Effects of Culture Conditions on Isoflavonoid Levels of Transformed and Non-Transformed Cultures of *Lupinus* – a Comparison of Suspension and Hairy Root Cultures

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Some highly productive suspension and hairy root cultures were found among several transformed cultures of *Lupinus polyphyllus* and *L. hartwegii*. A transformed suspension culture Lupo 30150 and a root culture Luha 15834 containing the highest specific isoflavone glucoside content were characterized and compared with normal phytohormone-dependent lines with respect to product stability as well as to their responsiveness to external triggers, *e.g.* response to changes in the medium. While phytohormone-dependent suspension cultures lost their initial ability to form increased levels of isoflavonoids on phytohormone-free medium, the transformed phytohormone-independent suspension Lupo 30150 remained a highly productive line, despite the fact that its specific levels decreased to 60% of the initial values during several years in liquid medium. Highest stability of product patterns and levels were noted for the transformed root culture. Phytohormones had little effect on growth and isoflavonoid levels in suspension cultures, while they reduced both strongly in root cultures. In the presence of 2,4-D the root culture changed into an aggregated low producing suspension culture from which the root state was recovered on phytohormone-free medium. As long as the root state was maintained, isoflavonoid levels could not be distinctly improved by media variation while specific isoflavonoid levels of suspensions were increased by stress factors such as phosphate depletion. When suspensions were transferred to fresh medium phenylalanine ammonia-lyase was greatly induced within 24 h, while the activity remained nearly unchanged in root cultures.

Introduction

Isoflavonoids belong to the class of secondary metabolites which have often been found to accumulate in morphologically undifferentiated suspension cultures [1, 2]. Though *Lupinus* species are known to accumulate a great variety of highly fungitoxic isoflavonoids in their leaves and roots [3, 4], cell cultures of *Lupinus* have not been investigated for their isoflavonoid patterns and levels. We have recently identified several isoflavone glucosides in transformed and non-transformed suspension and hairy root cultures of *L. polyphyllus* and *L. hartwegii* [5]. When isoflavonoid levels in suspensions and root cultures were compared, the root cultures in general contained higher levels of isoflavone glucosides. However, among the low producing *Lupinus* suspensions, a few were detected accumulating rather high levels of isoflavonoids. Lumpy, aggregated cultures regularly formed higher product levels, but the best producer was a fine phytohormone-independent transformed suspension culture. Indeed this line, Lupo 30150 was not only unique because of its high isoflavonoid content but also because it had a completely different pattern to all other suspensions analyzed.

It has often been shown that secondary metabolite levels of suspension cultures are strongly affected by the media composition [6]. In particular deletion or alteration of phytohormones may have large effects on the metabolite accumulation. As cell cultures transformed with wild type strains of *Agrobacterium* grow without any phytohormones, it was of interest to see whether transformed suspension cultures respond to the same extent to media alterations as phytohormone-dependent cultures. A great advantage of undifferentiated cell suspensions is their tendency to respond rather

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthaleneacetic acid; BAP, 6-benzylaminopurine; Kin, kinetin.

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rapidly to changes in their environment. Therefore we wanted to analyze whether root cultures show similar responses as suspension cultures to external triggers. The experiments described here were performed over a period of 4 years and thus some conclusions can be made with respect to the stability of product patterns in normal and transformed suspension and root cultures.

Materials and Methods

Cell cultures

Initiation, maintenance and isoflavone glucosides of the *Lupinus* cell lines studied here in more detail have been given in the preceding paper [5]. The cell lines Lupo *(L. polyphyllus)* (fine) and Luha *(L. hartwegii)* (aggregated/lumpy) are normal phytohormone-dependent green suspension cultures maintained on MS-medium [7] with 2 μM 2,4-D. The line Lupo 30150 is a fine white suspension culture, while Luha 30150 is green culture, both of which were produced by transformation with *A. tumefaciens* DSM 30150, growing on MS-medium without phytohormones. Luha 15834 and Lupo 15834 are hairy root cultures transformed with *A. rhizogenes* 15834. For media experiments 10–12 days old suspensions or 2–3 weeks old root cultures were transferred to the specified media for the periods indicated.

