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# *Phaeomoniella chlamydospora* infection induces changes in phenolic compounds content in *Vitis vinifera*

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**Summary.** The effect of *Phaeomoniella chlamydospora* infection was evaluated in three grapevine cultivars, in relation to the variation in phenolic compounds within the host. Young plants of *Vitis vinifera* (cv. Chardonnay, Touriga Nacional and two clones of cv. Aragonez) were infected using two strains of *Pa. chlamydospora* and were harvested after five months from inoculation. Overall, a localised increment in the amount of total phenolics within a methanolic extract of grapevine wood tissue was observed following infection. Such pattern was also manifested by a localised increase in non-tannin phenolics. HPLC analysis was used to identify the host phenolic compounds, which were mostly affected by the host-pathogen interaction. *trans*-Resveratrol was found to augment very significantly in both cv. Chardonnay and cv. Touriga Nacional following infection. ε-Viniferin also increased considerably in infected plants of cv. Touriga Nacional. Significant differences were encountered among infected plants depending not only on the cultivar, but also on the site of inoculation. Overall, cv. Chardonnay and cv. Touriga Nacional contained a higher amount of infection-induced phenolic compounds than both cv. Aragonez clones. Infection of young shoots also led to a greater accumulation of phenolic compounds than that of the trunk. Results are discussed in view of understanding the role of phenolic compounds and especially *trans*-resveratrol in grapevine defences against *Pa. chlamydospora*.

**Key words:** Petri disease, esca disease, plant defences, *trans*-resveratrol, ε-viniferin.

## Introduction

Grapevine (*Vitis vinifera*) represents one of the most economically important fruit crops worldwide, due to its widespread cultivation and its high commercial value (Vivier and Pretorius, 2002).

*Phaeomoniella (Pa.) chlamydospora* is associated with the occurrence of Petri and esca diseases, two of the most destructive ailments affecting grapevine.

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Esca has long been known across grapevine growing regions. It was first recognized and described in France (Ravaz, 1898) and California (Anonymous, 1895) and is nowadays affecting vineyards all over the world with a wide variety of symptoms.

Larignon and Dubos (1997) suggested that esca was probably caused by the interaction of several fungal species, acting sequentially. *Pa. chlamydospora* has been the pathogen more often associated with typical Petri disease symptoms (Mostert *et al.*, 2006) and appears also to be the most common pathogen in mature esca-infected vines. Petri disease occurs in grapevine during the first years of establishment,

following the use of infected propagation material, as well as following infection of the root system from soil-borne inoculum or infection of pruning wounds from aerial inoculum (Mostert *et al*., 2006). In the root system, the pathogen penetrates into the cortical tissue and subsequently into the vascular tissue causing necrosis of the rootstock (Mugnai *et al.*, 1999; Gubler *et al.*, 2004). Esca disease affects primarily mature plants. Infection occurs through pruning wounds by airborne spores (Ferradino *et al*., 2000; Larignon and Dubos, 2000; Sofia *et al*., 2005) and occasionally through the roots (Gubler *et al.*, 2004).

It has been suggested that infection of grapevine incited by *Pa. chlamydospora* and/or *Phaeoacremonium* spp. might predispose the vines to wood rots by breaking down toxic phenolics and other host-produced substances which are inhibitory to wood-rot fungi (Gubler *et al*., 2004). However, the mechanisms mediating the incidence of the disease symptoms and the changes occurring in the host and the pathogen during infection are poorly understood.

After the banning of sodium arsenite, there is no effective treatment against grapevine wood diseases, with emphasis on Petri and esca diseases. A wide variety of alternative options have been suggested to the use of sodium arsenite. These include stress prevention (Gubler *et al*., 2004), pre-treatment of propagation material with benomyl, hot water and/or *Trichoderma* spp. (Fourie and Halleen, 2004), wood protection with *Trichoderma* spp. or broad-spectrum fungicides, fosetyl Al applied as trunk injections (Di Marco *et al*., 2000) and chitosan treatment (Nascimento *et al.*, 2007). Despite the tentative use of these alternatives to sodium arsenite to control Petri and esca diseases, no long-term and effective control was attained to date. The only really effective solution remains that of uprooting and burning the infected vines. The lack of a specific treatment against *Pa. chlamydospora* reflects the little knowledge available on the effects that the onset of the disease causes at biochemical and genetic level in host.

Phenolic compounds are important plant defence metabolites. In grapevine callus cultures, the level of total phenolic compounds was found to be negatively associated with host susceptibility to *Pa. chlamydospora* and *Togninia minima*, the teleomorph of *Pm. aleophilum* (Bruno and Sparapano, 2006a). *trans*-Resveratrol has also been observed to cause growth inhibition in *Pa. chlamydospora* and *Phaeoacremonium angustius*, thus highlighting how the presence of this compound may induce resistance in grapevines (Santos *et al.,* 2006a).

To this date, however, very little evidence is available concerning the variation in the phenolic compounds of esca-infected grapevine wood. The work of Püssa *et al.* (2006) attempted to elucidate the variation in phenolic compounds present across different grapevine cultivars. Experimental evidence is even more reduced once the effects of Petri and esca disease on phenolics accumulation are considered. Amalfitano *et al.* (2000) showed that resveratrol and ε-viniferin content is higher in brown-red wood than in healthy wood taken from the same vine plant. Brown-red wood is considered an early symptom of esca in grapevine, but it is unlikely that asymptomatic tissues taken from the same plant might be considered as a true negative control. Furthermore, several esca-associated fungi are known to cause brown-red wood as one of their symptoms. Until now, no attempt has been reported to evaluate which fungus or fungi are more effective in triggering the phenolic compounds accumulation.

We strongly believe that it is becoming imperative to study the molecular interactions between grapevine and the esca fungi, to understand the biology of pathogenesis and to select potential targets to control the disease. In this respect, it seems particularly appropriate to perform the –omics analyses. However, whilst genomic, transcriptomic and proteomic analyses may still be considered fundamental tools to study plant-pathogen interaction, an ultimate and novel insight is provided by metabonomics. Understanding these variations will be the key to the development of a specific treatment against both Petri and esca diseases.

In this study, we have applied biochemical characterisation techniques to identify and quantify changes in plant phenolic compounds following infection of vine plants by *Pa. chlamydospora*. To our knowledge, this constitutes the first attempt to evaluate such variation in the plant. The results were analysed in view of the role that such changes may fulfil in disease susceptibility or resistance in grapevine.

## Materials and methods

### Fungal material

*Phaeomoniella chlamydospora* cultures (strain PH9 and PH13) were isolated from naturally occurring infections and were maintained in 90 mm

vented Petri dishes containing 20 ml 3.9% potato dextrose agar (Difco, Becton, Dickinson and C, Franklin Lakes, NJ, USA) at 23°C for up to three weeks prior to use.

For mycelium harvesting, *Pa. chlamydospora* was grown in 90 mm vented Petri dishes containing 20 ml 2.4% potato dextrose broth (Difco). Each plate was inoculated with a 5 mm inoculum plug from the actively growing margin of three week old colonies of *Pa. chlamydospora*, with the fungal mycelium facing upwards. After 2 weeks the mycelium was harvested by filtration through a sintered glass spore filter and washed three times with approximately 20 ml sterile deionised water prior to freeze drying. Freeze-dried mycelium was stored at -80°C until further use.

