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Influence of Berry Shrivel on Concentrations of Resveratrol, Catechin, Epicatechin and ε-Viniferin in Berries of *Vitis vinifera* L. Cultivars 'Zweigelt', 'Pinot Blanc' and 'Pinot Gris'

K. Bachteler[1,2)], M. Riedel[1)], C. Tisch[1)], N. Merkt[2)], R. Amann[1)] and J.-N. Wünsche[2)]
([1)]State Institute of Viticulture and Enology, Freiburg, Germany and [2)]University of Hohenheim, Institute of Crop Science, Stuttgart, Germany)

Summary

The effect of berry shrivel, a physiological disorder in grapevine, on the concentration of phenolic compounds in the cultivars 'Zweigelt', 'Pinot Gris' and 'Pinot Blanc' berries was studied. Specific phenolic compounds were analysed by HPLC and compared to concentrations in healthy berries of the same varieties and between the varieties. As resveratrol is often used as a marker for infection or stress induced defence mechanisms in grapevine, it was postulated that resveratrol concentration in the grape berries is positively correlated with berry shrivel. Other phenolic substances such as catechin, epicatecin and viniferin were also analysed to evaluate their potential effect on berry shrivel. Healthy berries from the variety 'Zweigelt' contained significantly more trans-resveratrol, trans-resveratrol-glucoside and ε-viniferin, whereas shriveled berries contained significantly higher concentrations of catechin and epicatechin. The phenolic concentrations were not significantly different between healthy and affected 'Pinot Gris' and 'Pinot Blanc' grape berries, respectively, except for 'Pinot Gris' with significantly higher catechin concentrations in healthy berries compared to shriveled berries. Cis-resveratrol could not be detected in any of the samples and cis-resveratrol-glucoside could not be definitely identified.

Key words. Phenolic compounds – physiological disorder – berry shrivel – grapevine

Introduction

Resveratrol is a phytoalexin and belongs to the group of the stilbenes. It is generated via the Shikimat biosynthesis pathway (Richter 1998). Different monomeric forms can be found in grapevines: trans- and cis-resveratrol and the trans- and cis-resveratrol glucosides (Soleas et al. 1995). In plants resveratrol is produced under abiotic stress conditions such as high UV-radiation or biotic stress induced for example by the pathogen *Botrytis cinerea* (Stein and Hoos 1984; Adrian et al. 1997; Bavaresco et al. 1997, 2007, 2009; Schouten et al. 2002; Favaron et al. 2009; Gonçalves van Zeller de Macedo Basto et al. 2011). It was found to be synthesized in different organs of grapevine such as leaves, shoots and berries (Langcake and Pryce 1977; Vezzulli et al. 2007; Bavaresco et al. 2007). In the berry, the production of resveratrol increases at the beginning of berry development and then ceases around veraison when the production of anthocyanins rises (Jeandet et al. 1991). Both anthocyanins and resveratrol share a similar synthesis pathway so that the constitutive chalcone synthase is competing against the inducible stilbene synthase (Jeandet et al. 1995). ε-Viniferin also belongs to the group of stilbenes and is a dimer of resveratrol (Bezhuashvili et al. 2010). It is biosynthesized by an oxidative process of resveratrol (Langcake 1977). Catechin and epicatechin are the flavonoids with the highest concentrations in red wines (Goldberg et al. 1998). They act as antioxidants and inhibit the formation of free radicals. It is also known that these flavonoids can inhibit the oxidation of human LDL (low density lipoproteins) and therefore help to prevent coronary artery disease (Nagao et al. 2005; Ribeiro de Lima et al. 2006). Usually only the diastereomers (+)-catechin and (−)-epicatechin can be found in noteworthy amounts. Butkhup et al. (2010) found 52.30 ± 1.13 mg 100 g^{-1} dry weight base for (+)-catechin content of whole grape extract and 27.92 ± 1.40 mg 100 g^{-1} dry weight base for (−)-epicatechin respectively. Catechin and epicatechin often form oligomers. Those procyanidins play an important role for the structure and astringency of wines (Ojeda et al. 2002).

