0273-2289/91/2829—0169$02.60

Oils from Wild, Micropropagated Plants, Calli, and Suspended Cells of *Euphorbia characias* L.

M. FERNANDES-FERREIRA,*[1] M. SALOMÉ S. PAIS,[2] AND J. M. NOVAIS[3]

[1]*Biologia, Universidade do Minho, 4719 Braga Codex, Portugal;* [2]*Dep. de Biologia Vegetal, Faculdade de Ciências de Lisboa, 1294 Lisboa Codex, Portugal; and* [3]*Laboratório de Engenharia Bioquimica, Instituto Superior Técnico, Av. Rovisco Pais, 1000 Lisboa, Portugal*

ABSTRACT

Micropropagated *Euphorbia characias* plants gave higher yields of crude oil than did wild ones. Leaves of either wild and micropropagated plants contained more oil than did stems.

Triterpenols, hydrocarbons, and free and esterified fatty acids are components of the crude oil produced by stems, young and mature leaves of wild and micropropagated *E. characias* plants, as well as by calli and suspended cells. With the exception of the free fatty acids fraction, all crude oil fractions were higher in micropropagated plants than in the wild ones. The crude oil content of leaves of either wild or micropropagated plants was higher than that of stems. However the triterpenols yields were higher in stems than in leaves, both in wild and micropropagated plants. The composition of the triterpenol fraction of the crude oil obtained from calli and suspended cells is quite different from that produced by any in vivo parent plant organ studied. Free fatty acids constitute the main fraction of the crude oil obtained from calli and suspended cells.

Index Entries: *Euphorbia characias;* micropropagation; calli; suspended cells; oil.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Euphorbia characias is a native species in marginal lands with mediterranean climate. Similar to other *Euphorbia* species, *E. characias* produces high amounts of latex rich in hydrocarbon-like compounds (*1*). Crude oil yields obtained from whole aerial parts of wild *E. characias* vary between 4.5 and 7.5% of the dry wt, depending on the season (*2*). These yields are comparable to those reported for species considered suitable candidates as energy crops (*3–5*). The oil content of calli and suspended cells is much lower than that of the wild parent plants. However, preliminary measurement performed on aerial parts of micropropagated *E. characias* plants grown in the field gave an oil content of 8.3% of the dry wt, which is markedly higher than that obtained from field-grown wild plants (*2*).

Calvin (*6*) suggested the use of genetic engineering as a way to improve yield and quality of the oil produced by *Euphorbia lathyris*. However, the most often used process to increase the production of crude oil has been the selection of high yield oil-producing plants presenting high growth rates.

Micropropagation provides the best way for clonal propagation of selected plants. Quantitative and qualitative variations of the crude oil content can also arise from physiological modifications introduced during in vitro manipulation. The effect of physiologically determinant factors, such as auxins and cytokinins, on the biosynthesis of oil compounds can be easily assayed through in vitro culture techniques, namely, of calli and suspended cells.

This paper deals with the study of the oil produced by leaves and stems of wild and micropropagated plants, as well as that obtained from young yellowish leaves, used as explants for induction of in vitro cultures, calli, and suspended cells. The aim is to compare the composition of the oil produced by each one of these plant components and in vitro cultures.

MATERIALS AND METHODS

Plant Material

All micropropagated plants, as well as in vitro cultures of calli and suspended cells, had origin from the several wild plants used in this study. Buds of those plants, growing wild at Rio de Mouro near Lisboa, were cultured on MS medium (*7*) supplemented with 0.5 mg/L giberelic acid. Rooted micropropagated plantlets were transfered to a mixture of 2/3 soil and 1/3 peat during 45 to 60 d. After that period, plants were transfered to the field at the location where the wild plants were growing. Ninety days after transfer to the field, 10 of those micropropagated plants were har-

vested and freeze-dried. By the same time, the aerial part of 10 wild *E. characias* plants were also harvested and freeze-dried. Young yellowish leaves, of the same type as those used for calli induction, as well as mature leaves and stems from all wild and micropropagated freeze-dried plants, were separately ground in a mill. The powdered material was submitted to extraction in a Soxhlet apparatus.

