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Low-Dose Micronized Biochar Enhances Vegetative and Physiological Traits of *Vitis vinifera* and Improves Soil Quality in Organic Cultivation: A Two Year-Experiment in Tuscany

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Abstract

A two-year field experiment was conducted in an organic vineyard in Tuscany (Italy), to evaluate the effects of micronized biochar (0.5% *v*/*v*) applied via fertigation on soil fertility/biological quality and *Vitis vinifera* performance. The biochar, derived from pyrogasified mixed wood, was compared to watered controls (CTR) following a randomized plot design. Soil chemical properties, dehydrogenase (DHA) and alkaline phosphatase (APA) activities, and plant parameters (biomass, leaf area, gas exchange, chlorophyll, flavonols, and foliar nutrients) were assessed in samples collected in July and September (2021 and 2022). Biochar did not significantly alter total and dissolved organic carbon contents or nitrogen fractions but enhanced DHA and APA activities, alongside increased available phosphorous content (+37.5%) and exchangeable potassium content (+7.1 and +19.7% in September 2021 and July 2022, respectively), indicating improved microbial activity and nutrient availability. Conversely, exchangeable calcium and magnesium contents decreased, likely due to biochar adsorption properties. Plant responses included increased leaf area and dry biomass in 2022, elevated net photosynthesis rate (+14.4%) and apparent carboxylation efficiency, and transient increases in foliar nitrogen, phosphorous and potassium contents, with reduced magnesium concentration (–27%) but stable chlorophyll levels. These findings suggest that low doses of micronized biochar may enhance soil quality and vine physiology, supporting its efficient and effective use in organic vineyards.

Keywords: nutrient availability; photosynthetic performances; pyrogasification; soil enzymatic activity; soil fertility

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1. Introduction

Climatic changes are dramatically undermining soil fertility and vineyard productivity worldwide. Severe drought periods alternated by extreme rainfall (including drought–flood abrupt alternation) or disaster events combined with anthropogenic activities are inducing degradation or compaction of soils and loss of Soil Organic Matter (SOM) and nutrient content, thus reducing plant productivity [1]. For these reasons, the management of vineyard soils is essential to preserve biodiversity, soil fertility and productivity. In these terms, conventional agriculture in vineyard, utilizing repeated tillage/plowing operations, crop residue removal, and strict weed and pest control treatments, worsens this scenario, inducing soil nutrient imbalances, salinization and sodification, contamination, acidification, waterlogging, and impacted soil structure and reducing air circulation, SOM content, soil microbial biomass and microbial-driven functions [2,3].

Grape processing stands out as one of the most important and promising sectors that has recently emerged within the field of organic agriculture. In 2020, the global organic vineyard area accounted for 506,400 hectares, representing 7.3% of the world vineyard surface. The vast majority of this—around 430,000 ha, i.e., 85% of the total—is in Europe (as reviewed by Homet et al. [4]). The management of organic vineyards entails restrictions on input use and the adoption of specific cultivation practices. Borsato et al. [5] evaluated indicators such as water footprint, carbon footprint, and other aspects of organic vineyard management. Results showed that conventional vineyards had higher water and carbon footprints and an overall environmental impact 11% greater than that of organic vineyards, considering factors such as fertilizer use, erosion, SOM depletion, soil compaction, pesticide application, and landscape effects [5]. Even in organic viticulture, as in conventional systems, significant weaknesses in soil management persist, particularly regarding the long-term decline in soil fertility [6]. Weed control through mechanical means poses another challenge, as it requires more frequent tractor passes, resulting in greater inter-row compaction than in integrated or conventional systems. Soil compaction reduces the available water capacity due to a decrease in pore volume [6]. To maintain yield and quality over time while reducing environmental impact, it is essential to adopt innovative, low-impact cultivation techniques and integrated soil management strategies.

The use of biochar, which is allowed in organic agriculture, has already been tested in vineyards by several authors [2,3,7–9], reporting encouraging results. For instance, García-Jamarillo et al. [2] found that the use of biochar (both 18 and 35 tons ha^{-1}) in two vineyards in Oregon characterized by different soil characteristics modified the chemical and physical composition of soils at both the studied locations, increasing the bioavailability of Total Organic Carbon (TOC) and dissolved inorganic nitrogen, their gravimetric water content and the concentration of plant available nutrients when compared with both no-tillage and tillage trials as controls. Moreover, Giagnoni et al. [3] in a two-year experiment in a vineyard amended with 22 tons biochar ha^{-1} showed a significant increase in respiration, TOC, nitrate and total phosphorous content in soil within the first year of the experiment, despite the long wait to highlight an increase in soil enzymatic activities, which occurred exclusively after two years of the experiment.

Biochar amendment can also affect plant physiological performance. Indeed, a meta-analysis conducted by Gao et al. [10], by including 965 pairwise comparisons from 135 studies, found that generally biochar application enhanced leaf photosynthetic rate by 23% on average. Recent studies suggest that the enhanced plant physiological responses of biochar-amended plants can be due to multiple factors, including improved soil water and nutrient availability, leaf nutrient concentrations, improved stomatal conductance and higher leaf chlorophyll concentration [10–14]. Moreover, an experiment conducted in the vineyard found that long-term biochar effects on plant physiology (i.e., higher photosynthetic rates compared to untreated plants) were still observable 10 years after application [11]. However, responses are not universally advantageous; for instance, in some vineyard trials, plant responses of biochar-amended plants were small or not statistically different from controls [2,9]. This suggests that outcomes could be highly context-dependent (i.e., site conditions, biochar particles size, biochar chemical properties such as pH), highlighting the need for further investigation into the topic.

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Given the significant quantities usually indicated for its use (8–45 tons ha^{-1}, [2,3,7]) and, consequently, the high and prohibitive cost of this kind of amendment, the consumption of biochar on the market is within the reach of agricultural production sectors with very high qualitatively/quantitatively marketable production. Therefore, the present two-year experiment aimed to assess the effect of micronized biochar, a product applicable with precision fertigation, aiming at lowering the biochar amount in the vineyard but still testing its positive effect on soil fertility and plant growth. We hypothesized that (i) micronized biochar enhances soil fertility and (ii) these improvements lead to higher plant biomass, enhancing plant physiological processes.

2. Materials and Methods

2.1. Experimental Site and Plant Growth Conditions

The experiment was realized in a vineyard located in Civitella Val di Chiana, Tuscany region, Italy (43°24′19′′ N 11°46′14′′ E). Two-year-old 'Ciliegiolo' vines (*Vitis vinifera* L.) were planted on May 2021; the trellis system was a single curtain with plant–row spacing of 0.8 and 2.4 m.