Berry shrivel (BS) is a physiological disorder of grapevines. Grapes affected by BS lose their turgescence at the onset of ripening. In red varieties the berries often remain

green and dull. The accumulation of sugars ceases and the must weight remains low around 10–15 °Brix. BS does not cause damage on the outside of the rachis and berries. This distinguishes BS from another common physiological disorder, the bunch stem necrosis. The rachis of grapes affected by bunch stem necrosis become necrotic and sometimes the whole bunch abscises. The factors causing both diseases may be related to nutrient scarcity in the soil or an unbalanced ratio of potassium and magnesium (Fardossi 2003). As no plant diseases caused by pathogens such as bacteria, phytoplasma or fungi could be associated with these symptoms (Reisenzein and Berger 2001; Keller 2008), abiotic stress was in the center of many researchers' attention investigating the causal factors for both non-pathological disorders. Temperature during ripening and flowering (W. Siegfried, personal comm. 2010), shortage or excess of water (Stücklin 2007) and yield control (Kührer and Gabler 2011) have been mainly suggested. The aim of this study was based on the hypothesis, that resveratrol might be a useful marker for presymptomatic detection of berries affected by BS. Possible differences in the concentration and composition of phenolic compounds between healthy and affected berries would offer some evidence as to which physiological mechanisms are likely associated with the occurrence of BS.

Material and Methods

Site description

Berry samples of 'Zweigelt' grapes were taken at a commercial vineyard in Weinstadt-Endersbach, Germany (48° 49' 05.54" N, 9° 22' 18.54" E, 246 m a.s.l.). The vineyard is orientated north-south and has an area of 1438.0 m². The soil type was classified as endostagnic Regosol (calcaric). The vines were all St 9 clones on 5BB rootstocks, planted in 2002 and trained as bended unilateral shoots with 10–11 buds.

The 'Pinot Blanc' site was located in Ehrenkirchen, Germany (47° 55' 33.22" N, 7° 44' 59.92" E, 297 m a.s.l.), orientated east-west and had a total area of 403.2 m². The soil type was classified as haplic Regosol (calcaric, siltic). The vines were 0212 clones on 5BB rootstocks, planted in 2000 and trained as unilateral shoots with 9–12 buds.

The 'Pinot Gris' site was located in Oberrotweil, Germany (48° 05' 30.54" N, 7° 36' 54.81" E, 198 m a. s. l.). The vines are unknown clones on 125AA rootstocks. The vineyard is orientated north-south and had an area of 1134.4 m². The vines are trained as unilateral shoots with 9–11 buds. The soil type was not classified but the parent rock material is volcanic tephrite.

Berry samples

At commercial harvest in 2010 berry samples of 'Zweigelt' were taken on 6 October and those of 'Pinot Blanc' on 7 October. In 2011 healthy and affected 'Pinot Gris' were collected 10 days after commercial harvest on 13 October, a delay that was due to miscommunication. The winegrowers' commercial harvest in both years took place at must weights of healthy berries of around 20.5 °Brix for 'Zweigelt' and 22.0 Brix for 'Pinot Blanc' and 'Pinot Gris'. Berries affected by BS showed must weights around 15.0 °Brix in all three varieties. Following harvest, healthy and shrivelled berries were frozen and stored at −25 °C until extraction. At harvest 12 samples of healthy and affected berries were taken for the variety 'Zweigelt', respectively, as well as 6 samples each of healthy and affected berries of both 'Pinot Gris' and 'Pinot Blanc'. Each sample had a total weight of about 20 g. Healthy individual berry weight was 3.2 ± 0.44 g, whereas one shrivelled berry weighed 1.1 ± 0.23 g.

Weather data

Weather data were collected by the Landwirtschaftliches Technologiezentrum Augustenberg, Germany, at meteorological stations near the experimental sites and provided by the program Vitimeteo (State Institute of Viticulture and Enology, Freiburg, Germany, and the Agroscope Changins-Wädenswil, Switzerland).

