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RELATION BETWEEN VALEPOTRIATE CONTENT AND DIFFERENTIATION LEVEL IN VARIOUS TISSUES FROM VALERIANEAE

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ABSTRACT.—Various tissues of valerianeae were cultured in vitro. Growth, morphological aspect, and valepotriate production were studied in callus and root differentiated tissue cultures on four media differing in plant hormone content, during 1½ year of growth. In vitro cultures showed a higher valepotriate content than the roots of control plants in several cases. Favorable valepotriate production in vitro was proved to be closely related to root differentiation.

The sedative action of the roots of valerian plants is mainly ascribed to the valepotriates (1-3). Valepotriates are triesters of freely unstable polyhydroxycyclopenta-(c)-pyrans with isovaleric (R_1), acetic (R_2), and β-acetoxy isovaleric (R_3) acid containing an epoxide. Valtrate ($2\times R_1$, $1\times R_2$), isovaltrate ($2\times R_1$, $1\times R_2$) and acevaltrate ($1\times R_1$, $1\times R_2$, $1\times R_3$) are conjugated dienes forming blue cyclopentapyrrilium salts with HCl; didrovaltrate ($2\times R_1$, $1\times R_2$) is a monoene valepotriate (4). Structure elucidation of valepotriates was performed by Thies (5-8).

As a source for secondary metabolites of valerian plants, in vitro production has been tested, hitherto mostly in undifferentiated callus cultures (9-14). Becker and coworkers determined valepotriate contents [0.5-1.8 g/100 g dry material (%)] in callus and suspension cultures of different valerianeae. In callus cultures of *Valeriana officinalis* L., however, they could not find valepotriates (9,10).

In previous work we reported a relatively small production of valepotriates in undifferentiated callus and suspension cultures of different valerianeae, including *V. officinalis* (12). A larger production was obtained in a root differentiated tissue culture of *Centranthus ruber* DC (3%) (12). These preliminary studies indicated that the plant hormone composition of the medium influenced both valepotriate content and differentiation level of the cultures. It was not clear, however, whether this influence was exerted primarily on the production of valepotriates or whether the degree of valepotriate production was a consequence of differentiation.

In the callus cultures we often found relatively greater amounts of valepotriates at the start of the cultures, diminishing quickly after subcultivation, and attaining a constant low production level in the well-growing callus.

In order to study the influence of plant growth regulators, we set up new cultures of *Centranthus macrosiphon Boiss, C. ruber* and *V. officinalis* on four media with different plant hormone content, determining at each subcultivation time (every 5 weeks for 1½ years) wet and dry weight, differentiation level, and valepotriate content.

As *C. macrosiphon* showed the clearest response, we studied the valepotriate production on the four media during one growth period between two subcultivations. The cultures were at that time 1½ years old. The results of a microscopical localization of val-

epotriates at the subcellular level and of a volatile oil study in various tissues (fresh and in vitro) are reported elsewhere (13,14).

EXPERIMENTAL

PLANT MATERIAL.—Cultures and control plants were grown from seeds of *C. macrosiphon* (National Botanical Garden, Meise, Belgium, 1981), *C. ruber* (Botanical Garden of the University of Leiden, Netherlands, 1981) and *V. officinalis* (Hortus Centralis Cultura Herbarium Medicarum, Brno, CSSR). Control plants were grown in the greenhouse. In vitro culures were initiated from aseptically grown seedlings. The seedlings were cut aseptically and all parts explanted on agar medium.

NUTRIENT MEDIA.—Common constituents for the four media were a basal B_5 medium (15) with mineral and organic constituents and as additional requirements sucrose (10 g/liter) and glycine (2.10^{-3} g/liter). pH was adjusted to 6.1 with 1N KOH and agar (10 g/liter) was added. The presence of two auxins, naphthylene acetic acid (NAA) and 2,4-dichlorphenoxyacetic acid (2,4-D), and one cytokinin, kinetin (kin), were tested as plant growth regulators in four media (I-IV), always in a concentration of 10^{-3} g/liter as follows: medium I, NAA; medium II, NAA, 2,4-D, and kin; medium III, NAA and 2,4-D; and medium IV, NAA and kin. NAA was present in the four media as it proved to be necessary for callus induction (12).