Determination of isoflavonoids

Cell extracts (80% MeOH) were analyzed by HPLC on a RP18-column as described [5]. The levels of the individual or total isoflavone glucosides were quantitated by comparison with a standard curve of genistein. The total isoflavonoid level is expressed as mg genistein equivalents/g dry mass.

Feeding and metabolism of 2,4-D

2,4-D (2–50 μM) were traced with 37 kBq 2,4-dichlorophenoxy-2-[^{14}C]acetic acid. Absorption was calculated from the decrease of radioactivity in the medium. Cells were extracted with MeOH after 7 d and chromatographed in the following systems on TLC-plates (silica gel): L1 BuOH:CH_3COOH:H_2O, 4:1:1; L2 $CHCl_3$:MeOH:H_2O, 60:40:5; L3 $(C_2H_5)_2CO$:petrolether:HCOOH, 50:50:2; L4 $(C_2H_5)_2CO$:C_6H_{14}:HCOOH, 70:30:2. Distribution of radioactivity was monitored by autoradiography.

Measurement of phenylalanine ammonia-lyase: Preparation of PD-10 (Pharmacia) purified enzyme extracts and the enzyme assay were performed as described [8]. Protein was determined by the Bradford method [9].

Results and Discussion

Comparison of a phytohormone-dependent with a phytohormone independent culture

Among the transformed suspension cultures was a white line Lupo 30150 containing 6–10 times higher levels of isoflavone glucosides than the corresponding green wild type strains. Though it was one of the first transformed lines we obtained, it has remained the highest producer of all our transformed *Lupinus* suspension cultures. Not only the high amount of isoflavonoids but also its unique isoflavonoid pattern attracted our attention. In contrast to all other suspension cultures and root cultures, this line accumulated high levels of **1** and **2** (Fig. 1). Initially **1** (2′-hydroxygenistein-7,2′-di-O-glucoside) accounted for up to 50% of

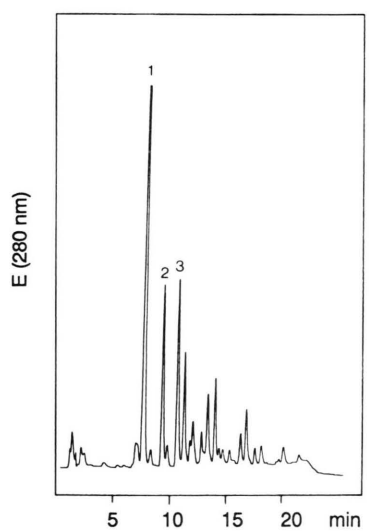

Fig. 1. Methanolic cell extract of Lupo 30150 chromatographed on a RP_{18}-column in a gradient from 20 to 60% aq. MeOH with 0.5% CH_3COOH in 20 min. **1** = 2′-hydroxygenistein-7,2′-di-O-glucoside; **2** = genistein-7,4′-di-O-glucoside; **3** = 2′-hydroxygenistein-7-O-glucoside.

all isoflavone glucosides in this line, followed by **2** (genistein-7,4′-di-O-glucoside) with 20%, while the amounts of the various isoflavone glucosides of wild type cells were all very similar. However, over a period of 4 years the quantitative ratio of the various compounds changed as indicated in Fig. 1. The levels of **1** and **2** decreased in relation to the other isoflavone glucosides so that these two compounds account at present for only 30–35% of all isoflavone glucosides in this line. During the same period the specific level of isoflavonoids of this line decreased by 40% but remained 6-fold higher compared to the corresponding wild type cell line. The low isoflavonoid levels of the phytohormone-dependent wild type cells did not decrease any further. Nevertheless, another "instability" was noted during the experimentation period as shown below.

Initially it was assumed that the overproduction of the transformed line was due to its independence of phytohormones. The first control experiments – induction of isoflavonoid formation in wild type cells after transfer to phytohormone-free medium – seemed to support this idea. However, the analyses of several other lines showed that many phytohormone-independent transformed suspension cultures contained similar levels of isoflavones as normal wild type cells.

