Universidade de Aveiro
2014

Departamento de Biologia

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Biochar amendment as a strategy to reduce hydric stress in *Eucalyptus*

Biochar como estratégia na redução do efeito da seca em *Eucalyptus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob orientação científica da Doutora Glória Catarina Pinto, investigadora no CESAM (Centro de Estudos do Ambiente e do MAR) e do Departamento de Biologia da Universidade de Aveiro, e co-orientação do Doutor Franciscus Gert Anton Verheijen, investigador em pós doutoramento do CESAM e Departamento de Ambiente e Ordenamento da Universidade de Aveiro

Apoio financeiro da FCT – Fundação para a Ciência e Tecnologia, no âmbito do projeto EXPLOCHAR (PEst-C/MAR/LA0017/2013)

Ao chão que piso, ao ar que respiro e às estrelas do céu…

o júri

Presidente	Professor Doutor João António de Almeida Serôdio Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro
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Agradecimentos

A concretização desta tese, concluindo mais uma etapa nesta vida, não teria sido possível sem a contribuição, direta ou indireta, de muitos aos quais devo um especial agradecimento:

Em primeiro lugar e aos mais importantes, ao papá e à mamã pela oportunidade e força que me deram e continuam a dar em seguir os meus sonhos e objetivos, pelas bases sólidas de valores humanos e éticos que me incutiram e por serem, indiscutivelmente, os melhores exemplos de vida a seguir. Ainda à Anita pelas gargalhadas, parvoíces e noites partilhadas e aos avós pelo carinho, preocupação e presença constantes, que em momentos de desânimo sempre me incentivaram a nunca a desistir.

Ao Dr. Jacob Keizer pela oportunidade e por me receber como parte da sua equipa de trabalho.

À Drª Glória Pinto e Dr. Frank Verheijen um enorme obrigado pela oportunidade e confiança depositadas, pela disponibilidade constante, cordialidade, recomendações e por serem, para mim, referências profissionais a seguir.

Agradeço também ao Grupo Leal & Soares – SIRO por me facultarem o espaço e condições necessárias à realização deste trabalho, em especial ao Eng. Hartmut Nestler pela amabilidade e disponibilidade constantes durante a realização do mesmo.

Um enorme obrigada à Cláudia Jesus, Joana Amaral e Pedro Monteiro pela preciosa e inesgotável alegria, força e espírito de companheirismo diários, fundamentais na execução deste trabalho. Ainda ao Flávio Silva e Hugo Vieira pela incansável ajuda prática e orientação estatística.

Ao Rui Costa, por me ter aparecido na vida e me ter mostrado o valor do trabalho, força e dedicação. Pelo carinho e incentivo contantes e por nunca ter duvidado de mim.

A eles, os melhores de todos: Catarina Domingues, Patrícia Fresco, Tânia Costa, Tânia Silva e Raul van Breda. Dos sorrisos às lágrimas e a todos os momentos de brincadeira, amizade e alegria que partilhamos. Obrigada pela vida e pela cor que trazem a todos os dias da minha existência!

palavras-chave Biochar, Ecofisiologia , *Eucalyptus globulus*, Seca, Nutrientes.

Resumo

As concentrações de CO_2 atmosférico têm atingido níveis alarmantes nas últimas décadas resultando no aquecimento global em conjunto com alterações climatéricas generalizadas. O *stress* hídrico é uma das maiores consequências destas alterações, constituindo um disruptor direto dos fluxos hídricos e de carbono com efeitos na produção primária e equilíbrio de ecossistemas terrestres. Afeta as plantas a diferentes níveis dependendo da intensidade, duração e níveis de progressão, conduzindo a respostas a níveis fisiológicos, bioquímicos e moleculares. Estudos recentes, suportam a aplicação de biochar nos solos como estratégia de mitigação, visando promover a retenção de água e nutrientes. Assumindo a importância económica da espécie *Eucalyptus globulus* em Portugal e o crescente aumento de períodos de seca na região mediterrânica, o objetivo deste estudo foi avaliar o efeito de mitigação do biochar em *Eucalyptus globulus* sujeitos a limitação hídrica. Comparamos também o seu efeito com a utilização de fertilizantes sintéticos e da conjugação da utilização de ambos. Deste modo, 136 plantas de *E.globulus* foram sujeitas a um período experimental de 6 semanas, divididas por 2 grupos (sem limitação de água – 80% de capacidade de campo e com limitação de água – 30% de capacidade de campo), cada um constituído por 4 tratamentos: sem biochar, com biochar (6%), sem biochar+ fertilizante e com biochar+fertilizante. No final do ensaio, as plantas foram avaliadas ao nível morfológico, fisiológico e bioquímico. Os resultados deste estudo indicam um decréscimo na maioria dos parâmetros analisados em condições de limitação de água, nomeadamente ao nível das trocas gasosas, potencial hídrico, altura, níveis de transpiração, fotossíntese, número de folhas, área das folhas, peso de biomassa aérea e peso das raízes, o que sugere que a limitação de água afeta severamente a performance da planta. A aplicação do biochar promove o aumento de área das folhas e níveis fotossintéticos, juntamente com uma diminuição no conteúdo de açucares totais (TSS) e um ligeiro aumento dos valores de potencial hídrico, sugerindo que em condições limitantes de água, a aplicação de biochar a 6% promove a habilidade de retenção de água pelos solos, reduzindo os efeitos gerais de stress nas plantas. Em condições de controlo, sem limitação de água, as plantas sujeitas a aplicação de biochar apresentaram sinais atípicos de murchidão apical, bem como baixos níveis de fotossíntese e morfológicos gerais, quando comparados com a utilização de fertilizantes, nas mesmas condições, sugerindo que em condições de rega abundante a aplicação de fertilizante é preferível ao biochar. Os resultados da conjugação do biochar+ fertilizantes mostraram baixa performance da planta, comparativamente à aplicação em separado, nomeadamente ao nível da área folear, número de folhas, peso das raízes, fotossíntese, trocas gasosas e carotenoides, em condições de limitação de água. Em conclusão, os nossos resultados indicam que o biochar tem um efeito mitigante em condições de limitação de água para *E.globulus*, no entanto a sua aplicação não representa uma mais valia em condições de rega abundante ou quando aplicado em conjugação com fertilizantes.

keywords Biochar, Ecophysiology, *Eucalyptus globulus,* Drought, Nutrients.

Abstract
keywords

Atmospheric CO_2 concentrations are higher than any time in the last decades, resulting in global warming along with general climactic changes. Drought stress is one of the most important effect of climactic alterations and directly disrupts water and carbon fluxes, with effects on primary production and terrestrial ecosystems equilibrium. It affects plants at different levels depending on intensity, duration and progression of drought, leading to responses at physiological, biochemical and molecular levels. The biochar application to the soils as a mitigation agent, by enhancing water and nutrient availability, has been well documented. Assuming the economic importance of *Eucalyptus globulus* in Portugal and the rising demand of dry periods in mediterranic region, the main goal of this study was to evaluate the ability of biochar to reduce the stress effects in *Eucalyptus globulus* plants, under water limiting conditions. We also evaluate the application of fertilizers in plant performance and the conjugation of both. For this propose, 136 plants of *Eucalyptus globulus* were subjected to a 6 weeks period assay, divided by 2 groups (well watered – 80% of field capacity and water stress - 30% of field capacity), which one with 4 treatments: without biochar, with biochar (6%), without biochar+fertilizer and with biochar+fertilizer. At the end of the experimental period, the plants were evaluated at morphological, physiological and biochemical levels. Our results showed a significant decrease in most of analysed parameters with water limitation, as gas exchanges, hydric potential, height, transpiration, lateral branches, leaf area and number of leaves, photosynthesis (except WSb), above ground biomass (fresh and dry) and weight of roots, suggesting that the water limitations severely affect plants performance. Biochar application improve leaf area and photosynthesis rates along with decreases the total soluble sugars content and slight higher values of water potential, suggesting that in water stress conditions it is beneficial to applied biochar into soils to enhance their ability to store and use water, reducing overall stress levels in the plants. In well watered conditions, the biochar showed wilting signals In well watered conditions the use of biochar promoted lower photosynthetic rates and lower values of all morphological parameters as above ground biomass, number of lateral branches and leaf area when compared with nutrients application, along with apical wilting signals suggesting that in in well water conditions, the use of biochar is not a better option. The results of conjugation of both biochar and fertilizers were contradictory. In well water conditions we found a higher values of Fv/Fm along with chlorophylls and lower values of number of leaves, leaf area, photosynthesis and TSS. Otherwise, in water stress conditions and besides the lower performance in all gas exchanges parameters, we noticed higher above ground biomass, when compared with biochar and fertilizer applied alone. In conclusion, our results shows that biochar amend water limitation conditions in *E.globulus*, but is not a main value for plant performance in well water conditions or when applied together with fertilizer. In either case, further studies are needed.

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List of Abbreviations

A – Photosynthesis

CEC – Cation exchange activity

Ci – Internal concentration of CO_2

Chla – Chlorophyll a

Chlb – Chlorophyll b

E – Transpiration

EC – Electric conductivity

Fv/Fm – Maximum quantum yield

gs – Stomatal condutance

ha – hectare

N - North

PCA – Principal component analysis

ϕPSII – Effective quantum yield

ROS – Reactive oxygen species

TSS – Total soluble sugars

UV – Ultra violet radiation

W – West

WS - (Water Stress – no biochar)

WSb - (Water Stress- with biochar)

WSbf - (Water Stress – with biochar and fertilizer)

WSf - (Water Stress – with fertilizer)

WW - (Well Watered – no biochar)

WWb - (Well Watered – with biochar);

WWbf - (Well Watered – with biochar and fertilizer)

WWf - (Well Watered – with fertilizer)

List of Elements and Chemicals

Ca – Calcium

CO_2 – Carbon dioxide

Cu – Copper

K – Potassium

MDA – Malondialdehyde acid

N - Nitrogen

P - Phosphorous

TBA – Thiobarbituric acid

TCA - trichloroacetic acid

Zn - Zinc

Chapter I

General Introduction, Research Aims and Relevance

Economic importance of Eucalyptus globulus

The world's total forest area (Figure 1) was estimated to be over 4 billion hectares in 2010 (about one-third of the total land area in the world), providing a complex array of vital ecological, social and economic goods and services (FAO, 2010). Forests constitute the main terrestrial biodiversity repository and currently, about one billion people around the world depending on their resources for survival supplies (FAO, 2010). Also, forests can store more than a 650 billion tons of carbon mostly in biomass and soil (FAO, 2010), and their destruction or degradation contribute to 10-30% of all CO_2 emissions to the atmosphere (Abril et al., 2011). Both wood and manufactured forest products contributed with more than $450 billion to the world economy each year (Abril et al., 2011).

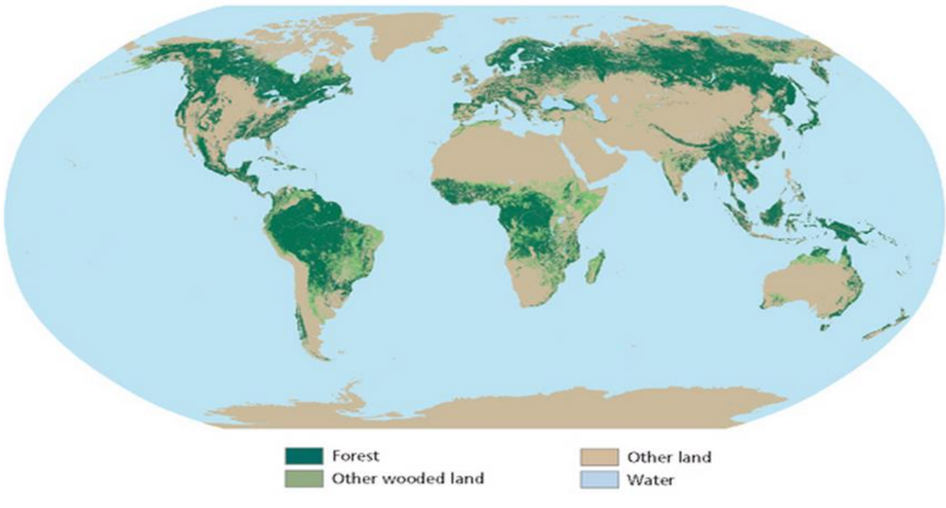

Figure 1- Representation of forest area, other wooded land, other land and water in the world (Source: FAO, 2010)

While sustainable management, planting and rehabilitation can conserve or increase the forests productivity, deforestation, degradation and poor forest management can reduce them (FAO, 2010). Industrial and agricultural revolutions, urbanization and other natural occurrences (such as fires and pests) have caused higher levels of deforestation, which caused in the last 20 years a loss of 95 million hectares of forests (Abril et al., 2011). Planted forests constitutes nearly 7% of world forests and they provide essential related forest products and services to follow the increasing growth demand population, allowing environmental benefits as a climate change mitigation (FAO, 2010).

The Eucalypt genus includes 900 species native to Australia. and is characterized by it fast growth and a high tolerance to arid environments, leading it to support many adverse conditions as drought, fire, insects and soil acidity, allowing to a high success as an exotic plant (Rockwood et al., 2008). *Eucalyptus globulus* is the main hardwood specie that grows in temperate Mediterranean regions and it is one of the most popular members of the genus in the forestry industry due its relatively high fibre yield and rapid growth (Warren et al., 2011). It was discovered in Tasmania in 1792 and it was the first eucalypt species to be formally described (Potts et al., 2004).

