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Resveratrol and the viniferins, their application to screening for disease resistance in grape breeding programs

by

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Resveratrol und die Viniferine — ihre Verwendbarkeit bei der Bonitierung der Pilzresistenz im Rahmen von Rebenzüchtungsprogrammen

Zusammenfassung. — Die Ergebnisse von Langcake und Price bezüglich der Phytoalexinbildung bei Reben wurden bestätigt; entsprechende Befunde ließen sich auch bei einer Ausdehnung der Untersuchungen auf Rebenarten, die eine hohe Pilzresistenz besitzen, aufzeigen. Bei diesen Species wurde sowohl innerhalb der Klone wie auch zwischen den Klonen eine abgestufte Resveratrolbildung beobachtet, die in Beziehung zum Auftreten von Pilzkrankheiten stand. Die angewandte Technik erfüllt die Anforderungen, die an eine Bonitierungsmethode zu stellen sind. Resveratrol und die Viniferine konnten nunmehr gesondert bestimmt werden. Hierzu sind nur geringe Mengen von Rebenmaterial erforderlich, und mittels Dünnschichtchromatographie in Verbindung mit UV- oder Fluoreszenzspektrophotometrie lassen sich rasch quantitative Ergebnisse erzielen.

Die Verwendung von UV-Licht zur Induktion der „Streßmetabolite" würde den Züchter der Notwendigkeit entheben, Pilzkulturen für Inokulationstests zu halten. Zum besseren Verständnis der Rolle, die diese Verbindungen bei der Pilzresistenz spielen, sind ebenso wie bezüglich ihrer Verwendung zur Resistenzvorhersage weitere Untersuchungen erforderlich.

Introduction

In breeding for resistance to any organism, the fundamental task is to develop a methodology which will identify susceptible and resistant individuals. The most frequent method of identifying disease susceptible progeny is to inoculate them with the disease organism in question and to monitor the development of the disease. The environment may or may not be modified in order to maximize the development (4). In breeding programs the most common practice is to inoculate young seedling populations while they are growing in a greenhouse, so that susceptible progeny may be identified and eliminated at an early stage. In our efforts to breed for increased levels of resistance to *Uncinula necator* (powdery mildew, oidium) at the New York State Agricultural Experiment Station this procedure has been used (1) and has been reported to predict the incidence of disease in seedling populations after transplanting to the field (2). The latter study, however, involved young vines growing in a nursery, an environment much different than that found in normal vineyard situations.

To further test the efficacy of the greenhouse inoculation screening procedure, the field incidence of powdery mildew in progenies from several crosses which had been previously screened in the greenhouse was rated for two consecutive growing seasons. During these two years the only fungicide used in the vineyard was Captan,

Table 1

Field incidence of powdery mildew in progeny from 30 grape crosses previously rated for powdery mildew susceptibility in the greenhouse

Das Auftreten von Oidium im Freiland bei den aus 30 Kreuzungen hervorgegangenen Rebsämlingen, die zuvor im Gewächshaus auf ihr Verhalten gegen Oidium geprüft worden waren

Field rating	Greenhouse rating		
	Resistant n (%)	Susceptible n (%)	Total n (%)
Resistant — n (%)	274 (20)	270 (20)	544 (40)
Susceptible — n (%)	256 (19)	557 (41)	813 (60)
Total — n (%)	530 (39)	827 (61)	1357 (100)

which has little, if any, effect on *U. necator*. The incidence of powdery mildew on the fruit and foliage was rated three times during the growing season using a five point scale with a rating of 1 indicating no infection and 5 severe infection (1).

In the initial screening 530 (39%) of 1357 seedlings had been rated as resistant (Table 1). When the field incidence of powdery mildew in these 530 vines was rated, only 274 (52%) were free of the disease. In the entire population 40% of the vines were rated as field resistant so the screening procedure resulted in only a 10% enrichment in resistant progeny. The benefit of eliminating the greenhouse susceptible progeny would have been to eliminate 827 progeny from the test which would have increased the apparent resistant population by only 10%. Thus, the technique as presently used does not seem to significantly contribute to our breeding program and is not currently used.

