Effect of Esca disease on the phenolic and sensory attributes of Cabernet Sauvignon grapes, musts and wines

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Abstract

Background and Aims: The impact of Esca, a cryptogamic disease affecting woody tissues of grapevines (trunk, branches and shoots), was investigated on phenolic and sensory qualities of Cabernet Sauvignon grapes, derived musts and wines.

Methods and Results: Global phenolic analyses revealed no major difference between healthy and affected grapes whereas high-performance liquid chromatography analyses showed decreases in the skin concentrations of catechin and epicatechin and of anthocyanins for the two vintages assessed. Proanthocyanidin characteristics, and particularly mean degree of polymerisation, were strongly decreased in skin proanthocyanidin polymeric fractions. Chemical analyses of wines made with different percentages of fruit from Esca infected grapevines confirmed the moderate impact of Esca on phenolic composition. Nevertheless, sensory analyses revealed a loss of wine sensory quality perceptible with as little as 5% of affected fruit in wines.

Conclusions: Phenolic variations in grapes and in derived wines may be related to changes to grapevine physiology caused by Esca fungi that impact on flavonoid metabolism. The chemical and sensory variations between wines can also be related to the delayed ripening delay of fruit from Esca infected grapevines.

Significance of the Study: Esca moderately affected the phenolic composition of grapes and decreased the sensory quality of wines, suggesting a dramatic increase in the economic importance of Esca if no control methods are found.

Keywords: *anthocyanin*, *Esca*, *grape*, *grapevine disease*, *must*, *proanthocyanidin*, *sensory analysis*, *total phenolics*, *wine*

Introduction

Esca is a complex cryptogamic disease widespread all over the world and homogeneously distributed in France (Fussler et al. 2008). The Esca syndrome's appearance can be of two forms, a severe (apoplexy) and a chronic form. The chronic form is characterised by foliage deterioration. The leaves have interveinal islands of chlorotic and yellowish tissue that later becomes necrotic. These external foliar symptoms are associated with internal symptoms consisting of changes to the photosynthetic apparatus and carbohydrate physiology (Petit et al. 2006). Grape bunches appear normal but berries do not fill properly and generally do not reach maturity (Calzarano et al. 2004). The severe form of the disease, also called apoplexy, is characterised by the sudden wilting and death of bearing vines or vine parts in midsummer. A complex of several fungi, involving mainly *Phaeomoniella chlamydospora* and *Phaeocraemonium aleophilum*, are considered to contribute successively to the characteristic necroses formed in different parts of the wood and the progressive destruction of the grapevine trunk (Larignon 2004, Valtaud et al. 2009). Studies conducted in various vineyards have gathered evidence that sensitivity to foliar symptoms expression was function of varieties (Larignon et al. 2009). For instance, Esca symptoms were found to be more easily expressed in Cabernet Sauvignon, Sauvignon blanc, Mourvedre, Ugni Blanc, Cinsault and Trousseau than in Merlot, Carignan, Roussane and Pinot Noir. In 1999, an Italian review reported that Esca had gained importance in all European vine-growing countries over the previous 10 years (Mugnai et al. 1999). At that time, Esca was estimated to have reached incidences of 20, 11, 15 and 19% in Portugal, Hungary, France and Germany, respectively, even though sodium arsenite was still allowed as a chemical control. In 2001, because of its high toxicity to humans and adverse environmental effects, the use of sodium arsenite was banned in France. Moreover in 2007, the French 'Grenelle de l'environnment' policy has advised the increase of organic farming surfaces and the reduction of chemical additives. As no alternative control method for Esca development is available, a dramatic increase in the economic importance of Esca is of major concern to grape growers and will require a consideration of alternative control methods.

Most of the scientific studies dealing with Esca attempt to elucidate the different mechanisms involved in this complex disease through identification of the responsible pathogens or changes to metabolism, with and overall aim of finding effective controls. Few studies have considered the effects of Esca on berry chemical composition and in particular phenolic composition

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(Calzarano et al. 2004, 2009a,b). Nevertheless, these compounds are of primary importance to wine quality.

The main classes of phenolic compounds in grapes affecting red wine quality are the anthocyanins and the condensed tannins, also called proanthocyanidins. Anthocyanins are the pigmented compounds responsible for red wine colour and are largely located in grape skins. Proanthocyanidins include a large range of phenolic compounds comprised of flavan-3-ol monomer subunits. Proanthocyanin structures vary in the nature of their constitutive subunits, mean degree of polymerisation (mDP) and linkage position. Grape seed proanthocyanidins comprise only procyanidins (subunits constituted of (+)-catechin (C) (–)-epicatechin (EC) and (–)-epicatechin gallate (ECG)), whereas grape skin proanthocyanidins include both procyanidins and prodelphinidins (subunits constituted of (–)-epigallocatechin (EGC)) (Prieur et al. 1994, Cheynier et al. 2006). Skin proanthocyanidins have a higher mDP and a lower proportion of galloylated subunits than seed proanthocyanidins. During red winemaking, proanthocyanidins and anthocyanins are extracted from seeds and skins. The wine phenolic composition will depend on the original grape composition but also on extraction related to winemaking techniques. Proanthocyanidins are of great importance to red wine quality because of their astringent and bitter properties (Gawel 1998, Peleg et al. 1999) and their role in long-term colour stability via chemical reactions with anthocyanins involving copigmentation and/or condensation (Somers 1971, Vivar-Quintana et al. 1999). The molecular size of proanthocyanidins (mDP) affects their relative bitterness and astringency (Robichaud and Noble 1990, Gawel 1998, Peleg et al. 1999, Vidal et al. 2003, Cheynier et al. 2006). Monomers are more bitter than astringent, whereas the reverse is true for large molecular weight derivates. The content of phenolic compounds in grape berries depends on climatic and geographical factors, cultural practices and stage of ripeness.

Aquitaine region's vineyards represent 17.3% of the total French vineyard area and this region is particularly concerned about the development of Esca. Thus, the first objective of this study was to determine the Esca effect (chronic form) on the phenolic composition of Bordeaux Cabernet-Sauvignon grapes. Secondly, wines were made from different percentages of affected grapes to investigate the impact on the chemical, phenolic and sensorial quality of derived musts and wines and to determine if there was a percentage of grapes from Esca affected vines that could be incorporated during the winemaking without modifying wine phenolic composition and sensory properties.