The explants used for calli induction were young yellowish leaves, still closed on the apical buds, withdrawn from wild plants at the end of January. Medium and environmental conditions for induction and maintenance of calli and suspension cultures were previously described (*8*). Eighteen calli and suspended cells of 18 suspension cultures, all at the stationary phase, were freeze-dried and submitted to extraction, as with parent plants.

Extraction

All the extractions were performed following the method of Buchanan et al. (*3*), modified in the partition step. Partition was performed between an isopropanol/water (72mL/56mL) phase and a *n*-hexane (5×80mL) phase in two stages: in the first, with the pH of alcoholic phase adjusted to pH 10, the neutral fraction of the oil was extracted, whereas in the second, with the pH of alcoholic phase adjusted to pH 3, the acid fraction of the oil, constituted mainly by free fatty acids, was extracted. The neutral fraction was saponified with an ethanolic solution of KOH at 5% during 2 h at 90°C. The unsaponifiable fraction was extracted with 5×50mL of a mixture of hexane/diethyl ether (1:1). Then, pH of the alcoholic phase was adjusted to pH 3 and the fatty acids extracted with 5×50mL of a mixture of hexane/diethyl ether (1:1).

Analysis

Either unsaponifiable fractions or both fatty acid fractions were derivatized by trimethylsililation prior to been analyzed by GLC and GC-MS. Extract samples of about 20 mg were dissolved in 200 μL of tetrahydrofuran (TFA) and 100 μL of hexamethyldisilasane (HMDS), and 5 μL of trimethylclorosilane (TMCS) were added. The solutions were left to react at 55°C during 2 h and then at room temperature overnight. GLC analysis of unsaponifiable fractions were performed as previously described (*1*). GLC analysis of the fatty acid fractions were performed with a GC instrument equipped with a flame ionization detector and a fused silica (30 m×0.50 mm) SPB-35 column coated with diphenyl-dimethyl polysiloxane (7:13). Chromatographic conditions used were as follows: injector and detector temperatures were 300 and 330°C, respectively; column 100 to 280°C at 5°C/min rate; carrier and makeup N_2 flows, 2.5 and 20 mL/min, respectively; air and H_2 flows, 300 and 30 mL/min, respectively.

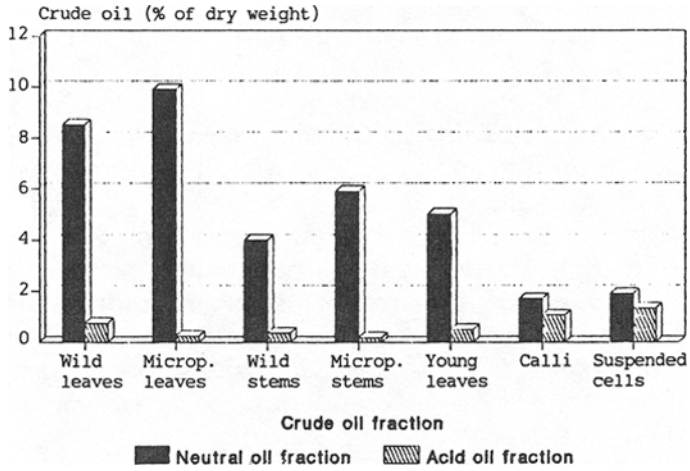

Fig. 1. Contents of neutral and acid crude oil fractions in calli, suspended cells, stems, mature, and young leaves of *E. characias*. Extractions were performed on 10 plants, 18 calli, and suspended cells of 18 batch cultures.

RESULTS

Mature leaves presented crude oil contents higher than stems. Leaves and stems of micropropagated plants showed neutral oil fractions yields higher than those obtained from wild plants (Fig. 1). Although the lowest contents of neutral oil fractions were found in calli and suspended cells, these cultures revealed the highest contents of the acid oil fraction. Contrary to the calli and suspended cells, the content of this fraction in leaves and stems of wild and micropropagated plants is much lower than that of the neutral oil fraction (Fig. 1).

