Inhibiting Effects of Resveratrol and Its Glucoside Piceid against *Venturia inaequalis*, the Causal Agent of Apple Scab

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Resveratrol is a phytoalexin produced in different unrelated plant species such as grapevine and peanut. The effect of resveratrol and its glucoside *trans*-resveratrol-3-*O*-β-D-glycopyranosid (piceid) against *Venturia inaequalis*, the causal agent of apple scab, was evaluated using a newly established test based on enzymatically isolated cuticular membranes (CMs) from apple (*Malus domestica* Borkh.) leaves. The test substances resveratrol and piceid were either sorbed to CMs before inoculation with spores or were applied simultaneously with the *Venturia inaequalis* spores to the CMs, and their effect on germination, appressoria formation, and penetration was examined. Resveratrol had no influence on spore germination but a significant inhibiting effect on penetration when applied simultaneously as well as before. A percentage inhibition of 89.7 ± 11.5 and 61.8 ± 35.1 was observed for simultaneous and preapplication, respectively. The resveratrol glucoside piceid had a significant inhibitory effect on germination and completely inhibited penetration of the fungus at concentrations between 200 and 400 μg mL^{-1} when applied simultaneously with the spores to the CMs. On piceid-enriched CM (preapplication), spores germinated but penetration was inhibited nearly completely ($96.1 \pm 5.1\%$). Thus, *in vitro* experiments showed that resveratrol and its glucoside in fact could contribute to improving the pathogen resistance of apple leaves.

KEYWORDS: fungal pathogen; isolated cuticular membrane; penetration; phytoalexin

INTRODUCTION

One important aspect of plant defense mechanisms is the accumulation of phytoalexins in response to pathogen attack. Phytoalexins are low-molecular-weight secondary metabolites with biological activity against a wide range of pathogens. Several unrelated plant species such as grapevine (*Vitis vinifera*) (*1*), peanut (*Arachis hypogaea*) (*2*), and blueberry (*Vaccinium corymbosum* and *Vaccinium angustifolium*) (*3*) are capable of synthesizing the phytoalexin stilbene (resveratrol). It is derived from the acetate−malonate and phenylpropanoid pathway of primary and secondary metabolism. The formation is catalyzed by the key enzyme stilbene synthase, which converts one molecule *p*-coumaroyl-CoA and three molecules of malonyl-CoA into 3,4′,5-trihydroxystilbene, commonly known as resveratrol. The production of this phytoalexin is related to fungal infection (*4*) and abiotic stimuli (*5*) such as UV light (*1*, *6*) and ozone (*7*, *8*).

Resveratrol has received much attention, because it has implications in both phytopathology and human health. The cardio protective, antiplatelet (*9*) and anticarcinogenic properties (*10*) stimulated research aimed at increasing the natural content of resveratrol in table grapes by UV irradiation of harvested fruits (*11*, *12*) to create "functional food" with improved inner quality. Phytopathological work demonstrated the significance of resveratrol in the resistance reaction of grapevine to *Botrytis cinerea* (*13*, *14*) and *Plasmopara viticola* (*15*). Antifungal properties of resveratrol have been studied in *in vitro* investigations (*16*, *17*, *18*, *19*, *20*) and by direct external application to plants (*21*).

Because of the fungistatic effect of resveratrol, this compound has attracted much attention as a candidate to increase resistance in transgenic plants. The overexpression of the key enzyme for resveratrol synthesis, stilbene synthase, leading to an accumulation of resveratrol in plant tissue, enhanced resistance against fungal pathogens in tobacco (*22*), wheat and barley (*23*), a grape rootstock (*18*), and alfalfa (*17*). Stilbene synthase was also successfully transferred into kiwi (*24*) and apple (*25*). In this context, the synthesis of resveratrol in transgenic fruits might also be regarded as an additional factor increasing pathogen resistance and improving "the inner quality" of the fruit. Besides endogenous enhancement of resveratrol accumulation, attempts were undertaken to apply resveratrol exogenously on fruits to exploit the antioxidant properties. Recent published studies (*21*, *26*) demonstrated indeed that the external application of resveratrol improved the postharvest quality of fruits. Hence, it can be regarded as a "natural fungicide".

