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RESEARCH ARTICLE

MICROPROPAGATION STUDY AND PRODUCTION OF TRANSGENIC CALLUS FROM DAUCUS GLABER FORSSK

[1]Rehab H. Abdullah, *,[1]Ehsan M. Abo Zeid, [2,3]Rashad M.Kebeish, [1]Rasha A. Attiah and [1]Samih I. El-dahmy

[1]Department of Pharmacognosy, Faculty of Pharmacy, University of Zagazig, Zagazig 44519, Egypt
[2]Plant Biotechnology Laboratory (PBL), Botany Department, Faculty of Science, Zagazig University, Zagazig, Egypt
[3]Taibah University, Faculty of Science Yanbu, Biology Department, Yanbu, KSA

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ABSTRACT

Daucus glaber Forssk, a wild annual herb, is distributed from the Mediterranean area to East Asia. This plant seems to be rich in important medicinally active secondary metabolites as volatile oils, phenylpropanoid esters, sesquiterpene lactones and flavonoids that have many biological activities. Experiments were carried out to optimize conditions for callus induction, somatic embryogenesis and organogenesis from leaf, stem and root explants of the *in vitro* germinated seeds of *Daucus glaber* Forssk. The study investigated the volatile constituents isolated from *in vitro* induced calli and micropropagated plant using gas chromatography-mass spectroscopy (GLC/MS), besides screening three nonvolatile compounds previously isolated from the wild plant (Angeloyloxylatifolone, Glaberin A and Talasin A) in induced calli and micropropagated plants using high performance liquid chromatography (HPLC). Some calli and micropropagated plant showed strong cytotoxic activity against HepG2 cell line and a significant anti-microbial activity against *Aspergillusfumigatus*, *Streptococcus Pneumonia, Bacillus subtilis* and *Escherichia coli*. An approach for a suitable transformation system was developed for *Daucus glaber* by co-cultivation of the seedlings with *Agrobacterium tumefaciens* GV 3101 that contains pTRA-K-TL-cTP plasmid carrying phytoene synthase (PSY) gene.

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INTRODUCTION

Daucus glaber Forssk.is an annual wild herb growing in the sand dune plains, and sea coast in the northern Nile Delta, along the Mediterranean, and in Sinai peninsula (Tackholm, 1974; Boulos, 1999). The plant is rich in medicinal active compounds as essential oils (Mansour *et al*., 2004), phenyl propanoid esters (Halim *et al.*, 1989; Mansour *et al*., 1990 and Sallam, *et al*., 2009), sesquiterpene lactones (Sallam*et al.*, 2010), sterols (Yousefbeyk *et al*, 2014) and flavonoids (Yousefbeyk *et al*., 2014). These components have many biological activities as anti-bacterial, anti-fungal, cytotoxic and antioxidant activities (Mansour *et al*., 2004; Baser *et al.*, 2009; Sallam *et al.*, 2010 and Yousefbeyk *et al*., 2014). *Daucus glaber* is considered a rare Egyptian plant, suffering from the threat of extinction at theMediterraneancoastalregion due to the expansion in construction which destroys its environment and decreases its numbers drastically. Recently, the *in vitro* propagation of rare plants is widely used to conserve the threatened plants from extinction (Kapai *et al.,* 2010). *In vitro* propagation is an easy, rapid and efficient method for banking of plant species. The micropropagation occurs from seeds of some rare and endangered plant species to investigate the possibility of transferring the *in vitro* regenerated plantlets to normal environmental conditions (Debnath, *et al.*, 2006). Recombinant DNA technology offered an alternative system to produce pharmaceutical proteins from plants via nuclear plant expression system. Genetic manipulation of higher plants depends essentially on the use of plant tissue culture and the establishment of protocols for stable, efficient and genotype-independent *in vitro* culture system. Vitamin A is defined as any natural or synthetic compound that performs the function of this vitamin. Vitamin A includes retinoids, and carotenoids which is the provitamin A (Bender, 2003). Carotenoids are lipophilic pigments found in all photosynthetic organisms.

Corresponding author: Ehsan M. Abo Zeid
Department of Pharmacognosy, Faculty of Pharmacy, University of Zagazig, Zagazig 44519, Egypt

They are characterized by an unsaturated 40 carbons skeleton with 15 double bonds (Busch *et al*., 2002: Taylor and Ramsay, 2005). Carotenoids include α-carotene, β-carotene, and their oxygenated derivatives the xanthophylls as zeaxanthin and violaxanthin. They are localized in the chloroplasts and chromoplasts in plants. In chloroplasts, they have a critical role in photosynthesis as they represent a part of light collecting complex, serving as electron transfer molecules by reducing chlorophyll. They are also photo protective agents that prevent chlorophyll damage by excess light energy. The carotenoids in fruits and flowers attract animals and insects for pollination and seed scattering. The phytohormoneabscisic acid, which has a role in stress response, is derived from carotenoid precursors (Cutler and Krochko, 1999). Phytoene synthase (PSY) is considered the major rate limiting enzyme in carotenoids biosynthesis in plants, because it irreversibly directed granylgranyl di phosphate (GGDP) towards carotenoid production; away from the production of many isoprenoids as chlorophylls, tocopherols, gibberellins and quinones (Cunningham and Gantt, 1998). The present study aimed to select the best conditions for micropropagation of *Daucus glaber* Forssk. Through *In vitro* germination of seeds, callus induction, formation of shoot and root, embryogenesis, acclimatization and transplantation of plantlets to the soil. This study also aimed to investigate the secondary metabolites isolated from different *in vitro* tissues through HPLC and GLC/MS prior to test their cytotoxic and antimicrobial activities besides Transformation of the plant using *Agrobacterium* mediated transformation method to investigate the most appropriate protocol and to study the effect of the inserted gene (PSY-gene) on the active carotenoids production.

MATERIAL AND METHODS

Seeds donor plant

The seeds used in this work, *Daucus glaber* Forssk. (Family: Apiaceae), were collected on September 2011 from SidiBarrani, North coast, Egypt. The donor plant was identified voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Sterilization of seeds

Seeds were sterilized by washing with 70 % ethanol for different time slots 3 and 5 minutes followed by shaking with 5% commercial hypochlorite solution (Clorox®) containing 2 drops of 1% tween 20 for different time slots 5, 10 and 15 minutes. The seeds were rinsed three times with sterile double distilled water before culturing on the germination media. In each condition, the seeds were cultivated over Murashige and Skoog (MS) medium (Duchefa, Germany) with 3% sucrose (Adwic, ARE) and 0.8% agar (purified agar for plant tissue culture, Bioworld, USA). The pH was adjusted at 5.6-5.8 and the jars were kept in the dark for a week at 25 °C. Decontamination of the seeds was investigated using the decontamination rate.

Seed germination

Sterilized seeds were transferred to four types of sterile solid media which are (4.4 g/L MS media ,30 g/L sucrose and 8 g/L agar), (4.4 g/L MS media, 30 g/L sucrose and 8 g/L agar and 100 mg/l gibberellic acid (GA3)) (Sigma Chemical Co, U.S.A), (4.4 g/L MS media, 30 g/L sucrose and 8 g/L agar and 1 mg/l 2, 4 D) and (4.4 g/L MS media, 30 g/L sucrose and 8 g/L agar and 1mg/l Kinetin). The pH was adjusted at 5.6-5.8 and the jars were kept in the dark for 3 days then incubated at 25 °C under white fluorescent lamp with a 16/8 hours light/dark period. Germination of seeds was investigated using seed germination percentage and seedling length after 4 weeks of cultivation.

Induction of callus from *in vitro* germinated seedlings

Uniformly sized explants (0.5-1 cm) were dissected from the germinated seedlings under sterile conditions to produce three types of explants the leaf, stem and root. The different explants were cultured in jars containing MS media with 3% sucrose 0.8% agar supplemented with different plant growth regulators (Medium I: 1 mg/l 2, 4 dichlorophenoxy acetic acid (2,4 D), Medium II: 0.5 mg/l Kinetin, Medium III: 1mg/l 2, 4 D + 0.5 mg/l Kinetin, Medium IV: 2mg/l 2, 4 D + 1 mg/l Kinetin, Medium V: 1 mg/l naphthalene acetic acid (NAA), Medium VI: 1mg/l NAA + 0.1mg/l benzyl amino purine (BAP) and Medium VII: 0.5 mg/l thidiazuron (TDZ) + 1 mg/l 2, 4 D + 0.1 mg/l BAP) (Sigma Chemical Co, U.S.A). The pH was adjusted to 5.6-5.8 and the cultures were incubated at 25 °C under white fluorescent lamp with light intensity (1000 μmol·m-2·sec-1), 16/8 hours light/dark periods for 5 weeks. The callus capacity [(no of explants produced callus/ total no. of explants cultured) *100] and callus dimensions were determined to select the most appropriate media for further investigations.