Meteorological parameters were collected constantly by an automatic weather station installed close to the experimental field. Figure 1 reports monthly precipitation (mm) and average of maximum and minimum temperatures (°C) from April to September (in 2021 and 2022).

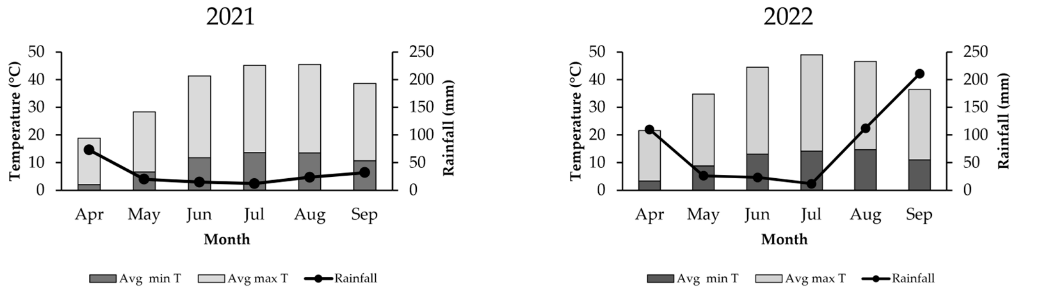

Figure 1. Weather conditions during the experimental period (from April to September during the years 2021 and 2022): average of minimum temperatures (Avg min T), average of maximum temperatures (Avg max T) and the rainfall.

2.2. Micronized Biochar and Experimental Trial

The micronized biochar utilized in the present experiment was a commercial product provided by BioDea (Civitella in Val di Chiana, Arezzo, Italy). The product was obtained by pyrogasification with partial combustion at 1200 °C of mixed woodchip forest residues (*Abies* sp., *Alnus* sp., *Castanea sativa* Mill., *Fraxinus* sp., *Quercus* sp., *Robinia pseudoacacia* L.). The combusted material was collected from the bottom of the reactor, micronized and solubilized in water, producing a liquid solution. Thanks to its aqueous characteristic and the microscopic (<Ø 40 µm) dimension of its particles, the micronized biochar can be utilized in fertigation. Indeed, the resulting micronized biochar was suspended into water at a dose of 300 mL L^{-1} in the stock solution, with a pH of 6.8 and Electric Conductivity of 1.10 mS cm^{-1}, TOC 65%, total N < 0.5%, total P 0.034%. This material was characterized by a high specific surface area (ranging from tens to several hundreds of m^2 g^{-1}) and a well-developed hierarchical pore structure, consisting of both micro- (<2 Ø nm) and mesopores (Ø 2–50 nm). Its surface was also enriched with oxygen-containing functional groups (e.g., carboxylic, phenolic, and carbonyl groups), contributing to its chemical reactivity and overall ability to interact with soil nutrients and organic matter.

A randomized plot experiment was established in 2021 and 2022 with two experimental conditions: (i) an untreated control group fertigated with water only (5 L plant^{-1}; CTR) and (ii) a treated condition consisting of fertigation with micronized biochar (0.5% *v*/*v*; 5 L plant^{-1} as treatment; MB). The fertigation was carried out every 15 days from April to September in both experimental years. A total of 10 fertigation events per year, for two years, were performed. Soil and plant sampling for each condition was performed on a row consisting of 150 young grapevine cuttings. Soil samples were obtained every 30 young grapevine cuttings, for a total of 5 soil samples for each condition.

2.3. Analysis of Soil Chemical Properties

Soil was sampled in July and in September in both 2021 and 2022. Soil samples were collected at a depth between 10 and 15 cm, refrigerated, air-dried, crushed and sieved with a specific Ø 2 mm sieve. Soil was characterized prior to the start of the experiment (Table 1). Physical and chemical properties of the soil, such as pH, texture, Cation Exchange Capacity (CEC), were determined by standard methods (Colombo and Miano, [15]). Water holding capacity was calculated by wetting the soil until saturation. For the total $CaCO_3$ content, a gas-volumetric analysis with a Scheibler calcimeter was performed.

Table 1. Soil characteristics of experimental vineyard. Abbreviations: F, infiltration rate; CEC, Cation Exchange Capacity; TOC, Total Organic Carbon; N_{tot}, nitrogen total content.

Soil Characteristic	Unit	Value
Sand	%	69.0
Silt	%	25.0
Clay	%	6.0
pH		6.1
F	%	35.0
CEC	meq 100 g^{-1}	38.0
$CaCO_3$	%	<1.0
TOC	%	1.13
N_{tot}	g kg^{-1}	0.98
C/N ratio		11.5
Available P	mg kg^{-1}	13.0
Exchangeable K	mg kg^{-1}	113.0
Exchangeable Mg	mg kg^{-1}	480.0
Exchangeable Ca	mg kg^{-1}	2111.0

For the other analyses, the same procedure was adopted for both soil characterization and leaf samples. Soil chemical parameters such as pH, TOC, dissolved organic carbon (DOC), total nitrogen (N_{tot}), nitrates (NO_3^--N), available phosphorous (available P) and exchangeable K, Ca and Mg (K_{ex}, Ca_{ex}, Mg_{ex}) contents, as well as enzymatic activities (Dehydrogenase; DHA and alkaline phosphatase; APA—detailed below) were performed in MB and CTR samples. Three or five replicates were performed for each assay.

N_{tot} and total C analyses were carried out using an Elementar Vario Micro Elemental Analyzer (Elementar Italia Srl, Lomazzo, Como, Italy). About 200 mg of leaf or 10 mg of soil powdered dry samples was analyzed. TOC was assayed by subtracting the $CaCO_3$ content from C content.

DOC was measured by agitating soil samples in distilled water (soil-to-water ratio of 1:20, *v*/*v*) for 24 h at ambient temperature. The resulting suspension was centrifuged at 10,000× *g* for 10 min, filtered through a 0.45 µm glass fiber membrane, and analyzed for carbon content using a liquid-sample OC analyzer (Hach QbD1200). Nitrate (NO_3^--N) concentrations were determined using a Dionex DX120 ion exchange chromatograph with IonPac AG4A and AS4A columns. The eluent (1.8 mM Na_2CO_3, 1.7 mM $NaHCO_3$) flowed at 2 mL min^{-1}, with a 50 mN H_2SO_4 regenerant (3–5 mL min^{-1}) and a conductivity detector (15–20 µS background). Soil (0.2 g) was extracted with 25 mL Milli-Q water at 60 °C for

1 h, using a 5 ppm $NaNO_3$ standard for calibration. Available P was determined spectrophotometrically (Perking Elmer Lambda 25, Italy, Mila) by the modified molybdenum blue method on acid extracts [15]. K^+, Ca^{2+} and Mg^{2+} content was quantified by extracting cations with ammonium acetate solution 1:50 and atomic absorption reading with Thermo Scientific ICE 3000 series (Thermo Scientific, Waltham, MA, USA).

