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The relationship of resveratrol production to infection of grapevine leaves by Botrytis cinerea

by

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Die Beziehungen zwischen der Bildung von Resveratrol und dem Befall von Rebenblättern durch Botrytis cinerea

Zusammenfassung. — Das Stilben Resveratrol gehört zu den Stoffwechselprodukten, die von der Rebe unter Streßbedingungen gebildet werden. An isolierten Blättern von *Vitis vinifera* wurde die Verteilung von Resveratrol in den sich ausbreitenden Läsionen, die durch *Botrytis cinerea* verursacht werden, geprüft. In einer bis zu 5 mm breiten scheinbar gesunden Gewebezone, die den visuell geschädigten Blattbezirk umgibt, wurden sowohl die höchsten Absolutmengen als auch die höchsten Konzentrationen von Resveratrol gefunden. Die Verteilung von Resveratrol entsprach dem Auftreten einer Blaufluoreszenz, die bei langwelliger UV-Bestrahlung (366 nm) makroskopisch beobachtet werden kann.

Die Beziehung zwischen der Resveratrolkonzentration, die im Umkreis der *Botrytis*-Läsionen an Rebenblättern vorliegt, und der Anfälligkeit dieser Blätter für die Pilzinfektion wurde untersucht. Die Pilzanfälligkeit nahm mit dem Alter der Blätter ab und variierte zwischen den untersuchten Rebsorten. In beiden Fällen stand die Resveratrolkonzentration im Umkreis der Läsion im umgekehrten Verhältnis zum Anfälligkeitsgrad. Die Rolle, die Resveratrol als Präcursor pilzfeindlicher Streßmetabolite, der Viniferine, bei der Krankheitsresistenz der Rebe spielen dürfte, wird diskutiert.

Introduction

The infection of detached grapevine (*Vitis vinifera* L.) leaves by *Botrytis cinerea* Pers. results in the development of spreading soft-rot lesions. When examined under long wavelength ultra violet radiation (366 nm), these lesions are seen to be surrounded by a zone of bright blue fluorescence (Fig. 1 a). Similar blue fluorescence is evident within 24 h of a brief (ca. 5 min) exposure of the leaves to short wavelength (254 nm) radiation (Fig. 1 b). The compound responsible for this fluorescence has been identified as the simple stilbene, resveratrol (3,4',5-trihydroxy stilbene) (7). The production of resveratrol by grapevine leaves is accompanied by the production of other stress metabolites (phytoalexins), the viniferins (8). In several plant species a relationship between phytoalexin production and resistance to disease has been demonstrated and it is widely believed that such phytoalexins are responsible in certain circumstances for preventing the growth of potential pathogens within the plant (6). Circumstantial evidence that resveratrol is the biosynthetic precursor of the viniferins has been presented (9), but the relationship of the production of resveratrol to disease resistance of grapevine has not been investigated previously. The distribution of resveratrol in *B. cinerea* lesions and the relationship between the amount of resveratrol produced in such lesions and resistance of the leaf to fungal invasion has been investigated here.

