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ORIGINAL ARTICLE

Photoprotection by foliar anthocyanins mitigates effects of boron toxicity in sweet basil (*Ocimum basilicum*)

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Abstract Boron (B) toxicity is an important agricultural problem in arid environments. Excess edaphic B compromises photosynthetic efficiency, limits growth and reduces crop yield. However, some purple-leafed cultivars of sweet basil (*Ocimum basilicum*) exhibit greater tolerance to high B concentrations than do green-leafed cultivars. We hypothesised that foliar anthocyanins protect basil leaf mesophyll from photo-oxidative stress when chloroplast function is compromised by B toxicity. Purple-leafed 'Red Rubin' and green-leafed 'Tigullio' cultivars, grown with high or negligible edaphic B, were given a photoinhibitory light treatment. Possible effects of photoabatement by anthocyanins were simulated by superimposing a purple polycarbonate filter on the green leaves. An ameliorative effect of light filtering on photosynthetic quantum yield and on photo-oxidative load was observed in B-stressed plants. In addition, when green protoplasts from both cultivars were treated with B and illuminated through a screen of anthocyanic protoplasts or a polycarbonate film which approximated cyanidin-3-*O*-glucoside optical properties, the degree of photoinhibition, hydrogen peroxide production, and malondialdehyde content were reduced. The data provide evidence that anthocyanins exert a photoprotective role in purple-leafed basil mesophyll cells, thereby contributing to improved tolerance to high B concentrations.

Keywords Chlorophyll fluorescence · Flavonoids · Photoabatement · Photoinhibition · Photo-oxidative stress · Protoplast

Abbreviations

Φ_{PSII}	Quantum yield of photosystem II
Chl	Chlorophyll
DCFH-DA	2′,7′-Dichlorofluorescein diacetate
DCF	2′,7′-Dichlorofluorescein
ETR	Electron transport rate
F_v/F_m	Maximal quantum yield of photosystem II
MDA	Malondialdehyde
MES	2-(*N*-morpholino)ethanesulfonic acid
NPQ	Non-photochemical quenching
PSII	Photosystem II
q_P	Photochemical quenching coefficient
ROS	Reactive oxygen species
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid

Introduction

Boron (B) is a microelement for which the window between essential and toxic concentrations is extremely narrow (Nable et al. 1997). B accumulation is particularly prominent in soils from arid and semi-arid environments, and in areas prone to geothermal activity; edaphic concentrations of B in soil solution between 5 and 100 mg L^{-1} are toxic for many species (Ryan et al. 1998). In the soil solution, B is found primarily as boric acid (H_3BO_4), which can

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either diffuse passively into root hair cells or else be taken up through channels (Dannel et al. 1998). The primary function of B is considered to be its contribution to the rhamnogalacturonan II complex in plant cell walls (Matoh et al. 2000).

Excessive uptake of B may: (1) impair cell division and development by binding to ribose, both as the free sugar and as a constituent of RNA; (2) interfere with primary metabolism by binding with ribose as a part of ATP, NAD(H) or NADP(H) and nucleotides; and (3) lower the pH of the cytosol, thereby affecting protein conformation and biosynthesis (Reid et al. 2004). As a result, key physiological processes such as photosynthesis are strongly perturbed (Cervilla et al. 2007; Landi et al. 2013c). Thus, because the capacity to process incident light energy is compromised, plants growing on B-rich soil are more likely to produce a surplus of reactive oxygen species (ROS) and consequently incur oxidative stress (Cervilla et al. 2007; Landi et al. 2013a). This, in addition to a reduction in photosynthetic area caused by necrosis of leaf margins, can greatly reduce net carbon assimilation rate (Lovatts and Bates 1984; Reid et al. 2004; Guidi et al. 2011).

It is evident that some plant species are more tolerant to high concentrations of edaphic B than are others (Reid 2007). This differential sensitivity to B can extend to differences between cultivars of the same species. For example, Landi et al. (2013b) reported that two purple-leafed cultivars of sweet basil, *Ocimum basilicum* L., were less sensitive than eight green-leafed cultivars to an excess of B. When supplied with B at 20 mg L^{-1} (approximately 1.8 mM B), the purple cultivars showed less necrosis, less oxidative damage and a smaller decline in photosynthesis. Nothing is known of the mechanism by which apparent tolerance to B is achieved in these purple cultivars. However, the presence of concentrated anthocyanins in the leaf epidermis and veins of their leaves is the most obvious difference between the two cultivars (Makri and Kintzios 2007). Given the putative protective functions of foliar anthocyanins in response to other stressors such as drought, wounding, pathogens, herbivores, extreme temperatures and ionic unbalance (reviewed by Gould et al. 2009; Andersen and Jordheim 2006), it is at least possible that these, or related flavonoids, are involved in the tolerance to excess B.

There are at least three possible mechanisms through which anthocyanins might mitigate the effects of B toxicity. First, anthocyanins might chelate to supernumerary B ions and sequester them in the cell vacuole. The ability of anthocyanins to chelate to metals has been reported for tungsten (Hale et al. 2002), aluminium (Elhabiri et al. 1997), copper (Somaatmadja et al. 2006), gallium (Elhabiri et al. 1997; Buchweitz et al. 2012), iron (Buchweitz et al. 2012), and molybdenum (Hale et al. 2001). However, there is as yet no experimental evidence for B-anthocyanin chelates in *Planta*. Second, anthocyanins may function indirectly by reducing the oxidative load on chloroplasts for which the ability to process light energy has been compromised through B toxicity. Anthocyanins are extremely potent antioxidants, capable of scavenging most species of reactive oxygen in vitro (Gould et al. 2002a; Gould 2004), and may well contribute to the antioxidant pool in purple-leafed basil (Landi et al. 2013a). However, because the anthocyanins reside exclusively in the abaxial and adaxial epidermises of basil leaves (Makri and Kintzios 2007), the pigments are not optimally located to scavenge chloroplast-derived ROS in this species. Third, by absorbing a proportion of the incident light energy, anthocyanins may diminish the generation of ROS by B-affected chloroplasts within leaf mesophyll. A possible protective effect of photoabatement by anthocyanins has been postulated many times (Neill and Gould 2003; Kytridis et al. 2008; Hughes et al. 2012; Hatier et al. 2013), but has never been evaluated in relation to B tolerance.

Here, we test the hypothesis that light-screening by epidermal anthocyanins diminishes the severity of photooxidative stress in subjacent mesophyll cells of sweet basil compromised by B toxicity. We report the responses of purple-leafed 'Red Rubin' (Fig. 1a) and green-leafed 'Tigullio' (Fig. 1b) basil cultivars to a combination of B and high light treatment, as measured by changes in chlorophyll fluorescence parameters, hydrogen peroxide production, and lipid peroxidation. Finally, we disentangle possible effects of anthocyanins as antioxidants from those as light filters by comparing the responses of green (acyanic) protoplasts isolated from the two cultivars.

