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**Tissue Culture Note**

# Triterpenoid levels are reduced during *Euphorbia tirucalli* L. callus formation

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**Abstract** In order to compare the profiles of hydrophobic secondary metabolites between the calli and plants of *Euphorbia tirucalli*, we analyzed their free sterol and free triterpenoid contents by GC-MS. We obtained the calli by culturing *E. tirucalli* internodes successively on solidified B5 medium containing hormones. The aerial parts of the plants or calli were extracted with $CHCl_3$-MeOH (2 : 1, v/v) and subjected to GC-MS analysis. In a plant extract, only two sterol peaks, for campesterol and $\beta$-sitosterol, and at least 11 peaks of the total ion chromatogram (TIC) with a $[M^+]$ of 426, including peaks for euphol, $\beta$-amyrin, and glutinol, were detected. The ratio of triterpenoid- and triterpenoid-like-peaks of TIC with a $[M^+]$ of 426 to the total of all peaks detected was ca. 86% (n=3) in the plant extract. In a callus extract, 6 sterols, campesterol, stigmasterol, $\beta$-sitosterol, isofucosterol, cycloartenol, and 24-methylenecycloartanol, and two triterpenoids with a molecular weight of 426 (euphol and $\beta$-amyrin) were detected. The ratio of triterpenoid- and triterpenoid-like peaks of TIC with a $[M^+]$ of 426 to the total of all peaks detected was only ca. 6.3% (n=3) in the callus extract. These results indicate that the ratio of free triterpenoids and free triterpenoid-like compounds (MW: 426) to free sterols is lower in the calli than in the plants of *E. tirucalli*.

**Key words:** Callus, *Euphorbia tirucalli*, petroleum plant, sterol, triterpenoid.

Sterols and triterpenoids are hydrophobic secondary metabolites with the same common precursor (Abe and Prestwich 1999). Phytosterols are constituents of the plant cell membrane (Hartmann 1998), raw materials for hormones, and cholesterol-analogues that can be used as dietary supplements. Triterpenoids are modified into terpenoid saponins, which are stored in the roots (Lee et al. 2004); can act as phytoalexins against pathogens (Hirai et al. 2000); and are major constituents of the flammable substance latex in oil plants (Nielsen et al. 1979; Rizk 1987).

Euphorbiaceae plants store abundant amounts of latex in an organ called the laticifer (Scassellati-Sforzolini 1916; Fahn 1979; Fahn 1990). The major constituents of latex are isomers of triterpenes with the molecular formula $C_{30}H_{50}O$ (MW: 426), such as euphol, tirucallol, glut-5-en-3-$\beta$-ol, cycloeuphordenol, euphorginol, $\alpha$-amyrin, lanosterol, cycloartenol, and others (McDonald et al. 1949; Nielsen et al. 1977; Nemethy et al. 1979; Nemethy and Calvin 1983; Hashimoto 2001). In this report, we refer to the sterol precursor cycloartenol (MW: 426) as a sterol, in contrast to triterpenoids with a molecular weight of 426, such as $\alpha$-amyrin, $\beta$-amyrin, and euphol.

Oil bodies have been identified in the calli of *Euphorbia tirucalli* (*Et*) by electron microscopy (Ohyama et al. 1984), and a plant regeneration protocol has been established for this plant (Uchida et al. 2004). Expressed sequence tag analysis (Kajikawa et al. 2004) and characterization of the genes for $\beta$-amyrin synthase (Kajikawa et al. 2005), squalene epoxidase (Uchida et al. 2007), and squalene synthase (Uchida et al. 2009) have also been performed. A previous report indicated that overexpression of the *Et* squalene synthase gene in *Et* calli increased the accumulation of phytosterols (Uchida et al. 2009). However, no comparative analysis of the sterol/triterpenoid profiles in *Et* shoots and calli has been performed. The amounts of minor triterpenoids in wild

Abbreviations: BA, 6-benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; EIC, extracted ion chromatography; *Et*, *Euphorbia tirucalli*; GC-MS, gas chromatograph-mass spectrometer; NAA, naphthaleneacetic acid; TIC, total ion chromatography.
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type calli have not been studied in detail (Uchida et al. 2009). Here, we analyzed the profiles of free sterols and triterpenoids by extracted ion chromatography (EIC) in *Et* calli and the aerial parts of the plants. This report will hopefully contribute to the development of future plant biotechnologies for producing phytosterols in calli, as a means of producing raw materials for hormones, insecticides, cosmetics, or dietary supplements.