Growth rate determination

The growth of callus was measured by evaluating the increase in fresh weight. Healthy, well growing calli were chosen to study the effect of plant growth regulators. The chosen calli of different explants were cultured in jars containing medium IV [2, 4 D (2mg/l) + Kinetin (1 mg/l)], VI [NAA (1mg/l) + BAP (0.1mg/l)] and VII [TDZ (0.5 mg/l) + 2, 4 D (1 mg/l) + BAP (0.1 mg/l)] which had the greatest callusing capacity and produced calli with the largest dimensions. In each media the pH was adjusted to 5.6-5.8. The cultures were incubated at 25 °C under white fluorescent lamp with a 16 hours photoperiod for 2-3 generations. Subcultures were made by cutting the calli produced in each case to one gram pieces and transferring them on a fresh medium with the same composition. Fresh weight was measured every 5-10 days for 40 days and the mean values of 3 readings were plotted against time to get the growth curve.

In vitro propagation of *Daucus glaber* Forssk

Regeneration of *Daucus glaber* callus via somatic embryogenesis

Eight-weeks- old calli with pre-embryogenic masses were selected from those grown on medium VI and VII and transferred to MS hormonal free semi solid media with 0.6% agar and 30 g/l sucrose. The pH was adjusted to 5.6-5.8; the cultures were incubated at 25 °C under white fluorescent lamp with 16 hours photoperiod.

Regeneration of *Daucus glaber* callus via organogenesis

Ten-weeks-old callus which grown on medium VII showed multishooted buds. The achieved shoots were cut and then transferred individually to jars containing rooting media which consists of MS with 30 g/l sucrose, 0.6% agar and 1mg/l NAA. The pH of the media was adjusted to 5.6-5.8., the cultures were incubated at 25 °C under white fluorescent lamp with 16 hours photoperiod.

Hardening and adaptation of the *in vitro* regenerated plantlets (acclimatization)

The regenerated plantlets, three-months-old, of about 10-15 cm long were first transferred into liquid MS media with 1mg/l NAA for three weeks for root hardening, washed to be free from media then were transplanted into pots containing sand:soil (1:1), covered with transparent perforated plastic bags which were removed a week later. Pots were maintained in green house.

GLC/MS analysis

Five grams of fresh calli (eight weeks old non organogenic calli of leaf, stem and root cultivated on media IV, VI, and VII) and micropropagated plant were separately macerated in diethyl ether, kept on a shaker overnight. After filtration the ether were evaporated under liquid Nitrogen. The GC/MS analyses were carried out at The Central Agricultural Pesticide Laboratory (CAPL), Cairo, Egypt; on Agilent 6890 gas chromatograph with fused silica capillary column PAS-5 ms (30 mm × 0.25 um film thickness). The carrier gas was Helium with1 ml/ min flow rate. The sample injection size was 1 µl. Oven temperature Program started at 50 ^{0}C then elevated to 280 ^{0}C at rate of 8 ^{0}C/ min. The injector temperature was adjusted at 250 ^{0}C while the detector temperature was at 280 ^{0}C. The detector used was Mass spectrophotometric, scanning from m/z 50 to 500, EI 70 ev. Identification of the components was based on matching the fragmentation pattern in the resulted mass spectra with the published data (Adams, 2007) and using Wiley and Nist 05 mass spectral data base.

HPLC analysis

The air-dried powdered calli (eight weeks old non organogenic calli of leaf, stem and root cultivated on media IV, VI, and VII) and micropropagated plant were separately grounded to fine powders then macerated in a mixture of n-hexane : diethylether : methanol in a ratio of (1:1:1) for three times. After filtration the solvent was distilled off under reduced pressure at 50^{o}C. The HPLC analyses were performed in bioavailability laboratory, faculty of pharmacy, Zagazig University on an Agilent 1200 series liquid chromatography. The mobile phase was a mixture of water, methanol and acetonitrile (55:40:5) delivered at a flow rate of 1 ml/min. Scatter analyses and calibration curves were established using different concentrations of each reference compound (Angeloyloxylatifolone, Talasin A and Glaberin A), (300, 200, 100, 50 and 25 µg/ml). Each concentration was repeated 3 times to ensure reproducibility.

Cytotoxic activity

Hepatocellular carcinoma cells (HepG2) (VACSERA, Egypt) was used to evaluate the cytotoxic effect of the tested extracts using cell viability assay. Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer and 50µg/ml gentamycin (Sigma/Aldrich, USA). The cells were seeded in 96- well plate at a cell concentration of 1x104 cells per well in 100µl of growth medium. Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Serial two-fold dilutions of the tested chemical compound were added to confluent cell monolayers dispensed into 96- well, flat-bottomed microliter plates (Falcon, NJ, USA) using a multichannel pipette. The microliter plates were incubated at 37°C in a humidified incubator with 5% CO2 for a period of 48 h. Three wells were used for each concentration of the test sample. Control cells were incubated without test sample and with or without DMSO. The little percentage of DMSO present in the wells (maximal 0.1%) was found not to affect the experiment. After incubation of the cells for 24 h at 37°C, various concentrations of sample (100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 µg) were added, and the incubation was continued for 48 h and viable cells yield was determined by a colorimetric method (Mosmann, 1998 and Vijayan *et al.,* 2004). The alcoholic extracts of the leaf calli grown on medium VI, VII and the micropropagated plants were dissolved in dimethyl sulphoxide (DMSO), then diluted thousand times in the assay to begin with the mentioned concentration. All experiments were repeated three times.

Antibacterial and antifungal activities

Antibacterial and antifungal activities of the alcoholic extracts of the leaf calli grown on medium VI, VII and the micropropagated plantlets were determined using the well diffusion method (Hindler *et al.*, 1994). The tested extracts were dissolved in dimethyl sulfoxide (DMSO) (Oxoid laboratories, UK) at concentration of 1mg/ml. The tested organisms were subcultured on nutrient agar medium for bacteria and Saboroud dextrose agar (Oxoid laboratories, UK) for fungi.

The microorganisms used in this study are *Aspergillusfumigatus* (RCMB 02568) and *Geotricumcandidum* (RCMB 05097) as fungi; *Streptococcus Pneumonia* (RCMB 010010) and *Bacillus subtilis* (RCMB 010067) as Gram positive bacteria; *Pseudomonas aeruginosa* (RCMB 010043), *Escherichia coli* (RCMB 010052), as Gram negative bacteria (RCMB at Antimicrobial Unit to test microorganisms, Al-Azhar University). Ampicillin and Gentamicin were used as a positive control against Gram positive and Gram negative bacteria, respectively. Amphotericin B was used as a positive control for fungi. The plates were done in triplicate. Bacterial cultures were incubated at 37°C for 24 h, while the fungal cultures were incubated at 37°C for 2-7 days. Antibacterial and antifungal activities were determined by measuring the diameter of the inhibition zone formed around the well (mm). Mean zone of inhibition and standard deviations were calculated. Results were expressed in mean zone of inhibition in mm ± standard deviation (SD) beyond well diameter (6mm) produced on a range of environmental and clinically pathogenic microorganisms.

Methods of transformation study

DNA isolation from *Synechococcus elongates* PCC6801

Based on the DNA sequences available in the database (http://www.ncbi.nlm.nih.gov/nuccore/74355276?from=46&to=612&sat=4&sat_key=66462477) and references there in, the PSY coding sequences is composed of 1050 base pairs. In order to clone the coding sequence of PYS total cellular DNA of *Synechococcus elongates* (obtained from Botany Department- Faculty of Science- Helwan University)was extracted using the method of (Murray and Thompson, 1980). For verification of quality and quantity, 2-5 µl of the total genomic DNA eluate were visualized on a 1% (w/v) agarose gel containing ethidiumbromide. DNA bands were visualized using UV transilluminator and gel doc 100 systems (Biorad, Germany).

