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Nutritional status of vines affected with esca proper

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Summary. A vineyard of the cv. Trebbiano d'Abruzzo located in Abruzzo, Italy, was monitored for more than ten years to distinguish healthy vines not only from vines with the visual leaf symptoms of esca, but also from those vines that were esca-infected but did not produce any visual symptoms for one or more growing seasons. In the period 2004–2006, leaves and berries were collected at four phenological growth stages from three groups of vines: healthy vines, infected vines showing esca symptoms, and infected vines that did not show symptoms. The macro and micro-elements of the leaves and berries, and the quality parameters of the must were determined. Esca did not seem to affect nutrient uptake in the vines. Nevertheless there were some differences in the nutrient levels of the leaves between healthy and diseased vines consistent with the degradation of the leaf blade caused by esca. Berries from symptomatic vines were less ripe at the time of harvesting and therefore had higher levels of mineral elements. These berries also had higher levels of nitrogen, which are thought to be associated with the defence response of diseased vines to esca, as are higher levels of iron in the leaves of diseased vines. The study confirmed earlier findings that fruit composition did not differ greatly between healthy and diseased-but-asymptomatic vines. In the three-year study period there were differences in the incidence of leaf symptoms and differences in nutrient levels attributable to fertiliser applications and rainfall. These differences suggested that the amount of mineral nutrients affected the onset of esca symptoms: a higher availability of nutrients in a growing season increased the proportion of diseased vines with symptoms and lowered the proportion of diseased vines without symptoms, whereas in a growing season with the lower levels of water and potassium, the yield was reduced, but this was accompanied by an increase in the proportion of diseased vines without symptoms. It is suggested that a higher availability of nutrients for diseased vines lowers the resistance of these vines and, by improving the nutrition not only of the vines themselves but also of the esca fungi, increase fungal virulence, as a result of which there is a greater incidence of diseased vines showing leaf symptoms.

Key words: esca, grape berries, leaf symptoms, macro-elements, micro-elements.

Introduction

Esca of grapevine is a serious and injurious disease in grape-growing countries all over the world, for which no effective control exists (Di Marco *et al.*, 2000).

Since esca causes various types of wood deterioration, especially in older vines, numerous studies have attempted to determine the causal agents of these types of deterioration, and how they interact with each other (Mugnai *et al.*, 1996c; Larignon and Dubos, 1997; Fischer, 2002). The findings of these studies have led to a name, esca proper, being given to a syndrome that comprises two distinct diseases, a trachaeomycosis caused by the fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum,* and a wood rot caused by the basidiomycete *Fomitiporia mediterranea* (Graniti *et al.*, 2000; Surico *et al.*, 2006). The division of one apparent disease into two diseases was made because it was found that these fungi acted inde-

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pendently of each other in space and in time and because the leaf symptoms were caused by the trachaeomycotic fungi (Mugnai *et al.*, 1999; Graniti *et al.*, 2000, 2001; Sparapano *et al.*, 2000, 2000a, 2001; Edwards *et al.*, 2001b; Feliciano *et al.*, 2004). There was no correlation between the severity of the leaf symptoms and the severity of the wood deterioration (Calzarano and Di Marco, 2007). Indeed, diseased vines sometimes do not present any leaf and fruit symptoms at all for one or more growing seasons in succession (Calzarano and Di Marco, 1997; Surico *et al.*, 2000; Cesari *et al.*, 2005; Marchi *et al.*, 2006). Attempts to explain this phenomenon of the occasional discontinuity of symptoms from one (or more) growing seasons to the next have so far not been successful; consequently, to identify a vine by its visual symptoms as infected with esca it is necessary to inspect it for several years in a row (Calzarano *et al.*, 2004; Cesari *et al.*, 2005).

Since, as was mentioned, esca cannot be controlled, it is important to determine the exact loss of yield quality that is caused by the disease. This has been the subject of a number of investigations in vineyards in Abruzzo, Italy (Calzarano *et al.*, 2001, 2004, 2007). In those studies leaf symptoms were inspected regularly over many years and this made it possible in the end to distinguish reliably between healthy vines, diseased-and-symptomatic vines, and diseased-but-asymptomatic vines. Thus it became possible to analyse and to compare some yield parameters at harvesting for these three groups of vines. However these initial results suggested that more extensive investigations were needed. This was done in the three-year period from 2004 to 2006, which is the subject of the present report (during which the vines still continued to be inspected for esca symptoms). In the growing seasons of this period the nutrient levels in the leaves and berries of each group of vines were measured and compared between groups during different phenological growth stages. The chemical properties of the musts were also determined and compared between groups. The aim of the analysis performed was to verify the nutritional status of the three groups of vines to ascertain possible imbalances of nutrients in diseased vines, and to evaluate whether these imbalances played a role in the mechanisms causing the leaf symptoms. Another aim was to confirm and define the loss in quality of the must from esca-affected vines as compared with the must from healthy vines.

