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Control of ascorbic acid synthesis and accumulation and glutathione by the incident light red/far red ratio in *Phaseolus vulgaris* leaves

Carlos G. Bartoli [a,*], Eduardo A. Tambussi [a], Fanello Diego [a], Christine H. Foyer [b]

[a] *Instituto de Fisiología Vegetal, Facultad Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, CCT-CONICET, cc327 1900 La Plata, Argentina*
[b] *School of Agriculture, Food and Rural Development, The University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, UK*

ABSTRACT

The effects of red/far red (R/FR) ratios on leaf ascorbate (AA) and glutathione (GSH) accumulation were examined in common bean (*Phaseolus vulgaris* L.). Growth under low R/FR ratios resulted in a "shade" phenotype and much lower leaf AA and GSH contents than high (R/FR) ratios. Photosynthesis rates were unaffected by changes in the R/FR ratio but leaf respiration rates, pyridine nucleotide pools and antioxidant enzyme activities were decreased under the low R/FR regime. The GSH pool changed slowly in response to altered R/FR ratios but leaf ascorbate acclimated over a single photoperiod. We conclude that light quality signals, particularly R/FR ratios, are important regulators of antioxidant synthesis and accumulation. These acclimatory changes are an early response to changing light environment.

1. Introduction

The red/far red (R/FR) ratio of incident light is a key environmental signal that allows leaves to perceive the presence of other leaves in the vicinity, either from the parent plant or from their near neighbours [1]. When exposed to a low R/FR ratio leaves orchestrate changes that enable them to avoid shading by competitors [2]. Plants experiencing a FR-enriched canopy typically show petiole elongation and or leaf area increment changes [3]. The phytochrome family of photoreceptors that absorb R and FR wavelengths in plants are signal transducers in these responses [3].

Ascorbic acid (AA) and glutathione (GSH) are ubiquitous and abundant metabolites participating of many protective mechanisms in leaves. AA functions not only as an antioxidant but it is also a cofactor in a number of enzymatic reactions [4]. In particular, AA is the co-factor involved in the catalysis of 2-oxoacid-dependant dioxygenase (2ODD) reactions in plants and animals [5]. The 2ODD family of enzymes are responsible for the synthesis of a wide range of secondary metabolites particularly hormones [5]. AA also participates in processes involved in the dissipation of excess excitation energy in the chloroplasts, such as xanthophyll cycle [6] and water–water cycle [7]. We have previously shown

that growth irradiance and respiration are important factors that regulate the AA content of Arabidopsis leaves [8]. We showed that increasing growth irradiance progressively elevated leaf AA contents. Moreover, we provided evidence that regulation of L-galactone-1,4-lactone dehydrogenase (L-GalLDH), which catalyses the last reaction of the synthetic pathway, by light and respiratory controls was an important determinant of the extent of leaf AA accumulation [8]. In this study we have further documented the effects of light, exploring the effects of light quality rather than quantity in determining AA accumulation in leaves.

In particular, we have analysed the effects of the R/FR ratio of incident light, which is a key shade signal, on the regulation of low molecular antioxidant contents in leaves.

2. Materials and methods

2.1. Plant material and experimental set-up

Phaseolus vulgaris L. (*cv*. TUC 500) plants were cultivated under two R/FR light ratios: (a) R/FR = 1.1 and (b) R/FR = 0.2 that simulate 'sun' and 'shade' light quality, respectively, while maintaining the same photosynthetic photon flux density (PPFD, 300 µmol photon $m^{-2}\,s^{-1}$). Plants were grown under a 12 h photoperiod at 30 °C for 7 or 11 days. At these times, some batches of plants were transferred from 'sun' (R/FR = 1.1) to 'shade' (R/FR = 0.2) growth

* Corresponding author. Fax: +54 2214233698.
E-mail address: carlos.bartoli@agro.unlp.edu.ar (C.G. Bartoli).

conditions for a further 5 or 1 days. The unifoliate leaves only were used in the following analyses after 12 days.

2.2. Light treatments

The light from tungsten halogen lamps was passed through solutions of Cu_2SO_4 or a green filter to generate 'sun' and 'shade', R/FR ratios, respectively. The wavelengths of absorption were chosen as previously described by other colleagues [9], with Cu_2SO_4 preferentially absorbing FR (725 nm) light while the green filter preferentially absorbs R (650 nm) radiation. Spectral energy distribution was continuously measured with a spectroradiometer (Instrumentation Specialities Co., Lincoln, NE, USA) and PPFD was measured with a quantum sensor (LI-190 SA, Li-Cor, Lincoln, NE, USA). Blue light radiation was similar under both R/FR treatments. The distance between lamps and the plants was adjusted daily in order to ensure comparable irradiance (PPFD) levels under both treatments.