PCR amplification of PSY gene

The coding sequences of PSY gene was cloned into the empty plant expression vector pTRA-K-TL-cTP. The PSY gene was amplified by PCR using 2µl of isolated *Synechococcus elongates* DNA as template, 0.5 µl of each two specific oligonucleotides primers for PSY gene flanked by Nco I and Xba I recognition sequences named Psy-Fw*Nco I* ($^{5`}$ACG TCC ATG GCT AAC GGT CAA TT TCT$^{3`}$) and Psy-Rev *Xba I* ($^{5`}$ACT ATC TAG ATT AAA GCA CCT GG CCC GCA$^{3`}$) (SibEnzyme, Estonia), 0.25 µl Taq DNA polymerase (Thermoscientific, USA), 2.5 µl of 10 X of PCR reaction buffer and 0.5 µl d NTPs (100 µM); total volume was adjusted to 25 µl. PCR amplification was carried out using a Master Cycler Gradient PCR (TechneProgene, USA). Each cycle began with 30 seconds at 94^{o}C for denaturation, followed by 30 minutes at an annealing temperature 56-60 ^{o}C, 90 seconds extension period at 72 ^{o}C completed each cycle. A total of 35 cycles were used before final extension and cooling to a temperature of 4^{o}C to avoid any degradation of amplified DNA. Primer melting temperature were calculated as 4 X (G+C) + 2 X (A+T). The PCR amplification products were visualized on a 1 % (w/v) agarose gel containing ethidium bromide.

Restriction of PSY PCR product and pTRA-K-TL-cTP vector

The amplified PSY PCR product was column purified using Spin-PCR purification kit (Thermoscientific, USA). The purified PCR product of PSY in addition to the expression vector pTRA-K-TL-cTP was subjected to a double restriction digestion using Nco I and Xba I together in one mixture in a single step. The restriction digestion was performed for 2 hours at 37 ^{o}C. The digested DNA samples were then column purified in order to remove the resulting small end fragments after enzymatic digestion. Aliquots of the purified products were visualized on 1 % (w/v) agarose gel.

Restriction of plasmid DNA

20-30 units of the respective enzymes were used for the double digestion of 15-20 µg of the plasmid DNA to get the desired vectors or inserts. The restricted samples were then excised and purified from gel using the Intron PCR Quick-Spin^{TM}purification kit (Intron, South Korea)**.**

Ligation of PSY into pTRA-K-TL-cTP Vector

Digested and purified PSY fragments were ligated into pTRA-K-TL-cTP vector. Ligation was performed using T4-DNA ligase (Genone, Germany) at 16 ^{o}C overnight.

***Escherichia coli* strains**

E. coli strain DH5α (Ausubel and Brent, 1994) that has dlacZ Delta M15 (lacZY A-argF) U169 recAlendAlhsdR 17 (rK` mK`) supE44 thi^{-1} gyrA96 mutations was used for the establishment and cloning of the plasmid constructs used in this study.

***Agrobacterium tumefaciens* strain**

GV 3101 (pMP90RK Gm^{R}, Km^{R}), Rif ^{R} (Koncz and Schell, 1986).

Preparation of competent *E. coli* (DH5α) for heat shock transformation

A single colony from LB plate containing bacterial colonies was inoculated into 5 ml LB medium and incubated overnight at 37 ^{o}C with continuous shacking (200 xg/ min). LB medium (200 ml) was inoculated with 1 ml of the overnight culture. The cells were left to grow at the same condition until the OD 600 reached 0.5-0.6. Cells were spun down for 10 min (4791 x g/4 ^{o}C), then re-suspended in 50 ml of 100 mM $CaCl_2$. The cells were then incubated on ice for 10 min and spun down by centrifugation for 10 min (4791x g/ 4 ^{o}C). The pellet was re-suspended afterwards in 1 ml of 100 mM $CaCl_2$ containing 80% glycerol and 100 µl-aliquots of the suspension were dispensed into pre-chilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -20 ^{o}C.

Transformation of competent *E. coli* (DH5α) by heat-shock:

As soon as competent cells were thawed, plasmid DNA (up to 100 ng) or 1-3 µl from the ligation products were mixed gently with the competent cells then were incubated on ice for 30 min. The cells were incubated at 42 ^{o}C for 90 seconds and placed directly on ice for 2 min, 1 ml of LB medium was added immediately to the tubes containing the heat shocked bacteria. The transformed cells were incubated at 37 ^{o}C for 45 min with continuous shaking (200 xg). Transformed cells (100 µl) were plated onto LB-agar plates supplemented with ampicillin and incubated at 37^{o}C overnight.

PCR analysis of plasmid DNA isolated from DH5 α transformed with pTRA-K -TL-PSY plasmid DNA

The isolated plasmid DNA from DH5α transformed with pTRA-K-TL-PSY construct was used as a template for PCR screening test. Two PSY vector specific primers named pS5` (13) (GAC CCT TCC TCT ATA TAA GG), pS3` (18) (CAC ACA TTA TTC TGG AGA AA) primers.

Preparation of competent *Agrobacterium tumifaciens*

A single colony from LB plate containing bacterial colonies was inoculated into 5 ml LB medium (supplemented with 50 µg/ml kanamycine and 100 µg/ml rifampicine) and incubated for 48 hours at 28°C with continuous shacking (200x g/ min). 200 ml LB medium were inoculated with 1 ml of the culture. The cells were left to grow at the same condition until the OD 600 reached 0.5-0.6. Cells were spun down for 10 min (4791x g/4 °C), then re-suspended in 50 ml of 100 mM $CaCl_2$. The cells were then incubated on ice for 10 min and spun down by centrifugation for 10 min (4791x g/ 4 °C). The pellet was re-suspended afterwards in 1 ml of 100 mM $CaCl_2$ containing 80% glycerol and 100 µl-aliquots of the suspension were dispensed into pre-chilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -20 °C.

Transformation of competent *A. tumefaciens* byheat shock

The same as in DH5α. The transformed cells were incubated at 28°C for 2 hours with continuous shaking (200 xg). 100 µl of the transformed cells were plated onto LB-agar plates supplemented with ampicillin, rifampicin and kanamycin then incubated at 28°C for 48 hours.

Transformation of *Daucus glaber* plants via *Agrobacterium tumefaciens* using floral dipping method

Daucus glaber seeds were planted in October in the experimental farm of Pharmacognosy Department, Faculty of Pharmacy, Zagazig University, Egypt. Flower buds started to appear in December. Standard protocol for culture of *Agrobacterium tumefaciens* and inoculation of plants according to (Clough and Bent, 1989) was carried out. *Agrobacterium tumefaciens*, carrying the plasmid pTRA-K-TL-PSY, was used in this method. Suspended in infiltration medium to a final OD600 of approximately 0.80 prior to use. The revised floral dip inoculation medium consisted of ½ strength Murashige & Skoog Basal Medium (Duchefa, Germany), 5.0% sucrose, 100 µM acetosyringone and 0.005% Silwet L-77; pH adjusted to 5.7. After seeds ripening 100 seeds were surface sterilized by washing with 70% ethanol for 5 min., then with 5% bleach (sodium hypochlorite) containing 0.05% Tween 20 for 15 min, followed by three rinses with sterile water. To select for transformed plants, sterilized seeds were plated on MS with 3% sucrose (Adwic, ARE), 0.8% agar (purified agar for plant tissue culture, Bioworld, USA) and 100 mg/l kanamycin. Seeds were left to grow for 4–6 weeks in a controlled environment at 25°C under 16 hour photoperiod. A positive control consisted of 20 seeds from inoculated buds were cultured on kanamycin free media (MS supplemented with 3 % sucrose and 0.8 % agar) to confirm the ability of the seeds to germinate.

