Stomatal limitation of photosynthesis in winter production of greenhouse tomato plants

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In May, greenhouse tomato (Lycopersicon esculentum Mill.) plants near the end of their winter production cycle were shown to exhibit a diurnal photosynthetic decrease. In order to identify the physiological causes of this decline, we compared in May the photosynthetic characteristics of the fifth youngest leaves from tomato plants of different ages corresponding to a winter production (11-month-old plants) and to a spring production (5-month-old plants). Although the leaves were developed simultaneously under the same environmental conditions, only the ones from the winter production showed a diurnal decline of the in situ CO2 assimilation rate (A CO2). This was accompanied by a decline of internal CO2 and stomatal conductance and by large accumulations of hexoses. When stomatal closure was relieved under saturated CO2 concentration (5%) using a leaf-disc electrode, it was shown to exhibit a diurnal photosynthetic decrease. In order to identify the physiological causes of this decline, we compared in May the photosynthetic characteristics of the fifth leaves of both tomato cultures had similar maximum quantum efficiency of O2 evolution (Fmax), light-saturated rate of O2 evolution (Pmax) and quantum efficiency of photosystem II (PSII) photochemistry (ΔF/Fm′, qP and qN). We concluded that the diurnal decline of A CO2 observed in winter tomato production during May originates from a stomatal limitation that is not dependent on environmental conditions but rather related to the developmental stage of the plants.

Introduction

An application of the well-documented effects of elevated atmospheric CO2 concentration on plant growth (Kramer 1981, Kimball 1983, Cure and Acock 1986, Curtis and Wang 1998) is the common use of CO2 enrichment in commercial greenhouses in order to improve crop productivity (Yelle et al. 1987, 1990, Dugal et al. 1990). The positive effects of elevated CO2 are directly related to an enhancement of photosynthesis owing to increased rates of carboxylation and decreased rates of oxygenation of ribulose-1,5-bisphosphate caboxylase/oxygenase (Rubisco) in C3 plants (Stitt 1991, Bowes 1996, Drake et al. 1997). However, the long-term response of photosynthesis to elevated CO2 is variable and dependent on the source-sink equilibrium of the whole plant, i.e. the balance between the production of carbohydrates in source leaves, their loading into and unloading from the phloem, and their utilization or storage in sink organs (Farrar and Williams 1991). In conditions where the rate of photosynthetic CO2 assimilation exceeds the capacity of sink organs, there is an accumulation of high levels of carbohydrates in leaves which triggers a down-regulation of photosynthesis involving a repression of several photosynthetic genes, namely Rubisco and thylakoid proteins (Yelle et al. 1989, Stitt 1991, van Oosten and Besford 1994, 1995, Stitt and Krapp 1999). Plant responses to elevated CO2 are strongly influenced by environmental factors. For instance, sufficient supply of

Abbreviations – A CO2, CO2 assimilation rate; β-Car, β-carotene; Ci, internal CO2 concentration; Cc, stomatal conductance; ΔF/Fm′, operational quantum yield of PSII electron transport; DW, dry weight; Fm′, minimal fluorescence with all reaction centres open after dark adaptation; Fm, minimal fluorescence with all reaction centres open after light adaptation; Fm′, maximal fluorescence with all PSII reaction centres closed after dark adaptation; Fm′, maximal fluorescence with all PSII reaction centres open after light adaptation; Fm′, fluorescence at steady-state; Fm′, maximum quantum yield of O2 evolution; ΦO2, quantum yield of O2 evolution; FW, fresh weight; LA, leaf area; Pm′, maximum photosynthetic rate; qN, non-photochemical quenching coefficient; qP, photochemical quenching coefficient; SLW, specific leaf weight; STP, spring tomato production; V + A + Z, the pool size of xanthophyll cycle components, violaxanthin, antheraxanthin and zeaxanthin; WTP, winter tomato production.

