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Efficiency of Corncob-Derived Biochar and Plant Growth-Promoting Rhizobacteria as Growth Promoters for *Coffea arabica* L. Seedlings

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Abstract

Corncob-derived biochar and plant growth-promoting rhizobacteria (PGPR) can improve soil fertility and plant performance, but their co-application in *Coffea arabica* L. nurseries remains poorly characterised. This study evaluated corncob-derived biochar combined with *Bacillus megaterium* S2-4a1 under well-watered greenhouse conditions. A completely randomised design with six replications was maintained for 180 days and included five treatments: control (T1), S2-4a1 alone (T2), and S2-4a1 combined with 1, 2, or 3% (w/w) biochar (T3-T5). Soil physicochemical properties, culturable microbial populations, soil microbial respiration (SMR), dissolved organic carbon (DOC), microbial biomass carbon (MBC), plant growth, nutrient status, and leaf physiological traits were assessed. T3 and T4 produced the highest soil pH, whereas cation exchange capacity increased with biochar rate and was highest in T5. T5 also had the highest organic matter, total carbon, exchangeable K, and culturable microbial population. T3 showed the highest SMR on day 1 and a secondary increase on day 18, together with the highest DOC and MBC. Plant height was not significantly affected, but T4 produced the greatest stem dry biomass and the highest relative water content, while co-application treatments generally lowered leaf H2O2 and membrane damage relative to the control. Under these non-stressed conditions, the results indicate that biochar rate altered the balance between soil microbial responses and coffee seedling performance. Rates of 2-3% with S2-4a1 were most consistently associated with improved soil chemical properties and seedling physiological status, supporting further evaluation in larger containers and field nurseries.

Keywords: *Bacillus megaterium*, S2-4a1, Coffee seedlings, Soil respiration

Introduction

Thailand's economy depends on agricultural products. The agricultural sector in the country produces an extensive amount of crop waste, such as rice husk, sawdust, corncobs, cassava stems, and cassava rhizomes. According to the data collected from 2006 to 2007 of the Department of Agricultural Extension indicated that Thailand made 4.40 million tons of corn and 17.6 million tons of cassava, of which 0.84 million tons were corncobs, 2.11 million tons were cassava stems, and 1.76 million tons were cassava rhizomes (Wijitkosum and Jiwnok, 2019). Farmers usually burn off agricultural residues before the next crop season starts, which releases a lot of pollution and greenhouse gases into the air (Nguyen *et al*., 2024). Therefore, many investigations aim to identify techniques for managing agricultural wastes.

Biochar is a rich carbonaceous material produced by heating agricultural wastes under oxygen-limited conditions (pyrolysis). Generally, it is used to improve soil fertility, water holding capacity, and carbon sequestration, which helps alleviate climate change (Lyu *et al*., 2020; Rambhatla *et al*., 2025). Biochar application improved soil aggregate stability, increased soil organic carbon (SOC), and enhanced microbial populations, leading to better soil fertility and maize yields in Northeast China in a 4-year field experiment (Wu *et al*., 2023). Similarly, previous studies revealed that biochar application with chemical fertilizer can significantly improve soil properties, with a 5.78 % increase in rice yield compared to control (Gu *et al*., 2022). Many physicochemical properties of biochar, including specific surface area, water retention capacity, and labile carbon, boost microbial activity and biomass by providing a habitat for microorganisms (Amoakwah *et al*., 2022).

Plant Growth-Promoting Rhizobacteria (PGPR) are beneficial microorganisms that inhabit the rhizosphere of plants and play a crucial role in enhancing plant growth and development, under

a wide range of environmental conditions (Barnawal *et al*., 2019; Grover *et al*., 2021; Jasso-Arreola *et al*., 2025). These bacteria are known for their ability to promote plant growth through diverse mechanisms, including nitrogen fixation, phosphate solubilization, and the production of phytohormones such as auxins and cytokinins (de Andrade *et al*., 2023). PGPR are gaining increasing attention in sustainable agriculture due to their potential to reduce the reliance on chemical fertilizers and pesticides, thereby promoting eco-friendly farming practices (Jeyanthi and Kanimozhi, 2018). Tea plants inoculated with *Streptococcus marcescens JW-CZ2* 11.37% increased stem diameter and 29.34% increased dry weight compared with the control after 180 days in a greenhouse experiment (Liu *et al*., 2022; Zhang *et al*., 2024). Additionally, biochar has been shown to positively influence microbial abundances in soil, enhancing both bacterial and fungal communities. It increases soil organic carbon and often pH, affecting soil enzymes, microbial biomass, and communities that support vital ecosystem services critical for soil quality (Ajema, 2018). Previous study reported that biochar increases soil enzyme activities, including invertase and urease, by up to 166% compared to untreated soils (Feng *et al*., 2021). Similarity, long-term studies from the years 2014 to 2017 indicate that biochar can sustain increased microbial biomass and activity, especially when combined with organic amendments like manure (Brtnicky *et al*., 2019). Biochar also provides physical protection and a large surface area for microbial colonization via a highly porous structure. These pores function as "secure niches" that protect bacteria from predation, desiccation, and mechanical disruption (Zheng *et al*., 2022). Besides, biochar has a significant variety of nutrients that are bioavailable and can improve soil fertility, especially when produced from nutrient-dense feedstocks like sewage sludge or plant biomass (Figueredo *et al*., 2017). Carbon serves as an energy source, while phosphorus and nitrogen are important nutrients, which collectively promote microbial expansion and activity in soil

ecosystems. Although biochar is known to positively influence the soil microbial community, there is still limited quantitative evidence supporting the hypothesis that its porous structure provides protective micro-niches for beneficial microbes as well as the efficiency of microbial functions and soil health. Furthermore, utilizing biochar as a carrier for inoculants is an innovative strategy in agricultural operations aimed at enhancing crop yield, due to characteristics that promote microbial activity (Bolan *et al*., 2023).

Coffee (*Coffea arabica* L.) is a major tropical crop whose growth, yield, and quality are sensitive to environmental conditions. Climate change is expected to alter the suitability of current coffee-growing regions and increase production risks through higher temperatures, irregular rainfall, pests, and diseases (Bunn *et al*., 2015; Wagner *et al*., 2021; Regassa, 2024). At the nursery stage, improving soil conditions and microbial functioning may help produce vigorous seedlings before field establishment. Recent studies show that biochar can act as a habitat and carrier for beneficial microorganisms and that co-application with PGPR can modify soil nutrient availability and beneficial bacterial communities (Bolan *et al*., 2023; Zou *et al*., 2024; Kesavardhini *et al*., 2026). However, these interactions depend strongly on biochar feedstock, pyrolysis conditions, application rate, soil properties, and microbial strain; biochar can also inhibit *Bacillus megaterium* growth or nutrient-solubilising activity under some conditions (Liu *et al*., 2026). The combined influence of corncob-derived biochar and S2-4a1 on coffee seedlings under well-managed, non-stressed nursery conditions therefore remains insufficiently understood. We hypothesised that the two amendments could exert complementary effects: biochar could improve nutrient retention and provide microsites for microbial persistence, while S2-4a1 could contribute phosphate and potassium solubilisation and indole-3-acetic acid production. Because matched biochar-only treatments were not included in the present experiment, these mechanisms are treated as plausible

explanations for co-application responses rather than as a statistically demonstrated biochar x PGPR interaction. Accordingly, this study evaluated the effects of corncob-derived biochar co-applied with S2-4a1 on soil physicochemical properties, microbial activity, and the growth and physiological status of *Coffea arabica* L. seedlings maintained under well-watered greenhouse conditions.

1. Materials and methods

2.1 Preparation of the inoculum

Bacillus megaterium (S2-4a1) was isolated from the rhizosphere soil of *Coffea arabica* L. in abandoned coffee plantations (coordinates: N18° 92′37, E99° 32′78E), referring to coffee plantations without management practices such as the application of chemical fertilizers, pesticides, herbicides, or soil preparation. The average age of the coffee plants at the site was 17 years. The S2-4a1 isolate was based on its demonstrated abilities to solubilize phosphate and potassium, synthesize indole-3-acetic acid (IAA), and function as a plant growth-promoting rhizobacterium (PGPR). The bacterial cultures were incubated in nutrient broth (NB) liquid medium at room temperature for 7 days prior to being incorporated into the soil together with biochar (Chromkaew *et al*., 2023).

2.2 Experimental arrangement

This study was conducted as a pot experiment at the Agricultural Innovation Research, Integration, Demonstration and Training Center, Faculty of Agriculture, Chiang Mai University, Thailand. The research center is located at 18°45'51.9"N latitude and 98°55'42.6"E longitude, at an elevation of 357 m. *Coffea arabica* L. cv. Catimor seedlings, 180 days old, were transplanted in February 2024 into 7-L pots (26 cm diameter × 19 cm height) in a greenhouse. The soil was obtained from the Faculty of Agriculture, Chiang Mai University to provide a reasonably uniform

growth medium. The experiment was arranged as a completely randomized design with six replications, giving 30 pots in total. The S2-4a1 inoculum was applied as a 100-mL liquid suspension per experimental unit. Treatments were: T1, control (no biochar and no S2-4a1); T2, S2-4a1 alone; T3, 1% biochar + S2-4a1; T4, 2% biochar + S2-4a1; and T5, 3% biochar + S2-4a1. Biochar rates were calculated on a dry soil weight basis (w/w). Biochar-only treatments using the same soil and Coffea arabica seedlings were evaluated previously by Kullachonphuri *et al.* (2025), which also reported the physicochemical properties of the biochar. Irrigation was applied approximately every 3 days, with a cumulative water application of 119.34 mm per pot during the 180-day growth period.

