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Esca in young and mature vineyards, and molecular diagnosis of the associated fungi

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Abstract Esca is one of the most important grapevine trunk diseases, and it can induce severe decline. In the past, the disease occurred mostly on mature vines, but today it is also present in young vineyards. The aim of this study was to investigate the incidence of esca in young (< 7 years old) and mature (> 11 years old) vineyards on cvs Montepulciano, Sangiovese, Verdicchio and Passerina located in the main viticultural areas of the Marche Region, central-eastern Italy. The average incidence of diseased plants was higher in mature (32.6%) than young (5.2%) vineyards, and Verdicchio and Passerina appeared to be the most sensitive among the cultivars considered, followed by Sangiovese and Montepulciano. The analysis of the spatial spread of esca carried out in two mature vineyards on cv. Verdicchio and a young vineyard on cv. Sangiovese showed a fluctuation in the numbers of infected plants over the three years of observation. The fungi associated with symptomatic plants were detected by classical and molecular tools. Isolation on agar media yielded colonies of *Phaeomoniella chlamydospora* (*Pch*), *Botryosphaeria* spp. (*Bot*), *Fomitiporia mediterranea* (*Fomed*) and, sporadically, *Phaeoacremonium aleophilum* (*Pal*). In samples from young plants, *Bot* and *Pch* were recurrent, while *Pch* and *Fomed* were found in mature vines and old rootstocks. Molecular detection with specific primer pairs for *Pch*, *Pal*, *Fomed*, and *B. dothidea* confirmed the data obtained using classical tools, and in some cases it was more sensitive. This study thus provides a further contribution to the association between causal agents and esca symptoms, and it confirms the importance of molecular tools for a sensitive detection of associated pathogens, which can also be present in propagative materials.

Keywords *Botryosphaeria* spp. · *Botryosphaeria dothidea* · *Fomitiporia mediterranea* · *Phaeoacremonium aleophilum* · *Phaeomoniella chlamydospora*

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Introduction

Esca is a complex disease that has been described as one of the most important grapevine trunk diseases, and can induce severe decline and dieback. Research on its etiology, which started at the end of the XIX century in France, continued from the beginning of 1900 in Italy and in California, USA, and was intensified further in

the 1990s (Mugnai et al. 1999). Over this period, esca spread in the main viticultural areas in Europe, and especially in Germany, Italy and Greece, where the arsenites that had been traditionally used to keep esca under control were banned. More recently, an etiological plurality has been hypothesised, according to which the fungi *Phaeomoniella chlamydospora* (*Pch*) *Phaeoacremonium aleophilum* (*Pal*) and *Fomitiporia mediterranea* (*Fomed*) are believed to be the main causal agents of the disease, being responsible for brown-red wood necrosis, dark streaks, and spongy wood, respectively (Mugnai et al. 1999).

The wide range of foliar symptoms seems to be due to interactions among the microorganisms involved, cultivar susceptibility, age of the vines, and pedoclimatic conditions (Graniti et al. 2000). Nowadays, esca is widespread in all vine-growing regions of Italy (Pollastro et al. 2000; Serra et al. 2000; Sidoti et al. 2000; Surico et al. 2000; Romanazzi et al. 2006; Michelon et al. 2007), and of the world, including California, USA (Scheck et al. 1998), Portugal (Rego et al. 2000), France (Larignon and Dubos 1997), Spain (Gimenez-Jaime et al. 2006), Australia (Edwards and Pascoe 2004), Greece (Rumbos and Rumbou 2001), New Zealand (Ridgway et al. 2002) and South Africa (Halleen et al. 2003). It commonly affects the old vineyards, but more often, the young vineyards.

The sanitary situation of Italian vineyards concerning esca is not dissimilar from that of the rest of the world. In some regions of central and southern Italy where epidemiological studies have been carried out, such as Tuscany, Marche, Abruzzi, Apulia, and Sicily, esca incidence has reached 60% to 80% in some old vineyards (Pollastro et al. 2000; Sidoti et al. 2000; Surico et al. 2000; Romanazzi et al. 2006; Calzarano and Di Marco 2007), while overall it was lower in northern Italy, in Trentino Alto Adige and Veneto (Michelon et al. 2007; Borgo et al. 2008), although with differences among cultivars. The presence of symptomatic vines in 2–3 year-old vineyards supports the assumption of the importance of infected propagative materials as a possible way of fungi spreading associated with the disease (Bertelli et al. 1998; Halleen et al. 2003; Edwards and Pascoe 2004; Aroca et al. 2006). Control strategies need to prevent (in the nursery) and/or reduce (in the field) the establishment and development of the disease (Di Marco et al. 2000; Fourie and Halleen 2006; Surico et al. 2006).

An important aspect of research into esca is the development of new sensitive, time-saving protocols for the detection and identification of the pathogens involved. *Pch*, *Pal* and *Fomed* are difficult to detect using traditional isolation methods due to their slow growth and lack of suitable selective media. Other fungi that are isolated together with *Pch* and/or *Pal*, e.g. *Botryosphaeria* (*Bot*) spp. and *Alternaria* spp., can overcome these fungi in growth, with subculturing often required, which makes the identification process longer. A certain number of symptomatic plants also harbour *Bot*, whose etiological role in esca is under discussion. Thirteen species of this genus can infect grapevines, including *B. dothidea*, and they can induce severe decline. Some are also found in apparently healthy mother plants (Halleen et al. 2003; Úrbez-Torres et al. 2006; Martos et al. 2008).

To speed up and facilitate the diagnosis of esca, sensitive DNA-based tools are required. Over the last few decades, conventional PCR, nested-PCR, one-tube nested-PCR, and quantitative PCR (SYBR-green, TaqMan, and Scorpion) have been developed for the identification of the main esca causal agents, and for their detection directly in wood, water and soil samples from nurseries and fields (Tegli et al. 2000; Overton et al. 2004; Abbatecola et al. 2006; Retief et al. 2006; Edwards et al. 2007).

