β-Carboline and Quinoline Alkaloids in Root Cultures and Intact Plants of *Peganum harmala*

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Alkaloid profiles of root and shoot cultures, seedlings and mature plants were analysed by capillary GLC and GLC-MS. β-Carboline alkaloids, such as harmine, harmaline dominate in normal and root cultures transformed by *Agrobacterium rhizogenes*, as well as in roots and fruits of the plant. In shoots, flowers and shoot cultures quinoline alkaloids such as peganine, deoxypeganine, vasicinone and deoxyvasicinone widely replace the β-carboline alkaloids. In root cultures, the formation of β-carboline alkaloids can be induced by methyljasmonate and several other elicitors indicating that these alkaloids are part of the reactive chemical defence system of *Peganum harmala*.

Key words: Root Cultures, Shoot Cultures, Alkaloid Induction

Introduction

Peganum harmala (Zygophyllaceae) occurs in arid habitats of the Mediterranean and Asia. It produces β-carboline and quinoline alkaloids as a major class of secondary metabolites. The β-carboline alkaloids have numerous pharmacological and toxic activities. The infamous properties include CNS stimulation and hallucinogenic activities which are linked to modulation of various neuroreceptors (such as 5HT-receptor) and inhibition of monoamine oxidase (MAO A) (Bergstrom *et al.*, 1997; Wink *et al.*, 1998; Wink, 2001). The idea of "flying carpets" might be related to the use of psychoactive β-carboline alkaloids (Mabberley, 1997). β-Carboline alkaloids are also known for their mutagenic, genotoxic, antimicrobial, cytotoxic, trypanocidal, and insecticidal activities (Albores *et al.*, 1990; El-Gengaihi *et al.*, 1997; Picada *et al.*, 1997; Wakabayashi *et al.*, 1997; Rivas *et al.*, 1999; Wink *et al.*, 1998; Wink, 2001). The cytotoxic effects, which are enhanced by UV light, are probably linked to DNA intercalation and resulting frame shift mutations (Wink *et al.*, 1998; Wink, 2001). The main quinoline alkaloids of *Peganum harmala* are peganine and vasicinone. They are formed by a different biosynthetic pathway than β-carboline alkaloids. Peganine shows hypotensive and bronchodilatory properties and acts as a respiratory stimulant. Besides anthelmintic properties it stimulates smooth muscles, shows uterotonic effects and is an abortifacient (Harborne and Baxter, 1993). Vasicinone shows bronchodilatory and weak hypotensive ionotropic activities and exhibits anthelmintic and antifeedant properties (Harborne and Baxter, 1993). *Peganum harmala* has been used since ancient times as an antiseptic and for treatment of skin and eye-disease, rheumatism and Parkinson's disease (El-Saad and El-Rifaie, 1980; Mabberley, 1997). The smoke of burnt plants has been used as a disinfectant (Al-Shama *et al.*, 1981). *Peganum* is also a source for oil and a dye (Turkey red) used for dyeing carpets and the hats are known as tarbooshes (Mabberley, 1997).

Because of the pronounced biological properties of β-carboline and quinoline alkaloids it is likely that they serve as potent defence chemicals against herbivores and microorganisms. The alkaloid formation is not static but under the regulation of internal and external factors. Upon attack or infections, secondary metabolism is often enhanced in many plants; either the biosynthesis of new compounds (phytoalexins) takes place or the concentration of already existing compounds is increased (reviews in Harborne, 1993; Roberts and Wink, 1998; Wink, 1999a, b).

Jasmonate has been found to be an important element of a signal pathway leading to the induction of secondary metabolites involved in defence against herbivores and microorganisms. Intracellular jasmonates transiently accumulate in cell sus-

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pension cultures that have been treated with elicitors implicating a role in the signal transduction system of the defence response (Mueller-Uri *et al.*, 1988; Gundlach *et al.*, 1992). If genes of the jasmonate cascade are silenced, plants were more vulnerable to herbivores (Kessler *et al.*, 2004). It has been established that treatment of *in vitro* cultures with exogenous methyljasmonate can elicit the accumulation of several classes of alkaloids (Gundlach *et al.*, 1992; Aerts *et al.*, 1996; Baldwin, 1999; Zayed and Wink, 2004). Fungal cell-wall elicitors and methyljasmonate can induce secondary metabolism in soybean cell cultures by different mechanisms (Enyedi *et al.*, 1992). Jasmonates have several other biological activities, including promotion of the stomata closure (Horton, 1991), acceleration of leaf senescence in oats and barley, pericarp senescence in soybean fruit, as a potent inducer of tendril coiling in *Bryonia*, and the stimulation of stem length and differentiated root system in potato plantlets (Weidhase *et al.*, 1987; Lopez *et al.*, 1987; Falkenstein *et al.*, 1991; Ravnikar *et al.*, 1992; Reinbothe *et al.*, 1992). Some of these effects are apparently mediated by controlling gene expression (Rickauer *et al.*, 1997).