2.3 Soil sampling and analyses

Soils were sampled at the beginning of the experiment and after 180 days at the end of the experiment and then air dried for further analysis. Subsamples were kept moist for microbial analyses. Basic physical and chemical soil properties were analyzed via the following methods: The measurement of electrical conductivity (EC) was conducted using an EC meter, with a soil-to-water ratio of 1:5 w/v (Rhoades, 1996). Soil pH was measured using a pH meter with a 1:2 ratio of soil to water (w/v) (Peech, 1965). The determination of soil texture was conducted using the hydrometer method as outlined by Gee and Or (2002). Total nitrogen (N_{tot}) was determined by the combustion method, following the protocol established by Page (1982). Available phosphorus (P_{ava}) was assessed using the Bray II extraction method. The extraction of exchangeable potassium, calcium, and magnesium (K_{exc}, Ca_{exc}, Mg_{exc}) were conducted using ammonium acetate (NH_4OAc), followed by measurement by atomic absorption spectrophotometry.

2.3.1 Soil microbial population

Soil microbial population was determined using the spread plate technique following standard microbiological procedures (Wollum, 1982; Somasegaran and Hoben, 2012). Fresh soil samples (1 g) were suspended in 9 mL sterile distilled water and subjected to serial ten-fold dilutions (10^{-1} to 10^{-6}). Aliquots of 100 µL from appropriate dilutions were spread evenly on nutrient agar plates using a sterile L-shaped glass spreader. Plates were incubated at 28 ± 2°C for 72 hrs under aerobic conditions. Visible colonies were counted, and microbial population density was calculated and expressed as colony-forming units per g of dry soil (CFU g^{-1} dry soil).

2.3.2 Soil microbial respiration

At 180 DAT, fresh soil was collected and incubated to assess carbon dioxide (CO_2) evolution. A 10-mL aliquot of 1 M NaOH was used to trap evolved CO_2 and was replaced with freshly prepared solution on days 1, 5, 7, 14, 18, and 21 to maintain trapping efficiency. CO_2 trapped in the NaOH solution was quantified by back-titration with standardized 0.1 M HCl. Aliquots (1.00 mL) of the NaOH trap solution were transferred to Erlenmeyer flasks, treated with 6–7 drops of $BaCl_2$ solution and 2–3 drops of phenolphthalein indicator, and titrated with HCl to the colorless endpoint. Three reagent blanks were titrated in parallel using the same procedure (Anderson, 1982).

2.3.3 Dissolved organic carbon and Microbial biomass carbon

After 60 days incubation, soil microbial biomass carbon (MBC) was determined using the chloroform fumigation-extraction method . Duplicate 5 g soil subsamples were prepared from each replicate pot: one fumigated with chloroform vapor for 72 h at room temperature to lyse microbial cells and release intracellular contents, and one non-fumigated control. Both fumigated and non-fumigated samples were extracted with 20 mL of 0.5 M K_2SO_4 solution by horizontal shaking at

200 rpm for 30 min, then filtered through Whatman No. 42 filter paper. Then, 4 mL of the extracts was determined by wet oxidation using the dichromate method. Each extract was mixed with 5 mL concentrated H_2SO_4 and 1 mL of 0.067 M $K_2Cr_2O_7$ solution, then back-titrated with standardized 0.0337 M ferrous ammonium sulfate [$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$] using 2 to 3 drops of phenanthroline indicator until the endpoint changed from blue-green to reddish-brown. Three reagent blanks containing only K_2SO_4 solution were titrated simultaneously to correct for background carbon. MBC was calculated from the difference in extractable carbon between fumigated and non-fumigated samples (EC) as MBC = EC/kEC, using kEC = 0.45 to account for incomplete extraction efficiency. Results were expressed as mg C kg^{-1} dry soil .

2.4 Biochar production and analysis

Corncobs to be used for biochar production were first sun-dried to reduce moisture content, as excessive moisture can negatively impact both the yield and quality of biochar. Then, corncob feedstock was situated in the space between the chamber and the outer chamber. Heat is generated inside the inner chamber, and the lid is tightly secured. Following a duration of 30 to 40 mins, the feedstock attained an elevated combustion temperature; the outer chamber commenced ignition after 60 mins. During that period, it emitted gases, resulting in a blue flame with minimal smoke. This suggests that there has been total combustion of the fuel. At the same time, thermocouple probes are arranged within the kiln in both radial and longitudinal orientations. The recorded temperature spans from 250 to 550 ^oC. The biochar is generated by combusting the firewood at the base of the biochar kiln, initiating the pyrolysis process for a duration of 4 hrs. The biochar was sieved through a 2 mm particle size prior to analysis.

Biochar structure and chemical properties were analyzed. To examine the structure of the biochar particles, a small quantity of samples was placed in a sample holder utilizing copper

conductive tape. Subsequently, the samples underwent characterization through High-Resolution FE-SEM analysis. The microscope features an Energy Dispersive X-Ray detector, which facilitates the investigation of the elemental composition of biochar (Figure 1). The measurement of EC was conducted using an EC meter, with a biochar-to-water ratio of 1:10 w/v (Rhoades, 1996). Biochar pH measurement was used by a pH meter with a 1:10 w/v ratio of soil to water (Peech, 1965). N_{tot} was determined by the combustion method, following the protocol established by Page (1982). The available phosphorus (P_{ava}) was assessed by using the Bray II extraction method. The extraction of exchangeable potassium, calcium, and magnesium (K_{exc}, Ca_{exc}, Mg_{exc}) were conducted using ammonium acetate (NH_4OAc), followed by measurement by atomic absorption spectrophotometry. The biochar used in this study was derived from corncob and was fully characterized in our previous work (Kullachonphuri *et al.*, 2025). As reported, this biochar exhibited 9.69 pH, 34.10 cmol kg^{-1} CEC, 0.43 mS cm^{-1} EC, 9.71 % OC, 17.84 % OM, 0.88 % N_{tot}, 0.62 % AP, 0.96 % K, 18.94 % Ca, 1 % Mg, and 60.95 % WHC.

2.5 Plant data collections

2.5.1 Coffee growth parameters

Fully expanded coffee leaves at the third node were tagged and monitored repeatedly throughout the experiment. Leaf length (L) and width (W) were measured to estimate observed leaf area (LA) following Unigarro Muñoz *et al.* (2015). Leaf chlorophyll status was measured non-destructively using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Inc., Japan). Measurements were taken on fully expanded, healthy leaves from the middle canopy during mid-morning (09:00–11:00) to minimize diurnal variation. Three readings were taken per leaf at different positions along the blade while avoiding major veins, and the mean was calculated for

each plant. Shoot height was measured from the soil surface to the apex of the main stem using a graduated measuring rod (±0.1 cm), and stem diameter was measured 15 cm above the soil surface using digital calipers (INSIZE model 1108-150, precision ±0.01 mm). Plants were harvested at 180 DAT for biomass determination. Whole plants were carefully removed from the pots, soil was gently washed from the roots, and plant material was separated into aboveground and belowground components. Samples were oven-dried at 70°C to constant mass (approximately 72 h; consecutive weights differing by <0.01 g) and weighed using an analytical balance (precision ±0.001 g) after cooling in a desiccator.

$$LA = 0.99927 \times (L \times (-0.14757 + 0.60986 \times W)) \quad (1)$$

Where, L is length of the leaf sample, W is width of the leaf sample.

2.5.2 Plant sampling and analyses

Leaf sampling (180 DAT) was conducted by collecting fully expanded leaves from the third node of Coffea arabica L. seedlings. Leaf samples were thoroughly washed with distilled water, oven-dried at 70°C for 48 hrs, and ground to pass through a 1 mm mesh sieve. For nutrient analysis, dried plant material (1 g) was digested using concentrated nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a microwave digestion system. The extraction of exchangeable potassium, calcium, and magnesium (K_{exc}, Ca_{exc}, Mg_{exc}) were conducted using acid digestion (nitric acid (HNO_3) and perchloric acid ($HClO_4$)), followed by measurement by atomic absorption spectrophotometry, while nitrogen concentration (N) was analyzed using the combustion method, and phosphorus (P) concentration was acid digestion followed by colorimetry.

2.5.3 Relative water content

Relative water content (RWC) was measured with 0.1 g of leaf sample that was immersed in Petri dishes holding 100 mL of distilled water after the measurement of leaf fresh weight. After 4 hrs, the leaf samples were extracted from the Petri plates, gently blotted dry prior to measuring the turgid weight, and subsequently oven-dried at 60°C, with the dry weight recorded after 2 days. RWC was calculated as follow Weatherley (1951) and Pappula-Reddy *et al*. (2024):

$$RWC = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100 \quad (2)$$

2.5.4 Membrane damage

To determine membrane damage (MD), 3 leaf discs of 1 cm² were excised from coffee leaves. The leaf discs were placed in 10 mL of distilled water and agitated at room temperature for 24 hrs using an orbital shaker. Subsequently, the initial electrical conductivity values (EC_1) of the samples were measured using an electrical conductivity meter. The samples were then autoclaved at 121°C for 20 mins to achieve complete membrane disruption, followed by cooling to room temperature. The second electrical conductivity measurements (EC_2) were recorded, and membrane damage was calculated using Equation 3 according to the method described by Lutts *et al.* (1996).

$$MD\ (\%) = (EC_1/EC_2) \times 100 \quad (3)$$

2.5.5 Chlorophyll content

Leaf chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids were quantified. Fresh leaves weighing 0.3 g were placed in 80% acetone for 24 hrs at 4°C. The absorbance of the supernatant was determined with a spectrophotometer at wavelengths of 663, 645, and 480 nm

(Hasan *et al*., 2015; Anwar *et al*., 2023). The subsequent formulas were employed to determine chlorophyll a and b concentrations, total chlorophyll content, and carotenoid levels:

Chlorophyll a (mg/g f. wt) = (12.7(OD 663) - 2.69(OD645) × V⁄100 × W) (4)

Chlorophyll b (mg/g f. wt) = (22.9(OD 645) - 4.68(OD663) × V⁄100 × W) (5)

Total chlorophyll (mg/g f. wt) = (20.2(OD 645) + 8.02(OD663) × V⁄100 × W) (6)

Carotenoids (mg/g f. wt) = Acar⁄Em × 100 (7)

Where, V is volume of the sample, W is weight of the fresh tissue, OD is Optical Density. Acar is OD 480 + 0.114(OD 663) - 0.638(OD 645), Em=2500

2.5.6 Determination of Proline Content

Proline content was determined spectrophotometrically following the modified method of Bates *et al*. (1973). Fresh leaf samples (0.25 g) were collected and homogenized in 3 mL of 95% ethanol (v/v). Then, the homogenized sample was transferred to microcentrifuge tubes and centrifuged at 6,000 rpm for 5 mins at 4°C using a refrigerated centrifuge. After that, an aliquot of 0.2 mL supernatant was mixed with 0.3 mL of distilled water and 2.0 mL of ninhydrin reagent (consisting of 1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid). The reaction mixture was incubated in a boiling water bath at 100°C for exactly 60 mins, with tubes covered with aluminum foil to prevent evaporation. After incubation, samples were immediately cooled in an ice bath for 2 mins to terminate the reaction. Absorbance was measured at 520 nm using a UV-visible spectrophotometer (UV-S2100, USA) against a toluene blank (Saidimoradi *et al*., 2019).

