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Original Article

Biotransformation of Monoterpenoids by Suspension Cultures of *Lavandula angustifolia*

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Abstract

Callus and suspension cultures of *Lavandula angustifolia* Mill. (Lamiaceae) were established and the effect of different culture media on growth rate was investigated. Terpenoids added to suspension culture to investigate their biotransformation. All samples were analyzed by gas chromatography (GC) and GC-mass spectroscopy (MS). Octan-1-ol, citronellol, linalool, borneol and geraniol were biotransformed products of octanal, citronellal, linalyl acetate, bornyl acetate and geranyl acetate, respectively. Citronellol, linalool, borneol, and menthol didn't change by *L. angustifolia* suspension cultures. Blue pigment production by cultures of *L. angustifolia* was also studied. Ester hydrolysis and oxidation were the main reactions which occurred in biotransformation process, which may be attributed to the presence of related or bifunctional enzymes. This technique is a possible way of the production of expensive or rare compounds from cheap and plentiful substrates.

Keywords: Biotransformation; *In vitro* cultures; *Lavandula angustifolia*; Terpenoids.
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1. Introduction

Plants are valuable sources for a variety of phytochemicals. Some of the biochemical processes occurring in plant cells is complex and can not be achieve by synthetic routs. Plant cell and tissue cultures possess considerable biochemical abilities to transforming foreign substrates administered exogenously. A wide variety of compounds including aromatics, steroids, alkaloids, coumarins, terpenoids and lignans can undergo biotransformation using plant cells, organ, and tissue cultures [1, 2]. Suspension culture techniques are preferred for biotransformation studies because they provide higher growth rate and greater ease of substrate

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addition, which increased chance of media and cell content.

Lavandula angustifolia Mill. (Lamiaceae) is a shrub with a well-developed woody base, growing to a height of about 2 ft, occasionally more. A native of the West Mediterranean region, but extending some way inland and up to 6000 ft; naturalized in parts of Central Europe [3]. *L. angustifolia* is the source of the true oil of lavender, which was once esteemed as a medicine for innumerable ills and also for making a tincture which was popular cordial. Many investigations have been done on *L. angustifolia* and essential oil composition had been analyzed previously. Anti-inflammatory, anxiolytic, mutagenic activities and analgesic property have been reported from its essential oil [4-6]. The essential oil has been suggested as natural repellant, pharmaceutical and industrial auxiliaries [7].

In this work, the main focus is on the establishment of *L. angustifolia in vitro* cultures under different conditions and study the biotransformation of terpenoids by cell suspension culture.

2. Material and methods

2.1. Plant material

Seeds of *Lavandula angustifolia* Mill. (Lamiaceae) were prepared from Mr. Fothergill's Seeds Ltd., Suffolk, UK.

2.2. Seed and explant sterilization and culture initiation

The seeds were soaked in 0.5% w/v H_2O_2 for 24 h and sterilized in 2% chloramine T containing 0.1% w/v Tween 20 for 20 min. following by washing with sterile water three times. When explants were used for culture formation the procedure for sterilization was as described for seeds. Two germination media were used in this investigation; 0.8% agar-water and diluted ½ Murashige and Skoog (MS) medium [8]. Cultures were kept in an incubator at 25±2 °C at the darkness.

2.3. Effect of different culture media on culture growth

Modified Tobacco (MT) [9], MS (Sigma, USA) media supplemented with 1.0 mg/l 2, 4-dichlorophenoxyacetic acid (2,4- D), 2.0 mg/l indole 3-acetic acid (IAA), 0.2 mg/l kinetin, 3% sucrose, 0.1% agar and 15% coconut water and SH (Schenk and Hildebrandt) [10] medium (Sigma, USA) supplemented with 1.0 mg/l 2,4- D, 2.0 mg/l IAA, 0.2 mg/l kinetin, 3% sucrose as well as complex vitamin solution which was used in MT medium, were used in order to optimize the growth rate of cultures and biotransformation of substrate in suspension culture. For suspension, culture media were prepared in the same manner of solid media, but without the agar as the solidification agent. Suspension cultures were continuously agitated using an orbital shaker at 120 rpm (5 cm throw). All cultures were kept in an incubator at 25±2 °C at the darkness.