Transformation of *Daucus glaber* seedlings via *Agrobacterium tumefaciens* using leaf disc method

Eight weeks old *in vitro* germinated seedlings of *Daucus glaber* cultivated on MS (Duchefa, Germany) media with 3% sucrose (Adwic, ARE) and 0.8% agar (Bioworld, UK) under sterile conditions were used. A single colony of transformed *Agrobacterium* strain was inoculated into 50 ml of LB medium with 100 mg/l kanamycin, 200mg/l rifampicin and 300 mg/l Ampicillin then left to grow for 48 hours at 28°C on a rotary shaker at 200 rpm. The culture was then diluted 1:50 in minimal medium (MMA) with kanamycin 100 mg/l and grow overnight under the same conditions. The bacterial suspension was then diluted in MMA (without kanamycin) to density of OD_{600} = 0.15, placed at 28 °C at 200 rpm, and allow to grow to a density of OD_{600} = 0.25-0.3. Aliquots (15 ml) of the culture were centrifuged at 3700 x g for 5 min at room temperature. The supernatant was discarded and the pellets were re-suspended in MS medium (pH 5.8) containing 100 µM acetosyringone and diluted to a final density of OD_{600} = 0.05 (Turk *et al.*, 1991). Epicotyl explants (1-cm long) from 6 weeks old aseptic *Daucus glaber* seedlings were pre-cultured for 5 days on MS medium supplemented with 3% sucrose, 0.8% agar, 1mg/l NAA and 0.1 mg/l BAP before transformation. After the pre-culture period, the explants were immersed in 10-25 ml of the bacterial suspension for 2 minutes, rinsed in liquid MS medium without sugar, dried over sterile filter paper and placed back on the pre-culture medium, Explants were co cultivated in the dark for 2 days at 25 °C. The infected explants were rinsed in sterile double distilled water containing 600 mg /l Claforan® three times then, dried over sterile filter paper and placed on selective medium (MS supplemented with 1mg/l NAA (Sigma, USA), 0.1 mg/l BAP (Sigma, USA), 300 mg/l Claforan® and 25 mg/l kanamycin). Jars of 5 explants each were incubated in the dark at 25 °C. Both positive and negative controls were incubated in the same condition. The positive control consisted of non-infected explants, cultured on kanamycin free media (MS supplemented with 1mg/l NAA and 0.1 mg/l BAP). The negative control consisted of non-infected explants cultured on selective medium. After 2 weeks, all explants were transferred to fresh selective medium with 600 mg/l Claforan® and 100 mg/l kanamycin and placed under white-florescent lamps with 16 hours light and 8 hours dark (photoperiod). The efficiency of transformation was established. Induced calli were sub-cultured on fresh selective media every 4 weeks, over 8 month period.

Total carotenoids determination

Spectroscopical analysis of carotenoid accumulation was undertaken to confirm the visual observations (yellow coloured calli). Carotenoids in calli samples were extracted by procedures described by (Herrero-Martinez *et al.*, 2006). Briefly, Calli were washed with sterile double distilled water and cut into small pieces. Four grams of each callus were blended separately with 2 g anhydrous sodium carbonate and mixed with a mechanical blender. Three grams of the mixture was transferred into a centrifuge tube, added with 5 ml of tetrahydrofuran (THF) and mixed for 2 min under cold water. The mixture was centrifuged at 5000 *g* for 5 min and the supernatant was collected. Extraction was performed by adding 4ml DCM and 4 ml of 10% w/v NaCl into the supernatant and shaken for 2 min. The extraction was repeated twice; organic layer was collected and evaporated under nitrogen steam. The residue was kept at -20 °C, reconstituted with 2 ml of dichloromethane (DCM) prior UV measurements. These procedures were performed separately for both transformed and non-transformed calli at different time intervals (30, 50, 70 days), for each investigation the average absorbance of three

examined samples were recorded (Karnjanawipagul *et al.*, 2010). The data represented as mean ± standard error (SE) of at least three independent experiments. The obtained data were analyzed statistically using independent sample *T-test* that was used to determinate significant differences among the data. Results were graphed using Microsoft- Excel 2010 and statistically analyzed in SPSS, V13.

RESULTS AND DISCUSSION

*Daucus glaber*Forssk., a wild rare Egyptian plant, is suffering from the threat of extinction at the Mediterranean coastal region due to the expansion in building. This study aimed to investigate the tendency of the *Daucus glaber* Forssk. seeds to germinate *in vitro*, the ability of the explants taken from the growing seedling to form stable calli then to be differentiated into organs or somatic embryos in order to continue the micropropagation process. The study of some secondary metabolites in the induced calli and micropropagated plants to compare them with those of the experimentally cultivated plant using GLC/MS and HPLC techniques besides screening the cytotoxic and antimicrobial activities of *in vitro* tissues. The study also evaluated the most appropriate conditions to inducetransformation via *Agrobacterium tumefaciens.*

In vitro germination of seeds

Seed germination of *Daucus glaber* Forssk.has not been reported in details. Many studies discussed the *in vitro* germination of the spiny seeds of *Daucus carota* L. seeds. (Pant *et al*, 2007; Tavares *et al*, 2009; Rabiei, *et al*, 2010 and Ojha*et al*, 2012). The contact time between the seeds and both ethanol (3 and 5 minutes) and hypochlorite (5, 10 and 15 minutes) showed a significant effect in the rate of seeds decontamination.

The best sterilization condition was achieved by immersing the seeds in 70% ethanol for 5 minutes then in 5% sodium hypochlorite (Clorox®) for 15 minutes (93% rate of decontamination). MS and MS with GA3 media showed the highest germination percentage (63 and 60 % respectively), however, no significant differences in the seedling lengths were observed after 4 weeks of growth (3-5 and 2-7 cm respectively). MS media supplemented with 1mg/l 2, 4 D and 0.5 mg/l kinetin showed very low germination percentage (8 and 4 %; respectively) with a very small seedling (0.3-0.8 and 0.3-0.5 cm length respectively).

Induction of callus formation from the *in vitro* germinated seedlings

The effect of explant type and plant growth regulators on the *in vitro* induced calli was studied and the callus induction percentages (callusing capacity), as well as, the morphological characters of three types of explants on seven different media after 5 weeks of cultivation (Fig. 1) were recorded in Tables (1 and 2). Callusing capacity is the percentage of calli formed to the total number of the explants cultured. Data in Table (1) showed that the explant type and the type of plant growth regulators caused significant influences on the callusing capacity of the explant.

Growth rate determination

For each type of explants (leaf, stem and root), one gram of five weeks old callus grown on selected media (IV, VI and VII), were sub cultured on media with the same composition. The fresh weight in each case was recorded at different time intervals (5, 10, 15, 20, 30 and 40 days). This was performed to study the effect of different hormonal combinations on the callus growth. The results were shown in (Fig.2).

Table 1. Callus induction percentages (callusing capacity) of different plant explants of the *in vitro* cultivated seeds of *Daucus glaber* Forssk. after 5 weeks of cultivation. 30 explants for each treatment

Explant / Media	Leaf	Stem	Root	Mean
Medium I	80	80	66.6	75.5
Medium II	20	33.3	-	26.6
Medium III	100	71.5	33	68.2
Medium IV	100	100	53	84.33
Medium V	66.6	60	86.6	71.1
Medium VI	100	100	86.6	95.5
Medium VII	100	100	66.6	88.9
Mean	80.9	77.8	56.1	

Table 2. Morphological characteristics and dimensions of the callus from different explants of the *in vitro* germinated seeds of *Daucus glaber* Forssk. after 5 weeks of cultivation

Explant / Media	Leaf	Stem	Root
Medium I	+Yellowish green, Compact	++Yellow white, Friable	++Yellowish white, Compact
Medium II	+Yellowish white, Compact	+Yellowish white, compact	-
Medium III	+++Yellowish white, Compact	++Yellowish white, Compact	+Yellowish white, Friable
Medium IV	+++Yellowish green, Compact	++Yellowish white, Compact	++Yellowish white, Friable
Medium V	++Yellowish white, Friable	+Yellowish white, Friable	++Yellowish white, Friable
Medium VI	++Yellowish green, Compact	+++Yellowish white, Compact	+++Yellowish white, Friable
Medium VII	++++Yellowish green, Compact	+++Yellowish white, Friable	++Yellowish white, Friable

+ Very weak growth (2-5 mm in diameter); ++ Moderate growth (5-10 mm in diameter);
+++ Good growth (10-15 mm in diameter) and ++++ Very good growth (15-20 mm in diameter).

