Physiologia Plantarum
An International Journal for Plant Biology

UV-B impairs growth and gas exchange in grapevines grown in high altitude

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Received 4 September 2012;
revised 5 November 2012

doi:10.1111/ppl.12012

We previously demonstrated that solar ultraviolet-B (UV-B) radiation levels in high altitude vineyards improve berry quality in *Vitis vinifera* cv. Malbec, but also reduce berry size and yield, possibly as a consequence of increased oxidative damage and growth reductions (lower photosynthesis). The defense mechanisms toward UV-B signal and/or evoked damage promote production of antioxidant secondary metabolites instead of primary metabolites. Purportedly, the UV-B effects will depend on tissues developmental stage and interplay with other environmental conditions, especially stressful situations. In this work, grapevines were exposed to high solar UV-B (+UV-B) and reduced (by filtering) UV-B (−UV-B) treatments during three consecutive seasons, and the effects of UV-B, developmental stages and seasons on the physiology were studied, i.e. growth, tissues morphology, photosynthesis, photoprotective pigments, proline content and antioxidant capacity of leaves. The +UV-B reduced photosynthesis and stomatal conductance, mainly through limitation in gas exchange, reducing plant's leaf area, net carbon fixation and growth. The +UV-B augmented leaf thickness, and also the amounts of photoprotective pigments and proline, thereby increasing the antioxidant capacity of leaves. The defense mechanisms triggered by + UV-B reduced lipid peroxidation, but they were insufficient to protect the photosynthetic pigments per leaf dry weight basis. The +UV-B effects depend on tissues developmental stage and interplay with other environmental conditions such as total radiation and air temperatures.

Introduction

The most reputed vineyards in Mendoza, Argentina, are located at ca. 1500 m a.s.l., where solar ultraviolet-B (UV-B) radiation levels are relatively high, with fluence rates that reach up to 0.40 W m^{-2} in summer (Berli et al. 2010). The UV-B (280–315 nm) radiation has enough energy to cause large photobiological effects on higher plants, some related to the plant's response to the evoked damage (nucleic acids, proteins and lipids are particularly sensitive) and others in response to the

Abbreviations – +UV-B, full UV-B treatment; −UV-B, minus UV-B treatment; ABA, abscisic acid; CHS, chalcone synthase; DAF, days after flowering; DW, dry weight; FW, fresh weight; IAA, indole acetic acid; LA, leaf area; LSD, least significant difference; MDA, malondialdehyde; OD, optical density; ORAC, oxygen radical absorbance capacity; PAL, phenylalanine ammonia lyase; PAR, photosynthetically active radiation; PCA, principal component analysis; PE, low density polyethylene; PET, clear polyester; PSII, photosystem II; RH, relative humidity; TE, trolox equivalents; UVAC, UV-absorbing phenolic compound; UV-B, ultraviolet-B.

perception of UV-B as an induced acclimation (Foyer et al. 1994, Rozema et al. 1997, Jansen et al. 1998, Frohnmeyer and Staiger 2003, Jenkins 2009, Berli et al. 2010, Pontin et al. 2010, Gil et al. 2012).

The impact of UV-B on various morphological, biochemical and molecular aspects has been studied, but most of the responses found were highly variable depending on the species, cultivars, experimental conditions, levels of UV-B and the relationship between UV-B and photosynthetically active radiation (PAR; Frohnmeyer and Staiger 2003, Brown et al. 2005). Regarding to the latter, it has been shown that under UV-B/PAR ratios higher than those found in natural conditions the UV-B effects can be exaggerated (Caldwell and Flint 1997).

The effects of UV-B on plant's vegetative growth are variable (Fiscus et al. 1999, Robson et al. 2003), but reductions in shoot length and leaf expansion were generally found (Searles et al. 1995, Mark et al. 1996, Zhao et al. 2003a, 2003b). It has also been observed that UV-B reduced plant's total biomass and photosynthetic capacity mostly through damages to the photosynthetic pigments and chloroplast structure (Teramura and Sullivan 1994, Day et al. 1996, Kakani et al. 2003), reductions in the activity of Rubisco (Sullivan and Teramura 1990, Jordan et al. 1992, Ziska and Teramura 1992) and inhibition of photosystem II (PSII; Teramura et al. 1991, Ziska et al. 1993, Allen et al. 1997). Additionally, photosynthesis could be indirectly affected though reductions in stomatal conductance (Dai et al. 1992, Day and Vogelmann 1995, Zeuthen et al. 1997) and total leaf area (LA) (Nogués et al. 1998, Pinto et al. 1999, Hofmann et al. 2001, Zhao et al. 2003a, 2003b), therefore limiting the plant's gas exchange capacity.

It has been shown that UV-B can change the leaf morphology, i.e. thicker leaves with more epidermal cells, epicuticular waxes and trichomes (Semerdjieva et al. 2003). Such responsive mechanisms augment the epidermal reflectance and reduce transmittance of the harmful radiation, and also increase the distance through the most sensitive internal tissues (Cen and Bornman 1993, Liu et al. 1995). However, a more general acclimation response to UV-B is the accumulation of phenolic compounds in epidermal cells (Caldwell et al. 1983, Tevini et al. 1991, Cuadra et al. 1997, Olsson et al. 1998, Burchard et al. 2000, Liakoura et al. 2001), and this mechanism was observed even in grapevine (Kolb et al. 2001, Berli et al. 2010). The phenols absorb UV-B and reduce its penetration trough underlying tissues (Li et al. 1993, Burger and Edwards 1996, Bieza and Lois 2001), but also act as antioxidants (Markham et al. 1998, Gould et al. 2002, Steyn et al. 2002).

We have previously studied the solar UV-B effects on *Vitis vinifera* cv. Malbec, the most important cultivar for red wine in Argentinean viticulture, and found that high altitude solar UV-B improves berry quality, mainly through accumulation of berry skin phenolic compounds, although the UV-B positive effects are accompanied with reduction in berry size and thereby in yield (Berli et al. 2011). The emerging hypothesis is that grapevine yield decreases are consequence of reductions in growth of vegetative tissues, lower photosynthesis and photochemical efficiency, related with an increased cellular oxidative damage. Also, as a defense mechanism against the UV-B evoked damage and/or environmental signal, the plants favor the production of antioxidant (secondary metabolites vs primary metabolites), and therefore reduce growth. Additionally, the UV-B effects will depend on tissues developmental stage and seasonal environmental conditions, being greater with combined higher stressful conditions [total radiation, air temperatures and relative humidity (RH)]. In this work, we investigate the effect of the elevated solar UV-B to which the plants are naturally subjected at high altitude vineyards, the effect of three different seasons and the effect of developmental stages (flowering to harvest), on the vegetative growth (assessed as shoot length, number of leaves and LA), physiological parameters (photosynthesis, stomatal conductance and chlorophyll fluorescence) and biochemical traits (photosynthetic and photoprotective pigments, lipid peroxidation, epicuticular waxes, proline levels and antioxidant capacity) of grapevines.

