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Antioxidative defence mechanisms in two grapevine (*Vitis vinifera* L.) cultivars grown under boron excess in the irrigation water

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Summary

The aim of this study was to investigate the impact of B excess on the antioxidative defence system in two grapevine cultivars differently sensitive to B, in order to evaluate in which way the different sensitivity was correlated to the activation of defence mechanisms. Two-year-old *Vitis vinifera* L. plants ('Merlot' and 'Sangiovese'), grafted on the same rootstock, were subjected to B treatment as potted vines, and biochemical determinations were employed to evaluate the antioxidative response. Compared to 'Sangiovese', 'Merlot' showed a much higher B accumulation and both leaf and peroxidative damages. In B-treated 'Sangiovese' the activity of superoxide dismutase did not change compared to control leaves, whereas in 'Merlot' a dramatic decrease in the enzyme activity was observed. B increased reduced ascorbate pools in both cultivars, but ascorbate peroxidase activity was enhanced only in 'Merlot'. In this latter cv. an enhancement of total phenols was also observed. 'Merlot' showed a lower ability to contrast B accumulation in leaves than 'Sangiovese' evidencing a higher oxidative stress. Even if defence mechanisms were generally activated in 'Merlot', they did not counteract efficiently metabolic damages likely due to the dramatic decrease in superoxide dismutase, the first enzyme involved in the detoxification of oxygen radicals.

K e y w o r d s : ascorbate; glutathione; oxidative stress; phenolic acids; reactive oxygen species.

Introduction

Boron (B) is an essential micronutrient for the growth and development of all higher plants and is taken up in the form of boric acid (Aquea *et al.* 2012, Gimeno *et al.* 2012). However, its function in plants is not yet fully understood. Boron is certainly involved in the formation of cell walls through the borate-diol bonding of two rhamnogalacturonan II molecules (Goldbach and Wimmer 2007). It is also involved in carbohydrate and phenolic metabolism and other biochemical activities and is responsible for the integrity of the membranes (Gimeno *et al.* 2012). Boron is frequently present at toxic concentrations in soils that have been exposed to B-contaminated irrigation water (municipal and other wastewater) or excess application of B-rich fertiliser, sewage sludge or fly ash, as well as from natural deposits found in semi-arid and arid environments throughout the world (Cervilla *et al.* 2007, Ardic *et al.* 2009, Ferro *et al.* 2010). In these areas high concentrations of B are often associated with high salinity (Reid 2007).

Signs of B toxicity, like formation of necrotic patches on leaves, are associated with accelerated senescence, reduced shoot and root growth, and with reduced crop yields (Nable *et al.* 1997, Papadakis *et al.* 2004, Yermiyahu and Ben-Gal 2006). A great number of physiological processes have been shown to be altered by B toxicity including degradation of leaf chlorophyll, inhibition of photosynthesis and lower stomatal conductance (Eraslan *et al.* 2007, Han *et al.* 2009, Gimeno *et al.* 2012), disruption of cell wall development (Reid *et al.* 2004), increased membrane leakiness, peroxidation of lipids and altered activities of antioxidant compounds (Gunes *et al.* 2006, Cervilla *et al.* 2007, Ardic *et al.* 2009, Ferro *et al.* 2010). These effects impair cellular electron transport chains so that molecular oxygen acts as an alternative acceptor for non-utilised electrons and light energy leading to reactive oxygen species (ROS) generation (Sgherri *et al.* 2013).

Despite the importance of the above nutritional disorders, mechanisms of B tolerance and toxicity are not completely clear. The physiology of tolerance to B toxicity includes both exclusion mechanisms and internal tolerance mechanisms. It has been suggested that exclusion represents the main defence system involved in the establishment of tolerance mechanisms in wheat and barley (Paull *et al.* 1992, Hayes and Reid 2004). In other species the defence systems against B damaging effects are based on antioxidative mechanisms (Gunes *et al.* 2006, Cervilla *et al.* 2007). The activation/induction of this antioxidative response plays a key role in protecting plants from the harmful enhanced production of ROS following a large number of environmental stresses (Pérez-López *et al.* 2009). Overproduction of ROS in the plant cell requires the intervention of defence antioxidant systems which include metabolites such as reduced ascorbate (AsA) and glutathione (GSH) as well as scavenging enzymes among which superoxide dismutase (SOD) and ascorbate peroxidase (APX). In cell compartments, including the apoplast (Sgherri *et al.* 2007), systems such as the AsA-GSH cycle bring about the reductive detoxification of ROS at the ultimate expense of NAD(P)H (Sgherri *et al.* 2013).

Grapevines have been defined a sensitive species to excess B by Maas *et al.* (1990) who observed a threshold value of B in the soil solution of 0.5 to 0.75 $mg \cdot L^{-1}$, above

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which vegetative growth was reduced. Little is known regarding the antioxidative response to high levels of B in grapevines. Furthermore, B toxicity mechanisms in grapevine show great differences from other crops since B is mobile in grapevine (Brown and Hu 1998). Indeed, differently from other crop species, B is mobile in the phloem of all species such as grapevine that utilise polyols as primary photosynthetic metabolites. Thus, B moves with the transpiration stream and, once entered into a leaf, tends to immobilise and to accumulate. Gunes *et al.* (2006) reported on the effects of increasing levels of B on the growth, B concentration and antioxidative response of grapevine. As result of B toxicity the concentration of H_2O_2 increased as well as membrane permeability and peroxidation. Compared to control plants, the activities of SOD and catalase were enhanced by B treatments, that of APX was impaired whereas both the ascorbate and glutathione pools were not investigated.