**Plant material, growth conditions and inoculation**

Cuttings of *Vitis vinifera* L. of the cultivars Touriga Nacional, Chardonnay and Aragonez (clones 52603 and RZ59) were supplied by Portuguese vine growers. Cuttings were rooted in water (cv. Chardonnay and cv. Aragonez) or soil (cv. Touriga Nacional) according to the routine methods used in our laboratory. Plants were then transferred to 1 l growing vessels containing a sandy soil mixture (1 part sand, 1 part peat, 2 parts soil). These were maintained in a ventilated greenhouse at 24°C, and irrigated twice weekly.

Plants were grown for one month under these conditions prior to being used for infection experiments with *Pa. chlamydospora*. Two different inoculation points were tested on the grapevine plants, one at the base of the primary shoot (between the first and second node) and one at approximately half way along the plant trunk. This aimed at establishing which area within the plant offered the best infection site. According to the location of the infection, two different inoculation methods were chosen. On the primary shoot, a wound was created by removing a small section of the bark with a scalpel. On the trunk, a wound was created by removing a 3 mm area of bark with a corkborer.

Inoculation plugs were cut from the actively growing margin of *Pa. chlamydospora* colonies. Infection took place by placing a 3 mm inoculation plug on the wound (mycelium side down). Each wound was then covered with moist cotton wool and sealed with Parafilm. Negative controls were treated in the same manner using 3 mm 3.9% potato dextrose agar plugs. Following inoculation, all plants were maintained under the conditions described above for a further five months prior to harvesting.

**Plant harvesting**

Five months after inoculation with *Pa. chlamydospora*, 10 plants were harvested for each treatment and for each fungal strain. Harvesting of the trunk-infected plants took place by removing 1 cm sections sequentially at either side the infection point, up to a total distance of 3 cm (6 segments). In the case of the primary shoot infected plants, the whole shoot was removed and subdivided in 1 cm sections, up to a total distance of 8 cm (8 segments). Corresponding sections of different plants from the same infection treatment were pooled. Each batch was ground to a powder in liquid nitrogen and stored at -80°C until further use.

**DNA extraction**

Genomic DNA extraction from plant material and fungal material used a simple and rapid method for high molecular weight plant DNA (Murray and Thompson, 1980). Approximately 1 g of the pulverised plant material or 0.2 g of freeze-dried mycelium was transferred to a 1.5 ml polypropylene microcentrifuge tube (Eppendorf, Hamburg, Germany). For DNA extraction from plant material, tubes contained approximately 100 mg polyvinylpolypirrolidone (PVPP) (Sigma-Aldrich, St. Louis, MO, USA). Plant material samples were instantaneously mixed with PVPP by thoroughly shaking.

Initially, 300 $\mu$l extraction buffer were added to the mixture mentioned above, which contained 0.35 M sorbitol, 0.1 M Tris-HCl (pH 8.2), 5 mM ethylenediamine tetra-acetic acid (EDTA), 0.38% (w/v) sodium bisulphite, 0.1% (v/v) β-mercaptoethanol. This was followed by the addition of 300 $\mu$l nuclei lysis buffer, containing 0.2 M Tris-HCl (pH 7.5), 50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, and 120 $\mu$l 5% (w/v) *N*-laurylsarcosine. The mixtures were vigorously shaken and incubated at 65 °C for 15 min. After incubation, the mixtures were cooled down to room temperature and 600 $\mu$l chloroform: isoamylalcohol (24:1) (v/v) were added. Samples were vigorously shaken and were then centrifuged for 15 min at 13,000 *g* and room temperature. The aqueous supernatants were carefully collected and transferred to new tubes. DNA was precipitated

with an equal volume of ice cold isopropanol and kept at -20°C for at least one hour before being centrifuged for 10 min at 13,000 *g* and room temperature. Pellets were air dried and washed with 70% (v/v) ethanol. Once the ethanol had evaporated, pellets were dissolved in 100 µl TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at 65°C for 5 min and stored at 4ºC until further use.

**PCR amplification**

The primer pair Pch1 (5´-CTCCAACCCTT-TGTTTATC-3´) and Pch2 (5´-TGAAAGTTGATA-TGGACCC-3´) were used for the amplification of a 360 bp fragment within the ITS region of *Pa. chlamydospora* DNA (Tegli *et al.*, 2000).

The PCR method was adapted from Tegli *et al.* (2000) as follows. Reactions were carried out in a total volume of 25 µl in 0.2 ml PCR tubes (Greiner Bio-one, Frickenhousen, Germany). Each reaction mixture contained 1 µl of DNA template, 0.5 µM of each primer (Pch1 and Pch2), 0.05 mM of each dNTP, 0.5 µl 50× BD Advantage™ 2 Polymerase Mix, 2.5 µl 10× BD Advantage™ 2 PCR Buffer (BD Biosciences Clontech, Palo Alto, CA, USA) and was made up to a total volume of 25 µl with MilliQ water.

PCR amplification was carried out in an automated thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). An initial denaturation step at 95°C for 3 min was followed by 25 amplification cycles: 30 s denaturation at 95°C, 20 s primer annealing at 45°C and 1 min extension at 72°C. A final extension step at 72°C for 5 min was performed. Amplified products were separated by electrophoresis in 1% (w/v) agarose gel. A 1 kb -Plus DNA Ladder (Invitrogen, Carlsbad, CA, USA) was used as molecular marker to evaluate the size of the PCR products. DNA fragments were stained with ethidium bromide (0.5 µg $ml^{-1}$) and visualised under ultraviolet light.

The presence or absence of *Pa. chlamydospora* infection was marked with a +/- scoring system.

**Extraction of phenolic compounds**

Phenolic compounds were extracted from infected and healthy plant material. All extractions were carried out in acid-washed borosilicate tubes, stoppered with Teflon caps. For each sample, 0.2 g of ground plant material was extracted in 2 ml HPLC-grade methanol. One hundred µg *p*-coumaric acid (Sigma-Aldrich, St. Louis, MO, USA) was added as an internal standard for subsequent HPLC quantification of individual phenolic compounds. Extraction took place over 40 min in a sonicating bath, with a 1 min interval after 20 min, during which samples were placed in ice to avoid overheating. After the extraction, 1.5 ml of the supernatant was removed and a further 1.5 ml fresh methanol was added and the extraction was repeated. This aimed at testing the extraction efficiency of the first extraction. After each of the two extractions, the supernatant was filtered through a 0.22 µm nylon filter and stored at -20°C until further analysis.