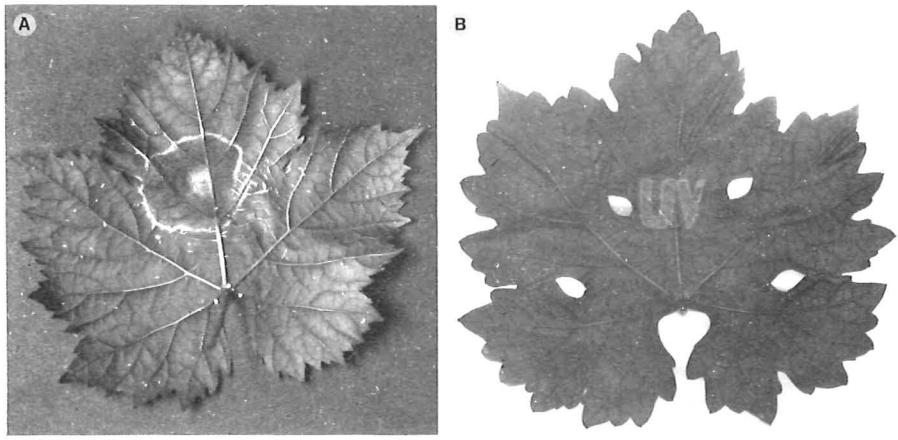

Fig. 1: Fluorescence of grapevine leaves. Photographs taken with illumination by long wavelength (366 nm) UV radiation. Blue fluorescent areas appear white. — A: Spreading lesion caused by *B. cinerea*. The rotted area is surrounded by a relatively narrow band of blue fluorescence. In some areas, streaks of blue fluorescence radiating from the lesion, principally along the veins are visible. Profuse growth of the fungus in the area of the inoculum drop (centre of the lesion) has given rise to some yellow fluorescence. — B: Grapevine leaf 24 h after a 5 min period of short wavelength (254 nm) irradiation through a card in which the letters „UV" had been cut out.

Fluoreszenz von Rebenblättern. Photographische Aufnahmen mit langwelliger UV-Strahlung (366 nm). Die Blaufluoreszenz erscheint weiß. A: Sich ausbreitende Läsion durch *B.-cinerea*-Infektion. Der nekrotische Bereich ist von einem relativ schmalen blaufluoreszierenden Saum umzogen. An einigen Stellen sind blaufluoreszierende Streifen zu erkennen, die hauptsächlich längs der Adern von der Läsion ausstrahlen. Starkes Pilzwachstum im Bereich des Inoculationstropfens (Mitte der Läsion) ist die Ursache für Gelbfluoreszenz. — B: Rebenblatt 24 h nach 5minütiger Bestrahlung mit kurzen Wellenlängen (254 nm) durch den Ausschnitt „UV" einer Karte.

Methods

Grapevine plants (vars. Clare, Gordo, Müller-Thurgau, Cabernet Sauvignon and Riesling × Sylvaner) were propagated from wood cuttings in the greenhouse and were approximately 60 cm high when used for experiments. Detached leaves were inoculated with *B. cinerea* as described previously (7). Harvested leaf tissue was weighed, then stored at −20 °C before extraction and assay for resveratrol.

Measurement of susceptibility to *B. cinerea*

Susceptibility, the extent to which a plant becomes diseased, was measured i) as fungal chitin content of the lesion (15); ii) as lesion diameter (the mean value of two readings taken at right angles to one another) and iii) as fresh weight of the excised lesion tissue.

Quantitative estimation of resveratrol

Resveratrol content of leaf samples was estimated either by GLC as described previously (7) or by the following spectrofluorometric method. Samples of tissue

from a single lesion were ground in a mortar with sand and 70 % methanol (10 ml). The homogenate was clarified by centrifugation, the supernatant was dried on a rotary evaporator and redissolved in absolute methanol (2 ml). The methanol-insoluble material was removed by centrifugation and the supernatant reduced to 0.2 ml volume. This solution was applied as a 2 cm band to the origin of a Whatman 3MM paper chromatogram (44 × 56 cm) and developed (approx. 4.5 h, descending) with 50 % aqueous methanol. The chromatogram was allowed to dry at room temperature overnight, the fluorescent area (Rf ca. 0.23) corresponding with resveratrol was located under UV light (366 nm), then cut out and eluted by standing for 5 h at room temperature in methanol (2 ml). Fluorescent intensity (arbitrary units) of the eluate was measured using an Aminco Bowman spectrofluorometer with excitation at 335 nm, emission measured at 390 nm. For each sample corrections were made for the fluorescence obtained by elution of a blank area of the chromatogram of similar size and for the fluorescence obtained in the above extraction procedure for an equivalent weight of non-fluorescent tissue taken from the same leaf. The relationship between fluorescent intensity and resveratrol concentration was not determined.

The extractions and analyses of resveratrol were carried out in subdued light to avoid *trans* to *cis* isomerisation.

Statistical analysis

The data for total resveratrol (or fluorescence) and concentration of resveratrol (or fluorescence) were transformed by $y = \log_{10}x$ before analysis in order to stabilise the variance.

The variations of susceptibility, total resveratrol (fluorescence) and concentration of resveratrol (fluorescence) with leaf position were analysed as well as the variation of concentration of resveratrol (fluorescence) with susceptibility. It seemed reasonable to assume linear relationships within each plant that were parallel but not colinear i.e. the lines for different plants were straight and had the same slope but not the same intercepts. Multiple regression analyses were therefore performed using dummy variables (cf. 16) to take account of the different intercepts.

The data of Table 4 were analysed using a randomised block analysis, regarding the replicates as blocks.