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nutrients, especially nitrogen (Stitt 1991, Sage 1994, Stitt and Krapp 1999), is required for the formation of new sink organs in order to maintain the source-sink equilibrium and thereby sustain stimulation of photosynthesis and plant growth under elevated CO₂ levels. Growth light intensities can also determine the extent of photosynthesis and growth stimulation by high CO₂: the stimulating effects of high CO₂ tend to decrease (Curtis and Wang 1998) and may even become negative (Dorais et al. 1996, Stutte et al. 1996, Louche-Teissandier et al. 1999) as growth light intensities increase.

The importance of the environmental factors mentioned above on crop response to elevated CO₂ have been rarely considered in greenhouse conditions. In northern latitudes, the period of production for several greenhouse crops, such as the so-called ‘winter tomato production’ (WTP), usually extends from early fall to late spring and is characterized by large variations of natural photosynthetic photon flux (PPF). These variations of PPF can therefore modulate plant responses to high CO₂ level. In a recent study on the diurnal and seasonal variations of photosynthetic activities of a WTP under those latitudes (Ayari et al. 2000), it was shown that from November to February, photosynthetic rates followed the variations of incident PPF and then became saturated in March when PPF at the canopy level reached 900 µmol m⁻² s⁻¹. In May, the photosynthetic rates in the fifth and the tenth tomato leaves were maximal early in the morning and then markedly declined during the day even though the incident PPF was still rising. These changes in photosynthetic activities were accompanied by large accumulations of carbohydrates in leaves, especially hexoses and starch. Taken together, these observations suggested that the combination of high CO₂ and high PPF in spring leads to a down-regulation of photosynthesis in greenhouse tomato plants. An alternative explanation would be that at some advanced developmental stage, the tomato plants become more susceptible to stomatal limitation owing to their long stems (12 m) or to a less efficient root system.

The objective of this study was therefore to determine whether the decline of photosynthetic activity observed in winter tomato plants results from a down-regulation of photosynthesis or from a stomatal limitation. To this end, we compared in May the photosynthetic characteristics of the fifth tomato leaves developed under the same environmental conditions but produced by plants of different developmental stages (5- and 11-month-old) and thus of different stem length (5 and 12 m long). Although the leaves from both cultures had similar maximum photosynthetic capacities and pigment contents, only the leaves from the older culture showed a diurnal photosynthetic decline related to a stomatal limitation.

Materials and methods

Plant material

Four-week-old plants of two greenhouse tomato productions were transplanted, respectively, on July 15, 1996 and on December 18, 1996 onto 13.5 l rockwool slabs (7.5 × 20 × 90 cm, Grodan, Denmark), each one receiving 3 tomato plants, in the same glasshouse at a density of 2.8 plants m⁻². Both cultures received complete nutrient solution (electrical conductivity = 2.0–2.8 mS cm⁻¹, pH 5.5–6.5) from a drip irrigation system. Supplemental lighting of 100 µmol m⁻² s⁻¹ provided by high pressure sodium lamps (HPS, 780/N 400, P. L., Lighting Systems Canada Inc., Ont., Canada) was used until the end of March. The period of day light extension was set at 14 h in mid-September and progressively increased (+ 1 min day⁻¹) to maximum (17 h) in December and January and then progressively decreased (−1 min day⁻¹) back to 14 h at the end of April. CO₂ enrichment at 800 ± 200 µmol mol⁻¹ (depending on ventilation) was provided during the photoperiod and CO₂ concentration was controlled by an infrared gas analyser (Priva computer).

By May 1997, the two plantings were 5- and 11-month-old with stems of 5 and 12 m long, respectively. In commercial greenhouse production, the dates of planting and subsequent production periods of the 5- and 11-month-old cultures correspond, respectively, to the so-called spring tomato production (STP) and WTP. For convenience, in the present study we used STP and WTP to designate these cultures.

