Comparative Investigation on Formation and Accumulation of Rare Phenylpropanoids in Plants and *in vitro* Cultures of *Pimpinella major*

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Unorganized callus and leaf/root-differentiating callus cultures of *Pimpinella major* have been established in liquid nutrient medium. Their capacity to accumulate rare phenylpropanoids such as epoxy-pseudoisoeugenol tiglate, epoxy-anol tiglate and anol tiglate was compared with that of seedlings and whole plants. The unorganized callus cultures were not able to accumulate any phenylpropanoids. In comparison, the leaf/root-differentiating callus culture promoted the accumulation of epoxy-pseudoisoeugenol tiglate (up to 90 mg/100 g fr.wt.) but not that of anol-derivatives. The accumulated amount of EPT in PMD-SH was comparable with that in plant seedlings.

Introduction

In former publications we could show that different species of the genus *Pimpinella* contain unusual substituted phenylpropanoids [1–3]. We called the 1-(*E*)-propenyl-2-hydroxy-5-methoxy-benzene skeleton of these compounds *pseudoisoeugenol* [1]. Other authors independently found the same class of compounds in various *Pimpinella* species (see Fig. 1) and thus confirmed our results [4–12]. Beyond the pseudoisoeugenol there also could be identified derivatives of 1-(*E*)-propenyl-4-hydroxy-benzene in some *Pimpinella* species [2, 9, 10]. Until now, pseudoisoeugenols have only been found in the genus *Pimpinella* and not in other *Apiaceae* or other plant families. These unusual substituted phenylpropanoids raise interesting questions about their biosynthesis and possible biological activities. Several pseudoisoeugenol derivatives isolated from various *Pimpinella* species were tested, for example, for their antigermination activity. All epoxy-pseudoisoeugenol derivatives were active against seeds from several different species (*e.g.* carrot, radish, lettuce), while compounds with olefinic groups instead of epoxy groups had no or only a minimal activity [10].

The unusual 2,5-dioxy substitution pattern of the pseudoisoeugenol derivatives is not consistent with the normally assumed biosynthetic pathway of known phenylpropanoids and requires its own explanation [13]. To learn more about the biogenesis of these phenylpropanoids in plant tissues we studied the formation and accumulation of pseudoisoeugenol- and anol-derivatives in plants and in various *in vitro* cultures of *Pimpinella major*.

Abbreviations: EPB, Epoxy-pseudoisoeugenol-(2-methylbutyrate); EPJ, Epoxy-pseudoisoeugenol isobutyrate; EPT, Epoxy-pseudoisoeugenol tiglate; EPA, Epoxy-pseudoisoeugenol angelicate; EAT, Epoxy-anol tiglate; PB, Pseudoisoeugenol-(2-methylbutyrate); PJ, Pseudoisoeugenol isobutyrate; PT, Pseudoisoeugenol tiglate; AT, Anol tiglate; Anol, 1-(*E*)-propenyl-4-hydroxy-benzene; PMD-SH, *P*impinella *m*ajor *d*iffer-enziert, *s*ubmers, *h*abituiert; NAA, Naphthyl acetic acid; M&S, Murashige and Skoog; fr.wt., fresh weight, TMS, Tetramethylsilane.

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Results and Discussion

Distribution of phenylpropanoids in plants and seedlings

The presence of pseudoisoeugenol-derivatives is characteristic for many species of the genus *Pimpinella* (Fig. 1). It is known from the literature that in roots of *Pimpinella major* EPT is the abundant compound, whereas EPB, PB and PT are only present in traces [1, 7, 9]. Besides EPT their exist also two other phenylpropanoids in the plant: EAT and AT [2, 8–10].

In order to examine the capacity of various plant tissues of *P. major* to accumulate phenylpropanoids we studied the occurrence and quantity of EPT, EAT and AT in plant materials of different

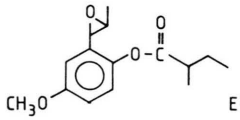
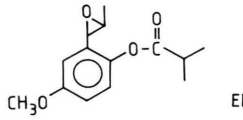
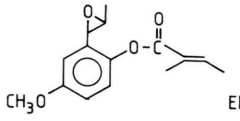
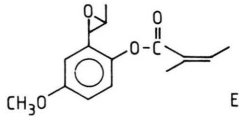
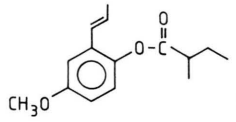
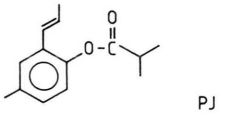
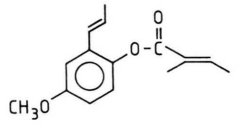
	Pimpinella anisum	*saxifraga*	*peregrina*	*major*	*diversifolia*	*tragium*
 EPB	+	+	+	(+)	+	+
 EPJ	–	(+)	+	–	+	–
 EPT	–	(+)	–	+	–	(+)
 EPA	–	–	–	–	+	–
 PB	+	+	+	(+)	–	–
 PJ	–	–	+	–	–	–
 PT	–	+	–	(+)	–	–