2.4. Extraction of blue pigment from the cultures

In suspension cultures of *L. angustifolia,* the blue pigments were extracted from the medium by addition of n-butanol and applying slight heat to the mixture whilst stirring. The organic fraction was separated and subjected to further analysis.

2.5. Estimation of dry and fresh weight

Every one or two days, the callus was removed under aseptic conditions and placed in the pre-weight sterile petri dish. The petri and callus were weighted and the weight of callus was calculated. The callus was then placed into the oven (60 °C) for 24 h, and stored in a desiccator containing silica gel. Then the dried samples were weighted. For each culture, 5 samples were tested and the average was calculated. For suspension cultures filter papers were dried by placing them in a drying oven (100 °C) for 10 h before being transferred to a desiccator

containing silica gel. A sample of 30 ml from the Erlenmeyer flask containing the suspension culture was removed and filtered through a pre-weighted, dry filter paper. The filter paper and the cells were weighted and the fresh weight per ml calculated. For the dry weight, the filter paper and the separated cells were placed in to dry oven (60 °C) for 24 h and then transferred to a desiccator to become cool. They were weighted again and dry weight of cells per flask of the culture was calculated.

2.6. Estimation of cell density and cell viability

A sample of 20 ml of suspension culture was taken and solid chromium trioxides were added to the sample to obtain a final concentration of about 2% (w/v). The culture was incubated at 80 °C until the solution turned dark brown/black (between 3-10 min.). The solution was passed repeatedly in and out of a Pasteur pipette until all the clumps had disintegrated. The cells were counted using a hemacytometer (Leitz Sm-Lux, Germany). The cell density was calculated as number of cells per ml of culture from an average of nine counts. When the cells were fed with fluorescien diacetate, live cells gave a green fluorescence under a fluorescence microscope, whilst dead cells were only visible under white light. Live cells have intact membrane through which the fluorescien diacetate is transported and the cells possess active esterase enzymes which cleave the acetate moieties from the fluorescien diacetate giving fluorescence in the cell. In this method, 500 mg of fluorescien diacetate was dissolve in 10 ml of acetone and the solution was stored at 4 °C. One drop of well-mixed culture was placed on a microscope slide and to this; one drop of fluorescien diacetate reagent was added. The slide was viewed under a fluorescence microscope (Leitz DMIL, Germany) fitted with blue barrier filter.

2.7. Study of biotransformation

Cell suspension cultures of *L. angustifolia* were investigated for biotransformation of octanal, citronellal, linalyl acetate, bornyl acetate, geranyl acetate, linalool, borneol, citronellol and menthol. Five mg substrates with more than 95% purity (Sigma or Aldrich,) were added to suspension cultures. The purity of substrates which were used for

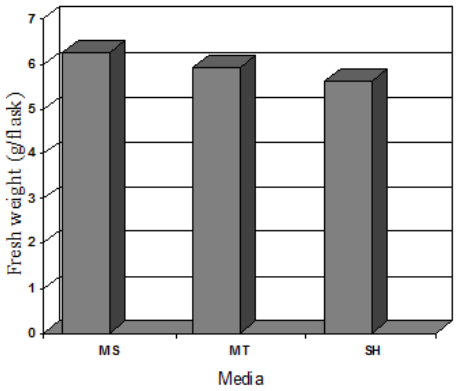

Figure 1. The growth of *Lavandula angustifolia* callus after 20 days in different media.

biotransformation studies was also checked by gas chromatography (GC). Control samples were made without the addition of substrates to cultures. Further controls, were established using substrates added to the cell-free medium. Controls and samples were incubated under the same conditions. Either a solution of substrate in ethanol or the undissolved substrates were added to flasks containing cell suspension cultures or to control with the aid of a pre-sterilized tip automatic pipette. After the incubation period, the flasks were swirled to ensure good mixing and then the cells and media separated by vacuum filtration through a filter paper. Cells and medium were extracted with dichloromethane and analysis by identical protocols. Controls were employed in every test as mentioned above. In biotransformation experiments, cultures were routinely checked for contamination with microbes and fungi by plating out samples of suspension cultures on the nutrient agar medium and also by microscopic examination. Cell suspension cultures of *L. angustifolia* together with controls were incubated for the required time. After the incubation, cultures were adjusted to pH 12 by adding 1 M KOH to hydrolyze ester forms of acidic products. In biotransformation studies when the cells were fed with substrates the ester hydrolysis was carried out for identification of acidic conversion products. The suspension culture was disintegrated using a blender and incubated for half an hour. The solid part was separated by using centrifuge (3000 rpm for 10 min.).

