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Impact of Elevated Carbon Dioxide and Planting Density on the Growth and Physiological Responses of *Stevia rebaudiana* Bertoni

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ABSTRACT

Stevia rebaudiana Bertoni produces steviol glycosides (SGs) which are 300-400 times sweeter than table sugar, non-caloric in nature, and used by diabetic patients worldwide. Increasing level of carbon dioxide (CO_2) in the atmosphere, due to environmental pollution and climate change, have the potential to influence crop growth and productivity including stevia. Additionally, planting density is another important agronomic factor that affects crop yield, but its interaction with CO_2 levels in stevia has not been adequately studied. Therefore, the current study was conducted with the objective to investigate the effects of elevated CO_2 (eCO_2) under different planting densities on the growth and physiological responses of *Stevia rebaudiana*. A factorial (2×3) experimental design was employed, with two CO_2 levels (400 ppm as ambient CO_2 [aCO_2] and 1200 ppm as eCO_2) and three planting densities: high-density vertical (HDV), high-density horizontal (HDH), and low-density horizontal (LDH). Growth data were collected monthly until the final harvest, while physiological parameters were recorded at 1st and 3rd month after planting (MAP). The results indicated that eCO_2 significantly enhanced plant growth, with the highest plant height (77.1 cm) observed in eCO_2-treated plants compared to aCO_2 (66.5 cm) at final harvest. Under aCO_2, HDH and HDV treatments resulted in taller plants than LDH at 1st MAP, but no significant differences were found at later stages. eCO_2-treated plants also showed increased branching (25%–28% more at 1st–4th MAP) compared to aCO_2-treated plants. Photosynthesis rates were 36% and 42% higher in eCO_2 plants at the 1st and 3rd MAP, respectively. LDH plants demonstrated better overall physiological performance, including higher photosynthetic rates and water use efficiency. In conclusion, eCO_2 significantly improves stevia growth and

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physiology, with LDH and HDH densities showing superior performance. These results suggest that optimizing both CO_2 levels and planting density can improve stevia productivity, particularly under future climate conditions.

Keywords: Antioxidant, natural sweetener, rebaudiosides, steviosides, stomatal conductance

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INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a perennial herbaceous plant species which belong to the family of Asteraceae, native to specific regions of South America, specifically Brazil and Paraguay (Geuns, 2007). However, the cultivation of Stevia has expanded globally, with significant production now occurring in countries like Argentina, Japan, China, Korea, Russia, Mexico, Indonesia, the United States, Malaysia, Tanzania, Canada and Thailand (Alvarez-Robles et al., 2016; Lemus-Mondaca et al., 2012; Sumon et al., 2008). Stevia produces special secondary metabolites which are known as steviol glycosides (SGs), that are unique to *Stevia rebaudiana*, distinguishing it from other plant species (Brandle et al., 1999). It has been reported that some of the steviol glycosides derived from Stevia are 300–400 times sweeter than sucrose (Moraes et al., 2013; Yadav et al., 2011), and these compounds are non-caloric, making them suitable for consumption by individuals with diabetes, as they do not affect blood glucose levels (EFSA, 2010; Moraes et al., 2013).

The Earth's climate has changed drastically because of human activities since the pre-industrial revolution. The earth's temperature is increasing, causing global warming due to increasing concentrations of greenhouse gases, such as CO_2, CH_4, and N_2O, in the atmosphere that traps the sun rays (Abzar et al., 2017; Solomon et al., 2009). The massive generation of greenhouse gases from rapidly expanding industrial and residential sectors has caused environmental contamination (Ramanathan & Feng, 2009; Tian, 2015). Environmental pollution has caused the concentration of CO_2 in the atmosphere to rise to 408 (ppm), from 280 (ppm) at the beginning of the twentieth century before the industrial revolution (Dong et al., 2018). The Intergovernmental Panel on Climate Change (IPCC) indicated that, before the end of this century, the CO_2 concentration will cross 1,000 (ppm) (Dong et al., 2018). Elevated CO_2 is a key component of climate change, with potential implications for plant growth and productivity. Generally, increased CO_2 concentrations enhance photosynthetic rates by providing more substrates for photosynthesis. This can lead to improved biomass accumulation and altered plant physiology (Thompson et al., 2017). Studies have shown that elevated CO_2 can increase the net photosynthetic rate in various crops (Ainsworth & Long, 2005; Leakey et al., 2009). For stevia, higher CO_2 levels might boost growth and sweetener yield, as enhanced photosynthesis can result in greater production of bioactive compounds (Ziska et al., 2016). Additionally, elevated

CO_2 can affect nutritional quality and secondary metabolite production in plants. For instance, changes in CO_2 levels may affect the concentration of steviol glycosides, which are responsible for the sweetness of stevia (Lemus-Mondaca et al., 2012). Understanding these effects is essential for optimizing the economic and nutritional values of stevia under changing atmospheric conditions.

Besides environmental factors, agronomics practices such as planting density also influencing plant growth and productivity. Zhang et al. (2021) reported that higher planting densities can lead to increased competition for resources, such as light, water, and nutrients, which might affect plant morphology, yield, and overall health. However, optimal planting densities can maximize space utilization and improve yield per unit area. Research on planting densities indicates that there is an optimal density range for various crops in which growth and productivity are maximized. For Stevia, adjusting the planting density can affect leaf production, stem diameter, and the concentration of sweet compounds. Dense planting may lead to increased shading and reduced leaf area, potentially affecting photosynthetic efficiency and steviol glycoside content (Kumar et al., 2019; Gomes et al., 2018). Conversely, well-spaced plants may have better access to resources, potentially enhancing their growth and sweetener yield. The recommended planting densities for obtaining higher yield and biomass is between 83,000 and 111,000 plants/ha (Kumar et al., 2014; Madan et al., 2010; Serfaty et al., 2013). Some authors have reported that higher stands may still provide biomass increases by area (Kumar et al., 2014). The ideal plant density for stevia varies greatly, depending on the climate and soil fertility of the growing region. The current study was conducted with the aim to investigate the impact of elevated CO_2 under different planting densities on physiology and growth of *Stevia rebaudiana* under Malaysian environmental condition. In more specific terms the study aims to understand that elevated CO_2 concentration due to the changing climatic condition affects the biomass accumulation, photosynthetic rate and production of steviol glycosides which are responsible for sweetness in stevia. Furthermore, the research explores how different planting densities affect stevia leaf production, growth and yield of the sweetener.

The significance of the study lies in its potential to improve the cultivation of stevia in response to changing environmental conditions, especially in Malaysia where the cultivation of stevia is not very common. By exploring the mutual effects of elevated CO_2 and planting densities, the study focuses on understanding how to improve the production of stevia, ensuring that its growth and the productivity of valuable bioactive compounds can be maximized under local environmental and agricultural conditions. This research could further contribute to enhancing stevia's economic and nutritional value, specifically as a non-caloric sweetener suitable for individuals with diabetes.