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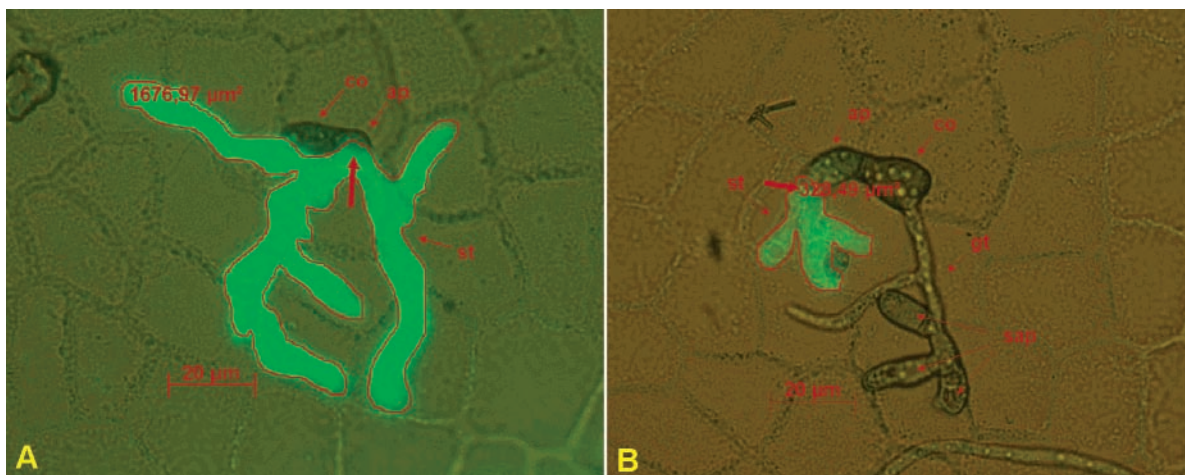† University of Bonn.
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Figure 1. V*enturia inaequalis* conidia with a short germ tube and penetration structures derived from the appressorium on control (A) and resveratrol-enriched (B) CM 72 h after spore application. Bright light and UV-excitation microscopy were combined to visualize both methylen blue-stained fungal structures such as conidio spores with appressoria located on the outer CM surface and greenish FDA-stained penetration structures (stroma) growing in the CM. (A) Extensively branched stroma originating from an appressorium of a conidio spore on control CM. Stroma sizes were exemplarily measured for the control with AxioVisison software from Zeiss. (B) Stroma with noticeably reduced size on resveratrol-enriched CM. Size in resveratrol CM reached only 24.3% in average of the control stroma. Absolute values are given in the figures. ap, appressorium; co, conidio spore; gt, germ tube; sap, secondary appressorium; st, stroma. The arrow indicates the penetration sites.

On the basis of this work, including the production of transgenic apple plants expressing the stilbene synthase gene from *Vitis vinifera* (*25*, *27*) as well as the possibility to use resveratrol as a "natural fungicide", it was the aim of the present study to investigate the antifungal activity of resveratrol against the fungus *Venturia inaequalis* (Cke.) Wint., the causal agent of apple scab, which is the most destructive disease of apple. Because of the fact that resveratrol in plants often occurs as a conjugate to sugar (*28*), we included the most common derivative *trans*-resveratrol-3-*O*-β-D-glycopyranosid (piceid) in our study in addition to resveratrol.

MATERIALS AND METHODS

Cuticle Isolation. For assays with cuticular membranes (CMs), astomatous adaxial CM were isolated enzymatically from *Malus x domestica* Borkh. cv. "Gloster" following the method of Schönherr and Riederer (*29*).