Materials and methods

Plant material

Seeds of *O. basilicum* cv. 'Tigullio' and cv. 'Red Rubin' were purchased from Franchi Sementi (Milan, Italy). Plants were grown from seed in a sandy soil–peat mixture (60:40, v:v) using 1.25 L pots, and watered with tapwater (which contained less than 18 μM B) for 14 d after sowing. Plants were then irrigated daily with B-enriched tapwater (1.8 mM B, added as boric acid) at pH 6.0 for 20 days. Control plants were watered only with tapwater, adjusted with sulphuric acid to the same pH. All plants were grown under natural light in an unheated glasshouse in Wellington, New Zealand (41°17′00″S 174°46′00″E) between March and July 2013. The minimum and maximum air

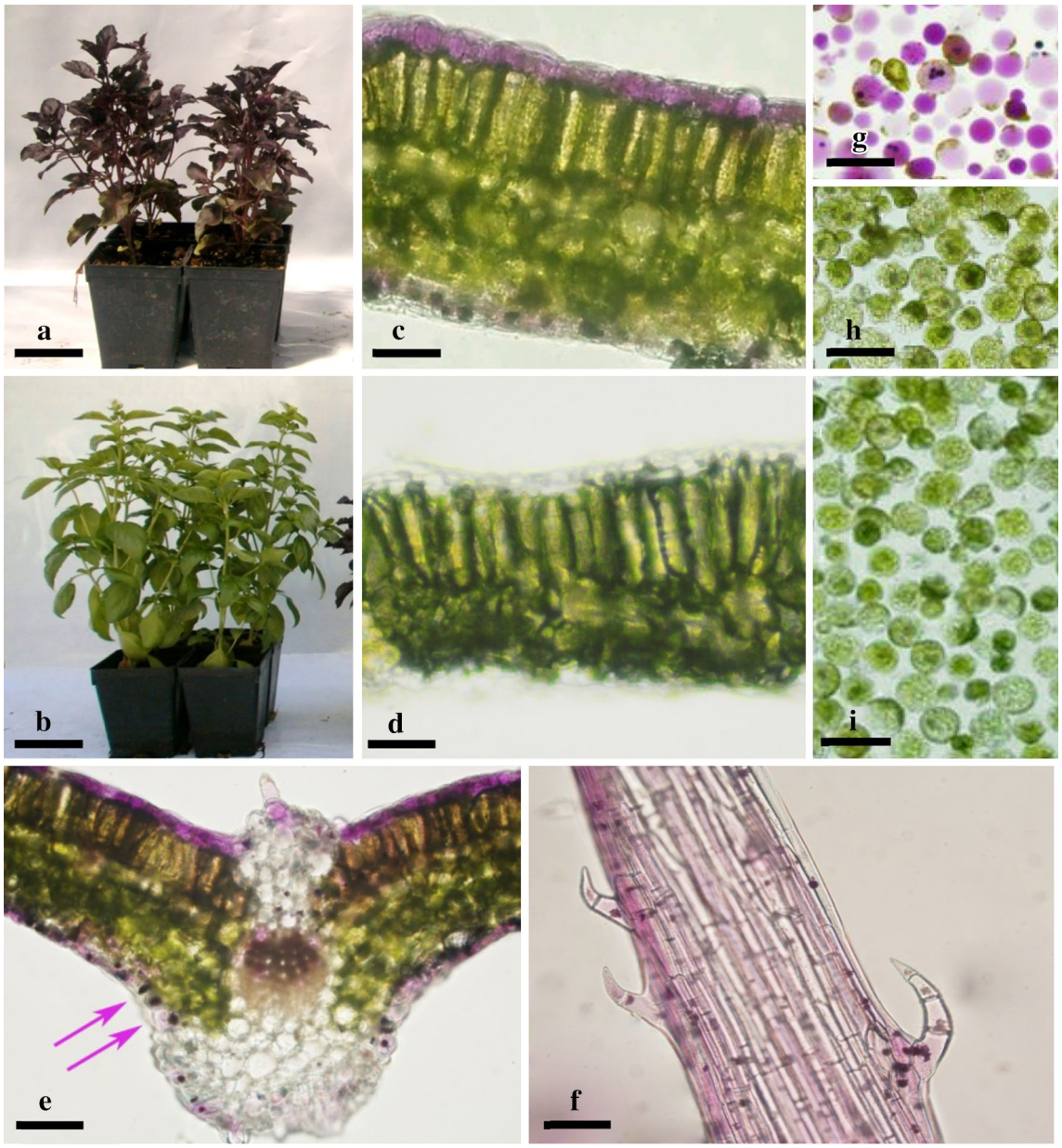

Fig. 1 Morphology and leaf anatomy of *Ocimum basilicum* cvs. 'Red Rubin' (**a**, **c**, **e**, **f–h**) and 'Tigullio' (**b**, **d**, **i**). Photographs of intact plants (**a**, **b**). Transverse sections through lamina (**c**, **d**) and midrib (**e**), showing anthocyanic vacuolar inclusions (*arrows*). Epidermal peel showing anthocyanins in trichomes (**c**). Protoplasts from leaf epidermis (**g**) and mesophyll (**h**, **i**). *Bars* 10 cm (**a**, **b**), 150 µm (**c**, **d**), 200 µm (**e**), 50 µm (**f–i**)

temperatures averaged 13 and 24 °C, respectively. Irradiance averaged 650 µmol m^{-2} s^{-1}, with peaks around 1,000 µmol m^{-2} s^{-1} at midday.

Leaf anatomy

Transverse hand sections were taken through fresh leaves, examined in an Olympus AX70 photomicroscope (Olympus Optical Co., Hamburg, Germany) and photographed using an Olympus DP70 digital camera.

Light treatments

Five B-treated and five control plants were randomly selected, and the third or fourth youngest fully-expanded leaf on each was irradiated on the adaxial surface with 1,000 µmol m^{-2} s^{-1} white light for 4 h at 23 °C. The light source was a bank of 12 × 1 W LEDs (Shaoxing Prolux Lighting Co., Zheijiang, China), which produced white light with a colour temperature of 6,500 K. To simulate the effect of epidermal anthocyanins in the green plants, one

leaf on each of five 'Tigullio' B-treated plants was covered with a purple polycarbonate film (Supergel Rosco no. 35 "light pink", Sydenham, UK) before being irradiated. The film attenuated approximately 30 % of the incident light, primarily in the green and yellow portions of the visible spectrum. This approximated the absorbance spectrum of anthocyanins from the 'Red Rubin' epidermis, as determined with an Ocean Optics USB2000 spectrometer (Ocean Optics Inc., Dunedin, FL, USA; data not shown). The transmittance and chromaticity coordinates of the film are available at: http://www.rosco.com/filters/supergel.cfm. An additional set of B-treated 'Red Rubin' plants ($n = 5$) was randomly chosen, from which one leaf per plant was irradiated with 1,300 µmol m^{-2} s^{-1} white light for 4 h at 23 °C. The 30 % additional light approximated the additional quantum flux that would have reached leaf mesophyll had not the leaves been equipped with anthocyanins.

Protoplast isolation

Acyanic (green) protoplasts were isolated from the leaf mesophyll of both 'Tigullio' and 'Red Rubin' control plants using the method by Bi et al. (2009) with some modifications. The abaxial cuticle was manually removed, and the exposed epidermis floated face down on a solution of 1 % cellulase from *Tricoderma viride* (1 U mg^{-1}, Sigma-Aldrich, Auckland, NZ) and 0.25 % pectinase from *Aspergillus niger* (1.7 U mg^{-1}, Sigma–Aldrich) in an isolation medium comprising 8 % mannitol, 10 mM $CaCl_2$, 20 mM KCl, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 5.7. Samples were gently agitated overnight under darkness at 23 °C. The partially digested leaf fragments were removed, and the remaining suspension centrifuged at 40*g* for 3 min. The pellet was suspended in Cell Protoplast Washing solution (CPW) containing 0.45 M sucrose (pH 5.8), and protoplast viability was assessed using Evans blue dye. Protoplasts were diluted using the same isolation medium without enzymes to obtain a suspension of about 2.5×10^{-6} protoplasts mL^{-1}. The suspensions were gently agitated under 20 µmol m^{-2} s^{-1} white light at 23 °C until required.

Purple protoplasts from 'Red Rubin' epidermis were isolated by incubating leaves without a cuticle in the enzymatic solution for 2 h as described by D'Onofrio et al. (1999). The protoplasts were counted with a hemocytometer and then re-suspended in CPW with sufficient 0.4 M sucrose in 0.1 M sorbitol to obtain 2.5×10^{-6} protoplasts mL^{-1}.

Protoplast treatments

Acyanic protoplasts were treated with 4 µM H_3BO_4 in 20 mM MES buffer (pH 5.7) for 30, 90 or 180 min at 23 °C. Control protoplasts were supplied with 20 mM MES buffer adjusted to pH 5.7 with HCl. Both B-treated and control protoplasts were irradiated with either 35 or 100 µmol m^{-2} s^{-1} white light for which the spectral characteristics were similar to that used to induce photoinhibition. Protoplasts were gently agitated during treatments.