Materials and methods

Nature of the vineyard and monitoring of foliar symptoms

The study was carried out in 2004–2006 in a vineyard located at Controguerra, in the province of Teramo, Abruzzo, Italy. The vineyard had been established for 32 years and was affected with esca proper. It had an area of 5984 m^2 and contained 740 vines cv. Trebbiano d'Abruzzo on 420A rootstock. Vines were trained to the Geneva Double Curtain (GDC) system with a planting arrangement of 2 (within rows) × 4 (between rows) and an average yield of up to 16.5 kg per vine in years of optimal yield. The vineyard had a clay-calcareous soil; when this soil sampled and analysed following the method of Violante (2000) it had an alkaline pH of 8.45 determined in a water suspension, an active limestone content of 16.5% and a SO content that was low at 1.25%. In 2004 and 2005 (but not in 2006) the vineyard was thoroughly fertilised with triple N-P_2O_5-K_2O fertiliser, with titres of 7-7-7 and 16-10-16 respectively, at a rate of 300 kg ha^{-1}. The yield of wine-producing grapes from the entire vineyard (including both healthy and esca-diseased vines) was 7880 kg in 2004, 11,400 kg in 2005 and 6500 kg in 2006.

The vines in the vineyard had been inspected for the leaf symptoms of esca in September every year ever since 1994, and these inspections were still continued in the 2004–2006 period studied here, in order to have a sufficiently ample basis to distinguish between healthy vines, diseased-and-symptomatic (DSY) vines, and diseased-but-asymptomatic (DAS) vines, and to collect samples from each of these distinct groups of vines for analysis. Symptoms were recorded as in previous studies (Calzarano *et al.*, 2007). DSY vines comprised 18.8% in 2004, 25.6% in 2005, and 9.1% in 2006, while DAS vines amounted to 47.4% in 2004, 42.9% in 2005 and 59.6% in 2006.

The number of rain events and amount of precipitation in the vineyard during the study period, which had been recorded by the Servizio Idrografico e Mareografico of the Abruzzo Region, was reviewed for correlations (Table 1).

Sampling

In each of the three years, one berry sample each was harvested from each of six individual vines in each vine group (healthy, DSY, DAS).

Table 1. Rainfall on 2004–2006 in Controguerra vineyard.

Year		Rainfall												
		January	February	March	April	May	June	July	August	September	October	November	December	Yearly total	March-September total
2004	mm	24.6	29.2	19.4	99.4	65.8	84	39.4	38.2	188.8	20.4	29.8	220.2	859.2	535.0
	Rainy days (No.)	6	8	9	11	10	8	4	3	5	3	9	12	88	50
2005	mm	72.8	100.8	45.8	64.8	36.2	33	19.2	84	51	41	78.4	0	627	334
	Rainy days (No.)	10	10	5	6	4	3	2	8	3	9	14	0	74	31
2006	mm	63.4	57.2	49.8	32.2	11	34.4	11.2	30.8	24.4	6.8	15.6	17.4	354.2	193.8
	Rainy days (No.)	10	6	10	6	5	7	2	7	7	1	3	2	66	44

Samples consisted of berries taken alternately from the wings, tips and centers of every cluster of each vine and each sample (from one vine) weighed about 0.5 kg.

Ten leaves were also taken from each of these vines. The leaves grew opposite some of the sampled grape bunches located at about the mid-point along the vine-shoot. Here too therefore one set of leaf samples was collected from each of six vines in each of the three vine groups. The symptomatic leaves collected all had the same amount of green area, which was about 35% of the entire leaf area.

In each growing season, the leaves were sampled at four phenological growth stages as defined in the BBCH classification (Lorenz *et al*., 2005): 77, 'berries beginning to touch' (26, 25 and 21 July in 2004, 2005 and 2006 respectively); 83, 'berries developing colour' (24, 22 and 21 August in 2004, 2005 and 2006); 85, 'softening of berries' (7 and 4 September in 2004 and 2006); and 89, 'berries ripe for harvest' (23, 20 and 19 September in 2004, 2005 and 2006). Fruit clusters were sampled each year only at the last three growth stages mentioned.

Analytical determination

Sample preparation

Immediately after collection, berry samples were weighed on an analytical scale, and then ground in a mortar. About half the homogeneous liquid sample obtained was taken under continued stirring for analysis of the macro- and micro-elements. The remaining part was manually pressed to separate the must from the skin and the seeds. The must obtained was used for the other chemical determinations. The leaves were washed in deionised water, air-dried on absorbent paper in a well-ventilated and protected environment, and then dried further in a stove at 70°C.

Must analysis

Total acidity, pH and reducing sugars of the musts from each of the samples were determined following EC Regulation 2676/90, appendix 13, 24 and 5 respectively (Anonymous, 1990).

Organic acids were determined on about 10 ml of each must as follows. The must samples were homogenised with an Ultra-Turrax (Ika, Heidelberg, Germany) and passed through filter paper. The filtrate was centrifuged at 6000 rpm for 3 min, after

which 1 ml of the supernatant was diluted with 9 ml of H_2SO_4 0.08 M, and again centrifuged at 6000 rpm for 3 min, after which the supernatant was analysed with a HPLC instrument (Series 200 System, Perkin Elmer, Monza, Italy) to determine the content of organic acids with a diode array detector (DAD) at channels of 210 and 220 nm. The analysis was carried out using an ion exchange column (Aminex HP87 H Ion Exclusion, Bio Rad, Milan, Italy; 300 mm×7.8 mm), thermostat set to 55°C with a pre-column (Cation H cartridge, 30 mm×4.6 mm; Bio Rad), using as the mobile phase H_2SO_4 0.08 M at a flow rate of 0.6 ml min^{-1} with an elution time of 22 min. With this method the peaks of the acids were well resolved and their retention times were: 8.83 min for tartaric acid, and 9.70 min for malic acid (SD 0.018 and 0.031; CV% 0.2 and 0.3). Measurements were made in duplicate by injecting 20 μl for each measurement. The calibration lines were found by injecting 20 μl of the standards at a known concentration of tartaric and malic acid in a solution of H_2SO_4 0.08 M. Five standards were prepared starting with a concentration of 1 mg ml^{-1} and successive standards by serial dilution at a ratio of 1:2.

