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The influence of chilling on photosynthesis and activities of some enzymes of sucrose metabolism in *Lycopersicon esculentum* Mill.

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Abstract

The effects of chilling stress on leaf photosynthesis and sucrose metabolism were investigated in tomato plants (*Lycopersicon esculentum* Mill. cultivar Marmande). Twenty-one-day-old seedlings were grown in a growth chamber at 25/23 °C (day/night) (control) and at 10/8 °C (day/night) (chilled) for 7 days. The most evident effect of chilling was the marked reduction of plant growth and of CO_2 assimilation as measured after 7 days, the latter being associated with a decrease in stomatal closure and an increase in Ci. The inhibition in photosynthetic rate was also related to an impairment of photochemistry of photosystem II (PSII), as seen from the slight, but significant change in the ratio of F_v/F_m. The capacity of chilled leaves to maintain higher q_P values with respect to the controls suggests that some protection mechanism prevented excess reduction of PSII acceptors. The results of the determination of starch and soluble sugar content could show that chilling impaired sucrose translocation. The activity of leaf invertase increased significantly in chilled plants, while that of other sucrose-metabolizing enzymes was not affected by growing temperature. Furthermore, the increase in invertase (neutral and acid) activity, which is typical of senescent tissue characterized by reduced growth, seems to confirm that tomato is a plant which is not a plant genetically adapted to low temperatures.

Introduction

Most tropical and subtropical plant species, including tomato (*Lycopersicon esculentum*), are sensitive to low, non-freezing temperatures (chilling) (Wang 1990, Keller and Steffen 1995).

Chilling results in a strong photoinhibition in tomato (Jung and Steffen 1997); moreover, the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and other enzymes of the Calvin cycle can be affected by chilling (Sassenrath and Ort, 1990). Also carbohydrate metabolism is influenced by chilling stress since respiration and growth-dependent sink carbon demand are minimized and source-to-sink photosynthate transport is reduced (Bruggemann *et al.* 1992). In several species chilling increases the leaf content of soluble sugars, such as sucrose and reducing sugars in tomato (Keller and Steffen 1995), or fructans in cereals (Calderon and Pontis 1985).

Both acid invertase and sucrose synthase have been implicated as playing a major role in cleaving sucrose, which in turn may regulate the rate of carbon translocation and accumulation. In a recent study increased activity of enzymes of sucrose and

fructans metabolism was observed in wheat plants subjected to chilling (Santoianni *et al.* 1993).

The aim of this work was to study in tomato plants how a period of chilling stress (7 days) altered the photosynthetic process and the activities of some enzymes involved in sucrose metabolism.

Materials and Methods

Plant material

Seeds of tomato (*Lycopersicon esculentum* Mill cv. Marmande) were germinated and grown in peat-filled pots in a greenhouse at 25/23 °C (day/night environment) during the summer. After 21 days, half of these plants were transferred to a chamber at 10/8 °C (day/night environment) for seven days. Illumination in the chambers was 400 μmol $m^{-2}.s^{-1}$ with a photoperiod of 12 hours and was provided by using a mercury vapour lamps Osram HQI-TS 250 W/NDN (Wembley, England). Relative humidity was around 70 %. The control plants were placed in a growth chamber at 25/23 °C with the same light and humidity conditions as above.

Sampling for physiological and biochemical analyses was carried out one day after the end of the chilling treatment.

Gas exchange measurements

Plants were analysed for CO_2 uptake using an open gas exchange system (Heinz Walz, Effeltrich, Germany). Gas exchange measurements were performed using an assimilation chamber with controlled conditions (CO_2 concentration 350 ppm; O_2 concentration 21 %; temperature 25 °C and relative humidity ranging from 45-60 %). CO_2 and water vapour were analysed using an infrared gas analyser (Binos, Leybold Heraeus, Germany). Further details of the gas exchange measurements are reported by Guidi *et al.* (1997).

The gas exchanges were determined on three fully expanded leaves in chilled and control plants at 350 μmol $m^{-2}.s^{-1}$ irradiance. Values of CO_2 assimilation (A), stomatal conductance to water vapour (G_w), transpiration rate (E) and leaf internal CO_2 concentration (C_i) were measured using the data acquisition software DIAGAS 3.01 (Walz, Germany).