Further acyanic protoplasts from the leaf mesophyll were exposed to 100 µmol m^{-2} s^{-1} light, obtained by passing white light through (1) the same purple polycarbonate filter used in the leaf photoinhibition experiments, or (2) a suspension of purple protoplasts isolated from 'Red Rubin' epidermis. Because of their lower density in a sucrose gradient, the purple protoplasts naturally settled on the top when mixed with acyanic ones, thereby presenting an opportunity to test for effects of these natural light filters. A 1:1 ratio of purple: acyanic protoplasts was found best to simulate the effects on optical paths of an anthocyanic epidermis in 'Red Rubin' leaves, as measured using the Ocean Optics spectrometer.

Chlorophyll fluorescence analysis

Modulated chlorophyll (Chl) *a* fluorescence analysis was conducted at 23 °C for 40 min in dark-adapted leaves using a PAM-2000 chlorophyll fluorometer (Walz, Effeltrich, Germany) connected to a Walz 2030-B leaf clip. Ratios of variable to maximum fluorescence (F_v/F_m) were measured, and then leaves were subjected to a light ramp comprising 12 steps from 0 to 1,850 µmol m^{-2} s^{-1} using the light source supplied by the fluorometer. For protoplasts, Chl *a* fluorescence was analysed using a Walz Water-PAM fluorometer (Walz), and F_v/F_m values were recorded after 20-min dark adaptation. Proportionate reductions in F_v/F_m were expressed relative to the maximum values obtained for protoplasts retained in the dark. Light-response curves for the protoplasts were performed using a light ramp of nine steps from 0 to 345 µmol m^{-2} s^{-1} using the light source supplied by the Water-PAM. Quantum yield of photosystem II (Φ_{PSII}) was calculated using the equations of Genty et al. (1989); photochemical (q_P) and non-photochemical quenching (NPQ) were calculated as described by Schreiber et al. (1986); and the apparent electron transport rate (ETR) was estimated according to Krall and Edwards (1992). For details of instrument settings and measured parameters see Guidi et al. (2010).

Chlorophyll determination

Chl levels in protoplasts and leaves were determined using a method modified after Jingxian and Kirkham (1996). Aliquots containing approximately 3.1×10^6 viable protoplasts collected after 180 min of treatments were centrifuged at 7,000*g* for 5 min, and then the pellet re-suspended

in 1 mL 80 % aqueous acetone. The suspension was centrifuged and eluted repeatedly until the pellet was completely decoloured. For leaves, 0.3 g (DW) was homogenised in 80 % aqueous acetone, and then the same procedure adopted. Concentrations of Chl *a* and *b* were calculated according to Lichtenthaler (1987) at 663 and 648 nm with an Ultrospec 2100 Pro UV–VIS spectrophotometer (GE Healthcare Ltd, UK).

Boron determination

The third and fourth youngest fully-expanded leaves were randomly collected from plants after 20 days of B treatment. Each sample was dried at 50 °C for 48 h, and 0.5 g (DW) digested with HNO_3 and $HClO_4$ at 230 °C for 2 h. B concentration was determined colorimetrically using the azomethine-H method (Wolf 1974) from absorbance measurements at 420 nm taken using an Ultrospec 2100 Pro UV–Vis spectrophotometer (GE Healthcare Ltd).

Malondialdehyde determination

Oxidative damage was estimated as malondialdehyde (MDA) accumulation by the thiobarbituric acid (TBA) reaction as reported in Guidi et al. (2011). Protoplasts were homogenised in 0.1 % trichloroacetic acid (TCA) and mixed with an equal volume of TBA (20 % of TCA and 0.5 % w/v TBA). The solution was heated at 90 °C for 25 min, and then cooled and centrifuged at 3,000*g* for 15 min. This method overcomes possible interference by anthocyanins in the TBA assay. Absorbances at 600, 532 and 400 nm were measured, and MDA levels calculated using the MDA extinction coefficient 155 mM^{-1} cm^{-1}. MDA concentrations were expressed per unit soluble protein, which was determined spectrophotometrically at 595 nm as described by Bradford (1976), with bovine serum albumin as a standard.

Anthocyanin quantification

Anthocyanins were extracted in acidified methanol (1.5 % HCl, v/v) overnight at room temperature, and their absorbance taken at 535 nm using an Ultrospec 2100 Pro UV–VIS spectrophotometer (GE Healthcare Ltd). Absorbance values were converted to cyanidin-3-*O*-glucoside equivalents using a calibration curve obtained for an authentic sample in the same solvent.

H_2O_2 production

To detect H_2O_2 production, protoplasts were incubated in 5 μM of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) dissolved in 15 % aqueous ethanol for 30 min at 23 °C under darkness. The protoplast solutions were gently agitated throughout the incubation (180 min). Green fluorescence, indicative of the oxidation by H_2O_2 of DCFH-DA to 2′,7′-dichlorofluorescin (DCF), was observed in a Leica TCS4D confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). A Krypton–Argon laser (488 nm) was used for excitation, and emission above 515 nm was collected. At least 30 protoplasts per treatment were examined. Autofluorescence of Chl was recorded at 648 nm.

To observe H_2O_2 production in foliage, the third or fourth youngest fully-expanded leaves were harvested, rinsed twice in double distilled water and then incubated for 30 min at 23 °C under darkness in loading buffer (50 mM Tris–HCl, pH 7.0) with 120 μM of DCFH-DA. After incubation, leaves were rinsed for 2 min in the loading buffer solution, mounted in fresh buffer on a microscope slide, examined for green fluorescence in an Olympus AX70 compound microscope (Olympus Optical Co., Hamburg, Germany), and photographed using an Olympus DP70 digital camera.

Statistics

Reported data are the mean ± SD of at least five replicates. DCFH-DA fluorescence images of infiltrated leaves are representative of at least 10 replicates per treatment, and those of protoplasts of at least 30 replicates. Means were compared by two-way ANOVA. Differences among treatment means were evaluated by Fisher's least significant difference test (LSD) for $P = 0.05$. Percentage values were arcsine transformed prior to analysis. All statistical analyses were performed using CoStat software package (CoHort™ Software, 1994; Berkeley, CA, USA).

Results

Histological location of anthocyanins

Anthocyanins in 'Red Rubin' leaves were found in the epidermis (Fig. 1c) and in the midrib. The purple pigmentation was not evident in any tissue in 'Tigullio' leaves, which were otherwise structurally similar to 'Red Rubin' (Fig. 1d). For the purple leaves, anthocyanins were present in the adaxial epidermal cells as vacuolar solutions; in the abaxial epidermis, they occurred both in solution and as aggregates known as 'anthocyanin vacuolar inclusions' (AVIs) in both the epidermal cells (Fig. 1e) and trichomes (Fig. 1f). Protoplasts isolated from the epidermis of 'Red Rubin' retained their purple pigmentation (Fig. 1g), whereas those from the mesophyll of 'Red Rubin' (Fig. 1h) and from both the epidermis and mesophyll of 'Tigullio' (Fig. 1i) invariably lacked anthocyanic pigmentation.

Table 1 Boron (B) content (μg mg^{-1} DW), total chlorophyll (Chl $a + b$) (μmol g^{-1} DW), Chl *a/b* ratio and anthocyanin concentration (mg g^{-1} DW) in leaves of *Ocimum basilicum* cvs

Treatment	B content	Chl $a + b$	Chl *a/b*	Anthocyanin
'Red Rubin'				
Control	48 (4.3)^{C}	9.8 (0.94)^{A}	1.2 (0.08)^{B}	2.2 (0.14)^{B}
B	344 (26.3)^{B}	7.9 (0.45)^{B}	1.3 (0.07)^{B}	2.9 (0.38)^{A}
'Tigullio'				
Control	55 (8.41)^{C}	9.2 (0.81)^{A}	1.7 (0.01)^{A}	n.d
B	560 (33.8)^{A}	7.4 (0.55)^{B}	1.8 (0.03)^{A}	n.d

'Red Rubin' and 'Tigullio' grown with negligible (control) or excess (1.8 mM) B. Means ± SD, $n = 5$

Different letters indicate significant differences at $P = 0.05$

Responses of plants to B and high light

Relative to the controls, foliar concentrations of B increased seven-fold in 'Red Rubin' and ten-fold in 'Tigullio' when plants were grown with B-enriched water (Table 1). Both cultivars showed a reduction in total Chl content after B treatment, though the ratio of Chl *a/b* remained unchanged. The Chl *a/b* ratio was, however, lower in 'Red Rubin' leaves. Anthocyanin levels increased by about 30 % in B-treated 'Red Rubin' leaves (Table 1).