The aims of this study were: (i) to investigate the incidence of esca in young and mature commercial vineyards on cvs Verdicchio, Sangiovese, Montepulciano and Passerina in the Marche Region; (ii) to isolate the causal agents from symptomatic grapevines and from rootstocks; and iii) to set up a protocol for the detection of *Pch*, *Pal*, *Fomed* and *B. dothidea* in wood samples.

Materials and methods

Field investigations

Twenty-seven commercial vineyards in the Marche Region were chosen for visual inspections and further analyses on sampled plants. These were both young (2–7 years old) and mature (≥11 years old) cultivated with the main white berry (Verdicchio and Passerina) and red berry (Montepulciano and Sangiovese) varieties (Table 1). A 30 year-old field of rootstocks

Table 1 Vineyards surveyed for esca symptoms in September 2005

Varieties	ID	Location (province)	Year of planting	Observed plants (no.)	Training system
Montepulciano	MP1Y	Osimo (AN)	2003	1,176	C. S.[a]
	MP2Y	Osimo (AN)	2002	1,568	C. S.
	MP1M	Falconara M. (AN)	1975	600	C. S.
	MP2M	Falconara M. (AN)	1985	580	C. S.
Sangiovese	SG1Y	Fiorenzuola di Focara (PU)	2003	900	C. S.
	SG2Y	Cartoceto (PU)	2003	1,152	D. G.
	SG3Y	Ripatransone (AP)	2001	900	C. S.
	SG1M	San Lorenzo in campo (PU)	1998	500	D. G.[a]
	SG2M	Falconara M. (AN)	1975	576	C. S.
	SG3M	Falconara M. (AN)	1985	500	C. S.
	SG4M	Falconara M. (AN)	1985	580	C. S.
	SG5M	Falconara M. (AN)	1998	400	C. S.
	SG6M	Ripatransone (AP)	1970	650	C. S.
Verdicchio	VD1Y	San Paolo di Jesi (AN)	2003	1,280	C. S.
	VD2Y	Cupramontana (AN)	2001	1,064	C. S.
	VD3Y	Montecarotto (AN)	2004	1,175	C. S.
	VD4Y	Montecarotto (AN)	2003	1,055	C. S.
	VD1M	San Marcello (AN)	1994	490	C. S.
	VD2M	Castelbellino (AN)	1990	564	C. S.
	VD3M	Cupramontana (AN)	1980	500	C. S.
	VD4M	Staffolo (AN)	1975	600	C. S.
	VD5M	Castelplanio (AN)	1975	864	C. S.
Passerina	PS1Y	Ripatransone (AP)	2002	800	C. S.
	PS2Y	Ripatransone (AP)	1999	1,050	C. S.
	PS3Y	Ripatransone (AP)	2001	500	C. S.
	PS1M	Ripatransone (AP)	1970	800	C. S.
	Total inspected plants			20,824	

[a] C. S.=cordon training with spur pruning; D. G.=double Guyot

(Kober 5BB, SO4, 420A, and 1103P) was also included in the analysis.

The evaluations of esca symptoms were first carried out in September 2005, with visual inspections in commercial vineyards. For each variety, one to six plantings were selected, with 500–1,568 vines for young vineyards and 400–864 vines for the mature ones (Table 1). Overall 20,824 plants were evaluated for the presence of esca symptoms. Disease severity was also recorded using an empirical scale with 7 classes: 0=symptomless plant, 1=1–2 symptomatic leaves, 2=3–9 symptomatic leaves; 3=symptoms on about 10 leaves; 4=symptoms on 50% of the canopy; 5=symptoms on >50% of the canopy; 6=apoplexy.

Spatial distribution of symptomatic plants over time

Among the plantings surveyed in 2005, two mature vineyards (~15 years old, with cv. Verdicchio) located at San Marcello (AN) (VD1M) and at Castelbellino (AN) (VD2M), and one young 'Sangiovese' (SG1Y) vineyard (established in 2003) at Fiorenzuola di Focara (PU), trained as cordons with spur pruning, were selected for an analysis of the spatial/temporal evolution of esca. These three vineyards were surveyed in September 2005, 2006 and 2007, and all of the plants showing esca symptoms were recorded. The data were used to set up a two-dimensional map showing the evolution of the disease symptoms in each vineyard.

Fungal detection: isolation on agar media

In September 2005, 2006 and 2007, 68 samples from the mature and young plants that showed esca symptoms and 12 rootstocks, most of which were apparently symptomless, were harvested and analysed for the presence of fungal pathogens. The uprooted vines were cleaned of the cortex, sectioned, surface-disinfested with 96% ethanol for 3 min, and dried over a flame. After this surface decontamination, pieces of the plants were cut longitudinally and any internal wood discolourations were noted. Under aseptic conditions, wood chips (2–5 mm) of both browned and apparently healthy wood tissues were placed in 90 mm diam Petri dishes containing potato dextrose agar (PDA, Difco Laboratories, Sparks, MD, USA), with added ampicillin (150 mg l^{-1}, Duchefa Biochemie, Haarlem, Netherlands) and streptomycin sulphate (150 mg l^{-1}, Merk, Darmstadt, Germany), and incubated at 25±1°C in the dark. At least twenty wood chips were examined for each sample. Fungal colonies emerging from the chip were subcultured by excising hyphal tips from the colony margins, and placing them onto fresh PDA plates.

The identification of colonies was based on morphological and cultural features, and on examination of fruiting structures and conidia under the microscope, comparing these with previously identified isolates of *Pch*, *Pal*, *Fomed*, and *B. obtusa*, kindly provided by Prof. F. Faretra (University of Bari, Italy), *Pch* CBS no. 229.95 and *B. obtusa*, kindly provided by Prof. G. Surico (University of Florence, Italy) and *B. dothidea*, kindly provided by Prof. S. Frisullo (University of Foggia, Italy).

