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# Stilbenes: biomarkers of grapevine resistance to fungal diseases

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## Abstract

Since the introduction of powdery and downy mildews in Europe in the late 19th century, breeding resistant cultivars by hybridizing *V. vinifera* (susceptible) with other *Vitis* species (resistant) has been largely used and led, in 1947, to the cultivation of > 350,000 ha (23%) of grapevine area in France. Because of the poor wine quality of this first generation of hybrids, legislation prohibited their cultivation for the production of quality wines. Recent investigations allowed sequencing the entire grapevine genome, but no precise resistance genes are yet known for further introduction in susceptible *V. vinifera* cultivars. At the molecular level, the use of QTL (Quantitative Trait Loci) as resistance markers is ongoing and could be correlated to resistant gene expression and further define metabolite production in resistance mechanisms. Stilbenic phytoalexins are key defence molecules implicated in the resistance of grapevine cultivars to three major fungal pathogens, *Botrytis cinerea* (grey mould), *Plasmopara viticola* (downy mildew) and *Erysiphe necator* (powdery mildew). HPLC analysis of stilbenes is an efficient method to evaluate the ability of the vine plants to inhibit the development of fungal pathogens. Resistant grapevine varieties react very rapidly to infections by producing high concentrations of the most toxic stilbenes, δ-viniferin and pterostilbene, at the sites of infection. Monitoring of such stress biomarkers is also of great interest for evaluating the efficiency of priming molecules at inducing the grapevines' natural defence responses. In addition, these compounds have various beneficial effects on human health, acting as anti-oxidants and also as potential chemopreventive agents. The diversity of stilbenes is intriguing, and new holistic analytical approaches, such as metabolomics, that are widely used for wine classification also have great potential for the comprehensive study of responses of Vitaceae to biotic and abiotic stress.

**Keywords**: breeding, resistance, fungal diseases, stilbenes, downy and powdery mildews, grey mould

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## Introduction

Stilbenes are a family of molecules whose chemical structure in both the monomeric and oligomeric states is constituted by a diphenylethylene group oriented in *cis* or *trans*. When exposed to UV light, they emit intense blue fluorescence. This characteristic is the origin of the name stilbene, which derives from the Greek word "στίλßοσ" (stilbos), translated as "shining". Stilbenes are natural phenolic compounds and have been isolated and identified in 25 different plant families and also in a Bryophyte and an Antarctic sponge. Stilbenes are secondary plant products that are produced through the phenylalanine/polymalonate pathway. Resveratrol was the first stilbene identified (Takaoka, 1939) and is the most studied. Stilbene synthase is the key enzyme for the formation of resveratrol and other stilbenes produced from various phenolic precursors. Plant-derived stilbenes are isolated as hydroxylated, methylated, esterified, glycosylated or prenylated monomers or as polymers. In Vitaceae, resveratrol and α-, ß- and ε-viniferin were identified to be phytoalexins, which are antimicrobial substances synthesised *de novo* by plants that accumulate rapidly at areas of incompatible pathogen infection. A methylated stilbene, pterostilbene, was later identified by Langcake *et al*. (1979). Resveratrol was first identified in *Veratrum grandiflorum* by Takaoka in 1939. Its name likely derives from an abbreviation of the class of molecules to which resveratrol belongs, i.e., resorcinol, the plant name *Veratrum* and ol indicating the presence of a hydroxyl group. Pterostilbene was first described by Späth and Schläger (1940) in *Pterocarpus santalinus*.

In Vitaceae, stilbenes represent defence biomarkers because they occur as phytoalexins that are produced dynamically in response to biotic or abiotic stress. Though resveratrol and its derivatives are present in lignified stem tissue (Pool *et al*., 1981; Dercks and Creasy, 1989), they are absent in the healthy green parts of the grapevine (leaves, young canes). Pterostilbene, however, is present in the healthy grape berries of *Vitis vinifera*.

The rate of synthesis of resveratrol after stress induction depends on the grape variety and provides a good metric for evaluation of the resistance of grapevine cultivars to grey mould and downy mildew (Pezet *et al*., 2004; Gindro *et al*., 2006). The synthesis of pure pterostilbene and resveratrol has allowed the study of the toxic effect of these stilbenes on *Botrytis cinerea*, one of the major fungal diseases that attack grapevines (Pezet and Pont, 1988). Enzymatic synthesis of δ-viniferin, as well as purification of ε-viniferin from lignified canes allowed toxicity testing of these resveratrol dimers on *Plasmopara viticola* (downy mildew) and *Erysiphe necator* (powdery mildew) (Pezet *et al*., 2004; Schnee *et al*., 2008). Because of these results, we have developed biological (artificial inoculation) and chemical methods (HPLC analysis of stilbenes in grape tissues) to evaluate the level of resistance of grapevine seedlings to downy and powdery mildew in breeding programmes. These tools have led to a significant reduction in the time and space required for such experiments.

