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# Production of oleanolic acid glycosides by hairy root established cultures of *Calendula officinalis* L.

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**In order to initiate hairy root culture initiation cotyledons and hypocotyls of *Calendula officinalis* L. were infected with *Agrobacterium rhizogenes* strain ATCC 15834 or the same strain containing pCAMBIA 1381Z vector with β-glucuronidase reporter gene under control of promoter of *NIK* (*Nematode Induced Kinase*) gene. The efficiency of induction of hairy roots reached 33.8% for cotyledons and 66.6% for hypocotyls together for both transformation experiments. Finally, eight control and nine modified lines were established as a long-term culture. The hairy root cultures showed the ability to synthesize oleanolic acid mainly (97%) as glycosides; control lines contained it at the average 8.42 mg·g$^{-1}$ dry weight in tissue and 0.23 mg·dm$^{-3}$ in medium; modified lines: 4.59 mg·g$^{-1}$ for the tissue, and 0.48 mg·dm$^{-3}$ for the medium. Additionally lines showed high positive correlation between dry/fresh weight and oleanolic acid concentration in tissue. Using the Killiani mixture in acidic hydrolysis of oleanolic acid glycosides released free aglycones that were partially acetylated in such conditions.**

**Key words**: Marigold, *Agrobacterium rhizogenes*, hairy roots, triterpenic saponins, oleanolic acid, transformation

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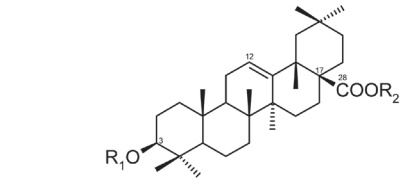
## INTRODUCTION

*Calendula officinalis* L. (*Asteraceae*) is an annual herb cultivated for centuries for ornamental and medicinal purposes. It is an important raw material for pharmaceutical, cosmetic and food processing industries. Extract from marigold flowers has been aproved by the Food and Drug Administration (FDA) as a food additive with a "generally recognized as safe" (GRAS) status. It can be added to food as a natural collorant with a strong antioxidant activity due to the presence of carotenoids and flavonoids (Piccaglia & Venturi, 1998; Guinot *et al.,* 2008). Marigold extract is also used in about 200 cosmetic formulations, mainly creams and shampoos (Andersen *et al.,* 2010). Therapeutical properties of *Calendula* have been known for centuries and successfully utilized in folk medicine. Today, phytochemical and pharmacological research has assigned specific healing activities to individual metabolites present in plant extracts. The chemicals produced by *C. officinalis* comprise steroids, terpenoids, phenolic acids, flavonoids, triterpenic alcohols, and others (EMEA, 1999; Muley *et al.,* 2009; Re *et al.,* 2009). Among these compounds pentacyclic triterpenes, i.e., oleanolic acid (OA) and its glycosides (triterpenic saponins) (Fig. 1) with glucose or glucuronic acid attached to the hydroxyl group at the C-3 position of the aglycone, are fundamental for numerous pharmacological properties: anti-viral (including HIV) (Kashiwada *et al.,* 1998), anti-bacterial, fungistatic (Szakiel *et al.,* 2005), antiparasitic (Szakiel *et al.,* 2008; Doligalska *et al.,* 2011), antitumor, cytotoxic, anti-inflammatory, hepatoprotective (Liu 1995; Liu, 2005), anti-ulcer and anti-diabetogenic. The main obstacle in their introduction in medical therapy is poor elucidation of their multidirectional activity




Oleanolic acid (OA) $R_1$=H; $R_2$=H

| | | $R_1$ | $R_2$ |
|---|---|---|---|
| Glucuronides | F | GlcUA | H |
| | D | GlcUA (3←1)Gal | H |
| | $D_2$ | GlcUA | Glc |
| | C | GlcUA(3←1)Gal | Glc |
| | B | GlcUA (3←1)Gal, (4←1)Glc | H |
| | A | GlcUA (3←1)Glc-Glc, (3←1)Glc-Glc | H |

| | | $R_1$ | $R_2$ |
|---|---|---|---|
| Glucosides | I | Glc | H |
| | II | Glc(4←1)Gal | H |
| | III | Glc(4←1)Gal-Gal | H |
| | IV | Glc (3←1)Glc, (4←1)Glc-Glc | H |
| | V | Glc (3←1)Glc-Glc, (3←1)Glc-Glc | H |
| | VI | Glc (3←1)Glc-Glc, (4←1)Gal-Gal | H |
| | VII | Glc (3←1)Glc-Glc-Glc, (4←1)Gal | H |
| | VIII | Glc (3←1)Glc-Glc, (4←1)Gal | Glc |

**Figure 1. Oleanolic acid glycosides found in *Calendula officinalis* (Szakiel *et al.,* 2005)**

at the molecular level. Additionally, standard methods of marigold compound acquisition are laborious and therefore expensive. Application of new biotechnological approaches will hopefully solve these problems.

