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Volatile organic compounds characterized from grapevine (*Vitis vinifera* L. cv. Malbec) berries increase at pre-harvest and in response to UV-B radiation

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ABSTRACT

Ultraviolet-B solar radiation (UV-B) is an environmental signal with biological effects in plant tissues. Recent investigations have assigned a protective role of volatile organic compounds (VOCs) in plant tissues submitted to biotic and abiotic stresses. This study investigated VOCs in berries at three developmental stages (veraison, pre-harvest and harvest) of *Vitis vinifera* L. cv. Malbec exposed (or not) to UV-B both, in *in vitro* and field experiments. By Head Space-Solid Phase Micro Extraction–Gas Chromatography–Electron Impact Mass Spectrometry (HS-SPME–GC–EIMS) analysis, 10 VOCs were identified at all developmental stages: four monoterpenes, three aldehydes, two alcohols and one ketone. Monoterpenes increased at pre-harvest and in response to UV-B in both, *in vitro* and field conditions. UV-B also augmented levels of some aldehydes, alcohols and ketones. These results along with others from the literature suggest that UV-B induce grape berries to produce VOCs (mainly monoterpenes) that protect the tissues from UV-B itself and other abiotic and biotic stresses, and could affect the wine flavor. Higher emission of monoterpenes was observed in the field experiments as compared *in vitro*, suggesting the UV-B/PAR ratio is not a signal in itself.

1. Introduction

Though ultraviolet-B radiation (UV-B; wavelength 280–315 nm) represents only a small portion of the solar spectrum reaching the Earth's surface, it has large biological effects. It can activate the plant defense system producing secondary metabolites in different tissues (Teramura, 2006), like leaves (Berli et al., 2010, 2013; Gil et al., 2012) and berries (Berli et al., 2011) of grapevine. Environmental UV-B levels are mainly regulated by cloudiness, season and latitude, but also increase with altitude as UV-B passes through a thinner atmosphere to reach the ground (McKenzie et al., 2003).

The effect of UV-B in tissues depends on the context in which UV-B treatments are given. That is, other environmental factors such as total radiation, temperature, water and nutritional status can interact with UV-B effects (Caldwell et al., 2003; Frohnmeyer and Staiger, 2003; Kakani et al., 2003). In general, lesser effects are observed when plants are submitted to contrasting UV-B situations under field trials than in controlled environmental conditions (Caldwell et al., 2003). It has been shown that under UV-B/PAR ratios higher than those found in natural conditions, generally in experiments performed in growth chambers with reduced photosynthetic active radiation (PAR), UV-B effects can be exaggerated (Allen et al., 1998; Björn, 1996), presumably because environmental PAR induce protective and repairing mechanisms that reduce UV-B damages (Jordan et al., 1992). In previous studies with *in vitro* grown grapevine plants related to gene expression (Pontin et al., 2010) and terpene metabolism (Gil et al., 2012), it has been found that under the same UV-B dose, relatively high UV-B irradiation (0.33 W m^{-2} during 4 h) triggered mechanisms of defense, while acclimation responses were induced by relatively low UV-B irradiation (0.08 W m^{-2} during 16 h). In Mendoza, Argentina's main wine producing region, the most reputed vineyards are located at high altitudes (*ca.* 1500 m a.s.l), where exposition to high levels of solar UV-B produce berries of high quality for winemaking by inducing synthesis of polyphenols (Berli et al., 2008, 2011). However, little is known regarding to production of volatile organic compounds (VOCs) purportedly involved in defense and acclimation of grapevine tissues (Gil et al., 2012; Pichersky and Gershenzon, 2002) and in the wine "bouquet" (Coelho et al., 2006; Palomo et al., 2007).

Plants synthesize and emit a large variety of VOCs with terpenoids and fatty-acid derivatives as the dominant classes (Pichersky and Gershenzon, 2002). In grapevine, the evolution of these

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aromatic metabolites at the level of reproductive tissues has been explored mainly in "floral" varieties like Muscat (Coelho et al., 2006; Palomo et al., 2007; Park et al., 1991). This is a biologically important aspect since VOCs can attract pollinators and may also protect reproductive tissues against pathogens (Pichersky and Gershenzon, 2002; Lücker et al., 2004; Kegge and Pierik, 2010). Different terpenoids VOCs (monoterpenes and sesquiterpenes) with antimicrobial activity have been found in *in vitro* cultured grapevine (Escoriaza et al., 2013). VOCs may also have a plant protective function towards abiotic stresses due to antioxidant activity that scavenge free radicals so reducing oxidative damage as it has been proposed by Aharoni et al. (2003) and Wei and Shibamoto (2007), as well as to induce integrity and stability of membranes (Beckett et al., 2012). An additional important aspect is that VOCs may be involved in the wine flavor (Coelho et al., 2006; Fang and Qian, 2005; Palomo et al., 2007).

In grapevine, the time elapsed between veraison (the moment in which the seeds become physiologically mature) and late ripening (when the fruit is harvested for winemaking) influences the berry composition, mainly regarding their varietal characteristics (Gómez et al., 1995). Luan and Wüst (2002) showed that the monoterpenes are synthesized in exocarp and mesocarp of the berry via the plastidial methyl erythritol phosphate pathway (MEP; Litchtenthaler, 1999). During berry development VOCs (mainly monoterpenes) fluctuate according to the environmental conditions (Peyrot des Gachons et al., 2005; Roujou de Boubée et al., 2000). Although monoterpenes, along with other VOCs such as aldehydes, alcohols and ketones are components of the aroma in grape berries (Girard et al., 2002; Mateo and Jiménez, 2000), no information is currently available about the effect of UV-B in VOCs composition of grape berries.

In this work, the evolution of VOCs at veraison, pre-harvest and harvest of grape berries exposed to different UV-B regimes were monitored by Head Space Solid Phase Micro Extraction–Gas Chromatography–Electron Impact Mass Spectrometry (HS-SPME–GC–EIMS). Field grown *Vitis vinifera* L. cv. Malbec plants of a high altitude (1450 m a.s.l.) vineyard in Mendoza, Argentina, were exposed (or not) to solar UV-B, from 15 days before flowering until harvest; also field grown clusters were challenged *in vitro* with one "field-like" dose of UV-B administered at two different intensities (low and high), in comparison with a treatment in which UV-B was filtered.