MATERIAL AND METHODS

Experimental Site and Planting Materials

The experiment was conducted under glass house conditions located in Putra agricultural centre (PAC) University Putra Malaysia (UPM) and Tenaga National Berhad Research Centre (TNBR) Kawasan Institusi Penyelidikan, Jalan Ayer Itam, Kajang, Selangor. The glasshouse with elevated CO_2 was designed in such a way that plants could receive a 12/h photoperiod throughout the growing season. The fully sealed 5 m × 3.67 m glasshouse was continually supplied with 99.8% pure CO_2 from high-pressure CO_2 cylinders for two hours a day, from 8:00 to 10:00 a.m. via a pressure regulator. During the CO_2 exposure time, the CO_2 concentrations were measured using air-sensing CO_2 sensors assigned to each chamber. The level of CO_2 inside the glasshouse increased from 400 (ppm) to 1200 (ppm) at two-week intervals, with an elevation of 400 (ppm) each time. The greenhouse was equipped with dripped fertigation for irrigation purposes. Seedlings were prepared by stem cutting. The seedlings with height of 7–8 cm were transferred to a medium containing coco-peat without soil in 16 cm × 16 cm (16 × 16) polyethylene bags. The temperature was maintained between 27 and 35°C during the day and between 18 and 21°C at night. The relative humidity was maintained at 50-60%. To allow the plant to grow under their natural environment stevia plants were grown under 50-60% shade (light intensity 225±50 μmol m^{-2} s^{-1}) using black netting.

Experimental Design

A nested design with RCBD in four blocks was performed to conduct the current experiment. The arrangement was made on factorial bases with two factors and three levels for each of the factor. Factor a representing two CO_2 levels (400 ppm) and (1200 ppm) while factor b represented three planting densities, high-density vertical (HDV) 78 plants/meter square, high-density horizontal (HDH) 25 plants/meter square and low-density horizontal (LDH) 12 plants/meter. Factor a (CO_2) was applied across the block, while densities were nested within factor a (CO_2).

Growth Attribute

Growth-related data such as plant height, number of branches, and number of leaves were assessed monthly starting from the first month of growth until the final harvest at month four. The plants standing upright were selected from the top of the coco-peat level of the polybag to the highest point of the plant to measure plant height and were expressed in centimetres (cm) using measuring tape. Primary, secondary, and tertiary shoots were considered the number of branches. The number of branches and leaves was counted visually.

Leaf Gas Exchange

Physiological characteristics, such as photosynthetic rate (µmol CO_2 m^{-2} s^{-1}), stomatal conductance (µmol CO_2 m^{-2} s^{-1}), transpiration rate (µmol H_2O m^{-2} s^{-1}), intercellular CO_2 (µmol CO_2 m^{-2} s^{-1}), and water use efficiency ((µmol CO_2 mol-^{1} H_2O), were examined by selecting healthy leaves at the completion of the first and third growing months. All of the above-mentioned parameters were measured using a portable infrared gas analyser (Li-Cor 6400, LI-Cor Inc., Lincoln, USA) adjusted at 400 µmol mol^{-1} $CO_{2,}$ 1000 µmol m^{-2} s^{-1} irradiance and saturated light condition (solar radiation > 1200 µmol. m^{-2} s^{-1}) on the abaxial surface of a leaf that has fully grown from the plant's tip. The ratio of photosynthetic rate to transpiration rate was used to calculate water use efficiency, following the methods of Condon et al. (2004).

Chlorophyll Estimation

Destructive method suggested by Lichtenthaler and Bushman (2001) were used for the estimation of Chlorophyll a, b, and total chlorophyll. Fresh and healthy leaves were collected from stevia plants (0.1 g), chopped and mixed with 10 mL of (80%) acetone in a flacon tube. To prevent light penetration into the samples the flacon tube was wrapped in aluminium foil and kept under dark conditions for 72 h. After 72 hours the solution was transferred to another tube and chopped leaves were allowed to settle. Absorbance for chlorophyll a, b and total chlorophyll was measured at 663.2, 646.8, and 470 nm using a spectrophotometric reader. For blank solution (80%) acetone was used, and the content of chlorophyll was expressed as µg mg^{-1} of fresh weight with the help following relationships:

$$\text{Chlorophyll a } (\mu g\ ml^{-1}) = (12.25 \times A663.2 - 2.79 \times A646.8)$$

$$\text{Chlorophyll b } (\mu g\ ml^{-1}) = (21.50 \times A646.8 - 5.1 \times A663.2)$$

$$\text{Total chlorophyll } (\mu g\ ml^{-1}) = (7.15 \times A663.2 + 18.71 \times A646.8)$$

Statistical Analysis

For every treatment, four replicates were created, and the results were presented as mean values. Significant variations in plant growth and physiology between the various treatments were found at a 5% probability using analysis of variance (ANOVA) and the Least Significant Difference (LSD) test.

RESULTS

Plant Height

The results from our experiment for plant height are shown in Figure 1a, which indicates that regardless of the planting density, the CO_2 treatment significantly increased the plant height consistently from the first month of planting (MAP) until the 4th MAP at the final harvest. At first MAP, the highest plant height (39.8 cm) was recorded for plants grown under elevated CO_2 (eCO_2), whereas plants grown under ambient CO_2 (aCO_2) only showed (27.6 cm) height. Following the same pattern, plant height was 16.5% higher in the 2nd month and 17% higher at 3rd MAP under eCO_2 than in plants grown under aCO_2 levels. After four MAP at the final harvest, the maximum height (77.1 cm) for stevia plants was recorded for eCO_2 treated plant compared with plants under aCO_2 (66.5 cm). Under aCO_2, different planting densities were found to be significantly different in terms of plant height at the early growth stage; however, at maturity, no significant differences were observed among the plants. In the first month, the plants under LDH showed the highest plant height (29.8 cm), followed by HDV (27.8 cm), while the HDH plants were seen with (25.2 cm) only. In contrast to month one the maximum plant height was recorded for plants grown under HDV (44.9 cm) while LDH was recorded with (44.2 cm) and HDH (44.5 cm) at 2nd month respectively. On the 3rd and 4th MAP, no significant difference in plant height was observed between all three densities. Under eCO_2, the pattern was observed to be the same as that under aCO_2 for different planting densities. However, the LDH plants showed significantly higher plant height in the 1st and 2nd months. At 1st MAP, LDH plants showed the highest plant height (42.6 cm), followed by HDV (41.2 cm) and HDH (35.5 cm). At 2nd MAP the LDH again was seen with highest plant height (56.5 cm), HDV (54.6 cm) and (46.5 cm) height was recorded for HDH plants. No significant difference was observed in the 3rd and 4th month among plants under different densities (Figure 1b).

