Limitations to photosynthesis at different temperatures in the leaves of *Citrus limon*

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The response of CO_2 assimilation rate (A) to the intercellular partial pressure of CO_2 (C_i) was measured on intact lemon leaves over a range of temperatures (10 to 40ºC). The A/C_i response shows how change in the leaf temperature alters the activity of ribulose-1,5-bisphosphate (RuBP) carboxylase-oxygenase (Rubisco) and RuBP regeneration via electron transport. The rate of A reached a maximum of 7.9 to 8.9 µmol m^{-2} s^{-1} between 25 and 30ºC, while dark respiration (R_d) increased with temperature from 0.4 µmol m^{-2} s^{-1} at 10ºC to 1.4 µmol m^{-2} s^{-1} at 40ºC. The maximum rates of carboxylation ($V_{c,max}$) and the maximum rates of electron transport (J_{max}) both increased over this temperature range from 7.5 to 142 µmol m^{-2} s^{-1} and from 23.5 to 152 µmol m^{-2} s^{-1}, respectively. These temperature responses showed that A can be limited by either process depending on the leaf temperature, when C_i or stomatal conductance are not limiting. The decrease in A associated with higher temperatures is in part a response to the greater increase in the rate of oxygenation of RuBP compared with carboxylation and R_d at higher temperatures. Although A can in theory be limited at higher C_i by the rate of triose-phosphate utilization, this limitation was not evident in lemon leaves.
Key words: A/C_i curves, gas exchange, lemon

Análise *in vivo* das limitações da fotossíntese, sob diferentes temperaturas, em folhas de *Citrus limon*: A resposta da taxa de assimilação de CO_2 (A) à pressão parcial de CO_2 (C_i) foi medida em folhas intactas de limão cravo, numa ampla faixa de temperaturas (10 to 40ºC). A variação na curva A/C_i mostrou como as mudanças na temperatura foliar alteram a atividade da ribulose-1,5-bisfosfato (RuBP) carboxilase-oxigenase (Rubisco) e a regeneração da RuBP, via transporte de elétrons. O valor máximo de A obtido foi de 7.9 a 8.9 µmol m^{-2} s^{-1}, entre 25 e 30ºC, enquanto a respiração mitocondrial (R_d) aumentou com a temperatura, de 0.4 µmol m^{-2} s^{-1} a 10ºC até 1.4 µmol m^{-2} s^{-1}, a 40ºC. A taxa máxima de carboxilação ($V_{c,max}$) e a taxa máxima de transporte de elétrons (J_{max}) aumentaram naquela faixa de temperatura, de 7.5 a 142 µmol m^{-2} s^{-1}, e de 23.5 a 152 µmol m^{-2} s^{-1}, respectivamente. A redução em A associada às altas temperaturas é, em parte, uma resposta ao maior aumento na taxa de oxigenação da RuBP, comparada à taxa de carboxilação, e de R_d sob altas temperaturas. Apesar de A poder ser, em teoria, limitada sob elevada C_i pela taxa de utilização de triose-fosfato, essa limitação não foi evidente nas folhas analisadas.
Palavras-chave: curvas A/C_i, limão cravo, trocas gasosas

INTRODUCTION

Temperature is one of the most variable environmental factors, which can suppress photosynthesis both at high and low values. Under global warming scenarios, the study of temperature effects on photosynthesis is essential to predict crop production in the future (Long, 1991). To examine the biochemistry of photosynthesis in leaves, measurement of CO_2 assimilation rate (A) in relation to chloroplast CO_2 partial pressures (C_c) would be ideal as this is the CO_2 pressure determining the Rubisco carboxylation. However, measuring C_c is

difficult. Therefore, it has become a common practice to calculate the CO_2 partial pressure in substomatal cavities (intercellular CO_2 partial pressure, C_i), based on measurements of gas exchange under different ambient CO_2 partial pressures (von Caemmerer, 2000; Long and Bernacchi, 2003). The response of A to C_i under different temperatures can be interpreted in terms of the biochemical processes controlling the response of A (Sage, 1994).