Materials and methods

Plant material and experimental design

The experiment was carried out during three consecutive seasons, the 2008–2009 (2009), the 2009–2010 (2010) and the 2010–2011 (2011), in a commercial high altitude vineyard (1450 m a.s.l., 69°15′37″W and 33°23′51″S), Gualtallary, Mendoza, Argentina. The grapevines were a selected clone of *Vitis vinifera* cv. Malbec, planted in 1997 on their own roots, trained on a vertical trellis system, arranged in north–south oriented rows spaced 2 m apart, with 1.20 m between plants on the row. The grapevines were pruned to 12 shoots when these shoots reached 100 mm long, leaving two bunches per shoot. Vines were maintained with no soil water restriction during the whole experiment by using a drip irrigation system.

A randomized complete block design with two UV-B treatments and five blocks was used (number of replicates, $n = 5$). The experimental unit consisted of

four plants selected on the basis of their homogeneity from six consecutive plants in the row. Two shoots per experimental unit were selected, marked and used for the non-destructive measurements (shoots length, number of leaves, LA, net photosynthesis, stomatal conductance and chlorophyll fluorescence), while the rest of the shoots were used for leaves sampling.

UV-B treatments

Two radiation regimens were set from 15 days before flowering, stage 23 (Coombe 1995), mid-November, until harvest at 133 days after flowering (DAF), in early April. The UV-B treatments were given during three consecutive seasons (2009, 2010 and 2011) and the dates maximum differences between the three seasons were ±5 days. Solar UV-B was filtered with 100 µm clear polyester (PET) filters (Oeste Aislante, Buenos Aires, Argentina) to produce minus UV-B treatment (−UV-B). The PET filter absorbed 78% of UV-B and 12% of PAR from the sunlight. A full UV-B treatment (+UV-B) was set with a 40 µm low density polyethylene (PE) cover to minimize environmental differences between −UV-B and +UV-B. This PE absorbed 10% of UV-B and 13% of PAR (transmitted most of the solar radiation). The UV-B differences between +UV-B and −UV-B treatments were 68% across the season. The plastic sheets were set 2.5 m above ground level, covering the entire grapevine canopy, and were replaced after breakdown or transmittance reduction, as controlled weekly with an Li-250 light meter and an Li-190SA quantum sensor (Li-COR Inc., Lincoln, NE) and a PMA2200 radiometer with a PMA2102 UV-B detector (Solar Light Co. Inc., Glenside, PA). Both treatments were protected with antihail nets (black polyethylene) that absorbed an extra 15% of UV-B and 17% of PAR. We have previously reported the PE and PET transmittance spectral characteristics (Berli et al. 2008) and a schematic representation of an experimental unit (Berli et al. 2011).

Meteorological data

The total solar radiation, air RH and air temperatures were registered during the 2009, 2010 and 2011 seasons, between September and April (from grapevine budburst to harvest), with an automatic weather station (iMetos ag, model IMT 300; Pessl Instruments, Weiz, Austria), located close to the experimental site. Measurements were registered every 60 min for 24 h a day and the daily averages were calculated. The total solar radiation was measured with a LI-200SZ pyranometer sensor (Licor Inc., Lincoln, NE), and the air temperatures (mean, maximum, minimum and thermal amplitude) and RH were measured with an Hygroclip 2 and PT100 Class A sensors (Rotronic Instrument Corp., New York, NY) installed at 1.5 m.

In planta vegetative growth measurements

The shoot length, number of leaves and midrib (main vein) length of all leaves from the two selected shoots per experimental unit (n = 5) were measured at 0, 38, 65, 96 and 133 DAF (the measuring date maximum differences between the three seasons were ±5 days). At harvest (133 DAF), all leaves on 10 randomly selected shoots were collected in nylon bags, kept on ice to prevent dehydration and taken to the laboratory where the length of the midrib, the weights of leaves and 100 mm^2 leaf discs were measured. Then, the LA was calculated based on the weights, and a linear regression model between the LA and the midrib length was generated. The correlation coefficient (r^2) was 0.91 and thus the model was used to transform the non-destructive measurements of midrib length into LA values. LA per shoot was calculated adding all the individual LA in the shoots. Basal LA and basal LA per shoot were calculated considering only the first 15 leaves from the shoot base. Internode length was determined dividing the shoot length by the number of leaves per shoot.

Sampling of leaves

Two fully expanded (9–10th from the apex) leaves exposed to West per experimental unit (n = 5) were collected at 0, 50, 70, 90, 110 and 130 DAF (the sampling date maximum differences between the three seasons were ±5 days). Sample leaves were protected in the field with aluminum foil and immediately frozen with liquid nitrogen, transported to the laboratory and kept at −20°C until further analysis.

Photosynthetic and photoprotective pigments

The chlorophyll (Chl) *a*, *b*, carotenoids (Car), total chlorophyll (T_{Chl}) and UV-absorbing phenolic compound (UVAC) were determined with a UV–vis spectrophotometer (Cary-50), measuring the optical density (OD) of leaf extracts as previously described (Berli et al. 2010). The photosynthetic and photoprotective pigment contents were calculated on the basis of leaf dry weight (DW; leaf discs dried at 40°C to a constant weight) and on the basis of LA.

Lipid peroxidation and epicuticular waxes

The malondialdehyde (MDA) content was measured in leaf samples following the procedure previously

described in (Berli et al. 2010), and was expressed on leaf fresh weight (FW) and LA basis.

A modification of the method described by Qaderi et al. (2002) was used to determine the leaves epicuticular waxes content. Eight discs (100 mm^2 each) from one defrosted leaf sample per experimental unit were immersed in 1 ml of chloroform and then it was gently stirred for 30 s. The obtained solution was transferred to a pre-weighed vial (1.5 ml) and evaporated to dryness using a Vacufuge apparatus (Eppendorf AG, Hamburg, Germany). Then, the vials were weighed again and the amount of extracted waxes was calculated. The epicuticular waxes content was expressed on the basis of leaf DW (leaf discs dried at 40°C to a constant weight) and on the basis of LA.