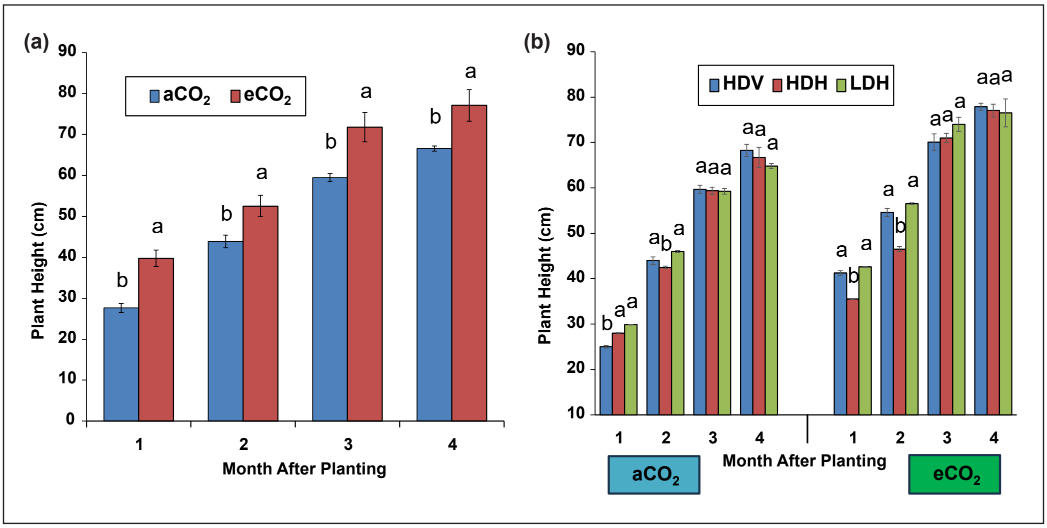

Figure 1. Plant height differences: (a) Between aCO_2 and eCO_2; (b) at different planting densities
Note. According to LSD, group means with the same letter do not differ significantly at $P \leq 0.05$. Error bars have been used to display the standard error of the mean

Number of Branches

The results from the current study show that elevated CO_2 treatment positively influenced the number of branches per plant, regardless of density. In the first month, the plants under eCO_2 produce (11.14) branches while the non-CO_2 treated plant produces (8.22) branches per plant. Similarly, the plants under eCO_2 were found to produce a 20% higher number of branches per plant at 2nd MAP, 30% at 3rd MAP, and 28% at month four than plants under aCO_2 levels (Figure 2a). Under aCO_2 levels, the HDH and HDV plants produced (9.2) and (8.2) branches per plant, respectively, which were significantly higher than LDH (7.2) in the first month. There was no significant difference recorded for plants under different densities at aCO_2 concentration at 2nd, 3rd and 4th month after planting in terms of branch number. For plants grown under eCO_2 at different densities, the results were almost the same as those for aCO_2. At first month of growth HDH and HDV produce (11.76) and (11.53) branches per plant while LDH produce (10.13) branches per plant which were significantly lower than HDH and HDV. In the months following until final harvest, no significant difference was recorded for different densities in terms of branch number under eCO_2 treatments (Figure 2b).

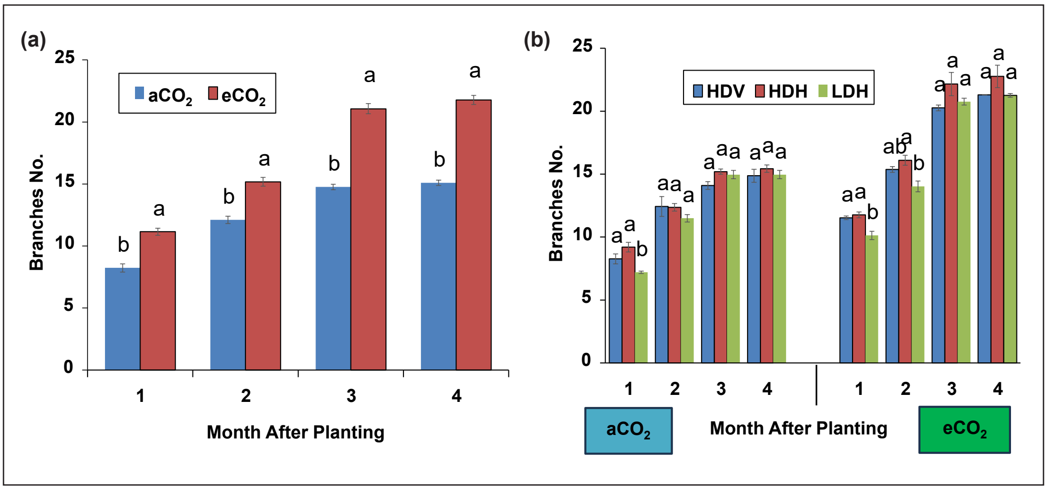

Figure 2. Effect on branching patterns of *Stevia rebaudiana* (Bertoni): (a) CO_2 levels; and (b) planting density
Note. Means with same letter in a group do not show a significant difference according to LSD at $P \leq 0.05$. Error bars indicate the standard error of means

Number of Leaves

The results summarized in Figure 3a show that, similar to other growth parameters such as plant height and branch number, leaf number was also found to be significantly higher under eCO_2 treatment than aCO_2 in normal glasshouse. The highest number of leaves per branch was recorded for plants exposed to eCO_2 throughout the growth period from 1st month of planting until the final harvest at the 4th MAP. In terms of leaf number per branch (18%), increments were seen for CO_2 treated plants at 1st MAP, (17%) in 2nd month, (20%) at 3rd MAP, and (18%) at 4th MAP compared to plants under aCO_2. The findings from the current experiment indicate that plants under different densities with aCO_2 levels do not show any significant difference in the number of leaves in the 1st and 2nd months of growth. At month three the highest number of leaves per branch was recorded for LDH (23.1), followed by HDH (20.03), and HDV (18.93). On the 4th MAP, the LDH plants had the highest number of leaves, and no difference was found between HDH and HDV plants. Under the eCO_2 treatment, the LDH plants produced (13.5), HDV (12.5) and HDH (11.8) leaves per branch. At 2nd month of growth, no significant difference was seen for HDV and LDH plants, whereas HDH produced (14.5) leaves, which were 12% higher than those of HDV and LDH. Similar trends were seen in the 3rd and 4th month as well, where LDH produced a higher number of leaves (Figure 3b).

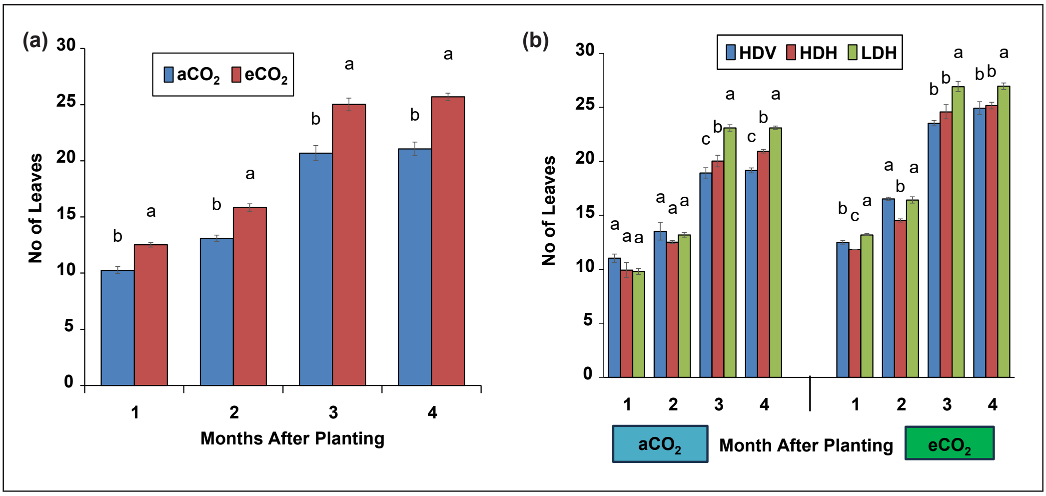

Figure 3. Variation in leaf number of *Stevia rebaudiana* (Bertoni): (a) Under ambient and elevated CO_2; and (b) at different planting densities

Note. Means with same letter in a group do not show a significant difference according to LSD at $P \leq 0.05$. Error bars indicate the standard error of mean

Photosynthesis (Pn)