Fig. 1. Occurrence of various pseudoisoeugenol-derivatives in different species of the genus. The figure summarize data of literature [1, 2, 4, 7–12].

origin (Fig. 2). For this purpose the flowering plants were harvested and separated into roots, stems, leaves, umbel pedicels, flowers, ovaries, and fruits. The different plant organs were extracted with chloroform and the compounds quantified by HPLC. The identity of the compounds were ascertained on the basis of their MS and ^{1}H NMR data. As Fig. 2 shows, the phenylpropanoids in the plant were accumulated mainly organ-specifically. EPT was especially accumulated in the root, while there

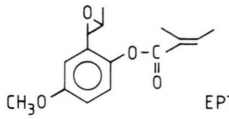
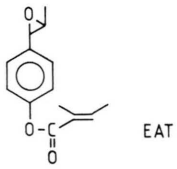
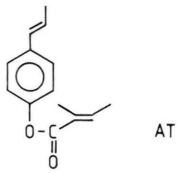
	Root	Stem	Leaf	Flower	Umbel pedicel	Ovary after blooming	Fruit
 EPT	10.7	0.5	0.6	0.4	3.1	3.1	–
 EAT	–	traces	traces	0.1	traces	2.1	7.0
 AT	–	–	–	–	–	–	5.3

Fig. 2. Distribution of EPT, EAT and AT in different organs of the whole plant (data in mg/g fr.wt.).

were essentially smaller quantities (0.4–0.6 mg/g fr.wt.) in stems, leaves, and flowers. In the region of the inflorescence EPT could be identified up to 3 mg/g fr.wt. in the umbel pedicels and in the ovary directly after blooming. This observation is very interesting, because EPT could not be identified in the fruit. This points to a repression of the corresponding genes of secondary metabolism at the transition zone from the ovaries to the fruits. Otherwise, both anol-derivatives would be mainly accumulated in the fruit. While AT could only be found in the fruits, EAT was also identified in the shoot and in the inflorescence in much smaller quantities (0.1–2.1 mg/g fr.wt.). The roots did not contain any anol-derivatives.

Examination of corresponding seedlings germinated and kept under sterile conditions revealed that EPT was accumulated even in this early stage of plant development. The roots comprised 0.4–0.7 mg/g fr.wt., the cotyledones 0.8–1.0 mg/g fr.wt. and the primary leaves 0.5–0.6 mg/g fr.wt. Anol-derivatives could not be identified. In relation to the adult plants, the roots and shoots of the seedlings comprise about the same quantities of EPT. Thus, in this early stage of plant development the roots are not the preferred accumulation organ for EPT.

Callus and suspension cultures

Callus and suspension cultures derived from seedlings germinated under sterile conditions grew very well. The suspension culture reached 9 times its initial weight after 24 days (from 0.8 g to 7.5 g/flask). Both *in vitro* cultures did not accumulate any phenylpropanoids.

Leaf/root-differentiating callus culture in suspension: PMD-SH

Morphological characterization: PMD-SH formed clumps of 1 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 and into roots.

Growth of PMD-SH and accumulation of EPT: PMD-SH were cultured in a liquid nutrient medium without phytohormones under continuous light (1500 lux, 26 °C). The growth behaviour and phenylpropanoid accumulation in the tissue were followed over a period of 28 days (Fig. 3). The *in vitro* culture achieved its maximum fresh weight of about 7.6 g at day 24 and increased its initial fresh weight 9.5-fold (from 0.8 g up to 7.6 g fr.wt./flask).

From the known phenylpropanoids of *P. major*, PMD-SH only produced EPT regularly over the

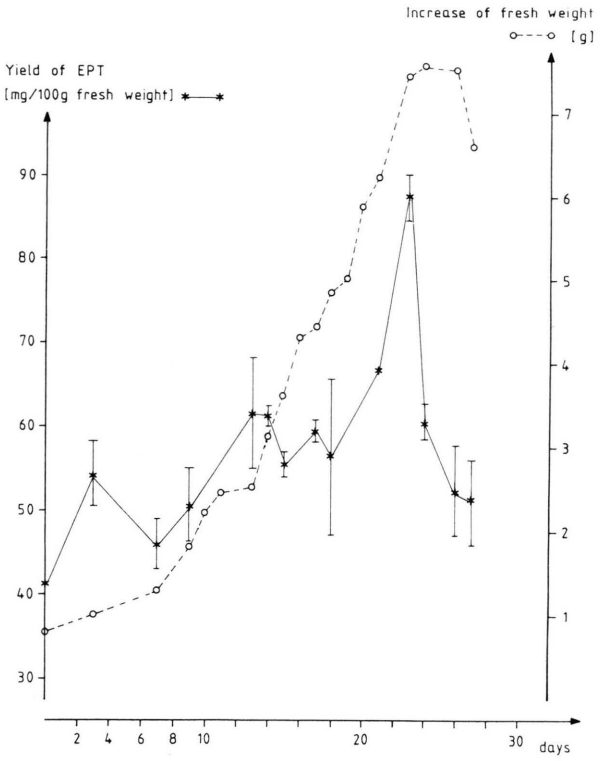


Fig. 3. Leaf/root-differentiating callus culture in suspension: PMD-SH. Correlation between the gain in fresh weight (g) and the accumulation of EPT in mg/100 g fresh weight.

entire length of the growth period. The increase in biomass and the accumulated amount of EPT/g fr.wt. rose more or less a parallel rate. Therefore a maximum of about 7 mg of EPT per culture flask could be detected on day 22 of culture period; this amount corresponded to about 90 mg/100 g fr.wt. Subsequent controls proved this behaviour to be consistent.

