Interactive effects between nitrogen fertilization and elevated CO₂ on growth and gas exchange of papaya seedlings

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Abstract

Elevated atmospheric CO₂ will change the requirements plants have for minerals, mainly nitrogen, altering the relationship between nutrient demand and plants growth. Here, we evaluated the interacting effects between two concentrations of CO₂ (390 or 750 $\mu\text{L L}^{-1}$) and two nitrogen (N) levels (3 mM or 8 mM) on gas exchange, N use efficiency (NUE), and dry mass responses of papaya seedlings. The study was conducted in a climate-controlled greenhouse using 3.5 L pots and six replicates for 62 days. No significant effects from N or CO₂ were noticed for leaf conductance to water vapor and transpiration. However, CO₂ elevation to 750 $\mu\text{mol mol}^{-1}$ increased assimilation rate for both N levels. Elevated CO₂ resulted in an increase of 52% and 16% of instantaneous water-use efficiency under high and low N, respectively, as compared to ambient CO₂ (390 $\mu\text{mol mol}^{-1}$). Lower N concentrations were observed for all the organs for plants grown under higher concentrations of CO₂, regardless of the N level used. However, plants grown under elevated CO₂ reached highest NUEs. There was an N $\times$ CO₂ interactive effect on the leaf, stem plus petiole, and root dry mass. However, no significant CO₂ effect on the root:shoot ratio was observed. Our results revealed that elevated CO₂ stimulated total dry mass accumulation of papaya plants, with the results being greatest for plants grown under lower levels of N.

Keyword - Gas exchange; Dry weight; Nitrogen-use efficiency; Leaf N concentration; Climate change.

Abbreviations: N – nitrogen, A - photosynthesis, g_s - stomatal conductance, E - instantaneous transpiration rate, NUE - nitrogen use efficiency, WUEi - instantaneous water-use efficiency, TDM - Total dry mass, SLA - specific leaf area, SEM - standard error of the mean

1. Introduction

Currently, ambient CO₂ concentrations are approximately 400 μL L⁻¹, and are projected to exceed 800 μL L⁻¹ by the year 2100 (Meehl et al., 2007). Since plants can be strongly affected by CO₂, there is tremendous interest in understanding how these increasing CO₂ levels might affect plant growth and agricultural production. Carbon assimilation (*A*) of C₃ plants is typically CO₂-limited at current atmospheric CO₂ concentrations and, therefore, should be benefited by an increase in atmospheric CO₂ (Bishop et al., 2014). In the long term, however, *A* may acclimate under elevated CO₂ concentrations, with higher CO₂ levels leading to reduced photosynthetic capacity. However, even with acclimation, *A* still tends to be higher in plants grown in a CO₂-enriched atmosphere than under the present-day ambient CO₂ levels (Leakey et al., 2009). While dry mass accumulation in C₃ plants typically benefits from an increase in the concentration of CO₂, the intensity of the response varies greatly among species (Ainsworth and Rogers, 2007) and even cultivars (Ziska et al., 1996). Furthermore, an increase in ambient CO₂ concentrations also reduces transpiration, resulting in improved efficiency of water use (Cruz et al., 2014). Therefore, it can be hypothesized that for C₃ plants, such as papaya, an increase in the CO₂ concentration should increase photosynthesis and dry matter accumulation.

The responses of plants to CO₂ are known to be influenced by nitrogen (N) availability (Stitt and Krapp, 1999; Yang et al., 2008; Anten et al., 2004). Plants cultivated under elevated CO₂ concentrations require more nutrients, especially N, to sustain the increased growth that accompanies the higher *A* (Calfapietra et al., 2003; Reddy et al., 2004). Therefore, a low N concentration in the growth medium may limit *A* and growth responses to CO₂ (Curtis, 1996). In fact, Reich et al. (2014) recently showed that while elevated CO₂ levels led to a significant increase in plant biomass when N was supplied at higher levels, it did not increase plant biomass when N was present at lower levels. Low N availability has been hypothesized to inhibit new organ formation and constrain the growth of existing organs, thus reducing the ability of plants to utilize the additional carbon that is fixed under elevated CO₂ (Stitt and Krapp, 1999; Isopp et al., 2000). This, in turn, can produce a source-sink imbalance, resulting in down-regulation of *A* due to low N. N is an important component that is present in several molecules, such as chlorophyll and proteins. More than half of the total leaf N is utilized by the photosynthetic apparatus (Makino and Osmond, 1991), which also helps explain the increased down-regulation of *A* in plants cultivated under elevated CO₂ and deficient N (Ellsworth et al., 2004). For Papaya, this aspect is important because it absorbs appreciable amounts of N (Cunha and Haag, 1980) and is, consequently, very sensitive to soil N concentrations (Oliveira and Caldas, 2004).