Fungal detection: molecular analysis

Twenty fungal isolates that were identified as *Pch* (5), *B. dothidea* (5), *Fomed* (5), and *Pal* (5) were selected for DNA extraction. Approximately 100 mg of mycelium was harvested from pure colonies, and ground to a fine powder in liquid nitrogen. These samples were then suspended in 800 µl preheated 2% CTAB buffer (200 mM Tris-HCl, pH 8.0, 50 mM EDTA, 2.2 M NaCl, 2% CTAB, 0.06% sodium metabisuphite) according to the DNA extraction protocol described by Doyle and Doyle (1990). A 1.2% agarose gel was run to determine the amounts and the integrity of the DNA.

Thirteen adult and fourteen young vines, infected by *Pch*, *Bot*, and/or Fomed (detected by classical tools), and all twelve rootstocks were selected for the direct DNA extraction from wood tissue. Fifty mg of phloem and xylem scrapings were homogenised in 700 µl extraction buffer (0.12 M Na_2HPO_4, 1.5 M NaCl, 2% CTAB) with 0.5 g glass beads (Abbatecola et al. 2006). The next steps were the same as those used for the DNA extraction from pure cultures. The DNA extracted was purified through spin columns with Sepharose CL-6B (Pharmacia, Peapak, NJ, USA), and the nucleic acids were stored at −20°C before being used in PCR and nested-PCR.

To detect *Pch*, 1 µl DNA was added to the PCR reaction mixture containing 1× PCR buffer (Promega Corporation, Madison, USA), 1.5 mM $MgCl_2$, 200 µM of each dNTP (EuroClone, Pero, Milan, Italy), 0.125 U *Taq* DNA polymerase (Promega) and 200 nM of each primer $OPA13_{844}$Df/$OPA13_{844}$Dr (Invitrogen, Life Technologies, Carlsbad, CA, USA) (Abbatecola et al. 2006). PCR was carried out in a thermal Bio-Rad iCycler (Bio-Rad, Hercules, CA, USA) programmed for an initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 60 s, with a final extension cycle at 72°C for 7 min. A second round of PCR (nested-PCR) was carried out in the presence of another *Pch*-specific primer pair $OPA13_{844}$Ff/$OPA13_{844}$Fr (Abbatecola et al. 2006) using the same PCR conditions except for the annealing temperature (Ta=60°C) and the number of cycles (30). Pal1N and Pal2R were used for the detection of *Pal* according to the PCR conditions suggested by Tegli et al. (2000).

The total DNA was also amplified with the primer pair EBdF and EBdR, reported as specific to *B. dothidea* according to the PCR conditions described by Ma et al. (2003). For the detection of *F. mediterranea*, the species-specific primers developed by Fischer (2006) were used.

The amplification products were separated by electrophoresis in 1.5% agarose (Molecular Biology Certified Agarose, Bio-Rad) gels in 1× Tris–boric acid–EDTA (TBE) buffer at 80 volts in a Sub-Cell Electrophoresis Cell (Bio-Rad). The PCR products in the gels were stained with ethidium bromide (0.5 µg ml^{-1}), and visualised under UV light at 312 nm on a transilluminator; the images were acquired with a digital camera (Canon Power Shot A95, Canon,

Tokyo, Japan). The expected lengths of the amplified DNA fragments were estimated by comparison with a 100 bp DNA Ladder (Invitrogen).

Results

Field investigations

During the surveys carried out in September 2005, esca symptoms were observed in mature vineyards and in most of the young vineyards, with the exceptions of SG1Y and VG5Y (see Tables 2 and 3). Mild symptoms, consisting of reduced growth and light chlorosis, were seen on some rootstocks. The incidence of plants showing esca symptoms was higher overall in mature (Table 2) than in young (Table 3) vineyards. In particular, in vineyards> 11 years old, the average incidence of symptomatic plants was 32.6%, and it reached its highest levels in the VD4M and SG6M vineyards (91.7% and 97.0%, respectively); the lowest infection levels were seen in the SG4M and VD5M vineyards (6.0% and 1.2%, respectively) (Table 2).

In mature vineyards, the red-berry varieties, Montepulciano and Sangiovese, were less infected overall than the white-berry varieties, Verdicchio and Passerina, reaching average incidences of 11.8% and 31.4% as compared to 39.0% and 47.0%, respectively. The average disease severity was between 1 and 2 for the Montepulciano and Sangiovese, while it was 2 or higher in three out of the five Verdicchio vineyards; the esca infection index was thus higher in Verdicchio than in Montepulciano and Sangiovese (Table 2).

In the young vineyards, 657 plants out of 12,620 showed esca symptoms, with an average incidence of 5.2%. This reached its maximum in the MP1Y and VG2Y vineyards (about 12%). Only in the SG1Y and in VG5Y vineyards esca symptoms could not be observed. The disease severity was between 1 and 2, with symptoms recorded on a few leaves for each plant (Table 3). In young vineyards the red-berry and white-berry varieties showed similar average incidences of infected plants, at around 5% for each. In both the mature and the young vineyards, no plants appeared heavily infected or died of apoplexy (classes 5 and 6).

Spatial distribution of symptomatic plants over time

The analysis of the spatial distribution of esca was carried out in two of the mature vineyards cv. Verdicchio (VD1M and VD2M) and in a young

Table 2 Frequency (F), severity (S) and McKinney's Index (I) of esca symptoms recorded in mature vineyards of Marche Region

Vineyard	Frequency			Severity			McKinney's Index		
	Infected plants (%)	A[a]	sd[b]	Infected plants (%)	A[a]	sd[b]	Infected plants (%)	A[a]	sd[b]
MP1M	13.2			1.7			3.8		
MP2M	10.3	11.8	2.0	1.5	1.6	0.1	2.6	3.2	0.8
SG1M	25.7			1.3			5.6		
SG2M	11.6			1.3			2.5		
SG3M	30.0			1.0			5.0		
SG4M	6.0			1.8			1.8		
SG5M	20.2			1.1			3.8		
SG6M	97.0	31.4	33.2	n.a.[c]	1.3	0.3	n.a.	3.7	1.6
VD1M	37.9			2.0			12.5		
VD2M	52.1			3.9			34.4		
VD3M	12.2			1.2			2.4		
VD4M	91.7			2.5			38.9		
VD5M	1.1	39.0	35.7	1.0	2.7	1.7	0.2	17.7	18.0
PS1M	47.0	47.0		n.a.			n.a.		
Overall Means	32.6			1.7			9.5		