The group of evergreen fruit trees includes numerous horticulturally and economically important crops, as *Citrus* spp., which are cultivated throughout most tropical and subtropical areas of the world. Although citrus tree thrive in hot, dry environments, leaf photosynthesis has a relatively low temperature optimum of $25^{\circ}C$ to $30^{\circ}C$ (Goldschimidt and Koch, 1996). The term evergreen relates to the nondeciduous nature of leaves and, as such, has immediate consequences for leaf longevity and photosynthesis. Broadleaf evergreen citrus leaves are relatively thick with a small proportion of leaf volume occupied by intercellular air space. They have a shiny waxy cuticle particularly on the adaxial surface and stomata are located almost exclusively on the abaxial surface (Goldschimidt and Koch, 1996). Therefore, citrus leaves have low rates of A (4 to 8 μmol CO_2 m^{-2} s^{-1} seem realistic under optimal conditions), and low stomatal and mesophyll conductances (Lloyd et al., 1992). In addition, its leaves act as a carbohydrate storage organ with slow rates of assimilates export, which in turn can feedback to reduce A (Syvertsen and Lloyd, 1994). Wullschleger (1993) made a retrospective analysis of the A/C_i curves of 109 C_3 species and concluded that the maximum rate of carboxylation ($V_{c,max}$) and the light-saturated rate of electron transport (J_{max}) were in general higher for herbaceous annuals plants than for woody perennials. Therefore, the aim of this study was to characterize the A/C_i response and the *in vivo* calculated photosynthetic parameters in *Citrus limon*, an evergreen plant with low A values even for a C_3 plant, estimated from the A/C_i response curves over a range of temperature.

MATERIAL AND METHODS

Three individuals of *Citrus limon* L. were germinated and grown in environmentally-controlled greenhouses located at the University of Illinois, Urbana, USA. Plants were grown in a soil-less growth medium (Sunshine Mix #1, SunGro Horticulture, Bellevue, USA) and were watered regularly. Nutrient additions were given weekly in the form of 300 μL L^{-1} of NPK 15:5:15 (Peters Excel, The Scotts Co., Marysville, USA) to pot saturation. Greenhouse temperature levels were set at $25^{\circ}C$ for the 16-h photoperiod and $18^{\circ}C$ for night.

Leaf gas exchange rates were measured using an open gas exchange system with independent $[CO_2]$ control using a 6 cm^2 clamp-on leaf cuvette (LI 6400, LI-COR, Lincoln, USA). The gas-exchange system was zeroed daily using CO_2-free air, and leakage of CO_2 into and out of the chamber, with a Citrus leaf inside, was determined for the range of CO_2 concentrations used in this study and used to correct measured leaf fluxes. The chamber was modified by replacing the peltier external heat sink with a metal block containing water channels, which in turn were connected to a heating/cooling circulating water bath (Endocal RTE-100, Neslab Instruments, Newington, USA). The modified heating/cooling blocks, used in conjunction with the peltier temperature controls, provided leaf temperature control at any preset value between 10-40$^{\circ}C$. Leaf temperature was measured using a chromal-constantin thermocouple pressed to the lower leaf surface. The temperatures reported by this particular thermocouple were cross-checked against standard mercury-in-glass thermometers in a controlled temperature chamber and found to be within $\pm0.4^{\circ}C$ (Bernacchi et al., 2003).

