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Polyploidy-mediated divergent light-harvesting and photoprotection strategies under temperature stress in a Mediterranean carnation complex

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Abstract:	Polyploidy can induce physiological novelties with adaptive potential, which may influence the range of environmental conditions that a neopolyploid tolerates. Dianthus broteri (Caryophyllaceae) is an autopolyploid complex that comprises four ploidy levels (2×, 4×, 6× and 12×) with separate distributions in the Iberian Peninsula, occupying different ecological niches along a gradient of temperature and aridity. We designed an experimental approach to disentangle the differential photochemical responses to temperature (from -3 ºC to 53 ºC) among D. broteri cytotypes by the measurement of leaf chlorophyll fluorescence. Our results showed higher energy fluxes, Fv/Fm and delayed fluorescence values along low and mild temperature levels in lower ploidies (2× and 4×) compared to higher ones (6× and 12×). This pattern would allow lower cytotypes to enhance their photosynthetic apparatus functionality in environmentally non-stressful habitats as those they inhabit. Contrarily, the 6× cytotype exhibited the overall lowest energy fluxes based on a reduced absorption while maximizing its flux ratios. Moreover, the 12× cytotype had notably high dissipation fluxes to ensure photoprotection, maintaining low but constant photochemical efficiency. These latter strategies would cause the reduction of photosynthetic capacities but help higher ploidies to tolerate the semi-arid Mediterranean environmental conditions with high temperatures under which they live.
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Highlights

- *D. broteri* showed inter-cytotype photochemical differences under heat and cold.
- An approach based on chlorophyll fluorescence in detached leaves was found suitable.
- 2× and 4× cytotypes showed overall higher energy fluxes than 6× and 12× ones.
- Light-harvesting and photoprotective responses to temperature stress were found.
- Each cytotype exhibited a divergent strategy related to its environmental niche.

Polyploidy-mediated divergent light-harvesting and photoprotection strategies under temperature stress in a Mediterranean carnation complex

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ABSTRACT

Polyploidy can induce physiological novelties with adaptive potential, which may influence the range of environmental conditions that a neopolyploid tolerates. *Dianthus broteri* (Caryophyllaceae) is an autopolyploid complex that comprises four ploidy levels (2×, 4×, 6× and 12×) with separate distributions in the Iberian Peninsula, occupying different ecological niches along a gradient of temperature and aridity. We designed an experimental approach to disentangle the differential photochemical responses to temperature (from -3 ºC to 53 ºC) among *D. broteri* cytotypes by the measurement of leaf chlorophyll fluorescence. Our results showed higher energy fluxes, F_v/F_m and delayed fluorescence values along low and mild temperature levels in lower ploidies (2× and 4×) compared to higher ones (6× and 12×). This pattern would allow lower cytotypes to enhance their photosynthetic apparatus functionality in environmentally non-stressful habitats as those they inhabit. Contrarily, the 6× cytotype exhibited the overall lowest energy fluxes based on a reduced absorption while maximizing its flux ratios. Moreover, the 12× cytotype had notably high dissipation fluxes to ensure photoprotection, maintaining low but constant photochemical efficiency. These latter strategies would cause the reduction of photosynthetic capacities but help higher ploidies to tolerate the semi-arid Mediterranean environmental conditions with high temperatures under which they live.

Keywords:

Autopolyploidy; *Dianthus broteri*; Photochemical efficiency; Energy fluxes; Temperature stress; Environmental constraints

1. Introduction

Polyploidy is the state of organisms with more than a pair of chromosome sets. In plants, it is extensively documented that polyploidy-associated novelties and subsequent local adaptation can alter phenotype and fitness through morphological, reproductive and/or physiological shifts (Levin, 2002; Ramsey and Ramsey, 2014). In fact, abiotic tolerances have been addressed as one of the key factors promoting habitat differentiation in polyploids relative to their diploid progenitors (Marchant et al., 2016). However, there is an evident lack of information in a context of physiology, functional traits or ecology regarding polyploid systems (Soltis et al., 2016). The few physiological studies on polyploids show an important bias towards the use of diploid-tetraploid comparisons (e.g. Li et al., 2011; Vyas et al., 2007) and, therefore, new approaches with high polyploid complexes are required to test the significance of increases in ploidy in such conclusions. Moreover, a common confounding factor in these studies is the allopolyploidy (polyploids originated from interspecific hybridization; e.g. Coate et al., 2012; Martínez et al., 2018) which produce transgressive phenotypes and diverse combinations of traits of the hybridizing partners (Seehausen, 2004). For this reason, new studies are also needed to ascertain if the adaptive advantage of allopolyploids over diploids across a broad range of environments could be extended to wild autopolyploids (polyploids originated from a single species), as recommended by Wei et al. (2019).

Dianthus broteri (Caryophyllaceae) is a recently radiated autopolyploid complex of perennial herbaceous plants with four different cytotypes (2×, 4×, 6× and 12×; Balao et al., 2010, 2009). Endemic to the Iberian Peninsula, this complex encompasses a considerable diversity of microhabitats, but each cytotype is distributed in a different geographic range and always in single-ploidy populations (Balao et al., 2009). Across *D. broteri* cytotypes, several polyploidy-associated shifts have been identified in morphological traits (vegetative and reproductive organs; Balao et al., 2011) and global cytosine methylation levels (Alonso et al., 2016). Furthermore, it has been addressed that *D. broteri* distribution is greatly constrained by abiotic factors, essentially temperature, water availability and soil properties. Thus, environmental gradients fostered niche evolution in this polyploid complex causing lower cytotypes (2× and 4×) to occupy broader niches with milder environmental conditions compared to the higher ploidies (6× and 12×), which are distributed in warmer, drier and more restricted niches

(López-Jurado et al., 2019a). Specifically, the dodecaploid cytotype (*Dianthus inoxianus*; Gallego, 1986) has received special attention because it is the highest-order polyploidy category in the genus. Moreover, it is considered threatened (Balao et al., 2007) and probably supports key vulnerable ecosystem functions (López-Jurado et al., 2019b). This cytotype also showed a remarkable high tolerance to physical damage to roots (López-Jurado et al., 2019b) and water stress (López-Jurado et al., 2016). In fact, an ecophysiological characterization of *D. inoxianus* under drought events revealed a great integrity of its photochemical apparatus, as indicated by the maintenance of photochemical efficiency, non-biochemical limitations and pigments concentrations stability (López-Jurado et al., 2016).

