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XXX CICLO

Morphological and 'omics' analysis of root systems in agronomics plant grown under biochar applications

Analisi morfologiche e "omiche" di sistemi radicali in piante agronomiche cresciute con biochar

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You always believe in yourself, even if it means walking alone.

Personal consideration

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Abstract

The continued increase of the demand for agricultural crops is responsible of a strong environmental deterioration due to soil fragmentation as well as, to a massive use of fertilizers uses that pollute both water and terrestrial ecosystems. In the future, this demand will growth up more and more. This explain why is crucial to find a new approach and new technologies to achieve greater yields with a lower environmental impact.

Biochar is a solid material obtained from a process called pyrolysis characterized by a thermal transformation of biomass at high temperature and in the absence of oxygen. This mineral transformation attributes to biochar a skeletal structure that looks like a carbon sponge, which allows a higher water retention. The addition of biochar to the soil reduces leaching of ammonium compared to untreated soil due to its characteristic sponge structure and recently, it has been observed that also the total nitrates/nitrites, ammonia and nitrogen content and the nitrogen fixation rate are affected.

The aim of this work is investigate some morphological and molecular response of plants treated with biochar. For this purpose, two of the most important crop species and one model specie have been tested: 1) *Solanum lycopersicum* (Cherry tomato of Pachino vr), 2) *Vitis vinifera* (Chardonnay cv) and 3) *Arabidopsis thaliana*.

Concerning Cherry tomato cultivar, the plant treated with biochar has shown an interesting modification of seedling and fruit traits – especially in the case of fruit quality.

In regard to *Vitis vinifera*, positive effects have been detected in both experiments (pot and field treatments) with biochar, improving the root length in pot experiment while the radial root growth in field experiment.

Finally, in *Arabidopsis*, biochar addition has induced positive effects in all the parameters measured under normal watering regime. In the case of seedlings affected by water stress, the presence of biochar seemed to inhibit strongly the plants growth.

Data obtained in this work throw light upon some of the most important aspects of plant nutrition and development. They indicate how those are modified in presence of biochar.

However, further studies are necessary to validate the effect of biochar as soil amendment for crop yield increase. The long-term aim is to use biochar to reduce soil degradation and to decrease the amount of fertilizers with a beneficial effect on environmental pollution.

Riassunto

La continua domanda di colture agricole è altamente correlata a un forte impatto ambientale dovuto alla frammentazione del suolo e agli usi di fertilizzazione che inquinano sia gli ecosistemi dell'acqua che degli ecosistemi terrestri. In futuro, la domanda di colture agricole crescerà sempre di più e questo implica un ulteriore importante impatto sull'ambiente. Al fine di evitare il deterioramento di una situazione già complicata, nel prossimo futuro sarà cruciale trovare nuovi approcci e tecnologie per ottenere maggiori rendimenti con minori impatti ambientali globali.

Il biochar è un materiale solido ottenuto da un processo chiamato pirolisi caratterizzato da una trasformazione termica della biomassa ad alta temperatura e in assenza di ossigeno. Questa trasformazione minerale attribuisce al biochar una struttura scheletrica che sembra una spugna di carbonio, che consente un'elevata ritenzione idrica. L'aggiunta di biochar al suolo riduce la perdita di ammonio rispetto al terreno non trattato a causa della sua caratteristica struttura a spugna.

Lo scopo di questo lavoro è studiare una certa risposta morfologica e molecolare delle piante trattate con biochar. A tal fine, sono state testate due delle specie di colture più importanti e una specie di modello: 1) *Solanum lycopersicum* (pomodoro ciliegio di Pachino vr), 2) *Vitis vinifera* (Chardonnay cv) e 3) *Arabidopsis thaliana*. Per quanto riguarda la cultivar di pomodoro ciliegio, le piante trattate con biochar hanno mostrato un elevato valore di tutti i tratti morfometrici e dei tratti riguardanti la frutta - in particolare i tratti qualitativi.

Per quanto riguarda la *Vitis vinifera*, sono stati rilevati effetti positivi in entrambi gli esperimenti (in vaso e in campo) dove la presenza di biochar ha migliorato la lunghezza radicale solo nell'esperimento in vaso mentre, nell'esperimento in campo, ha migliorato fortemente il diametro radicale.

Infine, per quanto riguarda *Arabidopsis*, i semenzali hanno mostrato come l'aggiunta di biochar nel terreno ha indotto effetti positivi in tutti i parametri considerati ma solo sotto il regime di irrigazione normale mentre, sotto condizioni di stress idrico, la presenza di biochar inibiva fortemente la crescita delle piante.

In conclusione, i dati ottenuti nel presente lavoro sono stati un passo iniziale verso la comprensione dei meccanismi coinvolti tra l'interazione tra il biochar – suolo e – lo sviluppo della pianta.

Tuttavia, ulteriori studi sono necessari per convalidare l'effetto del biochar come emendamento del terreno per l'aumento delle produzioni agricole. L'obiettivo a lungo termine è quello di utilizzare il biochar in modo tale da ridurre il degrado del suolo e per diminuire la quantità di fertilizzanti utilizzati negli ultimi anni, con un conseguente effetto benefico sull'inquinamento.

Chapter I

Introduction

In the XX century, characterized by the global population expansion, the demand of agricultural crops is dramatically growing resulting in an increase of intensive use of land, which in turn cause the soil quality deterioration and the need of higher amount of fertilizer inputs (Tilman et al. 2011). As consequence, in the last decade, this massive use of fertilizers caused pollution of water and terrestrial ecosystems (Sachs et al. 2010). Therefore, it is crucial for the next future, to find new solutions to obtain greater yields with lower global environmental impacts. Biochar may play an important role for this purpose. Indeed, it is produced by the heat plant biomass in the absence of oxygen (pyrolysis) and its structure, similar to a sponge, allows higher water retention. In several research study, has been observed how its additions to the soil affects positively the total nitrates/nitrites, ammonia and other compounds content improving the quality and the structure of soil and consequently, the increase of agricultural crops yield (Rondon et al., 2007; Van Zwieten et al., 2008).

1.1 Effect due to climate change and Biochar use as possible environmental restoration

In the last decades, the exponential social and economic growth due to the industrialization has increased the gas emissions into the atmosphere, which is now considered as the main factor responsible for climate change and its related disastrous effects on the environment.

Management strategies for the building soil organic matter (SOM) have been considered as possible measures to mitigate global change (Lal, 2004; Smith et al., 2008). As for example the reducing of SOM susceptibility to the decomposition seems to decrease the release of CO_2 in the atmosphere (Powlson et al., 2011). Another measure could be produce biochar by the heating of plant biomass in the absence of oxygen (pyrolysis) and then store it in agricultural or forest lands (Laird et al., 2009).

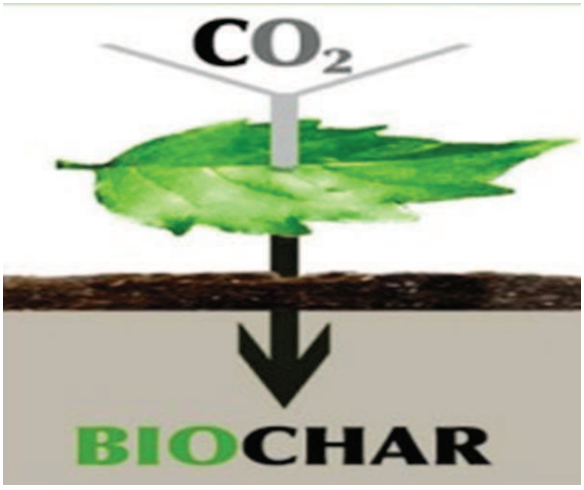

Figure 1.1 Biochar power to mitigate climate change; adopted from www.pronatura.org site

The International Biochar Initiative (IBI) promotes ubiquitous use of biochar as a soil amendment, it advocates for inclusion of provisions favourable to biochar use in national and international polices aimed to mitigate global change effects, promoting also biochar commercialization, and aspiring to a global system that sequesters 2.2 Gt C/yr by 2050 (International Biochar Initiative, 2013).

Currently in literature there are discordant opinions about the positive biochar effects; for example, some researchers believe that biochar could have adverse effects on environment by releasing toxic substances such as heavy metals into soil or by reducing the pesticides efficiency (Kookana et al., 2011).

1.2 Biochar properties

Biochar is a highly porous carbon that, if added to soil, could improve soil quality by reducing biomass emissions. Due to its aromatic structure, biochar is resistant to decomposition and could therefore account for a significant portion of all carbon present into the soil (Kuhlbusch et al., 1996). Biochar addition to the soil promotes food safety and soil biodiversity, improves the quality and amount of water in the soil, reduces the leaching of nutrients and so it improves the availability of nutrients and reduces environmental pollution from chemicals (Figure 1.2) (Yamato et al., 2006).

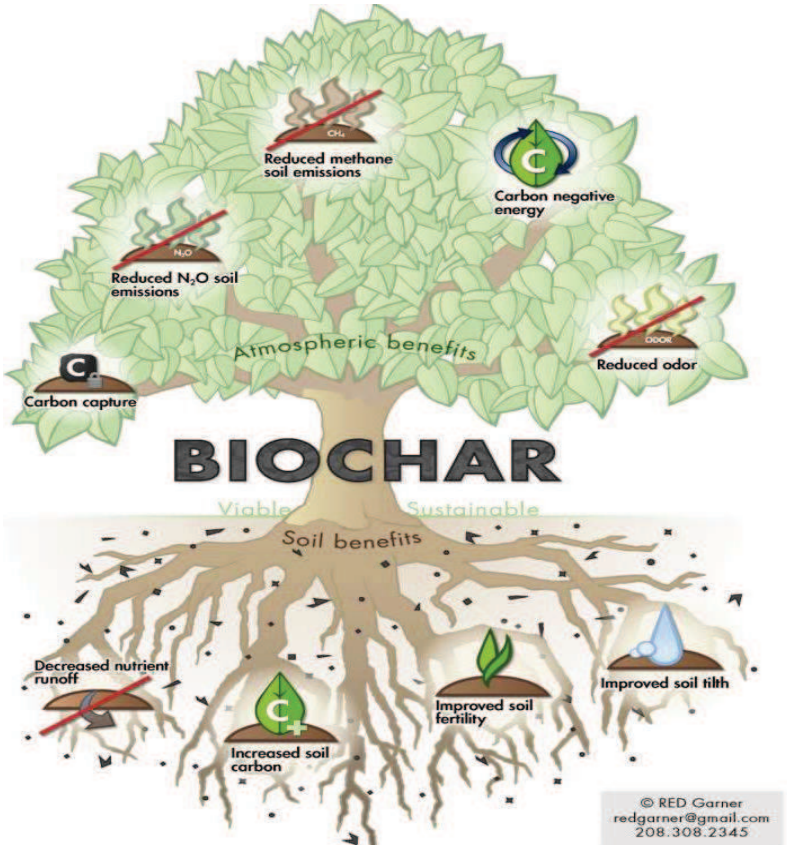

Figure 1.2 Global biochar effects: atmospheric and soil benefits; adopted from www.bettercarbonsolutions.com site

Moreover, cation exchange capacity (CEC) of biochar is consistently higher than that of the soil (Lehmann et al., 2003; Liang et al., 2006).

Some studies (Rondon et al., 2007; Van Zwieten et al., 2008) attribute the positive plant response to the effects of biochar on nutrients availability and to its ability in increasing or maintaining soil pH through calcination. Furthermore, the dark-colored biochar mixed with soil changes the soil-surface albedo with consequent variation of soil temperature (Meyer et al. 2012) which alters the rate of root growth and development (Figure 1.3).

The influence of biochar on roots may induce variation in plant biomass and fruit production.

Figure 1.3 Raw material color change when its biochar transformation occurs; adopted from www.charchive.org site

Hossain et al. (2010) reports that the biochar presence into the soil could also reduce heavy metals absorption from the soil, whereas other authors suggest that biochar increases soil fertility (Van Zwieten et al., 2008), crops yield (Yamato et al., 2006) and improves the fertility of sandy soil. Nevertheless, the debate regarding biochar properties is still intense and this fact explains the interest of the international scientific community for this topic. In particular, there is a considerable interest of the possibility to use biochar to store CO_2 in the soil.

In Italy, research involving biochar started in 2007 when the first papers about its effect when used as amendment in the fields- and on the lab started to be published. In the light of the first confirmations and promising results, in 2012, the ICHAR Association initiated a process aimed at the Italian recognition of biochar as a common fertilizer for agriculture. Since then, a number of reports, ministerial hearings, and protocol revisions have been published so that today, at a distance of 5 years, it becomes more and more concrete the possibility that biochar exits from its experimental state to be recognized (and appreciated) for its benefits in favor of agro-industrial, agro-food, economy. Moreover, a more recent development of biochar research starts to investigate its role in mitigating climate change effect through immobilization of carbon for long periods.

1.2.1 The biochar discovery

Plant biomass conversion to biochar is a process which has been known for a number of years (Seifritz et al., 1993), but few were the studies regarding its use as soil amendment. The discovery of biochar as soil amendment it took place when it was observed that the dark soil (called “dark earth” or “preta earth” by the locals) that characterizes the Amazon basin was provided by a considerable fertility (Figure 1.4).

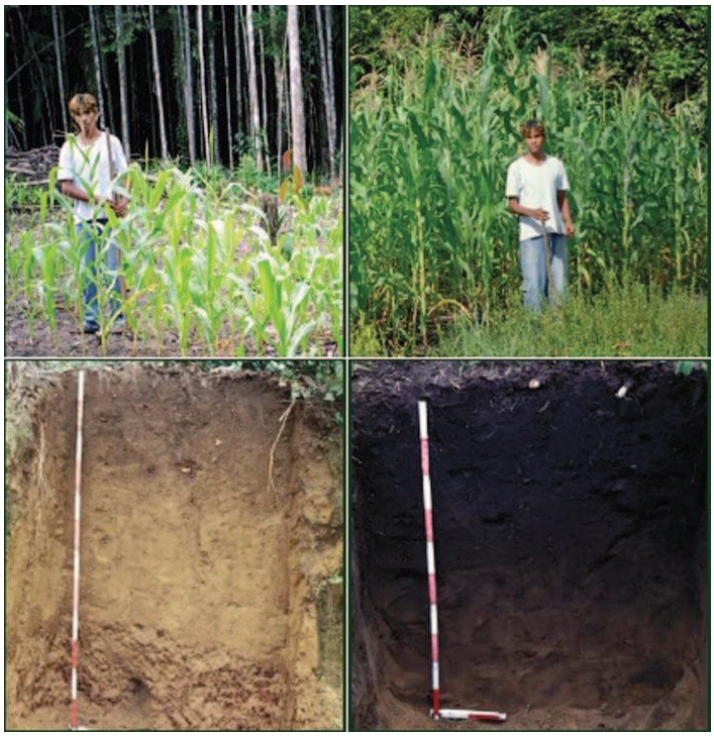

Figure 1.4 Different poor soil profile (left) and dark earth soil (to the right) of the Amazon rainforest; adopted from www.biochar.org site

Normally Amazonian soil is very poor in nutrients due to leaching, but this negative event is almost completely absent in the dark earth which it can be considered a soil that has been a stabilized for millennia (Kurt, 2013). This soil presents today fertile properties down to 2 meters below soil -surface. However, despite the attempt done to date, formation of preta earth it is still unclear and therefore it is unknown its generation has been an intentional or unintentional event (Heckenberger et al 2003, Meggers 2003, Stokstad 2003). Glaser and Birk (2012) have suggested that two hypotheses remains acceptable: a) an intentional generation of the dark earth to improve soil quality in home gardens, and b) an unintentional generation through casual deposition in the soil of biological materials such as: bones, stools, ashes, organic materials incompletely incinerated and other waste.

1.2.2 The production of biochar: pyrolysis process

Pyrolysis is a chemical decomposition of biomass that is carried out in the absence or limited presence of oxygen at a range of temperature comprised between 300 ° C (slow pyrolysis) and 500 ° C (fast pyrolysis) (Bridgwater et al., 2007). The parental material used is plant material and organic waste, with a humidity not exceeding 30%.

During pyrolysis (Figure 1.5), complex molecules are splitted into simpler molecules such as gas (syngas), liquid (bio-oil) and carbon material (biochar) (Mohan et al., 2006).

With this processes, approximately 50% of carbon contained in the original biomass source could be retained in the biochar, however this percentage is highly dependent on the pyrolysis process adopted (FAO 1985; Daud et al., 2001; Demirbas, 2001; Baldock & Smernik 2002; Lehmann et al., 2002; Laird, 2008). The main parameters to be considered during the pyrolysis process are: heating speed, highest treatment temperature, pressure, reactions residence time, reaction vessel pre-treatments, the flow of accessory components (nitrogen, CO_2, air, steam etc.) and post-treatments. Lua et al. (2004) have evaluated the relative importance of temperature, residence time, nitrogen flow, and the rate of heating provided during pyrolysis by considering the standard deviation and the coefficients of variation of the different physical parameters, finding that the main role was played from the pyrolysis temperature and, to a lesser extent, by the rate of heating, nitrogen flow, and residence time.

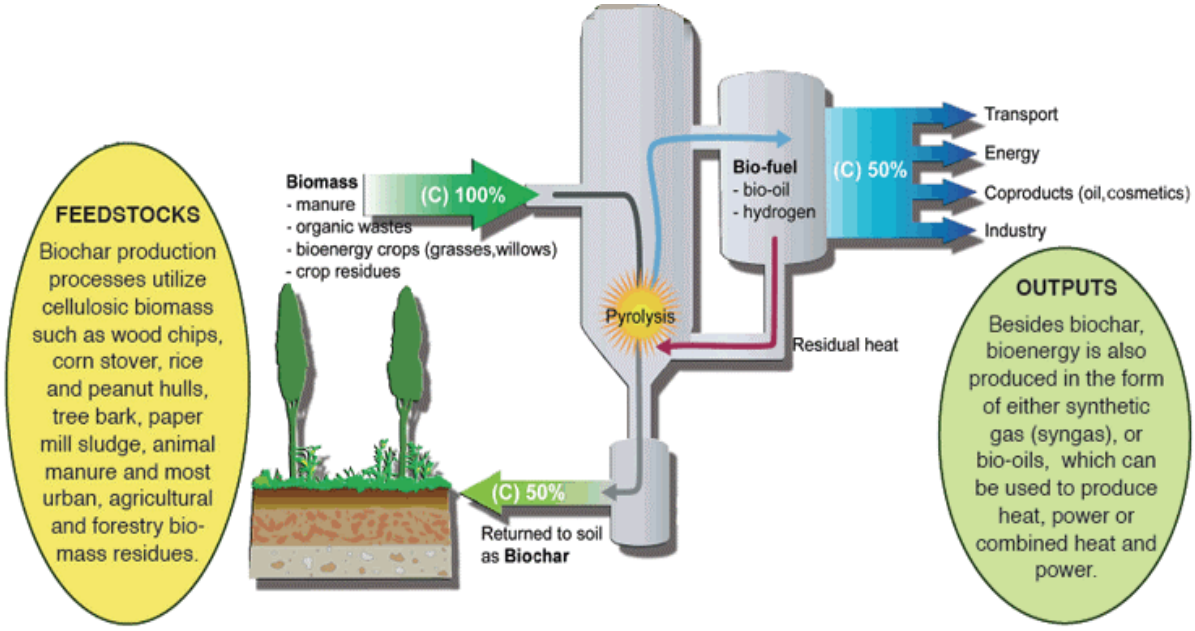

Figure 1.5 Production process of biochar; adopted from www.biochar.org site

1.2.3 Chemical - physical properties of biochar

The knowledge of the chemical-physical properties of the biochar are necessary in order to better understand how this material exerts its amendment potential.

Biochar is a carbonaceous material containing aromatic hydrocarbon polycyclics with a number of other functional groups (Krull et al., 2009).

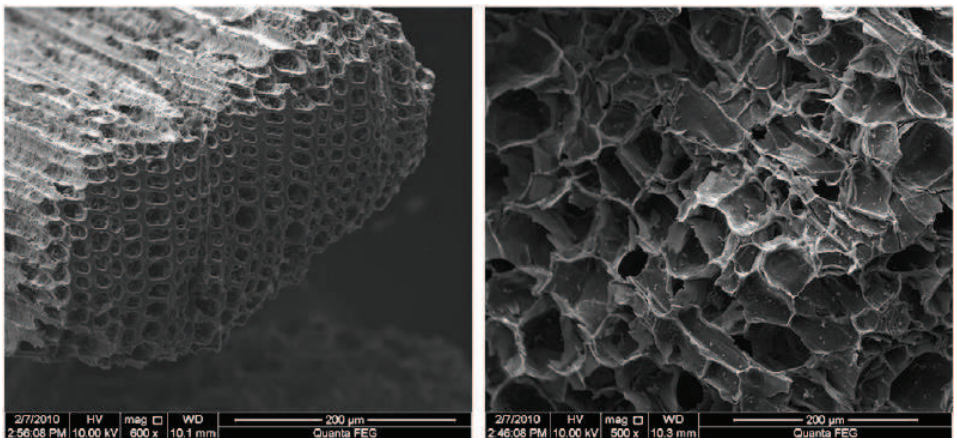

Figure 1.6 Image of biochar structure obtained by scanning electron microscope (SEM) (Sohi et al., 2009).

Its structure is highly porous and could contain detectable quantities of extractable humic and fulvic acid; these substances are important as mineral nutrition of plants for cation exchange and for the high buffering power of pH variations. The amount of humic and fulvic acid found in vegetable carbon depends on the temperature reached during pyrolysis (Trompowsky et al., 2005). Obviously biochar naturally contains basic nutrients such as Nitrogen, Sulfur, Phosphorus.

Biochar has a high degree of chemical stability which ensures its very slow degradation, and that it explains why carbon remains deposited in the ground (Cheng et al., 2008). Solomon et al. (2007) has shown that the biochar stability is due to the pyrolysis-induced anthropogenic origin of highly refractory aryl-C structures. Having a very heterogeneous composition of vegetable charcoal, biochar could exhibit on its surface different properties (hydrophilic, hydrophobic, acidic, basic, etc.) and thus it could interact with all the substances present in the soil in different ways. The variability in the chemical characteristics depends mainly on the parental material, or on the waste material from which it is obtained (Lua and Yang 2004).

As already mentioned, temperatures used for the production of vegetable charcoal are influenced by different characteristics; a study by Chan et al. (2009) has shown that even by starting with the same substrate different products with various pH, CO_2, N, P concentrations (Chan et al., 2009) can be obtained by using different pyrolysis temperatures. The size of the biochar particles also varies greatly depending on the rate of water loss during dehydration; the porosity is not uniform but portions of different size ranging from <0.9 nm to> 50 nm can be obtained (Figure 1.6). Macro-pores, due to their large size, represent a habitat that can be colonized by microorganisms and other beneficial organisms such as mycorrhizas. Micropores, on the other hand, are involved in the absorption and transport of molecules present in the soil (Downie et al., 2009). All these properties explain why the biochar porosity and surface have important repercussions on the nutrients retention through the possibility to bind cations and anions (Liang et al., 2006, Chan and Xu 2009). In addition, within the biochar pores, carbon dioxide, ammonia and water combine to form ammonium bicarbonate, a potent nitrogen fertilizer. (Winsley et al., 2007).

It is evident from what reported above that the biochar physical and chemical properties depend not only on the parental material but also by the production process; therefore it is very important to establish a regulation that certify its productive process for future marketing and utilization in agriculture activities.

1.2.4 Biochar and root traits

Roots are responsible for nutrient and water uptake from the soil but they play also the major role in the anchorage of the plant to the soil.

In regard of C sequestration (Matamala et al., 2003), roots transfer photosynthetically fixed C to soil organic matter pools (Jackson et al., 1997). The fact that biochar amendment has important effects on plant growth and development derives from the interaction between biochar and roots (Prendergast-Miller et al., 2013) that influences considerably root activity (Laird, 2008).

Studies aimed to investigate the interaction between biochar and roots have shown that root traits affected are: biomass and morphology (Prendergast-Miller et al., 2011; Brennan et al., 2014), nutrient root concentration (CHN), and root-association with microbes (Rondon et al., 2007). For example, it has been shown that root length is associated with water and/or nutrient acquisition, whereas root diameter is associated to biomass accumulation (Eissenstat & Yanai, 1997). However, reports about biochar effects on the root traits are highly variable, when not even contradictory, and that it explains why it is necessary to deepen our knowledge about the interaction between biochar amendments and roots

1.2.5 Biochar and soil biota

Different studies have been carried out on the chemical-physical properties of the biochar and on the effects of its application in agronomic fields; in recent years, studies are trying to characterize also the biological aspect of biochar, by observing the consequences of amendment operation on plant populations and microbes living in the soil. To study the role of biochar influence on soil biota, several methods have been used, such as: total genomic DNA extraction (O'Neill, 2007; Grossman et al., 2010), counts of cultured microorganisms (Jackson, 1958; O'Neill et al., 2009), extraction of fatty acid from membrane phospholipids (PLFA) (Birk et al., 2009), coloring and direct observation of single biochar particles (Jackson , 1958, Pietikäinen et al., 2000; Warnock et al., 2007; Jin, 2010).

In some soil treated with the biochar addition, an increase of the rate of microbial reproduction has been observed (Pietikäinen et al., 2000; Steiner et al., 2004). This effect could be explained with the observation that changes in the availability of C and nutrients could both increase and reduce microbial biomass, depending on the actual availability of nutrients and C, the extent of change and the population of microorganisms (Cheng et al. 2008).

Kasozi et al., (2010) have discovered that the absorption of certain molecules into biochar could inhibit or increase microbial growth. This effect could be explained with the observation that fungal and bacterial populations react differently to pH variations. As for example bacteria can increase their biomass when pH increases to 7, while fungi may have no change in total biomass (Rousk et al. , 2010), or they could drastically reduce their growth to higher pH values (Rousk et al., 2009).This fact highlights the importance of investigating the effect of biochar amendment on pH variations in the soil that could affect microbial population. In fact, it has been reported that the type of carbon used and the pyrolysis temperature used to make biochar affects the growth of colonies of Gonococcus and Meningococcus (Glass & Kennett, 1939).

1.2.6 Biochar effects on plant organisms

Several studies aimed to investigate effects of biochar amendments on plants have focused on crop species grown in pot, in greenhouse, or in the field. These studies have suggested that biochar affects yield and productivity of the crop species investigated. Effects observed after biochar amendments regard particularly soil parameters (i.e, an increase in nutrient concentration, pH enhancement, reduction of total leaching, etc.) and the resident soil flora and fauna.

In Colombia, the amendment of soil with vegetable charcoal has led to a considerable increase in *Zea mays* yield, which went from 24% (in the second year) to 140% (in the fourth year) compared to an control plants grown in absence of biochar (Major et al., 2010). The same remarkable increase in crop yield was observed when adjusting an orchard in the Mediterranean basin before cultivating *Triticum durum* (Vaccari et al., 2011).

While some authors attribute the benefits obtained after biochar addition to its fertilizer power and to an increase of nitrogen availability, other authors suggest that its beneficial effect is related to its buffering power and to the ability to make alkaline pH the main cause of improved plant growth conditions (Van Zwieten et al., 2008; Yamato et al., 2006). Moreover, Ahmad et al. (2012) highlighted that pH alkalization could lead to a reduction in lead assimilation and hence to an increase in seed germination in contaminated soils. Concerning germination in uncontaminated soils, biochar does not appear to have any influence on germination capacity or coleoptile length (Free et al., 2010).

In regard to element concentration, it has been reported that during the pyrolysis process, the availability of phosphorus in the biochar increases when maintaining the temperature around 450/500°C; however, if the temperature exceeds 700°C, then the volatilization occurs (De Luca et al., 2009). For this reason, when melting a soil at a low-temperature, vegetable charcoal makes more available phosphorus, which it stimulates root growth (Chan et al., 2008). In different studies, using biochar amendment, it has been observed that increase in availability and absorption regards not only phosphorus, but also potassium, calcium and zinc (Lehmann et al., 2003; De Luca et al., 2009; Major et al., 2010). Given the complexity of the existing interaction between biochar, soil, microorganisms and plants, it is not easy to understand the nature of the specific biochar property responsible for a certain effect observed in the plant. In general, it possible to stress that beneficial effects are the consequence of both the improvement of the physical chemical soil properties and the effects on fungi and bacteria (particularly those involved in nitrogen fixation) (Graber et al., 2010).

