

Processes contributing to photoprotection of grapevine leaves illuminated at low temperature

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Photoinactivation of photosystem II (PSII) and energy dissipation at low leaf temperatures were investigated in leaves of glasshouse-grown grapevine (*Vitis vinifera* L. cv. Riesling). At low temperatures (< 15°C), photosynthetic rates of CO₂ assimilation were reduced. However, despite a significant increase in the amount of light excessive to that required by photosynthesis, grapevine leaves maintained high intrinsic quantum efficiencies of PSII (F_v/F_m) and were highly resistant to photoinactivation compared to other species. Non-photochemical energy dissipation involving xanthophylls and fast D1 repair were the main protective processes reducing the 'gross' rate of photoinactivation and the 'net' rate of photoinactivation, respectively. We developed an improved method of energy dissipation analysis that revealed up to

75% of absorbed light is dissipated thermally via pH- and xanthophyll-mediated non-photochemical quenching at low temperatures (5–15°C) and moderate (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) light. Up to 20% of the energy flux contributing to electron transport was dissipated via photorespiration when taking into account temperature-dependent mesophyll conductance; however, this flux used in photorespiration was only a relatively small amount of the total absorbed light energy. Photoreduction of O₂ at photosystem I (PSI) and subsequent superoxide detoxification (water-water cycle) was more sensitive to inhibition by low temperature than photorespiration. Therefore the water-water cycle represents a negligibly small energy sink below 15°C, irrespective of mesophyll conductance.

Introduction

Low temperatures can limit light- and CO₂-saturated photosynthetic capacity of leaves in many plant species because the proportion of absorbed light that is excessive to photochemistry is increased (Allen and Ort 2001). This can drastically reduce growth and yield of crop species grown in many different climates. The photosystems are the primary targets for chilling-induced photoinactivation. In some chilling-sensitive plant species inhibition of photosynthetic electron transport can occur, despite relatively minimal reductions in F_v/F_m due to net photoinactivation of PSI rather than PSII

(Terashima et al. 1994; Tjus et al. 1998; Sonoike 1999) when leaf temperature drops to around 10°C. Inhibition of PSI commonly occurs in species that have evolved in tropical or subtropical climates and appears to indicate a high degree of chilling sensitivity. Under these circumstances the F_v/F_m assessment could underestimate the impact of chilling in the presence of light on leaf photosynthesis.

Low temperature-induced stress has been shown to limit growth of grapevine (Buttrose 1969), which is an economically important C₃ crop in many parts of the

Abbreviations – ΔpH , trans-thylakoid pH gradient; Φ_f , quantum yield of fluorescence; Φ_D , quantum yield of light-independent thermal energy dissipation; QY_I , quantum yield of PSII photoinactivation; Φ_{NPQ} , quantum yield of light-dependent and ΔpH - and xanthophyll-mediated thermal energy dissipation; Φ_{PSII} , quantum yield of PSII reaction centre photochemistry; C_c and C_i , CO₂ concentration in the chloroplast and intercellular airspace, respectively; F_v/F_m , intrinsic quantum efficiency of PSII photochemistry; I_A , PAR absorbed by the leaf; J_{PSII} , J_{NPQ} and $J_{\text{T,D}}$, rate of energy dissipation via linear photosynthetic electron transfer, light-dependent thermal processes, and light-independent thermal processes and fluorescence, respectively; NPQ, non-photochemical quenching; PAR, photosynthetically active radiation; T_{leaf} , leaf temperature.

world. However, grapevine leaves in the field remain relatively resilient to low temperature-induced net photo-inactivation of photosystem II based on sustained, high variable chlorophyll fluorescence (F_v/F_m) (Chaumont et al. 1997; Flexas et al. 1999; Hendrickson et al. 2003). This implies one or more highly efficient energy dissipation mechanism(s) are induced in grapevine leaves by the combination of low temperature and high light. Photoprotective mechanisms for avoiding photoinactivation of PSII during chilling include photorespiration (Hendrickson et al. 2003), the water-water cycle (Flexas et al. 1999) and the xanthophyll cycle (Chaumont et al. 1995; Hendrickson et al. 2003). The relative contributions of these processes to photoprotection at chilling temperatures need to be quantified for understanding their relative impact on PSII function and to further elucidate mechanistic details. Moreover, it remains to be investigated to what extent photoinactivation of PSI under low temperature-high light conditions plays a role in grapevine leaf photosynthesis and whether rates of photosystem II repair are sufficiently high to explain maintenance of relatively high F_v/F_m . The D1 protein in the PSII reaction centre is a primary target for oxidative damage by reactive oxygen species, i.e. singlet molecular oxygen, 1O_2 and the superoxide anion, O_2^- , and the generation of $P680^+$ during illumination (Aro et al. 1993). Plants that are capable of sustaining high rates of replacement of damaged D1 protein are likely to show little reduction in quantum efficiency of O_2 evolution or F_v/F_m (Andersson and Aro 2001). However, it has been found in higher plants that D1 repair is generally limited at low temperatures because D1 proteolysis is slowed down, thus preventing the integration of newly synthesized D1 protein and consequently increasing photoinhibitory damage (Aro et al. 1990). For grape vine leaves, Chaumont et al. (1995) demonstrated that 80% of initial D1 pool size was retained during a 5°C and 1500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ treatment over several hours, suggesting that the rate of repair is still considerable at low temperatures in contrast to the results of other C_3 plants.

In this paper we characterize the combined effects of chilling and light on energy dissipation in the leaves of grapevine (*Vitis vinifera* L.) in order to determine what process(es) are the major contributors to photoprotection in this species. The relative importance of photoprotective strategies such as photorespiration, the xanthophyll and water-water cycles, PSI inactivation and D1 repair for limiting net photoinactivation of PSII in grapevine has been evaluated qualitatively as well as quantitatively.