In this communication we report the establishment of hairy root cultures of *Peganum harmala* via transformation with *Agrobacterium rhizogenes*. These transformed root cultures are valuable and versatile systems for the study of secondary metabolism (Wildi and Wink, 2002). Alkaloid profiles of normal and transformed root cultures as well as of shoot cultures were compared to those of intact plants. Furthermore, the production of β-carboline and quinoline alkaloids in normal and transformed root cultures of *Peganum harmala* was analysed by capillary GLC-MS after treatment with potential inducing compounds, such as methyljasmonate, quercetin and other secondary metabolites which had elicitor properties in other systems (Wink, 1985).

Experimental

Establishment of in vitro tissue cultures

Mature seeds of *P. harmala* (maximally 6 month old; harvested in Southern Sinai, Egypt) were surface-sterilized for 1 to 5 min in 1% sodium hypochlorite; then they were rinsed twice with sterile distilled water. Afterwards the seeds were treated for 5 min with 70% EtOH and rinsed twice with sterile distilled water. The seeds were left overnight in sterile water and then incubated on agar plates until germination. The germinated seeds were transferred to hormone-free WP solid-medium (Lloyd and McCown, 1980). Root cultures (RC) were established from excised root tips; 3-week-old roots were cut off and subcultured in hormone-free WP liquid-medium. The flasks were kept on a gyrator shaker, 110 rpm, at 25 °C in an illuminated culture room. The cultures were subcultured frequently, maximally every week. After 2 months of good growing roots had been obtained. Growth kinetics of root cultures were monitored by measurement of fresh (FW) and/or dry (DW) weight (Fig. 1). Morphology of cultures: After the sterilized seeds had germinated on WP solid-medium, primary white roots appeared on day 2 and increased in length. On day 7, the white lateral roots that had many root hairs increased in number and length. By day 25–30, the growth entered the stationary phase. After 8–10 weeks of subculturing the cultures and their medium acquired a reddish-brown colour, growth stopped and finally the cultures died.

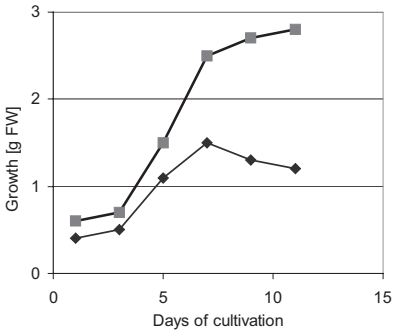

Fig. 1. Growth kinetics of normal and transformed root cultures of *Peganum harmala*. Lower line, normal root cultures (RC); upper line hairy root cultures (HRC); each data point is a mean of two independent experiments.

Hairy root cultures (HRC) were initiated by infecting 4-week-old seedlings with *A. rhizogenes* strains 15834 (kindly supplied by Prof. Dr. W. Alfermann, University of Düsseldorf, Germany). After 5–7 h the roots were surface-sterilized for 1 min with 1% sodium hypochlorite and placed on antibiotic-free agar-medium. The best growing roots were transferred into hormone-free WP liquid-medium to which claforan was added as an antibiotic (5 mg/10 ml medium). This procedure was repeated several times until *Agrobacterium*

was completely eliminated. Then the roots were subcultured every weak in fresh WP liquid-medium without antibiotic; growth kinetics are shown in Fig. 1. Morphology: HRC have a different morphology as compared to RC. They have a yellowish-white colour and are thinner and longer. They have many root hairs and much more branches than RC. The most important advantage of the transformed root cultures is their long-term stability.

After complete germination of the seedlings (3 weeks), shoots were excised and subcultured in WP liquid–medium in light (5000 lx). The shoots grew quickly in the first 2 weeks, especially their leaves, then they acquired a yellowish colour and the growth stopped in the third week. Thus, the shoot cultures (SC) should be investigated only during the first week after culture initiation.