1.3 The effects of Biochar on plant nutrition and stress response: tested genes

Nitrogen is one of the most important elements for all plant species. During the early growth stages, young developing roots capture the nitrogen present in the soil to convert it in amino acids in leaves. These amino acids will be used in future for the proteins and enzymes synthesis mainly involved in the erection of plant architecture and in the several components of the photosynthetic system (Richardson et al., 2009). Combined with nitrogen, also phosphorus has an important role

in plant productivity. In particular, it promotes root development and increases fruits yield (Filgueira et al. 2000). These two elements allow the correct growth of the plant and when one of these fail the plant health and growth suffer. It is known that biochar application increases soil nutrient content. In particular, has been demonstrated that biochar significantly increases the content of total nitrogen, extractable phosphorus and cation-exchange capacity (CEC) (Mustafa et al. 2010). Such changes in soil physical, chemical and biological properties, in turn, may significantly influence plant health and growth. In order to determine the effect of changing soil properties on plant growth, we analyzed the expression of the most important genes codifying root transporters of nitrogen (AMT1.1, AMT1.2, NRT2.1 and NRT1.2 gene; Ludewig et al., 2002; Gansel et al 2001), phosphate (PT1 and PT2 gene; Liu et al., 1998) and water (PIP2.2 gene; Jang et al., 2004). Moreover, in order to test the plant health status, expression of genes related to the stress response (BH7 and BH12) has been analyzed (Perotti et al., 2017).

1.3.1 Genes encoding nitrogen transporter

AMT1.1 and AMT1.2 belong both to the ATM genes family (ammonium transporter).
AMT1.1 has a higher affinity for the ammonium in NH_4^+ form than NH_3 form and its expression is predominantly dependent on the local N status of the roots. Indeed, it is mostly stimulated in the portion of the root system directly experiencing N starvation; therefore, it is possible to claim that it is up regulated in nitrogen deficiency condition (Ludewig et al., 2002).
AMT1.2 is expressed only in hair roots and it is involved in root ammonium transport but in contrast to AMT1.1 has not a specific affinity regarding the ammonium form. This gene is up-regulated when the nitrogen resource (NH_4^+ and NH_3) is present in high concentration in the soil. In recent studies (Lauter et al., 1996; Wang et al., 2001), it has been observed that AMT1.2 in *Solanum lycopersicum* is induced by both ammonium and nitrate resupply.

NRT2.1 and NRT1.2 genes are involved in the nitrate absorption.
NRT2.1 belongs to the family of HATS genes (high affinity transport system). Its expression is controlled by shoot-to-root signals of N demand under N deficiency condition. Gansel et al (2001) identified NRT2.1 as the first gene of the long-distance signaling that informs roots of the entire plant's status.
NRT1.2 belongs to the family of LATS genes (low affinity transport system) and, in *Solanum lycopersicum*, Ono et al (2000) observed that NRT1.2 is induced by NO_3^- in root hairs and that its expression increased after prolonged N starvation.

1.3.2 Genes encoding phosphate transporter

PT2 and PT1 genes are directly involved in phosphate root transport. These two genes belong to a family that codify for 12 membrane-spanning domain proteins and show a high degree of sequence identity to known high-affinity Pi transporters. Both genes are highly expressed in roots and PT1 also in leaves. In *Solanum lycopersicum* and *Arabidopsis thaliana*, their expression is markedly induced by Pi starvation. Their transcripts are primarily localized in root epidermis and PT1-mRNA was also observed in palisade parenchyma cells of Pi-starved leaves (Liu et al., 1998).

1.3.3 Genes encoding water transporter

PIP2.2 gene is an aquaporin belonging to a highly conserved group of membrane proteins called major intrinsic proteins. These proteins facilitate water transport across biological membranes. In the specific, PIP2.2 belongs to the plasma membrane intrinsic protein (PIP) sub group that are involved in plant response to environmental stimuli.
Moreover, PIPs are the main genes that response to water stress condition. In particular, PIP2.2 is up-regulated in roots when these are under water stress condition (Jang et al., 2004).

1.3.4 Genes involved in plant stress response

Homeobox is a class of genes that act as regulators of different aspects of organisms development both plants and humans. Moreover, several research works showed their essential role from the embryogenesis to the latest stages of cell differentiation (Kmita and Duboule 2003, Morgan 2006, Wang et al. 2009). The superfamily of homeodomain-leucine zipper (HD-Zip) –genes, belonging to the Homeobox genes, have been found only in plants and seems to have specific functions in their development (Perotti et al., 2017). To date, several links have been reported between homeobox genes and plant hormones such as the abscisic acid (ABA), which is one of the most important hormones in plants (Son et al., 2010). In particular, this phytohormone is produced when plant undergoes to stress conditions such as water deficit, inducing a number of physiological changes. In *Arabidopsis*, ATBH6 gene (Söderman et al. 1999), ATBH7 gene (Söderman et al. 1996) and ATBH12 gene (Lee and Chun 1998, Henriksson et al. 2005), are highly induced by ABA as well as water stress. In the present work, we have chosen BH7 and BH12 genes specifically active in water deficit conditions with a consequently negative regulation of growth (Söderman et al., 1996; Hjellström et al., 2003). Moreover, in *Arabidopsis*

thaliana, the BH12 is closely related to BH7 sharing over 80% identity in the deduced amino acid sequence of their HDZip motives (Olsson et al., 2004).

1. 4 Agronomic and model plants chosen to evaluate the effect of biochar

The plants chosen in this thesis concern agronomic plants such Cherry tomato of Pachino vr and Chardonnay cv. grape and *Arabidopsis thaliana*.
Both Cherry tomato and Chardonnay plants are two crops with a high commercial and agronomic impact on Italian economy and *Arabidopsis thaliana* is a genomic model plant.

1.4.1 *Solanum lycopersicum* (L.) (Cherry tomato of Pachino vr)

Tomato is a plant belonging to the Solanacee family. It is native of northern Chile and Ecuador and it was introduced in Europe in the 1540 by Spanish Hernán Cortés and today tomato is a plant species with a great commercial importance for the Italian agronomy (Leonardi et al., 2000 a, b).
In general, in Mediterranean region, cherry tomato plants is grown in passive solar greenhouses and this type of cultivation affects the qualitative and quantitative properties of tomato fruits. In particular, it has been shown that the content of antioxidants in the fruits can be affected considerably by environmental factors (Dumas et al. 2003).
Tomato plants produce a climacteric fruit, represented by an edible berry with a red color and with a variable size and shape which, after its complete ripening phase, is characterized by approximately 93 - 96% of water content. In the berry there is a smooth and thin epicarp, a fleshy mesocarp, and an endocarp subdivided into two or more logs. In each log, immersed in placental tissue, there are seeds, more or less numerous, small, flattened, yellow and rich in oil.
Tomato berry presents a relatively small genome, and more than 1000 molecular markers have been identified. (Manning et al., 2006). The resulting genetic map has been used in the identification and localization of quality traits (QTL), which influence the development and maturation of many fruits (Giovannoni 2007).
In recent times, tomato berries has been valued for their nutritional, dietetic and health traits. From several recent medical researches, the key-role of tomato berries has emerged as a supplier of antioxidant compounds essential in human metabolism such as like cis-lycopene, trans-lycopene and β-carotene (Figure 1.9) and other carotenoids.

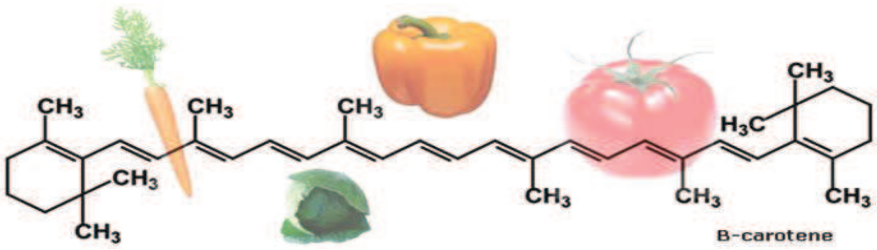

Figure 1.9 Chemical structure of β-carotene; adopted from www.sicurezzanutrizionale.org site

Biochar effects on tomato plant growth

Due to both agro-food and socio-economic importance of this plant species, many studies have been conducted on tomato plants to evaluate the potential effects of biochar amendment on their growth and harvest yield (Figure 1.10). In this regard, Graber et al., 2010, has observed an increase in the length of the stem and in the leaf area when tomato plants were grown on soil treated with biochar.

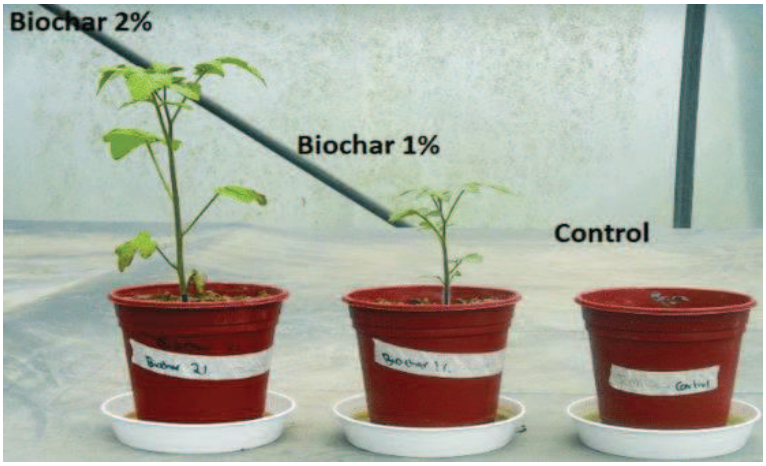

Figure 1.10 Tomato plants with different biochar rate, adopted from www.researchgate.net site

Moreover, recent studies have described an increase in overall organic matter obtained from bio-cultivated tomato cultivars (Yilangai et al., 2013). This result suggests that biochar could be used as an excellent improver of plant biomasses and therefore is not unreasonable to develop a technology that could allow the reuse of vegetable waste obtained from tomato industry. In past studies, it has been shown that the biochar addition to the soil could improve the agricultural production of the Pietrarossa variety (5%). Moreover, this treatment seems to increase nutrient concentration in the soil through a reduction of the imbalance present in the soil-plant system (typical of intensive agricultural areas) which leads to a slow development and a reduced production of plant biomass (Ichar.it).

1.4.2 *Vitis vinifera* (L.) (Chardonnay cv)

Grapevine (*Vitis vinifera* L.) is a perennial woody vine that produces the most economically important fruit crop in the world. In particular, among the great number of wine varieties, Chardonnay seems to be more tolerant to water deficit and salinity changes (Figure 1.12).
The grapevine fruit, the grape, is an infruttescence, that is, a group of fruits, called bunch.

Figure 1.12 *Vitis vinifera*, Chardonnay vr.; adopted from www.aisitalia.it site

The cluster is composed by a large number of small size berries with a light color in white grape while with dark color in case of black grappa.
The truss, or rachis, is the center of the cluster, branched in ramioli and then in pedicels, which carry the flowers and then the fruits, the grapes.
The grapes are mainly used for wine production and also for dry or fresh fruit consumption; finally, from the grape is possible also obtain a non-alcoholic beverage (juice) and grape seed oil from the seeds.
The constituents of grapes and wine such as polyphenols, anthocyanins, flavonols, have been studied for several years since they play an important role not only in the quality of grapes and wines but also in their beneficial effects on human health linked to their antioxidant properties. Epidemiological studies conducted in the early 1990s have shown that in France, where wine consumption is high and nutrition is based on a fat-rich diet, mortality following cardiovascular disease was reduced compared to other countries. This phenomenon, called the "French paradox", was positively correlated with wine consumption and its antioxidant compounds such as proanthocyanidins and resveratrol, which, among the various properties, have the ability to lower the levels of LDL cholesterol in the blood (Renaud and De Lorgeril, 1992; Frankel et al., 1993; Teissedre et al., 1996).

Recently, numerous epidemiological studies have shown that these secondary metabolites protect from the onset of chronic and degenerative pathologies, especially for cardiovascular system, due to their antioxidant, anticancer, anti-inflammatory and antimicrobial properties. These bio-components, once extracted from various parts of the plant, could also find important applications as active ingredients in pharmaceutical products, in fortified foods, dyes, and as natural preservatives for the food industry.

Biochar effects on grapevine plant growth and fruit quality

Baronti et.al, 2014 studies throw light upon the impact of biochar on soil-plant water relation in a perennial crops, and they demonstrate that biochar could effectively be used to increase water content in the soil. This effect upon water content it reduces the chance of a plant to undergo water stress and it increases consequently the photosynthetic activity without affecting soil hydrophobicity. Study by Genesio et al. 2015, shows that biochar application increased soil water content and plant available water and, this can be involved in the substantial increase of productivity (yield, average cluster weight and berry size) in all harvests.

Given that grapevine is a plant species with a great commercial importance for Italian and worldwide food economy, many studies are evaluating the potential effects of biochar on grapevine growth, harvest yield and fruit quality. Unexpectedly, no significant effects were observed on grape quality parameters and this suggests that the increased plant water availability due to biochar has a complex mechanism of action on plant physiology and on fruit tissues differentiation (Bonilla et al. 2015).

1.4.3 *Arabidopsis thaliana* (L)

Arabidopsis thaliana (Figure 1.13) is a flowering annual plant belonging to the Brassicaceae family. It represents the major model plant for its several advantageous traits, such as: a) simplicity in the cellular organization, b) rapid life cycle (6 weeks) and c) easy cultivation in restricted space and in non-soil media, d) efficient transformation methods when *Agrobacterium tumefaciens* is used (Hochholdinger and Zimmermann, 2008; Petricka et al., 2012).

Figure 1.13 *Arabidopsis thaliana* model plant; adopted from www.nature.com site

Its small genome (sequenced 114.5 Mb of total 125 Mb) allows to carry out depth genetic study about important genes (The Arabidopsis Genome Initiative, 2000). However, despite some progress made with other model crop, *Arabidopsis thaliana* remains the best characterized experimental system for studying the effects of biotic, abiotic stress and/or induced factors.
Due to such important characteristics, this plant species has been chosen as model plant in the present work.

Arabidopsis thaliana DR5:GUS line

In our research work, we have used *Arabidopsis thaliana* DR5:GUS seeds. In order to study auxin signalling the promoter DIRECT-REPEAT5 (DR5) has been used, - as a synthetic promoter that responds directly to the presence auxin.
DR5 is the site-where the directed mutation in the 5' end of the D1-4 AuxRE has been realized (Ulmasov et al., 1997).
This synthetic DR5 promoter has higher activity than natural AuxREs and for its property it is very useful when studying genes whose expression is auxin-responsive.
Moreover, DR5 promoter is widely used to study the auxin signalling through molecular marker such as GUS.
GUS (β-Glucoronidase) is a reporter gene that can be transcribed or translated under the direction of the controlling sequences of another gene called controller and, in the specific, this hydrolase enzyme catalyses the cleavage of a wide variety of β-glucuronides (Jefferson et al, 1986).
The above-mentioned reporter system has been developed in order to detect in plants the transgene tissues that, put into a X-Gluc solution, they colour them with a blue colour – as a result of the enzymatic activity (Jefferson et al., 1987).
The transgenic line of *Arabidopsis thaliana* was a wild-type expressing promoter:GUS constructs to characterize the pattern of the DR5 gene (an unpublished line from Riccardo Siligato and Ari Pekka Mähönen, University of Helsinki),
This union of the DR5 controller and the GUS reporter gene has been allowed us to individuate the stained root tips (a region in which the auxin is more expressed) and consequently we have able to count the roots when these were treated with the GUS solution.

1.5 The cost of using biochar in agronomy

Beside the positive effects of biochar in terms of crop yield, the effective costs-benefit ratio for its application still need to be discussed. According to Wrobel et al. (2015) biochar production from organic waste is a potential method for carbon sequestration and for the residual management costs. In the research work of Harsono et al. (2013) the Net Present Value (NPV) in Selangor and Malaysia for the production of biochar is greater than zero, which indicates that the investment for biochar production is economically advantageous and feasible. Kung et al. (2013) studied the benefits / costs ratio of biochar production asserting that the slow pyrolysis is more profitable than the fast due to the biochar on site value and the GHG reduction. Thus, to date

seems that the value of biochar application as soil amendment is higher than its value in terms of production energy process.

1.6 Possible negative effects of the application of biochar

There is still debate on the positive effect of biochar on plant growth and crop productivity (Jeffery et al. 2011). In the early 80's Kishimoto and Sugiura (1985) found yield reductions of soybean in plant treated with biochar and attributed this effect to the reduction of nutrients due to lower pH conditions. In particular, lower values of soil pH may increase the availability of aluminum element (Al), which is responsible for the phytotoxicity of the soil. In fact, in acidic soil, high concentrations of aluminum stop the growth of plant roots, block the absorption of calcium, and limit the plant productivity and development. Furthermore, aluminum is also expected to inhibit the cellular division process, damaging DNA and interrupting the plant growth (De Manzi et al., 1984). Graber et al. (2010) identified a set of chemical compounds contained in biochar and that negatively affect microbial growth and survival. These compounds include ethylene glycol and propylene glycol, hydroxy propionic acid and butyric acid, benzoic acid and o-cresol, quinones (resorcinol and hydroquinone), and 2-phenoxyethanol. Low levels of these toxic compounds in the soil promote the selection of less-sensitive microorganisms that, without competitors, proliferate at the expense of the microbial population useful for plant growth (Graber et al. 2010). In addition, biochar contains potentially toxic elements that could negatively affect the soil properties (CEC, 2008). Between these several compounds, the Potentially Toxic Elements (PTEs -heavy metals natural presents in the biochar parental material that increase in concentration after the pyrolysis process,) and the polycyclic aromatic hydrocarbons (PAHs - organic compounds produced during the pyrolysis process) are worthy of mention (Koppolu et al. 2003). Both these compounds have the potential to interfere with soil quality and may be absorbed from plants, included in fruits, and consequently end up in food products for human consumption (Badger et al., 1960; McGrath et al., 2001). Finally, Lehmann et al., (2011) reported that a decrease in tensile strength of the soil after the application of biochar facilitates the movement of invertebrates, causing higher predation of roots. It is still unclear whether this tensile strength reduction has a negative or positive effect on the root system of the plant.

1.7 Aim of work

In literature, there is a large amount of studies about biochar and its possible applications in agronomic field.

Never the less, studies of biochar effects on biological processes in plant species, on their relative molecular profile and on qualitative profile of fruit are very scant when not completely absent.

Among all possible candidate crop, *S. lycopersicum* and *V. vinifera* have been chosen in this thesis, and this choice is due to their economic interest and to a wide availability of bibliographic references and database transcripts. *A. thaliana* has been chosen to be used as model plant.
In this context, research activities have been carried out to understand the effect of biochar upon different morphological and molecular traits with particular attentions to the above- and below-ground biomass distribution in fruits and roots. Biochar rate was 30t ha^{-1} according to Baronti et al. (2010) that found an important improvement of *Triticum durum*, *Zea mays* and *Lolium perenne* plant yields.

The initial hypothesis was that the biochar amendment could reduce soil nutrient leaching and could improve plant growth.

In regard of molecular mechanisms affected by biochar a particular attention has been given to the expression of LeAMT1.1, LeAMT1.2, LeNRT1.2, LeNRT2.1 and LePT1, LePT2 genes in *S. lycopersicum* with the aim to evaluate the nitrogen and phosphate root absorption. Meanwhile, expression of AtBH7, AtBH12, AtAMT1.1, AtNAR2.3, AtNRT1.2 and AtNRT2.1 genes has been analyzed in *A. thaliana* in order to evaluate the nutrients absorptions and the plant health status during biochar treatment.

Finally, in order to quantify the biochar effect on fruit development and quality, we have evaluated fruit dry biomass, alcoholic juice potential and fruit antioxidant content.

Chapter II

Effect of biochar on the response of *Arabidopsis thaliana* (L.) to water stress

2.1 MATERIALS AND METHODS

In summary, with the aim to investigate the influence of biochar on *Arabidopsis thaliana* seedlings, we have investigated the following morphological and molecular parameters with seedlings that were grown under normal or water stress conditions:

- Above and below-ground biomass compartments

- dry weight of roots, stem and leaves

- Leaf

- Leaf area, leaf number
- chlorophyll *a*, *b*, *total* content
- Leaf relative water content (LRWC)
- Leaf anatomy

- Root

- Root length
- Root tips number
- Root anatomy

- Soil

- Soil water content (SWC)

- Gene expression

- AtAMT1.1, AtNRT2.1, AtNAR2.1, AtPIP2.2, AtBH7, AtBH12, AtACT2

2.1.1 PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis thaliana DR5:GUS seeds were sown in 42 treated pots (350 cc of volume) filled with commercial soil (sandy soil) mixed with woody biochar (vineyard parental material to obtain a ratio 30 t ha^{-1} (Baronti et al. 2010). Other 42 pots were filled only with commercial soil and were considered as controls. Afterwards, all pots were exposed to a photosynthetically active radiation (PAR, 400–700 nm) of 120 μmol m^{-2} s^{-1}, air temperature of 25°C a humidity regime of 75%. In the case of water stress treatments a calibration curve was developed relating a known soil water

content by weighing the pots with a direct water potential (MPa) measurements by gypsum block. Different levels of soil water potential (- 0.003 and - 0.09 MPa, values of soil water potential corresponding to a middle stress in the calibration curve) were applied to 21 treated and 21 control pots induced by watering withdrawing. In order to keep constant the level of soil water stress, daily measurements of soil water content were carried out. The water stress experiment lasted 32 days from germination but seedlings were water stressed only for 20 days only when seedlings were well developed. Four different treatments were applied, named: water-no biochar, water-biochar, no water-no biochar and no water-biochar.

2.1.2 BIOMASS DISTRIBUITION IN ABOVE AND BELOW-GROUND COMPARTIMENTS

To evaluate root dry weight (g) of roots (RDW) and leaf/above dry weight (ADW), samples were collected separately and dried at 70°C until constant weight was achieved.
RDW was measured after roots sample were repeatedly rinsed under running tap-water.

2.1.3 LEAF

The total leaf area and the leaves number of each plant were measured by means of a scanner at a resolution of 400 dpi. Successively, images were analyzed by WinRhizo Pro V. 2007d software (Regent Instruments Inc. Quebec).
To determine the chlorophyll (*a*, *b* and *total*) content (µg ml^{-1}), leaves were chopped to obtain 0.5 g of ground material which was homogenized in 10 ml of 80% acetone solution for 30 min.
The extract was then centrifuged at 2500 rpm for 5 minutes and 1 ml of supernatant was collected and diluted by adding 9 ml of 80% acetone. The diluted sample was measured by spectrophotometer at 663 nm and 645 nm. Chlorophyll contents were calculated according to Arnon equation (1949).

In order to determine the relative water content (LRWC) of the leaves a specific quantity of each leaf sample was placed in a pre-weighed airtight vial and then weighed to obtain the initial weight (W). The samples were then hydrated to full turgidity for by leaving them 3-4 h under normal room light and temperature in the vials. After hydration, the samples were taken out of water and dried by filter/tissue paper before being immediately weighed to obtain the weight under turgor conditions (TW). Samples were then dried in oven at 80°C for 24 h and weighed (after being cooled down in a desiccator) to determine dry weight (DW). Relative water content was obtained

with the following equation (Barr et al., 1962):
RWC (%) = [(WDW) / (TWDW)] x 100,

W – Fresh Weight
TW – Turgid Weight
DW – Dry Weight.

For anatomical analysis, leaves samples were fixed overnight in 25% (v/v) glutaraldehyde and 37% (v/v) formaldehyde in 0.05 mol/L sodium phosphate (FIX solution). Afterwards pieces of entire leaf and cross sections of 10 μm were placed on glass slides with distillated water before being observed with an optical microscope (OLYMPUS BX63) at a 4X, 10X and 40X magnification. The images acquisition was performed by means of a digital camera (OLYMPUS DP72) and images were processed with Image J software to determine leaf thickness and stomata number.

2.1.4 ROOT

In order to measure the biochar effects on the root growth, roots sample were repeatedly rinsed under running tap-water and after, scanned by a calibrated scanner (Epson, Expression 10000 XL) at a resolution of 800 dpi.
Afterwards, the acquired images were analyzed using WhinRhizo Pro V. 2007d software (Regent Instruments Inc. Quebec).
The extracted data were processed to obtain information about the root length.
To determine the root tips number *Arabidopsis* roots were subjected to histochemical GUS staining.
Arabidopsis roots were fixed with 90% acetone for 1 hour on ice and washed in 0.05 M Sodium Phosphate buffer (NaPi buffer with pH 7.4) three times within 1 hour on ice. Afterwards, they were vacuum-infiltrated for 10 min in GUS buffer (1 M NaPi buffer, Triton X-100, potassium ferricyanide, potassium ferrocyanide, and X-Gluc dissolved in DMF). Subsequently, samples were incubate in a fresh GUS buffer in the dark at 37°C until blue color was visible. After GUS protocol, the roots were scanned by a scanner (Epson, Expression 10000 XL) at high resolution and the acquired imagines elaborated with Image J software to count the root tips number.
Root anatomy was carried out to investigate the effects of biochar on root morphology so, root portions pre-fixed in a FIX solution, were dehydrated in different diluted ethanol-water solutions (30% - 50% - 70% - 90% - 100% x 2) and pre-infiltrated overnight with a solution of absolute

ethanol/glycol resin (1:1 ratio). The samples thus obtained were fixed on a support with a polyethylene glycol-basic resin (Leica Historesin, Leica Biosystem, Germany) and sections (7 μm thick) were cut with a sliding microtome (Leica 2400). The sample slices were stained with toluidine blue over glass slides and afterwards, they were observed with the optical microscope (OLYMPUS BX63) with a magnification of 10X and 40X. The acquired images with the digital camera (OLYMPUS DP72) were then analyzed by Image J software.

2.1.5 MEASUREMENT OF SOIL WATER CONTENT

To evaluate the biochar effect on the water availability in the soil, daily measurements were performed by weighting each pot.
Afterwards, pot and soil weight were subtracted to the total pot weight in order to obtain the soil water content (g).

2.1.6 EVALUATIONS OF RELATIVE GENE EXPRESSION IN MARKER GENES FOR NITROGEN AND PHOSPHATE ABSORPTION FROM SOIL IN PRESENCE OF BIOCHAR

In order to evaluate the relative gene expression of the specific marker genes such as AtAMT1.1, AtNRT2.1, AtNAR2.1, AtPIP2.2, AtBH7, AtBH12, AtACT2, primers were designed by using Primer3 Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with setting conditions such as to obtain a good quality primers.
Table 2.1.1 shows: primers sequences, melting temperatures, amplicons length for each PCR products.

Gene	**Primers** 5' –> 3'	**Product** (bp)	**Melting T°**
AtAMT1.1	F: TCCTGTTGGGTCCTAATGCCA R: TGACCAGAACCAGTGAGAGACGAC	500	55°C
AtNRT2.1	F: AGTCGCTTGCACGTTACCTG R: ACCCTCTGACTTGGCGTTCTC	190	55°C

AtNAR2.1	F: CCAGAAGATCCTCTTTGCTTCACT R: CCCAATCGAGCTTAGCGTCCA	199	55°C
AtPIP2.2	F: GGCAACTTTGCTTGTAAAACTATGC R: AGTACACAAACATTGGCATTGG	102	55°C
AtBH7	L: TGTTTGAGTCTGAGACAAGG R: ATCTGGTTCTTCCTCAAACC	503	55°C
AtBH12	L: AGGTTCAGGTAGCTAGAGAG R: ACCAGTTAGGGTAATTGCTG	522	55°C
AtACT2	L: TCACAGCACTTGCACCAAGCA R: AACGATTCCTGGACCTGCCTCA	161	55°C

Table 2.1.1 Primers sequences, melting temperatures and amplicons length for each PCR products

In order to preserve the single-strand structure of RNA from degradation, it was necessary to sterilize tools and environmental by UV rays for 30 min, before the RNA extraction. In addition, the working bench was sterilized by pure ethanol and RNaseZap Sigma in order to eliminate any possible trace of ribonucleases.

MirPremier microRNA isolation kit (Sigma) was used to extract RNA samples from root.

Samples powdered in N_2 liquid were aliquoted (0.07 g) in pre-cooled Eppendorf and 750 µl of Lysis solution \ 2ME (10 µl of β-mercapto-ethanol per ml of Lysis solution) was added. After 5 min incubation at 55°C, samples were centrifuged at 14.000 rpm was carried out for a time of 3 min freed from the supernatant and transferred to a "Filtration Column". The filtrate was centrifuged at 14.000 rpm for 1 min, recovered and 1.5 volumes of "Binding Solution" was added before vortexing. The solution was then filtered several times with the "Binding Column" for one min at 14.000 rpm, until the solution was exhausted. Subsequently the filter membrane was washed three times to remove any traces of phospholipids, proteins and carbohydrates with these steps: 1) 700 µl pure ethanol; 2) 500 µl of Binding Solution; 3) in the end with 500 µl pure ethanol. An additional centrifugations was carried out to remove any ethanol residue from the membrane.

30 µl of H_2O Nuclease-free was added to the membrane to extract acid nucleic and then a centrifugation at 14.000 rpm was carried out. To obtain a pure RNA elution, the genomic DNA was removed with RNase-Free DNase Set and as suggested by the kit protocol, then samples

were incubated at room temperature for 10 min.
The RNA was quantified by a spectrophotometer at 260 nm protein contamination was measured at 280 nm.