2.5.7 Determination of total soluble sugar content

Total soluble sugars (TSS) were quantified in leaf tissues using the phenol-sulfuric acid method with 95% ethanol extraction. Fresh leaf samples (1.0 g) were homogenized in 1 mL of 95% (v/v) ethanol using a mortar and pestle, then extracted in a water bath at 75°C for 15 mins to ensure complete sugar dissolution. The homogenate was centrifuged at 12,000 rpm for 15 mins at 4°C, and the clear supernatant was collected for analysis. For colorimetric determination, 250 µL of supernatant was mixed with 250 µL of 80% ethanol, followed by rapid addition of 1.25 mL concentrated H_2SO_4 and 250 µL of 5% (w/v) phenol solution (freshly prepared in distilled water). The reaction mixture was vortexed immediately for 30 seconds and incubated in darkness at room temperature for exactly 20 mins to allow complete color development. Absorbance was measured at 490 nm using a UV-visible spectrophotometer against a reagent blank (DuBois *et al*., 1956). TSS concentration was determined using a D-glucose standard curve (0 to 100 µg mL^{-1}) prepared fresh on the day of analysis with regression coefficient > 0.998. Each sample was analyzed in triplicate with coefficient of variation < 5 % required for data acceptance.

2.5.8 Hydrogen peroxide (H_2O_2)

Leaf hydrogen peroxide content was estimated by according to method of Velikova *et al*. (2008). Fresh leaf tissue (1.0 g) was homogenized in an ice-cold mortar and pestle with 3 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution. The homogenization was performed in an ice bath with continuous grinding for 3 to 5 mins to ensure complete tissue disruption while maintaining low temperature to preserve H_2O_2 stability. The homogenate was transferred to pre-cooled microcentrifuge tubes and centrifuged at 10,000 rpm for 15 mins at 4°C using a refrigerated centrifuge. Then, 0.5 mL of the supernatant was combined with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI solution. Absorbance was measured at 390 nm

using a UV-visible spectrophotometer against a reagent blank containing all components except the plant extract (replaced with 0.1% TCA).

2.6 Statistical analysis

Treatment effects were evaluated using one-way analysis of variance (ANOVA) after checking assumptions of normality and homogeneity of variance. When the ANOVA was significant, treatment means were separated using Tukey's HSD test at $p < 0.05$. Results are reported as means +/- standard error (SE). Statistical analyses were performed using Statistix version 10.0 (Analytical Software, Tallahassee, FL, USA). Figures were prepared using SigmaPlot version 15.0 (Systat Software Inc., San Jose, CA, USA) and R version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria) with ggplot2. Pearson correlation analysis was used to assess associations among final soil properties, plant nutrient concentrations, growth traits, and physiological variables. Correlation coefficients (r) were visualized as a correlation matrix, with values ranging from -1 to +1. These correlations were interpreted as associations and not as evidence of causal relationships.

3 Results

3.1 Biochar and soil properties

At 180 DAT, soil chemical properties differed significantly among treatments (Table 2). Soil pH was highest in T3 and T4, while cation exchange capacity increased with biochar rate and was greatest in T5. T5 also produced the highest organic matter, total carbon, and exchangeable K, whereas Ca and Mg showed non-monotonic treatment responses. The culturable microbial population increased following inoculation and was highest in T5 by day 7 (Figure 2). These results

indicate that the soil response depended on both the parameter considered and the biochar application rate rather than following a uniform dose-response pattern.

3.2 The effect of S2-4a1 and biochar on soil carbon cycle

Soil microbial respiration was highest during the early incubation period and declined thereafter, with a secondary increase around day 18 in the inoculated treatments (Figure 3). T3 showed the greatest SMR on day 1 and again had the highest value at day 18. T3 also had the highest DOC and MBC among the treatments (Figure 4), indicating that the 1% biochar plus S2-4a1 treatment produced the clearest short-term response in the measured soil carbon pools and microbial activity.

3.3 Plant growth characteristics

Plant height did not differ significantly among treatments during the experiment (Figure 5a). Treatment effects on stem diameter and observed leaf area were time-dependent, with the clearest stem-diameter separation occurring at 90 DAT (Figure 5b). Observed leaf area peaked around 90 DAT and declined thereafter across treatments (Figure 5c). SPAD values differed at selected dates but not consistently throughout the experiment (Figure 5d). At harvest, T5 had the highest leaf N and K concentrations, while P did not differ significantly among treatments and Ca and Mg showed variable responses (Figure 6). Stem dry biomass was significantly increased by the amended treatments, with T4 producing the greatest stem biomass, whereas root dry biomass did not differ significantly among T2-T5 (Figure 7).

3.4 Plant biological characteristics

Leaf biochemical and physiological traits responded to the co-application treatments at 180 DAT. Higher biochar rates were generally associated with greater chlorophyll pigment concentrations, proline, and total soluble sugars (Figures 8 and 9). Leaf H_2O_2 was significantly

lower in all amended treatments than in the control, while T4 and T5 maintained the highest relative water content. Membrane damage was highest in T1 and lowest in T4, with the other amended treatments also showing lower values than the control (Figure 10). Because no environmental stress treatment was imposed, these responses are interpreted as differences in physiological status under well-watered nursery conditions rather than as direct evidence of stress mitigation.

3.5 Correlation plot of soil microbial respiration and related soil parameters

The exploratory correlation analysis among SMR, DOC, and MBC showed that the direction and strength of the associations varied with biochar rate (Figure 11). Positive associations between early SMR and carbon pools were most apparent at the lower biochar rate, whereas weaker or inverse relationships occurred at higher rates. These patterns are treated as descriptive associations and do not by themselves demonstrate substrate limitation, metabolic saturation, or causal changes in microbial carbon-use efficiency.

3.6 Correlation between soil properties and plant traits

Pearson correlation analysis revealed coordinated relationships among soil fertility, plant nutrient status, growth, and physiological traits (Figure 12). The photosynthetic pigments were closely interrelated: chlorophyll a was positively correlated with chlorophyll b and total chlorophyll ($r = 0.86$ for both), while chlorophyll b and total chlorophyll showed a near-perfect positive association ($r = 1.00$). Several soil fertility indicators were also strongly interrelated. Soil CEC was positively correlated with soil organic matter ($r = 0.86$), total carbon ($r = 0.86$), and exchangeable K ($r = 0.90$), while soil organic matter was strongly associated with exchangeable K ($r = 0.81$). Plant physiological traits showed clear associations with these soil variables.

Chlorophyll b was positively correlated with soil CEC (r = 0.83) and exchangeable K (r = 0.86), and proline was positively correlated with soil CEC (r = 0.74), organic matter (r = 0.75), and exchangeable K (r = 0.80). Aboveground biomass was positively associated with soil CEC (r = 0.68) and exchangeable K (r = 0.70). In contrast, leaf H_2O_2 was negatively correlated with soil exchangeable K (r = -0.60) and Mg (r = -0.67), while membrane damage was negatively associated with exchangeable K (r = -0.50) and Mg (r = -0.48). These patterns indicate that improved soil fertility tended to coincide with more favorable plant nutritional and physiological status, although the correlations do not establish causal relationships.

Table 1 The physical and chemical characteristics of initial soil used for the pot experiment.

Chemical parameters	**Mean ± SD (*n* = 6)**	**Class/categorize**	**Classification reference**
Soil pH (1:2)	5.91 ± 0.04	Slightly Acidic	USDA (2017)
EC 1:5 (dS m^{-1})	0.12 ± 0.01	Non-saline	USDA (2014)
CEC (cmol kg^{-1})	10.91 ± 0.04	Low	Landon (1991)
OM (%)	4.02 ± 0.14	High	Walkley and Black (1934)
N_{tot} (%)	0.22 ± 0.01	Medium	Landon (1991)
OC (%)	2.03 ± 0.08	Low	Brady and Weil (2002)
P_{ava} (mg kg^{-1})	4.83 ± 0.72	Low	Olsen (1954)
K_{exc} (mg kg^{-1})	184.65 ± 0.90	High	Landon (1991)
Ca_{exc} (mg kg^{-1})	321.64 ± 8.65	Low	Laboksi and Peters (2012)
Mg_{exc} (mg kg^{-1})	111.28 ± 1.17	High	Landon (1991)
Particle size distribution			
Sand (%)	68.3 ± 2.88	-	Kettler *et al*. (2001)

Silt (%)	26.0 ± 2	-	Kettler *et al*. (2001)
Clay (%)	5.6 ± 1.52	-	Kettler *et al*. (2001)
Textural class	Sandy loam	-	USDA (2017)

Abbreviations: Chemical variables and soil nutrients estimated were as follows: pH, electrical conductivity (EC), cation exchange capacity (CEC), organic matter (OM), total nitrogen (N_{tot}), organic carbon (OC), available phosphorus (P_{ava}), exchangeable potassium (K_{exc}), and exchangeable calcium (Ca_{exc}), and exchangeable magnesium (Mg_{exc})

