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Synthesis of Benzylglucosinolate in *Tropaeolum majus* L.[1]

Isothiocyanates as Potent Enzyme Inhibitors

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Benzylglucosinolate accumulates in mature plants of *Tropaeolum majus* L. The biosynthetic capacity for synthesis of benzylglucosinolate and the total content of benzylglucosinolate have been investigated during plant development and in different tissues. The content increased from 5 mg of benzylglucosinolate in the fresh seed to between 200 and 400 mg in the adult plant, depending on size. The biosynthetic capacity was measured using L-[U-^{14}C]phenylalanine as precursor. Incorporation levels of approximately 30% were obtained with green leaves, whereas the incorporation levels obtained with other tissues were in the range of 0 to 5%. Leaves were the primary site of benzylglucosinolate synthesis. The high amounts of benzylglucosinolate accumulated in other tissues (e.g. developing seeds) reflected transport of benzylglucosinolate from the leaves. The initial steps in the biosynthesis of glucosinolates and cyanogenic glycosides are thought to be similar and to be localized on microsomal membranes. However, a microsomal system prepared from *T. majus* was biosynthetically inactive. Inclusion of *T. majus* plant material during preparation of sorghum microsomes also inhibited their activity. Benzylisothiocyanate, generated by degradation of benzylglucosinolate during the homogenization procedure, strongly inhibited the sorghum enzyme system, and its presence may thus explain why the isolated *T. majus* microsomal system is inactive.

Glucosinolates (Fig. 1, I) are a group of higher plant constituents found mainly within the order Capparales (Kjær, 1980). Several glucosinolate-containing species within the family Brassicaceae are important food crops and vegetables. The presence of glucosinolates in rape, *Brassica napus* and *Brassica campestris*, lowers the quality of the oil and limits the amount of oilcake to be used as animal feed. A glucosinolate-free variety may be developed using molecular biology and knowledge of the intermediates and enzymes involved in glucosinolate biosynthesis (Poulton and Møller, 1993). Like plants producing cyanogenic glycosides, plants that produce glucosinolates may show efficient conversion of the primary amino acid to the glucosinolate without accumulating detectable amounts of intermediates (Poulton and Møller, 1993). In studies of the biosynthesis of cyanogenic glycosides, in vitro experiments in which microsomal enzyme preparations are used have contributed greatly to the characterization of the intermediates involved (Møller and Conn, 1979; Halkier and Møller, 1989; Halkier et al., 1989, 1991; Conn, 1991). In view of this, it would obviously be desirable to obtain a similar microsomal enzyme system catalyzing the biosynthesis of glucosinolates.

We have chosen *Tropaeolum majus* L., garden Nasturtium, as the model plant. This species of the family Tropaeolaceae contains a single glucosinolate, benzylglucosinolate (Fig. 1, Ia), which is biosynthetically derived from L-Phe (Kjær et al., 1978). Studies in which labeled precursors are used and trapping experiments have previously shown *N*-hydroxyphenylalanine (Kindl and Underhill, 1968), 1-nitro-2-phenylethane (Matsuo et al., 1972), phenylacetaldehyde oxime (Tapper and Butler, 1967; Underhill, 1967), phenylacetothiohydroxamate (Underhill and Wetter, 1969), and desulfobenzylglucosinolate (Underhill and Wetter, 1969; Glendening and Poulton, 1988) to be precursors of benzylglucosinolate (Underhill et al., 1973). In the present study, the production of benzylglucosinolate has been followed from seeds to mature plants of *T. majus*. The main site of synthesis is the green leaves. It was not possible to obtain an active microsomal system from *T. majus* catalyzing the in vitro synthesis of benzylglucosinolate. One distinct possibility is that benzylisothiocyanate (Fig. 1, IIa), formed during plant homogenization, inactivates one or more of the enzymes involved.

