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Biochar as a sustainable nano-fertilizer

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Biochar as a sustainable nano-fertilizer

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List of abbreviations

2-AA	2-Amino Anthracene
Ă	Angstrom
AA	Amino Acid
AAS	Atomic Absorption Spectrometry
ACN	Acetonitrile
ANOVA	Analysis of Variance
As	Arsenic
ASTM	American Society for Testing Materials
AWCD	Average Well Color Development
BG	Borgotaro Grigio
C	Correggio
C_{org}	Organic carbonium
Ca	Calcium
CAT	Catalase
Cd	Cadmium
CEC	Cation Exchange Capacity
CFU	Colony Forming Unit
CH_4	Methane
Chl	Chlorophyll
CLPP	Community-Level Physiological Profiling
CO	Carbon monoxide
Co	Cobalt
CONS. C	Consortium Chosen
C_{org}	Carbon
Cr	Chrome
Cu	Copper
CV	Crystal Violet
cv	Cultivar
DIN	Deutsches Institut für Normung
DMC	Dry Matter Content
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EBC	European Biochar Certificate
EC	Electrical Conductivity
EDX	Energy Dispersive X-ray
ESEM	Environmental Scanning Electron Microscopy
EU	European Union
FA	Fatty Acid
Fe	Iron
FTIR-ATR	Fourier Transformed Infrared Spectroscopy with Attenuated Total Reflection
GC	Gas Chromatography
GI	Germination Index
H/H_2	Hydrogen
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
Hg	Mercury
HIS	Histidine
HNO_3	Nitric acid
IAA	3-indole-acetic-acid

IBI	International Biochar Initiative
IChar	Italian Biochar Association
ICP	Inductively Coupled Plasma
IPCC	International Panel on Climate Change
ISO	International Organization for Standardization
ISTD	Internal Standard
K	Potassium
KCl	Potassium chloride
LAI	Leaf Area Index
LB	Luria-Bertani medium
LCA	Life Cycle Assessment
MC	Moisture Content
MeOH	Methanol
$MgCl_2$	Magnesium chloride
Mg	Magnesium
Mn	Manganese
MS	Mass Spectrometry
MS	Murashige and Skoog medium
MT	Modena Tomaselli
N/N_2	Nitrogen
Na	Sodium
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NAM	Nucleabase/-side/-tide
NaOH	Sodium Hydroxide
Ni	Nickel
NO_2	nitrite
NO_3	nitrate
O/O_2	Oxygen
OA	Organic Acid
OD	Optical density
OECD	Organization for Economic Cooperation and Development
OM	Organic Matter
p	Statistical significance
P	Phosphate
Pa	Pascal
PAH	Polycyclic Aromatic Hydrocarbon
Pb	Lead
PCA	Principal Component Analysis
PCA	Plate Count Agar
PDA	Potato Dextrose Agar
PGPF	Plant Growth Promoting Fungi
PGPM/PGPR	Plant Growth Promoting Microoganisms/Rhizobacteria
Pheo	Pheophytin
ROS	Reactive Oxygen Species
RSG	Relative Seeds Germination
S	Siemens
S	Sulfur
SA	Sugar/Sugar Alcohol
SDG	Sustainable Development Goal
Si	Silica
SOD	Superoxide Dismutase

SRI	Shoot/Root Index
STD	Standard Deviation
TGA	Thermo Gravimetric Analysis
UK BQM	United Kingdom Biochar Quality Mandate
UNI EN	Ente Nazionale Italiano di Unificazione
US EPA	United States Environmental Protection Agency
UV	Ultraviolet
V	Volt
WHC	Water Holding Capacity
XRD	X-Ray Diffraction
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt
Zn	Zinc

CHAPTER 1 Biochar as sustainable nano-fertilizer

1.1 Introduction

1.1.1 Biochar: state of the art

As the European Biochar Certificate (EBC, 2012) Foundation guideline stated, biochar is a stable solid charcoal-like substance obtained from vegetative biomass and agricultural waste and treated under controlled conditions with heat clean technology which involves either a limited or absent oxygen supply. The term "biochar" is therefore closely related to its applications and to its original raw material. This terminology makes it possible to distinguish the term biochar from the more common "char" (or charcoal) which is instead the term generally used to define the solid product resulting from the pyrolysis of any organic material (Lehmann and Joseph, 2009). Actually, it can be produced from several feedstocks (i.e., plastic, compost, sewage sludge, animal manure), and has various applications going from energy production, to building materials, as a nutrient for animal feed, the aesthetic and cosmetic industry, as a component of adsorbent filters, and in metallurgical applications, as well as in agriculture and soil management (Schmidt, 2013; Weber and Quicker, 2018; Nartey and Zhao, 2014).

The amount of the production and use of biochar could materially help fostering a sustainable development. The Agenda 2030 and its 17 Sustainable Development Goals (SDGs) adopted by the United Nations in 2015 act as an international guide (UN SDG Action Campaign, 2015), and biochar emerges as a functional tool to achieve these goals, in order to protect the planet and ensuring prosperity for all. Biochar might participate in at least 12 of the 17 goals, ranging from lower fertilization costs for agricultural producers as the biochar can be produced from processed plant waste, and helping to make primary resources like water cleaner by acting as filter material, to reducing nutrient leaching and, thus, the impact on soil eutrophication. The latter are few general examples, but focusing on the agronomy and environment, in order to better understand the potential applications of the biochar within sustainable agriculture management, it is important to know the

real contribution of the biochar in these contexts. Global climate warming is effective and numerous long-term climate changes have been observed across the world. Agriculture is highly vulnerable to the impacts of climate change due to unsustainable agricultural management practices which, over the last decades, have led to environmental problems such as loss of fertility, soil erosion, in some cases desertification, variations in temperatures, extreme events like drought, heavy rainfall, heat waves and intensity of tropical cyclones. An innovative method to increase the stability of carbon stored in soil and mitigate greenhouse gas emissions (Wang, Xiong, and Kuzyakov, 2016) is the biochar (Nam *et al*., 2018), which has also been presented as a new green tool for the environmental behaviour because of the thermal process of its production that decomposes parts of the biomass but retains a large part of its carbon content. Its importance has also been reiterated in the International Panel on Climate Change report (IPCC, 2018). However, the increasing interest on biochar is due not only to its role in climate change mitigation, acting as a carbon sequestrator (IPCC, 2018; Ghani *et al.*, 2013), but also in water and soil remediation where it is emerging as sorbent for contaminants (Munera-Echeverri *et al*., 2018), in waste management achieving the zero-waste goal and creating a new valuable product from organic waste (Lee, Kim, and Kwon, 2017). The biochar is a porous material that contains inside itself a wide area organized by numerous cavities. These cavities form spaces of different sizes: macro, micro, and nano. The biochar's nano-compartmentalization allows it to be recognized as a nano-fertilizer (Maestri and Marmiroli, 2019). Porosity is one of the main and most important characteristics of the biochar as it is linked to fundamental properties for the wellbeing of the plant such as the ability to retain water and to release it gradually and to host nutrients and beneficial organisms. In agriculture, biochar improves soil characteristics by increasing soil pH value, water storage and nutrient supply, enhancing microbial dynamics in the rhizosphere and suppressing plant diseases *(Munera-Echeverri et al., 2018; Blok et al*., 2016; Hale *et al*., 2014) which all contribute to obtain increasing crop yield (Saxena, Rana, and Pandey, 2013; Ajeng *et al.*, 2020), soil fertility (Hale *et al*., 2014) and better quality *(Igalavithana et al.*, 2015; Ajeng *et al.*, 2020). For all these good reasons, biochar has also been proposed as an

effective tool for a sustainable agriculture, ensuring food security by preventing the loss of nutrients because of its liming effect and the cation exchange capacity (CEC) (Rajkovich *et al.,* 2011) and thereby protecting water resources, product quality, ameliorated nutritional status in food products and minimizing negative environmental impacts on biodiversity and ecological functions (Schröder *et al.,* 2018) also by reducing the utilization of chemical fertilizers.

The production and use of biochar as a fertilizer for agronomic purposes has increased in the last 10 years (Wu *et al.*, 2020). The dehydrogenation process and biomass conversion during the production of biochar allow the maintenance of the internal porous structure and contribute to give the biochar a characteristic physical and chemical functionality (Lee, Kim, and Kwon, 2017; Hale *et al.*, 2014) on which the advantages from the biochar use depend. However, the properties of biochar depend on many variables ranging from production characteristics (including temperature, temperature rising rate, total process time, chemical settings of thermal conversion), to the type of producing plant and to the nature of the raw materials (Marmiroli *et al.*, 2018; Lee, Kim, and Kwon, 2017; Nartey and Zhao, 2014). The biochar's specific characteristics give it several advantages in agriculture making it a potentially revolutionary tool. Recently, the biochar has become part of the new fertilizers allowed in the cultivations of the European territories (EU Regulations n° 2019/1009, 2019/2164). The effects of the biochar in soil have been proven for a long time and are mainly evident in the modulation of the physical chemical characteristics of the soil, helping it to increase its quality and fertility (Adekiya *et al.*, 2020), and providing a valid sustainable response to the growing and alarming problem of global pollution. Indeed, in recent decades agriculture has become increasingly specialized and has undergone a reversal in production techniques. We have moved from traditional agriculture to modern, intensive agriculture. The latter bases its activities on the full presence of mechanical tools, speciation, and the use of fertilizers. However, although these techniques, especially the use of synthetic fertilizers, lead to positive results in the short term as higher yields and guaranteed harvests, the side effects in the medium to long term are much more worrying. The resulting aridity of the soil is one of the distinctive features of this type of agriculture and it is an

aspect that must be decreased. One of the methods proposed to face the problem is to act on soil fertility through the study and use of microorganisms. The soil microbiome is the set of all the microorganisms present and all the relationships between them that contribute to provide a valid environment for the life and growth of living organisms. In particular, plant growth promoting microorganisms (PGPM) have been extensively studied. Their effect on the promotion of soil quality, the increase of its fertility and the benefits on the productive yield of various types of cultivation and on the quality of the plant's phytochemical compounds has been proven (Prasad, Kumar, and Varma, 2015). For this reason, the association between biochar and plant growth promoting rhizobacteria (PGPR) and the combination of their positive effects in agriculture has been verified in terms of environmental sustainability of the ecosystem (Prasad, Kumar, and Varma, 2015). This new combined device is potentially revolutionary because it makes obvious improvements in agriculture and is a valuable tool for operators. In this way, it also contributes to the social and economic sustainability of the food production system. One of the most important vegetable plants from an agronomic point of view, and the most cultivated (Rothan, Diouf, and Causse, 2019) and commercialized all over the world (Quinet *et al.*, 2019), and also both in Europe and in America, where the food industries occupy an important economic sector, is the tomato (*Solanum lycopersicum* L.). Studying the effects of new fertilizers on tomato cultivation is therefore a more focused study but at the same time also aimed at covering a larger portion of the agricultural sector. In addition, the tomato has already been extensively studied during the observation of the effects that the biochar has in agriculture and to try the study of the biochar functionalized with PGPR means to provide a more detailed description of the use, effects, and applications of the biochar.

1.1.2 Current regulatory legislation on biochar application as soil improver

Within the European agricultural management (EU Regulations n° 2019/1009, 2019/2164) and Italian (D.lgs. n° 75/2010, implementation of EU Regulations n° 2019/1009 and 2019/2164) regulatory systems, biochar use is legally permitted as soil amendment, compost additive, and organic fertilizer.

Since different natural factors and settings among the process parameters of biochar production (i.e. feedstock nature, rate of temperature increase, core temperature and process duration) influence the resulting type of biochar in its structural and chemical composition, and biological properties (Marmiroli *et al.* 2018; Igalavithana *et al.* 2015), any biochar sample must be characterized and accomplish to certified biochar requirements prior to application to soil (EBC, 2012). The potential importance of biochar use in agriculture has been increasingly understood over the years. Therefore, the interest has grown greatly towards the biochar so much that the rate of publication on this topic has grown exponentially (Wu *et al.,* 2020). Moreover, from an application point of view, an attempt has been made to regulate the use of the biochar in order to ensure safety (Domene *et al.,* 2015). Currently, there are several national and international bodies of certifying biochar quality with the aim of promoting sustainable biochar production and applications for environmental, agricultural, and industrial use worldwide (EBC, 2012; Schmidt *et al.,* 2012; IBI,2012; BQM, 2013; IChar). In order to establish the biochar risk assessment as soil amendment, those quality certifying bodies state that biochar requires physical, chemical, and suggested biological analyses to be evaluated before applying it to soil. In Europe, the EBC (2012) is responsible for providing certifications and references for the analyses of biochar samples. On an international scale, biochar must be studied on its characteristics following the International Biochar Initiative directory (IBI, 2012).

1.1.3 Aim of the project

The recent concern for the planet has generated a reassuring flow of study towards new materials, new techniques and new applications that would make an effective contribution to the fight against global warming and climate change. In agronomic and food-based points of view, enormous efforts have been made to change the method of cultivation towards a less impactful and more sustainable approach. The focus has been on a type of agricultural management practices that restricted the use of chemical fertilizers, increased biodiversity in ecosystems, and did not involve varietal speciation.

Biochar has been proposed for years as a potential soil improver and adjuvant in the cultivation of plants of agronomic interest, horticultural and ornamental plants. It has aroused much interest in the great advantages it has shown over the years in improving soil physical characteristics and, sometimes especially in marginal land, soil chemicals but also in maintaining soil microbial biodiversity providing a valuable habitat for the microbiome. Moreover, biochar has also shown positive effects in the cultivation of plants in terms of yield production, biomass quality, water retention and quality of fruits grown.

The sustainability of the biochar is given by the concept of reducing or potentially eliminating the presence of processing waste in agricultural industries through the use of a clean technology that transforms biomass waste into biochar (Ayaz *et al.,* 2021; Matustík *et al.,* 2020; Dutta and Raghavan, 2014). As part of a circular economy, within the European regulatory system biochar is no longer considered a waste (EU Directive 2008/98/CE) and is a very eco-friendly tool helping to achieve the "*end of waste*" goal (D.lgs. n° 128/2019) by recovering waste and valorising it. In fact, the biochar is produced by plant processing waste that is heat-treated with special processes. The biochar then returns to the field again to be used as a potential adjuvant for plant growth. The Life Cycle Assessment (LCA) of biochar refers not only to the economic viability of the full life-cycle of the "biochar system" but also to the environmental impact (Dutta and Raghavan, 2014). In fact, biochar has been proposed as a significant amendment helping to tackle the environmental, social, and economic outcomes of the sustainability of agriculture and environment (Ayaz *et al.,* 2021; IBI, 2012). Therefore, biochar has been recently added to the list of fertilizers permitted to European agriculture (D.lgs. n° 75/2010, All.2), including the organic production (D.lgs. n° 2019/2164).

The importance of the biochar as soil improver and its impact on the environment require a full understanding of its properties, the mechanisms that control its activity in soil, and its whole functioning as innovative tool for agricultural practices.

Therefore, this doctoral project has set several challenging objectives:

- The physical, chemical, and biological characteristics of biochar must be controlled and the potential toxicity of the biochar arising either from the source material or from the technical specifications of the production process was investigated.
- One of the initial purposes of this work was to investigate the relationship between the biochar's properties and the production temperature, also between the specific biochar elemental composition and its surface chemical environment, and, finally, to provide a detailed description of the biochar in order to define its relative structural organization and potential interactions with chemical and microbiological elements.
- The safety risk assessment of biochar must be investigated through the study of its potential ecological toxicity by observing the biological effects on monocotyledons and dicotyledons and also assessing the mutagenicity of toxic compounds possibly present in organic extracts of biochar. This is fundamental to understand the effects of the biochar that might come into contact with animals, plants, men in all the various stages of its processing and management such as the production, storage, transport, spreading it in pot or field by hand or possible respiration.
- Furthermore, interest in food quality and its effects on health has grown rapidly in recent years. The opportunity for an added value of a food product associated with increasing nutritional value is drawing attention to this issue and soil, and crop researchers are also beginning to take an interest in how to economically increase nutritional content of foods. Nutritional quality of foods is related to a healthy and fertile soil with its dynamic interactions between roots, soil microorganisms, and macroorganisms like invertebrates (Shaikh Abdullah Al Mamun *et al.*, 2017). Microorganisms that play a crucial role to assess and assure environmental balance and promote plant positive behavior are known PGPM and PGPR (Shaikh Abdullah Al Mamun *et al.*, 2017). The study of the activities related to the PGPM

naturally present in soil and the use of the same in association with the cultivation of plants has aroused much interest in relation to the positive results obtained both for the crop productive yields and for the increased quality in fruits. They have been also proposed as an alternative to synthetic fertilizers and a smart way to sustain eco-friendly agricultural practices (Shaikh Abdullah Al Mamun *et al.*, 2017; Prasad, Kumar, and Varma, 2015). PGPM have several mechanisms to enhance plant growth. They can act as biocontrol agents against pesticides and pathogens, and preventing plant diseases, as biostimulants by producing phytohormones and chemical compound which can stimulate plant growth and as biofertilizers improving the soil nutrient and water uptake, and as promoters of root development, mineral nutrition, seeds germination (Shaikh Abdullah Al Mamun *et al*., 2017; Prasad, Kumar, and Varma, 2015). For these reasons, another main objective was to evaluate the feasibility of the association of PGPM and, then, a pool of selected microorganisms (i.e. microbial consortium) with surface and porous cavities of the biochar. The biochar could be used as a substrate for the growth of microorganisms and the possibility that they form a biofilm on the biochar surface was investigated.

- The effects of a microbial consortium, specifically designed with PGPM commonly present in soil and whom positive role within the nitrogen cycle is well known needs to be evaluated in order to establish its use as a biofertilizer. The study was conducted on tomato plants whose physiological and phytochemical aspects must be examined. Moreover, the effects on the microbial composition and adaptability of treated soil were considered.
- In addition, another competitive goal set within this work was to observe whether and what kind of variations might occur in the plant at the level of metabolites and proteins resulting from the treatments. In fact, after the characterization of the biochar including the assessment of the safety of its application, and after the "functionalization" (i.e. to use biochar as a substrate to grow microorganisms) of it with a microbial consortium of PGPM, next step was to set up an *in vaso* experiment in the greenhouse. Two varieties of *Solanum lycopersicum*,

Ailsa Craig and *Heinz 3402*, were grown with soil, with soil and biochar, with soil and biochar functionalized with the microbial consortium, and with soil and seeds functionalized with the same microbial consortium. Effects on physiological and enzymatic properties and metabolomic response of plants exposed to treatment were evaluated.

CHAPTER 2 Biochar characterization

2.1 Introduction

2.1.1 Biochar production with gasification thermal technology

Three biochar samples (Borgotaro Grigio BG, Correggio C, and Modena Tomaselli MT) from three different areas of the Emilian Tuscan Apennines produced with the same gasification technology were evaluated for this work. They were all produced at the temperature range of 900-1000°C, except for BG which was produced at lower temperature compared to the others (Table 1).

Gasification is a thermochemical conversion of solid or liquid biomasses into a combustible gas (H_2, CO and CH_4) in a controlled experimental condition with a limited oxygen-supply or adequate supply of oxidizing agents (air, air enriched with oxygen or pure oxygen), and at high temperature ranges (McKendry, 2002). The fine grain size material and high porosity produced by gasification can be used as soil improver. Due to the complexity of the whole conversion, it could be fairly said that gasification is strongly involved at the molecular scale on the physical structure formation and surface chemical organization. Indeed, significant differences in the products could be found, related to the variety of the operatation conditions (heating rate, gas residence time, temperature of the reactor, cooling procedure) (Marmiroli *et al.*, 2018; Blok *et al.*, 2016). In my study, an AGT (Advance Gasifier Technology company, Rome, Italy) Gasifier down-draft, open core, fixed bed biochar storage system has been employed.

Table 1 Raw materials and production settings for biochar samples

BIOCHAR	FEEDSTOCK	PROCESS RANGE TEMPERATURE	TYPE OF PROCESS
Borgotaro grigio (BG)	Mixed Broadleaf	500-600°C	Gasification
Correggio (C)	Mixed Broadleaf	900-1000°C	Gasification
Modena Tomaselli (MT)	Mixed Broadleaf	900-1000°C	Gasification

2.1.2 Biochar characterization

Biochar refers to a biological product obtained from heat-treated vegetative biomass with the final aim of being used in soil for agriculture (Lehmann and Joseph, 2009). Because biochar is derived from a very large variety of source biomasses, and because the quality of the source biomass varies greatly in relation to the environment of origin, the type of specific instruments and techniques used to collect it and also the type of instrument used to produce biochar as well as the setting parameters for production, the biochar must be subjected to chemical-physical and biological screening before it can be used as a fertilizer and adjuvant for crops. The production and management of the biochar in agriculture comply with detailed legislations at both national and European level. With reference to the D.Lgs. 75/2010 All.2 those in Table 2 are the chemical and physical analyses to be done compulsorily before the use of biochar in agriculture. Italian legislative measures on this subject follow the European ones.

Table 2. Mandatory analyses to certify biochar quality according to National Legislation.

Feature	Unit of measure	Value required	Method reference
pH		4-12	UNI EN 13040
Electrical conductivity	mS/m	≤1000	UNI EN 13040
C_{org}	% d.m.	≥20 and ≤30 III grade >30 and ≤60 II grade >60 I grade	UNI EN 13654-2
Moisture	%	≥20	UNI EN 13040
Ash	% d.m.	>40 and ≤60 III grade ≥10 and ≤40 II grade <10 I grade	UNI EN 13039
H:C_{org} ratio		≤0,7	D.lgs. 7276 31/05/2016 Suppl. 13 n.2
Metals	mg kg^{-1}	Lead 140 Cadmium 1,5 Nichel 100 Zinc 500 Copper 230 Mercury 1,5 Chromium 0,5	UNI EN 13650
PAH	mg kg^{-1}	6	D.lgs. 7276 31/05/2016 Suppl. 13 n.2
Particle-size	mm	0,5-2-5	UNI EN 15428

2.1.3 Risk assessment of the potential mutagenic property of biochar

Despite many studies promoting biochar application to soil as a soil amendment, mainly centered on agronomical benefits, little attention has been paid to potential unintended effects (Kookana *et al*., 2011), such as the ecotoxicological risks of its application to soils and possible toxic effects on agricultural operators. Since biochar is produced from biomass, including polluted organic wastes, pollutant content such as heavy metals, polycyclic aromatic hydrocarbons (PAHs) and dioxins (Schimmelpfennig and Glaser, 2012) in biochars might be noticeable and present potential environmental risks when applied to soils. Safety assessment of the potential risk of using biochar as biological soil fertilizer was investigated by the microbial mutagenicity assay: the Ames test.

The Ames test is a rapid test specifically designed to detect genetic mutations that occur on the bacterial histidine operon caused by a wide range of toxic compounds. It is usually used as an initial screening for new molecules, undefined compounds, carcinogenic and toxic substances but also for drugs, dyes, reagents, cosmetics, chemicals, and environmental samples such as wastewater, pesticides and others (Vijay *et al.,* 2018). Although this test does not provide direct information on the mutagenic and carcinogenic potency of a substance, it has been demonstrated that many chemicals which result positive in this test also exhibit mutagenic activity in other tests (Maron and Ames, 1983). The Ames test is often preferred because of its quick response and visually clear results as well as its ease of execution. Several *Salmonella typhimurium* strains could be employed as standardized in the original protocol of Maron and Ames (1983). They all carry different mutations in the histidine operon which make them auxotrophic to the relative amino acid. Since the mutation that makes them auxotrophic also partially falls into the biotin operon, the bacteria that carry such mutation turn out to be auxotrophic also to the amino acid biotin. *Salmonella* can only grow on growth media in the presence of histidine and biotin. However, when a mutagenic substance faces the bacterial strains, it can produce a genetic mutation at the operon level by turning auxotrophy into prototrophy metabolism for both amino acids histidine and biotin, restoring

the original gene function. At this point, newly mutated bacteria reverted to histidine independence can grow on minimal media in the absence of histidine and biotin. In fact, this test is also called a "reversion assay" as the functional capability of the bacteria to synthetize the histidine amino acid has been restored. These specific *Salmonella typhimurium* strains are currently employed in detecting point DNA damages which involve substitution, addition or deletion of one or a few DNA base pairs, because they carry mutations that act as "hot-spots" for mutagenic chemicals and thus are highly respondent to them (Tejs, 2008). Moreover, the number of reverted colonies is dependent on the dose of carcinogenic compounds present in the test sample (Tejs, 2008).

International testing guidelines have been established in order to ensure chemical risk assessment (OECD 471). The Ames test has been proposed among the ecotoxicological analyses to be performed in order to monitor biochar quality (Piterina *et al.*, 2017).

2.1.3.1 Genetic characteristics of *Salmonella typhimurium* tester strains

The bacterial strains that have been used for this test were *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA100. A summary of their genotypes is listed in Table 3. They differ by the type of mutation carried on the histidine operon. In addition to the mutation in histidine operon, strains also have other mutations that significantly increase bacterial sensibility to mutations and, therefore, they are able to provide a more accurate investigation for toxic compounds. One of these additional mutations is a *rfa* mutation and refers to a partial loss of the lipopolysaccharide barrier present on the bacterial surfaces that allows a major permeability even to large molecules such as those with aromatic rings which have bigger steric bulk and which could not penetrate otherwise in the normal cell wall (Vijay *et al.*, 2018). Bacteria also bring *uvrB* mutation deriving from a deletion of a gene coding for the DNA excision repair system and therefore providing enhanced sensitivity in showing effect of many mutagens. This deletion partially occurs within the bacterial biotin operon and, for this reason, both *Salmonella typhimurium* strains also require biotin for growth (Vijay *et al*., 2018). Finally, both bacteria contain the R-factor plasmid, *pKM101*, that improve the

ability to detect mutagens by enhancing an error-prone DNA repair system normally present in *S. typhimurium* strains (Maron and Ames, 1983).

The higher permeability, the lower accuracy in the mechanism of damage repair at the level of nucleic acids, the presence of the R-factor plasmid result in greater sensitivity and in a higher number of revertant colonies on minimal medium.

2.1.3.1.1 *Salmonella typhimurium* TA 98

The histidine mutation within the correspondent operon is the *hisD3052* gene mutation. These gene codes for histodinol dehydrogenase. The plasmid is able to show frameshift mutagens in repetitive sequences as "hot spots" resulting in a frame shift mutation which results in a restoration of the original function to synthetize histidine.

2.1.3.1.2 *Salmonella typhimurium* TA 100

The *his* G46 mutation in the hisG gene in TA100 codes for the first enzyme of histidine biosynthesis (Ames *et al.,* 1975b) and provides a substitution of a proline (-GGG-) for leucine (-GAG-). Using the bacterial reverse mutation assay, potential point mutations in this strain that occurs as substitutions in one of the G-C pairs (Maron and Ames, 1983) can be detected. The R-factor plasmid is the key factor that enhances its ability to detect mutagens at a single basepair substitutions level.

Table 3. Genotype of the used Salmonella typhimurium tester strains, mutations in their histidine operon and additional mutations.

Salmonella typhimurium strains	Mutation in the histidine operon	Type of mutation	Genotype		
TA98	hisD3052	Frameshift	rfa	uvrB	pKM101
TA100	his G46	Substitution	rfa	uvrB	pKM101

2.1.3.2 Spontaneous Reversion Control

Each bacterial *Salmonella* strain has a characteristic spontaneous reversion (Table 4), both with and without the mammalian liver enzymes activation. The revertant frequency is described in the literature and can be confirmed experimentally. Since the reversion frequency is not strictly reproducible although relatively constant (Tejs, 2008), it is best to indicate a reversion range for each bacterial strain. In addition, different solvents can influence and change spontaneous reversion. The spontaneous reversion can be expressed as number of reverted colonies per plate (Maron and Ames, 1983).

Table 4. Spontaneous revertant control values for various strain types and number of revertant (Mortelmans and Stocker, 1979)

Strain type	Spontaneous	Revertants
	With S9	Without S9
TA98	20-50	20-50
TA100	75-200	75-200
TA102	100-300	200-400
TA104	200-300	300-400
TA1535	5-20	5-20
TA1537	5-20	5-20

2.1.3.3 The Rat Liver Extract use

Many toxic and potentially mutagenic compounds occur biologically in the inactive or pro-active form (Tejs, 2008). To accomplish their toxicity, they must be activated. Generally, activation is carried out enzymatically during the metabolism of higher organisms. Many other compounds, however, that are already present as mutagens and carcinogens are deactivated with metabolism. Thus, as *Salmonella* bacteria can not metabolize substances because of the absence of an enzymatic metabolic system, a mammalian metabolic pattern, the rat liver extract, which contains several metabolic enzymes with catalytic function, is also introduced in the Ames test (Vijay *et al*., 2018; Tejs, 2008). The microsomal activation solution consists of a rat liver extract homogenate (Trinova Biochem, GmbH, Germany) of a Sprague Dawley which contains liver soluble enzymes and cofactors belonging to monooxygenase and cytochromeP_{450} groups. The homogenate was prior induced

with Aroclor 1254 (Monsanto KL615) in order to concentrate the metabolic enzymes system. The oxygenase requires the reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADP) which is generally *in situ* by the action of glucose-6-phosphate dehydrogenase and reducing NADP both work as cofactors in assay.

2.1.3.4 S9 mix

A mixture solution was prepared following the Ames protocol. It was prepared fresh every time the assay has been performed. The concentration of S9 can vary according to the specificity of some test compound (Maron and Ames, 1983). The concentration used in this assay was of 4%.

2.2 Materials and methods

2.2.1 Feedstock and biochar production

Feedstocks used in this study were collected from different areas of Emilian Tuscan Apennine: Borgotaro grigio (BG), Correggio (C), and Modena Tomaselli (MT). The origin of feedstocks was mixed broadleaf. Biochars were produced by gasification in a fixed-bed, down-draft gasifier (Modena, Italy) at different temperatures. BG was produced at the range of 500-600°C low constant temperature and both C and MT were produced at 900-1000°C, high constant temperature. Biochar samples and feedstock compositions are summarized in Table 1.

2.2.2 Sample preparation

All samples were prepared according to ISO 13909-4: 2016 and to the American Society for Testing Materials (ASTM) D1752-84 which is recommended by the IBI guidelines (2015). After homogenization biochar samples were divided into representative portions. All air-dried samples were sieved in a vibratory mill with progressive 4mm, 2mm, 0.71mm, 0.06mm sieves before any analysis (in order to determine their particle size distribution according to UNI EN 15428:2008 with minor modifications).

2.2.3 Biochar characterization

2.2.3.1 Physical and chemical analyses

***pH and Electrical Conductivity*.** The pH and electrical conductivity (EC) values of each sample were measured with a glass electrode (SevenCompact Duo, Mettler-Toledo) in a 1:5 (v/v) biochar/deionized water mixture after 1 h shaking and stabilization, according to DIN ISO 10390:2005 and UNI EN 13038:1999 respectively.

Values of pH and EC were also measured in soil-biochar mixture at 0,5, 1, 3, and 5% (w/v) concentrations. Measures were taken immediately after the mixture preparation and after 30 days in order to establish whether chemical changes occur in the soil with the presence of biochar and how much those changes influence soil properties. EC values were expressed as $\mu S\ cm^{-1}$.

Bulk density. The bulk density provides information about the biochar mass related to the volume unit, considering the air interstices present in the solid matrix. It is not an intrinsic property of the material, but depends on size, shape and compaction of the particles. It is important in materials handling, production yield and application considerations. Bulk density was evaluated according to ISO 23499:2008 protocol with minor modifications. The samples were filled into a graduated cylinder with a capacity of 100 mL and the mass is determined by weighting. A plunger of 650 g with the same diameter of the cylinder was placed on the top for 3 min. After the compaction time, the plunger was removed, and the sample weight and volume measured. The biochar bulk density therefore results from the mass-volume ratio and results were expressed in $g\ cm^{-3}$.

Moisture content, Water Holding Capacity and Dry Matter Content. Moisture content (MC) and water holding capacity (WHC) were measured according to DIN ISO 14238 and The American Society for Testing and Materials ASTM standard protocol D2216-10 "Laboratory determination of water (moisture) content of soil and rock by mass". Samples of known weight were placed onto vessel and oven-dried at 105°C for a standard drying time of 24 h or until percent moisture readings over 1 h periods are less than 0.1%. In addition, a saturation of 1 day will provide homogenization of water content throughout the samples. Each sample evaluated was saturated with deionized water to establish biochar's WHC. Water was applied to each mixture until excess water was observed. Samples were then allowed to sit for 24 hrs to assure homogeneity of water content throughout the sample. After that, samples were drained by gravity for another 24 hrs through a Whatmann filter paper n. 41. Samples were weighted to determine wet mass. They were then dried at 110°C for 24 hrs using a convection oven and remassed to determine the dry mass. Results yielded the amount of water

being held by each mixture. Dry matter content (DMC) was calculated according to UNI EN 13040:2008 protocol. Briefly, no less than 50 g of samples were put in a tray spreading it to an even depth not exceeding 2 cm and, then, in a convection oven at 103°C for 24 hrs and then remassed.

The size 0.71-2 mm of each biochar showed good characteristics (Table 5) in order to be used in soil as fertilizer. The response of this size to the features observed (i.e. pH, EC, bulk density, WHC, MC, and DMC) was generally positive than the other two sizes considered. Therefore, the study of the biochar continued with more in-depth analyses only with this dimension.

***Elemental composition*.** Elemental analysis was conducted for each sample to determine C_{org}, H, N, O contents of biochars according to UNI EN 13654-2. Dried biochar samples were milled through a 1-mm sieve (Cutting mill SM 300, Retsch® mbH, Haan, Germany). In order to determine the total C_{org}, H, and N content, ground samples of 0.15 g were loaded into tin foil cups and analyzed. The analysis was performed with LECO Truspec® CHN Analyzer (LECO Corporation; Saint Joseph, Michigan, USA). In addition to the elementary composition, it is important to consider the associated elementary ratios. The H/C and O/C_{org} ratios provide important information on the structure of biomass.

Organic Matter Content and Ash content. Organic matter (OM) and ash content were evaluated according to UNI EN 13039:1999 European Standard protocol. Biochar samples were oven-dried (M710 Thermostatic Oven, F.lli Galli, Milan, Italy) at 103°C to a known constant weight, and then incinerated at 450°C in a muffle furnace (Model A022, Matest S.p.A, Bergamo, Italy) for 15 h. After incineration, the residues were weighted, and OM and ash were calculated as the difference between the fresh and final incinerated weights. Results were expressed as a percentage of the initial total dry weight.

Volatile Matter Content. The volatile matter content was evaluated according to UNI EN ISO 13039:2011 dispositions, with minor modifications. The thermogravimetric analysis (TGA) on biochar samples was performed by using a Perkin Elmer Pyris 1 TGA thermal analyzer under static-air/N_2 flow with the following temperature ramping scheme: temperature equilibration at 30°C, followed by a linear heating at a rate of 30°C min-1 from 30°C to 900°C.

Metals. The concentration of metals was determined by Atomic Absorption Spectrometry (AAS) method according to the EBC (2015). Metals evaluated were: Cd, Ni, Cr, Cu, Pb, Fe, Co, As, Hg, and Zn. Biochar samples were incinerated in ceramic crucibles in a muffle furnace at 500°C for 16 h. Then, all the ash samples were retrieved from the crucibles and digested with a three-steps method with nitric acid 65% (Carlo Erba, Milan, Italy) at 165°C for 30 min, 200°C for 30 min, and finally 230°C for 30 min in a heated digester thermoblock (DK20, Velp Scientifica, Usmate Velate, MB, Italy). Digested solutions were diluted with deionized water to 30% (v/v) acid concentration. Metals concentration was recorded using flame atomic absorption spectrometry (AA240FS, Agilent Technologies, Santa Clara, CA, United States). Calibration curves for each metal were prepared using 1,000 ppm certified standard solutions (Agilent Technologies, Santa Clara, CA, United States). Three instrumental and biological replicates were performed on each sample. The metal concentrations were expressed in mg kg^{-1} biochar. Heavy metal concentrations in BG have also been investigated with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique by Neotron S.p.a (Modena, Italy).

Polycyclic Aromatic Hydrocarbons. PAHs in biochar were extracted with 300 ml hexane/acetone mixture using a Soxhlet extraction system and analyzed by a gas chromatography (Neotron S.p.a., Modena, Italy) with a mass spectrometer (GC-MS). Briefly, the extraction solution was concentrated in a silica gel column with internal diameter of 0.53 mm for cleanup and then, the eluate was transferred to a 30 m capillary column with internal diameter of 0.25 mm. Data were collected with 70 eV scan mode (41-440 m/z). A 16 US EPA PAH mixture solution was used as a reference.

FTIR-ATR. Fourier Transfer Infra-Red Spectroscopy with Attenuated Total Reflection modality (FTIR-ATR) was analyzed on a Nicolet 5700 FT-IR Spectrometer. 1 mg of dried pulverized biochar was inserted in a diamond crystal gate and the following broad-band assignment was used (Chen and Chen, 2009): the infrared light absorption range from 4000 cm^{-1} to 3500 cm^{-1}, free and H-bonded O-H stretching vibrations of hydroxyl groups from phenols, alcohols and organic acids, 3500 cm^{-1} to 3000 cm^{-1}, alkenes, alkynes, arenes, broad H-bonded O-H stretching vibrations, weak intensity of primary and secondary amines, and very broad O-H stretching vibrations of carboxylic acids and derivatives; 3000 cm^{-1} to 2000 cm^{-1}, alkanes, alkynes, medium intensity C-H bonds of aldehydes and ketones, nitriles, thiols and isocyanates, isothiocyanates, diimides, azides and ketenes; 2000 cm^{-1} to 1000 cm^{-1}, weak C-H bending of aromatic compounds, aldehydes and ketones, carboxylic acids and derivatives, esters, amides, nitriles, sulfates, aromatic amines and esters, primary and secondary alcohol, and O-H stretching of phenolic compounds; 1000 cm^{-1} to 460 cm^{-1}, alkenes, C-H and O-H bending and NH_2 scissoring.

ESEM. An Environmental Scanning Electron Microscope ESEM FEG2500 FEI (FEI Europe, Eindhoven, Netherlands) was used in order to investigate the macro, micro and nano spaces of the biochar structures (Marmiroli *et al.*, 2018). ESEM observations were carried out with Bruker XFlash®6 | 30 X-ray detector. An environmental low-vacuum (60 Pa) with a large field detector allowed optimal secondary electron imaging that was performed at 5 kV and 10 kV with a beam size of 2.5 μm. The working distance was approximately 10 mm, and the scanning time 1–3 μs.

Zeta potential. The chemical environment of biochar surfaces was investigated with zeta potential according to Batista and Hong (Batista *et al*., 2018; Hong *et al.*, 2019) and Samsuri *et al.* (2014) protocols. A 0.0500 g sample of biochar that had passed through a 0.5 mm sieve was weighed into 250 mL polyethylene bottles, into which 200 ml of 0.15 mmol L^{-1} $NaNO_3$ was added. The suspensions were dispersed ultrasonically for 1h and then divided into six parts, which were then individually poured into 100 ml plastic bottles. The pH of the suspensions was adjusted within the range from 3

to 11 using NaOH or HNO_3. After the pH had stabilized, the suspensions were allowed to stand overnight. Then the zeta potential was measured using a Zetasizer Nano Series ZS90 (Malvern Instruments, Malvern, UK).

X-Ray Diffraction. Powdered biochar samples were put into the instrument gate and XRD measurements were conducted using standard powder diffraction procedures with Thermo Electron diffractometer (X-ray Diffractometer Bruker Smart Breeze, Bruker AXS Inc., Madison, WI) using through the range of 2-55° 2Θ in a transmission mode, a wavelength of about 1.54 Ă and a thin film attachment detector and a parabolic mirror tube with a scan rate of about 6.

2.2.3.2 Biological analysis

2.2.3.2.1 Germination test and root elongation assay

The germination test and root elongation assay were carried out following the ISO 11269-1: 2012 and ISO 11269-2:2012 protocols with minor modifications. Seeds of *P. sativum* L. and *H.vulgare* L. were used. Four doses of biochar (0.5, 1, 3, and 5% w/v) were used and added to MS agar media plates (0,5% Murashige and Skooge basal medium, 2% sucrose) (Sigma Aldrich, St. Louis, USA), media were then autoclaved at 121°C for 20' and then poured into Petri dishes and let solidify. Seeds were surface sterilized using 70% (v/v) ethanol for 3 min and 50% (v/v) sodium hypochlorite (Sigma Aldrich, St. Louis, USA) for 30 min, rinsed with deionized water and air dried. All the seeds were chosen after a previous screening to control germination rate (>80 %). Nine seeds were placed on each MS + biochar media plate. Sealed plates were incubated in a growth chamber at 25°C for 72 h in darkness.

Germinated seeds were counted and the relative seeds germination (RSG%) derived, as below.

$$RSG\% = (Gt / Gc) \times 100$$

where Gc is the number of germinated seeds of the control and Gt is the number of germinated seeds of the treated samples.

Moreover, root length was observed and germination index (GI%) was derived following Marmiroli *et al*. (2018) protocol, and root/shoot index (SRI%) was calculated, as below.

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

where Gc = germinated seeds in the control; Gt = germinated seeds in the treatments; Lc = main root length in the control; Lt = main root length in the treatments.