2.4. Soil Enzymatic Activities

Dehydrogenase (n = 3) activity followed the method of Tabatabai [16], based on the spectrophotometric measurement at 488 nm of 2,3,5-triphenyformazan. Therefore, the metabolic potential index (MPI) was calculated as a ratio between DOC and DHA activity [17].

Alkaline phosphatase (n = 5) activity was assessed following the method described by Eivazi and Tabatabai [18] based on the hydrolysis of *p*-nitrophenyl phosphate by soil enzymes and spectrophotometric measurement at 410 nm.

2.5. Plant Biomass and Biometric Parameters

Five plants from each condition (CTR and MB) were collected from the field trial, transported in the laboratory and samples for the biometric measurements. Dried plant biomass (n = 5) was obtained by weighing the leaf, branches and trunk biomasses, separately. Moreover, for each plant, the total number of leaves (n = 5) was annotated, and the leaf area (LA; n = 5) was measured using a ruler, measuring the length of the midrib and the width of the leaf blade and utilizing the following formula [19]:

$$LA = -0.465 + 0.914\ (\text{Length} \times \text{Width}) \tag{1}$$

Then, shoots and leaves, separately, were oven-dried (Memmert GmbH Co. KG Universal Oven UN30, Schwabach, Germany) at 105 °C until reaching a constant weight and the shoot and leaf dry weight was annotated. Specific leaf area (SLA; n = 5) was calculated as the ratio between LA and leaf dry weight.

2.6. Leaf Gas Exchange Parameters

Leaf gas exchange measurements were performed using a portable infrared gas analyzer (LI-6400XT System, Li-Cor, Lincoln, NE, USA). Measurements (n = 7) were taken on fully expanded leaves between 10:00 and 12:00 a.m. under ambient light conditions with a photosynthetic photon flux density of 1700 µmol m^{-2} s^{-1}. Within the leaf chamber, CO_2 concentration was maintained at 400 µmol mol^{-1} using a CO_2 mixer, and the flow rate was set to 500 µmol s^{-1}. Once steady-state conditions were achieved, net photosynthetic rate (P_n), stomatal conductance to water vapor (g_s), intercellular CO_2 concentration (C_i) and apparent carboxylation efficiency (P_n/C_i) were recorded.

2.7. Leaf Chlorophyll (Chl) and Flavonol (Flav) Content

The DUALEX® Scientific analyser (Force-A, Orsay, France) was used to analyze the chlorophyll content and flavonol index through non-destructive measurements (n = 70). The measurements were carried out on the adaxial leaf side.

2.8. Concentration of N, P, K, Ca and Mg in Leaves

Total nitrogen content was determined with the same method utilized for soil samples. P in leaves was measured spectrophotometrically by an Ultrospec 2100 Pro spectrophotometer (GE Healthcare Ltd., Little Chalfont, UK) following the molybdenum blue method according to Benini et al. [20]. For leaf cation content, about 0.2 g of dry powdered leaf samples was 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 to a final volume of 25 mL, reached by the addition of double-distilled water. After mineralization, cation content was measured using the atomic absorption spectrometer.

2.9. Statistical Analysis

The obtained data were elaborated and checked for normality of distribution (Shapiro–Wilk test, 95% confidence interval) and for homoscedasticity (Bartlett's test). Data are expressed as mean ± standard deviation. Results were compared with a two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. Data not normally distributed (i.e., g_s in July 2021, C_i in September 2021, P_n in July 2022 and C_i in July 2022) were subjected to Welch's *t*-test prior to further analysis. Chl and Flav data were analyzed using the non-parametric Mann–Whitney test. The statistical analyses were conducted using GraphPad 9.0 (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Soil Analysis

Figure 2 shows the TOC and DOC of soil sampled during 2021 and 2022 (in July and September) in the vineyard irrigated with water (CTR) or with micronized biochar (MB). No significant differences were found between CTR and MB in both TOC and DOC determinations in either year, nor in either season.

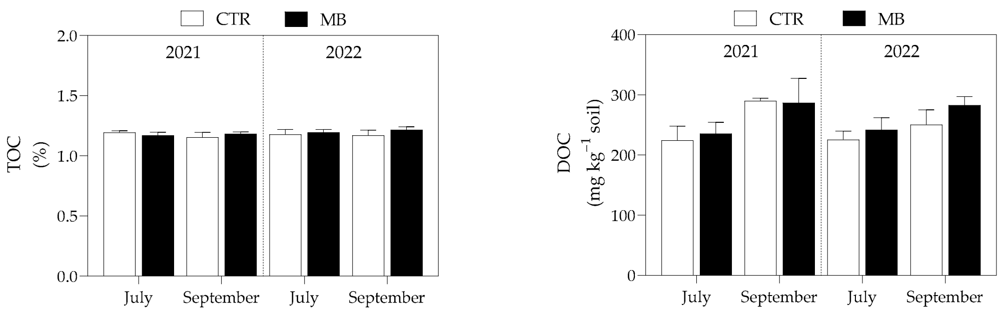

Figure 2. Total Organic Carbon (TOC, on the (**left**)) and dissolved organic carbon (DOC, on the (**right**)) content in soil irrigated with only water (CTR) or with micronized biochar (MB) and sampled in July and September in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The lack of asterisks indicates no significant differences following the cited statistical analysis.

In contrast, both DHA activity and MPI exclusively revealed a significant difference between CTR and MB in September of the second experimental year (+26.9% and +17.1%, respectively), whilst no significant differences were reported during July in both experimental years for both DHA activity and MPI and in September 2021 for DHA activity (Figure 3).

Similarly to DOC and TOC contents, total N and nitrates content did not report significant differences between CTR and MB soils in both July and September of both experimental years (Figure 4).

Differently, available P content was increased by the MB treatment in both seasons and in both experimental years, showing the highest values in September 2021 (26.0 mg kg^{-1} soil) and in July 2022 (26.3 mg kg^{-1} soil; Figure 5). Moreover, APA activity was increased by MB treatments in soils sampled in September 2021 (+49.9%) and in both July (+56.8%) and September in 2022 (+54.8%; Figure 5).

