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YEAST EXTRACT INDUCED ACCUMULATION OF BENZOFURANS IN CELL SUSPENSION CULTURES OF *AGERATINA ADENOPHORA*

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Key Word Index—*Ageratina adenophora*; Asteraceae; benzofurans; cell suspension culture; yeast extract.

Abstract—Cell suspension cultures of *A. adenophora* accumulate benzofurans not present in differentiated plants of this species. The amounts of benzofurans accumulating can be increased three- to four-fold by addition of yeast extract to the cell suspension culture. The major portion of the benzofurans synthesized following addition of yeast extract is found in the liquid growth media. The highest induction of benzofuran accumulation is achieved upon addition of yeast extract at the exponential growth phase. The accumulation of benzofurans in response to yeast extract results in maximal accumulation 24 hr after addition of yeast extract followed by a gradual decline. The induction of increased benzofuran accumulation can be repeated by a second dose of yeast extract. The accumulation of benzofurans following application of yeast extract is due to a *de novo* synthesis as suggested by PAL-inhibitor experiments and by feeding of [^{14}C]phenylalanine.

INTRODUCTION

2,2-Dimethylchromenes and 2-isopropenylbenzofurans are characteristic metabolites of many species of the Asteraceae, primarily from the tribes Astereae, Eupatorieae, Heliantheae, Inuleae and Senecioneae [1, 2]. Recently we demonstrated [3] that chromenes and benzofurans are also accumulated by cell suspension cultures of *Ageratina adenophora* (Spreng.) K. & R. (Asteraceae, tribe Eupatorieae) that is a widely adventive weed in many tropical and subtropical parts of the world [4]. Seedlings of *A. adenophora* elaborate only chromenes but no benzofurans [5]. An increased accumulation of benzofurans in cell suspension cultures of *A. adenophora* could be achieved by adding the chromene encecalin that is present only in seedlings [3]. Furthermore preliminary experiments suggested that yeast extract was effective in inducing an increased accumulation of benzofurans [3]. We have now studied in detail the induction of benzofuran accumulation in cell suspension cultures of *A. adenophora* following addition of yeast extract and results obtained indicating a *de novo* biosynthesis of the benzofurans.

RESULTS AND DISCUSSION

The cell suspension culture of *A. adenophora* was in continuous culture for one year at the beginning of the experiments. For optimum growth of the culture, light (16 hr white light daily) was found necessary (Fig. 1). Cultures grown under this light/dark regime reached stationary growth phase *ca* 12 days after inoculation of the cells into fresh medium. When the cultures were kept in continuous dark the growth was slow within the first 10 days. The stationary growth phase was reached at *ca* 17 days and the total increase of biomass was always smaller than in the cultures grown under the light/dark regime.

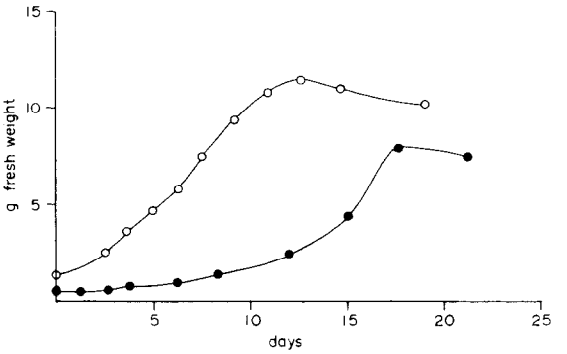

Fig. 1. Growth curve of cell suspension culture of *Ageratina adenophora*. ○ Grown under light/dark rhythm, ● Grown under continuous dark.