The RNA quality was verified by electrophoretic analysis with 1.5% agarose gel (1,5 g of agarose, 100 ml of TAE 1X buffer and 10 µl of SYBR Safe Gel Stain). In this case two bands corresponding at the 18S and 28S ribosomal subunits showed to be well separated on gel and that confirms in our samples the RNA was not degraded.
The RNA extract was immediately converted to cDNA by using the ImProm-II Reverse Transcription System (Promega) kit. The Retro-transcription (RT) reaction was carried out in two steps. In the first 10 µl of extracted RNA, 1 µl of Primer Oligo (dt) and 1 µl of dNTP were mixed in a micro-centrifuge tube and then positioned inside a thermocycler for 5 min at 70°C. To arrest the process the sample was kept at 4°C for 5 min. In the second stage 8 µl of master mix (20 µl of ImpromII 5X Reaction Buffer, 10 µl MgCl2, 5 µl of RNasin Ribonuclease Inhibitor Recombination and 5 ml of ImpromII Reverse Transcriptase) was added and then solution placed in a thermocycler for 5 min at 25°C, 60 min at 50°C, 15 min at 70°C.
The retro-transcript samples were then stored at -20°C.
To perform a PCR reaction, a mix solution was prepared with:

- 5 µl 5X Green GoTaq® Flexi Buffer
- 1,5 µl $MgCl_2$
- 15,9 µl H_2O Nuclease free
- 0,5 µl dNTP
- 1 µl primers (F+R)
- 0,1 µl GoTaq® DNA Polymerase (5u/µl)

In PCR tubes, 24 µl of this solution was mixed together with 1 µl of cDNA.
The PCR reaction was started by setting the thermocycler with the following program:

- 95 °C for 1 min
- 94 °C for 45 sec
- 60 °C for 1 min
- 72 °C for 1 min

} 35 cycles (25 for housekeeping gene)

- 72 °C for 5 min

PCR products were loaded (12µl template + 5µl Marker Rapid-Load PCR) on an agarose gel (2%) and as marker, the 1 kb DNA Ladder was used.
After the electrophoresis, the gel picture was obtained by means of ChemiDoc.

2.1.7 STATISTICAL ANALYSIS

For the data with a number of 5 replicates for each parameter a two-tailed t-test was applied with a significance level equal to 95% ($p < 0.05$). For the data with a no-normal distribution, before assessing their significance, normality tests were performed (Test Kolmogorov-Smimov and Test Shapiro-Wilk). This approach will enable us to apply statistical parametric or non-parametric tests on different data. For the data that did not meet the normal distribution neither square root or log transformed and, for both parameters, non-parametric static test was carried out (analysis of two independent samples) and the test Mann - Whitney U was applied as post-hoc test. The remaining have rather met the normal distribution condition and therefore, parametric statistical test was implemented (one- tailed log rank test); Bonferroni post-hoc test was subsequently applied.
Parametric and no-parametric analysis were applied to a significance level of 95%.
All data obtained by semi-quantitative PCR were normalized using the expression values of the housekeeping gene ACT2 and then treated samples were compared with control samples.
For the images obtained by the semi-quantitative PCR analysis were analyzed using Image J software. In this analysis, the optical density of pixels of each amplified band on the electrophoresis gels was calculated. The density values were calculated before for ACT2 and then for the marker genes. The normalization was calculated making a difference between density values of ACT2 and density values of each genes. The difference values obtained the different expressions of marker genes among the treated samples.
T test was applied to test the statistical significance of expression among marker genes differences in different samples.
All statistical analysis were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL,USA).

2.2 RESULTS

2.2.1 SEEDLING TRAITS

The highest RDW and ADW biomass values were observed in Biochar - water treated seedlings. Both root and shoot dry biomass, showed for biochar-water treatment values twofold higher than values measured for biochar – no water treatment (Figure 2.2.1).

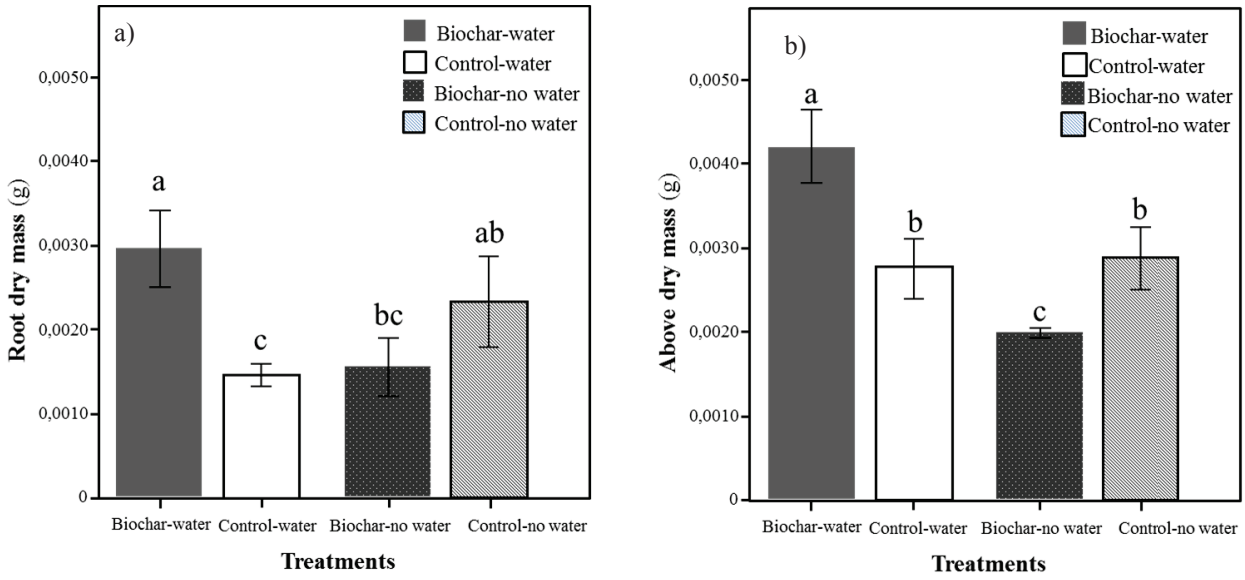

Figure 2.2.1 Dry biomass of root a) and above b) compartments. Each value represent the mean of (n=21) ±SE. a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

Total leaf area (2.2.2.a) showed no significant difference between Biochar-water, Control-water, and Control -no water treatment while Biochar- no water treatment showed the lowest value. Values measured in plant with Biochar – water treatment were twofold higher than values measured in plant with Biochar – no water treatment. Leaves number (figure 2.2.2 b) showed a significant difference only when biochar was added to seedlings being under normal watering regime. However, the lack of total leaf area increase indicated that the leaves of seedlings treated with biochar had slightly smaller dimension. Chlorophyll contents showed no significant result in the different treatments (Figure 2.2.2.c).

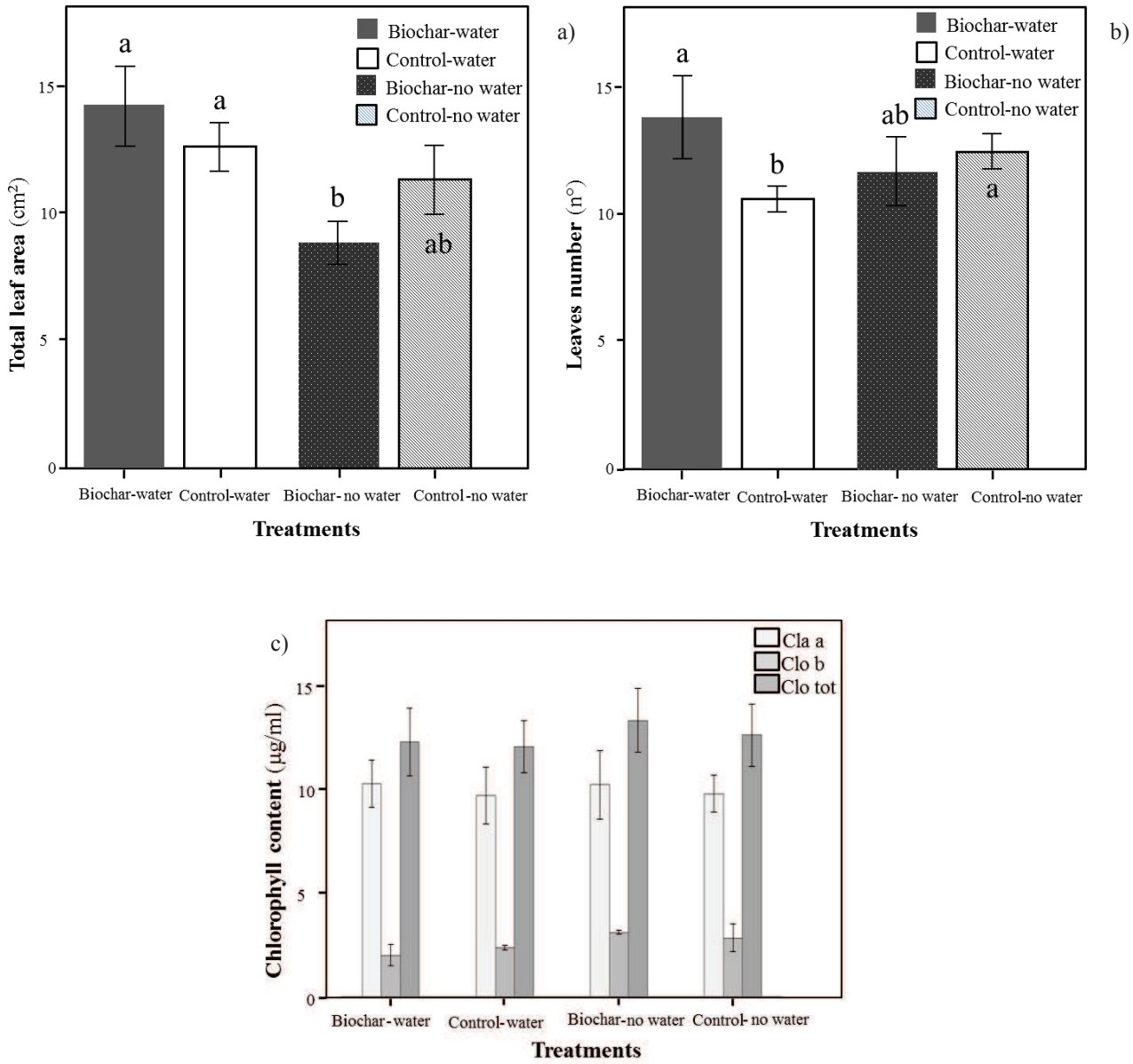

Figure 2.2.2 Leaf parameters: Total leaf area a), leaves number b) and Chlorophyll content c). Each value represent the mean of (n=21) ±SE. a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

The highest Leaf Relative Water Content (LRWC) values were measured in Control-water while the lowest were measured in control-no water treatment. In the case of normal watering regime control plants has the higher value. In the case of water withdrawn, the higher value was measure in biochar treatment (Figure 2.2.3).

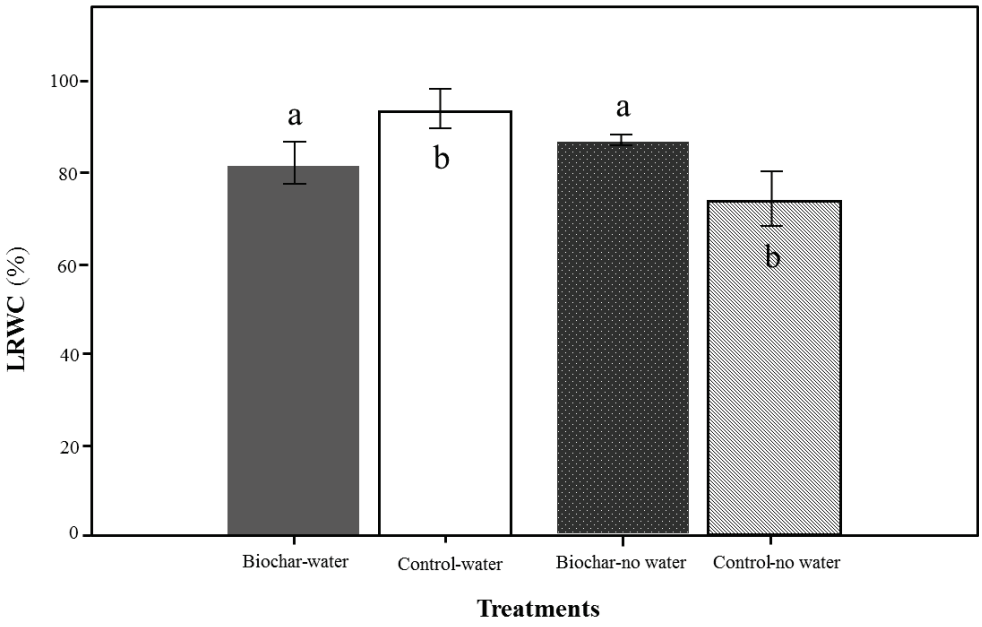

Figure 2.2.3 Leaf Relative Water Content values. Each value represent the mean of (n=21) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

In conclusion, regarding seedling traits is evident a general biomass increase of plants treated with biochar under normal watering regime. Therefore, the most important evidence of our results is the negative effect that the biochar exercises on the plants when applied under stress watering regime.

2.2.2 LEAF ANATOMY

About leaf anatomy, the highest values of leaf thickness (Figure 2.2.4) were observed under water stress condition but the biochar presence seemed to induce a slight thickness reduction in respect to the control only under normal watering regime.

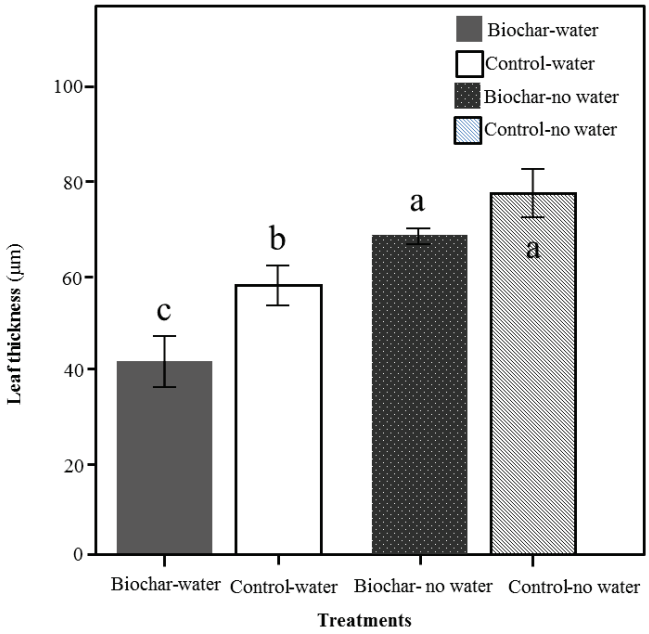

Figure 2.2.4 Leaf thickness. Each value represent the mean of (n=21) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

In regard to stomata number (Figure 2.2.5), the highest values were found under a normal watering regime independently from the presence of biochar in the soil.

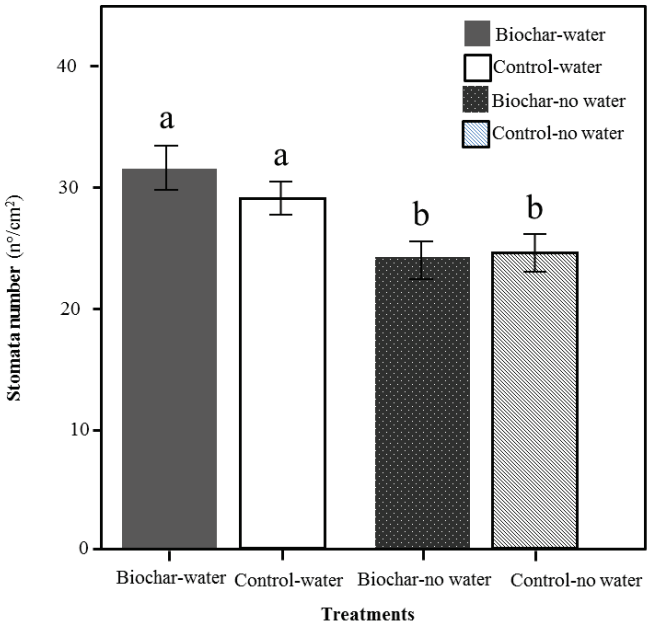

Figure 2.2.5 Leaf parameter: stomata number. Each value represent the mean of (n=21) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

2.2.3 ROOT ANATOMY

Fine root length showed the highest values in presence of biochar despite the number remained the same in control. Under water stress, the root length remained unaltered independently from biochar presence whereas the root tips number decreased (Figure 2.2.6.a, b).

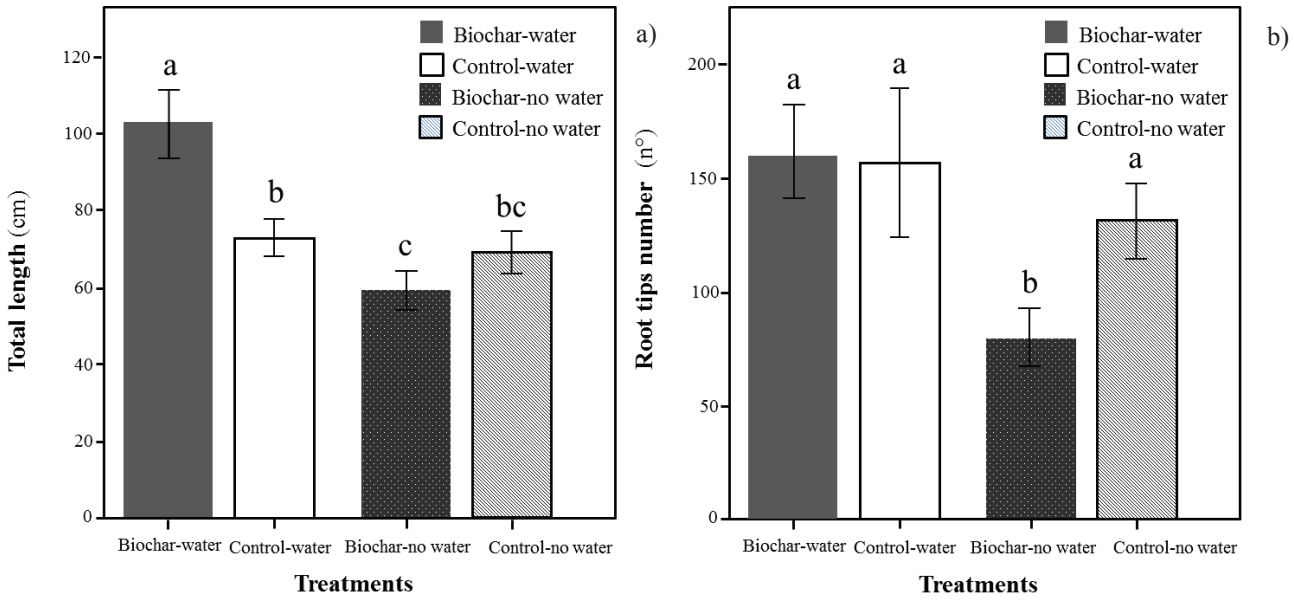

Figure 2.2.6 Root parameters: total root length a) and Root tips number b). Each value represent the mean of (n=21) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

Regarding root anatomy, the highest number of vessels present in the vascular cylinder (n°/µm2) (Figure 2.2.7) was observed in control when seedlings were under water stress regime.

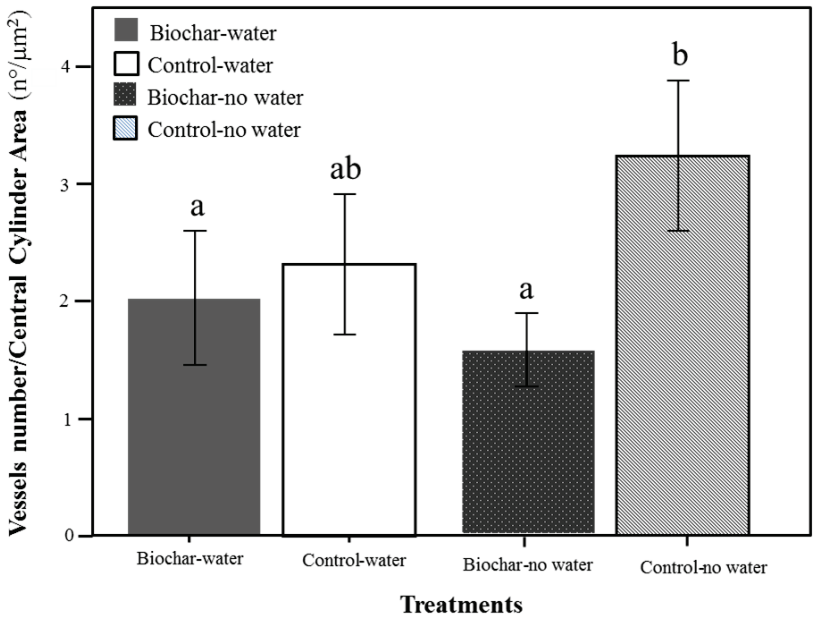

Figure 2.2.7 Vessels number in the vascular cylinder. Each value represent the mean of (n=21) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

2.2.4 SOIL TRAIT

The soil water content resulted to be higher in biochar treated pots under stress condition at all sampling point (Figure 2.2.8).

This confirmed the hypothesis that biochar retains water and prevents its loss.

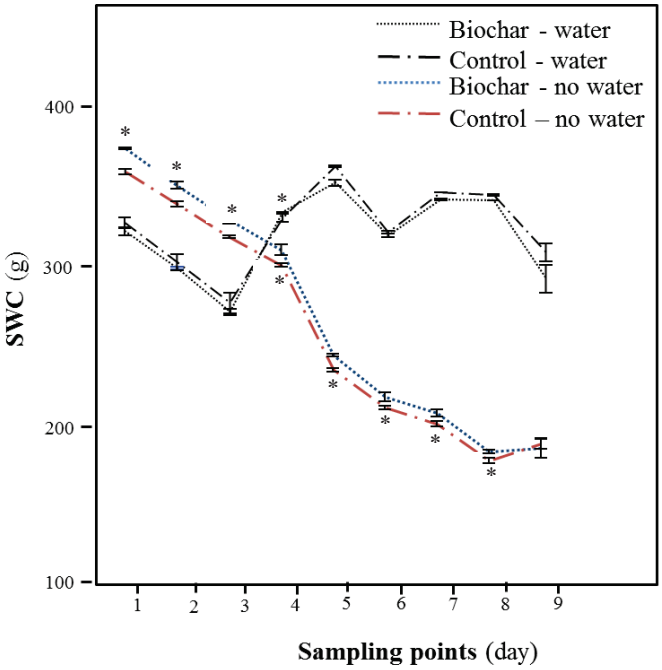

Figure 2.2.8 Soil Water Content (SWC) of pots. Each value represent the mean of (n=21) ±SE. An asterisk indicates a statistically significant difference among treatments ($p < 0.05$).

2.2.5 RELATIVE GENE EXPRESSION

Molecular analysis showed important difference regarding the relative gene expression of marker genes when plants were treated with biochar amendment.

BH7 marker gene (Figure 2.2.9.a) showed a higher value in the Control-no water treatment while in the other treatments no difference was detected.

BH12 marker gene (Figure 2.2.9.b) showed higher values in Control-no water and Biochar-no water treatment while no difference were detected between the two treatments under normal watering regime.

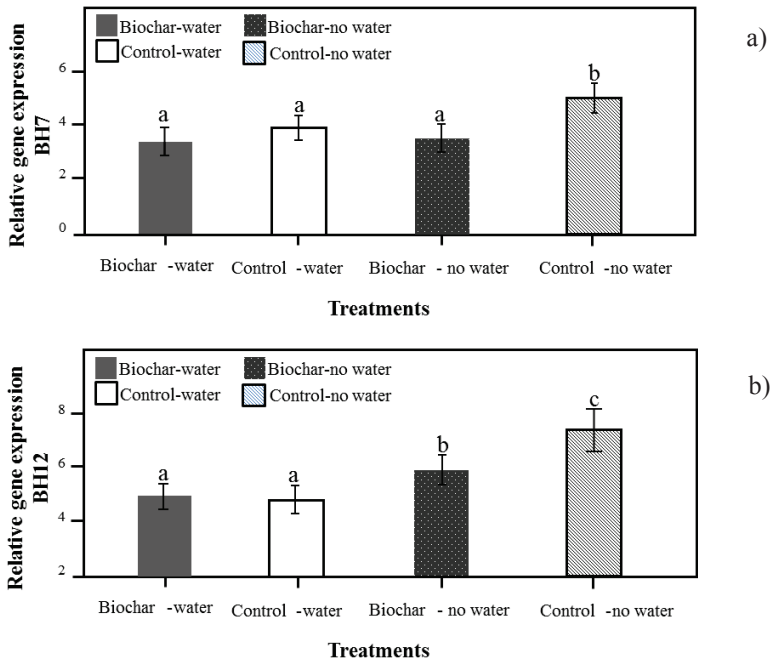

Figure 2.2.9 BH7 a) and BH12 b) relative gene expression. Each value represent the mean of (n=10) ±SE. a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

The relative expression of the AMT1.1 gene (Figure 2.2.10) showed a higher value in Control-water treatment with a significant difference than Biochar-water treatment. Concerning the gene expression in water stress condition, the gene showed a higher value in Biochar-no water treatment.

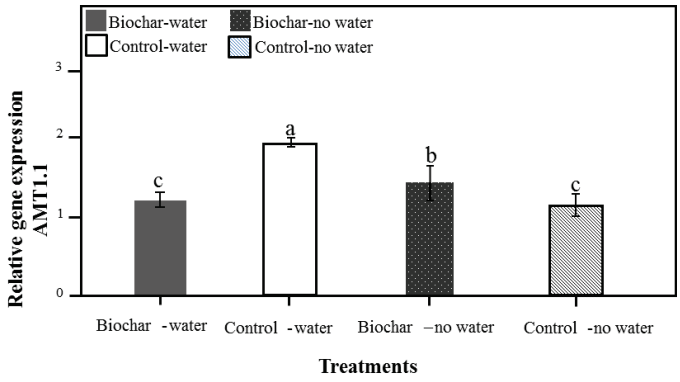

Figure 2.2.10 AMT1.1 relative gene expression. Each value represent the mean of (n=10) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

In the case of NRT1.2 gene (Figure 2.2.11) a higher value was observed in Control-water treatment while in stress water condition, a higher value was observed in Biochar-no water treatment.

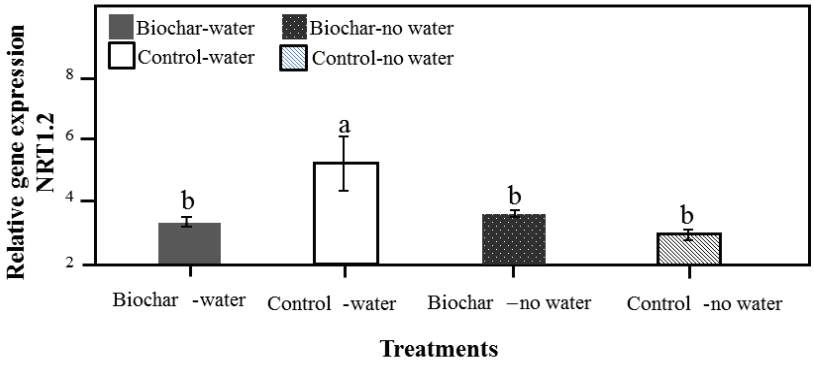

Figure 2.2.11 NRT1.2 relative gene expression. Each value represent the mean of (n=10) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

Relative expression of NRT2.1 gene (Figure 2.2.12) showed a higher value in Control-water treatment, while also in this case, in water stress condition the higher expression value was observed in the Biochar-no water treatment.

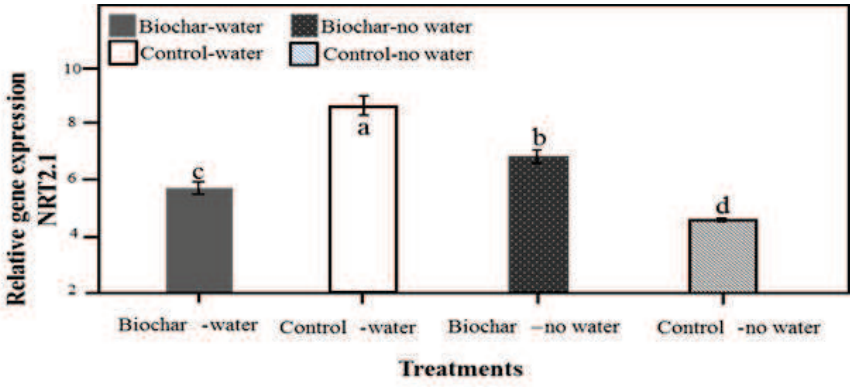

Figure 2.2.12 NRT2.1 relative gene expression. Each value represent the mean of (n=10) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

Finally, the relative expression of PIP2.2 (Figure 2.2.13) showed high values in Control-water and Biochar-no water treatment while the expression of this gene in Control-no water showed a minor expression.

