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PHYTOECDYSTEROID OVERPRODUCTION IN *POLYPODIUM VULGARE* PROTHALLI

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Key Word Index—*Polypodium vulgare*; Polypodiaceae; fern; phytoecdysteroids; 20-hydroxyecdysone; *in vitro* cultures; water-stress; temperature-stress.

Abstract—*Polypodium vulgare* prothalli, from *in vitro* cultures, showed an increase of phytoecdysteroid production when immersed in water. The degree of overproduction depended on water temperature and time used for the treatment. Although the presence of water at 25° caused an increase of phytoecdysteroid production, the highest production was detected at 45°. At higher water temperature a decrease of ecdysteroid production was observed as well as tissue necrosis, when prothalli were transferred to culture medium. A temperature increase in the absence of water did not change the phytoecdysteroid production. Using a labelled precursor we proved that *de novo* biosynthesis of ecdysteroids occurred when *P. vulgare* prothalli were immersed in water at 45°. The presence of water at 45° for 10 h increased 15 and 23 times the total production of ecdysteroids, depending if prothalli were maintained in the same water vial or changed three times during the experiment, respectively. At room temperature 20% of ecdysteroid total production was excreted into the water, whereas at 45° this amount increased to more than 97%. These results suggest that diffusion of ecdysteroids into water may occur, originating a dilution of ecdysteroid content in prothalli and an increase of ecdysteroid biosynthesis in an attempt to restore its normal intracellular levels. Different factors that may contribute to this observed ecdysteroid overproduction are discussed. © 1997 Elsevier Science Ltd

INTRODUCTION

Phytoecdysteroids are a family of polyhydroxylated steroids produced in many plant species, analogues of 20-hydroxyecdysone (20E), the insect moulting hormone. These compounds have been tested in different insect bioassays, showing moulting hormone activity as well as effects in regeneration, metamorphosis, reproduction and tissue differentiation [1–4]. Ecdysteroids also exhibit interesting activities in mammals, including diuretic, tonic, hypocholesteremic or insulin regulation effects [5, 6].

In vitro plant tissue culture is a widely used technique for the production of secondary metabolites. In our laboratory, *in vitro* root cultures of *Ajuga reptans* in a hormone supplemented solid medium were established and the phytoecdysteroid production found was higher than 0.3% of the dry weight [7]. Likewise, we isolated very high amounts of ecdysteroids from *in vitro* cultures of *Polypodium vulgare* prothalli (0.7% dry wt). Among these ecdysteroids 20E was the most abundant one and the other minor components were: abutasterone (20,24E), polypodine B (5,20E), inokosterone (25d20,26E), 24-hydroxyecdysone (24E), pterosterone (25d20,24E) and ecdysone (E) [8–10]. We have recently reported that *P. vulgare* prothalli cultured in the presence of tritium labelled ecdysone incorporated ^{3}H to all 25-hydroxyecdysteroids present in this tissue, with 20E the main labelled ecdysteroid detected [9].

Different approaches can be used to improve secondary metabolite production in plant tissue cultures, such as variation of culture media, temperature, illumination, use of elicitors, etc [11]. In this context, a three-fold increase of phytoecdysteroid production has been achieved by Matsumato and Tanaka [12] using *in vitro* culture in liquid medium of hairy root of *A. reptans* transformed with *Agrobacterium rhizogenes*. Moreover, product excretion to the medium can be induced by changing pH or components of the liquid medium. As example, Macek and Vanek [13] reported that half of the ecdysteroid production in *Pteridium aquilinum* cells (0.05% dry wt) was excreted to the medium, after 8 weeks of culture.

In the present paper we report on the induction of

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phytoecdysteroid overproduction and the increase of product release into the medium in *P. vulgare* prothalli immersed in water. We observed that both phenomena were water temperature and time dependent.

