

Preliminary histological observations on grapevine affected by esca disease

Research Article

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Abstract: Esca is a destructive disease of the woody tissues of grapevine (*Vitis vinifera* L.) and due to the complexity of disease many aspects of host-pathogen interactions are not clearly understood. The histological characteristics of esca symptomatic petioles and internodes, collected from Cabernet Sauvignon and Sangiovese grapevine were studied. The tissues were fixed in FAA, dehydrated and embedded in Histoplast. To identify the lignified cell walls the sections were stained by Crystal violet and Erythrosin B and observed using an optical microscope. The main feature of tissues infected by esca disease was the minor lignification of vascular tissues, which was observed in petiole tissues before appearance of esca symptoms. The opportunity to utilize the histological examination of tissues as a method for the early detection of esca infections is hypothesized for the future application.

Keywords: Shoot • Leaf • Genotype • *Vitis vinifera* L.

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1. Introduction

Esca is a complex and destructive disease of the woody tissues of grapevine (*Vitis vinifera* L.) leading to substantial losses in wine production. Multiple fungal species, such as *Phaeoconiella chlamydospora*, *Phaeoacremonium aleophilum* [1,2], and *Fomitiporia mediterranea* [3,4], are found to be associated with the development of the disease. Symptoms arise from structural and physiological changes that cannot be explained by a simple scheme of cause and effect and the unpredictable discontinuity in symptom expression increases the difficulty of studying esca [5], which is further exasperated by symptoms that look alike may have different causes [6]. Esca symptoms appear in severe or chronic forms [7-10] although both can occur alone or together on the same plant [1]. Esca symptoms are associated with a general decline that includes deformed and chlorotic leaves, precocious fading, lack of vigour, plugging of the xylem vessels

and trunk dieback due to the formation of cankers in the vascular tissue [11]. Generally, external symptoms in the Northern Hemisphere develop between June and September, either through the entire vine or on single branches [5,6].

Histochemical examination of vine plantlets inoculated with *P. chlamydospora* revealed that the fungus spread through the wood tissue, albeit slowly, probably a consequence of mechanical and biochemical barrier production. The main indication of defence response is the production of tyloses with the resulting occlusion of the xylem vessels. Chemical changes observed in the cell walls of the paratracheal parenchyma and the xylem fibers near the vessels invaded by *Pch.*, may be a consequence of the deposition of stress metabolites produced by the vine [12].

Light microscopy observations of roots and shoots inoculated with *Phaeoacremonium spp.* showed a rapid spread of the fungus through the vascular tissues and intercellular spaces. Spores were seen in the pith

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area as well as in the xylem. Remote from the point of inoculation, hyphae were seen in the epidermis, cortex, pith, and vascular tissues without noticeable change in appearance of the invaded cells. Plants growing in the field are not expected to be as susceptible to penetration as the plantlets grown in laboratory, although the young shoots regularly produced by grapevine could be potential infection sites [13].

Many aspects of the interaction between the host and the pathogen in esca vine disease are not clear yet. Biochemical and molecular studies need to be associated with histochemistry to locate damage induced by the esca disease. In this view, adult grapevine of cultivars presenting variable susceptibility to the disease were studied and the structure of petiole and internode in shoot affected by esca was analysed.

2. Experimental Procedures

2.1 Plant material

The trial was carried out on vineyard (25-years-old) located at the Colignola experimental station of University of Pisa (altitude 6 m, 43°02' N, 10°36' E). The vineyard is constituted by Cabernet Sauvignon and Sangiovese cultivars found throughout Italy. The Cabernet Sauvignon is a genotype known to be very susceptible to esca disease [14,15], while Sangiovese shows variable susceptibility to esca. Materials were collected during a three-year period (2005-2007) from: symptomatic vines; vines that have never shown symptoms during the experimental trial (as a control); vines that in previous years showed symptoms and that could be expected to show again them (PYS). Each group was constituted by five homogeneous plants.

Before esca appearance (from flowering to fruit-set starting of 2007) on PYS vines of Cabernet Sauvignon three shoots/vine with similar number of nodes were selected (ranged from 24 to 27). In order to sample material with the same age, the petioles (10 replicates) were collected from the same basal and apical portions of each shoot constituted by 3 nodes. When the first leaf symptoms appeared on the basal portion of selected shoots the petioles were collected from the apical asymptomatic leaves.

