SAMUEL DE SOUZA PINTO

GROWTH AND MORPHOPHYSIOLOGICAL RESPONSES OF EUCALYPTUS SEEDLINGS EXPOSED TO HIGH [CO_2] AND ELEVATED TEMPERATURE

Dissertation submitted to the Universidade Federal de Viçosa, as part of requirements in Plant Physiology Graduate Program for obtaining the *Magister Scientiae* degree.

VIÇOSA
MINAS GERAIS - BRASIL
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

P659g
2018

Pinto, Samuel de Souza, 1989-
Growth and morphophysiological responses of eucalyptus seedlings exposed to high [CO2] and elevated temperature / Samuel de Souza Pinto. - Viçosa, MG, 2018.
v, 46 f. : il. (algumas color.) ; 29 cm.

Texto em inglês

.

Orientador: Wagner Luiz Araújo.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Referências bibliográficas: f. 33-38.

1. Eucalipto - Mudas - Crescimento. 2. Troca gasosa em plantas. 3. Eucalipto - Metabolismo. 4. Mudanças climáticas. I. Universidade Federal de Viçosa. Departamento de Biologia Vegetal. Programa de Pós-Graduação em Fisiologia Vegetal. II. Título.

CDD 22. ed. 583.766

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APPROVED: July 31th, 2018

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ACKNOWLEDGMENTS

First of all, I thank God for always guiding me and giving me wisdom.

To my parents, Francisca Alves de Souza Pinto, "Xixica", and José Raimundo Pinto, "Zeca", who have always believed in me and above all, they are the main responsible to my character formation.

To my siblings and relatives for making the challenges easier while providing the necessary support in difficulty times.

To my girlfriend, Letícia de Melo Ferreira, for love, help and patience (including when I could not even talk).

To the Universidade Federal de Viçosa (UFV), in particular the Plant Physiology Graduate Program at the Department of Plant Biology and the Department of Agricultural Engineering for providing the necessary conditions to carry out this work.

To CAPES for financial support.

To my adviser, Prof. Wagner L. Araújo for giving me the opportunity to be part of his team, and for always supporting me, helping and encouraging.

To my supervisor "DORA" for always receiving me and saying "c*alm, let's find the error*" and the immense contributions to this work.

To all the members of my evaluation committee for accepting to judge my work and to contribute for its improvement.

To Prof. Edgard Picoli and Samuel Martins for the teachings and contributions to this work.

To my friends, Iale, Iagor, Luiz Felipe, Marciel, Elias, Franciele, Leonardo, Bruno and Rodrigo for all contributions during this time.

To all members of my working group - UCP for the coexistence and contributions, especially Italo, Jaciara, Daniele and Regina for the friendship.

Finally, to all who have contributed at some point to the realization of the work described here, many thanks.

SUMMARY

ABSTRACT

PINTO, Samuel de Souza, M.Sc., Universidade Federal de Viçosa, July, 2018. **Growth and morphophysiological responses of eucalyptus seedlings exposed to high [CO_2] and elevated temperature.** Adviser: Wagner Luiz Araújo. Co-adviser: Edgard Augusto de Toledo Picole.

The area planted with tree species is growing significantly in recent years and the main genus used is *Eucalyptus*. However, the adequate development of the crop is dependent on several factors such as concentration of carbon dioxide ([CO_2]) and temperature. Climate changes, mostly related to increases in [CO_2] and atmospheric temperature are expected, which will most likely result in changes in plant growth and development. Although the effects of [CO_2] have been extensively studied, and recent works indicated that high [CO_2] might mitigate the impacts of high temperature, the interaction between these two environmental factors have not been addressed in eucalyptus to date. Thus, the objective of this work was to investigate responses in eucalyptus seedlings exposed to high [CO_2] and elevated temperature using mini-FACE system. To this end, seedlings of two eucalyptus clones were cultivated for sixty-two days in environment with High [CO_2] and Heating either combined or isolated. Throughout this period, gas exchanges analyzes were performed. At the end, morphological analyzes and curves of response to light and CO_2 were realized, as well as, plant material was harvested for biochemical and anatomical analyzes. The results showed lower growth in response to environmental warming, associated with metabolic changes such as reductions in leaf levels of fructose and starch. On the other hand, increased [CO_2] contributed positively to growth and plant biomass accumulation. Plant exposure to these two factors simultaneously resulted in [CO_2] mitigation of the negative effects caused in response to plant warming. Therefore, it was possible to conclude that in a global climatic changes scenario, with increase in both [CO_2] and atmospheric temperature, metabolic acclimation responses would occur and contribute to the maintenance of seedling growth.

RESUMO

PINTO, Samuel de Souza, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Respostas morfofisiológicas e de crescimento em mudas de eucalipto expostas à elevada [CO_2] e aumento na temperatura.** Orientador: Wagner Luiz Araújo. Coorientador: Edgard Augusto de Toledo Picoli.

A área plantada com espécies arbóreas vem crescendo significativamente nos últimos anos e o principal gênero utilizado é o *Eucalyptus*. No entanto, o desenvolvimento satisfatório da cultura é dependente de fatores como a concentração de dióxido de carbono ([CO_2]) e temperatura. De acordo com projeções, aumentos tanto na [CO_2] quanto da temperatura são esperados, o que possivelmente resultará em alterações no crescimento e desenvolvimento vegetal. Nesse sentido vários estudos vêm sendo realizados elevando-se a [CO_2] e, recentes trabalhos mostram que o CO_2 atua mitigando os efeitos danosos causados por altas temperaturas, todavia pouco se sabe sobre a interação de desses fatores em eucalipto. Assim o objetivo desse trabalho foi investigar respostas apresentadas por mudas de eucalipto expostas a elevada concentração de [CO_2] e aumento da temperatura por meio de sistema mini-FACE. Para isso, mudas de dois clones de eucalipto foram cultivadas por sessenta e dois dias em ambiente com elevada [CO_2] e temperatura elevada, de forma combinada ou isolada. Ao longo do período de exposição aos tratamentos foram realizadas análises de trocas gasosas. Ao final, análises morfológicas, curvas de resposta à luz e ao CO_2, bem como coleta de material vegetal para análises bioquímicas e anatômicas das plantas foram realizadas. Os resultados mostraram menor crescimento em resposta ao aquecimento, associado a alterações metabólicas como reduções nos níveis foliares de frutose e amido. Por outro lado, o aumento na [CO_2] contribuiu positivamente para o crescimento e acúmulo de biomassa das plantas. A exposição das plantas a esses dois fatores simultaneamente resultou na mitigação dos efeitos negativos causados pelo aquecimento às plantas. Diante disso, pôde-se concluir que em um cenário de mudanças climáticas com aumento tanto na [CO_2] quanto na temperatura atmosférica, respostas metabólicas de aclimatação possivelmente ocorrerão de modo a contribuir para a manutenção do crescimento das mudas.

1. INTRODUCTION

The area used to commercial plantation trees in Brazil reached about 7.84 million ha in 2016, which the vast majority is destined to the production of pulp and paper (34 %), followed by independent producers and fomentations (29 %), steel and charcoal (14 %), among others (IBA, 2017). In order to attend this enormous demand, *Eucalyptus* and *Pinus* genus are mostly used (Binkley et al., 2017). It is important to mention that *Eucalyptus* is the most planted forest species in Brazil due to its characteristics such as rapid growth and high productivity. Moreover, the predominance of these species occurs due to privileged climate and environment conditions found in Brazilian lands, associated with corrects silvicultural practices that largely favors their establishment (Binkley et al., 2017). Nevertheless, the optimal development of those crops depends on both biotic and abiotic factors such as temperature and [CO_2] (Albert et al., 2011), water availability and nutrients (Wang et al., 2003). These factors might be able to individually or combined significantly influence plant development.

During the last 20 million years of their evolutionary history, plants have never experienced such intensification in [CO_2] (Pearson and Palmer, 2000). It should be noted that, by the end of this century, a [CO_2] of approximately 985 ppm is expected, which represents more than twice the current concentration. At the same time, temperature increases between 0.8 and 4.8 °C is also expected (IPCC, 2014). It is equally important to mention that increases in atmospheric [CO_2] are closely associated with global warming (Kimball et al., 1993; Körner, 2006), leading also to increases in temperature.

Different physiological and metabolic impacts are usually observed in C_3 plants following elevations in [CO_2]. First, increases in photosynthetic rate leading to higher productivity is normally observed; second, reductions in stomatal conductance, which culminated with lower water demand is verified (Saxe et al., 1998; Körner, 2006). In addition, changes in growth, development and metabolism (Klaiber et al., 2013; Oehme et al., 2013), as well as in the pattern of gene expression (May et al., 2013) are also observed. Moreover, there is a higher ratio between carbon dioxide and oxygen near the carboxylation sites of Rubisco, thus reducing carbon losses due to the reduction of the photorespiratory process (Long et al., 2004; Leakey et al., 2009).

High temperatures effects in plants are generally associated with other abiotic stresses such as drought and salinity. However, the individual characterization of plants responses to increased temperature is of crucial importance from a physiological point of view given that effects are depending on the species, as well as development stage. In this context, the first damage following exposure to high temperatures is associated with variations in membrane fluidity, protein and cytoskeletal instability, changes in chromatin structure, and enhancements on production of reactive oxygen species (Bita and Gerats, 2013). Accordingly, high temperatures induce also chlorophyll degradation and foliar abscission, reduction of both shoot and root growth, resulting, in general, in losses in productivity (Vollenweider and Günthardt-Goerg, 2005). Moreover, supra-optimal temperature leads to increased photorespiration since the proportion of O_2 available is increased (Dusenge et al., 2018). Furthermore, high temperatures are known to impact several physiological processes related to both primary and secondary metabolism (Bita and Gerats, 2013).

Due to expected changes in the composition of atmosphere (IPCC, 2014), it seems of pivotal significance to further understand plants' behavior under modified environmental conditions. In this sense, it has been demonstrated that CO_2 is an important factor involved in mitigation of damages caused by other stresses, including high temperatures (Sage and Kubien, 2007; Taub et al., 2000; Wang et al., 2008; Rodrigues et al, 2016). Remarkably, the responses commonly noticed are increases in metabolism of draining organs and thus stimulation of photosynthetic rate (Ainsworth and Rogers, 2007), recovery of growth (Figueiredo et al., 2015). It has been also suggested that greater tolerance to such stresses may be related to different metabolic routes activated under conditions of high availability of CO_2 (Yu et al., 2017).

One method currently used to study effects of CO_2 is based on Free Air CO_2 Enrichment (FACE) systems. Using this system, [CO_2] is elevated in natural environment, allowing the possibility to carry out studies directly in field, a fact that clearly minimize possible disturbances to plants. Noteworthy, this system has been successfully used since the eights in both United States and Europe (Hendrey and Kimball, 1994; Kimball et al., 1995; Miglietta et al., 1997).

Although interesting results are usually obtained employing FACE system, some problems such as high cost for realization and maintenance of experiments are also evident (Tubiello and Fischer, 2007). In fact, an alternative widely adopted in order to minimize the expenses nowadays is the so called mini-FACE systems. This technology allows the realization of experiments involving climate changes in relatively smaller areas, reducing the need for high investments (Miglietta et al., 2001). It is important to mention that FACE methodology have been used in several studies with different species, including eucalyptus (Carey, 2016). Nevertheless, most of such studies have only investigated short-term responses. Therefore morphophysiological and metabolic responses of eucalyptus seedlings growing in a system allowing the enrichment of atmosphere with CO_2 (mini-FACE) and ambient heating during 2 months were investigated in order to obtain a detailed understanding of isolated and combined effects of high temperature and elevated [CO_2] on the behavior of these plants in a possible global climate change scenario. Overall, the results obtained demonstrate that eucalyptus seedlings exposed to simultaneous temperature and [CO_2] enhancements can develop a differential acclimation that potentiates their metabolic mechanism allowing plants to mitigate to negative impacts of high temperatures due positive effects of high [CO_2].

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

The experiment was conducted in experimental field belonging to the Department of Agricultural Engineering, Federal University of Viçosa, in Viçosa (20º45′ S, 42º54′ W), south-eastern Brazil. According Köppen classification, the climate is warm temperate mesothermic (Cwa), with rainy summers and dry winters. The average annual rainfall is 1.220 mm, with average maximum temperature of 26 °C and average minimum of 14 °C. Two eucalyptus clones, one from *E. grandis* x unknown father (Clone 01), and the other from *E. grandis* x *E. urophylla* (Clone 02), were used at approximately 180 days after replication. These clones also display differential tolerance to Vale do Rio Doce dry pointers (Harguindeguy et al., 2018). Uniform seedlings were cultivated in 8 L pots containing dystrophic red latosol; each pot received 50 g of NPK 06-30-06. In addition, magnesium chloride (50 mg dm^{-3}), zinc sulfate (4 mg dm^{-3}), manganese sulfate (3 mg dm^{-3}), boric acid (0.81 mg dm^{-3}) and copper sulfate (5 mg dm^{-3}) were applied via fertigation at 0 and 30 days after transplanting. There was no apparent restriction on root development as judged by root examination at the end of the experiment.

2.2. Free air CO_2 enrichment system (mini-FACE)

The mini-FACE system was based on POPFACE system developed previously (Miglietta et al., 2001). For CO_2 injection, each mini-FACE consisted of a 25 L CO_2 cylinder, two pressure regulators (SM37, SM Industry, Brazil), connections in general and five solenoid valves. Environmental sensors (infrared gas analysers, anemometers, sensors for air and soil temperature, humidity, solar radiation and precipitation) were installed at the central region of each system. Each system was coupled to a datalogger (CR1000, Campbell Scientific Instruments, Utah, USA). Two mini-FACEs were installed at 5 m of distance between each other. Among these, a sonic anemometer (wind sonic, option 4, Gill Instruments) were installed to determine wind speed well and direction. Each mini-FACE used was constituted by a triangular structure fixed to three metal rods with 2 m of height. This was accomplished by using steel cables and a system of pulleys to adjust the system of injection of CO_2 at the height of the plants canopy. In order to provide homogeneity between the supplied CO_2 and atmosphere, a 0.5 inch PVC ring with 2 m diameter, laser drilled every 5 cm (hole diameter equal 3 mm) was fixed to triangular structure, being associated with high gas pressure. To CO_2 application optimize, each ring was subdivided into 3 half, so that each half was controlled by an individual valve to compensate for wind direction and a flow control device to compensate for wind speed changes. The rate control CO_2 release was performed as function of occurrence of variation between wind speed and direction, using a programmable logic controller, all data being stored in computer. CO_2 average on mini FACE was 718 ppm, only injected over the during daylight hours (from 06:00 a.m. to 06:00 p.m) corresponding to the period when the plants are photosynthetically active. In one mini-FACE, five resistances were added to raise the air temperature by 2°C above that of the environment (mini-TFACE), in this treatment CO_2 average was 710 ppm. Heating system, which operated over the entire course of the experiment, was installed at 30 cm above the plant canopies.

2.3. *CO_2 and heating* treatments application

At 30 days after transplanting, four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 ºC and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm). Measurements were performed from May to July 2017 during the period of reduced vegetative growth and the treatments were maintained for over 62 days.

2.4. Gas exchange parameters *measurements*

The net rate of carbon assimilation (A), stomatal conductance to water vapor (g_s), leaf transpiration (E) and internal CO_2 concentration (C_i) were measured in first fully expanded leaves (fifth or sixth leaf pair from the branches counted from apex to base of the plants) at 0, 10, 17, 25, 32, 52 and 62 days after treatments application (DAT). All measurements were made using two cross-calibrated infrared gas analyzers (LI-6400XT, Li-Cor, Lincoln, NE, USA), on clear-sky days between 08:30 and 11:30 a.m. (solar time). To improve uniformity over course the day, measurements were conducted at leaf level using humidity between 40 and 70 % and at an artificial photosynthetically active radiation (PAR) of 1400 µmol photons m^{-2} s^{-1}. This PAR intensity is sufficiently high to saturate the photosynthetic machinery without causing photoinhibition and approximated the ambient irradiance intercepted by the sampled leaves at their natural angles in most measurements. At the end of the experiment (62 days after treatment), we used plants from control and high CO_2 treatments to analyze gas exchange parameters during four time periods: 08:00-10:00 h, 10:00-12:00 h, 12:00-14:00 h and 14:00-16:00 h (solar time) using ambient radiation, measured by a thermo sensor associated to the IRGA. We additionally used these plants to perform the response of *A* to light (*A/PPFD*) and CO_2 (A/C_i). Briefly, the $A/_{PPFD}$ curves were performed at ambient CO_2 concentration (C_a) of 400 µmol mol^{-1} in control plants, while in plants cultivated at high CO_2 treatment, CO_2 concentration of 700 µmol mol^{-1} was used. In order to perform the curve, 12 points different photon flux density (1400, 2000, 1700, 1000, 800, 600, 400, 300, 200, 100, 50 and 0 µmol m^{-2} s^{-1}), in this order. Upon completion of the measurements, light compensation point (*LCP*), light saturation point (*LSP*) and apparent quantum yield were obtained.

