Biologia Futura (2023) 74:327–335
https://doi.org/10.1007/s42977-023-00182-3

ORIGINAL PAPER

Fight against cold: photosynthetic and antioxidant responses of different bell pepper cultivars (*Capsicum annuum* L.) to cold stress

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Received: 15 November 2022 / Accepted: 12 September 2023 / Published online: 27 September 2023

Abstract

The special metabolites of bell pepper (*Capsicum annuum* L.) leaves can protect the plant under possibly damaging circumstances, such as high light, UV, unfavorable temperatures, or other environmental effects. In this study, we examined the cold stress tolerance of three different Hungarian pepper varieties (Darina, Édesalma, Rekord), focusing on the antioxidant and photosynthetic responses. The plants were developed in growth chambers under optimal temperature conditions (day/night 25 °C/20 °C) until the leaves on the fourth node became fully developed, then half of the plants received a cold treatment (day/night 15 °C/10 °C). Via a detailed pigment analysis, the PS II chlorophyll fluorescence responses, gas exchange parameters and total antioxidant capacities, leaf acclimation to low temperatures has been characterized. Our results display some of the developing physiological and antioxidant properties, which are among the main factors in monitoring the damaging effects of cold temperatures. Nevertheless, despite their differences, the tested pepper varieties did not show different cold responses.

Keywords Phenolics · Antioxidants · Cold treatment · Pepper · Photosynthesis

Abbreviations

A	Photosynthetic carbon assimilation
E	Transpiration
FCR	Folin–Ciocalteu reactivity
FRAP	Ferric reducing antioxidant power
F_v/F_m	Maximum quantum efficiency of PS II
gsw	Stomatal conductance
IRGA	Infrared gas analyzer
q_P	Photochemical quenching
TAC	Total antioxidant capacity
UV	Ultraviolet radiation
Y(II)	Photosystem II quantum efficiency
Y(NO)	Non-regulated non-photochemical quenching
Y(NPQ)	Regulated non-photochemical quenching

Introduction

One of the most significant challenges of the upcoming decades for agriculture is to evolve an efficient acclimation to the changing climate. Extreme or atypical weather events are to come (McGregor et al. 2005); thus, complex analyses of plant acclimative responses to unfavorable conditions are required. The bell pepper (*Capsicum annuum* L.) is a worldwide-grown important agricultural plant. The general practice of bell pepper cultivation at mid-latitudes consists of two phases: (1) From germination until early development plants are usually kept in greenhouses or plastic foil tents at relatively warm temperatures during early spring, (2) followed by the transplantation or translocation outdoors mid-spring (Rego et al. 2018). During the post-transplantation period, unpredictable cold outbreaks (Shongwe et al. 2007) can easily delay or inhibit seedling development. However, transplantation may include a sudden change in other environmental conditions, such as increasing wind speed, drought, or higher photon flux. All can be potential stress factors that may affect plant growth and development and increasing the vulnerability to biotic stressors or influencing fruit quality negatively (Leskovar and Cantliffe 1993).

The present study compares the cold tolerance of three bell pepper cultivars: 'Darina,' 'Édesalma,' and 'Rekord.' The experiment was performed in growth chambers; hence, the effects of low temperatures were studied under controlled conditions. Results were intended to serve as a model for future multifactor experiments and to identify cold tolerance markers.

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One of the main groups of plant polyphenols is the phenolic acids, which are the first biochemical products in the phenolic pathway; they further develop and regroup into various flavonoids, the key compounds of cold tolerance (Francini et al. 2019). Nevertheless, low temperatures have been known to enhance polyphenol biosynthesis in various plant species (Bilger et al. 2007; Watanabe and Ayugase 2015; Petridis et al. 2016).

Several studies demonstrated that polyphenols are potent antioxidants in vitro (Rice-Evans et al. 1996; Grace and Logan 2000; Agati et al. 2012; Csepregi et al. 2016), the connection between their production and their role in stress responses is not always unambiguous (Hernández et al. 2009). Cold was shown to have contrasting, species and stress-dependent effects on phenolic contents and antioxidant capacities. For example, it caused a decrease in total phenolics and in leaf radical scavenging capacity in both cold sensitive and susceptible grapevine cultivars (Król et al. 2015). On the other hand, Cansev et al. (2012) found in olive leaves that low temperature treatments resulted in significantly increased total phenolics and less affected antioxidant capacities. The main flavonoids of bell pepper leaves are flavones, apigenin and luteolin derivatives (Soo-Yeon et al. 2020). Genzel et al. (2021) showed that moderate cold treatment increased the levels of luteolin glycosides, and a recent study showed that several other flavonoids were also stimulated by low temperature (Reimer et al. 2022).