## Criteria for early selection of resistant grape cultivars to downy and powdery mildew

Grapevine breeding is one of the most promising methods to preserve genetic diversity, but it also allows the selection of specific genetic traits, such as resistance to pathogens. Each cross produces a large number of germplasms (collection of genetic resources), and a rapid way to estimate the downy mildew resistance level is absolutely necessary to avoid long and tedious field observations. For this purpose, artificial inoculations of seedlings with *P. viticola* sporangia or the conidia of *E. necator* and estimation of the development of the disease after one week of incubation is a very useful method. The production and the density of sporangia resulting from these artificial inoculations are widely accepted as good criteria for the estimation of grapevine resistance to pathogens. Disease-resistance tests of grape cuttings in climate chambers or greenhouses are not always representative of the real resistance level in the vineyards, even if a good correlation has been demonstrated between artificial inoculations in glasshouses or climate chambers and field observations (Brown *et al*., 1999). Other resistance criteria must be stringently tested in seedlings to correlate the resistance in greenhouse or *in vitro* tests and in vineyards.

The first model of downy mildew resistant grape variety studied at Agroscope was Solaris [Merzling x (Saperavi severneyi x Muscat Ottonel)], which was obtained from the Weinbauinstitut Freiburg, Germany. In the case of *P. viticola*, two physiological events are representative of resistance to mildew. One is callose synthesis in stomata at seven hours post-infection with *P. viticola* zoospores (Gindro *et al*., 2003). The second is the synthesis of stilbenic phytoalexins, especially resveratrol and its subsequent oxidation products, ε- and δ-viniferins and the production of pterostilbene. Callose, a sugar polymer that consists of (1-3)-ß-D-glucose subunits, is a known constituent of papillae (raised thickenings

in the cuticle), which have long been known to serve as plant defences. When attacked, plants physically reinforce their cell wall to stall or prevent pathogen penetration. It is known that callose deposits play a role in the ability of grapevines to tolerate downy mildew (Kortekamp *et al*., 1997). More recently, rapid synthesis of callose in the stomata of grapevine leaves after *P. viticola* infection has been described. This phenomenon stops downy mildew penetration into the stomata and is only visible in resistant cultivars. In downy mildew-susceptible varieties, no callose synthesis has been observed, whereas the number of stomata with callose deposition is generally well correlated with the observed resistance of germplasms to mildew after artificial inoculations.

In addition to callose deposition in stomata, oxidised resveratrol derivatives and pterostilbene are produced in the leaves of resistant cultivars at the site of infection after artificial inoculation and can be analysed and quantified by HPLC. One of the oxidation products of resveratrol was determined to be ε-viniferin, and, more recently, an isomer of this product, δ-viniferin, was described as one of the major stilbenes present in stressed grapevine leaves (Pezet *et al*., 2003). Until now, pterostilbene, the naturally produced stilbene with the highest toxicity towards downy and powdery mildew and grey mould, has only been produced in high quantities in specific vine genotypes and backcrosses. Use of these genotypes must be prioritised to promote resistance. In susceptible cultivars, resveratrol is mainly glycosylated to form piceid. This addition of glucose to resveratrol protects it from further oxidation. This is particularly important when we consider the respective toxicity of the different stilbenes towards each fungal pathogen. Glycosylated resveratrol (piceid) is not toxic, while ε- and δ-viniferins and pterostilbene are highly toxic. Qualitative and quantitative analysis of stilbenes in the leaves of grapevine seedlings at 48 hours post-inoculation is also highly predictive of the level of *P. viticola* resistance genotypes. In *Muscadinia rotundifolia*, the necrotic areas consist of a number of small necrotic spots located under the infection droplet surface. This means that samples must be taken very carefully, just around the developing necrotic areas under the magnifying glass; otherwise, the intensity of the stilbene signals is diluted and therefore not representative of the real local accumulation. In this cultivar, the infection process is stopped before the development of either vesicles or infective structures and results in a rapid accumulation of considerable amounts of stilbenes (Alonso-Villaverde *et al*., 2011). Not all these stilbenes are equally toxic to *P. viticola*, as described previously. δ-viniferin and pterostilbene are considered to be the most toxic to downy mildew. Pterostilbene is generally absent or present at concentrations that are too low to have a significant effect. In *Muscadinia*, at 24 hours after infection, δ-viniferin and pterostilbene are present at levels 24 and 42 times higher than their respective ED50s (i.e., the concentration that inhibits 50% of pathogen development). Therefore, the most important step in the inhibition of disease in *Muscadinia* may be the rapid induction of metabolic responses, which occur before any haustorium can appear. More recently, some new stilbenic phytoalexins implicated in the overall resistance of grapevine against fungal diseases were identified in lignified canes (Schnee *et al*., 2013) showing different toxicity against *P. viticola*. Among them, one can cite ampelopsin A, hopeaphenol and E-vitisin B.

