Facultad de Farmacia
Departamento de Biología Vegetal

Studies on the terpene metabolism in *Lavandula latifolia* Medicus

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D030-01 PROGRAMA OFICIAL DE POSTGRAU EN BIOTECNOLOGIA

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Valencia, 2013

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CERTIFICAN: que la presente Tesis Doctoral titulada "Estudio del Metabolismo de los Terpenos en *Lavandula latifolia* Medicus.", presentada para optar al grado de Doctor en Biotecnología, ha sido realizada bajo nuestra dirección por la licenciada en Bioquímica y en Biología ISABEL MENDOZA POUDEREUX

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Agradecimientos

Esta tesis se financió gracias al proyecto (PROMETEO/2009/075) de la Generalitat Valenciana. Además debo agradecer con entusiasmo la concesión de una beca predoctoral de la Fundación Ramón Areces y posteriormente de una beca FPU del extinto Ministerio de Ciencia e Innovación, ahora Ministerio de Educación, Cultura y Deporte, que me permitieron vivir de mi actividad investigadora. Parte de los experimentos de esta tesis se hicieron utilizando las instalaciones del SCIE, en concreto el invernadero.

Thanks to the people at the Technische Universität München, Lehrstuhl für Biochemie in Garching, very specially but not only to Dr Wolfgang Eisenreich, Erika Kutzner and Dr Claudia Huber for their work and help in the labeling section of this dissertation. Especially the RMN work would not be there without your implication. *Vielen Dank!*

Gracias a la Dra. Isabel Arrillaga y al Dr. Juan Segura por su dedicación, cariño y por enseñarme lo que es el trabajo científico. Los años que he pasado entre el laboratorio 3.15 y el 3.25 trabajando y aprendiendo serán siempre una parte importante de mi vida.

Gracias al Dr Jesus Muñoz, sin cuya intervención esta tesis sería seguro otra distinta. En especial por su implicación en la generación de las distintas líneas transgénicas T0 que se han utilizado.

Gracias a Alicia Navarro por su trabajo en la caracterización de descendencias de líneas *LIS* y por ser una gran compañía el tiempo que pasó con nosotros en el laboratorio.

Gracias a todos los compañeros del laboratorio, tanto becarios como profesores, técnicos y colaboradores, por hacer las horas de cada día más fáciles y amenas.

Gracias a mi familia por hacerme saber, que ocurra lo que ocurra, estarán de mi lado, incluso si me equivoco o fallo.

Gracias a mis amigos, a los que están cerca y a los que están muy lejos, por ayudarme a ver más allá del laboratorio cuando sólo podía ver lo que tenía delante.

Gracias a Juanma, por todo.

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Resumen

Resumen

Estudio del metabolismo de los terpenos en *Lavandula latifolia* Medicus

1. Justificación y objetivos

La biosíntesis de los dos precursores universales de los terpenos vegetales, el isopentenildifosfato (IPP) y el dimetilalildifosfato (DMAPP), es un proceso complejo en el que intervienen dos rutas metabólicas independientes (Rodríguez-Concepción y Boronat, 2002; Lange y Ahkami, 2013): La ruta del mevalonato (MVA) que opera en el citosol, retículo endoplasmático y peroxisomas; y la ruta del metil-D-eritritol-4-fosfato (MEP) que opera en los plastos. La primera de ellas es la principal responsable de la biosíntesis de sesquiterpenos y esteroles mientras que la segunda lo es de la producción de monoterpenos, diterpenos y carotenoides (Lichtenthaler, 1999). Esta compartimentalización no es absoluta, dado que metabolitos comunes a ambas rutas pueden ser intercambiados a través de la membrana plastidial (Eisenreich y col., 2004; Bouvier y col., 2005; Lange y Ahkami, 2013).

Los mecanismos que regulan las rutas MVA y MEP no están totalmente dilucidados, aunque en ambos casos parece existir un control a nivel transcripcional de las enzimas clave (McConkey y col., 2000). La ruta MVA está regulada, principalmente, al nivel de la 3-hidroxi-3-metilgluratil-coenzima A (HMG-CoA) reductasa, HMGR (Manzano y col., 2004, Enfissi y col., 2005). De hecho, la actividad HMGR regula tanto el flujo metabólico a través de la ruta MVA como la síntesis de los productos finales (Rodríguez-Concepción, 2006). En contraste, la regulación de la ruta MEP parece ser más compleja, ya que ésta puede ser regulada por varias enzimas, incluyendo la 1-deoxi-D-xilulosa-5-fosfato (DXP) sintasa (DXS), la DXP reductoisomerasa (DXR) y la hidroximetilbutenil 4-difosfato (HMBPP) reductasa (HDR). Esta última enzima es responsable de convertir directamente el HMBPP en IPP y DMAPP en el ultimo paso de la ruta MEP.

Los resultados publicados hasta la fecha demuestran que la enzima DXS regula el flujo metabólico a través de la ruta MEP en varias especies vegetales (Rodríguez-Concepción, 2006), incluyendo el espliego (*Lavandula latifolia* Medicus ; Muñoz-Bertomeu y col., 2006). Así mismo, la actividad de la enzima HDR es limitante para la biosíntesis de isoprenoides en varios organismos, incluyendo bacterias y plantas (Rodríguez-Concepción, 2006). En contraste, el papel regulador de la enzima DXR está sometido a controversia, aunque hay pruebas de que puede limitar la biosíntesis de algunos isoprenoides plastidiales

en algunas plantas (Mahmoud y Croteau, 2001; Carretero-Paulet y col., 2006). Por lo tanto, el flujo metabólico a través de la ruta MEP está controlado por varias enzimas, siendo las principales la DXS y la HDR, que son reguladas tanto a nivel transcripcional como post-transcripcional en respuesta a cambios metabólicos, ambientales y del desarrollo (Rodríguez-Concepción, 2006).

El espliego es un arbusto aromático cuyo aceite esencial, formado mayoritariamente por monoterpenos, es sintetizado y acumulado en tricomas glandulares especializados. Nuestro grupo de investigación ha sobreexpresado, por separado, en espliego los genes *HMG1* y *DXS* de *Arabidopsis*, que codifican respectivamente a las enzimas HMGR y DXS (Muñoz-Bertomeu y col., 2006 y 2007a). En esta misma especie también se ha sobreexpresado el gen de la limoneno sintasa de la menta (*MsLS*), que convierte el geranil difosfato (GPP) en limoneno. Las contribuciones más importante de estas investigaciones previas al conocimiento de la biosíntesis de monoterpenos en espliego son:

a) Las rutas MEP y MVA están reguladas, al menos en parte, a nivel transcripcional ya que la sobreexpresión de los genes *DXS* (ruta MEP) o *HMG1* (ruta MVA) aumenta significativamente la producción de aceites esenciales.

b) El contenido en aceite esencial fue siempre mayor en las plantas de espliego que sobreexpresan el gen *DXS*, sugiriendo que la ruta MEP es la principal donadora de precursores C5 para la síntesis de monoterpenos. No obstante, los resultados con las plantas que sobreexpresan el gen *HMG1* también apoyan la participación de la ruta MVA en la biosíntesis de estos compuestos. De cualquier modo, son necesarias más investigaciones para averiguar si el incremento en la producción de aceite fue el resultado de la inducción de una ruta MVA latente, bloqueada al nivel de la HMGR, o una activación de una ruta MVA ya existente.

c) La sobreexpresión del gen *MsLS* causó alteraciones cuantitativas y cualitativas en el perfil de monoterpenos del aceite esencial, especialmente un incremento de limoneno.

Partiendo de los resultados anteriormente expuestos, los objetivos de esta tesis son:

1) Obtener plantas de espliego transgénicas que sobreexpresen el gen *DXR* de *Arabidopsis*. La caracterización molecular y fenotípica de estas plantas ayudará a clarificar si la enzima DXR, que cataliza el segundo paso de la ruta MEP, controla la producción de aceite esencial en esta especie.

2) Obtener plantas de espliego transgénicas que sobreexpresen el gen de la linalol sintasa (*MsLIS*) de *Clarkia breweri*, que convierte el GPP en linalol. Los aceites esenciales de flores de espliego más apreciados son aquellos con alto contenido en linalol. Por tanto, la generación de plantas de espliego que sobreexpresen el gen de la linalol sintasa puede ser una aproximación válida para aumentar la calidad de su aceite esencial. El linalol está presente únicamente en trazas en el aceite de las hojas, lo que puede facilitar el análisis fenotípico de las plantas transgénicas.

3) Estudiar si la co-expresión de genes que codifican las enzimas reguladoras de las rutas MVA y MEP y de genes que codifican monoterpeno sintasas maximizaría la producción de monoterpenos concretos en el aceite de espliego; esta aproximación sería de interés para producir plantas de espliego con valor añadido. Específicamente se obtendrán las siguientes plantas doble transgénicas: 1) plantas con los genes *HMGR* y *DXS*; 2) plantas con los genes *DXS* y *LIS* (*linalol sintasa*).

4) Dilucidar la posible contribución de las rutas MVA y MEP a la biosíntesis de los monoterpenos en espliego. El estudio se llevará a cabo mediante dos aproximaciones experimentales complementarias: utilizando inhibidores específicos de cada una de las rutas y con experimentos de marcaje con U-^{13}C-glucosa, $^{13}CO_2$ y ^{13}C-mevalonato.

Todo ello ayudará a diseñar nuevas aproximaciones biotecnológicas para mejorar la síntesis de terpenos en espliego.

2. Métodos

2.1 Material Vegetal

El material vegetal inicial utilizado consistió en semillas de espliego (*Lavandula latifolia* Medicus) suministradas por Intersemillas SA (Valencia, España) u obtenidas mediante polinización manual de plantas crecidas en el invernadero. Estas semillas se germinaron *in vitro* para obtener plántulas de las que se usaron discos de hojas (0.5 cm2) y/o tallos (1-2 cm de longitud) como explantos primarios en los experimentos *in vitro*.

La denominación T_0 de las líneas transgénicas se refiere a plantas regeneradas de explantos infectados con *Agrobacterium tumefaciens*. Las plantas T_1 (primera generación) se obtuvieron de semillas producidas por autopolinización o polinización cruzada de plantas T_0.

2. 2. Medios de cultivo y condiciones

El medio basal (BM) utilizado en los experimentos contiene sales y vitaminas MS (Murashige y Skoog, 1962), 3% de sacarosa, 0.8% de agar (Pronadisa) y un pH de 5.7. Los reguladores del crecimiento se añadieron antes del autoclavado (20 min a 120ºC, 10^5 Pa). Todos los antibióticos e inhibidores metabólicos se esterilizaron por filtración y fueron añadidos al medio previamente autoclavado. Si no se indica lo contrario, los cultivos *in vitro* se mantuvieron en cámaras de crecimiento a 25 ± 2 °C con un fotoperiodo de 16 h de luz (60 $\mu mol.m^{-2}.s^{-1}$ irradiancia al nivel del cultivo) proporcionado por tubos de luz blanca fluorescente Sylvania (GTE gro-lux, F36W/ GRO, Erlangen, Alemania).

2. 3. Obtención de plantas transgénicas con los genes *DXR* o *S-Linalol sintasa*

Para introducir los genes *DXR* y *S-linalol sintasa* en espliego se utilizó el cocultivo con *Agrobacterium tumefaciens*.

La cepa C58 de *Agrobacterium tumefaciens* que contenía el plásmido pLBI1DXR10 con el gen *DXR* (AF148852) de *Arabidopsis thaliana* fue proporcionado por el profesor Albert Boronat (Departamento de Bioquímica y Biología Molecular, División III, Facultad de Química, Universidad de Barcelona) (Figura 7 del apartado "Materials and Methods"). Por otra parte, el cADN de *Clarkia breweri* (A.gray) Greene que contiene la secuencia codificante del gen de la S-linalol sintasa fue proporcionado por el Profesor Pichersky (Departamento de Biología Molecular, Celular y del desarrollo, Universidad de Michigan) y recibido en el plásmido pBluescript II SK (+). Antes de ser utilizado para la transformación de plantas de espliego esta secuencia tuvo que ser insertada en el plásmido pBI121, dando lugar al plásmido de 13.3 kb pBILIS (Figura 8 del apartado "Materials and Methods"), y posteriormente introducido en la cepa C58 de *A. tumefaciens.*

2. 4. Transformación genética de *Lavandula latifolia* Medicus

Se siguió el protocolo descrito por Nebauer y col., (2000). Para cada experimento de transformación se utilizaron entre 300 y 500 explantos de hoja.

Las plantas regeneradas se trasplantaron a macetas de 100 ml con una mezcla de turba y perlita (1/1) y tras el proceso de aclimatación fueron transferidas al invernadero.

2. 4. 3. Obtención de progenies de plantas de espliego

Las progenies se obtuvieron por auto polinización o polinización cruzada manual. Se autopolinizaron plantas de las líneas transgénicas DXR y LIS. Las polinizaciones cruzadas se realizaron con líneas de espliego transgénicas para los genes *DXS* (Muñoz-Bertomeu y col., 2006), HMGR (Muñoz-Bertomeu y col., 2007a) y LIS mantenidas en el invernadero. La línea DXS6 se utilizó como donante de polen. Las líneas transgénicas que actuaron como receptoras fueron HMGR1, HMGR4, HMGR3, LIS1, LIS2 y LIS8.

Los frutos maduros se recogieron en Octubre. Después de su aislamiento, se procedió a la germinación *in vitro* de las semillas. Tras 2 meses las plántulas obtenidas se cultivaron en botes con medio ½ BM y se recogieron muestras para los análisis de PCR.

2. 4. 4. Análisis moleculares de las plantas

2. 4. 4. 1. Análisis PCR

Para la extracción de ADN se utilizó el protocolo CTAB descrito por Doyle y Doyle (1990), con ligeras modificaciones. La amplificación de ADN se realizó en volúmenes de 50 µL con 75 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.1 mM de cada dNTP, 0.25 mM de cada primer (ver Tabla 2 del apartado "Materials and Methods"), 50 ng de ADN y 4 U de Taq polimerasa (Biotools, España).

Los parámetros de amplificación de todos los genes fueron: 3 min a 94ºC, seguido de 30 ciclos de 1 min a 94ºC, 2 min a 60ºC y 2 min a 72ºC, y finalmente 7 min a 72ºC.

2. 4. 4. 2. Southern Blot análisis

El análisis Southern Blot se realizó con sondas marcadas con 11-dUTP-digoxigenina.

Para la extracción de ADN se uso el protocolo CTAB descrito por Doyle y Doyle (1990), con ligeras modificaciones partiendo de una muestra de 2 g de hojas.

Las endonucleasas utilizadas fueron *Eco*RI para los genes *DXR, DXS* y *HMG1* y *Bam*HI para el gen *LIS*. La digestión se realizó siguiendo las

instrucciones del fabricante. Las muestras digeridas fueron separadas mediante electroforesis a 60 voltios en TBE 1X con 0.8% agarosa.

Para transferir el ADN a las membranas, el gel se lavó con agua MilliQ, y posteriormente se incubó 40 min en 0.25 M HCl, dos veces durante 30 min en 0.5 M NaOH, 1.5 M NaCl, y dos veces durante 30 min en tampón de neutralización [0.5 M Tris-HCl (pH 8.0) + 1.5 M NaCl]. La transferencia del ADN desde el gel a la membrana de Nylon (Boehringer Mannheim) se consiguió por capilaridad durante 12-16 h usando SSC 20X como conductor. Tras la transferencia, la membrana se secó y el ADN se unió covalentemente usando radiación UV (Biolink BLX); finalmente se lavó en agua MilliQ, se secó y conservó a 4ºC.

La membrana se equilibró en tampón de prehibridación y se incubó a 60ºC en tampón de hibridación con 200 ng de la sonda. Seguidamente, la membrana se lavó, se incubó con el anticuerpo, se equilibró con el tampón de detección y se añadió CSPD (1:100 de CSPD) durante 5 min. Tras este paso se secó e introdujo en una funda de plástico hasta su exposición y revelado.

2. 4. 4. 3. Northern Blot

La expresión de los transgenes se determino por Northern Blot utilizando sondas de ADN marcadas con [α-^{32}P]dCTP.

La extracción del ARN se realizó siguiendo una modificación del protocolo propuesto por Tripure Isolation Reagent (Roche Applied Sciences), partiendo de 0.4 g de hojas.

La electroforesis del ARN se realizó bajo condiciones desnaturalizantes en 2.2 M de formaldehido de acuerdo con Maniatis y col., (1982).

La transferencia y fijación del ARN a una membrana de nylon Hybond-H (Amershan) se llevó a cabo de manera equivalente al realizado para el Southern Blot.

La sonda se marcó con [α-^{32}P]dCTP. por random printing utilizando la subunidad Klenow de la ADN polimerasa. Tras 6 horas de marcaje a temperatura ambiente, la reacción se paró añadiéndole 200 µL of 1X TE; finalmente se desnaturalizó a 95ºC durante 10 min y se mantuvo en hielo hasta su uso.

La hibridación con la sonda se realizó durante 12 horas a 65ºC. Seguidamente se lavó varias veces para eliminar el exceso de sonda y finalmente la membrana se transfirió a una funda de film transparente y se

colocó en una cámara oscura hasta su exposición y revelado.

2. 4. 4. 4. Western Blot

La detección de la proteína codificada por el transgén *DXR* se realizó mediante Western Blot. El anticuerpo policlonal usado fue proporcionado por el Dr Michael H. Walter del Leibniz-Institut für Planzenbiochemie, Alemania.

El extracto de proteínas se obtuvo de una muestra de 1 g de hojas homogeneizada en nitrógeno líquido. El extracto se centrifugó dos veces a 25000 rpm, 30 min y 4ºC y se resuspendió en tampón de extracción con un coctel de inhibidores de proteasas (Sigma, P-9599). La concentración de proteínas se determinó mediante recta patrón utilizando la tinción de Bradford. Finalmente los extractos se diluyeron a una concentración final igual con el tampón de extracción y tampón de carga 5X Laemmli.

La electroforesis tuvo lugar a 50 voltios en un tampón de 1.92 M glicina y 1% SDS a pH 8.3 en un gel doble: el gel empaquetador (125 mM Tris-HCl, pH 6.8, SDS 0.1%, 3.3% acrilamida/bisacrilamida, 0.14% persulfato amónico y 12 mM Temed), y el de separación (375 mM Tris-HCl, pH 8.8, SDS 0.1%, 8% acrilamida/bisacrilamida, 0.066% persulfato amónico y 5.7 mM Temed).

Las proteínas del gel se transfirieron a una membrana Immune-Blot de polifluoruro de vinilideno (PVDF) de BioRad usando el Mini Tran-Blot Cell (BioRad) según instrucciones del fabricante.

La detección inmunológica se realizó mediante el ECL Western Blotting Analysis System kit (Amersham Biosciences) siguiendo las instrucciones del fabricante.

2. 4. 5. Análisis fenotípico

2. 4. 5. 1. Análisis del aceite esencial con hexano como solvente

El análisis de aceite esencial se realizó según lo descrito en Muñoz-Bertomeu y col., (2006 y 2008), tanto en muestras frescas de los distintos verticilos (Figura 9 del apartado “Materials and Methods”) como en muestras secas de los verticilos 4 a 10.

