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Production of biochar from olive mill waste and remediation of heavy metal contaminated soil

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Dedication

It is my pleasure to dedicate this modest work

To the soul of my beloved mother …

To my father with my great appreciation …

To my brother and sister…

To Anni.

Amine

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Chapter 1

Introduction

1. Introduction

Climate change imposes to our world one of the most systemic problems and opens challenge we are facing today. Carbon dioxide (CO_2), methane (CH_4) and nitrous oxides (NO_x) released from burning fossil fuels, as well as decomposition of biomass and organic matter above and below ground, are the main causes of the anthropogenic greenhouse effect. Besides, agricultural activity contributes to these emissions from a variety of management practices such as intensive tillage, use of fertilizers, livestock production, etc. In some areas, agriculture and intensive tillage have caused a loss in organic C from 30 to 50% in agricultural soils in the last 100 years (Lehmann *et al.*, 2006; Lee *et al.*, 2010).

Population growth and intensive agricultural production are among the major reasons behind the rapid increase in volume and types of wastes generation. In a worldwide scale, around 140 billion metric tons of wastes per year are generated from agriculture (UNEP, 2009). The improper management of agricultural wastes contributes also to the emission of greenhouse gases. Annually huge quantities of agricultural wastes are burned in open fields. This practice results in a marginal and short term increase in soil fertility and in an immediate release of biomass organic C as CO_2 (Neto *et al.*, 2009). In contrast to combustion, more rational organic biomass residues management is assured through composting, anaerobic digestion, fermentation etc. which are all ways to immobilize part of the organic C into stable forms.

In the aim of reducing emissions of anthropogenic greenhouse gases, agricultural organic residues, still thought as waste, should be considered as a valuable source of organic carbon, renewable energy, a tool for carbon sequestration and, consequently, as a help in reversing the global warming process. In response to this complex problem, Lehmann *et al.* (2006) found that converting biomass into biochar can lead to sequestration of around 50% of the biomass carbon content. The biochar production through pyrolysis and its incorporation into soil can establish a long term sink for CO_2 sequestration and be a significant approach for global carbon capture (Dominic *et al.*, 2010; Lee *et al.*, 2010). Pyrolysis is defined as “The heating of a biomass in absence or under limited availability of oxygen produces a thermal decomposition of the organic material”. The final products of this carbon-negative process are: a solid residue rich in carbon (char or biochar), a gaseous mixture of hydrogen, methane and monoxide carbon (syngas) and a liquid phase (tar or bio-oil) (Basu, 2010). Beside reducing emissions and increasing sequestration of greenhouse gases, soil addition of biochar can improve soil fertility by maintaining or

increasing stable soil organic C pools and bringing nutrients back into agricultural fields. Through this process carbon sequestration, agricultural productivity and environmental quality can be sustained and improved if the biomass is transferred into inactive stable carbon pool and incorporated into agricultural soils (Lehmann *et al.*, 2006; Lehmann, 2007; Gaskin *et al.*, 2008). Biochar was identified in ancient soils of the Amazon basin, known as *Terra preta*. These dark, C-rich soils have high agricultural productivity in an area characterized by nutrient-poor soils (Lehmann, 2007). Biochar is a porous carbonaceous solid with physiochemical properties suitable for the safe and long-term storage of carbon in the environment and for soil improvement (Shackley and Sohi, 2011). It is estimated that diverting as little as 1% of net annual plant uptake into biochar would mitigate almost 10% of current anthropogenic C emissions (Lehmann and Joseph, 2009). Biochar has been shown to benefit crop growth and yield, and it is a promising soil amendment. However, as it is the case for any other soil amendment, its production, properties and efficiency must be well understood coupled with targeted uses and applied to a variety of cropping systems under definite optimal application rates. The biochar chemical and physical characteristics are highly affected by the choice of the feedstock, its properties and the pyrolysis process conditions basically the pyrolysis temperature, the heating rate and the residence time of the feedstock (Antal and Grønli, 2003; Cetin *et al.*, 2004; Angın, 2013). All these properties have an effect on the biochar interactions within the environment.

A wide range of different kind of biomasses can be used as feedstock for pyrolysis to produce biochar. In the Mediterranean region in general, and in Italy in particular, one of the most interesting candidates for biochar production is the waste produced by the olive mills. The olive oil industry is one of the most heavily polluting ones. Olive-mill waste, the byproduct of the olive oil production, which is a very important economic activity, particularly for Spain, Italy and Greece, causes a relevant environmental problem (Arvanitoyannis and Kassaveti, 2007; Morillo *et al.*, 2009). The treatment of such residues, characterized by high content in organic matter, mainly phenolic compounds, and high salt concentration, is limited by technical and economical constraints and by the scattered pattern of olive mills location in the concerned regions. Pyrolysis might offer efficient recycle of this phyto-toxic biomass. This biomass conversion technology represents a valuable tool to

(1) Manage the olive mill solid waste, reducing its impact on the environment;

(2) Produce biochar which sequester relevant quantity of C;

(3) reduce the emission of greenhouse gases;

(4) Produce a soil amendment in the aim of improving soil physical chemical and biological properties and can be used in the remediation of heavy metal contaminated soils.

2. Objectives and scope

The main objective of this research work was to produce biochar from solid olive mill waste through slow pyrolysis under different process conditions and to valorize its use as soil amendment to remediate heavy metal contaminated soil. In order to achieve this objective the following tasks were carried out:

1. Production of biochar from solid olive mill waste through slow pyrolysis under different temperatures and heating rates.
2. Chemical and physical characterization of the produced biochar.
3. Evaluation of the effect of biochar amendment in reducing nickel content uptake and translocation in tomato grown on perlite under controlled conditions.
4. Evaluation of the capacity of biochar in remediating a zinc smelter contaminated soil.

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Chapter 2

Literature review

1. Olive, olive oil and mill waste

1.1. Olive and olive oil production

The olive tree belongs to the family *Oleaceae* and it is an endemic plant of the Mediterranean region. The productive olive trees occupy a total surface of about 9.634.576 ha worldwide, with a total production of olives of 19.845.300 Mg (FAOSTAT, 2011). In the Mediterranean basin the olive cultivation is an important local economic and cultural heritage with a valuable rural economic activity and represents one of the main pillars of the agricultural sector. The countries of the Mediterranean region provide for more than 90% of both the total cultivated surface area of olive tree and the total olive oil production (Michael and Constantinos, 2005). Spain is the world leader in the sector. Its total olive trees crop surface is of 2.330.400 ha and its olive production is of 6.940.230 Mg. Italy comes second with 1.144.420 ha cultivated and 3.182.200 tons of olives produced, followed by Greece with a cultivated surface of 850.000 ha and an olive production of 2.000.000 Mg. Turkey, Morocco, Syria and Tunisia produce respectively 1.750.000, 1.364.690, 1.095.040 and 863.000 Mg of olives (FAOSTAT, 2011).

Regarding the olive oil, the total world production related to the harvesting year 2010/2011 was 3.075.000 Mg. The European Union (EU) provided the highest production of olive oil with a percentage of 71.8% (2.209.100 tons) of the world production in 2010/2011. The statistics of olive oil production (IOC, 2013) for the harvesting years 2011/2012 and the estimations for 2012/2013 are reported in the table 1 (IOC, 2013).

Table 1: Olive oil production in different countries

Country	2009/2010	2010/2011	2011/2012*	2012/2013**
Spain	1401.5	1391.9	1613.4	820
Greece	320	301	295	350
Italy	430	440	450	490
Portugal	62.5	62.9	76.2	68.6
Morocco	140	130	120	100
Syria	150	180	198	198
Tunisia	150	120	180	220
Total EU	2224.5	2209	2444	1739
Total world	2973.5	3075	3408.5	2718

*** Provisional**
****Estimated**

1.2. Olive-mill wastes production and characteristics

In the Mediterranean basin, olive oil production is one of the oldest agricultural activities with a great importance from both the economical and social perspective. This industry, even being seasonal, generates a huge quantity of bio-toxic wastes with high organic load, in a short period of time, usually 3 months (André *et al.*, 2005; Morillo *et al.*, 2009; Georgia O. *et al.*, 2010). This waste is composed by a solid component (pomace) and a liquid part (olive mill wastewater) (Morillo *et al.*, 2009). Olive-mill waste is composed mainly of sugars, volatile acids, polyalcohols, pectins, nitrogenous compounds, fats and polyphenols. Such a mixture is known to be highly polluting, phytotoxic and resistant to biological degradation. The disposal of the olive mill waste is particularly environmental concern due to the potential threat for the fauna, flora, soil and water of the dumped areas (Schieber *et al.*, 2001; Roig *et al.*, 2006).

The traditional extraction process, known as the pressing process, is not used anymore because of its low efficiency, the high quantity of liquid waste produced and the environmental legislation, in force in the concerned countries, which forbids its use (André *et al.*, 2005). Olive oil industry moved then from the traditional pressing system to a continuous system using horizontal centrifuges, called 3-phase decanters, reducing this way the amount of mills wastewater. Starting from the nineties, a further step towards the increasing of efficiency and the reduction of liquid waste was made by implementing the 2-phase extraction system. The 2-phase continuous process recycles the vegetation water and produces more solid waste and less liquid effluent (Tab.2) (André *et al.*, 2005; Lafka *et al.*, 2011).

Table 2 : Solid and liquid wastes produced by the 2- and 3-phase olive oil extraction systems

Waste	2-Phase system	3-Phase system
Pomace (%)	82.5	47.8
Wastewater (%)	14.5	49.5

Approximate values from Altieri (2007)

The pomace generated can be classified depending on the extraction system, as follows:

- Press pomace: moisture content 25 to 35% and oil content 4 to 7%.
- 3-phase pomace: moisture 45% and oil content 2-3%.
- 2-phase pomace: 70% of moisture and 2-3% of oil content.

This waste can undergo a secondary extraction after being dried to extract the remaining oil, with the use of solvents, and the water is lost as vapor into the atmosphere. The residual oil, commercialized under the name of olive-pomace oil, is

characterized by a low quality. Its demand is in continuous decrease and leads to the shut-off of the extraction plants (Nasini *et al.*, 2013).

1.3. Olive mill waste management

The management (treatment or disposal) of olive mill wastes is one of the most important environmental problems in the Mediterranean region (Morillo *et al.*, 2009). The current environmental legislation in force in Italy compels the treatment of liquid olive mill residues before its release into the environment. Considering that the high cost of liquid waste treatment could have an economic negative impact on the olive oil sector, new additional rules have been enacted (Regulation No 574 of 1996 and Ministry Decree of 6th July 2005) allowing land disposal of this waste under controlled conditions (Azbar *et al.*, 2004). These laws allow respectively a disposal of 50m^3 and 80m^3 of liquid wastes per hectare in case of discontinuous and continuous oil extraction processes, respectively. No specific limits are set for the use of pomace as soil amendments, in case of pomace characteristics compliance to a specific law enacted to regulate the use of soil amendments.

This direct land application offers a simple and economically feasible opportunity. Several studies reveal that olive mill wastes might be a promoting source of plant nutrients, can increase soil organic matter, total and soluble nitrogen and available phosphorus (Azbar *et al.*, 2004; Rodríguez-Lucena *et al.*, 2009; Lozano-García *et al.*, 2011). On the other hand, several authors point out number of agronomical and environmental deteriorations caused by the acidic pH, the high content of phytotoxic and anti-microbial agents (tannins, fatty acids, phenols, mineral salts content, etc.) of this waste, leading to higher mobility of heavy metals and lixiviation of nitrate and sodium into deep soil (Kavdir and Killi, 2008; Mechri *et al.*, 2009).

In Spain around the eighties the government economically encouraged the construction of storage ponds intended for evaporation of the olive mill waste during the summer period. This technique gave rise to important odor problems and air pollution (Azbar *et al.*, 2004). Later on this waste was mainly used to produce biogas, to recover useful chemicals, to produce compost, but large amounts of it are still evaporated in ponds. Both evaporation and sedimentation systems concentrate the semi-solid wastes by 70-75%, but in both systems there are emissions of odors and the remaining sludge still require further treatment (Federici *et al.*, 2009; Zaharaki and Komnitsas, 2009). From another perspective, evaporation ponds, inverse osmosis, ultra-filtration and thermal concentration are facing technical and economical limitations which impair their efficiency (André *et al.*, 2005; Hanifi and Hadrami, 2009).

Technical and economical limitations, together with the scattered pattern of olive mills location across the Mediterranean basin, make the waste treatment strategies difficult. In addition, all the techniques currently used, such as evaporation ponds, thermal concentration, composting, aerobic and anaerobic digestion and

combustion are coupled with the emissions of greenhouse gases (GHG) mainly CO_2 and CH_4.

2. Pyrolysis

2.1. Definition

Pyrolysis is defined as a thermal decomposition of organic matrix in total absence of oxygen. The pyrolysis temperature, being maintained for a sufficient time, allows the transformation of the feed in a host of useful products of solid, liquid and gaseous nature. The word 'pyrolysis' derives from the Greek words 'pyro' meaning fire and 'lysis' meaning decomposition or breaking down into constituent parts (Yaman, 2004; Decker *et al.*, 2007; Basu, 2010; Verheijen *et al.*, 2010). Laird *et al*., (2009) define pyrolysis as 'a thermo-chemical process that can be used to transform low-energy density organic matter (~1.5 GJ m^{-3}) into a high-energy-density liquid known as bio-oil (~22 GJ m^{-3} or ~17 MJ kg^{-1}), a high-energy-density solid known as biochar (~18 MJ kg^{-1}), and a relatively low-energy- density gas known as syngas (~6 MJ kg^{-1})'.

2.2. Pyrolysis process

The reactions occurring during a pyrolysis process are very complex and depend on both the nature of the biomass and the reactor conditions (Laird *et al.*, 2009). To simplify the complex reactions driving the pyrolysis of biomass, three main steps can be defined:

$$\text{Biomass} \longrightarrow \text{water} + \text{Unreacted residue} \quad (1)$$

$$\text{Unreacted residue} \longrightarrow (\text{Volatile} + \text{Gases})_1 + (\text{char})_1 \quad (2)$$

$$(\text{Char})_1 \longrightarrow (\text{volatile} + \text{Gases})_2 + (\text{char})_2 \quad (3)$$

In the first step moisture and some volatile compounds are lost. In the second step the primary bio-char forms followed by a slower step including some chemical rearrangements of the bio-char. During the third step, the latter decomposes at a very slow rate and carbon-rich residual solid forms. The formation of secondary charring makes the char less reactive (Demirbas, 2004).

Going more into details, typically pyrolysis of biomass is carried out in a relatively low temperature, ranging from 300 to 650 °C (Basu, 2010). The heating of the particles leads firstly to moisture evaporation, called drying stage, and then with raising temperature the large complex hydrocarbon molecules undergo a thermal scission of the chemical bonds. This primary decomposition phase generates a vapor phase (volatiles) and a residual solid phase (char or biochar) (Fig.1).

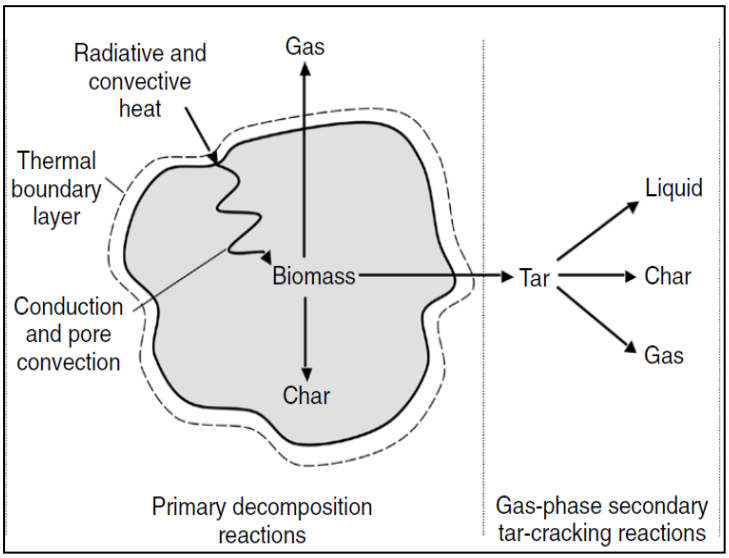

Figure 1 : Pyrolysis of biomass particle (Basu, 2010)

Later on, under cooler conditions, the pyrolytic volatiles, polar and high-molecular-weight compounds, condense out to form the liquid (bio-oil or tar) which is a complex mixture of organic chemicals and water, while the low-molecular-weight and volatile compounds remain in the gas phase to form the syngas. Beside these described reactions, shrinkage and fragmentation of the biomass particles may take place (Fig.2).

During the secondary decomposition phase several reactions can occur either homogeneously (gas phase) or heterogeneously (gas-solid phase) such as cracking, reforming, dehydration, oxidation, polymerization. The condensable gas or tar can participate further in a variety of secondary decomposition reactions. It can generate non-condensable gases (CO, CO_2, H_2, CH_4, C_2H_6 and C_2H_4), tar and char. In addition, the char formed from the primary decomposition phase can participate in catalyzing the conversion of organic vapors to light gases (cracking) and secondary formation of char (polymerization). It is not possible to distinguish perfectly between the primary and secondary decomposition reactions because they may take place simultaneously in different parts of the biomass particles (intra-particle and extra-particle). (Yaman, 2004; Laird *et al.*, 2009; Basu, 2010; Verheijen *et al.*, 2010; Neves *et al.*, 2011)

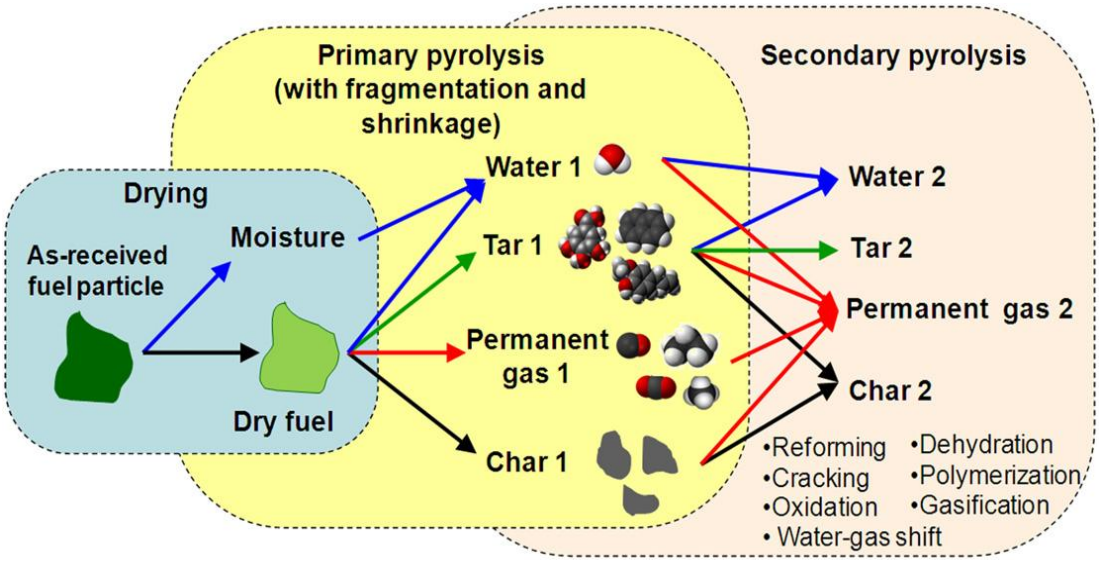

Figure 2: Thermal degradation of a solid biomass particle. The arrows indicate the main routes for the formation of products (Lehmann *et al*., 2006; Lee *et al*., 2010).

2.3. Types of pyrolysis

The pyrolysis can be classified based on the given specific operating conditions such as the heating rate, the process time, the medium in which pyrolysis is carried out. Generally the literature classifies the pyrolysis into slow pyrolysis and fast pyrolysis. Two other types of pyrolysis, mainly used for chemicals production are hydrous pyrolysis (in H_2O) and hydro-pyrolysis (in H_2).

2.3.1. Slow pyrolysis

Slow pyrolysis can be defined as '*The slow heating in the absence of oxygen to temperatures in excess of 400°C which induces the thermal decomposition of lingo-cellulosic biomass producing approximately equal masses of syngas, bio-oil, and biochar*" (Laird *et al.*, 2010b). The biomass, in absence of oxygen, is heated to a relative low temperature (300-450°C) with low heating rates (1-10°C min^{-1}) for a specified period of time reaching several days for a maximization of char formation (Decker *et al.*, 2007; Basu, 2010). This process has been used for a long time to transform wood in coal.

Slow pyrolysis can be divided into two types: carbonization and conventional. Thousands of years ago, pyrolysis of biomass has been practiced through its oldest form known as carbonization used for the aim to produce char or charcoal (Yaman, 2004). Figure 3 shows the typical beehive oven where large wood pieces were filled and covered by a clay wall. Heating is provided by firing at the bottom insuring an insulated closed area. The long process time of carbonization allows the conversion of condensable vapor into char and non-condensable gases (Basu, 2010). In such charcoal kilns the syngas and the vapors are released into the atmosphere causing

serious air pollution. The biochar produced this way is mainly used in domestic cooking or heating and in the metallurgical industry (Laird *et al.*, 2010b).

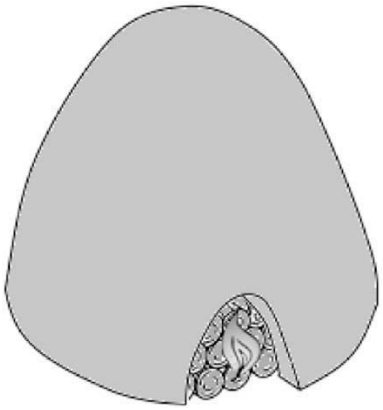

Figure 3: Beehive oven for charcoal production through slow pyrolysis of wood (Basu, 2010).

Conventional pyrolysis involves all three types of pyrolysis products - gas, liquid and char. The heating rate is qualified to be moderate and the process occurs in a moderate temperature (~600 °C). The product residence time is on the order of minutes (Basu, 2010). In the new pyrolysers the volatiles produced are either captured to be used as source of chemicals or burned directly to produce heat. Those slow pyrolysers, in comparison to other thermo-chemical conversion technologies, present the advantages of being small, inexpensive and can be fed by different types of feedstock. They can be optimized to produce a high quality biochar with low quantities of syngas and bio-oil (Laird *et al.*, 2010b).

2.3.2. Fast pyrolysis

Contrary to slow pyrolysis, fast pyrolysis is used to produce bio-oil and/or gas. It is characterized by high heating rate and rapid appeasing of the liquid product to avoid the secondary decomposition phase which maximizes the tar yield (Yaman, 2004). The biomass is heated rapidly (<1s) to reach the peak pyrolysis temperature of 400 to 700°C if the desired product is bio-oil or to reach 1000°C if the product of interest is gas. To achieve the rapid heating of biomass, the particle size of the feedstock must be less than 2mm. The biomass decomposes generating, gases, and aerosols, and less charcoal. After cooling and condensation of the volatile products, a dark brown liquid is formed characterized by a heating value about half that of conventional fuel oil (Decker *et al.*, 2007; Basu, 2010; Laird *et al.*, 2010b).

2.3.3. Pyrolysis in the Presence of a Medium

Based on the specific medium where pyrolysis is conducted, pyrolysis can be classified as three main types: 1) pyrolysis carried out in the absence of air (slow and

fast pyrolysis), 2) pyrolysis carried out in water (called hydrous pyrolysis) and 3) pyrolysis carried out in hydrogen atmosphere (known as hydro-pyrolysis).

Hydrous pyrolysis is a thermal decomposition of biomass carried out in water at high-temperature. It is used to convert biomass into light hydrocarbon that can be used for production of fuel, fertilizer or chemicals.

In hydro-pyrolysis the biomass is decomposed thermally under high pressure in hydrogen atmosphere. Hydropyrolysis is used to increase the volatile yield and the proportion of lower-molar-mass hydrocarbons (Basu, 2010).

2.4. Factors affecting pyrolysis products

Multiple factors can influence the pyrolysis process. The proportion and characteristics of the output products can vary depending on the chemical and physical characteristics of the feedstock used and the different processing parameters such as heating rate, pyrolysis temperature and residence time.

2.4.1. Feedstock characteristics

Feedstock is one of the most important factors controlling the properties of the pyrolysis products. The chemical and structural composition of the biomass feedstock relates to the chemical and structural composition of the output products and, therefore, reflects on their components and functions. Feedstock is the term conventionally used for describing the type of biomass that undergoes the pyrolysis to be turned into biochar (Verheijen et al., 2010). Generally, any organic feedstock can be pyrolysed due to the pyrolysis high temperature which decomposes organic toxins and destroys pathogens in the feedstock.

Biomass chemical properties have a significant influence on the products yield and quality. Biomass is composed of three main polymeric materials: cellulose, hemicelluloses and lignin. According to literature, the analysis of data from thermo-gravimetric apparatus (TGA) differential thermo-gravimetry (DTG) (Fig.4) shows that each of the mentioned constituents has its optimum ranges of temperature to initiate pyrolysis. Hemicellulose is the first compound to decompose. The process starts at 220°C and is completed at 315°C. Cellulose decomposes in the range between 240 and 350°C, and lignin begins to decompose at 160°C in a slow process extending to 900°C (Yang *et al.*, 2007; Lehmann and Joseph, 2009; Basu, 2010).

On heating to pyrolysis temperature the components of the feedstock contribute differently to product yields. Cellulose and hemicellulose present mainly the source of volatiles. Condensable vapors are provided mainly by the decomposition of cellulose, smaller parts of those vapors in addition to non-condensable vapors are generated from hemicelluloses. On the other hand, lignin decomposes slowly and presents the major contribution to char formation. Lignin contributes also to liquid and gaseous products but with lower quantities in comparison to its contribution to char production. Lignin yields about 40% of its weight as char, almost 35% as liquid and 10% as gaseous product (Yang *et al.*,

2007; Lehmann and Joseph, 2009; Basu, 2010). Demirbas (2004), mentions that biomass with high lignin contents have produced the highest biochar yields. The fact that the lignin is more resistant to thermal decomposition, its loss is generally less than half of the cellulose loss.

Mineral content of biomass can affect also the pyrolysis products. Pyrolysis of wood-based feedstock produces coarser and more resistant biochar rich up to 80% of carbon content with low ash content (<1% by weight) (Winsley, 2007). Biomass with high mineral content such as grass, grain husks and straw generally produce ash-rich biochar. The mineral content of the feedstock can be retained and concentrated in the produced biochar (Antal and Grønli, 2003; Demirbas, 2004; Lehmann *et al.*, 2011).

Regarding physical properties of biomass feedstock, its size and shape influence the pyrolysis product. The finer is the biomass particle, the more uniform is the heating rate. This enables the drying and the primary decomposition to take place uniformly throughout the biomass particle. This will present lower resistance to the releasing of moisture and volatile compounds. In the case of feedstock with larger particle size, the heating is non-uniform and consequently the primary and secondary decomposition phases occur simultaneously. This higher retaining energy of volatiles compounds will promote the secondary decomposition and lead finally to a maximization of char formation (Antal and Grønli, 2003; Basu, 2010; Neves *et al.*, 2011).