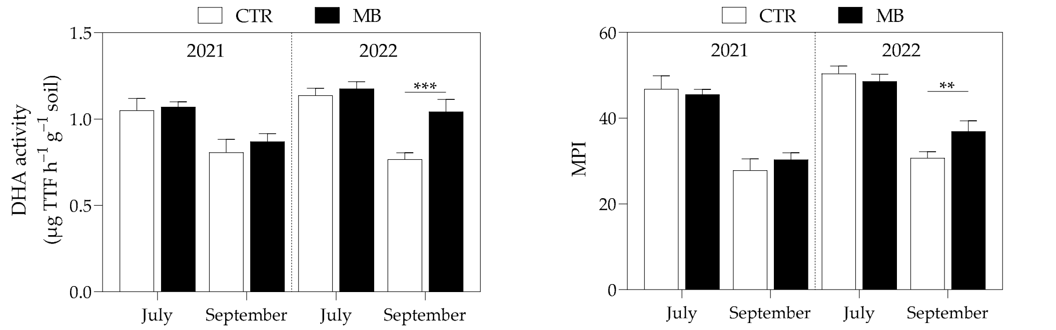

Figure 3. Dehydrogenase activity (DHA activity; on the (**left**)) and metabolic potential index (MPI; on the (**right**)) in soil irrigated with only water (CTR) or with micronized biochar (MB) and sampled in July and September in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The presence of asterisks indicates significant differences among means, whilst the lack of asterisks indicates no significant differences among means. **: $p \leq 0.01$; ***: $p \leq 0.001$.

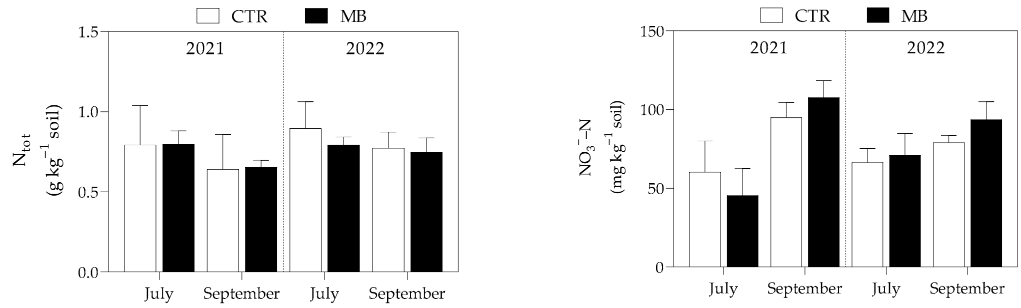

Figure 4. Total nitrogen (N_{tot}; on the (**left**)) and nitrates (NO_3^-–N; on the (**right**)) contents in soil irrigated with only water (CTR) or with micronized biochar (MB) and sampled in July and September in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The lack of asterisks indicates no significant differences among means following the cited statistical analysis.

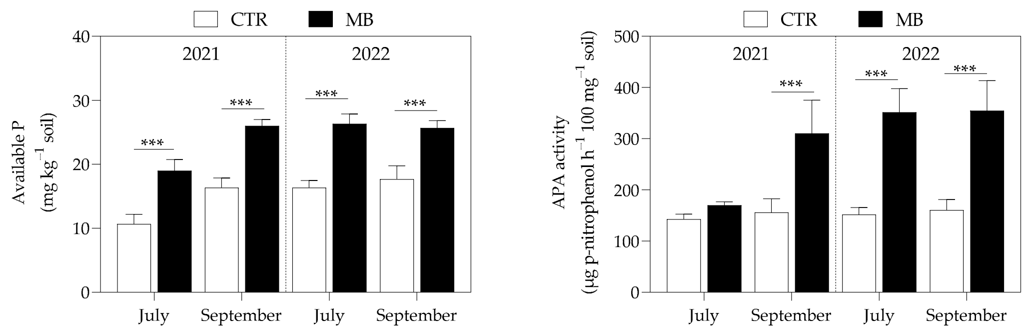

Figure 5. Available phosphorus (on the (**left**)) content and alkaline phosphomonoesterase (APA activity, on the (**right**)) in soil irrigated with only water (open bars) or with micronized biochar (closed bars) and sampled in July and September in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The presence of asterisks indicates significant differences among means, whilst the lack of asterisks indicates no significant differences among means. ***: $p \leq 0.001$.

Lastly, K_{ex} content was increased by the MB treatment in September 2021 (+7.1%) and in July 2022 (+19.7%). Ca_{ex} content was slightly reduced by MB treatment during July 2022 and Mg_{ex} was reduced by MB treatment during both seasons in both experimental years with an average decrement of 18.4% (Figure 6).

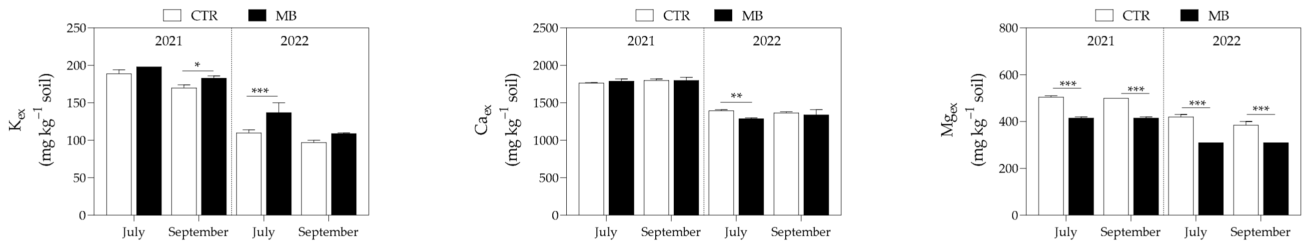

Figure 6. Exchangeable potassium (K_{ex}, on the (**left**)), calcium (Ca_{ex}, in the (**middle**)) and magnesium (Mg_{ex}, on the (**right**)) contents in soil irrigated with only water (CTR) or with micronized biochar (MB) and sampled in July and September in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The presence of asterisks indicates significant differences among means, whilst the lack of asterisks indicates no significant differences among means. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

3.2. Plant Analyses

Table 2 shows leaf, branch, trunk and total dry biomasses of vine plants cultivated in soils irrigated with only water (CTR) or treated with micronized biochar (MB) and totally sampled in 2021 and 2022. No significant differences were found in dry branches and trunk biomasses in both experimental years, whilst leaf dry biomass increased exclusively in 2022 in MB vines (+43.9%), inducing a significant increase in total dry biomass in the same year (+23.9%; Table 2). Leaf length was increased in leaves of MB-treated plants in both experimental years, whilst the leaf width was higher in MB-treated plants when compared to leaves of control plants exclusively during 2022. Furthermore, although no significant differences were shown in leaf number in both experimental years between CTR and MB vines, an increase in LA was observed in MB respect to CTR vine plants in both years (+21.8% and +31.0%, respectively; Table 2). Moreover, an increase in SLA was observed in MB-treated plants when compared with controls exclusively during 2021.