SRI% = (Ls / Lr) * 100

Where Ls = average shoot length and Lr = average root length.

Statistical analyses

Statistical analyses for pH, EC, bulk density, MC, WHC, DMC, OMC, ash content and metals were collected in triplicate and calculated using Past v.4.0 software. An analysis of variance (ANOVA) was carried out on the data, followed by Tukey's post hoc test.

2.2.3.2.2 The risk assessment of biochar through bacterial reverse mutagenic assay: the Ames test

There are currently very few studies that test the mutagenicity and carcinogenicity of the biochar. Biochar is an inert material, but although the release of chemical compounds is limited and gradual, it must be tested for environmental risk assessment. Not only, but also the assessment of possible effects in higher living organisms such as animals, plants and human must be investigated. Before each set of experiments, bacteria have to be prepared freshly and the genotype of each *Salmonella typhimurium* strain must be checked.

2.2.3.2.2.1 Control of bacterial strain genotype

All the marker genes must be verified before assessing each experiment. The histidine dependence could be analyzed by plating each bacterial strain onto minimal medium plate in absence and presence of the amino acid (i.e. Vogel-Bonner minimal medium E and glucose). The amino acid can be added directly into liquid medium agar before pouring it into Petri dishes or it can be spread out on the surface of solid medium plate. For each plate 0,1 ml of 0,1M of histidine and 0,1 ml of 0,5 mM biotin

are suggested (Maron and Ames, 1983). Control plate have been prepared with biotin and without histidine. The strains are examined for growth in biotin/histidine plates.

The *rfa* mutation in *S. typhimurium* is expressed through sensitivity to crystal violet dye (Sigma Aldrich, St. Louis, USA). The control of *rfa* character followed the protocol of Maron and Ames with minor modifications. Both S. *typhimurium* strains have been plated onto rich nutrient agar plates. Then, a filter paper disc soaked in crystal violet has been located on the center of plate. Plates were incubated at 37°C. After 12 hrs a clear growth inhibition zone should be observed near the filter paper disc indicating the presence of the mutation that allows large molecules like crystal violet to enter the cells and kill the bacteria.

Strains having *uvrB* mutation have been tested for UV light sensitivity. Each strain was allowed to grow up by streaking it on the surface of a nutrient agar plate in parallel stripes. Under a laminar flow hood, a half part of test plate has been covered with a piece of cardboard in such a way as to block the passage of UV rays. Test plates were irradiated with a UV-lamp of 15W for 8 sec. Then, plates were incubated for 12-24 hrs and the growth of bacterial cells has been verified knowing that the cells with the mutation can grow only in the part of the non-irradiated plate.

The plasmid confers ampicillin resistance, which is a convenient marker to detect the presence of the plasmid (Mortelmans, 2006). Briefly, each bacterial culture has been grown up onto ampicillin plates and incubated at 37°C for 12-24 hrs. Bacteria with the mutation will grow up.

2.2.3.2.2.2 Preparation of biochar extracts in DMSO

Biochar samples were added with Dimethylsulfoxide (DMSO) (Fisher Scientific Italia, Milan, Italy) to extract any mutagens and pro-mutagens soluble in organic solvent. 5 g of each air-dried sample were extracted with 300 ml of 1:1 acetone:hexane mixture for ten hours with Soxhlet instrument. Then, the solvent was evaporated by rotavapor and the resulting fraction was weighed. It was then

recovered by means of 2 ml of 1:1 acetone:hexane mixture and finally dried. The extract was then resuspended in DMSO, used as internal standard and negative control for the assay at final concentration of 1 μg ml^{-1}.

2.2.3.2.2.3 Growing bacterial cultures

Salmonella cultures were grown in OXOID n.2 (Oxoid Ltd., Basingstoke, Hants, UK) specific rich liquid medium and incubated at 37°C for 12-16 hrs (Ames *et al*., 1975b) to avoid the loss of viability of cells. Each bacterial strain must be taken from the master plate or from frozen cultures and revitalized. While incubated, it must be insured adequate aeration with agitation of 120 rpm. Once reached the desired optical density measured spectrophotometrically (Varian Cary 50 UV-Vis Spectrophotometer, Agilent Technologies, The Netherlands) at 600 nm, bacteria in tubes have to be kept first at room temperature to allow their temperature to decease slowly and avoid thermal shock when they will be in contact with top agar at 45°C and then in ice bath. Taking bacteria in ice bath is necessary in order to avoid the loss of viability of the strains (Maron and Ames, 1983).

2.2.3.2.2.4 Preparation of S9 mix

S9 mix with rat liver microsomal enzymes and cofactors was prepared according to Maron and Ames (1983). This solution was made fresh and always kept in ice during the whole duration of the assay. 4% rat liver S9 (Trinova Biochem, Giessen, GmbH, Germany) previously treated with Aroclor 1254 inducer, was added to a solution with 2% $MgCl_2$-KCl, 0,5% 1M glucose-6-phosphate, 4% 0,1 M NADP, 50% 0,2 M phosphate buffer pH 7,4 and deionized water.

2.2.3.2.2.5 The preincubation procedure

In the preincubation test, all the samples tested were placed in a solution with *Salmonella*, buffer or rat liver extract (S9 mix) and left to react for a short period between 20 and 30 minutes in the incubator at 28°C while shaking. The experiment was conducted separately for each bacterial strain. The pre-

incubation mixture has been prepared as below: 0,1 ml of fresh bacterial culture, 0,5 of sterile buffer or S9 mix and 0,1 ml of diluted test samples or 0,1 ml of control sample. After the incubation time, the samples were added to the top agar freshly prepared which contains histidine and biotin (AppliChem GmbH, Ottoweg, Darmstadt, Germany), and then poured into the Petri dish. The top agar was allowed to dry and then test plates were turned upside down and incubated at 30°C for 48 hrs. This procedure has been shown to be more sensitive to mutagenic than standard incorporation methodology (Tejs, 2008).

2.2.3.2.2.6 The mutagenic assay

The Ames test has been carried out following the Maron and Ames protocol (Maron and Ames, 1983). Fresh cultures of bacteria have been grown up and brought to a final concentration of 10^6 - 10^7 cells ml^{-1}. Bacterial concentration can be verified by light optic microscope and a Bürker counting chamber. *Salmonella typhimurium* TA98 and TA100 have been grown in OXOID n.2 broth liquid medium and incubated at 37°C.

The pre-incubation procedure was chosen for this work. Three sub-cytotoxic doses have been tested. After the incubation period, the total solution with the test sample has been mixed with 2 ml of top agar and poured onto minimal medium plates. The top agar contains 0,5% NaCl, 0,6% agar and 0,5 mM histidine/biotin solution to allow for a few cell divisions and it was kept at 45°C in a heating block (Eppendorf ThermoMixer® C, Eppendorf AG, Hamburg, Germany). Top agar and pre-incubation mixture were gently mixed and poured onto Petri plates. Plates were incubated at 37°C for at least 48 hrs. Positive and negative controls were tested as well, and all the sample were tested with and without S9 rat liver mix. Revertant colonies were counted and compared to the number of spontaneous revertant colonies on solvent control plates. The test samples and the control substances have been properly dissolved in organic solvent. Positive control was 2-aminoanthracene (2-AA). Negative control was DMSO, both purchased to Sigma Aldrich (USA). They were diluted prior to

treatment and the final concentration used was 1µg/ml. Positive and negative control have been used fresh to avoid problems due to the chemical stability.

2.2.4 Data collection and reporting

After 48 hrs of incubation, reverted colonies were marked for each plate and set of experiments, both for TA98 and TA100. All plate colonies, including positive and negative control colonies, were hand-counted to avoid any problem of inaccuracy due to the presence of precipitates in the top agar or misinterpretation of the data due to minimal color contrast between the colonies and the growth medium (Tejs, 2008). Data were reported as mutagenic indices expressed as the ratio of number of reverted colonies in tested plates on the number of reverted colonies in the negative control plates as indicated in Piterina *et al.* (2017) and following the Vargas *et al.* (1995) directions. In this way, inaccurate measurements due to the presence of spontaneous reverting colonies were reduced.

2.3 Results and discussion

2.3.1 Physical and chemical analyses

Table 5. Physical and chemical characterization of biochar samples and their different partide sizes.

		BG			C			MT		
	Units									
Particle sizes	mm	0.063-0.71	0.71-2	2-4	0.063-0.71	0.71-2	2-4	0.063-0.71	0.71-2	2-4
pH	pH	13,04 ± 0,17 a	10,17 ± 0,11 b	9,98 ± 0,18 b	10 ± 0,22 a	9,28 ± 0,13 b	9,0 ± 0,28 b	12,03 ± 0,06 a	10,38 ± 0,04 b	10,32 ± 0,31 b
EC	µS/cm	36,93 ± 0.11 a	4581,5 ± 2.12 b	3531,3 ± 9.07 c	1047,3 ± 3.06 a	476,8 ± 0.99 b	299,47 ± 2.34 c	2009,3 ± 111.54 a	792,05 ± 4.6 b	820,8 ± 2.54 b
Bulk density	g/cm^3	0,69 ± 0.014 a	0,17 ± 0.002 b	-	0,45 ± 0.006 a	0,25 ± 0.01 b	0,20 ± 0.01 c	0,27 ± 0.009 a	0,14 ± 0.008 b	0,11 ± 0.006 c
WHC	%	23,83 ± 90.2 a	80,16 ± 17.7 a	64,07 ± 102.6 a	170,42 ± 94.2 a	45,37 ± 10.5 a	40,05 ± 51.9 a	44,37 ± 6.2 a	70,87 ± 12.9 a	76,94 ± 115.4 a
MC	%	1.22 ± 0.79 a	2.69 ± 1.69 a	-	2.69 ± 2.36 a	7.19 ± 0.27 b	7.83 ± 1.03 b	3.59 ± 2.05 a	6.02 ± 4.05 a	3.72 ± 1.46 a
DMC	%	98,80 ± 0.77 a	97,39 ± 1.59 a	-	97,42 ± 2.23 a	93,30 ± 0.23 b	92,74 ± 0.89 b	96,56 ± 1.93 a	94,41 ± 3.69 a	96,43 ± 1.35 a

The physical and chemical properties of biochar samples are reported for each particle-size expressed in mm. The investigated properties are: pH, Electrical Conductivity (EC), bulk density, Water Holding Capacity (WHC), Moisture Content (MC), and Dry Matter Content (DMC).
Means of three biological replicates are reported in Table 5 with standard deviations. Letters mean significant differences at $p < 0.05$ (Tukey HSD test).

The alkalizing properties of biochar were known, and all the pH values were greater than 8 for all the biochar samples and all their particle-size dimensions. It was important to emphasize that the size of the biochar strongly influenced the pH of the solution and, therefore, of the soil. In all biochar samples, pH changed in relation to the size as shown in the Table 5. Higher pH values corresponded to smaller and finer sizes while values gradually decreased with increasing biochar sizes. Moreover, within each biochar sample, the difference of pH values between powdered biochar and bigger solid biochar was statistically significant. Furthermore, analyzing all the samples and focusing on the same size, the three biochars showed significant different pH values in 0.063-0.71 mm and 0.71-2 mm sizes, while in the 2-4 mm size only C biochar showed a significant difference with the other biochar samples. Also, the process temperature significantly affected pH values of biochar samples.

Modulations of pH values in the soil-biochar mixtures were shown in Fig. 1. The pH values of the mix in both time intervals were higher than that of the soil and were not dose-dependent showing good ability to adapt to the natural conditions of the soil. However, the pH values at time T_1 (after 30 days) decreased compared with those at T_0. This result was important because it showed that the soils did not become alkaline with the addition of biochar but managed to return to the original condition, without making changes to the ecosystem. Therefore, w/w 5% and 1% BG pH were significantly greater than the soil but also very lower than that at T_0. W/w 5% and 0,5% C had the same behavior than BG and were greater than the soil at T_1 but lower than their correspondent values at T_0.

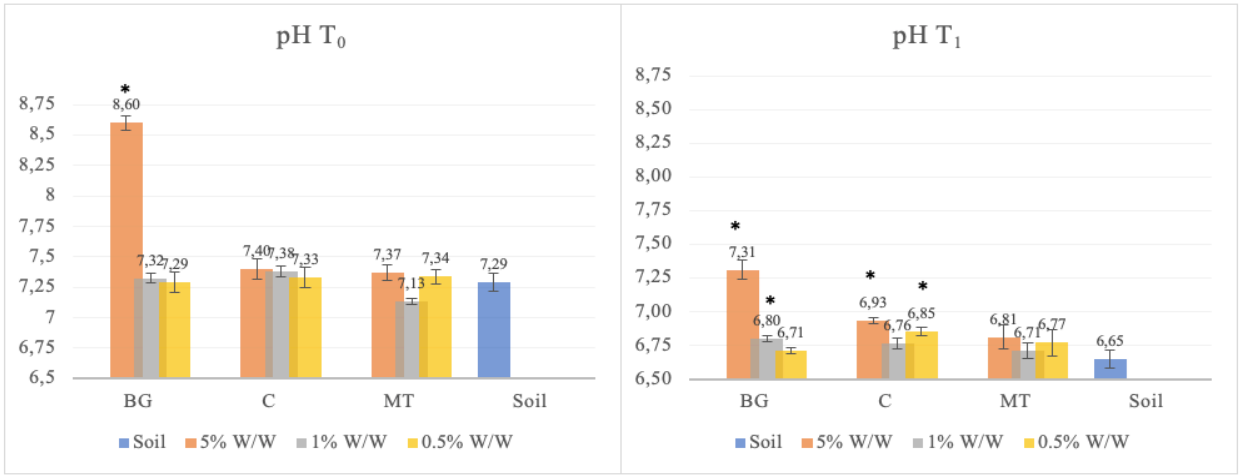


Fig. 1. Values of pH in soil-biochar mix with increasing biochar concentrations (w/w 0,5, 1, 3, 5%) at two time intervals: T_0 and T_1 (30 days). Data shown as means ± SD of triplicate.

The EC is an intrinsic property of the biochar and it was extremely variable both compared between all samples and between the sizes of the same biochar sample. In addition, for the MT biochar sample there was a significant difference in the values between the finest and the larger grain sizes (Table 5).

The EC of soil-biochar mix showed a dose-dependent trend and this can be attributed to the specific chemical environment present on the surface of the biochar. Salinity increased with increasing biochar doses, especially in BG and MT samples where the differences when compared to the soil were significant both at time T_0 and T_1. These values were much larger than that present in soil in all samples except for C sample which showed values similar to those present in soil at time T_0 and decreased at time T_1.

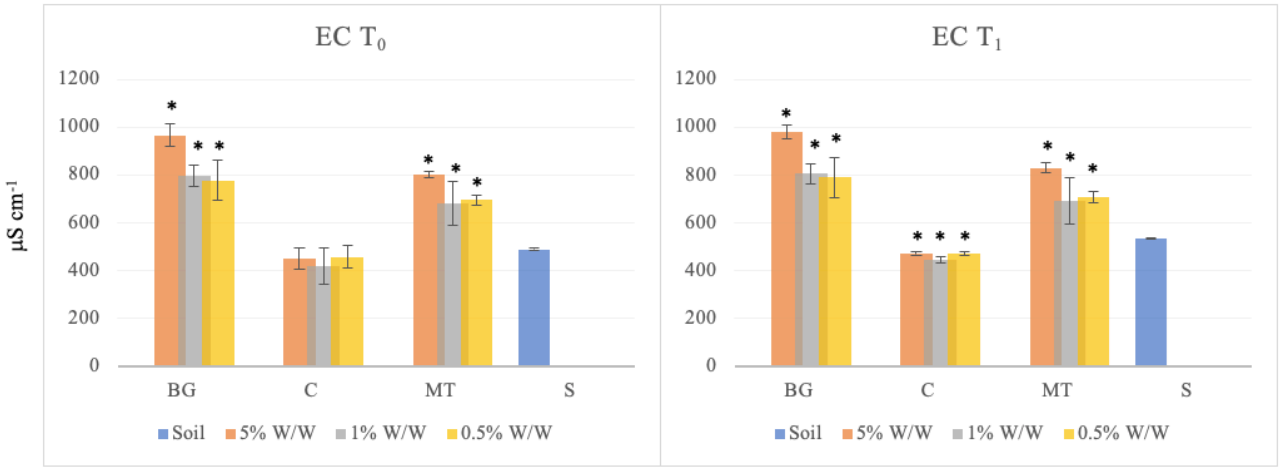


Fig. 2. Values of electrical conductivity (EC) in soil with increasing biochar concentrations (w/w 0,5, 1, 3, 5%) at two time intervals: T_0 and T_1 (30 days). Data shown as means ± SD of triplicate.

As expected, bulk density values were inversely proportional to dimensions. This occurred for each biochar sample (Table 5). Bulk density of biochar samples in this study varied from 0,11 to 0,69 g cm $^{-3}$. The highest bulk density recorded in the smallest fraction of the particle-sizes (0.063-0.71 mm) was in BG (0,69 g cm^{-3}), followed by C (0,45 g cm^{-3}), and MT (0,27 g cm^{-3}) while, considering the intermediate particle-size (0.71-2 mm) the highest bulk density was recorded in C (0,25 g cm^{-3}), followed by BG (0,17 g cm^{-3}), and MT (0,14 g cm^{-3}). The different bulk density of biochar of various dimensions can bring physical changes to soil texture. Depending on biomass structure, biochar bulk density is strictly related to size and compaction of the particles (IBI, 2015). Typically, biochar decreases soil particle density, especially low bulk density soil which are used for compacted soils,

and its effectiveness increases as the amount of biochar applied increases (Blanco-Canqui, 2017). Density and porosity almost certainly play an important role in biochar transport within the soil and are likely to determine the ecosystem services provided by biochar amendments to soil. They are taken into consideration to better investigate both short- and long-term effects of biochar on soil environment. Unfortunately, we were not able to provide data for the biggest size of BG sample because of the little amount of this specific particle size, but for each sample the bulk density was statistically different among the different sizes.

Despite the fact that biochars have been studied extensively, the interaction with water is not well understood and contradicting findings exist in the literature. Hydrophobicity is a result of the surface functional groups, whereas water holding capacity also depends on the porosity of the biochar's bulk volume. These properties can have counteracting or overlapping effects and are therefore sometimes not clearly distinguishable from each other. It is believed that a further increase in gasification temperature results in a decrease of the hydrophobic character of the biochar (Zornosa *et al*., 2015), that can maintain its polar surface functional groups otherwise lost by employing different thermal techniques as pyrolysis (Weber and Quicker, 2018).

The values of WHC were variable and were not related to the size of the biochar but only to the nature of the biochar source material. No value showed significant differences. However, all samples with the only exception of BG (0.063-0.71 mm) have shown excellent percentage of water retention.

MC refers to the water content of the biochar, and as measured may include some highly volatile organic compounds that evaporate along with the water. It can vary greatly, depending on how the biochar is produced and whether it has accumulated moisture during storage or shipping. In order to assess and compare properties of various biochars on a uniform "dry basis" (Chevali and Kandare, 2016; Brennan *et al.*, 2001; Nartey and Zhao, 2014), it is necessary to determine how much of the biochar is not water. The moisture measurement may include not only water content but also other

low-boiling organic solvents. MC in biochar samples did not change in relation to size for BG and MT samples. This is explained by the fact that the porous structure of the biochar has a macro, micro and nano sizes. Therefore, the pores of the biochar find accommodation for the water even in the smallest ones. In C sample, it was observed significant differences between the MC value in 0.063-0.71 mm dimension and the MC values found in the other dimension groups. Those latter were higher than the other one.

The DMC did not show differences between sizes of the same biochar sample with the only exception of C biochar. In fact, there was a significant difference in mass between the smallest size and the greater ones. The value denoted the purity of biochar which must contain at least 50% following international directives. Higher DMC values reflected greater and better biochars.

The elemental composition is reported in Table 6. The values of each element and the elemental ratios that provided information about the composition and the structure of the biochar and about its relationship with the employed production technique. All the samples showed a carbon percentage (C) considered optimal by the IBI and the EBC standards. The H/C, O/C and C/N ratios refers to the character of biochars. H/C and O/C ratios resulted of the dehydration and decarboxylation reactions. O/C ratio indicated the polarity and abundance of polar oxygen containing surface functional groups in biochar, higher the ratio more were the polar functional groups. Also, these groups actively took part in adsorption of heavy metals. H/C ratio indicated the aromatically and stability of the biochar and it characterizes the biochar when it is less than 0.5.

Biochar C/N ratio varies widely between 17 and 7242. This ratio is often used as an indicator of the ability of organic substrates to mineralize and release inorganic N when applied to soil. Since most of the biochar was composed of organic C of biological origin, which is not easily mineralized, this can cause the immobilization of N by its high C/N. ratio. However, the remaining part of organic C

(with even higher C/N ratio) did not cause mineralization-immobilization reactions due to its high degree of recalcitrance.

Table 6. Elemental composition of biochar samples.

%	C	H	N	S	O	H/C	O/C	C/N
BG	58,29	3,75	3,40	0,00	34,56	0,06	0,59	17,14
C	54,75	1,54	0,48	0,00	43,23	0,03	0,79	114,06
MT	72,42	1,05	0,01	0,00	26,52	0,01	0,37	7242,00

OM and ash content showed significant differences between the BG sample with C and MT (Table 7). In any sample, all values were high, leading to the good quality of biochar. In fact, according to EBC indications, a quality biochar contains an organic matter of at least 50% of its dry weight and here all the samples showed to exceed that percentage (Table 7). The quantity of ash is an indicator often linked to pH values in a proportional way due to the proportion of minerals and inorganic substances. They, as well as moisture content and volatile matter content registered by TGA analysis, are strictly dependent factors of the properties displayed by biomass feedstock (Nartey and Zhao, 2014).

Table 7. Organic matter content (OMC) and ash content in biochar samples.

		BG	C	MT
OMC	% (dry weight)	64.16 ± 19.62 a	86.45 ± 2.34 b	90.86 ± 4.54 b
Ash content	% (dry weight)	35.84 ± 19.62 a	13.55 ± 2.34 b	9.14 ± 4.54 b

TGA results showed that at temperature 750°C the thermal decomposition was almost complete (Fig. 3). The first mass loss ($50 < T < 150$°C) was due to moisture and evaporation of certain volatile compounds. The second mass loss ($200 < T < 360$°C) was related to the thermal degradation of hemicellulose and cellulose. The first degradation occurred at lower temperature in the sample BG in both the air and nitrogen flows: it started the decomposition at 300°C while the other samples required at least 400°C. Lignin is a more stable component presenting a large range of thermal degradation (from 250 to 500°C or even higher temperatures, depending on biomass), and in this way the third degradation step ($360 < T < 600$°C) was attributed to lignin degradation. The TGA analysis provided information on the quantity of ash, organic matter and volatile matter contents expressed as

percentages of the dry weight. Data about ash and organic matter content have been confirmed to ones carried out by the UNI EN 13039:1999 European Standard protocol. All biochar samples showed to have great stability at high temperatures. The thermostability of the biochar depends on the temperature at which it has been generated: in fact, more the temperature increased, more stable forms of carbon with a high resistance to heat originated inside the material (Kim *et al.*, 2012). Although BG sample was produced with lower process temperature compared with the other evaluated biochar samples, its carbonaceous intrinsic material showed great stability. All biochar produced at lower temperatures than 500°C recognized high structural instability. In order to verify whether the residual weight % is effectively due to more stable forms of carbon produced during gasification, thermogravimetric analyses were carried out in air atmosphere considering that organic compounds, unlike inorganic compounds, tend to give combustion reaction in the presence of oxygen. The thermograms of the samples analyzed showed a lower percentage by weight due to the combustion of the more stable forms of carbon (Fig. 3).

After degradation has been completed, as for the non-combustible fraction, the percentage of weight, expressed as percentage on dry basis, of inorganic compounds or metals is noted. BG sample has a higher proportion of inorganic substance (20%) than the other samples (10% and 5%). This fraction barely contains Si, S, P, O, K, Ca, Na, Mg, Fe, Mn, and Ni and possible metal complex species such as oxides, phosphates, hydroxides, and carbonates (Lu *et al*., 2020). All the curves shown have been blank curve corrected (Fig. 3).

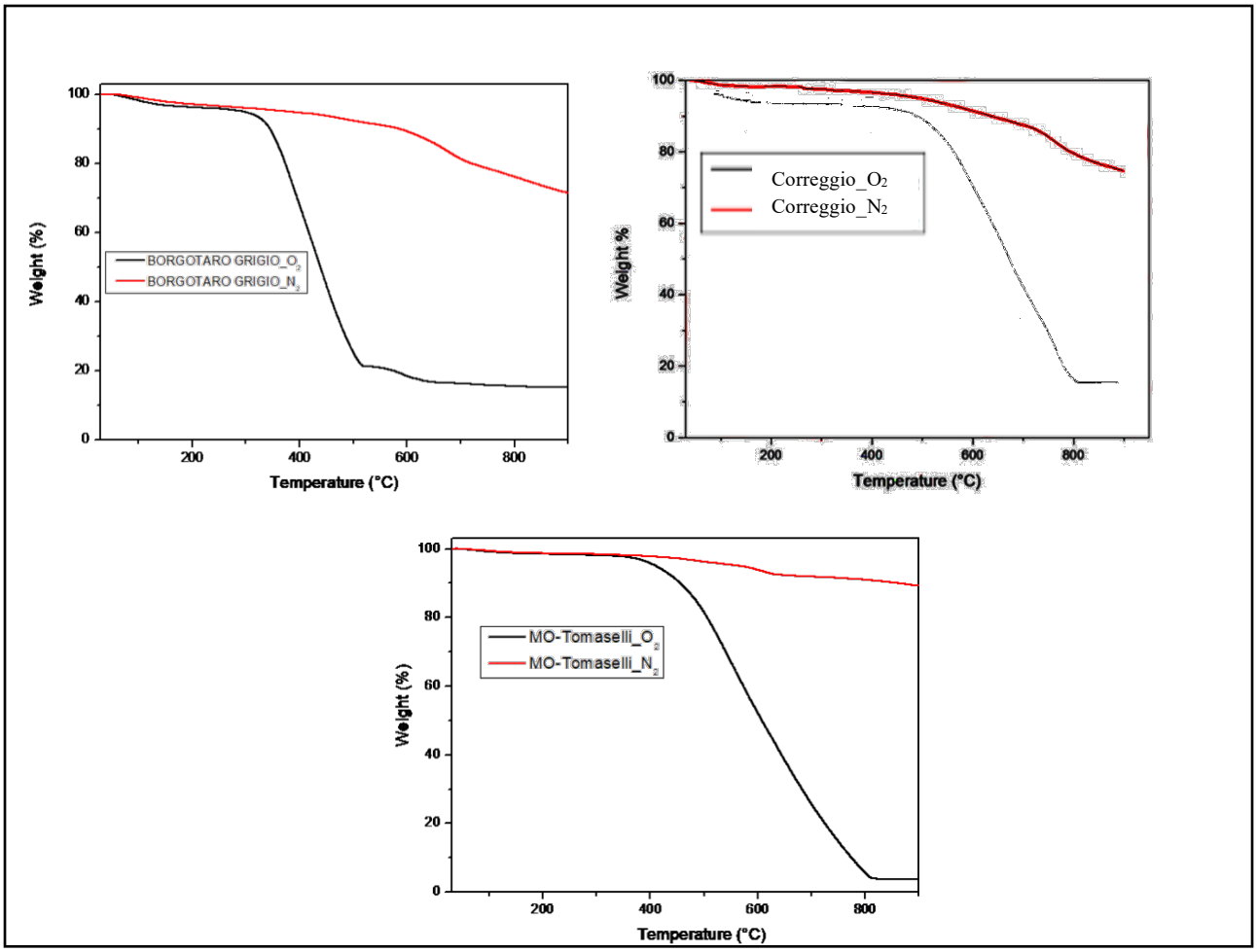


Figure 3. Thermograms of biochar samples in air (red line) and nitrogen (N_2) atmosphere (black line).

FTIR-ATR spectra are indicative of biochar surface chemistry, including surface-adhering aromatic and aliphatic pyrolysis condensates, as well as the degree of material aromaticity, which provides information on its value as a substrate (Chevali and Kandare, 2016; Brennan *et al*., 2001). The FTIR-ATR spectra and biomasses were shown in Fig 4. There was a variety of functional groups on the graphene sheet surface. Hydrogen, nitrogen, oxygen, phosphorus, sulfur are incorporated into the aromatic rings as heteroatoms (Brennan *et al.*, 2001). The presence of heteroatoms created a certain heterogeneity in surface chemistry, caused mainly by the difference in electronegativity of heteroatoms compared to that of carbon. Groups such as OH, NH_2, OR or O(C=O)R are classified as donor electrons, due to the presence of α and π electrons. Groups such as (C=O)OH, (C=O)H or NO_2 are classified as acceptor electrons due to the presence of empty orbitals. Carboxylic groups are strong acids; less strong acid groups are carbonyl and phenol groups. As highlighted from Fig. 4, there was

quite difference in substances and in the composition of the biochar's surface between samples obtained with high and low temperature. The spectra of all the biomasses of the samples showed a broadband from 4000-3500 cm^{-1} which was attributed to –OH from H_2O or phenolic groups, an absorption in the region 2900-2800 cm^{-1} attributed to -CH stretching from aliphatic functional groups, and another broadband from 2500 to 1800 cm^{-1} linked to carboxyl, carbonil acids and carbon monosubstituted alkynes stretching. The ATR spectra of all the samples revealed distinct and pronounced peaks in 1700 – 1200 cm^{-1} and 900 – 500 cm^{-1} ranges. The first range was related to the presence of carboxyl and carbonil groups, sulfones and azo compounds. All the aromatic compounds were included in the second range. BG biochar showed greater peaks of C=O, C=C, C=N, S=O, N=N stretching compared with the other samples. As expected, an increase to process temperature during the production of biochar leaded to an increased percentage of aromaticity (Lian and Xing, 2017).

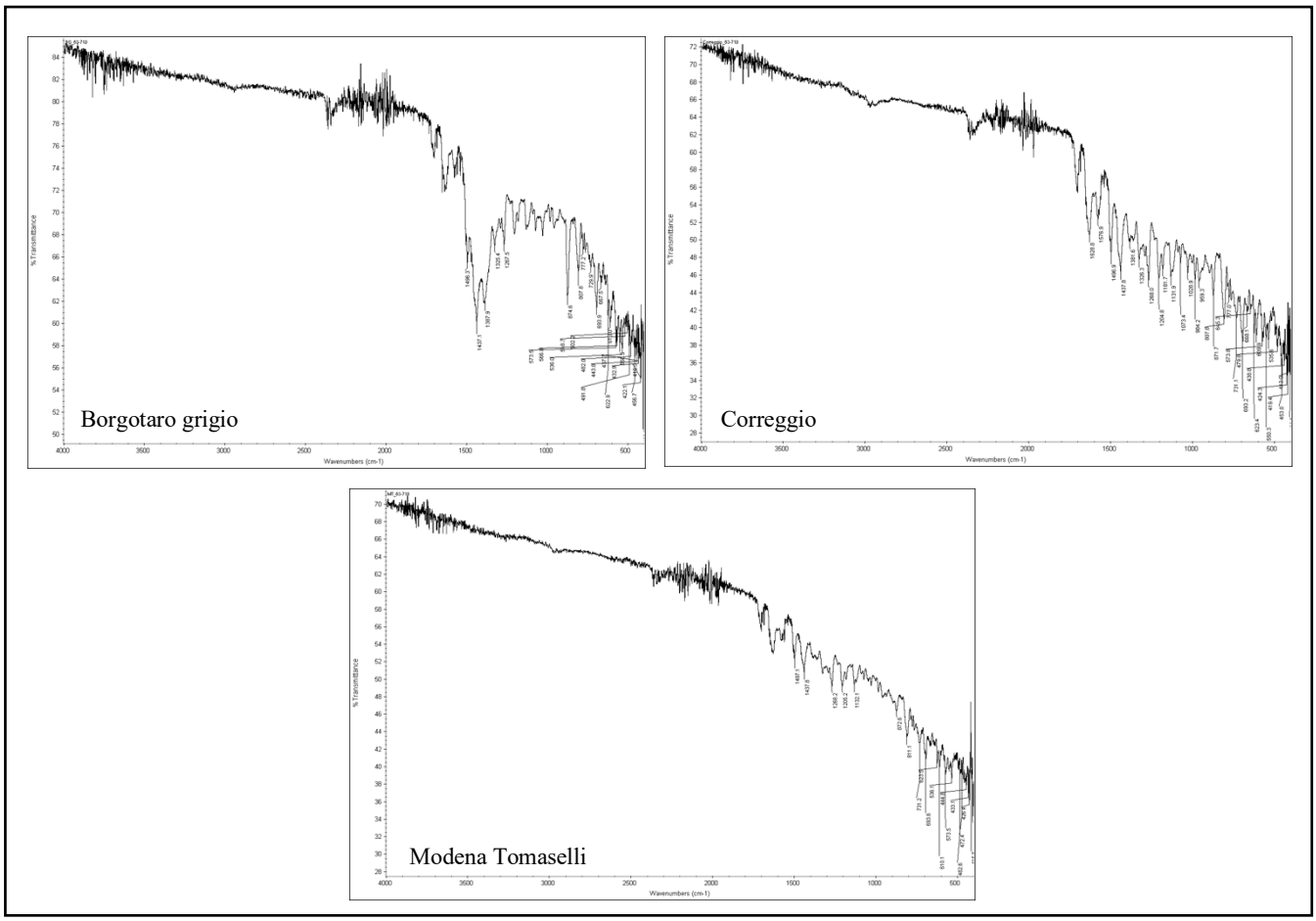


Figure 4. FTIR-ATR spectra of biochar samples.

The chemical environment was also investigated with a zeta potential analysis which provided information about absorption mechanisms (Fig. 5). A greater zeta potential value often coincides with a lower content of acidic groups on the surface that might result from the presence of $CaCO_3$, which has been identified by TGA and XRD analysis. The biochar's surfaces became predominantly negative charged because of the deprotonation of oxygen-containing surface groups (i.e., -COOH and -OH groups), favoring the absorption of cationic ions from the solution through electrostatic attraction and vice versa. Notably, C and MT samples shared the similarity in the absorption trend indicating that they might exhibit the identical absorption mechanisms.

Zeta Potential (mV)

pH	BG	C	MT
7	-18.29 ± 0.52	-35.16 ± 1.47	-31.03 ± 2.42
8	-17.31 ± 0.20	-28.30 ± 1.22	-33.54 ± 1.40

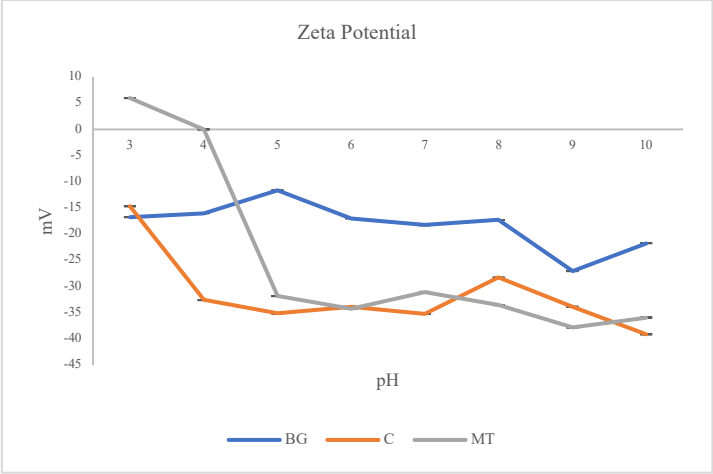


Figure 5. Zeta potential (mV) of the three biochar samples (BG: Borgotaro Grigio; C: Correggio; MT: Modena Tomaselli) at different pH values with a focus at mV values at pH 7 and 8. Data shown as means ± SE of instrumental triplicate.

The XRD analysis was used for the characterization of the inorganic constituents of biochar samples and to evaluate the existence of crystalline structures in the samples with the aim to detect all mineral phases present in the biochars. As clearly showed from the X-Ray diffraction patterns (Fig. 6), three crystal phases for all biochar samples can be observed. In BG sample, the lower production temperature corresponded to a more disordered structure linked to the many peaks of carbonate groups. At the opposite, C and MT biochars had a more ordered structures and less peaks of carbonate groups. Indeed, the temperature production is one of the features that mostly determined structure composition of biochars. In the ideal biochar structure development, low temperature of production correlates to an increased proportion of aromatic carbon while high temperature to growing sheets of conjugated aromatic carbon, becoming graphitic (Downie *et al.,* 2009). $CaCO_3$ abundance as well as

pH values of biochar have been recently rediscovered as essential tools related to estimate available nutrients in biochar and predict crop responses (Phillips *et al.*, 2020). Plus, the quality of crystal substances varied among samples. The three crystal phases observed in BG were fairchildite ($K_2Ca(CO_3)_2$) at 13.5°, 19.5°, 20.5°, 27°, 28°, 33°, 34°, 39.5°, 40°, 40.5°, 42°, 44.5°, 48°, 53°, 53.5°, calcite ($CaCO_3$) at 23°, 29°, 31°, 36°, 39°, 43°, 47.5°, 48°, and calcium carbide (CaC_2) at 25.5°, 26°, 30°, 32°, 43°, 45°, 47°. It was possible to notice the presence of other two peaks at 2θ angles of 35° and 37° probably linked to hydrogen (H_2) diffraction pattern. The presence of hydrogen in the inner structure of biochar may be referred to the reaction of thermal decomposition of hemicellulose and cellulose. The C and MT diffraction spectra showed a strong similarity in composition of different crystal substances: calcite ($CaCO_3$) at 23°, 29°, 36°, 39°, 43°, 47°, 48°, barbosalite ($FeFe_2(PO_4)2(OH)_2$) at 18°, 26°, 27°, 34°, 39° and calcium silicate ($CaSiO_3$) at 36°, 45°, 52.5° and other peaks with a very weak intensity. The dominant materials found in BG were calcite and fairchildite while both in C and MT samples was calcite.

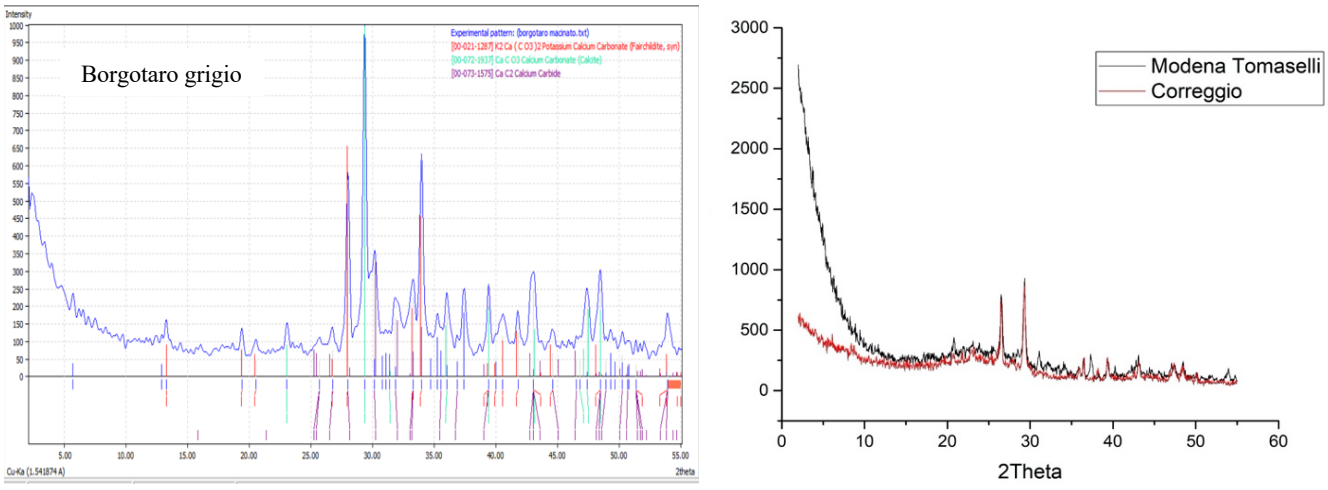


Figure 6. X-ray powder diffraction patterns of biochars produced at different temperature ranges: BG at 500-600° C (left), C, and MT at 900-1000° C (right). Vertical lines correspond to regions attributed to broad peaks. Mineral phases are labeled based on database matches.

The chemistry of biochar is linked to its reactivity with other compounds as pollutants, metals, nutrients, gases, and microorganisms. The sorption is one of the possible ways of interactions *(Lu et al.,* 2020).

Biochar characteristics depend on the way they are produced. They may contain several compounds originating as by-products of thermal process. PAHs are among the most widely discussed pollutants of biochar. While the study of PAH formation has been deeply focused on combustions or pyrolysis and it has been well understood (Bucheli, Hilber-Schöb, and Schmidt, 2015), the study of PAH formation during the gasification is still rather limited. In order to increase the safety of the use of biochar as an amendment, European legislation has established that concentration of PAHs should be controlled and should be below the threshold of 6 mg kg^{-1} (sum of 16 US EPA PAH) (D.Lgs 75/2010), depending on the extraction solvent in use. In this work, biochars were produced under different conditions, with temperatures varying from 500°C to 1000°C. Many works showed a linear trend between the increase in temperature process and the concentration of PAHs. The sum of the 16 US EPA PAHs was examined, and all the samples reported values below the threshold established by International guidelines (< 4 mg kg^{-1} for EBC and 6-300 mg kg^{-1} for IBI Biochar Standards V2.0). As shown in Table 8, samples BG and MT indicated phenanthrene amounts of 0.25 and 0.15 mg kg^{-1}, respectively which were both far below the allowed value. All the other PAHs were under the detection limit and, consecutively, the allowed permitted amounts.

