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Ontogenic variations in *n*-alkanes during somatic embryogenesis of flax (*Linum usitatissimum* L.)

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Summary

Hypocotyl segments of flax seedlings germinated in vitro, were used to induce indirect somatic embryogenesis on solid medium. The composition and distribution of *n*-alkanes in flax tissues collected at different developmental stages

were studied by GC and GC-MS. During induction and development of callus from hypocotyl tissues a decrease in the percentage of total lipids was observed. In all types of tissue sampled - hypocotyl segments used as primary explants, hypocotyl segments with differentiating calli at the cut ends, embryogenic and non-embryogenic calli and somatic embryos - a skewed-normal distribution of *n*-alkanes with a low mass range (C_{13}-C_{21}) were found. The highest content of *n*-alkanes occurred in the primary hypocotyl explants and in the early stages of callus development. Longer carbon chain *n*-alkanes were observed only in the mature or differentiated tissues of hypocotyls and somatic embryos. Although the *n*-alkane contents decreased with time, in somatic embryos and calli, a significantly lower *n*-alkane content was observed in embryogenic calli when compared to non-embryogenic calli independent of the time in culture. These results suggest the utilisation of *n*-alkanes for heterotrophic cellular growth as well as its mobilisation from embryogenic calli to developing somatic embryos.

Abbreviations: 2,4-D - 2,4-Dichlorophenoxyacetic acid; EC – Embryogenic callus; GC - Capillary gas chromatography; GC-MS - Capillary gas chromatography - mass spectrometry; HS – Hypocotyl segments; HSC – Hypocotyl segments with but incipient callus at the cut ends; ID – Internal diameter; MS – Murashige and Skoog; NEC – Non-embryogenic callus; SE – Somatic embryos; ZEA - Zeatin.

Introduction

n-Alkanes (C_nH_{2n+2}, abbreviated C_n) are widely distributed in the plant kingdom, especially associated with epicuticular leaf waxes in higher plants. These highly hydrophobic aliphatic molecules are important components of the

cuticles in maintaining plant water balance [1], although their physiological function within the plant cells is poorly understood. Several studies during the last 30 years have shown that superimposed to the wide *n*-alkanes content and distribution patterns variability, leaf waxes are basically composed of the homologous series C_{17}-C_{35}, typically with an odd carbon chain predominance and with C_{29} and/or C_{31} as the major components [2]. Internal *n*-alkanes have typically shorter chain lengths, and the distribution of the homologous series is generally flatter or gaussian-like, lacking alternation between an even and odd number of carbons. A vast number of studies on leaf *n*-alkanes explored these compounds as a taxonomic aid [3-8] mainly due to the high species-specific coherence in the *n*-alkane distribution patterns.

Lipid composition is controlled by both genetic and environmental factors [9, 10]. It was reported that the leaf age [11-13], the type of organ in a plant [5], the type of tissue [14] and the species position in the evolutionary scale [6, 15], among other factors, could influence the content and/or the distribution pattern of *n*-alkanes. It is well documented that lipid biosynthesis is developmentally regulated in plants [10]. Several references describing lipid accumulation and utilisation during somatic embryos germination can be found in the literature [16-18], but little is known about lipid modifications that occur during the onset of embryogenesis [19]. There are no reports concerning the *n*-alkanes changes during somatic embryogenesis. The main objective of the present work was to study the ontogenic variations in content and distribution patterns of total *n*-alkanes during the induction and expression of somatic embryogenesis from flax hypocotyl explants.

