Pergamon

PII: S0031-9422(96)00336-6

Phytochemistry, Vol. 43, No. 3, pp. 597–602, 1996

0031-9422/96 $15.00 + 0.00

BIOSYNTHESIS OF ECDYSTEROIDS IN *IN VITRO* PROTHALLI CULTURES OF *POLYPODIUM VULGARE*

NATÀLIA REIXACH, JOSEP IRURRE-SANTILARI, JOSEFINA CASAS, ENRIC MELÉ,* JOAQUIMA MESSEGUER* and FRANCISCO CAMPS†

Department of Biological Organic Chemistry, CID (CSIC). J. Girona, 18.08034, Barcelona, Spain;
*Department of Plant Genetics, IRTA, Centre de Cabrils, Ctra. de Cabrils s/n. 08348, Cabrils, Spain

(*Received in revised form* 27 *March* 1996)

Systematic names for mentioned ecdysteroids: abutasterone (20,24E): 2β,3β,14α,20*R*,22*R*,24*S*,25-heptahydroxy-5β-cholest-7-en-6-one: ecdysone (E): 2β,3β,14α,22*R*,25-pentahydroxy-5β-cholest-7-en-6-one; 5-hydroxyabutasterone (5,20,24E): 2β,3β,5β,14α,20*R*,22*R*,24*S*,25-octahydroxycholest-7-en-6-one; 20-hydroxyecdysone (20E): 2β,3β,14α,20*R*,22*R*,25-hexahydroxy-5β-cholest-7-en-6-one; 24-hydroxyecdysone (24E): 2β,3β,14α,22*R*,24*S*,25-hexahydroxy-5β-cholest-7-en-6-one; inokosterone (25d20,26E): 2β,3β,14α,20*R*,22*R*,26-hexahydroxy-5β-cholest-7-en-6-one; polypodine B (5,20E): 2β,3β,5β,14α,20*R*,22*R*,25-heptahydroxycholest-7-en-6-one; ponasterone A (25d20E): 2β,3β,14α,20*R*,22*R*-pentahydroxy-5β-cholest-7-en-6one; pterosterone (25d20,24E): 2β,3β,14α,20*R*,22*R*,24*S*-hexahydroxy-5β-cholest-7-en-6-one.

Key Word Index—*Polypodium vulgare*; Polypodiaceae; phytoecdysteroids; tissue cultures; prothalli; biosynthesis.

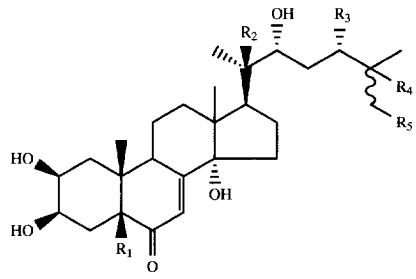
Abstract—The concentration of ecdysteroids in *Polypodium vulgare* has been studied in different parts of the wild plant as well as in the two *in vitro* micropropagated tissues. The ecdysteroid composition (abutasterone, polypodine B, 20-hydroxyecdysone, inokosterone, 24-hydroxyecdysone, pterosterone and ecdysone) was the same for all the sources investigated, but quantitative differences were found among them, with the exception of spores that did not produce these compounds. In contrast, the *in vitro* culture of spores originated the formation of haploid (prothalli) and diploid (sporophytes) tissues with a common feature: a proportion of 25-deoxyecdysone derivatives higher than that found in the wild plant. Prothalli micropropagated *in vitro*, generated from spores collected in different European areas, exhibited characteristic phytoecdysteroid contents that were constant over the four-year period studied. On the other hand, results on incorporation of radiolabelled ecdysteroid precursors, such as mevalonate, cholesterol or ecdysone, indicated that our *in vitro* system is adequate for biosynthetic studies. Labelled mevalonate and cholesterol were incorporated into all identified ecdysteroids, and labelled ecdysone was biotransformed into other 25-hydroxyecdysteroids present in this plant. Based on the different rates of incorporation of these precursors, a biosynthetic pathway for the last steps of ecdysteroid biosynthesis in *P. vulgare* was proposed.

