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ALKALOIDS FROM *CERATOCAPNOS HETEROCARPA* PLANTS AND *IN VITRO* CULTURES

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Key Word Index—*Ceratocapnos heterocarpa*; Papaveraceae; plant cell culture; isoquinoline alkaloids; cularines; berbines; ribasines.

Abstract—The production of isoquinoline alkaloids related to the crassifoline metabolism by plants of the genera *Ceratocapnos*, *Sarcocapnos*, *Fumaria*, *Platycapnos* and *Rupicapnos* was investigated. Callus and cell suspensions of *Ceratocapnos heterocarpa* were initiated and their alkaloid composition compared with that of the plant. In the absence of organogenesis, only reticuline related alkaloids (protopine, ribasine and dihydrosanguinarine) were produced. However, organogenic calli regained their ability to biosynthesize cularines and 1,2-berbines.

INTRODUCTION

Cularines (**1**–**3**), isocularines (**4**, **5**) and 1,2-berbines (**6**–**8**) form a group of isoquinoline alkaloids and have so far been isolated from some species of the genera *Ceratocapnos*, *Corydalis*, *Dicentra* and *Sarcocapnos* (Papaveraceae) [1]. Biosynthetically, these substances are related to crassifoline (**9**), a 1-benzylisoquinoline with an unusual 7,8-oxygenation pattern at the isoquinoline nucleus [2], as shown by incorporation of radioactive crassifoline into cularine (**2**) [3].

The biosynthesis of **9** and its substitution pattern can be accounted for in at least three ways, i.e. (i) by hydroxylation at position 8 followed by dehydroxylation at position 6 of reticuline (**10**), the 1-benzylisoquinoline precursor for most isoquinoline alkaloids; (ii) by condensation and cyclization of tyramine and a phenylacetaldehyde to give a 7-hydroxy-1-benzylisoquinoline and subsequent hydroxylation at position 8, (iii) by condensation of dopamine and an appropriate phenylacetaldehyde, followed by regioselective cyclization to the most hindered position. Hypotheses (i) and (ii) were recently discarded after the work of Müller and Zenk [4], who showed crassifoline to be biosynthetically formed by an unusual and enzymatically catalysed Picted–Spengler *ortho*-cyclization that follows the condensation of dopamine with the aldehyde.

Our interest in the synthesis and biosynthesis of crassifoline related alkaloids [5, 6] prompted us to study the production of this type of compound by plant cell cultures. This paper deals with the alkaloid composition of *Ceratocapnos heterocarpa* as the selected plant species. Alkaloids were identified and quantified in callus and cell suspension cultures, and the effect of phytohormones on metabolite production was studied.

RESULTS AND DISCUSSION

The isoquinoline alkaloids from several Papaveraceae genera in the Mediterranean region of the Iberian peninsula and Morocco were comparatively analysed. GC-MS was used for qualitative and quantitative determination of alkaloids. The use of standards allowed confirmation that both phenolic and non-phenolic reduced forms of cularines, isocularines and 1,2-berbines were appropriately resolved and quantified. Under identical conditions, most of the alkaloids involved in the reticuline metabolism, aporphines, protopines, 2,3-berbines, *N*-benzylisoquinolines and the reduced forms of benzophenanthridines were also analysed. Since these alkaloids account for over 85% of the tertiary bases of the plants, little attention was given to the fact that same oxidized forms, e.g. *seco*-cularines, oxocularines, cularine *N*-oxides, berbine *N*-oxides and berberinium salts were not detected. There were no significant differences between our GC-MS results (Table 1) and the reported data for the isolated alkaloids in any case.

The absence of crassifoline derived alkaloids was confirmed in the genera *Platycapnos* [7], *Rupicapnos* [8] and *Fumaria* [9] (more than 20 species of this last genera exhibited an alkaloid profile quite similar to that described for *F. macrosepala*). The genera *Sarcocapnos* [10–13] and *Ceratocapnos* [6, 14–18] proved to be good sources of cularines and isocularines, and contained a high proportion of reticuline-derived alkaloids in most

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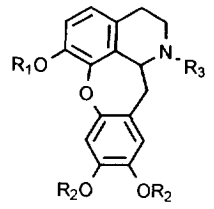
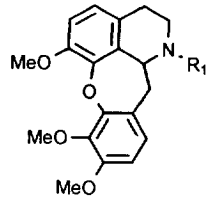
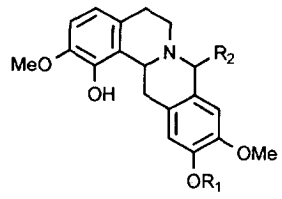


