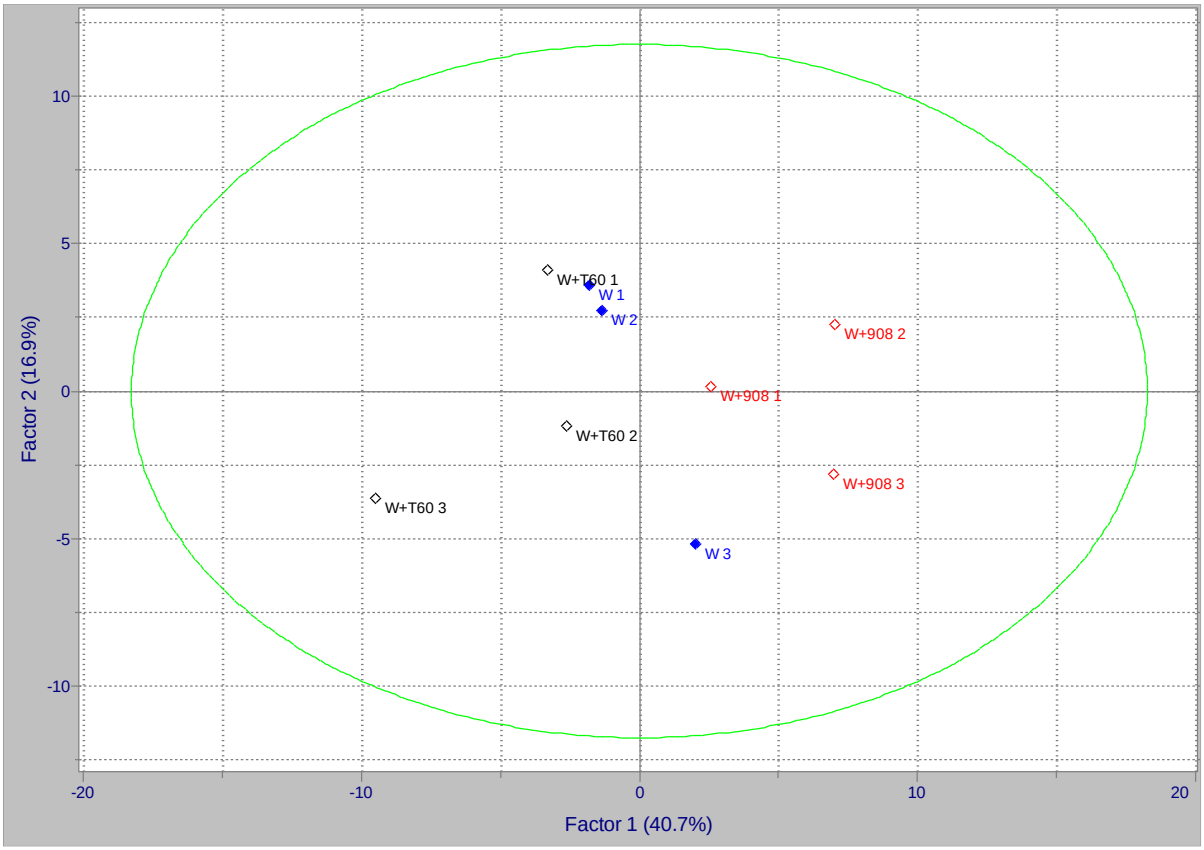


Figure 27 PC1 vs. PC2 scatter plot for poplar samples not inoculated (control, W 1-3) and inoculated with *Trichoderma* strains ITEM 908 and T60 (W+908 1-3; W+T60 1-3) and grown in absence of cadmium.

