Hairy Root Cultures of *Peganum harmala* II. Characterization of Cell Lines and Effect of Culture Conditions on the Accumulation of β-Carboline Alkaloids and Serotonin

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Hairy root cultures of *Peganum harmala* established by genetic transformation with *Agrobacterium rhizogenes* wild type strains A4, 15834 or carrying the binary vector pLTCgus1, respectively, were analyzed with respect to their tryptophan-derived metabolites. Beside the previously detected β-carboline alkaloids harmine, harmol, harmaline and harmalol all hairy root cultures were found to contain the β-carboline glucoside ruine and serotonin. The appearance of the root cultures and the levels of β-carboline alkaloids were greatly affected by the nitrogen source of the medium. In the presence of higher levels of ammonium ions (as in MS medium) the roots were short and thick, while in B50 medium (low ammonium) they were thin and long. Ammonium ions had a negative effect on the levels of β-carbolines. The best root cultures accumulated on B50 1.5–2% β-carbolines on a dry mass basis and in the presence of 6% sucrose *ca.* 10 mg β-carbolines/L × d were formed. In the presence of phytohormones root cultures were converted into low producing suspension cultures, from which the highly productive roots were reinduced on phytohormone-free medium.

Introduction

Fine cell suspension cultures of *Peganum harmala* produce only low levels of β-carboline alkaloids or even lack them [1]. From aggregated cultures root formation was sometimes induced in the absence of phytohormones and such untransformed root cultures produced β-carboline levels of 1–2% of dry mass [2]. However, low growth rates and the loss of the capability for root induction in long-term cultures hampered their use. Recently, transformed and thus stable root cultures were established showing distinctly better growth rates and reasonable alkaloid production [3]. In that work successful transformations were only obtained from plant cells infected with *Agrobacterium rhizogenes* A4 [3]. As will be shown here, another wild type strain of *A. rhizogenes*, as well as a constructed strain carrying a selectable marker gene, have also been very efficient in establishing hairy root cultures.

While looking for a suitable liquid medium for the transformed root culture, it was noted that the appearance of the roots on phytohormone-free MS medium and B50 medium [4] was quite different and that distinctly different alkaloid levels accumulated in such different roots. This prompted us to study the effect of various culture conditions on growth and alkaloid formation to devise optimal conditions for further biochemical studies.

Materials and Methods

Bacterial strains

The widely used wild type strains *Agrobacterium rhizogenes* A4 and 15834 were from our own culture collection. The strain *A. rhizogenes* Gus1 carrying the binary vector pLTCgus1 was constructed as follows: A 2600 bp fragment of pRT104gus [5] containing the *gus* gene, flanked by the 35S promoter and the corresponding polyadenylation signal of cauliflower mosaic virus, was obtained by digestion with *Hind*III. This fragment was ligated

Hairy root cultures of *Peganum harmala*, I. see Kuzovkina *et al.*, Z. Naturforsch. **45c**, 727–728 (1990).

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into the *Hind*III linearized binary vector pLX222 [6] which contains a selectable *npt-II* antibiotic resistance marker gene under the control of the nopaline synthase promoter. Restriction analysis revealed that the *gus* and the *npt-II* gene have opposite orientations in this new vector, named pLTCgus1. This plasmid was introduced from *E. coli* JM 109 into *A. rhizogenes* C 58 CI pRiA 4 [7] by triparental mating using *E. coli* RK 2013 as helper strain [8] to give *A. rhizogenes* Gus 1.

Root cultures

Initiation of the root culture transformed with *A. rhizogenes* A 4 (PH A 4) and its maintenance on MS-agar or -viscose medium with 1/2 strength of the nitrogen source has been described [3]. Root cultures transformed with *A. rhizogenes* 15834 (PH 15834) and *A. rhizogenes* Gusl (PH Gus) were established in the same way on MS- or B 50-medium by infection of pieces of sterile grown seedlings [3, 9]. Selection for kanamycin resistant roots was performed on media with 100 µg kanamycin/ml. For maintenance, 1.5 g roots were inoculated into 70 ml/200 ml Erlenmeyer flasks and were grown on MS or B 50 at 90–95 rpm for 3 weeks. All media variation experiments were performed with PH A 4 [3].