The aim of the present study was to explore correlations between leaf polyphenol content, antioxidant properties, and cold tolerance in bell pepper plants. Total antioxidant capacities (TAC) were shown to reflect stress induced changes in a variety of plants (Petridis et al. 2012; Csepregi et al. 2016, Popovic et al. 2016), and successful acclimation is usually characterized by higher TAC. Because different TAC assays may have different re-activities to the same phenolic compound (Csepregi et al. 2016), two different TAC methods were used. Leaf phenolic content can also be characterized by the ultraviolet radiation (UV) absorption of ethanolic extracts. All phenolic compounds absorb UV, but to different extents in UV-A (315–400 nm) and UV-B (280–315 nm). Phenolic acids had the strongest UV-B absorbing capacity among all compounds, approx. 10-times higher than anthocyanins. Flavones, the main representatives of flavonoids in pepper leaves (Soo-Yeon et al. 2020; Genzel et al. 2021), are weaker UV absorbers than phenolic acids, but stronger than anthocyanins. On the other hand, flavones have relatively lower UV-A abs: UV-B abs ratio than either phenolic acids or anthocyanins (Csepregi and Hideg 2018). Therefore, UV-B absorption of leaf extracts can be regarded as an approximation of phenolic acid content and UV-A absorption characterizes further phenolics.

The applied TAC measurements and UV photometric phenolic estimating methods are simple, low cost and high throughput assays, our results showed that these methods can complement the physiological characterization of cold stress responses of young bell pepper plants.

Materials and methods

Chemicals

Pure phenolic compounds were purchased from Merck (Merck Industrial and Laboratory chemicals, Darmstadt, Germany), and other chemicals were purchased from VWR (VWR International Kft., Debrecen, Hungary).

Experimental design

Bell pepper (*Capsicum annuum* L. cv. Darina, Édesalma, and Rekord) plants were grown on garden soils in 8 × 8 cm rectangular plastic pots in a growth chamber (Fitotron, SGC 120 Plant Growth Chamber, Weiss Technik UK, Loughborough, UK) under 110 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) under long-day conditions (16/8 h, control temperature: 25/20 °C; cold treatment: 15/10 °C) and 65% relative humidity for 5 weeks. For this treatment, 5-week-old plants were side-by-side under a Philips TL20 W/01RS tube (Philips Electronics, Roosendaal, The Netherlands) to 5 days. Two fully developed leaves from the 3rd node of each plant were analyzed with non-invasive methods. Following these fresh weights were measured; and then, leaves were frozen in liquid N_2 and stored at − 60 °C for further analyses.

Non-invasive measurements

Infrared gas analyzer (IRGA, LI-6800, LI-COR Biosciences GmbH, Bad Homburg, Germany) measurements were taken at midday (11:00–15:00) on fully developed intact leaves. Saturating light conditions (600 µmol photons m^{-2} s^{-1}) were applied on leaves by using a red–blue LED light source (red 9: blue 1) attached to the instrument. The humidity in the leaf chamber was set to 60%. The concentration of carbon dioxide was 400 µmol m^{-1} in the reference chamber. Stomatal conductance (gsw) and transpiration (E) were expressed in mol H_2O m^{-2} s^{-1}, and photosynthetic carbon assimilation (A) was expressed in µmol CO_2 m^{-2} s^{-1}.

Chlorophyll-*a* fluorescence (MAXI-version of the Imaging PAM, Heinz Walz GmbH, Effeltrich, Germany) was used to characterize leaf photochemistry and quenching. Plants were kept in darkness for 30 min; then, fluorescence yields were measured prior to (minimal fluorescence, F_0) and during (maximal fluorescence, F_m) a saturating pulse. The maximum photosystem (PS) II quantum yield was calculated from these data as $F_v/F_m = (F_m - F_0)/F_m$. Following this, leaves were illuminated by blue actinic light corresponding

to 110 µmol m^{-2} s^{-1} PAR for 5 min to record F and F'_m fluorescence yields. The light acclimated, effective PS II quantum yield was calculated as $Y(II) = (F'_m - F_0)/F'_m$, and the non-regulated ($Y(NO)$) and regulated non-photochemical ($Y(NPQ)$) quenching were characterized as $Y(NO) = F/F_m$ and $Y(NPQ) = F/F'_m - F/F_m$, respectively (Klughammer and Schreiber 2008). The efficiency of energy transfer from PSII antenna toward the opened PSII reaction centers (PSII trapping efficiency) was characterized by the $Y(II)$: photochemical quenching (q_P) ratio (Rosenqvist and van Kooten 2003), where $q_P = (F'_m - F)/(F'_m - F'_0)$ (Bilger and Schreiber 1986).