Eucalypt wood has been applied as a source of energy, once its biomass can be converted to produce petrochemical products such as a lubricants, textiles, biodegradable plastics, celluloses (Sims et al., 2010), plywood, veneer (Gorrini et al., 2004), essential oils and providing natural shade and windbreaker (Foley et al., 2004). Once eucalypt has a fast growth, its productivity is maximized by short rotations. Some species also have wood properties (high density) that allow its application for charcoal production, paper manufacturing and lumber (Rockwood et al., 2008). *Eucalyptus* s*pp*. plantations have been estimated to cover a total area of about 20 million ha around the world in South America, South Africa, Asia, Australia and Southwestern Europe (Flynn et al. 2010). In Portugal, 700,000 ha of *E. globulus* has been established as a key species (Chaves et al., 2003), and due its higher growth rates, superior pulp properties and environmental adaptability, it has been extensible used for pulpwood production (David et al., 1996), representing 6% of the total value of national exports, constituting a major value in the country’s economy (Silva et al., 2004). Due to increased anthropogenic activities and the consequent effect on global climacteric patterns, water limitation has become a main disruptor of terrestrial ecosystem dynamics once it will affect the textural properties and compositions of the soils influencing the water retention ability (Pereira et al. 2007).

Water deficit and its impacts on forests

The increasing need to supply the growing population in the last decades has dramatically increased the general world pollution, leading to global warming which largely contributes to alterations in the composition and equilibrium of the atmosphere and land around the globe. Extreme events such as rainstorms and drought periods are predicted, increasing the risks of wildfires which may severely impact on plant productivity, causing the mortality of trees and contributes to soil degradation (Santos et al., 2001).

The 21st century is characterized by increasing drought events (Barriospedro, et al., 2010) and the consequences for the plant water relations and general yield has been studied and is well documented (Leuzinger et al., 2005, Bréda et al., 2006, Granier, et al., 2007). According with the economic, agronomic and ecological importance of agriculture and forestry, drought periods and its effects on plants has evoked interest beyond scientific proposes, namely at governments and environmental organizations (Trigo et al., 2000). Along with the influences in agricultural aspects, the incidence of pests and diseases affected by climacteric alterations may influence the geographical distribution of crops (Santos et al., 2001) and recurrent severe droughts over the time may change the composition of species in ecosystem (Granier et al., 2007).

Mainland Portugal is characterized by a mild temperate climate, with average annual temperatures varying from 7°C to 18°C and average annual precipitation ranging from 500 mm to over 3000 mm (Santos et al., 2001). In the last years, the climacteric patterns exhibited temporally and spatially irregular behaviours (Daveau et al. 1977; Trigo et al., 2000), with severe winters and lower precipitation rates in spring, summer and autumn months, leading to more and severe drought periods (Guidi et al., 2013).

Plants often deal with biotic and abiotic stressors that may compromise their health and performance (Leuzinger et al., 2005). Among all variation factors of temperature, salinity and nutrients, hydric stress is probably the most limiting factor. It is determined by the increasing temperatures and/or heat waves associated with low levels of precipitation, leading to a long dry periods and high demands of plant transpiration rates (Ryer et al., 2013). Drought stress is a direct disruptor of water and carbon fluxes with effects on primary production and terrestrial ecosystems dynamics once it could affect the soil functions namely at organisms activity level (Bréda et al., 2006).

The lack of water supplies affect plants at different levels depending on intensity, duration and progression of drought, and it can lead to responses at physiological, biochemical and molecular levels (Seyed, S. et al., 2012), as can be seen in Figure 2. As a primary response, plants tend to close the stomata and reduce foliar areas, leading to lower levels of transpiration by avoiding water losses (Pita et al., 2001). Also, when the stomata close, the gas exchanges in plants are seriously compromised leading to a lower CO_2 uptake (Anjum et al., 2011) affecting photosynthetic rates and, consequently, tissue growth and differentiation (Shao et al., 2008).

The water balance of any plant is driven by their ability to absorb water from the soil and the efficiency of transport along the vessels, both ensured by transpiration rates. The water flow continuum between soil-plant is a result from a water potential gradient between roots-soil and leaves-transpiration, leading to a decrease in leaf water potential as evaporation increases and also as soil dries (Martinková et al., 2014).

The efficiency of soil water absorption largely depends both by extension and density of root systems and their distribution and growth also depends on physical soil properties such as bulk density or moisture content, directly linked with climate dynamics (Bréda et al., 2006). Thus, in drought conditions, plants develop a deep and dense root system, providing access to larger soil water reserves (Leuschner et al., 2004). Enhancing root system could also be described by a recovery strategy to re-establish the water potential equilibrium over night, when the deeper roots reach the water displayed as a gradual downward shift as the soil dries and supports transpiration supplies during stomatal closure periods (Bréda et al., 1995). Maintenance of turgor values provides a chance to preserve metabolic processes and growth. Turgor is a result from osmotic adjustments or decreases in cell wall elasticity, which allows plants to take up water at low soil water potentials (Pita et al., 2000). The enhancement of root systems is reported by some studies, which emphasise an increasing of root/shoot ratio under drought conditions (Xu et al., 2010; Chaves et al., 2003).

Plants react to hydric stress by avoiding losses of water also by growth inhibition as a way to maintain water levels and plant carbon assimilation at healthy rates. To minimize water losses, plants also reduce the light absorbance through rolled leaves, by a dense trichome layer which increase reflectance rates, steeping leaf angles, decreasing canopy leaf area or shedding older leaves (Chaves et al., 2003). They also respond by accumulating somatically active compounds also known as compatible solutes, which ensure a physical replacement of water (hydrophilic nature) near cellular compounds, preventing desiccation (Bohnert et al., 1999), stabilizing the photosystem II complex, protecting enzymes and proteins, maintaining membrane integrity and lower levels of ROS (Chen et al., 2007).

Elevated soluble sugar content (TSS), known as the most important compatible cellular solute in plants, are reported by many authors as a main physiological response of hydric deficit, once it will lower the hydric potential of cells which may facilitate the absorption of water from de soils. Increasing the TSS content may also maintain cell turgor, gas exchanges levels and growth rates at stress conditions (Chaves et al., 2003; Guarnaschelli et al., 2006), support biological stabilization of membranes, proteins and enzymes and it is related with a repression of the photosynthetic-associated genes expression (Chaves et al., 2003).

Cellular growth is the main direct physiological process constrained by drought conditions. With reduction of turgor pressure and assimilation of carbon, cells cannot ensure their development, limiting plant performance and productivity. Thus, during the acclimation phase, water stress typically leads to low growth rates. Some studies show that aboveground biomass can decrease in order to carbon partitioning favour root system development (Silva et al., 2004, Charp and Davis, 1979). Some studies in *Eucalyptus spp.* also reported significant decreases in shoot, basal diameter, total leaf area, foliage and number of branches (Demming-Adams et al., 1996, Chaves et al, 2003). Chlorophyll are thylakoids pigments responsible for the absorption and delivery of the light energy to the photosynthetic apparatus, along with carotenoids and anthocyanins that protect photosynthetic machinery from excess of light or UV light, respectively. Carotenoids are also an essential compounds of thylakoid membranes, supplying a variety of functions as a reactors with quench triplet chlorophyll, singlet oxygen and superoxide anions, harvest light to photosynthesis process and play an important role as a precursor for abscisic acid biosynthesis (growth regulator) (Cunningham and Gantt, 1998). When subjected to stress conditions, namely water limitations, plants tended to loose chlorophyll being forced to divert the absorb light by other ways like thermal dissipation to protect the photosynthetic apparatus, by carotenoids (Reddy et al. 2004). Due to their importance for the leaf and photosynthetic success, variation on pigment content may provide important information about the physiological state of the leaves. Usually, the values of both pigments decrease with hydric stress (Alberte et al., 1977).

The decrease of chlorophyll content under hydric stress is well documented as a result of photo-oxidation and degradation (Anjum et al., 2011), nevertheless Michelozzi et al. (1995) and Correia et al. (2014) found an increasing of chlorophylls and carotenoids content under drought conditions along with the maintenance of their volume, which seems to be a defence mechanism response in *E.globulus*, to prevent chloroplasts injury from toxic concentrations of ions.

The lack of water under stress conditions will increase the solute concentrations once the protoplast volume shrinks, which can cause serious structural and metabolic consequences. As a direct effect from desiccation are the membranes integrity and proteins damages, which leads to metabolic dysfunctions in lipid bilayer causing displacement of membrane proteins which, with solute leakage, contributes to a loss of selectivity and activity of membrane-based enzymes (Anjum et al. 2011).The Malondialdehyde acid (MDA), is a secondary product of lipid peroxidation, produced by oxidation and enzymatic degradation of cellular membranes in the presence of Reactive Oxygen Species (ROS) and it has been an extensively studied indicator of the prevalence of free radicals reactions and tissue damages in plants. *ROS* are commonly produced and accumulated in response to stress, which may cause oxidative damages to lipids, proteins and nucleic acids, seriously compromising cellular health. It is used as a direct indicator of oxidative stress (Arcoverde et al., 2011). Some studies have reported increasing MDA contents in response to ROS production as a consequence of moderate and sever hydric stress in plants (Arcoverde et al., 2011, Cechin et al., 2010, Lima et al., 2010) and Correia et al. (2014) reported a direct relation between increasing hydric stress levels and the MDA content, in *Eucalyptus globulus.*

Studies have been made in *Eucalyptus spp*. to access the water limitation stress levels:

- Significant decreases in most of morphological parameters such as growth rates, total biomass, leaf area, number of lateral branches and root length (Silva et al. 2004, Li et al. 2003);
- Decline in stomatal conductance (gs), photosynthesis (A) and transpiration reates (E) were also reported under drought stress (Guarnaschelli et al. 2006, Lima et al. 2003);
- Decreasing water potential in response to water limitations has been also reported for *Eucalyptus globulus* plants (Guarnaschelli et al. 2006, Correia, et al. 2014);
- Increasing total chlorophylls and carotenoids concentration with increasing water deficit (Michelozzi et al.1995, Correia et al.2014). Regarding to MDA content, Correia

et al. (2014) also reported an increase of MDA content in *E.globulus* exposed to different levels of water deficit.

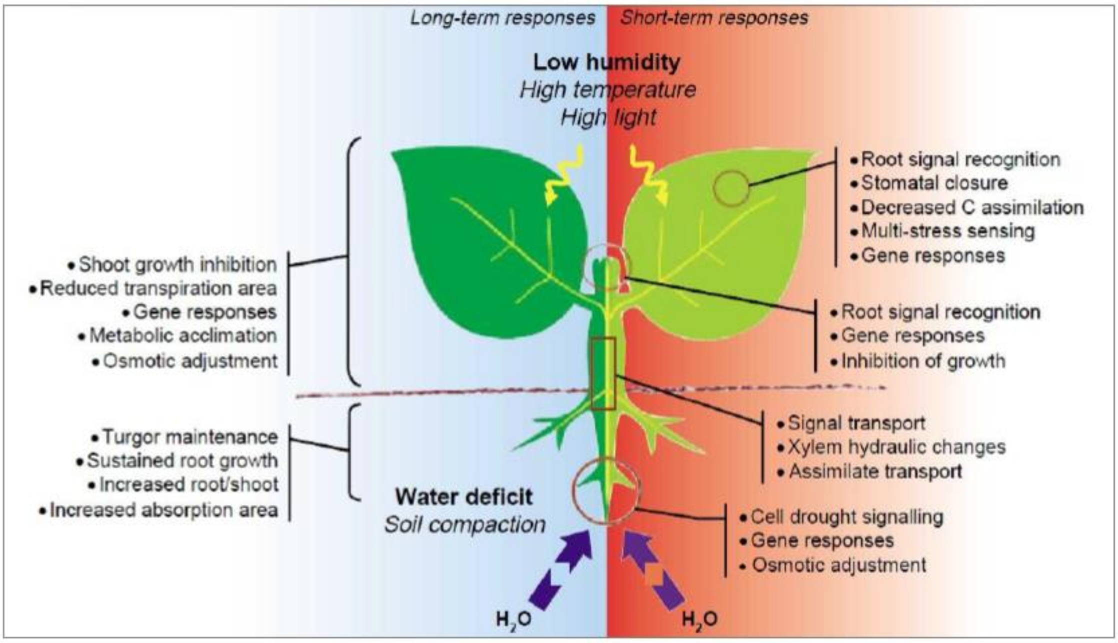

Figure 2- General plant responses to drought stress (short and long term). Source: Chaves et al.2003

Biochar as a strategy to mitigate hydric stress

Biochar is considered as a new and promising approach to improve soil quality and crop productivity. After application, biochar can influence soil aggregation, porosity and density, which in turn affect the soil aeration, water holding capacity, microbial and nutritional rates. Some studies reported biochar benefits in climate mitigation, reducing the effects of global warming and drought conditions (Lehman et al., 2007, Ogawa et al., 2006, Laird et al., 2008, Woolf et al., 2010).

Biochar is usually defined as a charred organic matter material, produced with the intent to be deliberately applied to soils to sequester carbon and improve their properties (Lehmann et al., 2009). It has a heterogeneous composition and its surfaces can exhibit both hydrophilic or hydrophobic, acidic or basic properties, allowing biochar to react with soil components (Amonette et al., 2009). Biochar is degraded on a timescale of centuries depending on original feedstock, production strategy, environmental and management conditions (Cheng et al., 2008, Lehmann et al., 2011).