The A/C_i were performed at saturating light irradiance of 1400 $µmol^{-2}$ s^{-1}. Briefly, the measurements started at ambient CO_2 concentration (C_a) of 400 µmol mol^{-1} for control plants, while for plants grown under high CO_2, C_a was 700 µmol mol^{-1}. After the stead state was achieved, C_a was decreased stepwise to 50 µmol mol^{-1}. Upon completion of the measurements at low C_a, C_a was returned to 400 or 700 µmol mol^{-1} to restore the original A_N. Next, C_a was increased stepwise to 2000 µmol mol^{-1} in different C_a values for control plants (400, 300, 200, 100, 50, 400, 500, 600, 700, 900, 1100, 1400, 1700 and 2000 µmol mol^{-1}) and for plants in high CO_2 treatment (700, 600, 500, 400, 300, 200, 100, 50, 700, 900, 1100, 1400, 1700 and 2000 µmol mol^{-1}). From A/C_i curves, the maximum carboxylation rate of RUBISCO (V_{cmax}) and electron transport rate (J_{max}) were calculated by fitting the mechanistic model of CO_2 assimilation according to (Farquhar et al., 1980) later modified by (Sharkey, 1985) and (Harley et al., 1992). All gas exchange analyzes were performed on the first fully expanded leaf located on fifth or sixth branch counted from apex to base of the plant.

2.5. Isotopic composition of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$)

Isotopic composition were performed on leaves, stem and root tissues collected at the end of the experiment. Samples were oven-dried at 70 ºC during 72 h and were subsequently ground (2.5 to 3.0 mg) before combustion at 950 °C under continuous O_2 flux. The obtained CO_2 was purified and its stable composition was determined in relation to international PDB standard using mass spectrometer (ANCA-GLS Sercom, Crewe, UK). Differences in carbon isotopic composition ($\delta^{13}C$) from each sample were below 2 ‰. Results were expressed as $\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000$, which *R* is the molar ratio ($^{13}C/^{12}C$) from sample and standard (Rodrigues et al., 2016) and nitrogen ($\delta^{15}N$): $\delta^{15}N = [(R_{sample}/R_{standard}) - 1 \times 1000]$, which *R* is the molar ratio ($^{15}N/^{14}N$) (Aranjuelo et al., 2015).

2.6. Growth and biomass parameters

At the end of the experiment, plant height, stem diameter, branch and leaf number were measured. In addition, specific leaf area was computed using the dry mass of nine leaf discs (1.11 cm diameter each) per plant. Total leaf area was measured using a leaf area meter (LI-3100C, Li-Cor Inc., Lincoln, USA). Plant tissues were oven dried at 70 ºC after which the root dry mass, shoot dry mass, total dry mass and shoot-to-root dry mass ratio were determined.

2.7. Biochemical analyzes

2.7.1. Samples collection and extraction procedures

Leaf samples were collected from 11:30 a.m. to 12:30 a.m., being immediately frozen in liquid nitrogen and then stored at -80 °C until further analyzes. Metabolite extraction was performed by rapid grinding in liquid nitrogen. Methanolic extraction was carried, and the obtained methanolic extract was stored at -20 °C until further quantification, except an aliquot of 150 µL, which was stored at -80 °C for subsequent derivatization and metabolic profile determination.

2.7.2. Chlorophyll content

Chlorophylls content (*a* and *b*) was determined immediately after methanolic extraction. For this, samples (25 µL extract + 175 µL methanol) were analyzed on a microplate reader according to (Porra et al., 1989). After, total chlorophyll content (*a* + *b*) as well as the chlorophyll *a*/*b* ratio were determined.

2.7.3. Sugars content

The levels of glucose, fructose and sucrose were determined in methanolic extract as described previously (Fernie et al., 2001), with alterations. 30 µL of extract were added in mix containing 0.1 M HEPES/KOH buffer pH 7, $MgCl_2$ (3 mM), ATP (120 mM), NADP (50mM), and glucose 6 phosphate dehydrogenase (G6PDH) (0.7 U/reaction). The absorbance at 340 nm was determined every minute until stability was achieved. After, hexokinase (1.5 U/reaction) was added to determine glucose level, phosphoglucose isomerase (0.7 U/reaction) to determine fructose level and invertase (5 U/reaction) to determine sucrose level. Addition of enzymes was performed in this order. The following equation was used: µmol NADPH = ΔOD/(2.85 * 6.22) to determine sugars concentration.

2.7.4. Starch levels

Starch levels were determined following (Fernie et al., 2001), with adaptations. Briefly, the remaining pellet from methanolic extraction was resuspended in 0.1 M NaOH and then neutralized with 1M acetic acid. Next, the degradation mix containing amyloglucosidase and α-amylase enzymes was resuspended in 50 mM sodium acetate pH 4.0. To each sample 60 µL of this mix was added to 40 µL of suspension, and then incubated at 55 °C for 60 min. Subsequently, the microplate was centrifuged for 10 sec at 13500 rpm. After, 25 µL of suspension from samples of control and high CO_2 treatments were transferred and 25 µL of methanol were add while 50 µL of suspension from samples of heating and heating + high CO_2 treatments were transferred to a new plate where it was added 160 µL of mix containing 1M HEPES/KOH buffer, pH 7.0, $MgCl_2$ (30 mM), ATP (120 mM), NADP (50mM) and glucose-6-phosphate dehydrogenase (0.7 U/reaction). The absorbance was read at 340 nm every minute until stabilization. After that, hexokinase (2 U/reation) was added, allowing the reaction to occur until stabilization. Finally, starch content was calculated following: µmol NADPH = ΔOD/(2.85 * 6.22).

2.7.5. Malate content

Malate content was determined as previously described by (Nunes-Nesi et al., 2007). The mix containing buffer Tricine/KOH 0.4 M, pH 9; $MgCl_2$ 10 mM, MTT (methylthiazolyldiphenyl-tetrazolium bromide) 10 mM, NAD^+ (60 mM), phenazinaetosulfato 20 mM, Triton x100 10 % (v/v) and H_2O was added to 10 µL of methanolic extract in microplate reader. Absorbances were read at 570 nm in one minute intervals. After stabilization, malate dehydrogenase (1U/reaction) was added. Malate concentration in samples was calculated based on standard curve.

2.7.6. Amino acids

Amino acid determination was performed according (Cross et al., 2006). Briefly, the mix consisting of 1M citrate buffer, pH 5.2, ascorbic acid 0.2 % (w/v), 50 µL of methanolic extract, and 100 µl of 1 % nihydrin solution (m/v in 70 % ethanol) was added to microplate and dark incubated for 20 min at 95 °C and centrifuged for 15 sec. At the end, absorbance was read at 570 nm, and amino acid content was determined based on standard curve.

2.7.7. Protein content

The protein content was determined according to methodology described by (Cross et al., 2006). To the remaining pellet, 400 µL of 0.1 M NaOH was added, and then suspension was incubated for one hour at 95 °C and 400 rpm. Subsequently, it was centrifuged at 13.300 rpm for 10 min. From supernatant, an aliquot of 5 µL was withdrawn, which was diluted 10-fold in NaOH and added to microplate containing 180 µL of Bradford reagent (1/5). Next, absorbance was measured at 595 nm and protein content was determined by calibration curve containing bovine serum albumin (BSA) as standard.

2.7.8. Extraction, derivatization and metabolites analysis by gas chromatography coupled to mass spectrometry (GC-MS)

About 50 mg of sample were divided into 1.5 mL tubes and extracted into a pre-cooled mixture of water, methanol and chloroform (1: 2.5: 1, v / v / v) and 60 µl of ribitol (0.2 mg/mL). Samples were vigorously shaken for 10 s for complete mixing. Samples were then incubated for 30 min with shaking of 1,246 g at 4 °C. After, samples were centrifuged at 16,200 g for 5 min at 4 °C. Supernatant was transferred to new tube, adding 750 µl of ultrapure water. samples were vigorously shaken again for 10 seconds following to further centrifugation at 16,200 g for 15 min at 4 ° C. The top layer (polar phase) was collected and aliquots of 200 µL removed. The samples were dried under vacuum and stored at -80 °C until further use. For derivatization, 40 µL of methoxyamine reagent was added to each sample and stirred at 16200 g for 2 h at 37 °C. Subsequently, 70 µL of N-

Methyl-N- (trimethylsilyl) trifluoroacetamide (MSTFA) and fatty acid methyl esters (FAMES) standards were added to samples and stirred at 1246 g for 30 min at 37 °C. Samples were transferred to glass vials and analyzed using gas chromatography mass spectrophotometry (GC-MS). The chromatograms and generated mass spectra were evaluated using the TAGFINDER software (Luedemann et al., 2008).

2.7.9. Pyridine nucleotides

NAD(H) and NADP(H) levels were determined using approximately 25 mg of fresh mass. Extraction of reduced and oxidized form was performed by preferential destruction in acidic and basic environments, respectively (Foyer et al., 1991). After, quantification was performed according (Hezirezaei et al., 2002), with modifications. Two aliquots of 50 µL of soluble extract were transferred to ELISA plate, the same volume being neutralized with 0.1 M KOH in 0.2 M TRIS/HCl pH 8.4 or $HClO_4$ in 0.2 M TRIS/HCl pH 8,4. Subsequently the material was diluted 5 and 30 times for quantification of NADP (H) and NAD (H), respectively. At the end, 100 µL of sample was transferred in duplicate to new ELISA plate and 50 µL of mix (15 µl Tricine/KOH 0.3 M pH 9.0; 6,0 µL Na^2 EDTA 12 mM; 0.5 µL PES 0.3mM; 10 µL MTT 1.8 mM; 4.4 µL ETOH 1.5 M; 0.45 µL 18 U/mL alcohol dehydrogenase, 18.45 µL ultrapure water). While for determination of $NADP^+$ and NADPH levels, 50 µL of mix (15 µL Tricine/KOH pH 9.0 0.3 M; 6.0 µL Na2 EDTA 12 mM; 0.5 µL PMS 0.3 mM; 10 µL MTT 1.8 mM; 2.5 uL glucose 6 phosphate; 0.645 µL Glucose 6 phosphate dehydrogenase grade I- 9 U/mL; 20,155 µL ultrapure water). The absorbance was read at 570 nm during 40 min. Nucleotide concentration determination was obtained by regression from standard curve. At the end, NAD(P)/NAD(P)H ratio was calculated.

2.7.10. Leaf anatomy

Samples from the median region of fully developed leaves from the fifth or sixth branch from the stem apex to base of the plant were collected. These samples were immediately fixed using FAA (formalin, acetic acid, 70% alcohol) 50 % v/v (Johansen, 1940) during 48 h and subsequently stored in 70 % aqueous ethanol. The samples was embedded in methacrylate (Historesin-Leica), according to the manufacturer's recommendations and transversely sectioned (5 µm thick) in auto-feed rotary microtome (model RM2155, Leica microsystems Inc., Deerfield, USA), and stained with toluidine blue (O'Brien *et al.*, 1964). Each treatment was represented by average of three replicates (sampled leaves) and each replicate was composed of three measures in different positions of the histological section. To study stomatal traits, epidermal impressions were obtained with instant adhesive glue (Super-Bonder®), with each treatment consisting of average 10 replicates. Also, leaf samples were clarified by using the diaphanization technique described elsewhere (Zsögön *et al.*, 2014) and modified for eucalyptus. To this, samples were clarified in methanol for 16 h and then in lactic acid for 16 h, both in water at 98 ºC (Johansen, 1940), to determine the vein density, with each replicate being represented by mean of six replicates. Material was photographed using a light microscope (AX-70 TRF, Olympus Optical, Tokyo, Japan) coupled to a digital photographic camera (Zeiss AxioCam HRc model, Göttinger, Germany) and computer with Axion Vision image capture program. In these images, measurements were performed using the Image-Pro® Plus software (version 4.1, Media Cybernetics, Inc., Silver Spring, USA). In intervein regions, palisade parenchyma, lacunar parenchyma, upper and lower epidermis thickness and intercellular space percentage were measured. In the main vein, number and total area of xylem vessel elements and the percentage of area occupied by them were quantified. Vein density, stomatal density and stomatal index (stomata/[stomata+cells]) were quantified on leaf surfaces.

2.8. Experimental design and statistical analysis

The experiment was set up in a completely randomized design with subdivided plots, containing four plots. The two eucalyptus clones (01 and 02) were allocated to each plot. Following an analysis of variance (ANOVA), mean comparisons were performed using the Tukey test ($P\leq0.05$).

The results concerning effects of CO_2, temperature and clone, as well as the interaction between factors, are presented and discussed according to statistical differences found (for details see ANOVA Tables S1, S2, S3 and S4).

3. RESULTS

3.1. Morphological and biomass analyzes

Plants that developed under elevated [CO_2] were taller than those grown under ambient [CO_2] (Fig. 1). Higher [CO_2] alone culminated in larger stem diameter in both clones, whereas for clone 01 heating alone resulted in lower values (Fig. 1C). In addition, increases in root, shoot and total dry mass of plants when grown in high [CO_2] alone. On the other hand, heating alone decreased biomass accumulation (Fig. 2). Interestingly, when high [CO_2] and heating were combined a recovery in plant growth occurs and these plants reached similar biomass to the control plants (Fig. 1C and Fig. 2). Leaf area was increased only for clone 02 in [CO_2] alone, when compared to plants grown in warmer environment (Fig. 1E). Both clones presented lower number leaves when exposed to heating, but when growing under high [CO_2] and heating, clone 01 recovered the number of leaves to control plants levels (Fig. 1D). Heating resulted in lower root/shoot ratio for clone 02 (Fig. 2D). The number of branches (Fig. 1B) and specific leaf area (Fig. 1F) did not differ significantly in response to [CO_2] and heating treatments.

