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Production of valepotriates by hairy root cultures of *Centranthus ruber* DC

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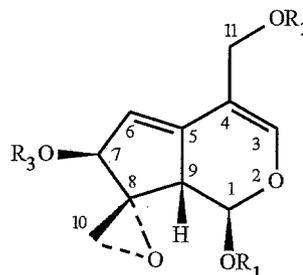
Abstract. Hairy root cultures of *Centranthus ruber* DC. were established by infection of sterile plantlets with *Agrobacterium rhizogenes*, strain R1601. The transformed roots were grown in 12 different, hormone-free liquid media, and valtrate, isovaltrate, 7-desisovaleroyl-7-acetylvaltrate, 7-homovaltrate, didrovaltrate and isovaleroxyhydroxydidrovaltrate were quantified by high performance liquid chromatography. The highest overall valepotriate content (3.0% dry wt) was observed in half-strength Gamborg B5 medium supplemented with 3% sucrose. This concentration is very similar to that found in the roots of parent plants grown in the field. The use of N,N-dimethylmorpholinium iodide, a plant bioregulator, was very detrimental to the hairy root growth and to the valepotriate production. The hairy roots cultured in half strength Gamborg B5 liquid medium supplemented with 3% sucrose for 45 days produced over 31 mg/g dry wt valepotriates.

Abbreviations

MS = Murashige and Skoog medium (Murashige and Skoog 1962); B5 = Gamborg B5 medium (Gamborg 1970); WP = McCown's woody plant medium (Lloyd and McCown 1980); H = Heller's medium (Heller 1953); 1/4 B5-7 = quarter strength B5 + 7% sucrose; DMI = N,N-dimethylmorpholinium iodide; VAL = valtrate; IVAL = isovaltrate; DIA-VAL = 7-desisovaleroyl-7-acetylvaltrate; HVAL = 7-homovaltrate; DI = didrovaltrate; IVHD = isovaleroxyhydroxydidrovaltrate (Fig. 1); NMR = nuclear magnetic resonance spectroscopy; HPLC = high performance liquid chromatography.

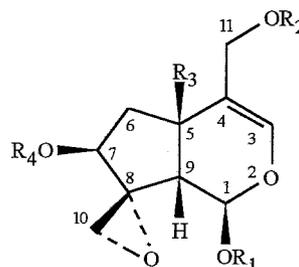
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Diene valepotriates



	R ₁	R ₂	R ₃
VAL	IV	Ac	IV
IVAL	IV	IV	Ac
HVAL	IV	Ac	MV
DIA-VAL	IV	Ac	Ac

Monoene valepotriates



	R ₁	R ₂	R ₃	R ₄
DI	IV	IV	H	Ac
IVHD	IV	IV-IV	OH	Ac

Abbreviations: Ac = acetyl; IV = isovaleryl;

IV-IV = α -isovaleryloxy-isovaleryl; MV = β -methyl-valeryl.

Fig. 1. Valepotriate structures

Introduction

Centranthus ruber DC. (*Valerianaceae*) possesses sedative properties reported for the first time by Paris and Moury (1963). This plant is well known to produce valepotriates (Thies 1968; Handjieva and Zaikin 1978) and the sedative activity of valepotriates have been shown in animal tests (Von Eickstedt and Rahman 1969; Wagner et al. 1980; Hölzl and Fink 1984). In addition, it was demonstrated (Wagner and Jurcic 1979; Hazelhoff et al. 1982) that these compounds have also spasmolytic properties. Recently, we reported on the production of valepotriates by hairy root cultures of *Valeriana officinalis* L. var. *sambucifolia* Mikan (Gränicher et al. 1992) and the encouraging results prompted us to continue our investigations on the species *Centranthus ruber*, another *Valerianaceae* producing valepotriates. Callus and root cultures of this species had been investigated by Becker and Schroll (1980) and Violon et al. (1983, 1984). In particular, Violon et al. (1983) reported that the valepotriate content was 16 times higher in the root culture of *C. ruber* (4.2% dry wt) than in a callus culture of the same species (0.25% dry wt). This result was confirmed by a more detailed study (Violon et al. 1984) in which it was demonstrated that *in vitro* valepotriate production was closely related to root differentiation. However there have been no reports on the establishment of *Agrobacterium*-mediated transformed roots. In this paper we report on the valepotriate production by hairy root cultures of *Centranthus ruber* DC.

