Effects of changes in the photosynthetic photon flux density on net gas exchange of *Citrus limon* and *Nicotiana tabacum*

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The objective of this study was to evaluate the effects of changes in the photosynthetic photon flux density (*PPFD*) on net gas exchange of *Citrus limon*, a woody species, and *Nicotiana tabacum*, an herbaceous species. When *PPFD* was increased from 50 to 350 μmol.m^{-2}.s^{-1} and returned to 50 μmol.m^{-2}.s^{-1} after 60 min, the CO_2 assimilation rate (*A*) increased and stabilized after 15 min in both species. Stomatal conductance (g_s), however, continued to increase. After returning to low *PPFD*, *A* immediately diminished to a low value for both lemon and tobacco. Stomatal conductance of lemon diminished slowly over 60 min, whereas g_s for tobacco took only 15 min to decrease. This difference in behavior is probably due to over-sensitivity of stomata of woody species, such as *Citrus*, when exposed to high light, retarding stomatal closure on return to low *PPFD*. Furthermore, when lemon, growing at a *PPFD* of 300 μmol.m^{-2}.s^{-1}, was submitted to a step increase of 600 μmol.m^{-2}.s^{-1}, there was an oscillatory behavior of *A* and g_s requiring 150 min to stabilize. The causes of this behavior are discussed with respect to improved intrinsic water use efficiency by stomatal closure.

Key words: CO_2 assimilation rate, gas exchange, stomata, intrinsic water use efficiency.

Oscilação cíclica da condutância estomática em *Citrus limon* e *Nicotiana tabacum*. O objetivo desse estudo foi avaliar o efeito da variação da densidade de fluxo de fótons fotossintéticos (*DFFF*) nas trocas gasosas de *Citrus limon*, uma espécie lenhosa, e *Nicotiana tabacum*, uma espécie herbácea. Quando a *DFFF* foi aumentada de 50 para 350 μmol.m^{-2}.s^{-1} durante 60 min, retornando em seguida a 50 μmol.m^{-2}.s^{-1}, a taxa de assimilação de CO_2 (*A*) aumentou e estabilizou após 15 min, mas a condutância estomática (g_s) continuou crescendo, em ambas as espécies. Quando se retornou à baixa *DFFF*, *A* diminuiu instantaneamente nas duas espécies, mas a g_s do limoeiro diminuiu lentamente durante 60 min, enquanto em tabaco, a g_s levou apenas 15 min para decrescer. Essa diferença nas respostas é provavelmente devida à hipersensibilidade dos estômatos de espécies lenhosas, como *Citrus*, quando submetidas à alta *DFFF*, retardando o seu fechamento, quando de volta à baixa *DFFF*. Além disso, quando o limoeiro, crescendo sob *DFFF* de 300 μmol.m^{-2}.s^{-1}, foi submetido subitamente a 600 μmol.m^{-2}.s^{-1}, iniciou-se uma flutuação de *A* e g_s, levando 150 min para se estabilizar. As causas dessa oscilação são discutidas em função do ajuste da eficiência intrínseca no uso de água, devido ao controle estomático.

Palavras-chave: eficiência intrínseca no uso de água, estômatos, taxa de assimilação de CO_2, trocas gasosas.

INTRODUCTION

Oscillatory transpiration is a rhythmic fluctuation that has been described in several species (Barrs, 1971), with an average cycle periodicity of 15 to 120 min (Kramer and Boyer, 1995). Stomatal cycling can be triggered by environmental signals such as changes in photosynthetic photon flux density (*PPFD*) (Zipperlen and Press, 1997) or changes in xylem water potential and hydraulic conductivity (L_p) (Rose and Rose, 1994). Stomatal cycling is common in *Citrus* under field conditions, when the *PPFD*, vapor pressure deficit

