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Chilling-induced reduction of photosynthesis is mitigated by exposure to elevated CO_2 concentrations

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Abstract

This work aimed to evaluate if chilling stress may be mitigated by elevated CO_2 (EC) in *Beta vulgaris* L. plants. Photosynthetic rate was measured at 21% and 2% O_2 after a short-term exposure of 5 h at four different treatments: 360 μmol(CO_2) mol^{-1}/25°C (AC); 360 μmol(CO_2) mol^{-1}/4°C (AC+LT); 700 μmol(CO_2) mol^{-1}/25°C (EC); 700 μmol(CO_2) mol^{-1}/4°C (EC+LT). Compared to AC+LT, EC+LT plants showed higher values of CO_2 fixation, photochemical activity, and Rubisco amount. These latter invest a higher portion of photosynthetic electron flow to O_2, differently from AC+LT plants that promote the regulated thermal dissipation processes. In EC+LT plants, the photosynthetic electron flow to O_2 acts as a safety mechanism against the excess of absorbed light, upon return to prechilling conditions, allowing photosynthetic apparatus to maintain its efficiency. In AC+LT plants, the increase of thermal dissipation processes was not adequate to guarantee the PSII photoprotection and the photosynthetic recovery after chilling.

Additional key words: chilling; elevated CO_2; partitioning of absorbed light to PSII; photosynthetic rate.

Introduction

Chilling represents a critical environmental stress that limits plant productivity and distribution in many regions of the world. In response to this constraint, plants have developed an elaborate defence system, namely cold acclimation, that confers chilling/freezing tolerance (Huner *et al.* 1993, Zheng *et al.* 2014). The acclimation to cold consists in reversible molecular, biochemical, and physiological modifications primed by the exposure to low temperatures in some periods of the day or season. From the ecological point of view, these changes are significant because allow many species to adapt successfully to own dynamic environment, maintaining the photosynthetic rate (P_N) over winter (Janská *et al.* 2010).

The reduction of photosynthesis induced by chilling is a typical response of both chilling-sensitive and chilling-tolerant plants (Martin *et al.* 1981, Venema *et al.* 2000). Such decrease might be observed not only during but also after chilling period because of the slow recovery of P_N due to both stomatal and nonstomatal limitations (Melkonian *et al.* 2004). A small drop in temperature, even if it does not produce visible damage may induce up to 50% reduction of plant productivity, because of chilling acts on important physiological processes, such as water uptake, mineral nutrition, photosynthesis, respiration, and total metabolism. The photosynthesis depression may be ascribed to different physiological constraints, such as stomatal and nonstomatal limitations to gas diffusion into the leaf, and biochemical constraints. More specifically, low temperatures inhibit photosynthesis either directly impairing PSII (Govindachary *et al.* 2004, Huang *et al.* 2010) and indirectly affecting Calvin-cycle enzymes (Venema *et al.* 1999, Allen and Ort 2001). The decline of photosynthesis at chilling temperatures may also be a consequence of photoinhibition and photooxidative damages

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Abbreviations: AC – ambient [CO_2]; [CO_2] – CO_2 concentration; Chl – chlorophyll; C_i – intercellular CO_2 concentration; EC – elevated [CO_2]; F_0 – minimal fluorescence yield of the dark-adapted state; F_0' – minimal fluorescence yield of the light-adapted state; F_m – maximal fluorescent yield of the dark-adapted state; F_m' – maximal fluorescence level in light-adapted state; F_v – variable fluorescence; F_v/F_m – maximum quantum yield of PSII photochemistry; F_s – steady-state fluorescence yield; g_s – stomatal conductance; J_f – electron transport rate; J_c – electron transport rate used for CO_2 assimilation; J_o – electron transport rate used for photorespiration; K – Kelvin; LT – low temperature; P_N – net photosynthetic rate; R_D – dark respiration; TCA – trichloroacetic acid; V_c – carboxylation rate of Rubisco; V_o – oxygenation rate of Rubisco; Φ_{PSII} – effective quantum yield of PSII photochemistry; Φ_{NPQ} – the yield of regulated energy dissipation; Φ_{NO} – the yield of nonregulated energy dissipation.

to the photosystems. In such circumstances, the balance between energy absorption and utilization is modified and detrimental effects are evident by increased lipid peroxidation, degradation of chlorophylls (Chl), carotenoids, and xanthophylls and reduced antioxidant capability (Fryer *et al.* 1998, Leipner *et al.* 2000, Alam and Jacob 2002).

Such situation can also continue after the chilling event as consequence of a delayed recovery of photosynthesis further enhancing the susceptibility of photosynthetic apparatus to photodamages.

Chilling-tolerant species show a reduction in photosynthesis during a chilling episode, but unlike chilling-sensitive species, recover more or less instantaneously with the return to permissive temperatures.

Most crop species have evolved a degree of cold tolerance, depending on the combination of the minimum temperature experienced and the length of exposure to cold (Janská *et al.* 2010).

Against cold, plants have developed two main strategies: invest more light energy in photochemical processes by increasing photosynthetic capacity or promote the nonphotochemical mechanisms that safely dissipate the excess of excitation energy as heat (Huner *et al.* 1993, Rapacz *et al.* 2008).