The main groups of compounds found in the oil obtained from *E. characias* were hydrocarbons, triterpenols, free, and esterified fatty acids. Figure 2 shows the amount of each one of these compound groups recorded in different organs and types of culture. Triterpenols are the main components of the oil extracted from leaves and stems of wild and micropropagated *E. characias* plants, whereas in calli and suspended cells, the main components are fatty acids (Fig. 2).

Although the triterpenol content of young leaves is higher than that of any other group of compounds, it is much lower than the one recorded for stems and mature leaves. On the contrary, the content of free fatty acids is higher in young leaves than in stems and well-matured leaves (Fig. 2).

The composition of the unsaponifiable fraction obtained from calli and suspended cells of *E. characias* is quite different from that obtained from stems, young, and mature leaves of the parent plants. Capillary GLC readily separated the hydrocarbons and trimethylsiliated triterpenols from unsaponifiable fractions (Figs. 3A and 3B). Analysis performed by

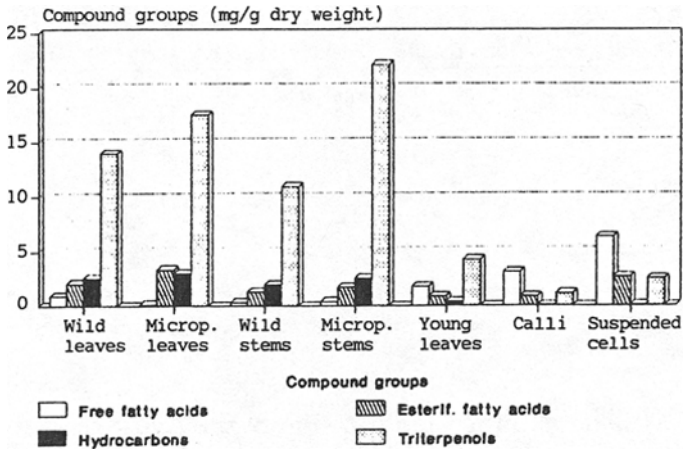

Fig. 2. Contents of the major oil compound groups analyzed in calli, suspended cells, stems, mature, and young leaves of *E. characias*. Extractions were performed on 10 plants, 18 calli, and suspended cells of 18 batch cultures.