Materials and methods

Plant material

Sampling for leaf pigments of the grapevine variety Riesling was undertaken at the same cool climate vineyard near Canberra, Australia, as described in Hendrickson et al. (2003). Grapevines (*V. vinifera* L. cv. Riesling)

were also grown in 10-l pots in an ambient irradiance (maximum daily $PAR = 1600 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$), temperature controlled (30°C/20°C day/night cycle) glasshouse at the Research School of Biological Sciences, Australian National University, Canberra, Australia. Vines were watered to field capacity daily and given complete, slow release fertilizer.

Carotenoid pigment analysis by HPLC

In the field and laboratory, leaf discs were taken from randomly selected mature Riesling leaves. Leaf discs were immediately frozen in liquid nitrogen. Total pigments were extracted from frozen leaf discs by grinding the tissue in 800 μl acetone: ethyl acetate 60:40 (v:v) extraction buffer in a microfuge tube and 640 μl of water were added. Samples were briefly vortexed and phases were separated by room temperature centrifugation (5 min at 12 000 g). The upper, pigment-containing phase (approx. 150 μl) was centrifuged (3 min at 12 000 g) again to pellet debris and 130 μl of the supernatant was used for carotenoid pigment analysis by reverse phase HPLC (Beckman system; Beckman CoulterTM, Fullerton, OA, USA) as described previously (Pogson et al. 1996). 20 μl extract were injected onto an Spherisorb ODS-2 C18 column (5- μm particles, Waters Corp., Milford, MA, USA). Carotenoids and chlorophylls were separated with a 2-step linear gradient changing from 100% solvent A (90% aqueous acetonitrile, 0.1% triethylamine (v:v)) to 66.7% solvent B (100% ethyl acetate) over 30 min at a flow rate of 1 ml min^{-1} followed by a change to 100% B within 12 s and elution at 100% B for 48 s at 1.5 ml min^{-1} flow rate. Peaks were detected and quantified at 440 nm based on absorption coefficients of pigment standards determined previously (B. Pogson, personal communication) and normalized to leaf FW and chlorophyll *a* (Chl *a*).

The relative de-epoxidation state of the xanthophyll cycle pigment pool (violaxanthin, V; antheraxanthin, A; zeaxanthin, Z) was calculated as ($DPS = \frac{[A]+[Z]}{[V]+[A]+[Z]}$) for each leaf discs from glasshouse-grown plants illuminated with 1500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ at 25°C and 9°C for 0–3 h.

Infra-red gas analysis

In the laboratory, photosynthetic gas exchange measurements were made on the youngest fully expanded leaf using an open circuit, temperature-controlled, infrared gas exchange system described elsewhere (Laisk and Oja 1998). Leaf temperature was controlled by contact thermostating. Gas-exchange parameters were calculated as described by von Caemmerer and Farquhar (1981). For modelling responses and predicting values, the following parameters were used for grapevine leaves (at 25°C): V_{cmax} between 85 $\mu\text{mol m}^{-2} \text{s}^{-1}$, $J_{\text{max}} = 130 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $R_{\text{d}} = 0.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the CO_2 compensation point in the absence of mitochondrial respiration, $\Gamma^* = 37 \mu\text{bar}$ (Schultz 2003). In addition,

these rates were calculated from measurements of CO₂ response curves of grapevine leaf photosynthesis at 1000 μmol quanta m⁻² s⁻¹ and 25°C. All other parameters were defined and corrected for different temperatures based on the specific activation energies according to von Caemmerer (2000), with the exception of Γ* (Bernacchi et al. 2001) and leaf mesophyll conductance for CO₂, g_m (Bernacchi et al. 2002). CO₂ partial pressure in the leaf mesophyll was calculated: C_c = C_i - A/g (von Caemmerer 2000). A g_m ~ 0.2 or 0.3 mol CO₂ m⁻² s⁻¹ bar⁻¹ at 25°C was derived from the relationship between A and g_m described in Evans and Loreto (2000), or g_m was varied between 0 and 1 mol CO₂ m⁻² s⁻¹ bar⁻¹. Correction for temperature dependence of g_m was made as detailed in Bernacchi et al. (2002). Where a mesophyll conductance was used, the derived quadratic equations for Rubisco-limited and RuBP-limited photosynthesis (von Caemmerer 2000) were used for photosynthetic modelling.

Leaf chlorophyll fluorescence: measurements and a new analysis

Leaf chlorophyll fluorescence parameters followed the nomenclature of van Kooten and Snel (1990). Chlorophyll fluorescence was measured simultaneously with gas exchange on the same area of the leaf using a PAM101 fitted with an ED101 BL emitter/detector that excites fluorescence with blue light and selectively measures the PSII fluorescence in the wavelength range 660–710 nm.

Utilization of photons absorbed by the PSII antennae in photosynthetic electron transport and thermal dissipation was assessed from the quantum efficiency (Φ) and flux (J) of photochemical energy dissipation (Φ_{PSII}; J_{PSII}), light-dependent (Φ_{NPQ}; J_{NPQ}) and light-independent thermal dissipation and fluorescence (Φ_{f,D}; J_{f,D}) energy dissipation according to L. Hendrickson et al. (2004) with Φ_{f,D} + Φ_{NPQ} + Φ_{PSII} = 1.

The equations for each dissipatory process are described as follows:

$$\Phi_{PSII} = 1 - \frac{F_s}{F_m'} \quad (1)$$

where F_s and F_m' are chlorophyll fluorescence yields in steady-state illumination or after closure of all PSII traps, respectively (Genty et al. 1989).

Φ_{f,D} = the sum of the fractions of light absorbed by the PSII antennae that is dissipated by either light-independent thermal dissipation (Φ_D) or via fluorescence (Φ_f) where:

$$\Phi_{f,D} = \frac{F_s}{F_m} \quad (2)$$

Φ_D is light-independent in the sense that short-term changes in light intensity do not alter its efficiency.

$$\Phi_{NPQ} = \frac{F_s}{F_m'} - \frac{F_s}{F_m} \quad (\text{Cailly et al. 1996}) \quad (3)$$

In an analogous calculation to the rate of electron transport utilized via photochemistry, J_{PSII}, it is possible,

using the quantum efficiencies Φ_{NPQ} and Φ_{f,D} to estimate the rate of energy dissipation via these processes. For example, the rate of energy dissipation via ΔpH- and xanthophyll-mediated thermal dissipation can be calculated as:

$$J_{NPQ} = \Phi_{NPQ} \times I_A \times 0.5. \quad (4)$$

where 0.5 is the assumed proportion of absorbed quanta used by PSII reaction centres (Melis et al. 1987) and I_A is the absorbed irradiance assuming an average leaf absorptance of 0.85 for grapevine leaves (Schultz 1996).

