Production and Accumulation of Phenylpropanoids in Tissue and Organ Cultures of *Pimpinella anisum*

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A leaf-differentiating callus culture and a root organ culture of *Pimpinella anisum* have been established in liquid nutrition medium. Their behaviour in accumulation of phenylpropanoids was shown to be different from that of the entire plant. This makes them, together with their special growth behaviour, suitable for labelling experiments with the biosynthesis of pseudoisoeugenols.

Introduction

Recently we reported the comparative studies on the production and accumulation of phenylpropanoids in plants and in cell cultures of anise [1, 2]. It was shown that cell cultures did not produce anethole, the main constituent in the fruit oil of anise, but instead regularly epoxy-pseudoisoeugenol-(2-methylbutyrate). We first identified this unusually substituted phenylpropanoid in the root of *Pimpinella saxifraga* [3, 4]. Pseudoisoeugenol derivatives are also common among other *Pimpinella* species [5, 6].

So far nothing is known about the biosynthesis of pseudoisoeugenols and the enzymes involved. Appropriate material for investigating their biosynthesis would be *in vitro* cultures of *Pimpinella anisum,* provided that the pseudoisoeugenols are both produced regularly in sufficient quantities and accumulated in culture.

In the following report we describe the phenylpropanoid production by a leaf-differentiating callus culture and a root organ culture of *Pimpinella anisum.* Their ability to synthesize phenylpropanoids will be compared with that of whole plants. Their suitability for studies on the biosynthesis of pseudoisoeugenols will be discussed briefly.

Results and Discussion

*Leaf-differentiating callus culture in suspension: PAD-SH**

Morphological characterization

PAD-SH was derived from a 3-year-old surface culture which was established from seedlings grown under sterile conditions. The 2-year-old *in vitro* culture was habituated and grew without phytohormons in a modified M&S liquid culture medium [7].

PAD-SH formed clumps of 2 to 3 cm in diameter. Their central parts were composed of relatively small volumes of callus tissue differentiating into small leaves with an area of 30 to 80 mm^2; occasionally roots were formed. Anatomical studies revealed that these leaves developed directly from the outer cell layers of the callus. These cell layers were composed of small meristematic cells without central vacuoles, but with large nuclei.

Growth of PAD-SH and accumulation of phenylpropanoids

Until the 14th day of cultivation, PAD-SH exhibited an almost constant growth rate related to fresh tissue weight. Starting at day 14, a remarkable increase of fresh weight was observed (Fig. 1). During this period, the dry weight always made up 6% of the fresh weight which means a real gain in bio-mass and not only an additional incorporation of water.

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* Names of the tissue cultures are german acronyms. PAD-SH: *Pimpinella anisum* differenziert, submers habituiert. PAW-SH: *Pimpinella anisum* Wurzel, submers habituiert.

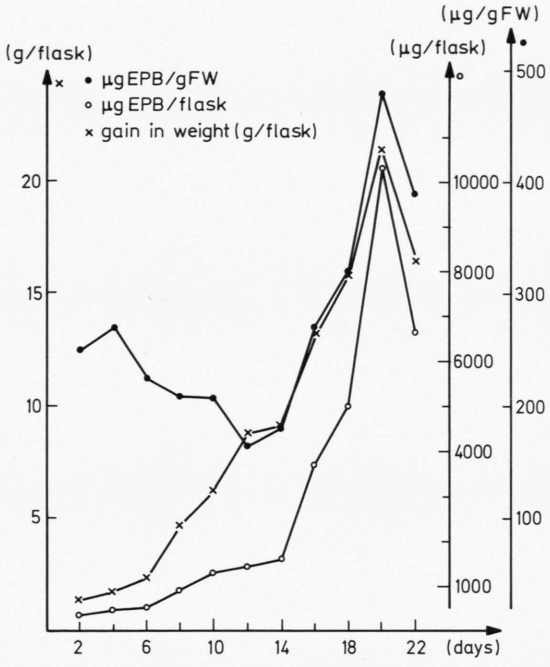


Fig. 1. Leaf-differentiating callus culture in suspension (PAD-SH): Correlation between the gain in fresh weight and the accumulation of epoxy-pseudoisoeugenol-(2-methylbutyrate).