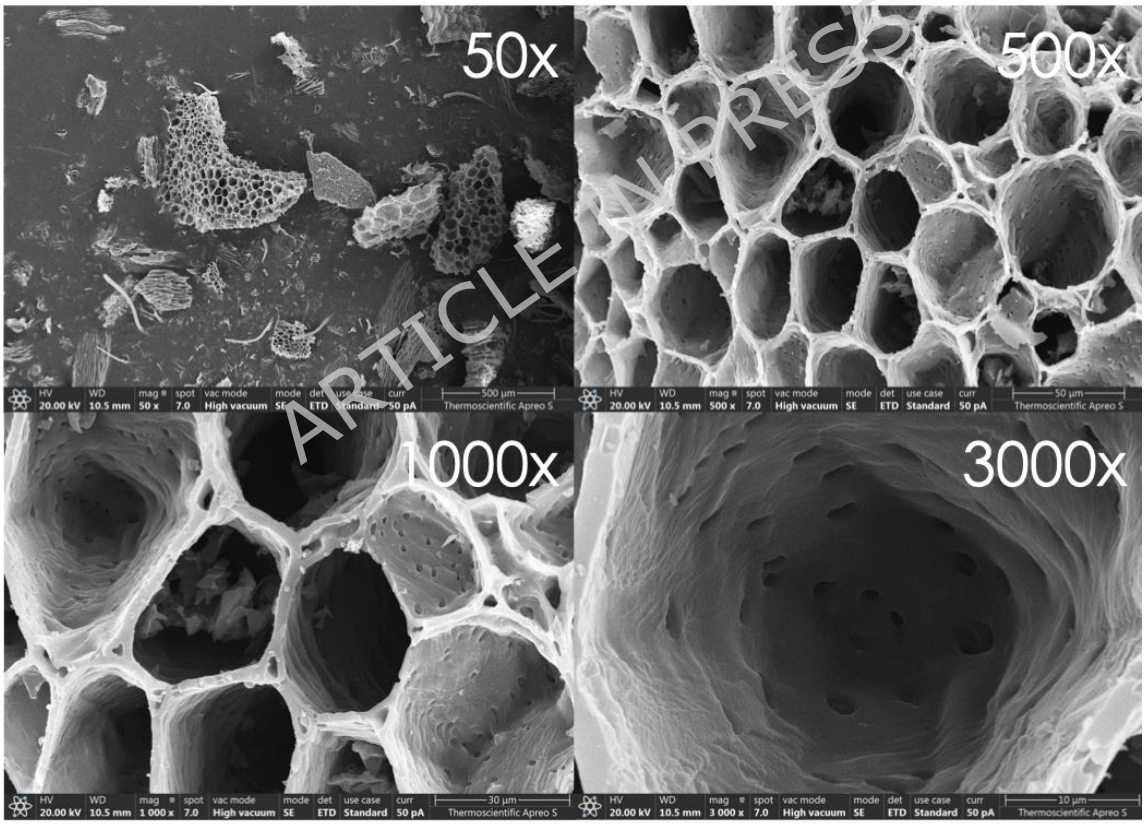

Figure 1. Scanning electron microscopy (SEM) images of corncob biochar.

Table 2 Mean ± SD (standard deviation) (n=6) of soil chemical property in various biochar rates application under coffee cultivation

Soil parameters	Control (T1)	S2-4a1 (T2)	Biochar-rate			F-test
			1% (T3)	2% (T4)	3% (T5)	***
Soil pH (1:2)	7.98 ± 0.02c	8.16 ± 0.03b	8.34 ± 0.02a	8.43 ± 0.01a	8.22 ± 0.02b	***
EC 1:5 (dS m^{-1})	0.13 ± 0.01d	0.15 ± 0.01c	0.22 ± 0.01a	0.20 ± 0.01b	0.21 ± 0.01b	***
CEC (cmol kg^{-1})	19.5 ± 0.3e	20.73 ± 0.2d	22.28 ± 0.2c	25.41 ± 0.33b	28.66 ± 0.15a	***
OM (%)	5.08 ± 0.07c	5.34 ± 0.08bc	5.44 ± 0.01b	5.49 ± 0.02b	6.32 ± 0.41a	**
N_{tot} (%)	0.33 ± 0.01b	0.33 ± 0.01b	0.33 ± 0.03a	0.33 ± 0.02a	0.29 ± 0.01c	**
TC (%)	3.22 ± 0.06b	3.22 ± 0.06b	3.27 ± 0.33b	3.60 ± 0.24b	3.77 ± 0.53a	**
P_{ava} (mg kg^{-1})	26.49 ± 1.04b	28.77 ± 0.85a	25.07 ± 0.13b	25.94 ± 0.32b	28.84 ± 0.8a	**
K_{exc} (mg kg^{-1})	49.11 ± 0.92e	107.08 ± 0.57b	75.46 ± 1.98d	95.98 ± 1.37c	120.67 ± 4.7a	***
Ca_{exc} (mg kg^{-1})	2323 ± 5.5bc	2524 ± 3.32a	2405 ± 74.12b	2261 ± 49.71c	2404 ± 35.2b	**
Mg_{exc} (mg kg^{-1})	155.9 ± 0.9b	181.0 ± 0.7a	175.6 ± 4.25a	176.9 ± 2.12a	184.6 ± 8.4a	***

Note: **, and *** differ significantly at 0.01, and 0.001, respectively. Different letters indicate statistically significant differences ($p < 0.05$), while the same letter suggests no significant difference between treatments.

Abbreviations: Chemical variables and soil nutrients estimated were as follows: pH, electrical conductivity (EC), cation exchange capacity (CEC), organic matter (OM), total nitrogen (N_{tot}), total carbon (TC), available phosphorus (P_{ava}), exchangeable potassium (K_{exc}), and exchangeable calcium (Ca_{exc}), and exchangeable magnesium (Mg_{exc}). T1, control; T2, soil with inoculated *S2-4a1*; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

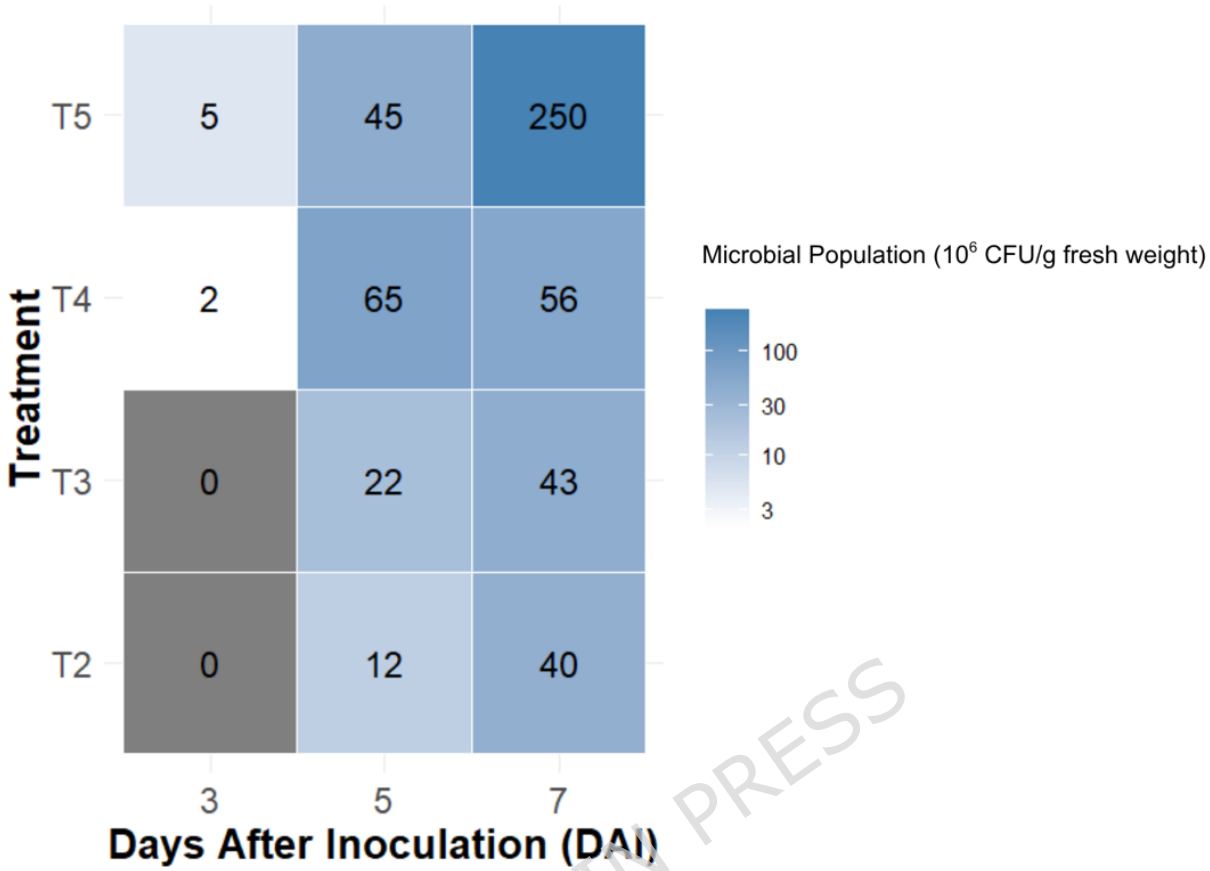

Figure 2. Culturable soil microbial population under the different treatments.