It is well known that plants exposed to short-term CO_2 enrichment increase photosynthetic capacity depending on plant species, developmental stage, and environmental conditions (Ainsworth and Rogers 2007, Duarte *et al.* 2014). Nevertheless, with long-term exposure to elevated CO_2, photosynthetic acclimation or the down-regulation of photosynthesis may occur, offsetting such stimulation (Moore *et al.* 1999, Urban *et al.* 2012, Sanz-Sáez *et al.* 2013).

Many studies have been performed to investigate the combined effects of elevated CO_2/high temperature (Hamilton *et al.* 2008, Gutiérrez *et al.* 2009, Qu *et al.* 2014) or high CO_2/low temperatures (Boese *et al.* 1997, Ziska 2001, Laing *et al.* 2002) on photosynthetic performance. Some studies show a lower chilling/freezing tolerance of photosynthetic apparatus in plants exposed to elevated CO_2 (Loveys *et al.* 2006, Bertrand *et al.* 2007, Martin *et al.* 2010), due to the higher leaf temperature as consequence of a reduced stomatal conductance (g_s). Other experiments report an improved resistance of photosynthetic machinery to low temperatures under elevated CO_2. This response has been ascribed to an increase in electron transport to carbon fixation (Ziska 2001, Laing *et al.* 2002, Dahal *et al.* 2014) or to a reduced g_s that limits chilling-induced water stress and photosynthetic depression (Boese *et al.* 1997, Drake *et al.* 1997). The cold tolerance mechanisms can also be modified by photosynthetic acclimation occurring both upon short- and long-term exposure to rising atmospheric CO_2 concentrations (Bigras and Bertrand 2006).

This work aimed to study the role of elevated CO_2 concentrations in mitigating chilling-induced photosynthetic depression in *Beta vulgaris* L., a winter crop widely cultivated in the Mediterranean agro-ecosystems. In particular, we investigated whether and how elevated CO_2 concentrations may affect plant response to chilling inducing a different regulation of leaf gas exchanges and PSII light energy partitioning.

Materials and methods

Plant material and growth conditions: The experiment was carried out during 2016 at the Department of Biology (University of Naples Federico II, Italy). Seeds of *Beta vulgaris* L. cv. Cicla were sown in 4-L pots filled with a 1:1 (v/v) mixture of peat and soil. Germinated seeds were transferred to plant growth chamber at the following conditions: 25°C, air relative humidity of 65%, 200 μmol(photon) m^{-2} s^{-1} PAR at the top of the canopy, and a photoperiod of 12 h. Irradiation was provided by a series of 28-W fluorescent tubes (*Nurdex T5-6500 K*). Plants were irrigated twice every week and fertilised once a week with a commercial nutritional solution (*Cifo, Italy*; N:P:K 20:20:20 + micronutrients). For the experiments, two different plant groups were used.

Experimental design: All plant groups were exposed for 5 h to four different temperature and CO_2 treatments at the irradiance of 200 μmol(photon) m^{-2} s^{-1} in a growth chamber equipped with LED lighting system. The lighting consisted of thirteen different wavelengths from ultraviolet (370 nm) to infrared (940 nm) and two types of phosphor-coated LEDs for white light (5000K–6500K). The CO_2 treatments inside the chamber were obtained flowing air at different CO_2 concentrations from gas bottles at specific [CO_2]. The CO_2 concentration in the chamber was monitored using a CO_2 analyser (*Qubit Systems*, Kingston, Canada). The performed treatments were:

CO_2 concentration [μmol(CO_2) mol^{-1}]	Temperature [°C]	Treatment	Assignment
360	25	-	AC
360	4	low temperature	AC+LT
700	25	elevated CO_2	EC
700	4	elevated CO_2 and low temperature	EC+LT

In the first group of plant, after each treatment, P_N-PPFD curves were performed at 25°C and ambient $[CO_2]$ to assess the value of saturation irradiance for photosynthesis.

On the second group of plants, before each treatment, the maximal quantum yield of PSII photochemistry (F_v/F_m) and the dark respiration (R_D) were determined on 30-min dark-adapted leaves. After the exposure for 5 h to different treatments, F_v/F_m ad R_D were measured again on 30-min dark-adapted leaves. Measurements of gas exchanges and Chl fluorescence emission in the light were carried out at 25°C and sub-saturating light intensity for photosynthesis [PAR of 200 μmol(photon) m^{-2} s^{-1}] under photorespiratory (21% O_2 and 0.036% CO_2) and nonphotorespiratory (2% O_2 and 0.036% CO_2) conditions. After each treatment, plants were darkened for 14 h and F_v/F_m ad R_D measured again to assess the recovery from chilling event.