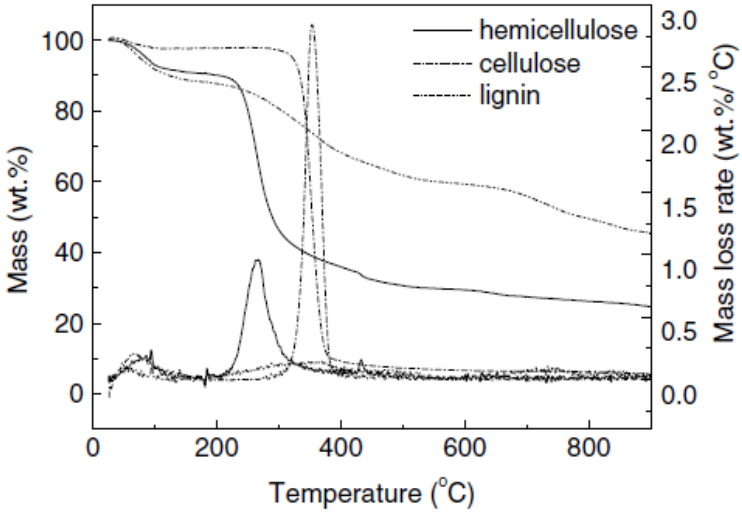

Figure 4: Pyrolysis curves of hemicellulose, cellulose and lignin in thermo-gravimetric analyzer (Yang *et al.*, 2007).

2.4.2. Heating rate, pyrolysis temperature and vapor residence time

The heating rate, the pyrolysis temperature and the vapor residence time have a major influence on the product yields and their composition. The product yields can change widely in quantity and quality depending on the process conditions.

The depolymerization and the secondary cracking are endothermic reaction and have higher activation energy than the dehydration of cellulose, which is an exothermic process. Longer vapor residence time, low pyrolysis temperature and slow heating rate (0.001-2.0°C s^{-1}) are conductive to a higher production of biochar. Long residence time, slow heating rate and higher temperature (700 to 900°C) favor the conversion of biomass into gas. To maximize the liquids production, moderate final temperature (450°to 600°C), short vapor residence and high heating rate are needed (Tab.3) (Decker *et al.*, 2007; Basu, 2010).

Sensöz *et al.,*(2006) revealed that using a heating rate of 10°C min^{-1}, the produced biochar from the pyrolysis of olive bagasse increased from 61.9% to 69.4% for the pyrolysis temperatures of 350 and 550°C. Increasing the heating rate to 50°C min^{-1} to reach the same pyrolysis temperatures of 350 and 550°C had decreased the yields in biochar from 35.3% to 30.6%.

From another perspective, to produce finer biochar material, higher heating rate (105-500°C/sec), shorter vapors residence time and small feedstock particles are preferred (Cetin *et al.*, 2004). Seeking for coarser biochar, Verheijen *et al* (2010), reported hat slow pyrolysis, heating rates of 5-30°C min^{-1} and large feedstock particles are needed. Basu (2010) suggested that low heating to a moderate temperature (400-500°C) produces more biochar. Under this slow heating the volatiles are more resident in the reactor which permits a secondary reaction to occur between char and volatiles leading to secondary char formation.

Table 3 : Typical product yields (%) obtained by different modes of pyrolysis

Mode	Conditions	Liquid	Biochar	Syngas
Fast pyrolysis	Moderate temperature ~500°C, short hot vapor residence time ~1 s	75	12	13
Intermediate pyrolysis	Moderate temperature ~500°C, moderate hot vapor residence time of 10-20 s	50	20	30
Slow pyrolysis	Low temperature ~400°C, very long solids residence time	30	35	35
Gasification	High temperature ~800°C, long vapor residence time	5	10	85

2.5. Pyrolysis products

Depending on the desired product, gas, liquid or char, the parameters of pyrolysis: heating rate, pyrolysis temperature and vapor residence time should be chosen accordingly. The adjustment of these parameters will lead to a formation of different proportions and properties of the out-products.

2.5.1. Pyrolysis tar

Pyrolysis liquid product, bio-oil or tar, is a dark brown free-flowing fraction with a distinctive acrid smoky smell due to the low molecular weight of aldehydes and acids (Bridgwater, 2006; I.E.A, 2006). The bio-oil contains different chemicals with

wide varying proportions. This liquid fraction is composed of a complex mixture of oxygenated aliphatic, aromatic compounds (75-80 weight %) and an appreciable proportion of water (20-25 weight %) from the original biomass moisture and the reaction process (Bridgwater, 2004; Laird *et al.*, 2010b).

Its high water content, low heating value, beside its high viscosity, high acidity and high ash content, makes it unstable and not suitable to be used directly as fuel. Upgrading techniques such as hydrodeoxygenation, catalytic cracking and steam reforming of the bio-oil can make it a suitable fuel. These techniques being complicated and highly depending on full developed reactors make this upgrading not economically viable (Zhang *et al*., 2007). Leibold *et al.,* (2008), revealed that hydro-treatment and catalytic cracking of bio-oil to produce liquid fuel and other co-products present potential efficient approach. From another perspective, bio-oil can substitute fuel oil or diesel in boilers, furnaces, engines and turbines for electricity generation or heating (Fig.5) (I.E.A, 2006).

The advantageous point about using bio-oil is that while burning it, due to the fact that plant biomass contains negligible amount of sulphur, very low and almost no significant amounts of SO_x is emitted. Beside this, bio-oil produces lower NOx than diesel (Bridgwater, 2004). In addition to the energetic possible uses, a range of chemicals can be extracted or derived from bio-oil such as food flavorings, resins, hydroxyaldehydes, hydroxyl ketones, carboxylic acids, phenolic compounds, agri-chemicals, fertilizers and emission control agents (I.E.A, 2006; Wang *et al.*, 2009).

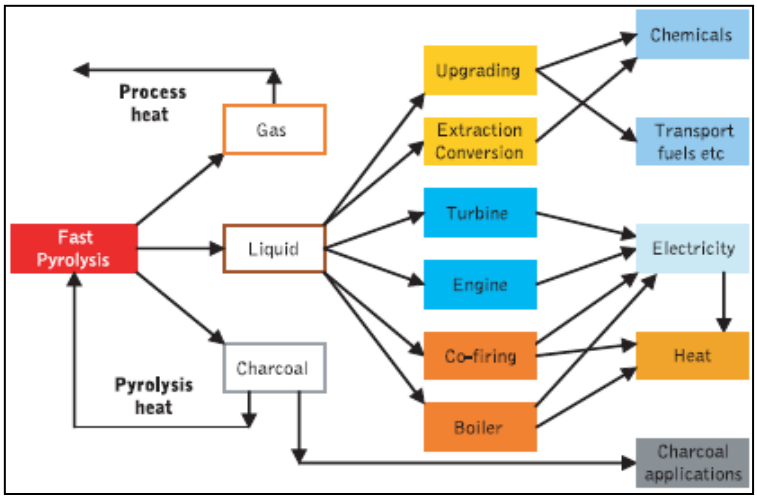

Figure 5: Applications of bio-oil products

2.5.2. Pyrolysis gas

Gasification is the term used for the thermal conversion of biomass to produce gas (syngas). This process present a valuable option for the valorization of biomass since the gas can be stored, easily transported and its final use can be decoupled from the production process (Efika *et al.*, 2012).

The pyrolysis gas mixture contains mainly low molecular weight gases such as carbon dioxide, carbon monoxide, hydrogen and minor amounts of higher hydrocarbons like methane, ethane, ethylene, propane and propylene (Yaman, 2004;

Basu, 2010). To generate a maximum yield of syngas it is necessary to decrease its condensable hydrocarbons content. To comply with it the gasification temperature should rise to higher levels, often above 900°C. This high temperature may induce a reduction in the total energy efficiency of the whole process. To cope with this problem, the use of catalysts, particularly nickel based, showed promising results by exerting a cracking effect which mainly after hydrogen formation, increases the tar decomposition and reduces the need for very high temperature (Encinar *et al.*, 2009; Efika *et al.*, 2012).

2.5.3. Pyrolysis char

Depending on the production method, the yield of charcoal can vary from 10 to 35 percent based on dry wood. Charcoal represents a very important fuel in developing countries and is still a highly desired reductant in the metallurgical industry because of its low sulfur and mercury content. Large amounts of charcoal are also used to produce activated carbons extensively used for cleaning water and air (Decker *et al.*, 2007). Pyrolysed char is called biochar when it is devoted to be applied to soil. Biochar characteristics, uses and impacts are discussed in details in the following section.

3. Biochar

3.1. Definition

Several authors defined biochar as a carbon rich material produced when organic matter (biomass) is heated in total or partial absence of air (pyrolysis or gasification). In more technical terms, biochar is produced by the thermal decomposition of organic material under limited or restricted supply of oxygen (O_2), and at relatively low temperature (<700°C) (Lehmann and Joseph, 2009; Jha *et al.*, 2010; Sohi *et al.*, 2010; Karhu *et al.*, 2011; Shackley and Sohi, 2011). The term ‘biochar’ refers specifically to the char produced and aimed to be used for soil application (Sohi *et al.*, 2010).

3.2. Biochar properties

3.2.1. Physical characteristics: structure, porosity and surface area

During pyrolysis, the degree of alteration, the formation of cracks and the original properties of the biomass used will influence its final structure, porosity and surface area properties. Under pyrolysis temperature the volatiles are lost, the biomass is objected to shrinkage and a volume reduction occurs (Downie *et al.*, 2009).

The biochar structure is essentially amorphous in nature, but contains some local crystalline structure of highly conjugated aromatic compounds (Qadeer *et al.*, 1994). These crystalline areas can be seen as flat aromatic graphene sheets linked in a random way giving to the biochar a good conductivity comparable to graphite.

The other non-conducting components forming biochar are aromatic-aliphatic organic compounds (complex structure) and the mineral fractions (inorganic ash).

Those components are complemented to the pores (macro-, meso- and micropores) formed during the process to complete the whole picture. With increasing pyrolysis temperature, the crystallines enlarge and become turbostratically arranged. For turbostratic arrangements, the layer planes are disposed more or less parallel and equidistant which increases the biochar surface area (Fig.6). The linkage of the crystallites and those within hexagonal planes present the origin for the development of pores of various sizes.

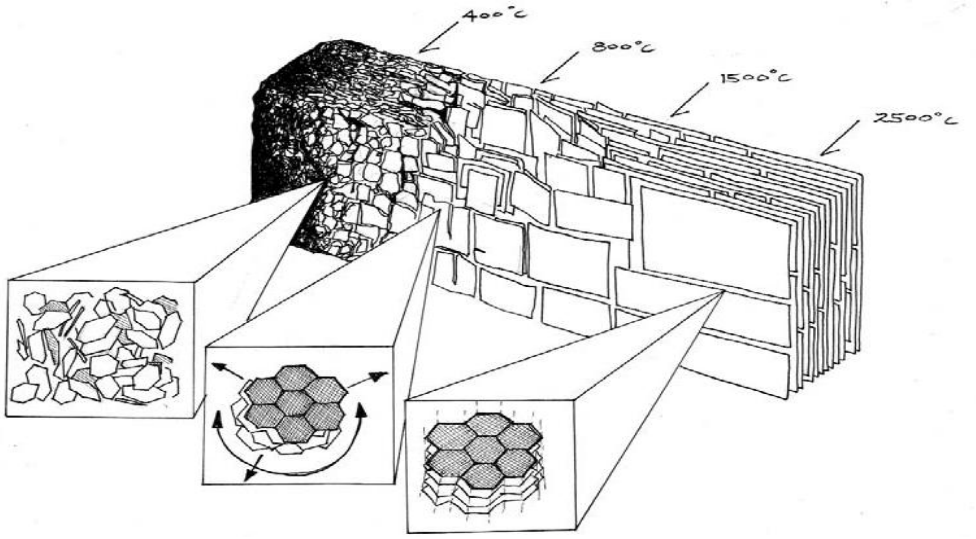

Figure 6: Ideal biochar structure development with the highest treatment temperature
(Left) increased proportion of aromatic carbon, highly disordered in amorphous mass;
(Center) growing sheets of conjugated aromatic carbon, turbostratically arranged;
(right) structure becomes graphitic with order in the third dimension (Downie *et al.*, 2009).

The various pores of a produced biochar are commonly determined through a scanning electron microscopy. Macropores (internal diameter > 50nm) are considered as feeder for the transport of adsorbate molecules to the meso- and micropores. They can provide suitable dimensions for microorganisms to inhabit (Fig.7). Mesopores (2nm < internal diameter < 50nm) are of main importance to many liquid-solid adsorption processes. Micropores (internal diameter < 2nm) are mainly responsible for the high adsorptive capacity of molecules of small dimensions and present the major contributor to the surface area of a biochar (Downie *et al.*, 2009).

The surface area of biochar is generally higher than sand but comparable to clay surface area (Chan *et al.*, 2007). Under increasing pyrolysis temperatures biochar surface area increases until reaching a pick temperature at which deformation occurs and consequently it starts to decrease (Brown *et al.*, 2006).

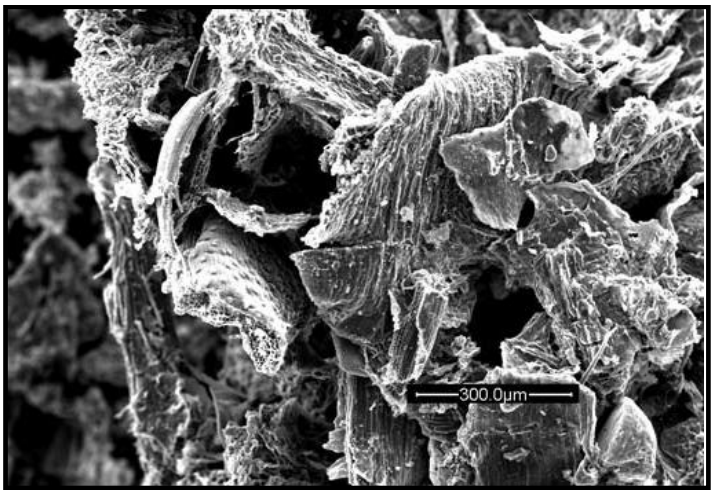

Figure 7 : SEM image showing macro-porosity in biochar produced using slow pyrolysis(Downie *et al.*, 2009).

3.2.2. Chemical characteristics

3.2.2.1. Organic and inorganic composition

Generally, the biochar composition can be splitted into relatively recalcitrant C, leachable C and ash. Biochar is characterized by a higher proportion of aromatic C in comparison with other organic matter, specifically the occurrence of fused aromatic C structures. The pre-cited structure of biochar can have variable forms depending on the pyrolysis temperatures. At low pyrolysis temperatures the amorphous C is dominant. On the other hand, under high temperatures, the C tend to aromatize and poly-condense into a poly-aromatic structures which allow increased formation of the turbostratic C. Those C structures are the main reason for the high stability of biochar. The C the N and other nutrients contained in the C structure will not be readily available to the microorganisms to be used as a source of energy (Lehmann *et al.*, 2011) this makes biochar chemically stable. In addition, the carbon atoms are strongly bound to one another making biochar resistant to decomposition. Unlike carbon in most of the organic matter, these aromatic forms of organic carbon are very stable and cannot be returned easily to the atmosphere as CO_2, even under favorable environmental and biological conditions, such as those that may prevail in the soil. By consequence, biochar can be a promising valuable tool for stabilizing carbon and storing it into the soil as a way of removing CO_2 from the atmosphere (Sohi *et al.*, 2010; Shackley and Sohi, 2011).

The biochar leachable or labile fractions can be mineralized and can stimulate microbial activity and proliferation. Those fractions are quantified generally by incubation studies. The third main component is resumed on the minerals present as ash inclusions in biochar. This fraction contains several macro- and micro-nutrients for biological uptake.

Their presence during the pyrolysis process can affect the biochar chemical structure. For example, pyrolysis of peat containing considerable amount of iron (Fe),

if carried out at temperature above 600°C, produces biochar with formation of Fe_3C bonds and small ferromagnetic iron clusters. For grasses, rice hulls and nut shells, known to contain quantities of amorphous silica (< 2% wt), biochar produced under pyrolysis temperature of 1200°C showed a formation of silicon carbide (SiC) taking part in cross-linking aromatic domains or crystallites (Freitas *et al.*, 2000; Lehmann *et al.*, 2011).

3.2.2.2. Elemental ratios as quality indicators

The major constituents of biomass are carbon, hydrogen, oxygen and nitrogen. Biochar production through pyrolysis is often coupled with changes in the elemental contents of C, H, O and N and their associated atomic ratios. These components are volatilized during pyrolysis with greater losses in H and O in comparison to C. The proportion of C in the solid phase increases from 40-50 % by weight in the feedstock to reach 70-80% by weight in the biochar after pyrolysis (Antal and Grønli, 2003).

The atomic ratios H/C and O/C are routinely used to define the degree of aromaticity and stability of the produced biochar (Baldock and Smernik, 2002; Hammes *et al.*, 2006) while (O+N)/C ratio is an indication of biochar surface polarity (Wang *et al.*, 2007). In general, H/C and O/C atomic ratios in biochars decrease with higher pyrolysis temperature and with higher heating rates. Indeed, H/C and O/C atomic ratios tend to be the highest in the low-temperature produced biochars and in partially charred plant materials. In contrary, biochar produced under high temperatures and vegetations fire residues reveal lower ratios (Baldock and Smernik, 2002; Almendros *et al.*, 2003). The decrease in H/C ratio reflects the formation of structures containing unsaturated C such as aromatic rings. This ratio can give an idea about the bonding arrangements. In peat the H/C ratio equal to 1.3 indicates that most of the C is directly bonded to a proton or connected to an OH group. An H/C ratio between 0.4 and 0.6 of a biochar indicates that every second to third C is connected to a proton (Knicker *et al.*, 2005). O/C and H/C ratios can be used also to indicate the biochar stability and by consequence to identify its suitability for carbon sequestration. Schimmelpfenning and Glaser (2012) revealed that biochars with O/C ratio<0.4 and H/C ratio<0.6 are effective as carbon sequestration agents when applied to soils.

Beside the atomic ratios, the CO_2 emission factor of the biochar can be used to evaluate the avoided amount of carbon emissions from biochar into the atmosphere (Anke, 2003).

3.2.2.3. Biochar pH, surface properties and sorption

The biochar pH is highly variable depending on the feedstock properties and the pyrolysis process parameters. Biochars with high ash content reveal greater pH values in comparison to those with low ash content. During pyrolysis, biochar pH

increases with higher pyrolysis temperature. Over time, the biochar pH may increase by dissolution of alkaline minerals or decrease by oxidation of carbon to form acidic carboxyl groups (Lehmann et al., 2011).

The changes occurring in the elemental composition of the biochar during pyrolysis reflect its molecular composition defining the surface functional groups. The ^{13}C nuclear magnetic resonance is generally used to determine the biochar surface chemistry. The low temperature produced biochar presents a quite rich and varied surface chemistry with heterogeneous compositions. Depending on the biomass composition and on the pyrolysis process parameters, the surface chemistry composition may show hydrophilic, hydrophobic, acidic or basic properties, affecting in consequence the reactivity of the biochar. The heterogeneity of the biochar functional groups is a result of the differences in the electro-negativity of the hetero-atoms relative to the carbon atoms. Those hetero-atoms can be formed by H, O, N, P and/or S and are inserted in the aromatic rings. By consequence, electron donors (OH, NH_2, O(C=O)R…) and electron acceptor groups ((C=O)OH, (C=O)H, NO_2…) can create the acidic and basic surfaces which might coexist on the surface and pores of biochar within micrometers. For example, carboxyl groups are strong bronsted acids, phenols and carbonyls are less acidic, chromenes and pyrones are basic functional groups (Lehmann and Joseph, 2009). The volatile compounds are lost gradually with the rise of pyrolysis temperature, both surface area and ash increase. In meanwhile, the surface functional groups providing the exchange capacity decrease (Guo and Rockstraw, 2007; Lehmann *et al.*, 2011).

With increasing pyrolysis temperature, the poly-condensation of carbon into aromatic rings and the insertion of hetero-atoms define the structure; enhance pore development and surface area of the biochar. Generally, biochar produced under high temperature contains large amount of carbon concentrated in form of aromatic rings and shows lower functional groups to generate surface charge and ion exchange due to de-carboxylation. These aromatic compounds can include the polycyclic aromatic hydrocarbons (PAH's) which are relatively recalcitrant and potentially toxic formed in incomplete combustion. Depending on the feedstock, it was revealed in literature that wood-based biochar contained higher PAH's content with increasing temperature while, PAH's content in straw based biochar showed a decreasing trend with higher pyrolysis temperature (Kloss *et al.*, 2012).

On the other side, low temperature produced biochars show significantly higher C=O and C-H functional groups, thus enhancing their sorption capacity (Glaser *et al.*, 2002; Hammes *et al.*, 2006).

The proportions of cellulose, hemicelluloses and lignin remaining along with the ash content, influence the reactivity of the produced biochar and the development of the physical structure which defines the biochar properties (Downie *et al.*, 2009). Biochars generated from sewage sludges and manures showed high abundance of nitrogen and sulfur functional groups in comparison to biochars derived from lignocellulosic biomass. An experiment conducted by Koutcheiko *et al*. (2007)

revealed that the major functional groups containing N in low temperature biochar, produced from chicken manure, were pyrrolic or pyridinic amines, whereas at high temperature biochar showed N functional groups with equal amounts of pyridinic and quaternary groups. The sulfur functional groups were sulphonates and sulphates, whereas thiophene and sulphide groups dominated in the high temperature biochar (Koutcheiko *et al.*, 2007). Biochars derived from high ash biomass content were presented to have higher cation exchange capacity (CEC) (Lehmann *et al.*, 2011; Downie *et al.*, 2009).

The heterogeneous functional groups composing the surface of biochar affect its sorption by their surface charge and electrons availability. The nature of the sorbate also affects its ability to sorb. For example, the non-transition metals (atransition element is a d-block element which forms one or more stable ions with incompletely filled d-orbitals) are strictly sorbed by electrostatic forces. Contrarily, the transition metals have exposed π-orbitals and can bond to π-electrons on the graphene sheets besides the electrostatic bonding at oxidized sites.

Several of these metals are amphoteric (ion that can react as an acid as well as a base), which makes the description of the sorption behavior more complicated (Amonette and Joseph, 2009). For example, the complex sorption behavior of lead (II) ions was described by Swiatkowski *et al.,* (2004). The authors revealed various ways of metal absorption on biochar. Biochar surface can adsorb lead cations through:

- Lewis base reaction; $C{:}H_3O^+ + PbOH^+ \text{-----> } C{:}PbOH^+ + H_3O^+$
- Cπ-cation interaction ; $C{:} + Pb^{2+} \text{-----> } C{:}Pb^{2+}$
- basic sites;

  $C\text{-}OH + Pb^{2+} + 3H_2O \text{-----> } COPbOH + 2H_3O^+$

  $C\text{-}O^{-*} + Pb^{2+} + 2H_2O \text{-----> } C\text{-}O\text{–}PbOH + H_3O^+$

  $N{:} + Pb^{2+} + 2H_2O \text{-----> } N\text{-}Pb(OH)^+ + H_3O^+)$
- and oxidized acidic sites;

  $C\text{-}COOH + Pb^{2+} + H_2O \text{-----> } C\text{-}COOPb^+ + H_3O^+$

  $(C\text{-}COOH)_2 + Pb^{2+} + 2H_2O \text{-----> } (C\text{-}COO)_2Pb + 2H_3O^+$

  $C\text{-}OH + Pb^{2+} + H_2O \text{-----> } C\text{-}OPb^+ + H_3O^+$

3.3. Biochar: motivations for environmental management

3.3.1. Management of wastes

Large quantities of animal and agricultural residues are produced annually worldwide. In the recent past these wastes were sold as fertilizers or simply spread over agricultural land which causes a significant environmental troubles leading to soil, air and ground water pollution. With the rising concerns toward the environmental protection these wastes can be better exploited as resources as pyrolysis feedstock for thermo-chemical conversion. Another interesting output from charring these wastes is that their volume and weight are significantly reduced (Lehmann and Joseph, 2009).

Biomass represents an available, renewable source and it is not likely to be depleted by consumption. In addition, slow pyrolysers have the advantage to be fed by different feedstocks. This offers a great opportunity to settle down an economic activity handling the wastes, producing energy and creating job opportunities. Biomass conversion plants can promote the development of associated activities such as biomass collecting and transporting (Basu, 2010). This thermo-chemical conversion technology has offered solutions toward the management of a wide range of agricultural residues and animal wastes. For example, the pyrolysis of the animal manures to produce biochar have revealed the total removal of pathogens, making this product hygienically safe (Lehmann and Joseph, 2009).

3.3.2. Soil amendment

It is estimated that the world population will reach 9.1 billion by 2050 (FAO, 2009). Therefore, the soil, being the fundamental resource for food, fibers and fuel, must be protected as a matter of urgency. Soils play a key role in the definition of sustainable land management since they represent the basis of food production. If soils are eroded or degraded to a large extent, a society may lose its fundament of safety and self-sufficiency (Pretty *et al.*, 2000). Huge areas of arable lands are degraded by overgrazing, deforestation and inappropriate agricultural practices. These changes are affecting a large part of the world population which is now suffering from hunger. According to FAO (2012) the number of hungry people remains unacceptably high with 870 million of undernourished. In Sub-saharan Africa and South Asia a lack of food security measured by undernourishment reaches respectively a percentage of 26.8 and 17.6 of the total population (FAO *et al.*, 2012).

The loss of soil productivity takes place under intensive use of agrochemicals linked with environmental risks for the soil and water sources. To cope with these problems, soil protection and improvement is of an important necessity. Under this issue, biochar was reported to be able to provide great opportunities to fight against soil degradation, to insure a better soil quality for agricultural activities and to enhance crop yielding. The biochar application to soil can:

(1) increase the net soil surface area (Chan *et al.*, 2007), enhancing CEC and pH. Biochar pH is mainly neutral to basic. When applied to soil biochar can increase the soil pH (up to one pH unit) due to its liming effect. In literature this liming effect is judged to be responsible for rising the CEC (up to 20%) (Glaser *et al.*, 2002) the plant nutrient availability (Glaser *et al.*, 2002; Laird *et al.*, 2010a) and by consequence the yielding of the crops (Verheijen *et al.*, 2010).
(2) improve soil water and nutrient retention (Verheijen *et al.*, 2010; Downie *et al.*, 2009). Glaser *et al.* (2002) found that in *terra preta* the water holding capacity was 18% higher than an adjacent soil where charcoal was absent. Experiments conducted by Laird *et al.* (2010b) and Karhu *et al.* (2011)

revealed that biochar could improve the water retention by 15 and 11% in 2 different soils.

(3) provide available mineral nutrients (Laird *et al.*, 2010a) thus improving the nutrient cycle by enhancing nutrients dynamics and protect groundwater (Glaser *et al.*, 2002). In an experiment conducted by laird *et al*. (2010a) the addition of biochar revealed an increase of total N (by 7%), organic C (by 69%) and Mehlich extractable P, K, Mg and Ca.

In addition to what have been mentioned, biochar has shown a capacity to affect the composition and abundance of soil biological communities. Due to its porous structure - with high surface area adsorbing inorganic nutrients and soluble organic matter - bacteria, actinomycetes and arbuscular mycorrhizal fungi find it a suitable habitat for colonizing and reproducing (Fig.8). By consequence, such change will affect the nutrient cycles, the soil structure, might have some symbiosis with the plants and then promote their growth and yielding (Janice and Matthias, 2009; Lehmann *et al.*, 2011).

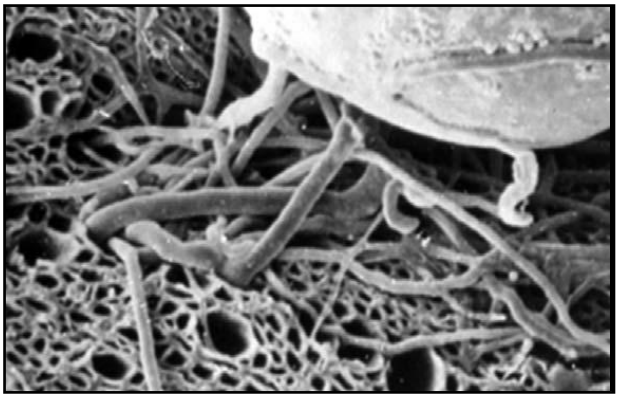

Figure 8 : Arbuscular mycorrhiza fungal hyphae growing into biochar pores from a germinating spore (Janice and Matthias, 2009)

From another perspective, literature revealed that biochar application might be of value for sequestering and retaining metals in contaminated soils and it can be reflected by improvement of the different features of the soil and the plant growth. In a experiment conducted by Buss *et al.* (2012) , applying biochar derived from forest green waste at rates of 2 and 4% in a sandy soil contaminated with 50 and 200 mg Kg^{-1} of Cu showed reduced plant stress and metal uptake indicating metal retention. Park *et al.* (2011) reported that chicken manure and green waste derived biochars had the potential to affect the behavior of the metals in soil by altering their solubility, availability and spatial distribution. They added that biochar application to the metal contaminated soil has the potential of in situ remediation by immobilizing metals. In addition, biochar improved the agronomical properties by increasing the nutrient availability and microbial activity.