The opulence and diversity of marigold biological compounds make it attractive for biotechnological applications. *In vitro* plant cultures have provide an opportunity to produce numerous valuable metabolites in a well-defined system. While designing such a system attention should be paid to somaclonal variation and generally low efficiency of metabolite synthesis. Hairy root

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**Abbreviations**: AcOA, 3-O-acetyl-oleanolic acid; CC, control lines obtained from cotyledon; CH, control lines obtained from hypocotyl; DW, dry weight; FW, fresh weight; GLC, gas-liquid chromatography; MC, modified lines obtained from cotyledon; MH, modified obtained from hypocotyl; MS, Murashige and Skoog (1962) medium; OA, oleanolic acid.

cultures seem to be the right choice, as they offer higher stability and productivity than undifferentiated cultures. Cultures induced by *Agrobacterium rhizogenes* are characterized by ample lateral branching, profusion of root hairs and absence of geotropism. Hairy root cultures are easy to maintain: a high growth rate can be obtained in media free of any plant growth regulators. Hairy roots can be grown in liquid medium, so the culture can be scaled up easily at will. New air-lift types of bioreactors allow exploiting such cultures for commercial production of valuable plant metabolites (Guillon *et al.,* 2006).

Previously we have reported a marigold suspension culture that synthesizing and releasing OA at a low level (Grzelak & Janiszowska, 2002). Addition of elicitors stimulated it to accumulate OA at a 10-fold higher level (Wiktorowska *et al.,* 2010). However, the yield was still less than that from the intact plant. Our first attempts at induction of a marigold hairy root culture induction were presented over three years ago (Długosz *et al.,* 2009), when only two lines at an early stabilization stage were analyzed. In this paper we present full documentation of stable hairy root cultures obtained from the previous experiment. Many of them contain more OA than native marigold roots (Kasprzyk & Fonberg-Broczek, 1967; Mrugasiewicz *et al.,* 1979; Dedio & Kozlowski, 1998).

The lines we have selected produce oleanolic acid glycosides at different levels and release them into the medium. The correlation of biomass growth with the accumulation of triterpenic saponins could be valuable for developing a strategy to maximize the release of such compounds into culture medium. The hairy root lines with the best growth rate can be useful for determining the influence of some physical, chemical and biological factors on production and secretion of saponins.

## MATERIAL AND METHODS

**Plant material**. Mature embryos of the marigold *Calendula officinalis* cv. Persimmon Beauty excised from seeds were sterilized for 20 min with a commercial hypochlorite preparation "Domestos" (Unilever) diluted with water (1:3, v/v). Subsequently the embryos were rinsed three times with sterile water and placed on solid (0.75% agar) medium Murashige and Skoog (1962), (MS) with concentration of macroelements halved (½ MS) for germination. Seedlings with two developed cotyledons and elongated hypocotyls were selected for transformation.

**Transformation and establishment of hairy root culture**. Wild type *Agrobacterium rhizogenes* strain ATCC 15834 bearing or not the pCAMBIA 1381Z vector (http://www.cambia.org/daisy/cambia/home.html) containing the β-glucuronidase (*GUS*) reporter gene under the control of the promoter (1058 bp upstream region from the ATG codon) of tomato *NIK* (*Nematode Induced Kinase*) gene (DFCI Tomato Gene Index TC218759) were used for induction of hairy roots (Święcicka *et al.,* 2009) and kanamycin resistant gene. This vector was kindly provided by Drs. Joanna Dąbrowska-Bronk and Marcin Filipecki (Warsaw University of Life Sciences, Department of Plant Genetics, Breeding and Biotechnology, Poland). Bacterial cultures were grown in liquid YEB medium to $OD_{600}$ = 1.5. The cultures were centrifuged at 5000 rpm for 20 min and resuspended in ½ MS liquid medium to $OD_{600}$ = 0.3. The cotyledons or hypocotyls dissected from the seedlings were placed for 30 min in a Petri dish containing 30 ml of bacterial suspension. After inoculation all explants were placed on solid ½ MS medium and cultivated for 3 days in the dark. Next, the explants were washed with ½ MS liquid medium supplemented with 300 mg/l of Timentin (GlaxoSmithKline) and placed on solid ½ MS supplemented with Timentin at the same concentration. Hairy roots were separated from the explants and placed on fresh solid medium with the antibiotic supplementation. Hairy root lines obtained were marked: CC or CH — control lines obtained from cotyledon (C) or hypocotyl (H) transformed with the bacteria carrying no plasmid and MC, MH — modified hairy root lines obtained by transformation with the bacteria carrying the reporter plasmid.

**Maintenance of hairy root lines**. Established lines were maintained on ½ MS solid medium free of antibiotics (C) or supplemented with kanamycine (M) at 100 mg/l and subcultured every 4–8 weeks in complete darkness at 24ºC for 6–30 months, depending on the line. For OA determination all selected lines (17) were transferred to 250 ml Erlenmeyer flasks containing 100 ml of liquid ½ MS medium and were maintained on a rotary shaker at 120 rpm in darkness for 30 days. All cultures in shaken flasks were inoculated with tissue derived from stationary culture maintained on solid medium. On the basis of preliminary results, the CC16 line was chosen for detailed studies. The growth of the culture in shaken flasks was determined by measuring fresh and dry weight every 3–4 days. Here, three flasks were analyzed for a single data point for fresh weight, dry weight, oleanolic acid content in tissue and in medium.