Analytical methods

a) Southern analysis of plant DNA

Isolation of plant DNA, hybridization probes of wild type strains and conditions of hybridization have recently been described in detail [9]. The integration of the *gus*-gene in the transformands was probed with the 1890 bp XhoI/XbaI restriction fragment of pRT 104 gus [5], which contains only sequences of the entire *gus* open reading frame.

b) Determination of NPT-II-protein

The presence of the NPT-II-protein was determined by a commercially available Elisa-test as recommended by the producer (5 Prime–3 Prime, Inc., West Chester, U.S.A.; ABCR Co., Karlsruhe, Germany).

c) Determination of β-glucuronidase (Gus) activity

100 mg roots were extracted with 150 µl extraction buffer [10] in a microvial and centrifuged. Aliquots (35 µg protein) of the supernatant was used in the assay [10] with *p*-nitrophenylglucuronide as substrate.

b) Isolation and identification of ruine

Fifty g freeze-dried roots were extracted 3 times with MeOH. The extract was reduced to a final volume of 30 ml, bound to 4 g silica gel, placed on a silica gel column (50 × 2.5 cm) and stepwise eluted with $CHCl_3$:MeOH 4:1, 3:1, 1:1, 2:3, and MeOH. The fraction containing the compound with the unknown harmine-like fluorescence was purified by preparative TLC ($CHCl_3$:MeOH:NH_4OH 4:1:0.1). The identity of the compound as ruine was deduced from its ^1H and ^{13}C NMR spectra. ^1H NMR (CD_3OD): δ = 8.17 [d, H-5, J(5–6) 5.4], 7.95 [d, H-9, J(9–10) 8.7], 7.88 [d, H-6], 7.11 [d, H-10], 4.99 [d, H-1′, J(1′–2′) 7.8], 4.04 [s, 11-OCH_3], 3.82 [dd, H-6′A, J(6′A–6′B) 12.0, J(6′A–5′) 3], 3.80 [dd, H-6′B, J(6′B–5′) 4], 3.69 [dd, H-2′, J(2′–3′) 9.0], 3.57 [dd, H-4′, J(4′–3′) 9.0, J(4′–5′) 9.0], 3.53 [dd, H-3′], 3.34 [m, H-5′], 2.85 [s, 3-CH_3]. Long-range couplings were detected in the 2D COSY spectrum between 3-CH_3 and H-5, 3-CH_3 and H-6, and 11-OCH_3 and H-10. ^{13}C NMR (CD_3OD): δ 153.23 (s, C-11), 142.93, 137.85, 136.65, 132.80, 130.47 (sx 5, C-2, C-3, C-8, C-12, C-13), 138.43 (d, C-5), 119.43 (d, C-9), 119.05 (s, C-7), 113.69 (d, C-6), 108.56 (d, C-10), 106.81 (d, C-1′), 78.23, 77.81 (dx 2, C-3′, C-5′) 75.72 (d, C-2′), 70.98 (d, C-4′), 62.09 (t, C-6′), 57.73 (q, 11-OCH_3), 19.77 (q, 3-CH_3). The assigments of the ^{13}C spectrum were made by comparison with the data for harmane [11].

Quantitation of metabolites: 25 mg dry mass were extracted with MeOH and were subjected to reversed phase HPLC-analysis. Conditions: Lichrosorb RP-18-column (250 × 4 mm, 10 µM), linear gradient from 20–80% aq. MeOH with 0.5% triethylamine formate (pH 8.5) in 15 min, then 10 min at 80%; flow rate 1 ml/min; detection for serotonin at 303 nm, for β-carbolines at 330 nm. For group determination (harmine + harmol + ruine; harmalol + harmaline; serotonin) the fluorescence assay of Sasse *et al.* [12] was used.