After the appearance of leaf symptoms (from fruit-set to veraison of 2005 and 2006) on symptomatic vine and control vines of Cabernet Sauvignon and Sangiovese samples were collected of apical and median internodes and petioles (10 replicates/organ/cv). To sample homogeneous material the petioles and internodes were taken from shoots selected from a similar number of nodes (ranged from 30 to 33) as describe above.

2.1.1 Observation of esca symptomatology

Beginning in 2005, visual observations were periodically made from June to September to determine the maximum incidence percentage of esca disease foliar chronic symptoms [(no. of symptomatic plants/total no. of plants)*100]. At each examination 44 plants of Cabernet Sauvignon and 85 plants and Sangiovese were examined.

2.2 Histochemical procedure

The tissues were fixed in FAA (45% ethyl alcohol, 5% glacial acetic acid, 10% formaldehyde; 8:1:1 v/v), dehydrated with an ethanol alcohol series and embedded in Histoplast. Cross sections (7 mm) were made by a Shandon microtome. To identify the lignified cell walls the sections were stained by Crystal violet and Erythrosin B [16]. The Crystal violet is a cationic dye with a high affinity for lignin [17] noting the presence by a blue purple staining. Histological observations were carried out at several magnifications (x100, x200, x400) using an optical microscope (Nikon, Fluophot) equipped with a digital camera (Olympus C-2000 z). For each sample of tissue 100 sections were examined and the percentage of sections characterised by delignification was determined.

3. Results

The mean of symptomatic grapevines recorded in the vineyard during three years was about of 35% without significant differences among years (Table 1). The Cabernet Sauvignon was more susceptible to esca disease than Sangiovese cultivars showing a more constant and higher esca incidence in the time: the percentage of symptomatic grapevines reached about 50%. The spread of disease on Sangiovese was characterised by a moderately annual fluctuation of symptoms from year to year and the percentage of diseased grapevines was, on average, about 50% lower respect to the Cabernet Sauvignon (Table 1). In relation to the vine health different histological characteristics in the internodes and petioles tissues were observed.

3.1 Observations before and at the beginning of esca symptoms appearance

When blooming began on Cabernet Sauvignon PYS vines, when the esca foliar symptoms had not yet appeared, petioles of basal and apical leaves were characterised by histological features analogous to the control tissues (Figure 1A, D). In the basal petioles the cell wall of vessel elements and xylem fibers were regularly lignified (Figure 1A), represented by a regular blue purple

	2005	2006	2007	$\bar{X}$
Sangiovese	28	18	21	22.3 (2.96) ^b
Cabernet S.	43	43	53	46.3 (3.33) ^a
$\bar{X}$	35.5 (7.5*) ^{ns}	30.5 (12.5) ^{ns}	37.0 (16.0) ^{ns}	34.3 (12.0)

Table 1. Percentage of symptomatic grapevines recorded each year, from 2005 up to 2007 in Sangiovese and Cabernet Sauvignon cultivars. Data from Andreini *et al.*, 2009 [24].

Mean values within a columns followed by different letters are significantly different (t test, $P \leq 0.05$).