Composition of J_{PSII} was resolved in more detail as processes including electron transport to carbon fixation (J_c), photorespiration (J_o) and alternative processes such as nitrate assimilation and the water-water cycle (J_A). J_c and J_o were calculated as the product of the rate of carboxylation (V_c) or oxygenation (V_o) and the minimum of 4 electrons required for fixation per mol CO₂ or O₂. J_A was calculated as the difference of the electron budget: [J_A = J_{PSII} - (J_c + J_o)].

The intrinsic quantum efficiency of PSII photochemistry, F_v/F_m , was derived from fluorescence measurements after 30 min of dark-acclimation with leaf clips, using a portable chlorophyll fluorometer (Plant Efficiency Analyser, Hansatech, King's Lynn, Norfolk, UK).

Photosynthetic response to irradiance and temperature

Using the laboratory based system described above, grapevine leaves were illuminated with 800 μmol quanta m⁻² s⁻¹ (PAR) at 25°C until the rate of net CO₂ fixation and fluorescence yield reached steady state (after c. 60 min) at 360 μbar CO₂ and 210 mbar O₂ in N₂. Leaves were then subjected to lower temperatures in 5°C increments while keeping irradiance and C_i approximately constant at 800 μmol quanta m⁻² s⁻¹ and 280 μbar CO₂, respectively, and leaf vapour pressure difference less than 0.5 kPa. At each temperature, the O₂ partial pressure was changed to 20 mbar O₂ for suppression of both oxygenation of RuBP by Rubisco and the direct photoreduction of O₂ via the water-water cycle. At 25°C and 10°C, the photosynthesis-light responses of grapevine leaves were measured. The percentage of absorbed PAR excessive to that required by Φ_{PSII} was determined using the method described by Schreiber et al. (1994) where actual J_{PSII} is compared to the maximum potential rate of PSII electron transport (assuming Φ_{PSII} is equal to F_v/F_m at all light intensities).

Photoinactivation experiments

Leaf discs were taken from glasshouse-grown grapevines and immediately floated on water at room temperature in darkness for 1 h before the experiment. Discs were then vacuum infiltrated (2 standard atmospheres) with either water (control) or inhibitors in aqueous solution.

After leaves lost excess intercellular water they were floated on the water or an inhibitor solution at 25°C or 9°C in a partially filled beaker. The hypostomatous leaf discs were orientated with the abaxial side-up to allow for un-inhibited gas-exchange and illuminated from below with 1300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the adaxial surface. The gas mixture supplied was varied to partially suppress photorespiration (700 $\mu\text{bar CO}_2$ in air) or photorespiration and the water-water cycle (20 mbar O_2 in air) within the leaf. Leaf discs were subsequently dark-acclimated for 30 min and F_v/F_m was measured (Hansatech Plant Efficiency Analyser). The quantum yield of photoinactivation of PSII (QY_I) was calculated as the number of PSII reaction centres lost after absorption of one mole photons. QY_I was estimated from the dynamic decline of F_v/F_m for leaf discs subjected to various treatments. In these experiments 1 mM of the non-specific lincomycin was used to inhibit chloroplast protein synthesis (Aro et al. 1994; Mulo et al. 2003) and compared with responses of leaf discs infiltrated with 1 mM dithiothrietol, DTT (Hendrickson et al. 2003) to eliminate xanthophyll interconversion by violaxanthin de-epoxidase (Demmig-Adams and Adams 1993).

Photorespiration and the water-water cycle

The proportion of electron flow to oxygen was derived from the ratio of Φ_{PSII} to the quantum efficiency of CO_2 assimilation, Φ_{CO_2} ($\Phi_{\text{PSII}} : \Phi_{\text{CO}_2}$; Genty et al. 1990) in photosynthetic temperature response curves at light saturation. Electron flux to O_2 via photorespiration (J_C) and the water-water cycle was suppressed by 2% O_2 conditioning. The proportion of that reduction in $\Phi_{\text{PSII}} : \Phi_{\text{CO}_2}$ attributed to photorespiration was estimated according to Sharkey (1988) and von Caemmerer (2000). The proportion of electron flux to the water-water cycle and other O_2 dependent electron consuming processes, collectively entitled, J_A , was then determined as above. Respiration of grapevine leaves in the light was estimated from measurements of dark respiration rates (Road) at each leaf temperature based the correlations established by Atkin et al. (2000) for what eucalypt leaves. The proportion of electron flow utilized in photorespiration at saturating irradiance (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) was estimated according to von Caemmerer (2000).

Table 1. Total carotenoid pool size, total xanthophyll pool size and the percentage breakdown of total carotenoid pool into the constituents: neoxanthin, lutein, β -carotene and the xanthophylls violaxanthin, antheraxanthin and zeaxanthin (V + A + Z) for mature, exposed field-grown and glasshouse-grown Riesling leaf. Different letters indicate a significant difference between rows. Each point is the mean \pm SE of 3–6 leaves.

