Effects of resveratrol, viniferins and pterostilbene on *Plasmopara viticola* zoospore mobility and disease development

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Summary

The effects of stilbenes (resveratrol, δ- and ε-viniferins, and pterostilbene) on the mobility of zoospores of *Plasmopara viticola* and on subsequent disease development were studied *in vitro*. δ-viniferin and pterostilbene are the most toxic stilbenes concerning zoospore mobility (ED_{50} : 14.6 and 28.3 μM) and disease development (ED_{50} : 14.7 and 12.7 μM). The analysis of stilbenes in leaf cells of resistant (Solaris) and susceptible (Chasselas) grape cultivars artificially inoculated with *P. viticola* has shown that very high amounts of stilbenic phytoalexins accumulate at the site of infection of the resistant cultivar compared to the susceptible one.

K e y   w o r d s : resveratrol, ε-viniferin, δ-viniferin, pterostilbene, *Plasmopara viticola*, *Vitis vinifera*.

Introduction

Since 1878, downy mildew (*Plasmopara viticola* [Berk. & Curt] Berl. & de Toni) has become one of the most serious diseases of grapevines in European vineyards and progressively in all viticultural areas in the world. The most cultivated grape varieties (*Vitis vinifera* L.*)* are sensitive to *P. viticola* and the control of downy mildew requires regular fungicide applications. More than 20 years ago, breeding programs were initiated in many countries to create grapevine genotypes resistant to downy mildew (Doazan 1980). Resistance to the pathogen was found in some *Vitis* species, mainly in native American species, which readily cross with *Vitis vinifera* L.; these genotypes have been used as a source of resistance to *P. viticola*. A variety of factors can be implicated in resistance, however, stilbene phytoalexins can be considered as one of the most important responses of grapevines to fungal infections (Langcake 1981; Dercks and Creasy 1989).

The effects of these compounds cannot be studied *in vitro* since *P. viticola* is an obligate parasite, which cannot be cultivated on artificial media. Nevertheless, effects of pure stilbenes on zoospore mobility can be measured, as well as the ability of these infectious cells, mixed with these compounds, to develop downy mildew on grapevine leaves. These observations have allowed to establish biocide effective doses of resveratrol, δ- and ε-viniferin and pterostilbene on *P. viticola* zoospores. However, to measure *in vivo* the effect of these stilbenes on *P. viticola* mycelium, growing inside the infected leaves, is impossible. But the high concentration of stilbenes at the site of infection of the resistant cv. Solaris, in which *P. viticola* does not develop spreading lesions, may suggest that these phytoalexins are toxic to the mycelium, too. Pterostilbene was considered the most toxic stilbene to fungi (Pont and Pezet 1990; Pezet and Pont 1995) but its concentration in infected grapevine leaves and berries is always very low and consequently its implication in defense mechanisms difficult to demonstrate. Recently, δ-viniferin was identified as one of the major stilbene synthesized by *P. viticola*-infected grapevine leaves (Pezet *et al*. 2003). We demonstrate here, that this molecule is as toxic as pterostilbene for *P. viticola* zoospores and plays an important role in resistance mechanism of grapevine to downy mildew.

Material and Methods

P l a n t   m a t e r i a l : Lignified cuttings of *Vitis vinifera* cvs Chasselas and Solaris (the latter resulting from a cross of Merzling x Gm 6493, carried out by the Weinbauinstitut of Freiburg, Germany) are sensitive and resistant to *P. viticola,* respectively; they were taken from the grape collection at the Swiss Federal Research Station for Plant Production of Changins, Switzerland.

The vines were maintained in growth chambers (16 h light at 22 °C, 8 h dark at 18 °C and 60 % relative humidity) until they had 10 fully developed leaves. They were then used for artificial inoculations.

O r i g i n   a n d   c u l t u r e   o f   *P l a s m o p a r a   v i t i - c o l a* : The pathogen was isolated from diseased leaves in the vineyards of Changins and was cultivated on rooted cuttings of Chasselas according to Gindro *et al*. (2003).