* Standard Error

ns - not significant

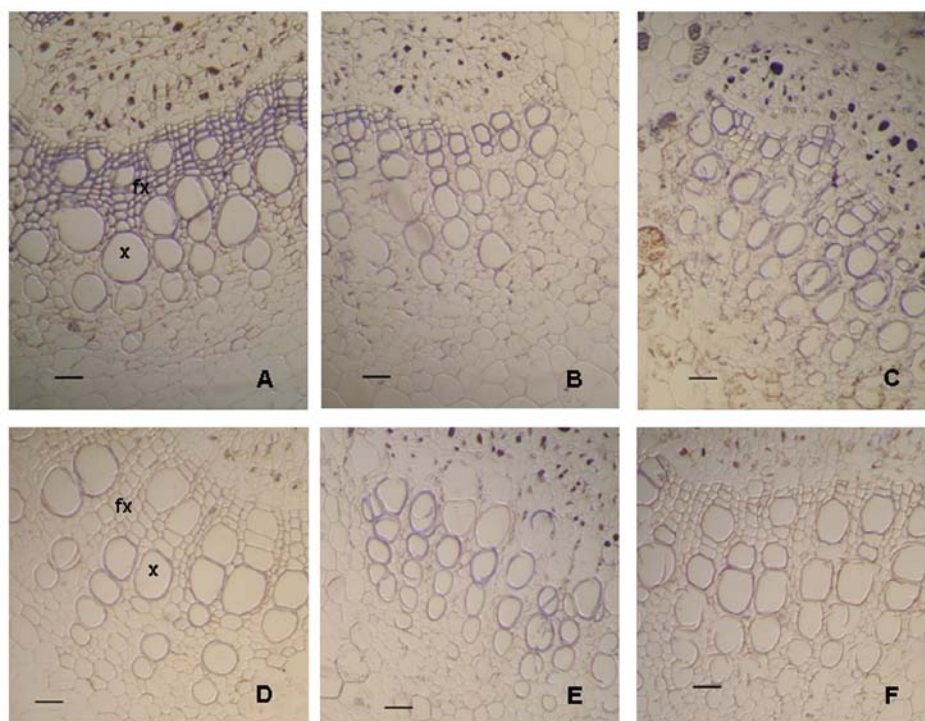


Figure 1. Cross sections of vascular bundle portion of Cabernet Sauvignon petiole belonging to vines that in the previous years were symptomatic. The petioles were collected from basal (A, B, C) and apical (D, E, F) part of shoots at blooming (A, D), fruit-set (B, E) and veraison (D, F) time. x: xylem vessel; fx: xylem fibers. Magnification 200x. Scale bars: 50 μ m.

staining, while in the apical petioles it was showed a weak staining in accordance of juvenile stage of leaves (Figure 1D). At the beginning of fruit set (Figure 1B, E) the PYS vines showed no esca foliar symptoms, while in the basal petioles the xylem tissues, particularly the xylem fibers, were poorly lignified in comparison to previous observations. In about 90% of the sections examined the poor staining of tissues revealed the minor presence of lignin in the cell walls (Table 2). At the beginning of veraison (Figure 1C, F), when the esca foliar symptoms were clearly visible only in the basal portion of shoots, the apical petioles of asymptomatic leaves were also poorly lignified.

3.2 Observations after appearance of esca symptoms

The cross sections of petioles collected at the veraison from Sangiovese and Cabernet Sauvignon vines with significant foliar symptoms, showed vascular elements were poorly lignified (Figure 2C, D and G, H), moreover, some areas of cortical parenchyma were characterised by cellular degradation (Figure 2D, G). Another type of tissue injury was observed in the cortical portion of petiole as the parenchyma cells showed damage as a minor degree of cell wall was stained (Figure 3C); while the cells of central parenchyma lost their turgidity and appeared almost collapsed (Figure 3D). In contrast, the

Cultivar	Internode			Petiol			Average
	Apical	Median	Average	Apical	Median	Average	
Cabernet S.	90	72	81	82	94	88	84,5
Sangiovese	78	85	82	80	87	84	83,0

Table 2. Percentage of sections characterised by tissue with poor lignification.