Biochar consists of a carbonaceous material with polycyclic aromatic hydrocarbons, with an array with other functional groups (Kookana et al., 2011). Heteroatoms can be found within the aromatic rings, such as hydrogen, oxygen, nitrogen, phosphorus and sulphur which largely contribute for the heterogeneous and reactive nature of biochar's surface. Carbonaceous material, volatile matter, mineral matter and moisture are the main compounds of any biochar, wherein carbonaceous and ash materials constitute the solid part of biochar and a mix of air and volatiles constitute the gaseous fraction (Verheijen et al., 2010).

In the distant past, Amazonian communities promoted in-field fires producing extremely productive soils with dark colour named 'Terra Preta do indio'. It is defined as an anthropogenic dark soil, characterized for enhancing the soil fertility and yield performance (Lehmann et al., 2007). Recently, the potential relevance of Terra Preta has been studied with the intent of developing strategies or soils whose properties would enhance those ancient findings. Darker soils may absorb more solar energy (Verheijen et al., 2013) increasing the overall soil temperature, directly affecting the physicochemical and biological processes. Sohi et al. (2009) reported that those characters may extend the growing season with optimal growing conditions.

Biochar is produced by thermal alteration of biomass under oxygen-limited conditions. This process is named pyrolysis and is characterized by using low levels of oxygen, with variable rates of heating and temperature peaks (Sohi et al., 2009). This process produces a solid structured material with a higher surface area, reduced oxygen and hydrogen contents and a higher nutrient concentration, compared to the original feedstock (Gaskin et al., 2008). The wide variability of biochar's chemical and physical properties depends on the feedstock used, as well as the pyrolysis dynamic between available oxygen and temperature achieved during the process. There is a large variety of different feedstock's used in biochar production, varying from crops residue (e.g. corn, cereals, wood, pellets, palm oil, oilseed rape, wheat straw, hazelnut shells) to manure or organic household wastes (Sohi et al., 2009).

The interaction of biochar with soil components is directly linked with the distribution and connectivity of pores in the soil and with the charged surface character dynamics over time (Joseph et al., 2010). Fresh biochar can show hydrophobic behaviour, but when natural oxidation occurs (in biochar contacts with air and water), it surface assumes a reactive hydrophilic nature (Basso et al., 2013). When in contact with soil, biochar surface oxidizes leading to a formation of hydrophilic carbonyl, carboxyl and phenolic surface groups, promoting further active interactions with soil biotic and abiotic constituents (Cheng et al., 2006). When oxidation occurs, the cation exchange capacity (CEC) increases by improving the reactivity at the surface of biochar particles (Cheng et al., 2006).

Biochar porosity determines its specific surface area. The size of biochar pores can vary from nano to macro pores (<0.2nm to >50nm) (Downie et al., 2009) and this feature characterizes its functionality in soils: aeration, hydrology and a niche for microorganisms such as mycorrhizae and bacteria (Pietikainen et al., 2000). Otherwise, the pores also allow molecular adsorption and transport (Thompson et al., 2005), improving the surface area and promoting nutrient retention (Liang et al., 2006). The porosity degree is determined by pyrolysis temperature and nature of feedstock. It will decline in the presence of lipids, fluvic and humic acids from original feedstock (Atkinson et al., 2010).

Soil aggregation can be increased by biochar application to soils due the interactions between biochar's internal and external surfaces with soil organic matter and surrounding biota (Abel et al., 2013). This promotes the decreasing of bulk density leading to a better aeration, drainage and water holding capacity (Lei et al., 2013, Mulkahya et al., 2013). Bulk density is one of the most important factors affecting the water infiltration in the soils and the ability of biochar to change this character will improve root systems and microbial respiration (Basso et al., 2013).

One of the most important agronomic benefits of biochar in soil application is the extraordinary sorption ability associated with the improvement of water retention (Atkinson et al., 2010), which may increase crop production in non-irrigated drought periods (Jeffery et al., 2011). The negative surface charge of oxidized biochar allows electrostatic bonding with the positively charged side of dipolar water molecules (Thomas et al., 2008). Some studies reported that irrigation requirements decline in biochar amended soils, when compared to controls (Novak et al., 2013; Chan et al., 2007), with more water retained, increasing the available moisture favouring crop development during critical drought stress in growing seasons (Laird et al., 2010).

Biochar addition increases overall accumulation of osmotic active substances in plant tissues (like K+), leading to an improvement of water uptake by plants (Gaskin et al., 2010). Furthermore, biochar may not only modify the availability of water in soils, but also the physical advantageous location of water within the soil matrix, near the plant roots (Sohi et al., 2009). When applied to soils, some studies reported that biochar can improve water availability in sandy soils (Basso et al., 2013; Busscher et al., 2010; Novak et al., 2009; Pereira et al., 2012), while for loamy soils a moderate improvement has been observed (Laird et al., 2010; Karhu et al., 2011). Bornemann et al. (2007) also recognized the ability of biochars to sorb pollutants in soil/sediments and reducing pesticide toxicity (Cui et al., 2009; Wang et al., 2010).

Biochar is also considered as a nutrient source when it provides macro and micronutrients to plants, conferring it a fertilizer character in short-term application (Thomas et al., 2013). Regarding the surrounding biota, this nutrients may not be available as an energy source but, they can be leached and mineralized and, therefore, stimulate microbial activity (Lehmann et al., 2011). Biochar has also been directly linked with increasing uptake rates of Phosphorus (P), Potassium (K), Calcium (Ca), Zinc (Zn) and Cupper (Cu) by plants (Lehman et al., 2006). Recent meta-analyses have shown that in most instances plant biomass increased after application of biochar into the soils (Jeffery et al., 2011; Liu et al., 2013). Oram et al. (2014) suggested that, in legumes after char application under N-limiting conditions, biological nitrogen fixation enhanced the plant performance and, thus increased the overall biomass.

The mechanical impedance is the most determining factors for root elongation and proliferation in soil. In conditions of water limitation, this may play an important role in reaching deeper moisture and maintain vital levels of water into the plant. Lehmann et al. (2011) also reported an overall reduced tensile strength of the soil when amended with biochar, leading to an easier way for plant roots to reach water.

Relevance and application of the results

Due the economic and environmental importance of *Eucalyptus globulus* around the world and assuming the increasing of drought periods arising from climate changes which constitutes a main disruptors to *Eucalyptus globulus* performance, it is extremely important to study and develop adaptation strategies to maintain or improve plant productivity, ensuring that environmental and human needs will be supplied along the time. The soil used in this study was a typical sandy soil from eucalypt plantations in Portugal and the drought stress imposed (30% of field capacity) represent a medium stress, equivalent to a dry periods in natural conditions in a temperate climate as Portugal.

Aim and objective of this study

The main objective of this study is to evidence that biochar can be used as an adaptation agent, diminishing drought effects of climate change in *Eucalyptus globulus*. Thus, plants with biochar exposed to hydric stress will show lower stress levels (at physiological, morphological and biochemical levels) compared to plants without biochar.

Hypothesis

Eucalypts plants will experience less hydric stress and show better physiological performance under water limitation conditions, when planted in biochar-amended soil and there is no need for additional fertilizer addition to improve this performance.

Organization of the thesis

This thesis comprises three parts. Chapter I introduces a brief literature review as a background and framework. Chapter II consists of a paper, where the experimental results of this work are presented and discussed, titled "Biochar amendment as a strategy to reduce hydric stress in eucalypt seedlings". Chapter III comprises the general conclusions and future perspectives on this work.

Chapter II

Biochar amendment as a strategy to reduce drought in eucalypt plants

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Abstract

Drought events are the most important disruptor in plant performance, due the climacteric alterations, with a straight effect on primary production and terrestrial ecosystem dynamics. It affects plants at different levels depending on intensity, duration and progression of drought, leading to responses at physiological, biochemical and molecular levels. It is well documented that the application of biochar as a strategy to amend soils in drought conditions, once it enhance water and nutrient retention. Thus, the main objective of this study was to evidence that biochar can be used as a mitigation agent, diminishing the drought effects in *Eucalyptus globulus* and there is no need further fertilizer addiction to improve that. The experimental design comprises 136 plants of *Eucalyptus globulus* exposed to a water limitation period (Water Stress - WS) of 6 weeks at 30% of field capacity and a control group at 80% of field capacity (Well Watered - WW), each one with four treatments: no biochar, biochar (4%), fertilizer and biochar+fertilizer. In the end of the experiments, 6 plants of each treatment were evaluated in morphological, physiological and biochemical parameters. Our results showed a significant decrease in levels of all measured parameters between well watered and water stress, leading us to conclude that the hydric stress severely affect plants performance. In water stress, biochar improve leaf area and photosynthesis rates along with lower values of total soluble sugars and slight higher values of water potential, suggesting

that in water stress conditions it is beneficial to applied biochar into soils to enhance their ability to store and use water, reducing overall stress levels in the plants. In well watered conditions the use of biochar promoted lower photosynthetic rates and lower values of all morphological parameters as above ground biomass, number of lateral branches and leaf area when compared with nutrients application, suggesting that under well-watered conditions, the use of biochar is not a better option. The results of conjugation of both biochar and fertilizers were contradictory. In well water conditions we found a higher values of Fv/Fm along with chlorophylls and lower values of number of leaves, leaf area, photosynthesis and TSS. Otherwise, in water stress conditions, besides the lower performance in all gas exchanges parameters, we noticed higher above ground biomass, when compared with biochar and fertilizer applied by their own. In either case, further studies are needed.

Introduction

Drought stress is a directly disruptor of water and carbon fluxes, with straight effects on primary production and network terrestrial ecosystems (Bréda et al., 2006). It results from climacteric alterations and directly affects the primary production and network terrestrial ecosystems (Granier et al., 2007). It affect plants at different levels depending on intensity, duration and progression of drought, and it can lead responses at physiological, biochemical and molecular levels. To avoid dehydration, plants have to deal with two main issues: minimizing water loss and maximizing water uptake (Chaves et al., 2003). To minimize water loss, plants tend to close the stoma and reduce foliar area, leading to a lower levels of transpiration and therefore preserve internal hydric levels, avoiding xylem cavitation and death (Chaves et al. 2003)). Plants also reduced the light absorbance by producing a dense trichome layer increasing reflectance, steeping leaf angles, decreasing canopy leaf area or shedding older leaves (Chaves et al., 2003; Bohnert et al., 1999). The hydric potential also decrease along with loss of turgor in cells in response to cell wall changes to osmotic adjustments (Shao et al., 2008). The stomatal closure limits the gas exchanges levels, resulting in a reduced transpiration and photosynthesis, which will constrict the assimilation of carbon and compromise overall growing and differentiation of plant tissues (Anjum et al. 2011).Silva et al. (2004) also reported an increasing in below ground biomass, with a development of deep and dense roots that may reach inaccessible moisture in the soil. Due to increased anthropogenic activities and the consequent effect on global climacteric patterns, the water limitations has become the main disruptor of terrestrial carbon sequestration variations and terrestrial ecosystem dynamics (Pereira et al. 2007).

Eucalypt species are native from Australia although is currently worldwide spread (Stackpole et al., 2011), established over than 18 million ha in 90 countries (FAO, 2005), covering the land area of 700 000 ha in Portugal (Chaves et al. 2003)

It shows a fast growth and a high tolerance to arid environments, leading it to support many adverse conditions (Rockwood 2008*).* *Eucalyptus globulus* is the main hardwood species that grows in temperate Mediterranean climate, around the world (Potts et al., 2004) and its success as a plantation tree species has been attributed to its high productivity and pulp quality (Guarnaschelli et al., 2006). Studies in *Eucalyptus spp.* reported the effects of hydric limitations, suggesting a decrease of growth rates, leaf area and total plant biomass (Pita et al. 2000; Metcalfe et al. 1990), low rates of photosynthesis, stomatal conductance, internal concentration of CO_2 (Warren et al. 2011; Silva et al. 2004), decrease in water potential (Correia et al. 2014), changes in chlorophylls and carotenoids content (Shvaleva et al. 2006) and increasing in MDA content (Correia et al. 2014) with huge consequences at crop productivity and cultures success. Biochar is charred biomass, produced by controlled conditions (pyrolysis) with the intent to be deliberately applied to soils to improve their properties and functions (Lehmann et al., 2009). Depending on feedstock and pyrolysis conditions, the resulting biochars can greatly differ in their properties and the effects on soils (Sohi et al., 2009). Biochar has been used as an amendment in soils with the purpose to enhance water and nutrients retention and availability to plants in drought periods (Chen et al., 2008). It appears to be the exchange of positively-charged ions on the negatively-charged biochar surface area, which is responsible for the most cases of mitigation of plant stress either by reducing the exposure of plants to contaminants or by ameliorating stress responses (Thomas et al., 2013). Along with this, some studies emphasised their ability to supply nutrients (Silber et al., 2010) and increase their retention by soils (Chen et al., 2007), to improve soil pH associated with dissolution of alkaline minerals from biochar's surface (Yamato et al., 2006) leading to an increase cation exchange capacity (Cheng et al., 2006), neutralization of phytotoxic compounds (Wardle et al., 2001), increased colonisation of mycorrhizal fungi (Yamato et al., 2006) and alteration in soil microbial equilibrium and functions (Graber et al., 2010).

