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MEDITERRANEAN INDUSTRIAL CROPS FOR PHYTOREMEDIATION AND BIOENERGY PRODUCTION IN HEAVY METAL POLLUTED SOIL

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- The accumulation of heavy metals in biomass requires special attention. Biorefinery process for contaminated biomass and bioenergy development appears to be a suitable way to add value to the biomass as a feedstock.
- Safflower can grow in heavy metal polluted soil, stabilizing the heavy metal mainly in the roots.
- Industrial hemp remediates heavy metal polluted soil and can tolerate high concentrations of heavy metal in the soil.
- Giant reed grew in contaminated polluted soil and is able to produce good quality biomass.
- African fodder cane is well adapted in heavy metal contaminated soil, producing good quality biomass.

Abstract

Using agricultural land to cultivate energy crops has increased the competition between fuel and food. To avoid this problem, marginal lands appear as an alternative to energy crop production without threatening food production. Marginal lands are generally defined as unproductivity lands exposed to stressed conditions such as limitations of nutrients or water or contamination by hydrocarbons or heavy metals. In the case of heavy metals, there are several methods to decontaminate the soil through different paths using physical, chemical, or biological techniques. Naturally, phytoremediation is a sustainable and renewable technique that uses plants to remediate the contaminated site; the plants should be tolerant of the contamination and capable of extracting or immobilizing the metals in the soil. The possibility of further utilization of phytoremediation biomass turns energy crops into an excellent option for this technique. Energy crops can produce biofuels, bioenergy, and bioproducts in a sustainable and renewable way, creating economic potential, mainly when cultivated in marginal lands. This work aimed to investigate the adaptability and the phytoextraction potential of four industrial crops, *Arundo donax* L., *Carthamus tinctorius* L., *Cannabis sativa* L. and *Saccharum spontaneum* L. ssp. *aegyptiacum* able to grow in different level of zinc, cadmium, lead and nickel contaminated soil in the mediterranean environment.

Keywords: Bioremediation, cadmium, zinc, nickel, lead, industrial hemp, giant reed, african fodder cane, safflower, oilseed crop, lignocellulosic crop, energy crops.

Riassunto

L'utilizzo di terreni agricoli per coltivare colture energetiche ha aumentato la competizione tra colture industriali e colture alimentari. Per evitare questo problema, i terreni marginali appaiono come un'alternativa alla produzione di colture energetiche senza minacciare le produzioni alimentari. I terreni marginali sono generalmente definiti come terreni improduttivi, esposti a condizioni di stress quali scarsità di nutrienti o acqua o contaminazione da idrocarburi o metalli pesanti. Nel caso dei metalli pesanti, esistono diversi metodi per decontaminare il suolo attraverso percorsi diversi utilizzando tecniche fisiche, chimiche o biologiche. Tra le tecniche biologiche il fitorisanamento è una tecnica sostenibile che utilizza le piante per decontaminare i siti inquinati; le piante utilizzate devono essere tolleranti alla contaminazione e in grado di estrarre o immobilizzare i metalli nel terreno. Inoltre, la possibilità di un ulteriore utilizzo della biomassa raccolta dopo il fitorisanamento, rende le colture energetiche una scelta ideale nell'utilizzo di questa tecnica. Infatti, esse possono essere utilizzate per produrre biocarburanti, bioenergia e bioprodotti in modo sostenibile e rinnovabile, creando un potenziale ritorno economico, anche se coltivate in terreni contaminati. Obiettivo di questa tesi è la valutazione della tolleranza e del potenziale di fitoestrazione di quattro colture industriali, *Arundo donax* L., *Carthamus tinctorius* L., *Cannabis sativa* L. e *Saccharum spontaneum* L. ssp. *aegyptiacum* in terreni contenenti diversi livelli di zinco, cadmio, piombo e nichel.

Parole chiave: biorimedio, cadmio, zinco, nichel, piombo, canapa da industria, canna comune, canna d'Egitto, cartamo, colture oleaginose, colture lignocellulosiche, colture energetiche.

1 Mediterranean industrial crops for phytoremediation and bioenergy production in heavy metal polluted soil

1.1 Introduction

Marginal land can be defined as land that has a low level of capacity to generate a profit. This can occur under several conditions, such as lack of water and nutrients in the soil, morphology of the site, extreme weather conditions, soil and water contamination due to natural events or human intervention (Muscat et al. 2022). In addition, the cultivation of crops on marginal lands can pose a risk to animal and human health, and thus the use of these lands needs to be done with specific considerations and farming systems (Bowyer et al. 2014; Nalepa and Bauer 2012) .

In many countries, the use of marginal land for food and feed production has been replaced by the cultivation of energy crops, providing an additional source of income for agriculture, creating new employment opportunities both in agriculture and in the energy sector, providing a sustainable and renewable alternative to petroleum in a refinery, and avoiding competition for land once food and feed cannot be produced on contaminated land (EU 2018).

The cultivation of industrial crops can provide environmental benefits, such as maintaining water and nutrients in the soil, improving biodiversity, promoting wildlife, and helping to combat CO_2 emissions (Scordia et al. 2017). .After harvesting, the biomass can be used to sustainably develop the economy through energy production (heat, biofuel, biogas) or in the bioproducts sector (textiles, paper, mats, bioplastics) (Fernando et al. 2015).

Among the contaminants that can affect the quality of soil are heavy metals, which can be dangerous to humans. Moreover, the production of food and feed in soil contaminated by heavy metals can introduce these contaminants directly or indirectly into the food chain, which becomes a public health issue because, although some of the heavy metals are used by humans and animals as micronutrients, the excess can cause serious health problems in the nervous system or generate tumours (Kumar et al. 2019).

Human activities are the main source of soil contamination by heavy metals. Residues from mining, pesticides and herbicides used in agricultural activities, residues from the petroleum industry or its derivatives or petroleum hydrocarbons, production of batteries and inappropriate disposal of electronic components are some of the human activities that lead to soil contamination by heavy metals (Li et al. 2019). Although the sources of heavy metals in the environment are diverse, some heavy metals are usually associated with specific activities. The most common metals from agriculture are Zn, As, Cd, Pb, Cu, Se and U. From the mining and smelting group, Cd, Pb, As and Hg are the most common, while from industry Cd, Hg, Cr, As, Cu, Co, Ni and Zn, from waste management As, Pb, Cu, Cd, Cr, Zn and Hg, and from atmospheric deposition the most common metals are As, Pb, Cr, Hg, Cu, Cd and U (Khalid et al. 2017).

Due to these potential problems, soil decontamination is a necessary step. Soil can be decontaminated in a number of ways, using chemical, physical or biological techniques, or a combination of these. For example, phytoremediation is a biological technique suitable for soil decontamination. The selection of plants needs to fulfil several conditions, such as crop tolerance to heavy metals, high biomass production, deep and extensive root systems, known agronomic techniques and low demand for agronomic inputs (Fernando et al. 2016). In this way, the cultivation of food crops in heavy metal-contaminated soils must be avoided, and the cultivation of industrial crops becomes a viable alternative due to their higher tolerance to the presence of contaminants, which allows the crops to grow without significant losses in productivity, and their higher capacity to accumulate the heavy metals, thus ensuring the remediation of the soil (Barbosa et al. 2015). When grown on heavy metal-contaminated soils, industrial crops (perennial, energy and fibre) can extract or stabilise the contaminants in the soil, making it possible to associate industrial crops with the remediation of the land (Cosentino et al. 2015).

1.2 The Mediterranean environment in climate challenge and the European legislation on renewable energy sources

In recent years, climate change has emerged as the most critical and widespread environmental challenge facing society, as its local, regional, national and global impacts are and will become increasingly evident over time (McKendry and MacHlis 2009).

Climate change has directly affected the distribution of a wide range of organisms, leading to a probable increase in extinction processes (Thomas et al. 2004), and the transformation process of the planet Earth has undergone a sudden acceleration. The Mediterranean basin is recognised as a biodiversity hotspot because of its rare and endemic plants (Alexopoulou et al. 2015; Cosentino et al. 2012)

. However, anthropogenic land use by humans, urbanisation and industrialisation have changed land use and led to the introduction of new plants able to grow in the Mediterranean environment. In addition, the growth of the world's population, inevitably accompanied by a greater consumption of resources, has led to profound imbalances within ecosystems, of which pollution is the most obvious consequence (Tóth et al. 2016) . Pollution can be defined as "the consequence of a human activity capable of modifying the characteristics of the conditions or the availability or quality of resources in a given interval of space and time". Contamination becomes pollution "when it reaches a level that causes adverse effects on organisms, populations and ecosystems" (Wang et al. 2013).

World population growth is projected to be around 9.2 billion by 2050 (FAO, 2012). This growth is expected to occur exclusively in developing countries, where urban areas will grow at the expense of rural areas.

Agriculture in the 21st century will face several challenges: producing more food to meet the demands of a growing population, contributing to development and poverty reduction in countries with agricultural economies, adopting more efficient and sustainable production systems while progressively reducing natural resources, and adapting to climate change. In terms of the interactions between agriculture and climate, future climate change is expected to lead to higher temperatures and more variable precipitation with an increase in extreme weather events, resulting in reduced water availability and crop stress, heat and cold waves, and an increase in the spread of animal and plant diseases (Gawel and Ludwig 2011; Tóth et al. 2016)

On the other hand, agriculture and forests can make a significant contribution to mitigating climate change through their role as "carbon sinks" and through opportunities to improve the management of agricultural land and livestock production systems. Public and private investment in sustainable agriculture, rural development and the environment is the future for coping with the impacts of climate change and ensuring sustainable management of water, forests and other natural resources.In addition, the role of renewable energy and biofuels produced from energy crops should be considered among the options for mitigating climate change (Bowyer et al. 2014).

The Directive of the European Parliament, adopted on 11 December 2018, (EU 2018), establishes a common framework for the promotion of energy from renewable sources. The Directive also requires Member States to collectively ensure the achievement of common targets for 2030: 1) Establishment of financial support schemes for energy from renewable sources. 2) Calculation of the share of energy from renewable sources The Directive sets a binding

Community target of a 32% share of energy from renewable sources by 2030 for the production of energy from renewable sources in the electricity, heating and cooling sectors. 3) Cooperation between Member States in joint projects. 4) Guaranteeing the origin of energy from renewable sources. 5) Network access and management: Member States shall require transmission and distribution system operators in their territory to publish technical standards in accordance with Article 8 of Directive 2009/73/EC, in particular as regards grid connection standards, including gas quality, vapourisation and pressure requirements. 6) Use of renewable energy in heating and cooling systems and in the transport sector. For the transport sector, Article 25 of the Directive (EU 2018) provides for the development of biofuels and supply chains. Circular economy, including these products in the calculation of each Member State's gross final consumption of energy from renewable sources, with a target of 14% by 2030. . 7) Consideration of sustainability criteria and reduction of greenhouse gas emissions:

The Directive pays much attention to the issue of biomass sustainability and the use of agricultural soils, or ILUC (indirect land use change). Indirect land use change occurs when the cultivation of biofuels, bioliquids and biomass fuels affects the traditional production of food and feed crops. Therefore, this Directive requires Member States to set a specific and gradually decreasing limit for biofuels, bioliquids and biomass fuels produced from food and feed crops in order to promote the use of biofuels, bioliquids and biomass fuels with a low risk of indirect land use change": the raw materials of which have been produced in systems that avoid the displacement of biofuels, bioliquids and biomass fuels from food and feed crops by improving agricultural practices and cultivating land not previously used for this purpose, and which have been produced in accordance with the sustainability criteria set out in the Directive.

In addition, in January 2020, the European Parliament decided to support the European Green Deal, a set of initiatives, strategies and legislation to become the first carbon-neutral continent by 2050, with zero net greenhouse gas emissions (Barredo et al. 2022).

The European Green Deal is not a law in itself, but a general policy strategy that outlines the ambitions and objectives in different policy areas through the achievement of specific targets and the adoption of the following measures: a) Raising the EU's climate ambition for 2030 and 2050; b) Providing clean, affordable and secure energy; c) Mobilising industry for a clean and circular economy; d) Building and renovating in an energy and resource efficient way; e) A zero-pollution ambition for a toxic-free environment; f) Preserving and restoring ecosystems and biodiversity; g) Farm to fork: a fair, healthy and environmentally friendly food system; and h) Accelerating the shift to sustainable and smart mobility (Sikora 2021).

1.2.1 Land Use Change

One of the main issues of land use change is the transition from a 'natural' use (such as forests and wetlands) to a 'semi-natural' use (such as farms) or - to an 'artificial' use (such as construction, industry, infrastructure) (Ahlgren and Di Lucia 2016). In addition to the loss of fertile land, which is in most cases permanent and irreversible, these transitions cause other negative effects such as fragmentation of the territory, reduction of biodiversity, alteration of the hydrogeological cycle and microclimatic changes (Muscat et al. 2022). Even if no binding targets have been set and no specific standards have been defined, in many countries the idea of reducing to zero the transformations for non-biospheric uses of the territory is being considered, since the space of the planet is neither a renewable resource nor replaceable (Gawel and Ludwig 2011). However, many agreements already provide incentives for the conservation of natural land use and to protect biodiversity by conserving natural habitats and wild flora and fauna, and by establishing and maintaining an ecological network of protected areas.

There are different scenarios of land use for each energy source and raw material used. In some cases, the biomass is grown locally. In other cases, they are imported, affecting land use in other countries. In other cases, they come from the waste of other processes and are integrated into agro-industrial supply chains (Scordia and Cosentino 2019). The two concepts used in the literature to study the relationship between food and energy (but not to assess the carbon footprint of agroenergy production) are direct and indirect land use change (dLUC and iLUC, respectively). The former refers to direct changes in land use, such as the substitution of one crop for another or the use of land previously used for food production for energy crops (or photovoltaic panels). The dLUC is easy to measure because it is visible. The iLUC, on the other hand, indicates the indirect change in the intended use of land and is a consequence of the dLUC (Wicke et al. 2012).

To solve the land use dilemma, it has been proposed to grow biomass crops on marginal land, i.e. land that has been abandoned for one or more reasons and is no longer used to grow food crops. However, there is still no precise and unambiguous classification of this type of land, so that marginal land can be objectively and measurably distinguished from arable land (Gawel and Ludwig 2011).

1.2.2 The marginal lands

Support for agriculture in areas with natural constraints aims to compensate farmers for the disadvantages resulting from possible adverse conditions. An ad hoc expert group led by the JRC (Joint Research Centre) has developed plausible combinations of parameters and biophysical thresholds. However, the experts also pointed out the uncertainties of this exercise due to the complexity of the soil-climate-plant interactions (Cossel et al. 2019).

The analysis work also identified cases where the sub-severe cut-off needs to be below the 20% level of the baseline (within a 20% margin) to imply a constraint on agriculture. However, strict application of the 20% margin around the initial threshold is not advisable in cases where data accuracy at national level is insufficient.

However, the JRC has not included in the list of potentially marginal soils those types of solo which have been degraded by anthropogenic activity and which are affected by local or widespread contamination, where it is not possible to cultivate food crops for reasons of food safety and quality (Nalepa and Bauer 2012).

1.2.3 A possible solution for the use of biomass

"Biomass" is a term that brings together many materials of a very heterogeneous nature. Biomass is everything that has an organic matrix and comes from the photosynthetic organisation of CO_2, with the exception of plastics and fossil materials (Foti and Cosentino, 2001). Biomass includes a wide variety of organic materials: wood and its derivatives, agricultural and forestry processing residues, residues from the agri-food industry, the organic fraction of waste, livestock effluent, energy crops (maize, sunflower, rape, beet, etc.), which can be used directly as fuel or converted in conversion plants into other substances (solid, liquid or gaseous) that are easier to use.

Biomass of agricultural origin for energy purposes is provided by the conventional cultivation of many starch- and oil-bearing sugar crops, some annual and perennial herbaceous species and short-rotation woody crops (SRF). Based on the product delivered at harvest, the different types of biomass can be classified as lignocellulosic, oilseed, sugar crops, starch crops and others.

However, the production of these crops is aimed at a higher energy content and their cultivation requires low-intensity and low-energy 'input' farming practices (Cossel et al. 2019). Biomass crops are therefore a very diverse group of species that can produce thermal and/or electrical energy from thermochemical processes, biofuels (biodiesel and bioethanol) and biogas from biochemical processes. The most appropriate type of bioconversion depends on the quality of the biomass and its composition in terms of simple and complex sugars, lipids, proteins, structural carbohydrates, lignin, moisture, calorific value, C/N ratio, ash and minerals. The world of bioenergy is evolving, and biomass crops other than food crops that are initially used for energy purposes are becoming more widespread. These non-food crops are known as "second-generation" biomass crops. The most common are Sorghum (*Sorghum* spp. Moench), giant reed (Arundo donax L.), switchgrass (*Panicum virgatum* L.), miscanthus (Miscanthus ssp.), cardoon (*Cynara cardunculus* L.), sunflower (*Helianthus annuus* L.).), sunflower (*Helianthus annum* L.), specific arboretums: poplar (*Populus* spp. L.), willow (*Salix alba* L.) and eucalyptus (*Eucalyptus* spp. Labill) (Alexopoulou et al. 2015; Fagnano et al. 2015; Fernando 2013; Gomes et al. 2022; Scordia et al. 2017). These species have the peculiarity of not competing with food species for edible products. Instead, they compete with food crops for already scarce arable land (Nocentini et al. 2015; Zanetti et al. 2019).

A critical priority is to understand how to involve farmers in bioenergy supply chain projects to ensure levels of profitability equal to or higher than those of alternative crops, and to involve them in the related activities of transformation and marketing of energy products. Policies for the development of bioenergy supply chains must be selective and define precise objectives in order to avoid the risk of market distortions and negative impacts on the "food" part of agricultural markets (Fernando 2013) . Furthermore, the widespread use of biomasses can have significant economic, environmental and occupational impacts, as they can guarantee

- The valorisation of agro-industrial residues.
- New development opportunities for marginal areas
- The possibility of developing new industrial initiatives.
- No contribution to the increase of CO_2 in the atmosphere.

- Possibility of producing energy with modest investment.
- Storage of significant amounts of carbon in the soil.

1.3 Industrial crops able to grow in a Mediterranean environment

The Mediterranean environment is characterised by dry and warm summers with water scarcity, and this constraint is known to limit the yield of most crops. Moreover, even if water can be used for irrigation, the reduction in water use efficiency in the crop is enormous due to the increased transpiration of crops in the semi-arid environment, especially for rainfed crops. Therefore, it is strategically important for the cultivation of industrial crops in the Mediterranean region to develop plants that (i) have increased yield potential, (ii) are better adapted to current and future environmental constraints, and (iii) are able to grow under water deficit conditions and with reduced inputs. Species characterised by high water use efficiency and low nitrogen demand, and therefore well adapted to the natural resources of a specific environment, could provide several solutions, such as also remediating polluted soils, reducing field emissions of CO_2 and N_2O due to reduced soil disturbance and fertiliser inputs, or indirectly by increasing soil C sequestration and offsetting fossil fuel emissions, to ensure biomass production without encroaching on agricultural, forest, highly biodiverse grassland or high carbon stock lands (Cosentino et al. 2014). The origin of the main industrial crops in the world can be observed in Figure 1.1.

Figure 1.1. Origin of the main industrial crops in the world

In this scenario, plant families of industrial crops, such as Poaceae, Brassicaceae, Asteraceae, Cannabaceae, Euphorbiaceae, Fabaceae, Linaceae and Malvaceae, contain species that are naturalised, endemic or able to grow in the Mediterranean basin, that are suitable for phytoremediation of polluted soils with low cultivation input and the potential to use the biomass for energy purposes.

For example, *Arundo donax* L. (Giant reed) or *Saccharum spontaneum* (L.) spp. *Aegyptiacum* (African fodder cane), two perennial rhizomatous grasses belonging to the Poaceae family, can be cultivated for industrial purposes (cellulose pulp, paper, second-generation bioethanol), giving high yields with reduced input supply (Cosentino et al. 2015; Scordia et al. 2013).

Giant reed is a C3 energy crop with the potential to produce high amounts of biomass. In addition to its agronomic potential, the chemical and energetic composition of this biomass meets EU market requirements for energy and advanced biofuels, paper and pulp, and construction materials (Alshaal et al. 2013; Liu et al. 2017). In addition, it grows spontaneously and abundantly throughout the Mediterranean and warm temperate world (Christou et al., 2001;

Cosentino et al., 2014. Copani et al., 2013).

The versatility of this crop allows its cultivation in soils with different stress conditions (Eid et al. 2016), such as salinity, pH, organic matter, nitrogen content, or heavy metal contamination in different concentrations, availability and biodisponibility. Furthermore, giant reed could be considered as a suitable metalliferous pioneer plant for the phytoremediation of heavy metal-contaminated soils, considering its mechanisms to resist, tolerate, grow and remediate toxic metalliferous soils (Papazoglou et al. 2005; Papazoglou et al. 2007).

African fodder cane is a perennial crop thought to have originated in North Africa. However, Saccharum is widely distributed in tropical and subtropical regions of Asia, growing up to an altitude of 1800m. It is also widely distributed in southern Asia, eastern Africa and the Mediterranean (Cosentino et al. 2015).

The search for energy crops that are well adapted to a specific climate, use abiotic resources efficiently, grow with reduced agronomic inputs, or thrive in poor soils is paramount for sustainable biomass production. Global climate change is predicted to increase the frequency and intensity of droughts in some geographical regions. In arid and semi-arid regions where evapotranspiration dominates, reduced water availability and drought duration are likely to increase (Cosentino et al. 2015; Scordia et al. 2017; Scordia et al. 2010).

Alternatively, *S. spontaneum* ssp. *aegyptiacum* is a fast-growing, wide-ranging, high-yielding perennial suitable for second-generation bioethanol production. It is attractive as a non-food energy crop due to its high biomass yield, relatively high carbohydrate composition, perennial growth and ability to grow well on marginal and non-agricultural land. (Scordia et. al. 2010) Oxalic acid pretreatment can release fermentable hydrolysate sugars from the hemicellulose and prepare the remaining solids for enzymatic saccharification or simultaneous saccharification and fermentation of

Saccharum to second-generation bioethanol.

In addition, thanks to a highly developed canopy and a deep root system, it covers the entire soil in the years following establishment, thus preventing the risk of soil erosion (Scordia et al. 2017). In addition, other industrial crops such as Panicum virgatum, Sorghum bicolor or *Miscanthus* spp. still belonging to the Poaceae family, but native to North America, Africa and Asia, have been cultivated in Mediterranean environments, showing a good range of adaptability to semi-arid conditions and great physiological characteristics capable of producing a good biomass yield suitable for low-input cultivation, can be used as dedicated crops for phytoremediation with environmental constraints. (Alexopoulou et al. 2015; Nocentini et al. 2015; Scordia et al. 2017b, 2020).

The Brassicaceae family includes several industrial crops that are cultivated as oilseeds, where the oil can be used for biofuel production, such as *Brassica carinata* L., *Brassica napus* L., *Thlaspi arvense* L., *Camelina sativa* (L.) Crantz and *Crambe abyssinica* Hochst. The last of these, crambe, is native to the Mediterranean region and is drought tolerant, well adapted to poor sandy soils and naturally resistant to insects. It also has favourable agronomic characteristics, such as a short growing cycle (Zanetti et al. 2016). However, the same characteristics are also found in Brassica spp. which are characterised by rusticity and adaptability to different environmental conditions. (Montemurro et al. 2016).

Moreover, *Camelina* varieties were tested and showed good adaptation to Mediterranean conditions, with a substantial crop yield and seed quality dependence on genotype and sowing time. *Camelina sativa* L. is native to southeast Europe and southwest Asia and is an oilseed crop in the Brassica family, recently an essential new player "fuelling" the alternative energy market. It grows in a temperate climate in Europe, throughout U.S. and Canada, where the oilseed is crushed to produce biodiesel. Camelina grows on marginal land, is unsuitable for food crops, and uses little moisture is suitable for growing on less fertilized, relatively drier soils with a light texture (clay soils are not suitable). However, this crop has a short growing cycle and oil content, representing up to 45%, an excellent option for biofuel production (Angelini et al. 2020).

The interest in the camelina crop is mainly due to its unique agronomic characteristics and the possibility to grow this plant on different types of soils, which are not suitable for food production (Park et al. 2015). Native to Eurasia, pennycress possesses many traits that can easily support its integration into existing European crops.

In the Asteraceae family, industrial crops native to the Mediterranean basin, such as *Calendula officinalis* L. and *Cynara cardunculus* L., appear suitable for growing on marginal land (Ierna and Mauromicale 2010), with high potential as biorefinery crops, producing bioproduct and bioenergy (Poveda-Giraldo and Cardona Alzate 2021). Sunflower is also grown as a spring-summer crop in the Mediterranean region, sown in March-April. Generally considered a drought-tolerant species and a valid option for regions where water resources are scarce, showed consistent productivity variation in the Mediterranean region, mainly related to rainfall occurring during the crop cycle (Ierna and Mauromicale 2010).

Safflower (*Carthamus tinctorius* L.), a member of the Asteraceae family, is an important oilseed crop grown in many areas of the world. The oil extract from the seeds is used for food and industrial application (Ciaramella et al. 2022; Zeng et al. 2019). Its high tolerance to drought makes it a viable alternative for arid agricultural areas and thus more likely to succeed than major crops in the context of global climate change (Yeilaghi et al. 2012). The adoption of early sowing in February could be profitable for cultivation in semi-arid regions such as Sicily, as it positively affects flower production and pigment content. (Patanè et al. 2020).

Cannabis sativa L. (hemp) is an annual crop, belonging to the Rosales order, sub-order Rosidae and family Cannabaceae, and is considered one of the oldest cultivated plants known to man. Its fibre can be used as a reinforcement in composites to produce insulation mats and automotive interior panels, and to reinforce expanded starch foams in the food packaging sector. Recently, the promotion of bioenergy production has stimulated research on the use of hemp for the production of ethanol, biogas and biomass for combustion (Amaducci et al. 2015; Cosentino et al. 2013).

The quality of the above products depends on the characteristics of the hemp fibre, in particular the morphology of the fibre bundles and the chemical composition of the crude fibre (Amaducci et al. 2015). The choice of a cultivar and sowing time in industrial hemp depends on the final destination of the crop, whether for fiber or biomass production. Considering that hemp harvesting for specific applications should be performed at flowering time, it is essential to hemp phenology prediction in decision support of cultivation practices. Identifying thermal and photoperiodic requirements to achieve high production may help individuate potential production areas in Mediterranean semiarid environments. As far as sowing dates are concerned, the plant has a flowering phase that is strictly controlled by the short photoperiod and temperature. In a Mediterranean environment, sowing before mid-April or later than the end of May shortens the "emergence-flowering" period, resulting in short stems and consequently lower yields. (Cosentino et al. 2012).

Another important industrial crop is the castor (*Ricinus communis* L.), a non-edible multipurpose oilseed of the Euphorbiaceae family, which is a fast-growing C3 crop. It is native to East Africa, probably Ethiopia, where it has the highest number of wild and semi-cultivated species in the world. It is a perennial crop that can produce high biomass without fertiliser. Among non-edible oils, castor oil is the most popular for various industrial, cosmetic and medical applications. The high proportion of ricinoleic acid in castor oil makes it suitable for the production of high quality lubricants (Bauddh et. al 2015). The high concentration of ricinoleic acid (12-hydroxy-9-octadecenoic acid) makes castor beans a promising source of oil (Barnes et al. 2009). India dominates the production of this crop with almost 60% of the market (Dias et al. 2013).

Castor has characteristics such as inedibility, high biomass productivity, tolerance to biotic and abiotic stresses such as heavy metals, salinity, drought, pests, persistent organic pollutants (POPs), etc. (Bauddh and Singh 2012). Furthermore, it is well documented that castor bean can grow quite well under the pedoclimatic conditions of the Mediterranean region (Papazoglou et al. 2020), as well as *Jatropha curcas* L., a tropical plant that can be grown in low soil water availability and can be used in marginal lands due to its lower sensitivity to seasonal temperature variation (Yadav et al. 2009).

Finally, regard to the Malvaceae, Fabaceae and Linaceae families, the main industrial crops belonging to this family appear to be suitable for growing in Mediterranean soils, an example being Kenaf (*Hibiscus cannabinus* L.), an annual fibre crop belonging to the Malvaceae family. It is widely grown in the Asia-Pacific region, with India, China and Bangladesh producing most of the world's supply. Kenaf grows rapidly and its biomass is enormous; it can reach a height of 4-6 m and produce up to 100-150 tonnes of fresh biomass per hectare within a four-month growing season (Corinzia et al. 2022).

Traditionally, kenaf is used for textiles, ropes and sacks. Several industrial uses of kenaf have been explored, including papermaking, building materials, biocomposites, insulation mats and recycled plastics. Kenaf also has excellent properties such as tolerance to drought, salinity, barrenness and heavy metals. Kenaf finds favourable temperature and light conditions for growth (Corinzia et al. 2022) and great potential for dry biomass production in Mediterranean-type climates (Patanè and Cosentino 2013). Another is white lupine, whose high productive performance and ability to fix N_2 varied significantly between locations and climatic conditions. However, the results indicate a remarkable N_2 fixation capacity of this grain legume grown under Mediterranean conditions, providing soil cover and reducing erosion in a Mediterranean area as a beneficial crop (Carranca et. al. 2009; Fumagalli et. al. 2014). Alternatively, flax, an indigenous oilseed crop that may be a suitable feedstock for both energy and non-energy applications, on which a biorefinery could be built in the Mediterranean region if significant environmental benefits can be demonstrated through further work on ecosystem services (Sertse et al. 2019).

1.4 Contamination and soil degradation

Soil provides irreplaceable ecosystem functions and services by producing food and other biomass, storing and transforming minerals, organic matter, water, energy and chemicals, and filtering water and pollutants.

Today, more than ever, it is essential and urgent to address these issues and to seek a fair balance between the economic benefits derived from the use of land by its primary users and the respect for the environment promoted by the adoption of European environmental directives and regulations. It is therefore in the public interest to require land managers to take precautionary and protective measures so that their use does not lead to degradation phenomena.

The term 'soil degradation' refers to the processes that lead to the loss of soil's ability to perform its functions and ecosystem services. For example, soil is subject to degradation through the following processes

a) Erosion: a natural process of removal of soil particles by water and wind, which can be significantly increased by some anthropogenic practices, leading to severe degradation effects.

b) Compaction: caused by excessive mechanical pressure from heavy machinery or overgrazing. After compaction, the soil loses its natural density, structure and porosity, reducing its permeability and fertility.

c) Contamination: the introduction into the soil of potentially toxic substances that can compromise its primary functions. The elements on which it is often appropriate to focus the investigation are Be, Cd, Cr, Co, Cu, Hg, Ni, Pb and Sb.

d) Sealing: anthropogenic action that determines the permanent covering of the soil through the construction of platforms, infrastructures, works and buildings; sealing prevents the soil from performing its ecosystem functions.

e) Salinisation: the accumulation of soluble salts in the soil as a result of natural events or human activity.

f) Desertification: All of the above risks, individually or in combination, can lead to acute soil degradation with the irreversible loss of most or all of the soil ecosystem functions and services.

All of these threats are the result of the abandonment of agricultural activities, in this case on marginal land, i.e. land whose characteristics of structure and grain size have been altered, making it unsuitable for the cultivation of crops of agricultural interest (Legislative Decree 1181 "Framework Law for the Protection and Sustainable Management of Soil",2013).

Soil contamination by potentially toxic heavy metals (HMs) and the abandonment of agricultural activities also pose serious risks to human health. At low concentrations, some HMs (e.g. copper, chromium, molybdenum, nickel, selenium and zinc) are essential for the normal functioning and reproduction of micro-organisms, plants and animals (including humans). However, high concentrations of these elements can be toxic to plants and animals. HMs, generally defined as heavy metals or trace metals of natural and anthropogenic origin, are present in soil in different forms that affect their mobility, potential toxicity and bioavailability (Fiorentino et al. 2017). For example, in soil, HMs can be present as free in solution, adsorbed on the surfaces of exchangers, and combined in the solid mineral and organic constituents.

In general, the total content of each element present is distributed among the different forms in proportions that vary with the nature and total amounts of the chemical elements involved, the characteristics and management practices of the soil, the type of vegetation, climatic conditions and time (Li et al. 2019). The free HMs in solution are ecologically important in the soil because they are mobile and readily bioavailable. However, while bioavailability is easy to define, it is difficult to determine. The bioavailable amount of HM is the fraction of the total amount of metal in the soil that is available for use by living organisms. The analytical methods commonly used to extract from the soil the amount of a nutrient or HM available for plant nutrition are based both on the use of chemical reagents capable of extracting that fraction of the total content of an element from the soil which, based on open field experiments, is significantly correlated with the amount available to the plant (functionally defined speciation) and on the use of chemical reagents classified on the basis of the metal form that can be separated from the soil (operationally defined speciation) (Purakayastha et al. 2008). The reagents used to extract from the soil that fraction of the total content of an element which, on the basis of field experiments, correlates significantly with the amount available to the plant, are mainly dilute solutions of mineral or organic acids (e.g, acetic acid, HCl, HCl + HNO_3), chelating agents (e.g. Na_2EDTA, EDTA, citric acid, NH_4-citrate), buffer salts (e.g. CH_3COONH_4), neutral salts (e.g. $CaCl_2$, $MgCl_2$, NH_4Cl, $NaNO_3$, NH-$4NO_3$) (Purakayastha et al. 2008).

1.4.1 Remediation of heavy metals contaminated soil

Given that contaminated land is a global problem, remediation is a necessary action. Soil remediation techniques can be grouped according to different criteria. Among the most common, methods are divided by ex situ or in situ, or by technological route (physical, chemical or biological) (Alloway 1995).

In terms of ex situ techniques, methods can be divided into three categories: physical, chemical and thermal. The physical ex situ method consists of removing the contaminated soil from the site and disposing of it in a safe landfill. Although this is a well-established technique, it is very expensive.

The choice of the most appropriate method depends on the nature of the contaminant, the characteristics of the soil and the distribution of the contaminant between the various phases (Liu et al. 2018). The economic factor and the expected remediation times are not negligible. There is, therefore, a strong need for low-impact remediation techniques capable of preserving and enhancing the natural fertility of soils. In situ chemical techniques consist of soil washing, which consists of washing the soil in situ using solvents that can be recovered and reused in the same process, and immobilisation, a technique very similar to solidification but applied in the contaminated site.

Although some chemical and physical soil remediation methods, such as ion extraction, soil washing, ultrafiltration and landfilling, are efficient in terms of site decontamination, they are not environmentally sound (e.g. landfilling only transfers the contaminated soil to another site) or economically viable. On the other hand, biological methods usually do not interfere with the site ecosystem, are more environmentally friendly, and are cheaper compared to other techniques (Barbosa et al. 2015).

As a biological technique, phytoremediation is a promising environmentally friendly technique that is receiving more attention every day. Phytoremediation is the use of plants to remove heavy metals from soil. This use is sometimes combined with bioremediation to remediate contaminated soils and wastewater (Yang et al. 2005).

1.4.2 Phytoremediation

Phytoremediation is a biological technique to promote soil or wastewater decontamination. It is a versatile method with different contaminants in high levels of single or multiple contamination (Cristaldi et al. 2017). The recent development in this field can be attributed to the improvement of biotechnology, with new methods to promote the stabilisation or volatilisation of the contaminants (Agnello et al. 2016).

This technique can be divided into different groups according to the biochemical mechanisms that the plant has shown to be able to remediate that can be observed in **Figure 1.2**.

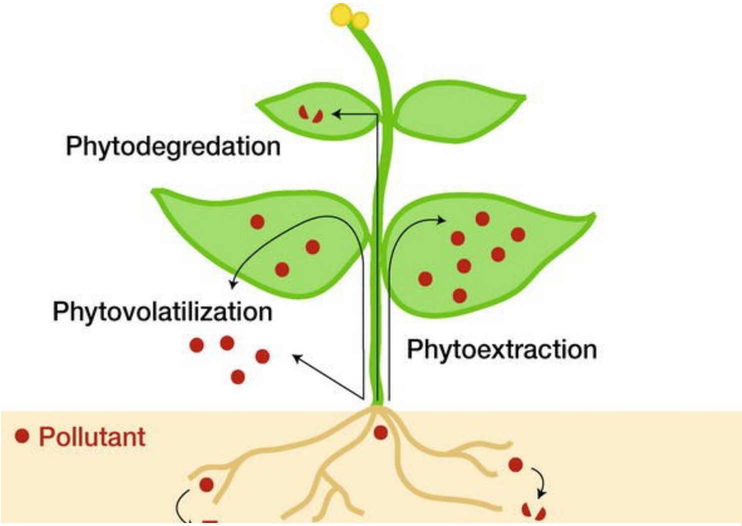

Figure 1.2 Possible pollutant fates during phytoremediation: the pollutant (represented by red circles) can be stabilized (phytostabilization) or degraded (phytostimulation) in the rhizosphere, sequestered or degraded (phytoextraction, phytodegradation) (Chandra and Hoduck 2018).