**GUS histochemical assay**. GUS expression in the hairy root was analyzed by histochemical staining as described by Jefferson *et al.* (1987) with slight modifications. Hairy root segments from both the putative transgenic and the control (10–20 mm in length) cultures were incubated for 24 h at 37°C in the dark in 50 mM phosphate buffer (pH 7.2) containing: X-GlucA Cyclohexylamonium (Sigma 0.1%, w/v), Triton X-100 (Sigma 0.1%, w/v) and methanol (20%, w/v). The roots were subsequently washed in ethanol at room temperature, observed under a microscope and photographed.

**Extraction of intracellular and extracellular oleanolic acid**. Air-dried roots (0.36–1.68 g of dry weight, DW) were ground with methanol and boiled for 60 min; the culture medium was extracted three times with 20 ml of 1-butanol. The methanolic and butanolic extracts were then evaporated under reduced pressure. Dried extracts were hydrolyzed with Killiani's mixture (Killiani, 1930), extracted three times with 20 ml of diethyl ether and separated by thin layer chromatography (TLC). Purified OA was methylated twice with an ethereal solution of diazomethane (Janiszowska & Kasprzyk, 1977). Detailed description of the extraction procedure was published previously by Wiktorowska *et al.* (2010). OA accumulation during the subculture of line CC16 was analyzed after hydrolysis with 10% hydrochloric acid (10%) in methanol.

**Quantification of oleanolic acid**. Quantitation of oleanolic acid methyl ester was performed by gas-liquid chromatography (GLC) at 259°C on a Shimadzu GC-2014 instrument equipped with a flame ionization detector (FID) and a 2-m × 3 mm 3% SE-30 Chromosorb WHP column (Supelco). The temperature of the injector and detector was 275°C. Nitrogen was used as the carrier gas at a flow rate of 30 $ml \cdot min^{-1}$. Peak identification and quantification of oleanolic acid were carried out by referring to a calibration curve prepared with an authenticated sample of methylated oleanolic acid as the standard (Wiktorowska *et al.,* 2010).

Unknown compounds detected on the GLC chromatograms just after the methyl ester of OA were identified by GLC-MS on an Agilent Technologies 7890A gas chromatograph coupled with a 5975C mass spectrometric detector. Samples (dissolved in 1–4 µl of a 5:1 diethyl ether:methanol mixture, v:v) were applied by split injection 1:10 on an HP-5MS 30 m × 0.25 mm, 0.25 µm column, (Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1 ml·min$^{-1}$. The following parameters were employed: column temp. 280ºC, inlet and flame ionization detector (FID) temp. 290ºC, MS transfer line temp. 275ºC, quadrupole temp. 150ºC, ion source temp. 230ºC, EI 70 eV, $m/z$ range 33-500; FID gas ($H_2$ from a hydrogen generator) flow 30 ml·min$^{-1}$, air flow 400 ml·min$^{-1}$. Identification was made by comparing the mass spectra with library data from Wiley 9th ED. & NIST 2008 Lib. SW (Version 2010) and, where available, by comparison of retention times and corresponding mass spectra with those of authentic standards. The inferred structures were confirmed by alkaline hydrolysis (10% NaOH in 80% methanol, 2 h, 100°C) and acetylation (acetic anhydride : pyridine 1:1, v:v, 0.5 h, 80°C). The source of artifacts (produced by cultures or generated during the procedure of OA isolation and quantification) was established by acid hydrolysis using 10% hydrochloric acid in methanol.

**Statistics**. Results are shown as means ± standard error (±S.E.). All statistical analyses were performed using Microsoft Office Excel 2007 and Statgraphics® 4.1.

## RESULTS AND DISCUSSION

### Induction of hairy root cultures

First hairy roots were observed on most explants two weeks after infection. The responses of cotyledons and hypocotyls were similar: at the site of cutting a single root or a root with some branching appeared (Fig. 2). In some cotyledons, two roots growing from a lump of callus were observed. Identification of such roots as a single or two independent transgenic events was difficult. The only one root 3 to 4 cm in length was cut off and placed on new plates separately and was marked as a line derived from a single independent transgenic event. So each explant can gave at a maximum two independent cultures of hairy roots.