Note: T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

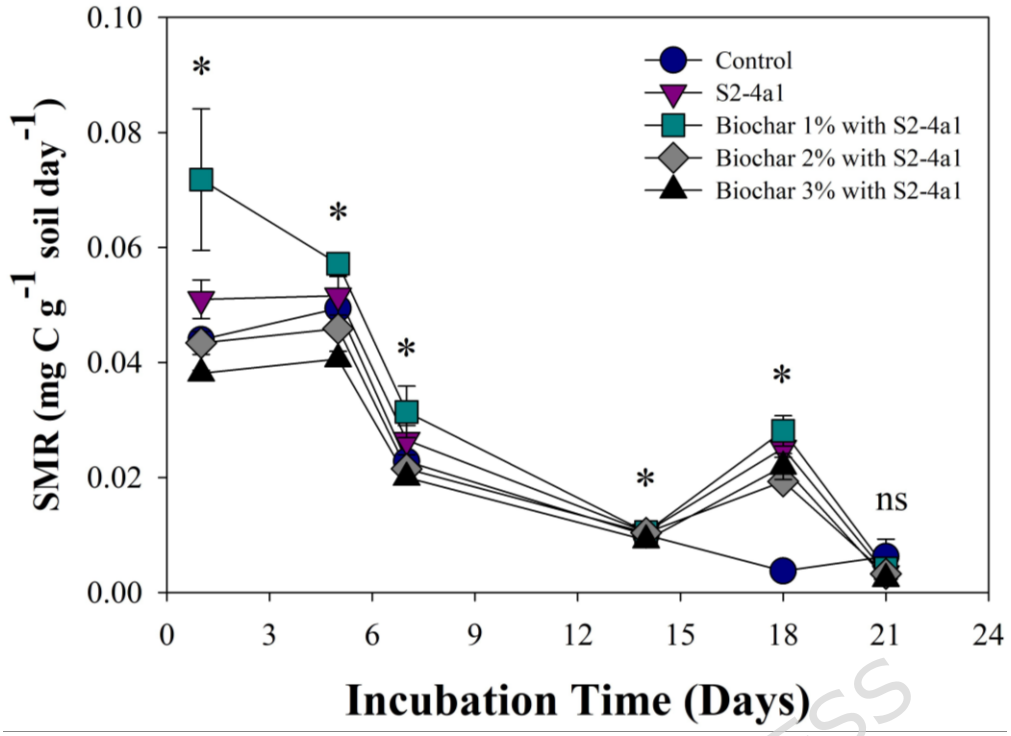

Figure 3. Soil microbial respiration (SMR) in various treatments.

Note: Points are means of six replicates ± SE. * indicates a significant treatment effect at $p < 0.05$; ns indicates a non-significant treatment effect at $p >= 0.05$.

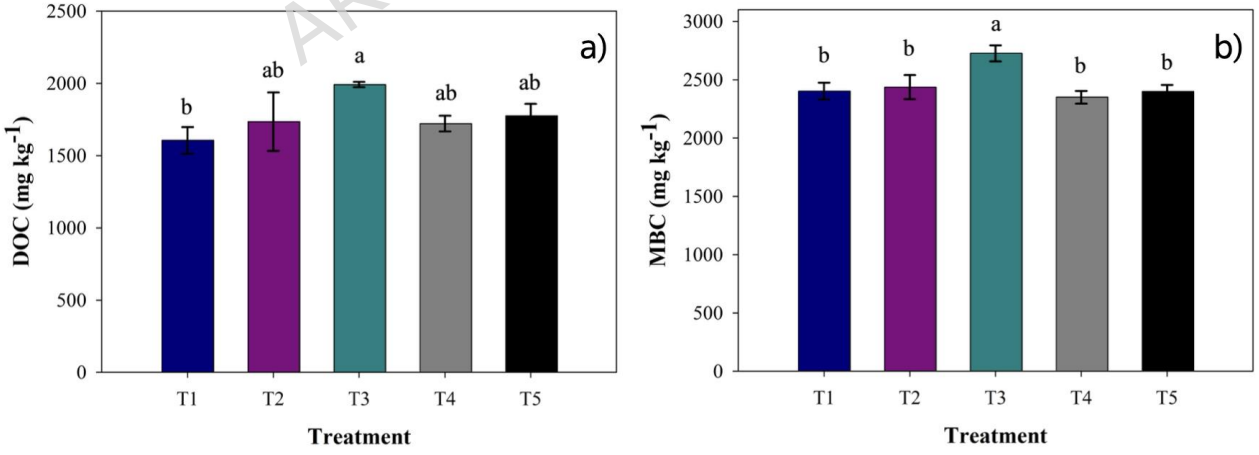

Figure 4. Soil dissolved organic carbon (DOC) (a) and microbial biomass carbon (MBC) (b) under the different treatments.

Note: Bars are means of 6 replicates ± SE. Difference letters on bars showed significant changes at $p \leq 0.05$. T1, control; T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

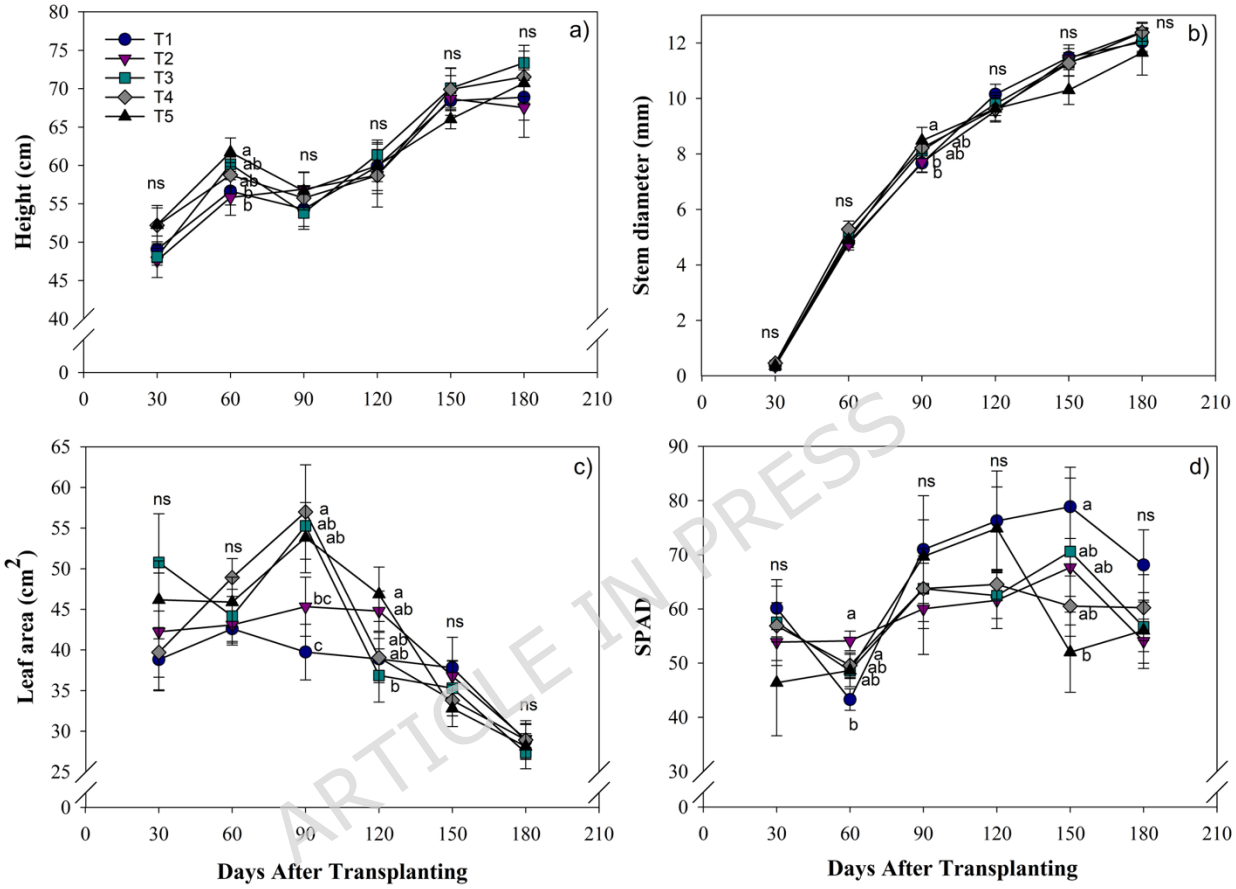

Figure 5. Plant height (a), stem diameter (b), leaf area (c), and SPAD (d) of *Coffea arabica* L. seedlings

Note: Bars are means of 6 replicates ± SE.

* Difference letters showed significant differences at $p \leq 0.05$. ns means non-significant differences at $p \geq 0.05$.

