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Metabolism of stilbene phytoalexins in grapevines: Oxidation of resveratrol in single-cell cultures

by

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Der Stoffwechsel von Stilbenphytoalexinen in Reben: Oxidation von Resveratrol in Einzelzellkulturen

Zusammenfassung: Um den Metabolismus der Stilbenphytoalexine Resveratrol und Viniferin zu untersuchen, wurden Einzelzellkulturen der pilzanfälligen Rebsorten Riesling und Optima und der resistenten Sorten Pollux und Sirius etabliert. Die kultivierten Zellen verschiedener Sorten zeigten morphologische Unterschiede (in Form und mechanischer Widerstandsfähigkeit), und ihre Empfindlichkeit gegen eine Behandlung mit Trockenpulver von *Botrytis*-Mycel entsprach der natürlichen Pilzanfälligkeit der Sorten im Freiland. Die meisten Zellen von Riesling und Optima wurden von toxischen Bestandteilen des Myzels innerhalb von 60 h abgetötet, während Kulturen von Pollux und Sirius nur leichte Schädigung zeigten.

Zwei Typen von Peroxidase wurden charakterisiert, die eine hohe Affinität zu Resveratrol als Substrat aufwiesen und dieses zu einem braunen Pigment oxidierten. Bei einem der Enzyme wurde Viniferin als Zwischenprodukt beobachtet. Die Peroxidaseaktivität war bei anfälligen Sorten geringer als bei resistenten. Die Oxidationsprodukte von Resveratrol hatten eine hemmende Wirkung auf die Peroxidasen.

Key words: cell, tissue culture, phytoalexin, phenol, enzyme, oxidation, resistance, variety of vine.

Introduction

Stilbenic substances (resveratrol, viniferins etc.) are normal constitutive components of woody parts of all Vitaceae. In green parts, however, they are only produced after induction by stress factors like UV-light or mechanical injury combined with certain inducing substances. It has been shown by several authors that both speed and intensity of stilbene formation are an indicator for the plant's resistance to fungi and thus resveratrol analysis is used as a tool for the selection of grapevine varieties resistant to *Botrytis cinerea* (Pool *et al.* 1981; Stein and Blaich 1985; Bavaresco and Eibach 1987).

Nevertheless, the underlying mechanisms of both induction and action of these substances are far from being understood. A direct fungitoxic or fungistatic activity has been observed by Langcake (1976, 1981), Stein and Bachmann (1982) and Stein and Blaich (1985), but other observations indicate that their main function might be on another level. Furthermore, the screening methods basing on stilbene analysis, although yielding satisfying results, do not take into account a possible turn-over of resveratrol.

Biochemical analysis of grapevine leaf material, in particular for studies of metabolic activities, is rather difficult because no continuous samples can be drawn and the high tannin content leads to denaturation of enzyme proteins, even if the tannin is masked by different methods. Liquid cultures of cells or protoplasts seemed more suitable for that kind of work.

To establish suspension cultures from grapevine tissue ARYA *et al.* (1962) and GOYAL and GOYAL (1973) tried phylloxera and crown galls. HAWKER *et al.* (1973) and AMBID *et al.* (1983) used cell suspensions derived from berry fragments. In this system AMBID *et al.* (1983) studied monoterpene metabolism. Testing *Botrytis* toxin in suspensions of stem callus, was described by STEIN and BACHMANN (1982). LEBRUN *et al.* (1985) started from anthers.

Protoplasts were produced from pericarp callus by SKENE (1974, 1975) who reports cell divisions and regeneration of callus tissue, and BROWN and COOMBE (1984) who used them to study the uptake of sugar. Production and culture of leaf protoplasts and sometimes biological and biochemical processes in these suspensions are described by BURGESS and LINSTEAD (1976), HASLER *et al.* (1982, 1983), BREZEANU and ROSU (1984), NISHIMURA *et al.* (1984), DE FILIPPIS and ZIEGLER (1985), SHIMIZU (1985), BESSIS *et al.* (1985), LEBRUN (1985), WRIGHT (1985).

Vitis vinifera peroxidase had been isolated from grape clusters by SCIANCALEPORE *et al.* (1985) with regard to its possible influence on wine quality. In this paper, results are presented which concern the properties of peroxidases involved in processing of resveratrol. They have been studied in continuous cultures of *Vitis* cell suspensions established from both fungus resistant and susceptible varieties.