*E. tirucalli* potted plants were sterilized as described elsewhere. Figure 1A shows a sterile *Et* plant that was cultured *in vitro*. The plants were maintained on solidified LS medium in 500-ml glass culture vessels and subcultured every three to four-weeks on the medium. *Et* internodes were inoculated onto solidified B5 medium containing $2\,mg\,l^{-1}$ NAA, $1\,mg\,l^{-1}$ 2,4-D, and $0.22\,mg\,l^{-1}$ BA and then subcultured successively, producing white calli (Figure 1B). The calli were then subcultured on the media. The plants and calli were grown at 27ºC under approximately $40\,\mu mol\,m^{-2}\,s^{-1}$ fluorescent illumination with a 16-h photoperiod.

Free sterols and free triterpenoids were extracted using $CHCl_3$-MeOH (2 : 1, v/v) overnight at 4ºC with gentle shaking. The extract was then mixed with 0.45% NaCl, and the organic phase was recovered, evaporated with $N_2$ gas bubbles, dissolved with acetonitrile, and injected into a Shimadzu GCMS-QP2010 (Shimadzu, Kyoto, Japan). The GC-MS was then fitted with a DB-1701 column ($0.25\,mm \times 30\,m$, $0.25\,\mu m$ film thickness, J W Scientific, Folsom, USA) and a helium carrier (flow rate 2.57 ml $min^{-1}$) and was operated at an ionization voltage of 70 eV with a scan range of 45–550. The sample size was $1\,\mu l$ with a splitless injection. The injector temperature was 280ºC. The column temperature was maintained at 50ºC for 1 min, elevated to 300 ºC at $20ºC\,min^{-1}$, and then held at 300ºC for 15 min.

The peaks detected in an extract of the aerial parts of a plant (plant 1 in Table 1), included two sterols (campesterol (a400) and $\beta$-sitosterol (c414)), three triterpenoids with a $[M^+]$ of 426 (euphol (B426), $\beta$-amyrin (F426), and glutinol (I426)), and 8 unidentified compounds with a $[M^+]$ of 426 (with prominent m/zs (rel. int.)): A426 (426(38), 411(47), 371(38), 137(100)); C426 (426(27), 274(100), 205(77), 137(88)); D426 (426(40), 411(100), 393(48), 259(20)); E426 (426(31), 412(31), 411(100), 393(40)); G426 (426(38), 247(67), 229(61), 205(100)); J426 (426(11), 229(35), 220(100), 205(41)); K426 (426(23), 411(17), 247(100), 229(58)); and L426 (426(90), 273(100), 205(90), 163(90)) (Figure 2). The three peaks at $[M^+]=426$, euphol, $\beta$-amyrin, and glutinol, were identified as triterpenoids by comparing them with authentic samples in the GC-MS. The 8 unidentified peaks mentioned above are referred to as triterpenoid-like peaks (compounds) in the present study. In the callus extract (callus 1 in Table 1), 6 sterol peaks (campesterol (a400), stigmasterol (b412), $\beta$-sitosterol (c414), isofucosterol (d412), cycloartenol (e426), and 24-methylenecycloartanol (f440)) and two major triterpenoid peaks with a $[M^+]$ of 426 (euphol (B426) and $\beta$-amyrin (F426)) were detected in the GC-MS as mentioned above. In triplicated experiments, the ratios of triterpenoid- and triterpenoid-like TIC-peaks with a $[M^+]$ of 426 to that for all detected peaks were 68.6%, 93.5%, and 96.2%, resulting in a mean of $86.1 \pm 12.4$ (mean$\pm$S.D.) % in the extract of the aerial parts of the *Et* plant, as compared with those of 6.32%, 0.443%, and 12.0%, with a mean of only $6.25 \pm 4.72$% in the *Et* callus (Table 1). This result indicates that callus formation from the aerial parts of the *Et* plant is accompanied by an approximately 14 times decrease in the ratio of total free triterpenoids and free triterpenoid-like compounds to all detected compounds. In biotechnologies used to provide the raw materials of phytosterols, a higher ratio of free sterols is preferable. The free sterols in *Et* calli comprise 86.0% of all compounds detected, in contrast to only 5.23% in the aerial parts of *Et* plants (Table 1). As far as we know, this is the first report to show an increase in the ratio of free sterols to free triterpenoids and free triterpenoide-like compounds in *E. tirucalli* callus formation. However, obligate amounts of free sterols are economically important. The total free sterol amount in *Arabidopsis thaliana* callus is 68% of that in mature plants (Schrick et al. 2004), and the free oleanolic acid content in *Fabiana imbricata* calli is 56% of that in plantlets (Schmeda-Hirschmann et al. 2004). Therefore, the obligate amounts of free sterols and free triterpenoids in *E. tirucalli* need to be evaluated in future. Decreases in the ratio of free triterpenoids to free sterols may be related to callus-specific phenotypes in this plant; active