RESULTS AND DISCUSSION

Previous results obtained in our laboratory showed that *in vitro* cultures in *P. vulgare* prothalli in liquid media released only 3% of its ecdysteroid production into the medium. As mentioned above, different strategies may be used to induce metabolite overproduction in plant tissue cultures and one of them was raising the temperature. Consequently, a study of the influence of water temperature and time on phytoecdysteroid production in *P. vulgare* prothalli was carried out. *P. vulgare* prothalli (line M-6. see Experimental Part) were submerged for 15, 30 or 60 min in water maintained at room temperature (25), 45, 50 or 60°. Since our interest was focused on the ecdysteroid production and on prothalli survival after these treatments, at the end of each experiment a prothalli fragment was transferred into solid medium and cultured under standard conditions, and the remaining portion was lyophilised. Likewise, phytoecdysteroids released into water and retained in the prothalli were extracted and separately quantified.

A proportional increase in total production of all detected ecdysteroids was generally observed (Fig. 1). However, only the production of 20E has been extensively considered throughout the present communication.

As shown in Fig. 2, when the temperature treatment lasted only 15 min no significant induction effect was observed, regardless of what temperature was used. However, after 30 min a clear increase on phytoecdysteroid production was observed at all tested temperatures. The highest production was detected when prothalli were immersed in water at 45° for 60 min; however at higher temperatures a slight total production decrease occurred. Likewise, after 15 min the relative ecdysteroid amount transferred in water

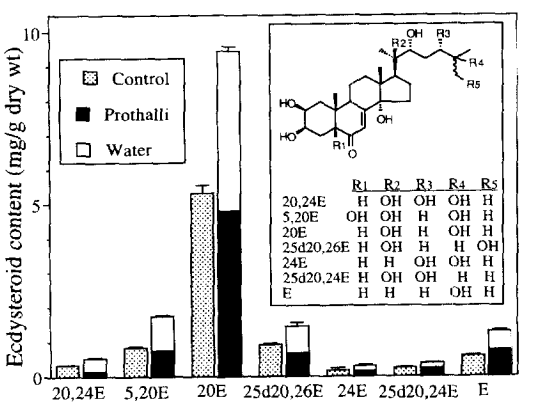

Fig. 1. Ecdysteroid production by *P. vulgare* prothalli (line M-6) immersed in water at 45° during 60 min. Control: Lyophilised prothalli extracted with methanol.

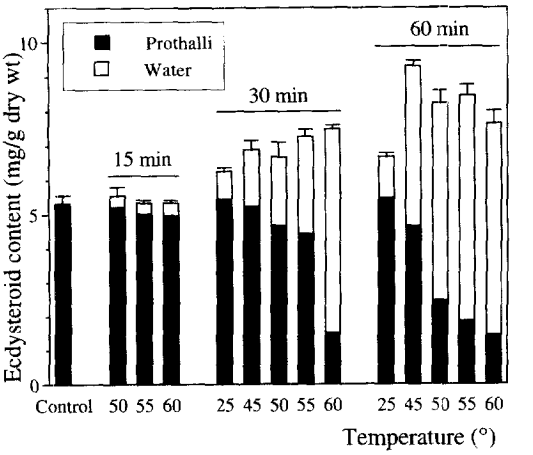

Fig. 2. Ecdysteroid production by *P. vulgare* prothalli (line M-6) immersed in water at different temperatures during 15, 30 or 60 min. Control: Lyophilised prothalli extracted with methanol.

was the same (6%) at the three tested temperatures, but under other assayed conditions it increased with water temperature and treatment duration, independently from the total amount of ecdysteroid production. In conclusion, a minimum of 30 min seems to be necessary to induce ecdysteroid overproduction and maximum production was obtained maintaining prothalli in water at 45° for 60 min.

On the other hand, prothalli immersed in water at 50°, or higher temperatures, for more than 30 min presented an irregular development and tissue necrosis when transferred into culture medium, indicating that *P. vulgare* prothalli submitted to extreme conditions did not survive. Finally, the fact that no differences in total ecdysteroid production were observed when lyophilised prothalli were submerged for 1 hr in water at 50° or 60° (control: 5.27 ± 0.13; treated: 5.61 ± 0.67 mg ecdysteroid/g^{-1} dry wt), and that maximum ecdysteroid overproduction was achieved with prothalli immersed in water at 45°, which remained alive, strongly suggest that biosynthesis of ecdysteroids was induced during this treatment.