Materials and methods

Bacterial strain. *Agrobacterium rhizogenes* strain R1601 (Pythoud et al. 1987) was used in the present study as described by Gränicher et al. (1992).

Plant material. Seeds of *Centranthus ruber* DC. obtained from Jelitto Staudensamen (Schwarmstedt, Germ.), were surface sterilized (15 min) in a 2% sodium hypochlorite solution supplemented with 2% v/v Triton X-100 (Fluka, Switzerland), washed three times (20 min) with sterile distilled water and left for germination on sterile wet filter paper at 25°C in the light (Osram-L-Fluora 77R; 700 lux). Under these conditions, the seeds started to germinate after 10 days. Two-week-old plantlets were again surface sterilized (1 min), washed three times (10 min) with sterile distilled water and transferred on solid MS-2 medium. They were grown for 6 weeks at 25°C in the light. Plants originating from the same batch of seeds and grown in the field under normal conditions were identified by comparison with the authentic herbarium specimens of the Conservatoire et Jardin Botaniques in Geneva.

Establishment of hairy root cultures. Stems of aseptically grown plantlets were wounded and infected with *A. rhizogenes*. Five to six weeks after infection, approximately 90% of the infected sites produced hairy roots, which were excised, transferred on MS-2 solid medium containing 0.25 g/l cefotaxime and 1 g/l ampicillin (Sigma, U.S.A.) and

subcultured every 10 days. After elimination of the bacteria, the hairy roots were cultured on B5-3 liquid medium and subcultured at four week intervals. To show the transformation, the opines specifically produced by crown gall and hairy root tissues (White et al. 1982; Clare 1990), were extracted and identified by paper electrophoresis according to the method of Petit et al. (1983). For culture experiments and time-course study, about 50 mg (fresh weight) of the hairy roots were inoculated into 50 ml liquid medium in 250 ml-conical flasks. All cultures were maintained in darkness at 25°C on a gyratory shaker at 80 rpm. All media were hormone-free and adjusted to pH 5.9 before autoclaving.

Preparation and addition of DMI. DMI was prepared by refluxing N-methylmorpholine with an excess of methyl iodide in methanol as described by Schölly (1989) and recrystallized in methanol. The purity was checked by NMR and mass spectrometry. According to the selected concentration, 0.01 - 1.0 ml of a 0.25% w/v sterile aqueous solution of DMI were added aseptically to sterile 1/2 B5-3 liquid media.

Extraction of valepotriates. The content of three flasks of each culture was harvested, and the fresh weight and dry weight, after lyophilization, were determined individually. The hairy roots were powdered and extracted as described previously (Gränicher et al. 1992). Liquid media and roots of non-transformed 8-month-old plants grown in the field were extracted using the same procedure.

Identification and quantification of valepotriates by HPLC. VAL, IVAl, DIA-VAL, HVAL, IVHD and DI were identified by comparison of their physical constants and spectral data with those of authentic samples isolated in our laboratory from a dichloromethane extract of *Centranthus ruber* non-transformed roots. The extracts were analysed using a HPLC method similar to that used for the valepotriate quantification in *Valeriana officinalis* hairy roots (Gränicher et al. 1994a). The quantification was based on the simultaneous estimation of the monoene and diene derivatives in a single HPLC run. Analyses were performed on a stainless steel Nucleosil C-18 column (25 x 0.4 cm; 5 µm; Macherey & Nagel, Germ.) fitted with a Nucleosil C-18 guard column (3 x 0.4 cm; 5 µm). An isocratic methanol-water (69:31) mixture was used as mobile phase at a flow rate of 0.7 ml/min for 10 min then at 1.4 ml/min for 30 min. The detection was performed at 208 nm. The filtered extract-internal standard (ethylbenzene) mixture (20 µl) was injected. Two calibration curves were established using VAL (previously isolated in our laboratory) for the calibration of diene type valepotriates and DI (Kalichemie, Germ.) for quantification of monoene type valepotriates.