Grapevines are often grafted onto specific rootstocks developed for their hardiness and tolerance to many different environmental conditions such as salinity and lime. In this respect, the rootstock Paulsen 1103 was selected for its strong drought tolerance and its ability to grow well on lime-based soils. Thus, 1103P is well suited for dry-farmed vineyards, and it is reported to have a good tolerance to salinity, a condition often present in B-rich soils (Walker *et al.* 2007). The knowledge of the response of different cultivars (grafted on the same rootstock) to B excess in the soil would allow the selection of the ones most suitable to maintain a good yield and high quality grapes.

In the present work we investigated the impact of B excess on the antioxidative defence mechanisms in two grapevine cultivars in order to provide a basis for developing strategies for reducing the risks associated with B toxicity and maintaining sustainable plant production. The cultivars taken into consideration were 'Merlot' and 'Sangiovese'. The first is a widespread cultivar that showed to adapt well to dissimilar geographical areas, whereas the latter is typical of the Tuscan-Emilian Apennines, but cultivated throughout Italy even if with different agronomical and qualitative results.

Material and Methods

Plant material: Two-year-old *Vitis vinifera* L. plants ('Merlot' and 'Sangiovese') grafted on 1103 P (*V. berlandieri* x *V. rupestris*) coming from a commercial nursery were grown as potted vine (non-draining PVC pots lined with polyethylene bag filled with 4 kg of air-dried soil) in a naturally lit greenhouse at the Dipartimento di Scienze Agrarie, Alimentari e Agroambientali, University of Pisa. Following a preliminary study, B concentration was chosen among those which induced an oxidative stress but allowed the survival of both plants, even in the presence of visible leaf damages. Relative leakage ratio was taken as parameter to evaluate oxidative damage to leaf cell membranes (Sgherri and Navari-Izzo 1995) due to its strict relation with membrane peroxidation (often evaluated also by malondialdehyde or thiobarbituric acid reactive substance production). On June 2012 one set of plants for each cv. was subjected to B treatment (30 $mg \cdot kg^{-1}$ soil) whereas the other set was not added with B and kept as a control. B was added to the soil as H_3BO_3 solution. The composition of the irrigation water was: pH 7.2, conductivity 940 µS, Na^+ 36 $mg \cdot L^{-1}$, K^+ 8 $mg \cdot L^{-1}$, Ca^{2+} 121 $mg \cdot L^{-1}$, Mg^{2+} 19 $mg \cdot L^{-1}$, Cl^- 68 $mg \cdot L^{-1}$, NO_3^- 17 $mg \cdot L^{-1}$, SO_4^{2-} 83 $mg \cdot L^{-1}$ and HCO_3^- 330 $mg \cdot L^{-1}$. During the experiment, soil was kept at approximately 75 % of the field capacity by watering with B-free tap water. After four weeks from treatment, leaves were collected and samples were frozen in liquid nitrogen and stored at -80 °C until analysis or oven-dried for dry weight and B determinations.

Boron: Dried ground material was microwave digested with a mixture of nitric acid and H_2O_2 (3:1 v/v) in capped Teflon pressure digestion vessels at 200 °C for 1 h (Markert 1996). After digestion B was determined by spectrophotometry at 420 nm (Varian Cary 1E UV-Vis, Palo alto, CA, USA) by the azomethine-H method (Lohse 1982). A standard curve in the range 2.5-10 $\mu g \cdot mL^{-1}$ B (as H_3BO_3) was used to calculate B concentration.

Thiobarbituric acid reactive substances (TBARS): Leaves were extracted with 50 mM potassium phosphate buffer (pH 7.8) containing 2 % (w/v) polyvinylpolypirrolidone, 0.1 mM ethylenediaminetetracetic acid (EDTA) and 5 mM cysteine. After centrifugation at 12100 × g for 15 min the supernatant was used for the determination of TBARS using the method described by Pérez-López *et al.* (2009). The TBARS concentration was determined by the extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$ after subtracting non-specific absorbance at 600 nm and sugar absorbance at 440 nm using the following formula: TBARS = (A – B), where A = $[(A_{532+TBA}) - (A_{600+TBA}) - (A_{532-TBA} - A_{600-TBA})]$ and B = $[(A_{440+TBA} - A_{600+TBA}) \times 0.0571]$.

Hydrogen peroxide: H_2O_2 concentration was evaluated following the method reported by Sgherri and Navari-Izzo (1995) based on the formation of the titanium-peroxide complex. Leaf tissue was homogenized at 4 °C with cold acetone and filtered. The precipitation of the complex was obtained by the addition to the extract of 5 % titanyl sulphate and of concentrated NH_4OH solution. After centrifugation at 12000 × g for 15 min, the supernatant was discarded and the pellet was washed with cold acetone. After 4 precipitations the pellet was dissolved with 1.5 N H_2SO_4 and the solution was read at 415 nm. H_2O_2 content was calculated using a standard curve in the 0.5-10 µmol range.

