Vitis **45** (4), 191–196 (2006)

Histological and biochemical criteria for objective and early selection of grapevine cultivars resistant to *Plasmopara viticola*

K. Gindro, J. L. Spring, R. Pezet, H. Richter and O. Viret

Swiss Federal Research Station for Plant Production of Changins (RAC), Nyon, Switzerland

Summary

Grapevine breeding is the most effective way to create cultivars resistant to downy mildew (*Plasmopara viticola*), and to reduce the number of fungicide applications.

Four criteria, including histological and biochemical analyses, based on the level of different mechanisms of resistance to grapevine downy mildew, were tested on 42 different cultivars. Plantlets were artificially inoculated with downy mildew and the sporangia density was measured spectrophotometrically 6 d after infection. Callose synthesis in stomata and δ- and ε-viniferin levels at the site of infection were recorded 48 h after inoculation. These observations have allowed the 42 cultivars to be divided into 5 groups: very resistant (VR), resistant (R), less susceptible (LS), susceptible (S) and highly susceptible (HS). All 4 criteria have to be applied to assign the resistance level closer to field conditions. This method allows to rapidly evaluate the level of resistance of seedlings to downy mildew thereby leading to a reduction in duration of the breeding program by several years.

K e y w o r d s : *Plasmopara viticola*, callose, stilbene, *Vitis vinifera*, breeding.

Introduction

Downy mildew (*Plasmopara viticola* (Berk. and M.A. Curtis) de Bary) is one of the most serious diseases in vineyards worldwide. In Switzerland, field observations have shown that downy mildew infection was rated as severe for 25 out of the last 50 years. Depending on meteorological conditions, 8-10 fungicide applications are necessary to control this disease (Viret *et al.* 2001). Chasselas, Gamay and Pinot noir, the major varieties in Swiss vineyards, are highly susceptible to *P. viticola*. The number of fungicide applications can be reduced only by an efficient forecast of downy mildew infection and special cultural practices such as integrated pest management (Viret *et al.* 2001).

Although a long-term endeavor, traditional breeding for resistance is an excellent way to create fungus resistant grape cultivars which allow to reduce the number of fungicide applications. Grape metabolic reactions induced in response to biotic stresses may be used as criteria to rapidly evaluate disease resistance in seedlings of progeny populations. For example, callose synthesis in stomata (Gindro *et al*. 2003), stilbene production at the site of *P. viticola* infection, or induced peroxidases (Kortekamp and Zyprian 2003) were thought to be of particular importance (Dercks and Creasy 1989; Pezet *et al*. 2003; Pezet *et al*. 2004 b; Hammerschmidt 2004).

In recent years, histological observations of callose and qualitative and quantitative micro-analysis of stilbenes in grape leaves some hours after artificial inoculation of *P. viticola*, as well as evaluation of sporangial density (Pezet *et al.* 2004 a) have been used to select resistant seedlings.

In Switzerland, a *V. vinifera* breeding program has released the red cultivars Gamaret and Garanoir (Gamay x Reichensteiner), which are highly resistant to *Botrytis cinerea.* However, to date, no red cultivars with resistance to downy mildew and with satisfactory wine quality have been bred. Therefore, a large number of crossings have been realized and selected the first year for their resistance potential. This paper describes the use of metabolic resistance criteria that have been used for the early selection of resistant seedlings from our cross-breeding program, to obtain new red and white cultivars with resistance to downy mildew.