O r i g i n   o f   s t i l b e n e s : Resveratrol was obtained from Sigma (Buchs, Switzerland). ε- and δ-viniferin were purified according to Pezet *et al*. (2003). Pterostilbene was synthesized according to Pezet and Pont (1988).

A s s e s s m e n t   o f   s t i l b e n e   e f f e c t s   o n   z o o - s p o r e   a n d   d i s e a s e   d e v e l o p m e n t : Sporangia of *P. viticola* were collected from sporulating lesions on Chasselas leaves by vacuum aspiration using a filtered pipette tip. They were suspended in distilled water and their concentration was adjusted to 10^5 ml^{-1}. After 1 h at room temperature with gentle agitation, zoospores were released from about 50 % of the sporangia, leading to a suspension containing $2.5 \cdot 10^5$ ml^{-1} mobile zoospores.

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Ethanolic stilbene solutions were added to the zoospores containing suspension so that ethanol was at 1 % (v/v) and stilbene concentration varied from 1 to 1000 µM. Controls consisted of pure water or 1 % (v/v) ethanol/water solutions. Five min after the addition of stilbenes, the number of mobile zoospores in an area of 0.11 mm² (microscope: Leica-Leitz, magnification 600x) was counted during a 30 s viewing period.

Three replicates of 20 droplets of 10 ml of zoospore suspension, prepared in the presence of stilbene solutions as described above, were also placed on the abaxial part of cut grapevine leaves (cv. Chasselas, 5th leaf of 10 fully developed leaves) and incubated in humid chambers (100 % RH) at 25 °C with 16 h d⁻¹ fluorescent light. After 7 d, the number of zones showing *P. viticola* sporulation at the sites of inoculation was counted, independently of the sporangiophore density.

S t i l b e n e a n a l y s i s : Leaves of cvs Chasselas and Solaris were inoculated with droplets of 10 ml of a zoospore suspension in water and incubated as described above. After 8, 24, 48, 72 and 96 h, three leaf pieces corresponding to the droplet surface (3 - 4 mm² and 1 - 1.5 mg fresh weight per piece) were cut and the concentration of stilbene analyzed by HPLC as described by Pezet *et al.* (2003).

Results and Discussion

A number of previous publications dealing with grapevine infected by *Botrytis cinerea* have suggested the importance of stilbenes in resistance (Langcake and McCarthy 1979; Stein and Blaich 1985; Pezet and Pont 1995; Schouten *et al.* 2002). However, little information is available concerning the role of stilbenes in the resistance of grapevines to *P. viticola*. Previously Langcake (1981) and Dercks and Creasy (1989) studied the influence of stilbenes on the release and mobility of zoospores. Our results confirm that resveratrol is the least toxic stilbene (Tabs 1 and 2) as was already demonstrated by Langcake and Pryce (1976, 1977) and Langcake *et al.* (1979), but there appears to be some divergence in the calculated ED$_{50}$ values. In addition, these authors did not publish effects of stilbenes on disease development. They estimated the ED$_{50}$ concentrations in µg ml⁻¹, which is not convenient comparing monomers to dimers: for the same weight, a dimer solution contains only half the molecules, which are the active units, compared to a monomer solution. A conversion of the values published by these authors into µM gives 877 µM for resveratrol, 27 µM for ε-viniferin, 11 µM for δ-viniferin (not identified in grapevine at that time and known as resveratrol dehydrodimer) and

T a b l e 1

Mobility of *Plasmopara viticola* zoospores suspended in increasing concentrations of the main grape leaf stilbenes. ED$_{50}$: calculation of concentration causing 50 % inhibition of zoospore mobility

Stilbenes	concentrations (µM)							ED50[b]	Corr. Coef.[b]
	1000	500	100	50	10	5	1	µM	R² =
trans-resveratrol	0[a]	0	7.3±1.6	10.0±3.6	13.0±1.2	13.0±1.2	13.3±1.6	192.00	0.9917
trans-ε-viniferin	0	0	5.3±2.3	8.0±1.7	11.3±3.2	14.0±1.0	12.3±4.0	73.00	0.9918
trans-δ-viniferin	0	0	0	0	9.6±1.5	12.0±1.7	13.0±1.0	14.60	0.9932
trans-pterostilbene	0	0	0	0	15.3±1.5	13.0±1.4	12.3±1.2	28.30	0.9281
Control Water	11.6±3.5								
Control EtOH 1%	12.3±1.1								