When Lupo wild type cells were transferred to a medium lacking phytohormones (Fig. 2) growth decreased from passage to passage while their specific isoflavonoid content increased greatly, reaching 80–90% of the level of the transformed line. The inducibility of the isoflavonoid levels of the wild type strain on phytohormone-free medium was repeatedly shown during the first 12–18 months after culture initiation. This result was not unusual, as removal of 2,4-D from the medium has often resulted in increased product levels of different pathways [6, 10–12]. As the above experiment indicated a negative effect of 2,4-D on the isoflavonoid level, the transformed line was transferred to 2,4-D containing media (Fig. 3). However, at 2,4-D concentrations used in cell culture media (1–5 μM) only a minor decrease of the isoflavonoid content and of growth was noted. Even at 50 μM 2,4-D, exceeding the concentration of the growth medium of wild type cells 25-fold, specific isoflavonoid levels of Lupo 30150 were still higher than in wild type cells.

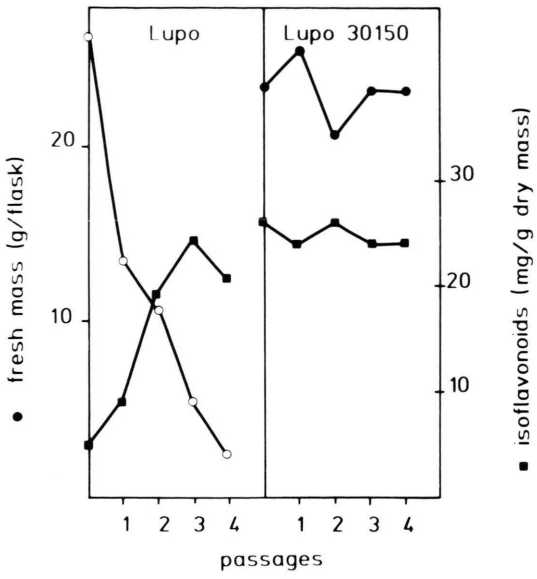

Fig. 2. Effect of depletion of 2,4-D on a phytohormone-dependent Lupo cell culture (maintained as suspension culture for 15 months) on growth and isoflavonoid levels. For comparison growth and isoflavonoid production of the transformed phytohormone-independent line Lupo 30150 is given. Inoculum was 2 g/70 ml and each passage lasted 10–12 days.

The low response of the transformed line to 2,4-D made us measure and compare 2,4-D uptake and metabolism by the normal and the transformed cell line (Fig. 4). A short delay in the uptake of 2,4-D by the transformed line was noted.

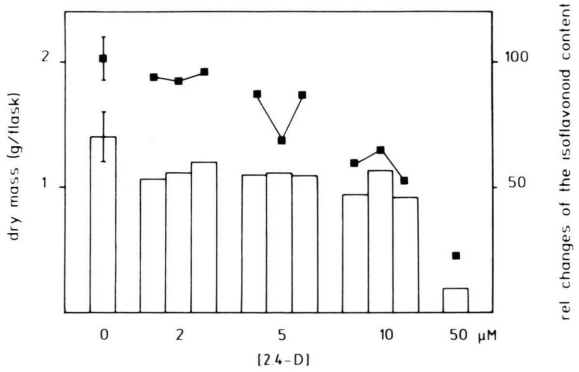

Fig. 3. Effect of 2,4-D on growth and isoflavonoid levels (■) of 18 months old Lupo 30150 cells. Inoculum: 2 g/70 ml. Harvest after 10 d. Cells were maintained for 3 consecutive passages on the 2,4-D containing medium. The 100% value of the specific isoflavonoid content was 32 mg/g dry mass.