INTRODUCTION

Production of ecdysteroids by *in vitro* plant cultures is becoming a subject of increasing interest to overcome the variation of concentration of these compounds in plants from different location and harvesting period. The formation of ecdysteroids in callus cultures was first described in seedling callus tissue from several *Achyrantes species* [1] and *Trianthema portulacastrum* [2]. Ponasterone A (25d20E) [3] and 20-hydroxyecdysone (20E) were isolated in very small amounts from culture filtrates of gametophytes of *Pteridium aquilinum* [4]. Recent studies show that *in vitro* cell cultures of this fern have considerably increased the production of these ecdysteroids and of ecdysone (E) [5]. In our laboratory, *in vitro* root cultures, in hormone supplemented solid medium, of *Ajuga reptans* were established and the phytoecdysteroid production found was higher than 0.5% (dry wt) [6–8]. Likewise, we isolated very high amounts of ecdysteroids from *in vitro* cultures of *Polypodium vulgare* prothalli [0.7% 20E, 0.1% polypodine B (5,20E) and 0.06% E] [9]. Recently, from these cultures of prothalli we have reported the isolation of five other phytoecdysteroids (5-hydroxyabutasterone (5,20,24E), abutasterone (20,24E), inokosterone (25d20,26E), 24-hydroxyecdysone (24E) and pterosterone (25d20,24E)) [10].

Phytoecdysteroid biosynthetic studies have been carried out using whole plants [11–13] as well as *in vitro* cultures [14, 15]. In the present paper we describe the production of all these ecdysteroids in different

†Author to whom correspondence should be addressed.



	R_1	R_2	R_3	R_4	R_5
Ecdysone (E)	H	H	H	OH	H
20-Hydroxyecdysone (20E)	H	OH	H	OH	H
Polypodine B (5,20E)	OH	OH	H	OH	H
Abutasterone (20,24E)	H	OH	OH	OH	H
5-Hydroxyabutasterone (5,20,24E)	OH	OH	OH	OH	H
24-Hydroxyecdysone (24E)	H	H	OH	OH	H
Pterosterone (25d20,24E)	H	OH	OH	H	H
Inokosterone (25d20,26E)	H	OH	H	H	OH

parts of *P. vulgare*, i.e. rhizomes, roots, fronds (diploid) and spores (haploid), as well as in two *in vitro* culture forms, i.e. sporophyte (diploid) and prothalli (haploid). In addition, the phytoecdysteroid production of *P. vulgare* prothalli derived from spores from different geographical origin, micropropagated *in vitro* for consecutive years, is also reported. Finally, the usefulness of these prothalli in biosynthetic studies is demonstrated by incorporation of different labelled precursors.

RESULTS AND DISCUSSION

As shown in Fig. 1, all ecdysteroids found in the different parts of the wild plant are present in the *in*