Cultivation of *Venturia inaequalis* and Production of Conidia. The *Venturia inaequalis* isolate originated from *Malus x domestica* Borkh. cv. "Elstar" and was maintained on potato-dextrose agar (PDA; Difco, Becton/Dickinson, Sparkes) at 20 °C in the dark. Conidia of *V. inaequalis* were produced on cellophane 235 P membranes (Pütz GmbH and Co Folien KG, Taunusstein, Germany), which were placed on PDA according to the method of Parker et al. (*30*). Conidia were washed from the cellophane by agitating in sterilized deionized water, and the liquid was filtered through Miracloth (Calbiochem/Merck Eurolab GmbH, Darmstadt, Germany). Spore density was quantified in a Kolkwitz plankton-cytometer (0.5 mL, Hydro-Bios, Kiel, Germany) (*31*) and adjusted to a final concentration of $0.5-0.8 \times 10^4$ conidia mL^{-1}.

Application of Resveratrol and Its Glucoside. Resveratrol (Sigma, Taufkirchen, Germany; purity, 99%) and its glucoside piceid (Apin Chemicals, Oxfordshire, U.K.) were applied in two different ways to CMs. Test substances were either applied together with the conidia to CMs (simultaneous application) or before inoculation with conidia to CMs that were first equilibrated with the test substances (preapplication). For equilibration with resveratrol or piceid, CMs were incubated in 50 mL of a saturated aqueous solution (100 μg mL^{-1} for resveratrol and 500 μg mL^{-1} for piceid) for 24 h on a rolling bench at room temperature (RT) in darkness. Incubation of CMs in water served as a control. In each experiment, 30 isolated CMs were used. The cap of the bottle (Schott, Germany) was lined inside with aluminum foil to prevent sorption of the test substances by the material of the cap. After incubation, CMs were dried on Teflon disks to flatten them.

CMs were put in glass Petri dishes with their inner morphological side facing 20 mL of saturated solutions of resveratrol, its glucoside, or water as a control. A 5 μL drop of spore suspension was pipetted in the center of the outer morphological side of each CM. Incubation was done at 20 °C in closed Petri dishes, which prevented evaporation of the drops containing the spores. After 24 h, half of the CM from each variant was randomly selected for determination of germination, while remaining CMs were used for penetration assays (see below).

In experiments with simultaneous application, *V. inaequalis* spores were centrifuged and resuspended in either saturated resveratrol or piceid solutions. For piceid, the following concentrations were tested as well: 50, 100, 200, 400, 500, and 1000 μg mL^{-1}. A 5 μL drop was pipetted in the center of each CM, and the samples were incubated as described above.

Determination of Germination. Conidia were stained with 2 μL of methylene blue (0.01%, Sigma) and observed by light microscopy (ZEISS, AxioPlan). The percentage of germination was assessed by subdividing conidia into three categories: germinated with appressorium, germinated without appressorium, and nongerminated.

Determination of Penetration. Penetration was determined 72 h after conidia application. The spores on the outer surface of the CM were stained with methylene blue as described above. Penetration hyphae were stained at the morphological inner side of the CMs by applying 40 μL of fluorescein diacetate (FDA) (10 μL of FDA in acetone added to 300 μL of Tris buffer). FDA is a metabolic dye, which is rapidly converted to fluorescein by esterases present in living cells. Fluorescein accumulating in the living cells was visualized under UV excitation (*32*, *33*) using a ZEISS AxioPlan 2 microscope equipped with a blue excitation filter (450−490 nm), a beam splitter FT 510, and a long pass filter LP 520 filter.

Because of the low permeability of the cuticle itself, FDA only stained hyphae at the inner side of the CMs. Structures located on the external surface of the CM, such as germ tubes and appressoria, were visible in light microscopy because CMs are translucent, but they were not stained by FDA. Therefore, hyphae grown through the CM or within the CM (**Figure 1**) were clearly distinguishable by their bright green fluorescence (**Figure 1**) during UV excitation. The penetration hyphae on the morphological inner side were counted and related to conidia with appressoria. When more than one penetration hyphae was developed, only one per conidium was taken into consideration. In some experimental series, a poor penetration on control CMs (less than 60%)

was observed. These experiments were completely discarded, because a poor performance on controls was due to a reduced viability of the spores.

Area of Penetration Structures. Besides germination and penetration, the size of penetration structures was also measured for two experiments when CMs were treated with resveratrol before inoculation. This was done by taking photographs of the infection structures, and the area covered by the hyphae was then measured using the AxioVision software from ZEISS.