Materials and methods

Experimental materials

Chemicals. Deionised water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA). Acetonitrile, ethyl acetate, chloroform, methanol, ethanol and acetone were of high-performance liquid chromatography (HPLC) grade and purchased from Scharlau (Sentmenat, Barcelona, Spain). (+)-Catechin (–)-epicatechin (–)-epigallocatechin (–)-epicatechin-3-*O*-gallate, B1 ((–)-epicatechin-(4β-8)-(+)-catechin), B2 ((–)-epicatechin-(4β-8)-(–)-epicatechin), gallic acid, Folin–Ciocalteu's phenol (2N), sodium metabisulfite, sodium carbonate, phloroglucinol, L(+)-tartaric acid, L-ascorbic acid, hydrochloric acid (37%), sodium hydroxide and acetic acid were purchased from Sigma Aldrich (Saint Louis, USA). B3 ((+)-catechin-(4α-8)-(+)-catechin) and B4 ((+)-catechin-(4α-8)-(–)-epicatechin) dimers, trimer (T) ((+)-catechin-(4β-8)-(+)-catechin-(4β-8)-(–)-epicatechin) were synthesised by the Laboratory of Organic Chemistry and Organometallic, Université Bordeaux 1 (Tarascou et al. 2006). Ammonia (25%), o-phosphoric acid (85%) and ammonium dihydrogen phosphate were from VWR-Prolabo (Fontenay sous Bois, France).

Fruit sampling and extraction. Grapes (*Vitis vinifera*, cv. Cabernet-Sauvignon) were collected from a vineyard near Bordeaux. The vineyard was planted on a sablo sandy-gravely soil and was grafted onto '3309' rootstock. The planting density was approximately 8333 vines/ha. Since 2004, Esca incidence (chronic form) in the vineyard was monitored each year by visual inspection. One hundred kilograms of healthy (from vines showing no foliar symptoms since 2004) and affected (from vines with visible foliar symptoms) fruit was harvested on the 8 October in 2009 and on the 4 October in 2010. A portion of each was frozen for chemical analyses with the remainder being used for small-scale winemaking.

Grape tannin extraction. Extracts were prepared in duplicate according to a previous study (Lorrain et al. 2011). Seeds and skins were removed by hand from grapes, lyophilised for 2 days and stored at −20°C. The frozen seeds and skins were finally ground in a ball grinder. A 6-g portion of the obtained powder was extracted using 55 mL of acetone/water (70:30, v/v) for 4 h and 55 mL of methanol/water (60:40, v/v) for 2.5 h. The centrifugal supernatants were combined and evaporated under reduced pressure at 30°C to remove organic solvents; the residue was dissolved in water and lyophilised to obtain a crude tannin extract.

A small fraction of this crude extract (equivalent to 1 g of dried skins or seeds powder) was conserved for further global phenolic analyses *(cf 'Global phenolic analyses section')* while the remaining (equivalent to 5 g of dried skins or seeds powder) was solubilised in 250 mL of water/ethanol (95:5, v/v) and extracted three times with chloroform (v = 250 mL) to remove lipophilic material. Then the aqueous phase was extracted three times with ethyl acetate (v = 250 mL) to obtain two distinctive fractions: a low molecular weight procyanidins fraction (monomeric/oligomeric tannins) in the organic phase and a high weight procyanidins fraction (polymeric tannins) in the aqueous phase. These two fractions were concentrated and lyophilised to obtain a dry powder.

Grape anthocyanin extraction. Anthocyanin extraction was adapted from previous studies (Sriram et al. 1999). A 1-g portion of dried skin powder was extracted with 40 mL of acidified methanol (0.1% HCl 12N) four successive times (for 4, 12, 4 and 12 h). The centrifugal supernatants were combined and evaporated under reduced pressure at 30°C to remove methanol; the residue was dissolved in water and lyophilised to obtain an anthocyanin powder.

Small-scale winemaking. Microvinifications were conducted in duplicate. In 2009, 0 (control), 5, 25, 50 and 100% of fruit from Esca-diseased vines was added to healthy fruit to make 10 kg ferments. In 2010, the same method was used except with 0 (control), 5, 15 and 25% of Cabernet Sauvignon grapes from Esca-diseased vines. Each '10-kg grapes' batch was mechanically crushed and destemmed and collected in a 10-L aluminum tank. Crusher pressure was adjusted to gain the same juice volume in all the tanks (constant ratio pomace/juice). Before addition of a 6 g/hL dose of aqueous bisulfite solution 18% (Laffort, Bordeaux, France) in each tank, a 60 mL-sample of must was collected and frozen for chemical analyses.

Saccharomyces cerevisiae yeasts (Zymaflore F15®, Laffort) were prepared by rehydratation in a warm aqueous mixture of sugar, nutrients (Thiazote®, Laffort) and an alcoholic fermentation activator (Superstar®, Laffort) for 15 min. Twenty mL of this leaven was applied to each fermentation tank (after a must maceration of 24 h) in order to reach concentrations of 15 g/hL for the activator, 20 g/hL for the nutrients and 20 g/hL for the yeasts. Fermentations were conducted at 20°C with temperature and density being monitored daily in each tank. After 14 days, densities were stable for each batch and concentrations of reducing sugars were lower than 2 g/L. Wines were separated from the pomaces by moderate manual pressing and poured into 1.5-L glass bottles. Malolactic fermentations were conducted by inoculating with a commercial lactic acid bacterium, Oenococcus *oeni* (Lactoenos 450 Preac®, Laffort) at 2 g/hL in mixture with its activator (Energizer®, Laffort) at 5 g/hL. The decrease in malic acid concentrations was followed by enzymatic kit (R-Biopharm, Saint Didier au Mont d'Or, France). When they were lower than 0.2 g/L, finished wines were racked in 1.5 L bottles and 6 g/hL of aqueous bisulfite solution 18% was added.

There was a total of six musts and six wines in duplicate ('control' or 0, 5, 25, 50, 75 and 100% of berries from Esca-diseased vines) for the 2009 vintage and of four musts and four wines in duplicate for the 2010 vintage ('control' or 0, 5, 15 and 25% of berries from Esca-diseased vines).

Chemical analyses

Classical enological analyses (musts and wines). In musts, total acidity, assimilable nitrogen, malic acid concentration, reducing sugars and consecutive probable alcohol were determined following the methods of OIV (OIV 2011).

For wine, reducing sugars, total acidity, malic acid, alcohol (% vol) and pH were measured by infra-red using Foss WineScan™ 79000 (Foss, Nanterre, France).