[a] A=Average; [b] sd=standard deviation; [c] n.a.=not assessed

Table 3 Frequency (F), Severity (S) and McKinney's Index (I) of esca symptoms recorded in young vineyards of Marche Region

Vineyards	Frequency			Severity			McKinney's Index		
	Infected plants (%)	A[a]	sd[b]	Infected plants (%)	A[a]	sd[b]	Infected plants (%)	A[a]	sd[b]
MP1Y	12.2			1.9			3.8		
MP2Y	2.0	7.1	7.3	1.6	1.7	0.2	0.5	2.2	2.3
SG1Y	0.0			0.0			0.0		
SG2Y	5.0			1.4			1.2		
SG3Y	5.5	3.5	3.0	n.a.		1.0	n.a.	0.6	0.8
VG1Y	5.3			1.7			1.5		
VG2Y	12.6			1.6			3.4		
VG3Y	9.7			1.8			2.9		
VG4Y	4.0			1.8			1.2		
VG5Y	0.0	6.3	4.9	0.0	1.4	0.8	0.0	1.8	3.4
PS1Y	5.6			1.0			0.9		
PS2Y	3.1			1.1			0.5		
PS3Y	2.8	3.8	1.5	1.0	1.0	0.0	0.5	0.6	0.3
Overall means	5.2			1.2			1.4		

[a] A=Average; [b] sd=standard deviation; [c] n.a.=not assessed

vineyard cv. Sangiovese (SG1Y). It showed a fluctuation in the numbers of symptomatic plants over the three years of observations. In the VD1M vineyard, the incidence of symptomatic plants showed relatively little change over time, at 37.9% in 2005, 34.5% in 2006 and 38.4% in 2007 (Fig. 1). Only 13.7% of plants showed symptoms for all of the 3 years, whereas 30.6% of the plants were symptomatic over two years, and 19.2% for just one year. The total cumulated esca was 63.5% over the three years (Fig. 2). In the VD2M vineyard, there was a slight decrease in the symptomatic plants between 2005 and 2006, and 2007, from 52.1% to 43.1%, and 41.5% in this last year (Fig. 3). In the VD2M vineyard, the cumulated esca was 66.3%, and about half of the plants showed symptoms in 2 out of the 3 years (Fig. 4). Moreover, in both VD1M and VD2M vineyards, the lowest percentages of plants showed esca in all 3 of the years of investigation (13.7% and 18.4%, respectively).

In the young SG1Y vineyard, no symptoms were observed during the survey carried out in 2005. In the following year, mild symptoms appeared on 48.0% of the plants, and on 31.4% in 2007. The plants showing symptoms only in 2006 were 28.4%, the vines that were symptomatic only in 2007 were 11.9%, while 19.6% of the vines showed symptoms in both years. Therefore, the total cumulated esca was 59.9% over the period considered (data not shown).

Fungal detection: isolation on agar media

From classical analyses carried out on 80 samples from the mature and young vineyards (Table 4) and from the rootstock mother plants (Table 5), the fungi isolated were mostly identified as *Pch*, *Pal*, *Bot* and *Fomed*. In the plants from mature vineyards, *Pch* was detected in 15, followed by *Bot* (8), *Fomed* (6) and *Pal* (4) (Table 4). *Bot* was mainly isolated from young vines, and only from cv. Montepulciano. Two samples were infected by *Pch* and one by *Pal*.

In the samples collected from the old rootstocks, *Pch* was frequently isolated (9 plants), followed by *Bot* (6, detected in the Kober 5BB, 1103P and 420A), *Pal* and *Fomed* (4). Co-infections of the fungi in different combinations were often recorded (see Tables 4 and 5). In a few plants, both mature and young, *Eutypa lata* and *Cylindrocarpon destructans* were also isolated (data not shown).

Fungal detection: molecular analysis

The DNA extraction from the mycelium of pure colonies yielded 100–200 $ng\,\mu l^{-1}$ nucleic acid for