Determination of Water Solubility. Stock solutions of resveratrol (228 μg mL^{-1} = 1 mM) and resveratrol glucoside (3890 μg mL^{-1} = 10 mM) were prepared by dissolving the compounds in water at 60 °C. After the solutions were stored overnight at 25 °C, saturated solutions over deposits of resveratrol and resveratrol glucoside were obtained. Aliquots of the saturated supernatant were diluted 2, 4, 5, and 10 times, and water was evaporated using a gentle stream of nitrogen. A total of 5 μg of tetracosane (99% purity; Fluka) was added as an internal standard to each sample. Samples were derivatized for 40 min at 70 °C using 20 μL of pyridine (99% purity; Fluka) and 20 μL of BSTFA (*N,N*-bistrimethylsilyltrifluoroacetamide; Macherey-Nagel, Germany). This converts the free alcohol groups of resveratrol to their corresponding methyl ethers. Finally, samples were diluted again with 150 mL of chloroform, and 1 μL of each sample was analyzed by gas chromatography (GC 5890 series II, Hewlett−Packard, PA) equipped with on-column injection and a flame ionization detector (GC/FID). The temperature program for analysis was injection at 50 °C, 2 min at 50 °C, 10 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, and then 16 min at 320 °C. The pressure program (H2 carrier gas) was injection at 50 kPa, 41 min at 50 kPa, 10 kPa min^{-1} to 150 kPa, and 24 min at 150 kPa. Resveratrol peaks were identified by gas chromatography coupled to mass spectrometry (GCMS 6890N, Agilent, Avondale; mass selective detector 5973, Agilent). Amounts of substance (in moles) detected via GC/FID were plotted versus the amounts of solvent (in liters; amount of water evaporated), and water solubilities [in moles per liter] of both compounds in their stock solutions were calculated from regression curves ($r^2 = 0.99$) fitted to these plots.

Determination of Cuticle/Water Partition Coefficients K_{CW}. Saturated solutions of resveratrol (Fluka, Neu-Ulm, Germany) were dissolved 2, 4, 5, and 10 times, giving final volumes of 25 mL. At each solution, an isolated and extracted cuticular polymer matrix membrane (MX) from *Stephanotis floribunda* Brongn. was immersed in the solution and rotated at 25 °C for 24 h overnight in the dark on a rolling bench (60 rpm). MXs were sampled and gently dried on filter paper, and sorbed resveratrol was re-extracted from the MXs in 1 mL of reactivials for 30 min at 25 °C using 1 mL of CHCl3 (p.a. Roth, Karlsruhe, Germany). As described above, tetracosane was added as an internal standard to each sample, and after evaporation of the solvent, samples were derivatized and diluted again with chloroform and 1 μL of each sample was analyzed by gas chromatography. In parallel aliquots of the external resveratrol, the solution was analyzed in the same way. Cuticle/water partition coefficients K_{CW} of resveratrol were calculated according to eq 1.

$$K_{CW} = \frac{\left(\dfrac{\mu g_{resveratrol}}{\mu g_{MX}}\right)}{\left(\dfrac{\mu g_{resveratrol}}{\mu g_{water}}\right)} \quad (1)$$

Data Analysis. For the analysis of the data of conidia germination and penetration, pair-wise t-test statistics were performed (t-test; $p < 0.05$) using the SigmaPlot software 8.0 from SPSS Science.