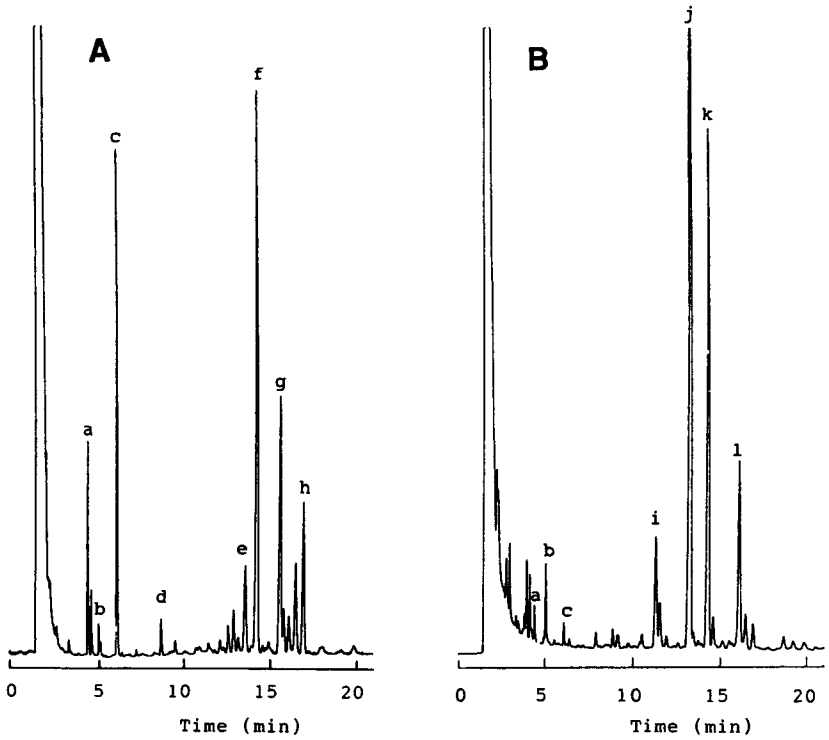

Fig. 3. Capillary gas chromatograms of unsaponifiable fraction of neutral crude oil from whole aerial part of wild plants (A) and from in vitro cultures of *E. characias* (B) after derivatization by trimethylsililation. Peaks (a) nonacosane, (b) squalene, (c) hentriacontane, (d) tritriacontane, (e) lanosterol, (f) lanosterol isomer, (g) cycloartenol, (h) 24-methylenecycloartanol, (i) campesterol, (j) β-sitosterol, (k) Δ^5-avenasterol, and (l) α-amyrin.

Table 1
Composition of Hydrocarbon Fraction in Different Plant Organs and Type of In Vitro Cultures (all units are μg/g dry weight; ± s. e.)

Hydrocarbons	Wild mature leaves	Micr. mature leaves	Wild plant stems	Micr. plant stems	Young leaves	Calli	Suspended cells
Nonacosane	768±53	408±13	517±41	617±14	98±1	t.a.	t.a.
Hentriacontane	1423±103	1986±35	1309±118	1732±54	197±5	t.a.	t.a.
Tritriacontane	182±15	280±8	96±7	100±6	26±5	t.a.	t.a.
Squalene	168±7	319±11	21±1	90±6	45±1	45±4	86±8

The values are derived from ten samples. Each sample was submitted to six measurements.
t.a. - trace amounts

capillary GC-MS enabled the identification of the hydrocarbons: nonacosane, hentriacontane, tritriacontane, and squalene, as well as the triterpenols: lanosterol, lanosterol isomer, cycloartenol, and 24-methylenecycloartanol, in the unsaponifiable fraction of oil extracted from stems, young, and mature leaves of wild and micropropagated plants of *E. characias* (Fig. 3A). However, as it was already reported (*1*), the triterpenols produced by calli and suspended cells of *E. characias* are campesterol, β-sitosterol, Δ^5-avenasterol, and α-amyrin. Only trace amounts of hydrocarbons were recorded in the unsaponifiable fraction of oil obtained from calli and suspended cells. Squalene is the main hydrocarbon produced by these in vitro cultures (Fig. 3B).

In vitro cultures produce the same fatty acids that are produced by leaves and stems of the parent plants. Those identified were: lauric acid (12:0), myristic (14:0), pentadecanoic (15:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidic (20:0), behenic (22:0), and lignoceric (24:0).

Table 1 shows the composition of the hydrocarbon fraction recorded for different plant components. Hentriacontane is the major hydrocarbon produced by stems, young, and mature leaves of wild and micropropagated plants. The highest levels of hentriacontane, tritriacontane, and squalene were recorded for mature leaves of micropropagated plants. However, the content of nonacosane in micropropagated plant mature leaves was lower than that recorded for mature leaves of wild plants and stems of wild and micropropagated plants. Squalene was the only hydrocarbon produced by calli and suspended cells in measurable amounts. Although the young leaves had revealed a total hydrocarbon content much lower than that of mature leaves, their composition is very similar. However, the ratio between squalene content and other hydrocarbons content is about twice higher in young leaves than in mature leaves.

The contents of main triterpenols produced by stems, young, and mature leaves of wild and micropropagated plants are shown in Table 2, and those of in vitro cultures in Table 3. The highest triterpenol content of

Table 2
Contents of the Main Triterpenols from Triterpenol Fraction Obtained from Different Organs of In Vivo Plants (all units are μg/g dry weight : ± s. e.)

Triterpenols	Wild mature leaves	Micr. mature leaves	Wild plant stems	Micr. plant stems	Young leaves
Lanosterol	531±25	1261±95	541±23	1627±88	204±5
Lanost. isomer	3500±159	5990±54	3916±261	8780±268	1893±15
Cycloartenol	2236±108	4644±95	2029±119	5150±233	850±9
24-Methylenecycloart.	935±30	2039±162	972±52	2335±94	348±6

The values are derived from ten samples. Each sample was submitted to six measurements.