2. 4. 5. 1. Contenido de clorofilas y carotenoides

El análisis del contenido en clorofilas y carotenoides de las hojas se llevó a cabo siguiendo el protocolo descrito en Muñoz-Bertomeu y col., (2006).

2. 5. Contribución de las rutas MVA y MEP a la biosíntesis de monoterpenos en espliego

Para dilucidar la contribución relativa de las rutas MVA y MEP a la biosíntesis de terpenos en espliego se utilizaron dos aproximaciones experimentales:

1) Tratamiento con inhibidores específicos de las rutas. En estos experimentos, explantos aislados de plantas control y transgénicas, crecidas *in vitro* o en invernadero, fueron tratadas con MEV o FSM. También se estudió el efecto del mevalonato sobre la recuperación fenotípica de plantas tratadas con inhibidores.
2) Experimentos de marcaje con $^{13}CO_2$, [U-$^{13}C_6$]glucosa y [1,2-$^{13}C_2$] mevalonato. En estos experimentos se utilizaron plantas crecidas *in vitro* y en invernadero.

2. 5. 1. Efectos de MEV y FSM en espliego

2. 5. 1. 1. Ensayos de germinación

Semillas de espliego, previamente esterilizadas, se cultivaron en tubos de vidrio con 15 ml de medio BM suplementado con MEV (0, 0,5, 1, 2 y 5 μM) o FSM (0, 10, 20, 30 o 40 μM). Para cada tratamiento se utilizaron 48 semillas. Tras 50 días de cultivo, se anotó el porcentaje de germinación, el número de hojas, la longitud de tallos y raíces y el contenido de clorofilas y carotenoides. Para la cuantificación de los pigmentos fotosintéticos se utilizaron 9 plántulas para cada concentración de inhibidor.

2. 5. 1. 2. Ensayos con tallos *in vitro* y *ex vitro*

Para los ensayos *in vitro*, se utilizaron ápices con 3 verticilos (1.5 cm), aislados de plántulas control de 2 meses de edad crecidas *in vitro*. En un primer experimento, los explantos se cultivaron durante 45 días en medio BM suplementado con las mismas concentraciones de MEV y FSM empleadas en los ensayos de germinación. Para cada tratamiento se utilizaron 24 tallos. En un

segundo experimento, se estudió si el MVA, precursor de la biosíntesis de terpenos, revierte el efecto de MEV y FSM. En este experimento, los tallos se cultivaron durante 28 días en medio BM suplementado con concentraciones crecientes de MVA (0; 0,3; 0,6; 1,2; 2,4 y 3,5 mM) sólo o en combinación con 1 μM MEV o 30 μM FSM. En el último experimento, se testó el efecto de MEV y FSM sobre plantas transgénicas de espliego. Se emplearon tallos de la línea HMGR5, que contenía 8 insertos del gen *HMGR* de *Arabidopsis thaliana*. Se cultivaron 24 explantos durante 42 días en medio BM suplementado con 1 μM MEV o 30 μM FSM. Como control se utilizaron ápices de plantas no transformadas.

En todos los experimentos, los cultivos se mantuvieron en la cámara de crecimiento y se determinó la longitud de la raíz y del tallo, el número de verticilos, el peso fresco y seco de la raíz y del tallo, y el contenido en clorofilas y carotenoides. En los experimento con MVA, también se analizó el contenido en aceite esencial, utilizando el hexano como agente extractor.

Los ensayos *ex vitro* se llevaron a cabo con tallos (5 cm de longitud y con 5 verticilos) de las líneas transgénicas de espliego HMGR, DXS, y HMGR-DXS crecidas en el invernadero. Una línea transgénica que sobreexpresa el gen *npt*II se empleó como control. En un primer experimento, los tallos aislados fueron colocados en macetas que contenían una mezcla 1:1 de turba y perlita. Antes de trasplantar, las bases de los tallos se sumergieron en talco que contenía 5000 ppm de IBA para inducir el enraizamiento. Tras un mes, las macetas con tallos enraizados se regados una única vez con la solución nutritiva Hoagland (Hoagland y Arnon, 1950). Al día siguiente las macetas se regaron con una solución acuosa de 1 μM MEV, 30 μM FSM o agua; este tratamiento se repitió cada dos días durante 15 días. Las plantas fueron muestreadas después de otros 15 días. En un segundo experimento, los tallos se regaron 2 veces a la semana durante 2 meses con las soluciones anteriormente indicadas. En ambos experimentos se analizó la longitud de la raíz y del tallo, el número de verticilos, el peso fresco, el peso seco, el contenido de pigmentos fotosintéticos y el aceite esencial. Se utilizaron al menos 10 tallos para cada línea y tratamiento.

2.5.2. Experimentos de marcaje

El cineol y el alcanfor son los monoterpenos mayoritarios en el aceite esencial de las hojas de espliego (Muñoz-Bertomeu y col., 2006). Por esta razón, estos dos compuestos fueron seleccionados para los análisis de NMR y

GC/MS en los experimentos de marcaje. Los precursores utilizados fueron $^{13}CO_2$, [U-$^{13}C_6$]glucosa y [1,2-$^{13}C_2$]mevalonato. Los experimentos fueron realizados con plantas control y la línea transgénica HMGR5, crecidas *in vitro* o en invernadero.

2. 5. 2. 1. Experimentos de marcaje con $^{13}CO_2$

Se utilizaron plantas de espliego control y transgénicas (línea HMGR5). Las plantas control procedían de semillas germinadas *in vitro* y las plántulas obtenidas fueron transferidas a bandejas con una mezcla de turba y perlita (7:3) y mantenidas en el invernadero en Dürnast (Weihenstephan, Technische Universität München, Alemania). Tras un mes, las plántulas se pasaron a macetas (15 cm) con el mismo sustrato y se mantuvieron en el invernadero durante 4 meses. Las plantas de la línea HMGR5 (de 3 meses de edad y aproximadamente 15 cm de altura) procedían de plantas crecidas *in vitro* y aclimatadas a condiciones de invernadero.

Para el marcaje con $^{13}CO_2$, las plantas (crecidas en maceta) se colocaron en una cámara de incubación cerrada (Biobox; GWS, Berlín, Alemania) a 25ºC e iluminada con luz blanca (Figura 10 en el apartado "Materials and Methods"). Antes del periodo de marcaje (fase de pulso), la cámara se llenó con aire sintético que contenía 700 ppm de $^{13}CO_2$. Durante este periodo de pulso la concentración relativa de $^{13}CO_2$ y $^{12}CO_2$ fue de aproximadamente 9:1. Después las plantas se transfirieron al laboratorio y se mantuvieron en las condiciones ambientales imperantes en el mismo. Los tiempos de pulso de cada experimento se presentan en el Apéndice. El patrón de marcaje del cineol y el alcanfor se determinó mediante técnicas de NMR y/o GC/MS.

Los experimento de marcaje con $^{13}CO_2$, así como los análisis de NMR y GC/MS fueron realizados en el laboratorio del Dr. Wolfgang Eisenreich en la the Technische Universität München, Departamento de Química (Garching, München).

2. 5. 2. 2. Experimentos de marcaje con [U-$^{13}C_6$]glucosa

En una primera serie de experimentos, se utilizaron plantas de espliego crecidas *in vitro*, tanto en medio líquido como sólido.

Para los experimentos en medio sólido, plántulas procedentes de semillas germinadas *in vitro* fueron cultivadas en botes de 200 ml (58 mm de diámetro, 92,5 de altura) con 20 ml de medio BM estéril (Sigma-Aldrich) con 30g/L de

sacarosa, 7,5 g/L de agar (Sigma) y 2 g/L de [U-$^{13}C_6$]glucosa y pH 5,7. Se sembraron 4 plántulas por bote y se prepararon un total de 80 botes. Los botes se incubaron en una cámara de crecimiento a 25ºC con un fotoperiodo de 16h. Tras 55 días, las plántulas se recogieron, se congelaron en nitrógeno líquido y se almacenaron a -20ºC hasta su uso.

Para los experimentos en medio líquido, las plántulas se colocaron en matraces de 100 ml (10 por matraz) con 30 ml de medio estéril (Medio BM con 30 g/L de sacarosa, y 2 g/L de [U-$^{13}C_6$]glucosa). Los cultivos se incubaron en una agitador (100 rpm) a 25ºC, con un fotoperiodo de 16 h. Tras 15 días de cultivo, las plántulas se trasfirieron a un nuevo matraz con medio fresco (100 ml) y se mantuvieron en las mismas condiciones durante otros 15 días. Tras 55 días, las plántulas enteras se recogieron, se congelaron en nitrógeno líquido y se almacenaron a -20ºC hasta su uso.

En un segundo experimento, tallos de espliego (1,5 cm de longitud) procedente de plántulas crecidas *in vitro* (control y línea transgénica HMGR5) se cultivaron en botes de 200 ml que contenían medio BM estéril con 30g/L de sacarosa, 7,5 g/L de agar (Sigma) y 2 g/L de [U-$^{13}C_6$]glucosa y pH 5,7. Se prepararon al menos 25 botes por línea con 4 tallos cada uno. Tras 7, 14, 21 y 28 días se anotó la longitud del tallo y número de verticilos. En cada uno de esos periodos se recogieron las plántulas de 5 botes y se almacenaron a -80ºC hasta la extracción del aceite esencial. Previamente se anotó el peso, número de raíces y longitud de la raíz.

2. 5. 2. 2. Experimentos de marcaje con [1,2-$^{13}C_2$]mevalonato

Tallos de 1 cm de longitud, aislados de plántulas procedentes de semillas germinadas *in vitro*, fueron cultivados en botes de 200 ml con 20 ml medio BM estéril con 30 g/L de sacarosa, 7.5 g/L de agar (Sigma) y 2 g/L de [1,2-$^{13}C_2$] mevalonato. Se prepararon al menos 25 botes con 4 tallos cada uno. Tras 7, 14, 21 y 28 días se anotó la longitud del tallo y número de verticilos. En cada uno de esos periodos se recogieron las plántulas de 5 botes y se almacenaron a -80ºC hasta la extracción del aceite esencial. Previamente se anotó el peso, número de raíces y longitud de la raíz.

2. 5. 2. 4. Extracción del aceite esencial

Dependiendo del método analítico (GC/MS o ^{13}C NMR), se utilizaron dos métodos de extracción diferentes.

Para los análisis GC/MS, muestras de entre 100- 200 mg se introdujeron en tubos de vidrio de 10 ml con 2 ml de cloroformo-d ($CDCl_3$). Después de una suave agitación, los tubos se mantuvieron a temperatura ambiente durante 15 min, se añadió una punta de sulfato de sodio anhidro y se dejo reposar una hora. Finalmente, 1 ml del extracto en cloroformo se traspasó a un vial de 1,5 ml para su medida en el GC/MS.

Para los análisis ^{13}C NMR, el material vegetal (600-1000 mg) se separó en 3 tubos de vidrio. Se añadieron 2 ml de cloroformo deuterado ($CDCl_3$) al primer tubo, se agitó suavemente y se mantuvo a temperatura ambiente durante 15 min. Después, el extracto clorofórmico se transfirió al segundo tubo y se repitió el proceso, transfiriéndose el extracto al tercer tubo. Seguidamente, se añadió una punta de sulfato de sodio anhidro y se dejó reposar 1 hora. Finalmente 0,6 ml del extracto clorofórmico se traspasaron a un tubo de NMR para los análisis de ^{1}H y ^{13}C.

2. 5. 2. 5. Medidas GC/MS

El cromatografo de gases (GC-17A y GC-2010), espectrómetro de masas (QP-5000 y GCMS-QP 2010 Plus), auto-inyector (AOC-20i) y software (Class 5000 y GCMSsolution) utilizados para estas medidas fueron adquiridos a Shimadzu (Duisburg, Alemania). Se empleó una columna capilar de sílice Equity TM-5 (30 m x 0,25 mm x 0,25 μm de grueso) de Supelco Inc. (Bellefonte, PA, USA). La temperatura del inyector y de la interface fueron de 230ºC y 250ºC respectivamente. Los ajustes de temperatura del horno fueron: 70ºC por 2 min, una rampa de 70-90ºC de 2ºC/min, 90-130 ºC con 5ºC/min y finalmente 250ºC por un min. EL programa de presión empezó a 76,1kPa con una velocidad lineal de 40,0 cm/sec. El control de flujo utilizado fue el de velocidad lineal. El flujo total fue de 16,1 ml/min mientras que el flujo de la columna permaneció a 1,19 ml/min. El ratio del Split fue de 1:10. Los voltios del detector fueron de aproximadamente 0,8 keV. El solvent cut se ajustó a 4 min y la velocidad de sampling fue de 0,15 sec. La anchura del escaneo se fijó en 0,1 u.

Cada muestra se analizó tres veces en modo SIM (monitorización de un único ión). Las intensidades relativas de los estándares (alcanfor y cineol) y las muestras obtenidas del análisis GC/MS (integración de picos) se procesaron siguiendo publicaciones previas (Lee y col., 1999; Pickup y McPherson 1976; Braumann 1966; y Korzekwa, 1990). Esta evaluación resulta en el exceso molar de los isotopólogos de carbono de cineol y alcanfor únicamente debidos al

enriquecimiento producido por el precursor ^{13}C.

2. 5. 2. 6. Medidas NMR

Para los espectros de ^{1}H se usó un Avance I 500 (UltraShield 500 MHz, SEI 500 S2 (5 mm, inverso con gradiente Z), Autosampler B-ACS 60) y un software TopSpin 2.1, de Bruker Instruments (Karlsruhe, Alemania).

Para los espectros 3C se utilizaron un Avance DXR 500 (Cryomagnet BZH 500 MHz, Autosampler B-ACS 60), o un Advance III 500 system con imán UltraShield PLUS 500 MHz y una cabeza de congelación para muestras (5 mm CPQNP, $^{1}H/^{13}C/^{31}P/^{19}F/\ ^{29}Si$ (Z-gradient), Autosampler B-ACS 120) de Bruker Instruments. El software instalado fue XwinNMR 3.1 y TopSpin 3.0, respectivamente (ambos de Bruker Instruments).

Las medidas se realizaron en campos magnéticos de 11,5 Tesla. Las frecuencias de resonancia de ^{1}H y ^{13}C fueron de 500,13 MHz y 125,77 MHz respectivamente y la temperatura fue de 300 ºK. El análisis de los datos se realizó con el software de MestReNova Software (Mestrelab Research, Santiago de Compostela, España), TOPSPIN o XWIN NMR.

Los espectros unidimensionales de ^{1}H y ^{13}C y los de dos dimensiones COSY, HSQC, HSQC-DEPTeditado, HSQC-TOCSY, NOESY (con 1 sec de mezcla), TOCSY (con 60 ms de mezclado) y HMBC fueron medidos siguiendo los sets de parámetros estándar de Brucker.

2. 6. Análisis estadístico

La significación de la variación en los parámetros fenotípicos entre los grupos control y los transgénicos, así como de los efectos de los distintos tratamientos se determinó mediante análisis de la varianza (ANOVA, SPSS 19 versión for Windows, SPSS Inc.). Las diferencias significativas entre medias se determinaron usando el test de Tukey (1953). Así mismo, y cuando se consideró apropiado se calcularon las desviaciones estándar (SD) y los errores estándar de la media (SE). La herencia de los transgenes se analizó mediante un análisis de chi-cuadrado con corrección de Yates (Zar, 1996).

3. Conclusiones

3.1 Sobreexpresión del gen *DXR* en plantas transgénicas de *Lavandula latifolia*

- Se han obtenido plantas transgénicas (T_0) de espliego que sobreexpresan el gen *DXR* de *Arabidopsis thaliana* que codifica la segunda enzima de la ruta MEP. Así mismo y por autopolinización controlada de las líneas T_0, que florecieron tras varios años en el invernadero, se han obtenido progenies T_1 de dos de las líneas.

- Una de las líneas transgénicas T_0 (línea DXR2) acumula significativamente más aceite esencial que los controles en las hojas más jóvenes. No obstante, el incremento en aceite esencial es menor que el observado en la línea DXS6, obtenida anteriormente en nuestro laboratorio, y que sobreexpresa el gen *DXS*, que codifica la primera enzima de la ruta MEP.

- El contenido en clorofila total y carotenoides en las plantas T_0 aumenta significativamente en las hojas más jóvenes en todas las líneas transgénicas en comparación a los controles, excepto en la línea DXR4 que no muestra diferencias y en la línea DXR5 que es menor.

- Todas las plantas transgénicas presentan un fenotipo visual idéntico al de los controles en términos de morfología, a excepción de la línea DXR1, que presenta características juveniles.

- Nuestros resultados sugieren que en el espliego la enzima DXR juega un papel menos relevante que la enzima DXS en el control de la biosíntesis de precursores de los monoterpenos a través de la ruta MEP.

3. 2 Sobreexpresión del gen *LIS* en plantas transgénicas de *Lavandula latifolia*

- Se han obtenido plantas transgénicas (T_0) de espliego que sobreexpresan el gen *LIS* de *Clarkia breweri* que codifica la enzima linalol sintasa, responsable de la formación de linalol. Así mismo, y por autopolinización controlada de las líneas T_0, que florecieron tras varios años en el invernadero, se han obtenido progenies T_1 de varias de dichas líneas.

- Los análisis de hojas de las plantas T_0 recogidas en distintos estados de desarrollo muestran que las hojas más jóvenes, tanto de plantas transgénicas como control, acumulan más linalol que las hojas maduras. Además, en las plantas transgénicas, estas hojas acumulan más linalol que las plantas control, lo que se correlaciona con los mayores niveles de transcritos del gen *LIS*.

- El fenotipo de contenido elevado en linalol observado en las hojas más jóvenes, se mantiene en las progenies T_1 que heredan el transgén *LIS*.

3. 3 Sobreexpresión simultanea de genes que codifican enzimas de la ruta de biosíntesis de terpenos en plantas transgénicas *Lavandula latifolia*

- La polinización cruzada de plantas transgénicas de espliego ha permitido la obtención de doble transgénicas que co-expresan los genes *DXS*-*HMGR* y *DXS*-*LIS*.

- Las plantas doble transgénicas *DXS*-*LIS* tienen un contenido inferior de aceite esencial que la planta T_0 DXS6 de la que descienden. El contenido del monoterpeno linalol es también inferior, posiblemente debido a efectos de co-supresión ligados a las estructuras de las construcciones utilizadas.

3. 4 Contribución de las rutas MVA y MEP a la síntesis de monoterpenos en espliego

4.

- La mevinolina (1 µM) afecta negativamente a la germinación y reduce el desarrollo de las plántulas de espliego. Por el contrario, no altera el contenido en pigmentos fotosintéticos y aceite esencial en hojas maduras.

- La fosmidomicina (30 µM) no reduce la germinación pero afecta negativamente el desarrollo de la parte aérea de las plantas de espliego. Además, disminuye significativamente el contenido de pigmentos fotosintéticos y aceite esencial, especialmente en las hojas más jóvenes.

- Concentraciones elevadas de mevalonato (3.5 mM) inhiben el desarrollo de tallos y raíces de espliego. Estas concentraciones también disminuyen el contenido en aceite esencial aunque incrementan el de pigmentos fotosintéticos en hojas jóvenes. Dependiendo de la concentración empleada, el mevalonato atenúa los efectos tóxicos de la fosmidomicina o revierte los de la mevinolina.

