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ROLE OF STILBENES AS A RESISTANCE FACTOR OF THE GRAPEVINE TOWARDS BIOTIC STRESS

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Presented on the recommendation of:
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Aos meus pais e à minha irmã

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Introduction

Worldwide, the grapevine and particularly viticulture have become of utmost importance.

Ever since spreading from Europe, where both cultivation and the know-how had been widely diffused by the Etruscan, Greeks and Romans whose influence led to the creation of a traditional, cultural and economical asset, viticulture and wine took over the world.

Viticulture as we know it today is distributed in areas where all vine requirements can be fulfilled. Soil, climate and varietal physiological adaptation have been crucial in determining viticulture's geographical world map.

Moreover, the properties of the vine and wine making techniques have played a fundamental role in determining both the broad distribution of viticulture and also the specific character of the wine trade at different periods during the past (Unwin, 1996). Currently, the latest data released by the Organisation Internationale de la Vigne et du Vin establishes worldwide under vine area around 7861mha. Wine production, without including juices and musts, reaches amounts between 266.6 and 272.3 Mio hl.

Although all its charm, the wine, object of passion, is also an economical product and even if a large interest developed for wine and wine tasting from the 90's on, due to social factors, as the widespread economical crisis, that overcame cultural traditions, the consumption of wine in Europe has been decreasing in the last years, greatly influencing world values that were encompassed between 237.1 and 248.7 Mio hl in 2008 (OIV report 2009).

Despite the differences that can be found within Old World and New World wine and viticulture, the true is that wine became a global phenomena evolving constantly.

But not all was easy in the viticultural globalisation process. In the late 1800's along with new vines from North America the worst parasites were imported in Europe: Oidium *tuckeri* (powdery mildew) a fungus found in London; the phylloxera of the vine, an insect found in the south of France and

finally the well known downy mildew or *Plasmopara viticola* (Fregoni, 2006). European viticulture crossed then great difficulties and although studies and increasing knowledge have allowed to partially overcome these problems, either by creation of anti-parasite treatments for fungus or by the exploitation of the indigenous North American vine species properties, natural developed resistances, through the grafting of European vine onto American rootstock, the pest and diseases of the grapevine are still a major source of concerns for viticulturists because no permanent solution were actually found and many other diseases, as Pierce's disease, may spread from the confinement areas to the rest of the world.

The grapevine defence mechanisms

The grapevine, as all living plants, is in constant change. From the time that the grafted vine is placed on a vineyard till it becomes fully formed and productive and during all its lifetime it will interact with the surrounding environment, whether with other plants, climatic conditions or cultural techniques adopted or even with several pathogens.

Many of these interactions are innocuous to the grapevine but others can cause different levels of damage to the plant ranging from reversible effects to morphological and physiological damages that can ultimately lead to the plant's death.

In order to overcome the adversities the grapevine has an elaborate defence system that relies on the joint action of barriers formed even before the attack has taken place, as well as induced barriers that are activate as soon as the plant senses any type of attack. The constitutive defence mechanism is an innate part of the plant's structure and is always active and functional, even before the plant is damaged by any kind of stress such as diseases and pathogens, climatic or cultural changes among others.

These defences lead to the production of multiple structural barriers like waxes, cutin, suberin and cellulose (Bell, 1981).

The general defence reaction is completed by the inducible defence mechanism and several additional barriers can form: papilla (callose, lignin, silicon and proteins between the cell wall and the cell membrane), tyloses and abcision zones (Agrios, 1997) lignifications (Hijwegen, 1963) phytoalexins (Müller and Börger, 1940; Paxton, 1981) synthesis of pr-proteins (van Loon and van Kammen, 1970; Gianinazzi, *et al*, 1970) or hypersensitive response - programmed cell death and oxidative burst (Pontier *et al*, 1998).

The induced plant defence system is a co-ordinated system of cellular, tissue and molecular responses.

This mechanism, unlike the pre-formed mechanism, is only activated upon recognition of a pathogen, via elicitors or pathogen associated molecular patterns. A basal level of induced resistance is created (Ton *et al*, 2002).

A common generic definition widely used to describe immune response of pathogen associated molecular (PAMP) defines exogenous elicitors or as microbe or plant derived molecules, generated by enzymatic degradation of plant components by the pathogen itself, during the first steps of infection that allow the plant cells to distinguish self from foreign cells (Jones *et al*, 2006).

Whether the pathogen is able to infect and develop at the expenses of the plant, it depends on the plant species and on the race of pathogen. Resistance is mainly defined as pathogen failure to invade the host cell but the most precise description would be to say that resistance can express itself at multiple stages of the infection process, from inhibition of penetration and germination to restriction of the pathogen after it has established.

When all genotypes of the pathogen are unable to overcome plant defences and the pathogen is incapable of growing in a certain plant species, the interaction is named non-host pathogen and in nature this is the most common outcome (Agrios, 1997). Only a handful of microbes are able to overcome the multiple resistance barriers that protect a given plant species from pathogen attack. Only those capable of breaching the resistances can colonize the plant successfully (Moerschbacher and Mendgen, 2001).

If the pathogen is allowed to grow in a plant, due to slow or insufficient response of its defence mechanisms, the interaction is referred to as host-pathogen. Hence the plant is susceptible to this pathogen. The expression of this interaction ranges from imperceptible consequences to utter plant death.

In this latter case the pathogen has been able to suppress, evade or manipulate host defences by action of effectors which are molecules that manipulate host cell structure and functions, facilitating infection or/and triggering defence responses (Díez-Navajas *et al*, 2008).

On one side plant/pathogen interactions depend on the plant species or more specifically in the genotypes found within a plant family. On the other side

they depend on the attacking pathogen, on the adaptation skills of a certain pathogen strain.

These interactions are without a doubt complex and bound to constant development as resistance techniques can emerge from the plant's side and new ways to avoid these defences can be found by the pathogen.

The activation of the inducible defence mechanism by a stimulus previously to a pathogen attack has proven to make the plant less sensitive to that attack, this is called induced resistance and the phenomena is known as systemically acquired resistance - SAR (Ross, 1961). In this case a broad spectrum resistance in tissue distant form the site of primary infection is achieved after contact with a pathogenic agent (van Loon *et al*, 1998).

Resistance activation in all parts of the plant can also be achieved by influence of non-pathogenic microorganisms; Induced Systemic Resistance (ISR) is another type of an induced state in which the plants show more resistance to challenge infection (van Loon *et al,* 1998).

A network of signal transduction pathways regulates induced responses in plants. Salicylic acid, jasmonic acid and ethylene act as secondary messengers organizing the expression of defence genes encoding antimicrobial proteins or enzymes needed for the production of defence metabolites. It has been showed, in Arabidopsis that the activation of defence mechanisms depends on the perceived attacker's nature since different pathways are differently effective against specific attackers. *Vitis vinifera* appears to have similar reactions to those displayed by Arabidopsis showing different regulation of defence and signalling genes by different secondary messengers and types of pathogens. (Chong *et al*, 2008). SAR is dependent on the accumulation of salicylic acid and associated with the induction of PR-proteins. In contrast ISR does not require SA, can occur without the PRs and is dependent on ethylene and jasmonic acid signalling (van Loon, 2001).

As the induced resistance depends not only on the defensive compounds produced after the inducing treatment but also on the better activation of the defence mechanisms, another phenomena called sensitization, potentiating or

priming has been described. Plants are naturally exposed to various elements and from this interaction it is likely that they become primed for resistance against further attacks. Due to traslocation, signals prime the plant against further pathogen attacks, probably by triggering a complex array of defense responses and the plant is then characterised by an increased sensitivity to certain stimuli due to prior challenge (Sticher et *al*, 1997).

Conrath et al (2001), emphasised its interest in plant defences by summarising various trials that highlight this phenomena.

It has been noticed that when resistance is induced by prior pathogen infection or treatment with an elicitor, some defences are expressed directly, while some defence mechanisms are not directly activated during induced resistance but that they are induced more quickly and efficiently after challenge inoculation with a pathogen (Heil and Bostock, 2002; Conrath et al, 2002).

Despite the fact that most primed plants show faster and stronger activation of defence response after further pathogen challenge. (Conrath et al, 2006), several outcomes have been described as possible after an induction treatment: (1) defences are activated, followed by further enhancement of these defences after pathogen challenge, (2) some defences are activated and after pathogen challenge, those defences plus other defence mechanisms are increased, and (3) no defences are activated until pathogen challenge occurs. (Walters and Boyle 2005).

Recently, a new class of chemicals, known as resistance inducers or plant activators, was defined. They turn the plant into a primed state, with broad-spectrum and systemic immunity, similar to, that triggered by pathogen-associated molecular elicitors. Apparently they mimic the matching between genes for host resistance (R) and those for pathogen avirulence (avr), giving rise to an induced resistant state (Gozzo, 2003).

Clearly when a plant is infected by a pathogen its metabolism is altered. Respiration is increased, photosynthesis is altered, water relations and transport processes are affected, photosynthesis is reduced, hormonal balance is shifted and stress metabolites are accumulated. A pathogen that subsequently encounters such a plant is confronted with a different host

environment compared to that encountered by a pathogen that attacks a healthy plant (van Loon, 2001).

Several special interaction cases have arisen, as is the case of some susceptible plant species within which some plant genotypes become resistant to a specific pathogen or race of pathogen. In these cases the infection is quickly stopped at the site of penetration by a hypersensitive response and programmed cell death (Schneider, 2002; Moerschbacher and Mendgen, 2001).

High fungal pressure shifts the balance between degraded and induced phytoalexins towards degradation reducing their concentration in the berries (Bavaresco *et al*, 2007).

Therefore the grapevine is able to show different degrees of induced resistance.

Stilbenes as phytoalexins

Among all barriers, the phytoalexins have deserved special attention worldwide as some of the compounds of this family have demonstrated positive human health effects.

Although the concept of phytoalexin was first laid out by Müller and Börger (1940), the current definition of phytoalexins as low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms was later redefined by the NATO Advanced Study Institute on "Active Defence Mechanisms in Plants" (Paxton, 1981).

Since phytoalexins do accumulate after pathogen infection a role of these compounds in plant defence can surely be assessed. Considering this, their involvement in disease resistance may be based upon the following criteria described by Hart (1981):

- **it must be present in those parts of the plants which are invaded by the pathogen**
- it must be present in high enough concentrations to inhibit the growth of the pathogen in vivo
- it must accumulate at the appropriate time to cause observed disease resistance
- changes in concentration in the plant must correspond to changes in susceptibility to the pathogen

The ability to accumulate these compounds has been observed in both monocots and dicots and more than 300 molecules have been identified as phytoalexins from approximately 900 species representing 40 plant families (Harborne, 1999).

Some of the produced compounds are closely linked to specific plant species and though differences may occur within a plant family, structurally similar phytoalexins are usually synthesized. Other compounds, like the stilbenes can be retrieved in plant species as divergent as the grapevine, peanut and pine.

Stilbenes are secondary metabolites that can be found in several species of the *Vitis* genus, including the *V. vinifera* L. whose importance is noted given its use for the production of grapes and wines worldwide.

In the grapevine, stilbenes can accumulate in different locations, leaves or berries for example, which lead to their first identification as they are responsible for the bright fluorescence that can be observes under long wavelength UV-light (Langcake and Pryce, 1976).

Nowadays, stilbenes are known to be fungitoxic phytoalexins at physiological concentrations against, for example, *Botrytis cinerea* while enhancing resistance to *Plasmopara viticola, Phomosis viticola* or *Rhizopus Stolonifer* (Montero *et al*, 2003).

Phenylpropanoid pathway

Considering the large number of phytoalexin molecules identified, the number of biosynthetic pathways and metabolisms used for their production is relatively small.

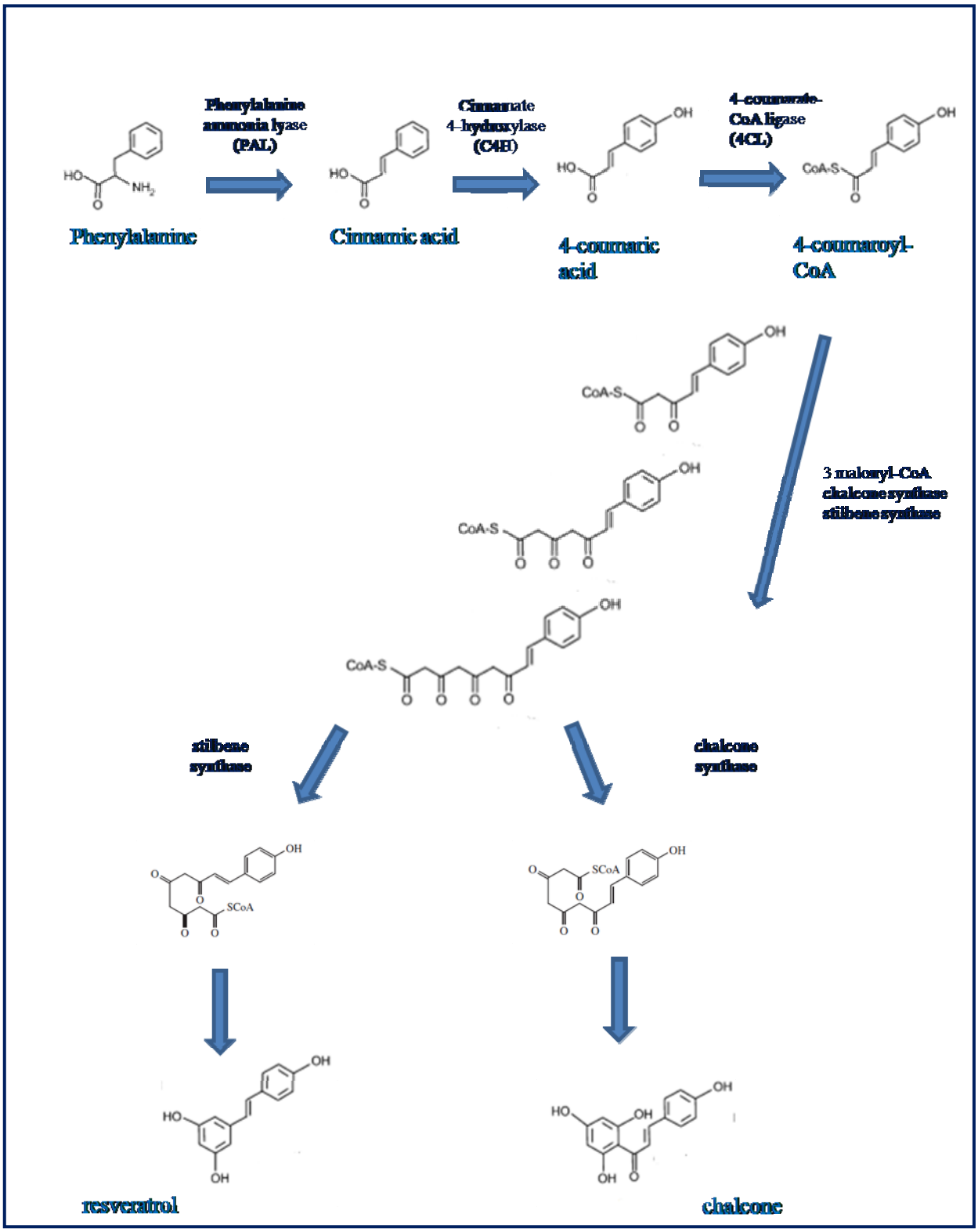
For the formation of stilbenes the activation of the phenylpropanoid metabolism is of at most importance (Dixon *et al*, 1995), particularly the branching between stilbene and flavonoid formation.

The first important enzyme of this metabolism is the phenylalanine ammonia-lyase (PAL) that catalyses the formation of cinnamic acid from phenylalanine, branching point from the primary Shikimate pathway (Herrmann, 1995) to the secondary phenylpropanoid metabolism (Harborne, 1999; Hahlbrock and Scheel, 1989; Lewis and Yamamoto, 1990; Dixon et al 1992).

In the last step of this metabolism the stilbene synthase and the chalcone synthase catalyse a single enzymatic reaction between ρ-coumaroyl-CoA and three units of malonyl-CoA units (Schröder *et al*, 1988).

This is a main point of the formation of stilbenes given the competition between the CHS (chalcone synthase) and STS for substract. These two enzymes share a common origin and catalyse the same type of reaction but form two very different products. The chain extension of 4-coumaroyl-CoA with 3-malonyl-CoA can assume different folding positions from which CHS forms chalcone, an intermediate for other routes, and STS forms simple stilbenes. (Dewick, 2009).

Lately it has been pointed out that it is not that simple to determine whether STS and CHS have separate origins or if they arose independently. It is likely that both possibilities occur depending on the plant species. (Goodwin *et al*, 2000).



Schematic representation of the phenylpropanoid pathway: stilbene synthesis

The synthesis of stilbenes only occurs if PAL and STS genes are induced (Jeandet *et al*, 2002). The PAL gene family has expanded in parallel to the STS gene family in *Vitis*. (Velasco *et al*, 2007) Although PAL and STS are parallel induced they reach a maximum at different times after elicitation (Melchior and Kindl, 1991). Stilbene synthases can be divided in 2 main groups: the pinosylvan synthases that prefer Cinnamoyl-CoA as a substrate and the resveratrol synthases that rather use 4-Coumaroyl-CoA as substrate. (Goodwin *et al*, 2000).

A member of the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) (Chong *et al*, 2009), the STS is encoded by a multigene family with and estimated number between 20 and 40 genes (Velasco *et al,* 2007) and approximately 20 different genes are expressed by the grapevine after infection with *Plasmopara viticola* (Richter *et al*, 2005).

Resveratrol

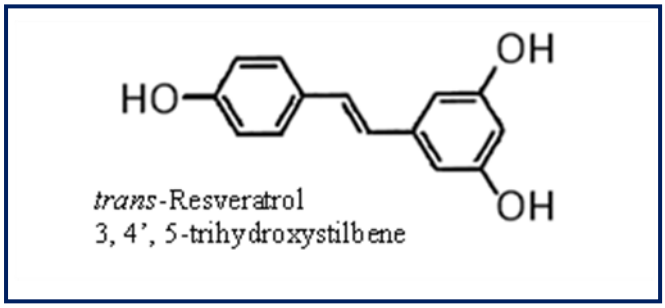
Resveratrol gained some notoriety a few years back as many of its health benefits became evident; the number of health studies on this compound increased greatly after the French paradox was noticed. This paradox stated that although the French have a high-fat basic diet and high cholesterol levels, in fact they have lower mortality rates related with coronary problems. One of the reasons given to explain the apparent contradiction was the high wine consumption of wine, characteristic of that nation. Despite common knowledge that life style, healthy diet and sports are of outmost importance to diminish disease risk, soon, wine became a must have for consumers and some of its components as resverarol became object of study by researchers.

Resveratrol seems to be involved in regulation of the cell cycle; mitochondrial energy production, vascular tone and cancer suppression and thus it can be used as treatment to several diseases also by slowing down cell aging processes and increasing cell lifespan (Howitz *et al*, 2003, Baur and Sinclair, 2006; Markus *et al*, 2008).

Animal studies have shown cancer inhibitory activity in a number of models, including adenoma, skin, breast, colon, esophagus, glioma, intestinal, liver, and neuroblastoma and humam and, even if, human testings are still necessary the current cancer chemopreventive profile of resveratrol anticipates favourable results (Pezzuto, 2008). Previous studies in cell lines provided support for the use of resveratrol in chemoprevention and cancer therapy trials (Joe *et al*, 2002). This compound also seems to have antioxidant activity, antiplatelet aggregation and vaso relaxing activity (Frémont, 2000).

Resveratrol, which is the basic stilbenic molecule, has a double function: it is a phytoalexin on its own and it also constitutes a precursor to other antimicrobial compounds (Jeandet *et al,* 2002).

In fact, the phytoalexins present in *Vitaceae* are relatively restricted to the stilbene family and most stilbenes derive from the basic unit *trans*-resveratrol: 3,4',5 -trihydroxystilbene (Langcake and Pryce, 1977_a).



Chemical structure of *trans*-resveratrol

Stilbenes can be found constitutively in grapevine roots (Mattivi *et al*, 1996; Bavaresco *et al,* 2000), canes (Langcake and Pryce, 1976,1977$_b$), seeds (Jeandet *et al*, 1995$_a$; Pezet and Cuenat, 1996) or ripe stems (Bourhis *et al,* 1996; Bavaresco *et al,* 1997a) while they are induced in flowers, leaves and berries (Bavaresco *et al*, 2009). The stilbenes usually found in *Vitis vinifera* stems are *trans*- and *cis*- resveratrol, piceatannol and *ε*-viniferin (Bourhis *et al* 1996).

Resveratrol content was found to be lower in berries when compared with that present in leaves. A reason for this might be that its production in berries is restricted to skin level and it is never detected in the fruit's pulp. Since the main defensive features can be found in the skin, when a fungus penetrates into the flesh, almost regardless of the amount of stilbenic compounds present, it will develop whatever the physiological stage of the grape (Jeandet *et al*, 1991).

The amount of this compound suffers a steady decrease during fruit ripening (Jeandet *et al,* 1991). The presence of this compound in ripe skins is correlated with the variety.

In leaves, resveratrol production seems to decrease as soon as the vegetative growth ceases (Jeandet *et al,* 1991).

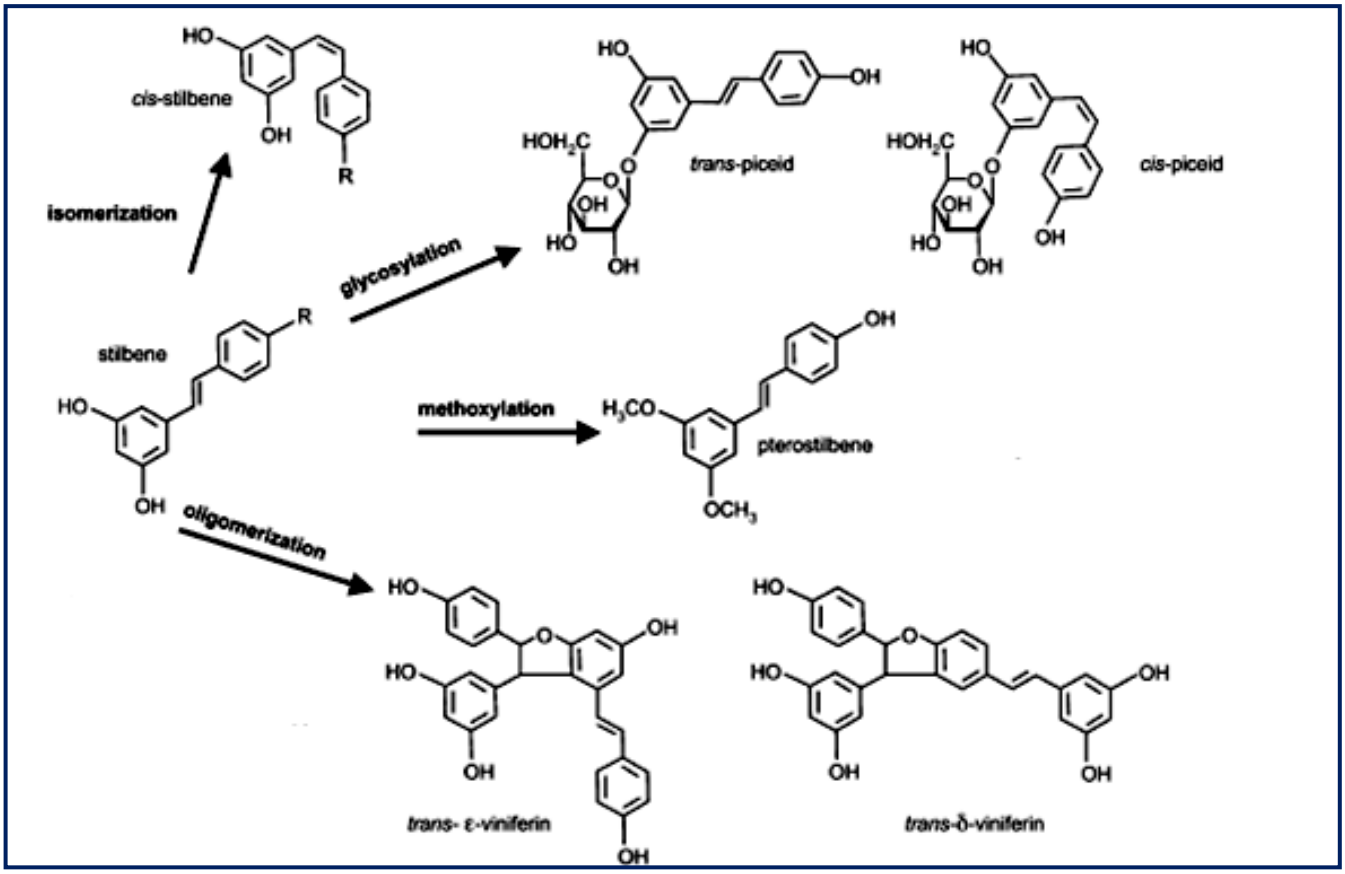
This may be due to a decreased use of the stilbene-type phytoalexins defence mechanisms leading to a decreased production of stilbenes as the development cycle advances.

Resveratrol concentration in both leaves and berries of *Vitis labrusca* appeared to be always higher than those found in *Vitis vinifera,* despite the developmental stage of the organ under study (Jeandet *et al,* 1991).

Moreover it has also been noticed that high resveratrol contents in wine have been associated with moderate fungal infection whereas extensive development may destroy the induced phytoalexin. Degradation might occur by laccase like stilbene oxidases or grapevine peroxidases (Versari *et al*, 2001).

Resveratrol derivatives

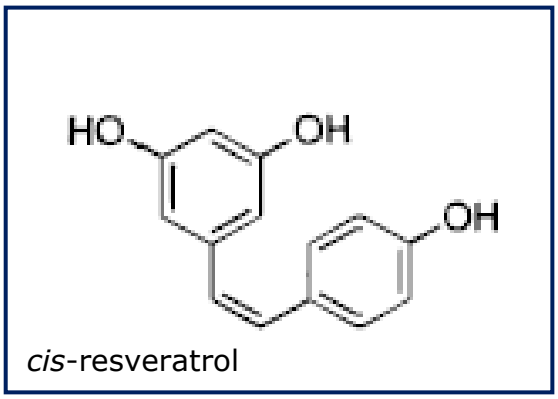
There are four main modifications that concur to the formation of new stilbenic molecules from the original resveratrol structure: isomerisation, glycosylation, methoxylationand oligomerization.



Common modifications of stilbenes (modified from Chong *et al,* 2009)

Cis-resveratrol

Cis-resveratrol, a isomerisation of the original *trans*-resveratrol molecule, is less stable than its precursor due to the steric hindrance between the aromatic rings (Chong *et al*, 2009).



Chemical structure of *cis*-resveratrol

As free *cis*-resveratrol is rare in *V. vinifera* berries, hypothesis to its presence are the isomerisation of the *trans*- form under UV irradiation (Roggero *et al,* 1995) and its hydrolysis from its *cis*-glucoside (Vrhovsek *et al*, 1997). *Cis*-resveratrol is rare in grapes and its presence in wine can be explained by photo isomerization or as a product of organic farming (Jeandet *et al*, 1995_b).

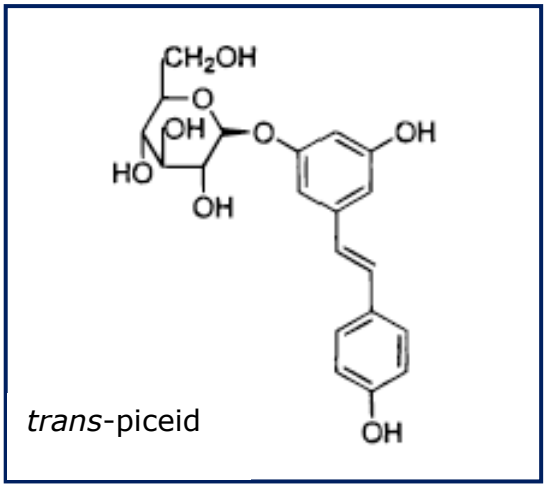
Other molecules formed from the original backbone can later be modified into their isomers depending on the surrounding conditions.

Piceid and astringin

Glycosylation is a common modification of secondary metabolites to which a carbohydrate moiety is added, altering several characteristics as hydrophilicity, stability localization and bioactivity (Gachon et al 2005).

The main glycosylated molecule from resveratrol is piceid, *trans*- and *cis*-resveratrol-3-*o*-*β*-D-glucopyranoside (Waterhouse *et al,* 1994; Waffo-Teguo *et*

al, 1996; Romero-Pèrez *et al,* 1999). This compound appears to be protected from enzymatic oxidation (Regev-Shoshani *et al,* 2003).



Chemical structure of *trans*-piceid

In plants that produce stilbenes all metabolites can be found in both free and glycosylated forms and this modification can be correlated with their storage, transport or protection from peroxidative degradation (Morales *et al,* 1998; Gatto *et al*, 2008).

In fact, piceid could be a form of storage or transport of resveratrol in the plant, a basal pool of immediately usable resveratrol mobilizable as primary defence (Douillet-Breuil *et al,* 1999).

It was also found that *trans*- and *cis*-piceid accumulate in healthy berries of high resveratrol producing varieties, which seem to be more constitutively expressed (Bavaresco *et al*, 2007; Gatto *et al*, 2008). In some susceptible cultivars, resveratrol is synthesized after infection but it is rapidly glycosylated into a non-toxic piceid (Pezet *et al*, 2004_a). Piceid is the main compound found in commercial wines (Romero-Pèrez *et al*, 1999).

Vitis vinifera cell suspensions of Gamay Teinturier evidenced the production of astringin a 3-OH-*trans*-piceid which appears to be up to 6 times more potent than trans-piceid (Mérillon *et al*, 1997).

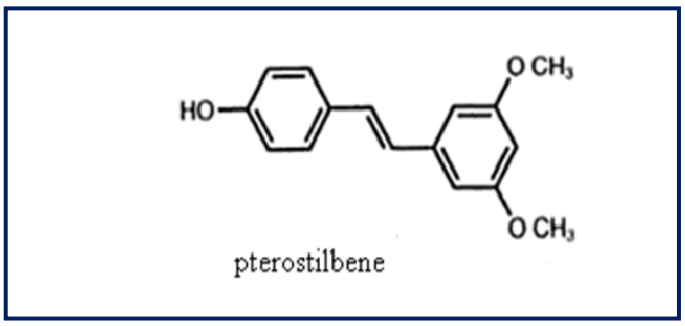
Pterostilbene

Methoxylation is a slow process that consists in the addition of methoxy (CH_3-O) groups. The best example is the pterostilbene, a 3,5-dimethoxy-4'-hydroxystilbene, a dimethylated derivative of resveratrol (Langcake et al, 1979; Langcake, 1981). Nonetheless and despite its proved fungitoxic activity (Pezet et al, 1990) its biosynthesis was only recently understood; Schmidlin et al (2008) characterized a grapevine resveratrol O-methyltransferase, ROMT, catalyzing the efficient biosynthesis of pterostilbene from resveratrol, both in vitro and in planta.

Despite this, the fungitoxic activities of the stilbene compounds indicate that the *in vivo* methylation of phenol groups can potentially lead to increased biological activity (Schultz *et al*, 1990).

This compound though clearly among the most toxic, 5-fold higher inhibitory activity than resveratrol against fungal growth *in vitro* (Pezet *et al,* 2004_b), is usually present in very low concentrations or even absent *in vivo* (Pezet *et al*, $2004_{a,b}$).

Pterostilbene is not produced by healthy leaves and is also not produced after bruising or cutting (Langcake *et al,* 1979).



Chemical structure of Pterostilbene

Viniferins

Oligomerization is a common modification and a large number of stilbenes are oligomers, dimmers, trimers and tetramers that arise from oxidative coupling of resveratrol or resveratrol derivatives.