Chemicals

Jasmonic acid (JA) and methyljasmonate (MEJA) solutions were prepared in DMSO or in 95% ethanol, salicylic acid (SA) in H_2O, and quercetin (Q) in MeOH. Other potential inducers (Wink, 1985) used were: alkaloids such as coniine, papaverine, quinine, biogenic amines such as cadaverine, spermine, spermidine and cyclic AMP. The metabolic inhibitors actinomycin D (AD, 5 μg/ml) and cycloheximide (CX, 1 μg/ml) were dissolved in 70% ethanol. Chemicals were obtained from Sigma-Aldrich-Chemie (Germany).

Cell culture media

In our investigation we have assayed different media for growth and maintenance of the cultures *e.g.* liquid and solid MS, WP media with and without phytohormone (Lloyd and McCown, 1980); furthermore we employed a "production medium" developed by Berlin *et al.* (1992). Hormone-free WP liquid- and solid-media as well as the "production medium" were optimal for RC and HRC. The pH of the medium was adjusted to 5.7 before autoclaving. For each subculture we have used 50 ml medium in 200 ml flasks. In induction experiments 10 ml medium in 100 ml flasks were employed. Yeast-Mannitol- Broth-medium (YMB) was used for growth and maintenance of *Agrobacterium rhizogenes* at 39 °C. It contains mannitol (10 g/l), yeast extract (0.4 g/l), NaCl (0.1 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), K_2HPO_4 (0.5 g/l) and agar (1%) at pH 7.2.

Induction experiments

RC, HRC and SC were treated with JA, MEJA, Q and other potential inducers to stimulate the accumulation of the secondary metabolite in plants. SA, CX and AD were used as inhibitors. The inducer and inhibitor solutions were filter-sterilized and added to 10 ml fresh medium in 100 ml Erlenmeyer flasks. Inhibitors were given 1 h prior to the addition of potential inducers. The flasks were kept on a gyrator shaker, 110 rpm, at 25 °C in an illuminated culture room.

Analytical methods

RC and HRC were harvested by Buchner vaccum filtration, weighed and kept at – 20 °C until extraction and phytochemical analysis. Frozen material was homogenized in a mortar with 20 ml 1 M HCl. The homogenate was left at room temperature overnight, then made alkaline with 6 M NaOH (pH 12). Solid phase extraction on Isolute column (20 g) was carried out with dichloromethane (three times, 20 ml each). The eluates were concentrated in a rotary evaporator, and kept at 4 °C until analysis. For analysis the dry alkaloid residues were taken up in 100–400 μl MeOH (according to the amount of the residue) and 1 μl was injected for gas chromatography. Samples from intact plants were processed identically. Medium samples were treated accordingly but the initial 1 M HCl step was omitted. Using GLC-MS, the alkaloids were identified (Al-Shama *et al.*, 1981; Table I). Harmine, peganine and harmaline were used as external standards for quantitative determinations.

The GLC-MS analyses were carried out on a Carlo Erba HRGC 4160 gas chromatograph equipped with a fused silica DB1 (30 m × 0.3 mm) column. The capillary column was directly coupled to a quadrupole mass spectrometer, Finnigan MAT 4500. EI-mass spectra were recorded at 40 eV. Conditions: injector 250 °C; temperature programme 150–300 °C, 15 °C min^{-1}; split ratio 1:5; carrier gas He, 0.5 bar (flow rate 2 ml/min).

Results and Discussion

Comparison of alkaloid profiles between Peganum harmala plants and corresponding organ cultures

Different organs (roots, shoots, flowers and fruits) from intact plants of *P. harmala* were harvested separately and investigated for their alka-

Table I. Identification of *P. harmala* alkaloids by GLC-MS.

Alkaloid	RI*	M^+	Characteristic ions (abundance %)
Peganine	1825	188 (50)	187 (100); 159 (20); 131 (23)
Deoxypeganine	1750	172 (48)	171 (100); 143 (3); 129 (5); 116 (6)
Vasicinone	1950	202 (100)	146 (95); 130 (14); 119 (63); 102 (13)
Deoxyvasicinone	1866	186 (82)	185 (100); 160 (3); 130 (9); 102 (6)
Harmaline	2157	214 (91)	213 (100); 199 (14); 170 (15); 107 (10)
Harmine	2190	212 (100)	197 (25); 183 (5); 169 (50); 106 (12)

* RI, Kovats retention index.