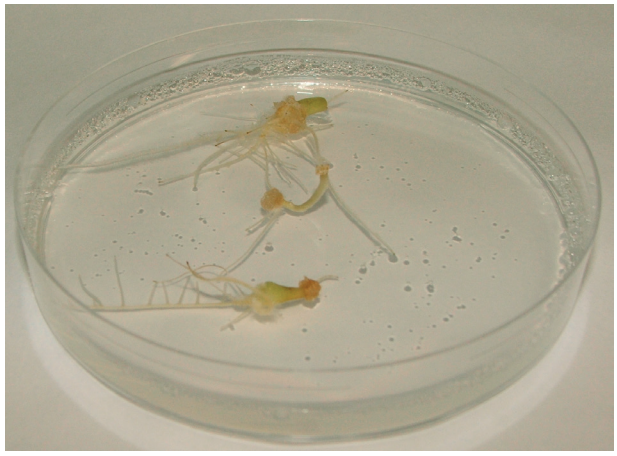

**Figure 2. Induction of hairy roots after transformation of *C. officinalis* cotyledons by *A. rhizogenes* strain ATCC15834 carrying pCambia 1381Z.**


**Figure 3. Diverse morphologies of hairy roots established on solid ½ MS medium; (a) line CH2 (wild strain) two weeks after subculture; (b) line MC5 (pCambia with *GUS* under *NIK* promoter) four weeks after subculture.**

The efficiency of root induction (transformation) determined initially was high: 66.6% for hypocotyls and 33.8% for cotyledons (regardless of whether empty (C) or plasmid-bearing (M) bacteria were used, see Material and Methods for details). In all, 42 lines coming from independent transformation were obtained: 26 of C lines (17 CH, 9 CC) and 16 of M lines (9 CH, 7 CC). In contrast to our observations, hairy root cultures were obtained from cotton cotyledons with a higher yield compared to hypocotyls (Triplett *et al.,* 2008). Authors used the same strain of *A. rhizogenes*. This strain was also used for transformation of four *Asteraceae* species, but unsuccessfully for *C. officinalis* (Pellegrino *et al.,* 1992).

Most stationary cultures of *C. officinalis* hairy roots required four-week subculturing. Generally C lines had thicker roots (Fig. 3a), grew better and required more frequent subculturing, while most M had thinner and finer roots (Fig. 3b) showed slower growth and could safely be subcultured every six or seven weeks. To increase the growth rate of the slowest cultures an inoculum comprising two fragments of lateral roots placed parallely in one direction was used. This method of inoculation stimulated root elongation and induction of lateral roots in lines with poor lateralization. A similar method of hairy root inoculation improved the dry weight and L-DOPA concentration in hairy root culture of *Stilozobium hassjoo* (Lin-Shiang & Shih-Yow, 2006).

Finally, 17 lines (8 control and 9 modified) were developed as a long-term hairy root culture. These lines showed stable and fast growth on both solid and liquid ½ MS medium (Fig. 4). Between ten and thirty passages

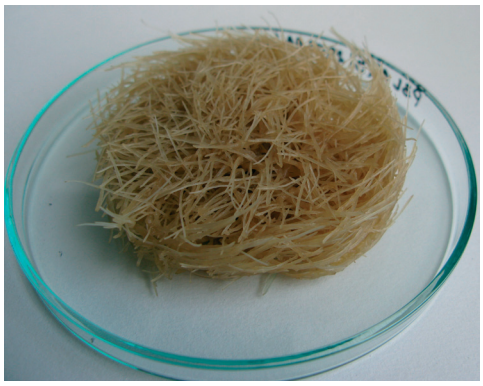

**Figure 4. Hairy root biomass of line CC16 after 30 days of culturing in shaken flasks.**

were required by individual lines for stabilization. Additionally, some lines exhibited changes in growth rate: after a period of slow biomass doubling, high growth intensification was observed.

Following preliminary analyses (not shown), the CC 16 lines showing one of the highest growth rates was chosen for a detailed study. Its kinetics in liquid culture is shown in Fig. 5. During the first 15-days of culture both fresh and dry weight increased slowly, and then undergo a short phase of exponential growth until day 20, linear increase up to day 20. The dry weight continues to accumulate rapidly until the day 30 and then drops slightly, while the accumulation of fresh weight slows down substantially but continues until the end of experiment (day 45). Interestingly, both FW and DW show a short phase of almost no growth between days 20 and 25, suggesting

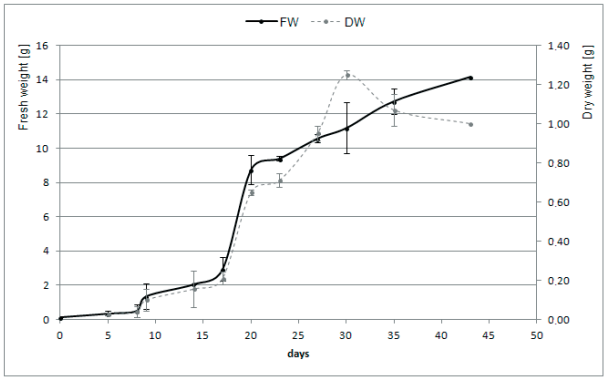


**Figure 5. Growth curve of hairy root line CC16 in liquid ½ MS medium. Data points are mean ± S.E. of three determinations.**