(*VPD*) of air or soil water deficit increase. This cycle has a period of about one hour, and increases in amplitude and decreases in duration as the daytime progresses (Levy and Kaufmann, 1976). Stomatal closure can also be induced by a non-hydraulic root signal from roots, usually ABA (Kramer and Boyer, 1995), or changes in L_p, a hydraulic signal, especially under high transpiration rates (Steudle, 2001). This change in L_p is caused, among others factors, by an apoplastic water movement through the endodermis cell wall in addition to that passing along the cellular pathway under high transpiration rates (Hartung et al., 2002). In maize under high transpiration rates, despite the increase in radial water flow, the ABA concentration in the xylem remained constant or even increased (Hose et al., 2001). This is due to transport of the root apoplastic and symplastic ABA by solvent drag (Hartung et al., 2002).

Low CO_2 assimilation rate (*A*) and leaf diffusive conductance (g_s) often characterize leaves of perennial species, as in *Citrus*. Although most agricultural and herbaceous C_3 plants have *A* in the range 20-40 μmol CO_2.m^{-2}.s^{-1}, in mature leaves of *Citrus*, under near optimal environmental conditions, *A* greater than 12 μmol CO_2.m^{-2}.s^{-1} is rarely observed (Lloyd et al., 1992). Although g_s of *Citrus* are also relatively low (generally less than 0.2 mol.m^{-2}.s^{-1}), under optimal or near optimal conditions intercellular CO_2 partial pressure (C_i) is typically in the range of 200-250 μmol.mol^{-1} (Lloyd et al., 1992), while for herbaceous plants C_i can vary from 250 to 350 μmol.mol^{-1} (Long and Hällgren, 1993). Thus, the stomatal limitation of *A* in woody species can be higher than 20%, as reported by Jones (1998) for herbaceous plants.

Citrus and others woody species have a low maximum rate of carboxylation (V_{cmax}) because of the low chloroplastic CO_2 partial pressure (Lloyd et al., 1992), resulting from a low mesophyll conductance to CO_2 diffusion (Syvertsen and Lloyd, 1994). In *Citrus*, Rubisco accounts for only 10% of the nitrogen in the leaf (Lloyd et al., 1987). Therefore, for long-lived leaves of woody shade-tolerant rain forest species exposed to light, the mean time to attain 90% full photosynthetic induction fell in the range of 11–36 min, as opposed to 1-3 min for short-lived species (Zipperlen and Press, 1997).

Presently in Brazil there is an increased interest in gas exchange studies with *Citrus*, especially after the incidence of citrus variegated chlorosis (CVC), a vascular disease caused by the bacterium *Xylella fastidiosa*, which causes reduction in photosynthesis and productivity of *Citrus* (Ribeiro et al., 2003). However, gas exchange measurements need to be carefully done to avoid environmental changes during measurements, which induce stomatal oscillations.

Thus, the aim of this study was to evaluate the different effects of changing *PPFD* on *A* and g_s behavior of lemon, an evergreen woody plant with low mesophyll conductance to CO_2 diffusion, compared to an annual herbaceous plant, specifically tobacco, with its higher mesophyll conductance to CO_2 diffusion.

MATERIAL AND METHODS

The plants of *Citrus limon* Burm. f. (two years old) and of *Nicotiana tabacum* cv. W38 (45 days old) germinated and grown in a greenhouse, in 1.5 L and 5 L pots, for tobacco and lemon, respectively, with a soil-less growth medium (Sunshine Mix #1, SunGro Horticulture, Inc., Bellevue, WA USA), at the University of Illinois, IL, USA (40º01"N, 88º16"W). The plants were watered regularly and were fertilized weekly with approximately 300 μL.L^{-1} of NPK 15:5:15 (Peters Excel, The Scotts Co., Marysville, OH USA) to pot saturation. Greenhouse air temperature was set at 25°C for the 16 h complemented photoperiod and 18°C for night. One week before measurements three plants were transferred to a cabinet (E15, Conviron, Winnipeg, Manitoba, Canada), with a *PPFD* of 300 μmol.m^{-2}.s^{-1} at canopy height, a photoperiod of 14 h, a day/night air temperature of 25/20°C, and a *VPD* of 0.7 kPa.