The cell suspension cultures accumulated several chromene and benzofuran derivatives including compounds **1** and **2**. The structure elucidation of the two benzofurans has been described [3]. The benzofurans **1** and **2** proved difficult to resolve either by HPLC or by TLC. Mass spectrometric analysis of the mixture of both benzofurans indicated that the ratio of **1**:**2** was 1:4. As HPLC was chosen as the method for quantification of benzofurans in cell extracts and growth media due to the sensitive UV-detection no clear distinction in the accumulation of the two individual compounds could be achieved and they were therefore quantified jointly as benzofurans **1**/**2** based on the absorbance of **2**. The methodical error thus introduced in the quantification of the absolute amounts of both benzofurans accumulated was estimated as 10–20%. The highest concentrations of both benzofurans (in the range of 0.05–0.07 μmol/g fresh weight) usually accumulated within the first four days

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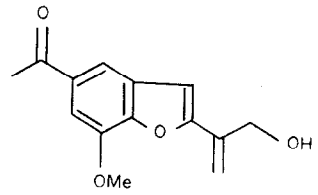

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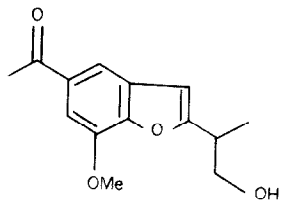

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following inoculation of the cells into fresh medium. A transient stimulation of natural product accumulation following inoculation of cells into fresh medium has also been described for other cell suspension cultures [6]. During subsequent growth of the cell cultures of *A. adenophora* the concentrations of the two benzofurans declined to 0.01 μmol/g fresh weight. These concentrations were two orders of magnitude smaller than those of structurally closely related chromenes found in seedlings of *A. adenophora* [5].

A clear increase in the concentration of benzofurans **1**/**2** could be achieved by addition of yeast extract to the cell suspension cultures. Yeast extract was previously shown to induce an increased natural product accumulation in cell suspension cultures of several plant species [7–11]. The increase of benzofurans in cell suspension cultures of *A. adenophora* was dependant on the growth stage of the cell culture as well as on the dose of yeast extract applied. The influence of growth stage on the benzofuran accumulation following addition of yeast extract is shown in Table 1. For this experiment a dose of 2 g of yeast extract/l growth medium was applied to cell suspension cultures after 3, 6 and 12 days growth following inoculation of the cells into fresh medium. Benzofuran accumulation at each growth stage was monitored at 6, 24 and 48 hr following addition of yeast extract. Induction of benzofuran accumulation relative to controls was observed when yeast extract was added at the lag phase and growth phase whereas no induction occurred at the stationary phase. The largest increase of benzofurans relative to controls was achieved upon addition of yeast extract during the growth phase resulting in an approximate three-fold increase of benzofurans (Table 1). The accumulation maxima of benzofurans **1**/**2** were usually found 24 hr after addition of yeast extract to the cell culture followed by a gradual decline at 48 hr.

The major part of the benzofurans accumulating upon addition of yeast extract was usually found in the liquid growth media (*ca* 50–70% of the total benzofurans). The decrease of benzofurans at 48 hr following addition of yeast extract compared to 24 hr was also most striking in the growth media. This decrease was also observed when growth media of cell suspension cultures that had received yeast extract 24 hr previously were separated from the cells and incubated for another 24 or 48 hr suggesting that the decrease of benzofurans was due to degradation (probably peroxidative destruction) by enzymes present in the growth media as frequently encountered with cell suspension cultures [12].

The induction of benzofuran accumulation in response to increasing doses of yeast extract was that of an optimum curve showing maximum induction at 6 g yeast extract/l growth medium (Table 2). Concentrations of yeast extract higher than 6 g/l resulted in comparison in lower benzofuran concentrations and in a brown coloration of the cells and liquid growth media. The concentrations of yeast extract required in inducing an increased benzofuran accumulation in cell suspension cultures of *A. adenophora* are comparable to those reported for example for the induction of isoflavone and pterocarpan accumulation in cell suspension cultures of *Cicer arietinum* (2.5 g/l) [7] or for the induction of chalcone derivatives in cell suspension cultures of *Glycyrrhiza echinata* (lg/l) [8].