In situ gas exchange measurements

Measurements of CO₂ assimilation rate (A₄CO₂), stomatal conductance (Cs), and internal CO₂ concentration (Ci) were made on the fifth attached leaves from the apex of 5 different plants from each tomato production, using a portable photosynthesis system (LI-6200, Li-Cor, Inc., Lincoln, NE, USA). Measurements were made 3 times a day, the first one at the beginning of the photoperiod (6:00 h), the second at midday (12:00 h) and the third measurement, 1 h before the end of natural diurnal period (18:00 h).

Specific leaf weight and leaf carbohydrate contents

The fifth leaves of 9 plants of each tomato production were removed and their leaf area (LA) was measured using a portable area meter (Li-3000, Li-Cor, Inc., Lincoln, NE, USA). Leaves were dried individually at 65°C for 2 days and weighed for dry weight determination. Specific leaf weight (SLW) was calculated as the dry weight/LA ratio.

For carbohydrate analysis, tomato leaves used for in situ gas exchange measurements were collected, frozen in liquid nitrogen and then ground in powder. Carbohydrate extraction was done according to Ozbun et al. (1973). Sucrose, glucose and fructose were quantified using HPLC. The insoluble pellet was resuspended in 0.02 M NaOH and boiled for 10 min at 100°C then amyloglucosidase (EC 3.2.1.3) was added. Samples were incubated in a water bath at 55°C overnight. Glucose content in starch was determined using YSI autoanalyser Model 7200 (Yellow Springs Co., Inc., OH, USA).

Measurements of O₂ evolution rates

Light saturation curves of O₂ evolution were measured 3 times each on the fifth leaves of each tomato planting using...
an LD2 leaf-disc electrode system (Hansatech Instruments Ltd., King’s Lynn, Norfolk, UK) under saturated CO₂ concentration (5%) according to Walker (1989). After a dark-adaptation of about 20 min to measure the rates of dark O₂ uptake and chlorophyll a (Chl a) fluorescence (see below), the leaf discs were adapted for 10 min to 250 μmol m⁻² s⁻¹ to completely induce photosynthesis as seen by a constant rate of O₂ evolution measured in real-time. Then, the O₂ evolution rates were measured after 5 min of illumination at different PPFDs increasing from 20 to 1250 μmol m⁻² s⁻¹. The maximum fluorescence level (Fₘ) was determined at saturating light intensity (Walker 1989).

Chl a fluorescence measurements

Chl a fluorescence was measured on leaf discs enclosed in the LD2 leaf-disc electrode system simultaneously with O₂ evolution measurements using a Pulse Amplitude Modulation (PAM 101/103) fluorometer (Walz, Effeltrich, Germany) connected to a computer with a data acquisition system (Q-data, Turku, Finland). The minimal fluorescence level (F₀) was measured with a weak non-actinic modulated light applied on a 20 min dark-adapted leaf disc, in which all the photosystem II (PSII) reaction centres were open, i.e. all the primary quinone electron acceptor QA was completely oxidized. The maximal fluorescence level (Fₘ) with all PSII reaction centres closed was determined by applying a 1 s saturating flash provided by a KL 1500 Schott light source (Scott, Mainz, Germany) to close all the PSII reaction centres. The steady-state (Fₛ) and the maximal (Fₘ) fluorescence levels were measured in a similar way to F₀ and Fₘ at the end of the 5 min of illumination periods used for O₂ evolution measurements. Immediately after the saturating flash, the actinic light was removed and the minimal fluorescence level (Fₐ) was measured in the presence of a far red light using a RG715 long-pass filter. Using both dark and steady-state fluorescence parameters, the photochemical quenching coefficient was calculated as qₚ = (Fₘ − Fₛ)/(Fₘ − Fₐ) and the non-photochemical quenching coefficient as qₚ = (Fₘ − Fₛ)/(Fₘ − Fₐ) (Schreiber et al. 1995). The operational quantum yield of PSII electron transport ΔF/Fₘ was calculated as (Fₘ − Fₛ)/Fₘ (Genty et al. 1989).