Proline

The method described by Bates et al. (1973) was used to determine the proline content in the leaves. Two discs (100 mm^2 each) from one defrosted leaf sample per experimental unit were ground and homogenized in 2.5 ml of 3% aqueous sulfosalicylic acid solution, using a tube for grinding with stainless steel balls (Ultra-Turrax Tube Drive System; IKA, Staufen, Germany). Extracts were added with 250 mg of insoluble polyvinylpolypyrrolidone, vigorously vortex-mixed for 30 s and clarified by centrifugation for 10 min at 10 000 *g*. Then, the supernatants were collected, mixed with 2 ml of glacial acetic acid and 2 ml of 2.5% acid ninhydrin solution, and then incubated 1 h at 100°C. The reaction was chilled in an ice bath and extracted with 4 ml of toluene (vortex-mixing vigorously during 1 min). The OD of the toluene phase was measured at 520 nm in 10 mm optical path cells (Cary-50). Proline content was determined from a standard curve and calculated on leaf FW and LA basis. The reactive used was purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Antioxidant capacity

The oxygen radical absorbance capacity (ORAC) of the leaves was determined according to Sandhu and Gu (2010). The leaf extraction solutions previously used to assess photoprotective pigments were diluted 1:600 v/v in 75 m*M* potassium phosphate buffer (pH 7.0). Aliquots (50 µl) of diluted samples and trolox standards (0, 3.125, 6.25, 12.5, 25 and 50 µ*M* solutions prepared with 75 m*M* potassium phosphate buffer pH 7.0) were added to a 96-well black plate. Then, 100 µl of fluorescein (20 n*M* solution in 75 m*M* potassium phosphate buffer pH 7.0) were added, and the mixture was incubated at 37°C for 7 min before the addition of 50 µl of the peroxyl radical generator AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride, 140 m*M* solution in 75 m*M* potassium phosphate buffer pH 7.0]. Fluorescence was monitored by using 485 nm excitation and 538 nm emissions at 1 min intervals for 60 min on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific Inc, Wilmington, DE). The area under the curve of the fluorescence decay during 60 min was calculated and the ORAC was expressed as µmol of trolox equivalents per leaf DW and LA basis. The reactive used was purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Photosynthesis, stomatal conductance and chlorophyll fluorescence

The determinations were done at midday and at veraison, only in the 2009 season, using the first fully expanded leaf (9–10th from the apex) of the two selected shoots per experimental unit. Net photosynthesis (P_n) and stomatal conductance (g_s) were measured using a CIRAS-2 portable photosynthesis system with an infrared gas analysis instrument (PP System, Amesbury, MA). The chlorophyll fluorescence was measured using a MINI-PAM portable chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany). The maximum quantum efficiency of PSII photochemistry (Fv/Fm), the maximal fluorescence in the light-adapted state (Fm′), the non-photochemical quenching of chlorophyll fluorescence (NPQ), the non-photochemical coefficient (qN) and the photochemical quenching coefficient (qP) were the variables analyzed. Fifteen minutes of dark adaptation with a leaf clip was used to allow various photosynthetic and photoprotective mechanisms and state transitions to relax.

Statistical analysis

A randomized complete block design was used and the effects of UV-B, seasons and DAF were determinate by multifactorial ANOVA and Fisher's least significant difference (LSD) test, with a significance level of $P \leq 0.05$ (STATGRAPHICS CENTURION XV version 15.0.10 software; Statpoint Technologies Inc., Warrenton, VA). For and overall interpretation of the results, data was standardized (centering and variance-scaling) because the units of measurement of the variables differ, and a principal component analysis (PCA) and biplot graphics were performed (INFOSTAT version 2009 software; Grupo InfoStat, Córdoba, Argentina). PCA was done using vegetative growth and leaf metabolites as variables and the different UV-B treatments and seasons as classification criteria.

For the meteorological data, the effects of the seasons and months were analyzed with multifactorial ANOVA

Table 1. Total solar radiation ($T_{Radiation}$; $W\,m^{-2}$), environmental relative humidity (RH; %), mean temperature (T_{mean}; °C), maximum temperature (T_{max}; °C), minimal temperature (T_{min}; °C) and temperature amplitude (T_{amp}; °C), in 2009, 2010 and 2011 seasons for September to April. All the meteorological data presented are the daily average of measurements registered every 60 min for 24 h a day. $P_{(SEASON)}$: effects of the different season; and $P_{(MONTH)}$: effect of the different months. Values are means (n = 5) for each factor and different letters between seasons and months indicate statistically significant differences ($P \leq 0.05$).

	$T_{Radiation}$	RH	T_{mean}	T_{max}	T_{min}	T_{amp}
Season						
2009	282.29a	50.12a	16.93a	23.89a	9.95a	13.94a
2010	266.70b	47.21a	16.15b	23.12b	8.94b	14.19a
2011	243.73c	49.69a	15.89b	22.76b	8.88b	13.89a
Month						
September	197.12d	56.07a	8.65e	15.10e	2.31f	12.79e
October	276.39b	43.01cd	13.65d	20.63d	6.16e	14.47abc
November	317.22a	39.14d	17.53c	24.85c	9.61d	15.24a
December	319.83a	47.22b	19.39b	26.17b	12.28b	13.88bcd
January	329.01a	45.92bc	20.84a	27.85a	13.57a	14.28abc
February	266.77b	54.82a	19.23b	25.84bc	12.89ab	12.95de
March	229.55c	56.29a	17.96c	24.76c	11.22c	13.55cde
April	178.04e	49.58b	13.36d	20.86d	6.00e	14.85ab
ANOVA						
$P_{(SEASON)}$	0.00001	0.05130	0.00130	0.00680	0.00001	0.57350
$P_{(MONTH)}$	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001

and Fisher's LSD test ($P \leq 0.05$). PCA and biplot graphics were performed with the different seasons and months as classification criteria.

Results

The meteorological data measured at the high altitude vineyard (1450 m a.s.l.) for the three seasons and for the months when grapevine vegetates are shown in Table 1. The PCA of Fig. 1 shows the association of the meteorological variables in the different seasons. The total solar radiation and air temperatures (mean, maximum and minimum) were statistically higher in 2009 and lower in 2011, while RH and thermal amplitude were not affected by the seasons (Table 1). The higher total radiations were registered between November and January, while the major air temperatures were measured in January, followed by December. The greater RH was found in March, February and September, and the maximum thermal amplitude was registered in November (Fig. 1B, Table 1).