- Phytoextraction for soil remediation. Plants can promote phytoextraction in several ways, such as phytoaccumulation, phytosequestration or photoabsorption. In this process, the plant's root system promotes the uptake of the contaminants, which can remain accumulated in the root system or be translocated to the aerial part of the plant (Cristaldi et al. 2017). Phytovolatilisation can be designed for different contaminated soils due to the characteristics of each plant: rapid growth, potential for metal accumulation, extensive root system, high tolerance to contaminants, wide geographical distribution, well adapted to prevailing climatic conditions, and repulsion of herbivores to avoid contamination of the food chain (Salt et al. 1995).
- Phytovolatilisation is the uptake of organic compounds by plants, followed by translocation and release of these VOCs from the stem/trunk and leaf surfaces. Indirect phytovolatilisation is due to increased volatile contaminant flux from the soil and water subsurface due to plant root activity.
- Phytofiltration refers to the approach of using plant biomass to remove contaminants, such as toxic metals, from polluted water by using plant roots (hemofiltration), seedlings (final filtration), or excised plant shoots (caulofiltration).
- Phytostabilisation/phytoimmobilization is the transformation of toxic compounds into non-toxic/less toxic forms that are fixed in the soil, thereby reducing the bioavailability of pollutants in the environment (Cristaldi et al. 2017). The immobilisation of heavy metals in soils using plants can be achieved by metal sorption by roots, precipitation, complexation or metal valence change/reduction in the rhizosphere. Rhizosphere bioremediation or rhizodegradation is the enhanced biodegradation of contaminants by root-associated bacteria and fungi. Due to increased microbial metabolic rates, rhizosphere bioremediation is efficient within certain plant species.

1.4.3 Mechanisms of tolerance to heavy metals in plants

To tolerate the presence of heavy metals in their tissues and normally carry out their metabolic functions, plants use two main mechanisms: Exclusion mechanisms and resistance mechanisms.

Exclusion mechanisms work by preventing the accumulation of toxic concentrations of heavy metals in sensitive areas of the cell, thus preventing adverse effects and not interfering with the biological mechanisms of the plant's vegetative cycle. The plant can use another mechanism, such as the root system, to secrete a metal chelating substance that enhances the extraction processes from the soil. In this scenario, the heavy metal is blocked in the soil, but the other plant cannot take up the heavy metal inside the plant. Another mechanism is symbiosis with other microorganisms that form the plant's rhizosphere. The micro-organisms allow the heavy metal to be chelated in their biological environment by chelating agents, such as the production of phenolic compounds or oxalic acid, or by sequestration in their cells to avoid uptake by the plant.

Other exclusion mechanisms include transport out of the plant by osmosis or binding through the cellulose, semi-cellulose, lignin or pectin in the cell wall and forming stable chelates with the sugars inside.

Cell wall binding refers to the binding of heavy metals to the major components of the cell wall, such as pectin, cellulose, hemicellulose and lignin. A significant number of heavy metals accumulate in the cell wall due to the binding of a divalent metal cation and trivalent metal cations with functional groups such as -COOH, -OH, and -SH in plant cells.

Another general recurring mechanism is the chelation of the metal by the ligand and the subsequent compartmentalisation of the ligand-metal complex. Several studies have shown that vacuoles are the site of accumulation of heavy metals, including Cd and Zn.

Resistance mechanisms involve the development of more resistant membranes or the activation of repair mechanisms that help plant cells to store the amount of heavy metal in the plant. In particular, several mechanisms are used by the plant to avoid ROS production, reduce competition between nutrients and heavy metal uptake, and avoid cell damage (Raza et al. 2020).

Plants cope with high levels of heavy metals by binding detoxifying metabolites, including metallothionein (MT), phytochelatins (PC), polyamines (Pas), the antioxidant enzyme system, abscisic acid (ABA), heat shock protein (HSP), and other metal chelating ligands (Raza et al. 2020). Metallothionein (MT), a low molecular weight cysteine-rich protein, is a ubiquitous non-enzymatic protein with an unusual amino acid composition.

The two best characterised heavy metal-binding polypeptides involved in the chelation and sequestration of heavy metals are the metallothioneins (MTs), which are small, gene-encoded, cysteine-rich polypeptides, and the phytochelatins (PCs), which are enzymatically synthesised, cysteine-rich peptides. Phytochelatin synthase (PC) is activated only in the presence of heavy metal ions, particularly Cd, Ag, Pb, Cu, Hg, Zn, Au and As. This ability to synthesise PCs is thought to be present in all higher plants.

Phytochelatins enable plants to tolerate heavy metals thanks to the sulphhydryl group (-SH) on cysteine. This allows heavy metals to be chelated, preventing their interaction with cellular components. Phytochelatins are low-molecular cytoplasmic proteins rich in cysteine (metallothioneine family). They are oligopeptides of three amino acids, including glutamine (Glu), cystine (Cys) and glycine (Gly), consisting of (y-glutamylcysteinyl)n-glycine, where n is the variable number of repetitions of the dipeptide y-glutamyl-cysteine, these repetitions can range from 2 to 11 (from PC2 to PC11). However, the most frequent are those between 2 and 5 (CP2-CP3-CP4-CP5). The detoxification mechanism of phytochelatins involves 2 phases: i) activation of the synthesis of phytochelatins, a process catalysed by the enzyme glutathione gamma-glutamyl-cysteinyl transferase, as a result of the increase in the intracellular concentration of heavy metals [by an enzyme (glutathione gamma-glutamyl-cysteinyl transferase)]; ii) chelation of heavy metals by the action of phytochelatins. The phytochelatin structure can be observer in **Figure 1.3**.

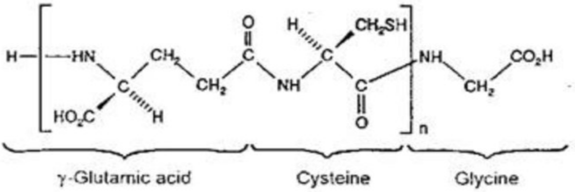

Figure 1.3. Phytochelatin structure

PCs play an essential role in eliminating the toxic effects of heavy metals in the cytosol (Li et al. 2013). PCs are transported into the vacuole as a complex when they are too heavy metal, thereby eliminating the toxic effects of heavy metals (Yang et al. 2005). Compartmentalisation can be achieved in the vacuole and epidermal cells, the subepidermal cell layer and the epitrichoderm.

Another strategy is used by heat shock proteins (HSPs), which are normally expressed in plants as a result of thermal stress, but when the heavy metals absorbed by the plant cause cellular lesions and secondary stresses such as osmotic and oxidative stress, plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance). HSPs are involved in the processes of protection, repair and degradation of damaged cellular components (Yang et al. 2005).

HSPs are classified according to their molecular weight as Hsp100, Hsp90, Hsp70, Hsp60 (or chaperone) and sHSP (small heat shock protein) .

Metal toxicity induces oxidative stress because metals are involved in several ROS-generating mechanisms. The plant can therefore activate several proteins that act as superoxide and hydrogen peroxide scavengers to prevent oxidative stress. These include superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX) and glutathione reductase (GR) (Yang et al. 2005). SOD converts free radicals to H_2O_2 and O_2, and CAT and POD further convert H_2O_2 to H_20, thereby reducing toxic effects. Under heavy metal stress, free radicals in plants induce denaturation of macromolecules such as proteins and nucleic acids and peroxidation of membrane lipids. Plant antioxidant enzymes are then activated to protect the plant from further damage. For example, SOD, POD and GSH are activated to scavenge free radicals generated by heavy metals. SOD, CAT and POD are maintained at a general level in heavy metal tolerant plants (Raza et al. 2020).

1.4.4 Index and factor in calculating for evaluating the tolerance or the potential of the biomass.

In order to better understand the tolerance and the phytoextraction capacity of the plants, to analyse the accumulation of the heavy metal in the plants in relation to the bioavailable heavy metal in the soil and the translocation in the different parts of the plant (roots, stems, leaves, seeds), it can be useful to evaluate the following index and factor.

- TI (Tolerance Index) is used to evaluate plants' susceptibility to the level of contamination present in the pot (Gomes et al. 2022; Yadav et al. 2009).

$$TI = \frac{\text{dry aboveground biomass weight of contaminated plants, g pot}^{-1}}{\text{dry aboveground biomass weight of control plants, g pot}^{-1}}$$

- mAI, the modified accumulation index, determines the ability of the plant to take up and accumulate a pollutant (Barbosa et al. 2015; Gomes et al. 2022).

$$mAI = \frac{\text{metal accumulation in the contaminated plant's mg kg}^{-1}}{\text{metal accumulation in the control plants, mg kg}^{-1}}$$

- The mBCF (modified bioconcentration factor) is used to assess the ability of the plant to extract and accumulate the metal in the aerial or radical fraction of the biomass. The content of bioavailable metal in the soil, determined by EDTA extraction, represents the amount of metal potentially bioavailable to the plant (Barbosa et al. 2015; Gomes et al. 2022).

$$mBCF = \frac{\text{metal concentration in the plant fraction, mg kg}^{-1}}{\text{bioavailable metal concentration in the soil, mg kg}^{-1}}$$

- The translocation factor (TF) estimates the capacity of the plant to translocate the metal in the aerial part, it is calculated based on the method of Mattina et al., 2003 (Mattina et al. 2003).

$$\mathrm{TF} = \frac{\text{metal conc. in the aboveground plant fraction, mg kg}^{-1}}{\text{metal conc. in the belowground plant fraction, mg kg}^{-1}}$$

Plants with mBCF and TF indices greater than one (> 1) are potentially usable in phytoextraction.

1.5 Promising industrial crops for phytoremediation

The most promising industrial crops and their metal tolerances can be observed in **Figure 1.4**. Considering the further use of the contaminated biomass, industrial crops are an excellent alternative for phytoremediation, in addition to the ability of these crops to extract, sequester and detoxify non-volatile compounds, and the possibility of producing bioproducts using the phytoremediated biomass (Barbosa et al. 2015). Another aspect of the association between industrial crops and phytoremediation is to be environmentally friendly, to decontaminate the soil, and to design specific hyper-tolerant and hyper-accumulator plants for each pollutant (Alloway 1995). This condition can be improved with the association of microorganisms (Wang et al. 2019).

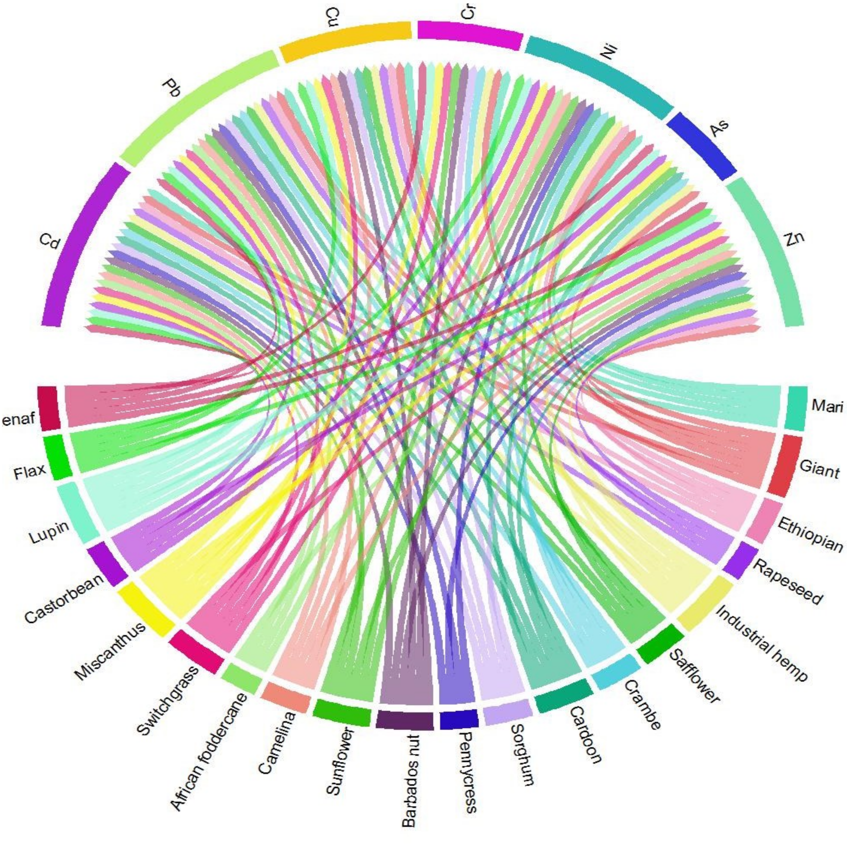

Figure 1.4 Chord diagram of the most important industrial crop and heavy metal tolerance.

Cadmium is a non-essential element that is detrimental to plant growth and development. It is recognised as a very important pollutant due to its high toxicity and high solubility in water. It can alter the uptake of minerals by plants by affecting the availability of minerals from the soil. Cadmium in nutrient solutions has been reported to affect stomatal opening, transpiration and photosynthesis. In addition, inhibition of root Fe(II) reductase by Cd led to Fe(II) deficiency and severely affected photosynthesis. Cd ions are normally retained mainly in the roots, but soil pH affects Cd uptake and transport. Plant accumulation of a given metal is a function of uptake capacity and intracellular binding sites. At each level, the concentration and affinities of chelating molecules are important. The presence and selectivity of transport activities also affect metal accumulation rates (Kabata-Pendias 2010).

Cadmium concentrations are generally measurable in roots and leaves, probably due to accidental uptake and translocation. The species of the Brassicaceae family (*Brassica carinata* L., *Brassica napus* L., *Crambe abyssinica* Hochst., *Thlaspi arvense* L., *Camelina sativa* (L.) Crantz) are well represented among the reported hyperaccumulators of Cd (Salt et al. 1995). Furthermore, plant species such as *Saccharum officinarum* can survive active CAT, SOD and GR (Fornazier et al. 2002), or Kenaf, possess a highly effective antioxidant system and are activated to cope with ROS damage under Cd stress (Chen et al. 2020; Deng et al. 2017). They suggest that kenaf could be cultivated in Cd-contaminated soils and used for phytoremediation.

Linger et al. (2012) showed that the photosynthetic pathway in hemp is affected by cadmium metal in two ways: (1) cadmium metal indirectly interferes with the uptake of water and ions by the plant, which affects the water status of the plant; (2) it directly affects the chloroplast apparatus after entering the leaf cells. Cd concentrations up to 72 mg/kg (soil) had no adverse effect on the germination of *C. sativa*. From the post-transplantation experiments, it was estimated that cadmium metal up to 100 ppm did not affect the morphological growth of *C. sativa* (Linger et al. 2002).

A study by Bauddha et al. (2015) compared castor with Indian mustard for phytoremediation potential of Cd in salinity and drought. The results showed that castor showed greater self-protection in the form of proline bioaccumulation in the presence of salinity and drought. Similar results were also observed for giant reed, safflower, cardoon, sorghum, barbados nut, sunflower, switchgrass, miscanthus, lupin, flax and pot marigold, which showed tolerance to growing in cadmium-contaminated soils.

Lead is a heavy metal with atomic number 82, belonging to group IV and the sixth period of the periodic table. The density of this metal is 11.4 g.cm^{-3}, with melting and boiling points of 327.4°C and 1725°C respectively. It is one of the most commonly used metals in industrial production because it is soft, highly malleable, ductile, has low conductivity, and is also very resistant to corrosion, but tarnishes when exposed to air. Pb does not play an essential role in plant metabolism, although if it is required for plant growth, the concentration should be less than 2 µg/kg dry biomass (Kabata-Pendias, 2011).

Soil Pb and extractable fractions were also reduced by giant reed in a 2-year outdoor experiment to assess the considerable potential of giant reed for phytoextraction and soil fertility restoration, confirming the ability of this crop to grow on polluted soils. (Fiorentino et al. 2017).

Buddha et al. 2015 reviewed the great potential of R. communis to accumulate a higher amount of Pb in its roots and shoots. *R. communis* accumulated 10.54-24.61 g Pb kg^{-1} dry weight of the plants, and according to Salt et al. (1995), *R. communis* was recommended as a hyperaccumulator species for Pb. The ability to accumulate lead has also been reported for industrial hemp, safflower, crambe, cardoon, common sorghum, pennycress, Barbados nut, common sunflower, camelina, african fodder cane, switchgrass, miscanthus, lupin, flax, kenaf.

Zinc is widely used in industry, particularly in the automotive, construction and battery industries. In plants, Zn has essential functions as a micronutrient in their metabolism. It is a building block for enzymes (Kabata-Pendias, 2011), metabolises carbohydrates, proteins and phosphates, and plays a role in RNA formation. Zn requirements vary from plant to plant, but excess of this heavy metal can be toxic to crops.Despite its functions in plant metabolism, excess Zn can interfere with nutrient uptake, be hazardous to enzymatic activities, affect photosynthesis and cause oxidative stress To avoid the harmful effects of excess Zn, plants accumulate the metal in cell walls and vacuoles, both below and above ground. This mechanism allows plants to tolerate high levels of zinc in the soil. Goodarzi et al. (Goodarzi et al. 2020) developed an experiment to study Zn-stressed safflower seedlings. Reduced levels of non-enzymatic antioxidants and decreased activity of enzymes involved in antioxidant defence and glyoxalase systems may also be associated with lower Zn concentrations in plants supplemented with MT, GSH and MT + GSH. In addition, studies are considering the application of antioxidants to increase the tolerance of the crop, thereby increasing the uptake potential (Namdjoyan et al. 2017).

Malik et al. show that *C. sativa* has a high translocation rate for the metal Zn compared to other species, and could be used as a potential hyperaccumulator of Zn. Due to this, the accumulation of metal Zn in shoots is high compared to other heavy metals showing hyperaccumulation in its different tissues from contaminated soil with different heavy metals in high status (Malik et al. 2010).

A study in Lisbon (Barbosa et al. 2015) tested the adaptability and phytoremediation capacity of giant reed and *Miscanthus* spp. on contaminated soil (under exposure of 450 and 900 mg kg^{-1} dry matter for Zn), showing their suitability for phytoextraction and accumulation. In particular, the results confirmed that the bioaccumulated Zn was easily transported and accumulated in the aerial fraction. Guidi et al. studied sunflower in contaminated soil and the result showed that the highest Zn concentration was observed in the stem. Similar behaviour was also observed in Barbados nut (Ko 2014). Good tolerance to the presence of Zn in soil was also observed in Ethiopian mustard, rapeseed, cardoon, common sorghum, pennycress, camelina, African fodder cane, switchgrass, castor bean, lupin, flax and kenaf.

Despite the natural presence of Ni in nature, some human activities can accelerate and imbalance Ni concentrations in the environment. Examples of anthropogenic Ni deposition in the environment include wastes from fossil fuel power plants, mining and smelting processes, emissions from the transport sector, industrial and urban wastes, and the steel and cement industries (Alloway 1995; Kabata-Pendias 2010). In plants, Ni is an essential micronutrient that is part of the plant nitrogen cycle through its presence in some enzymes such as urease and hydrogenase. The absence of Ni in the soil prevents the plant from completing its growth cycle, although the excess of Ni can lead to several harmful effects on the plant in morphological, physiological and biochemical aspects. In some cereals, the excess of Ni can be detected by some plant characteristics, such as interveinal chlorosis in new leaves, grey-green leaves, and brown and stunted roots (Kabata-Pendias, 2011), and it can be responsible for yield reductions of 20% in some plants.

Baran et al. 2022 showed that Carthamus species accumulated Ni in descending order of root > stem > leaf. Under all toxic treatments, the Ni content in the root and shoot tissues of both species increased progressively in a concentration-dependent manner. These results showed that large amounts of Ni were mainly accumulated in the roots than in the rest of the plant, while a small amount of Ni was gradually translocated to the aerial organs in safflower species.

Citterio et al. (2005) reported that mycorrhizal inoculation caused a slight reduction in plant growth, but Cannabis sativa accumulated higher concentrations of Ni in its shoots. The yield of Brassica spp. in Costa et al. was slightly affected by the presence of Ni in the soil. Furthermore, Camelina production was not affected in Ni-contaminated soil.

High tolerance at moderate levels of nickel has been reported for pot marigold, giant reed, Barbados nut, African fodder cane, switchgrass, miscanthus, castor bean, lupin and flax.

Among all the uses of chromium, stainless steel production is one of the most important due to stainless steel applications in the chemical, energy, and manufacturing sectors. The toxicity of Cr in the environment depends on the concentration of the metal and its oxidation state. Cr occurs in the environment as Cr(III) or Cr(VI), the latter being highly toxic to plants. Cr can affect the plant in different ways. It can reduce or even inhibit seed germination and the growth of roots, stems and leaves. The Cr uptake mechanism transports essential nutrients such as Fe, S and P into the plant, reducing the plant's nutrient uptake (Alloway 1995; Kabata-Pendias 2010).

Tang et al. 2021 show that Cr in Kenaf cultivation reduced plant height, root length, biomass and root cell viability compared to the control. In addition, the activities of antioxidant enzymes (SOD, POD and CAT) increased under Cr stress and peaked at Cr concentrations of 500, 300 and 400 μM, respectively. Finally, Gomes et al. 2022 studied giant reed and switchgrass in Cr-contaminated soils (300 and 600 mg kg^{-1}).The result showed that giant reed accumulated a certain amount of this heavy metal, especially in the roots, while switchgrass did not grow in the contaminated pots.

In Barzin et al. 2022, pot marigolds treated with 100 and 200 μM Cr decreased total dry weight by 19 and 35% and height by 21 and 37%, respectively. In addition, Cr toxicity induced a significant up-regulation of GR activity compared to control plants. Good tolerance in the presence of Cr was also observed in industrial hemp, crambe, common sorghum, Barbados nut, common sunflower, miscanthus and lupin.

Copper (Cu) metal is mainly used to make pipes and electrical cables, which are also essential in the construction and transport sectors. In plants, Cu is both an essential and toxic element, depending on its concentration. It acts as a cofactor for metalloproteins, but in excess it inhibits plant growth and interferes with some critical cellular processes. To maintain the required Cu levels, plants have developed mechanisms to regulate its homeostasis according to changes in the environmental conditions in which they are grown (Kabata-Pendias 2010).

Hu et al. (2015) tested the germination of crambe seeds in the presence of Cu (0.7 mM or ≥44.8 mg·L^{-1}) and found that the germination rate increased from 9.30% to 30.90% at 1.2 mM Cu, suggesting that crambe seed germination is moderately tolerant to Cu.

Several studies have been reported in the literature on the efficacy of *Saccharum* spp. in soils contaminated with heavy metals: in Sereno et al. (2007) sugarcane plantlets were able to tolerate up to 100 mmol/L copper in the nutrient solution for 33 days with no significant reduction in fresh weight and accumulated 45 mg/kg Cu on a shoot dry weight basis.

Calendula officinalis showed that translocation factor (TF) and bioconcentration factor (BCF) were more significant than 1. In addition, it showed a positive effect of salicylic acid as a chelate in soil on translocation, accumulation and bioconcentration factor. Phytoextraction potential was also observed in giant reed, Ethiopian mustard, rapeseed, industrial hemp, safflower, cardoon, sorghum, Barbados nut, camelina, switchgrass, miscanthus, lupin and flax.

1.6 Production of bioenergy with Industrial crops used in phytoremediation

The concept of biorefinery, which is similar to that of green chemistry, tends to overcome the limitations of a purely energetic use of non-food crops by proposing a potentially integrated use of plant biomass as a basis for the production of chemical molecules with reduced environmental impact, such as an alternative to products of petrochemical origin, using marginal land that is therefore not diverted from agriculture for food and by converting the same plants for oil refining.They are systems that integrate biomass conversion processes of a chemical, physical or microbiological nature to obtain energy products, materials and chemicals with high added value.

Biorefineries have been identified as the most complete and promising way to create an industry based on products derived from materials of biological origin, capable of enhancing the different chemical components of biomass through the production of multiple compounds.

Despite the contamination, the biomass produced is still a source of components that could be used sustainably to produce energy, fuels and products. Furthermore, the use of contaminated biomass in biorefineries completes a sustainable cycle from soil decontamination to the use of a renewable resource.Contaminated biomass can be processed in different ways in biorefineries, either physico-chemically or biologically. For example, gasification (Ancona et al. 2019; Dastyaret al. 2019; Nzihou and Stanmore 2013; Šyc et al. 2011), pyrolysis (Dastyar et al. 2019; He et al. 2019; Lievens et al. 2008; Zeng et al. 2019), and combustion (Dastyar et al. 2019; Hunce et. al. 2019; Nzihou and Stanmore 2013) of biomass are options for energy and fuel production, although treatment steps need to be implemented, such as ash treatment in the case of combustion, or monitoring the effects of heavy metals in the machinery, as in gasification.

The possibility of using industrial crops after phytoremediation opens up a large field of research, as different parts of the plant can be treated in different biorefinery ways, depending on the accumulation of heavy metals. As shown in **Figure 1.5**, industrial crops such as safflower, industrial hemp, giant reed and flax have already been tested in the biorefinery process, indicating its potential.

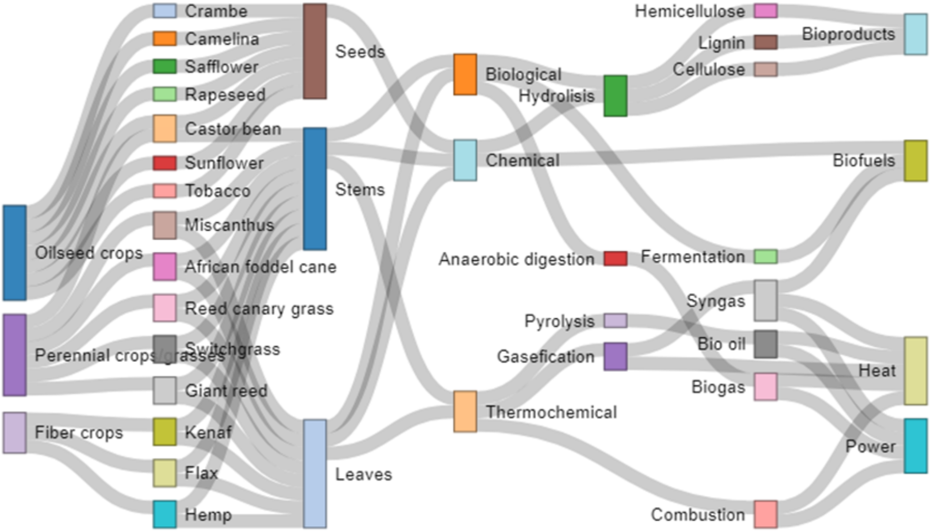

Figure 1.5 Biorefinery process of the main industrial crops.

1.6.1 Biological and chemical ways

Biological path uses microorganisms to valorise the biomass to produce biofuels, bioproducts or even to promote its pre-treatment. The chemical pathway promotes the use of chemical reagents, starting from biomass, to produce biofuels. The main paths of these two techniques are presented in the following sections.

1.6.1.1 Hydrolysis: Hemicellulose, Cellulose, and Lignin to produce bioproduct

Lignin is one of the most abundant substances in nature. Unlike cellulose, it has an amorphous structure made up of benzene groups. It is obtained as a by-product of hydrolysis, once this by-product was used as a fuel, whereas today it is obtained as lignosulphonates (by sulphonation with sodium sulphite at 150-200°C or with phenethylamine) and used to obtain other derivatives. Lignosulphonates are used as surfactants, dispersants, sequestrants, humectants and stabilisers, in adhesives and dyes, in the cement and paperboard industries. Acetic and formic acids can be obtained from the conversion of lignocellulosic materials. It is also possible to separate hemicellulose and cellulose from lignocellulosic materials by extraction with alkali. Cellulose is a D-glucose polymer with a well-defined crystalline structure and is the main constituent of the fibrous part of plants (40-60%) by separating cellulose from lignin and subsequent mechanical, semi-chemical and chemical processes. Cellulose is used in the manufacture of paper and boarding; its pure form, in the manufacture of man-made fibres. It is also used to make cellulose derivatives (rayon and cellulose acetate) and other polymers (cellophane, ethers and cellulose esters). Finally, hemicellulose is also a polysaccharide, poorly soluble and associated with cellulose. It is composed of glucose and other sugars, and cellulose and hemicellulose are the most abundant renewable materials. In addition, hemicelluloses can modify the properties of water, such as viscosity, surface tension and tendency to gel.

1.6.1.2 Fermentation

The main sugars, mono- and disaccharides, that can be extracted from plant components include D-glucose, D-fructose, D-L-galactose, sucrose, lactose and maltose, which can be obtained mainly from starch, cellulose and hemicellulose. They are used in the food industry and in the preparation of fermentation substrates. The alcoholic fermentation process, carried out by numerous microorganisms, allows the conversion of the carbohydrates contained in plant production into ethyl alcohol (bioethanol) (Scordia et al. 2010).

1.6.1.3 Bioethanol

Bioethanol can certainly be used in internal combustion engines, and among the alternative products derived from biomass conversion currently available, it offers the best compromise between price, availability and performance. In some South American countries, it is used purely in normal internal combustion engines or with petrol additives. The most tested and widespread crops are sugar cane, wheat and maize, but any crop rich in sucrose, starch and lignocellulose materials is suitable for bioethanol production (Scordia et al. 2010).

1.6.1.4 Anaerobic digestion

Anaerobic digestion is a biochemical conversion process carried out in the absence of oxygen by microorganisms, more precisely anaerobes, which break down complex organic substances such as lipids, carbohydrates and proteins contained in plants, but also animal by-products, manure, municipal waste, etc. Anaerobic digestion makes it possible to produce biogas from plant biomass (but also from organic residues of animal origin). The biomass is placed in a fermenter, where micro-organisms develop and, together with the fermentation of the organic matter, produce the so-called biogas, which consists of 50-70% methane (Piccitto et al. 2022).

1.6.1.5 Biogas

The biogas produced is collected, dried, compressed and stored, and can be used to drive gas boilers to produce heat, or perhaps coupled to turbines to produce electricity, or used to drive gas engines. In addition, at the end of the fermentation process, the main nutrients (nitrogen, phosphorus and potassium) already present in the raw material are almost intact. This makes the effluent an excellent fertiliser, once the dangerous substances that cannot be broken down have been removed. Once the biogas has been purified of the other components, especially carbon dioxide, it can be used as biomethane for vehicle transport, combustion in boilers, heating and the production of electrical or thermal energy. In this case, too, the CO_2 produced by the combustion of the biomethane makes it possible to offset the carbon dioxide emitted into the atmosphere; in fact, the amount of CO_2 emitted by the combustion of biogas is equal to the amount fixed by the plants (Piccitto et al. 2022).

1.6.1.6 Production of biofuels such as Biodiesel

Biodiesel is a biofuel derived from renewable sources such as vegetable oils (from the seeds of oil plants such as rapeseed, soya, sunflower and castor) and animal fats, similar to diesel oil derived from petroleum. Vegetable oils are converted into biodiesel through a chemical process called "transesterification", in which the triglyceride molecules are broken down using an alcoholic catalyst (methanol, ethanol), converting the base oil into the desired ester. Thanks to technologies that operate at low temperatures and pressures, two products are obtained: biodiesel and glycerine, a by-product that can be used in various applications, particularly in cosmetics or in the production of lubricants. The green fuel obtained can be used in the new generation of diesel engines. Biodiesel has many advantages over diesel fuel, particularly from an environmental point of view: it does not contribute to the greenhouse effect, since it returns to the air only the amount of carbon dioxide that was removed from the atmosphere during the growing cycle, thus reducing the health risks associated with "air pollution"; it is biodegradable within a few days and, when blended with diesel, it is three times more biodegradable. It is also safe to transport and handle, as it is non-flammable and can be stored and pumped using the same methods and equipment as diesel (Kadir et al.

2020).

1.6.2 Thermochemical conversion

It is possible to extract and convert energy in various forms through various thermo-chemical, combustion and aerobic/anaerobic digestion processes.

1.6.2.1 Pyrolysis

Pyrolysis is another process of thermochemical decomposition obtained by applying heat between 400 and 800°C, which, unlike gasification, takes place in the complete absence of oxygen. This makes pyrolysis particularly suitable for the valorisation of poorly biodegradable waste biomass (i.e. wood biomass, digestate, municipal and agricultural waste).

Thus, under anaerobic conditions and in a hermetic environment to prevent the escape of gases, the product undergoes a splitting of the bonds with the formation of simpler molecules.

The products obtained are both gaseous (syngas), both liquid (pyrolysis oil) and biochar, depending on the pyrolysis method (fast, slow or conventional) and reaction parameters, and can be used as fuels or raw materials for other conversion processes; for example, when the pyrolysis process is operated at low temperatures and long gas retention times (i.e. slow pyrolysis), the biochar is the main product. Furthermore, as the temperature increases, the conversion of the substrate to pyrolytic gas is enhanced. However, the longer the vapour residence time and the lower the heating rate, the lower the proportion of bio-oil formed. Conversely, high temperatures and heating rates and short gas retention times promote bio-oil production (i.e. fast and flash pyrolysis) (Dastyar et al. 2019).

1.6.2.2 Gasification

Gasification is a thermochemical process that allows biomass to be converted into gaseous fuels suitable for various applications. During gasification, the still wet biomass is placed in a dryer to evaporate excess moisture; once dried, it is transferred to the actual gasifier, where it undergoes pyrolysis at temperatures between 700 °C and 1200 °C, resulting in the formation of synthetic gas (syngas), composed mainly of molecular nitrogen (N_2), water vapour, carbon monoxide (CO), carbon dioxide (CO_2) and methane, as well as a small fraction of heavier hydrocarbons. The composition of synthesis gas varies, with the main components being H_2, CO and CO_2. In addition, syngas may contain other minor impurities (i.e. sulphur and nitrogen compounds, hydrocarbons and tars) depending on the type of substrate processed through a gasifier. If the substrate contains sulphur, nitrogen and chlorides, the sulphur is converted to H_2S, the nitrogen to NH_3 and the chlorides to HCl.

The gas is then cooled and filtered to remove dust, organic compounds and waste produced during the process, and can then be used as a fuel gas, for example to drive engines or turbines to generate electricity, or converted into liquid fuels such as ethanol.

1.6.2.3 Production of Hydrogen

The conversion of hydrogen to biomass can take place via various routes, such as gasification and pyrolysis, as well as various biological processes, depending on the characteristics of the feedstock. Biomass feedstocks used for hydrogen production include lignocellulosic biomass (second generation), agricultural residues and biowaste. Nowadays, pyrolysis and gasification are the most common phenomena for hydrogen production. However, it is still costly due to higher energy input and poor formation of hydrogen-rich syngas from biomass.

Hydrogen production from syngas also depends on the parameters of the gasification process, such as residence time, oxidizing agent, pressure, temperature, feedstock composition and type of gasifier (Heidenreich and Foscolo 2015).The composition of syngas varies, with H_2, CO and CO_2 as the main components. The use of steam as an oxidant can improve the H_2 yield once the oxygen in the water oxidises the carbon monoxide in the syngas, producing hydrogen in the process.Hydrogen can be produced from pyrolysis gas, or pyrogas, which contains mainly H_2, CO, CH_4 and CO_2. The abundance of these pyrolytic gas components varies depending on the feedstock, pyrolysis unit configuration, and process parameters. The potential of the pyrolysis gas for further biological conversion to CH_4 depends on its composition and the amount of reducing equivalents (i.e. CO and H_2). As the temperature of the pyrolysis process increases, a higher content of CO and H_2 in the pyrolysis gas can be achieved (Hossain and Davies, 2013).

1.6.2.4 Combustion

Biomass energy can be recovered in waste-to-energy plants through its direct combustion and through processes to improve its efficiency.

However, this use would require the availability of a suitable plant equipped with appropriate devices to reduce atmospheric emissions (e.g. electrostatic precipitators) (Lewandowski et al., 2006). The specially treated and selected plant residues are used as fuel: when burned, they produce steam that drives a turbine, which is connected to an alternator that produces electricity.

1.7 Conclusions

Biomass is a renewable and sustainable source of energy that contributes to the diversification of energy supply, reduces dependence on fossil fuels and mitigates greenhouse gas (GHG) emissions. Therefore, biomass production on marginal soils is recommended to reduce land use and change ethical issues related to competition with food crops. Furthermore, although the marginal conditions of the soils, such as contaminated soils, may hinder the cultivation of traditional crops, industrial crops manage not only the restoration of the contaminated areas, but the same way allowed to obtain a benefit from several perspectives, obtaining technological and environmental performance suitable. Once again, in this context, understanding the tolerance and phytoremediation of each species to a single contaminant helps us to clean up the polluted areas better, and it can be useful for organising protocols aimed at the reclamation of these areas, reducing time and management costs, providing a renewable source of energy and bioproducts.

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Aim of the thesis

The present thesis aims to investigate an economically efficient approach to clean up heavy metal polluted soil with the possibility of further utilization of phytoremediation biomass turns energy crops in a great option for this technique. Energy crops infact are known for their ability to grow with low agriculture input and later the biomass product can be used to produce biofuels, bioenergy, and bioproducts in a sustainable and renewable way, creating economic potential, mainly when cultivated in contaminated soils. In this experiment, *Arundo donax* L., *Carthamus tinctorius* L., *Cannabis sativa* L. and *Saccharum spontaneum* L. ssp. *aegyptiacum* are tested in soil contaminated with different concentrations of Zinc, Cadmium, Lead and Nickel, seeking for effect in productivity and the uptake of the heavy metal. Furthermore, it was investigate the concentration of the heavy metals in the different parts of the plant (roots, stems, leaves and seeds), in order to better undersand the best reuse of the contaminated biomass as feedstock and give a potential suistanable and economic return to phytoremediation technique.