Table 3
Contents of the Main Triterpenols Produced by Calli and Suspended Cells (units: μg/g dry wt.; ± s. e.)

Triterpenols	Calli	Suspended cells
Campesterol	87±5	168±7
β-Sitosterol	553±8	970±16
Δ^5-avenasterol	317±16	921±14
α-Amyrin	105±7	161±9

The values are derived from ten samples.
Each sample was submitted to six measurements

in vivo plants was obtained in micropropagated plant stems, and the lowest in young leaves. Lanosterol isomer was the main triterpenol produced by any of these plant components (Table 2). The triterpenol composition of wild and micropropagated plant stems revealed a percentage of lanosterol isomer higher than mature leaves. However, the percentage of lanosterol isomer recorded for triterpenol composition of the young leaves was higher than that of stems of the same plant. The ratio of cycloartenol/24-methylenecycloartanol decreased from young leaves to mature leaves, and from these to stems.

The content of the triterpenol fraction obtained from suspended cells was higher than that of calli (Table 3). The main difference between the triterpenol composition of these types of cultures was observed for the relative amounts of β-sitosterol and Δ^5-avenasterol. The ratio of β-sitosterol/Δ^5-avenasterol recorded for suspended cells was lower than that recorded for calli.

Table 4 shows the composition of the free fatty acids fraction obtained from stems, young, and mature leaves of wild and micropropagated plants, as well as calli and suspended cells. The composition of esterified fatty acids fraction obtained from the same plant components is shown in Table 5.

Table 4
Composition of Free Fatty Acids Fraction in Different Plant Organs and Type of In Vitro Cultures (all units are in μg/g dry weight; ± s. e.)

Fatty acids	Wild mature leaves	Micr. mature leaves	Wild plant stems	Micr. plant stems	Young leaves	Calli	Suspended cells
12 : 0	4±0.5	7±1	2±0.2	1±0.1	7±0.9	8±0.3	18±1
14 : 0	13±2	3±0.4	9±2	7±0.6	17±1	20±1	73±4
15 : O	3±0.2	0.7±0.1	2±0.4	5±0.2	7±0.8	52±2	186±10
16 : 0	142±17	37±5	90±4	187±9	360±11	840±10	2851±138
18 : 0	21±2	4±0.7	17±3	14±2	79±3	102±2	322±21
18 : 1	17±1	6±0.7	18±1	75±3	99±3	82±3	395±21
18 : 2	95±5	22±2	55±3	121±7	411±5	763±10	1396±81
18 : 3	589±51	62±7	113±7	93±3	715±9	1160±13	622±43
20 : 0	11±2	1±0.3	6±0.5	8±0.9	24±2	10±0.5	92±8
22 : 0	14±3	1±0.4	13±1	6±1	26±3	23±0.6	114±4
24 : 0	8±0.5	t. a.	7±0.4	2±0.1	13±3	49±2	302±9

The values are derived from ten samples. Each sample was submitted to six measurements

Palmitic acid was the main saturated fatty acid produced independently of the plant organ or type of culture (calli and suspended cells). With the exception of the micropropagated plant stems and suspended cells, linolenic acid was the main unsaturated fatty acid produced. The content of linoleic acid in micropropagated plant stems and suspended cells was higher than that of linolenic acid.

Except for 12:0 and 14:0, the contents of all free fatty acids recorded for calli and suspended cells were higher than those of esterified ones. On the contrary, all fatty acids produced by leaves and stems of wild and micropropagated plants were mostly esterified.

The highest content of free fatty acids was obtained for suspended cells. Free fatty acids content of calli was higher than that of any wild or micropropagated plant component. The total content of free fatty acids recorded for wild plant mature leaves was higher than those of micropropagated plant mature leaves and stems, but lower than that of young leaves.

Linolenic acid represents more than 64%, and palmitic acid about 16% of the free fatty acids fraction from wild plant mature leaves. However, even these two free fatty acids are found at higher levels in young leaves than in wild plant mature leaves (Table 4).