Results and Discussion

Transformation of plant material

As the final goal of our research on *P. harmala* cell cultures is to manipulate the biosynthesis of

β-carboline alkaloids and serotonin by the introduction of foreign genes, we need to transform this plant not only with wild type but also with constructed strains of *A. rhizogenes*. The initial observation that only *A. rhizogenes* A 4 was successful in transforming *Peganum* [3] was not particularly satisfactory. Hence another widely used wild type strain, *A. rhizogenes* 15834, and also a constructed strain *A. rhizogenes* GusI were tested for transformation efficiency. Indeed with both strains more than 50% of the infected pieces showed root formation regularly after 14–21 days. Leaves and stems of 4–8 weeks-old sterile grown seedlings proved to be the best plant material for transformation. Most often only roots appeared at the site of infection, although occasionally a mixture of callus and roots was obtained and the mixed character of such cultures was maintained for long periods. Overall *P. harmala* is a plant that is rather easily transformed with *Agrobacterium* strains. The constructed strain *A. rhizogenes* GusI carries the *npt-II*-gene as selectable marker for kanamycin resistance and the *gus*-gene. When transformation with this strain was performed under non-selective conditions, approximately 25% of the formed roots were kanamycin resistant. All kanamycin resistant cell lines contained the NPT-II-protein and Gus-activity (Table I). The expression of the transferred genes, however, varied distinctly among the various transformands. Though a close relationship between the extent of kanamycin resistance and the activity of the marker gene may not necessarily be found, kanamycin is a very suitable selectable marker in transformation experiments in which desired foreign genes are to be transferred to *P. harmala* cells.

Molecular characterization of transformands

Transformation of *P. harmala* root cultures was proven by Southern hybridization. In digests of total DNA from root cultures transformed with *A. rhizogenes* 15834 and *A. rhizogenes* GusI EcoRI and HindIII fragments of about 4 kb (Fig. 1 A, lane 1 and 2)) were detected with pLJ 1 EcoRI fragments 15 (4.3 kb) and 36 (1.85 kb) as probes [13]. These probes are internal of the T_L-DNA fragments of pRiHRI which is integrated into the plant genome after agroinfection. As DNA isolated from an untransformed *P. harmala* suspension culture did not hybridize to this probe (Fig. 1; lane A 4), the signals found with the transformed cultures are caused by cross-hybridization of the pRiHRI T_L-DNA probe to the T-DNA region from pRiA 4 and pRi 15834 integrated in the plant DNA. Hybridization signals of Southerns of root cultures transformed with Agrobacteria carrying pRiA 4 or pRiA 4/pLTC-GusI were stronger than in root cultures established with pRi 15834 reflecting the closer relationship of the hybridization probe to pRiA 4 as has recently also been shown for the corresponding transformed root cultures of *Lupinus* [9]. Very strong hybridization signals of 8.6 kb, 3.8 kb and 1.8 kb were obtained with DNA from PH A 4 (Fig. 1 A, lane 3). The 3.8 and 1.8 kb EcoRI fragments detected here are in the same size range as the probe fragments used. The weaker ad-

Tab. I. Kanamycin-resistance, NPT-II-protein and β-glucuronidase-activity of hairy root cultures transformed with *A. rhizogenes* A 4, *A. rhizogenes* 15834, and *A. rhizogenes* GusI. Initial inoculum 0.5 g roots/20 ml B 50-medium. Kanamycin concentration 100 µg/ml. Roots were harvested and analyzed after 18 days.

Root Culture	Biomass increase (fold) Kanamycin minus	plus	NPT-II [ng/mg protein]	β-Glucuronidase [nkat/mg protein]
PH 15834	7	0	n.d.*	n.d.
PH A 4	7	0	n.d.*	n.d.
PH Gus a	3	3	170	12.1
PH Gus b	7	6	65	0.4
PH Gus c	11	12	32	0.2
PH Gus d	8	8	201	11.3
PH Gus e	4	0	2	n.d.