Thesis Outline

Chapter 1: Mediterranan industrial crops for phytoremediation and bioenergy production in heavy metal polluted soil

An introductory chapter that focuses on the impact of mediterranean environment in land degraded by heavy metal pollution. This chapter review the possibility to use industrial crops, promoted also by the RED II and the Green Deal, able to grow in mediterranean environment, in order to remediate this contaminated soils with phytoremediation technique exploring the possibility to produce bioenergy or bioproducts though biorefinery paths.

Chapter 2: Phytoremediation of Heavy metal contaminated soils Using Safflower

In this chapter Safflower was tested in soils contaminated with zinc, cadmium, lead, and nickel at different concentrations to evaluate the effects on yield and heavy metal content in the different parts of the plant. The experiment highlights the tolerance of Safflower to cultivation in heavy-metal-polluted soil; in fact, a low reduction in biomass yield was observed. Among the evaluated heavy metals, the higher susceptibility was observed at the highest zinc and cadmium concentrations. Generally, safflower concentrates heavy metals in the belowground biomass. The relatively low concentrations of heavy metals in some parts of the aboveground biomass could suggest the possibility of using it as a feedstock for bioenergy conversion.

Chapter 3: Adaptability of industrial hemp to different levels of heavy metal in soil

In this chapter, two varieties of industrial hemp, Futura 75 and Kc Dora, were tested in different concentrations of Cadmium, Lead, and Zinc, seeking the physiological response and the mechanism of tolerance of this species to the heavy metals contaminated soil.

Chapter 4: Phytoremediation of heavy metal polluted soil by industrial hemp.

In this chapter two monoecious industrial hemp varieties. Futura 75 and Kc Dora, a medium-late ripening cultivar originated in France and Hungary, were tested to grow in three different levels of cadmium, lead and nickel, evaluating the productivity performance, the concentration of the heavy metals in the different parts of the plant, and the phytoremediation index.

Chapter 5: Physiological tolerance of perennial grasses to heavy metal contaminated soils

This chapter aimed to study the physiological response of plants tolerant to heavy metals, in particular, focused the attention on two non-food lignocellulosic perennial grasses: giant reed (*Arundo donax* L.) and African fodder cane (*Saccharum spontaneum* L. ssp. *aegyptiacum*), in soils contaminated by four heavy metals (Cd, Pb, Ni, Zn) with the purpose to observe the physiological response and the mechanisms of resistance in the increasing concentrations of the four soil heavy metals.

Chapter 6: A comparison study on the phytomanagement potential of two lignocellulosic crops

In this chapter, two industrial crops were chosen according to their potential to provide biomass suitable for different applications. Two lignocellulosic perennial grasses, *Arundo donax* L. and *Saccharum spontaneum* L. ssp. *aegyptiacum* were tested in contaminated soil with different concentrations of Zinc, Cadmium, Lead, and Nickel, seeking the productivity performance, and concentration of the heavy metal in the different parts of the plants. Moreover, the quality of the biomass was investigated and the energy potential was calculated.

Chapter 7: General discussion and conclusions

A conclusive chapter focused on the main result obtained in the different research activities involved in the PhD path.

2 Phytoremediation of Heavy Metal Contaminated Soils Using Safflower

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Phytoremediation of Heavy Metal Contaminated Soils Using Safflower

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Abstract: The promotion and gradual replacement of fossil fuels with renewable sources increase the competition between food and fuel. Therefore, energy crops could be produced on unproductive marginal land due to unfavorable conditions, such as nutrient and water availability limitations or contaminants such as hydrocarbons or heavy metals. In the case of soils contaminated with heavy metals, one option could be using plants to extract or immobilize the contaminants in the soil in a process called phytoremediation. Carthamus tinctorius L. is an annual herbaceous plant with a deep root system, and the oil extracted from the seeds is an excellent oil for conversion into biofuel. Therefore, it appears suitable to be used in the phytoremediation process, increasing the opportunity to valorize polluted areas and reducing the risk of abandonment of these lands. In this study, *C. tinctorius* was tested in soils contaminated with zinc, cadmium, lead, and nickel at different concentrations to evaluate the effects on yield and heavy metal content in the different parts of the plant. The experiment highlights the tolerance of Safflower to cultivation in heavy-metal-polluted soil; in fact, a low reduction in biomass yield was observed. Among the evaluated heavy metals, the higher susceptibility was observed at the highest zinc and cadmium concentrations. Generally, safflower concentrates heavy metals in the belowground biomass. The relatively low concentrations of heavy metals in some parts of the aboveground biomass could suggest the possibility of using it as a feedstock for bioenergy conversion.

Keywords: marginal lands; zinc; cadmium; nickel; lead

2.1 Introduction

The soil must be considered a non-renewable resource, and the preservation of its ability to provide ecological, economic, and social services is relevant to the well-being of future generations. Therefore, it must be used sustainably. It is estimated by Toth et al. (2016) (Tóth et al. 2016) that 137,000 km^2 of European agricultural land needs to be remediated from heavy metal contamination. Generally, heavy metals in contaminated soils are defined as chemical elements whose density is greater than 7 g/cm$_3$ or, based on their atomic weight, as an element whose atomic weight is greater than 20 (Adriano 1969; Alloway 1995). Heavy metals are naturally present in the soil, but due to industrialization and the discarding of electronic waste, for most of them (for example, Pb, Cd, Zn, As, Ni, and Hg), the concentration in the soil has been increasing, leading to severe concerns regarding health issues for human beings, animals, and plants. Several remediation techniques have been developed concerning the typology of chemicals contaminating the soil, involving both physical and biological processes. Among biological processes, phytoremediation uses plants to extract, stabilize, volatilize, and degrade pollutants. This approach has a lower environmental impact and economic cost than other chemical and physical techniques (Salt et al. 1995). Furthermore, the economic suitability can be enhanced with the possibility of cultivating energy crops, overcoming the inefficient use of the land (Cossel et al. 2019; Liu, Xiao, and Guo 2019; Nalepa and Bauer 2012).

Plants’ rhizospheres, along with the pH, climatic conditions, and organic matter, affect the bioavailability of heavy metals. As a result, metals in soils can be available, unavailable, or exchangeable (Shen et al. 2022), promoting the remediation of these soils. Therefore, the crop selection needs to fulfill several conditions, such as the tolerance of the crops to heavy metals, high production of biomass, deep and extensive root systems, well known agronomic techniques, and a low requirement for agronomic input. In addition, the possibility of converting biomass for bio-energy production, including ethanol, biogas, and biodiesel, represents an additional benefit of phytoremediation crops (Barbosa et al. 2015a; Fernando et al. 2015).

The bio-energy conversion allows for controlling the dispersion of the contaminated biomass or the contaminated ashes deriving from the uncontrolled combustion of the biomass and answers the request for the production of sustainable energy (RED II) (EU 2018), leading to a fair mediation between the economic profit deriving from the use of the land and the respect for the environment promoted through the adoption of European directives and regulations in environmental matter.

In this scenario, safflower (*Carthamus tinctorius* L.), an oilseed plant from the Asteraceae family that originated in southern Asia, appears to be an excellent candidate crop for heavily polluted soils. It is an annual or biennial herbaceous plant cultivated in different climatic conditions, including the Mediterranean area, thanks to its excellent tolerance to drought (Manvelian et al. 2021) and salinity (Gengmao et al. 2015). It is a spring–summer, long-day plant grown in high-intensity light and high temperatures during all phases of the biological cycle. It is currently cultivated in North America, Asia, and Russia.

Safflower has been cultivated for the yellow and red pigments obtained from the flowers, which have been used as coloring agents in food, clothes, cosmetics, and medicine. In addition, safflower seed has been recognized as a promising oil source for biodiesel production because of its high oil content of between 26 and 45% and the high oleic and linoleic acid (Gongora et al. 2022). Further bioenergy applications include the production of biogas or bioethanol from safflower straw and the development of biorefinery processes based on the whole safflower plant (Sajad, Karimi, and Mirmohamadsadeghi 2019; Sajad, Mirmohamadsadeghi, and Karimi 2020).

The hypothesis investigated in this work is that safflower can grow in contaminated heavy metal soils and remediate these soils. These abilities were evaluated under cadmium, lead, nickel, and zinc contamination, investigating two different concentrations for each metal. The remediation of these elements is essential due to their wider spread than other heavy metals and their negative impacts on the ecosystem and human health. The environmental contamination by these elements derives from various sources, such as industry, mining, smelting, and agriculture (Briffa, Sinagra, and Blundell 2020; J. Yang et al. 2022; Y. Yang et al. 2022). To define the possibility of reusing biomass for bioenergy purposes, the heavy metal accumulation in the different parts of the plants (stems, leaves, seeds) has been measured.

2.2 Materials and Methods

The experiment was carried out at the University of Catania, Department of Agriculture, Food and Environment (Sicily, Italy), in two subsequent years (2020 and 2021). Carthamus tinctorius L. was grown in pots containing 12 kg of soil (30 cm diameter and 30 cm height), previously contaminated with four heavy metals at two concentrations (low and high). The concentrations of the contaminants applied to the soils are reported in **Table 2.1**. Pots containing untreated soil were used as a control group. Pots were arranged in a completely randomized experimental design with 3 replications. The soil was contaminated using nitrate of Zinc [$Zn(NO_3)_2$], nitrate of cadmium [$Cd(NO_3)_2$], nitrate of nickel [$Ni(NO_3)_2$], and nitrate of lead [$Pb(NO_3)_2$].

Table 2.1. Amounts of contaminants supplied to the soil in the different studied factors.

Trial	Zinc (mg.kg^{-1})	Cadmium (mg.kg^{-1})	Nickel (mg.kg^{-1})	Lead (mg.kg^{-1})
Low level	450	4	110	450
High level	900	8	220	900

The soil (Andisol, USDA) used for the experiment was collected from a farm in the Etna area, taken from a depth of 30 cm. It was characterized at the beginning of the experiments by sampling 1 kg of soil, which had been dried in an oven at a temperature between 25 and 30 °C and then sieved through a 2 mm mesh. Particle size distribution was measured, and conductivity was determined in 1:1 soil/distilled water suspensions after 1 hour with conductivity electrodes. A pH-meter PH 7 Vio XS measured the pH value (H_2O). The soil organic matter was determined using the Walkley–Black method (Nelson and Sommers 1983).

The uncontaminated soil characteristics are reported in **Table 2.2**. The texture of the soil was characterized by a high sand content (92.9%). The organic matter content was low (0.86%). The pH was neutral–lightly alkaline and had a high conductivity.

The total metal content (Cd, Ni, Zn, and Pb) of the soil was quantified by atomic absorption spectrometry (AAnalyst 200 AA Spectrometer, Perkin Elmer) on the aqua regia digested samples, according to ISO 11466 (ISO, 1998) (Standard 1998) before the experiment and after the harvesting of the plants. In addition, the bioavailable heavy metal content in the soil was determined following ISO 17402 (ISO, 2008) (ISO 707:2008 2003) using EDTA concentration of 0.05 M, pH 7.5 (near to soil pH) to a volume ratio of 1:20 in 1 g of soil, under stirring for 24 h. To quantify the available heavy metals, the filtrate solution was measured by atomic absorption.

Table 2.2 Soil physical and chemical characteristics.

Physical Characteristics	
Clay (%)	3.0
Silt (%)	4.1
Sand (%)	92.9
Texture	Sandy
Conductivity (µS/cm)	34.2
Chemical characteristics	
pH	7.4
Organic matter (%)	0.86
Fe (mg kg^{-1})	23.6
P (mg kg^{-1})	7
Mn (mg kg^{-1})	0.1
Cu (mg kg^{-1})	21.8

The seeds were germinated in Petri dishes, and each germinated seed was planted into peat pots and, after two weeks, transplanted into the contaminated pots (eight plants per pot). During the crop cycle, plants were maintained in well water conditions. A meteorological station nearby recorded the main meteorological parameters, observed in **Figure 2.1**.

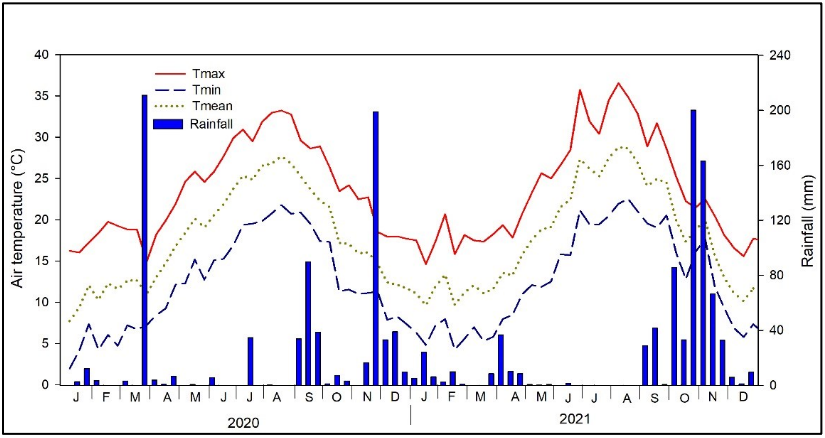


Figure 2.1 Meteorological data for the two years. Tmax and Tmin represent the 10 days average of the daily maximum and minimum temperature, respectively.

During the two growing seasons (March–July) the minimum temperature ranged from 6.7 °C to 19.8 °C and from 5.3 °C to 21.1 °C in the first and second years, respectively. On the other hand, the maximum temperature ranged from 14.9 °C to 31.9 °C and from 17.4 °C to 35.7 °C in the first and second years, respectively. The growing seasons' cumulative rainfall was 266.4 mm in the first year and 67.0 mm in the second year. The high rainfall in the first year was related to extreme events in the third week of March 2020 (Table S1).

The harvest was performed at the complete seed ripening stage, and the plants in each pot were collected and fractionated into stems, leaves, and seeds. Then, the biomass was weighed and dried in an oven at 65 °C until constant weight. In addition, the roots were collected and washed with ultra-pure water to remove soil particles and weighed and dried at 65 °C in the oven until constant weight.

Each sample was ground with a mill at a 1 mm sieve (IKA M20), and 1 g of biomass was reduced to ashes in the muffle furnace at 550 °C for 5 h. The heavy metal digestion of the biomass samples was performed in a water bath with 10 mL of nitric acid solution 1:1 (Nitric Acid 65%, Sigma-Aldrich, Burlington, MA, USA). The sample was filtrated through the Whatman paper. The heavy metal concentration of the extract was quantified in a specific volume by the atomic absorption spectrometer (AAnalyst 200 AA Spectrometer, Perkin Elmer, Waltham, MA, USA).

2.2.1 Data Analysis

To evaluate the tolerance of safflower, the potential phytoextraction, and the transportation of the heavy metals into the plants, the tolerance index (TI), the bioconcentration factor (BCF), the accumulation index (mAI), and the translocation factor (TF) were calculated according to Barbosa et al. (2015) (Barbosa et al. 2015a; Gomes et al. 2022).

- TI (Tolerance index) was used to evaluate the tolerance of plants at different levels of contaminants in the soil

(Girdhar et al. 2014; Gomes et al. 2022; Yadav et al. 2009):

$$TI = \frac{\text{dry aboveground biomass weight of contaminated plants, g pot}^{-1}}{\text{dry aboveground biomass weight of control plants, g pot}^{-1}}$$

- mAI (modified Accumulation Index) was calculated to evaluate the plant’s ability to uptake the heavy metal from the soil (Barbosa et al. 2015a):

$$mAI = \frac{\text{metal accumulation in the contaminated plant's mg kg}^{-1}}{\text{metal accumulation in the control plants, mg kg}^{-1}}$$

- mBCF (modified bioconcentration factor) was used to evaluate the ability of the plant to extract and accumulate the metal in the aerial or radical fraction of the biomass. The content of the bioavailable metal in the soil, determined by EDTA extraction, represents the amount of metal potentially bioavailable for the plant. Therefore, this factor can represent more realistically the translocation capacity of the metal in plants (Barbafieri et al. 2011; Barbosa et al. 2015a; Mattina et al. 2003):

$$mBCF = \frac{\text{metal concentration in the plant fraction, mg kg}^{-1}}{\text{bioavailable metal concentration in the soil, mg kg}^{-1}}$$

- TF (Translocation Factor) is expressed as the ratio between the metal concentration in the aboveground fraction of the plant (mg kg^{-1}) and the metal concentration in the root fraction of the plant (mg kg^{-1}) (Barbosa et al. 2015a; Gomes et al. 2022; Mattina et al. 2003):

$$TF = \frac{\text{metal concentration in the aboveground plant fraction, mg kg}^{-1}}{\text{metal concentration in the belowground plant fraction, mg kg}^{-1}}$$

Plants with mBCF and TF indices greater than one (>1) are potentially suitable for phytoextraction.

The data were statistically analyzed using R software (4.2.0, R Core Team, 2013). Contaminants and their concentrations were considered the main factors, and Tuckey’s HSD test separated the means. The Shapiro test was used to verify the normality of the residual distribution. Finally, the ANOVA tested the difference in productivity and heavy metal concentration over the years.

A principal component analysis (PCA) was used to visualize and interpret the multidimensional data. The PCA was based on Person’s correlation matrix calculated upon the biomass fraction yields and heavy metal concentration in the biomass fractions (Pidlisnyuk et al. 2019).

2.3 Results

2.3.1 Soil Characterization

The results showed in **Table 2.3** present that bioavailable Zn, Cd, and Ni concentrations in the soil were higher in the first year of the experiment (2020), while Pb had a higher bioavailable concentration in the second year (2021) (Table 3). Considering the first year, the bioavailability was 59.2% and 61.6% for Zn450 and Zn900, 54.1% and 61.6% for Pb_{450} and Pb_{900}, 55.7% and 65.5% for Cd_4 and Cd_8, 65.1% and 70.4% for Ni_{110} and Ni_{220}. In the second year, the bioavailability increased to 65.3% and 66.9% for Zn_{450} and Zn_{900}, respectively. In the Pb treatment, a slight decrease in the bioavailability was observed (53.1%) in the low level of contaminant (Pb_{450}), while an increase was observed in the high level (66.7%). In addition, the bioavailability of Cd underwent a considerable decrease, reaching 39.2% for Cd_4 and 51.8% for Cd_8. In Ni treatments, a lower bioavailability was observed in the second year, with 47.7% and 69.9% for Ni_{110} and Ni_{220}, respectively.

Table 2.3 The total and bioavailable heavy metals (HM) concentration in the contaminated and control soils.

HM	Concentration (mg kg^{-1})	2020		2021	
		Total HM (mg kg^{-1})	Bioavailable HM (mg kg^{-1})	Total HM (mg.kg^{-1})	Bioavailable HM (mg kg^{-1})
Control-Zn	-	35.98±2.72	9.51 ± 1.16	36.89±10.50	4.57 ± 1.41
Zn	450	478.61±5.40	283.18 ± 2.00	496.85±14.01	324.68 ± 13.69
Zn	900	883.70 ±22.33	544.60 ± 1.35	803.30±45.41	537.35 ± 6.16
Control-Pb	-	11.01 ±2.13	2.32 ± 1.16	17.45±6.96	3.76 ± 1.01
Pb	450	447.80 ±3.99	242.45 ± 1.63	464.96±53.44	247.02 ± 62.63
Pb	900	840.13±12.62	517.59 ± 5.85	876.20±69.86	584.38 ± 31.66
Control-Cd	-	0.43±0.11	0.11 ± 0.05	0.69±0.10	0.16 ± 0.001
Cd	4	4.43 ± 0.22	2.47 ± 0.19	3.74±0.20	1.47 ± 0.002
Cd	8	8.86± 0.80	5.80 ± 0.10	9.88±2.12	5.11 ± 0.26
Control-Ni	-	33.53 ± 3.79	12.96 ± 1.83	20.92 ± 4.76	4.72 ± 1.82
Ni	110	121.19 ± 7.93	78.87 ± 14.64	119.39 ± 13.22	56.98 ± 2.86
Ni	220	248.20 ± 18.82	174.80 ± 5.13	207.27 ± 6.23	144.82 ± 7.72

2.4 Biomass Production

The aboveground biomass was affected by heavy metals concentration in soil with different behaviors according to the contaminant, presented in **Figure 2.2**. In particular, Zn treatments significantly affected the aboveground biomass production in both years, with higher re-duction at higher Zn content. Pb contamination led to a minor reduction in aboveground biomass.

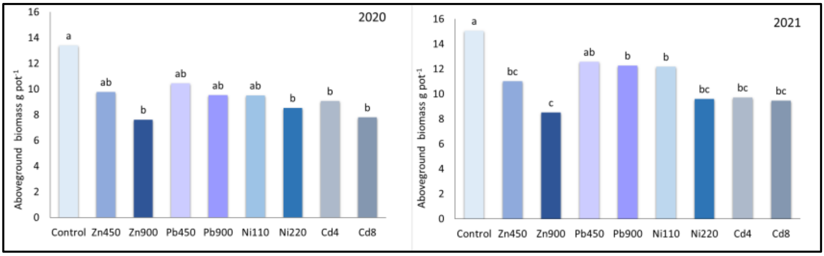


Figure 2.2. Aboveground DM (g.pot$^{-1)}$ of Safflower concerning the different studied treatments. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

The aboveground productivity was higher in the second growing season than in the first. A slight increase was observed in the Control, while the productivity increased from 9.8 to 11.0 g pot^{-1} in Zn_{450} and from 7.6 to 8.5 g pot^{-1} in Zn_{900}. The aboveground productivity in Ni_{220} reached 9.5 and 12.2 g.pot^{-1} in the first and second years, respectively. No significant effects were observed among different concentrations of Pb. In Ni-contaminated pots, the reduction in the yield was 19.1% and 36.3% for Ni_{110} and Ni_{220}, respectively, while in Cd trials, the reduction was 35.4% and 37.1% for Cd_4 and Cd_8, showing that Cd has a high effect on safflowers' productivity.

The contaminants' effect on the biomass fractions yields can be observed in **Table 2.4**. Regarding root yield during the first year, Zn induced the highest reduction. The highest productivity reduction was observed in the roots of Zn_{900}, with a reduction of 56% and 63% in the first and second years, respectively.

Table 2.4 Total biomass yield and plant fractions (roots, stems, leaves, and seeds) in the two years and relation to HM treatments. Within the same column, values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

	2020					2021				
	Roots (g pot^{-1})	Stems (g pot^{-1})	Leaves (g pot^{-1})	Seeds (g pot^{-1})	Total DM (g pot^{-1})	Roots (g pot^{-1})	Stems (g pot^{-1})	Leaves (g pot^{-1})	Seeds (g pot^{-1})	Total DM (g pot^{-1})
Control	1.8 ^{a}	5.4 ^{a}	3.7 ^{a}	4.3 ^{a}	15.2 ^{a}	5.3 ^{a}	7.0 ^{a}	4.8 ^{a}	3.2 ^{a}	20.4 ^{a}
Zn450	1.0 ^{b}	3.7 ^{b,c}	2.9 ^{a}	3.2 ^{a,b}	10.7 ^{a,b}	4.5 ^{a,b}	5.4 ^{a,b,c}	3.5 ^{a,b}	2.1 ^{a}	15.5 ^{b}
Zn900	0.8 ^{b}	2.8 ^{c}	2.4 ^{a}	2.5 ^{a,b}	8.4 ^{b}	2.0 ^{c}	3.6 ^{c}	2.6 ^{b}	2.2 ^{a}	10.5 ^{c}
Pb450	1.1 ^{ab}	3.7 ^{b,c}	3.5 ^{a}	3.2 ^{a,b}	11.6 ^{a,b}	3.9 ^{a,b}	5.6 ^{a,b}	3.5 ^{a,b}	3.4 ^{a}	16.5 ^{a,b}
Pb900	1.5 ^{a,b}	3.6 ^{b,c}	3.1 ^{a}	2.9 ^{a,b}	11.0 ^{a,b}	3.0 ^{b,c}	5.1 ^{b,c}	4.2 ^{a,b}	3.0 ^{a}	15.3 ^{b}
Ni110	1.2 ^{a,b}	3.7 ^{b,c}	2.5 ^{a}	3.3 ^{a,b}	10.7 ^{a,b}	3.5 ^{a,b,c}	5.5 ^{a,b,c}	3.5 ^{a,b}	3.2 ^{a}	15.7 ^{b}
Ni220	1.1 ^{a,b}	3.3 ^{b,c}	2.7 ^{a}	2.6 ^{a,b}	9.7 ^{b}	3.4 ^{b,c}	4.4 ^{b,c}	3.0 ^{b}	2.2 ^{a}	13.0 ^{b,c}
Cd4	1.2 ^{a,b}	4.3 ^{a,b}	2.6 ^{a}	2.2 ^{a,b}	10.3 ^{a,b}	3.2 ^{b,c}	4.2 ^{b,c}	2.7 ^{b}	2.8 ^{a}	12.9 ^{b,c}
Cd8	1.3 ^{a,b}	3.2 ^{b,c}	2.9 ^{a}	1.7 ^{b}	9.1 ^{b}	3.3 ^{b,c}	4.1 ^{b,c}	2.9 ^{b}	2.4 ^{a}	12.7 ^{b,c}
AVG	*1.2*	*3.7*	*2.9*	*2.9*	*10.7*	*3.6*	*5.0*	*3.4*	*2.7*	*14.7*

The stem productivity reduction due to heavy metal contamination was significant in 2020 and reached the highest magnitude in Zn$_{900}$. In 2021, the reduction in stem productivity was significant for Pb$_{900}$, Ni$_{220}$, and all Cd concentrations, with a reduction of 30%. The highest reduction (49%) was observed in Zn$_{900}$.

Heavy metal contamination affected seed yield only in 2020, when Cd$_{8}$ achieved the lowest seed yield and a 60% reduction compared to the uncontaminated plants. In 2021, the effect on seed yield was not significant.

2.4.1 Heavy Metal Uptake per Plant and Amount of Heavy Metal Extracted

The heavy metal concentration in the plants showed differences among the four contaminants. Higher heavy metal concentration in the soil induced a higher concentration of the contaminant in plant organs, denoting the bioaccumulation potential of safflower. In addition, different heavy metals induced a variation in the partitioning of heavy metals taken into the plant organs caused by the different mobility of the contaminants.

In Zn-contaminated soils showed in **Figure 2.3**, the highest concentration was observed in roots compared to leaves, stems, and seeds.

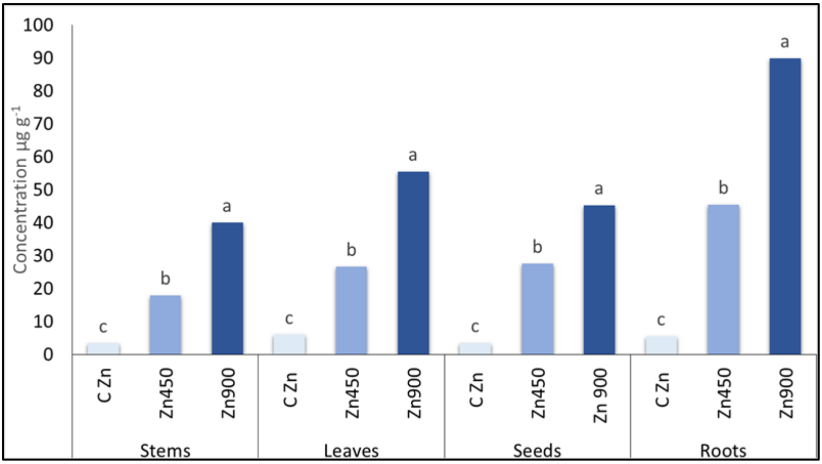


Figure 2.3. The concentration of Zn (μg g^{-1}) in the different fractions of the plant was the average of two years. Multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

The two levels of Zn led to a significant reduction in all biomass components, presented in **Table 2.5**. In the first year, the concentration of Zn in the roots was seven times higher in the treatment Zn_{450} than in the Control, and in Zn_{900}, the root concentration was twice Zn_{450}. The same trend was observed in the second year's root partitioning when zinc concentration was ten times larger than in Control in Zn_{450} and more than twenty-one times larger in Zn_{900} than in Control.

Table 2.5. Two-way ANOVA of the main factor and interaction for zinc concentration in stems, leaves, seeds, and roots during the two years of the experiment (* significance level at $p \leq 0.05$, ** significance level at $p \leq 0.005$, * significance level at $p \leq 0.001$).**

Zinc	Stems	Leaves	Seeds	Root
Level	0.0088 ***	0.0479 ***	0.0007 ***	0.0013 ***
Year	0.0010 **	0.2842	0.0002 ***	0.0197 *
Level x Year	0.0005 ***	0.1955	0.0535	0.4405

A similar result was observed in Pb-contaminated soil, in **Figure 2.4**, where the highest heavy metal concentration was observed in roots (Table 6). The concentration of Pb in soil affected the concentrations in stems, leaves, and roots, while it was not significant for the concentrations of Pb in the seeds. The accumulation in Pb_{450} plants was 30.3% of the total Pb uptake in the roots, 28.2% in the stems, 22.7% in the leaves, and 18.8% in the seeds. The accumulation in Pb_{900} plants was 36.0% of the total Pb uptake in the roots, 28.9% in the stems, 23.2% in the leaves, and 11.9% in the seeds.

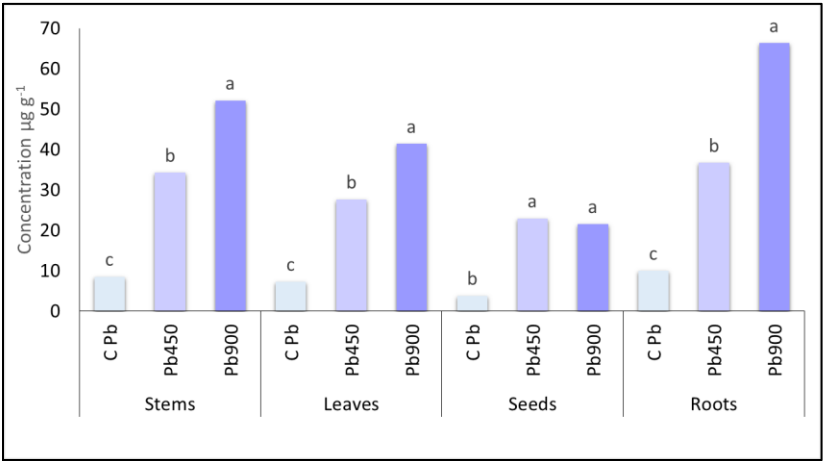


Figure 2.4. The concentration of Pb (µg g^{-1}) in the plant's different fractions is the average of two years. Multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

Table 2.6 Two-way ANOVA of the main factor and interaction for the concentration of lead in stems, leaves, seeds, and roots during the two years of the experiment (* significance level at $p \leq 0.05$, * significance level at $p \leq 0.001$).**

Lead	Stems	Leaves	Seeds	Roots
Level	0.0001 ***	0.0008 ***	0.5092	0.0495 ***
Year	0.3076	0.0639	0.0315*	0.1637
Level: Year	0.1376	0.4255	0.1100	0.6013

Compared with the control, the Pb accumulation in the different parts of the plant was four to seven times higher for Pb_{450} and Pb_{900}, respectively.

A different result was observed in Cd treatments, observed in **Figure 2.5**, where the highest heavy metal concentration was observed in the leaves, indicating the ability to translocate the uptaken Cd from the roots to the leaves.

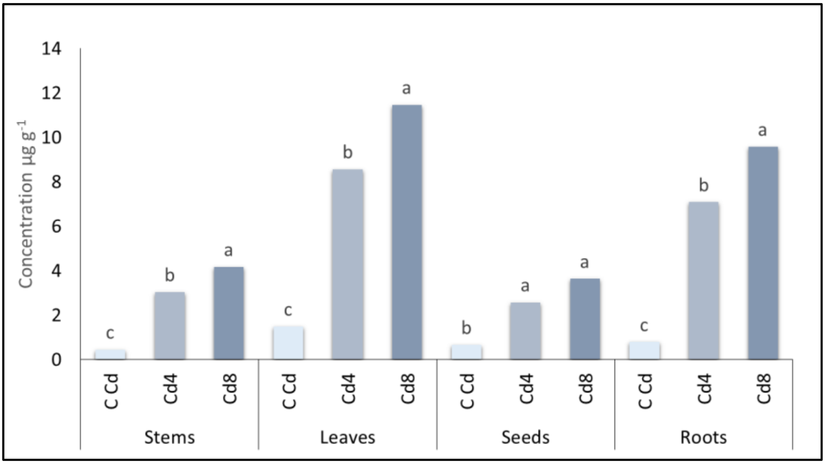


Figure 2.5 Concentration of Cd ($\mu g\ g^{-1}$) in the different fractions of the plants as the average of two years. Multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

The concentration in the stem, leaves, and roots showed significant differences between the two years of the experiment and between low and high concentrations in the soil, while the concentration in the seeds did not differ significantly between low and high concentrations in the soil (Table 7).

Table 2.7 Two-way ANOVA of the main factor and interaction for the concentration of cadmium in stems, leaves, seeds, and roots during the two years of the experiment (* significance level at $p \leq 0.05$, ** significance level at $p \leq 0.005$, * significance level at $p \leq 0.001$).**

Cadmium	Stems	Leaves	Seeds	Roots
Level	0.0002 ***	0.0023 **	0.0841	0.0041 **
Year	0.0000 ***	0.0002 ***	0.0016 ***	0.0006 ***
Level: Year	0.0003 ***	0.0068 **	0.1370	0.0056 **

The accumulation in the leaves was 40.2% and 39.9% of the total Cd uptake for Cd_4 and Cd_8, respectively. The accumulation in roots was lower, 33.3% and 33.2% for Cd_4 and Cd_8, respectively. Seed cadmium translocation was lower than 15% of the total uptake in both soil concentrations.

In Ni-contaminated soils, the highest heavy metal concentration was observed in the roots, followed by leaves, stems, and seeds, observed in **Figure 2.6**. In the Ni_{220} treatment, the concentration of Ni in the roots was six to eight times more than in the control treatment.

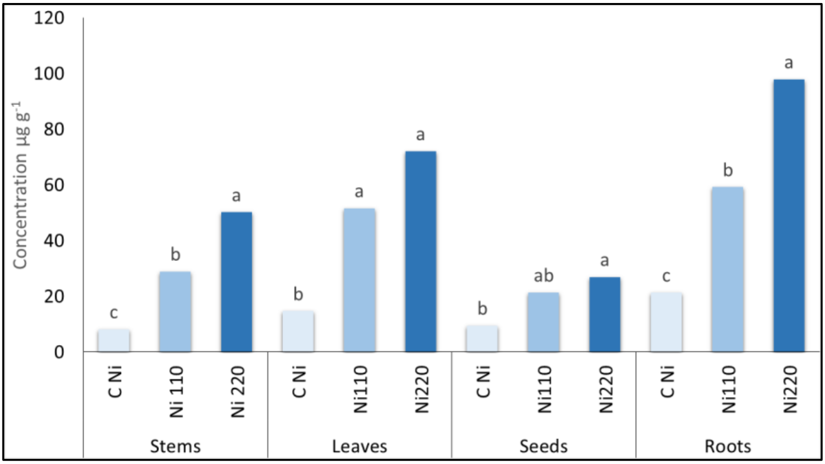


Figure 2.6. Concentration of Ni (µg g^{-1}) in the different fractions of the plants as average of two years. The multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

The concentration in the stems and roots showed significant differences between the two years of the experiment and between low and high concentrations in the soil, while the concentration in leaves and seeds did not differ significantly between low and high concentrations in the soil observed in **Figure 2.8**.

Table 2.8 Two-way ANOVA of the main factor and interaction for the concentration of nickel in stems, leaves, seeds, and roots during the two years of the experiment (* significance level at $p \leq 0.05$, ** significance level at $p \leq 0.005$, * significance level at $p \leq 0.001$).**

Nickel	Stems	Leaves	Seeds	Roots
Level	0.0338 *	0.1268	0.2217	0.0017 **
Year	0.0332 ***	0.0000 ***	0.0002 ***	0.0005 ***
Level x Year	0.0528	0.3382	0.5072	0.0053 **

In Ni_{110} treatment, the plants accumulated the heavy metal mainly in the roots, reaching 36.8% of total Ni uptake. On the other hand, the amount of Ni accumulated in the leaves reached 31.8%.

Roots accumulated a higher share of total Ni uptake in the Ni_{220} treatment, reaching 39.6%.

The amount of Ni stored in the seeds was below 15% of the total uptake in all the contaminated treatments, reaching the lowest value of 11.0% in Ni_{220}.

2.4.2 Tolerance Index, Translocation Factor, Modified Accumulation Index, and Bioconcentration Factor of the Aboveground and the Belowground

The tolerance index shows the adaptability of safflower to being grown in soils contaminated with increasing concentrations of Zn, Pb, Cd, and Ni (**Table 2.9**). The tolerance index decreased at the highest concentration for all the contaminants. The highest tolerance index has been found in Pb treatments, followed by Ni and Zn, both at low concentrations. The lowest scores of the tolerance index have been observed for Zn_{900}, Cd_8, and Ni_{220}.

Table 2.9 Index and Factors calculated concerning heavy metals in the soil and the plants.