Chlorophyll fluorescence and chlorophyll content

Measurements at 25 °C of chlorophyll *a* fluorescence were performed on attached leaves using a Pulse Amplitude Modulation Fluorometer (PAM 2000, Heinz Walz, Germany; see Guidi *et al.* 1997 for details). The nomenclature of the fluorescence parameters used is according to van Kooten and Snel (1990). Photochemical and non-photochemical fluorescence quenching coefficients were calculated as described by Schreiber *et al.* (1994). The overall quantum yield of photochemical energy conversion (Φ_{PSII}) was determined as ($F_m' - F_t$) F_m' (Genty *et al.* 1989) while the efficiency of excitation capture by open PSII reaction centres ($\Phi_{exc.}$) was calculated as F_v'/F_m'.

The Chl content was determined from 1 cm diameter leaf discs extracted with N,N- dimethylformamide overnight at 4 °C (Moran 1982).

Osmolite contents

The concentration of osmolites that was centrifugally-extracted from frozen tissue was measured with a freezing-point osmometer.

Soluble sugar and starch determination

0.5 g of fresh tissues were sampled from four fully expanded leaves, then smashed, placed in pierced 1.5 ml Eppendorfs vials and filled with 1ml of distilled water. The vials were centrifuged at 14,000*g* for 5 min and percolated water from the pierced vials was recovered to measure the soluble sugar content using the method described by Guy *et al.* (1992) with minor modifications.

The solid phase in the Eppendorfs vials was washed 3 times to eliminate any residue of soluble sugar, then 0.25 g of each residual sample were incubated at 80 °C for 12 h in a solution containing: 2.5 ml of acetate buffer (50 mM pH4.5), 12 units of α-amylase (EC 3.2.11) and 42 units of amyloglucosidase (EC 3.2.1.3). At the end of the incubation time, temperature was switched to 120 °C for 15 min. Samples were cooled and then centrifuged at 6,000 *g* for 5 min. The supernatant was recovered

and 50 µl of each sample was placed in an Eppendorf vial with 1 ml of anthrone (20 mM in sulphuric acid 97 %) and 150 ml of distilled water. The vials were heated at 60 °C for 15 min, then absorbance (λ=620 nm) was read.

Samples for soluble sugar analysis were taken in the morning, while those for starch analysis were taken at dawn and in late afternoon.

Measurements of enzyme activity

Crude extract to measure activity of sucrose synthase (SS), sucrose phosphate synthase (SPS), acid invertase (AI) and neutral invertase (NI) was obtained as described by Huber *et al.* (1989). Samples were obtained from the fully expanded leaves. Sampling was carried out in the morning. SS ands SPS activities were assayed as described by Guy *et al.* (1992). Assays for AI and NI in crude extracts were done as previously described (Huber *et al.* 1989).

Results

The most evident symptom in chilled tomato plants was a strong reduction of plant growth (Fig. 1). Also the leaf area was strongly reduced. The specific leaf area (SLA) was 386 $cm^2 \cdot g^{-1}$ in the control and 200 $cm^2 \cdot g^{-1}$ in the chilled plants (P=0.05).

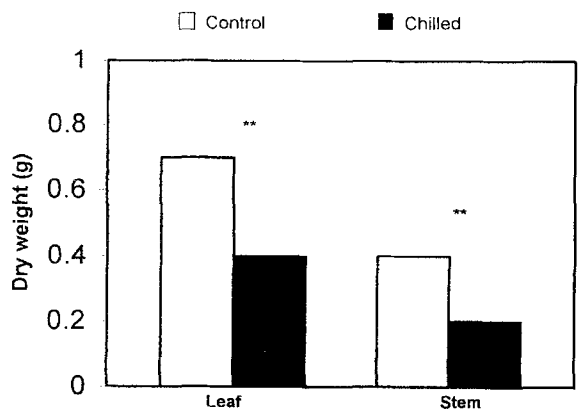

Fig. 1. Dry weight of tomato leaves and stem in plants subjected to 7-day period of chilling stress (10/8 °C, day/night) in comparison to controls maintained for the same period at a temperature of 25/23 °C. Each value is the mean of 10 replications. In the Figure is reported the significance of the difference between the two averages following the Student *t* test (**: P=0.01)