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, NE USA). The gas-exchange system was calibrated for CO_2 with a source of air containing a precisely known concentration of CO_2 in the range to be analyzed, and for H_2O vapor with a dew point generator (LI-610, LI-COR, Lincoln, NE USA), as proposed by Long and Hällgren (1993). It was zeroed daily using CO_2-free air, and the *VPD* in the cuvette was maintained between 0.5 and 1.0 kPa to prevent stomatal closure. All the measurements were carried out at 25°C and 360 μL.L^{-1} of CO_2 on the youngest fully expanded leaf prior to stem elongation.
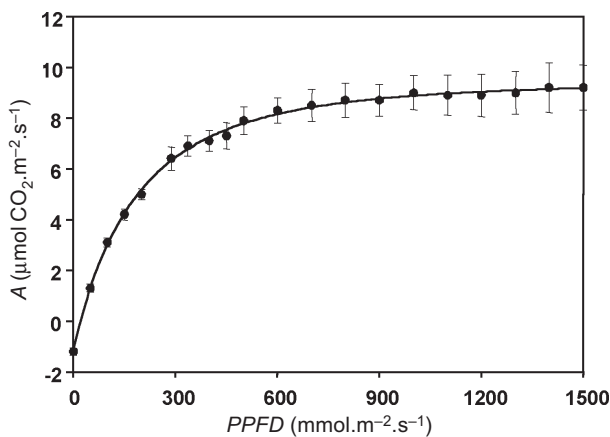
The lemon response of CO_2 assimilation rate (*A*) to *PPFD* (the A/Q curve, as stated by Long and Hällgren, 1993) was conducted on plants from the cabinet, using an artificial quartz halide light source (LI-6400-02 LED light source, LI-COR, Lincoln, NE USA) controlled with a quantum sensor located inside the leaf cuvette. The A/Q curve was obtained after one hour at the higher *PPFD* used. It was done diminishing the *PPFD* by 100 μmol.m^{-2}.s^{-1} after 5 min at each value of *PPFD*,

from 1,500 to 500 μmol.m^{-2}.s^{-1}, so as to avoid photoinhibition, and by 50 μmol.m^{-2}.s^{-1} from 500 to zero μmol.m^{-2}.s^{-1}, to obtain the curve inflexion. On the other hand, lemon and tobacco responses of A, g_s, C_i and Intrinsic Water Use Efficiency ($IWUE = A.g_s^{-1}$) were evaluated under a change of $PPFD$ from 50 to 350 μmol.m^{-2}.s^{-1}, after stabilizing under 50 μmol.m^{-2}.s^{-1} (data shown for the last 10 min). The $PPFD$ was lowered again to 50 μmol.m^{-2}.s^{-1} after 60 min under 350 μmol.m^{-2}.s^{-1}, as used by Barradas and Jones (1996) on *Phaseolus vulgaris*, an annual herbaceous plant. Finally, the cycling of A and g_s measurements in lemon were obtained after gas exchange parameters were stabilized (after at least 2 h) at 300 μmol.m^{-2}.s^{-1} (data showed for the last 20 min), and then increasing the $PPFD$ to 600 μmol.m^{-2}.s^{-1} during 130 min.

The measurements were all carried out with one leaf using three replicate plants in a completely randomized statistical design. Data were subjected to analysis of variance.

RESULTS AND DISCUSSION

Typical of a C$_3$ plant, the photosynthetic light response curve (A/Q curve) of *C. limon* (figure 1) produced a saturation of A at 600 to 800 μmol.m^{-2}.s^{-1}, which represents 25-30% of full sunlight (Long and Hällgren, 1993). Consequently, high light intensities over 600 to 800 μmol.m^{-2}.s^{-1} may cause photoinhibition reducing A (Bolhàr-Nordenkampf and Öquist, 1993), and therefore, for environmental-controlled assays with *Citrus*, the ideal light intensities for maximal A are in this range (Bernacchi et al., 2001). An increase of 20% of the integrated daily A was obtained with nursery trees of *Citrus sinensis*, by using a reflective net over the greenhouse during midday to avoid photoinhibition by high $PPFD$ (Medina et al., 2002).

