Biochar as a soil amendment in the tree establishment phase: What are the consequences for tree physiology, soil quality and carbon sequestration?

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HIGHLIGHTS

- Biochar amendment increased the P and K contents in the soil.
- Biochar increased the soil water holding capacity and availability to trees.
- Trees grown in biochar amended soil were more tolerant to water stress.
- The better quality of biochar soil enhanced the tree carbon sequestration.
- Trees grown in biochar amended soil increased their total biomass.

GRAPHICAL ABSTRACT
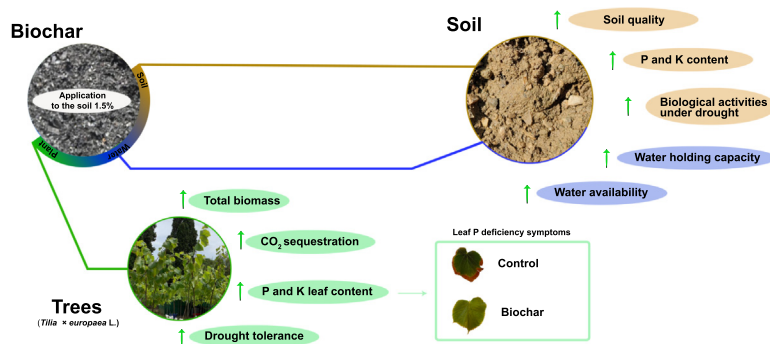
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ABSTRACT

Trees play a pivotal role in the urban environment alleviating the negative impacts of urbanization, and for this reason, local governments have promoted strongly tree planting policies. However, poor soil quality and neglect tree maintenance (e.g., irrigation and fertilization) can seriously mine the plant health status during the tree establishment phase. The use of biochar to provide long-lasting C to the soil and, at the same time, improving soil properties (e.g., improved water holding capacity), soil enzymes activities and NPK concentrations, is a promising research field. Therefore, with a two-step experiment, the study aimed to assay the physiological responses of a commonly used urban tree species (*Tilia × europaea* L.) to 1.5 % (w/w) biochar amendment (B), and secondly, to assess the ability of trees, grown in biochar amended soil, to tolerate a period of drought. Biochar amendment increased P and K availability in the soil, resulting in higher P and K concentrations in B than control leaves, according to the leaf stage. This induced B trees, higher values in both total biomass than controls (+ 22 %) in well-watered plants. Moreover, the higher water availability in soil amended with biochar helped B trees to tolerate water stress, with better leaf photosynthetic performances and a faster recovery than stressed controls after the re-watering. This study highlights the dual function of the biochar, improving CO_2 sequestration and soil properties, and at the same time, enhancing plant physiological responses to environmental constraints. The use of biochar at the tree planting, especially in an urban environment, is a feasible and environmentally sustainable strategy to improve the success during the tree establishment phase.

1. Introduction

Urban trees play a pivotal role as ecosystemic actors in alleviating some urbanization side effects e.g. by improving air quality and CO_2

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sequestration (Klingberg et al., 2022; Ozdemir, 2019; Shadman et al., 2022), mitigating the heat islands (Solecki et al., 2005) and providing a series of other benefits to public health (van den Berg et al., 2015; Venter et al., 2022).

Not by chance, local governments promote green strategies to create new urban forests and green spaces (McDonnell and MacGregor-Fors, 2016). However, cities that embrace strong tree planting policies should estimate the costs of such strategies that are not limited to the tree planting cost but also include maintenance expenses (e.g., pruning and irrigation, fertilization) that cities may fail to address (Pincetl et al., 2013; Smith, 2019). It was reported that the highest mortality level occurs for newly-planted trees in urban green spaces due to scarce planting conditions and poor maintenance at the tree establishment phase (Smith, 2019; Vogt et al., 2017). Indeed, in the urban environment, the establishment phase is the most critical phase for trees due to the reduced tree root system (Roman et al., 2015), water availability, and, in many cases, poor soil quality conditions, e.g., low nutrient availability, soil compactness with reduced water infiltration, storage and availability and consequently reduced soil gas exchanges and biological activities (Moore, 2012; Smith, 2019; Vogt et al., 2017).

Water stress is frequently experienced by the plants in Mediterranean areas during the hot summer periods. Water stress can induce in tree leaves an early stomatal closure reducing the rate of CO_2 fixation and affecting the efficiency of the photosynthetic apparatus sensu *lato*. Indeed, under abiotic stresses such as drought, the light absorbed by chlorophyll pigments becomes excessive for the requirement of the photosynthetic machinery increasing the possibility of photoinhibition (Guidi et al., 2019). This condition results in low primary productivity with reduced plant biomass and, if prolonged, drought can be lethal for the new-planted tree (Dervishi et al., 2022; Moore, 2012; Roman et al., 2014). On the other hand, poor edaphic conditions promote nutrient imbalances leading to plant metabolic limitations that inhibit plant growth and development, making them more susceptible to biotic or abiotic stress (Ferrini et al., 2017; Pregitzer et al., 2016). However, chemical fertilizers should be very carefully applied in an urban environment to avoid potential water pollution due to leaching or runoff phenomena (Ferrini et al., 2017). Nevertheless, a possible way to ameliorate the soil properties is the application of soil conditioners to the native soil (Sax et al., 2017; Somerville et al., 2020). Among these, the use of biochar to improve urban soil properties is an emerging research field (Kim et al., 2021; Scharenbroch et al., 2013; Somerville et al., 2020; Yoo et al., 2020).

Biochar is a C-rich porous material resulting from pyrolysis of organic matter at temperatures from 300 to 1000 °C (Kavitha et al., 2018). Biochar is a promising material to sequestrate carbon dioxide from the terrestrial ecosystem (Lehmann et al., 2006). Moreover, biochar can influence other soil quality and health indicators by stimulating changes in the microbial ecology, plant/fungal symbioses and different soil enzyme activities (Khadem and Raiesi, 2019; Khadem and Raiesi, 2017; Scharenbroch et al., 2013; Zhang et al., 2020). Among soil enzymes, it was reported a positive influence of the biochar on the alkaline phosphatase activity (Khadem and Raiesi, 2019), which plays an essential role in mediating organic P mineralization, making it readily available for plants (Li et al., 2021). This ability, combined with the readily available amount of P and K provided by biochar material (Gao et al., 2021; Jin et al., 2016; Jindo et al., 2020), can constitute an appreciable amount of macro-elements for tree growth. Furthermore, biochar can increase the water-holding capacity of native soil thanks to the high porosity of macro-aggregates typical of this material (Kim et al., 2021). All these physico-chemical characteristics are favorable key factors to increasing the success of tree establishment in the urban environment, ensuring the ecosystem services given by trees and limiting the cost of maintenance and tree replenishment.

Therefore, the present study aimed to i) assay the physiological responses of a widely used urban tree species in Italy (*Tilia × europaea* L.) to biochar amendment (applicated at a concentration of 1.5 %), using a soil characterized by low P availability during a whole vegetative season (experiment 1); ii) test the ability of biochar to enhance tree water availability during an induced water stress (experiment 2). This multilevel analysis provides new insights on the consequences of biochar amendment on tree physiology, soil quality and C sequestration.

2. Materials and methods

2.1. Soil, plant material and experimental design

A set of 50 individuals of 3-year-old, 100-cm-height lime trees (*Tilia × europaea* L.) were purchased from a plant nursery (Vivai Guagno, Padova, IT). Trials were conducted under uncontrolled field conditions at the Department of Agriculture, Food and Environment, University of Pisa, Italy (43°42′N; 10°25′E).

The sandy loam soil used was collected in January 2021 at Fiano di Pescaglia (Lucca, Italy; 43°56′N; 10°26′E; for soil properties, see Table 1). This soil was characterized by a weak sub-acid pH (6.5) with low P availability (Table 1).

During wintertime (February), before the leaf emergency, trees (25 trees per treatment) were transplanted into 9.5-L pots (ø 24 × H 21 cm), and divided into two different treatments: not amended (9.0 kg of soil per pot; i.e., Cnt) and amended with biochar (9.0 kg of soil previously amended with 1.5 % of biochar, the latter sieved to 2–5 mm; i.e., B). Biochar was produced from virgin wood belonging to a consortium of selected tree species (*Abies* sp., *Alnus* sp., *Castanea sativa*, *Fraxinus* sp., *Quercus* sp. and *Robinia pseudoacacia*) by pyrolysis process (Bio-Esperia Srl., AR, Italy). The parameters for the biochar characterization were analyzed through certified methods approved by Italian regulations (D.lgs. 75/2010) and reported in Becagli et al. (2021). The average heating rate before reaching a peak of 1100 °C was 75–80 °C min^{-1}.

In exp. 1, non-destructive physiological analyses were performed monthly, from leaf emergence (April 2021) until the senescent leaf stage (October 2021; Fig. A.1).

During the experiments, trees were kept well-watered without fertilization. Analyses for leaves were performed at three different leaf stages: young (1 week after the leaf emergence; in April), mature (on fully-expanded leaves; early July) and senescent (during leaf-fall; in October). Soil samples were collected at the same time as leaf sampling. At the end of the experiments (November 2021), part of the trees was used for dendrometry analyses.

Table 1

Physical and chemical characteristics of soil (Cnt) used for the two experiments. For chemical characteristics, also data for amended soil with 1.5 % of biochar (B) are reported.