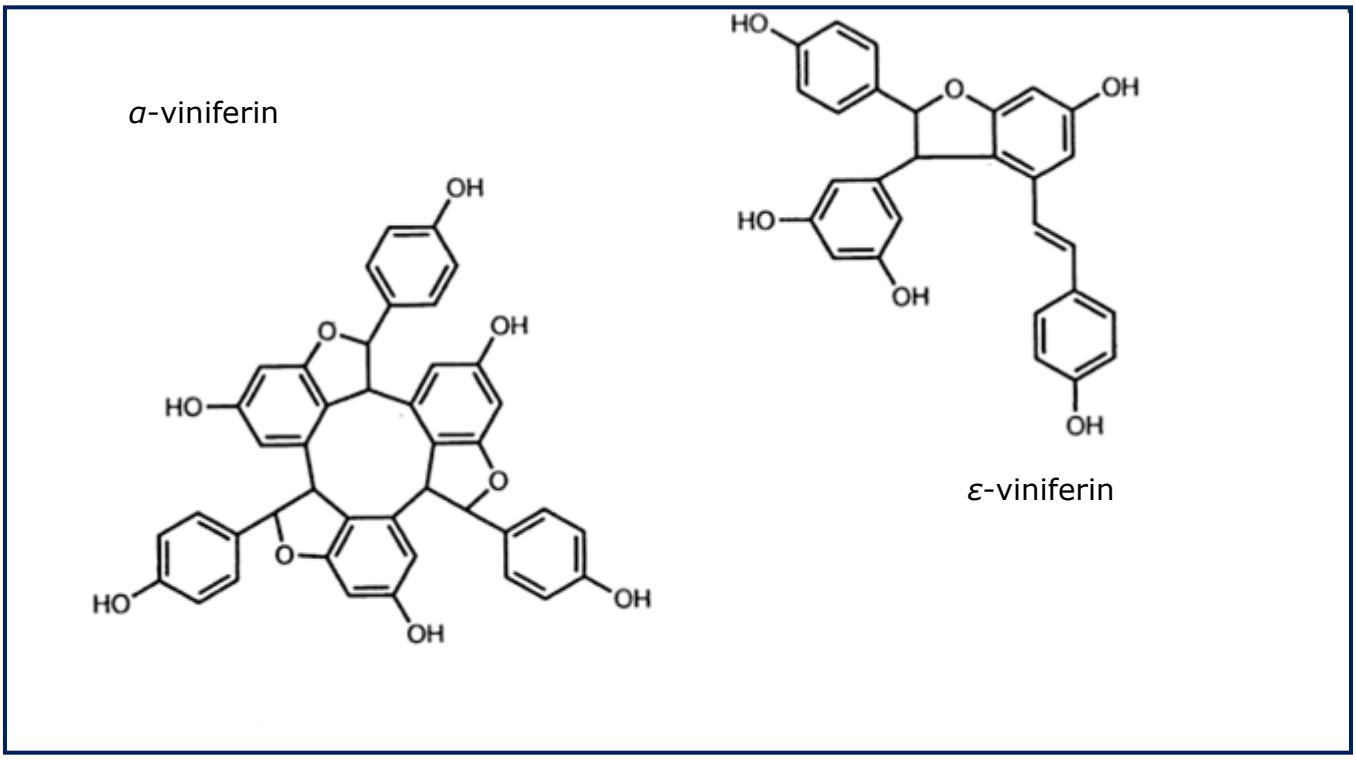
A major group of resveratrol oligomers is called viniferins. They are the product of the oxidation of the basic stilbenic structure by 4-hydroxystilbene peroxidases (Calderón *et al*, 1993; Morales *et al*, 1997) that are localized in the vacuoles, cell-wall and apoplasts of grapevine berry cells (Ros Barceló *et al*, 2003).

The main components of this group are ε-viniferin, a cyclic dehydrodimer of resveratrol, *α*-viniferin, a cyclic dehydrotrimer of resveratrol, *β*-viniferin, a cyclic dehydrotetramer of resveratrol, *γ*-viniferin a more polymerized oligomer of resveratrol and *δ*-viniferin, an isomer of the resveratrol dehydrodimer.

The first viniferins to be identified were ε-viniferin (Langcake and Pryce, 1977_b) and *α*-viniferin (Pryce and Langcake, 1977). More recently *δ*-viniferin was carefully described (Pezet *et al*, 2003).

ε-viniferin is the major stilbene synthesized after *Botrytis cinerea* attack in the berries *(*Bavaresco *et al,* 1997_b*).*

ε-viniferin and *δ*-viniferin are the major resveratrol dimmers to be synthesized by *Plasmopara viticola* infection or UV irradiated leaves. (Pezet *et al,* 2003). The main difference between these two compounds is that δ-viniferin is 5 times more toxic than *ε*-viniferin. In fact, *δ*-viniferin is as toxic as pterostilbene but produced in much higher amounts (Pezet *et al,* 2004 $_a$).



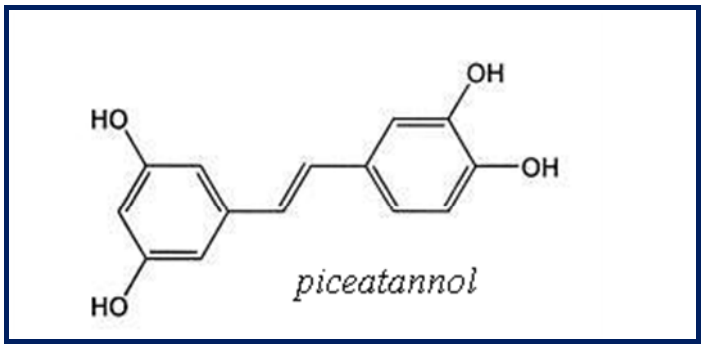
Chemical structure of *α*-viniferin and *ε*-viniferin

Other stilbenic compounds

Another compound that can also be found in grapes is the piceatannol, a hydroxylated analogue of resveratrol (trans-3, 4, 3', 5'-tetrahydroxystilbene) (Bavaresco *et al,* 2002). This stilbene has been shown to be a potent inducer of apoptosis in lymphoma cells and in primary leukemic lymphoblasts (Wieder *et al,* 2001). Investigators concluded that piceatennol can be considered a chemopreventive or anticancer agent (Wolter, 2002). This compound has also demonstrated chemosensitizing effects in the treatment of drug resistant phenotypes of lymphoma cells (Alas, 2003).

Piceatannol concentrations were found to increase significantly as the berry development advanced, in fact, this compound was only found during ripening in Barbera berries infected with *A. Ochraceus* (Bavaresco *et al,* 2003)

Astringinin (3,3',4',5-tetrahydroxystilbene), is another resveratrol analogue which has showed some considerable antioxidative activity and free radical scavenging capacity (Hung *et al*, 2001), that might be correlated with its antioxidant activity and upregulation of NO production.



Chemical structure of Piceatannol

Several other isomeric or glycolysed forms of the basic stilbenic compounds have been identified: hopeaphenol, a cyclic symmetric tetramers (Ito *et al*, 1997), resveratroloside, resveratrol-4'-o-β-D-glucopyranoside, has been identified (Waffo-Teguo *et al*, 1998), resveratrol di- and triglucoside derivatives have been recently isolated from *Vitis vinifera* (Decendit *et al*, 2002; Larronde *et al,* 2005).

Stilbenic role in resistance

The role of the stilbenic compounds in fungus/grapevine interaction can be shortly summarized for susceptible plants as a rapidly glycosylation of resveratrol into the less toxic compound piceid and for resistant plants as a quick oxidization of resveratrol to very toxic viniferins (Chong *et al*, 2009). Confirmation arises from the presence of higher levels of peroxidase in older leaves that show more resistance to *Plasmopara viticola* (Reuveni, 1998). On the other hand it must be said that resistance to infection or the lack of it depends also on the balance production/degradation of phytoalexins following pathogen attack (Darvill and Albersheim, 1984.).

STS induction and stilbene production in different grape organs

Studies have showed that the production of resveratrol can be detected after 24, culminates at 48h and then decreases (Douillet-Breuil, 1999) while

mRNA of stilbene synthase can be detected within 4h of elicitation (Adrian *et al*, 1997).

Parallel to the presence of resveratrol studies have showed that STS can be found in berry exocarp at all developmental stages and that outer hypodermis cells were STS-positive. No signal presence could be found in the berry's mesocarp. At cellular level it was found within vesicles, along the plasma membrane and in the cell wall, suggesting protein secretion in the apoplast. (Fornara *et al*, 2008)

Several types of stress can activate STS and stilbene accumulation in developing berries is highly regulated by the level of STS gene transcript. (Pan *et al*, 2009).

STS could be detected before and after veraison. The amount detected decreased in ripe healthy berries as well as the level of stilbenes accumulated. In fact, in ripe berries only STS fractions could be found. It is possible that due to degradation stilbene accumulation decreases overtime (Fornara *et al,* 2008). Despite these findings, the study of healthy berries collected from 78 *Vitis vinifera* varieties showed an STS expression profile of increasing concentration from veraison to ripening. It was also found that high resveratrol producing varieties accumulated higher gene transcript levels as well as stilbenic compounds overtime (Gatto *et al*, 2008).

Different results in STS induction obtained after UV irradiation or wilting processes suggest that there might be several mechanisms that modulate STS mRNA induction and stilbene synthesis (Versari *et al,* 2001). Although what stated before (Gatto *et al,* 2008), the differences in STS mRNA concentration between high and low resveratrol producing varieties decreased during the development of healthy berries although resveratrol content remained 10-fold higher in high producing varieties. The fact that other factors must be involved in stilbene accumulation is thus supported.

Efficient stilbene biosynthesis may be ensured by the coordinated activation of STS and upstream production of the enzymes in this pathway, such as PAL and C4H (Fritzemeier *et al,* 1981). In fact, an upregulation of

several defence genes until ripening was also noticed elsewhere (Gatto *et al,* 2008).

In UV induced berries stilbene concentration is related to accumulated STS enzyme at protein level and STS mRNA accumulation, being thus possible to state that it may be the result of STS gene expression at transcription and translational levels (Pan *et al,* 2009).

In vitro plantlets of *Vitis rupestris, Vitis vinifera* cvs Pinot noir and Chardonnay treated with three different elicitors were used to observe stilbene synthase expression behaviour. Under UV treatment the tolerant variety presented a constant level of expression and reached one single peak. The sensitive cultivars also remained constant but presented two concentration peaks overtime. All varieties showed similar resveratrol accumulation patterns highlighting the correlation between sts induction and stilbene production (Borie *et al,* 2004).

In *Vitis vinifera* cv Optima 7 STS genes were identified and gene expression was proven to differ in magnitude in response to various elicitors. Two waves in the accumulation of the corresponding mRNAs could also be seen in suspension cells treated with *Phytophthora cambivora* (Wiese *et al*, 1994). It was also advanced that the two peaks of accumulation could be related to early STS gene with unstable mRNA and later sts genes with more stable mRNA. In fact, cell suspensions of *Vitis vinifera* cv. Optima indicated that the expression of sts genes differs in scale in response to different elicitors (Wiese *et al,* 1994).

Nonetheless, elicitation with aluminium chloride and *Botrytis cinerea* induced irregular results in all varieties (Borie *et al*, 2004).

Elicitation with *Aspergillus carbonarius* induced the expression of the stilbene synthase gene and a higher production of stilbenes in Barbera, a susceptible variety, and Castor, a resistant variety (Vezzulli *et al,* 2007).

It was also noticed that high resveratrol accumulation seems to be related with a down regulation of the genes that play some role in fruit ripeness (Gatto *et al,* 2008).

Stilbene production and variety

Several trials have tried to highlight the differences between varieties.

The comparison of the stilbenic compounds produced in the berries of three varieties, Barbera, Croatina and Malvasia di Candia aromatica, revealed that *trans*-piceid was the highest compound (103 µg.kg-1 fw), while *trans*-resveratrol was lowest (57 µg.kg-1 fw). Barbera and Croatina showed higher *trans*-resveratrol (71 and 76 µg.kg-1 fw) against the 4 µg.kg-1 fw levels of Malvasia. Barbera also presented the highest levels of *trans*-piceid and *cis*-piceid (235 and 136 µg.kg-1 fw) (Bavaresco *et al,* 2007).

Under identical environmental condition and cultural practices the berries of four different varieties Tempranillo, Cabernet Sauvignon, Bobal and Crujidera, were used to analyze the stilbenic composition during ripening and the results showed that the production of these compounds was highly related with the variety (Navarro *et al*, 2008).

Studies conducted in 14 Croatian, both red and white varieties, established average stilbenic values of white skinned varieties as: 0.24 $mg.kg^{-1}$fw for *trans*-resveratrol, 0.30 $mg.kg^{-1}$fw for *cis*-resveratrol, 0.48 $mg.kg^{-1}$ fw for piceid and 0.60 $mg.kg^{-1}$ fw for astringin. In red skinned varieties the values found were: 0.59 $mg.kg^{-1}$fw for *trans*-resveratrol, 0.80 $mg.kg^{-1}$fw for *cis*-resveratrol, 0.37 $mg.kg^{-1}$ fw for piceid and 0.53 $mg.kg^{-1}$ fw for astringin (Katalinic *et al*, 2009).

The type of stilbenic compounds present and their resistance related nature seems to be genotype dependent (Gatto *et al*, 2008).

After short UV induction, the berries of 4 red varieties: Red globe, Flame, Crimson and Napoleon and 3 white varieties: Superior, Dominga and Moscatel italica have revealed that *trans*-resveratrol was the most induced compound. Flame presented the highest contents of both *α*-viniferin and *ε*-viniferin and Superior was the second highest. Red globe, Crimson, Moscatel italica, Dominga and Napoleon had *ε*-viniferin. *Cis*-resveratrol quantified in Red globe, Napoleon, Superior and Dominga. *Trans*-piceid was not found in Red globe and Crimson,

and *cis*-piceid and pterostilbene were not found in any of the varieties (Cantos *et al,* 2002).

The study of the skin of 36 white, rose and red varieties grown in Japan, revealed that the concentration of trans-resveratrol ranged from 0.5 to 14.1 $\mu g.g^{-1}$ fw (Piazzutello bianco and Müller-Thurgau respectively), showing an average value of concentration for the 36 varieties of 4.12 $\mu g.g^{-1}$ fw (Okuda and Yokotsuka, 1995).

The leaves of white and red grapevine crossings were analysed and white crossings presented higher *trans*-resveratrol levels even if the difference diminished over time while berries from the red crossings presented higher levels (Bábíková *et al,* 2008).

Stilbenes in wine

Wine is the most important consumption vehicle of resveratrol and given its cultural importance it is only natural that several reports from different countries point out the stilbenic values found in the most diverse wines.

Resveratrol in wine is highly affected by the climate, geographical area of cultivation, cultural technique, varieties used in vinification, wine making and storage conditions. A so, a considerable variety of resveratrol levels should be expect even in wines produced with the same varieties (McMurtey, 1996).

When comparing wines produced with thick or thin skinned berries average *trans*-resveratrol levels were not correlated with this factor. It is then difficult to highlight a single region or variety as the highest producing combination; Comparison of *trans*-resveratrol content depending on the latitude of the producing region showed that the high north of the Northern Hemisphere and regions close to the equator in the southern hemisphere show higher levels of it in the wines (Stervboa *et al*, 2007).

Skins of two autoctonous Portuguese varieties, Castelão and Tinta Roriz, and an international variety Syrah, all red, showed that showed no significantly different *trans*-resveratrol contents but very different levels of *trans*-piceid (67.2, 11.6 and 10.4 µg gdw-1, respectively). *Cis*-Piceid, was found only in

Castelão (58.9 µg gdw-1). All the stilbenic compounds were present in the bunch stems of Castelão whereas only *trans*-resveratrol was found in the seeds of this variety (Sun *et al*, 2006).

Spanish wines produced with Pinot noir, Merlot and Grenache were found to have levels of 5.13 $mg.l^{-1}$, 3.99 $mg.l^{-1}$ and 2.43 $mg.l^{-1}$ respectively (Lamuela-Raventòs *et al*, 1995).

The major Greek red and white wines were compared and the red wines presented higher concentrations of trans-resveratrol from 0.352 to 1.99 $mg.l^{-1}$, while white wines showed concentrations between 0.005 and 0.57 $mg.l^{-1}$ (Gerogiannaki-Christopoulou *et al*, 2006).

Regular Vidal and Seyval Blanc wines, both made from grapes of French hybrid cultivars, had very low to non-detectable concentrations of both resveratrol isomers, but its late harvest gave values comparable to those of *Vitis vinifera* cultivars Chardonnay and Riesling. Pinot Noir wines were significantly lower in mean *cis*-resveratrol concentration than were Cabernet Sauvignon wines but not Merlot wines. The mean concentrations of total resveratrol isomers ranged between 0.98 mg/L (Cabernet Franc) and 3.20 mg/L (Merlot), following the same pattern as *trans*-resveratrol (Soleas et al, 1997).

A study conducted on 120 Portuguese and French wines revealed that values in white wines ranged from 0.6 to 23.5 mg/L and from 2.3 to 53.5 mg/L in red wines. The levels of the *trans*- and *cis*-piceid were found to exceeded those of the free isomers and *trans*-astringin was also identified (Ribeiro de Lima *et al*, 1999).

Elicitors

Firstly, elicitors were defined as molecules that signal plants to begin the process of phytoalexin synthesis (Darvill and Albersheim, 1984) and accumulation (Keen et al, 1972).

More recently, elicitor is the term used to describe all molecules that stimulate any plant defence mechanism (Dixon, 1986; Ebel, 1986).

These molecules are able to trigger immediate plant and there are several known abiotic and biotic elicitors.

Abiotic elicitors

Abiotic elicitors are non-living chemical or physical factors such as UV-irradiation, Aluminium chloride or methyl jasmonate among others.

Exposure to Uv-irradiation induces the production and accumulation of resveratrol and viniferins in leaves, in fact, after treatment Chardonnay leaves produced 400 µg/g and Rupestris du Lot 800 µg/g of resveratrol within the first day after exposure. In the first variety the compound disappeared after 3 days and in the second after 6 days (Bonomelli *et al,* 2004). Trials using the cv. Perlette showed that after UV-irradiation, resveratrol levels peaked 18 h after exposure and declined rapidly thereafter while pterostilbene peaked after approximately 40 h (Sarig *et al,* 1997).

Recently more selective studies were carried out to determine the effect of different wavelengths in the production of stilbenes in table grape berries. Two wavelength close to the known maximum of absorbance were selected, 302.1nm and 300 nm, and it was found that in equal conditions the 302.1nm irradiation enhances the resveratrol content in grapes by up to six times more than that of 300nm irradiation (Sánchez *et al*, 2007). Among other defence responses, UV-C treatment induced higher PAL and STS expression and resveratrol accumulation in bunch-stems of both inflorescences and clusters following the treatments. Despite this, no defence processes were induced in grapevine flowers following UV-C exposure, whatever the stage analyzed and no resveratrol synthesis was noticed in berries induced at fruit set. Nonetheless in fruits slightly smaller than pea-size resveratrol accumulation and induction of CH3, PAL, and STS increased after UV-elicitation (Petit *et al*, 2009).

Trials in which Methyl jasmonate was used in low concentrations showed that this compound is capable of stimulating stilbenes in berries until 15 days after veraison, period after which there is no enhancement effect on stilbene

production. Therefore, it is possible to say that berries in more advanced phenological stages become irresponsive to its presence. The same type of treatment also enhances stilbene production in leaves, organ upon which the phenological phase has no significance over the produced concentration of stilbenes (Larronde *et al*, 2003). In Cabernet Sauvignon control leaves where no treatments were preformed there were no traces of stilbenes while in leaves treated with methyl jasmonate (Meja) after 12h it was possible to find *trans*-resveratrol and *trans*-piceid. Both compounds reached a plateau after 48h. After 18h also *ε*-viniferin could be found and at that point pterostilbene and *δ*-viniferin were at their peak. All values decreased after reaching maximum accumulation values (Larronde *et al*, 2003).

Overtime MeJa spray treatments on Barbera berries at different phenological phases, fruit set, veraison and ripening, increased the content of resveratrol and *ε*-viniferin (Vezzulli *et al*, 2007).

Cabernet Sauvignon leaves produced and accumulated several types of phytoalexins upon MeJa elicitation. Meja seems to enhance PAL and STS induction leading to an increased production of stilbenes (Belhadj *et al*, 2006).

Benzothiadiazole, a functional analogue of the endogenous hormone-like salicylic acid that is completely translocated and degraded in plant tissue, enhances by 40% the total production of resveratrol when used in pre-harvest treatments. Although the concentration of both isomers increases *trans*- was always higher than *cis*-resveratrol (Iriti *et al,* 2004). Along with an increased concentration of stilbenes BTH also enhances the amount of antocyanins and protoantocyanins produced. As so it is possible that BTH has some effect on the two metabolic branches of the phenylpropanoid pathway, inducing PAL production and therefore diminishing substrate competition (Iriti *et al,* 2004, 2005). The timing of the treatments is also important since this plant activator has little efficiency at the moment of application (Kolher *et al*, 2002).

Leaf treatments with Aluminium chloride, after 15h of incubation, revealed the presence of *trans*-resveratrol. Concentrations necessary to induce its production were higher in *Vitis vinifera* (superior to 22 mM) than in *Vitis rupestris* (above 7mM) and concentrations higher than 90mM led to toxicity and decreased stilbene production (Adrian *et al,* 1996).

While studying the effect of ozone in post-harvest decay caused by *Rhizopus Stolonifer* it was noticed that the phytoalexins resveratrol and pterostilbene were elicited by ozone treatments, at levels similar to those produced by uv-c irradiation and that resveratrol accumulated in greater quantities than pterostilbene. The combined effects of *R. Stolonifer* inoculation with ozone treatments raised the total levels of both stilbenes (Sarig *et al*, 1996).

Grape *trans*-resveratrol content can be enhanced trough short duration anoxic treatments at room temperature. The berries maintained in time the levels produced and no organoleptic damages were found in the berries or in the wines produced with these berries (Jiménez *et al*, 2007).

Lanthanum, europium, calcium, silver and cadmium are metal ions capable of inducing phytoalexins biosynthesis in plant cell cultures (Radman *et al,* 2003).

Biotic elicitors

Many biotic elicitors, by definition related to, produced by or caused by living organisms are known to stimulate the production of stilbenic compounds. Several examples can be found in literature.

	Elicitor	**Grapevine organ**	**Content ($\mu g.gfw^{-1}$)**
***trans*-resveratrol**	*Botrytis cinerea*	leaves berry skin unseeded berries	4.8-9.4 4.0-7.0 1.3-6.6
	Plasmopara viticola	leaves	10
	Bacillus sp.	leaves	31.1-78.3
	Aspergillus spp.	unseeded berries	1.4-4.3
***α*-viniferin**	*Botrytis cinerea*	leaves	26.7-72.4
***ε*-viniferin**	*Botrytis cinerea*	leaves unseeded berries	9.6-15.8 2.3-10.3
	Plasmopara viticola	leaves	100.0
***δ*-viniferin**	*Plasmopara viticola*	leaves	156.0
***trans*-pterostilbene**	*Botrytis cinerea*	unseeded berries	0.1-0.2
	Plasmopara viticola	leaves	22.0
Piceatannol	*Aspergillus spp.*	unseeded berries	<0.3

Stilbenic compounds biotic induction (modified from Bavaresco *et al*, 2009)

There are several reports of the influence of *Botrytis cinerea* as an elicitor. Trials conducted have allowed the determination of *trans*-resveratrol as the major component responsible for the blue fluorescence of grapevine leaf tissue following fungal infection or exposure to ultraviolet light. The compound was not detectable in healthy leaves but accumulated to between 50 and 400 µg/g fresh weight in infected or u.v.-irradiated leaves (Langcake and Pryce, 1976, 1977).

Since resveratrol is typically present in high concentrations shortly after *Botrytis cinerea* infection, resveratrol probably plays an important role in the regulation of the *Botrytis cinerea*— grapevine interaction (Adrian *et al*, 1997).

In leaves infected by *Botrytis cinerea*, the main stilbenes detected were *trans*-resveratrol, *ε*-viniferin and *α*-viniferin. In the area surrounding the lesion, resveratrol could always be found and studies have showed that susceptibility of the leaves diminishes with leaf age (Langcake *et al*, 1979).

Cell culture trials (Liswidowati *et al,* 1991) demonstrated that *Botrytis* elicitation induces the expression of STS and increases the production of resveratrol and some derivatives which are known to be fungitoxic, that is, more toxic towards pathogens.

A *Botrytis cinerea* conidia assay revealed that pterostilbene reduced the percentage of germination even at low concentrations whilst resveratrol at very low concentrations did not affect the percentage of conidia germination or the mycelial growth of *B. cinerea.*

Studies conducted with the *Botrytis* sensitive cultivar Huxelrebe and the resistant cultivar Castor have shown that the later produces more *trans*-resveratrol and *ε*-viniferin than the first (Bavaresco *et al*, 1997_b).

While comparing the ability of eight isolates of *Botrytis cinerea* to degrade the stilbene phytoalexins, resveratrol and pterostilbene, and comparing their pathogenicity to grapevines, resveratrol seemed to be somehow degraded. The strains that degraded resveratrol and pterostilbene were pathogenic to in vitro cultures of grapevines (*Vitis rupestris*) and in all cases the hydroxystilbene-degrading activity was related to the presence of laccase activity in the culture filtrates (Sbaghi *et al*, 1996).

Conidia incubations of *Botrytis cinerea*, the causal agent of the grey mold disease, with low concentrations of resveratrol revealed the presence of brown pigmentation after 48h; this is due to enzymatic oxidation of resveratrol by a laccase-like enzyme produced by the fungus. This mediated oxidation occurs in the cytoplasm and the products accumulate in the vacuole resulting in the visible discoloration (Adrian *et al*, 1998).

In 1989 the influence of stilbenes over the *Plasmopara viticola* – grapevine interaction was proved (Dercks and Creasy, 1989). In cultivars where no sporulation, limited sporulation, or heavy sporulation of the parasite was present, resistance was positively correlated with the capacity for the synthesis of resveratrol This study also showed that the necrotic reaction of plant tissue after inoculation was due to phytoalexin accumulation and that in fact phytoalexin response was dependent on inoculum density as was the velocity of infection and colonization by the pathogen.

New techniques for the detection of stilbenes in *Plasmopara viticola* infected leaves have revealed the presence of nine viniferin analogues: *trans*-piceid, *cis*-piceid, *trans*-resveratrol, *trans*-*ε*-viniferin, *cis*-*ε*-viniferin, *α*-viniferin, *trans*-*δ*-viniferin, *trans*-pterostilbene and *cis*-pterostilbene (Jean-Denis *et al*, 2006).

In Syrah and Rv1 (*V.rotundifolia* x *V.vinifera* hybrid), younger leaves produced more toxic stilbenes respect the older ones. In these varieties the higher the stilbene content the lower was the sporulation: At 0.4mg.g-1dw of stilbene in Syrah there were 65000 sporangia present while in RV1 there were only 26000 sporangia (Poutaraud *et al,* 2006).

Not all stilbenes are equally toxic to *Plasmopara viticola* zoospores, resveratrol is not so toxic, piceid has never showed any toxic activity against *Plasmopara viticola* zoospores while the viniferins are quite active. *δ*-viniferin is more toxic that *ε*-viniferin and equally toxic to pterostilbene although it is produced in much higher amounts than the later compound (Pezet *et al*, 2004_b). In fact *δ*-viniferin was indentified in leaves as the major resveratrol

dimer synthesized after *Plasmopara viticola* infection. After seven days of infection it was the most abundant stilbene in Chasselas leaves (Pezet *et al,* 2003).

It was also demonstrated that in diverse Vitis species the phytoalexins produced and accumulated were characteristic for each cultivar (Dercks and Creasy, 1989)

Phytoalexin accumulation in the berries of the cv. Perlette was studied using *Rhizopus Stolonifer* as an elicitor and the results show that resveratrol production was 4 times higher than that of pterostilbene. Both stilbenes presented their highest values after24h and after 50h, when decay was advancing, the content of resveratrol dropped 60% and that of pterostilbene 20%. *Rhizopus Stolonifer* elicited higher levels of resveratrol and lower levels of pterostilbene than *Botrytis cinerea* and turned out to be more sensitive to them as well (Sarig *et al,* 1997).

Ochratoxin-A producing *Aspergilli* are able to increase the amount of produced stilbenes. Resveratrol presented the highest total values but it decreased from veraison to ripening while *trans*-piceid had an opposite behaviour and apparently wasn't correlated with the fungus presence. Piceatannol concentration depends on the growth stage of the fungus and low values are found at ripening. The same study also showed that punctured berries have higher values of *trans*-resveratrol (Bavaresco *et al,* 2003).

During noble rot development in the varieties Sauvignon and Semillon the levels of *trans*-resveratrol and *trans*-piceid were very low (Landrault *et al,* 2002).

Suspension cell cultures elicited with *Tricoderma viride* responded with synthesis and accumulation of resveratrol and traces of *ε*-viniferin (Calderón *et al* 1993).

BABA, beta-aminobutyric acid, a non-protein amino acid, used pre-*Plasmopara viticola* infection reduced sporulation and induced *trans*-piceid, *trans*-resveratrol, *trans-δ- and trans-ε*-viniferin and *trans*-pterostilbene in the sensitive variety Chasselas and *trans*-resveratrol, *trans*-pterostilbene and *trans-δ*-viniferin in the resistant variety Solaris (Slaughter *et al,* 2008).

Treatments using FABE (*Frangula alnus* bark extract) and emodin (a methylated anthraquinone) have revealed that this compounds are able to stimulate phytoalexin production, an result that id enhanced when the plant material is also infected with *Plasmopara viticola*. Another compound used, RPRE (*Rheum palmatum* root extract), was only able to induce stilbene production in the presence of *Plasmopara viticola.* In leaves of Chasselas this compounds lead to the production of *trans*-resveratrol (below 50 $\mu g.g^{-1}$ fw), *ε*-viniferin (below 100 $\mu g.g^{-1}$ fw), *δ*-viniferin (below 50 $\mu g.g^{-1}$ fw) and pterostilbene (below 10 $\mu g.g^{-1}$ fw) being that all values were improved by the presence of *Plasmopara viticola* (Godard *et al,* 2009).

A soil bacterial strain was used successfully to control *Botrytis cinerea* and at the same time induced the production of resveratrol in both a sensitive variety, *Vitis vinifera* Chardonnay, and in a resistant variety, *Vitis rupestris* (Paul *et al,* 1998).

Also some cyclic oligosaccharides, the cyclodextrins can act as biotic elicitors (López-Nicolás *et al,* 1995).

Other elicitors

Mineral elements are a part of all defences as cell component, reaction substrates, and electron carriers or as activators, inhibitors and regulators of metabolism. Usually mineral supplies do not affect directly the defence but it has been noticed that an increase of nitrogen reduces resistance to downy

mildew, powdery mildew and decreases stilbene synthesis (Bavaresco and Einbach, 1987; Bavaresco *et al,* 2001).

Soil and climate can also influence the plant's production of phytoalexins. Calcareous soils induce the accumulation of stilbenes in the fruits, in fact under lime-stress condition, berries of the variety Merlot showed high concentrations of *trans*-resveratrol followed by piceatannol, *trans*-piceid and *ε*-viniferin (Bavaresco *et al,* 2005). Most stilbenic compounds increase from elevation from 150m to 320m and then decrease at 420m of elevation. *Cis*-piceid is affected by the climate of a given years, it will increase with relative humidity and decrease with the degree days (Bavaresco *et al,* 2007).

Cultural practices can also influence the contents displayed by the grapevine, leaf removal for example, allied with cool meteorological conditions increased *trans*-piceid in grapes of Barbera and decreased *trans*-resveratrol and *cis*-piceid in Croatina and Malvasia di Candia aromatica while the same procedure was ineffective over the stilbenic concentrations of the grapes of those varieties in warm and dry climatic conditions (Bavaresco *et al,* 2008). In Chardonnay inflorescences only *trans*-resveratrol was found while in Cabernet Sauvignon inflorescences, 7 stilbenic compounds were measurable. Photosynthetically active leaves are necessary for the supply of sucrose as an energy source and precursor for successful stilbene production, their removal will reduce the amount of stilbenes accumulated in inflorescences (Keller *et al,* 2000).

Plasmopara viticola

Worldwide the downy mildew has become one of the major concerns of all grape growers, whether because of the severe damages it can cause to the leaves and fruits or because the management cost necessary to control it cannot be neglected.