Material and Methods

Cuttings were obtained from grapevines (*Vitis vinifera* L. cvs and interspecific grape cvs) of the experimental vineyards of Agroscope-RAC. Rooted plants were grown in glasshouse as described before (Pezet *et al*. 2004 a). At the 10-developed-leaf-stage they were placed in a growth chamber and kept at a photoperiod of 16 h (22 °C) and a dark period at 18 °C. Relative humidity was 60 %. For inoculum, leaves infected with *P. viticola* were harvested in a vineyard in Perroy (canton Vaud, Switzerland) and sporangia were collected by vacuum aspiration, as described by Pezet and Pont (1990). They were stored in cryotubes at -80 °C until use. Eight cultivars, Solaris, Bronner, Johanniter, Seyval blanc, Pinot noir, Gamaret, Gamay and Chasselas, known to differ in their susceptibility to downy mildew (Pezet *et al*. 2004a) were chosen as references. The other 34 cultivars tested in this work represent new germplasms, whose level of resistance to downy mildew had not been determined under field conditions. Their origin and characteristics are described in Tab. 1.

D e t e r m i n a t i o n o f c a l l o s e p r o d u c t i o n : Callose synthesis in stomata was observed and quantified as described by Gindro *et al*. (2003). Thin sections of leaves,

Correspondence to: Dr. K. Gindro, Swiss Federal Research Station for Plant Production of Changins, Secteur 1 - Mycologie, Postal box 254, 1260 Nyon 1, Switzerland. Fax: +41-22-362-1325. E-mail: katia.gindro@rac.admin.ch

Table 1

Origin, parentage, characteristics and resistance level of cultivars

No.	RL	Cultivars	RW	Breeder	Parentage
1	nd	IRAC 2021	R	Agroscope RAC (CH)	Bronner x Gamaret
2	nd	IRAC 2074	W	Agroscope RAC (CH)	Bronner x Gamaret
3	nd	IRAC 1999	R	Agroscope RAC (CH)	Gamay x Solaris
4	+++	Solaris	W	Freiburg (D)	Merzling* x (Saperavi severneyi x Muscat ottonel)
5	+++	Bronner	W	Freiburg (D)	Merzling* x (Saperavi severneyi x St. Laurent)
6	nd	IRAC 2027	R	Agroscope RAC (CH)	Bronner x Gamaret
7	nd	IRAC 1933	R	Agroscope RAC (CH)	Bronner x Cornalin
8	nd	IRAC 2091	R	Agroscope RAC (CH)	Gamaret x Bronner
9	nd	IRAC 1997	R	Agroscope RAC (CH)	Gamaret x Solaris
10	nd	IRAC 2213	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
11	nd	IRAC 2062	R	Agroscope RAC (CH)	Bronner x Gamaret
12	nd	IRAC 2261	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
13	nd	IRAC 2014	R	Agroscope RAC (CH)	Bronner x Gamaret
14	nd	IRAC 2060	W	Agroscope RAC (CH)	Bronner x Gamaret
15	nd	IRAC 2385	R	Agroscope RAC (CH)	Garanoir x Seyval blanc
16	+	Johanniter	W	Freiburg (D)	Riesling x (SV 12481 x (Pinot gris x Chasselas))
17	nd	IRAC 2052	R	Agroscope RAC (CH)	Bronner x Gamaret
18	nd	IRAC 2003	R	Agroscope RAC (CH)	Gamaret x Solaris
19	nd	IRAC 2034	R	Agroscope RAC (CH)	Bronner x Gamaret
20	nd	IRAC 2226	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
21	nd	IRAC 2208	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
22	nd	IRAC 2055	R	Agroscope RAC (CH)	Bronner x Gamaret
23	nd	IRAC 2142	R	Agroscope RAC (CH)	Gamaret x Chambourcin
24	nd	IRAC 2020	R	Agroscope RAC (CH)	Bronner x Gamaret
25	nd	IRAC 2278	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
26	nd	IRAC 2253	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
27	nd	IRAC 2289	W	Agroscope RAC (CH)	Seyval blanc x Gamaret
28	—	Seyval blanc	W	Seyve-Villard (F)	Seibel 5656 x Rayon d'Or
29	— —	Pinot Noir	R	old cultivar	unknown
30	nd	IRAC 2276	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
31	nd	IRAC 2285	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
32	nd	IRAC 2070	R	Agroscope RAC (CH)	Bronner x Gamaret
33	nd	IRAC 1959	R	Agroscope RAC (CH)	IRAC 3219** x Solaris
34	nd	IRAC 2029	R	Agroscope RAC (CH)	Bronner x Gamaret
35	nd	IRAC 2461	R	Agroscope RAC (CH)	Rondo x Gamaret
36	nd	IRAC 1915	R	Agroscope RAC (CH)	Bronner x Cornalin
37	— —	Gamaret	R	Agroscope RAC (CH)	Gamay x Reichensteiner
38	— —	Gamay	R	old heirloom cultivar	Pinot Noir x Gouais
39	nd	IRAC 2185	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
40	nd	IRAC 2292	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
41	nd	IRAC 2239	R	Agroscope RAC (CH)	Seyval blanc x Gamaret
42	— —	Chasselas	W	old cultivar	unknown

