Production and Release of Isoflavonoids by Lupin Cell Cultures

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Cell cultures derived from white lupin (*Lupinus albus*) radicles consist of a mixture of friable cell aggregates and finely divided cells. Both types of tissue accumulate two isoflavonoid aglucones, genistein and 2'-hydroxygenistein, their 7-O-glucosides, and their respective 6-, 8-, and 3'-monoprenyl-, as well as the 6,3'-diprenyl derivatives. Most of these metabolites are differentially released in the culture medium. The relative amounts of aglucones, glucosides and prenylated derivatives that are produced by the aggregates and cells, as well as those excreted into the medium, are discussed in relation to culture growth.

Introduction

Plant tissue and cell cultures have the potential to produce a wide variety of plant secondary metabolites [1–6]. With few exceptions, however, plant cell cultures have failed to synthesize their characteristic metabolites. Even when the latter are expressed, they are produced at concentrations far below those found in the intact plants from which the cultures were derived.

Of the variety of natural products which have been produced by cell cultures, flavonoid compounds are the least represented [7]. The few examples reported in the literature are the isoflavonoids which are formed in response to biotic or abiotic elicitors ([8] and refs. cited therein). There is an evident need for cell culture systems which have the inherent ability to produce those flavonoids characteristic of their intact tissues. Such cultures would be very useful in studying the biosynthesis and regulation of these metabolites.

The roots of white lupin (*Lupinus albus*) represent a model system which accumulates a variety of isoflavonoid compounds which are based on two simple aglucones, genistein and 2'-hydroxygenistein and their respective 7-0-glucosides, as well as the 6-, 8-, 3'-mono- and 6,3'-diprenylated derivatives [9–11]. In addition to the latter, 17 other minor isoflavonoids have been identified in

white lupin roots which contain prenyl, pyrano, and dihydrofurano groups [12]. In contrast with other leguminous species, which produce prenylated isoflavonoids only after fungal infection, or after treatment with elicitors [13], lupin roots have the remarkable ability for the constitutive expression of isoflavonoid prenylation under normal conditions of growth. It was considered of interest, therefore, to investigate the potential of a cell culture derived from the radicle of white lupin for the production of its characteristic isoflavonoids.

Materials and Methods

Cell Culture

Seeds of white lupin (*Lupinus albus* L. *cv*. Kievskij Mutant) were surface sterilized and germinated on filter paper in sterile petri dishes. Callus cultures from 5–6 day-old radicles were initiated on B 5 agar medium [14] containing 2% sucrose, 1 ppm 2,4-D, and 0.1 ppm kinetin. Suspension cultures were established by transferring the callus formed to special rotating one-liter nipple flasks [15] containing 250 ml liquid medium of the same composition, and has been maintained in culture for more than two years. Batch cultures containing 8 ml of cell inoculum (*ca*. 2 g fresh weight) in 25 ml of culture medium were maintained on a rotary shaker. Culture growth was determined by measuring the fresh weight and protein content of the cells over a 20-day period.

Extraction of isoflavonoids

Where mentioned, cell clumps were harvested by filtration on a nylon mesh, and the finely divid-

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ed cells were separated from the medium by centrifugation. Cells were extracted twice with 80% aq. MeOH at room temperature. The combined extracts were concentrated *in vacuo* at 30 °C to an aq. residue, then extracted twice with EtOAc. The organic layer was evaporated, redissolved in 80% MeOH, and then used for HPLC analysis. Isoflavonoids were extracted from the culture media with EtOAc and treated as described above.

Chromatographic analysis

HPLC analysis was carried out with a Waters system (Millipore, Milford, MA) fitted with a Lichospher-100 RP-18, 5 µm column (250 mm × 4 mm I.D.) and spectrometric detection at 254 nm. Samples (20 µl in 80% MeOH) were passed through 3 mm, 22 µ filters before injection through the column. Elution of compounds was achieved at a flow rate of one ml.min^{-1} using isocratic 45% solvent A (0.1% methanolic HOAc) in 55% solvent B (0.1% aq. HOAc) for 2 min. This was followed by a gradient increase to 100% solvent A in 80 min, and continued for a further 5 min under the same conditions. Equilibration of the column, necessary to reach the initial conditions, was achieved using 45% A for 15 min before injection of a new sample. Peak areas were integrated with a Waters 810 Baseline Workstation, and molar concentrations of individual compounds were calculated. Characterization of isoflavonoids was achieved by cochromatography with authentic samples [10–12], and their retention times.