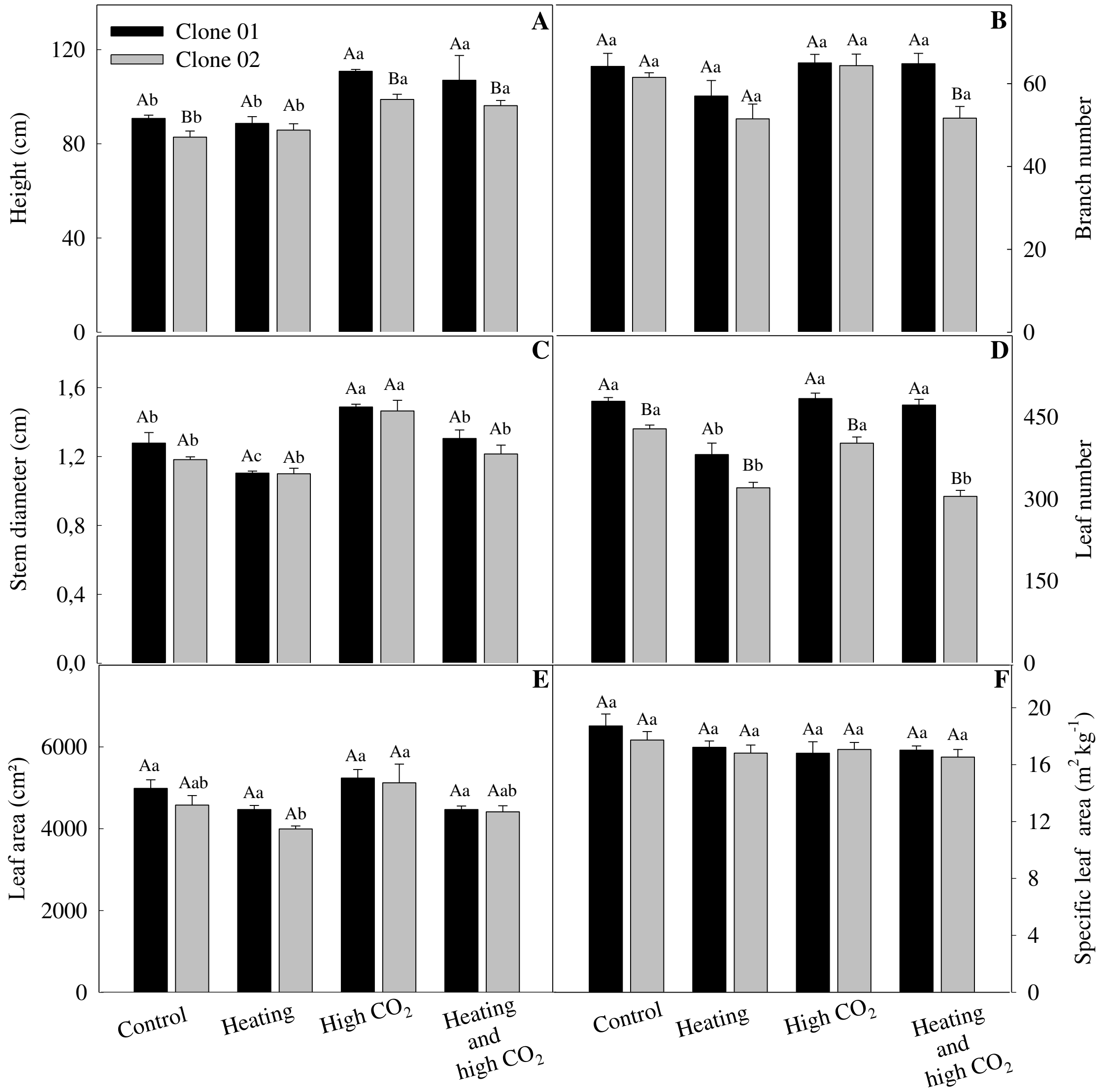

Figure 1: Changes in growth parameters in response to increase in [CO_2] and temperature in two eucalyptus clones. The parameters investigated include (A) Height. (B) Branches number. (C) Stem diameter. (D) Leaf number. (E) Leaf area. (F) Specific leaf area. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 ºC and environment [CO_2]), High CO_2 (environment temperature and [CO_2] to 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] to 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P< 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

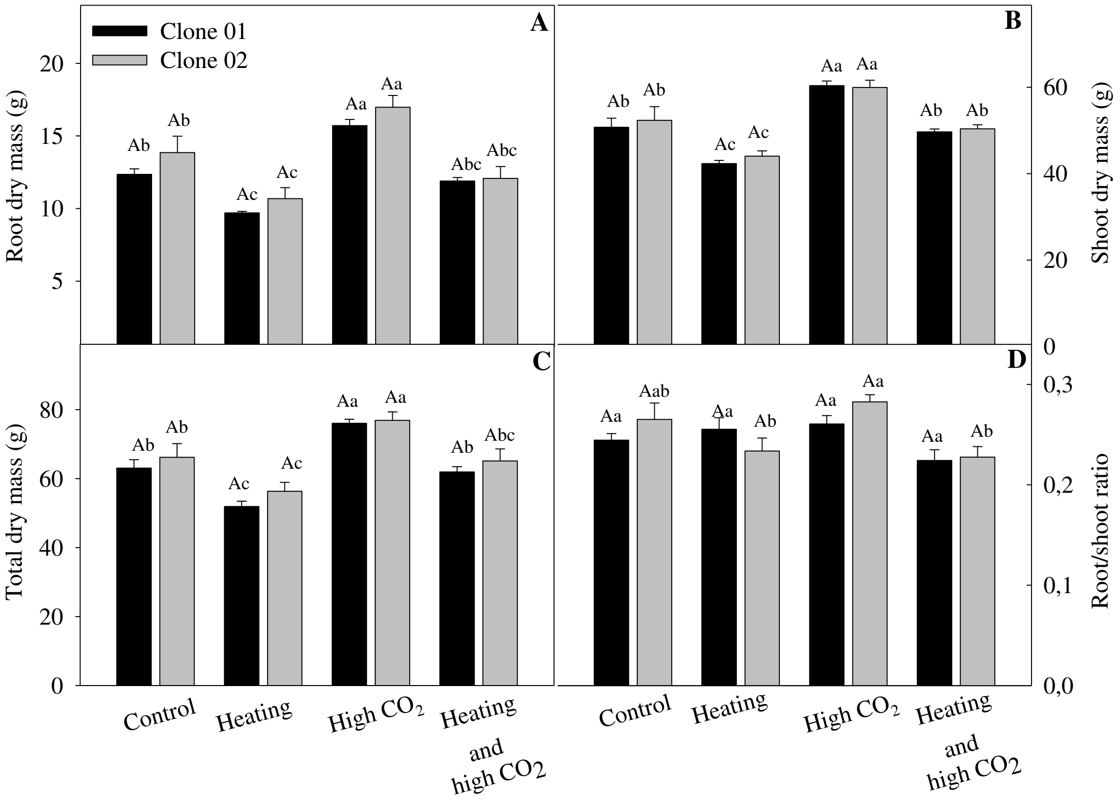

Figure 2: Variation in biomass parameters in response to increase in [CO_2] and temperature in two eucalyptus clones. The parameters investigated include (A) Dry mass root. (B) Dry mass shoot. (C) Total dry mass. (D) Root/shoot ratio. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 ºC and environment [CO_2]), High CO_2 (environment temperature and [CO_2] to 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] to 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P < 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

3.2. Physiological analyzes

Overall, A was significantly higher in plants under elevated than ambient [CO_2] over the course of experiment in both clones (Supplemental Fig. 4 and 5). Clone 01 presented lower stomatal conductance (g_s) and C_i/C_a ratio under high temperature (independent of [CO_2]), while the transpiration rate increased when it developed under heating and high [CO_2] simultaneously, and highest value of VPD was also observed in this treatment (Supplemental Fig. 4B - E). On the other hand, only g_s was lower in clone 02 when developing in warm environment and with elevated [CO_2] simultaneously. In this clone, it was also observed higher Ci/Ca ratio in plants grown under high [CO_2] alone (Supplemental Fig. 5B and D). At the end of the experiment, both clones presented higher A during the day when developing in high [CO_2] (Supplemental Fig. 6 - 7A). However, under [CO_2] clone 01 presented lower g_s and transpiration between 8 and 10 a.m.

(Supplemental Fig. 6B - C). At the end of the experiment, punctual gas exchange measurements (Fig. 3) demonstrated lower *A* in clone 01 under ambient [CO_2] (Fig. 3A). Also, the combination of factors (heating and high [CO_2]) resulted in higher transpiration when compared with either heating or high CO_2 alone (Fig. 3C). C_i/C_a ratio decreased in plants under heating (both alone and in combination with high [CO_2]) when compared to control for clone 01 (Fig. 3D). Lower g_s and C_i/C_a ratio were observed in clone 02 following simultaneous exposure to heating and high [CO_2] (Fig. 3B and 3C).

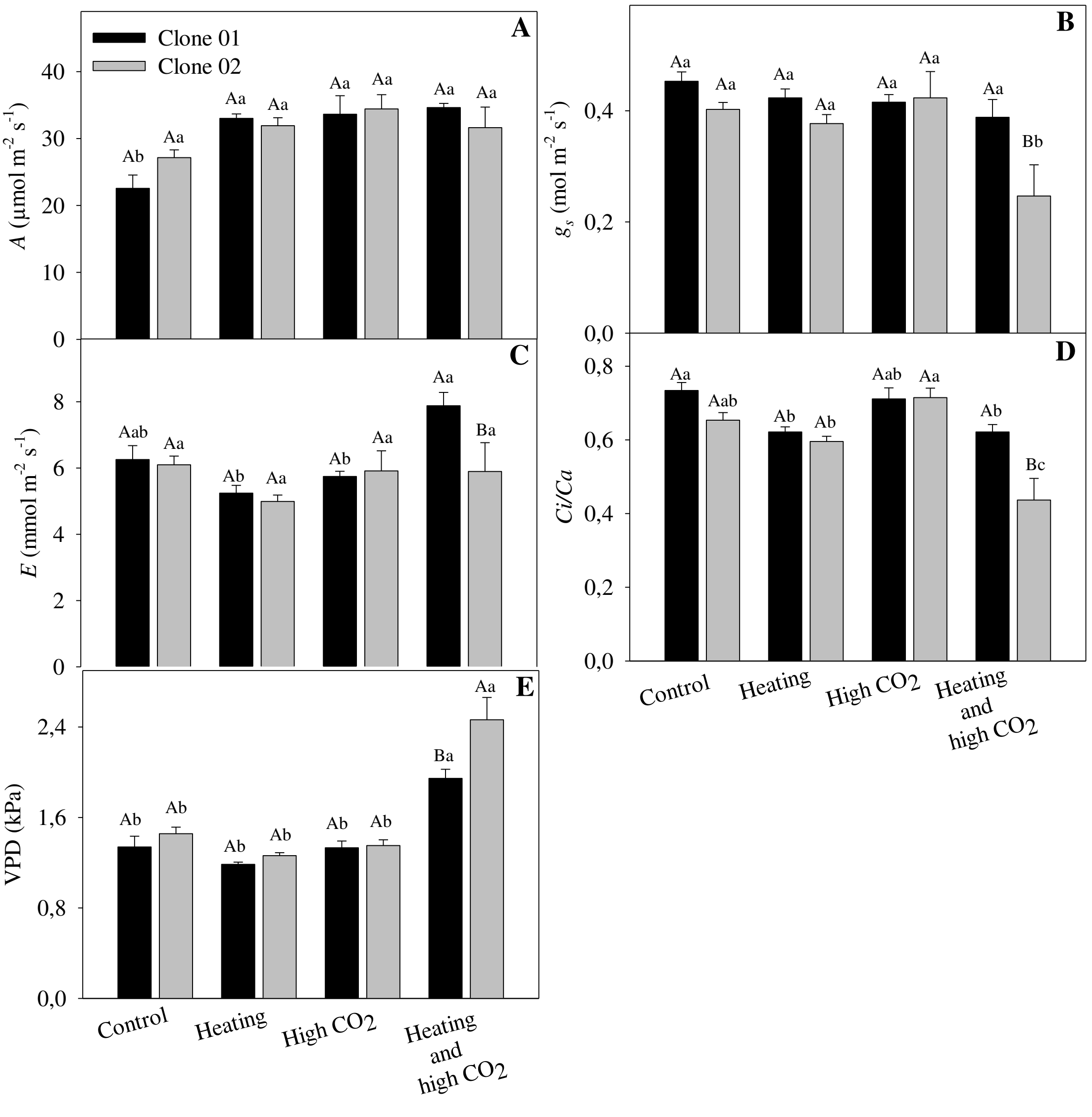

Figure 3: Variation in gas exchange parameters in response to increase in [CO_2] and temperature in two eucalyptus clones. The parameters investigated include (A) Photosynthetic rate. (B) Stomatal conductance. (C) Transpiration. (D) Ci/Ca ratio. (E) Vapor pressure deficit. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 °C and environment [CO_2]), High CO_2 (environment temperature and [CO_2] to 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] to 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P <$ 0.05). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

Regardless of clone and tissue (leaf, stem or root) studied, elevated [CO_2] (alone or in combination with heating) reduced carbon isotope composition, and lowest values were observed when the plants were cultivated under [CO_2] alone, except for clone 01, that maintained similar values for all plants maintained in elevated [CO_2]. In the stem, control plants of clone 01 presented lowest nitrogen isotopic composition than plants grown under elevated [CO_2], either alone or combined with heating. For clone 02, higher values were observed in plants growing under ambient [CO_2] compared to environment enriched [CO_2]. In root system, clone 02 presented lowest nitrogen isotopic composition in control plants, in detriment to those grown in warmer environment both in ambient [CO_2] and elevated [CO_2] (Table 1).

Table 1: Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic composition values in different tissue types of two eucalyptus clones exposed to high [CO_2] and heating.

	Clone	Treatment	Leaf	Stem	Root
$\delta^{13}C$ $^{0}/_{00}$	01	Control	-33 ± 0.32 Bb	-32 ± 0.24 Aa	-31 ± 0.00 Aa
		Heating	-31 ± 0.40 Aa	-31 ± 0.50 Aa	-31 ± 0.24 Aa
		High CO_2	-38 ± 0.37 Ac	-37 ± 0.24 Ac	-36 ± 0.24 Ac
		Heating and high CO_2	-38 ± 0.32 Ac	-36 ± 0.32 Ab	-35 ± 0.40 Ab
	02	Control	-32 ± 0.00 Aa	-32 ± 0.24 Aa	-31 ± 0.37 Aa
		Heating	-31 ± 0.24 Aa	-31 ± 0.20 Aa	-31 ± 0.24 Ba
		High CO_2	-39 ± 0.48 Bc	-38 ± 0.20 Ac	-37 ± 0.24 Ac
		Heating and high CO_2	-37 ± 0.20 Ab	-35 ± 0.40 Ab	-34 ± 0.00 Ab
$\delta^{15}N$	01	Control	-6.0 ± 0.55 Ba	-4.8 ± 0.24 Ab	-3.0 ± 0.00 Aa
		Heating	-5.0 ± 0.45 Aa	-4.0 ± 0.24 Aa	-2.2 ± 0.20 Aa
		High CO_2	-6.4 ± 0.40 Aa	-3.4 ± 0.24 Aa	-3.4 ± 0.24 Aa
		Heating and high CO_2	-5.0 ± 0.32 Aa	-3.6 ± 0.24 Aa	-2.4 ± 0.24 Aa
	02	Control	-3.4 ± 0.24 Aa	-4.6 ± 0.00 Ab	-3.4 ± 0.51 Ab
		Heating	-4.0 ± 0.32 Aa	-4.6 ± 0.24 Ab	-1.8 ± 0.49 Aa
		High CO_2	-5.0 ± 0.63 Aa	-3.4 ± 0.24 Aa	-2.8 ± 0.37 Aab
		Heating and high CO_2	-5.0 ± 0.77 Aa	-3.6 ± 0.24 Aa	-1.6 ± 0.24 Aa

Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 °C and environment [CO_2]), High CO_2 (environment temperature and [CO_2] to 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] to 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P < 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

3.3. Metabolic responses following enhanced temperature and [CO_2]

Heating alone increased chlorophyll *a*/*b* ratio for clone 02 (Fig. 4D). Chlorophyll *a*, chlorophyll *b* and total chlorophyll (*a*+*b)* content were unresponsive to the treatments (Fig. 4A – C).

Metabolism of two clones studied here presented different responses to imposed environmental conditions. Following the exposition to combination of factors (heating and high [CO_2]) increased levels of glucose and fructose were observed in clone 01, however, it should be noticed that glucose levels were higher only when compared to control plants (Fig. 5A - B). Starch levels were also higher in control plants of clone 01 compared to those grown in warm environment (both alone and combination with high [CO_2]), with lowest starch levels found in plants simultaneously exposed to heating and high [CO_2] (Fig. 5D). Protein levels did not differ significantly from control plants of clone 01, however, heating alone resulted in elevated protein levels compared to plants that developed at high [CO_2] (both at environment temperature and heating) and furthermore amino acid levels were not altered by treatments applied (Fig. 5E, F). The highest malate levels were found at elevated [CO_2] (both alone and combined with high temperature) in comparison with control plants of the clone 01 (Fig. 5G).

It was not observed any changes in glucose or sucrose levels as function of treatments application for the clone 02 (Fig. 5A, C), but fructose content was reduced following the application of heating alone in comparison to control plants (Fig. 5B). Furthermore, starch levels were lower following exposition of plants simultaneously to heating and high [CO_2] (Fig. 5D). For protein, amino acids and malate levels no variation was observed as function of treatments application compared to control in plants of clone 02 (Fig. 5E-G).