Gas exchange and Chl fluorescence measurements were performed simultaneously on 30-d-old fully expanded leaves, under photorespiratory and nonphotorespiratory conditions at ambient $[CO_2]$ of 360 μmol(CO_2) mol^{-1} using a portable photosynthesis and fluorescence system (*Li6400* combined with a *Li6400-40* leaf chamber fluorometer, *Licor*, USA). Gas-exchange parameters (*i.e.* net photosynthetic rate, P_N; stomatal conductance to H_2O, g_s; intercellular $[CO_2]$, C_i) were calculated according to von Caemmerer and Farquhar (1981) by the software operating in *Li6400*. The apparent carboxylation efficiency (P_N/C_i) was used as an indicator to evaluate the occurrence of stomatal and nonstomatal limitations.

On dark-adapted leaves, minimal fluorescence yield of the dark-adapted state, F_0, was induced by weak modulated measuring beam of 0.03 μmol(photon) m^{-2} s^{-1}. The maximal fluorescence yield of the dark-adapted state, F_m, was measured by an 800-ms saturating light pulse of 8,000 μmol(photon) m^{-2} s^{-1}; the maximal quantum yield of PSII photochemistry (F_v/F_m) was calculated as $(F_m - F_0)/F_m$. The steady-state fluorescence yield, F_s, was measured upon illumination. The maximal fluorescence yield of the light-adapted state, F_m', was measured by applying an 800-ms saturating flash of 8,000 μmol(photon) m^{-2} s^{-1}; the minimal fluorescence yield of the light-adapted state, F_0', was obtained turning off the actinic light and applying a far-red pulse.

The partitioning of absorbed light energy within PSII was calculated following the model of Kramer *et al.* (2004). The effective quantum yield of PSII photochemistry (Φ_{PSII}) was estimated following Genty *et al.* (1989) as: $\Phi_{PSII} = (F_m' - F_s)/F_m'$. The yield of regulated energy dissipation was calculated as: $\Phi_{NPQ} = 1 - \Phi_{PSII} - 1/[NPQ + 1 + q_L (F_m/F_0 - 1)]$, the yield of nonregulated energy dissipation was estimated as: $\Phi_{NO} = 1/[NPQ + 1 + q_L \times (F_m/F_0 - 1)]$. The nonphotochemical quenching, NPQ, was expressed according to Bilger and Björkman (1990). The PSII "excitation pressure" or the relative reduction state of PSII was calculated as $1 - q_L$, where q_L represents the photochemical quenching (Kramer *et al.* 2004).

The electron transport rate measured by chlorophyll fluorescence (J_f) was calculated following Krall and Edwards (1992) as: $\Phi_{PSII} \times PPFD \times 0.5 \times 0.84$, where 0.5 is a factor implicating an equal distribution of energy between photosystems (Krall and Edwards 1992) and 0.84 the assumed leaf absorbance for *B. vulgaris*. Light-response curves under nonphotorespiratory conditions (2% O_2) were performed to calibrate the relationship between fluorescence-derived electron transport rates and photosynthesis.

In this study, we used J_f/P_N ratio as an indicator of electron transport to acceptors other than CO_2, as previously reported by other authors (Krall and Edwards 1992, Flexas *et al.* 1998, Medrano *et al.* 2002). According to Epron *et al.* (1995), J_f was divided into two components: $J_f = J_c + J_o$.

J_c is the fraction of J_f used for CO_2 assimilation ($J_c = 1/3\ [J_f + 8(P_N + R_L)]$) and J_o is the fraction of J_f used for photorespiration ($J_o = 2/3\ [J_f - 4(P_N + R_L)]$). This approach assumes that all the reducing power generated by the electron transport chain is used for photosynthesis and photorespiration, and Chl fluorescence gives a reliable estimation of the quantum yield of electron transport. P_N-PPFD curves performed at 2% O_2 showed that J_f/P_N ratio was 4–5 in the all utilized light range (data not shown), in agreement with the theoretical value (Krall and Edwards 1992). Based on this assumption, the processes consuming reductive power other than photosynthesis and photorespiration are negligible in *B. vulgaris* grown under our experimental conditions.

The rates of carboxylation (V_c) and oxygenation (V_o) of Rubisco were calculated as: $V_c = 1/6\ [J_f/2 + 4(P_N + R_L)]$ and $V_o = 1/6\ [J_f - 4(P_N + R_L)]$, respectively (von Caemmerer 2000). Day respiration, R_L for calculation of J_{fc}, J_{fo}, V_c, and V_o was assumed to be half of R_D (von Caemmerer 2000).

Rubisco expression: Leaf protein extraction was carried out following the procedure reported in Arena *et al.* (2017). Leaf tissues (1 g), were ground in a mortar under liquid nitrogen. The powder was suspended in solution 10% TCA/acetone, centrifuged at 16,000 × *g* for 3 min at 4°C and then washed first with methanol (80%) and after in acetone (80%). After drying (50°C for 10 min), the pellet was re-suspended in 1:1 phenol (pH 8.0)/SDS (v:v) buffer and centrifuged at 16,000 × *g* for 3 min. The upper phenol phase was treated with methanol containing 0.1 M ammonium acetate, stored overnight at 20°C and centrifuged again. The pellet was washed once with 100% methanol and once with 80% acetone and after resuspended in an SDS sample buffer. The protein quantification was carried out by *Quick Start^{TM}* Bradford protein assay (*Bio-Rad Laboratories* S.r.l., Segrate, Milano, Italy).