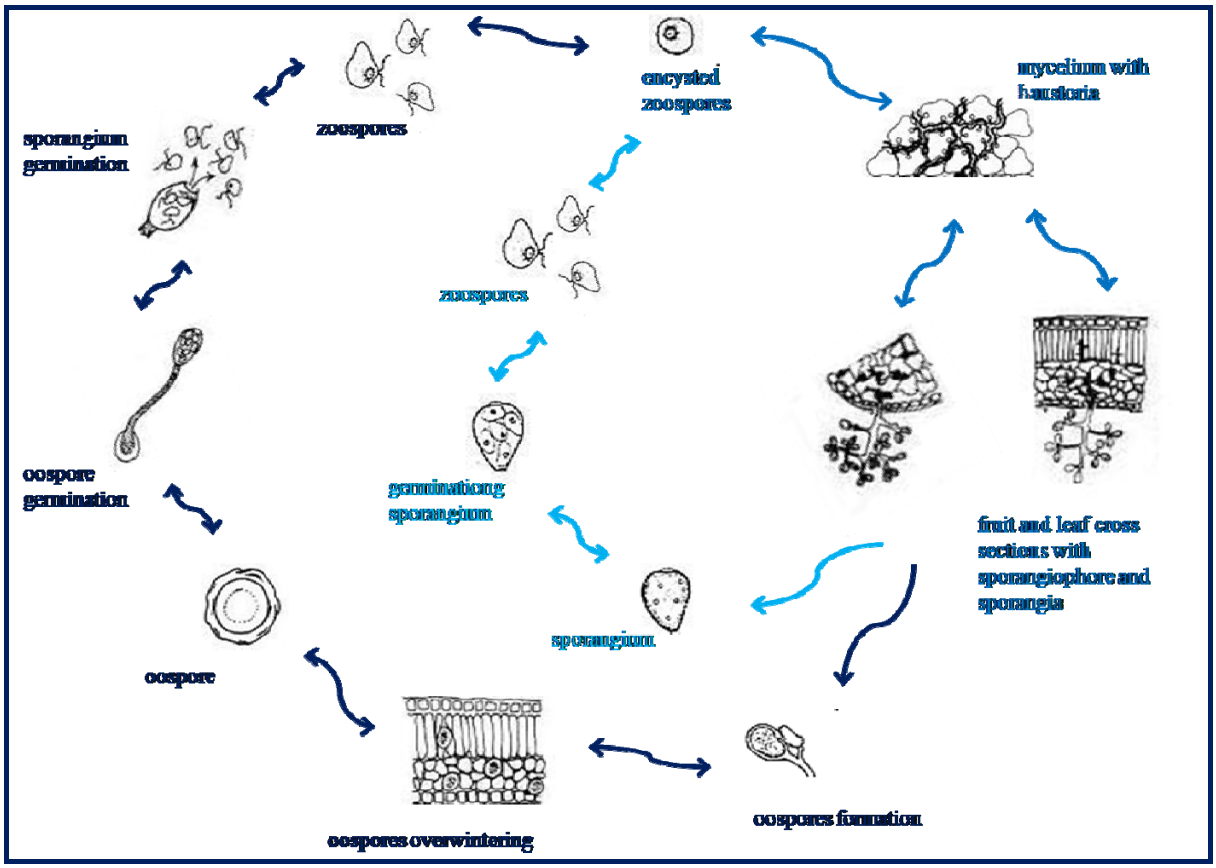
To viticulturist controlling this disease is crucial as it attacks almost all organs. The first symptoms of infection are the oil spots, yellow-brown oily spots visible in the leaf's upper surface. In young leaves the oil spot has a halo which fades in time and the centre of spot becomes brown as it dries up. In older leaves the oil spots are smaller and commonly bound by the veins creating a mosaic like pattern.

As soon as sporulation occurs, a cotton-like mass is visible on the lower surface of the leaf, in correspondence to the pre-formed oil spot. The number of lesions per leaf depends on the intensity of the attack and if they are numerous enough they can cause defoliation of the canopy. Young shoots, tendrils and stems, when infected, become oily brown and afterwards whitish due to the sporulation. Typically the tips of the young shoots will curl. Infection of inflorescences, young berries and bunches appears as oily brown areas that in time are covered in white. Inflorescences and young bunches tend to wither and turn brown while young berries stop growing and become hard later turning dark and falling from the bunch.

The downy mildew causal agent, *Plasmopara viticola*, is a highly adapted *oomycete* to its host: the vine. Specially adapted to the *Vitacea* family and the subgenus *Euvitis*, this *oomycete* is an obligate biotrophic (host cell supplies nutrients for parasitation) organism that depends exclusively on the plant cells to grow and propagate. Endemic in North America where most varieties are resistant to its action, it arrived in Europe in the end of the 19th century, spreading quickly over the continent where all traditional varieties revealed to be sensitive to this pathogen. Firstly named *Botrytis cana* was then called *Botrytis viticola* and at last it was renamed *Plasmopara viticola* by Berlese and

De Toni, hence the scientific name *Plasmopara viticola* (Berk. et Curt.) Berlese and De Toni.

The *Plasmopara viticola* life cycle includes sexual and asexual propagation phases. During the sexual propagation phase genetic recombination occurs and perennial oospores are formed while asexual propagation is a fast propagation phase during which a quick spreading is obtained by the production of sporangia (Kassemeyer *et al,* 2002).



Plasmopara viticola life cycle

This pathogen overwinters in the form of oospores in death leaves in the vineyard floor or in other grapevine organs as for example in pruning wood.

The dormant oospores need a certain periods to germinate and release sporangia after maturation. The length of this period is determined by environmental factors (Gobbin *et al,* 2005), usually after 5-to 18 days with optimum conditions of 18ºC and saturating humidity (Burruano, 2000). Under field conditions the rule of thumb is usually applied to predict the beginning of

the cycle 10:10:24, 10ml precipitation, 10 º C minimum temperatures over a 24h period (Fisher *et al,* 2007).

Under favourable humidity conditions the primary infection starts and 4 to 8 zoospores are released from the sporangia (Kortekamp *et al,* 1998).

Sporangia and zoospores are disseminated by action of water or wind. A thin moisture film is necessary over the leaf's surface since these zoopores are motile and use it to reach the stoma (Unger, 2007).

As soon as the pathogen enters in contact with the stoma of its possible host, the infection process begins. After they have encysted, a penetration peg is formed and host invasion has started.

A substomatal vesicle is formed and an incubation period follows, in which the intracellular space is colonized and haustoria are formed. These specialized organs differentiate in the parenchyma cells of the infected tissue and absorb the nutrients from the host cell without killing them. Only in advance stage of the disease does necrosis take place (Vercesi, 2009). Lesions caused by the *oomycete* development appear between 7 to 12 days after the infection started. The first symptom is known as oil-spots, yellow-brown spots form on the upper part of the leaves.

At last, the sporangiophores protrude trough the stoma and branch out in a species-typical manner. Subsequently the oomycete sporulates, forming sporangia on the tips of these branched structures. The downy mildew cottony effect becomes visible. For sporulation to take place darkness is necessary, since white light irradiation prevents the formation of sporangia (Rumbolz *et al,* 2002). Also important are temperature, that should be higher than 13ºC, and high relative humidity.

These sporangia will assure the secondary infection and the continuation of the propagation and infection of the pathogen as long as water is present in the environment.

Temperatures between a minimal temperature of 9 to 10ºC and a maximal temperature of 33 to 35ºC are necessary for fungal growth.

The complete infection cycle is thus a highly coordinated temporal and spatial process in which pathogen and host interact. An epidemic will only truly progress if several repeated infection cycle take place, that is, if an infection chain ensures proper propagation and spreading of the *oomycete* (Kassemeyer *et al,* 2002).

When discussing the infection the stoma gain a renewed importance, especially in berries, as they are necessary for the first step of infection since downy mildew *oomycete*s are only able to reach intercellular spaces on the mesophyll trough the stoma which should influence the recognition time of the plant and the triggered defence mechanisms (Díez-Navajas *et al,* 2008).

Timing becomes very import as stoma develop into lenticels by the time berries are pea-size even if the possibility of infection trough the pedicel exists (Gregory, 1915). Stoma developmental studies suggest a range of time during which the changes can occur, from 1 to 6 weeks after bloom, in most varieties till 20 after fruit set, after which the *oomycete* will not be able to enter the berry and even if it is already present in the berry it will not be able to support sporulation (Kennely *et al,* 2005).

As grapevine varieties tend to have different numbers of stoma in berries and leaves, the variety is also an important factor for successful infections.

In leaves, also the presence of hairs in the lower part of the leaf surrounding the stoma seems to have some importance to the proceedings of the infection as they repel water and hence prevent the encystations of the zoospores (Kortekamp and Zyprian, 1986).

A major role is also played by solar radiation that affecting temperature or humidity can therefore also influence the microorganisms by killing fungal spores with insufficient protective pigments (Dalla Marta *et al,* 2008). Being necessary for all chemical reaction that takes place in the plant it will lead to an increased production of defence compounds influencing greatly the pathogen's progression possibly enhancing defences in non-shaded plants over shaded ones.

Plasmopara viticola seems to modulate host cell defences through effectors, molecules that manipulate host cell structure and functions, both

apoplastic – secreted in the extracellular space and that interact with the plant cell surface receptors – and cytoplastic – enter the plant cell and interact with cytoplasmic targets – secreted during the first stages of infection either on host or nonhost plants (Díez-Navajas *et al,* 2008).

Resistance to *Plasmopara viticola*

Along the years many authors have worked to establish the differences between the species regarding their reaction to this pathogen.

Considering only the effects of the *oomycete* on the species the following classification was made *Vitis candicans, V. cinerea, V. cordifolia, V. monticola, V. riparia, V. rotundifolia* and *V. titania* are recorded as highly resistant, *V.lincecumii, V. vulpina* as partially resistant, *V. aestivalis, V. arizonica, V. berlandieri, V. doniana, V. palmate* and *V. rupestris* as partially susceptible while all *V. vinifera* varieties are considered highly susceptible (Boubals, 1959; Galet, 1977; Wiedemann-Merdinoglu et al, 2009). Nonetheless comparisons among *V. vinifera* cultivars reveal that some are far more susceptible than others: Riesling, Pinot noir, Pinot blanc and Cabernet Sauvignon seem to be little susceptible; Müller-Thurgau presents medium susceptibility and Alvarinho an Touriga Nacional are highly susceptible (Boso and Kassemeyer, 2008).

In other studies (Derck and Creasy, 1989) groups were created to describe the relation between susceptibility to this *oomycete* and production of phytoalexins:

High phytoalexin production and high resistance	Castor, Pollux, *V. riparia V. doaniana*
Intermediate phytoalexin production and moderate resistance	*V. rupestris,* cv Vignoles, *V. andersonii*
Intermediate to low phytoalexin production and susceptibility	Müller-Thurgau, Chardonnay, Riesling, *V. treleasei, V. Acerifolia, V. Argentifolia*
Low phytoalexin production and resistance	*V. cinerea, V. champini,* cv Bacchus

Intraspecific hybrids and their backcrossings with *V. vinifera* also show some degree of resistance.

The hybrid Solaris seem able to reduce the length and the number of haustoria and hyphae during colonization, with formation of necrosis (Dai *et al,* 1995; Kortekamp and Zyprian, 2003), phytoalexins or even callose (Gindro *et al,* 2003) attributed to a hypersensitive defence reaction.

Lesion size, necrosis and sporulation capacity were found to be the main differences present in the interaction between *Plasmopara viticola* and three different varieties with distinct levels of reaction. On the highly resistant plants, *Vitis rotundifolia*, the stomal tissue underwent a necrosis process and the *oomycete* was no longer able to grow on this tissue. *Vitis rupestris*, showed and intermediate response and the restriction of hyphal growth is probably due to the production of resveratrol, flavonoids and lignin as defence reactions. The susceptible *Vitis vinifera* cv Grenache allowed the fungus to grow extensively as the defence reaction were too late and too weak to cease the widespread of the *oomycete* (Dai *et al,* 1995).

Materials and Methods

The trials conducted aimed to verify the relation between resistant and susceptible varieties, berry and leaves stilbenic production within the same varieties and STS induction and stilbene production in different varieties.

Plant material

Sangiovese/Sirius trial

Grapevines from the varieties Sangiovese and Sirius, a hybrid, planted respectively in 2003 and 2002 were grown in pots in an outside open platform under a hail-protective net, with drip-irrigation from the beginning of the vegetative growth until harvest. They were Guyot pruned and spray treatments were ceased soon enough to prevent any effects on the trials. The 4th or the 5th leaves were harvested, washed, 3 leaf discs for each replicate were then prepared and placed in square petri boxes covered with filter paper and water, inoculated with *Plasmopara viticola.* The boxes were kept in climatic chamber under controlled humidity, temperature and light conditions and then collected at specific times, at inoculation, 1dpi, 2dpi, 4dpi, 6dpi, 8dpi and 11dpi.

Cabernet Sauvignon clones trial

Ten different clones of Cabernet Sauvignon, 169, 191 338, 341, 685, ISV2, ISV105, ISV117, R5, and VCR8 grown in 50L pots, grafted in SO4, and planted in 2003, were kept in the outside open platform under a hail-protective net, with drip-irrigation from the beginning of the vegetative growth until harvest.

The plants were kept separated from the other plants present in the platform and were covered when the treatments were carried out so that no treatments were ever used applied on them. Both leaf and berry behaviour was studied.

The 4th or the 5th leaves were harvested a little before flowering (stage 15 of the Eichhorn and Lorenz phonological scale), washed, placed in square petri

boxes covered with filter paper and water and inoculated with *Plasmopara viticola.* Stilbene production was then evaluated in all clones at specific times, at inoculation, 2dpi, 3dpi, 5dpi, 6dpi, 7dpi, 8dpi. 3 leaf discs were used for each replicate.

Aiming to study the berries before the stoma in their surface had closed; the berries were harvested before ten days had past from fruit set. Bunches were harvested and cut in smaller pieces after washing, including always berries and a part of bunch stem and placed in square petri boxes filled with an agar solution to prevent dissecation of the small bunch pieces. The berries were then inoculated with *Plasmopara viticola* and harvested at pre-established times: at inoculation, 1dpi, 3dpi, 12dpi and 19dpi.

All boxes, from leaves and berries were kept in climatic chamber under controlled humidity, temperature and light conditions.

Multiple varieties trial

Rooted cuttings of Chardonnay, Cabernet Sauvignon, Pinot noir, Sangiovese, Müller-Thurgau and Solaris, a hybrid, were grown in a greenhouse. The plants were kept in an isolated open chamber so that no spray treatments that could influence the development of the future inoculation were used. As soon as the plants had developed a sufficient number of both old and young leaves, minimum of 12 total leaves, the whole plants (3 replicates/variety) were inoculated with *Plasmopara viticola* L (25.000sporangia/mL water).

Sampling followed a pre-determined time course and young and old leaves were harvested and 3 leaf discs for each replicate were prepared at 0, 12, 24, 72 hours and after sporulation (after abundant sporulation was clearly visible at sight) and used for 2 different types of analysis: stilbene synthase relative quantification, stilbene byosinthesis production and for microscopic analysis.

<u>Estimation of the number of sporangia in a solution</u>

The number of sporangia was counted in the 16 (4x4) squares inserted in the big square of a microscope counting grid, their average was multiplied per

5000 and the number of sporangia/mL was obtained. All inocula were prepared with an estimated number of 25000 sporangia/mL. Suspension volume varied according to the grapevine material necessary to develop each trial.

Propagating *Plasmopara viticola*

In all the trials conducted in Italy, infected leaves with enough mycelium for all the trials were supplied by the Pathology department of the Milano University and were characterised by Italian *Plasmopara viticola* strains.

As for the *Plasmopara viticola* used in the multiple varieties trials, the inoculum was characterised from German strains that I collected from several fields and afterwards propagated as follows: after spraying the whole plant, in the late afternoon, the plants were covered with humid plastic bag. The bag was removed the morning after. When oil spots were visible, the leaves were sprinkled with water and the plant covered with a plastic bag for the second time. The following morning, sporulation which occurred overnight was visible and ready to be harvested.

Visual symptoms evaluation

At each sampling time the percentage of sporulation in the surface of each leaf disc was estimated and the value of a given replicate was obtained averaging the values of the 3 leaf discs that formed that replicate.

Likewise, at each sampling time, the percentage of sporulation present in the surface of each berry was estimated. Averaging these numbers allowed obtaining the percentage of sporulation for each replicate.

In both cases the value indicated for each time is the average of three replicates.

Preparing microscope slides

Following a method perfected in the Statliches Weinbauinstitut of Freiburg, Germany the leaf discs were prepared as follows:

After the leaves were harvested and well washed, the leaf discs were made.

The leaf discs were placed in a tube with KOH, boiled for 10min and then let to discolorate in the light. As soon as they have changed colour the leaf discs were placed in distilled water for 1 min.

Using a spatula and water they were carefully placed in the microscope slide. All water was removed and the staining solution, aniline blue, was added for around 10 min. The staining solution was removed and the cover slips were placed on top.

The slides, after being prepared can be maintained inside a Petri box covered with wet paper if kept inside a fridge (max 1 week).

Resveratrol extraction method from leaves

The small leaf disks, about 1.8 cm diameter, were collected from the previously selected shoots (preferably 4th and 5th leaves from the apex).

Maceration of the tissues was obtained using a mortar, 1.5 (or 2 depending on the microcentrifuge tubes) mL of methyl alcohol.

The result of the maceration, liquid and solid, was centrifuged for 10 min at 14.000 rpm.

The supernatant liquid was gathered and placed in small vials. The vials remained in the freezer until HPLC was performed.

Resveratrol extraction from berries

Approximately 20g of frozen berries were used.

The berries were macerated in a mortar and 30mL of methanol 95% were added. The result was placed into an Erlenmeyer flask and was stirred for 20 min.

As soon as the agitation period was over, filtration using Wathman GF/A filters took place. All liquid evaporated into a round bottom flask. The liquid fraction evaporated under vacuum with a Rotavapor at 35°C, to favor methanol evaporation.

To the liquid that remained in the round bottom flash (water), 5mL of sodium bicarbonate 5% and 5mL of ethyl acetate were added. The liquid inside the flask was stirred carefully and then transferred to a test tube. The tube was shaken vigorously to achieve a phase separation.

The organic fraction was recovered; transferred to a new round bottom flask, while the water was re-extracted with 5mL of ethyl acetate to recover, after the phase separation, the remaining organic phase.

The ethyl acetate solution was then evaporated under vacuum with a Rota-vapor until the complete evaporation of the sample.

The walls of the round flask were then washed in 2 successive phases, very slowly and carefully, with methanol 100% using in the first time 2mL and in the second 1mL, concentrating the resveratrol in 3mL of methanol 100% in glass vials.

RNA extraction method

The leave discs were collect and placed in a 2mL microcentrifuge tube along with some clean glass beads. The samples were frozen in liquid nitrogen and placed in the horizontal shaker for 15 to 20sec. Under the hut the buffer solution was added and shaken for 10m at 65°C. 800µL of isoamyalcohol/chloroform were added and centrifuged at 13000rpm for 10m. The upper part was separated and 800µL of isoamyalcohol/chloroform were added. The samples were then centrifuged at 13000rpm for 10m. The upper phase was pipetted and to each vial 1/4 of its volume of LiCl was added. All samples stayed overnight in a fridge at 4°C. After 30 min at 13000rpm (<4°) centrifuging, the microcentrifuge tubes were emptied with a tip-burned Pasteur pipette and 1mL of ethanol (70% at -20°C) was added, followed by another round of centrifuging: 10m at 13000rpm. The content of the microcentrifuge tube was re-aspirate with the Pasteur pipette and the microcentrifuge tubes were placed at 65°C till completely dry. 50µL of ddp water were added and the samples were stored in a 4°C fridge.

Reverse transcription

The reverse transcription was obtained using the iScript ^{tm}cDNA Synthesis kit from Bio Rad. The maximal amount of template was used for each sample and all samples, complete mix, were incubated in a termomixer for the times established in the protocol: 5 min at 25ºC,30 min at 42ºC and then the reaction was inactivated at 85ºC for 5 min. The samples were the stored at 4ºC.

Polymerase chain reaction (PCR)

Amplification was performed in an IQ5 Bio-Rad multicolor Real time PCR detection system. Using a total of 25µl per sample, of which, 1 µl was of template and 24 µl of SYBR Green Pcr Mastermix including a RT-STS reverse primer 5' CAA CTA AAG AGT CCA AAG CAT C 3' and RT-STS forward primer 5' GGT GGA ACT GTC CTT CGA ACC 3' encoding for a *Vitis* Stilbene Synthase gene.

Amplification was carried out starting with an initiation time for the polymerase of 15min at 95ºC, the 40 cycles of denaturation of 3sec at 95ºC and then 60sec at 60ºC for annealing and extension.

The results were analysed using the IQ5 optical system software version 2.0.

Standards

The trans-resveratrol (trans-3,4',5-hydroxystilbene) and piceatannol purchased from Sigma (St. Louis, MO) were used as standards; cis-resveratrol was prepared from the standard of *trans*-resveratrol by photoisomerization. ε-viniferin (dimer of trans-resveratrol) was kindly supplied by G. Hoos (formerly BAZ, Institut für Rebenzüchtung Geilweilerhof, Siebeldingen, Germany). *Trans*-piceid was kindly supplied by F. Mattivi (IASMA, Italy). The purity of each stilbene was controlled by HPLC, and the identity was confirmed. Working standards of stilbenes were prepared by dissolving them in methanol and diluting the solution with acetonitrile/water (40:60; v/v). The standards were also used in Germany for the trial preformed there.

HPLC Conditions

The HPLC system used to quantify the stilbenes from the 2007 trials, Sirius-Sangiovese plus leaves of Cabernet Sauvignon, was a Agilent HP 1100 series (Waldbronn, Germany) with an autosampler and diode array detector (DAD) set at 306 and 325 nm. A 250 × 4.6 mm i.d., 5µm, C 18 Supelco Supelcosil ABZ plus column was used, eluting with a gradient of methanol(A) and 0.01 M potassium phosphate monobasic adjusted to pH 2.5 with phosphoric acid (B). The gradient was of 40 to 85% of A at a flow rate of 1.0 mL/min. The instrumental limit of detection was 0.05 mg/L. The injection volume was of 50 µL.

In 2009, Cabernet Sauvignon berries trial, a new column was used: Phenomenex Gemini 3µm C18 110A, 100x4.6mm. The gradient was of 40 to 85% of A at a flow rate of 0.6 mL/min. The instrumental limit of detection was 0.006 mg/L.

In 2008, multiple varieties trial, a Waters HPLC system with a diode array detector and autosampler (Hewlett Packard 1050 series) set for 210, 255, 285, 306 and 370 nm. The column used was a LiChrospher RP-C18, 250 x 4 mm, 5 µm (Merck, Germany). Injection volume was of 25µl per each sample and the flow rate was kept at 2.5ml min^{-1}. Mobile phase solvents at 1 mmol were: A – H_3PO_4 and B – CH_3CN.

Stilbenes quantification.

Amounts of stilbene standards between 1 and 500 ng were injected. Quantification was on the bases of peak areas using the PC software.

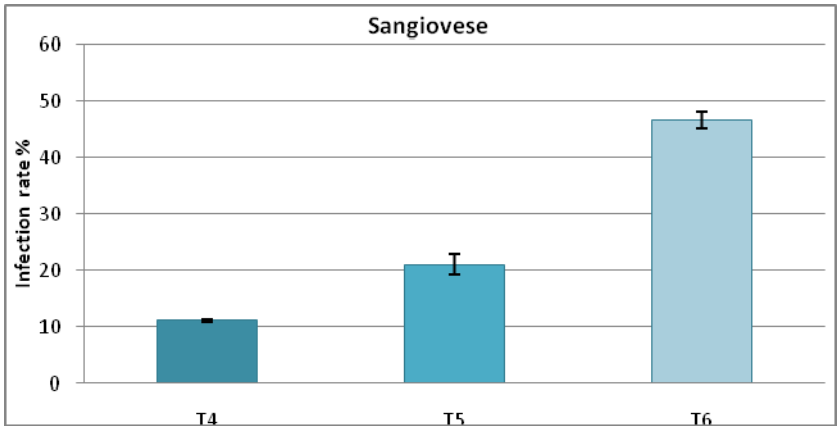
Statistical analysis

Using SPSS version 15.0.1(22 November 2006), licensed for USCS Piacenza, the data obtained were analysed. First Fisher's F was determined and then, when appropriate and according to the pertinence to the studied trial, Standard error, LSD, SNK or the Waller-Duncan test were used.

Results

Sirius-Sangiovese trial

The first trial conducted confronted two varieties, Sirius which is considered resistant, and Sangiovese which is considered sensitive to *Plasmopara viticola*. The inoculated leaf discs of Sirius never really presented any visible sporulation, only some necrotic spots, while those of Sangiovese started presenting visible signs of it at T4, 6 days post-inoculation (dpi). The amount grew from around 10% of covered leaf disc surface to a total of roughly 45% on the last time sampled, T6, which was 11dpi.

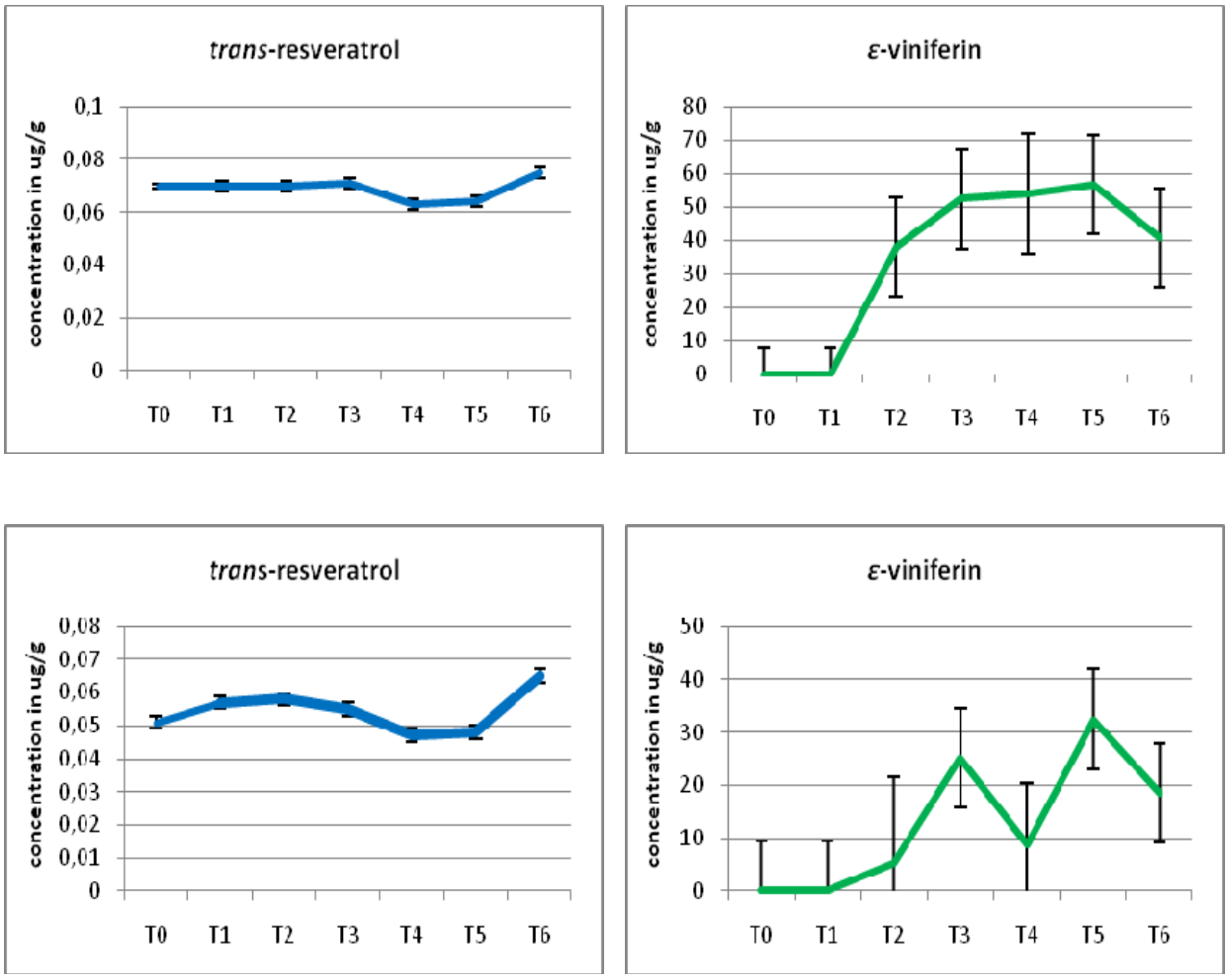


Infection rates in *Plasmopara viticola* infected leaf discs of Sangiovese

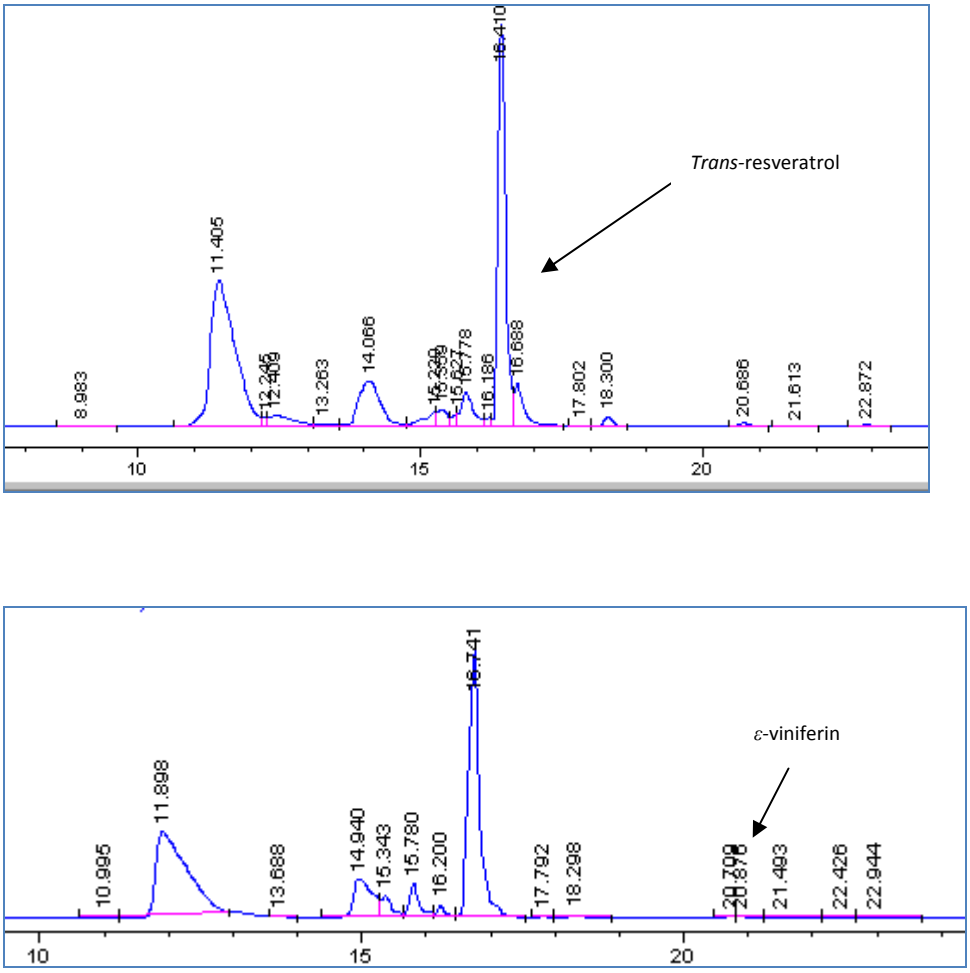
HPLC analysis revealed only the presence of *trans*-resveratrol and *ε*-viniferin. During that period, and despite no sporulation could be seen, the concentration of *ε*-viniferin increased greatly in the Sirius leaf discs. The highest increase occurred between the 1st and the 2nd dpi and high amounts of this compound could be found until T5, 8dpi, point after which the values decreased. Sangiovese leaf discs also revealed a first increase during the 1st and 2nd days but then, at T4, the values decreased and a second peak of accumulation was found at T5. After this a new low *ε*-viniferin value was found.

The amounts of ε-viniferin were always considerably higher in Sirius leaf discs.

For what concerns *trans*-resveratrol overtime behaviour in both varieties, the values remain stable until T4, at which point there is a decrease that prolongs until T5 and at T6 a new increase can be seen. Although the values are always higher in Sirius leaf discs the total values are always extremely low, never reaching 0.08 µg.g-1 fw.



Sirius (above) and Sangiovese (below) *trans*-resveratrol and *ε-viniferin* concentrations in *Plasmopara viticola* infected leaves



Chromatogram of 2 samples of Sirius (above) and Sangiovese (below)

Cabernet Sauvignon trial – leaf discs

Ten different clones were inoculated with *Plasmopara viticola* and the ongoing visual symptoms of this *oomycete* were visually assessed.