Due the economic importance of *Eucalyptus globulus* around the world and considering estimate increases in drought periods arising from anthropogenic activities, it is extremely important to study and develop adaptation strategies to maintain or improve productivity, while ensuring that environmental and economic needs will be maintained. Thus, biochar application to soils could be an answer to allow eucalypt plants an adaptation to a drier climates.

Thus, the main objective of this study is to investigate if biochar can be used as an adaptation strategy, diminishing drought effects of climate change in *Eucalyptus globulus*. The methodology to explore this objective was a greenhouse randomized pot experiment of eucalypt plants (clones) in pots filled with a typical local sandy soil and pots where this soil had been amended with biochar. It was hypothesized that plants with biochar exposed to water limitations would show lower stress levels (at physiological, morphological and biochemical levels), compared to plants without biochar. We also evaluated the fertilizer character of biochar by including a fertilized treatment in the methodological design.

Materials and methods

Location and soil type

The soil was collected at a *Eucalyptus globulus* plantation in Vagos, placed in the inner-dune complex of the coastal zone of central Portugal, at 6km distance from the Atlantic Ocean and approximately 10km south of the city of Aveiro (440 42' N and 80 42' W). This soil is a Haplic Arenosol Dystric (IUSS, 2006), a sandy and structurless soil. This area has a subhumid meso-Mediterranead climate, with a 15oC of mean annual temperature and 950mm of rainfall (Drarn-centro, 1997).

Soil and Biochar Pre-treatment

About 130kg of soil were collected into plastic bins from the field site at approximate depth pf 20cm, using a pickax and shovel, during a 2 months period. Both biochar and soil were air-dried to ensure uniform moisture contents at ambient temperature in a maximum of 5cm thick layer in cardboard boxes e a closed room. Soil was also sieved (porous lower than 2mm) to take off plant debris, stones, and other particles.

Soil and Biochar Pre-treatment and Characterization

Biochar was purchased from Swiss Biochar gmbh and was produced from mixed wood sieving feedstock in a Pyreg 500 III pyrolysis unit, 620°C maximum temperature, 20 minute duration, 80% C content, H/C 0.18. Loss on ignition was used to measure soil organic matter content. Approximately 10 g of soil sample was placed in a crucible, weighed on a decimal balance and placed in an oven at 105°C for 24 hours. Then, it was placed in a desiccator for 1 hour, weighed and placed in a muffle at 550oC for 4 hours and finally placed in a desiccator for 1 hour and weighed again on the same four decimal balance. The soil organic matter content was expressed as the percentage weight loss at 550°C relative to the oven-dry weight (at 105°C).

For bulk density the soil was placed in a metal cylinder of 251cm^3 (soil) and 87cm^3 (biochar) and weighed. The cylinders were then placed in the oven for 24 hours at 105oC. The bulk density values were calculated by dividing the oven-dry weight by the volume of the cylinder.

To measure the particle size distribution, the dry soil samples of known weight were sieved over a range of sieves with apertures between 5mm and 50µm.

To achieve the field capacity of the substrates, the pots were filled with oven-dry soil (1097g) and oven-dry soil + biochar (797g + 32g), weighed and saturated with water (from the bottom up) and allowed to freely drain overnight and weighed again. Subsequently, pots dried in an oven for one week at 60^{o}C. The moisture content at field capacity was calculated by dividing the weight of the water after overnight draining by the oven-dry weight of the soil or soil-biochar mixture. . These values supported the determination of 80% and 30% of well-watered and stress treatments that were used in this experiment.

Experimental Design

The randomized, factorial (3 factors) pot experiment was conducted at the research greenhouse of Siro (Grupo Leal & Soares, S.A.) located in Mira, Portugal. The light, temperature and humidity conditions were controlled and monitored, 48.4 W/m^2 mean total solar irradiance, 17.2^{o}C mean temperature ranged by 5.2^{o}C to 37.8^{o}C, and 78.6% mean relative humidity (ranging from 61.5% to 95.7%). On the sampling days the mean of total solar irradiance was 67 W/m^2 (308 W/m^2 maximum), the mean temperature was 20.2^{o}C and mean relative humidity was 69.4%. A total of 136 one litre pots with eucalypt plants were monitored. Pots were filled with soil and soil+biochar. Half (68) were filled with soil (1097g) and the other half with biochar-amended-soil at a concentration of 4% of oven-dry biochar (797g soil and 32g of biochar). A 4% biochar concentration in the top 15 cm of soil is roughly equivalent to a biochar application rate of 90 t ha^{-1} (assuming a bulk density of 1.5 kg dm^{-3} from literature), which is within the range reported by Jeffery et al. (2011). The pots were saturated with water and fertilizer (5ml/L Complesal – Bayer, according to the young eucalypt needs recommendations) from the bottom by immersion in a larger container, to ensure homogeneous wetting and planted with five months old rooted cuttings of *Eucalyptus globulus* plants (AL-18) from Viveiros do Furadouro in Óbidos, Portugal). These plants were watered daily, to 80% of field capacity, during a 3 month acclimation period. The pots were fertilized once a week and randomly moved around every two days, to ensure that every plant had similar growth conditions.

Two water conditions were tested: Well-Watered (80% of field capacity) and Water Stressed (30% of field capacity). Each water condition comprised of 4 treatments with 17 replicates each:

i) WW (Well Watered – no biochar);
ii) WWb (Well Watered – with biochar);
iii) WWf (Well Watered – with fertilizer);
iv) WWbf (Well Watered – with biochar and fertilizer);
v) WS (Water Stress – no biochar);
vi) WSb (Water Stress- with biochar);
vii) WSf (Water Stress – with fertilizer);
viii) WSbf (Water Stress – with biochar and fertilizer).

The pots were weighed and monitored every day during the six week treatment period, and the values of soil water content were maintained by adding the amount of water lost by evapotranspiration every day. Wilting symptoms and plant survival rates were recorded throughout the experiment.

Plants were harvested six weeks after the beginning of the experiment. At this point, six random plants were evaluated for *in vivo* measurements (gas exchanges and chlorophyll fluorescence parameters) and hydric potential. Additionally six randomly-selected, homogenous leaves were immediately frozen in liquid nitrogen and kept at -80^{o}C for further physiological and biochemical analysis (pigments, total soluble sugar and lipidic peroxidation). Additionally, measurements were made of plant height, number of leafs and lateral branches by direct counting. The dry weight of aerial portion and roots were measured after drying for 48hours in a 80^{o}C oven. Leaf area was determined by taking photographic images of picked leaves followed by image analysis using the software Image J.

Water potential

Midday shoot water potential (Ψmd) was measured using a Scholander-type pressure chamber (PMS Instrument Co., Corvallis, OR). Measurements were performed in 6 individuals per treatment at 12h30 (solar time) after 6 weeks of experiment

Gas exchange

Leaf gas exchange measurements were performed on fully expanded leaves using an LCpro-SD infrared gas analyzer (ADC BioScientific Ltd., UK). Measurements at saturation light intensity were performed at 350 µmol m-2s-1 and photosynthetic rates – A (µmol$CO_2$$m^{-2}s^{-1}$), transpiration –E (mmol$H_2O$$m^{-2}s^{-1}$), internal concentration of CO_2 – Ci (ppm) and stomatal conductance – gs (mol$H_2O$$m^{-2}s^{-1}$) were determined. Data were recorded when the CO2 remain at constant values.

Chlorophyll fluorescence

The Chlorophyll fluorescence was measured on the same leaves as gas-exchange measurements with the support of a portable pulse amplitude modulation fluorometer (Mini-PAM, Walz, Effeltrich, Germany). Steady-state fluorescence (F) and maximal fluorescence (Fm) were measured in light adapted leaves, by which ϕPSII (Effective Quantum Yield) was determined (equivalent to Fm-F0/Fm). Minimal fluorescence (F0) was measured in 30 minute dark-adapted leaves by applying weak modulated light and maximal fluorescence (Fm) was measured after applying a 0.7 s saturating pulse of white light (> 1500 µmolm-2s-1). Leaves were then dark-adapted for at least 30 minutes to obtain F0 (Minimum Fluorescence), Fm (Maxium Fluorescence), Fv (Variable Fluorescence, equivalent to Fm-F0) and Fv/Fm (Maximum Quantum Yield of PSII photochemistry).

Photosynthetic pigments content

Chlorophyll a, b and carotenoids were quantified according to Sims and Gamon et al., (2002). Chlorophyll a, b and carotenoids were extracted with cold acetone: 50 mM Tris buffer pH 7.8 (80:20) (v/v) and centrifuged for 5 min at 10000g. Supernatant's absorbance was read at 470, 537, 647 and 663 nm (Thermo Fisher Scientific Spectophotometer, Genesys 10-uv S, Waltham, MA). Pigments contents were calculated according as it described by Alves et al. (2011).

Lipid peroxidation

The level of lipid peroxidation was estimated by assessing the amount of malondialdheyde (MDA), following the procedure described by Hodges et al. (1999). About 50 mg of leaves were ground in 2.5 ml cold 0.1% trichloroacetic acid (w/v) and centrifuged. The supernatant was divided in two 250 µl aliquots. To 1 ml of 20% TCA (w/v) in 0.5% TBA (w/v) was added (positive control) whereas to the other 1 ml of 20% TCA (w/v) was added (negative control). Both aliquots were heated at 95ºC for 30 min and the reaction was stopped immediately, and the tubs were placed on ice. After centrifugation, absorbance was read at 440, 532 and 600 nm and MDA equivalents (nmol mL-1) were calculated as (A – B/157 000) × 106, where A = [(Abs 532+TBA) – (Abs 600+TBA) – (Abs 532-TBA – Abs 600- TBA)], and B = [(Abs 440+TBA – Abs 600+TBA) × 0.0571].

Total soluble content of sugars

Total soluble content of sugars (TSS) was determined by using the anthrone method, as described by Irigoyen et al. (1992): TSS extraction from frozen leaves was performed using 80% (v/v) ethanol at 80ºC for 1h. After centrifugation, the supernatant was mixed with 1.5 ml of anthrone and incubated at 100ºC during 10 min period. Absorbance was read at 625 nm and TSS content was calculated against a D-glucose standard curve.

Statistical Analysis

One way ANOVAS (Analysis of Variance) were made separately for Well watered conditions and Water stress (four treatments per group) followed by post hoc multiple comparison test (using Tukey when appropriate) was performed to estimate the significance of the results. The statistical procedures were performed using SIGMAPLOT (Systat Software, Inc. SigmaPlot for Windows 11.0), showed in plots where the different lowercase letters indicates differences in water conditions and the asterisks indicate significant differences between water conditions ($p \leq 0.05$).

The PCA – Principal component analysis was performed with R programming language running with an open-source software RStudio: Integrated development environment for R (RStudio Boston, MA, available from http://rstudio.org/. The PCA was carried out from a matrix data to a bidimentional plot that explains the highest proportion of data variation.

Results

1. Soil and Biochar characterization

The chemical and physical characteristics of soil and biochar used in the experiments are reported in Table 1 and Figures 3. In table 1 we can see that the total moisture content of the biochar was 35% and 88,9% of organic matter. The soil had a moisture content of 0.3%, and a soil organic matter content of 0.8%. Regarding to bulk density, soil showed higher values than biochar, from 1,65 to 0,17 respectively. Also, both pH and EC (Electric Conductivity) showed higher values in biochar when compared to soil, as 4,71 to 8,13 for pH, and 38 to 1496 from Electric conductivity, respectively. We didn’t found any difference between Soil and Soil+Biochar (4%) in Field capacity volumetric values, which shows 23% for both.

As we can see in Figure 3, both soil and biochar showed very different patterns in particle size distribution. Thus, biochar showed a very heterogeneous nature regarding to particle size characteristics, with particles varying from 5000 to 50 µm. The particle size distribution of the soil was more homogeneous, varying from 1000 to 250µm.