**Determination of total phenolics, non-tannin phenolics and extraction efficiency**

Estimation of total phenolic compounds within the methanolic extract was carried out according to the method of Makkar *et al.* (1993) and quantified as tannic acid (Sigma-Aldrich) equivalents. Hereafter, total phenolic compounds within the methanolic extract will be referred to as total phenolics. Reactions took place in 2 ml polypropylene microtest tubes (Greiner Bio-one, Frickenhousen, Germany). For each reaction, 50 µl of the phenolics containing extract was diluted in 0.45 ml MilliQ water. Subsequently, 0.25 ml 50% (v/v) Folin-Ciocalteau reagent (Merck, Darmstadt, Germany) and 1.25 ml of 20% (w/v) sodium carbonate were added. The reaction mixture was shaken vigorously, and kept in the dark for 40 min at room temperature. Samples were centrifuged for 5 min at 1300 *g* prior to reading the absorbance at 725 nm on a Shimadzu UV-2100 spectrophotometer. All blanks and the samples used to prepare the calibration curve contained an amount of *p*-coumaric acid equivalent to that found in the plant samples, as *p*-coumaric acid was added to the latter for subsequent HPLC analysis.

For each plant material sample, the total phenolics content was estimated for each of the two extractions, in order to estimate the efficiency of the extraction procedure.

Estimation of non-tannin phenolics also employed the method described above. This time, however, an additional step for tannin removal was introduced as the initial step of the reaction. To each 2 ml polypropylene microtest tube, 100 mg PVPP were added, followed by 0.5 ml extract and 0.5 ml MilliQ water. Tubes were shaken vigorously and stored at 4°C for 15 min, prior to being shaken vigorously again and centrifuged at 3000 *g* for 10

min. The supernatant was removed and used for non-tannin phenolics determination. Once again, all blanks and the samples used to prepare the calibration curve contained an amount of *p*-coumaric acid equivalent to that found in the plant samples. This time, however, *p*-coumaric acid also underwent the same preparation as the samples, prior to the reaction taking place. Data relating to both total phenolic compounds and non-tannin phenolic compounds content were used for the determination of tannins. This occurred by subtracting the latter value from the former.

**HPLC analysis**

The reversed-phase HPLC analytical separation was performed according to the method of Püssa *et al*. (2006) using a Waters 1525 binary pump linked to a Waters 2996 photodiode array detector (PDA) and a Waters column heater. The HPLC equipment was coupled with a Waters Symmetry C18 column (4.6 mm id, 250 mm length, 5 $\mu$m particle size) with a guard column filled with the same type of sorbent in a gradient mode of 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml $min^{-1}$ at 35°C. Elution was started with a linear gradient of B from 10 to 30% by 20 min, then to 90% by 40 min, and finished isocratically with 90% of B for 10 min. The sample injection volume was 10 $\mu$l. The PDA detector was set at an interval of 220–600 nm. The eluate optical density was monitored at the maximum absorbance wavelength for each peak ($\lambda_{max}$). These were 282 nm for resveratrol dimer and resveratrol tetramer A, 305 nm for *trans*-resveratrol, 309 nm for *p*-coumaric acid, 320 nm for resveratrol tetramer B and 323 nm for ε-viniferin.

Quantification of individual peaks was achieved by comparison to the sample internal standard. Identification of the chromatographic peaks was performed by comparison to known standards: *trans*-resveratrol, *trans*-resveratrol glucoside, catechin, epicatechin, epigallocatechin gallate, tannic acid, gallic acid, caffeic acid, quercetin and narigin. These were all obtained from Sigma-Aldrich. Selected samples were analysed by LC-MS/MS coupled with a PDA detector using an ESI-ion trap MS system LCQ ion trap mass spectrometer (Thermo Finnigan, CA, USA) equipped with electrospray source. The following conditions were used in experiments with ESI source in positive and negative mode: temperature of the heated capillary, 250ºC, source voltage 4.6 kV. Nitrogen was used as sheath gas and auxiliary gas. The sheath and auxiliary gas flow rates were 80 and 20 arbitrary units, respectively. LC-MS/MS was performed in the full scan mode. All the fragmentation experiments were done with 40% collision energy.

The results were compared to the findings of Püssa *et al*. (2006) and used to identify any remaining peaks, which did not match our standards. Data was analysed with Waters Empower software connected to the analytical instrumentation.

**Statistical analysis**

Data were analysed using InStat 3.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA) for the implementation of ANOVA analyses. Tukey post-testing was used to compare the statistical significance of the variation observed across treatments and across individual cultivars.

## Results

**Determination of plant infection**

*Phaeomoniella chlamydospora* was never detected in control plants, thus confirming the absence of this pathogen as a latent infection. A difference was observed in the colonisation of grapevine plants by the pathogen according to the infection method. Overall, following infection in the trunk, the pathogen was encountered up to 3 cm from the inoculation point. When infection of the stem was taken into consideration, this distance increased to about 3-4 cm according to the cultivar (Tables 1 and 2).

In cv. Touriga Nacional, strain PH13 grew to a greater distance than strain PH9. To the contrary, in cv. Chardonnay, strain PH9 grew further than strain PH13. This was also the case in cv. Aragonez 52603, whilst in cv. Aragonez RZ 59 no obvious difference was observed in the colonisation capacity of the two fungal strains.

**Determination of total phenolics, non-tannin phenolics and extraction efficiency**

A single methanolic extraction of phenolic compounds was able to yield on average 95.1±4.9% of the total soluble phenolics within the plant tissue. This result was based on the observation that multiple sequential extractions did not support any significant increase in the amount of total phenolic compounds. Control plants from cv. Chardonnay

and cv. Touriga Nacional, contained double the amount of total phenolics as compared to both cv. Aragonez clones ($P<0.001$). No significant difference was observed for cv. Chardonnay between the phenolic content of trunk and shoot tissue (Fig. 1).

Upon infection, a significant increase in the amount of total phenolic compounds within the wood tissue was generally observed. However, differences were detected in the way plants from individual cultivars responded to infection. Chardonnay plants showed the least response to infection. When challenged with strain PH13, a 30% increase in the amount of total phenolics was observed in colonised wood tissue within the trunk of infected plants ($P<0.001$), as compared to controls (Fig. 1). A similar but opposite pattern was observed when strain PH13 was used for the infection of shoot tissue. In this case, no variation was observed in the total phenolics content of the colonised tissue. However, a significant reduction of approximately 30% in total phenolics was observed in the uncolonised tissue from diseased plants.

Infected plants from cv. Touriga Nacional, displayed a significant increase of more than 30%

Table 1. Occurrence of infection in trunk tissue from plants from cv. Touriga Nacional (T. Nacional) and cv. Chardonnay (Char) challenged with *Pa. chlamydospora* strains PH9 and PH13. Results from control plants (Ctrl) are also shown. Distance expressed in cm above (+) and below (-) the inoculation point (IP) section (1 cm).

| Sample | Trunk section | | | | | |
|---|---|---|---|---|---|---|
| | -3 | -2 | -1 | IP | +1 | +2 |
| T. Nacional Ctrl | - | - | - | - | - | - |
| T. Nacional PH13 | - | + | + | + | + | + |
| T. Nacional PH9 | - | - | + | + | + | - |
| Char Ctrl | - | - | - | - | - | - |
| Char PH13 | - | + | + | + | + | + |
| Char PH9 | + | + | + | + | + | + |

[a] For each treatment the presence (+) or absence (-) of the fungus are reported;

