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DAURICINE PRODUCTION IN CULTURED ROOTS OF *MENISPERMUM DAURICUM*

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Key Word Index—*Menispermum dauricum*; Menispermaceae; root culture; bisbenzylisoquinoline; dauricine.

Abstract—Root cultures of *Menispermum dauricum* were established from adventitious roots formed on the leaf segments. These roots produce dauricine as a major constituent and the optimum conditions for dauricine production in cultured roots were investigated. A dauricine content of more than 0.5% dry weight was achieved in Gamborg's B5 medium containing 3% sucrose and 7.5 μM NAA. Tyrosine and tyramine, possible precursors of dauricine, increased dauricine formation. There was a positive correlation between dauricine content and root growth.

INTRODUCTION

The bisbenzylisoquinolines constitute one of the most important groupings of isoquinoline alkaloids. A variety of structural patterns arise in bisbenzylisoquinoline molecules. This diversity is due to differences in substituents on the aromatic rings, the number and the nature of the linkages between the benzylisoquinoline halves of the molecule and the absolute configurations of two asymmetric centres.

Cultured plant tissues, capable of producing desired secondary products, are an excellent material for the study of their biosynthesis [1, 2]. We have established root cultures of *Stephania cepharantha* [3] which produce aromoline and berbamine, bisbenzylisoquinolines with two ether linkages in the molecule. By feeding experiments of the labelled precursors into the root cultures, we demonstrated that both alkaloids were composed of four molecules of tyrosine [4–6]. Zenk *et al.* [7] have reported that intermolecular oxidative phenol coupling of two benzylisoquinolines, by which bisbenzylisoquinolines are formed, was catalysed by a cytochrome P-450-linked, NADPH and O_2-dependent, microsome-bound enzyme. Previous reports [7–9] have shown only preliminary information regarding the enzymatic coupling mechanism of benzylisoquinolines. Establishment of culture systems, producing simple bisbenzylisoquinoline with higher efficiency, is required to investigate the enzymatic coupling mechanism in more detail.

Dauricine (1) is built up of two molecules of (*R*)-6, 7-dimethoxy-1-*p*-hydroxybenzyl-2-methyl-1, 2, 3, 4-tetrahydroisoquinoline (armepavine) linked by an ether bridge between OH-11 and C'-10. This alkaloid has been isolated from several sources: the rhizomes of *Menispermum canadense* [10] and *M. dauricum* (Menispermaceae) [11], the leaves of *Polyalthia nitidissima* (Annonaceae) [12] and the bark of *Popowia pisocarpa* (Annonaceae) [13]. Dauricine is an ideal alkaloid to study the phenol oxidative coupling of benzylisoquinolines since it is a homodimer of (*R*)-armepavine and all the hydroxyl groups in the isoquinoline portions are methylated. Therefore, only coupling between the benzyl portions can take place.

We report herein the establishment of dauricine-producing cultured roots of *M. dauricum*. Effects of plant growth regulators, nitrogen, phosphate and sugars on growth and dauricine formation were studied. Several possible precursors of bisbenzylisoquinolines were also tested for their promotive effect on dauricine formation in *M. dauricum* root cultures.

RESULTS AND DISCUSSION

Identification of dauricine

Sterilized leaf segments of *M. dauricum* were inoculated on Linsmaier–Skoog (LS) agar medium [14] containing 10 μM NAA and 1 μM BA. After one month of incubation in the dark at 27°, callus was induced. After another one and half months of incubation, adventitious roots had formed. Thus, roots were obtained and cultured on a rotary shaker (70 rpm) in the dark at 27° in liquid B5 media [15] containing various concentrations of IBA. Preliminary experiments showed that in the media containing 10 μM IBA the total mass of the root was higher than in the media containing 1 μM IBA. But in the media containing lower amounts of IBA, root elongation was faster than in the media with higher IBA. We selected the B5 medium with 1 μM IBA since long roots handled

favourably, and maintained the cultured roots in the media for three years. Root cultures were transferred to fresh media every one to two months depending on their growth. The roots were harvested and dried. Alkaloids were extracted from the dried roots with methanol and analysed.