3.3.3. Climate change mitigation

Anthropogenic carbon dioxide (CO_2) emissions have risen by more than 3% annually from the year 2000. This rising emissions are causing disorders in Earth ecosystems revealed by a dangerous and irreversible rapid climate change (Dominic *et al.*, 2010). The existing technologies of biomass management are usually coupled with methane and nitrous oxide released into the atmosphere as the biomass decomposes (Lehmann and Joseph, 2009). To cope with this existential issue, humanity need to find solutions to reduce those emissions, to keep them under a threshold level and to settle down mitigation strategies to bring down the amount of CO_2 from the atmosphere (Dominic *et al.*, 2010). One of the most promoting ways cited by the literature is biochar production through pyrolysis. Biochar production and its incorporation into agricultural soils has been suggested to sequester atmospheric carbon, reduce greenhouse gas emissions and by consequence mitigate the climate change (Chan *et al.*, 2007; Lehmann and Joseph, 2009; Kammann *et al.*, 2011; Karhu *et al.*, 2011; Ippolito *et al.*, 2012).

To form biomass, plants use CO_2 from the atmosphere. After decay these plants, trees and residues decompose at different rates releasing CO_2 and might release CH_4 if they decompose in water (Basu, 2010). CH_4 and N_2O are potent greenhouse gases which can be released at high levels in intensively fertilized agriculture, by legumes, urine and manures of grazing animals.To avoid these gases from being released back to the atmosphere, the idea of transforming this biomass into stable biochar that decomposes much more slowly will drive the carbon into a much slower biochar cycle (Lehmann and Joseph, 2009). Lehmann and Joseph (2009) advanced more that the larger amounts of CO_2 are in cycles between the atmosphere and plants on annual basis. Diverting 1 % of this annual plant uptake into biochar can mitigate almost 10% of the current anthropogenic carbon emissions. Biochar added to soil can have a significant reduction in N_2O by facilitating the last step of denitrification. A decrease of N_2O emissions by 10 to 90% in 14 different agricultural soils was reported (Cayuela *et al.*, 2013). In other findings, under laboratory conditions, it was reported that biochar amendment suppressed soil CO_2 emissions by 53% and net soil CO_2 equivalent (eq.) emissions (CO_2, N_2O, CH_4) by 55%. Passing to field conditions the CO_2 emissions were reduced by 33% and annual net soil CO_2 eq. emissions by 37% over 2 years (Case *et al.*, 2013). The figure 9 shows the sustainable concept of biochar for mitigating the climate change. The biomass produced by removing CO_2 from the atmosphere is converted by pyrolysis to generate bio-oil, syngas and biochar. Both bio-oil and syngas can be combusted to give energy and CO_2. This energy and heat can be used to offset the fossil carbon emissions while the biochar produced will store carbon under very stable form for a longer period in comparison with the biomass left to decay. Emissions of methane and nitrous oxide are also avoided by preventing biomass decay. As a final outcome the production of biochar and its incorporation into

agricultural soils is qualified to be a carbon negative process (Mathews, 2008; Dominic *et al.*, 2010).

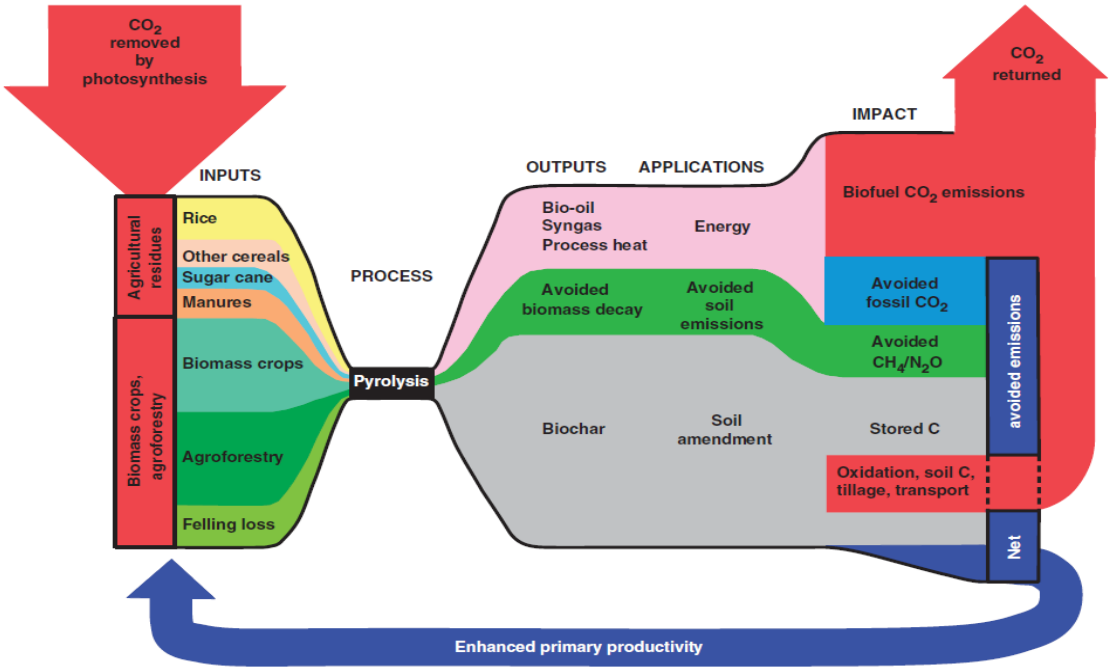

Figure 9: The sustainable biochar concept (Dominic *et al.*, 2010)

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Chapter 3

Production and characterization of biochar from de-oiled three-phase olive mill waste through slow pyrolysis

1. Abstract

Biochar production through slow pyrolysis presents a carbon negative process with high potential in transforming part of atmospheric CO_2 into a stable form of carbon that can be sequestrated in soil. The properties of biochar can vary with the pyrolysis parameters. In this chapter the slow pyrolysis technique applied on solid de-oiled three-phase olive mill waste is considered.

Various pyrolysis temperatures and heating rates impacts on the yield, morphological and physic-chemical characteristics of the produced biochar are investigated. Pyrolysis runs were performed in a downdraft gasifier using three ranges of pyrolysis temperatures (400-450°C, 450-500°C and 500-550°C) and three heating rates (25°C min^{-1}, 35°C min^{-1} and 45°C min^{-1}). The results showed that the raising of the temperature and the increasing of the heating rate decreased the biochar yield but increased its carbon concentration. Temperature had higher effect on the biochar yield in comparison to the heating rate. The highest biochar yield of 41.43% can be obtained from olive mill waste pyrolysed at 400-450°C and heating rate of 25°C min^{-1}. The elemental analysis showed that biochar aromaticity and stability increased with higher temperatures and heating rates. The measured heating values of biochar indicate its possible use as a fuel and for production of active carbon. In addition its high concentration in carbon (70-85%), low electrical conductivity and its high CO_2 emission factor make it suitable for incorporation into agricultural soils for carbon sequestration.

2. Introduction

In developing countries the agricultural sector presents one of the main economic activities and produces high amount of biomass. In the Mediterranean basin, olive and olive oil productions present one of the most important agricultural activities, source of employment, livelihood and income (Owen *et al.*, 2000; Zabaniotou *et al.*, 2000). The olive oil production is increasing over the years, and it is coupled with increasing generation of considerable amount of waste. This waste is heavily polluting and can cause number of environmental problems (Azbar *et al.*, 2004; Kavdir and Killi, 2008). As pyrolysis is an attracting and efficient tool for biomass conversion (Laird *et al.*, 2009; Basu, 2010), it is interesting to investigate the use of pyrolysis for the transformation and valorization of the olive oil industry waste in order to alleviate to the pollution problems engendered by it and transform it into a

valuable by-products (Encinar *et al.*, 1998; Schieber *et al.*, 2001; Dominic *et al.*, 2010). Biochar has attracted growing interest from scientists due to its promising use to improve soils, crop yielding, carbon sequestration with consequent global warming mitigation and boost agricultural productivity (Antal and Grønli, 2003; Lehmann *et al.*, 2006; Steiner *et al.*, 2007).

The study reported here, is aimed at investigating the effects of various slow pyrolysis temperatures and heating rates on the yield, morphology and physico-chemical properties of biochar produced from de-oiled three phase solid olive mill waste.

3. Materials and Methods

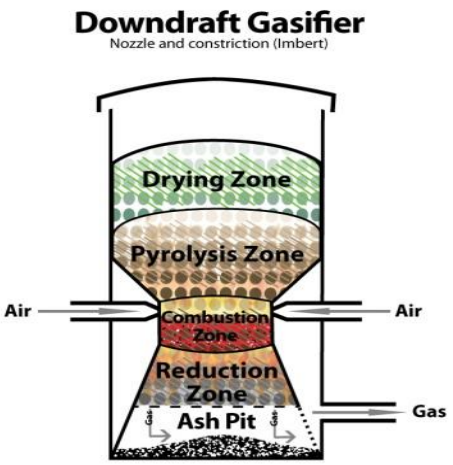
3.1. De-oiled three-phase olive mill waste

The olive oil mill solid waste generated from the 3-phase extraction system contains a considerable amount of oil 4.5 to 9%. Therefore, this waste undergoes a second extraction after being dried to get the seed oil. The solid waste of this seed oil extraction consists mainly of lignin and cellulose and it's called 'de-oiled three-phase olive mill solid waste. The de-oiled three-phase olive mill waste used in this study was collected from the Apulia region. This biomass (47% carbon, 5.7% hydrogen, 1.1% nitrogen, 27.7% oxygen) was left to dry at ambient air temperature then oven-dried for 24 hours prior to its use as feedstock for pyrolysis.

3.2. Gasifier and slow pyrolysis

Slow pyrolysis was applied to the solid olive mill waste using the gasifier experimenters kit (GEK) (All Power Labs, Berkeley, California). The GEK is designed in a modular fashion for easy switch out between common reactor types and operating situations. It can be used to run an expertly configured downdraft gasifier for fueling engines and generating electricity and heat. It can be used also to create a multi-mode pyrolysis reactor for biochar making. In this kit, biomass is fed from the top and air is fed from the bottom. The air or oxygen is usually admitted to the heating bed through intake nozzles (Fig.10 and Fig.11). The produced gas flow downwards through the reactor enabling the pyrolysis gases to pass through a throated hot bed of char, large complex hydrocarbon molecules thermally decompose releasing a vapor phase and a residual solid phase (biochar). On cooling the pyrolysis vapor, polar and high-molecular weight compounds condense out as liquid (tar or bio-oil) which generally contains small amounts of water, while low-molecular-weight volatile compounds remain in the gas phase (syngas). The condensable gas may break down further into non-condensable gases (CO, CO_2, H_2, and CH_4), liquid and char. This decomposition occurs partly through gas-phase homogeneous reactions and partly through gas-solid phase heterogeneous thermal reactions. In gas-phase reactions, the condensable vapor is cracked into smaller molecules of non-condensable permanent gases such as CO and CO_2. The

remaining solid component after pyrolysis is charcoal, referred as biochar when it is produced with the intention of adding it to soil. High char conversion, low ash carry over, lower tar level, quick response to load different solid wastes and simple construction are some of the most important advantages of the downdraft gasifier over other fixed bed gasifiers (Laird *et al.*, 2009; Basu, 2010; Verheijen *et al.*, 2010).



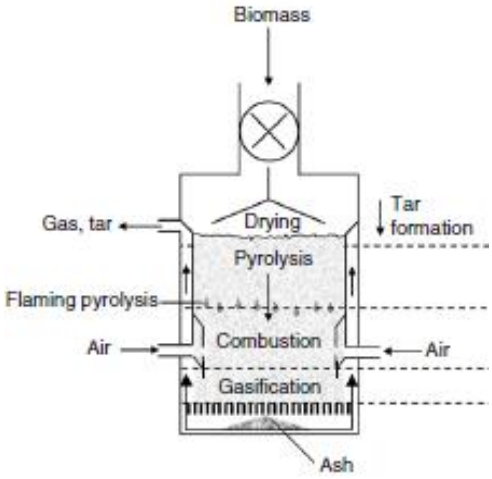


Figure 10 (left) and Figure 11 (right): Downdraft gasifier (Basu, 2010)

3.3. Biochar production, yield and its characterization

After assembling the gasifier, several test trials were launched to calibrate the equipment for an optimal use. The optimal feedstock upload was set to 800 g per production cycle. The feedstock residence time was set to 30 minutes. Three ranges of temperature (400-450°C, 450-500°C and 500-550°C) and three heating rates (25°C min^{-1}, 35°C min^{-1} and 45°C min^{-1}) were investigated to assess their effect on the biochar yield and its characteristics. To determine the biochar yield, the following equation was used:

Biochar yield (%) = [weight of biochar generated (g) / Oven dry weight of raw material (g)]* 100

To characterize the different types of biochar produced the following analyses were performed: pH , EC , ash content, elemental analyses (N, C, H, O contents), gross and net heating values, CO_2 emission factor, solid state ^{13}C nuclear magnetic resonance (NMR), and scanning electron microscopy images. To assess the safe use of biochar in soil and its potential phytotoxic effect, a germination and root elongation test of *Lepidium sativum* was performed.

3.3.1. pH, Electrical conductivity (EC) and Ash content

To measure pH, the biochar was soaked in ultra-pure water at a ratio of 3:50 solid/water for 2 hours with frequent agitation. The pH measures were determined using a pH meter (Crison Basic 20) provided with a glass electrode Crison 52-00.

The EC was measured on filtrates at 1:10 biochar/water ratio using a conductimeter XS cond 510. Ash content was measured using a modified ASTM method (D-1762-84). This measure is based on determination of weight loss. Briefly, about 5 g of oven-dried samples biochar (at 105°C for 24 hours) were weighed and then combusted at 750°C for 6h (Peng *et al.*, 2011). The samples were cooled to room temperature in desiccators and weighed again. The ash content was calculated as follows: Ash content (%) = [Weight of ash (g) / dry mass of biochar (g)] *100

3.3.2. Elemental analysis

Elemental carbon, nitrogen and hydrogen concentrations of biochar were determined by a dry oxidation using an elemental analyzer FLASH 2000 series CHNS/O Analyzer, Thermo Scientific, UK, operating according to the dynamic flash combustion. Biochar samples were weighed in a tin capsule and introduced into the combustion reactor by an auto-sampler. At the entering of the reactor, the sample reach a furnace heated at 900 – 1000°C. A small volume of pure oxygen is added to the system to help to burn the material, converting the sample into elemental simple gases. A separation column and TCD detector allows the determination of the elemental composition of the sample. Oxygen content was determined by difference (Demirbas, 2004; Novak *et al.*, 2009; Angın, 2013) as follows: O%= 100%-(C%+H%+N%+Ash%).

3.3.3. Biochar heating values and CO_2 emission factor

The heating value of any fuel is defined as ‘the energy released per unit mass or per unit volume of the fuel when the fuel is completely burned (Boundy *et al.*, 2011). The gross heating value (GHV) known also as the higher heating value, accounts all the released heat during combustion in addition to the heat that might be carried away with water vaporization while the net heating value (NHV), or the lower heating value, is excluding the latent heat of water formed during combustion. To express the efficiency of a thermal system, European countries use normally NHV, in contrary, in USA and Canada GHV is used (Basu, 2010; Boundy *et al.*, 2011).

CO_2 emission factor indicates the amount of carbon which can be emitted from any fuel under complete combustion. This factor gives an idea on the amount of carbon emission biochar prevents to release into the atmosphere (Anke, 2003).

The heating values and the CO_2 emission factor are calculated automatically by the elemental analyzer.

3.3.4. Scanning electron microscopy

Morphological characterization of the biochar was performed through a scanning electron microscopy images (SEM Zeiss Supra 40). The samples produced under different heating rates and under the same pyrolysis temperature were mixed to form a representative sample. Therefore, we investigated solely the effect of pyrolysis temperatures on the morphology of the produced biochars without considering the heating rate.

3.3.5. Nuclear magnetic resonance (NMR)

The 3 representative samples of biochar produced were analyzed through ^{1}H and ^{13}C NMR spectroscopy by recording mono (1D) and multidimensionnel (2D) spectra. The biochar samples were pulverized in a porcelain mortar. For each sample, 2 aliquots of 1g each were mixed. The first aliquot was added to 100 ml of $CHCl_3$ and the second to 100 ml of solution of H_2O/MeOH (1:1). The mixtures were shacked for 1 hour, filtered on whatman filter papers and then with sterile filter membrane of cellulose acetate (∅ 0.45 µm). The samples were then dried using a rotating evaporator. The solid residues of the extracts in $CHCl_3$ were dissolved in 700µL of $CDCl_3$ and the solid residues of the extracts in H_2O/MeOH were dissolved in 1ml of D_2O using TPS (3-trimethylsilyl-1-propane sulfonic acid) as internal standard. Each solution was transferred in ∅ 5 mm NMR tube.

NMR spectra were recorded using a Bruker Avance III operating at 400.13 MHz at 25°C. For each sample 1D ^{1}H, 2D ^{1}H COSY, ^{1}H *Jres*, ^{1}H-^{13}C HSQC and HMBC were recorded.

The 1D ^{1}H spectra were recorded with domain time of 32K on a spectral width of 6009.615 Hz, 64 scans with a relaxation delay of 2s. The 2D ^{1}H *Jresolved* spectra were recorded with a domain time of 4K on a spectral width of 6009.615 Hz for the axix F2 and 200.056 Hz for the axis F1, 32 scans in 128 experiments and a relaxation delay of 1.5s. 16 scans were performed in vacuum before recording. The 2D ^{1}H COSY spectra were recorded with a time domain of 4K a spectral width of 6009,615 Hz, 32 scans in 256 experiments, a relaxation delay of 2 s and 16 scans in vacuum prior to record. The spectra HETCOR 2D ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC NMR spectra were recorded with a time domain of 4K on a spectral width of 6009,615 Hz for the nucleus ^{1}H and 25154.953 Hz for the nucleus ^{13}C, 64 scans in 512 experiments, a relaxation delay of 2s and 16 scans in vacuum prior starting.

3.3.6. Phytotoxicity germination and root elongation test

The seed germination and root elongation test is a simple method of environmental biomonitoring. This method was applied to assess the phytotoxicity of the char produced on the germination and root elongation of *Lepidium sativum*.

Both non washed and washed char samples were used. The weighed samples of biochar were washed by a volume of water 10 times equal to their weight, and then dried in oven at 60°C. The washed and non washed biochar samples were brought to 60% humidity and centrifuged for 10 minutes at 5000 RPM at 10°C. The solution was filtrated at 0.2 µm, diluted to 10% and 30% and used as a germination media. This test was carried out in Petri dishes with a filter paper (80mm Waatman N°1 filter) on the bottom. Each dish contained 1.5 ml of diluted solutions and 10 seeds. The Petri dishes were then wrapped by parafilm then placed in germination chamber at 25°C for 48 hours. The number of germinated seeds and the root elongation for control and for each treatment were measured to calculate the germination index through the following formula:

$$GI(\%) = (Gt*Lt/Gc*Lc)*100$$

Gc: average number of germinated seeds in control
Lc: average seeds elongation in control
Gt: average number of germinated seeds in treatment
Gt: average seeds elongation in treatment

3.3.7. Statistical analysis

Analysis of variance (ANOVA) was used to assess the effects of pyrolysis temperatures and heating rates on the production yield of biochar. The Duncan-Waller test (α= 5%) was applied to assess the differences among the means of the replicates. Statistical analysis was performed using the software SPSS V.17.

4. Results and discussion

4.1. Effects of pyrolysis temperatures and heating rates on biochar yield

Table 4 shows the results of the analysis of variance at P<0.05 of the obtained biochar yield. This analysis pointed out a significant effect of pyrolysis temperatures and heating rates. The combined effect of these parameters was not significantly different.

Table 4 : ANOVA analyses of the biochar yield (P < 0.05)

Source	df	F	Sig.
Pyrolysis temperature (Pt) (°C)	2	27,656	0,000
Heating Rate (Hr) (°C min^{-1})	2	6,387	0,005
Pt * Hr	4	1,465	0,238

Figures 12 and 13 show the effect of pyrolysis temperature and the effect of heating rate on the biochar yielding, respectively. Means with different letters indicate significant difference between values (Duncan-Waller test; P < 0.05). Figure 12 shows the biochar yield as a function of pyrolysis temperature. As it was expected,

the biochar yield decreased as the pyrolysis temperature raised. Increasing the pyrolysis temperature from 400-450°C to 500-550°C induced a reduction in biochar yield from 38.06% to 29.20% (8.86% decrease). Therefore, lower temperatures should be selected to obtain a higher biochar yield. Indeed, Şensöz *et al.,* (2006) found out that the char yield of olive bagasse pyrolysis decreased from 35.3% to 30.6% as the pyrolysis temperature increased from 350 to 550°C. Similar results were recorded on olive husk pyrolysis (Demirbas, 2004). Our data are in agreement also with other studies done with cellulosic and lingo-cellulosic biomass. This decrease of biochar yield can be explained by higher primary decomposition of the biomass beside the possible secondary decomposition of the biochar itself to generate more gas (Parihar *et al.*, 2007).

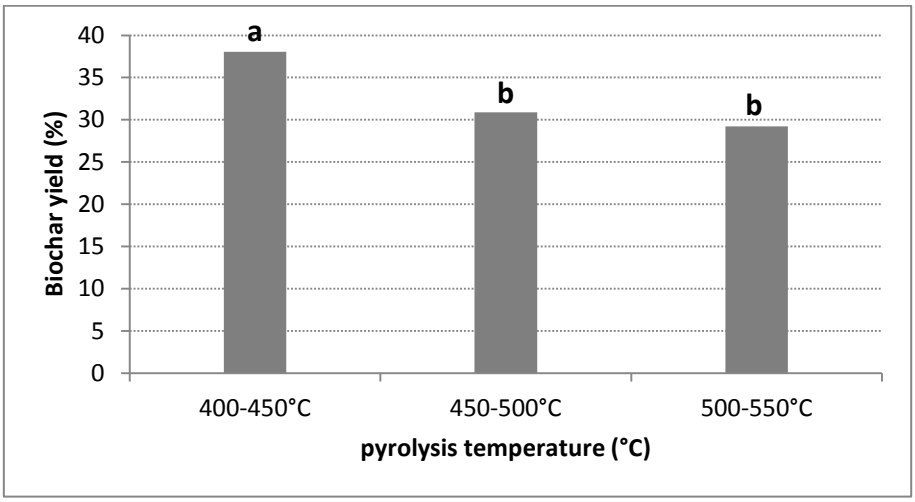


Figure 12: Effect of pyrolysis temperature on biochar yield. Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05).

The effect of heating rate on the yield of biochar is shown in Figure 13. According to what was observed for the raising of pyrolysis temperature on biochar yield, similar effect was recorded with the increase of the heating rate. Shifting from 25°C min^{-1} to 45°C min^{-1}, the biochar yield decreased from 35.08% to 30.38% (4.7% decrease). The higher yield of biochar under low heating rate in comparison to the yield obtained under higher heating rate can be explained by the cellulose decomposition. The exothermic decomposition of cellulose into a more stable form of anhydro-cellulose produces biochar. While heating the biomass rapidly (high heating rate) the dehydration of cellulose into anhydro-cellulose is slowed down and consequently favors gas production (Chen *et al.*, 1997; Liu *et al.*, 2005; Şensöz *et al.*, 2006; Brown, 2009).

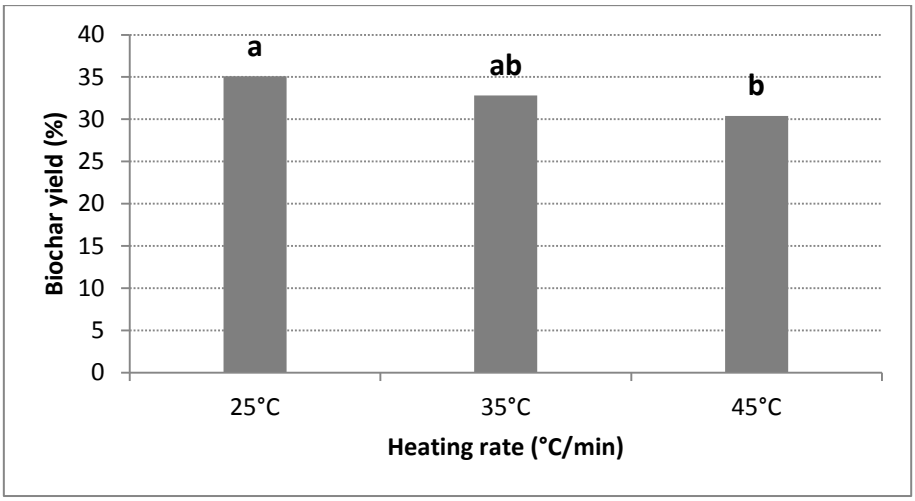


Figure 13: Effect of the heating rate on the biochar yield. Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05).

Figure 14 shows the trends of biochar yield at the heating rates of 25, 35 and 45°C min^{-1} in relation to increasing of pyrolysis temperatures. At the heating rate of 25°C min^{-1}, an increase of pyrolysis temperatures from 400-450°C to 500-550°C, leads to a biomass conversion of 58.57% and 65.61% respectively. At the same temperatures, biomass conversion of 65.51% and 73.14% was obtained with a feedstock heated at 35°C min^{-1}. At a heating rate of 45°C min^{-1} the biomass conversion was of 70.69 and 71.12%, respectively. According to these values, the most convenient pyrolysis parameters to choose, if the aim is reducing the weight of the polluting solid olive mill wastes, would be a heating rate of 45°C min^{-1} reaching a final temperature of 450-500°C.