For Western blotting analysis, 5 μg of proteins from the cytoplasmic fraction together with RbcL protein standard (2 μg) were analysed on 12% polyacrylamide slab gels in the presence of 0.1% sodium dodecyl sulphate (SDS) and electrotransferred onto 0.2 µm pore-size nitrocellulose membrane (*Bio-Rad Laboratories S.r.l.*, Segrate, Milano, Italy) at 100 V for 4 h at 4°C in the transfer buffer (Tris-glycine, methanol, H_2O). The nitrocellulose membrane was treated for 1 h with blocking solution (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (v/v) *Tween 20*, TBST) and 5% (w/v) dry milk. The incubation with commercial rabbit anti RbcL (type 1 + type 2) antibody (*Agrisera*, 1:10,000, v/v) was performed for 1 h at room temperature in the same solution supplemented with 5% (w/v) dry milk. The blot was then washed several times with buffer TBST and antibody binding detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (*Pierce*; 1:5,000; v/v). HRP reaction used a kit for chemiluminescence (*Westar Supernova*, *Cyanagen S.r.l.*, Bologna, Italy) and *Chemidoc* apparatus (*Bio-Rad*). Actin was used as loading control. Each Rubisco band was normalized to the appropriate actin band. Density values are expressed in arbitrary units and represented as bar diagrams that are pixel volumes of Rubisco bands. The data are processed with *Quantity One 1-D Analysis* software *(Bio-Rad)*, after minimising background effect.

Statistical analysis: Statistical significant differences among treatments were analysed by two-way analysis of variance (*ANOVA*) using CO_2 and temperature as independent factors, to assess the interaction between CO_2 and temperature. *Shapiro-Wilk* and *Kolmogorov-Smirnov* tests were performed to check for normality. The *Holm-Sidak* test was applied for all multiple comparison procedures based on a significance level of $p<0.05$. Differences between photorespiratory and nonphotorespiratory conditions were assessed by *t*-test based on a significance level of $p<0.05$. The package *Sigma-Plot 12.0* (*Jandel Scientific*, San Rafael, CA, USA) was used for graphical and statistical data processing.

Results

Recovery from treatments: F_v/F_m ratio, measured on 30-min dark-adapted leaves after returning to prechilling conditions, decreases by 18% in AC+LT plants and 7% EC+LT plants (Table 1). Both plant groups also showed the higher F_0 and the lower F_m values compared to the initial value (Table 1). After 14 h of darkness, F_v/F_m ratio returned to the initial values in both AC+LT and EC+LT plant groups (Table 1).

P_N–PPFD curves: In all treatments, the light saturation point for photosynthesis was 800 µmol(photon) m^{-2} s^{-1} PAR (Fig. 1). The highest P_N values were measured in EC plants, whereas the lowest values in AC+LT plants. P_N in AC and EC+LT exhibited intermediate values, being in EC+LT significantly higher than that in AC+LT plants (Fig. 1).

Gas exchanges and Chl fluorescence measurements: Net photosynthetic rate (P_N) measured at 21% O_2 was significantly lower than that measured at 2% O_2 in all treatments (Fig. 2*A*).

The exposure to low temperatures determined a significant reduction of P_N in AC+LT and EC+LT plants at both O_2 concentrations. The chilling-induced reduction of P_N was higher in AC+LT than that in EC+LT plants (Fig. 2*A*).

Stomatal conductance to CO_2 (g_s) (Fig. 2*B*) and P_N/C_i ratio (Fig. 2*C*) showed a similar trend of P_N: these parameters decreased in AC+LT and EC+LT compared to AC and EC plants at both O_2 concentrations. The reduction was higher in AC+LT than that in EC+LT plants.

The carboxylation rate (V_c), measured at 21 and 2% O_2, significantly decreased in AC+LT and EC+LT plants compared with control (Fig. 2*D*); the lowest value was measured in AC+LT plants. The oxygenation rate (V_o) at 21% O_2 decreased in AC+LT compared to other plant groups. The highest value was measured in EC+LT plants (Fig. 2*D*).

Table 1. Recovery of F_0, F_m,, and F_v/F_m, in *B. vulgaris* leaves after a dark period of 30 min and 14 h from different treatments. AC – ambient [CO_2]; AC+LT – ambient [CO_2] and low temperature; EC – elevated [CO_2]; EC+LT – elevated CO_2 and low temperature. Values are means ± SE ($n = 6$). *Different letters* indicate statistically significant differences among treatments at 21% O_2.