Ascorbate (AsA) and dehydroascorbate (DHA): Leaf tissue was homogenized in ice-cold 5 % (w/v) trichloroacetic acid containing 4 % (w/v) polyclar AT using a cold mortar and pestle. AsA and total ascorbate (AsA + DHA) were determined in the supernatant following the method of Wang *et al.* (1991). Total ascorbate was determined through the reduction of DHA to AsA by 0.97 mM dithiothreitol, and DHA levels were estimated on the basis of the difference between total ascorbate and AsA contents. Two separate calibration curves for

AsA and total ascorbate, covering the range of 5-50 nmol were used.

Reduced (GSH) and oxidized glutathione (GSSG): Leaf tissue was homogenized at 4 °C in ice-cold 5 % (w/v) trichloroacetic acid and centrifuged at 12000 × g for 15 min. The supernatant was used for total (GSH + GSSG) and GSSG determinations by the 5,5'-dithio-bis-nitrobenzoic acid (DTNB)-GSSG reductase recycling procedure as reported by Sgherri and Navari-Izzo (1995). GSSG was determined after GSH had been removed by derivatisation with 2-vinylpyridine. Absorbance of the reaction mixtures was detected at 412 nm at 25 °C. The amount of GSH was calculated by subtracting the GSSG amount, expressed as GSH equivalents, from the total glutathione amount. A standard calibration curve in which GSH equivalents (1-10 nmol) were plotted against the rate of change in A_{412} was used.

Enzyme extraction and assays: Leaf tissue (0.5 g) was ground at 4 °C in a cold mortar with sand using a specific buffer for each enzyme as outlined below. The homogenate was centrifuged at 12000 × g for 15 min and enzyme assays were carried out in the supernatant. Conditions for all assays were chosen so that the rate of reaction was constant during the time used and proportional to the amount of enzyme added. Proteins were determined according to Bensadoun and Weinstein (1976) using bovine serum albumin as a standard.

Glutathione reductase (GR; EC 1.6.4.2) was extracted with 0.1 M potassium phosphate buffer (pH 7) containing 1 mM Na_2EDTA and 2 % (w/v) polyvinylpolypirrolidone. GR activity was determined at 30°C by the loss in absorbance at 340 nm as NADPH was oxidized by GSSG (Gillham and Dodge 1986). The assay mixture contained 0.2 M potassium phosphate buffer (pH 7.5), 1.5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 0.25 mM GSSG, 25 µM NADPH and 50 µL enzyme extract. The reaction was initiated by NADPH addition and followed for 1 min. Corrections were made for the background absorbance at 340 nm without NADPH.

The extraction of APX (EC 1.11.1.11) was carried out using 50 mM potassium phosphate buffer (pH 7) containing 1 mM AsA to avoid inactivation during extraction and assay. APX activity was assayed by measuring the oxidation of AsA by H_2O_2 at 290 nm according to Wang *et al.* (1991). The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.6), 1 mM AsA, 0.4 mM Na_2EDTA and 50 µL enzyme extract. The reaction was started by adding 0.4 mM H_2O_2 to the reaction mixture after 1 min incubation at 25°C. Corrections were made for the low, non-enzymatic oxidation of AsA by H_2O_2 and for the oxidation of AsA in the absence of H_2O_2.

SOD (EC 1.15.1.1) activity was assayed in the same supernatant obtained for the determination of APX activity by measuring the inhibition of the photochemical reduction of nitroblu tetrazolium (NBT) (Beauchamp and Fridovich 1971). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 mM NBT, 0.1 mM Na_2EDTA, 2 mM riboflavin and 5-20 µL enzyme extract. Samples were illuminated for 5 min by a 150 W tungsten lamp (Osram *R 80*, Milan, Italy) and the absorbance at 560 nm was read against not illuminated samples. One unit of SOD was calculated as the amount of enzyme extract that gives half of the maximum inhibition.

Total phenols: Determination of total phenolic compounds was performed on methanolic extracts following the method reported by Nguyen and Niemeyer (2008). Briefly, extract (50 µL), deionised water (450 µL), Folin-Ciocalteu phenol reagent (250 µL), and 20 % sodium carbonate (1.25 mL) were added in a test tube, mixed, and allowed to incubate at room temperature for 20 min. Absorbance of the samples was then measured at 735 nm, and calculations were performed using a calibration curve prepared with gallic acid as standard.