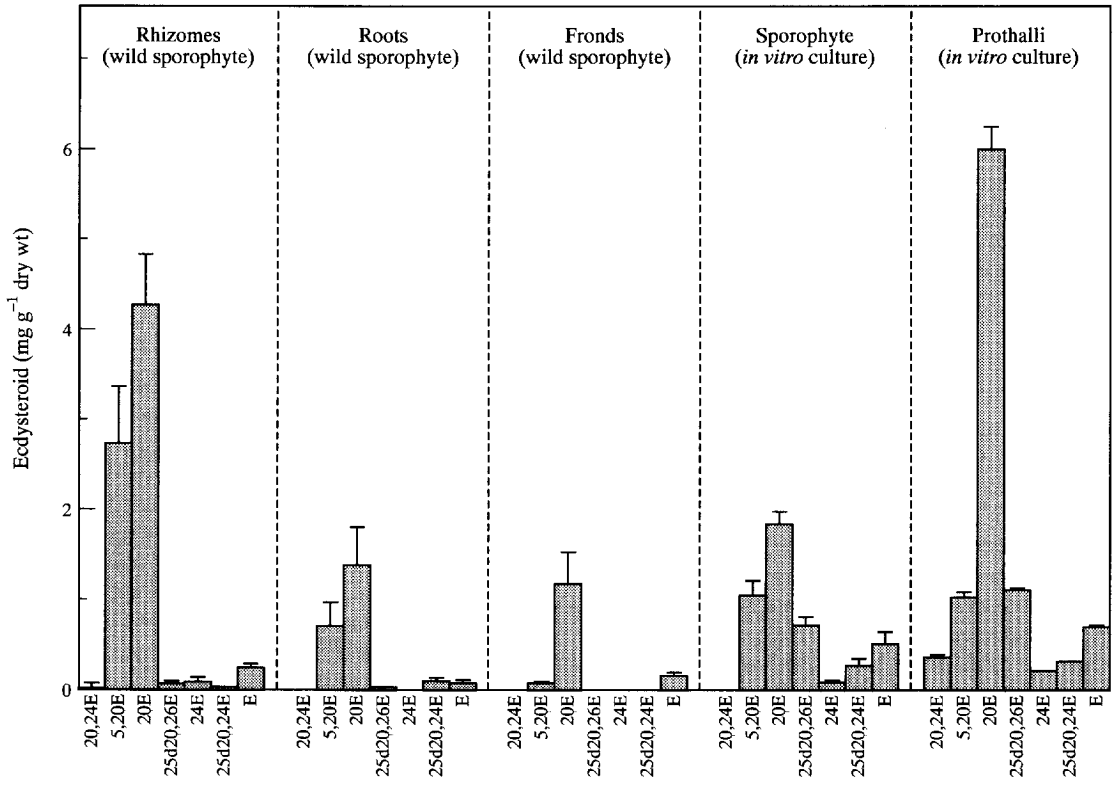


Fig. 1. Phytoecdysteroid content (mg g^{-1} dry wt, mean±s.d., $n = 6$) in roots, rhizomes and fronds of *P. vulgare* as well as in *in vitro* micropropagated sporophyte and prothalli derived from *P. vulgare* spores.

vitro cultured tissues and vice versa. Rhizomes and roots exhibited the same profile, but the total production of ecdysteroids was higher for the former case. On the other hand, the methanolic extracts of spores did not contain ecdysteroids within the detection limit of the analytical method used (10 μg ecdysteroid g^{-1} dry wt). However, the *in vitro* culture of this non-ecdysteroid productive material gives rise to diploid and haploid tissues which are capable of biosynthesizing these compounds.

The *in vitro* cultured sporophytes present a morphological shape and ecdysteroid profile similar to those of the wild plant. Regarding the most abundant ecdysteroids in the wild plant, the 20E:5,20E ratio of the *in vitro* cultured sporophyte (1.76) is closely related to that obtained considering together all parts of the wild sporophyte (1.86) (Experimental). This fact suggests that the biosynthesis of ecdysteroids in the *in vitro* cultured sporophyte could be comparable to that of the wild plant in terms of balance of compounds and total activity (4.42 vs. 4.77 mg ecdysteroid g^{-1} dry wt, respectively). As previously reported, the highest production of ecdysteroids was achieved in the *in vitro* micropropagated prothalli [6], and, as shown in Fig. 1, the contents of the new recently identified ecdysteroids do not change this assertion. The ecdysteroid profile of prothalli could be considered similar to that obtained from wild fronds; both tissues produce the highest proportion of 20E in comparison with the contents of the other ecdysteroids. The relative amounts of E and especially of 25d20,26E, in both *in vitro* sporophyte and prothalli cultures, are remarkably high when compared with the corresponding contents in the wild plant. Finally, the main difference between the two micropropagated *in vitro* tissues is the higher amount of 20E and 20,24E found in the prothalli cultures. As mentioned in Experimental, the *in vitro* sporophytes were derived from prothalli cultured with a higher concentration of agar, as the only difference from standard conditions. This fact might cause a decrease of the biosynthetic activity leading to 20E and 20,24E and their precursor(s) without altering too much the production of the 25dE derivatives of their precursor(s).