Plants grown under high CO₂ usually experience a substantial reduction in the concentration of N in their tissues (Curtis and Wang, 1998; Jablonski et al., 2002). Two principal factors may be involved in this CO₂-induced lowered tissue N concentration: dilution of N in plant tissues due to accelerated growth; and reduced N assimilation (Kant et al., 2012). Photosynthetic acclimation has been associated with plants acquiring and assimilating insufficient N at elevated CO₂, leading to N limitation in plant tissues and lower C acquisition (Kant et al., 2012). However, some species can direct more photosynthate to roots when grown under high CO₂, which allows greater exploitation of the soil (Rogers et al., 1994), thereby supporting

greater water uptake and improving the absorption of N. In rice, a C₃ plant, elevated CO₂ increased root volume, root dry weight, adventitious root length, and adventitious root number at all developmental stages by 25–71%. These changes were mainly associated with an increased rate of root growth and a lower rate of root senescence (Yang et al., 2008)

The aim of this study was to evaluate the effect of the elevated CO₂ levels and its interaction with N on the growth, gas exchange, and N use efficiency (NUE) of papaya seedlings. This study is necessary because papaya is an important fruit crop of the Brazilian agribusiness and, to date, there are no publications of the interactive effects between N fertilization and elevated CO₂ on the physiology and growth of this specie.

2. Material and Methods

2.1 Experimental Conditions and Plant Culture – This study was conducted in a climate-controlled greenhouse at the USDA-ARS Crops Research Laboratory in Fort Collins, Colorado. Tainung #1 F₁ Hybrid seeds, which are currently widely cultivated for commercial purposes, were planted in 3.5 L plastic pots. The growth medium was a 2:2:1 mixture of perlite, peat moss, and washed sand. Seeds were selected for uniformity, and five seeds were sown in each pot. Pots were irrigated with water for 14 days, after which the less vigorous seedlings were discarded and the experiment commenced with only one seedling per pot. After this procedure, 12 pots were transferred to a greenhouse with an ambient CO₂ concentration (390 ± 10 ppm), while the remaining 12 paired pots were transferred to an adjacent greenhouse with an elevated CO₂ concentration (750 ± 20 ppm). On the same day, N treatments (as nutrient solution) were applied to the pots. For the elevated CO₂ concentration condition, fumigation with CO₂ was performed between 06:00h and 18:00h and the CO₂ supply was continuously computer-controlled (Argus Control Systems Ltd., White Rock, BC). During the experimental period, the CO₂ concentrations inside the greenhouses ranged from 380 to 400 µL L⁻¹ for the ambient CO₂ treatment conditions, and from 730 to 770 µL L⁻¹ for the elevated CO₂ treatment conditions. The plants were irrigated daily and fertilized once a week with 1.4 L of modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) in order to provide the following two N treatments: (a) 8 mM NO₃⁻ and (b) 3 mM NO₃⁻. Original Hoagland's solution was modified to vary the N content while maintaining the same concentration of all the other nutrients (Cruz et al., 2007). Every seven days, all of the pots were thoroughly flushed with tap water to avoid substrate salinization, and freshly-constituted nutrient solutions for the two N treatment conditions were then applied to the appropriate pots. To avoid possible spatial and greenhouse differences, pots were re-positioned within each greenhouse once a week, and every 15 days, the CO₂ treatments and corresponding pots were switched between the two adjacent greenhouses. Air temperature inside both the greenhouses was maintained between 27 and 30 °C during the day, and between 23 and 25 °C at night using computer-controlled air conditioners and heaters (York International, York, PA). The relative humidity was maintained at 35 ± 4%. Light inside the greenhouse was supplemented (12:12h day:night photoperiod) with 600 W lights (PL Light Systems,

113 Beamsville, Ontario) to maintain a light intensity at between 1100 and 1200 $\mu\text{mol photons m}^{-2}\text{s}^{-2}$ at bench
114 level.