All statistical tests were two-sided and based on the Student t distribution with the appropriate degrees of freedom.

Results

Measurement of susceptibility

Grapevine leaves were found to have a remarkably consistent weight per unit area (ca. 13 mg fresh weight/cm^2) regardless of leaf age or variety. Since both the rotted area and the blue fluorescent zone pass right through the leaf from the upper to the lower surface, it seemed likely therefore that a two dimensional measure of lesion size would provide a reliable estimate of susceptibility. To test this, the relationships between chitin content, lesion diameter and lesion fresh weight were examined for a sample of 15 lesions of widely differing sizes. On the assumption that the most reliable estimate of susceptibility is the chitin content of the lesion

(15), the degree of correlation between chitin content and the other measures of susceptibility was examined by regression analyses. The best correlation was found between chitin content and lesion diameter ($r = 0.978$, $P \leqslant 1\ \%$) while the correlation between chitin content and lesion fresh weight was less good ($r = 0.958$) although highly significant. For this reason, lesion diameter was used as a measure of susceptibility throughout these experiments.

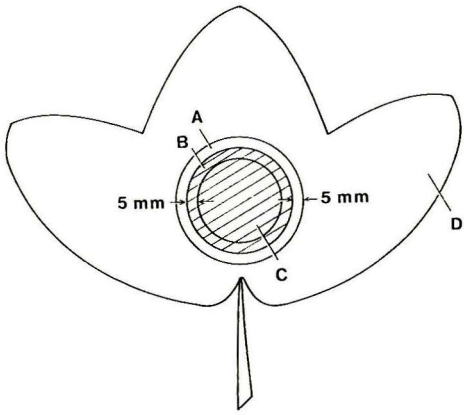

Fig. 2: Diagrammatic representation of the zones of tissue excised from *B. cinerea* lesions. See text and Table 1. The shaded area represents the brown rotted region of tissue.

Schematische Darstellung der Gewebezonen, die aus *B.-cinerea*-Läsionen isoliert wurden. S. Text und Tabelle 1. Schraffiert: Brauner, nekrotischer Gewebebezirk.

Distribution of resveratrol in spreading lesions caused by *B. cinerea*

Leaf tissue in and around lesions on grapevine leaves (var. Sultana) was excised as shown in Fig. 2, 5 d after inoculation. The resveratrol content of each of the regions (A to D) was then measured by the gas chromatographic method (Table 1). Mean lesion diameter (± standard error, $s\bar{x}$) was 30.1 ± 2.3 mm.

Table 1

Distribution of resveratrol in spreading lesions caused by *B. cinerea* on grapevine leaves (var. Sultana) · For the location of the various regions of tissue see Fig. 2 · Each result is the mean of 6 replicate determinations

Verteilung von Resveratrol in sich ausdehnenden Läsionen, die durch *B. cinerea* an Rebenblättern verursacht wurden (Sorte Sultana) · Lage der einzelnen Gewebebezirke s. Abb. 2 · Jeder Wert stellt das Mittel aus 6 Wiederholungen dar

Tissue region	Resveratrol content (µg)	Fresh weight (mg)	Resveratrol concentration (µg/g fresh weight)
A	2.56 ± 0.31	74.1	34.5
B	0.60 ± 0.14	59.1	10.2
C	0.25 ± 0.12	25.3	9.9
D	1.51 ± 0.47	584.3	2.6

Clearly, resveratrol was present predominantly in the apparently healthy tissue surrounding the lesion, the greatest concentration being in the first 5 mm outside the rotted area (region A). The resveratrol content of region D was particularly variable. In 3 of the 6 lesions examined, there was virtually no resveratrol present, but in the remaining 3 lesions there was almost as much resveratrol present as in region A. This corresponds with the variability in the width and distribution of the fluorescent zone around the lesion which can be observed macroscopically under UV light. Frequently streaks of blue fluorescence can be seen radiating from the lesion, predominantly along the major veins (Fig. 1 a).