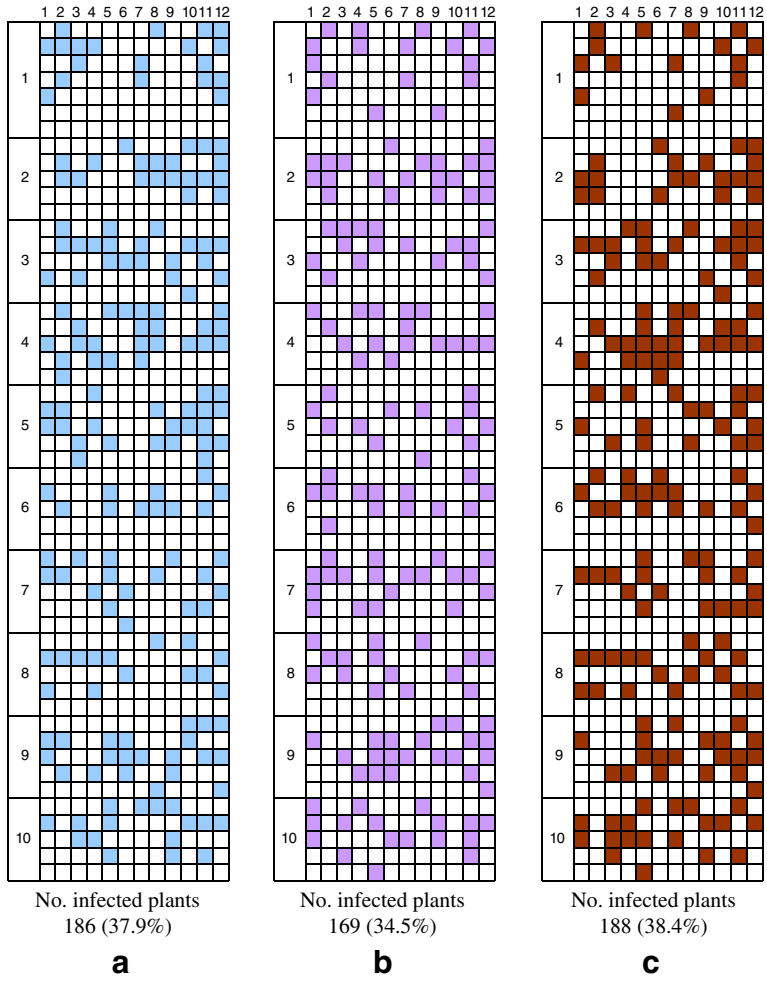

Fig. 1 Distributions of plants showing esca symptoms in a mature vineyard of cv. Verdicchio (VD1M), located at San Marcello (AN), during the surveys carried out in September 2005 (**a**), 2006 (**b**) and 2007 (**c**)

each isolate in about 2 h. The DNA extracted was amplified with specific primers for *Pch*, *Pal*, *Fomed*, and *B. dothidea*. Each specific primer pair showed a high detection efficiency (100%) even in the first PCR reaction, yielding the expected amplicons and confirming the identity of the fungal colonies assessed on their morphological properties. Direct extraction was also carried out from 30 cuttings, although this was more laborious due to the additional step of purification by Sepharose spin columns; this provided good DNA quality and quantity (50–100 ng μl^{-1}). For the detection of *Pch* directly from the tissues, it was necessary to run nested-PCR, which allowed the amplicon (498 bp) to be obtained (Fig. 5) even in samples that were not infected by classical analysis. Most samples (11/13) collected from the mature vineyards proved to be infected by *Pch* in nested-PCR (Table 4). In the young vineyards, three samples were infected by *Pch* and two by *B. dothidea*. Moreover, the *Pch*-primers provided a specific amplicon in 11 out of 12 rootstock mother plants (Table 5). A product of approximately 550 bp was obtained using *Fomed*-specific primers in 6 samples analysed among rootstocks and mature vines. The primer pair specific for *Pal* did not amplify in any sample.

Discussion

The importance of grapevine in the Italian economy and the recurrence of esca that has been recorded over the last years in different viticultural areas, have stimulated plant pathologists to intensify their research into this complex disease, which now occurs more frequently even in young vineyards. The present study was carried out on the four main cultivars

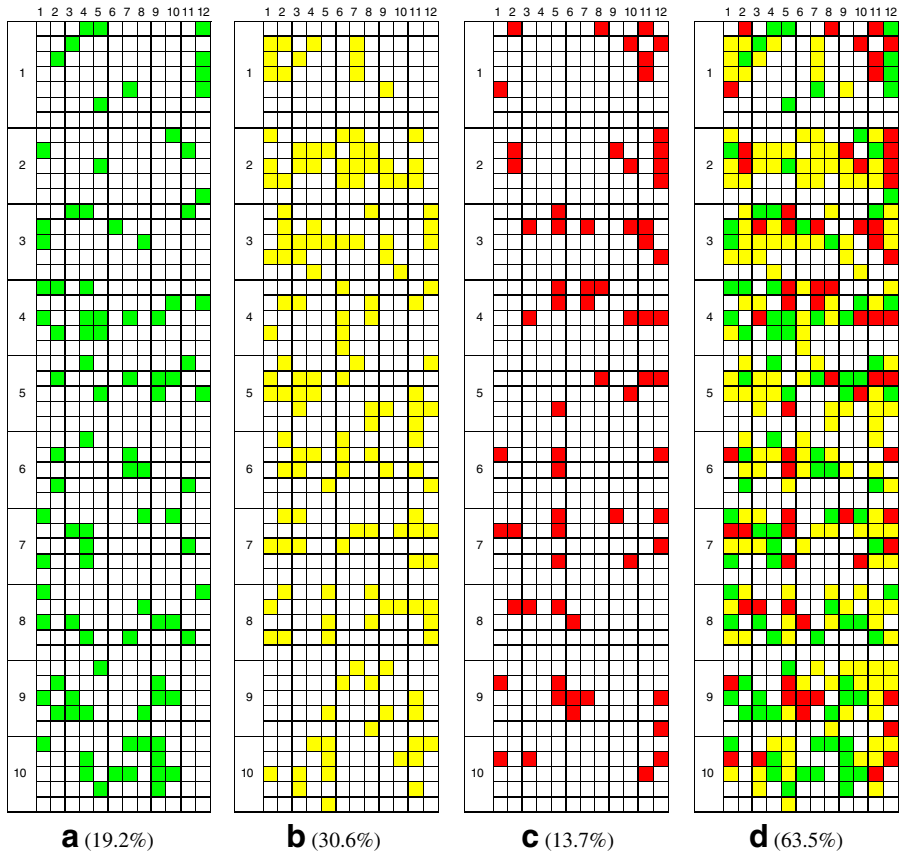

Fig. 2 Spatial patterns of esca in a mature vineyard of cv. Verdicchio (VD1M) located at San Marcello (AN). The green (**a**), yellow (**b**) and red-coloured squares (**c**) indicate the positions of esca-diseased vines showing symptoms over one, two and three years of inspection, respectively. The last map (**d**) represents the cumulative frequency of esca-diseased vines over the sampling period