* Merzling, Weinbauinstitut Freiburg (D), Seyval blanc x (Riesling x Pinot Gris)

** IRAC 3219, Agroscope RAC (CH), Gamay x Chancellor

RL: resistance level, +++: very resistant, ++: resistant, —: susceptible, — —: higly susceptible.

RW: W = white cultivar, R = red cultivar, nd = not detected

infected by application of 10 µl drops of an aqueous suspension of sporangia ($2x10^4$ sporangia·ml^{-1}), were cut with a razor blade. They were placed for 1 min in an aqueous solution of aniline blue (0.2 % in 5 % $NaHCO_3$) and observed with an epifluorescence microscope (Leitz filter A (UV), excitation 340 nm, emission 380 nm, stop filter LP 430 nm) according to the method of Kortekamp *et al.* (1997). Results are expressed as means with standard deviation in % of stomata with callose deposits counted on 100 infected stomata. The experiment was done in triplicate.

Stilbene analysis: Fourty-eight h post-infection (hpi), three pieces of leaf corresponding to the droplet

surface (see before), were cut from each inoculated leaf. Three replicates were made for each cultivar. Leaf samples were weighted and placed in a microfuge tube (1.5 ml) and 50 ml of MeOH were added. The tightly closed tubes were placed in a thermo-regulated shaker at 60 °C for 10 min, then placed in an ice bath for 5 min. The methanolic extracts (30 µl) were analyzed for stilbenes as described by Pezet *et al*. (2003). Results are expressed as means with standard deviation in µmol·mg^{-1} FW. Experiments were done in triplicate.

Sporangia density: Five leaf disks (diameter 1 cm) were excised from each cultivar, placed in humid chambers at room temperature and inoculated by spraying 1 ml sporangia suspension (see before) on each of the 5 leaf disks. The leaf disks were used to determine sporangial density. As control, two additional leaf disks were sprayed with sterile distilled water. Six d after inoculation, the sporulation density was measured by turbidimetry with a spectrophotometer at 400 nm, according to Gindro and Pezet (2001). For this test, each leaf disk was put in a 1.5 ml plastic tube containing 1 ml of distilled water and shaken for 1 min. The resulting sporangial suspension was collected and used for measurements. The controls were treated in the same way and used for calibration. Results are expressed as means with standard deviation in sporangia·mm^{-2}. The experiments were done in triplicate.

Results

The initial determination of the level of downy mildew resistance of new cultivars obtained by cross-breeding was made by counting the sporangial density on leaves, 6 d after inoculation with *P. viticola* (Figure, A). Cultivars were