Plants exposed to eCO_2 treatment were found to be significantly higher in terms of photosynthetic rates (Pn) compared to plants grown under aCO_2 conditions, regardless of density. On average, Pn was found to be (38%) higher under eCO_2, whereas in the first month, the plants treated with eCO_2 showed (36%) increment while in the 3rd month, the difference was recorded to be (42%) higher than that of plants under aCO_2 concentration (Tables 1 and 2). Our results indicate that plants grown under aCO_2 concentrations with different densities show significant differences in the first month of growth; however, no significant differences were observed in the 3rd month of growth. In the first month of growth, the LDH plants showed the highest values for photosynthesis (12.96 µmol CO_2 m^{-2} s^{-1}), HDV (9.9 µmol CO_2 m^{-2} s^{-1}), and HDH (8.1 µmol CO_2 m^{-2} s^{-1}). On 3rd MAP the higher values were recorded for LDH (6.23 µmol CO_2 m^{-2} s^{-1}) followed by HDH (5.09 µmol CO_2 m^{-2} s^{-1}) and HDV (4.66 µmol CO_2 m^{-2} sec^{-1}) however, the least significant difference test (LSD) at the probability threshold of (≤ 0.05) indicated that the values were not significant. The results summarized in Tables 3 and 4 also show that under the eCO_2 treatment, highly significant differences were observed among plants grown at different densities. The plants grown under LDH were found with highest value of Pn (20.6 µmol CO_2 m^{-2} s^{-1}) followed by HDH (15.3 µmol CO_2 m^{-2} s^{-1}) and

Table 1

Effect of CO_2 levels on leaf gas exchange parameters of Stevia rebaudiana *regardless of planting densities at 1st month of planting*

Parameters	**Treatments**	
	aCO_2	**eCO_2**
Pn (µmol CO_2 m^{-2} s^{-1})	10.32b ± 0.761	16.20a ± 1.212
gs (µmol CO_2 m^{-2} s^{-1})	0.418a ± 0.045	0.243b ± 0.046
TR (µmol H_2O m^{-2} s^{-1})	2.49a ± 0.095	1.75b ± 0.112
Ci (µmol CO_2 m^{-2} s^{-1})	241b ± 82.44	503a ± 57.33
WUE (µmol CO_2 mol^{-1} H_2O)	4.26b ± 0.450	9.59a ± 0.898

Note. The similar letters given after the number in a column do not show any significant difference according to LSD at 5% probability level. Numbers were followed by ± standard deviation. aCO_2 = Ambient carbon dioxide; eCO_2=Elevated carbon dioxide; HDV=High density vertical; HDH= High density horizontal; LDH=Low density horizontal; Pn=Photosynthetic rate; gs=Stomatal conductance; TR=Transpiration rate; Ci=intercellular CO_2; WUE=Water use efficiency

Table 2

Effect of CO_2 levels on leaf gas exchange parameters to of Stevia rebaudiana *regardless of planting densities at 3rd month of planting*

Parameters	**Treatments**	
	aCO_2	**eCO_2**
Pn (µmol CO_2 m^{-2} s^{-1})	5.33b ± 0.366	9.33a ± 0.554
gs (µmol CO_2 m^{-2} s^{-1})	0.335a ± 0.027	0.156b ± 0.006
TR (µmol H_2O m^{-2} s^{-1})	5.40a ± 0.097	4.31b ± 0.131
Ci (µmol CO_2 m^{-2} s^{-1})	393b ± 49.18	804a ± 36.58
WUE (µmol CO_2 mol^{-1} H_2O)	0.99b ± 0.077	2.21a ± 0.185

Note. The similar letters given after the number in a column do not show any significant difference according to LSD at 5% probability level. Numbers were followed by ± standard deviation. aCO_2 = Ambient carbon dioxide; eCO_2 = Elevated carbon dioxide; HDV = High density vertical; HDH = High density horizontal; LDH = Low density horizontal; Pn= Photosynthetic rate; gs= Stomatal conductance; TR= Transpiration rate; Ci= intercellular CO_2; WUE= Water use efficiency

Table 3
Effect of CO_2 levels on leaf gas exchange parameters to of Stevia rebaudiana *under different planting densities at 1st month of planting*

Treatments		Parameters				
		Pn (µmol CO_2 m^{-2} s^{-1})	**Gs (µmol CO_2 m^{-2} s^{-1})**	**TR (µmol H_2O m^{-2} s^{-1})**	**Ci (µmol CO_2 m^{-2} s^{-1})**	**WUE (µmol CO_2 mol^{-1} H_2O)**
aCO_2	HDV	9.9b ± 0.35	0.53a ± 0.03	2.7a ± 0.14	254b ± 43	0.83a ± 0.07
	HDH	8.1c ± 0.06	0.40b ± 0.03	2.5a ± 0.14	201b ± 79	0.93b ± 0.34
	LDH	13.0a ± 0.88	0.312c ± 0.02	2.2a ± 0.03	467a ± 40	1.21b ± 0.08
eCO_2	HDV	12.6c ± 0.58	0.30a ± 0.01	1.90a ± 0.36	430b ± 14	7.31a ± 1.2
	HDH	15.3b ± 0.66	0.29a ± 0.02	1.69a ± 0.04	635a ± 6.9	9.28ab ± 1.3
	LDH	20.6a ± 0.68	0.13b ± 0.03	1.66a ± 0.04	677a ± 14	12.2b ± 1.6

Note. At the 5% probability level, the number in a column that has the same letter is not significantly different by the least significant difference. Thus, numbers were followed by ± standard deviation; aCO_2 = Ambient carbon dioxide; eCO_2 = Elevated carbon dioxide; HDV = High density vertical; HDH = High density horizontal; LDH = Low density horizontal; Pn= Photosynthetic rate; gs= Stomatal conductance; TR= Transpiration rate; Ci= intercellular CO_2; WUE= Water use efficiency

Table 4
Effect of CO_2 levels on leaf gas exchange parameters of Stevia rebaudiana *under different planting densities at 3rd month of planting*

Treatments		Parameters				
		Pn (µmol CO_2 m^{-2} s^{-1})	**Gs (µmol CO_2 m^{-2} s^{-1})**	**TR (µmol H_2O m^{-2} s^{-1})**	**Ci (µmol CO_2 m^{-2} s^{-1})**	**WUE (µmol CO_2 mol^{-1} H_2O)**
aCO_2	HDV	4.6a ± 0.19	0.40a ± 0.01	5.6a ± 0.21	299b ± 96	3.2a ± 0.8
	HDH	5.1a ± 0.58	0.37a ± 0.00	5.5a ± 0.04	426a ± 32	3.7ab ± 0.6
	LDH	6.2a ± 0.76	0.24b ± 0.04	5.1a ± 0.09	453a ± 56	5.9a ± 1.0
eCO_2	HDV	7.7b ± 0.90	0.17a ± 0.016	4.8a ± 0.079	679b ± 77	1.61b ± 0.4
	HDH	9.3ab ± 0.21	0.15a ± 0.003	4.1b ± 0.049	803a ± 58	2.26ab ± 0.5
	LDH	10.8a ± 0.61	0.15a ± 0.004	4.0b ± 0.093	929a ± 10	2.74a ± 0.8

Note. The number followed by the same letter in a column is not significantly different by the LSD at 5% probability level. Numbers are followed by ± to show standard deviation; aCO_2 = Ambient carbon dioxide; eCO_2 = Elevated carbon dioxide; HDV= High density vertical; HDH= High density horizontal; LDH= Low-density horizontal Pn: Photosynthetic rate gs: Stomatal conductance TR: Transpiration R ate Ci: intercellular CO_2 WUE: Water use efficiency

HDV (12.63 µmol CO_2 m^{-2} s^{-1}) at first MAP. At the 3rd month, the LDH plants were found to be significantly higher than HDV but non-significant to HDH, whereas the HDH plants were not significantly affected by either LDH or HDV in terms of photosynthesis.