MATERIALS AND METHODS

Plant Material

Seeds of *Tropaeolum majus* L. cv Empress of India were obtained from Dansk Havefrøforsyning (Kolding, Denmark). For experiments with etiolated seedlings, seeds were allowed to imbibe and germinate in vermiculite in complete darkness at 25°C. The seeds were germinated for 5 to 10 d depending on the experiment. Younger and older plants used for estimation of total benzylglucosinolate content were grown in vermiculite and peat moss, respectively, in a greenhouse.

Biosynthetic Experiments

Biosynthetic experiments were carried out by administering L-[U-^{14}C]Phe (1 μCi, 479 mCi mmol^{-1}; Amersham Ltd.) to single excised parts of *T. majus*. All experiments were re-

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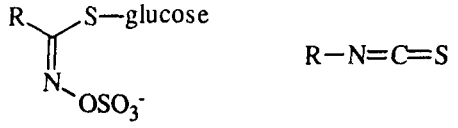

I II

Ia, R = Ph-CH_2 IIa, R = Ph-CH_2

Figure 1. I, Glucosinolate. Ia, Benzylglucosinolate. II, Isothiocyanate. IIa, Benzylisothiocyanate.

peated at least twice. The plant material was allowed to absorb the tracer and kept for 24 h in continuous light with water added as needed. The plant material was then extracted twice with boiling 90% methanol (approximately 30 mL g^{-1} fresh weight) for 15 min. The combined methanolic extracts were concentrated in vacuo, and the residue was dissolved in a known volume of 50% methanol.

Benzylglucosinolate Content of *T. majus* Seeds

After an initial 3-min microwave treatment (650 W) to destroy any enzyme activity, five seeds were crushed in a mill and extracted with 50 mL of 70% methanol. Following further homogenization using a Polytron, the suspension was refluxed for 15 min, cooled, filtered, and evaporated to dryness in vacuo. The residue was dissolved in 50 mL of water, washed with ether (2 × 10 mL), filtered (0.22 μm), concentrated in vacuo, and dissolved in water to a final volume of 3.50 mL.

Benzylglucosinolate Determination

Quantitative determination of benzylglucosinolate was carried out by HPLC analysis using 50-μL aliquots of seed/tissue extracts and an authentic sample of benzylglucosinolate for calibration. The HPLC system was equipped with an LKB 2249 gradient pump and an LKB 2141 variable wavelength monitor, both connected to a personal computer for collection and analysis of data. The column of Nucleosil 100–10C_{18} was operated at a flow rate of 1.0 mL min^{-1} using an isocratic mixture of 10% (v/v) solvent B (70% methanol) in solvent A (0.1 M ammonium acetate). A washing procedure for 5 min with 100% solvent B was included at the end of every run to clean the column. All liquids used for HPLC were filtered (0.22 μm) before use. UV absorption was measured at 235 nm. For quantitative determination of incorporation of radiolabeled Phe into benzylglucosinolate, the HPLC was coupled to a Berthold LB-506 C1 radioactivity monitor, which was fed liquid scintillator (Monoflow 2; National Diagnostics, Manville, NJ) at a rate of 3.0 mL min^{-1}. The incorporation percentages reported are corrected for loss of the carbon atom of the carboxyl group. Authentic benzylglucosinolate elutes at 13.00 min.

Microsomal Preparations of *T. majus* and *Sorghum bicolor*

Microsomal preparations were made by a modification of the method described by Møller and Conn (1979). Etiolated sorghum seedlings, *S. bicolor* (L.) Moench (20 g), obtained as described by Halkier and Møller (1989), were combined with different amounts of etiolated *T. majus* seedlings. The plant material was homogenized using a mortar and pestle in 2 volumes (v/w) of 250 mM Suc, 100 mM Tricine (pH 7.9), 50 mM NaCl, 2 mM EDTA, 2 mM DTT, and 0.5 mM PMSF. Polyvinylpolypyrrolidone (0.1 g g^{-1} fresh weight) was added before homogenization. Inclusion of the adsorbents Amberlite XAD-4 and Amberlite MB-1 revealed no beneficial effects. The homogenate was filtered through a 22-μm nylon cloth and centrifuged (12 min, 17,000*g*). The resulting supernatant was centrifuged (1 h, 165,000*g*), and the microsomal pellet obtained was resuspended and homogenized in isolation buffer using a Potter-Elvehjem homogenizer fitted with a Teflon pestle. After the material was recentrifuged and rehomogenized in 50 mM Tricine (pH 7.9) containing 2 mM DTT, the homogenate (5.0 mL) was dialyzed overnight against 50 mM Tricine (pH 7.9), 2 mM DTT in a nitrogen atmosphere.