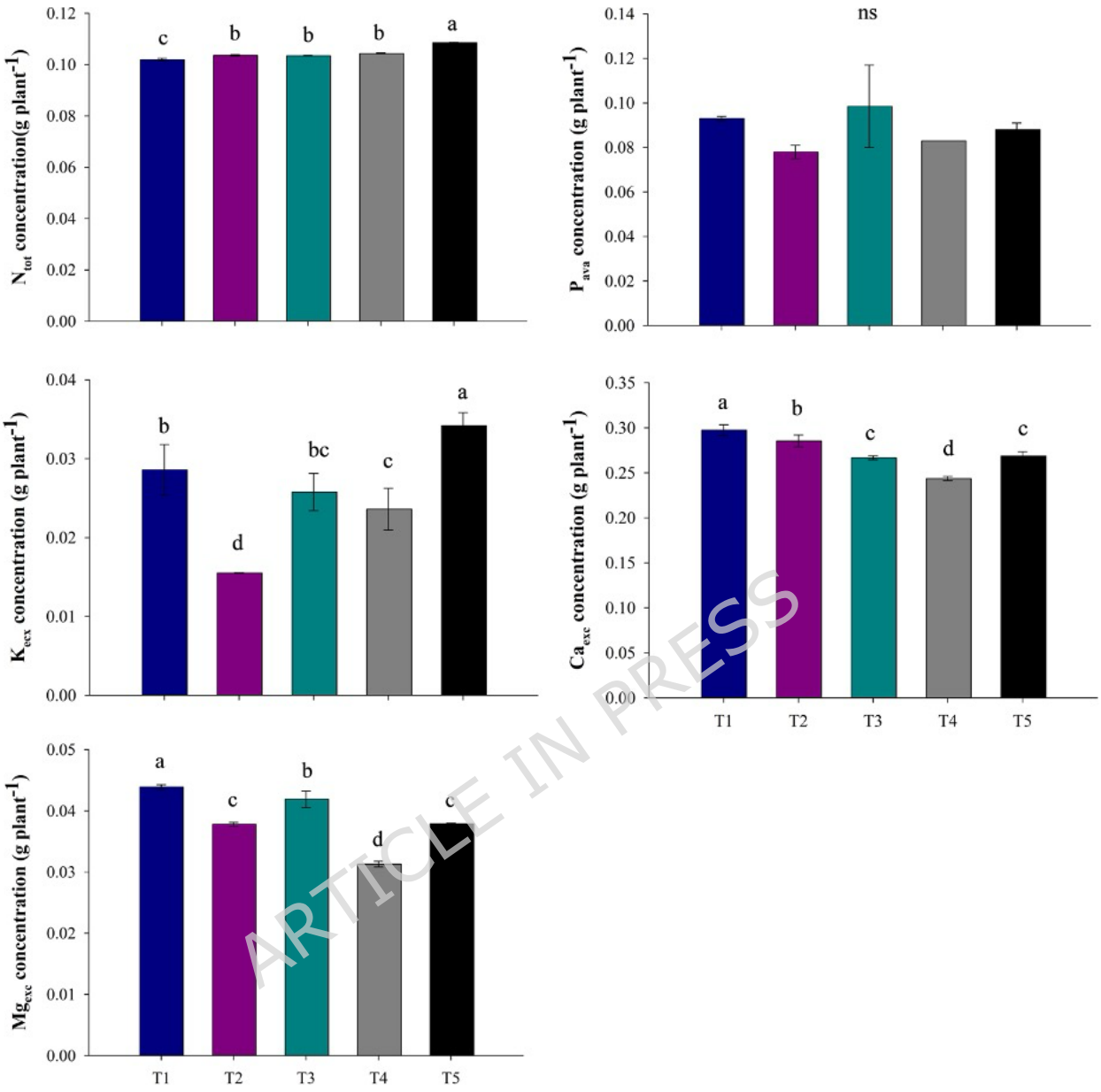

Figure 6. Nutrient concentrations in *Coffea arabica* L. leaves under the different treatments.

Note: Bars are means of 6 replicates ± SE. Difference letters on bars showed significant changes at $p \leq 0.05$. ns means non-significant differences at $p \geq 0.05$.

Abbreviations: total nitrogen (T_{tot}), phosphorus (P_{ava}), potassium (K_{exc}), and calcium (Ca_{exc}), and magnesium (Mg_{exc}). T1, control; T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

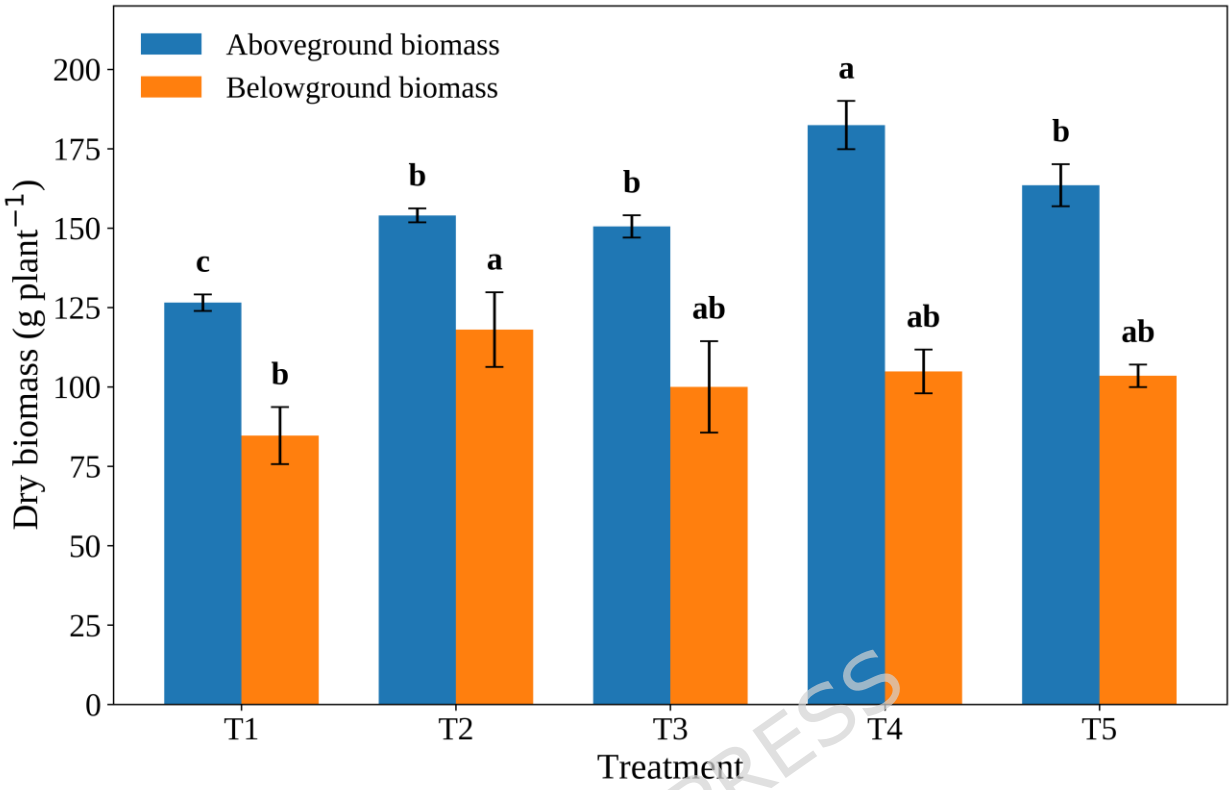

Figure 7. Aboveground and belowground dry biomass of *Coffea arabica* L. seedlings under the five treatments.

Note: Bars show means of six replicates ± SE. Different letters within each biomass component indicate significant differences among treatments according to Tukey's HSD test ($p < 0.05$).

Abbreviations: T1, control; T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

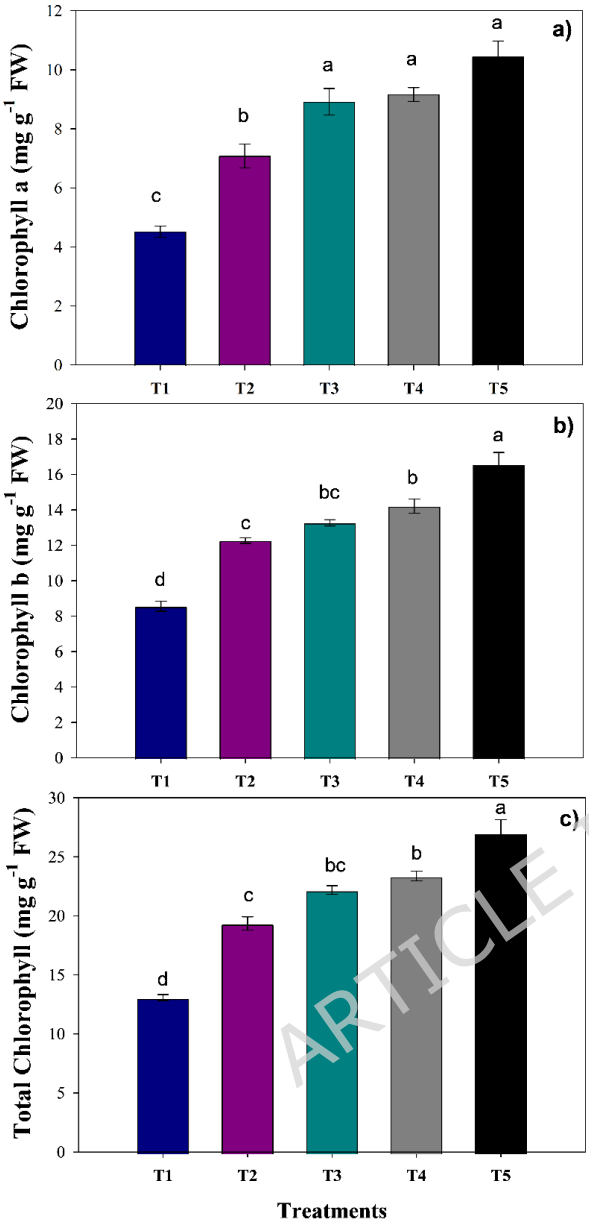

Figure 8. Effects of treatments on (a) chlorophyll a, (b) chlorophyll b, and (c) total chlorophyll in *Coffea arabica* L. leaves.

Note: Bars are means of 6 replicates ± SE. Difference letters on bars showed significant changes at $p \leq 0.05$. **Abbreviations:** T1, control; T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

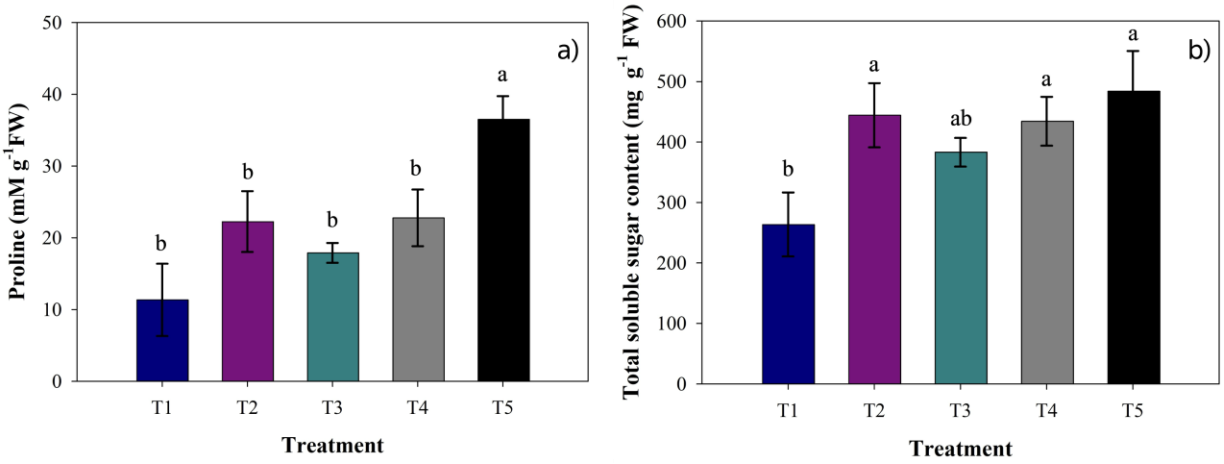

Figure 9. Proline content (a), and total soluble sugar content (b) of *Coffea arabica* L. leaves