Increase in [CO_2] and atmospheric temperature simultaneously resulted in higher NAD^+ and NAD^+/NADH level compared to control plants in clone 01. On the other hand, clone 02 had higher levels of NAD^+ and NADPH when growing in high [CO_2] (Fig. 6).

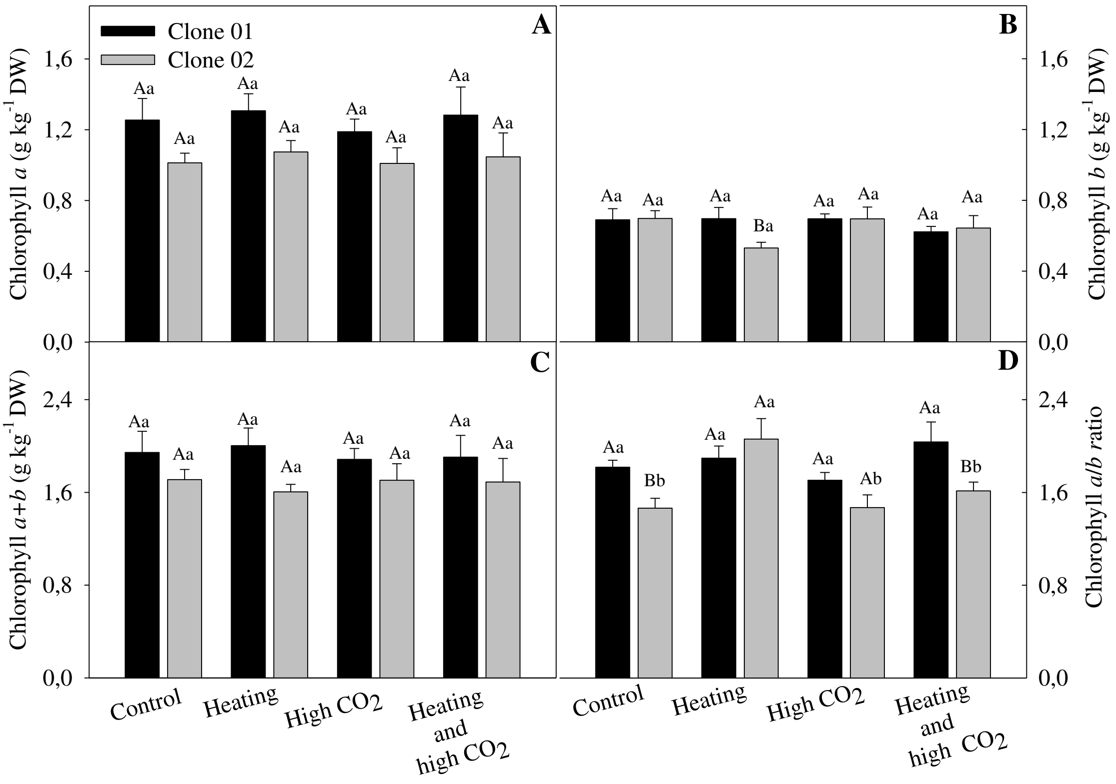

Figure 4. Variation in chlorophyll content in response to increase in [CO_2] and temperature in two eucalyptus clones. The parameters investigated include (A) chlorophyll *a*. (B) chlorophyll *b*. (C) chlorophyll *a*+*b*. (D) chlorophyll *a*/*b* ratio. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 ºC and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P < 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

To expand the study regarding metabolic alterations in response to treatments application, a more detailed investigation of the primary metabolism was carried out via metabolite profiling. This analysis allowed identify 13 amino acids (Fig. 7), 13 sugars (Fig. 8), 7 organic acids (Fig. 9) and 5 metabolites as other compounds (Fig. 10).

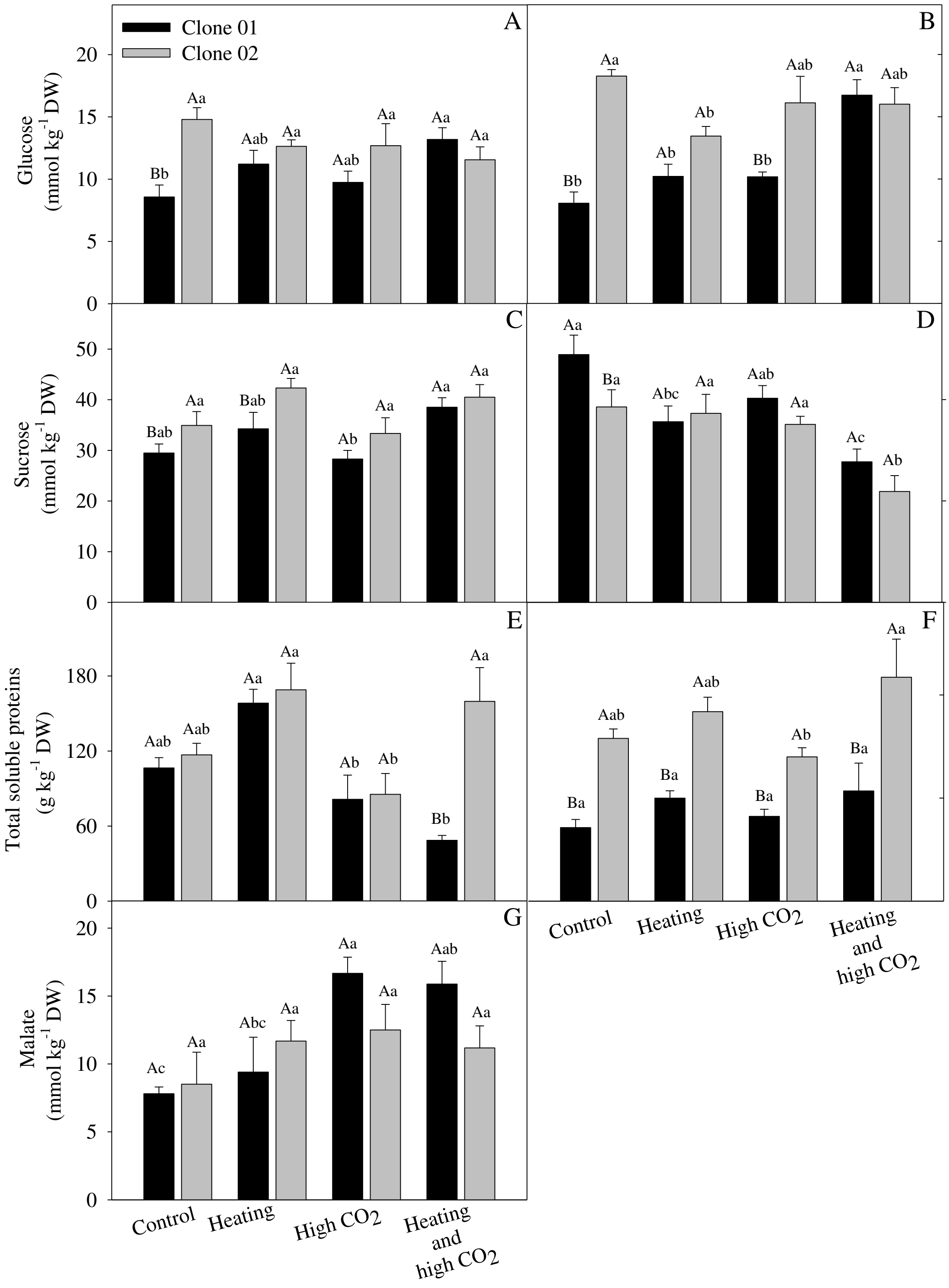

Figure 5. Variation in primary metabolites levels in response to increase in [CO$_2$] and temperature in two eucalyptus clones. The parameters investigated include (A) Glucose. (B) Fructose. (C) Sucrose. (D) Starch. (E) Proteins. (F) Amino acids. (G) Malate. Four different treatments were applied: Control (environment temperature and [CO$_2$]), Heating (increase of 2 ºC and environment [CO$_2$]), High CO$_2$ (environment temperature and [CO$_2$] of 700 ppm), Heating and High CO$_2$ (increase of 2 °C and [CO$_2$] of 700 ppm). Means (*n*=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P<$ 0.05). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

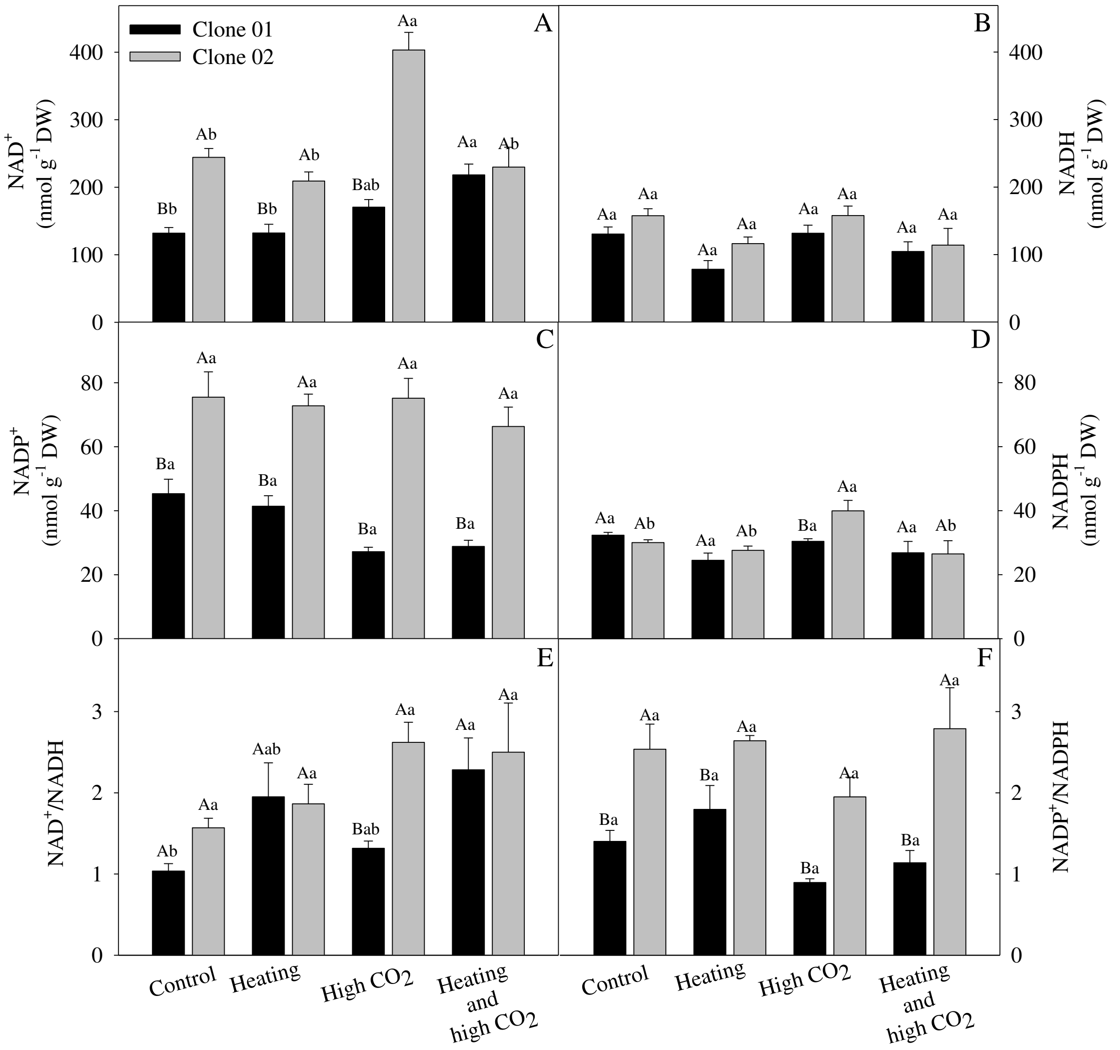

Figure 6. Variation in pyridine nucleotides levels in response to increase in [CO_2] and temperature in two eucalyptus clones. The parameters investigated include (A) NAD^+. (B) NADH. (C) $NADP^+$. (D) NADPH. (E) NAD^+/NADH ratio. (F) $NADP^+$/NADPH ratio. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 °C and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P< 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

Regarding amino acid pattern, increased levels of isoleucine, glutamate, phenylalanine, proline, threonine and valine were observed (clone 01 only) simultaneously exposed to heating and high [CO_2] in relation to control plants. Furthermore, threonine levels were higher in plants cultivated in heating alone (Fig. 7A). For plants of clone 02 simultaneously exposed to heating and increased [CO_2] higher levels of alanine, isoleucine, glutamate, phenylalanine, proline, serine, tyrosine, threonine, tryptophan and valine in comparison to control plants were observed. In addition, proline, serine, tyrosine and threonine levels were also higher than plants grown under high [CO_2] alone (Fig. 7B).

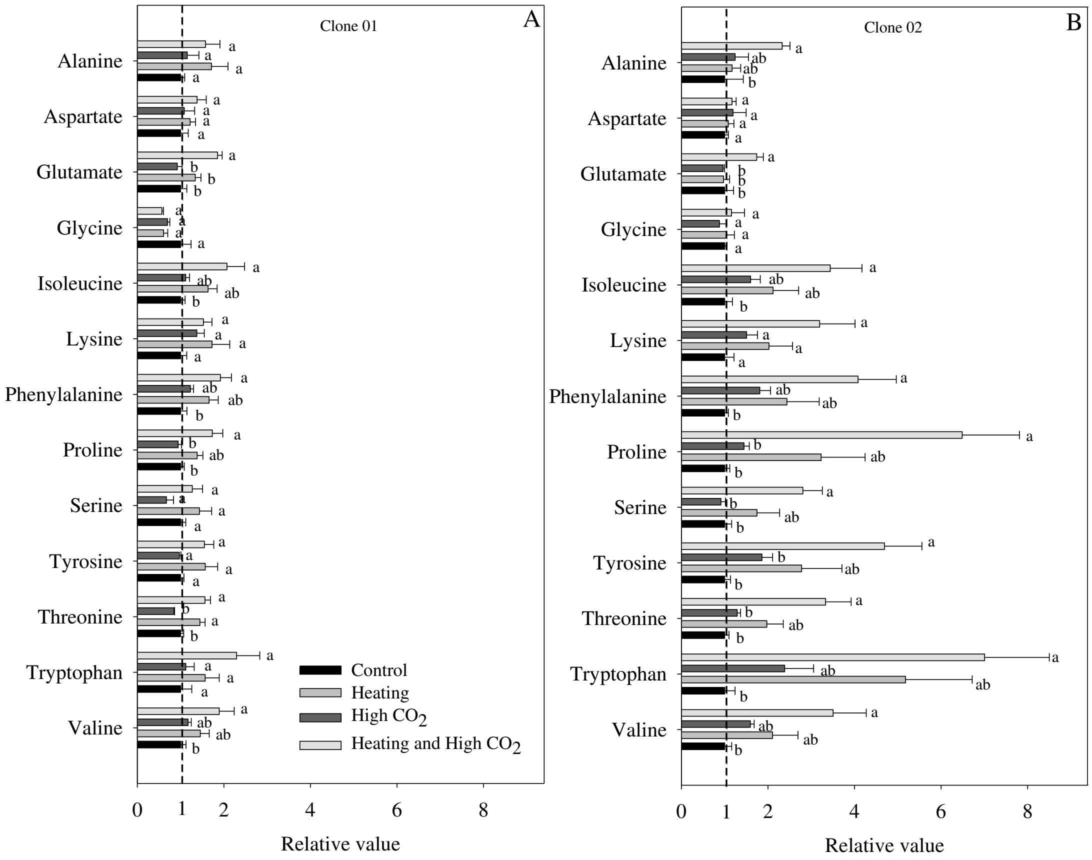

Figure 7. Variation in amino acids levels obtained by GC-MS in response to increase in [CO_2] and temperature in two eucalyptus clones. (A) Relative value to clone 01 (B) Relative value to clone 02. Means (*n*=5 ± SE, except in high [CO_2] treatment; n= 4) followed by different letters were determined by Tukey's test to be statistically different ($P < 0.05$). Letters compare treatments within each clone. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 ºC and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 ºC and [CO_2] of 700 ppm).

