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Elevated CO_2 Influences the Growth, Root Morphology, and Leaf Photosynthesis of Cacao (*Theobroma cacao* L.) Seedlings

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Abstract: Growing quality seedlings is a challenge for sustainable cacao production as the survival rate of young seedlings is strongly influenced by environmental factors that affect the productivity of cacao farmers. In this study, cacao (*Theobroma cacao* L.) seedlings were cultivated in a nursery, and the effects of elevated CO_2 concentrations (approximately 800 ppm) applied to cacao seedlings during daytime (6:00–17:59) on the root growth, morphology, and leaf photosynthetic capacity were examined. Treatment with elevated CO_2 significantly improved root growth, dry matter weight, and root/shoot ratio. Three-dimensional imaging of roots showed that lateral roots grew longer horizontally, lateral roots and fine roots were distributed over a larger area, and root surface and root volume increased significantly under elevated CO_2 treatment. Accurate quantification of root morphology using X-ray CT indicated that the treatment with elevated CO_2 concentrations may significantly affect root quality during the seedling stage by expanding the distribution range of lateral and fine roots, which increases the ability of lateral roots to elongate and absorb water and nutrients from the superficial layers. The photosynthetic characteristics of the aboveground leaves of cacao seedlings exposed to elevated CO_2 concentrations showed a tendency to adapt to elevated CO_2 concentrations by increasing light-use efficiency and CO_2-use efficiency. Therefore, the treatment of cacao seedlings with elevated CO_2 concentrations improved the growth quality of seedlings due to the characteristics of the roots as large sinks.

Keywords: quality seedlings; nursery; root volume; root surface; 3D image

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1. Introduction

Cacao (*Theobroma cacao* L.) is a tropical tree native to the upper reaches of the Amazon River [1,2]. It is a perennial crop grown in the understory of rainforests in regions with an annual precipitation of 1500–2000 mm [3]. Cacao cultivation is concentrated in West Africa, Central and South America, and Southeast Asia, where it is an important commercial crop. Cacao beans are the main ingredients of chocolate, and 5.24 million tons were produced in 2020/2021, approximately 10% higher than the 4.74 million tons produced in 2010/2011 [4,5]. However, the demand for cacao beans is expected to increase more rapidly than the production rate. Sustainability is a key issue, and the productivity of cacao beans requires major improvement [6]. The cacao seedlings are grown in nurseries, and the survival rate of young seedlings is strongly influenced by environmental factors, such as soil moisture, temperature, and light levels, which affect cacao productivity [7–11]. Therefore, growing quality seedlings is a major challenge in sustainable cacao production.

Atmospheric CO_2 concentrations are predicted to rise to 421–936 ppm by the end of this century [12]. An increase in CO_2 concentration increases the photosynthetic rate of plants and often improves their water-use efficiency by reducing their stomatal conductance and transpiration rates [13,14]. When CO_2 concentrations increased from 380 ppm to approximately 550–700 ppm, the photosynthetic rate of C3 plants increased by approximately 38% [15,16]. In a previous study on the tropical crop cacao, increasing CO_2 concentrations from approximately 400 to 700 ppm increased the dry matter weight of the aboveground leaves in ten-month-old cacao seedlings [17]. However, studies on the morphology and distribution of underground roots are lacking. Baligar et al. measured the instantaneous leaf photosynthetic response of 1.5-year-old cacao seedlings at different CO_2 concentrations and reported that the photosynthetic rate increased by 33%, stomatal conductance decreased by 65%, and water-use efficiency increased at CO_2 concentrations between 370 and 680 ppm [18]. However, few studies have focused on the photosynthetic capacity of cacao leaves under continuous treatment with elevated CO_2 concentrations [17,19].

Reportedly, 40% of the carbon fixed by photosynthesis is invested underground, including in rhizomes [20]. Thus, quantifying the root system is essential to understanding root growth in detail. Roots can be compared manually [21]; measuring root weight including that of the lateral roots and root length; and calculating the specific root length (ratio of root length to weight). The vertical distribution of the root system in cacao has also been reported by comparing the distribution of root length density and root density below the soil surface [22–24]. Root mass has been measured in rice plants using the monolithic method [25] and in pepper plants by placing a grid on a soil section, driving a metal cylinder perpendicular to the center of a grid perpendicular to the section, and then collecting soil samples containing roots [26]. Recently, neutron radiography and other methods have been explored; however, these require special facilities and are difficult to implement [27]. A simple and rapid measurement method employing a 2D optical scanner to acquire images followed by software-based analysis has been recently used to study root mass. [28]. In this method, the roots were washed with water to separate them from the culture medium, placed in a tank to loosen them and avoid overlapping lateral and fine roots, and 2D images were captured and analyzed. This method is efficient to measure roots with planar morphology; however, accurately measuring roots with a 3D morphology is challenging due to physical overlap. Three-dimensional images are more effective than 2D images, particularly for volume measurements. The imaging time, reconstruction time, and resolution of X-ray computed tomography (CT) scanners have improved, and the use of X-ray CT scanners for root phenotypic classification has major potential applicability [29,30].

This study aimed to examine the effects of treating cacao seedlings with and without elevated CO_2 (approximately 800 ppm) for 124 days during the daytime (06:00–17:59) on the growth and morphology of the aboveground and underground parts of the seedlings. The morphology and distribution of the underground roots were observed using 3D imaging, and their volume and surface area were quantified separately for the main, lateral, and fine roots.

2. Materials and Methods

2.1. Plant Materials and Growing Environment

The experiments were conducted in a greenhouse at Tokyo University of Agriculture and Technology (Fuchu City, Tokyo, Japan). *Theobroma cacao* L. (Variety, Trinitario) seedlings grown from seeds were purchased (Okuhida Farm Co., Ltd., Gifu, Japan). In March 2021, the first-year plants were repotted into 8.8 L pots (270 mm diameter, 236 mm depth, Kaneya Co., Ltd., Aichi, Japan) filled with 1.6 ± 0.1 kg of water-retentive and aerated acidic pumice and 3.7 ± 0.1 kg of natural coconut shell fiber swollen with water (Kaneko Seeds Co., Ltd., Gunma, Japan). Plant height, stem diameter at 100 mm from the base of the plant, and the number of leaves were measured on 24 April 2021, and 15 plants with similar growth were selected for subsequent analysis. Six plants were treated with elevated CO_2 and six plants were treated with ambient CO_2 for 124 days. Growth of the three plants was measured at the beginning of the experiment. The plants were treated with 0.5 to 0.6 L/day commercial nutrient solution (50% Otsuka-A solution; OAT Agrio Co., Ltd., Tokyo, Japan) via drip application throughout the growth period. The electrical conductivity (EC) and pH of the nutrient solutions ranged from 0.5 to 0.7 mS/cm and 6.0 to 7.0, respectively. The soil moisture content was controlled at 31.2–40.4%. The CO_2 application system was controlled using a Breath 4100 CO_2 controller (Omnia Concerto Co. Ltd., Tokyo, Japan). In the elevated CO_2 area, CO_2 was applied to the cacao seedlings from 06:00 to 17:59 through porous tubes from a liquefied CO_2 gas cylinder. In the elevated and ambient CO_2 areas, the CO_2 concentration was continuously measured at two points between the plants using a sensor (Murata Manufacturing Co., Ltd., Kyoto, Japan). The temperature and humidity were continuously measured at two points between the plants using a sensor (Sensirion AG, Zurich, Switzerland). The plants were grown under two different CO_2 treatments for 124 days between 1 May 2021 and 2 September 2021. As two plants in the ambient CO_2 area died because of a defect in the liquid fertilizer tube by 2 September 2021, four plants in the ambient CO_2 area and six plants in the elevated CO_2 area were chosen for further analysis.