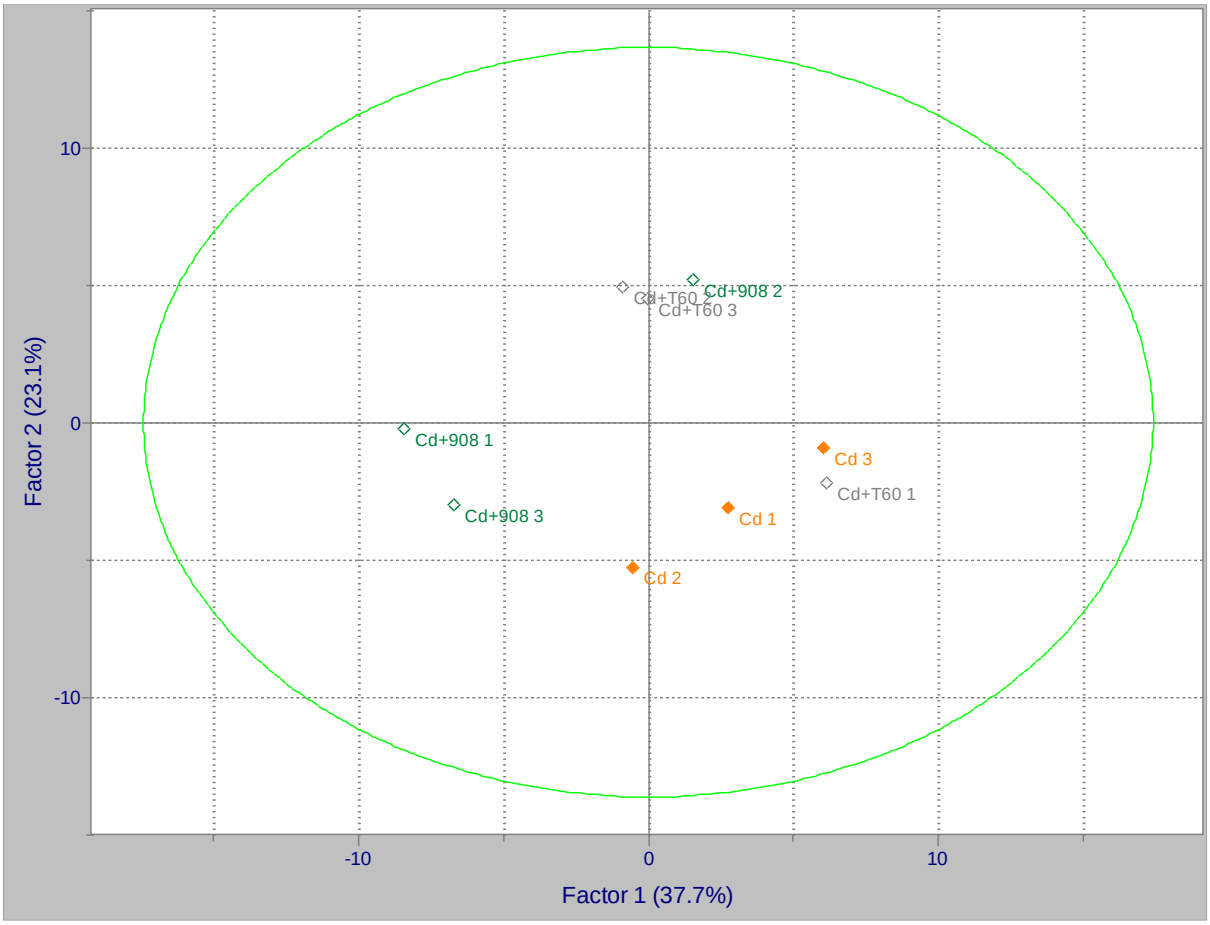


Figure 28 PC1 vs. PC2 scatter plot for poplar samples not inoculated (control, Cd 1-3) and inoculated with *Trichoderma* strains ITEM 908 and T60 (Cd+908 1-3; Cd+T60 1-3) and grown in presence of cadmium.

Subsequently, one-way ANOVA analyses followed by a *post hoc* Dunnett's test (Winer et al., 1991) were performed in order to assess differences between VOC contents in poplar samples in relation to: i) presence or absence of cadmium; ii) their fungal treatments with the *Trichoderma* strains ITEM 908 and T60 with respect to the controls in both tested case studies (i.e. presence or absence of cadmium).

For poplar samples grown in presence of cadmium a significant reduction ($p < 0.05$) of the contents of five VOCs, i.e. α-pinene, camphene, benzaldehyde, benzyl alcohol and nonanoic acid, was observed with respect to the poplar plants grown in absence of cadmium.