Maximum quantum yield of photosystem II (PSII), as estimated by F_v/F_m values in dark-acclimated leaves, declined only slightly in response to B treatment in both the cultivars (Table 2). When those B-treated plants were exposed to high light (1,000 μmol m^{-2} s^{-1}) for 4 h and then returned to darkness for 40 min, F_v/F_m values declined further, significantly more so in the green-leafed 'Tigullio' than in the purple 'Red Rubin'. However, when a polycarbonate filter, the optical properties of which approximated those of 'Red Rubin' anthocyanins, was inserted between the 'Tigullio' leaf and light source, differences in the decline of F_v/F_m values between the two cultivars were abolished. In contrast, when B-treated 'Red Rubin' was given 30 % additional light to compensate for the light-attenuating properties of the purple pigments, the plants did not show any further decline in F_v/F_m value (Table 2).

Light-response curves for PSII quantum yield (Φ_{PSII}) and non-photochemical quenching (NPQ) did not significantly vary ($P > 0.05$) between control and B-treated 'Red Rubin' plants (Fig. 2a, b). For 'Tigullio', too, Φ_{PSII} profiles did not change appreciably across treatments (Fig. 2c), although the light-saturated values of Φ_{PSII} were significantly lower in 'Tigullio' than in 'Red Rubin' ($P < 0.05$). In contrast, NPQ patterns were far greater in 'Tigullio' than in 'Red Rubin', especially at irradiances above 950 μmol m^{-2} s^{-1} (Fig. 2d). The imposition of a purple polycarbonate filter between the light source and the B-treated 'Tigullio' leaf lamina reduced the effect of high light on NPQ values (Fig. 2d). Nevertheless, NPQ remained greater than in the 'Tigullio' controls.

Table 2 Maximal photochemical efficiency of PSII (F_v/F_m) in leaves of *Ocimum basilicum* cvs

Treatment	F_v/F_m
'Red Rubin'	
Control	0.82 (0.01)^{AB}
B	0.81 (0.02)^{ABC}
L	0.79 (0.04)^{CD}
B + L	0.79 (0.02)^{CD}
B + L + L	0.79 (0.02)^{CD}
'Tigullio'	
Control	0.83 (0.01)^{A}
B	0.81 (0.03)^{ABC}
L	0.77 (0.04)^{D}
B + L	0.77 (0.03)^{D}
B + L + PF	0.80 (0.02)^{BC}

'Red Rubin' and 'Tigullio' after 20 days with 1.8 mM boron (B) and/or 4 h ×1000 μmol m^{-2} s^{-1} white light (L). Some 'Tigullio' plants were shielded by a purple polycarbonate filter (PF); some 'Red Rubin' plants were exposed to stronger light 1,300 μmol m^{-2} s^{-1} for 4 h (L + L). Means ± SD, $n = 5$

Different letters indicate significant differences at $P = 0.05$

Epifluorescence signals from leaves infiltrated with DCFH-DA indicated that 'Red Rubin' generated less H_2O_2 following high light and/or B treatments than did 'Tigullio' (Fig. 3a–j). In 'Red Rubin' leaves, fluorescence was predominantly located around the midrib and major veins; neither the distribution nor the intensity of fluorescence varied greatly across treatments (Fig. 3a–d). In contrast, for 'Tigullio' H_2O_2 was evident both in the veins and the lamina tissue (Fig. 3f–i); fluorescence was most intense when a high light treatment was applied to B-treated plants (Fig. 3h). When the polycarbonate purple filter was interposed between leaves of 'Tigullio' and the light source, a slight reduction in H_2O_2-induced fluorescence was observed after 180 min in comparison to B-treated plants exposed to full light (Fig. 3i, h). Control plants exhibited zero or extremely low levels of fluorescence (Fig. 3e, j).

Responses of protoplasts to B and high light

Protoplasts isolated from both cultivars appeared more sensitive to light stress than were intact leaves; irradiances as low as 100 μmol m^{-2} s^{-1} were sufficient to diminish the maximal quantum yield appreciably (Fig. 4). The reduction in F_v/F_m was less pronounced for 'Red Rubin' (29 %) than for 'Tigullio' (52 %). The addition of B to protoplast suspensions augmented the decline of F_v/F_m in both cultivars (Fig. 4; Table 3).

When a layer of purple protoplasts isolated from 'Red Rubin' epidermis was positioned over acyanic mesophyll

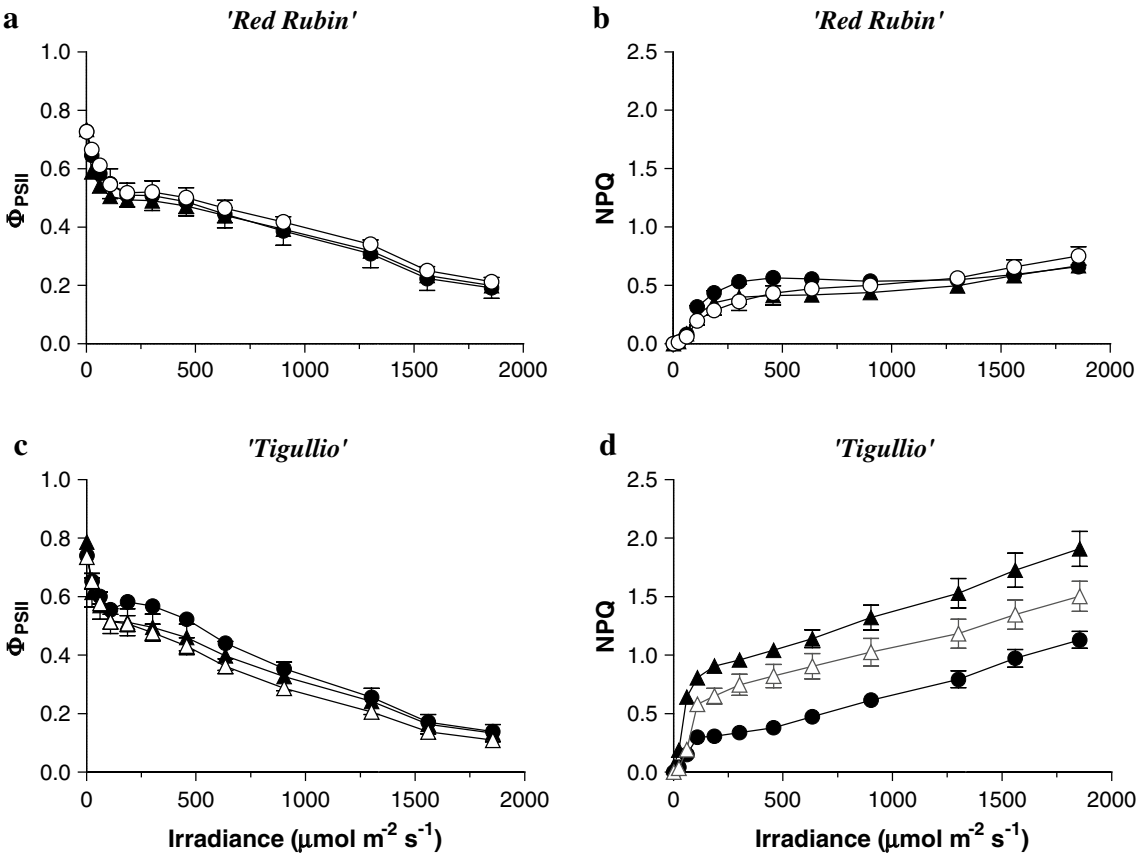

Fig. 2 PSII photochemical efficiency (Φ_{PSII}; **a**, **c**) and non-photochemical quenching (NPQ; **b**, **d**) in control (*filled circle*) and B-treated plants of *Ocimum basilicum* cvs. 'Red Rubin' and 'Tigullio'. B-treated plants were subjected to 4 h white light at 1,000 µmol m^{-2} s^{-1} (*filled triangle*) or 1,300 µmol m^{-2} s^{-1} (*open circle*). Some B-grown 'Tigullio' plants were shielded by a purple polycarbonate film (*open triangle*). Means ± SD, $n = 5$

protoplasts and the mix irradiated from above, F_v/F_m declined less, irrespective of B treatment or cultivar (Fig. 4). For 'Red Rubin', the light-filtering effects of a protoplast layer were similar to those of a purple polycarbonate filter, while for 'Tigullio', the polycarbonate filter had the greater ameliorative effect (Fig. 4).