Materials and Methods

Plant material and growth conditions

Flax hypocotyl segments from one-week-old in vitro grown seedlings were inoculated on an embryogenic medium [20] and cultivated in a growth cabinet at 24 °C under a 16 h photoperiod of 50 μmol. $m^{-2}.s^{-1}$ for 7 weeks. The embryogenic medium consisted in the MS basal medium [21] supplemented with 1.8 μM 2,4-D + 7.3 μM ZEA that were added before autoclavation of the medium. During the growth period no tissue transference were made and the samples were taken at time zero (t_0), after 2 (t_2), 5 (t_5) and 7 (t_7) weeks. The t_0 samples consisted of hypocotyl segments (HS); the t_2 samples of hypocotyl segments with incipient callus differentiation at the cut ends (HSC); the t_5 samples of embryogenic calli (EC), derived somatic embryos in a pos-cotyledonary stage (SE) and apparently non-embryogenic calli (with no visible somatic embryos) (NEC); and the t_7 samples of non-embryogenic calli (NEC), embryogenic calli (EC) and derived somatic embryos (SE). At t_7 somatic embryos were already developing to small plantlets. All samples were replicated and each replicate represents a pool of several cultures consisting of various numbers of plant materials: 50 of HS; 40 of HSC; 8 of NEC; 25 of EC and 110-138 derived somatic embryos (SE). The material was stored at – 80 °C and then freeze-dried.

Lipid extraction and analysis

The freeze-dried powdered material was extracted for total lipids by *n*-hexane during 48 h in a Soxhlet apparatus. Neutral lipid fractions (N) were obtained from known amounts of total lipid extracts by *n*-hexane:propanol

partition at pH 12. These N fractions were evaporated to dryness by a rotary evaporator, under nitrogen flow, and dissolved in a fixed volume of *n*-hexane.

For GC quantitative analysis of the *n*-alkanes present in the N fraction, a known amount of 5-α-cholestane (pure Sigma standard) was added to each sample as an internal standard. At least 3 analyses were made per replicate. The analyses were performed with a GC instrument (Perkin Elmer 8600) equipped with a split-splitless injector at 300 °C, a flame ionization detector at 320 °C and a BP5 column (25 m long x 0.22 mm ID x 0.25 μm film thickness of 5 % phenyl dimethyl siloxane). The set temperature program was from 100 °C to 280 °C at a rate of 5 °C/min. followed by an isothermal for 15 min. H_2 was used as carrier gas, with a column head pressure of 12 PSI.

To identify the *n*-alkanes, a mixture of 18 *n*-alkane Sigma standards (C_{14}-C_{32} with the exception of C_{30}) was used together with the internal standard. A correction factor that accounted for both the response factor and split discrimination was estimated from 5 analyses of that standard mixture. The quantitative output values were then corrected by the factor $0.98^{(Ci+1)}$, where C_i is the difference in carbon number between C_{32} and the alkane i.

The identification of peaks was confirmed by GC-MS (Perkin Elmer 8500), equipped with a 30 m long x 0.25 mm ID DB5 column (0.25 μm film thickness of 5 % phenyl dimethyl siloxane) connected to an Finnigan MAT ion trap detector (IDT) operating in Electron Impact (EI) mode at 70 eV. Split-splitless injector, interface transfer line and ion source temperatures were 300 °C, 260 °C and 220 °C respectively. The oven temperature program was as described above. He_2 was used as carrier gas with a column head pressure of 14 PSI.

Statistical analysis

Mean differences in total lipids and total *n*-alkanes between samples were analysed running the one way analysis of variance test (ANOVA), and *post hoc* comparisons were made with the Duncan test ($P < 0.05$). All the variables were homoscedastic (Bartlett's test with $P > 0.05$) with the exception of the total *n*-alkanes as a percentage of total lipids. To meet this assumption a square root transformation of this variable was made. A correlation analysis was performed to evaluate the relation ($P < 0.05$) between the *n*-alkane contents in the different types of tissues. Only the *n*-alkanes that were common in both tissues were used for this analysis. The standard deviations in figures 1, 2 and 3 were in average 15 % of the mean with a minimum and a maximum of 2 and 30 %, respectively. All the statistics were performed with the software Statistica@ 4.1 from StatSoft.

Results and Discussion

Lipid content changes in flax tissues during somatic embryogenesis

A gradual decrease in the total lipids content of flax tissues was observed over the sampling period from the hypocotyl segments to the 7 week-old calli tissue (Table 1). This decrease was related with calli formation (HSC) since calli cultures had a lower lipid content when compared to somatic embryos and hypocotyls (Table 1). As already described for flax [22], the lipid content of calli was 2-3 times lower than that of somatic embryos. In both t_5 and t_7 sampling times no significant differences were detected in the total lipid content between embryogenic and non-embryogenic calli (Table 1). From t_5 to t_7, although the general trend for a decline in total lipids has been maintained, it was not significant for either NEC, EC or SE (Table 1).