The induction of benzofuran accumulation by yeast extract could be repeated when cell suspension cultures that had received a first dose of yeast extract at six days growth were treated with a second dose after nine days growth. When yeast extract (2 g/l) was added to cell suspension cultures of *A. adenophora* at six days growth the induced accumulation of the benzofurans was *ca* two-fold higher in this experiment than in the controls (Table 3). At nine days growth a second dose of yeast extract (2 g/l) was added to these cultures (at this time the

Table 1. Induction of benzofuran (**1**/**2**) accumulation by yeast extract in cell suspension cultures of *Ageratina adenophora**

Time of addition of yeast extract (day following inoculation of cells into fresh medium)		Accumulation of benzofurans **1**/**2** in cells and growth media at 6 hr	24 hr	48 hr following addition of yeast extract
		μmol/g	μmol/g	μmol/g (given as $\bar{x} \pm$ S.D.)
3 day	+yeast extract	0.038 + 0.005	0.117 + 0.030	0.019 + 0.007
	−yeast extract	0.040 + 0.010	0.079 + 0.011	0.024 + 0.010
6 day	+yeast extract	0.047 + 0.007	0.120 + 0.017	0.040 + 0.009
	−yeast extract	0.044 + 0.010	0.038 + 0.010	0.024 + 0.006
12 day	+yeast extract	0.028 + 0.009	0.047 + 0.005	0.014 + 0.006
	−yeast extract	0.031 + 0.008	0.048 + 0.009	0.024 + 0.005

The dose of yeast extract applied was 2 g/l. μmol/g are given on a fresh weight basis; $n = 10$.

Table 2. Dose-response of benzofuran (**1/2**) accumulation for increasing amounts of yeast extract applied to cell suspension cultures of *Ageratina adenophora*

Cell suspension cultures	Benzofuran (**1/2**) accumulation in cells and growth media 24 hr following application of yeast extract μmol/g (given as $\bar{x} \pm$ S.D.)
control	0.045 + 0.020
+ yeast extract (2 g/l.)	0.121 + 0.020
+ yeast extract (4 g/l.)	0.121 + 0.015
+ yeast extract (6 g/l.)	0.173 + 0.019
+ yeast extract (8 g/l.)	0.102 + 0.019
+ yeast extract (10 g/l.)	0.084 + 0.010

*μmol/g are given on a fresh weight basis. Yeast extract was applied at day six. $n = 5$.

Table 3. Accumulation of benzofurans (**1/2**) by cell suspension cultures of *A. adenophora* following single and repeated addition of yeast extract*

Time of addition of yeast extract (days following inoculation of cells into fresh medium)	Accumulation of benzofurans **1/2** in cells and growth media 24 hr following addition of yeast extract μmol/g (given as $\bar{x} \pm$ S.D.)	
6 day	+ yeast extract	0.105 + 0.010
	− yeast extract	0.050 + 0.015
6 days + 9 days	+ yeast extract	0.040 + 0.005
	− yeast extract	0.025 + 0.004
9 days	+ yeast extract	0.042 + 0.001
	− yeast extract	0.025 + 0.004

*The dose of yeast extract applied was 2 g/l. μmol/g are given on a fresh weight basis; $n = 5$. Cell suspension cultures were transferred to the dark on day five following inoculation of the cells into fresh medium.

concentration of benzofurans in the cultures having received the first dose of yeast extract at six days growth had already substantially decreased and was not distinguishable from that of the controls) as well as to cell suspension cultures that received yeast extract for the first time at this growth stage. Both treatments (addition of yeast extract at 6 and 9 days or at 9 days only) resulted in a comparable induction of benzofuran accumulation at the later growth stage that was *ca* 60% higher than in the controls.