Table 2. Leaf, branch, trunk and total dry biomass, leaf area, leaf width, leaf length, leaf number and specific leaf area of vines cultivated in soils irrigated with only water (CTR) or with micronized biochar (MB). Vine plants were sampled in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The presence of asterisks following means indicates significant differences among means, whilst the lack of asterisks indicates no significant differences among means. *: $p \leq 0.05$; **: $p \leq 0.01$. DW: dry weight; LA: leaf area; SLA: specific leaf area.

	2021		**2022**	
	CTR	**MB**	**CTR**	**MB**
Leaf biomass (g DW plant^{-1})	20.1 ± 5.2	17.0 ± 2.0	20.5 ± 5.3	36.6 ± 5.7 *
Branches biomass (g DW plant^{-1})	21.0 ± 2.7	25.0 ± 5.5	24.8 ± 3.1	30.1 ± 6.6
Trunk biomass (g DW plant^{-1})	40.6 ± 5.9	46.5 ± 9.9	46.7 ± 6.8	54.4 ± 11.6
Total biomass (g DW plant^{-1})	81.7 ± 12.5	88.5 ± 15.6	91.9 ± 11.1	121.1 ± 20.7 *
Leaf number (n° plant^{-1})	88.4 ± 16.2	76.8 ± 19.2	116.0 ± 12.4	124.6 ± 9.9
Leaf length (cm)	7.41 ± 0.55	8.35 ± 0.58 *	7.54 ± 0.56	8.95 ± 0.20 **
Leaf width (cm)	5.65 ± 0.47	6.35 ± 0.50	5.60 ± 0.54	6.94 ± 0.34 **
LA (cm^2)	40.1 ± 6.3	51.3 ± 7.1 *	40.4 ± 0.5	58.6 ± 0.3 *
SLA (cm^2 g DW)	2.10 ± 0.57	3.04 ± 0.50 *	2.05 ± 0.53	1.62 ± 0.20

Figure 7 represents the results of gas exchange in vine plants cultivated in soils irrigated with only water (CTR) or amended with micronized biochar (MB). For this analysis, samplings were collected in September and July in the years 2021 and 2022.

P_n was higher in MB vines with respect to CTR ones in both sampling months in both years with an average increase of 14.4%, averaging the increase percentages during the comparison between MB-treated vines and CTR vines for each season and each year of the experiment, separately (Figure 7a). However, g_s increased in MB-treated vines exclusively in July 2021 (+14.3%; Figure 7b). C_i was reduced by B treatment in September 2021 (–5.0%) and July 2022 (–10.3%; Figure 7c). Finally, similar to P_n, the P_n/C_i ratio resulted increased by MB treatment in both sampling months in both experimental years with an average increase of 20.6% (Figure 7d).

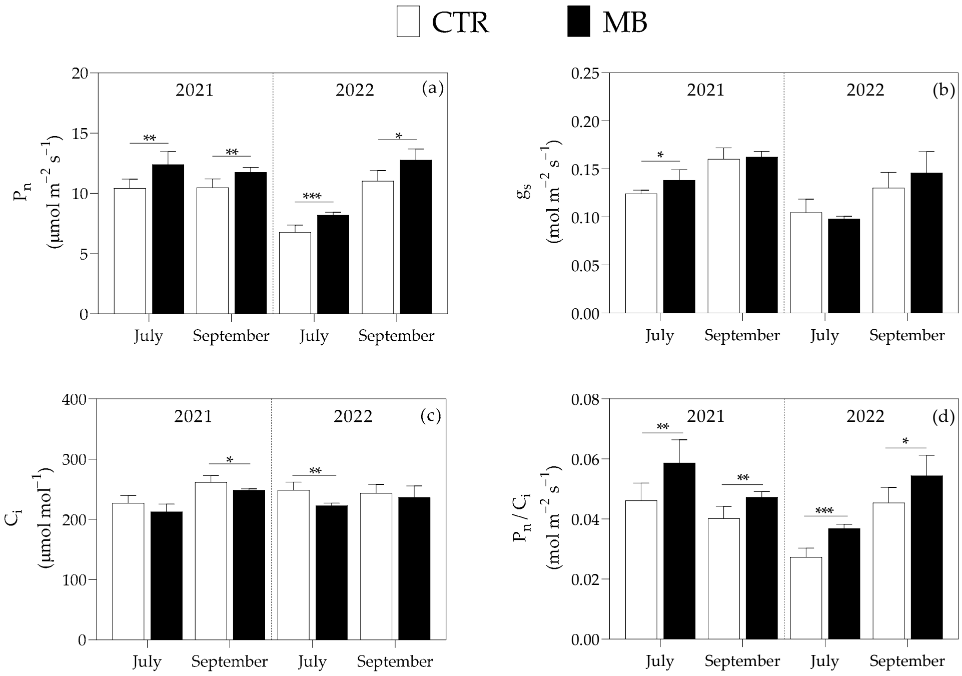

Figure 7. Gas exchanges parameters analyzed in vines cultivated in soils irrigated with only water (CTR) or with micronized biochar (MB). Vine plants were sampled in July and September in the years 2021 and 2022. Gas exchanges were determined in ambient light and CO_2 conditions: net photosynthesis (P_n; (**a**)), stomatal conductance (g_s; (**b**)), intercellular CO_2 concentration (C_i; (**c**)) and apparent carboxylation efficiency (P_n/C_i; (**d**)). Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The presence of asterisks indicates significant differences among means at $p \leq 0.05$, whilst the lack of asterisks indicates no significant differences among means. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.

Figure 8 shows Chl and Flav content determined using a Dualex® instrument. A significant decrease in Chl content was noted in MB-treated leaf vines during July 2021, whilst no significant differences were noted in the other experimental months in both 2021 and 2022. Flav content was lower in MB-treated leaf vines when compared to CTR plants during 2021 in both experimental months (July and September). No significant differences were found in Flav content during 2022 (Figure 8).

Finally, the leaf N content increased in MB vines in September 2021 (+5.2%) and July 2022 (+4.2%; Figure 9a), whilst no significant differences were found in leaf N content during July 2021 and September 2022. Leaf P increased exclusively in July 2022 (+19.0%; Figure 9b). In contrast, K content increased in leaves of MB vines at all the sampling dates with an average increase of 23.8% in 2021 and of 31.4% in 2022 (Figure 9c). Ca content decreased in leaves of MB-treated vines exclusively in July 2022 (Figure 9d), whilst Mg content decreased in leaves of B vines at all the sampling dates with an average decrease of 29.0% and the decrease of 45.2% during September 2021 (Figure 9e). In general, the highest values of leaf P, K, Ca, and Mg contents were found during the second experimental year (2022).