Extraction

Phenolic compounds were extracted following the procedure of Bavaresco et al. (1997). Frozen berries were cut in half and seeds were removed to avoid extraction of constitutive resveratrol. Subsequently the berries were crushed in a mortar with 20 ml of 95 % methanol. The homogenate was transferred in an Erlenmeyer flask with another 10 ml 95 % methanol and then agitated on a shaker (Innova® 40, New Brunswick Scientific, USA) at room temperature for 20 min. After filtration through a filter paper (Macherey-Nagel GmbH & Co. KG, Germany), the methanol was vaporized in a rotary evaporator (BÜCHI Rotavapor-RE, Essen, Germany) at 80 mbar and 35–40 °C. The aqueous extract was mixed with 7 ml ethyl acetate and 7 ml sodium bicarbonate and vortexed (IKA® MS1 Minishaker, Germany) until phase separation was observed. The upper phase was collected, another 5 ml ethyl acetate was added and the sample was again vortexed. After phase separation, the upper phase was collected again and vaporized until complete dryness. The dry material was resolved with 100 % methanol and stored in a freezer at −25 °C until analysis.

HPLC-Analysis

Phenolic compounds were analysed using high performance liquid chromatography (HPLC; Agilent 1260 Infinity, USA) with Diode Array Detector according to the method of Vrhovsek et al. (1995). The following standards were used: (+)-catechin hydrate (Fluka® Analytical,

Switzerland), (−)-epicatchin (Lancaster Synthesis, USA), trans-resveratrol (Sigma-Aldrich®, Germany), trans-resveratrol-glucoside (specs, Netherlands) and ε-viniferin (AK Scientific Inc., USA). Calibration of cis-resveratrol and cis-resveratrol glucoside: Aqueous solutions of the pure trans compounds were partially transformed to cis/trans mixtures by exposition to daylight for several days. Afterwards the chromatograms contained two peaks, peak 1 = remaining trans compound and peak 2 = cis compound formed by isomerization. The amount of remaining trans compound was determined at 306 nm with the existing calibration curve. The difference to the concentration before isomerization gave the amount of formed cis compound. The ratio of peak area to concentration for the cis compound at 285 nm was calculated to create a calibration curve.

The injected volume of each sample was 5 µl. The samples were separated with a LiCrospher^{R} 100 RP18e column (250 × 4 mm; 5 µm) with a LiCrospher 100 RP18e guard-column (4 × 4 mm; 5 µm). 0.1 % phosphoric acid (A) and a mixture of of 0.1 % phosphoric acid plus 80 % acetonitrile (B) were used as mobile phase. The flow rate was 0.8 ml min^{-1} and UV-absorption was measured from 200 to 400 nm. Catechin and epicatechin were quantified at 210 nm, trans-resveratrol and trans-resveratrol-glucoside at 306 nm, cis-resveratrol and cis-resveratrol-glucoside at 285 nm. Between two cycles the column was washed for 15 min with solvent A. See Fig. 1 for exemplary chromatograms.

Detection of Botrytis cinerea

To ensure that the analysis of resveratrol was not affected by *Botrytis cinerea*, the samples were tested with the LOEWE®FAST Kit *Botrytis cinerea* (Loewe® Biochemica GmbH, Sauerlach, Germany). Samples were mashed, the grape juice mixed with the buffer and then applied onto the test cassettes containing specific antibodies. After a few minutes, besides a control band, a red band can be observed when an infection with *Botrytis cinerea* exists.

Statistical analysis

12 affected and 12 healthy samples of Zweigelt berries as well as 6 affected and 6 healthy samples each of Pinot Gris and Pinot Blanc berries employed to evaluate treatment differences (variety and BS) using a bi-factorial analysis of variance (ANOVA, SAS® Institute INC, USA). Least significant differences (LSD) were computed and significance was indicated at a probability level of $P > 0.05$.

Results

Phenol analysis

The results indicate a great variability of phenol concentration in berries of the different varieties (Table 1).