Global phenolic analyses (grape crude extracts, musts and wines). Total polyphenol, tannin and anthocyanin contents were determined from grape skin and seed extracts, from musts and wines. Grapes crude extracts were solubilised in a model solution composed of water/ethanol (90:10, v/v; pH 3.5 with tartaric acid) at specific concentrations. For total phenol content (TPC) and total anthocyanin determination, concentrations were 2 g/L for the seed extracts and 6 g/L for the skin extracts. For total tannin determination, they were of 0.25 g/L and 1 g/L for seed and skin extracts, respectively. For musts, a centrifugation of samples (10 min at 2500 rpm) was necessary to eliminate solid residues and. Liquid supernatant was used with no previous dilution for analyses. For total tannin determination in musts, it was first necessary to remove sugars in order to avoid artifact reactions during acidic hydrolysis at 100°C. To this end 9 mL of musts was fractionated on a 60 mL-10 g LC-C18 gel column (Supelco, Bellefonte, PA, USA) previously conditioned with methanol and distilled water. After loading the sample, the column was first washed with 50 mL of distilled water (removing of sugars) and then tannins were eluted with 50 mL of methanol. After evaporation of the organic solvent under reduced pressure at 30°C, the residue was dissolved in 9 mL of a pH 3.5 water/ethanol solution (90:10, v/v) and used for total tannin determination. For wines samples, 1/10 and 1/50 dilutions were necessary for TPC and total tannin measurements, respectively.

Global analyses procedures. TPC was determined by Folin–Ciocalteu test with the following solutions being introduced in 50 mL volumetric flasks: 2.5 mL Folin–Ciocalteu solution, 0.5 mL of sample, 10 mL Na_2CO_3 solution and water up to the 50-mL mark. After 30 min, absorbance was measured at 760 nm on a UV-vis spectrophotometer (Jenway-6305) (Jenway, Stone, Staffordshire, United Kingdom). Gallic acid was used as a standard in order to express the results as mg of gallic acid equivalents (GAEs). The total tannin content was measured by acidic hydrolysis of proanthocyanidin resulting in carbocation formation partially converted into red cyanidin (Ribéreau Gayon and Stonestreet 1966). The procedure required the preparation of two samples, each containing 4 mL of sample, 2 mL of water and 6 mL of HCl (12 N). One tube was heated at 100°C in a waterbath for 30 min and 1 mL of pure ethanol was added. The other sample was not heated but received 1 mL of ethanol. The difference of absorbance was measured at 550 nm on a 10-mm optical path. The concentration was obtained in g/L by the calculation: $19.33 \times \Delta A$ for wines and musts and $19.33/50 \times \Delta A$ for grape extracts. Anthocyanin content was determined by the SO_2 bleaching procedure (Ribéreau Gayon and Stonestreet 1965).

Proanthocyanidin and anthocyanin HPLC analyses (grape purified extracts and wines)

Proanthocyanidin monomer and oligomer analyses. Monomeric/oligomeric tannin extracts were solubilised in a methanol/water solution (50:50, v/v) at concentrations of 1 g/L for seed extracts and 6 g/L for skin extracts. Wines were filtered (0.45 µm) and directly injected for HPLC analyses.

The equipment used for HPLC analysis consisted of a Thermo-Finnigan UV-vis detector (UV-vis 200), a Thermo-Finnigan autosampler and a Thermo-Finnigan (San Jose, CA, USA) ternary pump coupled to an Xcalibur data treatment system. Separation was performed on a reversed-phase Agilent C18 (250 mm × 4 mm, 5 µm) column. The mobile phases were 50 mM dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid (solvent A), 20% solvent A with 80% acetonitrile (solvent B) and 0.2 M orthophosphoric acid adjusted with ammonia to pH 1.5 (solvent C) at a flow rate of 0.5 mL/min. Initial A and B were set at 97 and 3%, respectively. The ternary mobile phase gradient was as following: 97% A and 3% B at 5 min; 92% A and 8% B at 15 min; 0% A and 8% B at 18 min; 0% A and 13% B at 30 min; 0% A and 20% B at 55 min; 0% A and 25% B at 60 min; 0% A and 30% B at 70 min; 0% A and 80% B at 75 min; 0% A and 97% B at 80 min; 97% A and 3% B from 82 min to 84 min. Eluting peaks were monitored at 280 nm. Identification of mean peaks was performed by comparison with injected external standards and previous results (Chira 2009). Calibration curves were established at 280 nm using external standards either commercial or synthesised (C, EC, ECG, B1, B2, B3, B4, T). The results were converted to milligrams of dried skin or seed weights and to mg/L of wine.

Determination of mDP. Proanthocyanidin mDP was determined for seed and skin extracts both in monomeric/oligomeric tannin fractions and in polymeric tannin fractions as well as in wines by phloroglucinolysis (Drinkine et al. 2007). The oligomeric and polymeric products were depolymerised in the presence of a nucleophilic agent (phloroglucinol) in an acid medium. Reaction products were analysed by HPLC-MS on a Hewlett-Packard 1100 series (Agilent, Massy, France) including a pump module and a UV detector and coupled to a Micromass Platform II simple quadruple mass spectrometer (Micromass-Beckman, Roissy Charles de Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in negative-ion mode. The source's temperature was 120°C, the capillary voltage was set at 3.5 kV and the cone voltage was −30 eV. Both systems

were operated using Masslynx 3.4 software (Waters, Milford, MA, USA). The absorbance was recorded at 280 nm and mass spectra were recorded in the range of 50 to 1500 amu. Separation was performed on a reversed-phase Waters XTerra RR C18 (Waters, Milford, MA, USA) (100 mm × 4.6 mm, 3.5 µm) column at room temperature. A binary gradient system was employed using 1% (v/v) aqueous acetic acid (solvent A) and MeOH (solvent B) at a flow rate of 1 mL/min. For grape seeds, the elution conditions were: 5% B at t0; 16% B at 1 min, 22% B at 7 min, 35% B at 8 min, 42% B at 15 min. The column was then washed with 100% B for 3 min and re-equilibrated with 5% B for 4 min before next injection. For grape skins and wines the elutions conditions were slightly modified: 5% B at t0; 5% B at 25 min, 20% B at 45 min, 40% B at 70 min, 100% B at 71 min. The column was then washed with 100% B for 9 min and re-equilibrated with 5% B for 4 min before the next injection.