2.8. Extraction of cell suspension cultures

Suspension cultures were filtered using Whatman no. 1 filter paper moistened with solvent. Known amounts of fresh cells were crushed using a blender or in a mortar and quantitively extracted with sufficient amount of solvent which was already spiked with the internal standard. The media were also similarly extracted. The extract was dried over a hydrous sodium sulphate and reduced under nitrogen gas to 200 µl and analyzed by GC. Alternatively, the total suspension culture was extracted by adding the solvent to the mixture and stirring it for 1 h using a magnetic stirrer. Extraction was carried out using dichloromethane, diethyl ether or methanol in a proportion of 10 ml solvent to each 10 µg of cells. The internal standard was pentadecane and was present in the extracting

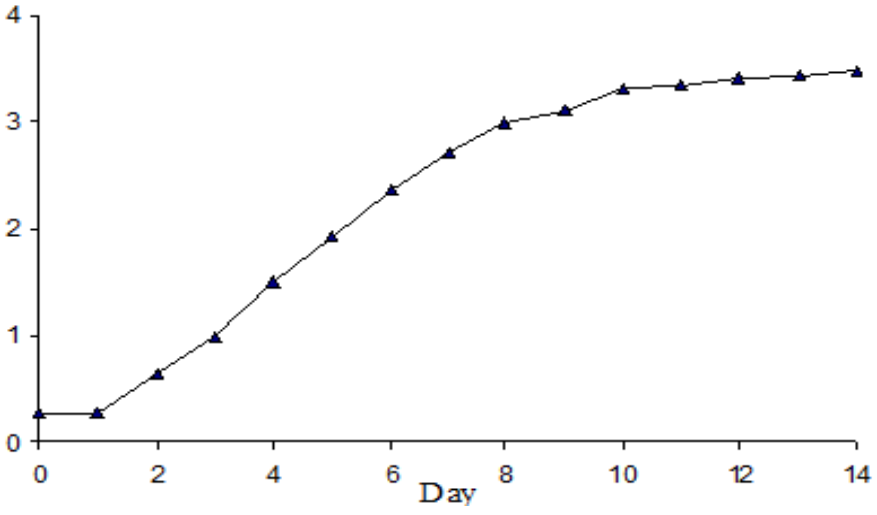

Figure 2. Increase in cell number of *Lavandula angustifolia* suspension culture.

solvent. In solid culture, after separating of the callus from media, the cells were crushed using a blender or in a mortar and quantitively extracted with the solvent spiked with the internal standard as above. In order to extract the media, water was added and the mixture was stirred while heat was slowly applied. These were extracted by the method described for cells. After extraction with an appropriate solvent, the extract was reduced under nitrogen gas. In all cases, 1 µl of sample was injected to the column.

2.9. Sonication

Sonication experiment was carried out to study the effect of sonication on bioconversion of the substrate which weren't biotransformed by *L. angustifolia*. A sonication experiment was also used for investigation rates of biotransformation of those substrates which were converted previously. Continuous wave 1 MHz ultrasound (Grant, UK) was used to release the biosynthetic products of cultured cells into the surrounding medium. The cells were fed by substrates and were exposed to ultrasound before incubation. As sonication may affect the substrate used in biotransformation experiments the combined media and substrates were also exposed to ultrasound wave as controls and extracted by the same solvent as the separated cells.