In general, sugar levels were slightly altered in both clones. Briefly, in clone 01, erythritol levels were higher in plants simultaneously exposed to heating and high [CO_2] in relation to other treatments whereas maltose levels were higher in plants developed in high [CO_2] alone compared to control plants and plants that developed under high temperature alone (Fig. 8A). On the other hand, higher levels of cellobiose and maltose were noticed in clone 02 developed under high [CO_2] alone in comparison to those developed under ambient [CO_2] and environment temperature as well as under high temperature (Fig. 8B).

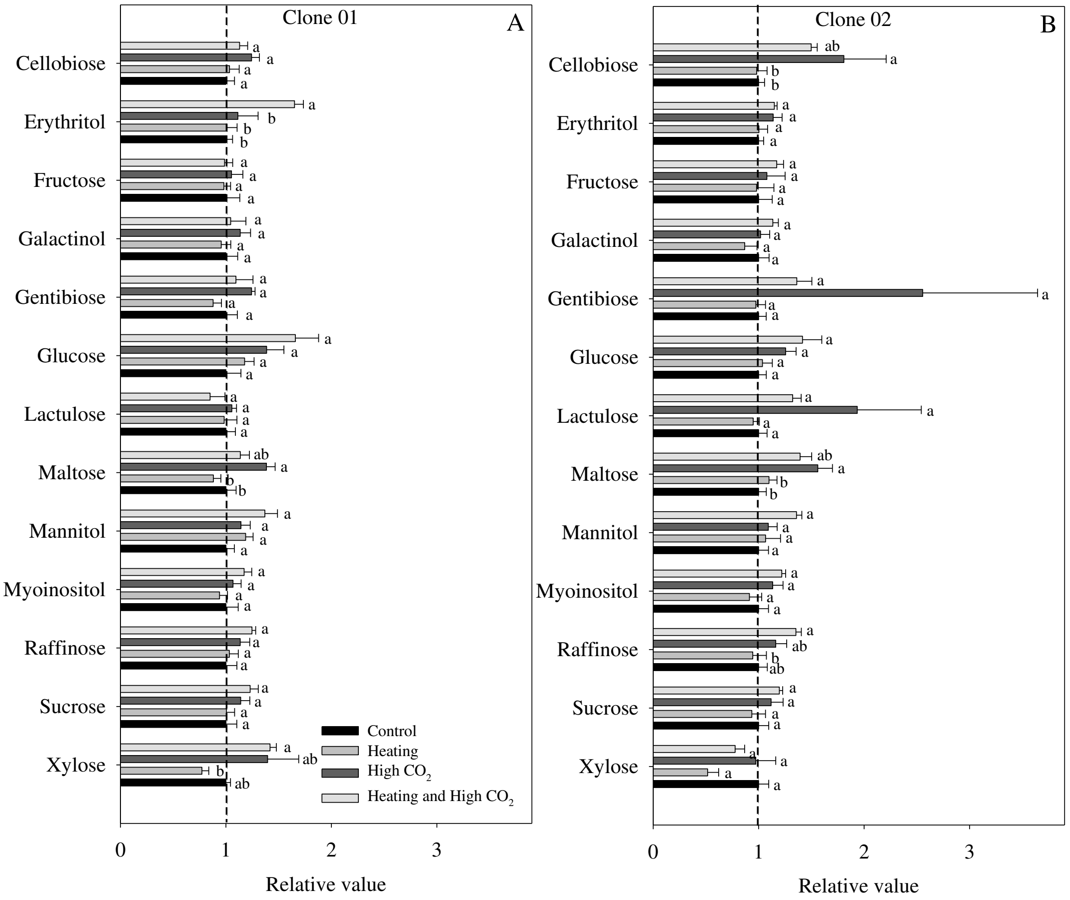

Figure 8. Variation in sugars levels obtained by GC-MS in response to increase in [CO_2] and temperature in two eucalyptus clones. (A) Relative value to clone 01 (B) Relative value to clone 02 Means (*n*=5 ± SE, except in high [CO_2] treatment; n= 4) followed by different letters were determined by Tukey's test to be statistically different (*P*< 0.05). Lower case letters compare treatments within each clone. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 °C and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm).

By analyzing organic acids group, it was possible to observe that clone 01 presented higher ascorbate levels under high temperature (regardless of [CO_2]) compared to control plants. In addition, dehydroascorbate levels were larger under high temperature and elevated [CO_2] simultaneously in comparison to control plants. Malate levels were lower in plants under ambient [CO_2] compared to plants that developed at high [CO_2] and heating simultaneously (Fig. 9A). For clone 02, increased [CO_2] alone resulted in higher malate and succinate levels when compared to plants under ambient [CO_2], regardless of temperature (Fig. 9B).

Both clones showed increase lactitol levels under high [CO_2] alone (Fig. 10A and 10B). Moreover, clone 02 under high [CO_2] and heating simultaneously showed higher urea contents compared to control plants (Fig. 10B).

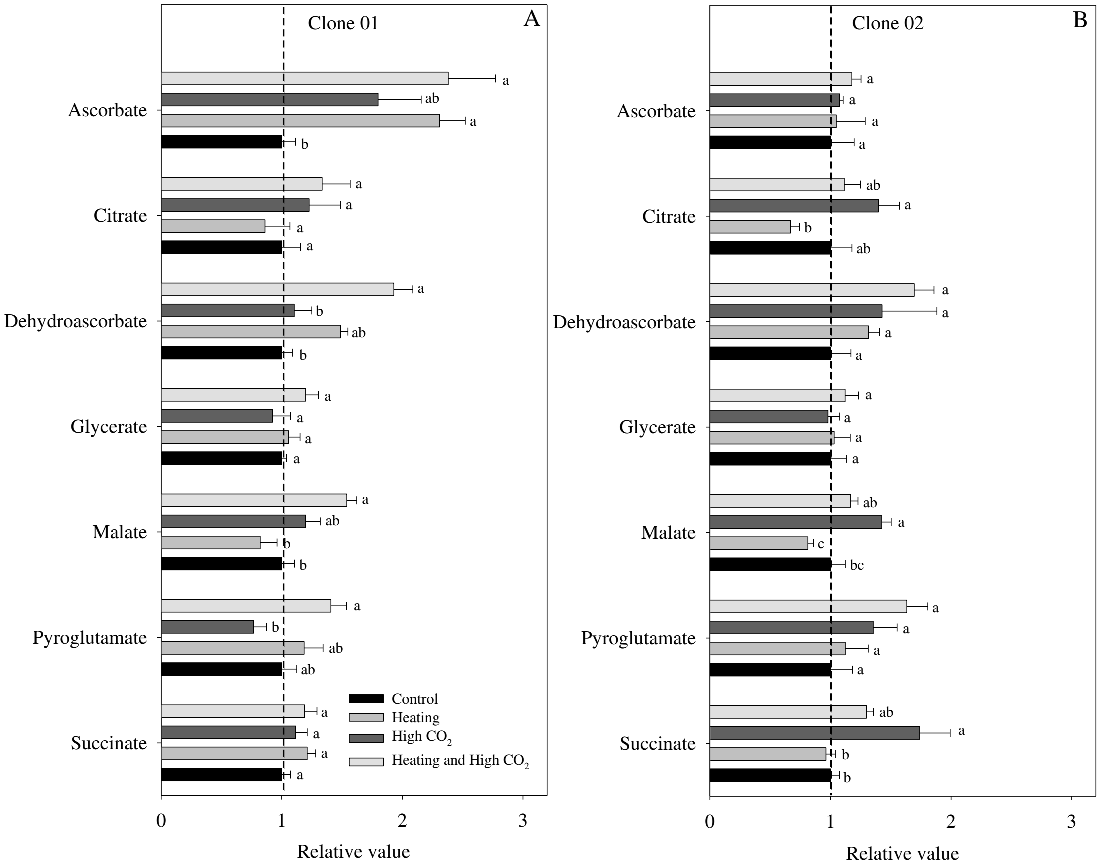

Figure 9. Variation in organic acids levels obtained by GC-MS in response to increase in [CO_2] and temperature in two eucalyptus clones. (A) Relative value to clone 01 (B) Relative value to clone 02. Means (*n*=5 ± SE, except in high [CO_2] treatment; n= 4) followed by different letters were determined by Tukey's test to be statistically different ($P< 0.05$). Lower case letters compare treatments within each clone. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 °C and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm).

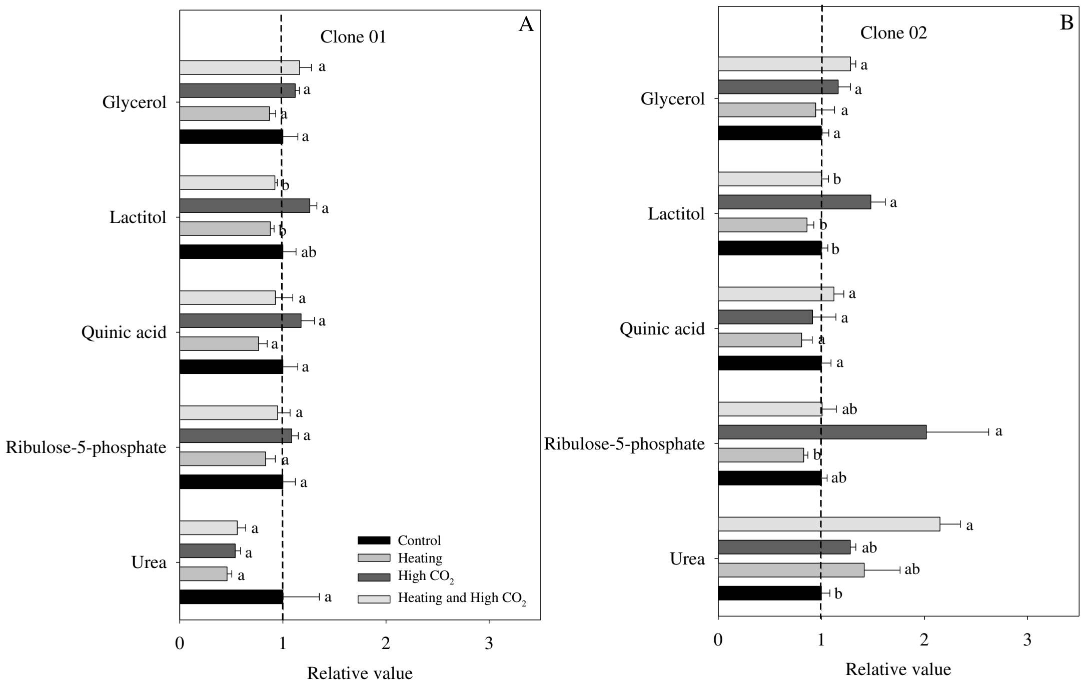

Figure 10. Variation in other compounds obtained by GC-MS in response to increase in [CO_2] and temperature in two eucalyptus clones. (A) Relative value to clone 01 (B) Relative value to clone 02. Means (n=5 ± SE, except in high [CO_2] treatment; n= 4) followed by different letters were determined by the Tukey's test to be statistically different ($P < 0.05$). Lower case letters compare treatments within each clone. Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 °C and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm).

3.3. Leaf anatomy

Clone 01 simultaneously exposed to heating and high [CO_2] were characterized by lower stomatal density, and when cultivated under high [CO_2] alone it was possible to notice reductions in thickness upper and lower epidermis in comparison to control plants. Interestingly, when the factors (heating and high [CO_2]) were combined, increased thickness of epidermis was further observed reaching similar values to control plants (Tab. 2).

Control plants of clone 02 were characterized by smaller vessels number when compared to plants developed under high [CO_2] alone. Notably, the changes presented in vessels number did not resulted changes in vessel area. Interestingly, changes in both temperature and [CO_2] did not result in significant alterations in stomatal index, vein density, parenchyma thickness and vascular bundles area in both clones (Tab. 2).

Table 2. Leaf anatomy parameters in eucalyptus clones exposed to high [CO_2] and heating alone and combined.

		Treatment			
	Clone	Control	Heating	High CO_2	Heating and High CO_2
Stomatal density (Nº mm^{-2})	1	417,42 ± 29,44 Aa	422,97 ± 15,47 Aa	472,95 ± 19,54 Aa	342,67 ± 1,524 Ab
	2	426,35 ± 7,435 Aab	402,24 ± 4,324 Aab	442,28 ± 6,900 Aa	367,92 ± 24,89 Ab
Stomatal index	1	0,2280 ± 0,005 Aa	0,2366 ± 0,007 Aa	0,2343 ± 0,003 Aa	0,2392 ± 0,006 Aa
	2	0,2043 ± 0,007 Ba	0,1961 ± 0,008 Ba	0,2069 ± 0,005 Ba	0,1973 ± 0,003 Ba
Vein density (µm mm^{-2})	1	0,0073 ± 0,002 Aa	0,0069 ± 0,003 Aa	0,0066 ± 0,001 Aa	0,0062 ± 0,003 Aa
	2	0,0062 ± 0,003 Ba	0,0062 ± 0,003 Aa	0,0063 ± 0,002 Aa	0,0055 ± 0,001 Aa
Palisade parenchyma (µm)	1	78,176 ± 3,528 Ba	74,025 ± 4,653 Ba	81,078 ± 5,273 Aa	82,754 ± 3,345 Aa
	2	95,509 ± 2,337 Aa	95,740 ± 6,952 Aa	94,198 ± 2,502 Aa	89,486 ± 6,089 Aa
Spongy parenchyma (µm)	1	139,02 ± 1,821 Aa	125,70 ± 5,766 Aa	147,54 ± 8,156 Aa	139,47 ± 11,28 Aa
	2	144,16 ± 3,137 Aa	147,09 ± 14,57 Aa	155,76 ± 9,026 Aa	150,53 ± 8,476 Aa
Upper epidermis (µm)	1	16,844 ± 0,480 Aa	15,840 ± 0,708 Aab	13,683 ± 0,557 Ab	13,916 ± 0,442 Aab
	2	14,271 ± 0,987 Ba	13,884 ± 0,803 Aa	14,288 ± 1,364 Aa	11,571 ± 0,791 Aa
Lower epidermis (µm)	1	11,871 ± 0,444 Aa	11,576 ± 0,880 Aa	9,1220 ± 0,477 Ab	10,259 ± 0,525 Aab
	2	10,966 ± 0,834 Aa	11,311 ± 0,738 Aa	9,6714 ± 0,588 Aa	8,9594 ± 0,409 Aa
Intercell space (%)	1	36,675 ± 1,695 Aa	35,600 ± 2,861 Aa	30,170 ± 0,950 Aa	30,819 ± 1,134 Aa
	2	29,691 ± 4,582 Ba	28,908 ± 1,764 Ba	27,558 ± 1,673 Aa	28,447 ± 1,151 Aa
Leaf thickness (µm)	1	246,20 ± 5,511 Aa	226,47 ± 6,908 Ba	250,70 ± 11,40 Aa	245,42 ± 13,10 Aa
	2	263,91 ± 3,146 Aa	267,57 ± 18,48 Aa	270,42 ± 11,02 Aa	260,77 ± 7,648 Aa
Vessels number	1	221,80 ± 19,04 Aab	174,47 ± 18,30 Bb	239,95 ± 54,04 Bab	306,73 ± 42,71 Aa
	2	194,87 ± 9,546 Ab	300,13 ± 42,83 Aab	346,80 ± 30,27 Aa	292,08 ± 10,30 Aab
Vessels area (mm^{-2})	1	0,0238 ± 0,003 Aa	0,0211 ± 0,001 Aa	0,0228 ± 0,0035 Aa	0,0282 ± 0,003 Aa
	2	0,0290 ± 0,002 Aa	0,0315 ± 0,002 Ba	0,0326 ± 0,002 Ba	0,0316 ± 0,003 Aa
Xylem area (%)	1	41,438 ± 0,447 Aa	39,666 ± 1,498 Aa	40,127 ± 1,537 Aa	41,269 ± 2,084 Aa
	2	36,812 ± 1,030 Aa	38,668 ± 1,079 Aa	39,608 ± 1,825 Aa	40,797 ± 0,886 Aa
Phloem area (%)	1	58,562 ± 0,447 Aa	60,334 ± 1,498 Aa	59,873 ± 1,537 Aa	58,731 ± 2,084 Aa
	2	63,188 ± 1,030 Aa	61,332 ± 1,079 Aa	60,392 ± 1,825 Aa	59,203 ± 0,886 Aa

Four different treatments were applied: Control (environment temperature and [CO_2]), Heating (increase of 2 ºC and environment [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm), Heating and High CO_2 (increase of 2 °C and [CO_2] of 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different (P< 0.05). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatment.