Chlorophyll, flavonoid, and anthocyanin contents were estimated by a non-invasive optical method on adaxial and abaxial leaf sides using the Dualex Scientific™ instrument (ForceA, Orsay, France) (Goulas et al. 2004; Cerovic et al. 2012).

Preparation of leaf extracts

Leaf samples for antioxidant capacity measurements were prepared from frozen leaves. Leaf samples were lyophilized (SCANVAC CoolSafe 110-4, LaboGene, Denmark), then extracted into aqueous alcohol (70:30, V:V, EtOH:H_2O). Extraction included sonication in a sonic bath (RoHS JP-020, Shenzhen, China) for 15 min, then centrifugation at 15,000×g for 10 min at room temperature (Thermo Fisher Scientific Inc., Waltham, MA, USA). The supernatant was collected, and the procedure was repeated two more times so that the three supernatants were subsequently combined.

Folin–Ciocalteu reactivity (FCR)

FCR is a widely used method for the spectrophotometric determination of the total flavonoid content of plant extracts. The assay is based on the molybdenum ion's oxidation by the secondary metabolites in the plant extracts. In this experiment, we used this method with a modification (Csepregi et al. 2013) of the original technique (Singleton and Rossi 1965), where 90 µL FC reagent (diluted 1:10 with distilled water) was added to 20 µL sample, and after 5 min, another 90 µL Na_2CO_3 (6% w/v) was added. After 90 min of incubation at room temperature, we measured the absorbance of the solution at 651 nm (Multiskan FC plate reader, Thermo Fischer Scientific, Shanghai, China). Gallic acid was used for calibration, and FRC of leaf extracts was given in mM gallic acid equivalents mg^{-1} leaf FW.

Ferric reducing antioxidant power (FRAP)

The method is based on the antioxidants' ability to reduce Fe^{3+} to Fe^{2+} resulting in a blue-colored product (Benzie and Strain 1996). The FRAP reagent was a mixture of 25 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL of 2,4,6-tripyridyl-*S*-triazine (10 mM TPTZ in 40 mM HCl), and 2.5 mL $FeCl_3$ (20 mM $FeCl_3$ in distilled water). A total of 10 µL of diluted leaf extract was added to 190 µL of FRAP reagent, and absorbance at 620 nm was measured with the above plate reader twice: first immediately after mixing, then after 30-min incubation at room temperature. The assay was calibrated with ascorbic acid (ASA), and results were given in mM ASA equivalents mg^{-1} leaf FW.

UV absorbtion

Leaf extracts were diluted in acidified aqueous alcohol (70:29:1, V:V:V, EtOH:H_2O:HCl) in order to obtain maximal UV absorbance between 1 and 1.5, and absorption spectra were recorded between 280 and 400 nm with a spectrophotometer (Shimadzu UV-1800, Shimadzu Corp., Kyoto, Japan). Spectra were corrected for background absorption of the solvent; then, total absorption per nm was determined separately in the UV-A (315–400 nm) and UV-B (280–315 nm) regions. Quercetin was used as reference compound, and UV absorbing pigment capacities of leaf extracts were characterized as mM quercetin equivalent mg^{-1} leaf FW units (Csepregi and Hideg 2018).

Statistical analysis

Each treatment group contained five plants. Two leaves from each plant were chosen for the analyses, and all measurements were performed in 3–4 technical repetitions. Results are presented as means ± standard deviations ($n = 10$). All data were normally distributed. Combined and single-factor effects of cold temperature and pepper varieties on the measured parameters were analyzed with two-way ANOVA and Tukey's post hoc test, differences were regarded significant when $p < 0.05$ in all methods. Correlations between means were analyzed with Pearson's method. Correlations were characterized by the correlation coefficient r and p values of t tests using the null hypothesis that there was correlation between parameters. This hypothesis was rejected for data sets with $p < 0.05$ and these were concluded to not correlated. All calculations were carried out using the PAST software (Hammer et al. 2001).