Incubation of the sorghum microsomal preparation with the cofactor NADPH and L-Tyr as substrate results in the formation of *p*-hydroxymandelonitrile, which can be dissociated quantitatively into hydrogen cyanide and *p*-hydroxybenzaldehyde (Møller and Conn, 1979). The biosynthetic activity of the sorghum microsomal preparation can therefore be monitored by a spectrophotometric cyanide assay. A typical enzyme assay contained 15 μL of microsomal preparation (10 mg of protein mL^{-1}), 30 μL of L-Tyr (5 mM), 10 μL of NADPH (5 mM), and 95 μL of buffer (50 mM Tricine [pH 7.9]). After incubation (30°C, 20 min), enzyme activity was stopped by addition of 40 μL of 6 M NaOH, and cyanide analysis was carried out as described by Halkier and Møller (1989).

RESULTS AND DISCUSSION

Seeds of *T. majus* contained approximately 5 mg of benzylglucosinolate (Fig. 2). During germination and through the seedling stage, the glucosinolate level was almost constant.

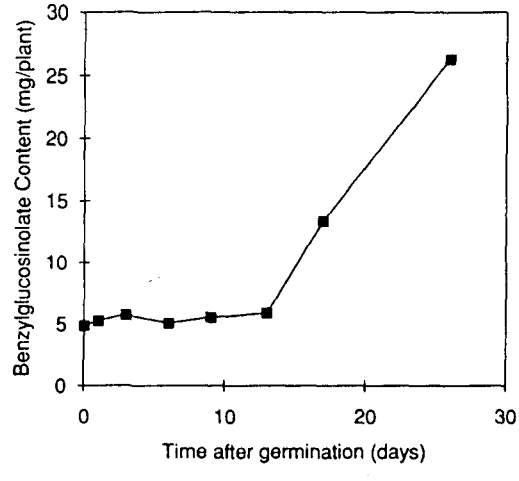

Figure 2. Total benzylglucosinolate content of *T. majus* during plant development.

The total content increased rapidly during the following weeks to reach 200 to 400 mg in adult plants (3 to 4 months old) depending on size. This result demonstrated the presence of an active benzylglucosinolate-synthesizing enzyme system during the later growth stages of *T. majus*.

The content of benzylglucosinolate, as well as the biosynthetic capacity in different parts of *T. majus*, was investigated during developmental stages from imbibition by the dry seed to seed formation on the adult plant using plant material grown in a greenhouse. The biosynthetic capacity was estimated semiquantitatively as the production of ^{14}C-labeled benzylglucosinolate upon administration of the ^{14}C-labeled precursor amino acid Phe to excised parts of *T. majus* plants. *T. majus* contained benzylglucosinolate in all parts of the plant (Fig. 3). Green leaves of *T. majus* were the major depositories of benzylglucosinolate and serve as the primary site of biosynthesis as well (Fig. 3). Most other tissues were either biosynthetically inactive or showed only marginal activity. These data suggested that benzylglucosinolate produced in the leaves was transported to other plant tissues. Shoots with small leaves or unexpanded stages of leaves were also biosynthetically active (Fig. 3).

The biosynthetically active microsomal system in sorghum is obtained from etiolated seedlings (Halkier and Møller, 1989). In *T. majus*, neither etiolated, red light-grown (Sylvania red fluorescent tubes, peak emission 660 nm, fluence rate 10 mmol m^{-2} s^{-1}), nor light-grown (Osram Lumilux daylight tubes, 60 μmol m^{-2} s^{-1}) seedlings incorporated Phe into benzylglucosinolate to any significant extent (Fig. 3). Correlated with the constant overall content of benzylglucosinolate in seedlings up to 13 d after germination (Fig. 2), the data demonstrate that neither de novo synthesis nor turnover took place at this stage of plant development. Thus, young seedling material is not suitable for the preparation of biosynthetically active microsomal systems of *T. majus*.