Anthocyanin analyses

Before injection, skin anthocyanin extracts were dissolved in water/methanol solution (50:50, v/v) at concentration of 10 g/L. Wines were filtered (0.45 µm) and directly injected for HPLC analyses. HPLC-UV analyses were performed by means of a Beckman System Gold (Beckman, Roissy Charles de Gaulle, France) with a 126 pump module and a 168 diode array detector and 32Karat 5.0 Software. Separation was performed on a reversed-phase C18 Licrospher column (250 × 4 mm, 5 µm). The injected volume was of 20 µL. The mobile phases were water/formic acid (95:5, v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B) at a flow rate of 1 mL/min. Initial solvent B was set at 10%. The mobile phase gradient was as following: 35% B at 45 min, 100% B at 46 min. The column was then washed with 100% B for 3 min and re-equilibrated with 10% B for 5 min before the next injection. Eluting peaks were monitored at 520 nm. Identification of mean peaks was performed by comparison with injected external standards and previous results (Chira 2009). Concentrations were expressed as malvidin 3-*O*-glucoside equivalent. The results were converted to milligram of dried skin weights and mg/L for wines.

Sensory analyses (wines)

Sensory analyses were carried out 3 months after the wines were bottled. The wines were evaluated by 20 judges, from the Oenology department of the University of Bordeaux. They were all selected on the basis of interest and availability as well as their experience in red wine sensory analysis. All analyses were performed in a specific room at 20°C with isolated booths. Three different triangle tests (IS0 4120 – 2007) were first set up in duplicate (two winemaking replicates), in order to determine any significant overall difference between wine control sample (0% of affected grapes) and wines containing different percentage of affected grapes (5, 25, 50, 75 and 100% in 2009 and 5, 15 and 25% in 2010). Twenty-millilitre samples were randomly presented in dark ISO approved wine glasses labelled with three-digit random codes to panellists. Panellists were asked to describe the aromatic profile of each wine. Four different 20 mL-wine samples were presented per session in the same conditions than for triangular tests (dark glasses, randomised presentation, duplication). After smelling and tasting, panellists marked the intensity of each chosen attribute on a 0- to 7-scale. The attributes were chosen from a list normally used for wine sensory description (fruitiness, vegetal-herbal like, reduced, oxidised, earthy-wild mushroom, acidity, bitterness, astringency). The panellists rinsed their mouth with water and rested for 30 s between samples. After checking that the factor 'panellist' was not significant (homogeneity of the panel), one-way analysis of variance (ANOVA) was performed to test the effects on each sensory attribute.

Statistics

All measurements were performed in duplicate. Results are expressed as means ± standard deviation. One-way ANOVA was performed to test the effects of factors (different samples) on each variable (TPC, total tannin, anthocyanin, phenol concentrations, mDP). If significant effects were found at a 95% confidence interval, ANOVA was followed by a Tukey's honestly significant difference and Duncan post hoc test to identify differences among groups. Statistical analyses (ANOVA, Tukey's honestly significant difference and Duncan's post-hoc tests) were performed using Statistica V.7 (Statsoft Inc., Tulsa, OK, USA).

Results and discussion

Analysis of berries

Seed phenolic composition. Some global analyses were first conducted in order to attempt the discrimination between grapes from healthy vines and grapes from Esca-affected vines. Procedures usually employed for wines analyses (TPC, total tannin and total anthocyanin) were applied to both kind of crude extracts (seed and skin) solubilised at appropriate concentrations in model solutions. For the seeds from 2009, a slight TPC decrease was observed between healthy and affected grapes (38.9 vs 30.3 mg GAE/g dw) but there was no affect on total tannins (Table 1). In 2010 there was, no significant difference between healthy and affected grapes for TPC and total tannin (Table 1).

With regard to proanthocyanidin composition, the flavan-3-ol monomers (C, EC, ECG) and oligomers (B1, B2, B3, B4 dimers and a trimer T) were identified and quantified in both seeds and skins (Table 1). For 2009, no significant differences were observed (Table 1). In 2010, significant differences between healthy and affected seeds were observed only for C and EC concentrations, which decreased from 46 and 43%, respectively.

Proanthocyanidin characteristics such as mDP, percentage of galloylation (%G) and percentage of prodelphinidins (%P) were also investigated for the two kinds of fractions (monomeric/oligomeric and polymeric tannin fractions) of seeds and skins of both grape types (Table 1).

With regard to seed tannin mDP (Table 1), 2009 and 2010 vintage results were in good agreement with only small deviations in mDP for 2009 vintage. No substantial differences between healthy and affected grape seeds in monomeric/oligomeric fractions as well as in polymeric fractions were observed. With regard to %G, both the 2009 and 2010 results confirm that Esca has little effect seed composition.

Looking all phenolic seed results, few variations in phenolic composition appeared between healthy and affected grape seeds for both vintages. Catechin and epicatechin concentrations varied between healthy and affected grape seeds. However, the contrasting trends observed for 2009 and 2010 vintages did not agree with a previous study (Calzarano et al. 2004).

Skin phenolic composition. No significant differences were observed between healthy and affected grapes for TPC and total tannin contents. Esca-affected grapes had a 15% lower total anthocyanin content than healthy grapes (Table 1). In the case of 2010-results, this pattern was repeated for all phenol analyses.

Table 1. Concentrations and structural characteristics of seed and skin phenolic compounds of healthy and Esca-affected grapes.