Macro- and micro-elements in the berries and leaves

Each ground berry sample was homogenised in an Ultra-Turrax and 100 ml of each sample was placed in a vial, frozen to -18°C and freeze-dried in an Alpha 2-4 freeze-drier (Christ, Osterode am Harz, Germany). The freeze-dried powders thus obtained were ground in an agate ball-mill (PM 200, Retsch, Haan, Germany), then dried in a desiccator under vacuum. The humidity of the dried powder was determined at 105°C. Five-hundred mg of each of the dried powders was mineralised with 8 ml of 67% HNO_3 in teflon tubes hermetically sealed. The samples were digested with a microwave digester (ETHOS900; Millestone, Shelton, CT, USA) programmed for the purpose. The cooled digestion solutions were brought to a final volume with Milli Q water in 25 ml plastic flasks and stored in polyethylene containers. The solutions were then analysed with an atomic absorption spectrophotometer using an air-acetylene flame (Analyst 700; Perkin Elmer, Waltham, MA, USA), after they had been diluted, and using highly purified standard solutions of the various elements. Ca, Mg, Zn, Mn and Fe were determined in absorption, K and Na in emission. Phosphorus was determined in the same digestion solutions by a colorimetric technique with a molybdenum reagent, measuring the absorbance of the solutions at 525 nm and interpolating the concentration values with calibration lines obtained from standard phosphate solutions of the requisite concentration (Murphy and Riley, 1962).

Nitrogen was determined on 1–2 mg of the powders (weighed accurately) using an HCNS elementary analyser (EA 1108; Fisons, Milan, Italy).

The dried leaves were ground in a ball mill in the same way as the berries had been after freeze-drying. The powders were kept for 12 hours in a drier under a vacuum produced with an oil pump before determining the macro- and micro-elements, which was done on 500 mg of powder, in the same way as already described for the berries.

All the determinations were carried out in duplicate and average results are shown.

Statistical analysis

Measurements on must, leaf, and berry samples from the six vines in each of the vine groups (healthy, DSY, and DAS), allowed data comparison of pairs (DSY-healthy; DAS-healthy) in each of the three study years and for four phenological growth stages. Data comparisons were subjected to analysis of variance and Student's *t*-test.

Results

Macro-elements in leaves and berries

The nutritional status of healthy cv. Trebbiano d'Abruzzo vines of the Controguerra vineyard used as reference for DSY and DAS vines was determined. This is usually performed by comparing the levels of nutrients and their ratios in the leaves with those of the optimal ranges of concentrations values for the same cultivar in the period between 'setting' and 'berries developing colour' (stages 77 and 83) reported in the literature. However, since these reference data are lacking for the cv. Trebbiano d'Abruzzo, the nutrient measurements recorded for healthy vines from the Controguerra vineyard in growth stages 77 and 83 (Table 2) were compared with the medium values published in international studies on a variety of cultivars, or with the levels from cultivars typically grown in Italian vineyards, as reported

by Fregoni (1998). Moreover, the trends of various element levels of Controguerra vines from budburst to harvest were compared with the corresponding trends reported in the same source.

Levels of the macro-elements potassium, nitrogen, phosphorus and calcium recorded in healthy vines at growth stages 77 and 83 were generally within the optimal range reported in the literature. There were exceptions in 2004 for potassium at growth stage 83, and for phosphorus at growth stages 77 and 83, which showed higher than 14 mg g-1 and 2.4 mg g^{-1}, respectively, which are the upper limits of the optimum level ranges. In 2004 and 2005, the levels of magnesium were higher than the average levels found in Italian vineyards, 3.1 mg g^{-1}, which was already close to the excess value (>3 mg g^{-1}) as compared with that estimated in international studies. In 2006, on the other hand, magnesium levels were near the optimum reference values (2.3–2.7 g^{-1}). Ratios between the macro-elements in vines at growth stages 77 and 83, which give an indication of the balanced nutritional status of leaves, are shown in Fig. 1. The ratio between K and Mg in healthy vines at these growth stages was near or below the lower limit of the optimal range (3–7) in all three years of the study, and the ratio between K and Ca was consistently below the optimum (0.45) in 2006, and in some cases below the optimum in 2004 and 2005. The other ratios, K/(Ca + Mg) and N/K, were more often within the optimal range (0.30–0.40 and 1.90–2.40 respectively).

In vine leaves, levels of magnesium and calcium usually go up during the growing season, and levels of potassium, nitrogen and phosphorus usually go down. These patterns were seen for healthy leaves in the Controguerra vineyard, with some exceptions in 2004, when magnesium and calcium levels went down somewhat as the season progressed, and when potassium, nitrogen and phosphorus increased from growth stage 77 to 83, although they gradually declined again in following growth stages. Levels of those elements in 2004, especially when passing from growth stage 77 to 83, were some of the highest recorded in the three years of the study, presumably because of their greater availability from the start of this growing season. This was probably due to the rainfall in April–July of that year, which was higher than that in the following two years (Table 1).

In the leaves of DAS vines the levels of macro-elements were similar to those in healthy vine leaves at all growth stages in every year, except for a few statistically significant differences, such as a lower level of magnesium at growth stage 83 in 2005 (Table 2). Levels of magnesium were always lower (though not significantly so) in DAS than in healthy vine leaves in 2004 and 2005. Levels of calcium on the other hand were always higher in DAS vines in 2004 and 2005, and this difference often became significant in 2005. In 2006 the only difference between healthy and DAS vine leaves was that DAS leaves always had lower (but not significantly lower) levels of potassium.