The lowest content of esterified fatty acids was found in young leaves and calli. The content of saturated esterified fatty acids in young leaves was slightly higher than that obtained from calli. However, the amount of

Table 5
Composition of Esterified Fatty Acid Fraction in Different Plant Organs and Type of In Vitro Cultures (all units are in μg/g dry weight; ± s. e.)

Fatty acids	Wild mature leaves	Micr. mature leaves	Wild plant stems	Micr. plant stems	Yong leaves	Calli	Suspended cells
12 : 0	48±5	55±7	21±0.7	43±6	23±1	10±1	46±7
14 : 0	187±5	207±5	72±2	103±2	40±2	20±2	79±9
15 : 0	7±0.3	9±0.7	11±0.6	25±1	10±1	24±2	48±6
16 : 0	610±9	607±9	336±6	484±8	277±15	198±12	840±83
18 : 0	96±2	102±2	52±2	81±5	98±5	27±2	108±13
18 : 1	149±3	213±2	144±2	281±3	60±3	41±3	317±25
18 : 2	245±3	909±9	246±4	391±8	128±4	185±14	598±56
18 : 3	563±5	1047±9	318±3	208±9	100±8	298±12	327±26
20 : 0	113±4	156±7	46±8	76±2	52±6	9±0.6	28±5
22 : 0	34±0.7	42±3	27±2	30±1	31±2	13±0.7	75±4
24 : 0	14±1	8±0.8	15±1	4±0.7	29±4	37±2	201±8

The values are derived from ten samples. Each sample was submitted to six measurements.

unsaturated esterified fatty acids produced by calli was higher than that provided by young leaves used in calli induction. Suspended cells showed the highest levels of saturated esterified fatty acids, whereas micropropagated plant leaves showed the highest yield of unsaturated ones (Table 5).

Although the content of unsaturated esterified fatty acids was higher in micropropagated plant mature leaves than in the wild plant mature leaves, the yield and composition of saturated esterified fatty acids were similar. The contents of both saturated and unsaturated esterified fatty acids were higher in leaves than in stems. With the exception of linolenic acid, micropropagated plant stems had higher contents of saturated and unsaturated esterified fatty acids than the wild plant stems. The level of esterified linolenic acid in micropropagated plant stems was lower than that of esterified oleic and linoleic acids. Young plant leaves and suspended cells also contained levels of esterified linolenic acid lower than those of esterified linoleic acid.

DISCUSSION

Lanosterol, lanosterol isomer, cycloartenol, and 24-methylenecycloartanol are the main hexane soluble compounds extracted from latex of *E. characias* plants (*1,9*). These triterpenols constitute about 8.7% of the fresh latex of *E. characias* plants. Although lanosterol, lanosterol isomer, cyclo-

artenol, and 24-methylenecycloartanol are the major compounds of the triterpenol fraction of the whole plant hexane extract, its GC-MS analysis shows more compounds than those found in the same fraction from latex. Although the relative amounts are different, these four triterpenols are also the major components of the hexane soluble fraction of *E. lathyris latex* (*10*). Similarly to what occurs with *E. characias,* the whole plant heptane extract from *E. lathyris* is also constituted mostly by triterpenols, but in a greater structural variety than that of latex triterpenols (*11*). Such diversity may mean that terpenoid synthesis occurs not only in the laticifiers but also in other parts of the plant.

Of all the triterpenols produced by calli and suspended cells, α-amyrin was the only one found in the whole plant hexane extracts. However, the amount of this compound in *E. characias* plants was very small, compared to that in the latex triterpenols.

Campesterol, β-sitosterol, and Δ^5-avenasterol were only found in calli and suspended cells. This may indicate that the biosynthesis of these compounds by *E. characias* cells can be shunted, depending on the medium conditions.

Squalene is precursor of triterpenols that arise via enzyme mediated cyclization of squalene 1,2-oxide followed by rearrangement sequences to produce the diverse interrelated C_{30} terpenoids. The immediate conversion of this compound can justify their low levels in the different plant organs and cultures studied.