Pigment analysis

Nine fifth leaves of each tomato planting were collected and frozen in liquid nitrogen. Samples were kept in the dark and at -80°C for pigment analysis. Pigment extraction was made under dim green light. Four extractions of leaf pigments were made with cold methanol followed by centrifugation at 10000 g for 5 min. Chl a, Chl b, β-carotene (β-Car), xanthophyll cycle components (violaxanthin + antheraxanthin + zeaxanthin, V + A + Z) were determined using reversible phase HPLC (Gilmore and Yamamoto 1991, Adams and Demmig-Adams 1992). The solvent system for isocratic separation was acetonitrile-methanol-Tris HCl buffer, 0.1 M, pH 8.0 (72:8:3) (solvent A) for 6 min followed by a 10 min linear gradient to 100% solvent composed of methanol-ethyl acetate (68:32) (solvent B). The column was re-equilibrated between samples for 10 min with solvent A.

Statistical analysis

Analysis of variance (ANOVA) was conducted using the SAS statistical program 6.12 (SAS Institute Inc., NC, USA). To satisfy the assumptions of the ANOVA model, the homogeneity of the variances was verified using Bartlett’s test. To identify differences on dependent parameters between tomato plantings, the LSD test was used at P = 0.05.

Results

Daily changes in in situ gas exchange measurements

In the fifth leaves of WTP, A₂CO₂ declined during a sunny day in May (Fig. 1A) as previously reported in Ayari et al. (2000). Here, we observed that this decrease of A₂CO₂ was accompanied by a noticeable decline of stomatal conductance Cs (Fig. 1B) resulting in a diurnal decline of internal CO₂ concentration Ci (Fig. 1C). In contrast to WTP, the A₂CO₂ (Fig. 1A) and Cs (Fig. 1C) of the fifth leaves of STP slightly increased during the day and Cₛ (Fig. 1B) decreased to a smaller extent than observed in WTP fifth leaves. However, the total daily photosynthesis appeared similar in both sets of tomato plants.

Leaf carbohydrate content and specific leaf weight

Higher glucose (Fig. 2A) and fructose (Fig. 2B) contents were observed in the fifth leaves of WTP compared to STP leaves. In the fifth leaves of WTP, the glucose concentration increased in the morning and remained constant thereafter, whereas the fructose leaf content increased throughout the day. In contrast to WTP, the STP fifth leaves had a constant hexose content (Fig. 2A,B). In addition, it is noteworthy that the fifth leaves of WTP had a higher initial hexose content (at 6:00 h) than in STP. Sucrose and starch contents in fifth leaves of WTP were slightly higher than in fifth leaves of STP (Fig. 2C,D). In the fifth leaves of WTP, the increase of sucrose (Fig. 2C) occurred in the morning and the starch increase (Fig. 2D) was observed in the afternoon. These increases of leaf carbohydrates in the fifth leaves of WTP contributed to their significantly higher SLW than those of STP (Table 1).

Light response curves of CO₂-supported O₂ evolution

Light response curves of photosynthesis under saturated CO₂ conditions were measured for the fifth leaves for each tomato culture (Fig. 3). The initial slope of the light saturation curves which estimates the apparent maximum quantum yield of O₂ evolution was similar in leaves of both tomato cultures (Φₚₘₐₓ = 0.0742–0.0784). Contrary to A₂CO₂, the maximum photosynthetic capacities (Pₘₐₓ) measured...
Changes in Chl a fluorescence characteristics

The decline with increasing PPF of the effective quantum yield of PSII electron transport expressed by $\Delta F/F_m$ (Genty et al. 1989) was similar in the fifth leaves of both tomato plantings (Fig. 4A). Complementary information on photochemical efficiency of PSII was provided by Chl a fluorescence quenching coefficients. Consistent with $\Delta F/F_m$ measurements, the decline of the photochemical quenching coefficient ($q_p$) as PPF increased was similar for both tomato cultures (Fig. 4B). The non-photochemical quenching coefficient ($q_N$) increased in the fifth leaves of WTP compared to STP leaves to a slightly lesser extent as PPF increased.