115 *2.2. Measurements* - The CO_2 assimilation, intercellular CO_2 concentration (C_i), stomatal conductance (g_s),
116 and instantaneous transpiration rate (E) were determined on the central lobe of the youngest fully-expanded
117 leaves 45 days after the start of treatment. Evaluations were performed using the CIRAS-1 steady state
118 portable gas analysis system with a PLC (U) leaf chamber (PP systems, United Kingdom). The CIRAS-1
119 system provided the temperature and vapor pressure control, which were adjusted to near growth conditions
120 on the day of measurement. The CIRAS-1 was set to measure gas exchange parameters using the CO_2
121 concentrations of 390 and 750 $\mu\text{L L}^{-1}$ for plants grown under both CO_2 treatment conditions. The PP system
122 was connected to an artificial light source to project a photosynthetic photon flux density of 1100 μmol
123 $\text{photons m}^{-2} \text{s}^{-1}$ on the leaf surface. The air flow was 250 mL min^{-1} and measurements were taken between
124 09:00h and 11:00h. Gas exchange measurements were recorded after a stable CO_2 exchange rate was
125 obtained. The instantaneous water-use efficiency was calculated as the ratio of A and E . All photosynthetic
126 parameters were calculated according to Farquhar et al. (1980).

127 The experiment was terminated 62 days after treatment initiation. The stem diameter was measured
128 10 cm from the base of the stem. Height was determined as the distance from the base of the stem to the
129 insertion of the apical bud. All leaves with >1.0 cm were counted. Total leaf area was estimated using the
130 same procedure described for papaya by Cruz et al. (2004). Plants were harvested and separated into leaf,
131 stem plus petiole, and roots. These were oven-dried at 75 $^{\circ}\text{C}$ for 96 h and subsequently weighed. Specific
132 Leaf Area (SLA) was determined as the ratio between the total leaf area of the plant and dry leaf mass (Cruz
133 et al., 2004). Each of the plant parts were crushed, and total concentrations of N in the dried tissues was
134 determined by the automated dry combustion procedures (Shepers et al., 1989). Using the dry mass data and
135 N concentration of each organ, the following parameters were calculated: (a) total plant N content, which
136 was calculated as the sum of the dry mass of each organ multiplied by the respective N concentration; and
137 (b) NUE, which was calculated as the ratio between the total dry mass and total plant N content.

138 *2.3 Experimental design and statistical analysis* - Plants were distributed over a completely randomized
139 design that involved a 2X2 factorial combination (two CO_2 concentrations x two N levels) with six
140 replicates. The treatments were: 750_8N (high CO_2 and high N); 750_3N (high CO_2 and low N); 390_8N
141 (ambient CO_2 and high N), and 390_3N (ambient CO_2 and low N). Each experimental unit was one plant.
142 Analysis of the variance was performed with the statistical analysis software SISVAR (Ferreira, 2008) and
143 the means were compared using the Student–Newman–Keuls (SNK) test when significant effects were
144 observed. Correlation analysis between some variables was also performed. The significance level for
145 rejecting the null hypothesis was $p \leq 0.05$. Data are presented as mean $\pm$ standard error of the mean (SEM).

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148 3. Results

149 The interaction between CO₂ and N was significant for the following variables: (i) total leaf area; (ii)
150 dry mass accumulation in the leaf, stem plus petiole, and root; (iii) total dry mass accumulation; and (iv)
151 instantaneous water-use efficiency (Table 1).

152 Under higher N availability, elevated CO₂ increased plant height, stem diameter, and leaf area over
153 plants grown under 390 µL L⁻¹ of CO₂ by 15.4%, 14.0%, and 26.8%, respectively (Figs. 1A, 1B, and 1C).
154 Similar effects of CO₂ were observed for the height and stem diameter of plants grown in 3 mM of N. There
155 was significant interaction to leaf area, with positive effect to plants grown under low N. The effect of
156 higher N concentrations on leaf area was stronger than the effect of elevated CO₂. Plants grown under
157 750_8N had 226% greater leaf area than the plants grown at 750_3N, while plants grown under 390_8N had
158 leaf areas that were 239% higher than plants grown at 390_3N. Plants grown under high N levels also had a
159 greater more leaves (Fig. 1D). However, elevated CO₂ levels did not appear to significantly influence this
160 characteristic.