2. Results

2.1. Identification of VOCs in berries

Monoterpenes, aldehydes, alcohols and ketones were identified by HS-SPME–GC–EIMS in berries at the three developmental stages

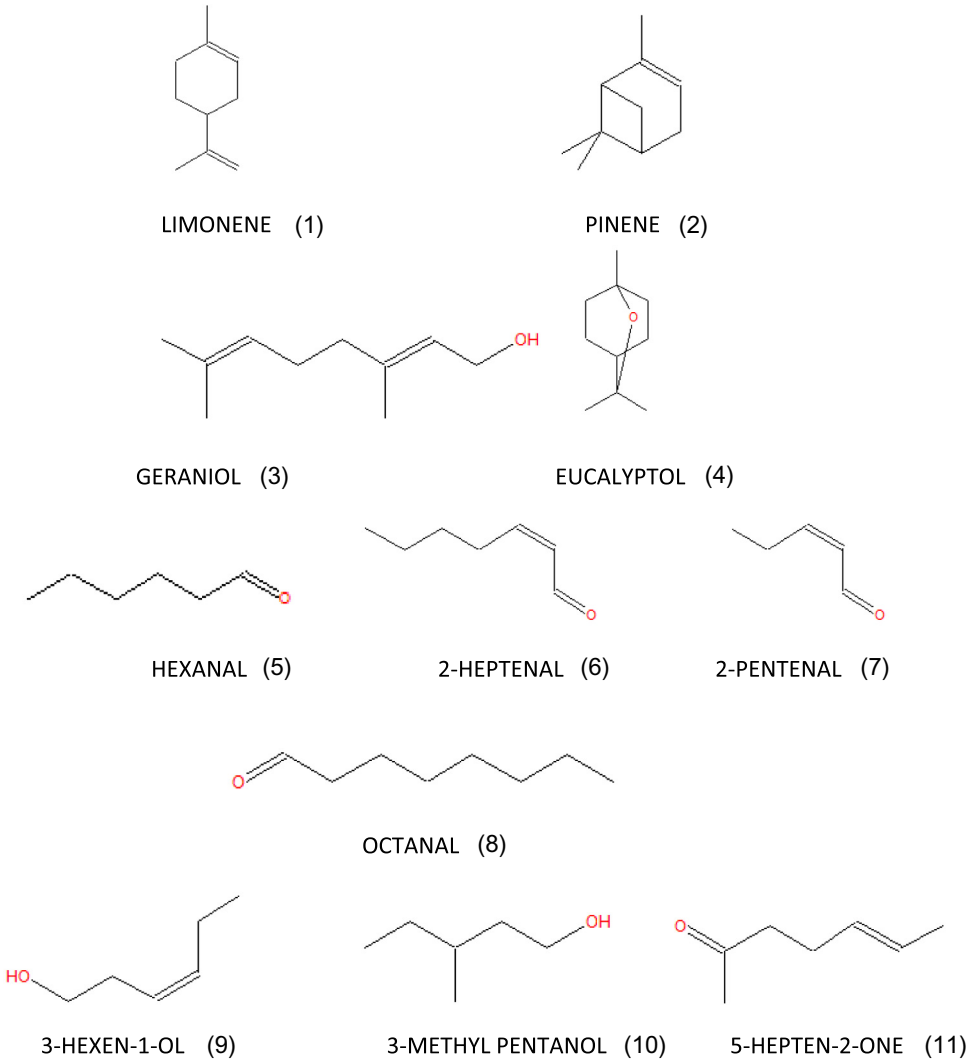

Fig. 1. Chemical structures of hydrocarbons monoterpenes, oxygenated monoterpenes, aldehydes, alcohols and ketone identified by SPME–GC–EIMS in grape berries.

analyzed (veraison, pre-harvest and harvest) in both, *in vitro* and field experiments (Fig. 1). Two hydrocarbon monoterpenes ($C_{10}H_{16}$; limonene (**1**) and pinene (**2**)), two oxygenated monoterpenes ($C_{10}H_{18}O$; geraniol (**3**) and eucalyptol (**4**)), three aldehydes (hexanal (**5**), 2-heptenal (**6**) and 2-pentenal (**7**)), two alcohols (3-hexen-1-ol (**9**) and 3-methyl pentanol (**10**)) and the ketone 5-hepten-2-one (**11**), were characterized in berries at mostly all developmental stages from the *in vitro* experiment (for full mass spectra see Figs. 1 and 2 of Supplementary material). In the field experiment the same VOCs were identified, plus the aldehyde octanal (**8**) which was not detected in the *in vitro* treatments, and that eucalyptol (**4**) and pinene (**2**) were only found in pre-harvest.

2.2. UV-B increases some terpenes during berry development

In berries treated *in vitro* with different UV-B levels (no UV-B, −UV-B; low intensity, +UV-B and high intensity, ++UV-B) the higher levels of limonene (**1**) were detected at pre-harvest, followed by harvest and veraison (Fig. 2a). At veraison and pre-harvest, levels of limonene (**1**) were increased in the +UV-B treatment as compared to ++UV-B, although the differences disappeared at harvest. Berries of +UV-B treatment showed a twofold increase in limonene (**1**) content as compared to the −UV-B treatment in the three different developmental stages. Geraniol (**3**) content was augmented under +UV-B (although only at $P \leqslant 0.07$) being pre-harvest the developmental stage in which the largest increases were recorded, but then the differences disappeared towards harvest (Fig. 2b). Eucalyptol (**4**) and pinene (**2**) levels did not show differences between UV-B treatments and berry developmental stages, and they were only identified at veraison and pre-harvest but not at harvest (data not shown).

In berries of the field experiment, limonene (**1**) levels were increased only at pre-harvest, and it was 2.7-fold higher in the ++UV-B treatment than in the −UV-B (Fig. 3a). In addition, ++UV-B increased geraniol (**3**) levels as compared to −UV-B at veraison and pre-harvest, and then decreased at harvest (Fig. 3b). The other monoterpenes identified in the berries subjected to field UV-B treatments were eucalyptol (**4**) and pinene (**2**); these were detected only at pre-harvest and, while eucalyptol (**4**) levels showed threefold higher under ++UV-B as compared to −UV-B (0.735 ng g^{-1} berries FW and 0.20 ng g^{-1} berries FW, respectively), pinene (**2**) levels did not show differences (data not shown).

It is noticeable that the absolute values of limonene (**1**) in field experiments exceeded 10-times those found *in vitro* (Figs. 2 and 3).

2.3. UV-B effects on aldehydes, alcohols and ketones during berry ripening

In berries treated *in vitro* with different UV-B irradiances hexanal (**5**) was the most abundant VOC, being 200-fold higher than