- Los tallos de las plantas transgénicas línea HMGR5 y dobles transgénicas DXS-HMGR presentan mayor tolerancia que los controles al tratamiento con 30 µM de fosmidomicina, especialmente en relación a su contenido en pigmentos fotosintéticos.

- El enriquecimiento del medio de cultivo con ^{13}C-mevalonato no incrementa el porcentaje en ^{13}C de los monoterpenos cineol y alcanfor, aunque promueve un aumento del contenido de estos dos monoterpenos, lo que sugiere una activación de la ruta MEP a otro nivel.

- El marcaje con [U-$^{13}C_6$]-Glucosa en plantas crecidas durante 55 días *in vitro* en medio sólido, produce porcentajes de exceso de abundancia de ^{13}C de 4,8 a 6,5 y de 5,3 a 6,1 para cineol y alcanfor, respectivamente. Los ratios M+2/M+3 y M+2/[(M+2) + (M+3)] obtenidos son superiores a los esperados si la síntesis de cineol y alcanfor fuera realizada exclusivamente a través de la ruta MEP. Después de 28 días de cultivo *in vitro* en medio sólido, el marcaje en tallos cultivados con [U-$^{13}C_6$]-Glucosa produce porcentajes de exceso de abundancia

de ^{13}C de 1,10 y 1,35 para cineol y alcanfor respectivamente en los controles, y de 1,53 y 1,63 para cineol y alcanfor, respectivamente, en la línea transgénica HMGR5. Los ratios M+2/M+3 y M+2/[(M+2) + (M+3)] de la línea HMGR5 fueron superiores a los de la línea control para ambos monoterpenos. Estos datos apoyan la existencia de una interconexión entre las rutas MEP y MVA, sugiriendo que los precursores derivados de la ruta MVA contribuyen a la síntesis de alcanfor y 1,8-cineol en *Lavandula latifolia*.

- El marcaje con [U-$^{13}C_6$]-Glucosa en plántulas cultivadas durante 30 días en medio liquido produce porcentajes de exceso de abundancia de ^{13}C de 10,6 y de 13,3 para cineol y alcanfor respectivamente. Los ratios M+2/M+3 y M+2/[(M +2) + (M+3)] obtenidos son exactamente los esperados en el caso de que la síntesis de cineol y alcanfor fuera realizada exclusivamente a través de la ruta MEP, lo que indica que en estas condiciones la síntesis de monoterpenos se realiza exclusivamente a través de esta ruta.

- El periodo óptimo de marcaje con $^{13}CO_2$ para conseguir incorporaciones significativas de ^{13}C en cineol y alcanfor en plantas de *Lavandula latifolia* debe ser superior a 5 horas. Así mismo, la incorporación de ^{13}C es genotipo dependiente, existiendo grandes diferencias de incorporación entre distintas plantas en las mismas condiciones. Los ratios M+2/M+3 y M+2/[(M+2) + (M +3)] obtenidos en plantas control son muy parecidos a los esperados en el caso de que la síntesis de cineol y alcanfor fuera realizada exclusivamente a través de la ruta MEP, lo que apunta a una contribución muy pequeña de la ruta MVA en la biosíntesis de estos monoterpenos en plantas cultivadas *ex vitro*.

- El análisis por RMN del aceite esencial de las hojas de plantas control de *Lavandula latifolia* confirma que sus componentes mayoritarios, los monoterpenos cineol y alcanfor, se sintetizan principalmente a partir de precursores procedentes de la ruta MEP. No obstante, el marcaje con $^{13}CO_2$ de plantas transgénica HMGR5 producen ratios M+2/M+3 y M+2/[(M+2) + (M +3)] para cineol y alcanfor superiores a los de la línea control en un tercio de las plantas estudiadas. Estos datos apoyan la existencia de una interconexión entre las vías MEP y MVA en *Lavandula latifolia*.

Introduction

I. INTRODUCTION

I. 1. Justification and objectives

The biosynthesis of the universal five-carbon precursors of plant terpenes, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), is a complex process involving two metabolic pathways (Rodríguez-Concepción and Boronat, 2002; Lange and Ahkami, 2013). These metabolic pathways are separately localized; the mevalonate (MVA) pathway operates in the cytosol, endoplasmic reticulum, and peroxisomes, and the methyl-D-eritritol-4-phosphate (MEP) pathway, operates in plastids. The former pathway is predominantly responsible for the biosynthesis of sesquiterpenes and sterols, whereas the latter involves the biosynthesis of monoterpenes, diterpenes and carotenoids (Lichtenthaler, 1999). This compartmentalized separation is not, however, absolute since metabolites common to both MVA and MEP pathways can be exchanged in any direction through the plastidial membranes (Eisenreich et al., 2004; Bouvier et al., 2005; Lange and Ahkami, 2013). Therefore, the relative contribution of each pathway to the biosynthesis of the different classes of isoprenoids remains unclear. By understanding this process, the biotechnological manipulation of the terpene synthesis would be much more efficient and fruitful.

Mechanisms regulating MVA and MEP pathways are far from being understood, although in both seems to operate a transcriptional control of the key enzymes (McConkey et al., 2000). The MVA pathway is primarily regulated at the level of the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, HMGR (Manzano et al., 2004; Enfissi et al., 2005), and it is known that HMGR activity regulates the metabolic flux through the MVA pathway and the eventual production of the isoprenoid end-products (Rodríguez-Concepción, 2006). In contrast, the scenario seems to be more complex in the case of the MEP pathway. In fact, the MEP pathway can be regulated by several enzymes, including 1-deoxi-D-xilulose 5-phosphate (DXP) synthase (DXS), DXP reductoisomerase (DXR), and hydroxymethylbutenyl 4-diphosphate (HMBPP)

reductase (HDR). The HDR enzyme directly converts HMBPP into IPP and DMAPP in the last step of the MEP pathway.

The results reported to date support a regulatory role for the DXS enzyme in controlling flux through the MEP pathway in plants (Rodríguez-Concepción, 2006), including *Lavandula latifolia* Medicus (spike lavender; Muñoz-Bertomeu et al., 2006). The role of DXR in the regulation of MEP pathway still remains unclear, although it might limit the biosynthesis of at least some plastidial isoprenoids in at least some plants (Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2006). In relation to the HDR, the activity of this enzyme has been demonstrated to be limiting for isoprenoid biosynthesis in several organisms, including bacteria and plants (Rodríguez-Concepción, 2006). In summary, the metabolic flux through the MEP pathway appears to be controlled by several enzymes (with a major contribution of DXS and HDR) regulated at transcriptional and post-transcriptional levels in response to metabolic (feedback regulation), environmental, and developmental cues (Rodríguez-Concepción, 2006).

Spike lavender is an aromatic plant producing essential oils, whose components (mostly monoterpenes) are synthesized and accumulated in specialized glandular trichomes. Our research group has overexpressed separately the *Arabidopsis HMG1* and *DXS* genes, encoding the respective HMGR and DXS enzymes, in spike lavender (Muñoz-Bertomeu et al., 2006 and 2007a). Also, our group was able to overexpress, in the same species, the spearmint limonene synthase gene (*MsLS*), which converts geranyl diphosphate (GPP) into limonene. The main contributions of this research to the knowledge of monoterpene biosynthesis in spike lavender are:

a) MEP and MVA pathways are regulated, at least in part, at the transcriptional level since the overexpression of *DXS* (MEP pathway) or *HMG1* (MVA pathway) significantly increases the production of essential oils.

b) Although essential oil yield was always higher in those transgenic spike lavender plants overexpressing the *DXS* gene, which suggest that the MEP pathway is the principal donor of C5 precursors for monoterpene biosynthesis, our results also support the involvement of the MVA pathway in

the biosynthesis of these compounds. Further investigation is needed, however, to know whether the increased essential oil yield resulted from either the induction of a latent MVA pathway blocked at HMGR or an up-regulation of an existing MVA pathway.

c) Overexpression of the *MsLS* gene caused quantitative and qualitative alterations in monoterpene profiles, particularly increased amounts of limonene.

On the basis of the results above mentioned, the objectives of this Thesis are:

1) To obtain transgenic spike lavender plants overexpressing the *Arabidopsis DXR* gene. Molecular and phenotypic characterization of these transgenic spike lavender plants should help to clarify whether the DXR enzyme, which catalyzes the second step of the MEP pathway, controls the essential oil yield in the species.

2) To obtain transgenic spike lavender plants overexpressing the *Clarkia breweri* linalool synthase (LIS) enzyme that converts GPP into linalool. The most appreciated flower spike lavender oils are those with a high content in linalool. Then, the generation of transgenic spike lavender plants overexpressing the *LIS* gene could be a valid approach to increase the quality of their essential oil. Linalool is present in trace amounts in the oil from leaves, which could facilitate the phenotypic analysis of transgenic plants.

3) To test whether the co-expression of genes encoding both regulatory enzymes of the MVA or MEP pathways and monoterpene synthase genes would maximize the levels of particular monoterpenes in spike lavender oil; this approach would be of interest to produce spike lavender plants of increased value. Specifically the following double-transgenic spike lavender plants will be obtained: 1) plants containing the *HMGR* and *DXS* genes; and 2) plants containing the *DXS* and *LIS* genes.

4) To elucidate the contribution of MVA and MEP pathways to monoterpene biosynthesis in spike lavender. The study will be undertaken by using two complementary approaches: specific inhibitors of both pathway and labeling experiments with U-^{13}C-glucose, $^{13}CO_2$ and ^{13}C-mevalonate.

All this will eventually help to design new biotechnological approaches to improve terpene synthesis in spike lavender.

I. 2. Terpene biosynthesis pathways in plants

The terpenes, or isoprenoids, are the largest and most diverse family of natural products. Within these compounds both primary metabolites, necessary for the well functioning and maintenance of the living organisms, and secondary metabolites, that are not directly involve in growth and/or development, can be found (Enfissi et al., 2005). Among the primary terpene metabolites are several hormones (gibberellins, cytokinins, abscisic acid, brassinosteroids and strigolactones), carotenoids, chlorophylls (phytol tail), and sterols, that all perform basic functions such as modulation of the development, light absorption, photo-protection, membrane fluidity and permeability control. The secondary terpene metabolites are involved in defense against herbivores and pathogens, allelopathic interactions and pollination (Dudareva et al., 2004; Baldwin at al., 2006; Tholl, 2006). They also have considerable commercial importance due to their uses in food, perfume, cosmetic and pharmaceutical industries (Verlet, 1993). Moreover, as the largest group of natural products, terpenoids provide a rich pool for exploring drugs and lead compounds (Cheng et al., 2007).

All terpenes originate from the universal C5 precursor IPP and its isomer DMAPP. The diverse types of terpenes are formed through condensation of additional IPP units by prenyl transferases; thus, monoterpenes are produced from geranyl diphosphate (GPP, C10), sesquiterpenes from farnesyl diphosphate (FPP, C15) and diterpenes from geranylgeranyl diphosphate (GGPP, C20). The larger isoprenoids are formed by condensation of these intermediates; thus, sterols come from the triterpene squalene (C30), which contains 6 C5 units achieved by condensation of two FPP molecules while carotenoids (C40) are formed through condensation of two GGPP molecules, achieved by condensation of 8 C5 units (Figure 1). Once the acyclic isoprenoids (GPP, FPP or GGPP) are formed, terpene synthases will catalyze the formation of the different groups of terpenes. Finally, a series of additional transformations

(oxidations, reductions and conjugations) are responsible for the great diversity of terpene compounds (Mahmoud and Croteau, 2002).

In plants, the IPP and DMAPP assembly is achieved through two pathways (Liu et al., 2005): the cytosolic MVA pathway, beginning with the condensation of acetyl-CoA, and the plastidial-located MEP pathway, starting with the reaction between pyruvate and glyceraldehyde-3-phosphate (G3P; Figure 2). However, some enzymes from the MVA pathway were shown, either *in vivo* or predicted *in vitro,* to reside in other locations such as the peroxisome, vacuole, plasma membrane, extracellular space, or nucleus (Vranová et al., 2012). For example, MVA-derived IPP is transported to the mitochondria for the biosynthesis of ubiquinone (Disch et al., 1998). Each one of these routes involves the activity of a series of enzymes that represent checkpoints for the biosynthesis of isoprenoids.

The enzyme HMGR catalyzes the first reaction of the seven-enzymatic steps of the MVA pathway (the irreversible conversion of HMG-CoA into mevalonate that is subsequently converted into IPP). It is accepted that HMGR activity regulates the flux through the MVA pathway and the subsequent production of the terpene end products. Additionally, onset of necrosis appears in a developmentally regulated manner that correlates with the decline of endogenous HMGR activity, thus indicating that this enzyme might be involved in necrosis avoidance (Manzano et al., 2004). The importance of HMGR is further emphasized by the existence of highly specific natural inhibitors (Alberts et al., 1980), which are explained more in depth in further paragraphs. Plant HMGRs are encoded by a small family of genes that are differentially expressed upon various internal and external stimuli; the transcriptional and post-transcriptional regulation of HMGRs has been studied (Dale et al., 1995; Learned, 1996; Newman and Chappell, 1997). The complete blockage of the MVA pathway in *Arabidopsis* results in male gametophyte lethality (Suzuki et al., 2009).

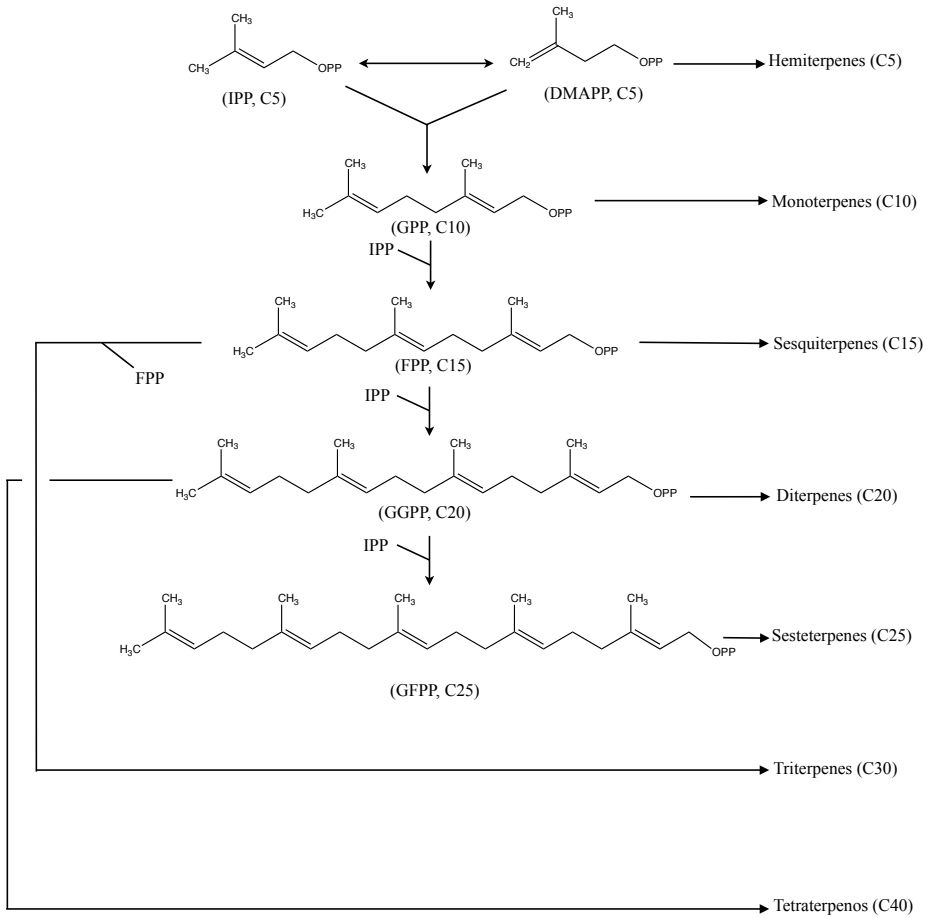


Figure 1: General scheme of the biosynthesis of terpenes. DMAPP: dimethylalyl diphosphate; FPP farnesyl diphosphate; GFPP, geranylfarnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate and IPP, isopentenyl diphosphate. Adapted from Dewick (2002).

The biosynthesis of IPP and DMAPP from acetyl-CoA involves seven different enzymes that are encoded in *Arabidopsis* by 12 different genes (Suzuki and Muranaka, 2007). Among these, *HMG1* and *HMG2*, encoding for two isoforms of the HMGR (Suzuki et al., 2004), as well as *IPI1* and *IPI2*, encoding for the IPP isomerase (Okada et al., 2008; Phillips et al., 2008; Sapir-Mir et al., 2008) have been analysed genetically. The IPI1 enzyme has been found in multiple subcellular locations: the cytosol (Okada et al., 2008), the plastid (Phillips et al., 2008) or the peroxisome (Sapir-Mir et al., 2008), and IPI2 appears in the three cited reports to be localized in the mitochondria.