Note: Bars are means of 6 replicates ± SE. Difference letters on bars showed significant changes at $p \leq 0.05$. **Abbreviations:** T1, control; T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

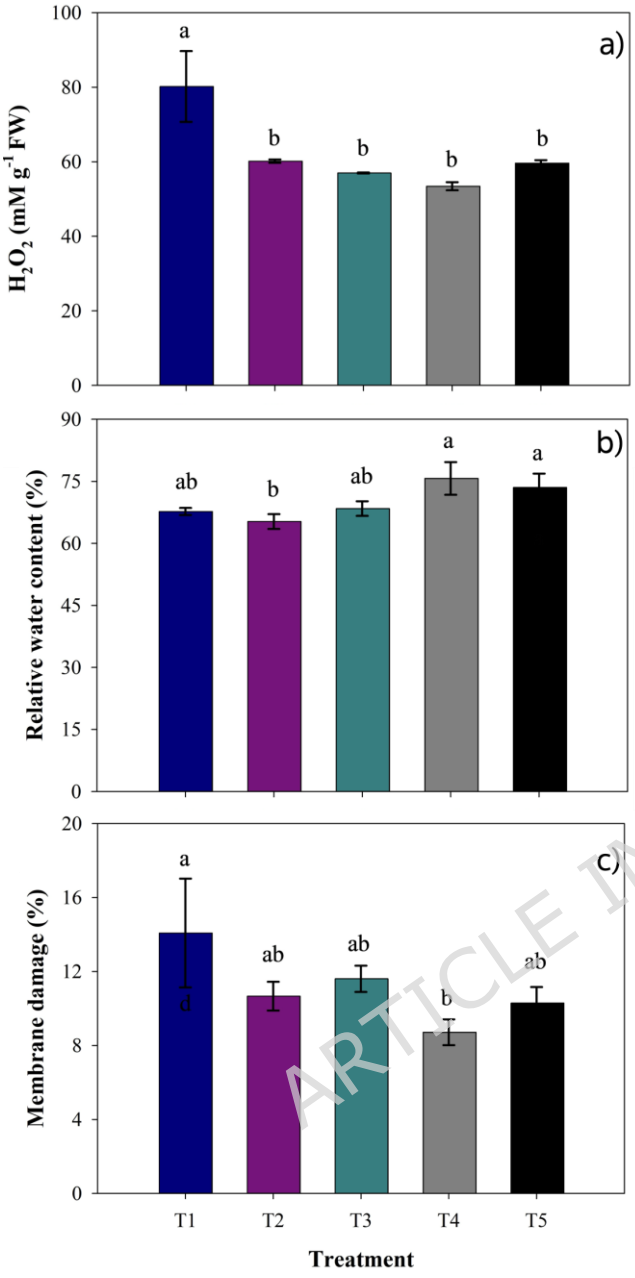

Figure 10. Leaf hydrogen peroxide (a), relative water content (b), and membrane damage (c) in *Coffea arabica* L.

Note: Bars are means of 6 replicates ± SE. Difference letters on bars showed significant changes at $p \leq 0.05$. **Abbreviations:** T1, control; T2, soil with inoculated S2-4a1; T3, Biochar 1% with inoculated S2-4a1; T4, Biochar 2%; with inoculated S2-4a1 T5, Biochar 3% with inoculated S2-4a1.

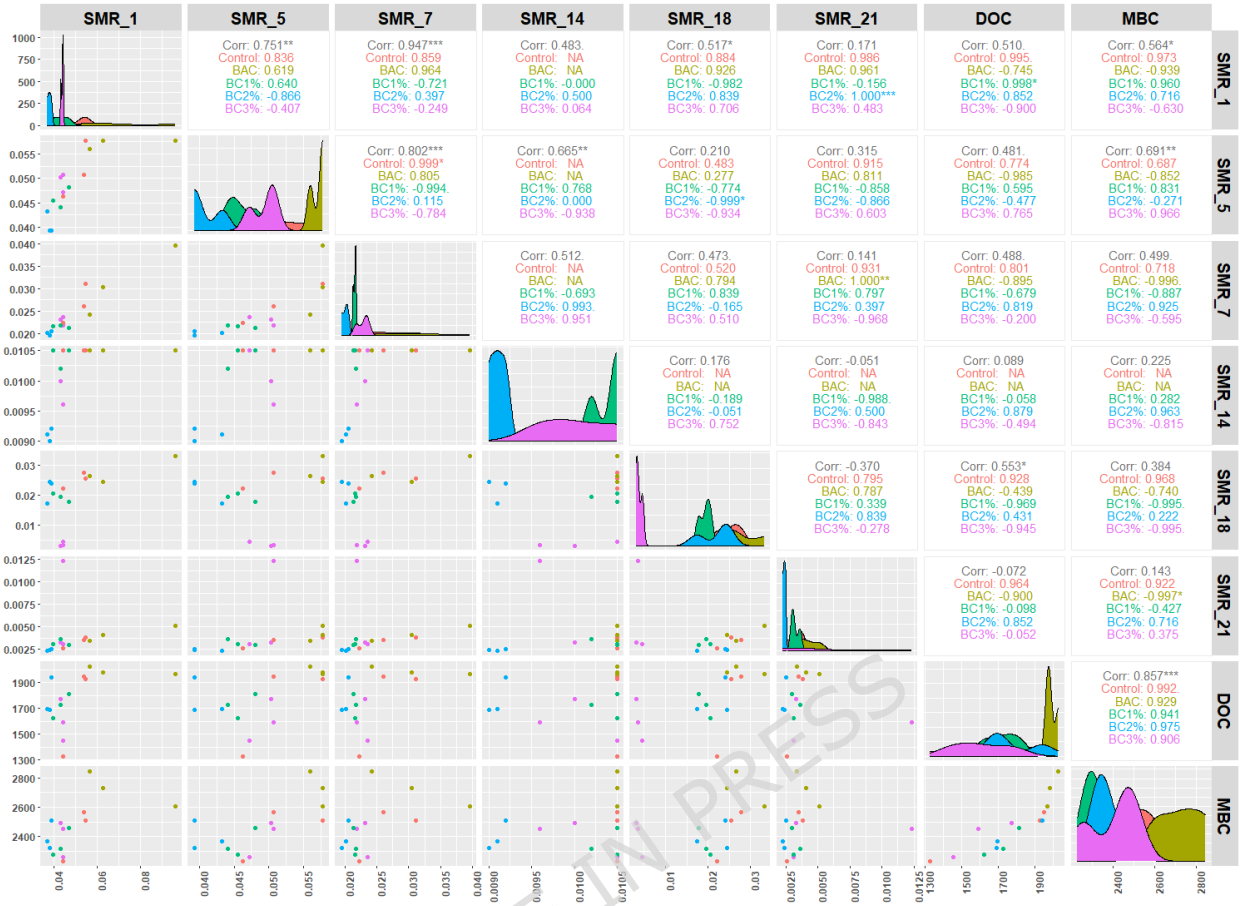

Figure 11. Correlation plot of soil microbial respiration and related soil parameters

Abbreviation: SMR_1, soil microbial respiration 1 days after incubation; SMR_5, soil microbial respiration 5 days after incubation; SMR_7, soil microbial respiration 7 days after incubation; SMR_14, soil microbial respiration 14 days after incubation; SMR_18, soil microbial respiration 18 days after incubation; SMR_21, soil microbial respiration 21 days after incubation; DOC, soil dissolved organic carbon; MBC, soil microbial biomass carbon.

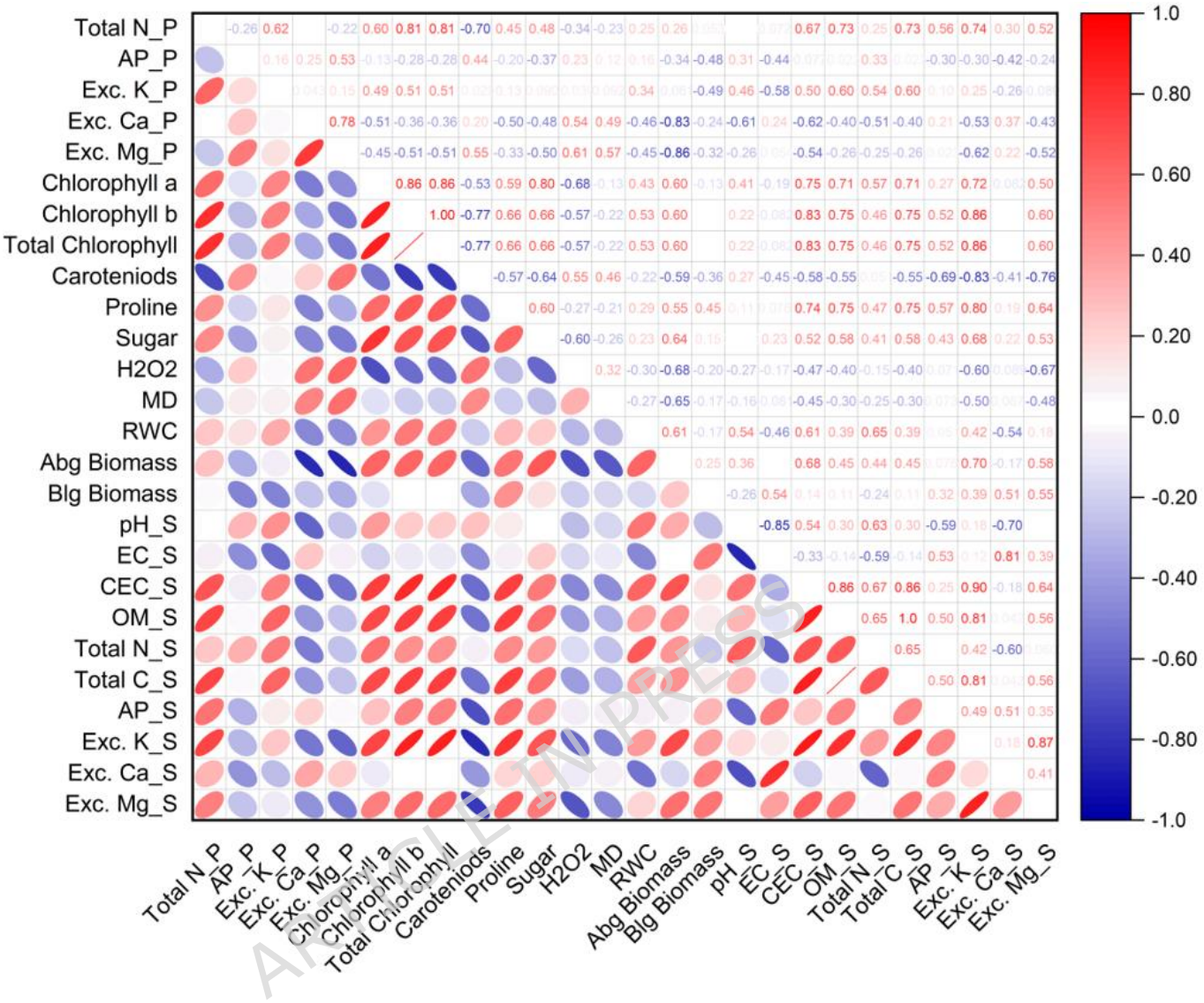

Figure 12. Pearson correlation matrix among soil properties, plant nutrient concentrations, growth traits, and physiological characteristics of *Coffea arabica* L. seedlings under biochar and PGPR treatments.