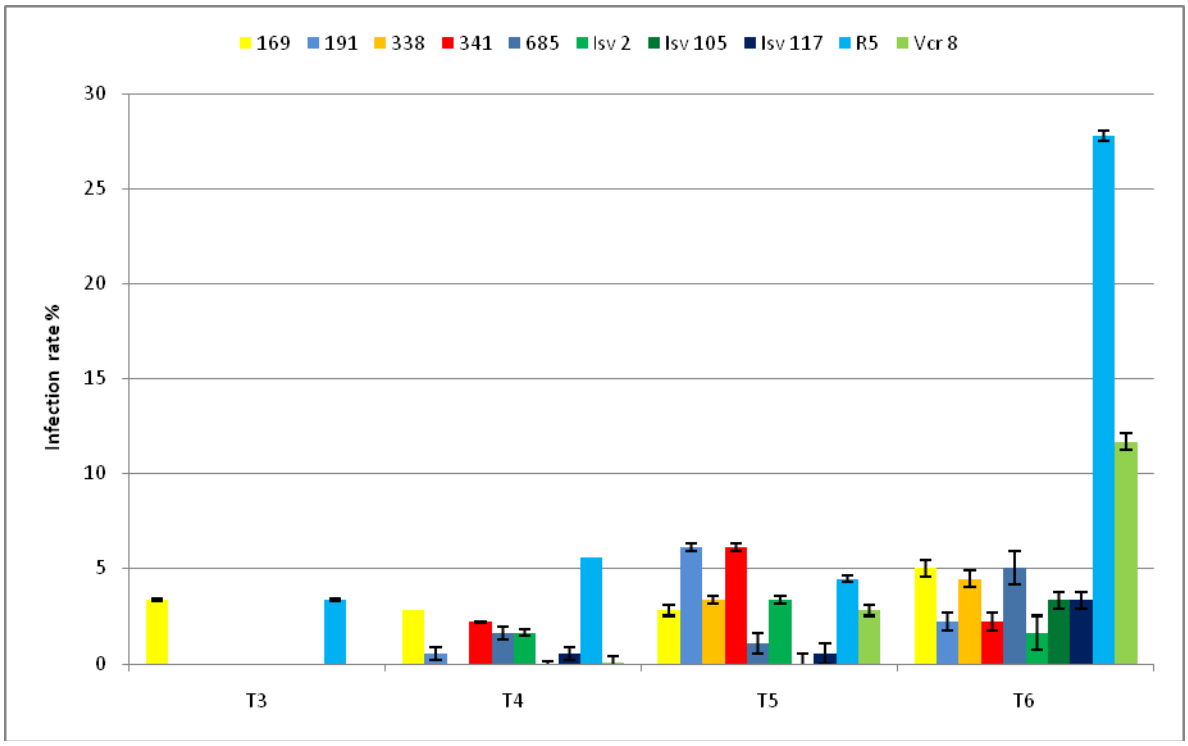
First, it is important to state that visually the clones appear to have a different response.

The development of the visual symptoms evolves over time but most of them maintain a relatively constant development of the visible cottony effect.

Only VCR8 and R5 clearly stand out in the last sampling time, showing higher visual symptoms, reaching from 12 to 28% of sporulation. It is also possible to see that at T6 the remaining clones form significantly different

groups: 169, 338 and 685 are different from 191, 341 and Isv2 which at there turn are different from the clones Isv105 and Isv117.

In the 169 and R5 clones, sporulation had occurred 1 day before being visible in the other clones while in the 338 and Isv105 it was only accountable afterwards.



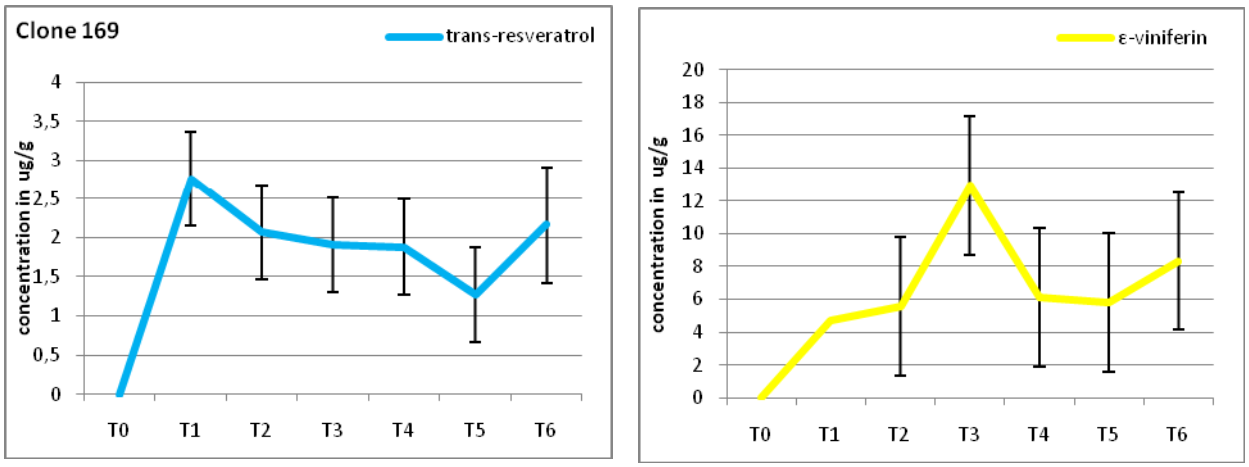
Infection rates in *Plasmopara viticola* infected leaf discs

The individual analysis of the stilbenic behaviour overtime in each clone shows that:

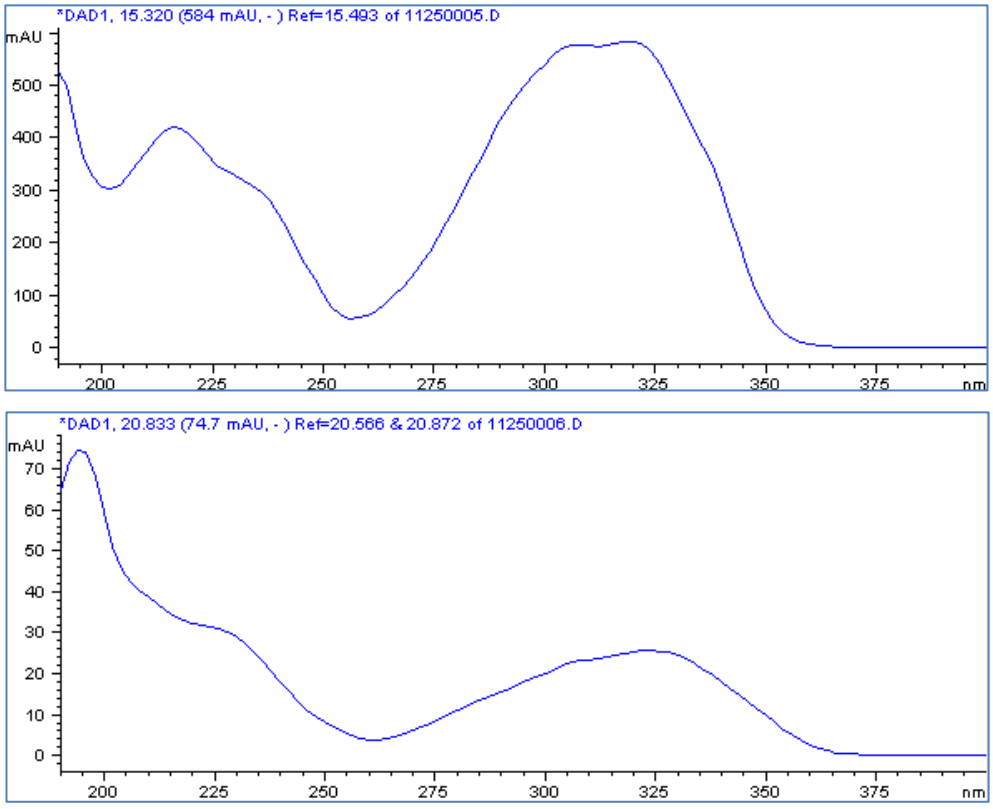
None of the 10 studied clones presented traces of any stilbenic compound at T0.

Clone 169: Following a fast increase from T0, after T1 *trans*-resveratrol steadily decreased till T5 showing an increase at T6 with concentration oscillating between 1 and 3 µg.g-1 fw while *ε*-viniferin values increased steadily until T3, the only peak and decreased afterwards with values ranging from 3 to 15 µg.g-1 fw.

None of the results, for both *trans*-resveratrol and *ε*-viniferin show any statistically significant differences between the times.

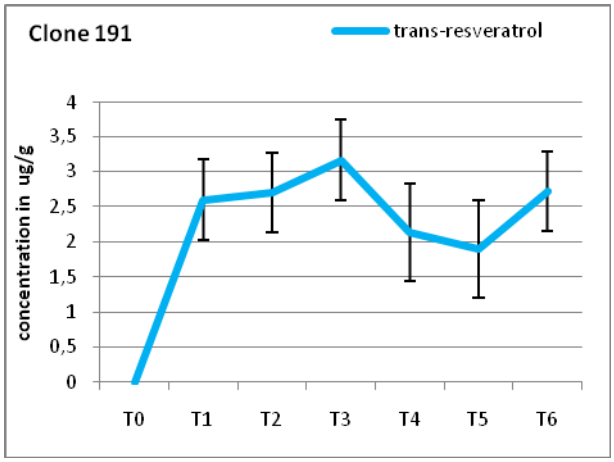


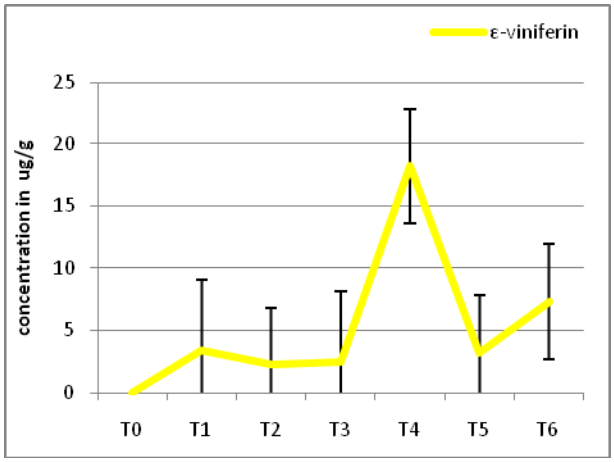
trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone 169 leaves. The bars present in the figure represent the value of 2 times the standard error



Absorption spectra of *trans*-resveratrol (above) and *ε*-viniferin (below)

Clone 191: Fast increase until T1, from T1 to T3, *trans*-resveratrol showed a slight increase and after the peak value, it decreased until T5. Another accumulation peak could be found at T6. However, none of the values observed in the different times showed any statistically significant differences between each other and values were always ranged between 2 and 3 $\mu g.g^{-1}$ fw.

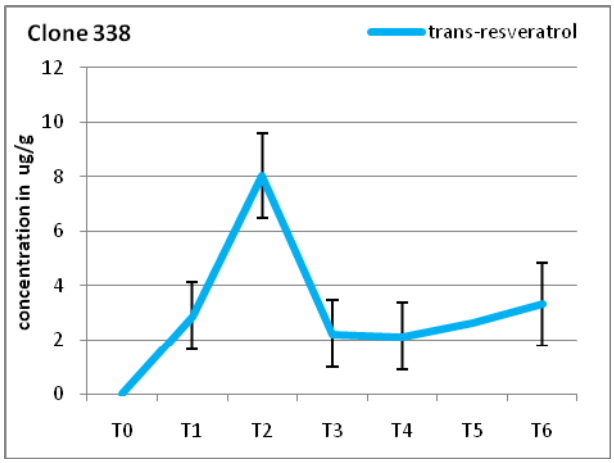


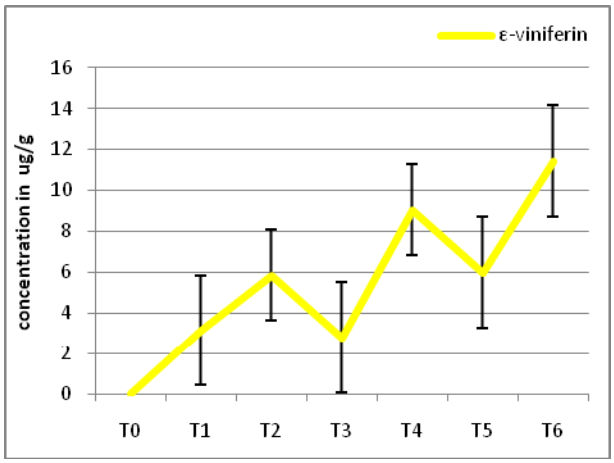


trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone 191 leaves. The bars present in the figure represent the value of 2 times the standard error

Meanwhile the *ε*-viniferin peak value, 18 μg.g^{-1} fw, at T4 is significantly different from all other times. No other significant differences can be seen over time, before T4 all values remained quite stable, below 5 μg.g^{-1} fw, and after this, only another point, T6 (7 μg.g^{-1} fw), stands out with higher accumulation but still considerably lower respect that of T4.

Clone 338: *trans*-resveratrol accumulation on this clone presented a clear statistical different peak at T2, reaching 8 μg.g^{-1} fw. After this the values remained very close for all the remaining sampling times, always between 2 and 4 μg.g^{-1} fw.

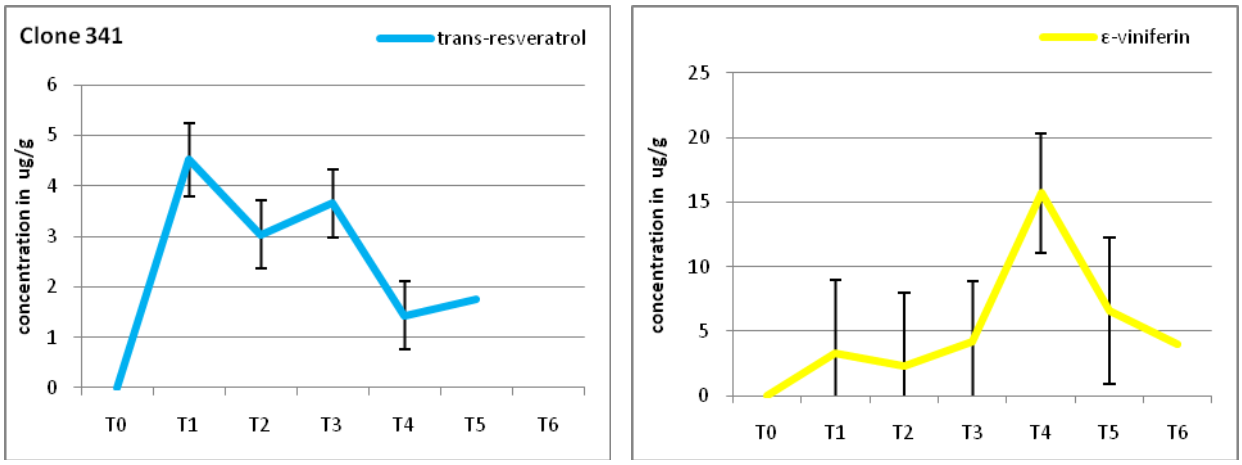




trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone 338 leaves. The bars present in the figure represent the value of 2 times the standard error

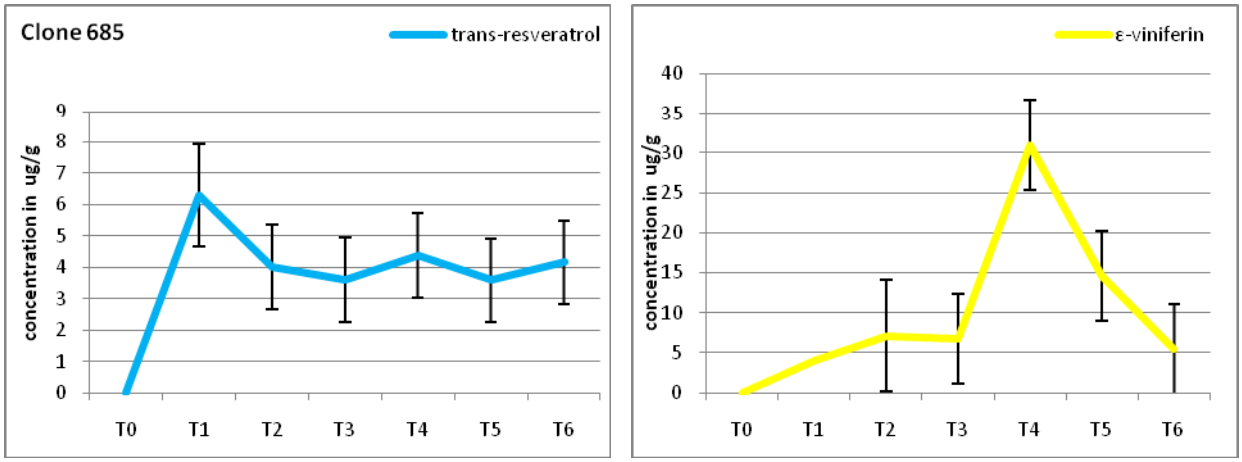
ε-viniferin shows a particular progress. From T0 to T2 an increase of accumulation could be seen, and then at T3 a new low value was found. At T4 a new peak is visible, 9 µg.g^{-1} fw, which is statistically different from the 2 low values of T1 and T2. At T5 a low values is again present and once again a peak values appears at T6, 11 µg.g^{-1} fw, significantly different from all values found before T4.

Clone 341: Apart from the initial increase in *trans*-resveratrol, in this clone, its presence decreased overtime, reaching a statistically significant minimum at T4, this, despite the fact that T3 showed a slight increased accumulation although not statistically significant.



trans-resveratrol and ε-viniferin in *Plasmopara viticola* infected clone 341 leaves. The bars present in the figure represent the value of 2 times the standard error

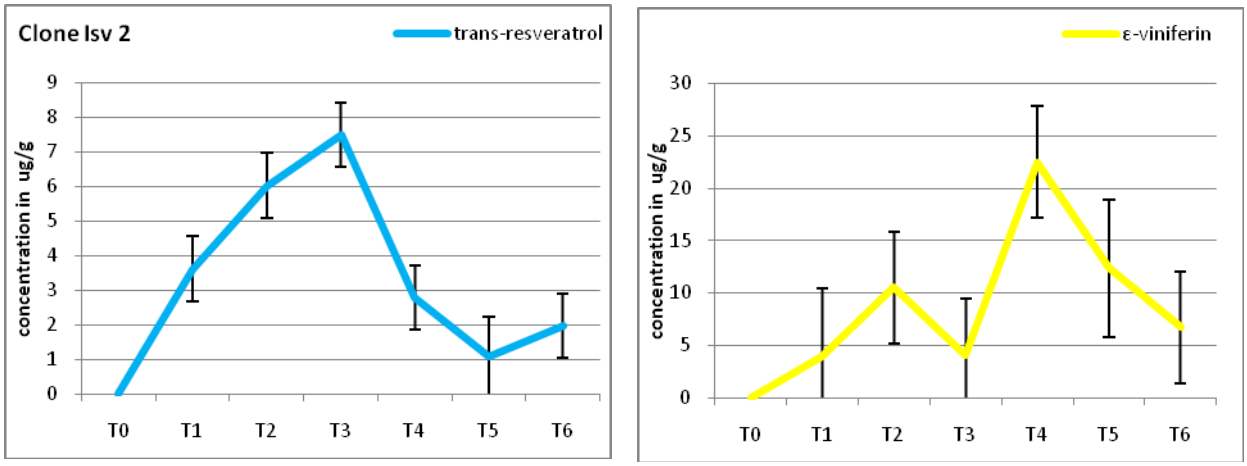
As for ε-viniferin overtime accumulation this clone presented mostly values between 4 and 6 µg.g^{-1} fw, not significantly different from each other, except in T4 where a statistically significant maximum peak of about 15 µg.g^{-1} fw could be seen. This value is almost the double of the 2nd highest value.



trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone 685 leaves. The bars present in the figure represent the value of 2 times the standard error

Clone 685: although values of *trans*-resveratrol were not significantly different they presented a steady decrease, with values always between 6 and 4 $\mu g.g^{-1}$ fw.

ε-viniferin values were also found to be relatively low in this clone at all times, except for T4 which was a statistically significant peak of more than 30 $\mu g.g^{-1}$ fw, almost the double of the 2nd highest value.

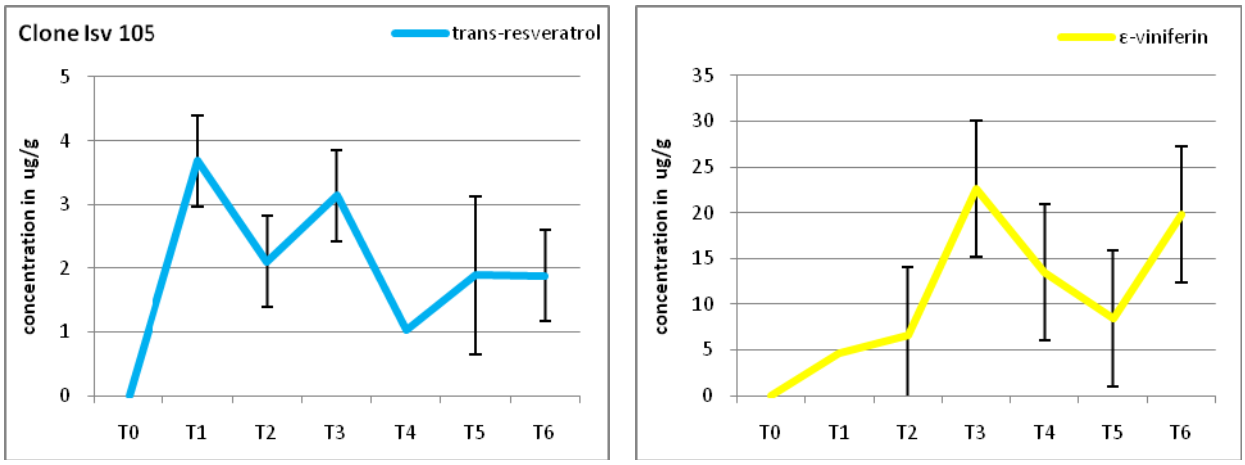


trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone Isv 2 leaves. The bars present in the figure represent the value of 2 times the standard error

Clone Isv2: this clone showed an increasing accumulation of *trans*-resveratrol from T0 to T3, being T2 and T3 statistically significantly different,

above all other values. After this point the accumulation of this compound decreased overtime.

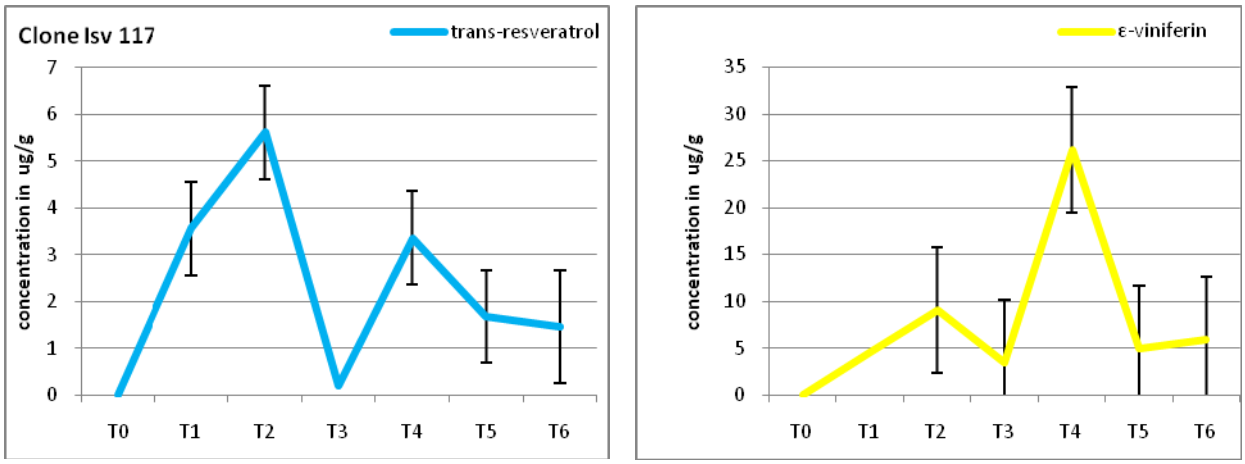
When analysing ε-viniferin behaviour, 2 peaks were found. The 1st at T2 and the 2nd at T4 but only this last, T4, was significantly different from the values found in T0, T1, T2, T3 and T6. The content of this compound oscillated from 5 μg.g^{-1} fw to around 20 μg.g^{-1} fw.



trans-resveratrol and ε-viniferin in *Plasmopara viticola* infected clone Isv 105 leaves. The bars present in the figure represent the value of 2 times the standard error

Clone Isv105: After increasing till T1, *trans*-resveratrol concentration decreased, and increased yet again at T3 but not in a statistically significant way. Afterwards the values decreased and kept stable. All values ranged from 1 and 3.5 μg.g^{-1} fw.

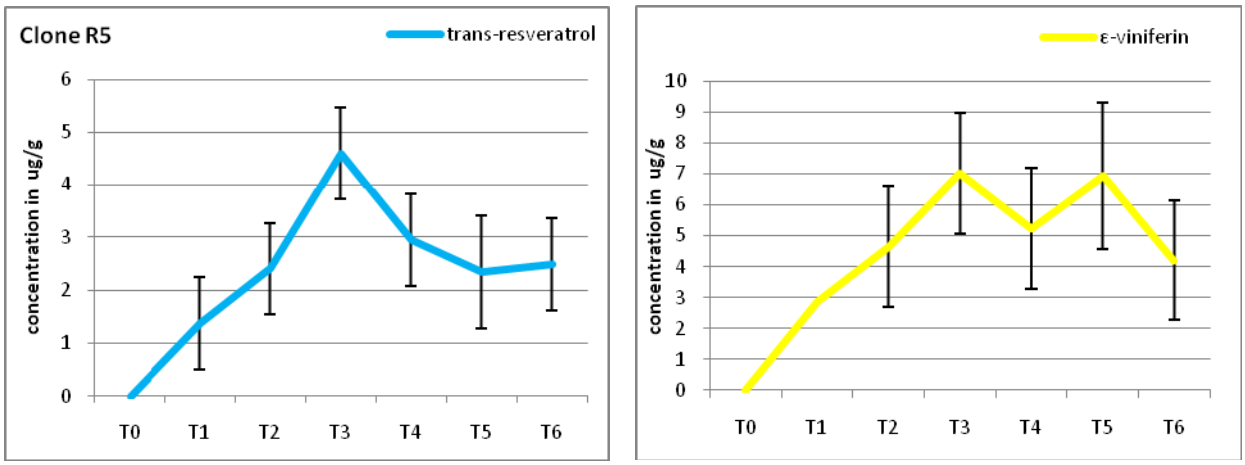
ε-viniferin values increased till T3, a maximum, significantly different from the values found in previous times. After this the values decreased, increasing only at T6 but no statistically significant differences were ever present. The values ranged from 5 to 23 μg.g^{-1} fw.



trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone Isv 117 leaves. The bars present in the figure represent the value of 2 times the standard error

Clone Isv117: two accumulation points can be seen at T2 and T4, being the 1st statistically different from all other points. After T4 however the values seem to constantly decrease. The concentration values ranged from 0 to 5.5 $\mu g.g^{-1}$ fw.

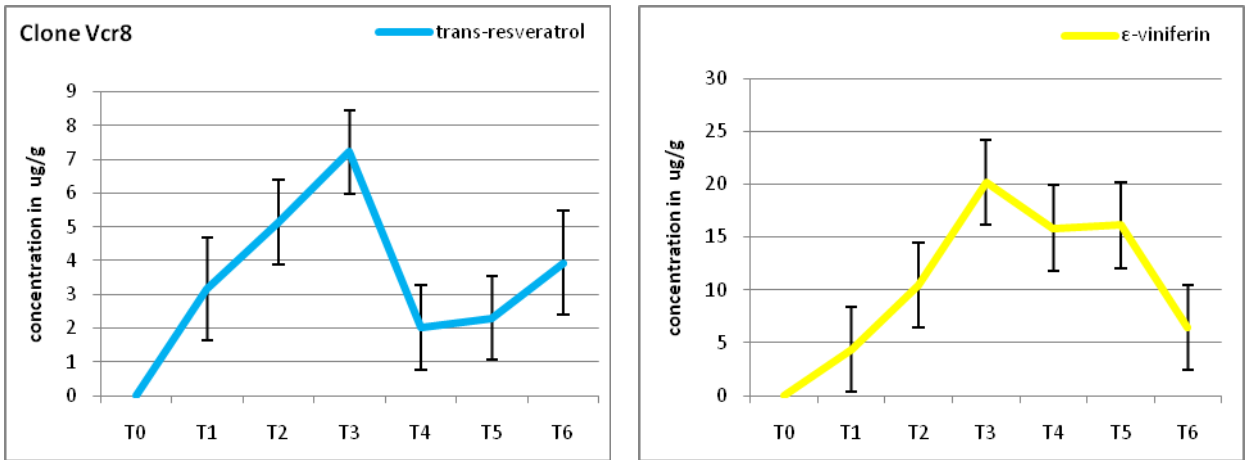
As for *ε*-viniferin, a statistically significant peak could be found at T4, reaching values above 25 $\mu g.g^{-1}$ fw. For all other sampling times the values were not significantly different and ranged from 5 to 10 $\mu g.g^{-1}$ fw.



trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone R5 leaves. The bars present in the figure represent the value of 2 times the standard error

Clone R5: One statistically different peak of accumulation of *trans*-resveratrol was found at T3, close to 4.5 $\mu g.g^{-1}$ fw. After an increase that led to this peak, the values decreased in the following sampling times.

ε-viniferin accumulation presented 2 higher accumulation points, T3 and T5 but the values were not statistically significantly different. All values were between 3 and 7 $\mu g.g^{-1}$ fw.



trans-resveratrol and *ε*-viniferin in *Plasmopara viticola* infected clone Vcr 8 leaves. The bars present in the figure represent the value of 2 times the standard error

Clone Vcr 8: *trans*-resveratrol accumulation increased until T3, point at which this clone presents a peak. After this point lower values can be found but with an increasing tendency.

Also *ε*-viniferin increased until T3, the only clear peak. T4 and T5 show similar levels of this compound, slightly lower than those of T3, and after that the concentration values drop.

When comparing the differences between the clones at a given sampling time some differences do arise.

In the case of *trans*-resveratrol accumulation in *Plasmopara viticola* infected leaves it is possible to see that there are only statistically significant differences at T2 and T3.

At T2, 2 groups with significant differences can distinguished. The clones with significantly lower values are 169, 191, 341, Isv105 and R5 while

significantly higher values were found in the clone 338. The remaining clones, 685, Isv2, Isv117 and Vcr8 have intermediate values respect the other clones.

At time T3, the clone Isv2 and Vcr8 present significantly higher values than the clones 169, 191, 338, 341, 685 and Isv105, which assume significantly lower values. The clone R5 presents values in between the lowest and the highest encountered.

	T1	**T2**	**T3**	**T4**	**T5**	**T6**
Clone 169	2,756	2,069 a	1,907 a	1,882	1,272	2,165
Clone 191	2,596	2,700 a	3,163 a	2,131	1,892	2,722
Clone 338	2,879	8,039 b	2,235 a	2,135	-	3,325
Clone 341	4,519	3,039 a	3,657 a	1,435	-	-
Clone 685	6,301	4,025 ab	3,599 a	4,392	3,593	4,160
Clone Isv 2	3,613	6,024 ab	7,476 b	2,770	1,077	1,981
Clone Isv 105	3,686	2,105 a	3,137 a	-	1,890	1,885
Clone Isv 117	3,557	5,610 ab	-	3,357	1,668	1,459
Clone R5	1,382	2,403 a	4,600 ab	2,949	2,352	2,491
Clone Vcr 8	3,159	5,133 ab	7,218 b	2,022	2,301	3,938
F	0,967	2,521	4,067	1,544	1,597	1,099
Sig.	n.s.	*	**	n.s.	n.s.	n.s.