Given the distribution range of *D. broteri* cytotypes in the western Mediterranean basin, climate change effects will exert a great constraint on them. In the Iberian Peninsula and the entire Mediterranean area, significant changes in temperature have been addressed (Fonseca et al., 2016; Giorgi and Lionello, 2008), as extreme temperature events (Abaurrea et al., 2018; Lau and Nath, 2014). Short heat and cold episodes trigger some well described mechanisms in plant metabolism and might have important consequences on plant phenology, reproduction and physiology (Hatfield and Prueger, 2015; Menzel et al., 2011; Pérez-Romero et al., 2019). These response processes are shared by both temperature stresses (Kaplan et al., 2004) and involve, among others, the synthesis of reactive oxygen species (ROS), antioxidants, abscisic acid (ABA), shock proteins and changes in fatty acids composition (Maeda et al., 2006; Penfield, 2008; Wahid et al., 2007). Eventually, the photosynthetic rate decreases due to the reduced RuBisCO activation and the dysfunction of photosystems (Ensminger et al., 2006; Hikosaka et al., 2006; Mathur et al., 2014).

One of the most powerful and widely used methods to monitor this type of fine-scale plant photosynthetic reactions is the chlorophyll fluorescence analysis (Ducruet et al., 2007). This technique provides a precise assessment of the photosynthetic apparatus performance in response to environmental stresses by means of simple, sensitive and non-invasive measurements (Baker, 2008; Kalaji et al., 2016). On the one hand, the "fluorescence transient" (i.e. Kautsky curve) estimated from prompt chlorophyll *a* emission kinetics is analyzed by the OJIP test, which informs about the stepwise energy fluxes (electron transport) through photosystems (Strasser et al., 2004). This test

represents an appropriate method to detect temperature stress (Xu et al., 2014) and, especially, the related maximum photochemical efficiency of PSII (F_v/F_m) has been addressed as a selection criterion for tolerant species (Sharma et al., 2014, 2012). On the other hand, when prompt fluorescence has decayed, delayed fluorescence is emitted in the red infrared region of the spectrum (Goltsev et al., 2009). This emission is associated with many photosynthetic reactions and explains light-induced electron transfer and related events not measurable by other methods (Buchta et al., 2007). For this reason, delayed fluorescence has been widely used to detect plant responses to environmental stresses, including the temperature one (Goltsev et al., 2009; Guo and Tan, 2013), and as a tool for assessing the degree of chilling sensitivity in plants (Ducruet et al., 2007). Therefore, the joint measurement of both types of fluorescence has been recommended to obtain further insights into the effects of abiotic stress in plants (Oukarroum et al., 2013; Salvatori et al., 2014). In addition, the use of detached leaves would also be helpful for screening numerous samples as well as, eventually, will allow us to characterize the physiochemical shifts caused by temperature excluding other abiotic factors such as light, water and nutrient supply (Sharma et al., 2014).

Thus, the main aim of this study was to uncover the differential temperature stress-triggered photochemical mechanisms across high polyploid series, using the *D. broteri* autopolyploid complex as a model. Specifically, we hypothesized that there would be a link between photochemical strategies and the thermal niche of each cytotype. Therefore, higher cytotypes would have developed strong adaptations to cope with extreme heat events whereas lower ploidies would show less marked photochemical responses and better performances under cold and mild temperatures.

2. Materials and methods

2.1. Plant material and experimental set-up

The experiment was conducted using mature *Dianthus broteri* plants (n = 5, per ploidy level) from seeds collected during late summer 2017 in two natural populations of each cytotype. These plants were grown for one year in 2.5 L pots filled with an organic commercial substrate (Gramoflor GmbH und Co. KG.) and perlite mixture (3:1) inside University of Seville Glasshouse General Services, with controlled temperature of 21-25 °C, 40-60% relative humidity, adequate irrigation with tap water and natural

illumination, being the maximum photosynthetic photon flux density (PPFD) level incident on leaves of 1200 µmol m^{-2} s^{-1}.

In June 2019, following the methodology employed by Brestic et al. (2012) and Perera-Castro et al. (2018), fully developed leaves from each *D. broteri* cytotype were randomly selected, detached and placed inside individual small sealable plastic bags with a hydrated atmosphere to avoid evaporative loss. They were subsequently exposed to target temperature levels by submersing them in a refrigerated circulating water bath (Frigiterm-TFT-10, JP Selecta, Spain) with an accurate temperature control (± 0.1 ºC) during 30 min. Subsequently, bags were removed from the bath and leaves were taken out of them and dried for 10 min at room temperature (c. 24 ºC). During this time, we placed leaf clips in the middle part of the leaf blades to perform chlorophyll fluorescence measurements. All this procedure was performed in darkness. Sample leaves of all cytotypes were replaced for each temperature treatment and specific fluorescence methodology.

A total of nine different temperature levels were selected from extreme cold (-3 ºC) to extreme heat (53 ºC) in steps of 7 ºC. Lowest and highest temperature levels were based on extreme events recorded in the Iberian Peninsula, where natural *Dianthus broteri* populations occur (De Castro et al., 2005). To reach the negative temperature in a liquid state, we added 30% glycerol to water, which disrupts the formation of ice (Chang and Baust, 1991).

2.2. Prompt fluorescence measurements

The chlorophyll *a* fast kinetics (by the OJIP test) was measured in dark-adapted leaves for each temperature and cytotype combination (n =10, two leaves per plant), using the pre-programmed OJIP protocols of FluorPen FP100 (Photo System Instruments, Czech Republic). Derived parameters for this fluorescence transient data were calculated according to Strasser et al. (2004).

Moreover, other dark-adapted sample leaves from each temperature and cytotype combination (n = 10, two leaves per plant) were used for the quantification of photosystem II efficiency parameters, using a portable modulated fluorimeter (FMS-2, Hansatech Instrument Ltd., UK) due to its higher saturating pulse intensity. Basal fluorescence in darkness (F_0) was measured using a modulated beam (<0.05 µmol m^{-2} s^{-}

^{1} for 1.8 μs) too small to cause significant physiological changes in the samples (Schreiber et al., 1986). The data stored were an average taken over a 1.6-second period. Maximum fluorescence (F_m) was then estimated by applying a saturating actinic light pulse of 10000 μmol m^{-2} s^{-1} for 0.8 s and it was recorded as the highest average of two consecutive points. Thus, F_0 and F_m values were used to calculate variable fluorescence ($F_v = F_m - F_0$) and maximum quantum efficiency of PSII photochemistry (F_v/F_m).

2.3. Delayed fluorescence measurements

Additional leaves from all cytotypes were stuck in a black cardboard and maintained in darkness after their removal from the bath for each temperature treatment (n = 4). Delayed fluorescence was detected using a plant imaging system (NightShade LB 985, Berthold Technologies, Germany) equipped with a deeply cooled CCD camera. Leaves were illuminated for 20 s with light supplied from far red (730 nm), red (660 nm), green (565 nm) and blue (470 nm) LED panels at 2, 105, 40 and 110 μmol m^{-2} s^{-1}, respectively. Immediately after switching off the LEDs, delayed fluorescence was measured and the recorded intensities of light were converted in counts per second (cps). Data were then normalized to each leaf area to obtain comparable cps values across cytotypes and temperatures.