Results and discussion

The fresh weight and the valepotriate content of the hairy roots cultured for 40 days in 12 different liquid media containing 2 - 7% sucrose were investigated and compared with the roots of 8-month-old non-transformed plants grown in the field (Tab. 1 and Fig. 2). In all the media tested, the transformed roots produced a spectrum of valepotriates which qualitatively mirrors that of non-transformed roots. VAL and HVAL were the major valepotriates of the hairy roots and of the non-transformed roots.

Quarter and particularly half strength B5 media stimulated the biosynthesis of DIA-VAL in the hairy roots, whereas this compound was detected only in small amount in the non-transformed roots. The IVAL content was unaffected by the various culture media tested and remained very low. The fastest growth (4.2 g) was observed in 1/2 B5-3 and B5-3 media, but this latter medium led to a poor valepotriate content (0.6% dry wt). In the 1/4 B5 medium, 2 - 5% sucrose had no significant effect on growth and valepotriate content, but 7% sucrose decreased substantially the valepotriate concentration. Except for B5 medium, the dilution of the investigated media to half or quarter strength had no effect on the growth and the valepotriate content. The highest concentration of valepotriates was observed in half and quarter strength B5-3 media and reached 3.0% dry wt. This value was in the same range as the valepotriate content of 8-month-old non-transformed roots (3.4% dry wt).

The effect of feeding DMI, a plant bioregulator (Lee et al. 1981; Förster and Becker 1987), on growth and valepotriate yield was investigated over a period of 40 days. Various concentrations of DMI (0.5, 1, 2.5, 5, 10, 25, 50 ppm) were added to the hairy root cultures on day 0 of cultivation. Table 2 shows that all the concentrations of DMI investigated had an unfavourable effect on the growth and reduced strongly the valepotriate production.

Culture media	Valepotriate content [% dry wt]	Fresh weight [g]
1/4 B5-2	2.7	2.6
1/4 B5-3	3.0	2.7
1/4 B5-5	2.7	2.7
1/4 B5-7	2.2	2.8
1/2 B5-3	3.0	4.2
B5-3	0.6	4.2
1/2 H-3	1.5	1.9
H-3	1.6	2.2
1/2 WP-3	0.8	2.5
WP-3	0.7	2.0
1/2 MS-3	0.5	2.4
MS-3	0.4	2.1
Control	3.4	---

Table 1. Fresh weight and valepotriate content of *Centranthus ruber* hairy roots cultured in different liquid media for 40 days. Valepotriate contents are the sum of the VAL, IVAL, DIA-VAL, HVAL, DI and IVHD. Untransformed plants (control) were cultured in the field during 8 months.