TLC of methanol extracts from cultured roots of *M. dauricum* indicated that several bases, which behave chromatographically like bisbenzylisoquinolines, were produced in the roots. The most abundant base was isolated as an amorphous solid and its ^{1}H NMR, IR and mass spectra and specific rotation were studied. All these parameters matched literature values of dauricine (**1**). The ^{13}C NMR spectrum also showed this base to be dauricine, which has not been reported in the literature. The ^{13}C NMR spectrum of *O*-methyldauricine (**2**) had been assigned by Jossang *et al.* [13]. We converted the isolated base to its methyl ether using diazomethane and measured its ^{13}C NMR spectrum; it matched that of **2**. Thus, dauricine (**1**) was identified in the cultured roots of *M. dauricum*. Parent plants of *M. dauricum* are known to produce other bisbenzylisoquinolines: dauricinoline, dauricoline, daurinoline, *N'*-desmethyldauricine and daurisoline [16, 17]. Other alkaloids in the cultured roots are under investigation. Culture of *M. canadense* failed because of heavy browning of the tissues.

Effect of plant growth regulators

Three auxins (NAA, IAA or IBA), each at 0.1, 1, 10 or 100 μM, were tested for growth promotion in cultured *Menispermum* roots (Fig. 1). NAA was the most effective for root growth promotion; IAA and IBA were less effective. Each auxin promoted root growth when present at 10 μM in the culture medium. At 10 μM NAA, cultured roots grew 28-fold in 40 days. With higher auxin concentrations, lateral roots were frequently induced and root elongation inhibited. At 10 μM auxin, lateral root induction and root elongation were well-balanced.

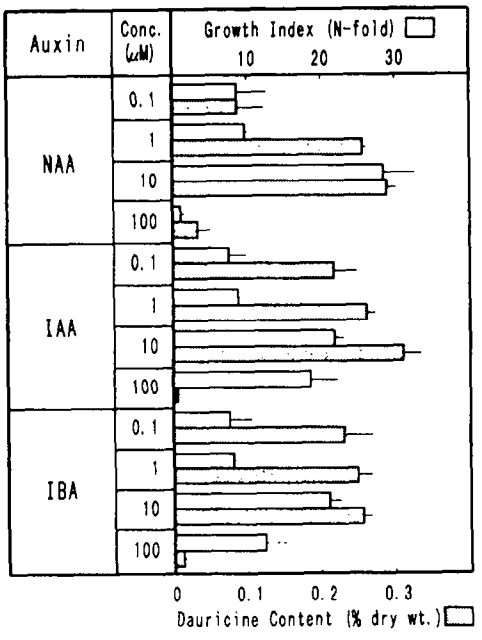


Fig. 1. Effects of auxin on growth and dauricine formation. Roots (0.1 g fr. wt) were cultured in 25 ml of medium with auxin for 40 days. Straight lines represent s.e. for three replicates.

The effects of auxin concentrations on dauricine content in cultured roots were also studied (Fig. 1). At the optimum concentration of each auxin for root growth, roots produced the highest amount of dauricine. High concentrations of auxins (100 μM) drastically inhibited dauricine formation.

NAA was most effective for dauricine production (Fig. 1.). The effects of NAA on root growth and dauricine content were examined in detail. As a result, cultured roots produced the highest amount of dauricine (**1**) at 7.5 μM. At this concentration, roots growth was 44-fold (40 days) containing 0.28% dauricine.

Other growth regulators were also studied in combination with 7.5 μM NAA for their effects on the growth and dauricine content of *M. dauricum* root cultures. The examined growth regulators were 6-benzyladenine as cytokinin, gibberellic acid (GA_3) and abscisic acid. These regulators were studied at 0.1, 1, 10 or 100 μM. None of them gave significant growth stimulation or increased alkaloid formation in cultured roots. At high concentrations (10 and 100 μM), each growth regulator completely inhibited root growth (data not shown).

Effects of nitrogen, phosphate and carbohydrate sources

Effects of the NH_4^+ to NO_3^- ratios in the culture medium on dauricine production in *Menispermum* roots were studied (Fig. 2A). The total amount of N was set equal to that in B5 medium, which has a ratio of NH_4^+ to NO_3^- of 1:14.6. Dauricine formation and root growth were inhibited by high concentrations of NH_4^+. The elimination of NH_4^+ from the medium did not reduce root growth significantly but dauricine formation was decreased. Changing the ratio of NH_4^+ to NO_3^- from half to twice that in B5 medium did not affect the dauricine production considerably. The original ratio in B5 medium gave the highest amount of dauricine (**1**).