Table 2. Occurrence of infection in shoot tissue from plants from cv. Chardonnay (Char) and cv. Aragonez clones (Ara 52603 and Ara RZ59) challenged with *Pa. chlamydospora* strains PH9 and PH13. Results from control plants (Ctrl) are also shown. Distance expressed in cm from the inoculation point (IP) section (1 cm).

| Sample | Shoot section[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IP | +1 | +2 | +3 | +4 | +5 | +6 | +7 |
| Char Ctrl | - | - | - | - | - | - | n.a. | n.a. |
| Char PH13 | + | + | + | - | - | - | n.a. | n.a. |
| Char PH9 | + | + | + | + | + | - | n.a. | n.a. |
| Ara 52603 Ctrl | - | - | - | - | - | - | - | - |
| Ara 52603 PH13 | + | + | + | - | - | - | - | - |
| Ara 52603 PH9 | + | + | + | + | + | + | + | + |
| Ara RZ59 Ctrl | - | - | - | - | - | - | - | - |
| Ara RZ59 PH13 | + | + | + | + | + | + | n.a. | n.a. |
| Ara RZ59 PH9 | + | + | + | + | + | + | n.a. | n.a. |

[a] See Table 1
n.a, not available data.

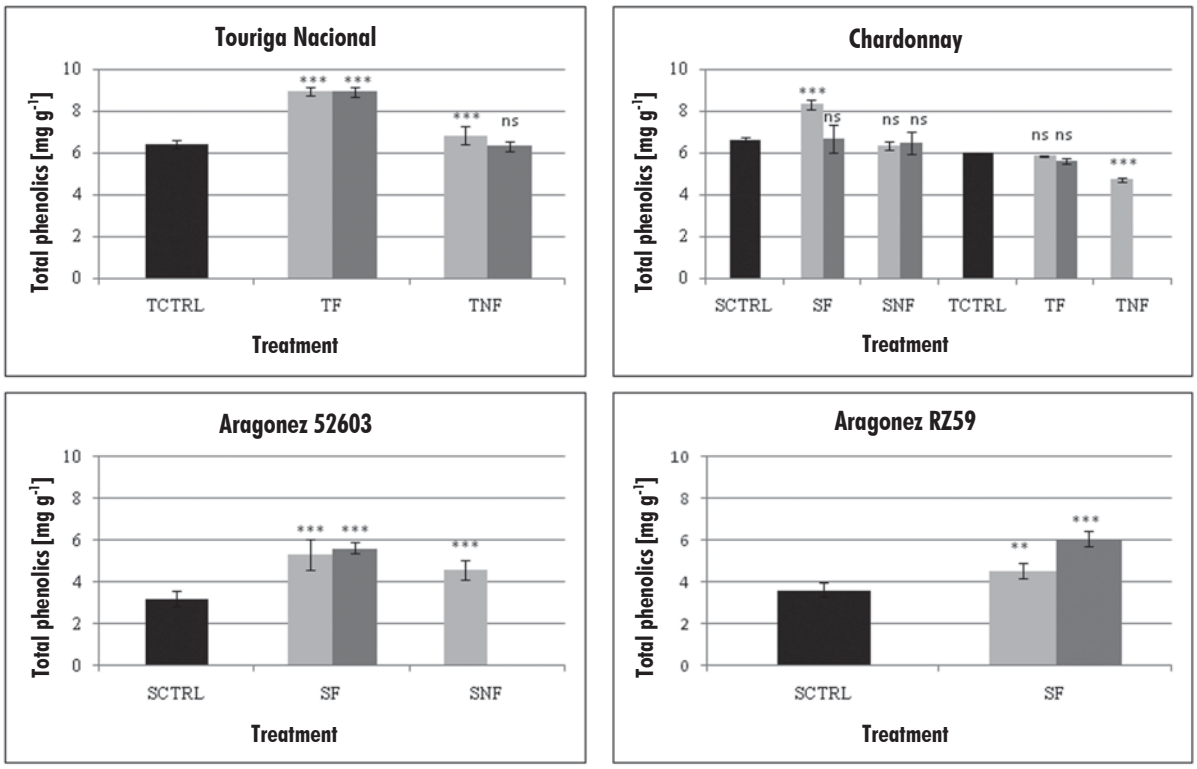



Fig. 1. Total phenolic compounds content within the methanolic extract from grapevine wood tissue from healthy and infected plants (cv. Touriga Nacional, cv. Chardonnay, cv. Aragonez [52603] and cv. Aragonez [RZ59]). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF). Error bars represent standard deviation (n=6). Level of statistical significance is also indicated as *, $P<0.05$); **, $P<0.01$; ***, $P<0.001$; ns, non-significant. Control (■), PH9 (■), PH13 (■). For control treatments, TCTRL refers to trunk control and SCTRL refers to shoot control.

in total phenolic content in the presence of both fungal strains ($P<0.001$). Such increase was not so pronounced within the uncolonised tissue of infected plants. In fact, when this tissue type was considered, a significant variation was only observed in the case of strain PH13 infection.

Total phenolic content in control plants of cv. Aragonez was approximately 3 mg $g^{-1}$of wood, that is 50% lower than that observed for cv. Chardonnay and cv. Touriga Nacional. Upon infection, the amount of total phenolic compounds almost doubled ($P<0.001$). This was independent of the fungal strain used for inoculation (Fig. 1). Once more, an increase in total phenolic content was also observed in uncolonised tissue of strain PH13 infected plants (no uncolonised tissue was available for strain PH9 infected plants and for cv. Aragonez clone RZ59). Despite being statistically significant ($P<0.001$), such increase was not as pronounced as that observed in colonised tissue.

Non-tannin phenolic compounds varied little between cultivars and treatments (Tables 3 and 4). Non-tannin phenolics represented approximately 13% of the total phenolic content, thus indicating that the greatest variation in total phenolic compounds was primarily linked to the variation in tannins content.

**Identification and determination of individual phenolic compounds by HPLC**

Data relative to the identification of individual peaks by HPLC (UV absorbance spectrum) and LC-MS/MS (molecular ion mass and main daughter ions following MS/MS fragmentation) are reported

Table 3. Mean non-tannin and tannin content (mg $g^{-1}$ wood) in healthy and infected plants from cv. Touriga Nacional (T. Nacional) and cv. Chardonnay (Char) infected at the trunk with *Pa. chlamydospora* strains PH13 and PH9. Results from control plants (Ctrl) are also shown.

| Sample | Pathogen detected [a] | | Pathogen undetected [a] | |
|---|---|---|---|---|
| | Non-tannin | Tannin | Non-tannin | Tannin |
| T. Nacional Ctrl | - | - | 0.82±0.31 | 5.59±0.18 |
| T. Nacional PH13 | 0.81±0.11 | 8.10±0.20 | 0.89±0.36 | 5.93±0.42 |
| T. Nacional PH9 | 1.07±0.39 | 7.84±0.21 | 1.06±0.46 | 5.26±0.24 |
| Char Ctrl | - | - | 0.80±0.27 | 5.19±0.03 |
| Char PH13 | 0.74±0.28 | 5.11±0.03 | 0.78±0.29 | 3.91±0.11 |
| Char PH9 | 0.60±0.22 | 5.00±0.13 | - | - |

[a] Mean ± SD (n=6).