Photosynthesis was measured after acclimation of the leaf to temperature (until A was steady-stated and total CV was lower than 0.3, at least after 2 h at each new temperature) at photosynthetic photon flux density (PPFD) between 600 and 800 μmol m^{-2} s^{-1}, which was light-saturating for this species. Photosynthetic photon flux density was controlled using an artificial quartz halide light source controlled with a quantum sensor located inside the leaf cuvette. The vapor pressure deficit in the cuvette was maintained between 0.5 and 2.0 kPa to prevent stomatal closure by passing the air entering the gas-exchange system through either anhydrous calcium carbonate (Drierite, W.A. Hammond Drierite Company, Xenia, USA) at lower temperatures when humidity was high or by bubbling the air through water for the higher temperatures. Values for A and C_i were calculated using the equations of von Caemmerer and Farquhar (1981). A

protocol commonly used in determining this A versus C_i response is: firstly, induce photosynthesis at the growth CO_2 concentration (36 to 380 µmol mol^{-1}) and saturating PPFD (between 600 and 800 µmol m^{-2} s^{-1}) until A is steady-stated (over a 5-min period). Values of A and C_i are recorded and then ambient CO_2 partial pressure (C_a) is decreased to 300, 250, 200, 150, 100 and 50 µmol mol^{-1}. Upon completion of this sequence, C_a is returned to growth CO_2 concentration to check that the original A can be restored and then is increased stepwise to 450, 550, 650, 800, 1000 µmol mol^{-1}. Steady-state photosynthesis needs to be obtained at each step (with a total CV lower than 0.3, at least after 5 min for each step).

Three replicate measurements of A/C_i curves (Figure 1: A/C_i curve at 25°C) were measured on different plants, at 5°C intervals between 10 and 40°C. The parameters $V_{c,max}$, J_{max} and R_d were estimated using regression analysis of the curves (Figure 2) based on the equations presented in the appendix (Long and Bernacchi, 2003). The temperature responses of $V_{c,max}$, J_{max}, and R_d were plotted from the results of the regression analysis at each measurement temperature from 10 to 40°C (e.g., Bernacchi et al., 2001). Data for photosynthesis measured at a CO_2 concentration of 370 µmol mol^{-1} was extracted from the curves and plotted as a function of temperature. Using the equations presented by Farquhar et al. (1980), based on A/C_i measurements (Figure 1) and thus the calculated response of $V_{c,max}$ (Figure 2) at the complete range of temperatures, estimations of the temperature response of photosynthesis under non-RuBP limiting conditions was also determined.

Data were subjected to analysis of variance (ANOVA) for temperature effects and means were compared by Student-Newman-Keuls test at 0.05 of probability, when significance was detected.

RESULTS AND DISCUSSION

Under light saturating conditions photosynthesis for C_3 plants is limited by Rubisco capacity, the Rubisco limited phase, as shown in Figure 1. As C_i increases above typical levels for this specie, photosynthesis will typically become limited by RuBP regeneration via electron transport, the RuBP limited phase, and by triose-phosphate utilization (TPU) at substantially higher C_i, the TPU limited phase (Sage, 1994; von Caemmerer, 2000).
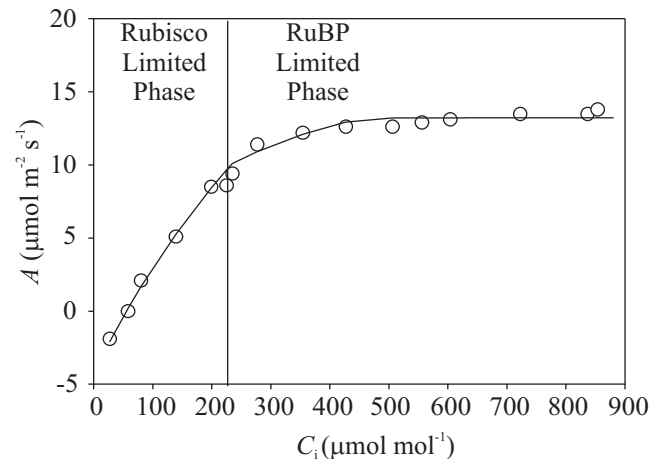
Figure 1. The A/C_i response of *Citrus limon* at 25°C. The actual rates of photosynthesis that would be achieved depending on whether Rubisco or RuBP are limiting (in this study triose-phosphate utilization was not limiting).