Material and methods

Grapevine material

Both protoplasts and single-cell cultures were derived from plantlets cultivated *in vitro* according to BLAICH (1977) in a medium (I) containing 0.03 mg/l BAP, and 0.01 mg/l NAA solidified with 8 g/l agar.

Medium (I) is composed of saccharose (30 g/l), meso-inositol (0.1 g/l), Fe-EDTA (50 mg/l), thiamin (0.4 mg/l), salt solution (50 ml/l), adjusted to pH 5.8.

Salt solution contains KNO_3 (38 g), NH_4NO_3 (16 g), $MgSO_4 \cdot 7\,H_2O$ (8 g), $CaNO_3 \cdot H_2O$ (9 g), KH_2PO_4 (5 g), $MnSO_4$ (0.4 g), H_3BO_4 (0.3 g), $ZnSO_4$ (0.1 g), $Na_2MoO_4 \cdot 2\,H_2O$ (5 mg), $CuSO_4$ (0.5 mg), $CoSO_4$ (0.5 mg), KJ (2 mg) per l.

Environmental conditions for all cultures were kept constant at 25 °C and 14/10 h light/dark.

Preparation of protoplasts: Tests with different enzyme combinations, enzyme concentrations and different temperatures, carried out on leaves (whole or cut in small strips of 2 mm) showed that the yield in vital protoplasts (as tested by a vital staining with fluoresceine diacetate, see STEIN and BACHMANN 1982) was best (73 %) at room temperature with Pectinase Rohament P5 (0.5 %), Cellulase TC (1.0 %), Cellulase Onozuka R10 (0.25 %) obtained from Serva (Heidelberg). The enzymes were applied to the leaves for 3 h dissolved in a preparation medium consisting of 0.3 M sorbitol, 0.1 M citric acid, 0.05 M ascorbic acid, 0.3 mM glutathion and 5 % of the salt solution mentioned above. After removal of the enzymes by repeated washing with the preparation medium, the protoplasts were separated from the debris by centrifugation in a Ficoll Paque (Pharmacia) density gradient (25—100 %). The culture was then continued in medium (I) supplied with the additives of the preparation medium (without additional salt solution), plus 10 mg/l BAP or coconut milk and 0.5 g/l PVP.

Single-cell cultures were obtained from small, sterile cuttings of *in vitro* plants. Upside down on medium (I) supplied with 1 mg NAA, 0.5 mg BAP and 8 g agar they formed a dense callus (partly with chloroplasts). After separation from the

cuttings and transfer to fresh agar medium the callus continued its growth. Part of the cells, though vital, produced a brown pigment. During a further growth phase of 2—4 weeks (depending on the grapevine variety) the callus formed zones exhibiting different consistencies (two types with a more or less dry appearance, either compact or spongy and a soft wet type).

If callus pieces were transferred into liquid culture (100 ml/300 ml Erlenmeyer flask on a rotary shaker 75 rpm) many cells died, others turned brown and only after some weeks enough single cells were detached from the pieces to provide a durable culture consisting of single cells and small cell aggregates. From this stage on the cultures could be maintained continuously without further difficulties.

The fresh weight production of the cultures was determined after washing the cells 2 times on a glass filter. Dry weight was calculated on the base of freeze dried material.

Determination of protein content of dried cells: 0.5 g were soaked over night in 15 ml of phosphate buffer (pH 7.0), then disrupted in a tissue homogenizer followed by ultrasonic treatment (3 times), and then centrifuged. Protein content was estimated by the colour reaction of BRADFORD (1976) using 10 µl of the supernatant in 2.5 ml test solution. Bovine serum albumin was used as a standard.

Enzyme analysis

Preparation of active cell extracts: Suspension cultures were filtered and washed with distilled water. For enzyme tests 1 g of fresh cells was then homogenized in 9 ml (for disc electrophoresis and gel chromatography 100 g/100 ml) of citrate-phosphate buffer (0.1 M, pH 4.5) at 0 °C using an Ultraturrax until 95 % of them were broken. The time for this procedure varied from 2 to 10 min/g callus depending on the grapevine variety. After centrifugation (20,000 *g* for 10 min), the supernatant was used as enzyme extract (E1).

Acetone precipitation (80 % v/v in the cold) of the 100/100 supernatant, and a second centrifugation (as above) yielded a pellet which was resuspended in 2 ml Tris-HCl buffer (pH 7.2, 0.1 M, + 0.001 M dithioerythrol) and treated with protamine sulfate (50 µl/ml of a saturated solution) to precipitate nucleic acids and other acid components. This resulted in extract E2.