* n.d. = not detected.

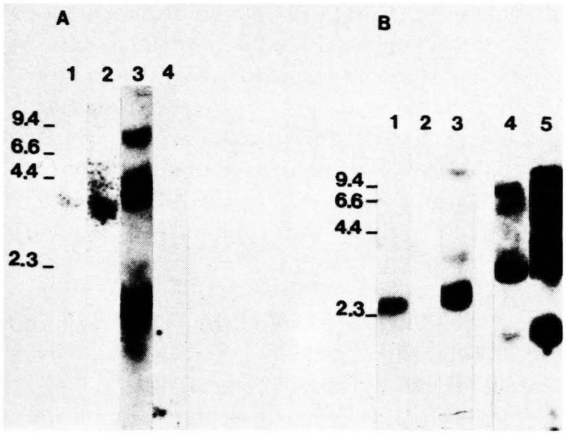

Fig. 1. Southern analysis of *P. harmala* root and suspension cultures. Hybridization probes used: A1–A4, internal T_L-DNA *Eco*RI fragments 15 (4.3 kb) and 36 (1.85 kb) from pRiHRI [13]; B1–B3, XhoI/XbaI fragment (1.9 kb) carrying the Gus gene; B4 and B5 *Eco*RI fragments 15 and 3a (T_L-DNA border fragment) from pRiHRI. DNAs loaded: A1 = *P. harmala* 15834 (*Eco*RI digest); A2 = *P. harmala* Gus (*Hind*III digest); A3 = *P. harmala* A4 (*Eco*RI digest); A4 = *P. harmala* untransformed suspension culture (*Hind*III digest). B1 = *A. tumefaciens* Gus1 (*Hind*III); B2 and B4 = *P. harmala* 15834 (*Hind*III); B3 and B5 = *P. harmala* Gus (*Hind*III). Fragment sizes (kb) of DNA markers are indicated on the left. 15 µg of total plant DNA was loaded per lane and 0.25 µg bacterial DNA was loaded on lane B1. Exposure times of X-ray films were 1 week in Fig. A and 6–16 h in Fig. B.

ditional fragment of 8.6 kb could be the result of hybridization of the T_L-DNA EcoRI border fragment 3a of pLJ1 which was present in the hybridization probe to a small extent.

The presence of the *gus*-gene in the root cultures transformed with *A. rhizogenes* GusI was shown with a HindIII-probe containing the whole *gus*-gene construct (Fig. 1B, lane 3), whereas the presence of the pRiA4 T_L-DNA was assayed with EcoRI fragments 15 and 3a (T_L-border fragment) from pLJ1 (Fig. 1B, lane 5). The hybridization pattern also obtained with the border fragment suggests multiple inserts of the T_L-DNA in the plant genome. In a control experiment the Ri T_L-DNA could be detected in a root culture transformed by *A. rhizogenes* 15834 whereas no hybridization signal was obtained with the probe for the β-glucuronidase gene (Fig. 1B, lanes 2 and 4).

Tryptophan derived metabolites of the root cultures

The presence of harmine, harmaline, harmol and harmalol in the root cultures of PH A4 has recently been shown [3]. Detailed analysis of the root extracts resulted in the isolation and identification of ruine (harmine-8-O-β-glucoside) and serotonin (Fig. 2), both are known as constituents of *P. harmala* [1, 14]. Harmine was the main β-carboline alkaloid in all root cultures under all conditions tested, accounting for 50–60% of the β-carbolines. The ratio of harmalol, ruine and harmol levels varied distinctly. Most often harmalol represented the second highest alkaloid content. However, sometimes ruine or harmol levels surpassed harmalol. Harmaline was always a minor component, often only detectable due to its intensive fluorescence.