Parameter	Unit	Treatments	
		Cnt	B
Sand	%	62.00	–
Silt	%	33.00	–
Clay	%	5.00	–
WHC	%	32.00 ± 2.00	36.00 ± 1.00*
pH		6.58 ± 0.07	7.52 ± 0.03***
$CaCO_3$ tot	%	0.80 ± 0.03	0.56 ± 0.03***
TOC	g kg^{-1}	23.2 ± 2.10	32.30 ± 3.40**
N	mg g^{-1}	2.10 ± 0.33	1.95 ± 0.34
N-NO$_3$	mg kg^{-1}	21.54 ± 3.60	21.37 ± 1.87
N-NH$_4$	mg k^{-1}	16.78 ± 0.06	16.13 ± 0.65
P$_{av}$	mg kg^{-1}	7.10 ± 1.20	11.80 ± 1.40**
K$_{ex}$	mg kg^{-1}	179.33 ± 4.49	306.33 ± 18.03***
C:N		11.00 ± 2.00	17.00 ± 3.00*
CEC	mmol kg^{-1}	143.00 ± 5.00	171.20 ± 2.10***

Each value is the mean ± SD of 4 replicates. Statistical analysis was conducted using an unpaired *t*-test.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

The water stress experiments (exp. 2 later on) were conducted in the second half of July (the hottest and driest month in Italy; Fig. A.1). Water irrigation was interrupted for nine consecutive days in eight trees of both treatments (Cnt-S and B-S, later on referring to stressed control and stressed biochar treatments, respectively), and physiological and biochemical analyses were performed every 3 days (T0, T1, T2 and T3) till the ninth day of the experiment (the aim was to reach predawn leaf water potential values of ~ -2 (± 5 %) MPa). On the tenth day, a re-watering (R) was done, and analyses were carried out 3 days after R. In this experiment, soil quality analyses were conducted before and at the end of water stress (T0 and T3, respectively).

In both experiments, at each sampling time, samples for biochemical analyses were collected at midday, immediately frozen in liquid nitrogen, and stored at -80 °C until investigation. In each experiment, three soil sub-samples were collected from 0 to 20 cm depth, and the sub-samples were merged to have a homogeneous sample ($n = 4$). Soil samples were stored at 4 °C in dark conditions to interrupt the N mineralization and microbial and enzymatic activity, then they were rapidly air-dried and sieved through a 2.0 mm mesh. Mean monthly temperatures and precipitations for the period are reported in Fig. A.2.

2.2. Biomass parameters

At the end of the first experiment, tree height and stem diameter (measured at root collar level, at 50 and 100 cm) were analyzed in Cnt and B trees ($n = 8$). Stem, branches and roots material (n = 8 for each treatment) was partitioned to determine the fresh weight (FW). Then, the plant materials were dried using a laboratory electric thermostatic oven (Memmert GmbH + Co. KG Universal Oven UN30, Schwa-bach, Germany) at 105 °C until constant weight (4 dd) and the dry weight (DW) was determined. Stem dry wood density was measured ($n = 8$ for each treatment) by measuring the volume and the dry weight of a portion of the stem.

2.3. Leaf gas exchange and chlorophyll a fluorescence analyses

Leaf gas exchange parameters were measured ($n = 6$) using a portable infrared gas analyzer LI-6400 system (Li-Cor, Lincoln, NE, USA).

All gas exchange measurements were conducted on randomly selected leaves from 11:00 to 14:00 (GMT) using a light intensity that varied from 1300 to 1800 μmol m^{-2} s^{-1}, according to the monthly external photosynthetic active radiation (PAR) measured at 12:00. Inside the leaf chamber, the CO_2 concentration was set to 400 μmol mol^{-1} by using the CO_2 mixer and the flow rate was 500 μmol s^{-1}. Once the steady-state was reached, net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), apparent carboxylation efficiency (P_n/C_i) and intrinsic water user efficiency (WUE_{int}; measured as P_n/g_s) were determined.

Chlorophyll *a* fluorescence parameters were measured using PAM-2000 fluorometer (Walz, Effeltrich, Germany). All measurements ($n = 6$) were conducted at the same time as gas exchange analyses in leaves that were homogeneous to those used for gas exchanges. After 30 min of dark adaptation, the photosystem II (PSII) maximum quantum yield (F_v/F_m) was measured.

2.4. Leaf chlorophyll content

The leaf chlorophyll content (Chl), was measured using a leafclip sensor DUALEX® (Force-A, Orsay, France). Starting from April to October, twenty-five randomly chosen leaves (young, mature or senescent) were measured monthly in each treatment at 12:00 (GMT) for exp. 1, while in the exp. 2, were measured every 3 days (T0, T1, T2 and R, respectively).

2.5. Leaf water potential and soil water content

During the exp. 2, the predawn (Ψ_{PD}) and midday (Ψ_{MD}) water potential were measured, using fully expanded leaves ($n = 4$ in each treatment)

by a Scholander pressure chamber (model 600, PMS Instrument, Albany, OR, USA). The soil water content was measured ($n = 3$ per treatment) weighing the pots at each sampling time of the water stress experiment.

2.6. Soil parameters, biological activity assays and N, NO_3, NH_4, P, K, and C analyses

The soil parameters such as particle size distribution, water holding capacity (WHC), pH in water and Cation Exchange Capacity (CEC) were measured following standard methods ($n = 4$) (Colombo and Miano, 2015).

Soil microbial biomass carbon (MB-C) was analyzed only in water stress experiments according to Wu et al. (1990), in which organic carbon (OC) was extracted from 10 g of fumigated and unfumigated soil by 1 N K_2SO_4 ($n = 4$). Extracted OC was measured with QBD1200 Laboratory TOC Analyzer (n = 4; Hach Company, USA) and the instrument data results were processed according to the following expression:

$$MB - C = (\text{Fumigated OC value} - \text{Unfumigated OC value}) \times \text{extraction efficiency coefficient (Kc} = 2.22)$$

All the enzyme activities were analyzed by spectrophotometric methods ($n = 4$). Alkaline phosphomonoesterase activity (APA) was measured in both experiments, at each leaf stage (exp. 1) and during the water stress experiments (exp. 2) according to Eivazi and Tabatabai (1977). Soil samples were incubated with *p*-nitrophenyl phosphate and buffered at pH 11. After 60 min at 37 °C, the released *p*-nitrophenol was measured at 410 nm. β-Glucosidase and urease activities (GA and UA, respectively) were only assayed in water stress experiments. GA was measured using a 4-nitrophenyl-β-D-glucopiranosyde substrate and, after an incubation period at 37 °C for 60 min, the produced *p*-nitrophenol was measured at 410 nm (Eivazi and Tabatabai, 1988). UA was measured spectrophotometrically at 690 nm according to Kandeler and Gerber (1988) by analyzing the production of ammonia after a 2 h incubation with urea substrate at 37 °C.

In water stress experiments, the influence of biochar on soil quality was evaluated by measuring the alteration index three (AI3) according to Puglisi et al. (2006). AI3 was measured through the procession of enzyme result data into the following expression:

$$AI3 = (7.87 \times \beta - \text{glucosidase}) - (8.22 \times \text{phosphatase}) - (0.49 \times \text{urease})$$

where enzyme activities were expressed in μmol of *p*-nitrophenol per gram of soil per hour (for β-glucosidase and phosphatase), and in μg of urea per gram of soil per 2 h (urease).

Leaf and soil total nitrogen (N), nitrates (NO_3), ammonia (NH_4), phosphorus (P), potassium (K), and carbon (C) contents ($n = 4$) were obtained as follows. Total N and C analyses were carried out by the use of an Elementar Vario Micro Elemental Analyzer (Elementaler, Comazzo, Co, Italy). About 2 mg of leaf or 10 mg of soil powdered dry samples were analyzed. Total organic carbon (TOC) was assayed by subtracting the $CaCO_3$ content from C content. Inorganic C ($CaCO_3$) was measured with a Scheibler apparatus. The MB-C:TOC ratio was calculated using the TOC measured in July (mature leaf stage). Nitrates were extracted by stirring the soil samples with distilled water (1:5) for 1 h. Then supernatants were collected and passed through filters (Whatman 42). NO_3 were detected by ion chromatography (Dionex DX120). Ammonia (NH_4) analysis on soil samples was conducted according to Kempers and Zweers (1986). Available phosphorus (P_{av}) in soils and in biochar was analyzed by using the Olsen method (Olsen et al., 1954). P in leaves was measured spectrophotometrically by an Ultrospec 2100 Pro spectrophotometer (GE Healthcare Ltd., Little Chalfont, England) following the molybdenum blue method according to Murphy and Riley (1962) modified by Benini et al. (2014). Exchangeable K (K_{ex}) in soils and in biochar material was measured using an atomic absorption spectrometer (Thermo Scientific ICE 3000 Series)

by extracting 2.5 g of the sample with ammonium acetate solution 7.0 buffered (Colombo and Miano, 2015). For leaf K, about 0.2 g of dry powdered leaf samples were put in Teflon tubes with 2 mL of H_2O_2 (30 %, w/v) and 8 mL of HNO_3 (70 %, v/v) and incubated overnight at room temperature. Then, samples were mineralized at 200 °C for 60 min using a microwave digestion system (Start D, Milestone Srl, Sorisole, BG, Italy). Samples were then transferred in a final volume of 25 mL, reached by the addition of double-distilled water. After mineralization, K content was measured using an atomic absorption spectrometer.

2.7. Lipid peroxidation analysis

Lipid peroxidation ($n = 3$) was evaluated by the thiobarbituric acid (TBA) assay as described by Hodges et al. (1999), in leaves of the water stress experiments. 0.1 g of fresh leaf material was homogenized in 6 mL of an ethanolic aqueous solution 80 % (v/v) and centrifuged at 10,000 rpm for 10 min at 4 °C. 1 mL aliquot of leaf extract was added to a test tube with 1 mL of either (i) –TBA ethanolic solution comprised of 20 % (w/v) trichloroacetic acid (TCA), or (ii) + TBA ethanolic solution containing the above plus 0.5 % TBA (w/v). Each mixture was incubated at 95 °C for 30 min and the reaction was stopped by transferring tubes on ice. Absorbances were spectrophotometrically read at 440, 532 nm and 600 nm. Malondialdehyde (MDA) equivalents were measured using the following equations (from Hodges et al., 1999 and corrected by Landi, 2017), considering ε of MDA as $157{,}000\ M^{-1}\ cm^{-1}$:

$$[(Abs532_{+TBA}) - (Abs600_{+TBA}) - (Abs532_{-TBA}) - (Abs600_{-TBA})] = A \quad (1)$$

$$[(Abs440_{+TBA} - Abs600_{+TBA})\ 0.0571] = B \quad (2)$$

$$MDA\ equivalents\ (nmol\ mL^{-1}) = (A - B/157{,}000) \cdot 10^6 \quad (3)$$

The level of lipid peroxidation was expressed as mmol MDA equivalents per g of fresh weight (mmol MDA g^{-1} FW).