Site	Field	Glasshouse
Total carotenoid pool size, $\text{mmol mol}^{-1} \text{Chl } (a + b)$	494 \pm 5 ^E	470 \pm 27 ^E
Total xanthophyll (V + A + Z) pool size, $\text{mmol mol}^{-1} \text{Chl } (a + b)$	138 \pm 4 ^H	131 \pm 23 ^H
Percentage of total carotenoid pool		
neoxanthin, %	12.7 \pm 0.1 ^L	11.0 \pm 0.2 ^L
lutein, %	37.9 \pm 0.3 ^J	38.1 \pm 1.9 ^J
β -carotene, %	21.4 \pm 0.3 ^L	23.5 \pm 1.1 ^L
V + A + Z, %	27.9 \pm 0.2 ^N	27.4 \pm 3.2 ^N
Carotenoid: Chlorophyll ratio	0.49 \pm 0.01 ^P	0.47 \pm 0.03 ^P

Photosystem I measurements

Leaf discs from glasshouse-grown Riesling grapevines were floated abaxial side-down on a 6°C water bath and illuminated at 1300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for 0–2 h. Leaf discs were then removed and photooxidation and re-reduction of PSI reaction centre chlorophylls (P700) was measured using a PAM 101-fluorometer (H. Walz, Effeltrich, Germany) with a single wavelength (820 nm) emitter/detector in the reflectance mode. Decreasing reflectance at 820 nm following illumination with saturating far-red light is due to increased absorbance from P700⁺ radical formation, and therefore represents a relative measure of photooxidizable P700 and, inversely, the relative level of PSI inactivation (Kim et al. 2001). Far-red light (\sim 8 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$), filtered by Schott RG9 and Calflex C heat filters, was passed through an electronic shutter for 8 s, and the redox signals of P700 were stored in a digital oscilloscope (Gould 1421, Essex, UK) and later plotted on a chart recorder. The far-red light-induced photooxidation signal was normalized to the baseline signal to give a percentage change in transmission of the 820 nm beam.

Results

Carotenoid pool size and composition in field- and glasshouse-grown grapevine

Constituents of the total carotenoid content were broken down into average percentages maintained as neoxanthin (N), lutein (L), β -carotene (βC) and the total xanthophyll cycle pigments (V + A + Z) (Table 1). Riesling leaves of both field and glasshouse-grown vines showed no significant difference between total carotenoid content, mean total V + A + Z pool or carotenoid to chlorophyll ratio despite considerably warmer minimum temperatures (20°C) for the glasshouse-grown vines (Table 1). The xanthophyll de-epoxidation state, DPS, of glasshouse-grown leaves treated at 9°C and 1300 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for 3 h was 0.73 \pm 0.03 (data not shown).

Responses of leaf photosynthesis and energy dissipation to temperature

The results of the photosynthesis-temperature response curve (Fig. 1A) demonstrate that light-saturated leaf

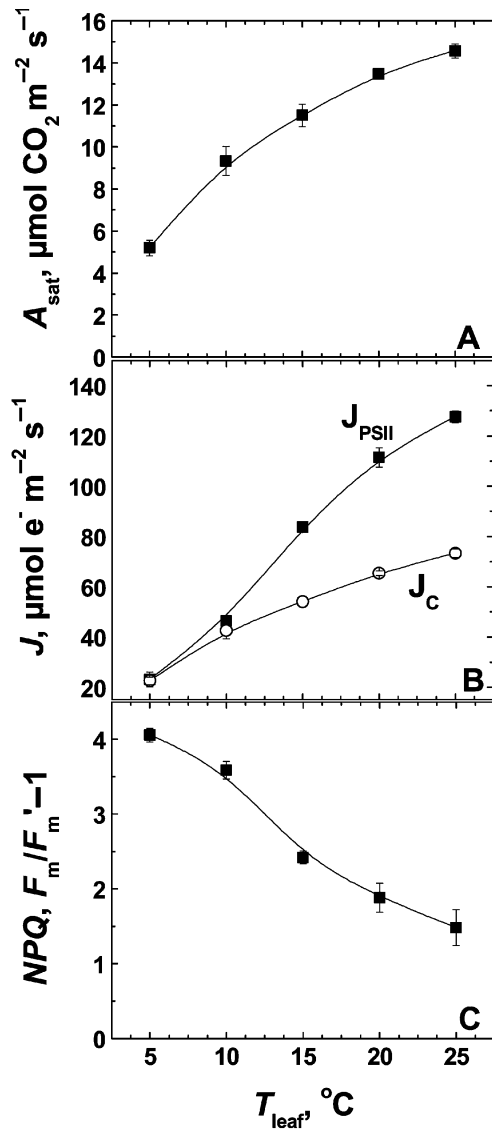


Fig. 1. Light-saturated ($800 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) rate of carbon assimilation, A_{sat} (A), total linear electron transport rate, J_{PSII} (B) and non-photochemical quenching, NPQ (C) of grapevine leaves plotted against leaf temperature. Each point is the mean \pm SE of 3–4 leaves. In panel B, total linear electron transport (■) and linear electron transport to carbon fixation (○; J_{C}) are presented. Measurements were undertaken at 210 mbar O_2 and 360 $\mu\text{bar CO}_2$. Intercellular CO_2 partial pressure was relatively constant and was not reduced by decreasing leaf temperature (See Table 2 for details). Parameters were estimated assuming a mesophyll conductance at 25°C ($g_m = 0.2 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$). Mean F_v/F_m subsequent to similar treatments varied from 0.834 ± 0.013 – 0.851 ± 0.002 for leaves treated between 25°C and 5°C , respectively.

photosynthetic CO_2 fixation rate (A_{sat}) was highly sensitive to changes in temperature showing an approximate Q_{10} of 1.7 ± 0.1 . Measurements of concurrent chlorophyll fluorescence (Fig. 1B) also revealed a strong temperature dependence of J_{PSII} at light-saturation ($Q_{10} = 2.5 \pm 0.1$). The photosynthesis-irradiance response varied with leaf temperature showing that, at light saturation ($800 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$), the percentage of absorbed light in

excess of that required for linear electron transport increased from $53 \pm 3\%$ to $83 \pm 1\%$ between 25°C and 10°C , respectively. Low temperature also reduced other electron-consuming processes (such as photorespiration and the water-water cycle), as evidenced by a reduction in the ratio of J_{PSII} to the rate of electron transport used for carbon fixation, J_{C} (Fig. 1B). Conversely, NPQ increased at lower temperatures under the same saturating irradiance ($800 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$) with a $Q_{10} = 0.6 \pm 0.1$ (Fig. 1C). These responses were not a result of sustained net photoinactivation of grapevine leaves as mean F_v/F_m remained relatively stable in response to treatments between 25°C and 5°C (0.834 ± 0.013 – 0.851 ± 0.002 between 25°C and 5°C , respectively).