Table 1. Average phenol concentration [µg kg^{-1} fresh weight] in healthy berries and berries affected by berry shrivel of 'Zweigelt', 'Pinot Gris' and 'Pinot Blanc' grapevines.

Variety	Healthy berries			Berries affected by BS		
Catechin						
'Zweigelt'	1392	a	A	5390	b	A
'Pinot Gris'	4790	a	B	2953	b	B
'Pinot Blanc'	2767	a	A	3408	a	B
Epicatechin						
'Zweigelt'	963	a	A	2425	b	A
'Pinot Gris'	1048	a	A	771	a	B
'Pinot Blanc'	656	a	A	350	a	B
Trans-resveratrol						
'Zweigelt'	443	a	A	17	b	A
'Pinot Gris'	62	a	B	31	a	A
'Pinot Blanc'	67	a	B	0	a	A
Trans-resveratrol-glucoside						
'Zweigelt'	469	a	A	219	b	A
'Pinot Gris'	102	a	B	56	a	B
'Pinot Blanc'	33	a	B	50	a	B
Viniferin						
'Zweigelt'	2035	a	A	621	b	A
'Pinot Gris'	162	a	B	183	a	B
'Pinot Blanc'	94	a	B	42	a	B

Means of healthy and affected berries of one variety followed by the same small letter in each line do not differ significantly at $P < 0.05$.
Comparing the three varieties, means of healthy berries or affected berries followed by the same capital letter in each column do not differ significantly at $P < 0.05$.

Comparing healthy and affected berries of the same variety, significant differences in phenol concentration were found in 'Zweigelt'. In this variety berries affected by BS had nearly four times higher catechin concentration and 2.5-fold higher epicatechin concentration than healthy berries. In contrast, affected 'Zweigelt' berries had concentrations of 96 % less trans-resveratrol, 53 % less trans-resveratrol-glucoside and 69 % less ε-vinferin when compared to healthy berries. For 'Pinot Gris' berries affected by BS had 38 % less catechin concentration than healthy berries. 'Pinot Blanc' berries did not show any significant differences between healthy berries and berries affected by BS. Cis-resveratrol could not be detected in any of the samples and cis-resveratrol-glucoside could not be definitely identified due to overlaying signals.