Comparison of leaf disc *trans*-resveratrol per clone after infection with *Plasmopara viticola.* Values with the same letter are not significantly different (α<0.05 WD Test) -: no stilbenes were detected in at least 2 of the replicates

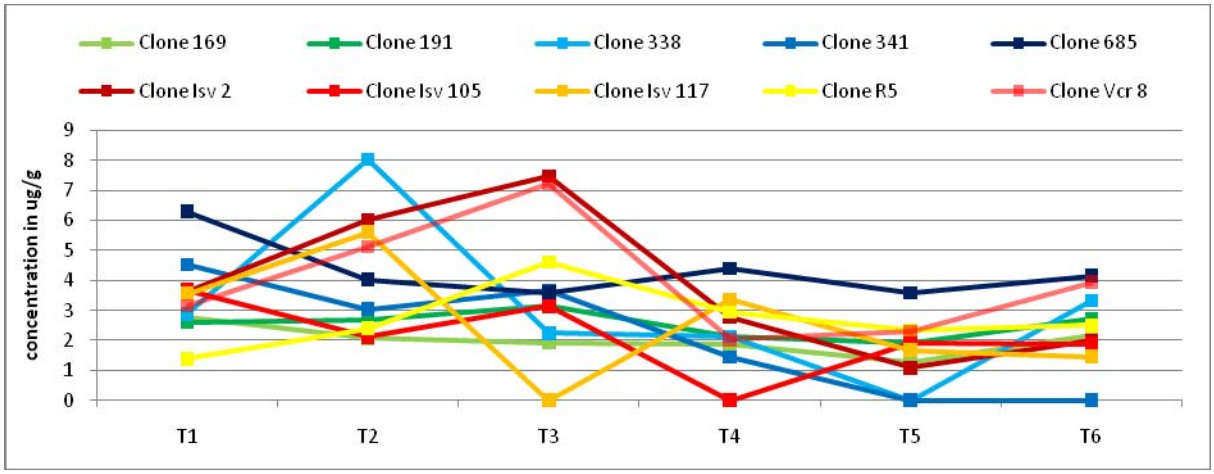
Analysing the graphic it is possible to individuate specific patterns within the clones:

Clones 169, 191, 341, 685 and Isv105 seem to have the same type of behaviour, slightly decreasing over time. All of the mentioned clones have very close values of *trans*-resveratrol although clone 685 always presents the highest ones within the graphic despite not being significantly different.

Clones 338 and Isv117 both peak at T2, even if only the first is significantly different, then they both decrease. An increase follows for both clones but at different sampling times, 338 at T6 and Isv117 at T4. No significant difference can be seen at these points.

Clones Isv2 and Vcr8 increase over time reaching significantly maxima at T3 with values above all other clones. Afterwards the concentration values of *trans*-resveratrol decrease for both clones.

The clone R5 although never statistically different from all others seems to increase concentration values until T3 and decrease after that point.



Comparison of leaf disc *trans*-resveratrol per clone after infection with *Plasmopara viticola*

The comparison per time of the clones' accumulation of *ε*-viniferin reveals significant difference at T3 and T5.

At T3 the clones 191 and 338 present the significantly lowest values while the Isv105 shows the significantly highest values for this sampling time. All other clones, 169, 341, 685, Isv2, Isv117, R5 and Vcr8 assume intermediate values placed between the significantly highest and lowest values.

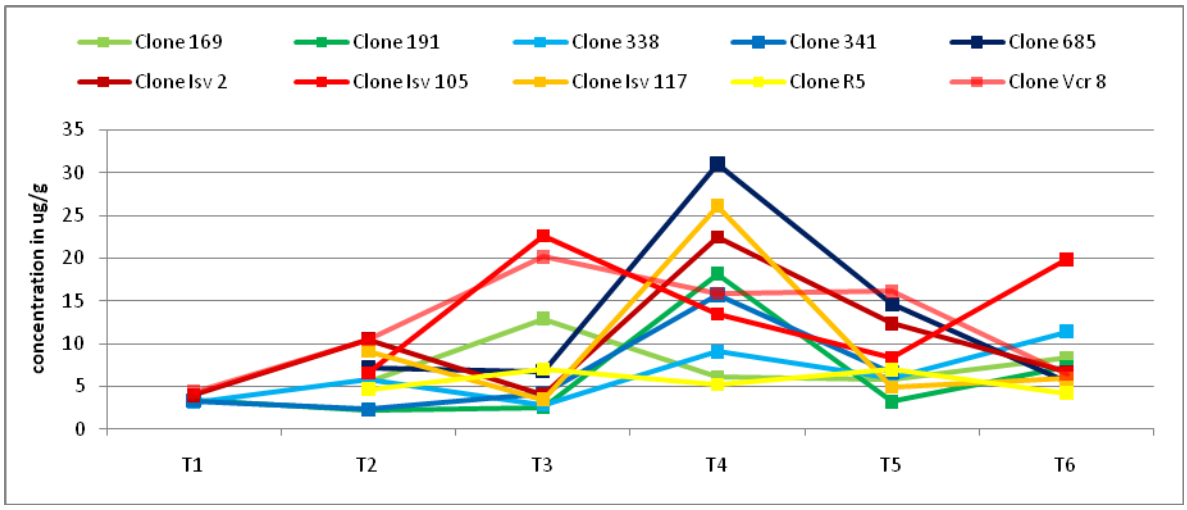
At T5 a more complex situation can be seen: the clone 191 has the significantly lowest value while Vcr8 has the significantly highest value. The clones 169, 338, 341, Isv2, Isv105 and R5 assume intermediate values. The clone Isv117 assumes intermediate low values significantly different from the highest ones while the clone 685 assumes intermediate values significantly different from the lowest values measured.

	T1	T2	T3	T4	T5	T6
Clone 169		5,571	12,926 ab	6,140	5,799 abc	8,348
Clone 191	3,388	2,230	2,504 a	18,190	3,184 a	7,318
Clone 338	3,147	5,864	2,787 a	9,057	5,979 abc	11,427
Clone 341	3,313	2,307	4,194 ab	15,722	6,602 abc	-
Clone 685	-	7,143	6,754 ab	31,002	14,614 bc	5,427
Clone Isv 2	3,949	10,530	4,069 ab	22,492	12,349 abc	6,715
Clone Isv 105	-	6,566	22,651 b	13,490	8,395 abc	19,863
Clone Isv 117	-	9,083	3,462 ab	26,143	4,902 ab	5,894
Clone R5	-	4,653	7,005 ab	5,231	6,927 abc	4,210
Clone Vcr 8	4,356	10,480	20,172 ab	15,857	16,114 c	6,444
F	0,078	1,144	2,093	1,184	2,500	0,801
Sig.	n.s.	n.s.	*	n.s.	*	n.s.

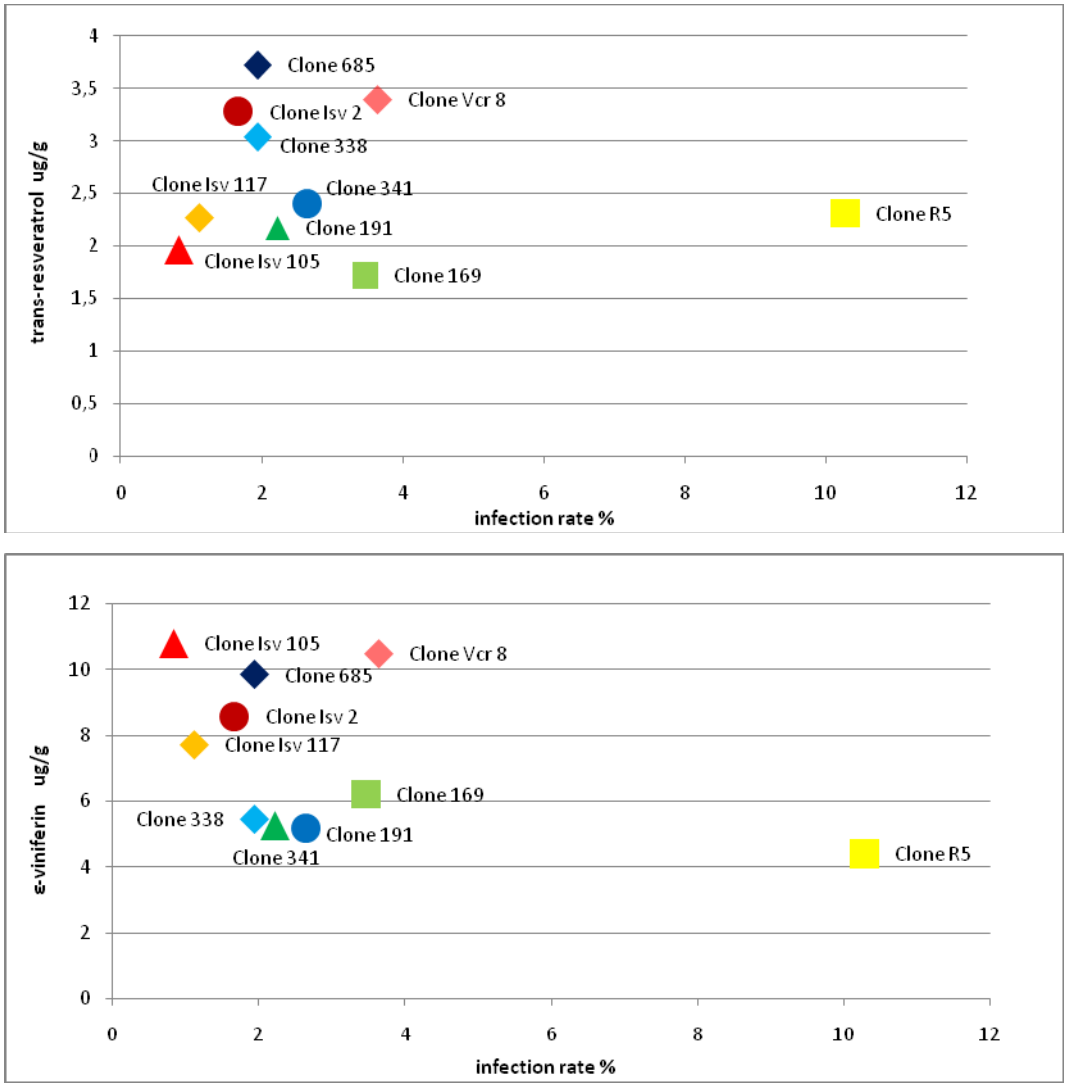
Comparison of leaf disc ε-viniferin per clone after infection with *Plasmopara viticola.* Values with the same letter are not significantly different (α<0.05 WD Test) -: no stilbenes were detected in at least 2 of the replicates

Evaluating the graphic of ε-viniferin accumulation we can see that from the 10 clones under study only 5 clones, 191, 338, 341, Isv2 and Vcr8, present any values at T1. The clone Isv105 has a significantly higher value than all other clones at T3 but the clones Vcr8 and 169 seem to follow the same type of behaviour, reaching a maximum at this point and decreasing afterwards.

At T4, several clones seem to reach their maximum value but no clone presents statistically significant differences. Instead, at T5, ε-viniferin values seem to decrease for all clones. At T6, Vcr8, 338, 169, 191 and even Isv117 seem to increase respect the values found in the previous sampling time.



Comparison of leaf disc ε-viniferin per clone after infection with *Plasmopara viticola*



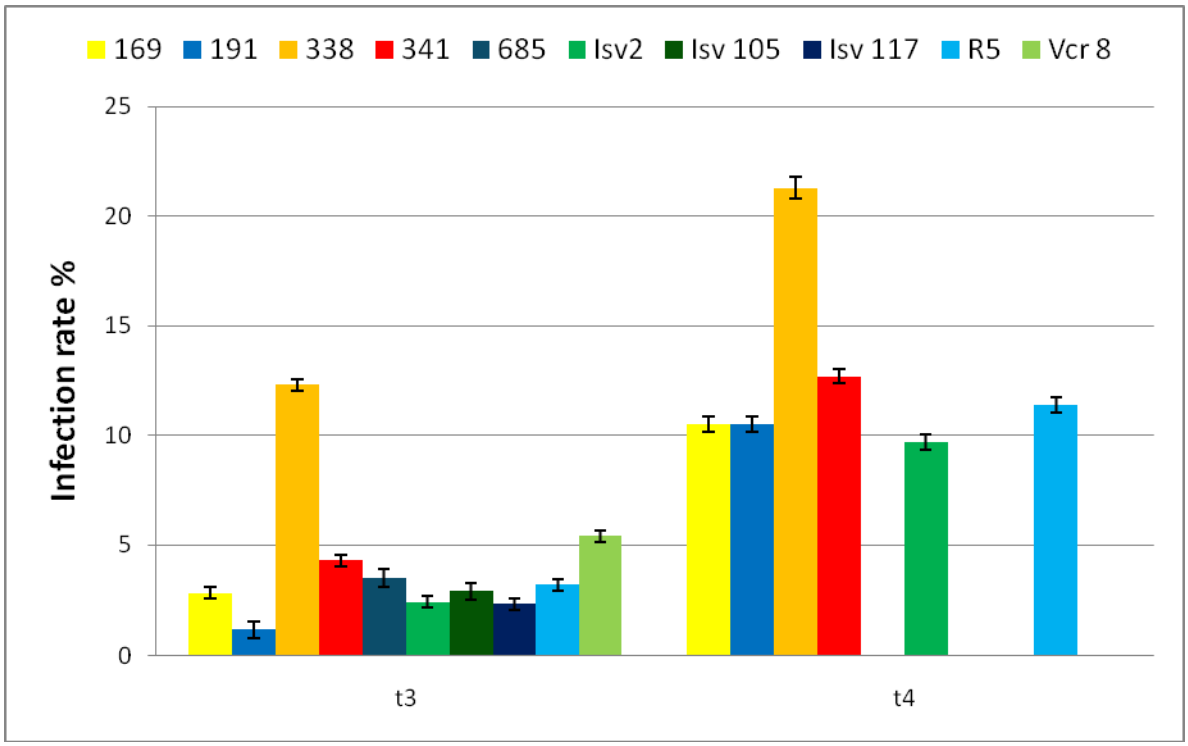
Relation between total average infection (%) and average *trans*-resveratrol (above) and *ε*-viniferin (below) accumulation in the leaf discs of the 10 clones

The relation between infection rate and *trans*-resveratrol and *ε*-viniferin showed that the clone R5 presented higher infection values respect all other clones and average low values of both stilbenic compounds. The remaining clones seem to group all together in a cloud on the upper left side of the graphs, showing that to relative low infection rates correspond to moderate *trans*-resveratrol values and relatively higher values of *ε*-viniferin accumulated.

Cabernet Sauvignon trial – berries

When analysing the berries of 10 Cabernet Sauvignon clones infected with *Plasmopara viticola* several differences can be noted. The clone 338 appears visibly more sensitive than the other clones. In fact, it presents the highest values both in the T3 and in the T4 samplings. The clones 169, 191, 341, Isv2 and R5 present an increase in infection rate, measured as visible sporulation, from T3 to T4.

The fact that some clones have no data in the T4 sampling time might be due to the fact that the berries used in this study are quite small and may in some cases not being able to resist to infection for long, which would explain the extended decomposition.

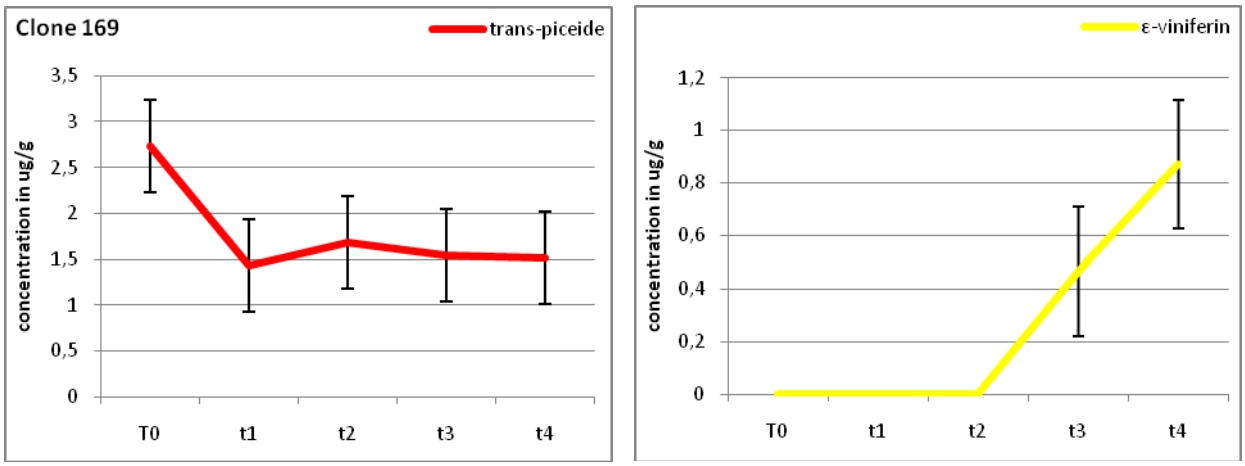


Infection rates in *Plasmopara viticola* infected berries

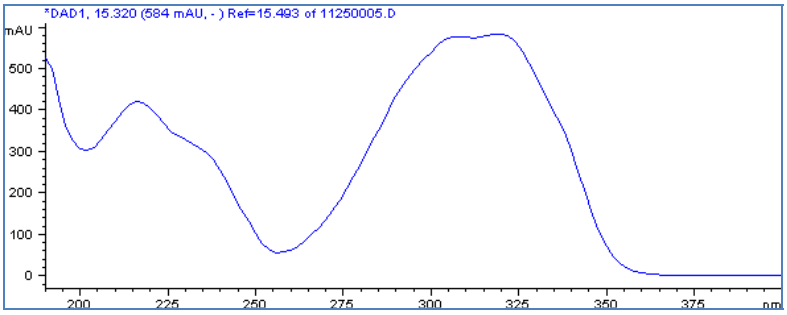
Trans-piceid and *ε*-viniferin were the only 2 stilbenic compounds found in the spectra of the 10 clones. Their accumulation however changed overtime for the different clones:

Clone 169: the higher value of *trans*-piceid was reported at T0 after which all values were lower and not significantly different among each other.

As for ε-viniferin levels, the presence of this compound could only be detected after T2 after which the concentration of ε-viniferin increased reaching values close to 1 μg.g^{-1} fw.



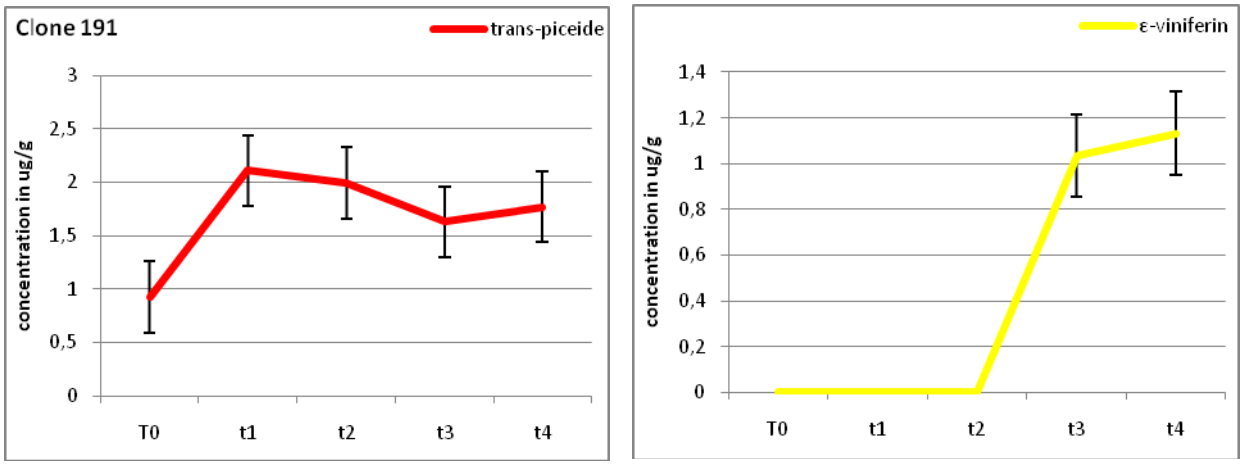
trans-piceid and ε-viniferin in *Plasmopara viticola* infected clone 169 berries. The bars present in the figure represent the value of 2 times the standard error



Absorption spectra of *trans*-piceid

Clone 191: at T0 the concentration of *trans*-piceid presented the lowest value, less than 1 μg.g^{-1} fw, significantly different from all other values. After this point the value increased at T2 and remained relatively stable after that point.

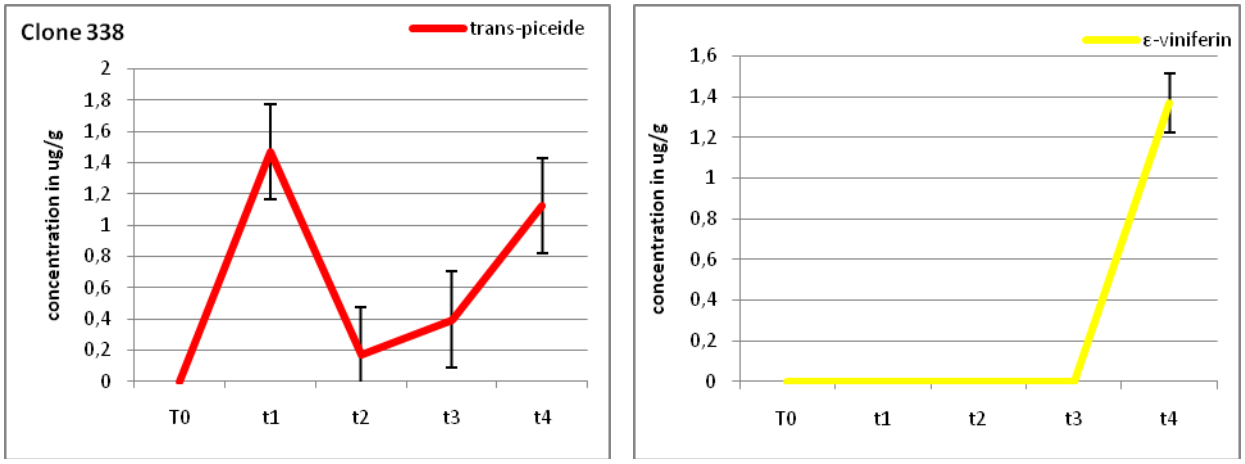
ε-viniferin levels could not be detected until T3 and T4, which presented values significantly higher, above 1 μg.g^{-1} fw.



trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone 191 berries. The bars present in the figure represent the value of 2 times the standard error

Clone 338: No *trans*-piceid could be found in the T0 sampling of this clone. T1 and T4 present 2 peaks, statistically significantly different from all other sampling times. The concentration increased from T0 to T1 and then decreased at 2, increasing again till reaching the second peak at T4.

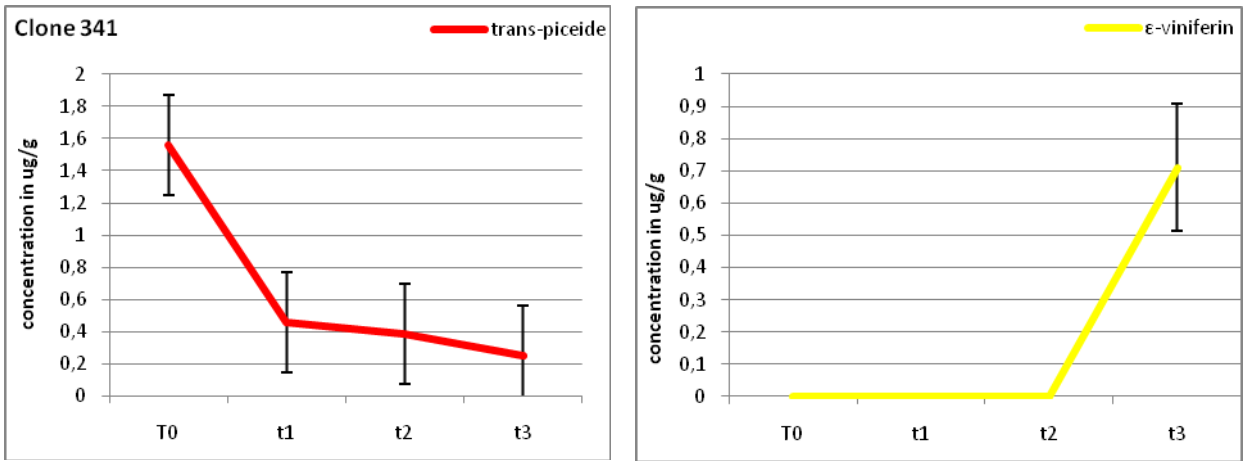
No *ε*-viniferin was detected until T3. At T4 a 1.4 $\mu g.g^{-1}$ fw value could be found for this clone.



trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone 338 berries. The bars present in the figure represent the value of 2 times the standard error

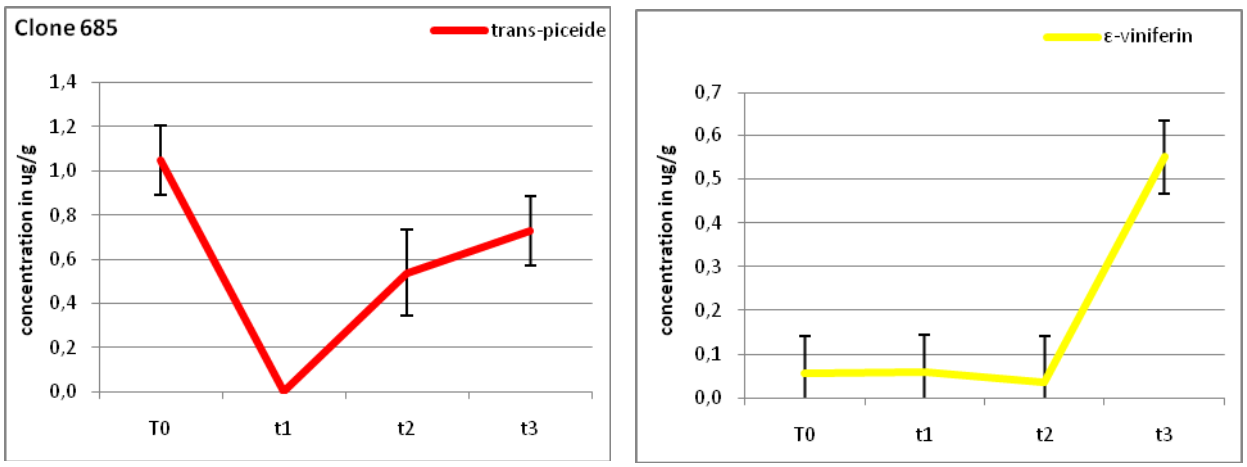
Clone 341: *Trans*-piceid concentrations 1.5 µg.g^{-1} fw, were significantly different at T0 and abruptly decreased at T1, maintaining considerably stable values in successive sampling times.

ε-viniferin was not detected until T2 and reached a maximum value of 0.7 µg.g^{-1} fw at T3.



trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone 341 berries. The bars present in the figure represent the value of 2 times the standard error

Clone 685: T0 values of *trans*-piceid were statistically significantly the highest ones. The following times, although characterised by lower values an increase respect the values found at T1.

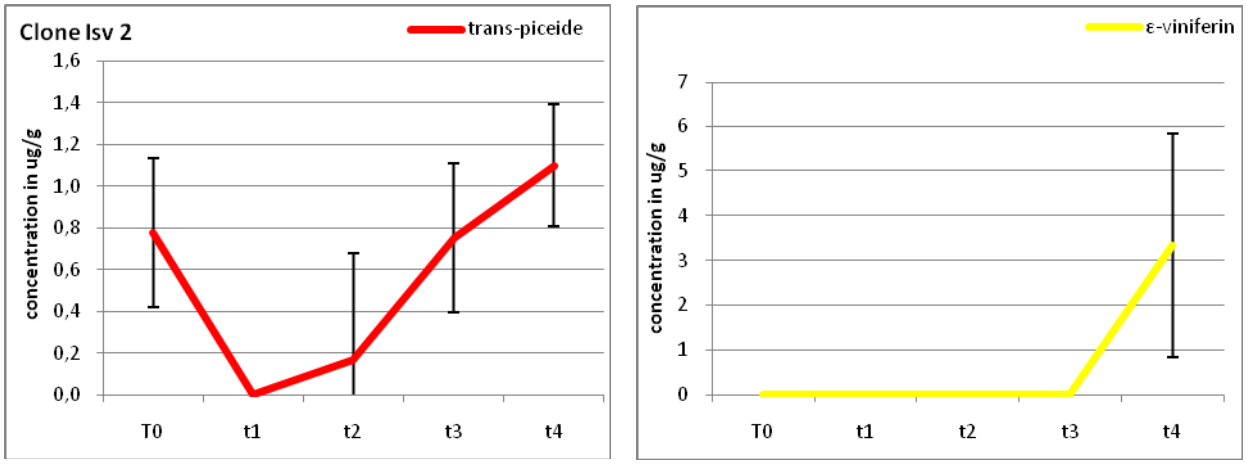


trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone 685 berries. The bars present in the figure represent the value of 2 times the standard error

T0 to T2 values of ε-viniferin were extremely low and at T3 a maximum of 0.5 µg.g^{-1} fw was found

Clone Isv2: *Trans*-piceid lowest values were found at T1 after which a steady increase could be seen. Concentrations ranged between 0 and 1.2 µg.g^{-1} fw

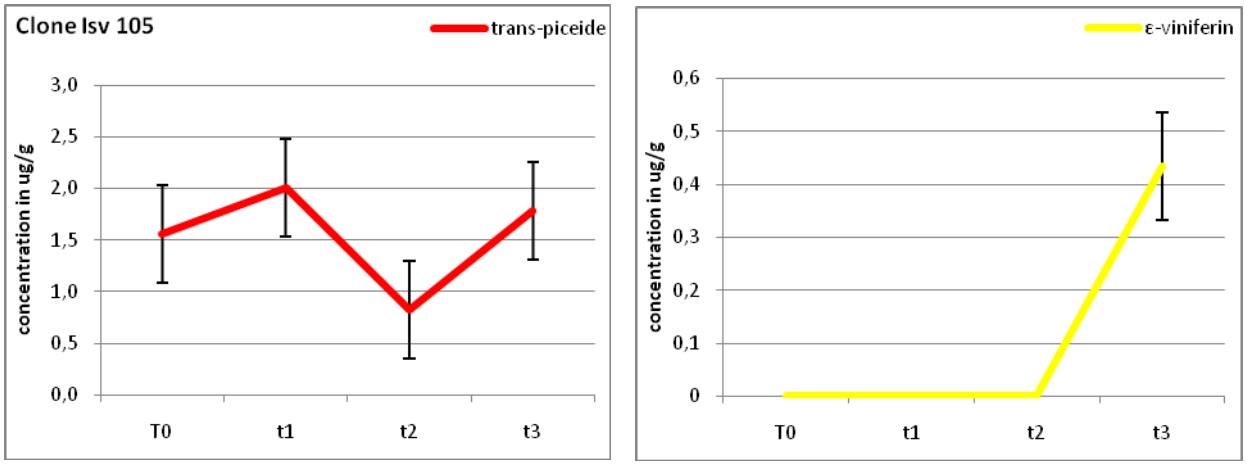
Also for this clone no traces were found until T4, time at which a concentration of over 3 µg.g^{-1} fw could be detected.



trans-piceid and ε-viniferin in *Plasmopara viticola* infected clone Isv 2 berries. The bars present in the figure represent the value of 2 times the standard error

Clone Isv105: two statistically significant different peaks, T1 and T3, were found for *trans*-piceid, respect the lowest values found at T2. All values ranged from 1 to 2 µg.g^{-1} fw.