Table 8. List of polycyclic aromatic hydrocarbons in biochar samples.

Sample Name	BG	C	MT
Units	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Naphtalene	< LOD	< LOD	< LOD
Acenaphthylene	< LOD	< LOD	< LOD
Acenaphthene	< LOD	< LOD	< LOD
Fluorene	< LOD	< LOD	< LOD
Phenanthrene	0,25	< LOD	0,15
Anthracene	< LOD	< LOD	< LOD
Fluoranthene	< LOD	< LOD	< LOD
Pyrene	< LOD	< LOD	< LOD
Benz(a)anthracene	< LOD	< LOD	< LOD
Chrysene	< LOD	< LOD	< LOD
Benzo(b)fluoranthene	< LOD	< LOD	< LOD
Benzo(a)pyrene	< LOD	< LOD	< LOD
Benzo(b)pyrene	< LOD	< LOD	< LOD
total of PAH	**0,25**	**< LOD**	**0,15**

Bioavailable concentrations in BG, C, and MT biochars of single PAH and sum of the 16 EPA-priority PAHs. Data shown as mg kg^{-1} and referred to EBC and IBI references. <LOD: under limit of detection.

The presence of the microelements has been investigated by AAS technique. Biochar has been recognized as a good soil improver as it carries many minerals on it. They are important for assuring

good health of plants and their growth. Applying the AAS technique, biochar samples showed (Table 9) all metal concentrations below the IBI and EBC guideline thresholds, but Cd and Ni seemed to be close to the upper limit for the EBC requirements (Cd < 1.5 mg kg^{-1} and Ni < 50 mg kg^{-1} for EBC and Cd 1.4 - 39 mg kg^{-1} and Ni 47 - 600 mg kg^{-1} for IBI) in BG sample. The elements contents of biochar samples were shown in Table 9. Elemental composition of BG biochar structure has been also evaluated by ICP-MS technique to provide a more accurate description (Fig. 7).

Table 9. Elemental composition of biochar produced with different feedstock and gasification temperatures.

Sample	Units	*Cd*	*Cr*	*Fe*	*Ni*	*Pb*	*Cu*	*Zn*	*Co*
BG	*mg kg^{-1}*	*2,29*	*0,39*	*1086,82*	*55,15*	*23,33*	*52,00*	*313,68*	*
C	*mg kg^{-1}*	*0,05*	*	*4104,94*	*12,14*	*22,70*	*38,69*	*134,13*	*
MT	*mg kg^{-1}*	*	*	*1155,69*	*1,71*	*42,54*	*19,37*	*22,13*	*

Metal concentrations in biochar samples detected by Atomic Absorption Spectroscopy (AAS) technique.

All the other metals tested (As, Hg) were below the detection limits.
* Metal concentration under limit of detection.

	As	Cd	Cr	Fe	Mg	Hg	Ni	Pb	K	Cu	Na	Zn
BG	< LOD	1,94	6,35	1310	1,52	< LOD	59	11,3	6,07	57,2	0,115	230

Figure 7. Metal concentration (mg kg^{-1}) in BG biochar sample with ICP-MS technique.

Values of elements were confirmed. There is no evidence that metals captured into biochar structures might be leached into ground. Therefore, bioavailability tests of inorganic micronutrients should be carried out to further verify the presence of dissolved metals in solution.

One of the fundamental characteristics of the biochar is its porous structure. Porosity gives the biochar many advantages and it is given by the presence of infinite inner cavities of different sizes. These can be of macro, micro and nano dimensions. The nano-size cavities that gave the biochar the characteristic of being a nano fertilizer. The porosity of the biochar was very well observed under the scanning electron microscope (Fig. 8).

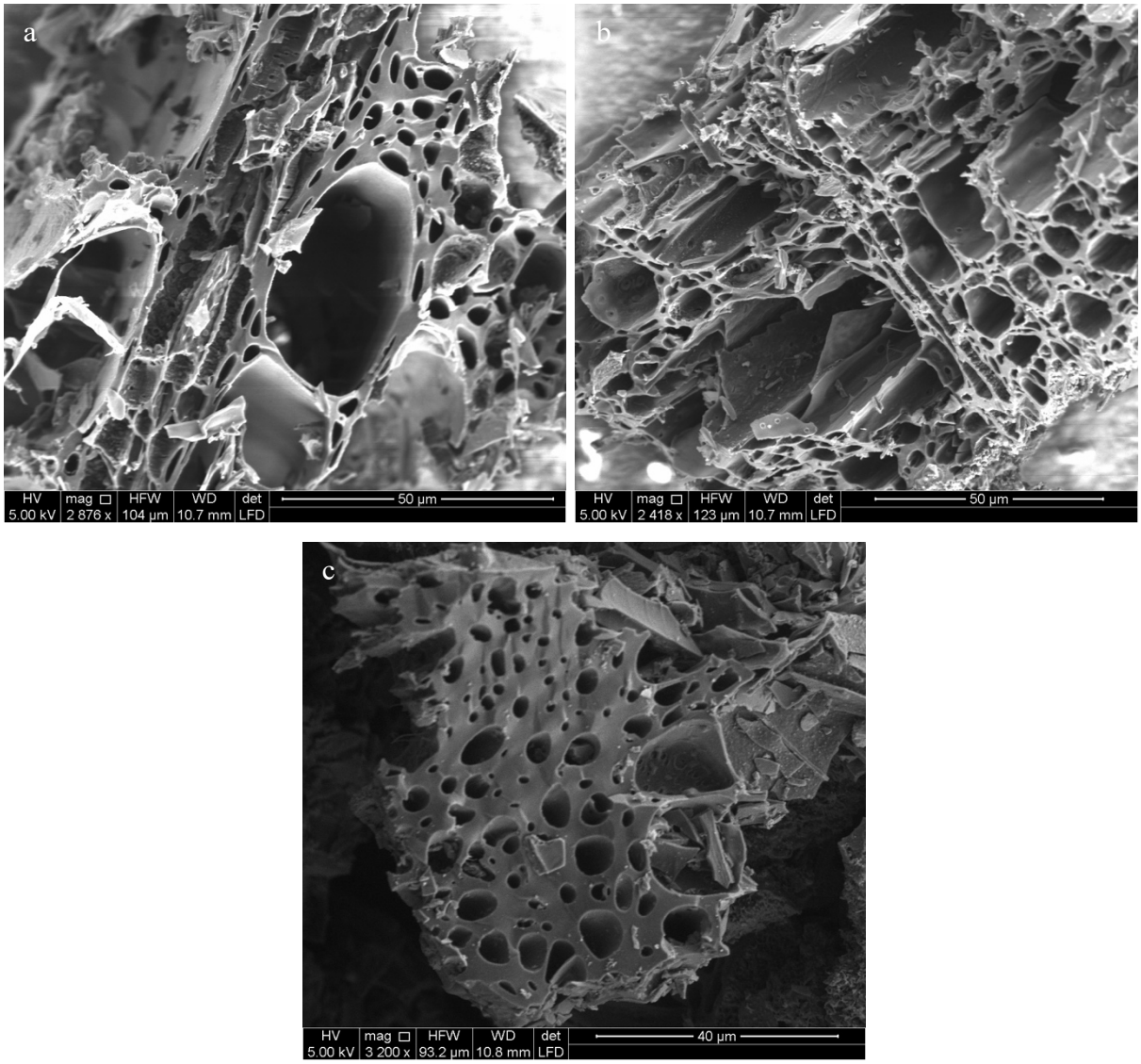


Figure 8. Images of biochars by the the environmental scanning electron microscope. a) BG; b) MT, and c) C samples.

2.3.2 Biological analyses

Crucial to plant health and yield was biological response to biochar treatment. Thus, even though biological analyses are not required to EBC guidelines, the germination assay was developed as early indicator of the effects of biochar, following the ISO 11269-1 and -2 protocols with two study models and with minor modifications. The test showed the results expressed in fig. 9.

Considering that all the following data were direct expression of the ratio between experimental treatments and controls as explained in the 2.2.3.2.1 paragraph, control references will not be shown on the next graphs as they must be intended as 100%. A similar dose-dependent behavior was observed for the RSG in both varietals: pea and barley (*Pisum sativum* L. and *Hordeum vulgare* L., respectively). Indeed, RSG values decreased as the biochar concentration in the medium increased although it remained relatively high in all biochar samples up to w/v 1% biochar, beyond which it decreased significantly compared with the control. The percentage of w/v 1% biochar was noticeably decreased but still as high as acceptable as seed germination index and it did not indicate acute toxicity of the biochar. Therefore, w/v 1% marked the threshold beyond which toxicity in seed germination could be observed. The only difference recorded among biochar samples was that MT biochar influenced the germination much more negatively than the other samples, especially in barley where RSG decrease was already significant at w/v 0,5%. Both the GI and the SRI were semi-linear, dose-dependent (Fig. 9), as RSG. The GI trends were similar comparing the two varietals. While biochar C had a slight decrease with increasing biochar doses, in BG and MT samples the germination index decrease was more marked, especially when testing w/v 3% and 5%, compared to lower concentrations. However, the values of GI in BG and C were similar. The biochar MT was mostly affected by the effect of the biochar at w/v 3% and 5% in pea and in all biochar tested doses in barley. The decrease in GI meant that the average root length decreased in a way inversely proportional to the biochar concentration. This occurred in both species studied.

Relationship between the length of the bud and the root could be seen (Fig. 9). Also in this case values decreased as biochar concentrations increased. This meant that a greater presence of biochar stimulated the growth and elongation of roots. This result was also visually confirmed by noticing the presence of many secondary radical hairs. The SRI easily reflected the effect of biochar treatments in both varieties and it had been heavily affected by the effect of w/v 3% and 5% biochar, respectively. In fact, except for C, there was no germination and root elongation in w/v 5% BG and MT biochars in both pea and barley and a very low shoot/root index in w/v 3% condition. The difference in SRI among the experimental doses for MT was evident in both varieties. There was a great difference between w/v 1% and 3% in BG (both in pea and barley). The same was for C in pea while the difference between the experimental conditions in barley was not marked. Resuming, it emerged that the w/v 1% biochar was a threshold limit dose for the phytotoxicity of the biochar, both for monocotyledonous (*Hordeum vulgare* L.) and dicotyledonous (*Pisum sativum* L.) species. Furthermore, the effects of the biochar were specific: different trends with great variability between the cultivars used and the tested doses had been found. RSG values showed that w/v 1% biochar was still the accepted dose for the use of biochar in agriculture and this was confirmed with both GI and SRI data. The varieties have been shown to be negatively affected by MT biochar even at low doses. They showed a similar behavior in SRI with BG and C biochars and, above all, showed to grow well on a medium that contained a maximum of w/v 1% biochar. Concluding, from all the graphs it emerged that w/v 1% was the limit dose for the potential biochar phytotoxicity.

Figure 9. Effects on Relative Seed Germination (RSG) (top), Germination Index (GI) (middle) and Shoot/Root Index (SRI) (bottom) of barley (*Hordeum vulgare* L.) (right) and pea (*Pisum sativum* L.) (left) treated with different doses (w/v 0.5, 1, 3, 5%) of biochar samples. * correspond to statistically different values compared with the control (one-way ANOVA, Tukey's test, $p<0.05$). Different colors refer to different colored samples. Control must be intended as 100%.

2.3.3 Effect of biochar extracts in DMSO with *S. typhimurium* TA98 and TA100

In this work the potential toxicity of biochar was evaluated for each sample by organic extracts in DMSO (Piterina *et al*., 2017; Anjum *et al*., 2014; Maron and Ames, 1983). Three different doses of organic extracts were evaluated, corresponding to 1 g ml^{-1} (Extract), 0,2 g ml^{-1} (DIL 5), and 0,01 g ml^{-1} (DIL 100), respectively. These doses were chosen arbitrarily, considering that the smallest one corresponded to the threshold limit dose found in biological analyses above which biochar phytotoxicity has been observed (Fig. 9). The results showed two slightly different situations when comparing the results obtained with the two different bacterial strains. This was understandable and due to the different sensitivity each strain has towards the chemical compounds and extracts analyzed. In fact, the different sensitivity of bacterial strains was already demonstrated in spontaneous reversions, which correspond to a significant difference in strain-related mutation frequency. It is a distinctive feature of each *S. typhimurium* strain. For this reason, the results have been shown separately for each bacterium.

In Fig. 10 there is an explanatory graph on the mutagenicity index of biochar extracts in DMSO for each tested sample and each microbial strain, in the absence (−S9) or presence (+S9) of rat liver microsomal fraction. On the top left it is shown the effect of biochar extracts with *Salmonella typhimurium* TA98 strain. First, it was clearly noticeable the great difference of mutagenic index between positive (2-AA) and negative (DMSO) controls. Then, data are separated by S9 activation (+S9) and absence of activation (-S9). Monitoring the effect on *Salmonella typhimurium* TA98, all biochar extracts of all tested doses did not show strong mutagenic properties considering that their mutagenic indices were much far below than the positive control threshold and were not at least twice as high as the negative control, as indicated by the "2-fold" rule in Piterina *et al.* (2017). In the case of BG and MT hundred times-diluted extracts, the (+S9) samples showed indices perfectly comparable to the negative control one, while C (+S9) biochar extracts seemed to act as a pro-mutagen since the mutagenic index was almost twice compared to the negative control one. On the top right instead, it is shown only the behavior of biochar extract samples without activation (-S9).

Even if in each dose considered they had a mutagenic index below the negative control limit, there were differences between the samples. BG showed the lowest index in 100-times diluted extract and it was the smallest value also when compared with C and MT (-S9) extracts. BG was the biochar produced at lower temperature than the others and it had a different trend than the other biochar extracts. MT was the biochar in which major reverting actions occurred, even in the case of the most diluted extract tested.

In Figure 10, on the bottom left it is represented the biochar extract effects on *Salmonella typhimurium* TA100. In general, no sample showed a strong mutagenic character as their mutagenicity indices were well below the positive control maximum. The three different (+S9) biochar extracts at higher doses (extract and DIL 5) showed a very weak potential mutagenicity as their indices were between positive and negative control values, but DIL 100 (+S9) extracts in all biochar samples indicated the absence of a mutagenic character as the mutagenic index was comparable with the negative control. Indeed, highlighting the case of BG in DIL100, even when DMSO extracts were activated by rat liver homogenate, the biochar had a lower index than the accepted threshold limit of non-mutagenicity. Instead, in the case of C and MT, when these samples were activated enzymatically by S9 mix, their mutagenicity index rose and became higher than the threshold level. However, according to the "2-fold" rule (Piterina *et al*., 2017), this difference was negligible and not sufficiently enough to attribute mutagenicity to biochar samples. On the bottom right of Fig. 10, all mutagenicity indices of not-activated samples are shown and they were all below the negative control mutagenic index but even in this case, it was possible to observe differences among samples. Biochar C showed the highest mutagenic index of all, followed by sample MT and, finally, BG.

The highest doses (Extract and DIL 5) shown in the previous graph were very high and they will not be ever used in any purpose for agriculture. The suggested and recommended doses were much lower and referable to 100-times biochar extracts dilutions (DIL 100).

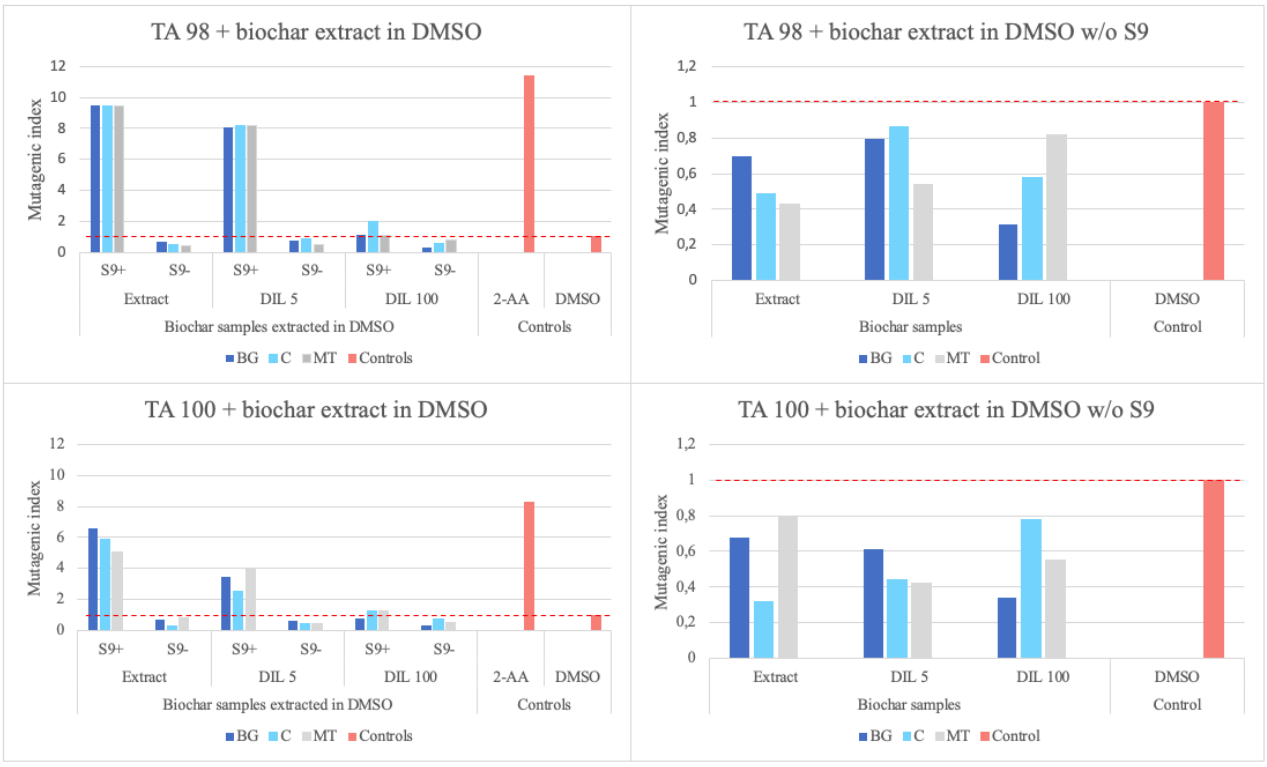


Figure 10. (Left) Mutagenic index of *S. typhimurium* strains TA98 (top) and TA100 (bottom) exposed to different doses of organic biochar extracts with (+S9) and without (-S9) metabolic activation. (Right) Particular of mutagenic index of *S. typhimurium* strains TA98 (top) and TA100 (bottom) exposed to different doses of organic biochar extracts without metabolic activation (-S9).

In Fig. 11 only the doses diluted 100 times (DIL 100) were taken into consideration.

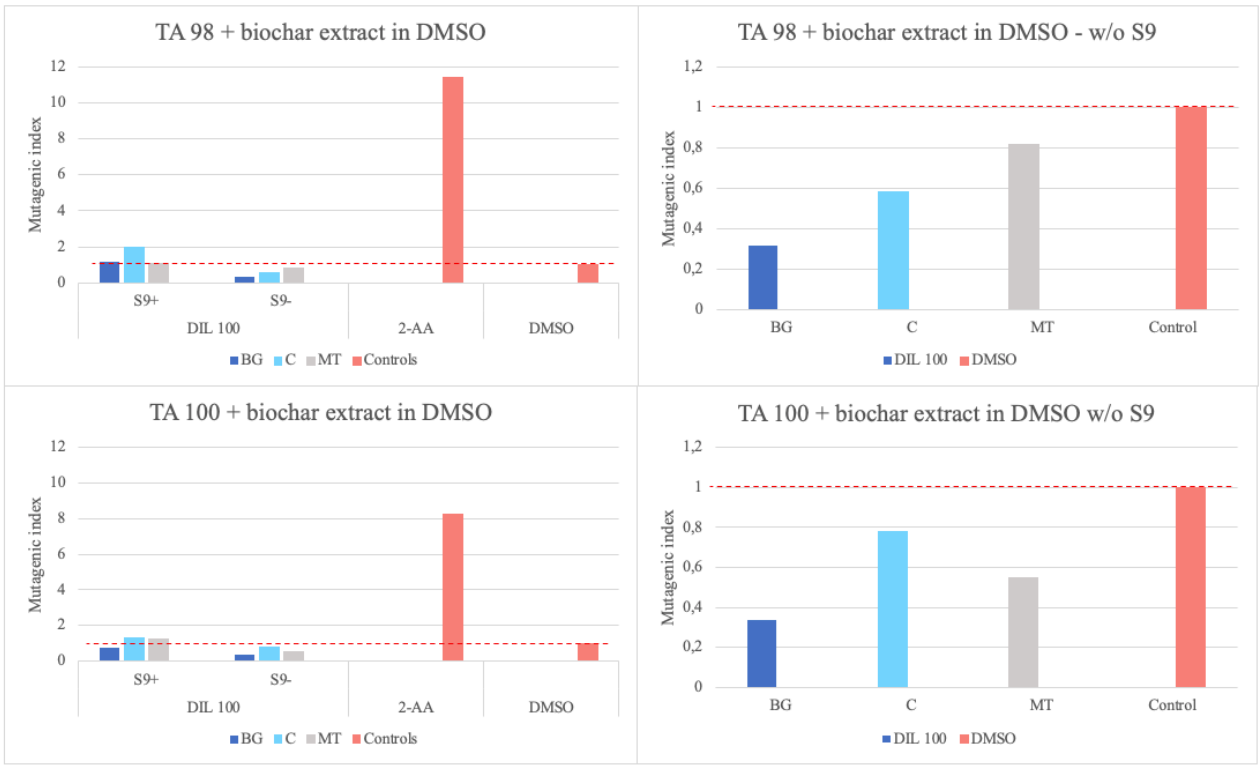


Figure 11. (Left) Mutagenic index of *S. typhimurium* strains TA98 (top) and TA100 (bottom) exposed to 100-times diluted organic biochar extracts (DIL 100) with (+S9) and without (-S9) metabolic activation. (Right) Particular of mutagenic index of *S. typhimurium* strains TA98 (top) and TA100 (bottom) exposed to 100-times diluted organic biochar extracts (DIL 100) without metabolic activation (-S9).

The 100-times diluted biochar extract was ascribable to the limit threshold that can be used without having phytotoxicity found in previous discussed biological experiments (Fig. 9). Summarizing all the data shown, there was no case of strong mutagenicity neither with *S. typhimurium* TA98 nor with TA100. The graph on the top left (Fig. 11) shows that the activated extracts did not exceed the value of the negative control with the sole exception of (+S9) C that has a behavior of a weak mutagen because the mutagenicity index was higher than the negative control but not at least twice than it and it was much less than the positive control value. If (-S9) extracts were considered, they were all lower than the negative control (Fig. 11, top right). Considering the mutagenic effect of biochar extracts revealed by *S. typhimurium* TA100, all mutagenicity indices were found to be low, both in (+S9) and (-S9) (Fig. 11, bottom left). In particular, the BG mutagenicity index was lower than the negative control in both activated and non-activated samples while the activated extracts of C and MT were slightly higher than the negative control. The inactivated extracts (Fig. 11, bottom right) showed no mutagenicity.

In conclusion, in the case of enzymatically activated (+S9) organic extracts, only the undiluted and 5-times diluted extracts of all biochar samples showed a weak mutagenicity, with both *S. typhimurium* TA98 and TA100. In cases where organic extracts were not enzymatically activated (-S9), all samples have been shown not to be mutagenic. In particular, the 100-times diluted dose was the one that could most commonly be used in agriculture and focusing the attention on it, it could be seen that all the extracts, activated and not, showed no toxicity and mutagenicity. It could therefore be concluded that there were no future negative implications on the use of these biochar samples in agriculture even the results did not show a consistent trend, and a dose-related co-linearity and were not reproducible. For these reasons, the tested biochar extracts in DMSO under the test conditions are not mutagenic in accordance with the Vargas *et al.* (1995) and Piterina *et al.* (2017) suggestions.

Finally, it appears from the all the analyses presented that biochar samples showed physical and chemical characteristics related to production specifications, especially production temperature, and

to the original plant organic material. These properties meant that the samples showed different characteristics and only one biochar sample had the suitable characteristics for use in later analyses and applications. The BG biochar was chosen for the qualities demonstrated as a valid material of further study and, in addition, concerning the results of the Ames test, among the biochar samples, BG was the one that showed better behavior than the others considering the trend of its mutagenicity index in relation to the dilutions.

CHAPTER 3 Biochar microbial engineering

3.1 Introduction

Nutrition has always been a fundamental and indispensable element in man life. Since ancient times, in fact, man was focused on procuring food for himself, also sharpening his wits to facilitate this task and, therefore, refining from time-to-time techniques of hunting, fishing, cultivation, breeding. However, over time man has undergone a turnaround, consuming food no longer for mere survival but for pleasantness and to satisfy his own personal taste. Nowadays, nutrition is mainly associated to the achievement of a gustatory pleasure. Nevertheless, nutrition is recognized as one of the factors that powerfully influence the quality of life of the population. A good diet, in fact, also preludes a good state of health and, conversely, an improper diet leads to an inadequate state of wellbeing that can eventually lead to pathology (Maestri, Marmiroli, and Marmiroli, 2016). For this reason, there is currently increasing attention in the world population to the nutritional values of food.

Agriculture provides much of the food that we consume and plays a key role in our survival, but if it is practiced unsustainably it can cause serious damage to the environment and the living beings that inhabit it. The degradation and pauperization of the soil, the enormous water consumption required by intensive agriculture, and the pollution of water by the massive use of pesticides and chemical fertilizers, in particular nitrogen fertilization, has caused negative agronomic, economic, social, and environmental impacts. Some examples are represented by nitrate contamination of surface water, global warming, soil eutrophication, salinization, and acidification. It is therefore necessary to move towards sustainable agriculture with the absence, or a reduction, of massive nitrogen fertilization, chemical fertilizers and synthetic substances which are all potentially harmful. This foreseen agriculture implies the use of elements which do not disrupt the natural habitat of the microorganisms and the ecosystem over time, and the long-term preservation of soil fertility, the protection of the health of operators and the consumers, and of the quality of the water resources.

3.1.1 Sustainable agriculture: state of the art

In past years, the tendency to prefer more intensive agriculture and the use of synthetic chemicals capable of increasing the physiological productivity of crops has led to a progressive depletion of land, which are now heavily polluted, rich in fertilizers, nitrogenous compounds, toxic and harmful elements, not anymore fertile and, actually, specialized only for the growth of certain crops. Over the years, global social events have led to a shift from traditional agriculture to a type of massive cultivation to try to increase profits and reduce handwork. The first step towards this innovation was marked by the change in agricultural techniques with the use of machinery that facilitated the task of workers. Therefore, modern agriculture is therefore based on mechanization, use of chemical fertilizers, selection of cultivated plants, and crop protection by applying pesticides. On the one hand, if modern agriculture has brought benefits in terms of increased productivity, on the other hand, certainly the same also brought loss of biodiversity, increased pollution of water and soil by leaching, resistance to pesticides and increased salinity of soils as a consequence of fertilization.

Sustainable agriculture is defined as all those agricultural systems that promote the production of food in a healthy social, economic and environmental way. These systems are based on the productive capacity of the soil's intrinsic fertility and respect for nature, soil, plants and animals. It avoids, or excludes, the use of synthetic chemicals for fertilization, by trying to preserve the agricultural production.

The achievement of the objectives below follows the European Codex Alimentarius Commission document (EC/GL 32/1999).

Here the objectives are:

-increasing biological diversity and soil biological activity;

-exploitation of local agricultural and natural resources (climatic, pedological, genetic-varietal), of the biogeochemical cycles, the natural biological processes, and the balances of the various ecosystems;

-maintenance of soil fertility in the long term;

-bypassing pollution from the use of synthetic chemicals;

-promotion of using renewable resources, minimizing that of fossil energy;

-promotion of correct use of soil, water resources and the atmosphere and the reduction as much as possible of any form of pollution which might result from farming and animal husbandry practices;

-maintenance of the biological integrity and essential qualities of the product at all stages.

Sustainable agriculture is growing, in relation to the prospects it can offer in relation to food security and environmental problems. In general, it uses only environmental-friendly interventions at all stages of production. Research has focused on testing different types of eco-friendly fertilization, including the application of PGPM. The latter can play a significant role in fixing atmospheric nitrogen and producing substances that promote plant growth, increase crop productivity, and contribute to soil health (Singh, Pandey, and Singh, 2011). Indeed, the functionality of PGPM has been studied for a long time with extremely interesting results such as the reduction or elimination of nitrogen or phosphate fertilizations, stimulation of the root system with a better absorption of water and nutrients, and the ability to act as biocontrol agents contributing to fight pathogens.

3.1.2 Environmental benefits of biochar's application to soil

3.1.2.1 Soil physical benefits

The high stability of the biochar in soil has generated much interest in its use in sustainable agriculture as adjuvant. The benefits of its use in soil are known and include important effects in a wide variety

of applications. Biochar has aroused much interest as a potential mechanism of carbon sequestration to reduce the atmospheric concentration of CO_2 (Ayaz *et al.,* 2021; Hui, 2021; IPCC, 2018), one of the most worrying greenhouse gases. Biochar application in soil leads to changes in soil physical (Asai *et al.*, 2009; Oguntunde *et al*., 2008), chemical (Deluca, Mackenzie, and Gundale, 2009), and biological (Lehmann *et al.*, 2011) properties. The greatest advantage in using biochar as a sustainable and biological soil improver is certainly its ability to procure soil nutrients directly or indirectly due to its ability to retain nutrients in soil and to reduce losses by leaching (Prendergast-Miller, Duvall, and Sohi, 2014; Ventura *et al*., 2013; Glaser *et al*., 2001), potentially resulting in increased nutrient uptake by attracting the system roots towards biochar (Prendergast-Miller, Duvall, and Sohi, 2014) and overall increasing crop productivity (Yin and Xu, 2009). Also, biochar could modulate the microbial community biodiversity according to its composition and to the chemical environment on its surface, acting as a nutrient for microorganisms or as inhibitor for their growth (Li *et al.*, 2020). The biochar's surface chemistry allows cation holding, thus reducing nutrient losses when applied to soil by retaining compounds by binding them. In addition, the natural and enormous porous cavity of the biochar, due to the source material, gives the biochar a high capacity for water retention by acting either as a water sequestrator or as a water procurator, as needed. Porosity can provide storage function for nutrients, minerals, various organic and inorganic compounds, and also for microorganisms.

3.1.2.2 Soil microbial community modulation

Biochar porosity can provide a valuable habitat for microorganisms or a refuge from predators, contributing to a more favorable environment for soil biota, whose support of plant growth is crucial for agricultural sustainability (Li *et al*., 2020). Whereas plant-microorganisms association has long been studied with great results in terms of sustainable agronomic and economic advantages and positive field applications (Arif, Batool, and Schenk, 2020) with ornamental, arboreal and herbaceous plants, the effect of the microbial engineering of biochar in association with plants still need to be

assessed. All the physical and chemical properties that characterize biochar also have effects on soil and its microbiome. The latter is strongly influenced by the soil pH, soil texture and porosity, and the cation exchange capacity even though the results about microbial diversity and abundance are straightly related to biochar type and application rate (Li *et al*., 2020).

3.1.3 Plant-bacteria relationship

The fate of the plants partially depends on the ability of the roots to communicate with other organisms present in the rhizosphere (Berg, 2009). The rhizosphere represents a complex and dynamic environment in which viruses, bacteria, fungi, microfauna establish a dense network of relationships and derive direct or indirect advantages (Woo and Pepe, 2018; Vacheron *et al.,* 2013) from the presence of radical exudates. The rhizosphere has an intense microbial activity that ensures soil quality (Schloter *et al.,* 2018), stability and productivity both in agricultural systems and in natural ecosystems. Most of the interactions between plants and microorganisms increases the availability of nutrients for the plant and defends against the action of pathogens (Enebe and Babalola, 2018). There are different types of such interactions, some negative harmful to the plant (collar tumor caused by several microorganisms like *Agrobacterium tumefaciens*), some positive as they allow plants to grow and/or expand their root system (mycorrhiza and PGPM) (Woo and Pepe, 2018).

3.1.4 Plant Growth-Promoting Microorganisms (PGPM)

PGPR are bacteria that populate the rhizosphere and commonly referred to bacteria belonging to the genera *Azospirillum, Bacillus, Pseudomonas, Serratia*, *Azotobacter, Trichoderma, Alcaligenes, Enterobacter, Burkholderia* (Pagnani *et al*., 2018), which are phosphate solubilizing microorganisms, nitrogen-fixing bacteria, biocontrol strains, endophytic bacteria (Calvo, Nelson, and Kloepper, 2014). The PGPR have been classified, depending on the beneficial effect they determine in the plant, in two groups: those involved in the metabolism of nutrients (biofertilizers and phytostimulants) and biocontrol agents of plant pathogens (Bashan and Holguin, 1998). The capacities of plant growth

promoting bacteria could be applied in sustainable agriculture as potential "biofertilizers" representing a viable alternative to chemical fertilizers or pesticides. Biofertilizers are living microorganisms which, when applied to seeds, plant surfaces or soil, colonize the rhizosphere or plant tissues and promote their growth by increasing nutrient availability and uptake by the plant. In addition, PGPM promote the development of the plant (height, shoot weight and tissues), and its physiology (nutrient content, chlorophyll content and crop yield) (Saharan and Nehra, 2011), and reduce the negative impact on the environment acting as bioprotectans. Moreover, PGPM produce phytohormones (auxins, cytokinins, and gibberellins) that enhance root growth, thus increasing the root surface area and the ability to access nutrients, and can be considered as “biostimulants” (Chennappa *et al*., 2019; Saharan and Nehra, 2011).

Plants usually live in close association with microorganisms, with which they can establish beneficial interactions (symbiotic and not) or harmful (pathogenic). Many PGP microorganisms have been identified. They are bacteria and fungi capable of interacting with the plants by exerting on them a direct and/or indirect beneficial action. The latter includes N_2 fixation that increases availability of nutrients in the soil and thus making them usable by the plant and potentially increasing growth even when the quantities of nitrogen (or phosphorous) in the soil are very low. Phytostimulating rhizobacteria are represented mainly by the genera Azotobacter, *Azospirillum*, *Alcaligenes*, and *Pseudomonas*, which directly promote the growth of the plant (Saharan and Nehra, 2011) also through the production of phytohormones. The microorganisms with indirect beneficial action, however, are those that inhibit the growth of pathogens that interfere with the normal processes of development of the plant.

Numerous are the techniques used in agriculture able to respect the environment and biodiversity: the use of insects or bacteria antagonists for the biocontrol of plant pathogens, the use of organic fertilizers, and the classic crop rotation. The techniques used according to these experimental plans are based on the use of bacteria for plant growth (Schloter *et al.,* 2018; Woo and Pepe, 2018). Indeed,

PGPM help to fix atmospheric nitrogen, improve the nutrient cycle by producing bioactive compounds such as vitamins, hormones and enzymes that stimulate plant growth, detoxify pesticides, suppress plant disease, and improve the decomposition of organic matter (Calvo, Nelson, and Kloepper, 2014).

Microbial inoculation therefore tends to rebuild, enhance, and improve the natural microbial balance of soil damaged by intensive agricultural practices, through genetic engineering. PGPR microorganism must first be rhizosphere competent, meaning capable of colonizing the expanding root surface. However, the root itself can control microbial populations through the active role played by radical exudates (Yuan *et al.,* 2018). This role includes activation, promotion and regulation of processes of interaction, colonization and protection by rhizobacteria that can promote beneficial associations or block the attack of harmful microorganisms (Vacheron *et al.,* 2013). Typically, exudates are low molecular weight molecules of a very varied chemical nature. Among these, important are sugars, organic acids, amino acids, phenolic compounds, but also many molecules of a composite chemical nature. These molecules spread around the root and modify the chemical-physical properties of the soil (Schloter *et al*., 2018), promoting beneficial symbiosis and tending to inhibit the attack of pathogenic microorganisms and/or the growth of neighboring competing plants (Marschner, 2012).

3.1.5 The microbial consortium design

The use of efficient inoculants is considered an important strategy for sustainable management in agriculture (Hayat *et al*., 2010). PGPM are important determinants of soil fertility and plant health for their potential to improve crop productivity, nutritional quality, as well as resistance to environmental stresses (Berg, 2009) and tolerance to abiotic stresses (Enebe and Babalola, 2018).

These microorganisms are able to promote plant growth and development through several mechanisms that could improve better opportunities for plants both in direct and/or in indirect

manner. Direct mechanisms involve atmospheric nitrogen fixation, nitrogen denitrification, siderophores production and mineral solubilization while indirect mechanisms are related to hormone production, the synthesis of several other growth-promoting compounds (e.g. enzymes) that act as biocontrol agents for other microorganisms and pathogens.

Most approaches for plant growth promotion imply the use of a single bacterial species as biofertilizer while only few consider a consortium of selected microorganisms. Microbial studies conducted in vitro without plants indicate that some mixtures allow the bacteria to interact with each other in a synergic manner, providing nutrients, removing inhibitory products, and stimulating each other through physical or biochemical activities that may enhance some beneficial aspects of their physiology, like nitrogen fixation. Compared to single inoculation, co-inoculation, frequently, increases growth and yield, providing the plants with more balanced nutrition, and improving absorption of nitrogen, phosphorous and mineral nutrients (Calvo Velez, Nelson, and Kloepper, 2014).

For example, *Azospirillum* sp. play an important role in the improvement of crop health and yield under several environmental and soil conditions (Bashan and de-Bashan, 2010), stimulating the root systems. Their potentialities as biofertilizer applied both alone or in consortium with other microbial species has been demonstrated (Rajasekar and Elango, 2011; Shahzad *et al.,* 2017). In particular, *Azospirillum* sp. has demonstrated its beneficial effects on *Solanum lycopersicum* L. plant growth and development.

The ability of beneficial *Azotobacter* strains to secrete plant growth promoting and regulating substances such as phytohormones, vitamins, and antifungal metabolites have been studied. Phosphate solubilization (Rojas-Tapias *et al.*, 2012; Hariprasad and Niranjana, 2009) and Fe mobilization (Rizvi and Khan, 2017) have been demonstrated in vitro and in soil, also under abiotic stress conditions (Viscardi *et al.,* 2016; Van Oosten *et al.*, 2017). Furthermore, the *Azotobacter*-

mediated synthesis of important enzymes such as superoxide dismutase (SOD) and catalase (CAT) can influence plant health and bring benefits to a wide variety of crops such as tomato *(Viscardi et al.,* 2016). *Pseudomonas* sp. and *Paracoccus* sp. have demonstrated effective increased plant growth and yield. The stimulatory effects of this PGPR strains on the yield and growth of these crops were attributed to the N_2 fixation ability, plant growth regulator production and phosphate solubilizing capacity (Cakmakci *et al.,* 2007). Native bacterial endophyte, *Alcaligenes faecalis*, significantly modulates primary plant productivity under pot and field conditions and greatly modulate soil health (Rehfuss and Urban, 2005).

Trichoderma was recognized as a good effective biofertilizer, biostimulant and enhancer of crop resistance to both biotic and abiotic stresses (Lorito and Woo, 2015). The potential of *Trichoderma* is related to the solubilization of important micronutrients (Altomare *et al.,* 1999), and the production of secondary metabolites (Vinale *et al.*, 2008; Spaepen, 2015), and hormones (Hermosa *et al.*, 2013) that stimulate plant development. Most of the chemicals produced by *Trichoderma* are bioactive and can affect the plant response to other microbes, by improving defense mechanisms, while stimulating plant growth and development, especially at the root level (Woo and Pepe, 2018).

3.1.6 Microbial strains: morphology, physiology, and biochemical characterization

3.1.6.1 *Azotobacter vinelandii*

Azotobacter are the most dominant species in the rhizosphere soil (Chennappa *et al.,* 2019) and are involved in nitrogen cycle as N_2-fixator, their effect as biofertilizer by producing plant-growth stimulating substances, and their detoxifying function towards pesticides and pollutants (Chennappa *et al.,* 2019; Noar and Bruno-Barcena, 2018). Among all the secondary metabolites they are able to produce, 3-indole-acetic-acid (IAA) and gibberellic acid are the most important as they are responsible for many primary vegetative functions of plants such as cell differentiation and organ developments (Chennappa *et al.,* 2019). Within the plant growth promoting effects, also the

production of siderophores and the biocontrol functions have to be subscribed. *Azotobacter*, as well as *Alcaligenes,* are able to produce hydrogen cyanide to suppress other microorganisms' growth and thus to affect plant growth and development (Chennappa *et al.,* 2019). Moreover, *A. vinelandii* has been proved to produce alginate as a coating protecting material for cells that is fundamental for cell survival under aerobic conditions (Noar and Bruno-Bárcena, 2018; Sabra *et al*., 2000). For all the above-mentioned benefits, *Azotobacter vinelandii* also showed important implications in improving soil sustainability and crop yields and nutritional value of crops by increasing their vitamin content and protein quality and quantity (Noar and Bruno-Barcena, 2018) and it has been employed to build up the microbial consortium.