In the case of poplar samples grown in absence of cadmium, among the identified compounds the contents of eight molecules (hexanal, β-myrcene, (Z)-2-hexenal, (E)-2-hexenal, 1-hexanol, 2-ethyl-1-hexanol, 1-nonanol and α-citral) were significantly ($p < 0.05$) higher in samples inoculated with the strain ITEM 908 than those measured in control samples. On the other hand, samples inoculated with the strain T60 showed significant differences only for three VOCs, with higher content for butanoic acid-methyl ester and a lower content for acetaldehyde and phenol than those observed for control samples.

For poplar samples growth in presence of cadmium (Figure 29), one-way ANOVA analysis showed few differences in VOC contents of inoculated samples with respect to those measured for the control samples. In particular, a significant reduction of VOCs content ($p < 0.05$) was observed for hexanal, 1-penten-3-ol, 1-pentanol, benzaldehyde and octanoic acid for samples inoculated with *Trichoderma* ITEM 908 and octanoic acid-methyl ester and benzaldehyde for those inoculated with *T. polysporum* T60. Furthermore, in these latter samples the content of (E,Z)-2-butenoic acid-hexenyl ester was higher than that found in control samples.

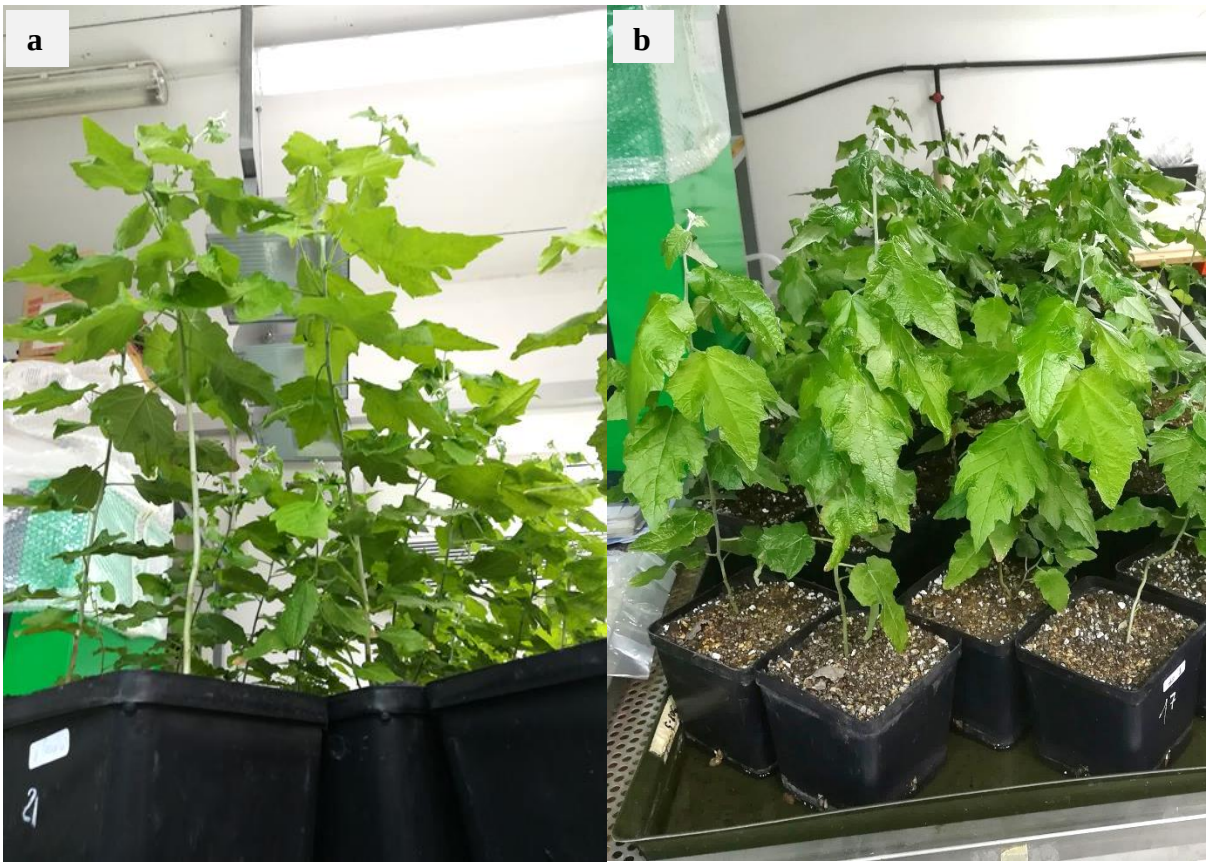


Figure 29 (a, b) Plants investigated for VOCs detection

Volatile Compound	Code	LRIlt [d]	LRIsp [d]	Volatile Compound	Code	LRIlt [d]	LRIsp [d]	Volatile Compound	Code	LRIlt [d]	LRIsp [d]
Hydrocarbons				**Alcohols**				**Esters**			
Undecane	15	1100	1099	Ethanol	7	944	944	Acetic acid, methyl ester	4	839	837
Dodecane	24	1200	1191	1-Penten-3-ol	20	1178	1179	Propanoic acid, methyl ester	6	911	917
Styrene	82	1256	1257	1-pentanol	31	1266	1266	Butanoic acid, methyl ester	9	998	998
				3-Heptanol	35	1306	1304	Butanoic acid, 2-methyl-, methyl ester	11	1018	1019
Terpenes				2-Penten-1-ol	37	1334	1334	Ethyl Acetate	5	901	901
α-Pinene	12	1026	1026	1-Hexanol	40	1364	1364	4-Methyl-2-pentyl acetate	17	1110	1112
Camphene	13	1069	1069	3-Hexen-1-ol, (E)-	41	1374	1375	Pentanoic acid, 4-methyl-, methyl ester	18	1136	1146