The grapevines grew vigorously as assessed by the number of leaves per shoot and shoot length until 38 DAF (early January), then slowed down until 65 DAF (beginning of veraison), and practically stopped growing after 96 DAF (Table 2). Internode length was not affected by the different developmental stages. The LA per leaf and basal LA per leaf and per shoot, augmented until 38 DAF, and then remained practically constant; while LA per shoot continued increasing until harvest (133 DAF). Filtration of UV-B augmented all the vegetative growth variables, i.e. the number of leaves per shoot, the shoot length and the internode length in the −UV-B treatment were 13, 24 and 9% higher than in the +UV-B treatment, respectively. The LA was also greater when solar UV-B was reduced, i.e. 21% in LA per shoot, 7% in LA per leaf, 6% in basal LA per shoot and 6% in basal LA per leaf. All the vegetative growth variables were also affected by the different seasons, being 2010 the season when plants grew more and 2011 when the plants grew less (Table 2). The PCA indicates that the PC 1 explained the 95% of the variance, and that all the growth variables measured were associated with the −UV-B treatment and with the 2010 season (Fig. 2).

The +UV-B treatment reduced markedly the gas exchange-related variables (16% P_n and 18% g_s), also decreased slightly the photochemical efficiency (2% Fv/Fm), and did not affected Fm′, qP, qN and NPQ (Table 3).

Table 4 shows that leaf DW per area (leaf thickness) increased 32% from 0 to 110 DAF, and then remained practically constant. It also augmented 5% in the +UV-B treatment, and was higher in the 2011 season. The UVAC augmented from 0 to 130 DAF (flowering to harvest) 46% per LA basis and 30% based on leaf DW. The UVAC was increased by +UV-B treatment (19% per LA basis and 15% per DW basis), and were also higher in the 2009 and 2011 seasons. The MDA content was not affected by UV-B or by the developmental stages, but it was reduced in the 2011 season in a per leaf weight basis. Epicuticular waxes content augmented 8% per LA basis, and decreased 16% per leaf DW basis

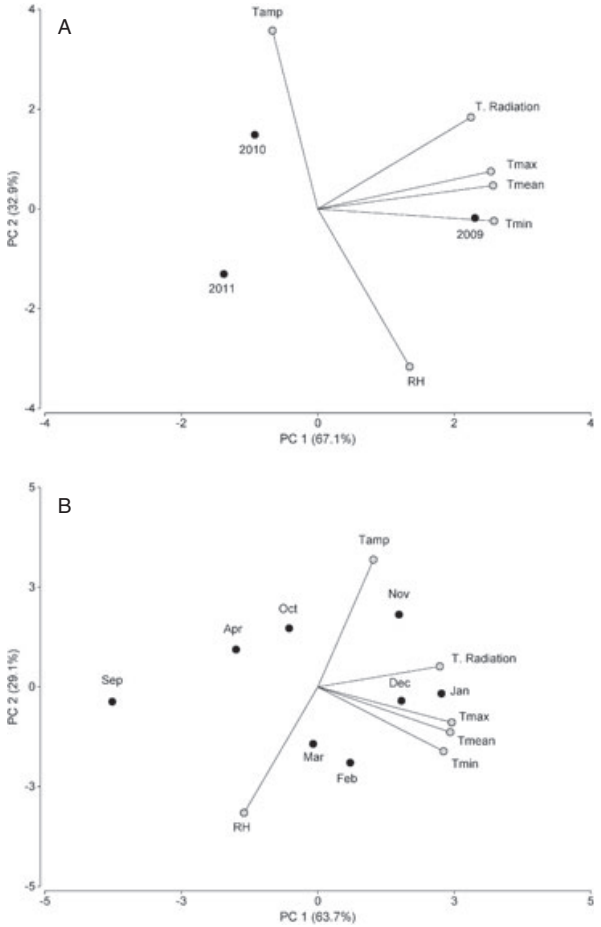

Fig. 1. Biplot display of the PCA of the meteorological determinations in the different seasons (A) and months (B).

from 0 to 130 DAF. In the 2009 season, waxes amounts were significantly increased, while in the 2011 season they were reduced. The waxes per leaf DW basis were reduced 6% by +UV-B treatment, while per LA basis were unaffected. Accumulation of proline per LA and per leaf DW basis at different developmental stages was reduced at 70 DAF (veraison). Proline was increased by +UV-B treatment (9% per LA basis and 5% per DW basis, respectively), and was higher in the 2011 season and reduced in the 2009 season. The ORAC augment markedly from 0 to 130 DAF (70% per LA basis and 53% based on leaf DW), and it was increased by +UV-B treatment (21% per LA basis and 19% per DW basis), being also higher in the 2009 season.

Table 5 indicates that the photosynthetic pigments (chlorophylls and carotenoids) per LA basis were markedly augmented from 0 to 70 DAF (flowering to veraison), and that reached their maximum accumulation at 110 DAF. The photosynthetic pigments per leaf DW basis also were markedly augmented from 0 to 70 DAF, but reached their maximum earlier (at 90 DAF) and then decreased toward harvest (130 DAF). The +UV-B treatment did not affect the photosynthetic pigments per LA basis, but reduced them per leaf DW basis. In the +UV-B treatment, the reduction per leaf DW basis was 5% for Chl *a* ($P = 0.0769$), 9% for Chl *b* ($P = 0.0019$), 4% for Car ($P = 0.0785$) and 5% for T_{Chl} ($P = 0.0293$). In the 2011 season, a higher accumulation of photosynthetic pigments per LA basis was obtained, but the seasonal effects disappear when the results were expressed per leaf DW basis. The T_{Chl}/Car ratio augmented from 0 to 90 DAF and then decreased, it was not affected by UV-B and was higher in the 2011 season.

The PCA for the different UV-B treatments shows that the PC 1 explained the 100% of the variance, that all the photosynthetic pigments per leaf DW basis and the epicuticular waxes content were associated with the −UV-B treatment; while the variables leaf DW mm^{-2} (leaf thickness), UVAC, MDA, proline, ORAC and most of the photosynthetic pigments per LA basis (except Chl *b*) were associated with the +UV-B treatment (Fig. 3A). The PCA for the different seasons indicates that the PC 1 explained the 67.5% of the variance and that most of the variables were higher in the 2011 season, except the waxes accumulation, ORAC, UVAC per leaf DW basis and MDA per leaf weight basis, that were more associated with the 2009 season (Fig. 3B).