RESULTS

Effect of the Test Substances on Germination. Resveratrol had no effect on germination of spores. Rates of germination on resveratrol-enriched CMs (preapplication) were 81.6% [standard deviation (SD) ±14.6%] compared to 87% (SD ±8.8%) of the control. When spores were added simultaneously
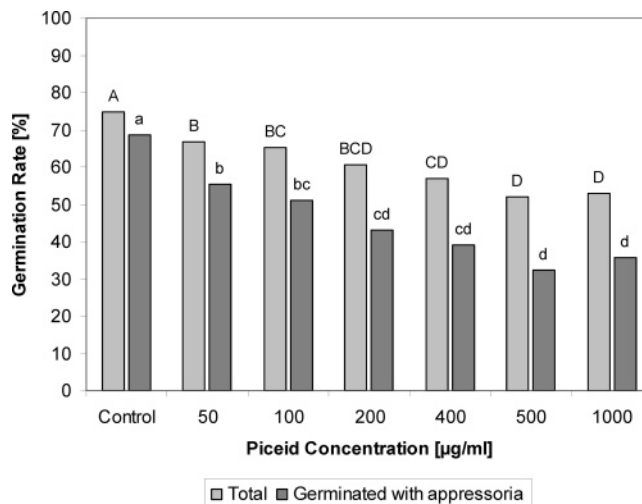

Figure 2. Influence of the resveratrol glucoside piceid on germination of conidia after simultaneous application to CM. Data represent the mean of five (for 1000 μg mL^{-1} four) independent experiments with five CM each. Values followed by the same letter did not differ significantly ($p < 0.05$).

with resveratrol, germination rates were 81.7% (SD ±11.3%) compared to 83.2% (SD ±8.3%) of the control.

When spores were simultaneously applied with the piceid, different concentrations were tested ranging from 50 to 1000 μg mL^{-1}. With all concentrations tested, germination rates of conidia were significantly reduced within a time of 24 h compared to the control (**Figure 2**). The proportion of germinated conidia with appressoria was also decreased (**Figure 2**). No effect on germination was detectable, when spores were added to piceid-enriched CMs. On control CMs, germination rate was 87.4% (SD ±6.5%), while 86.1% (SD ±0.3%) germinated on piceid-enriched CM.

Influence of the Test Substances on Penetration. In penetration assays, resveratrol showed a strong inhibiting effect on the development of penetration structures on both pretreated CMs (61.8 ± 35.1% inhibition) and on CMs inoculated with spores and resveratrol together (89.7 ± 11.5% inhibition). Besides reduction of penetration rates, the size of the mycelia grown after successful penetration was also affected by resveratrol sorbed to the CM. FDA-stained mycelium was looking different from the germ tubes (**Figure 1**). These hyphae were branched and similar to the penetration structures described by Ortega et al. (*34*). In both experiments ($n = 111$), the area of the mycelia after penetration was significantly different from the control CMs ($p < 0.05$). When the average area covered by the mycelia grown on the inner side of control CMs was set to 100% for both experiments, the mycelia grown in resveratrol-enriched CMs was only about one-fourth (24.3%).

A nearly 100% inhibition of penetration was observed when saturated piceid solutions were applied simultaneously with the spores to the CM. Penetration constantly decreased at concentrations from 50 to 200 μg mL^{-1}, and it was nearly completely inhibited at concentrations between 400 and 1000 μg mL^{-1} (**Figure 3**). A nearly 100% inhibition (96.1 ± 5.1%) was obtained for penetration of *V. inaequalis* spores on enriched CM (preapplication). All inhibitory effects of piceid and resveratrol on germination and penetration compared to the controls are summarized in **Table 1**.

Water Solubilities and Partition Coefficients. Water solubility of resveratrol was 52.2 μg mL^{-1} with a SD of ±2.3 μg mL^{-1} (=2.29 × 10^{-4} mol L^{-1} ± 1.01 × 10^{-5} mol L^{-1}), whereas
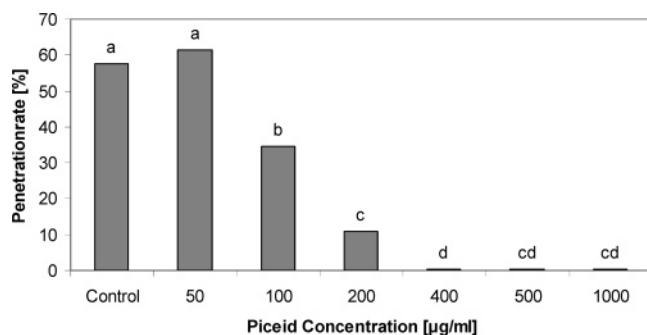
Figure 3. Effect of the resveratrol glucoside piceid on penetration of spores when applied simultaneously. Data represent the mean of three independent experiments with a minimum of five CM per experiment. Values followed by the same letter did not differ significantly ($p < 0.05$).