[a] Number of active zoospores counted on a surface of 0.11 mm² during 30 s (mean of 3 replicates + standard error).
[b] calculated from a sigmoidal Dose-Response (variable slope) [y=min+(max-min)/(1+10$^{(\log EC50-x)Hillslope}$)] (Sigmaplot regression wizard module) + correlation coefficients.

T a b l e 2

Downy mildew development (presence of sporangiophores) after inoculation of grapevine leaves (cv. Chasselas) with zoospore suspensions in the presence of various stilbenes. ED$_{50}$: calculation of concentration causing 50 % of disease expression

Stilbenes	concentrations (µM)							ED50[b]	Corr. Coef.[b]
	1000	500	100	50	10	5	1	µM	R² =
trans-resveratrol	0[a]	0	12.0±3.2	20	20	20	20	145.00	0.9833
trans-ε-viniferin	0	0	8.3±1.3	12.0±3.1	17.0±2.1	20	20	71.20	0.9967
trans-δ-viniferin	0	0	0	2.0±0.6	15.3±4.7	10.6±3.2	20	14.70	0.9866
trans-pterostilbene	0	0	0	0	17.3±3.2	12.3±5.6	11.6±6.6	12.70	0.9443
Control Water	20								
Control EtOH 1%	20								

[a] Number of sporulating colonies developed from 20 zoospore containing droplets per leaf (mean of 3 replicates + standard error).
[b] calculated from a sigmoidal Dose-Response (variable slope) [y=min+(max-min)/(1+10(logEC50-x)Hillslope)] (Sigmaplot regression wizard module) + correlation coefficients.

8.9 µM for pterostilbene. The differences with our results (Tab. 1) can be explained by the method of the antifungal bioassay used by these authors; they dried acetonic solutions of stilbenes on glass slides before adding water. This technique is not suitable for a sufficient solubilization of low polar stilbenes in water. The method described in this paper, mixing an ethanolic stilbene solution with water, allows a total solubility for resveratrol and viniferins and forms colloidal solution for the least polar pterostilbene, even at higher concentrations. One percent of ethanol in water does not affect zoospore mobility and disease development. In addition, the dilution series we have used has allowed a more precise calculation of the ED_{50} values as confirmed by the high correlation coefficient of each sigmoidal calculation curve.

In our experiments, effects of stilbenes on zoospore mobility were comparable with those of disease expression (Tab. 2), confirming that only swimming zoospores can infect leaves. However, when natural infection occurs, stilbenes are still not present in leaf cells and the mobility of zoospores is therefore not inhibited. Synthesis of stilbenes only occurs if PAL and stilbene synthase genes are induced (JEANDET *et al*. 2002), here probably by germinating encysted zoospores during *P. viticola* infection processes. In the resistant grape cvs Orion and Phoenix, infection of leaves was very similar to that observed in susceptible cvs Kerner and Riesling (KORTEKAMP *et al*. 1998), however fungal development was stopped in these resistant cultivars three d after inoculation. Our observations on Chasselas and Solaris are in agreement with this. Thus on the susceptible cv.

Chasselas, *P. viticola* produced abundant sporangiophores 6 d after inoculation, whereas only small brown spots without sporangiophores were observed on Solaris leaves at the sites of inoculation.

The analysis of stilbenes, restricted to the infected cells to avoid dilution by the surrounding healthy cells free of phytoalexins (results not shown), have shown important differences between both cultivars (Tab. 3). Forty-eight h after inoculation, the resistant cv. Solaris contained 11-fold higher levels of resveratrol and more than 60-fold higher levels of viniferins than the susceptible cv. Chasselas. Furthermore, stilbene concentrations in Chasselas were always lower than those required for *P. viticola* zoospore mobility inhibition except for δ-viniferin at 96 h post-incoculation (Tab. 1). On the contrary, stilbene concentrations in Solaris, 48 h after inoculation, were much higher than those required to inhibit infection of leaves by *P. viticola in vitro*.