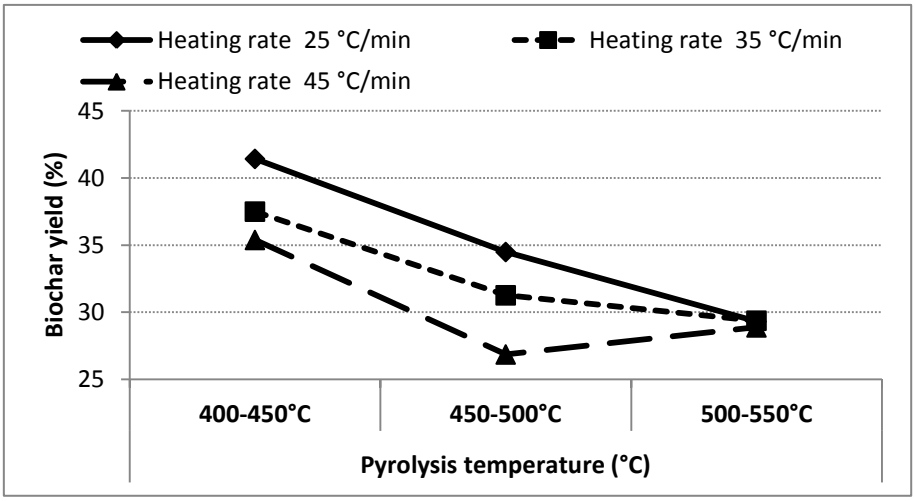


Figure 14: Effects of pyrolysis temperature and heating rate on biochar yield

4.2. pH, Electrical conductivity and ash content

The pH, EC and ash content of the produced biochars are reported in Table 5. These values are the mean of three replicate measurements. All the biochars had a basic pH. The lowest pH (8.8) measured on biochar produced at pyrolysis

temperature of 450°- 500°C and heating rate of 35°C min^{-1} and the highest one (9.7) on the biochar produced at 400°- 450°C and heating rate 25°C min^{-1}. No clear effect of pyrolysis temperature and heating rate was revealed. Contrastingly with the results obtained by Angın (2013) who found that the biochar pH continued to increase with the increase of temperature, the pH values obtained in our experiment were on a range between 8.8 and 9.7. On the other hand, the EC of the produced biochars increased with increasing pyrolysis temperature and also with increasing heating rates. For the heating rate of 25°C min^{-1} the EC values of biochar increased from 0.28 dS/m to 0.38 dS/m for pyrolysis temperature of 400°- 450°C and 500°- 550°C, respectively. Similar results were observed for the heating rates of 35°C min^{-1} and 45°C min^{-1}. The lowest EC value (0.28dS m^{-1}) was recorded on biochar produced at 400°- 450°C and at heating rate of 25°C min^{-1}. The highest one (0.47dS m^{-1}) was recorded on biochar produced at a temperature of 500°- 550°C and heating rate of 45°C min^{-1}. These values are considerably low in comparison to manure derived biochar, which reach 2.2dS m^{-1} (Cantrell *et al.*, 2012) and consequently wouldn't cause any unfavorable salts effects in case that high quantities of biochar are incorporated into soil (Lehmann and Joseph, 2009).

We recorded an increase in the ash content by raising the pyrolysis temperature. For the heating rate of 25°C min^{-1}, the ash content of biochar increased from 7.87% to 9.72% for the pyrolysis temperature 400 - 450°C and 500 - 550°C, respectively. These results were also reported in the literature for various types of biomass such as oak wood, corn stover and poultry litter (Nguyen and Lehmann, 2009). On the contrary, increasing the heating rate, while maintaining the same pyrolysis temperature, led to a slight decrease of the biochar ash content. Under pyrolysis temperature of 450-500°C the ash content decreased from 8.81% to 8.48% for heating rates of 25°C min^{-1} and 45°C min^{-1}, respectively.

Table 5: pH, EC and Ash content of the produced biochars.

	Pyrolysis temperature (°C)		
	400°-450°C	**450°-500°C**	**500°-550°C**
Heating rate of 25°C min^{-1}			
pH (1:50)	9.7	8.8	9.1
EC (dS/m)	0.28	0.34	0.38
Ash content (%)	7.87	8.81	9.72
Heating rate of 35°C min^{-1}			
pH (1:50)	9.1	8.8	9.2
EC (dS/m)	0.29	0.38	0.40
Ash content (%)	7.71	8.60	9.65
Heating rate of 45°C min^{-1}			
pH (1:50)	9.0	8.9	9.4
EC (dS/m)	0.31	0.36	0.47
Ash content(%)	7.50	8.48	9.52

4.3. Elemental analysis

The elemental analysis was performed on micronized samples of the various produced biochars. C (%), N (%), H (%), Oxygen (%) and the atomic ratios H/C, O/C and (O+N)/C are reported in the Table 6. The biochar carbon content increased with both the raising of pyrolysis temperature and heating rate. Carbon content increased from 70.23% to 84.14% by raising pyrolysis temperature from 400 - 450°C to 500 - 550°C. Similar effect was noticed by increasing the heating rate and maintaining the same pyrolysis temperature. Shifting from a heating rate of 25 to 45°C min^{-1} to reach a pyrolysis temperature of 450-500°C, the carbon content raised from 75.31% to 80.49%. N, H and O contents decreased with increasing pyrolysis temperature and heating rate. Similar trends were obtained using olive husk indicating a higher carbon content coupled with a greater losses in H and O while increasing temperatures (Demirbas, 2004).

H/C molar ratio is an indicator of the degree of aromaticity of biochar. As temperature and heating rate rose, the atomic ratio H/C decreased gradually. This indicates that biochars became increasingly more aromatic and carbonaceous. These trends are assigned first to a higher degree of carbonization resulting in more aromatic structures formation and second to the elimination of polar surface functional groups. Therefore, increasing pyrolysis temperatures generates more aromatic and less polar biochar (Lehmann and Joseph, 2009; Cantrell *et al.*, 2012). H/C atomic ratio can give an idea about the structure of the biochar. For example, an H/C ratio between 0.4 and 0.6 of the aromatic portion of the char indicates that every second to third C is connected with a proton. H/C ratio <0.1 indicates a more graphite-like structure. The H/C ratio of cellulose and lignin is nearly equal to 1.5. Black carbon is defined by having an H/C<0.2 and present a continuum, starting from partly charred material to graphite with no general boundaries (Krull *et al.*, 2009). By comparison to those values, it is obvious that the produced biochar present a high degree of aromaticity, having the majority of H/C values between 0.2 and 0.6. The O/C ratio of the biochar produced from olive mill waste decreased with increasing pyrolysis temperature as well as with increasing the heating rate indicating that the biochar surface is becoming less hydrophilic. The O/C values were all below 0.2.- Spokas (2010) reported that the O/C ratio can provide a robust indicator of biochar stability and values lower than 0.2 appears to provide, at minimum, a 1000-year biochar half-life.

Similar to the H/C and the O/C ratios, the (O+N)/C ratio decreased with increasing pyrolysis temperature and heating rates indicating a reduction in the biochar content of polar functional groups (Chen *et al.*, 2008).

Table 6 : C (%), N (%), H (%), O (%), atomic ratios H/C, O/C and (O+N)/C of the various produced biochars.

	Pyrolysis T° (°C)		
	400°-450°	**450°-500°**	**500°-550°**
Heating rate of 25°C min^{-1}			
C (%)	70.23	75.31	84.14
H (%)	4.97	3.64	1.89
N (%)	1.02	0.94	0.73
O (%)*	16.10	11.29	3.51
H/C **	0.85	0.58	0.27
O/C **	0.17	0.11	0.03
(O+N)/C	0.18	0.14	0.12
Heating rate of 35°C min^{-1}			
C (%)	74.65	79.77	77.49
H (%)	3.82	3.08	3.25
N (%)	0.94	0.88	0.88
O (%)*	12.89	7.67	8.72
H/C **	0.61	0.46	0.50
O/C **	0.13	0.07	0.08
(O+N)/C	0.12	0.08	0.08
Heating rate of 45°C min^{-1}			
C (%)	76.92	80.49	77.78
H (%)	3.51	2.82	3.10
N (%)	0.90	0.78	0.91
O (%)*	11.16	7.44	8.68
H/C **	0.55	0.42	0.48
O/C **	0.11	0.07	0.08
(O+N)/C	0.04	0.09	0.09

***calculated by difference. **atomic ratio**

4.4. Biochar heating values and CO_2 emission factors

The gross heating values, the net heating values and the CO_2 emission factors of the produced biochar are reported Table 7. The heating values indicate the potential use of biochar as fuel. Both measures of GHV and NHV of the produced biochar showed comparable values. The measured heating values of the produced biochar under different temperature ranges and heating rates, are all comparable to the levels of solid fuels ranging from lignite to anthracite (Raveendran and Ganesh, 1996).

Regarding the CO_2 emission factor, the amount of carbon emitted to the atmosphere in complete combustion depends on its composition in C, H, N, O, and ash (Anke, 2003). Taking into account that the biochar produced in this study is all generated from the same feedstock, then the differences in the CO_2 emission factors can be explained by the effects of pyrolysis temperatures and heating rates. The increasing pyrolysis temperature and heating rates increased the CO_2 emission factor. This can be linked to the results of elemental analysis which indicate higher amount of carbon with increasing temperatures and heating rates.

Table 7 : Heating values and CO_2 emission factors of the produced biochars.

	Pyrolysis temperature (°C)		
	400°-450°	**450°-500°**	**500°-550°**
Heating rate of 25°C min^{-1}			
Gross heating value (MJ/kg)	31,28	31,09	31,63
Net heating value(MJ/Kg)	30,21	30,31	31,22
CO_2 emission factor (TCO_2/TJ)	85,20	91,07	98,79
Heating rate of 35°C min^{-1}			
Gross heating value (MJ/kg)	31,12	31,83	31,28
Net heating value(MJ/Kg)	30,30	31,17	30,58
CO_2 emission factor (TCO_2/TJ)	90,29	93,82	92,86
Heating rate of 45°C min^{-1}			
Gross heating value (MJ/kg)	31,47	31,69	31,17
Net heating value(MJ/Kg)	30,71	31,08	30,50
CO_2 emission factor (TCO_2/TJ)	91,79	94,91	93,47

4.5. Scanning electron microscopy

The micro-structural features of the biochars produced at the three temperature ranges were investigated by means of scanning electron microscopy (SEM). The samples produced at 400-450°C showed low visible porosity. Instead, the presence of crystalline phases with cubic, tubular and elongated shapes on the particles surfaces was obvious making the particles rough and grainy. As the pyrolysis temperature increased (450-500° and 500-550°C) the biochar particles showed smooth surfaces and the porosity increased. The pore sizes were not uniform and were in the range of tens of nanometers to several tens of microns (Fig.15).

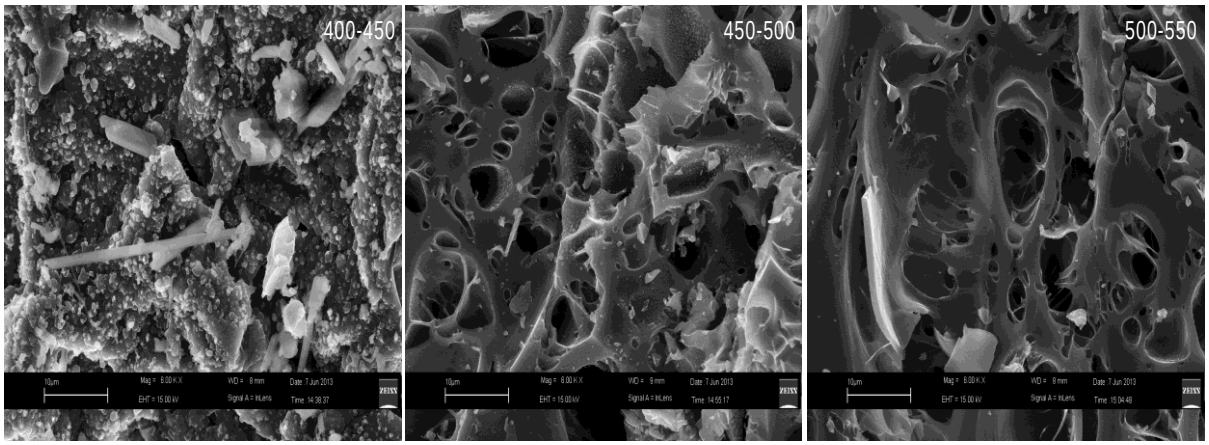


Figure 15: Scanning electron micrograph of biochar produced at 400 - 450°C (Left) , at 450 - 500°C (Center) and at 500 - 550°C (Right).

4.6. Nuclear magnetic resonance (NMR)

The NMR spectra of the 3 biochar samples extracted in water (Fig.16) are quite similar to each other. The biochar produced at 400 - 450°C shows a spectrum with high intensity of signals. Raising the pyrolysis temperature, the biochar produced

at 450 - 500°C and at 500 - 550°C showed a lower intensity of the peaks. The spectrum signals were identified and attributed to low molecular weight metabolites. At low frequencies (0.90, 1.06, 1.56 and 2.19 ppm) the observed signals were assigned to free fatty acids which became soluble probably due to water/methanol mixture. At 1.34 ppm a doublet signal is assigned to methyl lactate. In the 2D ^{1}H COSY spectrum, the latter, coupled with the quartet signal attributed to (CH) at 4.12 ppm. The singlet at 1.92 ppm was assigned to the methyl of acetic acid and the singlet at 2.41 ppm was attributed to the succinic acid.

At 2.04 and 2.51 ppm the multiplets are assigned respectively to glutamic acid and glutamine. At 2.74 and 2.82 ppm the singlets are relative to the methyl groups of the dimethylamine (DMA) and trimethylamine (TMA). The singlets at 3.26 and 3.36 ppm were assigned respectively to the trimethylamine N-oxide (TMAO) and the choline. The signals at 3.15 and 3.94 ppm to creatine. At 3.56 and 3.64 the doublet of doublets are assigned to the free glycerol. At 4.18 and 4.27 ppm the signals identified were related to the doublet of doublets at threonine and proline. At higher frequencies the detected signals were linked to phenylalanine (7.31, 7.36 and 7.47 ppm). At 8.45 ppm a very intense singlet was recorded and assigned to formic acid and at 9.38 the signal was attributed to aldehydic groups which decrease considerably in the biochars produced at 450-500°C and at 500-550°C.

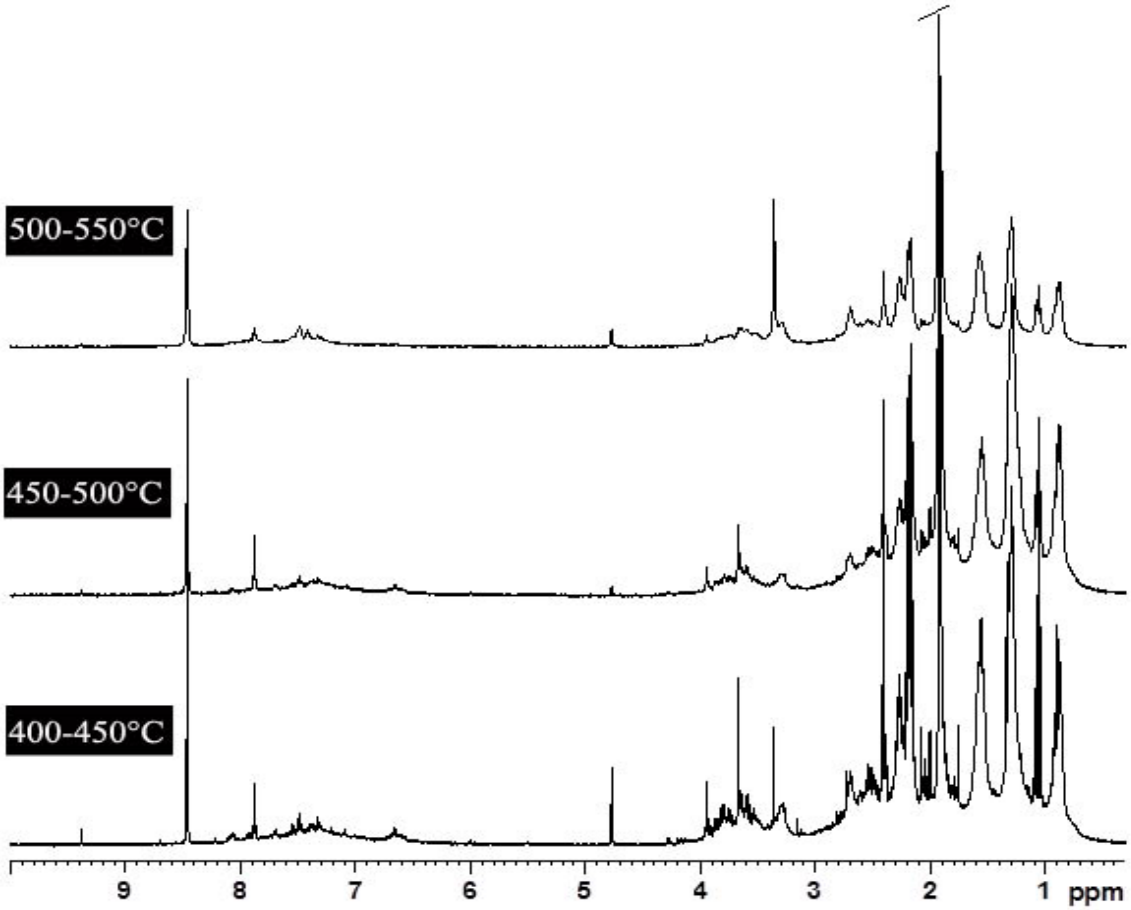


Figure 16: 1D ^{1}H NMR spectra in D_2O of biochar water extracts.

The NMR spectra of the biochar extracts in chloroform (Fig.17) show differences between the extract of biochar produced at 400-450°C and the other 2 extract samples (450-500°C and 500-550°C). On the ^{1}H NMR spectrum the typical NMR profile of fatty acids of the olive oil is observed. The spectrum shows a triplet at 0.86 ppm indicating the presence of methyl terminal group. The multiplet at 1.26, 1.58, 1.99 and 2.30 ppm indicate respectively the presence of aliphatic CH_2 chain, β CH_2 carbonyl, CH_2 double bound, CH_2 carbonyl. A multiplet at 5.32 ppm linked to vinyl unsaturated fatty acids. Similar to the olive oil, made of about 80% of oleic acid, in this sample the unsaturated fatty acids seems to be the most represented by oleic acids. No relevant intensity signals were seen (about 2.7ppm) for bis allyl linoleic and linolenic acids.

The major part of fatty acids are not estrified as triglycerides. However, in the 2D ^{1}H COSY spectrum (Fig.18) some peaks can be observed revealing intersection between the signals at 4.12, 4.27 and 5.20 ppm assigned to 2 groups of CH_2 and to the CH group of the glycerol in the triglycerides. Moreover, in the 2D ^{1}H ^{13}C HMBC spectrum two carbons are observed at 173 and 176 ppm respectively assigned to the carbonyl groups of esterified fatty acids and the free fatty acids. By integrating the 2 signals the calculation revealed that the free fatty acids are approximately 70% higher than the esterified acids. For the NMR spectra in $CDCl_3$ of the biochars produced at 450-500°C and 500-550°C, it was not possible to detect the signals of fatty acids. The spectrum was complicated, showing a remarkable broadening of signals and the presence of aromatic signals between 6.5 and 8.0 ppm which in the 2D ^{1}H ^{13}C HSQC mate with carbon in the range 125-130 ppm.

Because of the higher pyrolysis temperature, it could have been formed the polymeric species of the type polycyclic aromatic hydrocarbon (PAHs), as well as widely reported in literature (Sharma *et al.*, 2004; Kloss *et al.*, 2012). Guo and Rockstraw (2007) and Lehmann *et al.* (2011) revealed that with the rise of pyrolysis temperature surface area and ash increase in meanwhile the surface functional groups providing exchange capacity decrease. It was reported also that biochar produced under high temperature contains large amount of carbon concentrated in form of aromatic rings and shows lower functional groups to generate surface charge and ion exchange due to de-carboxylation. On the other side, low temperature produced biochars show significantly higher C=O and C-H functional groups, thus enhancing their sorption capacity (Glaser *et al.*, 2002; Hammes *et al.*, 2006).

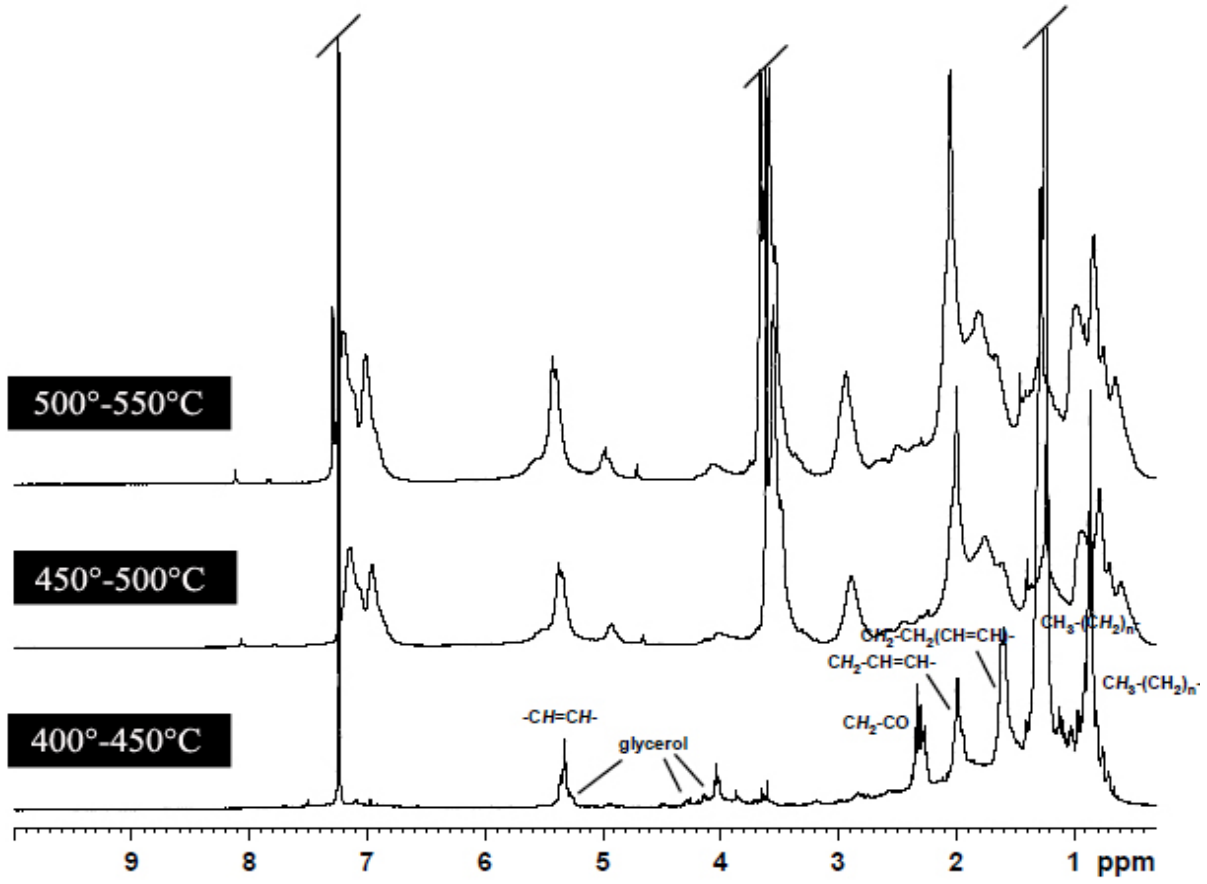


Figure 17 : 1D ^{1}H NMR spectra in CDCl3 of the biochar chloroform extracts.

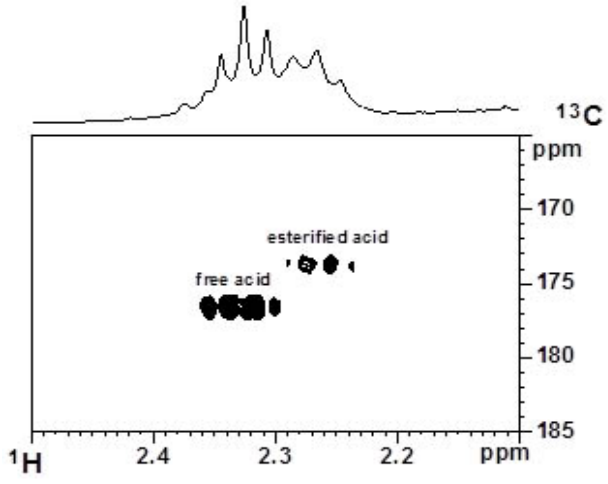


Figure 18: 2D ^{1}H ^{13}C HMBC in $CDCl_3$ of the biochar chloroform extract.

4.7. Phytotoxicity germination and root elongation test

The assessment of biochar phytotoxicity through the germination and root elongation test carried out with *Lepidium sativum* seeds revealed no phytotoxic effect (Tab.8). The Non washed biochar produced at 400-450°C, 450-500°C and 500-550°C, were characterized by germination indexes of 68, 64 and 65% respectively. Water washed biochar produced at the same pyrolysis temperature, raised the germination indexes to 71, 68 and 67%, respectively. The highest germination indexes, all above the phytotoxic levels (77 and 71%) were recorded on both extracts (10 and 30%) of the biochar produced at 400-450°C. The other washed biochar shown germination indexes above the phytotoxicity threshold, even thought with lower values. This lower germination indexes confirms the results obtained by the NMR analysis. In fact, the origin of this reduction might be linked to the formation of polycyclic aromatic hydrocarbons (PAH) produced under higher pyrolysis temperature. The higher indexes obtained in the washed samples can be only explained by the fact that washing the biochar with water leached some of the PAH's phytotoxic compounds.

Table 8: Germination index of *Lepidium sativum* (NW: non-washed biochar. W: washed biochar).

	Biochar 400°-450°C			
	NW	**NW**	**W**	**W**
Dilluted aqueous extract (%)	10	30	10	30
GI(%)	72	68	77	71
	Biochar 450°-500°C			
	NW	**NW**	**W**	**W**
Dilluted aqueous extract (%)	10	30	10	30
GI(%)	69	64	71	67
	Biochar 500-550°C			
	NW	**NW**	**W**	**W**
Dilluted aqueous extract (%)	10	30	10	30
GI(%)	65	65	68	66

5. Conclusion

Interest in biochar is continuously rising among scientists, policy makers, agriculture experts and lay people. Solid olive mill waste can be well managed through pyrolysis. This process, allowing a reduction of this waste up to 73%, presents a promising solution to manage this kind of waste. As already said, the pyrolysis experiments indicated that the properties of biochar were influenced by the

pyrolysis temperature and the heating rate. The increase of those parameters led to a decrease in the biochar yield and its nitrogen, hydrogen and oxygen contents. The surface functional groups decreased, but the carbon content increased. All biochars obtained showed high heating values and can, by consequence, be directly used like fuel. The ones produced at low temperature are highly concentrated in C, show low electrical conductivity, high CO_2 emission factor and higher surface functional groups can be devoted for soil incorporation to improve the agricultural soils fertility, absorb pollutants such as metals as well as offering a long term carbon sequestration.

Biochar can represent a key element for a new green revolution; it can be one of the most convenient tools to mitigate the global warming, an effective way to revalorize the degraded and low fertile soils and a promising amendment to counter balance the pollution of soils and waters.

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Chapter 4

Effect of biochar amendment in reducing nickel absorption, uptake and translocation in tomato grown on perlite under controlled conditions

1. Abstract

Soil application of organic amendments is one of the management practices used in the remediation of toxic compounds in soil. A wide range of soil amendments has been studied in this framework. The main aim of the experiment reported in this chapter is to investigate the effect of biochar, produced from olive mill waste through slow pyrolysis, in reducing Ni absorption of tomato plants. To achieve this goal, tomato plants were cultivated in perlite amended with biochar at three rates (0, 5 and 10% W/W). Plants were grown under controlled conditions in a growth chamber and irrigated with half strength Hoagland solution spiked with 3 different Ni concentrations (0μM; 0.1μM and 0.2μM). Vegetative growth parameters were monitored and Ni content in shoots and roots was measured to evaluate the effect of biochar on the nickel content in plant tissues, the total uptake and the translocation factor. In the presence of biochar, increasing nickel concentrations showed better plants' growth. Adding biochar, both in absence and presence of nickel led to higher number of leaves, internodes and higher shoots and roots dry weights. Increasing biochar amendment rate lowered Ni concentration in tomato shoots and roots indicating lowered absorption of this metal. The translocation factors indicated negligible amounts of Ni transferred from roots to shoots in presence of biochar.

2. Introduction

The potential benefits of biochar in agriculture and environment have attracted significant attention in recent years. Beside its potential to mitigate climate change, improve soil properties and enhance plant growth, biochar is getting more interest for its ability to retain and immobilize metals in soil.

Various types of biochars derived from different feedstock materials can have variable properties and consequently behave differently in soil. In deed various feedstock derived biochars used as amendment in contaminated soils resulted in wide variability regarding metals sorption capacities.

Therefore, it is of high importance to investigate the ability of a particular biochar to adsorb metals in order to define its suitability to be used as soil amendment to immobilize metals and limit their availability to plants.