CULTURE CONDITIONS.—The cultures were started, maintained, and regularly subcultured on Petri-dishes on their own medium every 5 weeks. They were incubated in permanent dark at 26°. The first passages were used to increase the amount of plant material. The inoculum was 0.7 g fresh material per dish. All plant cultures grew well on the four media except *C. ruber*. It was not possible to initiate cultures of *C. ruber* on media II and III.

DETERMINATION OF DIFFERENTIATION LEVEL, GROWTH PARAMETERS, AND PER CENT DRY WEIGHT.—The differentiation level was evaluated macroscopically and microscopically (13). The harvested fresh material was weighed (FW). This material was then dried (<40°) and weighed again (DW). Percent dry material is defined as DW/FW × 100. As growth parameter (GP) we have taken the ratio between the fresh weight of the harvested material at the subcultivation time, after 5 weeks of growth, and the fresh weight of the corresponding inocula.

VALEPOTRIATE ANALYSIS.—A sample of dried and pulverized (250 μm pore size) material was extracted twice with 20 ml of CH_2Cl_2. The CH_2Cl_2 extract was evaporated (<40°) in vacuo and the residue dissolved in CH_2Cl_2. Valepotriates were determined by spectrophotometry after tlc separation. Acevaltrate, (iso) valtrate, and didrovaltrate were quantitated in each sample. As stationary phase for the tlc we used Silicagel G 60 F_{254}-plates from Merck; the mobile phase was hexane-ethyl-methylketone (70:30), used twice. Standard (iso)valtrate, acevaltrate, and didrovaltrate were gifts from Dr. B. Hazelhoff, Groningen, Netherlands, Dr. J. Van Meer, Utrecht, Netherlands, and Dr. P.W. Thies, Kali-Chemie, Hannover, W. Germany.

Standard curves were made on the plates (10, 30, 60 μg of each valepotriate). Standard addition of the lowest standard was used on the plate. The amount of plant material and of CH_2Cl_2 for dissolving the residue and the amount spotted were adapted for the different samples to obtain a valepotriate amount between the lowest and the highest standard. Spots were located under UV_{254} and with dinitrophenylhydrazine-reagent according to Stahl and Schild (16). Spots were scraped off, as well as a blank on each plate and eluted with MeOH (5 ml). After centrifugation the methanolic solutions were measured spectrophotometrically at 254 nm [(iso)valtrate and acevaltrate] and 208 nm (didrovaltrate). Linear regression curves of the standards were calculated with the least square method and unknown concentrations calculated on these curves. The analyses were performed three times for each sample. Variations were always within 6%.

GROWTH PERIOD ANALYSIS OF *C. MACROSIPHON*.—We analyzed 1½-year-old in vitro cultures of *C. macrosiphon* (on the four media) during one 6-week growth period. At the beginning of the test two × 15 dishes were taken at random from each medium. These were used to determine the FW of the inocula per dish in order to check whether the sampling was statistically justified, which was the case. The FW of the inocula was 0.7 g. Every week, 15 dishes per medium were sampled at random for determination of FW, DW, and valepotriate content.

RESULTS

V. OFFICINALIS.—The evolution of valepotriate content during several passages is represented in Figure 1A. In all cases the cultures grew as white to yellow callus with

more or less extended root formation. Root differentiation disappeared totally on medium IV after the seventh passage; at that time the culture began to grow better (GP from 4 to 6.5), the valepotriate content diminished quickly (see Figure 1A) and the % dry material diminished from 6 to 3.5. On media I and II callus grew with little root formation. The GP was 4; and % dry material, 5.6. Valepotriate content was within the range found in roots of control plants (see Table 1).

TABLE 1. Valepotriate Content of the Roots of Valerianeae Control Plants

Plant	Valepotriate Compounds (g/100g dry material)		
	(iso)Valtrate	Acevaltrate	Didrovaltrate
C. macrosiphon	3.5	0.2	0.2
V. officinalis	0.8	0.1	0.1
C. ruber	1.0	0.1	0.1

On medium III roots were still formed on the callus after more than one year of growth, and a relatively high production of valepotriates was observed [2 g/100 g dry material (%)]. The GP was 4; and % dry material, 6.

The differentiation response to the different media was not so obvious as for the *Centranthus* species (see below). We observed that same phenomenon in *V. officinalis* cultures obtained from seeds of different origin. We suppose that the tested media (plant hormone composition and concentration) were not suitable for complete suppression of differentiation or, on the other hand, for induction of only root differentiation.