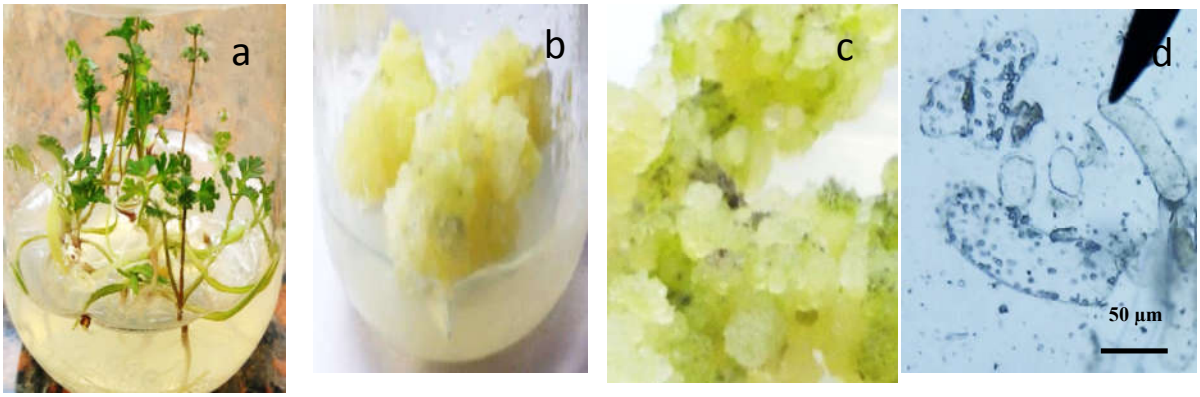

Fig. 1. Seeds germination, callus induction; a: Eight weeks old seedlings; b: Leaf calli on medium VII after 5 weeks of cultivation; c: Surface view of 8 weeks old callus on medium VI; d: Different forms of somatic embryos including globular, heart shape, torpedo shaped after 4-6 weeks on hormonal free medium

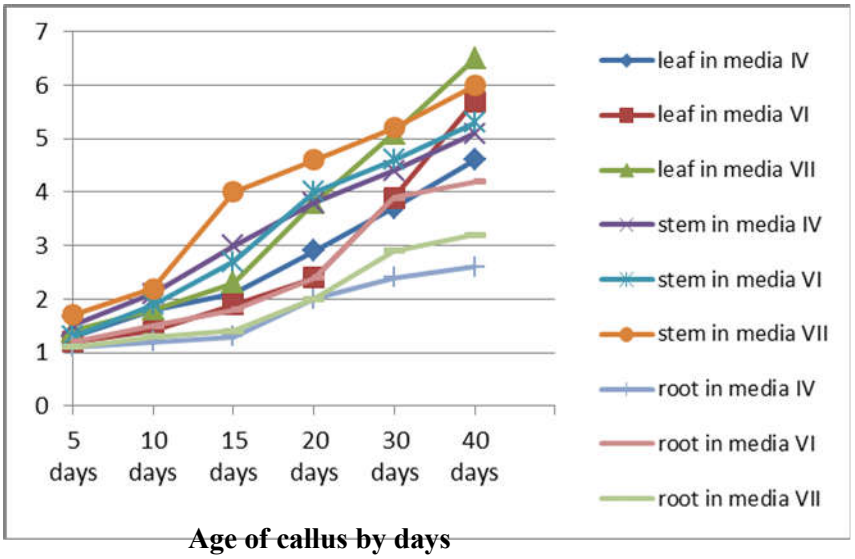

Fig. 2. The effect of different hormonal combinations on callus fresh weight (g)

In vitro propagation of *Daucus glaber* Forssk

Regeneration of *Daucus glaber* callus via somatic embryogenesis

Embryogenic calli were obtained through two steps. The first step was to induce the callus using media VI [NAA (1mg/l) + BAP (0.1mg/l)] and media VII [TDZ (0.5 mg/l) + 2, 4 D (1 mg/l) + BAP (0.1 mg/l)]. Eight weeks old calli grown on media VI in media VII, were greenish yellow in colour. The calli at this stage showed pre-embryogenic masses and globular embryos (Fig. 1).

When these calli were transferred to hormonal free media the second stage took place and further embryo development occurred. In the second stage the calli became more nodular and many spots with dark colour appeared, the callus were transferred to fresh media every 3 weeks. After 4-6 weeks on hormonal free media, different forms of somatic embryos including globular, heart shape, torpedo shaped and cotyledonary form were detected (Fig. 3a). After three months on hormonal free MS media small plantlets appeared (Fig. 3c).

Regeneration of *Daucus glaber* callus via organogenesis

The multishooted buds (Fig. 3b) were cut and then transferred individually to jars containing rooting media which consists of MS supplemented with 3% sucrose, 0.6 % agar and 1mg/l NAA. Roots started to appear after 2 weeks. After three weeks on rooting media plantlets were transferred to MS hormonal free media supplemented with 3% sucrose, 0.6 % agar to continue their growth.

Hardening and adaptation of the *in vitro* regenerated plantlets (acclimatization)

50 regenerated plantlets, 3 months old, were transferred to liquid MS media supplemented with 3% sucrose and 1 mg/L NAA for three weeks for root hardening. The plants were washed then transplanted into soil, covered with transparent perforated plastic bags which were removed a week later. Pots were maintained in green house. Only three plants can survive to two months in the soil after transplantation in a percentage of 6%. (Fig. 3e) shows plants after two months of transplantation.

GLC/MS study of the volatile contents of some *in vitro* tissues of *Daucus glaber* Forssk

The composition of the essential oil of the fruit, leaf and stem of the wild plant *Daucus glaber* Forssk.has previously been studied by GC/MS (Mansour *et al.*, 2004). This study is concerned with the analysis of essential oils of *in vitro* induced calli and micropropagated plants. Ether extracts of the calli of leaf, stem and root growing separately on media IV, VI and VII were subjected to GLC/MS. The GLC/MS revealed the absence of any essential oil components in calli extracts, except that of the root callus growing on medium VI. The GLC/MS of this extract revealed the presence of trans-Caryophyllene in a percentage of 0.2% of the total. The ether extract of micropropagated plants was subjected to GC/MS which revealed the presence of 84 components; eight of them were recognized as essential oil components representing 5.1 % of the total [α-Pinene (0.61%), Sabinene (0.9%), Limonene (1.04%), trans-Caryophyllin (0.15%), β-Bisabolene (0.76%) and γ-Bisabolene (0.22%)].

The application of these HPLC procedures aimed to investigate the presence of these compounds in micro propagated plants and calli. The samples were analyzed by HPLC under the same conditions as the standards. The results were compared using retention time and capacity factor (K) with the references. K is the most important parameter in the chromatography for determining the behavior of the columns (Watzig *et al.*, 1991 and Rouessac, *et al*, 2007). K= t_R(retention time of the compound) / t_M (dead time). The presence of any compound in any of the tested extracts was confirmed by spiking the samples with the suspected authentic. The number and intensity of peaks were investigated before and after the addition of the reference compound.

Quantitative analysis by HPLC Chromatography:

Scatter analyses and calibration curves were established using different concentrations of Angeloyloxylatifolone to determinate its concentration in different extracts.

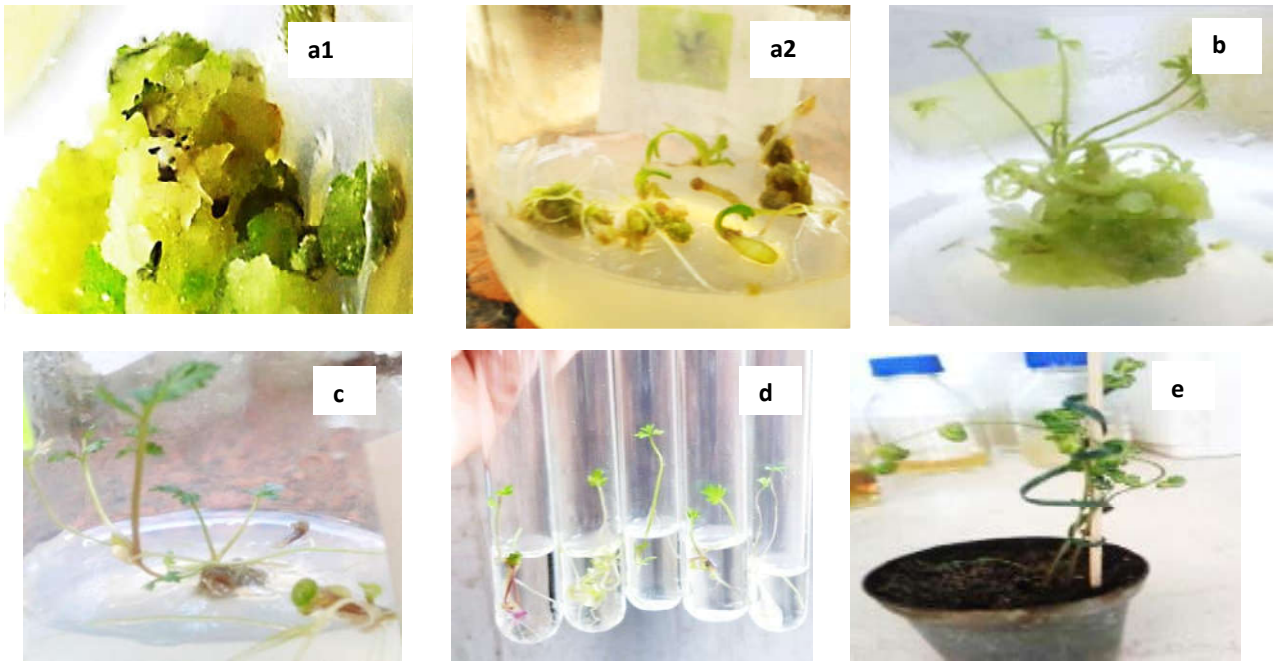

Fig. 3. Formation and development of somatic embryos from the calli of *Daucus glaber* Forssk; a1: Clusters of different forms of somatic embryos showing torpedo shaped and cotyledonary form embryos; a2: Green somatic embryos with elongated radicle and apical shoots; b: Twelve weeks old multishooted callus on medium VII; c: A developed plantlets after 3 weeks on hormonal free medium; d: One month old regenerated plantlets on liquid rooting medium; e: Two months old regenerated plant after transferring to the soil