2.4. Statistical analyses

Among the numerous OJIP-derived parameters, we selected those related to energetic pathways due to the useful information under stress conditions that they provide (Duarte et al., 2015). Specifically, we assessed PSII efficiency using F_0 as well as F_v/F_m and we used electron probability variables (flux ratios; φ_{Eo}, φ_{Do} and ψ_0). In addition, we employed the energy transduction fluxes on a leaf cross-section basis (phenomenological fluxes; ABS/CS, TR/CS, ET/CS and DI/CS) because in a preliminary analysis we found significant differences in the density of reaction centers (RC/CS) within temperatures and cytotypes (two-way analysis of variance, ANOVA: $P < 0.05$; see Table 1 for parameter description). Finally, we examined the proportion of active oxygen-evolving complexes (OECs) as they are the most sensitive components of the photosynthetic machinery to high temperatures (Allakhverdiev et al., 2008, and references cited therein).

Thus, the influence of ploidy level and temperature (both treated as factors; a total of 36 combinations) on these variables as well as delayed fluorescence were analyzed using two-way ANOVAs. Given that temperature largely affected all the response variables ($P < 0.05$), one-way ANOVAs using ploidy level as explanatory variable were also performed within each temperature. Significant test results were followed by *post hoc* Tukey's HSD tests ($\alpha = 0.05$) to detect pairwise differences among ploidy levels. All statistical analyses were performed in R software ver. 3.6.1 (R Core Team 2019).

Table 1

Glossary of chlorophyll fluorescence parameters used in this study.

Photosystem II efficiency	**Description**
F_0	Basal fluorescence in dark-adapted leaves
F_v/F_m	Maximum quantum efficiency of PSII photochemistry
Energy fluxes (Kautsky curves)	
φ_{Eo}	Probability that an absorbed photon will move an electron into the electronic transport chain
φ_{Do}	Quantum yield of the non-photochemical reactions
ψ_0	Probability of a PSII trapped electron to be transported from Q_A to Q_B
ABS/CS	Absorbed energy flux per leaf cross-section
TR/CS	Trapped energy flux per leaf cross-section
ET/CS	Electron transport energy flux per leaf cross-section
DI/CS	Dissipated energy flux per leaf cross-section

3. Results

All chlorophyll fluorescence parameters were broadly affected by temperature, especially by elevated temperature levels (two-way ANOVAs: $P < 0.05$). Besides, there was a widespread inter-cytotype divergence in terms of photosystems functionality and energy fluxes, with remarkable differences in the Kautsky curves (Fig. S1). Along almost the whole temperature range, the greatest fluorescence values in all OJIP phases as well as energy fluxes (ABS/CS, TR/CS, ET/CS and DI/CS) were found in diploids and the lowest values were exhibited by hexaploids (Figs. 1A-D, S1). Oppositely to these constant trajectories, dodecaploids, and especially tetraploids, showed a considerably variable fluorescence emission under the changing temperature conditions (Figs. 1, S1, S2). Thus, the major effects mainly occurred under extremely high temperatures (i.e. 46 ºC and 53 ºC), when the shape of Kautsky curves changed and fluorescence decreased in the four cytotypes especially affecting the J-I-P (thermal) phase (Fig. S1). In fact, immediately prior to this phase, heat stress caused the appearance of the additional K band in Kautsky curves at around 1000 µs (Fig. S1). This step was found for all the cytotypes but it was more pronounced in tetraploids due to their lower values of active oxygen-evolution complexes (OECs) at 53 ºC compared to the rest of ploidies, especially the 12× cytotype (one-way ANOVA, $P < 0.05$; Fig. 1E).

The mentioned differences among cytotypes were accompanied by a marked divergence in electron probability variables (ψ_0, φ_{Eo} and φ_{Do}) at extreme temperatures (-3 ºC and 53 ºC) and at 18 ºC (Fig. 1F-H). At the minimum temperature of exposure (-3 ºC), the 4× cytotype showed enhanced flux ratios in terms of primary photochemistry, being the probability of an absorbed photon moving an electron into the electron transport chain (φ_{Eo}) as well as of its transport from Q_A to Q_B (ψ_0), significantly higher than those of the other cytotypes (ANOVA: ploidy level, $P < 0.05$; Fig. 1F, G). Furthermore, the 4× cytotype had the lowest quantum yield of non-photochemical reactions (φ_{Do}; Fig. 1H). In comparison with tetraploids, diploids showed greater absorbed (ABS/CS), trapped (TR/CS) and dissipated (DI/CS) energy fluxes (Fig. 1A, B, D), but not transported ones (ET/CS; Fig. 1C). At subsequent temperatures (4 ºC to 25 ºC), the 4× cytotype decreased its electron probability variables of primary photochemistry (Fig. S2F, G) but increased the four phenomenological energy fluxes,

making them similar to those of the 2× cytotype (Figs. 1A-D, S2A-D). Under 32 ºC, we found the opposite pattern to the one exhibited under -3 ºC. In this case, the 4× cytotype showed significantly higher energy fluxes compared to the 2× cytotype, especially ET/CS (ANOVA: ploidy level, $P < 0.05$; Fig. S2A-D). Moreover, the exposure to high temperatures leaded again to increasing higher energy fluxes of diploids compared to tetraploids, with greater ABS/CS, DI/CS and primary photochemistry ratios under 53 ºC (ANOVA: ploidy level, $P < 0.05$; Figs. 1, S2).

Regarding the two higher ploidy levels, the 6× cytotype showed a generalized pattern of low energy fluxes based on its notably low ABS/CS values (Fig. 1A-D). Moreover, this cytotype exhibited a clear tendency to higher electron probability rates relative to photochemical reactions compared to the rest of cytotypes from 18 ºC to extreme heat, being significantly higher at 18 ºC and 53 ºC (ANOVA: ploidy level, $P < 0.05$; Fig. 1F, G). Likewise, the 12× cytotype mirrored a trend towards non-photochemical processes, as highlighted by enhanced DI/CS (Fig. 1D). It tended to be more pronounced with increasing temperatures (significant higher values at 32 ºC and 53 ºC; Fig. S2D; Fig. 1D), being this dissipated energy flux also accompanied by a greater quantum yield (φ_{Do}) at 18 ºC and 53 ºC (ANOVA: ploidy level, $P < 0.05$; Fig. 1H). Dodecaploids also showed a comparatively high ψ_0 under the highest temperature condition (Fig. 1F). Finally, the described pattern of high dissipation under extreme heat congruently occurred also with the greatest absorbed energy flux (ANOVA: ploidy level, $P < 0.05$; Fig. 1A) and fluorescence values in the Kautsky curves (Fig. S1), both of them similar to those of the 2× cytotype.