Percentage calculated on 100 sections for each sample of organ

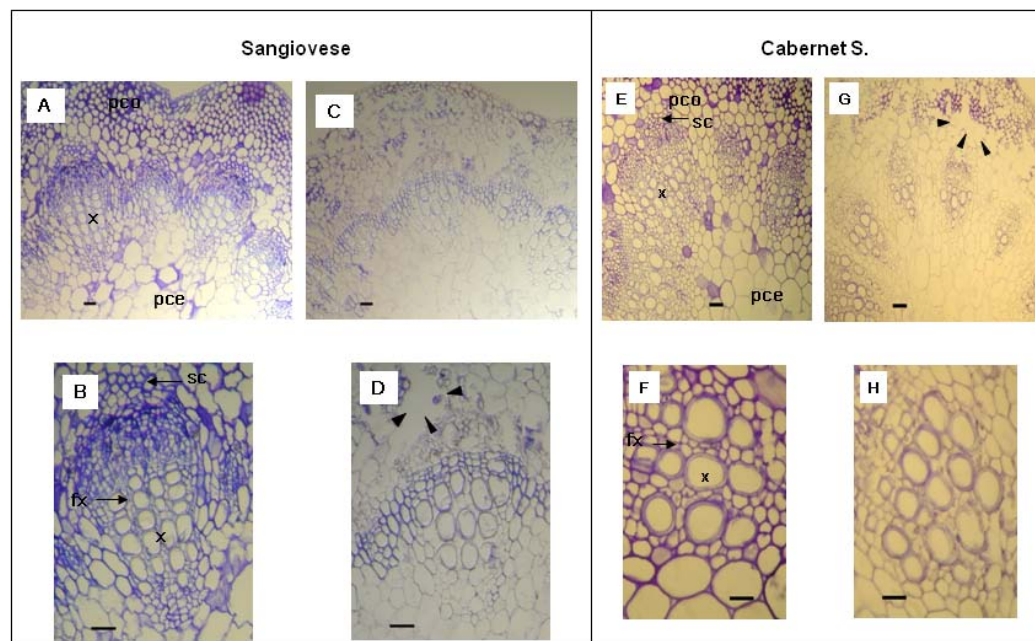


Figure 2. Cross sections of vascular bundle portion of petioles collected at veraison from Sangiovese (left) and Cabernet Sauvignon (right) vines: health (A, E); symptomatic (C, G) and their respective details (B, F and D, H). Black arrows indicate degradation cells area. sc: sclerenchyma cells; pco: cortical parenchyma; x: xylem vessel; fx: xylem fibers; pce: central parenchyma. Magnification: A, C, E, G 100x; B, D, F, H 400x. Scale bars: 50 μ m for all Sangiovese sections; for Cabernet Sauvignon A, C: scale bars 70 μ m. B, D: scale bar 30 μ m.

control sections of the petioles showed a regular stain displaying a good turgidity of tissues (Figure 3A, B).

Representative cross sections of median (Figure 4A, B) and apical (Figure 4E, F) healthy internode showed the good staining of tissues, while the tissues affected by esca disease showed irregular staining. The histological characteristics related to the esca disease were similar in Cabernet Sauvignon and Sangiovese cultivars (Table 2). The main feature of tissues affected by esca was the weak staining of tissues, in particular of the vascular elements. The minor lignification of internode collected at the veraison (Table 2), was clearly observed on the over 80% of thin sections when the shoots showed the foliar symptoms such as the tiger stripes (Figure 4 C, G). The tissues of median internodes were characterised by a minor degree, or by a lack, of cell walls staining of xylem vessel (x), xylem rays (r) (Figure 4C) and

sclerenchyma cells (sc) (Figure 4D). This feature was particularly evident at level of the sclerenchyma cells (sc) in apical internodes (Figure 4H).

4. Discussion

The symptomatology observations confirm that the esca foliar symptoms start to appear during the fruit-set [6] proceeding from the base toward the shoot apex. The cultivars examined, despite their different susceptibility of esca disease [14,15], showed a similar symptomatic appearance on shoots. The histological observation of infected tissues, showing a weak stain for lignin, suggests a general poor lignification of cell walls. This feature was found in both cultivars studied and was confirmed during three years period. Moreover this result was partially verified by another stain (Safranin

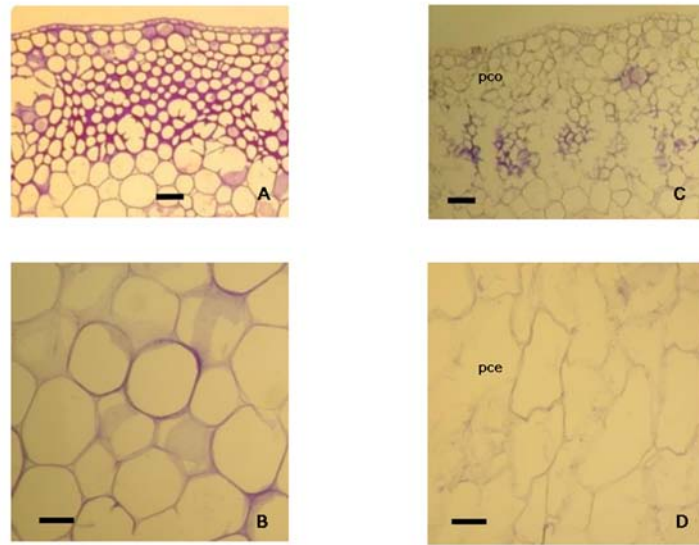


Figure 3. Particulars of cortical and central parenchyma cells in cross sections of petioles collected at veraison from Cabernet Sauvignon vines: health (A, B) and symptomatic (C, D). pco: cortical parenchyma; x: xylem vessel; pce: central parenchyma. Magnification 200x. Scale bars: 50 μm.