Soluble phenolic acids (free and conjugated): Soluble free phenolic acids were extracted from leaves with 70 % methanol containing 1 % HCl for 4 h under continuous stirring. After centrifugation at 12100 × g for 15 min, the supernatant was collected and the extraction was repeated again twice on the pellet. Before analysis, extracts were passed through a 0.45 µm filter (Sartorius Minisart, Goettingen, Germany) to remove any suspended material. Qualitative and quantitative analyses were performed by RP-HPLC (Talcott and Howard 1999). Twenty µL of extract were injected into a HPLC system (Waters model *515*, Milford, MA, USA) fitted with a 3.9 mm × 150 mm Nova-Pak C18 column (Waters). Detection was at 280 nm using a Waters 2487 dual λ UV-Vis detector. Mobile phase A contained 98 % water and 2 % acetic acid, and mobile phase B contained 68 % water, 30 % acetonitrile and 2 % acetic acid. A linear gradient of 10-95 % mobile phase B was run for 70 min at 1 mL min^{-1}. The identity of the phenolic acids was confirmed by co-chromatography on HPLC with authentic standards (Sigma, St. Louis, MO, USA), and quantification was performed using a standard curve in the range of 0.2-2 µg of standard mixtures containing gallic, protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, caffeic, siringic, *p*-coumaric and ferulic acids. Chromatogram analysis was performed by the software Millennium 32 (Waters).

Soluble conjugated phenolic acids were obtained by hydrolysis of the methanol/HCl extract (400 µL) adding a solution (1:1 v/v) of 4 M NaOH containing 1 % AsA and 10 mM Na_2EDTA (Nardini *et al.* 2002). The mixture was incubated for 1 h at room temperature in the dark under a flux of nitrogen and then conc. HCl (160 µL) and ethylacetate (1 mL) were added. After centrifugation the supernatant was collected and transferred in a flask. The operation was repeated twice. The combined organic phases were vacuum dried, resuspended with 2 % acetic acid and injected into the HPLC system as reported above.

Statistical analysis: The homogeneity of variances for all the studied parameters was evaluated by Barlett's test. Results were expressed as means ± SE, standard error of means. The statistical analysis was carried out with the CoStat version 6.400 (CoHort Software). One-way analysis of variance was independently applied to the data to evaluate the B effect. For each cv. significant differences among the mean values were assessed on the basis of the least significant difference test at 0.05 level of

significance. When necessary, an arc sin or angular transformation was applied before statistical analysis was performed.

Results

After four weeks from B treatment, 'Merlot' leaves showed visible signs of injury represented by brownish necrotic areas, both diffused and like a spot (Fig. 1A and B). On the contrary, 'Sangiovese'-treated leaves did not show any difference compared to control (Fig. 1C and D). The phenological evidence of B accumulation in 'Merlot' was also reflected by the decrease in leaf dry weight (from 29.9 to 26.9 % in control and B-treated leaves, respectively). In contrast, 'Sangiovese' did not show any reduction maintaining its dry weight values about 23.6 %.

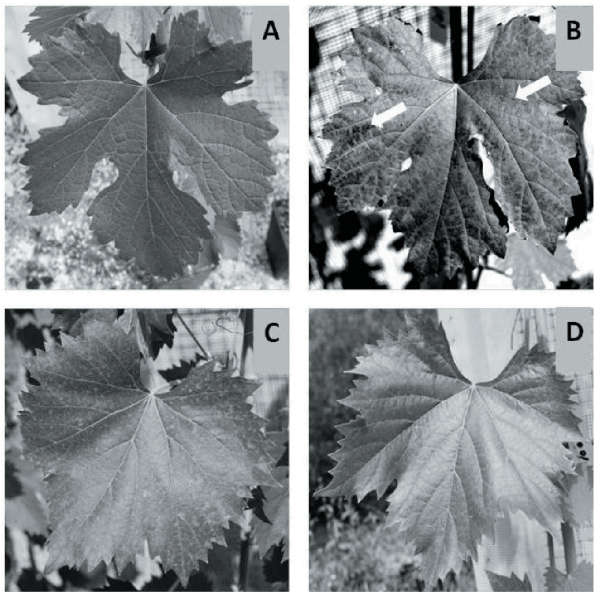

Fig. 1: Control and B-treated leaves of 'Merlot' (**A**, **B**) and 'Sangiovese' (**C**, **D**). The arrows indicate diffuse and spot-like necrotic areas caused by the treatment.

In both grapevine cvs. the treatment resulted in an increase in leaf B concentration compared to control plants (Fig. 2), 'Merlot' showing a much higher accumulation (about 27-fold) than 'Sangiovese' (about 6-fold). The untreated leaves of both cvs. did not evidence any difference in B concentration. Boron accumulation caused an enhancement of TBARS only in 'Merlot' (2-fold), indicating the establishment of a peroxidative damage at membrane level (Fig. 2).

In grapevine leaves H_2O_2 concentration was differently affected by B toxicity (Fig. 3). Whereas in control and treated 'Merlot' samples it did not show any change, in 'Sangiovese' B supply caused an increase in H_2O_2 concentration (+ 65 %). As regards SOD activity, the effects of B excess on grapevine leaves were opposite to those observed for H_2O_2. Indeed, the activity of the antioxidative enzyme decreased by 3.6-fold in 'Merlot' whereas remained constant in 'Sangiovese' (Fig. 3). The untreated leaves of the two cvs. presented values of SOD remarkably different between them (1.8-fold higher in 'Merlot').