Stomatal Conductance (gs)

The results from our current experiment demonstrate that elevated CO_2 has a positive influence on stomatal conductance (gs) of our stevia plant by significantly reducing its frequency. The gs for plants exposed to eCO_2 was found to be reduced by (41%) at the 1st MAP and (53%) at the 3rd MAP compared to plants under aCO_2 treatment, regardless of the density (Tables 1 and 2). Our results shown in Tables 2 and 4 indicate that under aCO_2, the LDH plants showed the lowest gs, followed by HDV, while HDH had the highest values at the 1st MAP. At 3rd month again the lowest values were recorded for LDH, which was significantly lower than HDH and HDV, while no significant difference was observed among plants grown in HDV and HDH. Under eCO_2 conditions, no significant difference was observed for plants grown in HDH and HDV, whereas LDH was found to be significantly lower than both HDV and HDH at 1st MAP. At the 3rd MAP no significant differences were observed between densities.

Transpiration Rate (Tr)

The results of our current study revealed significant alterations in the transpiration dynamics of stevia plants under eCO_2 concentrations. Our results indicate that transpiration rates (Tr) were lower in plants grown under eCO_2 at the 1st MAP and even 3rd MAP than in plants grown under aCO_2 levels (Tables 1 and 2). At the 1st month of growth, the Tr reduction was recorded at (29%) and at the 3rd month of growth, it was (20%) lower than that of plants exposed to aCO_2 level, irrespective of planting density. Plants grown at different planting densities under aCO_2 levels did not show any significant difference in terms of transpiration. The results are summarized in Tables 2 and 4 which illustrated that plants exposed to eCO_2 did not show any significant differences between different planting densities in the first month of growth. However, in the 3rd month, the HDV plants were observed to have the highest Tr values and were found to be significantly higher than HDH and LDH.

Intercellular Carbon Dioxide (Ci)

Intercellular CO_2 (Ci) was recorded for stevia plants under two different CO_2 conditions (aCO_2 and eCO_2) of 400 ppm and 1200 ppm. Measurements were recorded on the 1st and 3rd months of growth. Tables 1 and 2 illustrates that Ci was significantly increased by (52%) in the 1st month of growth and (51%) at 3rd MAP for plants grown under eCO_2 in comparison with plants grown under aCO_2 concentrations, despite the different planting densities. Under aCO_2 concentrations, significant differences were observed for different planting densities. In the first month of growth, the highest value was recorded for LDH plants (466.88 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$), which was significantly higher than HDV (254.73 $\mu mol\ CO^2\ m^{-2}\ s^{-1}$) and HDH (201.35 $\mu mol\ CO_2\ m^{-2}\ s^{-1}$). At 3rd MAP the trend was found to be the same; however, no significant differences were found between LDH and HDH. HDV was found to be significantly lower in terms of Ci than in the LDH and HDH plants. Under eCO_2, the HDV plants were found to have significantly lower Ci than HDH and LDH in the first month; however, the trend was almost the same for all treatments as aCO_2 in the 3rd month of growth (Tables 3 and 4).

Water Use Efficiency (WUE)

The result from the experiment indicates that eCO_2 has positively influenced water use efficiency (WUE) by comparing it with aCO_2 regardless of differences in planting densities. The eCO_2 treated plants observed (55%) increment in WUE in the 1st and 3rd months of growth in plants grown under aCO_2 (Tables 1 and 2). Regarding the planting density, LDH planting showed maximum WUE (5.88 μmol CO_2 mol^{-1} H_2O) followed by HDH (3.19 μmol CO_2 mol^{-1} H_2O) and HDV (3.71 μmol CO_2 mol^{-1} H_2O) under aCO_2 at 1st MAP. The results are summarized in Tables 3 and 4, which shows that at the 1st MAP, the LDH plant showed a significantly higher value for WUE than for HDV. However, no significant difference was observed between HDH and HDV, whereas LDH was not significantly different from HDH. Under eCO_2, the LDH plants showed maximum values for WUE (12.19 μmol CO_2 mol^{-1} H_2O), followed by HDH (9.28 μmol CO_2 mol^{-1} H_2O), which were not significant for HDV (7.30 μmol CO_2 mol^{-1} H_2O) according to LSD at the 5% probability level. The plants showed a similar trend in the 3rd month to the first month (Tables 3 and 4) under eCO_2.

Chlorophyll Content

The results from the current study showed that elevated CO_2 levels positively influenced total chlorophyll content by significantly increasing chlorophyll a and b. Regardless of differences in planting densities, a significant increase in chlorophyll a was observed, which was 25% and 19% higher under eCO_2 treatments than under aCO_2 at the 1st and 3rd MAP respectively (Figure 4a). Under aCO_2 concentration, no significant differences were observed among the different densities at the 1st MAP; however, at the 2nd MAP the HDH plants were found to produce (30.61 µg/mg) non-significant to HDH (28.64) but significantly higher chlorophyll a than HDV (19.95 µg/mg). Significant differences were seen among all three densities under eCO_2 treatment. At 1st MAP no significant differences were observed for LDH and HDV; however, HDH plants significantly higher than LDH and HDV plants in terms of chlorophyll a. At 3rd MAP the HDV plants were seen with significantly lowest values (25.57 µg/mg) than HDH (5.68 µg/mg) and LDH (36.59 µg/mg) for chlorophyll a production (Figure 4b).

Similar to chlorophyll a, eCO_2 concentrations were observed to be increasing chlorophyll b. The highest values (10.72 µg/mg) and (13.96 µg/mg) at 1st and 3rd MAP were recorded for plants grown under eCO_2 level while the plants grown under aCO_2 concentration were seen with significantly lowest values (8.14 µg/mg) and (11.01 µg/mg) for chlorophyll b (Figure 5a). Under aCO_2 concentration no significant difference was observed for all the three densities at 1st as well as 3rd MAP. However, under eCO_2 LDH plants showed significantly higher amount of chlorophyll b than HDV and HDH at both 1st and 3rd month of growth (Figure 5b).