4. DISCUSSION

Similarly to all sessile plants, during their evolution trees have also developed a range of mechanisms that enable them to cope with variable environmental conditions (Chinnusami et al., 2009). It seems reasonable to anticipate, however, that long life span of trees most likely compromise genetic adaptation to environmental changes, rending *Eucalyptus* plantation particularly susceptive to climate changes. Furthermore, both tree die-off and climate-driven vulnerability are growing concern to the sustainability of forest worldwide (McDowell et al., 2011; Allen et al., 2015). Research efforts have clearly improved our knowledge regarding how, and to which extent, abiotic stress, including heat (Yu et al., 2017), [CO_2] (Xu et al., 2014) and water deficit (Correa et al., 2014) impact plant growth and development. It should be mentioned however, that such abiotic stress factors have been mostly investigated alone and under well-controlled conditions (Mittler and Blumwald, 2010). By sharp contrast, has been give less attention to combined effects of such abiotic stress. It should mentioned that expected increase in [CO_2] will most likely not occur alone but associated with water limitation, higher temperature and high light conditions (Yu et al., 2017; Florian et al., 2014; Dusenge et al., 2018). For instance, plants challenged with the combination of drought and heat stress must respond adequately by regulating stomatal behavior to simultaneously preventing excessive water loss and leaf cooling by transpiration (Mittler and Blumwald, 2010). Having that in mind, here physiological and metabolic responses of eucalyptus seedlings were investigated following simultaneous or isolated exposition to enhancements of temperature and [CO_2]. The results obtained demonstrated the development of differential acclimation that mitigates the negative impacts of high temperatures due to positive effects of high [CO_2]. It is also important to note that most of observed responses are common to two clones studied (Figure 11), which may be important to understand the differential tolerance presented by clones to Vale do Rio Doce dry pointers.

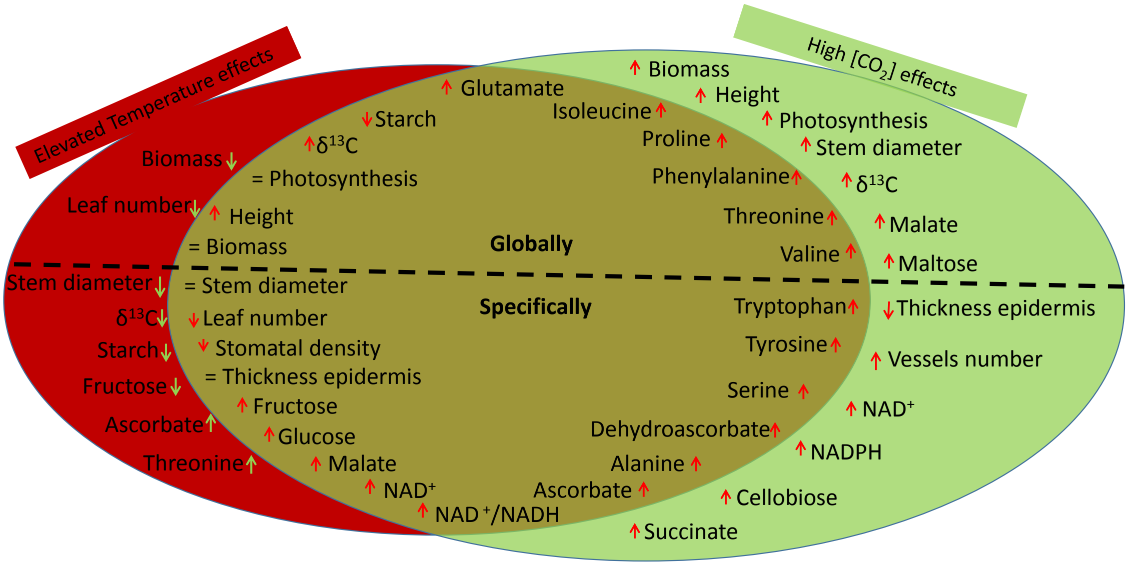

Figure 11: Venn diagram showing commons changes (globally) and individual (specifically) responses in two eucalyptus seedlings exposed to heating (red color), High [CO_2] (green color), Heating and High CO_2 combined (intersection between sets) in relation to the Control plants. Up arrow indicates increase, down arrow indicates decrease, equality indicates recovery after being exposed to high [CO_2] and heating simultaneously.

Eucalyptus seedlings are negatively affected by environment heating

Although optimal temperature is one factor of great significance responsible for plant growth and development, high temperatures usually result in significant losses in crop yield. Thus, to investigate responses of eucalyptus seedlings to an environment with 2 ºC higher than ambient temperature a mini-TFACE system was used. Notably, significant increases are expected in the mean temperature of planet during the next decades (IPCC, 2014). Increased temperature culminated in reductions of both growth and dry matter accumulation of plants (Fig. 1 - 2). The first negative effects caused by high temperature are related to structural alterations in chloroplast protein complexes and reductions in the activity of several chloroplastidic enzymes (Yamori et al., 2014). It is worth to mention that such enzymes usually display high sensitivity to increases in temperature, being inhibited even under small increases in the temperature (Maestri et al., 2002). In spite of lower growth, increased temperature did not result in significant reductions in A, E, and g_s at the end of the experiment (Fig. 3A - C), although lower C_i/C_a (Fig. 3D) in clone 01 was observed. It is important to mention, however, that in general this clone presented lower g_s and C_i/C_a over the course of the experiment (Supplemental Fig, 4B, D) suggesting that stomatal limitation is likely to occur with this clone under this condition. Remarkably, this is strongly correlated with the higher isotopic carbon composition in leaves of this clone (Table 1). This fact indicates that over time there were less carbon available at carboxylation site of RUBISCO under higher temperature. It should be noted that the stomata represent the main gateway for carbon entry in the cell, being closely related to the photosynthetic rate (Medeiros et al., 2015; Matthews et al., 2017; Vialet-Chabrand et al., 2017). Interestingly, the heating resulted in larger ^{15}N composition in the root system for the plants of clone 02 (Table 1), and this may be related to changes in the N-assimilation process by nitrate reductase. However, further studies should be carried out to elucidate this enzyme behavior in environment modified by high temperatures.

An interesting response observed in plants growing at enhanced temperature is related to decreased number plants' leaves (Fig. 1D) without changes in total leaf area (Fig. 1E). Taken together, these data indicate that increase in leaf size that is likely to increase the self-shading and it tends to minimize negative effects arising from high light intensity associated with increased temperature. In addition, both clones exhibited changes in their metabolism in response to increased temperature, the clone 1 reduces starch (Fig. 5D) and clone 02 reduces fructose level (Fig. 5B). According to (Ruan et al (2010) changes in metabolism are usually observed in plants exposed to high temperatures. In fact, reductions in starch synthesis were found in *Arabidopsis thaliana* at high temperature for short period of time (Florian et al., 2014). Starch represents a transient form of carbon storage in leaves that increases throughout the day, being remobilized to support growth and metabolism at night (Smith and Stitt, 2007; Stitt and Zeeman, 2012). Accordingly, reduction in fructose levels displayed by the clone 02 may have occurred due to the rapid usage of this sugar at high temperature (Florian et al., 2014). It is known that signaling via sugars is of extreme importance in the regulation of carbon metabolism and thus the activity of source forces such as photosynthesis, mobilization and export of nutrients are positively regulated in situations of low sugar (Paul and Foyer, 2001). Thus, reduction observed in level of these metabolites in each clone likely contributes to the occurrence of unbalance between growth and carbon metabolism, resulting in losses of productivity. In addition, when evaluating the metabolic profile of the plants, it can verify that heating increased the relative values of threonine in clone 01 (Fig. 7A). These effects may have occurred due to lower activity of the enzyme threonine-3-dehydrogenase, which directs the metabolism of threonine to the TCA cycle, and by lower activity of the threonine-ammonia-lyase enzyme that directs the metabolism of threonine to synthesis of others amino acids such as valine, leucine, isoleucine, among others. This hypothesis is suggested because there has been no change in metabolites previously mentioned. In addition, branched chain amino acids (BCAAs) may be used to contribute energy supply due increase in temperature. In fact, in *A. thaliana* BCAAs are considered an important alternative for maintaining metabolism under stress conditions (Barros et al., 2017, Pires et al., 2016).

Growth of eucalyptus seedlings is enhanced under high availability of CO_2

Carbon dioxide is the main substrate used by RUBISCO during photosynthetic process, and projections such as IPCC (2014) point out that the concentration of this gas in atmosphere may reach values of 985 ppm at the end of this century, a point that will clearly culminate in changes in plant metabolism (Xu et al., 2013b). Thus, to elucidate the physiological and metabolic responses displayed by eucalyptus seedlings grown in CO_2 enriched environment during 62 days in mini-FACE system a detailed metabolic analysis was undertaken.

The increased plant growth observed here (Fig. 1) is in good agreement with previous observation (Xu et al., 2013b). Nevertheless, the same authors demonstrated that in experiments using the FACE system these differences do not always occur. This fact may occur due difficulty in environmental controlling. In addition, increase in [CO_2] culminated in lower leaf carbon isotopic composition (Table 1). It is worth mentioning that lower isotopic carbon composition are related to higher isotopic discrimination, and this occurs due to diffusive aspects of CO_2 and also by biochemical aspects of RUBISCO. Increases in carbon dioxide also favored the maintenance of g_s (Fig. 3B), stomatal density and stomatal index at the end of the experiment (Tab. 2). The lack of changes in these parameters are associated to maintenance in V_{cmax} and J_{max} (Supplemental Tab. S2) and *Ci/Ca* ratio, as observed here (Fig. 3D). Another conspicuous feature is malate accumulation (Fig. 5G, 9B). This fact is related to maintenance of g_s in high [CO_2] (Fig. 3B) and it has been previously shown that malate and fumarate are directly related to stomatal opening and closure (Nunes-Nesi et al., 2007; Araújo et al., 2011; Penfield et al., 2012). It seems reasonable therefore to suggest that in eucalyptus seedlings high concentrations of malate are capable of minimizing stomatal closure under high [CO_2] (Lee et al., 2008). Noteworthy, malate is considered to be a key metabolite in guard cell movements (Hedrich and Marten, 1993). Therefore, further research will clearly be required to increase our understanding of functions of organic acid metabolism and transport in guard cells of *Eucalyptus*, particularly under stressful conditions.

Higher *A* was observed both over time and throughout the day (Supplemental Fig. 4 - 7A), showing that eucalyptus plants respond positively to increased [CO_2]. Increased photosynthetic rate in response to high [CO_2] were also observed in others species (Kubiske and Pregitzer, 1996; Ameye et al., 2012). In C_3 plants, such as *Eucalyptus*, elevated [CO_2] increases the diffusion from atmosphere to chloroplast, contributing to increase the photosynthetic rate (DaMatta et al., 2010) and also contributes to reduction in the photorespiratory rate (Long et al., 2004). To support the higher *A*, a higher apparent quantum yield was noticed (Supplemental Tab. S5), indicating greater use of light energy during photosynthetic process under this condition. Still, according with the preview results, increases in NAD^+ (Fig. 6A) and NADPH (Fig. 6D) occurred, although not significant for clone 01. NADPH is an important product of photochemical step of photosynthetic process and is involved in routes as glycolysis, cycle of tricarboxylic acids, respiratory chain, among others (Geigenberger and Fernie, 2014). On the other hand, the pool of NAD^+ is important because it is used as coenzyme in enzymatic reactions involved in several cellular processes and it is important in regulation of central metabolism in plants (Gakière et al., 2017).

Maintenance on sugar levels in leaves (Fig. 5A - C) indicates that photoassimilates was rapidly used in plant sink organs. Taken together with the increased respiration (Supplemental Tab. S5), data obtained indicate greater ability to respire the newly assimilated carbon. This is crucial to avoid carbon accumulation, which may result in retroinhibition of photosynthesis and/or physical damage to the chloroplast (Li et al., 2013). Nevertheless, plants of indeterminate growth, like *Eucalyptus*, due to their habit of growth, manage to use most of carbon assimilated to sustain growth. Remarkably, to further deeper the metabolic studies in response to fluctuation in [CO_2], in manner consistent, the metabolite profile presented relatively few variations under this condition. These results obtained here show a fine metabolic regulation, without altering the sugar balance in response to the higher [CO_2], thus potentiating the plant growth.

Interestingly, development under conditions of high [CO_2] led to increased number of xylem vessel elements for clone 02, despite no changes in the area occupied by them (Tab. 2). It seems reasonable to assume therefore that these plants present greater resistance to cavitation, without damage to water distribution in tissues, since alterations in venation density were not observed (Tab. 2), a feature of high importance in environments with limited water availability. On the other hand, decrease in vessel diameter is likely able to causes lower hydraulic conductivity (Saadaoui et al., 2018). Intriguingly, both upper and lower epidermis was reduced in plants of the clone 01 (Tab. 2). Variations in epidermal thickness reflect adaptation increasing survival capacity in response to altered environmental conditions (Ali et al., 2009). This observation may be related to lower energy investment in these structures, since they are responsible for the protection of leaves to adverse conditions (dry, high luminosity, pathogens). This study allowed concluding that were few anatomical alterations in response to higher concentration of [CO_2], however, the observed variations are great importance during plant development. Thus, smaller vessel diameter is important because it increases cavitation resistance, whereas reductions in abaxial and adaxial epidermal thickness may favor the CO_2 fixation, facilitating the entry of CO_2 via the stomatal pore.

Role of CO_2 as mitigating agent for damages arising from the increase in atmospheric temperature in eucalyptus seedlings

Anthropogenic interference has considerably increased [CO_2] as well as other gases in the atmosphere. These changes result also in increased temperature, changes in precipitation regime, occurrence of acid rain, and others (IPCC, 2014). Taken together, these factors can culminate in significant modifications in plant growth, development and productivity. Therefore, morphophysiological and metabolic variations of two eucalyptus clones grown during two months in environment with 700 ppm CO_2 and temperature 2 ºC higher than ambient temperature using mini-TFACE system were investigate.

Plants development at high [CO_2] and heating simultaneously culminated with higher plant height (Fig. 1A). In addition, both clones were characterized by root, shoot and total dry mass similar to control plants, showing that [CO_2] mitigated the negative effects of high temperature alone (Fig. 2A - C). For clone 01, there was still recovery in stem diameter and leaf number (Fig. 1C - D). Increased [CO_2] in heat environment has been associated with synergistic effect (Albert et al., 2011b; Milcu et al., 2012), as observed here. It is important to mention, however, that this effect is more evident in plants well irrigated or under moderate water deficit (Xu et al., 2014). Based on our results, it seems that the negative effects of high temperatures are likely mitigated by increased [CO_2], indicating that in scenario of climate changes there will be either minor or no damages to the development of eucalyptus seedlings. In any case, whereas our data clearly indicate a role of elevated [CO_2] on the morphophysiology of *Eucalyptus* seedlings developed at high temperature, more research should be performed to examine plant growth at other locations and under varied environmental conditions.