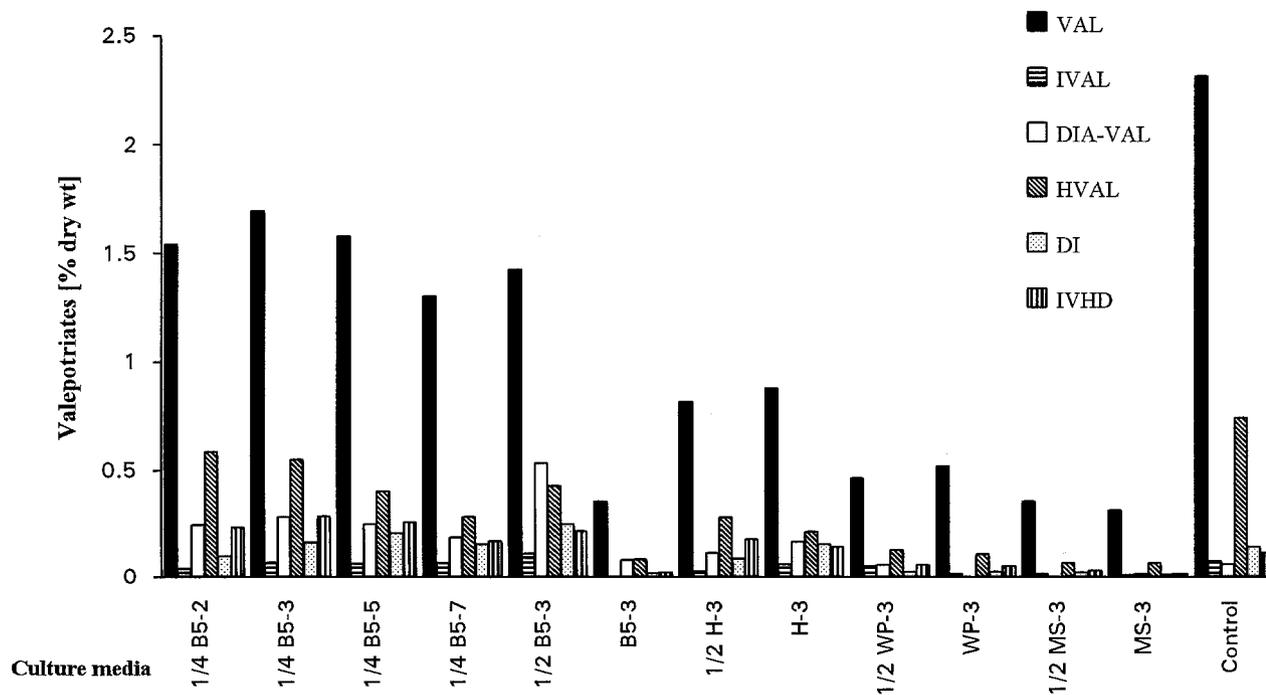


Fig. 2. VAL, IVAL, DIA-VAL, HVAL, DI, IVHD contents of *Centranthus ruber* hairy roots cultured in different liquid media for 40 days. Untransformed plants (control) were cultured in the field during 8 months.

It is noteworthy that the decrease of the valepotriate content is related to the increase of the DMI concentration. These results are not in line with those reported by Förster and Becker (1987) for cell suspension cultures of *Valeriana wallichii*.

In a further experiment, the time-course of growth and valepotriate production in 1/2 B5 medium supplemented with 3% sucrose was investigated (Fig. 3). At 5-day intervals, three flasks were harvested and the growth and the valepotriate production were measured. During a 45 day culture period, the fresh weight of the hairy roots increased from the original inoculum of *ca* 50 mg to reach 4.2 g (growth index: 84). The maximum biomass was already reached by 35 days of culture. On a mg/g dry wt basis, the overall valepotriate content reached 31.4 mg/g, representing a mean accumulation rate of 0.7 mg/g/day. Between day 10 and day 25, the growth rate was maximum and reached 80% of the final fresh wt. VAL, DIA-VAL and HVAL were the major constituents of the valepotriate mixture synthesized by the hairy roots. The VAL content increased rapidly and regularly between the 10th and the 45th day of culture to reach 1.5% dry wt. DIA-VAL and HVAL contents increased continually between the 20th and the 40th day of culture to reach a constant level of about 0.5% and 0.4% dry wt, respectively. DI, IVHD and IVAL were also detected but their levels remained low.

DMI content [ppm]	Valepotriate content [% dry wt]	Fresh weight [g]
control (no DMI added)	3.0	4.2
0.5	1.0	3.7
1	0.8	3.4
2.5	0.6	2.3
5	0.4	2.2
10	0.3	2.1
25	0.3	2.2
50	0.3	2.1

Table 2. Fresh weight and valepotriate content of *Centranthus ruber* hairy roots cultured for 40 days in 1/2 B5-3 liquid media supplemented with different concentrations of DMI. Valepotriate contents are the sum of the VAL, IVAL, DIA-VAL, HVAL, DI and IVHD. Each value is the mean of three separate determinations.