This last limitation, however, was not observed in this experiment with lemon (Figure 1), as is frequent in field-based measurements (Adams et al., 2000). During the electron transport limitation, the RuBP limited phase, CO_2 uptake still increases because CO_2 out-competes O_2 for the available RuBP, but during the triose limitation photosynthesis is no more CO_2 dependent (von Caemmerer, 2000).

The results obtained with lemon under temperatures from 10°C to 40°C showed a maximum values of A between 25 and 30°C, with 8.9 and 7.9 µmol m^{-2} s^{-1}, respectively, while R_d increased significantly with temperature from 0.4 to 1.5-1.4 µmol m^{-2} s^{-1} (obtained as in Figure 2). The range of temperature for maximal A measured (Figure 3A) is in agreement with Golschmidt and Koch (1996), who stated that the genus *Citrus*, which originated in tropical and semitropical regions, have an optimal temperature between 25°C to 30°C. The highest value of A (8.9 µmol m^{-2} s^{-1}; Figure 3A) was obtained at 25°C and A decreased both above and below this temperature. This is in contrast to R_d, which rose with temperature (Figure 3B). The low value of A for the perennial woody *Citrus* spp. when compared to annual herbaceous plants (Wullschleger, 1993), but also to other perennials fruit crops as *Prunus persica*, might be attributed to a lower mesophyll conductance (g_m) or/and low leaf nitrogen present as Rubisco (Lloyd et al., 1992), which can in turn reduce $V_{c,max}$ (Long, 1991).
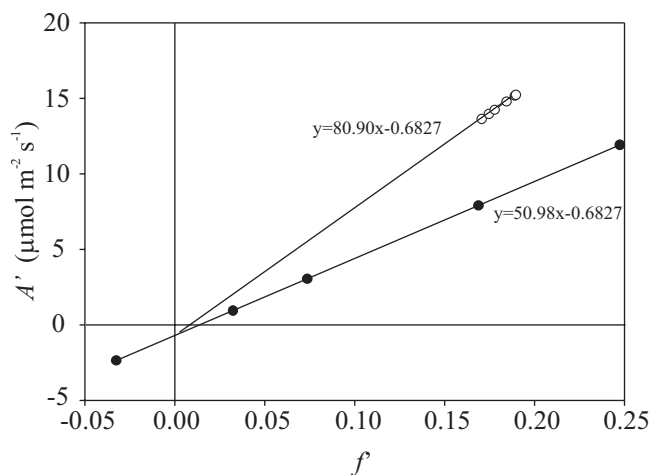
Figure 2. The adjusted CO_2 assimilation rate (A') plotted as a linear function of f' (which is a function of C_i in the Rubisco limited part of the A/C_i response curve; closed circles) and g' (which is a function of C_i in the RuBP limited part of the A/C_i response curve; open circles) of *Citrus limon* at 25°C. $V_{c,max}$ is obtained by the slope and R_d is the intercept of the A x f' linear function, while R_d values is used to solve for J_{max} in the A x g' linear function, as stated by Long and Bernacchi (2003). $n = 3 \pm SD$.

Potentially 50 to 70% of carbon assimilated in plant biomass is released back to the atmosphere as CO_2 during subsequent plant respiration (Baldocchi and Amthor, 2001). The response of A to temperature is parabolic and its decrease at high temperature occurs through numerous potential processes, including increases in R_d, decrease in membrane stability, decrease in the specificity factor of Rubisco and an accumulation of carbohydrates (Baldocchi and Amthor, 2001). In addition, the limitation of A imposed by an increase in g_m with temperature suggests that the dominant process(es) determining g_m is not physical, but probably protein-mediated, possibly involving a carbonic anhydrase or aquaporins (Bernacchi et al., 2002; Long and Bernacchi, 2003).