2.2. Growth Measurement and Root Extraction Methods

Above-ground parts were removed and separated into leaves and stems, and underground parts were removed from the pot at room temperature (23.1 °C, RH 68%). The pumice, natural coconut shell fibers, and roots were removed from the pots, and the pumice at the bottom was separated from the roots using tweezers or other tools. Natural coconut shell fibers were carefully removed from the roots, and the roots were separated by hand shaking to remove natural coconut shell fibers. This process occurred for approximately 2 h per plant and the roots that maintained their 3D structures were extracted. Each plant part was dried at 80 °C for 72 h, and the dry matter weight of each part was measured. The number of leaves, stem diameter (using calipers; 100 mm from the base of the plant), and stem length were measured. The growth ratios were calculated as follows: Relative growth rate (RGR, g g^{-1} day^{-1}) = [ln $(Wt_2/Wt_1)/(T_2 - T_1)$], where Wt is the total plant dry matter weight (shoot + root), T is the time in days, and 1 and 2 refer to the beginning and end of the treatment, respectively. The root/shoot ratio (R/S) = [Wr/Ws], where Wr is the root dry matter weight and Ws is the shoot dry matter weight. Leaf/shoot ratio (L/S) = [Wl/Ws], where Wl is the leaf dry matter weight and Ws is the shoot dry matter weight.

2.3. Leaf Stomatal Measurement

For the leaves, one leaf per plant was observed under a microscope (TM-3000, Hitachi High-Tech Co., Ltd., Tokyo, Japan) to estimate the stomatal number and area, which were quantified using ImageJ-Fuji software. The number of stomata per unit area was counted, and the stomatal area was calculated as the lowest height area at the center of the stomata per unit area of leaves collected during the day. Measurements were acquired from the youngest, fully mature, and stiffest leaves generated under the experimental treatments for each plant. Images were obtained 120–121 days after the start of treatment.

2.4. Three-Dimensional Root Imaging and Root Volume and Surface Measurement

X-ray CT imaging was performed using a Phoenix v|tome|x m300 (Baker Hughes Company, Houston, TX, USA) owned by the JMC Corporation. The equipment specifications and imaging conditions are listed in Tables 1 and 2. The volume and surface area of the main, lateral, and fine roots were analyzed from the volume data.

Table 1. Specifications of the X-ray CT used in this study.

Items	Specifications
X-ray tube type	Open directional microfocus X-ray tube, equipped with 2 tubes
Maximum voltage/Maximum power	300 kV/500 W
Geometrical magnification	1.3× to 100×
Minimum voxel size	2 µm
Minimum detection size	4 µm
Detector type	Dynamic 41 ∣ 100, Flat panel detector, 410 × 410 mm, 4048 × 4048 pixels, pixel size 100 µm
Focus–detector distance	800 mm
Maximum focus object distance	600 mm
Manipulation	Granite-based precision 4-axis manipulator
Rotatable angle	0–360°
Equipment	Phoenix V ∣ tome ∣ x M300
Software	Phoenix datos ∣ x

Table 2. Imaging conditions of the X-ray CT system. The root was placed in a cardboard box, fixed with a cotton ball, rotated from 0° to 360°, and imaged using an X-ray CT.

Items	Conditions
Voxel size	0.0673 mm
Number of pictures	2700–3600 sheets
Exposure time	334 ms
Voltage	100 kV
Power	150 µA

2.5. Leaf Photosynthetic Rate Measurement

Leaf photosynthetic curves of one leaf per plant were measured between 09:00 and 16:40 h using a portable photosynthetic system with red and blue LED lights (465 and 670 nm, respectively) as light sources (LI-6400, LI-COR, Inc., Lincoln, NE, USA). Measurements were acquired for the youngest, fully mature, and stiffest leaves generated under the experimental treatments for each plant. CO_2-response curves were measured 70–76 days after the start of treatment. The photosynthetic rate was measured at eight different CO_2 concentration levels (2000, 1500, 1000, 700, 400, 200, 50, and 0 µmol mol^{-1}) at a photosynthetic photon flux density of 1000 µmol m^{-2} s^{-1}. The measurements were recorded after stabilization (for 8 min) at each CO_2 concentration. Measurements were recorded at a chamber temperature of 25.0 ± 0.0 °C, relative humidity of 79.5 ± 2.7%, and airflow rate of 500 µmol s^{-1}, and the CO_2 concentration was reduced in steps. Light response curves were measured 99–115 days after treatment initiation. Photosynthetic rates were measured at eight photosynthetic photon flux densities (1000, 700, 500, 300, 200, 100, 50, and 0 µmol m^{-2} s^{-1}) at a CO_2 concentration of 400 µmol m^{-2} s^{-1}. The measurements were performed after stabilization (for 8 min) at each photosynthetic photon flux density. Measurements were recorded at a chamber temperature of 25.0 ± 0.0 °C, relative humidity of 74.8 ± 2.8%, and airflow rate of 500 µmol s^{-1}, and the photosynthetic photon flux density was reduced in steps.

2.6. Statistical Analysis

Data were analyzed for variance in the measured parameters between the ambient and elevated CO_2 treatments using the F-test in Microsoft Excel for Microsoft 365 MSO (Version 2305 Build 16.0.16501.20074) 64-bit (Microsoft Corporation, Redmond, Washington, USA). Significant differences were determined using Student's *t*-test for equal variance and Welch's *t*-test for unequal variance.