	2009		2010	
	Healthy	**Affected**	**Healthy**	**Affected**
Seeds				
TPC	38.9 ± 0.3[a]	30.3 ± 0.9[b]	40.4 ± 2.3[a]	43.3 ± 2.2[a]
Total tannin	100.3 ± 3.1[a]	96.3 ± 2.2[a]	131.3 ± 9.6[a]	122.7 ± 11.2[a]
C	1.414 ± 0.017[a]	1.512 ± 0.019[a]	2.505 ± 0.204[b]	1.354 ± 0.143[a]
EC	1.571 ± 0.020[a]	1.497 ± 0.021[a]	1.903 ± 0.153[b]	1.085 ± 0.105[a]
ECG	nd	nd	0.069 ± 0.007[a]	0.051 ± 0.000[a]
B1	0.134 ± 0.001[a]	0.131 ± 0.003[a]	0.148 ± 0.017[a]	0.098 ± 0.017[a]
B2	0.581 ± 0.013[a]	0.560 ± 0.007[a]	0.617 ± 0.062[a]	0.427 ± 0.056[a]
B3	0.221 ± 0.006[a]	0.214 ± 0.000[a]	0.250 ± 0.018[a]	0.144 ± 0.017[a]
B4	0.686 ± 0.012[a]	0.659 ± 0.007[a]	0.291 ± 0.031[a]	0.187 ± 0.028[a]
T	0.042 ± 0.005[a]	0.039 ± 0.002[a]	0.143 ± 0.017[a]	0.080 ± 0.006[a]
Monomeric/oligomeric fraction				
mDP	3.1 ± 0.0[b]	2.7 ± 0.0[a]	2.0 ± 0.0[a]	2.0 ± 0.0[a]
%G	36.4 ± 2.1[a]	31.9 ± 0.3[a]	16.1 ± 0.5[a]	17.9 ± 1.7[a]
Polymeric fraction				
mDP	18.6 ± 0.2[a]	20.0 ± 0.3[b]	10.0 ± 0.2[a]	9.6 ± 0.9[a]
%G	60.0 ± 1.6[a]	59.7 ± 0.5[a]	25.8 ± 2.2[a]	30.9 ± 2.0[b]
Skins				
TPC	27.6 ± 3.9[a]	24.8 ± 2.3[a]	29.6 ± 0.6[a]	30.8 ± 2.6[a]
Total tannin	84.3 ± 13.5[a]	82.2 ± 4.2[a]	106.1 ± 5.2[a]	94.8 ± 8.2[a]
Total anthocyanin	25.6 ± 0.4[b]	21.7 ± 0.1[a]	29.8 ± 0.1[b]	25.6 ± 0.6[a]
C	0.025 ± 0.000[b]	0.020 ± 0.000[a]	0.022 ± 0.001[b]	0.018 ± 0.000[a]
EC	0.006 ± 0.000[b]	0.003 ± 0.000[a]	0.006 ± 0.001[b]	0.003 ± 0.000[a]
ECG	nd	nd	nd	nd
B1	0.012 ± 0.001[a]	0.012 ± 0.001[a]	0.015 ± 0.001[a]	0.014 ± 0.001[a]
B2	0.006 ± 0.000[a]	0.005 ± 0.000[a]	0.006 ± 0.000[a]	0.006 ± 0.001[a]
B3	0.006 ± 0.000[a]	0.007 ± 0.001[a]	0.007 ± 0.000[a]	0.007 ± 0.001[a]
B4	nd	nd	nd	nd
T	0.001 ± 0.000[a]	0.002 ± 0.000[b]	0.011 ± 0.001[a]	0.012 ± 0.001[a]
Monomeric/oligomeric fraction				
mDP	nd	nd	6.7 ± 0.2[a]	7.1 ± 0.5[a]
%G	nd	nd	57.8 ± 2.8[a]	41.5 ± 3.5[a]
%P	nd	nd	18.8 ± 1.0[a]	24.6 ± 13.6[a]
Polymeric fraction				
mDP	nd	nd	19.2 ± 0.5[b]	14.2 ± 0.3[a]
%G	nd	nd	21.8 ± 7.3[a]	12.4 ± 1.7[a]
%P	nd	nd	31.1 ± 5.4[a]	46.9 ± 0.7[a]
Dp	1.587 ± 0.087[b]	1.193 ± 0.017[a]	3.950 ± 0.299[b]	2.176 ± 0.027[a]
Cy	0.173 ± 0.001[b]	0.117 ± 0.008[a]	0.875 ± 0.056[b]	0.379 ± 0.013[a]
Pt	1.071 ± 0.020[b]	0.849 ± 0.015[a]	2.253 ± 0.079[b]	1.511 ± 0.050[a]
Pn	0.847 ± 0.060[a]	0.763 ± 0.015[a]	1.665 ± 0.091[b]	1.183 ± 0.056[a]
Mv	6.370 ± 0.506[a]	5.835 ± 0.298[a]	10.381 ± 0.909[a]	9.709 ± 0.247[a]
Ac	3.033 ± 0.151[a]	3.509 ± 0.060[a]	0.856 ± 0.063[a]	0.921 ± 0.043[a]
Coum	1.010 ± 0.062[a]	0.977 ± 0.021[a]	1.209 ± 0.083[a]	1.069 ± 0.084[a]

Data are means of duplicate determination. In units of mg/g dw seed or skin ± standard deviation over the two replications in one grape sample. Analysis of variance to compare data: for each vintage, values with different letters within each row are significantly different (Duncan's test, $P < 0.05$). %G, percentage of galloylation; %P, percentage of prodelphinidins; Ac, sum of peonidin-3-*O*-acetylmonoglucoside and malvidin-3-*O*-acetylmonoglucoside; Coum, sum of peonidin-3-(6-*O*-*p*-coumaroyl)monoglucoside and malvidin-3-(6-*O*-*p*-coumaroyl)monoglucoside; Cy, Cyanidin-3-*O*-monoglucoside; Dp, delphinidin-3-*O*-monoglucoside; mDP, mean degree of polymerisation; Mv, malvidin-3-*O*-monoglucoside; nd, not detected; Pn, paeonidin-3-*O*-monoglucoside; Pt, petunidin-3-*O*-monoglucoside; TPC, total phenol content.

Proanthocyanidin composition analysis revealed significant reductions of 20% and 50% of C and EC concentrations respectively between healthy and affected grapes in 2009 (Table 1). Moreover, a 50% increase of trimer concentration was noticed. For 2010 vintage, 16% and 45% lower concentrations of EC and C were measured in affected grapes confirming the 2009 trend. For the 2010 vintage, no further effects on individual proanthocyanidin concentrations were seen.