HPLC study of some contents of *in vitro* tissues of *Daucus glaber* Forssk

The HPLC method using Hypersil Gold column, a Methanol (40%): Water (55%): Acetonitrile (5%) as mobile phase and diode array detection at 254and 220 nm was applied to reference compounds previously isolated as the major components of the n-hexane fraction of the wild plant of *Daucus glaber* Forssk. (Sallam, *et al.*, 2009 and Sallam, *et al.*, 2010) (Angeloyloxylatifolon, Glaberin A and Talasine A) at concentrations of 0.5 mg/ml. The retention time and capacity factor were determinated and listed for each compound in (Table 3).

The qualitative and quantitative HPLC analyses were showed that Angeloyloxylatifolone can be detected in the leaf calli those grown on medium VI and VII and micropropagated plant in a percentage of 0.2, 0.04 and 0.02 respectively (% of the investigated extract).

Table 3. Retention time and capacity factor of the reference compounds

Reference compounds	Retention time in minutes	Capacity factor (K)
Angeloyloxylatifolone	3.26	2.81
Talasine A	5.40	4.66
Glaberin A	5.85	5.04

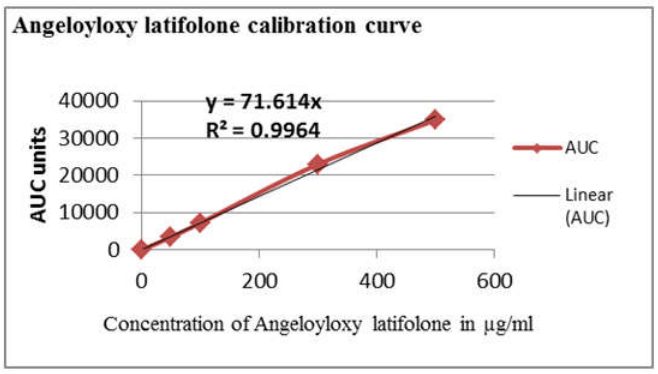

Fig. 4. Calibration curve of Angeloyloxylatifolone

Results of biological activities

Cytotoxic activity

As shown in (Table 4), the phenylpropanoid ester, Angeloyloxylatifolone showed the strongest cytotoxic activity against HEp-2 cells from the isolated compound under the investigation (IC_{50}3.67), followed by Glaberin A (IC_{50}6.7), while the sesquiterpene lactone, Talasin A showed the least activity (IC_{50} 50). The total alcoholic extracts of leaf calli grown on medium VI and VII as well as micropropagated plant showed strong cytotoxic activities against HEp-2 cells in comparison to doxorubicin standard (IC_{50} 8.94, 11.4 and 11.9 respectively).

A preliminary study of the antibacterial and antifungal activities

As shown in (Table 5), in comparison to amphotericin B, ampicillin and gentamicin, the total alcoholic extracts of leaf calli grown on medium VI and VII as well as micropropagated plant of *Daucus glaber* Forssk. had a significant antifungal and antibacterial effect against the tested microorganisms except for *Candida albicans* (RCMB 05036) and *Pseudomonas aeruginosa* (RCMB 010043).

Result and discussion of transformation study

Based on the DNA sequences available in the database, the PSY coding sequences is composed of 1050 base pairs. In order to clone the coding sequence of PYS, genomic DNA was isolated from *Synechococcuselongatus* PCC6803 as described in materials and methods. The isolated DNA was visualized on a 1 % (w/v) agarose gel as shown in (Figure 5). This genomic DNA was used as template for PCR amplification of the desired PYS gene. The PSY gene was amplified by PCR using the isolated *Synechococcus elongates* DNA as template. Two specific oligonucleotides for PSY gene flanked by Nco I and Xba I recognition sequences were also used in the PCR mixture. The PCR amplification products were visualized on a 1 % (w/v) agarose gel. Single band in the expected range (1050 bps) was amplified as shown in (Figure 6). The amplified PSY PCR product was column purified using Spin-PCR purification kit.

Table 4. Evaluation of cytotoxicity against HepG2 cell line of some different extracts of in vitro tissues of Daucus glaber Forssk

Tested material	Plant part used	Extract concentration (µg/ml)									
		0	0.39	0.78	1.56	3.125	6.25	12.525	25	50	100
		% Cell viability									
Alcoholic extracts	Callus*	100	-	-	90.13	78.79	63.27	32.41	21.94	10.48	-
	Callus **	100	100	99.08	97.12	86.12	69.38	45.92	24.86	15.24	-
	Micropropagated plant	100	-	-	-	92.97	78.89	59.20	43.74	25.29	10.34
Isolated compounds	Angeloyloxylatifolon	100	-	-	76.55	52.63	37.54	24.85	13.48	6.73	
	Talasin A	100	100	100	100	100	98.89	97.51	90.46	79.87	-
	Glaberin A	100	-	-	89.37	73.24	49.65	36.94	24.07	16.52	
Doxorubicin standard		100	-	57.44	48.25	30.32	21.03	16.90	14.29	10.95	-

Callus* = 12 weeks old leaf callus growing on MS media with 30 gm. /l sucrose, 8gm. / l agar , 1 mg/l NAA and 0.1 mg/l BAP (medium VI).
Callus** = 12 weeks old leaf callus growing on MS media with 30 gm. /l sucrose, 8gm. / l agar , 0.5 TDZ, 1 mg/l 2,4 D and 0.1 mg/l BAP (medium VII).

Table 5. Results of antibacterial and antifungal activities of some different extracts of *in vitro* tissues of *Daucus glaber* Forssk

	Fungi		Gram positive bacteria		Gram negative bacteria	
	Aspergillusfumigatus (RCMB 02568)	*Candida albicans* (RCMB 05036)	*Streptococcus pneumonia* (RCMB 010010)	*Bacillus subtilis* (RCMB010067)	*Pseudomonas aeruginosa* (RCMB 010043)	*Escherichia coli* (RCMB 010052)
Callus *	17.6±0.58	NA	20.3±0.423	21.4±0.53	NA	16.9±0.25
Callus **	16.3±0.44	NA	17.3±0.63	20.2±0.44	NA	15.9±0.37
Alcoholic extract of micropropagated plant	17.8±0.63	NA	20.6±0.63	22.4±0.44	NA	16.9±0.58
Standard antimicrobials:						
Amphotricin B	23.7±0.1	25.4±0.1	-	-	-	-
Ampicillin	-	-	23.8±0.2	32.4±0.3	-	-
Gentamicin	-	-	-	-	17.3±0.1	19.9±0.3

The purified PCR product of PSY in addition to the expression vector pTRA-K-TL-cTP was subjected to a double restriction digestion using Nco I and Xba I together in one mixture in a single step. The restriction digestion was performed for 2 hours at 37 ^{o}C. The digested DNA samples were then column purified in order to remove the resulting small end fragments after enzymatic digestion. Aliquots of the purified products were visualized on 1 % (w/v) agarose gel. (Figure 7) shows the restricted and purified PSY PCR product (1050 bps). Figure 8shows pTRA-K-TL-cTP plant expression vector (7820 bps) after NcoI and Xba I restriction. Digested and purified PSY fragments were ligated into pTRA-K-TL-cTP vector. The ligation mixtures were transformed into DH5α bacteria. In order to investigate the efficiency of the cloning strategy and the results of the ligation step described above, the resulting colonies of the DH5α bacterial transformation were subjected to a colony PCR screening using gene specific and vector specific primers named pS5` (13), pS3` (18) primers were used for this PCR test. The positive clones are expected to produce a PCR product of 1217 bps. Figure 9 shows the results of this colony PCR screening test after visualization on 1 % (w/v) agarose gel All the tested colonies gave the right product as expected (1217 bps).