From the known phenylpropanoids of the anise plant, PAD-SH produced epoxy-pseudoisoeugenol-(2-methylbutyrate) (EPB) regularly over the entire length of the growth period (Fig. 1). Most EPB was accumulated in the last third of the growth period. The increase of fresh weight and the accumulated amount of EPB/g fresh tissue rised at a parallel rate at this stage of culture growth. Therefore a maximum of more than 10 mg of EPB per culture flask could be detected on the 20th day of culture; this amount corresponded to about 480 µg EPB per gram fresh weight. Subsequent controls proved this behaviour to be consistent.

In addition to EPB, the corresponding olefinic derivative pseudoisoeugenol-(2-methylbutyrate) as well as anethole were accumulated in suspension culture from the 6th day of culture. The detected amounts in the tissue were much lower than those of EPB. Pseudoisoeugenol-(2-methylbutyrate) reached a maximum of up to 20 µg/g f.w. and anethole a maximum of up to 10 µg/g f.w.

Our established PAD-SH thus definitely promoted the production and accumulation of EPB, whereby anethole and the olefinic derivative of EPB were diminished. This behaviour was similar to that of root cells in the whole plant (Table I). Compared with undifferentiated callus and suspension cultures of anise which we reported earlier [1], PAD-SH accumulated higher amounts of EPB (80–100-fold).

Quantitative distribution of EPB in PAD-SH

Additionally we investigated the distribution of EPB in the different tissue types of PAD-SH. For this purpose the described clumps were separated into morphologically distinct callus, leaf and root tissues. In single leaves, roots and callus pieces (1 to 10 mg each) EPB was quantified by HPLC after extraction using an especially elaborated micromethod. As is obvious from Table II, the accumulation was strongly related to the degree of differentiation and seemed to take place in the basic tissue, since no specific regions of accumulation have been found microscopically. The degree of differentiation in the suspension culture seemed to be insufficient for a regular accumulation of anethole in larger amounts. These results correspond with the behavior of whole plants, where the highly developed degree of fruit differentiation is likewise associated with the accumulation of anethole in large amounts (Table I).

Root organ culture in suspension: PAW-SH

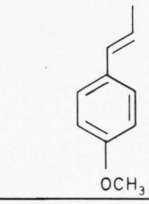
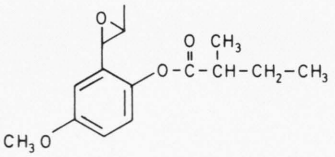
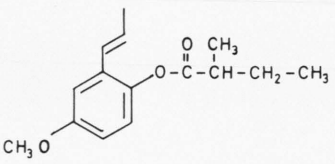
Morphological characterization

In addition to investigating PAD-SH we also examined the ability of a root organ culture to accumulate phenylpropanoids. PAW-SH was established from an anise surface culture which regenerated roots spontaneously. The roots were cut off and then transferred to a modified M&S liquid culture medium where it grew under habituated conditions. PAW-SH consisted of typical roots with cross-sections of 0.2 to 1 mm, reaching a length of up to 15 cm. They grew unipolar in length, partially spread out in branches and did not possess root hairs. Towards the end of the cultivation period the older parts started to produce callus which was removed on subculturing.

Accumulation of phenylpropanoids

PAW-SH almost exclusively accumulated EPB. Anethole was undetectable, pseudoisoeugenol-(2-

Table I. Distribution of anethole, epoxy-pseudoisoeugenol-(2-methylbutyrate) (= EPB) and pseudoisoeugenol-(2-methylbutyrate) in whole plant and *in vitro* cultures of anise; data in µg/g f.w. PAD-SH = leaf-differentiating callus culture; PAW-SH = root organ culture.

	Fruit	Shoot	Root	PAD-SH	PAW-SH
	21300	180	2	10	–
	4800	890	1350	480	850
	6600	140	Traces	20	Traces

methylbutyrate) was present only in traces. This behavior was similar to that in the normal plant root (compare Table I). The highest accumulation rate for EPB lied between the 3rd and 8th day (compare Fig. 2); up to 850 µg/g f.w. could be detected on the 6th day, decreasing to 400 µg/g f.w. on the 29th day. The maximum amount of EPB per flask was about 1 mg around days 13 and 24 and therefore about 10-fold lower than in PAD-SH. The observed fluctuations of the EPB-content cannot be explained as yet.