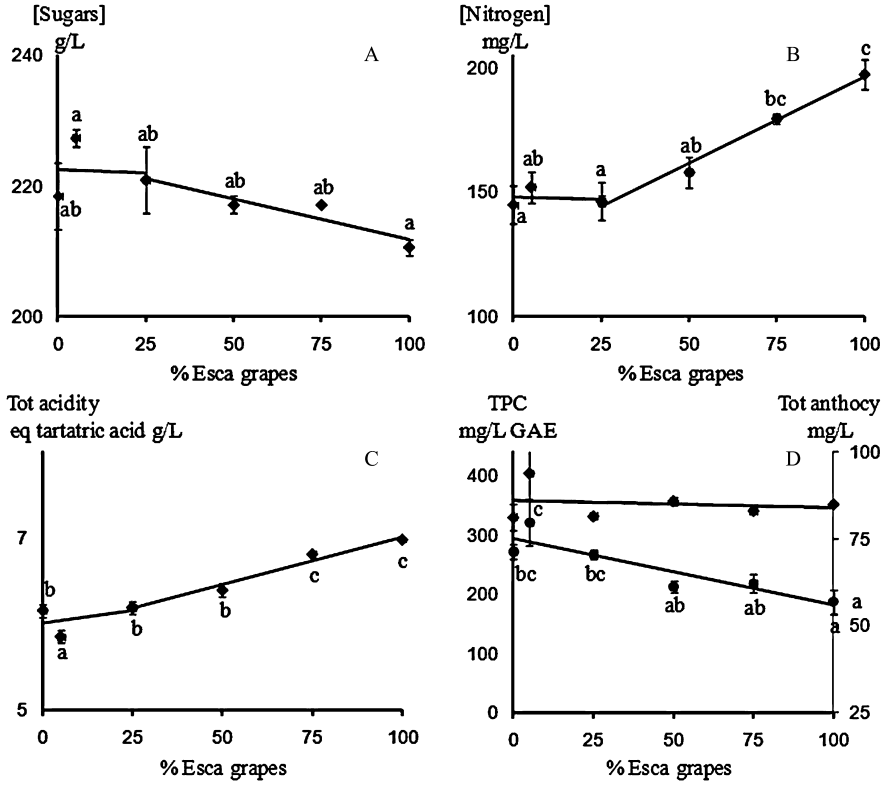

Figure 1. Reducing sugar content (A), nitrogen amount (B), total acidity in tartaric acid equivalent (C), total phenol content (TPC) in gallic acid equivalent (GAE; diamond) and total anthocyanin (circle) (D) of musts containing different percentages of Esca fruit for the 2009 vintage. For each parameter, values with different letters are significantly different (Duncan's test, $P < 0.05$).

Skin mDPs were assessed for the two kinds of proanthcyanidin fractions only for the 2010 vintage (Table 1). In the monomeric/oligomeric fraction, mDP values were around 7 (6.7 and 7.1) whether grapes were affected or healthy. Conversely, in polymeric fractions, healthy grapes had an mDP of 19 while affected grapes had a considerably lower mDP (14).

Finally, skin anthocyanin decreased from 10 to 30% in 2009 and from 30 to 60% in 2010. Results for four major monglucosilated anthocyanins (delphindin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and paeonidin-3-*O*-glucoside) were in good agreement with total anthocyanin amounts (Table 1).

In summary for skin phenolic composition, C and EC skin concentrations appeared to be appropriate tools for discriminating healthy and Esca-affected grapes, independently of the vintage. Variations of mDP in skin polymeric fraction also could also be useful in this regard but these preliminary results would need to be confirmed for other vintages. Esca also has an impact on anthocyanin molecules known to be sensitive compounds.

Esca impact appears to be moderate on seed phenolic composition but more pronounced on skin phenolics, and tannin characteristics. Several studies have reported that Esca developement in vine affects carbohydrate physiology, water transport through xylem and photosynthesis with a consequent adverse impact on carbon metabolism (Petit et al. 2006, Christen et al. 2007). Considering these results, it is conceivable that flavonoid pathway responsible for tannin and anthocyanin synthesis was also affected by Esca, resulting in lower phenolic compound concentrations and reduced polymerisation. A recent study showed that polypeptide fractions secreted *in vitro* by *P. chlamydospora* and *P. aleophilum* induced activation of the plant secondary metabolism and modified anthocyanin synthesis (Luini et al. 2010). On the other hand, and for the first time, a laccase was recently purified from a *Fomitiporia mediterranea* fungus, the main wood rot agent of the grapevine disease Esca (Abou-Mansour et al. 2009). This enzyme is able to oxidise a large range of different structural phenolic compounds indicating that some oxidative processes could be related to Esca symptoms. Thus, phenolic compounds and particularly proanthocyandins and anthocyanins could be involved in defense mechanisms, react with this extracellular fungal enzyme and be consecutively degraded. This may be an alternative explanation for the lower phenolic compound concentrations observed in Esca-affected grapes if a direct contact between laccase and phenol occurs.

Analysis of musts. Different 10-kg batches of fruit containing 0 (control), 5, 25, 50, 75 and 100% of Esca-affected grapes for the 2009 vintage and 0 (control), 5, 15 and 25% for the 2010 vintage were vinified in duplicate. Musts before maceration and SO_2 addition were analysed.

In 2010, no differences were observed between control, 5, 15 and 25% of Esca grape musts for all the analysed classical parameters (i.e. reducing sugars, pH, assimilable nitrogen, malic acid, total acidity, data not shown). In 2009, no differences were observed when up to 25% of Esca was added. However, for the higher percentage batches (>25% of Esca fruit) some interesting trends emerged (Figure 1). First, a progressive reducing sugars content decrease was related to an increase in affected grape percentages, reaching a 4% reduction between the control musts and the 100% Esca fruit musts. On the contrary, a proportional increase of total acidity up to 15% (100% batch) was linked with increasing Esca grapes percentages. Similarly, a 39% rise of assimilable nitrogen was observed between the control and 100% of affected fruit. These results might be due to the observation that healthy grapes and Esca-affected grapes were not at the same maturity level when harvested. These data are in good agreement with previous studies indicating that Esca caused delayed ripening through reduced photosynthesis (Calzarano et al. 2009a).

Regarding phenol, no variations were observed in 2010 for TPC, total tannin and anthocyanin. In 2009, differences in total anthocyanin were observed for musts containing more that

25% of Esca-affected grapes. A 20% decrease in total anthocyanin was recorded between the control and the 100% Esca treatment.

These results reflect the moderate differences in grape phenolic composition between healthy and Esca-affected fruit. The decrease in anthocyanin concentrations is consistent with a ripening delay in Esca-affected grapes. Taken together, these data seem to indicate that a '25% of Esca-affected grapes' threshold is necessary before there are any adverse effects on chemical and phenolic composition before maceration.

Analysis of wines. After micro-scale winemaking, six different wines in 2009 (containing 0, 5, 25, 50, 75 and 100% of Esca-affected fruit) and four different wines in 2010 (containing 0, 5, 15 and 25% of Esca-affected fruit) were obtained. Classical analyses (alcohol % volume, pH, total acidity, volatile acidity, tartaric acid concentration) were performed by means of infra-red technique (Foss WineScan™ 79000) (Table 2). In 2010, control wines and 25% batch wines did not show any significant differences for any of the classical parameters. In 2009, a significant reduction of alcohol percentage from 12.7 to 11.9 (vol %) between the control and 100% Esca fruit treatment was observed, reflecting the sugar content decrease previously observed in musts. Interestingly, pH was slighly increased while total acidity slightly decreased between the two extreme batches.