Pigments and xanthophyll pool

There are no significant differences in Chl $a + b$ concentrations, $\beta$-Car, lutein (Lut) and xanthophyll pool sizes between the fifth leaves of WTP and STP (Table 2). However, the Chl $a/b$ ratio was significantly higher in fifth leaves of WTP. Although not statistically different, the xanthophyll pool size of the fifth leaves of WTP was slightly higher than in STP leaves.

Discussion

Our results on in situ photosynthetic activity showed a marked diurnal decrease of $A_{CO_2}$ in the fifth leaves of WTP whereas the $A_{CO_2}$ of STP remained constant during the day (Fig. 1). Similar diurnal decrease of $A_{CO_2}$ was previously observed in the fifth and tenth leaves of greenhouse winter tomato plants during May (Ayari et al. 2000). Here, we demonstrated that this $A_{CO_2}$ decrease was related to a reduction of $C_s$ accompanied by a decrease in $C_i$, thereby suggesting a stomatal limitation of photosynthesis in WTP (Cornic and Briantais 1991, Brestic et al. 1995).

At this point, the cause of stomatal limitation remains unclear. A clue that may lead to its origin could be the large diurnal accumulation of hexoses in WTP leaves although we cannot yet determine if there is a cause and effect relationship between stomatal closure and hexose accumulation. Diurnal accumulation of hexoses is typical of tomato leaves (Galtier et al. 1995) and reflects the increasing activity of foliar invertase during the day (Kingston-Smith et al. 1998). However, the much larger hexose accumulation in WTP leaves relative to STP leaves may suggest a decreased export of hexoses due to insufficient sink activities in WTP plants or an altered metabolism with different relative rates of sucrose and starch turnovers. Changes of cellular and apoplastic concentrations of different ions ($K^+$ or $Pi$) or metabolites (malate, sucrose) may affect directly stomata opening or alter their sensitivity to abscisic acid (Correia et al. 1999, Ewert et al. 2000). Therefore, the diurnal rise of hexose concentration may be a symptom of an altered metabolism that would induce stomatal limitation of photosynthesis.

On the other hand, hexose accumulation related to stomatal closure was already observed in leaves subjected to water stress (Zrenner and Stitt 1991, Kameli and Lösel 1996, Foyer et al. 1998, Thomas and James 1999) during which growth is generally inhibited prior to the decrease of $CO_2$ assimilation (Thomas and Evans 1989, Busso et al. 1990, Chaves 1991). The large hexose accumulation in WTP leaves may therefore be a consequence of a mild water deficit rather than the cause of stomatal limitation. Indeed, water transport may be more restricted in the 12 m long stem of WTP plants than in STP plants which have a much shorter stem (5 m). Alternatively, an older and less efficient root system to absorb water can induce a stomatal closure in WTP leaves even if roots are provided with ample water.
Finally, stomata may close in response to hormonal signal (abscissic acid) from roots that are subjected to stress other than water deficiency (Davies et al. 1986, Zhang and Davies 1990). In a next experiment, it will be important to measure leaf water potential in leaves of both cultures in order to identify the origin of the observed stomatal limitation.

