Vitis **43** (3), 145–148 (2004)

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

R. Pezet, K. Gindro, O. Viret and H. Richter

Agroscope-RAC, Swiss Federal Agricultural Research Station of Changins, Nyon, Schweiz

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.

Correspondence to: Dr. R. Pezet, Agroscope-RAC, Swiss Federal Agricultural Research Station of Changins, Case Postale 254, Route de Duillier, 1260 Nyon, Switzerland. Fax: +41-22-363-4394. E-mail: roger.pezet@rac.admin.ch

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 mm^2 (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.

Stilbene analysis: 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 mm^2 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

Table 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	R^2 =
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 mm^2 during 30 s (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.

Table 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	R^2 =
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 additing 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*

		trans-resveratrol µM		*trans*-ε-viniferin µM		*trans*-δ-viniferin µM		*trans*-pterostilbene µM	
	Hours[a]	Mean[b]	Std error	Mean[b]	Std error	Mean[b]	Std error	Mean[b]	Std error
Control	24	9.46	0.90	0.07	0.01	0.02	0.00	0.00	0.00
Chasselas	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	24	10.06	0.32	13.11	0.16	0.13	0.01	0.00	0.00
Chasselas	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	24	3.89	0.78	0.00	0.00	0.03	0.01	0.00	0.00
Solaris	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	24	10.39	0.93	0.41	0.05	0.46	0.02	0.00	0.00
Solaris	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.

Acknowledgements

The authors thank I. DE GROOTE for helpful technical assistance and Dr. I. B. DRY, CSIRO Plant Industry, Glen Osmond, Australia, for reviewing the manuscript. We also gratefully acknowledge financial support from the National Center of Competence in Research (NCCR): Plant Survival in Natural and Agricultural Ecosystems.

References

DAI, G. H.; ANDARY, C.; MONDOLOT-COSSON, L.; BOUBALS, D.; 1995: Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera, V. rupestris* and *V. rotundifolia* and the downy mildew fungus, *Plasmopara viticola*. Physiol. Mol. Plant Pathol. **46**, 177-188.

DERCKS, W.; CREASY, L. L.; 1989: The significance of stilbene phytoalexins in the *Plasmopara viticola*-grapevine interaction. Physiol. Mol. Plant Pathol. **34**, 189-202.

DOAZAN, J. P.; 1980: The selection of grapevine genotypes resistant to fungus diseases and their use under field conditions. In: Proc. 3rd Int. Symp. Grape Breed., 324-331. Univ. California, Davis.

GINDRO, K., PEZET, R.; VIRET, O.; 2003: Histological study of the response of two *Vitis vinifera* cultivars (resistant and susceptible) to *Plasmopara viticola* infections. Plant Biol. Biochem. **41**, 846-853.

JEANDET, P.; DOUILLET-BREUIL, A. C.; BESSIS, R.; DEBORD, S.; SPAGHI, M.; ADRIAN, M.; 2002: Phytoalexins from the *Vitaceae*: Biosynthesis, phytoalexin gene expression in transgenic pants, antifungal activity, and metabolism. J. Agric. Food Chem. **50**, 2731-2741.

KORTEKAMP, A.; WIND, R.; ZYPRIAN, E.; 1998: Investigation of the interaction of *Plasmopara viticola* with susceptible and resistant grapevine cultivars. J. Plant Dis. Prot. **105**, 475-488.

LANGCAKE, P.; 1981: Disease resistance of *Vitis* spp. and the production of the stress metabolites resveratrol, ε-viniferin, α-viniferin and pterostilbene. Physiol. Plant Pathol. **18**, 213-226.

LANGCAKE, P.; CORNFORD, C. A.; PRYCE, R. J.; 1979: Identification of pterostilbene as a phytoalexin from *Vitis vinifera* leaves. Phytochemistry **18**, 1025-1027.

LANGCAKE, P.; MCCARTHY, W. V.; 1979: The relationship of resveratrol production to infection of grapevine by *Botrytis cinerea*. Vitis **18**, 244-253.

LANGCAKE, P.; PRYCE, R. J.; 1976: The production of resveratrol by *Vitis vinifera* and other members of the *Vitaceae* as a response to infection or injury. Physiol. Plant Pathol. **9**, 77-86.

LANGCAKE, P.; PRYCE, R. J.; 1977: A new class of phytoalexins from grapevines. Experientia, **33**, 151-152.

PEZET, R.; PERRET, C.; JEAN-DENIS, J. B.; TABACCHI, R.; GINDRO, K.; VIRET, O.; 2003: δ-viniferin, α-resveratrol dehydrodimer: One of the major stilbenes synthesized by stressed grapevine leaves. J. Agric. Food Chem. **50**, 2731-2741.

PEZET, R.; PONT, V.; 1988: Mise en evidence de pterostilbene dans les grappes de *Vitis vinifera*. Plant Physiol. Biochem. **26**, 603-607.

PEZET, R.; PONT, V. R.; 1995: Mode of toxic action of *Vitaceae* stilbenes on fungal cells. In: M. DANIEL, R. P. PURKAYASTHA (Eds.): Handbook of Phytoalexin Metabolism and Action, 317-331. Marcel Dekker Inc. New-York, Basel, Hong-Kong.

PONT, V.; PEZET, R.; 1990: Relation between the chemical structure and the biological activity of hydroxystilbenes against *Botrytis cinerea*. J. Phytopathol. **130**, 19-30.

SCHOUTEN, A.; WAGEMAKERS, L.; STEFANATO, F. L., VAN DER KAAIJ, R. M.; VAN KAN, J. A. L.; 2002: Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase. Mol. Microbiol. **43**, 883-894.

STEIN, U.; BLAICH, R.; 1985: Untersuchungen über die Stilbenproduktion und Botrytisfälligkeit bei *Vitis*-Arten. Vitis **24**, 75-87.

Received April 28, 2004