Results

Nature and kinetics of cell growth

Lupin cell cultures consist of a mixture of friable cell aggregates, 2–3 mm in diameter, and finely divided cells in suspension. The cell aggregates are partly greenish, but mostly reddish-brown in colour, as are the cells that are derived from them. The latter undergo cell division during culture growth, and eventually coalesce into small cell clumps.

Using B5 medium, containing 2% sucrose and supplemented with 2,4-D (1 ppm) and kinetin (0.1 ppm), resulted in a relatively slow growing cell culture which exhibits an approximately 2-fold increase in fresh weight after 20 days of culture (Fig. 1). For practical purposes, therefore, the culture growth period has been devided into five stages, I–V.

In spite of the slow growth of cultured cells, they exhibit a marked increase in protein content soon after subculture which subsequently decreases, and remains stable thereafter (Fig. 1). This phenomenon is common to many cultured cells, and the increase in protein level has been attributed to the dilution effect of subculture in a fresh nutrient medium [16].

Isoflavonoid production by the cultured tissue increases slightly during early growth, reaches its maximum in stage IV cells, then markedly declines (Fig. 1). Furthermore, lupin cell culture growth is characterized by the release of isoflavonoids into the culture medium at all stages of growth, especially stages II–IV (Fig. 1). This phenomenon is usually associated with the development of a reddish-brown coloration of the culture medium, 3–4 days after subculture, and intensifies during later stages of culture growth.

Isoflavonoid HPLC pattern

The pattern of isoflavonoids, whether produced by the cultured tissue or released in the medium, is qualitatively similar. It consists of two aglucones, genistein (**1**) and 2′-hydroxygenistein (**1a**) and their 7-*O*-glucosides (**2, 2a**), as well as their respec-
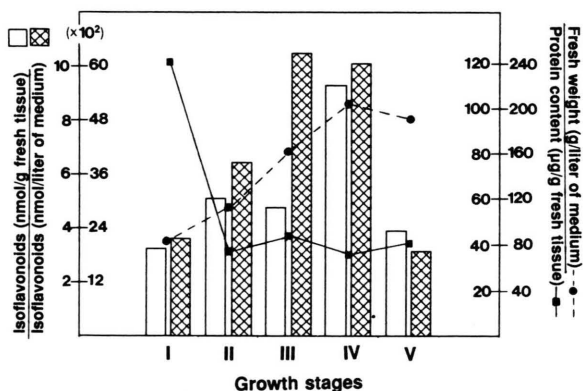

Fig. 1. Parameters of lupin culture growth: – –●– –, fresh weight; –■–, protein content; open columns, isoflavonoid accumulation; shaded columns, isoflavonoid secretion. Growth stages: I, 0–4; II, 5–8; III, 9–12; IV, 13–16; V, 17–20 days in culture.

tive 6-prenyl-(wighteone, **3** and luteone, **3a**), 8-prenyl-(lupiwighteone, **4** and 2,3-dehydrokievitone, **4a**), 3′-prenyl-(isowighteone, **5** and licoisoflavone A, **5a**) and 6,3′-diprenyl-(lupalbigenin, **6** and 2′-hydroxylupalbigenin, **6a**) derivatives (Scheme I). It should be noted, however, that the extraction procedure used in this work does not permit the recovery of the malonate esters of isoflavone glucosides which are known to be natural constituents of intact [17] and cultured [18] lupin tissues.
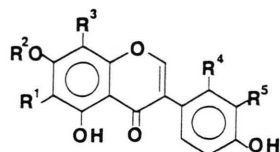
1: $R^1 = R^2 = R^3 = R^4 = R^5 = H$, genistein
1a: $R^1 = R^2 = R^3 = R^5 = H$, $R^4 = OH$, 2′-hydroxygenistein
2: $R^1 = R^3 = R^4 = R^5 = H$, R^2 = glucosyl, genistein 7-*O*-glucoside
2a: $R^1 = R^3 = R^5 = H$, R^2 = glucosyl, $R^4 = OH$, 2′-hydroxygenistein 7-*O*-glucoside
3: $R^1 = CH_2CH=C(Me)_2$, $R^2 = R^3 = R^4 = R^5 = H$, wighteone
3a: $R^1 = CH_2CH=C(Me)_2$, $R^2 = R^3 = R^5 = H$, $R^4 = OH$, luteone
4: $R^1 = R^2 = R^4 = R^5 = H$, $R^3 = CH_2CH=C(Me)_2$, lupiwighteone
4a: $R^1 = R^2 = R^5 = H$, $R^3 = CH_2CH=C(Me)_2$, $R^4 = OH$, 2,3-dehydrokievitone
5: $R^1 = R^2 = R^3 = R^4 = H$, $R^5 = CH_2CH=C(Me)_2$, isowighteone
5a: $R^1 = R^2 = R^3 = H$, $R^4 = OH$, $R^5 = CH_2CH=C(Me)_2$, licoisoflavone A
6: $R^1 = R^5 = CH_2CH=C(Me)_2$, $R^2 = R^3 = R^4 = H$, lupalbigenin
6a: $R^1 = R^5 = CH_2CH=C(Me)_2$, $R^2 = R^3 = H$, $R^4 = OH$, 2′-hydroxylupalbigenin