β-Pinene	16	1106	1106	3-Hexen-1-ol, (Z)-	43	1398	1398	Hexanoic acid, methyl ester	23	1188	1188
β-Myrcene	19	1155	1155	2-Hexen-1-ol, (E)-	45	1418	1418	2-Penten-1-ol, acetate, (Z)-	28	e	1228
d-Limonene	21	1184	1184	1-Octen-3-ol	46	1458	1458	3-Hexenoic acid, methyl ester	30	1260	1262
Eucalyptol	25	1203	1203	1-Heptanol	47	1463	1463	Acetic acid, hexyl ester	32	1273	1273
Myrtenal	59	1632	1631	1-Nonanol	61	1666	1667	2-Hexenoic acid, methyl ester, (E)-	33	1305	1293
Myrtenol	68	1800	1800	1-Hexanol, 2-ethyl-	52	1496	1497	3-Hexen-1-ol, acetate	36	1313	1311
Nerol	69	1809	1809	o-Cresol	75	2010	2010	2-Hexen-1-ol, acetate, (E)-	38	1338	1338
Geraniol	70	1858	1858	Phenol	76	2012	2013	Octanoic acid, methyl ester	42	1390	1391
Linalool	55	1555	1555	Benzyl alcohol	73	1891	1891	2-Hexen-1-ol, propanoate, (E)-	44	1392	1404
Camphor	53	1517	1517	Phenylethyl Alcohol	74	1927	1927	Butanoic acid, 3-hexenyl ester	48	1464	1464
Borneol	64	1709	1709	o-Guaiacol	72	1876	1876	Butanoic acid, 2-hexenyl ester	50	1476	1477
Eugenol	81	2117	2094					Nonanoic acid, methyl ester	51	1493	1494
								Hexanoic acid, 3-hexenyl ester, (Z)-	60	1662	1659
Aldehydes				**Acids**				Decanoic acid, methyl ester	56	1599	1598
								2-Butenoic acid, 3-hexenyl ester, (E,Z)-	57	1606	1605
Acetaldehyde	1	712	717	Acetic acid	49	1475	1475	Methyl salicylate	67	1784	1784
Hexanal	14	1092	1092	Nonanoic acid	80	2119	2091	Benzoic acid, ethyl ester	62	1673	1673
2-Hexenal, (Z)-	26	1209	1209	Octanoic acid	77	2039	2039	2-Propenoic acid, 3-phenyl-, methyl ester	78	2050	2049
2-Hexenal, (E)-	27	1226	1226	Hexanoic acid	71	1865	1865	3-Hexen-1-ol, benzoate, (Z)-	79	2074	2073
1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	58	1624	1624								
Benzaldehyde	54	1531	1531					**Organosulfur compound**			
Benzaldehyde, 2-hydroxy-	63	1686	1686								
								Dimethyl sulfide	2	755	756
Ketones											
Acetone	3	825	826								
3-Pentanone	8	986	988								
Methyl Isobutyl Ketone	10	1015	1016								
3-Octanone	29	1255	1255								
Acetoin	34	1299	1299								
5-Hepten-2-one, 6-methyl-	39	1343	1343								
α-Citral	66	1741	1742								
2(3H)-Furanone, 5-ethyldihydro-	65	1714	1714								