Roughly 60 different root cultures derived from *A. rhizogenes* 15834 or GusI have been inspected for β-carboline alkaloids and serotonin. Table II shows alkaloid and serotonin levels of well established root cultures with low or no callus like structures. Thus without any special treatment one will find many lines accumulating alkaloids and serotonin in the range 1% and 0.5%, respectively.

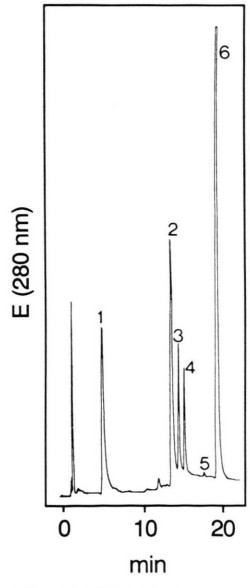

Fig. 2. HPLC chromatogram of a methanolic extract of a root culture grown on B50 medium. Conditions see Materials and Methods. **1** = serotonin, **2** = harmalol, **3** = ruine, **4** = harmol, **5** = harmalol, **6** = harmine. Detection of serotonin at 303 nm, of β-carbolines at 330 nm.

Table II. Some β-carboline alkaloid and serotonin levels of hairy root cultures derived from different *Agrobacterium* strains. The cultures had been subcultured on B 50-agar medium for at least 3 months and were transferred before analysis to liquid B 50 medium for three weeks.

Culture	β-Carbolines		Serotonin
	Aromatic	Dihydro	
	[mg/g dry mass]		
PH 15834 a	5.6	2.9	6.1
b	6.1	2.6	3.7
c	9.9	2.3	10.3
d	7.9	2.0	4.3
e	8.1	4.2	5.1
PH Gus 1	11.1	2.7	6.8
2	4.1	1.5	1.4
3	7.8	3.6	5.6
4	7.0	1.4	2.1
5	9.4	4.1	4.5

Root cultures with callus areas usually contained lower levels ranging from 0.2–0.5% for β-carbolines and 0.2% for serotonin. Independent of the *Agrobacterium* strain used for transformation or the source of plant material all root cultures showed similar qualitative patterns of tryptophan derived metabolites. The ratio of fully aromatic indole alkaloids *versus* dihydro-β-carbolines was usually 2–3:1 (Table II). The question arises whether the different levels of alkaloids found are indications of variant lines. As similar variations of specific content were found within one line analyzed several times during several months of subculture, the different lines may have to be followed for several passages before variant lines may be found. The levels of the line PH A 4 [3], maintained for more than 2 years in liquid medium and used for the experiments described below, accumulated even under seemingly controlled maintenance conditions variable levels between 1.2 and 2.0% β-carbolines. Growth and productivity may vary greatly depending upon the preculture. Compared to suspension cultures, the inoculum of root cultures is necessarily not as uniform. Thus variation may also be introduced by the inoculum itself, even if it originates from the same flask.

Serotonin is always present in the root cultures. However, its content is strongly dependent upon the cultural conditions and may be lower than or equal to the β-carboline alkaloids. The levels obtained after 3 weeks of cultivation (Table II) do not represent the maximum specific content of serotonin (see below). As reported for suspension cultures, in which tryptamine is readily hydroxylated to serotonin, tryptamine levels are also very low in the root cultures [15–16].

Effects of medium and culture conditions on β-carboline and alkaloid levels

When *P. harmala* root cultures were grown permanently on MS-medium or B 50-liquid medium the appearance of the roots differed greatly. On MS-medium the roots were rather thick and short (Fig. 3), white coloured and showed a strong yel-

