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In vitro incorporation of radiolabelled cholesterol and mevalonic acid into ecdysteroid by hairy root cultures of a plant, *Serratula tinctoria*

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***Serratula tinctoria*, Asteraceae, phytoecdysteroids, hairy roots, metabolism, cholesterol, mevalonic acid**

Abstract. After the transformation of stems with *Agrobacterium rhizogenes*, hairy root cultures have been obtained from *Serratula tinctoria* (Asteraceae), a plant containing very high amounts of 20-hydroxyecdysone (20E) and numerous other phytoecdysteroids. These cultures were found to grow regularly in vitro, either in liquid or solid media, and to maintain a high ecdysteroid production (c.a. 0.1–0.2% dry weight). 20E was the predominant ecdysteroid, together with the corresponding 3-acetate (20E3Ac). Ecdysteroids were not secreted in the medium but remained in root tissues. Moreover, a concentration gradient was observed in transformed hairy roots, as in plant roots, characterized by a higher ecdysteroid content in the meristematic zone. In vitro incubations with two radiolabelled precursors, [^{3}H]-cholesterol and [^{14}C]-mevalonic acid, have been performed, showing an efficient incorporation of these molecules into ecdysteroids: with labelled cholesterol, 20E indeed reached 0.25 to 0.67% of the radioactivity recovered in hairy roots after several days of culture and, with labelled mevalonate, 0.4 to 2.7%. Thus, hairy roots of *S. tinctoria* undoubtedly constitute a very promising tool for the analysis of ecdysteroid biosynthesis and functions in plants.

INTRODUCTION

More than 100 phytoecdysteroids have been described in different plant species and, though it is frequently suggested that they play a defensive role against insects or nematodes, their function remains an open question (reviews in Horn & Bergamasco, 1985; Lafont & Horn, 1989; Lafont et al., 1991). Ecdysteroid concentrations have been analyzed in different organs of various plant species (e.g., Girault et al., 1988; Dinan, 1992), but their localization does not appear to obey a clear rule. Moreover, the precise sites of biosynthesis and their regulations are almost completely unknown.

Several studies have emphasized the presence of very high amounts of 20-hydroxyecdysone (20E) and other phytoecdysteroids, in particular 20-hydroxyecdysone 3-acetate (20E3Ac), in the roots of *Serratula tinctoria* (Bathori et al., 1986, Rudel et al., 1992). In order to develop in vitro systems for the study of sterol and ecdysteroid biosyntheses in this plant, cultures from calli and isolated cells of *S. tinctoria* were previously developed, retaining a significant production of ecdysteroids in vitro, but at a concentration more than 100-fold lower than the parent plant (Corio-Costet et al., 1993a). Therefore, another possibility was engaged, in order to obtain transformed "hairy root" cultures from

S. tinctoria, which could keep more satisfactory capacities to produce ecdysteroids in vitro. This was confirmed by the following study and in particular by the incorporation of two putative radiolabelled ecdysteroid precursors, [^{3}H]-cholesterol and [^{14}C]-mevalonic acid.

MATERIAL AND METHODS

PLANT MATERIAL. Hairy roots cultures of *Serratula tinctoria* were initiated from seedlings obtained by germinating seeds under sterile conditions and transformed with *Agrobacterium rhizogenes* (strain A4, provided by Dr Tepfer), according to Tepfer (1984), as previously described (Corio-Costet et al., 1994). They were maintained at 25°C, in constant darkness, on MS medium (Murashige & Skoog, 1962), without hormone.

CHEMICALS. Reference unlabelled ecdysteroids as 20E, 20E3Ac and polypodine B or 5,20-dihydroxyecdysone (5,20E), according standardized abbreviations from Lafont et al. (1993), were gifts from Prof. R. Lafont (Paris). [1α,2α(n)-^{3}H]-cholesterol (40 Ci/mmol) and [2-^{14}C]-mevalonic acid (50 mCi/mmol, tested either under acid salt or lactone form, without significant difference) were purchased from Amersham.

INCUBATIONS. Labelled compounds (ca. 10 µCi per culture) were added, after solubilization with 50 µl dimethylsulfoxyde, in 50 ml medium. They were used in separate experiments. Various incubation times (1 to 5 days and 1 to 4 weeks) were tested.

