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# *cis*-Vaccenic Acid and Squalene in the Mitochondria of the *Sauromatum guttatum* Appendix


By

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With 3 figures

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Summary

SKUBATZ H. & HOWALD N. 2002. *cis*-vaccenic acid and squalene in the mitochondria of the *Sauromatum guttatum* Appendix. – Phyton (Horn, Austria) 42 (2): 189–198, with 3 figures. – English with German summary.

The fatty acid composition of the thermogenic mitochondria of the appendix of *Sauromatum guttatum* inflorescences changes during development. Four days prior to heat production the level of oleic acid (18:1 Δ9) starts to decline whereas the level of *cis*-vaccenic acid (18:1 Δ11) increases; each acid accounts for about 5% of total fatty acids at that time. On the day of heat-production, oleic acid level drops to about 2%



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and that of *cis*-vaccenic acid increases to about 15%. Evidence is also provided which indicates that squalene, a triterpene, is present in these mitochondria during anthesis.

## Zusammenfassung

SKUBATZ H. & HOWALD N. 2002. *Cis*-Vaccensäure und Squalen in den Mitochondrien des Appendix von *Sauromatum guttatum*. – Phyton (Horn, Austria) 42 (2): 189–198, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die Fettsäurezusammensetzung der wärmeerzeugenden Mitochondrien im Appendixgebilde von *Sauromatum guttatum* verändert sich während der Entwicklung. Vier Tage vor der Wärmeerzeugung beginnt der Ölsäuregehalt (18:1 Δ9) abzusinken, hingegen steigt der cis-Vaccensäuregehalt (18:1 Δ11) an; jede dieser Säuren trägt rund 5% zum Gesamtfettsäuregehalt zu diesem Zeitpunkt bei. Am Tag der Wärmeproduktion verringert sich der Ölsäuregehalt auf 2%, der der *cis*-Vaccensäure steigt auf rund 15%. Es gibt auch Anzeichen dafür, dass das Tripeptid Squalen während der Anthese in diesen Mitochondrien vorkommt.

Abbreviations: D-day, day of inflorescence opening and heat production; D-1, D-2, etc, days before D-day; D+1, one day after heat-production. FAME, fatty acid methyl esters

## Introduction

On the day of heat-production and inflorescence-opening (D-day), the appendix of the *Sauromatum guttatum* inflorescence (a 20-40 cm-long, slender organ) becomes warm, reaching a 32 °C temperature (SKUBATZ & al. 1991) and it emits more than 100 compounds including terpenes and fatty acid derivatives (SKUBATZ & al. 1996). Changes in the levels of the alternative oxidase forms in the mitochondria precede the production of heat and are detectable 2 days before heat-production (SKUBATZ & HAIDER 2001). On the day of heat-production, the ultrastructure of the mitochondria changes and they become elongated and intensively stained with osmium (SKUBATZ & KUNKEL 2000). It is in connection with the events that precede the metabolic flare-up that our data on the lipid content of the tissue has relevance (SKUBATZ & al. 1995). The composition of total fatty acids of the appendix tissue starts changing on D-3. The percentage of oleic acid from total fatty acids dropped from about 9% in the morning of D-3 to 6 in the morning of D-2 and the percentage of *cis*-vaccenic acid, the positional isomer of oleic acid, increased from 3 to 11%.

The goal of the present study is to investigate (a) the presence of *cis*-vaccenic acid in the appendix mitochondria and (b) the presence of isoprenoid compounds in these mitochondria. We report here on the presence of *cis*-vaccenic and squalene in the appendix mitochondria during anthesis. The acid level gradually increases during anthesis and it stayes high until the inflorescence dies. Squalene is also detected in the mitochondria and mitoplasts as well.

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## Material and Methods

### Plant material

Inflorescences of *Sauromatum guttatum* were allowed to develop in a growth chamber under long-day conditions as previously described (SKUBATZ & al. 1993). The developmental stage of the appendix was determined retroactively with respect to the day of inflorescence-opening and heat-production (D-day).

