Acta Bot. Neerl. 36(1), February 1987, p. 71–80

SITES OF IN-VITRO TRITERPENE SYNTHESIS IN EUPHORBIA LATEX

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SUMMARY

Latices of *Euphorbia* species utilize labelled acetate and mevalonate for triterpene synthesis under in vitro conditions. Triterpene synthesis was detected at two sites in fractionated latex, viz. in particle-containing vesicles and in a fraction consisting of amyloplasts, often associated with a tubular membrane system. Attempts to purify these fractions lead to a loss of triterpene synthesis in vitro.

Latex particles were successfully purified by means of gel filtration procedures. Radioactive triterpenes were traced in these purified particles after incubation of the latex with (2-^{14}C)acetate or (2-^{14}C) mevalonate. The newly synthesized triterpenes were firmly attached to the particles of all occurring sizes. Although the fraction containing the amyloplasts was found to be very active in triterpene synthesis, no radioactive latex particles could be isolated from this fraction.

1. INTRODUCTION

Studies have shown that the latex of *Euphorbia cyparissias, E. helioscopia,* and *E. erythrea* can synthesize triterpenes in vitro (PONSINET & OURISSON 1967, 1968). BIESBOER & MAHLBERG (1979) demonstrated the conversion of mevalonic acid into squalene and triterpenes by freshly tapped latex from *E. tirucalli.* Recently, NEMETHY et al. (1983) reported the in vitro synthesis of triterpenols and esterified triterpenes from labelled precursors of *E. lathyris* latex.

GROENEVELD (1976) in in vitro studies with latices of *E. lathyris* and *E. pulcherrima* showed that the ^{14}C distribution among the triterpenols and esterified triterpenes did not reflect the mutual mass ratios of the latex lipids regardless of precursors incorporated into the latex. In contrast, in vivo experiments with labelled sucrose revealed labelling patterns that corresponded with the existing latex triterpene composition (GROENEVELD et al. 1976, 1982, 1983). From these studies he postulated the possible existence of a dual mechanism for triterpene synthesis in the latex.

Triterpenes occur in the latex as solid particles, in which always some rubber can be detected. HEINRICH (1967, 1970) provided ultrastructural evidence that in articulated laticifers the terpene particles were located in the cytoplasm. In the non-articulated laticifers these particles were found in the large central vacuole. This hypothesis was shown to be correct for many non-articulated latici-

fers in the Euphorbiaceae. Laticifers of *E. lathyris* (MORITZ & FROHNE 1967), *E. pulcherrima* (SCHNEPF 1964), *E. characias* (MARTY 1968) appeared to have a large central vacuole containing the particles. MARTY showed that these particles were assembled in the thin wall-lining cytoplasm and subsequently secreted into the vacuole. The detailed sequence of this secretion process remained unsolved.

The latex obtained after incision of the mentioned euphorbias should consequently be regarded as a vacuolar sap. The wall-lining cytoplasm is not exuded and the subcellular structures involved in triterpene particle synthesis are retained by the laticifer. Nevertheless, the vacuolar part appeared to convert several water soluble precursors into triterpenes. The ready availability and manipulation of this latex makes it a desirable system for studies on terpene synthesis under cell free conditions. In this paper we provide new data on the sites of triterpene synthesis in latex of *Euphorbia* and an initial characterization of the components associated with the process of biosynthesis.

2. MATERIALS AND METHODS

Plants.

Euphorbia lathyris L., *E. tirucalli* L., *E. aphylla* L., and *E. pulcherrima* Willd. were grown and sampled under greenhouse conditions at Indiana University. Latex samples were obtained from mature plants by incision at internodes and tips of growing shoots.

Isolation of latex particles.

Latex samples were diluted five-fold with a phosphate buffer (0.2–0.25 M; pH 4.5–5.0) and chromatographed over Sephadex in the same buffer. The phosphate buffer was removed by subsequent gel filtration of the particle fraction in water. The derived aqueous suspension of latex particles was stable for several days.

Scanning electron microscopy (SEM).