2.8. Nitrogen resorption efficiency and total sequestered CO_2 from trees and biochar material

Nitrogen resorption efficiency (N_{res}) was measured as $(N_m - N_s)/N_m \times 100$, in which N_m is N content in mature leaves (leaves sampled in July) and N_s is that contained in the senescent leaves (leaves sampled in October).

The sequestered CO_2 from trees (S-$CO_{2\text{-Tree}}$) was measured by the following equation:

$$S\text{-}CO_{2-Tree} = (TC\text{-}R + TC\text{-}S + TC\text{-}B) \times 3.66 \quad (1)$$

in which 3.66 is a conversion index measured from the ratio of the molecular weights of CO_2 (44.009 g mol^{-1}) and C (12.011 g mol^{-1}) and TC-R, TC-S and TC-B were the total carbon content (g) in dry tree root, stem and branches, respectively. TC-R, TC-S and TC-B were obtained from the measure of the % of C contained in each tree portion (the %C was ~43 %; data not shown) by the use of an Elemental Analyzer (for details see Section 2.6). The TC content (g) was measured as follows:

$$TC = (Biomass_{(dry\ root,\ stem\ or\ branches)} \times \%C)/100 \quad (2)$$

For the calculation of CO_2 sequestered by biochar (S-$CO_{2\text{-Biochar}}$) Eq. (1) was utilized. TC-Bio was measured using the grams of dry biochar and the %C was taken from Becagli et al. (2021).

$$TC\text{-}Bio = (Biochar_{(dry)} \times \%C)/100 \quad (3)$$

2.9. Statistical analysis

Data obtained from physiological (gas exchanges, Dualex, chlorophyll a fluorescence parameters), water balance (leaf water potential and soil water

content) and biochemical analyses (N, NO_3, NH_4, P, K, C, MDA and APA) were analyzed by two-way analysis of variance (ANOVA) using the treatments and time as the source of variation. Soil quality data (APA, GA, UA, AI3, MB-C and MB-C:TOC), obtained during the water stress experiments were analyzed by two-way ANOVA using treatments and time as the source of variation. All the means were separated by Fisher's least significant difference (LSD) post-hoc test ($p = 0.05$). The normality of data was tested using Shapiro-Wilk test, while the homoscedasticity was tested using Bartlett's test. Percentage values were arcsine transformed prior analyses. The tree biomass parameters, nitrogen resorption and CO_2 sequestration data were compared between treatments using Student's t-test. All statistical analyses were conducted using GraphPad (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Experiment 1: vegetative season

3.1.1. Tree biomass parameters

Tree biomass data are summarized in Table 2. At the end of the vegetative season (November), B trees showed higher values of stem diameter at each measuring point with respect to Cnt trees (+9, +15 and + 30 %, for diameters measured at collar, 50 cm and 100 cm of height, respectively). Consequently, also the tree biomass (roots, stem and branches) was higher in B trees (Table 2). On a dry weight basis, B trees improved root biomass by 23 %, stem by 18 % and branches by 37 %, contributing to increasing the total tree biomass by 22 % with respect to Cnt individuals. Dry matter percentage was significantly higher in B compared to Cnt only in roots (+7 %). The dry wood density did not differ between treatments.

3.1.2. Leaf gas exchange and chlorophyll a fluorescence analyses

During the vegetative season (starting from April to October), the trend of P_n was similar for both Cnt and B trees (Fig. 1a). In July, a strong decrease in P_n values was observed in both treatments, with respect to values detected in June, and the reduction was significantly greater in Cnt than B trees (−50 and − 39 %, respectively).

Statistically significant variations in g_s values were observed across the experimental period, but irrespectively to the biochar treatments (Fig. 1b). The C_i trend was similar for both treatments with values of C_i increasing as the leaf physiological stages progressed (from young to senescent; Fig. 1c).

Table 2

Height, diameters and tree biomass parameters of *Tilia × europaea* plants at the end of the vegetative season (November).

Biomass parameters		Treatments	
		Cnt	B
Height	(cm)	142.29 ± 11.57	146.14 ± 9.24
Ø$_{collar}$	(cm)	2.30 ± 0.06	2.51 ± 0.24*
Ø$_{50cm}$	(cm)	1.16 ± 0.05	1.34 ± 0.13**
Ø$_{100cm}$	(cm)	0.69 ± 0.07	0.90 ± 0.10***
Roots	FW (g)	336.49 ± 44.06	386.80 ± 38.70*
	DW (g)	88.00 ± 13.20	108.57 ± 10.71**
	DM (%)	26.15 ± 1.97	28.08 ± 0.80*
Stem	FW (g)	124.07 ± 14.54	145.87 ± 19.20*
	DW (g)	56.30 ± 6.30	66.74 ± 9.13*
	DM (%)	45.40 ± 0.37	45.73 ± 0.39
Branches	FW (g)	17.01 ± 2.86	23.20 ± 5.06*
	DW (g)	7.21 ± 1.21	9.88 ± 2.20*
	DM (%)	42.39 ± 0.40	42.54 ± 0.49
Total biomass	FW (g)	477.57 ± 49.62	555.84 ± 49.65*
	DW (g)	151.51 ± 17.89	185.19 ± 17.33**
Stem dry wood density	(kg m^{-3})	597.80 ± 38.94	589.93 ± 76.91

Cnt = control trees grown without biochar, B = trees grown in soil amended with 1.5 % of biochar. Each value is the mean ± SD of 8 replicates. Statistical analysis was conducted using an unpaired t-test.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.
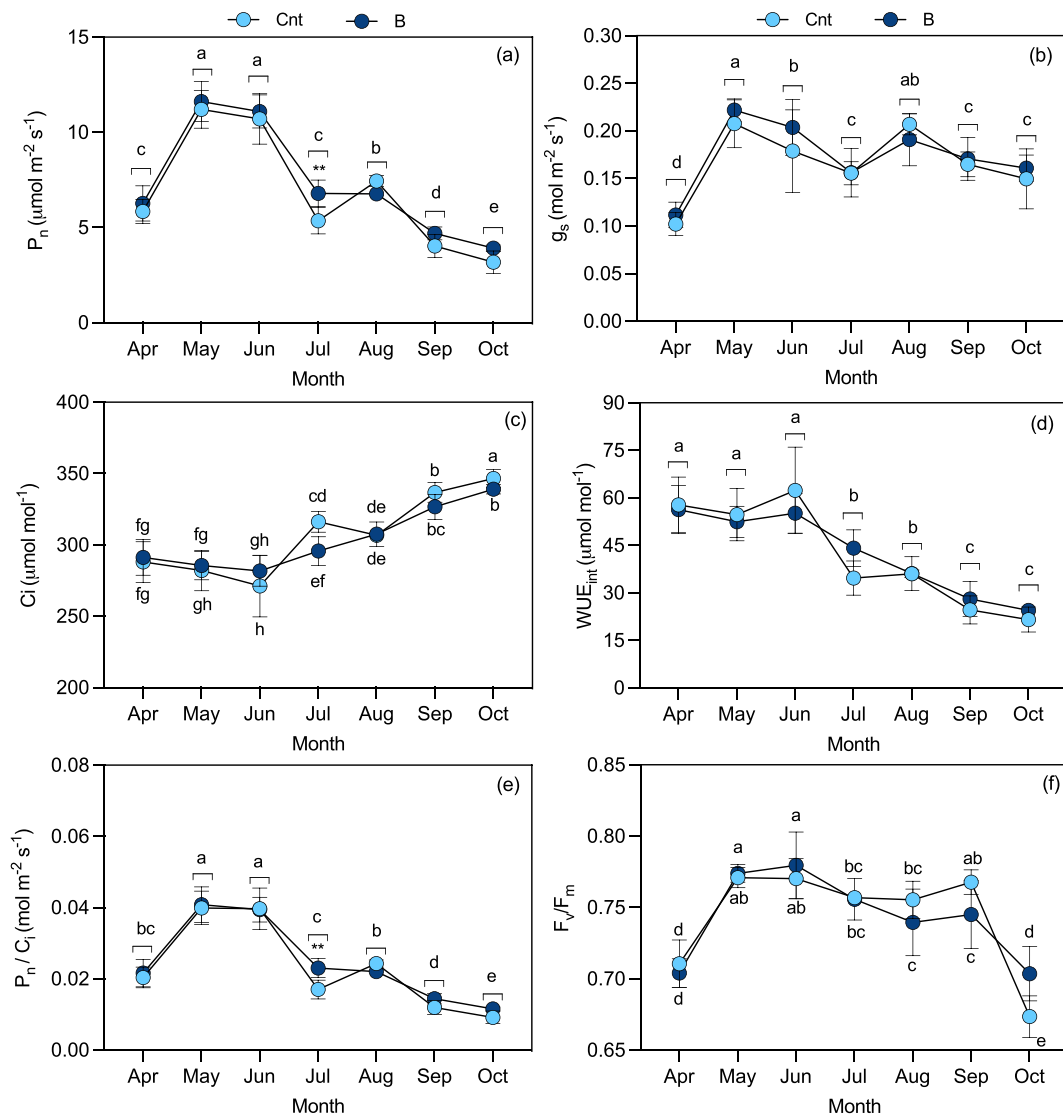
Fig. 1. Monthly gas exchanges and chlorophyll *a* fluorescence parameters analyzed in controls (Cnt) and 1.5 % biochar amended (B) trees (*Tilia × europaea*). Gas exchanges were determined at ambient light and CO_2 conditions: net photosynthesis:(P_n; a), stomatal conductance (g_s; b), intercellular CO_2 concentration (C_i; c), intrinsic water user efficiency (WUE_{int}; d), apparent carboxylation efficiency (P_n/C_i; e) and photosystem II maximum photochemical efficiency (F_v/Fm; f). The Cnt leaves are represented by cyan circles, while B leaves by blue circles. Means ($\pm$ SD; $n = 6$) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. Means flanked by the same letter are not statistically different for $p = 0.05$ after Fisher's least significant difference post-hoc test. When the F ratio of the interaction between the variability factors is not significant, letters indicate statistically significant differences between means over time. Differences between treatments were indicated by asterisks ($p < 0.05$: *; $p < 0.01$: **).