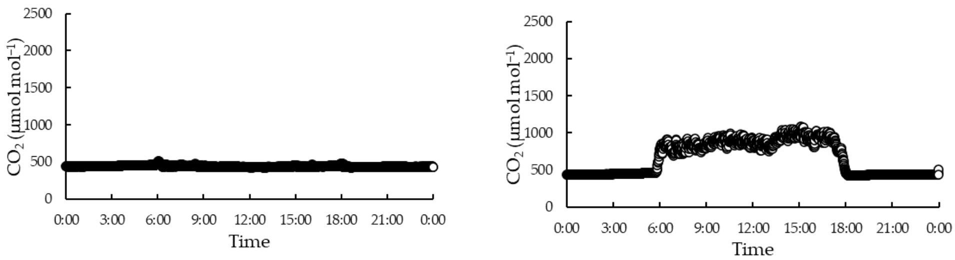
3. Results

3.1. Growing Environment

Table 3 shows the environmental conditions during the 124 days of treatment in the ambient CO_2 and elevated CO_2 areas. Under ambient CO_2, the temperature was 31.0 ± 3.3 °C, relative humidity was 57.6 ± 12.7%, and CO_2 concentration was 512.5 ± 112.6 ppm during the day. Meanwhile, under the elevated CO_2 treatment, the temperature was 30.6 ± 3.3 °C, relative humidity was 58.0 ± 12.8%, and CO_2 concentration was 801.6 ± 178.8 ppm during daytime. Temperature and relative humidity under the elevated CO_2 treatment were not significantly different from those under ambient CO_2; however, the CO_2 concentration under the elevated CO_2 treatment was 60% higher than that under ambient CO_2 during the day. Meanwhile, no significant differences were present between the two areas during the night (18:00–05:59). The diurnal changes in CO_2 concentrations were similar to those under ambient CO_2, but approximately 800 ppm of CO_2 was applied to the elevated CO_2 treatment during the daytime (Figure 1).

Table 3. Average temperature, relative humidity, and CO_2 concentration during the daytime and nighttime when cacao seedlings were grown in a greenhouse under ambient and elevated CO_2 conditions. Data presented as mean value ± SD, with daily means averaged over 124 days.

		Temperature (°C)	**Relative Humidity (%)**	**CO_2 Concentration (μmol mol^{-1})**
Ambient CO_2	Daytime (06:00–17:59)	31.0 ± 3.3	57.6 ± 12.7	512.5 ± 112.6
	Nighttime (18:00–05:59)	25.0 ± 2.9	73.9 ± 7.1	513.3 ± 83.5
Elevated CO_2	Daytime (06:00–17:59)	30.6 ± 3.3	58.0 ± 12.8	801.6 ± 178.8
	Nighttime (18:00–05:59)	24.7 ± 2.9	75.5 ± 7.3	517.1 ± 87.0



(**A**) (**B**)

Figure 1. Diurnal changes in CO_2 concentration for cacao seedlings grown in a greenhouse under ambient and elevated CO_2 conditions. CO_2 concentration was measured every minute under (**A**) ambient and (**B**) elevated CO_2 conditions. Data are presented as minute-by-minute averages over 18 days within the treatment period.

3.2. Growth

The elevated CO_2 treatment significantly increased the dry matter weight of the plants (Figure 2A). Plant dry matter increased by approximately 30% ($p < 0.05$). More assimilates were allocated underground than aboveground, and the root dry matter weight increased by 62%, on average ($p < 0.01$). The relative growth rate (RGR) increased by 20%, on average ($p < 0.05$) and the root/shoot ratio (R/S) increased by 30%, on average ($p < 0.05$) (Figure 2C). However, elevated CO_2 treatment did not affect the rate of increase in leaf number, stem diameter, stem length, or aboveground leaf/shoot ratio (L/S) (Figure 2B).

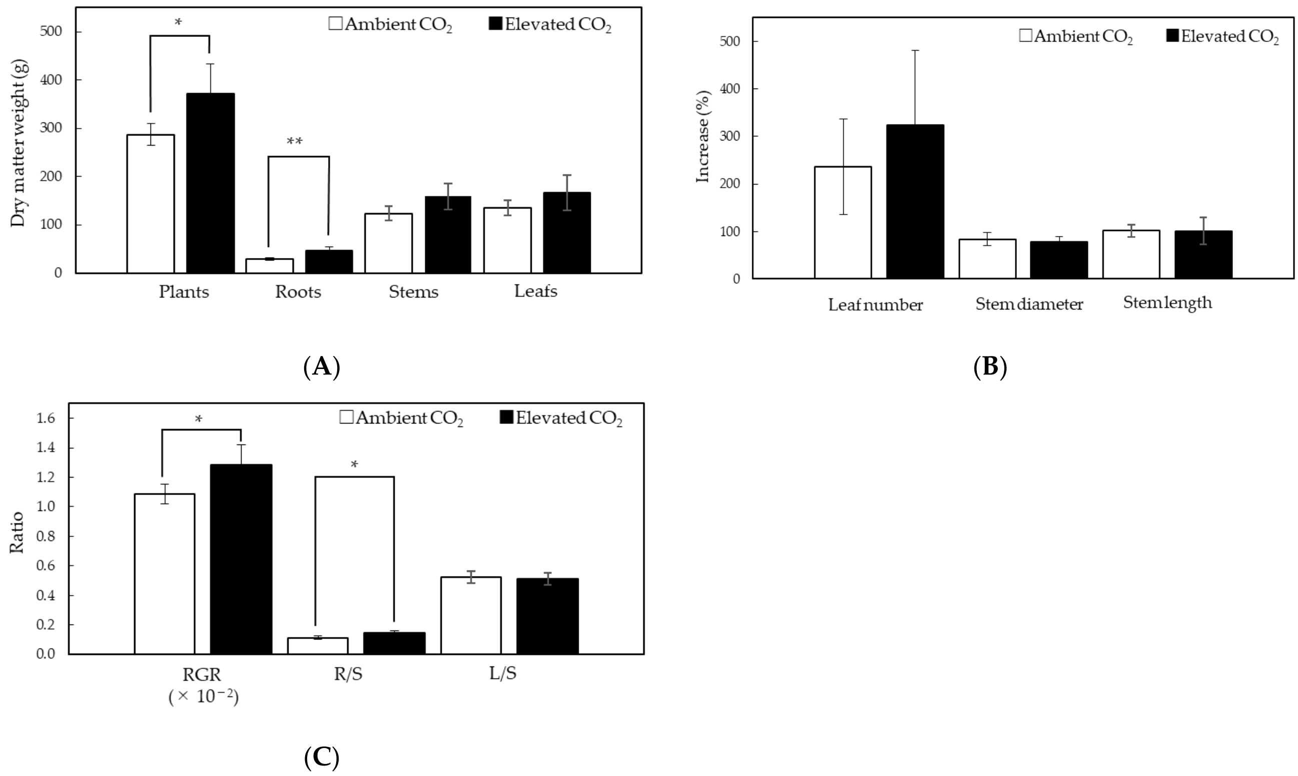


Figure 2. Growth response of cacao seedlings grown under ambient and elevated CO_2 conditions. (**A**) Dry matter weight in cacao seedlings, (**B**) percentage increase in aboveground of cacao seedlings, and (**C**) relative growth rate (RGR), root/shoot ratio (R/S), and leaf/shoot ratio (L/S) of cacao seedlings. The ambient and elevated CO_2 concentrations in the greenhouse are represented by the white and black bars, respectively. Data are presented as the mean value ± SD, n = 4 (ambient CO_2), n = 6 (elevated CO_2); * indicates a statistically significant difference ($p < 0.05$, *t*-test), ** indicates a statistically significant difference ($p < 0.01$, *t*-test), and the vertical bars indicate SD.