Leaf gas exchange and chlorophyll fluorescence

CO_2 assimilation rate and stomatal conductance measured at 350 µmol $m^{-2} \cdot s^{-1}$ irradiance were strongly depressed in chilled plants (Table 1). Transpiration rate was reduced, while the intercellular CO_2 concentration was not affected by low temperature.

Table 1. Gas exchange parameters of the last fully expanded leaves of *Lycopersicon esculentum* plants subjected to chilling stress (10/8 °C, day/night) for 7 days in comparison to the controls (25/23 °C, day/night). Gas exchange measurements were carried out at ambient CO_2 and O_2 concentration (345 ppm and 21 %, respectively) and at 350 µmol $m^{-2} \cdot s^{-1}$. Each value is the average of 3 replicates. The last column indicates the significance of the differences between control and stressed plants determined with Student's *t* test (*: P=0.05; NS: not significant).

	Control	Stressed	P
A	8.33	5.19	*
E	1.22	0.86	*
G_w	92.66	62.86	*
C_i	203	216	NS

A = CO_2 assimilation rate (µmol CO_2 $m^{-2} \cdot s^{-1}$)
E = transpiration rate (mmol H_2O $m^{-2} \cdot s^{-1}$)
G_w = stomatal conductance to water vapour (mmol H_2O $m^{-2} \cdot s^{-1}$)
C_i = intercellular CO_2 concentration (ppm)

Variable fluorescence, F_v, as well as maximal fluorescence F_m were not significantly different in control and chilled seedlings, while the ground fluorescence F_0 significantly increased (Table 2). The maximum efficiency of PSII estimated by the chlorophyll fluorescence ratio F_v/F_m was slightly, but significantly, reduced in chilled plants. Quenching of chlorophyll fluorescence under steady-state photosynthesis at a light intensity of about 350 µmol $m^{-2} \cdot s^{-1}$, which is due to photosynthetic electron transport (photochemical quenching coefficient, q_P) or to events not directly concerned with photochemistry (non-photochemical quenching coefficient, q_{NP}), was measured by pulse amplitude modulated fluorometer. The q_P, which reflects the capacity of PSII reaction centres to compete for Chl excited states and is related to the redox state of Q_A (Genty *et al.* 1989, Van Kooten and Snell 1990), increased in chilled

seedlings, thus indicating an increase in the fraction of open PSII reaction centres (Table 2).

In chilled plants the non-photochemical quenching

Table 2. Fluorescence parameters in the last fully expanded leaves of *Lycopersicon esculentum* plants subjected to chilling stress (10/8°C, day/night) for 7 days in comparison to the controls (25/23°C, day/night). Fluorescence measurements were carried out at ambient CO_2 and O_2 concentration (345 ppm and 21 %, respectively). Each value is the average of 3 replications. The last column indicates the significance of the differences between control and stressed plants determined with Student's *t* test (*: P=0.05; **: P=0.01; NS: not significant).

	Control	Stressed	P
F_0	76	84	*
F_v	353	362	NS
F_m	429	447	NS
F_v/F_m	0.823	0.811	**
q_P	0.868	0.905	*
q_{NP}	0.223	0.391	*
Φ_{PSII}	0.678	0.646	*
Φ_{exc}	0.781	0.713	**

(q_{NP}) also rose significantly (Table 2). The q_{NP} originates from lumen acidification as well as from sequestering of protons within the thylakoid membrane (Ruban and Horton 1995), and it has been considered as a photoprotective mechanism which contributes to dissipation of excitation energy (Demmig-Adams and Adams 1992).

Chilling reduced excitation capture efficiency of PSII (Φ_{exc}) as well as the actual quantum yield of PSII (Φ_{PSII}).