No significant differences between treatments were observed for WUE_{int}, and the only change occurred across months, with the highest values detected during spring (April, May and June; ~54.0 µmol mol^{-1}). The only difference between the treatments in the apparent carboxylation efficiency (P_n/C_i) was detected in July, with higher values (+35 %) observed in B compared to Cnt trees (Fig. 1e).

Values of F_v/F_m varied significantly among months and treatments (Fig. 1f). In October a significant reduction in the F_v/F_m ratio was observed in both treatments, with a higher decrease detected in Cnt compared to B trees (−12 and −5 %, respectively, with respect to September values).

3.1.3. Leaf chlorophyll content
The analysis of leaf total chlorophyll content evidenced significant differences between treatments and across the experimental period (Fig. 2). In July, B leaves showed higher content in chlorophylls than Cnt ones (+9 %). Then, a reverse trend was observed starting from August, generally showing higher values for Cnt leaves compared to B leaves.

However, this trend was statistically different only at the leaf senescent stage (October).

3.1.4. Leaf N, P, K and C contents
Total leaf N values were highest in April (young leaf stage), without any difference observed between treatments (Fig. 3a). In July (fully-expanded leaves), a decrease in N concentrations was reported for both treatments, which was higher in Cnt trees with respect to B individuals (−57 and −48 %, respectively). A further decrease was also observed in October (senescent leaf stage), although B trees showed significantly lower concentration values in N than Cnt trees (−19 %).
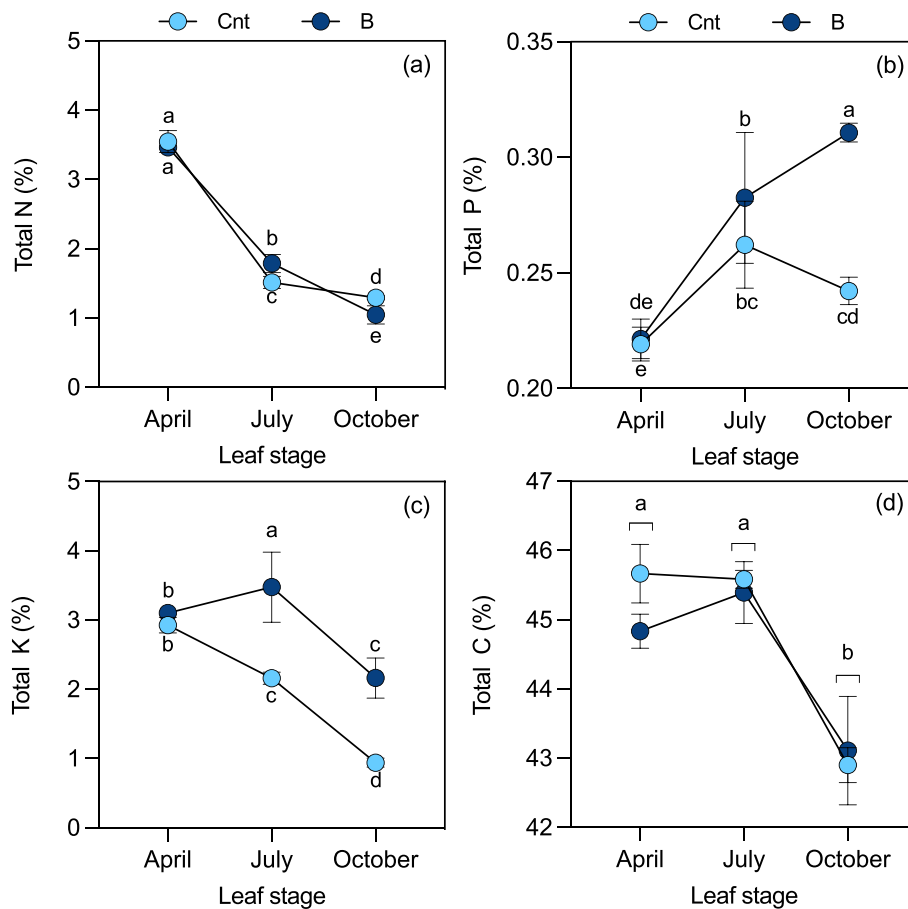
In April, the leaf P concentrations were similar between treatments (Fig. 3b). In July, the P contents similarly increased in both treatments. However, in October, B leaves showed higher concentration values of P than Cnt tree leaves (+29 %).

In April, K concentrations were similar between treatments (Fig. 3c). In July, K concentration decreased in Cnt (−26 %, with respect to April values), but significantly increased in B leaves (+12 % with respect to
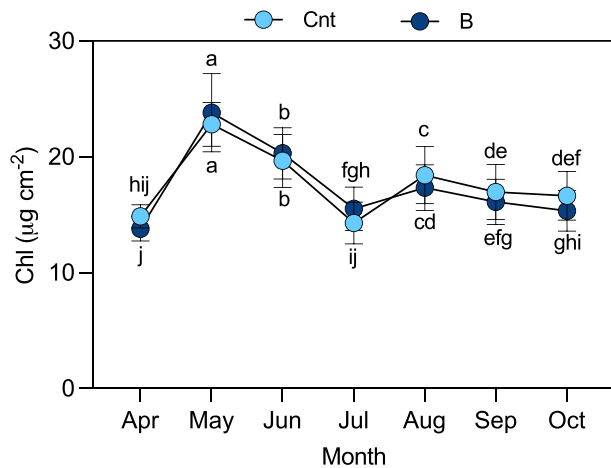
Fig. 2. Monthly chlorophyll content (Chl) analyzed in controls (Cnt) and 1.5 % biochar amended (B) trees (*Tilia × europaea*). The Cnt leaves are represented by cyan circles, while B leaves by blue circles. Means (± SD; n = 25) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. Means flanked by the same letter are not statistically different for p = 0.05 after Fisher's least significant difference post-hoc test.

April values). In October, K concentrations decreased in both treatments, although B leaves showed higher K concentration values compared to Cnt (+129 %). Total C content reached the highest values in April and July

and the lowest in October, but in any case, it was independent of biochar treatment (Fig. 3d).

3.1.5. Soil N, NO₃, NH₄, P, K contents and APA

No differences were observed for total N, nitrate and ammonia concentrations between treatments (Fig. 4a,b,c).

Variations of P_{av} concentrations across months were similar between treatments, with the highest values observed in July, intermediate in October and the lowest in April (Fig. 4d). Nevertheless, at each sampling time, P_{av} concentrations were consistently higher in B than Cnt soils (+66, +67 and + 36 %, in April, July and October, respectively).
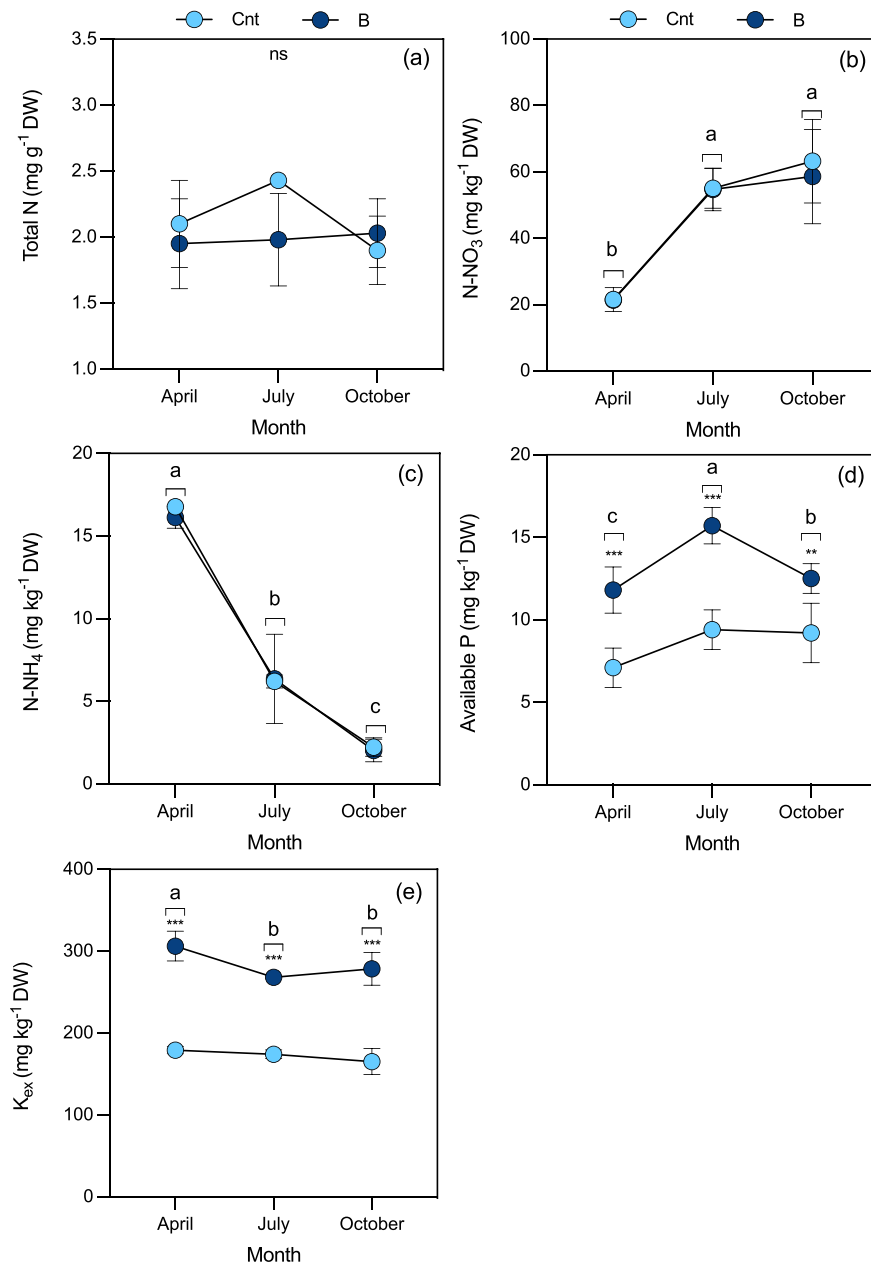
K_{ex} contents were higher in B than Cnt soils at each sampling time (+71, +54 and +68 %, in April, July and October, respectively; Fig. 4e), showing the highest contents in April.