Employing root cultures of *Eupatorium cannabinum* we recently demonstrated that the aromatic ring and the acetyl function of benzofurans (C_6C_2 part of the molecule) originate biogenetically from phenylalanine and cinnamic acid [13]. Amino-oxyacetic acid (AOA) is known as a competitive inhibitor of the phenylalanine-ammonia-lyase (PAL) [14]. Joint application of yeast extract (2 g/l) and of AOA (0.125 mM) to cell suspension cultures of *A. adenophora* at six days growth resulted in a drastically reduced accumulation of benzofurans **1/2** when compared to cell cultures that had received yeast extract only (Table 4) indicating that yeast extract probably caused a stimulated benzofuran accumulation by an increased enzymatic activity of the PAL. This was corroborated by feeding L-[U-^{14}C] phenylalanine (37 kBq, 0.5 mM) in addition to yeast extract (6 g/l) to cell suspension cultures of *A. adenophora* at six days growth. Controls received only [^{14}C]phenylalanine (same concentration as above). The incorporation of [^{14}C] phenylalanine into the benzofurans **1/2** was calculated as 0.23% (relative to the applied dose of [^{14}C]phenylalanine) for those cell cultures that had been treated with the precursor and with yeast extract whereas no measurable incorporation into the benzofurans was found in the cell cultures treated with [^{14}C]phenylalanine only.

Table 4. Suppression of benzofuran (**1/2**) accumulation by joint application of aminooxyacetic acid (AOA) and yeast extract to cell suspension cultures of *A. adenophora**

Cell suspension cultures	Benzofuran (**1/2**) accumulation in cells and growth media 24 hr following application of yeast extract μmol/g (given as $\bar{x} \pm$ S.D.)
− yeast extract − AOA	0.035 + 0.010
+ yeast extract − AOA	0.156 + 0.021
− yeast extract + AOA (0.125 mM)	0.052 + 0.001
+ yeast extract + AOA (0.125 mM)	0.055 + 0.003

*The dose of yeast extract was 2 g/l. μmol/g are given on a fresh weight basis; $n = 10$. Yeast extract was applied at day six.

The experiments with the PAL-inhibitor AOA and [^{14}C]phenylalanine suggest that the increased accumulation of benzofurans **1/2** caused by addition of yeast extract to cell suspension cultures of *A. adenophora* is due to a *de novo* synthesis of the benzofurans. Cell suspension cultures of *A. adenophora* therefore respond to the addition of yeast extract with an increased accumulation of constitutively formed natural products. Similar behavior has also been observed for example with cell suspension cultures of *Glycyrrhiza echinata* (increase of retrochalcone accumulation) [8] or of *Thalictrum rugosum* (increase of berberine accumulation) [9]. At present the benzofurans **1/2** have to be regarded as cell suspension culture metabolites only, as seedlings of *A. adenophora* accumulate exclusively chromenes and no benzofurans even when subjected to 'stress conditions' such as mechanical injury and/or treatment with yeast extract.

EXPERIMENTAL

The cell suspension culture of *A. adenophora* (established from young sterile seedlings grown from achenes) was grown according to ref. [13] except that the concentrations of 2,4-D and kinetin were changed to 0.033 and 0.25 mg/l, respectively. The

culture was maintained in the light (16 hr white light daily, 9 000 lux) at 25° with shaking at 120 rpm. Prior to the start of the experiments the culture was kept *in vitro* for one year.

In order to avoid formation of light-induced artefacts of the benzofurans the cell suspension cultures were transferred to the dark 24 hr prior to the addition of yeast extract. Incubation with yeast extract was also in the dark. The experiments were repeated 5–10 times. Averaged data ($x \pm S.D.$) are reported. HPLC was used for quantification of the benzofurans. The HPLC system employed was the same as described in ref. [3]. The separation column (125 × 4.6 mm) was prepacked with Nucleosil 5 C8 (5 μm). The analyses were run isocratically with a mixture of MeCN–H_2O (7:13) containing 1% H_3PO_4. Quantification was by the external standard method based on the UV-absorption of **2**. The flow was at 1 ml/min and detection was at 254 nm. TLC was carried out with silica gel plates with CH_2Cl_2–MeOH (49:1) as solvent system. Detection was under UV_{254} nm.

L-[U-^{14}C]Phenylalanine was purchased from Amersham Buchler (Braunschweig, F.R.G.). Aminooxyacetic acid was from Sigma (Deisenhofen, F.R.G).

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