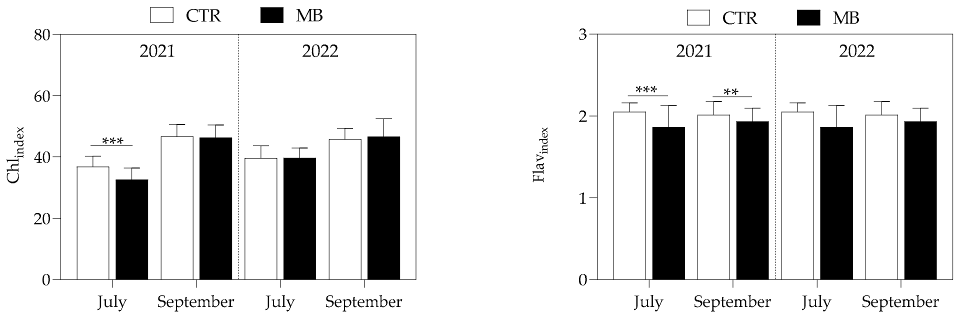

Figure 8. Chlorophyll index (Chl_{index}, on the (**left**)) and flavonoid index ($Flav_{index}$, on the (**right**)) content in leaf vines cultivated in soil irrigated with only water (CTR) or with micronized biochar (MB) and sampled in July and September in the years 2021 and 2022. Means were subjected to the non-parametric Mann–Whitney test. The lack of asterisks indicates no significant differences among means. **: $p \leq 0.01$; ***: $p \leq 0.001$.

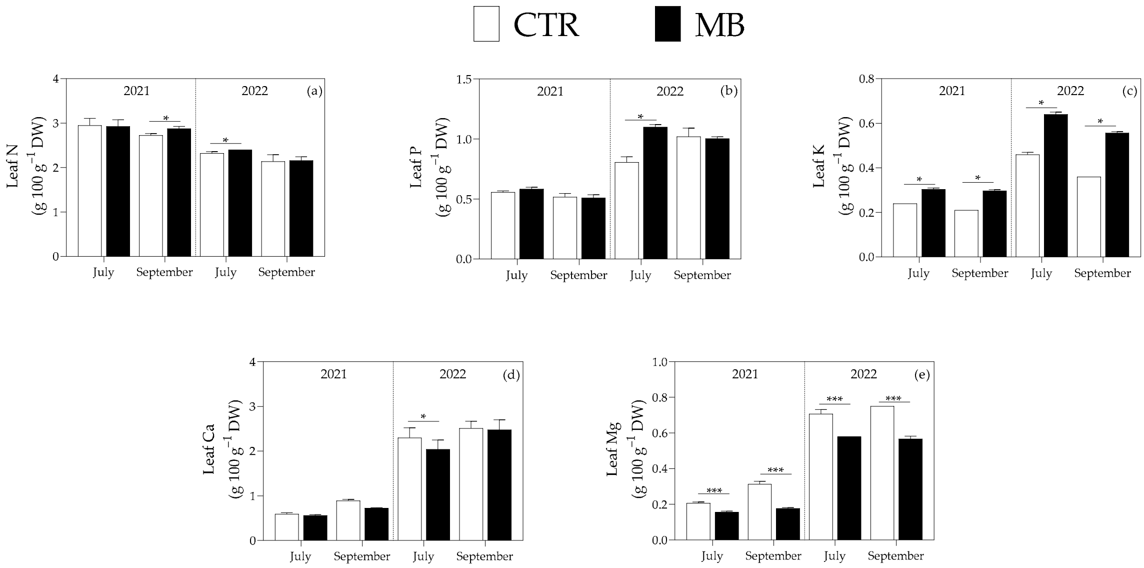

Figure 9. Total nitrogen (N, (**a**)), phosphorous (P, (**b**)), potassium (K, (**c**)), calcium (Ca, (**d**)) and magnesium (Mg, (**e**)) content of leaves of vine plants cultivated in soils irrigated with only water (CTR) or with micronized biochar (MB). Vine plants were sampled in July and September in the years 2021 and 2022. Means were subjected to two-tailed Student's *t*-test using a significance level of 0.05, considering the treatment with the micronized biochar as variability factor. The presence of asterisks indicates significant differences among means, whilst the lack of asterisks indicates no significant differences among means. *: $p \leq 0.05$; ***: $p \leq 0.001$.

4. Discussion

This study thoroughly assessed the effects of MB (0.5% v/v) application on the soil–plant system in a Tuscan vineyard in Italy in a 2-year experiment with 10 fertigation events with MB per year. Although no differences were noted in terms of SOM and N (total and nitrate contents), the soil quality was positively influenced by MB as revealed by enhanced enzymatic activity, and the plant performances were improved in terms of net photosynthesis rate and leaf biomass. The latter increased exclusively during 2022. Interestingly, the application of MB reduced the Mg availability in the soil with a consequent decrease in the Mg uptake by plants that, however, did not influence the Chl content. Below, the mechanistic explanation for these ameliorative aspects of MB application in the soil–plant system is depicted.

4.1. Soil P and K Availability and Soil Enzymatic Activity Were Enhanced by the Application of Micronized Biochar

The results of the present experiment on TOC and DOC in a vineyard treated with MB at 0.5% v/v (at doses comparable to 0.9 t ha^{-1}) over two years showed no significant changes compared to CTR, contrasting with previous studies. Research by Idbella et al. [8] and Giagnoni et al. [3] reported significant increases in TOC in vineyards amended with solid biochar at higher doses (18–35 t ha^{-1}) over longer periods (up to 10 years), due to biochar ability to stabilize soil OC and protect plant-derived inputs. Moreover, García-Jaramillo et al. [2] observed elevated TOC within six months of biochar application (34 t ha^{-1}), while Hagemann et al. [21] noted that biochar aging forms a nutrient-rich organic cover, enhancing OC stabilization. The short experimental duration in the current study likely limited OC mineralization changes, as biochar slow mineralization rate (10–100 times slower than uncharred biomass) supports long-term carbon sequestration [22]. Similarly, no significant DOC increase was observed, unlike findings by Becagli et al. [23], who reported a 50% DOC increase in biochar-amended soils, and Schulz and Glaser [24], who demonstrated biochar's capacity to adsorb and stabilize labile OC. Hailengnaw et al. [25] and Koga et al. [26] highlighted that biochar's impact on DOC depends on pyrolysis temperature, feedstock type, and soil properties (e.g., texture, pH, CEC), with higher biochar doses and specific soil conditions enhancing DOC retention. The minimal biochar dose and micronized form used here likely explain the lack of observed effects, suggesting that longer-term studies with higher doses could better elucidate biochar's role in OC dynamics when applied via fertigation.