Table 15 Volatile compounds (n = 81) identified by HS-SPME/GC–MS analysis of poplar seedlings as result of bioinoculation with *Trichoderema* strains

d: LRI_{lt}: Linear Retention Index reported in literature by www.pherobase.com, www.flavornet.org, www.chemspider.com and www.nist.gov; LRI_{sp}: Linear Retention Index calculated against n-alkanes (C5–C14 and C8-C40) on VF-WAXms column.

e: not available

4.6 Discussion

4.6.1 Effect of *Trichoderma* inoculation on plant tolerance and Cd uptake

Poplars are considered suitable trees in Cd phytoremediation from soils with relatively high Cd contaminations but metal tolerance and uptake is highly dependent on the cultivars and clones of the plant. Variability of cadmium tolerance and distribution in plant organs from different species, hybrids and genotypes was investigated in several study (Di Baccio et al 2014; Di Lonardo et al 2011; Yang et al 2014). The evaluation of different sensitive clones to excess Cd is interesting as it allow to select the most promising genotypes adapt to proliferate in polluted areas and capable of ameliorating or reducing the negative environmental effects of this toxic metal.

In the present study, an autochthonous and less studied white poplar clone ("Querce") was screened for its phytoremediation potential against great Cd dose using a semi-controlled experimental system.

Previous investigation carried out by Di Lonardo et al (2011) on variation in arsenic, cadmium, copper, and zinc tolerance, accumulation and translocation in "Querce" clone and other two poplar clones ("Fiorentini" and "Vllafranca") through an *in vitro* screening proved "Querce" marked tolerance to the Cd concentrations used (0, 5, 50 and 250 μM $CdSO_4$), such as metal phytostabilization capability at root level. On the other hand, however, "Querce" clone showed slower growth compared to commercial clones (i.e "Villafranca"). Thus, in this work, the beneficial effect of two selected *Trichoderma* bioinoculats (T1 and T2) was assessed with regard to "Querce" growth stimulation and metal translocation improvement from root to shoot under high Cd stress (250 μM).

The results showed that *Trichoderma polysporum* T1 and *Trichoderma harzianum* T2 strains used in the present study did not effectively stimulate the growth of young poplar plants (approximately 2-months old saplings) in terms of biomass, either in uncontaminated (data not show) or in contaminated soils. In spite of this, roots growth promotion was found in plants grown in the contaminated soil and bioinoculated with *Trichoderma* T2.

Unlike the data shown in our experiment, other studies reported the enhancement of plant growth under Cd stress. Babu et al. (2014b) showed that inoculation with *T. virens* PDR-28 increased the dry weight of maize shoots by 56 %, while Adams et al. (2007) found that *T. harzianum Rifai* 1295-22 inoculum increased crack willow dry weight by 39 %.

In this study, “Querce” coupled with *T. harzianum* T2 showed raise in fresh root biomass by 44.3% and enhancement of tolerance to high level of heavy metal and Cd-translocation from root to shoot.

Specifically, after the addition of inoculation agents T1 and T2, poplar metal translocation efficiency showed no significant variation under T1 inoculation while increased by 17.60 % in T2 treated plants (Table 13). In fact, much of the Cd accumulated by the T2 treated plants had been found in the shoot that were characterized by 55.34 % of Cd content.