Figure 1. Response of CO$_2$ assimilation rate (A) to intercepted photosynthetic photon flux density ($PPFD$) in *Citrus limon*: the A/Q curve.

The response of A, g$_s$ and C_i of lemon to changing $PPFD$ from 50 to 350 and returning to 50 μmol.m^{-2}.s^{-1} (figure 2), produced values in the same range as those for bean (Barradas and Jones, 1996). After 15 min of changing $PPFD$ from 50 to 350 μmol.m^{-2}.s^{-1}, A stabilized around 10.0 μmol CO$_2$.m^{-2}.s^{-1} (figure 2A). On the other hand, the *Citrus* g$_s$ values increased constantly when changing the $PPFD$ to 350 μmol.m^{-2}.s^{-1} reaching a value of 0.17 mol.m^{-2}.s^{-1} at 60 min (figure 2B). However, when returning the $PPFD$ from 350 to 50 μmol.m^{-2}.s^{-1}, A decreased instantly to 2.5 μmol CO$_2$.m^{-2}.s^{-1} and stabilized
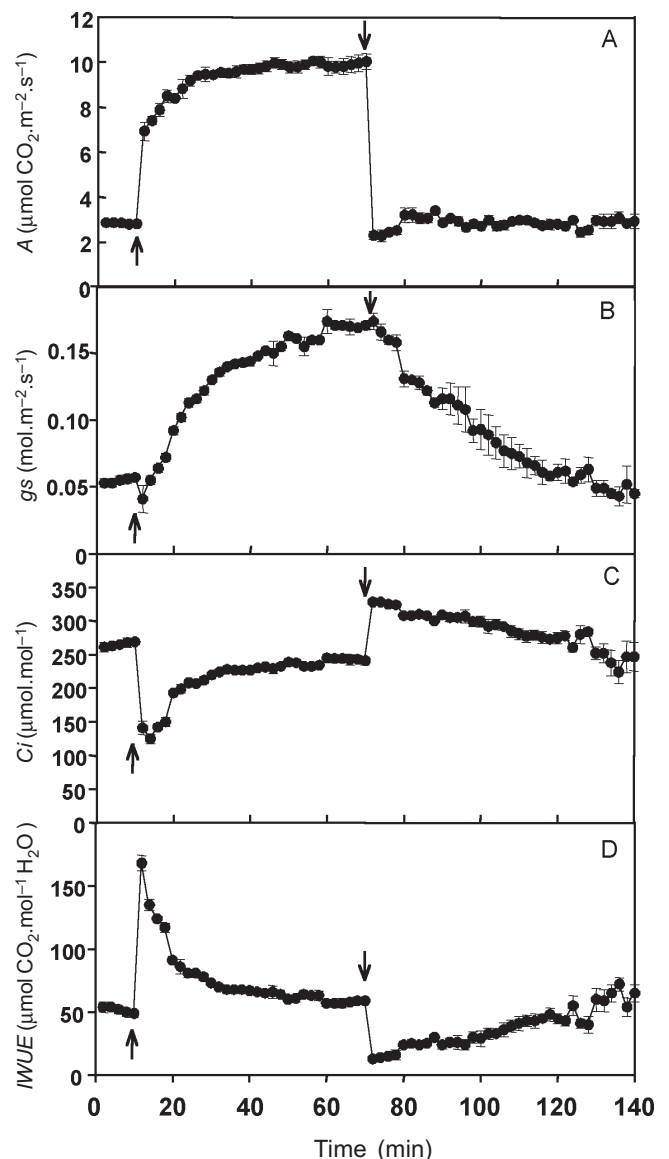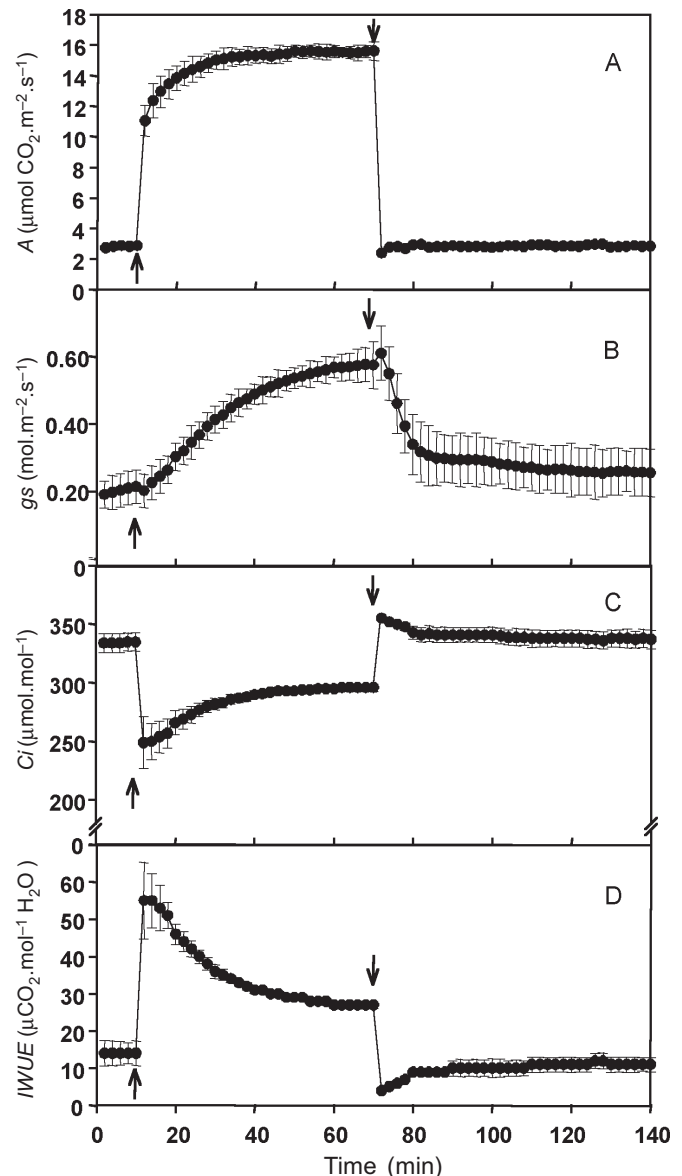