Discussion

Vegetative growth is reduced by UV-B through reduction in LA

The grapevine's vegetative tissues grew predominantly until the phenological stage of veraison, which is the onset of berry ripening, when berries soften and start to accumulate sugars and color develops in red cultivars. The plant tissues compete for photoassimilates and, depending on the developmental stages, these photoassimilates will be distributed to growing vegetative tissues, reproductive development or starch accumulation in wood and root tissues (Zapata et al. 2004). The perception of relatively high solar UV-B levels reduced all the vegetative growth variables, but the shoot length and the LA per shoot were more affected (>20% reductions as compare with the treatment where UV-B was reduced). The LA per leaf variable is related to the leaf expansion, and the LA per shoot variable depends on the leaf size but also depends on the number of leaves per shoot. Previous results indicates that different plant species vary widely in their response to ambient UV-B, although vegetative growth decreases by solar UV-B were observed in lettuce, mung bean,

Table 2. Leaves per shoot, shoot length (mm), internode length (mm), leaf area (LA; mm^2 per shoot and mm^2 per leaf; considering all the leaves in the shoot) and basal leaf area (basal LA; mm^2 per shoot and mm^2 per leaf; considering only the first 15 leaves from the shoot base) for +UV-B and −UV-B treatments at 0, 38, 65, 96 and 133 DAF in 2009, 2010 and 2011 seasons. $P_{(UV\text{-}B)}$: effect of UV-B treatments; $P_{(SEASON)}$: effects of the different season and $P_{(DAF)}$: effect of the different growth and development stages. Values are means (n = 5) for each factor and different letters between UV-B, seasons and DAF indicate statistically significant differences ($P \leq 0.05$).

	Leaves per shoot	Shoot length	Internode length	LA per shoot	LA per leaf	Basal LA per shoot	Basal LA per leaf
UV-B							
+UV-B	29.55b	1365.5b	45.1b	377251b	13397b	212320b	14586b
−UV-B	33.41a	1690.0a	49.1a	455461a	14266a	224970a	15435a
Season							
2009	30.72b	1466.5b	46.4b	413844b	13834b	212863b	14618b
2010	34.60a	1895.9a	54.0a	502781a	15477a	232589a	16356a
2011	29.17b	1221.0c	40.8c	332442c	12184c	210483b	14058b
DAF							
0	18.72c	855.5b	45.7a	190923c	12346b	182562b	12922b
38	31.07b	1552.4a	49.1a	413375b	14429a	230132a	15430a
65	35.20a	1726.0a	47.5a	481034a	14224a	227328a	15596a
96	36.21a	1752.0a	46.5a	497651a	14068a	226530a	15547a
133	36.21a	1752.9a	46.6a	498796a	14090a	226673a	15557a
ANOVA							
$P_{(UV\text{-}B)}$	0.00001	0.00001	0.00010	0.00001	0.00070	0.00840	0.00670
$P_{(SEASON)}$	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001
$P_{(DAF)}$	0.00001	0.00001	0.19550	0.00001	0.00001	0.00020	0.00001

maize and cucumber (Krizek et al. 1997, Pal et al. 1997, Krizek et al. 1998). Also, more compact and shorter plants, mainly due to shorter internodes rather than fewer nodes, were found (Santos et al. 1993, González et al. 1998, Zhao et al. 2003a, 2003b). However, the results presented here for grapevines show that both, the internodes length and the number of nodes and leaves were affected by UV-B which may be a characteristic response of the species. Mark and Tevini (1996) speculated that the mechanisms for shoot elongation reductions by UV-B in sunflower and maize might be due to changes in the indole acetic acid (IAA) levels, one of the phytohormones responsible for shoot elongation. Ros and Tevini (1995) demonstrated that IAA levels can be reduced by photooxidation in sunflower seedlings exposed to relative high levels of UV-B. Also, it has been claimed that the abscisic acid (ABA), a phytohormone that regulates plant responses to various stressful abiotic and biotic factors, is responsible for vegetative growth reduction in grape (Dry et al. 2000). However, in *Ilex paraguariensis* shoot elongation was promoted by keeping a better tissue turgor via ABA application (Sansberro et al. 2004). In a previous work with pot-grown Malbec grapevines with comparable UV-B treatments, we found that ABA levels increased in leaves exposed to UV-B and intermediated many of the defensive responses to UV-B (Berli et al. 2010). Thus, UV-B may be augmenting the ABA levels in the leaves and indirectly reducing grapevine vegetative growth.

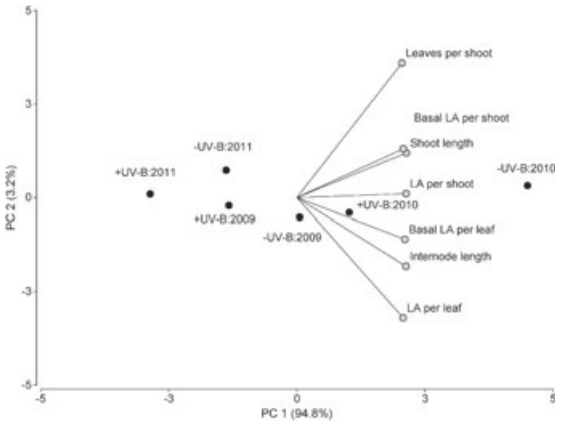

Fig. 2. Biplot display of the PCA of the vegetative growth measurements in the different UV-B treatments and seasons.

Our results are in correspondence with those who indicated that LA is a very sensitive growth parameter that responded to elevated UV-B reducing the cell division, the leaf expansion and the number of leaves (Staxén et al. 1993, Nogués et al. 1998, Zhao et al. 2003a, 2003b). Ballaré et al. (1995) and Grant (1999) also found that when plants were exposed to UV-B, the LA was lower because of both, smaller leaves and lower number of leaves. These morphogenic responses would be possibly part of an acclimatization mechanism to reduce the interception of the UV-B harmful radiation (Jansen 2002). Notwithstanding, more detailed studies

Table 3. Net photosynthesis (P_n; $\mu mol\, m^{-2}\, s^{-1}$) and stomatal conductance (g_s; $mmol\, m^{-2}\, s^{-1}$), determined at 75 DAF in 2009 season for +UV-B and −UV-B treatments. The maximum quantum efficiency of PSII photochemistry (Fv/Fm), maximal fluorescence in the light-adapted state (Fm′), photochemical quenching coefficient (qP), non-photochemical coefficient (qN) and non-photochemical quenching of chlorophyll fluorescence (NPQ) measured at 120 DAF in 2009 season for +UV-B and −UV-B treatments. All the measurements were done in fully expanded leaves (9–10th from the apex). $P_{(UV\text{-}B)}$: effect of UV-B treatments. Values are means (n = 5) and different letters indicate statistically significant differences ($P \leq 0.05$).