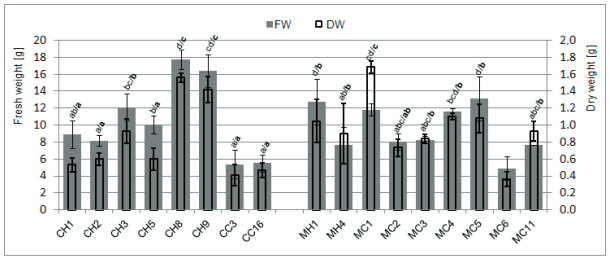


**Figure 6. Fresh and dry weight of hairy root cultures after 30 days of culturing in liquid medium.**
Denote lines obtained from cotyledons (**C**) or hypocotyls (**H**) by transformation with *A. rhizogenes* non-carrying (**C**) and or carrying (**M**) the CAMBIA 1381Z plasmid. Values are mean ± S.E. of three determinations. Values marked by same letters are not significantly different at $P \leq 0.05$ according to Fisher multiple range test.

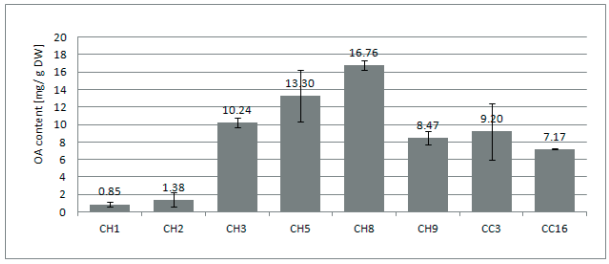


**Figure 7. Oleanolic acid accumulation in hairy root cultures obtained by transformation with plasmid no-carrying *A. rhizogenes* strain 15834 (CH-hypocotyl, CC-cotyledon).**
OA accumulated in tissue after 30 days of culturing was quantitated in air-dried roots after derivatization on GLC (for details see Material and methods). Values are mean ± S.E. of three determinations. Values marked by the same letters are not significantly different at $P \leq 0.05$ according to Fisher multiple range test.

a catabolic switch from an exhausted component of the medium to another one (possibly secreted by the biomass). After 40 days the culture shows first symptoms of dying: covering with callus, darkening of the medium and finally tissue fragmentation.

The hairy root lines exhibited differences in fresh and dry weights (Fig. 6). The lines derived from hypocotyl showed 37% higher concentration of FW than lines from cotyledons at significant difference. The type of construct or explant source had no influence on DW values. Faster growth of C lines was confirmed only in case of FW: 10.53 g/flask contrary to M lines 9.51 g/flask. The average value of DW in lines M was 0.97 g/flask (10.09%) and in C lines 0.81 g/flask (7.54%).

## Determination of oleanolic acid in roots and culture medium

A preliminary qualitative analysis of methanolic and butanolic extracts by TLC detected free oleanolic acid at a very low level: on the border of visibility. The appropriate area of the plate was scraped off, eluted and methylated. Gas chromatography confirmed that free oleanolic acid was present as a minor constituent: 1.51–2.15% of the dry mass of the tissue and 0.00–0.42% of medium (over 97% of oleanolic acid was incorporated into glycosides). The overall content of triterpenoid glycosides in tissue and medium was determined after acid hydrolysis and expressed as the aglycone, oleanolic acid. Oleanolic acid was detected at different concentrations in all of the long-term hairy root lines in both the tissue and the medium. Among of the control lines CH5 and CH8 showed

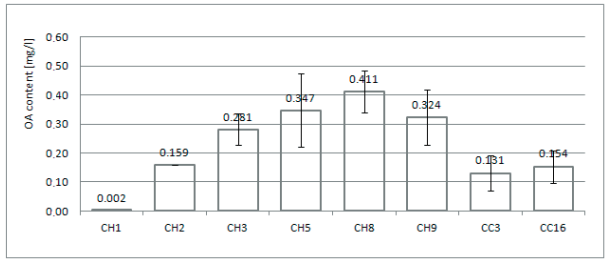


**Figure 8. Oleanolic acid secretion by hairy root cultures obtained by transformation with plasmid no-carrying *A. rhizogenes* strain 15834 (CH-hypocotyl, CC-cotyledon).**
OA released into liquid medium during 30 days of culturing was quantitated in filtrated medium after derivatization on GLC (for details see Material and methods). Values are mean ± S.E. of three determinations. Values marked by the same letters are not significantly different at $P \leq 0.05$ according to Fisher multiple range test.

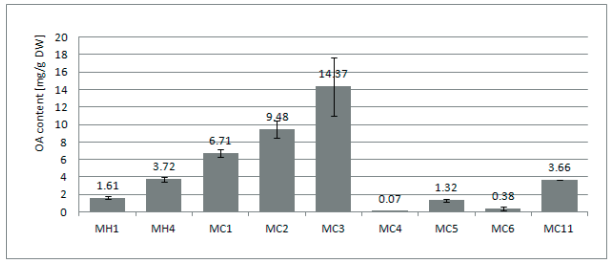


**Figure 9. Oleanolic acid accumulation in modified hairy root cultures obtained by transformation with plasmid pCAMBIA 1381Z (NIK gene promoter:*GUS*;) carrying *A. rhizogenes* (CH-hypocotyl, CC-cotyledon).**
OA accumulated in tissue after 30 days of subculture of culturing was quantitated in air-dried roots after derivatization on GLC (for details see Material and methods). Values are mean ± S.E. of three determinations. Values marked by the same letters are not significantly different at $P \leq 0.05$ according to Fisher multiple range test.