Table 4. Mean non-tannin and tannin content (mg $g^{-1}$ wood) in healthy and infected plants from cv. Chardonnay (Char) and cv. Aragonez clones 52603 (Ara 52603) and RZ59 (Ara RZ59) infected at the shoot with *Pa. chlamydospora* strains PH13 and PH9. Results from control plants (Ctrl) are also shown.

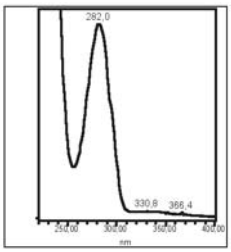
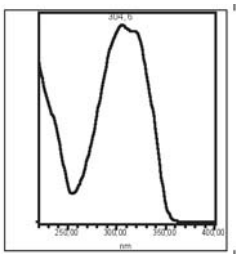
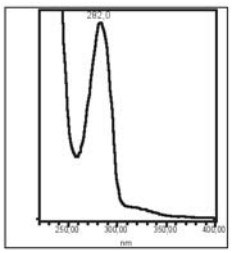
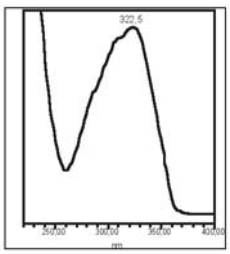
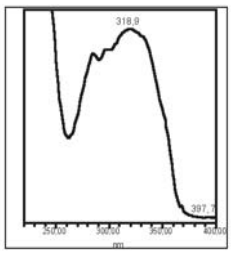
| Sample | Pathogen detected [a] | | Pathogen undetected [a] | |
|---|---|---|---|---|
| | Non-tannin | Tannin | Non-tannin | Tannin |
| Char Ctrl | - | - | 0.45±0.21 | 6.19±0.10 |
| Char PH13 | 1.00±0.44 | 7.30±0.26 | 0.96±0.37 | 5.39±0.18 |
| Char PH9 | 0.82±0.22 | 5.84±0.66 | 0.88±0.45 | 5.60±0.52 |
| Ara 52603 Ctrl | - | - | 0.61±0.15 | 2.61±0.39 |
| Ara 52603 PH13 | 0.54±0.10 | 4.77±0.73 | 0.60±0.18 | 4.00±0.47 |
| Ara 52603 PH9 | 0.55±0.16 | 5.08±0.25 | - | - |
| Ara RZ59 Ctrl | - | - | 0.59±0.19 | 3.03±0.33 |
| Ara RZ59 PH13 | 0.58±0.24 | 3.96±0.37 | - | - |
| Ara RZ59 PH9 | 0.57±0.22 | 5.48±0.36 | - | - |

[a] See Table 3.

in Table 5. HPLC analysis of healthy and infected plant material revealed no qualitative differences in phenolic compounds. Overall, *trans*-resveratrol and its oligomers were the main phenolic compounds identified within grapevine wood tissue, with ε-viniferin, other resveratrol dimers, and resveratrol tetramers being the most represented.

The most abundant phenolic compound in all cultivars was ε-viniferin. Cv. Touriga Nacional contained overall the highest ε-viniferin content (0.4 mg $g^{-1}$ of wood), which was significantly ($P$<0.001) reduced in all other cultivars (Fig. 2). Both clones from cv. Aragonez contained the least amount of ε-viniferin (approximately 0.1 mg $g^{-1}$ of wood). Cv. Touriga Nacional was also the only cultivar in which fungal infection by both strains caused a significant ($P$<0.001) increase in ε-viniferin accumulation, which accounted for approximately 0.5 mg $g^{-1}$ of wood. In the case of PH9 infection, this accumulation extended to uncolonised tissue. In all other cases,

Table 5. Characteristics of the main phenolic compounds extracted from *Vitis vinifera* wood tissue. Identification was based on retention time, peak characteristics (UV absorption spectrum) and LC-MS/MS data (molecular ion mass in positive ionisation mode $[M+H]^+$ and main daughter ions from MS/MS fractionation).

| Compound | Retention time [min] | Peak characteristics | $[M+H]^+$ | Main daughter ions |
|---|---|---|---|---|
| Resveratrol dimer | 33.4 |  | 455 | 349; 361; 437; 215; 343; 199; 255; 267 |
| *trans*-Resveratrol (compared to known standard) | 35.1 |  | 229 | 141; 183; 199 |
| Resveratrol tetramer A | 35.4 |  | 907 | 559; 813; 361; 453; 651; 541; 801; 783 |
| ε-Viniferin | 36.7 |  | 455 | 349; 361; 437; 215; 343; 199; 255; 267 |
| Resveratrol tetramer B | 37.9 |  | 907 | 559; 813; 361; 453; 651; 541; 801; 783 |

infection had no effect on ε-viniferin accumulation, although a significant decrease in its content was observed in the uncolonised trunk tissue from cv. Chardonnay plants infected with strain PH13.

The second most abundant phenolic compound was *trans*-resveratrol (Fig. 3), which was significantly affected by the presence of the fungus within the wood tissue. The most remarkable results were observed in the case of infected cv. Chardonnay shoots. In this instance *trans*-resveratrol was only observed following infection by both *Pa. chlamydospora* strains ($P$>0.001). Only plants from cv. Aragonez, clone RZ59 did not seem to vary significantly in their *trans*-resveratrol content, independently of infection. Generally, increases in *trans*-resveratrol content were limited to the infected tissue. Only occasionally, an increase was also observed in uncolonised plant tissue. Specifically this occurred for plants infected with strain PH13 for both cv. Chardonnay (shoot tissue) and cv. Aragonez clone 52603.

Significant differences ($P$<0.001) were also observed in the relationship between individual fungal strains and *trans*-resveratrol accumulation. Strain PH13 was always associated with higher *trans*-resveratrol amounts than strain PH9 (Fig. 3).

The accumulation of other resveratrol dimers was significantly lower ($P$<0.001) in cv. Aragonez than in any of the other cultivars (Fig. 4), with a difference of approximately 10-fold. Furthermore, within cv. Chardonnay, a significant difference ($P$<0.001) was observed in the accumulation of other resveratrol dimers in trunk and shoot tissue, with the former containing approximately half the amount of resveratrol dimers than that observed in the latter. Nevertheless, accumulation of these phenolics compounds was not affected by infection. Only in one case, in fact, a significant increase was observed ($P$<0.001), which corresponded to PH13-infected tissue from cv. Chardonnay. In this case, however, such increase