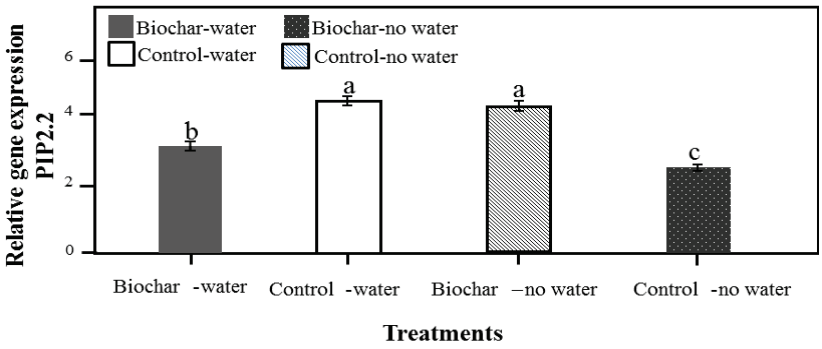

Figure 2.2.13 PIP2.2 relative gene expression. Each value represent the mean of (n=10) ±SE.
a, b, c indicate a statistically significant difference among treatments ($p < 0.05$).

In summary, our data suggest that the biochar use under normal watering regime affects positively the plant response to water and nutrients in the soil while, under stress condition, these responses are negatively affects by the biochar presence into the soil.

2.3 SHORT DISCUSSION

In the present work we found that biochar amendment had a positive effects on plant growth for all measured parameters but only under normal watering regime. This effect might be due to the higher nutrient content of biochar treated soil. Indeed has been observed that under water stress conditions biochar addition inhibits the growth of plants. This effect could be due to the water binding property of biochar (Conte et al., 2013). Thus, it is reasonable that under water stress condition, soil-biochar water potential has a lower value than the untreated soil so that both cell walls and epidermis cells of root hair are not able to overcome the strength.

Chapter III

Effect of biochar on the response of *Solanum lycopersicum* (L.) (Cherry tomato of Pachino vr)

3.1 MATERIALS AND METHODS (30t ha^{-1} experiment)

In summary, with the aim to investigate the influence of biochar on *Solanum lycopersicum* seedlings (Cherry tomato of Pachino vr), we have investigated the following morphological and molecular parameters with seedlings, which were grown under normal or water stress conditions:

- Above and below-ground biomass compartments

- dry weight of roots, stem and leaves

- Plant CHN analysis

- Leaf

- Leaf area
- Leaf number
- Chlorophyll *a*, *b*, *total* content

- Root

- Root length
- Specific root length (SRL), the root tissue density (RTD), Mean Diameter

- Fruit

- Number of Flowers and Fruits
- Morphometric parameters
- Titratable acidity
- Total soluble solids content
- Trans-lycopene, cis-lycopene and beta-carotene content
- Pannel test

- Soil

- Chemical analysis
- CHN and ICP analysis

- Gene expression

- LeAMT1.1, LeAMT1.2, LeNRT1.2, LeNRT2.1, LePT1, LePT2, LeTUB

- **Plant health status**
    - DNA extraction
    - Detection of *Phytophthora infestans* presence in leaf

3.1.1 PLANT MATERIAL AND GROWTH CONDITIONS

Six seeds of Cherry tomato were sown in 30 cylindrical pots (h24 cm, Ø21 cm lower and upper Ø 26 cm, 9000 cc of volume) filled with soil (sandy soil) mixed with woody biochar (vineyard parental material) to obtain a ratio of 30 t ha^{-1} (Baronti et al., 2010). Other 30 pots were filled only with commercial sandy soil and were considered as controls. After treated and control pots were maintained at field capacity under controlled conditions (temperature 25 ° C, humidity 75% and PAR 400/700 mol m^{-2} s^{-1}). After germination, seedlings were thinned and only one was left to grow in each pot. 10 treated plants (sandy soil with Biochar - B) and 10 control plants (only sandy soil - C) were collected at each of the three sampling time-points including the whole plant life cycle; early stage (ES), vegetative stage (VS) and fruit stage (FS). In order to maintain constant temperature and water potential during the experiment, measurements were carried out every 2 days. The pot experiment was carried out for a total of 20 weeks.

3.1.2 BIOMASS DISTRIBUITION IN ABOVE AND BELOW-GROUND COMPARTIMENTS (ES, VS and FS)

To evaluate biomass distribution dry weight (g), root (RDW), shoot (SDW) and leaves (LDW) were separately collected and dried at 70°C until constant weight was achieved, for each sampling point.
RDW was measured after roots samples were rinsed under repeatedly running tap water.

3.1.3 PLANT CHN ANALYSIS (ES, VS and FS)

The roots and leaf sampled during ES, VS and FS were firstly ground in liquid N_2 with mortar and pestle and after they were dried at 80 °C in order to eliminate any humidity trace. After the previous preliminary operations, the samples were analyzed for C and N concentrations with a CHN elemental analyzer (Perkin Elmer, 2400 series, II CHNS/O elemental analyzer).
The analyzer was calibrated with the atropine standard, and every 10th sample the calibration was again carried.

3.1.4 LEAF (ES, VS and FS)

The total leaf area and the leaves number of each plant were measured using a high resolution camera (OLYMPUS DP72) in order to acquire the images and after the acquisition the images were analyzed with the Image J software.
To determine the *a*, *b* and *total* chlorophyll content (µg ml^{-1}), leaves were chopped to obtain 0.5 g of ground material which was homogenized in 10 ml of 80% acetone solution for 30 min.
The extract was centrifuged at 2500 rpm for 5 min and 1 ml of supernatant was collected and diluted by adding 9 ml of 80% acetone.
The diluted sample was measured by spectrophotometer at 663 nm and 645 nm. Chlorophyll contents were calculated according to Arnon equation (1949).

3.1.5 ROOT (ES, VS and FS)

In order to measure the biochar effects on the root growth, roots of each sampled plant were rinsed repeatedly under running tap water and after, scanned by a calibrated scanner (Epson, Expression 10000 XL) at a resolution of 800 dpi.
Afterwards, the acquired images were analyzed by WhinRhizo Pro V. 2007d software (Regent Instruments Inc. Quebec).
The extracted data were processed to obtain information about the root length, the specific root length (SRL, m g^{-1}), the root tissue density (RTD, g cm^{-3}), the roots distribution in the main diameter size classes (0 - 0.25 mm, very fine roots; 0.25 - 2.0 mm, fine roots) and information regarding the mean diameter class.

3.1.6 MEASUREMENT OF FRUIT TRAITS

During the phenological phase of flowering and fruit development, the number of flowers and fruits per plant was monitored.
In order to valuate fruits parameters, tomato fruits were harvested at point 5 of the ripening color chart (USDA 1975).
To determine fruit biomass and fruit water content, 40 tomatoes for each treatment were weighed before (fresh weight, FW, g) and after (dry weight, DW, g) drying at 70°C for 48 h. The tomato plant fertility was determined considering the seeds number and the dry seeds weight of 10 tomatoes for each treatment.
Morphometric fruit parameters, such as polar and equatorial diameters, epicarp thickness, right

and left mesocarp thickness were measured by a scanner at high resolution and after analyzed with ImageJ software (20 tomato fruits were sampled and processed for each treatment).

In order to determine qualitative parameters tomato fruits were homogenized by homogenizer (VWR Collection, VDI 12).

The titratable acidity (TA), expressed as percentage of citric acid, was measured using the titration method at pH 8.1 with NaOH (0.1 N) (Petruccelli et. al 2015).

The pH was measured using an electronic pH meter (pH meter Eutech Instruments pH 700, 2013).

Finally, the total soluble solids content (TSSC), expressed as °Brix, was measured by refractometer (HANNA Instruments, HI 96813), prior homogenization and centrifugation at 13.000 g for 20 min at 8°C. (George et. al 2004).

The lycopene and β-carotene content was determined to quantify antioxidant properties.

Six g of the homogenate were extracted in dark condition using 60 ml of a hexane-methanol-acetone (2: 1: 1 volume/volume/volume) mixture with 2.5% of BHT at 4°C and for 30 min.

Subsequently, 10 ml of distillated water was added and the polar phase (hexane) was recovered.

On the polar phase spectrophotometric readings were carried out respectively at 472 nm (maximum absorbance peak of the trans-lycopene), 502 nm (maximum absorbance peak of the cis-lycopene) (George et al. 2004) and 453 nm (maximum absorbance peak of β-carotene) (Bohm et al. 2002).

The analysis above mentioned were carried out for 5 replicates per each treatment.

In order to evaluate the tomato taste, a panel test was performed. The sensory panel consisted of 7-10 trained subjects, panellists who were able to detect a significant flavor difference between two tomato fruit samples (biochar treated and control fruits) when presented as a paired comparison. Each panellist received 16 samples to assess different parameters on 1-9 scale (eg 1 = very weak aroma intensity and 9 = very strong aroma intensity).

The valuated parameters were aroma, acidity, consistency, juiciness and T-Test was performed to evaluate the statistically significance of the obtained data.

3.1.7 SOIL (ES, VS and FS)

The chemical soil analysis were carried out to measure total organic C and N content, available phosphorus (P_{av}), CE (electrical conductivity), pH and exchangeable cations content (CEC).

Total C and total N soil contents were determined by dry combustion Elemental Analyzer (Thermo Fisher Science). The P_{av} content determination was carried out by reference to the indications of the Olsen method (Arduino et al. 2000).

The CE was measured using the saturation method with barium-chloride at pH 8.5, where the adsorbed barium was shifted with a 0.05 M $MgSO_4$ solution and finally, the exceeded Mg was titrated with a 0.025 M EDTA solution (Gessa et al. 2000).
Soil pH has been measured using the potentiometric method with a 1:2.5 soil-water suspension. The CEC was measured using a barium chloride-triethanolamine buffered solution at pH of 8.2 and after, the parameter quantification was performed using a flame atomic absorption spectrophotometry. Finally, the total elements concentrations were determined for subsamples dried at 105°C for 24 h according to the EPA method 3052 and the filtered solutions were analyzed using an ICP-OES spectrophotometer (Varian Inc., Vista MPX).

3.1.8 EVALUATIONS OF BIOCHAR EFFECT UPON THE GENE EXPRESSION OF MARKER GENES FOR NITROGEN AND PHOSPHATE ABSORPTION FROM SOIL

In order to evaluate the relative gene expression of the specific marker genes such as LeAMT1.1, LeAMT1.2, LeNRT1.2, LeNRT2.1, LePT1, LePT2, LeTUB, primers were designed by using Primer3 Plus software (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with setting conditions such as to obtain a good quality primers.
Table 3.1.1 shows: primers sequences, melting temperatures, amplicons length for each PCR products.

Gene	**Primers** 5' –> 3'	**Product** (bp)	**Melting T°**
LeAMT1.1	F: GGCGCATATAATCCAGATAC R: ATTCCTAATTTCGGATCCTC	208	55°C
LeAMT1.2	F: AACAAGACATGGAGGATTTG R: CTTTGAGATTTGACGTTTCC	217	55°C
LeNRT2.1	F: ACAGAAGACGGGTACTGTTG R: CAATGACGTTGTCTGTTGAC	230	55°C
LePT1	F: ACCATCACTGGACTCTCAAG R: GCGTATAAGAATCCAAATGC	208	55°C

LePT2	L: GCTGCACAAAGTAAAGATCC R: GTTTCTTGTGATGCTTCCTC	167	55°C
LeTUB	L: TGACGAAGTCAGGACAGGAA R: CTGCATCTTCTTTGCCACTG	90	52°C

Table 3.1.1 Primers sequences, melting temperatures and amplicons length for each PCR products

In order to preserve the single-strand structure of RNA from degradation, it was necessary to sterilize tools and environmental by UV rays for 30 min, before the RNA extraction. In addition, the working bench was sterilized by pure ethanol and RNaseZap Sigma in order to eliminate any possible trace of ribonucleases.

MirPremier microRNA isolation kit (Sigma) was used to extract RNA samples from root.

Samples powdered in N_2 liquid were aliquoted (0.07 g) in pre-cooled Eppendorf and 750 µl of Lysis solution \ 2ME (10 µl of β-mercapto-ethanol per ml of Lysis solution) was added. After 5 min incubation at 55°C, samples were centrifuged at 14.000 rpm was carried out for a time of 3 min freed from the supernatant and transferred to a "Filtration Column". The filtrate was centrifuged at 14.000 rpm for 1 min, recovered and 1.5 volumes of "Binding Solution" was added before vortexing. The solution was then filtered several times with the "Binding Column" for one min at 14.000 rpm, until the solution was exhausted. Subsequently the filter membrane was washed three times to remove any traces of phospholipids, proteins and carbohydrates with these steps: 1) 700 µl pure ethanol; 2) 500 µl of Binding solution; 3) in the end with 500 µl pure ethanol. Additional centrifugations was carried out to remove any ethanol residue from the membrane.

30 µl of H_2O Nuclease-free was added to the membrane to extract acid nucleic and then a centrifugation at 14.000 rpm was carried out. To obtain a pure RNA elution, the genomic DNA was removed with RNase-Free DNase Set and as suggested by the kit protocol, then samples were incubated at room temperature for 10 min.

The RNA was quantified by a spectrophotometer at 260 nm and protein contamination was measured at 280 nm.

The RNA quality was verified by electrophoretic analysis with 1.5% agarose gel (1,5 g of agarose, 100 ml of TAE 1X buffer and 10 µl of SYBR Safe Gel Stain). In this case two bands corresponding at the 18S and 28S ribosomal subunits showed to be well separated on gel and that confirms the integrity of RNA in the samples.

The RNA extract was immediately converted to cDNA by using the ImProm-II Reverse

Transcription System (Promega) kit. The Retro-transcription (RT) reaction was carried out in two steps. In the first 10 µl of extracted RNA, 1 µl of Primer Oligo (dt) and 1 µl of dNTP were mixed in a micro-centrifuge tube and then positioned inside a thermocycler for 5 min at 70°C. To arrest the process the sample was kept at 4°C for 5 min. In the second stage 8 µl of master mix (20 µl of ImpromII 5X Reaction Buffer, 10 µl $MgCl_2$, 5 µl of RNasin Ribonuclease Inhibitor Recombination and 5 ml of ImpromII Reverse Transcriptase) was added and the solution placed in a thermocycler for 5 min at 25°C, 60 min at 50°C, 15 min at 70°C.
The retro-transcript samples were then stored at -20°C.

To perform a PCR reaction, a mix solution was prepared with:

- 5 µl 5X Green GoTaq Flexi Buffer
- 1,5 µl $MgCl_2$
- 15,9 µl H_2O Nuclease free
- 0,5 µl dNTP
- 1 µl primers (F+R)
- 0,1 µl GoTaq DNA Polymerase (5u/µl)

In PCR tubes, 24 µl of this solution was mixed together with 1 µl of cDNA.
The PCR reaction was started by setting the thermocycler with the following program:

- 95 °C for 1 min
- 94 °C for 45 sec
- 60 °C for 1 min
- 72 °C for 1 min

} 35 cycles (25 cycles for housekeeping gene)

- 72 °C for 5 min

PCR products were loaded (12µl template + 5µl Marker Rapid-Load PCR) on an agarose gel (2%) and as marker, the 1 kb DNA Ladder was used.
After the electrophoresis, the gel picture was obtained by means of ChemiDoc.

3.1.9 PLANT HEALTH STATUS

In order to verify the presence/absence of the pathogen *Phytophthora infestans*, tomato leaves were collected from greenhouse-grown plants at vegetative stage (VS). Fresh leaf tissue was ground to fine powder in liquid nitrogen with a mortar and pestle.
50 mg of plant tissue was subsequently transferred to a micro-centrifuge tube containing 150µl of Plant DNAzol and the solution was gently mixed a few times by inversion.
After an incubation at 25°C with shaking for 5 min, 150µl chloroform was added and the solution was vigorously mixed and another incubation phase was carried out at 25°C with shaking for 5 min. The aqueous phase obtained by a centrifuge at 13400 rpm for 10 min was transferred to a fresh tube and then it was mixed with 110µl of 100% ethanol.
The DNA was precipitated at 7000 rpm for 5 min after an incubation at room temperature for 5 min and the resulting supernatant was removed.
The DNA solubilization from extract was carried out by adding 50 µl of sterile milliQ water, after two important DNA washing operations.
In order to verify the presence/absence of the pathogen *P. infestans*, sequences primer were chosen containing complementary sequences of the pathogen genome.

Gene	**Primers** 5' –> 3'	**Product** (bp)	**Melting T°**
PiO8-3-3	L: CAATTCGCCACCTTCTTCGA R: GCCTTCCTGCCCTCAAGAAC	1500	55°C

Table 3.1.2 Primers sequences, melting temperatures and PCR products amplicons length of *P. infestans* pathogen

To perform a PCR reaction, a mix solution was prepared with:

- 5 µl 5X Green GoTaq Flexi Buffer
- 2 µl $MgCl_2$
- 13,8 µl H_2O Nuclease free
- 1 µl dNTP
- 1 µl primers (F+R)
- 0,2 µl GoTaq DNA Polymerase (5u/µl)

In PCR tubes, 23 µl of this solution was mixed together with 2 µl of cDNA.
The PCR reaction was started by setting the thermocycler with the following program:

- 95 °C for 1 min
- 94 °C for 45 sec
- 55 °C for 1 min
- 72 °C for 1 min

} 35 cycles

- 72 °C for 5 min

PCR products were loaded (12µl template + 5µl Marker Rapid-Load PCR) on an agarose gel (2%) and as marker, the 1 kb DNA Ladder was used.
After the electrophoresis, the gel picture was obtained by means of ChemiDoc.

3.1.10 STATISTICAL ANALYSIS

For the data with a number of 5 replicates for each parameter a two-tailed t-test was applied with a significance level equal to 95% ($p < 0.05$). For the data with a no-normal distribution, before assessing their significance, normality tests were performed (Test Kolmogorov-Smimov and Test Shapiro-Wilk); this enabled us to apply statistical parametric or non-parametric tests on different data. For the data that did not meet the normal distribution neither square root or log transformed and, for both parameters, non-parametric static test was carried out (analysis of two independent samples) and the test Mann - Whitney U was applied as post-hoc test. The remaining data have rather met the normal distribution condition and therefore, parametric statistical test was implemented (one- tailed log rank test); Bonferroni post-hoc test was subsequently applied.
Parametric and no-parametric analysis were applied to a significance level of 95%.
All data obtained by semi-quantitative PCR were normalized using the expression values of the housekeeping gene TUB and then treated samples were compared with control samples.
For the images obtained by the semi-quantitative PCR analysis were analyzed using Image J software. In this analysis, the optical density of pixels of each amplified band on the electrophoresis gels was calculated. The density values were calculated before for TUB and then for the marker genes. The normalization was calculated making a difference between density values of TUB and density values of each genes. The difference values obtained the different expressions of marker genes among the treated samples.
T test was applied to test the statistical significance of expression among marker genes differences in different samples.
All statistical analysis were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL,USA).

3.2 RESULTS (30t ha^{-1} experiment)

3.2.1 SEEDLINGS TRAITS

Mean biomass for root, stem and leaves (Figure 3.2.1.a, b, c) did not show any significant difference between treated and control plants at the ES stage, whereas significant differences with were found at both VS and FS with highest values found in biochar treated plants.

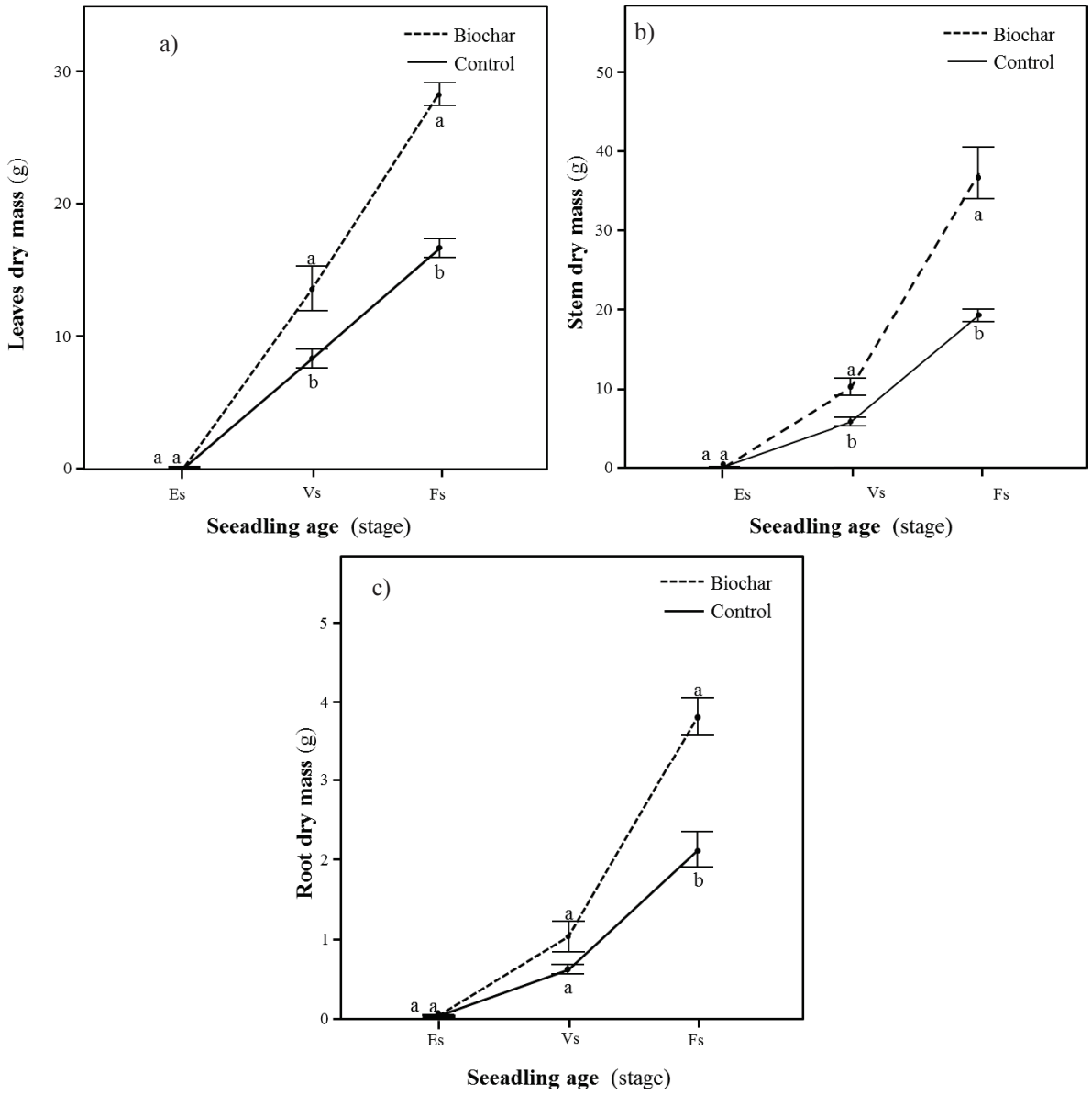

Figure 3.2.1 Biomass of leaves (a), stem (b) and root (c). Each value represent the mean of (n=10) ±SE.
a, b indicate a statistically significant difference between treatments ($p < 0.05$).

In regard of N_{tot} and C_{tot} root content (%) (Table 3.2.1) data indicated the absence of a significant difference between treated and control plants at ES and VS stage whereas at FS stage the highest value of N_{tot} was found in treated plants while, the highest value of C_{tot} was found in control plants. A slight difference was found between the life cycle phases in the sense that N_{tot} seemed to decrease with the time whereas C_{tot} seemed to increase. In both cases, such variations were observed independently from biochar treatment.

Root		Es		Vs		Fs	
Parameter	Unit	Control	Biochar	Control	Biochar	Control	Biochar
Ntot	%	3,4 ± 0,07 ax	3,7 ± 0,10 ax	2,27 ± 0,1 ay	2,1 ± 0,22 ay	1,3 ± 0,0 az	1,7 ± 0,16 bz
Ctot	%	37,9 ± 0,7 ax	37,8 ± 0,25 ax	40,5 ± 0,15 ay	40,2 ± 0,7 ax	42,9 ± 0,3 az	41,3 ± 0,75 by

Table 3.2.1 Root CHN data. Each value represent the mean of (n=10) ±SE.
a, b indicate a statistically significant difference between treatments ($p < 0.05$).
x, y, z indicate a statistically significant difference ($p < 0.05$) among the different sampling point (Es, Vs, Fs).

N_{tot} and C_{tot} content (%) in leaves (Table 3.2.2) did not show any significant difference between treated and control plants at Vs and Fs.

Leaves		Es		Vs		Fs	
Parameter	Unit	Control	Biochar	Control	Biochar	Control	Biochar
Ntot	%	6,1 ± 0,03 ax	6,91 ± 0,01 bx	3,6 ± 0,45 ay	4,5 ± 0,4 ay	1,45 ± 0,3az	1,9 ± 0,3 az
Ctot	%	36,1 ± 1,45 ax	32,5 ± 1,9 bx	35,3 ± 3,55 ax	36,12 ± 2,9 ax	35 ± 1,50 ax	36 ± 1,9 ax

Table 3.2.2 Leaves CHN data. Each value represent the mean of (n=10) ±SE.
a, b indicate a statistically significant difference between treatments ($p < 0.05$).
x, y, z indicate a statistically significant difference ($p < 0.05$) among the different sampling point (Es, Vs, Fs).

At the ES, treated plants showed that N_{tot} content was higher than the control plants while, unlike C_{tot} content which was higher in control plants.
In analogy with roots also in leaves the N_{tot} showed decreasing values with time with a significant difference of the trend in both treatments.

Total leaf area (Figure 3.2.2) showed higher values in the biochar treated plants at VS and FS sampling point. This result is in accordance with the results of mean biomass for root, stem and leaves.

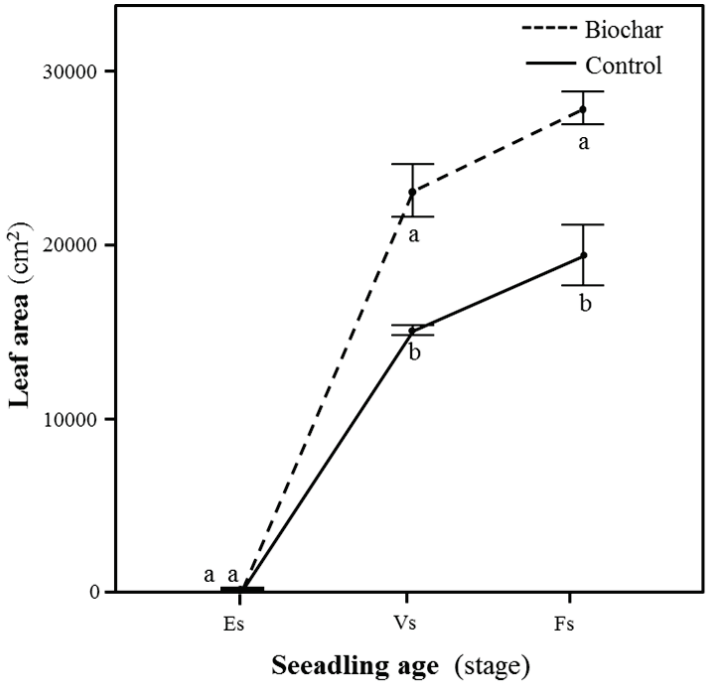

Figure 3.2.2 Total leaf area at ES, VS and FS. Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Chlorophyll *a* (µg ml^{-1}) and *b* (µg ml^{-1}) content (Figure 3.2.3.a, b) did not show any significant differences between treated and control plants at the ES time. Biochar treated plants showed a higher value of *b* chlorophyll than control plants at the VS. At the FS time, the highest value of both *a* and *total* chlorophyll (Figure 3.2.3.a, c) was found in control plants.