The analyses conducted on the biochar material showed that P_{av} and K_{ex} concentrations were 141 ± 12.01 and 8970.13 mg kg^{-1}, respectively.

During the time of the experiment, both the treatments showed similar trends in APA (Fig. 5). Nevertheless, in B soil, APA activity was significantly higher than Cnt in April and July (+37 and +68 %, p < 0.05 and 0.001, respectively).

3.1.6. Leaf N_{res} and sequestered CO_2 from trees and biochar material

At the leaf senescent stage (October), the N_{res} efficiency was higher in B than Cnt leaves (41 and 14 %, respectively; Table 3). The sequestered CO_2 by B trees increased by 22 % compared to Cnt ones, (about 0.24 g per day were sequestered more than Cnt) (Table 3). Sequestered CO_2 by biochar was 258.87 g CO_2 eq.

Fig. 3. Total leaf nitrogen (N; a), phosphorus (P; b), potassium (K; c) and carbon (C; d) contents in controls (Cnt) and 1.5 % biochar amended (B) trees (*Tilia × europaea*), measured in April (1 week after the leaf emerge), in July (on fully-expanded leaves) and in October (during leaf-fall). The Cnt leaves are represented by cyan circles, while B leaves by blue circles. Means (± SD; n = 4) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. Means flanked by the same letter are not statistically different for p = 0.05 after Fisher's least significant difference post-hoc test. When the F ratio of the interaction between the variability factors is not significant, letters indicate statistically significant differences between means over time.

Fig. 4. Total soil nitrogen (N; a), nitrates (NO_3; b), ammonium (NH_4; c), available phosphorus (P_{av}; d) and exchangeable potassium (K_{ex}; e) in controls (Cnt) and 1.5 % biochar amended (B) soils, measured in April (1 week after the leaf emerge), in July (on fully-expanded leaves) and in October (during leaf-fall). The Cnt leaves are represented by cyan circles, while B leaves by blue circles. Means (± SD; $n = 4$) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. When the F ratio of the interaction between the variability factors is not significant, letters indicate statistically significant differences between means over time. Differences between treatments were indicated by asterisks ($p < 0.01$: **; $p < 0.001$: ***).

3.2. Experiment 2: water stress and re-watering

3.2.1. Soil quality parameters

Soil quality parameters are summarized in Table 4. Starting from the beginning (T0) to the end (T3) of 9 days of water stress, APA decreased in both -S treatments compared to T0 conditions, but with a lesser intent in B-S than Cnt-S (-8 and -25 %, respectively). At each sampling time, B-S showed higher values in APA than Cnt-S ($+32$ and $+85$ % at T0 and T3, respectively).

GA values at T0, were lower in B-S compared to Cnt-S soil (-47 %). However, at T3, GA values decreased only in Cnt-S soil by 20 %, remaining higher than B-S ($+39$ %). The water stress reduced the UA values in both treatments. However, B-S values were higher than Cnt-S in each sampling time.

At T0 and T3, the AI3 values were more negative in B-S than Cnt soil (-30 and -92 % at T0 and T3, respectively). At T3, AI3 values increased

in both treatments, and the higher increases were shown in Cnt-S with respect to B-S soil ($+38$ and $+8$ %, respectively).

At T0, MB-C content values were not significative different between treatments, but at T3, were reduced more in Cnt-S than B-S (-40 and -21 %, respectively). The MB-C:TOC ratio values at T0 were lower in B-S than Cnt-S soils (-30 %). At T3, no statistically significant differences between treatments were detected. However, MB-C:TOC ratio values decreased in both treatments, but a higher reduction was observed in Cnt-S than B-S (-41 and -22 %, respectively).

3.2.2. Leaf water potential and soil water loss

During water stress experiments (July), the Ψ_{PD} decreased by 34 %, at T1, only in Cnt-S with respect to Cnt (Fig. 6a), whereas at any other sampling time (T2, T3 and R) only water stress induced changes. Indeed, Ψ_{PD}
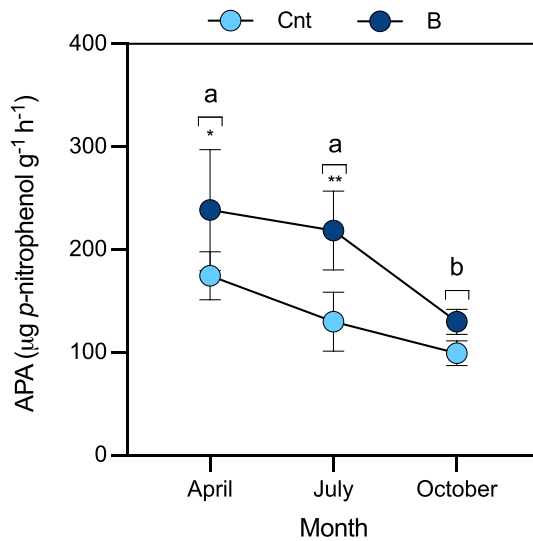
Fig. 5. Alkaline phosphomonoesterase activity (APA) in controls (Cnt) and 1.5 % biochar amended (B) soils, measured in April (1 week after the leaf emerge), in July (on fully-expanded leaves) and in October (during leaf-fall). The Cnt leaves are represented by cyan circles, while B leaves by blue circles. Means (± SD; n = 4) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. When the F ratio of the interaction between the variability factors is not significant, letters indicate statistically significant differences between means over time. Differences between treatments were indicated by asterisks ($p < 0.05$: *; $p < 0.01$: **).

decreased in both -S treatments at T2, reaching the lowest values at T3 (∼ −1.9 MPa). After 3 days of re-watering (R), values of Ψ_{PD} strongly increased reaching similar levels of their relative non-stressed counterparts. At T1, the Ψ_{MD} decreased in both -S treatments compared to non-stressed ones, but with a lower extent in B-S compared to Cnt-S trees (−48 and −62 %, respectively; Fig. 6b). At T2 Ψ_{MD} further decreased in both stressed treatments showing similar values (−1.3 MPa). The lowest Ψ_{MD} values were reached -S treatments at T3, but Cnt-S trees showed lower values than B-S (−2.2 and −2.0 MPa, respectively). After 3 days of re-watering, no differences were reported between -S treatments and their relative controls.

The % of soil water loss steeply negatively increased from T1 to T3 in both stressed treatments (Fig. 6c). However, at T2 and T3, in B-S soils higher values in % of soil water loss soils was observed with respect to Cnt-S soils (+17 and +15 % more than Cnt-S in T2 and T3, respectively). After re-watering, no differences were observed between -S treatments and their relative counterparts.

Table 3

Nitrogen resorption efficiency in senescent leaves of *Tilia × europaea* plants, CO_2 sequestrated by trees at the end of the vegetative season (November) and by biochar.

Parameters	Unit	Cnt	B
N_{res}	%	14.38 ± 3.53	41.46 ± 4.84***
S-$CO_{2\text{-}Tree}$	g CO_2 eq.	232.90 ± 27.57	284.75 ± 26.62**
S-$CO_{2\text{-}Biochar}$	g CO_2 eq.	–	258.87
S-$CO_{2\text{-}Tot}$	g CO_2 eq.	232.90	543.62

Cnt = control trees grown without biochar, B = trees grown in soil amended with 1.5 % of biochar. N_{res} = Nitrogen resorption efficiency, S-$CO_{2\text{-}Tree}$ = sequestered CO_2 by trees, S-$CO_{2\text{-}Biochar}$ = sequestered CO_2 by biochar, S-$CO_{2\text{-}Tot}$ = total sequestered CO_2 by trees and biochar. N_{res} value is the mean ± SD of 4 replicates, S-$CO_{2\text{-}Tree}$ value is the mean ± SD of 4 replicates. Statistical analysis was conducted using an unpaired *t*-test.

** $p < 0.01$.

*** $p < 0.001$.

3.2.3. Leaf gas exchange and chlorophyll a fluorescence analyses

Values of P_n decreased in -S treatments at T1 with respect to their relative non-stressed trees, with a lower extent observed in B-S compared to Cnt-S trees (−24 and −57 %, respectively; Fig. 7a). However, at T2 and T3, no differences between -S treatments were observed with similar decreasing trends. After re-watering, the P_n was almost fully recovered in B-S with respect to Cnt-S trees (differences between water-stressed and relative non-stressed trees, −32 and −19 % for Cnt-S and B-S trees, respectively).

At T1, the stomatal conductance was reduced under water stress conditions in both treatments (Fig. 7b). Nevertheless, B-S trees showed a lower reduction in g_s with respect to Cnt-S trees (−24 and −165 %, respectively). In both stressed treatments, a further decrease in g_s values was observed at T2 and T3 without any statistical differences between treatments (Cnt-S and B-S). After re-watering, the stomatal conductance values increased in both Cnt-S and B-S treatments; however, they did not reach the values recorded in Cnt and B trees.

Significant differences in C_i levels were observed only at T2 and T3 in Cnt-S trees, with higher values than Cnt trees (+8 and + 16 %, at T2 and T3, respectively; Fig. 7c). After re-watering, C_i levels in Cnt-S returned at values similar to those observed in Cnt trees, whereas in B-S trees were significantly reduced by 9 % compared to B trees.

WUE_{int} values increased in Cnt-S trees at T1 with respect to Cnt trees (+32 %), then strongly decreased at T2 and T3 (−30 and − 64 %, respectively; Fig. 7d). However, after re-watering, WUE_{int} values were higher than those recorded in Cnt trees (+28 %). In B-S trees, differences in WUE_{int} values were observed only after re-watering, when B-S showed higher values than B trees (+46 %).