161 Increases in leaf, stem plus petiole, and root dry mass at elevated *versus* ambient CO₂ levels were
162 49%, 54%, and 73%, respectively, for plants growing under high N availability (Figs. 2A, 2B, and 2C). For
163 plants grown under low N, growth at 750 compared to 390 µL L⁻¹ of CO₂ increased the biomass of leaf, stem
164 plus petiole, and root dry mass by 58%, 63%, and 78%, respectively. Total dry mass was 25.9 g for plants
165 grown under 750_8N, and 16.5 g for plants grown under 390_8N, resulting in an increase of 56.6% (Fig.
166 2D). However, for plants grown under low N, the elevated CO₂ caused a 64.1% increase in the total dry
167 mass accumulation. These results indicate that the intensity of the response for dry mass accumulation in
168 each plant organ was slightly higher for those plants grown under lower N. The interaction for total dry mass
169 was small, but highly significant (p <0.01; Table 1). Plants cultivated under elevated CO₂ and either high or
170 low N showed an increase of 14% and 11%, respectively, in the root:shoot ratio when compared to the
171 plants grown at ambient CO₂ (390 µmol mol⁻¹; Fig. 2E). However, these differences were not statistically
172 significant (Table 1). The increase in the CO₂ concentration reduced the SLA, which represents leaf area per
173 unit of leaf mass, for plants grown under 8 mM and 3 mM N by 14.9% and 16.9%, respectively (Fig. 2F).
174 SLA was lower for plants grown under lower N.

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181 Table 1. Anova table showing the effects of CO₂ and N levels on characteristics of papaya seedlings

Parameters	Units	CO ₂		Nitrogen (N)		CO ₂ x N	
		F	p	F	p	F	p
Height	cm	37.5	0.004	226.4	0.004	0.7	0.414
Diameter	mm	78.4	0.004	739.7	0.003	0.2	0.683
Leaf area	dm ²	46.3	0.001	892.0	0.003	10.8	0.004
Leaf number	number	0.0	0.992	118.4	0.003	0.5	0.473
Leaf dry mass	g	123.4	0.002	706.5	0.002	23.5	0.003
Stem + petiole dry mass	g	55.5	0.004	335.0	0.003	13.7	0.005
Root dry mass	g	38.7	0.002	166.4	0.004	11.7	0.003
Total dry mass	g	70.0	0.003	377.0	0.002	16.7	0.003
Root:shoot ratio		3.405	0.080	5.571	0.029	0.15	0.703
Specific leaf area	dm ⁻² g ⁻¹	59.6	0.003	28.5	0.002	0.0	0.952
Stomatal conductance	mmol m ⁻² s ⁻¹	0.2	0.641	0.5	0.484	1.6	0.222
Transpiration	mmolH ₂ O m ⁻² s ⁻¹	0.1	0.721	0.1	0.684	2.5	0.133
CO ₂ assimilation	μmol m ⁻² s ⁻¹	44.2	0.004	0.5	0.503	1.8	0.203
Water use efficiency	μmol CO ₂ mmol H ₂ O ⁻¹	56.1	0.002	0.0	0.952	12.0	0.004
Leaf N concentration	% in dry mass	875.7	0.004	0.4	0.544	1.5	0.232
Root N concentration	% in dry mass	56.0	0.003	0.3	0.611	1.9	0.181
Stem + petiole concentration	% in dry mass	109.2	0.003	12.86	0.003	0.2	0.644
Leaf N content	g	0.0	0.99	990.6	0.004	0.2	0.693
Root N content	g	24.2	0.002	142.7	0.002	10.0	0.002
Stem + petiole N content	g	12.4	0.001	353.1	0.003	3.1	0.094
Total N content	g	8.8	0.002	544.9	0.003	2.3	0.144
Nitrogen use efficiency	g dry weight gN ⁻¹	505.2	0.002	5.0	0.043*	0.7	0.423

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Fig. 1 Effect of ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO₂ at 3 or 8 mM N on height (A), stem diameter (B), total leaf area (C), and leaf number (D) of papaya plants. Values given are means $\pm$ SEM (n=6). Means followed by the same letter are not significantly different ($p < 0.05$).