3.3. Leaf Stomata

The number of stomata in the leaves grown under elevated CO_2 decreased by an average of 5%, and the stomatal area of these leaves decreased by an average of 17%. The elevated CO_2 treatment had no significant effect on leaf stomatal density and area (Figure 3).

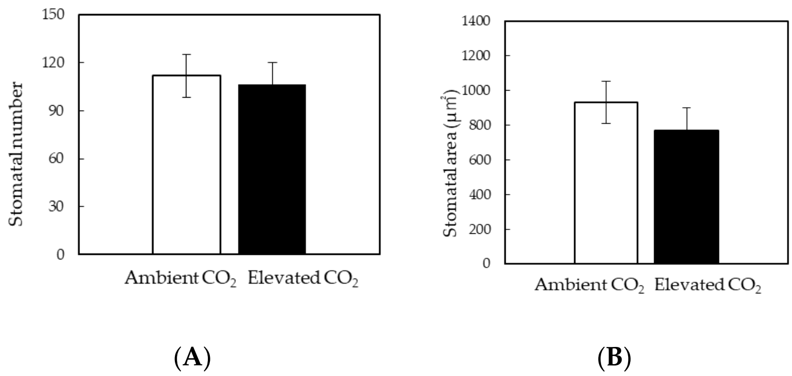


Figure 3. Growth response of cacao seedlings under ambient and elevated CO_2 conditions. (**A**) Stomatal number per unit area of cacao leaves, and (**B**) stomatal area per unit area of cacao leaves. The ambient and elevated CO_2 concentrations in the greenhouse are represented by the white and black bars, respectively. Data are presented as the mean value ± SD, n = 4 (ambient CO_2), n = 6 (elevated CO_2); the vertical bars indicate SD.

3.4. Three-Dimensional Root Imaging, Root Volume, and Surface Area

Underground roots were extracted while maintaining their 3D structure (Figure 4). Three-dimensional images were created by reconstructing the volume data obtained using X-ray CT, with a voxel size of 0.0673 mm (Figure 5). The main roots were thick and some were bent and vertically elongated (green areas in Figure 5A–C). Lateral roots developed horizontally from the periphery of the main root and stem (arrows in Figure 4), and fine roots developed around the lateral roots throughout the root. Roots grown under elevated CO_2 had longer lateral roots and a more extensive distribution of lateral and fine roots in all corners of the pot than those grown under ambient CO_2 (Figures 4C,D and 5C,D); in contrast, roots grown under ambient CO_2 had a narrower and more skewed distribution of lateral and fine roots (Figures 4A,B and 5A,B). The elevated CO_2 treatment significantly increased the total root volume (Figure 6A). Roots grown under elevated CO_2 conditions exhibited an increased total root volume by an average of 67% ($p < 0.01$) compared to roots grown under ambient CO_2. The volume of the main roots increased by an average of 52% and that of the lateral and fine roots increased by an average of 88% ($p < 0.05$). Treatment with elevated CO_2 increased the surface area of the entire root by an average of 77% ($p < 0.05$) (Figure 6B). The surface area of the main roots increased by an average of 37%, whereas that of the lateral and fine roots increased by an average of 81% ($p < 0.05$).

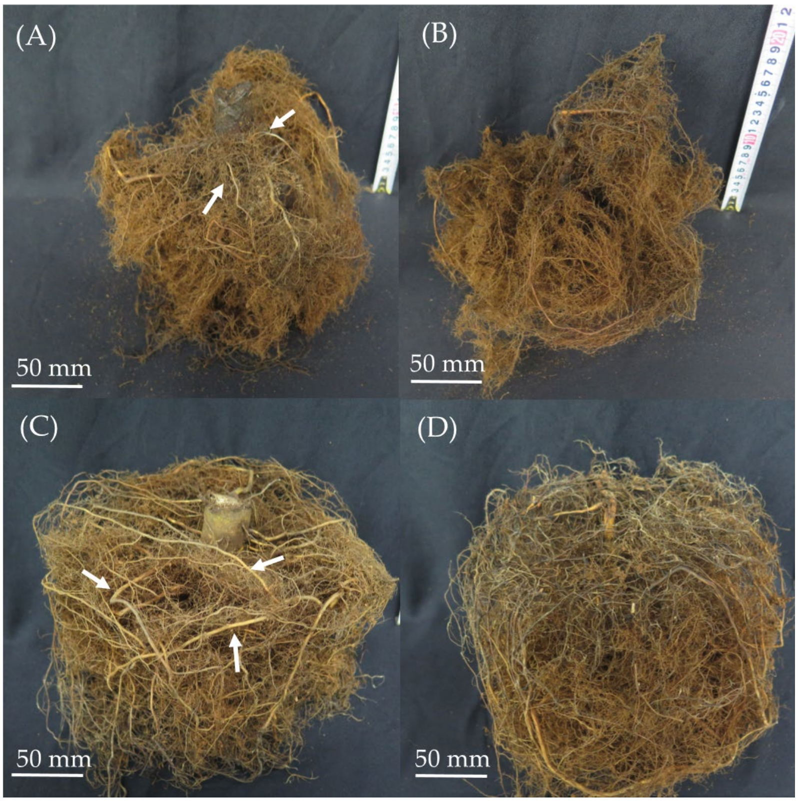


Figure 4. Comparison of root system excluding the culture soil (natural coconut shell fiber and pumice) of the underground part of cacao seedlings grown for 124 days under ambient and elevated CO_2 conditions. (**A**) Ambient CO_2, from upper side; (**B**) ambient CO_2, from bottom side; (**C**) elevated CO_2, from upper side; and (**D**) elevated CO_2, from bottom side. Arrows indicate lateral roots that develop horizontally from the periphery of the main root and stem.