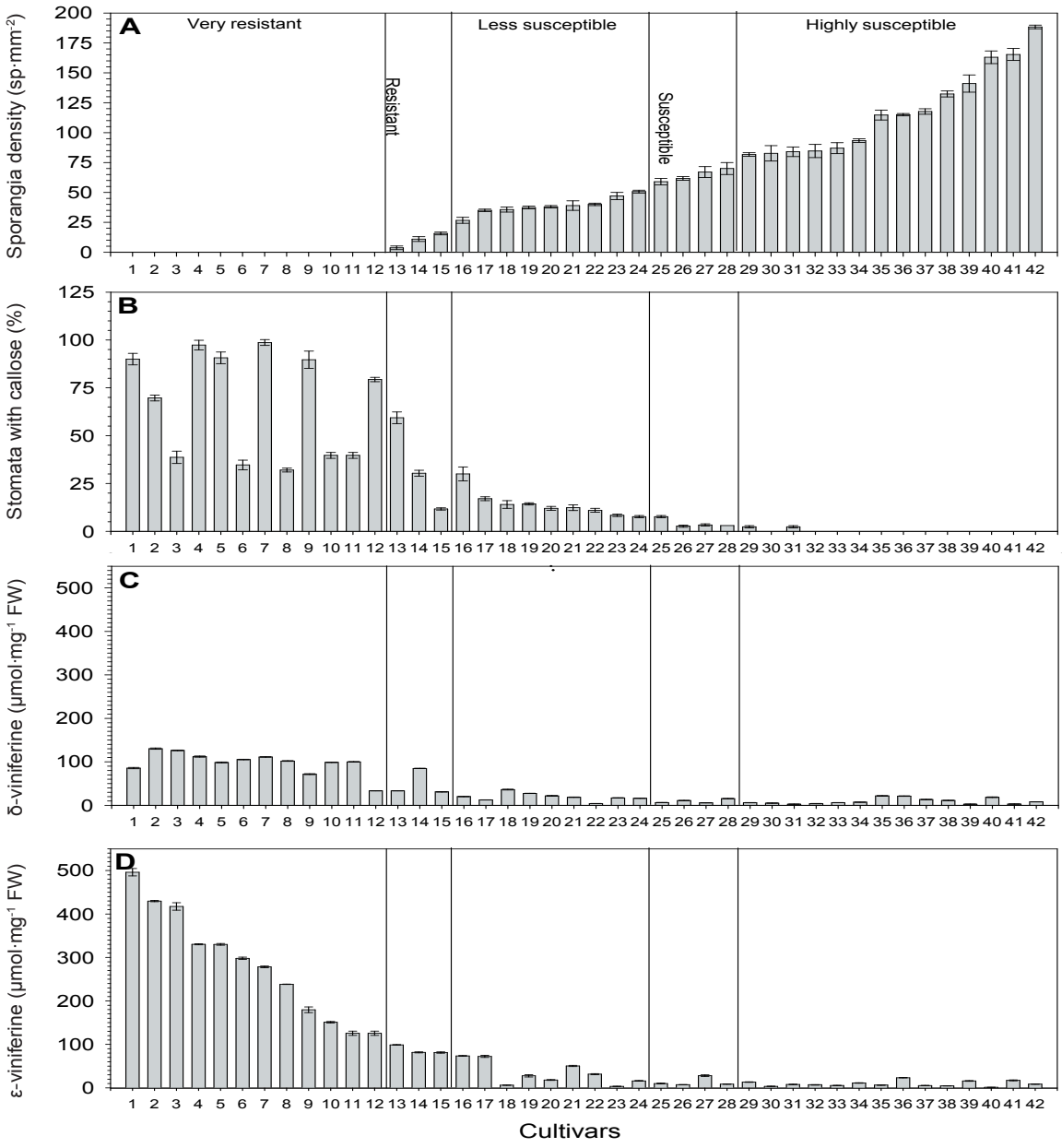

Figure: **A**: Sporangia density in artificially inoculated leaves of various grapevine cultivars 6 d after inoculation. Each value is expressed as mean of 3 replicates with standard deviation. **B**: Percentage of stomata having synthesized callose 48 h after infection of leaves. **C**: Concentration of δ-viniferin in leaves at the site of infection 48 h after infection. **D**: Concentration of ε-viniferin in leaves at the site of infection 48 h after infection.

ranked from 1 to 42 according to the density of sporangia observed. Twelve of the cultivars tested did not produce any sporangia and displayed only necrotic spots at the site of infection. These cultivars (numbered 1-12; Tab. 1) included the control cvs Solaris and Bronner and were classified as very resistant cultivars (VR).