In plants, long straight chain saturated hydrocarbons are considered waxes components. In wild and micropropagated *E. characias* plants, high amounts of nonacosane, hentriacontane, and tritriacontane were recorded. From these hydrocarbons, hentriacontane and tritriacontane were reported also for *Euphorbia lathyris* (*11*). The low levels of these compounds in calli and suspended cells of *E. characias* may be correlated with the absence of cuticular structures in these in vitro cultures.

Fatty acids, such as palmitic, estearic, oleic, linoleic, and linolenic acids, are widespread components of membrane lipids. Sucrose added to MS medium may be responsible for the higher amounts of free fatty acids found in calli and suspended cells. The easier access to exogenous sucrose in suspension cultures could explain the higher production of fatty acids by suspended cells when compared with calli. Sucrose may be converted to acetyl-CoA and malonyl-CoA by plant cells. These compounds are the substrates for the synthesis of fatty acids, performed by a plant fatty acid synthase system constituted by seven or eight enzymes (*12*). The levels of esterified fatty acids either in calli or suspended cells are much lower when compared with the levels of free fatty acids. That suggests that the intensive synthesis of fatty acids in calli and suspended cells of *E. characias* is not accompanied by a corresponding intensive synthesis of lipids.

Nonphotosynthetic tissues, such as roots (*13*), etiolated leaves (*14*), and heterotrophic white calli (*15*), contain smaller amounts of chloroplast

lipids than photosynthetic green tissues. Chloroplast lipids (i.e., monogalactosylidglyceride, digalactosyldiglyceride, sulfoquinovosyldiglyceride, and phosphatidylglycerol) have high relative amounts of 18:3. Lipids, such as phosphatidylcholine and phosphatidylethanolamine, that are membrane components of organelles other than chloroplasts, have high relative amounts of 18:2 and lower relative amounts of 18:3, when compared with chloroplasts lipids (*16*). A correlation between the contents of chlorophylls and those of chloroplast lipids and 18:3 may exist. That correlation has been observed in several plants during greening (*17,18*). Photomixotrophic green calli of tobacco revealed higher amounts of chlorophylls, chloroplast lipids, and 18:3 than the white ones. Our results obtained by analysis of the esterified fatty acids fractions from young yellowish leaves, mature green leaves, stems, green calli, and white suspended cells of *E. characias*, are in agreement with the correlation between the content of chlorophylls and that of linolenic acid.

Comparing the contents and composition of esterified unsaturated fatty acids from leaves and stems of micropropagated plants with those of wild ones, it seems clear that lipid biosynthesis was affected by micropropagation techniques. The amounts of fatty acids produced by leaves of micropropagated plants are almost completely esterified, witnessing a high amount of lipids produced. Wild plant leaves showed higher levels of unsaturated free fatty acids but lower levels of unsaturated esterified fatty acids, which means that the amount of lipids produced is lower. In the esterified fatty acids fractions obtained from micropropagated plants, the relative amounts of 18:3 are lower, and those of 18:2 higher than in esterified fatty acids fractions extracted from wild plants. This suggests that, in micropropagated plants, the biosynthesis of lipids having high relative amounts of 18:2 was increased, relatively to the chloroplast lipids.

The low levels of esterified fatty acids, and specially that of linolenic acid of young yellowish leaves, may indicate that chloroplast lipids as well as thylakoids are scarce.

It is not easy to explain the higher contents of triterpenols, hydrocarbons, and esterified fatty acids in micropropagated plants, relatively to the wild ones. After transfer to the field, micropropagated plants revealed lower apical dominance than the wild ones. The increase of the contents of these compounds is probably correlated with that type of growth. Micropropagated plant stems were shorter and less lignified than wild ones. As the yields are expressed in terms of dry wt, the higher contents of the oil components obtained in micropropagated plant stems may be as a result of the low percentage of lignocellulosic material in these stems. However, this hypothesis can hardly explain the higher yields obtained in micropropagated plant leaves when compared with those of the wild ones. Therefore, it is possible that micropropagation created conditions to increase the production of crude oil compounds. This hypothesis is supported by some results, not yet published, on *E. characias* calli that indi-

cate that the biosynthetic rate of triterpenols is strongly influenced by the type and concentration of auxins and cytokinins. Triterpenols are the most important fraction of the crude oil of *E. characias* plants. The possible alteration of the levels of endogenous phytohormones occurring as a consequence of micropropagation, and their effect on the biosynthetic rates of crude oil compounds, namely, triterpenols, is a hypothesis that should not be ruled out.