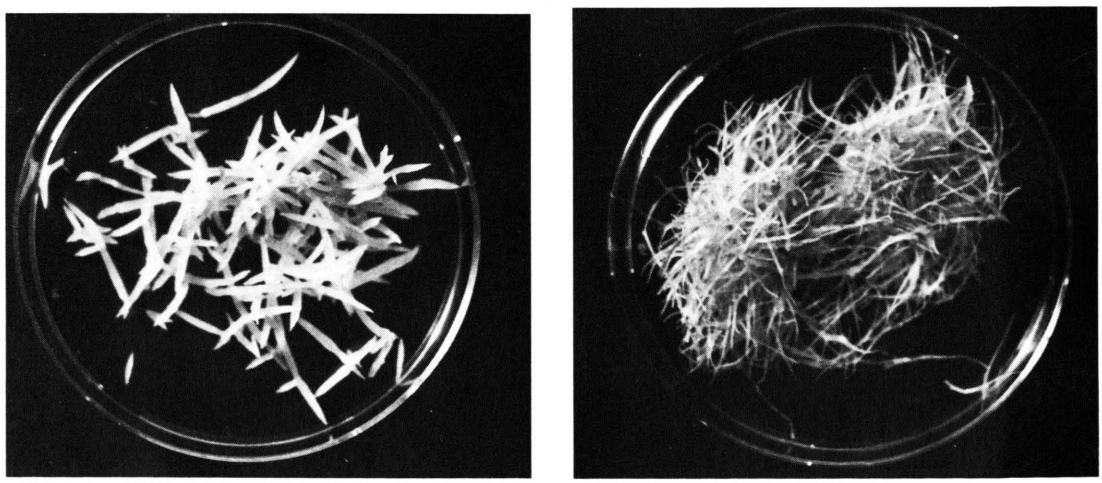

Fig. 3. Appearance of PH A 4 roots permanently maintained on liquid MS medium (left) and B 50 (right).

low/greenish fluorescence. On B 50-medium the roots were long, thin and interlacing (Fig. 3), and often roots and medium were reddish/brownish coloured thus suppressing the typical fluorescence of harmine or harmalol. While the biomass increase was quite similar on both media, alkaloid formation was quite different. Cultures permanently maintained on B 50-medium usually contained between 1.2–2.0% β-carboline alkaloids on a dry mass basis, while on MS-medium levels between 0.4 and 0.8% were noted. On B 50-medium the ratio β-carbolines:serotonin was normally 2–3:1 at the end of a 3 week culture period. Serotonin levels (5–10 mg/g dry mass) were quite similar on MS and B 50. However, due to the decrease of β-carboline levels on MS-medium the ratio changed to *ca.* 1:1 or with serotonin levels occasionally being a little higher than β-carbolines. It should be noted that β-carbolines did not show distinct changes of specific alkaloid concentrations over a growth period of 3 weeks. In contrast serotonin levels showed a maximum with up to 20 mg/g dry mass 3–5 days after transfer to fresh medium [17].

A MS-culture (Fig. 3) could be changed into a B 50-culture and vice versa by growing the cells for 3–4 growth cycles on the other medium. Even during the first growth cycle on the new medium the second root type appeared. The morphological changes were also reflected by rapid changes in the alkaloid levels (Table III). After 3–4 growth cycles the MS culture had reached its high level β-carboline production as on B 50-medium. The most important difference between the composition of B 50- and MS-medium is the different amount of ammonium ions which are very low in B 50. When the roots were maintained on MS-medium devoid of ammonium nitrate (20 mM NH_4NO_3), alkaloids increased to the levels found in B 50-medium (Table IV). Not only the production but also growth was severely inhibited when ammonium was the sole nitrogen source. Nitrate as sole nitrogen source (5–40 mM) did not change the specific alkaloid levels but affected growth, with 20 mM KNO_3 yielding the biomass obtained with B 50 or MS. Thus, nitrate as sole nitrogen source at the concentration of the B 50-medium (25 mM) gives optimal results with respect to growth and alkaloid production.

