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Cultured Cells of *Arachis hypogaea* Susceptible to Induction of Stilbene Synthase (Resveratrol-forming)

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ABSTRACT

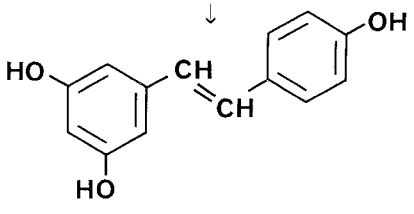
Callus cultures were established from cotyledons of *Arachis hypogaea*. The cultured cells were shown to produce stilbene synthase (resveratrol-forming) if the cells were irradiated with 254 nm UV light. The lag period after which the stilbene synthase activity appeared was similar for cultured cells and intact cotyledons.

INTRODUCTION

Studies aimed at the elucidation of enzymes involved in stilbene formation showed that in most cases an external stimulus, e. g. UV light, was necessary to elicit the formation of measurable amounts of stilbene synthase. Inducible stilbene synthases have been found in *Vitaceae* (Fritzemeier and Kindl 1981) and in *Pinus sylvestris* (Schöppner and Kindl 1979). With respect to a possible function of stilbenes as phytoalexins it was desirable to include plant cell cultures both for examining the properties of the enzymes and for studies on the enzyme induction under well defined conditions. It is intended to examine whether an elicitor or another stimulus eventually leads to an enhanced level of mRNA's coding for the enzymes which participate in the stilbene synthesis (Fig. 1).

Phenylalanine → Cinnamic acid → *p*-Coumaric acid → *p*-Coumaroyl-CoA

3 Malonyl-CoA + *p*-Coumaroyl-CoA



Resveratrol

Fig. 1. Pathway of resveratrol biosynthesis. The stilbene synthase catalyzes the condensation of *p*-coumaroyl-CoA with 3 molecules of malonyl-CoA to 3,5,4'-trihydroxystilbene (resveratrol).

We established cultured cells from cotyledons of ground-nut seedlings *(Arachis hypogaea)* and demonstrated their analogous behaviour to the original plant tissue with respect to the ability of synthesizing stilbene synthase upon illumination with UV light.

MATERIALS AND METHODS

Callus Formation and Culture Methods: Sterilized seeds of *Arachis hypogaea* were germinated at 26°C for 14 d. Parts of the cotyledons were placed on B5 medium (Gamborg et al. 1968) supplemented with 0.7 % (w/v) agar and 1 µg g^{-1} 2,4-dichlorophenoxy-acetic acid. The cultures were then kept at 26°C on the medium of Schenk and Hildebrandt (1972) solidified with 0.7 % agar. Callus was transferred to fresh plates every 2 weeks, and maintained for one year.

For the induction studies, calluses were grown for 14 d and then subjected to UV treatment for 15 min (254 nm, Camag inspection lamp, without filter). Subsequently, the cultures were kept at 26°C for the time indicated.

Cotyledons Induced by UV Light: Cotyledons of 8-d-old sterile seedlings were excised, placed under sterile conditions in petri dishes on moistened filter paper, and treated with UV light for 10 min on each side. Then, the cotyledons were incubated for 30 h, while aliquot samples were removed at the times indicated. Controls did not receive UV light.

Enzyme Preparations: One gram of cultured cells or 2 g of cotyledons, respectively, were homogenized at 2°C with 1.5 g polyclar AT in 10 ml of 150 mmol l^{-1} Hepes-NaOH buffer, pH 7.5, containing 5 mmol l^{-1} dithiothreitol and 10 % (w/w) sucrose. Cell debris was removed by centrifugation at 20 000 x g for 20 min. In most cases this crude extract was used as enzyme source. When partial purification was desired, fractions in a range between M_r 50 000 - 80 000 were taken after molecular sieving.