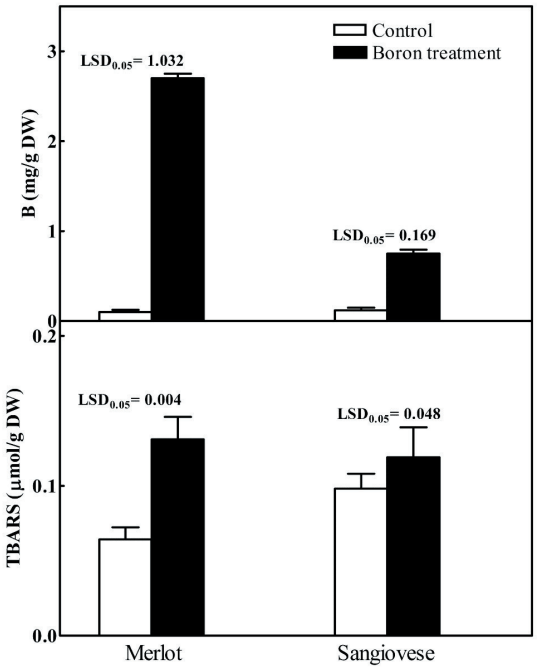

Fig. 2: Boron accumulation and thiobarbituric acid reactive substances (TBARS) concentration in grapevine leaves following B treatment. Data are means ± SE, n = 3. For each parameter ANOVA and the following least significance difference (LSD) were performed. The LSD values at the 0.05 P levels are shown, df = 5.

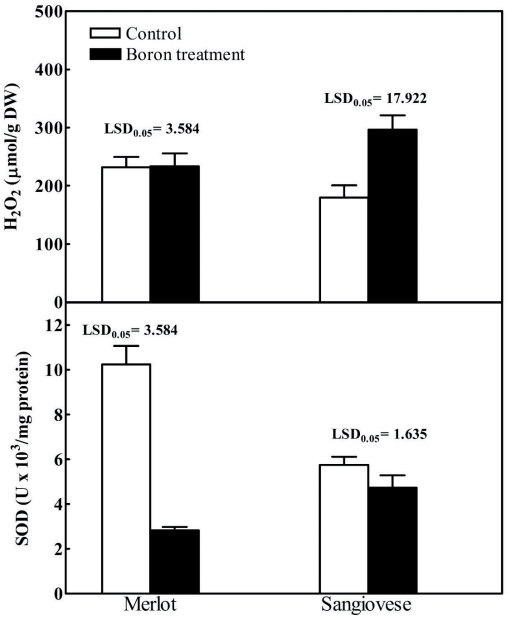

Fig. 3: Hydrogen peroxide concentration and superoxide dismutase (SOD) activity in grapevine leaves following B treatment. Data are means ± SE, n = 3. For other details see Fig. 2.

Total ascorbate (AsA + DHA) and AsA concentrations were enhanced in both cvs. following B addition (Fig. 4). In comparison with the controls, total ascorbate increased by 23 and 33 % in 'Merlot' and 'Sangiovese', respectively, whereas AsA concentration increased by 1.8 and 1.5-fold, respectively. Both control and treated leaves showed similar ascorbate values in the two cultivars. Boron toxicity resulted in a higher APX activity in 'Merlot' (+40 %) unlike

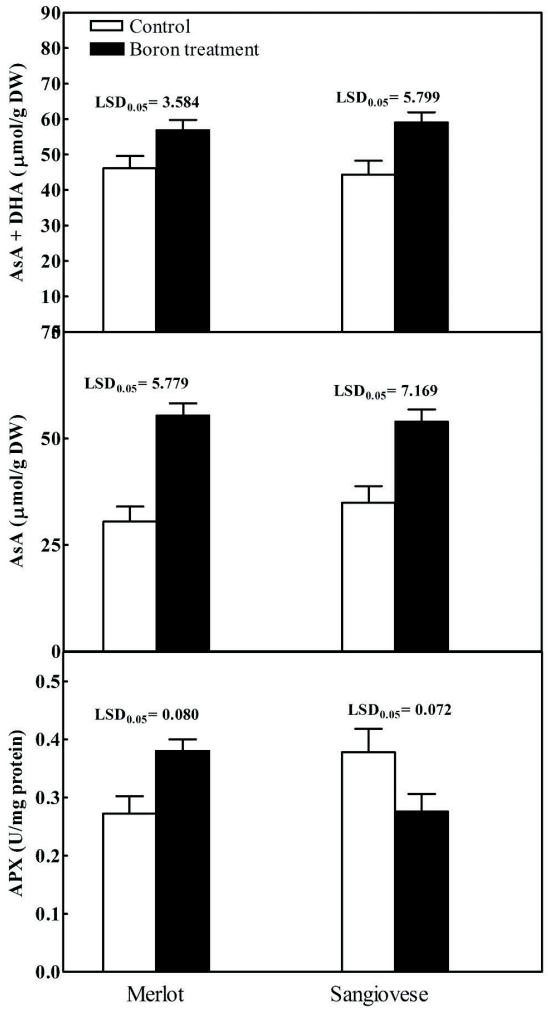

Fig. 4: Total ascorbate (AsA + DHA) and reduced ascorbate (AsA) concentrations, and ascorbate peroxidase activity (APX) in grapevine leaves following B treatment. Data are means ± SE, $n = 3$. For other details see Fig. 2.