3.1.6.2 *Alcaligenes faecalis* subspecies *phenolicus*

A. faecalis is a phenol-degrading bacterium with potential bioremediation property (Rehfuss and Urban, 2005) that commonly dwell in soil, water, and environment (Basharat *et al.*, 2018). Its ability to degrade phenanthrene and other polyaromatic hydrocarbons and pollutants combined with its capability to eventually tolerate heavy metals has been already proven (Basharat *et al*., 2018). *A. faecalis* J^T, which is the strain used in the present project, is able to grow on several types of carbon sources while for nitrogen it has denitrification capacities thanks to the presence of the nitrite reductase gene (nirK) which catalyzes the reduction of NO_2^- to NO (Rehfuss and Urban, 2005). It can use nitrite as finale electron acceptor in aerobic conditions, but it is not able to reduce nitrate. It is catalase and oxidase positive, and produces alkali from peptone (Rehfuss and Urban, 2005). *Alcaligenes faecalis subspecies phenolicus* is non-pathogenic and eco-friendly bacterium marked to have many great environmental applications.

3.1.6.3 *Pseudomonas fluorescens*

Pseudomonas fluorescent, used as liquid and seed inoculant, is very effective in improving soil health, stimulating plant growth and crop yield (Saharan and Nehra, 2011). It has been proved to colonize

both internal and external root surfaces and to shut down the vitality of pathogens therefore functioning as a biocontrol microbe (Panpatte *et al.,* 2016; Thrane *et al.*, 2001). *P. fluorescens* presence in soil does not represent any risk to microbiome in soil (Johansen and Olsson, 2005) but instead it brings great advantages to soil fitness (Panpatte *et al*., 2016). It grows on several carbon sources and is able to synthetize antibiotics, hydrolytic enzymes, phytohormones and siderophores, to fix nitrogen, and to solubilize nutrients like phosphorous. *P. fluorescens* is one of the most characterized growth promoting bacteria and its effective advantage in association with other microorganisms is investigated in the application in soil for the cultivation of plants of agronomic interest.

3.1.6.4 *Azospirillum brasilense*

Azospirillum brasilense is a gram-negative bacterium able to colonize the roots of plants by improving their growth and increasing the intake of water and minerals by the plant. This bacterium has a respiratory-type metabolism with oxygen as an electron terminal acceptor; in the absence of oxygen they can use nitrate, nitrite or nitrous oxide as an electron acceptor in the respiratory chain. Within the species of *Azospirillum brasilense* two groups can be distinguished, referred to as nir+ nir- , with and without denitrification capacity, respectively. However, microorganisms of both groups possess the enzyme nitrate reductase. The bacterium participates in all stages of the nitrogen cycle, excluding nitrification: N_2 fixation, reductive assimilation of nitrate and nitrite, NO_3-dependent nitrogen fixation. It apparently has no special nitrogenase protection mechanism; it can only fix nitrogen under microaerophilic conditions (Okon, 1985). The typical pink color of the colonies seems to be due to the presence of carotenoids. An interesting feature of these bacteria is their ability to produce phyto-stimulating substances such as auxins, cytokines, gibberellins, and abscisic acid (Bar and Okon 2011; Hartmann and Zimmer, 1994; Bar and Okon, 1993). Microaerophilic and nitrogen limitation conditions generally present in soil, favor the production of IAA (Lambrecht *et al*., 1998; Kolb and Martin, 1985;), as well as nitrogen fixation. Its ability to produce bacteriocins (Hartmann

and Zimmer, 1994) can inhibit the growth of other bacterial strains, conferring an ecological advantage of strategic importance within the rhizosphere (Del Gallo and Fabbri, 1991). A last characteristic is the production of siderophores: in conditions of iron deficiency, it is capable of producing siderophores, such as spirillobactins (Bachhawat and Ghosh, 1987), which are an important survival factor in a highly competitive environment such as rhizosphere (Bashan, 1999; Puente *et al.*, 1999; Holguin and Bashan, 1996; Negi, Sachdev, and Tilak, 1990).

3.1.6.5 *Paracoccus denitrificans*

P. denitrificans is a gram-negative bacterium capable of using molecular hydrogen in denitrification (Nokhal and Schlegel, 1980). It is catalase and oxidase positive and its growth is possible with a great variety of carbon source (Davis *et al.*, 1970). As it can be imagined from its name, it has the functionality to reduce nitrate to gaseous nitrogen via nitrite and nitrous oxide, both of which can be used as terminal electron acceptors to support anaerobic growth, a process also known as denitrification (John and Whatley, 1978). *P. denitrificans* is a facultative aerobe microbe, it prefers to use oxygen instead of nitrate (Berks *et al*., 1995). Biological denitrification is a major process that produce gaseous molecular nitrogen and it can influence soil parameters such as pH, temperature, water content and nitrate or carbon substrate availability (Olaya-Abril *et al*., 2018).

3.1.6.6 *Trichoderma harzianum*

The successful role of *Trichoderma spp.* in agricultural applications has been recognized worldwide, especially when it is combined with other physical and biological elements. It positively affects plant growth, bioremediation and contributes to reduce plant diseases, especially root diseases (Zin and Badaluddin, 2020; Woo and Pepe, 2018). Indeed, effects on stimulating both primary and secondary root hairs growth resulting in a better effectiveness of nutrient uptake by the plant have been demonstrated (Cai *et al.*, 2015). Moreover, effects as biocontrol agent in fighting phytopathogens by secreting specific chemical compounds (i.e. hydrolytic enzymes, antimicrobial peptides, secondary

metabolites and other proteins) and as detoxifying agent against pesticides and herbicides are well known.

Trichoderma is effective to colonize root surfaces which represent a favorable habitat for the fungus and this association is good to provide plant benefits (Zin and Badaluddin, 2020). However, it belongs to endophytic plant-growth-promoting-fungi (PGPF) and can also penetrate to root surfaces. As PGPF, it is able to produce secondary metabolites and plant hormones that greatly influence plant development and crop productivity (Hyakumachi, Kubota, and Arora, 2004). *Trichoderma spp*. use by improving soil productivity may support the reduction of chemical fertilizer application in agriculture (Hermosa *et al*., 2013). Moreover, plant quality could be improved as *Trichoderma spp*. effects on increased seed germination, growing biomass (Stewart and Hill, 2014), chlorophyll content and yield, improved nutrient uptake and translocation, enhanced plant biomass due to an efficient use of micronutrients and NPK have been established (Woo and Pepe, 2018). Moreover, *Trichoderma harzianum* has the potential to give greater bioactivity when associated to other positive PGPM (Stewart and Hill, 2014).

3.1.7 Aim of biochar functionalization with PGPM

In agriculture, the effect of biochar in soil and PGPR microorganisms has been amply demonstrated. One of the objectives of this study was to increase the potential of the biochar as soil improver by combining it with a specific consortium of microorganisms. The microorganisms in the consortium were selected carefully as described above. Then, we wanted to investigate the effects on the modulation of the microbial community of the soil and the physiological and biochemical effects on the cultivation of plants of important agronomic interest. The aim of this biochar functionalization was to combine the already proved positive modulations by PGPM linked to their applications, resulting in soil improvement by higher pollutant-degrading property and increased soil fertility by the means of much more content of micro- and macro-nutrients (Tao *et al*., 2020).

Taken into consideration all the data deriving from literature, a list of PGPR has been issued and selected and the consortium (i.e, the composition of microbes) has been produced based on the compatibility test and on their specific role into the nitrogen cycle. Among the microbes, all the functions present in the nitrogen cycle and all those necessary to uplift the vitality of the plants (i.e, nitrogen fixation, biocontrol activity, P solubilization, nitrogen denitrification and plant growth promoting activity) have been foreseen and the microorganisms have been chosen in such a way that these functions could be carried out. After ascertaining the type of relationship between microorganisms, in vitro tests and microscopic observations were carried out. Then the microorganisms were added to the biochar matrix and the ability to colonize was verified.

3.2 Materials and Methods

3.2.1 Microbial strain and their growing media requirements

Azotobacter vinelandii (DSM2289), *Alcaligenes faecalis* subspp. *phenolicus* (DSM16503), *Paracoccus denitrificans* (DSM413) were purchased from DSMZ Leibniz Institute (DSMZ, Braunschweig, Germany). *Pseudomonas fluorescens* (DR54) was kindly provided by Prof. Nicholeisen (University of Copenhagen, Copenhagen) and *Azospirillum brasilense* Cd was gently provided by Prof. Del Gallo (University of L'Aquila, Coppito, AQ). *Trichoderma harzianum* TH01 was purchased from Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands). Bacterial strains grow on LB medium (10 g L^{-1} NaCl, 10 g L^{-1} Tryptone, and 5 g L^{-1} yeast extract) + 2% glucose. *Trichoderma harzianum* grows on PDA medium (20 g L^{-1} dextrose, 4 g L^{-1} potato extract, and 15 g L^{-1} agar). All microbial strains grow at 28°C. Bacteria can be incubated for 12-16 hrs while the fungus must grow in darkness for 3-4 days until the mycelium reached the edges of the Petri dish.

3.2.2 Competition test

In order to test competition among microorganisms, 150 µl of a single microbial culture (grown o/n at 28°C and diluted to OD 0,3 which correspond to 10^8 cells/ml) were sown on a PCA plate, allowed to dry and a number of discs of paper Whatman n.41 corresponding to the number of microbial species whose relationship with the plated strain needed to be investigated was placed on it. 4 µl of culture (also grown o/n to 28°C and diluted to OD 0.3) were inoculated (Moran *et al.,* 2016; O'Toole 2011) on each disc. The growth of microorganisms was controlled after incubation at 28°C o/n. If the two species in contact have a competitive relationship, a halo of inhibition will be visible around the disk; otherwise, if the relationship is of cooperation or neutrality, there will be no halo of inhibition and the species will grow without any kind of competition (Fig. 12).

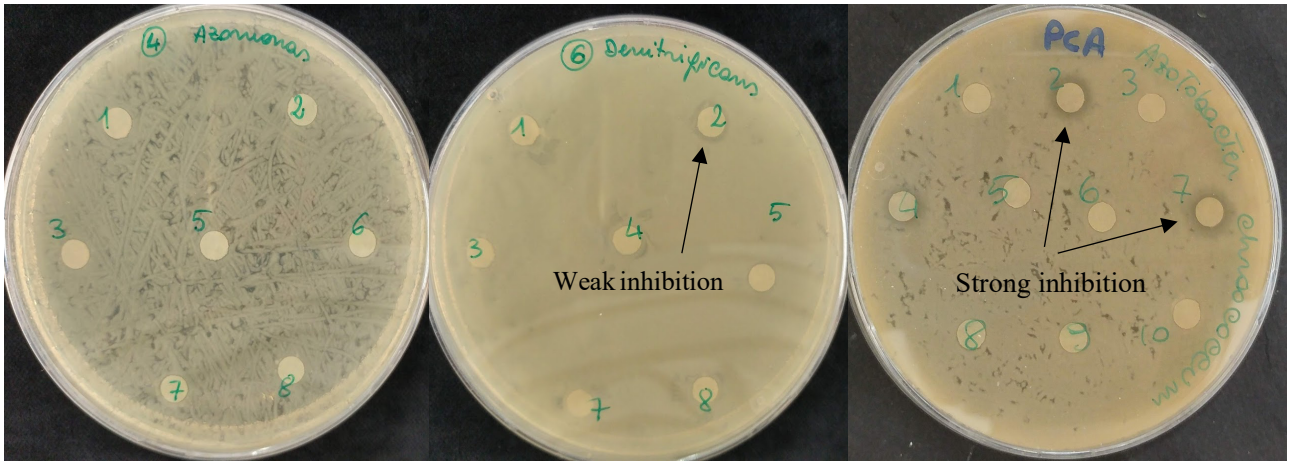


Figure 12. Example of relationship assignment in microbial competition test.

3.2.3 Biofilm production

In 96-well plates, all individual microbial species are inoculated at OD of 0,05 and after overnight growth at 28°C the culture in each well is replaced with a 0,1% (w/v) crystal violet (CV) solution and incubated at room temperature for 30 minutes, according to O'Toole (2011). Such incubation is necessary for the dye to bind perfectly to the biofilm. At this stage, the biofilm appears as a ring attached to the wall of the well. Finally, the excess dye is washed and the wells of the plate are treated with 30% (v/v) acetic acid in order to detach the biofilm ring from the wall and solubilize it. Using a spectrophotometer, the absorbance of the solution is measured at 595 nm. 30% (v/v) acetic acid was used as blank.

3.2.4 Biochar functionalization with microorganisms

Preliminary studies have been carried out to the biochar's functionalization with the aim of verifying whether this process could take place and to select the microorganisms suitable for this process from those present in the laboratory's microbial collection. Growth of microorganisms occurred in LB soil with 2% glucose. Each culture was grown overnight separately at 28°C and then they were added into the a 96-wells microplate at the same concentration (OD 0,05). Biochar crystals were added into the microplate and all the samples were allowed to grow another 24 hrs at 28°C with mild agitation.

3.2.4.1 *In vitro* study of biochar functionalization

The ability to colonize biochar surface was investigated. Three biochar samples were utilized: BG, C, and MT. Functionalization of matrices was observed in vitro, with the fluorescence microscopy (Zeiss, GmbH, Koenigsallee, Goettingen, Germany) and electron microscope (ESEM FEG2500 FEI, FEI Europe, Eindhoven, Netherlands).

In 96-well plates, 200 µl of 0,05 OD culture of each bacterium and different matrices were added into the wells. The microplate was then incubated at 28º C for 24 hours. Subsequently, the matrices were removed from the wells, moved into a new plate and 100 µl of XTT solution (90µL of XTT 1mg/ml + 10µl of phenazine metasulfate 0,34 mg/ml) was added to each sample. Plate was incubated for 1 h in the dark. The tetrazolium salt reacted with the present microbial metabolisms' products on the surface of the matrices, producing formazan, the colored compound. The amount of formazan is directly related to the number of metabolically active cells on the matrix. When the matrices were removed, the OD of the solution was read with a spectrophotometer (Varian Cary 50 UV-Vis Spectrophotometer, Agilent Technologies, The Netherlands) at a wavelength of 450 nm. In addition, the experiment was repeated with species that have shown to adapt better to the biochar, evaluating the values of XTT at different time intervals (24, 48, 72, 120 hours) to understand what is the optimal time for the functionalization of the biochar.

3.2.4.2 Fluorescence microscopy

The functionalization of the matrices was controlled also by fluorescence microscopy, using the 5µM Syto9 as fluorochrome. After the matrices were functionalized, as described above, the dye was allowed to interact with the matrices for 30 minutes in the dark and then the samples were observed with the microscope.

3.2.4.3 Environmental Scanning Electron microscopy

Microbial functionalization of the biochar was also observed with the electron microscope. Moreover, all individual microorganisms that colonized both the surfaces and inner spaces of the biochar and associations of two microorganisms (consortium) were displayed. Initially, the association of two microorganisms was tested to assess the real effect on the biochar. Indeed, the association of two microorganisms allowed to test the actual effect in terms of colonization of the biochar and the relationship that existed between the two microorganisms. The associations were assembled in order to have microorganisms that had different roles within the nitrogen cycle and also to have different shapes of microorganisms. In this way, observation was possible under the electron microscope and, also, the evaluation of the potential dominance of one microorganism over the other. The microbiological functionalization of the biochar of the individual strains and consortia created was observed by electron microscope ESEM FEG2500 FEI (FEI Europe, Eindhoven, Netherlands). Electron imaging was performed at 5kV and 10kV in an environmental low-vacuum (60 Pa). The working distance was approximately 10 mm, and the scanning time 1–3 μs.

3.3 Results and discussion

3.3.1 Microbial competition

The interspecies relationships can be summarized in Fig. 13.

	Rhodococcus kyotonensis	*Sphingomonas wittichii*	*Azospirillum brasilense*	*Azomonas agilis*	*Alcaligenes faecalis*	*Paracoccus denitrificans*	*Pseudomonas fluorescens*	*Pseudomonas putida*	*Azotobacter chroococcum*	*Azotobacter vinelandii*	*Trichoderma harzianum*
Rhodococcus kyotonensis	++	N	N	N	N	N	N	N	+	N	N
Sphingomonas wittichii	++	++	++	++	N	++	++	++	--	N	N
Azospirillum brasilense	N	N	++	N	N	N	N	N	+	+	N
Azomonas agilis	N	N	N	++	N	N	N	N	+	+	N
Alcaligenes faecalis	++	N	++	++	++	++	++	++	+	+	--
Paracoccus denitrificans	N	-	N	-	-	++	N	N	--	N	N
Pseudomonas fluorescens	-	-	N	N	N	N	++	N	+	N	N
Pseudomonas putida	N	-	N	+	-	+	+	++	-	N	N
Azotobacter chroococcum	+	--	+	+	+	--	+	-	++	++	N
Azotobacter vinelandii	N	N	+	+	+	N	N	N	++	++	-
Trichoderma harzianum	N	N	N	N	--	N	N	N	N	-	++

Legend

++	Cooperation
+	Weak cooperation
-	Weak inhibition
--	Inhibition
N	No inhibition/ no cooperation

Figure 13. Summary of the relationships among microorganisms through the competition test.

Cases of strong competition or inhibition of other organisms' growth were few (red). There were no strong competition relations between the studied strains, except for *Azotobacter chroococcum* with *Sphingomonas wittichii* and *Paracoccus denitrificans* and *Trichoderma harzianum* with *Alcaligenes faecalis.* Considering the results of inter-species cooperation and/or positive relationship and connecting this information with the characteristics of each strain and their role in the nitrogen cycle and information on their possible positive and negative combinations in the literature, associations of microorganisms (named consortia) were built. Initially, consortia were of two organisms and then a consortium of 6 microorganisms was created, which was then tested as a potential fertilizer for plants.

3.3.2 Biofilm production

The biofilm is a set of microorganisms (belonging to the same genre or different genres) wrapped in an extracellular matrix of polysaccharide nature produced by the micro-organisms themselves which has the characteristic of being in close contact with the colonized surface and which provides an adhesive and protective structure for the micro-organisms inside. In order to understand if the microbial species of my microbial collection were able to form biofilms and, possibly, to verify how much biofilms they were able to form, a spectrophotometric test was performed using a crystal violet (CV) dye (Sigma Aldrich, St. Louis, USA) that binds the polysaccharides of the biofilm in the extracellular matrix and, therefore, is able to provide reliable information on the presence and quantity of biofilms biomass that the microorganism manages to form (O'Toole 2011). In Figure 14 the absorbance values linked to biofilm production observed for each microorganism were reported.

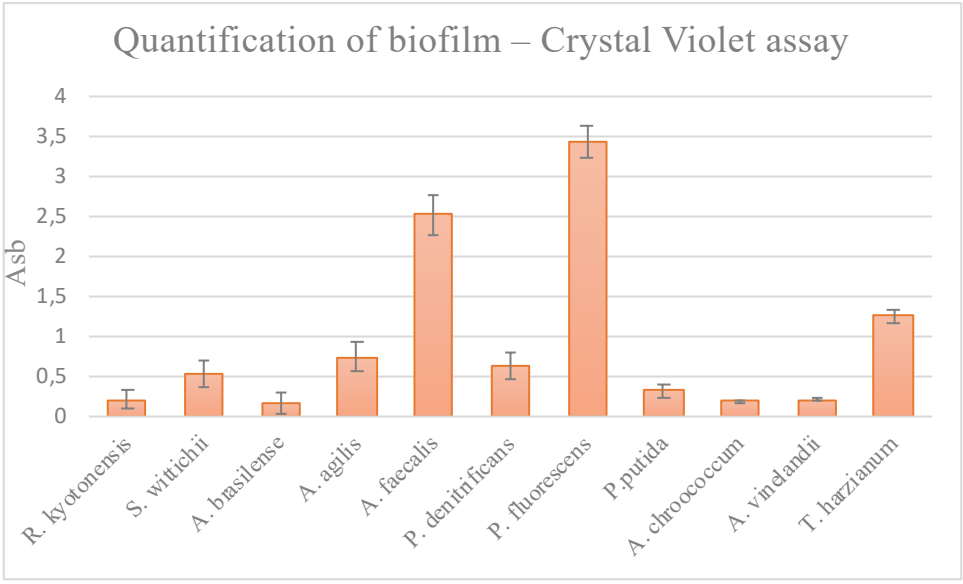
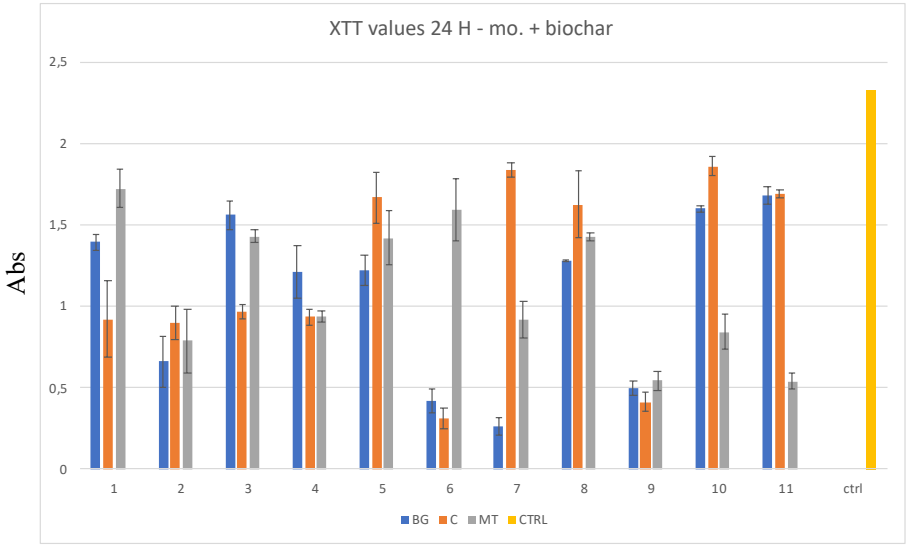


Figure 14. *In vitro* microbial biofilm quantification by absorbance values of crystal violet (CV) dye. The samples were run in triplicate, averaged, and the error bars represent standard deviation (± SD).

This was great qualitative information which we referred to build up the microbial consortium. All microorganisms were able to form biofilms on rich medium, although some have shown a better ability.

3.3.3 Biochar colonization

In vitro. In Fig. 15 were the results of the absorbance for each strain and each matrix.



1	*Rhodococcus kyotonensis*
2	*Sphingomonas wittichi*
3	*Azospirillum brasilense*
4	*Azomonas agilis*
5	*Alcaligenes faecalis*
6	*Paracoccus denitrificans*
7	*Pseudomonas fluorescens*
8	*Pseudomonas putida*
9	*Azotobacter winelandii*
10	*Azotobacter chroococcum*
11	*Trichoderma harzianum*
Ctrl	*P. fluorescens in LB medium*

Figure 15. *In vitro* evaluation of biochar surface's colonization by 11 microorganisms after 24 hrs by absorption at 600 nm of XTT fluorescent dye metabolized by microorganisms. Mean Abs of triplicate ± standard deviations.

From the observation of these results, it appeared that the microorganisms which best adapt to all the matrices tested were: *Rhodococcus kyotonensis, Azospirillum brasilense* and *Pseudomonas putida*. Therefore, the experiment was repeated only with the above mentioned species, evaluating the values of XTT at different times: 24, 48, 72, 120 hours.

1	*Rhodococcus kyotonensis*
3	*Azospirillum brasilense*
8	*Pseudomonas putida*

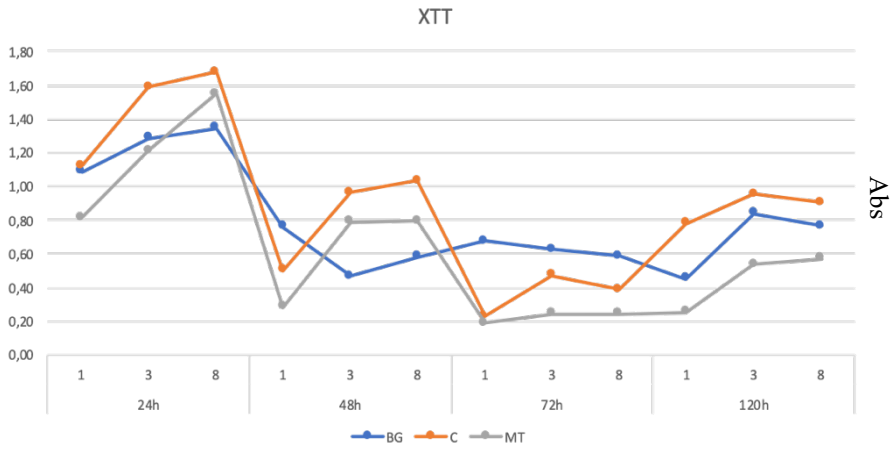


Figure 16. Absorbance values of XTT in three bacterial strains (1: Rhodococcus kyotonensis; 2: Azospirillum brasilense; 3: Pseudomonas putida) grown on BG, C, and MT biochars at different time intervals (24, 48, 72, and 120 hrs).

These results in Fig.16 showed that the functionalization of the matrices required a sufficient growth and colonization time of 24 hours. A longer time is not necessary and, on the contrary, would risk leading to the phase in which the biofilm could spoil (Moran *et al*., 2016).

Fluorescence microscopy.

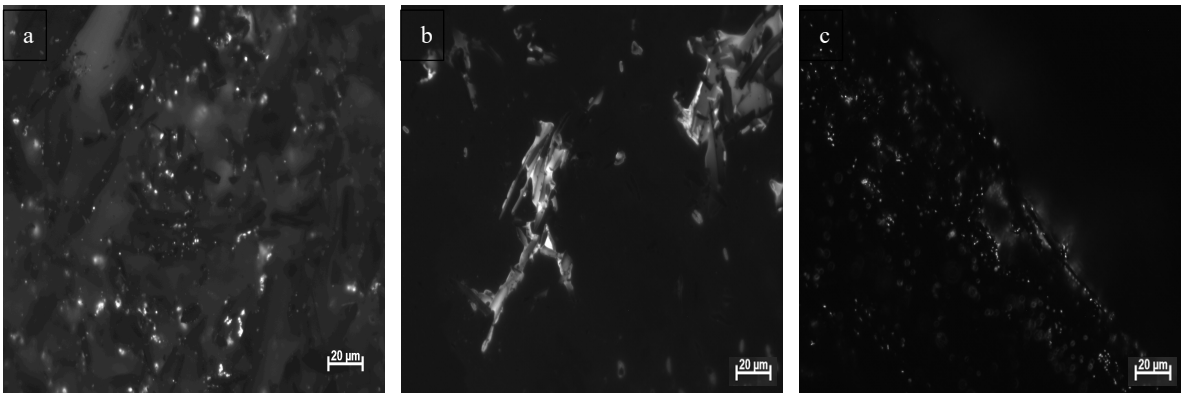


Fig 17. Images at the fluorescence microscope of functionalized biochar (BG) with Syto9. Scale bar is 20 µm. a), b) BG + *Azospirillum brasilense*; c) MT + *Pseudomonas putida*.

Colonization of biochar surfaces has also been demonstrated with fluorescence microscope observations (Fig. 17). The dye bound the nucleic acids of the bacteria and allowed easy observation. All microorganisms were able to use the biochar as a growth substrate. More detailed analyses of their presence on the biochar matrix were carried out successively with electron microscope.

Observations with the electron microscope confirmed the results reported in the in vitro tests (Fig. 17). The surface of all biochar samples could be colonized by microorganisms.

Electron microscopy. The functionalization of the matrices with all microorganisms was also confirmed by observations with the electron microscope, and subsequently with the aim of studying the effect of the functionalized biochar on crops of agronomic interest, it has been decided to create specific consortia to strengthen the activity of the biochar. Based on the previously recorded

relationships between strains, and the role of individual microorganisms within the biogeochemical nitrogen cycle, the following consortia have been established and tested:

• *Trichoderma harzianum* + *Azospirillum brasilense*

• *Pseudomonas putida* + *Sphingomonas wittichii*

• *Azomonas agilis* + *Rhodococcus kyotonesis*

• *Azotobacter chroococcum* + *Paracoccus denitrificans*

• *Pseudomonas fluorescens* + *Alcaligenes faecalis*

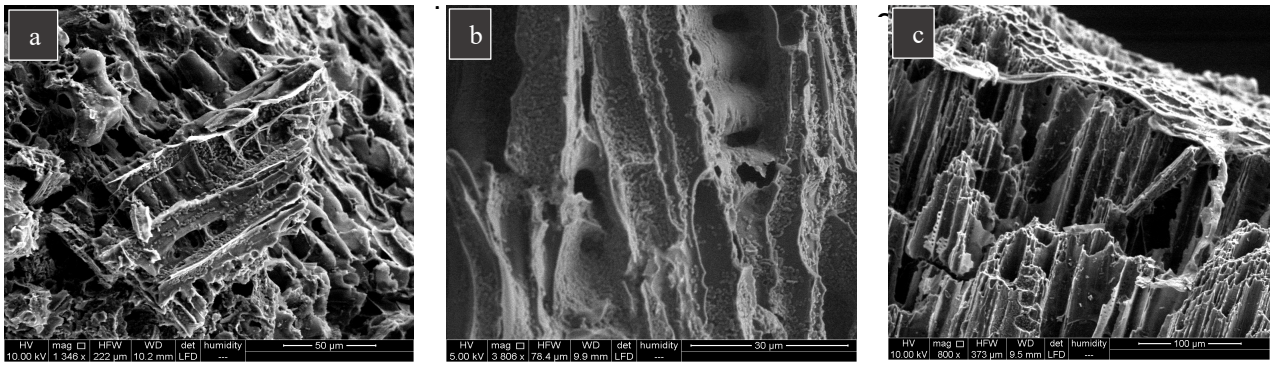


Figure 18. Images at the electron microscope of functionalized biochar. a) *P. fluorescens* (bacillus); b) *R. kyotonensis* (coccus); c) *T. harzianum* (fungus).

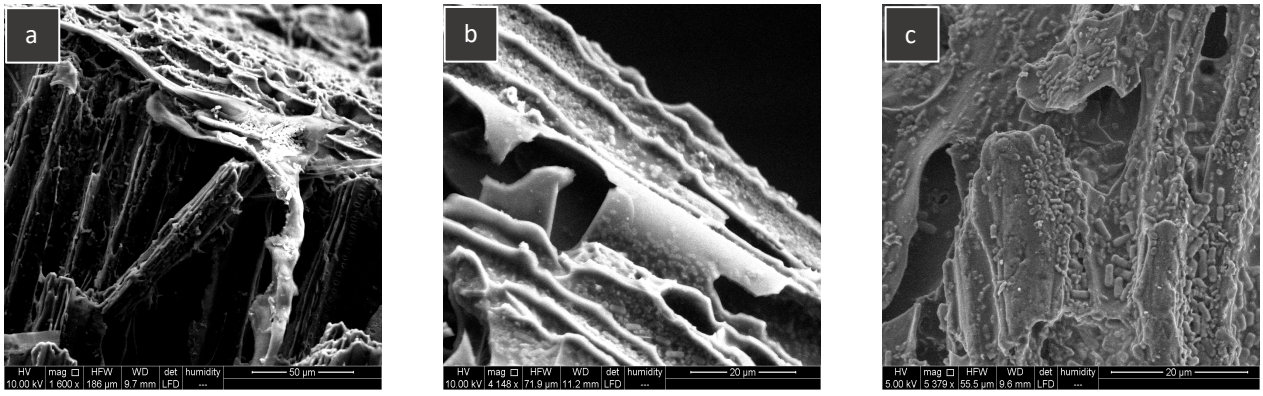


Fig 19. Images at the electron microscope of functionalized biochar with consortia. a) *T. harzianum* (fungus) + *A. brasilense* (bacillus); b) *P. putida* + *S. wittichii*; c) *P. fluorescens* (bacillus) + *A. faecalis* (coccus).

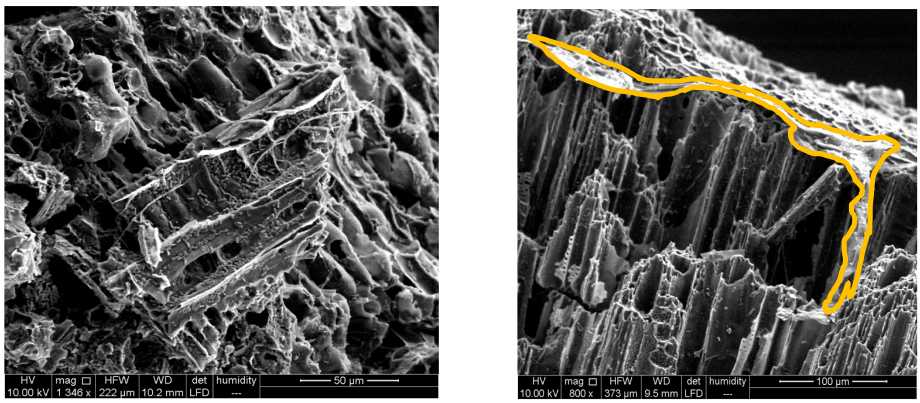


Figure 20. (Left) Particular of biochar colonized by *P. fluorescens*; (right) Particular of biochar colonized by *T. harzianum* which hold *A. brasilense* in its hyphae.

The electron microscope images showed an optimal colonization of the microorganisms of the surfaces of all the biochar samples and also the internal structures (Fig. 18, 19, 20). The high resolution of the images and their quality allowed to distinguish the type of microorganism from their shape (bacillus or coccus) (Fig. 18, 19). Especially in the presence of a microbial consortium formed by two microorganisms with different forms, it was possible to distinguish the two and also to evaluate the prevalence of one over the other (Fig. 20). The relationship between microorganisms could be clearly observed in images with the electron microscope.

Therefore, the data showed the ability of microorganisms to create biofilms, to colonize the internal and external surfaces of the biochar. This allowed the study of the initial association of two microorganisms to assess the actual colonization of the biochar. The latter and the relationship between microorganisms living in the same habitat have been evaluated and positive results have allowed the subsequent creation of a consortium of 6 microorganisms that was used to functionalize the biochar. This new tool could be used as a potentially revolutionary fertilizer in agriculture.

CHAPTER 4 Biochar effect in *in vaso* experiment

4.1 Introduction

4.1.1 *Solanum lycopersicum* L.

An *in vaso* experiment was conducted in order to investigate the effect of biochar as valuable amendment and the effect of a special microbial consortium applied to biochar and tomato seeds (*Solanum lycopersicum* L.). Tomato belongs to *Solanum* genus in the *Solanaceae* family and it has been studied as one of the most representative crops in the world (Bergougnoux, 2014). It is native to South America and was introduced to Europe in the 16th century (Bergougnoux, 2014). The varieties considered were *Ailsa Craig* and *Heinz 3402*. Growth parameters, photosynthetic pigments, some antioxidant nutrition contents, and the metabolite changes were analyzed as the main results of plant response to potential stress. Tomato is one of the most important plants in the world and its nutritional quality has been widely observed and it has become increasingly crucial over time for its role in human health (De Vos, Hall, and Moing, 2011) by acting as an anticancer, preventing cardiovascular risks and slowing down cellular aging (Al-Amri, 2013), thanks to its content in antioxidant compounds and vitamins. For these reasons, the effects of the treatment of cultivations with biochar and the microbial consortium as well as their association have been investigated with the aim of improving soil composition, health and balance from a physical, chemical and microbiological point of view and also the composition of the plant and the nutritional quality of the fruits. In order to study the effects of this new organic soil improver named biochar and its possible application in the field, we chose the tomato as a model plant. Not only that, but tomato is also one the most widely consumed and cultivated plant in the world and high-quality fields are a fundamental objective to be achieved for the economic success of producers around the world. Europe and America represented the most important producers worldwide (Bergougnoux, 2014). As *Ailsa Craig* cv. is one of the most widely used tomato varieties for both human consumption and industrial

tomatoes at European scale, it has been used in this work and has been compared with one of the most widely used and commercialized varieties in America, the *Heinz 3402* cv.

4.1.2 Study of the physiological effects on *Solanum lycopersicum* due to biochar application as fertilizer

The present thesis had set challenging objectives including the study of the effectiveness of an innovative sustainable fertilizer (biochar) and its interaction with a pool of microorganisms (consortium) in terms of vegetative production and quality of the plant both physiologically and phytochemically on two different varieties of tomato. The experimental plan included these treatments:

- application of biochar as a soil amendment;
- application of a specific consortium of selected microorganisms (PGPM);
- application of the association of this microbial consortium of PGPM combined with biochar.

Plants are the primary producers in the terrestrial ecosystem and their plasticity and their ability to adapt to internal and external inputs that disturb metabolic balance can be noticed by observing physiological features. These characteristics also provide information on plant welfare and phytonutrient values. Hence, one of the aims of this experiment has been to verify the effect of processing tomato crops with biochar, functionalized biochar and functionalized seeds focusing on the investigation of physiological parameters and measuring and interpreting nutrient assimilation data.

4.1.3 Important physiological analysis to be monitored

Physiological parameters need to be monitored in order to evaluate the impact of the treatments performed. Among these parameters we have:

- Fresh and dry biomass
- Shoot height and root length
- Number of leaf and leaflets
- Leaf area
- Water content
- Chlorophyll and carotenoid content
- Pheophytin content
- Anthocyanin content
- Catalase activity
- H_2O_2 estimaton

4.1.3.1 Fresh and dry biomass

Fresh and dry biomass: they provide information that can also be viewed with the naked eye easily. High values of both are desirable in a well-being condition of the plant.

4.1.3.2 Shoot height and root length

The shoot height: high values correspond to optimal metabolic function.

Root length also shows that a plant is in an optimal growing condition to plant if it shows that it is in a stress situation (generating secondary radical hairs and proving to be in search of new nutrients).

4.1.3.3 Number of leaf and leaflets

Leaf: monitoring the number of leaves and leaflets is a first screening to get information about plant health functioning. Indeed, leaves are a special organ of plant in which photosynthesis and transpiration processes are localized.

4.1.3.4 Leaf area

Leaf area: one of the most indicative indices for observing the primary net production, the use of water and nutrients by the plant and the consumption of carbon (Bréda, 2008) and energy through the mass exchange. In combination with chlorophyll content, it provides information about plant vitality, vegetative vigor, and photosynthetic potential. Leaf area index (LAI) underlies the plasticity of the plant, the ability to produce organs, the variability in shape and size, and the flexibility to react to internal and external inputs.

4.1.3.5 Water content

Plant water content: it affects the total amounts of available nutrients and it is a powerful estimation value for energy status of the plants. It strongly influences plant functioning and total productivity and carbon balance within the ecosystem (Huang *et al.*, 2020).

4.1.3.6 Chlorophyll and carotenoid content

Leaf chlorophyll: provides valuable information about physiological status of plants such as growth vigor, photosynthesis rate, and transpiration. Moreover, chlorophyll pigment located in chloroplast, helps to define the nutritional value of the plant and, where appropriate, of the fruits. The most common chlorophyll present in nature is chlorophyll a (Chl A), while chlorophyll b (Chl B) pigment is accessory. Both chlorophylls are key players in photosynthesis process and their ratio varies according to plant species and environmental conditions (Lichtenthaler, 1987).

Carotenoid: pigments which refer to derivatives of the *alpha* and *beta* forms of carotene including lutein, xanthophylls, zeaxanthines, violaxanthines and anterazanthins. Carotenoids present on plant photosynthetic organs are called primary carotenoids (Lichtenthaler, 1987).

Chlorophylls and carotenoid refer to prenyl pigments but differ by subclass as chlorophylls are isoprenilic molecules while carotenoids are tetraterpenoids.

4.1.3.7 Pheophytin content

Pheophytins: their content gives an idea of the phytochemical activity of the plant (Klimov, 2003). Indeed, they are chlorophyll-derivative products in which Chl A and B lost their magnesium ions by the effect of a weak acid. Pheophytins serve in the early steps of photosynthetic solar energy conversion as first electron acceptor and carrier within the respiration process in photosystem II (RC II) driving electron from light source to a quinone.

4.1.3.8 Anthocyanin content

Anthocyanins: water-soluble vacuolar phenolic compounds pigments with a single aromatic molecule known as cyanidin. They are present predominantly in the leaf in all stages of vegetative development. The protective function of anthocyanins has long been known: they are able to protect the plant from various stresses and can also give information on the adaptation of the plant to stresses (Gitelson, Merzlyak, and Chivkunova, 2001). The color ranges from red to blue of visible spectrum.

4.1.3.9 Catalase activity

Catalase: is a tetrameric heme enzyme known for its antioxidant activity in metabolizing stress-provoked reactive oxygen species and monitoring their effect on cellular metabolism and functions (Anjum *et al.*, 2014). It is located mainly in peroxisomes of leaves, cotyledons, and roots but also in mitochondria of some plant species (Anjum *et al.,* 2014). When reactive oxygen species (ROS) occur

in plants, the catalase enzyme reacts catalytically transforming two hydrogen peroxide molecules to water and oxygen as the following:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Catalase activity was observed trough both qualitative visual approach *(Anjum et al., 2014; Iwase et al.,* 2013) and spectrophotometric method (Poli *et al.*, 2018; Apodaca *et al.*, 2017).