C. RUBER.—*C. ruber* cultures grew only on media I and IV. At the first subcultivation passage the cultures showed formation of callus, shoots, and mostly roots. They developed afterwards to mainly root differentiated cultures. The GP was 3-4; and % dry material, 7-8. Valepotriate formation was relatively stable and high (2%) compared with the roots of control plants (see Table 1). This situation persisted after more than one year of growth on medium I. On medium IV, however, after the tenth subcultivation passage (50 weeks) roots disappeared gradually leaving well-growing callus (GP of 7) with much lower % dry material (from 7 to 3%) and quickly diminishing valepotriate content (see Figure 1B).

C. MACROSIPHON.—The evolution of valepotriate content is given in Figure 1C. The cultures grown on media I and IV, both without 2,4-D, showed differentiated structures from the start; at the first passage we saw very little callus, many roots, and in some cases shoot formation. Shoots disappeared promptly, and the cultures became clearly root differentiated tissue cultures. GP was 5-6 and % dry material was 6. Valepotriate content was high (5-8%). On media II and III well-growing white callus was formed immediately and maintained thereafter. GP was 6 and % dry material was lower (3%) than on media I and IV. Initial valepotriate content was much lower than on media I and IV and tended to diminish steadily during further passages (from 1 to 0.1%).

In order to evaluate and compare the growth and valepotriate production from the cultures on the different media, we followed these parameters every week during one growth period. Figure 2 shows fresh weight, dry weight, and valepotriate content—all expressed as comparable absolute amounts starting with equivalent fresh weight inocula. It is clear from this figure that media I, II, and III are equivalent concerning the increase of biomass (FW). Medium IV is a little less growth stimulating. The amount of dry weight is higher on the media without 2,4-D (I and IV) during the whole growth period, especially on medium I. Considering the increase of biomass on the four media,

valepotriate yield is much higher from the root differentiated tissue cultures (media I and IV) than from the callus cultures (media II and III). The root differentiated tissue cultures on medium I are most productive; starting with an inoculum of 15 g FW, 100 g of biomass containing 0.375 g valepotriates are obtained after 4 weeks. The production by the calli on media II and III is 100 times less.

DISCUSSION

From the results of this work it is clear that valepotriates can be obtained from in vitro cultures of valerianeae in amounts that are, in several cases, higher than in roots of corresponding plants.

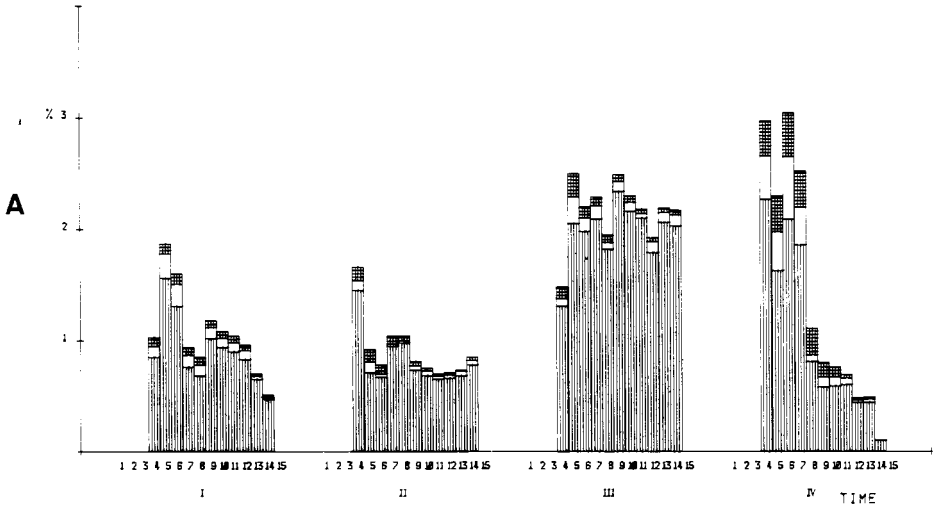

FIGURE 1A. *V. officinalis*. Additive valepotriate content of in vitro cultures, expressed as valepotriate g/100 g dry material (%), on four media (I to IV) during 70 weeks of cultivation. Arabic numbers in the abscis indicate the passage number. ▨=(iso)valtrate, □=acevaltrate, ▩=didrovaltrate.