Lack of net photoinactivation of PSI at low temperature

Under more extreme conditions of 6°C , $1300 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ and restricted gas exchange (leaf discs floated on water abaxial side down), it was possible to elicit a more rapid decline in F_v/F_m to a value below 0.3 after 2 h of treatment (Fig. 2). In contrast, measurements of the relative content of photooxidisable P700 in photosystem I, $\Delta\text{Transmission}$, during treatment under low temperature and high light conditions revealed that PSI was resilient to inactivation for at least 2 h under these conditions (Fig. 2).

Photorespiration and the water-water cycle at low temperature

The ratio $\Phi_{\text{PSII}}:\Phi_{\text{CO}_2}$ is an estimate of the quantum requirement for linear PSII electron flow per CO_2 fixed and has a theoretical minimum of 8 (Oberhuber and Edwards 1993). Experimental values are expected to be greater than the minimum requirement because of

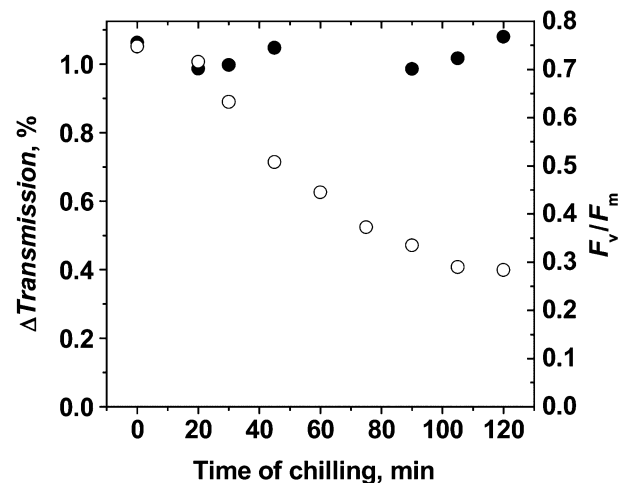


Fig. 2. Intrinsic quantum efficiency of PSII, F_v/F_m (○), and the relative content of photo-oxidizable P700 in PSI, $\Delta\text{Transmission}$ (●) of grapevine (*Vitis vinifera* cv. Riesling) leaf discs illuminated at $1300 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ and 6°C over time.

electron flow to other electron consuming processes such as photorespiration, the water-water cycle or nitrate/sulphate assimilation. Measurements of this ratio were undertaken at varying leaf temperatures and at two oxygen partial pressures (210 mbar and 20 mbar; Table 2). Not surprisingly, the quantum requirement for electron transfer to CO₂ was higher under ambient O₂ partial pressures compared to low (20 mbar) O₂ partial pressures (Table 2). The percentage reduction of the quantum requirement of CO₂ fixation reduced by low O₂ partial pressure was 46% and 2% at 25°C and 5°C, respectively. At low O₂ partial pressures, the quantum requirement for electron flow to CO₂ was relatively low between 9 and 10. At ambient O₂ partial pressures, lowering leaf temperature reduced the minimum quantum requirement by 48% such that at 5°C the quantum requirement was again lowered to around 9–10. From the photosynthetic equations of von Caemmerer and Farquhar (1981), it can be calculated that at 25°C the electron flow to photorespiration is around 29% of total electron transport (Table 2). In our experiments this potentially leaves a balance of 17% of electron flow that may be utilized by other energy-consuming processes. At room temperature the water-water cycle is saturated at approximately 210 mbar O₂ in air and half-saturated at approximately 6.5–8.4% O₂ in air (Furbank et al. 1982; Asada 1999). Thus, the O₂-sensitive water-water cycle can be considered a component of the decline in electron requirement seen in Fig. 4, brought about by the reduction in O₂ partial pressure.

The calculated percentage of electron flow to photorespiration declines with temperature but not to the same extent as the total reduction in the quantum requirement for CO₂ fixation seen here (Table 2). We must conclude that the balance, i.e. the proportion of electron flow to alternative processes consuming reducing equivalents (including the water-water cycle) was reduced from 17% 25°C to negligible proportions of total electron flow at 5°C.

Quantum yield of photoinactivation at low temperatures and varying conditions

Using F_v/F_m for the estimation of grapevine functional PSII (Hendrickson et al. 2003), the decline in F_v/F_m over time under varying treatments was measured for leaf

discs (Fig. 3). From F_v/F_m measurements the quantum yield of photoinactivation of PSII (QY_I) was calculated, assuming that all leaf discs initially had $0.95 \pm 0.04 \mu\text{mol PSII m}^{-2}$, which was the average value calculated from flash yield measurements of the O₂ yield per flash given to untreated grapevine leaves. This QY_I reflects the absolute number of initial PSII reaction centres that is lost after the accumulation of one mole of absorbed photons. Leaf discs exposed to warm (25°C) conditions under saturating (1300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) light exhibited a very low QY_I of $0.0049 \pm 0.0015 \mu\text{mol PSII (mol quanta)}^{-1}$ (i.e. one PSII per 2×10^8 quanta; Fig. 3). Under saturating light and chilling (9°C) temperatures the rate of photoinactivation was enhanced. During 2 h of exposure to 9°C, the mean QY_I was $0.0181 \pm 0.0042 \mu\text{mol PSII (mol quanta)}^{-1}$. The presence of 700 $\mu\text{bar CO}_2$ or 20 mbar O₂ did not appreciably affect the rate of photoinactivation during cold treatment under saturating light as demonstrated by the similar QY_I. In contrast, the rate of photoinactivation was dramatically increased with the addition of either 1 mM DTT solution, which inhibited violaxanthin de-epoxidase or 1 mM lincomycin, which inhibited chloroplast-encoded protein synthesis (Fig. 3).