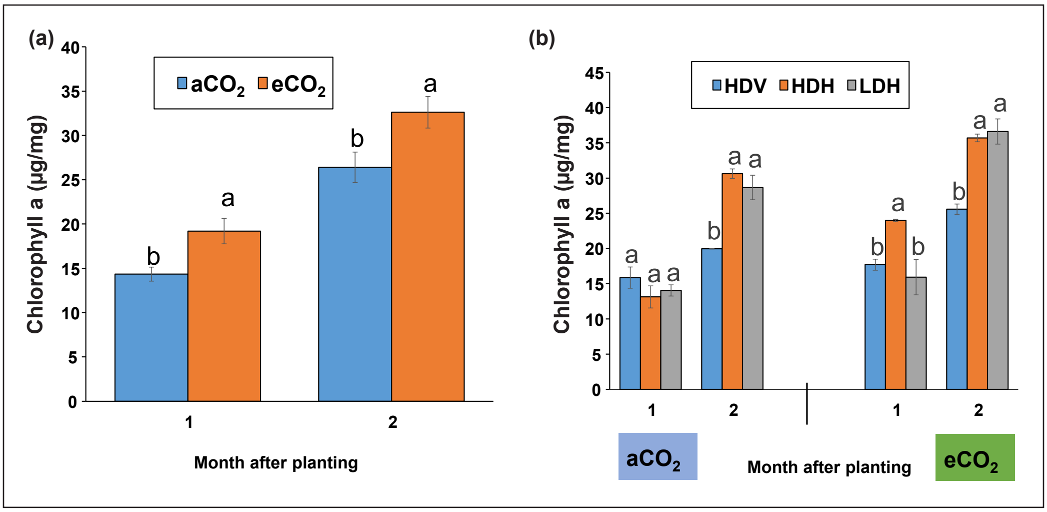

Figure 4. Variation in chlorophyll a concentration: (a) Under ambient and elevated CO_2; and (b) at different planting densities

Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

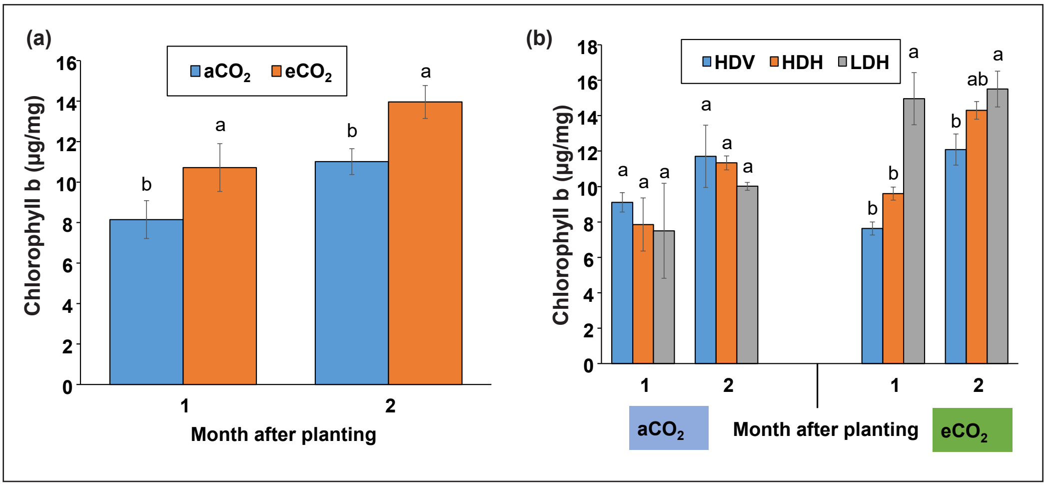

Figure 5. Variation in chlorophyll b levels: (a) Under ambient and elevated CO_2; (b) at different planting densities

Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

The maximum total chlorophyll was recorded for eCO_2 treated plants at 3rd MAP (46.91 µg/mg) and (29.92 µg/mg) at 1st MAP while the aCO_2 plants produced (37.22 µg/mg) at 3rd MAP and (22.49 µg/mg) of total chlorophyll at 1st MAP which were significantly lower than eCO_2 treatment (Figure 6a). The plant exposed to aCO_2 with different planting densities does not show any significant difference in the 1st month of growth while at 3rd

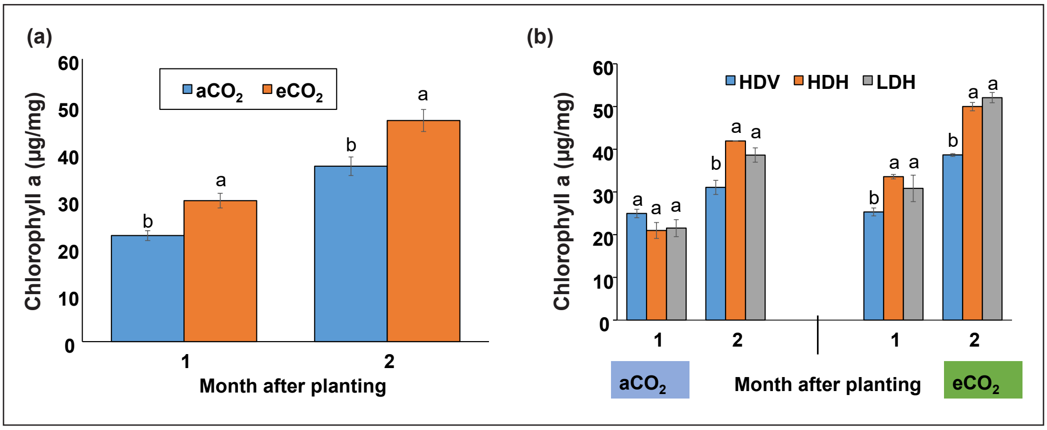

Figure 6. Impact on total chlorophyll content of *Stevia rebaudiana* (Bertoni): (a) CO_2 levels; and (b) plant density
Note. Means with same letter in a group do not show a significant difference according to LSD at P≤ 0.05. Error bars indicate the standard error of mean

month of growth the LDH and HDH plants were observed with significantly higher total chlorophyll than HDV. Under eCO_2 no significant was observed between HDH and LDH at 1st and 3rd MAP. However, HDV was seen with significantly lowest total chlorophyll at 1st as well as 3rd MAP (Figure 6b).

DISCUSSION

The results of the current experiment show that plant growth parameters specifically, plant height, branch number, and number of leaf were positively affected by elevated CO_2 (eCO_2) treatment compared to ambient CO_2 (aCO_2) in *Stevia rebaudiana*. As shown in Figures 1, 2, and 3, the eCO_2 treated plants were observed with maximum values for plant height, branch number, and leaf number throughout the growth period, from the first month after planting (MAP) until the final harvest at the fourth MAP. Increase in photosynthetic rates is the primary mechanism that promotes plant growth. This process also helps plants undergo morphological changes, such as changes in the ratio of roots to shoots, an increase in number of leaves and the production of tillers (Salihi et al., 2023). It is a common knowledge and has been revealed by various scientific reports that eCO_2 enrichment boosts photosynthesis in most of the C_3 crop like stevia by increasing the rate of carboxylation reaction and reduces oxygenation, hence producing more sugar, leading to an increase in plant growth. CO_2 reduces oxygenation reaction which alternately prevents the production of glycolate due to which plant saves more energy and spends those extra ATP, s for plant growth (Zhang et al., 2013; Zhu et al., 2010). An increase in height may be because of the fast growth of *Stevia rebaudiana* under CO_2 enrichment which is results of increase in cell division and elongation as Maity et al. (2019) reported similar results for rice plant height from 76.9 cm under aCO_2 to