Cont.	Conc.	TI	mAI	mBCF Abov.	mBCF Below.	TF
Zn	450	0.73 ± 0.10	3.87 ± 0.33	0.07 ± 0.012	0.15 ± 0.02	0.51 ± 0.10
Zn	900	0.57 ± 0.07	6.01 ± 0.39	0.09 ± 0.001	0.17 ± 0.01	0.52 ± 0.03
Pb	450	0.81 ± 0.11	3.55 ± 0.44	0.12 ± 0.020	0.15 ± 0.02	0.79 ± 0.07
Pb	900	0.76 ± 0.11	4.61 ± 0.48	0.07 ± 0.006	0.12 ± 0.01	0.61 ± 0.08
Cd	4	0.66 ± 0.11	3.80 ± 0.56	2.26 ± 0.3	3.79 ± 0.27	0.63 ± 0.08
Cd	8	0.61 ± 0.11	5.21 ± 0.82	1.19 ± 0.1	1.74 ± 0.15	0.68 ± 0.08
Ni	110	0.76 ± 0.14	6.17 ± 0.19	0.48 ± 0.1	0.89 ± 0.19	0.55 ± 0.11
Ni	220	0.64 ± 0.10	7.29 ± 0.25	0.32 ± 0.0	0.62 ± 0.06	0.54 ± 0.09

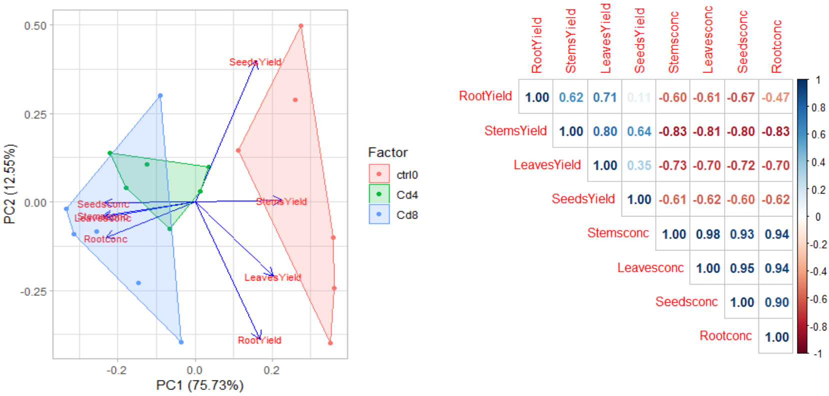
The ability to accumulate heavy metals in plant biomass was verified through the modified accumulation index, which relates the accumulation of heavy metals in the whole plant with the accumulation in the whole plant grown in the control soil. The mAI increased at higher concentrations for all four heavy metals tested. In the Pb treatment, a slight increase was observed, while at the highest concentration of Zn in the soil, the mAI was almost double compared to the lower concentration, showing that a Zn concentration of 900 mg kg^{-1} is not limiting for plant uptake.

The bioconcentration factor highlights the potential of safflower to accumulate heavy metals in the different parts of the plant, concerning the presence of bio-available heavy metals in the soil. The mBCF calculated for whole aboveground biomass showed a reduction at increasing concentrations of contaminants in the soil, except for Zn. The same trend was observed for the mBCF calculated on the belowground biomass.

The translocation factor represents the ability to translocate the pollutant from the roots to the aboveground biomass. This index did not vary significantly between the two concentration levels of each contaminant except for Pb.

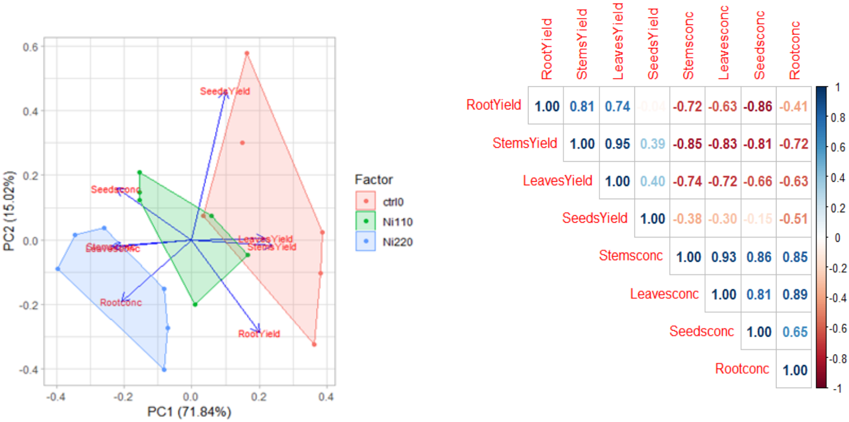
2.4.3 Tolerance and Phytoextraction Traits Evaluated with Principal Components Analysis and Multivariate Analysis

A principal component analysis (PCA) has been performed to evaluate the effect of the metal contaminants at several concentrations on several highly correlated variables (**Figure 2.7**, **Figure 2.8**, **Figure 2.9**, **Figure 2.10**). In particular, the biomass yield components (stem, leaves, and root) are mutually correlated and inversely correlated to the contaminant concentration in the plant fractions (stem, leaves, seeds, and root yield). In addition, seed yield appears to be weakly correlated to the other biomass components' yield, particularly root yield.



	RootYield	StemsYield	LeavesYield	SeedsYield	Stemsconc	Leavesconc	Seedsconc	Rootconc
RootYield	1.00	0.62	0.71	0.11	-0.60	-0.61	-0.67	-0.47
StemsYield		1.00	0.80	0.64	-0.83	-0.81	-0.80	-0.83
LeavesYield			1.00	0.35	-0.73	-0.70	-0.72	-0.70
SeedsYield				1.00	-0.61	-0.62	-0.60	-0.62
Stemsconc					1.00	0.98	0.93	0.94
Leavesconc						1.00	0.95	0.94
Seedsconc							1.00	0.90
Rootconc								1.00

Figure 2.7 Principal component analysis and correlation matrix in cadmium-contaminated soil in the two years.



	RootYield	StemsYield	LeavesYield	SeedsYield	Stemsconc	Leavesconc	Seedsconc	Rootconc
RootYield	1.00	0.81	0.74	-0.04	-0.72	-0.63	-0.86	-0.41
StemsYield		1.00	0.95	0.39	-0.85	-0.83	-0.81	-0.72
LeavesYield			1.00	0.40	-0.74	-0.72	-0.66	-0.63
SeedsYield				1.00	-0.38	-0.30	-0.15	-0.51
Stemsconc					1.00	0.93	0.86	0.85
Leavesconc						1.00	0.81	0.89
Seedsconc							1.00	0.65
Rootconc								1.00

Figure 2.8 Principal component analysis and correlation matrix in nickel-contaminated soil in the two years.

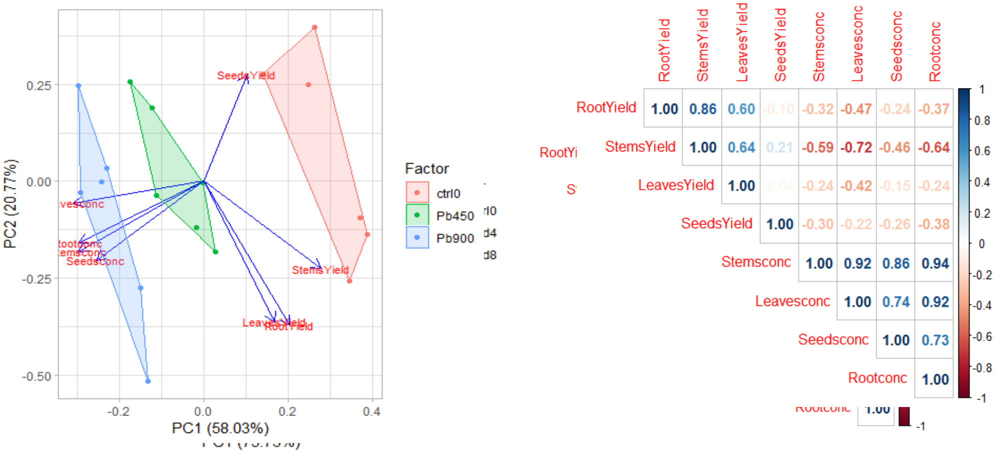


Figure 2.10. Principal component analysis and correlation matrix in lead-contaminated soil in the two years.

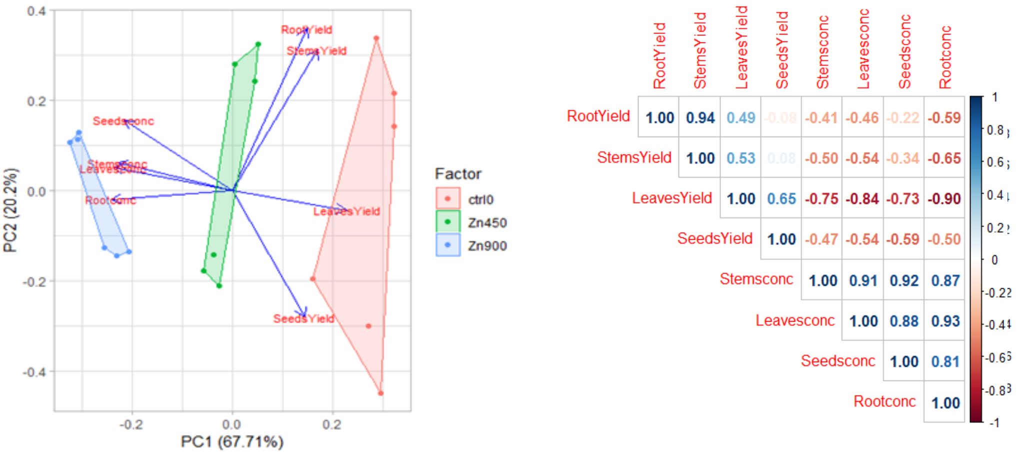


Figure 2.9. Principal component analysis and correlation matrix in zinc-contaminated soil in the two years.

The first component of the PCA (PC1) describes between 73.5% and 61.6% of the total variance in the data in the Cd- and Pb-contaminated samples, respectively. PC1 direction coincides with higher biomass yield and lowers contaminant concentration in the plant organs.

For all metal contaminants, the control group does not overlap with the contaminated groups. The control group has high values of PC1, indicating higher biomass yield components and lower contaminant concentration in the plant organs. The contaminated groups have lower PC1 scores concerning the concentration factor: the groups with the highest concentration factor have the lowest PC1 scores. In contrast, the groups with the lowest concentration factor have PC1 scores that are intermediate between the control group and those with the highest concentration factor. Considering the first and the second PCs, no overlapping has been observed between groups, excluding the samples contaminated with Cd.

In Ni-, Pb-, and Zn-contaminated samples, the root yield is the least influenced by the contaminant concentration, while flower and stem yields show the strongest negative correlation to the contaminant concentration. Cd contaminant concentration appears to be strongly anticorrelated with all biomass yield components.

2.4.4 The trend of the Four Heavy Metals Tested in Safflower

Safflower showed different behaviors regarding the accumulation and translocation of the heavy metals tested in this study.

Increasing concentration of contaminants in the soil led to an increase in the concentration of contaminants accumulated in all the organs of the plant for all the heavy metals tested.

Roots are the organs that accumulate most of the uptaken Pb, Ni, and Zn, while Cd has been translocated primarily on the leaves, decreasing the amount stored in the roots.

Concerning the aboveground biomass, leaves are the organs where most heavy metals are translocated, except for Pb, which is translocated mainly to the stems. Ni, Cd, and Pb concentrations were observed in the seeds, while Zn was translocated evenly among the aboveground organs, seeds included.

2.5 Discussion

In general, heavy metals can be found in soils in different forms, such as free heavy metals and soluble metal complexes, associated with the organic matter in the soil as oxides, hydroxides, carbonates, or incorporated into silicate mineral structures (Sajad et al. 2020; Shen et al. 2022). To be absorbed by plants, heavy metals must be bioavailable in the soil. Bioavailability is determined by several factors: pH, soil organic matter, redox potential soil texture and structure, water flux, and soil microorganisms. This experiment was performed in sand soil with a high concentration of Fe and neutral pH. As reported by Huang et al. (2020), (Huang and Hartemink 2020) pH, together with the presence of other metals in soils, has a main role in determining the availability of heavy metals in sandy soils and, therefore, the concentration of the metal ions. This experiment showed that the heavy metals bioavailable concentration in soils was at least 30% lower than the amount added to the soil.

Several studies confirm that Zn and Cd have similar chemical properties (Alloway 1995). However, the results showed that Cd bioavailability was strongly reduced due to the soil proprieties. Even if Cd has higher mobility than Zn at a pH lower than 7.5, Cd absorbed in soils is not easily available (Alloway 1995).

Ni content was high in the control soil (33.53 mg/kg), in agreement with Kabata-Pendis (Kabata-Pendias 2010). However, due to the affinity with metallic Fe, they found higher content in semiarid regions, up to 53.8 mg/kg in soil with 4.6% clay content.

The content of bioavailable Pb in the experiment soil was low, causing its low mobility when compared with other trace metals. One of the reasons that contribute to the low mobility is soil pH. In alkaline or sub-alkaline soils, Pb may precipitate as hydroxides, phosphates, or carbonates, forming stable Pb–organic complexes. Therefore, the decrease in soil pH may increase Pb solubility.

A decrease in safflower yield was observed during the first year, caused by a one-month delay in sowing time, as reported by Patane et al. 2020 (Patanè et al. 2020), who found that safflower is significantly affected by the time of sowing, showing an optimum in late winter and a decrease in plant productivity for later sowing.

Regarding the productivity in the contaminated pots, a decrease in yield was observed, particularly in Zn-contaminated soil, in both years of the experiment. A similar result was also observed in Namdjoyan et al. (2017) and Manvelian et al. (2021) (Manvelian et al. 2021; Namdjoyan et al. 2017), where Zn treatment significantly reduced roots and shoot biomass production compared to the controls. Zn inhibits photosynthesis in several ways, such as substituting Mg^2 in chlorophyll (Chl) or inducing Fe deficiency (Kabata-Pendias 2010).

The highest Zn accumulation in safflower was measured in the roots, followed by the leaves, the seeds, and the stems. Goodarzi et al. (2020) (Goodarzi, Namdjoyan, and Soorki 2020) experimented with studying Zn-stressed safflower seedlings. The depletion of non-enzymatic antioxidants content and the decreased activity of enzymes involved in the antioxidant defense and the glyoxalase systems may also be associated with the lower Zn concentration in the plants supplemented with salicylic acid, nitric oxide, and melatonin, which are signaling molecules that can increase plant ability to tolerate the HMs' stress-induced toxicity (Mattina et al. 2003).

In Ni-contaminated soil, safflower showed significant yield reduction, particularly at the highest concentration in the soil. Al Chami et al. (2015) (Al Chami et al. 2015) reported that Ni was more toxic than Pb and Zn in the studied plant species: no growth of safflower was observed at Ni concentration above 10 mg L^{-1}. In Baran et al. (2022) (Baran and Ekmekçi 2022), Carthamus species accumulated Ni in descending order as root > stem > leaf under all tested concentrations. Ni contents in root and shoot tissues of both species progressively increased in a concentration-dependent manner. These results demonstrated that large amounts of Ni were accumulated primarily in the roots and marginally in the plant’s other organs due to the low translocation between plant organs. In this experiment, a similar concentration of Ni was observed in roots and leaves at a low soil concentration. Whereas, at a high concentration of Ni in the soil, the highest concentration of the heavy metal within the plant organs was observed in the roots. Baran et al. (2022) report that Ni acts as a plant micronutrient at low soil concentrations; however, it can cause toxic effects, reducing shoot and root lengths and yield at high concentrations. In general, the bioavailability of Ni depends on its oxidation state. Afzal et al. (2021) and Hassan et al. (2019) (Afzal et al. 2021; Hassan et al. 2019) found that absorbed Ni is effectively stored in leaves and seeds.

Cadmium induced the highest stress on safflower plants, significantly reducing the dry weight at high soil concentrations. Z. Amjadi et al. (2021) [38] reported a severe decrease (56%) in shoot and root biomass yield in Cd-stressed safflower in comparison with the control. Namdjoyan et al. (2011) (Namdjoyan, Namdjoyan, and Kermanian 2012) reported a direct relationship between Cd-induced toxicity tolerance with higher accumulation of this element in roots and the prevention of its transfer to the plant's aboveground parts.

The concentration of Cd in safflower differed from Shi et al.'s (Shi and Cai 2009) experimental results. They reported good adaptability of safflower to Cd-contaminated soil and demonstrated that most Cd was found in the roots. However, substantial amounts were still found in the shoots, showing that it is a hyperaccumulator.

Finally, safflower yield was not particularly affected in Pb-contaminated soil. One reason for the Pb tolerance in safflower can be ascribed to the low mobility between roots and aboveground organs. For example, Al Chami et al. (2015) (Al Chami et al. 2015) reported that safflower was not affected in all the productivity parameters in soil contaminated by 5 and 10 mg kg^{-1} of Pb; while at higher concentrations (25 mg L^{-1}), the plants were not growing.

The tolerance and accumulation indices, the mBCF, and the TF indicate the ability of safflower to tolerate and accumulate heavy metals. The accumulation index increased at higher soil concentrations of Cd, Ni, Pb, and Zn. In contrast, the tolerance index decreased at higher soil concentrations for the four heavy metals.

The TF showed a differentiated behavior in relation to the heavy metal: no response to concentration was observed for Zn and Cd. In contrast, a decrease at higher concentrations was observed for Pb ad Ni. The mBCF decreased at higher concentrations of Cd, Ni, and Pb, while the belowground mBCF increased at higher Zn concentrations.

Results showed that safflower could grow in a wide range of heavy-metal-induced abiotic stresses. Depending on the heavy metal, safflower has adopted different mechanisms to accumulate or stabilize the contaminant through the different fractions of biomass in the soil. However, exploring the data through phytoremediation index and factors, PCA analysis, and correlation matrix helped to understand the behavior of safflower in soil contaminated with Zn, Ni, Cd, and Pb. Leaves and stems yields showed the strongest negative correlation to the contaminant concentration, while the root yield was the least influenced by the heavy metal concentration, explaining that safflower can still store the heavy metals in the belowground biomass at raised concentrations of pollutants in the soil. This result was also confirmed by the accumulation index that increased with the level of heavy metal in the soil and from the translocation factor, which showed that safflower accumulates more contaminants in the belowground biomass, reducing its ability to transport to the aerial part of the plant. Cd contamination concentration appears to be strongly anticorrelated with all biomass yield components.

This study highlights the possibility of further research concerning bioenergy production using contaminated safflower as a feedstock, as Gomes et al. (2022) (Gomes et al. 2022) investigated the potential of bioenergy production of contaminated biomass. Some studies have already proposed safflower biomass for biogas or bioethanol production, while the oil extracted from safflower seeds has been suggested for biodiesel production (Kadir et al. 2020). In addition, several methods to produce biogas or bioethanol have been investigated (Namdjoyan et al. 2012) and can be applied to contaminated safflower biomass to produce bioenergy.

2.6 Conclusions

The results showed safflower's suitability for biomass production under heavy metal contamination. Moreover, the plant's ability to accumulate heavy metals and translocate them to aerial biomass highlights its potential for the phytoextraction process.

Low heavy metal accumulation in safflower seeds suggests the possibility of using the seeds in the bioenergy conversion process, avoiding the concerns about contaminant dispersion. The possibility of valorizing safflower residues such as stems and leaves by converting them into biofuels or bioproducts increases the interest in this crop. It is essential to explore the economic viability of its utilization for the phytoremediation process. Future investigation on bioenergy products produced with the contaminated biomass of safflower could help obtain valuable feedstock from soils unsuitable for food crops.

Supplementary Materials: Table S1

2.7 References

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3 Adaptability of industrial hemp to different levels of heavy metal in soil

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Adaptability of industrial hemp to different levels of heavy metal in soil

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ABSTRACT: Soils give rise to many environmental problems, such as heavy metals pollutant that reduce water resources, leading to serious health problems for humans, directly or indirectly, by the contamination of the food crops. Moreover, the cultivation of industrial crops in heavy metals contaminated land improves several ecosystem services, such as biodiversity maintenance and nutrient and water cycling, while promoting soil decontamination through a phytoremediation technique. This biological technique uses plants to remediate the contaminated land while generating economic value in land that used to be unproductivity. However, industrial crop selection is crucial for good results of phytoremediation. In this way, hemp appears suitable for heavy metals contaminated soil.

Moreover, it is possible to obtain high-quality cellulose (stems), oil, and hemp proteins using different varieties. Still, it requires special attention for several physiological features, crop management, and site-specific selection of genotypes. This work aimed to evaluate the tolerance of two genotypes of industrial hemp (Futura 75 and Kc

Dora) in soils contaminated by three heavy metals (Ni, Cd, Pb). the experiment was carried out in pots adopting a factorial completely block-randomized experimental design with three replications.

KEYWORDS: biomass; bioenergy; industrial hemp; marginal land; tolerance; heavy metal; physiological response.

3.1 Introduction

Climate change and energy demand are the key points in the future to stabilize or stop global warming. In this regard, the cultivation of industrial crops appears as an alternative (Scordia and Cosentino 2019). Furthermore, industrial hemp now turns out to be a viable alternative due to the possibility of being involved in numerous industrial processes such as the textile industry, bioplastics production, and the construction industry. Thanks to research developed in recent years, more than 50 genotypes are registered in the European Union plant register, and by adopting suitable hemp varieties, it is possible to obtain high-quality cellulose (stems), essential oils, and valuable resins (inflorescences) as well as high-quality oil and protein from the seeds (Amaducci et al. 2015). Due to its characteristics, industrial hemp can increase crop diversification and improve farmers' agronomic and economic sustainability.

Concerning environmental constraints, several studies have shown that, in Europe, the optimal allocation of fiber hemp for no-food purposes is mainly in the Center. Its introduction in Southern Europe requires the selection of an appropriate genotype, management practices, and planting times for hemp cultivation. Based on its temperature requirements, hemp should be grown during the spring-summer season, so in Mediterranean areas characterized by low rainfall and high temperatures in spring-summer, the crop requires irrigation (Cosentino et al. 2013).

Furthermore, in a recent study (Shi et al. 2012; Wang et al.

2021), industrial hemp can grow in soil contaminated by heavy metals and fulfill several conditions, such as deep and extensive root systems, low request for agronomic input, high production of biomass, and short vegetative cycle. More, the use of industrial hemp opens a new perspective on managing contaminated soil, thanks to the economic incoming that the farmers can have and the environmental benefit of the soil's remediation. Using a plant to decontaminate polluted soil is known as phytoremediation, where the plants show different abilities to extract, stabilize, volatilize and degrade the pollutant from the soil. The strategy used by the plants to restore the soil depends on the contaminant in the soil. In the case of heavy metals, not all plants tolerate all the heavy metals and can extract them from the soil (Liu et al. 2018, Öztürk et al. 2015).

Plants employ specific strategies to tolerate harmful levels of heavy metals in the soil, highlighting the possibility of studying physiological and biochemical mechanisms for heavy metal tolerance (Salt et al. 1995). In addition, most plants have developed a complicated process for acquiring relatively unavailable micronutrients like Zn, Mn, Cu, Fe, and Ni from the soil (Papazoglou et al. 2005).

The mechanism of tolerance to metal stress in plants may range from the exclusion of toxic metal and inclusion and accumulation at inert places, with may vary from species to species (Raskin and Ensley, 2000).

In this experiment, two varieties of industrial hemp, Futura 75 and Kc Dora, were tested in different concentrations of Cadmium, Lead, and Zinc, seeking the physiological response and the mechanism of tolerance of this species to the heavy metals contaminated soil.

3.2 Material and methods

3.2.1 Trial Description

The trial was carried out during the spring-summer 2021 at the Department of Agriculture, Food and Environment of the University of Catania. To evaluate the tolerance of two varieties of industrial hemp (Futura 75 and Kc Dora) in a soil contaminated by three heavy metals (Ni, Cd, Pb), compared to untreated control, a trial was performed in 25 L pots (diameter of 40 cm and height of 30 cm) adopting a factorial completely block-randomized experimental design with three replications. The varieties were monoecious, and in particular, Futura 75 was developed in French, while Kc Dora was developed in Hungary.

The heavy metals were applied to the soil as nitrate of cadmium [Cd (NO_3)], nitrate of lead [Pb (NO_3)], and nitrate of nickel [Ni (NO_3)].

The amount of the single contaminant in the soil was decided according to the Italian legal limit referred to sites for commercial and industrial use (D.Lgs 3 aprile 2006 n.152 2006), imposing the legal limit as the first concentration, one and a half the legal limit concentration as the second and the double concentration as the third level for lead (1000 mg kg^{-1}, 1500 mg kg^{-1}, and 2000 mg kg^{-1}) and nickel (500 mg kg^{-1}, 1000 mg kg^{-1}, 1500 mg kg^{-1}). Besides, all the concentrations of cadmium used in this study were higher than the legal limits (from 4 up to 10 times: 60, 90, 120, 150 mg kg^{-1}). The plants were maintained in well-watered conditions during the experiment, and biometric characteristics and gas exchange were measured monthly. At the end of the growing season, all the plants were harvested. Plant biomass was weighed after drying in a ventilated oven at a temperature of 65° C until constant weight.

3.2.2 Measurements and calculations

During the growing season, the leaf photosynthesis (A, µmol CO_2 m^{-2} s^{-1}), the transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and stomatal conductance (mmol m^{-2} s^{-1}) were measured on three representative leaves in each genotype under treatment, on the third fully expanded leaf. A portable instrument (LCi-SD, ADC BioScientific, Great Amwell, Hertfordshire, U.K.) was used at a flow rate of 500 mL min^{-1} and under CO_2, temperature, and ambient humidity conditions during cloudless days.

The instantaneous water efficiency (iWUE) was calculated as the ratio between photosynthesis rate and leaf transpiration (μmol CO_2 mmol H_2O^{-1}).

3.2.3 Statistical analysis

The physiological data were statistically analyzed using R software (R Core Team, 2013). Genotypes and concentrations were considered the main factors, and the means were separated by the Student-Newman-Keuls (SNK) test at a 95% confidence level. The resistance mechanism to increasing soil pollutant concentration of Futura 75 and Kc Dora was evaluated through linear regressios between the pollutant concentration and stomatal conductance, with the b-value of the linear regression representing the plant response to increasing heavy metal concentration. The Shapiro-Wilk test was used to verify the normality of the residual distribution. Regression coefficients were considered significant when $P \leq 0.05$, and the goodness of fit was estimated by calculating the R^2.

3.3 Results and discussion

3.3.1 Photosynthesis rate

The ANOVA showed significant differences in photosynthesis rate compared with the treatments (**Table 3.1**). The genotype (G) was significant for Pb but not for Cd and Ni, while the concentration (C) of the heavy metal in the soil was significant for all the heavy metal (Cd, Pb, and Ni). The genotypes and the concentration showed significantly different photosynthesis rates in Pb but not Ni and Cd.

Table 3.1 P-value from the analysis of the variance of the main factors and interactions for the net photosynthesis of Futura 75 and Kc Dora under increasing levels of pollutants in the soil.

Photosynthesis rate	Cd	Ni	Pb
Genotype (G)	0.5913695	0.5046	5.161e-06
Concentration (C)	0.0001651	1.378e-06	0.0001811
G X C	0.3038328	0.5876	0.0045653

Across the average of measurements, compared to the controls, Kc Dora (average 8,92 µmol CO_2 m^{-2} s^{-1}) showed a higher photosynthesis rate than Futura 75 (average 8,60 µmol CO_2 m^{-2} s^{-1}), observed in **Figure 3.1**.

In cadmium-contaminated treatments, both Futura 75 and Kc Dora were at increasing levels of heavy metal in the soil, corresponding to a slight decrease in the photosynthesis rate. Futura 75 photosynthesis rate ranged from 11.39 µmol CO_2 m^{-2} s^{-1} in Cd_{60} to 8,10 µmol CO_2 m^{-2} s^{-1} in Cd 150. Kc Dora ranged from 10,56 µmol CO_2 m^{-2} s^{-1} in Cd_{60} to 7.41 µmol CO_2 m^{-2} s^{-1} in Cd_{150}.

In nickel-contaminated treatments, both species showed a decreasing photosynthetic rate with the increasing concentrations of the metal studied. A significant decrease in photosynthesis rate was shown in Ni_{1500}, with the value for Futura 75 equal to 5.70 µmol CO_2 m^{-2} s^{-1} and Kc Dora equal to 4.57 µmol CO_2 m^{-2} s^{-1}.

In the end, in all the lead-contaminated treatments, Kc Dora showed the highest value than Futura 75. In particular, for Futura 75, no significant differences were observed in different levels of lead concentrations, with a value of 8.79 µmol CO_2 m^{-2} s^{-1} in the treatment Pb_{1000} and a value equal to 8.27 µmol CO_2 m^{-2} s^{-1} in the treatment Pb_{2000}. However, even if Kc Dora showed a higher value than the control in Pb_{1000} and Pb_{1500}, a significant reduction of photosynthesis rate was recorded in Pb_{2000} with 8.88 µmol CO_2 m^{-2} s^{-1}.

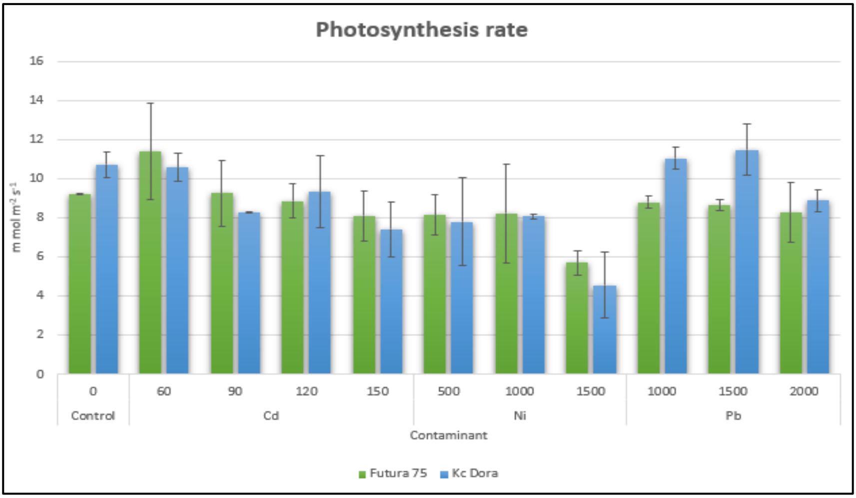

Figure 3.1. Leaf photosynthesis (µmol CO_2 m^{-2} s^{-1}) of Futura 75 and Kc Dora in relation to the different studied treatments.

3.3.2 Transpiration rate

The ANOVA showed significant main effect differences in the transpiration rate presented in **Table 3.2**.

Table 3.2 P-value of the main factors and interactions for Futura 75 and Kc Dora leaf transpiration rate under increasing levels of pollutants.

Transpiration rate	Cd	Ni	Pb
Genotype (G)	0.00688	0.98608	0.925503
Concentration (C)	9.228e-07	0.06332	0.051916
G X C	0.02973	0.29333	0.001425

Figure 3.2 presents the transpiration rate considering different trials. The genotype was only significant in Cd, while the heavy metal concentration affected the transpiration rate in Cd, Ni, and Pb, and the interaction between the genotypes and the concentration was significantly affected in Cd and Pb, while it was not significant for Ni.

The two genotypes under study showed a distinct tendency to transpire depending on the soil's contaminants and their concentration. Compared to the performance of both controls, in Futura 75, the transpiration rate was less than the control of Kc Dora. Still, at the rising concentration of Cd, the transpiration rate increased more in Futura 75 (2.01 mmol H_20 m^{-2} s^{-1} in Cd_{150}) than in Kc Dora (1.69 mmol H_20 m^{-2} s^{-1} in Cd_{150}).

Regarding the different levels of Ni, a higher transpiration rate was observed in Futura 75 than in Kc Dora, with a reduction of transpiration at the increasing levels of Ni for both genotypes.

Finally, among the level of lead-contaminant, the transpiration rate in Futura 75 was slightly higher in the concentration of Pb_{1000} (3.22 mmol H_20 m^{-2} s^{-1}) compared to Pb_{1500} and Pb_{2000}. At the same time, there was no significant difference in the transpiration rate for Futura 75 at the concentration of Pb_{1500} and Pb_{2000}. Furthermore, Kc Dora did not show a difference in the transpiration rate of the concentration of Pb_{1000} and Pb_{1500}, while the transpiration rate of Pb_{2000} (2.42 mmol H_20 m^{-2} s^{-1}) was lower compared with the previous two levels of Pb.

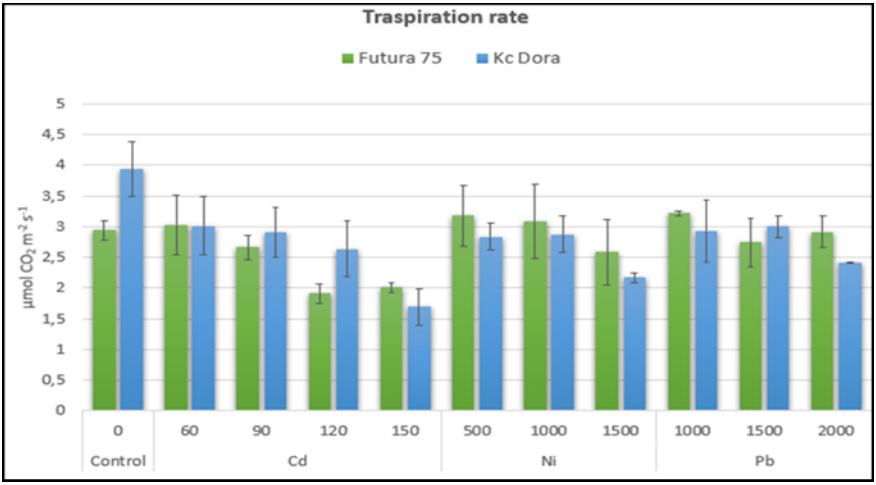

Figure 3.2. Leaf transpiration rate (mmol H_20 m^{-2} s^{-1}) of Futura 75 and Kc Dora in relation to the different studied treatments.

3.3.3 Stomatal conductance

The ANOVA showed significant differences concerning the stomatal conductance of the treatments under study, can be observed in

Table 3.3: P-value of the main factors and interactions for the stomatal conductance Futura 75 and Kc Dora under increasing levels of pollutants in the soil.

Stomatal conductance	Cd	Ni	Pb
Genotype (G)	0.1622	0.7075	0.521991
Concentration (C)	1.506e-05	0.0100	0.008851
G X C	0.4217	0.2792	0.051070

. Specifically, the stomatal conductance was significantly affected by the Cd, Ni, and Pb levels. No significative differences were observed among genotypes.

The trend of the stomatal conductance in both genotypes and contaminants was similar to the transpiration rate, obtaining higher values in the Control pots than in the contaminated pots for both genotypes.

Table 3.3: P-value of the main factors and interactions for the stomatal conductance Futura 75 and Kc Dora under increasing levels of pollutants in the soil.

Stomatal conductance	Cd	Ni	Pb
Genotype (G)	0.1622	0.7075	0.521991
Concentration (C)	1.506e-05	0.0100	0.008851
G X C	0.4217	0.2792	0.051070

Stomatal conductance was slightly lower in the Futura 75 than in Kc Dora, presented in **Figure 3.3**. Regarding cadmium-contaminated pots, there was a decreasing stomatal conductance in the different levels in both genotypes, with the lowest value obtained in Cd_{150}, with a value of 0.07 mol m^{-2} s^{-1} for Futura 75 and 0.06 mol m^{-2} s^{-1} for Kc Dora.

In the nickel contamination, a slightly higher stomatal conductance was observed in Futura 75 than in Kc Dora. However, no difference was observed in Futura 75 between Ni_{1000} and Ni_{1500}. On the contrary, in Kc Dora, in the highest level of Ni (1500), the stomatal conductance consistently decreased, with a value equal to 0.06 mol m^{-2} s^{-1}. Regarding lead contamination, there was a decreasing trend for Futura 75 at rising levels of Pb. Still, for Futura 75, the value was not lower than Kc Dora, which not showed a difference in raising levels of Pb.

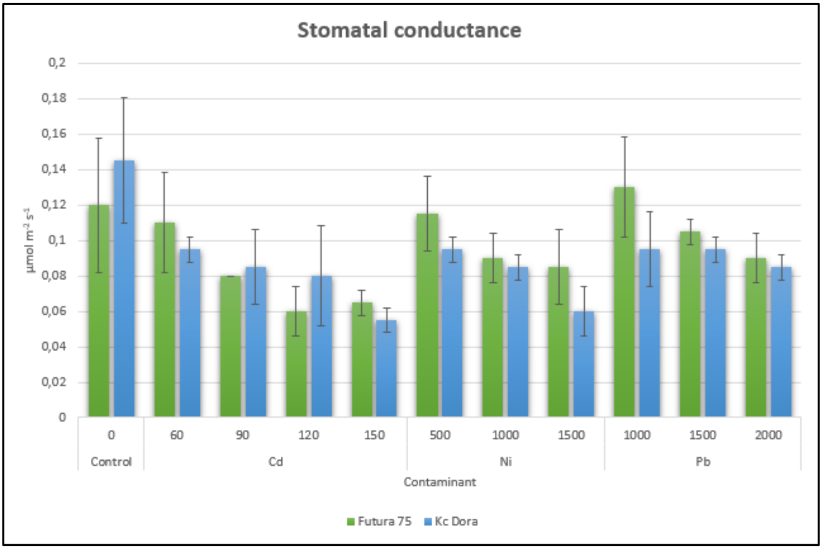

Figure 3.3. Stomatal conductance (mol m^{-2} s^{-1}) of Futura 75 and Kc Dora in relation to the different studied treatments.