Prothalli derived from spores collected in different European locations [Ahrensburg (Germany), Espoo (Finland) and Montseny (Spain)] have also been micropropagated *in vitro* for six to eight years. The fact that the growth ratio and the shape of the prothalli cultures had been maintained for more than 60 subcultures indicated that this culture could be considered morphologically stable for all three sources tested. The total ecdysteroid production of prothalli from different origins is summarized in Table 1. The phytoecdysteroid production also appeared to be stable, since it was maintained at high levels during the years analysed. The observed differences were not significant and were probably due to variations in the subculturing process, which was established for prothalli production approximately every six weeks. Nevertheless, the ANOVA analysis of these data detected significant differences, depending on the origin; thus, the phytoecdysteroid productions of prothalli derived from Espoo spores or Montseny spores were similar, whereas prothalli derived from spores from Ahrensburg were less productive.

Figure 2 shows the separate phytoecdysteroid content for four consecutive years in *P. vulgare* prothalli derived from spores collected in the above mentioned areas. No significant differences for each ecdysteroid were detected among the different years analysed. In relation to the produced phytoecdysteroids, although 20E is the major component, ranging from 65 to 70% for all the sources studied, the percentage of the other phytoecdysteroids is distinctive for each origin and thus can be used as a fingerprint character. Thus, ecdysteroid production in prothalli generated from Ahrensburg spores (Fig. 2A) shows higher amounts of E and 24-hydroxylated derivatives (20,24E; 24E and 25d2024E) and lower contents of 5,20E and 25d20,26E. Conversely, prothalli derived from Montseny spores (Fig. 2C) present an inverted profile related to the former, with a higher proportion of 5,20E and 25d20,26E and a lower one for 24-hydroxylated derivatives. Finally, ecdysteroid production in prothalli generated from Espoo spores (Fig. 2B) exhibits a higher abundance of polar ecdysteroids (20,24E and 5,20E) as well as E, and a very low amount of all the other compounds.

Based on the above results, prothalli derived from *P. vulgare* spores collected in Montseny or Ahrensburg were used for biosynthetic studies. Topical application of [23,24-^{3}H]-ecdysone on these prothalli revealed varying levels of ^{3}H incorporation into more polar

Table 1. Total ecdysteroid content in prothalli derived from spores of *P. vulgare* from different geographical origin micropropagated *in vitro* for four consecutive years*

	Ahrensburg (Germany)	Espoo (Finland)	Montseny (Spain)	Average
1990	5.85	9.76	9.14	8.25[a]
1991	8.01	10.39	9.40	9.27[a]
1992	6.45	8.99	9.55	8.33[a]
1993	6.83	10.58	9.20	9.11[a]
Average	6.84[b]	9.93[c]	9.44[c]	

*Data are given in mg ecdysteroid g^{-1} dry wt (mean, $n = 6$).

Statistical significance among means is indicated by different letters (ANOVA followed by Tukey's LSD test, $P = 0.01$).