According to these results, a two-step procedure is used to select seedlings (Figure 1) that are resistant to downy mildew: 1) artificial inoculation, in the greenhouse, of whole plantlets by spraying an aqueous suspension of downy mildew zoospores and elimination of sporulating plants after one week incubation; and 2) application of histological and biochemical criteria (sporulation, callose in stomata, ε- and δ-viniferins as well as pterostilbene production) to classify the detached leaves of remaining plants.

Leaf samples must be excised under the infection droplets areas. The combination of these criteria have permitted us to establish threshold values for sporulation (< 15 sporangia $mm^{-2}$), callose (> 15% of infected stomata presenting callose deposits), and stilbene levels (> 40 µmol $mg^{-1}$ FW δ-viniferin and > 50 µmol $mg^{-1}$ FW ε-viniferin) that can be used to identify resistant seedlings, which are then transferred to the hybridiser and can be planted in the vineyard for further agronomical and oenological evaluation. Currently, more than 58 crosses have been performed, generating about 22,000 plantlets, of which 900 have been selected using the early tests described before (mean of 4% of the initial plants). 33 varieties (30 red and 3 white) have been propagated to obtain 20 plants, from which 13 have been successfully planted in extended performance trials. Finally, one variety was announced for DHS (Diversity Homogeneity Stability) registration in 2009 and is registered since 2014 under the name DIVICO (red variety) (Figure 2) and another one (white variety) will soon be registered.

More recently, the resulting resistant seedlings have been screened for powdery mildew resistance using histological and biochemical criteria. However, no


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**Figure 1. Grapevine plantlets produced after grapevine cross-breeding. (A) Young plantlets still showing cotyledons and (B) plantlets showing six fully developed leaves which corresponds to the stage used to select seedlings.**

plantlets were eliminated before final validation of the correlation between field observations and laboratory results. A one-step procedure was developed to evaluate the susceptibility of the remaining seedlings. First, leaf fragments are taken and fixed under osmium vapours for scanning electron microscopy (SEM) or environmental scanning electron microscopy (ESEM) analysis (Figure 3). The grapevine adaxial leaf surface has revealed that the crystallisation pattern of epicuticular waxes varies between susceptible and resistant grape varieties (Schnee, 2008). The susceptible *V. vinifera* cv. Chasselas displays a relative smooth surface and some scattered protuberances. However, the surface of *V. candicans*, which is very resistant to powdery mildew, is densely covered by platelet-shaped crystals that protrude perpendicularly from the leaf plane. The width and the crystallisation patterns were confirmed by transmission electron microscopy. The platelets exhibit thin margins and relative triangular shapes on which no haustoria could be observed. Though these results are interesting, SEM as well as ESEM observations are quite time-consuming and expensive techniques and therefore unsuitable for a rapid evaluation of seedling resistance in our breeding programme. Further artificial inoculations were performed on leaf disks incubated under optimal conditions for *E. necator* growth. Observation of its development and quantification of the conidia germination rate, level of appressoria formation, mycelial network density and sporulation level at six days after inoculation are efficient epidemiological criteria to determine the level of susceptibility of seedlings to *E. necator*. A phenomenon specific to *E. necator* infection is the production of mycelium strictly on the host surface. Consequently, the induction of defence metabolites increases only during the development of infectious structures (appressoria, infection peg and haustorial differentiation). The haustoria of *E. necator* infect only the first epidermal cell layer, while *P. viticola* develops an intercellular mycelium that invades the mesophyll and forms many infective structures in the cells. Because of the local synthesis of stilbenic phytoalexins at the sites of infection, the quantification of stilbenes induced by powdery mildew infections must be linked to the number of appressoria and infective structures.