Table 1. Percentage Inhibition of Germination and Penetration by Preapplication and Simultaneous Application of Resveratrol and Piceid[a]

	preapplication		simultaneous application	
	piceid (%)	resveratrol (%)	piceid (%)	resveratrol (%)
germination	no effect	no effect	25.4 ± 5.3	no effect
penetration	96.1 ± 5.1	61.8 ± 35.1	94.9 ± 9.1^{b}	89.7 ± 11.5

[a] For piceid, concentrations at and above its saturation were taken into account. Values represent the mean ± SD of three independent experiments for simultaneous application and six and two independent experiments for preapplication of resveratrol and piceid, respectively. [b] Mean value ± SD of all concentrations above saturation, i.e., 200, 400, 500 and 1000 μg mL^{-1}.

water solubility of resveratrol glucoside was 161 μg mL^{-1} with a SD of ± 5.7 μg mL^{-1} ($= 4.14 \times 10^{-4}$ mol L$^{-1} \pm 1.47 \times 10^{-5}$ mol L^{-1}). The cuticle/water partition coefficient K_{CW} of resveratrol was 3079 (SD ± 686). Using the prediction equation

$$\log K_{CW} = 1.118 - 0.596 \log S_{water} \qquad (2)$$

from ref *35*, which allows the calculation of cuticle/water partition coefficients from water solubilities S_{water} of lipophilic molecules, a K_{CW} of 1939 was calculated for resveratrol and a K_{CW} of 1370 was calculated for piceid.

DISCUSSION

Test Method. Here, a newly developed test method based on isolated astomatous CMs of apple leaves was used to investigate the effect of resveratrol and its glucoside piceid on the fungus *V. inaequalis*, the causal agent of apple scab. Opposite to agar- or liquid-based tests methods, where conidia from *V. inaequalis* germinate and produce long germ tubes without appressoria, this method allows us to study effects of different substances on the development of appressoria and penetration structures (*36*). This is due to the fact that the CM represents the natural habitat for the apple pathogen *V. inaequalis*, where the fungus behaves in a way similar to that on leaves of susceptible apple cultivars: the spore germinates under high humidity; the germ tube contacts the cuticle; an appressorium is formed; and penetration occurs below the appressorium (*37*). Obviously, specific signals on plant surfaces must be responsible for the different behavior of *V. inaequalis* on CM compared to artificial agar or in liquid media, where the fungus germinates readily but does not produce appressoria. Hard-surface contact has been known to be necessary to induce infection structure formation in many plant pathogenic fungi (*38, 39*). These signals

include components of the leaf surface, leaf surface topography, or hardness and hydrophobicity of surfaces (*40, 41*).

Effect of Resveratrol. Because resveratrol had no effect on spore germination and appressoria formation, the inhibiting effect on the following penetration must be due to disturbance in fungal development after appressoria formation. According to the fairly high K_{CW} of 3079, most of the resveratrol will be accumulated within the CM, forming a lipophilic compartment, and resveratrol concentration in the water drop in which the spores were added will be low. Because of the water solubility of 52.2 μg mL^{-1}, resveratrol concentration in the drop, where the spores germinate, is limited and it might not be sufficient to inhibit the process of germination and penetration. However, during penetration, resveratrol develops its antifungal activity because it causes significant reduction in penetration rates and the size of the penetration structures. In regards to penetration, resveratrol concentration within the CM is relevant for the inhibition of fungal growth. The inhibitory effect after simultaneous application (89.7% $\pm$ 11.5) was more pronounced in comparison to preapplication (61.8% $\pm$ 35.1), but here the SD was extremely high.

The structure of the plant cuticle is heterogeneous, and the main transport-limiting barrier of the CM is formed by the waxes, deposited in a highly ordered, partially crystalline manner to the outermost part of the cuticle (*42*). However, most of the resveratrol will be accumulated in the inner parts of the cuticle forming a sorption compartment for lipophilic compounds (*35*), and thus, it will hardly effect germination and appressorium formation occurring on the surface of the CM. However, during penetration, fungal hyphae will come in close contact with resveratrol, which accumulated in the CM.