In DSY vines, levels of magnesium, potassium and nitrogen in the leaves were often significantly lower than in the leaves of healthy vines (Table 2). This was true for magnesium at all growth stages in 2004 and 2005; for potassium at growth stages 83 and 85 in 2006, when it sank below the sufficiency threshold (<10 mg g^{-1}); and for nitrogen, in most growth stages of 2004 and in growth stage 77 in 2005. In any case, levels of these elements, as well as of phosphorus and calcium, were often lower in the leaves of DSY vines, even though the difference was not always significant. Levels of calcium were significantly lower in DSY vines at growth stage 77 in 2004.

Only in 2006 were the ratios K/Mg, K/Ca, and K/(Ca+Mg) in the leaves of both DAS and DSY vines in the first three growth stages lower (though not significantly) than those in healthy vines. In that same year, however, the leaves of DSY vines had a higher N/K ratio than either DAS or healthy vines (Fig. 1).

When berries ripen, the levels of mineral elements in them tend to go down; and this is what mostly happened in the three years of the study in berries from healthy vines. In the same way, at the time of harvesting the levels of phosphorus and nitrogen were also lower than they were at earlier growth stages (Table 3).

Berries from DAS vines had levels of macro-elements not unlike those from healthy vines, except for some rare cases when they had significantly lower levels of nitrogen (Table 3).

Levels of all the macro-elements were always higher, and often significantly higher, in DSY vines at growth stages 83 and 89 (Table 3).

Micro-elements in leaves and berries

In the leaves, levels of sodium, a non-essential

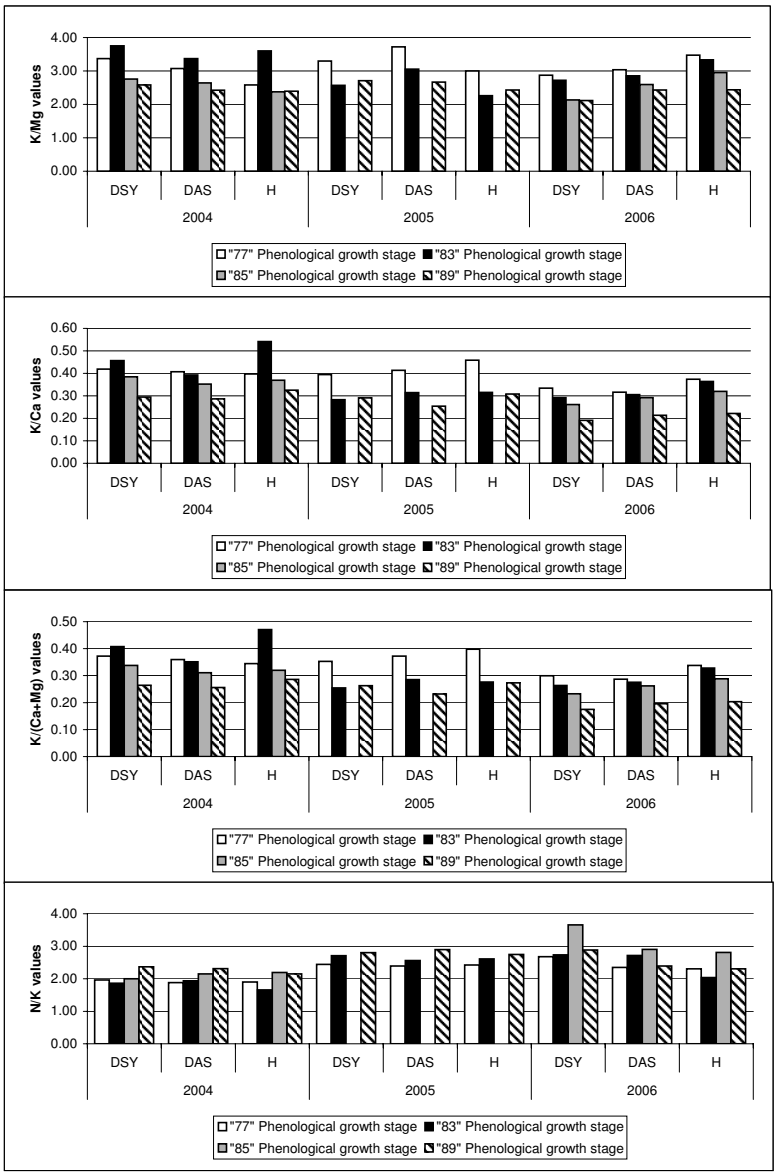

Fig.1. K/Mg, K/Ca, K/(Ca+Mg) and N/K ratios in leaves from symptomatic (DSY), asymptomatic (DAS) and healthy vines (H). Data were collected at different phenological growth stages according to BBCH classification (Lorenz *et al.*, 1995): "77", "Berries beginning to touch"; "83", "Berries developing colour"; "85", "Softening of berries"; "89", "Berries ripe for harvest".

element with a role in osmoregulation, were similar in all three vine groups, with a tendency to lower levels in 2004, probably due to the greater rainfall of that year (Table 1). The sodium levels were lower than optimal levels, which are 240–260 mg kg^{-1} (Table 2).