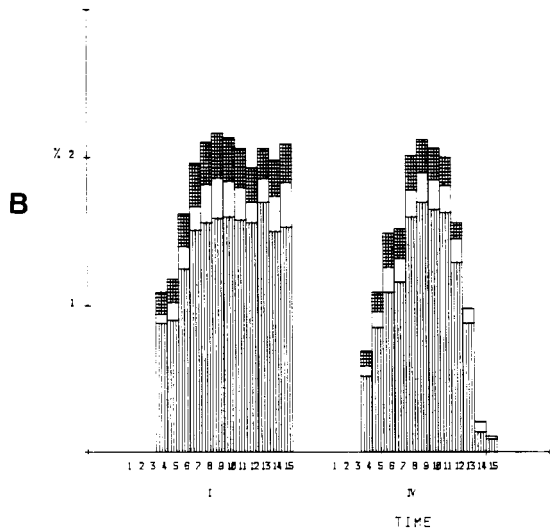

FIGURE 1B. *C. ruber*. Additive valepotriate content of in vitro cultures, expressed as valepotriate g/100 g dry material (%), on four media (I and IV) during 70 weeks of cultivation. Arabic numbers in the abscis indicate the passage number. ▨=(iso)valtrate, □=acevaltrate, ▩=didrovaltrate.

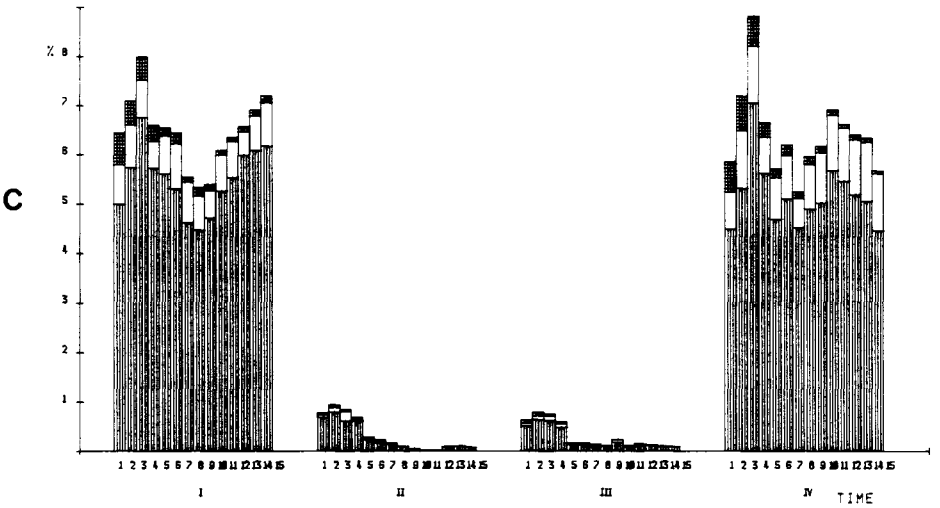

FIGURE 1C. *C. macrosiphon*. Additive valepotriate content of in vitro cultures, expressed as valepotriate g/100 g dry material (%), on four media (I to IV) during 70 weeks of cultivation. Arabic numbers in the abscis indicate the passage number. ▥=(iso)valtrate, □=acevaltrate, ■=didrovaltrate.

The highest and stablest valepotriate production in *C. macrosiphon* was obtained on media without 2,4-D (I and IV).

Considering the increase in biomass and the valepotriate yield, the most productive cultures were differentiated root organ cultures of *C. macrosiphon* on medium I (containing only the auxin NAA). High production and root differentiation went together with a high % dry material.

Root differentiated tissue cultures of *C. ruber*, obtained on media without 2,4-D, also gave a high yield of valepotriates in comparison with the control plants. After the tenth passage (50 weeks) on medium IV (NAA kinetin), differentiation disappeared, leaving well-growing but far less productive callus.

For *V. officinalis* the best and stablest production was obtained on a medium with the auxins 2,4-D and NAA. Morphologically, this culture showed callus formation with root differentiation.

A stable, high valepotriate content was maintained during several passages (1½ year) in differentiated root tissue cultures. In one of these cultures (*C. ruber*, medium IV), differentiation disappeared steadily after one year. Simultaneously valepotriate content diminished. From this we conclude that aging of the culture is important in relation to production only in so far that it is correlated with differentiation changes.