In cold-treated plants a significant decrease in Chl *a* and *b* contents was observed, whereas no significant differences were found between controls and chilled plants in terms of Chl *a*/*b* ratio (Table 3).

Sugar and osmolite contents

Sucrose content did not change in chilled plants, while a strong increase in the reducing sugars content was observed (Fig. 2A). The starch content was higher in treated plants compared to the control and a significant interaction was found between the time of sampling and the stress (Fig. 2B). The high-

Table 3. Chlorophyll *a*, *b* and total content ($g \cdot cm^{-2}$) and ratio in the last fully expanded leaves of *Lycopersicon esculentum* plants subjected to chilling stress (10/8 °C, day/night) for 7 days in comparison to the controls (25/23 °C, day/night). Each value is the average of 3 replications. The last column indicates the significance of the differences between control and stressed plants determined with Student's *t* test (*: P=0.05, **: P=0.01; NS: not significant).

	Control	Stressed	P
Chl *a*	26.42	17.35	**
Chl *b*	9.48	6.21	**
Chl tot	35.90	23.56	*
Chl *a*/*b*	2.84	2.83	NS

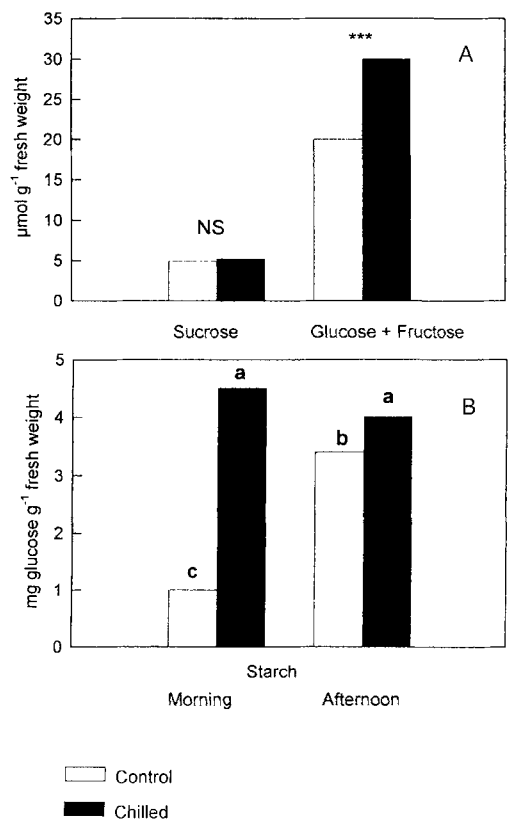

Fig. 2. Relative carbohydrate levels in leaves of plants subjected to 7-day period of chilling stress (10/8 °C, day/night) in comparison to controls maintained for the same period at a temperature of 25/23 °C. A. Soluble sugar content (sucrose, glucose+fructose) collected in the morning. B. Starch content determined in the morning and afternoon. Each value is the average of 8 replications. In Figure 2A is reported the significance of the difference between the two averages following the Student *t* test (***: P=0.001; NS: not significant). In Figure 2B values with the same letters are not significantly different following the ANOVA test using the time of sampling (morning and afternoon) and the treatment (chilling and control) as factors.

est value of starch content was recorded in the morning in chilled plants, while the lowest value was observed in control plants at the same sampling time.

No changes were observed in the osmolite content in treated plants as compared to the controls (*data not shown*).

Enzyme activities

Sucrose phosphate synthase (SPS) and sucrose synthase (SS) activities did not change in leaves of plants subjected to low temperature as compared to the controls (Table 4). By contrast, a strong increase was observed in AI and NI activities in chilled plants (Table 4).

Table 4. Sucrose-phosphate synthase (SPS), sucrose synthase (SS), acid and neutral invertase (AI and NI) activities ($mmol \cdot mg^{-1}protein \cdot h^{-1}$) in extracts from last fully expanded leaves of *Lycopersicon esculentum* plants subjected to chilling stress (10/8 °C, day/night) for 7 days in comparison to the controls (25/23 °C, day/night). Enzymatic assays used 40 µl of desalted, crude extract. Each value is the average of 5 replications. The last column indicates the significance of the differences between control and stressed plants determined with Student's *t* test (**: P=0.01; NS: not significant).