Quantitative distribution of EPT in PMD-SH: We were interested to discover if all different tissue types of PMD-SH were able to accumulate EPT in the same manner. For this purpose the described clumps were separated into morphologically distinct callus, leaf and root tissues. In single leaf, root and callus pieces EPT was quantified by HPLC after extraction using a special micromethod [15]. Leaves accumulated 360 mg/g fr.wt., roots 1000 µg/g fr.wt. and callus only traces (0 to 20 µg/g fr.wt.) of EPT. The yield of EPT in leaves and roots of PMD-SH was comparable with that in corresponding organs of seedlings.

Conclusion

The comparative investigation on formation and accumulation of phenylpropanoids in plants and *in vitro* cultures of *P. major* clearly showed that the occurrence of phenylpropanoids *in vitro* was closely linked with tissue differentiation. The more or less undifferentiated callus culture was not able to accumulate EPT. In comparison, the established PMD-SH definitely promoted, in a very constant manner, the production and accumulation of EPT (up to 90 mg/g fr.wt.). Consequently PMD-SH is suitable for examination of the biosynthetic pathway of EPT *in vitro*.

However, the degree of differentiation in PMD-SH seemed to be insufficient for a regular accumulation of anol-derivatives. These results correspond with the behaviour of whole plants, where the highly developed degree of fruit differentiation is likewise associated with the accumulation of EAT and AT in large amount.

Materials and Methods

Plants

Pimpinella major grows in the Botanical Garden of the University of Heidelberg. Flowering plants were harvested and separated into roots, stems, leaves, umbel pedicels, flowers, fruits and ovaries.

Seedlings

Fruits were surfaced sterilized by 1% $HgCl_2$-solution. After germination under sterile conditions the seedlings were transferred to an agar culture medium of Murashige and Skoog [14] without phytohormones and solidified with 1% Difco agar. After 10 days growth under constant illumination (1500 lux, 26 °C) roots and leaves were extracted separately by means of a special micromethod described elsewhere [15].

Callus and suspension cultures

Callus and suspension cultures were established from seedlings germinated under sterile conditions and kept under constant illumination (1500 lux, 26 °C). The medium used for *P. major* cultures was derived from Murashige and Skoog medium [14]

containing 30 g/l sucrose, 7 mg/l NAA and 1 mg/l kinetin (pH 6.0). Agar media were solidified with 1% Difco agar. Suspension cultures were kept in 200 ml Erlenmeyer flasks with 50 ml culture medium at 26 °C on a gyratory shaker at 110 rpm. Suspension cultures were transferred to fresh medium every 18–20 days and callus cultures every 10–12 days. The cultures examined were six month old.

Leaf/root-differentiating callus culture in suspension: PMD-SH

PMD-SH was derived from a 1 year old callus culture established from seedlings grown under sterile conditions. The suspension culture PMD-SH was habituated and grew without phytohormones in a modified M & S liquid medium [14]. All other culture conditions were the same as described above.

Identification of phenylpropanoids

For the identification of phenylpropanoids the compounds were first isolated and their structure verified by MS and ^{1}H NMR spectroscopy. The spectroscopical data found for the isolated compounds were consistent with data from literature [1, 8, 10].

Quantification of phenylpropanoids

Epoxy-isoeugenolisobutyrate (750 µg) was added as internal standard to the biomass of one culture flask. After addition of 5 ml of chloroform the mixture was homogenized (Ultra Turrax) and filtered, an aliquot of the extract was then subjected to HPLC. The quantification of the phenylpropanoids in whole plants and seedlings was conducted in an analogous manner [15]. All data given in the figures represent averages from 3 independent experiments.

HPLC equipment

LDC/Milton Roy (Hasselroth, F.R.G.); 2 pumps Constametric I and III; injector: Rheodyne 7125, 20 and 200 µl-loop; column: Lichrospher 100 CH 18/2, 5 µm; solvent: methanol + water (70–75), isocratic; flow: 1.2 ml/min; detector: Spectro-Monitor D, 278 nm.

Mass spectroscopy

Mass spectra were obtained by electron impact at 100 eV, direct inlet on Finnigan MAT 711.

^{1}H NMR spectroscopy

^{1}H NMR spectra were run on a Bruker WM 200 with $CDCl_3$ as solvent and TMS as internal standard. The spectra were compared with data from literature and authentic compounds.

Acknowledgement

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