The effect of 2,4-D in the growth media of *C. macrosiphon* cultures was obvious; callus formation had low valepotriate content in the presence of 2,4-D and root differentiation had high valepotriate content in the absence of 2,4-D. Because *V. officinalis* cultures, on the other hand, grew as callus with roots and relatively high valepotriate content in the presence of 2,4-D (medium III), we can conclude that 2,4-D does not inhibit primarily valepotriate production. Valepotriate production is only inhibited or suppressed if differentiation is suppressed simultaneously by the plant hormone content of the medium.

As we cultured in permanent dark, it is clear that light is not an indispensable physical condition for valepotriate formation.

We previously showed (13) that valepotriates are present in intracellular lipid droplets in both roots and in vitro material. In the in vitro material no real essential oils were present but only lower fatty acids (14). We suppose that differentiated structures of the

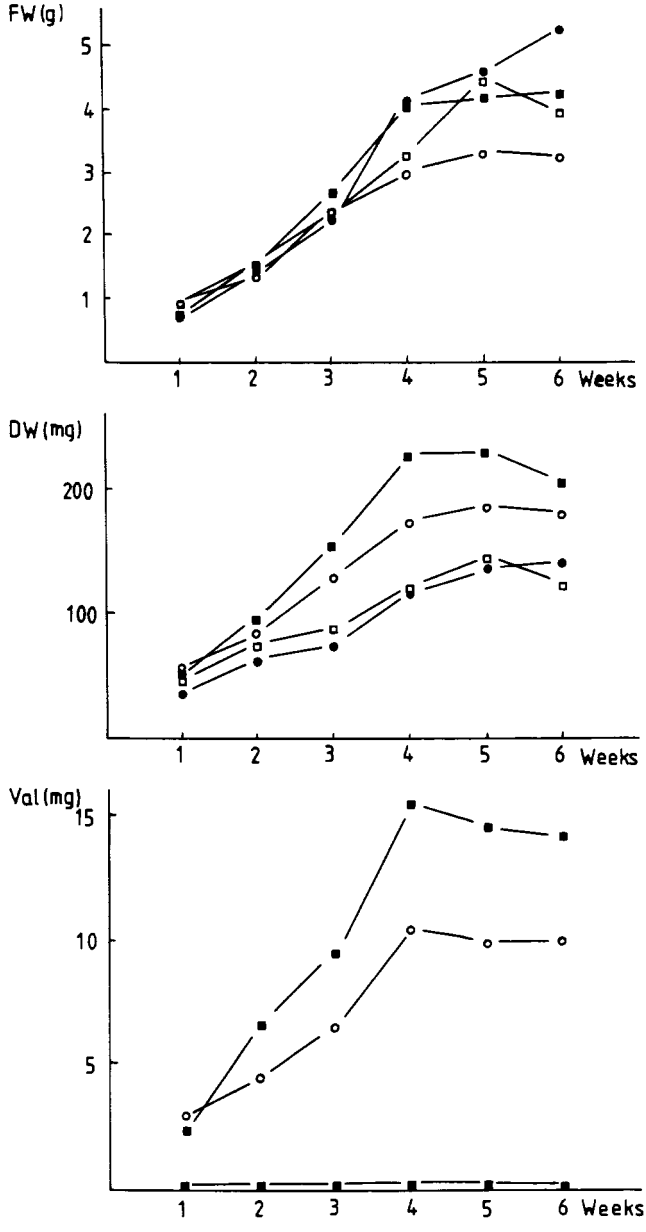

FIGURE 2. Fresh weight (g), dry weight (mg), and valepotriate content (mg) of *C. macrosiphon* cultures expressed as absolute amounts during a growth period on four different media; ■=medium I, ●=medium II, □=medium III, and ○=medium IV.

roots facilitate formation of these droplets and, consequently, valepotriate production. Indeed, valepotriates are present to a large extent in roots and rhizomes of valerianeae and are very scarce in the aerial parts of the plants (17,18).

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NOMINATIONS FOR THE AMERICAN SOCIETY OF PHARMACOGNOSY RESEARCH AWARD

The American Society of Pharmacognosy announces the establishment of a new research award to recognize members of the Society for their outstanding contribution to research in the field of natural products.

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A nomination may be made by any member and must include a curriculum vitae of the nominee, a list of publications, and a statement emphasizing the nominee's accomplishments and their significance. Attachment of representative reprints may be helpful. Honorary Members of the Society would normally be excluded from consideration, because their achievements have been acknowledged already by the Society.

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