Results

In the absence of cold stress all varieties had the same maximum photochemical yields (F_v/F_m). Light acclimated yields, on the other hand were different, the Darina had the highest $Y(II)$ and there was no difference between the two other varieties (Table 1). Low t temperature decreased F_v/F_m in all varieties. $Y(II)$ decreased in Darina and Rekord by ca. 40% but did not change significantly in Édesalma. Although,

Table 1 Effects of low temperature on maximum (F_v/F_m) and 110 µmol m^{-2} s^{-1} PAR acclimated effective (Y(II)) quantum yields; the regulated (Y(NPQ)) and non-regulated (Y(NO)) non-photochemical quenching of PSII

Plant variety, treatment	F_v/F_m	Y(II)	Y(NPQ)	Y(NO)	Y(II): q_P
F ($df1=1$, $df2=24$) and p values (variety)	3.7, n.s.	12.7, n.s.	9.0, n.s.	0.7, n.s.	4.5, $p<0.05$
F ($df1=2$, $df2=24$) and p values (low temp.)	247.5, $p<0.05$	70.2, n.s.	48.9, n.s.	106.5, $p<0.05$	44.9, $p<0.05$
F ($df1=2$, $df2=24$) and p values (interaction)	7.3, n.s.	6.5, n.s.	1.5, n.s.	1.3, n.s.	5.7, $p<0.05$
Darina, control	0.825 ± 0.037^{a}	0.555 ± 0.052^{a}	0.219 ± 0.062^{a}	0.225 ± 0.036^{a}	0.674 ± 0.116^{a}
Édesalma, control	0.797 ± 0.044^{a}	0.379 ± 0.032^{b}	0.314 ± 0.087^{a}	0.307 ± 0.098^{a}	0.601 ± 0.046^{a}
Rekord, control	0.798 ± 0.039^{a}	0.438 ± 0.024^{b}	0.303 ± 0.045^{a}	0.258 ± 0.053^{a}	0.602 ± 0.057^{a}
Darina, low temperature	0.501 ± 0.060^{a}*	0.337 ± 0.059^{a}*	0.093 ± 0.039^{a}*	0.569 ± 0.088^{a}*	0.466 ± 0.029^{ab}*
Édesalma, low temperature	0.624 ± 0.037^{b}*	0.316 ± 0.050^{a}	0.134 ± 0.028^{a}*	0.549 ± 0.070^{a}*	0.412 ± 0.032^{a}*
Rekord, low temperature	0.531 ± 0.045^{a}*	0.273 ± 0.063^{a}*	0.206 ± 0.030^{b}	0.522 ± 0.053^{a}*	0.560 ± 0.028^{b}*

The Y(II): q_P refers to the PSII trapping efficiency. Results of two-factor ANOVA are shown as F and p values. Replacing a p value with n.s. indicates that although the corresponding F value was higher than F_{crit} (2.24) = 3.4 but factor significance was not confirmed by Tukey's post hoc test

Lower case letters: variety effect; significant ($p<0.05$) difference between varieties under the same conditions

*Low temperature effect: significant ($p<0.05$) difference between control and treated plants of the same variety

Y(II) parameters were not uniformly affected by cold: The observed, significant, approx. 50% increase in non-regulated non-photochemical quenching Y(NO) indicated stress in all varieties. Decrease in regulated non-photochemical quenching Y(NPQ) was observed in two varieties (Darina, Édesalma) confirming stress. The PSII trapping efficiency (Y(II): q_P) was decreased by 30–35% in Darina and Rekord, and by 12% in Édesalma, indicating impeded energy flow between LHCII and PSII.

Leaf gas exchange measurements showed no significant differences either among varieties or treatment groups in general (Table 2). Although, relatively high variances within groups mask differences cold seemed to tend to limit stomatal opening and consequently gas exchange, especially in the Rekord variety.

Comparison of leaf pigments showed that all varieties had the same epidermal flavonol index and anthocyanin indexes were very similar, but chlorophyll were more diverse, and followed a Darina > Édesalma > Rekord order. Cold treatment decreased leaf chlorophyll content in all varieties on both adaxial and abaxial leaf sides. Untreated leaves were characterized by approx. 30% higher adaxial than abaxial flavonol indexes, which were not significantly affected by cold in either cultivar. Anthocyanin contents, on the other hand, were always 15–25% lower on the adaxial than the abaxial side, and cold treatment increased both indexes by ca. 20% (Table 3). The instrument calculates flavonol index from 375 nm absorption (Goulas et al. 2004). Therefore, flavonoids other than flavonols, which are characterized by higher UV-B than UV-A absorption (Csepregi and Hideg

Table 2 Effects of low temperature on net carbon assimilation (A, µmol m^{-2} s^{-1}), transpiration (E, mol m^{-2} s^{-1}), stomatal conductance (gsw, mol m^{-2} s^{-1}) and water use efficiency (A/gsw)