Table 1 - Contents of total lipids and total *n*-alkanes in HS, HSC, NEC, EC and SE obtained during the period of induction and expression of somatic embryogenesis from flax hypocotyl explants[a].

Plant material	Total lipids (% of dry weight)				Total *n*-alkanes (% of total lipids)			
	Sampling time				Sampling time			
	t_0	t_2	t_5	t_7	t_0	t_2	t_5	t_7
HS	15.6 a				8.09 a			
HSC		7.03 c				8.13 a		
NEC			3.41 bd	2.03 b			4.54 b	1.53 c
EC			3.45 bd	2.31 b			1.49 c	0.31 d
SE			6.17 c	5.37 cd			8.48 a	2.7 [b]

[a] Data represent the mean of two replicates, and each replicate is the average of 3 GC analyses. For each measured variable, means followed by the same letters were not statistically different ($P > 0.05$).

[b] It was not possible to include this value in the statistical analysis due to the loss of a replicate.

The proportion of *n*-alkanes varied from 0.3 to 8.5 % of the total lipids. Although there were no significant changes during the early steps of tissue dedifferentiation and calli production (t_0 -> t_2), there was a sharp decrease in the content of those compounds in all types of tissues (NEC, EC and SE), from t_5 to t_7 (Table 1). Contrarily to what was found for total lipids, there were highly significant differences in *n*-alkane content between EC and NEC, and the ratio NEC/EC found in t_5 almost doubled in t_7 (Table 1). From these results, the *n*-alkane ontogenic variation can be pictured in two perspectives: in time, there is a decline associated with cellular multiplication and growth; and in space, there is a carbon mobilisation from EC, probably related to somatic embryos differentiation. This view is somewhat supported by the results obtained by Joy et al. [17] with white spruce embryos, that showed that suspensor cells and the basal region of the embryos played an important role in channelling nutrients to the developing embryos.

Although no quantification of the wax fraction has been made, it is assumed that the higher *n*-alkane content found in HS, HSC and SE relative to calli was mainly due to the presence of an incipient epicuticular wax. The following results on the n-alkane profile in SE further support this point.

Aliphatic hydrocarbon profiles

Two basic patterns of hydrocarbon distribution in plant tissues have been described in the literature [1, 14, 15, 23 and references therein]. Following the classification adopted by Carriere et al. [23], type A pattern is composed of short chain-length hydrocarbons, with a gaussian-like distribution and a site of biosynthesis in parenchyma tissues. Type B pattern is composed of longer chain-length hydrocarbons, with a dominance for long-chains with an odd-number of carbons, mainly located in the epicuticular waxes and synthesized in epidermal cells. The denomination Population II [14] and Group A [15] are also found in the literature as synonyms of the type B pattern.

In our flax system, the *n*-alkanes were distributed within a wide carbon length range, from C_{13} to C_{31}, and qualitative and quantitative changes occurred in the pattern of *n*-alkanes distribution during somatic embryogenesis.