### Isolation of mitochondria

Washed and Percoll-purified mitochondria were obtained as previously described (SKUBATZ & al. 1989), except that 31% (v/v) Percoll was used to form the gradient instead of 29% (v/v). The intactness of the mitochondrial preparation was high : between 85 to 99% (SKUBATZ & al. 1989). Our mitochondrial preparation strongly interacts with a monoclonal antibody against the alternative oxidase on a Western blot (SKUBATZ & HAIDER 2001).

### Mitoplast preparation

Percoll-purified mitochondria were treated with freshly prepared 1% digitonin in 0.4 M sucrose. The digitonin solution was added to mitochondrial suspensions to give a digitonin : protein ratio of 1:5. At this low ratio, the outer membrane is separated from the inner membrane (DAY & WISKICH 1974, JANCSIK & al. 1988). The suspension was incubated at 4 °C for 30 min and centrifuged at 40,000 g for 15 min to obtain the mitoplasts.

### Electron microscopy

Percoll-purified mitochondria and mitoplasts were fixed in a fixative buffer (0.4 M sucrose, 0.1 M sodium cacodylate, pH 7.4) containing 0.25% glutaraldehyde and 1% osmium tertroxide for 2 hr at 4 °C. The mitochondria and mitoplasts were pelleted in an IEC cytospin. The fixative was removed and the mitochondria and mitoplasts were washed 4 times for 10 min in 0.1 M cacodylate buffer, pH 7.4. Subsequently, the mitochondria and mitoplasts were dehydrated through grades of ethanol: a brief wash in 15% and 50%, 3 times in 70% for 5 min each, followed by 2 washes for 2 min each with propylene oxide. After the washes, they were incubated in Medcast : propylene oxide mixture in a 1:1 ratio for 1 h, and in 3:1 ratio overnight. Next day, the mitochondria were incubated 2 times for 1 h in 100% Medcast following a third change, spin and placed in 60 °C for 2 days. The blocks were sections and stained with 7% uranyl acetate in 50% ethanol for 30 min followed by 3% lead citrate for 10 min.

### Fatty acid extraction and methylation

Mitochondria (1 to 5 mg total protein) were extracted in 0.5 mL of methanol / 1 N HCl (Supelco) in the presence of the internal standard triheptadecanoin (1,2,3-triheptadecanoyl glycerol, 50 µg per sample) at 80 °C for 1 h. Half mL of 10% KCl and 0.5 mL of hexane were added and fatty acid methyl esters (FAME) were extracted with hexane 3 times. The pooled hexane phases were then concentrated under nitrogen to 50 µL and was used for GC analysis.

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Squalene extraction

Mitochondrial preparations were extracted 3 times with 0.5 mL of hexane. The hexane phases were concentrated under nitrogen to 5 μL from which 1 μL was taken for GC-MS analysis.

GC-MS analysis

GC-MS analyses were performed on a Fisons VG MD quadrupole mass spectrometer directly interfaced with a Carlo Erba 8000 gas chromatograph equipped with a capillary split-splitless and on-column injector (Fisons Instruments, Manchester, UK). Squalene analyses were carried out using a DB-1 fused-silica capillary gas chromatograph column (30 m × 0.32 mm I.D., 0.25 μm film thickness; J & W Scientific, CA, USA) operated with helium as a carrier gas (head pressure, 10 psi). FAME analyses were carried out using a fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) coated with the bonded stationary phase, DB-23 (J & W Scientific) using helium as a carrier and makeup gas with a column flow of 1 mL min-1. FAME and squalene were identified by comparison of their retention time and mass spectra with authentic standards purchased from Sigma (St. Louis, USA). The relative percentage of each FAME was calculated as the ratio of its integrated peak area to the total integrated peak area of all FAME in the total ion chromatogram.

Protein determination

Protein amount was determined by the BRADFORD procedure 1976 using BSA as the standard.