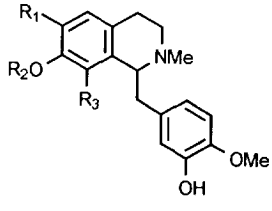
	R_1	R_2	R_3
1	H	Me	Me
2	Me	Me	Me
3	H	CH_2	Me



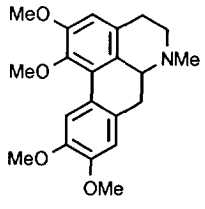
	R_1
4	Me
5	H



	R_1	R_2
6	H	H
7	Me	H
8	H	CH_2OH



	R_1	R_2	R_3
9	H	Me	OH
10	OMe	H	H



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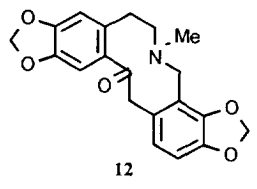

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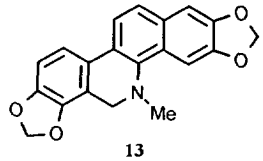

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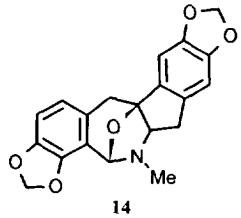

14

Sarcocapnos species. Inspection of Table 1 also reveals two main features of the alkaloid composition of *Ceratocapnos heterocarpa*, the sole species found to produce substantial amounts of both cularines [18] and 1,2-berbines [6, 17], and the higher ratio of crassifoline/reticuline derived alkaloids. For these reasons, a thorough study of *C. heterocarpa* alkaloids was started some time ago; moreover, callus and cell suspension cultures were also initiated.

Alkaloids in the plant

Over 25 isoquinoline alkaloids have so far been isolated from the plant, the more significant of which are listed in Table 2.

Cularidine (**1**) and caseamine (**6**) are the major alkaloids and they account for over 50% of the total alkaloid fraction, while reticuline-derived alkaloids account for barely 7%, glaucine (**11**) and protopine (**12**) being the most abundant. The consistency with the results obtained by isolation and GC-MS is quite significant. The major differences found in the concentration of norsarcocapnine (**5**) can be attributed to its oxidation to oxosarcocapnine, a ready process facilitated by the extensive manipulation involved in the isolation process. The divergence in the concentrations of caseamine (**6**) can be due to its low EIMS response. No significant differences in the alkaloid contents were found between two populations of *C. heterocarpa* collected regularly between 1987 and 1990.

Alkaloids in callus and cell cultures

Initially, flower buds were used to establish callus cultures in the presence of IAA. At 3 $mg\,l^{-1}$, 40% of the explants formed callus, whereas lower and higher concentrations resulted in much lower percentages. No callus was formed in the absence of auxin. Occasionally (e.g. with cell cultures of *Cephaelis ipecacuanha* [19] and *Rhazia stricta* [20]), a synergistic effect of IAA and kinetin on growth has been encountered; we thus studied the effect of kinetin in this callus after several subcultures in the presence of 3 $mg\,l^{-1}$ IAA. Addition of 1 $mg\,l^{-1}$ kinetin increased growth 1.5 times relative to the control; as expected [21], this cytokinin gave rise to shoots. This organogenic callus was multiplied to establish stock A (Table 3). Schneider and Wightman [22] suggested an antagonistic effect of strong auxins on organized growth. Addition of 3 $mg\,l^{-1}$ picloram to the IAA-kinetin containing medium completely inhibited organogenesis and in such a way stock B callus was established.

Table 1. GC-MS determination of alkaloids from Fumariaceae

Botanical source	CUL	ICL	1BE	NBN	2BE	APO	PRT	BNZ
Ceratocapnos claviculata (= *Corydalis claviculata*)	+ + +	+ +	—	(+)	+	(+)	+ +	+
Ceratocapnos heterocarpa	+ + +	+ + +	+ + +	+	(+)	+	+	+
Platycapnos spicata	—	—	—	—	+	+ + +	+	(+)
P. saxicola	—	—	—	—	+	+ +	—	(+)
Rupicapnos africana	—	—	—	—	+	(+)	+ +	+ +
Fumaria macrosepala	—	—	—	—	+	+	+ +	+
Sarcocapnos baetica								
subsp. *baetica*	+ +	+ + +	—	—	—	+	+ +	(+)
subsp. *integrifolia*	+ + +	+ + +	—	—	—	+	+	(+)
subsp. *ardalii*	+ + +	+ +	—	—	—	+	+ +	+
Sarcocapnos enneaphylla	+ +	+ + +	—	+	(+)	+ +	+ + +	+ +
Sarcocapnos saetabensis	+ + +	+ + +	—	+ +	(+)	+	+ + +	+ +
Sarcocapnos crassifolia								
subsp. *crassifolia*	+ + +	+ + +	—	—	—	+	+ + +	+ + +
subsp. *speciosa*	+ + +	+ + +	—	—	(+)	+	+ + +	+