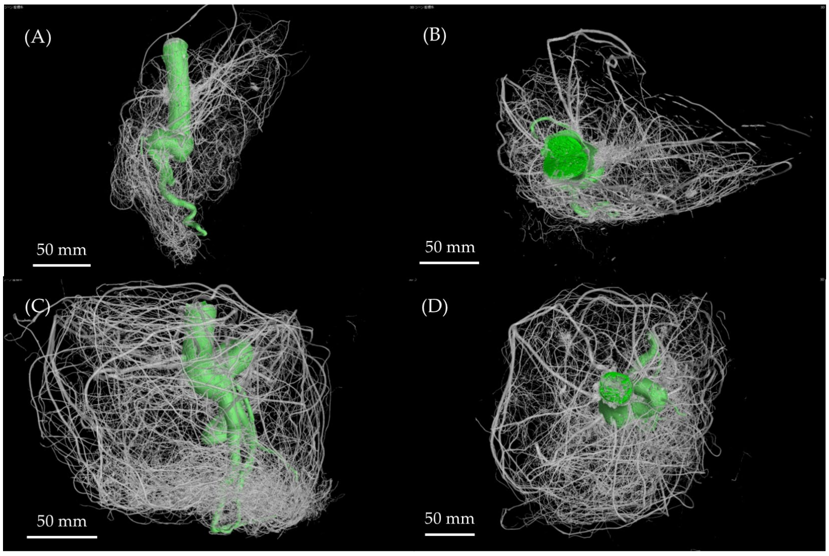
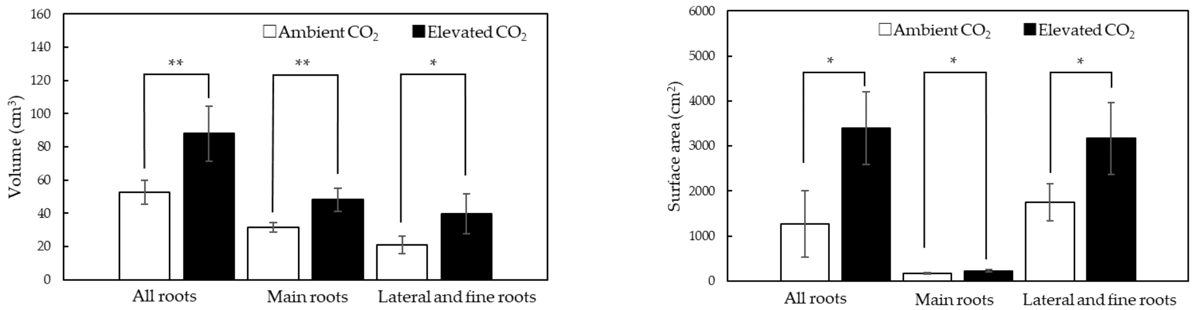


Figure 5. X-ray CT comparison of 3D root images of the roots shown in Figure 4. (**A**) Ambient CO_2, X-axis direction; (**B**) ambient CO_2, Z-axis direction; (**C**) elevated CO_2, X-axis direction; and (**D**) elevated CO_2, Z-axis direction. Voxel size 0.0673 mm, green areas indicate main roots and white areas indicate lateral and fine roots.

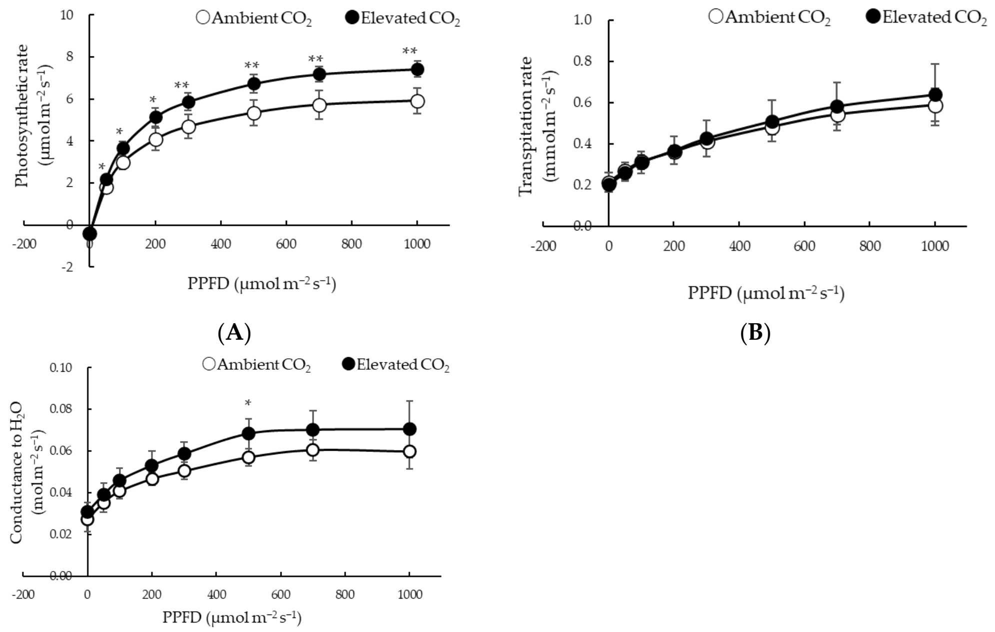


(**A**) (**B**)

Figure 6. Three-dimensional X-ray CT images highlighting differences in root volume and surface area measured for the roots of cacao seedlings grown for 124 days under ambient and elevated CO_2 conditions. (**A**) Volume of roots and (**B**) surface area of roots. The ambient and elevated CO_2 concentrations in the greenhouse are represented by the white and black bars, respectively. The data are presented as the mean value ± SD, n = 4 (ambient CO_2), n = 6 (elevated CO_2); * indicates a statistically significant difference at $p < 0.05$ (*t*-test), ** indicates a statistically significant difference ($p < 0.01$, *t*-test), and the vertical bars indicate SD.