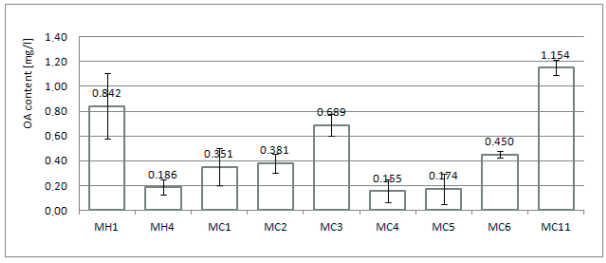


**Figure 10. Oleanolic acid secretion by modified hairy root cultures obtained by transformation with plasmid pCAMBIA 1381Z (NIK gene promoter:*GUS*;) carrying *A. rhizogenes* (CH-hypocotyl, CC-cotyledon).**
OA released into liquid medium during 30 days of culturing was quantitated in filtrated medium after derivatization on GLC (for details see Material and methods). Values are mean ± S.E. of three determinations. Values marked by the same letters are not significantly different at $P \leq 0.05$ according to Fisher multiple range test.

the highest OA accumulation in roots lines: 16.76±0.57 and 13.30±2.96 mg·g$^{-1}$ DW, respectively (Fig. 7). Line CH8 released the saponins into medium at the highest efficiency among all C lines: 0.41±0.07 mg·dm$^{-3}$ (Fig. 8).

As a rule, the M lines accumulated OA glycosides in roots at significantly lower levels than the C lines (Fig. 9). The MC3 line showed the best results: 14.37±3.36 mg·g$^{-1}$DW. The level of saponins released into the medium was not significantly different among the selected M lines; the highest OA concentration in medium was noted for MC11: 1.15±0.06 mg·dm$^{-3}$ (Fig. 10). Our hairy root cultures produced triterpenoid saponins at the highest yield among all *C. officinalis* cultures, even those after elicitation. A young (2 months) suspension culture of undifferentiated cells accumulated about 0.09 mg of oleanolic acid·g$^{-1}$ FW (Grzelak & Janiszowska, 2002). Wiktorowska and co-workers (2010) investigated the influence of five popular biotic elicitors on the OA accumulation in a suspension culture of *C. officinalis*. The control old culture (16 months) reached over tenfold the OA acumulation (0.09 mg OA per g of DW), and after supplementation with the strongest elicitor jasmonic acid, the OA production was 0.84 mg·g$^{-1}$ DW. Also the concentrations of saponins in the medium were determined: 0.10 mg·dm$^{-3}$ for control and 0.43 mg·dm$^{-3}$ after yeast extract addition. The concentration of oleanolic acid in some hairy root cultures was similar to that in native plants (inflorescence) (Kowalski, 2007), higher than in native roots (Kasprzyk & Fonberg-Boczek, 1967; Mrugasiewicz *et al.,* 1979; Dedio & Kozłowski, 1998). Such results are promising for commercial exploitation of hairy root cultures, especially in case of saponins fluctuation in intact plants dependent on many environmental factors (Dedio & Kozłowski, 1998; Szakiel *et al.,* 2011).

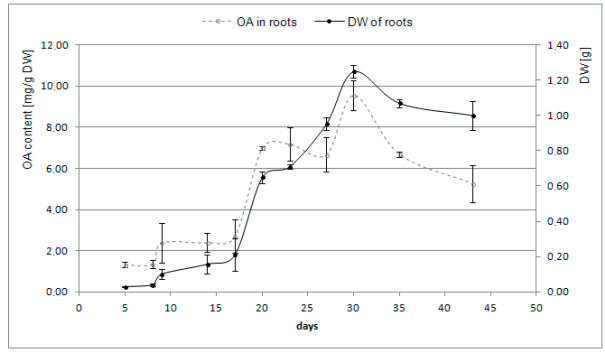


**Figure 11. Oleanolic acid (OA) accumulation and dry weight of hairy root culture of *C. officinalis* hairy root line CC16 culture.**
Data points are mean ± S.E. of three determinations.

In reports concerning production of plant metabolites diverse modes of expression of efficiency are used: mg per fresh weight or dry weight, mg per dm$^{-3}$ of culture, etc. When the compounds investigated are highly unstable their concentration should be determined in fresh tissue. The oleanolic acid glycosides that we determined after acid hydrolysis are of sufficient stability to be determined after drying of the biological material. Additionally, the root dry mass is easier to homogenize, which improves extraction. Crucial culture parameters comprise dry weight (DW) and fresh weight (FW). Their proportion provides valuable information about the culture status: a higher water content suggest aging of the culture, higher content of DW might indicates metabolite production. The data reported here are OA concentrations determined in 30-day cultures, that is at the time of their highest DW content (Fig. 5).