Levels of iron, manganese and zinc were within optimal range (as reported in the literature) in the leaves of all three vine groups, except zinc in 2006, which was often below the sufficiency threshold (<20 mg kg^{-1}) (Table 2). Leaves of DSY vines tended to have higher levels of iron than healthy vines as the season progressed in each of the three study years. Levels of iron were higher (though not significantly) in 2004 from growth stage 85 onwards, and in 2006 from growth stage 83 onwards. In 2005 iron levels were always higher in DSY vines than in healthy vines, and the difference became significant from growth stage 83 onwards. Levels of manganese in the vine leaves presented a less consistent picture in the three study years. They were significantly higher in DSY than in healthy vine leaves at growth stages 83 and 85 in 2004, lower in DSY than in healthy vine leaves (though not significantly) in 2005, and broadly the same in all groups of vines in 2006. Levels of zinc were much the same in all three vine groups in the three years, except that they were significantly lower in DAS vines at stage 83 in 2004 and 2006.

As was the case with the macro-elements, the berries of DSY vines always had higher levels of the micro-elements iron, manganese and zinc than the berries of healthy vines. The difference in micro-element level between DSY and healthy vines often reached statistical significance. Berries of DAS vines, on the other hand, had levels of micro-elements that were not much different from those in healthy vines, except in some cases where the difference became significant (Table 3). Sodium levels too were sometimes higher in berries from DSY vines than in those from healthy vines, but the difference here became significant only once, at growth stage 89 in 2006.

Table 2. Macro- and micro-elements in leaves from symptomatic (DSY), asymptomatic (DAS) and healthy (H) vines in the Controguerra vineyard affected by esca proper. Each value is the mean of 6 replications of the leaf data individually obtained from 6 vines for each grapevine group. The statistical analysis separately compared leaf data from each grapevine group in every phenological growth stage of each year (following Lorenz *et al.*, 1995). Values of symptomatic and asymptomatic vines followed by the letter statistically differ respect to the healthy vines according to the Student's *t* –test.

Growth stage [a]	Vine group	Mg (mg g^{-1})			K (mg g^{-1})			N (mg g^{-1})			P (mg g^{-1})			Ca (mg g^{-1})			Na (mg Kg^{-1})			Fe (mg Kg^{-1})			Mn (mg Kg^{-1})			Zn (mg Kg^{-1})		
		2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006
77	DSY	3.06 [c]	2.93 [a]	2.97	10.3	9.67	8.52	20.2 [a]	23.6 [a]	22.8	2.02	1.84	1.93	24.6 [b]	24.5	25.5	86.7	131	145	136	129	240	74.1	111	59.3	26.2	29.7	23.7
"	DAS	4.66	2.96	2.94	14.3	11.0	8.93	26.9 [a]	26.3	21.0	3.08	1.98	1.95	35.1	26.6 [a]	28.2	85.0	111	147	150	110	262	85.8	115	68.2	33.0	29.1	23.5
"	H	4.96	3.53	2.91	12.8	10.6	10.1	24.3	25.7	23.3	2.65	1.84	1.98	32.2	23.1	27.0	92.3	122	141	165	117	243	70.0	119	55.5	32.5	29.8	22.6
83	DSY	3.12 [b]	3.46 [b]	2.82	11.7	8.90	7.67 [a]	21.8 [a]	24.1	21.0	2.42	1.72	1.43	25.6	31.5	26.3	81.7	178	136	130	223 [a]	119	80.6	117	52.4	29.8	28.1	20.3
"	DAS	3.68	3.21 [b]	2.99	12.4	9.81	8.54	24.0	25.1	23.2	2.90	1.86	1.53	31.6	31.2	28.0	78.7 [a]	131 [a]	134	144	160	108	76.7	149	58.3	25.4 [b]	34.7	18.6 [a]
"	H	4.50	4.32	3.12	16.2	9.77	10.4	26.8	25.5	21.2	2.99	1.77	1.54	29.9	31.0	28.6	92.0	157	139	160	165	109	68.4	153	48.8	34.7	35.1	21.2
85	DSY	3.63 [a]	n.d.	2.78	10.0	n.d.	5.93 [b]	20.0 [a]	n.d.	21.7	1.68	n.d.	1.32	26.0	n.d.	22.7	93.3	n.d.	111	194	n.d.	103	96.9 [a]	n.d.	50.7	29.9	n.d.	18.6
"	DAS	3.82	n.d.	2.88	10.1	n.d.	7.47	21.7	n.d.	21.7	1.80	n.d.	1.41	28.7	n.d.	25.6	87.3	n.d.	121	188	n.d.	101	80.6	n.d.	64.7	27.1	n.d.	18.1
"	H	4.29	n.d.	2.86	10.2	n.d.	8.44	22.4	n.d.	23.7	1.87	n.d.	1.34	27.6	n.d.	26.4	91.7	n.d.	125	174	n.d.	98.8	69.5	n.d.	51.1	29.3	n.d.	21.7
89	DSY	3.17 [a]	3.08 [a]	3.38	8.19	8.35	7.14	19.4	23.4	20.6	1.77	1.91	1.49	27.8	28.7	37.4	82.1	145	95.4	167	233 [a]	132	77.1 [a]	116	69.8	24.8	31.6	23.9
"	DAS	3.84	3.19	3.51	9.31	8.50	8.52	21.5	24.6	20.4	1.90	1.97	1.93	32.5	33.4 [a]	39.9	69.8	93.9 [a]	104	147	123	129	71.7	114	88.9	22.6	28.4	24.0
"	H	3.99	3.67	3.72	9.55	8.91	9.07	20.5	24.5	20.9	1.92	1.92	1.68	29.4	28.9	40.9	74.1	125	93.1	131	160	114	56.2	144	71.6	22.7	35.6	24.9

[a] $0.05>P>0.01$.
[b] $0.01>P>0.001$
[c] $0.001>P$.
n.d., not determined.