Particle suspensions or fractions obtained by ultracentrifugation were fixed with OsO_4 vapour for 3 h. Small drops of these suspensions were placed on a stub, frozen, freezedried, and coated with gold-palladium or droplets were transferred to a milliporefilter (0.01 μm), dried, mounted onto a stub and coated as above. Preparations were examined with a Cambridge 250 SEM at 10 kV.

Transmission electron microscopy (TEM).

Samples were fixed with OsO_4 vapour for 3 h, concentrated to a pellet by centrifuging (30,000 g, 30 min) and embedded in 1% liquid agar (44 °C). Agar blocks (2–3 mm^2) were rinsed in water and post-fixed in 3% glutaraldehyde (TURNER 1970). Sections were gridstained with uranylacetate and lead citrate and viewed in a Philips 300 TEM.

Detergent treatment.

Suspensions of purified latex particles were treated with different concentrations of Tween-80 for 15 hr. These samples were mixed with sucrose to make an end concentration of 1.5 M sucrose and centrifuged for 2 h at 300,000 g. The sticky mass (particle coagulate) was extracted with acetone and saponified. The total amount of triterpenes was determined with a Hewlett-Packard 5710A gas liquid chromatograph on OV-1 on 100/120 mesh Supelcoport. The instrument was operated isothermally at 285 °C; 5α-cholestane was used as an internal standard. Compounds were quantitated with a Hewlett-Packard 3380A integrator.

Incorporation experiments.

Freshly tapped latex (200–400 mg) was incubated with (2-^{14}C)acetate or (2-^{14}C)mevalonate at room temperature for up to 30 h. Incubations were terminated by adding 3 ml of ice cold phosphate buffer and an equal volume of acetone. Latex lipids were extracted with light petroleum (b.p. 40–60 °C) and separated into free triterpenols and triterpene esters on silica gel G thinlayer plates developed in cyclohexane/ethylacetate (5:1, v:v), or saponified with 5% KOH in methanol/benzene (19:1, v:v) at 90 °C for 1.5 h prior to TLC. Radioactivity was detected with Kodak XAR-5 film, exposed for 1–2 wk and developed in D–19 for 5 min at 20 °C. Scrapings of the ^{14}C-spots were counted in omnifluor with a liquid scintillation counter. (2-^{14}C) acetate (58.3 mCi/mmol) and (2-^{14}C) mevalonate (53 mCi/mmol) were purchased from Amersham.

3. RESULTS

The water insoluble triterpenes in latex occur in association with submicroscopic particles coated with proteins (*fig. 1*). This particle suspension in exuded latex readily coagulates into a sticky mass when diluted with water. However, a stable suspension of these particles is obtained when the latex is collected into a phosphate buffer with a pH 4.5–5.0 and adequate ionic strength, a phenomenon supporting the interpretation that they possess a proteinaceous coat. When these particles are separated from other latex constituents by gel fitration a stable suspension of pure particles is obtained. This fraction viewed by SEM shows a population of spherical particles of different sizes (*fig. 1*). The average diameter may differ among taxa, measuring 0.20 μm in *E. lathyris* and 0.24 μm in *E. tirucalli*. When viewed in TEM, only solid spherical structures were observed, having no membrane like structure at the periphery.

Centrifugation at 120,000 g for 1 h separated whole latex into three fractions (*fig. 7*): a toplayer containing an uppermost zone of coagulated particles on top of a zone of concentrated particles in suspension, a clear serum, and a bottom fraction containing amyloplasts with associated membranes as observed by microscopy (phase contrast). The particle suspension from the top layer was purified by gel filtration to yield pure particles. If this suspension was fixed in OsO_4 and glutaraldehyde, TEM revealed vesicle like structures containing various