The failure of the screening technique to adequately identify susceptible progeny leaves us with two options, 1. develop new inoculation procedures or identify incubation conditions which will improve our ability to identify susceptible materials, or 2. search for another basis upon which susceptible progeny may be identified. In New York both of these options are being pursued. The first by Dr. H. Aldwinckle and the second, our efforts which form the basis for this paper.

In 1976, Langcake and Pryce reported that a fluorescent compound was formed by grape leaves following challenge by *Botrytis cinerea* (7). This compound was isolated and shown to be the stilbene, *trans*-resveratrol. They also reported that, while resveratrol was found only in infected leaves, it was constitutive in grape canes. Resveratrol was tested for its ability to suppress germination, growth or zoospore motility of *B. cinerea, Cladosporium cucumerinum, Piricularia oryzae, Fusarium oxysporum* and *Plasmopara viticola*. It did not prove to be a very potent microstat (7).

In a second paper the same authors extended this work and showed that in addition to resveratrol, three other fluorescent compounds were formed in response to *Botrytis* infection. These were isolated and two of them were shown to suppress growth or germination of the organisms tested above. Because of this activity it was proposed that they constituted a new class of phytoalexins, the viniferins. The two were named α- and ε-viniferin. The structure of ε-viniferin was proposed as

being a dimer (8) and *a*-viniferin a trimer (10) of resveratrol. A separate paper reported that viniferins were induced in response to irradiation by short wave UV radiation (9)[1]).

Because these data suggested a possible mechanism for resistance of grape leaves to fungus attack, we began to study the relationship between resveratrol and its associated compounds in relation to disease resistance in grapevines. Because previous work has been almost exclusively on *V. vinifera* and because any program with the goal of producing disease tolerant clones must involve other grape species we have concentrated our efforts on non-*vinifera species*. These appear to have significantly different resistance interaction mechanisms than *V. vinifera* (3). The thrust of our efforts has been to investigate the potential use of these compounds in a screening program to identify disease resistant progeny.

Materials and methods

Extraction, isolation and identification of resveratrol and ε-viniferin

Plant tissue (fresh leaves or freeze-dried xylem from canes) was ground and extracted with 70% methanol. Methanol was distilled off *in vacuo*, the residue taken up in water, and partitioned into ethyl acetate. For gas chromatography (GC), the trimethylsilyl (TMS) derivatives were made. Octacosane was added before partitioning and served as an internal quantitative standird for GC. In addition to GC. LH-20 column, high pressure liquid (HPLC) and thin layer (TLC) chromatography were used to separate resveratrol and ε-viniferin. The validity of the standard methodology was verified using resveratrol purified from grape canes. Following partition, the cane extract was purified by LH-20 chromatography, HPLC and TLC (Table 2). UV absorption spectra matched that reported (9) and GC : mass spectroscopy of the TMS derivative confirmed resveratrol. Similarly, ε-viniferin was confirmed by LH-20 chromatography, HPLC, TLC, UV spectroscopy and direct inlet mass spectroscopy. Quantification was by UV absorption or by peak area determination (GC).

Resveratrol concentrations in canes (xylem) of grape species

To investigate the potential relationship between constitutive resveratrol concentration in wood and the disease resistance of grape foliage in diverse species, internode pieces from the mid-cane region of the following vines growing at the New York State Agricultural Experiment Station were collected in January: *V. andersonii, V. argentifolia, V. berlandieri, V. champini, V. cinerea, V. cordifolia, V. labrusca, V. longii, V. riparia, V. rubra, V. rupestris, V. treleasei, V. vinifera* cv. Sultanina. The *V. vinifera* wood was taken from dormant vines growing in a ground bed in a cool greenhouse, other wood was collected from 8-year-old vines growing in the field. All vines except *V. vinifera* received no sprays which would control powdery mildew and mature foliage infection was rated in September using a five point scale (1 = no infection to 5 = severe infection).