3.5. Leaf Photosynthetic Rate

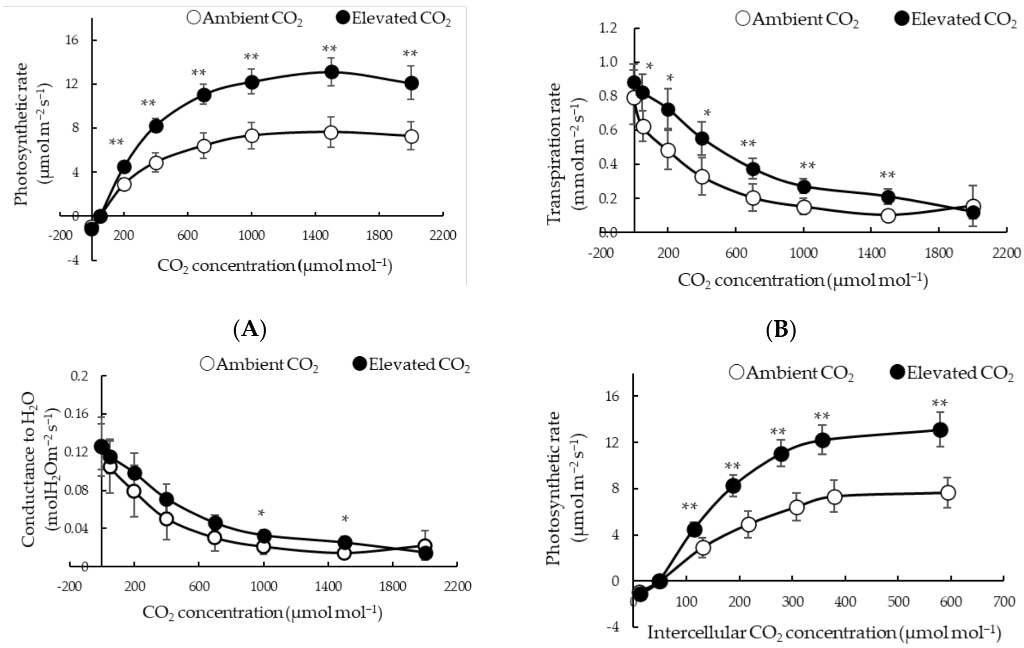
The photosynthetic rate of leaves grown under elevated CO_2 significantly increased at photosynthetic photon flux densities of 1000, 700, 500, 300, 200, 100, and 50 µmol m^{-2} s^{-1} (Figure 7A) ($p < 0.01$); however, no significant differences were observed in either transpiration rate or stomatal conductance, except for the photosynthetic photon flux density of 500 µmol m^{-2} s^{-1} (Figure 7B,C). The photosynthetic rate of leaves grown under elevated CO_2 was significantly increased at CO_2 concentrations of 2000, 1500, 1000, 700, 400, and 200 µmol mol^{-1} (Figure 8A), and the transpiration rate was also significantly increased (Figure 8B) ($p < 0.01$). However, no significant differences were observed in stomatal conductance, except at the CO_2 concentrations of 1500 and 1000 µmol mol^{-1} (Figure 8C). The A/Ci curves showed that the photosynthetic rate was significantly increased in leaves grown under elevated CO_2 at leaf intercellular CO_2 concentrations of approximately 114–580 µmol mol^{-1} compared with that in leaves grown under ambient CO_2 (Figure 8D) ($p < 0.01$).



(**A**) (**B**)

(**C**)

Figure 7. Photosynthetic light–response curves of cacao leaves grown under ambient and elevated CO_2 conditions. (**A**) Leaf photosynthetic rate, (**B**) leaf transpiration rate, and (**C**) leaf stomatal conductance. Data are presented as the mean value ± SD, n = 4 (ambient CO_2), n = 6 (elevated CO_2); * indicates a statistically significant difference at $p < 0.05$ (*t*-test), ** indicates a statistically significant difference at $p < 0.01$, and the vertical bars indicate SD.



(**A**) (**B**)

(**C**) (**D**)

Figure 8. Photosynthetic response curves of cacao leaves grown under ambient and elevated CO_2 conditions. Responses of (**A**) leaf photosynthetic rate, (**B**) leaf transpiration rate, (**C**) leaf stomatal conductance, and (**D**) leaf photosynthetic rate to intercellular CO_2 concentration of cacao leaves. Data are presented as the mean value ± SD, n = 4 (ambient CO_2), n = 6 (elevated CO_2); * indicates a statistically significant difference at $p < 0.05$ (*t*-test), ** indicates a statistically significant difference at $p < 0.01$, and the vertical bars indicate SD.

4. Discussion

In this study, we investigated the effects of treating cacao seedlings with elevated CO_2 levels (approximately 800 ppm) for 124 d during the daytime (06:00–17:59) on the growth and morphology of the aboveground and underground parts of the seedlings. The experiment was conducted for 124 days under a daytime CO_2 concentration of 801.6 $\pm$ 178.8 ppm in the elevated CO_2 treatment area, or 512.5 $\pm$ 112.6 ppm under ambient CO_2 (Table 3, Figure 1). The total dry matter weight increased significantly after the treatment period ($p < 0.05$). The average dry weights of leaves, stems, and roots increased by 24%, 28%, and 62%, respectively (Figure 2A). Lahive et al. reported that the aboveground dry matter weight increased by 28.5% on average when young 4-month-old cacao seedlings were grown under elevated CO_2 (approximately 700 ppm) for 154 days; however, they did not measure the underground roots [17]. Baligar et al. reported significant increases in total and root dry matter weights when young cacao seedlings of seven genotypes were grown under elevated CO_2 (approximately 700 ppm) for 90 d [31]. Comparing the results of the present study with those of Baligar et al., the total dry matter weight, especially that of the roots (average 62% increase), increased significantly, albeit for different ages of the seedlings (Figure 2A). In addition, the relative growth rate and R/S increased significantly, whereas the aboveground L/S did not differ. Morphological changes induced by the treatment were more prominent in the roots than shoots (Figure 2).

Baligar et al. analyzed the total root length in 2D when young cacao seedlings were grown under elevated CO_2 concentrations (approximately 700 ppm) for 90 d after planting [31]. However, because this study used seedlings that were older than one year, we assumed that accurately quantifying root morphology and distribution would be challenging from 2D images without physical overlap. Considering the morphological reason for the significant increase in root dry matter weight by observing the 3D structure of the roots, X-ray CT was used to obtain clear 3D images of cacao roots, observe their 3D structure, and analyze the root volume and surface area (Figure 6). The main, lateral, and fine roots were observed by color coding the images. The lateral roots extended horizontally from the periphery of the main root and rhizome, and the fine roots were distributed around the lateral roots and throughout the main root (Figure 5). The results of this study indicate that the elevated CO_2 treatment may have greatly affected the distribution of cacao roots, especially in the surface layer, because the roots grown under the elevated CO_2 treatment had thicker and longer lateral roots that grew horizontally, and lateral and fine roots were widely distributed throughout the pot. The promotion of root elongation by elevated CO_2 treatment should be investigated in the future from the perspective of the distribution of photosynthetic assimilates and hormones involved in root elongation and cap formation.

The elevated CO_2 treatment increased root surface area and volume by an average of 77% and 67%, respectively, with those of the lateral and fine roots increasing more than those of the main roots (Figure 6). These results indicate that continuous treatment with elevated CO_2 increased the number of lateral and fine roots as well as the surface area for increased nutrient and water absorption, and enhanced rooting. Santos et al. reported that drought stress on cacao seedlings decreased leaf area but increased root dry matter weight and volume, demonstrating that they adapted to survival conditions [32]. Therefore, treatment with elevated CO_2 may help decrease the drought stress by increasing root volume and surface area, thereby increasing the chances of obtaining water at the soil surface and in deeper layers. The amount of nutrients taken up by plants depends on their root structure and growth rate [33]. Elevated CO_2 treatment of cacao seedlings is expected to accelerate their establishment and growth in cacao plantations.