The temperature dependence of photoprotective mechanisms

Utilization of absorbed photons was analysed in more detail in Fig. 4, which describes the temperature response of all energy dissipation mechanisms implicated in the photoprotection of light-saturated grapevine leaf under varying temperature. Using the quantum efficiencies (Φ) thylakoid electron transport and thermal dissipation described in this paper and elsewhere (Genty et al. 1989; Cailly et al. 1996), it was possible to calculate the flux (J) of absorbed light energy through these various pathways by multiplying Φ by absorbed light energy partitioned through PSII in an analogous fashion to the photosynthetic electron transport (J_{PSII} , often referred to as ETR). Photochemistry (J_{PSII}), light-dependent ΔpH - and xanthophyll-mediated non-photochemical thermal dissipation (J_{NPQ}) and the sum of light-independent heat dissipation and fluorescence ($J_{\text{f,D}}$) were engaged to varying extents in energy dissipation at saturating light intensity (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and varying leaf temperature (Fig. 4).

Table 2. CO₂ compensation point (Γ_*), mean \pm SE intercellular CO₂ partial pressure (C_i), the mean quantum requirement for electron transport for CO₂ fixation at 210 mbar O₂ and 20 mbar O₂ partial pressures, the minimum percentage of total electron transport used by photorespiration (R_L) and the remaining electron use by other O₂ consuming processes at saturating light (800 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$) and five leaf temperatures in grapevine (*Vitis vinifera* L.). Estimations of chloroplastic CO₂ partial pressure were made assuming a mesophyll conductance, $g_m = 0.3 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ at 25°C.

$T_{\text{leaf}}, ^\circ\text{C}$	$\Gamma_*, \mu\text{bar}$	$C_i, \mu\text{bar}$	$\Phi_{\text{PSII}}: \Phi_{\text{CO}_2}$ (210 mbar)	$\Phi_{\text{PSII}}: \Phi_{\text{CO}_2}$ (20 mbar)	Reduction by 20 mbar O ₂ , %	$R_L, \%$	Other processes, %
25	37	257 \pm 25	17 \pm 1	9.2 \pm 0.6	46	29	17
20	32	263 \pm 3	17 \pm 1	9.7 \pm 0.2	42	26	16
15	27	253 \pm 7	14 \pm 1	8.9 \pm 0.6	36	24	12
10	23	258 \pm 11	10 \pm 1	8.3	22	21	1
5	19	340 \pm 16	9 \pm 1	8.6 \pm 0.5	2	14	–

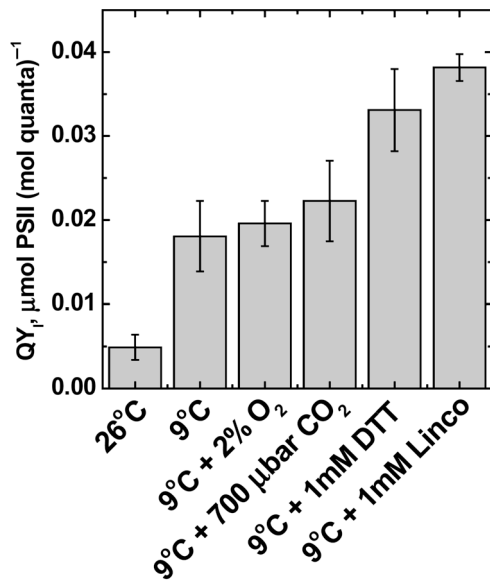


Fig. 3. Average quantum-yield of photoinactivation of PSII (QY_1) in glasshouse-grown *Vitis vinifera* L. cv. Riesling leaf discs after illumination for 2 h at $1300 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ under varying experimental conditions. Quantum yields are calculated assuming that a linear relationship exists between F_v/F_m and the percentage of functional PSII reaction centres and that all leaf discs start with $0.95 \pm 0.04 \mu\text{mol PSII m}^{-2}$. Each rate is the mean \pm SE of 3–9 measurements. Different letters indicate a significant difference between rows. The results for the $9^\circ\text{C} + 1 \text{ mM DTT}$ treatment was calculated from Hendrickson et al. (2003).

Similar to Fig. 1, as leaf temperature was reduced below 25°C , J_{PSII} decreased in response to a reduction in the capacity of A_{sat} (Fig. 4). Additionally, $J_{\text{r,D}}$ declined as temperatures were reduced and J_{NPQ} increased from $37 \pm 3\%$ to $75 \pm 1\%$ accounting for the reduction in J_{PSII} and $J_{\text{r,D}}$ from 25°C to 5°C , respectively (Fig. 4).

The rate of electron transport through PSII (J_{PSII}) can be subdivided into the Rubisco dependent processes of carboxylation (J_c) and oxygenation (J_o) and other alternative light-dependent electron consuming processes including the water-water cycle, nitrate assimilation and sulfate assimilation (J_A). As demonstrated by Fig. 1 and Table 2, the relative importance of J_A in energy dissipation was significantly reduced at low leaf temperatures in grapevine (Fig. 4). This result was further evaluated using three different temperature-dependent mesophyll conductance values to predict J_A (Fig. 5) demonstrating a lack of significant J_A at low temperature regardless of contrasting mesophyll conductance. This variation increased with temperature, predicting large J_A closer to 25°C (Fig. 5).

Discussion

Net photoinactivation is negligible in grapevines despite limited photosynthesis

Despite a dramatic increase in the proportion of all excess absorbed light by 30% under light-saturating irradiances, there was negligible net photoinactivation