loid content and composition. Alkaloids were analysed by GLC and identified unequivocally by GLC-MS. Two types of alkaloids were detected: β-carboline and quinoline alkaloids (Table I). The alkaloid contents and patterns varied in the different organs (Table II). Harmine was the major alkaloid in roots and fruits/seeds whereas peganine and vasicinone dominated in shoots and flowers. The alkaloid profile of organ cultures showed almost the same alkaloid profile as the respective organs of the intact plant: harmine represented the major alkaloid of the root cultures and peganine and vasicinone were the major compounds of the shoot culture. Harmine had also been found by Kuzovkina *et al.* (1990) and Berlin *et al.* (1992) in hairy root cultures of *Peganum harmala.* This observation confirms that alkaloid formation in *in vitro* cultures closely corresponds to the situation in the intact plant. This indicates that differential expression of the genes of alkaloid biosynthesis in root cultures is still under similar control as in the intact plant, in contrast to the situation of undifferentiated cell cultures which often fail to produce secondary metabolites (Wink, 1987; Wildi and Wink, 2002).

Table II. Alkaloid profiles (major compounds) of various organs of intact plant and corresponding *in vitro* cultures (RC, HRC and SC) (total alkaloid = 100%).

Alkaloid	RC	HRC	SC	Fl	R	Sh	Ft
Harmine	95	98	28	–	100	16	50
Harmaline	–	2	–	–	–	–	50
Peganine	2	–	41	77	–	60	–
Deoxypeganine	–	–	–	20	–	–	–
Vasicinone	3	–	31	1	–	24	–
Deoxyvasicinone	–	–	–	2	–	–	–
Alkaloid content*	0.7	0.9	0.48	0.6	0.8	0.9	1.5

Fl, flowers; R, roots; Sh, shoot; Ft, fruits.
–, not detected.
* mg/g DW of intact plants and mg/g FW of cultures.

The alkaloid patterns of developing seedlings

The seeds of *P. harmala* contain harmine as the major and harmaline and peganine as minor alkaloids. During germination and seedling development, a rapid decrease of total alkaloids, represented by harmine and harmaline was observed (Fig. 2). After the early developmental stages, when seedlings had produced first leaves (stage 3), alkaloid contents were very low. When both roots and shoots started to grow (from stage 4 onwards), harmine and peganine levels increased until the

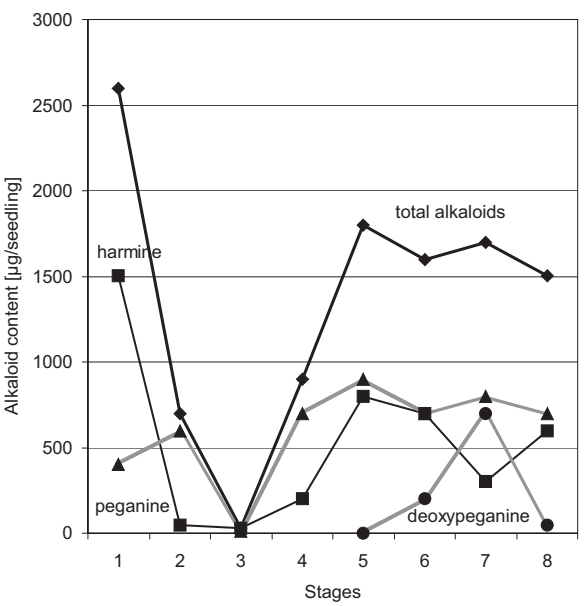

Fig. 2. Variation of alkaloid patterns and total alkaloids during germination and seedling development. The seeds were germinated under aseptic conditions on WP solid-medium, incubated at 25 °C and under continuous light. The data points are means of 3 seedlings. Stage 1: seeds; 2: roots appear; 3: roots and cotyledons present; 4: first leaves appear; 5: root elongation; 6: roots with branches, several leaves; 7: roots with many branches, growth of leaves; 7: many roots and leaves/seedling. Values are means of 3 individuals; the standard deviation was always lower than 10%.

6th developmental stage. Deoxypeganine levels increased during stage 6 and 7. The reduction of total alkaloids in *P. harmala* seedlings may either be due to alkaloid secretion into the environment (here the culture medium), or to their degradation. In this experiment, we found no evidence for secretion of the alkaloids into the medium, thus the alkaloids were probably degraded and their nitrogen reused as a nitrogen source by the seedlings, as has been observed with seedlings of *Lupinus* and other legumes (Wink and Witte, 1985).