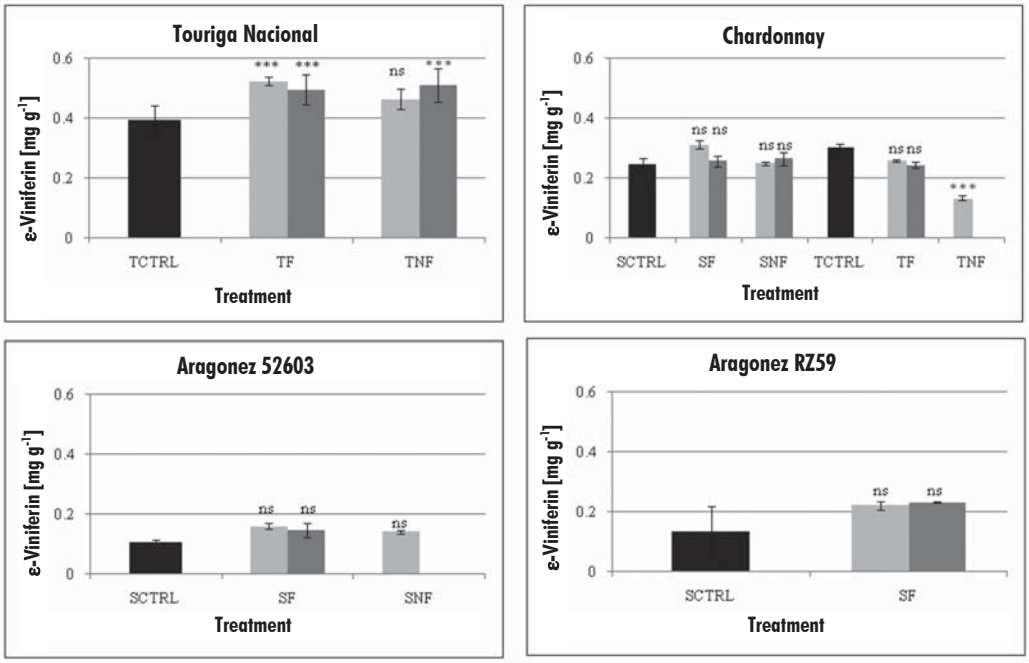



Fig. 2. ε-Viniferin content in healthy and infected plants, from cv. Touriga Nacional, cv. Chardonnay, cv. Aragonez (52603) and cv. Aragonez (RZ59). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF). Error bars represent standard deviation (n=4). Level of statistical significance is also indicated as *, $P$<0.05; **($P$<0.01); ***, $P$<0.001; ns, non-significant. Control (■), PH9 (□), PH13 (■). For control treatments, TCTRL refers to trunk control and SCTRL refers to shoot control.

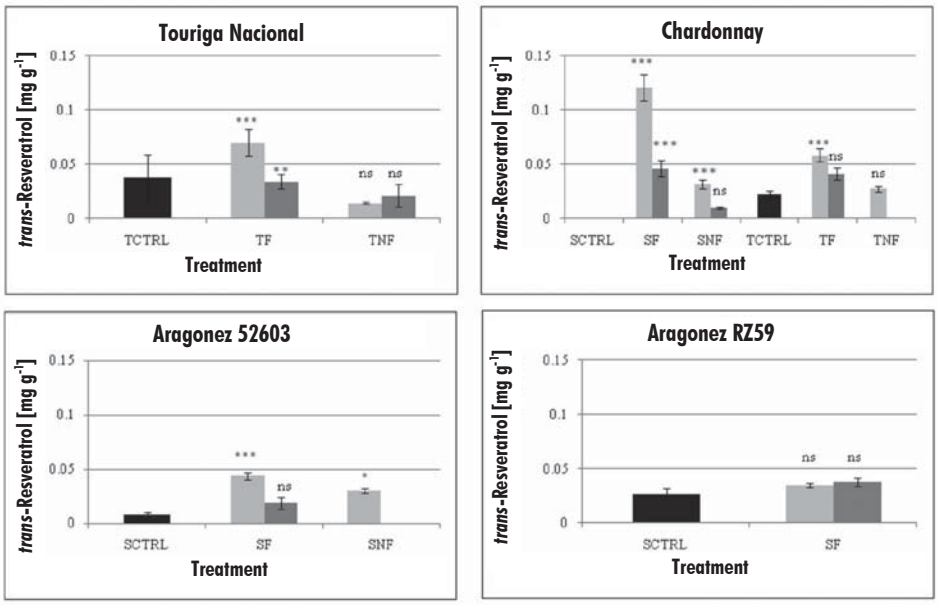



Fig. 3. *trans*-Resveratrol content in healthy and infected plants, from cv. Touriga Nacional, cv. Chardonnay, cv. Aragonez (52603) and cv. Aragonez (RZ59). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF). Error bars represent standard deviation (n=4). Level of statistical significance is also indicated as *, $P<0.05$; **($P<0.01$); ***, $P<0.001$; ns, non-significant. Control (■), PH9 (■), PH13 (■). For control treatments, TCTRL refers to trunk control and SCTRL refers to shoot control.

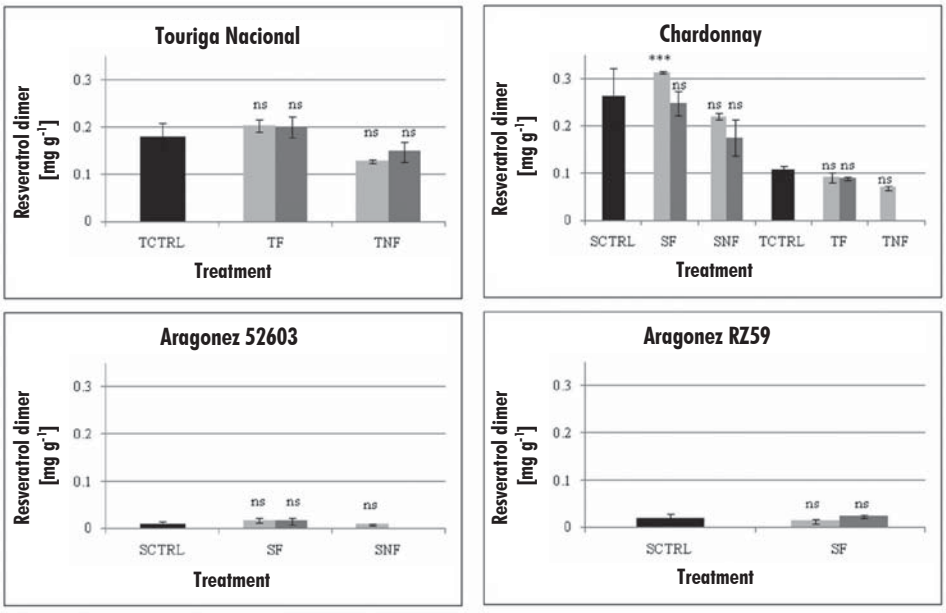



Fig. 4. Resveratrol dimer content in healthy and infected plants, from cv. Touriga Nacional, cv. Chardonnay, cv. Aragonez (52603) and cv. Aragonez (RZ59). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF). Error bars represent standard deviation (n=4). Level of statistical significance is also indicated as *, $P<0.05$; **($P<0.01$); ***, $P<0.001$; ns, non-significant. Control (■), PH9 (■), PH13 (■). For control treatments, TCTRL refers to trunk control and SCTRL refers to shoot control.

only accounted for approximately 10% relative to control plants.

Two resveratrol tetramers were identified. Cv. Touriga Nacional and cv. Chardonnay contained significantly higher amounts of resveratrol tetramer A (0.05 to 0.1 mg $g^{-1}$ of wood; $P<0.001$) than either of the cv. Aragonez clones (0.02 to 0.05 mg $g^{-1}$ of wood). Nevertheless, in most cases considered, no significant variation was observed in resveratrol tetramer A content as a result of fungal infection (Fig. 5). The only exceptions were observed in cv. Chardonnay. In this case, a significant decrease in resveratrol tetramer A was observed in shoot tissue, beyond the area of fungal colonisation. Similarly, in trunk tissue, a significant reduction in resveratrol tetramer A content was observed in plants challenged with strain PH13. This reduction was recorded in both colonised and uncolonised tissue.