For preparative gelchromatography we applied 1 ml of E2 to a Fractogel (TSK HW 55 F, Merck) column (16 × 650 mm, Tris-HCl buffer as above at 1.8 bar).

Disc electrophoresis and isoelectric focusing were carried out in cylindric gels as described in BLAICH (1978) or on Servalyt Precotes 3—10 (Serva, Heidelberg) on a LKB 2117 Multiphor.

Peroxidase activity was determined either by TLC analysis (2 µl probes on S & S F1700 micropolyamide plates; eluant: MeOH/ethylacetate 2 : 1; Shimadzu High-Speed TLC-scanner) of resveratrol degradation and formation of ε-viniferin under different conditions (stopped by adding methanol to 50 % at the appropriate time) or by spectrophotometric measurements (655 nm) of the oxidation of tetramethylbenzidine (100 µg/ml, citrate phosphate buffer pH 4.5 + 0.06 % H_2O_2, 1 min) according to ANDREWS and KRINSKY (1982).

Botrytis mycelium

To prepare an elicitor substance 100 g of mycelium (grown 10 d on 3 % malt extract, for details see STEIN 1985) were frozen in liquid nitrogen and then homogenized with 200 ml of methanol at −40 °C. The slurry was then filled up with methanol to 1000 ml and left over night at room temperature. After centrifugation and air drying a fine powder (9 g) was obtained.

Results and discussion

Establishing a test system

Homogenized leaf material from the field had been used for preliminary tests. It turned out that the phenol content of these extracts had a deleterious effect on enzyme proteins which could only partly be overcome by PVP, PEG, and reducing agents such as ascorbic acid, DTT, or glutathion.

Protoplasts were therefore used for further tests. It was, however, difficult to prepare them from field material according to the methods published for tobacco, as neither upper nor lower epidermis could be removed to allow the access of cellulolytic or pectinolytic enzymes.

These difficulties could be overcome using leaves of *in vitro* plants which — in contrast to field plants — are sterile and exhibit a spongy leaf structure even within the palisade parenchyma.

This allowed the degradation of whole leaves by infiltration of the enzyme solution under high pressure within 3 h. A high buffering capacity of preparation solution and culture media was important to avoid damage due to pH shifts. Pectinase concentration could not be increased without deleterious effects to the protoplasts. The addition of salt solution reduced the formation (due to osmotic shocks) of phenols like resveratrol, the rest of which was inactivated by antioxidants and PVP. Sorbitol proved to be a better osmoticum than mannitol, which killed 30—50 % of the protoplasts within 24 h.

The optimized method allowed to get 5—10 · 10^7 protoplasts/g leaf from 8 different grape varieties. In some cases the formation of cell walls could be observed after 5 d — in particular if the culture had been taken from the 25 % Ficoll interphase of the density gradient. The addition of coconut milk proved to be negative. Although the cells could be kept alive for at least 14 d, neither continuous cell division nor even continuous growth was possible.

Suspension cultures of cells taken from callus material proved to be by far the most suitable system for investigations of stilbene metabolism.

Growth and subculture of 4 varieties have been studied in detail. Because there were no major varietal differences only the typical growth of a Pollux culture is given in more detail (Fig. 1).

If cultures were diluted 10-fold with fresh medium (B) (Fig. 1 B) the onset of both fresh weight and dry weight production showed a 3—4 d delay as compared to 1 : 3 dilutions (A) (Fig. 1 A). This held true for all varieties. Sirius showed a more or less parallel growth of fresh mass and dry mass, whereas in the other varieties vacuolization seemed to continue after the production of dry mass had come to an end. This seemed to be due to morphological pecularities of the Sirius cells (Fig. 2 c) which showed a typical unidimensional growth. The protein content started to grow 4 d ahead of the dry weight in (B) and even earlier in (A). Optima and Riesling calli produced less protein in (A) than in (B). After some weeks, though, all cultures reached a steady state at a content of 50—60 g fresh cells/100 ml of liquid. 1 : 10 subcultures of Riesling and Sirius led to pigmentation and sometimes to the death of the cells. Inoculates taken from young