Results have shown a strong induction of viniferins, which are very toxic towards *E. necator*, in resistant grapevine cultivars. For this reason, the quantification of viniferins at 48 hours after inoculation could be an important measure of resistance towards powdery mildew. The choice and the relevance of each

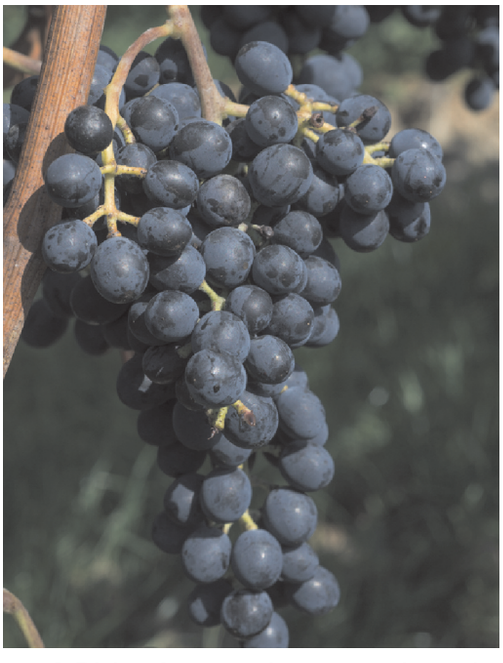

**Figure 2. Divico, the new resistant grapevine variety of Agroscope.**

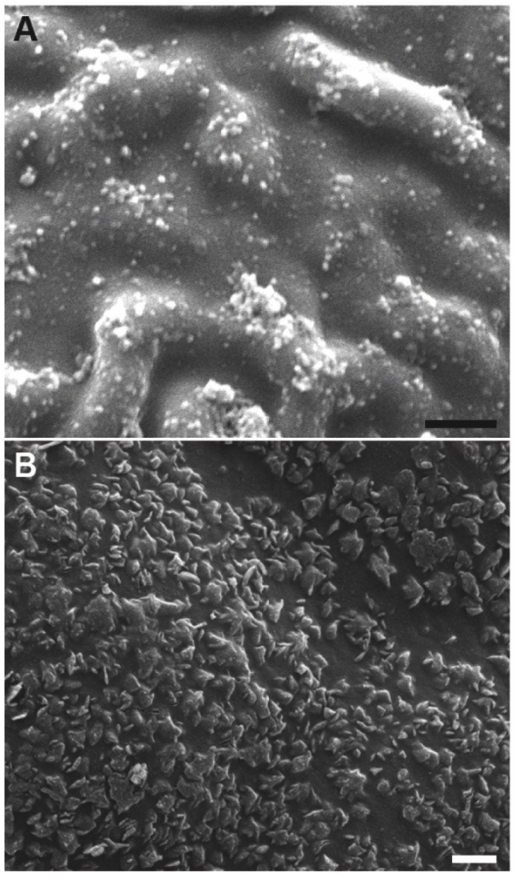


**Figure 3. ESEM observations of the smooth surface of the susceptible grapevine *V. vinifera* cv. Chasselas (A) in comparison to the resistant *V. candicans*, showing platelet-shaped crystals (B). Scale bars represent 1 µm.**

criterion of resistance must still be correlated to the real resistance of the grapes in question towards *E. necator* in field trials. The chemical and microscopical analyses of the epicuticular waxes as well as the developmental patterns of *E. necator* on new grapevine crosses, showing a very high level of resistance to powdery mildew, are in progress.

## Conclusions

Since Langcake identified stilbenic phytoalexins in the Vitaceae family in 1976, other types of molecules, such as pathogenesis-related (PR) proteins, have been described as enzymes present on host cell walls that can efficiently degrade fungi. However, these proteins probably play a secondary role in resistance. Many years of pioneering research on grape stilbenes at Agroscope in Changins and other research institutes have demonstrated that some of these molecules, such as viniferins and pterostilbene, are at the centre of grapevine defence mechanisms against fungal pathogens. Very good spatial correlations were found between the synthesis of these compounds and the sites of infection in grapevine leaves and berries. The higher their concentration, the higher the level of pathogen resistance of cultivars obtained from our breeding programmes. Screening new grape plantlets for their natural resistance, using artificial inoculation and micro-analysis of stilbenes, is a very efficient tool for identifying and breeding grapevines that are resistant to fungal diseases. Experiments aiming to characterise new stilbenic phytoalexins or stilbenic constitutive compounds that may be implicated in grape host defences using MS-based metabolomics (UHPLC-TOF-MS) are underway. The objective is to better understand the induction and biochemical synthesis of grape stilbenes and also to improve the actual breeding tools, both of which will prove very useful for the selection of new grape varieties with disease resistance and good organoleptic properties. As this has been discussed, stilbenes and resveratrol play a key role in plant defence and are also of interest to human health. The fine-tuning of stilbenic phytoalexins production by Vitaceae is necessary for the proper response to pathogen infection. A good understanding of the molecular mechanisms that regulate this response is important for improving the resistance of grapevine cultivars. With the knowledge acquired over the years, patterns of stilbene production are on their way to becoming good diagnostic and predictive tools for both grapevine cultivar selection and defence priming optimisation. All of these scientific advances should provide ways to produce wine with optimal oenological characteristics in a sustainable manner.

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