Induction of alkaloid accumulation

JA or its derivative MEJA (which is better absorbed by cells than JA) are considered to be compounds involved with the signal transduction system, that induces particular enzymes catalysing biosynthetic reactions of defense compounds (Pena-Cortes *et al.*, 1993; Doares *et al.*, 1995; Kessler *et al.*, 2004). We have studied whether alkaloid formation in RC, HRC and SC can be stimulated by MEJA and JA.

In vitro cultures were incubated for 48 and 120 h with MEJA and JA. As can be seen from Fig. 3, JA and MEJA stimulated the formation of alkaloids approx. 5-fold in RC and approx. 7- to 8-fold in HRC as compared to the untreated control. The highest harmine level of 0.7 mg/flask was reached after 120 h of MEJA application in PM-medium and about 7 mg/flask in WP liquid-medium. A secretion of alkaloids into the culture medium was not observed. Alkaloid composition did not change after induction (data not shown). When the shoot cultures were treated with JA and MEJA, only a small induction effect by MEJA was detected; but a secretion of peganine (the major alkaloid of SC) into the medium was apparent. MEJA was more effective than JA to induce alkaloid formation in RC and HRC which is probably due to its better bioavailability. Applying different doses of MEJA, the maximum level of alkaloids was obtained with a MEJA concentration of 10 μM (data not shown).

Root cultures and hairy root cultures were treated with various other compounds (salicylic acid; alkaloids such as coniine, papaverine, quinine; amines such as cadaverine, spermine, spermidine; cAMP), besides JA and MEJA, which are known to elicit alkaloid formation in lupin cell cultures (Wink, 1985). As can be seen from Table III a variety of alkaloids and amines, which are biogenetically different from β-carboline alkaloids (and therefore cannot act as precursors), induced β-carboline alkaloid formation substantially similar to the situation in lupin cell cultures (Wink, 1985). Salicylic acid, however, had inhibitory properties

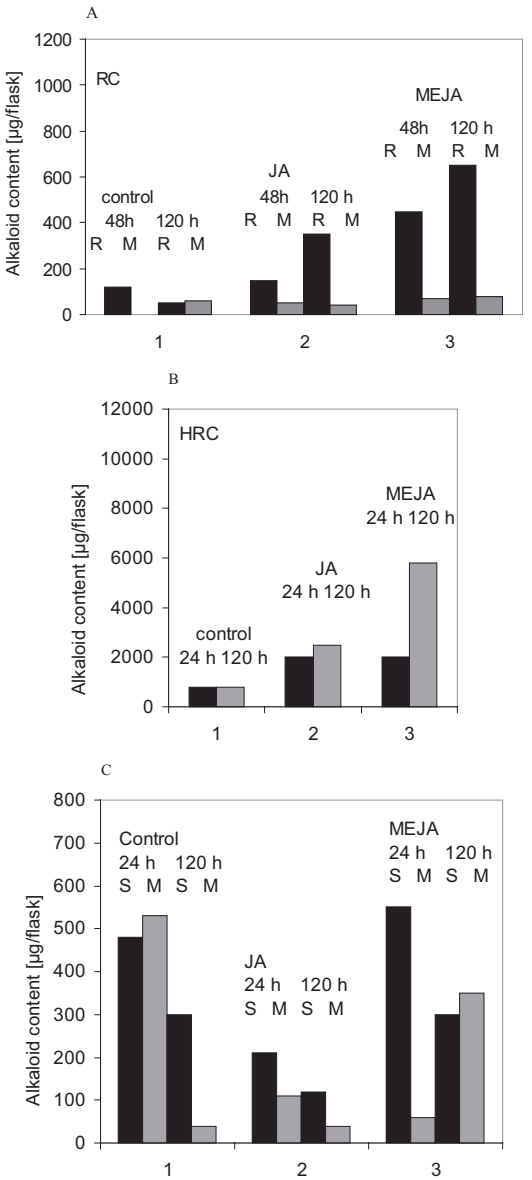

Fig. 3. Effect of JA and MEJA on alkaloid formation in *P. harmala* root cultures after 48 and 120 h of incubation. A) Root cultures; B) hairy root cultures; C) shoot cultures; S, shoots; R, roots; M, medium; values are means of 3 experiments; the standard deviation was always lower than 10%.