In a series of other studies, inhibitory effects of resveratrol against a series of different fungi were observed (*43*). Adrian et al. (*16*) used an *in vitro* liquid system to evaluate the biological activity of resveratrol against the *Botrytis cinerea*, the causal agent of gray mold, and they found significant reduction in spore germination when applying 60−160 μg mL^{-1} resveratrol. Ali et al. (*19*) reported that only half of the concentration, 40.8 μg mL^{-1}, was sufficient to reach 50% growth inhibition of *B. cinerea*, while a similar concentration (78.3 μg mL^{-1}) was necessary for a 50% growth inhibition of *Penicillium digitatum*. In agar-based bioassays, the inhibitory effect of resveratrol against the grape pathogens *Botrytis cinerea* and *Eutypa lata* (*18*) were investigated. For both fungi, a significant growth inhibition, expressed as the diameter of mycelium spreading from the inoculum point, was observed, but no total inhibition was achieved despite the application of more than 205 μg mL^{-1}. Hipskind and Paiva (*17*) tested the activity of resveratrol in a concentration of 50 μg mL^{-1} against mycelia growth of *Phoma medicaginis*, leading to more than 50% hyphal growth reduction. In plate tests, Seppänen et al. (*20*) applied resveratrol to 16 species of fungi. Partly, there was a significant reduction in radial growth of fungi, but all except one were still able to grow at 400 μg mL^{-1}. All of these studies confirm the rather unspecific, in relation to pathogen toxicity nonselective, antifungal character of this polyphenolic phytoalexin.

Effect of Piceid. In contrast to resveratrol, the resveratrol glucoside piceid significantly affected germination of *V. inaequalis* in comparison to the control, when spores were applied to the CM in a solution containing the piceid. The values above saturation, that is 161 μg mL^{-1}, did not differ significantly among each other. An average inhibition of germination of 25.4 $\pm$ 5.3% and of appressorium formation of 45.2 $\pm$ 6.8% was

obtained from 200 to 1000 μg mL^{-1} (**Figure 2**). Because the glucoside has a 1.8-fold higher water solubility than resveratrol, spores faced higher concentrations compared to resveratrol when inoculated together with piceid. This could explain the inhibitory effect found with piceid and not with resveratrol when applied simultaneously. When piceid-enriched CMs were inoculated with spores, germination was not affected, which indicates that the piceid sorbed to the CM did not affect the spores on the outer surface of the CM. Opposite to resveratrol, partially inhibiting the penetration of *V. inaequalis*, the piceid inhibited penetration by nearly 100%, independent of the way the piceid was added (**Figure 3**). This indicates that the piceid had a stronger effect as an antifungal compound on *V. inaequalis* than resveratrol.

Reports in the literature, obtained with transgenic plants transformed with the stilbene synthase, are somewhat contradictory related to the antifungal activity of the piceid. Whereas transgenic kiwi, synthesizing resveratrol and the glucoside piceid, did not show increased disease resistance against *Botrytis cinerea* (*24*), transgenic alfalfa accumulating the glycosid piceid showed a significant reduction in the size of necrotic lesions after the infection with the pathogen *Phoma medicaginis* (*17*). This inhibitory of the glucoside against this fungus was confirmed in plate tests (*17*).

Estimation of Resveratrol Activity in Planta. In all of the examples described above including the results of this study, it is evident that the highest possible aqueous resveratrol and piceid concentrations at or even above their water solubilities of 52.2 μg mL^{-1} for resveratrol and 161 μg mL^{-1} for piceid were used in the experiments. In most cases, only then were significant effects observed. This raises the questions of whether these concentrations can ever be reached in plants naturally producing resveratrol or synthesizing resveratrol after transformation with the stilbene synthase. To compare externally applied resveratrol and piceid concentrations with those naturally occurring in leaf and fruit tissues, it is assumed that the concentrations given in μg g^{-1} fresh weight of investigated plant sample are directly comparable to experimental concentrations given in μg mL^{-1}. Because leaf and fruit tissues on average are composed of about 90% water, this assumption is justified.