In this framework, this experiment was set to assess the effect of biochar, produced from olive mill waste, in reducing the tomato absorption of Ni grown on perlite under controlled conditions. The effect of biochar amendment rate and Ni

concentrations in the growth media were monitored through the plant growth parameters, the plant tissues Ni content, the plant Ni uptake and the Ni translocation factor.

3. Materials and Methods

3.1. Tomato seeds

The tomato seeds (*Lycopersicum esculentum*) used in this experiment belongs to the hybrid variety 'Costanza' produced by the Japanese company 'Seed ASAKII professional hybrid'. This indeterminate growth variety is characterized by a high resistance to diseases and suitable for production under greenhouse.

3.2. Growth media

Perlite, known to have a very low cation exchange capacity bordering to zero, was selected to be the inert growth media. 'Perlite Agro' was purchased from the Greek company 'Vioryp' and the product is qualified being chemically inert, sterile and free of diseases and weed seeds. The perlite was amended with biochar produced from olive mill waste through slow pyrolysis at a temperature range of 400-450°C.

3.3. Chemicals and nutrient solution

The chemicals used to prepare the half strength Hoagland stock solution (nutrient solution) and their final concentrations are reported in the table 9. The chemicals were purchased from Sigma Aldrich - Germany. Deionized water (Elix; Millipore Corporation) was used for nutrient solution preparation. Ultra-pure water (18.2 MΩ cm-1 - Milli-Q; Millipore Corporation) was used for chemical analysis.

Table 9 : Half strength Hoagland's nutrient stock solution (Millner and Kitt, 1992)

Nutrients	Final concentration
$Ca(NO_3)_2.4H_2O$	2.5mM
KNO_3	2.5mM
$MgSO_4.7H_2O$	1.0mM
KH_2PO_4	20.0µM
$Na_2MoO_4.2H_2O$	0.2µM
H_3BO_3	10.0µM
$ZnSO_4.7H_2O$	1.0µM
$CuSO_4.5H_2O$	0.5µM
$MnCl_2.4H_2O$	2.0µM
NaFe EDTA	50.0µM

3.4. Experimental design

The standard guidelines of US Environmental Protection Agency (EPA) for plant uptake and translocation test were followed to perform this experiment. The statistical experimental design adopted was a completely randomized design with five replicates as each replicate was composed by 3 plants.

3.5. Seed germination and transplantation

Tomato seeds were placed over moist filter paper in Petri dishes sealed with parafilm, incubated at 25°C for three days until germination. The seedlings were transferred to a half strength Hoagland solution (Tab.9) without Ni. Later on, three uniform seedlings with 2 to 3 cm root length were selected to be transplanted into the growth media.

3.6. Growth media, nutrient solution and experimental conditions

The growth media was composed of perlite amended with three rates of biochar 0, 5 and 10% weight/weight. The seedlings were irrigated with half strength Hoagland solution at three different concentrations of Nickel sulfate hexahydrate ($NiSO_4.6H_2O$) - 0µM (control), 0.1µM (treatment1) and 0.2µM (treatment2). Glass beakers (250mL) wrapped with aluminum paper were used as pots. The beakers were placed in a growth chamber (FDM mod. C1500S; F.lli Della Marca S.r.l- Italy) in a completely randomized design with five replicates for each treatment (Fig.19 and Fig.20). The following conditions were maintained for the whole test period until a sufficient biomass was developed: photoperiod of 16h light and 8h darkness, day/night temperatures at 25/22°C and relative humidity equal to 60% during light periods and 75% during dark periods. Irrigation was scheduled and applied based on daily controls of the nutrient solution levels inside the beakers to guaranty permanent root contact with the spiked nutrient solutions. The nutrient solutions were renewed weekly. Total amounts of used solutions were recorded for further calculations.

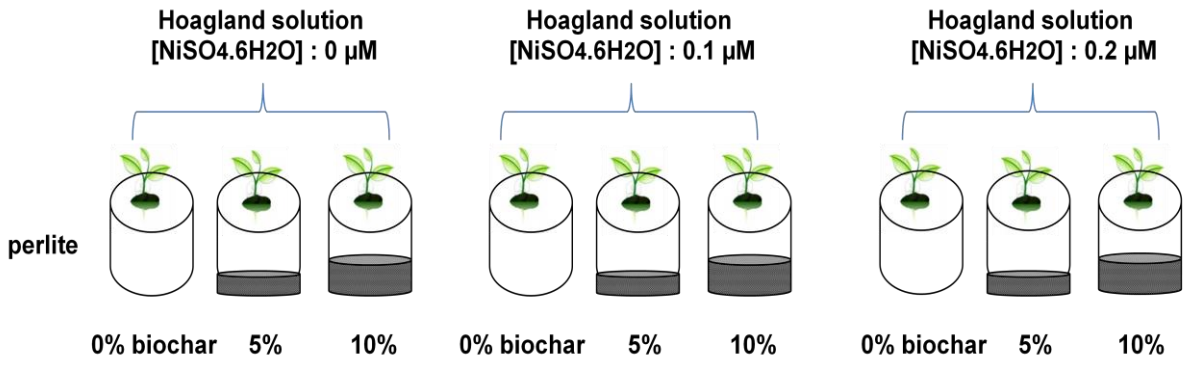

Figure 19: Schematic design of the experiment.

Figure 20: Tomato plants inside the growth chamber.

3.7. Plant growth parameters

The tomato plants were grown for a period of 30 days. At the end of the growth period the leaf chlorophyll content was measured using a portable chlorophyll meter (Minolta SPAD-502). The number of leaves and internodes were counted. Shoot and root length was measured and weighed (fresh weight). Roots were immersed in a solution of 0.05 M $CaCl_2$ at pH=3 adjusted with HCl for half an hour in order to remove adsorbed nickel on root surface. Later on, roots were washed with tap water, rinsed with distilled water and placed in oven to dry. Dry weights of shoots and roots were measured after incubation in oven at 60°C until constant weights were recorded.

3.8. Ni content of plant tissues

The dried plant tissues were grinded using an agate mortar and pestle to avoid contamination. A wet digestion of the dried grinded samples (1ml H_2O_2 + 5ml HNO_3 for 20 minutes at 190°C) was used to extract the nickel content using a microwave digester (CEM model, MARS Xpress). Samples were filtered by filter paper whatman N°42 and the filtrates were diluted (1:25) with ultra pure water. Nickel content, expressed in mg kg^{-1} of dry weight, was determined using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Thermo Electron ICAP 6300).

3.9. Translocation factor and total uptake

The translocation factor (TF) was used to evaluate the translocation of nickel from the roots to the shoots. TF is defined as the ratio of the metal concentration in the shoots to that in the roots (Yu and Zhou, 2009). It is used to measure the effectiveness of plant in transferring a chemical from roots to shoots (Sun *et al.*, 2009). The total uptake (TU) of nickel was determined to assess indirectly the effectiveness of the biochar in reducing the metal uptake by the plants. TF was calculated by multiplying the metal concentration in shoots or roots by the shoots or roots biomass. The calculation of these indexes is shown by the following equations:

TF= (µg metal/ g shoots dw)/ (µg metal/ g roots dw)

TU= µg metal g^{-1} shoots or roots dw X g shoots or roots dw per plant

3.10. Statistical analysis

To assess the effects of biochar rate and Ni concentrations on the dependent variables (number of leaves, number of internodes, chlorophyll content, shoots and roots length, fresh and dry weights and their nickel content) multivariate analyses of the recorded data were performed using SPSS version 17. Comparison of means was done by Duncan-Waller test at a level of 0.05.

4. Results and discussion

4.1. Effects of biochar rate and Ni concentrations on the growth parameters

Results of the statistical analysis related to the effects of Ni and biochar and their interactions on the measured growth parameters are given in Table 10. Results show that Ni had statistical significant effect on the chlorophyll content, root fresh and dry weight. Biochar affected significantly all the measured parameters except the chlorophyll content. Interactions between nickel concentrations and biochar rates were not statistically significant for all the measured parameters.

Table 10 : ANOVA Multivariate analysis of the growth parameters

Source of variance	df	Number of leaves	Number of internodes	Chlorophyll content	Shoot lenght	Root lenght	Shoot fresh weight	Shoot dry weight	Root fresh weight	Root dry weight
Nickel	**2**	0.321	0.737	**0.002**	0.229	0.261	0.447	0.081	**0.004**	**0.001**
Biochar	**2**	**0.000**	**0.000**	0.111	**0.000**	**0.012**	**0.000**	**0.000**	**0.000**	**0.000**
Nickel * Biochar	**4**	0.422	0.479	0.356	0.389	0.640	0.333	0.339	0.676	0.712

Data represents P values of ANOVA (P < 0.05 are significant)

In the absence of biochar, the increasing concentrations of Ni had both positive effects on some plant growth parameters and negative influence on some others (Fig.21 and Fig.22). At a concentration of 0.1µM of Ni the number of leaves (NL) and the number of internodes (NI) were not significantly different to the control. Leaves chlorophyll content (Chc), shoot length (SL), root length (RL), shoot dry

weight (SDW) and root dry weight (RDW) showed lower values than the control by 6, 5, 11, 14 and 41% respectively. At higher Ni concentration (0.2µM) the NL, NI and SL recorded values were higher than the control by 20, 17 and 11 % respectively. On the contrary, for RL, SDW and RDW the results were lower than the control by 8, 9 and 14%, respectively.

Adding biochar at rates of 5 and 10% to the different concentrations of Ni showed no significant effect on the Chc, reduced the tomatoes' RL and improved all the other growth parameters.

In the absence of Ni, the addition of biochar at 5 % induced higher NL and NI respectively by 43 and 40%. Same effect was revealed on SL, SDW and RDW which increased by 30, 79 and 89%, respectively. Only the RL showed a lower value in comparison to the control (15% lower). The plant growth responses to the addition of 10% biochar were positive and higher than the values observed on 5% biochar addition. The NL, NI, SL, SDW, RDW registered higher values than the control by 66, 62, 31, 89 and 73%, respectively. For the RL the same reduction, as while adding 5% biochar, was recorded and reached 14%.

At 0.1µM Ni the addition of 5% and 10% biochar induced an increase in NL, NI, SL, SDW, RDW and a decrease in RL. The same effects were observed by adding biochar at a Ni concentration of 0.2µM. The recorded RL for all the treatment showed lower values in comparison to the control. This might give a prejudgment that the Ni and biochar amendment inhibited the development of the root. In contrary, while examining the digital photos of the plants (Fig.23) it became obvious that the roots were denser and developed horizontally on the upper layer of the growth media. This was confirmed by recording a higher RDW over passing the control treatment by 73, 28, and 80% respectively for the treatments (0µM Ni*10%B), (0.1µM Ni*10%B) and (0.2µM Ni*10%B).

These positive impacts of biochar on the growth of plants are in agreement to other results reported in literature. Testing 7 woody species Chidumayo (1994) reported that adding charcoal to soil improved seed germination by 30%, shoot heights by 24% and biomass production by 13%. Similar results were obtained on lettuce and cabbage cultivated on soil amended with biochar produced from rice husk. High increase in biomass roots and shoots production, plant heights and number of leaves were recorded (Carter *et al.*, 2013).

The improvement of plant growth by adding biochar can be explained also by the essential nutrients released from the biochar itself. Mukherjee and Zimmerman(2013) demonstrated through a batch extraction and column leaching experiments using a variety of fresh and aged biochar, pure and mixed with soil, that biochars contained plenty of nutrient forms and had different release rates explaining the biochars effect on soil fertility over time.

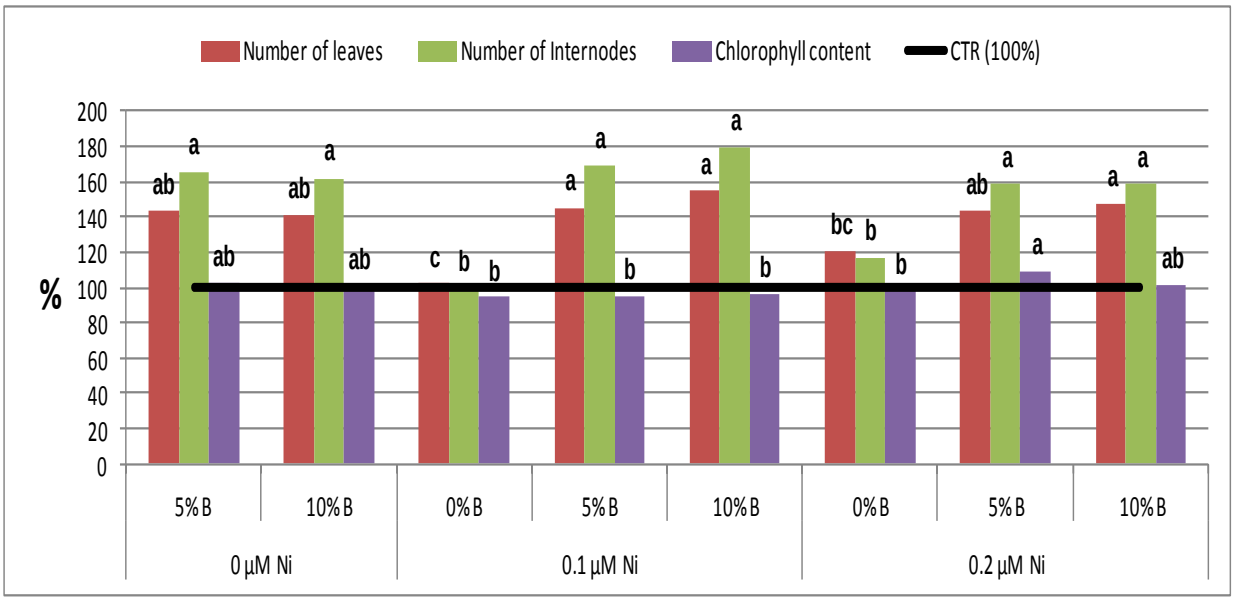

Figure 21 : Effects of Ni concentrations and biochar amendment on tomato number of leaves, internodes and leaf chlorophyll content. Values are means in comparison to the control (100 %). Means with different letters indicate significant difference between values (Duncan-Waller test; P < 0.05).

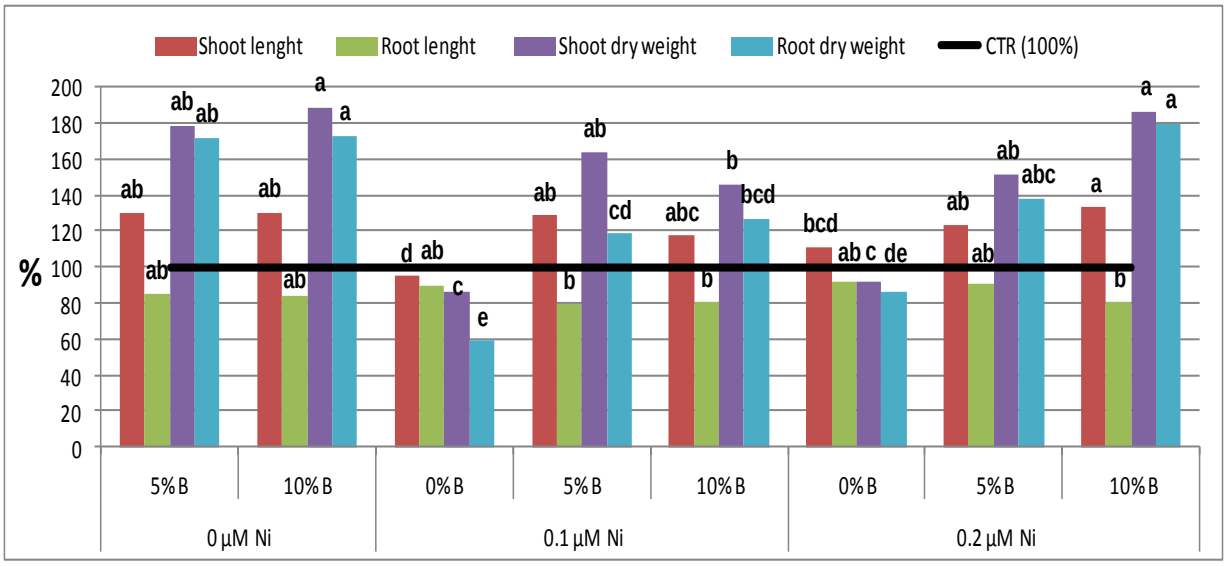

Figure 22: Effects of Ni concentrations and biochar amendment on tomato shoot length and dry weight, root length and dry weight. Values are means in comparison to the control (100 %). Means with different letters indicate significant difference between values (Duncan-Waller test; P < 0.05).

0µM Ni*0%B

0µM Ni*5%B

0µM Ni*10%B

0.1µM Ni*0%B

0.1µM Ni*5%B

0.1µM Ni*10%B

0.2µM Ni*0%B

0.2µM Ni*5%B

0.2µM Ni*10%B

Figure 23: Tomato plants' responses to biochar and nickel treatments.

4.2. Nickel content, uptake and translocation factor

The mean values of Ni content in tomato roots and shoots are presented respectively in Figures 24 and 25. Ni concentration in plant tissues grown as control treatment (0µM Ni* 0% biochar) were 3.9 mg Kg^{-1} dw and 0.8 mg Kg^{-1} dw in roots and shoots, respectively. These levels of Ni in the control plants can be explained by the impurities of the chemicals used to prepare the Hoagland solution and probable contamination caused by the operational tools.

The recorded values of Ni content in tomato roots showed higher amounts in comparison to the levels recorded in shoots. In this experiment, the roots were in permanent contact with the nutrient solution spiked with Ni which explains the higher contents of Ni in roots. It was reported in the literature that metals accumulated more in the roots in comparison to the shoots when the Ni is applied in the root feeding solution. An experiment, conducted by Cash and Leone (1987), investigating the accumulation of Ni in tomato plants under Ni foliar application and root applied Ni, revealed that shoot-treated plants accumulated more Ni in leaves than in roots and the inverse was true for root treated plants. In addition, roots present the primary route for the metal ions penetration into the plant tissues (Piechalak *et al.*, 2002).

In absence of Ni in the growing media, the roots of the plants grown on the perlite amended with 5 and 10% of biochar accumulated concentrations of Ni of 27.2 and 23.7 mg Kg^{-1} dw, respectively. The latter observation indicates that biochar contained some amount of Ni released in the growing solution and assimilated by the plants.

The highest accumulations of nickel in the roots were recorded on the treatment (0.1µM Ni*0%B) and (0.2µM Ni*0%B) reaching a mean values of 84 and 84,2 mg Kg^{-1}dw respectively. The biochar addition decreased considerably the Ni content in roots. At the rate of 5% biochar addition, a reduction of Ni root content by 28 and 26% were recorded respectively on the plants irrigated with 0.1µM and 0.2µM of Ni. The same trend was observed also for an amendment of biochar at a rate of 10%. The reduction percentages of Ni roots content recorded were 53 and 75% respectively for the treatments 0.1µM and 0.2µM Ni (Fig.24).

Both, plant physiological factors and growing media properties, affect Ni uptake by plants. The most important factor is the influence of growing media pH. The uptake is reduced while increasing alkalinity (Kabata and Mukherjee, 2007). In fact, the added biochar had a pH value of 9 which has raised the pH of the root growing environment leading to the reduction of Ni uptake by roots. Studying the uptake of Ni by cereals and vegetables from soils amended with sewage sludges and artificially contaminated with nickel revealed that under low pH values of the soil, the plants were able to uptake the highest amounts of Ni (Sauerbeck and Hein, 1991). In addition the porous structure of the biochar, shown by the scanning electronic microscopy images, and its richness in functional groups, revealed by the NMR analysis, have both a key role in adsorbing a considerable amount of Ni. The

literature supports these results. An application of chicken manure and green waste derived biochars showed a significant reduction in Cd and Pb accumulation in Indian mustard plants (Park *et al.*, 2011). A broiler litter derived biochar formed at a pyrolysis temperature of 350°C improved the immobilization of Cu, Cd and Ni (Uchimiya *et al.*, 2010). Another experiment conducted on rice plantations revealed that biochar produced from rice plants (straw, husk and bran) could decrease noticeably the concentrations of Cd, Zn and Pb in rice plants up to 98, 83 and 72% respectively (Zheng *et al.*, 2012).

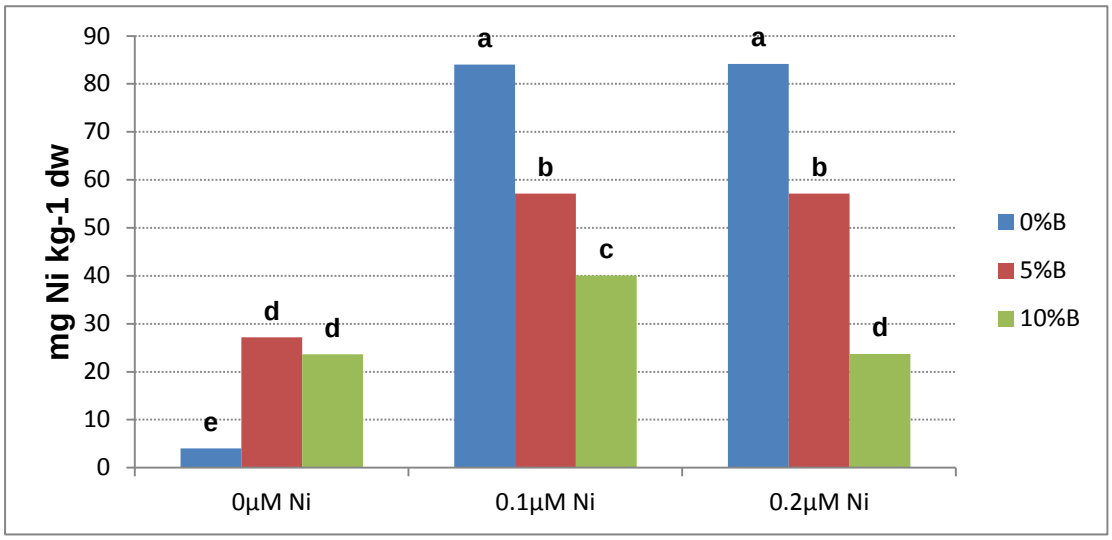

Figure 24: The effect of biochar amendment on nickel root content. **Means with different letters indicate significant difference between values (Duncan-Waller test; P < 0.05).**

The Ni concentrations in the shoots (Fig.25) were relatively low in comparison to the levels recorded in the roots. The highest recorded level of Ni content reached 10.8 mg Kg^{-1} dw, which is under the threshold of toxicity level comprised in the range from 25 to 50 mg Kg^{-1} dw (Gouugh *et al.*, 1979). For the Ni concentration of 0.1µM, the biochar added at 5% was coupled with higher Ni content in shoots of 19%. At 10% biochar added, the Ni content was reduced by 61%. For the treatment 0.2µM, adding biochar at rates of 5 and 10% reduced the Ni content of shoots by 8 and 47%.

The recorded results related to the roots and shoots content in Ni indicate that biochar addition has reduced the plant absorption of this metal.

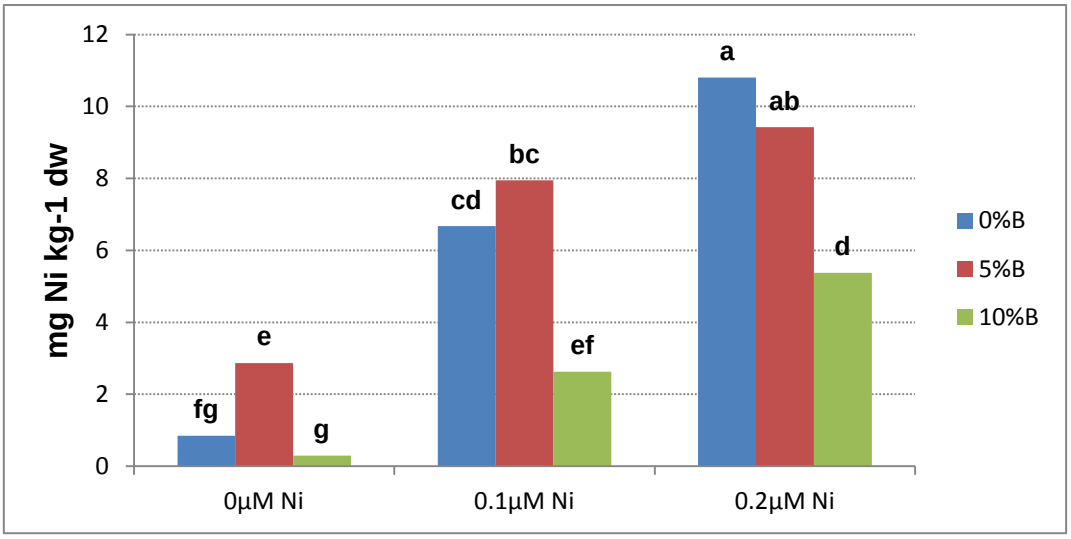

Figure 25: The effect of biochar amendment on nickel shoot content. **Means with different letters indicate significant difference between values (Duncan-Waller test; P < 0.05).**

Ni uptake values are presented in Table 11. For all tested treatments the values of Ni were very low and almost negligible. The highest recorded uptake values were recorded on roots in comparison to shoots. These results confirm that the roots are the main accumulation site of Ni. Translocation factors are presented in Table 12. The transport and the storage of Ni by plants is metabolically controlled (Kabata and Mukherjee, 2007). Studying the Ni uptake by tomato plants in a sandy loam soil contaminated with a range from 58 to 168 mg kg^{-1} dw Poulik (1999) found that the Ni absorbed by the tomato plants was translocated at a level of 75% from the roots to the shoots. Singh et al. (2010) found that Ni translocation factor of 0.94 on naturally spontaneous grown plants over a contaminated site.

In contrary, the recorded results of this experiment revealed much lower translocation factor (Tab.12). The highest translocation factor was recorded in the treatment (0.2µM Ni*10%B) with a value of 0.23 of Ni absorbed by roots passed to shoots. The lowest translocation factor was recorded in the treatment (0µM Ni*10%B) reaching the value of 0.01. According to the literature, the biochar amendment can reduce the translocation and mobility of the metals in the plant tissues. It was reported that straw char application decreased significantly the plant transfer coefficients of Cd, Zn and Pb into rice shoots (Zheng *et al.*, 2012). In this experiment, the Ni total uptake and the translocation factor were very low indicating adsorption of Ni by the biochar.

Table 11 : Nickel uptake by roots and shoots of tomato plants.

Treatments	Nickel uptake of roots (µg plant^{-1} dw)	Nickel uptake of shoots (µg plant^{-1} dw)
0µM Ni*0%B	0.079	0.068
0µM Ni*5%B	0.952	0.438
0µM Ni*10%B	0.837	0.048
0.1µM Ni*0%B	1.011	0.487
0.1µM Ni*5%B	1.071	1.095
0.1µM Ni*10%B	1.042	0.322
0.2µM Ni*0%B	1.485	0.837
0.2µM Ni*5%B	1.611	1.207
0.2µM Ni*10%B	0.869	0.854

Table 12: Nickel translocation factor.

Treatments	Translocation factor
0µM Ni*0%B	0.21
0µM Ni*5%B	0.11
0µM Ni*10%B	0.01
0.1µM Ni*0%B	0.08
0.1µM Ni*5%B	0.18
0.1µM Ni*10%B	0.06
0.2µM Ni*0%B	0.13
0.2µM Ni*5%B	0.16
0.2µM Ni*10%B	0.23

5. Conclusion

In this laboratory scale experiment the biochar was used as amendment in an artificially contaminated perlite growth media. The aim was to identify if biochar can reduce the Ni absorption of tomato plants. Through monitoring the plant growth parameters, the results showed that biochar addition at different rates to non-spiked and spiked perlite with low levels of Ni had improved the vegetative growth. Increasing rate of biochar amendment induced higher values of NL, NI, SL, SDW and RDW.