	P_n	g_s	Fv/Fm	Fm′	qP	qN	NPQ
UV-B							
+UV-B	10.64b	164.80b	0.7851b	1978.90a	0.7263a	0.0253a	0.0201a
−UV-B	12.31a	193.70a	0.7972a	2067.65a	0.7262a	0.0253a	0.0208a
ANOVA							
$P_{(UV\text{-}B)}$	0.00640	0.00690	0.04000	0.23740	0.99810	0.99040	0.77690

should be performed to determine if UV-B affects cell division, the cell expansion or both.

The LA per shoot was highly affected by +UV-B because it adds the UV-B effect in the leaves number per shoot and the individual LA. The LA per leaf and the basal LA (considering only the first 15 leaves from the shoot base) per leaf and per shoot were also affected by +UV-B, but less markedly because they are affected only by the UV-B effect in the leaf size.

Photosynthesis is reduced by UV-B through limitation of leaves gas exchange in leaves

The solar UV-B at high altitude vineyards reduced P_n and g_s, both physiological variables related to gas exchange and dependent on the stomatal aperture. The phytohormone ABA is responsible for stomatal closure also in grapevine (Stoll et al. 2000) and the stomatal regulation is an important process that limits the photosynthesis (Radin et al. 1988, Hetherington and Woodward 2003). Reduced stomatal conductance in response to UV-B was found (Dai et al. 1992), and UV-B may be augmenting the ABA levels in the leaves, as previously found (Berli et al. 2010), therefore indirectly reducing stomatal aperture and limiting gas exchange.

In UV-B exclusion experiments with dwarf shrub at high-arctic regions it has been reported that ambient UV-B is a significant plant stressor that reduce net photosynthesis, PSII performance, i.e. the Fv/Fm ratio (Albert et al. 2008, Albert et al. 2011a) and stomatal conductance (Bredahl et al. 2004), compared to responses in reduced UV-B. In our experiment, measuring leaves exposed to high UV-B (first fully expanded leaf, 9–10th from the apex) and despite the significant UV-B reduction of the Fv/Fm, the values ranged from 0.78 to 0.79 in both UV-B treatments. Accordingly, the Fv/Fm values of healthy and sunny leaves are around 0.8 ± 0.5 (Critchley 1998). The Fv/Fm is a physiological variable that represents the maximum potential quantum efficiency of PSII when all capable reaction centers are open, so the value obtained in the +UV-B treatment (0.785) may indicate that the grapevines were not experiencing important stressful conditions. The optimal range observed indicates that PSII can afford the excess of radiation and/or the excess of UV-B adequately. The Fv/Fm is a direct measurement of the PSII efficiency that excludes the indirect effect of UV-B reducing stomatal aperture and thereby limiting gas exchange.

There was no statistically significant change of values for photochemical quenching (qP) with the UV-B exposition for Malbec variety. This indicates that the proportion of the reaction centers remained open even with UV-B exposition. As Fv/Fm showed no detrimental changes in its efficiency after the exposition to UV-B, we may assert that the antennae were protected against photo damage through an effective energy dissipation mechanism (qN and NPQ). Thus, though UV-B could affect photosynthesis through impedance of gas exchange, it was not effective in causing an impairment of the photochemistry apparatus.

Morphological, biochemical and antioxidant effects

A lower leaf expansion because of the solar UV-B perception and/or because of the 2011 season environmental conditions resulted in thicker leaves. Our data are consistent with the work of Day and Vogelmann (1995) in which UV-B increased leaf mesophyll thickness in pea leaves, and with the work of Albert et al. (2011b) in high-arctic dwarf shrub plants. The photoprotective pigments were continuously accumulated in the leaves during the different developmental stages, and their accumulation was promoted by UV-B. When the results are expressed per LA basis the differences in leaf thickness affect them, but the concentration effect can be excluded from the analysis expressing the results per DW basis, which is related to a higher biosynthesis and/or accumulation. The thicker leaves obtained in the +UV-B treatment and in the 2011 season contribute to augment the UVAC per LA basis, but the greater UVAC per DW basis in the +UV-B treatment and in the 2009 and 2011 seasons indicates that the UVAC biosynthesis and/or accumulation was also directly augmented. We previously found that in the grapevine Malbec leaves the UVAC are mainly phenols, such as flavonols (quercetin and kaempferol) and hydroxycinnamic acids (caffeic acid, *p*-coumaric acid and ferulic acid), and that quercetin and kaempferol were significantly enhanced

Table 4. Leaf dry weight (DW; μg mm^{-2}), UV-absorbing phenolic compound (UVAC; OD_{305} mm^{-2} leaf and OD_{305} mg^{-1} leaf DW), malondialdehyde (MDA; pM mm^{-2} leaf and pM mg^{-1} leaf FW), epicuticular waxes (Waxes; μg mm^{-2} leaf and μg mg^{-1} leaf DW), proline (pg mm^{-2} leaf and ng mg^{-1} leaf FW) and oxygen radical absorbance capacity (ORAC; μmol of TE mm^{-2} leaf and μmol of TE mg^{-1} leaf DW) for +UV-B and −UV-B treatments at 0, 50, 70, 90, 110 and 130 DAF in 2009, 2010 and 2011 seasons. All the measurements were done in fully expanded leaves (9–10th from the apex) exposed to West. $P_{(UV\text{-}B)}$: effect of UV-B treatments; $P_{(SEASON)}$: effects of the different season; and $P_{(DAF)}$: effect of the different growth and development stages. Values are means (n = 5) for each factor and different letters between UV-B, seasons and DAF indicate statistically significant differences ($P \leq 0.05$).