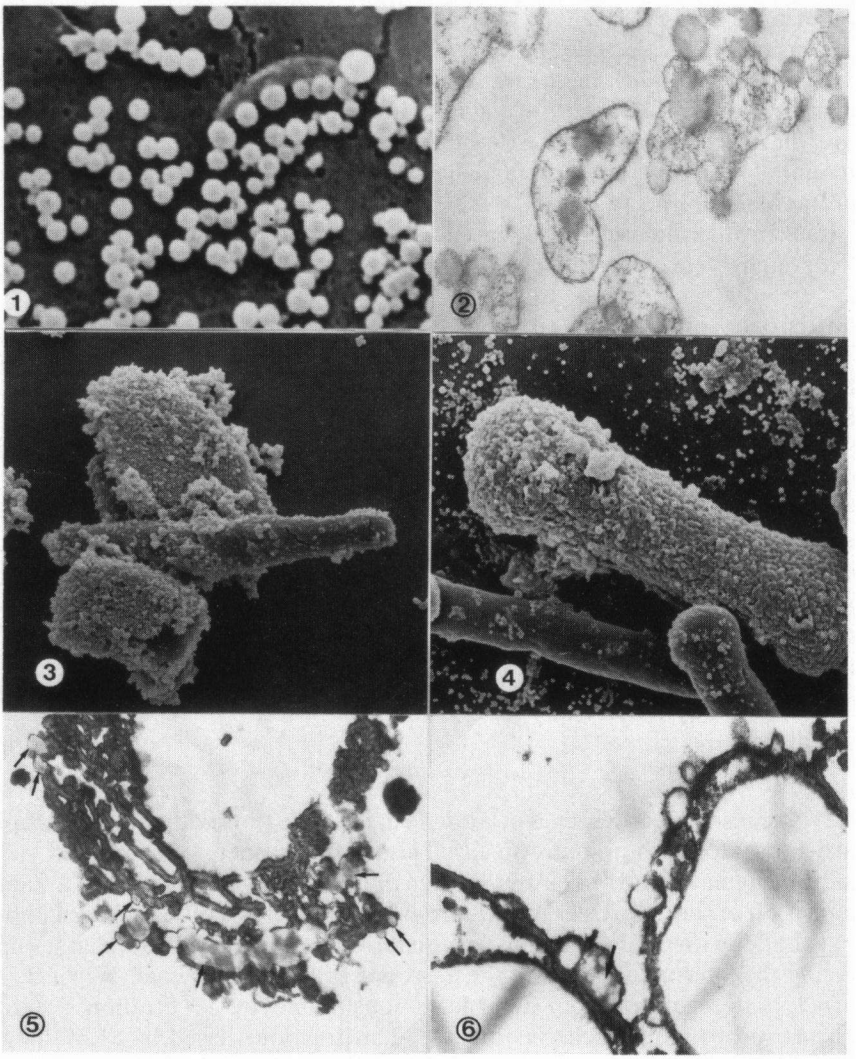


Fig. 1. SEM of latex particles (*E. lathyris*) isolated and purified by gel filtration. Particle diameters range from 0.07–0.5 μm. × 8,450

Fig. 2. TEM of the particle suspension in the top layer (obtained by centrifugation of pure latex, *fig. 7*). Triterpene particles are only present in vesicles or surrounded by vesicle membranes. × 39.260

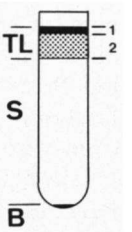
Fig. 3. SEM of the *E. lathyris* bottom fraction. Amyloplast with associated membranes and lipid droplets. The structure at the lower left consists of tubular membranes and lipid droplets only; the amyloplast is missing. × 1,625

Fig. 4. SEM of the *E. tirucalli* bottom fraction. Amyloplast with tubular membrane system and lipid bodies at the surface. Amyloplast with smooth membranes at lower left. × 2,355

Fig. 5. *E. lathyris* bottom fraction. TEM of tubular membrane system, associated with amyloplasts. Arrows mark the lipid droplets in the anastomosing tubular system. × 16,950

Fig. 6. *E. tirucalli*, bottom fraction. Amyloplasts (S) with a relatively smooth surface. Lipid droplets in between electron dense membraneous envelopes (arrows). × 21,125

Fig. 7. Fractionated latex (*E. lathyris, E. tirucalli*) after centrifugation at 120,000 g for 2 h. TL: top layer or upper fraction consisting of 1) a sticky mass of coagulated particles and 2) a particle suspension, S: clear serum, B: bottomfraction.



amounts of particles (*fig. 2*). These vesicles were lost in gel filtration; the enclosed particles were recovered.