4.1.3.10 H_2O_2 estimation

Hydrogen peroxide: it refers to oxygen reactive species that could be dangerous for higher organisms reacting with cellular membrane phospholipids and procuring damages to DNA eventually.

4.2 Materials and Methods

4.2.1 Plant growth and treatments

In table 10 all the experimental conditions and selected plant species are resumed. Two tomato cultivars, *Ailsa Craig* and *Heinz 3402*, were used in this experiment. The detailed experimental procedure outline is shown in Fig 21.

Table 10. *Solanum lycopersium* L. species and *in vaso* experimental treatments.

Solanum lycopersicum cv.	Treatments			
Ailsa Craig (AC)	Control (Ctrl)	Biochar (BG)	Functionalized biochar (BG cons C)	Functionalized seeds (SEEDS cons C)
Heinz 3402 (H)	Control (Ctrl)	Biochar (BG)	Functionalized biochar (BG cons C)	Functionalized seeds (SEEDS cons C)

Fig 21. *In vaso* experiment workflow.

4.2.2 Experimental facility

The study was conducted at Bren School of Environmental Science & Management of University of California, Santa Barbara, UCSB, California, CA, USA. 90% 1 cm mesh sieved Canadian sphagnum peat moss (Sun Gro Horticulture Inc. Agawam, MA, USA) was used as soil in combination with 10% sand in 14x14x21 cm pots filled up to 750 g of mixed soil.

The early vegetative stage of plant growth was located in mini-pots in a growth chamber for 21 days, until the germination occurred in all the samples. They were supposed to be in a light (16 hrs) and dark (8 hrs) cycle. Chamber air temperature was controlled to avoid temperature fluctuations. The high constant temperature of 24°C was set and water irrigation was provided manually to keep water content in vase at least 60% of its capacity. After the germination period, samples were first transplanted to bigger pots (750 g mixed soil) and then moved to greenhouse. The light and dark cycle

and temperature replicated the outer atmospheric conditions. Average day temperature was 30º C while the relative humidity values ranged between 45 and 50%.

4.2.3 Sterilization of seeds

Heinz 3402 seeds were kindly provided by Kraft Heinz Company (Irvine, CA, USA), while *Ailsa Craig* seeds were purchased to Victory Seeds (Molalla, Oregon, USA). Tomato seeds were washed twice with deionized water and surface sterilized with 70% v/v ethanol with mild agitation for 3 mins, washed twice with sterile deionized water and, then with 10% v/v sodium hypochlorite with agitation for 10 mins. Then, seeds were thoroughly washed three times with sterile deionized water and transferred to a Petri dish until they will be transferred into mini pots.

Six sterilized seeds were placed into a Petri dish with LB medium to check their surface sterilization and their vitality.

The seeds were divided into three parts: an aliquot was added to liquid LB medium containing the microbial consortium mentioned above in order to functionalize the seeds with those microorganisms (see below); another part was added to a Becker including the same microbial consortium and also biochar for its functionalization (see below in the treatments); and the last part was directly transferred to a 5 cm diameter pots filled with mixed soil (90% sieved sphagnum peat moss and 10% sand) and incubated in a growth chamber at 24°C for three weeks with 16hrs light and 8hrs dark.

4.2.4 Preparation of the microbial consortium

Fresh pre-cultures of each bacterial microorganism have been prepared into liquid LB medium. After overnight incubation at 20°C with mild agitation, their optical densities were established by reading the absorbance at 600 nm. The microbial consortium was built up by adding $2x10^7$ cells ml^{-1} for each culture for biochar functionalization and $2x10^8$ cells ml^{-1} for seeds functionalization.

About fungal preparation of *Trichoderma harzianum*, a small amount of the mycelium was transferred in the center of a fresh PDA medium plate and allowed to grow at 20°C until the mycelium reaches the edges of the Petri dish. When ready, 10 ml of deionized water and 0,01% Triton were added to the surface and then with a sterile brush the homogenate created was scraped from the ground and filtered in order to remove residual hyphae. A concentration of $4x10^7$ CFU ml^{-1} was employed both for biochar and seeds functionalization.

4.2.5 Biochar functionalization

The amount corresponding to 0,1% w/w biochar (considering the soil in pots) was first weighted and sterilized by autoclave, fresh microbial consortium was prepared as described above. Each culture was grown overnight separately and then they were added into the same flask diluting them to the right concentration and to the optimal final volume of 10X (ml) per g biochar. Microorganisms and biochar were allowed to growth again 24 hrs at 20°C with mild agitation.

4.2.6 Seeds functionalization

Fresh pre-cultures were prepared in LB liquid medium and incubated at 20°C overnight. Each strain is diluted to a final concentration of 10^8 cells ml^{-1} (or CFU ml^{-1}) and combined in the same tube were seeds, previously sterilized, were added for 24 hrs at 20°C. Liquid was removed with a sterile mesh and seeds sowed immediately into mini pots.

4.2.7 Plant growth and treatments

Pots for the experiment were filled up with 90% sieved sphagnum soil, 10% sand, and, in the foreseen experimental conditions, 0,1% (w/w) biochar. Biochar was mixed to soil and sand before filling the pots. For the specific treatment requiring it, the biochar was first functionalized with the microbial consortium, then mixed to soil and sand before being added to pots. Microbial consortium was also used to functionalize tomato seeds for the corresponding condition. Pots were filled up with 750g of

mixture soil. Twelve seeds were planted for each treatment and for each cultivar resulting in a 96 total plants experiment.

Tomato seeds were surface sterilized as indicated previously. Part of the seeds were grown into LB medium with microbial consortium and then transferred straightly to mini pots. One seed per pot was sown. Tomato seeds were let grown in the growth chamber with constant air temperature of 24°C (day/night) with 14 h light and 10 h dark. After 21 days, seedlings were transplanted to bigger pots (14x14x21 cm) filled up with 750 g of mixed soil and moved to greenhouse. Plants were harvested 50 days after sowing (DAS). Plants did not have fruits at 50 DAS. After the harvest, plants were cut into organs which were washed and immediately used for analyses or instantly frozen for future experiments.

4.2.8 Physiological analysis

The fresh weight was taken immediately after the collection of the samples for both the epigeal and the hypogeal parts. Samples were then dried with a dryer for 24 hrs under vacuum atmospheric conditions. Data were expressed as g.

Data have been collected immediately after the sampling of the plants and are expressed in cm.

The total number of leaflets and leaves were counted by hand.

Leaf area was measured through digital image analysis with ImageJ 1.53a software as suggested by O'Neal (2002) protocol. Data were reported in mm^2.

The total water content was accurately estimated using the difference of fresh weight tissue and dry weight tissue. Data of relative quantity of water were reported as g.

4.2.9 Chlorophyll and carotenoid content

Both Chl A and B, and also carotenoids were investigated in this experiment according to Zhang *et al.* (2015) protocol. 100 mg of fresh leaves were collected before plant harvest and immediately frozen with liquid nitrogen. Leaves were then grinded with a pestle and 80% acetone. The homogenate was collected in 2 ml tubes and centrifuged at 5000 rpm for 10 mins. The process was repeated until the pellet was colorless. The final volume was noted and the absorbance was measured at 663, 646, 750, and 470 nm by UV-1800 SHIMADZU Spectrophotometer (Schimadzu Corporation, Camby, OR, USA).

The concentrations of Chl A, Chl B, and total carotenoids were then calculated using the equations of Lichtenthaler (1987) and expressed as μg g^{-1} fresh weight.

4.2.10 Pheophytin content

The concentration of pheophytin a (Pheo A) , pheophytin b (Pheo B) and total pheophytins (Pheo tot) was observed following the Lichtenthaler (1987) advices and it was expressed as mg g^{-1} fresh weight. Briefly, total pigments were extracted as described in 4.2.9 paragraph. Then, for pheophytin extraction, one drop of 25% acqueous HCl solution was added to 5 ml of the extract. The solution was allowed to settle and the absorbance was measured at 470, 653, and 665 nm.

4.2.11 Anthocyanin content

Fresh leaves and roots were immediately frozen with liquid nitrogen and then added into 10 ml of acidic methanol (1% v/v HCl) and the mixture was incubated overnight at room temperature. Anthocyanins were extracted from the total pigment content by adding 10 ml of chloroform and 9 ml of deionized water. Samples were shaken gently, and the solutions were read spectrophotometrically at 505 (Haida and Hakiman 2019), 530, and 657 nm. Anthocyanin content was normalized and expressed as gram fresh weight according to Bharti and Khurana (2003).

4.2.12 Catalase activity

For extractions, 200 mg of fresh leaf and root samples were grinded with 1800 µl of a 25 mM phosphate buffer solution (Apodaca *et al*., 2017) with minor modifications. The extracts were collected, centrifuged for 10 mins at 4°C and 10000 rpm. Supernatants were employed for the catalase assays.

Qualitative estimation. This assay is a qualitative approach for measuring catalase activity which underlies the principle of that oxygen bubbles will appear when enzymatic catalase pool faces hydrogen peroxide molecules. These bubbles were then visualized as foam, the test-tube height of which was measured to quantify the enzymatic activity. Leaf and root catalase activity was assessed and expressed as oxidized catalase units per g fresh weight.

Spectrophotometric assay. Test solutions were prepared according to Poli *et al.* (2018) with minor modifications. It consisted of 1,5 ml phosphate buffer solution, 0,6 ml 10 mM H_2O_2 solution, 0,6 distilled water, and 0,3 ml extract. After gently inverting quartz cuvettes by hand, the absorbance was measured at 240 nm every 30 seconds for 3 mins. The catalase activity was expressed as µmol H_2O_2 oxidized per minute per gram fresh weight.

4.2.13 H_2O_2 estimation

Total H_2O_2 content was extracted grinding both fresh leaf and root samples (0,2 g) with 2 ml of 25 mM phosphate buffer solution. The homogenate was centrifuged at 6000 g for 15 mins at 4°C according to Haque *et al.* (2018). The absorbance was read at 390 nm against the blank solution and hydrogen peroxide contents were estimated per g fresh weight.

4.2.14 Statistical analysis

LAI measurements were performed among ten replicates for each experimental condition. The leaf number and leaflets were carried out based on the total amount of samples. All the remaining physiological analyses were set up in biological replicate of six plants for each treatment. Data were analyzed using one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$ using Past v.4 software. Tukey's test followed one-way analysis for multiple comparisons of treatments. Multivariate statistical analysis was performed by Principal Component Analysis (PCA) and data were represented through heatmap illustrations. Heatmaps were designed utilizing the software R version 4.0.2.

4.3 Results and discussion

4.3.1 Fresh and dry biomass

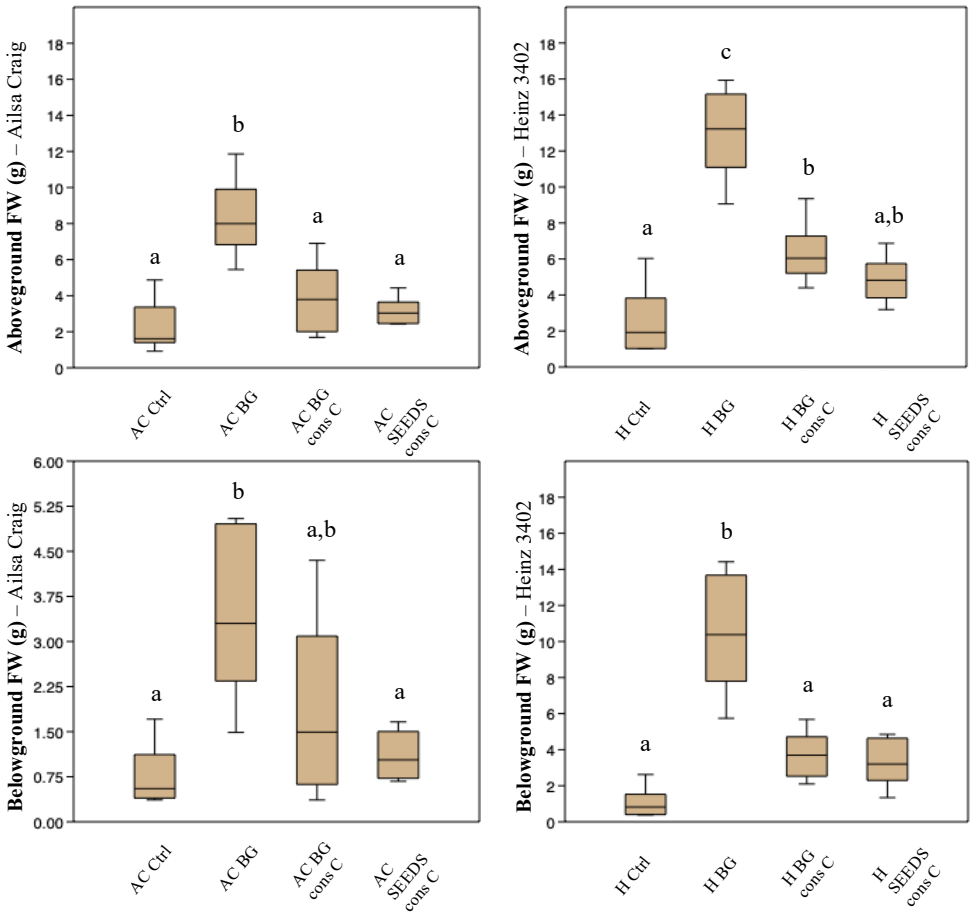


Figure 22. Aboveground fresh weight biomass (g) of *Ailsa Craig* (top left) and *Heinz 3402* cv (top right). Belowground fresh weight of *Ailsa Craig* (bottom left) and *Heinz 3402* cv (bottom right). Results are expressed as mean values (n=6) with standard deviation (±SD). AC: *Ailsa Craig*; H: *Heinz 3402*; Ctrl: control; BG: biochar; BG cons C: functionalized biochar with consortium C; SEEDS cons C: functionalized tomato seeds with consortium C. Different letters correspond to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$).

In *Ailsa Craig* cultivar, both aboveground and belowground fresh biomasses of treated plants were greater than the control ones but only the treatment with biochar was statistically different from the control (Fig. 22). Actually, there was a 73% increase in AC BG, followed by 20% (AC BG cons C), and 11% (AC SEEDS cons C) increases respectively compared with the control for the aboveground fresh weight and 78% (AC BG), 32% (AC BG cons C), and 10% (AC SEEDS cons C) increases for the belowground biomass fresh weight. Even in *Heinz 3402* cultivar, all treated plants showed greater biomasses compared with the control both in above- and belowground. In particular, the differences

in percentage were higher: 81% (H BG), 29% (H BG cons C), and 18% (H SEEDS cons C) increases respectively compared with the control for the aboveground biomass fresh weight, and 90% (H BG), 25% (H BG cons C), and 22% (H SEEDS cons C) increases respectively for the belowground fresh weight. Also in this case, plants treated with biochar showed statistical difference compared to the control for both the belowground and the aboveground parts. However, only for the aboveground part plants treated with functionalized biochar showed significantly greater biomass than the control (Fig. 22).

4.3.2 Shoot height

In *Ailsa Craig* cultivar all experimental treatments did not show significant differences with the control due to high variability among samples although differences in increasing mean height values in treated plants were found and were 14% (AC BG), 28% (AC BG cons C), and 33% (AC SEEDS cons C) respectively, compared with the control (Fig. 23). Treatments with microbial functionalized seeds allowed for larger growth in height for plant stems, followed by the treatment of microbial consortium associated to biochar and, finally, by the treatment with biochar. In *Heinz 3402* tomato cultivar no statistic differences have been highlighted even though it seemed that the averaged shoot heights of treated plants with functionalized biochar were greater than the control (there was a slight 6% increase H BG cons C).

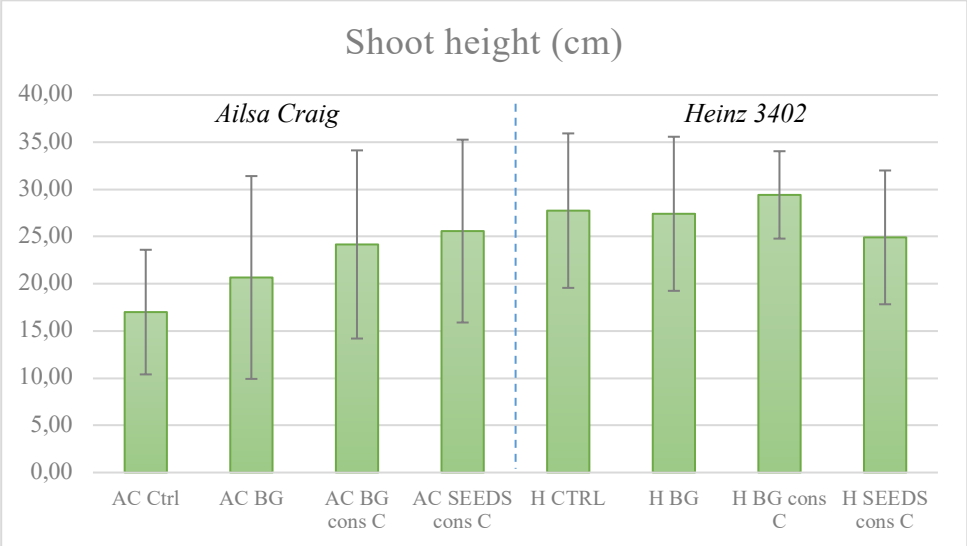


Figure 23. Shoot height of *Ailsa Craig* (left) and *Heinz 3402* (right) cv. Data shown as mean (n=6) ± SD.

4.3.3 Root length

In *Ailsa Craig* cultivar, root lengths of all the treatments were greater than the control one but only the root length of plants treated with biochar showed significant difference from the one of control plants (35% increase).

In *Heinz 3402* cultivar, all the treatments were significantly different from the control showing a much greater length of roots. They showed similar values and no significant differences have been found among treatments but there were 27% (H BG), 35% (H BG cons C), and 33% (H SEEDS cons C) increased root lengths in treated plants compared with the control (Fig. 24).

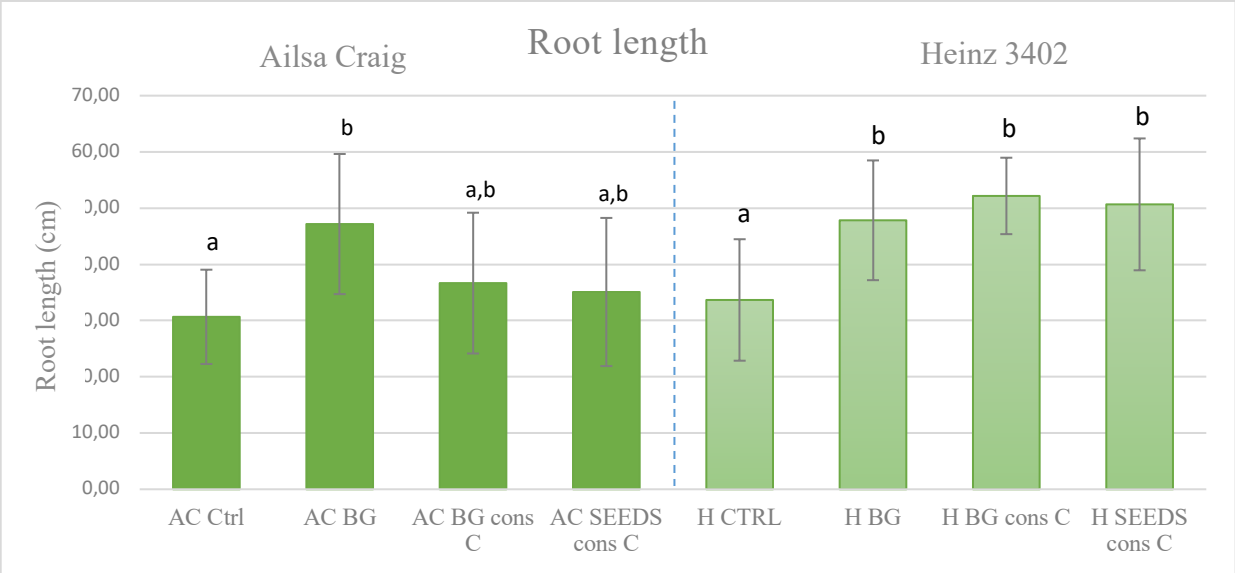


Figure 24. Root length of *Ailsa Craig* (up) and *Heinz 3402* cv (bottom). Data shown as mean (n=6) ± SD. Different letters correspond to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$).

4.3.4 Number of leaf and leaflets

Total leaf number did not show significant differences between treatments with the only exception of *Heinz 3402* plants treated with biochar which had a total leaf number highly greater than the others and the control. Leaflets number followed the total leaf number data for *Ailsa Craig*

cultivar. In *Heinz 3402* statistical differences from the other experimental conditions has been reported in plants treated with biochar and functionalized biochar (Fig. 25).

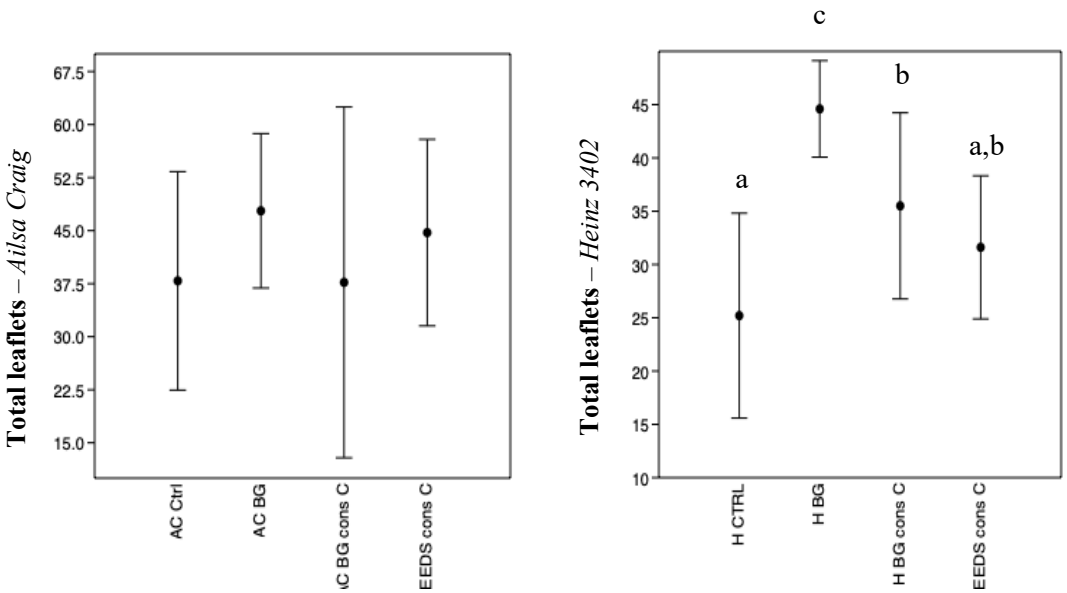


Figure 25. Total leaflets in *Ailsa Craig* samples (left) and *Heinz 3402* cv (right). Values refers to means with standard deviation. Different letters correspond to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$).

4.3.5 Leaf area

It was possible to notice that the leaf area trends were opposite within each tomato cultivar (Fig. 26, next page). In *Ailsa Craig* the leaf area was relatively constant with a significant decrease in plant leaf treated with functionalized biochar.

In *Heinz 3402* plants, the leaf area increased slightly in plants treated with functionalized biochar and more consistently in those treated with seeds engineered with the microbial consortium while in plants treated with biochar the increase was statistically significant.

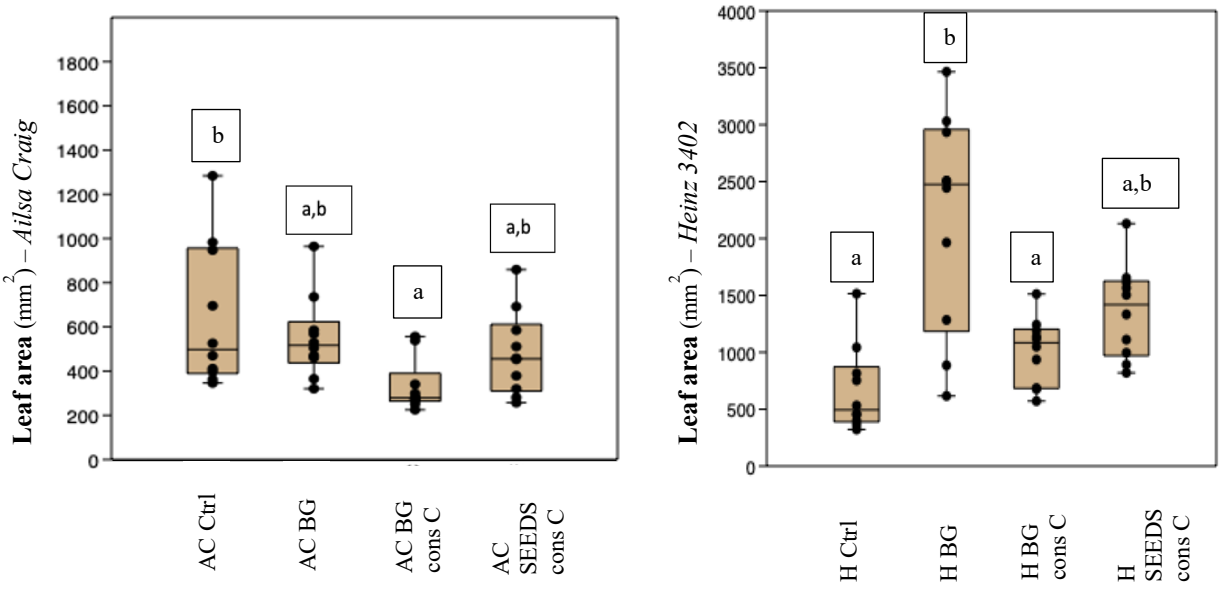


Figure 26. Leaf area of *Ailsa Craig* (left) and *Heinz 3402* (right) cv. Results are expressed as mean values (n=10) with standard deviation (±SD). Different letters correspond to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$).

4.3.6 Total water content

Biochar is well known for its capability to hold water. Plants treated with biochar showed the greatest ability; functionalized biochar also showed an important characteristic of water retention, followed by functionalized tomato seeds and then the control. As shown in the Fig. 27, *Heinz 3402* cultivar seemed to be the plant variety that retained the most water comparing the two tomato cultivars, except for plants treated with functionalized seeds in which *Ailsa Craig* showed higher capacity to hold water molecules than *Heinz 3402*. However, both cultivars had the same trend, considering each experimental condition separately. Plants treated with biochar were those which hold water the most, both in above- and below-ground. They were followed by plants treated with functionalized biochar that showed lower capacity to hold water but at the same time greater than the ones of control plants and those with functionalized seeds.

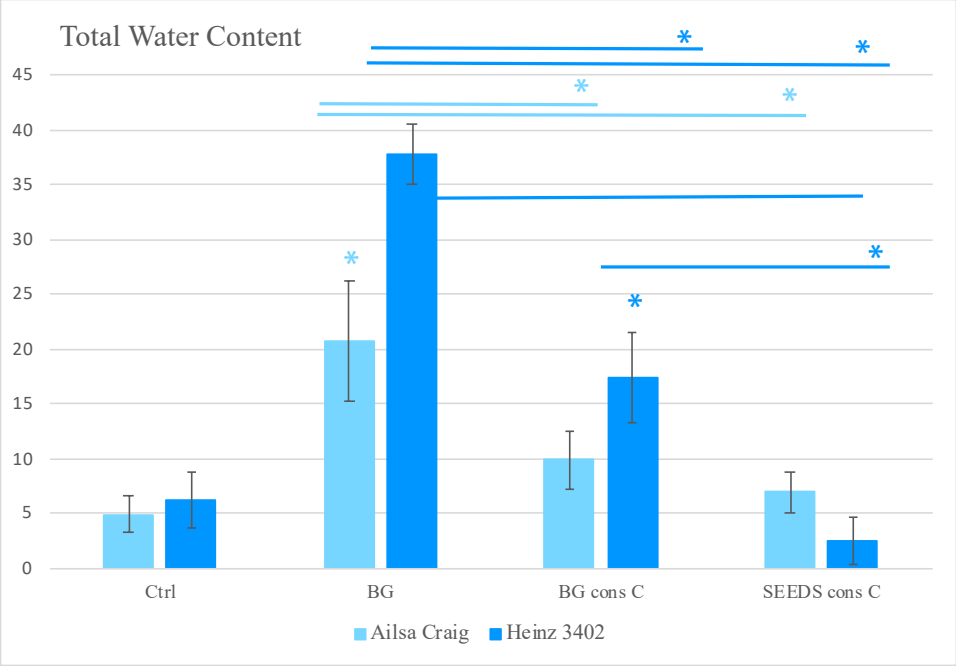


Figure 27. Total water content of *Ailsa Craig* and *Heinz 3402* cv. Results are expressed as mean values (n=6) with standard deviation (±SD). * corresponds to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$). Different colors follow the legend.

4.3.7 Chlorophyll and carotenoid content

In *Ailsa Craig* cultivar, the quantity of Chl A, Chl B and the total chlorophyll content remained almost constant, although in some cases there were slight increases in treated plants (Fig. 28). Also the carotenoid content registered values greater than the control although they were not significant. Focusing on the Chl A content, which is the major occurring chlorophyll in nature, in *Heinz 3402* cultivar (Fig. 28) there have been positive trends in all treated plants but significant increases only in those treated with biochar and with functionalized seeds. Plus, Chl A of biochar-exposed plants was greater than the relative quantity in plants with functionalized seeds. The content of Chl B was statistically different comparing the control condition with the other experimental treatments. Carotenoid content differed statistically from the control only in plants treated with biochar and in those with functionalized seeds. They also showed a significant difference between each other.

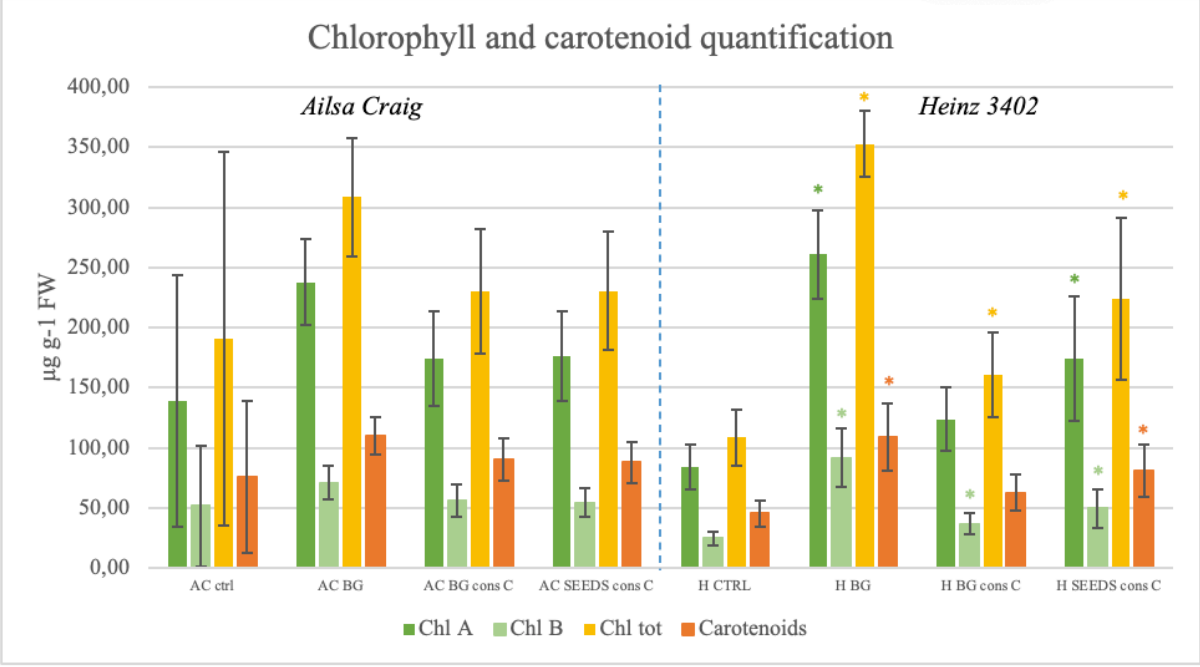


Figure 28. Chlorophyll a (Chl A), chlorophyll b (Chl B), total chlorophyll (Chl tot), and carotenoid content in *Ailsa Craig* (left) and *Heinz 3402* (right) cv. Data shown as mean (n=6) ±SD. * corresponds to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$). Different colors follow the legend.

4.3.8 Pheophytin content

The concentration of Pheo A, Pheo B and total pheophytin (Pheo A + B) were investigated. In both tomato cultivars, the sum of pheophytin a and b (Pheo A +B) was significantly greater in all the experimental treatments compared to the control.

With the reference to Pheo A content, significant differences were observed between all the treatments and the control both in *Ailsa Craig* and in *Heinz 3402* cultivars. In *Heinz 3402* the Pheo A content also significantly differed to the other treatments and the relative quantity in plants exposed to biochar amendment was greater than the others (Fig. 29).

In *Ailsa Craig*, Pheo B was significantly much more abundant in both plants treated with biochar and those with functionalized seeds compared to the control and plants exposed to functionalized biochar. In *Heinz 3402* all the treatments had a significant difference compared to the control. The condition H BG cons C and H SEEDS cons C showed similar levels of pheophytins (Pheo A, Pheo B, and Pheo A + B) but lower than the level in plants treated with biochar (Fig. 29).

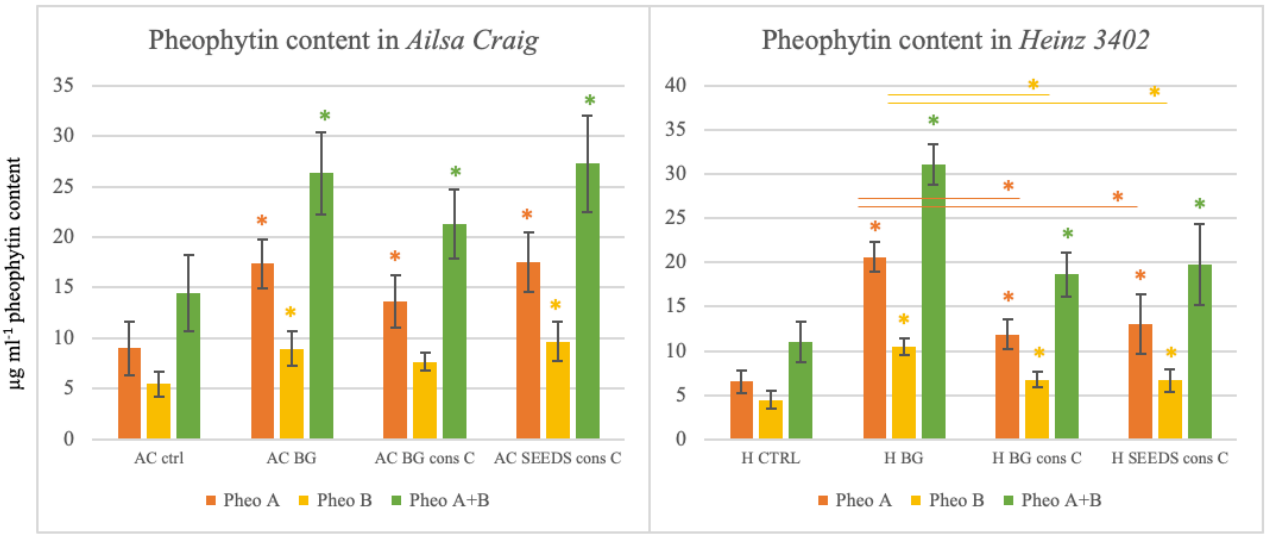


Figure 29. Pheophytin a (Pheo A), pheophytin b (Pheo B) and total pheophytin (Pheo A + B) content in *Ailsa Craig* (left) and *Heinz 3402* cv (right). Data shown as mean (n=6) ±SD. * corresponds to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$). Different colors follow the legend.

4.3.9 Anthocyanin content

The anthocyanin content has been evaluated both in leaf and root samples in each cultivar.

In *Ailsa Craig* cultivar, both in leaf and root samples plants treated with biochar and functionalized biochar showed a significant increase of anthocyanin content from the control. In *Heinz 3402* cultivar, it has been observed a significant decrease of pigment content in plants with functionalized seeds only for leaf samples while in root samples only plants treated with biochar showed a significant increase of anthocyanin content (Fig. 30).

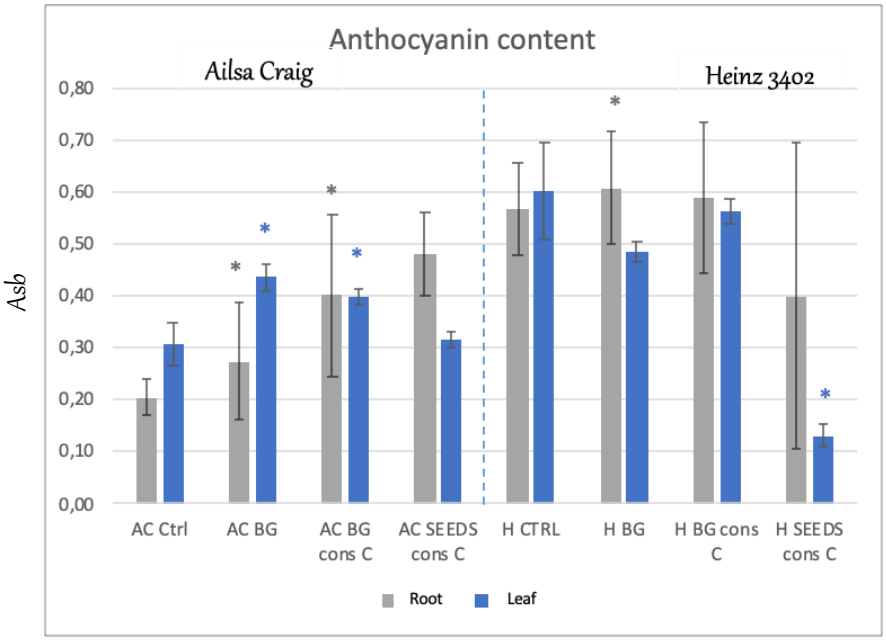


Figure 30. Anthocyanin content in root (gray) and leaf (blue) in *Ailsa Craig* (left) and *Heinz 3402* (right). Data shown as mean (n=6) ±SD. * corresponds to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$). Different colors follow the legend.

4.3.10 Catalase activity

The activity of catalase enzyme in the vegetative metabolism of plants has been observed both in leaf and root samples with two methods. First it was estimated evaluating the height of foam produced after combining the vegetative aqueous extract with a solution of hydrogen peroxide. Data were expressed in mm (Fig. 31).

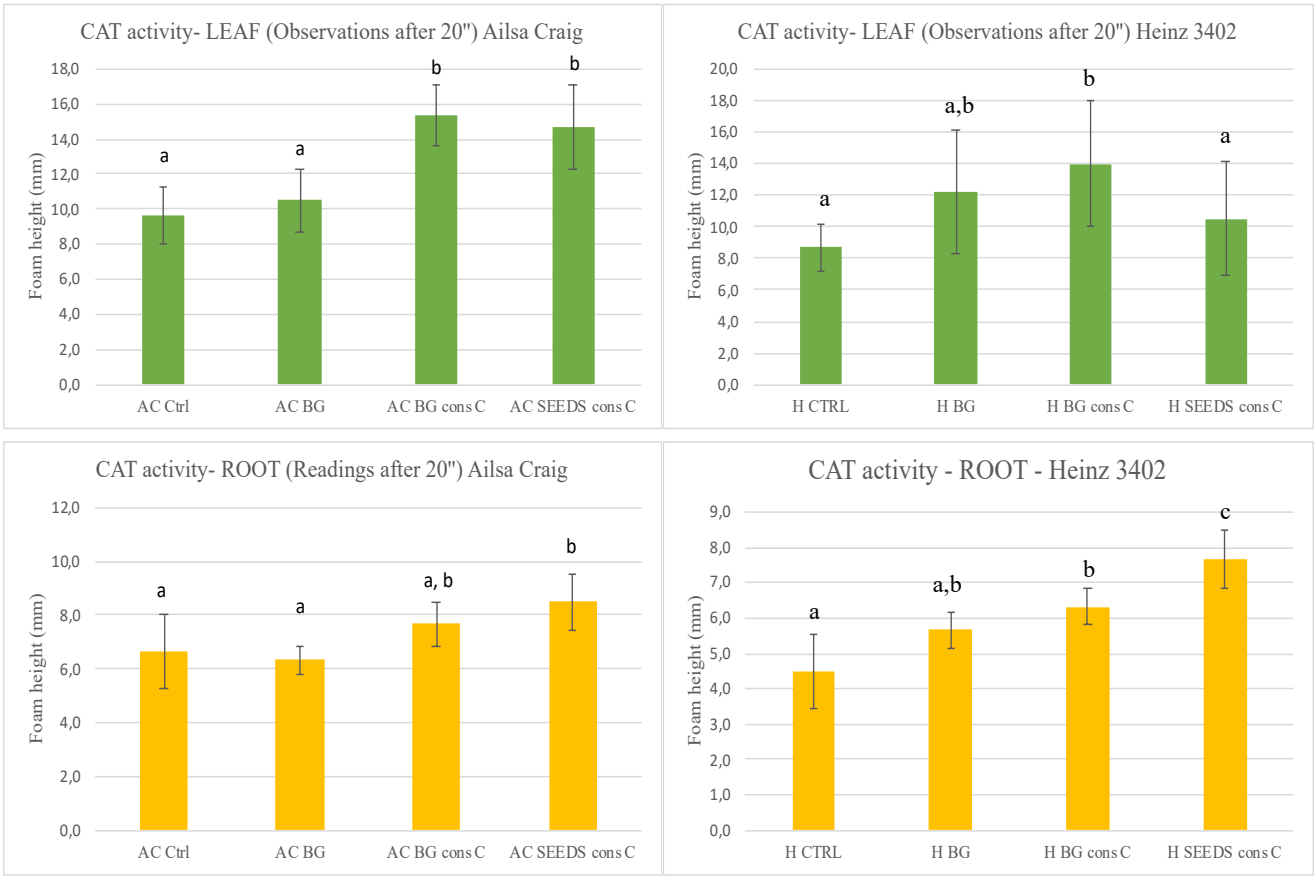


Figure 31. Catalase activity from foam height observations in *Ailsa Craig* leaf and root (up and bottom left respectively), and *Heinz 3402* leaf and root (up and bottom right). Data shown as mean ± standard deviation of triplicate. Different letters correspond to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$).