Table 2

Variation of susceptibility (lesion diameter), total fluorescence and concentration of fluorescence (fluorescence per 10 mm lesion diameter) around lesions with leaf position for grapevine plants (var. Müller-Thurgau) infected with *B. cinerea* · Each value is the mean of 6 replicates

Anfälligkeit gegen *B. cinerea* (Läsionsdurchmesser-, gesamte Fluoreszenz im Umkreis der *B.*-Läsionen sowie Fluoreszenz je 10 mm Läsionsdurchmesser in Beziehung zur Insertionshöhe der Blätter (Sorte Müller-Thurgau) · Jeder Wert stellt das Mittel aus 6 Wiederholungen dar

Leaf position	1	2	3	4	5
Lesion diameter (mm)	19.8 ± 0.7	16.8 ± 1.6	16.1 ± 1.1	11.6 ± 1.5	9.2 ± 1.5
Total fluorescence	0.092 ± 0.020	0.117 ± 0.027	0.205 ± 0.054	0.209 ± 0.059	0.187 ± 0.065
Concentration of fluorescence	0.049 ± 0.012	0.072 ± 0.018	0.129 ± 0.032	0.187 ± 0.053	0.323 ± 0.177

Table 3

Variation of susceptibility (lesion diameter), total resveratrol content and concentration of resveratrol (resveratrol content per 10 mm lesion diameter) around the lesion with leaf position for grapevine plants (var. Sultana) infected with *B. cinerea* · Each value is the mean of 6 replicates

Anfälligkeit gegen *B. cinerea* (Läsionsdurchmesser), gesamter Resveratrolgehalt im Umkreis der *B.*-Läsionen und Resveratrolgehalt je 10 mm Läsionsdurchmesser in Beziehung zur Insertionshöhe der Blätter (Sorte Sultana) · Jeder Wert stellt das Mittel aus 6 Wiederholungen dar

Leaf position	1	2	3	4
Lesion diameter (mm)	30.3 ± 3.1	26.5 ± 3.1	22.7 ± 4.2	15.3 ± 1.4
Total resveratrol (μg)	1.53 ± 0.14	2.87 ± 0.73	3.33 ± 0.77	3.30 ± 0.44
Concentration of resveratrol (μg/10 mm lesion diameter)	0.53 ± 0.07	1.16 ± 0.30	1.63 ± 0.32	2.23 ± 0.40
Concentration of resveratrol (μg/g fresh weight)	9.5 ± 1.4	20.9 ± 6.7	23.9 ± 5.9	30.6 ± 6.1

Table 4

Variation of susceptibility (lesion diameter), total fluorescence and concentration of fluorescence (fluorescence per 10 mm lesion diameter) for grapevine leaves of different varieties infected with *B. cinerea* · Leaves were taken from position 2 · Each value is the mean of 5 replicates

Anfälligkeit gegen *B. cinerea* (Läsionsdurchmesser), gesamte Fluoreszenz sowie Fluoreszenz je 10 mm Läsionsdurchmesser bei Blättern verschiedener Rebsorten · Die Blätter stammen jeweils von Insertion 2 · Jeder Wert stellt das Mittel aus 5 Wiederholungen dar

Variety	Mean susceptibility (lesion diameter, mm)	Detransformed mean[1]) total fluorescence	Detransformed mean[1]) concentration of fluorescence
Clare	21.0	0.29	0.14
Gordo	11.2	0.65	0.60
Müller-Thurgau	21.7	0.22	0.10
Cabernet Sauvignon	18.9	0.58	0.31
Riesling × Sylvaner	20.6	0.36	0.18
LSD between two predetermined two	3.4		
LSD between any two means	4.9		
Least significant ratio between two predetermined means		2.42	2.54
Least significant ratio between any two means		3.58	3.83

[1]) Detransformed mean = $\text{antilog}_{10} \frac{(\log_{10} x_1 + \log_{10} x_2 + \ldots \log_{10} x_5)}{5}$, where $x_1, x_2 \ldots x_5$ are the 5 observations on a given variety.

Relationship between resveratrol content around lesions and susceptibility to *B. cinerea* for leaves of different ages

Consecutive leaves down the stems of each of six grapevine plants were detached. The leaf at position 1 was the youngest and was approximately 20 cm below the apex. Leaves were inoculated with *B. cinerea*. Susceptibility (lesion diameter) was measured after an incubation period appropriate for the lesions to develop (60 h for 1st experiment, 6 d for 2nd experiment), then the fluorescent tissue surrounding the lesions (approx. 10 mm zone corresponding to region A of Fig. 2) was excised and stored at −20 °C before extraction and assay for resveratrol content by the spectrofluorometric (1st experiment) or gas chromatographic (2nd experiment) methods.