Plant variety, treatment	A	E	gsw	A/gsw
F ($df1=1$, $df2=24$) and p values (variety)	1.7, n.s.	0.6, n.s.	0.1, n.s.	6.1, n.s.
F ($df1=2$, $df2=24$) and p values (low temp.)	24.7, n.s.	7.4, n.s.	9.9, n.s.	0.8, n.s.
F ($df1=2$, $df2=24$) and p values (interaction)	0.3, n.s.	4.3, n.s.	3.3, n.s.	1.0, n.s.
Darina, control	$1.41\pm6.03\ 10^{-1}$	$1.17\ 10^{-4}\pm5.46\ 10^{-5}$	$7.19\ 10^{-3}\pm3.20\ 10^{-3}$	194.96 ± 147.26
Édesalma, control	$1.02\pm3.48\ 10^{-1}$	$1.46\ 10^{-4}\pm1.75\ 10^{-5}$	$8.64\ 10^{-3}\pm1.08\ 10^{-3}$	118.97 ± 40.48
Rekord, control	$1.11\pm3.16\ 10^{-1}$	$1.78\ 10^{-4}\pm7.47\ 10^{-5}$	$1.04\ 10^{-2}\pm4.32\ 10^{-3}$	113.02 ± 18.42
Darina, low temperature	$0.637\ 10^{-1}\pm0.210$*	$2.60\ 10^{-4}\pm8.04\ 10^{-5}$*	$7.29\ 10^{-3}\pm2.44\ 10^{-3}$	80.57 ± 16.46
Édesalma, low temperature	0.434 ± 0.156	$1.97\ 10^{-4}\pm6.00\ 10^{-5}$	$5.44\ 10^{-3}\pm1.53\ 10^{-3}$	80.79 ± 28.29
Rekord, low temperature	0.307 ± 0.116*	$1.64\ 10^{-4}\pm1.69\ 10^{-5}$	$4.55\ 10^{-3}\pm6.01\ 10^{-4}$*	64.68 ± 30.69

Replacing a p value with n.s. indicates that although the corresponding F value was higher than F_{crit} (2.24) = 3.4 but factor significance was not confirmed in Tukey's post hoc test

*Low temperature effect: significant ($p<0.05$) difference between control and treated plants of the same variety

Table 3 Effects of low temperature on estimated chlorophyll indexes (Chl AD, Chl AB), flavonoid indexes (Flav AD, Flav AB) and anthocyanin indexes (Anth AD, Anth AB)

Plant variety, treatment	Chl AD	Chl AB	Flav AD	Flav AB	Anth AD	Anth AB
F ($df1=1$, $df2=24$) and p values (variety)	72.2, $p<0.05$	59.9, n.s.	10.1, n.s.	8.6, n.s.	48.2, n.s.	38.1, $p<0.05$
F ($df1=2$, $df2=24$) and p values (low temp.)	82.6, $p<0.05$	50.6, $p<0.05$	11.7, n.s.	4.2, n.s.	67.7, $p<0.05$	65.8, $p<0.05$
F ($df1=2$, $df2=24$) and p values (interaction)	1.4, n.s.	0.7, n.s.	11.1, n.s.	2.9, n.s.	0.5, n.s.	1.2, n.s.
Darina, control	24.20 ± 2.33^{a}	23.23 ± 3.16^{a}	0.73 ± 0.12^{a}	0.55 ± 0.11^{a}	0.14 ± 0.02^{a}	0.18 ± 0.02^{a}
Édesalma, control	21.54 ± 2.04^{b}	20.99 ± 2.31^{a}	0.76 ± 0.14^{a}	0.61 ± 0.12^{a}	0.15 ± 0.02^{a}	0.20 ± 0.01^{b}
Rekord, control	17.34 ± 1.91^{c}	16.21 ± 1.76^{b}	0.77 ± 0.13^{a}	0.60 ± 0.09^{a}	0.18 ± 0.02^{b}	0.22 ± 0.02^{c}
Darina, low temperature	17.77 ± 2.29^{a}*	18.80 ± 2.31^{a}*	0.83 ± 0.15^{a}	0.46 ± 0.09^{a}	0.17 ± 0.02^{a}*	0.21 ± 0.02^{a}
Édesalma, low temperature	18.81 ± 1.98^{a}*	17.99 ± 2.17^{a}*	0.99 ± 0.16^{b}*	0.64 ± 0.11^{b}	0.18 ± 0.02^{a}*	0.23 ± 0.02^{a}
Rekord, low temperature	13.74 ± 1.38^{b}*	12.78 ± 1.85^{b}*	0.70 ± 0.18^{a}	0.50 ± 0.12^{a}	0.20 ± 0.01^{b}*	0.24 ± 0.02^{b}