The remaining 30 cultivars tested demonstrated at least some level of sporulation and there appeared to be a continuum of downy mildew sensitivities, based on sporangia density, from the resistant cv. (R) IRAC 2014 which had only 5 sp·mm^{-2} up to the highly susceptible cv. (HS) Chasselas with a sporangia density of 185 sp·mm^{-2}. Each of these 42 cultivars were then examined for the production of various host factors such as callose (Figure, B) and stilbenes (Figure, C, D) in response to downy mildew inoculation to determine the potential role of these host resistance mechanisms to the observed level of downy mildew susceptibility.

The synthesis of callose in stomata at the sites of infection is considered a characteristic of resistant cultivars. This is confirmed by our observations, showing the presence of abundant callose within the stomata of resistant cultivars but little or no callose in those of highly susceptible cultivars. Very resistant cultivars (cvs 1-12) were characterized by the presence of callose in at least 30 % of their stomata. The only exception to this rule was IRAC 2014 (cv. 13) which was found to have callose in 60 % of stomata but still showed a very low level of sporangia production.

Previous results have shown that the phytoalexins ε-and δ-viniferin are toxic to *P. viticola.* The possible role of these phytoalexins in the degree of resistance observed in these 42 cultivars was determined by measuring the levels of viniferin at the sites of inoculation 48 h post-inoculation (hpi) (Figure, C, D). Eleven of the resistant cultivars were found to have concentrations of δ-viniferin of at least 80 µmol mg^{-1} FW whereas IRAC 2261(cv. 12) contained only 30 µmol·mg^{-1} FW of δ-viniferin (Figure, C). All the susceptible cultivars, except for IRAC 2060 (cv. 14), were found to have δ-viniferin levels of ≤35 µmol mg^{-1} FW of δ-viniferin at the site of inoculation.

A similar relationship was observed between resistance to downy mildew and the minimum level of ε-viniferin synthesis at the site of infection (Figure, D). Resistant cultivars were found to contain ε-viniferin at concentrations of ≥110 µmol·mg^{-1} FW whereas susceptible cultivars contained ε-viniferin at levels of ≤100 µmol·mg^{-1} FW. We have established thresholds for the 4 criteria that characterize the 5 categories of cultivars (Tab. 2). No sporulation must appear on the leaves of VR cultivars and consequently, the limit for this criteria is 0. In addition, no less than 30 % of stomata must contain callose and the lower limit for δ-viniferin is fixed at 80 µmol and for ε-viniferin at 100 µmol·mg^{-1} FW. Eleven cvs (1-11) fulfilled these conditions. Only one (cv. 12) contained a lower δ-viniferin concentrations (35 µmol·mg^{-1} FW), but the three other criteria are in the VR (very resistant) category (Figure). The thresholds for the other groups, R (resistant), LS (less susceptible), S (susceptible) and HS (highly susceptible) were established according at first to the level of sporulation and then to the other criteria. Some of the cultivars are positioned near the border of one or another category. For example the cv 13 (R) which presents a weak sporulation level, cannot be placed in the VR category, even if the percentage of stomata with callose is higher than the that required for VR cultivars. On the base of these considerations we propose thresholds for each criterion, allowing a rapid and safe classification of the cultivars tested according to the methods described here (Tab. 2).

Discussion

Resistance to *P. viticola* has been observed in many *Vitis* species, especially native North American species, which have been commonly used as the source of resistance in grape breeding programs (Doazan 1980). A large number of different genotypes is generated from each cross between different grape varieties and a method for the rapid estimation of the downy mildew resistance level at the seedling stage would significantly reduce the time and effort required for selection of resistant progeny. Our results demonstrate that artificial inoculation of seedlings with *P. viticola* sporangia and estimation of disease development one week after incubation is a very efficient method for screening progeny populations for downy mildew resistance. The production and the density of sporangia arising from these artificial inoculations are widely acknowledged as an important indicator of a resistance estimation of grapevine (Dercks and Creasy 1989, Dai *et al.* 1995, Liu *et al.* 2003). However, using only sporulation as a criterion to test the resistance of potted grape cuttings in climate chambers or glasshouses may not always be representative of the real level of field resistance. Therefore, other resistance criteria must also be checked to give satisfactory con-