	Leaf DW mm^{-2}	UVAC mm^{-2}	UVAC mg^{-1}	MDA mm^{-2}	MDA mg^{-1}	Waxes mm^{-2}	Waxes mg^{-1}	Proline mm^{-2}	Proline mg^{-1}	ORAC mm^{-2}	ORAC mg^{-1}
UV-B											
+UV-B	34.6a	1.00a	0.27a	85.1a	587.82a	17.5a	212.23b	291.2a	1.70a	2909.8a	79950a
−UV-B	32.8b	0.81b	0.23b	83.2a	587.79a	17.7a	224.75a	265.8b	1.62b	2307.0b	64730b
Season											
2009	30.7b	0.94a	0.27a	81.0a	628.64a	25.6a	308.22a	258.6b	1.64b	3064.0a	89390a
2010	31.2b	0.80b	0.23b	84.2a	601.67a	16.1b	213.65b	234.1c	1.48c	2478.5b	71420b
2011	39.3a	0.97a	0.24b	87.3a	533.11b	11.1c	133.60c	342.9a	1.86a	2282.9b	56210c
DAF											
0	28.8d	0.69d	0.20b	91.3a	636.70a	16.9c	236.97a	281.2a	1.72a	1954.6c	56210d
50	32.1c	0.79c	0.25a	77.9a	532.97a	17.5bc	231.15a	276.6a	1.68a	2192.5c	71320bc
70	32.5bc	0.93b	0.26a	83.7a	606.89a	17.6b	225.76ab	242.6b	1.52b	2633.6b	75670abc
90	34.5b	0.94b	0.26a	83.0a	590.87a	17.5b	214.84b	287.8a	1.71a	2782.3b	78100ab
110	37.9a	1.04a	0.25a	83.2a	567.98a	18.1ab	202.70c	287.5a	1.64a	2764.4b	66470cd
130	36.5a	1.01ab	0.26a	85.8a	591.43a	18.2a	199.50c	295.3a	1.69a	3322.7a	86280a
ANOVA											
$P_{(UV\text{-}B)}$	0.00270	0.00001	0.00001	0.60170	0.99880	0.46430	0.00040	0.00480	0.03490	0.00001	0.00001
$P_{(SEASON)}$	0.00001	0.00001	0.00010	0.34480	0.00280	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001
$P_{(DAF)}$	0.00001	0.00001	0.00001	0.41610	0.17650	0.00080	0.00001	0.01430	0.01870	0.00001	0.00001

Table 5. Chlorophyll *a* (Chl *a*), b (Chl *b*), carotenoids (Car) and total chlorophyll (T_{Chl}) contents on the basis of leaf DW (μg mg^{-1} leaf DW) and LA (ng mm^{-2} leaf), and T_{Chl}/Car ratio for +UV-B and −UV-B treatments at 0, 50, 70, 90, 110 and 130 DAF in 2009, 2010 and 2011 seasons. All the measurements were done in fully expanded leaves (9–10th from the apex) exposed to West. $P_{(UV\text{-}B)}$: effect of UV-B treatments; $P_{(SEASON)}$: effects of the different season and $P_{(DAF)}$: effect of the different growth and development stages. Values are means (n = 5) for each factor and different letters in UV-B, seasons and DAF indicate statistically significant differences ($P \leq 0.05$).

	Chl *a* mm^{-2}	Chl *b* mm^{-2}	Car mm^{-2}	T_{Chl} mm^{-2}	Chl *a* mg^{-1}	Chl *b* mg^{-1}	Car mg^{-1}	T_{Chl} mg^{-1}	T_{Chl} Car^{-1}
UV-B									
+UV-B	271.9a	93.4a	75.8a	365.3a	7.79a	2.67b	2.18a	10.47b	4.81a
−UV-B	265.9a	95.1a	73.5a	361.0a	8.14a	2.90a	2.26a	11.04a	4.88a
Season									
2009	237.7b	87.8b	66.5c	325.5b	7.75a	2.87a	2.17b	10.61a	4.85b
2010	254.3b	81.2c	71.9b	335.5b	8.16a	2.60b	2.32a	10.76a	4.64c
2011	314.7a	113.8a	85.4a	428.6a	7.99a	2.90a	2.16b	10.89a	5.04a
DAF									
0	186.3d	61.8c	55.2d	248.1d	6.50c	2.13d	1.95c	8.63d	4.54c
50	226.0c	81.9b	64.7c	307.9c	7.09c	2.57c	2.03c	9.66c	4.75bc
70	282.4b	102.2a	76.3b	384.5b	8.68ab	3.15a	2.35ab	11.83ab	5.01a
90	307.6a	108.5a	82.7a	416.1a	9.00a	3.18a	2.42a	12.18a	5.02a
110	314.5a	109.2a	85.1a	423.7a	8.34ab	2.88b	2.25b	11.22b	4.97ab
130	296.8ab	102.1a	83.8a	398.8ab	8.19b	2.81bc	2.30ab	11.00b	4.76bc
Anova									
$P_{(UV\text{-}B)}$	0.38650	0.49920	0.17410	0.64030	0.07690	0.00190	0.07850	0.02930	0.27140
$P_{(SEASON)}$	0.00001	0.00001	0.00001	0.00001	0.22290	0.00160	0.00900	0.69610	0.00001
$P_{(DAF)}$	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001	0.00001	0.00010

by similar +UV-B treatments (Berli et al. 2010). The increases in UVAC have been reported as acclimation responses to protect the plant by specifically absorbing in a broader wavelength region (280–340 nm) that includes UV-B and UV-A (Winkel-Shirley 2001). It has been shown that the increase of phenolic compounds in leaves occurs because UV-B activates the expression of genes encoding some enzymes of the phenylpropanoids and flavonoids pathway, such as phenylalanine ammonia lyase and chalcone synthase, respectively

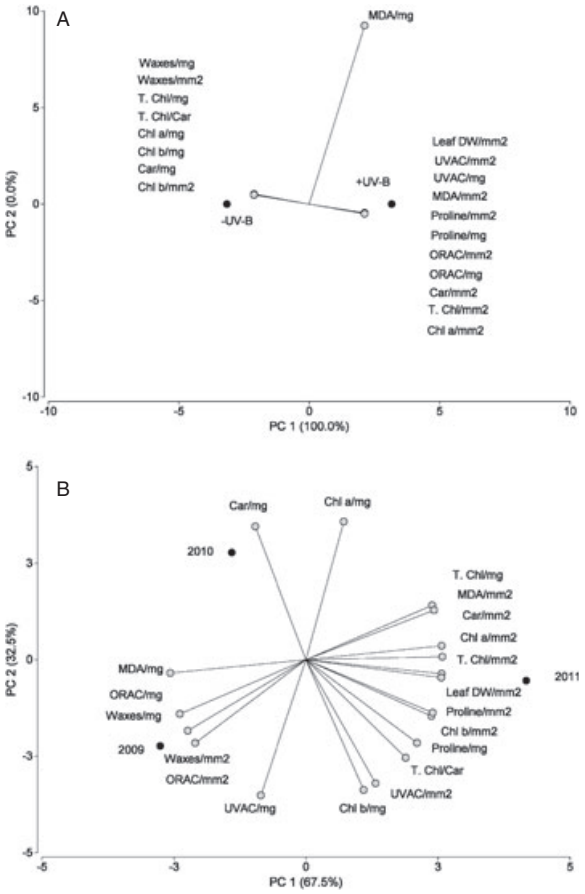

Fig. 3. Biplot display of the PCA of the leaves metabolites and antioxidant capacity in the different UV-B treatments (A) and seasons (B).