Under non-limiting environmental conditions, *in vitro* Rubisco activity ($V_{c,max}$) for the activated enzyme extracted from citrus leaves is generally in the range of 300 to 400 µmol CO_2 mg chlorophyll^{-1} h^{-1} (Vu and Yelenovsky, 1988). These authors equate a $V_{c,max}$ value of about 75 µmol CO_2 mg chlorophyll $^{-1}$ h^{-1} for leaves of "Valencia" orange (*Citrus sinensis* [L.] Osbeck) having a maximum A of 8 mmol CO_2 m^{-2} s^{-1}. On the other hand, from an A/C_i plot, Syvertsen and Lloyd (1994) obtained a value
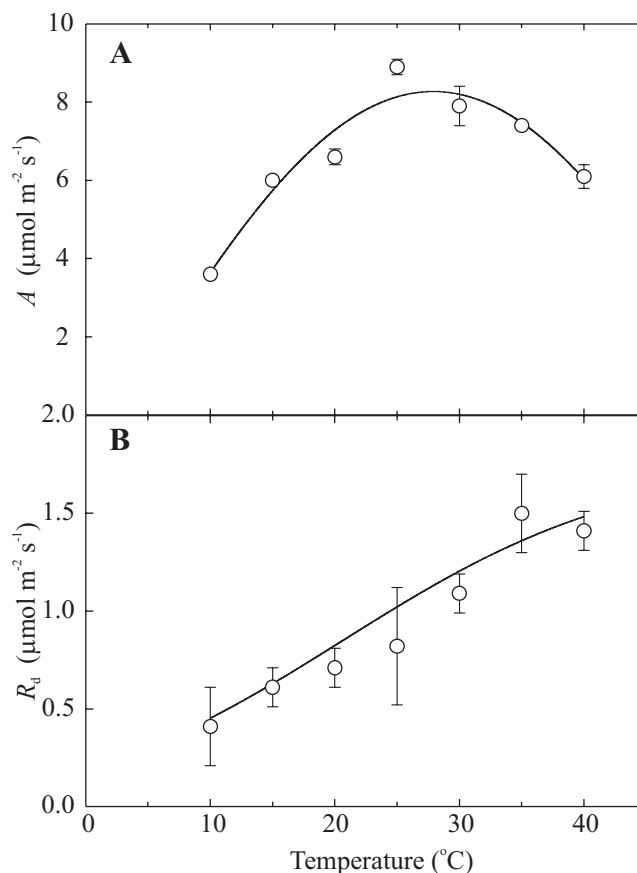
Figure 3. Temperature response of (**A**) CO_2 assimilation rate (A) and (**B**) dark respiration (R_d), determined from gas exchange measurements on *Citrus limon*. $n = 3 \pm SD$.

for $V_{c,max}$ varying from 75 to 106 µmol m^{-2} s^{-1}, and a value for J_{max} varying from 130 to 140 µmol m^{-2} for "Marsh" and "Ruby Red" grapefruit at 25°C, respectively. The *in vivo* values of $V_{c,max}$ and J_{max} for *C. limon* at 25°C were 55 µmol m^{-2} s^{-1} and 87 µmol m^{-2} s^{-1} (Figure 4A,B), respectively. These values of $V_{c,max}$ and J_{max} at 25°C are close to the mean values obtained by Wullschleger (1993) for perennials species, i.e. 44 and 97 µmol CO_2 m^{-2} s^{-1}, respectively, whereas the mean values of these parameters for herbaceous annuals plants are 75 and 154 µmol CO_2 m^{-2} s^{-1}, respectively.