81.7 cm with eCO_2 exposure. It has been reported by Seneweera et al. (2011) that 50% increment was seen for Jarrah rice variety under eCO_2 (700 ppm) which support our current results for increased in branches number, as Stevia is C_3 crop and most of the C_3 crop showing similar response to environmental factors like CO_2. Our results in term of leaves show similarity to Costa et al. (2003) where they reported that leaf number and LAI were significantly higher under eCO_2 (570 ppm) than ambient CO_2 (370 ppm). Our results are also in agreement with those of Saha et al. (2015) who observed that height for chickpea plants increased when exposed to eCO_2 concentration. Thilakarathna et al. (2015) also suggested that under eCO_2 level there will be high carbon supply to plant cells which may accelerate its division and expansion in meristematic tissues which enhance growth and development of the plant. Additionally, eCO_2 by positively enhancing the expression of genes which is responsible for growth, cell division and cell wall properties in plants. For example, eCO_2 up regulates genes that encode for expansins, xyloglucan, endotransglucosylase and pectin esterase which play an essential role in cell wall loosing and ultimately helping leaf expansion in plants (Desouza et al., 2008; Wei et al., 2013). Likewise, the ribosomal protein genes that control the cytoplasmic development of plant cells and cell cycle genes encoding cyclin, cyclin-dependent protein kinase, tubulin, and cyclin-dependent protein kinase regulator were shown to be up-regulated at eCO_2 level (Ainsworth et al., 2006; Wei et al., 2013). Plant cells under eCO_2 may divide, develop, and expand more often due to the up regulation of these enzymes involved in the cell cycle and cytoplasmic proliferation which contribute to maximizing growth and development of the plant.

Besides the enhancement in photosynthesis and gene expression under elevated carbon dioxide, it has also been reported that plant hormones are playing an essential role in modification of plant growth and development (Desouza et al., 2008; Teng et al., 2006; Wei et al., 2013). Some of the fundamental plant's hormones are auxins, gibberellic acids and cytokinin's that are mutually stimulating and regulate the division and expansion of the cells as well as control shoot meristem development and stem elongation (Cato et al., 2013; Gamage et al., 2018). Teng et al. (2006) used *Arabidopsis thaliana* to study the effects of eCO_2 on plant hormones and reported that there was a significant increase in gibberellic acid, zeatin riboside, dihydrozeatin riboside, isopentenyl adenosine of the class cytokinin, and indole-3-acetic acid, one of the common plant hormones in the auxin class. It has also been suggested by several other studies that eCO_2 significantly increases transcript abundance of genes that are related to synthesis and transport of auxins, gibberellic acid and cytokinin (Desouza et al., 2008; Gamage et al., 2018; Wei et al., 2013). All of these findings may explain why all the growth-related parameters like plant height; number of branches and leaves were significantly higher under eCO_2 concentration in comparison to aCO_2 for *Stevia rebaudiana* in the current experiment.

Different planting densities did not show any significant effects on plant height and branch development, specifically at the later growth stages under ambient CO_2 (aCO_2). However, at the first month after planting (MAP), plants grown under high-density vertical farming (HDV) showed reduced plant height, and at the second month, high-density horizontal planting (HDH) plants showed significantly lower plant height compared to low-density horizontal planting (LDH) and HDV. Under elevated CO_2 (eCO_2) concentration, only HDH plants exhibited significantly lower plant height at both the first and second MAP. No significant differences were observed in plant height across all planting densities at the third and fourth MAP under eCO_2. Results from current experiments are being supported by Benhmimou et al. (2017) where they observed that narrow spacing with plant populations of 142857 plants ha^{-1} resulted low plant height by comparing with wider spacing with a plant population of 47619 plants ha^{-1}. However, results from eCO_2 enrichment showed higher plant height in HDV plants which are supported by Mahajan and Pal (2021) by reporting that plant height was improved with dense planting. Similarly, the number of branches were observed with significant difference at 1st MAP only where the LDH plant produce significantly lower branches than HDH and HDV under both aCO_2 and eCO_2 while no significant were found at 2nd , 3rd and 4th MAP respectively. The higher plant height at early growth stages under high plant density could be due to the receiving of less radiation at ground/basal portion, therefore elongation of bottom internodes increases. Under dense canopy the light received is poor in red radiations and enriched with far-red radiation which strengthens apical dominance by stimulating internodes elongation (Rajcan & Swanton, 2001; Xue et al., 2016). Ozer (2003) reported that plant height in *Brassica napus* was higher under high plant density and similarly, higher plant height was also documented for cotton (*Gossypium hirsutum* L.) by several authors under high density planting (Clawson et al., 2006; Gwathmey & Clement, 2010; Khan et al., 2020). In this study, for high density planting, the number of branches were higher under eCO_2 at 1st and 2nd MAP which could be possible due to CO_2 enrichment as the plants were not suffering from resource limitations, specifically plant nutrition. Leaf is an important and economical part of stevia rebaudiana as steviol glycosides are present in higher amount in leaves than other parts of the plant. Under aCO_2 significant difference were seen at late growth stages where the LDH plants produce higher leaves followed by HDH and HDV produce significantly lower leaves. Similarly, the leaves number were higher for low density planting under eCO_2 conditions. Under low density planting, the competition for available resources is less due to less inter-row and intra-row spacing hence there is higher chance for plant to develop greater number of leaves than high density planting. The results for higher leaves number under LDH show similarities to Kumar et al. (2014) and Tadesse et al. (2016) where they reported that wider spacing produced significantly higher number of leaves per plant in comparison to narrow spacing. However, the high number of leaves per unit area can be achieved from high density planting which is due to the increasing number of plants per unit area.

Results from the current study showed that leaf gas exchange parameters were positively influenced by eCO_2 despite the differences in planting densities. Photosynthetic rate (Pn), intercellular CO_2 (Ci) and water use efficiency (WUE) were significantly increased under eCO_2 while transpiration rate (Tr) and stomatal conductance (gs) decreased with increasing the concentration of CO_2. Carbon dioxide shows the first and most direct effect on photosynthesis of the plants. Because CO_2 is the raw material in the process of photosynthesis that is significantly affecting production of the plant (Zhang et al., 2013). There are three main phases in light independent reaction of photosynthesis which are carboxylation, reduction and regeneration. The Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) enzyme is responsible for fixing CO_2 to ribulose-1,5-bisphosphate (RuBP), resulting in a 3-phosphoglycerate (PGA) molecule (Dusenge et al., 2019). Due to the low affinity of Rubisco to CO_2 it can bind to O_2 and lead to photorespiration producing glycolate, as the carboxylation reaction of RuBP is not saturated at the current atmospheric CO_2. Therefore, in the C_3 crop, eCO_2 induced photosynthesis to increase in two different ways. First, improves carboxylation level, Second, CO_2 prevented the oxygenation/photorespiration reaction and reduces glycolate production which is wasteful in terms of energy, as it costs the plant more energy and does not lead to any gains in energy or carbon (Dusenge et al., 2019; Peterhansel et al., 2010). The similar results to our findings for high Pn under eCO_2 were also reported by Ji et al. (2015). Our results also show similarity to Javaid et al. (2022) where they reported that Pn was increased by 25% with eCO_2 by comparing to that of aCO_2 concentration. Additionally, it is commonly believed that CO_2 enrichment increases photosynthesis in plants, even under stressful environments (Dekauwe et al., 2021; Ghahramani et al., 2019). According to Park and Runkle (2018), the Pn rate was increased under eCO_2 concentration which supports our current results.