Enzyme Tests: Stilbene synthase (resveratrol-forming) was assayed as follows: 4 kBq malonyl-CoA (specific activity 2 GBq/mmol) were incubated with 33 µmol l^{-1} *p*-coumaroyl-CoA and 280 µl enzyme preparation in the

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presence of 5 mmol l^{-1} dithiothreitol and 10 % sucrose (Rupprich and Kindl 1978).

After incubation for 30 min at 30°C, the reaction was terminated by addition of 1 ml 0.1 mol l^{-1} HCl and 30 µg resveratrol. The mixture was 3-times extracted with ethyl acetate. The organic phase was reduced in vacuo and applied onto a thinlayer chromatography plate (0.2 mm, Merck, Darmstadt). Chloroform - ethyl acetate - formic acid (5:4:1) was used as solvent system (resveratrol; R_f = 0.68). For further identification, the respective zones were eluted and rechromatographed in toluene - methanol (9:1; R_f of resveratrol: 0.11) and compared with authentic samples. The zones of the respective chalcone and flavanone (naringenin) were also assayed for radioactivity (Schröder et al. 1979; Kreuzaler and Hahlbrock 1975).

Phenylalanine ammonia-lyase (EC 4.3.1.5) was tested according to Kindl (1970) with L-[1-^{14}C]phenylalanine as substrate.

Radioactivity on thinlayer chromatograms was visualized by scanning, absolute radioactivity was determined after elution of the compounds.

Materials: Coenzyme A esters of cinnamic acids were prepared as described (Stöckigt and Zenk 1975). [2-^{14}C]Malonyl-CoA was a product of Radiochemical Centre, Amersham, U.K. Seeds of *Arachis hypogaea* were obtained from M. Meyer, Frankfurt, FRG.

RESULTS AND DISCUSSION

The studies were aimed at establishing cultured cells which were capable of synthesizing stilbenes. After several passages, a line of cultured cells could be selected which had under normal conditions in the presence or absence of white light only an extremely low amount of resveratrol and its derivatives. But the cells were found to be sensitive to enzyme induction upon UV treatment. Stilbene synthase, being virtually absent during the first 2 weeks of callus growth, appears 10 h after the stimulus.

Fig. 2 shows the profile of enzyme activity from cultured cells which were grown in the presence of light. The specific activity of stilbene synthase shortly after UV treatment was 3.6 nkat kg^{-1}. A value of 50 nkat kg^{-1} was determined for cultures grown in darkness. In both cases, however, the maximal value, reached after 10 h, was 250 nkat kg^{-1}.

The reaction of callus originally derived from cotyledons and of cotyledons towards UV light was similar. In both cases, the enzyme activity of stilbene synthase was virtually not expressed unless the unphysiological, external stimulus was applied. The time after which the maximal value was reached was somewhat longer with the intact tissue. Furthermore, it can be depicted from Fig. 2 that the profile of phenylalanine ammonia-lyase did not coincide with the one of stilbene synthase when we investigated the induced callus.

In order to integrate the enzymatic activity detected into the scheme of stilbene syntheses known at present and of other enzymes catalyzing the reaction of various cinnamoyl-CoA esters with malonyl-CoA, we tested two C_6C_3-substrates and isolated the products. Table 1 demonstrates the enzyme preparation from *Arachis hypogaea* representing a resveratrol-synthesizing enzyme with only a small amount of chalcone synthase (determined as formation of naringenin). Chalcone synthase was found to be separable from stilbene synthase (data not shown).

A selectivity of 100:30 (resveratrol to pinosylvin) is typical of resveratrol-synthesizing stilbene synthases (Rupprich et al. 1980; Fritzemeier and Kindl 1981) while a ratio 30:100 was shown for the pinosylvin-synthesizing stilbene synthase (Schöppner and Kindl 1979).