2.3. Chlorophyll determinations

Chlorophyll was determined after extraction with dimethyl-formamide [10].

2.4. Antioxidant determinations

Reduced and oxidised ascorbic acid (AA and DHA, respectively) were determined as described in [8] and reduced and oxidised glutathione (GSH and GSSG, respectively) were determined as described in [11]. L-GalLDH was extracted and measured according to [12]. Dehydroascorbate reductase (DHAR) and glutathione reductase (GR) were measured essentially as described in [13,14].

2.5. Nucleotide pyridines determinations

NAD(P)/NAD(P)H contents were measured spectrophotometrically as described in [15].

2.6. Photosynthesis and respiration measurements

The net CO_2 assimilation rates of unifoliate leaves were measured using an infra red gas analyser (Ciras-2, PP Systems Ltd.) with a standard leaf cuvette (PLC6). Photosynthetic electron transport rates (*ETR*) and photochemical quenching (*qP*, the percentage of open PSII centres) were measured in the same leaves with a portable modulated chlorophyll fluorometer (FMSII, Hansatech, UK). The *ETR* and *qP* parameters were calculated as described in [16]. The net CO_2 assimilation rates and chlorophyll *a* fluorescence measurements were performed under the growth conditions (temperature, PPFD and R/FR ratio) for each treatment.

Respiration was measured using a Clark-type electrode (Hansatech, UK) in leaf disks harvested from dark-adapted leaves [8].

2.7. Inhibition of photosynthetic electron transport by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)

Photosynthetic electron transport was inhibited by supplying 50 μM DCMU via the roots for 48 h prior to the analysis. The extent of inhibition of the photosynthetic electron transport chain was determined using the *ETR* measurement, as described above.

2.8. Statistical analysis

The data were analysed using a standard ANOVA method. The data shown are the mean values obtained from at least four independent experiments with a $P \leqslant 0.05$.

3. Results and discussion

3.1. Leaf responses to different R/FR ratios

The common bean plants showed typical responses to the different light environment treatments: leaves produced under light enriched in FR had lower chlorophyll contents and lower specific leaf weights. They also had longer petiole lengths than leaves produced under high R/FR ratio regime (Table 1). Plants that were transferred from growth in the 'sun' (R/FR 1.1) environment to the 'shade' (R/FR 0.2) environment for 5 days prior to measurement displayed an intermediate plant 'sun/shade' phenotype (Table 1).

3.2. Effects of the R/FR ratio on leaf antioxidant contents

Leaves under 'shade' (R/FR 0.2) environment had on average 38% lower AA content than leaves under 'sun' (R/FR 1.1) environment (Fig. 1A). The amounts of DHA were similar under all light treatments. When plants were transferred from the 'sun' to the 'shade' R/FR ratio regime the leaf AA content decreased (Fig. 1A). In addition, leaf GSH contents were lower when plants were grown under the 'shade' (R/FR 0.2) environment (Fig. 1B) conditions, and the level of GSSG was similar under both regimes. The content of AA was reduced to about a 50% at the end of the dark period (3.3 and 2.5 $\mu mol\,g^{-1}$ FW for 'sun' and 'shade' light environment, respectively) and the oxidised state was similar for leaves under both treatments showing 20% of the oxidised form.

The concept that light is a key environmental factor controlling leaf antioxidant contents is widely accepted [17]. However, while the effects of light quantity are well documented, there is no literature information on the effects of light quality on the abundance of leaf antioxidants. The data presented in Fig. 1 demonstrate that high R/FR ratios favour accumulation of AA and GSH. The high R/FR ratios simulate the light quality experienced by the plant under conditions when plants might be exposed to full sunlight, a situation where the potential for excess irradiance and photoinhibition is high. In this context high AA and GSH contents would confer a pre-emptive advantage so that the photosynthetic tissues could support light-induced increases in oxidative load. AA is essential for the production of zeaxanthin and thermal excitation energy dissipation by non-photochemical quenching [18]. AA and GSH participate the water–water cycle which is a mechanism for the dissipation of excess reducing power in chloroplasts [7].