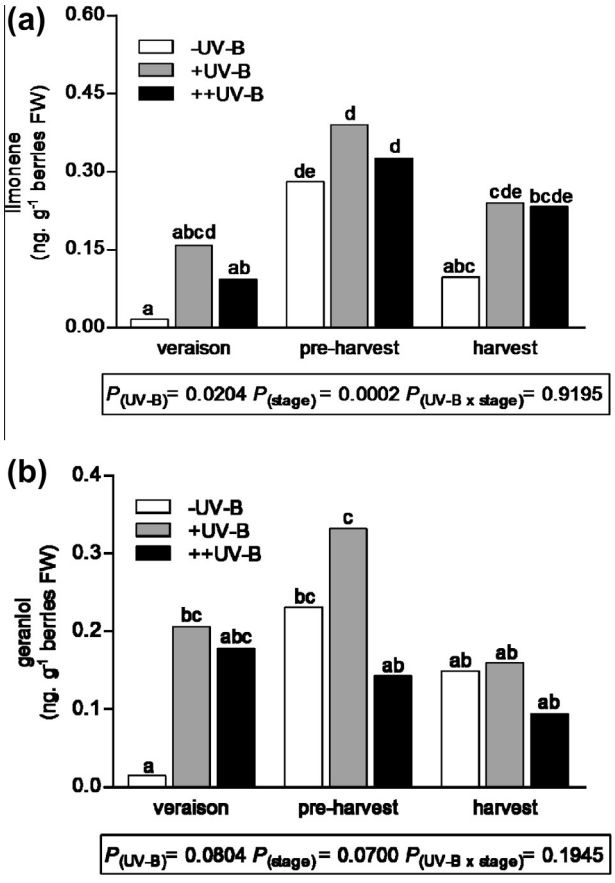

Fig. 2. Limonene (**1**) (a) and geraniol (**3**) (b) levels assessed by SPME–GC–EIMS (ng g^{-1} berries FW), in grape berries subjected to *in vitro* treatments. UV-B treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low intensity (+UV-B, 0.08 W m^{-2} during a 16 h per day photoperiod), high intensity (++UV-B, 0.33 W m^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a treatment in which UV-B was filtered (−UV-B). Determinations were done at three developmental stages (veraison, pre-harvest and harvest). $P_{(UV\text{-}B)}$: UV-B effect; $P_{(stage)}$: berry developmental stage effect; $P_{(UV\text{-}B\times stage)}$: UV-B × berry developmental stage interaction effect. Values are means ± SE, $n = 3$.

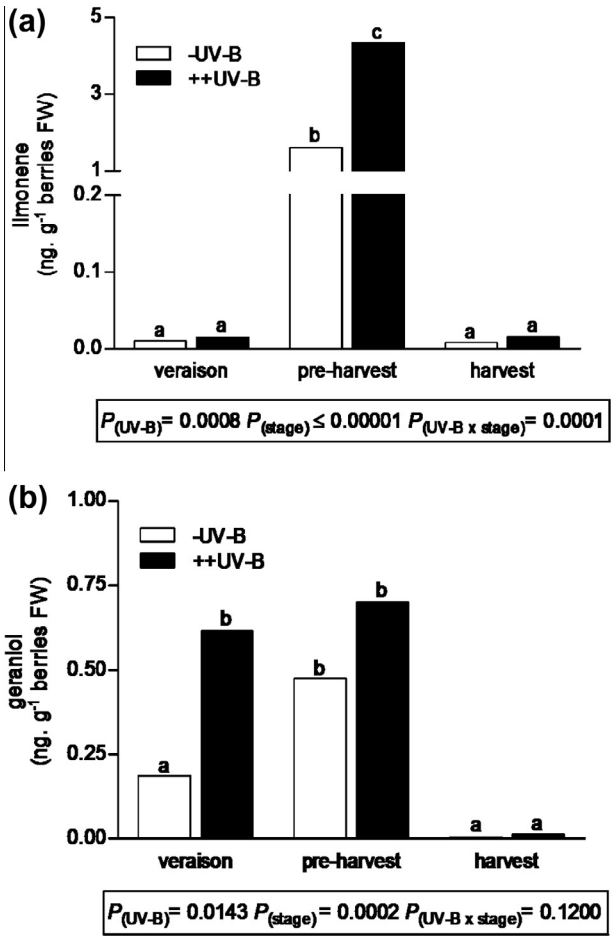

Fig. 3. Limonene (**1**) (a) and geraniol (**3**) (b) levels assessed by SPME–GC–EIMS (ng g^{-1} berries FW), in grape berries exposed or not to solar UV-B (++UV-B and −UV-B, respectively) in the field experiment at three developmental stages (veraison, pre-harvest and harvest). $P_{(UV\text{-}B)}$: UV-B effect; $P_{(stage)}$: berry developmental stage effect; $P_{(UV\text{-}B\times stage)}$: UV-B × berry developmental stage interaction effect. Values are means ± SE, $n = 3$.

the alcohols and the ketone (Figs. 4 and 6). The highest levels were observed at harvest under ++UV-B, where hexanal (**5**) augmented twofold as compared to the −UV-B and +UV-B treatments (Fig. 4a). UV-B had no effect on 2-heptenal (**6**) and 2-pentenal (**7**) levels, which increase at harvest (Fig. 4b), while the level of 2-pentenal (**7**) decreased from veraison towards harvest (Fig. 4c).

In berries from the field experiment hexanal (**5**) was also the most abundant VOC found (*ca.* 200-fold, Figs. 5 and 7), with no significant differences between UV-B treatments or along the developmental stages (Fig. 5a). The levels of 2-heptenal (**6**) under ++UV-B respect to −UV-B were twofold at veraison, without differences at pre-harvest, and then reached up to 2.7-fold at harvest (Fig. 5b). The VOC 2-pentenal (**7**) attained the higher levels at veraison and pre-harvest, decreasing then at harvest (Fig. 5c). The levels of octanal (**8**) (compound not detected in the *in vitro* experiment) was not affected by UV-B and reached the higher values at pre-harvest (Fig. 5d).

Two volatile C6 alcohols, the 3-hexen-1-ol (**9**) and 3-methyl pentanol (**10**), and the ketone 5-hepten-2-one (**11**) were identified by HS-SPME–GC–EIMS analysis from both, the *in vitro* and the field experiments (Figs. 6 and 7). In berries from the *in vitro* experiment the levels of 3-hexen-1-ol (**9**) were only affected by the developmental stages without significant differences among UV-B treatments (Fig. 6a). Their values decreased from veraison to pre-harvest and then increased at harvest. The levels of the most abundant alcohol, 3-methyl pentanol (**10**), were not affected by UV-B, and their highest levels were detected at veraison, but then decreased towards harvest (Fig. 6b). In contrast to alcohol levels, the ketone was affected by UV-B treatments but not by the berry developmental stage. Under ++UV-B, 5-hepten-2-one (**11**) levels were increased threefold respect to the other UV-B conditions (Fig. 6c).

In berries from the field experiment, the 3-hexen-1-ol (**9**) levels were increased at veraison and harvest by ++UV-B when compared to −UV-B, with no differences between UV-B treatments at pre-harvest (Fig. 7a). In contrast, the 3-methyl pentanol (**10**) levels were affected by the berry developmental stage, with higher values at harvest, without being affected by the UV-B treatments (Fig. 7b). The 5-hepten-2-one (**11**) levels under ++UV-B increased 3.8-fold at veraison and twofold at harvest when compared to −UV-B. However, they decreased from veraison towards pre-harvest and harvest (Fig. 7c).