The results of the two experiments (Tables 2 and 3) indicate the same conclusions. Thus, susceptibility to *B. cinerea* was found to decrease with leaf age (increasing leaf position number). Conversely, the resveratrol content per lesion (fluorescence per lesion) increased with leaf age. Since resveratrol was predominantly present as a relatively narrow zone around the periphery of the lesion, its concentration will be related to the length of the perimeter. For this reason, resveratrol concentrations were expressed in terms of lesion diameter. Accordingly, the resveratrol concentration (resveratrol content or fluorescence per 10 mm lesion diameter) was found to increase markedly with leaf age. Similar relationships were obtained when resveratrol concentrations were expressed on a fresh weight basis (Table 3).

Regression analyses of the data from the 1st experiment showed the following relationships:

i) Susceptibility $= 22.7 - 2.72\ (\pm 0.29) \cdot$ (leaf position)
ii) $\log_{10}$ (total fluorescence) $= -1.15 + 0.089\ (\pm 0.23) \cdot$ (leaf position)
iii) $\log_{10}$ (concentration of fluorescence) $= -1.57 + 0.183\ (\pm 0.020) \cdot$ (leaf position)
iv) $\log_{10}$ (concentration of fluorescence) $= -0.27 - 0.052\ (\pm 0.0093) \cdot$(susceptibility)

These relationships were similar to those found for the 2nd experiment:

v) Susceptibility $= 35.9 - 4.9\ (\pm 0.69) \cdot$ (leaf position)
vi) $\log_{10}$ (total resveratrol) $= 0.12 + 0.11\ (\pm 0.027 \cdot$ (leaf position)
vii) $\log_{10}$ (concentration of resveratrol) $= -0.47 + 0.20\ (\pm 0.028) \cdot$ (leaf position)
viii) $\log_{10}$ (concentration of resveratrol) $= 0.87 - 0.035\ (\pm 0.0052) \cdot$ (susceptibility)

For each case, the slopes of the regression lines were highly significant ($P \leqslant 0.01$). The slopes of susceptibility on leaf position differed significantly ($P \leqslant 0.05$) between the two experiments in which different varieties of grapevine were used, but this was not so for the other three relationships examined. Equations (iv) and (viii) clearly show that there was an inverse relationship between susceptibility and log of the concentration of resveratrol (fluorescence), i.e. resistance of the leaves to the spread of lesions caused by *B. cinerea* was correlated with the concentration of resveratrol produced in response to infection.

Relationship between resveratrol content around lesions and susceptibility to *B. cinerea* for leaves of different varieties of grapevine

In this experiment, the resveratrol contents of *B. cinerea* lesions on leaves of different grapevine varieties were compared. Five replicate leaves for each variety were used, each from leaf position 2 (see above). Lesion diameters (susceptibility) were measured and the fluorescent tissue surrounding the lesions (approx. 10 mm

zone corresponding to region A, Fig. 2) was excised 4 d after inoculation. Resveratrol content was estimated by the spetrofluorometric method.

The results (Table 4) show that of the five varieties examined, four varieties (Clare, Müller-Thurgau, Cabernet Sauvignon and Riesling × Sylvaner) were of similar susceptibility to *B. cinerea*. The fifth variety, Gordo, was significantly less susceptible to infection. Considerable variation between replicates was found for the values of total fluorescence and there were no significant differences between the detransformed means although the value for Gordo, the least susceptible variety, was the highest. There was somewhat less variation for the values of concentration of fluorescence where there were no significant differences between the detransformed means for the four "susceptible" varieties Clare, Müller-Thurgau, Cabernet Sauvignon and Riesling × Sylvaner. The least susceptible variety, Gordo, gave the highest value for the concentration of fluorescence and this was significantly greater than the corresponding values for the more susceptible varieties Clare and Müller-Thurgau, although not for Cabernet Sauvignon and Riesling × Sylvaner. This experiment further suggests therefore that a high concentration of resveratrol around lesions is associated with reduced susceptibility to infection by *B. cinerea*.

Discussion

The results show that in grapevine leaves of decreasing susceptibility increasing total amounts of resveratrol per lesion are produced in response to infection by *B. cinerea*. It follows therefore, given the distribution of resveratrol as indicated in Table 1, that the concentration of resveratrol around the less susceptible lesions is greater than that around the more susceptible lesions, and this was found to be the case whether resveratrol concentrations were expressed in terms of lesion diameter or fresh weight (e.g. Table 3).