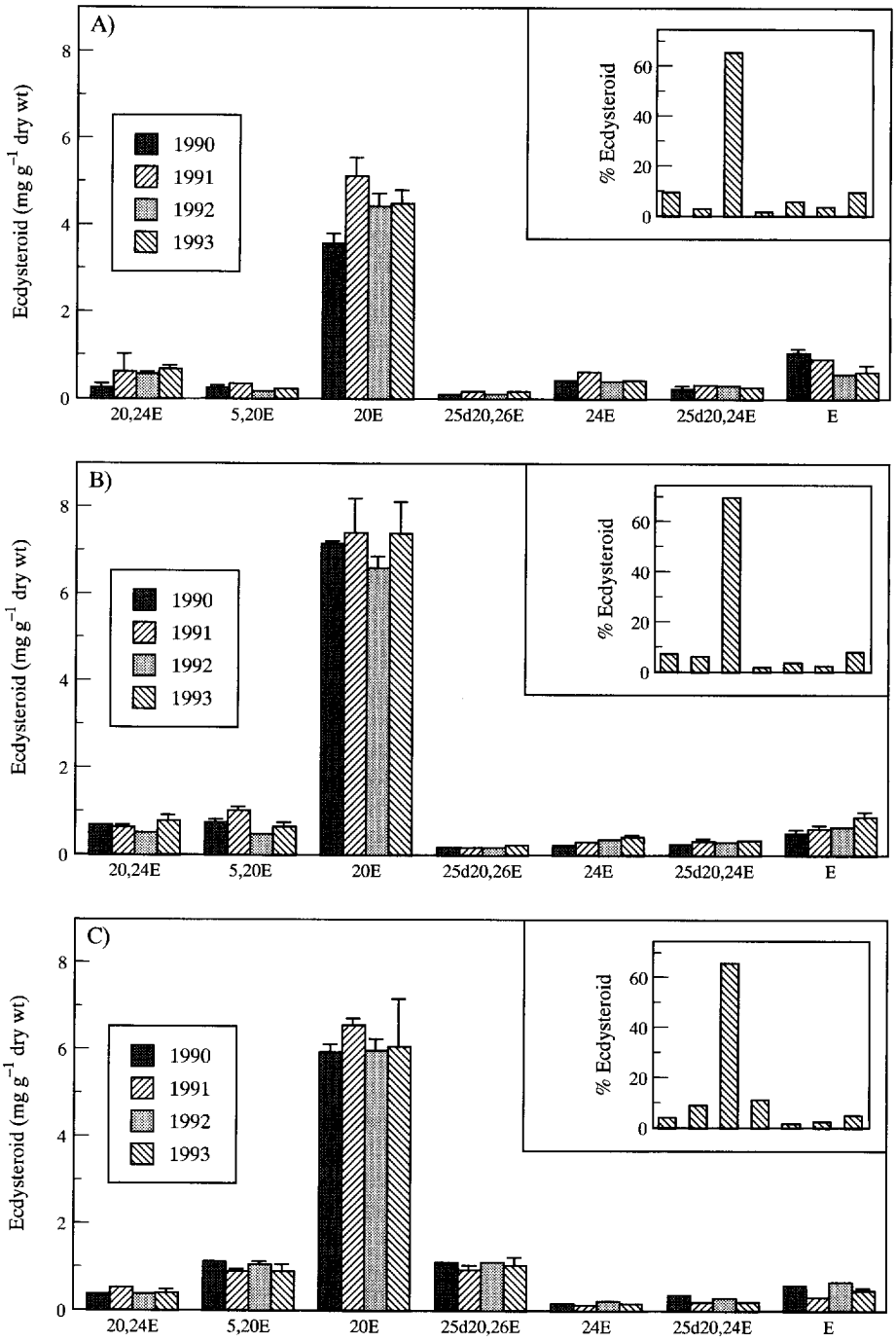


Fig. 2. Phytoecdysteroid content (mg g^{-1} dry wt, mean±s.d., $n = 6$) in *P. vulgare* prothalli derived from spores collected in (A) Ahrensburg, (B) Espoo and (C) Montseny, and micropropagated *in vitro* for four consecutive years. The percentage of ecdysteroid production in 1993 is shown in the figure insets, with the same abscissa axis as the main figure.