Replacing a p value with n.s. indicates that although the corresponding F value was higher than F_{crit} (2.24) = 3.4 but factor significance was not confirmed in Tukey's post hoc test

Lower case letters: variety effect; significant ($p<0.05$) difference between varieties under the same conditions

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2018) may not be fully represented in results. Table 4 shows UV absorption values measured in our samples. Neither UV absorbing capacities showed cultivar dependence in untreated plants. Cold stress decreased UV-A and UV-B absorbing parameters by approx. 50% and 30%, respectively. Although strong UV absorbing phenolic compounds are known to have high antioxidant capacities, cold treatment evoked opposite changes in TAC values than in UV absorption (Table 4). Both FRAP and FC assays detected significant, 35–50% increases in TAC, in all cultivars.

Discussion

Our results show that even a short-term cold period is capable to initiate stress responses in bell pepper seedlings. Significantly reduced photochemical yields (Y(II)) and decreased regulated energy dissipating pathways (Y(NPQ)) indicated cold-triggered light stress in all varieties. Cold temperatures can limit the activation of violaxanthin de-epoxidase enzyme and thus obstruct the efficient energy dissipating in PSII antennas (Arvidsson et al. 1997; Latowski et al. 2003). In our experiment, this was accompanied by an increase in non-regulated non-photochemical quenching (Y(NO)), suggesting elevated production of ROS due to cold. The light-induced ROS may damage light-harvesting antenna complexes and result in less effective energy transfer between the LHCII and the PSII, as indicated by the cold-induced reduction of PSII trapping efficiency (Y(II): q_P) in all cultivars (Table 1). The negative correlation between Y(NPQ) and the chlorophyll index in cold-treatment but not in control plants (Fig. 1) also suggests photo-inhibition in the former.

Table 4 Effects of low temperature on antioxidant and UV absorbing (FRAP, FC, UV-A abs, UV-B abs and total UV abs) properties

Plant variety, treatment	FRAP	FC	UV-A abs	UV-B abs	Total UV abs
F ($df1=1$, $df2=24$) and p values (variety)	5.4, n.s.	3.8, n.s.	2.3, n.s.	3.9, n.s.	2.7, n.s.
F ($df1=2$, $df2=24$) and p values (low temp.)	62.7, $p<0.05$	36.9, $p<0.05$	49.9, $p<0.05$	34.6, n.s.	45.6, $p<0.05$
F ($df1=2$, $df2=24$) and p values (interaction)	0.3, n.s.	0.1, n.s.	0.3, n.s.	1.0, n.s.	0.5, n.s.
Darina, control	0.677 ± 0.047	0.859 ± 0.080	6.888 ± 1.549	7.242 ± 1.648	6.995 ± 1.566
Édesalma, control	0.698 ± 0.092	0.951 ± 0.108	8.445 ± 1.146	9.050 ± 1.230	8.672 ± 1.141
Rekord, control	0.876 ± 0.228	1.018 ± 0.279	8.316 ± 2.479	9.881 ± 2.639	8.786 ± 2.517
Darina, low temperature	1.056 ± 0.134*	1.172 ± 0.121*	3.941 ± 0.296*	5.177 ± 0.425	4.312 ± 0.304*
Édesalma, low temperature	1.064 ± 0.045*	1.276 ± 0.112*	4.719 ± 0.595*	5.735 ± 0.596*	5.025 ± 0.588*
Rekord, low temperature	1.180 ± 0.071*	1.386 ± 0.120*	4.578 ± 0.741*	6.048 ± 0.722*	5.020 ± 0.734*

Replacing a p value with n.s. indicates that although the corresponding F value was higher than F_{crit} (2.24) = 3.4 but factor significance was not confirmed in Tukey's post hoc test

*Low temperature effect: significant ($p<0.05$) difference between control and treated plants of the same variety