Scheme I. Isoflavonoid derivatives in lupin culture.

The choice of column support and the elution program described in the Methods section resulted in an efficient separation of the individual aglucones, glucosides and prenylated derivatives (Fig. 2). The order of elution of isoflavonoids followed a sequence of decreasing polarity, whereby glucosides eluted first, which were followed by aglucones, monoprenyl- and diprenyl derivatives. For each group of compounds, 2′-hydroxygenistein derivatives eluted before those derived from genistein (Fig. 2). The availability of an elaborate library of reference compounds of lupin isoflavon-
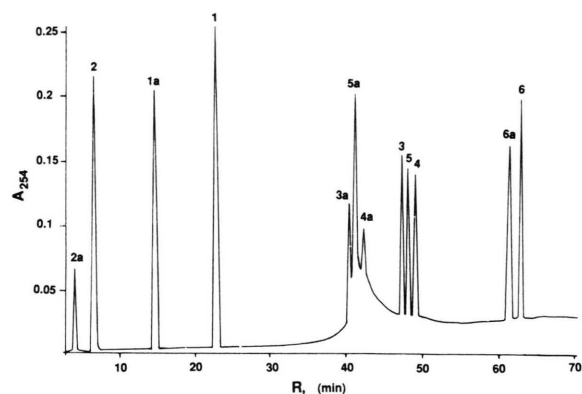

Fig. 2. HPLC profile for the elution of authentic samples of isoflavonoid derivatives. Compounds **1–6** and **1a–6a** are designated in Scheme I.

oids [10–12] allowed for identification of the metabolites in cultured cells, and those released in the medium without ambiguity. For practical purposes, the quantitative changes in isoflavonoids of the cultured aggregates and cells, as well as those released into the medium during culture growth, will be discussed as groups of metabolites, namely aglucones, glucosides, monoprenyl and diprenyl derivatives (Figs. 3–5) rather than as individual compounds.

Isoflavonoid accumulation by cultured tissues

Analysis of the isoflavonoids of tissue aggregates and finely divided cells indicates distinct qualitative and quantitative differences between both cell populations (Figs. 3, 4) that warrant separate treatment.
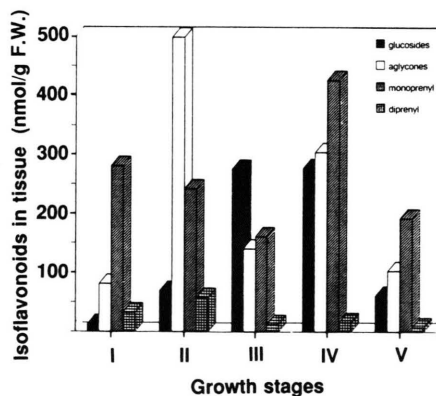

Fig. 3. Isoflavonoid accumulation (nmol/g fresh weight) by tissue aggregates at different stages of culture growth.

The isoflavonoids profile of tissue aggregates consists mainly of aglucones, glucosides and monoprenylated derivatives, which increase invariably during growth stages I–IV and markedly decrease in stage V cells (Fig. 3). The three groups of metabolites prodominate during growth stages II–IV, especially the aglucones which are the substrates destined for further glucosylation and/or prenylation. However, the amount of diprenylated derivatives, which are the least abundant metabolites, seem to decrease during culture growth (see below).

The isoflavonoid pattern of finely divided cells, on the other hand, consists predominantly of monoprenylated derivatives which reach a maximum in growth stage IV, then decline to half that amount in stage V cells. In contrast, the other groups of metabolites are present in trace amounts during all stages of culture growth (Fig. 4). It is interesting to note that, of all four groups of metabolites, the ratios of genistein-derived compounds (**1–6**) to those derived from 2-hydroxygenistein (**1a–6a**) are almost equal during culture growth, except for the aggregates of stage IV and the cells of stage V, which amount to 0.6 and 3.7, respectively (data not shown).