## Results

### *cis*-Vaccenic acid in the appendix mitochondria

Table 1 shows the presence of *cis*-vaccenic acid (18:1Δ11) and oleic acid (18:1Δ9) in mitochondria purified on Percoll gradients. Figure 1 clearly demonstrates the changes in the level of *cis*-vaccenic acid during anthesis. Its level increased from D-4 to D-day, whereas level of oleic acid decreased somewhat. The increase in the percentage of *cis*-vaccenic acid was higher than the decrease in oleic acid, suggesting that only some of *cis*-vaccenic acid could be synthesized directly from oleic acid.

Table 1.
The presence of *cis*-vaccenic acid in the appendix mitochondria of *Sauromatum guttatum*. Percoll-purified mitochondria were prepared from D-1 and D-day appendices. Values represent means ± s.d. of n experiments in parentheses.

| Mitochondrial Preparation | Fatty Acid Composition (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 16:0 | 16:1 | 18:0 | 18:1 Δ9 | 18:1 Δ11 | 18:2 | 18:3 |
| D-day (n=3) | 29±5 | 5±4 | 15±5 | 3±0 | 18±3 | 30±6 | 0±0 |
| D-1 (n=4) | 29±6 | 2±1 | 16±9 | 4±2 | 11±3 | 38±9 | 1±0 |

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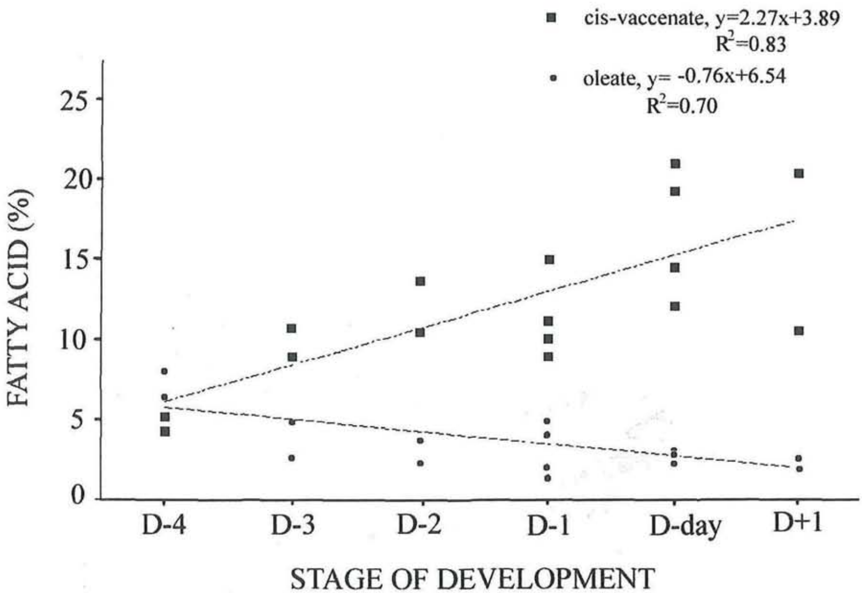


Fig. 1. The level of *cis*-vaccenic acid in the *Sauromatum* mitochondria during development. The persentage of the acid in different preparations was calculated and the best fit graph was plotted after mediation of the data. Each point represents one mitochondrial preparation.

Table 2.
Squalene in Percoll-purified mitochondria from a D-day *Sauromatum* appendix. The percentage of squalene represents the integrated area of the squalene peak in a total ion chromatograph per mg of mitochondrial protein. The amount of protein is: 4.8 mg of washed mitochondria; 3 mg of Percoll-purified mitochondria; and 0.75 mg of digitonin-treated mitochondria.

| Mitochondrial Preparation | Squalene (%) |
|---|---|
| Washed | 100 |
| Percoll-purified | 10 |
| Percoll-purified, treated with digitonin | 333 |

## Squalene in the appendix mitochondria

Squalene was found in hexane extracts of Percoll-purified mitochondria prepared from D-day appendices (Tab. 2). Treatment of Percoll-purified mitochondria with digitonin at a concentration of 0.2 mg/mg protein resulted in a 30-fold increase in the amount of extractable squalene (Tab. 2). At this concentration of digitonin, the outer membrane of the mitochondria was separated from the mitochondria and the mitoplast