In this study with lemon, the *in vivo* values of $V_{c,max}$ and J_{max} increased significantly with temperature from 7.5 to 142 µmol m^{-2} s^{-1} (Figure 4A) and from 23.5 to 152 µmol m^{-2} s^{-1} (Figure 4B), respectively. In addition, the increase in $V_{c,max}$ with temperature is greater than $V_{o,max}$ (Figure 4A,C), and $V_{o,max}$ values varied from 2.4 to 35.7 µmol m^{-2} s^{-1}
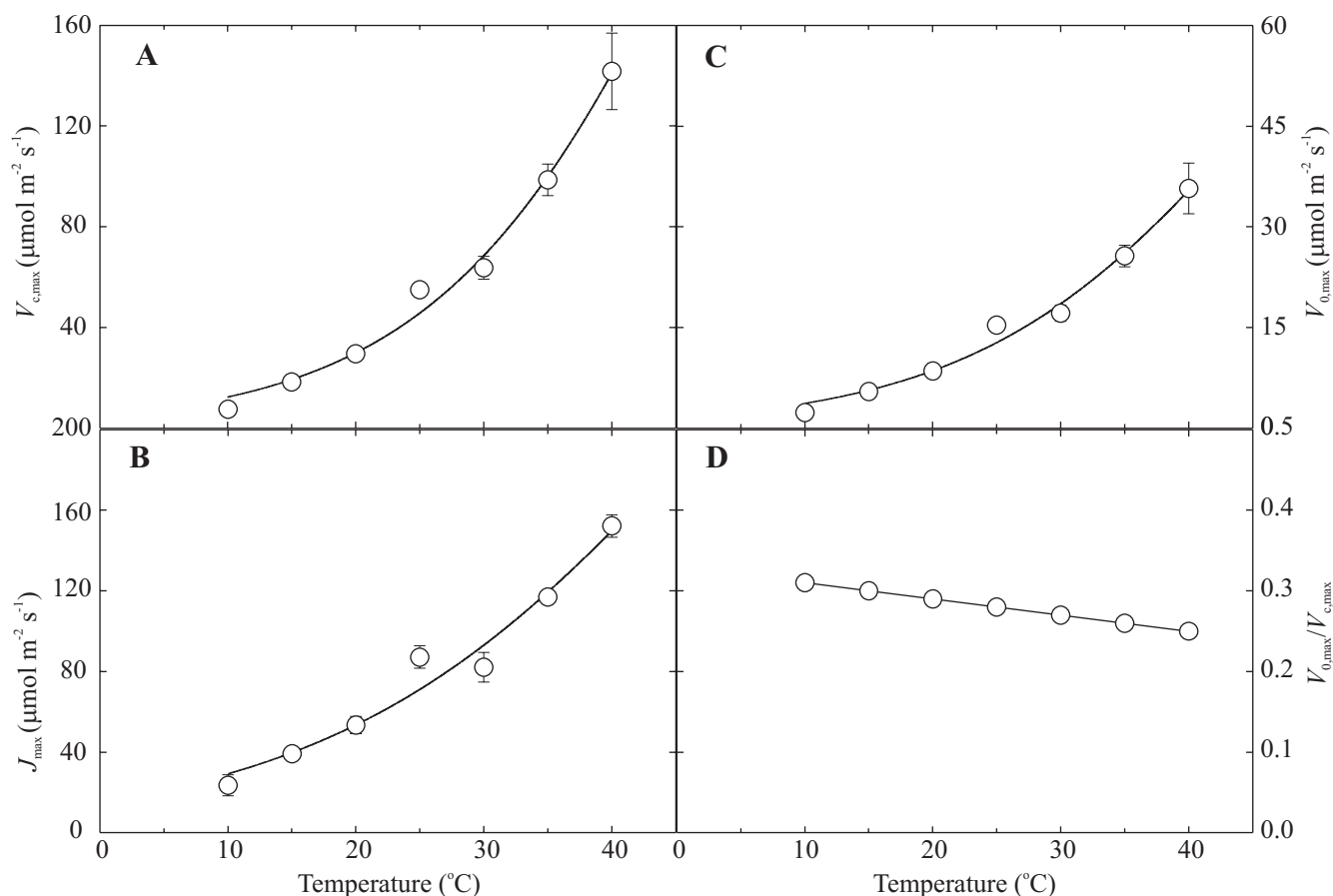
Figure 4. Temperature response of four parameters describing photosynthesis on *Citrus limon*: (**A**) response of $V_{c,max}$ (the maximum rate of carboxylation); (**B**) response of J_{max} (light saturated rate of electron transport); (**C**) response of $V_{o,max}$ (the maximum rate of oxygenation); and (**D**) and the ratio of $V_{c,max}$ / $V_{o,max}$ versus temperature. $n = 3 \pm SD$.