grown in the Marche Region, and has allowed us to estimate the average incidence of esca as 32.6% and 5.2% in mature and young vineyards, respectively. These levels of esca infection are of the same magnitude as those recorded in surveys carried out in Apulia and Tuscany (Pollastro et al. 2000; Surico et al. 2000). In mature vineyards, the two tested red-berry varieties examined (Montepulciano and Sangiovese) were less sensitive than the white-berry varieties, as was observed in Veneto by Borgo et al. (2008), even if they were considering different cultivars. These different reactions of the plants appears to be due to the defence responses of vines, which are based on the accumulation of phenols (especially of condensed tannins) in the tissues adjacent to the vessels invaded by *Pch*. Indeed, within white-berry cultivars, the high levels of variability in the phenolic content of cvs Italia (susceptible) and Matilde (intermediate resistant) are strongly related to their susceptibility to esca-associated pathogens (Bruno and Sparapano 2006). In young vineyards, there were no evident differences in the esca frequency between white and red varieties, probably due to the relatively low numbers of symptomatic plants. Indeed, overall, it was not possible to establish statistical differences in esca incidence between the tested cultivars because of the lack of uniformity and the high variability in the training systems, pedoclimatic conditions, the rootstocks, and ages of the vineyards and their general management.

In the 27 surveyed vineyards, the severity of the symptoms were estimated to be from degrees 1 to 4, with averages of 1.2 and 1.8 for the young and the mature vineyards, respectively. To have an accurate picture of the true frequency of esca infection in a vineyard, it is necessary to consider the cumulative disease incidence over a period of observation of 3–5 years (Surico et al. 2000). Indeed, leaf symptoms that are clearly expressed around the end of the summer to the beginning of autumn do not appear constantly every year as they are affected by exogenous factors (e.g. rain and temperature) (Surico et al. 2000). We thus included an analysis of the spatial and temporal spread of esca in two of the mature

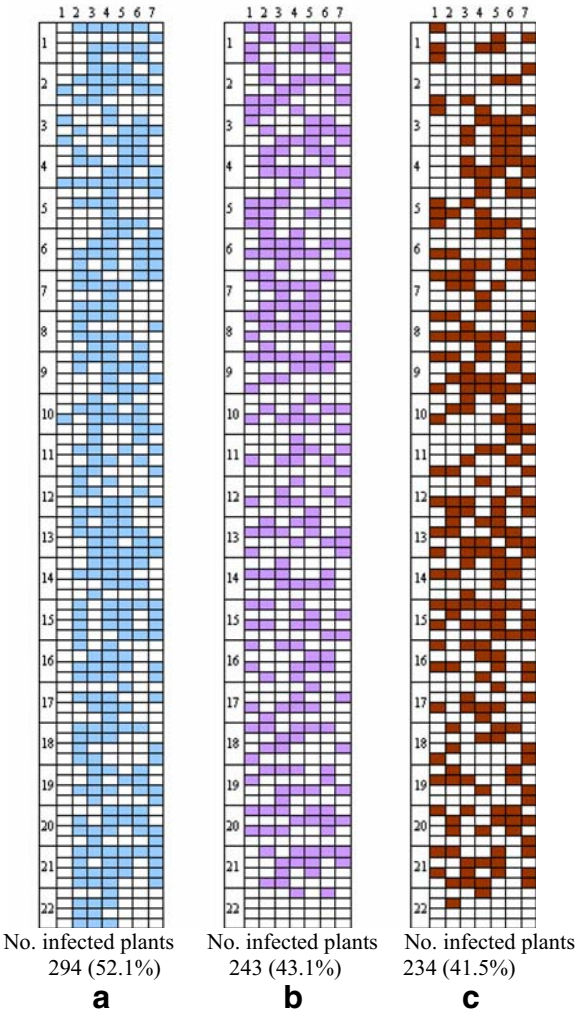

Fig. 3 Distributions of plants showing esca symptoms in a mature vineyard of cv. Verdicchio (VD2M), located at Castelbellino (AN), during the inspections carried out in September 2005 (**a**), 2006 (**b**) and 2007 (**c**)

vineyards on cv. Verdicchio and in one of the young Sangiovese vineyards, for a three-year observation period (2005–2007). All vines that showed esca symptoms at least once during the selected period were included in the map for yearly and cumulated esca. In surveyed vineyards the cumulative disease frequency was 66.3% and 63.5% for the mature VD1M and VD2M vineyards and 59.9% for the young SG3Y vineyard. The frequency of infected plants constantly showing symptoms over the three year period was 13.8% and 18.4% in the two mature Verdicchio vineyards, which is the same magnitude of data obtained by Mugnai et al. (1999) in Tuscany. In the young SG1Y vineyard, disease symptoms, which were apparently not evident in 2005, showed a dramatic increase from 2006 to 2007, reaching a cumulate incidence of 59.9%, which is not dissimilar to that observed in old Verdicchio vineyards. The wide fluctuation in the expression of foliar symptoms appears to be due to climatic conditions, and can be ascribed to the amount of rainfall during the late spring and summer. At present the precise role of rainfall in the expression of esca symptoms has not yet been established, although a correlation between amount of rainfall in July and the incidence of apoplexy was seen. Potentially, a greater and more constant flow of water towards the leaves favours the accumulation of higher levels of phytotoxic substances in leaf tissues, leading to the appearance of symptoms as soon as a critical threshold is reached (Marchi et al. 2006). Indeed, the present study showed an overall reduction in the number of symptomatic plants in 2007 with respect to 2006 in two vineyards out of three, and this behaviour could be ascribed to the climatic conditions, characterised by few rainfall events, occurred in 2007.

In addition to the climatic factors, the symptom expression seems linked to different susceptibility of the varieties, influence of the rootstocks, features of the vineyards, to training systems and stress conditions (Surico et al. 2000; Marchi 2001; Corti et al. 2004; Surico et al. 2004). Even the evolution of the control strategies for the main grapevine fungal diseases and the cessation of sodium arsenite use and later also of DNOC, which was prohibited in European countries from 1999 (Council Directive 91/414/EEC of 15 July 1991) could have contributed to the outbreak of esca.