The diurnal increase of hexoses observed in the fifth leaves of WTP led to their long-term accumulation as seen by the higher initial hexose content at the beginning of the day (at 6:00 h) in WTP compared to STP leaves (Fig. 2). Since $P_{\text{max}}$ was similar or even slightly higher in WTP than in STP leaves when measured in the presence of high CO$_2$ concentrations (1–5%) that relieves any stomatal limitation (Fig. 3), this long term accumulation did not induce an acclimation of photosynthesis occasionally observed in plants growing under elevated CO$_2$ (700–800 ppm) that involves a repressed expression of several photosynthetic

![Fig. 2. Diurnal variation of glucose (A), fructose (B), sucrose (C) and starch (D) content on the fifth leaves of WTP (closed squares) and STP (open squares). Samples were taken 3 times a day at 6 h intervals. Data are the mean of 5 independent replicates ± sd.](image)

![Fig. 3. Light saturation curves of O$_2$ evolution under saturated CO$_2$ concentration (5%) measured on the fifth leaves of WTP (closed squares) and STP (open squares). Data are the mean of 3 independent replicates ± sd.](image)

**Table 1.** Vegetative characteristics of the fifth leaves of winter and spring tomato productions. Values are the mean of 9 observations ± sd. Within each column, means followed by the same superscript are not significantly different at $P = 0.05$ according to an LSD test.

<table>
<thead>
<tr>
<th>Tomato production</th>
<th>LA (cm$^2$)</th>
<th>DW (g)</th>
<th>SLW (mg cm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTP</td>
<td>$271 \pm 86^a$</td>
<td>$1.56 \pm 0.45^a$</td>
<td>$5.8 \pm 0.9^a$</td>
</tr>
<tr>
<td>STP</td>
<td>$277 \pm 57^a$</td>
<td>$1.26 \pm 0.52^a$</td>
<td>$4.5 \pm 1.4^b$</td>
</tr>
</tbody>
</table>
Fig. 4. Variations of the operational quantum yield of PSII electron transport (ΔF/Fm%; A), photochemical (qP; B, squares) and non-photochemical (qN; B, circles) quenching coefficients in response to PPF levels on the fifth leaves of WTP (closed symbols) and STP (open symbols). Measurements were taken simultaneously with the light saturation curves of O2 evolution. Data are the mean of 3 independent replicates ± SD.

Table 2. Pigment concentrations of the fifth leaves of winter and spring tomato productions. Values are means of 9 observations ± SD. Within each column, means followed by the same superscript are not significantly different at P = 0.05 according to an LSD test.

<table>
<thead>
<tr>
<th>Tomato production</th>
<th>Chl a+b (mg [g FW]⁻¹)</th>
<th>Chl a/b</th>
<th>β-Car (mg [g FW]⁻¹)</th>
<th>Lut (mg [g FW]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTP</td>
<td>2.4 ± 0.2ᵃ</td>
<td>3.8 ± 0.2ᵃ</td>
<td>0.10 ± 0.03ᵃ</td>
<td>0.13 ± 0.01ᵃ</td>
</tr>
<tr>
<td>STP</td>
<td>2.5 ± 0.2ᵃ</td>
<td>3.6 ± 0.2ᵇ</td>
<td>0.12 ± 0.02ᵃ</td>
<td>0.12 ± 0.01ᵃ</td>
</tr>
</tbody>
</table>

and non-photosynthetic genes, namely Rubisco (Yelle et al. 1989, Stitt 1991, van Oosten and Besford 1994, Stitt and Krapp 1999). This is supported by the Chl a fluorescence quenching analysis made simultaneously with measurements of O2 evolution, which indicated that both tomato cultures had an identical effective quantum yield of PSII electron transport ΔF/Fm and the fluorescence quenching coefficient qP (Fig. 4).

No significant differences were detected between the pigment contents (total chlorophyll, β-Car, Lut and V + A + Z) of WTP and STP (Table 2). However, it is noteworthy that the Chl a/b ratio of the fifth leaf was significantly higher in WTP than in STP. This may suggest a different composition of the photosynthetic apparatus in WTP characterized by more PSII units with smaller light-harvesting antennae relative to PSI (Demmig-Adams 1990, Anderson et al. 1993).

In conclusion, we demonstrated that the diurnal decline of ACO2 observed in WTP during May (Ayari et al. 2000) originates from a stomatal limitation that is not dependent on environmental conditions but rather related to the developmental stage of the plants.

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References


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