Measuring the plant tissues Ni content, the plant uptake and the Ni translocation factor indicated that biochar was able to reduce the plant Ni absorption. The Ni content in roots and shoots of tomato showed considerable reductions with

increasing biochar rate. The total Ni uptake measured on the plants' tissues showed very low levels in order of 0.6 to 1.6 µg.plant^{-1} dw. The translocation factor itself showed very low values indicating a very low translocation of Ni from the roots to the shoots.

Olive mill waste derived biochar amendment showed promising results in reducing the plant absorption of Ni. It improved the vegetative growth, reduced the metal content in the plants' tissues, its uptake and also its translocation from the roots to the shoots.

Combined with its capacity to improve the physical and chemical properties of the soil, olive mill waste derived biochar can be a good candidate to be used as soil amendment for *in situ* remediation of metal contaminated soils.

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Chapter 5

Capacity of biochar produced from olive mill waste in remediating a zinc smelter contaminated soil

1. Introduction

Metals are naturally occurring in low concentrations in the Earth's crust. With the fast growth of the industrialization, human activities such as mining and smelting activities, in addition to fossil fuel combustion and use of cadmium containing phosphate fertilizers resulted in excessive anthropogenic metal emissions. Exceeding by far the natural sources emissions, anthropogenic emissions have distorted dramatically the geochemical cycles and the biochemical balances of the metals (Sebastiani *et al.*, 2004). Emitted into the atmosphere and deposited in soils and waters, metals – persistent and non-biodegradable, were and still are responsible for many environmental problems as this threats human health as well as ecosystems. Lead, chromium, cadmium, copper, zinc and mercury are among the most frequently observed metal contaminants.

The accumulation of metals in soils at toxic levels can even completely destroy the natural vegetation resulting in a bare soil. Moreover, metals can percolate in the soil profile and consequently pollute the surface and groundwater. They can enter the human food chain through plants and animals as they tend to bio-accumulate. Agricultural products containing increased amounts of toxic metals may lead to serious health problems and possible acute or chronic toxicities (Vangronsveld *et al.*, 1995a; Vangronsveld *et al.*, 1995b; Callender, 2003; Ruttens *et al.*, 2010; Uchimiya *et al.*, 2010; Beesley *et al.*, 2011; Park *et al.*, 2011; Zheng *et al.*, 2012).

In arable soils the presence of metal contaminants can limit and change their use for agricultural production. Contamination with metals can lead to considerable decreases in soil microbial activity, alter soil fertility and can cause high losses in plant productivity (Yang *et al*., 2005). In plants, metals provoke biochemical and physiological disorders modifying several metabolic processes (MacFarlane et al., 2003). The photosynthetic rate is reduced dramatically which leads to a decrease in growth and productivity (Van Assche *et al*., 1988). At the cellular level, exposure of plants to toxic metals leads to the production of reactive oxygen species (ROS). At moderate level the latter serve as signals for the plants to activate antioxidant responses for detoxification. At higher levels ROS can perturb the cellular redox balance and cause a strong damage (Keunen *et al*., 2011).

To cope with metal contaminated soils, in recent years the *ex situ* traditional technologies, known to be expensive, labor-intensive and destructive for the structure and biological activities of the soil (Mulligan *et al*., 2001), are being replaced by *in situ* technologies. The latter do not involve soil removal and by consequence do not

destroy the biological and the functional integrity of the soil. Qualified to be sustainable processes with lower effective costs and moreover ecologically viable, phytoremediation, bioremediation and the use of metal immobilizing soil amendments have shown promising results for treating contaminated soils (Cohen *et al.*, 2004; Vangronsveld *et al.*, 2009; Beesley and Marmiroli, 2011; Amer *et al.*, 2012).

Scientific experiments conducted up to now, testing the effect of various soil amendment materials on metals immobilization, have shown attractive and promising results being easily applicable and successful in restoration and revegetation of contaminated sites. Among a wide range of soil amendments, composted materials, cyclonic ashes, lime, steel shots, red mud and many others, positive results have been observed in reducing metal mobility, availability and phytotoxicity (Bolan *et al.*, 2003; Adriano *et al.*, 2004; Gray *et al.*, 2006; Ruttens *et al.*, 2006; Ruttens *et al.*, 2010; Amer *et al.*, 2012).

Recently, increasing interest was devoted to another soil amendment known as 'biochar'. The 'biochar' was defined as black carbon formed by the pyrolysis of biomass by heating it under oxygen-free atmosphere so that it is not subject to complete combustion (Jha *et al.*, 2010). Depending on the biomass material source and the pyrolysis process parameters, key physical and chemical characteristics (pH, CEC, functional groups, total surface area, porosity, etc.) of the produced biochar may vary considerably and by consequence behave differently in the soils where it is applied. Attracting the scientists' attention, due to numerous positive effects while applied to soil, biochar showed promising capacities for improving the physicochemical and biological soil functions besides sequestrating carbon. It can increase the net soil surface area (Chan *et al*., 2007), hence the cation exchange capacity (CEC) and pH, improve the soil water and nutrient retention (Verheijen *et al*., 2010; Downie *et al*., 2009). It was also reported that biochar can provide nutrients in their available forms (Laird *et al.*, 2010a), decrease their leaching in soils, thus improving the nutrient cycle by enhancing nutrients dynamics and protecting groundwater (Glaser *et al*., 2002).

Beside all these features, the high porous structure of biochar and the richness of its surface functional groups (Lehmann and Joseph, 2009; Namgay *et al.*, 2010) form its potential to adsorb and sequester toxic metals (Liu and Zhang, 2009; Steiner *et al*., 2007) a valuable tool to be used as a soil additive for remediation of metal contaminated soils.

Until now, no general recommended application rate for soils was presented in literature due to the insufficient field data to generalize its use for a specific soil type, a specific crop or a specific aim. In addition, the high variability of biochar material characteristics and mineralization rates make it difficult to specify a common application rate. More thorough experiments testing different biochar application rates need to be conducted in order to gather more information concerning its behavior, its short- and long-term effects and to draw a clear picture of appropriate doses for pre-defined aims (Chan *et al.*, 2007; Quilliam *et al.*, 2012). Therefore, our experiment

aimed to evaluate the effect of biochar produced from solid olive mill waste, an important biomass highly abundant in the Mediterranean basin, for remediating a metal contaminated agricultural soil sampled 500 m NE from a zinc smelter in the region of Lommel, Belgium (Ruttens *et al.*, 2011).

1.1. Mind mapping and performed tasks

In order to investigate the capacity of biochar for sequestrating the metals, the metal contaminated soil was amended with increasing rates of biochar and stabilized for two different periods (aiming to evaluate the biochar aging effect). Initial and final total and $Ca(NO_3)_2$ exchangeable metal concentrations were determined. This chemical analysis (selective extraction) is routinely used to monitor soil phytotoxicity, but it can lead to misinterpretations since plant availability in and consequently uptake of metals from the substrate are function of several soil parameters, e.g. pH, organic matter, cation exchange capacity and chemical form of the metal and also of plant parameters (*e.g.* root exudation of organic acids, siderophores, *etc.*). Therefore, an evaluation of the soil phytotoxicity through a biological test, using plant species is of interest to obtain more reliable and accurate results. In metal contaminated soils, the assimilation of toxic amounts of zinc, cadmium, copper, nickel and other metals is reflected by inhibiting plant growth and reducing biomass production (Vangronsveld and Clijsters, 1994). Stunted growth, leaf epinasty and chlorosis are visible symptoms of strong metal toxicity. However, at lower degrees of soil pollution, these visible symptoms are less pronounced or can even be absent, although reduction of plant quality and inhibition of biomass production persist. For that reason, monitoring some stress-induced metabolic processes in the plant tissues can be relevant to detect potential phytotoxicity resulting from the interference of the metals assimilated through the roots with these metabolic processes. The presence of phytotoxic amounts of metals in plant cells leads to inhibition of several enzymes and increases the capacity of others (Vangronsveld and Clijsters, 1994). This induction of the capacity of a particular group of enzymes is strongly correlated to shoot growth inhibition and biomass reduction (Van Assche *et al*., 1988). It is considered to play an important role in the stress metabolism caused by toxic metal concentrations in the cell (Vangronsveld and Clijsters, 1994).

Induction of peroxidases is related to oxidative reactions, while the activities of several enzymes of the intermediary metabolism (*e.g.* malic enzyme, isocitrate dehydrogenase, glutamate dehydrogenase) and glucose-6-phosphate dehydrogenase (key enzyme of the oxidative pentose-phosphate pathway) are possibly stimulated to compensate for the decrease of ATP and NADPH normally provided by the metal sensitive photosynthetic reactions (Vangronsveld and Clijsters, 1994) or to enhance the cellular reducing capacity. This increase of enzyme capacity, together with morphological parameters (e.g. length, biomass, leaf area), can be applied as diagnostic criteria for an integrated evaluation of soil phytotoxicity due to

several metals (Vangronsveld and Clijsters, 1992). In this work, reduction of phytotoxicity by soil treatment with biochar was evaluated by this test system. Therefore, the treated soil was used to grow *Phaseolus vulgaris* on which growth parameters and capacities of the pre-mentioned stress enzymes were measured. In addition, the metal concentrations and the soluble protein contents in primary leaves and roots were determined as additional potential indicators.

Besides monitoring the soil phytotoxicity using the above-mentioned test, an assessment of the microbiological status of the treated soil can be of high interest to get a deeper view on the efficiency of biochar in sequestering the metals and restoring the different functional features of the soil. Therefore, after harvesting the bean plants the recuperated soil was used to conduct a Biolog Ecoplate test. This test provides a rapid screening of soil communities in terms of Ecocarbon use. We used the Biolog Ecoplates to obtain a snapshot of the metabolic activity of the bacterial assemblages in the metal contaminated soil without and with addition of biochar. The fast and high consumption of Eco-carbon sources are an indication of higher soil microbial communities. The more Eco-carbon sources that can be used indicate a more diverse microbial community.

In order to create a more complete overall picture of the biochar efficiency reducing the metal stress in soil and creating a better environment for the soil living organisms, a standardized soil eco-toxicology test using soil invertebrates was performed (OECD, 1984). Soil earthworms also called ‘Ecosystems engineers’ play an important role in degrading dead organic matter and have a positive effect on bacteria and fungi in soils. Their survival and proliferation in soils are performant indicators for soil quality. Therefore, a survival and reproduction test of red earthworms *Eisenia foetida* was performed firstly to determine the acute and chronic toxicity of the treated soil and secondly to investigate any potential negative effects of biochar application on this soil living organism, which were quite often reported in literature (Liesch *et al.*, 2010; Li *et al.*, 2011; Weyers and Spokas, 2011).

2. Materials and Methods

2.1. Experiment 1: Effects of biochar amendment and stabilization period on soil pH, EC, total and $Ca(NO_3)_2$ exchangeable metals

2.1.1. Sampled soil and used biochar

The soil used in this experiment originated from an experimental field located in Lommel (Belgium) 500 m NE of a metal smelter in Balen (Belgium). This experimental field site was a former maize field (out of production since 1999). In the North East of Belgium and in the South of the Netherlands (Campine region), an area of about 700 km² is contaminated with metals due to past activities of different pyrometallurgical metal smelters. Metal mobility in soils of this region is relatively high, due to the sandy texture and an acid soil pH. A shift from pyrometallurgical to hydrometallurgical process technologies in the early 1970s drastically reduced emissions of metals to the environment. However, historic soil contamination still is responsible for a continued metal exposure of people and ecosystems in the area (Staessen *et al.*, 1999; Nawrot *et al.*, 2006; Hogervorst *et al.*, 2007).

The soil is currently characterized by total concentrations of 0.8 to 17.0 mg kg^{-1} for Cd and several hundreds mg kg^{-1} for Zn and Pb (Ide, 1992), while background metal levels in these soils are in the range of 0.1-0.5 mg Cd kg^{-1}, 25-70 mg Zn kg^{-1}, and 5-40 mg Pb kg^{-1} (De Temmerman *et al.,* 2003). A large portion of this area is currently in agricultural use, but several local vegetable harvests (*e.g.* carrot, scorzenera) cultivated for food industry have already been confiscated by the Belgian Federal Agency for Food Safety (FAVV) because Cd concentrations in the crops were exceeding legal threshold values for human consumption (Ruttens *et al.*, 2011).

Soil sampling was based on an unsystematic sampling “X” scheme. Five subsamples were taken using a shovel at a depth of 0-20 cm after eliminating the top two cm of the soil. The subsamples were mixed to form a representative sample. The soil was air-dried and then sieved through a 1cm sieve. Stones and gravels were excluded. In this experiment a sandy loam non contaminated soil was used as reference.

To determine the pH, soil was soaked in distilled water at a ratio of 1:2.5 (weight/volume) and shaken overnight. The samples were filtered using a filter paper Whatman N°1 and pH was measured using a WTW Multi 197i equipped by an electrode Sen Tix 51. On the same filtrates the electrical conductivity was measured at 20°C, expressed in dS m^{-1}, using a WTW microprocessor conductivity meter LF537 equipped with an electrode Tetrocon 96. pH was determined also in saline solution of potassium chloride KCl (1M). Same procedure was followed, as previously described, with just 1 h of shaking samples.

Soil total metal content was determined by a wet digestion of 0.5 g of air-dried 2 mm sieved soil, in 4 ml aqua regia (1 ml HNO_3 and 3 ml HCl) using a microwave (Milestone 1200 Mega). Samples were filtered by filter paper Whatman N°40 (Ashless) and the filtrates were diluted (1:50) with ultra pure water. The exchangeable fractions of the metals were extracted with calcium nitrate -. 25 ml of

0.1M $Ca(NO_3)_2$ were added to 5 g of soil. The mixture was shacked for 2 h, filtered by filter paper Whatman N°40 (Ashless) and then 200 µL of super pure HNO_3 was added to the filtrates. All metal contents expressed in mg kg^{-1} were determined using Inductively Coupled Plasma Optical Emission Spectrometry 700 Series ICP-OES Agilent Technologies.

The biochar used in this experiment as soil amendment was produced through slow pyrolysis of olive mill waste. This biochar was pyrolysed in a downdraft gasifier (GEK - All power labs) at a temperature range of 400 - 450°C with a feedstock residence time of 30 min. Some key characteristics of this biochar are presented in the following table 13.

Table 13: Key characteristics of the used biochar

Biochar characteristics	pH (1:50)	EC (dS m^{-1})	Ash (%)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	H/C *
	9.0	0.31	7.50	76.92	3.51	0.90	0.55

***atomic ratio**

2.1.2. Soil-biochar mixtures preparation and analyses

To investigate the effects of the biochar and its stabilization in soil the following biochar rates were incorporated as soil amendments: 0, 5, 10 and 15% (w/w). Biochar was mixed with soil using a concrete mixer to obtain 5 replicates per treatment. The soil mixed with biochar at different rates was placed in a greenhouse , and moistened until saturation point every 15 days with distilled water, and left for stabilization for periods of respectively 30 (SP_{30}) and 90 (SP_{90}) days. At the end of the stabilization periods, sufficient quantities of the mixtures were recuperated for analysis. The air dried soil was separated from biochar first through sieving then through electrostatic separation. Soil pH, EC, total and exchangeable metal concentrations were determined as pre-described after the 30 and 90 days stabilization periods.

2.2. Experiment 2 : Growing of *Phaseolus vulgaris* and analysis of the soil microbial communities

2.2.1. Growth chamber experiment

Dwarf beans *Phaseolus vulgaris* cv. Limburgse vroege were sown in the non-contaminated soil, the untreated soil and in the biochar amended stabilized soils in 400ml polyethylene pots. For each treatment, five pots were sown, each with three seeds. The pots were placed in a climate chamber (Philips GreenPower LED research modules) in a fully randomized design. The plants were grown for 15 days. The following growth conditions were maintained for the entire period: photoperiod of 12h light and 12h darkness, day/night temperature of 22°/18°C, relative humidity 65% ±10%, photosynthetically active radiation of 170 µmol m^{-2} s^{-1} (light provided by Philips Green-Power LED modules (blue, red and far-red modules)). The plants were

irrigated on the first and the seventh day of the growth period with half strength Hoagland solution using 50ml/pot and with de-ionized water for the rest of the growth period.

2.2.2. Plant harvest and analyses

2.2.2.1. *Phaseolus vulgaris* vegetative growth and metal contents

By the end of the growth period (15 days), the bean plants were harvested and shoot length, fresh weights of roots and leaves were determined. The roots were carefully washed with tap water to remove the small attached biochar particles. Later on, roots' and leaves' dry weights were determined after drying in an oven at 60°C until constant weight.

Metal contents of roots and primary leaves were determined using a wet digestion in a digester heating block. The oven dried plant material was ground in a mill (Retch Type MM 2000) and homogenized thoroughly prior to metal extraction. Aliquots of 200 mg dry plant material were wet digested in hot HNO_3 (70-71%). At the end the tubes' content was dissolved with 0.5 ml supra pure HCl (20%) and 4.5 ml Millipore water. Blanks and certified reference material (trace elements in spinach, n° 1570a of the National Institute of Standards and Technology, U.S Department of Commerce) were included for quality control of the data. Metals in the extracts were analyzed using Inductively Coupled Plasma Optical Emission Spectrometry 700 Series ICP-OES Agilent Technologies.

2.2.2.2. *Phaseolus vulgaris* activities of anti-oxidative enzymes and soluble protein content

To determine the activities of anti-oxidative enzymes and soluble protein content, 0.5g fresh tissue of leaves and roots were snap frozen separately in liquid nitrogen and stored at -80°C until analyses. Root and leaf samples were ground in a mortar at 0°C, with a ratio of 1:5 (w/v), in Tris-HCl extraction buffer (0.1M) containing 1mM dithiotreitol (DDT) and 1mM EDTA with adjusted pH equal to 7.8. The homogenates were filtered and centrifuged at 13500 RPM for 10 minutes at 4°C using an Eppendorf 5810R centrifuge. The supernatants were used to determine the enzyme activities (Bergmeyer, 1974) expressed in mU per gram of fresh weight (FW) and the soluble protein (Bradford, 1976) expressed in mg g^{-1}FW. The capacities (the potential activity measured *in vitro* under non-limiting reaction conditions) of malic enzyme (ME), peroxidase (GPOD), iso-citrate dehydrogenase (ICDH) and glutamate dehydrogenase (GIDH) were determined spectrophotometrically, in a total volume of 1 ml, using a SHIMADZU UV-spectrophotometer UV-1800, following the method described by Van Assche *et al.* (1988).

Malic enzyme (E.C.1.1.1.40) activity was determined by monitoring the increase of absorbance at 340nm (with an extinction coefficient of NADP equal to 6.22 cm^2.µmol). The reaction mixture contained 0.1M L-Malate (pH 7), 15mM Tris-

buffer (pH 7.3), NADP, 36mM $MnSO_4$ and the extract. For peroxidase (E.C.1.11.1.7), the reaction mixture was composed by 0.1M KH_2PO_4 buffer, 8mM H_2O_2, 18mM guaiacol and the extract. The enzyme capacity was estimated by following the increase in absorbance at 436nm with an extinction coefficient of guaiacol equal to 25.5 cm^2.µmol. Iso-citrate dehydrogenase (E.C.1.1.1.42) capacity was determined from the reduction of NADP at 340nm. The reaction mixture consisted of a buffer composed by 0.1M Tris, 4.6mM DL-isocitrate and 52mM NaCl in addition to 0.12M $MnSO_4$ and NADP and the enzyme extract. For glutamate dehydrogenase (E.C.1.4.4.2) the reaction mixture was composed by 0.1M Tris buffer (pH 7.5), 1M NH_4Cl, 0.3M Alfa-KGA (pH 7) and NADH. The capacity was measured following the absorbance at 340nm.

The soluble protein content was determined with Bradford G-250 reagent (Bradford, 1976) using bovine serum albumin (BSA) as a standard. Briefly, the extracted root and leaf samples were diluted 1/5 and 1/20 respectively to be in the range of the standard (0-25µg/µl). Later on, 20µl of the standard and the diluted samples in triplicates were filled in 96 well plates. To each well 180µl of filtered 1/5 diluted Bradford was added. The plate was firstly shaken for 60 seconds then the measurements were performed after 30 minutes using a FLUOstar Omega BMG LABTECH spectrometer. The measurement parameters were as follows: Greiner 96 F-bottom, 0.5sec positioning delay, 20 flashed per well, absorbance at 595nm wavelength, shake duration before plate reading 10 sec 700rpm (Double Orbital).

2.2.2.3. Integrated evaluation system of the soil phytotoxicity

As pre-described the biological integrated system is based on the analysis of the morphological parameters (length, weight) together with the variation of the stress enzymes capacities in leaves and roots of the 15 day-old bean seedlings (Vangronsveld and Clijsters, 1992). The measured values of shoot length, root length and the different enzymatic capacities in primary leaves and roots were ‘transposed’ into phytotoxicity classes. Each of the pre-mentioned parameters was used to classify the soil in a given Phytotoxicity class. A reduced version of this classification system is presented in the table 14. The ‘Phytotoxicity index’ is calculated by summing up the toxicity class number obtained for each parameter and dividing this sum by the total number of parameters and rounding off to unity.

Table 14: Classification of biological data into phytotoxicity classes. Ranges apply to the relative (percentage) values of the results as compared to the respective control values.

Parameters		Degree of phytotoxicity (class number)			
		Not toxic (1)	Slightly toxic (2)	Moderately toxic (3)	Strongly toxic (4)
Shoot length		>85	85-70	70-50	<50
Root weight		>85	85-70	70-50	<50
Enzyme capacity					
Leaf	GPOD	<150	150-325	325-500	>500
	ME, ICDH	<125	125-175	175-250	>250
Root	GPOD, ME, GIDH	<125	125-175	175-250	>250

ME: malic enzyme; GIDH: glutamate dehydrogenase; ICDH : iso-citrate dehydrogenase ; GPOD : peroxidase.

2.2.3. Soil microbial community analyses

Biolog ECOplates were used to study the responses of the soil microbial community towards the changes occurring in the metal contaminated soil due to biochar incorporation and the stabilization period. Briefly, 5g of fresh soil was mixed with PBS-Buffer with pH adjusted to 6.8 at a ratio of 1:10. The flasks, and the buffer solution were autoclaved and the inoculation was done under a flow hood to avoid contaminations. The mixtures were shaken for 5 min manually then left to deposit for 10 min. 1ml of the resulting solution was diluted in 20ml of P-Buffer then used to inoculate each well with 120µl using an 8-channel pipette. The Ecoplates were sealed in a plastic bag and incubated at 30°C in the dark. The purple color development was measured at 590nm using a FLUOstar Omega BMG LABTECH spectrometer each 24 hours for 7 days.

The average well color development over time (AWCD) was calculated according to Garland and Mills (1991) using the formula:

$$AWCD=\sum(C-R)/n$$

where C is the color production in each well, R is the absorbance value of the plates' control well and n is the number of carbon sources equal to 31. Biolog readings data at 72 h of incubation were used to calculate the richness (R) and the Shannon Weaver index since it represented the shortest incubation time allowing a good resolution among the treatments. The R values were calculated as percentage of the oxidized carbon sources using an OD of 0.25 as threshold for positive response and Shannon Weaver indexes (H) were calculated (Garland, 1996;1997; Gomez *et al.*, 2004) using the formula:

$$H =-\sum P_i * \ln P_i \text{ , with } P_i=n_i/N$$

where n_i equals the AWCD of a particular carbon source and N is the sum of the AWCD for all carbon sources at 72h.

2.3. Earthworms' toxicity, growth and reproduction test

This test was conducted to evaluate the effects of long exposure of earthworms to the non-treated and treated soils on mortality, growth and reproduction of *Eisenia foetida.* The worms were cultivated to reach adult phase with 0.4 to 0.6g of individual weight. After depuration for 24h on filter paper, hydrated with distilled water, the earthworms were transferred to the 500ml wrapped jars filled with soil (10 worms/400g of soil) containing different rates of biochar (0, 5, 10 and 15% (w/w)) for a test period of 28 days. The biochar amended soil was stabilized for a period of 120 days. The initial weight of the 10 worms (average 5.25±0.25g) introduced to each jar was recorded to monitor weight changes. All soil and soil biochar mixture treatments were moistened to 80% of their water holding capacity 24h prior to starting the test to allow optimum moisture conditions for the earthworms. The jars were weighed at the start, and then moistened each week by adding the suitable amount of water to keep the moisture at 80%. To feed the worms, 5g of dried and ground cow manure were added on the soil surface each week. Each treatment was replicated five times and the jars were kept in a lighted room at 20°±1°C for the duration of the experiment.

At the end of the experiment the surviving worms were recuperated by emptying the soil from each jar in a tray. Cocoons' numbers were counted to assess worm reproduction. The worms were rinsed with distilled water, depurated for 24h and then the number and weight of the survived worms were recorded to assess mortality and growth.

2.4. Statistical analysis

Using SPSS version 17.0 software, the data were subjected to analysis of variance (ANOVA) and comparison of means was determined using the Duncan-Waller test.

3. Results and discussion

3.1. Effects of biochar amendment and stabilization period on soil pH, EC, total and $Ca(NO_3)_2$ exchangeable metal contents

3.1.1. Effects on soil pH and EC

The obtained results concerning the effects of biochar amendment and stabilization period on soil pH and EC are reported in table 15. These parameters were determined after 30 days and after 90 days of stabilization. The reported values present the means of three replicate measurements. Both pH and EC showed progressive increasing trends and statistical analysis revealed significant effect of both treatments. After incorporating biochar with raising rates soil pH rose from 6.2 in non-amended soil (0% biochar) to reach 7.3 in the soil amended with 15% biochar after 30 days and from 6.2 to 7.5 on the amended soil stabilized for 90 days. For all

biochar amendment rates, soil pH showed higher increases for the samples stabilized for 90 days.

Regarding the soil EC similar increasing trends were observed. Increasing the biochar amendment from 0 to 15% EC showed an increase from 0.07 to 0.17 dS m^{-1} after 30 days and from 0.06 to 0.19 dS m^{-1} after 90 days. Examining the effect of the stabilization period, it appeared that for a fixed amount of biochar amendment, raising its stabilization in soil resulted in higher increases in soil EC.

The obtained results are in accordance with various studies reporting the liming effect of biochar on different types of soils (Van Zwieten *et al.*, 2010; Cui *et al.*, 2011; Lehmann *et al.*, 2011; Alburquerque *et al.*, 2013). In a study conducted by Granatstein *et al.* (2009), the biochar produced from herbaceous feedstock such as switchgrass, digested fiber peanut hull and from woody material such as softwood bark and wood pellet, had pH values ranging from 8.3 to 9.6 for the herbaceous materials and from 6 to 8.4 for the woody ones. These biochars were incorporated in sandy and silt loamy soils respectively; it was found that the herbaceous derived biochars possessed a greater liming impact raising the pH of sandy soils from 7.1 to 8.1 and in silt loamy soils – from 4.3 to 5.3. The woody derived biochars showed lower effects shifting the pH of sandy soils from 7.1 to 7.6 and the pH of silt loamy soils from 4.4 to 4.8. Similar results were found when using a rice-husk biochar possessing a pH of 7.79, which increased the soil pH by 1.2 units (Carter *et al.*, 2013). The increase in soil pH and EC observed after biochar application can be attributed to ash accretion (Chirenje and Lena, 2002), to the high surface area and the porous structure of the biochar increasing the cation exchange capacity of the soil (Nigussie *et al.*, 2012).

Table 15: Effects of addition of 5, 10 and 15% of biochar and stabilization period (30 and 90 days) on soil pH and electrical conductivity (EC).

		pH (H_2O)	pH (KCl)	EC (dS m^{-1})
SP	**BA (%)**			
SP_{30}	**0**	6.2 f	5.1 f	0.07 ef
	5	6.5 e	5.2 e	0.08 e
	10	7.0 c	5.4 c	0.13 c
	15	7.3 b	5.6 b	0.17 b
SP_{90}	**0**	6.2 f	5.2 e	0.06 f
	5	6.8 d	5.3 c	0.11 d
	10	7.4 b	5.7 b	0.16 b
	15	7.5 a	6.0 a	0.19 a
Reference soil		7.2	6.8	0.21

Means with different letters among the same column indicate significant difference (Duncan-Waller test; P < 0.05). BA: biochar amendment. SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period.