The results of this study enable us to consider *E. characias* as an energetic renewable resource with value similar to other species considered suitable candidates as energy crops (*3,4,19*). This species constitutes also an excellent source of *n*-alcans, fatty acids, and mainly, triterpenols. This study demonstrated also that it is possible to change the pathways for the biosynthesis of triterpenols performed by cells of *E. characias*. Triterpenols produced by calli and suspended cells are different from those of the parent plant latex, probably because of the absence of laticifers in these in vitro tissues. However, as for parent plants, triterpenols are an important fraction of their crude oil. Some triterpenols produced by *E. characias* calli and cells can be precursors of economically more important sterols than the triterpenols produced by parent plants. For example, by biotransformation of sitosterol, steroids such as pregnelone can be produced. Calli and suspended cells would also be preferred relatively to the parent plants for the production of fatty acids. Additionally, these in vitro cultures constitute an excellent material to assay the effect of different medium and environmental factors on the production of crude oil compounds.

ACKNOWLEDGMENTS

We are grateful to Higuinaldo Neves for permission to make the mass spectra in his laboratory, and to Ana Vasconcelos for the GC-MS analysis. We thank LNETI for permission to make part of this research work in its Renewable Energies Laboratory. We also thank INIC for the grant awarded to MF-F.

REFERENCES

1. Fernandes-Ferreira, M., Novais, J. M., and Pais, M. S. S. (1990), *Phytochemistry* **29**, 1855.
2. Fernandes-Ferreira, M., Pais, M. S. S., and Novais, J. M. (1990), *Biomass for Energy and Industry*, 5th E. C. Conference, Grassi, G., Gosse, G., and Santos, G., eds., vol. 1, pp. 517, Elsevier, London.
3. Buchanan, R. A., Cull, I. M., Otey, F. H., and Russel, C. R. (1978), *Economic Botany* **32**, 131–153.

4. Ng'eny-Mengech, A. and Kihumba, S. N. (1985), *Energy from Biomass*, 3rd E. C. Conference, Palz, W., Coombs, J., and Hall, D. O., eds., pp. 749, Elsevier, London.
5. Wiatr, S. M., (1985), *Biomass* **4,** 59.
6. Calvin, M. (1983), *Science* **219,** 24.
7. Murashige, T. and Skoog, F. (1962), *Physiol. Plantarum* **15,** 473.
8. Fernandes-Ferreira, M., Novais, J. M., and Pais, M. S. .S (1989), *Biotechnol. Lett.* **4,** 259.
9. Nielsen, P. E., Nishimura, H., Otvos, J. W., and Calvin, M. (1977), *Science* **198,** 942.
10. Nielsen, P. E., Nishimura, H., Liang, Y., and Calvin, M. (1979), *Phytochemistry* **18,** 103.
11. Nemethy, E. K., Otvos, J. W., and Calvin, M. (1981), *Pure & Appl. Chem.* **53,** 1101.
12. Stumpf, P. K. (1987), *JAOCS* **64,** 1641.
13. Soimajarui, J. and Linko, R. R. (1979), *J. Agric. Food Chem.* **27,** 1279.
14. Hirayama, O. and Suzuki, T. (1968), *Agric. Biol. Chem.* **32,** 549.
15. Kates, M. (1970), *Adv. Lipid Res.* **8,** 225.
16. Matsuzaki, T., Koiwai, A, Nagao, T., Sato, F., and Yamada, Y. (1984), *Agric. Biol. Chem.* **48,** 1699.
17. Leese, B. M. and Leech, R. M. (1976), *Plant Physiol.* **47,** 789.
18. Bahl, J., Francke, B., and Moneger, R. (1976), *Planta* **129,** 193.
19. Adams, R. P., Balandrin, M. F., Brown, K. J., Stone, G. A., and Gruel, S. M. (1986), *Biomass* **9,** 255.