[1]) More recently, the influence of various inducer substances on resveratrol excretion by wounded leaves was investigated by Blaich, R. and Bachmann, O., 1980: Die Resveratrolsynthese bei Vitaceen. Induktion und zytologische Beobachtungen. Vitis 19, 230—240.

Table 2

Thin layer chromatography R_f values of *trans*-resveratrol and ε-viniferin
R_f-Werte von *trans*-Resveratrol und ε-Viniferin bei Dünnschichtchromatographie

Adsorbant	Solvent	R_f *trans*-resveratrol	R_f ε-viniferin
Silica gel	TEFFA[1]) (5:4:1)	0.36	0.22
Silica gel	Methylene chloride : methanol (4:1)	0.80	0.80
Cellulose	50 % methanol	0.40	0.80

[1]) TEFFA = toluene : ethyl formate : formic acid.

Resveratrol induction by short wave UV radiation

UV radiation was reported to induce resveratrol production by vine leaves, but only when the lower (abaxial) surface was exposed (9). This was tested by taking 14 mm leaf disks from mature leaves of a vine growing in the greenhouse. Disks were floated on water in petri dishes with either their adaxial or abaxial surfaces uppermost. Four dishes were continuously incubated in the dark and four each of ad- and abaxial exposed surface dishes were exposed to short wave UV radiation (0.6 m W/cm^2) for 10 min and then incubated in the dark. After 48 h, the disks were frozen, ground and resveratrol was extracted. The resveratrol content was measured by GC.

Long and short term resveratrol production following exposure to UV radiation

14 mm disks taken from mature, greenhouse grown *V. rupestris* leaves were incubated after a 10 min exposure of the abaxial surface to UV radiation. Resveratrol was extracted at 0, 1.5, 3, 4.5, 6, 9 and 12 h after exposure, or, in a separate experiment, at 0, 1, 2, 3, 4, 5, 6 and 8 d after exposure. All extractions were performed in duplicate and resveratrol was quantified by GC.

Resveratrol and ε-viniferin production following inoculation with a spore suspension of *B. cinerea*

The adaxial surface of leaves of a *V. riparia* vine in a growth chamber were sprayed with *B. cinerea* spores suspended in dilute malt extract broth. Disks (14 mm) were taken daily for 6 d following inoculation. Resveratrol and ε-viniferin were measured by UV absorption after extraction and separation by TLC.

The effect of leaf position on the shoot on resveratrol production following leaf exposure to UV light

14 mm disks were taken from successivly older leaves on shoots of *V. riparia* and *V. rupestris* growing in the greenhouse. The youngest leaf selected was the smallest that would still provide 5 disks. The disks were floated on 0.1 M sucrose, given a 10 min exposure to UV radiation and incubated in the dark. Following extraction and derivatization, resveratrol concentration was determined by GC.

Resveratrol production following inoculation with *B. cinerea* spores in leaves of different ages and in plants of differing susceptibility

Two different vines growing in the greenhouse were used. One vine was a clone of *V. cinerea* and the other was a seedling from the cross Chelois × Ives (Seibel 10878 × *V. labrusca)*, which had previously been field rated as disease susceptible. The potted vines had two shoots. All leaves on one shoot/vine were sprayed with *B. cinerea* spores suspended in malt extract broth, the other shoot sprayed with M. E. B. without spores. The vines were inoculated on May 24, 1978, and necrosis began to appear 2 d later. Symptoms were most severe on younger leaves and were considerably lighter on *V. cinerea* than on the susceptible progeny. At 2 and 5 d after inoculation disks were removed from one-half of selected leaves. On the smallest leaves whole or entire halves were sampled. Resveratrol was determined by GC.

Results and discussion

Resveratrol concentration in xylem

Although the variation among samples was large, there was a relationship between resveratrol concentration and field powdery mildew infection (Table 3). However, the hypothesis that constitutive resveratrol in xylem might be positively correlated with the ability of leaves to produce resveratrol or related compounds and that this in turn might be related to disease resistance was not confirmed. More tolerant species had less rather than more resveratrol in the wood. It may be that resveratrol in the xylem reflects accumulation in response to disease infection sites on the shoots or leaves. Langcake and McCarthy indicated that resveratrol or resveratrol-like compounds are to some extent translocatable within leaves (6).