	Control	Stressed	P
SPS	19.77	13.85	NS
SS	6.42	7.79	NS
AI	13.73	27.74	**
NI	15.11	31.06	**

Discussion

Tomato is a chilling-sensitive species and, as expected, in our experiments growing the plants at 10/8 °C day/night temperature resulted in a strong inhibition of leaf area expansion and biomass accumulation. This result has already been reported by other authors (Bruggemann *et al.* 1991, Brugemann *et al.* 1992).

The reduction in photosynthetic rate in chilled plants was probably due to the reduction in stomatal conductance, but the fact that C_i did not change indicated that an impaired mesophyllic capacity also contributed to photosynthetic limitation. Data from chlorophyll fluorescence analysis confirm this hypothesis. In fact, F_v/F_m ratio slightly decreased in chilled tomato, indicating however a diminished efficiency of PSII as already reported (Janda *et al.* 1996, Szalai *et al.* 1996). The increase in F_0 was mainly responsible for the lowering of the F_v/F_m ratio and it may indicate an impaired energy trapping efficiency in the PSII reaction centres or a partial disconnection of the antennae from the centres. In chilled plants light absorbed by PSII is in excess of that which can be utilized by the Calvin cycle. Photoinhibition acts as a photoprotective mechanism when excess absorbed excitation energy is dissipated as heat within the PSII antennae, decreasing the F_v/F_m ratio (Bjorkman and Demmig 1987).

The capacity of chilled tomato plants to maintain higher q_P values than control leaves suggests that protection mechanisms are present allowing the leaves to prevent excess reduction of PSII acceptors. Moreover, it has been reported that this feature is related to high content of xanthophyll zeaxanthin (Jung and Steffen 1997). Other authors (Demmig-Adams and Adams 1996) reported that zeaxanthin is related to excess energy dissipation as heat in the antennae and, probably, it is responsible for the reduced efficiency of excitation energy capture by open PSII reaction centres ($\Phi_{exc.}$) when compared to controls. Similar results have already been reported by other authors in maize (Fracheboud *et al.* 1999). Furthermore, the reduction of Φ_{PSII} in chilled plants is largely due to a reduction in $\Phi_{exc.}$ and its decrease contributes also to the increase in q_{NP} observed in chilled plants. Thus chilling in tomato plants caused inhibition of CO_2 assimilation, but did not affect the capacity of energy-dependent quenching. The reaction centres were kept open in the steady-state conditions and this is probably achieved by increased thermal deactivation as demonstrated by increased q_{NP}.

The starch content was very different in control leaves according to whether the sampling was carried out in the morning or in the afternoon. The higher starch content in leaves of control plants recorded in the afternoon was the product of photosynthesis; during the night this sugar is hydrolized and translocated and so a low value is recorded in the morning. In chilled plants this behaviour was al-

tered: the starch content did not change during the night. The starch accumulation and the reduction in dry weight recorded in chilled tomato plants were interpreted as a result of chill-induced inhibition of sucrose translocation. Other authors reported similar results in sugarcane exposed to temperature below optimum (Ebrahim *et al.* 1998). The partition mechanism is a very complex problem it involves the activities of enzymes of starch degradation and sink-source relationship. Thus further investigations are required in this direction.

The glucose plus fructose content increased significantly in treated plants, while sucrose did not change. It has been reported that soluble sugar levels increase in many plant species during exposure to low temperature (Alberdi and Corcuera 1991, Keller and Steffen 1995). A high rate of soluble sugar synthesis could be related in chilled plants to the necessity to increase osmotic potential in the cells, but this was not the case in the present experiment. Therefore, these results confirm also that tomato is a plant which is not genetically adapted to low temperature. Indeed, it is known that sucrose accumulation during cold acclimation is often correlated with freezing tolerance (Guy *et al.* 1992).

Furthermore, the increase in AI and NI activities, which is typical of senescent tissue characterized by reduced growth, seems to confirm this hypothesis.

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