Continuous treatment under elevated CO_2 concentrations resulted in a slight increase in the number of leaves and a decrease in the number of stomata per unit area. However, the photosynthetic capacity of leaves per unit area remained high, indicating that more assimilates were produced. In this study, the leaves grown under the elevated CO_2 treatment showed significantly increased photosynthetic rates with an 11–18% increase in stomatal conductance at a photosynthetic photon flux density of 50–1000 mmol m^{-2} s^{-1}

compared with that in leaves grown under ambient CO_2, even at the same CO_2 concentration and photosynthetic photon flux density in the chamber (Figure 7A). The maximum photosynthetic rate at the leaf level is achieved under relatively low photon flux densities of approximately 400 mmol m^{-2} s^{-1} in cacao [18,34], and the photon flux density in agroforestry systems, where cacao is typically grown, is 243–1273 mmol m^{-2} s^{-1} [35]. This study showed that leaves grown under elevated CO_2 concentrations significantly increased photosynthetic rates at a photosynthetic photon flux density of 300–1000 mmol m^{-2} s^{-1}, and that an increase in light-use efficiency occurred even at low photon flux densities, such as those in shaded areas, which is a typical cacao growth condition on farms. Lahive et al. reported that the light-saturated photosynthetic rate (Pmax) of 4-month-old young cacao seedlings grown under elevated CO_2 (approximately 700 ppm) was higher than that of seedlings grown under ambient CO_2 (approximately 400 ppm) by an average of 105%, and that water-use efficiency was significantly increased [17]. In contrast, Baligar et al. reported a significantly increased photosynthetic rate, decreased stomatal conductance and transpiration rate, and significantly increased water-use efficiency in 4-month-old young cacao seedlings grown under elevated CO_2 (approximately 700 ppm) compared to that in seedlings grown under ambient CO_2 (approximately 400 ppm) [31]. In this study, the leaves grown under the elevated CO_2 treatment significantly increased the photosynthetic rate by 55–72% and the transpiration rate by 30–100% compared to those in leaves grown under ambient CO_2 at the same CO_2 concentration of 200–2000 mol mol^{-1} in the chamber. (Figure 8A) ($p < 0.01$) However, no significant differences were observed in stomatal conductance, except for CO_2 concentrations of 1500 and 1000 µmol mol^{-1} (Figure 8C). The continuous treatment of leaves with elevated CO_2 concentrations is expected to improve CO_2-use efficiency and increase leaf photosynthetic capacity. The A/Ci curves showed that the photosynthesis rate was significantly increased in leaves grown under the elevated CO_2 treatment at intercellular CO_2 concentrations in leaves of approximately 114–580 µmol mol^{-1} compared with leaves grown under ambient CO_2 (Figure 8D). Leaves treated continuously with elevated CO_2 concentrations showed significantly higher photosynthetic capacity and more efficient use of CO_2 for photosynthesis, even though the concentrations of CO_2 diffusing into the leaves were similar. Improvement in the photosynthetic capacity of leaves under continuous treatment with elevated CO_2 should be investigated in future studies in terms of the response of RuBisCO to CO_2 and O_2 [36].

Additionally, we observed the roots using 3D imaging with X-ray CT and quantified their volume and surface area. X-ray CT is widely used to nondestructively measure the internal structure of industrial metal parts and the bone structure of biological specimens owing to its reproducible measurement capability [37]. When roots with high moisture content are imaged by X-ray CT, a small amount of moisture evaporates during the imaging process, causing the roots to move and resulting in a lack of clear images. To obtain a clear image, the roots must be dried while maintaining their 3D structure. The roots were separated from the soil by shaking to maintain their 3D structures. The natural coconut-shell fiber used as the soil contained a large liquid phase, large vapor phase, and small solid phase. It is easily separated from the roots and allows X-rays to pass through because of its low density. Further studies are required to determine the environmental conditions and soil types that can aid in the separation of roots from soil. The Pearson correlation coefficient between root dry matter weight and volume analyzed using X-ray CT was $r = 0.94$, indicating a significant correlation ($p < 0.01$). Root analysis using X-ray CT was effective in qualifying and quantifying roots. However, X-ray CT is expensive in terms of equipment and maintenance.

In conclusion, in this study, we examined the effects of elevated CO_2 concentrations applied to cacao seedlings during daytime (6:00–17:59) on the root growth and morphology. We demonstrated that treatment with elevated CO_2 concentrations significantly affects root quality during the seedling stage by expanding the distribution range of lateral and fine roots, which increases the ability of lateral roots to elongate and absorb more water and nutrients from the superficial layers. Our findings may be valuable for obtaining quality seedlings with a high R/S ratio for sustainable cacao production.