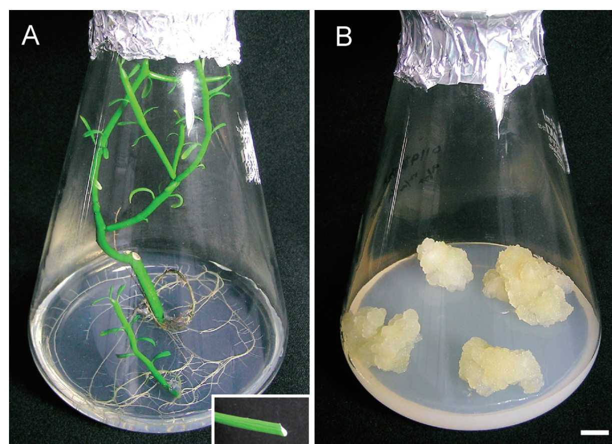


Figure 1. *In vitro* culture and callus of *Euphorbia tirucalli* L. Potted plants were sterilized, and an *in vitro* culture was obtained (A). The inset shows a latex-exuding stem, immediately after it has been cut. The internodes of the *in vitro* plants were inoculated onto B5 agar medium supplemented with $2\,mg\,l^{-1}$ NAA, $1\,mg\,l^{-1}$ 2,4-D, and 0.22 $mg\,l^{-1}$ BA every month. After successive cultures, white calli were obtained (B). The bar represents 1 cm.

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Table 1. Percentages of TIC peaks detected in calli and the aerial parts of *E. tirucalli* plants.

| Compounds[a] | Callus1 | Callus2 | Callus3 | Plant1 | Plant2 | Plant3 |
|---|---|---|---|---|---|---|
| Campesterol (a400) | 2.12 | – | 0.888 | 1.06 | 0.196 | – |
| A426 | – | – | – | 0.792 | 53.2 | 0.666 |
| Euphol (B426) | 3.92 | – | 1.85 | 25.6 | 0.379 | 45.5 |
| Stigmasterol (b412) | 34.3 | 5.94 | 30.3 | – | – | – |
| 414 (N.I.) | 2.65 | – | 0.190 | – | – | – |
| C426 | – | – | – | 2.03 | 15.9 | – |
| D426 | – | – | – | 7.38 | 15.0 | 11.4 |
| $\beta$-sitosterol (c414) | 8.87 | 2.93 | 10.7 | 14.4 | – | – |
| 416 (N.I.) | 5.85 | 0.310 | 6.24 | – | – | – |
| Isofucosterol (d412) | 26.3 | 90.3 | 20.7 | – | – | – |
| 428 (N.I.) | – | – | – | 10.6 | 5.97 | – |
| E426 | – | – | – | 1.80 | 1.08 | 4.36 |
| $\beta$-Amyrin (F426) | 2.40 | – | 10.1 | 6.15 | 1.76 | 10.5 |
| Cycloartenol (e426) | 6.01 | – | 13.6 | – | – | – |
| G426 | – | – | – | 4.25 | 0.824 | 2.13 |
| Lupeol (H426) | – | 0.443 | – | – | – | – |
| Glutinol (I426) | – | – | – | 17.1 | 4.34 | 15.9 |
| 408 (N.I.) | – | – | – | 4.23 | 0.294 | 1.20 |
| 24-Methylenecycloartanol (f440) | 1.98 | 0.0191 | 2.93 | – | – | – |
| 424 (N.I.) | – | – | – | 0.851 | – | 2.47 |
| J426 | – | – | – | 0.532 | 0.182 | 0.897 |
| 410 (N.I.) | 1.16 | – | 0.685 | – | – | – |
| 412 (N.I.) | 2.20 | – | 0.773 | – | – | – |
| K426 | – | – | – | 0.832 | 0.191 | – |
| L426 | – | – | – | 2.14 | 0.600 | 4.84 |
| 410 (N.I.) | 2.05 | – | 0.739 | – | – | – |
| Total of triterpenoid- and triterpenoid-like compounds with a TIC [$M^+$] of 426 | 6.32 | 0.443 | 12.0 | 68.6 | 93.5 | 96.2 |
| Total sterols, which include cycloartenol | 79.6 | 99.2 | 79.1 | 15.5 | 0.196 | – |

| [b] Means of total amounts | Calli | Plants |
|---|---|---|
| Triterpenoid- and triterpenoid-like compounds with a TIC [$M^+$] of 426 | $6.25 \pm 4.72$ | $86.1 \pm 12.4$ |
| Sterols | $86.0 \pm 9.36$ | $5.23 \pm 7.26$ |

[a] The major peaks detected and the percentages of the corresponding peak areas relative to the total peak area are listed in accordance with their retention times. Note that cycloartenol (MW: 426) is not included in the total area of triterpenoid- and triterpenoid-like peaks with a TIC [$M^+$] of 426. The TIC and EIC of *Et* callus 1 and plant 1 are shown in Figure 2. N.I., not identified; –, undetectable; capital letters, peaks for triterpenoids, or triterpenoid-like compounds; lower case letters, peaks for sterols. [b] Means of percentages ($\pm$SD.) (n=3).

cell division; or the absence of differentiation in various organs, such as roots, laticifers, the cortex, and the cambium. Since the oxidization (Seki et al. 2008) and glycosylation (Lee et al. 2004) of triterpenoids are important in certain plant parts, especially in the roots, seeds, and lignified barks, a future study of *E. tirucalli* secondary metabolites should include an analysis of the chemically modified triterpenoids and sterols present in calli and plants.