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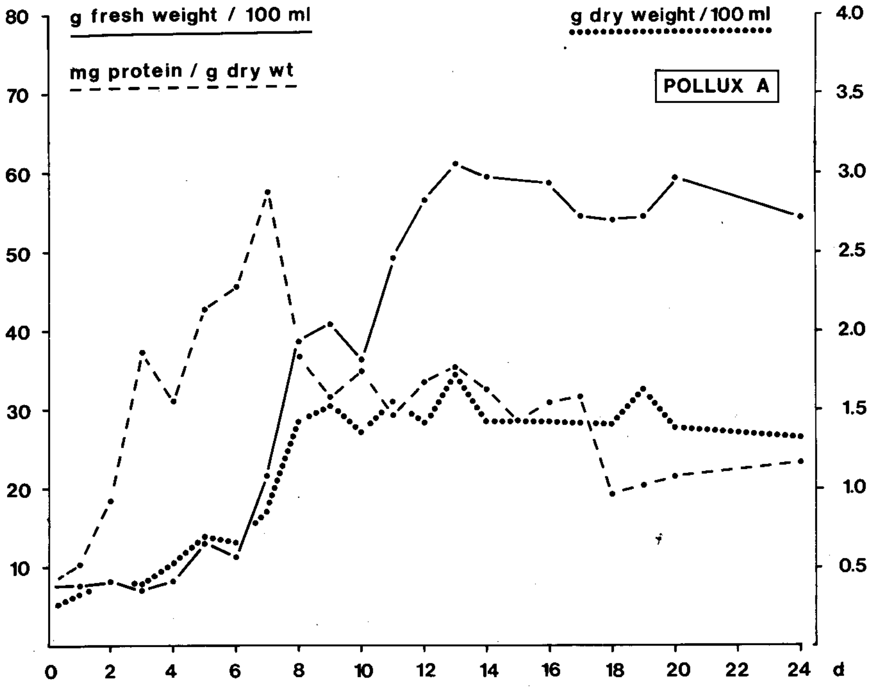
Fig. 1: Growth of callus suspension cultures (*Vitis* cv. Pollux) starting at different initial cell densities. The inoculum of Pollux A is diluted 3-fold as compared with B where the culture starts with 7.5 g living cells per 100 ml of liquid medium.

Wachstumsverlauf von Kallussuspensionskulturen (*Vitis,* Sorte Pollux) mit unterschiedlicher Zelldichte bei Kulturbeginn. Das Inokulum von Pollux A ist 3fach verdünnt, verglichen mit B, wo die Kultur mit 7,5 g lebenden Zellen je 100 ml Kulturmedium beginnt.


80
70
60
50
40
30
20
10
g fresh weight / 100 ml
mg protein / g dry wt
g dry weight / 100 ml
POLLUX A
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1.5
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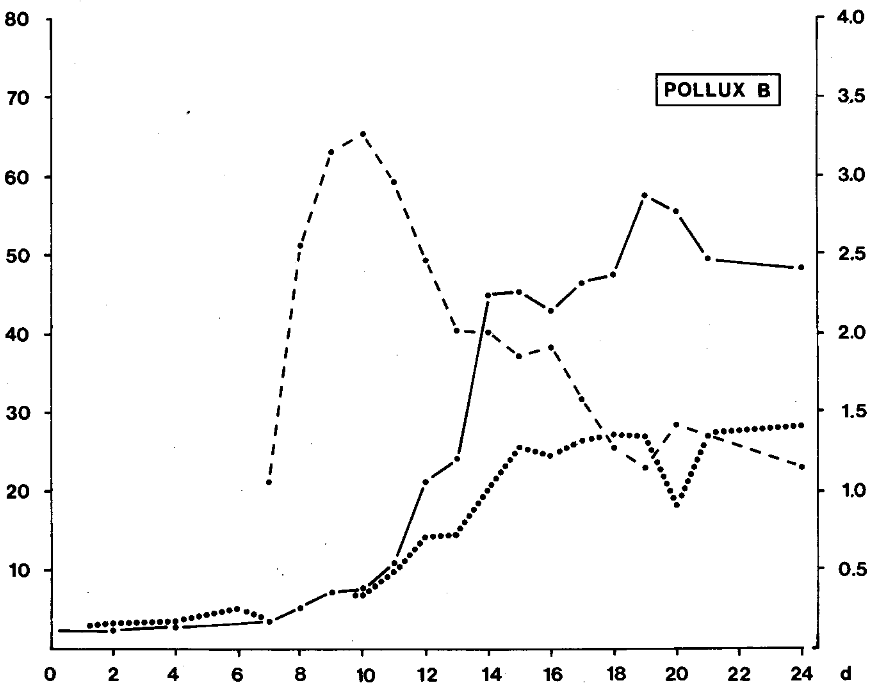
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POLLUX B
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cultures started to multiply earlier than those of old ones. Therefore a dilution of 1 : 5 of 2—3 week cultures was used for standard propagation. To initiate cell divisions a BAP concentration of > 0.3 mg/l was necessary; > 3 mg/l BAP or > 1 mg/l NAA reduced fresh weight production.