	AC	AC+LT		EC		EC+LT	
	Initial value	30 min	14 h	30 min	14 h	30 min	14 h
F_0	0.224 ± 0.002^{a}	0.297 ± 0.008^{b}	0.219 ± 0.008^{a}	0.219 ± 0.003^{a}	0.229 ± 0.008^{a}	0.267 ± 0.002^{c}	0.216 ± 0.002^{a}
F_m	1.292 ± 37.642^{a}	0.926 ± 0.003^{b}	1.180 ± 37.964^{a}	1.167 ± 18.023^{a}	1.213 ± 44.681^{a}	1.065 ± 24.973^{c}	1.188 ± 24.024^{a}
F_v/F_m	0.826 ± 0.006^{a}	0.679 ± 0.007^{b}	0.814 ± 0.003^{a}	0.812 ± 0.005^{a}	0.811 ± 0.003^{a}	0.749 ± 0.004^{c}	0.818 ± 0.003^{a}

Compared with control, the fraction of electron transport used for CO_2 assimilation, J_c, decreased in both AC+LT and EC+LT plants under photorespiratory and nonphotorespiratory conditions. This decrease was 42% in AC+LT and 17% in EC+LT plants; conversely, no difference between control and EC plants was found (Fig. 3*A*). The fraction of electron transport used for photorespiration, J_o, showed the lowest value in AC+LT and the highest value in EC+LT plants (Fig. 3*A*). The J_f/P_N ratio measured at 21% O_2 did not show any differences between treatments, except for EC+LT plants, where an increase of 20% compared with control was found (Fig. 3*B*). At 2% O_2, the J_f/P_N ratio did not vary among treatments and was near to the theoretical value (4–5).

The partitioning of absorbed light energy within PSII, measured at 21% O_2, showed in AC+LT plants the lowest Φ_{PSII} and the highest Φ_{NPQ} and Φ_{NO} values compared to other plant groups. AC+LT and EC+LT plants exhibited comparable values of Φ_{NO} (Fig. 3*C*).

Rubisco expression and content: The Rubisco protein expression was differently modulated in the different treatments (Fig. 4*A*); in particular, AC+LT treatment caused in leaves a significant decrease of Rubisco content compared with control and EC treatments. In particular, the EC treatment stimulated the Rubisco expression compared with control. The combination of elevated CO_2 and low temperature (EC+LT), mitigated the reduction of Rubisco contents compared with AC+LT treatment (Fig. 4*B*).

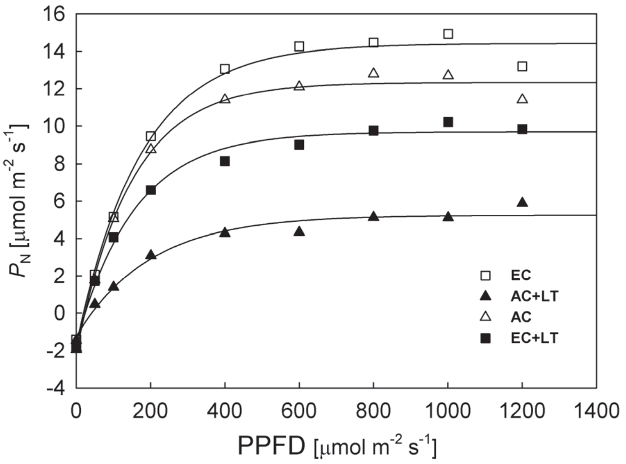

Fig. 1. P_N–PPFD curves performed at 25°C and ambient [CO_2] on *Beta vulgaris* plants after 5-h exposure at each treatment. Values are means ± SE (n = 6). AC – ambient [CO_2]; AC+LT – ambient [CO_2] and low temperature; EC – elevated [CO_2]; EC+LT – elevated [CO_2] and low temperature.

Discussion

Gas exchanges in response to different treatments: Changes of temperature modify the balance of energy absorbed during the light phase of photosynthesis and the energy utilized in dark processes. The reduction of photosynthesis induced by cold may continue after chilling period because of the occurrence of persistent stomatal and nonstomatal limitations (Melkonian *et al.* 2004). Our experiment indicated that the exposure to elevated CO_2 successfully mitigated the chilling-induced reduction of P_N in *B. vulgaris* plants (EC+LT) upon return to prechilling conditions. It may be stated that elevated CO_2 by reducing stomatal conductance, may improve plant