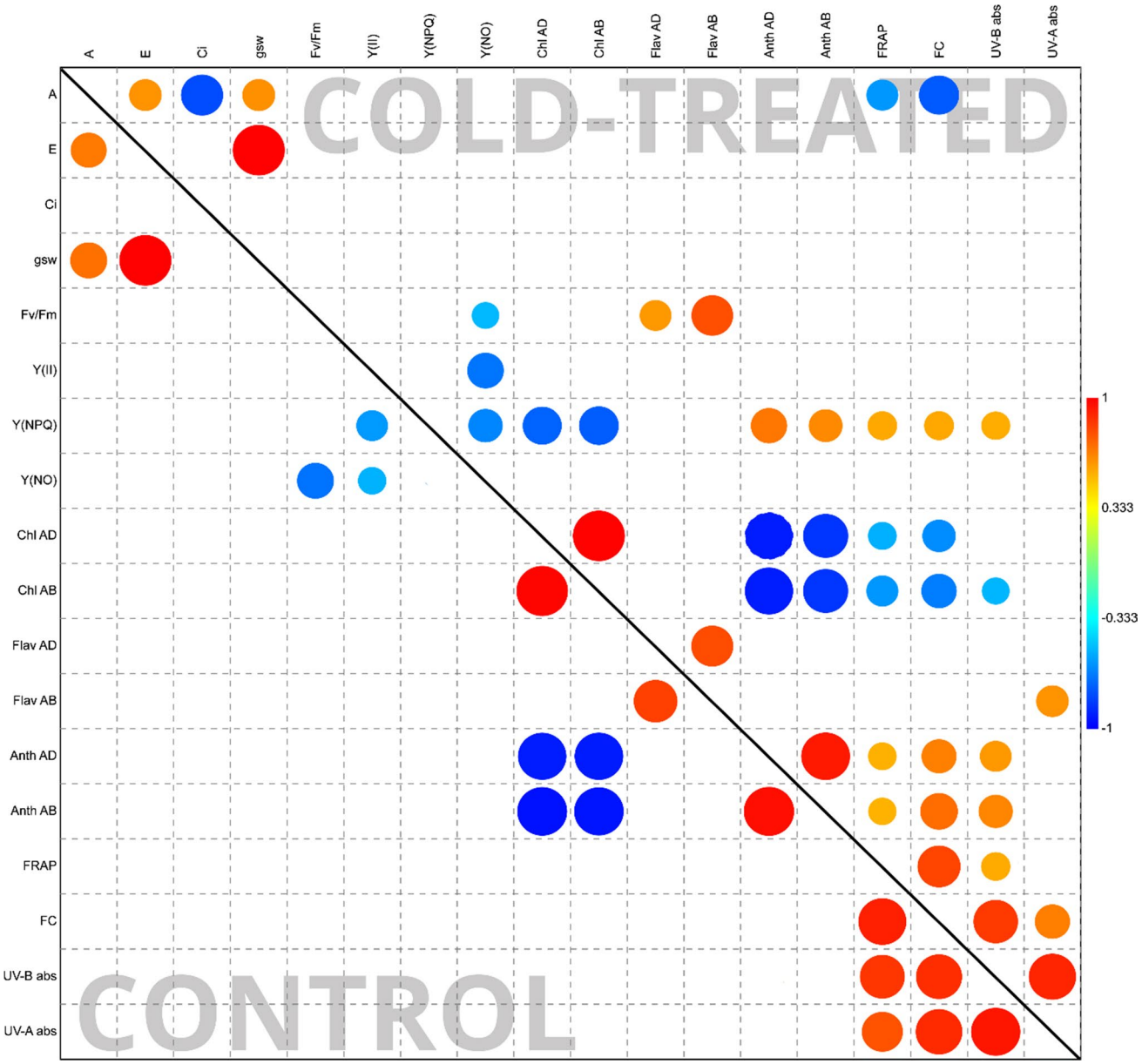

Fig. 1 Pearson correlation analysis between all measured variables in treated and untreated plants. Blank squares indicate no significant correlation (p 0.05) and squares with colored circles indicate significant correlation (p 0.05). The cold–warm color scale indicates the positive or negative value of the correlation coefficient. The following abbreviations are used in the correlation table: A, photosynthetic carbon assimilation; E, transpiration; Ci, intercellular CO_2 concentration; gsw, stomatal conductance; F_v/F_m, maximum quantum efficiency of PS II; Y(II), photosystem II quantum efficiency; Y(NPQ), regulated non-photochemical quenching; Y(NO), non-regulated non-photochemical quenching; Chl AD, adaxial chlorophyll index; Chl AB, abaxial chlorophyll index; Flav AD, adaxial flavonoid index; Flav AB, abaxial flavonoid index; Anth AD, adaxial anthocyanin index; Anth AB, abaxial anthocyanin index; FRAP, Ferric reducing antioxidant power; FC, Folin–Ciocalteu reactivity; UV-B abs, UV-B absorbing properties; UV-A abs, UV-A absorbing properties