2.10. Analysis of bio-converted substrates

GC analyses were carried out using a Perkin Elmer (model 8320B) GC. Capillary columns BP-1, BP-5 and BP-20 were used. In all experiments, the FID detector was used and the carrier gas was He at a linear velocity of 30 m/s. The instrument was operated as follows: The oven temperature was adjusted from 50 to 250 °C at 7 °C/min. (for BP-20 oven temperature rang was from 50 °C to 250 °C). Injector and detector temperature were set at 250 °C and 280 °C, respectively. The identification of products and substrates was confirmed by co-chromatography and GC-MS analysis. A β-cylodex column (Chrompack, UK) which is normally used for chiral separation was used when identification of enantiomers was required. The specification of column was as follows: 25 m length and 0.22 mm internal diameter fused silica capillary column with permethylated β-cyclodextrin suspended in a moderately polar stationary phase in 0.25 m film thickness equivalent to BP-10. In order to estimate the amount of compound, especially in the bioconversion experiment, pentadecane was used as internal standard. GC/MS analyses were performed using a model 5890 Hewlett-Packard GC unit connected to model 5972 Hewlett-Packard mass spectrometer. The separation on GC-MS unit was carried out using a BP-5 column with the same specification as described above. Culture extracts were prepared and then reduced to 200 µl. One µl of extract was subjected to GC. The GC conditions were as described above. Mass spectral data was obtained under the following conditions: Ionization current 1 A, ionization potential 70 eV, source temperature 150 °C, resolution 1000. Identification of the sample component was based on retention time, retention index electron-impact-mass spectral (EI-MS) data. Comparison was made with relevant reference standards and the visual check with references [11, 12].

2.11. Quantitative analysis

Peak areas were used for quantitative analysis and internal standard used in quantitative studies was at a concentration range of 1-4 mg/ml. Pentadecane were injected as a standard at the concentration rang of 1-4 mg/ml and area under each peak were calculated (8.77, 15.75, 21.41, 24.90, respectively), and then area under each sample peak was compared with the quantity of standard injected.

3. Results and discussion

The best result for germination in *L. angustifolia* was obtained when seeds were sterilized by chloramine T. With another sterilization method either no germination was observed or seeds become infected. The time required for seeds to germinate was 3-7 days for *L. angustifolia* and more than 80% of the seeds on each plate germinated. Seeds plated in ½ MS medium germinated faster and length of the radical and hypocotyl was longer, however contamination risk of seeds increased. The faster seed germination was obtained in darkness. Calli cultures were obtained from seedlings, but the sterilized explants of seeds and stems were also used to initiate callus cultures. The cultures obtained from seedlings and explants were similar

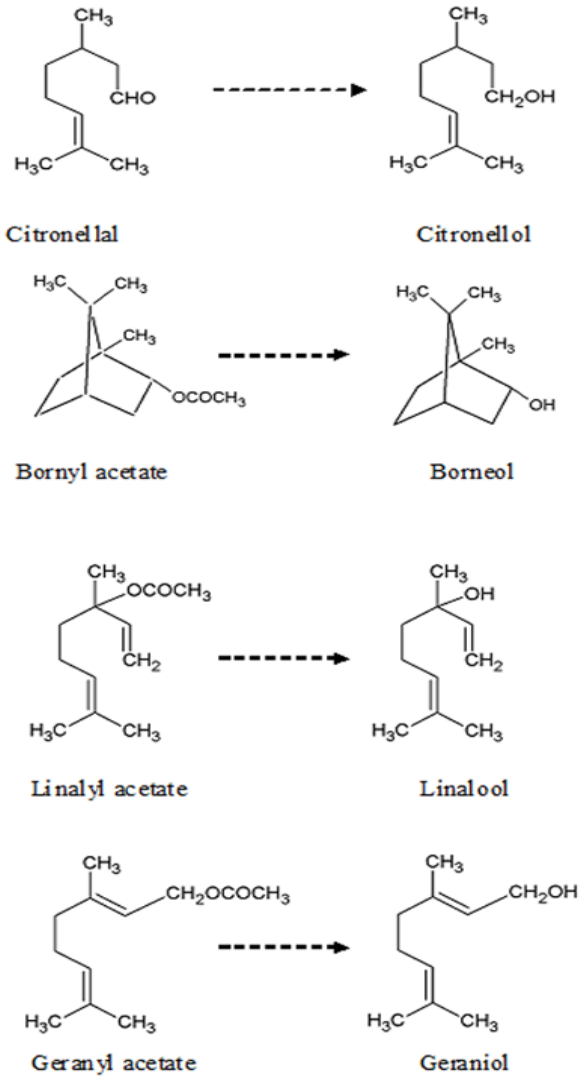

Figure 3. Biotransformation of terpinoids by *Lavandula angustifolia* cell suspension culture.

both in appearance and biosynthetic products even after several passages. The appearance of *L. angustifolia* callus was green/ pale brown and compact and the appearance of suspension cultures were blue/green using MS media.