In general, over time A remained similar to control plants in clone 02, but increased in clone 01. However, higher transpiration and lower g_s in clone 01 than in clone 02 were also observed (Supplemental Fig. 4 and 5). High transpiration rate is an important mechanism that provides greater loss of latent heat, avoiding damages due to heating (Teskey et al., 2015). In both clones, lower isotopic composition in relation to control plants indicates that there was greater availability of CO_2 for assimilations (Table 1). This parameter provides also a good indication of higher carbon fixation over time in plants. By analyzing the metabolic profile of these plants, it was possible to verify an increased level of several amino acids for both clones (Fig. 7A and B). Increases in amino acid levels may be association with up regulation of the expression of genes related to amino acid synthesis, with the aim of conserving energy under limited conditions (Baena-

González and Sheen, 2008; Bunik and Fernie, 2009; Sulpice et al., 2009). Furthermore, increased heating tolerance has been associated with higher alanine, valine, serine, glutamate and proline contents in several species (Merewitz et al., 2011; Xu et al., 2013a). This increase was probably induced by high [CO_2] and occurs only under stress conditions since the effect of factors alone (heating or CO_2) did not lead to occurrence of such changes. In addition increases in proline and glutamate levels (Fig. 7A – B and 9A - B) may be related to modulation in 2-oxoglutarate flux for amino acid metabolism. This fact suggest a strong metabolic regulation to sustain the amino acid metabolism coordination in same way as previously observed in different species (Foyer et al., 2003; Zhu and Galili, 2003; Sweetlove and Fernie, 2005). Clone 01 also showed increase in organic acids values, such as ascorbate and dehydroascorbate (Fig. 9A), which are substrate and product of enzyme ascorbate peroxidase, an important component of antioxidant system (Cakmak and Horst, 1991), involved in elimination of reactive oxygen species. Therefore, it seems reasonable to anticipate that further studies are still required to elucidate the significance of this mechanism under the climate change scenario.

In both clones, lower levels of starch were observed (Fig. 5D) and this may occur as function of increased respiratory rate, as observed for others species (Farrar and Williams, 1991; Qaderi et al., 2006; Ge et al., 2012). Notably, this represents the main path for carbon loss, especially under high temperatures. For clone 01, increases in glucose, fructose levels (Fig. 5A - B) may be related to increases in malate levels (Fig. 5G) and this in turn can be used as precursor in synthesis of amino acids during stress. It should also be mentioned that there is a strict relationship between photosynthesis and respiration in order to maximize plant development under both optimal and stress conditions (Nunes-Nesi et al., 2010; Araújo et al., 2014). Thus observed changes demonstrate great metabolic flexibility of eucalyptus plants that can be important strategy for their growth in climate change scenario.

5. CONCLUSIONS

Both clones studied here presented a group of common responses when exposed to enhancements of temperature and [CO_2], revealing that in a climate change scenario most likely there will be either minor or no damage to plant growth in *Eucalyptus* seedlings. The cultivation of *Eucalyptus* seedlings in warmer environment culminated in significant changes in metabolism that most likely explain the reductions in both plant growth and accumulation of biomass under this condition. The increase in [CO_2] availability resulted in higher growth and accumulation of biomass in eucalyptus seedlings. Acclimatative responses to high [CO_2] occurred following long-term exposition to this environmental condition. The greater availability of [CO_2] also caused important anatomical changes related to the use of water by the *Eucalyptus* seedlings. Elevated [CO_2] not only mitigated but also modified the negative effects caused by heating in both clones studied by morpho-physiological changes that are important under stress conditions. This behavior may also be extremely important in environments with limited water availability, which is also a factor target due global climate change.

Collectively, the results described here further highlights that elevated [CO_2] will be a key factor for the sustainability of *Eucalyptus* seedlings in face of predicted climate change and global warming scenarios, which may be important during a seedling production phase in nurseries. Further investigation is still required to fully elucidate the mechanisms behind this intriguingly behavior under the climate change scenario.

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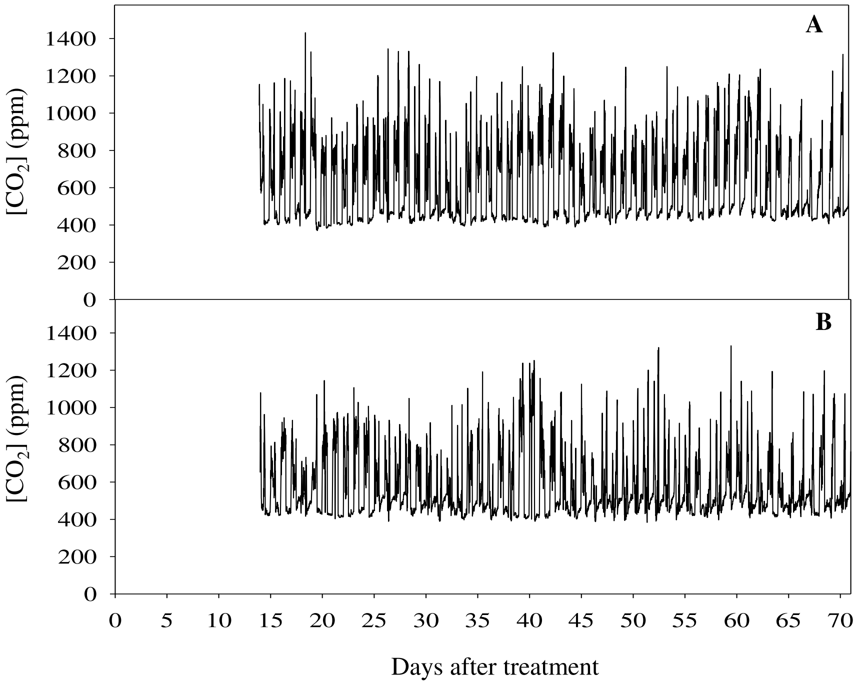
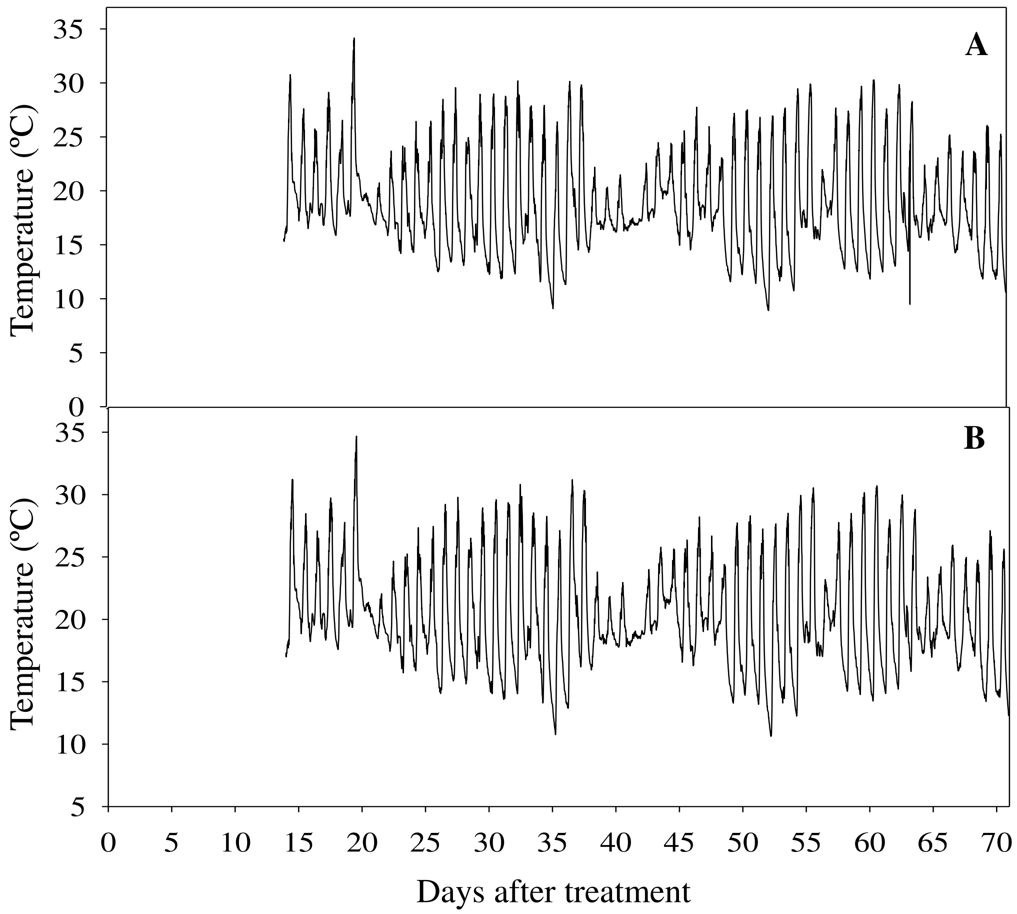
7. SUPPLEMENTAL DATA

Supplemental Figure 1. Overview of the experimental area at 62 days after treatments application. (A) Control. (B) Heating. (C) High [CO_2]. (D) Heating and High [CO_2]. White bar indicates the scale



Supplemental Figure 2. Oscillations of [CO_2] average over day during the experiment. (A) High [CO_2] treatment, value average was 718 ppm. (B) Heating and High CO_2 treatment, value average was 710 ppm during the day.



Supplemental Figure 3. Daily averaged air temperature over the course of the experiment. (A) High [CO_2] treatment, value average was 18.7 ºC. (B) Heating and High CO_2 treatment, value average was 20.2 ºC.

Supplemental Table 1: ANOVA values to gas exchange parameters during the day obtained at 62 days after the treatments application.

	Clone 01			Clone 02		
Parameter	Treatment	hour	Treatment x hour	Treatment	hour	Treatment x ho
A	**	ns	**	**	ns	*
g_s	*	ns	ns	ns	ns	ns
E	ns	**	ns	ns	**	ns
Ci/ca	ns	ns	ns	*	ns	ns
VPD	ns	**	ns	ns	**	ns
PAR	ns	**	ns	**	**	ns

(*) P < 0,05; (**) P < 0,01; (ns) no significant.

Suplemental Table 2: ANOVA values obtained at 62 days after the treatments application.

Parameter	**Treatment**	**Clone**	**TreatmentxClone**
Height	**	**	ns
Branch number	ns	*	ns
Stem diameter	**	ns	ns
Leaf number	**	**	**
Leaf area	**	ns	ns
Specific leaf area	ns	ns	ns
Dry mass root	**	*	ns
Dry mass shoot	**	ns	ns
Dry mass total	**	ns	ns
Root/shoot ratio	**	ns	ns
A	**	ns	ns
g_s	**	*	ns
E	**	ns	ns
Ci/Ca	**	**	*
DPV	**	**	*
δ^{13}C leaf	**	ns	*
δ^{13}C stem	**	ns	ns
δ^{13}C root	**	ns	ns
δ^{15}N leaf	ns	**	ns
δ^{15}N stem	**	ns	ns
δ^{15}N root	**	ns	ns
Chlorophyll *a*	ns	ns	ns
Chlorophyll *b*	ns	ns	ns
Chlorophyll *a+b*	ns	*	ns
Chlorophyll *a/b*	**	*	ns
Glucose	ns	**	**
Fructose	**	**	**
Sucrose	**	**	ns
Starch	**	*	ns
Protein	**	**	**
Amino acids	*	**	ns
Malate	**	ns	ns
NAD+	**	**	**
NADH	**	*	ns
NAD+/NADH	*	*	ns
NADP$^+$	ns	**	ns
NADPH	**	ns	ns
NADP$^+$/NADPH	ns	**	ns
Stomatal density	**	ns	ns
Stomatal index	ns	**	ns
Vein density	ns	**	ns
Palicadic parenchyma	ns	*	ns
Spongy parenchyma	ns	ns	ns
Upper epidermis	*	*	ns
Lower epidermis	**	ns	ns
Intercell space	ns	*	ns
Leaf thickness	ns	**	ns
Vessels number	*	*	*
Vessels area	ns	**	ns
Xylem area	ns	ns	ns
Floem area	ns	ns	ns
R_{dark}	**	ns	ns
$1/\phi$	**	ns	ns
LCP	ns	ns	ns
LSP	ns	ns	ns
V_{cmax}	ns	ns	ns
J_{max}	ns	ns	ns

(*) P < 0,05; (**) P < 0,01; (ns) no significant.

Suplemental Table 3: ANOVA values to parameters obtained by GC-MS at 62 days after the treatments application.

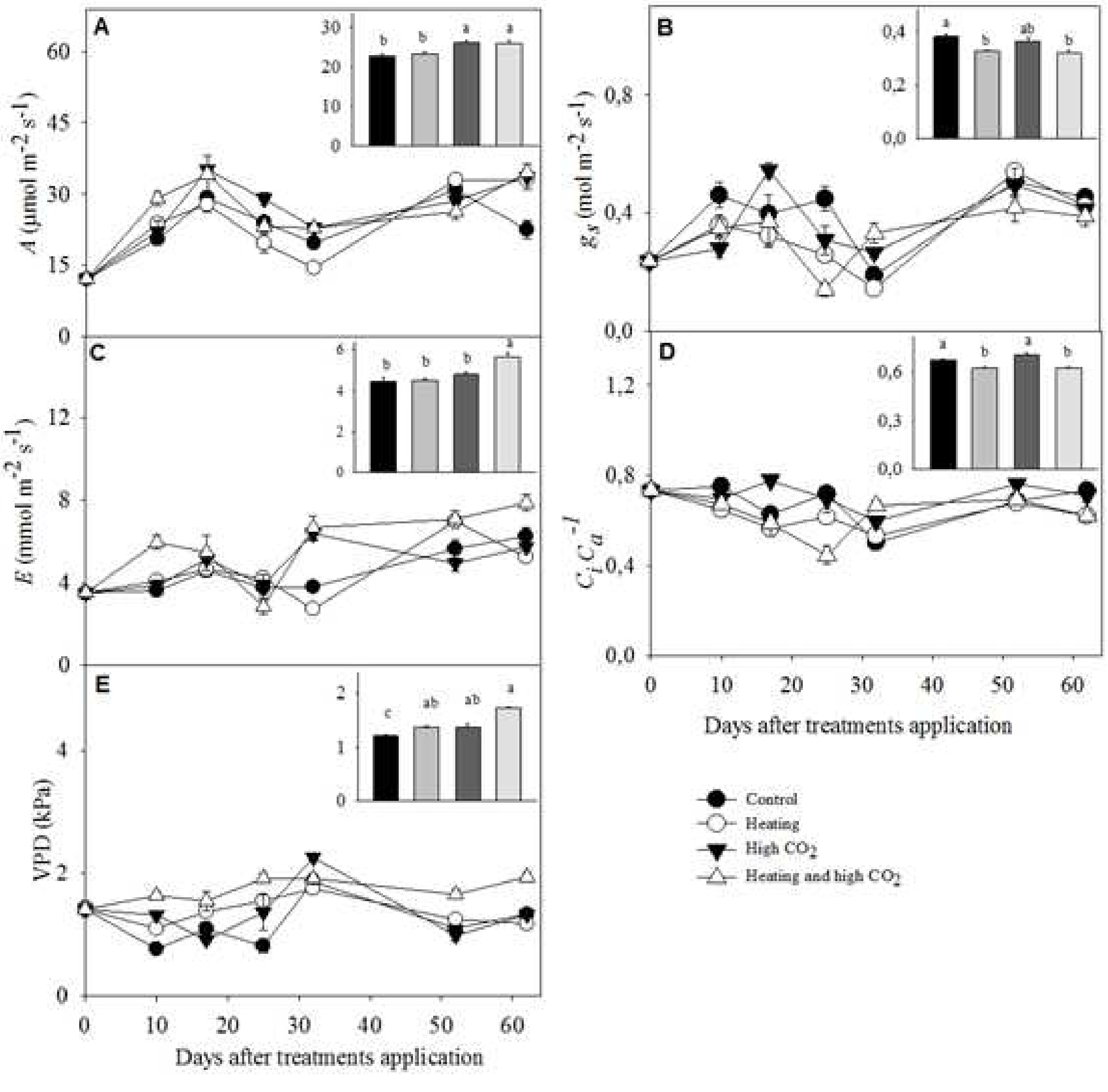
Parameter	Clone 01	Clone 02
Alanine	ns	*
Aspartate	ns	ns
Glutamate	**	**
Glycine	ns	ns
Isoleucine	*	*
Lysine	ns	ns
Phenylalanine	*	*
Proline	**	**
Serine	ns	*
Tyrosine	ns	**
Threonine	**	**
Trytophan	ns	**
Valine	*	*
Cellobiose	ns	*
Erythitol	**	ns
Fructose	ns	ns
Galactinol	ns	ns
Gentiobiose	ns	ns
Glucose	ns	ns
Lactulose	ns	ns
Maltose	**	**
Mannitol	ns	ns
Myoinositol	ns	ns
Raffinose	ns	*
Sucrose	ns	ns
Xylose	*	*
Ascorbate	*	ns
Citrate	ns	*
Deydroascorbate	**	ns
Glicerate	ns	ns
malate	**	**
Piroglutamate	*	ns
Succinate	ns	**
Glycerol	ns	ns
Lactitol	*	**
Quinic acid	ns	ns
Ribulose-5-phosphate	ns	*
Urea	ns	*

(*) P < 0,05; (**) P < 0,01; (ns) no significant.