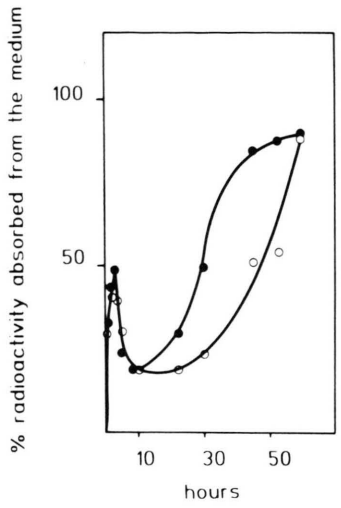

Fig. 4. Comparison of the absorption of 20 μM 2,4-D by Lupo (filled symbols) and Lupo 30150.

However, after 48–72 h 90% of 2,4-D were absorbed by both lines. Even after 7 d 95–100% of the absorbed radioactivity were extracted from the cells by MeOH. Chromatographic analyses revealed that 90–95% of the radioactivity were stored in both cell lines unmetabolized (not shown). Thus, an altered metabolism of 2,4-D was not detected in the transformed line.

The effects of other phytohormones (kinetin/NAA/BAP alone and in combinations (1–10 μM)) on Lupo and Lupo 30150 were also tested. Whenever a phytohormone composition led to a growth decrease, isoflavonoid levels increased in wild type cells in a similar manner as shown for 2,4-D while in the transformed cell line neither growth nor isoflavonoid levels were distinctly altered by any physiological concentrations of phytohormones (data not shown). Evidently, Lupo 30150 cells must have acquired complete metabolic independence of phytohormones absorbed from the medium.

From experiences with other cultures it has been known that responses to production media may change in long-term culture. For example, Berlin and Sasse [14] found that 5 year old *Peganum* suspension cultures had lost their previous inducibility of tryptophan decarboxylase and did not accumulate serotonin. Thus it was of interest to see whether the response of wild type cells and transformed cells to phytohormone changes would be reproducible in a 24–30 months old suspension culture (Fig. 5). The appearance, growth rates and productivities of the two lines had not distinctly changed during this period. The levels of the transformed line had decreased by 20–30% and the pattern resembled that of Fig. 1. The great biochemical difference between the 12–18 and the 24–30 months old wild type cells only became evident when they were transferred to phytohormone-free medium. During the first passage on this medium a small increase in biomass was generally noted before growth decreased steadily during the next passages (Fig. 5A). However, in contrast to the younger culture (Fig. 2) isoflavonoid levels were not distinctly enhanced in the growth inhibited cultures. After 5–7 passages on phytohormone-free medium a few green colonies grew out of the brown aggregates. After the 9th passage a poorly growing, aggregated green culture had been established. This apparently habituated line had the same or even lower levels of isoflavonoid glucosides than the original wild type on the nor-

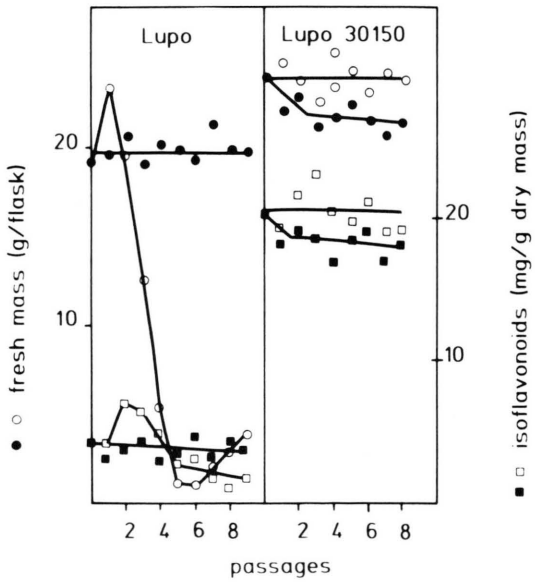

Fig. 5. Effect of A) depletion of 2,4-D on Lupo cells maintained for more than 30 months on liquid medium, and of B) addition of 2,4-D to the medium of the transformed line Lupo 30150, on growth and isoflavonoid levels. Filled symbols give data in the presence of 2 μM 2,4-D, while open symbols indicate data for the phytohormone-free medium. Generally the inoculum of each 10-d-passage was 2 g/70 ml. In the case of complete growth inhibition all aggregated cells were transferred and the culture period was extended to 3–4 weeks.

mal growth medium. Clearly, the wild type line had lost its initial ability to respond to phytohormone depletion with increased productivity. The previous reversed relationship between growth and isoflavonoid production present in the young cultures was decoupled in the older cultures.