The Φ_{PSII} and q_P both declined when acyanic protoplasts from 'Red Rubin' and 'Tigullio' were treated with strong light and/or B (Table 3). A purple filter placed between light source and protoplasts partially mitigated these effects of light on 'Red Rubin' protoplasts, but had little impact on 'Tigullio' (Table 3).

NPQ values were greater in acyanic protoplasts from 'Red Rubin' than those from 'Tigullio' across most of the treatments, though high light caused a greater proportionate increase in NPQ in 'Tigullio' (Table 3). The purple filter effectively lowered NPQ in protoplasts of both cultivars. NPQ values were consistently lower for protoplasts than for intact leaves. Maximum PSII electron transport rates were about 32 % higher for 'Tigullio' than for 'Red Rubin' control protoplasts at saturated light (Fig. 5a, b). All treatments resulted in a decline in ETR; the different treatments affected ETR to a similar degree in 'Red Rubin', but in 'Tigullio' ETR appeared most affected by B (with or without high light).

Total Chl content did not differ ($P > 0.05$) between protoplasts of both cultivars, but decreased following light and B treatments (Table 4). Chl *a*/*b* ratios were lower for 'Red Rubin' than for 'Tigullio' protoplasts. Of note, Chl *a*/*b* values for 'Tigullio' protoplasts, when shielded by a purple filter, declined to a ratio comparable to that for 'Red Rubin' control protoplasts (Table 4).

Oxidative stress in protoplasts

MDA accumulated in green protoplasts within 180 min of both B and high light treatments. The combination of the two stressors elicited more MDA than did either stressor alone or the effect was greater for 'Tigullio' than for 'Red Rubin' ($P < 0.05$; Fig. 6). The polycarbonate purple filter placed between the protoplasts and light source effectively reduced the extent of lipid peroxidation. Those results

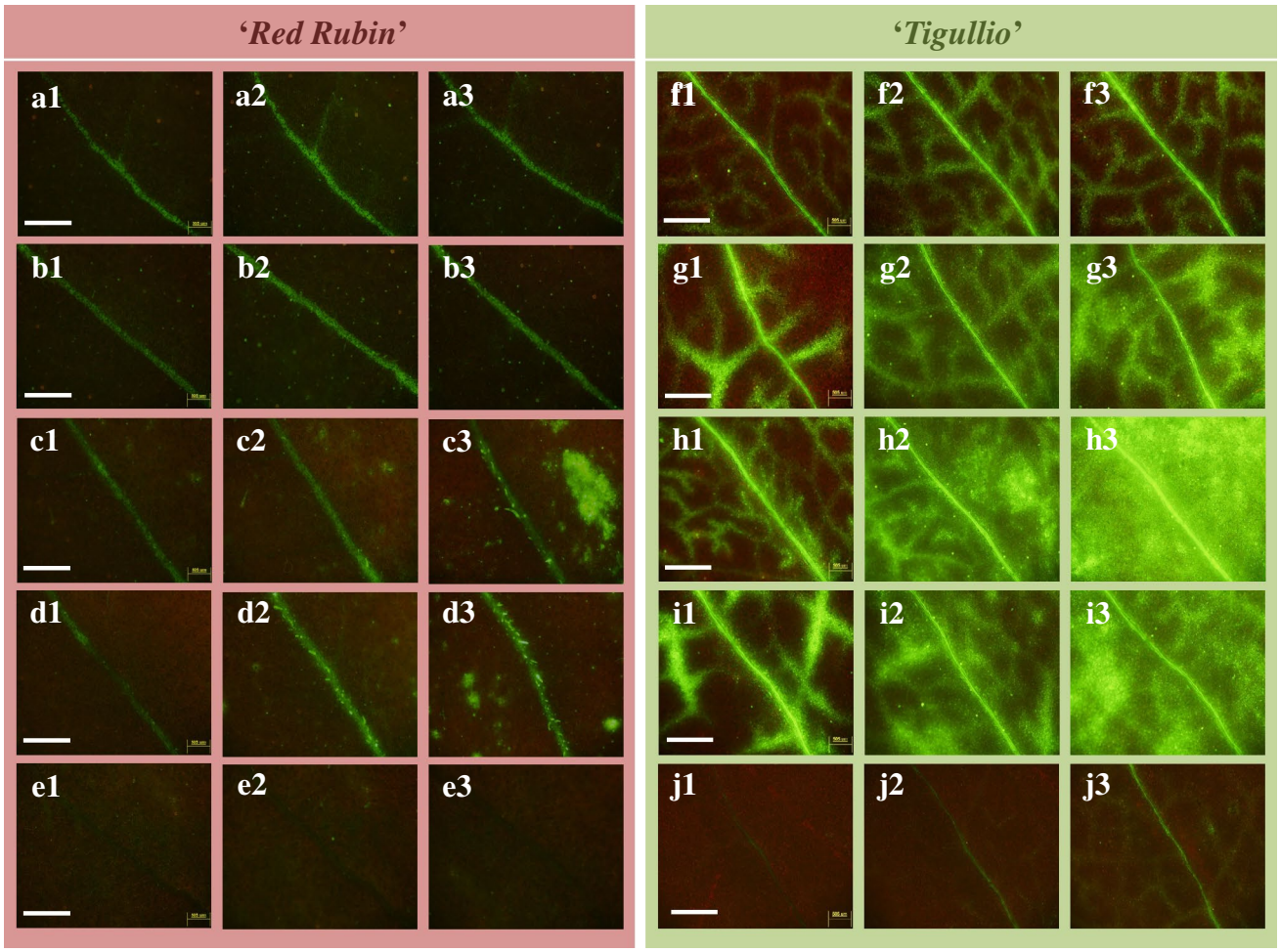

Fig. 3 Epifluorescence micrographs of DCFH-infiltrated leaves of *Ocimum basilicum* cvs. 'Red Rubin' (**a**–**e**) and 'Tigullio' (**f**–**j**). Plants were treated with boron (B) and white light at 400 µmol m^{-2} s^{-1} (**a**, **f**); 1,000 µmol m^{-2} s^{-1} white light (**b**, **g**); B and 1,000 µmol m^{-2} s^{-1}(**c**, **h**); B and 1,300 µmol m^{-2} s^{-1} white light (**d**); B and 1,000 µmol m^{-2} s^{-1} shielded by a purple filter (**i**); or 400 µmol m^{-2} s^{-1}(**e**, **j**). *Columns 1, 2, 3* represent 30, 60 and 180 min of treatments, respectively. Images are representative of at least 10 replicates. *Bars* 150 µm

were corroborated by observations of H_2O_2 production, seen as epifluorescence from protoplasts infiltrated with DCFH-DA. For both cultivars, the strongest fluorescence was observed under the combination of high light and B (Fig. 7). H_2O_2 production was consistently higher in 'Tigullio' than 'Red Rubin' in acyanic protoplasts, irrespective of treatment (Fig. 7). Chloroplast numbers and dimensions within protoplasts were similar between the two cultivars, and no appreciable changes in chloroplast conformation and distribution were found across all the treatment as revealed by chlorophyll autofluorescence (Fig. 7).