Table 1- Soil, biochar and biochar amended soil characteristics. Values with "" were measured by Oliveira et al. (2014); na= not available*

	Soil	**Biochar**	**Soil + Biochar (4%)**
Moisture content (%)	0,3	35	na
Bulk density	1,65 (±0.04)	0,17 (±0.01)	na
pH	4,71 (±0,03)*	8,13 (±0,04)*	7.63 (mean)
EC (µS cm^{-1})	38 (±11)*	1496 (±43)*	na
Organic matter (%)	0,8 (±0.06)	88,9 (±2.79)	na
Field capacity volumetric (%)	23%	na	23%

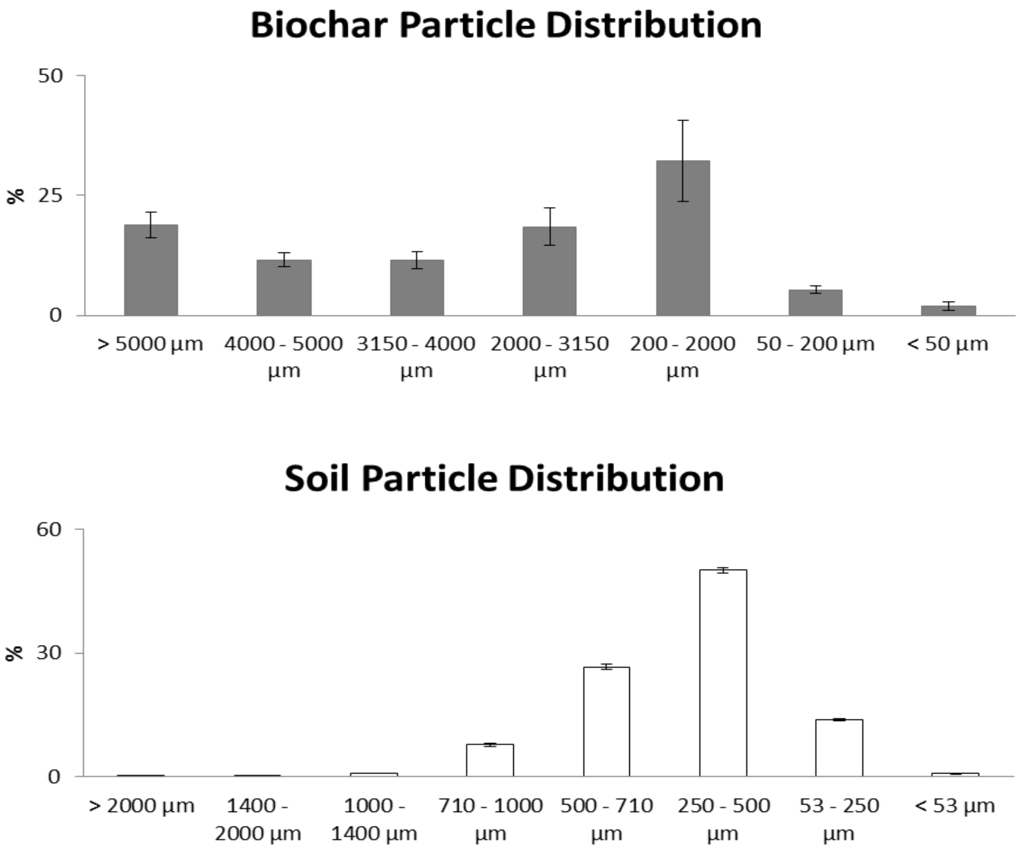


Figure 3 - Particle size distribution of soil and biochar (%)

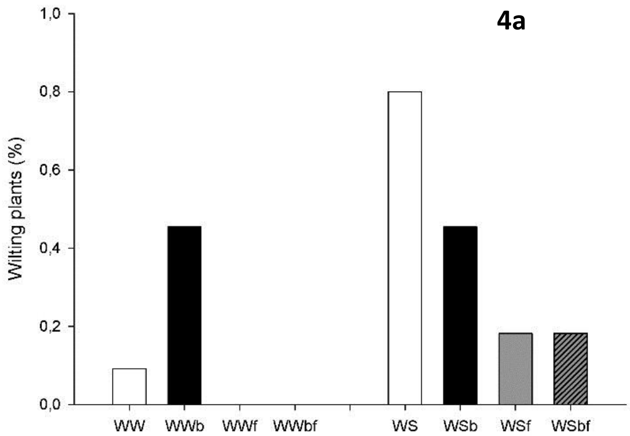
2. Plant performance

There was made a first sampling point at 4 weeks of experiment where we measured fluorescence of chlorophylls and gas exchanges after four weeks of experiment, without any significant differences. Then, the experiment were kept till six weeks, when all the morphological, physiological and biochemical measurements were taken and evaluated.

2.1 Wilting and Survival Rates

Tree days later from the beginning of stress assay, the plants showed wilting signals with 80% of incidence in WS treatment and only 45% in WSb treatment. This values decrease in treatments with fertilizer, to 18% for WSf and WSbf. In well watered condition, WWb treatment showed higher values of wilting (45%), against 9% for WW. In this condition, neither WWf nor WWbf showed wilting signals (Figure 4a). There were also observed 67.71 % of WS plants of survival rates against 100 % survival rates in other treatments (Figure 4b).

Also, the plants WWb showed a wilting signals on the top of the plant, characteristic for this treatment. Along with wilting signals, we recorded an atypical wilting symptoms at WWb treatment (Figure 5), where all the plants of this treatments showed only wilting signals at apical leaves. Also, this symptom is not the same wilting that can be seen in water limiting conditions, suggesting that these plants were not suffering from water limitation.



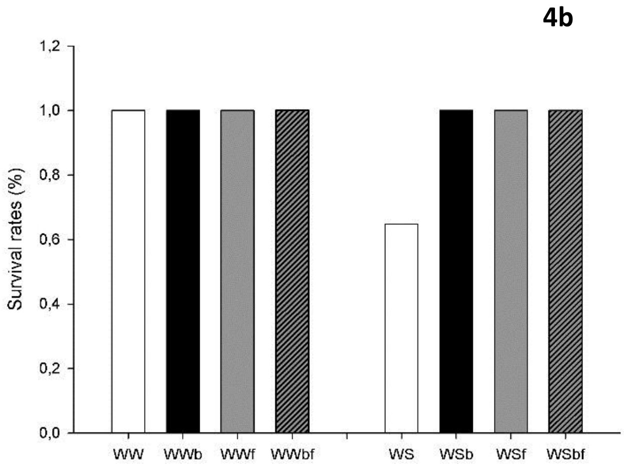


Figure 4- Wilting (4a) and Survival Rates (4b), expressed in percent of Well Watered (80%FC) and Water Stress (30%FC) treatments. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

Figure 5- Detailed view of WWb treatment and the apical wilting

2.2 Morphological Parameters

In Figure 6 are represented the general visual differences between all treatments.

In Figure 7 are represented the results for morphological parameters in this study. As we can see in Figure 7a, the number of lateral branches was also significantly lower in all treatments of water stress when compared to well-watered conditions, except in Biochar+Fertilizer treatment. There were no differences between treatments in water stress treatment but, under well-watered conditions, WWf treatment showed significant differences compared to the lower values of WWb and WWbf. Underground biomass, represented in Figure 7b by dry roots weight, was lower under water stress than well-water conditions, except for the 'no biochar' treatment where no significant differences were found. Between well-watered treatments, WWf showed significant higher values than WW and WWbf. There were no significant differences found between treatments under water stress conditions. The values for leaf area (Figure 7c) were lower in water stress compared to well water condition, with statistical differences in all treatments. In well watered condition the WWf treatment showed significantly higher leaf areas than the other treatments and in water stress condition there was found a statically higher leaf area values in WSb when compared to WS. The above ground biomass fresh (Figure 7d) showed a decrease from well watered to water stress, with a significant differences between all treatments. In WWf treatment show significant higher value than WWb and in WSbf show, as well, significant higher values than WS. The number of leaves was significantly lower in water stress when compared to well watered, in all treatments (Figure 7e). There were no differences between water stress treatments, but under well-water conditions, the WWf treatment showed significantly higher number of leaves than the other treatments.

As can be seen in Figure 7f, the height of above ground biomass suffers a significant decrease from well watered to water stress conditions, with a statistical relevance in all treatments ($p<0.05$). There were no observed differences between treatments among water status.

Figure 6 – General visual aspect between Soil and Biochar treatments. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

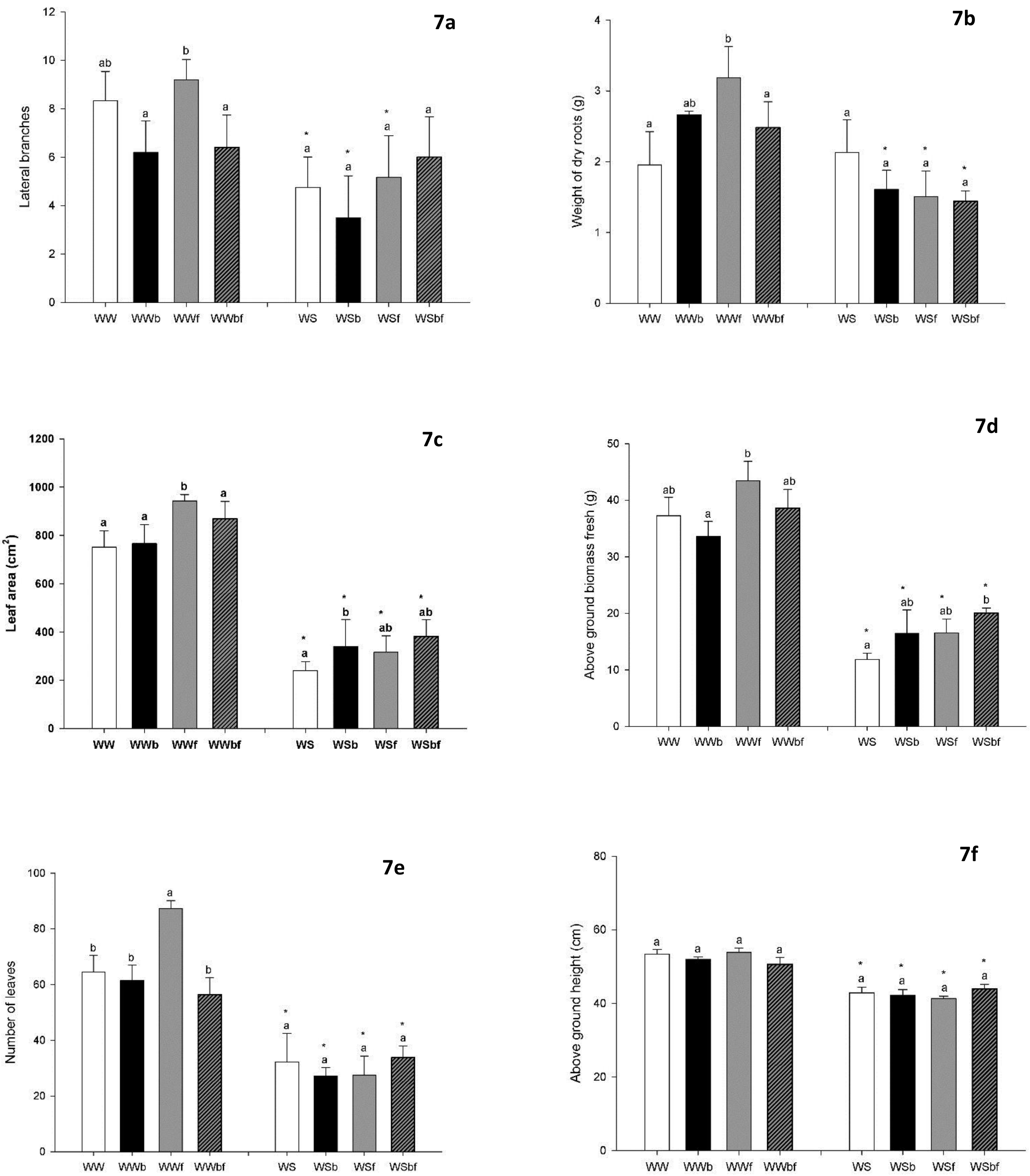


Figure 7 - Morphological Parameters in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity conditions in Eucalyptus globulus clones (MB 43) after 6 weeks of water limiting stress period: Lateral brances (7a), Weight of dry roots (7b), Leaf area (7c), Above ground biomass fresh (7d), number of leaves (7e) and above ground height (7f). Different letters indicate significant differences within water treatments and asterisks indicate significant differences between water treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WW_b – well watered with biochar, WW_f – well watered with fertilizer, WW_{bf} – well watered with biochar and fertilizer; WS – water stress without biochar, WS_b – water stress with biochar, WS_f – water stress with fertilizer, WS_{bf} – water stress with biochar and fertilizer

3. Hydric Potential

There were no observed differences for plant water potential within water treatments (Figure 8). However, there was a trend of lower plant water potential values for the water stressed plants, with significant differences between biochar and fertilizer treatments.

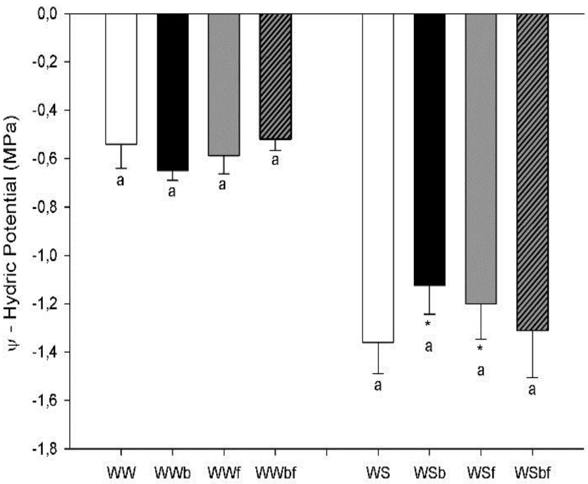


Figure 8 -Hydric Potential in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity) conditions, of Eucalyptus globulus after 6 weeks of water limiting stress period. Different letters indicate significant differences within water treatments and asterisks indicate significant differences between treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

4. Gas Exchanges

As we can see in Figure 9a, the internal concentrations of CO_2 (Ci), significant differences were observed between biochar and fertilizer treatments, which showed lower values under water stress compared to well-watered conditions. Moreover, no biochar treatment showed significant higher values than the other treatments under both water conditions. Regarding the standard deviations, WSbf had a higher standard deviation compared to the other treatments, i.e 6 times higher than WSf and 13 times higher than WWb.

Lower values were found for transpiration rate (E) – Figure 9b, under water stress compared to well-watered conditions in, with statistical differences in all treatments. There were significant differences between WSb and WSf, under water stress conditions and no differences between treatments under well-watered condition.

For stomatal conductance (gs), represented in Figure 9c, there can be seen a clear decrease from well-watered to water stress, with significant differences between all treatments. The WW and WWbf were significantly different, under well-watered conditions, where WWbf showed lower values than the WW treatment. Also, in this treatment, large standard deviations were observed in some treatments, namely in WW, WWf and WWbf, where for example WWf is 5.8 times larger than WWb.