EXTRACTIONS AND ANALYSES. Enzyme immunoassay (EIA) of ecdysteroids was adapted from Porcheron et al. (1989), but using a peroxidase tracer (Delbecque et al., in prep.). Ecdysteroids were extracted and analyzed using high performance liquid chromatography (HPLC), as previously reported in Corio-Costet et al. (1993b). Radioactivity was measured on aliquots at the various extraction steps and counted with 10 ml of a scintillation cocktail (BCS, Amersham) on a Beckman LS 6000 scintillation spectrophotometer. RP-HPLC (reverse phase) using Merck Lichrospher RP-18 column (125 × 4 mm), with 12 to 38% acetonitrile in water at 1 ml/min during 20 min then purging with 100% acetonitrile, was more suitable for on-line radioactivity measurements, which were monitored with a Flo-One β detector (Radiomatic), beside a classical UV detector (244 nm). NP-HPLC (normal phase) using Merck Lichrospher Diol column appeared more suitable for qualitative analyses, allowing the complete separation of 20E and 5,20E, but was generally not used for on line radioactivity measurements, as the mobile phase (5 to 20% ethanol gradient in dichloromethane at 1 ml/min during 20 min) induced more quenching; in this case, solvent evaporation was recommended before counting fractions.

RESULTS

The hairy roots induced by *A. rhizogenes* were generally found to grow regularly in vitro, from 0.5 to 2.8 mm per day, making frequent nodules, with rare extensive branching. After successive subcultures for ca. 8 months, several clones were selected for their more rapid growth (ca. 2 to 2.8 mm elongation per day) and then subcultured regularly during more than one year, without appreciable loss of activity. They were maintained either in liquid or solid MS media, in the absence of phytohormone.

The ecdysteroid content in these hairy roots in vitro was found to reach 0.1 to 0.2% of the dry weight, as estimated using EIA or HPLC. Ecdysteroids were totally retained in hairy roots: only neglectible immunoreactivity was found in either liquid or solid media.

Interestingly, ecdysteroids were not found to be randomly distributed within hairy roots. Measurements cm per cm clearly showed a concentration gradient, with the higher amounts at the tip, i.e. the growing part or meristem. A similar distribution was also observed in plant roots (Fig. 1).

RP- and NP-HPLC analyses indicated that 20E was the predominant ecdysteroid, but also revealed the presence of 20E3Ac and 5,20E, together with other minor compounds,

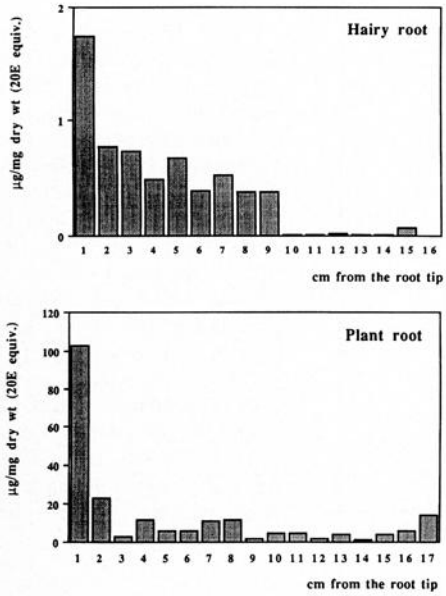

Fig. 1. Examples of ecdysteroid concentrations, measured cm per cm with EIA, in hairy root (upper box) or plant root (lower box) of *S. tinctoria*, showing higher concentrations in the tip. Concentrations are expressed in µg/mg dry weight (ordinate). Root fragments are numbered from the tip (abscissa).

which have not yet been identified. Fig. 2 gives an example of NP-HPLC analysis, showing that 20E, completely separated from 5,20E, is more abundant than 20E3Ac.

Biosynthesis of ecdysteroids in hairy roots of *S. tinctoria* in vitro was confirmed by separate incorporations of two radiolabelled precursors, ^{3}H-cholesterol and ^{14}C-mevalonic acid. After variable incubation times, the roots were extracted and analyzed using RP-HPLC. Efficient incorporations of radioactivity into ecdysteroids were easily observed, for incubation times over 4 days: indeed radiolabelled ecdysteroids more or less appeared to accumulate in hairy roots with increasing incubation times.

Examples of ecdysteroid patterns, using RP-HPLC, are given in Figs 3 (mevalonate) and 4 (cholesterol), showing, with both precursors, the presence of a major labelled peak co-migrating with 20E (detected by UV absorbance, Fig. 3, upper box) and suggesting the presence of several other ecdysteroids, among which 5,20E and 20E3Ac were possibly present but not definitely identified. These and possible other ecdysteroids, together with probable apolar intermediates retained on Sep-Pak during extraction, should be identified in more details in future studies. However, the identification of 20E was confirmed in NP-HPLC, as the main radioactive peak was also found to migrate like the reference compound in this second system (not shown).