Resveratrol induction by UV light

Resveratrol concentration increased significantly only when the abaxial surface was irradiated (Table 4). This confirms the findings of Langcake and Pryce (9). The apparent but non-significant increase in resveratrol following illumination of the adaxial surface conceivably may have been due to transmission through the leaf to the lower surface or due to reflection of UV light to the lower surface.

The lack of induction by upper surface irradiation raises questions about the localization of resveratrol synthesis and accumulation within grape leaves. Several important fungi primarily attack the upper leaf surface (as *U. necator)*. Evidence suggests that unknown factors in Concord *(V. labruscana)* tend to restrict powdery mildew infection to the upper epidermal cells (5). We intend to utilize these leaves to determine the site of synthesis as well as the site of induction.

Long and short term resveratrol production following exposure to UV radiation

Resveratrol was not formed for the first 7 h following irradiation. After that time, the concentration increased linearly for 2 d (Figs. 1 and 2). After 2 d there

Table 3

Resveratrol concentrations of mature grape internode xylem
Resveratrolkonzentration im reifen Rebholz

Species	Powdery mildew infection Rating[1])	Resveratrol (mg/g)
V. champini	1	0.040
V. cinerea	1	0.030
V. riparia	1	0.028
V. rupestris	1	0.040
Mean		0.048
V. andersonii	2	0.061
V. argentifolia	2	0.030
V. berlandieri	2	0.101
V. cordifolia	2	0.043
V. cordifolia	2	0.022
V. labrusca	2	0.030
V. longii	2	0.080
V. rubra	2	0.137
V. treleasei	2	0.113
Mean		0.0684
V. cordifolia	3	0.098
V. vinifera	3—5	0.191
Mean		0.145

[1]) 1 = Most resistant, 5 = least resistant.

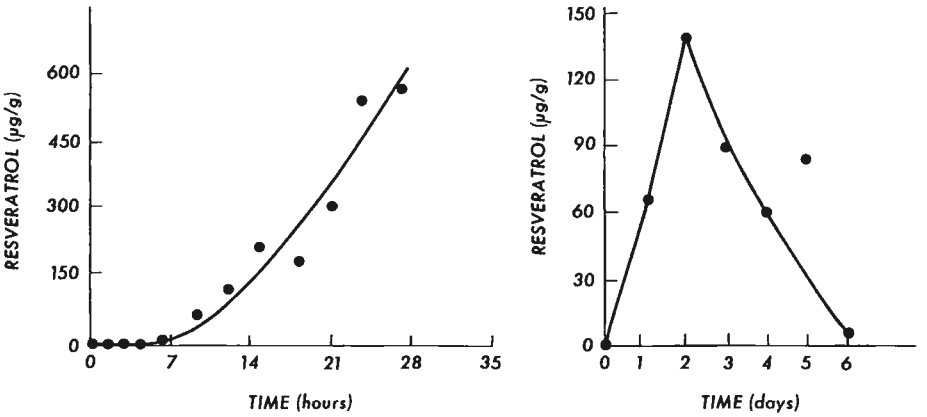
Table 4

Leaf disk resveratrol concentration 48 h after exposure of the upper (adaxial) or lower (abaxial) leaf surface to short wave UV light
Resveratrolkonzentration in Blattscheiben nach 48stündiger Bestrahlung der oberen (adaxialen) oder unteren (abaxialen) Blattseite mit kurzwelligem UV-Licht

Disk side exposed to UV light	Resveratrol (μg/g) TLC	GC
No exposure	2.86[b1])	0[b]
Adaxial	5.59[b]	6.9[b]
Abaxial	45.7[a]	55.2[a]

[1]) Within a column means with the same superscript do not differ (P = 0.05).

was a decline in resveratrol concentration. At 6 d resveratrol concentration had reached the level found before irradiation. The grossly different levels found in the two experiments are typical of our findings and those of others (9). Although the same clone was used for both experiments, there may have been differences in leaf age or other factors that were responsible for the different levels found. Regardless of absolute amount found, the time course was similar in these and other experiments.