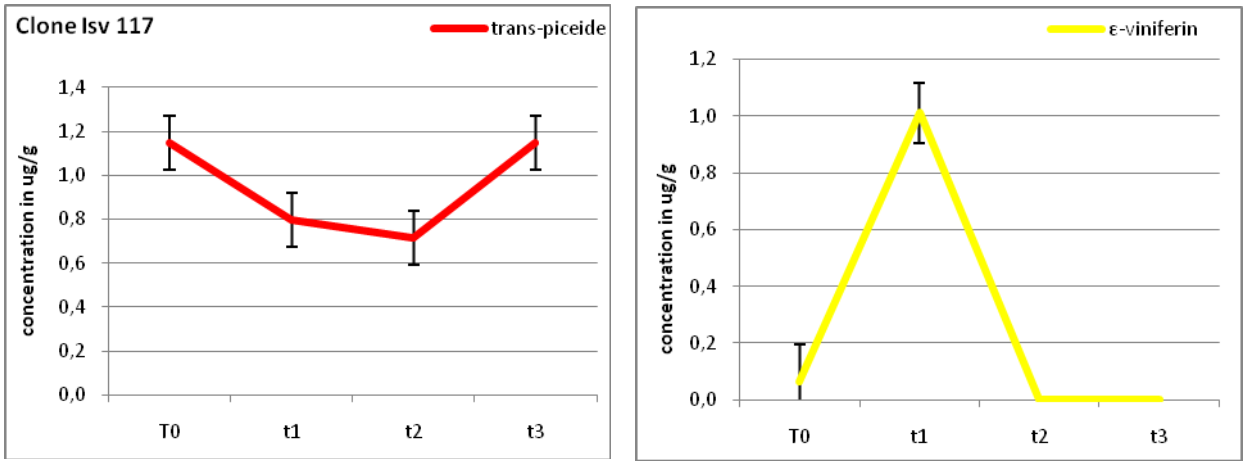
ε-viniferin values were untraceable till T2 and reached 0.4 µg.g^{-1} fw at T3



trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone Isv 105 berries. The bars present in the figure represent the value of 2 times the standard error

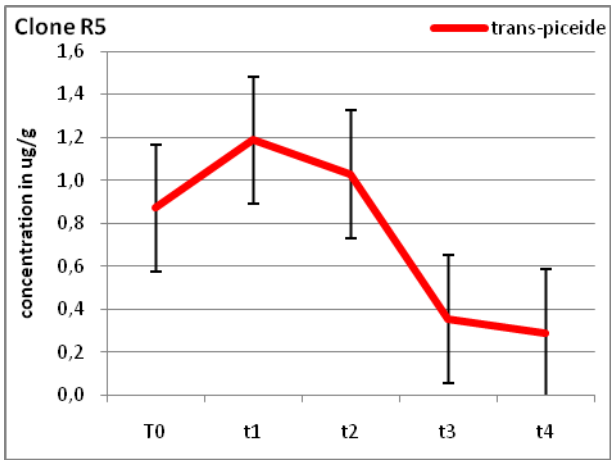
Clone Isv117: T0 and T3 present statistical different levels of *trans*-piceid, peak close to 1.20 µg.g^{-1} fw respect the 2 low points of concentration that rounded 0.80 µg.g^{-1} fw.

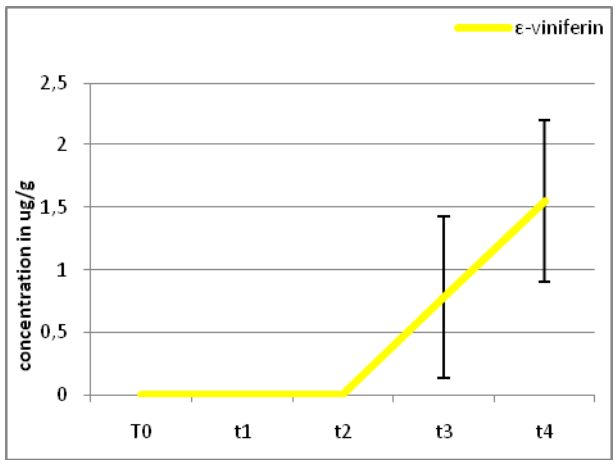
Unlike al other clones, Isa117 presented high values of *ε*-viniferin at T1, the only peak, of 1 µg.g^{-1} fw and was virtually undetectable thereafter.



trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone Isv117 berries. The bars present in the figure represent the value of 2 times the standard error

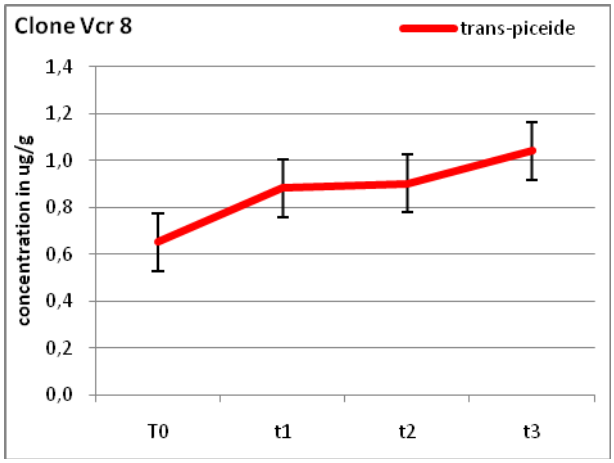
Clone R5: values from T0 to T2, placed between 0.80 and 1.2 µg.g^{-1} fw, were significantly above T3 and T4 values, around 0.20 and 0.40 µg.g^{-1} fw of *trans*-piceid.As for *ε*-viniferin, it was only found at T3 and T4 and it reached 1.5 µg.g^{-1} fw.

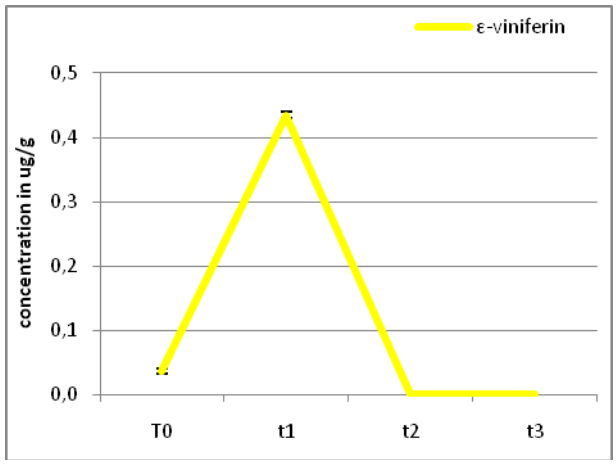




trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone R5 berries. The bars present in the figure represent the value of 2 times the standard error

Clone Vcr8: T1 till T3 values were the highest, and all significantly different from the lowest values found at T0. The concentrations of *trans*-piceid were found to continuously increase with time in this clone.





trans-piceid and *ε*-viniferin in *Plasmopara viticola* infected clone Vcr8 berries. The bars present in the figure represent the value of 2 times the standard error

As seen in another clone, *ε*-viniferin was close to zero at T0, peaked at a value of 0.45 $\mu g.g^{-1}$ fw at T1 and could not be found after that in Vcr8.

Although the presence of *trans*-piceid could not be measured in all clones for a given time, the statistical analysis gives interesting results.

Unlike what could be seen in the analysis previously presented of the leaves of the clones of Cabernet Sauvignon, the results found in the berries, revealed the presence of stilbenic compounds at T0.

	T0	T1	T2	T3	T4
Clone 169	2,732 b	1,435 ab	1,682 d	1,544 b	1,512 b
Clone 191	0,921 a	2,108 b	1,991 d	1,628 b	1,771 b
Clone 338	-	1,467 ab	0,170 a	0,396 a	1,124 ab
Clone 341	1,563 ab	0,460 a	0,387 ab	0,250 a	-
Clone 685	1,049 ab	-	0,538 abc	0,730 ab	-
Clone Isv 2	0,777 a	-	-	0,753 ab	1,099 ab
Clone Isv 105	1,555 ab	2,003 b	0,829 bc	1,782 b	-
Clone Isv 117	1,148 ab	0,796 ab	0,715 abc	1,147 ab	-
Clone R5	0,871 a	1,186 ab	1,028 c	0,355 a	0,289 a
Clone Vcr 8	0,652 a	0,882 ab	0,903 bc	1,041 ab	-
F	2,061	2,561	10,764	3,290	3,817
Sig.	*	*	***	*	*

Comparison of berry *trans*-piceid per clone after infection with *Plasmopara viticola.* Values with the same letter are not significantly different (α<0.05 WD Test) -: no stilbenes were detected in at least 2 of the replicates

Only clone 338 shows no traces of any *trans*-piceid. Clones 191, Isv2, R5 and Vcr8 showed the lowest statistically significant values (a), between 0.6 and 0.9 $\mu g.g^{-1}$ fw while the clone 169 had the highest significant (b) value, close to 2.8 $\mu g.g^{-1}$ fw. All other clones reached values in between these maximum and minimum values.

At T1 *trans*-piceid was undetectable in tow clones, both the clone 685 as well as the Isv2 clone. The clone 341 presented the significantly lowest value (a) of 0.46 $\mu g.g^{-1}$ fw while the clones 191 and Isv105 the significant highest values (b), above 2 $\mu g.g^{-1}$ fw. All other clones presented values in between.

At T2 the clone 338 has the lowest significantly different value. The clones 341 (ab), Isv105 and Vcr8 (bc) and Isv117 (abc) show intermediate values between the clones 338 (a) and R5 (c). The clones 169 and 191 present the highest statistically significant values.

At T3, three clones show significantly lower values (a), 338, 341 and R5, between 0.25 and 0.39 $\mu g.g^{-1}$ fw, while three others show significant higher

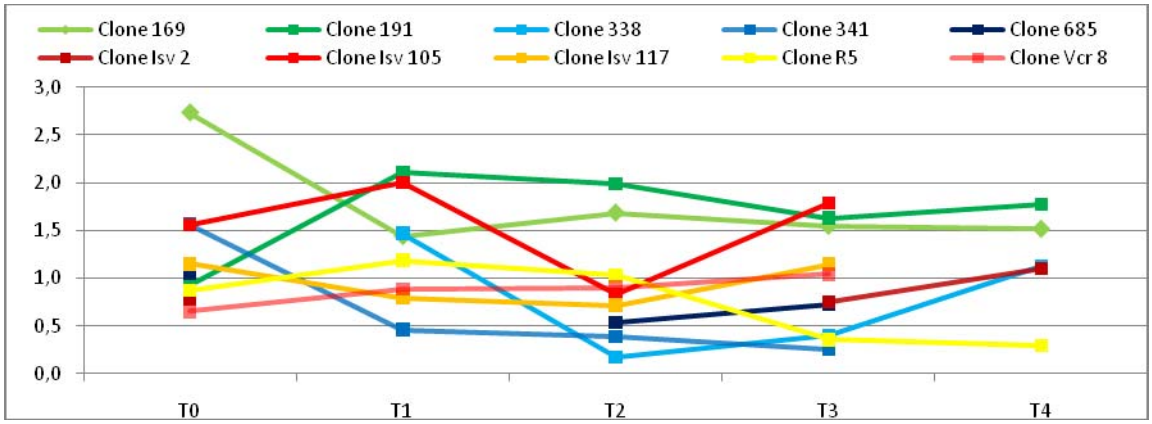
values (b), 169, 191, Isv105, between 1.5 and 1.8 µg.g^{-1} fw. The remaining clones 685, Isv2, Isv117 and Vcr8 present intermediate values.

The clones 169 and 191 also show the significant highest values of *trans*-piceid accumulation at T4, above 1.5 µg.g^{-1} fw. The 338 and Isv2 reach intermediate values between the highest accumulating clones and the lowest accumulating clone, the R5 which accumulates only around 0.2 µg.g^{-1} fw.

Analysing the graphic it is possible to try to separate some visual groups. Clones 169 and 341 ability to accumulate *trans*-piceid overtime seems to decrease from the highest values found at T0. Clone 191 accumulation is enhanced overtime and remains relatively stable, closer the highest value of T1.

Both the Isv105 as the 338 clones seem to have two accumulation peaks, one at T1 and the other at T2 for the Isv105 clones and one at T1 and the other at T3 for the 338 clone, with a severe decrease in between these sampling times.

All other clones seem to maintain relatively stable values of *trans*-piceid overtime.



Comparison of berry *trans*-piceid per clone after infection with *Plasmopara viticola.*

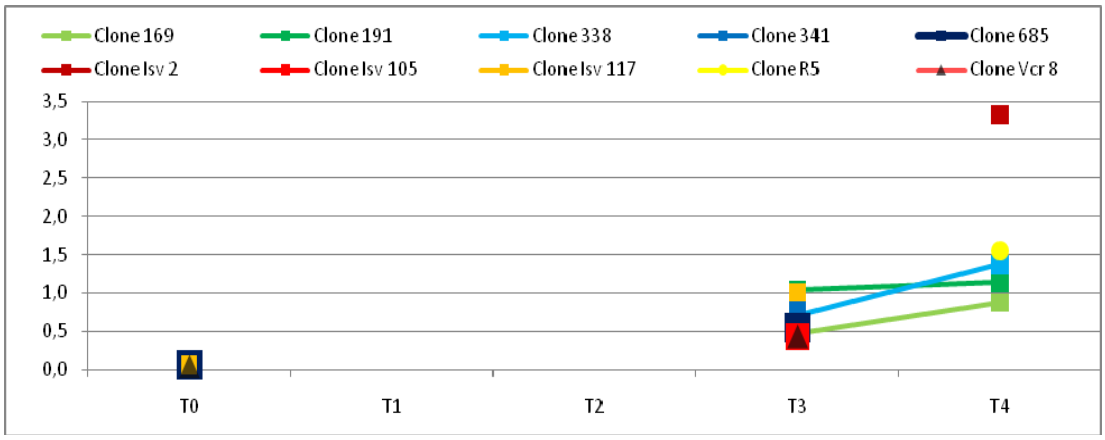
	T0	T1	T2	T3	T4
Clone 169	-	-	-	0,465	0,872
Clone 191	-	-	-	1,036	1,133
Clone 338	-	-	-	0,710	1,369
Clone 341	-	-	-	0,781	-
Clone 685	0,057	-	-	0,551	-
Clone Isv 2	-	-	-	-	3,327
Clone Isv 105	-	-	-	0,435	-
Clone Isv 117	0,064	-	-	1,010	-
Clone R5		-	-	-	1,552
Clone Vcr 8	0,037	-	-	0,435	-
F	1,288			2,220	0,591
Sig.	n.s.			n.s.	n.s.

Comparison of berry *ε-viniferin* per clone after infection with *Plasmopara viticola.* Values with the same letter are not significantly different (α<0.05 WD Test) -: no stilbenes were detected in 2 of the replicates

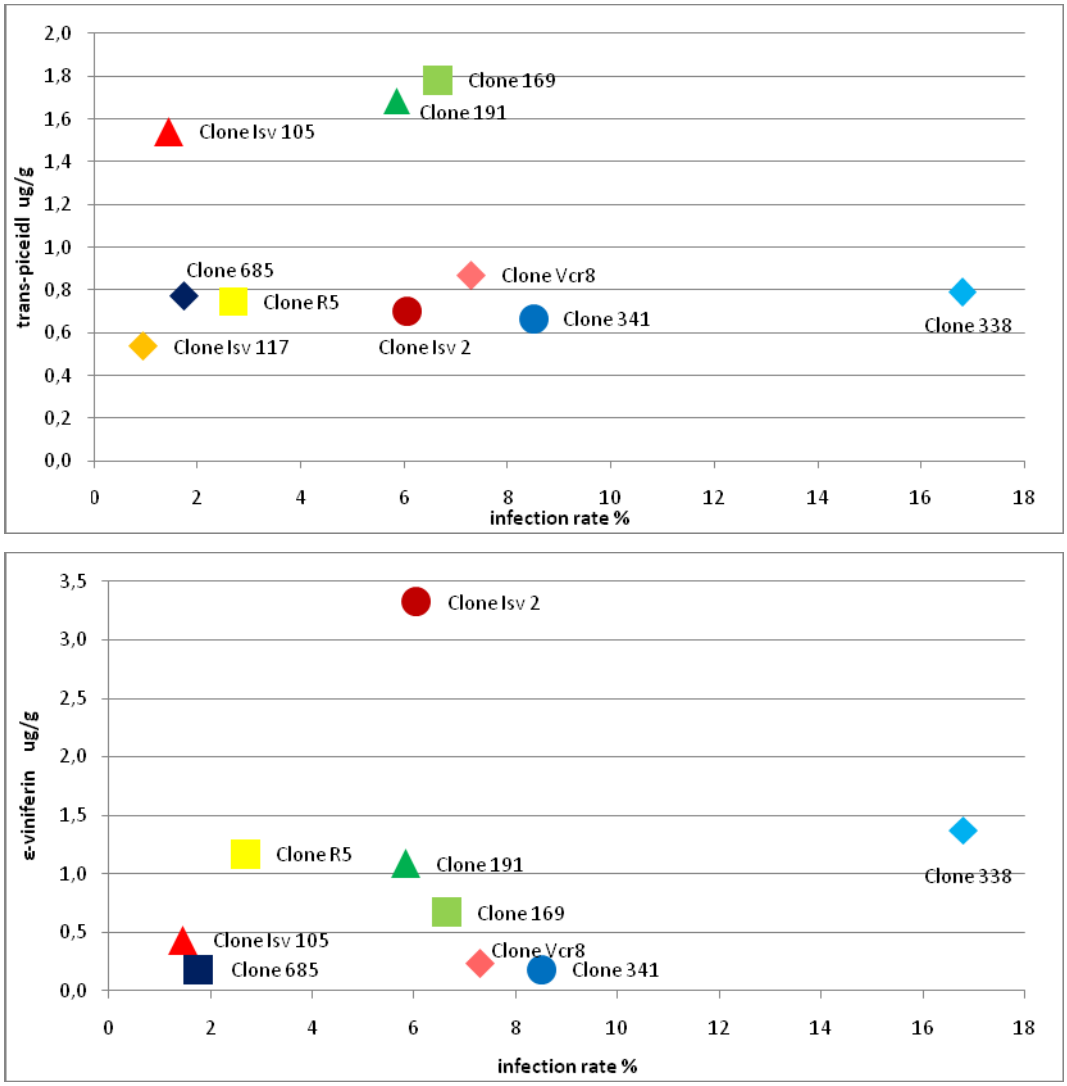
No statistically significant differences could be found at any of the sampling times between the studied clones for ε-viniferin.

It is only possible to say that for most clones values can be traced only in 2 sampling times.

It can be stated that small berries as the ones used in this study may not be able to induce the production ε-viniferin before a certain time as passed after the inoculation. They seem to have a delayed reaction time, especially when compared to the results obtained in leaves.



Comparison of berry *ε*-viniferin per clone after infection with *Plasmopara viticola*



Relation between total average infection (%) and average *trans*-piceid (above) and *ε*-viniferin (below) accumulation in the berries of the 10 clones

From the relation of total infection and stilbenes accumulation, it was possible to see that berries of the clone 338 are more susceptible to the pathogen presenting higher infection rates and only moderate concentrations of *trans*-piceid or *ε*-viniferin. While in the *trans*-piceid no other main differences could be seen between the remaining clones, in the *ε*-viniferin-infection relation, the clone Isv2 also standed out. It presented an average infection but considerably higher amounts of *ε*-viniferin when compared with the other clones.

Multiple varieties trial

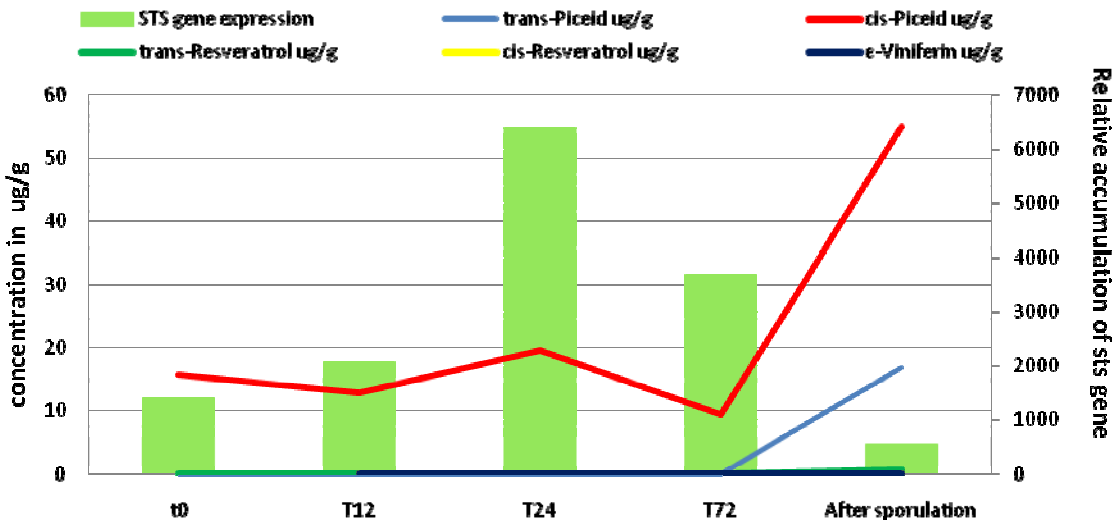
Six varieties were used to observe the stilbene synthase gene accumulation as well as stilbenic compounds production respectively in young and old leaves.

Chardonnay

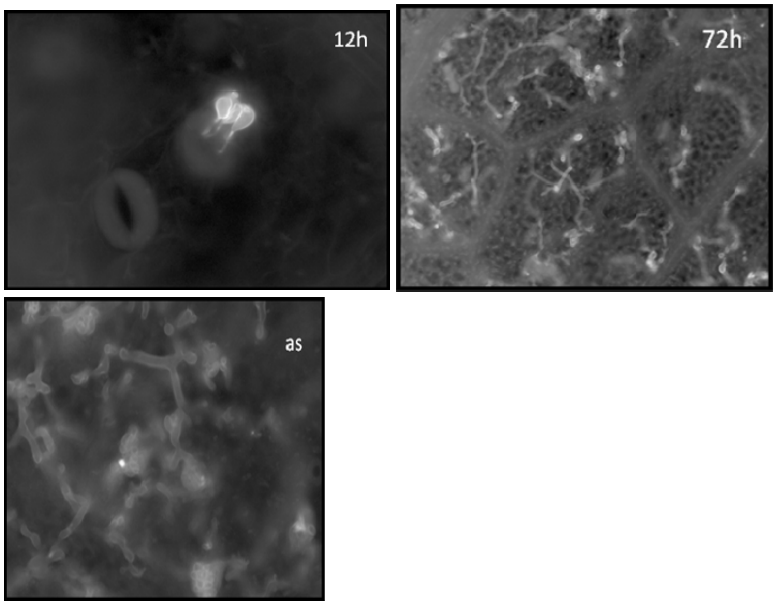
The 1st variety, Chardonnay presented some differences between young and old leaves.

In young leaves, STS induction could already be accounted for at T0 and after a maximum of induction found at 24hpi, the induction values started to decrease.

Meanwhile *cis*-piceid showed a moderate increase until T24, decreasing at T72, with values between 10 and 20 µg.g^{-1} fw. In the after sporulation sampling time, *cis*-piceid reached values of 55 µg.g^{-1} fw. At this point, also *trans*-piceid could be detected and reached values of 18 µg.g^{-1} fw.



Stilbenic production and STS induction in young leaves of Chardonnay infected with *Plasmopara viticola*



Young leaves of Chardonnay infected with *Plasmopara viticola*

There is a clear progression in the physiological development of the *oomycete* that is highlighted by the microscope slides of the several varieties.

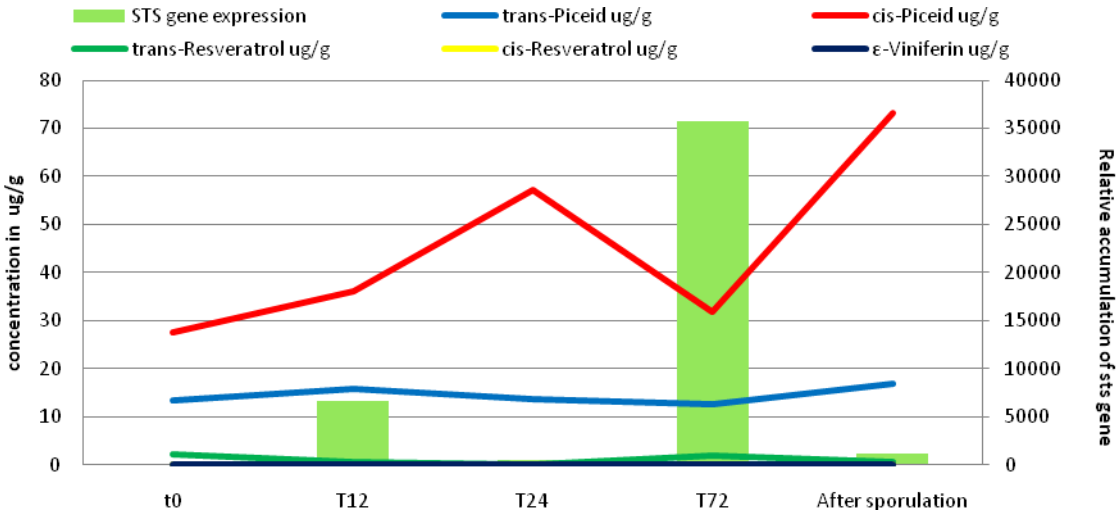
In the slides of young leaves of Chardonnay, at 12hpi, it was possible to observe fully ongoing development.

At 72hpi, in young leaves the development is high, in fact, when comparing with that of Cabernet Sauvignon young leaves it is possible to say that in this case the sporulation is completely widespread and considerably more advanced in its development.

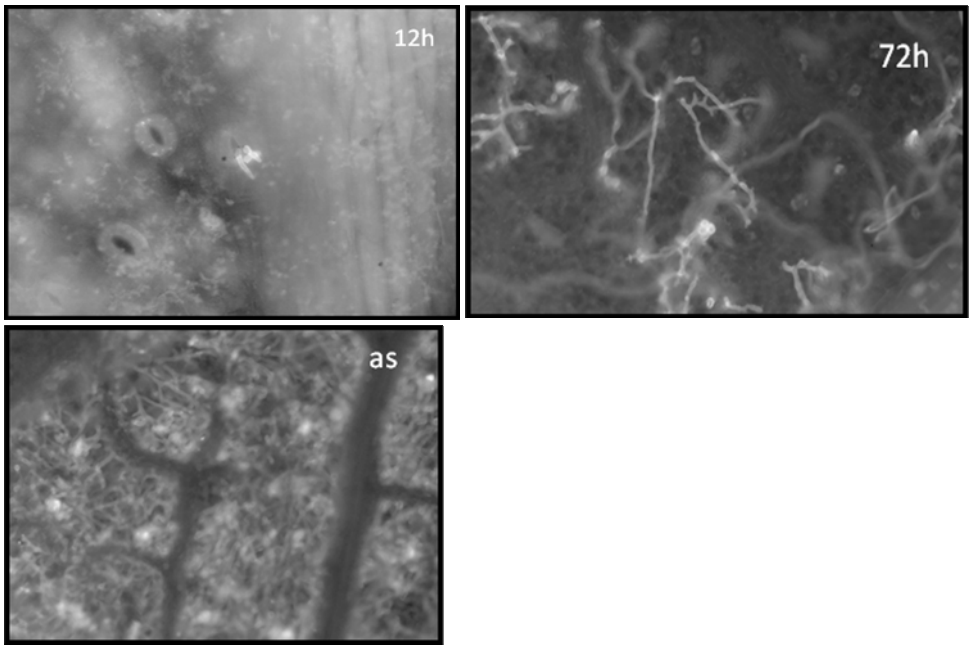
In fact, sporangiophore expansion is more vast, more dense and with several branches visible. Despite this more advanced development at 72hpi, in the after sporulation sampling, the effect is the same in both varieties; the sporulation covers the surface of the leaves.

A different induction pattern was found in old leaves where some induction was found at T12 and a maximum level appeared at T72. At T24 and In the after sporulation sampling times almost no induction could be accounted for.

As for stilbenic accumulation, *cis*-piceid showed a similar behaviour as in young leaves while this time *trans*-piceid presented a constant level throughout all sampling time and *trans*-resveratrol was detected at T72.



Stilbenic production and STS induction in old leaves of Chardonnay infected with *Plasmopara viticola*



Old leaves of Chardonnay infected with *Plasmopara viticola*

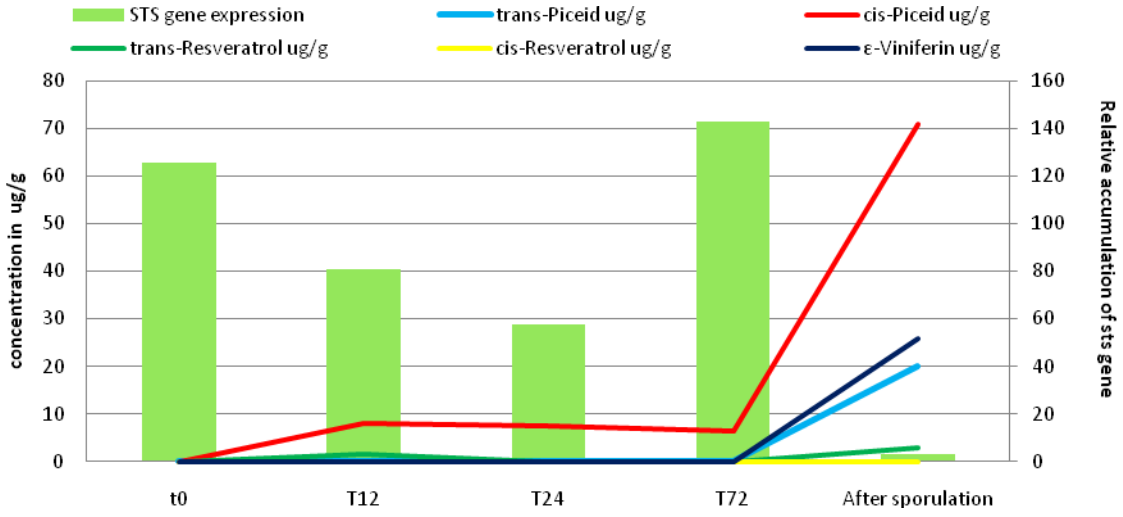
In old Chardonnay leaves, it is also possible to confirm that one stoma can house multiple infection points. A slower development could be noted upon comparison against Chardonnay young leaves. At 72h the sporulation was as developed as in young leaves but less dense. While in the after sporulation sampling, the leaf inner spaces were completely covered, although, a clear division caused by the main veins was visible; the sporulation is clearly contained by them.

Cabernet Sauvignon

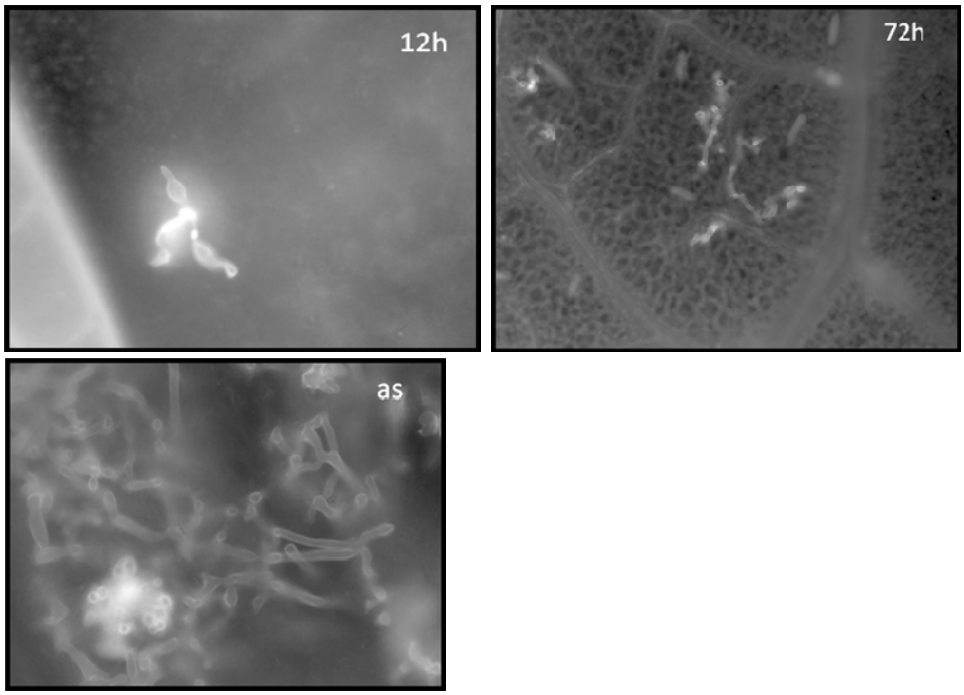
In young leaves of Cabernet Sauvignon STS induction was found as soon T0 and a maximum of induction was found at T72 after which induction became very low.

The only compound present at all times was *cis*-piceid, even if at levels below 10 $\mu g.g^{-1}$ fw.

In the after sporulation sampling time, corresponding to the sampling time after the highest induction, almost all compounds analysed could be found, *cis*-piceid reached 70 $\mu g.g^{-1}$ fw, *ε*-viniferin and *trans*-piceid close to 20 $\mu g.g^{-1}$ fw, and concentrations below 5 $\mu g.g^{-1}$ fw of *trans*-resveratrol and *cis*-piceid were detected.