Despite no significant differences in DOC and TOC content, enzymatic activities (i.e., DHA and APA activities) were positively affected by MB fertigation, especially in the second year of experiment. After a meta-analysis, Pokharel et al. [27] noted that DHA activity is usually enhanced by biochar applications. It has been reported that activity was increased by biochars produced in similar conditions to those applied for obtaining our micronized biochar (low pyrolysis temperature; <350 °C C/N ratio < 50) [27]. The DHA/DOC ratio defines the MPI as a soil quality indicator, reflecting the vitality of the microbial biomass through its enzymatic activity on organic compounds [17]. Higher MPI values, as found in the present experiment during the second year in MB-treated soil, indicate greater microbial activity and, consequently, enhanced microbiological fertility. The DHA and MPI enhancement in September 2022 can be linked to the increase in rainfall during this month in the second experimental year. This led to a higher retention of water induced by the presence of MB fertigation and higher diffusion of microbial substrates thanks to the presence of rainwater in the soil porosity, inducing an increment in microbial community.

The responses of APA activity, which facilitates the breakdown of P-containing organic compounds, to biochar application exhibited significant variability in the literature [28]. This variability in enzyme activity responses is linked to differences in soil characteristics and biochar properties, as already seen for DHA activity. In the present experiment, APA activity was enhanced in September 2021 and during 2022 (in both seasons), and this enhancement can justify the continuous P availability during both experimental years. Indeed, MB treatment promoted the growth of microbial population responsible for the APA catalysis, leading to the release of $H_2PO_4^-$ e HPO_4^- from SOM. Results are consistent with the scientific literature; Idbella et al. [8] registered a significant increase in available P with growing MB doses, while Becagli et al. [23] underlined that APA enzyme is specific for the substrate and attributes the increase in available P to several factors such as the promotion of a stimulant environment for specific microbial populations and/or biochar retention of mobile P forms. Carril et al. [29] demonstrated that the association of biochar with liquid vermicompost (i.e., highly active microbiological material) enhanced APA activity and P availability, probably due to a synergic effect of the interaction between vermicompost and biochar which improved environment for microbial community, confirming the positive response of APA activity to biochar application in soil.

In the present experiment, the nitrification process was not affected by MB application to the soil, with no significant variations in N_{tot} and NO_3^--N content during both experimental years. These results are in line with the results of other experiments which utilized different doses of biochar [23,30,31]. For instance, Sorrenti et al. [30] reported no effect on N fractions after three years of investigation, while Liu et al. [31] studied the effect of biochar application on nitrification/ammonification, reporting that biochar can contain a little amount of inorganic N, while the organic N fraction is recalcitrant to availability; thus biochar cannot be considered an efficient N source, explaining the results of the present experiment. Becagli et al. [23] reported a NO_3^--N increase with MB treatment, probably due to microbial community associated with nitrification, but also lower nutrient leaching. The lower NO_3^--N leaching through the MB use was also confirmed in paddy fields by Chen et al. [32].

K_{ex} showed a significant increase during the experiment in MB-treated soil, confirming the positive effect on soil chemical fertility. Lo Piccolo et al. [13] reported the same behavior in a study conducted on *Tilia × europaea* L. treated with 1.5% (*w*/*w*) biochar, observing that solid biochar brings beneficial changes in bacterial community, accelerating the conversion of slowly available K in K_{ex}. Xiu et al. [33], in an experiment with growing rates of biochar (0, 10, 20, and 30 $g \cdot kg^{-1}$ soil), reported an increase in available, water-soluble, exchangeable and non-exchangeable K, demonstrating not only that biochar is effective in increasing this nutrient but also that this effect is dose-dependent. All together, these findings confirm that MB treatment can be an effective solution to increase K availability in low-fertility soils.

In parallel with the increase in available P and K_{ex}, we noticed that the contents of Ca and Mg were reduced in MB-treated soils. Levels of Ca were lower in July 2022, whilst Mg content declined in both the experimental years and seasons. The decrease in Ca is in contrast with the previous literature, since some authors hypothesized that the Ca bridging, facilitated by Ca ions derived from biochar, can facilitate the formation of the biochar–SOM–soil particle complex during aggregate formation [34]. This Ca-based bridge is of the upmost importance for SOM stabilization (as reviewed by Lützow et al. [35]).

The decline in Mg content in the soil was noted during all sampling dates. This trend was also reported by Sadowska et al. [36] who found the lowest Mg content in the soil treated with a biochar dose of 45 t ha^{-1}. These authors hypothesized that the adsorption/desorption Mg^{2+} processes were probably responsible for the observed concentrations of cation in the soil, reducing the soluble Mg [36]. Likely, biochar alkaline nature or its interaction with soil pH and other nutrients (e.g., Ca or K) could alter Mg solubility or uptake competitiveness, leading to a temporary decrease in Mg in plant tissues as described in the next paragraph.

4.2. Leaf Biomass and Net Photosynthetic Process Were Improved by Continuous B Application in Both Experimental Years

In the present experiment, total dry biomass was enhanced by MB treatment in the second year of the experiment. Similarly, Lo Piccolo et al. [13] recorded that the total biomass of biochar treated plants was 22% greater than that of controls. The authors correlated this biomass growth to the improved physiological status observed in treated trees, promoted by greater macronutrient availability relative to controls, as observed during the present experiment. Despite this trend, in our case study, analyses of shoot biomass show no statistically significant differences between CTR and MB and, thus, the increment in total biomass was attributable to the marked difference in leaf biomass. Indeed, analyses performed on leaf sampling showed highly positive results for MB-treated plants in 2022. The leaves were not only larger in size but also thicker than those of CTRs, despite the total number of leaves per plant being similar for both conditions. In fact, the LA in MB-treated plants was greater than that in untreated plants, justifying the values obtained in 2022 for biomass of MB leaves. This latter result can be attributed to the several rainfall events present during the second experimental year. Moreover, Liao et al. [37] demonstrated that biochar (specifically from lignocellulosic biomass) increased the LA in *Rosa tomentosa* Sm. and *Cattleya edithiae* Lindl., while Guo et al. [38] further confirmed the same pattern in *Schefflera arboricola* (Hayata) Merr. Indeed, the addition of lignocellulosic biochar to the soil provided benefits to the physiological processes of leaf development in grapevine cuttings, acting on multiple key factors. These included (i) the water retention capacity by biochar [37–40] and (ii) the capability of biochar to result in plant hormonal changes, as recent studies indicate that biochar stimulates growth pathways associated with change in auxins, key hormones regulating cell elongation and leaf development [41]. Moreover, the LA data were also supported by the increase in SLA during 2021. Indeed, the increment in this parameter highlights the higher investment of plants in leaf expansions with the consequence of the major capability to light interception. Despite these interesting results in terms of plant biomass, in the present experiment, the vines were only 2–3 years old (still young), so representative fruit quality data could not be obtained. Therefore, data on plant yield may be obtained in further and longer experiments.