Figs. 1 and 2: Resveratrol production by *V. rupestris* leaf disks following UV irradiation.
Die Bildung von Resveratrol durch Blattscheiben von *V. rupestris* nach UV-Bestrahlung.

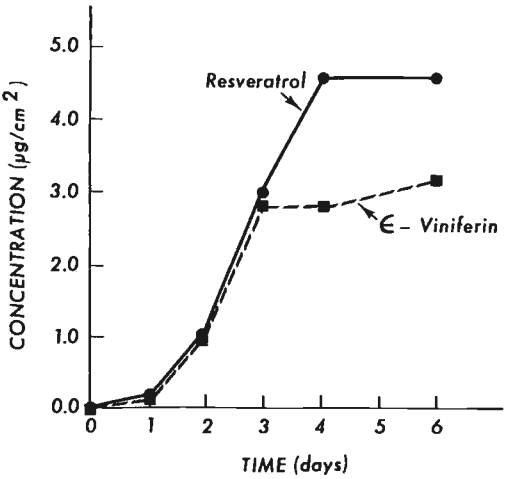


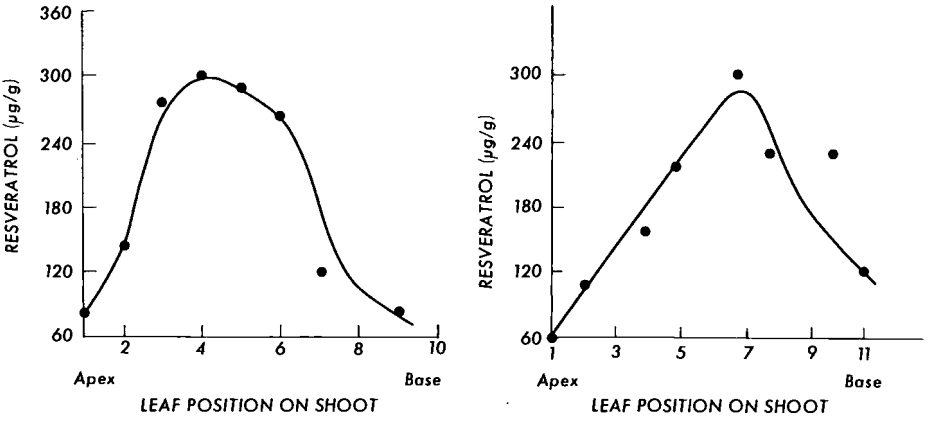
Fig. 3: Resveratrol and ε-viniferin concentration in *V. riparia* leaves following inoculation with *B. cinerea*.

Die Konzentration von Resveratrol und ε-Viniferin in Blättern von *V. riparia* nach Inokulation mit *B. cinerea*.

Time course of resveratrol and ε-viniferin production in response to *B. cinerea*

Resveratrol and ε-viniferin concentration increased 1 d after inoculation and continued to increase for several days (Fig. 3). Unlike the response to UV radiation, the concentrations did not decline, probably because in contrast to the brief irradiation, the inducing principle(s) remained present in the leaves.

The time course experiments indicate that both the parent compounds, resveratrol and its more biologically active condensation product, ε-viniferin, are produced in a time scale consistent with disease establishment and/or spread.



Figs. 4 and 5: Effect of leaf position on the shoot on resveratrol concentration in leaves of *V. rupestris* (left) and *V. riparia* (right) following irradiation with UV light.

Der Einfluß der Insertionshöhe auf die Resveratrolkonzentration in den Blättern von *V. rupestris* (links) und *V. riparia* (rechts) nach Bestrahlung mit UV-Licht.