Higher level of photosynthesis (A) were found in WSb treatment, with a clear significant difference ($p<0.05$), compared to other treatments under water stress. In all other cases the values of photosynthetic rates decrease from well watered to water stress, where significant differences were found between no biochar, fertilizer and biochar+fertilizer (Figure 9d).

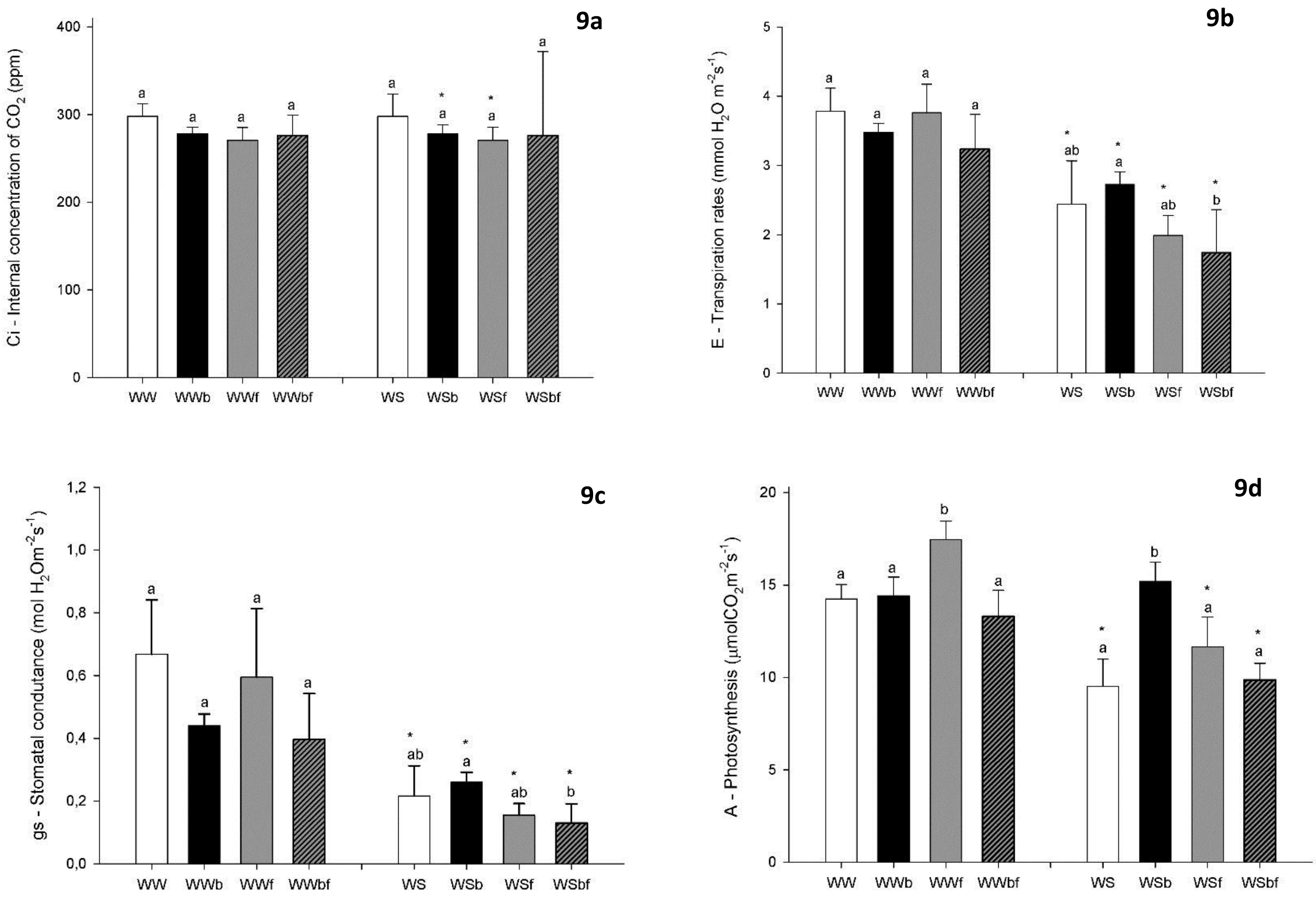


Figure 9 - Gas Exchanges Rates: Internal Concentration of CO2 (Ci) – 9a, Transpiration (E) – 9b, Stomatal Conductance (gs) – 9c and Photosynthesis (A) – 9d in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity) conditions of Eucalyptus globulus clones (MB 43) after 6 weeks of water limiting stress period. Different letters indicate significant differences within water treatments and asterisks indicate significant differences between water treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

5. Chlorophyll fluorescence

There was no differences between water conditions or between treatments in Effective Quantum Yield parameter (Figure 10a).

As we can see in Figure 10b, under well-watered conditions, Fv/Fm was significantly lower for WW than for WWbf. Under water stress conditions, Fv/Fm was significantly higher for WS than for WSf. Between water conditions (WW vs WS), only biochar showed significant differences ($p<0.05$), and displayed higher values in stress WS condition. There were no significant differences observed in water treatments (80% and 30% FC) or between treatments.

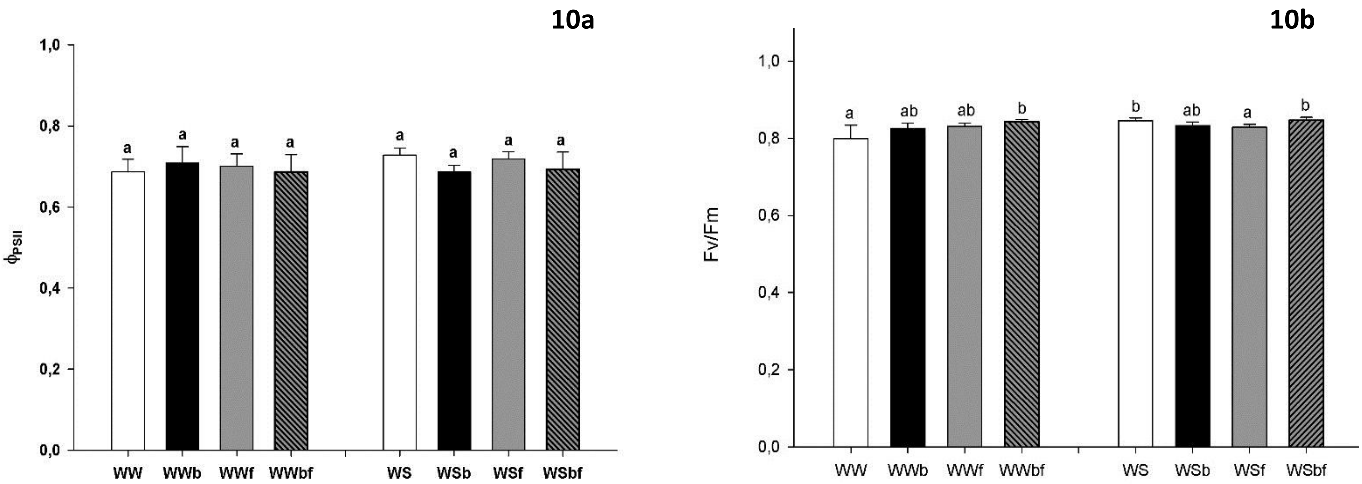


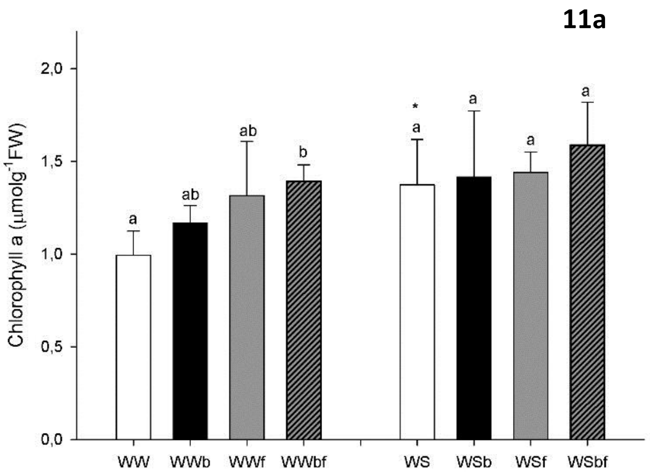
Figura 10- Chlorophyll fluorescence: Effective quantum yield ϕPSII (10a) and Maximum quantum yield Fv/Fm (10b), in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity) conditions of Eucalyptus globulus after 6 weeks of water limiting stress period. Different letters indicate significant differences within water treatments and asterisks indicate significant differences between water treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

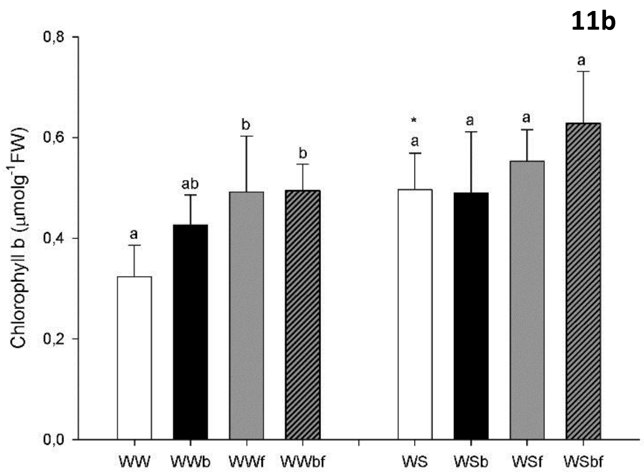
6. Photosynthetic Pigments Content

There was a general increase of 'chlorophyll a' content from well watered to water stress condition, with a significant difference in no biochar treatments (WW and WS) ($p<0.05$). Among well watered conditions, there was a significant difference between WW and WWbf, which show higher content of chlorophyll a than other treatments (Figure 11a). Under water stress condition, there were no significant differences between treatments.

The Chlorophyll b (Figure 11b) content did not show any significant differences among water stress treatments, but significant differences were found between WW and the fertilised well-watered treatments (WWf and WWbf). Despite no significant differences were found under water stress conditions, the WSb treatment did show lower values than the other treatments, although its standard deviation was also larger than, for the other treatments. No biochar treatment showed significant differences between the two water conditions, where the chlorophyll b content showed higher values under water stress conditions.

For carotenoids (Figure 11c) content no significant differences were found between treatments under well-watered conditions. Under water stress conditions, WSb and WSbf showed significantly higher ($p<0.05$) carotenoid content. There was a clear trend of increasing carotenoid content from well-watered to water stressed, with a significant difference between no biochar and fertilizer treatments. Carotenoid contents also showed varying standard deviations, with a 2.8 times difference between WWf and WWbf, and 3.8 times between WSf and WSb. For carotenoids content analysis, no significant differences were found between treatments under well-watered conditions. Under water stress conditions, there were significant differences ($p<0.05$) between WSb and WSbf, which showed higher carotenoid content. There was a clear trend of increasing carotenoid content from well-watered to water stressed, with a significant difference between no biochar and fertilizer treatments.





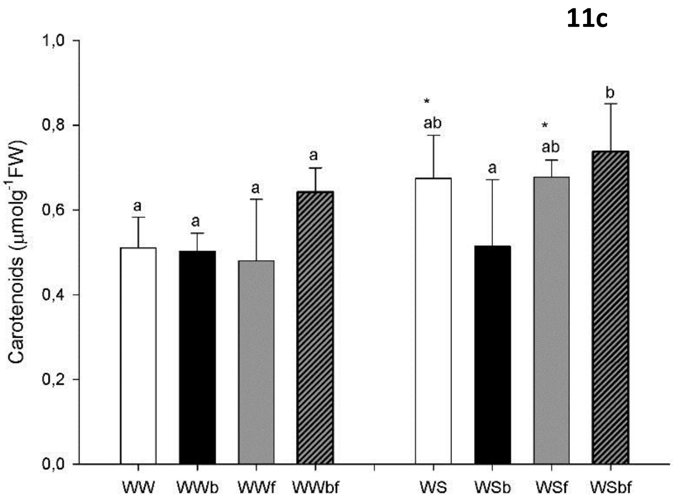


Figure 11- Total Content of Pigments – Chlorophyll a (11a), Chlorophyll b (11b) and Carotenoids (11c), in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity) conditions, of Eucalyptus globulus clones (MB 43) after 6 weeks of water limiting stress period. Different letters indicate significant differences within water treatments and asterisks indicate significant differences between water treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

7. Lipid Peroxidation

MDA content was similar for all treatments, except an overall trend of greater MDA contents for the water stressed treatments (Figure 12).

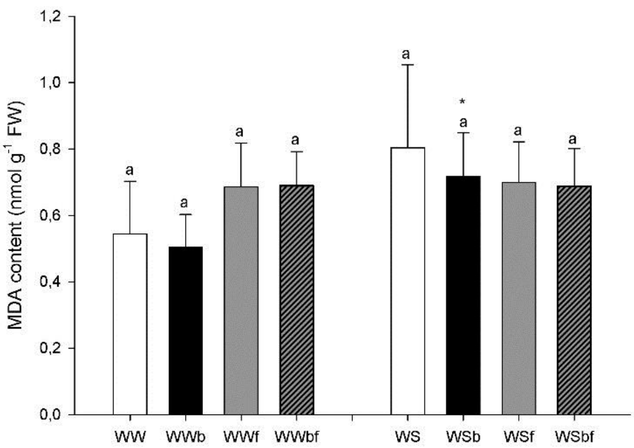


Figure 12– MDA content in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity) conditions of Eucalyptus globulus clones (MB 43) after 6 weeks of water limiting stress period. Different letters indicate significant differences within water treatments and asterisks indicate significant differences between water treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

8. Total Soluble Sugars (TSS) content

Under well watered conditions WWb had significantly higher TSS contents than the remaining treatments. Under water stress conditions the opposite pattern merged, with WSb showing significantly lower TSS contents than the remaining treatments (Figure 13).