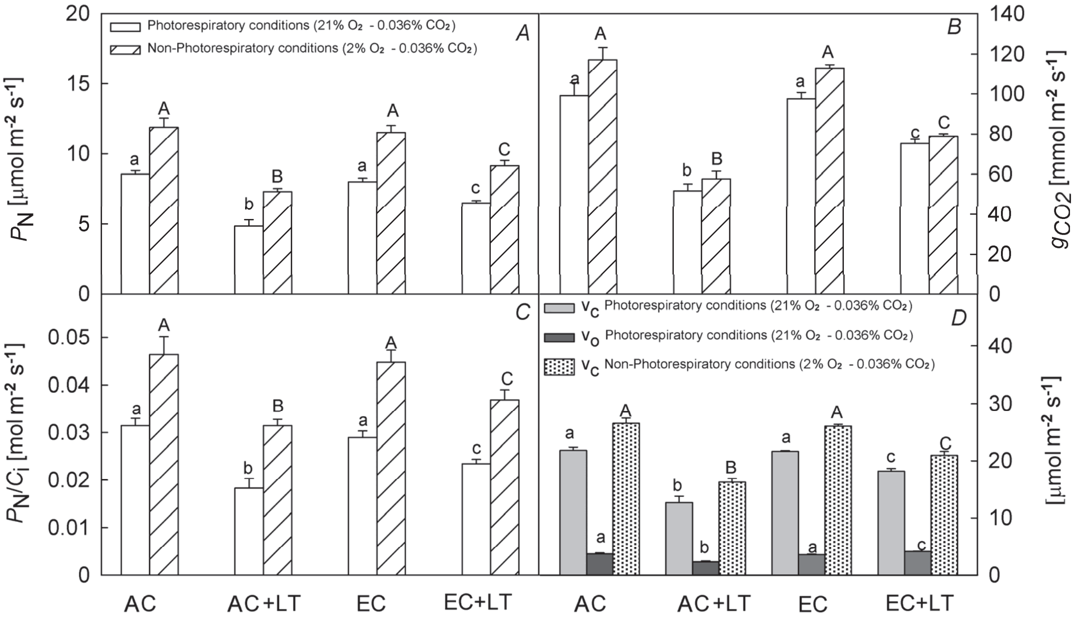

Fig. 2. (*A*) Net photosynthetic rate (P_N), (*B*) CO_2 stomatal conductance, (g_s), (*C*) apparent Rubisco efficiency (P_N/C_i), and (*D*) carboxylation (V_c) and oxygenation (V_o) rates of Rubisco, measured before and after different treatments under photorespiratory (21% O_2 and 0.036% CO_2) and nonphotorespiratory (2% O_2 and 0.036% CO_2) conditions. Values are means ± SE (n = 6). AC – ambient [CO_2]; AC+LT – ambient [CO_2] and low temperature; EC – elevated [CO_2]; EC+LT – elevated [CO_2] and low temperature. *Small and capital letters* indicate statistically significant differences between treatments under photorespiratory and nonphotorespiratory conditions.

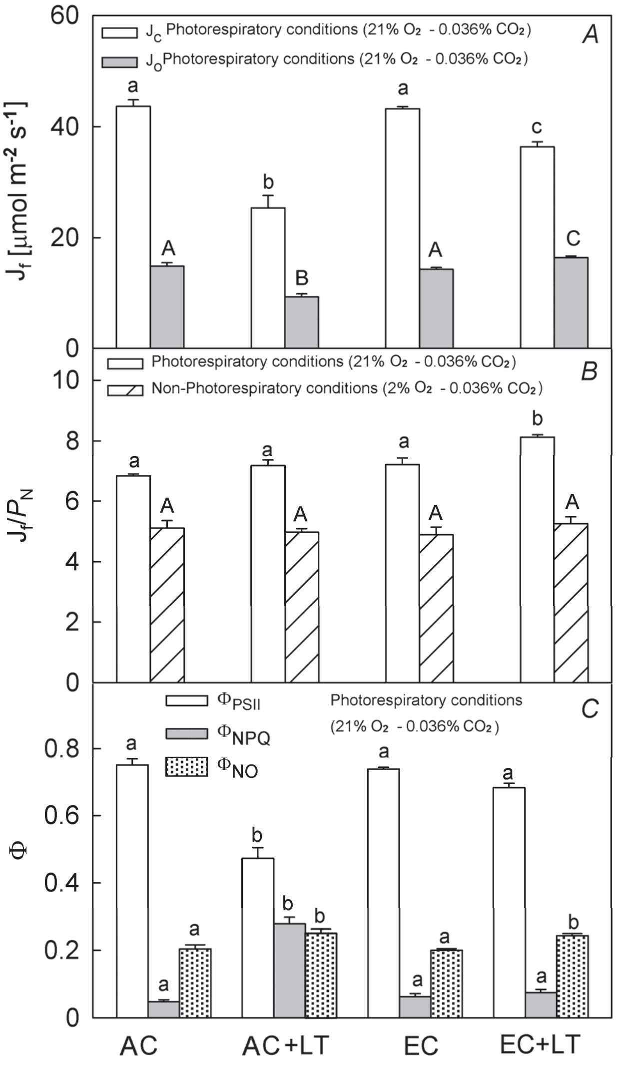

Fig. 3: (*A*) Fraction of electron transport (J_f) used in CO_2 assimilation (J_c) and in photorespiration (J_o), (*B*) ratio of electron transport and net photosynthesis (J_f/P_N), (*C*) effective quantum yield of PSII photochemistry (Φ_{PSII}), yield of regulated energy dissipation (Φ_{NPQ}), and nonregulated energy dissipation (Φ_{NO}), measured before and after different treatments under photorespiratory (21% O_2 and 0.036% CO_2) and nonphotorespiratory (2% O_2 and 0.036% CO_2) conditions. Values are means ± SE ($n = 6$). AC – ambient [CO_2]; AC+LT – ambient [CO_2] and low temperature; EC – elevated [CO_2]; EC+LT – elevated [CO_2] and low temperature. *Small and capital letters* indicate statistically significant differences between treatments under photorespiratory and nonphotorespiratory conditions.

water relations during the chilling event and mitigate the photosynthetic depression as well as chilling damages (Boese *et al.* 1997, Drake *et al.* 1997).