Resveratrol production by leaves of differing ages in response to UV irradiation

Similar results were obtained on both *V. rupestris* (Fig. 4) and *V. riparia* (Fig. 5) vines. Younger leaves produced less resveratrol than recently mature leaves and older leaves in turn produced less resveratrol. This pattern is consistent with the infection pattern we find when Concord or White Riesling leaves are inoculated with *U. necator*.

Resveratrol production by leaves of different ages and susceptibilities in response to inoculation with *B. cinerea*

The pattern of production was similar between the two species tested (Fig. 6) and resembled that found in response to UV radiation (Figs. 4 and 5). Younger and older leaves produced less resveratrol than mid-shoot leaves. With leaves near the apex or at mid-shoot, resveratrol production was inversely related to disease symptom development. That is, symptoms were greatest for young leaves and less for older leaves. Similarly, symptom development was much greater and resveratrol production was much less on the susceptible seedling clone as compared to the *V. cinerea* clone.

Summary

We have confirmed the findings of Langcake and Pryce and extended them to include species which have significant resistance to disease. Using these species we have observed differential production of resveratrol both within and among clones in relation to the incidence of disease. The techniques we have used fit the requirements of a screening technique. Unlike the analytic technique most recently used by Langcake and McCarthy (6) resveratrol and the viniferins are separated in our procedures. Minimal amounts of tissue are required, and TLC combined with either

UV or fluorescence spectrophotometry give rapid quantitative results.

The use of UV to induce the production of these "stress metabolites" would free the breeder from the necessity of maintaining fungus cultures for inoculation tests. Further study must be done to elaborate the role of these compounds in disease resistance and the efficacy of their role in predicting resistance.

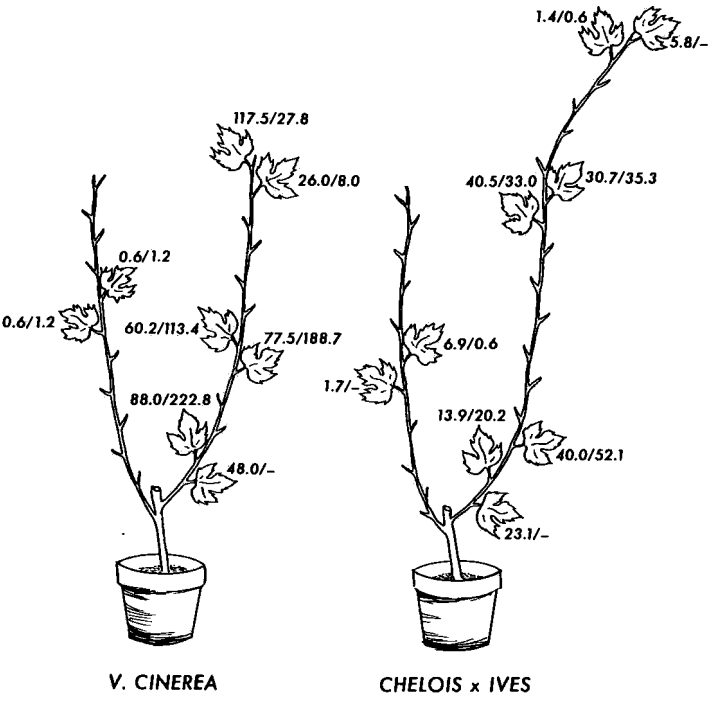


Fig. 6: Reveratrol concentration in different leaves of *V. cinerea* (more resistant) and a seedling from the cross, Chelois × Ives (less resistant) following inoculation with *B. cinerea* (right shoots only). The first number is the $\mu g/cm^2$ 2 d following inoculation and the second number is the concentration 5 d after inoculation.

Die Resveratrolkonzentration in verschiedenen Blättern von *V. cinerea* (resistent) und einem Sämling aus der Kreuzung Chelois × Ives (weniger resistent) nach Inokulation mit *B. cinerea* (jeweils nur rechter Trieb). Erste Zahl: Konzentration ($\mu g/cm^2$) 2 d nach der Inokulation; zweite Zahl: Konzentration 5 d nach der Inokulation.

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