Comparison of PAD-SH and PAW-SH

If both the leaf-differentiating culture PAD-SH and the root organ culture PAW-SH are compared with respect to their suitability for biosynthetic studies, PAD-SH is superior to PAW-SH.

Due to the absolute amount of accumulated EPB, PAD-SH is suitable for labelling experiments using only one or two flasks per experiment. Furthermore, the steep rise in the accumulation rate starting at day

Table II. Accumulation of EPB in callus, leaflets and roots of PAD-SH; data in µg/g f.w.

Leaf-primordium	Green leaflet	Pale leaflet	Root	Callus
300–500	200–400	60–100	600–800	0–20

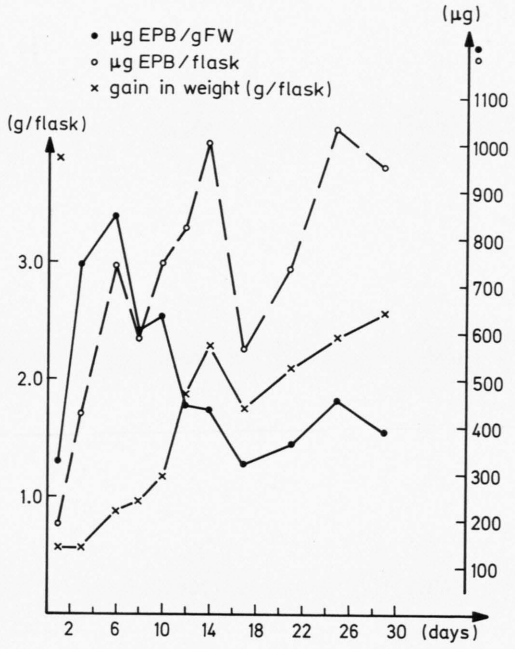


Fig. 2. Root organ culture in suspension (PAW-SH): Correlation between the gain in fresh weight and the accumulation of epoxy-pseudoisoeugenol-(2-methylbutyrate).

14 of the culture period allows high incorporation rates to be expected if labelling is conducted during this period. Initial labelling experiments confirm this assumption.

Materials and Methods

Origin and breeding of the anise plants

The fruits of *Pimpinella anisum* were ordered from the Müggenburg Company in Hamburg (Type: 06913). One portion of these fruits was sown in the botanical garden at the beginning of June 1986 and harvested before blooming in July/August. The plants (about 10 cm in height) were then separated into roots and shoots and treated separately.

Cultivation conditions

Both suspension cultures were habituated. Cultivation was carried out in 200 ml Erlenmeyer flasks containing 50 ml culture medium (pH 6) according to Murashige and Skoog [7], containing 30 g/l sucrose, without phytohormones. The flasks were kept under constant illumination (1500 lux) at 26 °C on a gyrotary shaker (Pilot Shake) at 110 rpm. 1.5 to 2.0 g tissue of PAD-SH was regularly transferred to new culture medium at day 22. About 0.7 g of PAW-SH was transferred to new medium at day 15. The day of transfer was counted as day 0.

Quantification of phenylpropanoids in tissue culture

An amount of 750 µg of epoxy-isoeugenol-isobutyrate (for synthesis see ref. [4]) as internal standard was added to the bio-mass of one culture flask. 20 to 30 ml of chloroform was added and the mixture homogenized. An aliquot of the chloroform layer was filtered and directly subjected to HPLC.

Quantification of the whole plant parts was conducted in an analogous manner.

Microanalysis of EPB in tissue: The tissues were weighed on a microbalance, transferred into small glass cylinders and 200 µl of a chloroform solution of internal standard (25 µg/ml) was added. After addition of a minute amount of purified sea-sand the mixture was homogenized by thorough grinding using a flat-ended glass rod. A Pasteur pipette was plugged with a small amount of cotton, the solution was filtered through it and subjected to HPLC analysis.

HPLC equipment (LDC/Milton Roy)

2 pumps Constametric I and III; injector: Rheodyne 7125, 20 µl loop; column: LiChrospher 100 CH 18/2, 5 µm; solvent: MeOH/water 80:20; flow: 1.2 ml/min; detector: Spectro-Monitor D, 278 nm.

Identification of phenylpropanoids

Identification of the phenylpropanoids was conducted by co-chromatography with authentic reference compounds as well as by GC-MS coupling and by ^{1}H NMR spectroscopy.

Acknowledgements

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