Phenolic analyses (TPC, total tannin and anthocyanin) showed no variation in composition due to addition of affected

Table 2. Classical enological parameters of wines containing different percentages of Esca-affected fruit.

	2009					2010				
	Alcohol	**pH**	**Total acidity**	**Volatile acidity**	**Tartaric acid**	**Alcohol**	**pH**	**Total acidity**	**Volatile acidity**	**Tartaric acid**
0%	12.71 ± 0.10^{bc}	4.05 ± 0.03^{a}	3.06 ± 0.04^{b}	0.31 ± 0.00^{a}	1.48 ± 0.11^{a}	13.14 ± 0.06^{a}	3.83 ± 0.04^{a}	3.49 ± 0.00^{a}	0.25 ± 0.02^{a}	2.48 ± 0.00^{a}
5%	12.87 ± 0.07^{c}	4.15 ± 0.01^{cd}	3.03 ± 0.01^{ab}	0.29 ± 0.01^{a}	1.53 ± 0.02^{a}	13.29 ± 0.05^{ab}	3.83 ± 0.04^{a}	3.61 ± 0.13^{a}	0.27 ± 0.01^{a}	2.56 ± 0.01^{ab}
15%	nd	nd	nd	nd	nd	13.35 ± 0.01^{b}	3.78 ± 0.03^{a}	3.69 ± 0.02^{a}	0.26 ± 0.01^{a}	2.70 ± 0.05^{b}
25%	12.68 ± 0.02^{bc}	4.07 ± 0.01^{ab}	3.13 ± 0.03^{b}	0.31 ± 0.01^{a}	1.49 ± 0.04^{a}	13.20 ± 0.05^{ab}	3.80 ± 0.02^{a}	3.69 ± 0.02^{a}	0.27 ± 0.02^{a}	2.70 ± 0.09^{b}
50%	12.39 ± 0.03^{bc}	4.09 ± 0.03^{abc}	3.06 ± 0.01^{b}	0.31 ± 0.01^{a}	1.52 ± 0.04^{a}	nd	nd	nd	nd	nd
75%	12.35 ± 0.21^{b}	4.13 ± 0.03^{bcd}	3.03 ± 0.02^{ab}	0.30 ± 0.02^{a}	1.50 ± 0.02^{a}	nd	nd	nd	nd	nd
100%	11.86 ± 0.23^{a}	4.18 ± 0.01^{d}	2.94 ± 0.04^{a}	0.29 ± 0.02^{a}	1.59 ± 0.08^{a}	nd	nd	nd	nd	nd

Data are means of duplicate determination. In units of volume % for alcohol, equivalent sulfuric acid (g/L) for total acidity, equivalent sulfuric acid (g/L) for volatile acidity, g/L for tartaric acid ± standard deviation over the two replications. Analysis of variance to compare data: for each parameter, values with different letters within each column are significantly different (Duncan's test, $P < 0.05$).

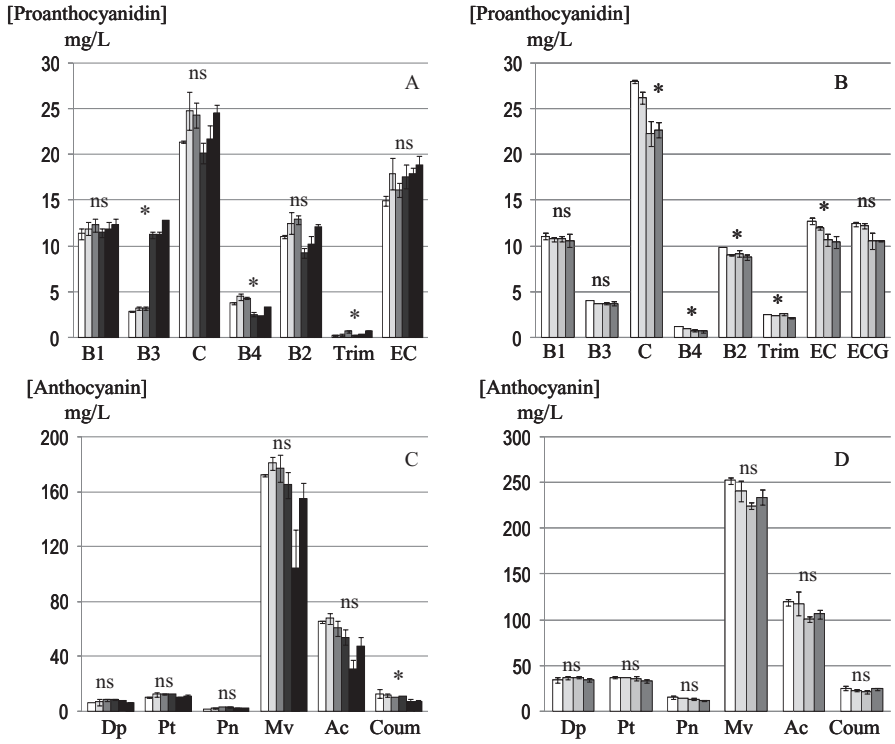

Figure 2. Individual proanthocyanidin concentrations ((A) 2009 vintage, (B) 2010 vintage) and individual anthocyanin concentrations ((C) 2009 vintage, (D) 2010 vintage) of wines containing different percentages of Esca fruit (grey colour gradations from blank, control or 0% to black, 100% with 5, 25, 50 and 75% intermediates for 2009 vintage. Grey colour gradations from blank, control or 0% to middle grey, 25% with 5 and 15% intermediates for 2010 vintage). Analysis of variance to compare data (Duncan's test, $P < 0.05$): for each compound. 'ns' means no significant difference between the different percentages, while '*' indicate significant differences at minimum between the two extreme treatments (0% vs 100% for 1009, 0% vs 25% for 2010). TPC, total phenol content; Dp, delphinidin-3-*O*-monoglucoside; Cy, cyanidin-3-*O*-monoglucoside; Pt, petunidin-3-*O*-monoglucoside; Pn, paeonidin-3-*O*-monoglucoside; Mv, malvidin-3-*O*-monoglucoside; Ac, sum of peonidin-3-*O*-acetylmonoglucoside and malvidin-3-*O*-acetylmonoglucoside; Coum, sum of peonidin-3-(6-*O*-*p*-coumaroyl)monoglucoside and malvidin-3-(6-*O*-*p*-coumaroyl)monoglucoside.