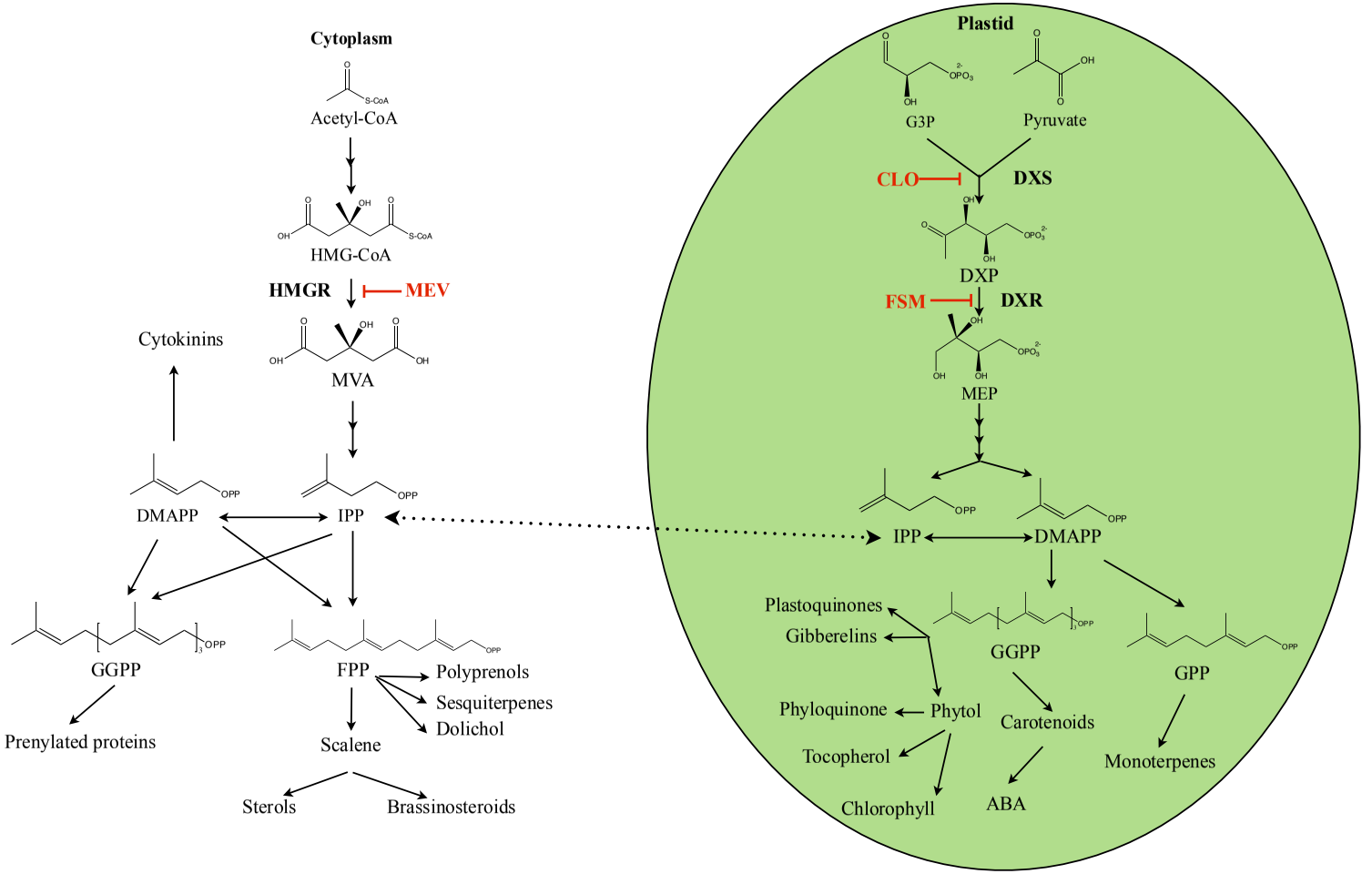


Figure 2. General scheme of the terpene synthesis pathways in plants and their inhibitors. ABA: abscisic acid. CLO: clomazone. DMAPP: isomer dimethylallyl diphosphate. DXP: 1-deoxi-D-xilulose 5-phosphate. DXR: DXP reductoisomerase. DXS: DXP synthase. FPP: farnesyl diphosphate. FSM: fosmidomycin .GGPP: geranylgeranyl diphosphate. G3P: D-glyceraldehyde-3 phosphate. HMG-CoA: 3-hydroxi-3-methylglutaryl-coenzyme A. HMGR: HMG-CoA reductase. IPP: isopentenyl diphosphate. MEP: methyl-D-eritritol-4-phosphate. MEV: mevinoline. MVA: mevalonate.

It is commonly accepted that HMGR activity regulates the metabolic flux through the MVA pathway and the eventual production of isoprenoid end-products (Rodríguez-Concepción, 2006; Tang et al., 2010).

The enzymes DXS and DXR catalyze the first and second steps, respectively, of the MEP pathway. DXS catalyzes the formation of DXP from piruvate and D-glyceraldehyde- 3 phosphate (G3P), whereas DXR converts DXP into MEP, which is considered the first committed precursor of plastid isoprenoids. After the conversion of MEP into methylerythritol 2,4-cyclodiphosphate, in three enzymatic steps catalyzed by CMS (4-diphosphocytidyl-methylerythritol synthase), CMK (4-diphosphocytidyl-methylerythritol kinase) and MCS (methylerythritol 2,4-cyclodi- phosphate synthase) enzymes, a reduction catalyzed by hydroxymethylbutenyl diphosphate (HMBPP) synthase (HDS) produces HMBPP, which is finally converted into a mixture of IPP and DMAPP by the enzyme HDR (Rodríguez-Concepción, 2006).

Since the identification of the first two enzymes of the MEP pathway (DXS and DXR), they both were proposed to be potential control points (Carretero-Paulet et al., 2006). Work in bacteria showed that isoprenoid biosynthesis was limited in this organism by the activity of DXS but not DXR (Harker and Bramley, 1999; Kuzuyama et al., 2000; Miller et al., 2000). The role of these enzymes in plants has been often studied using model systems, where an increased production of plastid isoprenoids in response to external or age-related signals was desired. Transgenic DXS *A. thaliana* and tomato plants confirmed the regulatory role of this enzyme in controlling the flux through the MEP pathway (Estévez et al., 2001; Enfissi et al., 2005). Expression pattern analysis during accumulation of different plastidial terpene products implied that increased *DXS* levels might be required to supply C5 units in several plants models, including *Arabidopsis thaliana* (Mandel et al., 1996; Estévez et al., 2000; Botella-Pavía et al., 2004), *Capsicum annuum* (Bouvier et al., 1998*)*, *Mentha* x *piperita* (Lange et al., 1998), *Catharanthus roseus* (Chaded et al., 2000; Veau et al., 2000; Burlat et al., 2004); *Solanum lycopersicum* (Lois et al., 2000) and the mycorrhizal fungi *Glomus intraradices* (Walter et al., 2000; Walter et al., 2002*)*. In the case of DXR, a direct correlation between plastid

terpene biosynthesis and transcript accumulation has been detected in some plant systems, such as *Glomus intraradices* (Walter et al., 2000), *Catharanthus roseus* (Veau et al., 2000), *Arabidopsis thaliana* (Carretero-Paulet et al., 2002; Hsieh and Goodman 2005), arbuscule-containing cells of maize (Hans et al., 2004), *Populus* x *canescens* (Mayrhofer et al., 2005) and *Medicago truncatula* (Bede et al., 2006). In contrast, no direct correlation has also been observed in other species, such as *Antirrhinum majus* (Dudareva et al., 2005). Overexpression of *DXR* gene in peppermint (*Mentha* x *piperita*) led to increased monoterpene levels in leucoplasts of the non-photosynthetic secretory cells of glandular trichomes (Mahmoud and Croteau, 2001). Still, it is possible that the MEP production might also limit the biosynthesis of other terpene in other plastid types. Modified DXR activity levels in transgenic *Arabidopsis* plants demonstrated that together with other enzymes of the MEP pathway, DXR controls flux to IPP and DMAPP in photosynthetic tissues (Carretero-Paulet et al., 2006) in this model plant.

The regulation of plant terpenoid biosynthesis is a complex matter that can be divided into two categories: the spatial and the temporal, the temporal one mainly due to light cycles (Dudareva et al., 2004). Formation of volatile compounds is spatially regulated and therefore specially adapted structures have evolved, like oil glands, glandular trichomes or resin ducts (Phillips and Croteau, 1999; Gershenzon et al., 2000; Miller et al., 2005), where large amounts or isoprenoids accumulate.

Generally, it is assumed that the MVA pathway is responsible of providing precursors for the synthesis of sesquiterpenes and triterpenes, while the MEP pathway donates precursors for monoterpenes, diterpenes and tetraterpenes (Rodríguez-Concepción and Boronat, 2002). However, experiments with labeled products and/or treatments with specific inhibitors (mevinoline and fosmidomycin that respectively block the MVA and MEP routes) illustrate that the compartmental division between the two pathways is not complete since common metabolites from both pathways can be exchanged, in both directions, through the plastidial membrane (Schuhr el al., 2003; Bouvier el al., 2005). Because of this, the relative contribution of the MVA and MEP pathways for the biosynthesis of plant terpenes remains uncertain. It is

assumed, however, that monoterpenes are primarily synthesized in the plastids via the MEP pathway-derived IPP and DMAPP (Mahmoud and Croteau, 2002).

Monoterpenes are crucial for many biological activities of plants, including defense against herbivores and pathogens, allelopathic interactions and pollination (Langenheim, 1994; Pichersky and Gershenzon, 2002; Baldwin et al., 2006; Dudareva et al., 2006; Tholl, 2006). Also, they have a high economic value due to their use in the agroalimentary (as flavorings), perfume and cosmetic (for their fragrance) and pharmaceutical industries (for their antimicrobial activities) (Verlet, 1993; Lubbe and Verpoorte, 2011). Also, essential oils are widely used for aromatherapy and other alternative healthcare products, and some are even used as insect repellents and as detergents (Lubbe and Verpoorte, 2011). As a consequence of consumer demand and the wide range of uses of monoterpenes, the global trade in essential oils is expected to expand during the early 21st century. Thus, spike lavender oil production alone has been estimated to be between 50-100 tonnes per year (Sangwan et al., 2001, Lubbe and Verpoorte, 2011). All these facts made metabolic engineering of terpenes an interesting field of research, by improving the quantity and/or quality of essential oils.

With few exceptions, monoterpene biosynthesis can be divided into four phases (Mahmoud and Croteau, 2002; Dudareva et al., 2004): (1) construction of the basic C5 units, IPP and DMAPP; (2) condensation of IPP and DMAPP by prenyltransferases producing geranyl diphosphate (GPP; C10); (3) conversion of GPP to the parent structure of the various monoterpene subfamilies, catalyzed by monoterpene synthases; for this conversion, GPP is first ionized and isomerized to produce linalyl diphosphate (LDP), which either produces the acyclic monoterpenes or the α-terpinyl cation, the universal intermediate for cyclic monoterpenes (Bohlmann et al., 1998; Dewick, 2002); and (4) transformation of the parent structures to various derivatives. This mechanism was elucidated largely by Croteau and co-workers by studies with substrate analogs, inhibitors, intermediates and analogs and native enzymes (Croteau, 1987; Wise and Croteau, 1999).

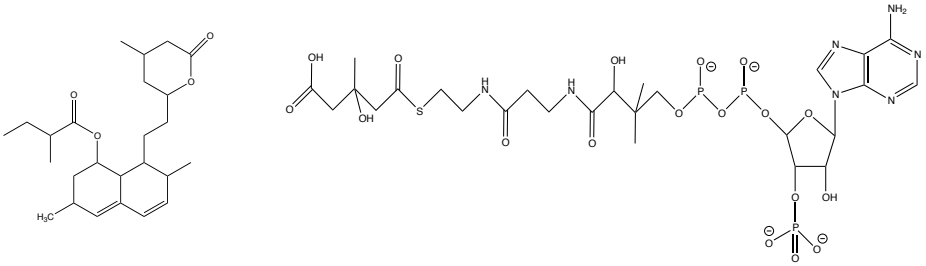
Theoretically, all four steps leading to the biosynthesis of monoterpenes can be engineered in order to increase yield and/or modify the essential oil

profile. Thus, the manipulation of the steps involved in construction of the basic C5 units resulted in significant increases in essential oils of peppermint (Mahmoud and Croteau, 2001) and spike lavender (Muñoz-Bertomeu et al., 2006), without change in monoterpene composition when compared with control plants. There are also examples of the production of transgenic plants overexpressing monoterpene synthases, a key control point in the biosynthesis of monoterpenes, like in the case of peppermint (Mahmoud and Croteau, 2002), spike lavender (Muñoz-Bertomeu et al., 2008) and *Eucalyptus camaldulensis* (Ohara et al., 2010). This approach has been also successfully undertaken in plants and/or organs that do not naturally produce these compounds, such as *Arabidopsis* and tobacco (Degenhardt et al., 2003; Aharoni et al., 2005; Wu et al., 2006). Nevertheless, approaches other than overexpression from the above mentioned enzymes may also be useful. Thus, monoterpene engineering could also be potentially achieved by using trichome cell-specific promoters due the cell-type-specific expression of transgenes in essential oil plants. Another option would be to modulate the expression of genes encoding regulatory proteins that control the development of glandular trichomes (Lange and Ahkami, 2013).

The compartmentalization of terpene synthesis is not only restricted to the cytosol for the MVA pathway and plastid for the MEP pathway. It is also possible that different plastids are being responsible for the synthesis of different terpenes (Lange and Turner, 2013). The plastids of the secretory cells of plant glands are often non-pigmented, in contrast to the chloroplast of adjacent mesophyll cells. The presence of amoeboid, non-pigmented plastids, called leucoplast, lacking thylakoid membranes and with few ribosomes, appears to correlate strongly with monoterpenes occurrence in the resulting essential oil (Cardé, 1984; Cheniclet and Cardé, 1985). Some plants, however, also possess chloroplast in their glandular cells (Cheniclet and Cardé, 1985; Nielsen et al., 1991; Duke and Paul, 1993; Göpfert et al., 2005; Heinrich et al., 2010; Cui et al., 2011). Nevertheless, Cheniclet and Cardé (1985) conducted a correlative study with 45 different plants species and proved a strong correlation between the number and volume of leucoplasts in gland cells and the quantity of monoterpenes in the essential oil produced.

I. 3. Use of inhibitors to study the terpene biosynthesis pathways

The availability of compounds that disturb the metabolic routes is a powerful tool that enables the study of metabolism in all living organisms (Damaj et al., 2006; Vercauteren et al., 2009; Böttcher et al., 2012; Kavitha et al., 2012). To study the flux and importance of each of the terpene synthesis pathways, several inhibitors are available. In the case of the MVA pathway the inhibitors known belong to the statin (e. g. mevinoline, MEV) group (Figure 3), while the inhibitors used to study the MEP pathway are the ones that work at the DXS (clomazone, CLM) or at the DXR enzyme level (fosmidomycin, FSM) (Figure 4).



Mevinoline 3-hydroxy-3-methylglutaryl-coenzyme A

Figure 3. Molecular structure of mevinoline and 3-hydroxy-3-methylglutaryl-coenzyme A.

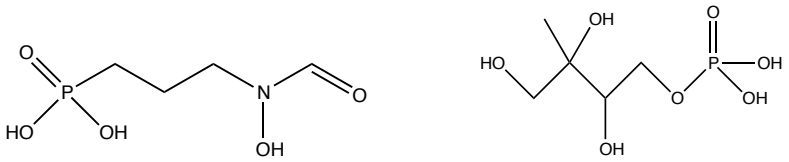


Figure 4. Molecular structure of fosmidomycin and methyl-D-eritritol-4-phosphate.

Statins (or HMGR inhibitors) are a class of drugs used to diminish the flux through the MVA pathway at the HMGR level. These compounds have been used for decades in medicine to decrease the cholesterol blood content (Shigi, 1989). The first identified statin was mevastatin (Endo et al., 1976), widely used in MVA pathway research, but never in humans (Mitchel et al., 2003). To date, the most widely used statin, both in hospitals and in plant research, is MEV (K_i: 0.64 nM), also known as lovastatin (Alberts et al., 1980).

As all statins, MEV acts as a competitive inhibitor to the HMGR enzyme mimicking its substrate HMGR-CoA (K_m= 4 μM). MEV has been used widely to study the MVA pathway in seedlings of several plant species like *Arabidopsis thaliana* (Bach and Lichtenthaler, 1983; Rodríguez-Concepción et al., 2006; Re, et al., 1995), *Raphanus sativus* and *Triticum spp.* (Bach and Lichtenthaler, 1983).

For the MEP pathway studies, the inhibitors usually employed are CLO (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone), and FSM (3-(N-formyl-N-hydroxyamino) propylphosphonic acid) that work on the DXS and DXR enzymes respectively.

CLO is a soil-applied herbicide tha inhibits the activity of the DXS enzyme. A bioactivation of this herbicide to produce ketoclomazone is needed in order to affect the synthesis of terpene dramatically. CLO treatment causes bleaching of plant seedlings (Flores-Pérez et al., 2010), and thereby a drastic reduction of several terpenes, such as the phytol side chain of chlorophyll, carotenoids, quinones, tocopherol and gibberellins (Ferhatoglu and Barret, 2006).

FSM is an anti-malarial drug that inhibits the second step (DXR enzyme) in the MEP pathway. The K_i value of FSM is 38 nM. It is a mixed-type inhibitor, acting in a competitive and non-competitive way simultaneously (Kuzuyama et al., 1998). FSM is the structural homolog to C-methyl-D-erythrose-4 phosphate or MEP (K_m=270 μM), the product of the DXR enzyme. As CLO, FSM also causes bleaching of plant seedlings (Rodríguez-Concepción et al., 2004).

I. 4. Use of ^{13}C-labeled metabolic products to study the terpene biosynthesis pathways

The incorporation of ^{13}C or ^{14}C-labeled compounds into the normal metabolism is one of the most powerful methods to determine the biosynthesis pathways, both for the primary and secondary metabolism. One of the most notable examples was the elucidation of the CO_2 fixation in the photosynthesis (Bassham et al., 1956). The use of isotope labeled tracers for the elucidation of

biosynthesis pathways is not a new concept, although very limited at first by the development of methods for producing, detecting and quantifying isotopes. The first trace studies were performed in the 1930s with deuterium, proving that it could be used and detected in living organism (Schönheimer and Rittenberg, 1935). After this, during the 50s-70s, radioactive compounds were available, being then responsible for the high sensibility of the studies (Rauschenbach et al., 1974). A disadvantage of working with radioactive isotopes was the difficulty to precisely determine the position of a labeled atom. Even in the case of very simple molecules, it was extremely challenging to clearly distinguish single carbons or hydrogens (Simon and Floss, 1967).

Tracer studies for explaining the biosynthesis pathways using stable isotopes were possible after the development of high sensitive Nuclear Magnetic Resonance (NMR) and Mass Spectrometric (MS) techniques. NMR techniques allow determining the exact position of the labeling, whereas the high sensitivity of the MS techniques allows the analysis of small sample quantities. In the case of minor labeling rates and large sample volumes, the NMR gives more accurate results than the MS. In the case of NMR, reliable detections can be achieved with a labeling 1.5 times higher than the natural ^{13}C enrichment (1.1%). In the NMR, only the neighbour atoms have an influence in the resulting signal; because of this, the method is most useful for C2 and C3 units. By coupling a ^{13}C nucleus with one or two direct ^{13}C neighbours, a resulting split of the signal with a specific coupling constant may be observed. In a ^{13}C-NMR spectrum, satellites are to be found around the central signal. With the MS techniques, the number of ^{13}C molecules can be established, but no decision can be made about their relative position.

Isotopomers and isotopologues compounds can be found in nature as a complex mixture. The International Union of Pure and Applied Chemistry or IUPAC defines isotopomers as molecules with the same number of isotopes and isotopologues as molecules with a certain isotope (e.g. ^{13}C) in varying quantities (McNaught and Wilkinson, 1997). From the distribution of the ^{12}C and ^{13}C atoms in a molecule pool, the isotopologues are exposed. The possible isotopologues are $z= 2^n$ where n is the number of carbon atoms in the molecule.

The natural occurrence of ^{13}C in molecules is H_n= 1.1 mol $\%^m$, where m is the number of the given ^{13}C atoms.

After the addition of the labeled precursors and its metabolization by the organism studied, an analysis from the metabolites produced follows. The detected ^{13}C isotopologues reflect the embarked paths and play a central role in the enlightenment of metabolic pathways and fluxes. In some cases, the obtained data accurately reflects the hypotheses. When this does not occur, the methods enable to identify new metabolic fluxes (Schäfer et al., 1989; Strauss et al., 1992; Sauer et al., 1997 and 2004; Werner et al., 1997; Petersen et al., 2000; Gunnarsson et al., 2004) or the discovery of new pathways (Fischer and Sauer, 2003; Jahn at el., 2007; Huber et al., 2008). Thus, the use of ^{13}C- and ^{2}H-labeling techniques, NMR spectroscopy, and GC/MS analyses, promoted the discovery of the non-mevalonate pathway during the 90s (Lichtenthaler, 1999). Subsequently, several studies shown that green algae (chlorophyta), higher plants, and other algal groups synthesize their plastidial isoprenoids (including isoprene) via a non-mevalonate pathway, that was later known as the MEP pathway (Lichtenthaler et al., 1995; Schwender, 1995; Schwender et al., 1995; Schwender et al., 1996; Arigoni et al., 1997; Lichtenthaler et al., 1997a; Lichtenthaler et al., 1997b; Lichtenthaler et al., 1997c; Schwender et al., 1997a; Schwender et al., 1997b; Zeidler et al., 1997; Schwender and Lichtenthaler, 1998).