2.4. Principal component analysis (PCA) of VOCs

For an overall interpretation of the results obtained, PCA analyses were performed. Fig. 8 shows the *biplot* graph for berries in the *in vitro* experiment. The matrix for the analysis consisted of nine cases corresponding to the combination of the three UV-B regimes (−UV-B, +UV-B and ++UV-B) and three berry developmental stages (veraison, pre-harvest and harvest), and 10 variables (VOCs assessed by HS-SPME–GC–EIMS). PC1 explained 36.8% of the variance of the remaining variables and associated the monoterpenes limonene (**1**), geraniol (**3**) and pinene (**2**) with pre-harvest, while eucalyptol (**4**) was associated with pre-harvest and veraison, not being affected by UV-B treatment. PC2 explained 33.2% of the variance and separated the aldehydes 2-heptenal (**6**) and hexanal (**5**) along with 5-hepten-2-one (**11**) associated with harvest, where hexanal (**5**) and 5-hepten-2-one (**11**) were also associated with ++UV-B. The alcohol 3-hexen-1-ol (**9**) was associated with veraison and harvest, the 3-methyl pentanol (**10**) and the aldehyde 2-pentenal (**7**) were associated with veraison, and none of them were affected by UV-B. A *biplot* graphic was made for the field experiment (Fig. 9) with six cases for the combination of the two UV-B treatments (−UV-B and ++UV-B) and three berry developmental stages, and 11 variables (VOCs assessed by HS-SPME–GC–EIMS). PC1 explained 52.2% of the variance and separates the pre-harvest time of the other developmental stages, associating monoterpenes and aldehydes to pre-harvest. Limonene (**1**), geraniol (**3**), eucalyptol (**4**) and pinene (**2**) levels were associated with pre-harvest and ++UV-B, where geraniol (**3**) was also related to veraison. The aldehydes 2-pentenal (**7**) and 2-heptenal (**6**), and the ketone 5-hepten-2-one (**11**) were related to veraison, the latter two being associated with ++UV-B. PC2 explains 24.7% of the variance, where the alcohol

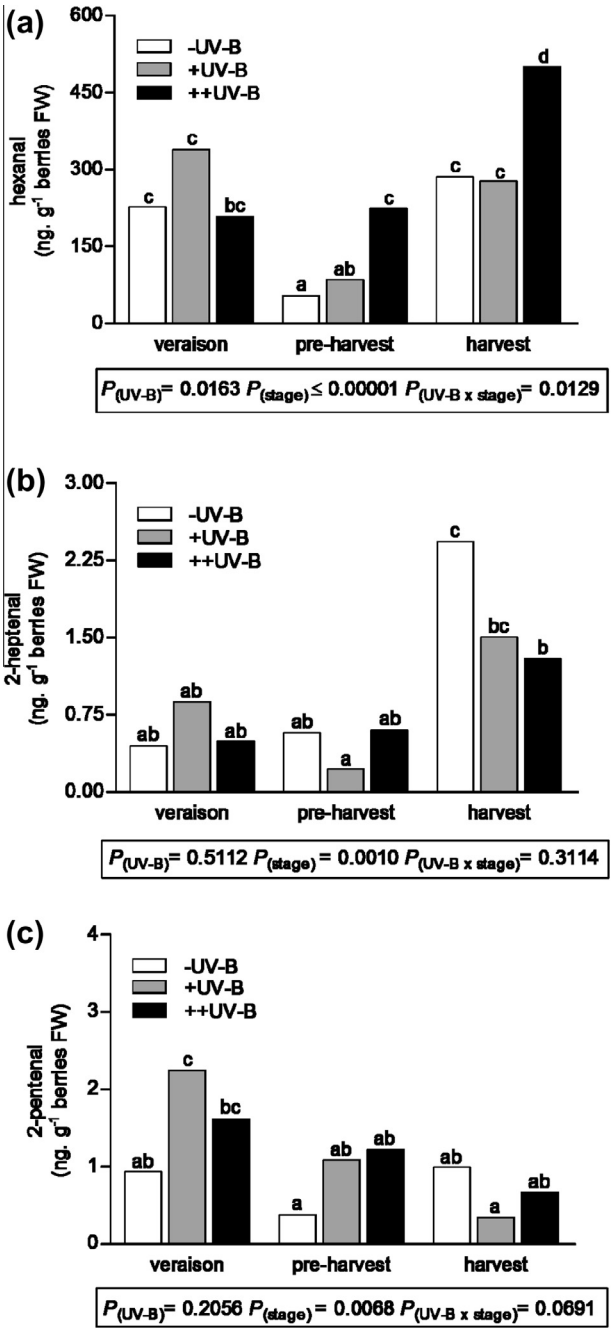

Fig. 4. Aldehyde levels assessed by SPME–GC–EIMS (ng g^{-1} berries FW). Hexanal (**5**) (a), 2-heptenal (**6**) (b) and 2-pentenal (**7**) (c) in grape berries subjected to *in vitro* treatments. UV-B treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low intensity (+UV-B, 0.08 W m^{-2} during a 16 h per day photoperiod), high intensity (++UV-B, 0.33 W m^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a treatment in which UV-B was filtered (−UV-B). Determinations were done at three developmental stages (veraison, pre-harvest and harvest). $P_{(UV\text{-}B)}$: UV-B effect; $P_{(stage)}$: berry developmental stage effect; $P_{(UV\text{-}B\times stage)}$: UV-B × berry developmental stage interaction effect. Values are means ± SE, $n = 3$.