3.3.4 Instantaneous water used efficiency

The ANOVA showed significant differences concerning the instantaneous water use efficiency (iWUE) of the treatments under study observed in **Table 3.4**. The genotype was significant for Cd and Pb, not Ni. Also, the concentration of the heavy metals was significant in the instantaneous water use efficiency in all the treatments (Cd, Ni, and Pb). The interaction between the genotype and the concentration was significant for Pb, not Cd and Ni.

Table 3.4. P-value of the main factors and interactions for the instantaneous water use efficiency of Futura 75 and Kc Dora under increasing levels of pollutants in the soil.

iWUE	Cd	Ni	Pb
Genotype (G)	0.046064	0.370111	6.686e-05
Concentration (C)	0.004565	0.008086	0.04362
G X C	0.222372	0.560519	3.248e-05

The iWUE, which represents the ratio between the photosynthesis and the transpiration rate, expresses the micromoles of CO_2 absorbed on the mole of transpiration H_2O, showing differences between the levels of the heavy metals presented in **Figure 3.4**. The highest iWUE was observed in Futura 75 then in Kc Dora among the two genotypes compared to the controls. There was an increasing trend in the different levels of Cd-treatments, with increasing iWUE for Futura 75 and Kc Dora. The highest iWUE for Futura 75 was observed in Cd_{120} (4.62 µmol CO_2 mmol H_2O^{-1}), whereas the highest iWUE for Kc Dora was observed in Cd_{150} (4.37 µmol CO_2 mmol H_2O^{-1}).

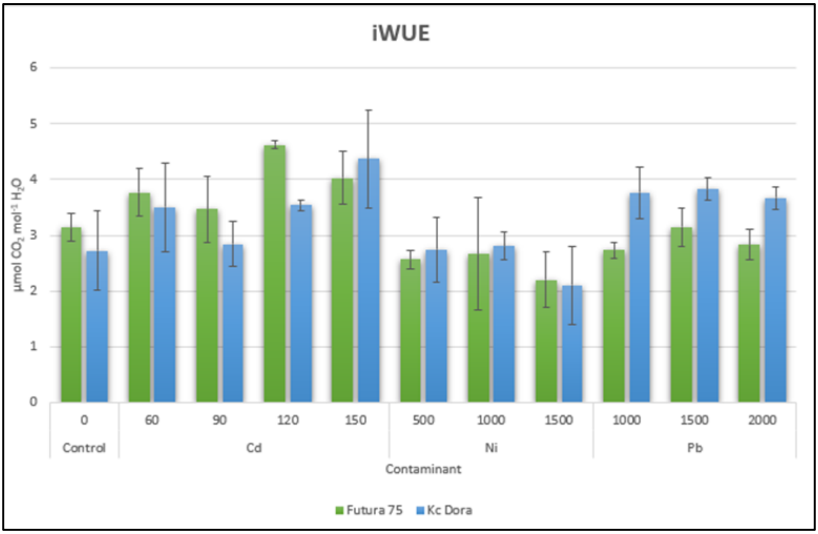

Figure 3.4. Instantaneous WUE of Futura 75 and Kc Dora in relation to the different studied treatments.

In Ni–treatments, there was no significant difference among the levels of nickel for both genotypes. However, the lowest value of iWUE was observed for both Futura 75 and Kc Dora in Ni_{1500} (2.20 and 2.10 µmol CO_2 mmol H_2O^{-1}, respectively).

In the Pb treatment, there was no significant difference in rising levels of Pb for both genotypes. However, the iWUE of Futura 75 was lower than the iWUE of Kc Dora.

3.3.5 Mechanisms of resistance

The linear regressions between soil contaminants at different levels and the stomatal conductance of Futura 75 and Kc Dora highlighted the different resistance mechanisms of these genotypes in response to soil heavy metal pollution showed in **Figure 3.5**, **Figure 3.6**, and **Figure 3.7**.

There was a linear and decreasing trend of stomatal conductance in all the linear regression considered as the concentration of contaminated soil increased.

Futura 75 showed a slightly lower stomatal conductance in relation to the concentrations of the Cd in the soil. In particular, the slope and intercept values of the linear relations were consistently lower, indicating a greater tolerance response.

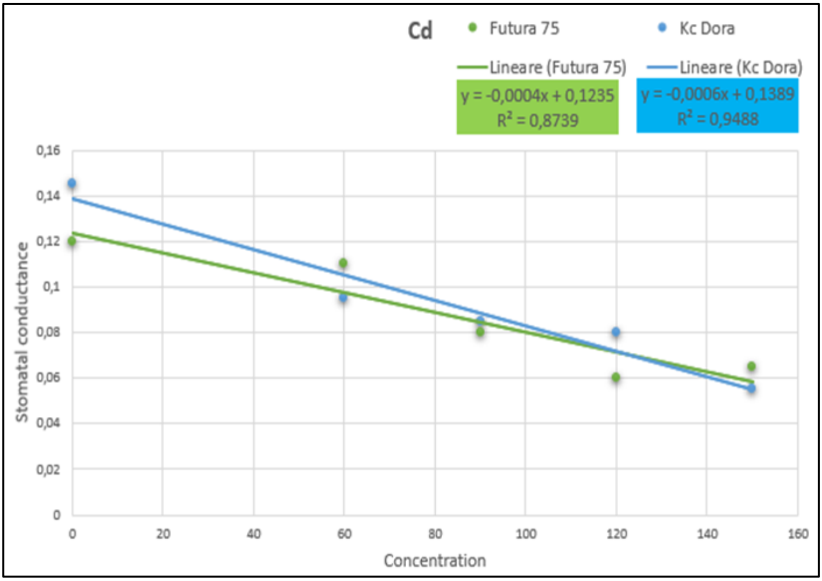

Figure 3.5. Linear regressions between contaminated soil and stomatal conductance (mol m^{-2} s^{-1}) in Futura 75 and Kc Dora in the different contamination levels of Cd.

Regarding the Ni concentration in the soil, Futura 75 showed higher stomatal conductance than Kc Dora. In particular, the slope and intercept values of the linear relations were consistently lower, indicating a higher tolerance response. Furthermore, for Futura 75, the R^2 was 0,91, and for Kc Dora was 0,92, indicating a strong correlation.

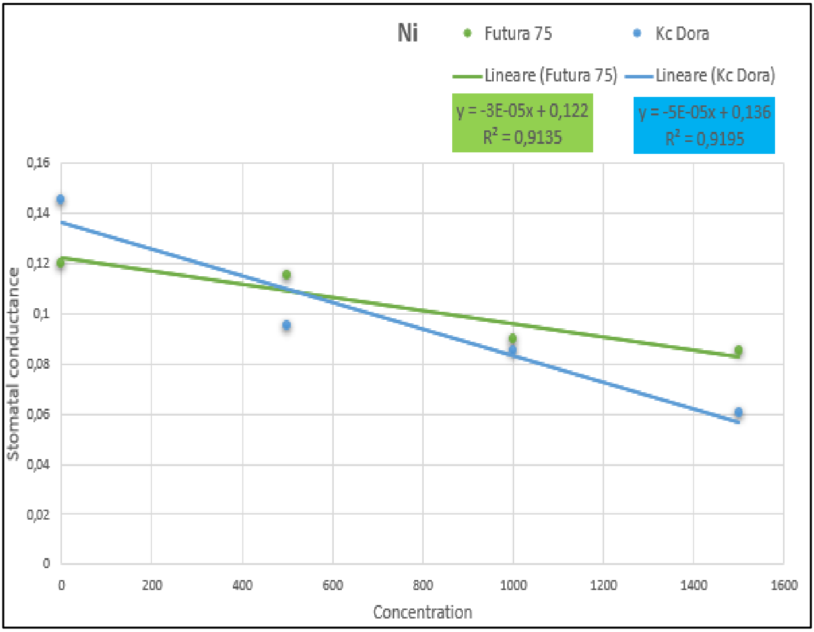

Figure 3.6. Linear regressions between contaminated soil and stomatal conductance (mol m^{-2} s^{-1}) in Futura 75 and Kc Dora in the different contamination levels of Ni.

Even in Pb-contamination treatments, Futura 75 showed lower stomatal conductance than Kc Dora. In particular, the slope and intercept values of the linear relations were lower in Futura 75 than in Kc Dora, indicating a greater tolerance response of Futura 75.

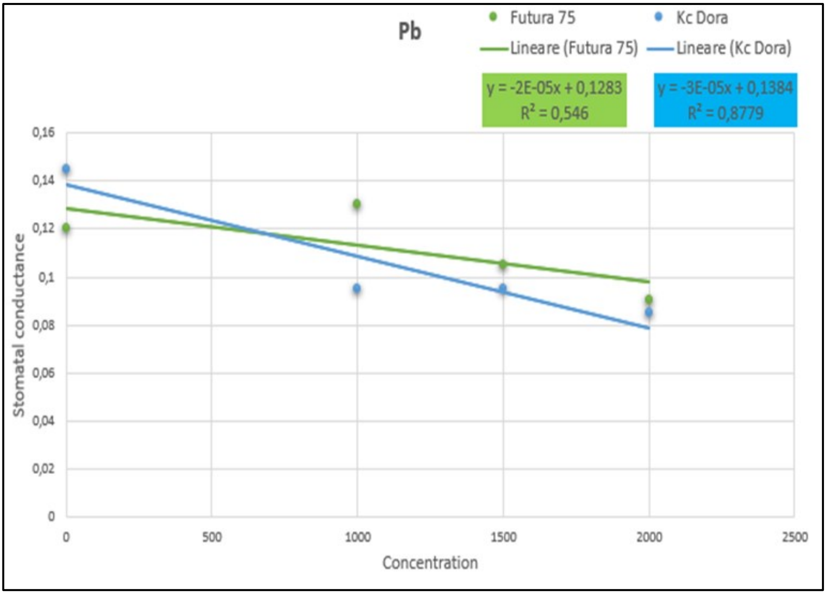

Figure 3.7. Linear regressions between contaminated soil and stomatal conductance (mol m^{-2} s^{-1}) in Futura 75 and Kc Dora in the different contamination levels of Pb.

3.3.6 Biomass yield

Figure 3.8 presents the differences were observed in the yield of the two genotypes. The yield of the two genotypes in the controls was higher than in the polluted treatments. Futura 75 showed productivity higher than Kc Dora for Cd, Pb, and Ni. Cd's presence did not affect Futura 75's productivity, while Kc Dora in Cd concentration decreased its productivity. The presence of lead in the soil did not affect Futura 75's productivity but caused significant losses in the Kc Dora biomass. Finally, the increasing nickel concentration affected the yield in both varieties.

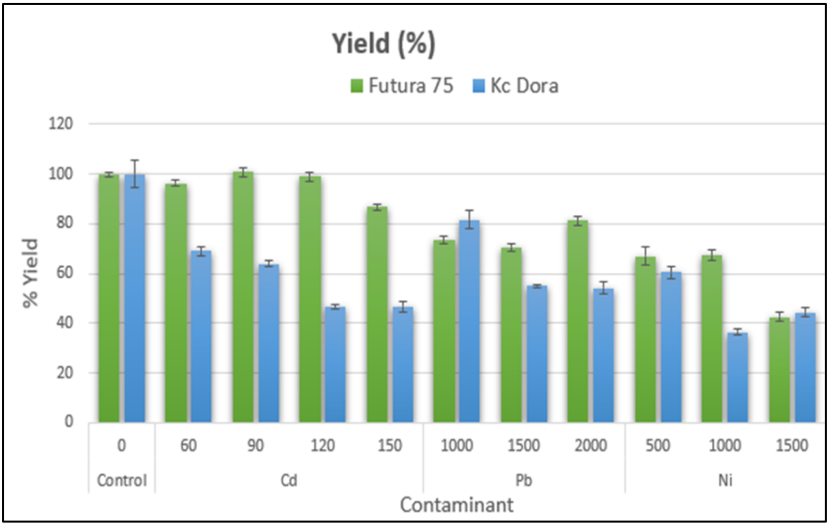

Figure 3.8. Yield (%) in relation to the control obtained from each pot of Futura 75 and Kc Dora in relation to the different studied treatments.

3.4 Conclusion

The present study's result highlighted industrial hemp's ability to tolerate heavy metals in the soil. The linear regressions between the increasing levels of soil pollutants and the stomatal conductance provide insight into the plant's stress condition. Generally, plants subject to increasing soil pollutants respond to the stress, gradually closing the stomata. A minimum reduction in the closure of the stomata indicates the plants' tolerance to heavy metals in soil. The difference observed in the reduction of the yield of the two studied genotypes suggests a greater tolerance of Futura 75 than Kc Dora.

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4 Phytoremediation of heavy metal polluted soil by industrial hemp

ABSTRACT

Restoring heavy metal polluted soils is important to recover ecosystem services. Evidently, phytoremediation technique is a sustainable and renewable biological method that consists in the utilization of the plants to remediate the contaminated site; the plants should be tolerant to the contamination and capable of extracting or immobilized the metals in the soil. Moreover, to define an economically efficient approach to clean up contaminated sites with the possibility of further utilization of phytoremediation biomass turns energy crops in a great option for this technique. Energy crops infact are known for their ability to grow with low agriculture input and later the biomass product can be used to produce biofuels, bioenergy, and bioproducts in a sustainable and renewable way, creating economic potential, mainly when cultivated in marginal lands. the aim of this work provide to test two monoecious industrial hemp varieties in different level of Cd, Pb and Ni.

4.1 Introduction

The challenges that agriculture will face in the next future will be shaped by the increase in the global population, which is directly associated with the rise of natural resources utilization, the finite availability of agricultural land, and climate change, which leads to higher temperatures and more significant variability in rainfall, with an increase in extreme weather events (Shen et al. 2022). Therefore, adopting sustainable production systems to regenerate the ecosystems while sequestering atmospheric carbon will be necessary to address these challenges.

Agricultural land suitability for food production can be hindered by soil contamination. Human activities are the primary source of soil contamination by heavy metals. For example, the residues from mining, pesticides, and herbicides used in agriculture activities, residues from the petroleum industry or its derivates, residues from battery production, and the inappropriate discard of electronic components are some of the human actions that result in soil contamination by heavy metals (Jaskulak, Grobelak, and Vandenbulcke 2020).

Among all the contaminants that can compromise the quality of the soil, heavy metals can be hazardous for human health and the ecosystem in general, despite some of the heavy metals being used by humans and animals as micronutrients due to the process of bioaccumulation in the food chain and the impossibility of degradation. Furthermore, excess absorption of heavy metals by humans and animals can cause serious health problems in the nervous system or generate tumors (Manno, Varrica, and Dongarrà 2006).

Soil decontamination can be attained by following different paths: using chemical, physical, biological techniques, or a mix of them. Phytoremediation is a biological technique for decontaminating the soil that uses plants to extract or stabilize the contaminants (Barbosa et al. 2015). Plants are selected to fulfill several conditions, such as the tolerance to heavy metals, high biomass yield, deep and extensive root systems, well know agronomic techniques, and suitability to low agronomic input (Gomes et al. 2022).

Many energy crops meet these requirements, and their biomass obtained on contaminated soils can be used as a feedstock for energy production (heat, biofuel, biogas) or in the bioproducts field (textile, paper, mats, bioplastics) with low environmental and health risks (Fernando et al. 2015). Nowadays, using land to grow crops for bioenergy has become an increasingly important policy objective designed in the RED II (2018/2001/E.U.) (EU 2018).

Different studies on industrial hemp (*Cannabis sativa* L.) demonstrated the ability to hyperaccumulate toxic trace metals such as lead, cadmium, magnesium, copper, chromium, and cobalt and therefore reclaim contaminated while offering different end uses for its biomass. Worldwide, Hemp can provide both an economical and sustainable solution for soil decontamination (Mihoc et al. 2012).

In the past, Hemp was traditionally grown for its long bast fibers and the seeds, though it can also be grown for its short fibers or energy production (Tang et al. 2017). Its high cellulose content and relatively high productivity make Hemp an attractive annual crop for second-generation bioethanol production.

Hemp can be grown under various agroecological conditions, varying in temperature, photoperiod, and soil water availability, by choosing planting date and variety according to the local condition (Amaducci et al. 2015; Wang et al. 2021). In addition, hemp varieties can be classified according to several attributes such as geographic origin, end use (fiber or seed), ripening time, and reproductive system (dioecious or monoecious) (Salentijn et al. 2015).

As reported by the European Environmental Agency in the industrial pollution country profiles, the most abundant heavy metals produced from industrial waste in Italy, considering the period from 2007 to 2016, were cadmium, lead, and nickel. For this reason, this research aims to evaluate the adaptability of two industrial hemp varieties. Futura75, a French late-ripening cultivar, is one of South Europe's most cultivated varieties of industrial Hemp due to its excellent acclimatization to high temperatures, and KC Dora, a Hungarian variety, growing in north-east Europe. Both varieties are monoecious and were tested under three different levels of cadmium, lead, and nickel soil contamination to assess the phytoremediation potential and the yield of these varieties in the Mediterranean environment.

4.2 Material and methods

The experimental trial was carried out at the University of Catania, Department of Agriculture, Food and Environment (Sicily, Italy), in two subsequent years (2020 and 2021). The following factors were studied in block-randomized experimental design in a pot with three replications in order to evaluate the tolerance of two varieties of *Cannabis sativa* L., Futura 75 and Kc Dora in soils contaminated by three heavy metals (Ni, Cd, Pb) applied in the soil as nitrate (Cd (NO_3), Pb (NO_3), Ni (NO_3)). The amount of the single contaminant in the soil was decided according to the legal law limit referred to sites for commercial and industrial use, reported in D.Lgs 3 aprile 2006 n.152 (2006) imposing the legal limit as first concentration, one and half the legal limit concentration as second and the double concentration as the third level for lead (1000 mg kg^{-1}, 1500 mg kg^{-1} and 2000 mg kg^{-1}) and nickel (500 mg kg^{-1}, 1000 mg kg^{-1}, 1500 mg kg^{-1}); while all the concentrations of cadmium used in this study were higher than the legal limits (up to 10 times: 60, 90, 120, 150 mg kg^{-1}). Untreated soil was also used as a control group.

The soil (Andisol, USDA) used for the experiment was collected from a farm in the Etna area, taken from a depth of 30 cm. It was characterized at the beginning of the experiments by sampling 1 kg of soil, which had been dried in an oven at a temperature between 25 and 30 °C and then sieved through a 2-mm mesh. Particle size distribution was measured, and conductivity was determined in 1:1 soil/distilled water suspensions after 1 hour with conductivity electrodes. A pH-meter P.H. 7 Vio X.S. measured the pH value (H_2O). The soil organic matter was determined using the Walkley-Black method (Nelson and Sommers 1983). The uncontaminated soil was tested as the Control group. The texture of the soil was characterized by a high sand content (92.9%). The organic matter content was low (0.86%). The pH was neutral – lightly alkaline, and had a high conductivity.

The total metal content (Cd, Ni, and Pb) of the soil was quantified by atomic absorption spectrometry (AAnalyst 200 AA Spectrometer, Perkin Elmer) on the aqua regia digested samples, according to ISO 11466 (ISO, 1998) (Standard 1998) before the experiment and after the harvest of the plants. In addition, the bioavailable heavy metal content in the soil was determined following ISO 17402 (ISO 707:2008 2003) using EDTA concentration of 0.05 M, pH 7.5 (near to soil pH) to a volume ratio of 1:20 in 1 g of soil, under stirring for 24 h, the filtrate solution was measured by atomic absorption to quantify the available heavy metals.

The seeds were germinated in Petri dishes, and each germinated seed was planted into peat pots and, after two weeks, transplanted into the contaminated pots (three plants per pot). During the crop cycle, plants were maintained in well water conditions. The main meteorological parameters were recorded by a meteorological station close nearby.

During the two growing seasons (April-September), the minimum temperature ranged from 6.7 °C to 19.8 °C and from 5.3 °C to 21.1 °C in the first and second years, respectively. On the other hand, the maximum temperature ranged from 14.9 °C to 31.9 °C and from 17.4 °C to 35.7 °C in the first and second years, respectively .

The harvest was performed at the complete seed ripening stage, and the plants in each pot were collected and fractionated into stems, leaves, and seeds. Then, the biomass was weighed and dried in an oven at 65°C until constant weight. Also, the roots were collected and washed with ultra-pure water to remove soil particles, weighed fresh, and dried at 65 °C in an oven until constant weight.

Each sample was ground with a mill at a 1mm sieve (IKA M20), and 1 g of biomass was reduced to ashes in the muffle furnace at 550 °C for 5 h. The heavy metal digestion of the biomass samples was performed in a water bath with 10 mL of nitric acid solution.1:1 (Nitric

Acid 65%, Sigma-Aldrich) The sample was filtrated through the Whatman paper. The heavy metal concentration of the extract was quantified in a specific volume by the atomic absorption spectrometer (AAnalyst 200 AA Spectrometer, Perkin Elmer).

4.2.1 Data analysis

To evaluate the tolerance of the two varieties of industrial Hemp, the potential phytoextraction, and the transportation of the heavy metals into the plants, the tolerance index (TI), the bioconcentration factor (BCF), the accumulation index (mAI), and the translocation factor (TF) were calculated (Barbosa et al. 2015; Gomes et al. 2022). Tolerance index (TI) was used to evaluate the tolerance of plants at different levels of contaminants in the soil (Barbosa et al. 2015; Gomes et al. 2022; Yadav et al. 2009). It was calculated divided the dry aboveground biomass weight of contaminated plants (g pot^{-1}) per the dry aboveground biomass weight of control plants (g pot^{-1}).

The modified Accumulation Index (mAI) was calculated to evaluate the plant's ability to absorb the soil's heavy metal (Gomes et al. 2022). It was obtained divided the metal accumulation in the contaminated plant (mg kg^{-1}) by the metal accumulation in the control plants (mg kg^{-1}).

The modified bioconcentration factor (mBCF) is used to evaluate the ability of the plant to extract and accumulate the metal in the aerial or radical fraction of the biomass. The content of the bioavailable metal in the soil, determined by EDTA extraction, represents the amount of metal potentially bioavailable for the plant. Therefore, this factor can represent more realistically the translocation capacity of the metal in plants (Barbosa et al. 2015; Gomes et al. 2022; Mattina et al. 2003). It was obtained as a ratio between the metal concentration in the plant fraction (mg kg^{-1}) and the bioavailable metal concentration in the soil (mg kg^{-1})

Translocation Factor (TF) is expressed as the ratio between the metal concentration in the aboveground fraction of the plant (mg kg^{-1}) and the metal concentration in the root fraction of the plant (mg kg^{-1}) (Barbosa et al. 2015; Gomes et al. 2022; Mattina et al. 2003). It was obtained by the ratio between the metal concentration in the aboveground plant fraction (mg kg^{-1}) and the concentration in the belowground plant fraction (mg kg^{-1}).

Plants with mBCF and TF indices greater than one (> 1) are potentially suitable for phytoextraction.

The data were statistically analyzed using R software (R Core Team, 2013). Contaminant and concentration were considered the main factors, and Tuckey's HSD test separated the means. The Shapiro test was used to verify the normality of the residual distribution. Finally, the ANOVA tested the difference in productivity and heavy metal concentration over the years.

A principal component analysis (PCA) was used to visualize and interpret the multidimensional data. The PCA was based on the Person's correlation matrix calculated upon the biomass fraction yields and heavy metal concentration in the biomass fractions (Ferrarini et al. 2021; Pidlisnyuk et al. 2019).

4.3 Result

4.3.1 Soil characterization

The soil has been characterized as sandy soil (Andisol, USDA), with neutral pH, low nitrogen, and high iron content, as presented in Table 4.1.

Table 4.1 Physical and chemical characteristics of the soil.

Physical characteristics	
Clay (%)	3.0
Silt (%)	4.1
Sand (%)	92.9
Texture	Sandy
Conductivity (µS/cm)	34.2
Chemical characteristics	
pH	7.4
Organic matter (%)	0.86
Fe (mg kg^{-1})	23.6
P (mg kg^{-1})	7
Mn (mg kg^{-1})	0.1
Cu (mg kg^{-1})	21.8

Soil bioavailable Cd, Ni, and Pb concentrations at the sowing time showed no differences between the pots used for Futura 75 and KC Dora showed in Table 4.2.

In Cd-contaminated soil, the bioavailability ranged from 60.2% at the lowest level of Cd-contamination (Cd_{60}) to 75.0% at the highest level of contamination. The bioavailability of Ni in soil underwent a considerable increase from low to the high level of contamination, ranging from 21.6.7% to 77.4%. In Pb-contaminated soil, the bioavailability ranged from 48.7% to 81.1%.

Table 4.2. Total and available heavy metal (mg kg^{-1}) in the soil.

		Total H.M. in soil (mg kg^{-1})	Available H.M. in soil (mg kg^{-1})
Cd	Control	1.7±0.1	1.1±0.1
	60	59. ±2.3	36.0±2.1
	90	88.2± 1.4	55.3±2.0
	120	119.4±1.4	80.2±2.3
	150	150.5 ±2.6	112.9±1.5
Pb	Control	39.6±0.0	19.3± 0.0
	1000	1075.5±46.9	570.9±7.1
	1500	1546.6±11.9	1116.2±57.9
	2000	1808.1 ± 32.3	1465.9±53.7
Ni	Control	40.3±5.7	8.7±1.9
	500	508.2 ±43.1	331.0±14.4
	1000	1047.3±44.5	753.6±29.5
	1500	1491.5±18.7	1153.9±16.1

4.3.2 Morphological measurement

The two hemp varieties studied differed in morphology but showed similar behavior in response to the heavy metal contamination.

All the plants of both Futura 75 and Kc Dora varieties sowed in uncontaminated soil survived until harvesting, while the plant survival rate decreased at high level of contamination, particularly at Cd_{150} and Ni_{1500}, with the rate of survival approaching 50%, as can be observed in Table 4.3

Table 4.3 Plant survival per pot, the height of the plant per pot, and basal diameter per pot.

Varieties	Cont	Conc.	Plant Survival (%)	Average height (cm)	Avarage diameter (mm)
Futura 75	Control		100 a	81.9 ± 9.6 a	4.8±1.0a
	Cd	60	100a	88.3±7.3a	4.6±0.2a
	Cd	90	93ab	75.4±7.7a	4.6±0.3a
	Cd	120	73ab	80.1±10.3a	4.5±0.8a
	Cd	150	57b	72.3±7.3a	4.3±0.4a
	Ni	500	93ab	66.3±4.1a	3.6±0.3a
	Ni	1000	87ab	63.0±2.3a	3.7±0.3a
	Ni	1500	53b	64.9±18.6a	3.7±0.8a
	Pb	1000	87ab	78.3±16.4a	4.8±1.5a
	Pb	1500	80ab	60.4±3.4a	3.7±0.6a
	Pb	2000	73ab	63.2±9.5a	3.5±0.6a
KC Dora	Control		100a	77.9±10.2a	4.2±1.9a
	Cd	60	93a	76.7±5.1a	4.9±0.5a
	Cd	90	93a	77.3±14.9a	4.5±1.1a
	Cd	120	73ab	65.3±2.3a	4.1±0.8a
	Cd	150	6ab	55.3±16.2a	3.6±1.3a
	Ni	500	87a	71.0±8.4a	4.4±0.9a
	Ni	1000	87a	62.1±12.7a	4.0±0.5a
	Ni	1500	47b	47.4±11.4a	3.0±0.6a
	Pb	1000	80ab	76.5±8.9a	4.8±0.9a
	Pb	1500	87a	75.4±11.4a	4.9±0.2a
	Pb	2000	73ab	69.9±4.6a	3.8±2.5a

In uncontaminated soil, Futura75 grew taller than KC Dora. Cd contamination did not reduce plant height and basal diameter, except for the highest concentration (Cd_{150}) in Futura 75 and at concentration higher than 120 mg/kg in Kc Dora. Ni-contamination induced the largest plant height and basal diameter reduction in both varieties. Both varieties were little affected by the lowest level of Pb contamination (Pb_{1000}), but a significant reduction of plant height and basal diameter was observed at the two higher concentrations (Pb_{1500}, Pb_{2000}).

4.3.3 Plant biomass production

Biomass production can be observed in **Figure 4.1**. The two hemp varieties did not differ in biomass productivity on uncontaminated soil. However, on heavy metals contaminated soil, Futura75 showed greater tolerance than Kc Dora, in particular at Cd150, Ni1500, and Pb2000, where the biomass yield reduction in comparison with the uncontaminated control was 32%, 38%, and 38%, respectively for Futura 75 and 47%, 71% and 44% for Kc Dora. Both industrial hemp varieties recorded the greatest reduction in biomass yield in Ni-contaminated soil.

Regarding the biomass production, a significative difference was observed in both varieties for the dry weight of stems and leaves, whereas a not significative difference was observed in the dry weight of the roots and seeds, presented in **Table 4.4**.

The dry biomass of the stems was significantly reduced at the concentration of Ni_{1500} and Pb_{2000} for Futura75 and Ni_{1000} and Ni_{1500} for KC Dora. The production of leaves in the two varieties was affected by the concentration of the heavy metals: a significative reduction was observed in Cd_{120} for Futura 75, and in in Ni_{1500} for KC Dora. Seed yield ranged between 0,4 and 1,5 g pot^{-1} for Futura75, while in KC Dora seed yield ranged between 0,3 to 1,2 g pot^{-1}. In both varieties, the highest productivity of seeds was recorded in the untreated pots.

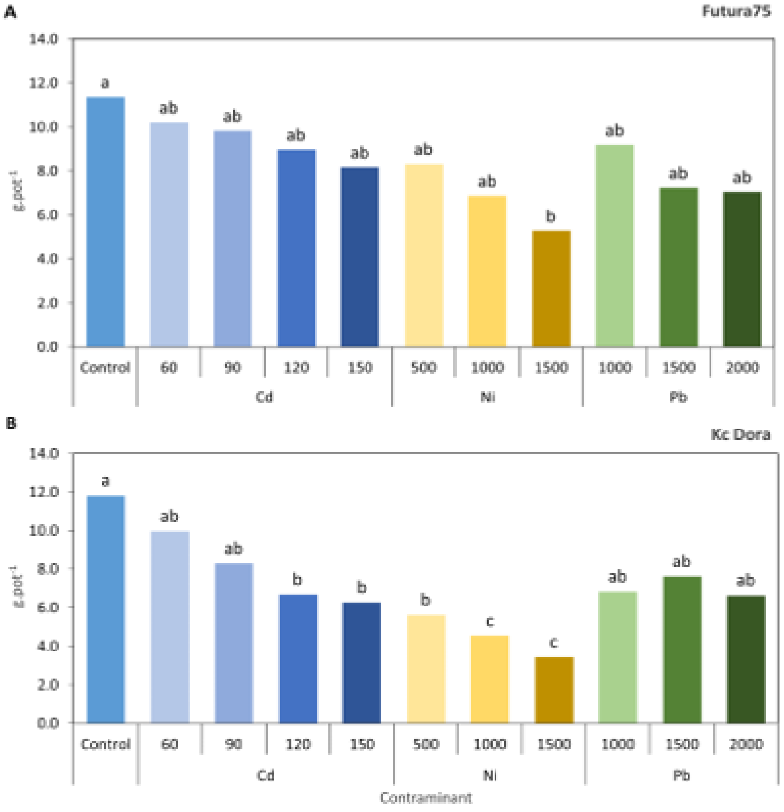


Figure 4.1 Aboveground biomass of Futura75 and Kc Dora.

Table 4.4 Average of the weight of the different compounds of the biomass (roots, stem, leaf, and seed)

Variety	Cont.	Conc.	Average dry roots (g)	Average dry stem (g)	Average dry leaf (g)	Average dry seed (g)
Futura 75	Control		1.6a	6.4ab	3.5a	1.5a
	Cd	60	3.2a	6.9a	2.7ab	0.6a
		90	1.4a	5.2ab	3.8ab	0.9a
		120	1.2a	5.3ab	3.3b	0.4a
		150	1.3a	4.3ab	3.1ab	0.7a
	Ni	500	1.1a	4.0ab	3.8ab	0.6a
		1000	1.2a	4.1ab	1.9ab	0.9a
		1500	0.9a	3.0b	1.6ab	0.7a
	Pb	1000	1.2a	5.7ab	2.7ab	0.7a
		1500	1.6a	3.9ab	2.5ab	0.8a
		2000	1.0a	3.4b	2.8ab	0.8a
KC Dora	Control		2.0a	6.6a	4.0a	1.2a
	Cd	60	1.6a	5.5ab	3.3ab	1.3a
		90	1.6a	5.2ab	2.3ab	0.8a
		120	0.9a	4.2ab	1.7ab	0.8a
		150	1.1a	3.9ab	2.1ab	0.3a
	Ni	500	0.9a	2.8ab	2.2ab	0.6a
		1000	0.7a	2.5b	1.5ab	0.6a
		1500	0.6a	2.0b	1.2b	0.3a
	Pb	1000	1.8a	4.2ab	2.0ab	0.7a
		1500	1.6a	4.3ab	2.3ab	1.0a
		2000	1.6a	3.8ab	2.0ab	0.9a

4.3.4 The concentration of heavy metals in the different parts of the plants.

At low level of cadmium contamination, the highest Cd concentration among plant organs in Futura 75 was observed in the leaves. At high level of contamination, above Cd_{120}, the plants decrease the translocation from the roots towards the aboveground organs, leading to higher concentration of cadmium in the roots. Kc Dora showed a larger translocation tendency for cadmium than Futura 75, which led to similar concentration in roots and leaves at all levels of soil contamination. Cadmium concentration in the aboveground organs did not increase linearly with the concentration in the soil, suggesting the existence of a limitation factor for the translocation. Cadmium concentration in the seeds was lower than 3 µg g^{-1} at any level of soil contamination.

Futura 75 showed higher nickel uptake and translocation than KC Dora: nickel concentration in the plant tissues was higher in Futura 75 than in KC Dora in both roots, leaves, stems, and seeds. The significative difference was observed in all the concentrations. Regarding the aboveground biomass, the highest concentration was observed in the leaves of Futura75, with a concentration of 26%, 57%, and 87% for Ni_{500}, Ni_{1000}, and Ni_{1500}, respectively. In comparison, the concentration of Ni in the leaves was increasing in KC Dora, with a percentage of 16%, 30%, and 31% in Ni_{500}, Ni_{1000}, and Ni_{1500}.

Lead translocation potential from roots to aboveground organs was low for both Futura 75 and KC Dora. Both varieties showed higher lead concentration in the roots, reaching over 100 µg.g-1 at Pb2000. Lead concentration was lower in the aboveground organs, staying below 40 µg.g-1 in the stem and the leaves and below 20 µg.g-1 in the seeds at the highest level of lead soil contamination for both varieties. The concentration of the contaminants can be observed for cadmium in Figure 4.2, for nickel in Figure 4.3, and for lead in Figure 4.4.