Molecules labeled in one or more atoms (or complex isotopes mixtures) can be used as tracers. The introduction of isotope labeled molecules in metabolic networks produce a perturbation of the ^{13}C pool, far away from the natural almost stochastic enrichment level. Through the conversion of the applied isotope labeled precursors, the perturbation spreads in every direction of the metabolic network. The metabolism, also in very simple organisms, is not a one-dimensional system but a complex x-dimensional network, a so-called scale free network. Theoretically, pathways and fluxes from every knot of the network can be followed to every other knot (Bacher et al., 1999).

The biosynthetic metabolites produced are a complex mixture of isotopologues that where formed through several pathways from a single labeled precursor. The supplied disturbance moves along the entire network.

The distribution of the labelings is not random but reflects the metabolic pathway followed by the metabolite. The answer to a disturbance in a metabolic network can be called relaxation. The answer to not expected results can be accomplished by studying as many metabolites as possible (Figure 5).

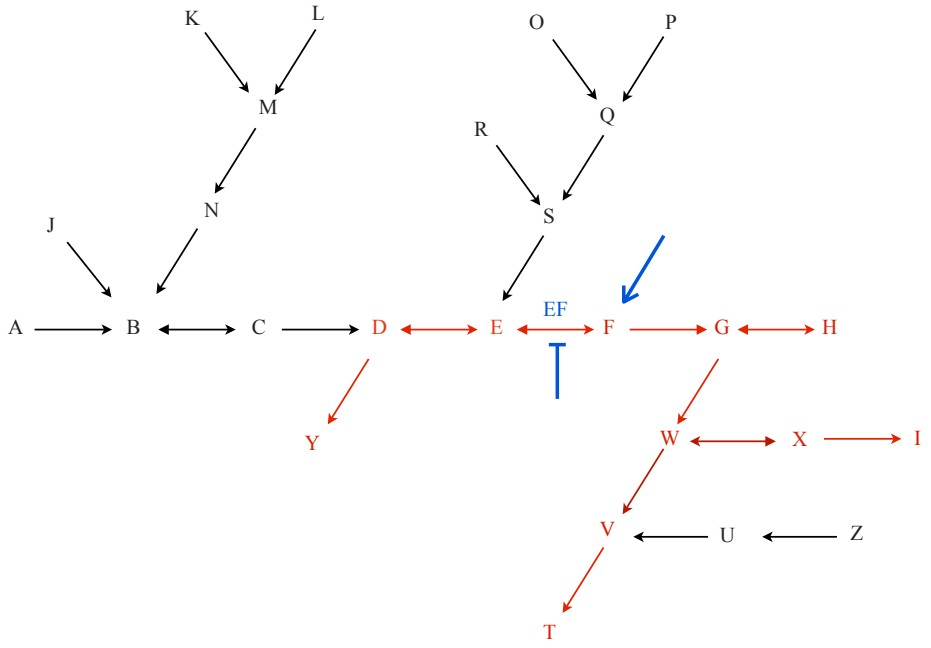


Figure 5. Scheme for a simple metabolic network. The perturbation could be due to the addition of the labeled F metabolite (promoting a new isotopologue distribution of the metabolites) or to the inhibition of the EF enzyme (supporting a decrease of all metabolites related).

I. 5. Spike lavender (*Lavandula latifolia* Medicus)

The genus *Lavandula*, of the Lamiaceae family, contains about 20 species almost exclusively to be found in the Mediterranean area (Segura and Calvo, 1991). They are shrubs (<200 cm) that usually grow in geographical areas with little summer rainfall and have obvious xerophytic trends. Their stems have a characteristic square shape and the leaves are almost linear to oblong-lanceolate and usually tomentose. Also, most aerial plant organs are covered in hairs and glands that content their essential oil.

Only three species of the *Lavandula* genus are considered to be of economic interest: *Lavandula angustifolia* (common or true lavender), *Lavandula latifolia* (spike lavender; Figure 6) and their hybrid (*L. angustifolia* x *L. latifolia*), commonly known as lavandin.

Figure 6. Spike lavender in its natural environment (Photo: J. Muñoz-Bertomeu).

The general essential oil market, specially pointed towards the flavour and fragrance industry, is one of the top world markets and growing every year (Schwab et al., 2008). As mentioned above, spike lavender is of considerable economic importance due to its essential oil. Is has been traditionally used in perfumery, cosmetics and food industries. Also, a great number of medical uses of its essential oil have been described: as antispasmodic, sedative, antihypertensive, antiseptic, healing and/or anti-inflammatory (Buchbauer, 2002; Cavanagh and Wilkinson, 2002; Hart and Lis-Balchin, 2002; Berdonces-Serra, 2007). The biocide action of *Lavandula* spp., has also been studied (Haig et al., 2009; Varona et al., 2010; Santana et al., 2012).

Traditionally, the essential oils of *Lavandula* species are obtained by steam distillation from the flowering spikes. The monoterpenes are the main components of the essential oil in *Lavandula latifolia* that has a weight per ml of 0.894-0.915 g (Harborne and Williams, 2002; Muñoz-Bertomeu et al., 2007b); like in other lamiaceae, these compounds are synthesized and

accumulate in glandular trichomes, especially in the peltated ones (Hallahan, 2000).

The main constituents in spike lavender oil from flowers are the monoterpenes linalool, cineol and camphor, while in the oil from leaves are cineol and camphor (Muñoz-Bertomeu, 2007). The essential oil of *L. angustifolia* flowers has a weight per ml of 0.878-0.892 g (Calvo and Segura, 1991). It normally contains not less than 35% esters, mostly linalyl acetate; other important constituents include linalool, cis-ocimene and lavandulyl acetate. The oils from the hybrid lavandin have a weight per ml of about 0.89 g and show characteristics of both common and spike lavender oils.

Despite spike lavender commercial interest and good adaptation to its natural environment, the culture of the species has been shifted in Spain in the past years by the most productive lavandin, which is characterized by a higher yield of essential oil per hectare (Renaud et al., 2001), although has a lower market price.

The composition and quality of spike lavender oil has been widely studied, due to its high economic interest (Muñoz-Bertomeu et al., 2007b). This topic has been extensive reviewed (Harbone and Williams, 2002). Some studies about chemical composition of some Spanish wild populations of spike lavender have also been carried out (Salido et al., 2004; Muñoz-Bertomeu et al., 2007b; Herraiz-Peñalver et al., 2013). All these studies revealed a great intraspecific variability in the chemical composition of oils that can be attributed to several variation sources: genotypic, climatic, geographical and/or seasonal (Guillén et al., 1996; Masotti et al., 2003; Angioni et al., 2006; Muñoz-Bertomeu et al., 2007b; Figueiredo et al., 2008; Herraiz-Peñalver et al., 2013).

Until recently, the selection of high-yielding essential oil spike lavender plants, has been based in conventional methods. Recent progress in plant biotechnology offers an alternative way to improve the production of essential oil in spike lavender using genetic engineering. Although knowledge about the regulation of the biosynthesis of monoterpenes is incomplete, several studies (Dudareva et al., 2004 and references therein) show that their production depends on both the supply of GPP units and the level of expression of each of

the monoterpene synthases. This, allows two possible strategies to undertake the metabolic engineering of monoterpene biosynthesis: (a) manipulation of the initial steps, that is, those implicated in the synthesis of IPP and DMAPP that will cause an increased amount of monoterpenes; and (b) the manipulation of the final steps of the pathway, that is, the monoterpene synthases catalyzing the synthesis of monoterpenes; this second approach would cause changes mainly in the qualitative profile of produced monoterpenes .

Any rational plant-breeding program should share both metabolic engineering approaches and conventional improvement techniques. This is particularly true for spike lavender, where the quantity and quality of its essential oil is related to the cultivar, the environmental conditions and type of cultivation (Harborne and Williams, 2002; Salido et al., 2004; Muñoz-Bertomeu et al., 2007b; Herraiz-Peñalver et al., 2013). This high variability means that the selection of suitable spike lavender genotypes for desired phytochemical traits can be achieved relatively easy.

Papers related with the *in vitro* culture of *Lavandula* species are summarize in Table 1 (see Segura and Calvo, 1991 and Gonçalves and Romano, 2013, for review). Most of these studies are related with the establishment and growth of calli, the isolation and culture of cell-derived calli, and plant regeneration from primary explants, calli and isolated cells. Although studies have been focused on the species with significant commercial interest (*L. angustifolia, L. latifolia* and lavandin), other species have been also investigated (*L. dentata, L. stoechas, L. viridis*, *L. pinnata* and *L. pedunculata*). In almost every published study, plant regeneration occurred through adventitious or axillary shoot organogenesis.

Not only plants, but also lavender secondary metabolites can be produced using the *in vitro* systems, including liquid culture (Segura and Calvo 1991; Trejo-Tapia et al., 2003; Gonçalves and Romano, 2013). The success of experiments aiming to increase essential oil yields or manipulate their chemical profiles suggests that commercial exploitation is possible. *In vitro* culture is therefore a valuable approach in all lavender species and 100% compatible with improvement and transformation programs.

To date, the published studies about genetic transformation of species of the genus *Lavandula* were limited to *L. latifolia* and the hybrid lavandin. In both cases, transformation was performed using *Agrobacterium tumefaciens* as a vector. In the event of *Lavandula latifolia*, the material used for the infection was leaves (Nebauer et al., 2000) or leaf-derived callus (Mishiba et al., 2000). The protocol for transforming spike lavender in a stable way was fine-tuned in our laboratory and until now has been used for several purposes in relation with the manipulation of the terpene synthesis pathway.

Table 1. Research summary on *in vitro* culture of genus *Lavandula* (Adapted from Muñoz-Bertomeu, 2007 and Gonçalves and Romano, 2013).

Species	Explant	Tissue answer	Reference
L.angustifolia Miller	Calli	Stems	Webb et al., (1984)
	Bud segment	Plants	Andrade et al., (1999)
	Leaf explant	Plants	Falk et al., (2009)
L.angustifolia 'Munstead'	Leaf explant	Plants	Wang et al., (2007)
L.angustifolia/ *L latifolia*	Leaf primordium	Callus, embryos	Quazi, (1980)
	Calli	Plants	
L. spica *	Hypocotyl	Callus	Trejo-Tapia et al., (2003)
L.dentata L.	Nodal auxillary buds	Plants	Jordan et al., (1998)
	Shoot tips	Plants	Sudriá et al., (1999)
	Nodal auxillary buds	Plants	Echeverrigay et al., (2005)
L. latifolia Medicus	Hypocotyl / Root / Cotyledon	Plants	Calvo and Segura, (1988)
	Isolated cells	Plants	
	Hypocotyl	Plants	Calvo and Segura, (1989a)
	Leaves	Plants	Calvo and Segura, (1989b)
	Calli / Isolated Cells	Plants	Jordan et al., (1990)
	Nodal auxillary buds	Plants	Sánchez-Gras and Calvo, (1996)
L. stoechas L.	Isolated cells	Callus	Gómez et al., (1987a)
	Hypocotyl	Callus	Gómez et al., (1987b)
	Hypocotyl / Root / Cotyledon	Callus	Calvo and Segura, (1988)
	Nodal auxillary buds	Plants	Nobre, (1996)
L. vera DC.**	Leaves / Calli	Stems	Tsuro et al., (2000)
	Nodal auxillary buds	Plants	Andrade et al., (1999)
L. viridis L'Hér	Nodal auxillary buds	Plants	Dias et al., (2002)
L. pinnata L.	Nodal stem segments	Plants	Shao et al., (2011)
L. pedunculata (Miller) Cav	Nodal explants	Plants	Zuzarte et al., (2010)
Lavandin	Nodal auxillary buds	Plants	Panizza and Tognoni, (1988)
	Shoot tips	Plants	Chambon et al., (1992)
	Nodal auxillary buds	Stems	Panizza et al., (1993)
	Micro cuttings	Plants	Mensualisodi et al., (1995)
	Calli	Stems	Panizza et al., (1997)
	Leaves	Plants	Dronne et al., (1999a)
	Leaves	Plants	Dronne et al., (1999b)

* *L. latifolia* or *L. angustifolia*, not specified by authors; ** *L. vera* and *L. angustifolia* are synonymous noums

Materials and Methods

II. MATERIALS AND METHODS

II. 1. Plant material

Initial plant material used in this thesis consisted of spike lavender mature seeds, provided by either Intersemillas SA (Valencia, Spain) or from manual-pollinated plants growing in the greenhouse. These seeds were germinated *in vitro* (see below) to obtain plantlets from which leaf discs (0.5 cm^2) and/or shoot-tips (1-2 cm in length) were isolated and used as primary explants in *in vitro* experiments.

Prior to germination, seeds were sterilised as previously described by Calvo and Segura (1988); briefly, after soaking in 0.5% H_2O_2 for 24 h, the seeds were surface-sterilised with 2% Chloramine T for 1 h, followed by three rinse cycles in distilled sterile water and finally germinated in Petri dishes on solid medium [0.7% Pronadisa agar (Madrid), 3% sucrose] at 26±2ºC in darkness. The seedlings were then placed in glass tubes (25 x 15 mm) covered with polypropylene closures (Wellco, Vineland, NJ, USA) containing 25 mL of basal medium (BM, see below).

Transgenic T_0 lines refer to plants regenerated from explants originally infected with *Agrobacterium tumefaciens*. T_1 plants (first generation) are seed-derived plants obtained from controlled self- or crosspollination of T_0 plants. Non-transgenic, wild type spike lavender plants were grown under the same conditions as controls.

Wild type and transgenic spike lavender plants, grown *in vitro* or in the greenhouse, were used for experiments aimed to know the contribution of MVA and DXP pathways to monoterpene biosynthesis in spike lavender. Both, flowers and either developing or fully expanded leaves were sampled for molecular and phenotypical analyses.

II. 2. Culture media and conditions

BM medium used in the experiments contained MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% of agar (Pronadisa) and a pH

of 5.7. In some experiments, MS salts and vitamins were employed at half-strength of its original concentration. Growth regulators were added to the media before autoclaving (20 min at 120ºC, 10^5 Pa). All antibiotics and metabolic inhibitors used were sterilised by filtration and added to the medium after autoclaving.

Unless otherwise stated, *in vitro* cultures were kept in growth chambers at 25±2°C and a 16 h photoperiod provided by Sylvania (GTE gro-lux, F36W/ GRO, Erlangen, Germany) cool-white fluorescent tubes (60 $\mu mol.m^{-2}.s^{-1}$ irradiance at culture level).

II. 3. Obtention of transgenic plants with *DXR* or *LIS* genes

Coculture with *Agrobacterium tumefaciens* was used to introduce *DXR* and *S-linalool synthase (LIS)* genes into spike lavender.

The strain C58 of *Agrobacterium tumefaciens* bearing the plasmid *pLBI1DXR10* with the *DXR* (AF148852) gene from *Arabidopsis thaliana* (Figure 7) was kindly supplied by Professor Albert Boronat (Biochemistry and Molecular Biology Department, Division III, Chemistry Faculty, University of Barcelona). In turn, the 2.760 kb cDNA from *Clarkia breweri* (A.Gray) Greene that contents the coding sequence for *LIS* gene was provided by Professor Pichersky (Department of Molecular, Cellular, and Developmental Biology, University of Michigan) and received in pBluescript II SK (+). Several steps were required before transformation with this cDNA.

Figure 7. Scheme of the T-DNA fragment of the plasmid (*pLBI1DXR10*) used in transformations with *Agrobacterium tumefaciens*. p, PCR amplified fragment; s, probe used in Southern Blots; n, probe used in Northern Blots.

II. 3. 1. Preparation of *Agrobacterium* construct with the *LIS* gene

The *pBluescript II SK* (+) plasmid that contained the *LIS* cDNA was digested with *Sal*I to achieve linear DNA. Then, using the Klenow enzyme, non-cohesive ends were produced. The DNA was then digested with *Dra*I and *Bam*HI enzymes producing a 2,760 bp fragment that contained the *LIS* gene.

The pBI121 vector was digested with *Bam*HI and *Ecl*136II releasing an 11.1 kb fragment that was separated by electrophoresis in 1X TBE 0.8% agarose and purified with QIAquick Gel Extraction Kit following manufacturer instructions.

The *LIS* gene fragment was introduced in the *Bam*HI-Ecl136II site, between the 35S promoter and the NOS terminator using T4 DNA ligase, achieving finally a 13.3 kb plasmid (*pBILIS*).

II. 3. 1. 1. *E. coli* competent cells preparation

A modified Sambrook protocol (2001) was used to prepare competent cells. Frozen samples (-80ºC) of an XL1Blue strain were cultured on petri dishes containing LB medium (10 g/L bactotryptone, 5 g/L yeast extract and 10 g/L NaCl) solidified with 1.5% agar (Pronadisa) and incubated in the dark at 37ºC. After 24 h, a single colony was transferred into a 100 mL flask with 25 mLLSOB (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 3 mM KCl, 10 mM $MgSO_4$; Hanahan, 1983) medium and cultured at 37ºC until an OD_{600} of 0.4 was achieved. Then, the bacterial suspension was transferred into 50 mL sterile tubes and cooled on ice for 10 min. After 10 min centrifugation (2,700 g, 4ºC) the pellet was resuspended in 30 mL of a cold (4ºC) $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$). After a second centrifugation, supernatant was discarded and the pellet was resuspended in 2 mL of a 4ºC $CaCl_2$ 0.1 M solution. Competent cells were frozen in liquid N_2 and stored at -80ºC until used.

II. 3. 1. 2. *E. coli* transformation and plasmid extraction

The bacteria transformation was performed following a modified procedure described by Mandel and Higa (1970), in which a heat shock (90 s at 42ºC) of the cell suspension in cold $CaCl_2$ stimulates the incorporation of exogenous DNA into the cells. The plasmid with the *LIS* cDNA was amplified in competent cells of the *E. coli* XL1Blue line. Fifty 50 ng of plasmid were used for every 100 µL of competent cells. Then, 0.8 mL LB were added and the tubes maintained for 1 h at 37ºC. Finally, cells were cultured in LB petri dishes with 50 mg/L of kanamycin (Kan) and the resistant colonies were selected after 16 hours.

For plasmid extraction, a modified Sambrook protocol (2001), based on the effect of anionic detergent and high pH values, was used. A single cell colony was cultured for 12-16 hours in 2 mL LB with 50 mg/L Kan. Then, 1.5 mL of this culture was centrifuged (13,000 rpm for 5 min at 4ºC), the pellet was resuspended in 100 µL of lysis solution (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA, pH 8.0) and 200 µL of fresh prepared alkaline solution was added (0.2 N NaOH, 1% SDS). The tubes were mixed gently and placed on ice. Subsequently, 150 µL of acid solution were added (60 mL potassium acetate 5 M, 11.5 mL glacial acetic and 28.5 mL water), gently mixed, cooled on ice for 5 min and maintained at room temperature for 2 min. The tubes were centrifuged (13,000 rpm, 10 min, 4ºC) and the supernatant was mixed with two volumes of ethanol 96%. Then, the mixture was centrifuged (13,000 rpm, 20 min) and the pellet, once dried, was resuspended in 25 µL of TE 1X with 0.4 µg/ µL of RNase.