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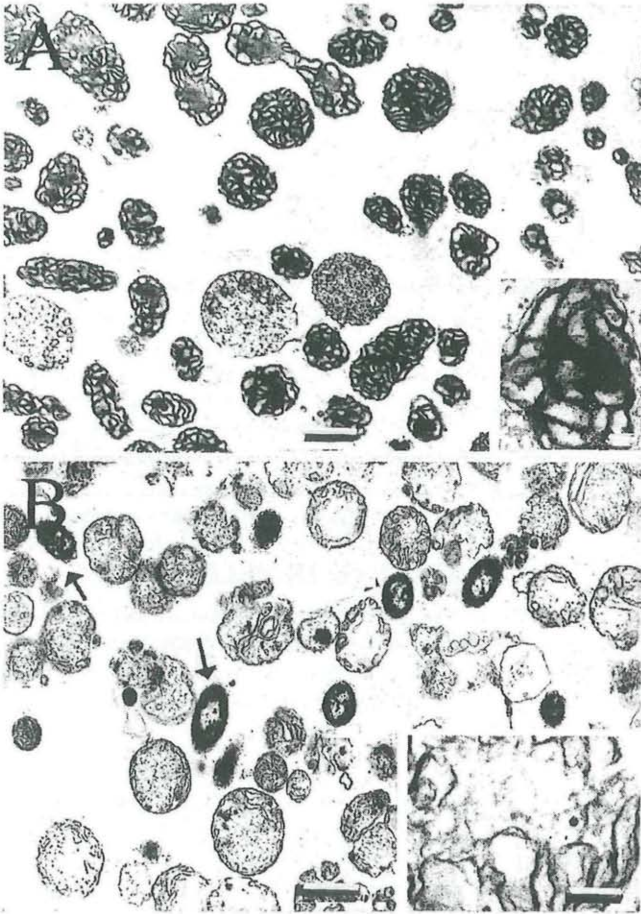


Fig. 2. Electron micrographs of mitochondria and mitoplasts from a D-day appendix. (A) mitochondria with an electron-dense matrix. The insert depicts a mitochondrion with a visible outer membrane. (B) mitoplasts formed by a digitonin treatment of a portion of unfixed mitochondria observed in A. Arrows point to osmiophilic structures that may represent electron-dense mitoplasts. The insert depicts a mitoplast with no visible outer membrane. Bars: 1 μm and 0.1 μm for the inserts.

membrane was clearly visible (Fig. 2B). This effect of digitonin on the extraction of squalene was also observed in 3 other preparations of mitochondria obtained from D-4 to D-day appendices (data not shown). This fact strongly suggested that squalene was inaccessible to hexane extraction because it was incorporated in the mitochondrial membranes. To check whether squalene is present in the inner membrane of the mitochondria, mitoplasts were prepared and extracted with hexane. Figure 3

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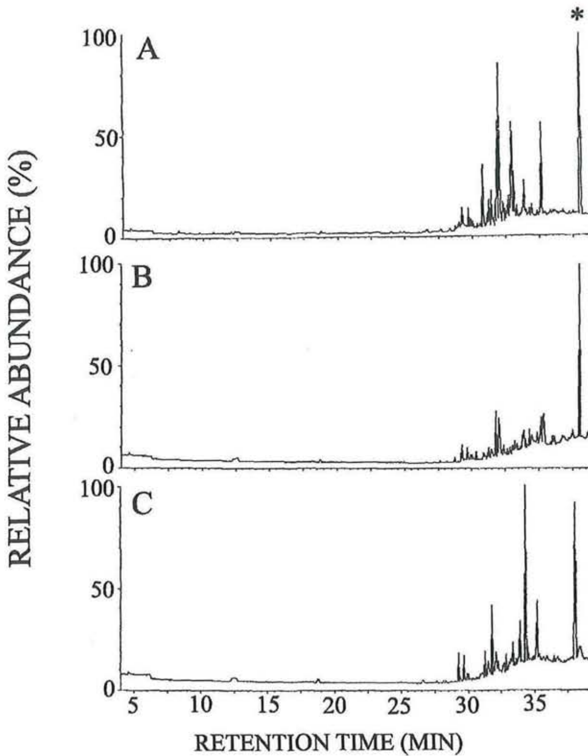