Seeds of *T. majus* contain high amounts of benzylglucosinolate. To establish whether the glucosinolate is produced in situ during seed development or translocated to the seed from other sources, biosynthetic experiments were carried out using *T. majus* in different stages of blooming and seed development. The content of benzylglucosinolate in *T. majus*

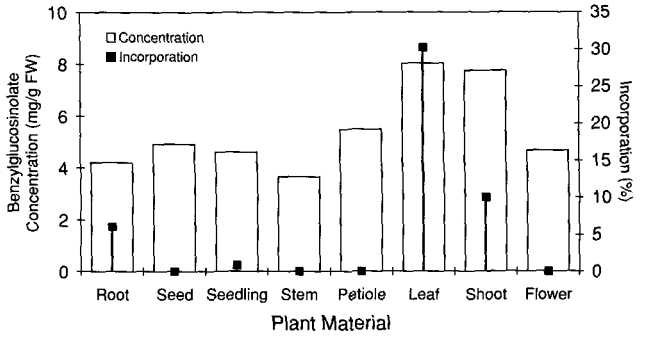

Figure 3. Benzylglucosinolate content and incorporation of L-[U-^{14}C]Phe into benzylglucosinolate by various tissues of *T. majus*. The biosynthetic experiments were carried out by administering 1 μCi of [U-^{14}C]Phe to single excised parts of *T. majus*. FW, Fresh weight.

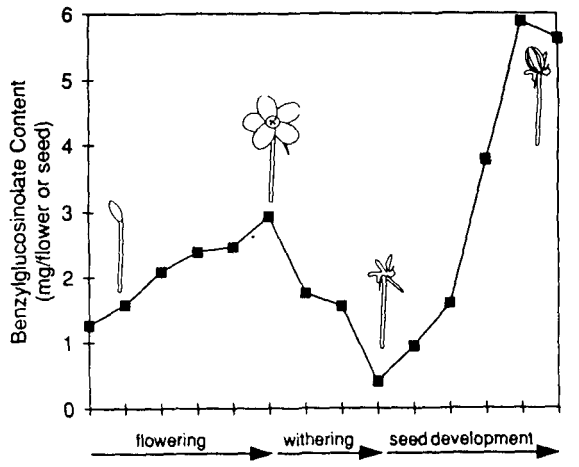

Figure 4. Benzylglucosinolate content of the developing flower and seed.

flowers increased rapidly during the process of flowering, decreased during flower withering, and increased again during seed formation (Fig. 4). The biosynthetic experiments revealed no detectable capacity for de novo synthesis of benzylglucosinolate in any of these tissues (see data in Fig. 3). The measured changes in benzylglucosinolate content at the different flower stages thus reflected transport of benzylglucosinolate from the leaves and shoots (Fig. 3). Biosynthetic experiments (data not shown) in which leaves neighboring developing seeds were used showed no detectable increase in their biosynthetic capacity compared with other leaves.

The determined incorporation of [U-^{14}C]Phe into benzylglucosinolate points to the leaves of *T. majus* as the obvious starting material for the preparation of biosynthetically active microsomal preparations. However, microsomal preparations from young leaves, fully expanded leaves, or etiolated leaves and shoots obtained from plants transferred to darkness were