Table 3. Macro- and micro-elements in berries from symptomatic (DSY), asymptomatic (DAS) and healthy (H) vines in the Controguerra vineyard affected by esca proper. Each value is the mean of 6 replications of the berry data individually obtained from 6 vines for each grapevine group. The statistical analysis separately compared berry data from each grapevine group in every phenological growth stage of each year (following Lorenz *et al.*, 1995). Values of symptomatic and asymptomatic vines followed by the letter statistically differ respect to the healthy vines according to the Student's *t*-test.

Growth stage	Vine group	Mg (mg g^{-1})			K (mg g^{-1})			N (mg g^{-1})			P (mg g^{-1})			Ca (mg g^{-1})			Na (mg kg^{-1})			Fe (mg kg^{-1})			Mn (mg kg^{-1})			Zn (mg kg^{-1})		
		2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006
83	DSY	0.84	0.88 [a]	0.62 [a]	15.5 [b]	21.3 [a]	13.4 [a]	8.65 [b]	10.7 [a]	8.36	1.76 [a]	2.01 [b]	1.06 [c]	1.76 [b]	2.31 [b]	1.99 [a]	18.3	33.0	56.1	21.0 [a]	24.9	20.7	8.06 [a]	17.7 [a]	7.88 [a]	10.6 [a]	9.48	11.9 [a]
"	DAS	0.64	0.66	0.49	12.2	17.2	12.0	6.80	5.48	6.52	1.48	1.13	0.83	2.11	1.51	1.30	18.2	34.6	45.2	17.2	30.8 [a]	17.8	5.07	14.0	5.33	7.18	7.46	8.11
"	H	0.65	0.69	0.45	12.6	17.5	11.7	6.47	6.36	7.60	1.49	1.28	0.81	1.49	1.42	1.20	17.3	39.1	45.1	17.8	23.4	15.0	4.78	15.1	4.17	7.51	8.35	8.71
85	DSY	0.62	n.d.	0.59	12.2	n.d.	12.6	8.64	n.d.	7.58	1.47	n.d.	1.03	1.73	n.d.	1.01 [a]	18.1	n.d.	46.0	23.9	n.d.	28.4 [a]	6.83 [a]	n.d.	4.87	11.0	n.d.	9.51
"	DAS	0.59	n.d.	0.50	10.3	n.d.	12.1	7.05 [a]	n.d.	6.56	1.22	n.d.	0.93	1.37	n.d.	0.70	18.4	n.d.	32.3	17.6 [a]	n.d.	24.0	4.94	n.d.	3.85	5.84	n.d.	7.44
"	H	0.61	n.d.	0.53	10.8	n.d.	11.4	8.49	n.d.	5.96	1.25	n.d.	0.90	1.40	n.d.	0.64	15.3	n.d.	31.0	20.7	n.d.	19.8	4.30	n.d.	3.78	9.20	n.d.	7.57
89	DSY	0.67	0.91 [a]	0.53	14.1 [a]	21.2 [c]	11.8	7.61 [a]	12.0 [a]	7.92	1.54	2.20 [b]	0.97	1.60	2.14 [a]	1.55 [a]	13.0	30.3	35.2 [a]	14.4	24.1 [c]	20.1	6.46 [b]	10.2 [a]	5.81	7.61 [a]	6.60 [a]	6.49
"	DAS	0.57	0.60	0.46	9.73	16.2	10.4	4.59	5.16 [a]	6.36	1. 22	1.13	0.77	1.20	1.07	1.10	15.7	33.3	29.3	11.3	15.2	17.9	3.78	7.43	4.97	4.55	4.66	6.03
"	H	0.61	0.65	0.41	11.9	15.6	10.6	4.74	6.16	6.42	1.26	1.20	0.75	1.32	1.17	0.88	11.7	35.8	27.4	13.6	11.9	16.5	3.99	5.57	4.36	4.65	4.78	4.99

[a], [b], [c] See Table 2.

Table 4. Chemical analyses carried out in Controguerra vineyard on musts from symptomatic (DSY) and asymptomatic (DAS) esca proper affected vines and from healthy (H) vines. Each value is the mean of 6 replications of the must data individually obtained from 6 vines for each grapevine group. The statistical analysis separately compared must data from each grapevine group in every phenological growth stage of each year (following Lorenz *et al.*, 1995). Values of symptomatic and asymptomatic vines followed by the letter statistically differ respect to the healthy vines according to the Student's *t*-test.