Significant differences have been highlighted in leaf samples of *Ailsa Craig* cultivar for treatments with functionalized biochar and functionalized seeds compared to the others. In *Heinz 3402* leaves slight increases were recorded in plants treated with biochar and functionalized seeds and a significant increase was recorded in plants treated with biochar in association with the microbial consortium. *Ailsa Craig* also showed increasing values in roots especially in plants treated with the microbial consortium both with biochar and seeds, but statistically significant difference was detected only in

seeds-functionalized plants. The enzyme activity was always increasing in all treated roots in *Heinz 3402* cultivar with valuable statistic differences in all plants treated with the microbial consortium.

Differences in the activity of the enzyme between the treatments and between the varieties of tomato could be observed. Therefore, differences in catalase activity have been investigated more in-depth with the spectrophotometric method (Fig. 32).

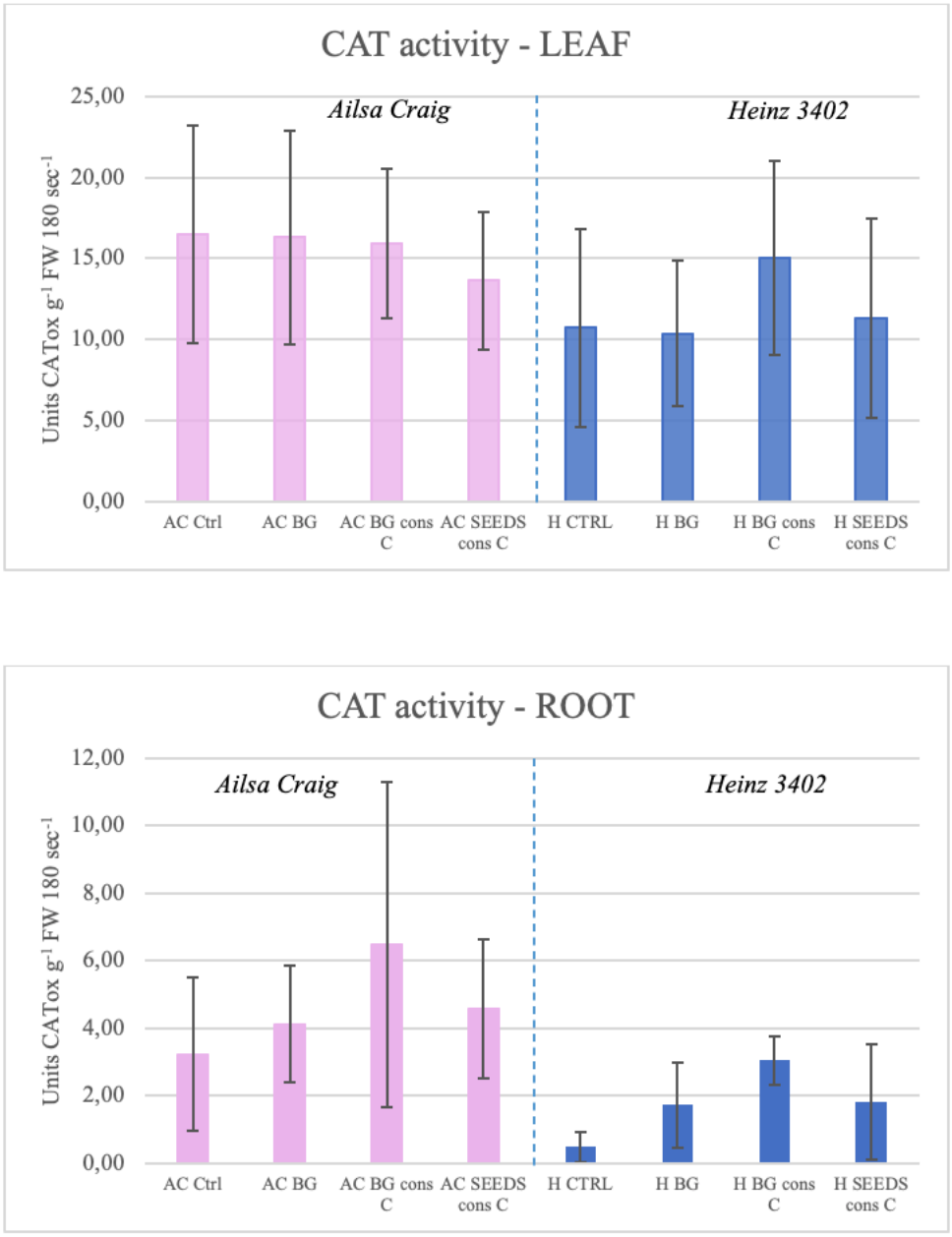


Figure 32. Catalase activity in *Ailsa Craig* (pink) and *Heinz 3402* (blue) of leaf (up) and root (bottom) samples through spectrophometric assay. Data shown as mean (n=6) ±SD. No significant differences ($p < 0,05$) have been highlighted.

As shown above, by spectrophotometric technique differences in catalase activity values have been confirmed especially in the *Heinz 3402* cultivar for plants treated with the microbial consortium even though it was not statistically significant. In *Ailsa Craig* instead the trend is confirmed only in the root samples showing an increase of activity in the plants functionalized. In *Ailsa Craig* leaf samples values remained relatively constant. The catalase activity has been calculated as Units of enzyme oxidized per gram of fresh weight per 3 min (Units CATox g^{-1} FW 180 sec^{-1}).

Even spectrophotometrically it was possible to see a positive variation of catalase activity both in leaf and root samples in each cultivar, no variation has been statistically significant (Fig. 32).

4.3.11 H_2O_2 estimation

With the only exception of *Ailsa Craig* leaf samples in which plants treated with functionalized seeds have shown a significant increase of hydrogen peroxide content, in all the other conditions the estimation of hydrogen peroxide was relatively constant. At the opposite, in the case of *Heinz 3402* cultivar, in leaf samples plants with functionalized seeds have shown a significant decrease while in root samples, a significant difference has been reported in *Ailsa Craig* cultivar in plants treated with biochar which showed a better response to the treatment and decreased the stress-related H_2O_2 content, and in *Heinz 3402* again, with a similar behavior observed in leaf samples of the same cultivar, plants with functionalized seeds and plants treated with biochar have shown a significant decrease in hydrogen peroxide content (Fig. 33, next page).

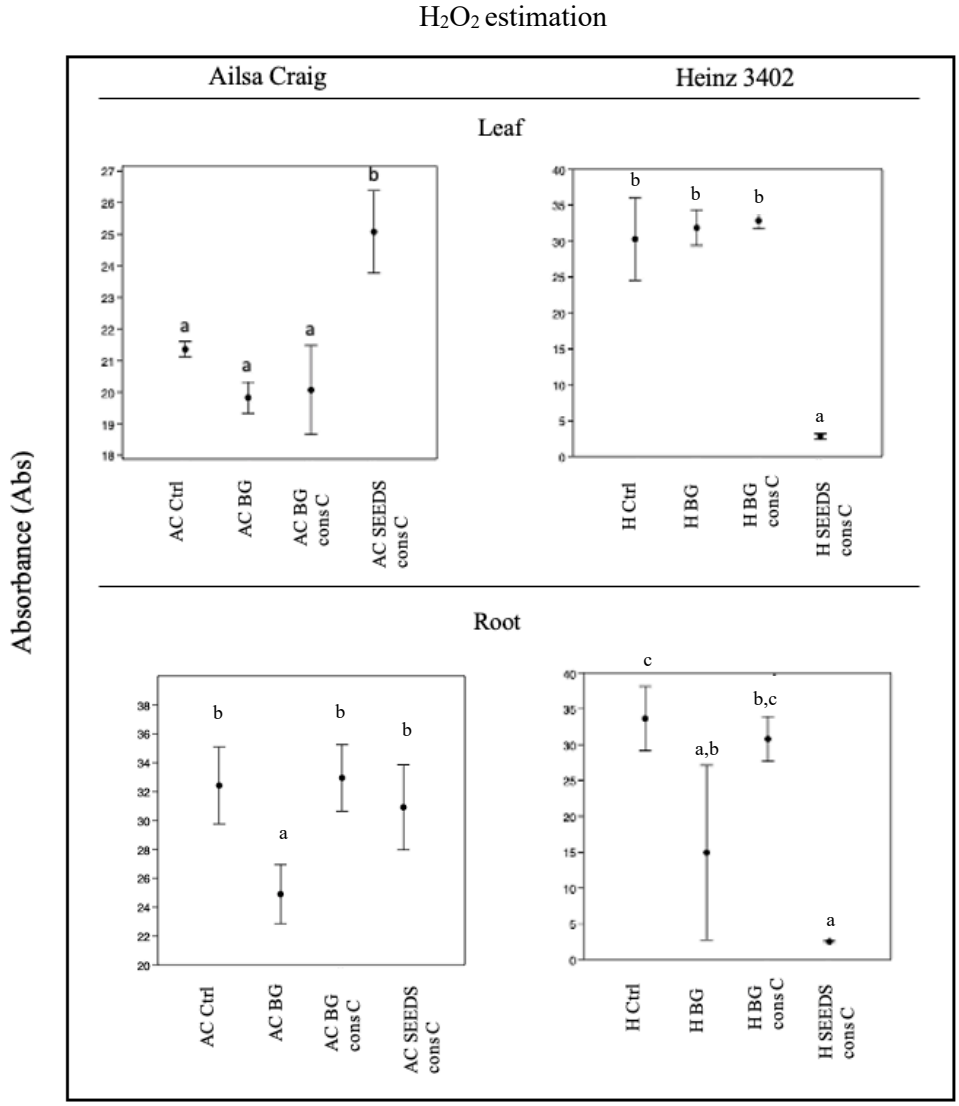


Figure 33. Estimation of hydrogen peroxide (H_2O_2) content in *Ailsa Craig* leaf (up left) and root (bottom left) and *Heinz 3402* leaf (up right) and root (bottom right). Data shown as mean (n=6) ±SD. Different letters correspond to statistically different values (one-way ANOVA, Tukey's test, $p<0.05$).

With the aim of combining the physiological and phytochemical effects observed in the plants of *Heinz 3402* and *Ailsa Craig* due to the experimental treatments, a Principal Component Analysis (PCA) was performed (Appendix: fig. 45, 46, 47, and 48). Then, in order to better investigate the effects of treatments on the well-being and functioning of the plant, multivariate statistical analysis was also performed by dividing the parameters observed in aboveground plant section and belowground plant section data. Data were illustrated by heatmaps (Fig 34 and 35).

Considering both physiological and phytochemical results obtained, it was noticed that there were differences in response to the treatments between the two tomato varieties considered. In detail, *Heinz 3402* showed to be better suited to treatments, especially treatment with the biochar, with and without microbial functionalization. However, although in a minor way, *Ailsa Craig* also showed satisfactory results. *Ailsa Craig* has shown to adapt better to the stimuli given by seeds treated with microbial consortium. Results of phytochemical analyses confirmed the trends in physiological effects.

Data were summarized in a heatmap (Fig. 34). All the experimental treatments yielded favorable results. Considering all data, the treatment that showed the most positive growth of the values and showed to influence better and more strongly both physiological and phytochemical parameters was that with biochar, in both tomato cultivars. Although the results were positive for both varieties considered, the effects were more evident in *Heinz 3402* compared with the ones collected in *Ailsa Craig* cv.

The treatment with the microbial consortium also showed positive trends in the growth of parameters compared with control. In *Ailsa Craig* cv, the microbial consortium had better effects when associated with seeds than with biochar association while it had better qualitative effects when associated with biochar than with *Heinz 3402* seeds association.

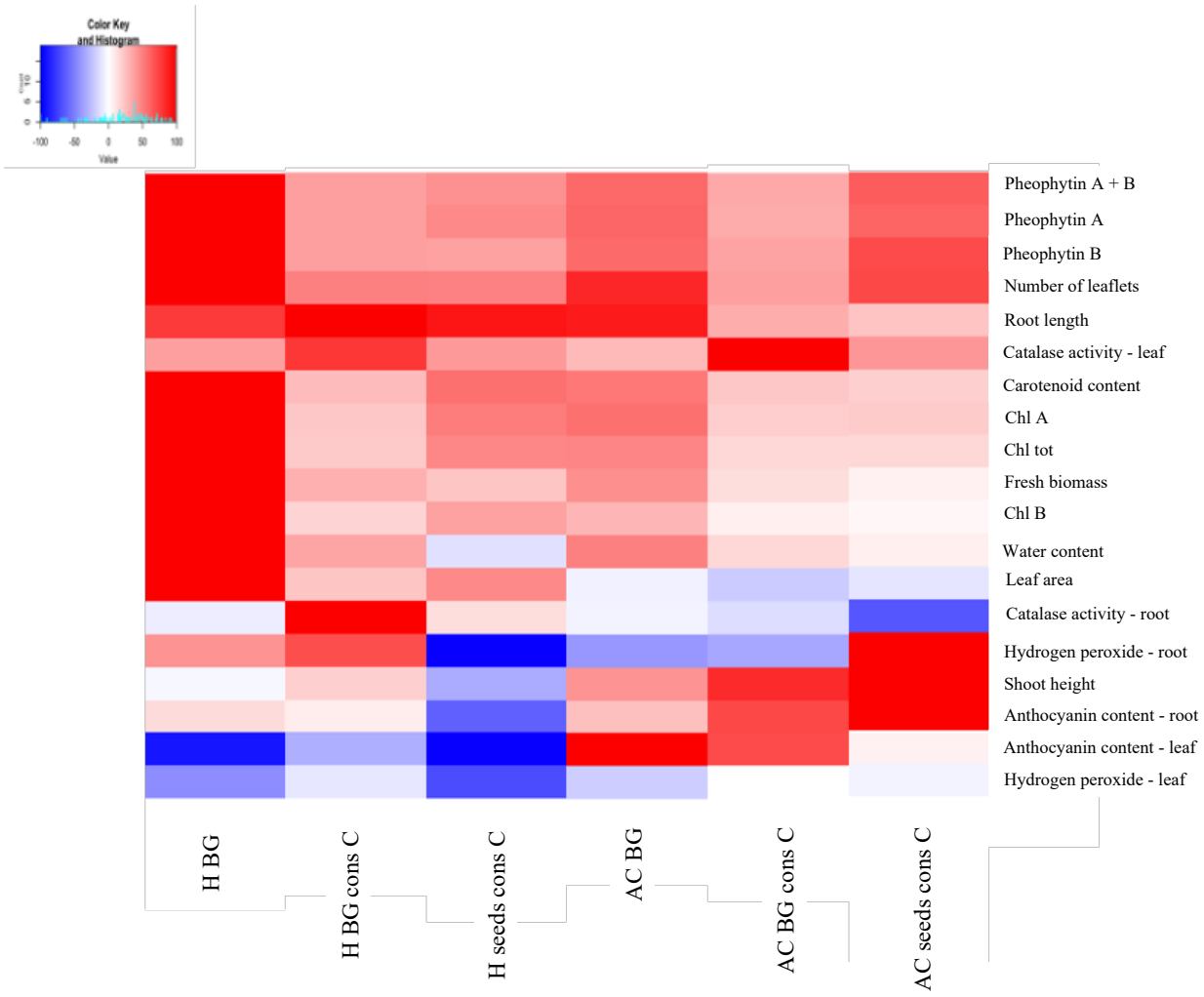


Figure 34. Heatmap of changes in physiological and phytochemical parameters in both *Heinz 3402* (H) and *Ailsa Craig* (AC) tomato cultivars. Red colors indicate higher concentrations, and blue colors indicate lower concentrations compared with the control.

Then, dividing the data into aboveground and belowground parts data, it was possible to observe that the positive growth trends in treatments were confirmed in both groups and for both varieties (Fig. 35) and that phytochemical values confirmed physiological data.

In aboveground parts, the growth trend was observed in all parameters analyzed except for the anthocyanin content in leaves that decreased in *Heinz 3402* experimental treatments compared to control. In all data set analyzed, the treatment with biochar showed to explain the almost the whole variability (about the 90%) (Appendix). All physiological and phytochemical data correlated positively with this treatment. In particular, by focusing on the evaluation of the *Ailsa Craig*

parameters, it was noted that despite the area of the leaves decreased with the treatments, it did not affect the content of chlorophyll, carotenoids, anthocyanins, and antioxidant activity. The only negative relationship was observed between the hydrogen peroxide content and all other values, as could be expected. In *Heinz 3402* cv, all phytonutrients data (chlorophyll, carotenoids, pheophytins, anthocyanins, antioxidant activity) correlated positively with leaf area, number of leaflets, fresh biomass, and water quantity and negatively with the hydrogen peroxide content (Fig. 35). Indeed, hydrogen peroxide, an indicator of oxidative stress, decreased in *Heinz 3402* and *Ailsa Craig* BG, and BG with consortium.

Even for the hypogeal part of the plant, the variability of the data was due to treatment with biochar for about the 99% and all parameters responded positively to treatment with biochar. In belowground parts data, the parameter most heavily influenced by all treatments was root length. Fresh biomass and water content were also increased by the experimental treatments. In *Ailsa Craig* cv, there were positive correlations between physiological and phytochemical parameters such as the relationship between root length and catalase activity and water content and anthocyanin content. In *Heinz 3402* cv, root length, water content, fresh biomass and antioxidant activity were positively correlated. In both tomato varieties observed, the hydrogen peroxide content was reduced compared to the control and it was negatively related to the rest of the physiological and phytochemical parameters investigated. This condition occurred in all treatments, demonstrating that they did not bring changes about the homeostasis of plants and did not stress them.

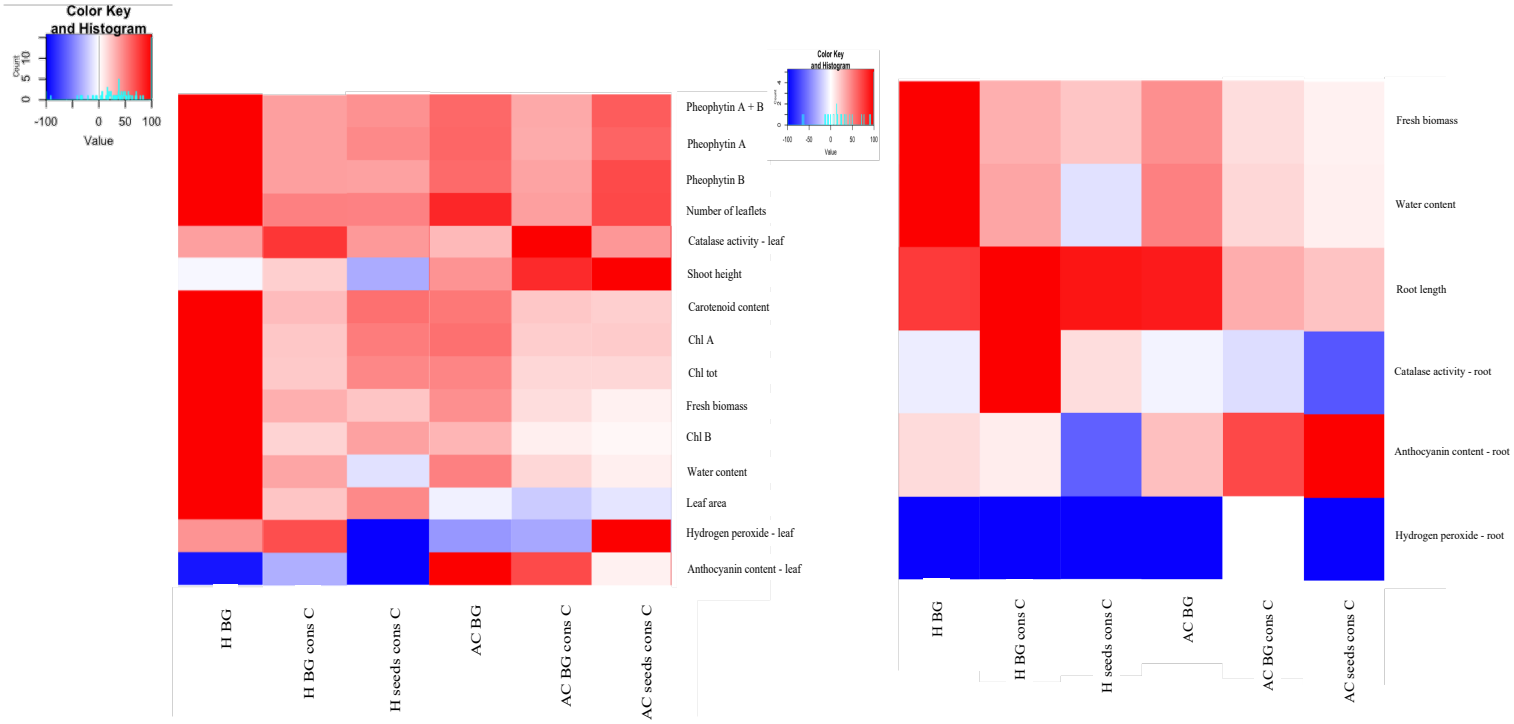


Figure 35. Heat maps of changes in physiological and phytochemical parameters due to experimental treatments. (Left) aboveground changes and (right) belowground changes.

Summarizing all physiological and phytochemical data, different effects could be observed between experimental treatments. However, each plant variety studied showed specific effects. In fact, considering *Ailsa Craig* the experimental treatment that provided the most significant positive effects was that with the biochar (+53%), followed by the functionalized seeds (+35%) and the functionalized biochar (+29%). Very few were the negative effects observed in treatments with functionalized biochar and seeds which were statistically significant (-6%), absent in treatment with biochar. In BG cons C negative effects referred to leaf area index while in SEEDS cons C significant negative effects referred to hydrogen peroxide estimation in leaf.

All the experimental treatments had more positive effects in *Heinz 3402* cv. where the most evident effects, both in physiological and phytochemical analyses, were given by the treatment with biochar (+82%), followed by both the treatment with functionalized biochar (+65%) and functionalized seeds (+65%). In *Heinz 3402* the significant negative effects were not recorded in the BG and BG cons C

treatments and only -6% for the SEEDS cons C treatment. This negative effect was related to the content of anthocyanins in the leaves.

Thus, treatments had more noticeable effects in *Heinz 3402* than in *Ailsa Craig* cultivar. However, in both the most effective and positive treatment was with BG. In both cultivars the negative effects were minimal.

CHAPTER 5 Soil microbial community profiling

5.1 Introduction

5.1.1 Soil

Soil is the surface layer covering the Earth's crust, consisting of a solid part (organic component and mineral or inorganic component), a liquid part and a gaseous part. During its evolution, the soil differentiates along its profile (the set of layers that form the profile) a series of horizons. Together with air, water and plants, it is both a natural and a vital source that is essential not only for the production of food for humans and other animals, but above all for the health of agroecosystems and the future of humanity (Karlen, Ditzler, and Andrews, 2003).

5.1.2 Rhizosphere

The rhizosphere is the portion of soil that is affected by the roots of plants, from which they absorb the essential nutrients and water they need for growing. In the rhizosphere, besides the roots, there are other biotic components such as symbiotic microorganisms, beneficial and pathogenic bacteria, micro and macroscopic fungi. The roots of the plants release in the soil various compounds and molecules secreted by the radical hairs that make this environment ideal for the life of many bacteria. These microorganisms are able to associate with the roots through a particular mutual relationship known as symbiosis. Microorganisms can develop both inside and outside the roots, creating a positive or negative influence of root growth. The radical surface, even if not colonized uniformly and completely, achieves a positive environment for the development of microbial niches intensely active and continuously supplied with nutrients, secreted by the roots of the plants such as exudates, that are low molecular weight compounds that passively escape from intact radical cells (e.g. sugars, amino acids, vitamins, etc.); secretions, compounds actively released by radical cells, plant

mucilages, and lysates: material released through the lysis of epidermal cells of the roots aged or flaked.

In the rhizosphere, the plants, through the release of a high number of molecules, can regulate not only the microbial community and establish symbiosis relationships, but also change the chemical-physical properties of the soil, inhibit the growth of competitive plant species. On the other hand, molecules produced by the organisms involved in the relationships that take place at the level of the roots influence, the activity, the growth, and the state of health of the plants.

5.1.3 Soil quality

Soil quality is a key parameter to be evaluated in order to assess the environmental behavior (Sharma *et al.,* 2010). It represents an increasingly used indicator as it provides information on the quality of the environment, food safety and the economic sustainability of the management of agricultural practices (Sharma *et al*., 2010). Besides the most common definition of soil quality as a fertile land serving for agricultural purposes and rising crop production (www.fao.org), there are many other definitions, and a single topic-related argument does not provide a complete description. The concept of soil fertility has been widely extended, including the importance of the biotic community functioning, of the climate and vegetation (Carter *et al*., 1997; Dumanski and Pieri, 2000), and of the evaluation of the ecosystem sustainability as the basis of direct or indirect impacts on animal, plant and human health (Bünemann *et al.,* 2018). Only recently, the importance of the soil biota and its biodiversity has been recognized. In particular, soil quality has been defined as "the capacity of a soil to function within ecosystem and land-use boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health" (Doran and Parkin, 2015; Doran and Parkin, 1996). Pedosphere is fulfilled of continuous interactions between soil, water, air and all their components. There are many indicators which establish the quality of soils and they are either qualitative or quantitative. Commonly, physical and chemical characteristics have always been

employed to analyze soil. Some of these soil threats are the salinization, the erosion, the compaction, the sealing, the organic matter cycling (carbon sequestration), the water cycling, the nutrient element cycling, the soil structure and decomposition. Some others focus on the soil biodiversity which connect abiotic soil properties to soil functions in term of biochemical and biophysical transformations and potential aboveground vegetation performance (Lehman *et al.*, 2015). The field of ecotoxicology is equipped to detect important changes in environmental health at multiple levels of biological complexity and organization, specifically the organism, population, community, or ecosystem (Moriarty, 1999; Newman and Clements, 2008).

Adverse agricultural practices have strong negative impacts on soil biodiversity and composition, due to decreased viability of microorganisms which cannot establish in deeply changed and potential contaminated biological niches (Nielsen *et al*., 2002). Macro- and micro-organisms are key indicators for soil quality as they can change soil physical and chemical features (Sharma *et al.,* 2010) and can provide quick responses to environmental changes due to many different natural pressure or human stress. The soil ecosystem parameters usually are the biodiversity and bioavailability, the biological activity, nutrient cycling (carbon and nitrogen), microbial biomass and quality. In the present study, an *in vaso* experiment was conducted with two varieties of tomato and several experimental treatments: biochar, biochar functionalized with microorganisms and direct microbial functionalization of tomato seeds. The soil of the different pots of this experiment which were exposed to different treatments was analyzed and the potential effects deriving from the experimental treatments have been investigated by observing the microbial biomass and their activity in soils. These analyses have been carried out performing the Community-Level Physiological Profiling (CLPP).

5.1.4 The Community-Level Physiological Profiling (CLPP)

The CLPP is a useful molecular technique for verifying the presence and adaptation of micro-organisms in a treated soil. It measures not only the spatial and temporal adaptation (Rutgers *et al*., 2016) of microorganisms and the relative abundance of each family and also it yields to information about adapted functions present in the soil (Weber and Legge, 2010).

The most common method to study CLPP is by utilizing the BIOLOG™ microplates *(Zhen Teng et al.,* 2020). The BIOLOG EcoPlate™ have been specifically designed (Garland and Mills, 1991) for applied environmental researches (Weber and Legge, 2010) and have been employed for this work. They improve our knowledge on microflora perturbations, microbiological activities, soil dynamics and impacts on soil due to any potential stress as enzymatic activities and metabolism in the soil are mainly of bacterial and fungal origin (Sharma *et al.*, 2010). Therefore, three main information could be taken from CLPP analysis: the rate of color development, the richness and evenness of the response among wells, and the pattern, or relative rate of utilization, among wells (Garland, 1997). EcoPlate™ have been used for a 7-days dynamic monitoring on the functional diversity of soil microbial community, and the Average Well Color Development (AWCD) values of carbon-source utilization and, consequently, the kinetic metabolic profiles were collected.

Each microplate contains 31 different carbon substrates and a blank well in triplicate. The different carbon substrates can be divided into six classes of nutrients: phenolic compounds, carbohydrates, amines, amino acids, carboxylic acids and polymers. Each substrate in each well is bonded to a freeze-dried dye that reconstitutes only in the presence of the solution with microorganisms. A (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium dye combines with an electron mediator (phenazine methosulfate, PMS) to yield a water-soluble purple product called formazan that can be measured spectrophotometrically. As microbes utilize the carbon source, through cell respiration mechanisms, they produce the reduced form of adenine dinucleotide

nicotinamide, NADH, generating an electron flow that reacts with tetrazolium salt, which acts as a final electron acceptor and is reduced to formazan, the colored compound visually observed (Weber and Legge, 2010). Simultaneously the NADH is re-oxidized to NAD+. As each sample was inoculated at known density and with the same volume, the rate at which the violet compound is formed is directly proportional to the metabolic flow and the intensity of the staining will be proportional to the use of the substrate and the relative abundance of the microorganisms. The tetrazolium reporter dye is reduced to form a visible purple color and the metabolic rate was reported. Communities of microorganisms will exhibit a characteristic reaction pattern, a metabolic fingerprint, that reflects the metabolic properties of the community. The color development is additive and directly proportional to the metabolism of each carbon source so the development of formazan can be followed over time. The intensity of purple color as a pattern in the wells corresponds to the metabolic footprint of the sample (Garland and Mills, 1991; Garland, 1997). Therefore, on each pure substrate, species capable of using the only source of carbon present will develop the color. The uncolored wells indicate the absence in the sample and, presumably, in the community of species capable of using that particular substrate. The use of metabolic profile analysis for characterization, on a functional basis, of soil microbial communities or other environmental matrices shall, however, be based on the assumption that the differences found in the footprint left by microbial cells on Biolog^{TM} plates in the form of differential oxidations of substrates, reflect real differences in the number or species present in the inoculum and in the matrix from which it was obtained and yield the fingerprinting of the potential functions of the microbial community (Garland and Mills, 1991). The color of the wells of each substrate is recorded and collected as the AWCD.

5.2 Materials and methods

5.2.1 The Average Well Color Development (AWCD)

The inoculum preparation required the passage in solution of a sufficiently representative number of cells, both qualitatively and quantitatively, of the populations actually present in the soil sample. The heterogeneity of the spatial distribution of microorganisms and the strong interactions between cells and organic and inorganic particles strongly conditioned the extractive yield. It was necessary to strike a balance between the need to use an extraction method strong enough to break the aggregates and release the microbial cells, but not so strong as to kill them or condition their viability. To obtain the representative inoculum of the microbial community, we proceeded to extract from the natural matrix the bacterial cells using the protocol given by the company (Biolog Inc., Hayward, CA).

To prepare for extraction, soil samples were roughly cleaned of macroscopic natural residues and subsequently sieved with a 1-cm mesh. In the 50 ml centrifuge tubes, 27 ml of 0,9% NaCl solution and 3 g of soil were added. Then, the samples were incubated for 30 minutes at room temperature at 250 rpm to detach the cells from the soil matrix, ensuring, in any case, their integrity. Once an emulsion has formed in the falcon, the samples were centrifuged at 2000 rpm for 3 minutes, to allow the soil to settle in the pellet, leaving the bacteria in suspension.

The supernatant formed was recovered and 100 μL of it directly inoculated in each well of a Biolog Ecoplate™ at know density. Absorbance reading of the plate at the spectrophotometer was done immediately (T_0) and every 24 hrs (0, 24, 48, 72, 96, 120, 144, 168 hours) for 7 days at 590 nm wavelength. Between readings, the plates were held in incubator at 30°C in such a way that microorganisms can grow on each substrate under the expected experimental conditions.

Optical density value for each well was subtracted by the density value of the control. The AWCD was calculated as the mean of the absorbance values for all the 93 response wells per reading time

(Guckert *et al*., 1996). Then, OD values were standardized by dividing them by the AWCD following the formula (Glimm *et al*., 1997) with minor modifications:

AWCD (j,t)= ΣOD(i,j,t)/93

Where OD(I,j,t) denoted the corrected OD for well i of plate j measured at time t.

Hence, the standardized value will be:

OD(I,j,t)= OD(i,j,t)/AWCD(j,t).

Time reading of 168 hrs has been chosen as a metric for this study and these results were used for assessing the microbial functional diversity and statistical analyses.

5.2.2 Statistical analysis

Data collected from the Ecoplate™ have been derived for AWCD values. An analysis of variance (ANOVA) using Past v.4.0 to explore the significant effect of the treatments on AWCD with a significance level of $p < 0,05$ has been carried out. Each Ecoplate™ contains sole-carbon sources in triplicate and each treatment has been analyzed in biological duplicate that enhance confidence of statistical analysis of the collected data. Moreover, multivariate analysis was performed by a PCA. To evaluate all plate data, 168h time point has been chosen as a metric in order to minimize any bias due to the different densities between samples as suggested by Garland (Garland, 1997). The total size of the data matrix composed of 31 columns (substrates) and 4 rows (treatments) and the complexity of all the interacting variables have been reduced by extracting an orthogonal set of new variables, called principal components. The new set designed by new linear subsets with maximum variance values concentrated on the first component (PC1) and values with lower variances on subsequent components. Then, a correlation matrix (Pearson's coefficient correlation) was calculated

among the principal components and the single variables (substrates) for each tomato cultivar. Each substrate has been reported as indicated from the Biolog Inc. industry.

The correlation coefficients of the variables with the main axes were also shown in the tables below (Tables 12 and 13). By observing the matrix of the components, it was possible to determine the weight of the different variables in the determination of the main components. By examining the components, various types of correlation can be distinguished:

- if $p>0$, the variables are directly correlated or positively correlated;
- if $p=0$, the variables are said to be incorrect;
- if $p<0$, the variables are said to be inversely related, or related negatively.

Therefore, in both cases of positive or negative correlations, it can be distinguished:

- if $p<0,1$ there is a weak correlation;
- if $p<0,2$ there is moderate correlation;
- if $p>0,2$ there is a strong correlation.

Then, from the correlation values and the variability of the main components it can be noticed how the consumption of the following substrates varied with the experimental treatments: β-Methyl-D-Glucoside (A2), D-Galactonic acid γ-Lactone (A3), L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Xylose (B2), D-Galacturonic acid (B3), L-Asparagine (B4), Tween 40 (C1), i-Erythritol (C2), 2-Hydroxy Benzoic acid (C3), L-Phenylalanine (C4), Tween 80 (D1), D-Mannitol (D2), 4-Hydroxy Benzoic acid (D3), L-Serine (D4), α-Cyclodextrin (E1), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), L-Threonine (E4), Glycogen (F1), D-Glucosaminic acid (F2), Itaconic acid (F3), Glycyl-L-Glutamic acid (F4), D-Cellobiose (G1), Glucose 1-Phosphate (G2), α-keto Butyric acid (G3), Phenylethylamine (G4), α-D- lactose (H1), D,L-α-Glycerol Phosphate (H2), D-Malic acid (H3), and Putrescine (H4).

5.3 Results and discussion

The profile of changes in the soil microbial community as a result of specific treatments with soil improvers was observed. Soil biodiversity changed as a response to adaptation to external stimuli (treatments). Soil near the roots in tested plants (see above) was collected and microbial communities analyzed.

5.3.1 Metabolic profiles of soil microbial communities: the AWCD index

The metabolic profiles obtained for the samples of the experimental plan were compared by analyzing the average staining of the wells, AWCD index, at different incubation times (0-168h). The following were, for each variety of tomato cultivar, the trends in metabolic profiles of the four conditions considered.

5.3.1.1 ***Ailsa Craig***

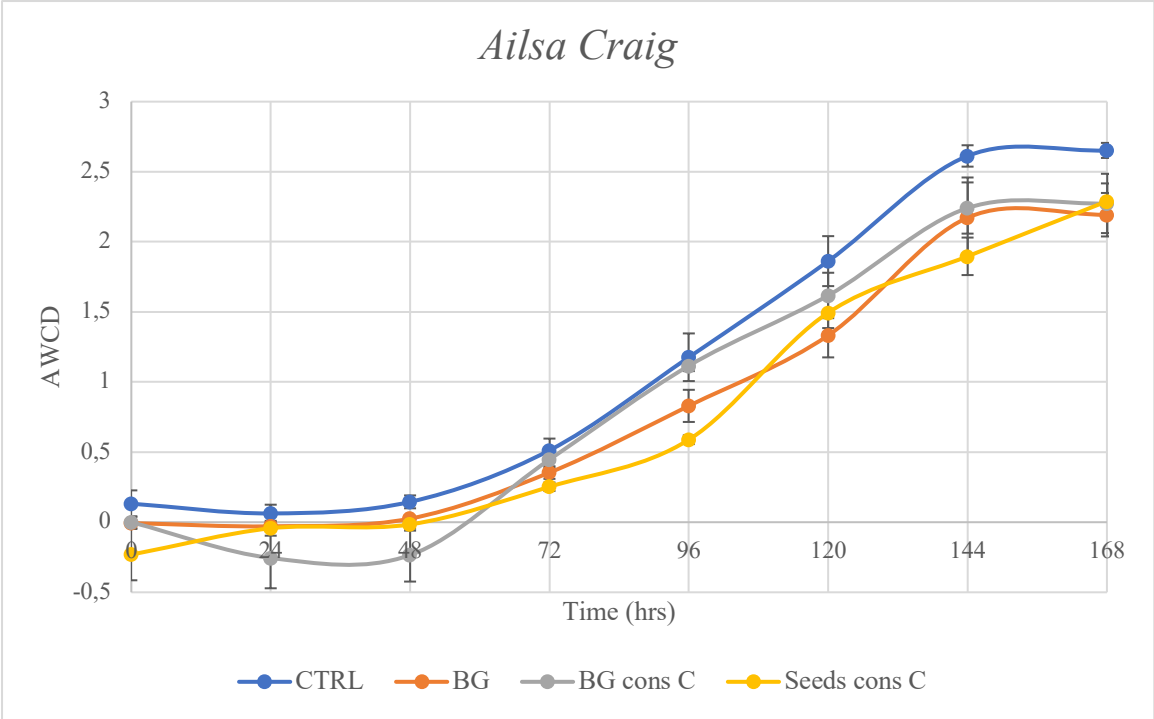


Figure 36. AWCD trend of *Ailsa Craig* treated soils at different time intervals (0, 24, 48, 72, 96, 120, 144, 168 hrs). Data shown as mean ± standard error of duplicate of sum of AWCD (n=93).

In all experimental conditions the same metabolic trend could be observed (Fig. 36). All conditions had a lower AWCD index when compared with the control, and microbial communities tended to stabilize at a time interval between 144h and 168h. Constant growth at all time intervals in the case

of the microbial soil community treated with functionalized seeds was observed. In soil treated with functionalized biochar, it seemed that microorganisms have greater access to nutrients and consume more substrate in the medium-time phases with semi exponential growth.