Phosphate concentration was changed from one-eighth to twice that in the B5 medium. Low concentration (1/2) of phosphate gave a higher dauricine content but slower root growth. Changing the phosphate concentration did not promote dauricine production significantly (Fig. 2B).

The effects of various sugars on root growth and dauricine formation were also studied. Two disaccharides, sucrose and lactose, and three monosaccharides, glucose, fructose and galactose, were used at 3%. The results of Fig. 2C show that root growth in a medium containing sucrose, glucose or fructose is significantly higher than medium containing galactose or lactose. Dauricine contents were 0.33, 0.24 and 0.14% as a result of sucrose, glucose and fructose, respectively. Galactose and lactose decreased both root growth and dauricine formation. Since sucrose was the most suitable for dauricine production, the effects of sucrose concentration on

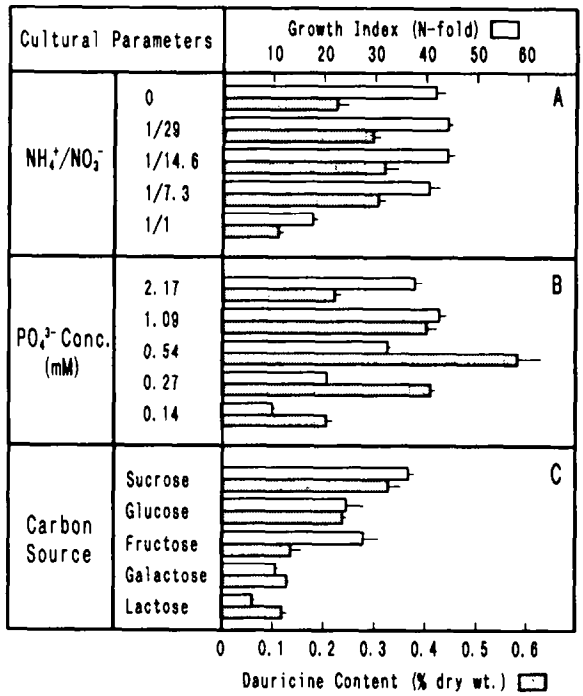


Fig. 2. Effects of NH_4^+ to NO_3^- ratios (A), phosphate concentration (B) and sugars (C) on growth and dauricine formation. Roots (0.1 g fr. wt) were cultured in 25 ml of modified B5 medium for 40 days. Straight lines represent s.e. for three replicates.

dauricine production were studied. The optimum concentration of sucrose for root growth was 3%, but, on the other hand, that for the dauricine content (per dry matter) was 4%. Overall, the optimum sucrose concentration for total dauricine production was 3% (data not shown).

Dauricine-production medium

As described above, we established the optimum medium for dauricine production in cultured roots of *M. dauricum*. This was found to be B5 medium with 7.5 μM NAA and 3% sucrose. We evaluated plant growth regulators, inorganic constituents or carbon sources, qualitatively and quantitatively. Only the auxin showed the promoting effect on dauricine production compared with other cultural parameters in the initial medium. Cultured roots appeared to be highly adapted to the originally chosen medium (B5 medium with 1 μM IBA and 3% sucrose) during more than three years maintenance. A typical time-course of root growth and dauricine content, for roots cultured in the optimized medium, is shown in Fig. 3. Cultured roots grew *ca* 40-fold in 50 days. Dauricine content was the lowest when roots were growing exponentially. In contrast, dauricine increased substantially at the late log-phase and stationary phase. The dauricine content of the cultured roots reached more than 0.5% of the dry wt. In the original plants, dauricine content in the aerial and underground parts were only *ca* 0.03 and 0.2%, respectively.

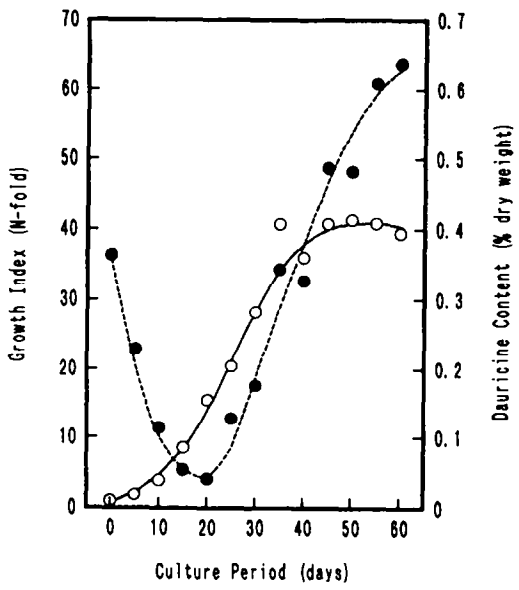


Fig. 3. Time-course of growth and dauricine content in root cultures of *M. dauricum*. The symbols denote root growth (—○—) and dauricine content (---●---).