In addition to affecting photosynthesis parameters and metabolite contents, cold stress also altered statistical correlations among these, implying old-induced changes in regulatory pathways. These are illustrated in Fig. 1. Positive correlations among assimilation, transpiration and stomatal conductance were observed both on control and cold-treated plants in accordance with earlier reports on cold treatment limiting stomatal opening and consequently gas exchange (Adamski et al. 2020; Wilkinson et al. 2001). Cold triggers stomatal closure and transpiration reduction for aiding the plants to withstand the suboptimal temperature. Just like the effect of drought, stomatal guard cells quickly close the stomata under unfavorable temperatures to prevent the plants from dehydrating (Agurla et al. 2018). The negative correlation between the

net photosynthesis and the internal carbon dioxide concentration (Ci) in cold-treated plants shows that the latter was a photosynthesis limiting factor in stressed plants only. The negative correlation of chlorophyll indexes (both adaxial and abaxial) with TAC, only observed in cold-treated leaves, indicates the role of elevated antioxidant capacities in protection against photooxidative stress leading to chlorophyll degradation. Interestingly, flavonoid indexes were not correlated to TAC, although flavonoids are good antioxidants. Because the Dualex instrument can only detect pigments in the epidermis or tissue layer very close to this, cold-inducible flavonoids concentrated in the mesophyll layers (Agati et al. 2012) and thus not likely to be detected.

After the cold treatment, leaf total antioxidant capacities increased, as well as anthocyanin levels, and the two parameters became positively correlated. Anthocyanins are the end products of the phenylpropanoid synthesis, which have better antioxidant properties than phenolic acids or flavonoids (Csepregi et al. 2016), and high UV-B but low UV-A absorbing capacities (Csepregi and Hideg 2018). These properties explain the strong positive correlation between the anthocyanin levels, TAC and UV-B absorbing capacity in cold-treated plants, which was not found in controls (Fig. 1). TAC parameters showed strong positive correlations with both UV-B and UV-A absorbing capacities of leaf extracts in control plants. With one exception, these statistical connections were also found in cold-treated leaves, in which UV-A absorption and FRAP were not significantly connected. This indicates a cold stress-induced metabolite rearrangement in favor of not only anthocyanins (see above) but also phenolic acids, which are very effective UV-B absorbers. These compounds are early products in the phenylpropanoid pathway and precursors of flavonoids and anthocyanins. Phenolic acids have higher FC than FRAP reagent reactivity (Csepregi et al. 2016); therefore, a selective increase in phenolic acid contents would explain the lack of correlation between FRAP and UV-B absorbing capacity in cold-treated leaves (Fig. 1). The cold-induced strong positive correlation of regulated energy dissipation with anthocyanin indexes and total antioxidant capacities suggests a combination of defense strategies: prevention of photoinhibition and ROS scavenging. An active role of anthocyanins in preventing photoinhibition was proposed to occur under extreme stress, when energy dissipation mechanisms are completely exhausted (Simkin et al. 2022), and our study suggests the involvement of these pigments in less harsh conditions, in low temperature facilitated light stress, too.

Conclusions for future biology

Even though the varieties of pepper examined were different (sweet, spicy), we found no difference in the response to low temperatures. It is conceivable that the low temperature responses are unified, which are not or only less affected by differences between species. Recommended indicator parameters for rapid testing of low temperature effects are chlorophyll fluorescence derived yield and non-photochemical quenching parameters, as well as the non-invasively estimated anthocyanin and chlorophyll indexes. Metabolite profiling, and a detailed analysis of cold-induced stomata closure are possible targets of future experiments. Stomatal closure is a crucial step of the development of cold tolerance in plants, in the regulation of which abscisic acid plays a prominent role (Chinnusamy et al. 2004). To learn more about the gas exchange responses against cold stress and to develop cold-tolerant genotypes, it is essential to include abscisic acid in future experiments. Results also display that phenolics are an extremely diverse compound groups with various properties. In addition, protect against the cold stress, phenolics can also play an important role in overcoming other stress effects; therefore, their further examination is essential.

Author contributions KC performed and analyzed the TAC measurements, AR analyzed the photosynthetic parameters and performed statistical analysis, GC designed the experiment, performed the photosynthetic parameters, and contributed to data analysis, NN and DK performed pigment estimation and contributed to the rest of the experiments, and ÉH contributed to discussions. KC coordinated the writing of the paper, to which all co-authors contributed.

Funding Open access funding provided by University of Pécs. This work was supported by the National Research, Development and Innovation Office (Grant Number NN-128806) and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00362/20/4).

Declarations

Conflict of interest The authors declare no competing interests.

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