5.3.1.2 ***Heinz 3402***

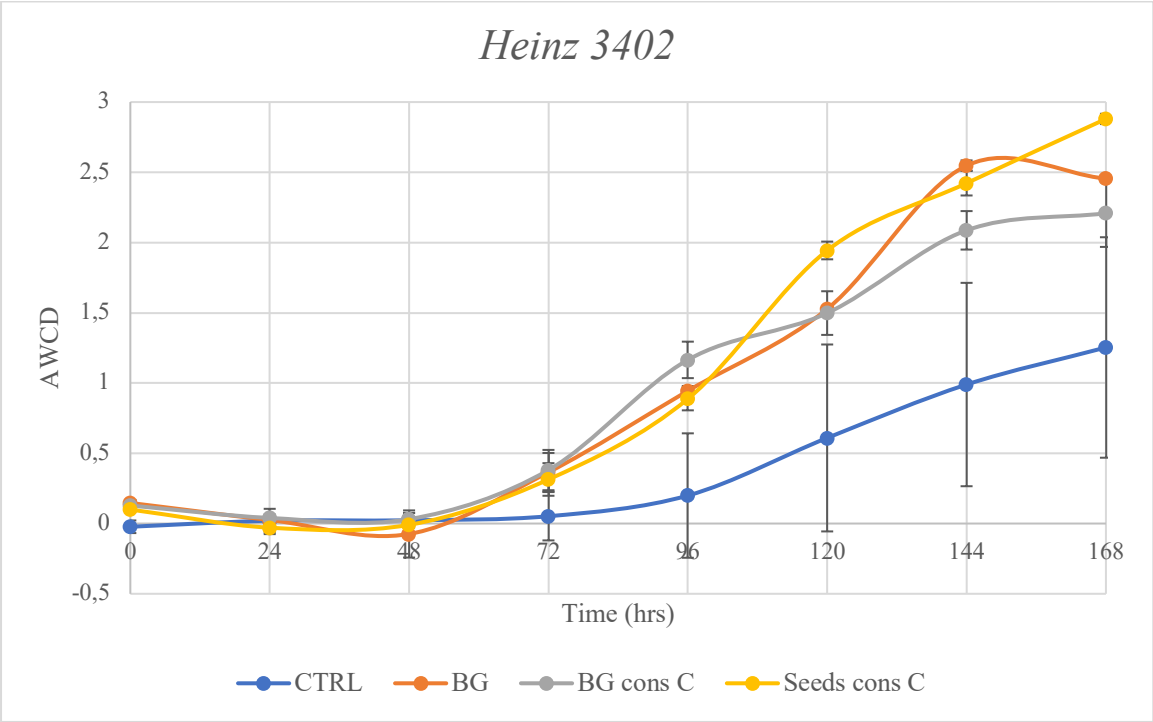


Figure 37. AWCD trend of *Heinz 3402* treated soils at different time intervals (0, 24, 48, 72, 96, 120, 144, 168 hrs). Data shown as mean ± standard error of duplicate of sum of AWCD (n=93).

In the case of the *Heinz 3402* tomato cultivar, it was noticed that all soil microbial communities in the experimental treatments had adapted better to the habitat (Fig. 37). In fact, the AWCD index was much higher than that of the control. In the control, microorganisms appeared to start consuming nutrients only in the median stages and then follow a slow positive growth. In both soil habitats in treatments with the biochar and biochar functionalized, the microbial soil community gradually stabilized and settles between 144 h and 168h. In the case of soil microorganisms in treatments with functionalized seeds, the positive growth trend seemed to be semi-exponential from 48h of growth. Below, however, are represented all the average coloring indices of all treatments and in both tomato cultivars (Fig. 38). The difference in metabolic growth between all conditions and control in the *Heinz*

3402 soil was still evident. In the cultivar *Ailsa Craig* there was no significant difference between control and treatments.

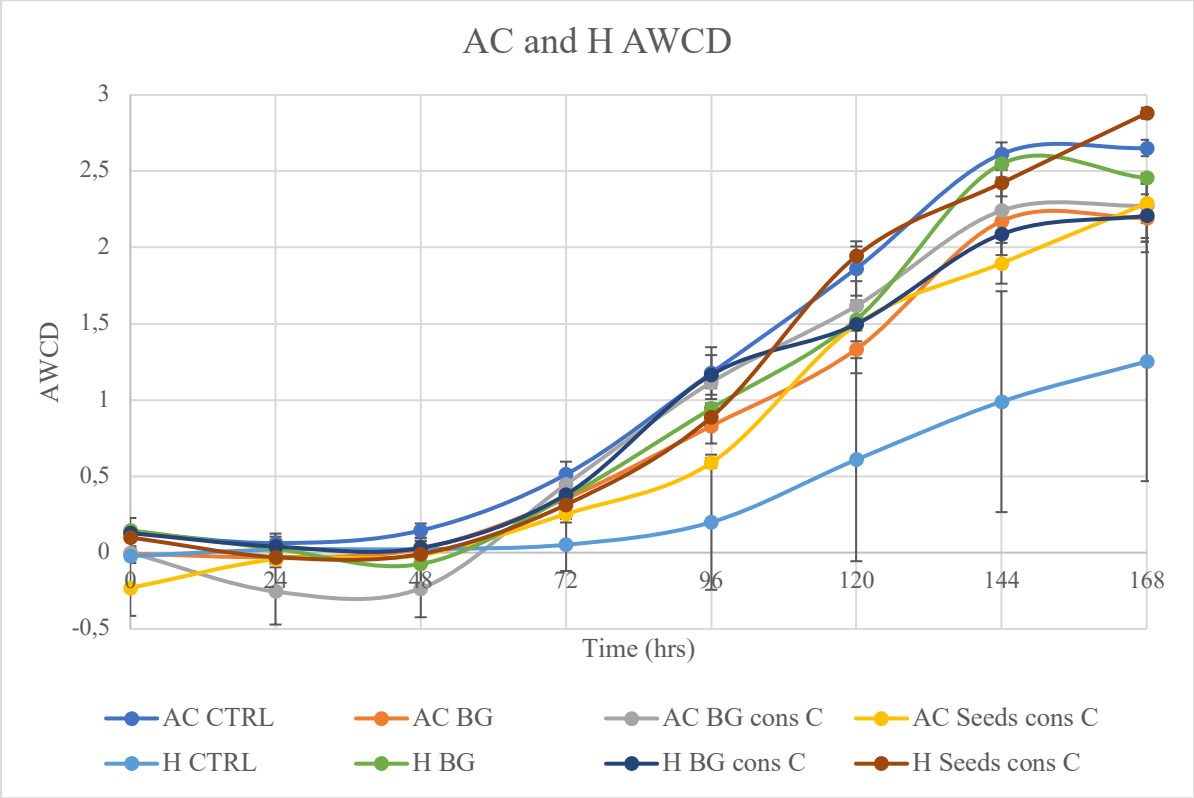


Figure 38. Averaged AWCD in both *Ailsa Craig* and *Heinz 3402* at different time intervals (0, 24, 48, 72, 96, 120, 144, 168 hrs). Data shown as mean ± standard error of duplicate of sum of AWCD (n=93).

Statistical analysis was carried out by performing the univariate statistic of AWCD index by the means of one-way ANOVA. Multivariate analysis was performed by the analysis of principal components (PCA). The principal component analysis revealed mean differences in microbial community modulations due to the experimental treatments (Fig. 39 and 40).

5.3.2 Principal Component Analysis (PCA)

5.3.2.1 ***Ailsa Craig***

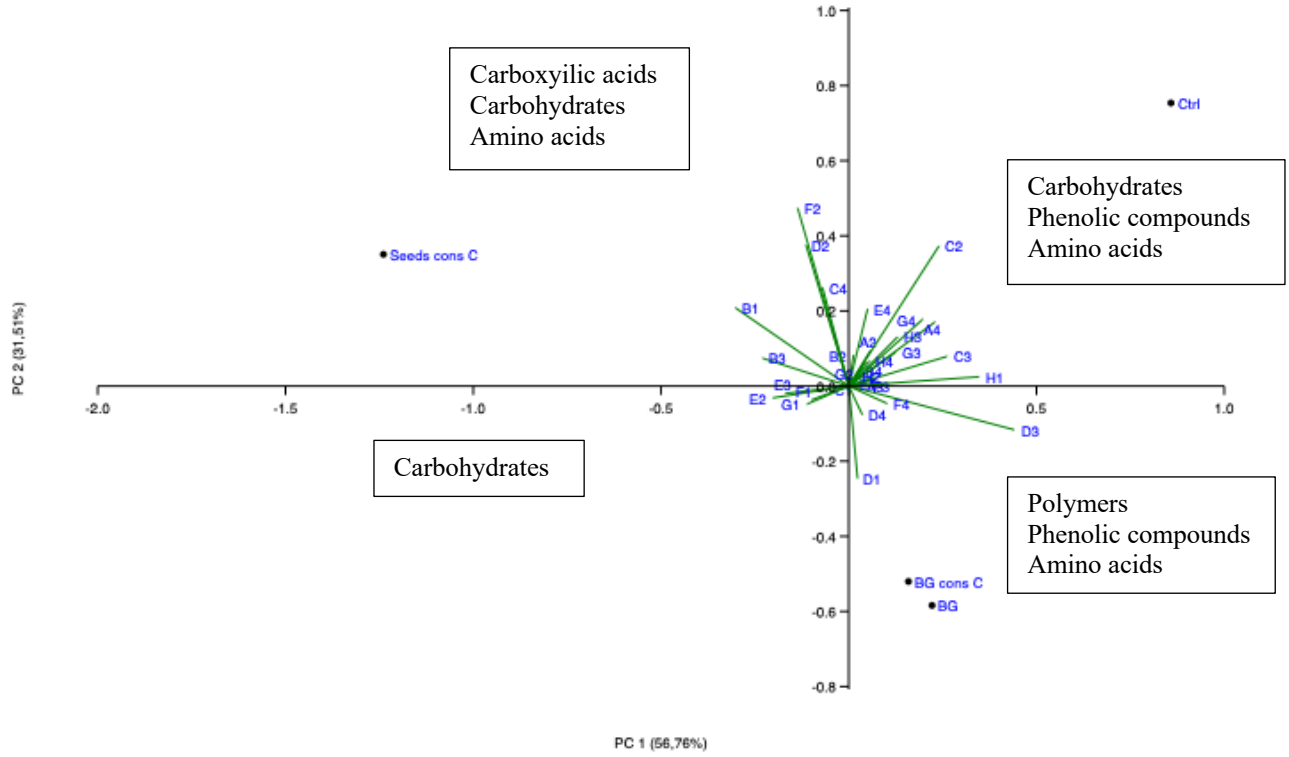


Figure 39. PCA of *Ailsa Craig* AWCD values. Carbon sources were: β-Methyl-D-Glucoside (A2), D-Galactonic acid γ-Lactone (A3), L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Xylose (B2), D-Galacturonic acid (B3), L-Asparagine (B4), Tween 40 (C1), i-Erythritol (C2), 2-Hydroxy Benzoic acid (C3), L-Phenylalanine (C4), Tween 80 (D1), D-Mannitol (D2), 4-Hydroxy Benzoic acid (D3), L-Serine (D4), α-Cyclodextrin (E1), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), L-Threonine (E4), Glycogen (F1), D-Glucosaminic acid (F2), Itaconic acid (F3), Glycyl-L-Glutamic acid (F4), D-Cellobiose (G1), Glucose 1-Phosphate (G2), α-keto Butyric acid (G3), Phenylethylamine (G4), α-D- lactose (H1), D,L-α-Glycerol Phosphate (H2), D-Malic acid (H3), and Putrescine (H4).

The whole variability of data from soil community analysis of *Ailsa Craig*'s tomato cultivar was explained by three principal components. The first two of them explained about the 88% of the total variability. The property that influenced the most the variance is shown by the PC1 (56,76%) and was the treatment with biochar and functionalized biochar. As shown in the graph above (Fig. 39), the soil microbial community changed over the different treatments. In particular, it can be observed how the metabolic activities change due to the microbial stabilization in the different treated soils. While microorganisms in control soil preferred to utilize carbohydrates as nutrients, followed by

phenolic compounds and finally amino acids, the microorganisms present in treated soil with biochar and functionalized biochar changed their nutrition preferring polymers such as glycogen and cyclodextrin first, then phenolic compounds and amino acids. The microbial metabolic activities were negatively correlated to the consumption of carbohydrates.

Table 11. Number of strong and moderate correlation values of different groups of carbon sources to each principal component.

	Ailsa Craig		
	PC 1	**PC 2**	**PC 3**
Polymers	**1**	**1**	**1**
Phenolic compounds	**2**	**1**	**2**
Carbohydrates	**5**	**3**	**4**
Amino acids	**2**	**3**	**5**
Carboxylic acids	**6**	**3**	**5**
Amines	**1**	**1**	**0**

Sum of positive (p>0,1) and negative (p<-0,1) strong and moderate correlations between carbon sources (collected in main chemical groups: polymers, phenolic compounds, carbohydrates, amino acids, carboxylic acids, amines) and principal components based on Table 12.

In fact, although from the resume of the influences of each nutrient group on the three principal components (Table 11) it emerged that carboxylic acids, carbohydrates, polymers, and phenolic compounds had a greater influence on the principal component 1 (PC 1), while carbohydrates, amino acids and carboxylic acids had a greater influence on the principal component 2 (PC 2), correlation might show a different effect for each component (Table 12). Analyzing the correlation values for each principal component (Table 12), it can be noticed that the substrates L-arginine (A4), i-Erythritol (C2), 2-Hydroxy Benzoic acid (C3), 4-Hydroxy Benzoic acid (D3), Glycyl-L-Glutamic acid (F4), α-keto Butyric acid (G3), Phenylethylamine (G4), α-D- lactose (H1), and D-Malic acid (H3) had strong and moderate positive correlation with the first principal component; the substrates Pyruvic acid Methyl Ester (B1), D-Galacturonic acid (B3), D-Mannitol (D2), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), Glycogen (F1), D-Glucosaminic acid (F2), and D-Cellobiose (G1) had strong and moderate but negative correlation with the first principal component.

The substrates β-Methyl-D-Glucoside (A2), L-arginine (A4), Pyruvic acid Methyl Ester (B1), i-Erythritol (C2), D-Mannitol (D2), L-Threonine (E4), D-Glucosaminic acid (F2), Phenylethylamine

(G4), D-Malic acid (H3) had strong-moderate and positive correlation with the second principal component while Tween 80 (D1) and 4-Hydroxy Benzoic acid (D3) negative correlation with PC 2.

Table 12. Correlation values of carbon with each principal component.

	PC 1	PC 2	PC 3
A2	0.062975	**0.10291**	-0.02179
A3	0.078364	0.018334	**0.12566**
A4	**0.2461**	**0.18258**	**0.15353**
B1	**-0.32262**	**0.2226**	-0.069579
B2	0.013499	0.088423	**0.41099**
B3	**-0.24562**	0.079353	**-0.22282**
B4	0.047339	0.06856	-0.041917
C1	0.031762	-0.0077118	**-0.11169**
C2	**0.25698**	**0.39865**	**0.1682**
C3	**0.28006**	0.084381	**0.4237**
C4	-0.074491	**0.2803**	**-0.17169**
D1	0.02521	**-0.2631**	0.056262
D2	**-0.12197**	**0.40182**	0.084823
D3	**0.47104**	**-0.12453**	**-0.24402**
D4	0.038789	-0.082029	**0.10455**
E1	0.029588	0.031021	0.015302
E2	**-0.21634**	-0.033779	**0.15919**
E3	**-0.1807**	-0.020823	**0.4299**
E4	0.053714	**0.21952**	**0.15919**
F1	**-0.1059**	-0.039338	0.04442
F2	**-0.14548**	**0.50661**	**-0.26526**
F3	0.069575	0.025217	-0.041928
F4	**0.10976**	-0.049912	**-0.15329**
G1	**-0.11904**	-0.051549	0.071485
G2	0.033123	0.034148	-0.012975
G3	**0.13425**	0.096897	-0.025662
G4	**0.21106**	**0.1902**	0.075217
H1	**0.37126**	0.026302	**-0.19948**
H2	0.038534	0.052999	-0.028593
H3	**0.13935**	**0.14018**	**-0.13575**
H4	0.059239	0.072057	0.021148

Carbon sources were: β-Methyl-D-Glucoside (A2), D-Galactonic acid γ-Lactone (A3), L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Xylose (B2), D-Galacturonic acid (B3), L-Asparagine (B4), Tween 40 (C1), i-Erythritol (C2), 2-Hydroxy Benzoic acid (C3), L-Phenylalanine (C4), Tween 80 (D1), D-Mannitol (D2), 4-Hydroxy Benzoic acid (D3), L-Serine (D4), α-Cyclodextrin (E1), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), L-Threonine (E4), Glycogen (F1), D-Glucosaminic acid (F2), Itaconic acid (F3), Glycyl-L-Glutamic acid (F4), D-Cellobiose (G1), Glucose 1-Phosphate (G2), α-keto Butyric acid (G3), Phenylethylamine (G4), α-D- lactose (H1), D,L-α-Glycerol Phosphate (H2), D-Malic acid (H3), and Putrescine (H4). Bold caracters: strong and moderate correlation values.

5.3.2.2 ***Heinz 3402***

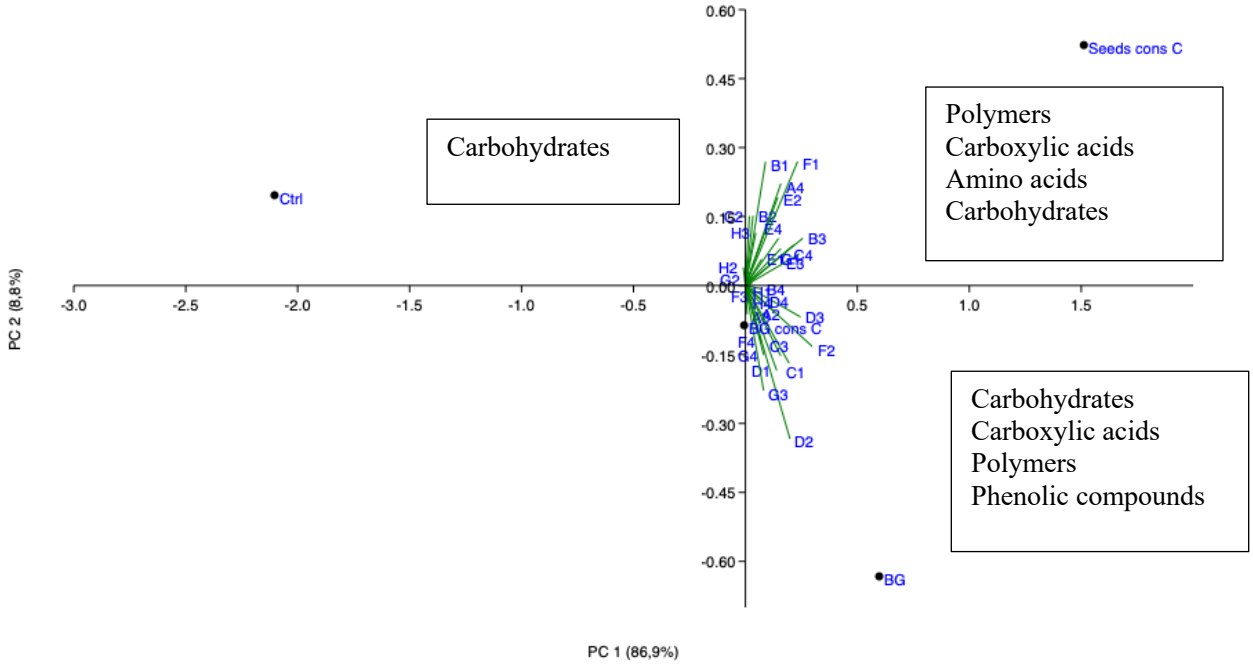


Figure 40. PCA of *Heinz 3402* AWCD values. Carbon sources were: β-Methyl-D-Glucoside (A2), D-Galactonic acid γ-Lactone (A3), L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Xylose (B2), D-Galacturonic acid (B3), L-Asparagine (B4), Tween 40 (C1), i-Erythritol (C2), 2-Hydroxy Benzoic acid (C3), L-Phenylalanine (C4), Tween 80 (D1), D-Mannitol (D2), 4-Hydroxy Benzoic acid (D3), L-Serine (D4), α-Cyclodextrin (E1), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), L-Threonine (E4), Glycogen (F1), D-Glucosaminic acid (F2), Itaconic acid (F3), Glycyl-L-Glutamic acid (F4), D-Cellobiose (G1), Glucose 1-Phosphate (G2), α-keto Butyric acid (G3), Phenylethylamine (G4), α-D- lactose (H1), D,L-α-Glycerol Phosphate (H2), D-Malic acid (H3), and Putrescine (H4).

The total variance of data deriving from the soil microbial community analysis was explained by three principal components. The first two of them explain about 95% of the total variability (Fig. 40). As occurred in *Ailsa Craig* tomato cultivar, even in this case the major part of the variance was due to the treatments with biochar and functionalized biochar. The control condition was very different from the experimental condition of treatment with functionalized seeds as well. The microorganisms in treated soil with biochar and functionalized biochar based their nutrition preferentially on carbohydrates, carboxylic acids, polymers and phenolic compounds. The ones in soil with functionalized seeds preferred polymers, carboxylic acids, amino acids and finally carbohydrates as carbon sources.

The different substrates influenced differently the variability and each principal component as the following:

Table 13. Number of strong and moderate correlation values of different groups of carbon sources to each principal component.

	Heinz 3402		
	PC 1	**PC 2**	**PC 3**
Polymers	**3**	**3**	**3**
Phenolic compounds	**2**	**1**	**2**
Carbohydrates	**3**	**4**	**5**
Amino acids	**4**	**4**	**5**
Carboxylic acids	**5**	**5**	**3**
Amines	**1**	**1**	**0**

Sum of positive (p>0,1) and negative (p<-0,1) strong and moderate correlations between carbon sources (collected in main chemical groups: polymers, phenolic compounds, carbohydrates, amino acids, carboxylic acids, amines) and principal components based on Table 14.

Resuming the correlation values considered strong or moderate (Table 13), it can be noticed that carboxylic acids, amino acids, carbohydrates, and polymers influenced the most the variability of the principal component 1 (PC 1), while carboxylic acid, carbohydrates, and amino acids influenced the most the principal component 2 (PC 2), but these correlations can be both positive and negative as showed later in Table 14. Reviewing the correlation values of each principal component, the substrates that had produced the most changes on the different principal components can be affirmed (Table 14). The L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Galacturonic acid (B3), Tween 40 (C1), 2-Hydroxy Benzoic acid (C3), L-Phenylalanine (C4), Tween 80 (D1), D-Mannitol (D2), 4-Hydroxy Benzoic acid (D3), L-Serine (D4), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), L-Threonine (E4), Glycogen (F1), D-Glucosaminic acid (F2), D-Cellobiose (G1), α-keto Butyric acid (G3), Phenylethylamine (G4) were all positively correlated with the first principal component. The L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Xylose (B2), D-Galacturonic acid (B3), i-Erythritol (C2), L-Phenylalanine (C4), N-Acetyl-D-Glucosamine (E2), L-Threonine (E4), Glycogen (F1), and D-Malic acid (H3) were positively correlated with the second principal component while Tween 40 (C1), 2-Hydroxy Benzoic acid (C3), Tween 80 (D1), D-Mannitol (D2),

D-Glucosaminic acid (F2), Glycyl-L-Glutamic acid (F4), α-keto Butyric acid (G3) and Phenylethylamine (G4) were negatively correlated with it.

Table 14. Correlation values of carbon sources with each principal component.

	PC 1	PC 2	PC 3
A2	0.089256	-0.051779	0.025204
A3	0.0092523	-0.07751	0.058856
A4	**0.19569**	**0.27401**	**0.21495**
B1	**0.11114**	**0.33291**	**-0.29521**
B2	0.041871	**0.18777**	**0.26512**
B3	**0.31807**	**0.12746**	**0.14984**
B4	0.093645	-0.0096566	-0.048488
C1	**0.24128**	**-0.20915**	**0.21454**
C2	0.021909	**0.18684**	**-0.12343**
C3	**0.19443**	**-0.18898**	**0.19556**
C4	**0.26668**	**0.10899**	**-0.18624**
D1	**0.17317**	**-0.22868**	**-0.2037**
D2	**0.2462**	**-0.41241**	**-0.45349**
D3	**0.30476**	-0.084699	**0.10601**
D4	**0.15569**	-0.067431	**0.3299**
E1	0.089364	0.071137	-0.016416
E2	**0.17852**	**0.2371**	**0.16785**
E3	**0.29747**	0.08438	**0.11601**
E4	**0.18305**	**0.12685**	**-0.24413**
F1	**0.28853**	**0.33363**	**-0.21652**
F2	**0.36956**	**-0.16415**	0.082142
F3	0.010199	-0.0045398	0.067421
F4	0.085871	**-0.15058**	**-0.21173**
G1	**0.19553**	0.099118	**-0.13076**
G2	-0.0013179	0.015874	0.089545
G3	**0.10042**	**-0.28268**	0.073627
G4	**0.10122**	**-0.18736**	0.083629
H1	0.039757	0.0075622	0.097107
H2	-0.011949	0.048699	-0.064905
H3	0.05737	**0.14183**	-0.094552
H4	0.039288	-0.019761	0.037634

Carbon sources were: β-Methyl-D-Glucoside (A2), D-Galactonic acid γ-Lactone (A3), L-arginine (A4), Pyruvic acid Methyl Ester (B1), D-Xylose (B2), D-Galacturonic acid (B3), L-Asparagine (B4), Tween 40 (C1), i-Erythritol (C2), 2-Hydroxy Benzoic acid (C3), L-Phenylalanine (C4), Tween 80 (D1), D-Mannitol (D2), 4-Hydroxy Benzoic acid (D3), L-Serine (D4), α-Cyclodextrin (E1), N-Acetyl-D-Glucosamine (E2), γ-Amino Butyric acid (E3), L-Threonine (E4), Glycogen (F1), D-Glucosaminic acid (F2), Itaconic acid (F3), Glycyl-L-Glutamic acid (F4), D-Cellobiose (G1), Glucose 1-Phosphate (G2), α-keto Butyric acid (G3), Phenylethylamine (G4), α-D- lactose (H1), D,L-α-Glycerol Phosphate (H2), D-Malic acid (H3), and Putrescine (H4). Bold caracters: strong and moderate correlation values.

Considering all the results of microbial community modulations, it was evident that the experimental treatments did not bring stress to the microbiome of treated soil. All samples showed satisfactory microbial growth and line-up with control growth. In *Ailsa Craig*, microbial communities developed in the early stages of growth showed a higher capacity for adaptation and faster colonization of soil habitat. From PCA analysis, it was evident that microbial communities stabilized in soils treated with biochar and biochar associated with microorganisms were stimulated by the consumption of phenolic compounds, polymers and amino acids and inhibited by the presence of carbohydrates. The microbiome present in soils treated with functionalized seeds preferred the use of carboxylic acids, carbohydrates, and amino acids.

In *Heinz 3402*, the development of the microbial community was faster and higher than that of the control. All treatments showed much higher growth than control, showing that microorganisms were present in larger amounts in experimentally treated soils. Between the treatments they had the same speed of adaptation to the stimuli. The PCA indicated that most variability was imputed to treatments. Microbial communities in soils treated with biochar and functionalized biochar preferred nutrient sources such as carboxylic acids, polymers, and phenolic compounds. Those of the soils treated with functionalized seeds preferred amino acids, polymers, and carboxylic acids. The microbiome in control soil grew well with the presence of carbohydrates. Such analyses certainly made an essential contribution to the understanding of the mechanisms of soil adaptations of the microbiome to experimental treatments. However, they need further study to better outline the modulation of microbial communities.

CHAPTER 6 Metabolomic analysis of two varieties of *Solanum lycopersicum* L. treated with biochar and PGPM

6.1 Introduction

In order to assess food quality, plant health, safety and the sustainability of new types of amendments, it is essential to understand whether modulations might result in plants of great agronomic interest treated with biochar and biochar engineered with a microbial consortium. Exposure to treatments underlies metabolic reprogramming, including responses to various external pressures which ranges from genetic polymorphism, gene regulation, epigenetic control, post-transcriptional regulation mechanisms to physiological mechanisms, and their observation can help the identification of gene functions. Therefore, a metabolomic approach is useful to understand changes in organisms that want to adapt to external stimuli. Its importance lies in the flow of biochemical functions and in all the cellular networks modifications and molecular variations (Majumdar and Keller, 2020; Zhang *et al*., 2018).

In the last years, the role of PGPM dwelling rhizosphere in shaping plant performance has been recognized (Chen *et al.*, 2020). The ecotoxicological mechanisms involved in biochar and PGPM treatments were investigated observing metabolisms pathways of amino acids (AA), fatty acids (FA), carbohydrates such as sugars and sugar alcohols (SA), organic acids (OA), nucleabases, -tides, and -sides (NAM) and antioxidants that result as a direct response of biochemical activities to abiotic stress (Kong *et al*. 2019). Metabolites are part of the basic molecular structure of the plant cells and are directly involved in the fundamental mechanisms for the life of an organism or in particular phases of its growth. Metabolism may be profoundly damaged by environmental stresses that require a "molecular" adjustment involving in particular the biosynthetic mechanism of carbohydrates, amino acids and amines, signalling mechanisms and physiological regulation of the plant. Metabolites were investigated by using liquid chromatography coupled to triple

quadrupole mass spectrometry (LC-MS/MS) capable to provide high-sensitive qualitative fingerprint and quantification at trace levels. A complete data set of the abundance and variance of secondary metabolites has been assembled. Metabolite profiling was provided from data collected from leaf, root, and stem samples of plants harvested at 50 days after sowing.

6.2 Materials and methods

6.2.1 Sample preparation

Root, leaf, and stems tissues were chosen for metabolic analysis. They were analyzed through quadrupole LC-MS/MS and samples were prepared as the following. At 50 days after sowing, roots, leaves, and stems were harvested and freeze-dried. Samples were then ground and homogenized into a fine powder and stored at -80°C until analysis was performed. For metabolites extraction, 100 mg of tissues were weighted into 1,5 ml microcentrifuge tubes, and 1 ml of 80% aqueous methanol (MeOH) with 2% formic acid was added. The tubes were vortexed at 3000 rpm for 20 min, sonicated for 20 min, and centrifuged at 20000 X g for 20 min. Then, 150 µl surnatant were transferred to LC vials and a specific ISTD for each class of metabolite but antioxidant was added. The mixture in vials for OA and NAM was diluted to 50% MeOH. Vials for AA and SA investigation were diluted to 80% aqueous acetonitrile (ACN) while those for FA analysis in an ACN:IPA:H_2O (65:30:5) solvent. Samples for AA, SA, and FA analysis were dried and then reconstituted before the LC-MS/MS analysis.

6.2.2 Liquid chromatography coupled to mass spectrometer (LC-MS/MS)

An Agilent 1260 Infinity UHPLC coupled to an Agilent 6470 triple quadrupole mass spectrometer was used to perform the metabolic pattern analyses. Analysis was provided in ESI method. An Agilent InfinityLab Poroshell 120 HILIC-Z (2.1 × 100 mm, 2.7 µm) column was used for chromatographic separation of amino acids. Stock aqueous solution was prepared in water with 200 mM ammonium formate and adjusted to pH 3 with formic acid. Mobile phase A (aqueous) was prepared by diluting the stock solution 9:1 in water, and mobile phase B (organic) was prepared by diluting the stock solution 9:1 in acetonitrile (final ionic strength of both mobile phases = 20 mM). Same column was used to detect sugars. Mobile phase A was prepared with 0.3% ammonium hydroxide in water, and mobile phase B was prepared with 0.3% ammonium hydroxide in acetonitrile. An Agilent ZORBAX StableBond 80 Å C18 (4.6 x 50 mm, 3.5 µm) column was used for antioxidant separation. Mobile phase A was prepared with 5 mM ammonium formate and 0.1%

formic acid in water, and mobile phase B was prepared with methanol. An Agilent Polaris 3 C18-Either (150 x 3.0 mm) was used for chromatographic separation of organic acids, nucleobase/side/tides, and fatty acids. Mobile phase A and B were the same for organic acids and nucleobase/side/tide and they were prepared with 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B), respectively. Mobile phase A for the detection of fatty acids was prepared with 40% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid in water, while mobile phase B was prepared with IPA:ACN (9:1), 10 mM ammonium formate, and 0.1% formic acid. The column was maintained at 25°C throughout the run. A dual eluent mobile phase was run at 500 µL/min for separation. The injection volume for each sample was 1 µL.

Mass spectrometry was performed on an Agilent 6470 triple quadrupole mass spectrometer. The optimization of the mass spectrometer was performed as indicated in Huang *et al*. (2019).

6.2.3 Metabolite profiling data analysis

Samples were analyzed in triplicate. Data were processed with Agilent MassHunter Workstation Software Quantitative Analysis (Version B.07.01/Build 7.1.524.0). Metabolites were baseline corrected to remove artifacts and aberrations due to experimental or instrumental variation, identified and interpretated. Univariate statistical analysis was performed through one-way analysis (ANOVA), followed by Tukey's test with PAST v.4.0 software. Analysis was carried out considering statistically significant P values less than 0.05. Method reporting limits (MRLs) were calculated as indicated in Huang *et al.* (2019). Data were normalized on the control, and used to perform heat map of changes due to experimental treatments.

6.3 Results and discussion

Treatments often bring stress to the plant generating cellular consequences (Maestri, Marmiroli and Marmiroli, 2016). In response to this, plants modify their metabolic pathways by modulating the presence and quantity of biochemical compounds. The systematic identification and quantification of all the metabolic products of a cell, tissue, organ, or organism under varying conditions were observed by the analysis of metabolites. The latter are the antioxidants whose role is known in defense from oxidizing compounds (Huang *et al.,* 2019), amino acids which are also important components of all living beings (Chennappa *et al.,* 2019), organic acids that are photosynthetic products as fixed carbon storage (Igamberdiev *et al.,* 2016), precursor of nucleic acids which provide information about how the energy is employed in plant metabolism, fatty acids with energy storage and structural organization properties, and sugars that influence food quality and participate in plant growth and development actions. The quantitative LC-MS/MS was a useful analysis for studying basic changes in metabolites' concentrations modulated by exposure to potential stress like the experimental treatments. In addition, this analytical method has been used as an optimal way to determine plant oxidative stress responses, metabolic mechanisms, and phytonutritional quality (Huang *et al.,* 2018). The method provided rapid screening and low-level (ng L^{-1}) quantification of metabolites with low MRLs, which were reported in Appendix.

All metabolites were investigated in samples of leaves, stems and roots in both *Heinz 3402* and *Ailsa Craig* tomato varieties. Metabolites that showed more marked changes than control were shown in heat maps that provided details more easily viewable. Data were presented divided by experimental treatment in order to better show the effects that each of them had on tomato varieties and on each analyzed organ (Fig. 41, 42, and 43).

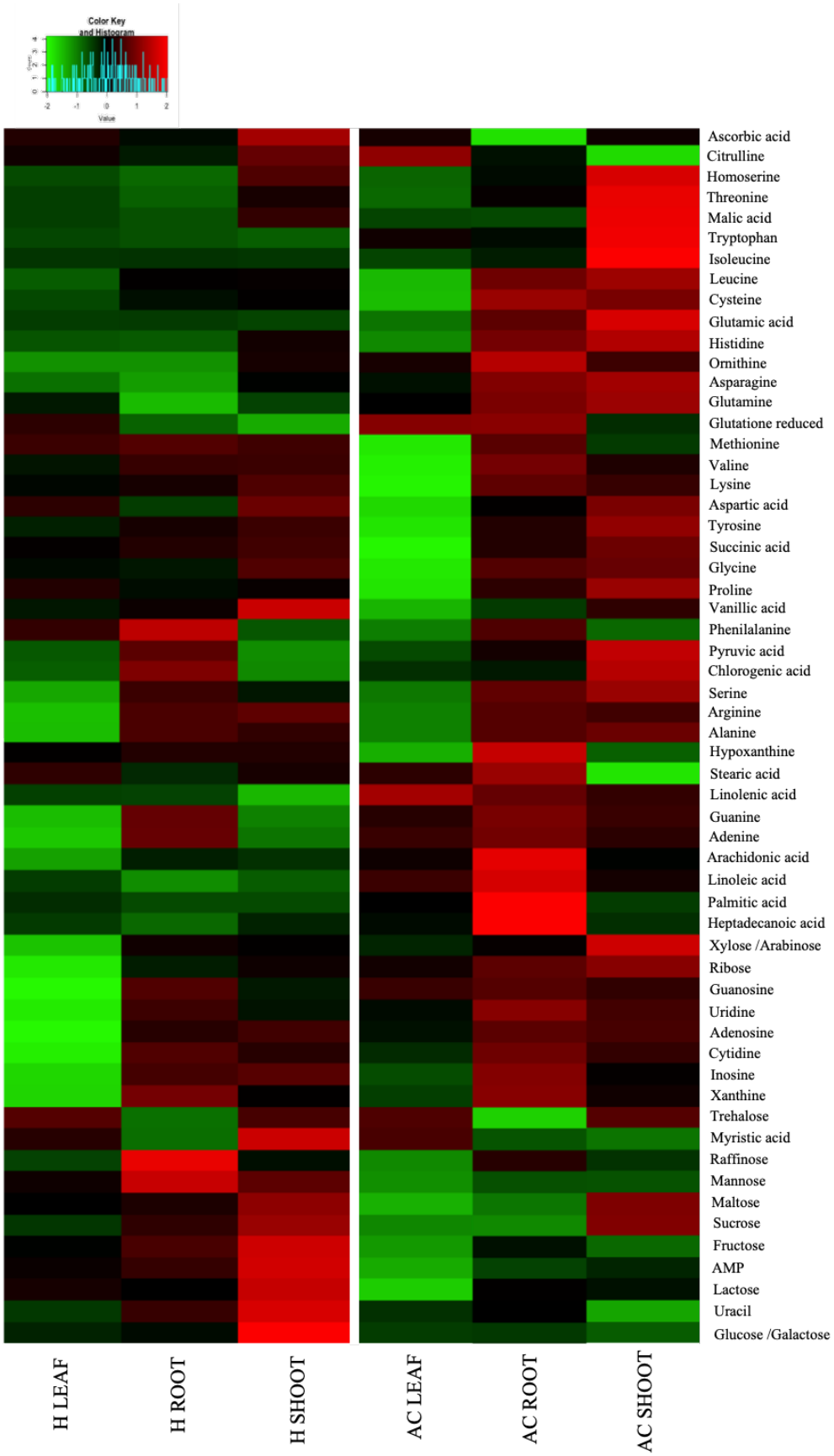


Figure 41. Heatmap of changes in *Heinz 3402* (left) and *Ailsa Craig* (right) organs due to biochar exposure. Red colors correspond to increased values and green colors correspond to decreased values compared with the control. H: *Heinz 3402*; AC: *Ailsa Craig*.

Concerning the treatment with biochar, metabolic responses in both tomato varieties were similar (Fig. 41). There was a partial decrease in metabolites' concentration in the leaves and a simultaneous increase in the metabolites in both stems and roots. The higher values of metabolites in the roots justified the direct interaction between roots, biochar and rhizosphere. Stem and leaf data indicated primary changes at the time of plant sampling that would probably also affect the leaves more in time after collection. In the *Heinz 3402* leaves, sugars and nucleosides and derivatives decreased in favor of increases in roots and stems. In particular, in roots mannose and raffinose increased compared to the control. In shoots, sugars increased compared to control, especially for maltose, sucrose, trehalose, lactose, fructose, and glucose/galactose. Even AMP, uracil, inosine, and adenosine were greater than the control. That result was clearly understandable as a direct source-to sink translocation (Fig. 44). The carbohydrate metabolism is a key feature to be monitored as plants capture photosynthetic energy and provide carbon needed to produce new tissues. Sugars are truly important for plant growth and development. In all *Heinz 3402* organs, fatty acids generally decreased except for myristic acid which increased in stems and stearic acid which increased in leaves. In the *Heinz 3402* leaves, the antioxidants remained almost constant except for a small increase in glutathione and a slight decrease in malic and pyruvic acid. Ascorbic, malic, aspartic, succinic and vanillic acids were the antioxidants that increased in *Heinz 3402* stems while pyruvic acid, chlorogenic acid increased in the roots compared with control. Among the amino acids, methionine, tyrosine, proline and phenylalanine increased weakly in the leaves; methionine, valine, tyrosine, phenylalanine, serine, arginine, alanine increased in the treated roots and citrulline, homoserine, methionine, valine, lysine, tyrosine, glycine, arginine, alanine increased though weakly compared to control. The increase in amino acid concentration indicated an increase in metabolic activity, demonstrating that exposure to the biochar stimulated interaction and metabolic production. Moreover, some of the amino acids that increased were precursors of antioxidants such as phenylalanine which normally participates in the metabolism of phenolic compounds by deamination reaction and proline which in addition to an activity of ROS-scavenging, is normally used for protein production and contributes to maintaining

redox balance. In the *Ailsa Craig* tomato cultivar, antioxidants remained constant compared to control with weak increases like for reduced glutathione. The concentration of amino acids in leaf tissues generally decreased compared to the control while both antioxidants and amino acids increased their concentration in samples of stems and roots. In addition, in the roots of *Ailsa Craig* the amount of fatty acids increased strongly compared to the control as happened in stearic acid, arachidonic, linoleic, palmitic, heptadecanoic. In these samples, the sugars remained constant except for trehalose, which decreased and ribose increased. The constituents of the nucleic acids did not show great variations. In *Ailsa Craig* shoots, fatty acids and sugars were constant except for maltose and sucrose that increased. The constituents of the nucleic acids increased weakly compared to the control. Therefore, in this specific harvest time of the tomato plants analyzed, the treatment with biochar produced positive effects especially in the roots and stems of the varieties considered.