The decline of P_N and P_N/C_i ratio, observed in AC+LT and EC+LT plants, suggests that Rubisco represents a critical factor in photosynthesis recovery of *B. vulgaris* plants. More specifically, the slow recovery of P_N, after returning to prechilling conditions, could be attributed not only to the decrease of stomatal conductance but also to the loss of Rubisco activity due to the reduction of its content. It is noteworthy that the harmful effects of low temperature on Rubisco activity are often associated with changes in its concentration (Galmés *et al.* 2013). Our data indicate that the exposure of plants to elevated CO_2, alone or in combination with low temperature, determined differences in Rubisco expression that might have affected its activity during recovery. Consistent with this hypothesis, we observed a decrease of photosynthesis in AC+LT plants together with an important reduction of Rubisco expression level.

Even if Rubisco is a very abundant and quite stable protein, its synthesis is limited under low temperatures (Meza-Basso *et al.* 1986). Thus, the high drop (56%) of Rubisco content observed under cold may be due to the slow capability of this enzyme to recover after a chilling event. More specifically, so drastic reduction observed in LT plants suggests that the Rubisco degradation during chilling exceeded its synthesis. This unbalance might negatively affect the carboxylase/oxygenase activity, as confirmed by the significant decrease of V_c and V_o.

The reduction of carboxylase activity is also accompanied by the decline of the oxygenase activity (Allen and Ort 2001). In our experimental conditions, the combination chilling/elevated CO_2 led to the reduction of carboxylation rate (V_c), likely due to an impairment of CO_2 assimilation, and to the increase of oxygenation rate (V_o). We hypothesise that the substantial decrease (56%) of Rubisco amount in AC+LT leaves, compared with control, was the result of an accelerated degradation of the protein induced by cold. On the other hand, the small reduction (9%) found in EC+LT leaves could be ascribed to a stimulatory effect of elevated CO_2 on Rubisco synthesis. It is noteworthy that short-term exposure to elevated CO_2 leads to an increase of leaf-level photosynthesis due to enhanced activity of Rubisco (Moore *et al.* 1999, Urbonavičiūtė *et al.* 2006). In our experiment, the highest Rubisco amount was found in EC leaves, suggesting the hypothesis of a direct stimulation of elevated CO_2 on enzyme activity (Seneweera *et al.* 2011, Jiang *et al.* 2012, Rai *et al.* 2016). In our experimental context, the elevated CO_2 concentration in combination with low temperature exerted a mitigating effect on reduction of Rubisco content induced by chilling. This allowed EC+LT leaves to maintain higher photosynthetic rates and reduce the adverse effect of low temperature on Rubisco oxygenase activity, upon return to prechilling conditions. The elevated CO_2 concentrations increased the Rubisco content, but it resulted in a significantly higher P_N only under saturating light levels, as shown by light-response curves, being photosynthesis limited by ribulose-1,5-bisphosphate (RuBP) regeneration at low irradiance levels. Our data demonstrate that the effect of elevated CO_2 concentrations on Rubisco protein content is evident and determines significant consequences not only after long-term growth but also after a few hours of exposure of plants to elevated CO_2 (Cheng *et al.* 1998).