Fig. 3. The present of squalene in D-2 to D-4 mitoplasts. The integrated area of the squalene peak (marked with an asterisk) per mg of mitochondrial protein was: 732, 752 for washed mitochondria in (A); 36% of squalene amount in (A) was present in the Percoll-mitochondria (B); and 90% of it was present in the mitoplasts (C). All other peaks in the chromatograms were not terpenes and could not be identified.

shows the chromatograph's profile and the retention time of squalene. The integrated peak area of squalene in mitoplasts (Fig. 3C) was higher than in Percoll-purified mitochondria (Fig. 3B), implying that squalene indeed is present in the mitochondria.

## Discussion

### *cis*-Vaccenic acid in *Sauromatum* mitochondria

In our previous report we have demonstrated the presence of an inverse linear correlation between *cis*-vaccenic acid and oleic acid in the *Sauromatum* appendix (Skubatz & al. 1995). In the appendix mitochondria, the level of *cis*-vaccenic is high and can reach 15% of total fatty acid. The level of oleic acid in the mitochondria is lower than 5%.

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The presence of *cis*-vaccenic acid in other plant mitochondria has not been reported suggesting that the lipid composition of thermogenic mitochondria may be different than the non-thermogenic ones, and that *cis*-vaccenic acid may have a role in these highly active mitochondria.

Plant mitochondria have high levels of palmitic and linoleic acids (DALZIEL & BREIDENBACH 1982, DAUM 1985, HARWOOD 1985, MOREAU & al. 1974), whereas the *Sauromatum* mitochondria have an unusually high level of *cis*-vaccenic acid and a low level of oleic acid. It has been shown that *cis*-vaccenic acid is synthesized mainly from palmitoleic acid by chain elongation and to some extent by double bond shifting by an isomerase from position 9 to 11 (SHIBAHARA & al. 1990). In the *Sauromatum* appendix the route of *cis*-vaccenic acid synthesis has yet to be determined.

The shift of the double bond to position 11 from 9 may increase the melting temperature of the membrane phospholipids and thus allowing the mitochondrial membrane to stay functioning during the thermogenic activity. In rat mitochondria, oleic acid is preferentially incorporated in the 2-position of triglycerides and phospholipids whereas, cis-vaccenic acid better occupies the 1- and 2- positions in phospholipids and 1- and 3-positions in triglycerides (SGOUTAS & al. 1976). This organization may influence and regulate enzymatic activity in the mitochondrial membrane (SANDERMANN 1978). However, it does not seem to have any affect on the proton permeability of the mitochondrial inner membrane (BROOKES & al. 1997, 1998).

Squalene in *Sauromatum* mitochondria

Squalene is an intermediate in sterol biosynthesis and usually does not accumulate in the cell and definitely not in the mitochondria. It is detectable, for example, in yeast mutants that lack unsaturated fatty acids (TUNG & al. 1991). *Sauromatum* mitochondria have an unusual high respiration rates (SKUBATZ & al. 1991) and squalene may be a scavenger of oxygen free radicals generated during respiration (KOHNO & al. 1995). Squalene may also affect the mitochondrial membrane fluidity because it promotes the formation of non-bilayer structures (LONHER & al. 1993).

Squalene synthase, an enzyme that condenses two molecules of farnesyl diphosphate, has been found in various compartments of the plant cells but not in the mitochondria (BELINGHER & al. 1991, KLEINING 1989). Plant mitochondria have only prenyl transferases that are capable of formation of the isoprenoid side chain of quinone (KLEINING 1989). Therefore, squalene may be synthesized in other cellular compartments and transported into the mitochondria (SCALLEN & al. 1985).

General consideration

This study is one additional step in the understanding the spectacular metabolic flare-up in the *Sauromatum* appendix. It is reasonable to as-

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sume changes in membrane composition of the appendix tissue during anthesis and on D-day. The presence of *cis*-vaccenic acid and squalene in mitochondria is unusual and they may affect the respiratory metabolism of the *Sauromatum* mitochondria.

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