3.1.2. Effects of biochar amendment and stabilization period on soil total and $Ca(NO_3)_2$ exchangeable heavy metal concentrations

The effect of biochar amendment rate and stabilization period on the metals was examined by monitoring the total and the $Ca(NO_3)_2$ exchangeable fractions. Tables 16 and 17 show the data of total and $Ca(NO_3)_2$ extractable Pb, Cd, Cu and Zn in all treatments. The metal contaminated soil like it was sampled from the field contained total concentrations of 317 mg Kg^{-1} DW for Pb, 42.4 mg Kg^{-1} DW for Cu, 7.1 mg Kg^{-1} DW for Cd and 411 mg Kg^{-1} DW for Zn indicating a moderate contamination. In this soil, about $1/6^{th}$ of the total Zn and and $1/7^{th}$ of the total Cd were exchangeable while for Pb and Cu the exchangeable amounts were very low in comparison to the total amounts. The $Ca(NO_3)_2$ exchangeable fractions were equal to 0.48 mg Kg^{-1} DW for Pb, 0.02 mg Kg^{-1} DW for Cu indicating a very low plant-availability. In contrast, the exchangeable amounts of Cd and Zn equal to 0.9 mg Kg^{-1} DW and 68 mg Kg^{-1} DW, respectively, suggest a quite high plant-availability. This can be due to the different affinities of the metals for soil surfaces. Indeed, Pb and Cu are strongly sorbed to the soil complex while Cd and Zn have lower affinities for soil sorption sites (Appel *et al.*, 2008).

Statistical analysis revealed that both the addition of biochar at increasing rates and the stabilization periods caused considerable reductions of both total and $Ca(NO_3)_2$ extractable heavy metals indicating dilution and immobilization of metals.

Biochar amendment rate had significant effects on all total amounts of metals. At 30 days of stabilization, rising the biochar amendment rate from 5 to 15% leads to a reduction of 19 and 27% of total Pb content, 1 and 5% of total Cd, 11 and 12% of total Cu and 10 and 13% of total Zn, in comparison to the non-amended soil. These reductions are most likely linked to a simple dilution effect of the soil by the addition of biochar (Houben *et al.*, 2013).

The $Ca(NO_3)_2$ exchangeable fractions showed considerable reductions and were much higher than the reductions of the total metal fractions. Raising the amount of biochar amendment had significant effects on all exchangeable metals except Cu. At 30 days of stabilization, increasing the biochar amendment rate from 5 to 15% led to a reduction of 14 and 37% for Pb, 14 and 37% for Cd and 19 and 49% for Zn, in comparison to the non-amended soil. Examining the results obtained after 90 days, the reductions of $Ca(NO_3)_2$ exchangeable metal concentrations were much higher than the ones observed after 30 days. The stabilization period obviously had a statistical significant effect on all the exchangeable metal concentrations indicating increased metal immobilization by biochar with aging. The highest reductions were observed at the highest biochar amendment rate (15%) stabilized for 90 days. In comparison to the non-amended soil, $Ca(NO_3)_2$ exchangeable metal concentrations showed lower values reaching 54% reduction of exchangeable Pb, 67% for Cd and 77% for Zn surpassing by far the reductions observed on the total metal amounts indicating increased metal immobilization.

The obtained results confirm that biochar addition considerably reduced the exchangeable fractions of the metals present in the soil. These encouraging results are in accordance with several reports providing sound data on the efficiency of biochar removing metals from soils. Beesley and Marmiroli (2011) reported that biochar applied in a multi-element contaminated soil could reduce the concentration of Cd and Zn by 300 and 45 fold. The same authors confirm that biochar amendment showed interesting capacities in immobilizing and retaining As, Cd and Zn. Another experiment, using a derived biochar from rice straw at a level of 3 and 5% w/w, revealed a reduction in the bioavailability of Cu and Pb by 19.7 and 100% and by 18.8 and 77%, respectively (Jiang *et al.*, 2012). Park *et al.* (2011) reported that chicken manure derived biochar reduced NH_4NO_3 extractable Cd and Pb by 88.4% and 93.5%, respectively. They also found that green waste derived biochar immobilized 30.3% of Cd, 22.9% of Cu and 36.8% for Pb.

The capacity of biochar for immobilizing metals can be attributed to various mechanisms. The pH increase due to the addition of the biochar amendment leads to a decrease of the metal mobility. Incorporation of biochar raises the overall negative charges of the soil complex, therefore the electrostatic attraction between the positive charged metal ions and the negative charged biochar surface is increased (Dong *et al.*, 2011; Peng *et al.*, 2011). The results of NMR analysis (Chapter 3 section 4.6) of the biochar used in this experiment showed a substantial richness in heterogonous functional groups (carboxylic, hydroxyl group, etc.). These groups, composing the surface of biochar, raise its sorption capacity by their surface charge and electrons availability binding the various metals in different and complex sorption behavior. Besides some metals can be strictly sorbed by electrostatic forces, some others have exposed π-orbitals and can bind to π-electrons on the biochar graphene sheets (Swiatkowski *et al.*, 2004; Amonette and Joseph, 2009).

Table 16: Effects of addition of 5, 10 and 15% of biochar and stabilization period (30 and 90 days) on soil total metal concentrations.

Treatment		Soil Total metal content (mg Kg^{-1} DW)							
		Pb	% DTC	Cd	% DTC	Cu	% DTC	Zn	% DTC
SP	BA (%)								
Sp_{30}	0	278 a	-	6.53 a	-	38.0 a	-	366 a	-
	5	226 ab	-19	6.46 a	-1	34.0 abc	-11	329 abc	-10
	10	217 ab	-22	6.13 bc	-6	33.2 abc	-13	317 abc	-13
	15	202 b	-27	6.23 ab	-5	33.3 abc	-12	320 abc	-13
Sp_{90}	0	274 a	-	6.10 bc	-	36.5 ab	-	348 ab	-
	5	222 ab	-19	5.93 bc	-3	31.6 abc	-13	306 bc	-12
	10	214 ab	-22	5.90 c	-3	30.8 bc	-16	303 bc	-13
	15	196 b	-28	5.56 d	-9	29.3 c	-20	288 c	-17
Reference soil		23	-	0.45	-	14.3	-	65	-

BA: biochar amendment. Sp_{30}: Stabilization period for 30 days. Sp_{90}: Stabilization period for 90 days.DTC%: difference in comparison to the control.Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05).

Table 17: Effects of addition of 5, 10 and 15% of biochar and stabilization period (30 and 90 days) on soil Ca(NO3)2 exchangeable metal concentrations.

Treatment		Soil $Ca(NO_3)_2$ extractable metals (mg Kg^{-1} DW)							
		Pb	% DTC	Cd	% DTC	Cu	% DTC	Zn	% DTC
SP	BA (%)								
Sp_{30}	0	0.49 a	-	0.86 b	-	0.018 a	-	62.8 a	-
	5	0.42 b	-14	0.74 c	-14	0.016 ab	-11	50.7 b	-19
	10	0.36 c	-27	0.63 e	-27	0.009 c	-50	40.1 d	-36
	15	0.31 cd	-37	0.54 f	-37	0.012 bc	-33	32.3 e	-49
Sp_{90}	0	0.54 a	-	0.93 a	-	0.008 c	-	63.8 a	-
	5	0.36 c	-33	0.68 d	-27	0.007 c	-13	42.4 c	-34
	10	0.28 d	-48	0.42 g	-55	0.010 c	+25	21.4 f	-66
	15	0.25 d	-54	0.31 h	-67	0.008 c	0	14.7 g	-77
Reference soil		3.32	-	0.10	-	0.017	-	6.6	-

BA: biochar amendment. Sp_{30}: Stabilization period for 30 days. Sp_{90}: Stabilization period for 90 days.DTC%: difference in comparison to the control. Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05).

3.2. Effects of biochar amendment and stabilization period on *Phaseolus vulgaris*

3.2.1. Effects on growth of *Phaseolus vulgaris*

The results of the statistical analysis on growth parameters revealed that both biochar amendment rates and stabilization periods significantly affected the three measured morphological parameters.

The recorded parameters on the plants grown on the non contaminated soil used as reference were the highest; 128mm of shoot length, 0.22g of root dry weight and 0.62g of primary leaves dry weight.

The plants cultivated on the non treated soil originating from the field site showed reduced growth with small leaves and a stunted root system. In the absence of biochar the plants showed the lowest values of shoot length, leaf and roots dry weights (Fig.26).

Applying biochar with increasing rates and its stabilization led to statistically significant effects on shoot length and leaf and root dry weight (Fig.26). All the measured growth parameters showed higher values. Increasing biochar application rate from 5 to 15% lead to higher shoot length by 41 and 84%, higher leaves dry weight by 53 and 98% and higher roots dry weight by 49 and 160% after 30 days of stabilization. After 90 days of stabilization similar improving trends were observed; the biochar application rate from 5 to 15% increased the shoot length by 62 and 86%, the leaves dry weight by 76 and 155% and the roots dry weight by 78 and 198%.

The rising trends of the plant growth parameters as a response to increasing biochar amendment were also reported on lettuce and cabbage (Carter *et al.*, 2013), on pepper and tomato (Graber *et al.*, 2010), on common beans (Rondon *et al.*, 2007) and many other crops. These positive responses were attributed to several direct and indirect effects of biochar addition: improvements in soil pH, increased CEC, improvement of soil water retention (Glaser *et al.*, 2002; Jeffery *et al.*, 2011), increased nutrient retention and supply (Silber *et al.*, 2010; Steiner *et al.*, 2007), promotion of mycorrhizal fungi (Rondon *et al.*, 2007; Lehmann *et al.*, 2011), alteration of microbial communities (Graber *et al.*, 2010) and immobilization of phytotoxic compounds (Uchimiya *et al.*, 2010; Park *et al.*, 2011).

The morphological parameters determined on the seedlings grown after 90 days of stabilization (Fig.27) were slightly higher than the ones recorded on the ones grown after 30 days of stabilization (Fig.28). These differences can be attributed to the aging of biochar coupled with further oxidation developing more negative charges leading to higher CEC. The latter enhances the interactions of biochar with the clay, silt, minerals, organic matter, improving its capacity to sorb more compounds and reflect by consequence positive effects on plants (Brodowski *et al.*, 2005; Hammes and Schmidt, 2009).

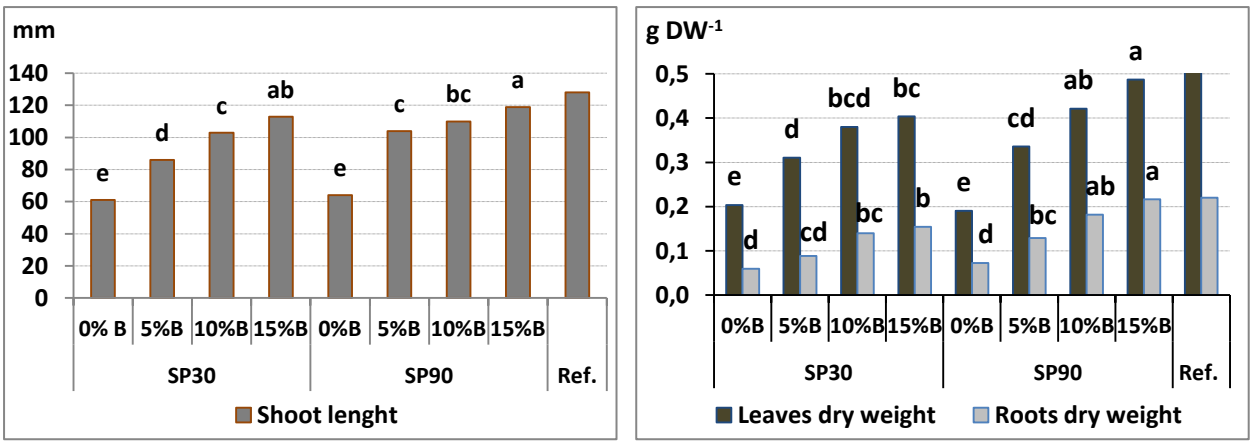

Figure 26: Effects of biochar application and stabilization period on 15-day-old Phaseolus vulgaris shoot length (mm; left), and dry weights (g) of leaves and roots (right). Means with different letters indicate significant difference (Duncan-Waller test; $P < 0.0$). x%B: biochar amendment rate. SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period.

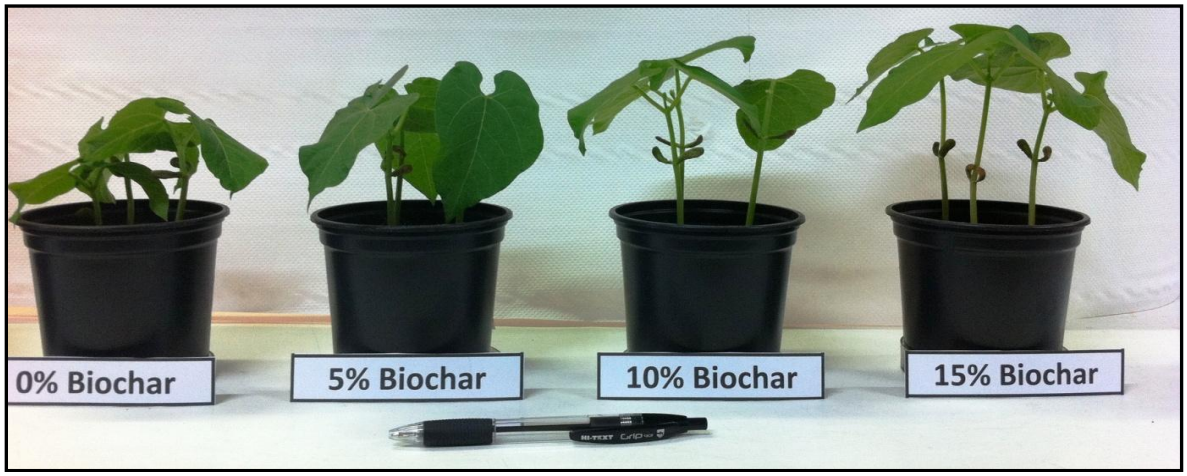

Figure 27: Bean plants after growing for 15 days on a metal contaminated soil amended with increasing concentrations of biochar stabilized for 30 days.

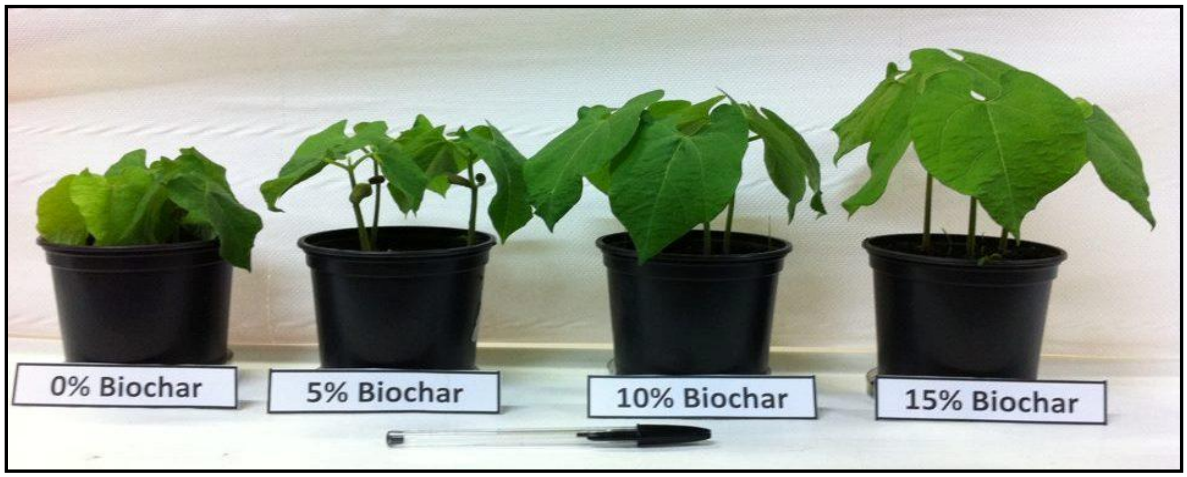

Figure 28: Bean plants after growing for 15 days on a metal contaminated soil amended with increasing concentrations of biochar stabilized for 90 days.

3.2.2. Effects on metal contents in roots and primary leaves of *Phaseolus vulgaris*

The effects of biochar application rate and stabilization period on the metal contents in roots and leaves of *Phaseolus vulgaris* are reported in tables 18 and 19. The statistical analysis showed significant differences between the treatments indicated with different letters among the same column. The bean plants grown on the non amended soil accumulated high amounts of metals; this was reflected in a considerable growth inhibition.

The incorporation of biochar with increasing rates in soil for the two stabilization periods resulted in considerable reductions of the metal contents in roots and leaves. For roots, the increasing application rate of biochar had the highest effect on Zn, followed by Pb, and then Cd. Raising biochar amendment from 5 to 15% induced a decrease in Zn roots content by 60 and 67% after 30 days of stabilization and by 55 and 78% after 90 days. Pb content decreased by 24 and 55% after 30 days of stabilization and by 29 and 50% after 90 days. Similar reductions were observed for the Cd content. Regarding Cu content in roots, the raising biochar application rates was coupled with slight reductions without being statistically different. Olive mill waste derived biochar showed higher reductions of roots metal contents when it was stabilized for 90 days in comparison to 30 days indicating an increasing of metal immobilization with biochar aging.

Concerning the leaves, the different treatments had the highest reducing effect on Cd followed by Zn, and then Pb. Cd content was below detection limit in the leaves of plants grown on the soil amended with 10% biochar stabilized for 30 days and also the ones amended with 10 and 15% biochar stabilized for 90 days. For soil amended with 15% biochar, after respectively 30 and 90 days of stabilization, Zn content in leaves was reduced by 67 and 81% and Pb by 60 and 57%.

Olive mill waste derived biochar applied to and stabilized in the multi-element metal contaminated soil lead to considerable reductions of metal contents in plants tissues. Matching the latter values with the results observed on the soil $Ca(NO_3)_2$ exchangeable metals (Tab.17), the lowest metal extractability recorded in the treated soils coincided with the lowest metal contents in leaves. Indeed, high correlation factors were obtained between the $Ca(NO_3)_2$ exchangeable metals in soil and their correspondent contents in the bean leaves. Pearson's correlation at 0.01 showed very high correlation for Pb (r=0.95), Cu (r=0.90) and Zn (r=0.94) and high correlation for Cd (r=0.83).

Our results are in accordance with the results obtained by Karami *et al.* (2011) who reported that the use of green waste derived biochar resulted in a lower plant availability of Ni, Zn, Cd and Pb. In another experiment, application of prune residues derived biochar in mine tailings reduced significantly the bioavailability of Cd, Pb and Zn (Fellet *et al.,* 2011). Considerable reductions were also recorded in Indian mustard shoots cultivated on both spiked and metal contaminated soils from the field

amended with biochar. Park *et al.* (2011) reported that applying 1, 5 and 15% of chicken manure derived biochar reduced metal uptake by 74.7, 79.6 and 88.0% for Cd, and 76.1, 82.2 and 96.3% for Pb, respectively.

Table 18: Effects of biochar amendment and stabilization period on metal contents (mg kg^{-1} dry weight) in roots of 15 days old Phaseolus vulgaris.

Treatment		Roots metal content (mg Kg^{-1} DW)							
		Pb	% DTC	Cd	% DTC	Cu	% DTC	Zn	% DTC
SP	BA (%)								
Sp_{30}	0	66.0 a	-	12.6 a	-	12.6	-	733 a	-
	5	50.3 ab	-24	12.0 a	-5	12.2	-3	291 bc	-60
	10	40.8 bcd	-38	10.2 ab	-19	11.3	-10	271 bc	-63
	15	29.4 cd	-55	10.3 ab	-18	10.2	-19	242 bc	-67
Sp_{90}	0	46.3 bc	-	10.0 ab	-	12.3	-	699 a	-
	5	32.9 bcd	-29	8.8 b	-11	11.5	-7	317 b	-55
	10	24.7 d	-47	4.4 c	-56	10.8	-12	174 bc	-75
	15	23.4 d	-50	5.4 c	-46	8.0	-35	154 c	-78
Reference soil		14.3	-	3.6	-	6.2	-	128	-

Values are means of 5 replicates. Means with different letters within the same column indicate significant difference (Duncan-Waller test; P < 0.05). BA: biochar amendment. SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period. %DTC : %difference in comparison to control.

Table 19: Effects of biochar amendment and stabilization period on metal contents (mg kg^{-1} dry weight) in primary leaves of 15 days old Phaseolus vulgaris.

Treatment		Leaves metal content (mg Kg^{-1} DW)							
		Pb	% DTC	Cd	% DTC	Cu	% DTC	Zn	% DTC
SP	BA (%)								
SP_{30}	0	6.3 a	-	0.86 b	-	8.7	-	247 a	-
	5	4.5 ab	-30	0.21 c	-76	8.3	-5	167 b	-32
	10	3.8 b	-39	0.08 c	-90	7.5	-14	123 bcd	-50
	15	2.5 b	-60	*	*	8.5	-3	81 cde	-67
SP_{90}	0	6.3 a	-	1.18 a	-	7.2	-	300 a	-
	5	4.4 ab	-30	0.09 c	-92	7.3	+2	140 bc	-53
	10	3.2 b	-49	*	*	7.3	+2	75 de	-75
	15	2.7 b	-57	*	*	7.0	-2	56 e	-81
Reference soil		1.9	-	*	-	5.5	-	65	-

Values are means of 5 replicates. Means with different letters within the same column indicate significant difference (Duncan-Waller test; P < 0.05). BA: biochar amendment. SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period. %DTC : %difference in comparison to control.

3.3. Effects of biochar amendment and stabilization period on the activities of stress enzymes and soluble protein contents in bean plants

3.3.1. Effects on stress enzymes

The capacities of ME, ICDH and GPOD in leaves and ME, GIDH and GPOD in roots are reported in table 20. Significant differences were found between the treatments and are indicated by means with different letters among the same column. In response to the oxidative stress, caused by the presence of metals in different concentrations, the induction of the stress enzymes is considered as one of the important cellular defense strategies (Chaoui *et al.,* 1997). The bean seedlings grown on the non amended soil, exposed to metals stress, showed the highest anti-oxidative activities of the measured enzymes and the smallest growth (shoot length, leaf and root weight) (Fig.26). Van Assche *et al.* (1988) confirm that poor growth and high capacities of antioxidant enzymatic activity in plant tissues are symptoms of phytotoxicity.

At both, 30 and 90 days of stabilization, the activities of the antioxidative enzymes decreased significantly with increasing biochar application rate, except for GPOD in roots. Increasing the biochar amendments from 5 to 15% lead to a significant reduction of ME activity in roots by 12 and 29% at 30 days and even stronger reductions at 90 days reaching values of 16 and 31%. GIDH activity also showed a decreasing trend with rising biochar application rate, but the differences were not significant. Roots of plants grown on the 90 days stabilized soil amended with 15% biochar showed the lowest GIDH activity. In contrast to ME and GIDH, GPOD activity in roots exhibited an increasing trend with increasing biochar rate. The highest activities were found in roots of plants grown on the soil amended with 15% biochar showing an increase of 24 and 46%, respectively for 30 and 90 days of stabilization.

Also in leaves the activities of ME showed a similar decreasing trend. In comparison to their respective controls, 5 to 15% biochar amendments lead to declines in activity by 28 and 42% after 30 days and by 32 and 53% after 90 days. The activity of GPOD in leaves decreased gradually with increasing biochar rate. Higher reductions were found in leaves of plants grown on 90 days stabilized in comparison to 30 days stabilized soils. After 15% biochar amendment, the GPOD activity in leaves was decreased by 87% after 30 days and by 82% after 90 days stabilization time of the amended soils. ICDH activity in leaves also decreased with increasing biochar application rate. In leaves of plants grown from 30 days after application of 5 and 15% biochar, the activity of ICDH was reduced by 27 and 51% respectively. After 90 days of stabilization, the highest reduction was observed with an amendment rate of 15% biochar reaching 39% reduction.

Various studies reported increase activities of stress enzymes in plants exposed to toxic metal concentrations (Shah *et al.*, 2001; Pandey *et al.*, 2009; Nadgorska-Socha *et al.*, 2013). Olteanu *et al.* (2010) reported that increasing

concentrations of lead induced increased anti-oxidant responses which were obvious by higher activities of superoxide dismutase, catalase and peroxidase in 7-day-old wheat seedlings. Verma and Dubey (2003) reported that rice seedlings exposed to up to 1000mM Pb showed concomitant increases of the activities of superoxide dismutase (87 to 100% increase), guiacol peroxidase (1.2 to 5.6 times increase) and ascorbate peroxidase (1.2 to 1.9 times increase). They also found that the roots maintained higher enzyme activities in comparison to the shoots. However, Chaoui *et al.* (1997) found that Cd and Zn stressed common beans (*Phaseolus vulgaris)* exhibited higher antioxidant enzymatic activities in stems and leaves rather than in roots.

With increasing biochar amendment rates most of the antioxidant enzymatic activities in bean plants showed decreasing values. This effect is linked to the metal immobilizing effect of biochar reducing the amounts of metals available for the plants. Vangronsveld and Clijsters (1992) and Ruttens *et al.* (2006) have shown that after incorporating compost, cyclone ashes and steel shots, the plant-availability of metals decreased and this was coupled with a reduction in activities of stress enzymes in roots and leaves of bean seedling grown on the amended soils. The higher reductions of the activities of stress enzymes that we observed in the plants grown on the biochar amended soil that were stabilized for 90 days in comparison to the ones stabilized for 30 days, are mainly due to the aging of the biochar allowing more immobilization of metals. The natural oxidation of the biochar increases oxygen functional groups giving the biochar higher capacities for metal immobilization and by consequence lower metal stress for the plants grown on the amended soil (Cheng *et al.*, 2008).

Table 20: Effects of biochar amendment and stabilization period on the anti-oxidant enzymatic activity.

Treatments		Enzyme capacity (mU g^{-1} FW)											
		Root						Leaf					
		ME	% DTC	GIDH	% DTC	GPOD	% DTC	ME	% DTC	ICDH	% DTC	GPOD	% DTC
SP	BA (%)												
SP_{30}	0	884 a	-	133	-	10513 bcd	-	844 a	-	907 a	-	1866 a	-
	5	778 ab	-12	123	-7	12161 abc	+16	608 b	-28	666 b	-27	555 b	-70
	10	665 bc	-25	103	-22	11967 abcd	+14	565 bc	-33	615 bc	-32	480 bc	-74
	15	632 bcd	-29	95	-29	13066 ab	+24	490 bc	-42	448 c	-51	242 cd	-87
SP_{90}	0	565 cd	-	102	-	8938 c	-	861 a	-	866 a	-	715 b	-
	5	474 de	-16	91	-11	9414 cd	+5	616 b	-28	667 b	-23	147 d	-79
	10	371 e	-34	88	-13	10062 bcd	+13	611 b	-29	640 b	-26	141 d	-80
	15	392 e	-31	83	-18	13721 a	+46	420 c	-51	527 bc	-39	129 d	-82
Reference		562	-	108	-	9871	-	522	-	538	-	1192	-

Each value represents the mean of 5 measurements. Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05). ME: malic enzyme; GIDH: glutamate dehydrogenase; ICDH : iso-citrate dehydrogenase ; GPOD : peroxidase ; BA: biochar amendment. SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period. %DTC: %difference in comparison to control.