In order to confirm that R/FR ratio of incident light was an important environmental trigger controlling the leaf AA and GSH contents as well as the reduced/oxidised ratios, we performed short-term transition experiments transferring plants from the high to the applied low R/FR ratio conditions, for short periods and the acclimation process was then followed. Accordingly plants grown under the 'sun' R/FR (1.1) were transferred to the 'shade' R/FR (0.2) on day 11 of the experiment in darkness at the end of

Table 1
The effect of different R/FR light ratios on the chlorophyll content, specific leaf weight and petiole length of common bean leaves. Plants were cultivated under either 'sun' (R/FR 1.1) or 'shade' (R/FR 0.2) ratios at the same PPFD (300 μmol photon $m^{-2}\,s^{-1}$) for 12 days. At the day 7 a batch of plants was transferred from 1.1 to 0.2 R/FR light ratio (R/FR 1.1–0.2) for a further 5 days.

R/FR ratio	Chlorophyll content (μg mg^{-1} FW)	Specific leaf weight (mg cm^{-2})	Petiole length (mm)
1.1	1.14 ± 0.03a	18.8 ± 0.4a	26.7 ± 1.5a
1.1–0.2	1.01 ± 0.04b	16.3 ± 0.3b	60.0 ± 4.0b
0.2	0.80 ± 0.01c	15.1 ± 0.3c	64.0 ± 6.0b

Data with a similar letter represent a statistically homogenous groups (ANOVA, $P \leqslant 0.05$). The results are the mean values obtained from four independent experiments.
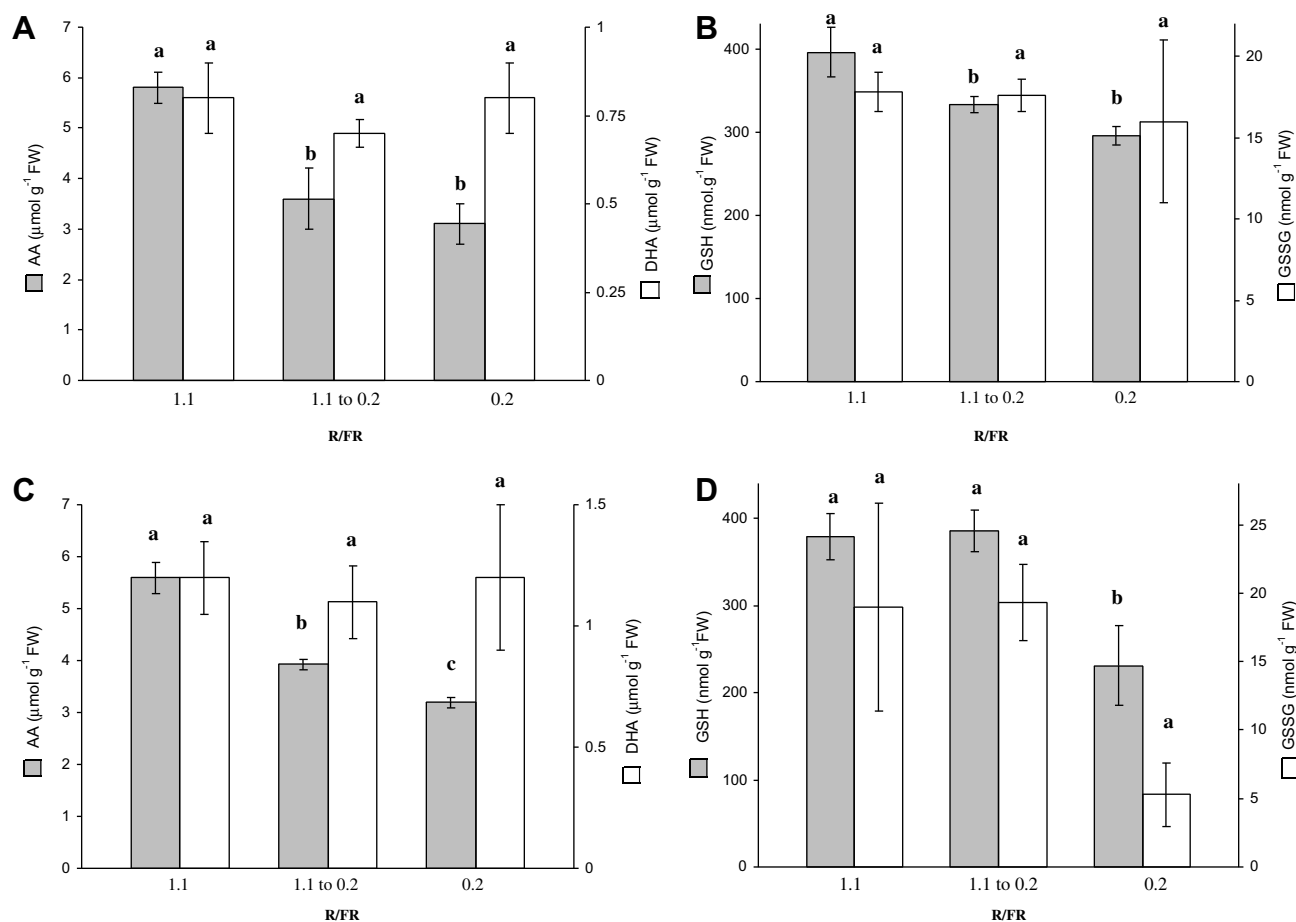
Fig. 1. The effect of the incident light R/FR ratio on the antioxidant contents of common bean leaves. In (A and B) plants were cultivated at an overall intensity of 300 μmol photon m^{-2} s^{-1} for 12 days. Some batches of plants were then transferred at day 7 from the high to the low R/FR light ratio (1.1–0.2) condition for 5 days. Leaf AA and DHA contents (A) and GSH and GSSG contents (B) were then measured. Results in (C) and (D) show the effect on leaf AA/DHA and GSH/GSSG contents, respectively, after transfer from high to low R/FR ratios for a single photoperiod (10 h). Plants were cultivated at R/FR ratio of 1.1 or 0.2 for 12 days. One batch of plants was transferred at day 11 from the R/FR ratio of 1.1 to R/FR ratio of 0.2 (1.1–0.2). The results are the mean values obtained from four independent experiments.