CUL, Cularines; ICL, isocularines; 1BE, 1,2-berbines; NBN, *N*-benzylisoquinolines; 2BE, 2,3-berbines; APO, aporphines; PRT, protopines; BNZ, benzophenanthridines, Symbols: — not detected, (+) minor alkaloid, + < 5%, + + < 10%, + + + > 10%. (Percentages refer to total alkaloids.)

Table 2. Isoquinoline alkaloids from *Ceratocapnos heterocarpa* (% of total tertiary bases)

Alkaloids	Isolated yields	GC-MS
Cularines		
Cularidine (**1**)	20.8	31.0
Cularine (**2**)	0.7	2.6
Isocularines		
Sarcocapnine (**4**)	6.9	12.4
Norsarcocapnine (**5**)	6.7	23.5
1,2,Berbines		
Caseamine (**6**)	39.0	26
Caseadine (**7**)	5.8	Not detected
Malacitanine (**8**)	1.01	Not detected
Aporphines		
Glaucine (**11**)	2.1	2.1
Protopines		
Protopine (**12**)	2.9	2.0
Benzophenanthridines		
Dihydrosanguinarine (**13**)	0.1	<1

Callus initiation was also accomplished from explants of stem segments. Seventy per cent of the cultures formed callus in the range 3–10 mgl^{-1} picloram. After several subcultures at these concentrations, the effect of kinetin was studied. Maximum growth of a compact nodular callus was obtained in the range 1–3 mgl^{-1} kinetin, and so, stocks C–E were established (Table 3). No organ formation was observed in the presence of picloram; however, replacement with 2,4-D resulted in emergence of shoots from the nodular callus (stock F).

Calli from stock B were extracted with methanol and the alkaloid fraction was isolated by acid–base treatment. GC-MS analysis of the extract revealed an extremely simple alkaloid profile: a peak at R_t 11.2 min identified as protopine (**12**), a broad signal at 16 min recognized as dihydrosanguinarine (**13**), and a third peak between the previous two ($[M]^+$ at m/z 351) not detected in the whole plant. The alkaloid extracts from several cultures were chromatographed and the compound responsible for the third peak was isolated and spectroscopically characterized as ribasine (**14**). Full identification was accomplished by comparison with an authentic sample isolated from *Sarcocapnos enneaphylla*. Comparable concentrations of the same alkaloids were obtained from stocks C–E. No alkaloids were found to be excreted to the medium in any case. It is worth emphasizing the absence of cularines and 1,2-berbines, the major alkaloids in the plant, as well as that of glaucine (**11**).

Both types of explants exhibited shoot formation in the absence of picloram (stocks A and F). The occurrence of morphogenesis in callus cultures is generally associated with enhanced production of the secondary metabolites normally associated with these organs in the mother plant [23]. While total alkaloid production in *C. heterocarpa* was essentially similar for organogenic and non-organogenic calli, the alkaloid profile was not. Cularines were present in significant amounts in stock A, particularly norsarcocapnine (**5**) and cularicine (**3**), the only known cularine alkaloid exhibiting a molecular ion at m/z 311 which has never been detected among the alkaloids in the plant. The alkaloid profile from organogenic calli in stock F was close to that of the plant; while ribasine (**14**) practically disappeared, the cularine levels rose, particularly those of norsarcocapnine (**5**), sarcocapnine (**4**) and cularidine (**1**), whereas that of cularicine (**3**) decreased (Table 4). No 1,2-berbines were detected, in spite of the fact that caseamine (**6**) is the most abundant alkaloid in the plant.