Suspension cultures of *L. angustifolia* were subcultured after 12-17 days. Cells in *L. angustifolia* suspension cultures didn't aggregate into clumps. Changes in the physical appearance of suspension cultures were not noticeable after 3 years subculturing. The callus obtained from three media used in this study (MT, MS, and SH) were not significantly different. However, the growth rate of solid cultures of *L. angustifolia* (measured as fresh weight) was slightly increased in MS medium (Figure 1).

Dry weight started to increase from the 4^{th} day and continued for 10 to 14 days in *L. angustifolia.* It has been reported [13] that in the fast growing phase plant cells synthesis cell wall material and starch from available carbohydrate, and even when they reach to the stationary phase starch is metabolized. Also, cells are large and tend to trap culture medium which leads to a high fresh weight. The increase in the number of cells of *L. angustifolia* and in suspension culture is presented in Figure 2.

An interesting observation of *L. angustifolia* cultures was the appearance of a blue pigment. In this work it was also observed that the blue pigments were found only in the media, presumably because they were either released to or were synthesized in the medium. Suspension cultures of *L. angustifolia* produced the intense blue pigment in first few passages, but in further passages the production decreased and finally this cell line lost its ability to produce blue pigment in suspension culture. The production of blue pigment in callus cultures decrease after about 60 passages in 3 years of subculturing. In any stage of subculture when the cells were transferred from solid culture to a suspension culture, the suspension culture still produced blue pigment in the media. A preliminary explanation for the occurrence of the pigment was that it is attributed to complexation with the Fe^{++} in the medium as a medium not containing Fe^{++} reminded colorless or slightly yellow. It was noted that making the blue medium strongly acidic caused the coloration to disappear. The appearance of the blue pigment has been reported by other investigators [14]. In this study the iron stock solution which contained Fe^{++} was omitted from the MT media and this resulted in a significant decrease in the biosynthesis of blue pigment. Both light and high concentration of sucrose slightly increased the production of the blue pigment.

The added substrates and converted compounds are listed in Table 1. Using suspension culture of *L. angustifolia* for biotransformation of linalyl acetate resulted in detection of linalool (64%) (Figure 3). The cells reduced 50% of the added citronellal to citronellol (50%) and the non-terpenoid aldehyde, octanal, was converted to octan-1-ol (84%). When bornyl acetate and geranyl acetate were added to *L. angustifolia* suspension cultures, borneol (80%) and geraniol (68%) were detected in a dichloromethane extract, respectively. Biotransformation of bornyl acetate and citronellal using suspension cultures of *L. angustifolia* were investigated previously and in both the results cases were in agreement with the results which were obtained in this study [2]. In sonication experiment there was no differences in biotransformation of substrates were observed when ultrasound was used before or after incubation of substrate and cells. It is suggested that the ultrasound only helps to release the content of the cells.

The data in this work indicates that the natural metabolite monoterpenoids such as linalool were not converted but linalool with added functional groups (linalyl acetate) was efficiently transformed using cells. *L.*

angustifolia suspension cultures were fed with the monoterpene alcohols such as linalool, borneol, citronellol and menthol but no conversions which could be attributed to addition of these monoterpenoids were observed. These finding are in a good agreement with the results of previous study on *Cannabis sativa* suspension cultures which was reported that linalool, borneol, citronellol and menthol were recovered unchanged from the suspension cultures [15]

In this study ester hydrolysis and oxidation were the main reactions which occurred in biotransformation process, which may be attributed to the presence of related or bifunctional enzymes. A coordinated approach of molecular studies on metabolic pathway engineering to understand the genes and enzymes involved may contribute towards the utilization of biotransformation *in vitro* for practical applications.

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