Figure 2. Response of CO$_2$ assimilation rate - A (A), stomatal conductance - g$_s$ (B), intercellular CO$_2$ partial pressure – C_i (C), and intrinsic water use efficiency – $IWUE$ (D) to a change in the photosynthetic photon flux from 50 to 350 μmol.m^{-2}.s^{-1} and returning to 50 μmol.m^{-2}.s^{-1} after 60 min, in *Citrus limon*. Arrows indicate changes in $PPFD$.

at this value. However, g_s diminished slowly, from 0.17 to 0.054 mol.m^{-2}.s^{-1}, tending to stabilize only after 60 min. These results are in agreement with those obtained with long-lived leaves (Zipperlen and Press, 1997), but contrast data for bean, an herbaceous plant, where a more rapid decrease of g_s was recorded when returning to 50 µmol.m^{-2}.s^{-1} (Barradas and Jones, 1996). The C_i values for lemon increased as for A when changing to a higher *PPFD* (figure 2C), but when returning to a low *PPFD*, the C_i values diminished more slowly than A. In addition, the lemon C_i values, after stabilization at low or higher *PPFD*, were low, around 250 µmol.mol^{-1}, in agreement with Lloyd et al. (1992). On the other hand, the *IWUE* shows a step increase when changing to a higher *PPFD* (figure 2D) because the increase of A is greater than that of g_s. After 60 min under high light, it diminished to values close to those under low light due to the slower increase of g_s compared to A. When returning to low light, the *IWUE* diminished rapidly but returned to initial values after the slow decrease of g_s.