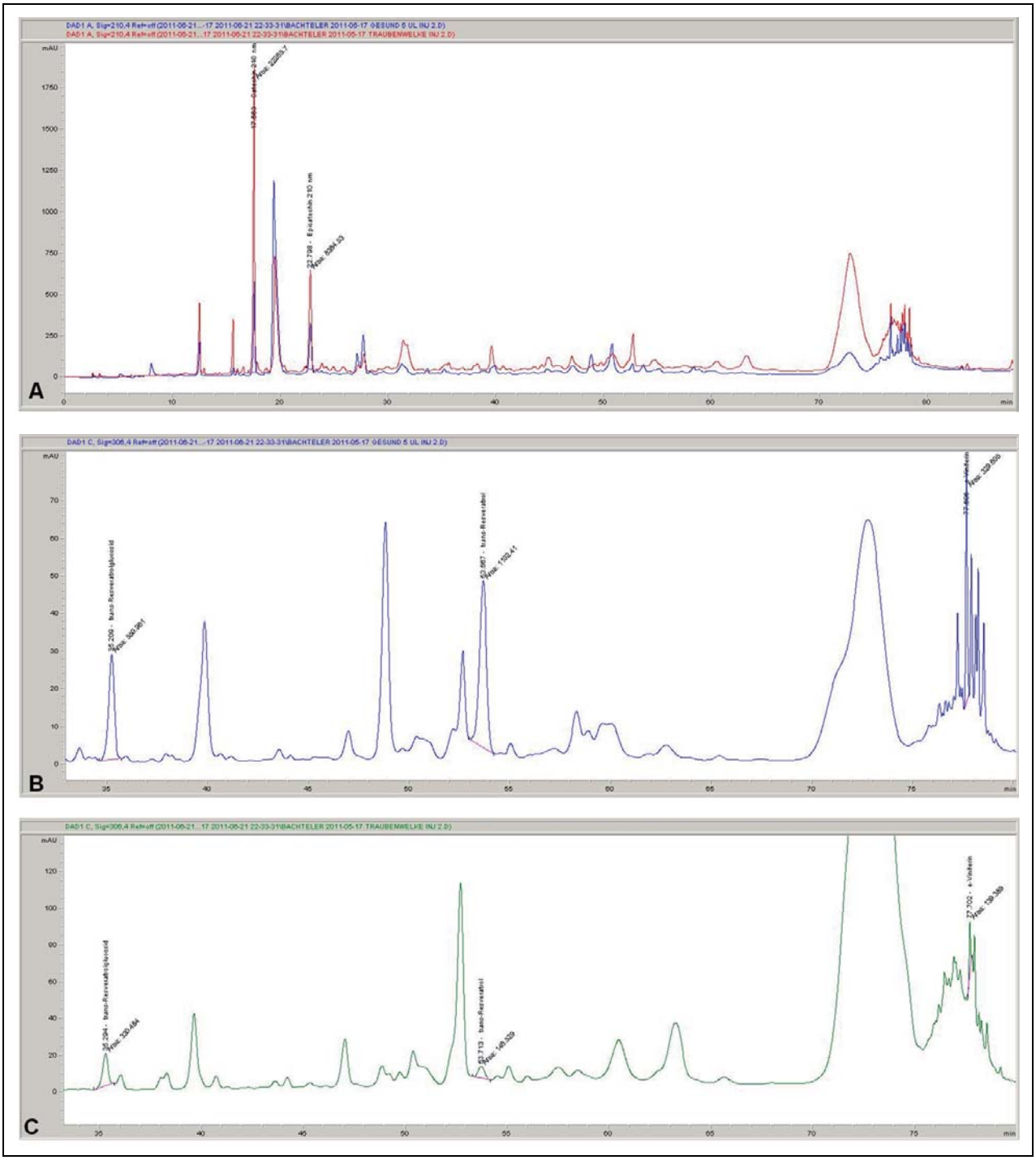


Fig. 1. Exemplary chromatograms of two 'Zweigelt' samples (healthy and affected by BS). A: Detection at 210 nm of catechin (17.5 min) and epicatechin (22.6 min) in healthy (blue line) 'Zweigelt' berries and berries affected by BS (red line); B: Detection at 306 nm of trans-resveratrol-glucoside (35.2 min), trans-resveratrol (53.6 min) and viniferin (77.6 min) in healthy 'Zweigelt' berries; C: Detection at 306 nm of trans-resveratrol-glucoside (35.2 min), trans-resveratrol (53.6 min) and viniferin (77.6 min) in 'Zweigelt' berries affected by BS.

Weather data

In 2010, the average daily temperature was 8.9 °C and 10.3 °C and the amount of precipitation was 772 mm and 888 mm at the 'Zweigelt' and 'Pinot Blanc' sites, respectively. At the 'Pinot Gris' site, the average daily temperature was 11.1 °C and the annual rainfall was 495 mm in 2011.

Detection of Botrytis cinerea

None of the samples tested with the Lateral Flow Test showed an infection with *Botrytis cinerea*.

Discussion

In this study, the concentration of trans-resveratrol was higher in healthy berries of all varieties compared to berries affected by BS of the same variety. The role of phenolic compounds such as catechin and epicatechin under abiotic stress conditions is not clear and with contradictory results in the literature. Some studies showed an inhibition of these substances e. g. in seeds of *Vitis californica* (Weidner et al. 2011) under osmotic stress. Others reported an increased production of phenols under different stress conditions such as pathogen attack, wounding, high light or low temperature (Dixon and Paiva 1995; Weidner et al. 2009). It has also been shown that vines under water deficit before and after veraison contain more flavan-3-ols than plants without or with less stress (Ojeda et al. 2002). If BS indicates another form of abiotic stress for the plant or if stress leads into BS symptoms, this may explain the increased production of catechin and epicatechin in 'Zweigelt' berries affected by BS. Another reason for the differences might be that healthy berries contain more anthocyanins than affected ones (Krasnow et al. 2009) so there is more conversion from catechins to procyanidins (Goldberg et al. 1998). The non-red varieties 'Pinot Blanc' and 'Pinot Gris' berries do not show an increased concentration of catechin and epicatechin when affected by BS. The fact that 'Pinot Gris' even shows significantly higher concentrations of catechin in healthy berries indicates that our results are not consistent with other findings (Balik et al. 2008).