Metabolite excretion

Fig. 5 shows the levels of the individual groups of isoflavonoids that are released by the cultured tissues into the medium. In spite of their low abundance, there was an exponential increase in
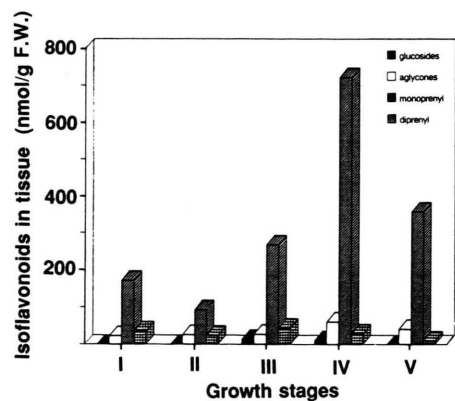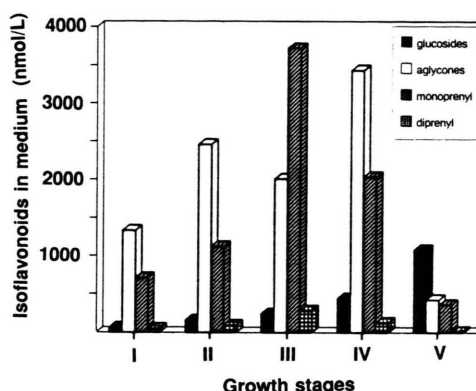
Fig. 5. Release of isoflavonoids (nmol/l) into the culture medium at different stages of culture growth.

the excretion of glucosides during culture growth. On the other hand, both aglycones and monoprenylated derivatives represent the bulk of metabolites released during growth stages I–IV, although they drop to $< 30\%$ of their maximum in stage V. Calculation of the amounts of isoflavonoids released in the medium relative to those produced by the tissue aggregates and finely divided cells indicates that the latter contribute to a much higher ratio of metabolite excretion especially during stages I–III of culture growth. In fact, stage II cells contribute to the bulk of isoflavonoids released into the medium (Table I).

Discussion

The biosynthetic potential for isoflavonoids by white lupin cell cultures is remarkable, and yields of up to $0.8 – 1$ μmol/g fresh tissue (Table I) are impressive, considering the very slow rate of culture growth. In contrast with other cell cultures which produce secondary metabolites in response to either nutrient limitation by a two-stage nutrient regime [19], or after plant regeneration [20, 21], lupin culture can synthesize its characteristic metabolites when grown in a simple nutrient medium such as B 5. In addition, this culture exhibits the typical inverse relationship between cell growth and product yield [21, 22]. Furthermore, whereas most cell cultures accumulate their metabolites late in the growth cycle, isoflavonoid production by lupin cells and aggregates begins early in the cycle and increases thereafter.

Fig. 4. Isoflavonoid accumulation (nmol/g fresh weight) by finely divided cells at different stages of culture growth.

Table I. Isoflavonoid accumulation by lupin cultured tissue and its release into the medium.

| Parameter	Growth stages				
	I	II	III	IV	V
Total isoflavonoids released into the medium as:					
[nmol/l][a]	2155	3840	6260	6045	1875
[nmol/g fresh tissue[b]]	24.5	33.0	39.0	29.6	9.9
Isoflavonoid accumulation (nmol/g fresh tissue) in:					
Aggregates	408	867	592	1062	365
Cells	231	142	352	822	408
Ratio of secretion to accumulation [%] by:					
Aggregates	6.0	3.8	6.6	2.9	2.7
Cells	10.6	23.2	11.1	3.6	2.4

[a] Values taken from Fig. 5;
[b] Calculated on the basis of tissue weight (g/l) as in Fig. 1.

Both the qualitative and quantitative differences in isoflavonoids of aggregates (Fig. 3) and cells (Fig. 4) are not unexpected, since the former are considered to represent a higher level of differentiation/organization [20]. It may be envisaged, therefore, that whereas the cell aggregates produce mainly a mixture of aglucones, glucosides and monoprenylated derivatives, the finely divided cells that are derived from these aggregates are specialized in the synthesis of mainly monoprenylated compounds. However, the paucity in production of the diprenylated compounds, **6** and **6a** by both types of cells is noteworthy, although both the 6- and 3'-prenylated intermediates are present in abundance, and are available for further prenylation. More work will be required to investigate the nutritional or hormonal conditions which favour the biosynthesis of diprenylated metabolites.