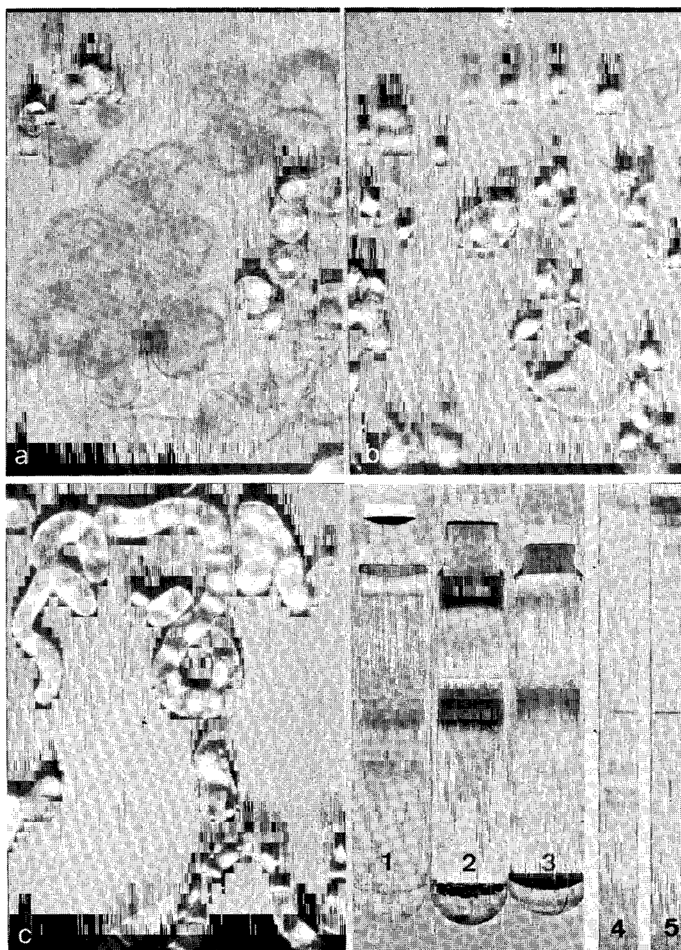


Fig. 2: a, b, c) Vital staining of callus cells in suspension cultures treated with debris of *Botrytis* mycelium 60 h before; fluorescence indicates living cells, dead ones are dark. a) Cv. Optima (Riesling cells look identical), b) cv. Pollux, c) cv. Sirius. — d) 1, 2, 3: Disc electrophoresis (anode at the bottom) of callus cell extracts. Staining for proteins (1) and for peroxidase activity with tetramethyl benzidine (2) and resveratrol (3) as substrates. — d) 4, 5: Thin layer electrofocusing (top pH 3.5, bottom pH 10) of fractions A (5) and B (4) of a gel filtration (see Fig. 3); staining like (2). The sharp band in the middle is the application site.

a, b, c) Vitalfärbung von Kalluszellen in Suspensionskulturen, die zuvor 60 h lang mit zerkleinertem *Botrytis*-Myzel behandelt worden waren. Fluoreszenz zeigt lebende Zellen an, dunkle sind tot. a) Sorte Optima (Rieslingzellen haben dasselbe Aussehen), b) Pollux, c) Sirius. — d) Diskelektrophorese (Anode unten) von Kalluszellextrakten. Färbung auf Protein (1) und auf Peroxidaseaktivität mit Tetramethylbenzidin (2) bzw. Resveratrol (3) als Substrat. — d) 4, 5: Dünnschicht-Elektrofokussierung (pH 3,5 oben, pH 10 unten) der Fraktionen A (5) und B (4) einer Gelfiltration (siehe Fig. 3); Färbung wie (2). Die scharfe Bande in der Mitte ist die Auftragstelle.