Values of P_n/C_i decreased at T1 in both -S treatments, with a lower decrease in B-S compared to Cnt-S trees (−29 and −59 %, respectively; Fig. 7e). At T2, a further decrease in P_n/C_i values was observed in both stressed treatments without any differences between them. The P_n/C_i values remained steadily low also at T3, while after re-watering increased in both -S treatments. Nevertheless, after re-watering, P_n/C_i values recovered to non-stressed levels only in B-S trees.

At T1, F_v/F_m values decreased in both -S treatments, but B-S trees showed lower decreases compared to Cnt-S trees (−5 and − 10 %, respectively; Fig. 7f). At T2, F_v/F_m values measured in B-S trees remained unchanged with respect to T1 values, whereas Cnt-S values increased to non-stressed levels. On the other hand, at T3 and after the re-watering, a strong decrease was observed only in Cnt-S trees with low F_v/F_m values (∼0.68). F_v/F_m values measured in B-S after the re-watering were not different to those measured in B trees.

3.2.4. Leaf chlorophyll content and lipid peroxidation

Leaf chlorophyll contents remained unchanged between -S and non-stressed plants till T3 (Fig. 8a). Indeed, a strong decrease was observed at T3 in both -S treatments, which was more severe in Cnt-S compared to B-S trees (−35.1 and −25.8, respectively). Then, Chl values remained lower also after re-watering as compared to Cnt and B trees.

Leaf MDA contents increased in both -S treatments at T1 (on average by ∼87 %; Fig. 8b). Although MDA content values remained higher in -S treatments than non-stressed ones, at T2 and T3, a reduction in MDA contents was observed with respect to T1 values (∼ − 21 %) in both treatments. Whereas, after re-watering, only B-S trees returned at non-stressed leaf MDA levels.

4. Discussion

4.1. Experiment 1

4.1.1. Biochar soil amendment promoted tree growth and improved macronutrient availability

In the last years, biochar has received considerable attention in the horticultural sector due to its beneficial effects on soil, promoting plant performances thus increasing the crop yield (Becagli et al., 2021; Kavitha et al., 2018; Semida et al., 2019). Furthermore, a recent meta-analysis

Table 4

Soil quality parameters before and at the end of the water stress performed in July.

	Cnt-S	B-S	$\overline{X}$
APA (μg p-nitrophenol g^{-1} h^{-1})			
T0	114.0 ± 4.1^c	$\mathbf{150.0 \pm 5.7^a}$	$\mathbf{132.0 \pm 19.8^A}$
T3	74.1 ± 10.2^d	137.3 ± 3.3^b	$\mathbf{105.7 \pm 34.6^B}$
$\overline{X}$	$\mathbf{94.1 \pm 22.6^B}$	$\mathbf{143.7 \pm 8.1^A}$	
GA (μg p-nitrophenol g^{-1} h^{-1})			
T0	77.0 ± 3.6^a	40.9 ± 2.9^c	$\mathbf{58.9 \pm 19.4^A}$
T3	61.7 ± 3.9^b	37.3 ± 3.4^c	$\mathbf{49.5 \pm 13.4^B}$
$\overline{X}$	$\mathbf{69.3 \pm 8.8^A}$	$\mathbf{39.1 \pm 3.5^B}$	
UA (μg NH_4 g^{-1} 2 h)			
T0	65.1 ± 4.3	104.3 ± 4.0	$\mathbf{84.7 \pm 21.4^A}$
T3	38.7 ± 5.4	70.9 ± 4.9	$\mathbf{54.8 \pm 17.9^B}$
$\overline{X}$	$\mathbf{51.8 \pm 14.7^B}$	$\mathbf{87.7 \pm 18.29^A}$	
AI3			
T0	-35.6 ± 2.2^b	-46.2 ± 2.2^d	$\mathbf{-40.9 \pm 6.0^B}$
T3	-22.1 ± 2.3^a	-42.5 ± 1.7^c	$\mathbf{-32.3 \pm 11.1^A}$
$\overline{X}$	$\mathbf{-28.9 \pm 7.5^A}$	$\mathbf{44.3 \pm 2.6^B}$	
MB-C (mg kg^{-1})			
T0	1056.7 ± 31.1^a	1058.0 ± 13.5^a	$\mathbf{1057.2 \pm 18.14^A}$
T3	632.0 ± 19.5^c	836.9 ± 32.4^b	$\mathbf{734.5 \pm 111.4^B}$
$\overline{X}$	$\mathbf{844.3 \pm 227.8^B}$	$\mathbf{947.5 \pm 258.0^A}$	
MB-C:TOC			
T0	0.046 ± 0.002^a	0.032 ± 0.003^b	$\mathbf{0.039 \pm 0.007^A}$
T3	0.027 ± 0.001^c	0.025 ± 0.002^c	$\mathbf{0.026 \pm 0.001^B}$
$\overline{X}$	$\mathbf{0.036 \pm 0.010^A}$	$\mathbf{0.028 \pm 0.004^B}$	

T0 = before the water stress period, T3 = at the end of the water stress period (9 days), Cnt-S = control soil without biochar subjected to water stress, B-S = soil amended with 1.5 % of biochar subjected to water stress, APA = alkaline phosphomonoesterase activity, GA = β-Glucosidase activity, UA = urease activity, AI3 = soil alteration index 3, MB-C = microbial biomass carbon, MB-C:TOC = microbial biomass carbon total:organic carbon ratio. Each value is the mean ± SD of 4 replicates. Means without letters are not significantly different following the two-way ANOVA test using the different times before and after water stress and the treatments (Cnt and B) as variability factors; means were separated by Fisher's least significant difference (LSD) post-hoc test. In the Table, the mean values (with the letter when significant) between and within groups are reported.

conducted in crop species by Gao et al. (2021) reported that biochar applications enhanced P_n by 23 % on average. However, few studies have examined the effect of biochar on the photosynthetic responses of tree species, so the influences of this type of soil conditioner are yet to be fully elucidated. In a multi-year experiment conducted on oaks, Tanazawa et al. (2021) reported a P_n increase in trees grown in biochar amended soil thanks to improvements in physiological activities (i.e., higher maximum carboxylation rate and maximum electron transport than not amended ones). However, the authors indicated that the positive effects of the biochar amendment on P_n faded gradually year by year. So, it is to be assumed that the effects we observed may only help the tree for a limited period (tree establishment phase). Moreover, a study conducted by Wang et al. (2014) on ornamental trees (*Malus hupehensis*) reported that biochar applications to the soil alleviated the stress from replanting, increasing the photosynthetic leaf performances by stimulating antioxidant enzymes and reducing lipid peroxidation. Our hypothesis that biochar amendment in lime trees improves photosynthetic parameters is partially supported by our results. Indeed, in general, P_n values were similar between treatments. However, in July, the hottest/driest period over the year in the Mediterranean area (Grassi and Magnani, 2005), the biochar amendment alleviated the detrimental effects caused by these environmental conditions (i.e., high temperatures). The reduction in P_n in leaves observed in July was mainly due to lower g_s and reduced P_n/C_i values (lower in Cnt than B trees). The substantial decline in P_n/C_i values observed in July could have been due to a partial deactivation of Rubisco caused by high temperatures (Grassi and Magnani, 2005; Sharkey, 2005). The higher N and K contents found in B leaves in July (mature leaves) have advantaged B trees to alleviate the reduction in the photosynthetic activity with respect to Cnt trees

(Fig. 3a). Indeed, key leaf photosynthetic biochemical traits such as chlorophyll *a* and Rubisco contents are strongly influenced by the leaf N contents (Xiong and Flexas, 2021). Given K is involved in H^+ efflux from the thylakoid lumen to the stroma through H^+/K^+ ion-exchange regulating photosynthesis, an adequate leaf K content can enhance plant tolerance to (a)biotic stress (Wang and Wu, 2017). Besides chemical changes at soil (e.g. pH, ionic interactions, etc.) which may promote nutrient mobilization, biochar has been proposed to increase nutrient availability in view of its release of some nutrients, including K (see results; Gao et al., 2021; Jindo et al., 2020). Moreover, it was observed that biochar amendment can stimulate beneficial changes in the bacterial community accelerating the conversion of slowly-available K to available K (Zhang et al., 2020). Therefore, our data confirmed that biochar amendment provided an extra K availability for trees, and could be used as a technique to increase available K in low fertile soils.

The positive effects of the biochar amendment were not only limited to increasing the K availability in soil but it also increased the edaphic availability of P, which was higher in B than Cnt soils at each leaf stage (Fig. 4d). This was due to a combined effect: i) a direct biochar contribution to P_{av} content; ii) increased APA in B soil. Indeed, it was reported that biochar has positive effects on APA by enhancing chemical (e.g., pH and nutrient contents), biological (e.g., microbial biomass and activity) and physical (e.g., gas exchanges, and water holding) properties of amended soil (Jin et al., 2016; Khadem and Raiesi, 2019; Lehmann et al., 2011; Yoo et al., 2020). The increased P_{av} contents in B soil, limited the leaf P deficiency symptoms in B leaves, which conversely, emerged in Cnt leaves at the end of August (Fig. A.3), showing necrotic leaf areas and darker green colour than B leaves. This condition brought out a positive role in plant P nutrition given by biochar supplementation. Moreover, the P deficiency in senescent Cnt leaves (October) could explain why considerably lower F_v/F_m values were observed in those leaves if compared to B ones. Indeed, P deficiency may reduce the phosphate content in the chloroplast stroma, inhibiting the ATP synthase activity, resulting in protons accumulation, lumen acidification, inhibiting the electron flow between PSII and PSI (Carstensen et al., 2018; Hernández and Munné-Bosch, 2015). Last but not least, it seems that P deficiency affected the leaf senescence processes, influencing the N resorption in Cnt trees since the senescent leaves showed higher content in chlorophylls and N contents with respect to B leaves (Fig. 2, 3a and Table 3). This could cause a loss of valuable nutrients for trees since they are not readily re-mobilized from leaf to trunk becoming unavailable for the following growing season.