At the present, esca is showing greater recurrence in young vines (< 10 years old), supporting the hypotheses that these vines are infected early due to an increase in fungal inoculum in the vineyard, or to the use of infected nursery materials. The commercialisation of infected propagative materials could be linked to the great demand of the 1980s, when esca disease also became more frequent (Surico et al. 2004). To support this hypothesis, it is known that the conidia of fungi associated with esca disease, such as *Pch* and *Pal,* can survive undetected in the xylem vessels of vines, and hence in the propagative materials collected from the canes of these vines (Halleen et al. 2003; Retief et al. 2006). The infected

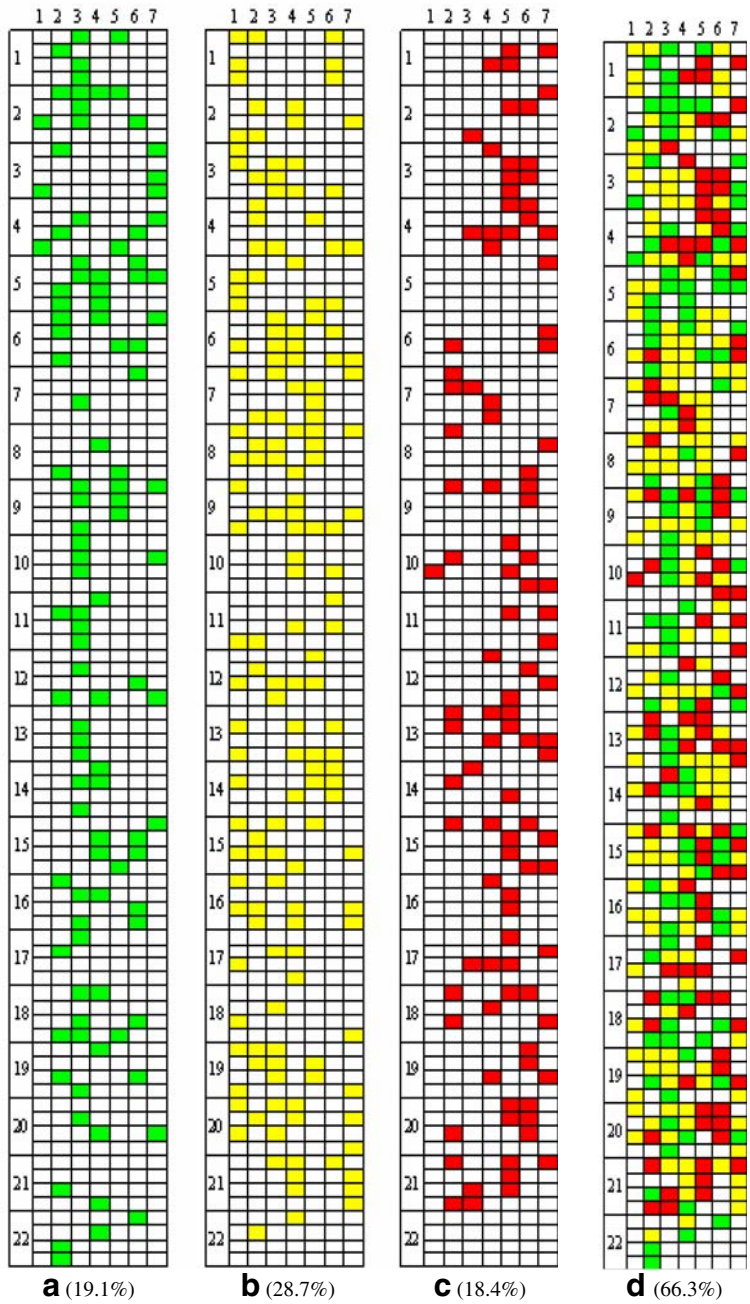

Fig. 4 Spatial pattern of esca in mature a vineyard of cv. Verdicchio (VD2M), located at Castelbellino (AN). The green (**a**), yellow (**b**) and red-coloured squares (**c**) indicate the positions of esca-diseased vines showing symptoms over one, two and three years of inspection, respectively. The last map (**d**) represents the cumulative frequency of esca-diseased vines over the sampling period

propagative materials may represent inoculum sources that can cause the spread of esca in the field through aerial and soil-borne dispersion of *Fomed* and *Pal* and *Pch*, respectively (Larignon and Dubos 1997; Cortesi et al. 2000).

On the other hand, Borgo et al. (2008) noted that in Veneto, northern Italy, where the rainfall during the summer is generally greater than in Marche, esca symptoms appeared on plants older than 8 years and then progressively increased, particularly on the susceptible varieties, with the ageing of vines and with a pruning system requiring more extensive cuttings. Also Zanzotto et al. (2007) have claimed that in Veneto, Petri disease has not yet been observed as a problem in young vines, and that the increasing incidence of plants affected by *Pch* in older grape-

Table 4 Fungi detected by classical and molecular tools from deteriorated wood showing symptoms of esca sampled in mature and young vines

Vineyards	Varieties	Fungal colonies isolated (no.)					Molecular detection				
		Samples (no.)	*Pch*	*Pal*	*Fomed*	*Bot*	Samples (no.)	*Pch*	*Pal*	*Fomed*	*B. dothidea*
Mature	Montepulciano	13	5	0	2	5	13	11	0	2	
	Verdicchio	22	5	1	0	3					
	Vernaccia	3	3	3	3	0					
	Trebbiano	1	1	0	0	0					
	Chardonnay	1	1	0	0	0					
	Sangiovese	1	0	0	1	0					
Young	Montepulciano	18	0	0	0	13	14	3	0	0	2
	Verdicchio	5	0	0	0	0					
	Lacrima nera	2	1	1	0	0					
	Traminer	2	1	0	0	0					
Total		68	17	5	6	21	27	14	0	2	2

vines would suggest a progressive infection of healthy plants in the field, or a delayed development of latent fungal infections that were already present in rooted grafts. For these reasons, the selection of propagative materials must be supported by efficient diagnosis techniques that can detect these agents even in asymptomatic plant materials.