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Fig. 2 Effect of ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO₂ at 3 or 8 mM N on dry masses of the leaf (A), stem + petiole (B), root (C), total leaf area (D), and SLA (E) of papaya plants. Values given are means $\pm$ SEM. Means followed by the same letter are not significantly different ($p < 0.05$).

Nitrogen and CO₂ treatments had no significant effect on E and g_s (Figs. 3A and 3B). However, whole plant transpiration may have been higher under elevated CO₂ since the leaf area was higher when compared to plants grown at ambient CO₂ levels. The increase in CO₂ allowed the plants to achieve a higher

230 A, even when grown under low N conditions (Fig. 3C). Plants grown under 750_8N and 750_3N conditions
 231 displayed similar values for A, and both treatments had higher A than the 390_8N and 390_3N plants. Under
 232 high N, higher CO₂ increased A by 31%, whereas under low N, the increase was only 24.1%. Plants grown
 233 under 390_8N conditions had higher A than the 750_8N plants when both were measured at concentration of
 234 750 $\mu\text{L L}^{-1}$ CO₂ on the leaves (Fig. 3C, 5TH column), characterizing a probable photosynthetic acclimation.
 235 For plants grown at the same N level, those grown under elevated CO₂ showed the highest instantaneous
 236 water-use efficiency, with the 750_8N treatment being superior to the 750_3N treatment (Fig. 3D). The
 237 elevated CO₂ concentration induced an increase of 52.7% in the instantaneous water-use efficiency of the
 238 plants grown under 8 mM N, and only 16% for those grown under 3 mM N.

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253 **Fig. 3** The effect of ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO₂ at 3 or 8 mM N on transpiration
 254 (A), stomatal conductance (B), photosynthesis (C; to identify possible acclimation, 390_8N plants also were
 255 measured with 750 $\mu\text{L L}^{-1}$ CO₂ [390_8N (750), last column]), and instantaneous water-use efficiency (D) of
 256 papaya plants. Values given are means $\pm$ SEM. Means followed by the same letter, or without letters, are not
 257 significantly different ($p < 0.05$).

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259 Nitrogen concentrations in the leaves, stems, and roots were lower for plants grown under higher
 260 CO₂ concentrations regardless of the concentration of N. Leaf N concentrations of plants grown under
 261 750_8N conditions were 34% lower than that of the 390_8N plants (Fig 4A). However, for plants grown
 262 under low N (750_3N x 390_3N), elevated CO₂ reduced the leaf N concentrations by 35%. Stem + petiole
 263 (Fig. 4B) and root N concentrations (Fig. 4C) were 24% and 10% lower, respectively, in plants grown at the
 264 higher CO₂ concentration. In contrast, the high CO₂ concentration had no effect on total N content in leaves
 265 (leaf dry mass x leaf N concentration; Fig. 5A). The CO₂ concentration increased the levels of total N in
 266 both the roots and the stem + petiole portions of the plants in the 8 mM N treatment group, but not in the 3
 267 mM N treatment group (Figs. 5B, 5C). N fertility had no effect on the concentration of N in leaf and root
 268 tissues (Figs. 4A, 4B). Surprisingly, plants fertilized with 3 mM N had higher stem + petiole N
 269 concentrations when compared to plants fertilized with 8 mM N (Fig. 4C). However, N fertilization
 270 substantially increased N content of all plant organs, as well as total plant biomass (Figs. 5A-D). The
 271 positive effect of N fertilization on root N content was significant for plants grown at ambient and elevated
 272 CO₂. However, elevated CO₂ increased root N content in plants with the high N supply, but not those with
 273 the low N supply.

287 **Fig. 4** Effect of ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO₂ at 3 or 8 mM N on leaf N concentration
 288 (A), root N concentration (B), and stem + petiole N concentration (C) of papaya plants. Values given are
 289 means $\pm$ SEM. Means followed by the same letter are not significantly different ($p < 0.05$). DW means dry
 290 weight.