Previous studies conducted on tree species found that biochar soil amendment promotes the increase of plant biomass (Lefebvre et al., 2019; Scharenbroch et al., 2013; Zoghi et al., 2019). Our results are in agreement with previous findings, supporting that the better physiological status of B trees due to the higher availability of macronutrients in B soils with respect to Cnt soils resulted, at the end of the growing season, in increased root, stem and branch biomass.

4.1.2. The use of biochar promoted the CO_2 sequestration

Biochar applications to the soil were proposed as an effective strategy to sequestrate in the soil significant amounts of atmospheric CO_2 from the ecosystems for long periods (Lehmann et al., 2006; Wu et al., 2016). Generally, the conversion of uncharred woody biomass to biochar is about 50 % of the initial C contents (Lehman et al., 2002; Lehmann et al., 2006). Although biochar C is more stable through the years than raw woody biomass, its lifetime depends on the feedstock used to produce biochar as well as environmental soil conditions in which biochar is used (Windeatt et al., 2014; Wu et al., 2016). These variables can influence the biochar C stability determining its lifetime in the soil from tens to thousands of years (Windeatt et al., 2014). In our pot experiments, the estimated CO_2 sequestration, given solely by biochar applications, was 258.87 g CO_2 eq. per pots. This aspect combined with the increase in tree biomass of B trees reached a total amount of 543.62 g CO_2 eq. per pot. Therefore, besides the direct capability of biochar to provide long-lasting C in the soil, it is also to be considered its
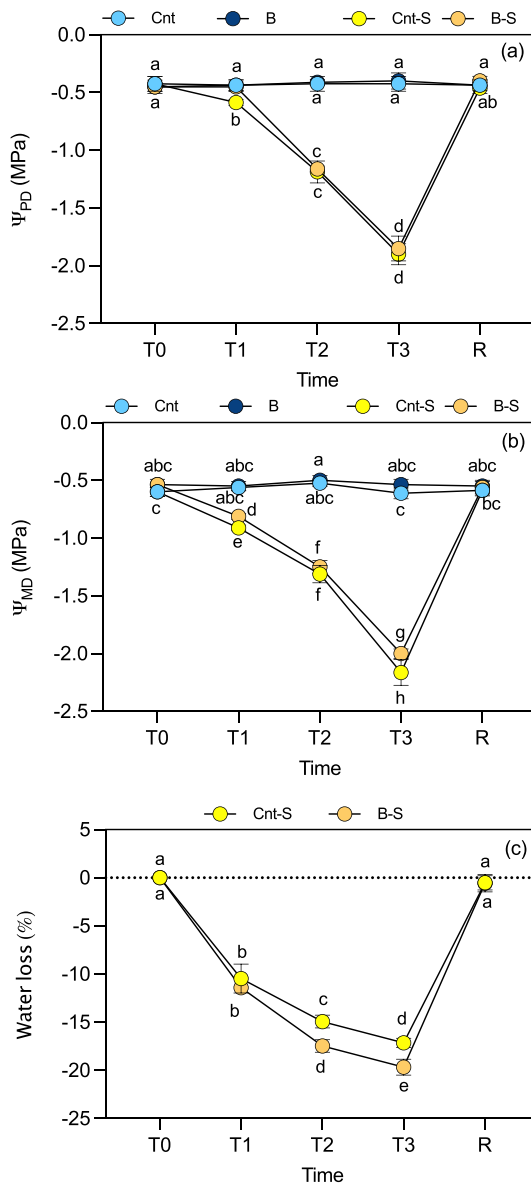
Fig. 6. Leaf predawn water potential (Ψ_{PD}; a), midday water potential (Ψ_{MD}; b) and soil water loss (c) in controls (Cnt), stressed controls (Cnt-S), 1.5 % biochar amended (B) and stressed 1.5 % biochar amended (B-S) trees (*Tilia × europaea*), measured before the water stress (T0), after 3 days (T1), 6 days (T2) 9 days (T3) of water stress and after 3 days of following re-watering (R). The Cnt leaves are represented by cyan circles and Cnt-S leaves by yellow circles, while B leaves by blue circles and B-S leaves by orange circles. Means (± SD; n = 4 for Ψ_{PD} and Ψ_{MD}; n = 3 for soil water loss) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. Means flanked by the same letter are not statistically different for p = 0.05 after Fisher's least significant difference post-hoc test.

potential indirect effect on trees (increase in CO_2 assimilation rates, resulting in higher biomass) as an overall strategy for CO_2 sequestration and storage. These indirect benefits provided by biochar applications suggest that the possible loss of C from biochar, due to decomposition, could be replaced by higher tree biomass over time. Furthermore, transporting this model to a city-scale, it is conceivable that biochar significantly contributes to sequestering large amounts of CO_2 from the environment thereby increasing the CO_2-uptake by urban trees. Moreover, if all the tree pruning and the wood from trees (at the end of the cycle) were used to produce biochar and the latter utilized for new tree plantings in the city, it would create a virtuous cycle of sustainability.

4.2. Experiment 2

4.2.1. Biochar improved the soil quality and provided better habitat for soil microbes and enzymatic activities during water stress

Many authors have reported that biochar applications increase the water holding capacity and the biological properties of the soil (Becagli et al., 2022; Khadem and Raiesi, 2019; Khadem and Raiesi, 2017; Scharenbroch et al., 2013). This study shows a positive effect of biochar applications on most soil quality parameters analyzed before the water stress, as enhanced APA and UA. In addition to the already listed effects of the biochar amendment on APA, the increase in the UA might be due to an intensification of N demand by microorganisms (Talbot and Treseder, 2012), possibly caused by the high C/N ratio of biochar that can lead to N immobilization in the soil (Bengtsson et al., 2003). However, the observed decrease in GA is in line with the results reported by Günal et al. (2018), suggesting that reduced GA was related to the modulatory activity of biochar on soil characteristics such as pH, soil moisture, carbon content or possible enzyme denaturation. In this case, the little availability of labile C fraction due to the high adsorbed ability of biochar could be the reason for the decrease of the GA. The AI3 is a valid marker of soil quality, since through the three microbially-secreted soil enzymes activities it is very sensitive to alteration in soil characteristics caused by agricultural practices or soil stressors (Puglisi et al., 2006). Generally, the more altered the soil, the higher the AI3 values are (Puglisi et al., 2006). Therefore, the lower AI3 values obtained in B than Cnt soil mean a better soil quality, confirming previous studies about the positive effects of the biochar amendment on soil fertility (Becagli et al., 2022; Becagli et al., 2021). The lack of C-MB increase could be due to environmental conditions (high temperatures of July), and/or to the high native TOC content of the soil without a consistent supply of labile C by biochar treatment (Kuzyakov et al., 2009; Zhang et al., 2014).

The water stress can also remarkably affect the plant-soil-microbe system, inhibiting most soil enzymes' activities by reducing soil water availability (Ali et al., 2021; Sardans and Peñuelas, 2005). The slowing of the enzymatic activities results in reduced nutrient mineralization which declines the nutrient supply to plants (Sardans and Peñuelas, 2005). In our study, biochar amendment played a pivotal role in preserving the biological soil activities during water stress. Indeed, the biochar application, in addition to inducing higher APA and UA levels than not amended soil, also reduced their decrease during the water deficiency resulting in slighter changes in AI3 values after the water stress. This was also confirmed by the minor reduction in MB-C and MB-C/TOC ratio values in B-S treatment than Cnt-S after the water stress. These results agree with other experiments that reported the ability of biochar to maintain soil quality under water stress (Ali et al., 2021; Ali et al., 2017) due to an enhancement of soil physico-chemical properties by biochar applications.