The fact that lupin cell cultures can reproduce the same isoflavonoid pattern of the intact root is remarkable, although the latter tissue usually contains trace amounts of the 8-prenylated compounds **4** and **4a,** as compared with the 6- and 3'-isomers (unpublished data). However, the isoflavonoid pattern of the cultured tissues constitutes the entire complement of intermediates that is involved in the biosynthesis of the diprenylated isoflavonoids lupalbigenin (**6**) and 2'-hydroxylupalbigenin (**6a**) (Scheme II). In the absence of biosynthetic studies of lupin isoflavonoids, it is not known whether prenylation of genistein and 2'-hydroxygenistein proceeds along separate routes, or

that some prenylated genistein intermediates may be further hydroxylated to their corresponding 2'-hydroxy analogs. However, in contrast with other cultures [18], the constitutive expression of white lupin cell culture for the biosynthesis of prenylated isoflavonoids characteristic of the root is remarkable, and their predominance over the aglucones or glucosides, especially in the finely divided cells, is noteworthy. This situation contrasts with other cultured tissues in which prenylation of such compounds can only be achieved after elicitation with biotic [23] or abiotic [13] elicitors. These features render lupin cell culture as a good source of enzymes for the study isoflavonoids prenylation.

Another characteristic feature of this culture is the release into the medium of a variety of lipophilic prenylated isoflavone derivatives, without the use of biotic or abiotic elicitors [24], immobili-
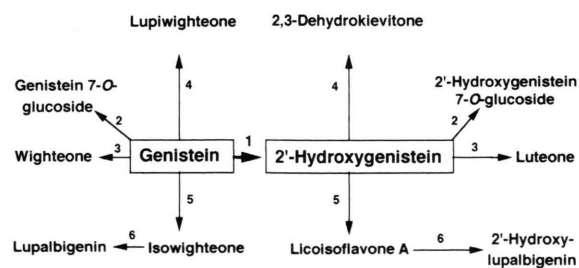
Scheme II. Proposed pathway for the biosynthesis of lupin isoflavonoids that are derived from genistein and 2'-hydroxygenistein: 1, 2'-hydroxylation; 2, 7-*O*-glucosylation; 3, 6-prenylation; 4, 8-prenylation; 5, 3'-prenylation; 6, 6-prenylation.

zation [25], or cell permeabilization [26]. The extent of metabolite excretion by lupin cells is notable, and amounts to more than 20% of the isoflavonoid production by stage II cells (Table I). This does not take into account possible degradation and/or polymerization of isoflavonoids as a result of secretion of various lytic enzymes into the medium, e.g. peroxidases [8]. The release of secondary products into the medium is considered a desirable biotechnological trait for cell culture metabolites, especially those with commercial importance. The dramatic increase in flavonoid secretion by lupin cultures is not due to lysis of cells, since microscopic examination indicated that the latter remain viable until the end of the culture period. On the other hand, the release of isoflavonoids into the medium may have a direct/indirect effect on cell division and cell growth, and may explain the slow growth rate of lupin culture. Labeling experiments with [14C]phenylalanine to 4- and 8-day cultured cells, for 24 h, resulted in incorporation of label into the aglucones and most prenylated compounds, indicating an active *de novo* synthesis of these flavonoids (unpublished data).

It is not known with certainty whether excretion of isoflavonoids into the medium involves preformed 'vacuolar' molecules, or *de novo* synthesized ones in 'cytoplasmic vesicles' [27]. The fact that several labeled prenylated flavonoids were re- covered from the culture medium in the above mentioned isotopic experiments tends to support the latter view. However, precise studies of the intracellular localization of lupin flavonoids will be required in order to better understand their compartmentation within the cell and their release into the medium. Furthermore, there is a lack of knowledge as to the mechanism(s) implicated in secondary metabolite secretion, and whether it involves simple diffusion or carrier-mediated transport [28, 29]. There is, at present, ample evidence to suggest that secondary metabolites are transported across membranes by specific carrier proteins ([28] and refs. therein). Assuming that a similar mechanism operates in the release of prenylated isoflavonoids, implies the existence in lupin cells of a number of 'isoflavonoid-carriers' with high selectivity for individual compounds, or specific binding of a general carrier for structurally related compounds, *e.g.* prenylated isoflavonoids.

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