(Figure 4C). From our measurements, we found that $V_{o,max}$ /$V_{c,max}$ declines with temperature. However, due to the differential effect of temperature on the velocity of carboxylation relative to oxygenation (V_c/V_o), observed A values will actually decline with increasing temperatures due to increased photorespiration (Long et al., 2004). Therefore, the ratio of $V_{o,max}$/$V_{c,max}$ is reduced (Figure 4D) at high temperature and there is a greater increases in $V_{c,max}$ compared to $V_{o,max}$, as stated by Bernacchi et al. (2001) and, thus, the proportion of potential carbon uptake lost to photorespiration increases (Long, 1991).

Therefore, depending on temperature A can be limited by very different processes. The amount and activation state of the photosynthetic enzymes, each representing a different limiting process to overall CO_2 assimilation, are integral for determining the temperature optimum of photosynthesis.

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APPENDIX

Model theory

Farquhar et al. (1980) presented a model of leaf level photosynthesis with two rate limiting steps with a third added by Harley and Sharkey (1991). This model states that at any given internal concentration of CO_2, photosynthesis is limited by the slower of three processes: 1) the maximum rate of Rubisco-catalyzed carboxylation (Rubisco-limited *A*); 2) the regeneration of RuBP controlled by electron transport rate (electron transport-limited *A*); or 3) the regeneration of RuBP controlled by the rate of triose-phosphate utilization (TPU-limited *A*). Both CO2 and O2 compete for the Rubisco binding site in the processes known as carboxylation and oxygenation, respectively (Farquhar et al., 1980). To account for the competitive inhibition between CO_2 and O_2, *A* is mathematically expressed as:

$$A = v_c - 0.5v_o - R_d, \qquad [1]$$

where v_c and v_o are the rates of carboxylation and oxygenation, respectively, and R_d is the mitochondrial respiration (Farquhar et al., 1980).

When *A* is Rubisco-limited (W_c) the velocity of carboxylation can be expressed as:

$$W_c = v_c = \frac{V_{c,max} \cdot C_i}{C_i + K_c(1 + O / K_o)}, \qquad [2]$$

where $V_{c,max}$ is the maximum rate of carboxylation, O is the oxygen concentration, and K_c and K_o are the Michaelis-Menten constants for CO_2 and O_2, respectively (Farquhar et al., 1980).

The velocity of carboxylation when limited by the rate of electron transport (W_j) is expressed as stated by von Caemmerer (2000):

$$W_j = v_c = \frac{J \cdot C_i}{4.5C_i + 10.5\Gamma*}, \qquad [3]$$

where J is the potential rate of electron transport and can be expressed as a function of light saturated rate of electron transport (J_{max}), as stated by von Caemmerer (2000):

$$J = \frac{J_{max} \cdot f \cdot I}{f \cdot I + 2.1 J_{max}} \, , \qquad [4]$$

where f is the fraction of light not absorbed by functional photosynthetic pigments and I is the photon flux hitting the leaf.

Triose phosphate utilization limited photosynthesis (W_p) was not a limitation in this study but can occur at low temperatures or high levels of CO_2 for others species and is expressed as:

$$W_p = v_c = 3 \cdot V_{TPU} \, , \qquad [5]$$

where V_{TPU} is the velocity of triose phosphate utilization, which is multiplied by three to represent three mol CO_2 that can be fixed for every mol of triose-phosphate made available (Harley and Sharkey, 1991).