Triterpene analysis of the fractions obtained in ultracentrifugation revealed one triterpene profile in gas-liquid chromatography, being characteristic for the species used. Quantitatively, a trace of triterpenes was detected in the bottom fraction; the particle suspension and the sticky layer contained 3–5% and 95–97% respectively of the lipids present in the latex sample used. No triterpenes were detected in the clear serum.

Incorporation experiments

Latex from four different *Euphorbia* species was incubated with (2-^{14}C) mevalo-

Table 1. Recovery of ^{14}C-triterpenes from particle suspension and bottom fraction after incorporation of 2-^{14}C mevalonate (2 µCi, 24 h, 22 °C) by latex of *Euphorbia* species.

Species	Whole latex, mg	Bottom fraction, dpm	Particle fraction, dpm
E. aphylla	250	757	2,057
E. lathyris	340	4,268	2,222
E. pulcherrima	280	4,677	8,511
E. tirucalli	370	127,096	17,206

Table 2. ^{14}C-triterpene distribution of the bottom fraction after isopycnic centrifugation at 90,000 xg for 2.5 h after 24 h incubation with 2.5 µCi 2-^{14}C mevalonate at 22 °C.

Sucrose*, M	*E. lathyris* shoot tip, dpm	*E. tirucalli* shoot tip, dpm	*E. tirucalli* stem base, dpm
0.15	1,227	19,960	1,360
0.60	1,200	41,450	1,410
0.90	320	2,560	230
1.40	110	1,340	340
1.70	40	110	100
2.00	25	2,412	330
2.50	915	21,945	3,080

*, made in 0.2 M phosphate buffer, pH 4.8

nate for 24 h at room temperature. After centrifugation (30,000 g, 2 h) the radioactivity in the triterpenes of the bottom fraction and the toplayer was measured. Results presented in *table 1* show that ^{14}C-triterpenes were detected in both fractions. No labelled lipids were recovered from the serum. The absolute yield of in-vitro synthesised lipids differed among the plant species used, but also the site of latex collection appeared to influence the percentage of incorporation. Latex obtained from growing shoot tips incorporated more ^{14}C from labelled acetate or mevalonate than latex collected from the older stem regions (*table 2*). This phenomenon is difficult to assess at the present time.

The bottom fractions contained less than 0.1% of the triterpenes present in the latex sample used, yet it may be concluded from the data in *table 1* that the most active site in triterpene synthesis is to be found in this precipitate. Fractionating of this bottom fraction (after mevalonate incorporation) over a sucrose gradient revealed ^{14}C-triterpenes in all the prepared density layers (*table 2*). About 25–45% of the labelled triterpenes were still recovered from the dense amyloplast containing fraction. However, no ^{14}C containing particles were obtained when this bottom fraction was filtrated over a Sephadex column.

^{14}C containing particles were isolated from the top layer. For that purpose the latex sample was diluted with an appropriate amount of phosphate buffer and centrifuged on top of 0.15 M sucrose in the same buffer for 10 minutes at 1200 g. A small fraction containing amyloplasts and associated membranes settled under the sucrose layer; a suspension of particles composed the phosphate layer. If this suspension was centrifuged at 30,000 g for an additional 3 h over 0.15 M sucrose in phosphate buffer, no other precipitate was obtained, indicating that the components of the bottom fraction had been removed completely from the toplayer.

Purification of the radioactive particles resulted in a slight decrease of specific activity (*table 3*). Apparently the newly synthesised triterpenes adhered tightly to these particles as was found after in vivo incorporation. Treatment of these particles with increasing concentrations of Tween-80 only resulted in a partial release of ^{14}C-triterpenes from these particles, the specific activity of these lipids in the particles remained constant. A gradual dissolving of latex particles was measured at increasing concentration of the detergent (*figs. 8a* and *8b*). About 15 mg of Tween was required to dissolve the particle equivalent of 1 mg triterpene from *E. lathyris* latex, and 1 mg of triterpene enclosed in the particles from *E. tirucalli* required 10 mg of Tween-80 to dissolve completely. SEM showed detergent treated particles increase two or threefold in diameter, indicating a considerable uptake of detergent before their structural collaps and dissolution in the higher detergent concentrations. Probably the smallest particles were the first to be dissolved, followed by those of progressively larger size. When ^{14}C-triterpenes containing particles in different detergent concentrations were examined for label content, the remaining intact particles maintained a rather constant specific activity, indicating that the newly synthesized triterpenes were equaly distributed among particles of all sizes. Freshly tapped latex showed a decreased level of triterpene synthesis upon dilution with phosphate buffers. In all cases at least