ecdysteroids, depending on the period of treatment and the source of prothalli used (Table 2). After one week, prothalli derived from Montseny spores metabolized 38% of the applied [23,24-^{3}H]-ecdysone to 20E, and a small amount (3%) of 5,20E was also formed. The biotransformation of the precursor increased up to 61% after three weeks and at that moment, radiolabelled 20,24E (3%) was also present. The fact that [^{3}H]-24E had not been detected may suggest that 20,24E is biosynthesized, at least in part, via a 24-hydroxylation of 20E and not 20-hydroxylation of 24E. For elucidating the biogenesis of 24E, prothalli derived from Ahrensburg spores, which are a good source of 24-hydroxylated ecdysteroids, were treated with [23,24-^{3}H]-ecdysone. As shown in Table 2, after three weeks all the detected 25-hydroxyecdysteroids, including 24E

Table 2. Percentage of incorporation of radiolabelled precursors (ecdysone, cholesterol and mevalonate) into ecdysteroids from *P. vulgare* prothalli micropropagated *in vitro*

		20,24E	5,20E	20E	25d20,26E	24E	25d20,24E	E
Montseny								
Control*		4	7	66	11	4	2	6
	1 week	—	3	38		—		59
[23,24-^{3}H]-E	2 weeks	—	4	48		—		48
	3 weeks	3	4	54		—		39
	1 week	3	10	40	18	4	9	16
[4-^{14}C]-Cholesterol	2 weeks	4	7	48	13	5	7	16
	3 weeks	4	8	53	12	5	4	14
	1 week	2	5	40	11	5	4	31
[2-^{14}C]-MVA	2 weeks	3	6	44	12	6	6	23
	3 weeks	3	6	52	12	4	2	21
Ahrensburg								
Control*		10	3	66	2	6	3	10
[23,24-^{3}H]-E	3 weeks	2	1	55		2		40

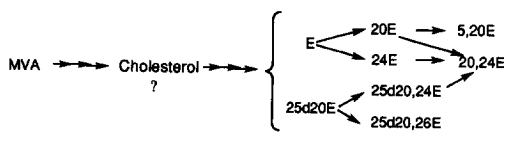
*Percentage of ecdysteroids produced in assays carried out in the absence of labelled precursors. These percentages are conserved through the three-week period.

(2%), showed ^{3}H incorporation. Nevertheless, the relative amount of labelled 20,24E with respect to the control (1:5) was the lowest when compared to the relative ratios of the other labelled ecdysteroids (1:3 as a minimum). This fact suggests that ecdysteroids other than 24E and 20E may be the precursor(s) for 20,24E (Scheme 1). On the other hand, as expected, the 25-deoxyecdysteroids were not labelled when [23,24-^{3}H]-ecdysone was used as precursor.

When an early biosynthetic precursor, mevalonate (MVA), was used, all identified phytoecdysteroids showed ^{14}C incorporation after one week in culture (Table 2). The extent of MVA incorporation differed among samples, ranging from 2 to 10% of ^{14}C incorporation into the ecdysteroidal fraction during the three-week period. Concerning the formation of 25-deoxyecdysteroids, [^{14}C]-25d20,26E was formed at the same rate as the unlabelled one. Conversely, [^{14}C]-25d20,24E was present in a higher amount than the control after the first week, but its contents decreased after three weeks to become similar to those of the unlabelled analogue. The fact that when [23,24-^{3}H]-ecdysone was used as precursor a longer time was needed to detect the formation of labelled 20,24E, together with the higher amount of [^{14}C]-25d20,24E formed from [^{14}C]-MVA cultures, may indicate that this compound could contribute to the biosynthesis of 20,24E (Scheme 1). On the other hand, a possible precursor of 25-deoxyecdysteroids (ponasterone A, 25d20E) was tentatively identified in very low amount (1%) in these assays, suggesting that it undergoes a rapid conversion. With respect to 25-hydroxy-ecdysteroids, [^{14}C]-E was present in high amounts during all of the assay, and although its relative contents decreased when the culture periods were prolonged, the value after the three-week period did not reach that of the control. Finally, as occurred with the treatment with [23,24-^{3}H]-ecdysone, the amounts of the more polar labelled ecdysteroids increased with time and came close to the control value.