To support this hypothesis, [23,24-^{3}H]-ecdysone was topically applied to *P. vulgare* prothalli (line M-27) and they were immersed in water at 45° for 1 hr. A 3.8-fold increase of ecdysteroid production was observed after this treatment (control: 0.89 ± 0.19, treated: 3.37 ± 0.99 mg ecdysteroid g^{-1} dry wt). On the other hand, the results on bioconversion of [23,24-^{3}H]-ecdysone into labelled 20E in control samples (Table 1) agreed with our previously reported data [9]. As shown, no significant differences in labelled 20E formation were observed between treated samples, which may indicate that the relative amount of labelled ecdysone that was absorbed by the tissue and was available to the enzymatic systems was similar in all cases, with the exception of 6 hr. This last point suggests that the applied precursor needed more than 6 hr to be incorporated in the biosynthetic pathway.

Table 1. Bioconversion of [23,24-^{3}H]-ecdysone into labelled 20E in *P. vulgare* prothalli after temperature treatment

Time (days)*	% of bioconverted [23,24-^{3}H]-E (mean ± sd) Control	Temperature treatment†
0.25	nd‡	nd
0.75	2.5 ± 0.02	34.4 ± 18.7§
1	3.0 ± 0.02	27.6 ± 10.6§
2	9.8 ± 1.2	48.7 ± 5.8§
5	25.4 ± 5.1	50.3 ± 9.4§

* Time period in which *P. vulgare* prothalli (line M-27) were cultured in the presence of labelled ecdysone, before temperature treatment.

† Prothalli were immersed in water at 45° for 1 hr.

‡ Not detected.

§ No significant differences between them (Student *t*-test, $p = 0.05$).

However, the main point to be considered from these results is that a higher formation of labelled 20E was observed in prothalli immersed in water at 45° than in control, and suggested that during the treatment an induction of ecdysteroid biosynthesis occurred.

The observed induction of ecdysteroid biosynthesis may be caused by one or several of the following factors: temperature or osmotic stress, dilution of ecdysteroids in water and *de novo* biosynthesis to restore the normal intracellular levels in prothalli, etc. To know which of the above factors contributed to the induction of ecdysteroid production and for how long this induction was effective, *P. vulgare* prothalli (line M-27) were immersed in water up to 10 hr at 25° and 45° (see Experimental Part). Results on ecdysteroid prothalli content, ecdysteroid released in water and ecdysteroid production (ecdysteroid content in the prothalli plus ecdysteroid released in water) are summarised in Table 2. When *P. vulgare* prothalli were maintained for 1, 2, 5 or 10 hr in the absence of water (Sets 1 and 4) prothalli ecdysteroid contents were similar to those of control regardless of what temperature was tested, indicating that only a temperature rise does not explain the observed induction.

The presence of water at room temperature for 1 hr up to 10 hr increased somewhat the ecdysteroid production in the same degree (Set 5). However, if prothalli were transferred into a new water vial during the experiment a progressive increase in ecdysteroid production was observed (Set 6). This increase was mainly caused by the prothalli biosynthetic capacity to maintain its normal intracellular ecdysteroid levels to balance the released ecdysteroid amounts (equivalent to 90% of normal prothalli content) in each vial change carried out.

On the other hand, an increase of prothalli ecdysteroid content was observed after water treatment at 45° for 1 hr (Sets 2 and 3). However, during the second hour this content decreased below control level and this tendency was followed until the end of the experiment, especially in prothalli that were transferred from time to time into different water vials (4% of control value after 10 hr, Set 3). The ecdysteroid amounts released in water increased all along the experiments causing an increase of ecdysteroid production, although after 2 hr a reduction of the ecdysteroid production rate was observed, a fact that occurred in the same time period when the prothalli content starts to be lower than that in control. As shown, the highest overall ecdysteroid production was achieved when prothalli were transferred into new water vials in the course of the experiment (Set 3), albeit ecdysteroid contents in prothalli were lower in samples submitted to vial changes than in those unchanged.