Table 3. Recovery of ^{14}C-triterpenes after incorporation of [2-^{14}C] mevalonic acid into particle and bottom fractions after 24 h at 22 °C in *E. tirucalli*.

Fraction	Triterpene, dpm	Triterpene, mg	Specific activity, dpm μg
Particle fraction			
In water	14,400	1.4	10.3
In buffer	64,100	5.53	11.7
In serum	57,280	4.58	12.5
Bottom fraction	463,980	0.63	740.0

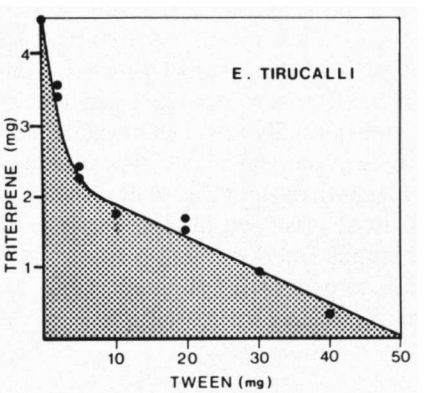


Fig. 8a. Disappearance of triterpene particles (shaded area) from *E. tirucalli* latex (containing 4 mg triterpene) with increasing concentrations of Tween-80. About 50 mg of Tween-80 is required to dissolve 4.5 mg triterpene particles suspended in 2 ml of water.

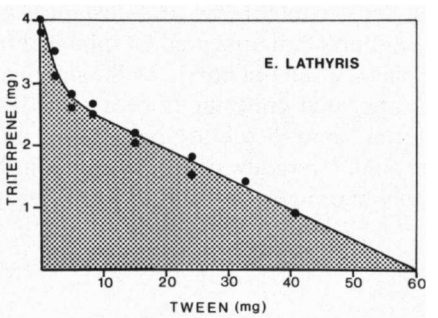


Fig. 8b. Disappearance of triterpene particles (shaded area) from *E. lathyris* latex with increasing concentrations of Tween-80. About 60 mg of Tween-80 dissolved the equivalent of 4 mg of triterpenes occurring as latex particles in 2 ml of water.

Table 4. In-vitro [2-^{14}C] acetate incorporation into triterpenes by the bottom and particle fractions suspended in serum and by whole latex incubated for 30 h at 22 °C. Latex samples, 250 mg.

Species	Whole latex, dpm	Bottom fraction, dpm	Particle fraction, dpm
E. lathyris	22,156	12,540	6,700
E. pulcherrima	67,360	10,780	15,980
E. tirucalli	101,300	7,290	18,176

a 75% reduction in incorporation was measured upon a 1:1 dilution of the latex, and this figure increased on further dilution. Other incubation media introduced flocculation and occasionally coagulation of the particles resulting in variation of the ^{14}C-triterpene yield. Best reproducible results were obtained by centrifuging the freshly tapped latex at low speed, followed by resuspending the particle suspension of the toplayer and the bottomfraction in samples of clear serum. Both fractions did synthesise triterpenes from acetate (*table 4*), but the yield of these ^{14}C-lipids was substantially lower than the labelled lipid yield derived from an equivalent sample of pure latex. The serum fraction alone did not convert acetate or mevalonate into insaponifiable lipids. Apparently triterpene synthesis can be achieved in at least two different sites: a light particle fraction and an amyloplast containing fraction. Always the amyloplast containing fraction produced labelled triterpenes with the highest specific activity (*table 3*); it is supposed to be the most effective site in this synthesis.

Ultrastructure.