Discussion

Our previous studies indicated that the purple-leafed sweet basil cultivars ('Red Rubin' and 'Dark Opal') were more tolerant of B toxicity than were other green-leafed cultivars, and we postulated that foliar anthocyanins were likely to play a part in the protective mechanism (Landi et al. 2013b). The greater tolerance of the purple morphs could not be attributed to a reduced uptake of B (Landi et al. 2013b). Rather, three sets of evidence presented here from both intact plants and isolated protoplasts indicate that anthocyanins serve to mitigate effects of photoinhibitory stress in leaves once the photosynthetic machinery is compromised by B toxicity.

First, when B-treated intact plants were given a photoinhibitory light treatment, the purple leaves showed a smaller decline in F_v/F_m, even when supplied with 30 % extra light, than did the green leaves (Table 2). Moreover, when we attempted to simulate the optical effects of a layer of anthocyanic epidermal cells by applying a purple polycarbonate film over the green leaves, the degree of photoinhibition

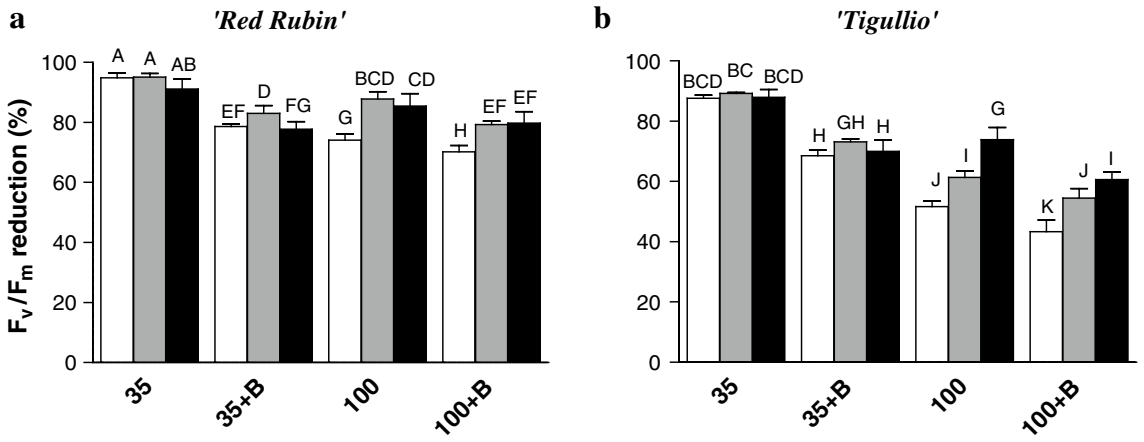

Fig. 4 Percentage reduction of maximum photochemical efficiency of PSII (F_v/F_m) in leaf mesophyll protoplasts of *Ocimum basilicum* cvs. 'Red Rubin' (**a**) and 'Tigullio' (**b**) as compared to controls (dark-held protoplasts). *White bars*, green protoplasts; *grey bars*, green protoplasts beneath a layer of anthocyanic protoplasts from 'Red Rubin' epidermis. *Black bars*, green protoplasts shielded by a purple polycarbonate filter. Protoplasts were treated with: 35 µmol m^{-2} s^{-1} (35); boron (B) and 35 µmol m^{-2} s^{-1} (35 + B); 100 µmol m^{-2} s^{-1} (100); or B and 100 µmol m^{-2} s^{-1} (100 + B). Means ± SD, $n = 5$. *Different letters* indicate significant differences at $P = 0.05$

Table 3 Maximal and actual PSII efficiency (F_v/F_m and Φ_{PSII}), photochemical quenching (q_P) and non-photochemical quenching (NPQ) in green protoplasts of *Ocimum basilicum* cvs

Treatment	F_v/F_m	Φ_{PSII}	q_P	NPQ
'Red Rubin'				
35	0.59 (0.01)^{B}	0.27 (0.03)^{B}	0.23 (0.01)^{A}	0.16 (0.02)^{B}
35 + B	0.47 (0.06)^{CD}	0.21 (0.03)^{C}	0.18 (0.02)^{BC}	0.14 (0.03)^{BC}
100	0.49 (0.04)^{C}	0.22 (0.02)^{C}	0.16 (0.03)^{CD}	0.20 (0.01)^{A}
100 + B	0.44 (0.04)^{D}	0.16 (0.02)^{D}	0.15 (0.01)^{D}	0.22 (0.02)^{A}
100 + B + PF	0.50 (0.01)^{C}	0.21 (0.01)^{C}	0.19 (0.01)^{B}	0.15 (0.03)^{BC}
'Tigullio'				
35	0.66 (0.05)^{A}	0.33 (0.05)^{A}	0.24 (0.01)^{A}	0.03 (0.01)^{F}
35 + B	0.42 (0.01)^{DE}	0.11 (0.02)^{E}	0.18 (0.03)^{BC}	0.14 (0.01)^{BC}
100	0.36 (0.04)^{E}	0.21 (0.02)^{C}	0.20 (0.02)^{B}	0.13 (0.01)^{CD}
100 + B	0.27 (0.01)^{G}	0.13 (0.02)^{DE}	0.20 (0.03)^{B}	0.08 (0.01)^{E}
100 + B + PF	0.40 (0.04)^{EF}	0.13 (0.02)^{DE}	0.19 (0.04)^{B}	0.11 (0.01)^{D}

'Red Rubin' and 'Tigullio'. Protoplasts were treated with white light at 35 or 100 µmol m^{-2} s^{-1} and/or 4 µM boron (B). Some protoplasts were shielded by a purple polycarbonate filter (PF). Means ± SD, $n = 5$

Different letters indicate significant differences at $P = 0.05$

diminished to a level approaching that of the purple-leafed cultivar (Table 2). The data are entirely consistent with a light-screening role for anthocyanins, preventing the over-excitation of chloroplasts for which the photosynthetic machinery has already been compromised by B. A photoprotective role for foliar anthocyanins has been reported many times (see reviews by: Chalker-Scott 1999; Steyn et al. 2002; Gould et al. 2009), but to our knowledge, this is the first demonstration of its use in B-stressed plants. As evidence of its effectiveness, in the presence of the purple filter, the requirement for NPQ in 'Tigullio' leaves apparently diminished (Fig. 2d). NPQ via the xanthophyll cycle is a key mechanism through which leaves dissipate excess excitation energy as heat (Demmig-Adams and Adams 2006); several reports have shown that anthocyanic leaves have a reduced pool of xanthophyll cycle components, suggesting that anthocyanins may constitute an alternative photoprotective strategy (Manetas et al. 2002; Kytridis et al. 2008; Hughes et al. 2012), although exceptions have been reported (Zeliou et al. 2009). Accordingly, in the absence of anthocyanins green leaves might be expected to have a greater reliance on xanthophylls; maximum NPQ values were 2–3 times greater in 'Tigullio' than in 'Red Rubin', irrespective of treatments (Fig. 2b, d). Tattini et al. (2014)