Fig. 5 Effect of ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO₂ at 3 or 8 mM N on leaf (A), root (B), stem + petiole nitrogen content (C), and total nitrogen accumulated (D) in papaya plants. Values given are means $\pm$ SEM. Means followed by the same letter are not significantly different ($p < 0.05$).

Significant, but minor, differences were observed in total N content (leaf plus stem + petiole plus roots) between plants grown at different CO₂ concentrations, but the same N levels. As a result, NUE was higher for plants grown under the CO₂-enriched environments, regardless of the N level in the substrate (Fig. 6), with the 750_8N and 750_3N plants showing NUE values 37.2% and 42% greater than those observed for the 390_8N and 390_3N plants, respectively (Fig. 6). When analyzed for all treatments, the correlation between root dry mass and total N content was high and positive ($r = 0.93^*$; Fig. 7).

4. Discussion

This study was designed to evaluate the effects of elevated CO₂ and N levels on important aspects of growth and physiology in papaya plants. To our knowledge, this is the first work to describe the interaction of N fertilization and elevated CO₂ favoring the production of more vigorous papaya seedlings. These results

are important because seedling production and establishment have significant importance for papaya production systems (Mendonça et al., 2006).

Fig. 6 Correlation between root dry mass and total N accumulated by papaya plants grown under ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO_2 at 3 and 8 mM N. *Indicates a significant correlation ($p < 0.01$).

Fig. 7 Effect of ambient (390 $\mu\text{L L}^{-1}$) and elevated (750 $\mu\text{L L}^{-1}$) CO_2 at 3 or 8 mM N on NUE of papaya plants. Values given are means $\pm$ SEM. Means followed by the same letter are not significantly different ($p < 0.05$). DW means dry weight.

As a C_3 plant, papaya growth is limited by the atmospheric CO_2 concentration. Therefore, we expected that elevated CO_2 levels would promote an increase in the dry mass accumulation, similar to other C_3 species (Ainsworth and Long, 2005). The total dry mass production of papaya plants grown in 750_8N was 56.6% greater than those grown in 390_8N. Surprisingly, at the elevated CO_2 concentration, papaya

plants grown under low N had increased total dry mass production when compared to plants grown in high N ($p < 0.01$). Similar results were observed for the dry mass of each plant organ. These results contradict several earlier studies, which indicated that low N reduces the plant's response to increased CO₂ levels. In fact, de Graaff et al. (2006) and Curtis and Wang (1998) analyzed a set of papers and observed that N deficiency reduced the response of herbaceous and woody plants to elevated CO₂ concentrations. Similar results were reported in a review on the responses of C₃ grasses to CO₂ levels (Wand et al., 1999). However, we found that the low N concentration substantially limited the biomass accumulation of plants grown at both elevated (-68%) and ambient CO₂ levels (-69%), indicating that papaya growth was much more responsive to the levels of N than CO₂ used in this experiment. The results presented here also indicate that an increase in the CO₂ level alleviated the effect of low N on dry matter accumulation in papaya, a result similar to that reported by Mohamed et al. (2013). The higher dry mass accumulation observed for both N levels when grown under 750 $\mu\text{L L}^{-1}$ CO₂ can be at least partially explained by the larger leaf area and higher rate of A per leaf area unit observed in these treatments.

The effect of CO₂ on total leaf area was greater than the effects obtained for stem diameter and height, with the increase due almost exclusively to leaf size since CO₂ levels had no significant effect on the number of leaves. Several studies have also reported an increase in the leaf area in response to elevated CO₂ levels (Mohamed et al., 2013; Smith et al., 2013; Rosenthal et al., 2012), and these results are explained by the faster plant growth associated with an increased rate of carbon assimilation (Manderscheid et al., 2003; Mohamed et al., 2013). This is important because leaf area, within certain limits, is directly related to the absorption of incident radiation, which can increase whole-canopy carbon assimilation capacity by itself (Lambers et al., 1990; Anten et al., 1995) and, consequently, enhance plant growth. Some have contended that an increase in the CO₂ concentration will only enhance total leaf area when plant-available N is at appropriate level (Hirose et al., 1996; Kim et al., 2003). However, our results show that seedling growth in a CO₂-enriched atmosphere contributed to an increase in the total leaf area, plant height, and stem diameter in papaya, even under conditions of low N availability. Further, the increase in total leaf area resulting from growth under CO₂ enrichment was somewhat greater for plants grown under low N as compared to high N (CO₂ by N interaction $p < 0.01$). This positive effect of CO₂ on papaya seedlings has not been described previously and suggests that an elevation in the CO₂ level will be beneficial to this specie.