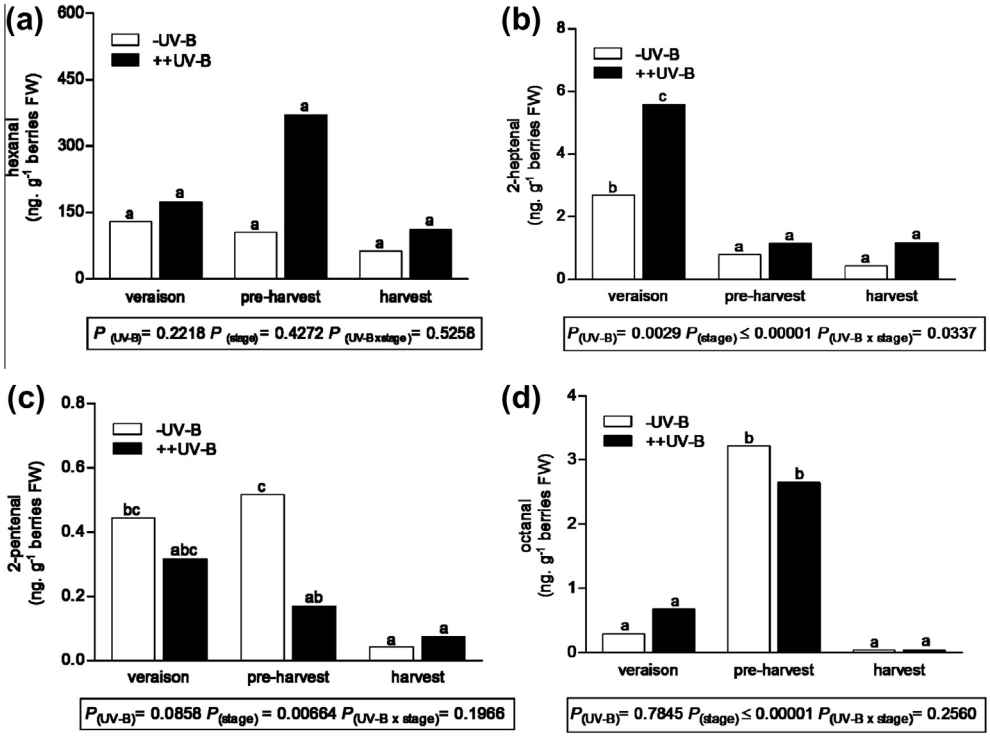

Fig. 5. Aldehyde levels assessed by SPME–GC–EIMS (ng g^{-1} berries FW). Hexanal (**5**) (a), 2-heptenal (**6**) (b), 2-pentenal (**7**) (c) and octanal (**8**) (d) in grape berries exposed or not to solar UV-B (++UV-B and −UV-B, respectively) in the field experiment at three developmental stages (veraison, pre-harvest and harvest). $P_{(UV\text{-}B)}$: UV-B effect; $P_{(stage)}$: berry developmental stage effect; $P_{(UV\text{-}B\times stage)}$: UV-B × berry developmental stage interaction effect. Values are means ± SE, $n = 3$.

3-methyl-pentanol (**10**) was associated with harvest and the 3-hexen-1-ol (**9**) with veraison and harvest. The aldehydes hexanal (**5**), octanal (**8**) and 2-pentenal (**7**) were related to −UV-B treatment.

3. Discussion

The VOCs identified and measured from grape berries in the present work coincide with those reported in the literature, where amongst the oxygenated monoterpenes geraniol (**3**) is considered one of the major free aromatics (Ribéreau-Gayon et al., 2006). Limonene (**1**) and pinene (**2**) have been previously found in the mesocarp and exocarp of grape berries (Carballeira Lois et al., 2001; Coelho et al., 2006), while eucalyptol (1,8-cineole) (**4**) is a derivative of limonene (**1**) and the two (eucalyptol (**4**) and limonene (**1**)) are associated with the fragrance of eucalyptus in red wines (Jackson, 2000). However, it is unclear whether these terpenes are potential precursors of aromatic compounds in winemaking, or are just end products due to high natural enzyme activity in this particular stage of development or in response to induced mechanisms of defense (Kalua and Boss, 2009). It has been reported that methyl jasmonate (as a signal of stress) promotes the synthesis and accumulation of terpenes in cell cultures of grape berries (Hampel et al., 2005). In addition, Deluc et al. (2009) showed that the isoprenoid metabolism in grape berries may be enhanced by water restriction. Likewise monoterpenes were higher in bunches exposed to sunlight as compared with those in the shadow (Reynolds and Wardle, 1989). In general, most grape varieties contain free and glycoside-bound terpenes (Mateo and Jiménez, 2000) and the combination of the aroma of each compound determines the wine bouquet (Clarke and Bakker, 2004). In fact, geraniol (**3**) has been found in Pinot noir wines contributing to floral and cherry flavors (Fang and Qian, 2005). This data agreed with our experiments where both limonene (**1**) and geraniol (**3**) were minimal at harvest suggesting conjugation or degradation. Some studies have demonstrated that monoterpene aroma compounds are favored by cool weather and high altitude (Rapp, 1998; Reynolds and Wardle, 1997). Winter (2002) suggests that light and temperature are important for aroma development because both drive leaf photosynthesis and sugar production. Reynolds and Wardle (1997) found that fully sun exposed fruit of the cv. Gewurztraminer had higher content of free volatile terpenes than those partially exposed or shaded. However, other experiments have shown that monoterpenes decrease with excess of exposure to sunlight (Reynolds et al., 1996). Such decrease could be caused by the destruction of some molecules through UV radiation associated with overexposure (Winter, 2002). However, the results in *in vitro* conditions at high irradiances did not correspond with those from Winter (2002) since limonene (**1**) and geraniol (**3**) concentrations were 10-fold and twofold higher, respectively, as compared with minus UV-B. Moreover, stimulation in the biosynthesis of terpenes by neighboring, herbivory, wounding or oxidative stress has been reported (Arimura et al., 2005; Beaulieu, 2007; Kegge and Pierik, 2010). Loreto et al. (2004) have suggested that isoprenoid VOCs may have antioxidant properties and their synthesis might be stimulated by stress-inducing conditions. Complementary, UV-B signaling may also trigger acclimation responses that prepare the tissues to cope with higher UV-B irradiances and/or other stresses (Gil et al., 2012; Pontin et al., 2010). Many monoterpene VOCs may rapidly combine with reactive oxygen species (ROS) as stated by Calogirou et al. (1999), and their emissions stimulated by high light and temperature conditions (Delfine et al., 2000; Duhl et al., 2008). Since UV-B stimulates the generation of ROS (Berli et al., 2010) these signals (ROS) may also trigger the formation of monoterpenes. In addition, UV-B activates synthesis of terpenoid antioxidant compounds that protect tissues from oxidative damage (Graßmann et al., 2005; Lee et al., 2005). In previous experiment with leaves of *in vitro* grown plants of cv. Malbec, it was found that the same monoterpenes characterized in the present work with