the photoperiod. Antioxidant contents were then measured at the end of the following photoperiod (*i.e.* 10 h into the photoperiod). While the leaf chlorophyll contents and the leaf petiole lengths were not modified significantly in this short period after transfer (data not shown), the amounts of AA and DHA responded rapidly to the altered 'sun' and 'shade' environments, reaching values that were similar to those attained in the previous studies on longer term transfer exposures to the different light quality environments (Fig. 1C). In contrast, GSH and GSSG contents were not modified in this short term experiment (Fig. 1D). The observed changes in leaf AA contents were achieved over a relatively short time period (i.e. 10 h). This observation suggests that light quality, i.e. R/FR ratio is a key determinant of the extent of leaf AA accumulation and that the regulatory effect of R/FR ratio on AA synthesis is triggered early in the acclimatization response to the changing light environment. Moreover, these short term transition experiments provide evidence that the observed R/FR ratio effects on AA occur in advance of (and are therefore largely independent of) other acclimatory modifications in for example leaf chlorophyll contents, specific leaf weight, etc.

3.3. Effect of R/FR ratio on respiration and photosynthesis

Leaf antioxidant homeostasis is directly influenced by photosynthesis and respiration. AA synthesis is tightly linked to the mitochondrial electron transport chain [11] because L-GalLDH,

which catalyses AA synthesis, is located in the inner mitochondrial membrane. The oxidation of L-galactone-1,4-lactone by L-GalLDH feeds electrons into the cytochrome *c* pool in the mitochondrial electron transport chain. This relationship is important in acclimation to environmental cues [19]. Leaves obtained from plants grown under high irradiances show increased respiration rates and they also have higher L-GalLDH activities and AA concentrations [8]. The leaf respiration rate is lower in leaves grown under 'shade' light conditions (Table 2), consistent with the low leaf AA levels observed in the same conditions (i.e. R/FR 0.2). Leaf respiration measured in plants transferred from 'sun' to 'shade' for only one photoperiod show an intermediate rate (data not shown). Leaf

Table 2
The effect of different R/FR light ratios on dark respiration and photosynthesis. Plants were cultivated under either 'sun' (R/FR 1.1) or 'shade' (R/FR 0.2) ratios at the same PPFD (300 μmol photon m^{-2} s^{-1}) for 12 days.

| R/FR ratio	Respiration rate (μmol O$_2$ g^{-1}FW h^{-1})	Net photosynthetic rate (μmol CO$_2$ g^{-1}FW h^{-1})	ETR (μmol electrons m^{-2} s^{-1})	*qP* relative units
1	28.8 ± 2.3a	191 ± 7a	95 ± 2a	0.91 ± 0.01a
0.2	19.5 ± 1.1b	184 ± 8a	98 ± 2a	0.92 ± 0.02a

Data with similar letter represent a statistically homogenous group (ANOVA, $P \leqslant 0.05$). The results are the mean values obtained from four independent experiments.