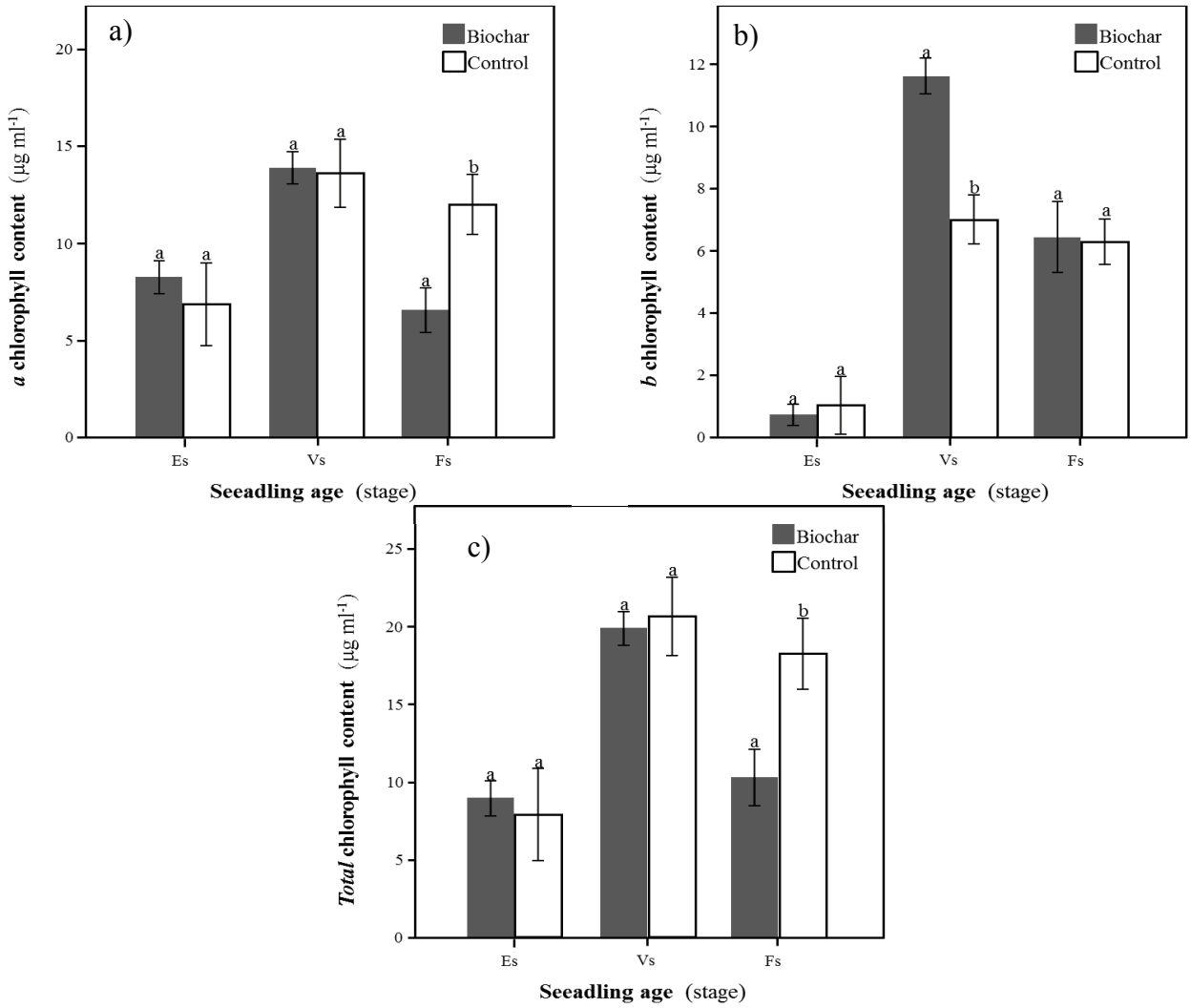

Figure 3.2.3 *a* chlorophyll a), *b* chlorophyll b), *total* chlorophyll c) content at ES, VS and FS. Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

The highest values of root length were found in treated plants throughout the experiment (Figure 3.2.4).

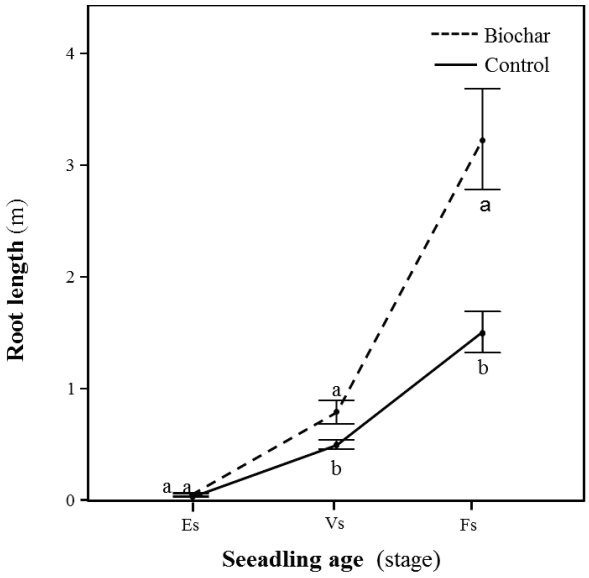

Figure 3.2.4 Root length at ES, VS and FS. Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

The SRL, RTD and the mean diameter class values have not shown any significant difference between treated and control plants at each sampling point (Figure 3.2.5).

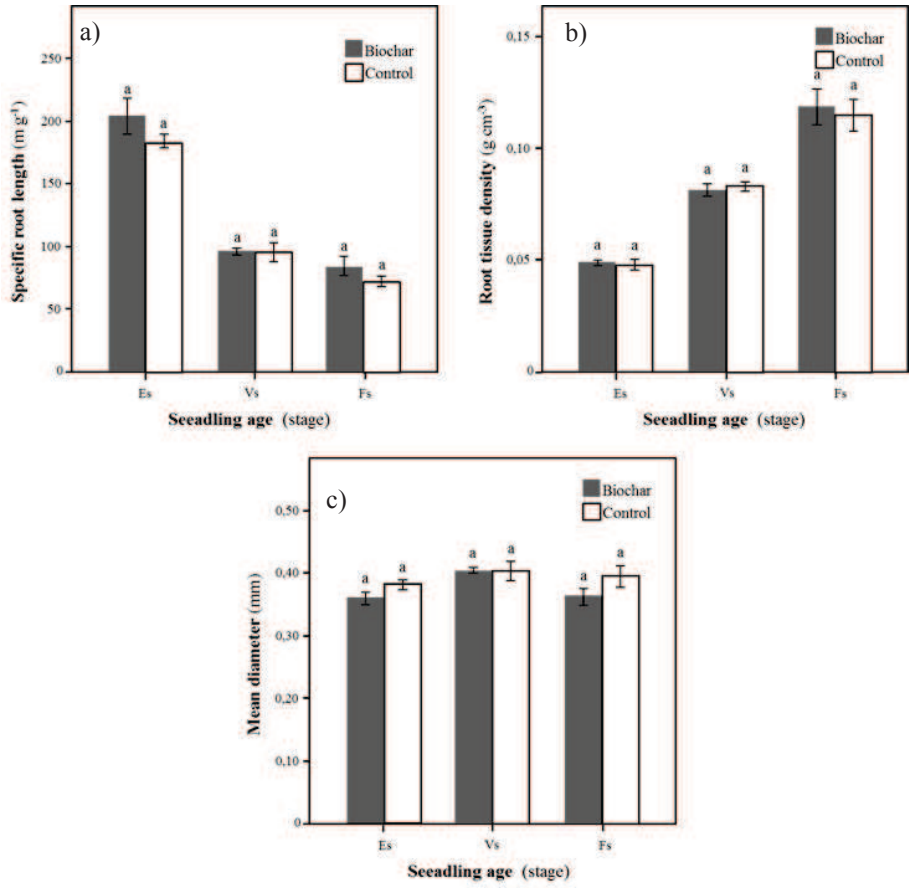

Figure 3.2.5 Specific root length (SRL) a), Root tissue density (RTD) b) and Mean root diameter c) at ES, VS and FS. Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Flower and fruit number were found in biochar treated plants respectively five and two fold higher (Figure 3.2.6.a, b) than in control plants.

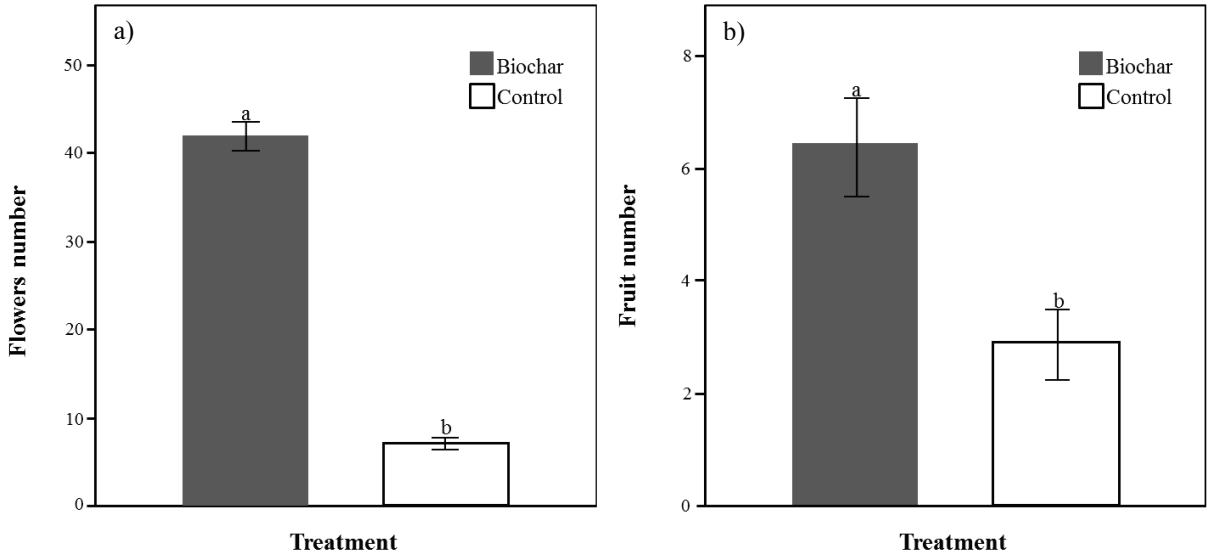

Figure 3.2.6 Flowers a) and Fruit b) number. . Each value represent the mean of (n=10) ±SE.
a, b indicate a statistically significant difference between treatments ($p < 0.05$).

All fruit morphometric parameters (Figure 3.2.7.a, b, c, d, e) did not show any significant difference between treated and control plants.

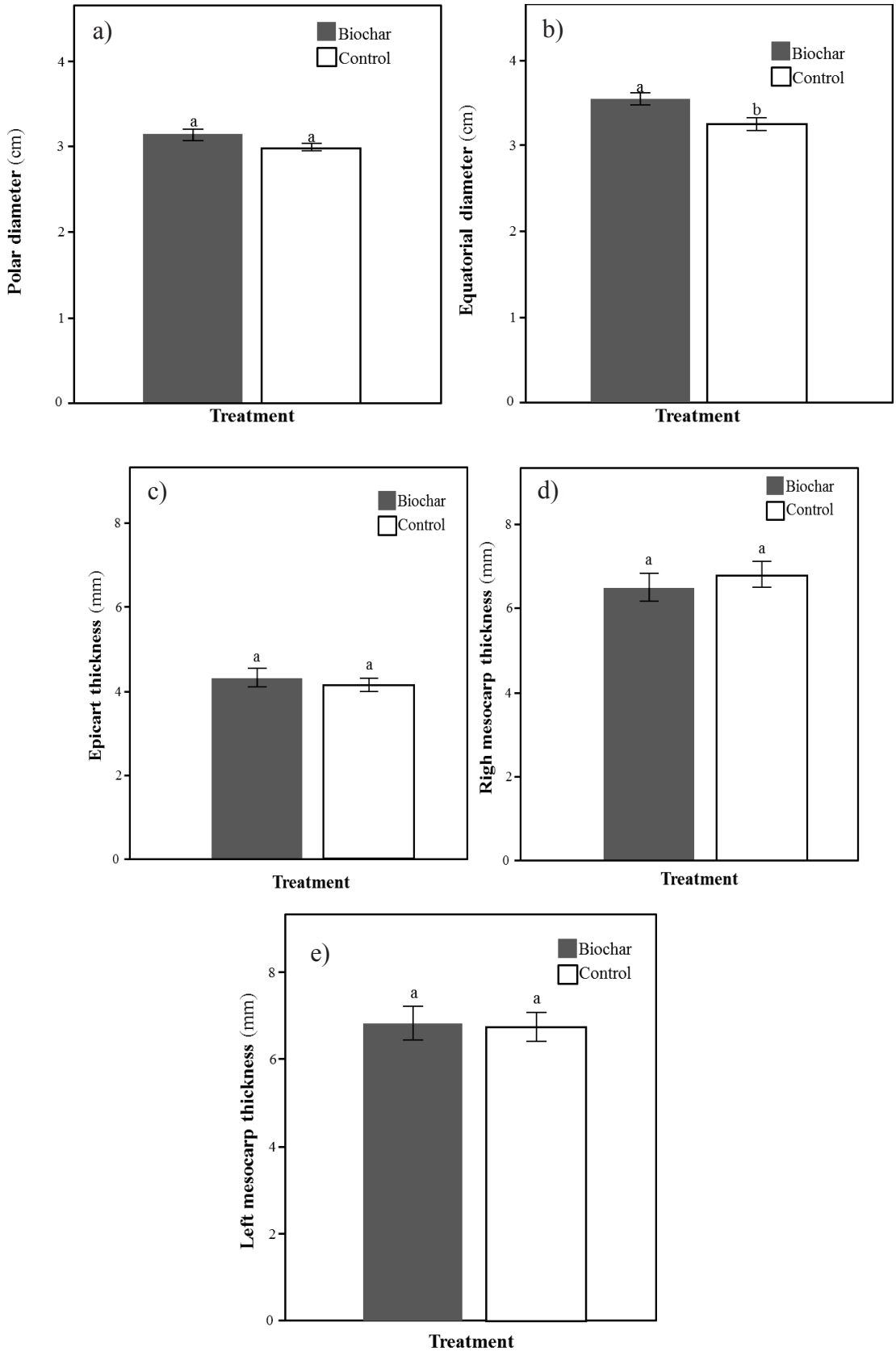

Figure 3.2.7 Morphometric fruit parameters: Polar a) and equatorial diameter b); Epicarp c), right mesocarp d) and left mesocarp e) thickness. Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Finally, treated plants showed higher values than the control in qualitative parameters such as titratable acidity (Figure 3.2.8,a), total soluble solids content (Figure 3.2.8.b), trans-lycopene, and cis-lycopene (Figure 3.2.8.c, d); no differences were detected for β-carotene (Figure 3.2.8.e).

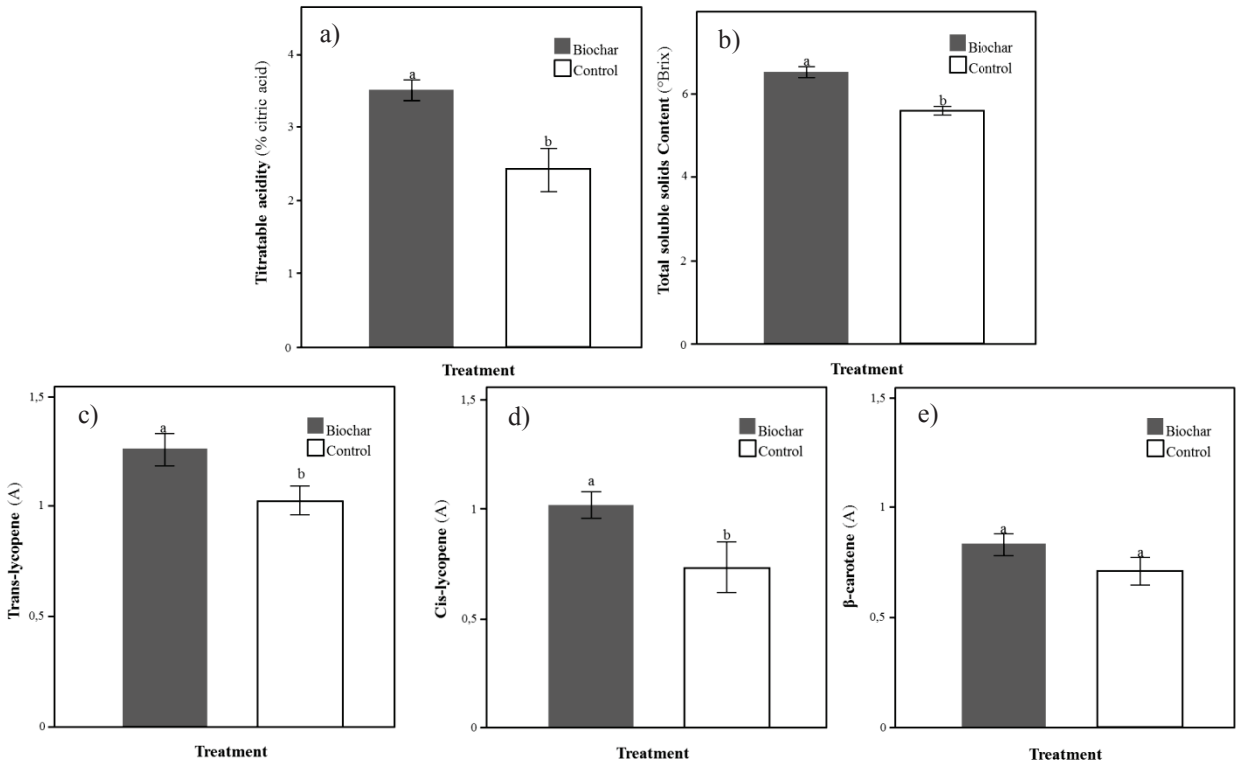

Figure 3.2.8 Qualitative fruit parameters: a) Titratable acidity (% citric acid) ; b) Total soluble solid content (°Brix); c) Trans-lycopene (A); d) Cis-lycopene (A); d) β-carotene content (A). Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

About Panel test, all sensory attributes did not show any significant taste difference (low significance: $p > 0.05$).

In summary, results obtained from this experiment confirm our hypothesis about the possible beneficial biochar effects on plant growth, fruit yield and fruit quality –one of the most important parameters.

3.2.2 SOIL TRAITS

In regard of chemical and physical soil parameters (Table 3.2.3) such as pH, EC and N_{tot} we did not notice significant difference in all treatments and plant stages examined. CEC (Cmol kg^{-1}) showed the highest value in treated plants at the FS stage. Also N_{av} content (mg kg^{-1}) showed a significant difference at the FS, where the control soil showed an high value than treated plants. P_{tot} (mg kg^{-1}) content showed a higher value in treated soil at the FS, while at the ES, the highest value of P_{av} (mg kg^{-1}) was found in control soil. No relevant differences were present at VS. About C_{tot} (g kg^{-1}) content, treated soil showed a higher value at the VS.

Table 3.2.3
Chemical-physical analysis performed on soil samples of control and biochar-treated pots at different sampling point (Es, Vs, Fs).
a, b indicate a statistically significant difference between the treatments.
x, y, z indicate a statistically significant difference among the different sampling point (Es, Vs, Fs).
Each value represent the mean of (n=6) ±SE

		Es		Vs		Fs	
Parameter	Unit	Control	Biochar	Control	Biochar	Control	Biochar
pH	-	7.35 ± 0.076ax	7.4 ± 0.077ax	7.44 ± 0.077ax	7.47 ± 0.079ax	7.47 ± 0.068ax	7.53 ± 0.078ax
EC	dS m^{-1}	0.8 ± 0.26ax	1.1 ± 0.36ax	0.9 ± 0.29ax	1.2 ± 0.4ax	1 ± 0.35ax	1.3 ± 0.43ax
CEC	Cmol kg^{-1}	18 ± 0.86 ax	19 ± 0.9ax	17 ± 0.82axy	18 ± 0.9ax	16 ± 0.77ay	18 ± 0.82bx
N_{tot}	g kg^{-1}	16 ± 1.52ax	14 ± 1.33ax	11 ± 1.1ay	12 ± 1.15axy	12 ± 1.18ay	10 ± 0.96ay
N_{av}	mg kg^{-1}	150 ± 16.6ax	140 ± 5.6ax	110 ± 4.45ay	110 ± 4.39ay	120 ± 4.85ay	100 ± 4.12bz
P_{tot}	mg kg^{-1}	544.9 ± 20.16ax	533.5 ± 19.78ax	440.2 ± 16.28ay	432.1 ± 16ay	346.5 ± 13az	411.9± 15.24by
P_{av}	mg kg^{-1}	42.64 ± 1.5ax	37.87 ± 1.33bx	38.67 ± 1.4ay	38.69 ± 1.3ax	23.9 ± 0.84az	27.62 ± 0.97ay
C_{tot}	g kg^{-1}	25.6 ± 0.62ax	25.61 ± 0.62ax	20.1 ± 0.48ay	23 ± 0.55by	19.8 ± 0.47ay	20.1 ± 0.5az

Es: Early stage; Vs: vegetative stage; Fs: fruit stage

3.2.3 RELATIVE GENES EXPRESSION

Molecular analysis conducted at the three different developmental stages showed interesting differences regarding the relative gene expression of marker genes (Figure 3.2.9.a, b, c).

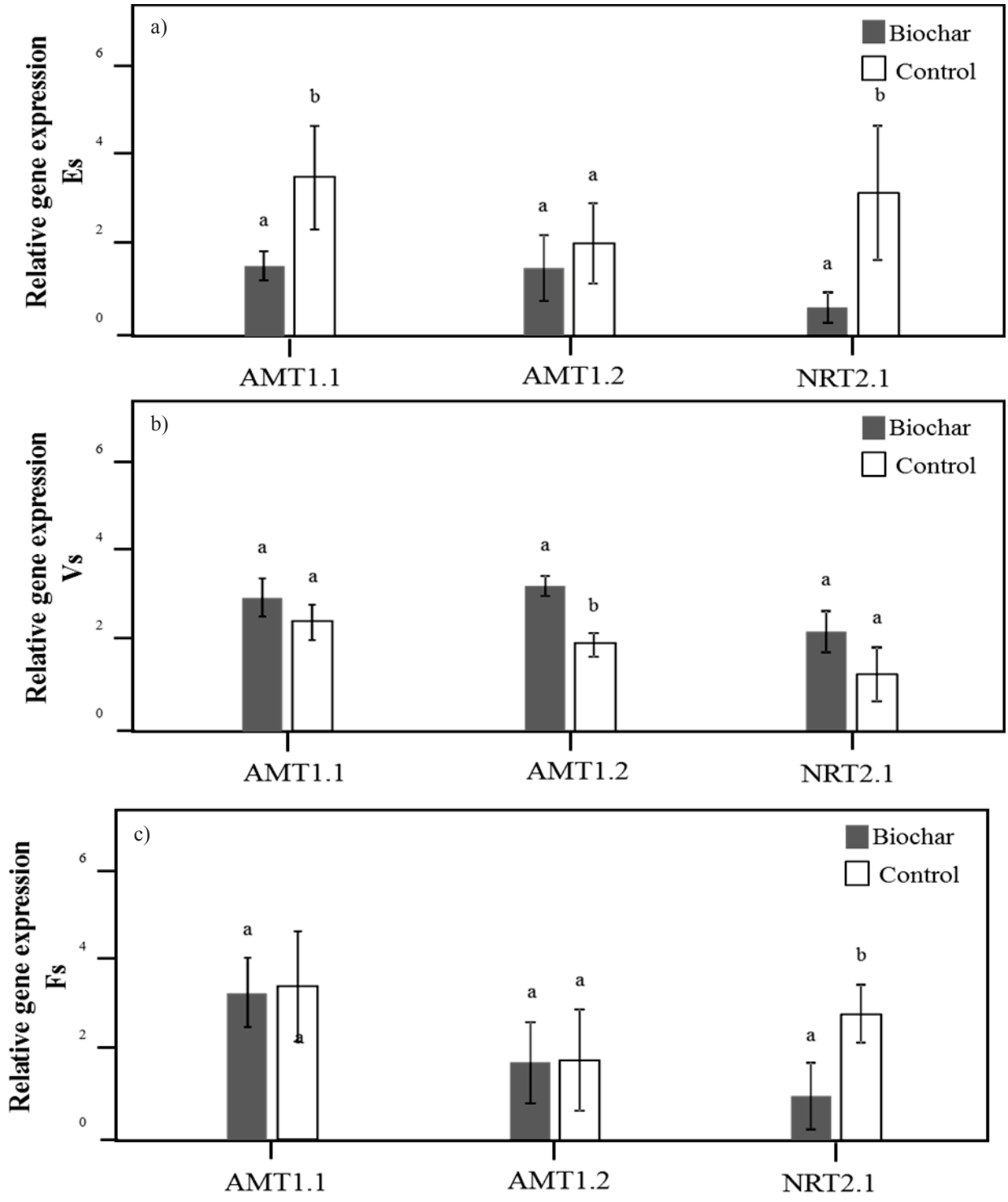

Figure 3.2.9 AMT1.1, AMT1.2 and NRT2.1 relative gene expression at ES a), Vs b) and FS c). Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

In fact, AMT1.1 marker gene showed its highest expression in control plant at ES, whereas no relevant difference emerged between control and Biochar treatment at VS and FS. AMT1.2 marker gene showed that its expression was twofold higher in treated plants at VS.
In regard to NRT2.1 marker gene, we found that it showed its highest expression in control plants at both ES and FS developmental stages.

PT1 marker gene showed its highest expression in control plants at ES, unlike PT2 marker gene which showed its highest expression values in control plants at ES and also at VS (Figure 3.2.10.a, b).

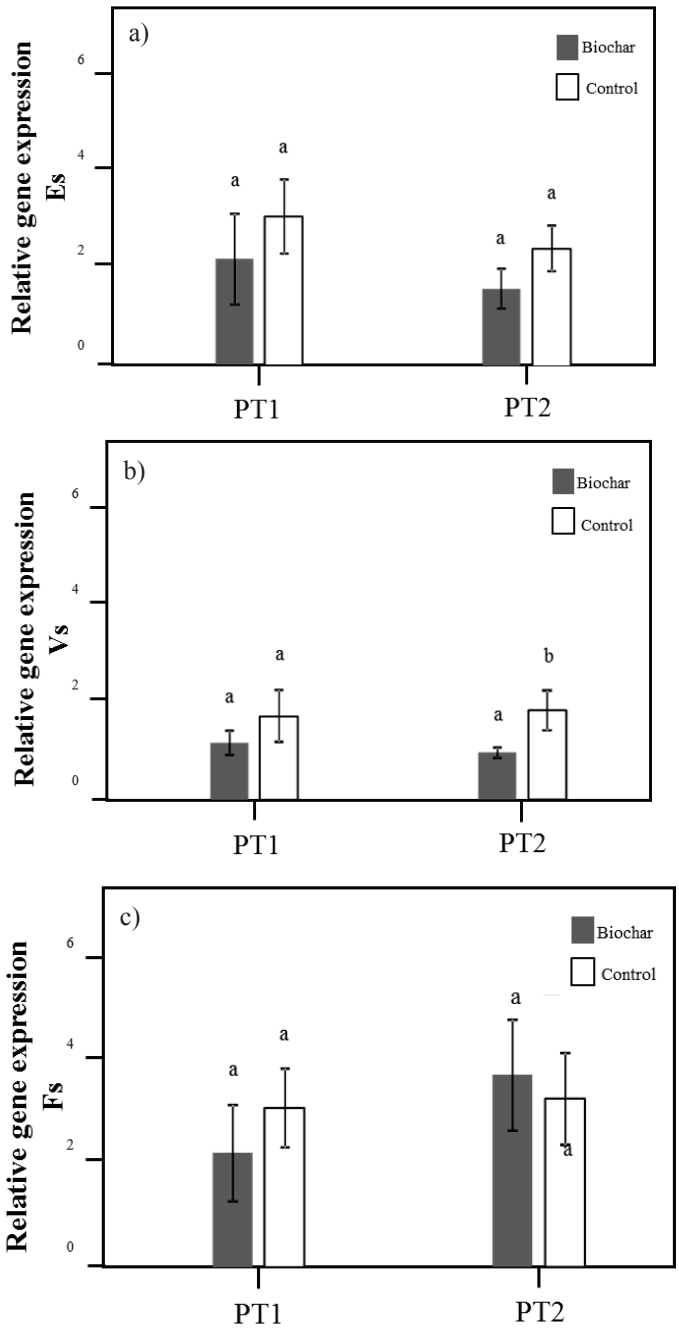

Figure 3.2.10 PT1 and PT2 relative gene expression at ES a), Vs b) and FS c). Each value represent the mean of (n=10) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

In summary, in regard the genes expression, our results highlight a higher expression of the AMT1.1, AMT1.2, NRT2.1 and PT2 gene during the different life stages of the plant treated with biochar.

3.2.4 PLANT HEALTH CONDITION

During the Vs, same tomato leaves of both treatments showed the presence of yellow colors and irregular spots above their surface.

In order to exclude the presence of *P. infestans* pathogen, after fungal DNA extraction, a PCR reaction was performed using PiO8-3-3 primers. The gel image obtained after the electrophoretic run (Figure 3.2.11) did not show the presence of *P. infestans* pathogen.