4.2.2. Influences of biochar soil amendment on the physiological traits of trees subjected to water stress

In an urban scenario, drought is the most affecting abiotic stressor that can severely impact the success of tree establishment, given that in some cases ~50 % of the newly planted trees can die due to water deficiency (Gilbertson and Bradshaw, 1985; Moore, 2012; Nowak, 1990). Water stress negatively affects plant growth by altering physiological (e.g., photosynthesis, plant hydraulic balance) and biochemical traits (e.g., reactive oxygen species contents and phytohormones) (Fang and Xiong, 2015; Tardieu et al., 2014). Many studies evidenced an alleviation of the water stress on plants thanks to the incorporation of biochar in the soil (Ali et al., 2017; Kim et al., 2021; Somerville et al., 2020). Nevertheless, there is a lack of knowledge about the effects of biochar amendment on leaf photosynthetic traits in tree species under water stress (Lyu et al., 2016; Zoghi et al., 2019). Generally, an early response of plants to water stress is to limit the stomatal conductance, which often results in an increase of diffusion resistance of CO_2, leading to the decline of P_n (Fang and Xiong, 2015). However, when drought becomes prolonged, non-stomatal factors become the major constraints to P_n, potentially damaging the
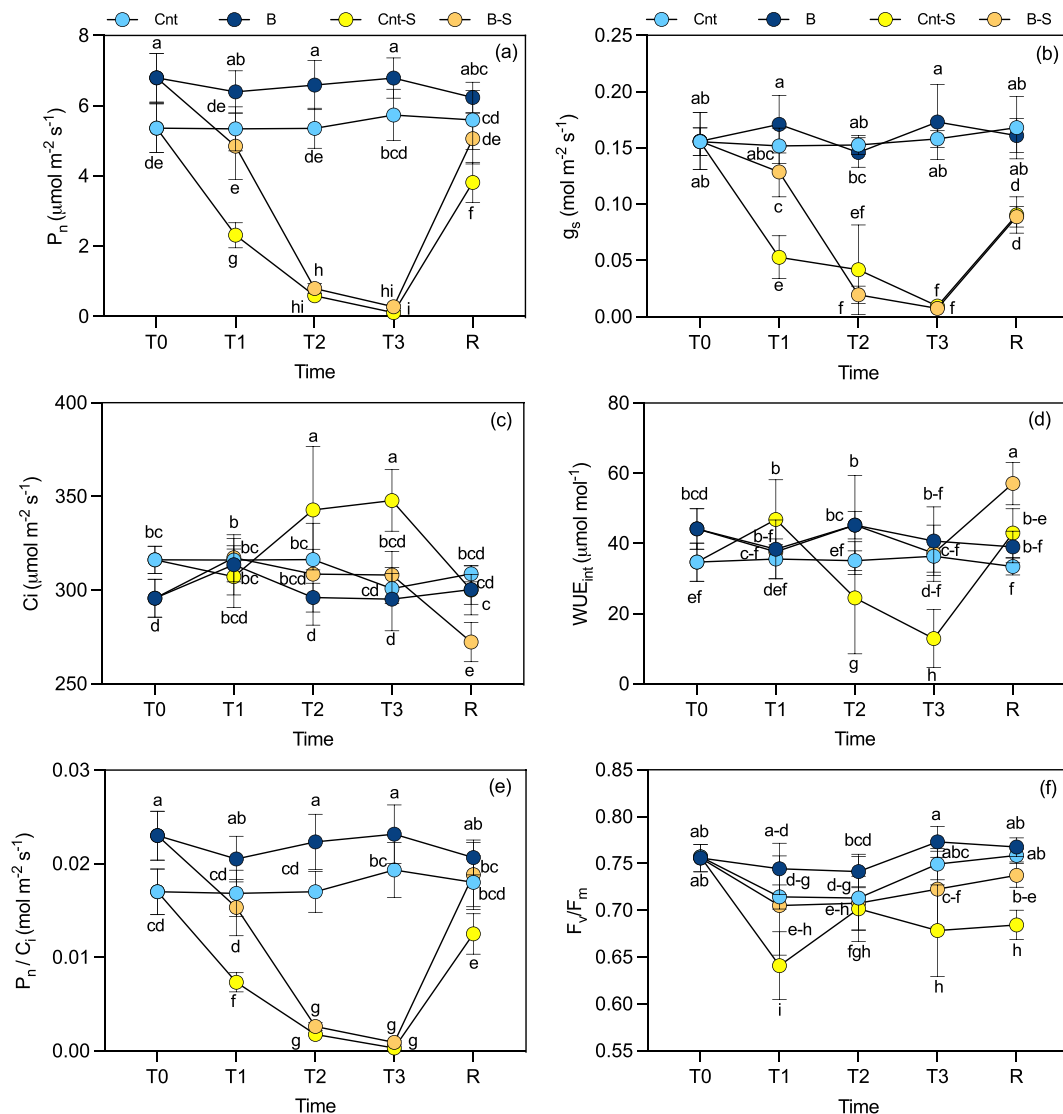
Fig. 7. Leaf gas exchanges and chlorophyll *a* fluorescence parameters determined at ambient light and CO_2: net photosynthesis (P_n; a), stomatal conductance (g_s; b), intercellular CO_2 concentration (C_i; c), intrinsic water user efficiency (WUE_{int}; d), apparent carboxylation efficiency (P_n/C_i; e and photosystem II maximum photochemical efficiency (F_v/F_m; f) in controls (Cnt; cyan circle), water-stressed controls (Cnt-S; yellow circle), 1.5 % biochar amended (B; blue circles) and water-stressed 1.5 % biochar amended (B-S; orange circles) trees (*Tilia × europaea*), measured before (T0), after 3 days (T1), six days (T2) nine days (T3) of water stress and after 3 days of re-watering (R). Means (± SD; $n = 6$) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. Means flanked by the same letter are not statistically different for $p = 0.05$ after Fisher's least significant difference post-hoc test.

photosynthetic apparatus, and consequently slowing the recovery after re-watering (Fang and Xiong, 2015; Flexas et al., 2004; Zhao et al., 2019).

In our study, at the early drought stage (T1), a lower stomatal conductance together with an alteration of the PSII photochemical efficiency limited the CO_2 assimilation rates in both -S treatments. However, these reductions were lower in B trees than Cnt trees thanks to the direct effects of the biochar amendment, such as higher initial K contents (B mature leaves) and a higher water availability for trees. Indeed, K has a key function related to the turgor pressure of plant cells since it is an important inorganic osmolyte (Wang and Wu, 2017). While the higher water availability in B soil was due to the biochar structure (high micropores) that increased the soil water holding capacity (as can be seen in Table 1; Student's *t*-test; $p < 0.05$), enhancing water availability for plants and microbials (Yoo et al., 2020). This capacity of B soils was indirectly highlighted also by higher values in Ψ_{PD} and Ψ_{MD} observed in B-S leaves with respect to Cnt-S ones. Nevertheless, it is worthy of note that Cnt trees tried to counteract the water stress, at the early stage, by transiently

enhancing the WUE, then decreasing the water consumption via evapo-transpiration (Zhao et al., 2019).

Generally, under water stress conditions, reactive oxygen species (ROS; e.g., O_2^-, $^1O_2$, H_2O_2, OH) levels in chloroplasts increase over physiological levels due to an imbalance between the generation and utilization of reducing power, i.e. NADPH (Reddy et al., 2004). Under prolonged stress conditions, if antioxidant systems do not effectively counteract this over-production, damages to the lipid membrane may further compromise the photosynthetic machinery (Lyu et al., 2016; Reddy et al., 2004). Therefore, it was not unexpected that MDA values increased in water-stressed trees (both Cnt and B individuals). However, our results are supportive of the fact that prolonged water stress conditions seriously damaged only the photosynthetic machinery in Cnt-S leaves. Indeed, at T2 and T3, only Cnt-S leaves showed increasing C_i values (with P_n/C_i values statistically different between Cnt-S and B-S for Student's *t*-test; $p < 0.05$); these conditions demonstrate that biochemical limitations (e.g., reduced maximum efficiency of PSII and Rubisco activity) become more limiting compared to stomatal ones
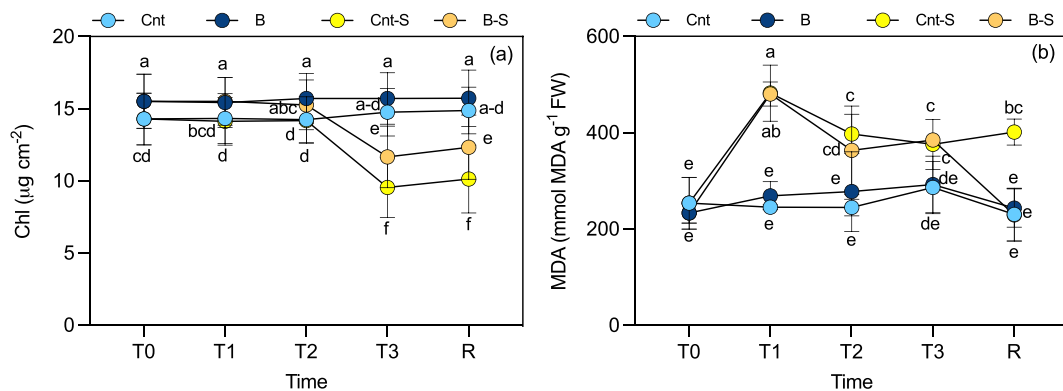
Fig. 8. Leaf chlorophyll content (Chl; a) and malondialdehyde content (MDA; b) in controls (Cnt; cyan circle), water-stressed controls (Cnt-S; yellow circle), 1.5 % biochar amended (B; blue circles) and water-stressed 1.5 % biochar amended (B-S; orange circles) trees (*Tilia × europaea*), measured before (T0), after 3 days (T1), 6 days (T2) 9 days (T3) of water stress and after 3 days of re-watering (R). Means (± SD; $n = 25$ for Chl; $n = 3$ for MDA) were subjected to two-way ANOVA with treatment and sampling time as the source of variations. Means flanked by the same letter are not statistically different for $p = 0.05$ after Fisher's least significant difference post-hoc test.

(Zahoor et al., 2017; Zhang et al., 2015). The better response of B-S trees at T2 and T3 could be related, once again, to the better water and initial nutritional status of B trees compared to Cnt ones. Indeed, it was reported that during water stress there is a considerable loss of K from the chloroplasts (Zahoor et al., 2017); then high K doses are necessary to maintain active the photosynthetic machinery (Zahoor et al., 2017). Therefore, we suggested that the higher starting K contents in mature leaves of B trees likely have enhanced the tree tolerance to prolonged water stress conditions. The higher ability to tolerate water stress of B-S trees is also evidenced by the complete recovery of P_n/C_i and F_v/F_m values after the re-watering (Fig. A.4) which are absent in Cnt-S trees.

5. Conclusion

The multidisciplinary approach adopted in the present work offers a clear picture of the effect of biochar soil amendment on tree physiology and soil quality. Indeed, the work provides the evidence that biochar applications significantly affect the soil properties (water holding capacity, enzyme activities and macronutrient content) by increasing the nutritional status and water balance of planted trees. Biochar amendment resulted in improved tree biomass at the end of the growing season, and better tree tolerance to water stress. These are critical aspects for a successful tree establishment in a challenging environment such as the urban one. Moreover, thanks to the stable C form of biochar and the positive effects on the tree biomass, its use in the urban environment improves the CO_2 sequestration from the atmosphere. However, biochar physico-chemical features, such as size, pH and C content, are strictly dependent to raw material and pyrolysis parameters. Therefore, though a potential application has been demonstrated herein, more research using different tree species, soil types and biochar is urgently needed to fully understand the potentiality of the biochar amendment in the urban environment, especially in a long-term period.

CRediT authorship contribution statement

Ermes Lo Piccolo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Michelangelo Becagli:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Giulia Lauria:** Investigation. **Valentina Cantini:** Investigation. **Costanza Ceccanti:** Investigation. **Roberto Cardelli:** Methodology, Writing – review & editing. **Rossano Massai:** Writing – review & editing. **Damiano Remorini:** Methodology, Writing – review & editing. **Lucia Guidi:** Data curation, Formal analysis, Methodology, Validation, Writing – review & editing. **Marco Landi:** Conceptualization, Methodology, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157175.

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