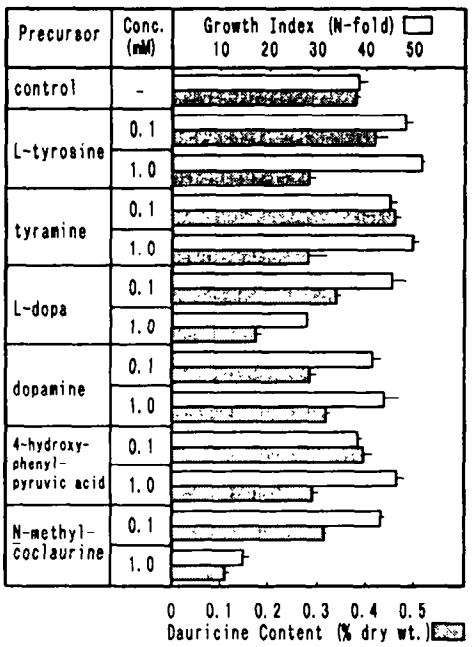


Fig. 4. Effects of possible precursors on growth and dauricine formation. Roots (0.1 g fr. wt) were cultured in 25 ml of medium with various precursors for 40 days. Straight lines represent s.e. for three replicates.

Effects of precursors

Effects of precursor feeding on dauricine formation were studied with possible precursors of benzylisoquinoline biosynthesis: L-tyrosine, tyramine, L-dopa, dopamine, 4-hydroxyphenylpyruvic acid and *N*-methylcoclaurine (Fig. 4). The first four precursors were reported

to be incorporated into the protoberberines. 4-Hydroxyphenylpyruvic acid is known to be a building block of the benzylisoquinoline skeleton [18]. L-Tyrosine, tyramine and *N*-methylcoclaurine have been reported to be incorporated into bisbenzylisoquinolines [8].

Precursors were added to the root cultures at 0.1 or 1 mM at the beginning of incubation. Tyrosine and tyramine (0.1 mM) effectively increased dauricine formation, which indicates that the supply of these precursors might be the limiting factor for dauricine formation more than the others. In previous studies [4, 6], exogeneous tyrosine was well incorporated into cultured roots of *S. cepharantha* and then stored in the form of tyramine. But neither tyrosine nor tyramine promoted alkaloid formation. Other precursors inhibited dauricine formation.

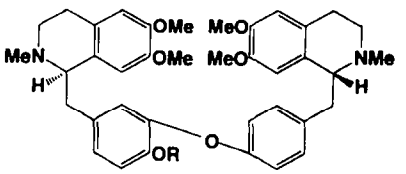
Relationship between dauricine content and growth

The relationship between dauricine content and growth rate was analysed for all the results described above. Statistically significant correlation was obtained. The correlation coefficient (r) was 0.584 ($P<0.01$). The positive correlation means that faster-growing roots contained more dauricine. This characteristic is very useful for selection in order to establish high dauricine-producing cultures.

EXPERIMENTAL

Plant material. Plants of *M. dauricum* were grown in a greenhouse. For root induction, sterilized leaf segments were incubated in the dark at 27° on LS medium containing 10 μM NAA, 1 μM BA, 3% sucrose and 1% agar. After 2.5 months of incubation, adventitious roots were induced. Excised roots were cultured on a rotary shaker (70 rpm) in the dark at 27° in B5 medium with 1 μM IBA and 3% sucrose.