The highest amounts of resveratrol tetramer B were also observed in cv. Touriga Nacional and cv. Chardonnay (0.2 and 0.1 mg $g^{-1}$ of wood, respectively). Once again, the two cv. Aragonez clones presented significantly lower levels of resveratrol tetramer B, which corresponded to a 5-fold reduction, as compared to cv. Chardonnay plants and approximately 10-fold reduction as compared to cv. Touriga Nacional. No significant differences were observed between healthy and infected plants across the treatments tested (Fig. 6).

## Discussion

The aim of the present study was to determine the effect of *Pa. chlamydospora* infection in young *Vitis vinifera* L. plants. Specifically, the study

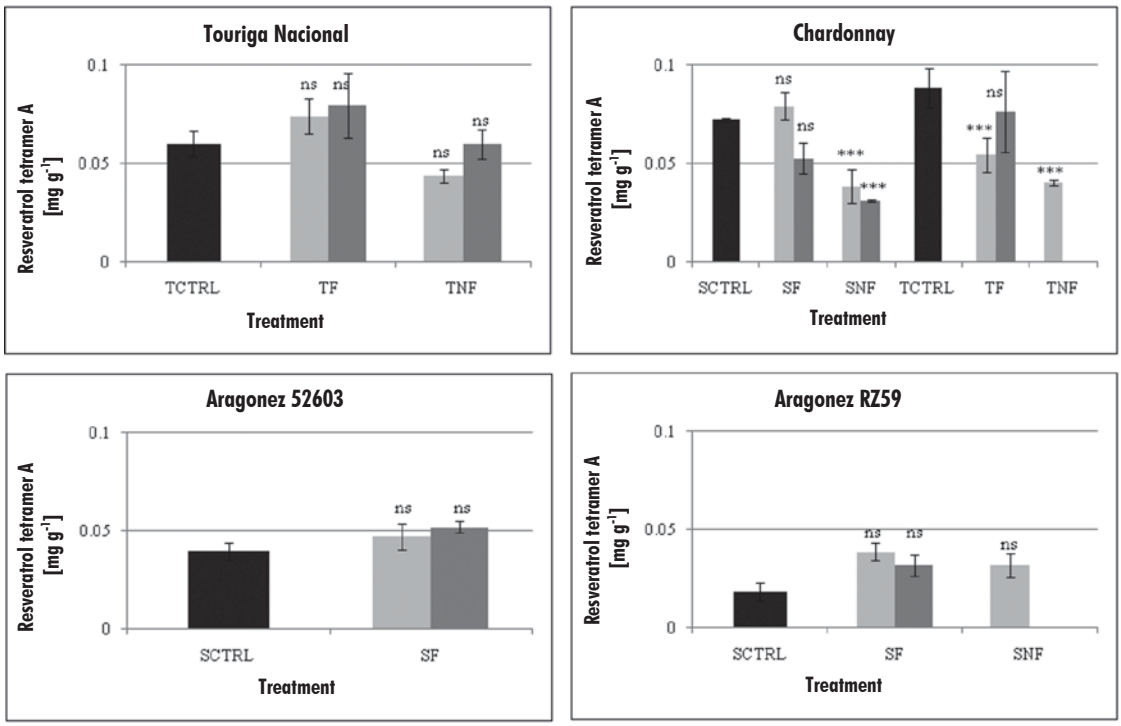



Fig. 5. Resveratrol tetramer A content in healthy and infected plants, from cv. Touriga Nacional, cv. Chardonnay, cv. Aragonez (52603) and cv. Aragonez (RZ59). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF). Error bars represent standard deviation (n=4). Level of statistical significance is also indicated as *, $P<0.05$; **($P<0.01$); ***, $P<0.001$; ns, non-significant. Control (■), PH9 (■), PH13 (■). For control treatments, TCTRL refers to trunk control and SCTRL refers to shoot control.

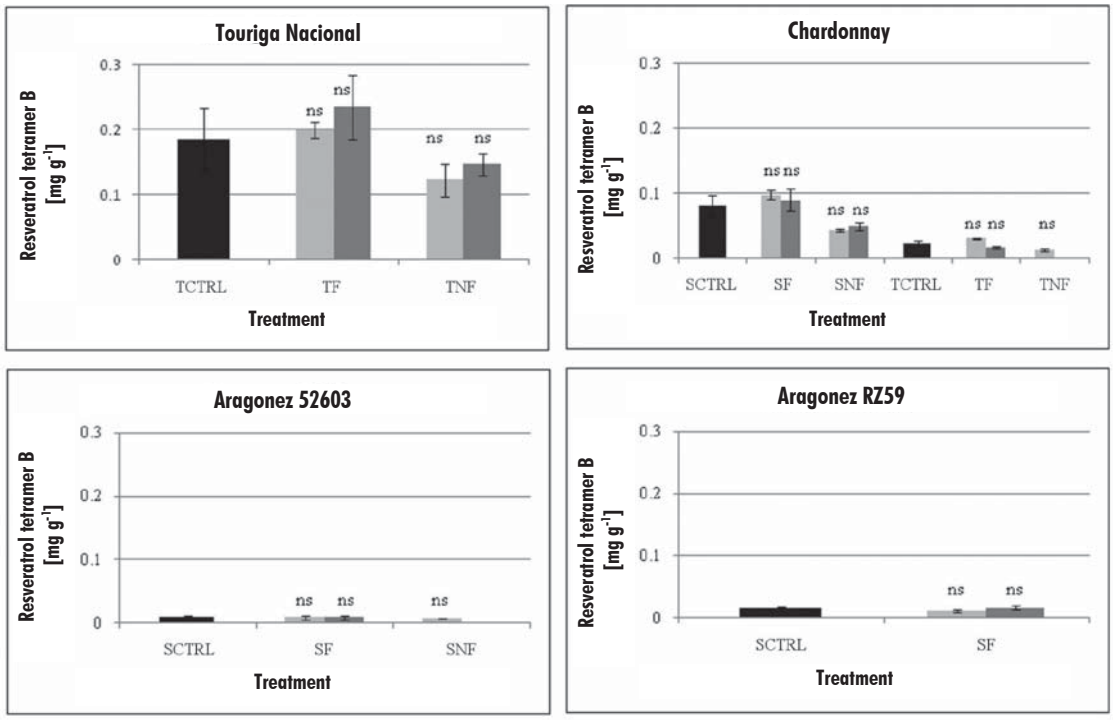



Fig. 6. Resveratrol tetramer B content in healthy and infected plants, from cv. Touriga Nacional, cv. Chardonnay, cv. Aragonez (52603) and cv. Aragonez (RZ59). Different tissue types are represented with infected plants: colonised trunk tissue (TF), uncolonised trunk tissue (TNF), colonised shoot tissue (SF) and uncolonised shoot tissue (SNF). Error bars represent standard deviation (n=4). Level of statistical significance is also indicated as *, $P<0.05$; **($P<0.01$); ***, $P<0.001$; ns, non-significant. Control (■), PH9 (■), PH13 (■). For control treatments, TCTRL refers to trunk control and SCTRL refers to shoot control.

aimed to elucidate the effect of fungal colonisation on the accumulation of phenolic compounds within infected and healthy wood tissues.