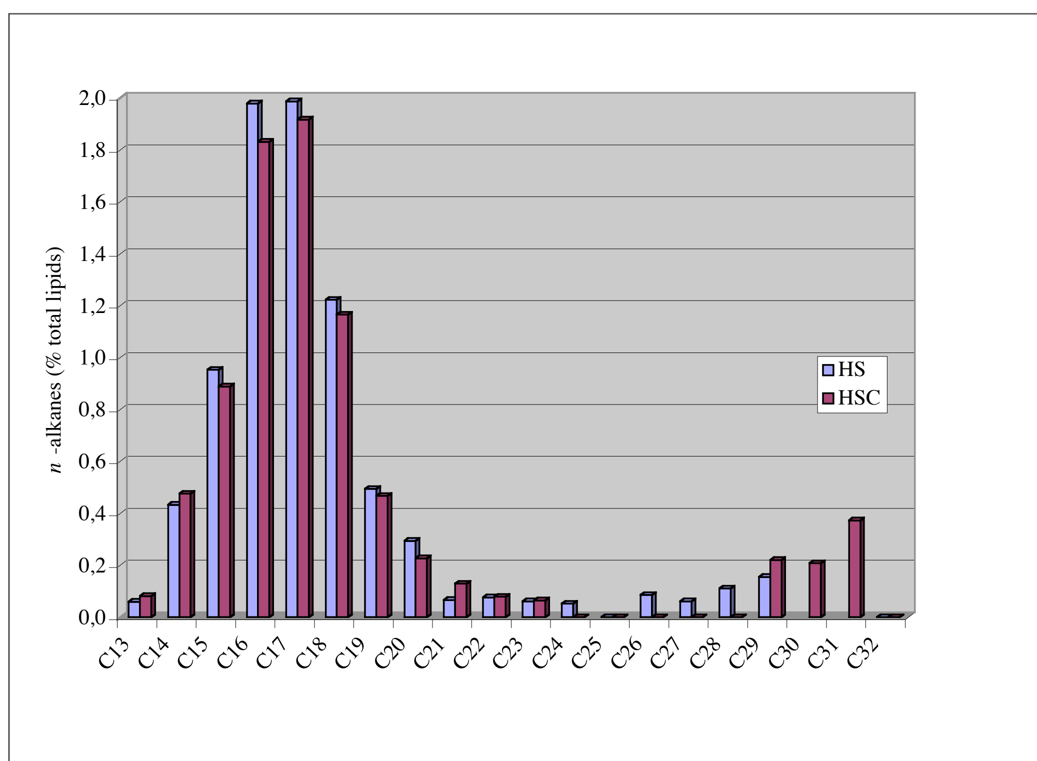

Figure 1 - Distribution of *n*-alkanes in HS (t_0) and in HSC (t_2) of flax. Hydrocarbons are described by their carbon chain length (number of carbon atoms). In the GC analysis the chromatogram showed an irregular base line near C_{30} and C_{31} RTs, however, these compounds were detected in trace amounts by GC-MS.

Young hypocotyl tissues (HS) consisted mainly of a skewed type A pattern with a dispersion range from C_{13} to C_{21} centered unimodally at C_{16}-C_{17}. In the medium-chain (C_{22}-C_{26}) and long-chain length range (C_{27}-C_{31}) the *n*-alkanes were present in low amounts and exhibited a flat distribution with no preference for odd or even carbon numbered *n*–alkanes. No typical type B pattern was observed in HS where C_{30} and C_{31} were absent (Fig. 1). In the early dedifferentiation steps of the hypocotyl tissue with calli formation in the cut ends (HSC), no significant differences were found in the type A pattern. However, the disappearance of the alkanes C_{24} to C_{28}, correlated with the maintenance of C_{29} and the rise of the long-chain alkanes C_{30} and C_{31} (Fig. 1). The emergence of a type B

pattern predicts a maturation of a cuticle covering the hypocotyl epidermal cells that are not yet involved in the dedifferentiation process.

In exponentially growing calli, independently from its embryogenic competence, only type A hydrocarbons were found, with the same dispersion range and maxima of those obtained in HS and HSC (Fig. 2). Similar results were obtained with *Euphorbia characias* cell suspensions in heterotrophic or photomixotrophic conditions [23]. Similar to that found for young hypocotyl tissues (HS), early germinating somatic embryos (t_5) displayed a well-defined type A pattern and a flat distribution for the medium and long-chain alkanes without even or odd-chain alternation (Fig. 2).