With B 50 (2% sucrose) and MS (3% sucrose) biomass increased 6–8-fold within 3 weeks. A rough calculation shows that roots on B 50-medium would accumulate approximately 100 mg alkaloids and 30 mg serotonin/L (de novo synthesized) during this period. Thus other parameters were sought for which might help to increase productivity. First the sucrose concentration of B 5-medium was varied from 1–10% (Fig. 4). *P. harmala* root cultures tolerated rather high levels of sucrose. Specific alkaloid and serotonin levels remained unaffected up to 6% sucrose and were lower only at 8–10%. As dry mass formation increased up to 6% sucrose, the roots should be grown at 5–6% sucrose for higher alkaloid yields. From this one could calculate that 200–250 mg β-carbolines/L may be formed on B 50 with 6% sucrose in 3 weeks or 10–12 mg β-carbolines/L × d.

Phosphate levels of the culture medium have often been shown to affect secondary metabolite formation in suspension cultures [18]. On B 50-medium, permitting the formation of high levels of alkaloids, no distinct stimulatory effect on specific

Table III. Changes of β-carboline levels in relation to changes in the medium. Roots of PH A 4 were grown for 3 weeks in the indicated medium. MS/B 50 implies that the MS culture had been maintained for one cycle on B 50.

Culture	Medium	β-Carboline alkaloids [mg/g]
MS	MS	4.0
MS	B 50	7.5
MS/B 50	B 50	10.0
MS/B 50	MS	3.9
B 50	B 50	14.2
B 50	MS	7.9
B 50/MS	MS	4.9
B 50/MS	B 50	8.2

Table IV. Alkaloid and serotonin accumulation on media with different levels of ammonium salts. The total nitrate levels varied from 25 mM (B 50) to 20–40 mM (MS). Roots of PH A 4 (initial inoculum) 1 g/35 ml were harvested after 18 d.

Ammonium [mM]		Biomass [g/flask]	β-Carboline alkaloids [mg/g dry mass]	Serotonin
B 50	0.5	6.3	17.2	6.6
MS	20.0	7.2	3.7	4.6
MS	10.0	5.8	4.0	4.3
MS	0	5.6	14.3	4.1

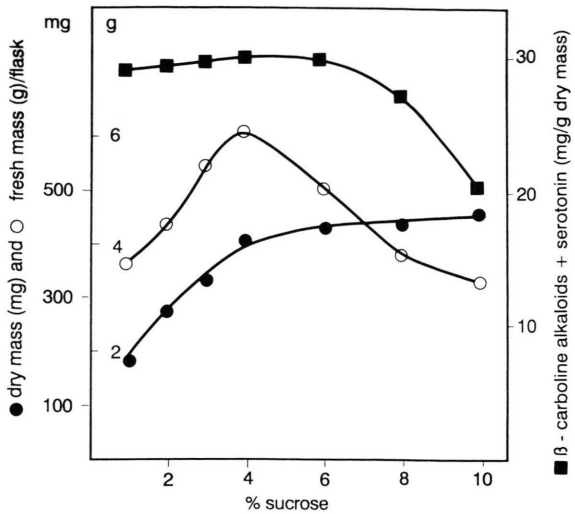

Fig. 4. Effect of sucrose concentration on biomass and product formation. Initial inoculum 0.75 g/35 ml B 50 medium. Roots were harvested after 3 weeks.

alkaloid levels was seen when phosphate levels were reduced (data not shown). Only the specific serotonin levels were slightly enhanced on B 50-media with lowered phosphate levels. In contrast, lowering of the phosphate levels enhanced both alkaloid and serotonin accumulation of the low productive roots permanently grown on MS-media (Table V). However, because of lower biomass production at lower phosphate levels even total alkaloid yields of the low producing root state were not greatly changed. Thus, one has to conclude, as in the case of isoflavonoid formation in *Lupinus* root cultures [19], that phosphate depletion is not helpful for improving product levels of root cultures. Changes of the ratio phosphate/nitrate did not alter the specific content of the alkaloids and serotonin.

As the specific product levels obtainable on B 50-medium were not readily improved by altering media components, we devised an improved growth medium in which more alkaloids were produced due to increased biomass formation. This medium is a B 50-medium with 4% sucrose and 2.5 mM phosphate. As it had similar beneficial effects on growth and total product levels of several other roots cultures (Linum, Nicotiana, Lupinus) it might be a useful medium for many other root cultures.