The question as to whether resveratrol should be considered a phytoalexin is clearly debatable. As currently used, the term phytoalexin denotes an antimicrobial compound produced by a plant in response to infection or other forms of stress. Evidence for their role in disease resistance is in several cases strong, albeit circumstantial. Antifungal activity of resveratrol can be demonstrated (5, 7), although in several tests the compound was apparently inactive (4), while in those tests in which antifungal activity was found, there was some doubt as to whether resveratrol was the active principle (7). The term stress metabolite, as used by Stoessl *et al.* (19), circumvents some of the semantic problems associated with the term phytoalexin since it encompasses both antifungal and non-antifungal compounds produced in response to various forms of stress, including infection, and which may or may not be involved in disease resistance. In most plants which have been studied adequately, a range of biosynthetically related compounds (stress metabolites) are produced in response to infection, some of which are antifungal in tests *in vitro*. The demonstration that a stress metabolite has only weak antifungal properties in tests carried out *in vitro* does not preclude a role for such a compound in disease resistance. For example, pisatin has very little effect on the germination of spores of *Erisyphe pisi in vitro* yet is very effective in preventing the infection of pea leaves by this pathogen (12).

The most likely explanation of the relationship between resistance and resveratrol concentration is that resveratrol functions as a precursor of the viniferins.

Evidence for the role of resveratrol as a precursor of the viniferins has been presented previously (9). In addition, recent experiments (LANGCAKE, in preparation) have shown that the resistance of grapevine leaves to *B. cinerea* is correlated with the accumulation of amounts of the antifungal stress metabolite α-viniferin (14), in excess of those required to inhibit growth of *B. cinerea*. Nevertheless, the relationship between resveratrol and the viniferins is undoubtedly complex. Resveratrol is quantitatively one of the major stress metabolites of grapevine but the relative proportions of resveratrol, ε-viniferin and α-viniferin vary considerably depending on the inducing stimulus, the time at which measurements are made and the region of tissue sampled relative to the lesion. For example, in non-spreading lesions caused by *B. cinerea* on grapevine leaves, the concentration of resveratrol is very low, the predominant component being α-viniferin (LANGCAKE, unpublished). In the experiments reported here, leaves were relatively susceptible although large differences in susceptibility were noted.

The observation that resveratrol is present in the apparently healthy cells, in some cases 10 mm or more in advance of the visibly rotted area of the lesion, is relevant to the controversy as to whether stress metabolites (phytoalexins) can be produced by living cells (11). Although it is possible that resveratrol had been transported out of the lesion to these cells, the fact that in UV-irradiated leaves, resveratrol is confined to those cells which had been irradiated (Fig. 1 b) shows that this compound does not readily move in grapevine leaves. It is more likely that an elicitor had diffused in advance of the rotted area. Thus, resveratrol is probably produced in cells which are apparently healthy. Similar observations have been made with respect to the production of the phytoalexins wyerone and wyerone acid in broad bean *(Vicia faba)* (11) and phaseollin in green beans *(Phaseolus vulgaris)* (13). However, it is likely that while stress metabolites may be produced by living cells, these cells are committed to dying in view of the possible phytotoxic effects of the stress metabolites themselves (1, 3, 10, 17, 18) and this could explain the commonly observed association between phytoalexin production and necrosis (e.g. 2).

Summary

The stilbene resveratrol is one of the stress metabolites produced by grapevine (7, 8). The distribution of resveratrol in spreading lesions caused by *Botrytis cinerea* on detached grapevine leaves has been examined. Both the highest absolute amounts and the highest concentrations of resveratrol were found in the apparently healthy zone of tissue up to 5 mm in advance of the visibly rotted area. The distribution of resveratrol corresponded with the distribution of blue fluorescence observable macroscopically under long wavelength (366 nm) UV irradiation.

The relationship between the concentration of resveratrol around *B. cinerea* lesions on grapevine leaves and susceptibility of those leaves to infection was investigated. Susceptibility decreased with age of the leaf and varied between the varieties of grapevine studied. In both cases, there was an inverse relationship between the concentration of resveratrol around the lesion and susceptibility. The probable role of resveratrol as the precursor of antifungal stress metabolites, the viniferins, in the disease resistance of grapevine is discussed.

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