Elevated values of Ti and TF suggested that *Trichoderma hatzianum* T2 influenced metal resistance and metal translocation in the plant.

This features marked by *Trichoderma harzianum* T2 is perhaps due to increasing root absorption area and stimulating the acquisition of plant nutrients including metals ions (Khan et al. 2000). Almost *Trichoderma* species, indeed, several study mentioned about their release of nutrients from soil (Altomare et al. 1999, Harman et al. 2004a, b), and development enhancement of root system architecture (Contreras-Cornejo et al. 2009; Harman et al. 2004b). However, the results about the inoculation of “Querce” by T2 in increasing the values of coefficients of TF are consistent such as *T. harzianum* T22 on crack willow (*Salix fragilis*) found by Adams et al. (2007) and *T. atroviride* F6 on mustard (*Brassica juncea* L.) reported by Cao et al. (2008). Unlike these studies, our research has not confirmed the increase in the accumulation of Cd in “Querce” clone by means of *Trichoderma* T2 inoculation.

Regarding the plant-root colonization ability of *Trichoderma* strains, T2 ($3.08x10^5$ CFU g^{-1} dw) did not reach high levels of concentration in the root after phytoremediation like T1 strain (2.08×10^6 CFU g^{-1} dw). T1 abundance on “Querce” roots demonstrated the good capacity of accretion and root colonization of this strain under heavy Cd concentration, even if this strain did not show interesting traits for phytoremediation purposes.

However, the colonization of “Querce” roots by means of *Trichoderma* agents seemed to have no effect in promoting photosynthetic activity in plants (Figure 21). This feature may be due to the most commonly observed consequences of Cd toxicity on chlorophyll lost (Ekmekci et al., 2008; Mobin and Khan, 2007).

4.6.2 VOCs emission

It is well established that the beneficial effects of *Trichoderma* species to plants are not limited to the direct control of plant pathogens, but are in part due to modifications of the plant physiology as a result of the plant-*Trichoderma* interaction. After establishing an intimate,

symbiotic relationship with the plant, *Trichoderma* enhances resistance to both biotic and abiotic stresses and promotes plant growth by both stimulating the spatial development of roots and boosting photosynthesis and uptake of CO_2 in leaves (Harman et al., 2004; Shoresh and Harman, 2008; Vargas et al., 2009). These effects are generated by the capability of *Trichoderma* to regulate the levels of plant phytohormones. For instance, the growth-promoting activity of *T. atroviride* on tomato seedlings is associated with the reduced ethylene production (Gravel et al., 2007). Also, *Trichoderma* spp. produce auxins (Contreras-Cornejo et al., 2009) and compounds with an auxin-like effect, such as harzianolide and 6-pentyl-*a*-pyrone (Vinale et al., 2008). Plants emit exclusive blends of volatiles in response to nonhost and host interactions, as well as to beneficial microbes and necrotrophic/biotrophic pathogens through a regulation mechanism that involves phytohormones (Sharifi et al., 2018). Plant volatiles are also formed through phytohormone-driven biochemical pathways in response to abiotic stresses (Loreto et al., 2004; Vickers et al., 2009). Changes in volatile emission patterns under stress conditions provide circumstantial evidence that volatiles are linked with stress responses. Under abiotic stress conditions the emission of VOCs has often been found to increase (Vickers et al., 2009). Under stress, VOCs biosynthesis is activated not for constitutive compounds, but also for stress-induced volatile compounds, which are made from several biochemical pathways (Mithofer and Boland, 2012).