Elicitor tests with dry powder of *Botrytis* mycelium (50 mg/100 ml of culture) which is a potent inductor for phenylalanine ammonia lyase and stilbene synthase (Hoos and Blaich in prep.) did not lead to clean-cut results in the case of peroxidase induction, probably due to its more or less drastic toxic effects on the cells:

After 60 h, a large percentage of Riesling and Optima cells were dead (Fig. 2 a), whereas most Pollux and Sirius cells survived (Fig. 2 b, c). Exact numbers cannot be given because the cells form small aggregates which makes counting rather difficult. The effect could be attributed either to the *Botrytis* toxin being associated with the mycelium (see Stein 1984) or to a self-poisoning of the cells due to the formation of autotoxic products after the induction of a defense reaction.

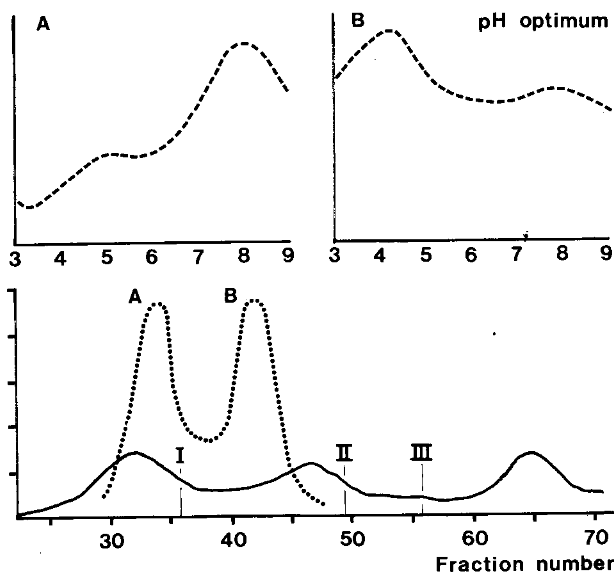


Fig. 3: Separation of a callus culture extract by gel chromatography (bottom). Absorption of fractions at 295 nm (——) and peroxidase activity (·····). Standards for MW estimation: I hemoglobin (68,000), II papain (23,400), III cytochrome c (12,300). Graphs A and B (on top) show the pH optimum of the activity peaks A and B, respectively, of the separation.

Gelchromatographische Trennung eines Kalluskultur-Extrakts (unten). Absorption der Fraktionen bei 295 nm (——) und Peroxidaseaktivität (·····). Eichproteine für die Molekulargewichtsschätzung: I Hämoglobin (68.000), II Papain (23.000), III Cytochrom c (12.300). Grafik A und B (oben) zeigen das pH-Optimum der Aktivitätspeaks A bzw. B der Trennung.

Characterization of peroxidases

The course of the intracellular peroxidase activity during growth was followed in Pollux cultures. It remained more or less proportional to the fresh weight of the cells; 10—14 d cultures were usually taken for the preparation of enzymes. The extracellular activity was about the same as the intracellular one.

The specifity of peroxidases was tested in crude extracts with different substrates both quantitatively with a spectrophotometer and after separation by disc electrophoresis (Fig. 2 d). After gel filtration (Fig. 3), two isoenzymes (A and B) were found which could be further separated by analytical isoelectric focusing (Fig. 2 d; for a discussion

of this microheterogeneity see BLAICH and ESSER 1974). The enzymes seem to be at least partially identical to those isolated from grape berries by SCIANCALEPORE *et al.* (1985).

Quantitative separation on a Fractogel column (Fig. 3) revealed that the isoenzymes differ in molecular weight and in pH optimum. Both enzyme groups were able to oxidize resveratrol, eventually forming a brown pigment (Fig. 2 d) which can also be demonstrated on TLC plates. The intensity of this reaction on disc gels was unexpected and seems to indicate a high substrate affinity of the enzyme which could, however, not be measured because of the low solubility of resveratrol (about 200 μg/ml) in the reaction buffer. According to tests with different resveratrol concentrations (Fig. 4), this is far from being the maximum velocity of the system.

The enzymatic oxidation of resveratrol was followed quantitatively analyzing the degradation and some of its products by means of TLC. Peroxidase A seems to form ε-viniferin as an intermediate product. It is, however, not clear whether this is due to substrate specifity or to the difference in pH. Previous results (BLAICH and BACHMANN 1980) as well as theoretical considerations (STERMITZ 1967; SCHOLZ *et al.* 1967; BLACKBURN and TIMMONS 1969) indicate that the oxidation involves an initial *cis-trans* conversion of the *cis*-resveratrol.