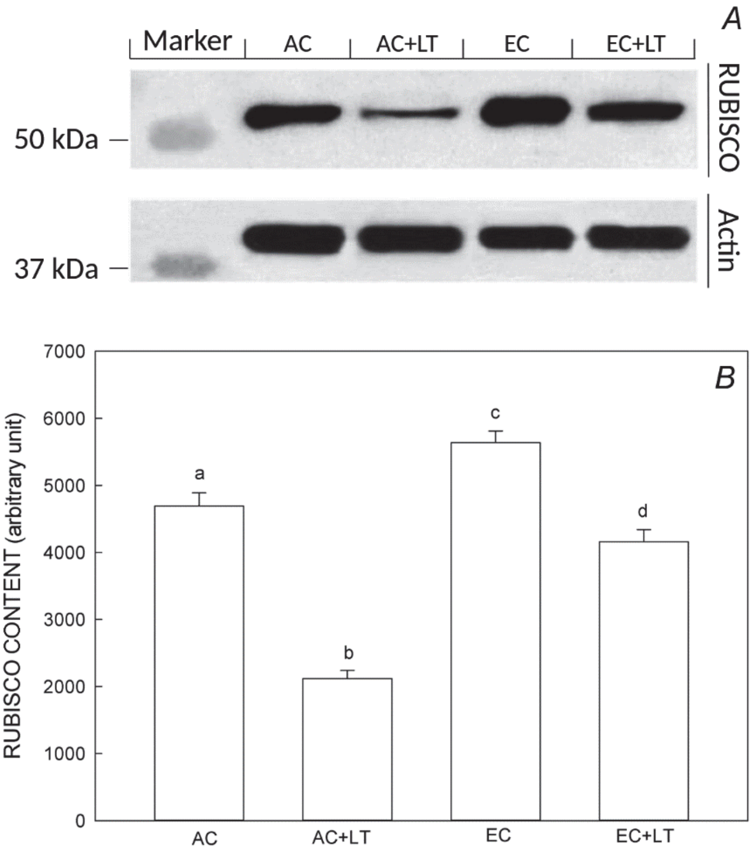

Fig. 4: (*A*) Rubisco expression (*B*) and content measured at the end of each treatment. Rubisco protein content in control and treated plants was determined by immunoblotting with protein-specific primary antibody. Actin was used as loading control. The bar diagrams represent pixel volumes of Rubisco bands normalised to the appropriate actin band. Values are means ± SE ($n = 6$). AC – ambient [CO_2]; AC+LT – ambient [CO_2] and low temperature; EC – elevated [CO_2]; EC+LT – elevated [CO_2] and low temperature. *Different letters* indicate statistically significant differences between treatments at 21% O_2.

Effects of different treatments on photochemistry and light energy partitioning: When CO_2 assimilation is limited by chilling, the excess of absorbed light must be dissipated to prevent or minimise the formation of reactive oxygen species, dangerous for the photosynthetic membranes. The exposure to chilling caused an instantaneous decline of PSII efficiency in both AC+LT and EC+LT plants accompanied by the reduction of F_v/F_m ratio; this was less pronounced in EC+LT plants.

Harmful conditions for photosynthetic apparatus may also persist after chilling, because of a delayed recovery of photosynthesis, enhancing the risks of photoinhibition or photooxidative damages (Melkonian *et al.* 2004). In our experiment, the recovery of F_v/F_m, F_0, and F_m within 14 h from chilling treatments indicated a reversible inactivation of PSII reaction centres in both AC+LT and EC+LT plants.

The capability of photosynthetic apparatus to recover after sudden temperature drops is associated to the activation of photoprotective pathways during recovery, ensuring the maintenance of plant productivity within certain limits. It may be crucial for many crop species, as chard, cultivated in Mediterranean areas, where short chilling episodes are typical during winter.

Even if both AC+LT and EC+LT plants showed the full recovery of photochemistry after 14 h from chilling, our results showed a significant chilling-induced reduction of photosynthesis in AC+LT compared to EC+LT plants, evidencing the positive effect of elevated CO_2 in mitigating chilling stress.

The different behaviour could be due to the different utilisation of the absorbed light in photochemistry. In AC+LT plants, the partitioning of reductive power between photosynthesis and alternative sinks consuming electrons, estimated by J_f/P_N ratio, did not vary compared to prechilling conditions (AC), indicating that the electron transport rate (J_f) was down-regulated by carbon metabolism. Our results are in contrast with data reported by other authors (Melkonian *et al.* 2004, Zhou *et al.* 2004) who found a higher electron flow to alternative photochemical sinks other than CO_2 fixation after returning to prechilling conditions.

Conversely to AC+LT, in EC+LT plants, the J_f/P_N ratio was higher compared to prechilling conditions, indicating a significant proportion of electron flew to processes other than CO_2 assimilation. The parallel increase of J_o in these plants, suggests that, among different sinks, a part of electron flow was directed to photorespiration.

The different behaviour between AC+LT and EC+LT plants was evident also from the analysis of the partitioning of absorbed light within PSII. As compared to the prechilling conditions, the exposure to low temperatures determined, in AC+LT plants, a reduction of light energy used in photochemistry (Φ_{PSII}), according to the P_N decrease. Contextually, the fraction of the regulated ΔpH- and xanthophyll-dependent thermal dissipation, Φ_{NPQ}, increased by about 80% compared to prechilling conditions, highlighting the importance of this pathway of thermal dissipation in consuming the excess of excitation energy when photochemistry and photosynthesis are reduced. However, Φ_{NPQ} was not sufficient to prevent the drastic reduction of maximal photochemical efficiency (F_v/F_m) observed in AC+LT leaves. In EC+LT plants, even if the contribution of Φ_{NPQ} was negligible compared to that occurred before the chilling conditions, only a slight reduction of F_v/F_m was observed. It may be hypothesised that in these plants, the increase of electron flow to O_2 successfully contributed to dissipate the excess of light energy to photosystems.

Conversely to $\Phi_{NPQ,}$ the nonregulated energy dissipation in PSII (Φ_{NO}) similarly increased in the two plant groups compared with control, indicating that a fraction of absorbed light was diverted to nonregulated energy conversion processes, favouring the production of reactive oxygen species (ROS) (Wang *et al.* 2009).

Nevertheless, even if the potential risk linked to the ROS production is similar for AC+LT and EC+LT plants, the dissipative processes activated by elevated CO_2 under low temperatures are more effective in limiting ROS action and are able to guarantee a higher photosynthesis and maximal photochemical efficiency in EC+LT plants.

In conclusion, the simultaneous short exposures of plants to low temperature and elevated CO_2 increased photorespiration upon to return to the prechilling conditions. Conversely, the exposure to low temperatures actively promoted the regulated thermal dissipation processes. These two dissipation mechanisms were not equally effective in PSII photoprotection as confirmed by the highest reduction of photosynthesis and maximal photochemical efficiency in AC+LT compared to EC+LT plants after chilling events.

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