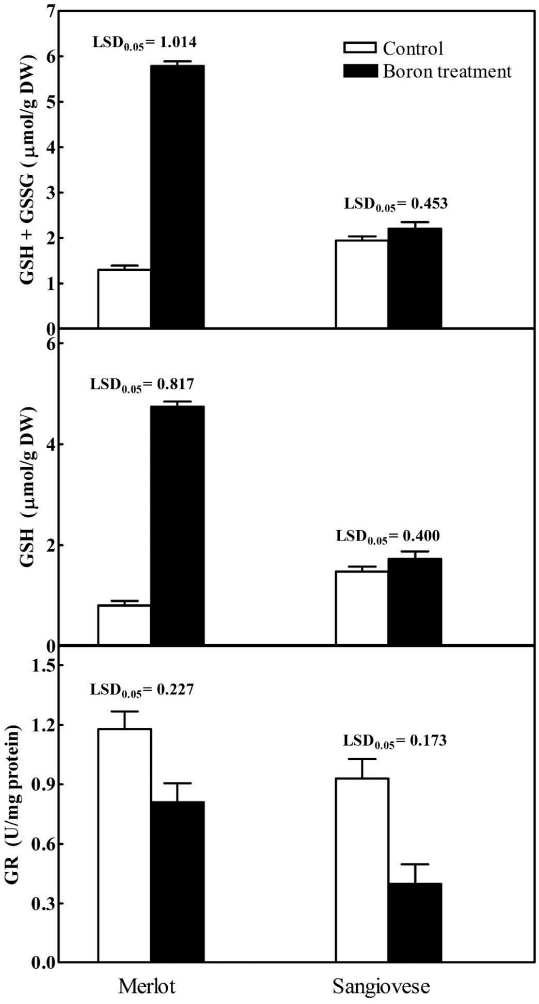

Fig. 5: Total glutathione (GSH+GSSG) and reduced glutathione (GSH) concentrations, and glutathione reductase activity (GR) in grapevine leaves following B treatment. Data are means ± SE, $n = 3$. For other details see Fig. 2.

'Sangiovese' in which APX activity was reduced by 27 % following B supply. The untreated leaves of this latter cv. showed a higher enzyme activity compared to the former one.

As for ascorbate, in the two cvs. the behaviour of total glutathione (GSH + GSSG) was the same of that observed for GSH, B treatment enhancing the concentrations in 'Merlot' (4.5 and 5.9-fold for GSH + GSSG and GSH, respectively) but not determining any change in 'Sangiovese' (Fig. 5). The accumulation of B in leaves reduced GR activity in both cvs. (31 and 57 % in 'Merlot' and 'Sangiovese', respectively).

Leaves of 'Merlot' plants grown in the presence of B excess showed a higher concentration of total phenols in comparison with untreated ones (Fig. 6), the increase being 26 %; in contrast, in 'Sangiovese' plants no significant increase could be observed following B excess. Soluble phenolic acids (free plus conjugated) did not show significant variations between control and treated samples in both cultivars. On average soluble phenolic acids represented about 5 and 7 % of total phenols in leaves grown in the presence or not of B excess. As shown in Tab. 1 almost all soluble free phenolic acids were represented by *p*-hy-

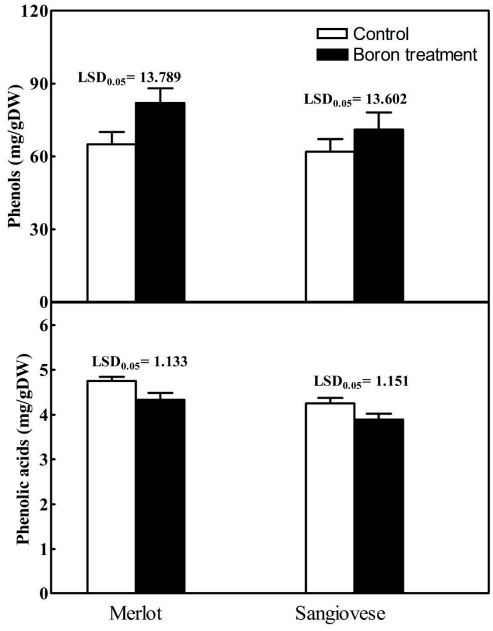

Fig. 6: Total phenol and phenolic acid concentrations in grapevine leaves following B treatment. Data are means ± SE, $n = 3$. For other details see Fig. 2.

Table 1

Soluble free phenolic acid composition (%) in leaves of 'Merlot' and 'Sangiovese' grown in the presence of B excess. Data are means ± SE, $n = 3$. For each parameter ANOVA and the following least significance difference (LSD) were performed. The LSD values at the 0.05 P levels are shown. M, 'Merlot'; S, 'Sangiovese'; tr, trace amounts; df, degrees of freedom

	M	M + B	LSD (df = 5)	S	S + B	LSD (df = 5)
Gallic acid	1.3 ± 0.1	3.0 ± 0.2	0.578	2.7 ± 0.2	6.6 ± 0.2	0.801
Protocatechuic acid	tr	0.7 ± 0.1		tr	tr	
p-OH-benzoic acid	98.0 ± 0.6	93.9 ± 0.5	2.156	96.7 ± 1.1	90.7 ± 1.1	4.534
Vanillic acid	tr	2.0 ± 0.1		tr	2.3 ±0.2	