TABLE 1. Substrate specificity of the stilbene synthase

Substrates	Products (relative amounts)
p-Coumaroyl-CoA + malonyl-CoA	Resveratrol (100) Naringenin (1)
Cinnamoyl-CoA + malonyl-CoA	Pinosylvin (30)

With *Arachis* cultures, high amounts of stilbene synthase were found besides trace amounts of chalcone synthase. This was in contrast to preparations from other sources, e. g. non-inducible cultured cells from *Picea excelsa* (Rolfs and Kindl, unpublished), where the chalcone synthase is the prevailing enzyme converting *p*-coumaroyl-CoA and malonyl-CoA.

The data presented provide further insights to explain earlier findings that stilbenes, e. g. isopentenylresveratrol in *Arachis hypogaea* (Keen and Ingham 1976; Aguamah et al. 1981) and methyl ethers of resveratrol in grape leaves (Langcake et al. 1979), could be synthesized only after attack of fungi or the application of artificial elicitors. That also alginate and mucic acid can elicit stilbene formation was shown by Blaich and Bachmann (1980).

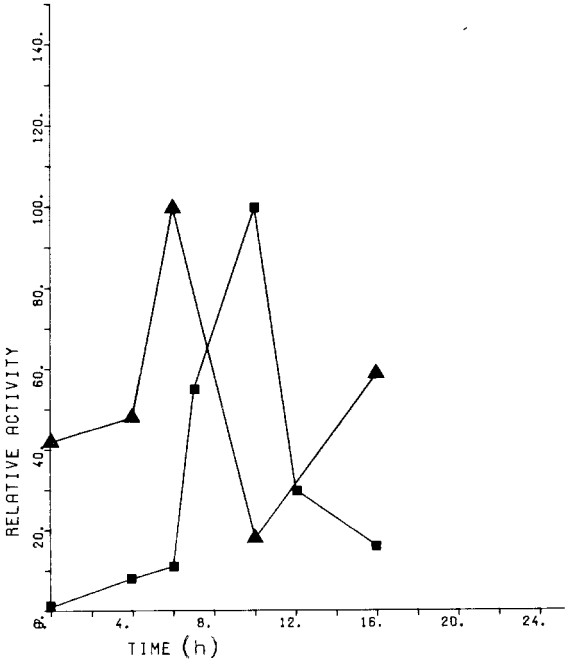

Fig. 2. Time course of enzyme activities after the callus received UV light for 15 min.

Relative activity (%) at different times (h) after induction. Stilbene Synthase (—■-■—) and phenylalanine ammonia-lyase (—▲—▲—).

Whether a coordinated induction of a series of enzymes including those responsible for the formation of stilbenes (Fig. 1) takes place, as was shown for the synthesis of stilbenes in intact leaves of *Vitacaea*, will be studied in more detail with cell suspension cultures.

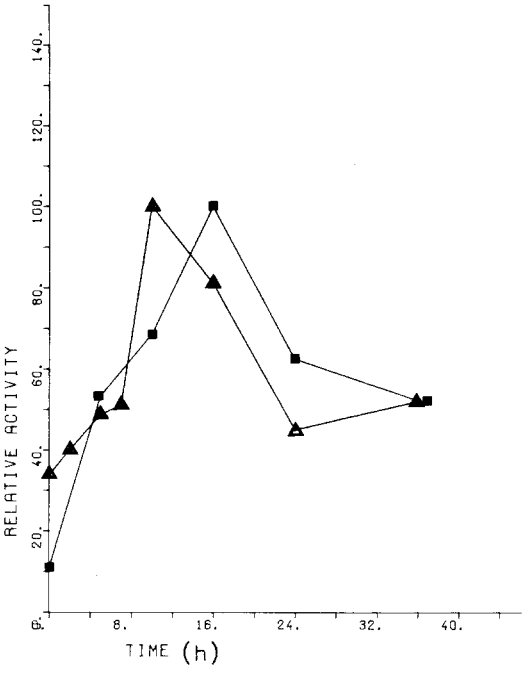

Fig. 3. Profile of stilbene synthase activity in intact cotyledons after UV light treatment.

Stilbene synthase (—■-■—) and phenylalanine ammonia-lyase (—▲—▲—).

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