The P_n values obtained in the present study were higher in MB-treated plants compared to CTRs, with a statistically significant variance between the two treatments in favor of MB in both years. During the first year of the present experiment, these data were supported by the increment in SLA values. According to Lo Piccolo et al. [13], the higher P_n value in MB justified the superior results in P_n/C_i ratio. Similarly, Tanazawa et al. [42] and Wang et al. [43] reported a greater ability of MB to increase P_n and P_n/C_i in oaks and Chinese apple, respectively. In the present experiment, the C_i was lower in MB compared to the CTR in both September 2021 and July 2022. Analyzing the trend of g_s over the two years of the experiment, the values recorded in MB-treated individuals were higher than those obtained for CTR plants exclusively in July 2021. On the other sampling dates, no significant differences were found for this parameter. The g_s values found in the present experiment highlight that, despite high summer temperatures, the stomatal overture of MB-treated plants is more efficient compared to CTRs. Zhang et al. [44] noted similar findings in terms of g_s values in rice growth under drought stress and treated with biochar coating. They observed an improving stomatal density and aperture in biochar-treated plants, justifying the higher or stable g_s level also at high temperatures [44].

Results for leaf Chl show no statistically significant variations between the two conditions, neither in 2021 nor in 2022. This is supportive of the lack of negative effects induced by lower level of Mg ions (core of tetrapyrrolic ring of Chl) in MB-treated plants. Bozzolo et al. [45], in a study on the effects of moderate and high doses of solid biochar on grapevine growth in a greenhouse, emphasized the importance of Chl content as a practical indicator of both potential photosynthetic productivity and plant vigor. This aspect also appears to be directly correlated with N concentration and is used as a measure of crop response to the application of N fertilizers and the soil nutritional status. The importance of macronutrients (N, P, and K) remains a critical indicator of plants' physiological state and their ability to withstand biotic and abiotic stresses. In this regard, Lo Piccolo et al. [13] demonstrate that plants treated with biochar exhibit a better physiological state and improved management of water due to a greater availability of N, P, and K observed in plants after treatment with biochar. More specifically, they proposed a direct correlation between the increased P_n rate in MB-treated plants and the higher N and K contents observed in biochar-treated plants [13]. This is also supported by Xiong and Flexas' [46] findings, who argue that key biochemical leaf characteristics for photosynthesis, such as Chl and ribulose-1,5-*bis*-phosphate carboxylase/oxygenase content, are strongly influenced by leaf N content.

The results of Lo Piccolo et al. [13] validate the effect of biochar in increasing soil properties [10,47], stimulating the soil bacterial community and thus accelerating the conversion of slowly available K into readily available K [48], as observed in the present experiment. The increase in P content was observed in the MB-treated plots in July 2022, which, however, was not sustained by September 2022, when P levels returned to values similar to those of CTR. This pattern does not indicate a P deficiency in the leaf tissues, as the recorded values were positive in both 2021 and 2022, considering the age of the young vines, which were two years old in July–September 2021 and three years old in July–September 2022.

Notably, the lower content in Ca and Mg found in leaves of MB plants did not result in any negative effect as, for example, the level of Chl was retained, suggesting some N compensatory mechanisms to biosynthesize these pigments. The persistent CEC of micronized biochar may have progressively immobilized available Mg in the soil–plant system [49]. The observed decrease in leaf Mg content without a corresponding change in Chl content may be thus explained by the buffering capacity and CEC of micronized biochar, which could have reduced Mg availability in the soil–plant system without inducing a critical deficiency in terms of Chl biosynthesis. As a result, Mg levels may have declined within leaf tissues without reaching a threshold sufficient to affect total chlorophyll concentration.

Despite the promising outcomes reported by the present experiment with the use of MB in vines, further investigations must be carried out in this direction, analyzing in-depth the nutrient release/adsorption by biochar and the effect of the micronized biochar form in long-term experiments.

5. Conclusions

The present two-year field experiment demonstrated that micronized biochar applied via fertigation at 0.5% (*v*/*v*) exerted selective positive effects on soil fertility and vine performance in an organic vineyard setting. These findings underscore micronized biochar potential as a low-dose, sustainable amendment in organic viticulture to mitigate soil degradation and enhance productivity, particularly in nutrient-limited systems. Moreover, micronized biochar applied via fertigation (dozens of times lower in quantity than the doses commonly used) can be considered effective and more economically sustainable since the results of the present experiment were obtained with lower doses of biochar when compared with the biochar doses usually utilized in the experiment present in the scientific literature. This is due exclusively to the micronization process. However, the experimental duration of the present experiment may have constrained long-term carbon sequestration and nutrient stabilization effects. Further studies should extend trial periods and investigate microbial community dynamics to optimize micronized biochar's role in resilient vineyard management and climate challenges. Moreover, long-term trials will also be needed to assess effects on grape yield and fruit quality.

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Abbreviations

The following abbreviations are used in this manuscript:

APA	Alkaline Phosphatase Activities
available P	Available Phosphorous
MB	Micronized Biochar
Ca_{ex}	Exchangeable Ca
CEC	Cation Exchange Capacity
Chl_{index}	Chlorophyll Index
C_i	Intercellular CO_2 Concentration
CTR	Control
DHA	Dehydrogenase Activities
DOC	Dissolved Organic Carbon
EC	Electric Conductivity
$Flav_{index}$	Flavonol Index
g_s	Stomatal Conductance to Water Vapor
K_{ex}	Exchangeable K
L	Length
Mg_{ex}	Exchangeable Mg
MPI	Metabolic Potential Index
NO_3^--N	Nitrates
N_{tot}	Total Nitrogen
P_n	Net Photosynthetic
P_n/C_i	Apparent Carboxylation Efficiency
SOM	Soil Organic Matter
TOC	Total Organic Carbon
W	Width

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