The effect of stilbenes on *P. viticola* hyphae development in leaf cells is unknown; only in a speculative way, the toxicity of these compounds on mycelial hyphae and that observed *in vitro* on zoospores can be correlated. The observations of DAI *et al*. (1995) confirm that abundant hyphae were present in leaves of susceptible grapevines whereas hyphal development was limited to necrotic areas in resistant cultivars. These authors also observed an intense blue fluorescence under excitation at 365 nm in the necrotic cells, identified the presence of resveratrol and attributed the restriction of *P. viticola* development to the presence of stilbenes and also of lignin; they did not identify flavonoids.

Table 3

Concentration of stilbenes in healthy or infected leaves of cv. Chasselas (sensitive) and cv. Solaris (susceptible) 24 - 96 h after inoculation with zoospores of *Plasmopara viticola*

	Hours[a]	*trans*-resveratrol µM		*trans*-ε-viniferin µM		*trans*-δ-viniferin µM		*trans*-pterostilbene µM	
		Mean[b]	Std error	Mean[b]	Std error	Mean[b]	Std error	Mean[b]	Std error
Control Chasselas	24	9.46	0.90	0.07	0.01	0.02	0.00	0.00	0.00
	48	7.43	0.71	2.06	0.17	0.00	0.00	0.00	0.00
	72	17.59	2.69	1.17	0.05	0.97	0.02	0.00	0.00
	96	13.48	0.63	2..83	0.28	4.35	0.28	0.00	0.00
Infected Chasselas	24	10.06	0.32	13.11	0.16	0.13	0.01	0.00	0.00
	48	67.63	10.37	1.03	0.10	0.09	0.01	0.00	0.00
	72	85.67	2.19	14.42	0.26	9.23	0.29	0.00	0.00
	93	28.65	4.30	34.21	0.94	27.80	0.98	0.00	0.00
Control Solaris	24	3.89	0.78	0.00	0.00	0.03	0.01	0.00	0.00
	48	6.92	0.36	0.02	0.00	0.01	0.00	0.00	0.00
	72	8.93	0.32	2.42	0.28	4.47	0.59	0.00	0.00
	96	5.10	0.26	0.22	0.04	0.87	0.07	0.00	0.00
Infected Solaris	24	10.39	0.93	0.41	0.05	0.46	0.02	0.00	0.00
	48	762.92	5.86	61.75	0.95	74.16	1.04	0.00	0.00
	72	407.47	10.74	268.86	1.48	169.70	2.55	1.77	0.43
	96	289.49	2.05	223.99	1.36	110.15	2.78	7.75	0.45

[a] Hours after infection set.

[b] Mean of three replicates + standard error.

Our experiments have shown that in infected leaves of Chasselas, with low concentrations of stilbenes, *P. viticola* can invade tissues and produce spreading lesions and sporangiophores, indicating intracellular mycelium development. On the contrary, in Solaris, high concentrations of stilbenes were analyzed at the sites of infection where only necrotic spots were visible, indicating an inhibition of growth of *P. viticola* mycelium in a stilbene-rich environment.

While the observed resistance of different grapevine varieties to *P. viticola* may be associated in some cases with factors other than stilbenes, as suggested by Dercks and Creasy (1989), our observations demonstrate that stilbenes have significant inhibitory effects on the mobility of *P. viticola* zoospores and on subsequent disease development.

The calculated effective concentration (ED_{50}) of different stilbenes demonstrates that δ-viniferin is as toxic as pterostilbene. The interesting difference between these two stilbenes is their respective concentration in infected leaves. Concentrations of pterostilbene, if present, are very low, whereas δ-viniferin is always present and in high concentration. This new information reinforces the role of stilbenes in resistance mechanisms.

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