Evaluation of the growth rate of *Pa. chlamydospora in vivo* was beyond the scope of this study. Nevertheless, an insight in the growth mode of the fungus was obtained whilst testing for infection, during the preparation of experimental material. Troccoli *et al.* (2001) had previously reported information concerning the growth of *Pa. chlamydospora in vivo* using one year old micropropagated plants. Despite the different nature of the plant material used in this study, a growth pattern similar to that described by the authors was detected here.

Our main observation was that the extent of fungal growth in infected grapevine plants was not only dependent on the cultivar or fungal strain, but also on type of tissue, where infection occurred. Overall, colonisation of the plant tissue took place at a higher rate in young shoots as compared to the trunk. Differences in the pattern of colonisation observed may depend on several factors. Among these, the ability of the pathogen to colonise different tissue types, as well as variations in the structural and chemical characteristics of the host tissue, are worth of note. The results presented here fit well with the observation of Santos *et al.* (2006b). They indicated that, whilst testing for extracellular metabolites of *Pa. chlamydospora* as a virulence factor, differences in the results were observed, which were dependent on the host cultivar and the pathogen strain tested.

Accumulation of phenolic compounds is a well known defence mechanism in the plant Kingdom (Jeandet, 2002). The importance of phenolic compounds has been acknowledged through an extensive body of literature available on this topic, which relates to a large variety of species including grapevine (Kortekamp and Zyprian, 2003; Bruno and Sparapano, 2006b; Kortekamp, 2006; Püssa *et al.*, 2006) and *Arabidopsis thaliana* (Soylu, 2006).

Whilst the assessment of total phenolic compounds content may be a valuable tool to gain an insight in the changes occurring during pathogenesis, it is worth of consideration that measurements of total amounts may hide ecologically meaningful variation in individual compounds (Keinänen *et al*., 1999). For this reason, coupling the determination of total phenolics with a more detailed characterisation of these seemed to be an appropriate choice in this study.

A great variability in phenolics content was observed between tested grapevine cultivars. Previous studies had reported that phenolic content in grapevine was cultivar-specific (Bruno and Sparapano, 2006b; Püssa *et al*., 2006) and that may be directly linked to host susceptibility to pathogenic attack. Plants from cv. Aragonez contained the lowest constitutive amount of total phenolic compounds. At the same time, these plants seemed to be the most susceptible to *Pa. chlamydospora* colonisation. Kortekamp and co-workers (Kortekamp and Zyprian, 2003; Kortekamp, 2006) suggested that resistance mechanisms against the fungal pathogen *Plasmopara viticola* were present in both resistant and susceptible plants. However, these mechanisms might simply fail to be activated during infection. The reasons for this may be several and may be related to host and pathogen specificity and combativeness.

*Pa. chlamydospora* might be able to break down toxic phenolic compounds and other host-produced metabolites (Gubler *et al*., 2004). This was recently confirmed as *Pa. chlamydospora* was observed to be able to grow in the presence of resveratrol and tannic acid as the sole carbon sources of a minimal nutrient medium. Among the fungi tested, *Pa. chlamydospora* was the least inhibited by both resveratrol and tannic acid, suggesting that it might be able to enzymatically convert these phenolic compounds to less toxic derivatives. It is possible therefore, that the differences observed in phenolic compounds accumulation during infection by *Pa. chlamydospora* strain PH13 and PH9, may be related to the different ability of each strain to break down and/or utilise these metabolites. The present study, did not attempt to elucidate whether such variation may also be due to a direct effect of the pathogen on the ability of the host to synthesise defence metabolites. This certainly remains an interesting question, which will be addressed in future work.

Interestingly, the increased accumulation in phenolic compounds (both as total phenolics and as individual stilbenes) seemed to be confined to colonised plant tissue. Often, in fact, two patterns where observed. When an increased accumulation of phenolic compounds occurred as a consequence of fungal colonisation, a significant increase was sometimes also observed in the uncolonised tissue (e.g. total phenolic compounds and *trans*-resveratrol content in cv. Aragonez clone 52603; Fig. 1 and Fig. 3). When little variation was observed in the phenolics content of colonised tissue, sometimes a decrease was observed in uncolonised tissue (e.g. total phenolic compounds and ε-viniferin content in cv. Chardonnay trunk tissue; Fig. 1 and Fig. 2).

The mechanisms responsible for this localised accumulation of phenolic compounds remain unclear. Considerable evidence is available concerning the compartmentalisation of phenolic compounds within plant tissue. It remains to clarify whether such accumulation may be caused by increased localised synthesis or active translocation from adjacent sites. Maize cells have been observed to accumulate phenolic-rich mucilage a short time after wounding, which may inhibit pathogenic growth (Crews *et al.,* 2003). Localised accumulation of phenolic compounds has also been reported in mycorrhizae (Weiss *et al.,* 1997, 1999) and in association to abiotic stress (specifically copper exposure, Santiago *et al.,* 2000), which provide evidence that a common response mechanism may be activated in response to both biotic and abiotic stress alike.

Results concerning the variation in individual phenolic compounds (*trans*-resveratrol and its oligomers) failed, in some cases, to point out a definitive upregulation mechanism responsible for their accumulation. Nevertheless, the results relating to the accumulation of *trans*-resveratrol monomers certainly offered a clear picture of how pathogenesis caused an increase in the accumulation of this stilbene. It is worth of note, however, that different mixtures of phenolic compounds may act additively or synergistically (McKey, 1979; Berenbaum, 1985). Thus, variation in the relative proportions of secondary compounds may have considerable ecological consequences (Langenheim, 1994). This may cause the simple observation of absolute values to be misleading.

Pezet *et al*. (2005) reported that resveratrol monomers showed no toxicity against *P. viticola*, yet their

synthesis was essential for the formation of resveratrol dimers (δ- and ε-viniferins) in resistant plants, as these were highly toxic to the fungus. This may also be the case in with *Pa. chlamydospora*, as its ability to degrade resveratrol has been reported previously (Bruno and Sparapano, 2006c). It appears therefore, that none of the cultivars investigated here showed true resistance against *Pa. chlamydospora*. Nevertheless, there seemed to be a correlation between the accumulation of resveratrol and its oligomers and the ability of the pathogen to colonise wood tissue.

From the results presented here, it can be concluded that the infection of young grapevine plants with *Pa. chlamydospora* induced an upregulation of plant defence mechanisms. This resulted in increased and localised accumulation of antifungal phenolic compounds, which are known to inhibit fungal growth. This would be a fundamental step in controlling the spread of the infection within the host. The results also emphasised how variation in host cultivar, pathogen strains and infection methodology should all be taken into consideration when designing pathogenicity studies, as this would allow drawing more widely applicable conclusions.

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