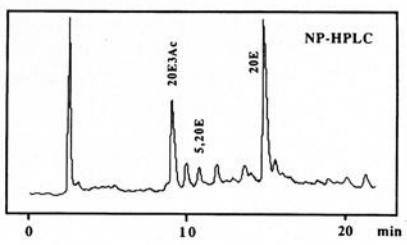

Fig. 2. Normal phase HPLC of an ecdysteroid extract from hairy roots of *S. tinctoria*. Standardized ecdysteroid abbreviations as given in the text, according to Lafont et al. (1993). Conditions: Merck Lichrospher Diol column (250 × 4 mm); 5 to 20% ethanol gradient in dichloromethane at 1 ml/min during 20 min. Abscissa: time in min. Ordinate: UV absorbance at 244 nm (full scale: 0.1 absorbance unit).

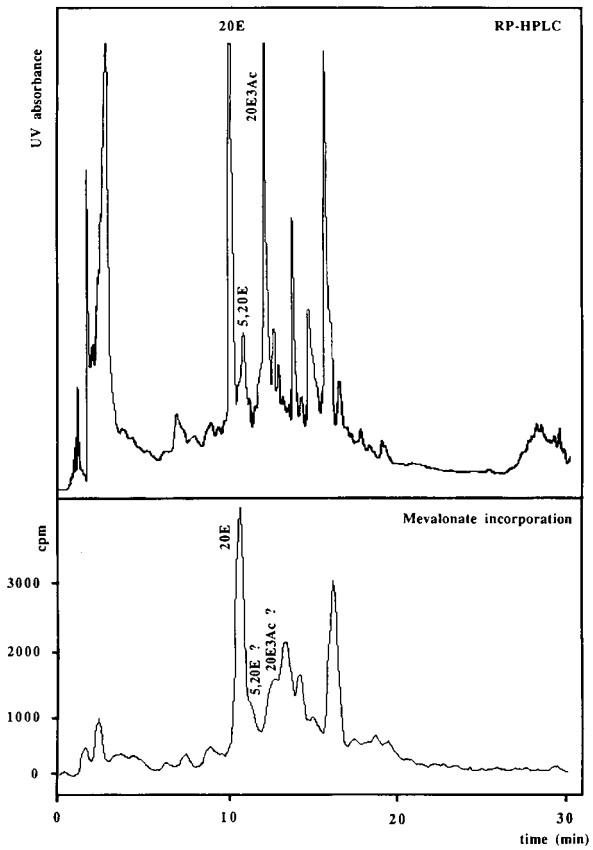

Fig. 3. Example of a reverse phase HPLC of an ecdysteroid extract from hairy roots of *S. tinctoria*, after incorporation of [^{14}C]-mevalonic acid for 2 weeks. The injected aliquot corresponded to ca. 6 mg root dry weight (one tenth of a culture). Abscissa: time in min for the two boxes. Upper box: UV profile at 244 nm (full ordinate scale: 0.1 absorbance unit). Lower box: on-line radioactivity measurements (cpm: counts per min). Abbreviations: see Fig. 2. HPLC conditions: Merck Lichrospher RP-18 column (125 × 4 mm); 12 to 38% acetonitrile in water at 1 ml/min during 20 min then purging with 100% acetonitrile.

In the experiments with labelled cholesterol, 20E was estimated to represent 0.25 to 0.67% of the radioactivity recovered in hairy roots after several days of culture, whereas in experiments with labelled mevalonate, 20E represented 0.4 to 2.7%. Of course, if 20E yields were expressed in percents of the 10 µCi initial doses, they appeared much lower (0.08 to 0.4% for cholesterol, 0.13 to 0.97% for mevalonate), but such a calculation is

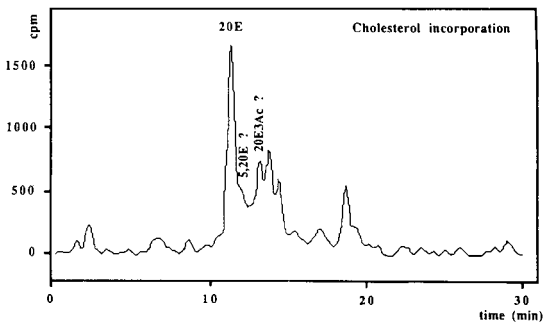

Fig. 4. On-line radioactivity measurements of a similar experiment as Fig. 3, but with [^{3}H]-cholesterol incubation for 2 weeks. Other conditions and legends as Fig. 3.

probably less relevant, as a great part of labelled precursors seemed to remain unchanged in the excess of culture medium and as no labelled ecdysteroid was found in this medium.