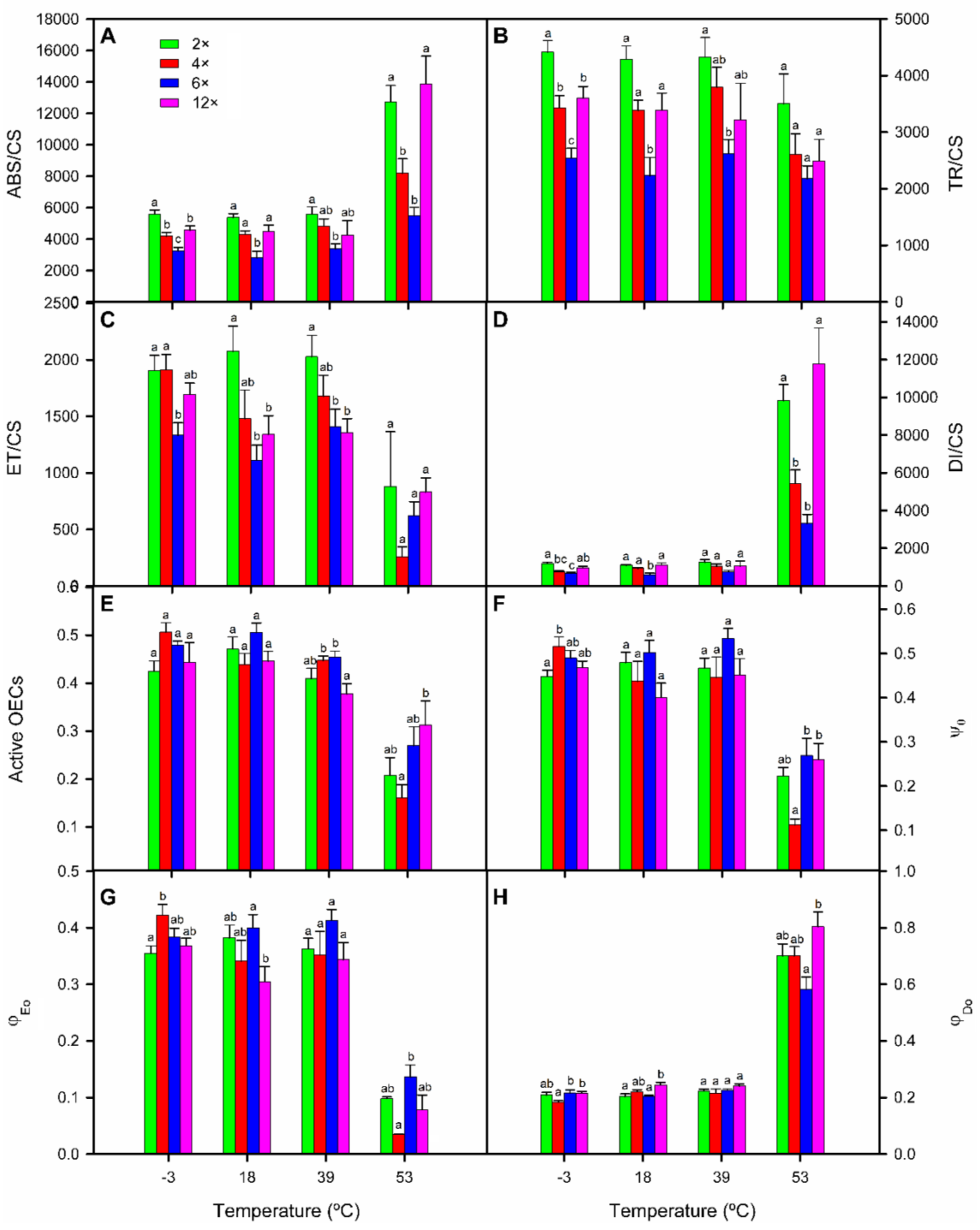

Fig. 1. Absorbed energy flux per leaf cross-section, ABS/CS (A), trapped energy flux per leaf cross-section, TR/CS (B), transport energy flux per leaf cross-section, ET/CS (C), dissipated energy flux per leaf cross-section, DI/CS (D), active oxygen-evolving complexes, OECs (E), probability of a PSII trapped electron to be transported from Q_A to Q_B, ψ_0 (F), probability that an absorbed photon will move an electron into the electronic transport chain, φ_{Eo} (G), and quantum yield of the non-photochemical

reactions, φ_{Do} (H), in dark-adapted leaves of the four *D. broteri* cytotypes exposed to four selected temperature levels of the complete range. Parameters were derived from measurements taken with FluorPen FP100. Values represent mean ± standard error. Different letters indicate cytotypes that are significantly different from each other within each temperature level (one-way ANOVA: $P < 0.05$, Tukey's HSD test: $\alpha = 0.05$).

Delayed fluorescence emissions were partly similar to prompt fluorescence ones. Along the temperature range, overall lower values were detected in 6× and 12× cytotypes, especially in the first one, compared to 2× and 4× ploidies (Fig. 2A). Nevertheless, at mild temperature levels as 18 ºC (Fig. 2C) and 25 ºC, no significant differences between cytotypes were found (ANOVA: ploidy level, $P > 0.05$; Table S1; Fig. 2A). Thus, delayed fluorescence became greater in the 4× cytotype than in the remaining ploidies (ANOVA: ploidy level, $P < 0.05$; Table S1) under extreme cold (-3 ºC; Fig. 2B) as well as under the high temperature levels 32 ºC and 39 ºC (Fig. 2A, D). Moreover, tetraploids showed higher values compared to at least one of the other cytotypes at cold temperatures (4 ºC and 11 ºC; Fig. 2A). However, unlike under extreme cold (Fig. 2B), the most elevated temperature (53 ºC) dramatically reduced delayed fluorescence to zero values for all cytotypes (Fig. 2A). Remarkably, in the previous temperature level step from 39 ºC to 46 ºC, lower ploidies (2× and 4×) suffered considerable delayed fluorescence decreases (of c. 22% and c. 57%, respectively) whereas higher ploidies (6× and 12×) experienced increases (of c. 49% and c. 29%, respectively; Fig. 2A).