Elevated CO₂ levels led to a reduction in the SLA, a result consistent with other studies (Markelz et al., 2014; Poorter and Navas, 2003). The importance of a lower SLA is complex. It can result either from larger mesophyll cellular layer or a larger cell size, which contributes to increased carbon fixation (Muralikrishna et al., 2013; Lin et al., 2001), and/or it can be related to the increase in fiber and carbohydrates content (Roumet et al., 1999; Teng et al., 2006; Brown and Byrd, 1997), without any positive influence on the A capacity of the plants. For papaya, the first mechanism appears relevant since an elevated CO₂ concentration promoted a reduction in the SLA and increased the CO₂ assimilation rate.

Elevated CO₂ levels reduced the N concentrations in all of the plant organs, with the most significant effect on leaf N. This is a common response in plants, and may be a plant mechanism involving N

380 redistribution to maximize carbon gain (Leakey et al., 2009), to inhibit nitrate assimilation into protein
381 (Bloom et al., 2014), and/or simply be the result of carbon uptake exceeding the capacity of the plant to
382 assimilate N, which is a more passive dilution response (Hodge, 1996; Kant et al., 2012). As a result, even a
383 substantial increase in N (from 3 mM to 8 mM) was insufficient to minimize the effect of elevated CO₂ on
384 the reduction of leaf N concentration. Plants grown under 750_8N and 750_3N conditions presented leaf N
385 concentrations of 1.89% and 1.84%, respectively. The observed leaf N concentration in 750_8N plants was
386 considerably less than the normal leaf N concentration for papaya seedlings, which is around 3% (Cruz et
387 al., 2004). A much higher N concentration than was used in the 8 mM N treatment may be needed to
388 maximize papaya growth in CO₂-enriched atmospheres, although such levels are likely not economically and
389 environmentally feasible (Kant et al., 2012). Further, a lower demand for N relative to C in papaya leaves at
390 high CO₂ levels may promote maintenance of present-day root N concentrations in CO₂-enriched
391 environments (Walch-Liu et al., 2001).

392 Despite lower plant N concentrations, total N content (the sum of the N in all plant organs) was
393 higher for the plants cultivated under the elevated CO₂ concentration. Plant growth at elevated CO₂ levels
394 can increase root growth, enabling greater absorption nutrients, such as N, from the soil (Yang et al., 2008).
395 In our experiment, the high and positive correlation between the root dry mass and total N content ($r =$
396 0.93^*) reinforces this idea. Additionally, the higher transpiration stream, due to the maintenance of *E* and
397 greater total leaf area, which increases delivery of N to the root system (Taub and Wang, 2008), and the
398 increased demand for N to sustain growth, may have also contributed to the increased N content (an
399 approximate measure of uptake) by the plants grown under elevated CO₂. The lack of interactions between
400 the CO₂ and N on total N content does not agree with the results presented by Stitt and Krapp (1999), who
401 reported that the uptake of N was enhanced by increased CO₂ concentrations only in situations of increased
402 N availability. The elevated CO₂ concentration also promoted greater NUE. Among other factors, CO₂
403 enrichment leads to a reduction in photorespiration of C₃ plants, so less photosynthetic leaf
404 protein is required to produce a given amount of dry matter (Stitt and Krap, 1999). This
405 can result in more efficient carbon fixation per N unit. However, the increase in NUE for
406 papaya plants grown under low N was slightly higher, although not significant, than that of plants grown
407 under conditions of higher N availability. This result in NUE can help to explain why papaya plants grown
408 under low N responded better, in terms of biomass production, than plants under higher N concentrations
409 did. In fact, Norby et al. (1986) indicated that mechanisms associated with an internal adjustment in the
410 distribution of N leading to an increase or maintained level of NUE can be an important factor in the
411 accumulation of dry mass in plants grown under conditions of elevated CO₂ and reduced N availability.