Table 2

Amounts of sporangia density, callose and viniferins of 5 different groups of resistance

Level of resistance	Sporangia density (sp·mm^{-2})	Callose (% of stomata)	δ-viniferin (µmol·mg^{-1} FW)	ε-viniferin (µmol·mg^{-1} FW)
Very resistant (VR)	0	≥30	≥80	>100
Resistant (R)	>0 and <15	≥15 and <30	≥40 and <80	≥50 and <90
Less susceptible (LS)	≥15 and <50	≥6 and <15	≥20 and <40	≥25 and <50
Susceptible (S)	≥50 and <80	≥2 and <6	<20	<25
Highly susceptible (HS)	≥80	<2	<20	<25

cordance between the downy mildew resistance observed in glasshouse and that observed in the field. Interestingly, Seyval blanc (cv. 28) which has been considered resistant based on field observations was found to be susceptible under our experimental conditions suggesting that the conditions used in these experiments are more severe than those normally experienced in the field.

Previous work has demonstrated that two biochemical processes are indicative of downy mildew resistance levels of grapevines. One is the synthesis of callose in stomata 7 hpi with *P. viticola* zoospores (Gindro *et al.* 2003) and the second is the synthesis of resveratrol and its subsequent oxidation in ε- and δ-viniferins (Langcake 1981, Pezet *et al.* 2003, Pezet *et al.* 2004 a).

Callose, a sugar polymer of (1-3)-β-D-glucose subunits, is a well known constituent of papillae produced in response to fungal infection (Aist 1976). When attacked, plants physically reinforce their cell wall to reduce the rate of pathogen penetration (Maor and Shirasu 2005). As demonstrated previously (or completely block), the rapid synthesis of callose deposits in stomata play an important role in the resistance of grapevine leaves soon after *P. viticola* infection (Gindro *et al.* 2003). This phenomenon stops downy mildew penetration *via* stomata and is only detectable in resistant cultivars. The results presented in this paper further demonstrate that the number of stomata in grapevine seedlings with callose deposition 48 hpi is also well correlated with the resistance to downy mildew following artificial inoculation.

In addition, resistant cultivars were found to rapidly accumulate high levels of oxidized derivates of resveratrol in the leaves at the site of infection, wheras resveratrol was glycosylated to piceide in susceptible grapevines (Pezet *et al.* 2004 a). One of the oxidation products of resveratrol was determined by Langcake (1981) 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). In downy mildew sensitive cultivars, resveratrol was found to be glycosylated to form piceide. The addition of glucose to resveratrol protects it from further oxidation (Regev-Shoshani *et al.* 2003). This is particularly important when we consider the different toxicities of various stilbenes in stressed leaves. Glycosylated resveratrol (piceide) is not toxic to *P. viticola* zoospores wheras ε- and δ-viniferin are highly toxic (Pezet *et al.* 2004 b). Our results confirm that qualitative and quantitative analysis of stilbenes in the leaves of grapevine seedlinds 48 hpi are also highly predictive of the level of downy mildew resistance of grape genotypes. Increased phytoalexin biosynthesis has also been correlated with enhanced resistance of other plant species to several pathogenic fungi (Maor and Shirasu 2005).

In summary, we have described 4 criteria which can be applied in the laboratory to select, at the seedling-stage, downy mildew resistant cultivars arising from cross-breeding. This method allows for the rapid selection of potentially resistant progeny within a segregating population at the seedling stage which can then be planted in the vineyard for further agronomical and oenological evaluation.

The use of this method will reduce the time span of the whole breeding program by several years and will replace time consuming steps and inconsistent observations often obtained under field conditions.