When [4-^{14}C]-cholesterol was fed as precursor, incorporation of radioactivity to all reported ecdysteroids was also observed. The relative incorporation profiles were similar to those obtained with MVA, but with a lower proportion of E.

In summary, the special and simple morphology of prothalli makes this material easily adaptable to *in vitro* culture. Their natural laminar growth allows an easy incorporation of biosynthetic precursors and/or modulators artificially supplied. On the other hand, their phytoecdysteroid production is constant and reproducible for all three sources tested. Labelled MVA, cholesterol or ecdysone can be incorporated and biotransformed by these tissues. Labelled ecdysone may be partially a biosynthetic precursor for 25-hydroxy-ecdysteroids, whereas MVA and cholesterol may be biotransformed into all known ecdysteroids including those recently identified (Scheme 1). From all these results it can be concluded that *P. vulgare* prothalli micropropagated *in vitro* are an excellent tool to utilize for biosynthetic studies.



Scheme 1. The last steps of the biosynthetic pathway of phytoecdysteroids in *P. vulgare* prothalli micropropagated *in vitro*.

EXPERIMENTAL

Plant material. Polypodium vulgare wild plants were collected in October in a beech forest from the Montseny area (Spain). The dry wt ratio between the different parts of the collected plants was 1:4:2 (roots:rhizomes:fronds).

In vitro *cultures*. (*i*) *Prothalli production*: This was carried out as described in ref. [9], in 1/2 MS cultured

medium supplemented with sucrose (30 g l^{-1}) and agar (8 g l^{-1}), adjusted to pH 5.7. Prothalli clusters were systematically divided into 3–4 sections every 6 weeks and cultured at 25±2° under 16 hr light. These cultures have been maintained since 1988. (*ii*) *Sporophyte production*: When prothalli clusters were cultured in the same medium, but increasing the agar conc up to 12 g l^{-1}, some sporophytes were isolated. This material was transferred into a fresh medium and systematically subcultured every 8 weeks. Sporophyte cultures could not be obtained by *in vitro* introduction of rhizome segments since it was not possible to sterilize the plant material.

Chemicals and supplies. Ecdysteroid standards were obtained from extracts of *P. vulgare* prothalli [10]. Solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany) and C_{18} reversed-phase cartridges (Sep-Pak) were from Waters (Millipore, Milford, U.S.A.). Water for HPLC was purified with a Milli-Q system (Millipore), filtered through 0.45 μm filters and degassed.

HPLC. Analyses were monitored at 242 nm with a diode array detector and recorded and quantified with chromatographic software. The reversed-phase HPLC column was a Spherisorb ODS2 (5 μm, 15 cm × 4.6 mm i.d.) maintained at 55° and eluted with *i*-PrOH–H_2O (7:93) for 10 min followed by a linear gradient (over 10 min) from 0 to 20% of MeCN in *i*-PrOH–H_2O (7:93) at 1.2 ml min^{-1}.

Analysis of samples. Lyophilized prothalli or sporophytes (microprogated *in vitro*), rhizomes or roots (50 mg) were extracted with MeOH (4 × 5 ml) and the combined extracts were evapd. The residue was dissolved in 10 ml H_2O and loaded on to a C_{18} reversed-phase Sep-Pak cartridge. After washing with H_2O (10 ml) and MeOH–H_2O (3:17) (10 ml), ecdysteroids were eluted with MeOH–H_2O (17:3) (5 ml). An aliquot of this eluate was mixed with 7-ethoxycoumarin (int. standard) and injected on to the HPLC system. Calibration curves for E and 20E in front of 7-ethoxycoumarin were obtained. For ecdysteroids other than E or 20E, values were modified with a correction factor corresponding to the different absorptions of ecdysteroids (ε = 12213 for 24E and values reported in ref. [16] for the other ecdysteroids). Lyophilized fronds (50 mg) were analysed as above, but an elution from the Sep-Pak cartridge with 0.1 N NaOH (10 ml) after loading the sample was also carried out, in order to obtain a cleaner HPLC profile [17]. Spores (150 mg) were ground in a mortar in liquid N_2 and the obtained powder extracted as described previously. Since 5-hydroxyabutasterone was present in very small amount in all the samples analysed and its quantification was not possible, this ecdysteroid has not been included in the present study.