Tobacco, with higher values of A, g_s and C_i than lemon, produced the same response patterns to *PPFD* as shown for bean (Barradas and Jones, 1996). After 15-20 min under 350 µmol.m^{-2}.s^{-1}, A was stabilized around 15 µmol CO$_2$.m^{-2}.s^{-1} (figure 3A), but g_s continued to increase reaching a value of 0.576 mol.m^{-2}.s^{-1} at 60 min (figure 3B). When returning to 50 µmol.m^{-2}.s^{-1}, A diminished to 2.8 µmol CO$_2$.m^{-2}.s^{-1} immediately and g_s took only 15 min to decrease to 0.307 mol.m^{-2}.s^{-1}, thereafter decreasing slowly to 0.255 mol.m^{-2}.s^{-1} over 70 min at 50 µmol.m^{-2}.s^{-1}. On the other hand, the C_i values for tobacco (figure 3C) were between 250 to 350 µmol.mol^{-1} after stabilizing at low or high *PPFD*, and on return to low *PPFD*, stabilized as for g_s. As seen for lemon, the *IWUE* produced a step increase under high light (figure 3D), and then diminished gradually during the 60 min under this condition, but it stabilized at a value greater than those under low light. On return to low light, it decreased suddenly but after a while, it returned to the same initial values. On the other hand, values of A, g_s and C_i for tobacco were much higher than for lemon, whereas *IWUE* was much lower than for lemon.

Thus, for tobacco, as for bean (Barradas and Jones, 1996), the changes of A in response to either increasing or decreasing *PPFD* were significantly faster than changes in g_s. However, this is not the case for a woody plant such as lemon, at least when changing the *PPFD* from a high to a low value showing that the changes in g_s of lemon are slower and the transpiration rates higher than for herbaceous plants. The slower reduction

of g_s in *Citrus*, compared to tobacco, when reducing the *PPFD* is probably due to over sensitivity of the stomatal response to light for woody species (Zipperlen and Press, 1997), which did not occur in tobacco or bean, both herbaceous plants. In *Citrus*, as for other woody species, the CO$_2$ mesophyll conductance is considerably lower (1.1 to 2.2 µmol CO$_2$.m^{-2}.s^{-1}.Pa^{-1}) than those reported for unstressed herbaceous plants (Lloyd et al., 1992). However, the low mesophyll conductance of *Citrus* can be due to the lower C_i values, which is due to the lower g_s values
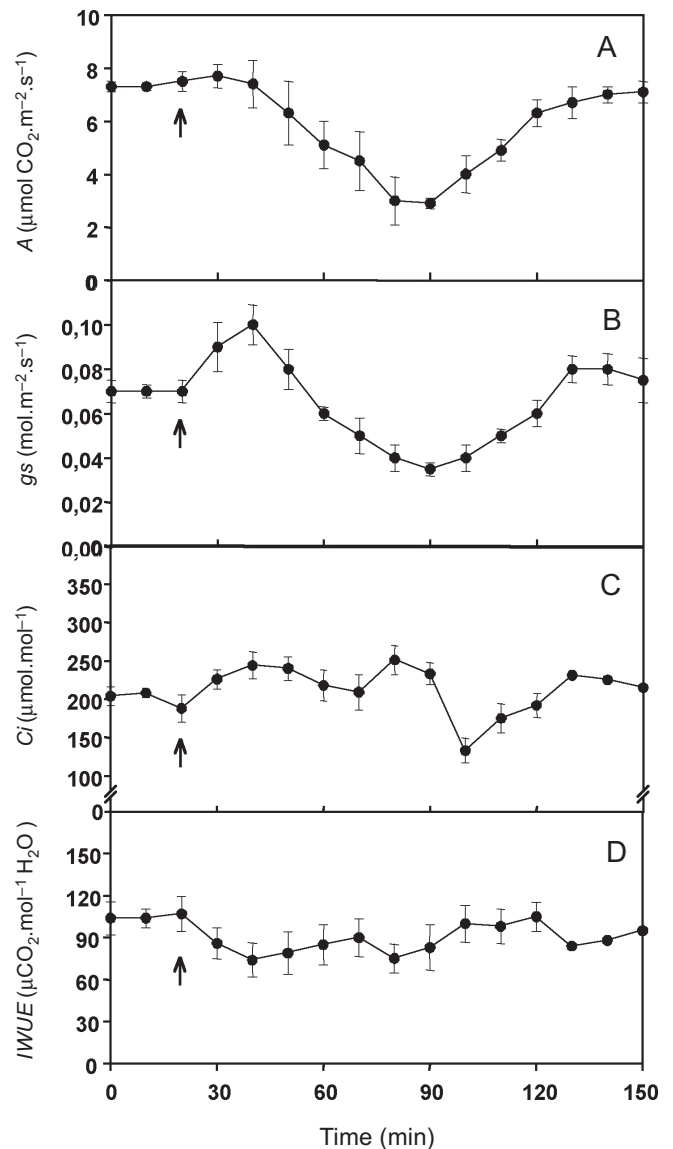