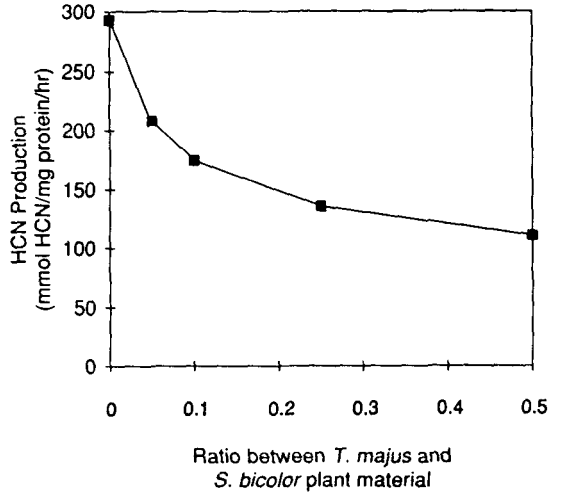

Figure 5. Enzymic activity of *S. bicolor* microsomal preparations after addition of various amounts of *T. majus* seedlings before homogenization. HCN, Hydrogen cyanide.

all inactive. Our failure to obtain a biosynthetically active microsomal system from leaves of *T. majus* prompted an investigation of the possible presence of an enzyme inhibitor in *T. majus*. This possibility was tested using the biosynthetically active microsomal system from *S. bicolor* thought to share many common features with the *T. majus* system (Ettlinger and Kjær, 1968; Poulton and Møller, 1993).

Microsomes were prepared from a constant amount of etiolated sorghum seedlings to which different amounts of etiolated seedlings of *T. majus* had been added. The presence of *T. majus* plant material inhibited the enzymic activity of the sorghum system (Fig. 5). Experiments in which a sorghum microsomal preparation was assayed in the presence of an added microsomal preparation from *T. majus* also showed decreased biosynthetic activity upon addition of increasing amounts of the *T. majus* preparation (data not shown). The initial concentration of benzylglucosinolate in a homogenate obtained using green leaves of *T. majus* is approximately 9 mM. The soluble thioglucosidase present in the homogenate catalyzes the conversion of benzylglucosinolate into benzylisothiocyanate (Fig. 1, IIa) (Underhill, 1980). Biosynthetic experiments (Møller and Conn, 1979) with sorghum microsomal systems prepared using isolation buffer supplemented with increasing amounts of benzylisothiocyanate demonstrated that benzylisothiocyanate is a potent inhibitor of enzyme activity (Fig. 6). The inhibitory effect was irreversible, because extended dialysis of the preparations did not result in recovery of enzymic activity. Most likely, benzylisothiocyanate reacts with amino, hydroxyl, or sulfhydryl groups (Reid, 1966; Tang, 1974; Satchell and Satchell, 1975; Takeguchi et al., 1983) essential for the catalytic activity of the microsomal system.

In the present study we have clearly identified green leaves of *T. majus* as the major site of benzylglucosinolate biosynthesis. If the enzyme systems catalyzing the biosynthesis of cyanogenic glucosides and glucosinolates are indeed very similar, it is tempting to speculate that the benzylisothiocyanate generated during homogenization of the *T. majus* leaves would also act as an inhibitor of the enzyme system responsible for benzylglucosinolate biosynthesis and thus prevent the preparation of an active microsomal system catalyzing in vitro synthesis of benzylglucosinolate. It cannot be excluded that other inhibitory components present in *T. majus* augment the inhibitory effect.

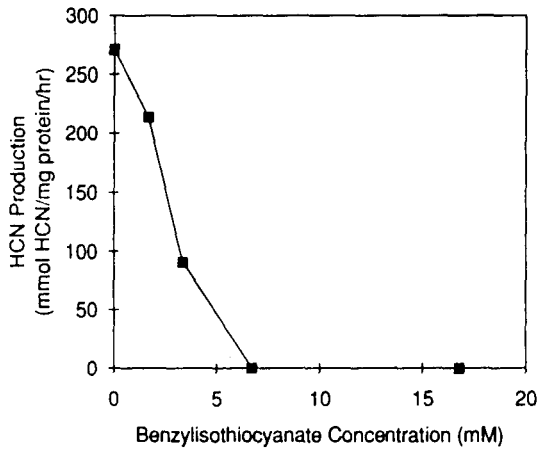

Figure 6. Inhibition of the enzymic activity of *S. bicolor* preparations by the presence of increasing amounts of benzylisothiocyanate in the isolation buffer. HCN, Hydrogen cyanide.

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