Each positive colony was inoculated into 15 ml of LB medium supplemented with ampicillin and incubated overnight at 37 ^{o}C for isolating the plasmid DNA, pTRA-K-TL-PYS (Figure 10) to be used for further analyses. Plasmid DNA mini kits were used to isolate plasmid DNA from DH5α bacteria that was transformed with pTRA-K-TL-PSY plasmid according to the instructions provided with those kits. Two PSY vector specific primers named pS5` (13), pS3` (18) primers were used for PCR analysis of plasmid DNA isolated from DH5 α transformed with pTRA-K-TL-PSY plasmid DNA. The positive result was expected to produce a PCR product of 1217 bps. Figure 11 shows the results of this PCR test after visualization on 1 % (w/v) agarose gel containing ethidium bromide. The plasmid DNA (pTRA-K-TL-PSY) isolated from transformed DH5α was transformed into competent *A. tumefaciens.* The resulting colonies of *A. tumefaciens* transformation with pTRA-K-TL-PSY construct were used as a template for the colony PCR screening test. Two vector specific primers named pS5` (13), pS3` (18) primers were used for this PCR test. The positive clones are expected to produce a PCR product of 1217 bps (Figure 12). Two *Agrobacterium* transformation protocols were under investigation. In the first protocol, Floral buds of *Daucus glaber* plant were transformed with *Agrobacterium tumefaciens* carrying the plant expression plasmid pTRA-KTL-PSY following the transformation protocol as described in materials and methods. After 6 weeks of cultivation of 100 seeds of the inoculated buds on MS media with 3% sucrose, 0.8 % agar and 100 mg/l kanamycin, no germination obtained in all jars while 70 % of seeds of the inoculated buds grown on the same media but without kanamycin were germinated within 4 weeks. Standard Floral dipping method (Clough and Bent, 1989) might not be suitable for transformation of *Daucus glaber* Forssk. In the second protocol via leaf disc method, Epicotyl explants of *Daucus glaber* plant were transformed with *Agrobacterium tumefaciens* carrying the plant expression plasmid pTRA-KTL-PSY following the transformation protocol described by (Hernandez, 1994) with some modifications, as described in materials and methods. Transformed *Daucus glaber* explants were allowed to grow on MS medium with 3% sucrose, 0.8% agar , 1mg/l NAA, 0.1mg/l BAP, Claforan® (300 µg/ml) to inhibit the growth of *A. tumefaciens* and Kanamycin antibiotic (100 µg/ml) for selection of transformed explants(Figures 13). Negative controls (non- transformed explants on selective medium) were carried out to ensure the sensitivity of the plant to this concentration of Kanamycin (Figure 14).

Positive controls (non-transformed explants on the same medium without Kanamycin) were carried out to ensure the ability of the media to induce calli(Figure 15). The efficiency of the transformation protocol was investigated by calculating the percentage of transformation after 6 weeks of culturing on selective media. The percentage of transformation was 16.4%. In order to confirm the presence of PYS in genomic DNA isolated from the transformed callus of *Daucus glaber*, genomic DNA was isolated from *Daucus glaber* transformed and none transformed callus as described in materials and methods. The isolated DNA were used as as templates. Two specific oligonucleotides for PSY gene flanked by Nco I and Xba I recognition sequences named Psy-Fw*Nco I* and Psy-Rev *Xba I* (see material and methods), were also used in the PCR mixture. The PCR amplification products were visualized on a 1 % (w/v) agarose gel containing ethidium bromide. Single band in the expected range (1050 bps) was amplified as shown in (Figure 18). The total carotenoids (determined as *β*-carotene) in, 30, 50, and 70 days old, transformed calli with PSY gene and non-transformed calli were spectrophotometrically determined at 460 nm, at least three replicates of each age were used. The data was recorded in (Table 6) and Figure (18). From Table (6) and Figures (19),it is clear that carotenoids concentrations appeared to be higher in transformed calli with PSY gene which grown on MS with 3% sucrose, 0.8% agar, 600 mg/l Claforan®, 100 mg/l Kanamycin, 1 mg/l NAA and 0.1 mg/l BAP of 30 days old while no carotenoids could be detected in non-transformed callus of the same age which grown on the same media but without Kanamycin. The percentage increase in carotenoids contents in 50 days old transformed calli was 81% in comparison to non-transformed calli while it became 26% in 70 days old calli. These results provide clear evidence of the functionality of PSY gene in transformed calli that results in increasing the carotenoids biosynthesis as expected.

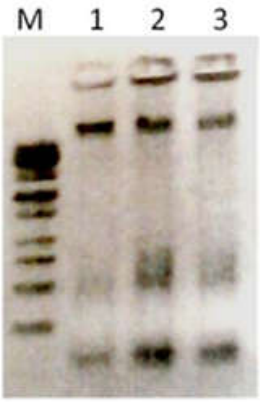

Fig. 5. Genomic DNA was isolated from *Synechococcus elongates* PCC6803

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation. The isolated *Synechococcus elongates* DNA appears after a 1 % (w/v) agarose gel in 1x TAE for 50 min at 100 V.; M: 1 kb DNA ladder (Intron Biotechnology, South Korea); lane 1-3: the isolated genomic DNA from *Synechococcus elongates* PCC6803 samples

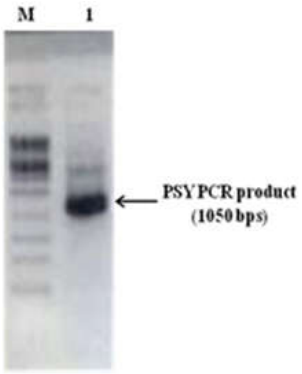

Fig. 6. PCR amplification products of PSY gene

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation. One band in the expected size range appears after a 1 % (w/v) agarose gel in 1x TAE for 50 min at 100 V; M: 1 kb DNA ladder; lane 1: amplified PCR product of PSY gene.

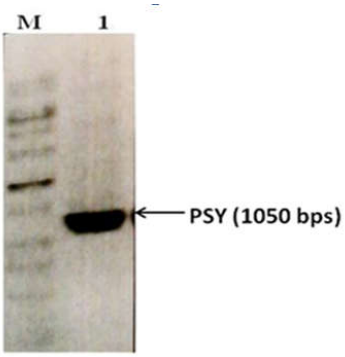

Fig. 7. The restriction digestion of PSY PCR product

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation, clear band for PYS PCR product with 1050bps (lane 1) after double restriction digestion with Nco I and Xba I enzymes were observed after separation on a 1% (w/v) agarose gel in 1x TAE for 50 min at 100 V. M: 1 kb DNA ladder; lane 1: PSY PCR product after restriction with Nco I and Xba I enzymes.

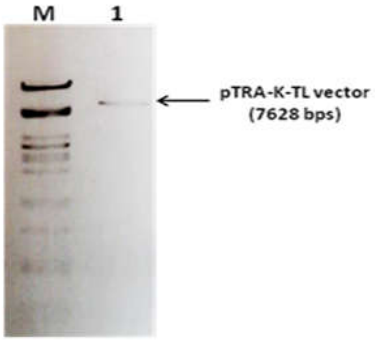

Fig. 8. The restriction digestion of pTRA-K-TL-cTP vector

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation, vector of 7628 bps, one clear band for pTRA-K-k with 7628 bps (lane 1) after double restriction digestion with Nco I and Xba I enzymes were observed after separation on a 1% (w/v) agarose gel in 1x TAE for 50 min at 100 V. M: 1 kb DNA ladder; lane 1: pTRA-K-TL after double restriction with Nco I and Xba I enzymes.