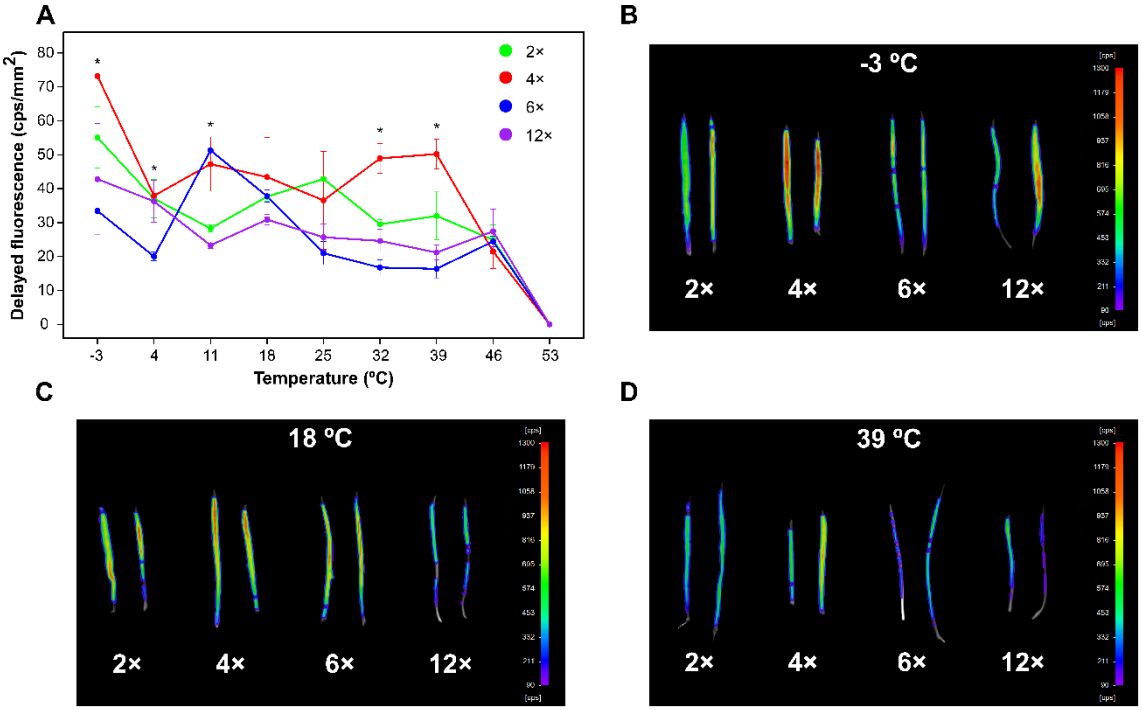

Fig. 2. Delayed fluorescence results in dark-adapted leaves of the four *D. broteri* cytotypes exposed to nine different temperatures. Panel A presents cps values for each ploidy level normalized to leaf area along the temperature range. Values represent mean ± standard error. Asterisks indicate significant differences among cytotypes at each temperature using one-way ANOVAs ($P < 0.05$). Panels B, C and D show photographs taken at -3 ºC, 18 ºC and 39 ºC, respectively, by the plant imaging system NightShade LB 985. The color scale mirrors the detected counts per second (cps) of delayed fluorescence emission in leaves.

As expected, the main pattern observed in the transient fluorescence emission was repeated in the basal fluorescence (F_0; Fig. 3A). Hexaploids exhibited the lowest values across the temperature range, which were significantly lower than the remaining cytotypes at 4 ºC, 25 ºC and, mostly, the three highest temperature levels (ANOVA: ploidy level, $P < 0.05$; Table S1; Fig. 3A). Moreover, all the cytotypes showed sharp F_0 increases from 39 ºC to 46 ºC (Fig. 3A). However, it is noteworthy that these values continued decreasing until 53 ºC in the lower cytotypes (2× and 4×) whereas they experienced subtle increases in 6× and 12× cytotypes (c. 19% and c. 7%, respectively; Fig. 3A). From 39 ºC to 53 ºC, in contrast to F_0 but similarly to prompt and delayed fluorescence emissions, an additional sharp decrease occurred in the maximum quantum

efficiency of PSII photochemistry (F_v/F_m; Fig. 3B). The four cytotypes shared this trend although the lower ploidies showed greater F_v/F_m reductions with drops of c. 43%, c. 49%, c. 29% and c. 37% for 2×, 4×, 6× and 12× leaves, respectively. For this reason, 6× and 12× ploidies had significantly higher F_v/F_m values than 2× and 4× at 46 ºC and 53 ºC, especially the hexaploid cytotype (ANOVA: ploidy level, $P < 0.05$; Table S1). At lower temperatures, little F_v/F_m differences among ploidy levels were recorded (only at 4 ºC, 11 ºC, 25 ºC and 39 ºC; ANOVA: $P < 0.05$). These inter-cytotype differences in F_v/F_m were mainly due to lower values of the 12× cytotype when compared to the 6× (Fig. 3B; Table S1). Nevertheless, from -3 ºC to 39 ºC, F_v/F_m values of all the cytotypes remained remarkably constant, with average values ranging between 0.81-0.84 (Fig. 3B).

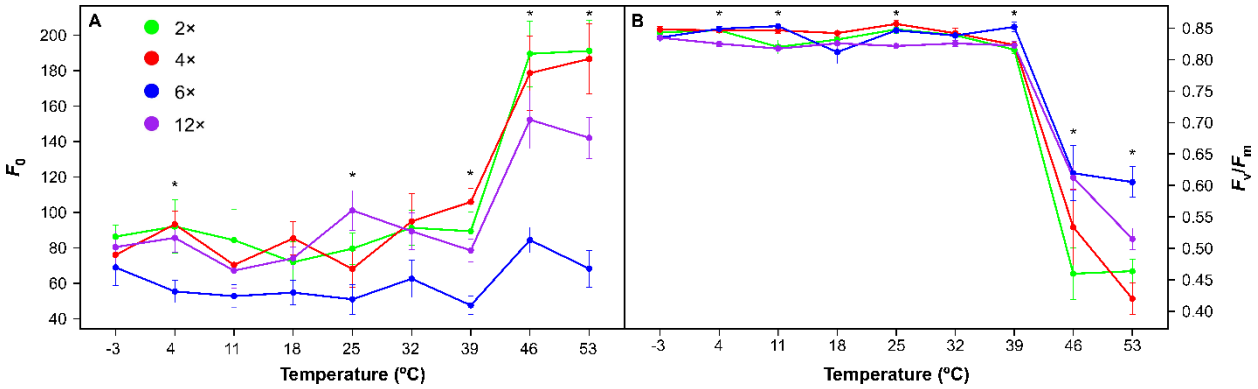

Fig. 3. Basal fluorescence, F_0 (A), and maximum quantum efficiency of PSII photochemistry, F_v/F_m (B), in dark-adapted leaves of the four *D. broteri* cytotypes exposed to nine different temperatures. Parameters were derived from measurements taken with the portable modulated fluorimeter FMS-2. Values represent mean ± standard error. Asterisks indicate significant differences among cytotypes at each temperature using one-way ANOVAs ($P < 0.05$).