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References

1. Motamayor, J.C.; Risterucci, A.M.; Lopez, P.A.; Ortiz, C.F.; Moreno, A.; Lanaud, C. Cacao domestication I: The origin of the cacao cultivated by the Mayas. *Heredity* **2002**, *89*, 380–386. [CrossRef]
2. Cheesman, E. Notes on the nomenclature, classification and possible relationships of cocoa populations. *Trop. Agric.* **1944**, *21*, 144–159.
3. Bertolde, F.Z.; Almeida, A.A.F.; Almeida, A.F.; Pirovani, C.P.; Pirovani, C.P.; Gomes, F.P.; Ahnert, D.; Baligar, V.C.; Valle, R.R. Physiological and biochemical responses of *Theobroma cacao* L. genotypes to flooding. *Photosynthetica* **2012**, *50*, 447–457. [CrossRef]
4. International Cocoa Organization. *ICCO Quarterly Bulletin of Cocoa Statistics, XLIX, 1, Cocoa Year 2022/23*; International Cocoa Organization: Abidjan, Côte d'Ivoire, 2023.
5. International Cocoa Organization. *ICCO Quarterly Bulletin of Cocoa Statistics, XLV, 1, Cocoa Year 2018/19*; International Cocoa Organization: Abidjan, Côte d'Ivoire, 2019.
6. World Cocoa Foundation. *Cocoa & Forests Initiative Annual Report Ghana 2021*; World Cocoa Foundation: Washington, DC, USA, 2021.
7. Wood, G.A.R.; Lass, R.A. *Cocoa*, 4th ed.; Wiley-Blackwell: Hoboken, NJ, USA, 2001; ISBN 978-0-470-69898-3.
8. Famuwagun, I.B.; Agele, S.O. Cacao growth and development under different nursery and field conditions. In *Theobroma Cacao, Deploying Science for Sustainability of Global Cocoa Economy*; IntechOpen: London, UK, 2019. [CrossRef]
9. Almeida, A.A.F.; Valle, R.R. Ecophysiology of the cacao tree. *Braz. J. Plant Physiol.* **2008**, *19*, 425–448. [CrossRef]
10. Zuidema, P.A.; Leffelaar, P.A.; Gerritsma, W.; Mommer, L.; Anten, N.P.R. A physiological production model for cocoa (*Theobroma cacao*). *Agric. Syst.* **2005**, *84*, 195–225. [CrossRef]
11. Lahive, F.; Hadley, P.; Daymond, A.J. The physiological responses of cacao to the environment and the implications for climate change resilience. A review. *Agron. Sustain. Dev.* **2019**, *39*, 5. [CrossRef]
12. Stocker, T.F.; Qin, V.; Plattner, G.K.; Tignor, M.; Allen, S.K.; Boschung, J.; Nauels, A.; Xia, Y.; Bex, V.; Midgley, P.M. *Climate Change 2013: The Physical Science Basis, in Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC)*; Cambridge University Press: Cambridge, NY, USA, 2013; pp. 1–30. [CrossRef]
13. Ainsworth, E.A.; Long, S.P. What have we learned from 15 years of free-air CO_2 enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO_2. *New Phytol.* **2005**, *165*, 351–372. [CrossRef]
14. Farquhar, G.D.; Sharkey, T.D. Stomatal conductance and photosynthesis. *Annu. Rev. Plant Biol.* **1982**, *33*, 317–345. [CrossRef]
15. Long, S.P.; Ainsworth, E.A.; Rogers, A.; Ort, D.R. Rising atmospheric carbon dioxide: Plants FACE the future. *Annu. Rev. Plant Biol.* **2004**, *55*, 591–628. [CrossRef]
16. Wang, L.; Feng, Z.; Schjoerring, J.K. Effects of elevated atmospheric CO_2 on physiology and yield of wheat (*Triticum aestivum* L.): A meta-analytic test of current hypotheses. *Agric. Ecosyst. Environ.* **2013**, *178*, 57–63. [CrossRef]
17. Lahive, F.; Hadley, P.; Daymond, A.J. The impact of elevated CO_2 and water deficit stress on growth and photosynthesis of juvenile cacao (*Theobroma cacao* L.). *Photosynthetica* **2018**, *56*, 911–920. [CrossRef]
18. Baligar, V.C.; Bunce, J.A.; Machado, R.C.R.; Elson, M.K. Photosynthetic photon flux density, carbon dioxide concentration, and vapor pressure deficit effects on photosynthesis in cacao seedlings. *Photosynthetica* **2008**, *46*, 216–221. [CrossRef]
19. Lahive, F.; Handley, L.R.; Hadley, P. Climate Change Impacts on Cacao: Genotypic Variation in Responses of Mature Cacao to Elevated CO_2 and Water Deficit. *Agronomy* **2021**, *11*, 818. [CrossRef]
20. De Kroon, H.; Visser, E.J. *Root Ecology*; Springer Science & Business Media: Berlin, Germany, 2003; p. 168.
21. Weaver, J.E.; Jean, F.C.; Crist, J.W. *Development and Activities of Roots of Crop Plants: A Study in Crop Ecology*; No. 316; Carnegie Institution of Washington: Washington, DC, USA, 1922.
22. Kummerow, J.; Kummerow, M.; Souza, S.W. Fine-root growth dynamics in cacao (*Theobroma cacao*). *Plant Soil* **1982**, *65*, 193–201. [CrossRef]

23. Moser, G.; Leuschner, C.; Hertel, D.; Holsche, D.; Kohler, M.; Leitner, D.; Michalzik, B.; Prihastanti, E.; Tjitrosemito, S.; Schwendenmann, L. Response of cocoa trees (*Theobroma cacao*) to a 13-month desiccation period in Sulawesi, Indonesia. *Agrofor. Syst.* **2010**, *79*, 171–187. [CrossRef]
24. Nygren, P.; Leblanc, H.A.; Lu, M.; Cristino, A.; Gómez, L. Distribution of coarse and fine roots of *Theobroma cacao* and shade tree Inga edulis in a cocoa plantation. *Ann. For. Sci.* **2013**, *70*, 229–239. [CrossRef]
25. Abe, J.; Morita, S. Growth direction of nodal roots in rice: Its variation and contribution to root system formation. *Plant Soil* **1994**, *165*, 333–337. [CrossRef]
26. Morita, S.; Toyota, M. Root system morphology of pepper and melon at harvest stage grown with drip irrigation under dessert conditions in Baja California Mexico. *Jpn. J. Crop Sci.* **1998**, *67*, 353–357. [CrossRef]
27. Nakanishi, T.; Matsubayashi, M. Nondestructive water imaging by neutron beam analysis in living plants. *J. Plant Physiol.* **1997**, *151*, 442–445. [CrossRef]
28. Arsenault, J.L.; Pouleur, S.; Messier, C.; Guay, R. WinRhizo, a root measuring system with a unique overlap correction method. *Hortic. Sci.* **1995**, *30*, 906.
29. Mooney, S.J.; Pridmore, T.P.; Helliwell, J.; Bennett, M.J. Developing X-ray computed tomography to non-invasively image 3-D root systems architecture in soil. *Plant Soil* **2012**, *352*, 1–22. [CrossRef]
30. Pfeifer, J.; Kirchgessner, N.; Colombi, T.; Walter, A. Rapid phenotyping of crop root systems in undisturbed field soils using X-ray computed tomography. *Plant Methods* **2015**, *11*, 41. [CrossRef] [PubMed]
31. Baligar, V.C.; Elson, M.K.; Almeida, A.A.F.; de Araujo, Q.R.; Ahnert, D.; He, Z. The impact of carbon dioxide concentrations and low to adequate photosynthetic photon flux density on growth, physiology and nutrient use efficiency of juvenile cacao genotypes. *Agronomy* **2021**, *11*, 397. [CrossRef]
32. Santos, E.A.d.; Almeida, A.A.F.d.; Branco, M.C.d.S.; Santos, I.C.d.; Ahnert, D. Path analysis of phenotypic traits in young cacao plants under drought conditions. *PLoS ONE* **2018**, *13*, e0191847. [CrossRef]
33. Barber, S.A. *Soil Nutrient Bioavailability: A Mechanistic Approach*; John Wiley & Sons: New York, NY, USA, 1995.
34. Balasimha, D.; Daniel, E.V.; Bhat, P.G. Influence of environmental factors on photosynthesis in cocoa trees. *Agric. For. Meteorol.* **1991**, *55*, 15–21. [CrossRef]
35. Niether, W.; Armengot, L.; Andres, C.; Schneider, M.; Gerold, G. Shade trees and tree pruning alter throughfall and microclimate in cocoa (*Theobroma cacao* L.) production systems. *Ann. For. Sci.* **2018**, *75*, 38. [CrossRef]
36. Salazar, J.C.S.; Melgarejo, L.M.; Casanoves, F.; Rienzo, J.A.D.; DaMatta, F.M.; Armas, C. Photosynthesis limitations in cacao leaves under different agroforestry systems in the Colombian Amazon. *PLoS ONE* **2018**, *13*, e0206149. [CrossRef]
37. Plessis, A.d.; Broeckhoven, C. Looking deep into nature: A review of micro-computed tomography in biomimicry. *Acta Biomater.* **2019**, *85*, 27–40. [CrossRef]