Table 3
The effect of different R/FR light ratios on leaf L-GalLDH, DHAR and GR activities. Plants were cultivated under either 'sun' (R/FR 1.1) or 'shade' (R/FR 0.2) ratios at the same PPFD (300 µmol photon m^{-2} s^{-1}) for 12 days. On days 7 and 11 after sowing some of the batches of plants grown under the "sun" conditions (R/FR 1.1) were transferred to 'shade' (R/FR 0.2) conditions, so that effects of the transition to shade R/FR ratios could be determined 5 days and 1 day after transfer, respectively. In all cases samples were harvested for analysis on the 12th day after sowing.

R/FR ratio	L-GalLDH activity		DHAR activity (µmol g^{-1}FW min^{-1})		GR activity	
1.1	0.144 ± 0.01a	0.159 ± 0.03a	4.4 ± 0.2a	3.4 ± 0.2a	1.32 ± 0.1a	1.20 ± 0.07a
1.1–0.2 1d	nd	0.10 ± 0.03ab	nd	2.7 ± 0.2b	nd	1.16 ± 0.06a
1.1–0.2 5d	0.096 ± 0.03ab	nd	2.8 ± 0.2b	nd	0.94 ± 0.1ab	nd
0.2	0.075 ± 0.01b	0.09 ± 0.015b	2.5 ± 0.3b	2.0 ± 0.24b	0.8 ± 0.05b	0.85 ± 0.1b

nd means not determined. Data with similar letter represent a statistically homogenous group (ANOVA, $P \leqslant 0.05$). The results are the mean values obtained from four independent experiments.
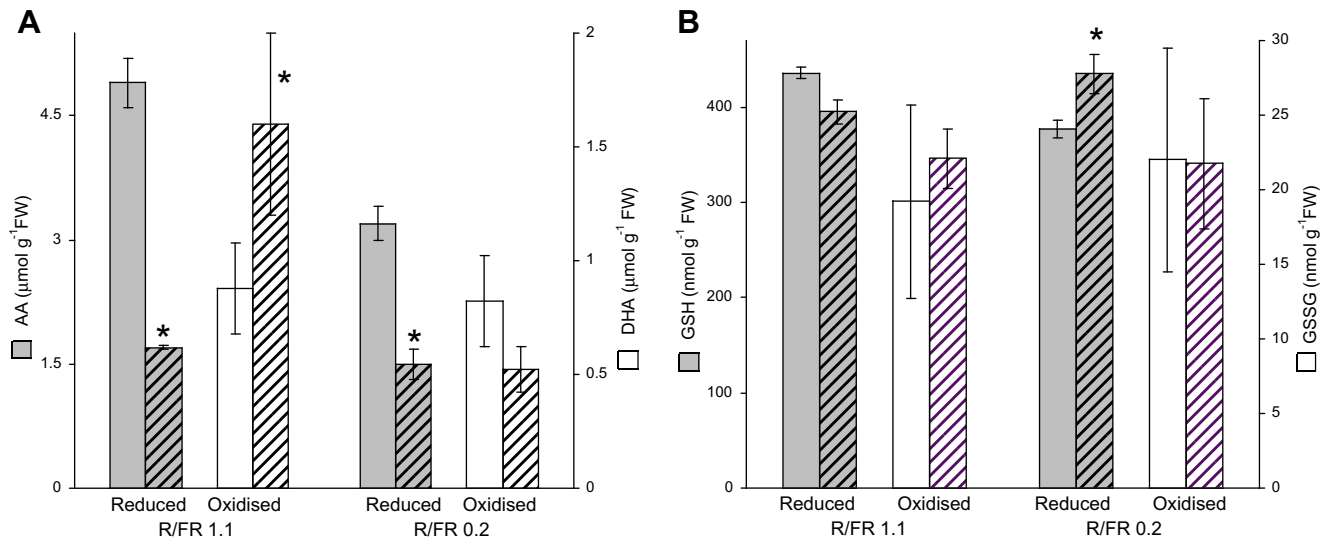
Fig. 2. The effect of the photosynthetic inhibitor DCMU on leaf antioxidant contents. Plants were grown for 12 days under the different R/FR light ratios and then one batch of plants was treated with 50 µM DCMU. Leaf antioxidant contents were measured 2 days later. (A) AA/DHA contents and (B) GSH/GSSG contents. Dashed bars represent the DCMU treatment. Asterisks denote the data that are statistically different from the control (without DCMU) (ANOVA, $P \leqslant 0.05$). The results are the mean values obtained from four independent experiments.