4. Discussion

Our results demonstrated the existence of significant photochemical differences among cytotypes through the entire temperature range tested. Notwithstanding specific differences at some temperature levels, F_v/F_m values from -3 ºC to 39 ºC reflected a

relatively unaffected carbon metabolism and conversion to chemical energy in photosynthesis (Sharma et al., 2012). However, between 39 ºC and 46 ºC there seems to be a threshold for photosystems functionality. The impairment in such a fundamental process will influence the decay of many other processes downstream from primary photochemistry (Poudyal et al., 2019), as delayed fluorescence (Badretdinov et al., 2004). This latter process is, in turn, caused by the failure of important inner-protein proton movements in the thylakoid membrane (Buchta et al., 2007) and would lead to the loss of photosynthesis efficiency (Wang et al., 2007). Despite this general trend, we detected differences in energy fluxes and efficiencies among cytotypes to be more prominent at extreme temperatures (particularly, -3ºC and 53 ºC). These temperature levels triggered specific physiological responses to cope with the mentioned stress consequences.

Diploids and tetraploids occur in colder habitats than those of hexaploids and dodecaploids (López-Jurado et al., 2019a), which would explain their limited capacity to face extreme heat, dramatically decreasing F_v/F_m and delayed fluorescence emission. Additionally, lower cytotypes sharply increased basal fluorescence (F_0) from elevated to severe heat (39 ºC to both highest temperature levels), which is a sign of the irreversible aggregation of light-harvesting complexes and their dissociation from PSII (Yamane et al., 1997), an early indicator for damage in PSII (Mathur et al., 2011; Pollastri et al., 2019). Nevertheless, these lower cytotypes were capable to maintain an optimal photochemical functionality at low temperatures and mild conditions. Diploids enhanced total energy fluxes (ABS/CS, TR/CS, ET/CS and DI/CS) to maximize photosynthesis as confirmed by their high fluorescence values across temperatures in Kautsky curves, which were not accompanied by high flux ratios. Therefore, they demonstrated an offsetting between the amount of energy involved in fluorescence reactions and its use efficiency at the temperature range from low to moderate. This mechanism under chilling stress is described as ‘photoacclimation’ and avoids the damage of photosystems by balancing the energy absorbed with the energy metabolized and dissipated (Ensminger et al., 2006). However, the elevated absorption rate at high temperatures unbalances these processes because collected energy exceeds the capacity for photochemical reactions and the protective energy dissipation becomes necessary, as indicated by the enhanced DI/CS at 53 ºC. This dissipated flux would cause the 2× cytotype to protect the donor side of PSII as demonstrated by its comparatively low

360 inactivation of OECs, which is the rate-limiting process in the photodamage to PSII
361 (Takahashi and Murata, 2008).

362 In contrast, the 4× cytotype tolerated cold stress by decreasing its energy fluxes
363 and maximizing their ratios for primary photochemistry and hence their delayed
364 fluorescence intensity (Zhang and Xing, 2008). A possible explanation to this
365 mechanism exhibited by the tetraploids would be the downregulation, redistribution or
366 dissociation of antenna systems in order to harvest a lower light flux and promote
367 energy-quenching mechanisms under these conditions (Rochaix, 2014; Xu et al., 2015).
368 This strategy has been described in plant species exposed to cold temperatures, mostly
369 in conifers distributed in boreal environments (Ensminger et al., 2004; Ottander et al.,
370 1995) but also in *Arabidopsis thaliana* (Nellaepalli et al., 2012). Moreover, delayed
371 fluorescence of this cytotype was constantly higher than the other ploidies and varied
372 without abrupt changes between 4ºC and 32 ºC. This pattern indicated that the shift in
373 light collection could be partly linked with the membrane fluidity of 4× thylakoids,
374 which would not suffer phase transitions influenced by temperature (Havaux and
375 Lannoye, 1983). Thus, this cytotype could be considered a chilling-resistant ploidy.
376 Nevertheless, tetraploids exhibited an increase in delayed fluorescence emission at 32
377 ºC and 39 ºC, which matches with a phase transition likely promoted by a remarkable
378 structural flexibility of thylakoid membranes (Garab, 2014). Notably, energy fluxes of
379 the 4× cytotype also increased and exceeded those of the 2× at 32 ºC. This complex and
380 flexible photochemical behavior of the 4× cytotype might be a consequence of its two
381 independent lineages with unrelated evolutionary processes (Balao et al., 2010) and
382 their different ecological niches (López-Jurado et al., 2019a). Recurrent origins of the
383 tetraploids would have caused more diverse genetic and physiological properties
384 (Weiss-Schneeweiss et al., 2013) which, in addition, would be especially regulated by
385 local adaptation processes (McIntyre and Strauss, 2017). However, in this cytotype,
386 severe heat caused a decrease of F_v/F_m, delayed fluorescence, energy fluxes and ratios
387 (with low dissipation) as well as active OECs. These results suggested an excessive
388 formation of reactive oxygen species (ROS), causing PSII photodamage (Yamamoto et
389 al., 2008), the inhibition of its repair (Murata et al., 2007) and the inactivation of OECs
390 (by lipid peroxidation; Song et al., 2006).

391 Additionally, 6× and 12× cytotypes exhibited straightforward mechanisms
392 focused on the response to the highest temperatures. In this case, both higher cytotypes

showed a subtle F_0 decrease at 53 ºC after the marked increase from 39 ºC to 46 ºC. This reduction under extreme heat would support the dark reduction of Q_A as the main cause of the basal fluorescence rise, as demonstrated at similar temperatures (c. 50 ºC; Yamane et al., 2000). Unlike the alleged irreversible changes attributed to lower ploidies, the redox state of Q_A would be reversed by reoxidation in higher ploidies (Yamane et al., 2000). This transition would also influence the greater maximum quantum efficiency of PSII photochemistry (F_v/F_m) of 6× and 12× cytotypes against heat stress compared to 2× and 4×. Furthermore, the two higher ploidies showed a high preservation of active OECs of PSII, which could be protected by bicarbonate (Klimov et al., 1997) or the association of small heat-shock proteins (HSPs) with thylakoids (Heckathorn et al., 2002). In order to successfully complete their strategies, both higher cytotypes would have to regulate their photosynthetic capacities to concentrate the higher productivity-related rates in narrow periods of opportunity (Demmig-Adams et al., 2017).

Hexaploids additionally had low energy fluxes per leaf cross-section (especially the absorbed one) at the entire range of temperatures, based on reduced prompt and delayed fluorescence values. This behavior suggested more efficient photochemical machinery in relative terms, depicted by enhanced flux ratios and a high overall F_v/F_m with a lessened reduction under heat stress. Jointly, these trends suggested that the 6× cytotype would base their photochemical strategy on the optimization of antenna size to prevent photoinhibition and increase photosynthetic efficiency (Adams and Demmig-Adams, 2004; Ort et al., 2011). This ‘avoidance’ adjustment of hexaploids was similar to the one exhibited by tetraploids under extreme cold but, in this case, seems to be a constitutive adaptation which allow their establishment in the highly nutrient-poor and light-exposed habitats that they inhabit (López-Jurado et al., 2019a, unpublished data). Congruently, the mentioned minimization of light-harvesting complexes has been addressed in other plants subjected to similar stresses (Logan et al., 1999; Morales et al., 2000).