The bottom fraction possessed starch bearing plastids, some covered with membraneous configurations, whereas these configurations were less evident on other plastids (*figs. 3* and *4*). Occasionally these membraneous structures were observed as single structures, having lost their starchy substrate (*fig. 3*). TEM preparations of the amyloplasts in the bottom fraction revealed membranes extended from the plastid surface in a complex anastomosing array of tubules containing an electron dense content (*fig. 5*). Substances accumulated at different positions in these tubules and appeared as spherical bodies, often at positions distant from the plastid itself (identified by the starch grain, S). The content of these small bodies appeared continuous with that of the tubules (*fig. 5*). Smooth amyloplasts often showed solitary particles of different sizes on their surface. These lipid droplets were always encountered in between membranes (*fig. 6*). No other organelles or micro-organisms were detected in this latex fraction.

4. DISCUSSION

Two different sites for triterpene synthesis were identified in fractionated latex

of *Euphorbia*, in that incorporation occurred in the upper particle fraction and the bottom fraction composed of amyloplasts and associated membranes. Both sites appear to accomplish this process independently. In the upper fraction many particle containing vesicles of different sizes were found, resembling those described for *Euphorbia characias* (MARTY 1968, 1971b). These vesicles are supposed to originate in the cytoplasm and released in the central vacuole. Similar vesicles are supposed to be involved in particle synthesis in *Euphorbia pulcherrima* (FINERAN 1982) and have been described for the latex of *Asclepias* (WILSON & MAHLBERG 1980). In our study these vesicles were recovered from the vacuolar part of the laticifer and apparently still able to convert acetate into triterpenes, which in turn were added to existing particles. This resembles the rubber synthesis in *Hevea* latex, where extension of the rubber molecule occurs at the particle surface (ARCHER & AUDLEY 1973).

The site of synthesis in the bottom fraction is less evident, because of the presence of both amyloplasts with a relatively smooth surface and those with an associated tubular membrane complex. The latter probably resembles the structures detected in the laticifers of *Euphorbia characias* and mentioned by MARTY as amyloplasts with a thick electron dense coating (MARTY 1971a). The involvement of amyloplasts in latex particle synthesis has been suggested many years ago (MILANEZ 1949, PROKOFJEV 1947, ULMANN 1951). Our study indicates the membranes associated with the starch grains to be active in the synthesis of latex triterpenes. The incorporation experiments demonstrate the conversion of acetate and mevalonate into triterpenes, the occurrence of lipid droplets is shown in EM. The presence of these lipid droplets along the tubular membranes suggest a secretion of lipid globules and sections of amyloplasts with a relatively smooth surface show lipid globules in between the enveloping membranes. Attempts to purify the active sites in this bottom fraction have failed so far. Isopycnic centrifugation over a sucrose gradient (*table 2*) revealed that about 50% of the ^{14}C-triterpenes remained associated with the dense amyloplasts. The other labelled triterpenes were recovered from the various sucrose layers and might be part of membranes or fragments of the tubular membrane system. The gel filtration procedure which was successfully used to purify the latex particles from the toplayer was inappropriate in the isolation of latex particles from the bottom fraction. Apparently, triterpene particles with physical properties similar to normal latex particles are not produced in this fraction. The amyloplast with associated membranes is therefore probably not a major site of latex particle synthesis, despite its high incorporation rate in triterpene synthesis from acetate or mevalonate.

An other pertinent observation from these and previous incorporation experiments is the unequal distribution of ^{14}C among free and esterified triterpenes. More esterified triterpenes were labelled compared with the free triterpenols (GROENEVELD 1976). Also it has been reported that the ratio of ^{14}C-triterpenol to ^{14}C-triterpene ester was time dependant and that the acyl moiety was not derived from acetate (NEMETHY et al. 1983). TLC analysis of our preparation showed that over 90% of the label co-chromatographed with long chain fatty

acid esters of the triterpenes. These compounds occur as traces only in the latices of these *Euphorbia* species. Therefore, under in vitro conditions the newly synthesised triterpenes may esterify with long chain fatty acids in a process that probably does not or hardly occur in vivo.

ACKNOWLEDGEMENTS

This work was funded by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) to H. W. Groeneveld and by the Indiana University Foundation to P. G. Mahlberg.

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