For *Vitis* resveratrol, values ranging from 16 ± 0.5 (*26*) to 330 μg g^{-1} in berry skin (*44*) depending on the variety and developmental stage were reported. UV irradiation increased the concentration to 810−1075 μg g^{-1} in berry skin. In total wine grapes, 1.69 ± 0.18 μg g^{-1} fw (*45*) of piceid was detected. The heterologous expression of a stilbene synthase gene from *Vitis vinifera* L. in transgenic apple plants led to the accumulation of a resveratrol glycoside (*25*). The highest amount measured was 34.7 μg g^{-1} fresh weight in transgenic apple leaf tissue, while about 13.0 μg g^{-1} was detected in transgenic apple fruit skin (our unpublished results). It has to be considered that the stilbene synthase gene in transgenic apple plants is under the control of an UV-, wound-, and pathogen-inducible promotor. Formation and accumulation of resveratrol was routinely induced by UV irradiation. The quantities given above represent the average amount of resveratrol glycoside found in the whole tissue. Because only those cells that have been induced by the UV light accumulate the resveratrol glycoside, it can be expected that local concentrations, e.g., in infected areas, might be much higher. Nevertheless, overall concentrations measured in leaf and fruit tissues show that the concentrations used in our experiments were not unrealistically high.

Exogenous Application of Resveratrol. From water solubility of resveratrol and its K_{CW}, it is possible to calculate that the maximum amount of resveratrol that accumulates in CM is 8.6 mg g^{-1} cuticle. We can assume that this must be the maximum amount of resveratrol that can accumulate in a plant cuticle in equilibrium with an aqueous phase saturated with resveratrol. Gonzáles et al. (*21*) and Montero et al. (*26*) applied resveratrol exogenously on grape and apple fruit by dipping them for a few seconds (5 s) into a 36.5 μg mL^{-1} (=1.6 × 10^{-4} M) resveratrol solution and investigated the effects on fruit conservation during storage as well as on ethylene production. The treated fruits maintained their postharvest quality for a longer time with clear differences from the untreated controls. They also found a significant decrease in the ethylene production in treated grapes (*26*), probably attributed to the action of *trans*-resveratrol on different microorganisms present on the grapes, which are also synthesizer of ethylene. After the volume loss of the solution was measured, they calculated that 7.64 μg g^{-1} grape skin was added. Their calculation was based on the amount of resveratrol added to the solution (36.5 μg mL^{-1}) not taking into account the water partition coefficients. Because we incubated the isolated leaf CM used in this study for 24 h in a saturated resveratrol solution, an equilibrium concentration between the external solution and the cuticle was established. According to the K_{CW}, the solubility of resveratrol in a leaf CM is approximately 3000 times higher than that in water, and this should be considered when determining the maximum amount that can be effective after exogenous application on leaves or fruits. Because of the high K_{CW}, most of the resveratrol will accumulate in the cuticle.

Conclusions. To our knowledge, this is the first time that the effect of resveratrol and its glucoside piceid was specifically tested against *V. inaequalis*, the causal agent of apple scab. The newly developed CM-based test system described here will allow for future investigation of the antifungal activities of further substances against plant pathogens, because the CM represents the natural habitat of the pathogens. Both substances tested here showed antifungal activity against *V. inaequalis*. The results confirm the rather unspecific antifungal properties of this polyphenolic phytoalexins, resveratrol, and its glucoside. The present study also underlines the capacity of resveratrol and its glucoside to increase fungal resistance by endogenous enhancement, because it can be found in transgenic plants expressing the responsible enzyme stilbene synthase, as well as its potential to be used as a fungicide by exogenous application. Because apple, forming the natural host of *V. inaequalis*, does not usually produce resveratrol, the fungus should not be able to detoxify or metabolize that novel antifungal compound.

ABBREVIATIONS

CM, cuticular membrane; MX, polymer matrix membrane

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362 *J. Agric. Food Chem.,* Vol. 53, No. 2, 2005

Schulze et al.

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