412 Exposure of papaya to elevated CO₂ for 45 days increased *A* in plants grown under 8 mM and 3 mM
413 of N by 30% and 24%, respectively. No interaction was observed between CO₂ and N levels, indicating that
414 low N availability did not limit the *A* response in papaya, which contrasts to several previous reports (Piva et
415 al., 2013; Sanz-Sáez et al., 2010; Stitt and Krapp, 1999). The plants grown under conditions of 750_8N and
416 750_3N exhibited similar *A*, and this may be related to the similar leaf N concentrations in these treatments.

CO₂ assimilation of the 390_8N plants was higher than that of the 750_8N plants when both were measured with 750 $\mu\text{L L}^{-1}$ CO₂. This indicates that the CO₂ assimilation rate of the papaya plants experienced a reduction in capacity, probably due to photosynthetic acclimation, resulting from long-term exposure to CO₂-enriched atmospheres. Lower leaf N concentrations observed for the 750_8N condition may have contributed to this possible photosynthetic down-regulation in plants grown at elevated CO₂, due to a reduction in the proteins associated with the CO₂ assimilation processes (Seneweera et al., 2011; Xu et al., 2011) and in the carboxylation capacity of Rubisco (Cruz et al., 2003).

The g_s and E were not affected by the elevated CO₂ concentration or low N, indicating that the increase in the instantaneous water-use efficiency (A/E) of the 750_8N plants was due to the increase in A . The lack of an effect of elevated CO₂ on g_s and E of the papaya plants is an atypical result (Ainsworth and Rogers, 2007; Morgan et al., 2004; Drake et al., 1997). The papaya plants did not exhibit an adaptive mechanism to minimize water loss by transpiration under elevated levels of CO₂. Diverse and interdependent factors are involved in stomatal control in response to the environmental factors (Ainsworth and Rogers, 2007; Wang et al., 2014), and it is still not fully understood why stomata fail to respond to the elevated external CO₂ levels. According to Haworth et al. (2013), species with little or no control of the stomatal aperture are more likely to exhibit a reduction in the stomatal density when grown under conditions of elevated CO₂ than species with active stomatal control. As greater CO₂ availability increased the total leaf area it is possible that in a scenario of rising CO₂ the canopy transpiration of papaya will increase, thus intensifying the demand for water. These results are concerning because in future climate change scenarios, water will be a valuable asset, making it more expensive, and its supply for irrigation will decrease.

There was no reduction in the leaf N concentration between 750_8N and 750_3N treatments, while the total leaf area of the 750_3N plants was reduced by 69.3% when compared to the 750_8N. This implies that the papaya plants grown under low N prioritize the reduction in the interception of solar radiation, while they maintain or minimize a reduction in leaf N concentration. By employing this strategy, these plants are able to minimize the negative effect of low N on the CO₂ assimilation capacity per unit leaf area. A similar strategy has already been described for potato (Vos and Van Der Putten, 1998) and another papaya variety (Cruz et al., 2007). In contrast, maize struggles to maintain leaf area per plant at the expense of decreased N concentration, thus leading to a reduction in the CO₂ assimilation rate (Vos et al., 2005). The ability of papaya seedlings to maintain photosynthesis and leaf N concentration, and increases the response of leaf area under low N contributes to the positive effect of elevated CO₂ levels on dry matter accumulation under such conditions. At this early growth stage, the increased N level produced the greatest effects on height, diameter, and leaf area than did the elevated CO₂ concentration. The primary effect of supplemental N is to increase plant growth, since several processes that contribute to the transverse and longitudinal growth of each plant organ are highly dependent on an adequate level N (Longnecker, 1994; Krouk et al., 2011).

In conclusion, an increase in the atmospheric CO₂ concentration was beneficial for dry mass production of papaya and alleviated the negative effects of N reduction in the substrate on papaya growth.

453 **Acknowledgments**

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455 This research was partially supported by United States Department of Agriculture/USDA-ARS and
456 Embrapa Cassava & Tropical Fruits. The authors gratefully acknowledge Deborah Pelacani Cruz for
457 valuable help in growing plants.

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