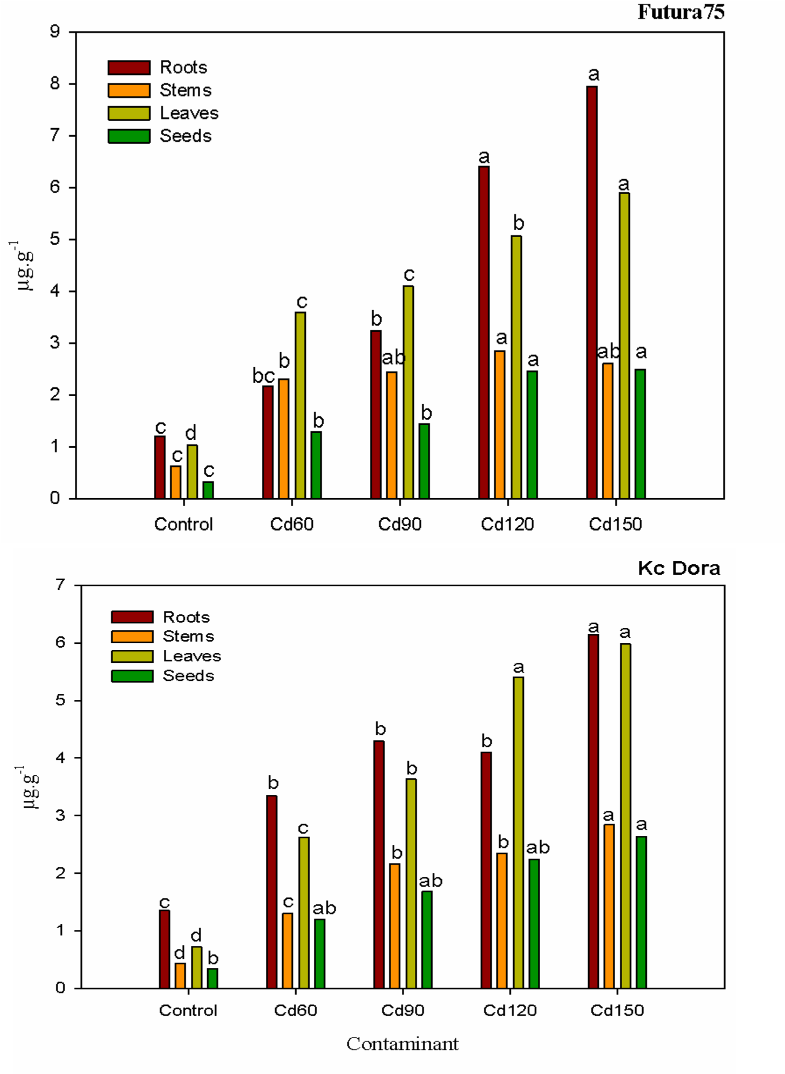


Figure 4.2 Concentration of cadmium (μg g^{-1}) in roots, stems, leaves and seeds in Futura75 and Kc Dora as average of two years. The multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

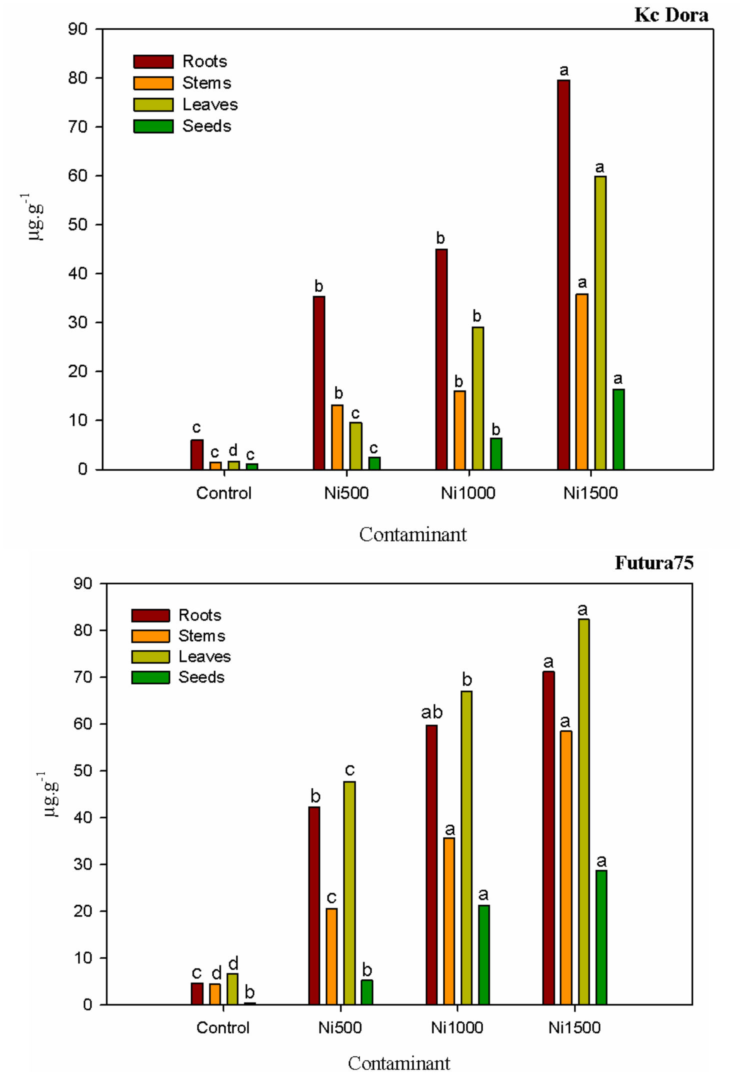


Figure 4.3 Concentration of nickel (µg g^{-1}) in roots, stems, leaves and seeds in Futura75 and Kc Dora as average of two years. The multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

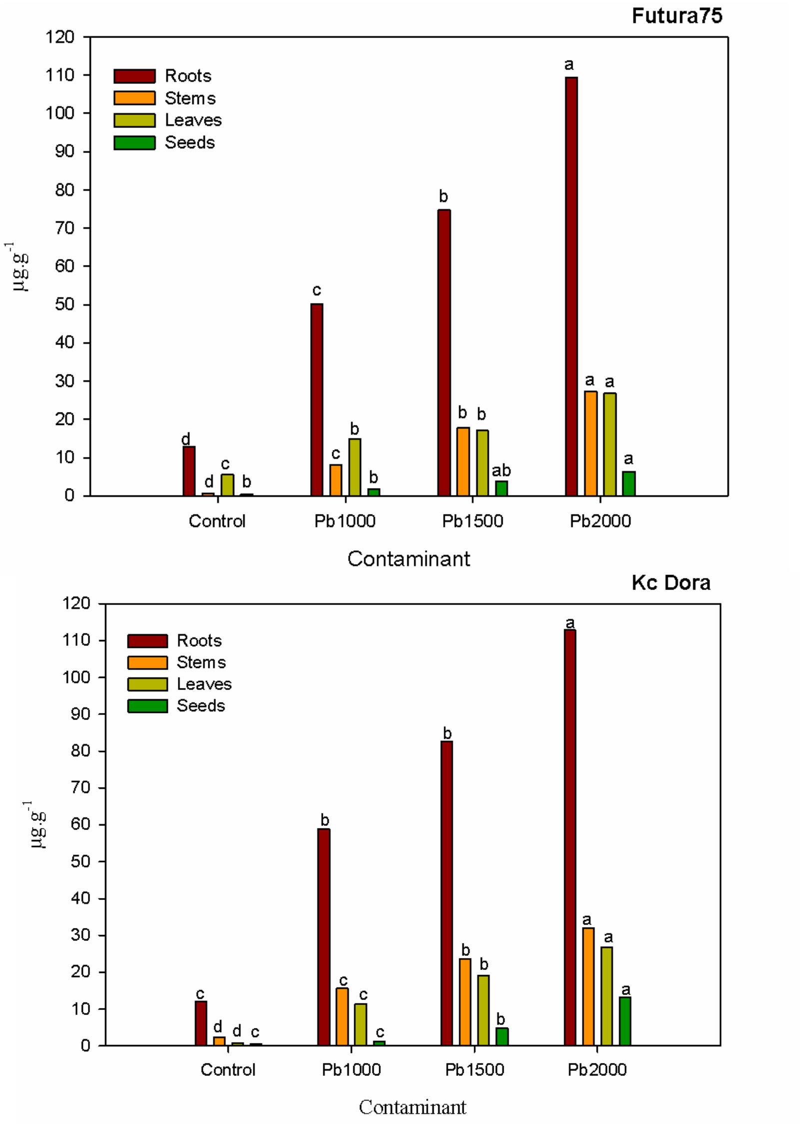


Figure 4.4 Concentration of lead (µg g^{-1}) in roots, stems, leaves and seeds in Futura75 and Kc Dora as average of two years. The multiple comparisons between the means have been performed within the plant fractions. Values with different letters indicate statistical significance (according to HSD at $p \leq 0.05$).

4.3.5 *Evaluating the tolerance and the potential phytoextraction by phytoremediation index and factors.*

The several indexes and factors can be calculated to evaluate the adaptability to soil contamination (TI) and the phytoextraction potential (mAI, aboveground and belowground mBCF and TF) **Table 4.5**. The TI shows the adaptability of the two varieties of industrial hemp to being grown in soils contaminated with increasing concentrations of cadmium, nickel and lead. The tolerance index decreased at the increasing level of soil contamination for both hemp varieties and all the heavy metals tested. The lowest TI score has been observed at Ni_{1500} (0.46 and 0.35 for Futura 75 and KC Dora respectively). Futura 75 showed higher TI than KC Dora for all the heavy metals at all the level of contamination.

The mAI, which assesses the amount of the heavy metal uptake, increased at the increasing level of soil contamination for Futura 75 and KC Dora, indicating that the plants can phytoextract higher amount of heavy metals from soil with high heavy metal concentration. The highest mAI score has been observed in KC Dora at Ni_{1500} e Pb_{2000}. KC Dora showed higher values of mAI than Futura 75.

The comparison of aboveground and belowground mBCF gives an insight on the heavy metal partitioning between plant organs. Both factor tend to decrease at high contamination levels. Under cadmium and nickel contamination, Futura 75 showed a higher aboveground mBCF than KC Dora, suggesting a better suitability for uptake and removal of the heavy metal from the soil.

The TF depends on the ability to translocate the heavy metals from the roots to the aboveground organs.

Under lead and nickel contamination, both Futura75 and KC Dora had increasing TF scores at the increasing soil concentration.

Under cadmium contamination, only KC Dora had increasing TF scores at the increasing soil Cd concentration, while the TF of Futura 75 decreased.

Table 4.5 Phytoremediation factors of phytoremediation extraction of Futura 75 and Kc Dora.

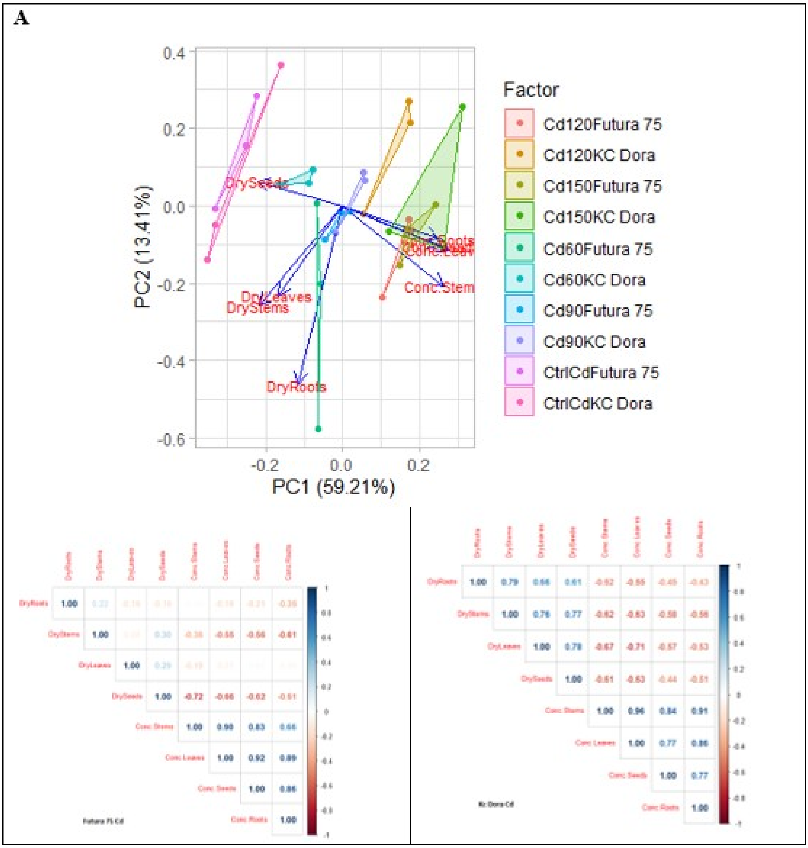
Varietà	HM – Conc		TI	mAI	mBCF Aboveground	TF	mBCF belowground
Futura 75	Cd	60	0.90	3.66	0.28	3.32	0.09
		90	0.87	4.06	0.20	2.47	0.08
		120	0.79	5.26	0.16	1.62	0.10
		150	0.72	5.58	0.13	1.38	0.09
	Ni	500	0.73	6.55	0.34	1.76	0.20
		1000	0.60	10.90	0.26	2.07	0.13
		1500	0.46	14.92	0.16	2.38	0.07
	Pb	1000	0.81	3.68	0.05	0.50	0.10
		1500	0.64	5.71	0.03	0.52	0.07
		2000	0.62	8.94	0.05	0.55	0.08
KC Dora	Cd	60	0.84	3.45	0.14	1.54	0.09
		90	0.63	5.03	0.14	1.74	0.08
		120	0.57	6.72	0.15	2.44	0.06
		150	0.53	8.40	0.15	2.04	0.07
	Ni	500	0.62	6.02	0.12	0.71	0.17
		1000	0.51	12.27	0.11	1.14	0.10
		1500	0.35	26.79	0.15	1.41	0.11
	Pb	1000	0.75	8.12	0.04	0.49	0.09
		1500	0.64	13.39	0.04	0.58	0.07
		2000	0.56	20.19	0.06	0.60	0.09

4.3.6 Principal component analysis and correlation of the main factor between the two varieties of industrial hemp

A principal component analysis (PCA) has been performed to evaluate the effect of the metal contaminants at several concentrations on several highly correlated variables for cadmium, nickel, and lead in **Figure 4.5**, **Figure 4.6**, and **Figure 4.7**, respectively. In particular, the biomass yield components (stem, leaves, and root) are mutually correlated and inversely correlated to the contaminant concentration in the plant fractions (stem, leaves, seeds, and root yield). In addition, seed yield appears to be weakly correlated to the other biomass components' yield, particularly root yield.

The first component of the PCA (PC1) describes between 59.21% and 66.14% of the total variance in the data in the Cd-, Ni-and Pb-contaminated samples, respectively. PC1 direction coincides with higher biomass yield and lowers contaminant concentration in the plant organs. For all metal contaminants, the control group does not overlap with the contaminated groups. Furthermore, between the Ni, Pb, and Cd levels, there is also no overlapping between the two varieties, showing different behavior in Futura 75 and Kc Dora.

In fact, in cadmium contaminated soil, in Futura 75, just the dry biomass of the stems and the seeds is strongly negatively affected by the concentration of cadmium in the different parts of the plant. In contrast, in Kc Dora, all the biomass of the plant's different parts is strongly negatively correlated with the concentration in the different parts of the plants. Similar behavior was obtained in nickel contaminated soil in the correlation matrix of Futura 75 and Kc Dora. Whereas in lead contaminated soil, the biomass of dry stems and dry leaves in Kc Dora was strongly negatively correlated with the concentration of Pb in the different parts of the plant. In Futura 75, the stem biomass was strongly negatively correlated with the concentration of the heavy metal in the plant.



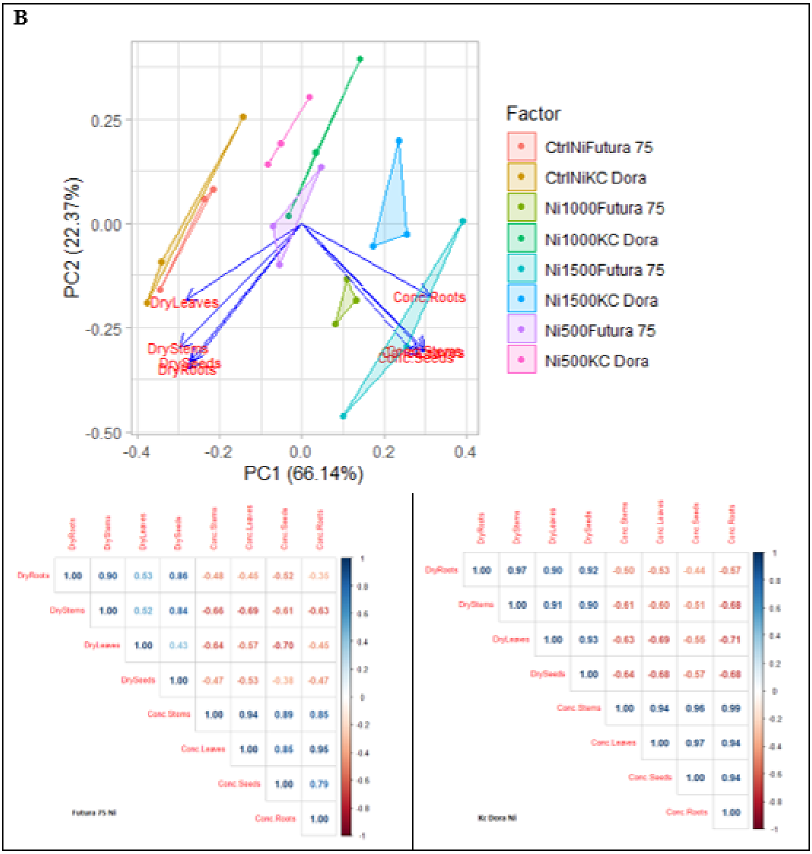
Futura 75 Cd

	DryRoots	DryStems	DryLeaves	DrySeeds	Conc.Stems	Conc.Leaves	Conc.Seeds	Conc.Roots
DryRoots	1.00	0.22	-0.16	-0.16	[illegible]	-0.16	-0.21	-0.36
DryStems		1.00	[illegible]	0.30	-0.36	-0.55	-0.56	-0.61
DryLeaves			1.00	0.29	-0.15	[illegible]	[illegible]	[illegible]
DrySeeds				1.00	-0.72	-0.66	-0.62	-0.51
Conc.Stems					1.00	0.90	0.83	0.66
Conc.Leaves						1.00	0.92	0.89
Conc.Seeds							1.00	0.86
Conc.Roots								1.00

Kc Dora Cd

	DryRoots	DryStems	DryLeaves	DrySeeds	Conc.Stems	Conc.Leaves	Conc.Seeds	Conc.Roots
DryRoots	1.00	0.79	0.66	0.61	-0.52	-0.55	-0.45	-0.43
DryStems		1.00	0.76	0.77	-0.62	-0.63	-0.58	-0.56
DryLeaves			1.00	0.78	-0.67	-0.71	-0.57	-0.53
DrySeeds				1.00	-0.61	-0.63	-0.44	-0.51
Conc.Stems					1.00	0.96	0.84	0.91
Conc.Leaves						1.00	0.77	0.86
Conc.Seeds							1.00	0.77
Conc.Roots								1.00

Figure 4.5 Principal component analysis and correlation matrix for Cd, of Futura75 and Kc Dora, using as variable the dry biomass yield of roots, stems, leaves and seeds, and the concentration of the heavy metal measured in the related part of the plant.



Futura 75 Ni

	DryRoots	DryStems	DryLeaves	DrySeeds	Conc.Stems	Conc.Leaves	Conc.Seeds	Conc.Roots
DryRoots	1.00	0.90	0.53	0.86	-0.48	-0.45	-0.52	-0.35
DryStems		1.00	0.52	0.84	-0.66	-0.69	-0.61	-0.63
DryLeaves			1.00	0.43	-0.64	-0.57	-0.70	-0.45
DrySeeds				1.00	-0.47	-0.53	-0.38	-0.47
Conc.Stems					1.00	0.94	0.89	0.85
Conc.Leaves						1.00	0.85	0.95
Conc.Seeds							1.00	0.79
Conc.Roots								1.00

Kc Dora Ni

	DryRoots	DryStems	DryLeaves	DrySeeds	Conc.Stems	Conc.Leaves	Conc.Seeds	Conc.Roots
DryRoots	1.00	0.97	0.90	0.92	-0.50	-0.53	-0.44	-0.57
DryStems		1.00	0.91	0.90	-0.61	-0.60	-0.51	-0.68
DryLeaves			1.00	0.93	-0.63	-0.69	-0.55	-0.71
DrySeeds				1.00	-0.64	-0.68	-0.57	-0.68
Conc.Stems					1.00	0.94	0.96	0.99
Conc.Leaves						1.00	0.97	0.94
Conc.Seeds							1.00	0.94
Conc.Roots								1.00

Figure 4.6. Principal component analysis and correlation matrix for Ni of Futura75 and Kc Dora, using as variable the dry biomass yield of roots, stems, leaves and seeds, and the concentration of the heavy metal measured in the related part

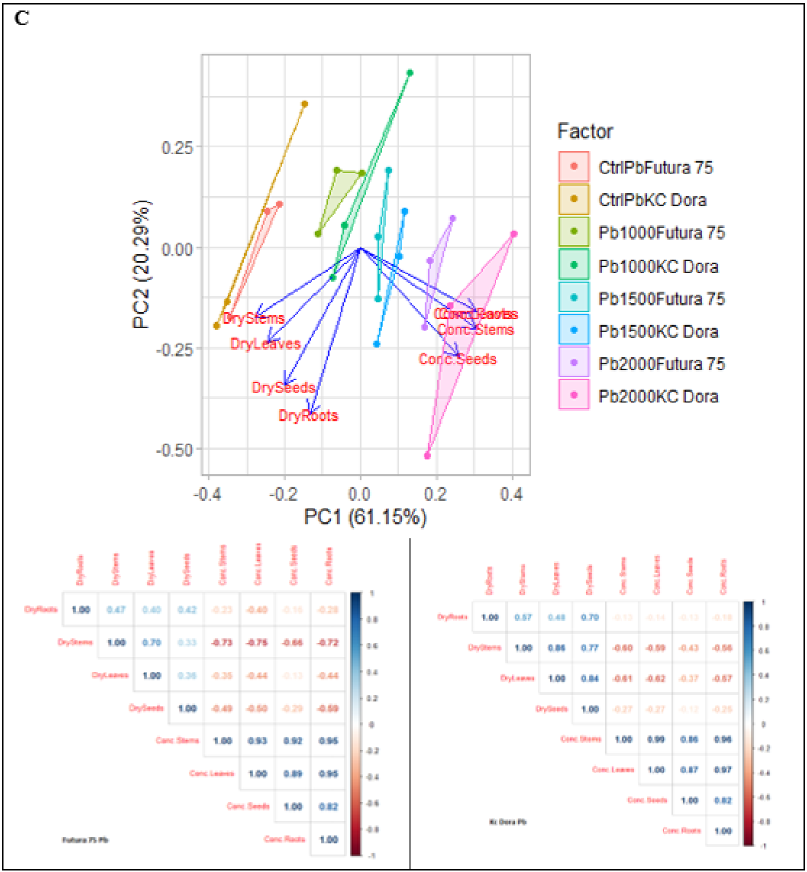


Figure 4.7. Principal component analysis and correlation matrix for Pb, of Futura75 and Kc Dora, using as variable the dry biomass yield of roots, stems, leaves and seeds, and the concentration of the heavy metal measured in the related part

4.4 Discussion

Industrial hemp can be grown in most of the world for its high environmental adaptability (Salentijn et al. 2015). The selection of the best suited genotype for a specific environment, climatic condition and agronomic management is crucial for crop success (Wang et al. 2021).

Different studies carried out on *C. sativa* showed its potential as hyperaccumulator for different toxic traces of metals such as lead, cadmium, magnesium, copper, chromium, and cobalt which pose a great risk to the ecological system (Golia et al. 2023; Rheay, Omondi, and Brewer 2021), allowing to reclaim contaminated while yielding fiber and/or seed (Tang et al. 2017). Worldwide, hemp can provide both an economic and sustainable solution to the contamination of soils (Mihoc et al. 2012). In this study, the productivity of stems in both varieties of hemp was affected by the raising level of heavy metal, while no significant difference was observed in the seed production. However, low levels of contamination were not detrimental to the overall aboveground biomass; morphologic parameters were not affected by the heavy metal in the soil. A similar result was observed by De Vos et al. (2023) , Pietrini et al. (2019) and Guidi Nissim et al. (2018), which reported no significant differences in stem height and stem diameter between the control and plants grown on low soil contamination.

The present study found that Futura 75, a late ripening variety, was more tolerant than Kc Dora, a early ripening variety; to high concentrations of cadmium, lead and nickel, confirming the results observed by Tang et al. (2016).

Cadmium is known to be one of the most phytotoxic heavy metals (Salt et al. 1995). Linger et al. (2002) showed that the photosynthesis pathway in hemp is influenced in two ways by cadmium: (1) cadmium indirectly disturbs the water and ion uptake by the plant, which harms the plant water status; (2) it directly affects the chloroplast apparatus after entering the leaf cells. Cd concentrations of up to 72 mg/kg (soil) had no negative effect on the germination of hemp. It has been estimated from the post-conduction experiments that, up to 100 ppm, cadmium does not affect the morphological growth of hemp (Linger et al. 2002). Shi et al. 2012 compared 18 hemp accessions cultivated on cadmium-contaminated soils for biodiesel production. It was observed that most of the hemp varieties, except USO-31, Shenyang, and Shengmu, could grow quite well under 25 mg of cadmium per kg of dry soil. Under this condition, the tolerance factor observed in hemp was high (68.6% - 92.3%) and the ability to store cadmium in the aerial fraction of biomass is suitable for phytoextraction, indicating that the production of this crop can be an alternative to valorize and remediate cadmium contaminated soils (Shi et al. 2012).

Hemp productivity was less affected by lead contamination when compared with the highest concentration of cadmium or nickel. The translocation of lead from roots to the aerial biomass was low, therefore the highest concentration was observed in the roots. A similar result was observed by Ahmad et al. (2016) and Angelova et al. (2004), which reported Pb concentrations in the following order: roots > stems > leaves > seeds; and by Pietrini et al. (2019), who reported that hemp tends to accumulate lead mainly in the roots, with minimal translocation to the aboveground biomass, which explains the relatively low BCF for Pb in the present study

Nickel soil contamination induced the highest reduction of biomass production among the heavy metals tested. Ferrarini et al. 2021(Ferrarini et al. 2021) reported that hemp yield reduction on soil contaminated by nickel (>500 mg kg^{-1}) in combination with copper (>150 mg kg^{-1}). Zhao, Guo, and Papazoglou (2022) reported a reduction in germination and biomass production even at low nickel concentrations (110 and 220 mg kg^{-1}), and both higher concentration in plant organs and higher translocation factor (TF) than the value observed for lead.

For cadmium and nickel, with the exception of Ni_{500}, the translocation factor was higher than 1, indicating the high suitability of hemp for phytoextraction processes, thanks to the accumulation of the heavy metals in the aerial part of the plant.

Despite the soil analysis indicated that the bio-availability of cadmium is low, the actual availability fo cadmium over time can increase due to the low tendency to form complexes, while bio-availability of lead and nickel have a higher complex rate, which reduces the bio-availability.

Hemp is not recognized as a hyperaccumulator plant, however, its high tolerance towards certain heavy metals in the soil, makes this plant a suitable alternative for contaminated soil valorization and remediation (De Vos et al. 2023)

4.5 Conclusion

This research highlighted the different phytoextraction capabilities among the two hemp varieties and explored the capability of industrial hemp varieties to translocate metals from the soils to the different parts of the plants. Industrial hemp showed the ability to complete its life cycle until seed ripening in heavily contaminated soils. This study suggests an interesting linear regressions between the metal content in the plant and soil and the biomass yield, highlighting a significative correlation

4.6 References

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5 Physiological tolerance of perennial grasses to heavy metal contaminated soils

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Physiological tolerance of perennial grasses to heavy metal contaminated soils

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ABSTRACT: There are several environmental advantages to using perennial crops, such as reduced soil erosion, increased soil organic matter, low fertilizer and agrochemical demands, and increased biodiversity. Bioproducts, biofuels, and bioenergy appear as an alternative to replacing petroleum-based products, and nowadays, using land to grow crops for bioenergy has become an increasingly important policy objective designed in the RED II (2018/2001/EU). In this context, marginal lands appear as an alternative for industrial crop production without threatening food production. Marginal lands are generally considered unproductivity lands that are exposed to stress conditions such as limitations of nutrients or water or contamination by hydrocarbons or heavy metals. Therefore, biomass production in marginal soils is recommended to lessen land use and change ethical issues linked with competition with food crops. Contaminated soils hinder the cultivation of traditional food crops, while industrial crops can provide several ecosystem services. However, understanding the tolerance and the phytoremediation of each species to a single contaminant helps us better clean up the polluted areas. It can be useful for organizing protocols to reclamation these areas, reducing time and management costs, and providing a renewable source for energy and bioproducts. The species suitable to heavy metals polluted soils can be divided into exclusion and resistance. The mechanisms of exclusion prevent the accumulation of toxic concentrations in sensitive sites within the cell, thus preventing negative effects. The resistance mechanisms generally concern the development of proteins that allow the plant to resist heavy metals, allowing the accumulation of the same contaminant in the aerial parts of the plant. This work aimed to study the physiological response of plants tolerant to heavy metals, in particular, focused the attention on two non-food lignocellulosic perennial grasses: giant reed (*Arundo donax* L.) and African fodder cane (*Saccharum spontaneum* L. ssp. *aegyptiacum*), in soils contaminated by four heavy metals (Cd, Pb, Ni, Zn) with the purpose to observe the physiological response and the mechanisms of resistance in the increasing concentrations of the four soil heavy metals.

KEYWORDS: biomass, bioenergy, perennial grass, marginal land; dryness; water footprint; energy product

5.1 Introduction

Lignocellulosic biomass can be used in various ways, such as an energy source, industrial feedstock, and raw material in integrated biorefineries (Scordia and Cosentino 2019). Nowadays, using land to grow crops for bioenergy has become an increasingly important policy objective designed in the RED II (2018/2001/EU), where Member States must supply a minimum of 14% of biofuels with at least 3.5% from advanced bioconversion by 2030. This target for the transport of biofuels is also contributing to a higher demand for biomass, which increases competition for land, threatening food security (EU 2018).

Using agricultural land to cultivate energy crops has increased the competition between fuel and food. To avoid this problem, marginal lands appear as an alternative to energy crop production without threatening food production (Cosentino et al. 2015). Marginal lands can be understood generally as unproductivity lands exposed to stressed conditions such as limitations of nutrients or water or contamination by hydrocarbons or heavy metals not suitable for food production. In heavy metals, there are several methods to decontaminate the soil through different paths using physical, chemical, or biological techniques. Naturally, phytoremediation is a sustainable and renewable technique that utilizes plants to remediate the contaminated site (Barbosa et al. 2015). The selection of the plant needs to fulfill several conditions, such as the tolerance of the crops to heavy metals, high production of biomass, deep and extensive root systems, well know agronomic techniques and low request for agronomic input. In this way, the cultivation of food crops in heavy metal contaminated soils must be avoided, and the cultivation of industrial crops becomes a viable alternative due to their higher tolerance to the contaminant presence, which allows the crops to grow without significant productivity losses, and their higher capability to accumulate the heavy metals, providing in this way, the remediation of the land (Papazoglou et al. 2005). Physiological and biochemical mechanisms of heavy metal tolerance have gained considerable insight during the last few decades.

Plants employ specific strategies to tolerate harmful levels of heavy metals in the soil (Liu et al. 2018). Most plants have developed a complicated process for acquiring relatively unavailable micronutrients like Zn, Mn, Cu, Fe, and Ni from the soil. The mechanism of tolerance to metal stress in plants may range from the exclusion of toxic metal and inclusion and accumulation at inert places, with may vary from species to species (Raskin and Ensley 2000). Metal tolerance or accumulation can be enhanced by producing binding proteins and peptides in plants-high specificity of these peptides or proteins for more significance in plants (Ryu et al. 2003). However, tolerance levels in plants can be grouped into sensitive, resistant excluder, tolerant non-hyperaccumulator, and hyper-tolerant hyperaccumulator species, each with specific physio-anatomical and molecular mechanisms for their resistance/tolerance to metal toxicity (Alloway 1995).

Moreover, the possibility of further utilizing biomass from phytoremediation turns energy crops into an excellent option for this technique and highlights the opportunity to enhance these areas that would be discarded with a consequent increase in environmental pollution (Scordia et al. 2014). In this scenario, Arundo donax L. and Saccharum spontaneum L. ssp. aegyptiacum are two perennial drought-tolerant grass species adapted to grow in marginal or sub-marginal lands, thus reducing competition with food crops for soil use, and the first results of experimental tests conducted in Sicily allow it to be indicated as a possible crop dedicated to the production of biomass for energy (Scordia et al. 2017, Cosentino et al. 2012). In this experiment, two lignocellulosic perennial grasses, Arundo donax L. and Saccharum spontaneum L. ssp. aegyptiacum were tested in contaminated soil with different concentrations of Zinc, Cadmium, Lead, and Nickel, seeking the physiological response and the mechanisms of resistance of these species to the heavy metals contaminated soils.

5.2 Material and methods

5.2.1 Trial Description

The present experiment was conducted in March-November 2020 at the University of Catania. The two lignocellulosic species, namely *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) and *Arundo donax* L. were grown in a contaminated pot of 12 kg, previously contaminated with 4 heavy metals in two different levels, high and low. In particular, the soil was contaminated using nitrate of cadmium [$Cd(NO_3)_2$], nitrate of lead [$Pb(NO_3)_2$], nitrate of Zinc [$Zn(NO_3)_2$], and nitrate of nickel [$Ni(NO_3)_2$]. The concentration of heavy metal in the soil was applied following EU guidelines for contaminated soil, following as the first concentration for each heavy metal the limit for contaminated soil, and as the second concentration the double of the limit. The nitric metal salt per pot was calculated by multiplying the molecular weight of the salt by the grams of metal contained per pot and dividing by the metal's molar weight. A nitrate fertilizer control was added to reach the same concentration of the highest level of $Zn(NO_3)_2$.

Clonal rhizomes of giant reed (*Arundo donax L.*) and African fodder cane (*Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) were collected at the Experimental Farm of the University of Catania, Italy (10 m a.s.l., 37°25’ N lat., 15° 03’ E long.), weighted and size reduced as homogeneous as possible and transplanted in the pots two months after the soil contamination. The irrigation was kept in optimal conditions for the whole crop cycle. Pots were arranged in a completely randomized design with 3 replications.

5.2.2 Measurements and calculations

During the growing season, the leaf photosynthesis (A, µmol CO_2 m^{-2} s^{-1}), the transpiration rate (E, mmol H_2O m^{-2} s^{-1}), and stomatal conductance (mol m^{-2} s^{-1}) were measured on three representative leaves in each species and treatment, on the third fully expanded leaf. A portable instrument (LCi-SD, ADC BioScientific, Great Amwell, Hertfordshire, UK) was used at a flow rate of 500 mL min^{-1} and under CO_2, temperature, and ambient humidity conditions during cloudless days.

The instantaneous water efficiency (iWUE) was calculated as the ratio between photosynthesis rate and leaf transpiration (µmol CO_2 mmol H_2O^{-1}).

5.2.3 Statistical analysis

The physiological data were statistically analyzed using R software (R Core Team, 2013). Species and pollutants were considered the main factors, and the means were separated by the Student-Newman-Keuls (SNK) test at a 95% confidence level. The mechanisms of resistance to increasing soil pollutant concentration of giant reed and African fodder cane were evaluated through linear regressions between the pollutant concentration and stomatal conductance, with the b-value of the linear regression representing the plant response to increasing heavy metal concentration. The Shapiro-Wilk test was used to verify the normality of the residual distribution. Regression coefficients were considered significant when $P \leq 0.05$, and the goodness of fit was estimated by calculating the R^2 (SigmaPlot11, Systat Software Inc., San Jose, CA, USA).

5.3 Results

5.3.1 Photosynthesis rate

The ANOVA showed significant differences in photosynthesis rate compared with the treatments Table 5.1. The measurement date was significant for Cd, Pb, and Ni but not for Zn. The species showed significant differences for Ni and Pb but not Cd and Zn. Pollutant concentration and first and second-order interactions did not show significant differences in photosynthesis rate.

Table 5.1 ANOVA of the main factors and interactions for the net photosynthesis of *Arundo donax* L. and Saccharum spontaneum spp. aegyptiacum under increasing levels of pollutants in the soil (significance level at $P \leq 0.05$).

Photosynthesis rate	Cd	Ni	Pb	Zn
Date (D)	0.019	0.000	0.001	0.061
Genotypes (G)	0.128	0.000	0.002	0.260
Concentration (C)	0.736	0.226	0.937	0.988
D x G	0.071	0.387	0.628	0.063
D x C	0.736	0.948	0.748	0.411
G x C	0.792	0.994	0.522	0.180
D x G x C	0.125	0.946	0.557	0.738

Across the average of measurements, Saccharum showed a higher photosynthesis rate than giant reed under the control and the different levels of contaminants used in the study, presented in **Figure 5.1**

In the cadmium-contaminated theses, the photosynthesis rate in Saccharum did not show significant differences through cadmium concentration equal to 4 mg kg^{-1} of soil and cadmium concentration equal to 8 mg kg^{-1} of soil, although values were lower than the controls. Arundo slightly decreased photosynthetic rate under Cd contamination but at the highest level as compared with the control (12 at Cd_8 and 14.5 μmol CO_2 m^{-2} s^{-1}, respectively). In the nickel-contaminated both species showed a decreasing photosynthetic rate with the increasing concentrations of the metal being studied. Furthermore, the lower values were recorded in the Ni 220 mg kg^{-1} in the soil in both species (13 and 11 μmol CO_2 m^{-2} s^{-1}, respectively, for Saccharum and Arundo). In the lead-contaminated, Saccharum showed greater sensitivity to this heavy metal and obtained a lower value in the photosynthesis rate, particularly with the highest level of lead in the soil (Pb900). Arundo showed lower photosynthetic rate values than the controls, but no significant differences were found between the two concentrations of a contaminant in the soil (average Pb levels, 11 μmol CO_2 m^{-2} s^{-1}). Finally, the photosynthesis rate was always lower than the controls for both specie in soil contaminated with zinc, but between the different levels of contaminant present in the soil, it was higher for Saccharum in the level of 450 mg kg^{-1} of zinc and lower in the concentration of 900 mg kg^{-1} of zinc in the soil (14 and 12 µmol CO_2 m^{-2} s^{-1}, respectively). Surprisingly Arundo increased leaf photosynthesis at 900 mg Zn kg^{-1} than in the concentration 450 mg kg^{-1} (10 and 13 µmol CO_2 m^{-2} s^{-1}, respectively).

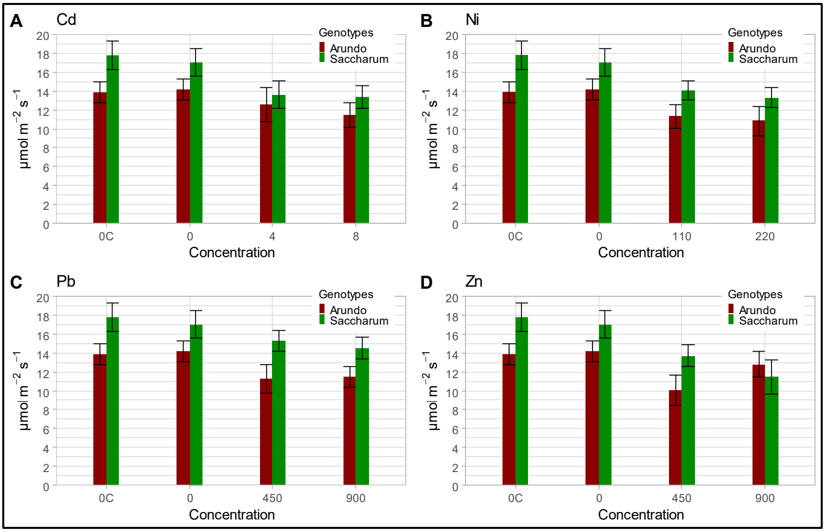

Figure 5.1 Leaf photosynthesis (µmol CO_2 m^{-2} s^{-1}) in Arundo donax and Saccharum spontaneum spp. aegyptiacum under different levels of contaminant (A: Cd, B: Ni, C: Pb; D: Zn) and control unfertilized (0) and control N fertilized (0C).