It is important that the activity of peroxidases on resveratrol be determined by measuring the initial velocity of the oxidation reaction because it is slowed down considerably after a certain amount of substrate has been consumed (Fig. 4). This effect can be counteracted to a certain extend if 'protectant proteins' like bovine serum albumine are added. Obviously their own reaction products are able to inhibit the enzymes after a certain time. Similar effects have been described by LEE *et al.* (1978) for horse radish peroxidase and MASON and WASSERMANN (1987) for red beet β-glucan synthase.

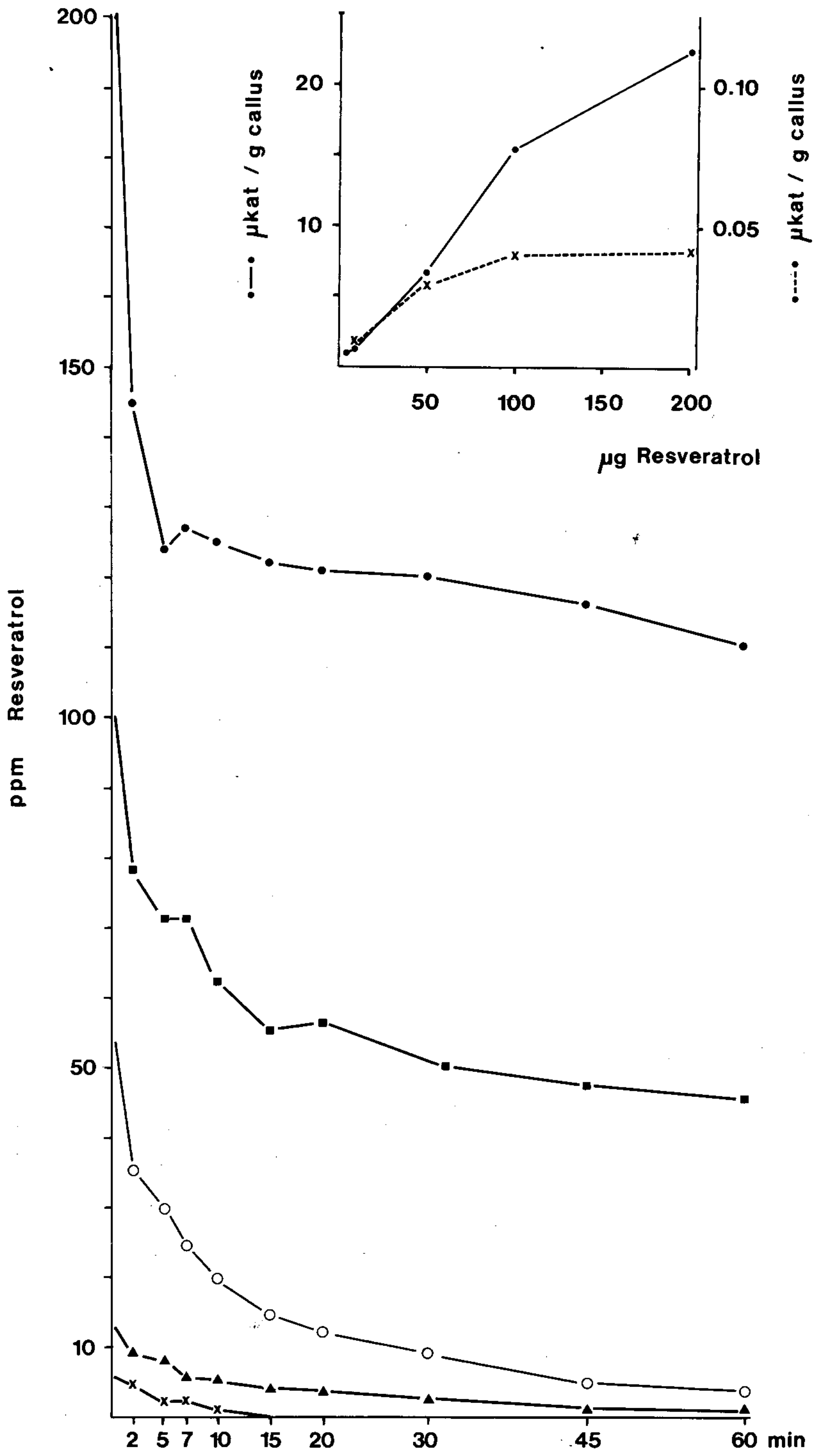
The interpretation of variety specific peroxidase activities is difficult due to self-inhibition (Fig. 4 a) and due to inhibitory effects of other phenolics, which occur even in single-cell cultures (mainly of resistant varieties). Nevertheless the peroxidase activity in cells of susceptible varieties is weaker than in resistant ones (Riesling 36, Optima 77, Pollux 113, and Sirius 276 μkat tetramethylbenzidine/g protein).