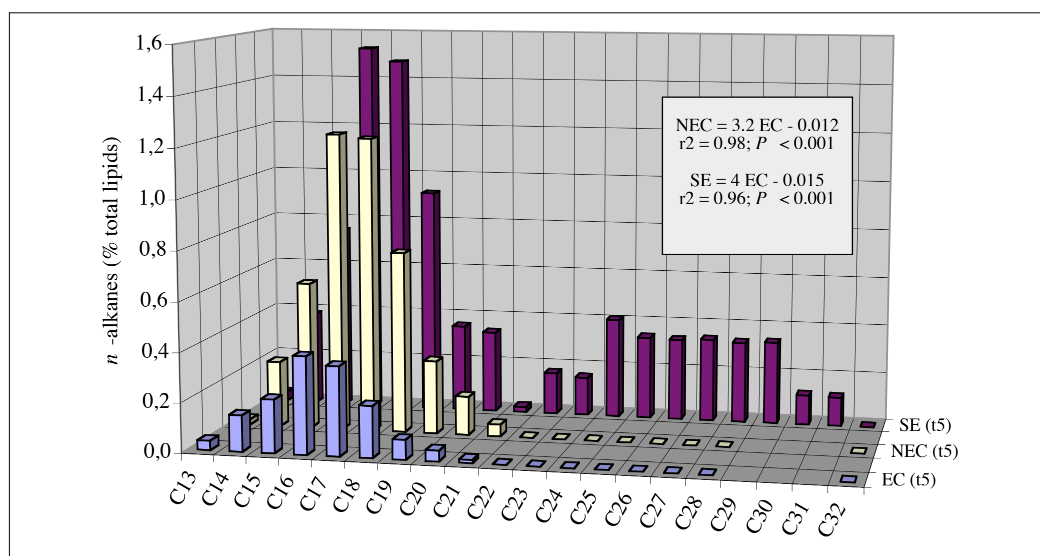

Figure 2 - Distribution of *n*-alkanes in NEC, EC and SE after 5 weeks of culture (t_5). Hydrocarbons are described by their carbon chain length (number of carbon atoms). The inserts show the equations, r^2 and *P* values from the regression analysis. The missing values were due to the occurrence of other unidentified constituents but, with the exception of C_{32}, they were detected in these samples in trace amounts by GC-MS.

Two weeks late (t_7), both types of patterns were present in somatic embryos (Fig. 3). As observed during the first stage of culture establishment (HS -> HSC), a decrease in medium-chain *n*-alkanes were paralleled by a relative

increase in the long-chain odd numbered hydrocarbons, C_{27} and C_{29}, from t_5 to t_7 (Fig. 3). The accumulation of type B long odd-chain *n*-alkanes, characteristic of epicuticular waxes, reinforces the idea of cuticle formation, even in culture conditions where the relative humidity is nearly saturation. The biosynthesis and transient accumulation of medium-chain odd or even *n*-alkanes remains unexplained.