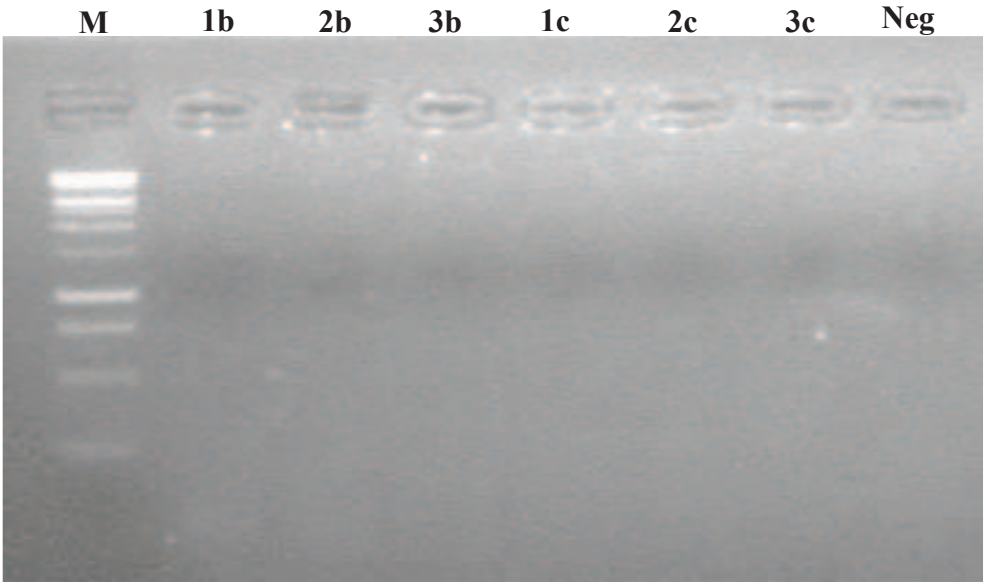

Figure 3.2.11 PCR gel image of genomic *P. infestans* extraction (M = molecular marker; 1b, 2b, 3b = biochar replicates; 1c, 2c, 3c = control replicates; Neg = negative control)

Data collected during this experiment are part of a draft paper entitled:

Biochar soil amendment enhances cherry tomatoes growth, fruit production and quality in controlled conditions.

For the full manuscript please see page 115.

3.3 MATERIALS AND METHODS (300t ha^{-1} experiment)

In order to test the influence of biochar on *Solanum lycopersicum* seedlings (Cherry tomato of Pachino vr) the following morphological were investigated: 1) Above and below-ground biomass compartmentation; 2) - in the case of leaves we measured leaf area, leaf number; 3) - in the case of roots we measured root length .

3.3.1 PLANT MATERIAL AND GROWTH CONDITIONS

Seeds of Cherry tomato were sown in rectangular pots (h 16 cm, width 20 cm and length 60 cm) and maintained at field capacity under controlled conditions (temperature 25 ° C, humidity 75% and PAR 400/700 mol m^{-2} s^{-1}). Each treated pot contained approximately 11,5 L of air dried soil mixed with biochar at a rate of 300 t ha^{-1} whereas control pots were filled with commercial sandy soil.

A number of 16 treated plants and 16 control plants were collected at the end of the experiment. In order to maintain temperature and water potential constant throughout the experiment, measurements were carried out every 2 days. After 22 days, morphological analysis were carried out on the sampled plants.

Figure 3.3.1 *Solanum lycopersicum* experimental set up

3.3.2 BIOMASS DISTRIBUITION IN ABOVE AND BELOW-GROUND COMPARTIMENTS AND MORPHOLOGICAL TRAITS OF LEAVES AND ROOTS

To evaluate biomass distribution we measured separately the dry weight (g) of roots (RDW), shoot (SDW), and leaves (LDW). Collected material was dried at 70°C until constant weight was achieved, for each sampling point. In the case of RDW roots were rinsed repeatedly under running tap water, slightly dried with paper before starting the drying procedure.
In the case of leaves, the total leaf area and the leaves number were measured using a high resolution camera (OLYMPUS DP72) in order to acquire the images and then they were processed using by Image J software. In the case of roots, these were rinsed repeatedly under running tap water and after, scanned using a calibrated scanner (Epson, Expression 10000 XL) at a resolution of 800 dpi. Afterwards, the acquired images were analyzed by WhinRhizo Pro V. 2007d software (Regent Instruments Inc. Quebec).

3.3.3 STATISTICAL ANALYSIS

For the data with a number of 5 replicates for each parameter a two-tailed t-test was applied with a significance level equal to 95% ($p < 0.05$). For the data with a no-normal distribution, before assessing their significance, normality tests were performed (Test Kolmogorov-Smimov and Test Shapiro-Wilk); this will enable us to apply statistical parametric or non-parametric tests on different data. For the data that did not meet the normal distribution neither square root or log transformed and, for both parameters, non-parametric static test was carried out (analysis of two independent samples) and the test Mann - Whitney U was applied as post-hoc test. The remaining have rather met the normal distribution condition and therefore, parametric statistical test was implemented (one- tailed log rank test); Bonferroni post-hoc test was subsequently applied.
Parametric and no-parametric analysis were applied to a significance level of 95%.
All statistical analysis were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL,USA).

3.4 RESULTS (300t ha^{-1} experiment)

3.4.1 SEEDLING TRAITS

Biochar treated plants showed lower root, stem and leaves biomass values than control plants and the same result was observed for total leaf area and fine root length parameters (Figure 3.4.1.a, b, c).

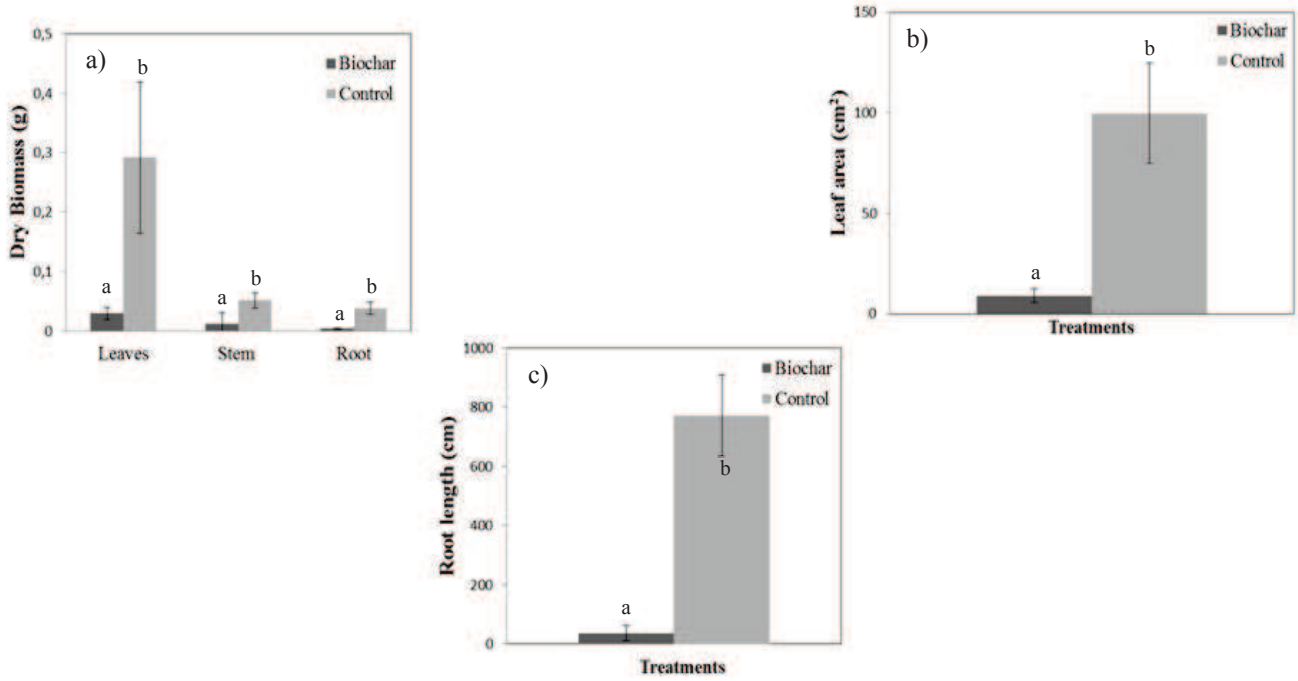

Figure 3.4.1 Compartmental biomass a), Leaf area b) and Root length c). Each value represent the mean of (n=16) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

3.5 SHORT DISCUSSION

In the present experiment plants of *Solanum lycopersicum* treated with a biochar rate of 30 t ha^{-1} showed a better growth performance in terms of both morphological and fruit traits. Our results highlight for the first time the positive relationship between biochar amendment and the increase of antioxidant compounds in the fruit. This result might be related to the increase of both nitrogen availability and carotenoid metabolic pathway (Delgado et al., 2004). Therefore, results obtained from this experiment support our hypothesis about the positive effect of biochar amendment on 1) plant growth, 2) fruit yield and quality and 3) soil nutrient availability. On the contrary in the case of plants treated with a biochar rate of 300t ha^{-1}, growth was strongly inhibited. This might be due to the large volume of biochar in relation to the soil, which is a reduction of the overall nutrient content. Moreover, the concentration of polycyclic aromatic hydrocarbons in the biochar composition could also negatively affect the plant growth at high concentration (Krull et al., 2009).

Chapter IV

Effect of biochar on the response of *Vitis vinifera* (L.) (Chardonnay cv)

4.1 MATERIALS AND METHODS (Pot experiment)

In order to test the influence of biochar on *Vitis vinifera* plants, we investigated the following traits in plants and soil:

- Leaf

- Leaf water potential (LWP)
- Chlorophyll fluorescence (Yield)

- Root

- Root length

- Soil

- Soil temperature (ST)
- Soil water potential (SWP)

4.1.1 PLANT MATERIAL AND GROWTH CONDITIONS

In order to test the influence of biochar on soil water and temperature and also on morpho-physiologal parameters of *Vitis vinifera* (Chardonnay cv), a pot experiment was set up. 3 years old plants of *Vitis vinifera* were placed in cubic pots (h 30 cm x 30 cm x 30 cm; 25 L) specifically modified and presenting a transparent acrylic side to allow the visual control of the root growth kinetics (Van Do et al. 2015). 5 pots were filled with agricultural soil mixed with woody biochar (vineyard parental material) at a rate of 30t ha^{-1} (Baronti et al. 2010; Mustafa et al. 2010). Other 5 pots were filled only with agricultural soil and considered as control samples. Both treated and control plants were grown from April to October 2016 under natural conditions. Time points were used to collect the samples and which coincided with 1) the annual maximum of solar radiation, 2) the highest peak of air temperatures, 3) the end of fruit growth, 4) the end of ripening phase. Soil temperature (ST) and soil water potential (SWP) were weekly measured at 5 and 10 cm soil depth. Experiment was established adjacent to a weather station (LSI Lastem) and air temperature, atmospheric humidity, wind speed and direction were measured.

4.1.2 LEAF

LWP measurements were carried out once a week by using the Bomb Scholander (SKPM 1400, Skye Instruments Ltd., UK).
A leaf attached to the stem (10 treated and 10 control) was placed inside a sealed chamber and pressurized gas was slowly added inside the chamber. When the applicate pressure forced the lymph out from the xylem drops became visible at the extremities of cut stem. The pressure required to get out the lymph was equal and opposite to the water potential of the leaf.
Leaf fluorescence measurements (10 treated and 10 control leaves) were performed once a week. For the abovementioned parameter, a portable modulator pulse fluorimeter (OS1 - FL, opti - sciences, inc. USA) has been used. Minimum fluorescence measurements (Fo) were carried out one hour before the lights impulse in dark conditions in order to determine the maximum fluorescence (Fm). The maximum fluorescence value was obtained by exposing the same leaves adapted to the dark at a short pulse of saturated white light (0.8 s about 600 μmol m^{-2} s^{-1}). The PSII quantum yield (Fv / Fm, with Fv = Fm - Fo) was then calculated as %.

4.1.3 ROOT

In order to measure the biochar effects on the root growth a non-destructive analysis were carried out. In regard to this, root images were acquired by using a calibrated scanner (Epson, Expression 6000 XL) at a resolution of 800 dpi. Afterwards, the acquired images were analyzed by using WhinRhizo Pro V. 2007d software (Regent Instruments Inc. Quebec); subsequently the extracted data were processed to obtain information about the root length, root surface area and root volume.

4.1.4 STATISTICAL ANALYSIS

Concerning the bioinformatics and the statistical analysis, see paragraph 3.3.3 of *Solanum lycopersicum* 300t ha^{-1} experiment, Chapter III.

4.2 RESULTS (Pot experiment)

4.2.1 LEAF AND ROOT TRAITS

LWP (Bar) (Figure 4.2.1.a) showed significant difference between treated and control plants in June 22th and from 3th August to 21th September where control plants showed higher values than treated plants. Chlorophyll fluorescence (Figure 4.2.1.b), express as Yield (% of Photosistem II rate) showed significant difference between treated and control plants from 13th July to 24th August with, treated plants showing higher values than control plants.

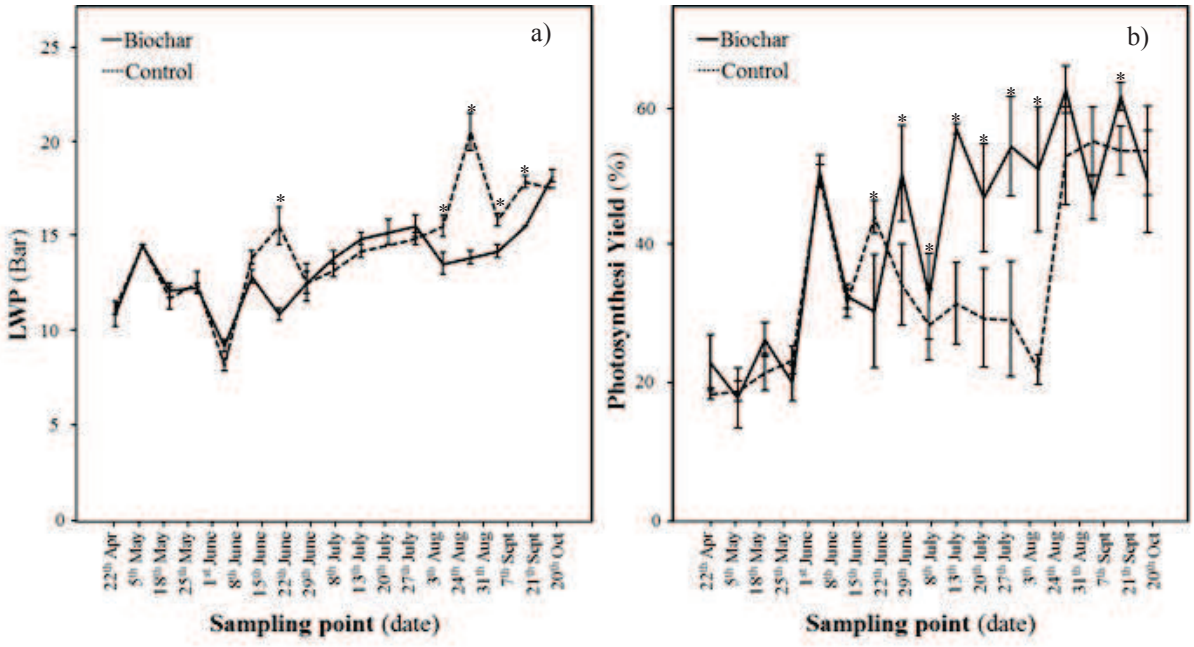

Figure 4.2.1 Leaf parameters: Leaf water potential values a) and Yield (% of Photosintetic rate) values b). Each value represent the mean of (n=5) ±SE. An asterisk indicates a statistically significant difference between treatments ($p < 0.05$).

Fine root length (cm) (Figure 4.2.2.a), showed significant difference between treated and control plants from 8th June to 20th October where, also here, treated plants showed higher values than control plants. Both the two treatments showed a typical seasonal root pattern.

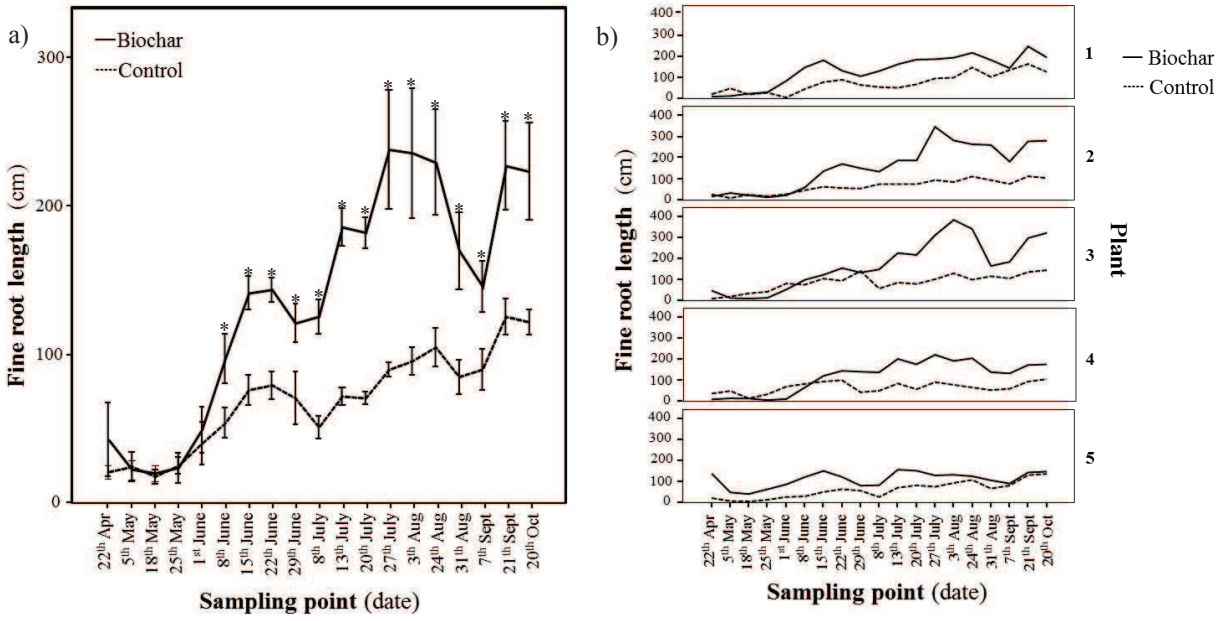

Figure 4.2.2 Root length: Mean root length a) and Specific plant root length b). Each value represent the mean of (n=5) ±SE. An asterisk indicates a statistically significant difference between treatments ($p < 0.05$).

4.2.2 SOIL TRAITS

Concerning ST (at 5 and 10 cm depth) (Figure 4.2.3.a, b) there was no significant difference between control and treated pots. The only exception was detected in July 20th with a higher temperature of 1.35 °C at 5 cm depth in treated plants.

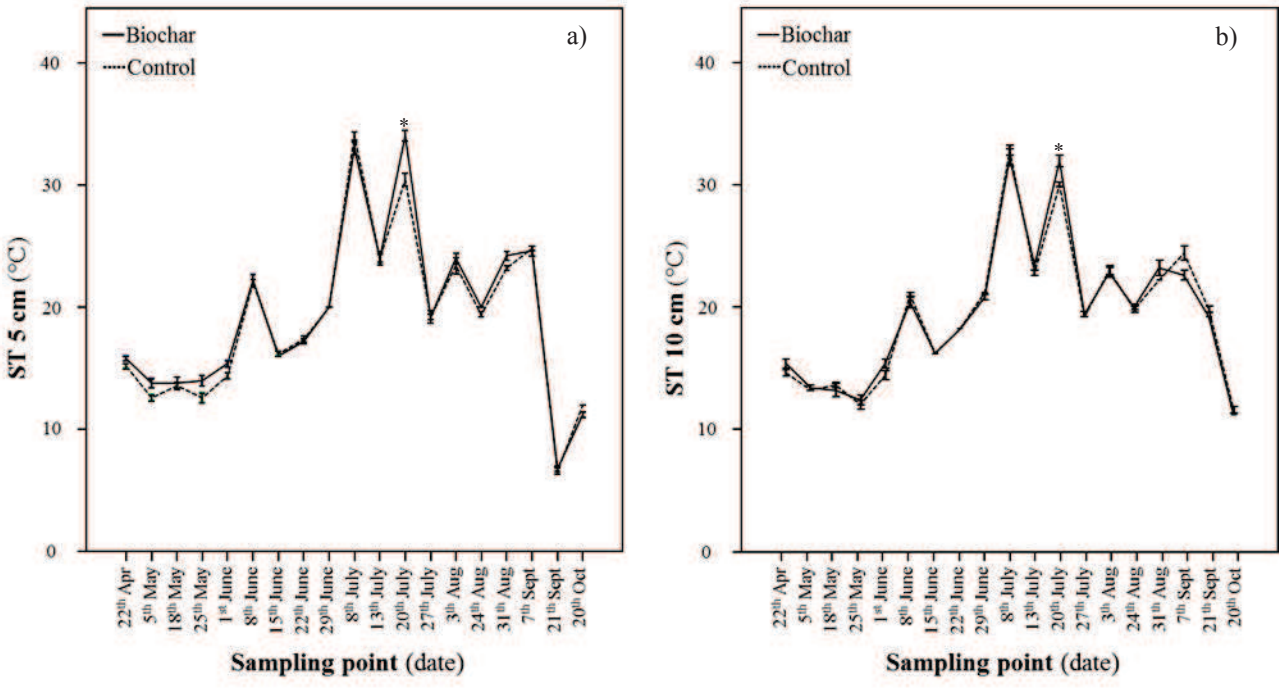

Figure 4.2.3 Soil temperature at 5 cm depth a) and Soil temperature at 10 cm depth b) values. Each value represent the mean of (n=5) ±SE. An asterisk indicates a statistically significant difference between treatments ($p < 0.05$).

SWP (at 10 and 20 cm depth) (Figure 4.2.4.a, b) did not show significant difference between treated and control plants.

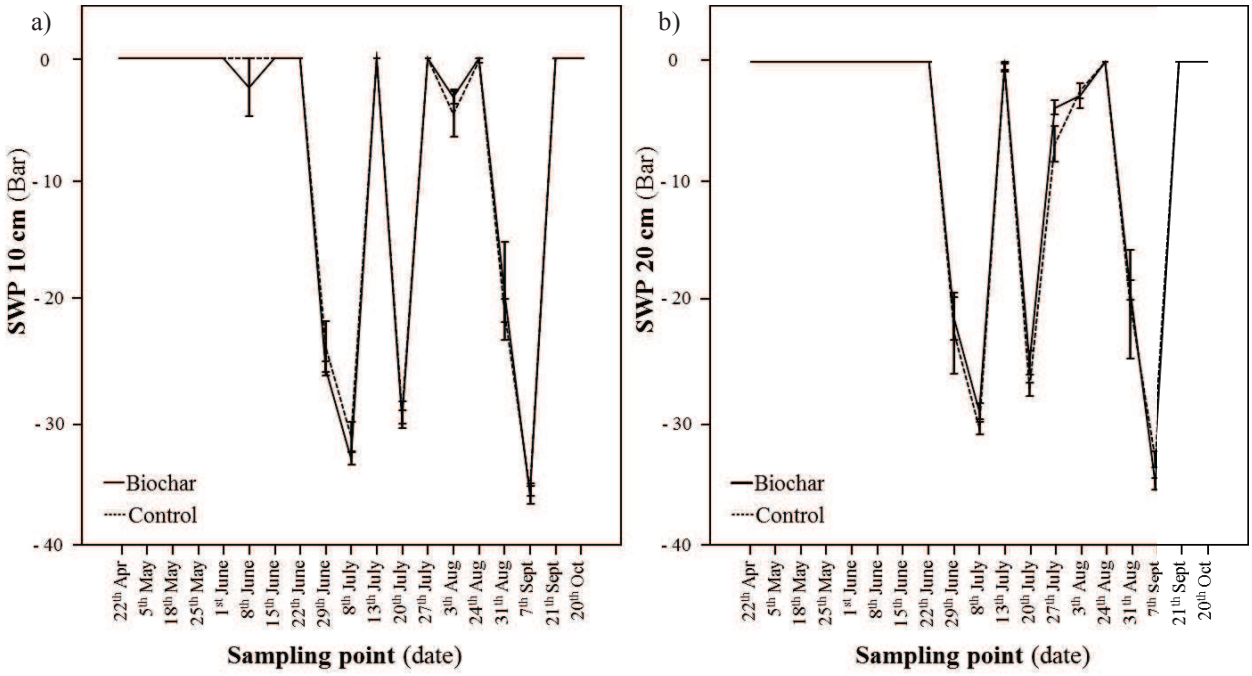

Figure 4.2.4 Soil water potential at 10 cm depth a) and Soil water potential at 20 cm depth b) values. Each value represent the mean of (n=5) ±SE. An asterisk indicates a statistically significant.

4.3 MATERIALS AND METHODS (Field experiment)

In order to test the influence of biochar on *Vitis vinifera* plants, we investigated the following morpho-physiological parameters:

- Leaf

- Leaf water potential (LWP)
- Chlorophyll fluorescence (Yield)

- Root

- Root length, root surface area and root volume

- Fruit

- Fruits yield
- Fruit biomass
- Fruit Fertility
- Titratable acidity
- Total soluble solids content or °Brix
- Fruit Antioxidant content

4.3.1 PLANT MATERIAL AND GROWTH CONDITIONS

In order to test the influence of biochar on morpho-physiological aspects of *Vitis vinifera* (Chardonnay cv) an open-field experiment was set up in December 2016. Thirty 3-years old plants of *Vitis vinifera* grown in pot were transplanted in the fields in two rows; fifteen plants represented the control row and fifteen plants represented the biochar treatment. Each row was in turn divided into 5 plot with 3 plants per plot. In treated plots, natural soil was mixed with woody biochar (parental material obtained from different fruit plant species) at the depth of 30 cm and with a concentration of 30t ha^{-1} as suggested by Baronti et. al 2010 and Mustafa et al. 2010. On a side of each row a trench was excavated with the aim of burying 5 plastic boxes. The boxes were partially buried but remained accessible from above-ground to enable the possibility to insert in them a scanner. The side of each box facing the plants was modified with a plexiglass sheet to enable a scan of the roots growing in contact with the box (Van Do et al. 2015). SWP (Soil Water Potential) were weekly measured at 10, 20 and 30 cm depth. The setting of this experiment included a weather station (LSI, Lastem) where air temperature, atmospheric humidity, wind speed and direction were measured. The morphological and physiological parameters were measured each 20 days.

4.3.2 LEAF

Leaf parameters measurements were carried out once a week; see paragraph 4.1.2 of *Vitis vinifera* pot experiment, Chapter IV for more information.

4.3.3 ROOT

In order to measure the biochar effects on the root growth a non-destructive analysis were carried out. Root images were acquired by using a calibrated scanner (Epson, Expression 6000 XL) at a resolution of 800 dpi.
Afterwards, the acquired images were analyzed using WhinRhizo Pro V. 2007d software (Regent Instruments Inc. Quebec); subsequently the extracted data were processed to obtain information about the root length, root surface area and root volume.

4.3.4 FRUIT

During the harvest fruit season, fruit quality parameters were measured in treated and control plant.
In order to determine the plot yield (n° bunch/plot), the bunch number and bunch dry biomass weight were measured for treated and control plot; also the berries number was calculated for bunch.
To determine fruit biomass 20 berries for each treatment were weighed before (fresh weight, FW, g) and after (dry weight, DW, g) drying at 70°C for 48 h.
The grape fertility was determined through a seed-count and through dry seeds weight (10 berries for each treatment).
In order to determine qualitative parameters berries were homogenized by homogenizer (VWR Collection, VDI 12) and then they were centrifuged at 13,000 rpm for 20 minutes at 8°C.
The titratable acidity (TA), expressed as percentage of tartaric acid, was measured using the titration method at pH 7 with NaOH (0.1 N) (Petruccelli et. al 2015).
The pH was measured using an electronic pH meter (pH meter Eutech Instruments pH 700, 2013).
Finally, TSSC (°Brix) and Alcohol potential (% v/v) was measured by refractometer (HANNA Instruments, HI 96813).
To quantified the grape antioxidant content the samples were kept frozen at -18 ° C for a period of 2- 3 weeks before the analysis. During the extraction phase, the berries were subdivided into

skin, seed and pulp in order to identify the compartmental distribution of the antioxidant content. The skin and seeds were grounded with liquid N and the pulp was blended.

For each treatment, 1 g of skin, seed and pulp samples was extracted with 10 ml of acetone/water solution (80/20, v/v) containing 0.1% (v/v) of concentrated HCl to preserve the polyphenols content and to improve the phenolic extraction; acidification was required for anthocyanin extraction because its solubility is dependent on the pH of the medium used.

For a more efficient extraction an ultra-sonication (Branson 3510EMTH, Danbury, USA) was performed for 15 minutes and then the samples were stirred for 30 min.

After centrifugation at room temperature and at 3000 rpm for 10 min, the obtained supernatant was collected and brought to a final volume of 25 ml with distilled water.

The diluted extract was filtered with 0.45 μm filter to improve the yield of the spectrophotometric determination of the antioxidants. The total polyphenolic content (TPC) of the skin, seed and pulp extracts was determined using the Folin–Ciocalteu method (Slinkard & Singleton, 1977).

The absorbance was measured at wavelength of 765 nm by using a cuvette with 1 cm of optical path. Gallic acid was used as a standard for construction of the calibration curve and the concentration of TPC was expressed as gallic acid equivalent (mg g^{-1} fresh mass).

For total anthocyanin (TA) analysis, the grape skin extracts were diluted with a solution of acidified aqueous ethanol (Di Stefano, Cravero, & Gentilini, 1989) and the absorbance was measured immediately at 540 nm (1 cm optical path). Results were expressed as malvidin-3-glucoside equivalents (mg g^{-1} fresh mass), calculated by using the following equation proposed by Di Stefano et al., 1989:

$$TA_{540nm} = A_{540nm} \cdot 16{:}7\underline{d}$$

where, A_{540nm} is the absorbance at 540 nm and $\underline{d}$ is the dilution.