Incorporating the three rate limiting steps into equation 1 yields:

$$A = [1 - \Gamma^*/C_i] \cdot \min(W_c, W_j, \frac{W_p}{1 - \Gamma^*/C_i}) \, , \qquad [6]$$

where the term Γ^* is the CO_2 compensation point in the absence of R_d. The term $[1 - \Gamma^*/C_i]$ represents photorespiration and is derived from the equation:

$$v_o = \frac{V_{o,max} \cdot O}{O + K_o(1 + C_i/K_c)} \, , \qquad [7]$$

where $V_{o,max}$ is the maximum rate of oxygenation (Farquhar et al., 1980; von Caemmerer 2000). Photosynthesis limited by W_p is insensitive to changes in CO_2 or O_2 and thus the term representing photoinhibition is removed (von Caemmerer, 2000).

From the Rubisco limited portion of the A/C_i curve (integrating equation 2 into equation 1), below the inflection point of the curve (obtained by the interception of the adjusted curves for Rubisco limited and RuBP limited phases), the values of $V_{c,max}$ and R_d can be calculated from the equation:

$$A = [(1 - \Gamma^*/C_i) \cdot \frac{V_{c,max} \cdot C_i}{C_i + K_c(1 + O/K_o)}] - R_d \, , \qquad [8]$$

where $\Gamma^* = 42.05$ μbar; $O = 20.9$ μbar; $K_c = 404.9$ μbar; and $K_o = 278.4$ mbar at 25°C (Long and Bernacchi 2003). For other temperatures, Γ^*, K_c and K_o are adjusted by the

equation parameter $= \exp(c - \Delta H_a/RT_k)$, where c and ΔH_a values for each parameter are presented in Bernacchi et al. (2001). The two unknowns $V_{c,max}$ and R_d can be solved, as shown by Long and Bernacchi (2003), by plotting A (below the inflection point: Rubisco limited) as a linear function of f' (Figure 2):

$$A = V_{c,max} \cdot f' - R_d \, , \qquad [9]$$

where:

$$f' = \frac{C_i - \Gamma^*}{C_i + K_c(1 + O/K_o)} \, , \qquad [10]$$

In this linear function, $V_{c,max}$ is the slope and R_d the intercept (Figure 2).

On the other hand, from the RuBP limited portion of the A/C_i curve (using equation 3 into equation 1) above the inflection point of the curve (obtained by the interception of the adjusted curves for Rubisco limited and RuBP limited phases), J_{max} can be calculated from the equation stated by Long and Bernacchi (2003):

$$A_{max} = [(1 - \Gamma^*/C_i) \cdot \frac{J \cdot C_i}{4.5 C_i + 10.5 \Gamma^*}] - R_d \, , \qquad [11]$$

Similarly for $V_{c,max}$, J_{max} can be obtained by plotting A (above the inflection point where it is RuBP limited) as a linear function of C_i (i.e. g'), but fixing the R_d value obtained from $V_{c,max}$ calculations (equation [9]) to avoid large errors in estimated R_d from a linear regression ($A \times C_i$, i.e. g'), due to small errors in the higher rates of A (RuBP limited). Thus, after solving for R_d from the Rubisco limited portion (together with $V_{c,max}$), the value for R_d can be used in a linear regression (Long and Bernacchi, 2003), together with the high values of A (from RuBP limited portion) in a linear function of g' (Figure 2):

$$A = J_{max} \cdot g' - R_d \, , \qquad [12]$$

where:

$$g' = \frac{C_i - \Gamma^*}{4.5 C_i + 10.5 \Gamma^*} \, , \qquad [13]$$

where J_{max} is the slope in this linear function (Figure 2).

Finally, $V_{o,max}$ can be solved by the equation:

$$V_{o,max} = \frac{V_{c,max} \cdot K_o \cdot \Gamma^*}{0.5 K_c \cdot O} \, , \qquad [14]$$

where $V_{c,max}$, K_o, Γ^*, K_c and O are either known or solved using previous equations.