At our knowledge there are no data available about the effect that the abiotic stress caused by exposure to Cadmium have on the emission of leaf volatiles in poplar. Also, it is not known if the inoculation of poplar roots with *Trichoderma* strains alters the emission of volatiles either in healthy or Cd-stressed poplar plants. For this reason, we undertook an investigation on poplar leaf volatolomics aimed at: 1) identify possible Cd-stress marker VOCs; 2) study the effect of *Trichoderma* inoculation on production of poplar VOCs; 3) investigate the response of Cd-exposed plants to *Trichoderma* inoculation in relation to VOCs emitted as markers of Cd-stress. A total of 81 volatiles were identified and subjected to PCA and one-way ANOVA analyses to discriminate treatments. As expected, the exposure of plants to Cd resulted in a significant variation in the VOCs emitted by stressed plants, compared to control plants. In poplar samples grown in the presence of cadmium a significant reduction ($p < 0.05$) of the contents of five VOCs, i.e. α-pinene, camphene, benzaldehyde, benzyl alcohol and nonanoic acid, was observed with respect to the poplar plants grown in absence of cadmium.

Likewise, inoculation with the two *Trichoderma* strains altered the profiles of poplar leaf VOCs, either by up-regulating or down-regulating biosynthesis of some compounds. Interestingly, the regulation of VOCs by *Trichoderma* appears species and/or strain dependant.

Few responses of plants are stress-specific. Most often, stresses elicit generic response, in particular, production of excess reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). ROS are important signaling molecules and also serve to initiate defense responses. The cellular balance of ROS is normally kept under control. However, when this control is lost, damage occurs. ROS cause direct damage to plant cells through oxidation of biological components (nucleic acids, proteins and lipids) and can instigate chain reactions resulting in accumulation of more ROS and initiation of programmed cell death. Certain volatiles belonging to the terpenoid family (also called isoprenoids) have been implicated in protection against oxidative and other abiotic stresses. The mechanisms by which isoprenoids exert their protective effect are not yet determined, but this is thought to be associated to stabilization and protection of the integrity of cell membranes (Vickers et al., 2009). In this study, an increase in the emission of the isoprenoids β-myrcene and α-citral was observed as a result of root treatment with the strain *T. harzianum* ITEM 908 in plants not exposed to Cd stress. However, the same thing was not observed in treated plants subjected to Cd-stress. Conversely, a decrease in the emission of aldehydes, alcohols and acids was found in this latter treatment.

Therefore, while elicitation of isoprenoid production seems not to be the mechanism by means of which the strains *T. harzianum* ITEM 908 and *T. polysporum* T60 increase tolerance of poplar plants to Cadmium.

4.7 Conclusion

The study clearly demonstrated that high tolerance and translocation ability showed by "Querce" clone coupled with *Trichoderma harzianum* ITEM 908 (T2) inoculation could be an excellent tool for efficient phytoremediation of Cd from the contaminated soil. However, further research is needed to evaluate the long-term effects of *Trichoderma* T2 on metal behaviour in the heterogeneous system under field conditions.

Regarding VOC emission, plants exposure to heavy dose of Cd induced significant variation in the VOCs emitted by stressed plants, compared to control plants. In detail, significant reduction ($p < 0.05$) of the contents of five VOCs, i.e. α-pinene, camphene, benzaldehyde, benzyl alcohol and nonanoic acid, was observed with respect to the poplar plants grown in absence of cadmium.

In the same way, poplar associated with the two *Trichoderma* strains showed alteration in the profiles of leaf VOCs, either by up-regulating or down-regulating biosynthesis of some compounds. By the way, *Trichoderma harzianum* T2 (ITEM 908) inoculum led to an increase in the emission of the isoprenoids β-myrcene and α-citral in uncontaminated plants; similar result was not observed in treated plants subjected to Cd-stress. Under this considerations, it is possible assert that elicitation of isoprenoid production seems not to be involved in the tolerance strengthening mediated by the strains *T. harzianum* ITEM 908 and *T. polysporum* T60 in poplar plants exposed to Cd; the significance of the observed variations in the VOCs emission in plants treated with the two bioinoculants and grown either in the presence or in the absence of Cd still remains to be ascertained.

CHAPTER V

5.1 General conclusion

Soil pollution by heavy metals is spread in more or less vast areas of all over the world arousing serious concern for the surrounding environment and human health. Therefore, new method for its remediation are claimed. Phytoremediation emergent use in the heavy metals contaminated soil reclaiming goals denotes a valid solution in their containment and removal. The skill of selecting plant species, which can accumulate great amounts of heavy metals and are resistant to heavy metals, would facilitate reclamation of contaminated soils.