In summary, only a temperature variation in absence of water did not modify the phytoecdysteroid content (Table 3, entry 1). At room temperature the presence of water during 10 hr increased 1.9 and 4.1 fold the total production of ecdysteroids, depending if prothalli were maintained into the same vial (entry 2) or not (entry 3) during the experiment, indicating that the immersion of prothalli into a hypotonic medium could contribute to the observed induction. Remarkably, if water was kept at 45° the ecdysteroid overproduction achieved was 15.2 fold higher (entry 2) or 23.1 fold higher than controls when prothalli were submitted to the vial changes mentioned above (entry 3). These results suggest that diffusion of ecdysteroids in water occurred, originating a diminution of ecdysteroid content in prothalli. This fact may produce an increase of ecdysteroid biosynthesis in prothalli in an attempt to restore its normal intracellular levels. Different factors may contribute to the ecdysteroid overproduction observed. The special morphology of prothalli that allows an easy exchange between its components and the aqueous medium; the high solubility of ecdysteroids in water, which increases with temperature; the prothalli viability at quite high temperature (45°); the potential biosynthetic capacity of prothalli that, in the absence of nutrients, are able to produce ecdysteroids up to 2.3% of dry weight. All these considerations make *P. vulgare* prothalli a potentially useful material to continue ecdysteroid research in plants with a promising interest for the production and study of other plant hydrophilic secondary metabolites.

EXPERIMENTAL

In vitro *cultures*. *Polypodium vulgare* prothalli production was carried out as previously described [8] in 1/2 MS culture medium supplemented with sucrose (30 gl^{-1}) and agar (8 gl^{-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. Two different culture lines, M-6 and M-27, were used in the present work. M-6 had high ecdysteroid production (0.7% dry wt) whereas M-27 exhibited a faster

Table 2. Ecdysteroid production by *P. vulgare* prothalli (line M-27) submitted to different treatments

Set	Temperature	Immersion in water	Vial change	Time (hr)	Ecdysteroid content (mg g^{-1} dry wt. mean ± sd) Prothalli	Released	Total
1	45	no	no	1	1.16 ± 0.09†		1.16 ± 0.09
				2	1.17 ± 0.02†		1.17 ± 0.02
				5	1.22 ± 0.02†		1.22 ± 0.02
				10	1.02 ± 0.19†		1.02 ± 0.19
2		yes	no	1	1.64 ± 0.36	3.71 ± 1.39	5.34 ± 0.86
				2	0.79 ± 0.01†	7.79 ± 0.24	8.58 ± 0.20
				5	0.59 ± 0.11†	12.04 ± 0.55	12.54 ± 0.50
				10	0.46 ± 0.10†	14.28 ± 0.85	14.73 ± 0.74
3		yes	yes	1	1.64 ± 0.36	3.71 ± 1.39	5.34 ± 0.86
				2	0.70 ± 0.07†	11.83 ± 0.81	12.33 ± 0.44
				5	0.16 ± 0.01	18.82 ± 0.59	19.11 ± 0.64
				10	0.04 ± 0.01	22.30 ± 1.83	22.36 ± 1.51
4	25°	no	no	2	1.27 ± 0.20†		1.27 ± 0.20
				5	1.31 ± 0.13†		1.31 ± 0.13
				10	1.12		1.12
5		yes	no	1	1.33 ± 0.06†	0.35 ± 0.07	1.69 ± 0.01
				2	1.28 ± 0.01†	0.28 ± 0.04	1.56 ± 0.04
				5	1.26 ± 0.26†	0.29 ± 0.10	1.55 ± 0.25
				10	1.44 ± 0.12†	0.41 ± 0.12	1.85 ± 0.01
6		yes	yes	1	1.33 ± 0.06†	0.35 ± 0.07	1.69 ± 0.01
				2	1.34 ± 0.04†	1.17 ± 0.01	2.51 ± 0.02
				5	1.05 ± 0.39†	2.09 ± 0.46	3.14 ± 0.60
				10	0.94 ± 0.52†	2.98 ± 0.53	3.93 ± 0.75
Control*					0.97 ± 0.36		0.97 ± 0.36

* Lyophilised prothalli (line M-27) extracted with methanol.
† No significant differences respect to control (Student *t*-test, $p = 0.05$).

growth with a lower ecdysteroid production (0.1% dry wt).