Incorporation of radioactive precursors. Small prothalli clusters (25 mg wet, *ca* 50 mm^2) were cultured in Petri dishes in the above medium. After 1 week, an aq. soln of [2-^{14}C]-MVA DBED salt (1 μCi), [23, 24-^{3}H]-ecdysone (0.1 μCi) or [4-^{14}C]-cholesterol (in Me_2CO–H_2O, 1:4, 1 μCi) was topically applied. After incubation for different time periods, samples were lyophilized and radiolabelled ecdysteroids extracted and sepd by HPLC as described above and quantified with a liquid scintillation counter. Experiments were carried out in triplicate using at least three different prothalli subcultures.

Statistics. Mean values shown in Table 1 were subjected to ANOVA followed by Tukey's LSD test.

Acknowledgements—We thank Dr J. Coll for a critical review of the manuscript. Financial support from CICYT (Grant BIO/92/0398) and Comissionat per a Universitats i Recerca (Generalitat de Catalunya GRQ 93-8016) and predoctoral fellowships from CIRIT-Generalitat de Catalunya (to N. R.) and the Ministerio de Educación y Ciencia (to J. I.-S.) are acknowledged.

REFERENCES

1. Hikino, H., Nomoto, K. and Takemoto, T. (1971) *Phytochemistry* **10**, 438.
2. Ravishankar, G. A. and Mehta, A. R. (1979) *J. Nat. Prod.* **42**, 152.
3. Lafont, R., Koolman, J. and Rees, H. (1993) *Insect Biochem. Mol. Biol.* **23**, 207.
4. McMorris, T. C. and Voeller, B. (1971) *Phytochemistry* **10**, 3253.
5. Svatos, A. and Macek, T. (1994) *Phytochemistry* **35**, 651.
6. Tomás, J., Camps, F., Claveria, E., Coll, J., Melé, E. and Messeguer, J. (1992) *Phytochemistry* **31**, 1585.
7. Tomás, J., Camps, F., Coll, J., Melé, E. and Messeguer, J. (1993) *Phytochemistry* **32**, 317.
8. Camps, F. and Coll, J. (1993) *Phytochemistry* **32**, 1361.
9. Camps, F., Claveria, E., Coll, J., Marco, M. P., Messeguer, J. and Melé, E. (1990) *Phytochemistry* **29**, 3819.
10. Coll, J., Reixach, N., Sánchez-Baeza, F., Casas, J. and Camps, F. (1994) *Tetrahedron* **50**, 7247.
11. de Souza, N. J., Ghisalberti, E. L., Rees, H. H. and Goodwin, T. W. (1970) *Phytochemistry* **9**, 1247.
12. Adler, J. H. and Grebenok, R. J. (1995) *Lipids* **30**, 257.
13. Devarenne, T. P., Sen-Michael, B. and Adler, J. H. (1995) *Phytochemistry* **40**, 1125.
14. Delbecque, J. P., Beydon, P., Chapuis, L. and Corio-Costet, M. F. (1995) *Eur. J. Entomol.* **92**, 301.
15. Nagakari, M., Kushiro, T., Yagi, T., Tanaka, N., Matsumoto, T., Kakinuma, K. and Fujimoto, Y. (1994) *J. Chem. Soc., Chem. Commun.* 1761.
16. Lafont, R. and Wilson, I. D. (1992) *The Ecdysone Handbook*. The Chromatographic Society Press, London.
17. Calcagno, M. P. (1995) Ph. D. Thesis. Universitat de Barcelona, Spain.