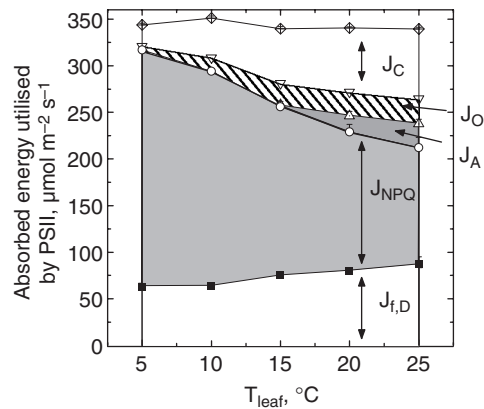


Fig. 4. The proportion of absorbed light transferred to the various energy sinks within the grapevine leaf at low temperatures assuming a constant mesophyll conductance ($g_m = 0.2 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$). The various dissipatory processes include total linear electron flow to CO_2 fixation (J_c), photorespiration (J_o) and alternative processes (J_A), and the rate of energy dissipation through light-dependent ΔpH - and xanthophyll-mediated thermal dissipation (J_{NPQ}) and the sum of fluorescence quenching and light-independent thermal dissipation ($J_{\text{r,D}}$). Measurements were made under constant illumination at $800 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ with C_i as described in Table 2 and $\text{O}_2 = 210 \text{ mbar}$ partial pressure. Each point is the mean \pm SE of 3 leaves. Calculations were made assuming that the fraction of incident light absorbed by the leaf does not change with reductions in leaf temperature and that approximately one half of total absorbed light is utilized by PSII. Mean F_v/F_m subsequent to similar treatments ranged between 0.832 ± 0.015 and 0.853 ± 0.016 for leaves treated from 25°C to 5°C .

of PSII observed following short-term exposure of attached leaves to high light and low temperature (Figs 1 and 3). Although low temperature does increase the rate of photoinactivation, by a factor of three, the quantum yield of photoinactivation (QY_1) is still relatively low ($QY_1 = 0.0181 \pm 0.0042$) compared to other species. Other studies reported higher maximum QY_1 of 0.039, 0.06, 0.1 and $0.3 \mu\text{mol PSII (mol quanta)}^{-1}$ for bean (Pätsikkä et al. 1998), pumpkin acclimated to very high light (Tyystjärvi and Aro 1996), capsicum (Lee et al. 1999) and pea leaf discs (Park et al. 1995), respectively, at room temperature. This would explain the absence of net photoinactivation of PSII in grapevine leaves under a variety of conditions and after varying treatments, particularly low temperature events in the field (Chaumont et al. 1995, 1997; Flexas et al. 1999; Hendrickson et al. 2003). However, it should be noted that grapevines are not immune to photoinactivation if conditions are severe enough (Fig. 3).

Several mechanisms contributing to grapevine photoprotection at low temperature have been proposed, including the xanthophyll cycle (Chaumont et al. 1995, 1997; Hendrickson et al. 2003), the D1 repair cycle (Chaumont et al. 1995) and the water-water cycle (Flexas et al. 1999). The present investigation into this phenomenon yielded three important results: (1) that photoinactivation of PSI was not responsible for the reduction in photosynthetic capacity at low temperatures and high light (Fig. 2); (2) that the intrinsic repair rate of

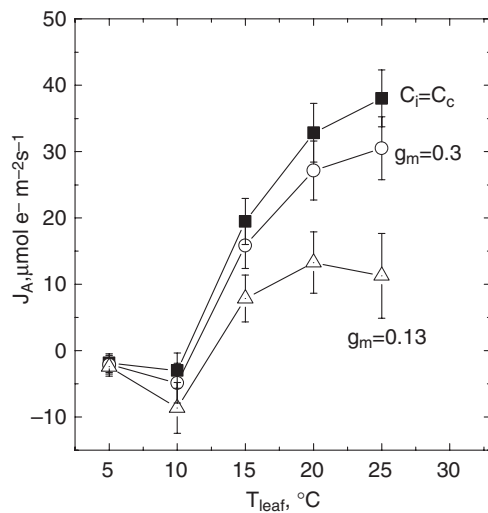


Fig. 5. The estimation of the proportion of absorbed light dissipated via alternative processes other than linear electron transport or thermal dissipation (J_A) at low temperature. Either no mesophyll conductance (g_m) where $C_i = C_c$ (■), $g_m = 0.3 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ (○) or $g_m = 0.13 \text{ mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ bar}^{-1}$ (△) was assumed. Measurements were made under constant illumination at $800 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$, and approximately constant C_i ($274 \pm 14 \mu\text{bar}$) and O_2 (210 mbar) partial pressures. Each point is the mean \pm SE of 3 leaves. Calculations were made assuming that the fraction of incident light absorbed by the leaf does not change with reductions in leaf temperature and that approximately one half of total absorbed light is utilized by PSII.

photoinactivated PSII reaction centres at low temperature was high enough to maintain a low rate of net photoinactivation (Fig. 3); and (3) that light-dependent thermal dissipatory processes were relatively more important than O_2 consuming processes for effective minimization of gross photoinactivation (Table 2; Figs 3 and 4).

Measurement of QY_I in the presence of lincomycin suggests that D1 protein repair is also a highly important process at low temperature in grapevine leaves (Fig. 3). This is surprising given that the D1 repair process has been reportedly suppressed by low temperature in *Cap-sicum* (Lee et al. 1999) and even completely inhibited at 4°C in pumpkin (Salonen et al. 1998). Under conditions similar to those used in this study, low light grown grapevines only lost 20% of D1 protein levels (Chaumont et al. 1995) suggesting that warm-grown grape leaves are capable of maintaining a high rate of turnover at low temperatures. From the doubling of the rate of loss of F_v/F_m in the presence of lincomycin (Fig. 3), we can infer that the rate of D1 repair was roughly equivalent to one-half of the rate of gross photoinactivation at 9°C and $1300 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. These conclusions must be tempered by the non-specific nature of lincomycin and its possible effect on the redox state of the chloroplasts (Mulo et al. 2003). Cold acclimation of plants includes a desaturation of glycerolipids within the thylakoid membrane that accelerates the processing of precursor D1 protein (Murata and Nishiyama 1998). It is therefore expected that cold-acclimated field-grown vines would

have an even greater resilience to photoinactivation at low leaf temperatures.