5.3.2 Transpiration rate

The ANOVA showed significant differences in the main effect on the transpiration rate showed in Table 5.2.

Table 5.2 ANOVA of the main factors and interactions for the leaf transpiration rate of *Arundo donax* L. and Saccharum spontaneum spp. aegyptiacum under increasing levels of pollutants in the soil (significance level at P≤0.05).

Transpiration rate	Cd	Ni	Pb	Zn
Date (D)	0.006	0.571	0.084	0.214
Genotype (G)	0.002	0.000	0.000	0.285
Concentration (C)	0.882	0.960	0.531	0.444
D x G	0.042	0.037	0.007	0.222
D x C	0.758	0.708	0.554	0.637
G x C	0.414	0.531	0.195	0.288
D x G x C	0.039	0.478	0.128	0.908

The date of the measurement was only significant for Cd. The genotype showed substantial differences for Cd, Ni, and Pb, while the pollutant concentration showed no differences. The data x genotype interactions were significantly different for Cd and Ni, and the second-order interaction was only for Cd. The two species under study showed a distinct tendency to transpire depending on the soil's contaminants and their concentration, similar to leaf photosynthesis. In Arundo, the transpiration rate detected was higher than in Sacchurum in accordance with the photosynthetic cycle of the crop, C4 in Saccharum, and C3 in Arundo,as observed in **Figure 5.2** .

A higher transpiration rate was obtained among the controls in all treatments, and in particular, the fertilized one had a higher transpiration rate than the unfertilized control. Among the cadmium – contaminant, similarly to the photosynthesis rate, the highest transpiration rate between the two levels of contaminant in the soil was found for Saccharum in the lower cadmium concentration equal to 4 mg kg^{-1}; for Arundo, no significant differences were found in the transpiration rate in both contaminant levels. Among the nickel-contaminated, the transpiration rate was lower than the controls. In Arundo, the transpiration rate decreased when the nickel concentration in the soil increased. In Saccharum, although not significant, the transpiration rate increased in the highest nickel concentration in the soil (220 mg kg^{-1}). Among the lead-contaminated trials, Arundo and Saccharum showed a higher transpiration rate in the controls than the two lead levels in the soil. Between the two contaminant levels, Arundo did not show significant differences (3.7 mmol H_2O m^{-2} s^{-1} in the average concentration). Saccharum instead showed a higher transpiration rate in the trial containing 450 mg kg^{-1} lead in soil and lower in 900 mg kg^{-1} of lead in soil (2.9 and 2.5 mmol H_20 m^{-2} s^{-1}, respectively). Finally, for treatments contaminated by zinc, the species showed a different trend within the levels of the heavy metal in the soil. Arundo showed an increasing transpiration rate at the higher contaminant level (4 mmol H_20 m^{-2} s^{-1} to 900 mg kg^{-1} of zinc in the soil versus 3.24 mmol H_20 m^{-2} s^{-1} at 450 mg kg^{-1} of zinc in the soil). Saccharum showed a decreasing transpiration rate in the level with the highest zinc concentration in the soil (2.9 mmol H_20 m^{-2} s^{-1} at 900 mg kg^{-1} of zinc in the soil).

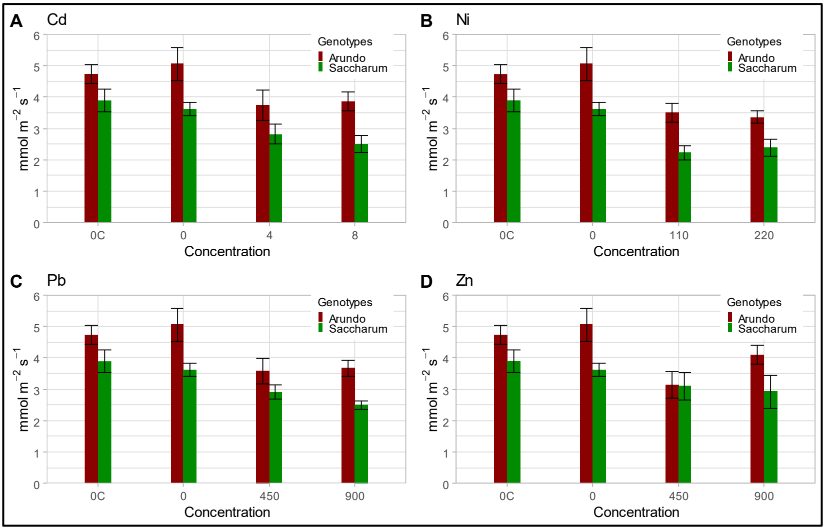

Figure 5.2. Leaf transpiration rate of *Arundo donax* L. and *Saccharum spontaneum* spp. *Aegyptiacum* in the different levels of contaminant (A: Cd, B: Ni, C: Pb; D: Zn) and control unfertilized (0) and control N fertilized (0C).

5.3.3 Stomatal conductance

The ANOVA showed significant differences concerning the stomatal conductance of the treatments under study (**Table 5.3**). The measurement date, the genotype, and the date x genotype interaction was significant in all the pollutants studied. The pollutant concentration and the first and second-order interactions did not show differences between the various pollutants. The stomatal conductance in both species and contaminants was similar to the transpiration rate and the photosynthesis rate, obtaining higher values in the control pots than in the contaminated pots for both species. Stomatal conductance was consistently lower in Saccharum due to the C4 photosynthetic cycle, as discussed in the transpiration rate (**Figure 5.3**). The trend of stomatal conductance in the Arundo showed higher values in the controls, in particular, was also higher in the non-fertilized control compared to the fertilized one (0.145 and 0.132 mol m^{-2} s^{-1}, respectively).

Table 5.3 ANOVA of the main factors and interactions for the stomatal conductance of *Arundo donax* L. and *Saccharum spontaneum* spp. *aegyptiacum* under increasing levels of pollutants in the soil (significance level at P≤0.05).

Stomatal conductance	Cd	Ni	Pb	Zn
Date (D)	0.000	0.000	0.000	0.003
Genotype (G)	0.000	0.000	0.000	0.030
Concentration (C)	0.649	0.769	0.395	0.414
D x G	0.000	0.004	0.007	0.034
D x C	0.669	0.882	0.345	0.653
G x C	0.397	0.817	0.691	0.501
D x G x C	0.086	0.737	0.265	0.989

The stomatal conductance in Arundo was higher in the pots with the higher levels of the metals, particularly in cadmium at 8 mg kg^{-1} and zinc at 900 mg kg^{-1}. Moreover, in the two levels of nickel and lead, the highest conductance values were recorded in the lower level of a contaminant, in particular at the concentration of 110 mg kg^{-1} of nickel in the soil (0.098 mol m^{-2} s^{-1}) and the concentration of 450 mg kg^{-1} of lead in the soil (0.091 mol m^{-2} s^{-1}).

The trend of stomatal conductance in Saccharum was higher in the controls, and the fertilized one showed a higher stomatal conductance than the non-fertilized controls (0.090 and 0.075 mol m^{-2} s^{-1}, respectively). In the pots contaminated by cadmium, lead, and zinc in relation to the two concentrations of heavy metal present in the soil, Saccharum showed higher stomatal conductance values in the low level of the heavy metal concentration. Except for the two levels of nickel concentration, where Saccharum does not show significant differences.

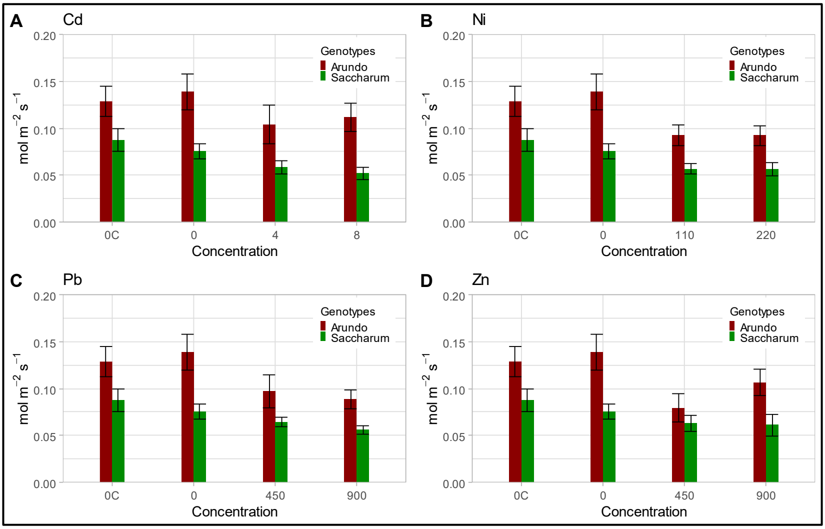

Figure 5.3. Stomatal conductance of *Arundo donax* L. and *Saccharum spontaneum* spp. *aegyptiacum* in the different levels of contaminant (A: Cd, B: Ni, C: Pb; D: Zn) and control unfertilized (0) and control N fertilized (0C).

5.3.4 Instantaneous water used efficiency

The ANOVA showed significant differences concerning the instantaneous water use efficiency (iWUE) of the treatments under study presented in **Table 5.4**. The measurement data was significant for Ni and Pb, the genotype for all pollutants. At the same time, the concentration and the interactions of the concentration with the other factors were insignificant. The data x genotype interactions showed differences only for Ni and Pb.

Table 5.4. ANOVA of the main factors and interactions for the instantaneous water use efficiency of *Arundo donax* and *Saccharum spontaneum* spp. aegyptiacum under increasing levels of pollutants in the soil (significance level at $P \leq 0.05$).

iWUE	Cd	Ni	Pb	Zn
Date (D)	0.506	0.000	0.000	0.291
Genotype (G)	0.001	0.000	0.000	0.000
Concentration (C)	0.926	0.115	0.463	0.377
D x G	0.974	0.005	0.003	0.913
D x C	0.758	0.795	0.88	0.469
G x C	0.163	0.251	0.467	0.640
D x G x C	0.495	0.32	0.342	0.431

The iWUE, which represents the ratio between the photosynthesis and the transpiration rate, expresses the micromoles of CO_2 absorbed on the mole of transpiration H_2O, showed lower levels in the controls compared to all polluted pots showed in **Figure 5.4**.

Among the species, Saccharum always showed higher iWUE values than Arundo, both in the controls and in the various levels of soil contaminant, by its C4 photosynthetic cycle. For cadmium, the highest iWUE value was obtained at the concentration of 8 mg kg^{-1} of soil in Saccharum, a value much higher than that of the controls (6.0 and 4.5 µmol CO_2 mmol H_2O, respectively). For Arundo, the higher value was obtained at the cadmium concentration equal to 4 mg kg^{-1} (4.0 µmol CO_2 mmol H_2O). For a nickel, the highest iWUE values were obtained in Saccharum, and in particular, the highest value was obtained at the nickel concentration equal to 110 mg kg^{-1}, followed by the concentration of 220 mg kg^{-1} of nickel in the soil (7.0 and 6.5 µmol CO_2 mmol H_2O, respectively). For Arundo, no significant differences were observed between the controls and metal concentrations in the soil (average 3.0 µmol CO_2 mmol H_2O). In the lead treatment, the higher values of iWUE were found in Saccharum at a concentration of 900 mg kg^{-1} of lead in the soil (5.8 µmol CO_2 mmol H_2O^{-1}), while for

Arundo, no significant differences were observed between study concentrations and controls (3.2 µmol CO_2 mmol H_2O^{-1}, on average). Finally, Saccharum and Arundo did not show significant differences for zinc between the different controls and the different contaminant levels, reaching average values of about 4.5 µmol CO_2 mmol H_2O^{-1} for Saccharum and 3.0 µmol CO_2 mmol H_2O $^{-1}$ for Arundo.

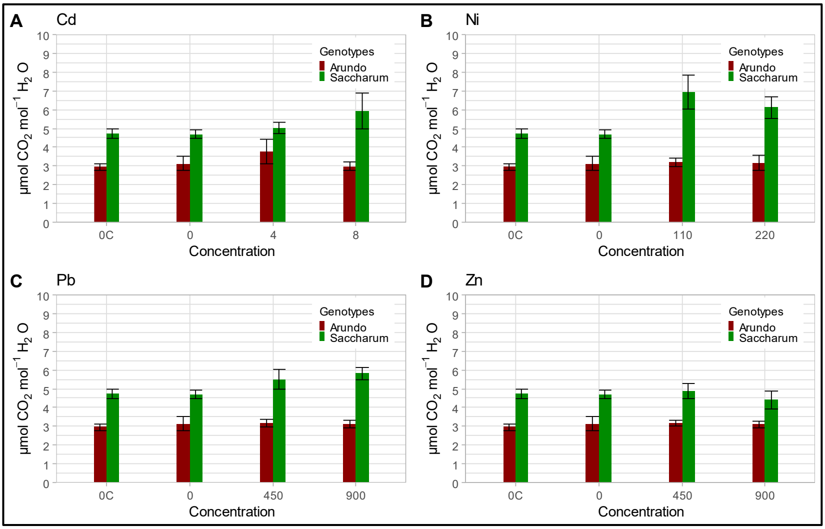

Figure 5.4. Instantaneous WUE of Arundo donax and Saccharum spontaneum spp. Aegyptiacum at different levels of contaminant (A: Cd, B: Ni, C: Pb; D: Zn) and control unfertilized (0) and control N fertilized (0C).

5.3.5 Mechanisms of resistance

The linear regressions between soil contaminants at different levels and the stomatal conductance of *Arundo donax* and *Saccharum spontaneum* spp. *aegyptiacum* was calculated on the average of the measurements made during the crop cycle and highlighted the different mechanisms of the species under study in response to heavy metal pollution of the soil (**Figure 5.5**).

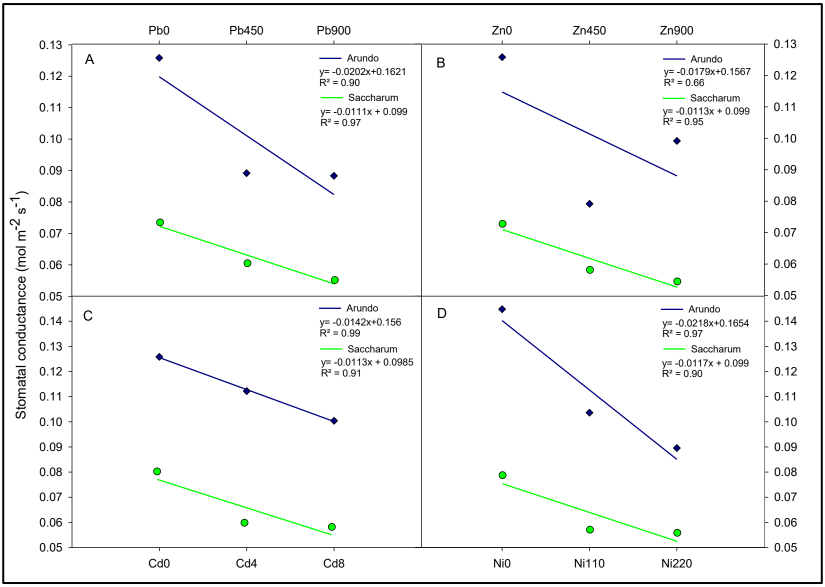

Figure 5.5. Linear regressions between contaminated soil and stomatal conductance (mol m^{-2} s^{-1}) in Arundo donax and Saccharum spontaneum spp. aegyptiacum in the different contamination levels (A: Cd, B: Ni, C: Pb, D: Zn).

In general, there was a linear and decreasing trend of stomatal conductance in all the linear regressions considered as the concentration of contaminated soil increased.

Saccharum showed a lower stomatal conductance in all relations than the Arundo, and the slope and intercept values of the linear relations were consistently lower, indicating a greater tolerance response. Considering the value of the slope, Arundo showed a decrease of 0.0202 mol m^{-2} s^{-1} with increasing Pb concentration with 90% adaptation of the estimated data compared to those observed, against a decrease of 0.0111 mol m^{-2} s^{-1} in Saccharum ($R^2 = 0.97$). In the soil contaminated with Zn, the Arundo showed a decrease of

0.0179 mol m^{-2} s^{-1} in stomatal conductance with increasing Zn concentration (R^2=0.66), against a decrease of 0.0113 mol m^{-2} s^{-1} were observed in Saccharum (R^2 = 0.95). In the case of soil contamination with Cd, the slope in Arundo reduced as the concentration of Cd increased (0.0142 mol m^{-2} s^{-1}) with an approximation of 99% and was relatively similar to Saccharum, which, however, recorded values similar to the previous regressions described (0.0113 mol m^{-2} s^{-1} and R^2 = 0.91). In the relationship between Ni concentration and stomatal conductance, the Saccharum slightly increased the slope value (0.0117 mol m^{-2} s^{-1}; R^2 = 0.90), while the Arundo showed the highest slope value as compared to the other regressions (0.0218 mol m^{-2} s^{-1}; R^2 = 0.97)

5.4 Discussion

Arundo donax L. and *Saccharum spontaneum* spp. *aegyptiacum* showed characteristics of physiological resistance in soils polluted by heavy metals (Cd, Pb, Ni, and Zn). Therefore, the development and the transition to the bio-economy sectors will mainly rely on the availability of sustainable biomass, in terms of yield per unit area and quality of raw material, on the competition for land, food, resources, and development of new biotechnologies (Cosentino et al. 2012). In this context, the crops studied could be suitable for supplying raw materials without competing for land, food, and resources with food crops and capable of enhancing land polluted by heavy metals that would otherwise be abandoned, with consequent risk for the surrounding ecosystem.

The studied species showed different physiological responses to soil contaminants. In particular, Saccharum spontaneum spp. aegyptiacum, a bioenergy grass with a photosynthetic cycle C4, showed higher photosynthesis a lower stomatal conductance and transpiration rates as compared to the C3 Arundo donax (Cosentino et al. 2016) and other C3 crops (Cosentino et al. 2013), thus showing an increased efficiency in the use of resources (mainly solar radiation and water available soil) in all conditions of heavy metals in the soil.

The linear regressions between the increasing levels of soil pollutants and the stomatal conductance provide an idea of how to cope with the stress adopted. In all the regressions considered, there was a linear and decreasing trend of the stomatal conductance when the concentration of contaminated soil increased, indicating that the plants gradually increased the contaminant, tending to close the stomata. Furthermore, the Saccharum showed in all the calculated regressions a lower stomatal conductance than the *Arundo* (highlighted by the lower values of the intercept), and in particular, a response contained in the reduction of the stomatal conductance while the concentration of soil pollutant increases (values lower than the slope), indicating a good ability to tolerate the experimental conditions imposed in the present work.

The *Arundo d.*, while undergoing greater stress in the higher level of pollutants in the soil, indicated by a greater reduction in stomatal conductance, continued to maintain a certain stomatal opening, thus ensuring normal gas exchanges with the atmosphere and consequently allowing to accumulate of dry matter in biomass thanks to the photosynthetic process (Cosentino et al. 2016; Cosentino et al. 2014) .

5.5 Conclusion

In conclusion, the present study highlighted the ability of the two-biomass species, no-food lignocellulosic perennial grasses, to tolerate the heavy metals in the soil and the physiological responses to increasing stress. The linear regressions between the increasing levels of soil pollutants and the stomatal conductance provide insight into the plant's stress condition. Stomatal conductance's linear and negative response to increasing soil contaminants indicated that the plants subject to increasing levels of soil pollutants suffer gradually increasing stress, tending to close the stomata. This is one mechanism of resistance that the plant adopted, showing the tolerance to the heavy metal of the two species. The smaller “b value” in all linear regressions suggests Saccharum is more tolerant than Arundo to increasing soil contaminants. However, physiological results must be confirmed when elaborating morphological and productive data.

5.6 Acknowledgements

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6 A comparison study on the phytomanagement potential of two lignocellulosic crops

6.1 Introduction

Agricultural land is a natural resource consisting of one of the most important indicators of economic growth. However, in the last decades, an inadequacy of available agricultural land has been observed due to rapid population growth, migration, land-use change, and abandonment (Akinci et al., 2013; Renwick et al., 2013; van Vliet et al., 2015). Besides, the increase in demand puts pressure on the food sector, requiring growth in productivity in an already limited agricultural area (Tramberend et al., 2019). Moreover, the expansion of the cities (Jiang et al., 2013) and soil contamination (Hou et al., 2020) are reducing the availability of agricultural land. In addition, the exploitation of agricultural land to produce biomasses for non-food purposes has become an issue. Indeed, using these soils to cultivate biomass to produce biofuels, bioenergy, or bioproducts should not be an option (Graham-Rowe, 2011) to not put food production at risk, which is critical to the development of society. Nevertheless, despite the competition with food crops, non-food crops play an essential role in transitioning to a more sustainable society. The versatility of non-food crops allows bioenergy and biofuel production, increasing energy security, allowing a fast response to the oscillations in demand, and serving as a source for different fuels and energy conversion techniques (Prasad et al., 2019). Moreover, non-food crops can also feed many industries, replacing fossil resources (need a reference here). Consequently, the cultivation, processing, and use of non-food crops impact the energy, transport, and industry sectors (Alper et al., 2020; Fernando et al., 2010; Hiloidhari et al., 2019). Furthermore, its production, either for bioenergy, biofuels, or biomaterials, offers environmental advantages by contributing to the reduction of greenhouse gases and energy savings, helping to combat climate change, and social benefits, especially in rural areas (Von Cossel et al., 2019). So, how can non-food crops be produced with limited impacts on land-use conflicts due to competition for food and feed? So far, several solutions have been presented to avoid the competition for agricultural land. One promising option is exploiting contaminated land to cultivate non-food crops releasing valuable fertile and healthy soils for food production (Evangelou et al., 2015; Barbosa et al., 2018; Rajendran et al., 2022). These lands are not viable for growing food due to the stressful conditions caused by the contamination, which also hamper the site's economic viability (Gomes et al., 2019). Besides, soil contamination gives rise to several environmental problems, such as desertification, water resources contamination, and food crops, which can lead to serious health problems for humans directly or indirectly. Cultivating non-food crops in contaminated soils and producing biomass may provide additional income for farmers. A decontamination action can also occur through phytoremediation, with all the associated benefits. Phytoremediation promotes soil decontamination by immobilizing the contaminants or extracting them from the land (Do Nascimento & Xing, 2006; Singh et al., 2020). In addition. The cultivation of industrial crops also promotes the increase of agricultural soils, collaborating with food security while providing a renewable feedstock to replace fossil resources.

Heavy metals deserve the spotlight among the contaminants capable of making a site unsuitable for food crops (Qin et al., 2021). Furthermore, the high concentration of heavy metals in soil represents a threat once these elements enter the food chain, causing different ecological problems and representing a risk to human health (Sall et al., 2020). Therefore, the cultivation of non-food crops in heavy metals contaminated soils represents an intelligent alternative to produce biomass for bioenergy, biofuels, and bioproducts and secure food production simultaneously.

This way, selecting the crops is essential to remediate the contaminated land and deliver an economically viable value chain. Moreover, the plants should tolerate contamination, maintaining similar productivity without losing quality (Pandey et al., 2016). Considering these characteristics, industrial crops represent an option due to their ability to resist stressful conditions, maintaining the yield while providing biomass for different applications needing a reference.

Understanding the effects of each heavy metal on non-food crops is necessary to select the most suitable crop for contaminated soil. Thus, the present work studies the effects of different heavy metals on perennial crop yield and biomass composition. The experiment followed the design addressed in Marginal Lands for Growing Industrial (MAGIC), E.U. – a funded project. The industrial crops were selected according to their potential to provide biomass suitable for different applications.

6.2 Material and Methods

The experiments were conducted in Italy at the University of Catania. Two lignocellulosic crops were evaluated for their phytoremediation potential, namely: giant reed (*Arundo donax* L.) and African fodder cane (*Saccharum spontaneum* L. spp. *aegyptiacum* Willd. Hackel) were grown as shown in Italy, Catania, University of Catania 37°31’ N, 15°04’ E, 75 m a.s.l.

The meteorological data during the cultivation periods is presented in **Figure 6.1**.

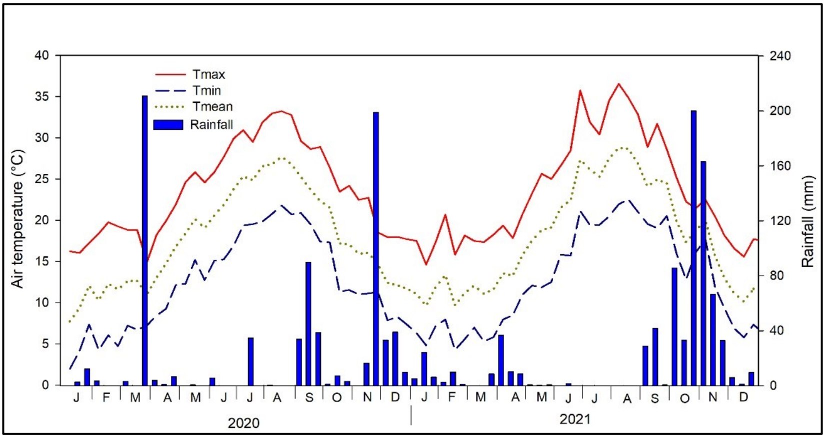

Figure 6.1. Meteorological data (from 1 January to 31 December, 2020 and 2021)

6.2.1 *Soil sampling and characterization*

The soil was collected from agricultural fields in Sicily, Italy, and taken from a depth of 30 cm. The soil was transferred to the experimental sites, homogenized, air-dried, and passed through a 2 cm sieve. According to the FAO soil protocol, selected soil physicochemical characteristics were determined, and presented 6.1. (Motsara & Roy, 2008). Particle size distribution was measured by the sieving and pipette methods. Soil pH and conductivity were determined in 1:1 soil/ distilled water suspensions after 1 hour with pH and conductivity electrodes.

Table 6.1. Soil physical and chemical characteristics per country

Physical characteristics	
Clay (%)	3.0
Silt (%)	4.1
Sand (%)	92.9
Texture	Sandy
Conductivity (μS/cm)	34.2
pH	7.4
Organic matter	0.86

6.2.2 Experimental setup and measurements

The research was carried out outdoors in pots with a capacity of 9.5 L, and each pot was filled with 10 kg of soil. Two months before sowing, the heavy metals Cd, Ni, Pb, and Zn were applied to the soil as water solutions of nitrate salts [$CdNO_3+4H_2O$, $Pb(NO_3)_2$, $Ni(NO_3)_2+6H_2O$, $Zn(NO_3)_2+6H_2O$] in order to achieve low and high treatments. Three different quantities of each metal nitrate salt were used to achieve the following final concentrations in the soil:

(i) Contamination with Cd: 0, 4, 8 mg kg^{-1}, referred to as Cd_0 (control), $Cd_{4,}$ and $Cd_{8,}$ respectively.

(ii) Contamination with Pb: 0, 450, 900 mg kg^{-1}, referred to as Pb_0 (control), $Pb_{450,}$ and $Pb_{900,}$ respectively.

(iii) Contamination with Ni: 0, 110, 220 mg $kg^{-1,}$ referred to as Ni_0 (control), $Ni_{110,}$ and $Ni_{220,}$ respectively.

(iv) Contamination with Zn: 0, 450, 900 mg kg^{-1}, referred to as Zn_0 (control), $Zn_{450,}$ and $Zn_{900,}$ respectively.

The experimental design was completely randomized, with three replicates for each treatment. After the germination of seed sown plants, a manual thinning was performed, leaving one plant per pot. Then, rhizomatous crops were propagated through rhizome cuttings, with 2-3 main buds each and rhizome weight as homogeneous as possible. Giant reed and African fodder cane rhizomes were collected from the germplasm collection at the Experimental farm of the University of Catania (37°24’N, 15°03’E, 10 m a.s.l.).

At the end of the experiment, growth measurements were recorded per pot: plant height, number of leaves, number of tillers, and fresh and dry weights. In addition, the moisture content (%) per plant was also calculated, as this is an important characteristic for the final uses of the produced biomass. Finally, the heavy metal content in the plant tissues was determined.

6.2.3 Plant and soil analysis

At harvest, the plants were subdivided into separate sections, i.e., shoots, leaves, and inflorescences. The sections were then thoroughly washed separately with tap water, weighed, oven dried (96 h at 60°C), and weighed again. Fresh and dry matter weights were recorded. All plant samples were ground using a cross-hammer beater mill and sieved with a 1 mm sieve. Determination of heavy metal content was carried out by means of atomic absorption spectroscopy (AAnalyst 200 AA Spectrometer, Perkin Elmer). Plants samples (1g dry weight) were digested with 10 ml HNO_3 solution. Then the samples were filtered through Sterile Mixed Cellulose Ester Filter with 0.2 um pore size. The sample volumes were adjusted to 50 mL using deionized water.

The soil samples were oven dried (96 h at 60 °C) and ground through a 2 mm sieve. Soil samples of 1 g (dry weight) were digested using the aqua regia method. Then the samples were filtered through Sterile Mixed Cellulose Ester Filter with 0.2 um pore size. The sample volumes were adjusted to 50 ml using deionized water.

To ensure the analysis's accuracy and precision, standard solutions of each metal were run before the samples in the atomic absorption spectroscopy .

6.2.4 Biomass analysis

Volatile material was calculated as the difference between the biomass weight and the residue obtained after the carbonization of the biomass at 900 ± 20°C for 7 minutes. (Chamberlain et al., 1974)

Ash content obtained from the residue obtained after the incineration of the biomass was obtained in the carbonization at a temperature of 550 ± 50 ºC for 3 hours—an adaptation of the ASTM D3172-13. (ASTM E1755 - (Reapproved 2015), 2015).

The fixed carbon was determined by subtracting the total dry biomass's volatile material and ash percentual. (Chamberlain et al., 1974).

The high heat value will be calculated using Yin's relation, which uses the fixed carbon and the volatile material to estimate the HHV (Yin., 2011).

The high heating value (HHV) expressed in $\mathrm{MJ.kg^{-1}}$ dry weight (d.w.) was calculated, taking into consideration both the volatile matter (VM) and the fixed carbon (FC), as given in Equation 1 (Yin., 2011):

$$\mathbf{HHV\ (MJ.kg^{-1,}\ d.w.) = 0.1905 \times [VM,\ \%\ w/w\ d.w.] + 0.2521 \times [FC,\ \%\ w/w\ d.w.]}$$

Equation 1

To evaluate the energy potential of each crop when submitted to thermochemical processes, the yield was taken into account, as observed in Equation 2:

$$\mathbf{Energy\ potential\ (MJ.ha^{-1}) = (HHV\ (MJ.kg^{-1})\ x\ Yield\ (g.m^{2}))\ x\ 10^{-1}}$$

Equation 2

6.2.5 Statistical Analysis

Statistical analysis was performed according to the experimental layout using R. As data followed a normal distribution, a one-way ANOVA for each treatment was performed to compare the growth and the ability of the two industrial crops as phytoremediators. In addition, the Tukey test was used to compare significantly different means at a confidence level of 95%.

6.3 Results

6.3.1 Plant growth response to heavy metals

Giant reed, it is a perennial, warm-season tall grass. The crop showed increased tolerance only under the Cd treatments for both years. Plant height, number of leaves, and number of tillers remained unaffected in Cd, and Pb-contaminated soil (**Figure 6.2** and **Figure 6.3**), while it was affected significantly in Ni_{220} and Zn_{900}. The dry weight of the biomass was significantly reduced by Pb_{900}, Ni_{220}, Zn_{450}, and Zn_{900} as compared with the control. In 2020, neither morphometric parameters nor moisture content was significantly affected by HMs treatments; on the other hand, fresh and dry weights were confirmed to be significantly affected by the highest concentration of Pb and Zn.

African fodder cane: it is a perennial, warm season pant of biomass. In 2019, Ni_{110} stems did not sprout from rhizomes in all three replications. This affected the means of the two years in morphological traits and biomass productivity. The number of tillers was mostly affected in Cd and Zn, while the plant height reduced in the highest concentration of Cd and Pb and the low concentration of Ni.

The total biomass yield, compared with the control, was affected by the high presence in the soil of Cd and Zn in the soil, with a reduction in productivity.

It is possible to observe that African fodder cane was higher than *Arundo donax* for all contaminated trials, also having a higher number of leaves. However, the number of tillers was similar for both crops. For *African fodder cane,* the height was affected by Cd contamination proportionally to the heavy metal concentration in the soil. For the Pb trial, the lower concentration had not affected the height f the plants. However, it was observed a reduction in the highest concentration. A different behavior was observed for Ni contamination, where the higher concentration had not affected the crop in terms of height, but the lower concentration of Ni reduced not only the height of the plant but also the number of leaves. For giant reed, the most affected trials were Ni and Zn in the highest concentration, showing a slight decrease in the number of leaves and a reduced number of tillers compared to the control.

Regarding the dry weight of the biomasses, the productivity was similar to both crops, showing that despite the African fodder cane having the highest plants, giant reed plants were more robust. The highest reduction in African fodder cane occurred in the high level of the Zn trial, mainly due to the reduction in the stems. Zinc high contamination showed a reduction around 40%. For giant reed, on the other hand, a slight reduction occurred in all contaminated trials (except in Cd lowest trial), affecting both stems and leaves.

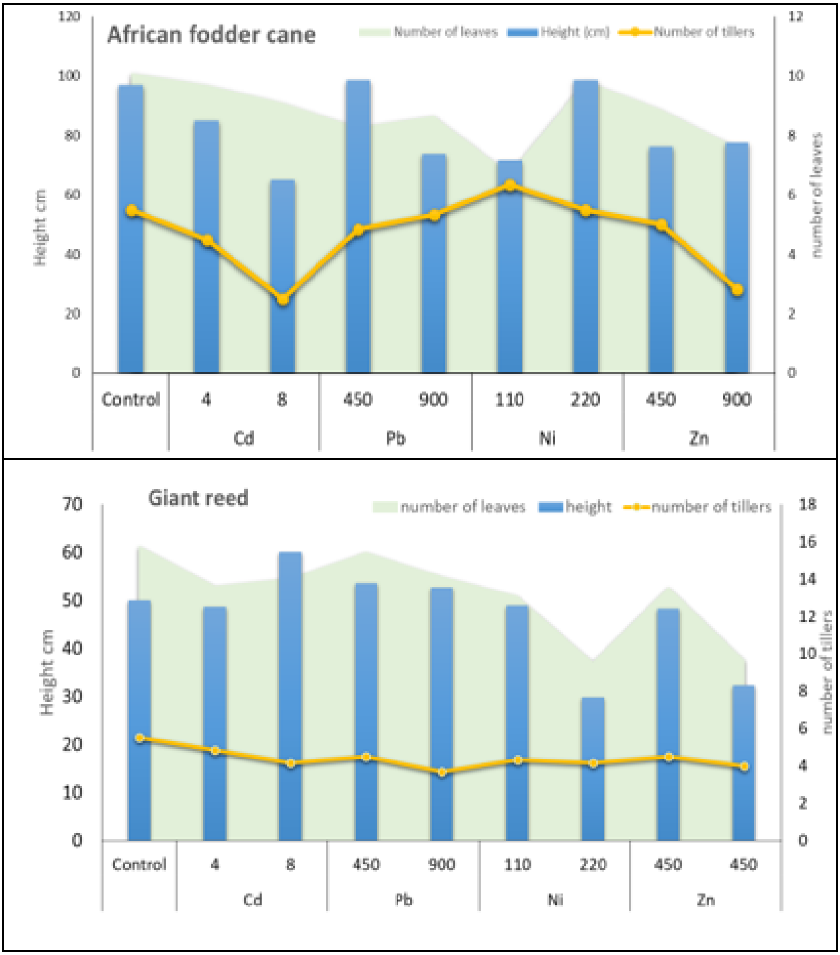

Figure 6.2. Plant height (cm), number of leaves, and number of tillers of the lignocellulosic crops subjected to different Cd, Ni, Pb, and Zn concentrations (mean values, n=3).