Isolation and identification of dauricine (**1**). Roots were cultured for 40 60 days in the above medium, then harvested and freeze-dried. A sample of the dried roots (17 g) was powdered and sonicated for 30 min in MeOH. The roots were then filtered from MeOH through filter paper. Treatment with MeOH was repeated ×3. Thereafter, the combined MeOH extracts were dried by evapn. The dry residue (6.5 g) was dissolved in 600 ml of 3% citric acid, then the acidic aq. soln was passed through filter paper. The filtrate was made alkaline with aq. NH_3 and partitioned with $CHCl_3$ ×4. The $CHCl_3$ extracts were dried by evapn. The basic residue (0.15 g) was sepd by silica gel TLC using $CHCl_3$ MeOH–aq. NH_3 (200:50:1). Dauricine was located by UV illumination and by spraying the plate with modified Dragendorff's reagent. The R_f value of **1** was 0.72. The band was scraped off and the alkaloids eluted with MeOH and dried (81 mg). Sepn by semi-prep. HPLC gave 28 mg of **1** as a powder. $[\alpha]_D^{22.4}$ −113.0° (MeOH; *c* 0.50) (lit. [10] −115.1° (MeOH; *c* 0.73)). ^{1}H NMR (200 MHz, $CDCl_3$ +TMS), IR and CI-(isobutane, 70 eV) and EI-MS matched lit. values [10, 19]. The column was Capcell Pak C_{18} (250 × 20 mm), and the solvent contained 70% MeOH with 0.2% aq. NH_3. The flow rate was 8 ml min^{-1}. A short pre-column (10 × 4.6 mm) was placed between the inj. and the sepn column. Dauricine (**1**) was detected by UV absorption at 283 nm and its R_t was 20.2 min. Methylation of **1** with CH_2N_2 resulted in only one product, which was sepd by semi-prep. HPLC using 75% MeOH with 0.2% aq. NH_3 as solvent. R_t of this product was 26.8 min. ^{13}C NMR (50 MHz, $CDCl_3$ +TMS) spectrum matched lit. values of *O*-methyldauricine (**2**) [13].



1 R = H

2 R = Me

Culture medium. Culture conditions were as follows: B5 medium was used as the basal inorganic soln with 1 μM NAA and 3% sucrose: auxin (NAA, IAA or IBA) at 0.1, 1, 10 or 100 μM, for all of them, was independently added to B5 medium containing 3% sucrose. NAA at 1, 2.5, 5, 7.5 or 10 μM was added to B5 medium containing 3% sucrose. Other growth regulators (BA, GA_3 or ABA) at 0.1, 1, 10 or 100 μM, for all of them, were independently added to B5 medium containing 3% sucrose and 7.5 μM NAA. NH_4NO_3 and KNO_3, as N sources (total amount of N 31.7 mM; NH_4^+ to NO_3^- ratio was 0, 1:29, 1:14.6, 1:7.3 or 1:1), were added to B5 medium minus N, containing 3% sucrose and 7.5 μM NAA. Sucrose, glucose, fructose, galactose or lactose as the carbon source at 3% was added to B5 medium containing 7.5 μM NAA. Sucrose at 2, 3, 4, 5 or 6% was added to B5 medium containing 7.5 μM NAA. Roots (0.1 g fr. wt) were inoculated in 25 ml of the medium and cultured on a rotary shaker (70 rpm) at 27° in the dark for 40 days. All expts were replicated 3 times.

Measurement of growth and analysis of dauricine (**1**). Root growth was measured by determining dry wt (freeze-dried). Dried roots (50 mg) were powdered and soaked overnight in MeOH. This macerated material was centrifuged for 5 min at 3000 rpm. This procedure was repeated once more and the combined MeOH extracts evapd to dryness at 40°. The dry residue was dissolved in 2 ml of 3% citric acid and the acidic aq. soln filtered through filter paper into a glass tube. A 1-ml portion of this acidic aq. soln was made alkaline (pH 10) with aq. NH_3 and loaded on to an Extrelut column (Merck). After 10 min, 3.5 ml of $CHCl_3$ was passed through the column twice. The $CHCl_3$ extracts were combined, then evapd to dryness at 35°. The dry residue was dissolved in 1 ml MeOH and analysed by HPLC. Dauricine contents were measured with HPLC at room temp. The stationary phase was Develosil ODS-3 (150 × 4.6 mm) and the solvent 75% MeOH containing 0.2% aq. NH_3. The flow rate was 0.3 ml min^{-1}. A short pre-column (30 × 4.6 mm) was placed between the inj. and the sepn column. Dauri-

cine (**1**) was detected by UV absorption at 283 nm. The R_t was 17.8 min. Peak areas were quantified with an electronic integrator.

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