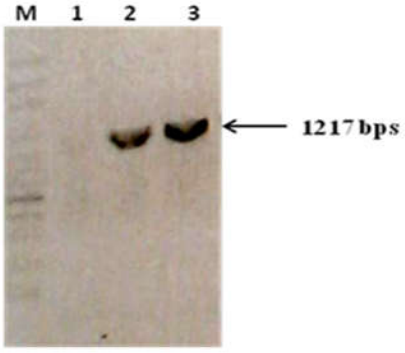

Fig. 9. Results of the colony PCR screening of the transformed DH5α with pTRA-K vector and PSY ligation mixture

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation. One clear band in the expected size range was observed (1217 bps). M: 1 kb DNA ladder; lane 1: Negative PCR control without template; lane 1(2 and 3): PCR products from 2 different transformed DH5α colonies.

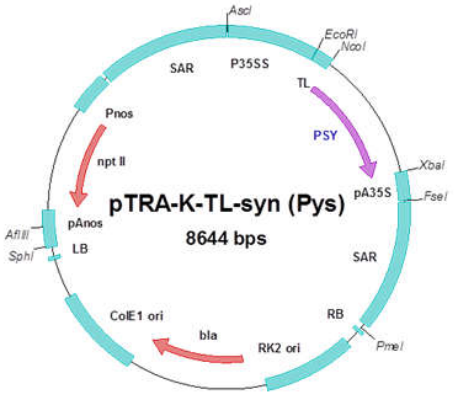

Fig. 10. Structure of pTRA-K-TL-PSY plasmid DNA

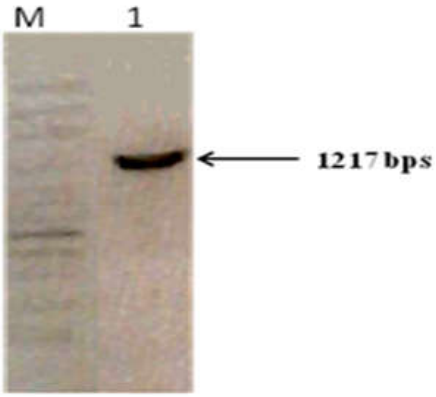

Fig. 11. PCR test of the plasmid DNA isolated from transformed DH5α bacteria

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation, after PCR test using pTRA-K-TL-PSY plasmid as template. clear band for PSY PCR product with 1217 bps (lane 1) was observed after separation on a 1% (w/v) agarose gel in 1x TAE for 50 min at 100 V. M: 1 kb DNA ladder, lane1: PSY PCR product.

Fig. 12. Results of the colony PCR screening of the transformed *A. tumefaciens* with pTRA-K vector

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation. The products of the colony PCR were separated on a 1 % (w/v) agarose gel in 1x TAE buffer for 1 hour at 12 V/cm. One clear band in the expected size range was observed (1217 bps). M: 1 kb DNA ladder; lane 1 and 2: two positive colonies of *A. tumefaciens* transformed with pTRA-K-TL-PSY. Lane 3: Negative PCR control without template.

Fig. 13. Transformed explants after 20 days from transformation, growing on selective media with 100 mg/l Kanamycin

Fig. 14. None-transformed explants after 20 days from cultivation on selective media with 25 mg/l Kanamycin

Fig. 15. None-transformed explants after 20 days from cultivation on MS media with 3% sucrose, 0.8% agar, 1% NAA, 0.1 % BAP and 300 mg/l Claforan®

Fig. 16. None transformed calli after 50 days from cultivation on MS media with 1mg/l NAA and 0.1 mg/l BAP

Fig. 17. Transformed calli after 50 days from cultivation on selective media with 100 mg/l Kanamycin

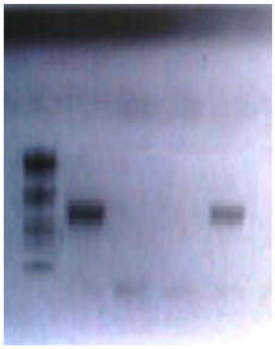

Fig. 18. PCR amplification products of PSY gene in transformed and non-transformed callus of *Daucus glaber*

Shown is the ethidium bromide mediated fluorescence of DNA fragments after UV excitation. One band in the expected size range appears after a 1 % (w/v) agarose gel in 1x TAE for 50 min at 100 V; M: 1 kb DNA ladder; lane 1&4: amplified PCR product of PSY gene from DNA of transformed callus; lane 2&3: negative PCR result of PSY gene from DNA of none transformed callus.

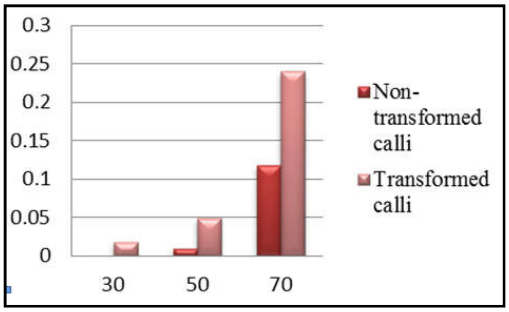

Fig. 19. UV absorbance of extracted carotenoids from transformed and none-transformed calli of *Daucus glaber* Forssk growing on MS media with 1 mg/l NAA and 0.1 mg/l BAP at different time intervals 30, 50 and 70 days

Table 31. UV absorbance of carotenoids isolated from transformed and non-transformed calli of *Daucus glaber* Forssk

Type of calli	None-transformed calli			Transformed calli with PSY gene		
Age of calli(days)	30	50	70	30	50	70
Mean absorbance at 460 nm	0	0.01	0.178	0.018	0.049	0.24

Conclusion

In this study, conditions for seeds sterilization, plant germination, callus production and plant micropropagation of *Daucus glaber* Forssk.were investigated. The best hormonal combinations for callus induction from leaf and stem explants were (TDZ 0.5mg/l + 2,4-D 1mg/l + BAP 0.1mg/l) while the best hormonal combinations for callus induction from root explants were (NAA 1mg/l + BAP 0.1 mg/l). The media with hormonal combinations of (TDZ 0.5mg/l + 2,4-D 1mg/l + BAP 0.1mg/l) and (NAA 1mg/l + BAP 0.1 mg/l) were the best for embryogenesis. The acclimatization and transplantation trials lead to the maintenance of the transplanted plantlets for about two months. The GLC/MS study of the ether extracts of induced calli revealed that only root callus grown on MS media with (NAA 1mg/l + BAP 0.1 mg/l) can produce trans-Caryophyllin while the ether extract of the micropropagated plant contains α-Pinene, Sabinene, Limonene, trans-Caryophyllin, β-Bisabolene and γ-Bisabolene. The HPLC investigation of the compounds Angeloyloxylatifolone, Glaberin A and Talasin A previously isolated from wild plant revealed that Angeloyloxylatifolone was detected in leaf calli grown on medium VI and VII besides the micropropagated plant. All extracts under investigation showed strong cytotoxic activity against HepG2 cell line and a significant anti-microbial activity against *Aspergillusfumigatus*, *Streptococcus Pneumonia, Bacillus subtilis*and *Escherichia coli*. Phytoene synthase (PSY) is considered the major rate limiting step in carotenoid biosynthesis because it irreversibly directed granylgranyl di phosphate (GGDP) towards carotenoid production (Cunningham and Gantt. 1998). The transformation of the plant using *Agrobacterium* mediated transformation method has been performed to investigate the most appropriate protocol for efficient transformation knowing that the transformation protocols of common model species such as Tobacco and Arabidopsis cannot be applied to this plant because of the inability of the *in vivo* germinated plant tissues to produce calli. In this study the cyanobacterial gene PSY (encodes for Phytoene synthase) was cloned into suitable plant expression vector (pTRA-K) in order to transfer the PSY activity into *Daucus glaber* Forssk. The PSY gene was PCR amplified using *Synechococcuselongatus* (PCC6803) as template and PSY gene specific primers flanked by *NcoI* and *XbaI* recognition sequences. The resulted PSY PCR product was further cloned into pTRA-K vector. The resulted pTRA-K-PSY plasmid DNA was used to transform *Daucus glaber* via *Agrobacterium tumefaciens* mediated plant transformation. The resulted explants after these transformation events were selected on medium with Kanamycin. Presence of PSY gene in transformed calli was confirmed using PCR. The effect of the inserted gene on accumulation of carotenoids in the callus was performed by comparing the UV absorption of the extracted carotenoids in transformed and non-transformed calli at different time intervals. The insertion of PSY gene in *Daucus glaber* Forrsk. significantly increased the accumulation of carotenoids (provitamin A) in callus cells.

Recommendation

The protocol used in this study may provide an efficient method to insert different specific genes into *Daucus glaber* in order to increase the yield of its active constituents as sesquiterpene lactones and phenylpropanoid esters.

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