Figure 3. Response of CO$_2$ assimilation rate - A (A), stomatal conductance - g_s (B), intercellular CO$_2$ partial pressure - C_i (C), and intrinsic water use efficiency – *IWUE* (D) to a change in the photosynthetic photon flux from 50 to 350 µmol.m^{-2}.s^{-1} and returning to 50 µmol.m^{-2}.s^{-1} after 60 min, in *Nicotiana tabacum*. Arrows indicate changes in *PPFD*.

of lemon, because the C_i values are proportional to g_s values (Long and Hällgren, 1993). Thus, for *C. limon* g_s increases can affect *A* probably because of the low mesophillic conductance and the need of very high P_{CO2} for saturation of *A* (Lloyd et al., 1992). As a result, the response of *A* and g_s, and consequently of *IWUE*, to changing *PPFD* from 50 to 350 and back to 50 µmol.m^{-2}.s^{-1} in *Citrus* (figure 2), a perennial woody species, was quite different from tobacco or bean (Barradas and Jones, 1996). The *A* and g_s values for lemon varied from 3 to 10 µmol CO_2.m^{-2}.s^{-1} and from 0.04 to 0.17 mol.m^{-2}.s^{-1}, respectively (figures 2A and 2B), whereas for tobacco, these values varied from 2.8 to 15 µmol CO_2 m^{-2}.s^{-1} and from 0.19 to 0.61 mol.m^{-2}.s^{-1} respectively (figures 3A and 3B). By contrast, the *IWUE* values for lemon varied from 15 to 170 µmol CO_2.mol^{-1}H$_2$O (figure 2D), while for tobacco, these values varied from 5 to 55 µmol CO_2.mol^{-1}H$_2$O (figure 3D). Therefore, in woody perennial species such as *Citrus*, with much lower g_s values than annual herbaceous species such as tobacco, the g_s control of *A* will be more important (Lloyd et al.,1992), especially when reducing the *PPFD*, improving their *IWUE*. Also, the *IWUE* of lemon was always higher than for tobacco, and under high light it stabilizes at values close to those under low light, but for tobacco, the *IWUE* values under high light are greater than that under low light.