Despite the fact that transformed phytohormone-independent lines of *Lupinus* did not necessarily yield higher productive cultures than might be found during a normal culture initiation programme [6], the observation with Lupo 30150 cells suggests inclusion of transformation with *A. tumefaciens* in such a programme and screening of transformed and non-transformed cell clones for the highest producers. According to the observations made with Lupo 30150 one could speculate that working with phytohormone-independent lines would be more reliable, especially when similar specific yields may only be obtained in growth-inhibited wild type cells after deletion of phytohormones.

Responses of hairy root cultures to phytohormones

While the phytohormone-independence of plant cells transformed with *A. tumefaciens* seems to result from the overproduction of auxin and cytokinine by T-DNA genes transferred into the plant genome, hairy root tumors seem to result from an increased sensitivity of the transformed cells to phytohormones [15]. Indeed the root cultures reacted quite differently to the phytohormone treatments (Table I) compared to the transformed suspension culture. In contrast to the transformed suspension culture growth of the roots was greatly inhibited by the various phytohormone compositions. During the second passage on a phytohormone containing medium growth ceased. In the case of 2,4-D callus formation was noted which resulted in an aggregated slowly growing suspension culture (biomass doubled within 4 weeks) after the 5th passage. The specific isoflavonoid content had decreased to 10–20% after the second passage and remained low in the suspensions. As growth did not improve during the next passages, the cells were returned to phytohormone free medium. It took 2–3 passages before the first roots reappeared from the aggregates. After two further passages the root culture was re-established. Its productivity was initially a little lower but recovered to the original levels. Thus it is possible to grow hairy root cultures of *Lupinus* as suspension or at least as callus like culture in the presence of phytohormones and re-induce the root state by deleting the phytohormones.

Table I. Growth and relative changes of isoflavonoid content of Luha 15834 root culture when transferred to phytohormone containing media for 3 weeks. Initial inoculum: 2 g/70 ml. Isoflavonoids are expressed as genistein equivalents.

Medium + phytohormone	Biomass [g/flask]	Isoflavonoids [%]
MS + none	15.8	100
+ 2 μM 2,4-D	3.4	48
+ 1 μM BAP	3.4	60
+ 5 μM NAA/1 μM Kin	4.6	67
B5 + 5 μM 2,4-D	3.3	100

This change into a suspension culture could be a useful step for the scale up of a root culture. The desired production state "root" need only be induced in the last fermenter. However, in the case of *Lupinus* we noted two problems – 1. growth of the phytohormone treated roots was disappointingly low and 2. reappearance of the roots was not uniform. If both factors can be improved, the way root–suspension–root may become interesting from a biotechnological point of view.

Effects of medium constituents on growth and isoflavonoid levels

Interestingly, the isoflavonoid content remained unchanged when B5 [16] was the medium to which 2,4-D was added (Table I). This indicated that ammonium ions may affect isoflavonoid levels in *Lupinus* root and suspension cultures. Indeed, it was clearly shown that ammonium ions have a rather negative effect on the isoflavonoid levels in normal and transformed suspension cultures (Table II) and in root cultures (Fig. 6). Growth of suspensions and roots could not been maintained when ammonium was the only nitrogen source. Even when comparing NH_4NO_3 and KNO_3 as nitrogen sources the negative effect of the ammonium ions remained evident. As biomass and isoflavonoid production are differently affected by the nitrate concentrations (Fig. 6), fine tuning of the nitrate level is required for optimal production.