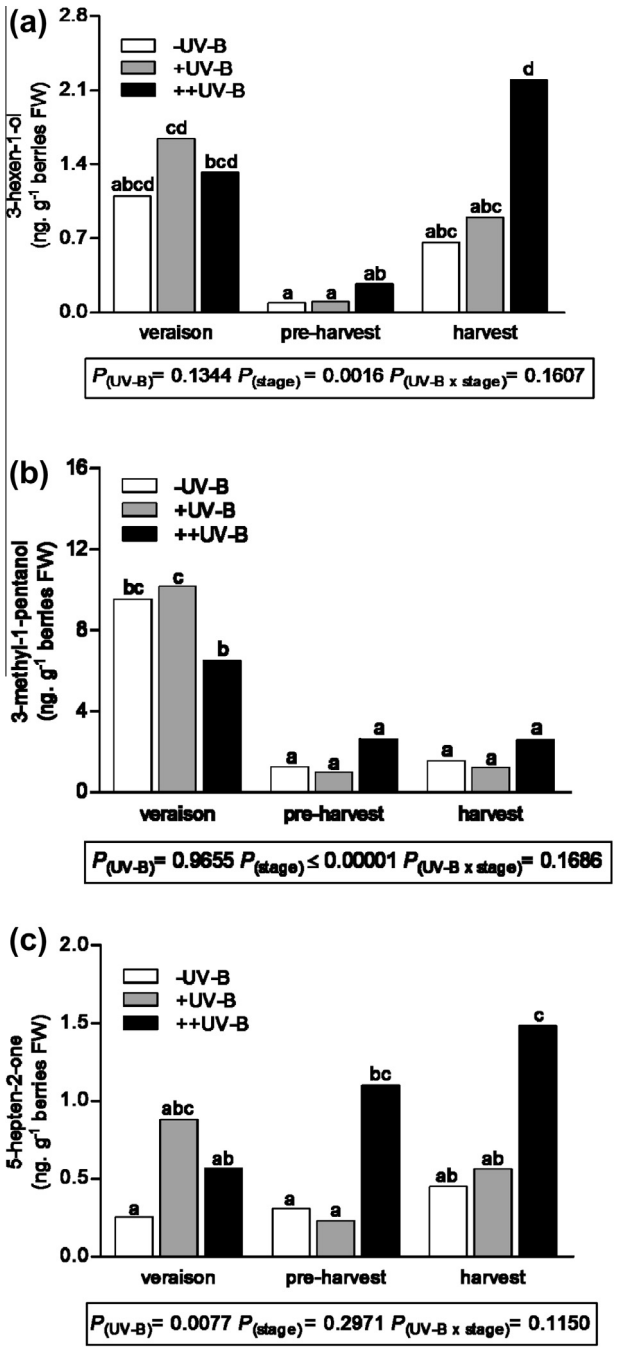

Fig. 6. Alcohols and ketone levels assessed by SPME–GC–EIMS (ng g^{-1} berries FW). 3-Hexen-1-ol (**9**) (a), 3-methyl pentanol (**10**) (b) and 5-hepten-2-one (**11**) (c) in grape berries subjected to *in vitro* treatments. Treatments: one dose (4.75 kJ m^{-2} - d^{-1}) of low intensity (+UV-B, 0.08 W m^{-2} during a 16 h per day photoperiod), high intensity (++UV-B, 0.33 W m^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a treatment in which UV-B was filtered (−UV-B). Determinations were done at three developmental stages (veraison, pre-harvest and harvest). $P_{(UV\text{-}B)}$: UV-B effect; $P_{(stage)}$: berry developmental stage effect; $P_{(UV\text{-}B\times stage)}$: UV-B × berry developmental stage interaction effect. Values are means ± SE, $n = 3$.

berries increased under UV-B (Gil et al., 2012). That is, the response to UV-B radiation was similar for both tissues (leaves and berries), although the absolute values of limonene (**1**) in field experiments exceeded 10-times those found *in vitro*. It is also important to notice that the same metabolites were assessed in different tissues both *in vitro* and in field conditions (commercial vineyard). The relevance of the latter is indubitably since it has been reported that UV-B treatments in a poor PAR background may give exaggerated results (Allen et al., 1998; Björn, 1996), and also the interaction with other environmental variables may affect the plant's response (Caldwell et al., 2003; Frohnmeyer and Staiger, 2003; Kakani et al., 2003).

The results obtained in this work show that the monoterpenes limonene (**1**) and geraniol (**3**) were increased by low irradiance UV-B (+UV-B) as compared either with no UV-B or high irradiance UV-B (−UV-B and ++UV-B, respectively). At pre-harvest the levels of these monoterpenes were the highest, and then decreased towards harvest. Under field conditions, limonene (**1**) and geraniol (**3**) increased under ++UV-B, with the highest levels of terpenes at pre-harvest. Eucalyptol (**4**), which is only detected at pre-harvest, also increased under UV-B radiation. Consistent with the results presented here, Lücker et al. (2004) observed that the expression of terpene synthase (TPS) genes takes place during the later stages of berry development, several weeks after veraison, and no transcripts for TPS were detected during the early stages of fruit development. Thus, the present results support the idea that low irradiance UV-B (+UV-B) trigger acclimation processes via induction of TPS leading to monoterpenes synthesis as it was suggested by Pontin et al. (2010) and Gil et al. (2012).

On the other hand, it has been reported that monoterpenes begin to decline in berries before the maximum accumulation of sugars is reached (Conde et al., 2007). Therefore, it is possible to assume that the odorant substances had been glycosylated as maturation proceeds and so reducing the possibility to volatize.

UV-B affects other metabolic pathways such as fatty acids, which also act in plant defense and stress responses (Deluc et al., 2009). The biosynthesis of ketones begins with the activation of phospholipases. These enzymes hydrolyze the phospholipids of the cell membrane and promote the removal of fatty acids from triglyceride. Consequently, an accelerated lipase activity results in a stimulated β-oxidation and so formation of carbonyl compounds. According to Lo et al. (2004) the lipase activity is strongly influenced by UV-B radiation. The ketone 5-hepten-2-one (**11**) was increased by UV-B and maintained constant throughout fruit development. This agrees with the results of Eichholz et al. (2011) in blueberries treated with UV-B. Hexanal (**5**) and 2-heptenal (**6**) levels were increased in berries towards harvest, where only hexanal (**5**) increased with high UV-B intensity (++UV-B treatment), while 2-pentenal (**7**) decreased since veraison. In turn, hexanal (**5**) can be converted into other VOCs, which are thought to be involved in defense signaling (Halitschke et al., 2004). Also, water stress increased the abundance of transcripts of several lipoxygenases (LOX) in other grapevine varieties, throughout ripening in the cv. Chardonnay and at maturity in the cv. Cabernet Sauvignon (Deluc et al., 2009). Although the alcohols 3-hexen-1-ol (**9**) and 3-methyl pentanol (**10**) were not affected by UV-B, their levels increased at harvest-time.

In field conditions, hexanal (**5**) levels did not change significantly during berry development or UV-B radiation regimens. In contrast, 2-heptenal (**6**) increased by UV-B and tended to decrease towards harvest, as well as 2-pentenal (**7**) and octanal (**8**). The 3-hexen-1-ol (**9**) showed no changes throughout all berry developmental stages, but were increased by UV-B, and the 3-methyl pentanol (**10**) levels increased toward late berry development without UV-B effect. The reduction of aldehydes towards late berry development along with the increase of alcohol is consistent with previous studies (Garcia et al., 2003; Kalua and Boss, 2009) where the decrease in aldehydes could be an indication that the enzyme alcohol dehydrogenase is more active or the hydroperoxide lyase enzyme is, by contrast, less active. Furthermore, aldehydes were the compounds that contributed more with the volatile composition of the cv. Malbec grapes at maturity. Hexanal (**5**) was the most abundant VOC not only in this class, but also in total compounds