In addition to the above factors we noted that shaking speed (aeration or stress?) or inoculum size seem to affect growth, colour of the roots and medium as well as alkaloid and serotonin production (data not shown). When optimizing the productivity of the root cultures one should consider also such parameters. In general, specific β-carboline levels were higher at lower biomass increases.

When root cultures were transferred to media containing phytohormones (*e.g.* 2,4-D, NAA/kinetin) they were rapidly covered with callus, in particularly those maintained on MS-medium. Suspension cultures were readily established from the friable callus material. However, the growth of these suspensions was very low compared to growth rates known for normal *P. harmala* suspension cultures [1]. Table VI describes the change of a B 50-culture into a suspension in the presence of 2,4-D and its return to the root state on B 50. The state of untransformed root cultures could be

Table V. Effect of the phosphate concentration on growth and metabolite production of PH A 4. Initial inoculum *ca.* 75 mg dry mass/35 ml medium, harvested after 3 weeks.

Medium	Dry mass	β-Carbolines		Serotonin	
	[mg/flask]	[mg/g]	[mg/flask]	[mg/g]	[mg/flask]
MS−PO_4	186	11.9	2.2	12.0	2.2
+0.1 PO_4*	302	10.2	3.0	12.7	3.8
+0.2 PO_4	331	9.2	3.0	12.8	4.2
+0.5 PO_4	360	11.3	4.1	9.2	3.3
MS	390	6.1	2.4	6.0	2.3
+2.0 PO_4	425	6.2	2.6	4.9	2.1

* 0.1 PO_4 means 1/10 of the PO_4 concentration of the MS medium which is 1.25 mM.

Table VI. Effect of 2,4-D on the formation of β-carboline alkaloids and serotonin of PH A 4. The root culture was maintained on B 50 medium before transfer to B 5 medium with 5 μM 2,4-D. The length of the passages was three weeks.

Medium	Appearance of the culture	β-Carbolines [mg/g dry mass]	Serotonin
B 50 control	thin roots	16.5	6.1
B 5 (5 μM 2,4-D)	callus-covered roots (2)*	4.1	2.6
	callus-free of roots (3)	1.8	2.2
	suspension (5)	0.6	2.1
Return of the suspension to B 50	lumpy suspension (1)*	1.0	2.8
	roots with callus (2)	2.1	2.4
	roots (3)	8.2	4.1
	roots (5)	13.8	5.0

* Number of 3-week passages on B 50 after return from the suspension state.

maintained for some time by manipulating with the phytohormone levels in the medium [2]. However, when such roots changed in friable callus or in the suspension state it was impossible to reinduce the root state on phytohormone-free medium. In contrast, the program for root formation introduced in the transformed cells can be suppressed by phytohormones but is immediately activated when the phytohormones are depleted. The correlation between root and β-carboline formation is evident (Table VI) and this fits with the observation that all transformed root cultures containing callus material accumulated lower levels of β-carbolines. The change of the transformed root culture into a suspension and its reversal was a rather long process of *ca.* 6 months. For practical, technological purposes the cultures should be maintained in the root/callus state in the presence of low phytohormone levels. Such cultures can be handled as lumpy, highly aggregated "suspension" culture from which the full root state can be rapidly recovered.

In conclusion, transformed root cultures of *P. harmala* containing β-carbolines levels of 1% and higher are readily obtained by cultivating the roots on media with low levels of ammonium ions. Specific alkaloid levels may change with the appearance of the roots, but are difficult to alter by factors (phosphate or sugar levels) which are known to affect secondary metabolites formed in suspension cultures. These cultures will now be used for biochemical studies to clarify the biosynthetic steps leading to the various β-carboline alkaloids [1]. It is expected that precursor feeding experiments will provide data about intermediates and the sequence of the pathway.

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