Table 3. Effect of growth regulators (mg l^{-1}) on callus production by *Ceratocapnos heterocarpa*

Explant	Callus stock	IAA	Picloram	2,4-D	Kinetin	Fr. wt (g)
Flower buds	A	3			1	1.6
	B	3	3		1	1.3
Stem segments	C		3		1	1.2
	D		10		1	1.3
	E		10		3	2.2
	F			3	1	1.4

Table 4. Alkaloids in callus from *C. heterocarpa*

Callus stock	Per cent alkaloids (dry wt)	Per cent of akaloids from GC-MS			
		Cularines	Protopine	Ribasine	Dihydro-sanguinarine
B	0.025	—	47	7	46
C	0.11	—	46	10	44
D	0.09	—	29	16	54
E	0.07	—	46	10	44
A	0.1	16	28	4	50
F	0.08	49	50	—	1

Cell suspensions obtained after culturing callus from stock C in basal liquid medium supplemented with NAA (3 mg l^{-1}) and kinetin (0.3 mg l^{-1}) were found to be poor sources of alkaloids. The contents of these compounds decreased with increasing subculturing (e.g. after three subcultures, ribasine disappeared, while, after seven subcultures, only protopine traces were detected). Decreased production of secondary metabolites with time has also been observed in many other cases (e.g. alkaloids in cultures of *Peganum harmala* [24]), and could be a result of the genetic instability of the cell cultures.

The absence of crassifoline-derived alkaloids observed from several calli that preserved the ability to produce **12**–**14** among the alkaloids biosynthesized in the reticuline pathway, cannot be related to the presence of precursors since they (dopamine and 3′-hydroxyphenylacetaldehyde) are common to both metabolic pathways [4, 25]. This behaviour and the fact that cularines are formed immediately after callus cultures exhibit morphogenesis, seems to indicate that specialized cells are responsible for their biosynthesis.

The presence of **14** (as well as protopine and dihydrosanguinarine) might be related to the ready formation of the methylenedioxy group frequently observed in callus cultures of Papaveraceae plants [26]. Based on the biosynthesis of this group from *ortho*-methoxyphenols, this result can be ascribed to the methylenedioxy bridge forming cytochrome P-450 dependent enzyme being strongly elicited under *in vitro* culture conditions [27].

This is the first report on the isolation of ribasine in cell cultures, and appears to be a suitable means for studying its biosynthesis, which has been interpreted in terms of a dipolar cycloaddition of a protopine ylide [28]. An aziridinium intermediate possibly produced from 6-hydroxy-13,14-dehydro-*N*-methylstilopine, which is related to the biosynthesis of sanguinarine [29] should also be considered in this respect.

EXPERIMENTAL

Plant material. All plants listed in Table 1 were collected from their natural habitats. Voucher specimens were deposited in the herbarium of the Department of Plant Biology (MGC), University of Malaga: *C. claviculata* (12 189), *P. spicata* (23 084), *P. saxicola* (27 676), *R. africana* (25 941), *F. macrosepala* (31 775), *S. baetica* subsp. *baetica* (23 047), *S. baetica* subsp. *integrifolia* (31 775), *S. baetica* subsp. *ardalii* (37 176), *S. enneaphylla* (31 779), *S. saetabensis* (36 003), *S. crassifolia* subsp. *crassifolia* (37 097), *S. crassifolia* subsp. *speciosa* (31 774). Seeds and plants of *C. heterocarpa* collected at different locations, namely Moron de la Frontera, Sevilla (10 000) and Motril, Granada (31 440) proved to be identical.

Culture initiation and establishment. Flowering plants of *C. heterocarpa* were obtained in the greenhouse after germinating seeds in a peat moss: perlite (1:1) substrate Two types of explant were used, namely, 1-cm-long stem segments and flower buds. Before culturing, both types of explant were sterilized in a 0.5% NaOCl soln for 10 min, followed by several rinses with sterile H_2O.

The basal medium used was that of Murashige and Skoog [30], supplemented with 30 g l^{-1} sucrose and 8 g l^{-1} Bacto-agar. The pH was adjusted to 5.7 before autoclaving at 121° for 15 min. Cultures were incubated in the dark at a constant temp. (25°).

Initially, the effect of auxin indole-3-acetic acid (IAA) at various concns (0, 0.3, 1, 3, 10 $mg\,l^{-1}$) was studied by using flower buds. After several subcultures at 6-week intervals, callus grown in the presence of 3 $mg\,l^{-1}$ was used to study the effect of kinetin (1 $mg\,l^{-1}$). Finally, the effects of adding 3 $mg\,l^{-1}$ picloram (4-amino-3,5,6-trichloro picolinic acid) to a medium containing 3 $mg\,l^{-1}$ IAA and 1 $mg\,l^{-1}$ kinetin were evaluated. Stem segments were used to study the effects of picloram at various concns (0, 0.3, 1,3 and 10 $mg\,l^{-1}$) on callus formation. After callus initiation, calli obtained from the higher auxin concns (3 and 10 $mg\,l^{-1}$) were subcultured at 6-week intervals. After 6 months, the effect of kinetin (1, 3 $mg\,l^{-1}$) on callus growth was evaluated. Finally, some callus cultures growing in the presence of 10 $mg\,l^{-1}$ picloram + 1 $mg\,l^{-1}$ kinetin were transferred to another medium in which picloram had been replaced with 3 $mg\,l^{-1}$ 2,4-D (2,4-dichlorophenoxyacetic acid). In all callus experiments, inoculum size was 0.3 g.