Table 3. Proanthocyanidin characteristics (mDP, %G, %P) of wines containing different percentages of Esca-affected fruit.

	mDP		%G		%P	
	2009	2010	2009	2010	2009	2010
0%	1.8 ± 0.0^{ab}	5.4 ± 0.2^{a}	2.9 ± 0.1^{a}	20.4 ± 0.5^{ab}	22.1 ± 0.2^{b}	36.1 ± 0.3^{a}
5%	1.9 ± 0.1^{b}	5.7 ± 0.1^{a}	5.7 ± 0.8^{b}	20,4 ± 1.1^{ab}	21.7 ± 0.3^{b}	40.3 ± 0.5^{b}
15%	nd	5.9 ± 0.1^{ab}	nd	18.6 ± 0.4^{a}	nd	41.7 ± 0.6^{b}
25%	1.9 ± 0.1^{ab}	5.4 ± 0.1^{a}	5.3 ± 0.1^{b}	22.2 ± 0.3^{a}	22.0 ± 0.6^{b}	34.4 ± 0.9^{a}
50%	1.8 ± 0.0^{ab}	nd	4.9 ± 0.3^{ab}	nd	21.5 ± 0.3^{b}	nd
75%	1.7 ± 0.0^{a}	nd	5.1 ± 0.1^{b}	nd	17.7 ± 0.8^{a}	nd
100%	1.7 ± 0.0^{ab}	nd	3.9 ± 1.1^{ab}	nd	18.4 ± 1.1^{a}	nd

Data are means of duplicate determination. Analysis of variance to compare data: for each parameter, values with different letters within each column are significantly different (Duncan's test, $P < 0.05$). %G, percentage of galloylation; %P, percentage of prodelphinidins; mDP, mean degree of polymerisation.

fruit during vinification in both 2009 and 2010 (data not shown). This reflects the moderate impact Esca had on the global phenolic content in grapes.

Nevertheless, individual proanthocyanidin concentrations (Figure 2a and b), differed for B3, B4 and trimer concentrations in 2009. B3 concentration substantially declined for the 50% fruit treatment while B4 concentrations declined for the 50% fruit treatment. In the case of the 2010 vintage, significant differences between the control and the 25% affected fruit treatment were also observed. This was in agreement with the fruit phenolic composition, C, B4, B2, EC and trimer concentrations, which followed a declining trend, proportional to the increase in affected fruit percentages in all cases except for trimer concentration.

In 2009 and in 2010, individual anthocyanin concentrations did not show significant differences between the treatments, except for coumaroylated anthocyanins, which slight decreased in 2009 (Figure 2c and d). Nevertheless, for both vintages slight concentrations decreases in relation to higher percentages of affected fruit in wine were be observed for the majority of anthocyanins, related with the skin phenolic composition.

Proanthocyanidin characteristics (mDP, %G, %P) of the different wines (Table 3) showed few significant variations. In spite of an observed decrease in polymeric mDP of grape skins, no mDP variation was observed in derived wines, even in the wines containing the highest percentages of Esca fruit. This suggests that low mDP tannins were in the majority of extracted tannins during maceration.

Investigation of sensory properties of Esca's wine by a triangle test showed that judges could distinguish a wine made from healthy grapes and a wine obtained from 50% of affected fruit in 2009. In 2010, judges could discriminate differences based on just 5% of Esca affected fruit.

In order to characterise each wine, judges were asked to evaluate wines on the criteria of fruit (nose and mouth), astringency and bitterness using a 0- to 7-point scale. In 2009, there was a significant difference between the control and 100% Esca wine, which was perceived as less bitter and less astringent. Esca wines were also perceived as reduced, phenolic and herbal.

In 2010, profile tests confirmed this previous trend, showing that the highest percentages Esca wines exhibited a significant decrease in fruity aroma in parallel with significant increases in earthy and herbal-like or vegetal characters (Figure 3). The ripening delay attributed to Esca-affected grapes may explain such 'green' sensory perceptions often related to 2-methoxy-3isobutylpyrazine, an herbaceous methoxypyrazine usually

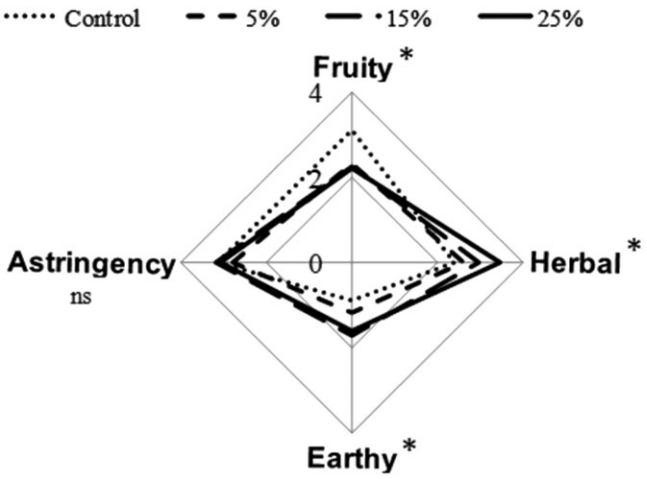

Figure 3. Sensory profile of 2010 wines containing different percentages of Esca fruit (control or 0, 5, 15 and 25%). Analysis of variance to compare data (Duncan's test, $P < 0.05$): for each descriptor. 'ns' means no significant difference between the different treatments, while '*' indicate a significant difference at minimum between the two extreme treatments (0% vs 25%).

most apparent when the grapes are not fully ripe. This is an important maturity index in Bordeaux Cabernet Sauvignon (Allen et al. 1995). No variation in astringency and bitterness was perceived by judges, in good agreement with the absence of mDP variation between the different wines.

Conclusions

Almost all the studied parameters were consistent and similar over the two assessed vintages indicating that Esca moderately affects the phenolic composition of grapes. In terms of skin composition: catechin, epicatechin and anthocyanin concentrations as well as the polymeric mDP of skin tannins were decreased in fruit from Esca-affected vines. The classical and chemical analyses of derived musts reflected a delay in ripening of Esca-affected fruit upon incorporation of more than 25% of affected fruit in musts. In wines, the impact of Esca on chemical composition was weak but the sensorial perception was more strongly affected. A loss of sensory wine quality was evident with just 5% of affected fruit with wines being perceived as having vegetal and herbaceous characters. Changes in grapevine physiology associated with Esca may have had an impact

on the flavonoid metabolism pathway. Alterations in polyphenols as a result of host defense mechanism against Esca may also, in part, explain these results.

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