A comparison of dry weight and OA accumulation in tissue during the culture growth cycle in liquid medium produced some interesting insights (Fig. 11). The CC16 line, after three years of maintenance on solid ½ MS medium, showed an excellent correlation between the dry weight and OA amount, 0.92, indicating a constant rate of triterpenic saponins synthesis in dividing cells. In contrast a suspension culture of marigold cells (Grzelak & Janiszowska, 2002) showed marked fluctuations of the biomass : saponins ratio; on the first days of culture the OA content reached the highest level, then decreased dramatically and then continued to grow to a second

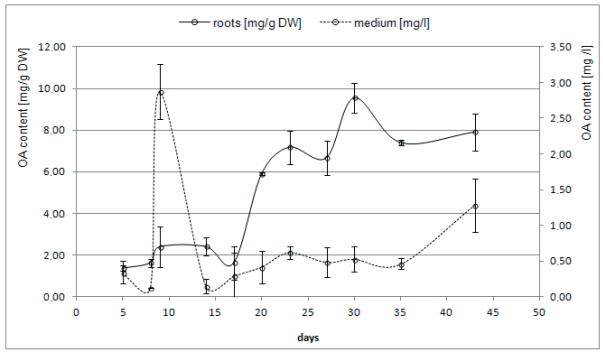


**Figure 12. Oleanolic acid (OA) content in liquid medium of hairy root line CC16 culture.**
Data points are mean ± S.E. of three determinations.

weak peak at the end of culture. Surprisingly, the kinetics of OA accumulation in the medium of the CC16 hairy root culture reminded that for the undifferentiated cell suspension culture rather than that of the hairy root biomass (Fig. 12). Again two peaks were observed: first one, probably in response to fresh medium or shaking, and the second — at the end of culture, possibly due to mechanical damage of the oldest root fragments, or influenced by depletion of carbon source, or accumulation of some (toxic) metabolite.

### Qualitative analysis of purified extracts using GLC-MS

Chromatogram analysis of the purified extracts using GLC on SE-30 column showed methyl ester of oleanolic acid and an additional compound with a retention time similar to that of methyl ester of ursolic acid (Fig. 13). That peak was observed for extracts from all hairy root lines at its high area comparable to that of the OA peak. Further analysis by GLC-MS showed that the compound was 3-O-acetyl-oleanolic acid methyl ester (olean-12-en-28-oic acid, 3-acetyloxy-, methyl ester) (AcOA) (Fig. 14). The MS spectrum showed fragment peaks at m/z of: 203 (100%), 262 (48%), 189 (29%), 202 (21%), 43 (19%), 204 (18%), 133 (15%), 190 (15%), 119 (13%), and 69 (11%). Alkaline hydrolysis removed the acetyl group from the C-3 hydroxyl group of OA, therefore only methyl ester of OA was observed on the chromatogram. A standard acetylation procedure was used to generate AcOA, which demonstrated an identical retention time and *m/z* profile by GLC-MS, thus confirming the identity of the compound in question. The AcOA identified in the extracts was not formed in the culture, but was formed during acid hydrolysis using the Killiani mixture. In samples treated with 10% hydrochloric acid in

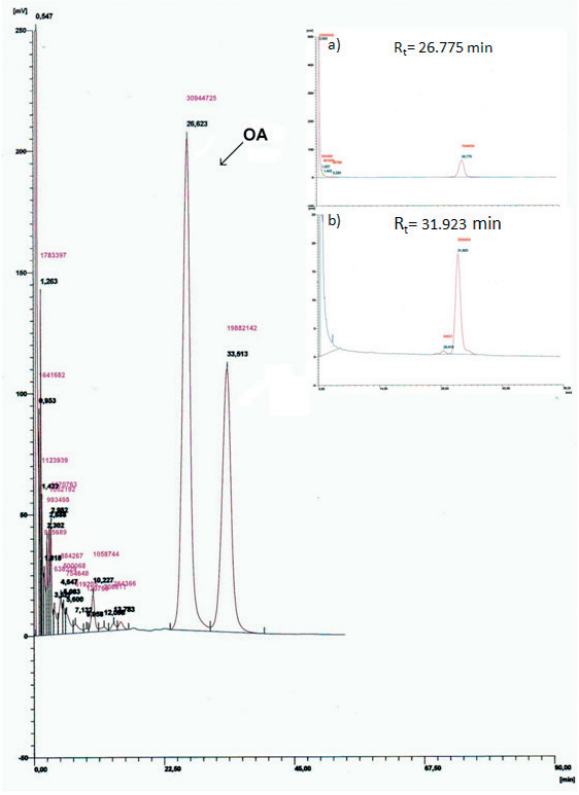


**Figure 13. GLC chromatogram of OA and unknown compound (methyl ester derivatives) accumulated in hairy root line CH8 culture. Insets show chromatograms of authentic standards of methyl ester of OA (a) and methyl ester of UA (b).**