Cell suspension cultures were established from callus previously grown in the presence of 3 $mg\,l^{-1}$ picloram and 1 $mg\,l^{-1}$ kinetin, in a liquid medium supplemented with 3 $mg\,l^{-1}$ NAA (naphthalene-2-acetic acid) and 0.3 $mg\,l^{-1}$ kinetin.

Identification and quantitation of alkaloids by GC-MS. An HP 5988 mass spectrometer coupled to a gas chromatograph equipped with a fused silica capillary column (HP-1, 12 m × 0.2 mm i.d., 0.33 μm film thickness) was used. Helium flow rate 1 $ml\,min^{-1}$. Injection temp. 250°; transfer line 280°; programme temp: 200°, hold 0.8 min, to 250° at 10° min^{-1} hold 24 min. EI-MS were obtained at 70 eV, and quantitation was based on the total ion current, corrected for the detector response to each individual alkaloid. Cularicine, R_t, 7.87; MS *m/z* 311 ($[M]^+$, 100), 294 (70). Sarcocapnine, R_t, 7.98; MS *m/z* 341 ($[M]^+$, 100), 326 (60), 310 (23), 308 (53), 176 (90). Norsarcocapnine, R_t, 8.37; MS *m/z* 327 ($[M]^+$, 100), 312 (51), 294 (49), 162 (68). Cularidine, R_t, 8.75; MS *m/z* 327 ($[M]^+$, 64), 312 (100). Cularine, R_t, 8.88; MS *m/z* 341 ($[M]^+$, 34), 326 (100). Glaucine, R_t, 9.76; MS *m/z* 355 ($[M]^+$, 75), 354 (100), 340 (50). Protopine, R_t, 10.73; MS *m/z* 353 ($[M]^+$, 5), 148 (100). Ribasine R_t, 11.64; MS *m/z* 351 ($[M]^+$, 75), 350 (100) 188 (55). Caseamine, R_t, 12.84; MS *m/z* 327 ($[M]^+$, 40), 178 (100), 150 (17). Dihydrosanguinarine R_t, 13.57; MS *m/z* 333 ($[M]^+$, 90), 332 (100).

Alkaloids from plants. Whole plants were dried, powdered (15 g) and were extracted with MeOH (100 ml) in a soxhlet to a negative Mayer's test. The resulting methanolic residue was taken up in 2.5% HCl and filtered. The acidic aq. soln was adjusted to pH 8 with NH_4OH and extracted with $CHCl_3$ (3 × 15 ml). The solvent was partially removed and final vols of the extracts were made to 25 ml.

Alkaloids from cultures. (i) The data listed in Table 4 were obtained from dry calli (1–3 g) extracted with refluxing MeOH (50 ml) for 3 hr. Alkaloid concns were determined by titration with a $CHCl_3$ soln of p-toluenesulphonic acid and Methyl Yellow as indicator. Methanolic residue was redissolved in MeOH, neutralized with dilute NH_3 (final vol. 1 ml) and used directly for GC-MS analysis.

(ii) Dry calli from stocks B–E, were found to be homogeneous in its alkaloid composition (GC-MS). A mixt. of such calli was extracted (56 g) with MeOH in a soxhlet for 48 hr. After solvent removal, the extract was taken in 2 M HCl (2 × 15 ml) and the acid fr. thoroughly washed with hexane. The acid layer was basified with NH_4OH (pH 8.5) and extracted with CH_2Cl_2 (3 × 10 ml). The extract was concd to *ca* 1 ml at red. pres. and subjected to prep. TLC (silica gel, eluent: $CHCl_3$–MeOH, 95:5). Three bands were sepd and extracted to afford dihydrosanguinarine (3 mg), ribasine (6 mg) and protopine (12 mg), which were identified by comparison with authentic samples (^1H NMR, MS, TLC).

(iii) Morphogenic calli from stock F (12 g dry wt) were extracted similarly. Prep. TLC (silica gel, eluent: $CHCl_3$–MeOH, 90:10) afforded an upper band consisting of a mixt. of *ca* 1:1 (5 mg) sarcocapnine–cularidine and a lower band composed mostly of norsarcocapnine (3 mg) impurified with protopine. In all cases, identification was confirmed by using authentic samples (^1H NMR, GC/MS, TLC).

(iv) Dried cell suspensions were extracted as above, and analysed by GC-MS.

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