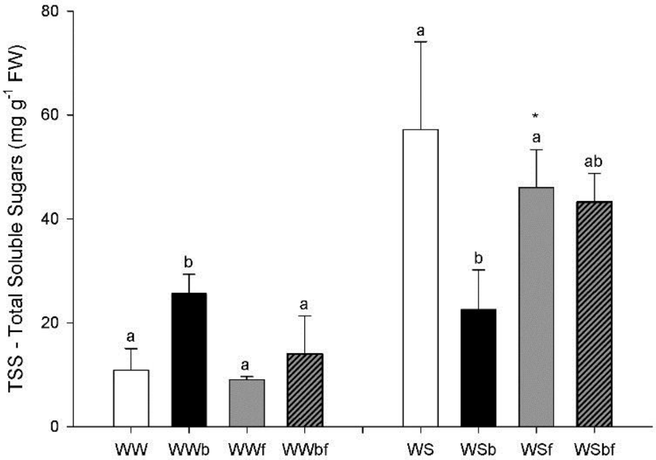


Figure 13- Total Soluble Sugars content in Well-Watered (80% of Field Capacity) and Water Stressed (30% of Field Capacity) conditions of Eucalyptus globulus clones (MB 43) after 6 weeks of water limiting stress period. Different letters indicate significant differences within water treatments and asterisks indicate significant differences between water treatments of different water status. Different treatments are symbolized by WW – well watered without biochar, WW_b – well watered with biochar, WW_f – well watered with fertilizer, WW_{bf} – well watered with biochar and fertilizer; WS – water stress without biochar, WS_b – water stress with biochar, WS_f – water stress with fertilizer, WS_{bf} – water stress with biochar and fertilizer

9. Overall Coefficient of Variation

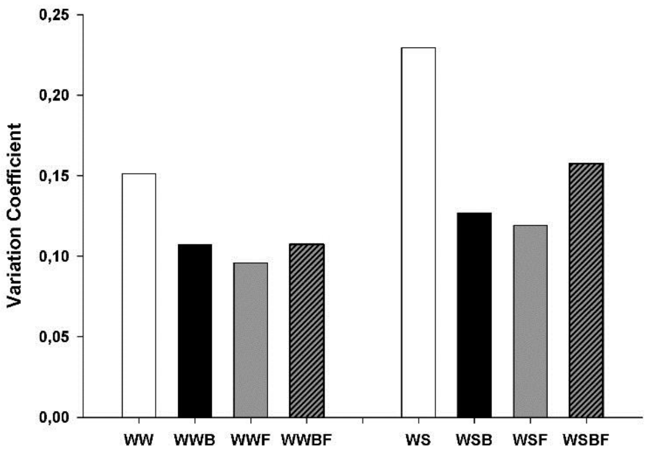


Figure 14- Coefficient of variation between treatments. Different treatments are symbolized by WW – well watered without biochar, WW_b – well watered with biochar, WW_f – well watered with fertilizer, WW_{bf} – well watered with biochar and fertilizer; WS – water stress without biochar, WS_b – water stress with biochar, WS_f – water stress with fertilizer, WS_{bf} – water stress with biochar and fertilizer

Figure 14 shows similar coefficients of variation (COV) between treatments (0.1 mean), except for the without biochar treatment (WW and WS) which showed greater variation, with 0.15 and 0.23 respectively. This appears to be mainly caused by variation in WS for the photosynthetic variables, i.e. gas exchanges rates CO_2 assimilation rate (A), Stomatal Conductance (gs), Transpiration (E) and Internal Concentration of CO_2 (Ci). For most of the other measured variables, the COV for WS was not greatly different than for the other treatments, and for plant water potential the COV for WS was even the smallest between treatments.

10. Principal Component Analysis (PCA)

Figure 15 shows the results of a Principal Component Analysis of all variables presented above, from 6 replicates randomly chosen for each treatment. Dimension 1 appears to be related to water condition since all water stress treatments appear in the negative part (left side) and the well-watered in positive (right side). Dimension 2 clearly divides the WSbf (green) and WW (dark blue) treatments.

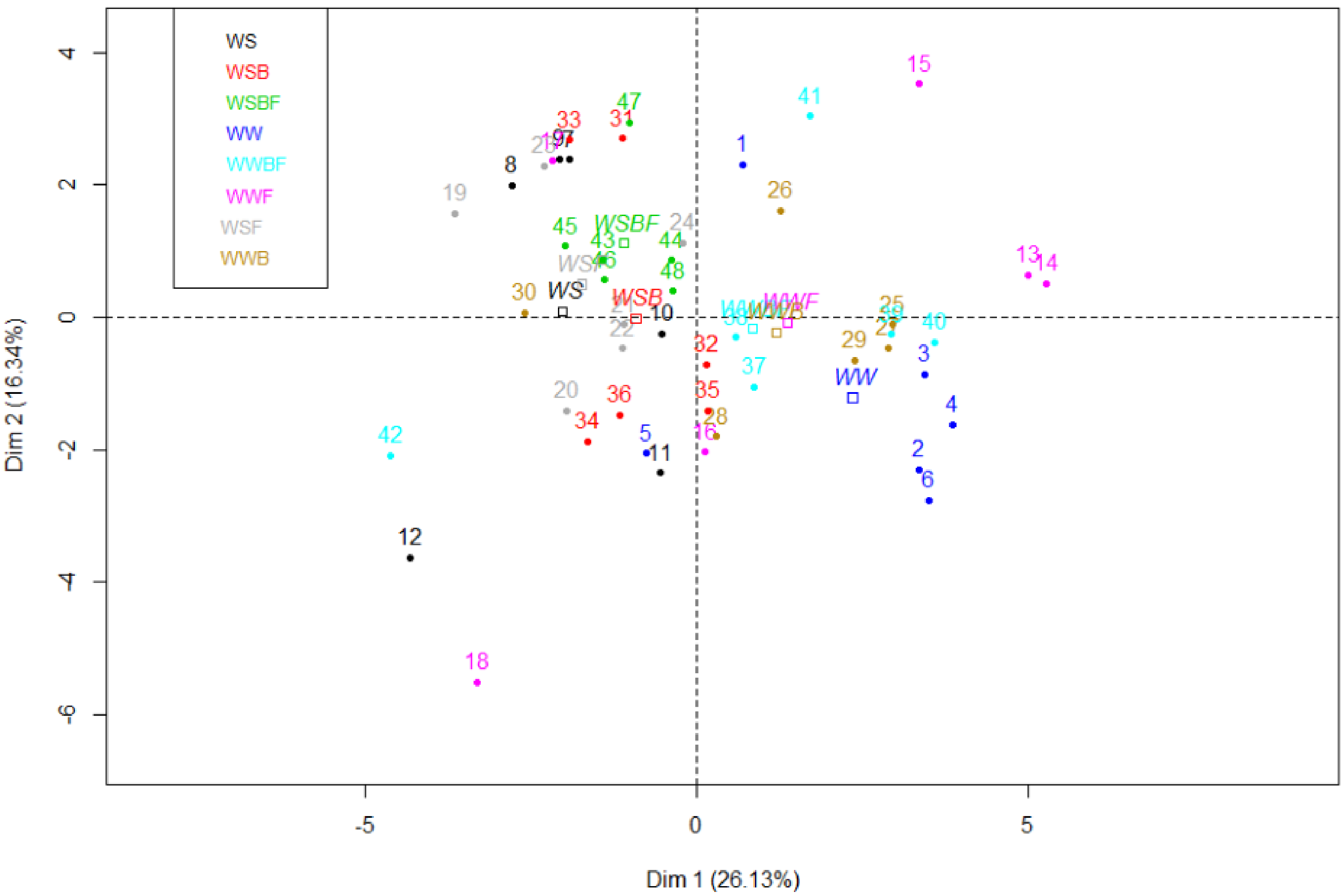


Figure 15- Principal Component Analysis. Different treatments are symbolized by WW – well watered without biochar, WWb – well watered with biochar, WWf – well watered with fertilizer, WWbf – well watered with biochar and fertilizer; WS – water stress without biochar, WSb – water stress with biochar, WSf – water stress with fertilizer, WSbf – water stress with biochar and fertilizer

Discussion

This work investigated the effect of biochar amendment on hydric stress conditions of *Eucalyptus globulus* clones in sandy soil. This discussion considers the three factors of the factorial experiment, i.e. water stress, biochar amendment and fertiliser addition.

WATER STRESS

As expected, this study confirmed that water limitation disrupts plant performance. In this study, water limitation resulted in a reduction in morphological parameters by decreasing plant height, number and size of leaves, number of lateral branches and above ground fresh weights. Other parameters as transpiration, hydric potential, stomatal conductance and photosynthesis also showed lower performance, indicating a disorder in levels of stress for plants under water limiting conditions. Similarly, Silva et al. (2004) also reported a general decrease in growth rates in two *Eucalyptus globulus* clones and Correia, et al. (2012) reported lower growth rates reflected in height, number of branches and total biomass reductions. Coopman et al. (2008) found that although drought acclimation treatments significantly reduced growth rates, some variables, such as stem diameter, height and root biomass, are less sensitive to drought. Additionally, the differences between water treatments are well defined in the PCA analysis, where well-watered and water stress were associated with dimension 1, explaining most (26%) of the variation.

BIOCHAR AMENDMENT

Some studies reported the increase yield productivity by improving general soil properties, in biochar amended soils (Masulili et al., 2010; Major et al., 2010). According to this, our results showed statistically significant higher levels of photosynthetic rates in WSb plants, compared to WS. The photosynthetic values from WSb were not different from WWb, which suggests that plants were not suffering from any stress at CO_2 assimilation rates, assuming that decreasing in photosynthesis is an indirect effect of hydric limitations on plants. Furthermore, our results were accomplished with the same internal concentration of CO_2 content for all treatments, suggesting that WSb plants have a beneficial biochar effect for CO_2 assimilation rates. This results is supported by Haider et al. (2014), who found higher levels of photosynthesis associated with an improvement in effective quantum yield (ϕPSII), which indicated a reduction in heat dissipation for biochar treatments compared to controls, in a maize yield study. In line with the results of higher photosynthetic rates, the WSb treatment also showed lower levels of carotenoids and total soluble sugars (TSS) compared to the other water-stress treatments, while both showed no statistical differences when compared with WWb correspondent treatment. Carotenoids function to protect the plant's photosynthetic machinery against excess of light, when the light has been absorbed but not used in photosynthetic proposes (Reddy et al. 2004). Total soluble sugars (TSS) are produced as a defensive plant response to hydric stress, to improve water absorption in water stress conditions (Bohnert et al. 1999). The lower values of TSS and carotenoids in WSb also support our hypothesis, which state that plants growing in biochar-amended soil will show lower stress levels under water limiting conditions. Conversely, Danish et al. (2014), found an improvement in carotenoids content, along with all photosynthetic pigments in a sandy soil amended with 1 and 2% of biochar, in *Triticum aestivum*. They suggests that biochar do not only improve the availability of nutrients but also improves the photosynthetic pigments production with direct effects on vegetative development and growth. In despite of that, our results don't show any significant increase in Chlorophyll contents but we can see an increasing in CO_2 assimilation rates, which were reflected in higher leaf area. Additionally, even without a significant difference, our results also shows a slight higher above ground biomass of WSb when compared to WS, which go through Danish et al. (2014) findings.

The results of this study also show a slightly higher hydric potential values in the WSb treatment, i.e. less stress, although not statistically significant. These observations confirm that under water limitation conditions, plants in biochar-amended soil have more available water, leading to a better overall performance of the plant, also supporting the hypothesis that biochar addition may be a useful strategy to condition soils as an adaptation to maintain or improve plant performance under potentially drier future climatic conditions. Biochar is known to have a high proportion of transmission pores, i.e., pores with >50µm (Greenland, 1977), which are responsible for air and water movement through the substrate, improving soil-water-plant relationships and promoting good soil structure conditions (Sohi et al. 2010). Possibly, the biochar used in the present study promoted the retention of available water in the soil near the roots, which were reflected by the ability of the plants to maintain its physiological performance under water-limited conditions. Improvement in plant hydric potential levels following biochar application were also reported by Baronti et al. (2014) in a study with two rates of biochar (22 and 44 ton ha^{-1}) on water plant relations in a *Vitis vinifera* field experiment, which substantially increased the leaf water potential during dry periods. Also, Basso et al. (2013) reported an increase in soil water availability and water holding capacity in a sandy soil with biochar (6%) and Kamman et al. (2011) found an improvement of water use efficiency by *Chenopodium quinoa* subjected to a 20% field capacity stress in sandy soils.