Contrastingly, the 12× cytotype maintained overall higher energy fluxes than 6×, mostly at mild temperature conditions (from 18 ºC to 32 ºC), so probably light-harvesting antenna complexes are preserved and chlorophyll degradation is not acting as an underlying defense mechanism. Its overall high non-photochemical fluxes and yields

(DI/CS and φ_{Do}), especially under severe heat, suggested that this cytotype is adapted to tolerate a considerable degree of photoinhibition. In fact, these joint results could reflect the well-addressed photoprotection of the photosynthetic apparatus via thermal dissipation to avoid ROS production (Demmig-Adams and Adams, 2006; Silva et al., 2015). In this 'photoprotective' strategy, excess energy would be dissipated through xanthophylls (zeaxanthin and antheraxanthin) retention and the rearrangement or degradation of PSII cores (Demmig-Adams and Adams, 2006). Together with the non-photochemical quenching (dissipated energy), linear electron transport downregulation (depicted here as ET/CS) is essential for protecting both photosystems and other chloroplast structures against photoinhibition damage (see Brestic et al., 2016 for temperature stress and Meng et al., 2016 for other abiotic stress -drought-). While the ET/CS decrease at high temperatures was shared by all cytotypes, the elevated DI/CS showed by 2× and 12× could be crucial to survive while absorbing excessive energy. The 'photoprotective' strategy and the previously mentioned F_0 pattern would cause the 12× cytotype to not suffer abrupt decreases in F_v/F_m under extreme temperatures and other abiotic stresses, as supported by the similar results obtained in a drought stress experiment (López-Jurado et al., 2016). Likewise, dodecaploids occur mostly in the understory of pine forests, with a much lower incoming radiation than hexaploids (López-Jurado et al., 2019b) so their strategy allows greater light absorption as well as photoprotection against environmental stresses.

Photochemical inter-cytotype differentiation is definitely associated with the environmental conditions of the niche that each cytotype occupies, which suggests an enhanced local adaptation and functional plasticity by means of polyploidization events (Ramsey, 2011) and epigenetic changes (Alonso et al., 2016; Song and Chen, 2015). These differences in physiological processes might be promoted by gene duplication as a result of polyploidization (Panchy et al., 2016). Duplicate retention of photosynthetic-associated genes, as those regulating the Calvin cycle and light-harvesting complexes, would then drive their functional divergence (neofunctionalization or subfunctionalization; Coate et al., 2011). The photochemical differentiation among *D. broteri* cytotypes was congruent with a previous pairwise diploid-polyploid comparison in citrus seedlings (Oustric et al., 2019) but cannot be generalized since there are examples of not a clear divergence (Pavlíková et al., 2017) as well as more effective adaptations in diploids (Guo et al., 2016). Furthermore, a similar study on cereals with

four different ploidy levels found greater photosynthetic capacities and more efficient antioxidant defense systems in higher ploidies under favorable conditions (6× and 8×; Mao et al., 2018).

Remarkably, although we detected that higher ploidies have developed specific photochemical processes to survive in extremely warm conditions, a divergence pattern was also found in favorable conditions (18 ºC). The reduced performance of higher cytotypes suggested that they would be less competitive in a common environment with mild temperatures. As proposed by He et al. (2013), species interactions could shift under stress to facilitation or reduction in competition. Therefore, 6× and 12× cytotypes would have adapted to the absence of inter-cytotype competition at their locations near to edges of the environmental gradients facilitating their establishment (López-Jurado et al., 2019a). However, the increase in frequency and severity of cold and heat episodes due to climate change, as indicated the most extreme predictions for Spain in the coming years (Furió and Meneu, 2011), could cause that 6× and 12× cytotypes would reach better performances compared to their low-cytotype counterparts. Consequently, the current distribution of all ploidy levels would be compromised under climate change projections.

To sum up, the high autopolyploid complex *Dianthus broteri* gave us the opportunity to unravel the effect of successive genome duplications on physiological divergence and plasticity. In this case, highly specific photochemical adaptations to extreme heat were found in higher ploidy levels, which are distributed in warm and semi-arid environments. Notwithstanding the pattern cannot be generalized, this approach would shed light on the segregation pattern of cytotypes within other polyploid complexes and it would also be useful to provide new insights into their environmental preferences, niche evolution and ecological interactions, as well as to predict the evolution of the different cytotypes under climate change scenarios.

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SUPPLEMENTARY MATERIAL

Table S1

Summary of inter-cytotype comparisons in F_0, F_v/F_m and delayed fluorescence (cps/mm^2) for the different temperature conditions. For each temperature, letters marked in bold indicate a significant effect of ploidy level on the response variable (one-way ANOVA: $P < 0.05$). Different letters show significant differences among cytotypes within each temperature level (Tukey's HSD test: $\alpha = 0.05$).

T (˚C)	F_0				F_v/F_m				Delayed fluorescence			
	2×	4×	6×	12×	2×	4×	6×	12×	2×	4×	6×	12×
-3	a	a	a	a	a	a	a	a	**ab**	**a**	**b**	**ab**
4	**a**	**a**	**b**	**ab**	**a**	**a**	**a**	**b**	**a**	**a**	**b**	**a**
11	a	a	a	a	**a**	**b**	**b**	**a**	**a**	**b**	**b**	**a**
18	a	a	a	a	a	a	a	a	a	a	a	a
25	**ab**	**ab**	**a**	**b**	**a**	**a**	**a**	**b**	a	a	a	a
32	a	a	a	a	a	a	a	a	**a**	**b**	**a**	**a**
39	**a**	**a**	**b**	**a**	**a**	**a**	**b**	**a**	**a**	**b**	**c**	**ac**
46	**a**	**a**	**b**	**a**	**a**	**ab**	**b**	**b**	a	a	a	a
53	**a**	**a**	**b**	**a**	**ab**	**a**	**c**	**b**	a	a	a	a