Depletion of phosphate stimulated the specific secondary metabolite content in many suspension culture systems (see for review [6]). Thus the in-

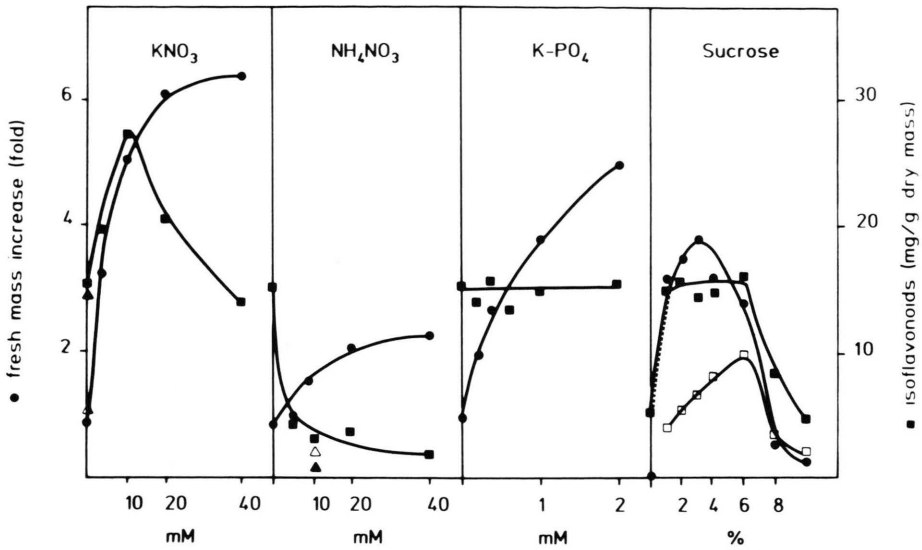

Fig. 6. Effects of media variation on growth and isoflavonoid levels of Luha 15834. Inoculum was 1 g/70 ml. Roots were harvested after 18 d. The open squares of the last column indicate the dry mass pattern at the various sucrose concentration.

Table II. Responses of Lupo and Lupo 30150 on growth and isoflavonoid levels to changes in the medium composition. Growth medium was for Lupo MS + 2 μM 2,4-D, for Lupo 30150 MS without phytohormones. Cells were harvested after 14 d. Isoflavonoids are expressed as genistein equivalents.

	LUPO		LUPO 30150	
	Biomass mg/flask	Isoflavonoids mg/g dry mass	Biomass mg/flask	Isoflavonoids mg/g dry mass
Growth medium (GM)	561	2.1	562	16.9
GM−P	140	8.5	137	27.4
GM−N	186	3.4	192	18.3
GM−N				
+ 10 mM $(NH_4)_2SO_4$	72	0.6	91	5.4
+ 10 mM NH_4NO_3	215	1.4	232	10.2
+ 10 mM KNO_3	275	2.7	306	19.8
+ 10 mM KCl	182	3.2	196	17.6
GM with 8% sucrose	272	2.9	275	22.4

crease of the specific isoflavonoid content of *Lupinus* suspension cultures by deletion of phosphate was nothing special (Table II). The specific content of the root culture, however, was not affected by a medium lacking phosphate (Fig. 6). Phosphate levels affected only growth of the roots. As we noted no stimulatory effect of phosphate depletion on secondary metabolite formation in other root cultures (*e.g.* β-carbolines in *Peganum* [17], one may conclude that phosphate depletion is not a reliable indicator of stress-inducible pathways for root cultures, in contrast to suspension cultures.

Higher sucrose concentrations (*e.g.* by causing osmotic stress) have also repeatedly been shown to increase secondary metabolite production in suspension cultures [6]. *Lupinus* cell suspensions contained also more isoflavonoids/g dry mass in the presence of 8% sucrose. In contrast, not only growth but also the isoflavonoid content of root cultures decreased greatly in the presence of high osmotic stress (Fig. 6).

While secondary product formation in suspension cultures was often shown to be induced by growth inhibition or stress factors [6], this principle may not be transferable to root cultures. The

specific levels or total yields of the root cultures may be improved by finding the best growth medium [18, 19] or by changing the appearance of roots *e.g.* by altering the NH_4^+/NO_3^- ratio [17, 20].