Increasing the light intensity to 600 µmol.m^{-2}.s^{-1}, for plants growing at 300 µmol.m^{-2}.s^{-1}, induce an oscillatory stomatal behavior in lemon (figure 4). The g_s values increased from 0.07 to a maximal g_s value of 0.1 mol.m^{-2}.s^{-1} in 10 min at 600 µmol.m^{-2}.s^{-1}, while *A* did not change and was maintained around 7.5 µmol CO_2.m^{-2}.s^{-1}. This response is in agreement with data obtained for long-lived leaves, where a faster increase for g_s than for *A* was found, which takes 11-36 min to reach 90% of full photosynthetic induction with increasing light intensity. This contrasts the 1-3 min for the species with short-lived leaves. In the former, g_s increased at a much greater rate than photosynthesis due to a low mesophyll conductance and thus a slower full photosynthetic induction (Zipperlen and Press, 1997). However, a high g_s value means a high transpiration rate, and if there is no parallel increase in *A* (figure 4A), it tends to diminish the *IWUE* (figure 4D). Afterwards, g_s decreased (figure 4B), due to excessive transpiration, and consequently *A* will also decrease, and the minimal *A* and g_s values were 3.0 µmol CO_2.m^{-2}.s^{-1} and 0.04 mol.m^{-2}.s^{-1}, respectively, after 60 min of saturating light intensity. The rapid initial increases in g_s at 600 µmol CO_2.m^{-2}.s^{-1}, without changes in *A*, lowers the *IWUE* because g_s increased (figure

4D). Then, there is an induction of stomatal closure and increase in *IWUE*, probably due to the increased amount of ABA carried by the increased transpiration stream (Hartung et al., 2002). Thus, the reduction in g_s, and consequently in transpiration rate, will improve the *IWUE* and prevent wilting. However, *A* became too low and g_s began to increase again because stomatal movement favours maximum possible *A* values with lower transpiration rates (Kramer and Boyer, 1995). Then, *A* and g_s stabilized at values of 7.1 µmol CO_2.m^{-2}.s^{-1} and 0.07 mol.m^{-2}.s^{-1}, respectively, and the total oscillatory cycle in *C. limon* took two and half hours.

Figure 4. Cyclic oscillation of the CO_2 assimilation rate - *A* (A), stomatal conductance - g_s (B), intercellular CO_2 partial pressure - C_i (C), and intrinsic water use efficiency – *IWUE* (D) in *Citrus limon*, after changing the photosynthetic photon flux from 300 to 600 µmol.m^{-2}.s^{-1}. Arrows indicate changes in *PPFD*.

The temporal dynamics of the circadian control of stomatal movements indicate that it may benefit the plant by increasing *IWUE* (Kramer and Boyer, 1995). The extra carbon obtained during peaks can outweigh the reduced carbon gain during troughs, resulting in greater net carbon assimilation with a lower loss of water (Webb, 1998) than would be obtained during a steady rise to a maximum, where transpiration would increase without an increase in *A* (Zipperlen and Press, 1997). The patterns of stomatal oscillations, obtained with potted *Citrus* trees in these assays, are in agreement with the observations in field-grown trees of *C. sinensis* (Levy and Kaufmann, 1976). This oscillation can occur in water-stressed plants and is initiated by a sudden shock such as water shortage in the soil, a short period of darkness, cooling of the soil, or changes in humidity and temperature (Kramer and Boyer, 1995).

Therefore, the results presented here for different g_s and C_i values and behavior when changing the *PPFD*, in the woody species *Citrus* as compared to the herbaceous species tobacco, highlight the necessity of great care when carrying out gas exchange measurements, especially with woody species. To avoid confusing cyclical plant response with random noise in measurements of an average gas exchange rate of a group of plants, especially when growing in pots, frequently determined by taking just one measurement per plant, repetitive measurements at 10 min intervals can determine whether transpiration is stable or oscillating when environmental conditions are varying (Rose and Rose, 1994). Also, simultaneous measurements of gas exchange with light adapted fluorescence analysis (the measurement of the efficiency of Photosystem II photochemistry - Φ_{PSII}) can also be used because fluorescence can vary faster than gas exchange, from static to oscillating, indicating stomatal oscillation and or patchy stomatal closure, or some environmental stress (Cardon et al., 1994).

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