II.3. 1. 3. *Agrobacterium tumefaciens* competent cells preparation and transformation

A. tumefaciens C58 strain was cultured in 50 mL LB medium with 50 mg/L ampicillin (LBAmp) until an OD_{600} of 1.0 was achieved. The bacterial suspension was centrifuged (4,000 rpm, 10 min) and the pellet resuspended in 1 mL of $CaCl_2$ 20 mM. These cells were divided into 0.1 mL aliquots, frozen and stored at -80ºC until used.

The plasmid *pBILIS* was introduced in the C58 strain of *Agrobacterium tumefaciens* following a modified protocol of the freezing and thawing method (Holsters, 1978). A 100 µL competent cells aliquot was used along with 1µg plasmid. The mixture was quick freezed in liquid N_2 and then maintained at 37ºC for 5 min. Subsequently, 1 mL LBAmp was added and the cell suspension was then incubated at 28ºC for 4 hours with gentle shaking. After centrifuging (4,000 rpm, 10 min), cells were resuspended in 100 µL LBAmp and cultured at 28ºC in darkness for 2-3 days in petri dishes containing LB medium with 50 mg/L Amp, 50 mg/L Kan (LBAmpKan) and 1.5% agar (Pronadisa). Colonies were resuspended in 25 mL LBAmpKan and after one day the plasmids were extracted following the *E. coli* procedure to check for the presence of the construct by PCR using 5´-GGGAGGAAGTTGATGAGAAGAAGC -3´ and 5 ´-CTTGTTAACCCCTTTCCCCAC -3´ primers (1,329 bp).

To check for the presence of the *pBILIS* plasmid in the kanamycin resistant colonies, plasmid was isolated and digested with *Hind*III. The expected fragment sizes were 12,371 and 3,255 bp. A scheme of the *pBILIS* plasmid is shown in Figure 8.

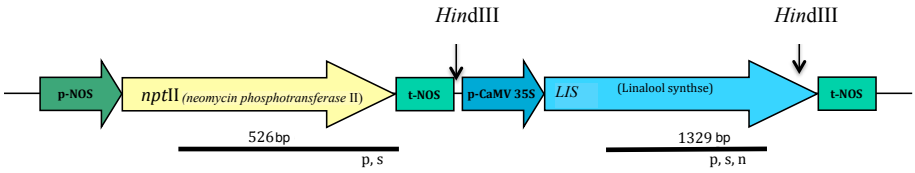


Figure 8. Scheme of the T-DNA fragment of the plasmid (*pBILIS*) used in transformations with *Agrobacterium tumefaciens*. p, PCR amplified fragment; s, probe used in Southern Blots; n, probe used in Northern Blots.

All strains were stored at -80ºC. For that, the aliquots of fresh bacterial cultures were mixed with 0.2 volumes of sterile glycerol and freezed in 1.5 mL tubes at -80ºC until used.

II. 4. Genetic transformation of *Lavandula latifolia* Medicus

II. 4. 1. *Agrobacterium tumefaciens* cultures

The bacterial cultures were initiated from glycerol stocks and grown in LBAmp medium (28ºC, horizontal gyratory shaker at 200 rpm and overnight). The cultures were then transferred to the same medium supplemented with 100 µM acetosyringone for 7-8 hours (28ºC, horizontal gyratory shaker at 200 rpm and overnight). The bacterial culture was centrifuged at 3,500 rpm for 15 min and resuspended and diluted to approximately 10^6 cells mL^{-1} (OD_{600} = 0.6) in liquid BM (pH 5.5) supplemented with 100 µM acetosyringone.

II. 4. 2. Leaf explant infection and production of transgenic plants

The protocol described by Nebauer et al., (2000) was followed with small modifications. For each transformation experiment, between 300 and 500 leaf explants were excised and precultured for 24 h in 15 mm × 100 mm petri dishes containing 25 mL of regeneration medium (RM) [BM medium with 0.6 µM IAA and 8.8 µM BA]. Then, the explants were immersed for 20 min in the diluted *Agrobacterium* suspension, blot dried between sterile filter paper, placed on RM, and incubated at 28±1ºC in darkness. After 24 h of cocultivation, the explants were transferred to selection medium (RM with 120 mg/L cefotaxime and 30 mg/L kanamycin) and maintained under a 16 h photoperiod. The explants were transferred to fresh medium weekly until adventitious buds differentiation was observed. After 30-50 days, buds were isolated and placed into tubes with 25 mL elongation medium (BM medium with 0.06 µM IAA, 8.8 µM BA, 20% of coconut milk and antibiotics). Coconut milk was prepared according to George (1993) using ripe coconuts from local fruit markets. For rooting, elongated stems were placed into jars with 50 mL of ½ BM medium (BM with nutrients and sucrose at half strength) with 120 mg/L of cefotaxime. Putative transgenic plants were cloned through axillary buds proliferation; for that, apical buds and nodes were isolated and cultured in the same medium. After 3-4 subcultures the cefotaxime was removed from medium.

Regenerated plants were transplanted to 100 mL pots containing a mix of 1:1 perlite/peat moss, placed into GA7 Magenta boxes (Sigma, St. Louis, Missouri, USA) and adapted to growth chamber conditions (75% RH, 25±1ºC and 80 μmol.m^2.s^{-1} irradiance) with gradual exposure to reduced relative humidity by progressively removing the Magenta cover over 2–3 weeks. Once acclimatisation was achieved, plants were transferred to the greenhouse. Finally, the plants were transplanted to 10 L pots containing the same substrate. Dripping irrigation provided moisture for maintenance of vigorous growth. The pots were regularly irrigated with half-strength Hoagland and Arnon (1950) nutrient solution.

II. 4. 3. Obtention of progenies from transgenic spike lavender plants

Progenies were obtained by manual selfing or by manual cross-pollination of those transgenic T_0 lines that flowered in the greenhouse. Crosses were performed with specific brushes for each line during flowering time (summer).

Selfing was performed with DXR and LIS transgenic spike lavender T_0 lines obtained in this work. Cross-pollinations were performed with transgenic spike lavender lines for the genes *DXS* (Muñoz-Bertomeu et al., 2006), *HMGR* (Muñoz-Bertomeu et al., 2007a) and *LIS* genes maintained in the greenhouse. Line DXS6 was used as pollen donor plant. The corresponding recipient transgenic lines were: HMGR1, HMGR4, HMGR3, LIS1, LIS2 and LIS8. In late October, mature fruits from crosses were collected. Subsequently, T_1 seeds were germinated and handled as previously described. After two months plantlets about 2 cm in length were cultured in vessels with 40 mL of ½ BM medium. At the same time samples were collected for PCR. After successive subcultures, some clones were transferred to pots, acclimatized and transferred to the greenhouse as previously described.

II. 4. 4. Molecular analysis of plants

Molecular analyses were performed in putative T_0 transgenic and control plants as well as in T_1 progenies by polymerase chain reaction (PCR), Southern Blot, Northern Blot and Western Blot analyses.

II. 4. 4. 1. PCR analyses

For DNA extraction a modified CTAB protocol, described by Doyle and Doyle (1990), was used. About 100 mg of fresh leaves from *in vitro*-grown plants were homogenised in tubes with 500 µL of extraction buffer [2% CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 1% of PVP (Mr 40000) and 0.2% β-mercaptoethanol] and then incubated at 65ºC for 30 min. Then, the extract was purified with 500 µL of chloroform: isoamyl alcohol (24:1 v/v) and isopropanol. After centrifuging (20 min, 13,000 rpm), the pellet was washed with 70% ethanol, dried, resuspended in 50 µL of TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and stored at 4ºC until used.

Table 2. Primers used for transgene amplifications

Gene	Primer sequence	Expected fragment
DXS	5´-GTTCATTTCATTTGGAGAGGAC-3´ 5´-TGGGAATTGTTGTTGGGTTTC-3´	320 bp
HMG1	5´- GTTCATTTCATTTGGAGAGGAC -3´ 5´-AGGGCAAACGCATACGCAC-3´	1430 bp
LIS	5´-GGGAGGAAGTTGATGAGAAGAAGC -3´ 5´-CTTGTTAACCCCTTTCCCCAC -3´	1329 bp
DXR	5´-GTTCATTTCATTTGGAGAGGAC-3´ 5´-CCTCTCCCTTGATTCCTCCTC-3	270 bp

The amplifications of DNA were performed with 50 µL reaction volumes containing 75 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.1 mM of each dNTP, 0.25 mM of each primer (see Table 2), 50 ng of DNA and 4 U of Taq polymerase (Biotools, Spain). The amplification parameters for all genes were: 3 min at 94ºC, followed by 30 cycles of 1 min at

94ºC, 2 min at 60ºC and 2 min at 72ºC, and finally 7 min at 72ºC. The amplification products of DNA were separated by electrophoresis in 1X TBE gels (90 mM boric acid, 90 mM Tris-HCl pH 8.0 and 2 mM of EDTA) with 1% agarose, 0.05 mg/mL of ethidium bromide.

II. 4. 4. 2. Analyses by Southern Blot

The Southern Blot analysis was performed with nonradioactive digoxigenin-11-dUTP labeled probes.

II. 4. 4. 2. 1. Genomic DNA isolation

Two grams of leaves, previously homogenised with liquid nitrogen, were transferred into centrifuge tubes with 10 mL of extraction buffer and incubated at 60ºC for 1 h. After washing with 10 mL chloroform: isoamyl alcohol (24:1 v/v), DNA precipitation was performed using the CTAB method. Pellet was resuspended in 2.5 mL of TE 1X NaCl and proteins were washed out with a phenol:chloroform: isoamyl alcohol mixture (25:24:1 v/v). The nucleic acids of the supernatant were precipitated at -20ºC for 2 h by adding 2 volumes of cold 96% ethanol. Finally, the pellet was resuspended in 200 µL MilliQ water with 200 ng/µL RNase and stored at 4ºC until used.

II.4. 4. 2. 2. Quantification, digestion with restriction enzymes, electrophoresis and transfer to membranes

DNA was quantified using a TBE 1X gel (0.8% agarose with 0.05 mg/mL of ethidium bromide). DNA concentration was estimated by comparing the bands brightness and thickness with the λ/*Hin*dIII marker bands. The endonucleases used were *Eco*RI for the *DXR, DXS* and *HMG1* genes and *Bam*HI for the *LIS* gene. Digestion was performed according to manufacturer's instructions. The digested samples (40 µL of sample + 8 µL of loading buffer 6X) along with the λ/*Hin*dIII marker were separated by electrophoresis at 60 volts in a TBE 1X gel with 0.8% agarose. Finally, the gel was stained with ethidium bromide.

For transferring the DNA to the membranes, the gel was washed out with MilliQ water and then incubated for 40 min in 0.25 M HCl, twice for 30 min in 0.5 M NaOH, 1.5 M NaCl, and twice for 30 min in neutralisation buffer [0.5 M Tris-HCl (pH 8.0) + 1.5 M NaCl]. The transfer of the DNA from the gel to the nylon membrane (Boehringer Mannheim) was achieved by capillarity for 12-16 hours using buffer SSC 20X as transfer. Once the gel was removed, the membrane was allowed to dry and DNA was covalently fixed covalently using UV irradiation (Biolink BLX); finally, the membrane was washed in MilliQ water, left to dry and stored at 4ºC until required.

II. 4. 4. 2. 3. Hybridisation and detection

The membrane was equilibrated in prehybridisation buffer; after removing this buffer the membrane was incubated overnight at 60ºC in 10 mL of hybridisation buffer containing 200 ng of the DNA probe. Next, the membrane was incubated for 5 min in washing buffer (0.1% maleic acid, 0.15 M NaCl and 0.3% tween 20), followed by 30 min in antibody buffer (0.1% maleic acid, 0.15 M NaCl and 1% of block agent) and for other 30 min in antibody buffer (1:10,000 of Anti-digoxigenin Fab fragments). Then, the buffer was removed and the membrane was equilibrated with detection buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl and 0.05 M $MgCl_2$). Subsequently, the membrane was placed into a plastic bag where it was covered with CSPD solution (1:100 of CSPD in detection) for 5 min. Finally, the membrane was placed into a new plastic cover, which was sealed by heat being ready for use.

II. 4. 4. 3. Analysis by Northern Blot

The transgene expressions were determined by Northern Blot, based on the hybridisation of RNA with [α-^{32}P]dCTP DNA using the random printing method.

II. 4. 4. 3. 1. RNA isolation

The RNA extraction procedure used is a modification of the protocol proposed by Tripure Isolation Reagent (Roche Applied Sciences). The plant material (0.4 g of leaves) from transgenic and control plants was frozen in liquid nitrogen and stored at -80ºC. For RNA extraction, the samples were homogenised with liquid nitrogen and the powder transferred into 15 mL tubes with 2 mL Tripure and maintained at room temperature for 15 min; next 0.4 mL of chloroform was added, mixed and maintained for 15 min at room temperature. Then, the tubes were centrifuged (12,000 g) for 15 min at 4 ºC and the supernatant transferred to a new tube in which RNA was precipitated by adding the same volume of isopropanol at room temperature for 10 min. Subsequently, the tubes were centrifuged (12,000 g) at 4ºC for 10 min and the pellet was washed out with 4 mL 75% ethanol, vortexed for 5 sec and centrifuged (7,500 g) for 5 min at 4ºC. After discarding the supernatant, the pellet was dried and resuspended in 50 µL of MilliQ water and stored at -80ºC until used. RNA was quantified by spectrophotometry (λ=260 nm).

II. 4. 4. 3. 2. RNA Electrophoresis

RNA electrophoresis under denaturing conditions in 2.2 M formaldehyde was performed according to Maniatis et al., (1982) using the MOPS buffer system. RNA under these conditions is fully denatured and migrates according to the $\log_{10}$ of its molecular weight.

To the RNA samples (30 µg), 8 µL of MilliQ water, 36 µL of loading buffer 1.25X [1.25 % MAE 10X (0.2 M MOPS, 50 mM sodic acetate, 10 mM EDTA, pH 7), 7.4% formaldehyde, 55% formamide, 8% glycerol and 0.5 mg/mL bromophenol blue] and 1 µL of an aqueous solution of ethidium bromide (1 µg/µL) were added. Prior loading, samples and the marker were heated at 56ºC and 5x loading buffer was added. The RNA was separated at 60 volts for 4 h in an agarose gel (MAE 1X buffer with 2.2% formaldehyde) immersed in MAE buffer 1X with 1.1% formaldehyde. Once electrophoresis was accomplished, the bands were observed under UV light and the gel was washed twice with SSC 10X for 20 min.

Transference of RNA to the nylon Hybond-H (Amershan) membrane was performed by capillarity for 12-16 hours in a 10X SSC buffer. Then, the RNA was fixed covalently using UV light for 3 min; the membrane was washed with 2X SSC, allowed to dry and stored at 4ºC until required.

II. 4. 4. 3. 3. Probe labeling

The probe was labeled with [α-^{32}P]dCTP by random printing techniques. The DNA template used for the labeling was a PCR-amplified fragment prepared as follows: The DNA template (approximately 50 ng), diluted in 15 µL MilliQ water, was denaturalised for 10 min at 95ºC and quickly cooled down on ice, the sample was centrifuged and 5 µL of the labeling oligonucleotides buffer were added [(0.2 mg/mL hexanucleotide random primers, 25 mM $MgCl_2$, 5 mM DTT, 100 mM Mes-Tris pH 6.8, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP), 2 µL of BSA 0.1%, and 2.5 µL of [α-^{32}P]dCTP (10 µCi/µL). After 6 hours of labeling at room temperature, reaction was stopped by adding 200 µL of 1X TE; finally, the probe was denaturalised at 95ºC during 10 min and maintained on ice until used.

II. 4. 4. 3. 4. Hybridisation and detection

The membranes, previously equilibrated for 20 min at 65ºC in 7 mL of hybridisation buffer (0.4 M NaH_2PO_4 pH 7.2, 1 mM EDTA and 7% SDS), were hybridised overnight at 65ºC in the same buffer with 227 µL of the labeled probe. Then, the membranes were washed twice for 10 min with 4X SSC/0.1% SDS at 65ºC and twice again for 5 min with 0.4X SSC/0.1% SDS at 65ºC. Finally, the membranes were transferred to a vessel with 4X SSC/0.1% SDS for 10 min at room temperature, dried using filter paper and sealed in a plastic bag. Next, the filter was placed for 4 hours inside a dark camera with the appropriate film at -80ºC and finally was developed as described by the manufacturer.

Each membrane was hybridised with the appropriate probe. The probe was then removed by washing the membranes in 0.1X SSC/0.1% SDS at 100ºC

during 10 min; after cooling and drying, the membrane was stored at 4ºC until next use.

II. 4. 4. 4. Western Blot analysis

The study of the presence of the protein codified by the transgene *DXR* gene was performed using the Western Blot method. The polyclonal antibody used for this was provided by Dr Michael H. Walter from the Leibniz-Institut für Planzenbiochemie, Germany.

II. 4. 4. 4. 1. Protein extraction

Protein extract was obtained by double centrifugation (25,000 rpm for 30 min at 4ºC) of 1 g of leaves homogenised in liquid nitrogen and resuspended in 1 mL of extraction buffer (50 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 100 mM KCL, 0.25 mM sucrose, 10% glycerol, 1% PVP 40,000, 1 mM DTT, 1 mM PMFS, 0.4% 2-mercaptoethanol) with 33.33 µL protease Inhibitor Cocktail (Sigma, P-9599). Protein content in the extracts was quantified with Bradford reagent (Bio-Rad) using bovine serum albumin as a standard. The protein extracts were diluted to the same final concentration with the protein extraction buffer and 5X LaemmLi loading buffer (7.5% SDS, 0.1 M DTT, 10 mM EDTA, 30% sucrose, 0.25 mg/mL of bromophenol blue and 0.3 M Tris-HCl pH 6.8) was added. Samples were stored at -80ºC until used.

II. 4. 4. 4. 2. SDS-PAGE electrophoresis

First, the samples (about 30 µg of proteins) were heated at 95ºC for 5 min and incubated at room temperature for 5 more min. The electrophoresis was carried out twice; each gel consisted of two parts: the packaging gel (125 mM Tris-HCl (pH 6.8), SDS 0.1%, 3.3% acrylamide/bisacrylamide, 0.14% ammonic persulfate and 12 mM Temed), which allows the concentration of all the proteins as a single band, and the separation gel (375 mM Tris-HCl (pH 8.8), SDS 0.1%, 8% acrylamide/bisacrylamide, 0.066% ammonic persulfate and 5.7 mM Temed), which allows the differential separation of the proteins by their

molecular weight. The electrophoresis was conducted at 50 volts using an electrode buffer containing 1.92 M glycine and 1% SDS, pH 8.3 adjusted with Tris.

To verify equal protein loading, one of the gels was stained for 1 hour with a solution of Coomassie Blue (0.05% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid) and then washed with a solution of 40% methanol and 10% acetic acid.