(Bieza and Lois 2001, Liakoura et al. 2001), also in Malbec grapevines (Pontin et al. 2010). The phenols are secondary metabolites with antioxidant capacity that respond to different environmental stressful conditions (Koes et al. 1994, Dixon and Paiva 1995) and the greater UVAC per DW basis in the 2009 season was likely associated with the higher stressful conditions found during this season (higher temperatures and total radiation).

The ORAC continuously augmented in the leaves during the different developmental stages, and their accumulation was promoted by the UV-B and by the 2009 season stressful conditions, in correspondence with the photoprotective pigments accumulation. The thicker leaves obtained in the +UV-B treatment and in the 2011 season contribute to augment the ORAC per LA basis, but the greater ORAC per DW basis in the +UV-B treatment and in the 2009 season indicates that it was also directly augmented. Majer and Hideg (2012) also found that UV-B increases the antioxidant capacity of grapevine leaves.

The lack of lipid peroxidation (oxidative damage) in grapevine leaves exposed to relatively high UV-B was unexpected., because we previously found that MDA concentration (per leaf weight basis) was significantly increased by similar +UV-B treatments (Berli et al. 2010). However, although the UV-B levels were comparable between these experiments, Berli et al. (2010) performed with younger plants (1 year old vs 11–13 years old), and the treatments were initiated at budbreak. On the other hand, the oxidative damage per leaf weight basis reduction during 2011 was expected, because the 2011 was the season with lower stressful conditions (air temperatures and total radiation).

The epicuticular waxes were continuously accumulated in the leaves during the different developmental stages and in correlation with the higher total solar radiation and air temperatures, were increased in the 2009 season, and reduced in the 2011 season. Particularly, the solar UV-B did not affect epicuticular waxes per LA basis, and reduced it accumulation per leaf DW basis. Therefore, the epicuticular wax layer mainly responded to the increased total radiation and air temperature as previously found by Rao and Reddy (1980) and Bondada et al. (1996). When the leaves were thicker (+UV-B treatment, 2011 season and the more advanced developmental stages), the waxes content per leaf DW basis were reduced. These compounds can act as an interface between environment and leaf internal structures providing the first line of defense, augmenting the proportion of the harmful radiation reflected (Clark and Lister 1975, Caldwell et al. 1983, Holmes 1997). Other authors found that UV-B can increase the leaf surface waxes content on LA basis in species like barley, bean (Steinmüller and Tevini 1985, Gonzalez et al. 1996) and cotton (Kakani et al. 2003), and that UV-B can also alter its chemical composition (Tevini and Steinmuller 1987, Barnes et al. 1996). Proline content was increased by solar UV-B and was also higher in the 2011 season and reduced in the 2009 season. The thicker leaves in +UV-B and in the 2011 season contribute to augment proline per LA basis, but the greater proline per DW basis in +UV-B and in the 2011 season indicates that proline accumulation was also directly augmented. The reduced proline content in the 2009 season was contrary to what was expected since it had been associated with higher stressful conditions in pea, white clover and faba bean (Alexieva et al. 2001, Shetty et al. 2002, Hofmann et al. 2003). Proline has many biological functions that include acting as an energy source, antioxidant and osmoprotectant in many plant species, including grapevines (Deluc et al. 2009).

Photosynthetic pigments were accumulated in the leaves during the different developmental stages, but reached their maximum after veraison and then

decreased toward harvest, indicating that leaves started to senesce. Their accumulation in leaves was reduced by UV-B expressing them per leaf DW basis, but UV-B did not affect them when the results were expressed per LA basis, meaning that light interception may not be affected. In the 2011 season, higher photosynthetic pigments per LA basis were obtained, but this effect disappears when the results are expressed per leaf DW basis. The thicker leaves obtained in the +UV-B treatment and/or because of the 2011 season conditions, and the concentration effect (more pigments per LA) may be masking the direct deleterious effect of UV-B on the photosynthetic pigments accumulation. We have also previously found a similar +UV-B treatment did not affect them, because the results were only expressed per LA basis (Berli et al. 2010). The UV-B could be directly damaging Chl molecules by photooxidation, as other authors found Chl reduction by UV-B in many plant species (Tevini et al. 1981, Mirecki and Teramura 1984, He et al. 1993). Also, structural damage to chloroplasts and changes in photosynthetic pigments may result in reduction of photosynthesis (Sullivan and Rozema 1999).

In conclusion, the solar UV-B reductions of P_n and g_s (all expressed per LA basis), mainly through limitation in gas exchange, plus the reduction in whole plant's LA by +UV-B treatment may have produced a lower carbon fixation per plant and thereby contribute to a reduced growth. The UV-B augments the leaf thickness, and also the photoprotective pigments and the proline content, and thereby the antioxidant capacity of leaves. The direct effects on their biosynthesis and/or accumulation are complemented with the concentration effect caused by the thicker leaves. The defense mechanisms triggered by UV-B reduced the lipid peroxidation expected, but were insufficient to protect the photosynthetic pigments. The combined higher stressful conditions (total radiation and air temperatures) were registered in 2009 season, and the grapevines increased photoprotective pigments, antioxidant capacity and epicuticular waxes of leaves, but a major oxidative damage was also observed.

Acknowledgements – This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PAE-PID 2007–00149 to R. B.), Secretaría de Ciencia y Técnica de la Nación (SECyT, PICT 2008–1666 to R. B.), Secretaría de Ciencia y Técnica de la Universidad Nacional de Cuyo and Bodega Catena-Zapata. The authors thank the technical assistance of L. Bolcato and M. Fucili.

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Edited by A. Krieger-Liszkay