Note: Red and blue denote positive and negative Pearson correlations, respectively; darker and more elongated ellipses indicate stronger relationships. Numerical values are Pearson correlation coefficients (r). Abbreviations: _P, plant; _S, soil; AP, available phosphorus; Exc., exchangeable; MD, membrane damage; RWC, relative water content; Abg Biomass, aboveground biomass; Blg Biomass, belowground biomass.

Discussion

The strongest soil responses to co-application were observed for cation exchange capacity, organic matter/total carbon, and exchangeable K. CEC increased progressively with biochar rate and reached its highest value in T5, while T5 also had the highest organic matter, total carbon, and exchangeable K. These changes are consistent with the addition of carbon-rich, alkaline biochar containing exchange surfaces and mineral constituents that can increase nutrient retention. The pH response, however, was not strictly dose dependent: T3 and T4 had the highest final pH, whereas T5 was lower. Moreover, the final control pH was substantially higher than the initial soil pH, indicating that temporal changes in the pot system, irrigation, nutrient cycling, or other experimental factors also contributed to the final pH. The treatment effect should therefore be interpreted relative to the final control rather than attributing the full pH increase to biochar. S2-4a1 alone also increased exchangeable K relative to the control, which is consistent with the previously characterised K-solubilising capacity of the isolate, although the present experiment does not directly identify the biochemical pathway responsible. Recent field evidence similarly shows that biochar-PGPR co-application can improve soil organic matter and plant-available nutrients, but these responses are context dependent (Zou *et al*., 2024). Importantly, biochar chemistry can also restrict *Bacillus megaterium* growth and P-solubilising activity, demonstrating that positive biochar-microbe interactions cannot be assumed for all feedstocks or pyrolysis conditions (Liu *et al*., 2026).

The microbial population data and SEM observations provide a plausible physical basis for the co-application response. The corncob biochar contained abundant pore spaces, and T5 supported the highest culturable microbial population by day 7. Biochar pores and surface functional groups can provide attachment sites, retain water and dissolved nutrients, and reduce

direct exposure of inoculated cells to environmental fluctuations, which is why biochar has been proposed as a carrier for microbial inoculants (Bolan *et al*., 2023; Kesavardhini *et al*., 2026). Consistent with this interpretation, Zou *et al*. (2024) reported that combined biochar and PGPR application increased the abundance of several beneficial bacterial groups and altered rhizosphere community diversity. Nevertheless, plate counts in the present study quantify culturable microorganisms rather than the persistence of S2-4a1 specifically. We therefore cannot conclude that the higher counts represent successful long-term colonisation by the inoculated strain. Strain-specific tracking or amplicon/metagenomic sequencing would be required to verify this mechanism.

The carbon-cycle measurements showed a different response pattern from the final soil chemical properties. T3 produced the highest early SMR and the highest DOC and MBC, suggesting that 1% biochar with S2-4a1 provided a favourable balance between accessible carbon and microbial biomass during the incubation. SMR showed a pronounced initial flush, declined during the following days, and then increased again around day 18 in the inoculated treatments while the control remained comparatively low. A cautious explanation is a two-phase pattern of substrate use: readily available carbon may have supported the initial respiration flush, after which microbial turnover, release or desorption of retained organic compounds, and use of less readily available substrates could have contributed to the later increase. Because S2-4a1 alone also showed a secondary rise, the day-18 response cannot be attributed to biochar alone. In addition, DOC and MBC were not measured specifically on day 18 and microbial community succession was not monitored, so this mechanism remains a hypothesis. This caution is consistent with recent evidence showing that biochar effects on respiration and carbon mineralisation vary with application rate, substrate quality, and microbial carbon-use efficiency (Kalu *et al*., 2024; Bekchanova *et al*., 2024).

Plant growth responses were more selective than the soil responses. Height was not significantly affected, whereas stem diameter differed at selected dates and stem dry biomass was greatest in T4. This indicates that co-application affected biomass allocation and stem development more clearly than final plant height. Observed leaf area peaked near 90 DAT and declined thereafter across treatments. Because this variable was estimated from dimensions of sampled leaves rather than total canopy leaf area, the decline may reflect changes in the developmental stage, position, or cohort of the sampled leaves rather than a reduction in whole-plant foliage. Leaf expansion and retention in *Coffea arabica* vary with leaf age, axis position, season, and water availability (Rakocevic and Matsunaga, 2018). The non-significant root dry biomass response also requires caution. A 180-day experiment in a finite rooting volume can constrain root elongation and architecture independently of treatment effects, and pot binding can alter plant morphophysiology and the interpretation of controlled-environment experiments (Hill *et al*., 2024). The present results therefore support a treatment effect on stem biomass but do not rule out unmeasured effects on root length, branching, surface area, or root function.

The physiological measurements should be interpreted within the well-watered, non-stressed conditions of this experiment. Higher biochar rates increased proline and soluble sugars, while amended treatments generally had lower H_2O_2 and membrane damage and higher relative water content than the control. These variables are involved in osmotic adjustment, carbon metabolism, redox regulation, and membrane stability, but their alteration does not demonstrate mitigation of drought, salinity, or another imposed stress because no such stress treatment was applied. The lower H_2O_2 together with reduced membrane damage is most appropriately interpreted as improved cellular redox status and membrane integrity under nursery conditions. A specific ROS-scavenging mechanism cannot be established because antioxidant enzymes such as superoxide

dismutase, catalase, ascorbate peroxidase, and peroxidase, as well as defence-related gene expression, were not measured. Recent studies provide mechanistic context: *Bacillus megaterium* can modulate antioxidant- and drought-responsive genes in rice under drought (Lee *et al*., 2024), and combined biochar with plant growth-promoting microorganisms has been associated with changes in H_2O_2, antioxidant enzymes, proline, and phenolic metabolism in tomato (Carlo *et al*., 2025). Those stress-focused studies support plausible pathways but are not direct evidence that the same mechanisms operated here. Similarly, the strong responses reported for biochar-PGPR combinations under salinity stress (Salehi *et al*., 2025) should not be extrapolated to the present non-stressed nursery system. Overall, the data support rate-dependent co-application effects on soil functioning and coffee seedling physiological status, while formal biochar x PGPR synergy, specific plant-microbe signalling pathways, and stress tolerance remain to be tested directly.

The correlation matrix further linked the soil and plant responses observed across treatments. The strong positive relationships of soil CEC, organic matter, total carbon, and exchangeable K with one another indicate that the main soil-fertility responses occurred as a coordinated set rather than as isolated changes. Their positive associations with chlorophyll pigments, proline, and aboveground biomass are consistent with the interpretation that improved nutrient-retention capacity and K availability were accompanied by a more favorable physiological status of the coffee seedlings. Conversely, the negative relationships of H_2O_2 and membrane damage with exchangeable K and Mg suggest that improved soil nutrient status coincided with lower indicators of oxidative and membrane disturbance under the well-watered nursery conditions. These associations strengthen the integration of the soil and plant datasets, but they should not be interpreted as direct evidence of causation because treatment effects can generate covariance among variables.

Conclusion

Co-application of S2-4a1 with corncob-derived biochar altered both soil and coffee seedling responses during the 180-day well-watered greenhouse experiment. The 1% biochar treatment produced the clearest response in DOC, MBC, and short-term microbial respiration, whereas 2-3% biochar with S2-4a1 more consistently improved CEC, organic matter/total carbon, exchangeable K, stem dry biomass, relative water content, and membrane integrity. Lower H_2O_2 and membrane damage indicate a more favourable physiological and redox status under nursery conditions, but they do not demonstrate stress mitigation because no environmental stress was imposed. Because the experiment did not include matched biochar-only treatments, formal biochar x PGPR synergy cannot be separated from co-application effects. In addition, finite pot volume may have constrained root development, and the proposed microbial and antioxidant mechanisms were not measured directly. Larger-container and field nursery trials, strain-specific microbial tracking, root architectural measurements, and direct assays of antioxidant enzymes or gene expression are therefore needed before broader agronomic recommendations are made.

CRediT authorship contribution statement

Sasiprapa Kullachonphuri: Writing – original draft, Investigation, Formal analysis, Data Curation, Writing – review & editing. **Thanabodee Sriwichaikaew:** Data Curation, Methodology. **Piyaphad Ninlaphong, and Phuangphet Hemrattrakun:** Formal analysis, Data Curation, Writing – review & editing. **Kesinee Iamsaard, Butian Wang and Yupa Chromkaew:** Supervision, Conceptualization, Formal analysis, Writing – review & editing. **M. Scott Demyan:** Writing – review & editing, Visualization, Supervision. **Nuttapon Khongdee:** Writing – review

& editing, Conceptualization, Resources, Supervision, Funding acquisition, and Final manuscript revision.

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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.

Data Availability

Data will be made available on request.

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