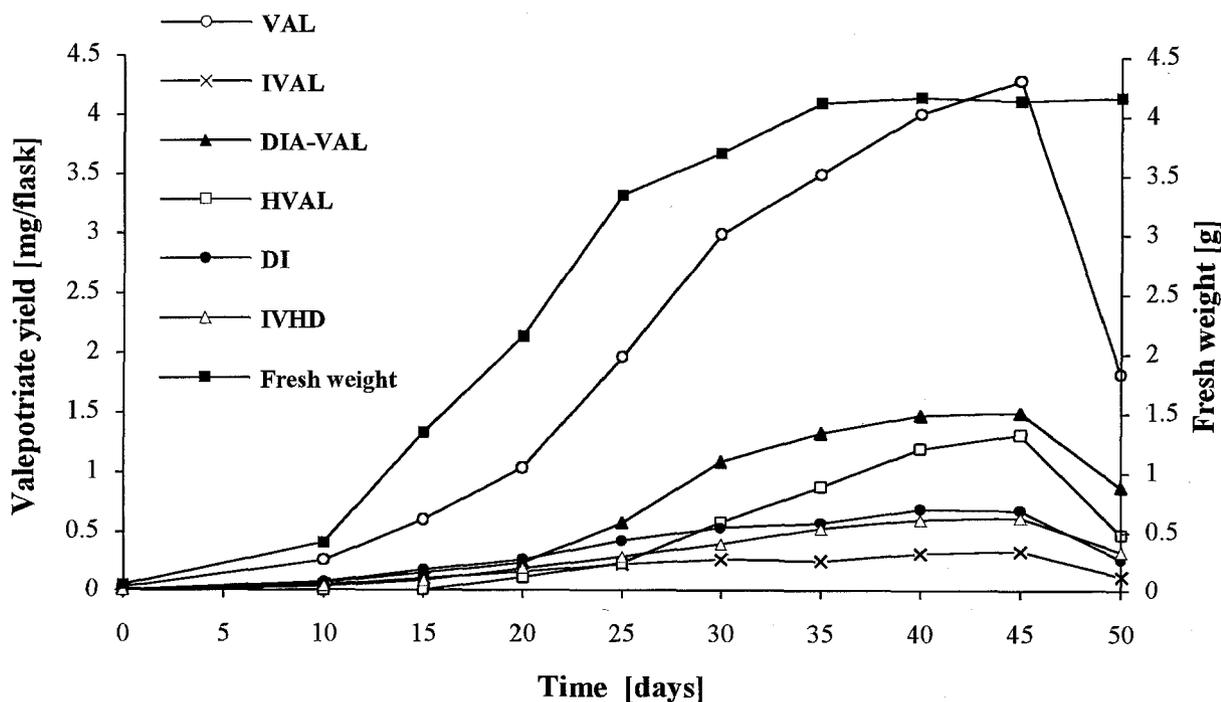


Fig. 3. Time-course study of growth and valepotriate production in *Centranthus ruber* hairy roots cultured in 1/2 B5-3 liquid medium.

The lack of accumulation of these valepotriates may be due to other biosynthetic pathways competing for common precursors or to a very low activity of one or more of the relevant biosynthetic enzymes. The medium was also examined for the presence of valepotriates. During the first 45 days of culture, neither valepotriates nor their decomposition products were detected in the culture medium. This suggests that all valepotriates were retained within the tissues. After 45 days of culture, the hairy roots turned brown and died, and the valepotriates were released into the culture medium.

The results reported in this paper differ significantly from those obtained with the hairy roots of *Valeriana officinalis* var. *sambucifolia*, another species belonging to the *Valerianaceae*. In the case of *V. officinalis*, the transformation by *A. rhizogenes* strain R1601 led to important modifications of the metabolic pathways. The spectrum of valepotriates was qualitatively and quantitatively modified. VAL was the most abundant valepotriate in the hairy roots, whereas it was IVAL in the non-transformed roots. IVHD, a minor compound in the non-transformed roots, reached 2.3% dry wt in the hairy roots (Gränicher et al. 1992). DIA-VAL and a new iridoid compound, named valdiate, were detected in the hairy roots only (Gränicher et al. 1994b). On the other hand, the hairy roots of *Centranthus ruber* biosynthesized a spectrum of valepotriates which mirrored quantitatively and qualitatively that of the non-transformed roots. The ratio VAL/IVAL and the monoene valepotriate content were not affected by the transformation with *A. rhizogenes* strain R1601. However some similarities have to be reported between the transformed roots of both species. The best yield in valepotriates was recorded in both cases with hairy roots cultured either in 1/4 B5 or in 1/2 B5 liquid media, the lowest yield occurring in MS liquid medium. Half or quarter strength Gamborg B5 liquid media supplemented with 2 to 3% sucrose are the most appropriate for the valepotriate biosynthesis in hairy root cultures.

The present study demonstrates that the genetically transformed root cultures of *C. ruber* may represent a valuable source of valepotriates, as the yield of valepotriates from a culture grown for 40 days in 1/4 or 1/2 B5-3 medium is comparable to that in roots of eight-month-old non-transformed plants grown in the field. Further investigations on the volatile compounds produced by the transformed roots of this species are in progress in our laboratory.

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