Concluding remarks

Single-cell cultures have proved to be a valuable tool for biochemical studies on defense mechanisms in general and for phytoalexin research in particular. In addition, it seems possible to screen *Botrytis* resistant cell lines. Although the regeneration of single cells to plants is not yet possible this might be a useful method for future breeding of resistant varieties.

Fig. 4: Oxidative degradation of different initial concentrations of resveratrol by callus peroxidase. It slows down considerably after 15—20 min indicating self-inhibition of the reaction by the oxidation products. The inset (top) shows the initial velocity of the oxidation (——) and the velocity after 20 min (·····) when 40—80 % of the initial amount of resveratrol are converted (different scales on the ordinate!).

Oxidativer Abbau unterschiedlicher Ausgangskonzentrationen von Resveratrol durch Kallus-Peroxidase. Er verlangsamt sich beträchtlich nach 15—20 min, was auf Selbsthemmung durch die Oxidationsprodukte hindeutet. Der Einsatz (oben) zeigt die Anfangsgeschwindigkeit der Oxidation (——) und die Geschwindigkeit nach 20 min (·····), wenn 40—80 % der Ausgangsmenge des Resveratrols umgesetzt sind (unterschiedliche Maßstäbe für die Ordinate!).



200
150
100
50
10
ppm Resveratrol
2 5 7 10 15 20 30 45 60 min
20
10
•—• µkat / g callus
0.10
0.05
•----• µkat / g callus
50 100 150 200
µg Resveratrol

The influence of phenolic compounds on enzyme studies in grapevines could be reduced considerably by the application of cell cultures. Nevertheless, phenolics are present and quantitative measurements should be interpreted cautiously, particularly if comparing resistant and susceptible varieties which show different phenol contents.

It must be pointed out that the rapid turnover of resveratrol and probably other stilbene phytoalexins could lead to wrong results if estimating stilbene production of different varieties by the analysis of leaf extracts. This would explain earlier observations that the resveratrol measured within leaves (inducted by UV-irradiation) was not correlated with the *Botrytis* resistance of over 100 varieties tested, whereas measurements with leaf pieces on filter cardboard containing elicitor solutions did correlate. In this case, resveratrol was measured which had leaked out from the leaf (apart from the peroxidases) and had been adsorbed by the cardboard due to its high affinity for cellulose fibers.

The inhibitory action of the oxidation products of stilbenes on enzymes indicates the necessity of a re-interpretation of earlier reports on the biological function of these compounds.

Stilbenes and their oxidation products show a high affinity for cell walls. Cytological investigations (HEINTZ, BLAICH and HOOS, in prep.) on host-parasite interactions between the grapevine and parasites like *Oidium* and *Botrytis* have shown that these metabolites are deposited together with other phenolic compounds during a lignification-like process in the surroundings of the infection site and which has been described in numerous publications on other plants (for literature e. g. VANCE *et al.* 1980). We feel that the stilbenes exercise a composite action in the defense system of the plant. On the one hand they are able to inhibit fungal growth of a fungus, on the other hand they are used as precursors for structural defense mechanisms.

Summary

Single-cell cultures of the fungus resistant grapevine varieties Pollux and Sirius and the susceptible ones Riesling and Optima have been established to study the metabolism of the stilbene phytoalexins resveratrol and viniferin. Cells of different varieties showed morphological differences (form and mechanical resistance) and their susceptibility to treatment with debris of killed mycelium of *Botrytis cinerea* reflected the varietal resistance under natural conditions. Most cells of Riesling and Optima were killed by toxic components of the mycelium within 60 h, whereas cultures of Pollux and Sirius showed only slight damage.

Two types of peroxidases have been characterized which can take resveratrol as a substrate converting it to a brown pigment. Viniferin was observed as an intermediate product. Peroxidase activity in suscpetible varieties was reduced as compared to the resistant ones. The oxidation products of resveratrol inhibited the activity of peroxidase.

The function of stilbene phytoalexins as precursors of lignin-like substances is discussed.

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