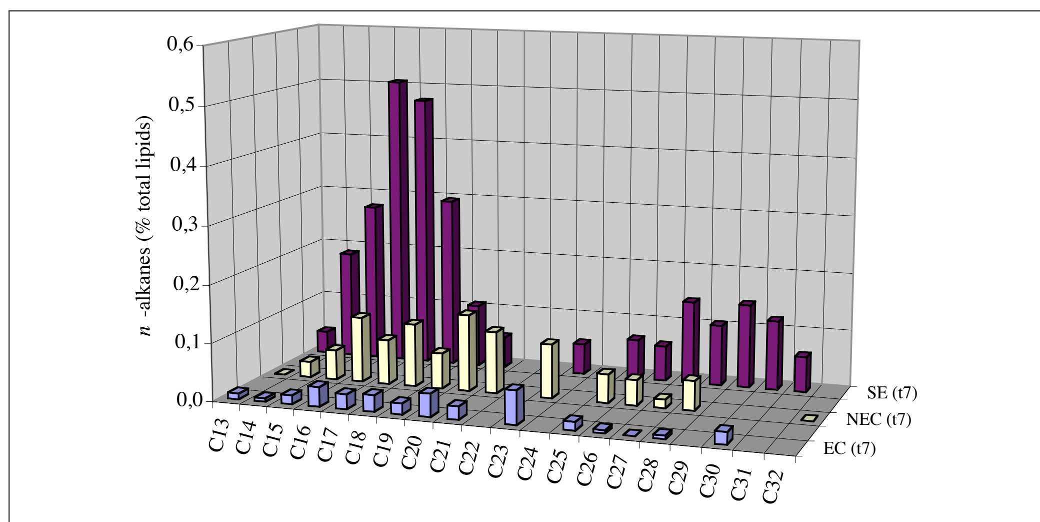

Figure 3 - Distribution of *n*-alkanes in NEC, EC and SE after 7 weeks of culture (t_7). Hydrocarbons are described by their carbon chain length (number of carbon atoms). The missing values were due to the occurrence of other unidentified constituents but, with the exception of C_{32}, they were detected in these samples in trace amounts by GC-MS.

It was observed that the content of each one of the type A hydrocarbons in somatic embryos was proportional to those of calli (NEC or EC). Moreover the accumulation of these hydrocarbons in SE could be explained by the decrease observed in EC relatively to NEC (Fig. 2, see equations). The *n*-alkanes in seed oil and in internal hydrocarbons may participate in maintaining inner cell structure [1, 24] and may act as energy storage components [24]. The proportional higher decrease of the type A hydrocarbons in EC relatively to NEC, eventually due to SE growth (Fig. 2), foresees a role for short-chain *n*-alkanes, as translocable,

reduced carbon reserves during the early steps of embryo germination from somatic tissues. Some functions for short-chain and long-chain length *n*-alkanes in the plant may be predicted from their different physical states at physiological temperatures. Phase diagrams of *n*-alkanes show that C_{16} and C_{17} had melting temperatures around 20 °C whereas C>20 had melting temperatures above 35 °C [25]. This fact, together with the even-odd effect in the melting points and crystallisation characteristics of long *n*-alkanes [25], could explain the importance of the long odd-chain *n*-alkanes as efficient water barriers, as well as the mobility of short *n*-alkanes in inner tissues.

Another interesting feature was the shift in *n*-alkane profile from the exponential (t_5) to the stationary EC or NEC growth phase (t_7). The skewed-normal distribution of *n*-alkanes observed at the short-chain range was replaced, at t_7, by a flatter and wider distribution, with a slight preference for even-carbon number hydrocarbons (Fig. 3), an uncommon type of *n*-alkane distribution in plants. Weete et al. [14] had similar results when comparing the *n*-alkane profiles of habituated callus (non-differentiated tissues) and teratoma tissues (with shoot and leaves differentiation) of tobacco cultures. As flax NEC at t_5, the habituated tobacco calli had a gaussian-like distribution in a range of C_{17} to C_{28} but with a slight even preference. Teratoma tissues had a flat distribution in the range C_{22}-C_{28} with C_{29} and C_{31} as the major components.

The mechanism of biosynthesis of hydrocarbons is poorly understood. Two pathways that operate in the biosynthesis of external *n*-alkanes have been described; the “condensation pathway”, postulated for the synthesis of tobacco hydrocarbons [26] and the “elongation pathway” proposed by Eglinton and Hamilton [27] and very recently by Somerville et al. [28] to explain the synthesis

of wax components. Both pathways, where a decarboxylation step of even-numbered fatty acid leads to the formation of odd-carbon numbered *n*-alkanes, accounts satisfactorily for type B *n*-alkanes [23]. The type A distribution, with lower *n*-alkanes and a broader carbon-numbered distribution with no odd-number preference, cannot be explained by that route. Fatty acids or their derivatives are generally considered to be closely related to the direct precursor for the hydrocarbons produced in plants [1] and the α-oxidation system had been described to participate in the synthesis of even-numbered *n*-alkanes [1, 29]. A recent study with *Arabidopsis* wax mutants [30] showed that the decarboxylation pathway cannot use fatty acids of short-chain lengths and therefore, only long-chain *n*-alkanes could be synthesized this way. It is possible that an intermediate α-oxidation pathway should produce type A *n*-alkanes. As already mentioned by Bognar et al. [29], the reason for finding a proportion of even-chain *n*-alkanes in the internal tissues higher than that usually found in the cuticular tissues might be that α-oxidation makes a more significant contribution to the former.

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