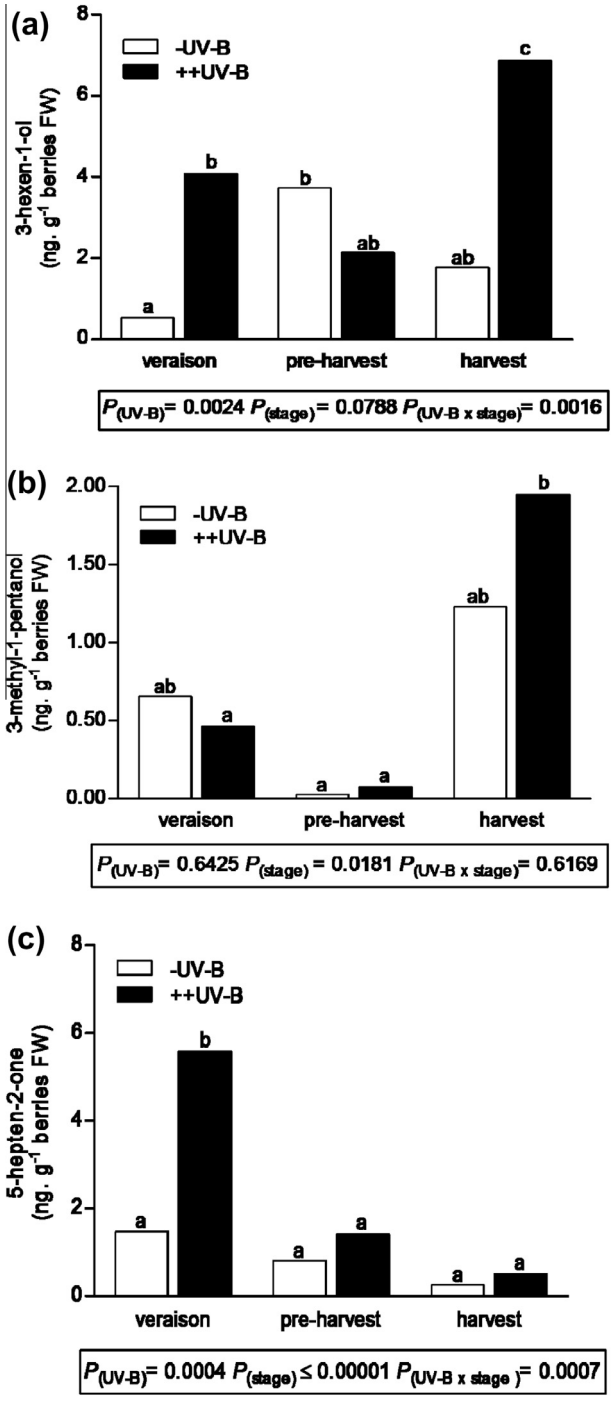

Fig. 7. Alcohols and ketone levels assessed by SPME–GC–EIMS (ng g^{-1} berries FW). 3-Hexen-1-ol (**9**) (a), 3-methyl pentanol (**10**) (b) and 5-hepten-2-one (**11**) (c) in grape berries exposed or not to solar UV-B (++UV-B and −UV-B, respectively) in the field experiment at three developmental stages (veraison, pre-harvest and harvest). $P_{(UV\text{-}B)}$: UV-B effect; $P_{(stage)}$: berry developmental stage effect; $P_{(UV\text{-}B\times stage)}$: UV-B × berry developmental stage interaction effect. Values are means ± SE, $n = 3$.

analyzed and in all sampling points. Beyond the observed differences between berry clusters irradiated *in vitro* and plants under field condition, a common monoterpenoid profile was assessed, with higher concentrations at pre-harvest. Then, monoterpenes tend to decrease towards harvest, where only limonene (**1**) and geraniol (**3**) were detected. This profile observed in both conditions may be a characteristic distinctive of the variety.

A final consideration is related to the different content of monoterpenes found between *in vitro* and field experiments, especially regarding the levels of limonene (**1**). These levels showed 10-times higher in field samples as compared with those *in vitro*, implying that the high UV-B/PAR ratio has an opposite effect of what was expected from the literature (Allen et al., 1998; Björn, 1996; Jordan et al., 1992). The explanation is that the treated tissues had already grown under high PAR and were only submitted to a single one-day treatment, showing that the high UV-B/PAR ratio is not a signal in itself but the tissues response depends on the absolute PAR during a long period.

4. Conclusions

The levels of monoterpenes in berries were increased by UV-B in both, *in vitro* and field conditions, with the highest level at pre-harvest. UV-B also modified in some cases the synthesis of aldehydes, alcohols and ketones. All these compounds may be involved in resistance to abiotic and biotic stresses. The results suggest a specific role of monoterpenes as defense in grape berries in response to UV-B. The consequence in that UV-B exposure could affect the flavor of grape berries and their products.

5. Material and methods

5.1. Experiment 1: in vitro UV-B treatments

Clusters were collected from a 12 year-old vineyard of *V. vinifera* L. cv. Malbec in Luján de Cuyo, Mendoza, Argentina (900 m a.s.l.; 68°52′ W and 33°3′ S), at three different developmental stages: veraison (72 days after flowering; DAF), pre-harvest (90 DAF) and harvest (126 DAF). Samples were placed in 250 mL "wide-mouth" glass flasks (120 mm height × 65 mm diameter) containing 50 mL of isotonic medium phosphate buffered saline (PBS; 3.6 mM KH_2PO_4, 4.8 mM K_2HPO_4, 0.97 mM $MgSO_4{\cdot}7H_2O$ and 0.13 M NaCl, adjusted to pH 6.1), and the flask tops were covered with low-density polyethylene, which transmitted most of the PAR and UV-B. After 10 h in darkness, clusters were irradiated in a controlled growth chamber at 25±2 °C with 80 μmol m^{-2} s^{-1} of PAR (cool fluorescent tubes) with a single 16/8 h photoperiod. Additionally, for different UV-B treatments, supplemental UV-B was given using a TL 100 W/01 tube (311 and 313 nm spectrum peaking; Philips, Eindhoven, The Netherlands) suspended 400 mm above the flasks. A single total effective dose of UV-B normalized at 311 nm of 4.75 kJ m^{-2} d^{-1} was provided in two different irradiation treatments: "low intensity UV-B" (+UV-B treatment; 0.08 W m^{-2} irradiance during a 16 h day photoperiod) and "high intensity UV-B" (++UV-B treatment; 0.33 W m^{-2} irradiance during the last 4 h of a 16 h day photoperiod). The UV-B was filtered by a clear polyester (100 μm), which absorbs more than 95% of UV-B, and transmitted most of the PAR to produce the minus UV-B treatment (−UV-B). The UV-B treatments were previously reported in Pontin et al. (2010) and Gil et al. (2012). PAR was measured with a Li-250 light meter (equipped with a Li-190 quantum sensor; Li-COR Inc., Lincoln, NE, USA), and UV-B irradiance was measured at the top of the flasks with a PMA2200 radiometer (equipped with a PMA2102 UV-B detector; Solar Light Company Inc., Glenside, PA, USA). Immediately at the end of the UV-B treatments, all the berries were separated from the rachis, weighed and used for analytical determinations. Three independent biological replicates ($n = 3$) were used for VOCs analyses.