Water repellence of soils may induce problems in water infiltration rates, enhance surface runoff and erosion and by forming preferential patterns of water flow in the soils (Keizer et al., 2005; Kawamoto et al., 2007), which may lead to increased nutrient leaching and reduced water availability to plants. Some studies reported that biochar reduced soil water repellence leading to a better hydric retention by soils (Dugan et al., 2010). Contrarily, Baronti et al. (2014) reported no hydrophobicity changes in biochar-treated soils along with Abel et al. (2013), who also found no direct impact on the wettability in a sandy soil study related with biochar application. Otherwise, Basso et al. (2012) found that the biochar may be hydrophobic in fresh, becoming more hydrophilic along the time after soil, air and water interactions, suggesting that biochar may not promote any adsorption of water immediately after its application. Our experiment was conducted by daily addition of a defined amount of water to the pots to maintain a constant water stress level (30% of field capacity) and some bypass flow were observed in all treatments, although water repellence was not measured but it could be a reason why plants with biochar showed better performance, due to an increased water holding capacity.

The results of this study also showed an atypical wilting signal in WWb, where only the apical leaves were affected (Figure 5), i.e., this symptoms were not visible in any other treatment. The WWb treatment also showed lower values of plant morphological and physiological parameters, e.g. above ground biomass, number of lateral branches, and hydric potential. Additionally, the higher values of TSS indicated that this plants were in higher stress when compared to other treatments. As described in literature, biochar addition to the soil increased the pH values and, our results showed a rise from 6.5 mean in soil without biochar leachates to 7.7 mean in biochar treatments leachates. Specifically, the WWb treatment showed 8.6 pH and it could explain why these plants showed those wilting signals. At this higher pH values, the availability of some macronutrients (namely phosphorus (P)) is affected by diminishing and, moreover, the availability of micronutrients such as Boron (B), Iron (Fe) and Manganese (Mn) are extremely reduced (Sarkar et al. 1982). The reduced availability of P could be attributed to a decrease in mycorrhizal fungi directly conditioned by the pH increasing (McElloigott, 2011). This symbiotic relation is important to plant performance once it can access to unavailable P through an extended deep network of hyphae, enlarging the total volume of soil explored. Nelly et al. (1996) reported an inhibition in ectomycorrhyzal activities as an effect of pH rising in *Eucalyptus uropylla*, and also suggests that this natural symbiotic association promotes the growth and development in soils with low nutritional status. Higher values of pH also limit Boron (micronutrient) availability to the plants and it could explain our wilting signals in WWb treatment. Although the main role of boron in plant physiology remains unclear, it is known for its importance to cellular wall synthesis, mainly in their compounds supply as pectine, cellulose and lingnine, leading to a serious physiological and biochemical alterations when absent (Ramos et al. 2009). Boron deficits were reported by Sakya et al. (2002) as a main micronutrient limiting problem to *Eucalyptus spp*. plantations, largely limiting their productivity. Mattielo et al. (2009), reported death of apical leaves as the main symptom of boron limitation, which support our results. Although, according to Baronti et al. (2014), the additional volume of water and nutrients stored in biochar (absorbed) only became available to the plants as the soil dries, inversely with an increasing metric potential, in sandy soils. So, the water limitation condition imposed to WSb treatment could be an advantage in terms of nutrients supply to *Eucalyptus globulus*, in a poor macro and micro nutrients media, as a result of pH increasing, which should be an explanation that why this wilting signals were not observed in WSb. Contrarily, Ramos et al. (2009) found that the availability of boron increased with moisture content of the soil in a *Eucalyptus citriodora* study with variations in water supply. Also, Ruiz et al. (1998), found that boron has a major role in N metabolism, namely in N fixation rates and has reported a significant reduce in foliar N content with boron addiction in a *Nicotiana tabacum L.,* as a consequence of nitract reductase activity reductions, which contradict our results. However, in this study plant and soil nutrients were not measured and remains a topic of further exploration.

FERTILIZER ADDITIONS

As expected, fertilizer addition improved plants performance in a nutrient-poor substrate as a sandy soil used in our study, namely at morphological levels and chlorophyll contents under well-watered conditions. Under water stress conditions, the fertilizer addition resulted in no significant improvement in studied parameters, except a lower performance in Fv/Fm rates. The Maximum Quantum Yield – Fv/Fm is a parameter of chlorophyll fluorescence which indicates the extension of stress effects in the photosynthetic apparatus (Maxwell el at. 2000) and some studies associated lower values of Fv/Fm to stress performance under water limitation conditions (Souza et al. 2004, Wright et al. 2009). Although, attending to similar levels of photosynthesis and chlorophyll contents, and according with our results that there is no difference in Fv/Fm between WWf and WSf, our results suggests no stress effects associated to fertilizer application under water limitation conditions. The NPK fertiliser (Nitrogen-Phosphorous-Potassium) used in this study (Complesal 5.8.10), and according to a water stress study in *Eucalyptus grandis* (Graciano et al., 2006), P fertilizers tend to allocate dry biomass from the roots to the leaves, leading to lower growth rates. Regarding our results in water limitation treatments, even without statistical significance, we found a lower dry weight roots and higher above ground biomass (fresh), suggesting that, as expected, P fertilizer is changing biomass allocation. Although, with the exception of WS, every water limiting treatment showed that allocation in biomass, even WSb which had no fertilizer added. Also, Lori et al. (2013) reported, in a meta-analysis study that the biochar addition itself could increase the above-ground productivity, with an increasing of macronutrients, as P, K, N and Ca, availability.

Moreover, even without statistical significance, we can see that WSf treatment shows higher water potential values when compared with WS, suggesting that these treatment shows lower hydric stress levels. Supporting these findings, Stoneman, G. et al. (1996) found an increase of growth rates but also an increase in tree water stress, which limited the leaf area extension in *Eucalyptus marginata*.

However, WSbf showed stress signals with higher carotenoids and TSS content when compared with other water limitation treatments. TSS content from WSbf was slightly higher than WSb but with no statistical significance, along with lower photosynthetic rates that WWf and WSb in controls and water limiting treatment respectively, suggesting that the additional effect of fertilizer produced a side stress response in the plants. This could be explained by the biochar adsorption characteristics. In this way, biochar significantly affects nutrient retention and availability and may play a key role in a wide range of biogeochemical processes in soils, especially in nutrient cycling (Liang et al., 2006). Along with raising pH in the soils, which increasing in nutrients availability to the plants, biochar can increase the CEC of soil. After biotic and abiotic reactions into the soils, the biochar surface becomes more negatively charged, improving its ability to sorb nutrients which will easily became available to the plants (Danish et al. 2014). In our study, the conjugation of biochar and fertilizer only obtained better performance than WW in the well watered conditions and only at chlorophyll (a and b) and Fv/Fm contents. According with our results, WWbf shows lower rates of number of leaves, dry roots and CO_2 assimilation rates than WWf, suggesting that in well-water conditions the plants perform worse with biochar and fertilizer instead when fertilizer is applied by itself. Additionally, under water stress conditions, WSbf shows lower CO_2 assimilation, transpiration and stomatal conductance than WSb. The carotenoids performed higher values than WSb, and this altogether suggest that WSbf treatment could not be an advantage from WSb or WSf. Macro and micro nutrients are the main compounds of most biochars and, when release to the soils these provide an extra source of nutrients to the plants. With biochar and fertilizer additions as a source of nutrients, there is an increased amount of reactive N, K and P in the media. However, nitrogen uptake is extremely sensitive to phosphorous availability and when it is present, the availability of N decreases (Oram et al., 2014). Also, the increase of pH by the biochar application will stimulate microbial activity, which will promote the mineralization and decomposition of organic matter. However, in our study the organic matter available is very low (0.8% for soil), and this seems to be unlikely to be the reason for more available nutrients. Zhang et al. (2010) found higher nutrient availability after biochar application in maize yield, along with Ghoneim et al. (2013) who found a significant increase in total soil N in rice amended cultures with biochar. Joseph et al. (2009) found that potassium is highly available to the plants with biochar, conversely to nitrogen (Amonette 2009), that largely depends on pyrolysis feedstock character and conditions. Rondon et al. (2007) also concluded that applications of biochar with fertilizers result in less available N content which support our results. Nitrogen constitutes a macronutrient, with main importance to the plants healthy maintenance and it is required in large and frequent amounts, constituting a limiting factor as it is an essential element of amino acids and proteins, a component of chlorophyll and may affect largely the photosynthesis occurrence when it was under appropriate levels (Evans et al. 1989). The lower availability of N promoted by biochar and fertilizer interactions, could explain our results in water stress treatment, which showed lower value of photosynthetic, transpiration and stomatal conductance. Additionally, the carotenoids display higher values suggesting that the plants are subjected to an oxidative stress. Supporting our results, Lehmann et al. (2003) study showed lower N availability in the presence of biochar application.

Regarding to the wilting signs on plants, they were reduced for WSb compared to WS. Although the treatments with fertiliser (WSf and WSBf) showed less wilting still, which indicated an additional effect of nutrient limitation on wilting. Villar-Salvador et al. (2013) reported that, in a *Pinus pinea L*. seedlings study, the addition of nitrogen nutrition reduces the seedling frost and tissue dehydration tolerance and, however, Graciano et al. (2005) suggested that fertilization only enhanced plant performance under good water supplies, in *E.grandis* study in sandy soils. Although, Harvey et al. (1997) showed that fertilization additions may affect hydric xylem conductivity, and specifically P fertilizers can reduce those effects, leading to a lower wilting signals in the plants, and Kleiner et al. (1992), also found that red oak seedlings subjected to fertilizer and water limitations exhibit higher osmotic adjustments than plants with lower nutrients availability, which also supports our results. Possibly, the increase in available nutrients on the biochar surface may have caused, in part, the reduction in wilting for WSB.

In any case, not much information is currently available on the effects of fertilization on susceptibility of plants amended with biochar in water limiting conditions, and this could be an interest area to further research in the future.

Concerning about the standard deviations values founded in our results, the overall increase in COV for WS may possible be caused by plants in the WS treatment being more stressed at the time of measurement of the photosynthetic variables, which took place on relatively hot and sunny days with some leaves already wilting, which is supported by the wilting data in Figure 4. Although, the smallest standard deviation values in hydric potential parameter could be due that this analysis was performed at stem level, and the wilting signals and higher variation were recorded at leaf levels, showing primary stress signals for some plants.

FACTOR SYNTHESIS

Our results show beneficial effects of biochar addition to general plant performance under water limitation conditions. This result could be explained by the improving of soil-plant relations with more available water to the plant, reducing the stress effects.

The apical wilting noticed in WWb treatment might be explained by the increasing of pH conferred by biochar addition. This would promote a decreasing in micronutrients availability namely of Boron, which is documented to promote this symptom in eucalyptus.

The plants subjected to a biochar and fertilizer treatment showed contradictory results, where even with lower gas exchange values, they present higher above ground biomass. This could be explained by the interactions of NPK fertilizer with soil nutrients, which along with lower N availability conferred by the increasing pH, will promote an N deficit media.

Although, further analysis is needed to validate these findings.

Conclusions

This study sought to test the hypothesis that eucalypt plants will experience less hydric stress and show better physiological performance under water limitations, when planted in biochar-amended soil and that there is no need for additional fertilizer addition to improve this higher performance. In view of findings of the present study, it can be concluded that *Eucalyptus globulus* is strongly affected by water limitations. Biochar could be successfully applied into soils to enhance the plant physiological performance under water limiting conditions by improving CO_2 assimilation rates, leaf area and, even with no significant difference, with a slight increasing in water potential. Additionally, the addiction of biochar seems to reduce plant stress at carotenoids and TSS content levels. The conjugation of biochar and fertilizer seems not to be a surplus value to amended water limiting conditions. Further studies are needed to better understand the effects of biochar in well watered and in water limiting conditions and its effect on nutrient limitations to safety apply biochar into soils, without compromising plants.

Chapter III

Concluding Remarks, On-going Research and Recommendations for Future Studies

Expected climate change outcomes have emphasized the need to investigate strategies to mitigate drought stress in *Eucalyptus globulus*, which has special economic relevance for Portugal. Biochar has been considered as a promising approach to amend stressed soils and, therefore, the aim of this study was to investigate the effects of biochar on hydric limitations in *Eucalyptus globulus* seedlings.

After analysing morphological conditions, water status, gas exchanges, lipid peroxidation, osmotic compounds accumulation and fluorescence chlorophylls profiles dynamics, it can be concluded that biochar had a positive effect under drought conditions on photosynthetic rates and leaf area, leading to lower cellular membrane damages. In well water conditions, the application of biochar presented lower performance than fertilizer application in the same conditions, namely at Above ground biomass, lateral branches, leaf area, number of leaves, photosynthesis, weight of dry roots and an increasing in TSS values, indicated stress.

Thus, the application of biochar to soils to improve plant performance under water limitations is promising, but further studies are needed. It is important to account that short-term effects of biochar studies may be not observed in the field, emphasis long term realistically occurrences to improve agronomic management decisions. More research is needed in drought stress, involving acute (cyclic) hydric stress, as this is more environmentally relevant. The determination of effects of different kinds of biochar contents and soil combinations, especially in field conditions are also required. Otherwise, little has been done in *Eucalyptus spp*.

In future, more studies must be taken in account to produce feasible data about the effects of biochar amending drought stress in Eucalyptus, regarding the content, period of exposition and the way of application. Also there must be accessed the intrinsic mechanisms on which biochar reacts in water limiting conditions and its interaction in plant biochemical and genomics/proteomic pathways, with and without fertilizer addictions.

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