During the surveys in old fields of rootstocks, fruiting bodies of *Fomed* were found at the crown of 1103P plants; abundant emission of black drops came from transverse sections of 420A and Kober 5BB plants, and black streaks were seen in SO4 wood. Although we chose a 30 year-old planting, with an increased possibility of infection, the presence of infection supports the possibility that fungi related to the esca complex (*Pch, Pal*, and *Fomed*) can be spread by infected propagative materials. A high incidence of *Pch*, *Pal*, and *Bot* (including *B. dothidea*) was also found in rootstocks in Spain (Aroca et al. 2006).

In the present study, two different diagnostic approaches were followed. The classical detection on artificial media allowed the isolation of *Pch*, *Fomed, Bot* and *Pal*. *Fomed* colonies, identified also by molecular tools, were obtained with high frequency from spongy wood, while *Pch* and *Bot* were prevalent in black and dark-brown wood, as reported in previous studies carried out on mature vineyards (Mugnai et al. 1999; Pollastro et al. 2000).

Different results have been obtained under different climatic conditions on propagative materials and in young vineyards infected by *Pch* and *Pal* (Abbatecola et al. 2006; Gimenez-Jaime et al. 2006; Zanzotto et al. 2007). Abbatecola et al. (2006) reported a strong

Table 5 Fungi detected by classical and molecular tools from wood of 30 year-old rootstock mother plants

Rootstocks	Location	Samples (no.)	Classical detection				Molecular detection			
			Pch	*Pal*	*Fomed*	*Bot*	*Pch*	*Pal*	*Fomed*	*B. dothidea*
Kober 5BB	Petritoli (AP)	3	2	2	0	2	2	0	0	0
1103P	Petritoli (AP)	2	2	0	2	2	2	0	2	0
420 A	Petritoli (AP)	3	3	0	0	2	3	0	0	0
SO4	Petritoli (AP)	4	2	2	2	0	4	0	2	0
Total		12	9	4	4	6	11	0	4	0

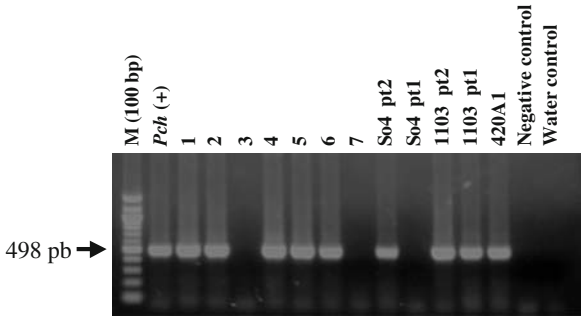

Fig. 5 Agarose gel (1.5%) showing the 498 bp fragments amplified by nested-PCR with the specific primer pairs for *P. chlamydospora*, from grapevine plants (lanes 1–7) and from rootstocks, as indicated. 1–3: Montepulciano, young vines; 4–7: Montepulciano, mature vines; *Pch*: positive control; M: 100 bp DNA Ladder Plus (MBI, Fermentas, GMBH, Germany)

positive correlation (0.94) between the severity of wood discolouration seen in grafted and ungrafted rootstocks and the presence of *Pch*. On the other hand, discolouration in the wood has also been observed in grafted rootstocks due to abiotic factors (Frisullo et al. 1992; Rumbos and Rumbou 2001). For this reason, the molecular detection approach is valid for the identification of the main causal agents, most of which arise from asymptomatic plants. DNA extracted from young and mature vines and old rootstock mother plants was amplified by specific primers for *Pch*, *Pal*, *Fomed,* and *B. dothidea*, already validated in previous studies (Tegli et al. 2000; Ridgway et al. 2002; Abbatecola et al. 2006; Fischer 2006) or developed for the detection of *B. dothidea* on pistachio (Ma et al. 2003) and tested in our investigations on grapevine samples. *B. dothidea* can also infect other crops, such as olive trees, which are often grown close to vineyards, at least in central and southern Italy (Lazizzera et al. 2008).

DNA extractions from plant tissues and the following amplifications with specific primers allowed the intermediate steps to be by-passed (i.e. *in vitro* cultures, subcultures and microscopial identification), thus obtaining results in a shorter period of time (Abbatecola et al. 2006). In particular, PCR efficiency appears to be more affected starting from wood scrapings, and for this reason an additional purification step was required to reduce the levels of polyphenol and polysaccharide compounds in the plant DNA extracts. However, molecular detection proved to be more sensitive than classical methods, allowing the detection of fungi in plants that had tested negative after isolation on agar media. In particular, in mature Montepulciano vines, 11 out of 13 samples proved to be infected by *Pch*, while only 5 samples were positive after isolation on agar medium. Moreover, in young vineyards, cv. Montepulciano proved to be infected only by *Bot* and *Pch* was also detected only by the molecular approach. In this 3 year-old vineyard, *Bot* was isolated both from rootstocks and scions, while *Pch* was found just in rootstocks. Two samples that were infected by *Bot* using classical detection, also tested positive to *B. dothidea* after molecular detection with specific primers; the *Fomed*-specific primer was also able to confirm the results obtained by classical tools. No *Pal* infections were detected with the specific primer pairs in vines and rootstocks. However, *Pal* has been found recurrently in Tuscany and Veneto, while it had an overall lower frequency in Marche, also reported in Apulia (Pollastro et al. 2000).

Too many questions remain to be resolved at present. It will be necessary to understand clearly the effective roles that nursery practices have in *Pch*, *Pal* and *Bot* infections in young vineyards, the influences of climatic factors on the spatial evolution of esca disease and symptom expression, and on the interactions that might develop among *Fomed*, *Pch*, *Pal* and *Bot* mixed infections. Further information on the different susceptibility of grapevine cultivars and rootstocks to fungal colonisation should emerge from GFP transformed isolates, which for *Pch* are now available (Bradshaw et al. 2005; Landi and Romanazzi 2006).

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