5.2. Experiment 2: in field UV-B treatments

The experiment was carried out in a commercial high altitude vineyard (1450 m a.s.l., 69°15′ W and 33°23′ S), at Gualtallary, Mendoza, Argentina. Plants of *V. vinifera* L. cv. Malbec were

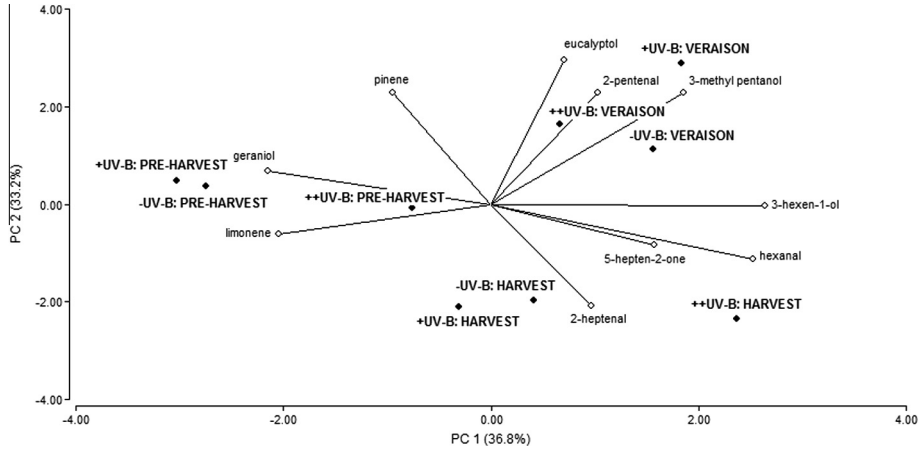

Fig. 8. *Biplot* display of the principal component analysis (PCA) for the grape berries subjected to *in vitro* treatments, using for classification the UV-B treatments (+UV-B, ++UV-B and −UV-B) and berry developmental stages (veraison, pre-harvest and harvest). Treatments: one dose (4.75 kJ m^{-2} d^{-1}) of low intensity (+UV-B, 0.08 W m^{-2} during a 16 h per day photoperiod), high intensity (++UV-B, 0.33 W m^{-2} during the last 4 h of a 16 h per day photoperiod) UV-B irradiance, and a treatment in which UV-B was filtered (−UV-B).

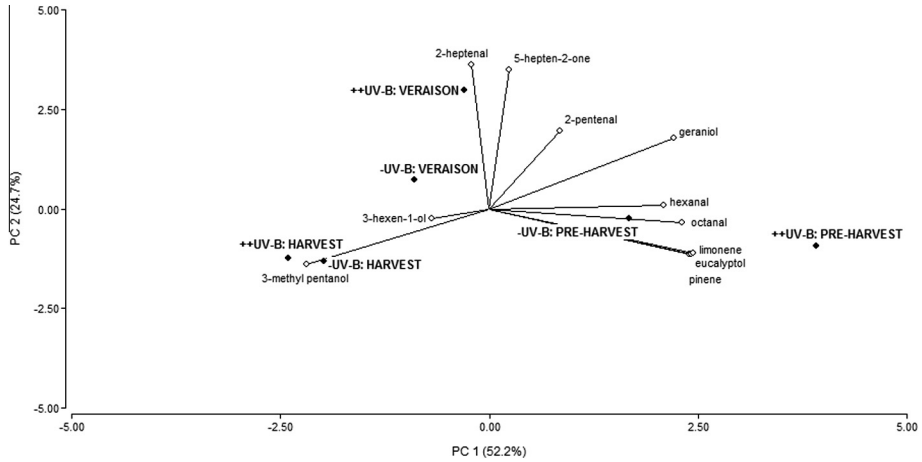

Fig. 9. *Biplot* display of the principal component analysis (PCA) for the grape berries exposed or not to solar UV-B in the field experiment, using for classification the UV-B treatments (++UV-B and −UV-B) and berry developmental stage (veraison, pre-harvest and harvest).

exposed to two UV-B radiation regimens by covering the grapevines with specific plastic sheeting, from 15 days before flowering until harvest. The experimental design and treatments were previously reported in Berli et al. (2011). Solar UV-B radiation was removed to produce the minus UV-B treatment (−UV-B) by using clear polyester (100 μm), which absorbs more than 95% of UV-B, and transmitted most of the PAR. Low density polyethylene (40 μm) transmitting most of the radiation from sunlight was used for the full UV-B treatment (++UV-B) and to minimize environmental differences between −UV-B and ++UV-B treatments. The plants in the ++UV-B treatment were exposed to UV-B irradiances that reach up to 0.40 W m^{-2} at midday. Plastics were located 2.5 m above ground level, covering the entire grapevine canopy, and were replaced after breakdowns or transmittance reductions. At veraison, pre-harvest and harvest, clusters were collected at midday ($n = 3$), immediately frozen with liquid nitrogen and kept at −80 °C until further analysis.

5.3. Analysis of berry volatiles

The extraction and concentration of volatile compounds from berries was performed by HS-SPME–GC–EIMS, as described by Yang et al. (2009) with some modifications. Five berries per experimental unit (clusters from the *in vitro* and field experiments) were separated and the seeds were removed. Then, seedless berries (berry skin and pulp) were weighed and placed in a HS-SPME vial (Varian, 20 mL) and 1 g of NaCl was added. The vials were closed with Teflon-coated screw caps and sonicated in three cycles of 5 min. Samples were spiked with an internal standard solution (4-methyl-2-pentanone) to obtain a final concentration of 2 μg mL^{-1}. They were placed into a 10 mL glass screw-top vial with polytetrafluoroethylene/silicone septa and placed on the magnetic stirrer (1000 rpm). Samples were allowed to equilibrate for 20 min at 40 °C. Then, the SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane, 50/30 μm Supelco) was exposed on headspace mode (30 mm) during 40 min. After extraction, the SPME fiber was withdrawn into the needle, removed from the vial and inserted into the injection port of GC–EIMS. All the analyses were performed in triplicate. Prior to sampling, the SPME fiber was preconditioned at 270 °C for 60 min in the GC injection port, according to the the manufacturer's instructions. VOCs were separated by GC–EIMS (same equipment as described for the analysis of terpenes in Gil et al., 2012) equipped with a capillary column (30 m × 0.32 mm × 0.25 μm, Varian CP-Wax 52CB) by applying the following temperature program: 35 °C for 5 min, then augmented to 70 °C at 3 °C min^{-1}, held for 2 min and augmented to

165 °C at 2 °C min^{-1}. Carrier gas was He at 1.0 mL min^{-1}, and the mass spectrometer was operated using electronic impact mode at 70 eV and a range of 35–200 atomic mass units was scanned. The identities of compounds were confirmed by comparison of their retention times and full scan mass spectra with those of authentic standards, and with mass spectra of the NIST library.

5.4. Statistical analysis

The statistical evaluation was performed using the software Statgraphics Centurion XVI version 15.0.10 (StatpointTechnologies Inc., Warrenton, VA, USA). Significance of differences was conducted with Fisher's LSD test ($P \leqslant 0.05$). Multifactorial ANOVA was used to analyze VOCs from berries across the different developmental stages. Results are reported as a mean of three independent replicated assays with their standard error (SE). Principal component analysis (PCA) was performed with the *InfoStat* software (*InfoStat version 2008*. Grupo InfoStat, Argentina), and the results of this analysis are presented as *Biplots*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.08.011.

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