Stomatal conductance and transpiration rate were found to be inversely proportional to photosynthesis with increase in CO_2 concentration. The plants under eCO_2 were observed with significantly lower values for gs and Tr than aCO_2 treated plants. The similar findings are reported by Yasutake et al. (2016) where they observed that the Pn was significantly increased while gs and Tr were decreased under eCO_2 concentration. The low gs could be the reason for the low Tr under CO_2 enrichment. Our results for gs and Tr are in line with Ahmed et al. (2022) where the reported low gs and Tr for Lettuce plants under elevated CO_2 concentration of (1000 & 1500 ppm) in comparison to (500 ppm). The similar results to our findings for reduction in Tr and gs were also reported by Lamichaney et al. (2021) under eCO_2 (566-630 ppm) in comparison to (379−423 ppm) for chickpea plants. Our results are also in line with Wang et al. (2018) who reported the decline of Tr and gs under CO_2 enrichment. The Ci was observed with a significant increment in response to

CO_2 enrichment. However, in the 3rd month of growth the Ci concentration was higher as compared to the 1st MAP. The reduction of Ci was related to photosynthesis. As mentioned in earlier section that a high Pn was recorded under eCO_2 at 1st MAP than 3rd MAP. When the plant performs high photosynthesis the concentration of CO_2 in leaves decreases because CO_2 is an essential element that is used in the process of photosynthesis. The results from our experiment are in line with Fathurrahman (2023) where he reported higher Ci with eCO_2 (800 ppm). The similar results to our finding are also reported by Javaid et al. (2022) with Ci enhancement under eCO_2 concentration. However, in contrast to our findings, Lamichaney et al. (2021) reported a decline in Ci under eCO_2 and explain that under eCO_2 conditions the reduction in Ci could be due to greater affinity of CO_2 binding with Rubisco enzyme leading to higher photosynthesis. The results for the current experiment showed significantly higher WUE upon the exposure of plants to eCO_2 concentration especially at early growth stages. The higher WUE may be due to low transpiration rate as results of low gs under eCO_2 treatment. In fact, it is clearly understood from our results along with finding from other researches that enhancement in WUE is the result due to higher Pn with low gs under elevated carbon dioxide concentration (Wei et al., 2022). Our finding is supported by Javaid et al. (2022) where they reported a 40% higher WUE for *Datura stramonium* under eCO_2 conditions in comparison to aCO_2. Similarly, another study by Walia et al. (2022) reported that eCO_2 increased WUE by decreasing Tr and gs due to partial closure of stomata. Our results are also in line with Robredo et al. (2007) where they found increased WUE for barely plants grown under eCO_2 than those grown under aCO_2 conditions.

Leaf gas exchange plays a central role in biomass and yield accumulation as these traits are the base of plant physiology. The Pn which reflects photosynthetic capacity and potential productivity is one of the most important physiological trait (Xia et al., 2020). In the current study Pn was significantly higher for LDH while HDV and HDH do not show any significant at 1st MAP however, at 3rd MAP all the three densities were non-significant under aCO_2. The similar results were seen for all densities under eCO_2. The low Pn could be due to the low quality of light intensity with increased canopy layer under high planting density and gradual development of shading due to increasing number of *Stevia rebaudiana* leaves under high planting density. The similar results are reported by Huang et al. (2021) with high Pn under low planting density for *Cunninghamia lanceolata*. Our results are also in agreement with Mwamlima et al. (2020) where they reported significantly higher Pn under low planting density for Soybean. Significantly higher Ci and WUE were recorded for low planting density. However, the Ci was higher at maturity while WUE was maximum at early growth stages. This might be due to high photosynthesis performance at early growth stages where the plant uses more CO_2 and water. On the other hand, at maturity low photosynthesis might be due to the lack of assimilating photosynthetic material which decreases WUE and increases CO_2 at the cellular level (Tang et al., 2015). The similar results for high WUE and Ci were reported by Huang et al. (2021) which are supporting our findings for the current experiment. The result from the current study showed maximum gs for HDV than LDH and HDH under aCO_2 while under eCO_2 the trend was similar to aCO_2 at 1st MAP but no significant difference was seen at 3rd MAP between all the three densities. In term of Tr the HDV was found to be significantly higher than HDH and LDH under eCO_2 at 3rd MAP while no significant were seen for aCO_2 between HDV, HDH and LDH. Our results for low gs from the current study agree with Koesmaryono et al. (1997), Zhou et al. (2011) and Moreira et al. (2015) where all of them reported that under low planting density the stomatal conductance decreased. Similarly, the non-significant results for Tr agree with Wilson et al. (2012) where they reported that varying planting density does not affect Tr.

Chlorophyll is an important pigment which is found in the chloroplast of the plant and plays a central role in the processes of photosynthesis, that's why it is very important for plant growth and yield (Ohmiya et al., 2017). Results from the current study showed that chlorophyll a, b and total chlorophyll were significantly increased under elevated CO_2. Our results for high photosynthesis in the earlier section might explain the increment in chlorophyll under CO_2 enrichment. Results from current study are consistent with previous finding from Song et al. (2020) who proved that CO_2 enrichment has positively enhanced photosynthesis by increasing chlorophyll content in cucumber. In order to find the reason for enhanced photosynthesis and chlorophyll increment under eCO_2, Song et al. (2020) screened 17 genes related to chlorophyll by transcriptome sequencing and found that most of the genes were upregulated under CO_2 enrichment which may explain the increase in chlorophyll under eCO_2 for our stevia plant as well. Our results are also supported by Samuolienė et al. (2006) where they reported that eCO_2 (1500 ppm) significantly increased chlorophyll content in radish. Results from our study are also similar to Yang et al. (2023) where they mention 7.6% increment in chlorophyll content under eCO_2. Naznin et al. (2015) reported increment in chlorophyll content in garlic under eCO_2 level which are supporting our current results for increased chlorophyll content in stevia. Regarding the differences in planting densities, no significant difference was found under aCO2 for chlorophyll a and total chlorophyll at first MAP. However, at 3rd MAP HDV were seen with low significant value than HDH and LDH. Overall chlorophyll b was found to be non-significant for all three densities. under eCO_2 HDV were found with significantly lower values of chlorophyll a and total than LDH and HDH at 1st as well as 3rd MAP. Under high density planting the number of leaves increased per unit area which cause shading lower down plant capacity to capture light. The minimizing of light capturing decreases the carboxylation capacity and mesophyll conductance which lead to low concentration of chlorophyll content per unit area of leaf. For the majority of plant species, reduced numbers of chloroplasts and lower fresh mass per area offset higher levels of chlorophyll per unit of fresh mass and per chloroplast in shaded leaves (Lambers et al. 2008). Our current results are in line with Gomes et al. (2018) where they reported a decrease in chlorophyll content under high plant density for Stevia rebaudiana. Similarly, Zhang et al. (2021) reported that chlorophyll content in maize leaves were respectively decreased under high planting density compared to low planting densities. On the other hand, Mahajan and Pal (2021) reported that overall, no significant effect was found in chlorophyll content under high and low planting density. However, chlorophyll content was slightly decreased with dense planting which are in support of our results.

CONCLUSION

The results from the current experiment show that elevated eCO_2 boosts the growth of *Stevia rebaudiana*, positively enhancing plant height, branch number, and leaf production, basically due to increased photosynthesis and water use efficiency. Additionally higher chlorophyll content supports the enhancement in growth pattern. While lower planting density promotes leaf production by reducing competition, eCO_2 improves growth across all densities. These results suggest that eCO_2 enrichment can optimize Stevia cultivation, enhance yield and plant productivity in horizontal farming planting setups rather than vertical farming.

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