II. 4. 4. 4. 3. Transfer and detection

The proteins were transferred onto Immune-Blot polyvinylidene difluoride (PVDF) membranes (BioRad) using the Mini Tran-Blot Cell (BioRad) for 16 hours at 40 V with the transfer buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% methanol). The efficiency of the protein transfer was checked by staining the membrane with Ponceau solution (0.1% Ponceau S, 1% acetic acid) and later washed-out with a solution of 1% acetic acid.

The immunological detection was performed with the ECL Western Blotting Analysis System kit (Amersham Biosciences) following the recommendations of the manufacturer under continuous agitation. First, the membrane was incubated in PBS-T buffer (20 mM NaH_2PO_4 2 H_2O, 80 mM Na_2HPO_4 12 H_2O, 100 mM NaCl, 0.1% Tween 20, adjusting the pH at 7.5 with NaOH) with 5% skimmed milk. The incubation with primary antibody (1:2,000 dilution) was performed in PBS-T for 1 hour at room temperature. Then, the membrane was washed 3 times for 5 min in PBS-T and after that incubated for 1 hour with the secondary antibody (1:7,500 dilution). After 3-5 min washing in PBS-T, the membrane was placed in a plastic cover and the detection solution was added. After 1 min the membrane was dried and placed in a new plastic cover, sealed by heat and placed over an appropriate film inside an exposure case for 1-20 min before developing.

II. 4. 5. Phenotypic analysis

II. 4. 5. 1. Essential oil analyses with hexane as solvent

Leaves and inflorescences from each examined plant were treated separately for essential oil extraction; tissues were manually crushed and mixed to ensure sample uniformity. When appropriate, samples were air-dried for 30 days. Two different types of extraction were performed: hydrodistillation and direct extraction with hexane.

For hydrodistillation, dried samples of leaves (0.5-1.0 g), consisting of a homogeneous mixture of leaves from the 4th to the 10th whorl of every plant, or flowers (0.5 g), containing inflorescences with 3-5 open flowers, were distilled in 100 mL of water in a Clevenger type apparatus for 1.5 hours, containing *n*-tetradecane and naphthalene as internal standards. Oils obtained were recovered with hexane, dried over anhydrous sodium sulphate, filtered through 0.22 µm PVDF (Millipore) membranes and adjusted to a final volume of 10 mL with hexane. In these conditions, the concentration of the internal standards was 10 ppm for n-tetradecane and 400 ppm for naphthalene. The samples were stored at 4ºC in sealed vials until analysed. All extractions were performed in triplicate.

The direct extraction in hexane was performed with fresh tissue. When greenhouse plants were analysed, single cohorts of leaves from different developmental stages were sampled (see Figure 9). Fresh leaves (100 to 300 mg) were transferred to 10 mL tubes containing 1 mL of hexane for each 100 mg plant material, with n-tetradecane and naphthalene as internal standards. For each 100 mg tissue, 100 mg of small glass beds (425-600 µm in diameter, Sigma) were incorporated into the mixture before shaking up in a vortex for 1 min. After 4 h incubation at room temperature, the extract

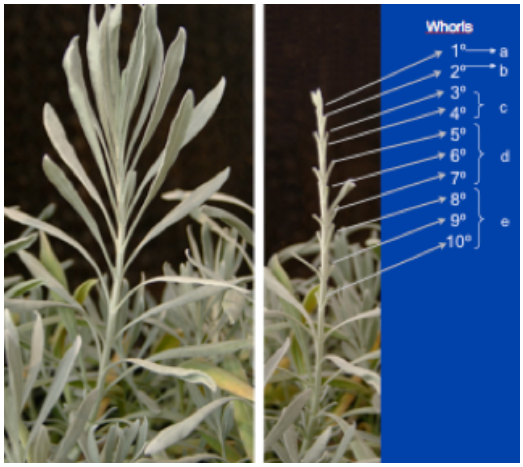


Figure 9. Different whorls used in all the experiments.

was filtered through PVDF (Millipore) membranes (0.22 μm), concentrated under a nitrogen stream to 1 mL, and stored at 4ºC in sealed vials until analyzed by GC. The essential oil analysis was performed with gas chromatography (GC) techniques using a Focus GC (Thermo Finnigan, Italia) equipped with a flame ionization detector (FID) and a capillary column BP-20 (polyethylene glycol, 30 m x 0.25 mm; SGE Europe Ltd, France); helium was used as a carrier (constant flow 1 mL/min).

The samples were injected automatically (AI 3000 Autosampler) in splitless mode (0.8 min). The initial temperature of the oven was set at 40ºC for 1 min, followed by a ramp of 4ºC/min to 130ºC, and finally held isothermal at 130ºC for 25 min. The temperatures of the injector and detector were 230ºC and 260ºC, respectively. The identified components of the essential oil were quantified (mg/g of dried tissue) using standard curves with the internal controls. The retention time of each compound was established by comparing with the pure standards. The relative peak area for individual constituents was determined using the Chrom-Card S/W program (Termo Finnigan).

II. 4. 5. 2. Chlorophyll and carotenoid content

Extraction and determination of total chlorophylls and carotenoids were conducted as described by Lichtenthaler, (1987). Extracts were obtained in 100% acetone from 80 to 300 mg of fresh material from spike lavender plants, either grown *in vitro* or in the greenhouse. Spectrophotometric quantifications (Table 3) were carried out in a Shimadzu UV-1203 spectrophotometer based on their absorbance for the wavelengths 470, 645, and 662 nm. All analyses were performed at least three times.

Table 3. Lichtenthaler Equations (1987) for determination of chlorophyll a (C_a), chlorophyll b (C_b), total chlorophylls (C_{a+b}) and total carotenoids (C_{x+c}) concentration (μg/mL)

C_a=	$11.24\ A_{662} - 2.04\ A_{645}$
C_b=	$20.13\ A_{645} + 18.08\ A_{662}$
C_{a+b}=	$7.05\ A_{662} + 18.08\ A_{645}$
C_{x+c}=	$(1{,}000\ A_{470} - 1.9\ C_a - 63.14\ C_b)/214$

II. 5. Contribution of MVA and MEP pathways to monoterpene biosynthesis in spike lavender

To elucidate the contribution of MEP and MVA pathways to monoterpene biosynthesis in spike lavender, the following experimental approaches were employed:

1) Treatment with specific inhibitors of these pathways. In these experiments explants from wild type or transgenic plants, grown *in vitro* or in the greenhouse, were treated with either MEV, an inhibitor of the HMGR enzyme from the MVA pathway, or FSM, an inhibitor of the DXR enzyme, a key step of the MEP pathway. The effect of MVA on phenotype recovery of the inhibitor-treated plants was also tested.

2) Labeling experiments with $^{13}CO_2$ (see II.5.2.1.) and [U-$^{13}C_6$]glucose (see II.5.2.2.) and [1,2-$^{13}C_2$]mevalonate. In these experiments, both *in vitro*- or greenhouse-grown transgenic or wild type plants were used. Also *in vitro* labeling experiments with [1,2-$^{13}C_2$]mevalonate were performed.

II. 5. 1. Effects of MEV and FSM on spike lavender

FSM (Invitrogen, Molecular Probes, Life Technologies) and MEV (Sigma-Aldrich) stock solutions were prepared as described by Rodríguez-Concepción et al., (2001). Briefly, FSM was diluted in MilliQ water to a final concentration of 1 mg/mL. In the case of MEV, 4 mg were dissolved in 750 µl of EtOH. After that 1.125 mL of 0.1 M NaOH were added. The mixture was incubated for 2 hours at 50ºC. Then, pH was adjusted with HCl to a value of 7.5. Finally, the volume was adjusted to 5 mL with MilliQ water giving a final concentration of 2 mM MEV.

II. 5. 1. 1. Germination assays

Spike lavender seeds, sterilised as previously described, were cultured in glass tubes containing 15 mL BM supplemented with MEV (0, 0.5, 1, 2 and 5 µM) or FSM (0, 10, 20, 30 or 40 µM). Cultures were maintained in a growth chamber under 16 h photoperiod. For each treatment 48 seedlings were used.

After 50 days, the percentage of germination, number of leaves, the stem and root lengths and the content of chlorophylls and carotenoids were recorded. For the photosynthetic pigment quantification 9 seedlings were used for each inhibitor concentration.

II. 5. 1. 2. *In vitro* and *ex vitro* stem assays

For the *in vitro* assays, shoot apices with 3 whorls (1.5 cm in length), isolated from either two-month old *in vitro* grown spike lavender wild type seedlings or transgenic shoot cultures maintained *in vitro* were employed as primary explants. In a first experiment, the explants were cultured for 45 days on BM medium supplemented with the same MEV or FSM concentrations employed in the germination assays. Twenty-four control explants were cultured for each treatment.

In a second experiment, we tested whether MVA, a precursor of terpene biosynthesis, overcomes the effect of FSM or MEV. In this experiment, shoot apices were cultured for 28 days on BM medium supplemented with increasing concentrations of MVA (0, 0.3, 0.6, 1.2, 2.4 and 3.5 mM) alone or in combination with 1 μM MEV or 30 μM FSM. Twenty-four explants were cultured for each treatment.

In the last experiment, the effect of MEV and FSM on transgenic spike lavender plants was tested. The transgenic spike lavender HMGR5 line, that contains 8 inserts of the *HMGR* gene from *Arabidopsis thaliana,* was used as shoot apices source. Thus, 24 explants were cultured for 42 days on BM with 1 μM MEV or 30 μM FSM. Non transformed shoot apices from *in vitro* proliferating spike lavender shoot cultures were employed as controls.

In all experiments, cultures were maintained in a growth chamber under a 16 h photoperiod and examined for root and stem lengths, whorl number, the number of new developed whorls, fresh and dry weight, and the content of chlorophylls and carotenoids. In the experiments with MVA, essential oil content was also analysed. In this case, a direct extraction with hexane of fresh tissues was employed.

Ex vitro assays were undertaken by using transgenic HMGR, DXS, and HMGR-DXS spike lavender lines, grown in the greenhouse, as a source of explants (stems 5 cm in length with 5 whorls). A transgenic line overexpressing the *npt*II (*neomycin phophotransferase* II) gene was used as control.

In a first experiment, isolated stems were individually placed in pots containing a mixture of 1:1 peat moss and perlite. Before transplanting, the stem bases were immersed in talc containing 5,000 ppm IBA to induce rooting. After 1 month, pots containing rooted shoots were first irrigated with a Hoagland (Hoagland and Arnon, 1950) nutrient solution (1x). After 1 day, the pots were irrigated with aqueous solutions of 1 μM MEV, 30 μM FSM or water every two days for 15 days, and plantlets were sampled after another 15 days. In a second experiment, the stems were treated as above but plantlets were irrigated twice per week for two months with the inhibitors.

In both experiments, cultures were examined for root and stem length, stem whorl number, fresh weight, dried weight and photosynthetic pigment and essential oil content. At least 10 rooted stems were used for each transgenic line and treatment.

II. 5. 2. Labeling experiments

Cineol and camphor are the most predominant monoterpenes in the leaf essential oil of spike lavender (Muñoz-Bertomeu et al., 2007b). Because of this, both compounds were selected for the NMR and GC/MS analysis of the labeling experiments aimed to study the biosynthetic origin of monoterpenes in spike lavender. $^{13}CO_2$, [U-$^{13}C_6$]glucose and [1,2-$^{13}C_2$]mevalonate precursors were employed.

The experiments were performed with wild type and transgenic HMGR lines growing either in the greenhouse or *in vitro*.

II. 5. 2. 1. $^{13}CO_2$ labeling experiments

Wild type and HMGR5 spike lavender plants grown in the greenhouse were employed. Wild type plants were established from freshly *in vitro*

germinated seeds (see II.1.) which were planted in pot-trays with a mixture of peat moss and perlite (7:3) and maintained in the greenhouse at Dürnast (Weihenstephan, Technische Universität München, Germany). After 1 month, plantlets were transferred to pots (15 cm) with the same substrate and kept in the greenhouse for 4 months. Three-month old HMGR5 stock plants (about 15 cm in length) were prepared from the transgenic T_0 HMGR5 line cloned *in vitro* and acclimatized to greenhouse conditions as described in II.4.2. Wild type plants with the same age and grown under the same conditions as the HMGR5 plants were also employed as controls.

For $^{13}CO_2$ feeding, three or four potted plants were simultaneously placed in a closed gas incubation chamber (Biobox; GWS, Berlin, Germany) at 25°C and illuminated with white light (Figure 10). Prior to the labeling period (pulse phase), the chamber was flushed with synthetic air containing oxygen and nitrogen until CO_2 was fully removed. The plants were then fed with synthetic air containing 700 ppm of $^{13}CO_2$. During this pulse period, the concentration of $^{13}CO_2$ and $^{12}CO_2$ was typically detected at a ratio of 9:1. Subsequently, the plants were transferred to the laboratory and kept under standard environmental conditions. The time settings of each experiment are listed in Appendix I. Subsequently the samples were analyzed to detect the labeling pattern of camphor and cineol using NMR and/or GC/MS techniques, as described below.

Figure 10. Gas chamber for incubation of plants with $^{13}CO_2$.

The $^{13}CO_2$ labeling experiments as well as the NMR and GC/MS analysis were performed in Wolfgang Eisenreich's laboratory at the Technische Universität München, Department of Chemistry (Garching, München).

II. 5. 2. 2. [U-$^{13}C_6$]glucose labeling experiments

In a first series of experiments, wild type *in vitro* grown spike lavender plants cultured in liquid or agar-solidified medium were employed.

For experiments on solid medium, *in vitro* germinated seeds were cultured in 200 mL vessels (58 mm diameter; 92.5 mm high) containing 20 mL of sterile BM medium (Sigma-Aldrich) with 30 g/L sucrose, 7.5 g/L agar (Sigma) and 2 g/L of [U-$^{13}C_6$]glucose, pH of 5.7. Four germinated seeds were cultured per vessel, and a total of 80 vessels were prepared. Cultures were incubated in a growth chamber at 25ºC under a 16 h photoperiod. After 55 days, whole plants were harvested, frozen in liquid nitrogen and stored at -20ºC until analyzed. This way, 59.4 g of plant material was achieved.

For experiments in liquid medium, germinated seeds were placed in 100 mL flasks (10 seeds per flask) with 30 mL of sterile liquid medium (BM medium with 30 g/L of sucrose, and 2 g/L of [U-$^{13}C_6$]glucose). Cultures were incubated in an orbital shaker (100 rpm) at 25ºC, with a 16 h photoperiod. After 15 days of culture, the seedlings were transferred to new flasks with fresh medium (100 mL) and maintained under the same conditions for another 15 days. Then, plants were harvested, frozen in liquid nitrogen and stored at -20ºC until analysis. A total of 8 g were achieved by this method.

In a second experiment, spike lavender stems (1.5 cm in length) from *in vitro* grown wild type or HMGR5 lines were cultured in 200 mL vessels containing solidified BM medium with 2 g/L of [U-$^{13}C_6$]glucose . At least 25 vessels per line with four stems each were prepared. After 7, 14, 21 and 28 days, stem length and whorl number were scored. At each of these periods, plants from 5 vessels were harvested and stored at -80ºC until essential oil extraction. Previously, plant weight, root number and length were scored.

II. 5. 2. 3. [1,2-$^{13}C_2$]mevalonate labeling experiments

Stems (1 cm in length) isolated from *in vitro* germinated spike lavender seeds, obtained as previously described, were cultured in 200 mL vessels containing 20 mL of solidified BM medium with 2 g/L [1,2-$^{13}C_2$]mevalonate. At least 25 vessels with four stems each were prepared. After 7, 14, 21 and 28 days, stem length and whorl number were scored. At each of these periods, plants from 5 vessels were harvested and stored at -80ºC until essential oil extraction. Previously, plant weight, root number and length were scored.

II. 5. 2. 4. Essential oil extraction from labeled material

Depending on the analytical (GC/MS or ^{13}C NMR) method, two different extraction protocols were employed.

Samples for GC/MS analysis were extracted as follows: samples (100-200 mg) were introduced into 10 mL glass tubes and 2 mL of chloroform-d ($CDCl_3$) was added. After a gentle shake, the tubes were maintained for 15 min at room temperature; subsequently, a spatula of anhydrous sodium sulfate was added and tubes were left for 1 hour at room temperature. Finally, 1000 µl of chloroform-extract were placed into a 1.5 mL autosampler vial suitable for GC/MS measurements.

For ^{13}C NMR analyses, the plant material (600-1,000 mg) was split into 3 different 10 mL glass tubes. Then, 2 mL of chloroform-d was added to the first tube, shaked gently and left at room temperature for 15 minutes. The chloroform was then transferred into the second tube and the whole process repeated. This procedure was also performed for the third tube. A spatula of anhydrous magnesium sulphate was added to this tube and left for an hour at room temperature. Finally, 600 µl of the chloroform extract were placed in a NMR tube for ^{1}H and ^{13}C analyses.

II. 5. 2. 5. GC/MS measurements

The Gas Chromatograph (GC-17A and GC-2010), mass spectrometer (QP-5000 and GCMS-QP 2010 Plus), auto injector (AOC-20i) and software (Class 5000 and GCMSsolution) used for these measurements were acquired from Shimadzu (Duisburg, Germany). The column used was a silica capillary column Equity TM-5 (30 m x 0.25 mm x 0.25 µm film thickness) from Supelco Inc. (Bellefonte, PA, USA).

The measurements were performed as follows: the injector and interface temperature were 230ºC and 250ºC respectively. The temperature settings of the oven were: 70°C for 2 min, then 70°C-90°C with 2°C/min, then 90°C-130 °C with 5°C/min and finally 250°C for 1 min. The pressure program started at 76.1 kPa with a linear velocity of 40.0 cm/sec. The flow control was set to linear

velocity. The total flow was 16.1 mL/min while the column flow stayed at 1.19 mL/min. The split ratio was 1:10. The detector volts were approx. 0.8 keV (according to the last tuning result of the MS). The solvent cut was adjusted at 4 min and the sampling rate at 0.15 sec. The micro scan width was fixed at 0.1 u.

Each sample was analysed three times in SIM (single ion monitoring) mode. The relative intensities of the standards (camphor and 1,8-cineol) and the samples obtained from GC/MS analysis (peak integration) were processed according to previous publications (Braumann, 1966; Pickup and McPherson, 1976; Korzekwa et al., 1990; Lee et al., 1991). This evaluation results in the molar excess of carbon isotopologues of the main components camphor and 1,8-cineol only due to the enrichments from the ^{13}C precursor.

II. 5. 2. 6. NMR measurements

NMR analyses were performed as follows: For ^{1}H spectra, an Avance I 500 (UltraShield 500 MHz, SEI 500 S2 probe head (5 mm, inverse with Z-gradient), Autosampler B-ACS 60) from Bruker Instruments (Karlsruhe, Germany) was used. The software installed was TopSpin 2.1 also from Bruker Instruments.

For ^{13}C spectra, either an Avance DXR 500 (Cryomagnet BZH 500 MHz, Autosampler B-ACS 60) or an Advance III 500 system with an UltraShield PLUS 500 MHz magnet and a cryo probe head (5 mm CPQNP, $^{1}H/^{13}C/^{31}P/^{19}F/^{29}Si$ (Z-gradient), Autosampler B-ACS 120) both from Bruker Instruments (Karlsruhe, Germany) were used. The software installed was XwinNMR 3.1 and TopSpin 3.0, respectively (all from Bruker Instruments, Karlsruhe, Germany).