Stilbenic production and STS induction in young leaves of Cabernet Sauvignon infected with *Plasmopara viticola*

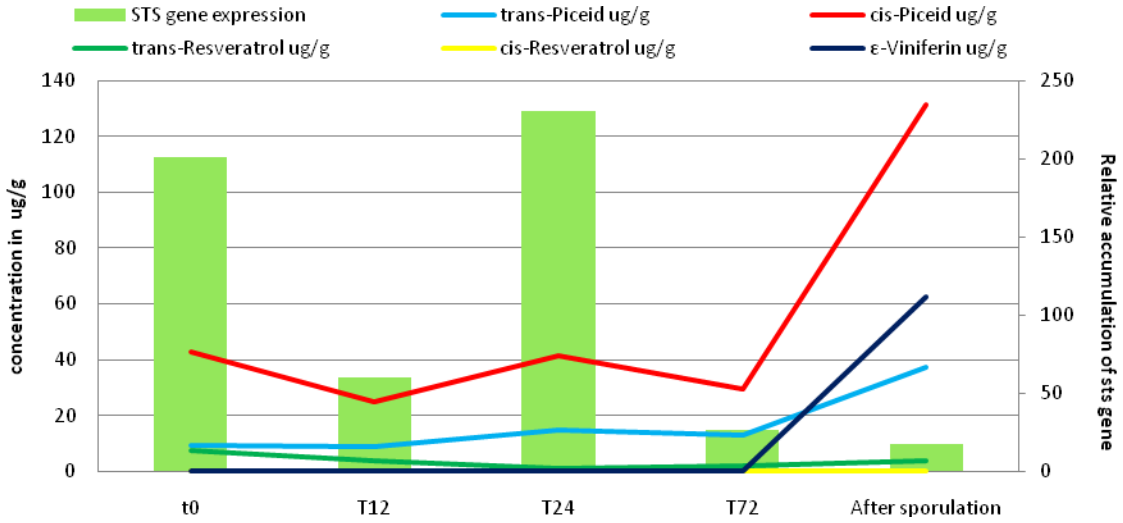


Young leaves of Cabernet Sauvignon infected with *Plasmopara viticola*

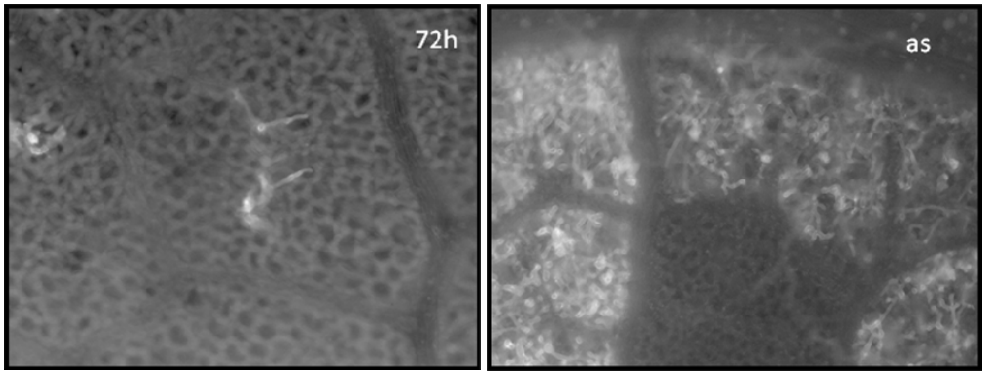
The microscope images concerning Cabernet Sauvignon are quite revealing. At the 12hpi it was possible to see that from one stoma more than one infection can start.

At 72hpi several inoculation points were visible and the sporangiophores had grown considerably. At the after sporulation sampling time, full covering of the leaf surface with *Plasmopara viticola* sporulation was visible.

Older leaves of Cabernet Sauvignon presented a very different behaviour. Induction was highest are T24 and quite high at T0. It was present but always lower in all other times. Values of *cis*-piceid and *trans*-piceid could be found at all times being those of *cis*-piceid higher. In the after sporulation sampling *cis*-piceid reached 130 µg.g^{-1} fw, *ε*-viniferin above 60 µg.g^{-1} fw and *cis*-resveratrol below 10 µg.g^{-1} fw.



Stilbenic production and STS induction in old leaves of Cabernet Sauvignon infected with *Plasmopara viticola*

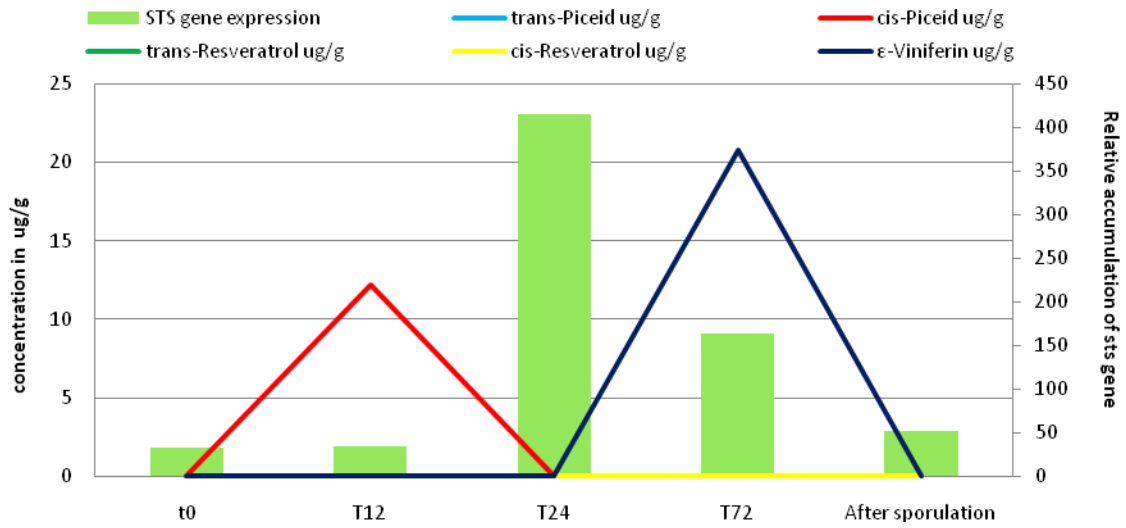


Old leaves of Cabernet Sauvignon infected with *Plasmopara viticola*

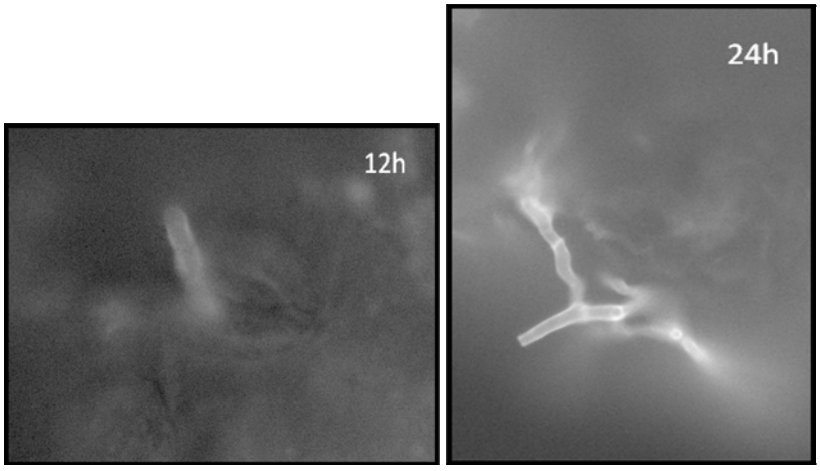
In old leaves of Cabernet Sauvignon, at 72hpi, it was also possible to see some infection focus but less in number respect those found in young leaves at the same time. They were also less developed, that is, with presence of less developed sporangiophores. At the after sporulation sampling time, the sporulation was highly developed and organized. It was easy to observe the enclosement of the sporangiophores within the main veins of the leaf.

Müller-Thurgau

In young leaves of Müller-Thurgau some induction could be seen at T0 and 12hpi, STS induction peaked at T24, decreasing afterwards. In this variety *cis*-piceid could be found already at T12 but it disappeared in the following times. At T72 a peak of *ε*-viniferin, 20 μg.g^{-1} fw, was found despite this compound was untraceable before and after this point.



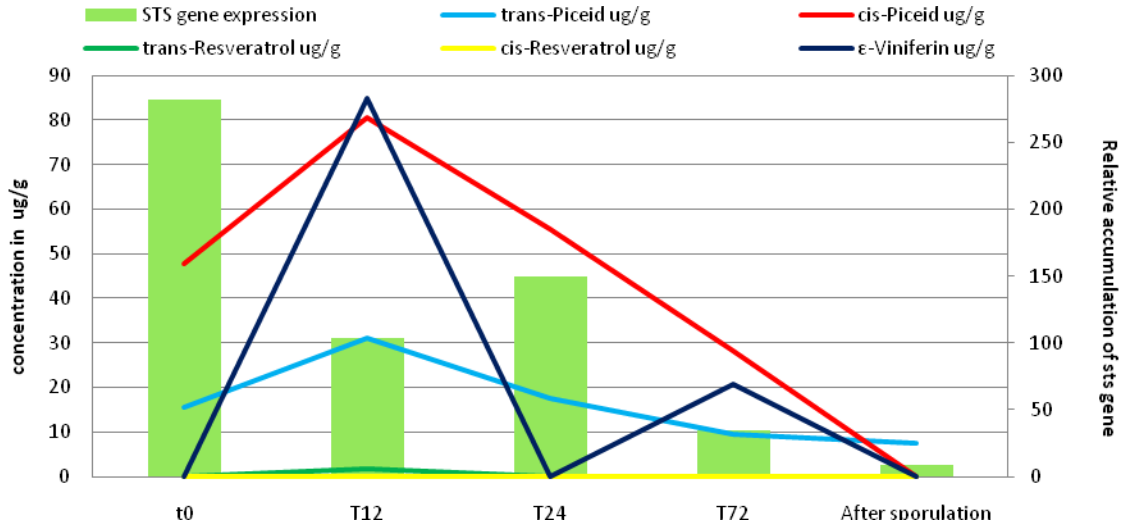
Stilbenic production and STS induction in young leaves of Müller-Thurgau infected with *Plasmopara viticola*



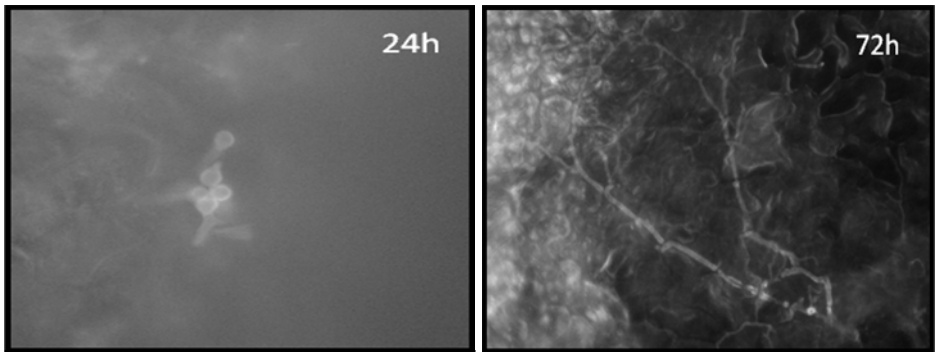
Young leaves of Müller-Thurgau infected with *Plasmopara viticola*

At 12hpi it was possible to detect several points of emergence from the stoma and at 24hpi, in young leaves, developed was visible.

In old leaves of Müller-Thurgau the induction patterns appears quite diverse from that of young leaves. T0 presented a high STS induction, which decreased until reaching a high values at T24 decreasing to lower values in the following times. Both *ε*-viniferin and *cis*-piceid presented high values at T12 above 80 µg.g^{-1} fw. After this point, *cis*-piceid values decreased abruptly reaching 0 after sporulation. *ε*-viniferin was not detectable at T24, peaked at T72, 20 µg.g^{-1} fw and vanished yet again. *Trans*-piceid was also present at T0 and T12 and steadily decreased until the after sprorulation sampling ranging between 30 and 8 µg.g^{-1} fw.



Stilbenic production and STS induction in old leaves of Müller-Thurgau infected with *Plasmopara viticola*



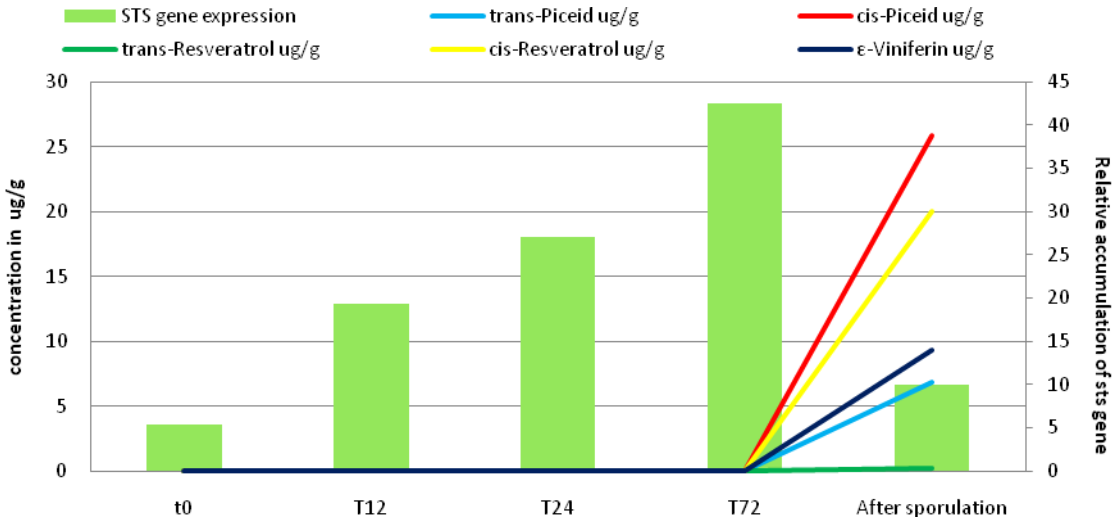
Old leaves of Müller-Thurgau infected with *Plasmopara viticola*

Older leaves seem, also in this variety, to lead to a slower development so that only primary sporangiophores could be seen while some branches were

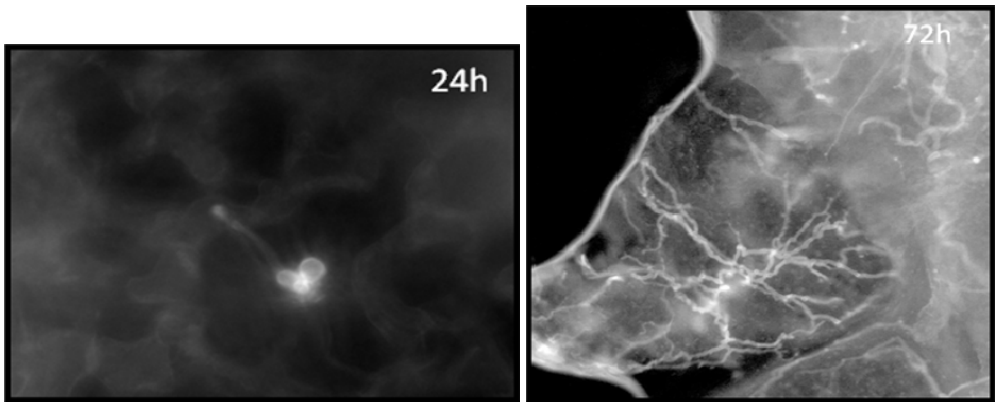
forming. At 72hpi, the sporangiophores had developed further. On the after sporulation sampling time (not seen), the sporulation had quickly expanded.

Pinot noir

Young leaves of Pinot noir inoculated with *Plasmopara viticola* showed an increase induction of the STS gene that only decreased In the after sporulation sampling time was visible in the leaves. No stilbenes were found before sporulation at which time *cis*-piceid (25 µg.g^{-1} fw), *cis*-resveratrol (20 µg.g^{-1} fw) and *ε*-viniferin and *trans*-resveratrol (between 5 and 10 µg.g^{-1} fw) were measurable.



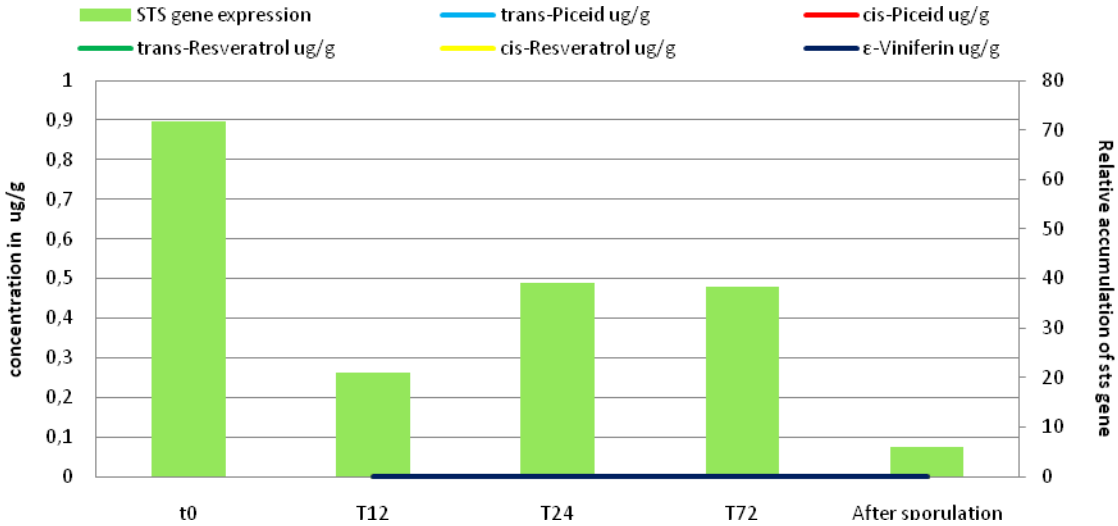
Stilbenic production and STS induction in young leaves of Pinot noir infected with *Plasmopara viticola*



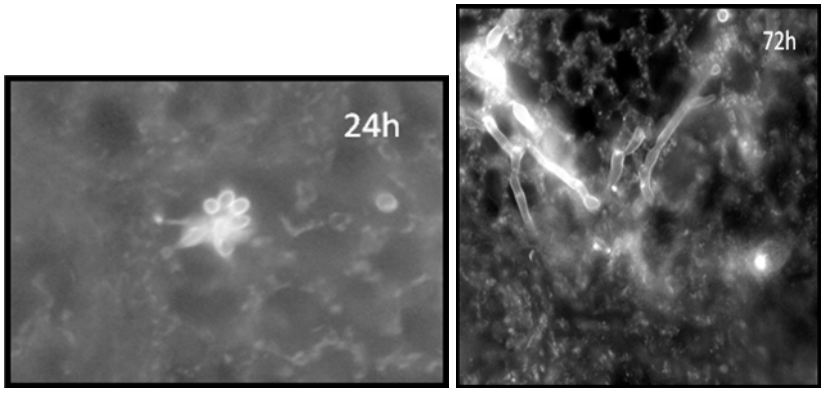
Young leaves of Pinot noir infected with *Plasmopara viticola*

Analysing the images of the Pinot noir young leaves, it appears that this variety leads to slower development of the *oomycete*. At 24 hpi, the primary steps of infection are still occurring. At 72hpi the sporulation seems far more developed but lot less dense then in the young leaves of the previously seen varieties.

In old leaves of Pinot noir and despite STS induction was high at T0 and relatively high at T24 and T72, no stilbenic compounds were found in any of the measured times.



Stilbenic production and STS induction in old leaves of Pinot noir infected with *Plasmopara viticola*



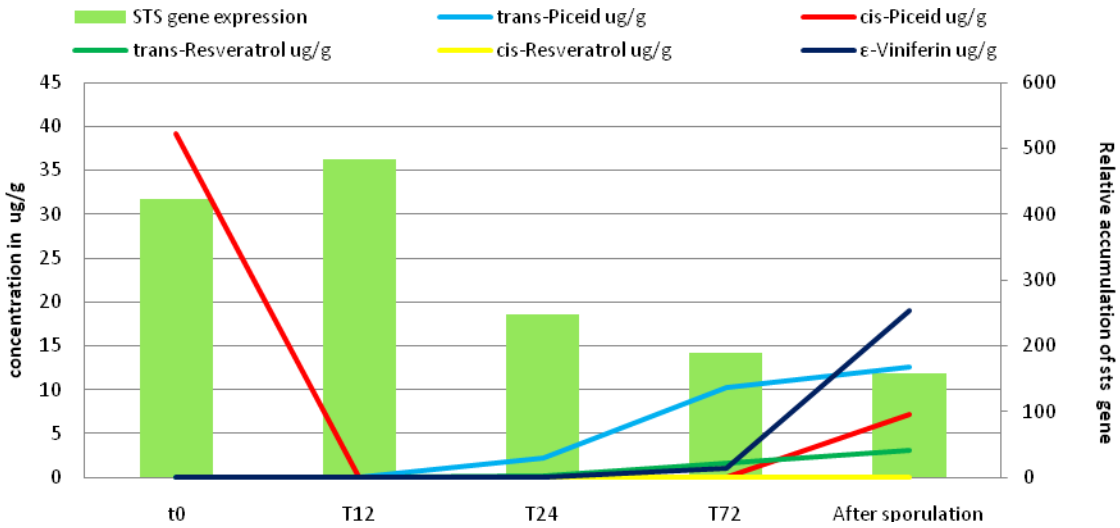
Old leaves of Pinot noir infected with *Plasmopara viticola*

Also in old leaves the slower development can be noticed since at a 24hpi the first protusions are still appearing.

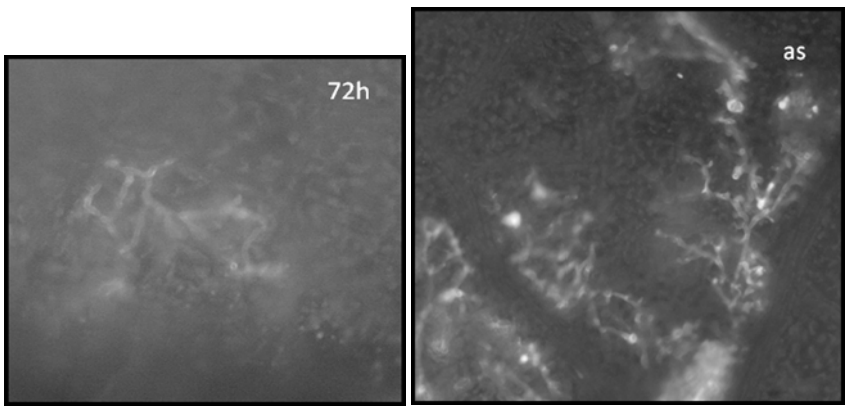
At 72hpi some more developed sporangiophores could be seen but still not so dense and little branching structures can already be seen

Solaris

STS inducation in young leaves of Solaris decreased from T12 until sporulation was visible. Nonetheless, at T0 *cis*-piceid and *trans*-piceid at T24 could already be measured and it increasingly accumulated in all measured times reaching 12.5 $\mu g.g^{-1}$ fw in the after sporulation time. *ε*-viniferin reached 19 $\mu g.g^{-1}$ fw, *cis*-piceid 7 $\mu g.g^{-1}$ fw and *trans*-resveratrol 3 $\mu g.g^{-1}$ fw.



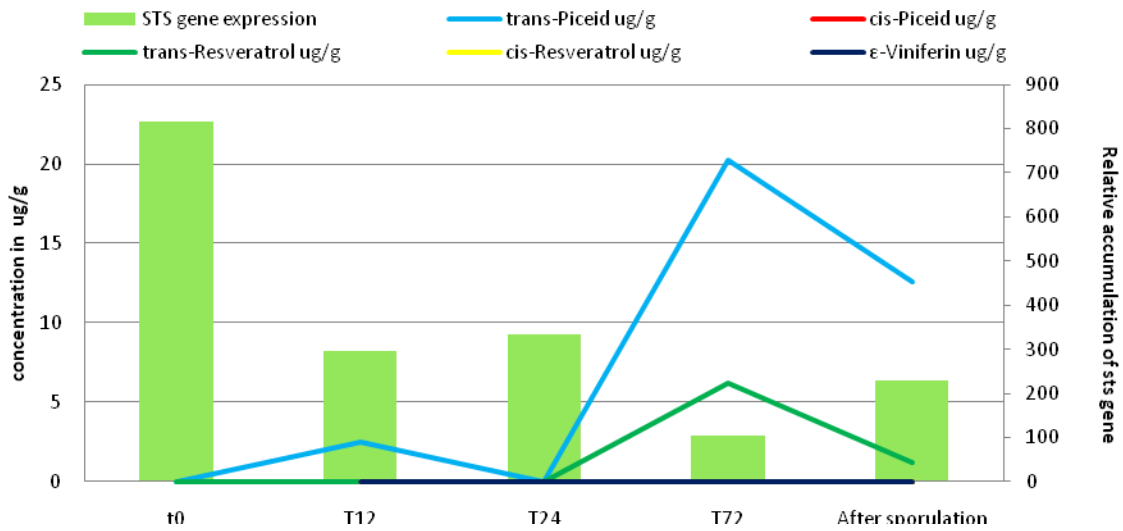
Stilbenic production and STS induction in young leaves of Solaris infected with *Plasmopara viticola*



Young leaves of Solaris infected with *Plasmopara viticola*

In young leaves of Solaris the development was highly contained, this time not in zones but in level of physiological expression. After 72hpi, only rudimental stages of development could be seen. Not many infection points and those developing were slow, appearing somehow atrophied respect to those found in other varieties. In fact, in the after sporulation sampling, a higher quantity of sporulation could be seen, but unlike on the other varieties, in this variety the development seems not very diffuse and with little sporangiophore expansion.

In old leaves STS induction was highest at T0, increased from T12 to T24, decreased at T72 and increased again after sporulation. *Trans*-resveratrol and *trans*-piceid peaked at T72 (6 µg.g^{-1} fw and 20 µg.g^{-1} fw respectively), even if another maxima of *trans*-piceid could also be seen at T12

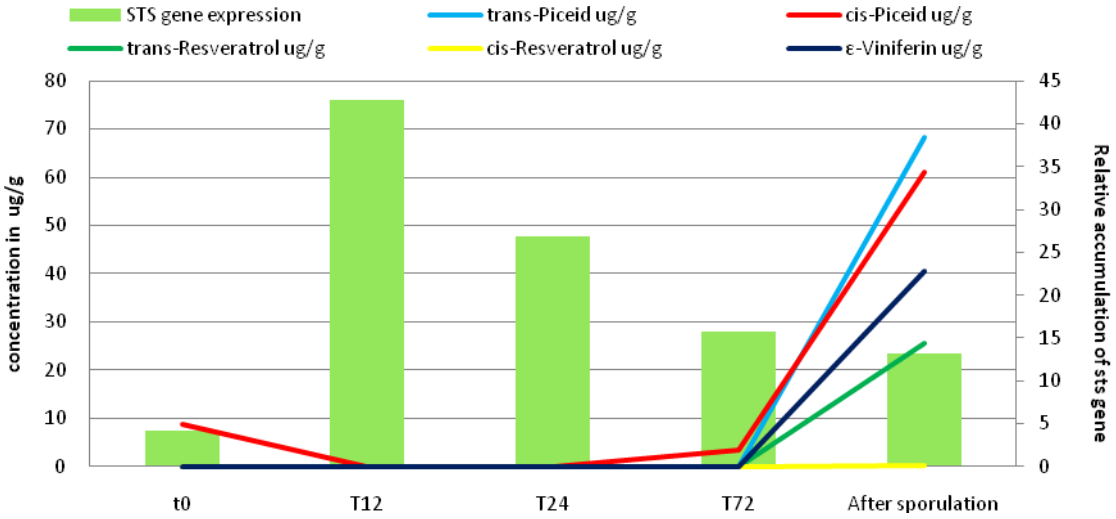


Stilbenic production and STS induction in old leaves of Solaris infected with *Plasmopara viticola*

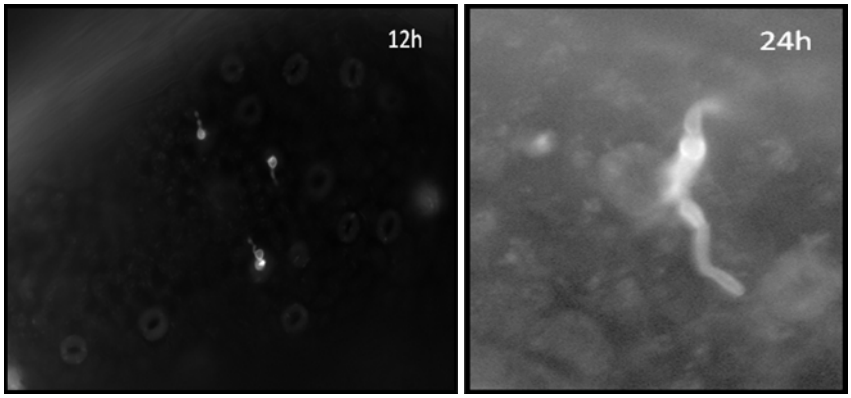
Sangiovese

STS induction in young leaves of Sangiovese decreased from a maximum at T12 until the after sporulation sampling. Apart from some *cis*-piceid at T0, no stilbenes could be found until after sporulation *trans*-piceid, almost 70 µg.g^{-1}

fw, *cis*-piceid 60 µg.g^{-1} fw, *ε*-viniferin 40 µg.g^{-1} fw and *trans*-resveratrol 25 µg.g^{-1} fw, could be found.



Stilbenic production and STS induction in young leaves of Sangiovese infected with *Plasmopara viticola*



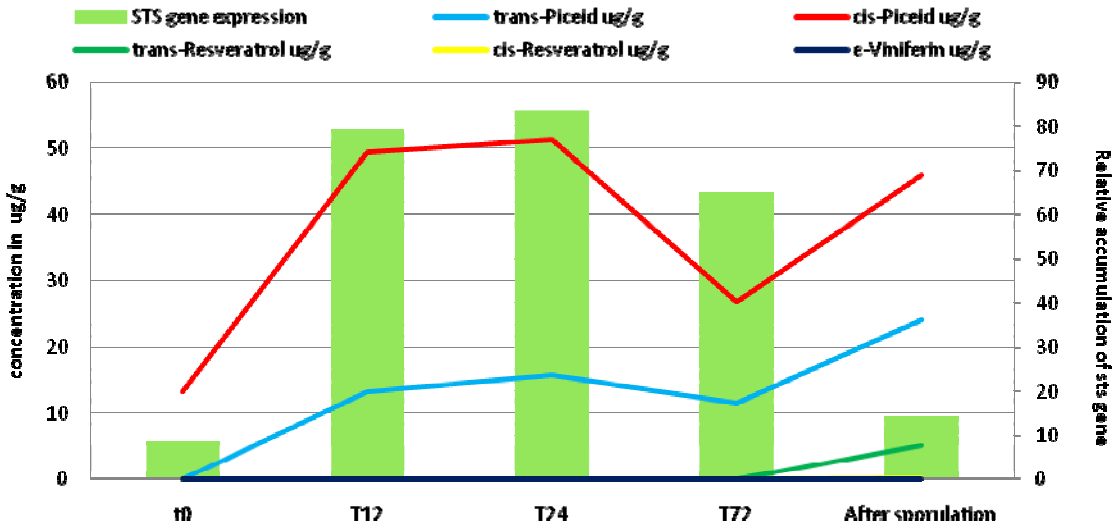
Young leaves of Sangiovese infected with *Plasmopara viticola*

In young leaves of Sangiovese showed several primary emergence points but tendentialy with a single or double emerging points per stoma. It seems that development between 12 and 24hpi it is relatively slow. After this point development becomes faster and the inner spaces of the leaf tissues eventually become completely covered.

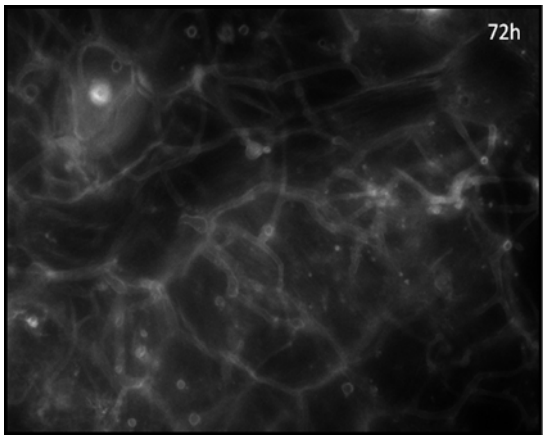
Unlike in younger leaves, in older leaves STS induction increased from T0 and kept stable from T12 to T24. After this point it started decreasing. High

values of *cis*-piceid, 50 µg.g^{-1} fw, could be found at T12 and T24, decreasing at T72, 28 µg.g^{-1} fw, and increasing again at the after sporulation time: 45 µg.g^{-1} fw.