Table 2

Soluble conjugated phenolic acid composition (%) in leaves of 'Merlot' and 'Sangiovese' grown in the presence of B excess. Data are means ± SE, $n = 3$. For each parameter ANOVA and the following least significance difference (LSD) were performed. The LSD values at the 0.05 P levels are shown. M, 'Merlot'; S, 'Sangiovese'; tr, trace amounts; df, degrees of freedom

	M	M + B	LSD (df = 5)	S	S + B	LSD (df = 5)
Gallic acid	1.9 ± 0.1	1.9 ± 0.1	0.358	1.2 ± 0.1	1.4 ± 0.2	0.661
Protocatechuic acid	tr	0.9 ± 0.1		0.9 ± 0.2	0.5 ± 0.1	0.717
p-OH-benzoic acid	2.5 ± 0.2	3.2 ± 0.1	0.507	3.1 ± 0.2	2.8 ± 0.1	0.717
Vanillic acid	tr	0.9 ± 0.1		0.5 ± 0.1	tr	
Chlorogenic acid	1.4 ± 0.1	2.2 ± 0.1	0.358	0.6 ± 0.2	0.7 ± 0.1	0.578
Caffeic acid	83.4 ± 1.1	78.6 ± 0.6	3.584	83.3 ± 1.1	82.6 ± 2.3	7.169
Syringic acid	3.6 ± 0.2	tr		tr	3.0 ± 0.1	
Coumaric acid	6.2 ± 0.6	9.7 ± 0.6	2.267	6.9 ± 0.3	7.4 ± 0.1	1.014
Ferulic acid	0.5 ± 0.2	2.4 ± 0.3	0.935	3.2 ± 0.3	1.5 ± 0.2	0.935

droxybenzoic acid (95 % on average) followed by gallic acid (about 3 %). Following B addition, *p*-hydroxybenzoic acid suffered a slight but significant decrease (4 and 6 % in 'Merlot' and 'Sangiovese', respectively) in favour of gallic and vanillic acids. The main component of soluble conjugated phenolic acids was caffeic acid (Tab. 2) which percentage was reduced under B supply in 'Merlot' (-6 %) but not in 'Sangiovese'. The other acids underwent different rearrangements depending on the cv. under observation: in treated 'Merlot' leaves coumaric and ferulic acids increased compared to the control, whereas in 'Sangiovese' ferulic acid was reduced.

Discussion

Despite the importance of nutritional disorders involved in B toxicity, mechanisms of B tolerance have not yet been understood (Cervilla *et al.* 2007). Usually, species and genotypes susceptible to B toxicity have higher concentrations of B in their leaves and shoots than tolerant ones (Ardic *et al.* 2009), and in the present experiment 'Merlot' behaved as a sensitive cv. Indeed, it accumulated more B in leaves and showed visible leaf burns in contrast to 'Sangiovese' (Figs. 1B and 2). Being the two *V. vinifera* cvs. grafted on the same rootstock (1103 P), tolerance mechanisms such as exclusion from roots and avoidance by means of a shallow root system cannot explain the different B accumulation. However, since B is transported to shoots through the transpiration stream (Marschner 1995), a different accumulation based on a higher B translocation rate in 'Merlot' can be suggested (Fig. 2).

Excess B operates inhibiting photosynthesis and decreasing CO_2 uptake (Eraslan *et al.* 2007, Han *et al.* 2009, Gimeno *et al.* 2012), conditions where electrons leakage towards molecular oxygen with generation of the superoxide radical (Sgherri *et al.* 1993) which starts a long chain of oxidative reactions leading to lipid peroxidation and oxidative damages. In this regard, B excess can be considered as an oxidative stress (Ardic *et al.* 2009), and we can classify tolerant genotypes as those which have a better ability to cope with B excess by inducing antioxidant defence systems. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage (Sudhakar *et al.* 2001). However, the antioxidant response to both abiotic (Pérez-López *et al.* 2009) and biotic (Sgherri *et al.* 2013) stresses is quite complex and a correct interpretation cannot be drawn without considering oxidative stress intensity (Sgherri and Navari-Izzo 1995). Indeed, literature reports a large number of different data regarding changes in the levels of antioxidant compounds and antioxidative enzymes, even if they are referred to the same species and the same type of stress. This variability does not agree with the general assumption that following the imposition of an oxidative stress there is an induction of the synthesis of antioxidants. For instance, this is not true anymore for high stress intensities (Sgherri *et al.* 1995). Thus, the only way to explain the different behaviour towards an oxidative stress, in dependence of the different cv. sensitivity, is to correlate data on antioxi-

dant levels with the concentration of the stress factor (B in our case) and the entity of oxidative damage (*i.e.* TBARS), namely to oxidative stress intensity. In 'Merlot' B excess increased TBARS content (Fig. 2), an indicator of oxidative stress and lipid peroxidation of membranes. The fact that TBARS values did not change in B-treated 'Sangiovese' compared to the control, together with the absence of leaf burns, suggests that 'Sangiovese' tolerated B stress better than 'Merlot', likely due to a 4-fold lower B accumulation in the leaves (Figs 1 and 2) with a consequent lower stress intensity.