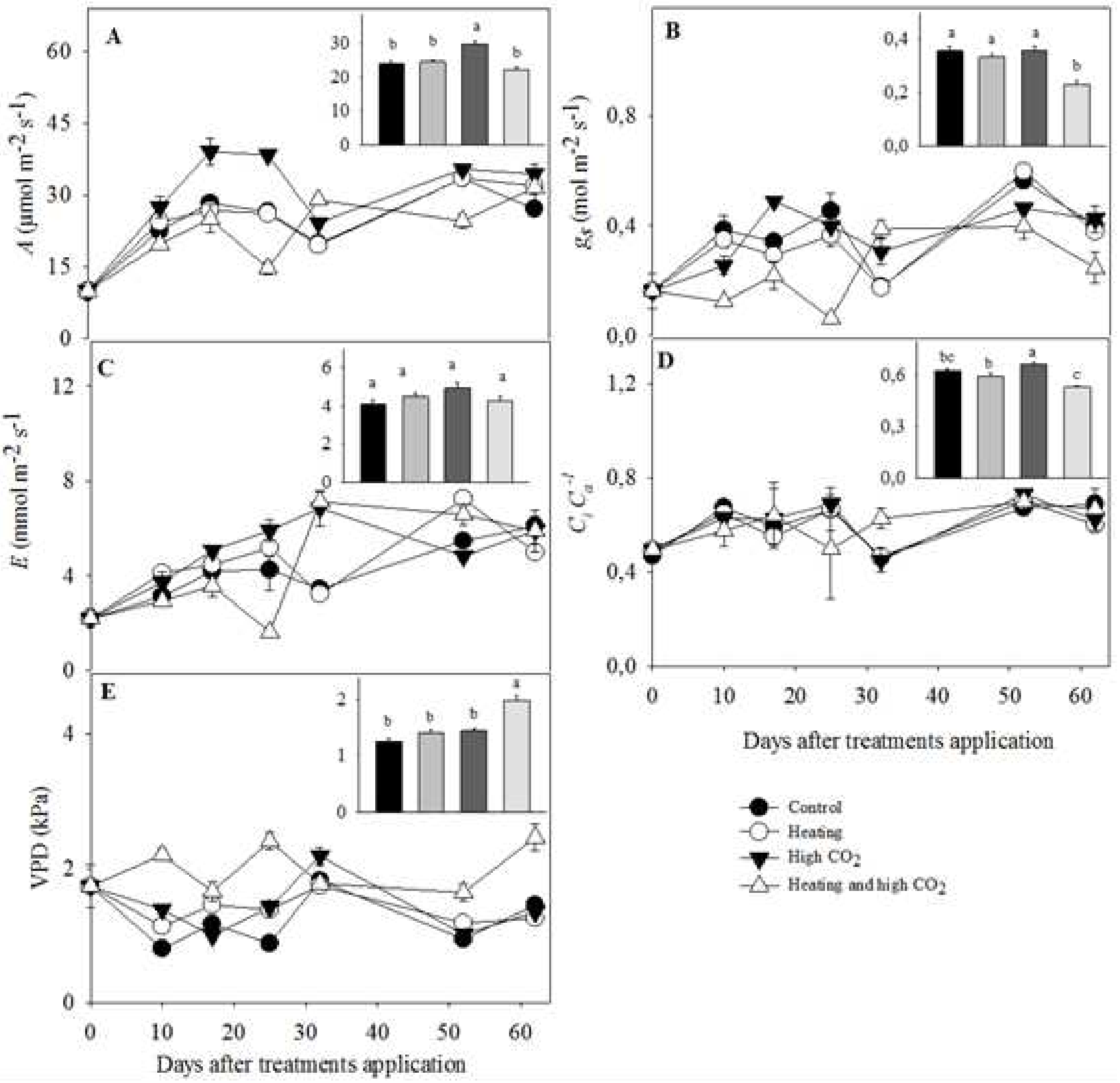
Suplemental Table 4: ANOVA values to gas exchange parameters over time.

Parameter	Clone 01	Clone 02
A	**	**
g_s	**	**
T	**	ns
Ci/Ca	**	**
VPD	**	**

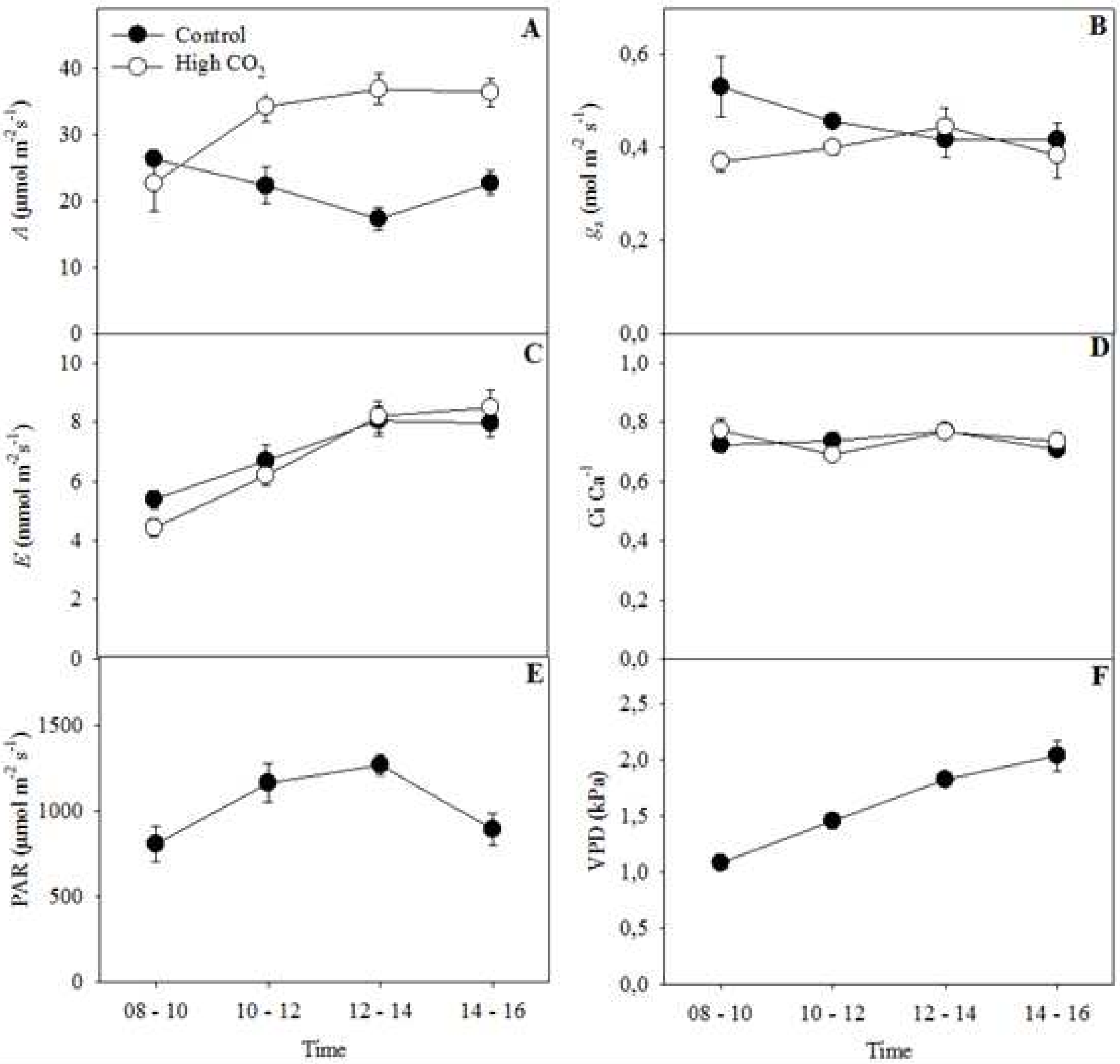
(*) P < 0,05; (**) P < 0,01; (ns) no significant.



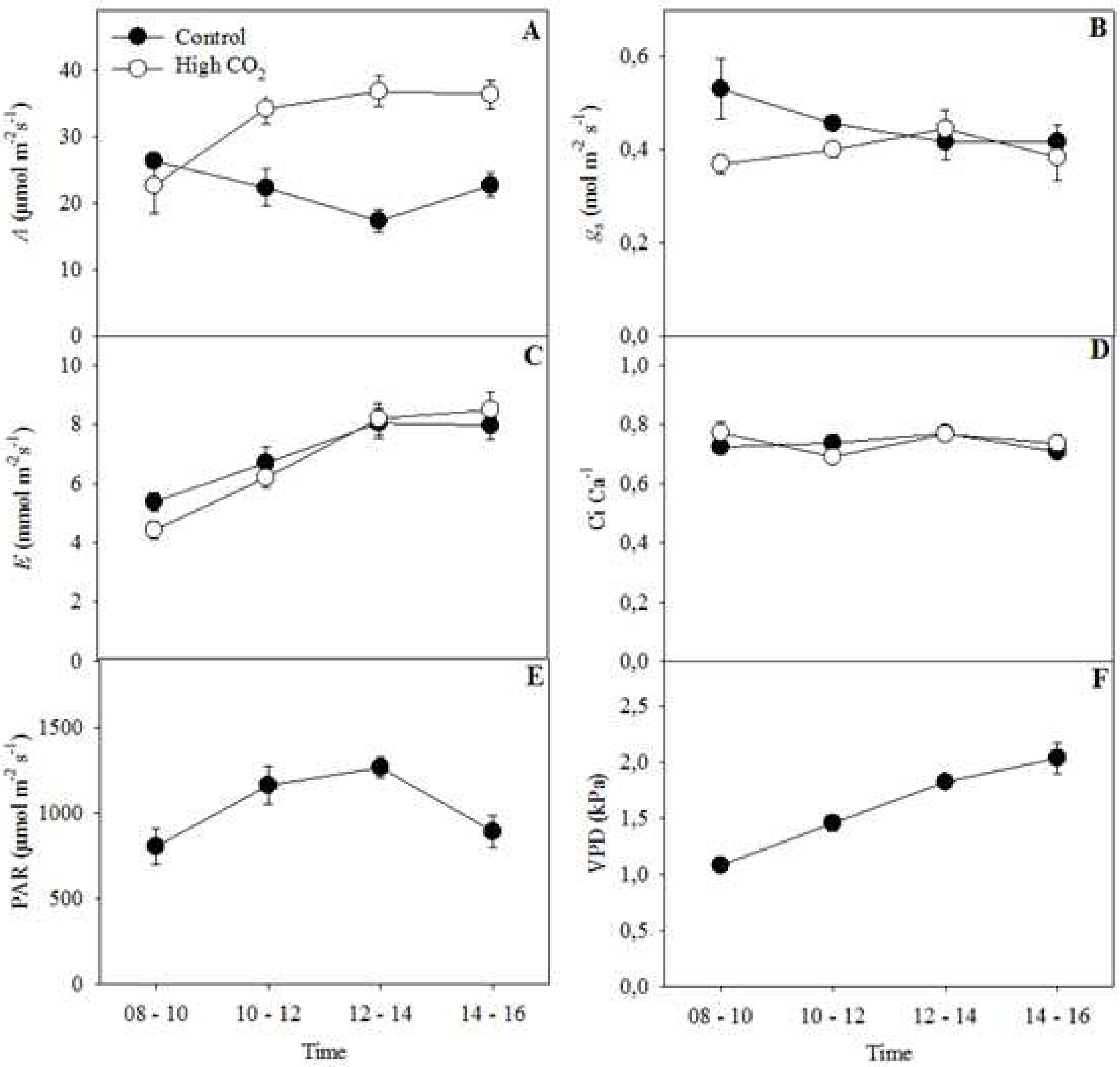
Supplemental Figure 4. Variation in gas exchange parameters over time in response to increase in [CO_2] and temperature in eucalyptus clone 01. The parameters investigated include. (A) Photosynthetic rate. (B) Stomatal conductance. (C) Transpiration. (D) *Ci/Ca*. (E) Vapor pressure deficit. Four different treatments were applied: Control (environment temperature and [CO_2]), heating (increase of 2 ºC and environment [CO_2]), high CO_2 (environment temperature and [CO_2] of 700 ppm), heating and high CO_2 (increase of 2 °C and [CO_2] of 700 ppm). Means (*n*=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different ($P < 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatments. Columns graph represents the average throughout the experiment Bars represent mean ± standard error of five replicates Letters compare treatments within each.



Supplemental Figure 5. Variation in gas exchange parameters over time in response to increase in [CO$_2$] and temperature in eucalyptus clone 01. The parameters investigated include. (A) Photosynthetic rate. (B) Stomatal conductance. (C) Transpiration. (D) *Ci/Ca*. (E) Vapor pressure deficit. Four different treatments were applied: Control (environment temperature and [CO$_2$]), heating (increase of 2 ºC and environment [CO$_2$]), high CO$_2$ (environment temperature and [CO$_2$] of 700 ppm), heating and high CO$_2$ (increase of 2 °C and [CO$_2$] of 700 ppm). Means (n=5 ± SE) followed by different letters were determined by Tukey's test to be statistically different (P< 0.05). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatments. Columns graph represents the average throughout the experiment Bars represent mean ± standard error of five replicates Letters compare treatments within each.



Supplemental Figure 6. Variation in gas exchange parameters during the day in response to increase in [CO_2] in clone 01. The parameters investigated include. (A) Photosynthetic rate. (B) Stomatal conductance. (C) Transpiration. (D) *Ci Ca*$^{-1}$. (E) Vapor pressure deficit. (F) Photon flux density. Two different treatments were analyzed: Control (environment temperature and [CO_2]), and High CO_2 (environment temperature and [CO_2] of 700 ppm.



Supplemental Figure 7. Variation in gas exchange parameters during the day in response to increase in [CO_2] in clone 02. The parameters investigated include. (A) Photosynthetic rate. (B) Stomatal conductance. (C) Transpiration. (D) $Ci\ Ca^{-1}$. (E) Vapor pressure deficit. (F) Photon flux density. Two different treatments were analyzed: Control (environment temperature and [CO_2]), and High CO_2 (environment temperature and [CO_2] of 700 ppm.

Supplemental Table 5: Parameters obtained from light and CO_2 curves in two eucalyptus clones after exposure to high [CO_2].

Clone	Treatment	R_{dark}	$1/\phi$	*LCP*	*LSP*	V_{cmax}	J_{max}
1	Control	2,60 ± 0,2 Ab	14,61 ± 0,5Aa	39,71 ± 4,4 Aa	1004,60 ± 35,1 Aa	168,42 ± 14,0 Aa	240,82 ± 2,4 Aa
	High CO_2	3,56 ± 0,3 Aa	11,66 ± 0,4 Ab	47,02 ± 6,2 Aa	1024,72 ± 42,1 Aa	180,18 ± 13,5 Aa	250,82 ± 9,4 Aa
2	Control	2,66 ± 0,2 Ab	15,37 ± 0,4 Aa	47,30 ± 6,7 Aa	1035,37 ± 33,6 Aa	154,46 ± 26,6 Aa	257,92±14,7 Aa
	High CO_2	3,57 ± 0,4 Aa	11,70 ± 0,5 Ab	41,91 ± 3,4 Aa	1006,71 ± 39,5 Aa	190,81 ± 12,2 Aa	233,99 ± 4,2 Aa

The parameters include. Respiration rate (R_{dark}). Apparent maximum quantum yield (Φ). Light compensation point (*LCP*). Light saturation point (*LSP*). Maximum carboxylation rate of Rubisco (V_{cmax}). Transport rate of electrons (J_{max}). Two different treatments were analyzed: Control (environment temperature and [CO_2]), High CO_2 (environment temperature and [CO_2] of 700 ppm. Means (n=5 ± SE except to *Vcmax* and *Jmax*, n=3) followed by different letters were determined by Tukey's test to be statistically different ($P < 0.05$). Lower case letters compare treatments within each clone. Upper case letters compare clones within each treatments.