Inducibility of PAL-activity in suspension and root cultures

Phenylalanine ammonia lyase (PAL) is an enzyme which is easily induced in many suspension culture systems when transferred to fresh medium or stress media [21, 22]. The sluggish response of the root cultures to the medium variation let us check how the activity of this enzyme would change in comparison to suspension cultures (Fig. 7). Independent of their productivity and their growth rates all suspension cultures showed a distinct increase in PAL activity within 24 h after the transfer and returned back to the low PAL-level within the next 24–48 h. In contrast, PAL-activity of both root cultures did not change in the fresh medium. However, their specific activity was generally a little higher than in the suspensions with the exception of the first 24–48 h. While specific product levels of the cultures varied over the growth cycle, the isoflavonoid levels of the root cultures remained constant. Thus the sluggish reactions of secondary metabolism of root cultures to external manipulations might result from the fact that in an organized tissue enzyme activities do not or can not change as freely as in suspension cultures. The sluggish reaction of organized tissues to manipulations might hamper their use in identifying regulatory controls of secondary pathways. On the other hand a correlation between PAL-inducibility and isoflavonoid levels of the various suspension cultures is hardly seen (Fig. 7) raising questions as to whether enzymes inducible in suspension cultures always provide reliable clues for regulatory controls. Indeed, without having analyzed potential factors influencing the accumulation pattern (turnover or dilution), it is not possible to establish how close PAL-activity is correlated with isoflavonoid synthesis which accounts for more than 90% of all soluble cinnamic acid derived metabolites in *Lupinus*.

In conclusion, whether root cultures can be used for biotechnological purposes is still disputed [23],

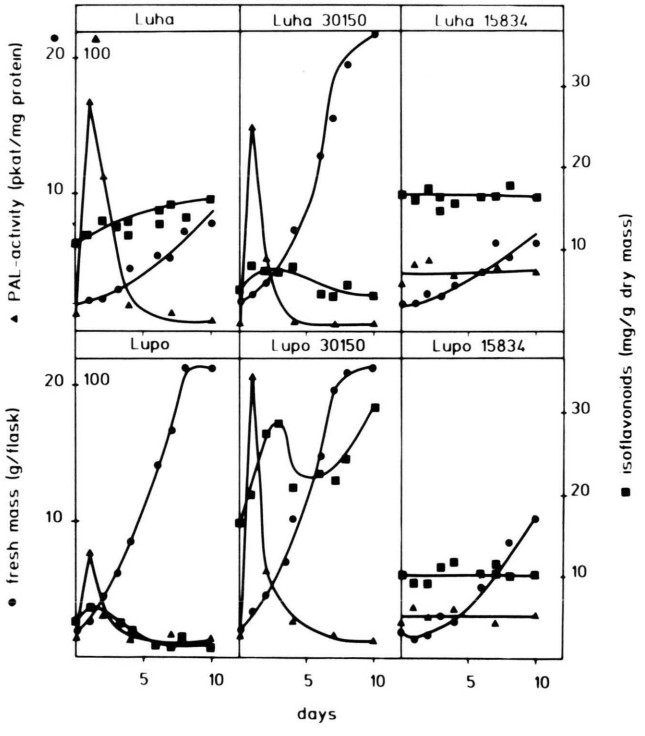

Fig. 7. Comparison of PAL-activity and isoflavonoid levels during a 10 d growth period of normal suspension cultures (Lupo, Luha), transformed suspension cultures (Lupo 30150, Luha 30150), and hairy root cultures (Lupo 15834, Luha 15834). Inoculum size was 2 g fresh mass/70 ml medium.

despite the fact that they produce reasonable levels of interesting compounds [24]. Our finding that transformed root cultures can be grown as suspension cultures may show a new avenue for overcoming the technical hurdle. Presently more important is the fact that root cultures often contain compounds which are not found in suspension cultures [24]. Even in the case of isoflavonoids the different quantitative pattern of roots and suspensions may favor one or other systems for a specific study. While for suspensions optimization of product formation by defining growth-limiting production media is often a worthwhile step, efforts for improving product levels of roots may concentrate on the effects of the N-source and N-concentration of the growth medium.

Acknowledgements

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