The total flavonoid content (TFC) was evaluated according to a colorimetric assay with aluminum chloride (Zhishen, Mengeheng, & Jianming, 1999).

The absorbance was measured against the blank (prepared in the same way with distilled water) at 510 nm using a 1 cm optical path. (+) -Catechin was used as a standard for the calibration curve and the TF content was expressed in mg g^{-1} fresh mass as catechin equivalent (Ivanova et al., 2011).

4.3.5 STATISTICAL ANALYSIS

Concerning the bioinformatics and the statistical analysis, see paragraph 3.3.3 of *Solanum lycopersicum* 300t ha^{-1} experiment, Chapter III.

4.4 RESULTS (Field experiment)

4.4.1 PLANT TRAITS

LWP (Bar) (Table 4.4.1) showed significant difference between treated and control plants from 23th June to 24th August. In the three measurements, control plants showed always higher values than treated plants.

LWP (Bar)	Biochar	Control
23th June	11 ± 0,8 a	14 ± 1,10 b
13th July	11,3 ± 0,8 a	15,12 ± 1,15 b
24th August	15,34 ± 0,5 a	17,3 ± 0,43 b

Table 4.4.1 Leaf water potential (LWP) values of treated and control plants. Each value represent the mean of (n=15) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Chlorophyll fluorescence (Table 4.4.2), express as Yield (% of Photosintetic rate) did not show significant difference between treated and control plants from 23th June to 24th August.

Yield (%)	Biochar	Control
23th June	0,58 ± 0,04 a	0,48 ± 0,10 a
13th July	0,39 ± 0,22 a	0,35 ± 0,18 a
24th August	0,42 ± 0,11 a	0,45 ± 0,11 a

Table 4.4.2 Yield (% of Photosintetic rate) values of treated and control plants. Each value represent the mean of (n=15) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Root length (cm) (Figure 4.4.1.a) and root surface area (cm^2) (Figure 4.4.1.b) did not show significant difference between treated and control plants at all sampling dates, unlike root volume (Figure 4.4.1.c), which showed that treated plants had a higher values than control plants at all sampling date.

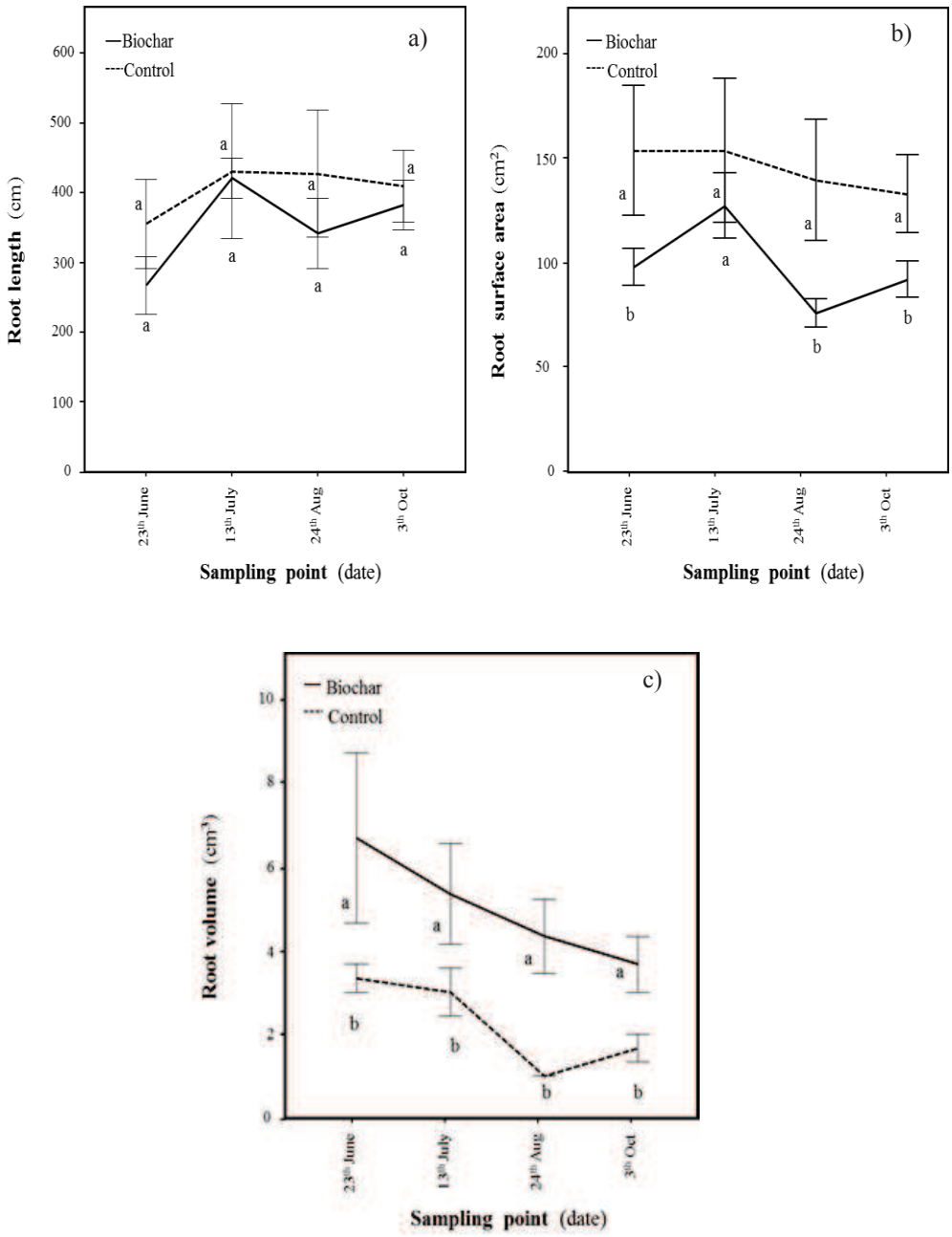

Figure 4.4.1 Root parameters: root length a), root surface area b) and root volume c). Each value represent the mean of (n=15) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

4.4.2 FRUIT TRAITS

Plot yield (n° bunch/plot) and bunch yield (n° berries/bunch) (Table 4.4.2.a) did not show significant difference between the treated and control plants.

Parameters	Unit	Biochar	Control
Plot yield	n° bunch/plot	5,25 ± 5 a	8,75 ± 7,2 a
Bunch yield	n° berries/bunch	27 ± 11 a	32,5 ± 7 a
Berries dry weight	g	0,18 ± 0,06 a	0,11 ± 0,05 b
Fruit fertility	n° seeds/berries	1,3 ± 0,5 a	1,7 ± 0,7 a

Table 4.4.2 Fruit quantitative parameters. Each value represent the mean of (n=15) ±SE.

a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Berries dry biomass (g) (Table 4.4.2) showed a higher value in treated plants while fruit fertility (n° seeds/berries) did not show significant difference. Fruit qualitative parameters (Figure 4.4.3.a, b) such as tartaric acid, pH, total soluble solids content and Alcohol potential did not show significant difference between the treated and control plants. However, despite statistical evaluation, we observed that the values of total soluble solids content and Alcohol potential were higher in treated than control in plants.

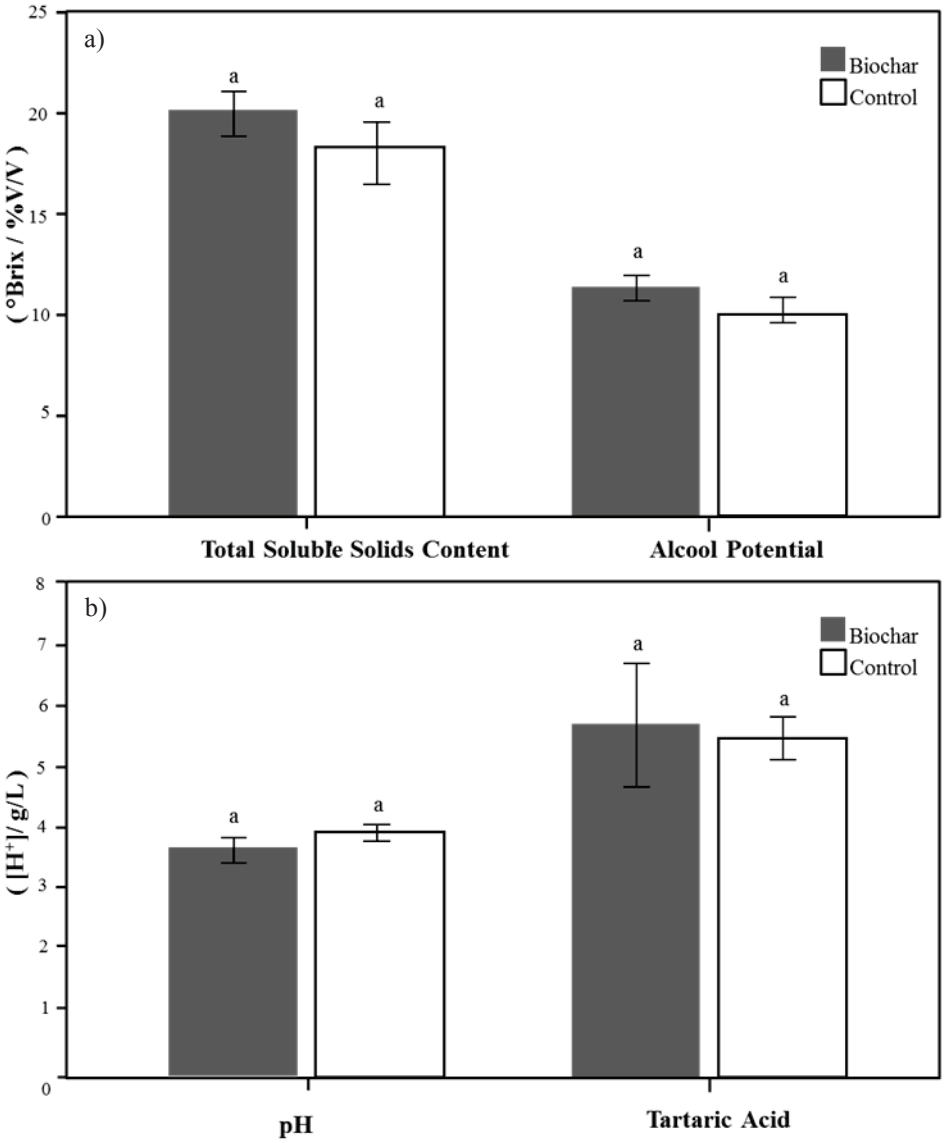

Figure 4.4.3 Fruit qualitative parameters: total soluble solids content (°Brix) and Alcohol potential (%V/V) a), pH (H^+) and tartaric acid (g/L) b). Each value represent the mean of (n=5) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

Grape content of antioxidant molecules (Figure 4.4.4) such as total polyphenolic, anthocyanin and flavonoid content were higher in biochar treated plants than in the control ones. PC (Polyphenol Content) was mainly present in seeds fraction while, AC (Anthocyanin Content) and FC (Flavonoid Content) were mainly present in seeds and skin fraction in both treatment.

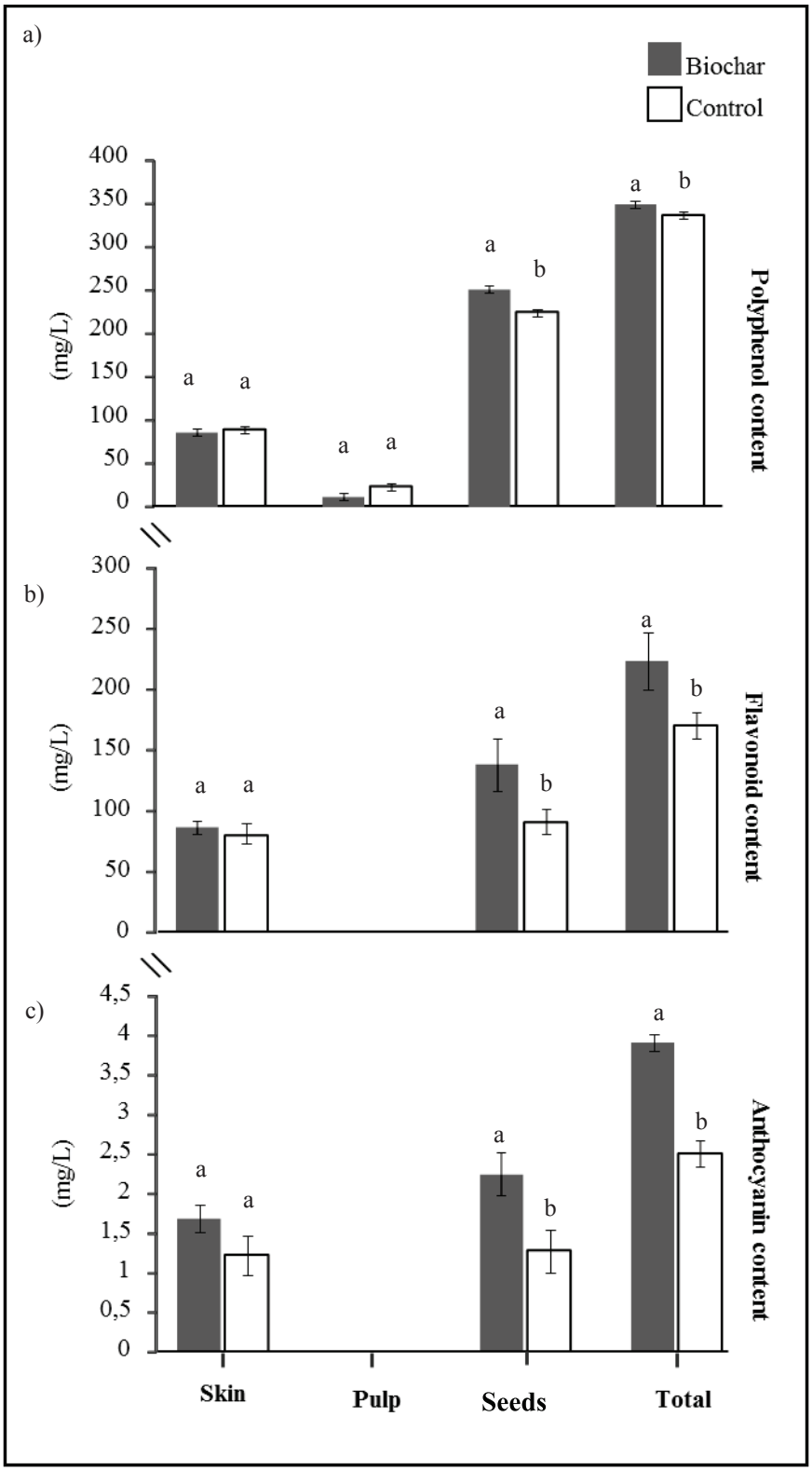

Figure 4.4.4 Grape antioxidant content: polyphenol content a), flavonoid content b) and anthocyanin content c). Each value represent the mean of (n=5) ±SE. a, b indicate a statistically significant difference between treatments ($p < 0.05$).

4.5 SHORT DISCUSSION

In the present work we found a higher photosynthetic efficiency of the Photosystem II (yield parameter) for potted *Vitis vinifera* plants treated with biochar. This result clearly indicated a higher plant performance probably due to the higher soil water and nutrient availability together with a higher root length (Chan et al. 2007). In particular, results referring to roots showed the occurrence of a considerable difference when biochar is present in the soil. The higher value of root number and length suggests the biochar effect on root production and biomass investment. Finally, in the case of *Vitis vinifera* plants growth in open field, biochar addition seemed to influence the radial growth of roots with an increase in root volume. In this case, fruit parameters were also measured showing a higher biomass of grape berries for plants treated with biochar probably due to the higher soil nitrogen availability (Bell et al., 2005). Analysis of fruit quality also showed a higher concentration of antioxidant substances and phenolic compounds for biochar treated plants. Also in this case the higher soil nitrogen availability may be responsible for the increase of polyphenols, anthocyanin and flavonoids (Delgado et al., 2004).

Chapter V
General Discussion

Amendment of soil by vegetal charcoal called biochar could help to remediate a number of environmental problems due to the modern style of life and to an increase of C emission in the atmosphere.

Biochar is a solid material obtained by pyrolysis process applied to different type of vegetal material and this fact explains its different properties which depend upon 1) the raw material used, and 2) the pyrolysis reaction parameters. It is obvious that the final result obtained after amendment it will depend also on the chemical and physical properties of the soil where this biochar is applied.

It is far from the scope of this thesis to make a review of all the types of effect ascribable to biochar amendments; nevertheless, in general terms it can be said that biochar has the capacity to influence positively the soil fertility and the productivity of plants (Chan et al. 2007). In particular, it is known that biochar effects on a plant are also the consequence of its beneficial action on the soil parameters such as: increased nutrients availability, pH enhancement, reduction of nutrient leaching, and effect on the life cycle of resident soil flora and fauna. In our experiments, the soil used to grow the plants shows that the addition of biochar does not change its field capacity for water when a normal watering regime is applied unlike when a water with holding regime is applied. In this last case, the addition of biochar delays the loss of water.

This effect of biochar on water retention explains why we have investigated different anatomical and molecular traits in three different plant species at various stages of development. The three plant species have been selected for different reasons: *Arabidopsis* represented the plant model useful for future molecular investigations by forward and reverse genetics approaches; tomato and grape have been selected as both represent the most important crops for the Italian agriculture and economy which could take advantage of biochar amendment.

5.1 *Arabidopsis thaliana*

In order to test the influence of biochar on *Arabidopsis thaliana* seedlings, we investigated different morphological and molecular parameters when the seedlings were grown under normal conditions or under water stress conditions.

5.1.1 SEEDLING TRAITS ANALYSIS

The higher values of RDM and ADM found in Arabidopsis seedlings when soil was treated with biochar amendment under a normal watering regime, could be attributed to the intrinsic-structural properties of biochar. In fact, the "sponge-structure" of biochar could have prevented leaching of water and nutrients from soil in respect to soil which had not been amended. The combination of these two elements (higher availability of water and nutrients) could have allowed a better growth condition and the accumulation of biomass in above- and below- compartments than control plants. At the same time data regarding plants grown under water stress conditions, the presence of biochar into the soil inhibits the overall (above- and below-ground) plant growth more strongly in respect to the control. This effect could be explained by the same strong binding property of biochar for water (Conte et al., 2013). In fact, it is reasonable to suggest that under water stress condition the water potential of soil with biochar becomes more negative than the soil without biochar with the consequence that cell walls of root hair and epidermis cells are not able to win the soil water potential (i. e., the strength, which binds water to soil-biochar particle mixture). The presence of biochar increases the number of leaves, when water stress is not applied. Interesting is the fact that under water stress condition total leaf area is reduced despite the number of leaves emitted remains constant; this suggests that smaller leaves are formed in presence of biochar. These results could be interpreted as an effect of biochar presence in the soil on the activity of the shoot apex, which could influence its ability to form new leaf-primordia but not in presence of water shortage. The explanation of this effect is too complex for the high number of factors which could be involved in this event, however a possibility could be that the activity of the shoot apex in presence of biochar is affected in the rate of the mitotic activity of stem cells in the SAM (Shoot Apex Meristem).

In literature has been reported that a strong event of water stress degrades the photosynthetic pigments (Mohammadkhani et al., 2007). In our experiment, we have never observed damage to the photosynthetic pigments and a possible explanation could be that the water stress duration in our experiments was too short for inducing a degradation of the photosynthetic pigments.

However, the importance of the metabolic control of the photosynthesis calls for further investigations of this aspect in order to understand better whether or not biochar amendment has an influence also in the photosynthetic process.

LRWC is an important parameter, which measures the relation existing in plant tissues between a) water content, b) osmotic adjustment between cells and c) amount of water loss due to stomata transpiration. In our experiment the value of LRWC in plants treated with biochar results to be independent from water availability and it is lower than in the control plants when water is available whereas becomes higher under water stress condition. This fact could be explained by suggesting that biochar addition to the soil becomes a stabilizing factor of relations between plants and water metabolism in the sense that protect the plants from the effect of water stress, at least for the value of water shortage achieved in our experiments. However, at this stage it is difficult to understand exactly if the effect of biochar observed by us is ascribable to variations in water uptake/transportation, in osmotic exchange between cells, or in water transpiration. Certainly, the data referring to the number of stomata seems to suggest that an effect of biochar upon water transpiration could be reasonably excluded as no variation in the number of stomata is present in respect to the control when water is available.
The number of stomata is reduced when plants grow under water stress whereas the presence of biochar in the soil does not induces any variation in respect to the control. This data referring to stomata suggest that an increase of rate of water transpiration cannot be responsible for the reduction of leaf thickness observed in our treatments.
In recent studies (Marenco et al., 2009) the leaf thickness has been correlated to the LRWC. Our data referring to leaf thickness confirm the possible stabilizing role of biochar in respect to the water metabolism when plants are under water stress condition. In normal watering regimes, the difference (the highest values were found in control plants) in leaf thickness found by us could have been the consequence of a reduction of the number and dimension of mesophyll cells, or by a reduction of their turgidity and consequently of their diameter.
Further studies will still have to be carried out to better understand the effects of biochar on morphometric leaf parameters.

Interesting are the data referring to the root compartment. In fact, the observed increase of root length could represent the need of the plant to explore an increased amount of soil in search for water as probably the biochar particles bind water molecules with a water potential so strong that cannot be won by the water potential of roots. Therefore, the plants must explore a major amount of soil to absorb the same amount of water. This hypothesis is supported by three observations: 1)

no difference in total root length exists when plants are maintained under water stress conditions as less water is available; 2) root tips do not variate when water is available indicating that there is no need to emit new roots in response to biochar presence into the soil but an increased root length is sufficient to absorb the water needed; 3) there is no need to increase the number of vessels in the central cylinder when plants are under water stress condition as observed in control plants because in presence of biochar in the soil water absorption depends closely on the root length increase.

In conclusion, we suggest that *Arabidopsis* seedlings grown under a normal watering regime receive a metabolic advantage by the biochar presence in a soil which induces a greater biomass accumulation whereas is very evident how its presence when plants are under water stressed condition affects strongly all morphological traits investigated in our experiments.

5.1.2 GENE EXPRESSION: MOLECULAR ANALYSIS

The use of biochar has been shown to have a lot of advantages including improvements in soil quality and consequently plant growth (Chan et al. 2007).
In particular, it has been demonstrated that biochar significantly changes many chemical properties of the soil and, among the most important, there are the increase of total nitrogen, the increase of extractable phosphorus and cation-exchange capacity (CEC) (Mustafa et al. 2010). Furthermore, the addition of biochar to the soil reduces leaching of ammonium compared to untreated soil and this effect is due to its already known “sponge-structure” (Lehmann et al. 2003). In recent experimental works have been reported that the total nitrates/nitrites, ammonia and nitrogen content and nitrogen fixation rate is increased by biochar addition into the soil (Baronti et al. 2010) that consequently improves the soil microbial communities and their activity, in particular the activity of nitrogen fixing bacteria (Lehmann et al. 2011). All these important evidences highlight that the biochar increases the soil nutrients and decreases the amount of nitrogen fertilizer input to soil.
In order to determine the effect of this soil improvement on the plant treated with biochar we have selected some genes codifying transporters of nitrogen metabolites and genes codifying genomic factors involved in the plant stress response.

In particular, AMT1.1 and AMT1.2 genes were chosen as it is known that they are up-regulated when the nitrogen resource (NH_4^+ and NH_3) is present in high concentration in the soil (Lauter et al., 1996; Wang et al., 2001). AMT1.1 is up regulated by the NH_4^+ form deficiency condition

(Ludewig et al., 2002) while AMT1.2 has not a specific affinity regarding the ammonium form. Due their specific affinity at the different ammonium forms the expression of these genes has been evaluated in order to deepen the biochar influence on these two ammonium forms.
In regard to nitrate transport we have chosen to test the expression of NRT2.1 and NRT1.2 genes. NRT2.1 has been selected as it belongs to the family of HATS genes (high affinity transport system) while NRT1.2 belongs to the family of LATS genes (low affinity transport system). Their induction seems to be stimulated by $NO3^-$ in root and their expression is increased after prolonged N starvation (Ono 2000). In our experiments, these genes have been tested for their specific different affinity to nitrogen compounds with the aim to understand if their expression is affected by biochar presence in the soil.
Concerning the genes involved in plant stress response, BH7 and BH12 genes have been chosen by us as both genes seems to be specifically active under water deficit conditions and they have been proposed to act as a negative regulator of growth (Söderman et al., 1996; Hjellström et al., 2003). BH7 and BH12 genes belong to the homeodomain leucine-zipper and are implicated in the plant response to water deficit as deduced from their transcriptional induction by water deficit condition. Finally, PIP2.2 gene (an aquaporin belonging to plasma-membrane intrinsic proteins) has been chosen for its susceptibility to multiple environmental stimuli (Jang et al., 2004).

Our data suggest that addition of biochar in the soil does not induces a change in the expression of BH7 and BH12 genes when plants are maintained under a normal watering regime. Under water stress conditions only the expression of BH12 gene is increased although at a reduced level in respect to the control. This last result confirms that biochar interferes with the water cycle in the soil probably by releasing the water bound to its particles when plants are under stress conditions.

AMT1.1 gene is a gene belonging to the ATM family (ammonium transporter) and it is characterized by a high affinity for the ammonium in its NH_4^+ form rather than its NH_3 form. In literature, it has been shown that this gene is up-regulated in nitrogen deficiency condition and its expression is predominantly dependent on the local N status of the roots (Ludewig et al., 2002). The higher AMT1.1 gene expression found by us in control plants under normal watering regime indicates that control plants need more nitrogen supplementation than plants growing in presence of biochar. Despite at present we do not have any indication of how the presence of biochar in the soil makes more available nitrogen to the roots, it remains that an increased nitrogen mobility in the soil could be an additional factor that together with the increased water availability discussed above could sustain the improved biomass accumulation observed by us in our experiments.

NRT1.2 gene belongs to the family of LATS (Low Affinity Transport System) whereas NRT2.1 gene belongs to the family of HATS genes (High Affinity Transport System). Both genes are up-regulated in condition of N-deficiency (Glass et al., 2000). The NRT2.1 gene expression is controlled by shoot-to-root signals of N demand and moreover, this gene is the first identified molecular target of the long-distance signaling informing the roots of the whole nitrogen plant's status (Gansel et al 2001). The higher expression of both genes that has been observed in our experiments when control-plant grow under a normal watering regime indicates that both biochar presence in the soil and the loss of water alter the binding of nitrogen to the soil particles and make the nitrogen more available for absorption by the roots (Zheng et al., 2013).

PIP2.2 gene codes for an aquaporin belonging to a highly conserved sub-group of membrane proteins called "plasma-membrane intrinsic proteins" PIPs.
PIPs are genes involved in plant response to environmental stimuli and especially they are the most important genes that respond to water stress condition by influencing water availability through a modification of the water channels. PIP2.2 is abundantly expressed in roots and under water stress condition, its expression is up-regulated (Jang et al., 2004).
Data presented here suggest that the presence of biochar in the soil down-regulates the PIP2.2 gene expression when plants are grown in normal watering regime while up-regulates it when plants are under water stress by returning its expression level to the same level as it was in control plants growing under a normal watering regime. This result confirms that biochar modifies the water cycle in the soil making more water available for the metabolic activity. Difficult to explain is the high expression value of this enzyme when water stress is applied to plants growing in presence of biochar.

In summary, data obtained by us in regard to expression of different genes, support the hypothesis that the biochar presence into the soil improves the nitrogen transport in the plant.

5.2 *Solanum Lycopersicum* (L.) 30t ha^{-1}

In order to test the influence of biochar on Cherry tomato cultivar, we have investigated several different morphological and molecular parameters.
Results obtained from this experiment confirm our hypothesis about the possible beneficial biochar effects on 1) plant growth, 2) fruit yield and quality and 3) soil nutrient availability.

5.2.1 SOIL ANALYSIS

Data presented in different research works claim that the effects of biochar on plant growth are indirect consequences of its beneficial effects on the soil parameters (increased nutrients, pH enhancement, less leaching, etc). In order to test this statement, in this thesis we have tested parameters such as CEC, N_{av}, P_{av} and C_{tot}.
CEC (Cation-exchange capacity) represents the power of soil to retain cations and organic acids; moreover, it stands for the cation exchange between the soil particles and the solution present within (Chapman, 1965). In literature, it is well known that the biochar applied into the soil reduces the leaching of nutrients/cations and is able to retain a major quantity of water; thus, the soil CEC increase found in our investigation is attributable to the combination of these two elements (Qambrani et al., 2017).
Moreover, the biochar particles into the soil are subjected to oxidation processes that contribute to increase the charge density on their surface with the consequence of increasing CEC value (Liang et al., 2006). This last hypothesis could explain the higher CEC value found in our plants when they were treated with biochar during the FS stage, in which the biochar particles are probably subjected to a higher degree of oxidation.
In regard to N_{av} content, the higher value found in control soil during the Fs could be due to a reduced microbial activity that does not use the available nitrogen present in control soil. Concerning the P_{av} content, a lower value was found in biochar-treated soil during the ES. This data could be attributed to a higher content of root exudates into the soil that, due to their chelating activity, do not allow the instrumental detection of the P_{av} element (Marschner et al., 1987). Finally, regarding the C_{tot} value, a higher value was found in biochar-treated soil during the ES. This value could be attributed to two important factors: 1) the initial and elevated microbial activity and their subsequent production of organic acids into the soil (Sood et al., 2003); 2) the conservative properties of biochar that keeps the soil carbon content constant – a value that, in normal soil, tends to naturally decrease over time (Laird, 2008).