Growth stage	Vine group	Reducing sugars g l^{-1}			Total acidity g l^{-1}			pH			Tartaric acid g l^{-1}			Malic acid g l^{-1}		
		2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006	2004	2005	2006
83	DSY	144	117 [b]	109 [a]	11.5	14.5 [c]	11.8 [a]	2.95	3.11 [b]	3.00 [b]	3.73	5.08 [c]	5.82	10.7	10.7 [c]	7.20 [a]
"	DAS	142	122	115	10.9	8.72	10.7	2.96	3.19	3.13	3.41	3.85	6.01	9.03	6.68	6.81
"	H	153	132	132	11.1	8.73	10.2	2.97	3.23	3.19	3.40	3.50	5.77	9.91	6.32	6.31
85	DSY	168	n.d.	128 [b]	9.25	n.d.	10.1 [b]	3.01	n.d.	3.16 [a]	2.97 [a]	n.d.	5.05	7.93	n.d.	5.80 [a]
"	DAS	162	n.d.	156	9.45	n.d.	9.40 [a]	3.02	n.d.	3.43	2.53	n.d.	5.34 [a]	7.41	n.d.	5.07
"	H	172	n.d.	168	9.12	n.d.	8.36	3.08	n.d.	3.42	2.28	n.d.	4.86	7.48	n.d.	4.88
89	DSY	181	164 [c]	150 [c]	5.97 [c]	5.68 [c]	8.17 [c]	3.58	3.32	3.34	2.62 [a]	3.52	4.73 [a]	4.18	3.87 [c]	4.45 [c]
"	DAS	183	181 [a]	190	5.53 [a]	5.06 [a]	6.26 [b]	3.41 [b]	3.39	3.53	2.33	3.28	4.45	4.26	3.16 [a]	2.81
"	H	187	190	199	4.92	4.29	5.53	3.61	3.41	3.57	2.20	3.27	4.43	3.74	2.62	2.50

[a], [b], [c] See Table 2.

Analysis of musts

Sugars

Sugar levels in the berries increased in all vines as the berries ripened. However, sugar levels were always lower in the berries of DAS and DSY vines, especially at growth stage 89 (harvest ripe). The only exception was in 2004, when sugar levels were the same across all vine groups. The differences found in 2005 and 2006 were greater between DSY vines and healthy vines than between DAS vines and healthy vines. The differences between DSY vines and healthy vines were always significant (Table 4).

Organic acids

The highest levels of tartaric acid were found in the musts of DSY vines and they were significantly different from tartaric acid levels in healthy vines at the last two growth stages in 2004, at growth stage 83 in 2005, and at growth stage 89 in 2006. Tartaric acid levels were also higher in DAS vines, but the differences were not significant, except at growth stage 85 in 2006.

Malic acid levels were higher in both DSY and DAS vines in 2005 and 2006, and the difference was always significant in DSY vines (Table 4).

Total acidity and pH

Total acidity of the musts went down in all vines as they ripened, as is normal. The reduction in total acidity was correlated with lower levels of free malic acid. Total acidity of the must of diseased, and especially DSY, vines, remained higher than total acidity in the must of healthy vines, especially at full ripening (growth stage 89).

The pH values were much the same in the musts of all vine groups. Sometimes, however, pH values were significantly lower in the musts of DSY vines than in healthy vines, and once in the musts of DAS vines (Table 4).

Discussion

On the basis of the thresholds reported in the literature for vine nutritional status as determined by foliar diagnosis at the first two growth stages (77, 83) (Fregoni, 1998), healthy vines of the Controguerra vineyard did not reveal any grave nutritional imbalances in any of the three years of the study. There were however slight deficiencies of potassium and phosphorus in 2005 and 2006, and of nitrogen in 2006. But in 2004, probably as a result of a greater rainfall from the start of the growing season that year, and of the annual basic fertilisation, there was a greater uptake of nutrients, as generally shown by the higher levels of macro-elements in the healthy vine leaves at the first two growth stages, as compared with the following two years. Total yield of wine grapes (including those from diseased vines) was greater in 2004 and 2005, and less in 2006, in which year the vineyard was not fertilised and rainfall was lower, especially at the start of the growing season. In concomitance with the greater yield that occurred in 2004 and 2005, attributable to a greater availability of nutrients, the incidence of esca symptoms was higher.

Diseased vine leaves (DSY, DAS) generally did not have lower levels of phosphorus and the micro-elements than healthy vine leaves, despite the difficulty of absorbing all these elements from an alkaline soil such as that of the Controguerra vineyard, indicating that even the roots of diseased vines retained a sufficient capacity to absorb and transport these elements.

In the leaves of DAS vines, specifically, levels of other minerals also did not differ significantly from those in healthy vines, nor did the mineral composition of the berries differ between DAS and healthy vines. Total acidity of the berries on the other hand was significantly greater in DAS than in healthy vines at harvesting stage, even though the sugar content of the berries was only slightly lower in DAS vines. These findings generally confirmed earlier findings that the only substantial difference between DAS vines and healthy vines was that total acidity was higher in DAS vines, though not significantly. Here too therefore the yield quality of DAS vines was somewhat lower than that of healthy vines, but the difference was slight and not important (Calzarano *et al.,* 2004, 2007).

Symptomatic vine leaves had lower levels of nitrogen, potassium and magnesium than healthy leaves and the differences were often significant. These elements are mainly involved in photosynthetic functions. Though these leaves were not examined before they developed symptoms, yet it can be assumed that those elements had already gradually translocated away from them well before the leaf blades became completely covered with the tiger stripes, since in other studies the PSII showed impairment before any of these symptoms appeared