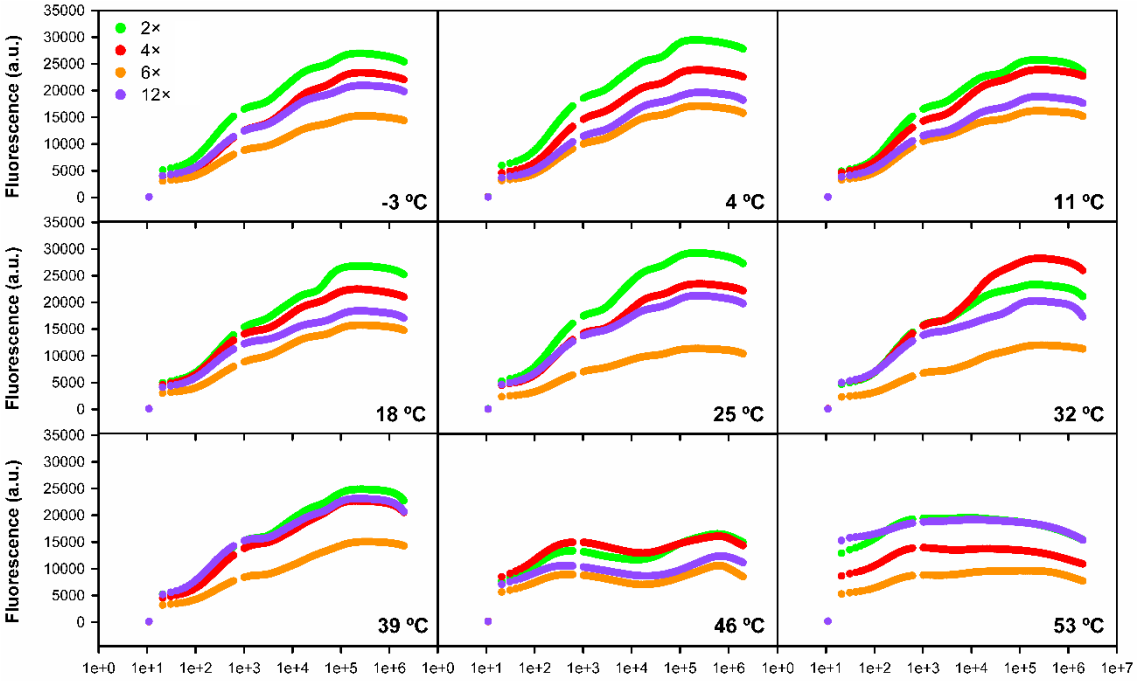

Fig. S1. Fluorescence transients (Kautsky curves) in dark-adapted leaves of the four *D. broteri* cytotypes exposed to nine different temperatures (average values).

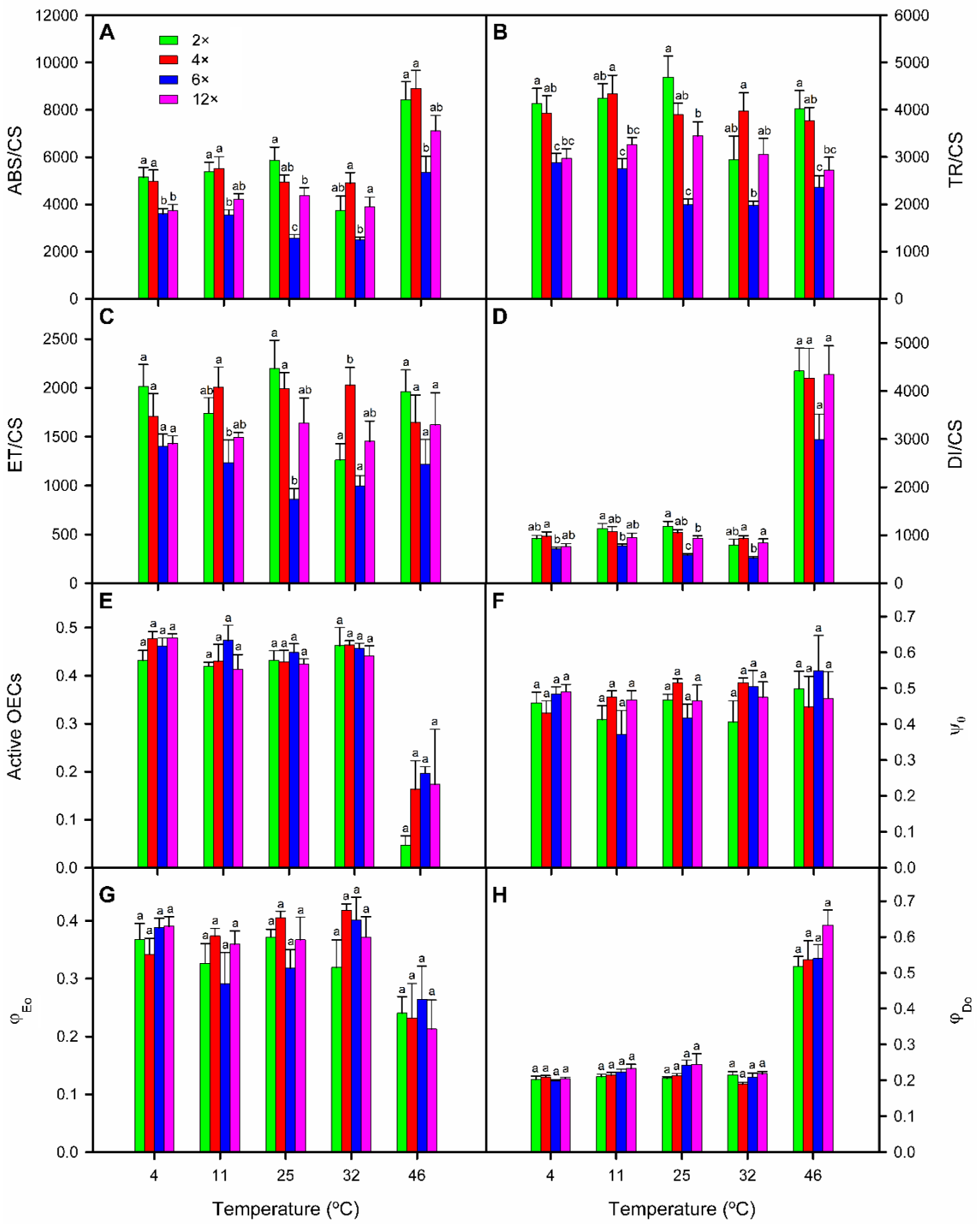

Fig. S2. Absorbed energy flux per leaf cross-section, ABS/CS (A), trapped energy flux per leaf cross-section, TR/CS (B), transport energy flux per leaf cross-section, ET/CS (C), dissipated energy flux per leaf cross-section, DI/CS (D), active oxygen-evolving complexes, OECs (E), probability of a PSII trapped electron to be transported from Q_A to Q_B, ψ_0 (F), probability that an absorbed photon will move an electron into the electronic transport chain, φ_{Eo} (G), and quantum yield of the non-photochemical reactions, φ_{Do} (H), in dark-adapted leaves of the four *D. broteri* cytotypes exposed to

five selected temperature levels of the complete range. Parameters were derived from measurements taken with FluorPen FP100. Values represent mean ± standard error. Different letters indicate cytotypes that are significantly different from each other within each temperature level (one-way ANOVA: $P < 0.05$, Tukey's HSD test: $\alpha = 0.05$).

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Javier López-Jurado: Methodology, Writing – original draft & review and editing, Formal analysis, Investigation. **Francisco Balao:** Conceptualization, Supervision, Validation, Writing – review and editing. **Enrique Mateos-Naranjo:** Conceptualization, Supervision, Validation, Writing – review and editing.