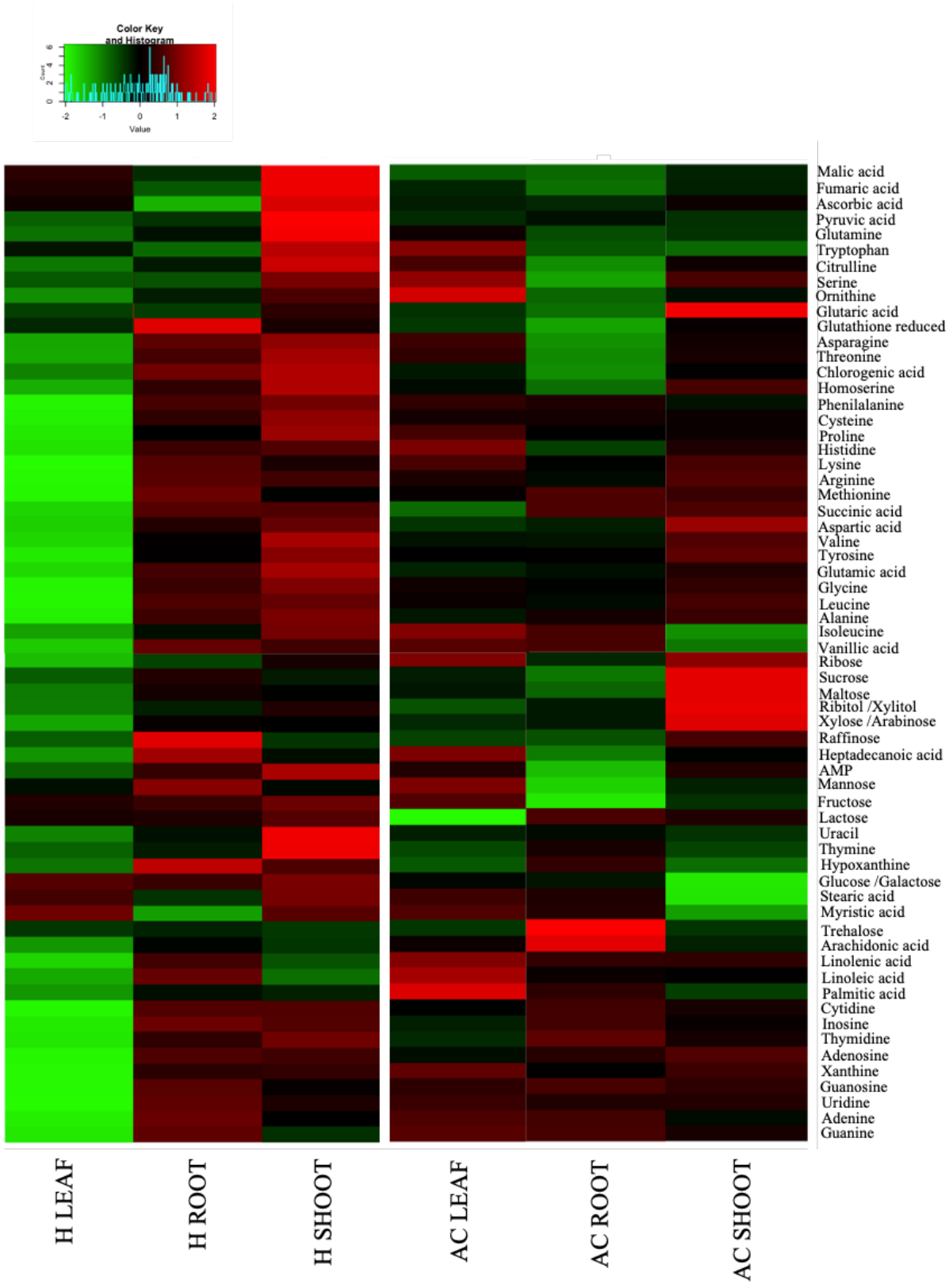


Figure 42. Heatmap of changes in *Heinz 3402* (left) and *Ailsa Craig* (right) organs due to microbial-functionalized biochar exposure. Red colors correspond to increased values and green colors correspond to decreased values compared with the control. H: *Heinz 3402*; AC: *Ailsa Craig.*

In the case of treatment with biochar associated with microbial consortium, the responses of tomato varieties to treatment were different and not fully comparable (Fig. 42). Specifically, in *Heinz 3402* the metabolites observed for the classes of antioxidants, organic acids, amino acids, sugars and nucleic acid precursors increased in roots and stems compared to the control. In *Ailsa Craig*, amino acids such as tryptophan, citrulline, serine, proline, histidine, lysine, isoleucine and ornithine increased in leaves, while lysine, arginine, methionine, valine and tyrosine increased in stems. In roots they remained comparable with control. Antioxidants increased in *Ailsa Craig* stems as glutaric acid, aspartic and in roots as succinic and vanillic which also increased in leaves. The fatty acids increased in *Ailsa Craig* leaves more visibly than the increase in nucleotides. Sugars, nucleotides and fatty acids also increased in roots with particular reference to trehalose and arachidonic acid. There was a great increase in sugar in the stems compared to the control with the only exception of glucose that decreased, while fatty acids decreased in the stems. In summary, the treatment of the biochar functionalized with microbial consortium showed a better adaptation of the *Heinz 3402* tomato variety which responded better to such treatment. In fact, there was a general increase in metabolites observed in both stems and treated roots. In *Ailsa Craig*, the experimental treatment also had effects on the leaves, although the effects were very variable among the organs.

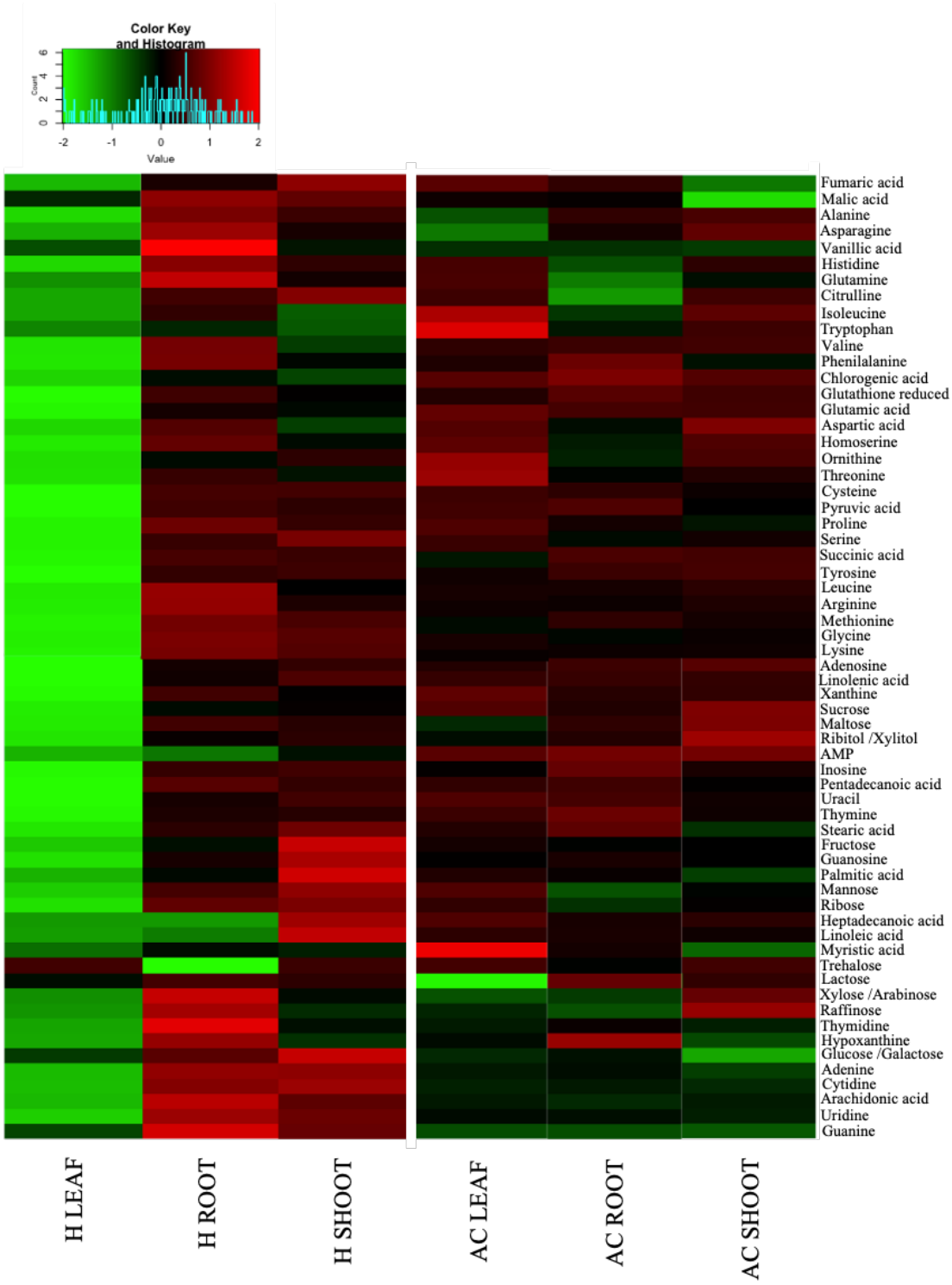


Figure 43. Heatmap of changes in *Heinz 3402* (left) and *Ailsa Craig* (right) organs due to microbial-functionalized seeds exposure. Red colors correspond to increased values and green colors correspond to decreased values compared with the control. H: *Heinz 3402*; AC: *Ailsa Craig.*

The heat map of the treatment with seeds functionalized with microbial consortium confirmed the behaviours observed in physiological and phytochemical parameters (Fig. 43). Treatment with microbial consortium directly on the seeds produced different effects by comparing the two tomato

varieties studied. In *Heinz 3402*, metabolites strongly decreased in exposed leaves with very few exceptions. However, all metabolites except for some sugars (fructose, trehalose) and fatty acids increased in treated roots. The most important increases in stems were found in sugars, fatty acids and some nucleic acid precursors such as fructose, mannose, ribose, palmitic acid, heptadecanoic, arachidonic and guanosine, adenine, cytidine, uridine and guanine respectively. Antioxidants, organic acids, and amino acids generally increased except in isoleucine and tryptophan, chlorogenic acid in which there was a slight decrease. The effects were best in the *Ailsa Craig* variety. Increases in metabolite concentration were observed in all organs studied. Increases in the content of antioxidants, organic acids and amino acids were visible in leaves, roots and stems with small exceptions. Sugars increased in the leaves, roots and stems of *Ailsa Craig,* while fatty acids increased in the leaves and decreased in the roots and stems. The nucleotides and derivatives were present in lower concentration compared with the controls on all of *Ailsa Craig*'s organs.

To resume all data, it was possible to affirm that changes in metabolites were visible and linked to the specific experimental treatment to which the plants had been exposed in the vegetative growth phase. As shown in Fig. 41, treatment with biochar produced a similar effect of translating some metabolites comparing the two varieties: in fact, it was observed that sugars (SA) and nucleotides/sides/bases (NAM) translocated from leaves to stems and roots. Same trend was also found for organic acids (OA) in *Heinz 3402* and AA in *Ailsa Craig*. In addition, treatment with biochar produced a decrease in fatty acids (FA), amino acids (AA) in *Heinz 3402* and in OA in *Ailsa Craig* both in leaves and roots. FA in *Ailsa Craig* increased both in leaves and roots. Antioxidants in *Ailsa Craig* roots slightly decreased.

Treatment with functionalized biochar led to a migration of SA, FA, AA, NAM and antioxidants from leaves to roots into *Heinz 3402* plants (Fig. 42). Organic acids were an exception to this trend as they decreased in both leaves and *Heinz 3402* roots. Treatment with BG cons C varied greatly in *Ailsa Craig*. In fact, the only translocation of metabolites that was observed was that of AA and antioxidants

from roots to leaves. SA and OA decreased in both leaves and roots, while nucleotides and fatty acids increased in both leaves and roots.

In *Heinz 3402*, the treatment with functionalized seeds allowed the translocation of all groups of metabolites (SA, FA, NAM, AA, OA, antioxidants) from leaves to roots (Fig. 43). Same trend was observed in *Ailsa Craig* for SA, NAM and antioxidants. FA, AA and OA in *Ailsa Craig* cultivar increased in both leaves and roots. Thus, the experimental treatments affected the groups of metabolites differently.

Generally, it was observed that in all the experimental treatments of *Heinz 3402*, most of the metabolites analyzed decreased in leaves and increased simultaneously in stems and roots suggesting a source-to-sink translocation (Fig. 44). Same trend was seen in *Ailsa Craig* biochar-treated plants. Metabolites in plants exposed to functionalized biochar and seeds increased in AC tomato variety. The two tomato varietals reacted differently to the fertilizer treatments used. *Heinz 3402* better adapted to treatment with biochar, both functionalized with microbial consortium than not. *Ailsa Craig* showed a preference for exposure to the microbial consortium directly associated with seeds, followed by the treatment with biochar. In both cases the primary and secondary metabolites studied led to hypothesize an adaptive behavior to treatments and a positive effect promoted by all experimental treatments. The effects, however, are varietal-related as already found in Marmiroli *et al.* (2017).

An important finding to point out was that although it was essential to study the translocations of metabolites among plant organs, it was also crucial to observe how these changed their concentrations within each organ. Therefore, even in cases where there was no translocation, variation of metabolites concentration can occur and meant a change of metabolism functioning in response to experimental treatments.

6.3.1 Behaviour of the different metabolites

Considerations on each group of metabolites needed to be done.

- Sugars and sugar alcohols (SA) migrated distinctly from leaves to stems and roots under all experimental conditions in *Heinz 3402*, and in treatments with biochar and functionalized seeds in *Ailsa Craig*. In fact, heatmaps (Fig. 41, 42, 43) revealed a significant decrease in sugar in leaves in favor of a great increase in roots and stems. In *Ailsa Craig* there was a significant decrease in leaves AC BG and AC SEEDS cons C, and a simultaneous increase in sugar concentrations in the roots of the same plants. Considering SA in all treatments, the most intensive translocation was in BG, followed by SEEDS cons C in both varietals and finally BG cons C only in *Heinz 3402*.
- Fatty acids (FA) behaved differently in the treatments and varieties considered. They decreased greatly in BG cons C and SEEDS cons C leaves of *Heinz 3402* to migrate to stems and roots where the concentrations of this group of metabolites increased, though more weakly. In H BG, FA decreased both in leaves and in roots. In *Ailsa Craig*, however, FA increased significantly under all experimental conditions in both leaves and roots. The increase of FA in AC was more marked in the roots treated with biochar and in leaves treated with biochar and seeds functionalized with the microbial consortium.
- Amino acids (AA) decreased in both *Heinz 3402* BG leaves and roots although an initial increase in biochar-treated root concentrations at the time of plant sampling was observed. In *Ailsa Craig* there was a contextual translocation of AA from leaves to roots treated with biochar while there were AA translocations from roots to leaves both in AC BG cons C and AC SEEDS cons C. Moreover, AA observed under H BG cons C and SEEDS cons C had the same pattern: they decreased in leaves and increased in roots.
- Nucleosides/tides/bases (NAM) have shown translocation in all *Heinz 3402* treatments from leaves to roots and stems. In *Ailsa Craig* there was a slight increase of NAM in biochar-treated roots and also an increase in both BG cons C leaves and roots. In AC SEEDS cons C there was a decrease in leaves concurrently with the increase in roots.

- Organic acids (OA) decreased in leaves H BG and H SEEDS cons C and increased in roots while decreased in both H BG cons C leaves and roots. In *Ailsa Craig* organic acids decreased in leaves and roots in both AC BG and AC BG cons C while increased in AC SEEDS cons C in leaves as well as roots and stems.
- The content of antioxidants in *Heinz 3402* decreased in leaves of all treatments and increased in roots while in *Ailsa Craig* the antioxidants decreased slightly in the roots treated with biochar and functionalized biochar. The investigated antioxidants instead increased in AC SEEDS cons C roots.

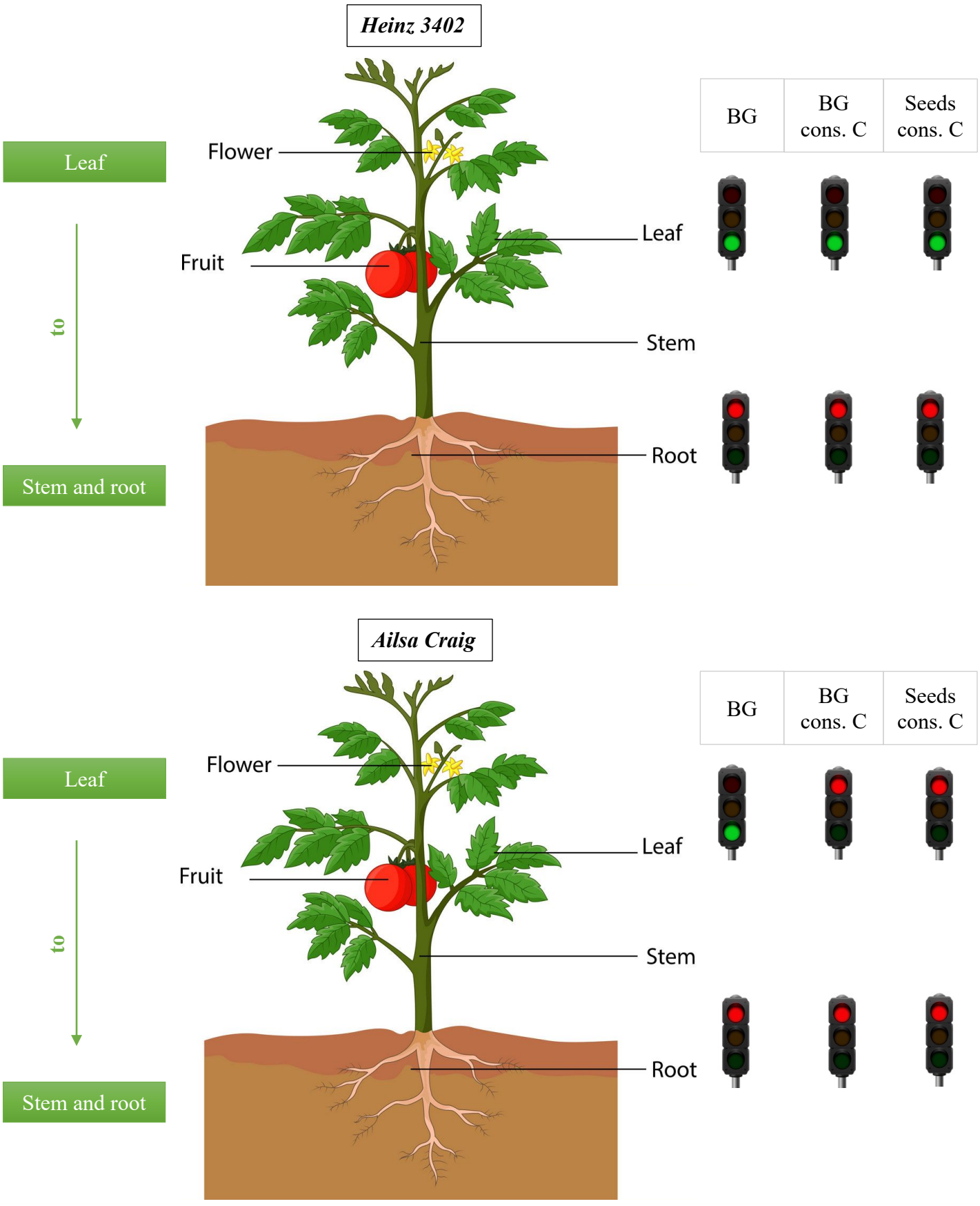


Fig. 44. Hypothesis of metabolites translocation in *Heinz 3402* (up) and *Ailsa Craig* (bottom) tomato cultivars. In *Ailsa Craig* cultivar, traffic light on the left refers to treatment with biochar, and traffic light on the right refers to treatments with functionalized biochar and seeds. Green traffic light refers to translocation of metabolites. Red traffic light means that translocation does not occur and metabolites accumulate *in situ*. Images are indicative: the plants of the *in vaso* experiment were sampled at 50 DAS and had neither flowers nor fruits. All the tests were carried out only on leaves, stems, and roots.

Conclusions

This doctoral project has set itself several challenging objectives. One of these was to potentially offer a valuable tool to agriculture to increase the quality of soil, its nature, fertility, and yield. The biochar was proposed as sustainable agricultural fertilizer to tackle these problems and also to make a contribution to the ever-increasing problem of climate change. Indeed, the first part of the work was focused on the study of the biochar characteristics as production parameters and biochar final features were related. It was found that the production temperature played a fundamental role in creating the biochar's intrinsic characteristics according to Xiao *et al*. (2018). A lower temperature production contributed to create a biochar with the highest amount of DMC, key parameter in determining biochar quality, and a structurally more disordered biochar with a reduced presence of ordered graphite layers, as shown in XRD patterns. This result is in agreement with Lehmann and Joseph (2009) and linked to the greater availability of functional groups inside the porous structure as visible in the FTIR-ATR spectra and zeta potential values: the chemical environment of both internal and external surfaces, its functioning (i.e. WHC, EC) and, consequently, its biological properties were greatly influenced by the technology of production and, especially, by the thermo-process temperature (Tomczyk *et al*., 2020). The evidences in the present study were all correlations between industrial production settings and biochar quality: all physical, chemical, and biological properties were assessed and were particularly favorable in the sample produced at lower temperature.

In order to offer a soil improver to agriculture, it was also necessary to assess biochar potential risk for crops, animals in field and also for operators who come into contact with it at all stages of its processing (from production, storage and sorting to field use). For this reason, the biochar's risk analysis was performed by the bacterial reverse mutagenic assay (the Ames test). This represented a novelty in testing the toxicity, application and use of biochar. All biochar samples showed no mutagenic effect at 0,01 g ml^{-1} concentration, even with metabolic activation, and represented a good, eco-friendly tool for agriculture. This result was in agreement with that found in biological analysis which outlined w/w 1% biochar as the threshold limit for safe biochar application in field.

Another important objective of this project was to combine the positive effects of the biochar, which was widely described in the literature and in the present work, with the extremely positive effects of PGPM used in agriculture to increase plant health, its phytochemical value and yield. The biochar was found to be an excellent substrate for the growth of selected microorganisms that were able to colonize its internal and external porous structure and to produce a biofilm on its surface. Moreover, a consortium of 6 different PGPM, produced with the aim of enhancing the powerful effect of an organic fertilizer such as the biochar, had interesting positive results in term of surfaces' colonization of biochar. This represented a revolutionary combination of two largely positive tools for soil wellbeing and its fertility. Nevertheless, enhancing the positive effects of two organic adjuvants made it possible to make a valuable input to sustainable agricultural practices.

The effect of the association of the two fertilizers (biochar and PGPM) had been studied on two tomato plants as tomato has a great importance in social, economic, and constitutes the model plant for climacteric plant studies. In addition, the effect of the microbial consortium alone, inoculated directly with seeds, was also studied. The physiological effects observed showed positive responses in all treatments, in both tomato varieties, with particular reference to treatments with biochar. Parameters which varied the most were FW, root length, total water content, Pheo A, Pheo B, Pheo A+B, anthocyanins in roots, and a great decrease of H_2O_2 content in roots in both varieties; anthocyanin content in leaf in *Ailsa Craig*; and, the number of leaflets, LAI, Chl A, Chl B, Chl tot, carotenoid content in *Heinz 3402* cv. Very few were the negative effects in *Ailsa Craig* (slightly increase of leaf H_2O_2 content in AC SEEDS cons C and decreased LAI in AC BG cons C) and *Heinz 3402* (slightly decrease of leaf anthocyanin content in H SEEDS cons C). No negative effects were recorded in treatment with biochar in either variety. Those results linked to a better phytochemical value of plants although further experiments focusing on the assessment of the quality of fruits might be desirable.

The effects of treatments with biochar and microbial consortium associated with biochar and tomato seeds were also evaluated in soil microbiology in order to establish whether fertilizers might bring

stress to the soil microbiome and to study the variations related to fertilizers' application. The present study demonstrated that experimental treatments had indirect effects by altering the microbial community in soil of *Heinz 3402* and the microbial richness increased (AWCD), showing that the microbial community adapted in soil and grew quickly.

Finally, metabolomics was evaluated to observe potential changes in the functioning of the plants as an indicator of plant physiological responses to experimental exposures.

Metabolomics in this study provided information on the variation of the major classes of metabolites in plant organs in terms of concentrations, illustrating physiological responses to treatment exposure. *Heinz 3402* roots recorded increases in metabolites especially in plants treated with SEEDS cons C (SA, FA, AA, NAM, OA, antioxidants), followed by BG cons C (SA, FA, AA, NAM, antioxidants) and, then, BG (SA, NAM and OA). However, decreases were also recorded in roots exposed to BG cons C (OA), while minimal decreases were shown in treatment with biochar (FA, AA). In *Ailsa Craig* the results of the study of the main metabolites showed that treatments with microbial consortium led to increases of metabolites in leaves (FA, AA, and OA in AC SEEDS cons C; FA and AA in AC BG cons C) while in the roots the most favorable treatment was that with functionalized seeds in which all metabolites increased, followed by that with biochar (SA, FA, AA, NAM increased). This data confirmed the findings of physiological and phytochemical analyses.

In addition, the metabolic study provided information on translocations of metabolites between plant organs. This was crucial in order to deepen our understanding of the plant physiological response to external stimuli. In *Heinz 3402* a great migration of metabolites was demonstrated under all experimental conditions, especially in plants with seeds (all groups of metabolites) and biochar (SA, FA, AA, NAM, Antioxidants) associated with microorganisms. In *Ailsa Craig* there was migration of metabolites from leaves to stems and roots only in plants treated with biochar (SA, AA, NAM) and in those treated with functionalized biochar in which translocation of AA took place from roots to leaves. The results of the migration of metabolites reflected exactly those obtained in physiological and phytochemical investigations in which the well-being and quality of plants was better in

treatments with biochar, followed by functionalized seeds and finally functionalized biochar. Finally, the two varieties studied showed a different adaptation to the treatments. *Ailsa Craig* showed more favorable results in terms of physiological aspects (FW, root length, water content), phytochemicals (chlorophylls, pheophytins, and antioxidants) and metabolic outputs (translocation of SA, AA, NAM from leaves to roots) in plants exposed to biochar, followed by treatment with functionalized seeds and functionalized biochar. In contrast, *Heinz 3402* showed better adaptability in terms of metabolites' translocation in SEEDS cons C and BG cons C than BG treatment, although all treatments showed positive results. The results were in line with the trend observed in both physiological, and phytochemical analyses in which *Heinz 3402* adapted very positively at all treatments and showed excellent results in many investigated parameters.

Metabolic responses of tomato plants were specific interactions and they varied depending on the tomato variety analyzed and depending on the organ studied. Metabolomics was a key analysis to understand the plant's phytochemical modulations and allowed to understand the changes in the plant vegetative growth phase due to exposure to external stress, observing the metabolic functioning. This study represented an innovative and transversal approach in the study of tomato in combination with biochar and PGPM application as fertilizers. However, tracking of metabolite flows gave indicative information and the specific responses of plants to the external stimuli represented by the experimental treatments could not be accurately established. Further in-depth investigations will be needed to understand deeper levels of response such as genetic and functional modulations of plant metabolism. Metabolomics has certainly been an initial screening in the study of further more in-depth analysis.

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Appendix

Method Reporting Limits (MRLs) for each metabolite analyzed. MRLs are reported in ng ml^{-1}.

Compound	MRL (ng/ml)
Amines	
Ethanolamine	498,42
2,4-DAS	881,48
m-PDA	1013,75
ANL	994,01
o-ANS	399,53
o-T	132,76
4,4-ODL	3473,55
4-CA	4154,42
2,6-DMA	1530,26
4,4-DPM	482,91
2-M-5-NA	4036,11
Diphenylamine	1755,07
Amino acids	
Leucine	1,67
Phenylalanine	166,67
Methionine	16,67
tryptophan	1,67
Isoleucine	1,67
Valine	133,33
Proline	333,33
Tyrosine	50,00
Cysteine	1666,67
Threonine	133,33
Serine	3333,33
Alanine	1,67
Homoserine	50,00
Glycine	5000,00
Glutamine	166,67
Asparagine	333,33
Glutamic acid	1,67
Citrulline	50,00
Aspartic acid	1,67
Histidine	83,33
Arginine	3,33
Lysine	1,67
Ornithine	3,33
Antioxidant	
Glutathione reduced	16,67
Chlorogenic acid	1,67
Curcumin	1,33
Vanillic acid	200,00
2-hydroxycinnamic acid	1,67
L-Dehydroascorbic acid	3,33
4-(Trifuoromethyl)cinnamic acid	0,33

a-Tocopherol	166666,67
Fatty acids	
Linolenic acid	8875,42
myristic acid	45442,02
Linoleic acid	8161,62
Pentadecanoic acid	43034,92
Palmitic acid	36357,71
Heptadecanoic acid	12467,25
Stearic acid	26187,49
Arachidic acid	11169,40
Nucleobase/side/tide	
Cytosine	2186,23
CMP	2328,29
Cytidine	2142,31
Adenine	2905,45
Guanine	1085,26
uracil	9307,30
AMP	9667,71
Hypoxanthine	2997,71
Uridine	1927,04
Xanthine	1366,77
Adenosine	1073,02
Thymine	5168,30
Inosine	873,07
Guanosine	755,33
Thymidine	8347,73
Organic acids/Phenolics	
glycolic acid	15339,31
malic acid	1719,66
Citric acid	11312,57
lactic acid	21308,01
succinic acid	1272,45
Pyruvic acid	22617,26
Gallic acid	5367,50
Glutaric acid	9487,69
fumaric acid	22643,43
ascorbic acid	1174,59
Caffeic acid	1930,92
p-coumaric acid	1140,29
ferulic acid	1133,05
benzoic acid	2700,98
Salicyllic acid	1153,40
Sugar/Sugar alcohols	
Ribose	13171,02
L-fucose	13033,31
Xylose/Arabinose*	10668,80
Ribitol/Xylitol*	2152,79
Fructose	8649,77
Mannose	6821,45
Glucose/Galactose*	10240,22
Sucrose	3586,72
Maltose	7424,02

Lactose	76473,39
Trehalose	2157,99
Raffinose	4186,50
Galactinol	14533,47

Principal Component Analysis (PCA) - *Ailsa Craig*

- Aboveground physiological and phytochemical parameters

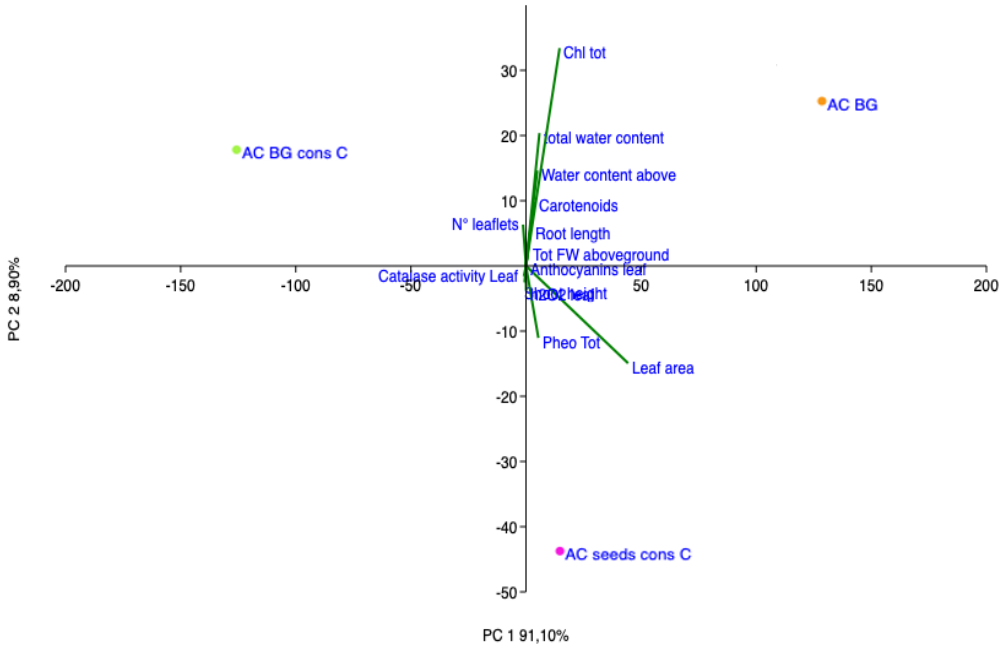

Figure 45. Principal component analysis (PCA) of aboveground physiological and phytochemical data of *Ailsa Craig* (AC). Principal component 1 (PC 1) explains the 91,1% of variability and principal component 2 (PC 2) the 8,9%. BG: biochar; BG cons C: functionalized biochar with microbial consortium; seeds cons C: functionalized seeds with microbial consortium.

- Belowground physiological and phytochemical parameters

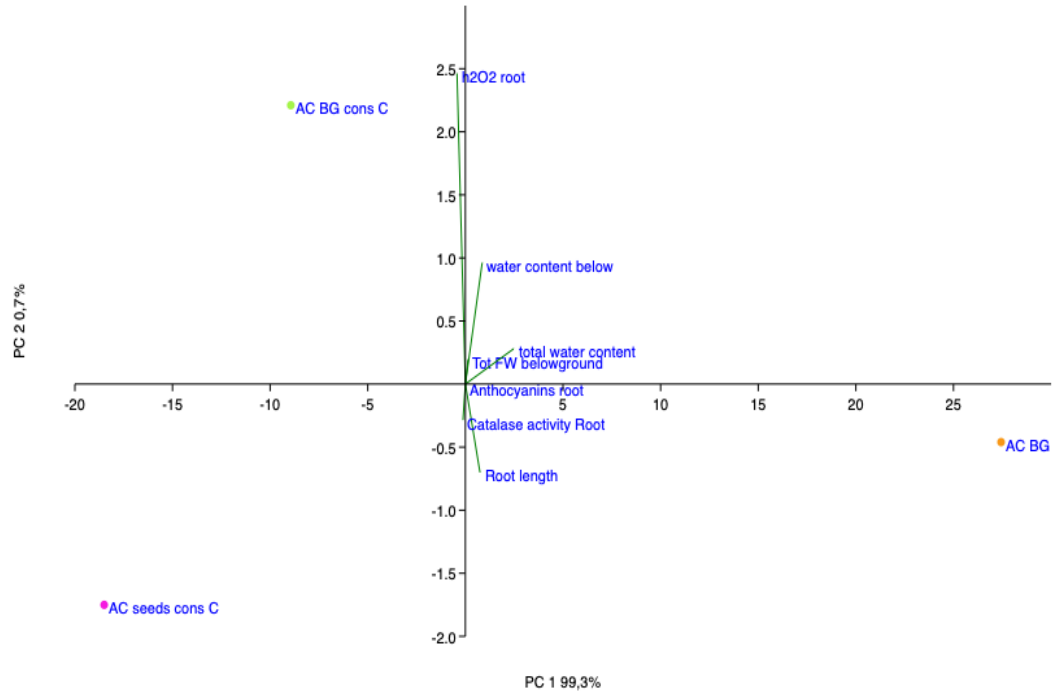

Figure 46. Principal component analysis (PCA) of belowground physiological and phytochemical data of *Ailsa Craig* (AC). Principal component 1 (PC 1) explains the 99,3% of variability and principal component 2 (PC 2) the 0,7%. BG: biochar; BG cons C: functionalized biochar with microbial consortium; seeds cons C: functionalized seeds with microbial consortium.

Principal Component Analysis (PCA) – *Heinz 3402*

- Aboveground physiological and phytochemical parameters

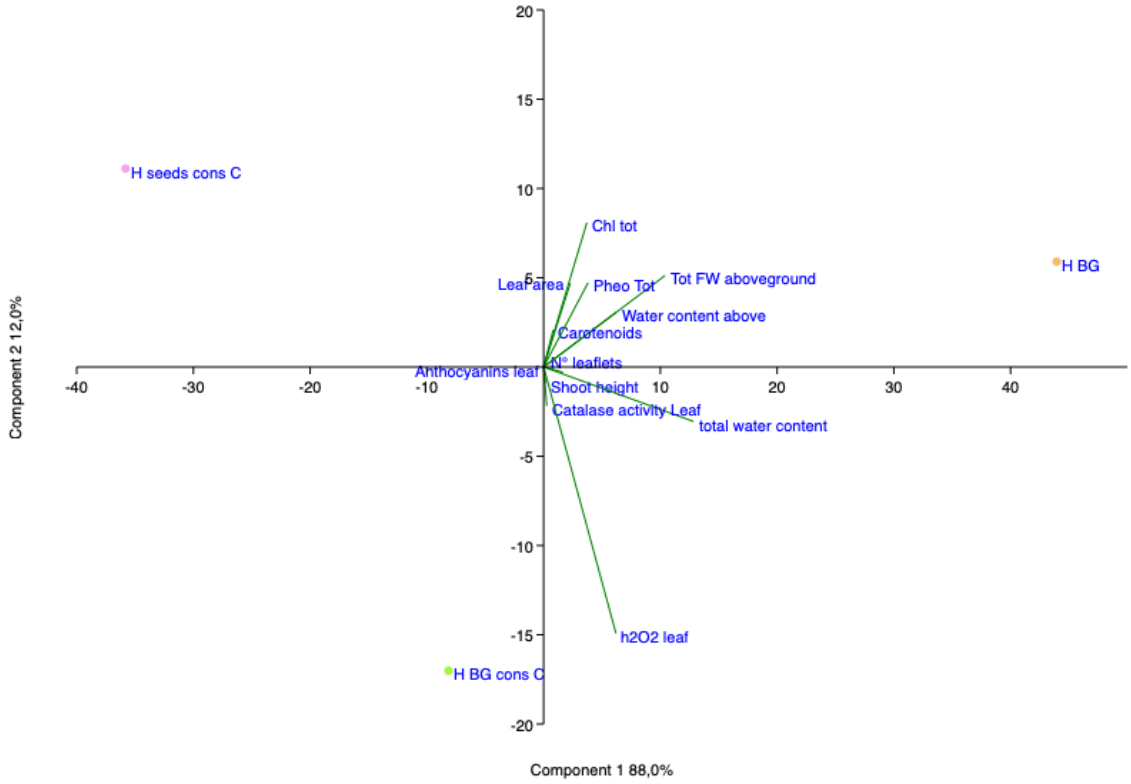

Figure 47. Principal component analysis (PCA) of aboveground physiological and phytochemical data of *Heinz 3402* (H). Principal component 1 (PC 1) explains the 88,0% of variability and principal component 2 (PC 2) the 12,0%. BG: biochar; BG cons C: functionalized biochar with microbial consortium; seeds cons C: functionalized seeds with microbial consortium.

- Belowground physiological and phytochemical parameters

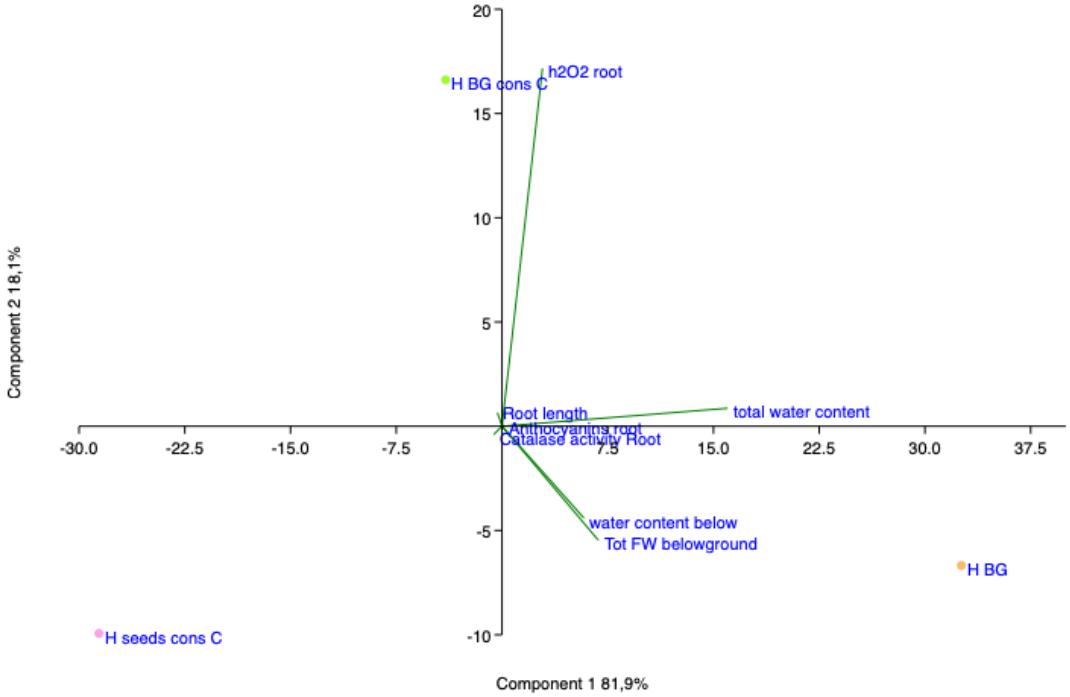

Figure 48. Principal component analysis (PCA) of aboveground physiological and phytochemical data of *Heinz 3402* (H). Principal component 1 (PC 1) explains the 81,9% of variability and principal component 2 (PC 2) the 18,1%. BG: biochar; BG cons C: functionalized biochar with microbial consortium; seeds cons C: functionalized seeds with microbial consortium.

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