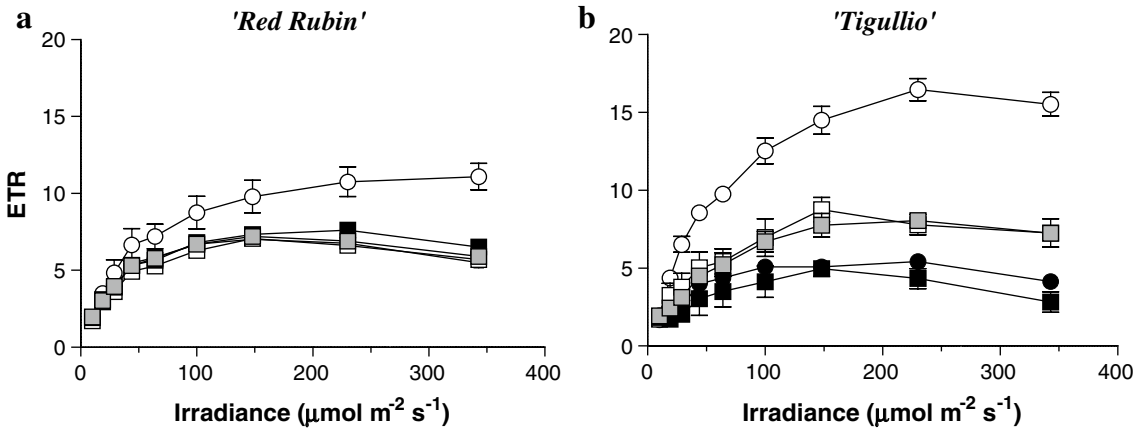

Fig. 5 Electron transport rate (ETR) in green protoplasts of *Ocimum basilicum* cvs. 'Red Rubin' (**a**) and 'Tigullio' (**b**). Protoplasts were treated with: 35 µmol m^{-2} s^{-1} white light (*open circle*); boron (B) and 35 µmol m^{-2} s^{-1} (*filled circle*); 100 µmol m^{-2} s^{-1} (*open square*); or B and 100 µmol m^{-2} s^{-1} with (*grey shaded square*) or without (*filled square*) a purple filter. Means ± SD, $n = 5$

Table 4 Total chlorophyll content (Chl $a + b$; µg mL^{-1}) and Chl *a*:*b* ratio in green protoplasts of *Ocimum basilicum* cvs

Treatment	Chl $a + b$	Chl *a*:*b*
'Red Rubin'		
35	82.34 (1.86)	1.63 (0.04)
35 + B	75.63 (3.15)	1.71 (0.12)
100	69.88 (1.69)	1.98 (0.11)
100 + B	62.96 (2.88)	2.04 (0.07)
100 + B + PF	68.73 (1.78)	1.65 (0.05)
'Tigullio'		
35	86.73 (1.66)	2.25 (0.12)
35 + B	83.12 (0.86)	2.29 (0.13)
100	72.93 (1.83)	2.19 (0.16)
100 + B	65.96 (2.50)	2.27 (0.09)
100 + B + PF	73.91 (2.81)	1.73 (0.08)

'Red Rubin' and 'Tigullio'. Protoplasts were treated with white light at 35 or 100 µmol m^{-2} s^{-1} and/or 4 µM boron (B). Some protoplasts were shielded by a purple polycarbonate filter (PF). Means ± SD, $n = 5$

Means without letters are not significantly different at $P = 0.05$

reported recently that ratios of xanthophyll to Chl are enhanced under high light much more so in 'Tigullio' than in 'Red Rubin' leaves, and that there are clear correlations between NPQ and xanthophyll levels. Under sub-saturating light, PSII photochemical efficiencies (Φ_{PSII}) might be expected to be comparable across the two cultivars because (1) in 'Tigullio', the excitation energy that exceeds photochemistry is channelled through the xanthophylls and dissipated as heat, while (2) in 'Red Rubin', anthocyanins abate quanta that would otherwise be absorbed by chloroplasts.

Second, to disentangle the effects of light-screening by epidermal anthocyanins from possible intrinsic differences between cultivars in light- and/or B-tolerance in the subjacent mesophyll, we measured the photoinhibitory responses of acyanic protoplasts from leaf mesophyll. That the B-treated protoplasts of both cultivars showed a smaller decline in F_v/F_m when shielded from high light by a purple polycarbonate film (Fig. 4, Table 3) or by a layer of anthocyanic protoplasts (Fig. 4), is further evidence of a direct and effective role of anthocyanins in light abatement. Anthocyanins are evidently able to reduce, though not entirely prevent photodamage as indicated by the F_v/F_m ratios. The data also confirm that an excess of light does indeed constitute a significant stressor for plant cells already compromised by B toxicity. Moreover, the lower ratio of Chl *a*:*b* in 'Red Rubin' than in 'Tigullio' protoplasts (Table 4) provides a good indication that the chloroplasts in purple leaves are the more shaded, most likely by the anthocyanic shield. Low Chl *a*:*b* is a characteristic feature of the leaves of shade plants (Lichtenthaler et al. 2000), and has been reported previously for anthocyanic leaf laminae (Gould et al. 2002b; Manetas et al. 2003; Kyparissis et al. 2007). That the Chl *a*:*b* ratios declined in both cultivars when illuminated through the polycarbonate filter suggests that the waveband absorbed by anthocyanins better protects Chl *b* than Chl *a* from photodamage.

Third, the B-treated leaf tissues produced less H_2O_2 (Figs. 3, 7) and incurred less lipid peroxidation (Fig. 6) when they were partially shielded from high light by a purple polycarbonate filter, indicating that photoabatement by anthocyanins translates into a measurable benefit. Because the DCFH probe used to visualise H_2O_2-generated green fluorescence, and because anthocyanins themselves absorb green light, it is at least possible that for the intact leaves of 'Red Rubin', part of the attenuation of the probe's signal could have resulted from anthocyanin absorption rather than altered H_2O_2 production per se (see: Gould et al. 2002b). However, this caveat cannot hold true for the protoplasts,

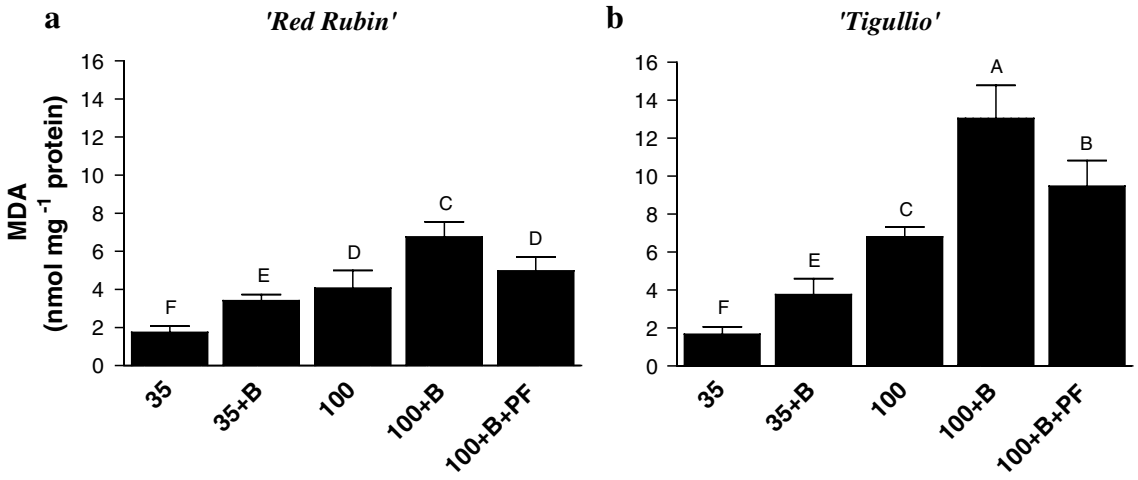

Fig. 6 MDA content in green protoplasts of *Ocimum basilicum* cvs. 'Red Rubin' (**a**) and 'Tigullio' (**b**). Protoplasts were treated with: 35 µmol m^{-2} s^{-1} white light (35); boron (B) and 35 µmol m^{-2} s^{-1} (35 + B); 100 µmol m^{-2} s^{-1} (100); B and 100 µmol m^{-2} s^{-1} (100 + B); or B and 100 µmol m^{-2} s^{-1} with a purple filter (100 + B + PF) for 180 min. Means ± SD, $n = 5$. *Different letters* indicate significant differences at $P = 0.05$