3.3.2. Effects on plant soluble protein contents

Table 21 shows the soluble protein contents of the roots and leaves as affected by the biochar amendment rate and stabilization period. The results represent the mean values of five measurements. Although statistical analysis revealed no significant differences between the treatments, the soluble protein content in both roots and leaves showed an increasing trend with increasing biochar application rates. The effect was more pronounced on the protein content in leaves compared to the roots. Biochar application was coupled with higher protein anabolism. Biochar application rates of 10 and 15% lead to increases in the mean protein content by 12 and 23% in roots of plants grown after a stabilization period of 30 days and by 6 and 8% after a stabilization period of 90 days. Increasing biochar application rate from 5 to 15% lead to an increase in soluble protein content by 9 and 23% in leaves of plants grown after 30 days of stabilization and by 13 and 34% after 90 days of stabilization.

Assessing the effect of different concentrations of Pb on *Phaseolus vulgaris* seedlings protein content, Hamid *et al.* (2010) found that exposure to increasing Pb concentrations was coupled with several physiological disruptions of the plants and was accompanied by decreases of total protein content, chlorophyll and carbohydrates. Vierstra (1993) mentioned that acceleration of protein degradation can be attributed to several forms of stress. Ericson and Alfinito (1984) mentioned that metal stress can promote the anabolism of some proteins and inhibit the synthesis of other proteins with a general declining trend of the overall content. The decreasing effect of metals on protein content was also reported in wheat (Olteanu *et al*., 2010), in *Lupinus albus* (Costa and Spitz, 1997), in *Lemna minor* (Mohan and Hosetti, 1997) and tomato (Djebali *et al*., 2008). The decrease in soluble protein content in *Phaseolus vulgaris* exposed to higher metal concentrations might be explained by the increased protease activity favoring protein degradation (Palma *et al*., 2002). It can be also linked to induction of lipid peroxidation and the increasing generation of the reactive oxygen species (Pinto *et al*., 2003). By consequence, the increasing trend of the soluble protein contents in leaves and roots that we found further supports that the biochar amendment reduces the metal availability and toxicity of the contaminated soil.

Table 21: Effects of biochar application and stabilization period on soluble protein contents in roots and leaves.

Treatment		Roots		Leaves	
		Proteins (mg g^{-1} FW)	% DTC	Proteins (mg g^{-1} FW)	% DTC
SP	BA (%)				
Sp_{30}	0	4.3	-	21.1	-
	5	4.2	-2	23.0	+9
	10	4.8	+12	24.0	+14
	15	5.3	+23	25.9	+23
Sp_{90}	0	5.0	-	21.3	-
	5	5.3	+6	24.0	+13
	10	5.3	+6	27.5	+29
	15	5.4	+8	28.5	+34
Reference		5.2	-	27.8	-

Each value represents the mean of 5 measurements. Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05). BA: biochar amendment. SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period. %DTC: %difference in comparison to control.

3.3.2.1. Integrated evaluation system of the soil phytotoxicity

The test plants grown on the non-amended contaminated soil originating from the field site showed the lowest values for shoot length, leaf and root dry weight (Fig.26). The phytotoxicity indexes were determinaed based on calculation of the relative (percentage) values of the results as compared to the reference values (non contaminated soil). The Phytotoxicity index of the soil originating from the field site showed a slightly toxic soil (class 2). Van Assche *et al.,* (1988) found that the threshold value for Zn toxicity in the primary leaves of *Phaseolus vulgaris* was 226 mg Zn Kg^{-1} DW above which phytotoxicity was observed. The Zn concentrations in the primary leaves of the plants grown on the non-amended soil were higher than this threshold level (Tab.19) explaining the growth inhibition and the increased capacities found for the stress enzymes that were investigated. The application of biochar amendment and its stabilization for the two respective periods of 30 and 90 days resulted in a reduction of phytotoxicity of the soil. A considerable improvement of the vegetative growth and important decreases of the stress enzymes capacities were found (Fig.26, Tab.19). The addition of biochar at a rate of 5% and stabilized for 30 days was not efficient in reducing the soil phytotoxicity index. Indeed the calculated index indicated a slightly toxic level (class 2) equal to the original contaminated soil. All the other treatments resulted in a complete elimination of the phytotoxicity (class 1) (Tab.22). Plant growth was strongly improved and capacities of stress enzymes

were considerably decreased indicating an efficient immobilization of the metals leading to a classification of the soil as no more phytotoxic.

Table 22: Classification of the biological data according to the classification system presented in table 14

			Enzymes capcities						
			Leaf			Root			
Soil sample	Shoot lenght	Root weight	ME	ICDH	GPOD	ME	GIDH	GPOD	Phytotoxicity index
SP_{30},0%B	4	4	2	2	2	2	1	1	2
SP_{30},5%B	3	4	1	1	1	2	1	1	2
SP_{30},10%B	2	3	1	1	1	1	1	1	1
SP_{30},15%B	1	3	1	1	1	1	1	2	1
SP_{90},0%B	4	4	2	2	1	1	1	1	2
SP_{90},5%B	2	3	1	1	1	1	1	1	1
SP_{90},10%B	1	2	1	1	1	1	1	1	1
SP_{90},15%B	1	1	1	1	1	1	1	2	1

SP_{30}: 30 days stabilization period. SP_{90}: 90 days stabilization period. x%B: biochar amendment rate. ME: malic enzyme; GIDH: glutamate dehydrogenase; ICDH : iso-citrate dehydrogenase ; GPOD : peroxidase.

3.4. Effects of biochar application and stabilization period on soil microbial communities

The Biolog data were analyzed first by calculating the color intensity development over time through calculation of the average well color development (AWCD) on each plate every 24h and second – by calculating the Shannon-Weaver index (H) and the Richness (R) (OD>0.25) at 72h presenting the shortest incubation time revealing a clear difference in the responses among the treatments. The results related to the effect of biochar application on the activity of soil microbial community assessed by calculating the AWCD over incubation time are illustrated in figure 29. The Shannon-Weaver diversity index (H) and the Richness (R) (OD>0.25) are presented in figures 30a and 30b, respectively. The color intensity indicating the microbial activity at 144h on the Biolog Ecoplates is illustrated in figure 31.

Soil microbial activity for all treatments increased with incubation time during the 7 days. During the first 24 h the AWCD values showed little changes. The shortest incubation time revealing clear responses among the treatments was 72 h except for the 15% biochar amended soil after 90 days of stabilization which showed a high distinctive AWCD at 48 h. At 144 h, the AWCD on the non amended soils for 30 and 90 days of stabilization reached values of 0.7 and 0.8. The soil microbial activity showed higher values on all amended soils. After 30 days of stabilization, the responses of the microbial communities were comparable for the three biochar amendment rates (5, 10 and 15%) reaching an AWCD around 1.1. After 90 days of stabilization, a clear increasing trend was recorded with increasing biochar

application rate to reach an AWCD of 2.2 on the soil amended with 15% biochar. The Shannon-Weaver index (H) at 72h, indicating the diversity of the microbial community, showed higher values in the treated soils in comparison to the non amended ones. It increased from 2.50 on the non amended soil to reach 2.57 and 2.60 in the 10% and 15% biochar amended soils, after 30 days of stabilization. After 90 days of stabilization, the Shannon-Weaver index (H) increased from 2.22 in the non amended soil to reach 3.27 in the 15% biochar amended soil. The Richness (OD>0.25 at 72h) showed considerable increases with increasing amendment application rate (Fig.30b). The richness value in the 15% biochar amended soil was equal to 45% after 30 days of stabilization, and 97% after 90 days of stabilization. The higher values of AWCD, H and R on the stabilized soils amended with biochar indicate a higher rate of carbon source utilization and a greater functional diversity in comparison to the non amended soil. These responses of the microbial soil community to biochar amendment and stabilization period can be linked to various reasons. The lower availability of metals that we already illustrated before (Tab 16), lead to a better environment and a more suitable habitat for microbes to colonize and grow. Indeed, an experiment conducted by Muhammad *et al.* (2005) investigating the effect of Pb and Cd nitrate on the soil microbial community revealed that increasing concentrations of these metals caused an abiotic stress indicated by a continuous decrease in the AWCD and a decline in the soil microbial biomass carbon and nitrogen. In addition to this role of biochar reducing the availability of metals, Janice and Matthias (2009) mentioned that the porous structure of the biochar, its considerable surface area, its effect in improving the soil water holding capacity and its effect on the soil pH are all parameters that are positively influencing growth, reproduction and activity of microbial populations.

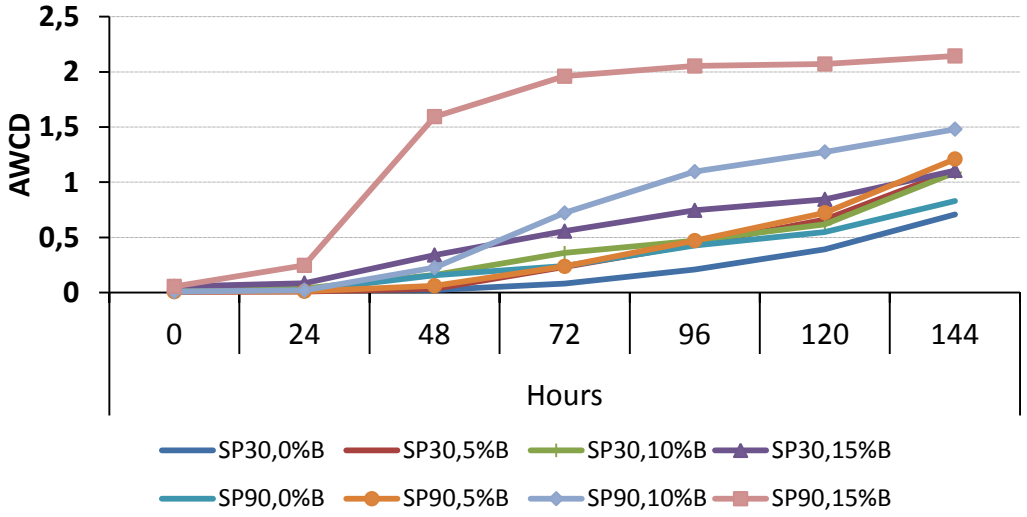

Figure 29: Effects of biochar application and stabilization period on the functional activity of soil microbial communities indicated by the average well color development (AWCD) over time at 590nm. SP30: 30 days stabilization period. SP90: 90 days stabilization.

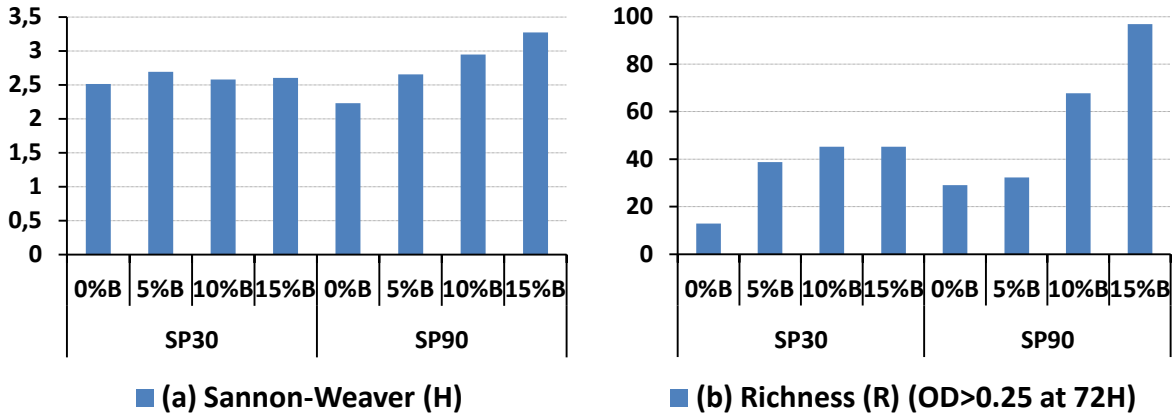

Figure 30: Effects of biochar application and stabilization period on the (a) Shannon-Weaver diversity index and (b) the Richness (R) (OD>0.25 at 72h). SP30: 30 days stabilization period. SP90: 90 days stabilization period. x%B: biochar amendment rate.

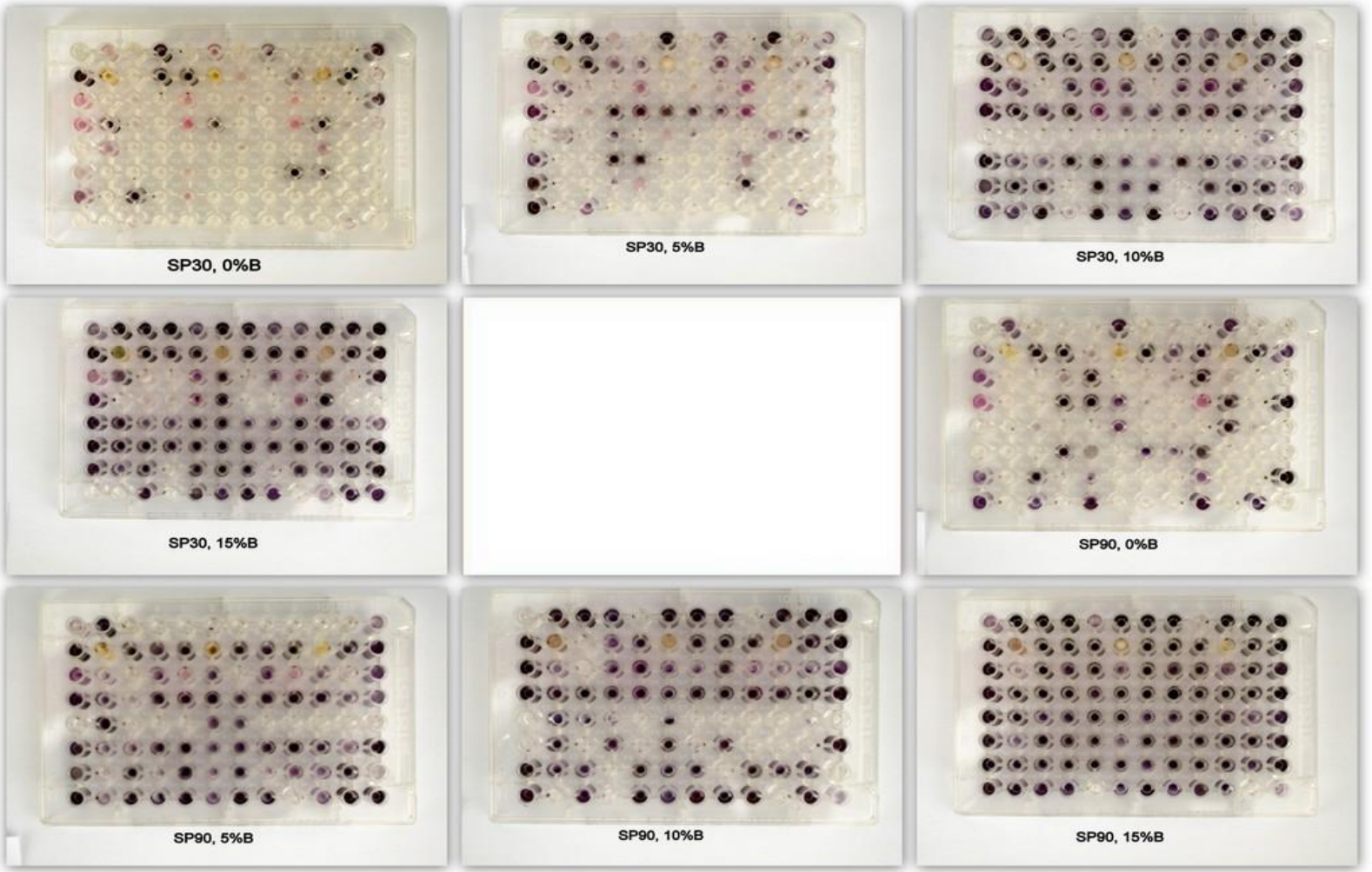

Figure 31: Effects of biochar application and stabilization period soil microbial activity illustrated by well color development at 144 h incubation time. **SP30: 30 days stabilization period. SP90: 90 days stabilization period. x%B: biochar amendment rate.**

3.5. Effects of biochar application on the earthworm's mortality, growth and reproduction

The effects of biochar application on the mortality, growth and reproduction of *Eisenia foetida* are presented in figure 32. The statistical analysis revealed significant effects of biochar application on the three studied parameters. Significant differences between the treatments are indicated with different letters. In this experiment the biochar particles having a minimum size of 1mm were stabilized in soil for 120 days.

In the non amended soil, the worms' mortality reached 12%, with a weight loss of 7.3% and a reproduction capacity of 30.8 cocoons (Fig.32). The lowest biochar application rate insured a 100% worms' survival which was also found after 10 and 15% biochar application. The worms' weight showed an increasing trend with increasing biochar application rate. The worms showed a weight increase of 17.8, 19, and 22.08% for 5, 10 and 15% biochar application rate respectively. The most pronounced effect of biochar was observed on the reproduction capacity assessed by counting the number of cocoons. The incorporation of biochar obviously allowed better habitat conditions for the worms, like already mentioned for both effects on phytotoxicity and microbial communities by reducing the availability of the metals in the soil; this was illustrated by a rise in cocoon numbers from 30.8 in the non amended soil up to 84.4, 85.6 and 88.4 found for the 5, 10 and 15% biochar application rate respectively. While counting the cocoons it was noticed that a considerable number of worms were hatching in the soils amended with biochar. These findings are in accordance with Denyes *et al.* (2012) who observed that biochar addition to a polychlorinated biphenyls (PCB) contaminated soil at a rate of 2.8% stabilized for 50 days was coupled with a reduction of PCB accumulation in the worms tissues by 53% allowing an increase in the survival rate by 17.5 times in comparison to the control. Referring to literature, amending soil with biochar lead to an avoiding behavior by the earthworms (Li *et al.*, 2011), caused mortality (Liesch *et al.*, 2010) and reduced their growth rate and reproduction (Weyers and Spokas, 2011). The latter authors reported that these negative impacts are only observed in short term after addition of dry biochar and disappeared with biochar aging. The short term negative impacts were attributed to rapid alterations of the soil pH, to potential physical damages arising from the dry material sticking to the earthworms' body and to worms' ingestion of powdered biochar. Due to these observations we decided to use in this experiment biochar particles having a minimum size of 1mm and allow a stabilization time of 120 days, which clearly allowed to avoid these negative impacts of biochar.

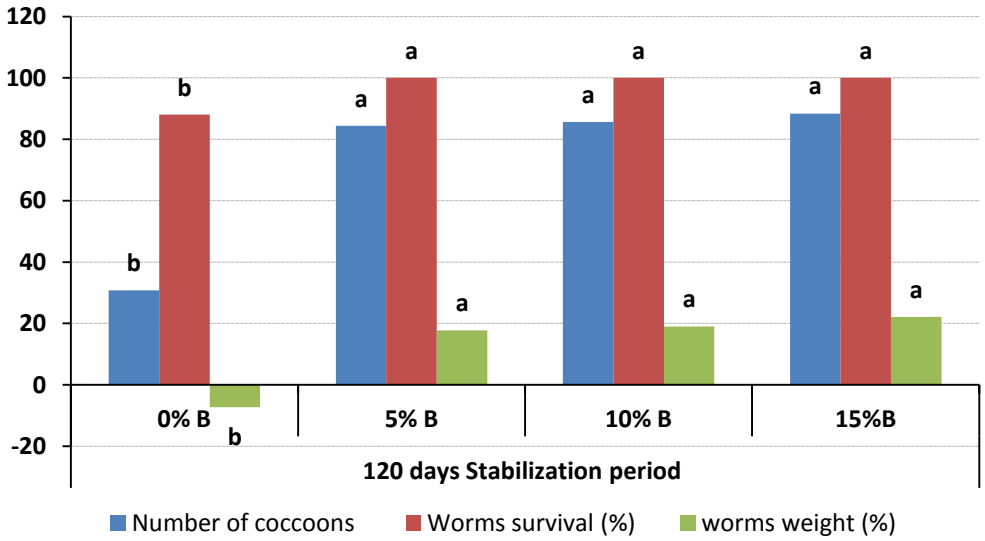

Figure 32: Effects of biochar application on mortality, growth and reproduction of Eisenia foetida. Means with different letters indicate significant difference (Duncan-Waller test; P < 0.05). x%B: biochar amendment rate.

4. Conclusions

Soil amendments can reduce the 'bioavailability' of a wide range of contaminants and consequently enhance the revegetation success and avoid contaminants spread by wind and/or water (Vangronsveld *et al.*, 1995a; Vangronsveld *et al.*, 1995b). In this study, the use of biochar, produced from olive mill waste through slow pyrolysis, as a soil amendment, showed interesting potentials for treating a multi-metal contaminated agricultural soil (Zn, Pb, Cd, Cu). Biochar amended soil at all levels stabilized for 30 and 90 days significantly reduced the total and $Ca(NO_3)_2$-exchangeable metal contents. The highest reductions were recorded on the soil amended with 15% biochar (w/w) and stabilized for 90 days. Total and exchangeable metal concentrations showed decreases by 28 and 54% for Pb, 9 and 67% for Cd, 17 and 77% for Zn. Due to a dilution effect after adding the biochar, total Cu was reduced by 20% while its exchangeable fraction was below detection limit.

Growing *Phaseolus vulgaris* on the amended soils lead to a better growth indicated by increases of shoot length and leaves and roots dry weight indicating a lowered phytotoxicity. Plant tissues contained lower metal concentrations. In leaves, the highest reduction effects were observed on Cd followed by Zn, and then Pb. For roots, the highest effects were recorded on Zn, followed by Pb, and then Cd. Regarding Cu, its total and exchangeable concentrations in the soil were within the normal ranges. In our experiment, Cu contents in the plants tissues decreased slightly. In case of higher reductions due to biochar application, symptoms of Cu deficiency might occur; therefore before applying biochar to a soil, deficiency risks need to be well studied to avoid such collateral effects.

The capacities of specific stress enzymes involved in the defense response of the plants to metal stress showed decreasing trends with increasing biochar application rates and a longer stabilization period indicating a lowered phytotoxicity. Further, the soluble protein contents of leaves and roots of plants grown on the amended soils increased considerably. The integrated phytotoxicity test revealed that the soil treatment was efficient in immobilizing the metals classifying the soil as no more phytotoxic. The responses of the soil microbial community to biochar application and its stabilization revealed higher AWCD, higher H index and higher R index indicating higher microbial activity and higher microbial richness and diversity. Earthworms reported in literature to refute added biochar showed positive responses in our experiment. Using biochar particles of minimum 1mm size and stabilizing it long enough in the soil was successful to avoid rapid changes in soil pH and possible ingestion of powdered biochar by the worms. Increasing biochar application rates in the contaminated soil allowed a total suppression of mortality of worms, improved their growth and their reproduction capacity indicating a safe use of biochar, an efficient immobilization of metals and a potential improvement of soil properties, leading to a more suitable habitat for the soil living organisms.

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Chapter 6

1. Conclusions and recommendations

1.1. Conclusions

The results presented in this thesis indicate that slow pyrolysis can be an efficient management process for the solid olive mill waste to reduce considerably its volume and weight. Biochar production through slow pyrolysis was revealed to be an interesting tool to transform this waste rather than leaving it for natural decay or spreading it as fertilizer in agricultural soils which causes a significant environmental deterioration leading to soil, air and ground water pollution.

Pyrolysis parameters showed significant effects on biochar yield and its characteristics defining by consequence its suitable use for specific fields. Increasing the pyrolysis temperature and the heating rate led to a continuous decrease in the biochar yield. Nitrogen, hydrogen and oxygen contents in biochar decreased while its concentration in carbon increased. The calculations of different atomic ratios (H/C, O/C and (O+N)/C) indicated higher aromaticity of the biochar, higher stability and lower polarity of its surface.

The heating values of the different biochar produced were not influenced by pyrolysis temperature or by the heating rate. The CO_2 emission factor increased indicating higher carbon sequestration capacity.

The morphological assessment showed that low temperature produced biochar had a complex structure with low porosity. Increasing pyrolysis temperature was coupled with formation of pores of the size tens of nanometers to several tens of microns. The biochar structure became more organized with smooth surfaces and higher surface area.

The analysis of ^{13}C NMR spectra showed rich surface functional groups of the biochar produced at low temperature. Increasing the pyrolysis temperature resulted in progressive elimination of the polar surface functional groups and a formation of aromatic compounds with phytotoxic effect evaluated through a germination and root elongation test.

The phytotoxic effect of the biochars produced at 450°-500°C and 500°-550°C limit their use as soil amendment. In meanwhile, their high heating values give them the potential to be used like fuel.

The low temperature produced biochar (400°-450°C) showed low electrical conductivity, high carbon content, high CO_2 emission factor and high surface functional groups. These properties make it suitable for soil incorporation to improve soil properties, to immobilize pollutants such as metals as well as offering a long term carbon sequestration and consequently mitigate the climate change.

In a laboratory scale experiment, the evaluation of the capacity of the biochar produced at 400-450°C in adsorbing nickel and in reducing its content, uptake and translocation in tomato grown in perlite was performed.

Biochar addition, at different rates, to non-contaminated and contaminated perlite with different levels of Ni has improved significantly the vegetative growth parameters. The Ni content in the tomato plants' tissues decreased significantly with increasing biochar amendment rate. The total Ni uptake measured on the plants' tissues showed very low levels. The translocation factor showed very low values indicating a very low translocation of Ni from the roots to the shoots.

Biochar amendment showed promising results in reducing the plant absorption of Ni, in improving the growth of tomato plants, reducing the metal content in the plants' tissues, its uptake and also its translocation from the roots to the shoots indicating the immobilization of this element reducing its plant-availability.

In the aim of building a wider picture of the biochar potential in immobilizing metals, a multi-element contaminated agricultural soil was amended with biochar and stabilized to evaluate later on its potential to immobilize the present metals through chemical and biological tests.

The results of this experiment showed that biochar amendment increased the soil pH, reduced considerably soil total and $Ca(NO_3)_2$-exchangeable metal contents. Higher amendment rates and longer stabilization period allowed the highest reduction in metals.

The treated soil used as growth medium for *Phaseolus vulgaris* resulted in better growth and lower metal content in the plants tissues. Using the morphological parameters (length, weight) together with the variation of the stress enzymes capacities in leaves and roots of the 15 day-old bean seedlings, as an integrated test to evaluate the phytotoxicity of the soil showed that phytotoxicity index dropped from class 2 to class 1 indicating a shift from a slightly toxic soil to a non-toxic soil.

The soluble protein content of the plant tissues showed an increase with higher biochar amendment rate and longer stabilization period indicating a lowered stress and better plant physiological status.

Evaluating the microbiological status of the treated soil showed that the microbial communities expressed higher activity, richness and diversity indicating biochar efficiency in sequestering the metals and restoring soil microbial life.

Increasing biochar application rates in the contaminated soil was efficient in total suppression of red worms mortality, improved their growth and their reproduction capacity indicating a safe use of biochar, an efficient immobilization of metals and a potential improvement of soil properties, leading to a more suitable habitat for the soil living organisms.

1.2. Recommendations

For future research works and in order to provide more sound data to draw wider picture and better understanding of biochar production, use and impacts, it is of high importance to:

- Conduct systematic field research to investigate the biochar quality derived from a wide range of feedstocks,
- Investigate the performance parameters of various biochar production systems,
- Investigate the effects of biochar incorporation into soils across different climates and soil gradients,
- Investigate any adverse effects related to biochar use in soil such a possible release of toxic substances or reducing efficiency of pesticides,
- Study the biochar stability under different environmental conditions, its effect on the decomposition of soil organic matter and its impact on net greenhouse gas emissions to the atmosphere for both short and long term scales,
- Conduct a Life cycle analysis integrating the different emissions factors associated with the whole production system from the feedstock supply source to the biochar application to soil to understand the impacts on terrestrial carbon stock and atmospheric greenhouse gas concentrations.