Chemicals. Ecdysteroid standards were obtained in our laboratory from extracts of *P. vulgare* prothalli [14]. Water for prothalli treatments was purified with a Milli-Q system and filtered through 0.45 μm filters.

Water treatment. Prothalli clusters (line M-6, around 500 mg fr. wt) were immersed into H_2O (5 ml) at different temps (25, 45, 50, 55 or 60°) during different times (15, 30 or 60 min). Once the assay was finished prothalli were lyophilised, extracted with MeOH (4 × 5 ml) and ecdysteroids were purified and quantified by HPLC as previously described [9]. H_2O samples, containing ecdysteroids, were analysed by HPLC without purification. Prothalli clusters that have been lyophilised and extracted with MeOH without previous treatment are referred to as control in the text. All experiments were performed at least in triplicate.

Prothalli clusters (line M-27, around 500 mg fr. wt) were immersed in water (5 ml) at 45° during 1, 2, 5 or 10 hr (Table 2, Set 2), whereas in another experimental set, prothalli were transferred into a new vial with 5 ml of water at 45° at different times (Set 3): when treatment lasted for 2 hr, prothalli were transferred into a new H_2O vial after 1 hr; when treatment lasted for 5 hr, prothalli were transferred after 1 and 2 hr; and when treatment lasted for 10 hr, prothalli were transferred after 1, 2 and 5 hr. Five different groups of reference samples were used: two experimental sets similar to those described above but performed with H_2O at 25° (Sets 5 and 6, respectively), prothalli kept at room temp without H_2O (Set 4), prothalli maintained at 45° in a vial satd with H_2O, but without being in contact with it (Set 1), and finally lyophilised prothalli extracted with MeOH, as control. All exper-

Table 3. Relative ecdysteroid production in *P. vulgare* prothalli (line M-27) submitted for 10 hr at the different treatments mentioned in Table 2, referred to ecdysteroid content in control *P. vulgare* prothalli

Entry	Immersion in water	Vial change	Temperature 25°	45°
1	no	no	1.1	1.2
2	yes	no	1.9	15.2
3	yes	yes	4.1	23.1

imental sets were performed in triplicate. Once the assay was finished prothalli were lyophilised, extracted with MeOH (4 × 5 ml) and ecdysteroids were purified and quantified by HPLC as previously described [9]. H_2O samples, containing ecdysteroids, were analysed by HPLC without purification. The released ecdysteroid amounts in experimental sets 3 and 6 were calculated by the addition of released ecdysteroids into H_2O of each vial change.

Incorporation of [23,24-^3H]-*ecdysone*. Small prothalli clusters (line M-27, around 140 mg fr. wt) were cultured in Petri dishes in the above medium. After one week, an aq. soln of [23,24-^{3}H]-ecdysone (0.1 μCi) was topically applied to each cluster. After incubation for different time periods, prothalli were immersed in H_2O (5 ml) at 45° for 1 hr. The ecdysteroid amounts released into H_2O and retained in prothalli were quantified by HPLC with an on-line radioactivity monitor for the detection of labelled ecdysteroids.

HPLC. Analyses were monitored at 242 nm with a diode array detector and recorded and quantified with a 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 *iso*-PrOH–H_2O (7:93) for 10 min followed by a linear gradient (over 10 min) from 0 to 20% of MeCN in *iso*-PrOH–H_2O (7:93) at 1.2 ml min^{-1} [9].

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