5.2.2 SEEDLING AND FRUIT ANALYSIS

In this experiment, the data referring to the below and above compartments have been found of particular interest. They could be interpreted as an effect of biochar addition on nutrient and water content in the soil. The increase of these two parameters could be attributed to two important events: a) soil biota activity that could have been improved by biochar addition (Castaldi et al., 2011); and b) reduced leaching of nutrient, which could be closely related to the intrinsic sponge property of biochar inducing an increase in water retention (Lehmann et al. 2003).

This hypothesis seems to be supported also by data referring to CHN plant content which confirm the higher content of nutrients in the soil when biochar is present, In fact CHN results suggest the occurrence of a higher metabolic activity and respiration in the roots during the fruit stage (Fs). This finding agrees with several works that show that the nitrogen concentration in fine roots is directly related to their metabolic activity and respiration (Ryan 1991, Pregitzer et al. 1998, Withington et al. 2006).

About the higher value of C_{tot} root content found in control plants at the Fs, one possible explanation could be due to an increase of the secondary metabolite content (i.e., lignin and tannins) in the thinnest fine roots (Harborne 1984); in fact, it is known for secondary metabolites to have a C content higher than compounds like cellulose and other sugars (Chua and Wayman 1979, Krässig, 1993). In regard to CHN analysis on leaf compartment, its increase suggests a higher photosynthetic activity of the plant during this first growth stage (Sinclair et al., 1987).

Unfortunately, this result contrasts with the higher value of *total* and *a* chlorophyll content which has been found in control plants. In regard to this result, the current literature does not provide a clear cut explanation about the influence of biochar on the leaf chlorophyll content. Thus, further investigations is necessary to understand whether biochar amendment has an influence in the photosynthetic process.

Interesting are the data referring to the production of flowers and fruits. The higher production of flowers and fruits in treated plants could be attributed to the higher value of phosphorus content in the soil. In fact, Poulton et al 2002 suggests that vegetative and reproductive properties of tomato plants are improved by a higher phosphorus content in the soil, as this nutrient is able to stimulate a root development that increases fruit production (Filgueira et al. 2000).

Regarding the morphological and morphometric traits of fruits, the lack of significant difference contrasts with data referring to traits such as TA, TSSC and cis- / trans-lycopene content that show a clear increase of the value. The increase of titrable acidity (TA) is of particular interest as it confirms that the addition of biochar to the soil increases the availability of potassium which

improves the TA during the fruit repining stage (Arah et al. 2015, Passam eta al., 2007).
This result has a practical consequence for the Agri-food industry that traditionally adds citric acid as a preservative. Therefore, a higher content of natural acids in tomatoes resulting after biochar amendment could lower the quantities of additional preservative necessary to preserve the final product.
However, it remains that in literature the complete knowledge about the biochar influence on fruit quality is still far from having been achieved, and this situation explains why we cannot give an appreciable explanation for the data presented here. We can only assume generically that biochar makes more easily available nutrients and that it makes an effect on the quality of fruits. In regard of antioxidant compounds, the current literature does not provide evidences which could help us to interpret the increase observed in presence of biochar; therefore it seems reasonable to suggest the occurrence of a possible interference between a possible increase of nitrogen availability and the carotenoid metabolic pathway. In conclusion, we may assert that the use of biochar has a positive effects on the fruit yield and quality of tomato plant. Similar results has been recently found by Agbna et al. (2017) on tomato plants subject to three different biochar rates and irrigation regimes.

5.2.3 GENE EXPRESSION: MOLECULAR ANALYSIS

Among the different genes involved in nutrients transport, genes codifying for the main transporters of nitrogen and phosphate metabolites have been investigated. The tested genes in our experiments confirm and support the biomass data showed above which indicate that when the plants are treated with biochar they show a growth rate higher than the control plants.

In particular, the choice of AMT1.1 gene is due to its involvement in in ammonium transport in the sense that it is up-regulated when the plants are in presence of low concentration of nitrogen (NH_4^+) (Ludewig et al., 2002). In fact, sometime the increase in the expression of this gene has been used as an indicator of a Nitrogen starvation in the plant (Gansel et al., 2001). In particular, it seems that AMT1.1 expression is predominantly dependent on the local N status of the roots, as it is mostly stimulated in the portion of the root system directly experiencing N starvation (Gansel et al., 2001).

In our experiments the higher expression level of this gene in control plants during the Es suggests the beneficial effect of biochar amendments. An indirect demonstration that our hypothesis is reasonable comes directly from the data regarding AMT1.2 gene that is up-regulated in presence of higher concentration of (NH_4^+ and NH_3) (Lauter et al., 1996; Wang et al.,

2001). In this case, our data present an increase of its expression in soil with biochar during the Vs confirming the effect of nutrient mobilization of this amendment.

In regard to the whole plant's N status indicator we have tested an indicator such as the level of expression of the NRT2.1 gene (one of the most important genes for the nitrogen transport -as suggested by Gansel et al., 2001).

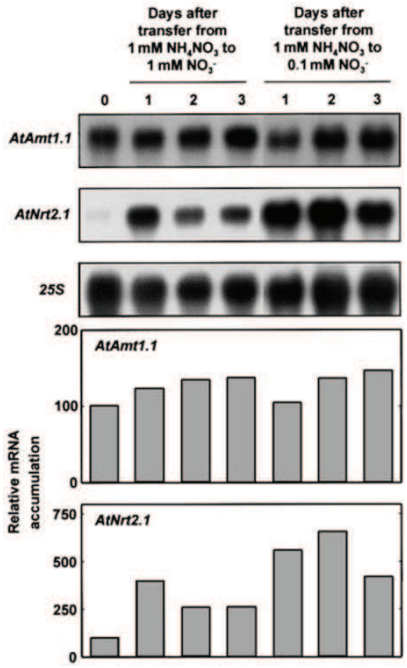

Figure 7.2.1 Both AtNrt2.1 and AtAmt1.1 have been shown to be induced by N starvation (Filleur and Daniel-Vedele, 1999; Gazzarini et al., 1999; Lejay et al., 1999; Rawat et al., 1999; Zhuo et al., 1999).

Our data show a higher expression value of this gene in treated plant during the Es and Fs: these results highlight how the presence of biochar into the soil improves the availability of nitrogen compounds that could be absorbed and used by plants.

Finally, in regard to phosphate transport, the genes tested (PT1-2) by us are known to be up-regulated in the presence of a low phosphate concentration in the soil (Liu et al., 1998).

In our research experience, PT2 gene shows a high expression value in control plants during the Vs, while the PT1 gene never shows differences in its expression. At present, it is difficult to explain why PT1 gene expression does not change in presence of biochar, whereas the higher expression of PT2 in the vegetative stage confirms that also for this nutrient the presence of biochar in the soil could increase its availability with the effect of supporting a better growth rate.

The importance of our data in regard to an improvement of nutrient availability in presence of biochar is bound to the fact that we confirm the occurrence of this effect in long-term

experiments, whereas all the demonstrations presented up to date in literature refer to short-term experiments.

5.3 *Solanum Lycopersicum* (L.) 300t ha^{-1}

Data presented here and referring to parameters such as above- and below-ground biomass show that biochar application in the soil at high concentrations affects negatively the plant growth.
In literature some problem in regard to biochar use have been reported, for example the binding and deactivation of agrochemicals (herbicides and nutrients) in soil, the excess of toxicants, the increase of EC and pH and finally, the negative impacts on germination (Kookana et al., 2011).
A possible explanation for our obtained results could be attributed to the high concentration and to the subsequently large volume that biochar particles occupy inside the pot. The pot without the biochar will have a larger quantity of soil and relative nutrients than the pot with the presence of biochar, therefore, the lower quantity of soil and the corresponding lower nutrient content do not enable an optimal growth to the treated plants. Another possible cause could be found in the chemical biochar composition; in fact, its aromatic hydrocarbon polycyclics at high concentration could affect negatively the overall plant growth rate (Krull et al., 2009).

5.4 *Vitis vinifera* (L.) (Chardonnay cv) Pot experiment

In order to test the influence of biochar on *Vitis vinifera*, we have investigated different morphological and physiological parameter in a pot experimental-setting.

5.4.1 SEEDLING ANALYSIS

The higher LWP value observed in control plants indicates that during treatment these plants have achieved a higher level of stress condition (Williams et al., 2002) in respect to plants treated with biochar. The stress condition could be attributed to higher leaf temperature developed during the experiment (Frank et al., 1973). From this result, we can assume reasonably that plants treated with biochar become more efficient in water utilization. This hypothesis is supported by the observation that unlike the LWP, the SWP value did not differ between treated and controls pots.

The photosynthetic efficiency of the Photosystem II (yield parameter) which is higher in plants treated with biochar indicates a better overall health status of plants probably due to a higher availability of water and nutrient in the soil, but due also to a higher root length value (Chan et al. 2007). This is particularly important in the period of July-August which coincides with the phase of ripening fruit, in which the plant absorbs the nutrients and water that will be used in the leaves for carbohydrates production.

However, literature published on higher plants does not report any research work that could be used to validate this our hypothesis on superior plants. Unlike studies in unicellular algae show that nitrogen deficit conditions affects significantly the photosynthetic efficiency of photosystem II leading to an effect on the overall relative health status (Berges et a., 1996).

Also for the *Vitis vinifera* the data referring to roots show the occurrence of a considerable difference when biochar is present in the soil. In particular, the higher value of root traits (number of roots and their length) suggests occurrence of an effect on the production of new roots or alternatively, on the biomass investment that could explain the length increase observed by us.

In regard to T5 and T10 cm the lack of difference between the treatments (and, accordingly, the biochar action on the soil surface albedo cannot be validated) could be due to a reduction of pot surface area and, consequently, to a decrease in the amount of solar radiation absorbed by the soil.

5.5 *Vitis vinifera* (L.) (Chardonnay cv) Field experiment

5.5.1 SEEDLING ANALYSIS

In analogy with the pot experiment also in the field experiment, a higher LWP values found in control plants in all the three sampling points and this could be attributed to a smaller amount of water present in the plant consequently due to the severe environmental water stress conditions. This data one again highlights how the biochar could provide a greater water availability to the plant (Van Zwieten et al. 2008).

Difficult to interpret is the lack of significative differences found in the photosynthesis efficiency of the photosystem II; nevertheless, we can suggest that in the field experiment unknown environmental factors could have quenched the potential effect of biochar.

Concerning the below ground biomass, a very important data has been observed for the root volume as the addition of biochar positively influenced only the radial growth of roots measured as volume of roots.

An explanation for this phenomenon could be attributed to a greater need for transporting water and nutrients leading to the production of higher amount of vessels in the xylem as suggested by the work of Amendola et al 2017, in which the presence of biochar in grape roots seems to increase their morphological plasticity and, as a consequence, a larger radial growth.

Beside a larger availability of water, the largest dry weight of grape berries treated with Biochar could also be attributed to a higher availability of nitrogen (Bell et al., 2005). From this factor could also depend the increase in the concentration of antioxidant substances and phenolic compounds such as polyphenols, anthocyanins and flavonoids as suggested by the work of Delgado et al., (2004).

Therefore, our data support the hypothesis that biochar improves also the nutrient uptake despite is not clear yet how the nitrogen increase availability can improve the grape quality as this property is affected by a complex interaction of several factors such as the type of cultivar, the rootstock, the site, the climate, the soil type and the season.

Chapter VI

Conclusion

The present study represent only a first step to understand the mechanisms involved in plant growth and development which could be effected when biochar is used for.

All morphological, physiological and molecular data presented here support the hypothesis that the biochar addition to the soil affects positively plant growth and the quantity and quality of fruits.

In particular, for both *Solanum* and *Vitis* species, the biochar presence has shown to improve the antioxidant fruit content and this result calls for attention given its importance for potential technological applications.

In regard to Cherry tomato cultivar, in all the three plant stages considered, the plant treated with biochar shows a higher value in the seedling and fruit traits.

Positive effects have been detected also in both *Vitis vinifera* experiments where the biochar presence improves the root length in the pot experiment while, in field experiment, it improves a radial root growth. However, the fact that in *Solanum lycopersicum* 300t ha^{-1} experiment, the biochar addition to the soil does not always produce positive effects calls attention on the right amount biochar to be added to the soil for amendment purpose. Interesting are the data obtained with the model plant. In regard to this *Arabidopsis* seedlings, the biochar addition into the soil shows positive effects in all the parameters considered but only under normal watering regime. In our experimental setting, it has emerged that the biochar presence under water stress condition inhibits strongly the growth of plants but does not influence the stress status of the plant. In any case, the presence of morphological, physical and molecular responses in the model plants indicates that in future all the hypothesis suggested in this work could be verified in the other plants. The use of forward and reverse genetic approaches could highlight the genes responsible for the variations observed in our experiment.

In conclusion, the data in this thesis confirm the hypothesis that the biochar presence improves the soil fertility and plant growth however, further investigations will be carried out to strengthen our results.

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Scientific paper in preparation

Data collected during the experiments will be published in two papers. Here we present the draft of the first one which will be sent to an international journal.

Biochar soil amendment enhances cherry tomatoes growth, fruit production and quality in controlled conditions.

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1. Introduction

Global demand of agricultural crops is connected to a strong environmental impact mainly due to habitat fragmentation and land clearing as well as fertilization uses that pollute both water and terrestrial ecosystems (Tilman et al. 2011). To meet the need of doubling the crops production forecasted for the 2050, will implicate a major impact on the environment (Sachs et al. 2010; Tilman et al. 2011). Therefore, in the next future will be crucial to find new methods and technologies to achieve greater yields with lower global environmental impacts. Biochar is a solid material obtained from a process called pyrolysis characterized by a thermal transformation of biomass at high temperature and in the absence of oxygen dramatically reducing gas emission (Bridgwater et al. 2002; Yanai et al. 2007). Moreover, so far, has been demonstrated that biochar application significantly changes most of the soil chemical-physical properties (Adeyemi and Idowu, 2017). In particular, biochar showed to increase both soil carbon and soil water content as well as macro aggregates, electrical conductivity (EC), total nitrates/nitrites, ammonia and nitrogen (Baronti et al. 2010; Amendola et al. 2017), extractable phosphorus and cation-exchange capacity (CEC) (Hossain et al. 2010). Furthermore, due to its skeletal-sponge structure, biochar reduce soil leaching of ammonium (Lehmann et al. 2003), improves rhizosphere microbial communities and activities with particular regards to both cellulose degrading and nitrogen fixing bacteria (Lehmann et al. 2011). All these important evidences highlights that biochar enhance important functions such as soil carbon sequestration and nitrogen soil retention becoming a good technological products for a future sustainable agriculture (Ying et al. 2014).

Although changes of soil characteristics due to the biochar application seems to have a general positive trend, with a mean yield increase of 10%, averaging different crops, soils and climates (Jeffery et al., 2011), results on the effects of biochar on crop development are still inconsistent (Biederman and Harpole, 2013). This is due to various factors such as differences in parental starting material, pyrolysis conditions and chemical-physical soil characteristics (Amonette et al., 2009; Mukherjee and Lal, 2014). Thus, for deeper understandings of the complexity of these

relationships more studies are required before introducing the biochar strategy among the common and sustainable agricultural practices (Lorenz and Lal, 2014). Tomato plants (*Solanum lycopersicum* L.) in Mediterranean region are optimally grown in passive solar greenhouses on well-drained, sandy loamy soils with pH values ranging between 6 and 7. Tomato is a plant species of a great commercial importance worldwide (Leonardi et al., 2000 a, b). Indeed, tomato is the most consumed nonstarchy vegetable with a global production of about 164 million tons (t) of fresh fruit harvested on a 4.7 million hectares (ha) surface (Burton-Freeman and Reimers, 2011; FAOSTAT, 2015 from Vaccari et al. 2015). From a health point of view, a large body of research supports an inverse relationship between consuming tomatoes and tomato products and risk of certain cancers as well as cardio-vascular disease, osteoporosis, ultraviolet light–induced skin damage, and cognitive dysfunction (Weisburger et al., 2002; Burton-Freeman and Reimers, 2011). Indeed, tomatoes are the most significant source of dietary lycopene, a powerful antioxidant, and in general, secondary metabolites like cis-lycopene, trans-lycopene and β-carotene and other carotenoids, which are directly involved in these protective actions (Burton-Freeman and Reimers, 2011). To date, there is still very poor information on the effects of biochar on tomato plants growth, fruit yield and antioxidant content (Hossain et al. 2010; Vaccari et al. 2015). Dumas et al. (2003) reviewed contradictory results and often-incomplete dataset, in various studies concerning the effects of environmental factors such water availability, mineral nutrients (nitrogen, phosphorus, potassium and calcium) and plant growth regulators on antioxidant content in tomato fruits. Given the above-mentioned multiple effects that biochar can have on soil characteristics, we hypothesized that biochar-derived changes in resource supply may play a crucial role in enhancing plant growth, fruit yield and antioxidant content. To test this hypothesis, after assessing the effects of biochar on soil physical-chemical properties, morphological parameters of shoot, root and fruit together with the number of fruit and their antioxidant content were investigated in a time-course pot experiment of cherry tomato plant (Pachino var.). The identification of possible relationships between any alterations of soil

chemical-physical properties, plant growth and fruit production may further contribute to elucidating the mechanisms of biochar actions and its use.

2. Materials and methods

2.1 Experimental set up

Six seeds of cherry tomato variety were sown each 9 L cylindrical pots (h 24 cm, Ø 21 cm lower and upper Ø 26 cm) filled with 1:2:1 mixture of peat, silica sand and bark humus (Table X) and placed in a growth chamber. Soil was maintained constantly wet, relative air humidity was 75 % with air temperature of 25 °C and Photosynthetically active radiation (PAR, 400–700 nm) at pot height of 500 μmol m^{-2} s^{-1}. After germination, only one seedling was left to grow for each pot. In treated pots, soil was mixed with biochar at a rate of 30 t ha^{-1} according to Baronti et al. (2010). A number of 10 treated plants (B) and 10 control plants (C) were collected at each of the three harvesting points defined as early stage (Es, 36 days after germination (a.g.)), vegetative stage (Vs, 84 days a.g.) and fruit stage (Fs, 140 days a.g.) for a total of 60 plants.

2.2. Biochar characterization

Biochar used in this study was produced by Romagna Carbone s. n.c. (Italy) from orchard pruning biomass through a slow pyrolysis process with an average residence time of 3 h at 500 °C in a kiln of 2.2 m in diameter and holding around 2 ton of feedstock. Measurement of pH was carried out by potentiometry (pH meter Eutech Instruments pH 700, 2013) according to IBI standards (2014). The electrical conductivity (EC) value was obtained by direct instrumental determination in 1:20 soil:water (w/v) extracts, according to IBI standards (2014). Cation exchange capacity (CEC) was assessed according to Mehlich (1938) using $BaCl_2$. Moisture content was calculated according to the Black method (1965) as the difference in sample weight before and after oven drying at 105 °C to constant weight.

Several parameters can be used to assess carbon stability in biochar. Calvelo Pereira et al. (2011)

used the thermo-labile fraction and the oxidation efficiency with potassium permanganate and potassium dichromate, while Enders et al. (2012) used a combination of volatile matter and H:C ratios corrected for inorganic C. In the present work, we referred to IBI standards (2014), which define carbon stability as the molar ratio of hydrogen to organic carbon (maximum 0.7).

Total nitrogen (N_{tot}), total carbon (C_{tot}), organic carbon (C_{org}) and hydrogen (H) contents were determined by dry combustion (Dumas, 1831) using a CHN elemental analyser (Carlo Erba Instruments, Mod 1500, series 2). In the case of C_{org}, combustion was carried out after the complete removal of inorganic C with acid. Available nitrogen (N_{av}) was determined by a modified Kjeldahl procedure using Devarda's alloy (Liao, 1981) as reducing agent to convert (NO_3) and (NO_2) into $(NH_4)^+$ and subsequent Kjeldahl digestion. Total phosphorus (P_{tot}) was detected by spectrophotometry (UV-1601 Shimadzu) according to the test method described by Bowman (1988). Available phosphorus (P_{av}) was extracted by a $NaHCO_3$ solution at pH 8.5 and evaluated by spectrophotometry according to the Olsen test method (1954). Alkalinity of samples with a pH value greater than 7.0 was determined by titrimetry according to the Higginson and Rayment method (1992).

2.3 Soil characterization

To assess soil chemical-physical properties and the effects of biochar on these characteristics, four soil samples were collected and analyzed before and after biochar application. Methods for the characterization of CEC, P_{tot} and P_{av}, N_{tot} and N_{av}, C_{tot} were described in the previous paragraph of the biochar characterization. The pH was determined by potentiometry (pH meter Eutech Instruments pH 700, 2013) according to Conyers and Davey (1988). EC was measured by direct instrumental determination according to Rhoades (1996). The different forms of available mineral nitrogen were determined by ion selective electrodes (Greenberg et al., 1985) on soil samples dissolved in deionized water. Only for soil samples, particle size distribution was analyzed by the Udden-Wentworth (1922) method. Furthermore, soil characteristics for control

and biochar-amended pots were determined at three different sampling points (Es, Vs and Fs). In this case, samples were collected at soil surface, at the middle (18 cm) and at the bottom of the pot (32 cm). Once freed from roots, soil samples were mixed together in one bulk sample, air dried until constant weight, passed through a 2 mm sieve and stored at 4 °C in dark until processed.

2.4 Plant traits analysis

At each sampling point, plant traits for stem, leaves and roots sectors were measured. In particular, leaves were detached from the branches, counted, scanned at a resolution of 400 dpi with a calibrated flatbed scanner coupled to a lighting system for image acquisition (Epson Expression 10000 XL). Successively, images were analyzed by WinRhizo Pro V. 2007d (Regent Instruments Inc. Quebec). In the case of both length and surface area of root, the root system was carefully washed from the soil, all roots collected, scanned and images analyzed as described above for leaf traits. Content of both chlorophyll *a* and *b* (μg ml^{-1}), was obtained by finely chopping 0.5 g of fresh leaf material, homogenized with 10 ml of 80% acetone solution and then centrifuged for 5 minutes at 2500 rpm. Afterwards, 9 ml of 80% acetone were added to 1 ml of concentrated extract handily shacked and subjected to spectrophotometric reading at the wavelengths of 663 nm and 645 nm. Arnon's (1949) equations were used for calculation of the extracted chlorophyll. Finally, in order to obtain biomass values as dry weight (g), root (RDW), shoot (SDW) and leaves (LDW) were separately oven dried at 70°C until constant weight and weighed.

2.5 Fruit traits analysis

The number of flowers and fruits were monitored for each plant during the relative phenological stages. In order to have a homogeneity in fruits collection, tomatoes were harvested at point 5 of the ripening color chart (USDA 1975). To evaluate fruit biomass and the fruit water content, 40

tomatoes for each treatment were oven drying at 70 °C for 48 h and weighed (FDW). Moreover, the tomato fertility was determined by both seeds number and dry weight on a samples of 10 tomatoes for each treatment. Morphometric fruit parameters such as polar and equatorial diameters, epicarp thickness, right and left mesocarp thickness, were measured on 40 tomatoes for each treatment by scanning the fruits and analyzing the images with ImageJ software (open source https://imagej.nih.gov/ij/). Furthermore, a number of five fruits were homogenized (VWR Collection, VDI 12) for the determination of the following qualitative parameters. The titratable acidity (TA), expressed as percentage of citric acid, was measured according to the titration method at pH 8.1 with NaOH (0.1 N) (Petruccelli et. al 2015). The total soluble solids content (TSSC), expressed as °Brix, was measured by refractometer (HANNA Instruments, HI 96813), after homogenate centrifugation at 13000 g for 20 minutes at 8°C (George et. al 2004). Finally, both cis- and trans-lycopene and β-carotene content were determined by extracting 6 g of homogenate with 60 ml of hexane-methanol-acetone (2:1:1 volume) with 2.5% of BHT for 30 minutes at 4 °C in dark condition (Martinez and Valverde 2002). Subsequently, 10 ml of distillated water were added and the polar phase (hexane) recovered. The polar phase was subjected to spectrophotometric reads at 472 nm (maximum absorbance peak of the trans-lycopene), 502 nm (maximum absorbance peak of the cis-lycopene) (George et al. 2004) and 453 nm (maximum absorbance peak of β-carotene) (Bohm et al. 2002).

2.6 Statistical analysis

The comparison of control and biochar-treated plants was tested for all measured parameters. Normality of data distribution was tested for each investigated parameter (Kolmogorov-Smimov and Shapiro-Wilk tests). Square root or log transformations were applied to ensure normal distributions and equal variances. For Chlorophyll content (a, b and total), n° of leaves, leaf area, dry weight of roots, stem and leaves, root length, SRL, RTD, mean diameter, n° of flowers and fruit quality parameters (% ac. Citric, ° Brix, content of cis-/trans- lycopene and β-carotene) a

two-tailed t-test was applied. For n° of fruits, seeds dry weight, fruit dry weight and water content, polar and equatorial diameter, epicarp thickness and right mesocarp thickness, a one-way ANOVA was performed followed by Bonferroni post-hoc test.

N° of seeds and left mesocarp thickness data did not meet the normal distribution and non-parametric statistic test was performed (Kruskal–Wallis test) followed by Mann–Whitney two samples test as post-hoc test.

Parametric and non-parametric analysis were applied at a significance level of 95%. Statistical analysis were performed using SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Biochar characteristics

The biochar tested was found to meet European Biochar Certificate (EBC, 2012) and IBI-Standard (2014) requirements with regard to C_{tot} and C_{org} content, respectively. Its C:H value, close to 0.7, ensure a good stability to the organic carbon. With regard to the conductivity value, the biochar used showed a higher salt content compared to soil. Moreover, available phosphorus and nitrogen represented 17.7% and 0.3% of total phosphorus and nitrogen, respectively (Table 1). Particles larger than 2 mm accounted for 11.9% of the total mass. Particles smaller than 2 mm were distributed as follow: 16.1% between 2 mm and 200 μm, 10.1% between 200 μm and 50 μm, 52.7% between 50 μm and 20 μm, 17.4% between 20 μm and 2 μm and 3.7% smaller than 2 μm (Table 1).

3.2 Soil characteristics

3.2 Plant characteristics

Leaves biomass (Figure 1a) showed a linear growth during the time while stem and root biomass (Figure 1b, c) showed an exponential growth throughout the experiment. For all the three plant sectors considered, at early stage (Es, day 36) biomass did not show any significant differences

between treated and control plants. Whereas, at both vegetative (Vs, day 84) and fruit stages (Fs, day 140) biochar treated plants showed significantly higher values ($p<0.05$) for all three plant sectors. Leaf number (Figure 2a), leaf area (Figure 2b), root length (Figure 2c) and root surface area (Figure 2d) increased significantly in time throughout the experiment and biochar treated plants showed significantly higher values than control plants at the second and third developmental stages (i.e. Vs and Fs).

Chlorophyll content did not show any significant differences between treated and control plants at day 36 (Figure 3a, b and c). Biochar treated plants showed a higher value of *b* chlorophyll content than control plants at day 84 (Figure 3b). At the last sampling point, higher values of both *a* and *total* chlorophyll content were found in control plants (Figure 3a, c).

3.3 Fruit characteristics

Both flower and fruit number (Figure 4a, b) showed respectively almost four-fold and three-fold higher values in the biochar treated plants than in control ones. Moreover, also both seed number and dry weight (Figure 4c, d) resulted significantly higher for biochar treated plants than control ones. Fruit dry weight (Figure 5a) and fruit morphometric parameters such as polar and equatorial diameter (Figure 5b, c), epicarp thickness (Figure 5d), mesocarp thickness right (Figure 5e) and left (Figure 5f) did not show any differences between biochar-treated and control plants. Fruit qualitative parameters such as citric acid (Figure 6a) and total soluble solids content (Figure 6b) resulted to be significantly higher in biochar treated plants than in control plants. Fruit content of antioxidant molecules such as trans-and cis- lycopene (Figure 7a, b) were also higher in biochar treated plants than in the control ones. Unfortunately, no differences were detected for β-carotene (Figure 7c).

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Interspecific variation in functional traits of oak seedlings(Quercus ilex, Quercus trojana, Quercus virgiliana) grown under artificial drought and fire conditions D. Chiatante1 · R. Tognetti2,4 · G. S. Scippa2 · T. Congiu3 · B. Baesso1 · M. Terzaghi1 A. Montagnoli1 Received: 10 December 2014 / Accepted: 13 February 2015 / Published online: 13 May 2015 © The Botanical Society of Japan and Springer Japan 2015

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