Trans-piceid was always present between 12 and 15 µg.g^{-1} fw, except after sporulation when it reached 25 µg.g^{-1} fw. *Trans*-resveratrol reached 5 µg.g^{-1} fw and could only be found In the after sporulation sampling time.



Stilbenic production and STS induction in old leaves of Sangiovese infected with *Plasmopara viticola*



Old leaves of Sangiovese infected with *Plasmopara viticola*

In old leaves a fully developed sporulation encosed by the veins can be seen, comparable to that seen in some of the other varieties, at the after sporulation sampling.

	ch		cs		mt		pn		sl		sg	
	yg	old	yg	old	yg	old	yg	old	yg	old	yg	old
Primary protrusions	++	+	++	+	++	+	+	+	+	+	+	+
sporangiophores	++	+	++	+	++	++	+	+	+	+	+	+
Branced sporangiophores	+	+	+	+	+	+	+	++	++	++	++	++
Infection expasion	++	++	+	+	+	+	++	++	++	+	++	+
Infection density	++	+	+	+	+	+	+	+	+	+	+	+
Total stilbenes accumulated (µg.g^{-1})	57,7	0,0	23,1	198,6	33,0	393,2	0,0	0,0	54,3	29,0	12,2	181,2

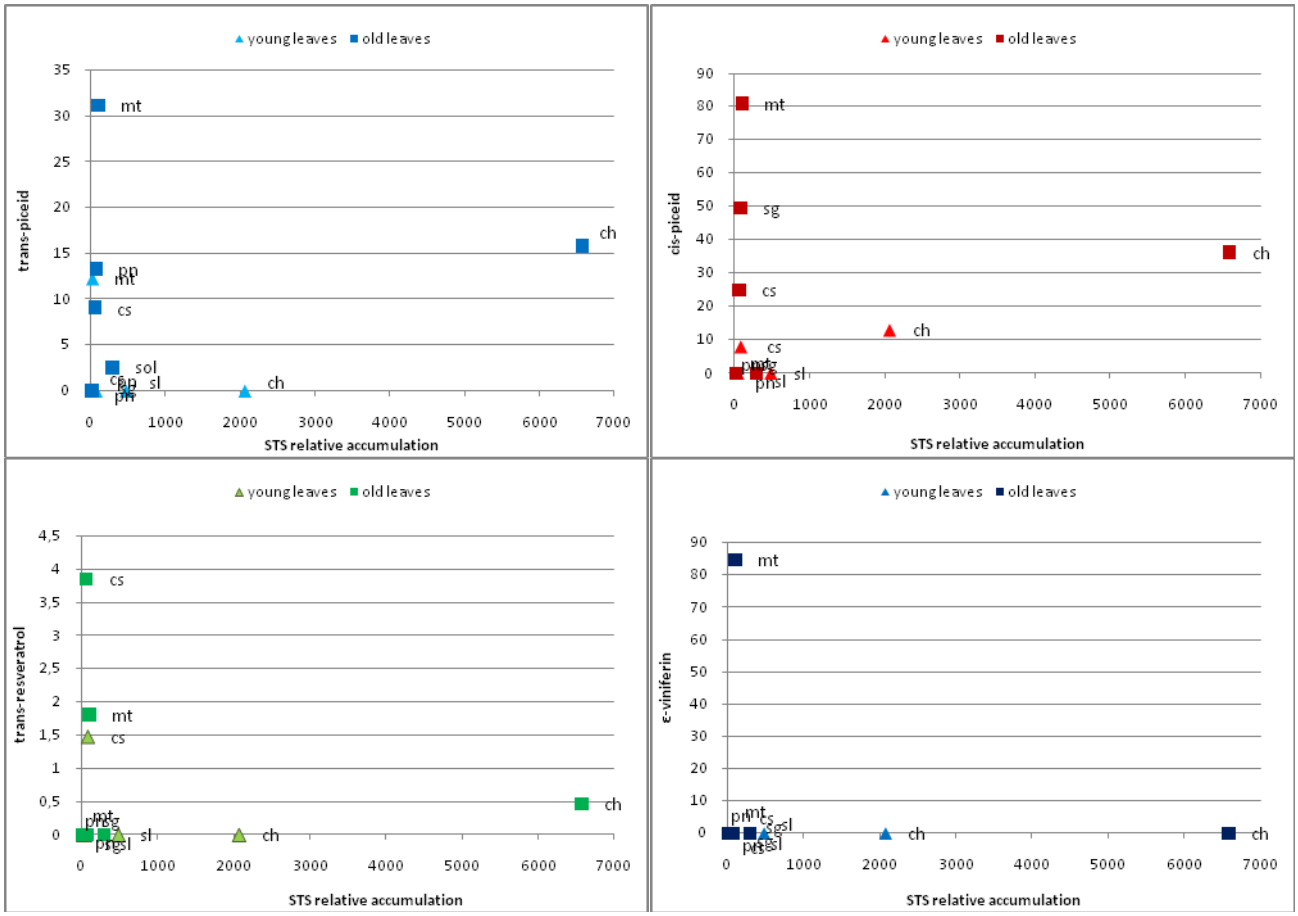
Infection development during the first 72hpi and corresponding total stilbenes accumulated. ch –Chardonnay; cs – Cabernet Sauvignon; mt – Müller-Thurgau; pn – Pinor noir; sl – Solaris; sg – Sangiovese; yg – young leaves; old – old leaves

	ch		cs		mt		pn		sl		sg	
	yg	old	yg	old	yg	old	yg	old	yg	old	yg	old
sporulation expansion	+++	+++	+++	+++	++	++	++	++	+	+	++	++
sporulation density	+++	++	++	++	++	++	+	+	+	+	++	+
Total stilbenes accumulated (µg.g^{-1})	72,6	119,3	119,3	235,1	0,0	7,6	62,3	0,0	42,0	13,8	196,0	75,3

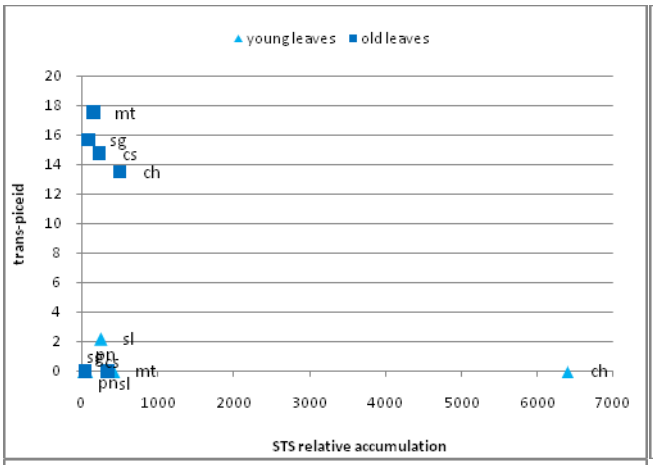
Infection development after visible sporulation and corresponding total stilbenes accumulated. ch –Chardonnay; cs – Cabernet Sauvignon; mt – Müller-Thurgau; pn – Pinor noir; sl – Solaris; sg – Sangiovese; yg – young leaves; old – old leaves

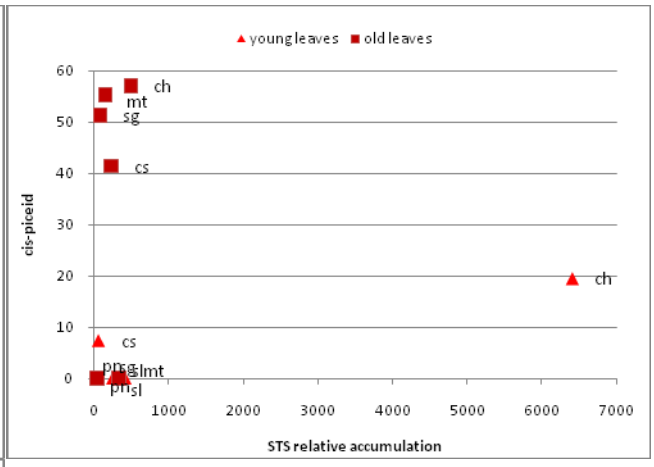
A closer look at infection development and corresponding total stilbenes accumulated, both from the first 72hpi and In the after sporulation sampling time, highlighted that there are considerable differences in both parameters between young and old leaves, even between the same varieties.

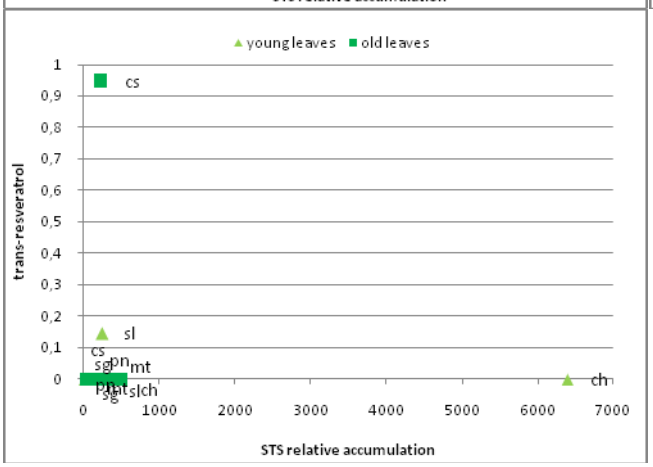
The fact that diverse infection developments induce different stilbenic values may only be explained by the action of other defence mechanisms that act simultaneous with the stilbenes.



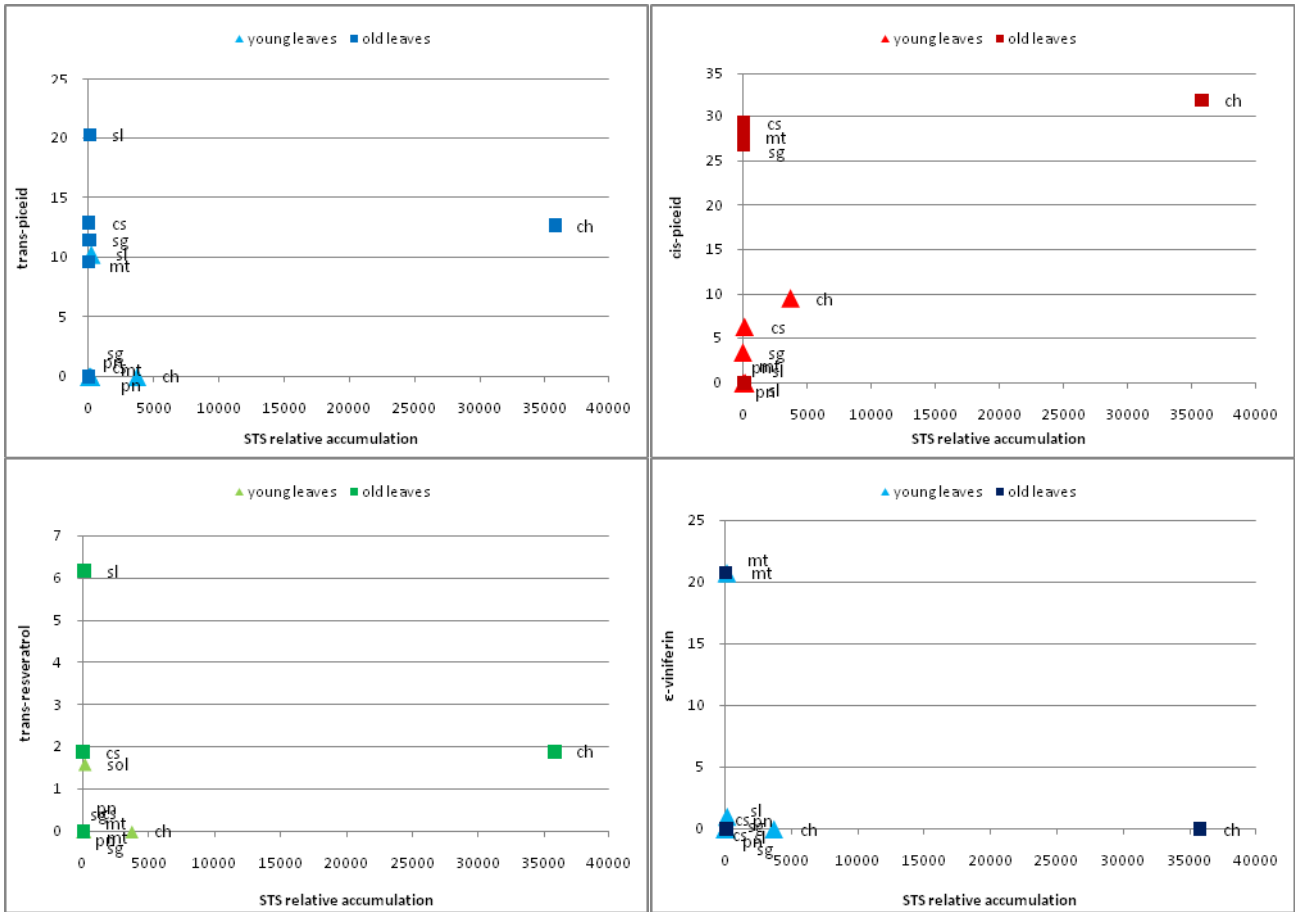
Relation between the concentration of stilbenes and STS induction at T12 for all clones ch –Chardonnay; cs – Cabernet Sauvignon; mt – Müller-Thurgau; pn – Pinor noir; sl – Solaris; sg – Sangiovese; Δ – young leaves; □– old leaves



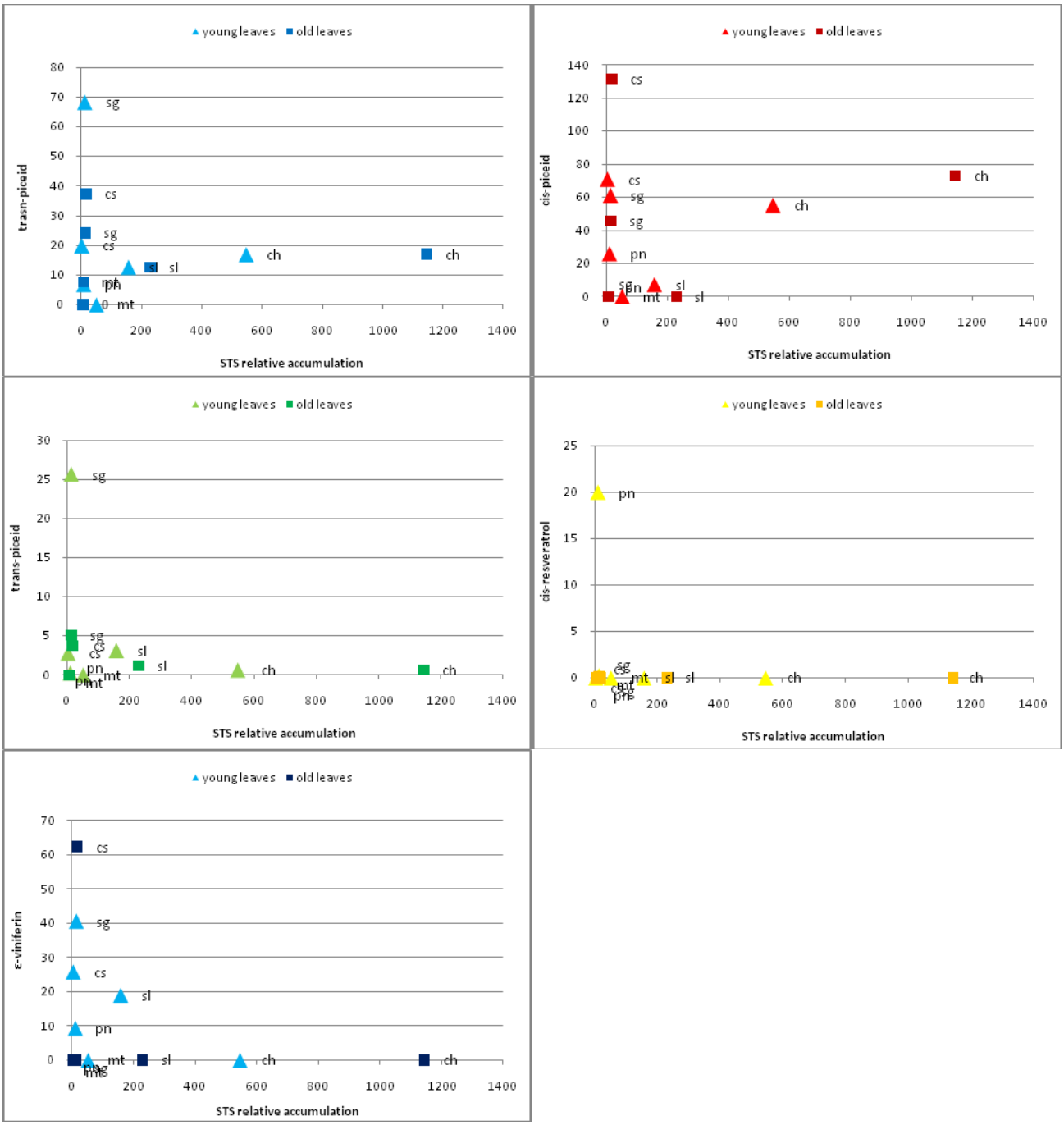




Relation between the concentration of stilbenes and STS induction at T24 for all clones ch – Chardonnay; cs – Cabernet Sauvignon; mt – Müller-Thurgau; pn – Pinor noir; sl – Solaris; sg – Sangiovese; Δ – young leaves; □– old leaves



Relation between the concentration of stilbenes and STS induction at T24 for all clones ch –Chardonnay; cs – Cabernet Sauvignon; mt – Müller-Thurgau; pn – Pinor noir; sl – Solaris; sg – Sangiovese; Δ – young leaves; □– old leaves



Relation between the concentration of stilbenes and STS induction at after sporulation for all clones ch –Chardonnay; cs – Cabernet Sauvignon; mt – Müller-Thurgau; pn – Pinor noir; sl – Solaris; sg – Sangiovese; Δ – young leaves; □– old leaves

The graphics above, describing the relation between stibenic compounds and STS induction, in the different sampling times, showed that there are no clear relations between STS induction and every single stilbenic compound found in the leaves in all the different sampling times. This observation applies to young and old leaves in all the varieties studied.

Discussion

It is interesting to notice in the first trial, which compares the stilbenic response in leaves of a variety considered resistant, Sirius, and a variety considered susceptible, Sangiovese, that after inoculation with *Plasmopara viticola,* similar patterns of *trans*-resveratrol production could be seen in both varieties. The same cannot be stated when analysing the *ε*-viniferin production in time, since the patterns created were clearly different.

Levels of *ε*-viniferin accumulation in Sangiovese increased in time and two accumulation peaks can be seen. In Sirius, this compound accumulated gradually and reached values of almost double of those found in the accumulation maxima of Sangiovese.

The low resveratrol levels can be due to a quick oxidization of resveratrol to viniferins (Chong *et al*, 2009). Considering that *ε*-viniferin has been previously described as a quite fungitoxic stilbene (Langcake and Pryce, 1977_b; Bavaresco *et al*, 1997_b; Pezet *et al*, 2003) this may partially ascertain the higher resistance found in Sirius leaves respect those of Sangiovese.

Since higher levels of stilbenes correspond to lower levels of sporulation (Poutaraud *et al*, 2006), the lack of sporulation in the leaves of Sirius could then be a result of the high levels of *ε*-viniferin found in the leaves.

The Cabernet Sauvignon trial, relates stilbene accumulation after *Plasmopara viticola* inoculation in both leaves and berries of the 10 different clones.

It is important to notice that in all berries, stilbenes were already present in T0, while in leaves, no stilbenes could be measured. In the leaves there is always an increase of accumulation from T0 to T1. Sampling T4 for berries was taken really late, 22 days pos inoculation and cannot really be compared with any of the leaves' sampling times.

When looking at both leaf and berry results for the same clone some things do stand out.

One of the first things that can be noticed is that *ε*-viniferin is the only common compound in both leaves and berries in these trials. Apart from this compound, *trans*-resveratrol could be found in the leaves of all 10 clones and *trans*-piceid in the berries of the same 10 clones under study.

The differences found between the 10 clones of Cabernet Sauvignon can be justified by the genotypical differences that can be found within the clones, a result of the genetical variation that characterises them. Others have also found such variabilities while studying a F1 population of Freiburg 993-60 x Teroldego segregating for the tolerance to *Plasmopara viticola.* Malacarne *et al* (2009) showed that in only 9 of the 105 genotypes of all the main stilbene were present and that they reached different values within the population.

Another noteworthy fact is that the absolute stilbenic values are definitely higher in the leaves where *trans*-resveratrol reaches up to 8 $\mu g.g^{-1}$ fw and *ε*-viniferin up to 30 $\mu g.g^{-1}$ fw. Meantime, in berries, the values rise only up to 3 $\mu g.g^{-1}$ fw of *trans*-piceid and up to 1.5 $\mu g.g^{-1}$ fw of *ε*-viniferin.

The presence of both resveratrol and *ε*-viniferin in leaves is supported by the trial of Jean-Denis *et al* (2006) that showed that resveratrol, *ε*-viniferin and *δ*-viniferin are the main stilbenes induced in *Plasmopara viticola* infected grapevine leaves. Also Godard *et al* (2009), using the same susceptible variety Chasselas, found that resveratrol and *ε*-viniferin were the highest accumulating stilbenes after inoculation. Still, other susceptible varieties present other stilbenes seen in Muscat Ottonel leaves inoculated with *Plasmopara viticola,* which presented high levels of *trans*-resveratrol and *trans*-piceid and lower values of *cis*-resveratrol, *ε*-viniferin and *δ*-viniferin (Poutaraud *et al*, 2007).

In the berries, the presence of *trans*-piceid and *ε*-viniferin can be supported by other trials that show that in susceptible varieties, resveratrol seems to be glycosylated into piceid and it is possible that the initial values of resveratrol are too low to allow the production of viniferins, in fact the viniferins tend to be detected only some time after inculation which can be due to the necessary presence of peroxidade synthase whose activation is slower than that of STS (Pezet *et al*, 2004_a).

Despite all this, little is known about the stilbenic levels of the berries soon after fruit setting, since most studies concentrate on their accumulation from veriason to ripening. One study reports that in fruits slightly smaller than pea-size resveratrol accumulation and induction of STS increased after UV-elicitation (Petit *et al*, 2009). Also in literature the results of more developed berries are contradictory, some state that stilbene accumulation decreases from veraison to ripening (Jeandet *et al* 2001; Bais *et al*, 2000) while other revealed that stilbenes concentration increases from veraison to ripening (Gatto *et al*, 2008).

The only common information reported is that several differences could be detected among genotypes, a fact that is also confirmed by the present study.

In their study Gatto *et al* (2008) also stated that high stilbene producers have higher values of glycosylated forms, constitutively expressed, while *trans*-resveratrol appears as an inducible component. If this is the case, it is possible to say that small berries as the ones used in this study, apart from having different amounts of *trans*-piceid, are also able to produce stilbenes as a reaction to the inoculation with *Plasmopara viticola,* in this case, *ε*-viniferin.

A possible separation of clones in relative groups that tend to have similar behaviour can be extrapolated: In the last sampling time the leaves of the clones Vcr8 and R5 have the highest sporulation percentage values and at the same time the lowest *ε*-viniferin accumulation values. The clones 338 and Isv105 are the last ones to present visual symptoms, only at T5, and they do present the highest *ε*-viniferin values in the last sampling times. As for berry evaluation, the clone 338 presents the highest visual symptoms and simultaneously some of the lowest *trans*-piceid values and a very low level of *ε*-viniferin which only appears at T4. In all other clones, both *trans*-piceid and *ε*-viniferin are quite low and the last only appears in the two latest sampling times.

Despite value differences, in clones 169 and 341 both *trans*-resveratrol (leaves) and *trans*-piceid (berries) seem to decrease over time while for 169 *ε*-

viniferin (leaves) has a maximum at T3 and 341 *ε*-viniferin (berries) only at T4. Both clones present *ε*-viniferin in berries in the later times.

The *trans*-resveratrol accumulation in the leaves of the clones Isv2, R5 and Vcr8 reaches maxima at T3 and then decreases while *trans*-piceid content of their berries remains stable in time. *ε*-viniferin in the leaves is quite diverse for these clones: Isv2 reached two maxima at T2 and T4, Vcr8 reached one maximum at T3 and R5 one maximum at T4. In the berries *ε*-viniferin was only present at T3 in the clone Vcr8 and at T4 in Isv2, which, as can be seen in the relation graphic, produces higher contents of this compound even at medium infection rates respect the other clones. Also R5 shows its highest accumulation at this point. Despite, this clone shows the highest infection rates only average stilbenic values.

In the leaves of the clones 191 and 338 *ε*-viniferin values increase and reach a maximum at T4 while in berries *ε*-viniferin values appear at T3 and increase at T4. As for *trans*-resveratrol in the leaves, it decreases in time in the clone 191 and reaches a maximum at T2 and then decreases in clone 338. Berry *trans*-piceid increased in time in clone 191 while it reached two maxima in clone 338.

The clones 685 and Isv117 also share some similarities: leaf *ε*-viniferin increases to a maximum at T4 and then decreases while in berries it only appears at T3.

Trans-piceid values in the berries remain stable while leaf *trans*-resveratrol decreases continuously for 685 and reaches a maximum at T2 decreasing afterwards in clone Isv117.

A closer look at the results of the multiple varieties trial shows differences between young and old leaves of a given variety:

In all varieties, regardless of the fact that all presented sporulation after some time, even if very reduced in Solaris leaves, the progress of the infection was different in all varieties. For Chardonnay, Cabernet Sauvignon and Müller-Thurgau it was faster in young leaves than in old leaves while for Pinot noir,

Solaris and Sangiovese the differences between young and old leaves are not as expressive.

Despite different STS inductions patterns, for the specific gene studied within the stilbene synthase multigene family, in both young and old Chardonnay leaves, *cis*-piceid could always be retrieved and it followed the same behaviour in both sets of leaves, increased between 12 and 24h, decreased at 72h and increased again in the after sporulation sampling time, although with relatively higher values in older leaves.

Trans-piceid and *trans*-resveratrol could be seen in several measuring times in old leaves while they were only present in the after sporulation sampling time in young leaves and similar values were then found in young and old leaves.

Cabernet Sauvignon induction seems to follow the same pattern but with one sampling time delay in young leaves, that is, maximum induction is found at 24h in young leaves and at 72h in old leaves.

Cis-piceid which appears constant in all sampling times in young leaves. In old leaves it reaches concentrations three fold higher after in all other times.

Also *ε*-viniferin, *trans*-piceid and *trans*-resveratrol are present in old leaves and, in the after sporulation sampling, in young leaves as well. Concentrations are always higher in old leaves.

In Müller-Thurgau leaves STS induction patterns are similar in young and old leaves, induction increases till 24hpi, were it reaches a maximum and then steadily decreases. In this case stilbene production appears little related with STS induction as in young leaves *ε*-viniferin and *cis*-piceid can only be accounted for at 12h while in old leaves *ε*-viniferin and *trans*- piceid reach a maximum at T12 and then decrease in time and *trans*-resveratrol is only detected at T12. It seems that the leaves react quickly to the attack; infection is quite fast on this variety but they are unable to properly defend themselves through stilbenes which seem somehow to disappear.

Pinot noir young leaves are able to induce increasing values of STS till a maximum is reached at 72hpi. Old leaves have longer maximum since induction

is high and stable from 24 to 72hpi. Awkwardly no stilbenic compounds were found at any of the sampling times in old leaves and in young leaves, only after sporulation sampling were them found. In this case *cis*-piceid, *cis*-resveratrol, *ε*-viniferin, *trans*-piceid and *trans*-resveratrol were present. The fact that no stilbenes were found in old leaves seems rather particular especially because a normal infection took place, but in fact, different varieties might react differently to infection. In a study conducted (Pezet *et al*, 2004_a) using leaves of several varieties, divided in susceptible, intermediate and resistant to downy mildew, showed different stilbenic behaviours after inoculation. The susceptible varieties, presented higher levels of piceid and resveratrol, even if for example Pinot noir presented extremely low stilbenic values.

At 12h STS induction was high in young leaves as well as in old leaves of Sangiovese. After that point it decreased in young leaves an it also decreased in old leaves but only after being identical to T12 at T24. *Cis*-piceid was identical at 12hpi and 24hpi in old leaves and present until after sporulation sampling as was *trans*-piceid but always with lower values. Stilbenes could only be detected in young leaves in later sampling times being that *trans*-piceid, *ε*-viniferin, *trans*-resveratrol and *cis*-resveratrol only the after sporulation time. *Cis*-piceid, even if low, was detected at 72hpi.

In the resistant variety Solaris, STS induction in young leaves was maximal at 12hpi and decreased afterwards. In old leaves only at 72h was a lower value of induction found. *Trans*-piceid was detectable from 24h on, *ε*-viniferin from 72h and *cis*-piceid and *trans*-resveratrol only in the after sporulation time. In old leaves only *trans*-piceid and *trans*-resveratrol could be measured, the 1^{st} in all times and the 2^{nd} only at the last sampling times.

Usually it is reported that intermediate varieties show an early activation followed by a rapid decrease and resistant varieties show a gradual activation of the defence genes (Malacarne *et al*, 2009). But in the case of Solaris STS induction does not appear all together significant (Slaughter *et al*, 2008), especially because it has been shown that stoma near the infection sites contained callose deposits in and around the stomatal openings which allied to

the presence of necrosis indicates a hypersensitive reaction to *Plasmopara viticola* (Gindro *et al*, 2003).

Similarly to what had been found by Poutaraud *et al* (2006), whose trial conducted using leaves of Syrah (susceptible), *Muscadinia rotundifolia* (totally resistant) and a hybrid called RV1 (partially resistant), showed that the 6th leaf of both resistant and susceptible varieties produces more stilbenes than younger leaves, also in the present trial the same phenomena can be seen for the main stilbenic compounds produced in most varieties.

The trials conducted in this study have helped to confirm the differences between infection progress, stilbenic accumulation and defence genes induction in susceptible and resistant varieties to pathogens.

Through the analysis of 10 different clones of Cabernet Sauvignon, 169, 191 338, 341, 685, ISV2, ISV105, ISV117, R5, and VCR8, it was possible to form an idea of how leaves and berries react to the infection of one of the main pathogens that attacks the grapevine, *Plasmopara viticola*.

It is clear now that the different clones have different reaction times and that they produce independent values of stilbenic accumulation even if they do tend to produce the same type of stilbenic compounds when comparing equal grapevine organs. The relations appeared as genotype dependent.

It was also possible to determine which compounds were in fact produced in attacked leaves and berries, as well as, to determine that leaves and berries of the same clone can display different susceptibilities and reactions.

Furthermore, this trial confirmed the possibility of very early infection of the berries. This pathogen is not only able to infect as early as fruit set, trough the stoma of the berries, but it is also able to exit the stoma and complete its cycle. This may be an important fact when planning the treatments in a given vineyard if the vintage climatic characteristics favour the development of *Plasmopara viticola.*

The last trial, conducted using six different varieties, has allowed confronting infection development, STS induction and stilbene accumulation in

young and old leaves of Chardonnay, Cabernet Sauvignon, Pinot noir, Müller-Thurgau, Sangiovese, and Solaris.

The fact that, STS induction for the specific gene studied within the stilbene synthase multigene family could be seen, in almost all young and old leaves studied, before infection, was unexpected and might be justified by the action of other elicitors even if the trials were conducted under controlled conditions. Other studies have showed that elicitation with Aluminium chloride and *Botrytis cinerea* also induced irregular results in different varieties (Borrie *et al,* 2004) and that STS induction and stilbene synthesis might be modulated by several mechanisms (Versari *et al*, 2001) and synthesis does require the coordinated activation of all the enzymes in the pathway (Gatto *et al*, 2009).

Plasmopara viticola inoculated leaves, from the same shoot of a grapevine, but arising from opposite extremities and having therefore opposite ages, have highlighted that the type of stilbene accumulating, the amount accumulated, the induction of defence genes and the ongoing progress of the infection itself can relate with the age of the leave that carries the infection. Young leaves tend to present faster infection cycles and stilbene accumulation appears to be delayed while older leaves present slower infection cycles with a constant presence of stilbenes over time.

This trial also confirmed the differences between resistant and susceptible varieties, but it also allowed seeing the differences that are present between susceptible varieties, as for example Cabernet Sauvignon, that despite being susceptible is more capable of reacting to the ongoing infection than Müller-Thurgau.

The present study has succeeded by means of the conducted trials to help complete our knowledge of the responses of the grapevine to pathogenic infection.

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