Thereby, the advantageous effect of two several clone of poplar trees on soil clean up by HMs has been widely studied in this work. Poplars secret lies in the naturally well-designed root system which take up large quantities of water in their fast growth and high biomass production and in their ability to tolerate and accumulate a wide variety of contaminants. However, these features may diverge depending on the poplar genotype.

Biotechnologies are now available for investigating this potential and enlarge the possibilities of exploitation of trees for remediation. The use of in vitro cultures, the role of beneficial fungi, the exploitation of in vivo experiment by pots, are some of the aspects focused in this paper that open prospects of global relevance for a better understanding of the processes related to the uptake of heavy metals by these woody plants.

In this study, in vitro screening of the poplar clone “Villafranca” was proved surely powerful tools allowing to investigate and evaluate the potential of phytoremediation in axenic condition. On the basis of other study, a completely new screening model was reported for the first time in the plant tissue cultures.

The research developed demonstrated the suitability of “Villafranca” clone to be micropropagated in autotrophic culture system and its promising exploitation in the phytostabilization of Cu contaminated sites and in phytoextraction of Cd by soil.

The large part of the study, dealing the activity of different *Trichoderma* strains on Cd, suggested the involvement of two main mechanisms in metal tolerance by these filamentous fungi.

Outcomes obtained by *in vitro* screening allowed to denote a first tolerance strategy based on the production of chelating compounds and solubilizer of insoluble phosphates, which are

engaged in the cell exclusion of the toxicant; and a second strategy residing in the intracellular compartmentalization, and/or enzymatic detoxification of Cd.

Based on their potential for remediation, *Trichoderma* strains presenting the first strategy mentioned above were taken into consideration due to their good ability for increasing bioavailability of Cd in soils and thus enhance phytoremediation.

The features of two of these strains (i.e *Trichoderma polysporum* T60 named T1 and *Trichoderma harzianum* ITEM 908 named T2), thereby, were studied at the rhizosphere level, with regard to a possible translocation of the uptaken Cd at the interface of the plant-Trichoderma symbiosis.

The pot-experiment clearly demonstrated the suitability of T2 inoculum on white poplar "Querce" clone in phytoremediation process enhancement.

The addition of the T2 bio-inoculant agent has indeed been effective in enhancing metal tolerance and translocation of the metal from roots to shoot in this autochthonous clone. Furthermore, the two *Trichoderma* strain coupled with "Querce" clone affected the profiles of leaf VOCs in absence of Cd. Likewise Cd proved variation in VOCs emission. The correlation of VOCs data between the several treatments, however, did not show the involvement of Trichoderma in the strengthening tolerance of poplar plants exposed to Cd.

Under this consideration it is possible ascertain that the experiments conducted have allowed the selection of poplar genotypes suitable for mitigation strategies in contaminated sites.

Furthermore, the results obtained confirmed the wide tolerance capacity of the two selected *Trichoderma* strains under Cd stress as well as their ability to colonize the roots of the "Querce" cultivar, thus presaging their potential use in strategies to restore contaminated sites. .

As a natural evolution of the presented study, it was started other tests at the ISPA institute in the CNR of Bari aimed at selecting *Trichoderma* strains from contaminated sites in association with poplar clones. These new trials will have as their purpose the evaluation of the potential / effectiveness of these strains in the contaminated site mitigation strategy.

Under this consideration it is possible ascertain that the experiments conducted have allowed the selection of poplar genotypes suitable for mitigation strategies in contaminated sites, proposing itself as a preparatory work for the use of these clones in the open field.

Furthermore, the results obtained confirmed the wide tolerance ability of the screened Trichoderma strains belonging to the ISPA collection site in the CNR of Bari under Cd stess and hence their potential use in land resoration strategies; as well as poplar roots colonization ability of the two *Trichoderma* bioinoculants coupled to "Querce" plants.

Nevertheless, other ongoing tests at the ISPA institute are aimed to select *Trichoderma* strains from polluted sites in order to value their potential in mitigation strategy of contaminated site in association with poplar clones.

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