L-GalLDH, which is an integral component of the respiratory electron transport chain, had much lower activities in leaves under the 'shade' light environment (Table 3). These results suggest that down-regulation of mitochondrial metabolism may be a key process contributing to the lower leaf AA contents under the 'shade' light regime.

The activity of the photosynthetic electron transport chain also influences AA accumulation in Arabidopsis leaves [20]. However, the data shown in Table 2 show that photosynthesis is not involved in the acclimation response to R/FR light ratio, as it was similar under both light quality conditions. Net CO_2 assimilation rates, and

Table 4
Effect of R/FR light ratio on the NAD(P)/NAD(P)H contents in common bean leaves. Plants were cultivated under either 'sun' (R/FR 1.1) or 'shade' (R/FR 0.2) ratios at the same PPFD (300 µmol photon m^{-2} s^{-1}) for 12 days. At the day 11 a batch of plants was transferred from 1 to 0.2 R/FR light ratio (1–0.2) and nucleotides were measured by the end of the photoperiod (i.e. 10 h).

R/FR ratio	NAD	NADH (nmol g^{-1}FW)	NADP	NADPH
1	74.5 ± 7a	55.6 ± 9a	58.2 ± 7a	56.7 ± 1a
1–0.2	79.7 ± 4a	35.7 ± 1b	61.8 ± 7a	44.0 ± 4ab
0.2	77.0 ± 4a	32.7 ± 3b	57.3 ± 8a	36.0 ± 5b

Data with a similar letter represent statistically homogenous groups (ANOVA, $P \leqslant 0.05$). The results are the mean values obtained from four independent experiments.

ETR and qP values were similar in plants grown under the 'sun' and 'shade' R/FR conditions. The differences in leaf AA content observed under different light treatments are therefore not directly linked to photosynthesis. When plants were treated with DCMU, an inhibitor of the electron transport chain for 48 h, photosynthesis was inhibited (data not shown) and leaf AA contents fell to similar values under both light regimes (Fig. 2A). These results suggest that a process linked to photosynthesis is required to support high levels of leaf AA accumulation.

Surprisingly, DHA content increased under 'sun' but not under 'shade' light environmental conditions when photosynthesis was inhibited. A candidate process that might be affected by the prevailing R/FR ratio of the light environment is the regeneration of AA from DHA through the AA/GSH cycle as this is a NADPH-dependent reaction sequence. The data in Fig. 1B show that leaf GSH contents are decreased when plants were grown under 'shade' R/FR ratio conditions. In addition, the 'shade' leaves have less DHAR and GR activities suggesting that the capacity for AA regeneration from DHA is decreased when the R/FR ratio is low (Table 3). Furthermore, an small GSH increase was observed in leaves under a 'shade' R/FR ratio when ETR is blocked (Fig. 2B). Under the 'sun' R/FR ratio it is possible that the flux of electrons to NADPH and hence to AA regeneration is greater than under the 'shade' R/FR ratios. In support of this hypothesis, the total leaf pyridine pools are increased and the NADH/NAD and NADPH/NADP ratios are higher under the 'sun' conditions of high R/FR light ratios (Table 4).

4. Conclusions

The data presented here demonstrate that the R/FR ratio of incident light modulates leaf antioxidant contents. The leaf AA pool (but not leaf GSH) was changed by the R/FR signalling system after a single photoperiod. We conclude that a shade acclimation response mediated by the R/FR signalling system has a major control over the extent of AA accumulation in leaves. The rapid acclimation of the AA pool to the R/FR ratio of incident light and the effects of the R/FR ratio on respiration, L-GalLDH, DHAR and GR activities suggest direct effects on AA synthesis as well the regeneration of AA from its oxidised forms. Taken together, these results suggest that the incident R/FR ratio might act as an alarm signal, conveying information relevant to the possibility of an enhanced risk of photoinhibition and oxidative load compared to that experienced in a shade environment. Finally, R/FR treatments similar to those used here might be applied not only to boost the content of leaf antioxidants but also as a postharvest treatment to maintain or enhance the antioxidant content of stored vegetables.

Acknowledgements

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