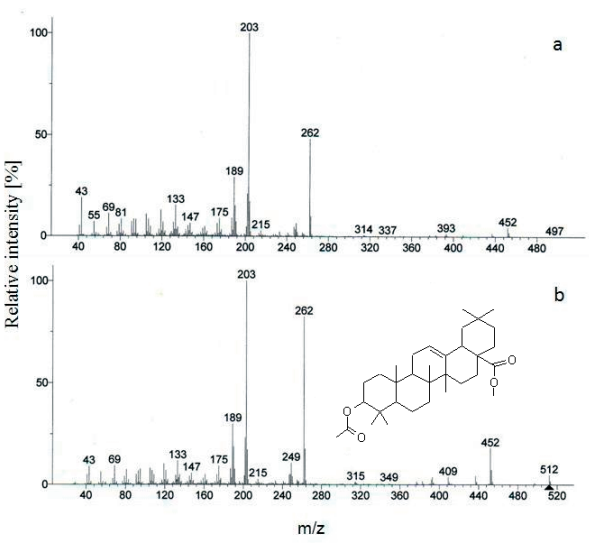


**Figure 14. Comparison of GLC-MS spectra of unknown compound found in *C. officinalis* hairy roots (a) and of 3-O-acetyl-oleanolic acid methyl ester, from library (Wiley 9th edn. & NIST 2008 Lib. SW, Version 2010) (b).**

methanol (without acetic acid, as in the case of Killiani mixture) the acetyl derivative was not detected, and the yield of the final products (OA), as integrated by GLC software, was by 25% higher than that obtained using Killiani mixture and contained 5% less degraded compounds (not presented). For this reason the areas of both peaks observed after Killiani hydrolysis were summed to quantitate the OA aglycone. In previous experiments (Grzelak & Janiszowska, 2002; Wiktorowska *et al.,* 2010), although the Killiani mixture was used consistently for hydrolysis of OA glycosides, the acetyl derivative was never observed. One possible explanation of this discrepancy is that the concentration of the OA glycosides studied in those reports were much lower than in the present case. In marigold plants only oleanolic acid and its derivatives (glycosides) were detected (Janiszowska & Kasprzyk, 1977). Studies in other plants show in fact AcOA is a naturally occurring oleanolic acid derivative, and is sought after for pharmacological sciences (Assefa *et al.,* 1999; Zhu *et al.*, 2001, Zhang *et al.,* 2008). Recently

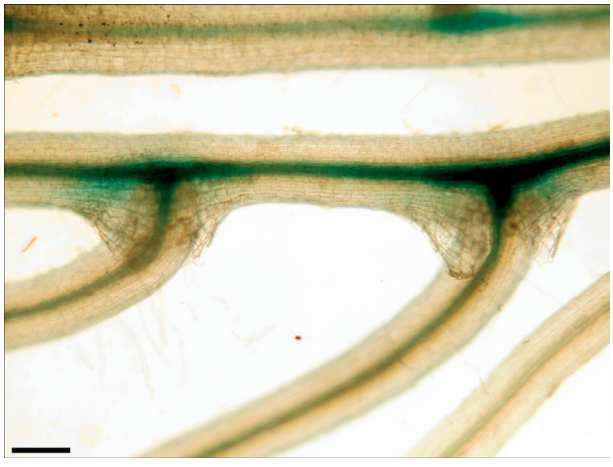

**Figure 15. Histochemical analysis of GUS activity in transgenic hairy root of *C. officinalis* line MC2.**
The *GUS* gen driven by tomato *NIK* promoter was introduced by transformation with *A. rhizogenes* carrying the CAMBIA 1381Z, (bar = 200 μm)

AcOA isolated from *Vigna sinensis* seeds has been shown to induce apoptosis in human colorectal carcinoma cells (Yoo *et al.,* 2012).

### GUS histochemical assay

To verify the expression of the GUS reporter gene under the control of the *NIK* gene promoter in the hairy root cultures, GUS activity was determined histochemically in nine putative transgenic (M) and two control (C) hairy root cultures. All the putative transgenic hairy root cultures stained blue to various intensities. Microscopic observation showed the highest staining in the cell division area and root elongation area restricted to the main vascular bundle (Fig. 15). The control (non-transgenic) hairy root samples showed no GUS activity. These results indicate that the reporter gene was integrated into the genome of the transgenic *C. officinalis* hairy root cultures.

## SUMMARY

In this article we presented the analysis of oleanolic acid glucosides (saponins) production and secretion into the medium by established hairy roots lines of *C. officinalis* obtained in a procedure described previously by our team in 2009. The hairy root lines selected in this experiment showed a good ability to accumulate oleanane type triterpenes and to excrete them into liquid culture medium. The maximum saponin concentration in roots ranged for individual lines between 0.38 and 16.77 $mg \cdot g^{-1}DW$ in ½ MS medium. These hairy root cultures seem a suitable starting point to achieve industrial-scale of the saponins production.

Further experiments are required to characterize the mechanisms underlying the synthesis and accumulation of saponins in *C. officinalis* hairy root cultures and their secretion into the medium and to establish how various biological, physical and chemical factors affect those processes.

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