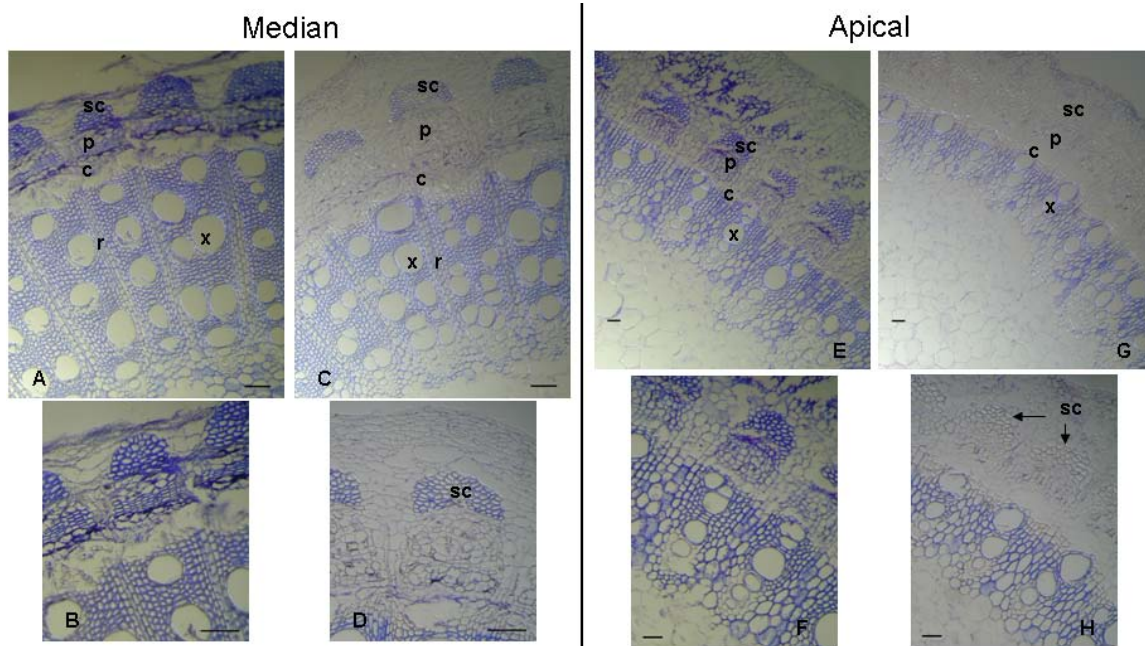


Figure 4. Portions of cross sections of median (left) and apical (right) internodes collected at veraison from vines: health (A, E) symptomatic (C, G) and their respective details (B, F and D, H). sc: sclerenchyma cells; p: phloem; c: cambium; x: xylem vessel; r: xylem ray. Magnification: A, C, E, G 100x; B, D, F, H 200x. Scale bars: 100 μm for median internodes; 50 μm for apical internodes. The sections are relative to the Sangiovese cv.

and Fast Green, data not shown). Our observations could show the initial phase of a process which will produce several different types of damage that can be found in the vines. In fact, the symptoms on shoots and branches can start in spring, with delayed and weak growth, and end in autumn, with reduced lignification of

the canes [6]. Furthermore, the various types of wood discoloration and decay in the trunk or main branches are the result of a number of structural and physiological changes. The main causes of these tissues alteration may be cellulolytic and ligninolytic enzymes produced by the fungi associated with esca [18]; vascular occlusion

due to gels and gums secreted by the diseased xylem parenchyma cells, and necrosis of vascular elements as the result of diffusion of the pathogen's toxins [19]. Similarly, the appearance of esca foliar symptoms has been linked to the presence of phytotoxins [20,21]. Phytopathogenic fungi produce several enzymes capable of hydrolysing macromolecules in plant tissues, namely cellulose, pectin, xylan and lignin [1,22,23]. The phytotoxic compounds, which reach the leaves by xylematic flow from the stems, were the cause of typical tiger stripes symptoms.

In relation to our qualitative observations we consider that more specific and quantitative experiments may be useful to confirm the relationship between the presence of esca disease and the minor lignification of cell walls. It would be also interesting to add biochemical analyzes to follow the variation of lignin and other wall polymers.

Considering these preliminary results it is possible to suggest that the alteration of tissues belonging to the vines affected by esca disease could be also detected before the foliar symptoms appearance. As consequence we

propose to further investigate the minor degree of vascular elements lignification, as it could be hypothesized that the histological examination could represent a valuable method for the early detection of esca.

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