Treatment	Total alkaloids (control = 100%) after 120 h	
	RC	HRC
Control	100	100
Salicylic acid		
1 μM	103	
10 μM	33	
50 μM	35	
Quinine (3 mM)		480
Cadaverine (3 mM)		480
Papaverine (1 mM)		520
Spermine (0.5 mM)		780
Coniine (3 mM)		1000
cAMP (0.3 mM)		1140
Spermidine (0.5 mM)		1360

Table III. Effect of various secondary metabolites on alkaloid production in root and hairy root cultures. Values are means from triplicate experiments. Alkaloids and amines were adjusted to pH 5.

(Table III). A possible mechanism may involve the inhibition of the enzymes of the jasmonate cascade as was postulated from other systems (Doares *et al.*, 1995; Pena-Cortes *et al.*, 1993).

The protein biosynthesis inhibitor cycloheximide (CX) and the transcription inhibitor actinomycin D (AD) were used to investigate whether transcription and translation of biosynthetic enzymes are required for the response to the different inducers. A few representative experiments are illustrated in Fig. 4 and Table IV. Alkaloid induction by quercetin that elicited sanguinarine alkaloid biosynthesis in suspension cell culture of *Sanguinaria canadensis* (Mahady and Beecher, 1994) or by other alkaloid elicitors was significantly suppressed by pre-treatment of the *in vitro* cultures with actinomycin D (5 μg/ml), cycloheximide (1 μg/ml) for one hour prior to the addition of quercetin or other inducers. Table IV shows that the most effective inhibitor was translation inhibitor CX. The induction effect of quercetin was completely blocked with CX, and by ca. 60% with AD. The inhibition effect by CX provides evidence that the observed induction involves protein biosynthesis, probably of enzymes that are part of alkaloid formation. The inhibition with SA indicates that quercetin probably triggers the jasmonate induction pathway, which is inhibited by SA.

Table IV. Effect of different inhibitors, salicylic acid (SA, 100 μM), actinomycin D (AD, 5 μg/ml) and cycloheximide (CX, 1 μg/ml) on the induction effect of quercetin (1 μM) in RC and HRC of *P. harmala*. The experiment was performed in triplicate and the values shown are means ± S.D.

	Harmine content [μg/flask] after 24 h	
	RC	HRC
Control	181 ± 14.4	753 ± 60
Quercetin	1786 ± 142.8	2992 ± 239
Quercetin + SA	979 ± 78	1557 ± 124
Quercetin + AD	951 ± 56.4	1477 ± 118
Quercetin + CX	63 ± 3	675 ± 54

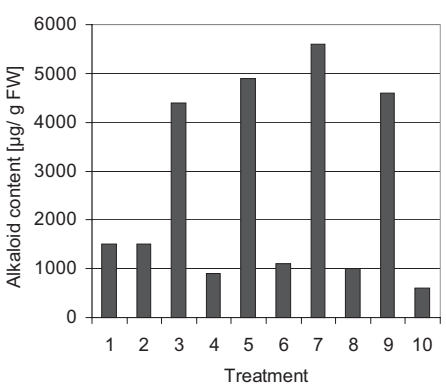

Fig. 4. Influence of cycloheximide on the induction of hairy root cultures by alkaloid and amine elicitors. Values are means from three experiments evaluated 24 h after addition of the substances. 1: untreated control; 2: + cycloheximide (CX) alone; 3: + 3 mM coniine; 4: 3 mM coniine + CX; 5: + 3 mM quinine; 6: 3 mM quinine + CX; 7: + 0.5 mM spermine; 8: 0.5 mM spermine + CX; 9: + 1 mM papaverine; 10: 1 mM papaverine + CX. Values are means of 3 experiments; the standard deviation was always lower than 10%.

Conclusion

Alkaloid profiles of root, hairy root and shoot cultures correspond closely to those of the intact plant. Alkaloid formation in root and hairy root cultures can be stimulated substantially with jasmonates, quercetin and various alkaloid/amines.

The induction can be inhibited by cycloheximide and actinomycin D, indicating that the apparent increase of alkaloid production appears to be related to an enhanced expression of genes coding for enzymes in the biosynthetic pathway of β-carboline alkaloids. These observations indicate that the regulation of alkaloid formation in root cultures closely corresponds to the situation in the intact plant. Root cultures are easy to maintain and therefore offer good systems to study the response of roots towards biotic and abiotic stress factors. Since β-carboline alkaloids exhibit a wide range of pharmacological and toxic properties, their enhancement by jasmonates and other compounds support the assumption that the alkaloids serve as part of the dynamic defence system against herbivores in *Peganum harmala.*

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