DISCUSSION

Several authors have developed in vitro systems with the aim to facilitate the production and/or study of phytoecdysteroids (e.g., McMorris & Voeller, 1971; Hikino et al., 1971; Ravishantar & Mehta, 1979; Vanek et al., 1990; Camps et al., 1990; Tomas et al., 1993; Svatos & Macek, 1994), including our previous studies (Corio-Costet et al., 1993a,b; Corio-Costet et al., 1994). However, it has been frequently observed, not only for ecdysteroids, that undifferentiated plant cells often produce lower amounts of metabolites than intact plants. Hairy root cultures generally appear as a valuable alternative to enhance the production of secondary metabolites (Herman, 1993) and such a possibility for phytoecdysteroids has been recently initiated by Matsumoto & Tanaka (1991) in several species: *Ajuga reptans*, *Achyranthes fauriei*, *Pfaffia iresinoides* and *Vitex stickeri*.

Our transformed hairy roots of *S. tinctoria* thus synthesize important amounts of ecdysteroids, which remain within the tissues and are not secreted into the culture medium. Though ecdysteroid quantities are only approximately one-tenth of the values encountered in plant roots (i.e. 0.1 to 0.2% vs. 1 to 2% dry weight), they are far higher than those previously obtained in the other in vitro systems derived from this plant, i.e. less than 0.01% dry weight in callus and cell suspension cultures (Corio-Costet et al., 1993a) and also higher than in most other in vitro systems developed in various species. Consequently, such hairy roots appear much more convenient for metabolic studies than isolated cells or calli in vitro.

The main ecdysteroid found in our transformed roots was 20E, with lower amounts of 20E3Ac, whereas the inverse situation was observed in normal roots (Corio-Costet et al., 1993a). This may be the consequence of tissue age: the older, as are normal roots, generally contain more 20E3Ac, than the younger, hairy roots included.

Interestingly, the transformed hairy roots of *S. tinctoria* appeared to keep the possibility to establish ecdysteroid concentration gradients in vitro, as the plant roots in vivo. The fact that ecdysteroids may vary during plant development (Grenebok & Adler, 1991) and make concentration gradients (Dinan, 1992) has been previously evidenced in other species and could be a general and interesting feature of phytoecdysteroids. Though the presence of higher amounts of ecdysteroids in the growing zones (which are probably the most fragile and the most attractive for phytophagous animals) can be interpretated as an increased defence against insects and nematodes, such gradients could also play developmental roles, and, if so, our in vitro system could be a valuable tool for studying the possible involvement of ecdysteroids in plant physiology.

The incorporations of two radiolabelled precursors, [^{3}H]-cholesterol and [^{14}C]-mevalonic acid, have in fact given very similar results, particularly on a qualitative viewpoint, in the ecdysteroid fraction: 20E, identified with two different HPLC systems, was the main labelled product, beside other compounds in lower quantities, which remain to be identified. The very similar incorporation patterns suggest that most of the mevalonate by-products in the ecdysteroid fraction are also cholesterol metabolites. The comparison of the two incorporation patterns will probably be more different in the apolar fraction (presently under investigation). For the qualitative aspect, cholesterol incorporations have

given remarkably high incorporation yields but mevalonate has given better results, undoubtedly due to its greater ability to penetrate plant tissues. Due to the lower specific activity of this [^{14}C]-precursor, a much higher molar concentration of mevalonate was used to get the same 10 μCi dose per culture as cholesterol. This fact undoubtedly increased the absolute levels of uptake and incorporation (the molar amounts of mevalonate really incorporated into 20E were indeed far higher than those of cholesterol) but probably not the incorporation rates expressed as radioactivity percentages: on the contrary, higher concentrations are generally supposed to influence incorporation percentages in a negative way. Finally, it is noticeable that such a use of a [^{14}C]-precursor in vitro could allow the obtainment of ecdysteroids labelled on the nucleus, which could be of interest for future metabolism experiments.

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