Thermal dissipation is the most significant photoprotective process at low temperatures

Thermal dissipation of absorbed light via xanthophyll-mediated non-photochemical quenching is the dominant photoprotective mechanism in grapevine at low temperature (Figs 3 and 4). At light-saturation the fraction of total absorbed light dissipated via ΔpH - and xanthophyll-mediated processes was up to 75% of total absorbed radiation in grapevine (Fig. 4), a value similar to the 76% reported by Demmig-Adams et al. (1996) for sun-acclimated *Vinca major* leaves. A study by Hovenden and Warren (1998) reported similar relative increases in thermal dissipation for chilled eucalypt leaves. A recent review by Flexas and Medrano (2002) quoted specific thermal dissipation fractions of 64% and 75–92% for irrigated and drought stressed grapevines, respectively, at high light and high temperatures. The irrigated control (25°C) grapevines in the present study are marginally lower than this range at equivalent light intensities ($50 \pm 3\%$) and is likely due to the higher leaf temperatures (33°C) reported by Flexas and Medrano (2002). The importance of the xanthophyll cycle in minimizing photoinactivation is supported by the relatively high xanthophyll de-epoxidation state, DPS, at 9°C and the doubling of QY_I at 9°C by DTT, an inhibitor of violaxanthin de-epoxidase (Fig. 3). It is clear that grapevine leaves rely heavily upon thermal dissipation under stressful conditions, including low leaf temperatures, and that the capacity for thermal dissipation is very high even at optimum leaf temperatures (35–37%).

Total xanthophyll pool sizes did not differ between glasshouse and field conditions implying that both excessive irradiance (glasshouse conditions) and additional low temperature stress (field conditions) stimulates the same accumulation of carotenoid pigments and is suggestive of a similar photoprotective capacity. Similar results were obtained by Medrano et al. (2002) who showed that total carotenoid pool sizes did not differ between irrigated and drought-stressed grapevines that were both high light-acclimated. The V + A + Z leaf concentrations comprising 20–28% of the total leaf carotenoid content in this study were similar to that reported for four sun-acclimated species at 25% of the total leaf carotenoid content and corresponding V + A + Z leaf concentrations of 114–141 mmol mol^{-1} Chl. (a + b) (Demmig-Adams et al. 1995).

Reduction of O_2 via photorespiration and water-water cycle is negligible at low temperatures

When the water-water cycle and photorespiration were suppressed by low O_2 partial pressure the rate of photoinactivation failed to increase (Fig. 3). This indicates that both processes contributed less to photoprotection under low temperature and high light conditions than at high

temperatures (Table 2; Fig. 3). This data is further supported by energy dissipation where the total flux of absorbed light energy through these two processes is less than a few percent. This result is remarkably similar to previous observations of *Epilobium hirsutum*, *Phaseolus vulgaris* and *C₃ Flaveria pringlei* that demonstrated, by use of the same technique as for this study, that alternative O₂ dissipative processes declined to negligible levels below 15°C (Oberhuber and Edwards 1993; Ghoshghaie and Cornic 1994). If the water-water cycle were increasingly important for photoprotection at low temperature then one would assume that the proportion of direct reduction of O₂ would increase at low temperature as reported by Flexas et al. (1999). In our study, however, the alternative dissipatory component (J_A) appears to be more temperature sensitive than photorepiration, implying that the importance of the water-water cycle for photoprotection was reduced at low temperature in grapevine. This calculation is in qualitative agreement with conclusions made by other authors working with C₃ plants (Wiese et al. 1998; Ruuska et al. 2000). This conclusion is consistent with the lack of PSI inactivation during chilling in the light (Fig. 2) and the lack of a significant increase in the QY₁ at low temperature and low O₂ partial pressure (Fig. 3). Jeong et al. (2002) demonstrated that in both chilling-sensitive and chilling-resistant varieties of rice, chilling in the light had little effect on the activities of the chloroplastic antioxidant enzymes ascorbate peroxidase and superoxide dismutase, two known target sites for chilling-induced PSI photoinactivation. The extent of photoinactivation of grapevine photosynthesis appears to then depend predominantly upon the capacities for xanthophyll-mediated photoprotection and repair of photosystem II D1 protein.

The estimates of photorespiration-dependent electron transport, J_o, and hence J_A shown in Fig. 4 depend upon several assumptions. Most importantly, it depends upon the CO₂ compensation point in the absence of mitochondrial respiration, Γ*, that in turn depends upon accurate calculations of C_i. Mesophyll resistance at low temperature may provide a considerable barrier against CO₂ diffusion favouring oxygenation over carboxylation (Laisk and Loreto 1996). Although a mesophyll diffusion conductance term was included in calculations of chloroplastic CO₂ partial pressures, this was taken from a non-specific relationship (Evans and Loreto 2000) and may not accurately reflect the CO₂ diffusion barrier of grapevine leaves. However, it should be noted that the relationship is very uniform between species of a mesophytic and sclerophyllous nature (Evans and Loreto 2000). The same issue arises when considering the temperature dependence of Γ* used (see Bernacchi et al. 2001) that may have under- or over-estimated Γ* for grapevine leaves at low temperatures. Regardless of the large variation in J_A induced by the lack of or use of different assumed mesophyll conductance this study clearly demonstrates that there is minimal alternative electron transport other than to photorespiration or CO₂ fixation below 15°C (Fig. 5).

Conclusions

We conclude that despite a reduction in energy dissipation by Rubisco-mediated processes at low temperature causing significant increases in the amount of light excessive to that utilized by photosynthesis, grapevine leaves maintain high intrinsic quantum efficiencies of PSII. Grapevine leaves were found to be more resistant to photoinactivation at high and low temperatures compared to other species and that xanthophyll-mediated non-photochemical quenching and a high intrinsic rate of D1 repair were responsible for conferring this high resistance by reducing the 'gross' rate of photoinactivation and the 'net' rate of photoinactivation, respectively. A detailed analysis of energy dissipation revealed that up to 75% of all absorbed light was dissipated thermally via ΔpH and xanthophyll-mediated non-photochemical quenching at low temperatures and moderate light. Energy dissipation via photorespiration was found to account for up to 20% of total electron transport when using an assumed temperature-dependent mesophyll conductance. However, the flux of light energy dissipated by O₂ consuming processes was minimal at low temperatures. The water-water cycle was more temperature dependent than photorespiration and was reduced to negligible proportions below 15°C, regardless of mesophyll conductance.

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