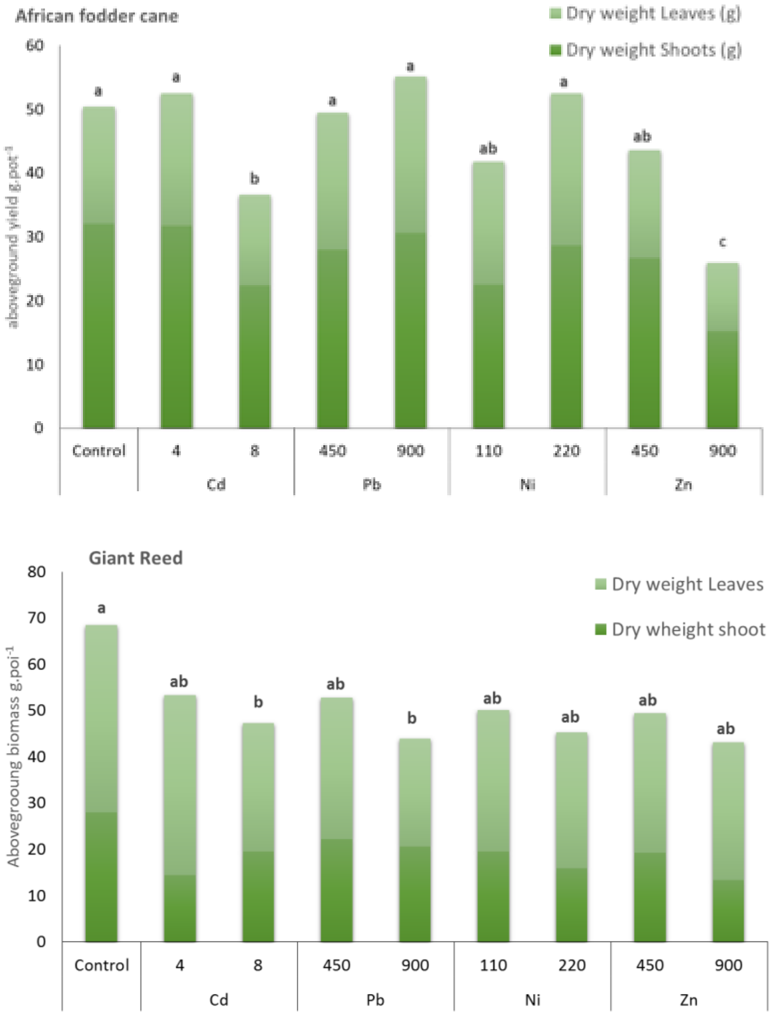

Figure 6.3. Plants' fresh and dry weights (g) and moisture content (%) of crops are affected by the heavy metal treatments.

6.3.2 The concentration of heavy metals (Cd, Ni, Pb, and Zn) in stems and leaves of the two lignocellulosic crops.

The accumulation of cadmium, lead, and nickel was similar for African fodder cane and giant reed; however, the accumulation of zinc was higher in African fodder cane, affected especially by the leaves accumulation potential, which was 50% higher in the lowest contamination and accumulated almost twice the amount of giant reed leaves in the highest concentration trial. The results are presented in **Figure 6.4** and **Figure 6.5**, for giant reed and African foddercane, respectively.

For zinc, lead, and cadmium, the accumulation in the stems is proportional to the concentration of heavy metals in the soil. The same behavior is observed for the leaves of cadmium and lead, while in the zinc trial, the concentration accumulation of the contaminant by the leaves increased with the presence of the heavy metal in soil but appeared to reach a limit, is not affected by the contamination level. On the other hand, in nickel contaminated soils, both stems and leaves absorbed the heavy metal. Therefore, the accumulation had not increased with the contaminant concentration in the soil. It is also important to indicate that the accumulation in giant reed for cadmium and nickel was higher in the leaves, while zinc and lead were slightly higher in the stems.

For African fodder cane, it was observed that for nickel, lead, and zinc, the accumulation potential of the crop is related to the concentration of the contaminant in the soil, while for cadmium, despite the presence of the contaminant, increased the accumulation in the crop, it reaches a limit, indicating that the plant is saturated. Furthermore, a higher accumulation was observed in the leaves for cadmium zinc and nickel, while a higher accumulation in the stems was observed for lead.

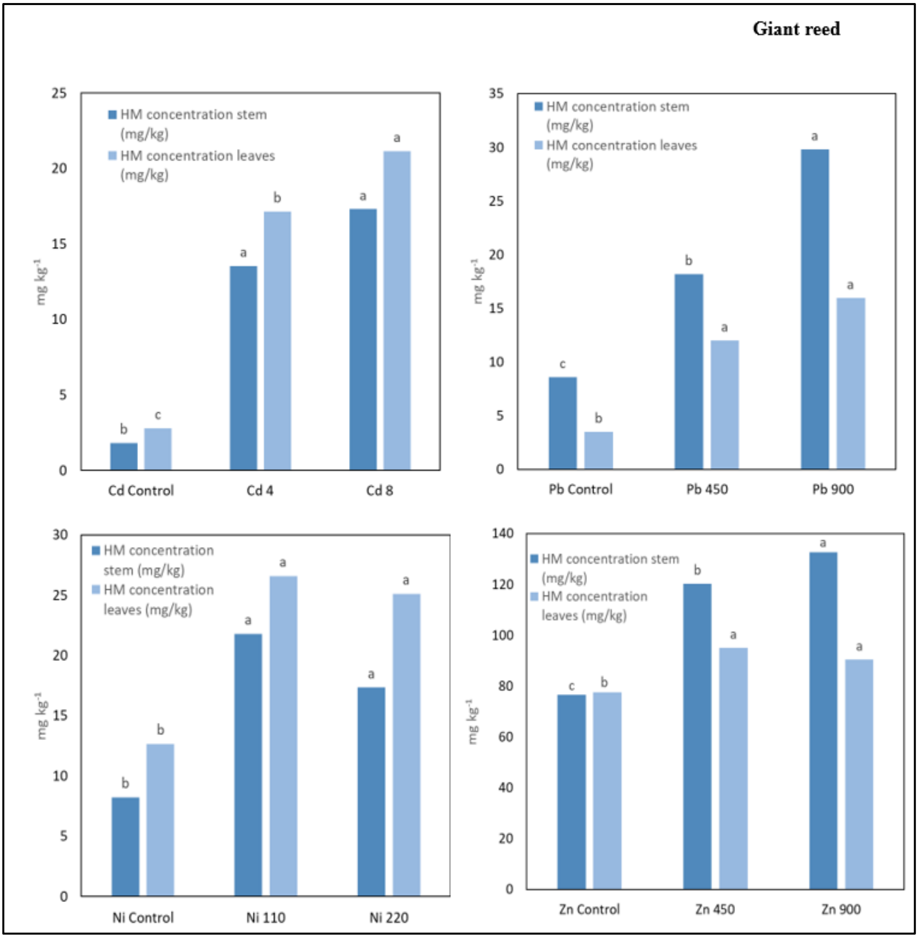

Figure 6.4. Heavy metal concentrations (mg kg^{-1}) in giant reed plants' aerial biomass.

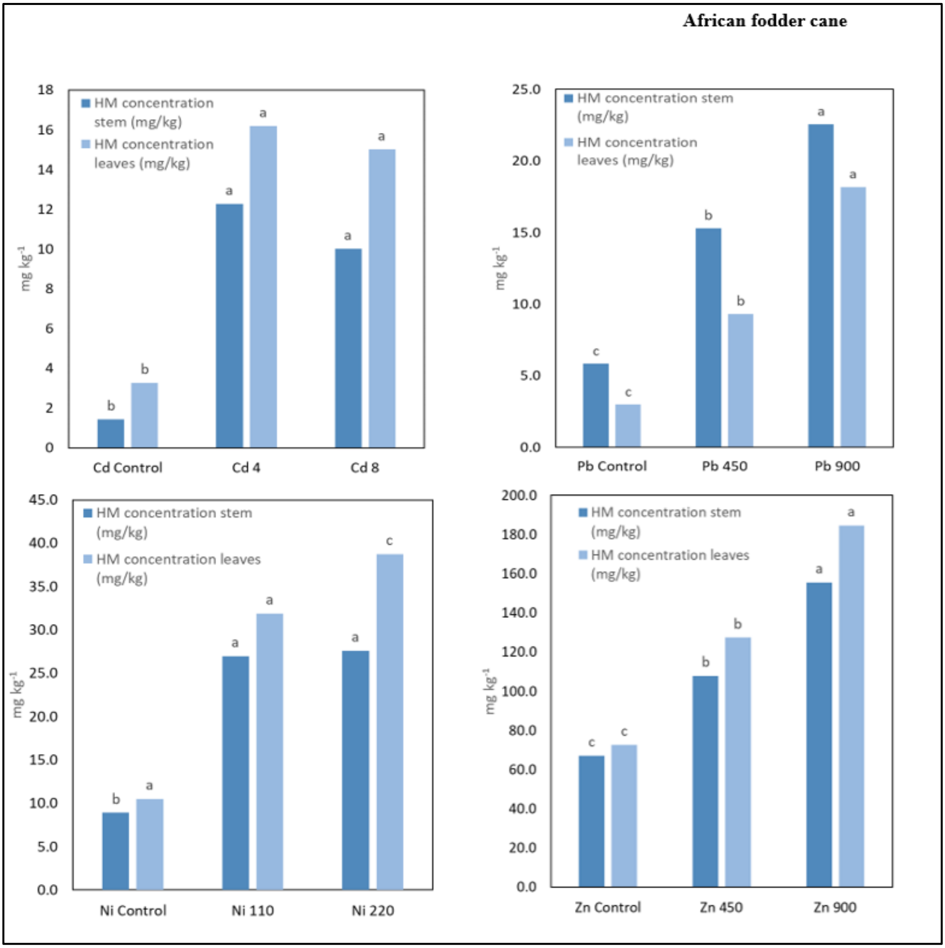

Figure 6.5. Heavy metal concentrations (mg kg^{-1}) in the aerial biomass of plants in African fodder cane.

6.3.3 Qualitative analysis of the fiber composition of the two lignocellulosic crops

Regarding the fiber composition of biomass, presented in **Figure 6.6**, a high value for cellulose was observed in both crops, indicating the potential for biological conversion pathways such as anaerobic digestion or fermentation due to the high sugar content, or even its use in the production of bioproducts such as cellulose and nanocellulose. Furthermore, the high amount of lignin can be used to produce bioproducts using lignin or nano-lignin or for energy in combustion, gasification and pyrolysis processes.

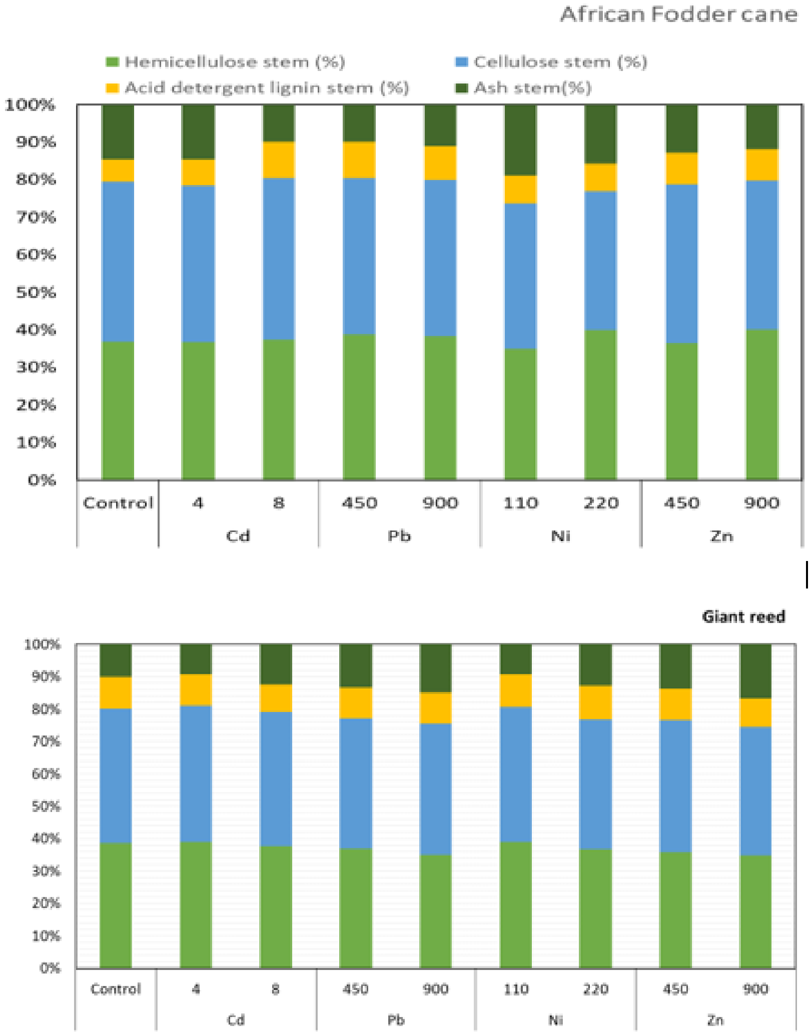

Figure 6.6 Qualitative analysis of the shoot biomass grew under heavy metal polluted soil

6.3.4 The energy potential of the biomass after the phytoremediation process

The energy potential of the biomass was calculated considering that the biomass was dried, palletised and used in a combined heat and power plant. The effects of plant contamination were calculated and simulated for a field trial. The high calorific value was calculated taking into account the measured ash content, volatile matter and fixed carbon. The energy potential of the two crops was similar in almost all trials, except for Ni_{220} and Cd_4, where African fodder cane showed a higher energy potential compared to giant reed. It was observed that zinc, nickel and cadmium were the contaminants that most affected the energy potential of the crops, but these reductions were caused by the low tolerance of the plants, which had a lower productivity. The HHV was not significantly affected in any of the trials, indicating that the tolerance of the plants to the contaminants is the factor to be considered in the energetic valorisation of the contaminated crops.

The energy potential for both crops can also be observed in the **Figure 6.7**, where the HHV of the biomass is analyzed with the productivity. This study showed that despite a reduction in the energy potential observed in some cases, the cultivation of both African fodder cane and a giant reed in heavy metal contaminated soils still must be considered. The highest reduction in the energy potential for both crops is observed in the highest concentration of Zn-contaminated soils. However, it is caused by the reduction in productivity, not HHV. Despite a lower potential in all trials compared to the Control, heavy metal contaminated biomass utilization also considers the ecological benefits of soil remediation, compensating for the energy gap.

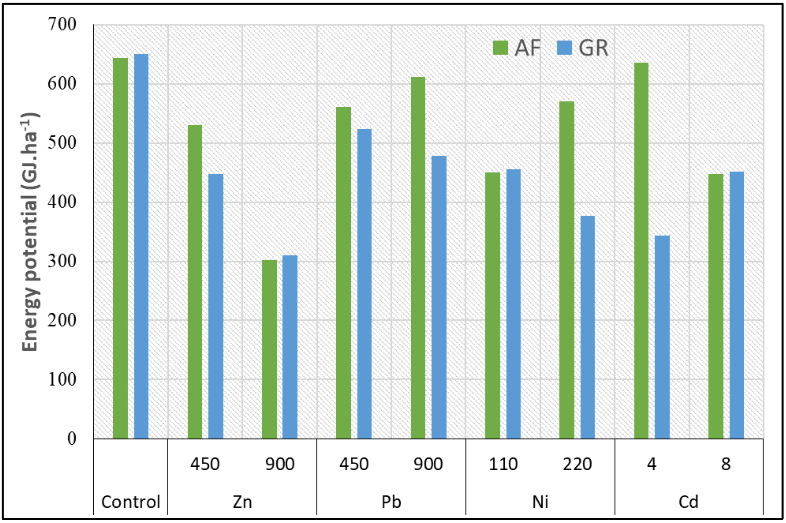

Figure 6.7 Energy potential of African fodder cane (AF) and giant reed (GR) after phytoremediation.

6.4 Discussion

African fodder cane (*Saccharum spontaneum* L. ssp. *aegyptiacum*) is a perennial crop thought to have originated in North Africa. However, Saccharum is widespread in tropical and subtropical regions of Asia, growing up to an altitude of 1800m. It is also common in southern Asia, eastern Africa and the Mediterranean region. Alternatively, african fodder cane is a fast-growing, widespread, high-yielding perennial suitable for second-generation bioethanol production. It is attractive as a non-food energy crop due to its high biomass yield, relatively high carbohydrate composition, perennial growth and ability to grow well on marginal and non-agricultural land. Oxalic acid pretreatment can release fermentable hydrolysate sugars from the hemicellulose and prepare the residual solids for enzymatic saccharification or simultaneous saccharification and fermentation of Saccharum to second generation bioethanol (Scordia et al. 2010).In this study, it was shown to be sensitive to cadmium contamination, affecting its height, number of tillers, number of leaves and stem and leaf weight, especially at the highest concentration where the effect of the contaminant is more intense. Despite the high effect of this heavy metal, the concentration of cadmium in African fodder cane stems and leaves increased compared to the control, but is not significantly affected by the concentration of the contaminant in the soil. The growth and heavy metal accumulation of sugarcane grown on artificially contaminated soil with different concentrations of Cd was also studied by Xia et al. (2009), who found that sugarcane with its large biomass has a high ability to tolerate and accumulate Cd. The feasibility of using edible sugarcane for rehabilitation of manganese mining sites was investigated and it was found that Cd and Pb concentrations in the edible parts of sugarcane were higher than the safety limits (Li et al. 2007).

Different behaviour was observed for giant reed, where the increase in cadmium in the soil reduces the number of tillers, but this is compensated by the increase in stem length. Giant reed is a C3 energy crop with the potential to produce large quantities of biomass. In addition to its agronomic potential, the chemical and energetic composition of this biomass meets EU market requirements for energy and advanced biofuels, paper and pulp, and construction materials. (Wang et al. 2019; Ashaal et al. 2014). The versatility of this crop allows it to be grown in soils with different stress conditions (Liu et al. 2017), such as salinity, pH, organic matter and nitrogen content, or heavy metal contamination in different concentrations, availability and bioavailability (Eid et al. 2016).

Considering its mechanisms to resist, tolerate, grow and remediate toxic metalliferous soils, giant reed could be considered as a suitable metalliferous pioneer plant for phytoremediation of heavy metal-contaminated soils (Papazoglou et al. 2007).

The weight of stems and leaves, in giant reed decreased in the cadmium-contaminated trials, showing that despite the increased height of the plants, the tillers were not as robust as in the control.The accumulation of cadmium in stems and leaves also increased compared to the control, but did not seem to be affected by the concentration of the contaminant in the soil, a possible explanation being that the plant has a concentration limit for the aerial accumulation of this heavy metal.

The presence of Pb contamination did not affect the number of tillers or the number of levels, but the be seen in the productivity, with the yield decreasing as the concentration level increases. The accumulation of lead in the biomass increased in both crops, but the accumulation in the stems stabilised with increasing concentration, whereas the accumulation in the leaves increased with increasing contamination.

In a previous restoration study, the biomass of giant reed seedlings in red mud and a mud-soil mixture (control) increased over time by

40.4% and 47.2%, respectively, and the concentrations of available Cd, Pb, Co, Ni and Fe in the soil all decreased at the same time (Wang et al. 2019). Soil Pb and Zn EDTA extractable fractions were also reduced by giant reed in a 2-year outdoor experiment to assess the potential of giant reed for phytoextraction and soil fertility restoration, confirming this ability of the crop to grow on polluted soils (Pilu et al. 2013). The addition of compost allowed the highest biomass production and consequently the highest metal uptake of giant reed (Fiorentino et al. 2017). Similarly, a study in Lisbon (Barbosa et al. 2015) tested the adaptability and phytoremediation capacity of giant reed and Miscanthus spp. on contaminated soil (under exposure of 450 and 900 mg kg^{-1} dry matter for Zn and Pb; 300 and 600 mg kg^{-1} dry matter for Cr), showing their suitability for phytoextraction and accumulation. In particular, the results confirm that bioaccumulation occurs mainly in the hypogeal part (i.e. rhizomes and roots), especially for Pb and Cr, while Zn is easily transported and accumulated in the aerial fraction. The application of soil amendments, including acetic acid, citric acid and ethylenediaminetetraacetic acid (EDTA), has also been shown to improve the growth and phytoremediation potential of giant reed (Fagnano et al. 2015).

Despite acting as a micro-nutrient in plants, the highest concentration of zinc drastically reduced the productivity of African fodder cane, while giant reed productivity was not affected by zinc concentration. However, in terms of biometric parameters, giant reed showed a reduction in height at the highest Zn contamination without significant changes in the number of tillers or leaves. African fodder cane, on the other hand, significantly reduced its number of leaves and tillers while maintaining its height. The effect of zinc on crop productivity can be related to its accumulation potential. In giant reed, the increase of the contaminant in the soil had not increased the concentration of heavy metals in plant tissues, thus indicating that a limited was reached, helping to mitigate the toxic effect of Zn in the highest concentrations, in African fodder cane the accumulation in plant tissues increased with the concentration of Zn in the soil, which could have made this crop more susceptible to the toxic effects.

Nickel did not significantly affect the productivity of African fodder cane, but it increased the length and number of leaves, slightly reduced the number of tillers. Regarding the accumulation potential, an increase was observed in the contaminated plots compared to the control, but the concentration of nickel in the soil did not affect the accumulation potential, which was slightly higher in the leaves. The same accumulation potential was observed for giant reed, where the presence of the contaminant also had no effect on the productivity of the crop. Regarding to the other biometric parameters, increasing the concentration of heavy metals in the soil significantly reduced the length of giant reed stems, but had no effect on the number of tillers or the number of leaves, indicating that giant reed at the highest concentration produced more robust plants. The accumulation of nickel in giant reed also showed no significant variation in either leaves or stems from the lowest to the highest concentration of Ni trials, however in both trials the accumulation of was higher than in the control. This behaviour may indicate that there is a limit to nickel accumulation in this crop.

Genetic differences have been reported among non-metallically contaminated giant reed populations. Therefore, the genetic diversity and differences between giant reed populations in heavy metal contaminated areas should be studied (Fiorentino et al. 2017). Therefore, it would be interesting to study the effect of heavy metal on the different giant reed populations, considering that they prove to be adaptable in this marginal land. Looking for energy crops that are well adapted to a specific climatic area and are able to use abiotic resources efficiently, grow with reduced agronomic inputs. Global climate change is predicted to increase the frequency and intensity of droughts in some geographical regions. In arid and semi-arid regions where evapotranspiration dominates, reduced water availability and drought duration are likely to increase (Cosentino et al. 2012; Stocker et al. 2013). Therefore, it is important to investigate drought-tolerant industrial crops for the Mediterranean region, able to grow in heavy metal contaminated soils, where water resources are limited (Scordia et al. 2017).

6.5 Conclusion

In conclusion, the present study highlighted the ability of the two-biomass species, no-food lignocellulosic perennial grasses, to tolerate the heavy metals in the soil and the physiological responses to increasing stress. On the other hand, Giant reed was not affected by heavy metal, while African fodder cane was slightly affected by Ni contamination in the soil. However, good quality of biomass was obtained, highlighting the possibility of these energy crops being used as feedstock to produce energy. Further studies could be focus on the transformation of this biomass into biofuel, in order to obtain high benefic in the remediation of polluted areas.

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7 General discussion and conclusions

Marginal lands are generally know as soil with low capacity to produce profits. This can be related to natural or anthropogenic causes such as the lack of water and nutrients in the soil, the morphology of the site, extreme weather conditions, soil, and contamination, due to natural events or human intervention (Mehmood et al., 2017). Cultivating food crops on contaminated marginal lands can represent a risk to animals and human health, especially when contaminants are present in the soil, such as heavy metals (Nalepa et al., 2012).

The cultivation of industrial crops in contaminated soils as a raw material becomes an essential point for providing the industry in the energy field the necessary amount to reach the growing demand while avoiding the competition for food land (European Union Parlament, 2018), and it reduces the environmental impacts caused by the presence of hazardous pollutants (Protásio et al., 2013). The valorization of the contaminated biomass must be through energy generation, which has increased rapidly in many countries since sustainability is the keystone for the future development strategy.

Attention is focused on biofuels, such as biogas, or bioethanol, produced from raw materials. Furthermore, using industrial crops in contaminated soil contributes primarily to decontamination, avoiding abandonment of these lands, reducing carbon emissions, and allowing to obtain valuable products to use in bioenergy production. Furthermore, studies in these directions with different biomass species help to add knowledge in phytoremediation techniques and improve the methodologies to valorize the contaminated biomass for industrial process.

This work aimed to evaluate better the tolarence and the phytoextraction ability of four industrial crops cultivated in heavy metal polluted soil to understand the impact assessment of the phytoremediation technique.

Among the lignocellulosic perennial grasses species, Giant reed and African fodder cane were tested seeking for the physiological responses, productivity and HMs concentration in the plants at increasing soil pollutants. The linear regressions between the increasing levels of soil pollutants and the stomatal conductance provide insight into the plant's stress condition. This relation showed linear and negative response at increasing soil contaminants indicated that the plants subject to different levels of soil pollutants suffer and tending to close the stomata. This is one mechanism of resistance that the plant adopted, showing the tolerance to the heavy metal of the two species. The smaller “b value” in all linear regressions suggests *Saccharum* is more tolerant than *Arundo* to increasing soil contaminants.

Industrial hemp showed an appreciable capability to grow until the seed matures in heavily industrial contaminated soils and unfavorable environmental conditions. The two varieties highlighted different phytoextraction capabilities and also translocate in different part of the plants the metals uptaked from the soils. Also safflower showed, in relation to the different heavy metals, different mechanisms to accumulate or stabilize the contaminant through the different fractions of biomass. However, exploring the data through phytoremediation index and factors, PCA analysis, and correlation matrix helped to understand the behavior of safflower in soil contaminated with Zn, Ni, Cd, and Pb. Leaves and stems yields showed the strongest negative correlation to the contaminant concentration, while the root yield was the least influenced by the heavy metal concentration, explaining that safflower can still store the heavy metals in the belowground biomass at raised concentrations of pollutants in the soil. This result was also confirmed by the accumulation index that increased with the level of heavy metal in the soil and from the translocation factor, which showed that safflower accumulates more contaminants in the belowground biomass, reducing its ability to transport to the aerial part of the plant.

The choise of the bioconversion process play a key role in the case of the use of these plants that contain heavy metals in order to avoid secondary pollution.

Since the methods that are used to recover HMs from plant biomass and/or the safe disposal of harvested plants are still limited, the production of these crops is actually concentrated in thermochemical conversion.

Therefore, biomass crops mean a very varied set of species that can produce thermal and/or electrical energy from thermochemical processes, biofuels (biodiesel and bioethanol), and biogas from biochemical processes. The most suitable type of bioconversion will depend on the quality of the biomass and its composition in terms of simple and complex sugars, lipids, proteins, structural carbohydrates, lignin, humidity, calorific value, C/N ratio, ashes, and minerals contained in it.

In polluted soil an alternative to feed and food crops could be the cultivation of industrial crops used for bioenergy production in order to generate an extra income source for agriculture, generating new job opportunities both in agriculture in industry and energy field while avoiding the competition for food land.

8 Annexes: Scientific Curriculum

8.1 Research and Professional experience

- **2019-2022: a Ph.D. course in Agricultural, Food, and Environmental Science**, University of Catania. Research project: “Mediterranean industrial crops for phytoremediation and bioenergy production in heavy metal polluted soil.” Under the supervision of Prof. Salvatore Luciano Cosentino and Prof. Giorgio Testa.
- **Mar 2019 - Sep 2019: Scholarship -** Department of Agriculture, Food and Environment, University of Catania, supervisor: Prof. Salvatore Luciano Cosentino. Research activities: Evaluation of the phytoremediation capacity of industrial herbaceous crops suitable for the Mediterranean environment.
- **Sep 2018 - Feb 2019: Scholarship -** Department of Agriculture, Food and Environment, University of Catania, supervisor: Prof. Salvatore Luciano Cosentino. Research activities: Evaluation of phytoremediation capacity of herbaceous biomass crops suitable for the Mediterranean environment.
- **Gen 2018 - Jun 2018: Postgraduate Internship -** Department of Agriculture, Food and Environment, University of Catania, supervisor: Prof. Giorgio Testa. Research activities: Carrying out analysis for the assessment of phytoremediation potential and estimation of the Phyto-extractive capacity of Mediterranean herbaceous crops

8.2 Education and Professional qualifications

- 2015-2017: MSc in Agricultural Biotechnology, University of Catania, LM 7 (magna cum laude), specialty on Biotecnologie delle colture erbacee. - University of Catania (Italy). Experimental thesis: “Phytoremediation of heavy metals polluted soil by industrial hemp.”
- 2011-2015: BSc in Herbal sciences and Nutraceutical products, L69 - the University of Catania, Catania (Italy). Thesis: “Herbal remedies for the treatment of cardiovascular diseases caused by cholesterol.”
- 2011: High School diploma: Istituto Tecnico Industriale statale Gallileo Ferraris, Acireale (CT).
- 2019: Qualification as Biology Doctor.

8.3 Memberships and IDs

- ORCID ID https://orcid.org/0000-0002-7857-7964

8.4 Scientific contributions

8.4.1 Published articles

- Ciaramella, B. R., Corinzia, S. A., Cosentino, S. L., & Testa, G. (2022). Phytoremediation of heavy metal contaminated soils using safflower. Agronomy, 12(10) doi:10.3390/agronomy12102302

8.4.2 In press, under review, and submitted articles

8.4.3 Conference Papers

- Piccitto A., Corinzia S. A., Scordia D., Calcagno S., Ciaramella B. R., Cosentino S. L., Testa G., 2020.Evaluation of the thermal pretreatment on the methanogenic potential of two lignocellulosic crops: A. Donax and S. Spontaneum. 28th European Biomass Conference and Exhibition (In press, Scopus indexed).

- Piccitto A., Corinzia S. A., Scordia D., Calcagno S., Ciaramella B. R., Patanè C., Cosentino S. L., Testa G., 2020. Biomethane potential of an old plantation of giant reed genotypes with two irrigation levels. 28th European Biomass Conference and Exhibition (In press, Scopus indexed).
- Scordia D., Calcagno S., Testa G., Copani V., Corinzia A., Piccitto A., Ciaramella B.R., Patanè C., Cosentino S.L., 2019. Biomass yield, water use efficiency, energy content, and energy return on investment of diverse perennial grasses in autumn and winter harvest regimes in the Mediterranean area. 27th European Biomass Conference and Exhibition, 27-30 May 2019, Lisbon (Portugal).
- Scordia, D., Scandurra, A., D'accorso, G., Corinzia, S. A., Testa, G., Ciaramella, B. R., Cosentino, S. L. (2022). Seed yield of brassica carinata lines in low and high organic fertilization levels. Paper presented at the European Biomass Conference and Exhibition Proceedings, 120-124.
- Ciaramella, B. R., Crapio, E., Scordia, D., Cosentino, S. L., Patanè, C., & Testa, G. (2022). Adaptability of safflower in heavy metal polluted soil. Paper presented at the European Biomass Conference and Exhibition Proceedings, 183-186.
- Viegas, C., Longo, A., Pires, J., Gomes, L., Ciaramella, R., Testa, G., Gonçalves, M. (2022). Bioremediation of effluents from biomass fractionation using microalgae:

a circular economy approach. Paper presented at the European Biomass Conference and Exhibition Proceedings, 1099-1103.

- Pires, J. R. A., Gomes, L. A., Pinheiro, J., Ventura, M., Ciaramella, R., Costa, J., Fernando, A. L. (2022). Characterization of residual liquors from lignocellulosic biomass fractionation and its exploitation for biomass production – closing the loop and contributing to the circular economy. Paper presented at the European Biomass Conference and Exhibition Proceedings, 406-410.
- Ventura, M., Gomes, L., Pires, J. R. A., Pinheiro, J., Ciaramella, R., Costa, J., Lapa, N. (2022). Anaerobic co-digestion of residual liquors from lignocellulosic biomass fractionation with a synthetic food waste for biogas production. Paper presented at the European Biomass Conference and Exhibition Proceedings, 773-777.

8.4.4 Conference Proceedings

- Ciaramella B.R., Corinzia S.A., Scordia D., Patanè C., Cosentino S. L., Testa G. Tolerance of Giant Reed To The Cultivation In Heavy Metal Polluted Soil. Società Italiana di Agronomia 50° Convegno Nazionale (SIA) dal titolo "Evoluzione dei sistemi agronomici in risposta alle sfide globali" 15-17 settembre 2021, Udine.
- Corinzia S.A., Ciaramella B.R., Piccitto A., Testa G., Patanè C., Cosentino S.L., Scordia D. Yield of lignocellulosic perennial grasses under different soil water availability Società Italiana di Agronomia 50° Convegno Nazionale (SIA) dal titolo "Evoluzione dei

sistemi agronomici in risposta alle sfide globali” 15-17 settembre 2021, Udine.

- Scordia D., Corinzia S.A., Piccitto A., Ciaramella B.R., Calcagno S., Testa G., Patanè C., Cosentino S.L. First harvest results of reduced and well-watered perennial grass clones, species and hybrids. Società Italiana di Agronomia 50° Convegno Nazionale (SIA) dal titolo “Evoluzione dei sistemi agronomici in risposta alle sfide globali” 15-17 settembre 2021, Udine.
- Ciaramella B. R., Corinzia S.A., Piccitto A., Scordia D., Calcagno S., Cosentino S. L., Testa G. Adaptability of Industrial Hemp to Increasing Level of Heavy Metals in Soil. XLIX Convegno della Società Italiana di Agronomia,16-18 settembre 2020.
- Corinzia S.A., Ciaramella B. R., Calcagno S., Scordia D., Piccitto A., Patanè C., Cosentino S. L., Testa G. Evaluation of Monoecious and Dioecious Hemp Genotypes in Southern Italy. XLIX Convegno della Società Italiana di Agronomia,16-18 settembre 2020.
- Testa G., Piccitto A., CorinziaS.A., Ciaramella B.R., Calcagno S., Scordia D., Cosentino S.L., 2019. Evaluation Of The Methanogenic Potential Of Lignocellulosic Crops Cultivated In Mediterranean Environment. XLVIII Convegno della Società Italiana di Agronomia, 18-20 settembre 2019, Perugia (Italia).
- Patanè C., Pellegrino A., Cosentino S.L., Ciaramella B.R., Scordia D., Testa G., 2019. Allelopathic Effects of Cannabis sativa L. Leaf Extracts on Durum Wheat and Barley Seed Germination. XLVIII Convegno della Società Italiana di Agronomia, 18-20 settembre 2019, Perugia (Italia).

- Testa G., Ciaramella B.R., Corinzia S.A., Scordia D., Patanè C., Cosentino S.L.,2019. Evaluation Of Different Hemp Genotypes In Mediterranean Environment. XLVIII Convegno della Società Italiana di Agronomia, 18-20 settembre 2019, Perugia (Italia).

8.5 Training in national or international universities and/or research institutions

Barbara Rachele Ciaramella is involved in a collaboration between University of Catania and National Center of Research to evaluate the CBD content in different hemp genotypes growing in the semi-arid Mediterranean environment. The inflorescence analysis of different hemp varieties was carried out, in the period March-April 2021, at the laboratory of Istituto di Chimica Biomolecolare – CNR (Catania). The inflorescences were collected at the experimental farm of the University of Catania, during the different phenological stage of the plant. The analysis was performed using HPLC (High-Performance Liquid Chromatography) technique.

Barbara Rachele Ciaramella missioned at the Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa (FCT/UNL), Portugal.The work conducted at FCT/UNL is in the framework of a closed collaboration between both universities (Universidade NOVA de Lisboa and University of Catania).

In the context of her activities, developed skills in the area of Energy Crops, Phytoremediation and Sustainability Issues. She also developed analytical skills in laboratory, namely regarding biomass analysis (total nitrogen, total phosphorus, ash content, metals content). During her stay in Lisboa, Barbara Rachele Ciaramella also contributed to the MAGIC project, coordinated in Portugal.

8.6 Attended Congresses / Workshops / Meetings

- “4th Joint Meeting of Agriculture-oriented Ph.D. Programs UniCT, UniFG and UniUd”, 3-7 October 2022, Paluzza, Udine (Italy).
- “2nd Joint Meeting of Agriculture-oriented Ph.D. Programs UniCT, UniFG and UniUd”, 14-16 September 2020, online.
- Final PANACEA Event' webinar organized by Panacea Project Team on February 22nd, 2021
- Annual and technical meetings of the Horizon 2020 project entitled “Marginal lands for growing industrial crops: turning a burden into an opportunity – MAGIC” meeting, virtual, 30th June 2021
- 7th Central European Biomass Conference, 18th to 20th of January 2023, Graz, Austria

8.7 Participation in research projects

- Collaboration in MAGIC project.
- Collaboration in WIRE COST action.

8.7.1 Attended Courses

- **“CAD, GIS, and participatory mapping Course,”** Prof. Teresa Graziano and Prof. Francesca Valenti, 8-18 February 2021 (45 hrs). Main topics: CAD Course and ICT for Participatory Mapping & Disseminating. Department of Agriculture, Food and Environment, University of Catania, Italy.
- **“Biometry and data analysis - Basic statistics with applications in R”** Dr. Corrado Dimauro, 18-22

November 2019 (40 hrs), Department of Agriculture, Food and Environment, University of Catania, Italy.

- **'R Programming** - Johns Hopkins University' training course by Coursera concluded on September 6th, 2021.
- **"Corso di Metodologia Statistica per le Scienze Agrarie «Dario Sacco»: I modelli lineari generali e generalizzati"** organized by Società Italiana di Agronomia (SIA), 2nd – 11th febbraio 2021
- **"B2 Level English Test"** OLS language test for Erasmus traineeship student, taken on 09/11/2021

8.7.2 Teaching activities

- Collaboration in didactic and research activities of the Section of "Agronomia generale e coltivazioni erbacee" (Di3A, University of Catania).
- Laboratory Training in phytoremediation

8.7.3 Thesis mentoring

Luigi Schillaci, MS thesis on the "Adaptability of industrial hemp to grow at raising level of cadmium and lead in soil" master's degree in Agricultural Biotechnology