which were entirely acyanic. Thus, the lower signal found for mesophyll protoplasts of the purple cultivar as compared to that for 'Tigullio' protoplasts provides a clear indication that cells of 'Red Rubin' generated less H_2O_2. The production of ROS by chloroplasts has long been observed in plants subjected to a variety of abiotic stressors, and this can lead to cell death when the abundance of ROS exceeds the scavenging capacities of the antioxidant pool (Asada 1999; Mittler et al. 2004). Anthocyanins themselves may directly scavenge a diverse variety of ROS including H_2O_2 (Yamasaki 1997; Neill and Gould 2003). However, a direct antioxidant role is likely to be less important when anthocyanins are located far from the source of ROS (Kytridis and Manetas 2006). It is evident from the DCFH-infused protoplast suspensions (Fig. 7) that chloroplasts in leaf mesophyll are primary sources of H_2O_2 following light and B stress; the epidermal anthocyanins in 'Red Rubin' leaves (Fig. 1c) are, therefore, not optimally located to scavenge mesophyll-generated ROS. However, there is another source of H_2O_2, in the midrib and major veins of the leaf lamina (Fig. 3). It has been shown for other species that the chloroplasts in bundle sheath cells are the primary source of veinal H_2O_2 (Mullineaux et al. 2006). In that case, anthocyanins in the xylary parenchyma (Fig. 1e) might well contribute directly to the antioxidant pool. Nonetheless, because the effects on F_v/F_m of a layer of anthocyanic protoplasts were similar to those of a purple polycarbonate filter (Fig. 4), a light-screening role rather than an antioxidant role for anthocyanins best explains our data in pigmented sweet basil.

Given that the mesophyll of 'Red Rubin' appears to be shade adapted as a result of the overlying anthocyanic cells, once the cells were exposed to high light following protoplast isolation we might have expected them to show more pronounced photoinhibition and greater oxidative damage than the presumably more light-adapted mesophyll protoplasts from 'Tigullio'. However, contrary to our expectations, the data indicate that green protoplasts of 'Red Rubin' continued to exhibit superior photosynthetic performance when subjected to B and/or light stress, as indicated by the retention of higher F_v/F_m and Φ_{PSII} values (Table 3). In addition, 'Red Rubin' protoplasts appeared to produce less H_2O_2 (less DCF fluorescence) than did 'Tigullio' protoplasts when subjected to B and/or high light (compare Fig. 7c, d with Fig. 7h, i). The ~50 % lower MDA levels in 'Red Rubin' than in 'Tigullio' protoplasts subjected to high light plus B (Fig. 6) suggests that 'Red Rubin' mesophyll cells are intrinsically better fortified against oxidative damage. Thus, we postulate that in addition to the photoprotective role of anthocyanins in 'Red Rubin' leaves, other mechanisms, including a higher antioxidant pool, contribute to its more robust performance under B and light stress. Levels of both ascorbate and glutathione have been demonstrated to be higher in leaves of 'Red Rubin' than in 'Tigullio' (Landi et al. 2013a).

In conclusion, this report represents the first demonstration of a photoprotective role for anthocyanins in mesophyll cells when chloroplast functionality has been compromised by B toxicity. The data do not preclude other possible functions of anthocyanins in B tolerance, such as sequestration of boric acid and/or borate anions in the cell vacuole; these anions are likely able to bind with *cis*-diols (Tate and Maister 1978) and cyanidin derivatives. Our work opens the possibility for future studies on anthocyanin-B interactions.

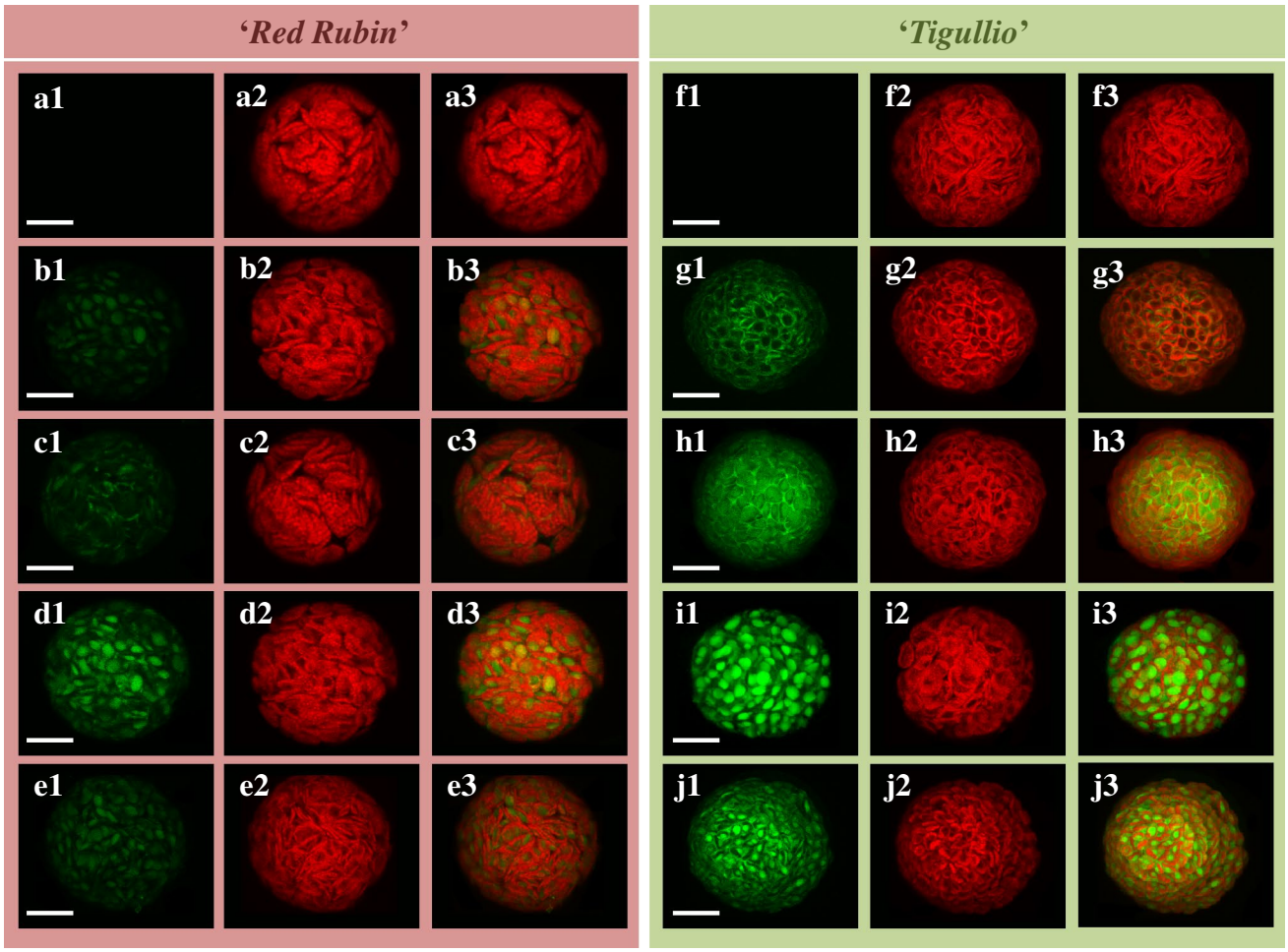

Fig. 7 Confocal laser scanning micrographs of protoplasts of *Ocimum basilicum* cvs. 'Red Rubin' (**a–e**) and 'Tigullio' (**f–j**) infused with DCFH. Protoplasts were treated with: 35 μmol m^{-2} s^{-1} of white light (**a**, **f**); boron (B) and 35 μmol m^{-2} s^{-1} (**b**, **g**); 100 μmol m^{-2} s^{-1} (**c**, **h**); B and 100 μmol m^{-2} s^{-1} (**d**, **i**); or B and 100 μmol m^{-2} s^{-1} with a purple filter (**e**, **j**). *Column 1*, DCF fluorescence, indicative of H_2O_2; *column 2,* chlorophyll autofluorescence; *column 3,* combination of DCF and autofluorescence. Images are representative of at least 30 protoplasts. *Bars* 10 μm

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