It is difficult to compare different varieties especially red and white ones. White varieties naturally contain less resveratrol and viniferin (Ector et al. 1996; Bavaresco et al. 2007) which becomes apparent when comparing 'Zweigelt' with 'Pinot Blanc' or 'Pinot Gris' berries. However, exceptions can be found too, as Soleas et al. (1995) and Okuda and Yokotsuka (1996) obtained results for resveratrol in white varieties that were in the range of red skinned varieties or even above. Differences between 'Zweigelt' and 'Pinot Gris' might be explained by the different times of sampling and the intermediate role of 'Pinot Gris' between white and red varieties. 'Zweigelt' berries were sampled at commercial harvest, whereas Pinot Gris berries were sampled after harvest. The later sampling date may have caused lower concentrations of resveratrol as the concentration decreases constantly after veraison (Jeandet et al. 1991; Eder et al. 2001). The effect of variety, e.g. 'Zweigelt', 'Blaufränkisch', 'Pinot Noir' and 'Cabernet Sauvignon' and harvest time on the concentration of resveratrol was shown by Eder et al. (2001).

Comparing these results with previous studies is difficult as concentrations of phenolic compounds differ with varieties, years, dates of sampling and trial site. The concentrations found in the present study for catechin and epicatechin are 20 times lower than those obtained by Zhao et al. (2006) in 'Cabernet Sauvignon' or those obtained by Butkhup et al. (2010) in dry berries of 'Shiraz'. The concentrations of trans-resveratrol found in earlier studies differ also substantially with those obtained in the present study. Jeandet et al. (1991) reported concentrations below 1000 μg kg^{-1} in 'Pinot Noir' at harvest and Okuda and Yokotsuka (1996) describe a range from 59–1795 μg kg^{-1} in white varieties and 92–1604 μg kg^{-1} in red or pink varieties. Others stated much higher concentrations such as Ector et al. (1996) with 6390–14000 μg kg^{-1} in 'Muscadine' berries or Bavaresco et al. (1997) obtaining 5000 μg kg^{-1} in 'Castor' berries and 2110 μg kg^{-1} in 'Huxelrebe'. For ε-viniferin little data can be found. Gonçalves van Zeller de Macedo Basto et al. (2011) indicated a minimum of 37 μg kg^{-1} just prior to an infection with *Plasmopara viticola* as elicitor and a maximum concentration of 3327 μg kg^{-1} after infection. The present results are within the range of these values.

If abiotic stress causes or increases symptoms of BS, it does not seem to increase the concentration of resveratrol in affected berries. Therefore, the use of resveratrol as a marker for an early detection of berries affected by BS is not suitable. Nevertheless, well ventilated soils, healthy roots, a suitable green cover and adapted yield targets can help to minimize abiotic stress for the vines in general and may also help to minimize the incidence of BS (Redl et al. 2009).

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Addresses of authors: Kristina Bachteler (corresponding author), State Institute of Viticulture and Enology, Merzhauser Str. 119, 79100 Freiburg, Germany and University of Hohenheim, Institute of Crop Science, Emil-Wolff-Str. 25, 70599 Stuttgart, Germany, Monika Riedel, Christine Tisch and Rainer Amann, State Institute of Viticulture and Enology, Merzhauser Str. 119, 79100 Freiburg, Germany, and Nikolaus Merkt and Jens-Norbert Wünsche, University of Hohenheim, Institute of Crop Science, Emil-Wolff-Str. 25, 70599 Stuttgart, Germany, e-mail (corresponding author): Kristina.bachteler@yahoo.de.