Notwithstanding 'Sangiovese' did not show visible damages, an increased H_2O_2 production was observed in treated samples (Fig. 3) indicating that B accumulation was sufficient to induce an oxidative stress, which was lower in intensity than in 'Merlot' since 'Sangiovese' leaves did not show any increase in TBARS levels (Fig. 2). One of the first defence reactions against ROS involves the activity of SOD which dismutates superoxide radicals into H_2O_2 and oxygen, thus representing one of the most important steps in protecting cells from oxidative damage. In treated 'Sangiovese' leaves the increase in H_2O_2 may be related to the decrease in APX activity, SOD remaining at the control level (Figs 3 and 4). In the absence of membrane injury it may be hypothesized that superoxide radicals, likely formed as a consequence of an enhanced B concentration, could have been scavenged also through a direct removal by some antioxidant compounds such as AsA (Fig. 4), not including necessarily the increase in SOD transcription or up-regulation. The importance of SOD in B tolerance was also suggested by the 4-fold decrease of SOD activity in stressed 'Merlot' leaves which suffered much higher stress intensity. Moreover, although control 'Merlot' leaves showed a double constitutive SOD activity compared to 'Sangiovese', following stress 'Merlot' presented a 40 % reduction of SOD compared to 'Sangiovese' (Fig. 3). In this case superoxide radicals, not being sufficiently dismutated, could have interacted with H_2O_2 with the consequent formation of the highly reactive hydroxyl radicals and the induction of lipid peroxidation (Fig. 2), hydroxyl radicals being the main responsible for oxygen toxicity in the cell (Azevedo *et al.* 2005).

Hydrogen peroxide is considered as signal molecule in the environmental stress response inducing the expression of a variety of defence genes when mild and high stress intensities depress antioxidant enzyme activities (Sgherri and Navari-Izzo 1995, Sgherri *et al.* 2007). In this study the increase in H_2O_2 in 'Sangiovese' was not sufficient to induce or sustain APX activity (Fig. 4). However, it should be taken in mind that damages to tissue occur when the capacity of detoxification of the antioxidative systems becomes lower than the rate of ROS production and in 'Sangiovese' the maintained SOD activity, together with the enhanced AsA pool, was sufficient to protect leaf cells from B toxicity without an increase in the glutathione levels. In contrast, notwithstanding 'Merlot' increased APX activity and accumulated AsA and GSH, likely contrasting the enhancement in H_2O_2 level, damages were observed (Figs 4 and 5). The decrease in GR activity (Fig. 5) in the leaves of 'Merlot' did not limit the reduction state of the glutathione pool, even if the cellular thiol-disulfide redox status is commonly altered under conditions of oxidative stress. This was likely due to an increase in glutathione synthesis. In fact, the initial oxidation of GSH to GSSG following the imposition of a stress is generally the trigger for the induction of glutathione synthesis as the release of feedback inhibition of GSH synthesis is involved (Smith 1985).

The antioxidant defence system of the cell is also represented by phenolic acids which, either directly or through the peroxide/phenol/ascorbate cycle of the vacuole or apoplast, can detoxify ROS and, in particular, H_2O_2. As previously observed in *Raphanus sativus* (Sgherri *et al.* 2003), both in 'Merlot' and in 'Sangiovese' AsA could have reduced phenoxyl radicals formed following the scavenging of H_2O_2 by a peroxidase, thus replenishing phenolic acids whose level was maintained to that of control plants. (Fig. 6). Free phenolic acid composition confirmed what was previously observed in *V. vinifera* cv. Trebbiano (Sgherri *et al.* 2013) where the most represented phenolic acid was *p*-hydroxybenzoic acid. However, the similar small changes in free phenolic acid composition in both cvs. (Tab. 1) suggest that these molecules did not have a role in explaining the different tolerance to B toxicity even if soluble conjugated phenolic acids could have some effects in the protection of 'Merlot' from oxidative stress (Tab. 2). In fact, despite a general unchanged composition in 'Sangiovese', in 'Merlot' protocatechuic, *p*-hydroxybenzoic and chlorogenic acids increased at the expense of caffeic acid which was utilised following B treatment (Tab. 2). The above phenolic acids showed to play a particular role during dehydration and rehydration of the resurrection plant *Ramonda serbica* (Sgherri *et al.* 2004), and caffeic acid is considered to be a more powerful antioxidant than the hydroxyl-derivatives of benzoic acid such as *p*-hydroxybenzoic acid (Rice-Evans *et al.* 1996).

Conclusions

In conclusion, *V. vinifera* 'Merlot' showed a lower ability to limit B accumulation in leaves than 'Sangiovese'. As a consequence, 'Merlot' evidenced leaf burns and membrane damages, both being symptoms of a higher oxidative stress. Moreover, even if antioxidative defence mechanisms were generally activated in 'Merlot', the dramatic decrease in SOD activity, in contrast to 'Sangiovese' where it was maintained at control level, could have played a key role in the generation of oxidative stress which was counteracted in 'Sangiovese'.

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