(Christen *et al*., 2007), and since the chlorophyll content was lower in asymptomatic leaves on shoots that were already symptomatic, and in those portions of symptomatic leaves that were still green, in which RuBisCo activity was also lower (Petit *et al*., 2006). There was further a greater closure in the stomata not only of symptomatic leaves, but also of asymptomatic leaves on symptomatic shoots (Petit *et al*., 2006). The berries of symptomatic vines may have benefited from this translocation of nitrogen, magnesium and potassium away from the damaged leaves; however, these berries had higher levels of all elements (compared with berries of healthy vines), including those elements that did not go down in DSY leaves. Further, in DSY vines the berries showed the normal tendency of levels of elements to go down as the growing season progressed, although the leaf symptoms of these vines usually became intensified. This suggests that the berries of symptomatic vines had already raised their levels of mineral elements even before the first sampling at growth stage 83, probably through the xylem, in an attempt to neutralise the higher levels of total acids. In spite of this, however, acid levels in DSY vines remained higher than those of the other vine groups, right up to growth stage 89, while at the same time sugar levels were significantly lower. The higher levels of nitrogen in the berries of symptomatic vines, on the other hand, are probably to be attributed to the higher levels of amino-acid substances, such as proline, in response to the stress caused by the disease (Calzarano *et al*., 2007), which also causes resveratrol levels in diseased grape berries to rise (Calzarano *et al*., 2004). However, higher resveratrol levels also occur in the leaves of diseased vines (Calzarano *et al*., 2009). This defence response at leaf level may also have affected the iron content in symptomatic leaves, which was generally higher than that in healthy vines. The greater iron content may be a sign that enzyme complexes, of which iron is a co-factor, had become more active, for example the superoxide-dismutases (SOD), which become active in response to stresses of various kinds (Marschner, 1998). However, higher iron levels were already reported in symptomatic leaves in earlier studies, when they were explained as having been mobilised by fungi that produce and stabilise complexes of iron in its ferrous form while they colonise the vines (Di Marco *et al*., 2001).

In symptomatic vine leaves, the lowest levels of magnesium and nitrogen occurred in 2004 and 2005, while potassium reached its lowest point in symptomatic vine leaves only in 2006, particularly at the hottest times of year. In 2006 potassium levels were also lower (though not significantly) in the leaves of DAS vines as compared with healthy vine leaves. These findings suggest that when nutrient uptake became critical, as it presumably did in 2006 because of the low rainfall and absence of fertilisation, potassium levels were most strongly affected. At times of water stress potassium is lost from the chloroplasts in the leaves (Marschner, 1998); consequently, the lower levels of potassium seen in diseased vine leaves during the hottest periods of the low-rainfall 2006, suggest that vines with esca were more sensitive to water stress than healthy vines; this was probably because the vessels had already been impaired. A reduced water availability, combined with lower levels of potassium, could limit still further the capacity of symptomatic vines to synthesise and translocate carbohydrates: and in fact, the lowest sugar content upon harvest in the berries of symptomatic vines was recorded precisely in the year 2006. In that year, the reduction in phloem flow from the source sites was also suggested by a failure of magnesium and nitrogen to retranslocate from the esca-damaged leaves, since the levels of these elements in DSY leaves were similar to those in healthy leaves, whereas in preceding growing seasons they were lower. In those seasons the greater availability of potassium and water may have been part cause of producing the retranslocation effects in symptomatic vines, especially in 2004, when the leaves of DSY vines had the highest levels of potassium at the onset of ripening, and when musts from those vines had levels of sugar comparable to those of musts from healthy vines.

In 2004 and 2005, when there seems to have been a better uptake of nutrients (as indicated also by the greater yield), the incidence of esca leaf symptoms was greater than it was in 2006. This was in line with the greater incidence of leaf symptoms that year in those vines of the vineyard whose leaves were treated with fertiliser (Calzarano *et al*., 2007). This greater incidence of leaf symptoms was also found in similar tests carried out in 2006 and 2007 (Calzarano, unpublished data). The nutrient regime of esca-affected vines

could therefore be implicated in the onset of leaf symptoms. There is perhaps a link between the leaf symptoms in the vine and the supply of nutrients available to the esca fungi: the fungi succeed in exploiting the nutrients that were intended for the vine. In that case a greater supply of carbohydrates translocated towards the infection sites could actually favour the fungi. When on the other hand water and nutrients, especially potassium, are scarce, the photosynthesis of the vines is reduced and sugars are translocated more to the reproductive organs, in order to enable them to mature, depleting the reserves in the trunk and branches where the esca fungi reside (Petit *et al*., 2006). Evidence for this supposition may have been provided in 2006, when yield was low and a large proportion of diseased vines remained asymptomatic (and hence apparently healthy): in that year no fertiliser was applied and rainfall was less than in previous years, this led to higher levels of sugars in the berries of healthy vines, as is normal in dry seasons (Fregoni, 1998).

Certain circumstances may also have raised the resistance of vines to esca fungi, and this may have been a determining factor in masking the symptoms of esca. Lignification interferes with the spread of esca fungi, and this process is hastened when the nutritional regime is poorer (as for example when nitrogen levels are lower): such a regime occurred in 2006. As regards the resistance offered by the vine plants, the finding that the leaves of DSY vines always have lower calcium levels (though not significantly lower) than the leaves of DAS vines, and that DAS vines often have higher calcium levels than healthy vines, deserves closer scrutiny. Calcium is an untranslocable element which strengthens the middle lamellae and the membranes (Legge *et al*., 1982), and it is a factor in modulating response to stress (Datnoff *et al*., 2007). Higher calcium levels also make various species of plants less susceptible to disease by acting directly on the disease agents themselves (Datnoff *et al*., 2007). Applications of calcium are effective in the control of powdery mildew and *Penicillium* rot in grapevine (Gadoury *et al*., 1994; Droby *et al*., 1997).

Consequently, when nutrients were plentifully available and the resistance of the vines was lowered, as in 2004 and 2005, the pectinolytic fungi *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* could have become more virulent and could have spread more, and this may have made it more likely for symptoms to appear by, for example, facilitating the release of phytotoxins (Evidente *et al*., 2000; Tabacchi *et al*., 2000).

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