Acknowledgements

The authors thank Dr. I. Dry (Horticulture Unit at CSIRO's Plant Industry Division, Adelaide, Australia) for critically reading the manuscript, Mrs. I. De Groote and Mr. J. Taillens for helpful technical assistance. We also gratefully acknowledge financial support from the National Center of Competence in Research (NCCR): Plant Survival in National and Agricultural Ecosystems.

References

Aist, J. R.; 1976: Papillae and related wound plugs of plant cells. Annu. Rev. Phytopathol. **14**, 145-163.

Dai, G. H.; Andary, C.; Mondolotcosson, L.; Boubals, D.; 1995: Histochemical-studies on the interaction between 3 species of grapevine, *Vitis vinifera, Vitis rupestris* and *Vitis rotundifolia* and the downy mildew fungus, *Plasmopara viticola*. Physiol. Mol. Plant Pathol. **46** (3), 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.; 2001: Effects of long-term storage at different temperatures on conidia of *Botrytis cinerea* Pers.: Fr. FEMS Microbiol. Lett. **204**, 101-104.

Gindro, K.; Pezet, R.; Viret, O.; 2003: Histological study of the responses of two *Vitis vinifera* cultivars (resistant and susceptible) to *Plasmopara viticola* infections. Plant Physiol. Biochem. **41**, 846-853.

Hammerschmidt, R.; 2004: The metabolic fate of resveratrol: Key to resistance in grape? Physiol. Mol. Plant Pathol. **65**, 269-270.

Kortekamp, A.; Wind, R.; Zyprian, E.; 1997: The role of callose deposits during infection of two downy mildew tolerant ant two-susceptible *Vitis* cultivars. Vitis **36** (2), 104-104.

Kortekamp, A.; Zyprian, E.; 2003: Characterization of *Plasmopara*-resistance in grapevine using *in vitro* plants. J. Plant Physiol. **160**, 1393-1400.

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

Liu, S.M.; Sykes, S.R.; Clingeleffer, P.R.; 2003: A method using leafed single-node cuttings to evaluate downy mildew resistance in grapevine. Vitis **42**, 173-180.

Maor, R.; Shirasu, K.; 2005: The arms race continues: Battle strategies between plants and fungal pathogens. Curr. Opinion Microbiol. **8**, 399-404.

Pezet, R.; Pont, V.; 1990: Ultrastructural observations of pterostilbene fungitoxicity in dormant conidia of *Botrytis cinerea*. J. Phytopathol. **129**, 19-30.

Pezet, R.; Perret, C.; Jean-Denis, J. B.; Tabacchi, R.; Gindro, K.; Viret, O.; 2003: δ-Viniferin, a resveratrol dehydrodimer: One of the major stilbenes synthesized by stressed grapevine leaves. J. Agric. Food Chem. **51**, 5488-5492.

Pezet, R.; Gindro, K.; Viret, O.; Richter, H.; 2004 b: Effects of resveratrol, viniferins and pterostilbene on *Plasmopara viticola* zoospore mobility and disease development. Vitis **43**, 145-148.

Pezet, R.; Gindro, K.; Viret, O.; Spring, J. L.; 2004 a: Glycosylation and oxidative dimerization of resveratrol are respectively associated to sensitivity and resistance of grapevine cultivars to downy mildew. Physiol. Mol. Plant Pathol. **65**, 297-303.

REGEV-SHOSHANI, G.; SHOSEYOV, O.; BILKIS, I.; KEREM, Z.; 2003: Glycosylation of resveratrol protects it from enzymic oxidation. Biochem. J. **374**, 157-163.

VIRET, O.; BLOESH, B.;TAILLENS, J.; SIEGFRIED, W.; DUPUIS, D.; 2001: Prévision et gestion des infections du mildiou de la vigne (*Plasmopara viticola*) à l'aide d'une station d'avertissement. Rev. Suisse Vitic. Arboric. Hortic. **33**, 1-12.

Received March 13, 2006