In a previous report, euphol and $\beta$-amyrin were detected in extremely low amounts in wild type calli (Uchida et al. 2009), as shown in callus 2 in Table 1. In the present study, we surveyed the EIC of triterpenoids and triterpenoid-like compounds in *E. tirucalli* calli. Euphol and $\beta$-amyrin were detected in calli 1 (Table 1 and Figure 2) and 3 (Table 1), while these two compounds were not detected, but lupeol (H426) was detected, in callus 2 (Table 1). The total amounts of free triterpenoids and free triterpenoid-like compounds with a [$M^+$] of 426 in calli 1, 2, and 3 were 6.32%, 0.443%, and 12.0%, respectively. This result may reflect physiological differences between each callus. *E. tirucalli* cells in suspension culture include oil bodies, which are observed as black spots under light microscopy, and these cells include sterols as well as triterpenoids and diterpenoids (Ohyama et al.1984). Black patches were also observed on the surfaces of calli grown on solidified medium (data not shown), and these black patches appeared to be rich in oil bodies. The sizes and densities of these patches were affected by the culture conditions, such as the inoculation interval; the presence or absence of agar; the differing purities of the agars used, which were obtained from several companies (Wako, Osaka, Japan; Becton, Dickinson and Company, Sparks, USA; Nacalai, Kyoto, Japan); and the hormones added to the media. In the above mentioned study (Ohyama et al.

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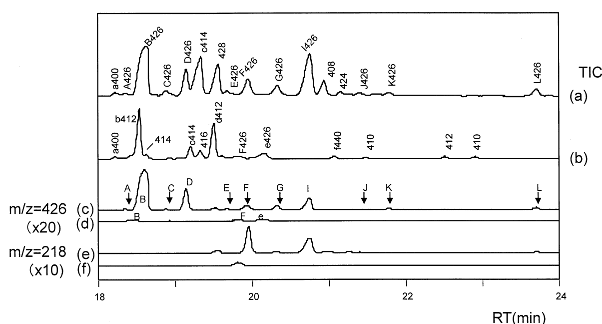


Figure 2. GC-MS analysis of *Euphorbia tirucalli* plant or callus extracts. (a, b), Total ion chromatograms (TIC) of a plant extract (a) and a callus extract (b). (c, d) m/z=426 extracted ion chromatograms (EIC) of extracts from the plant (c) or the callus (d). (e, f) m/z=218 EIC chromatogram of extract from the plant (e) or the callus (f). The amplitudes of (c, d) and (e, f) were magnified 20 and 10 times more than those of (a) and (b), respectively. Capital lettrs and lower case letters have the same meanings as in Table 1. The EIC m/z=218 chromatogram is shown to identify peaks belonging to the triterpenoid $\beta$-amyrin (F426).

1984), the relative amounts of steroids in the oil bodies and the other parts of the cells were not measured separately by GC-MS. In future, the ratio of sterols to triterpenoids in black patches and the other parts of *Et* calli should be compared in detail by EIC analysis, with attention paid to the culture conditions.

At least 3 triterpenoids and 8 triterpenoid-like compounds with a $[M^+]$ of 426 were detected in the aerial parts of *E. tirucalli* plants (Table 1 and Figure 2). This is the first report to list the major m/z signals of these eleven compounds, which were extracted and analyzed by GC-MS at the same time. The identification of these peaks should be performed with reference to the previously reported *Euphorbia* compounds with a molecular weight of 426: euphol (Hashimoto 2001; Nes et al. 1984), tirucallol (Nes et al. 1984), glut-5-en-3-$\beta$-ol (Khan et al. 1987), cycloeuphordenol (Khan et al. 1988), euphorginol (Rasool et al. 1989), $\alpha$-amyrin (Malik et al. 1980), lanosterol (Nemethy and Calvin 1983). Functional analyses of the synthases of triterpenoids that accumulate in the laticifers of *E. tirucalli* plants should also be performed, as has been done in plants that do not possess laticifers (Sawai et al. 2006; Suzuki et al. 2006; Ohyama et al. 2009).

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