The measurements were performed at magnetic fields of 11.75 Tesla. The resonance frequencies of ^{1}H and ^{13}C were 500.13 MHz and 125.77 MHz, respectively, and the temperature was 300ºK. Data analysis was performed with the MestReNova Software (Mestrelab Research, Santiago de Compostela, Spain), TOPSPIN or XWIN NMR.

The one-dimensional ^{1}H and ^{13}C NMR spectra, and the two-dimensional COSY (magnitude mode or phase-sensitive), HSQC, HSQC-DEPTedited, HSQC-TOCSY, NOESY (with 1 sec mixing), TOCSY (with 60 ms mixing) and HMBC spectra were measured with standard Bruker parameter sets.

II. 6. Statistical analysis

Significance of the variation in phenotypic parameters between control and transgenic groups, as well as of treatment effects were determined using analysis of variance (ANOVA, SPSS 19 version for Windows, SPSS Inc.). When appropriate, significant differences among treatments were determined using Tukey's procedure (1953) which makes use of the studentized range and is applicable to pairwise comparisons of means. Also, means and standard variation (SD) or standard error of the mean (SE) were used when appropriate. Inheritance observed data were compared to the expected ratios using a chi-squared analysis with Yates's correction (Zar, 1996).

Results and Discussion

III. RESULTS AND DISCUSSION

III. 1. Generation and characterization of transgenic plants overexpressing genes of the terpene biosynthetic pathways

The selection of spike lavender plants with a high production of essential oil has been primarily based on conventional breeding methods. However, in the last few years newer, more sophisticated, procedures have been shown to be valuable complementary options for breeding purposes. Thus, transgenic spike lavender plants overexpressing the *Arabidopsis DXS* cDNA demonstrate that metabolic engineering of the MEP pathway is an appropriate approach for spike lavender breeding as it has been shown to provide significantly enhanced essential oil yields without apparent detrimental effects on plants (Muñoz-Bertomeu et al., 2006). To further investigate the metabolic engineering potential of the MEP pathway we targeted the second step (Hsieh and Goodman, 2005) of this pathway by overexpressing the *Arabidopsis DXR* cDNA in spike lavender. Previous studies in peppermint (*Mentha* x *piperita* L.) reported that an overexpression of the *DXR* gene led to an enhanced production of essential oil (Mahmoud and Croteau, 2001).

From an economical point of view, the biotechnological breeding of spike lavender should be addressed to improve both yield and quality of the essential oil. This can be achieved modifying the profile of the essential oil by the overexpression of monoterpene synthases. In spike lavender essential oil, a high percentage of linalool determines a good quality and thereby a higher price. Then, we studied whether the up-regulation of the LIS enzyme, catalyzing the synthesis of linalool from Geranyl-PP (Lavy et al., 2002), may modify the monoterpene profile of spike lavender. To this end, we generated transgenic plants overexpressing the *LIS* cDNA from *Clarkia breweri*. In other species, like in *Dianthus caryophyllus* (Lavy et al., 2002), *Solanum lycopersicum* (Lewinsohn et al., 2001) and *Petunia hybrida W115* (Lücker et al., 2001), *LIS* overexpression increased production of linalool and/or their derivatives (*trans*-linalool, 8-hydroxylinalool and S-linalyl-β-D- glucopyranoside respectively).

As a final approach to improve both quantity and quality of the spike lavender essential oil, we generated, by controlled crosses, transgenic plants bearing two of the genes involved in the terpene biosynthesis. Specifically we obtained the following double-transgenic plants: 1) plants containing the *HMGR* and *DXS* genes; and 2) plants containing the *DXS* and *LIS* genes.

III. 1. 1. Generation of transgenic plants overexpressing the *DXR* gene

Spike lavender plants were engineered by inserting a *DXR* gene from *Arabidopsis thaliana,* using the transformation protocol described by Nebauer et al., 2000. The efficiency of transformation (measured as the percentage of explants that produce at least one transgenic plant) with *DXR* gene was low (lower than 1%). Nevertheless seven kanamycin-resistant plants were generated. These plants were first screened by PCR for the presence of the *neomycin phosphotransferase* II (*npt*II) and *DXR* genes (data not shown). All *npt*II$^+$/*DXR*$^+$ plants were cloned, acclimatized to *ex vitro* conditions, and transferred to the greenhouse for further analyses. Finally, five independent primary lines (T_0), designated as DXR1 to DXR5, were obtained.

The external phenotype of these transgenic *DXR* plants was similar to the non-transformed (WT) control plants, except for line DXR1 that had less branched shoots bearing leaves with longer petioles and wider leaves (Figures 11 and 12).

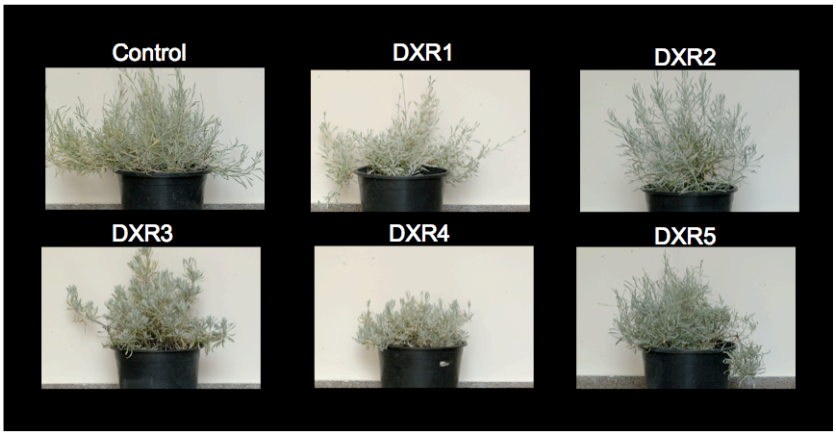


Figure 11. Phenotype of control and transgenic *DXR* spike lavender plants grown in the greenhouse.

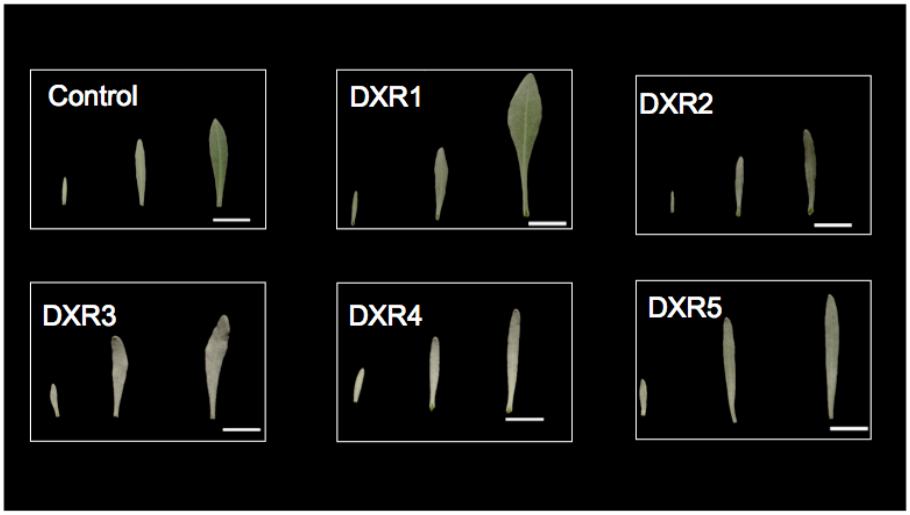


Figure 12. Leaf morphology of control and transgenic *DXR* spike lavender plants. Each panel shows (from left to right) leaves of the 1st, 2nd and 3rd whorls. Bar=1cm.

III. 1. 1. 1. Molecular analyses of DXR T_0 transgenic plants

The number of transgene inserts in each of the five lines was determined by Southern blotting using DXR probes. As shown in Figure 13, different patterns of insertions were observed. Lines DXR1 and DXR5 had 1 insertion each; line DXR3 had 4 insertions while lines DXR2 and DXR4 had 5 and 8 insertions, respectively.

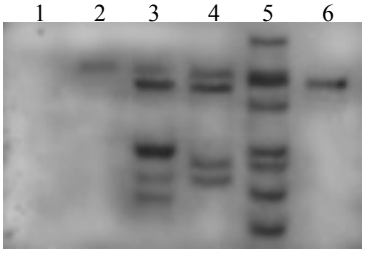


Figure 13. Southern Blot hybridization analysis of the *DXR* transgene in spike lavender plants. Lane 1, Control; lane 2, DXR1; lane 3, DXR2; lane 4, DXR3; lane 5, DXR4; lane 6, DXR5.

Northern Blot analysis was employed to determine *DXR* expression levels in two Sets of leaf samples, one containing leaves from the first and second

whorls and the other with leaves from the third whorl (Figure 14). Samples from the transgenic DXS6 line, previously obtained in our lab (Muñoz-Bertomeu et al., 2006), were also included. Leaf development did not affect *DXR* transgene expression since the transcript levels were similar in both Sets of samples (Figure 14A). In contrast, *DXS* expression was higher in the youngest leaves (Figure 14B). *DXR* transgene expression was line dependent, including lines with low (DXR3 and DXR4), middle (DXR2) and very high (DXR1 and DXR5, with one insert each) transcription levels (Figure 14A).

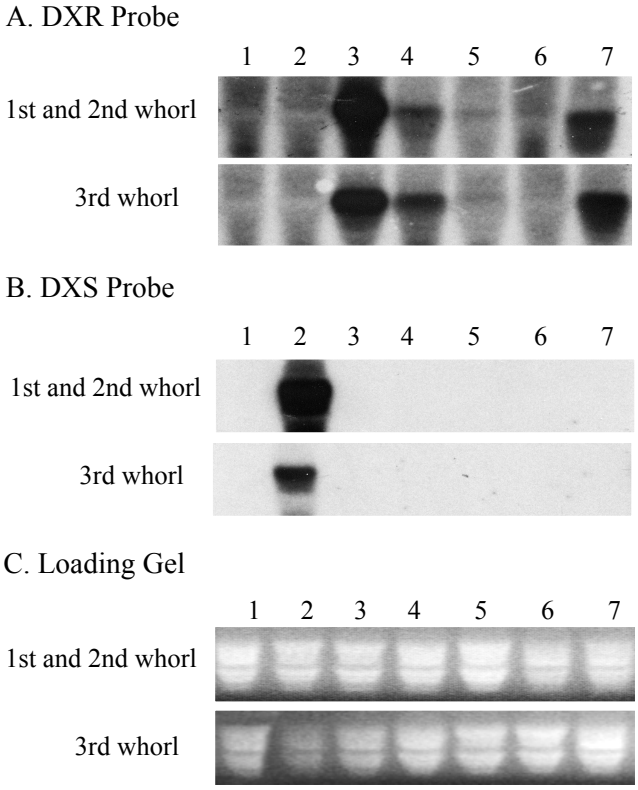


Figure 14. Expression of *DXR* (A) and *DXS* (B) genes in leaves (1st+ 2nd and 3rd whorls) of transgenic DXR, DXS and control spike lavender plants. (C), gel loading control. Lane 1, Control; lane 2, DXS6; lane 3, DXR1; lane 4, DXR2; lane 5, DXR3; lane 6, DXR4; lane 7, DXR5.

Western blotting was performed on extracts obtained from both young (whorl 1st to 3rd) and mature (whorl 4th to 10th) leaves. As shown in Figure 15 the level of the DXR protein depended on the developmental stage of the leaf being higher in the youngest leaves, including those from the control.

Transgenic DXR1 and DXR5 lines showed the higher protein content; note that these lines also presented a high mRNA transcript level. DXR4, showed the smallest content of protein among the transgenic plants. DXS6 line showed an increased amount of the DXR protein, suggesting that *DXS* overexpression promotes the synthesis of the DXR protein.

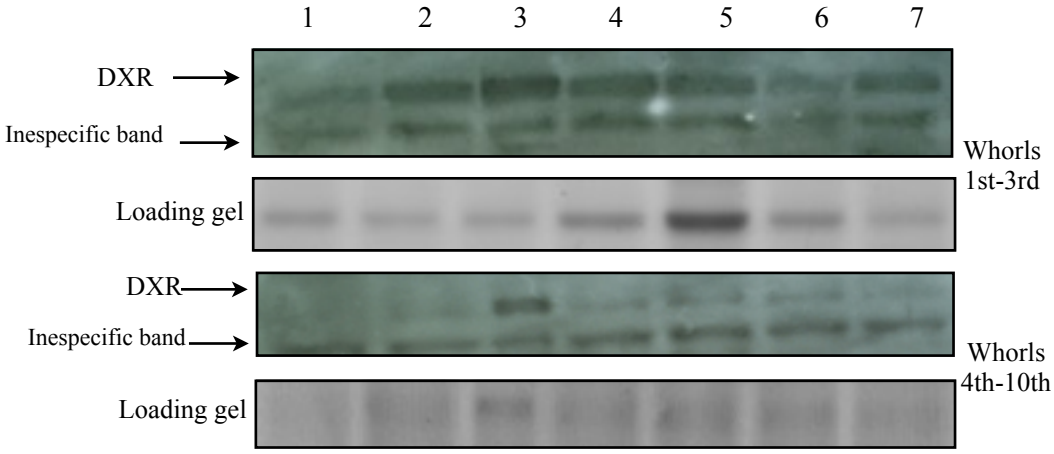


Figure 15. Western Blot analysis of the DXR protein in leaves from young and mature whorls of transgenic DXR, DXS and Control spike lavender plants. A Rubisco loading control is also shown. Lane 1, Control; lane 2, DXS6; lane 3, DXR1; lane 4, DXR2; lane 5, DXR3; lane 6, DXR4; lane 7, DXR5.

III. 1. 1. 2. Phenotypic analyses of DXR T_0 transgenic plants

Transgenic T_0 and control plants were screened for both essential oil and photosynthetic pigments content.

III. 1. 1. 2. 1. Essential oil content

The essential oil analysis was performed by direct extraction in hexane or by hydrodistillation, followed by gas chromatography (GC) separation of components and quantification using internal standards. Identification of the oil components was corroborated by GC/mass spectrometry (MS). Hexane extraction was performed in two Sets of leaf samples, one containing leaves from the first and second whorls (Set I) and the other with leaves from the third

whorl (Set II). Hydrodistillation was performed with air-dried flowers and leaves from 4-10th whorls (Set III).

Irrespective of the extraction method, most of the components found in spike lavender oils were present in the oil samples analyzed (Harborne and Williams, 2002) (Figure 16).

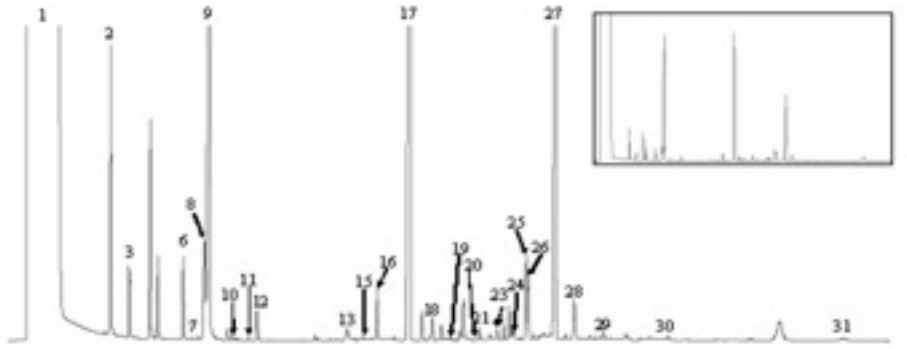


Figure 16. Typical chromatogram for the spike lavender essentail oil from leaves extracted by hydrodistillation. 1, hexane (solvent); 2, α-pinene; 3, camphene; 6, mircene; 7 α-terpinene; 8, limonene; 9, cineol; 10, γ- terpinene; 11, p-cymene; 12, terpinolene; 13, n-tetradecane (internal standard); 14, methyl butyrate; 15, octenol; 16, sabinene hydrate; 17, camphor; 18, linalool; 19, t-caryophylene; 20, lavandulol acetate; 21, myrtenal; 24, lavandulol; 25, α-terpineol; 26, borneol; 27, naphtalene; 28, geranyl acetate; 29, myrtenol; 30, geraniol; 31, caryophylene oxide.

In both transgenic and control lines, the essential oil constituents were within the range already described in spike lavender (Tables 4 to 9). Twenty-four constituents were identified, accounting for 87.10% to 93.53% of the total oils. In both transgenic and control plants, the essential oils determined consisted mainly of monoterpenes (85.26%-91.16% and 83.72%-91.42% in leaves and flowers, respectively). The most abundant fractions were oxygenated and hydrocarbon monoterpenes. The oxygenated monoterpenes ranged from 81.15% to 88.02% in leaves and from 81.45% to 87.86% in flowers. The hydrocarbon monoterpenes were less abundant, ranging from 2.71% to 4.91% in leaves and from 1.23% to 3.56% in flowers. The sesquiterpene fraction ranged from 0.62%-2.28%, in leaves and from 1.26% to 3.05% in flowers.

The most frequent monoterpenes in leaves for all lines were camphor (33.49-46.12%) and cineol (31.36-41.46%) except for the DXR4 line, that had higher camphor (60.47%) and borneol (12.57%) and lower cineol (3.85%). In flowers, the major monoterpenes among the determined fraction of essential oil were camphor (20.27-36.18%), cineol (4.32-22.79%), linalool (19.86-49.29%) and borneol (2.87-4.91%) except for the DXR4 line, characterized by a very low content in cineol (0.80%). Note that linalool, a major constituent in spike lavender oils from flowers (19.86-49.29%), is a minor constituent in the leaf oils (0.21-0.65%).

Data on essential oil yield and monoterpene production in leaves from Set I and Set II of transgenic DXS, DXR and control plant lines are summarized in Table 6, 8 and 9. ANOVA of these data demonstrated that both essential oil yield and monoterpene (hydrocarbons and oxygenated) production in transgenic and control plants were significantly affected by the line and leaf age, being also evident a significant interaction between both factors (Table 6).

The youngest leaves (Set I) from all lines produced the highest amount of essential oil (Table 6), but only Set I leaves from DXR2 and DXR5 transgenic lines accumulated significantly higher amounts of essential oil than controls (2.07- and 1.64-fold in DXR2 and DXR5 respectively). The remaining DXR transgenic lines produced similar amounts of leaf essential oils.

With the exception of DXR1 line, that presented high transcript levels but low essential oil production, a correlation among transgenic *DXR* mRNA and essential oil production was evident at least for the younger leaves (Figure 14 and Table 6). Note that DXS6 line produced the highest essential oil yield (2.76 fold compared to control) that was highly correlated to the transcription level of the gene (Figure 14 and Table 6).

Results and Discussion

Table 4. Percentage of essential oil constituents in hydrodistilled leaves (4^{th}-10^{th} whorls) from control and transgenic T_0 DXR spike lavender plants transformed with the *Arabidopsis DXR* gene. Reported values represent the means ± SD of three measurements.

	Control	DXS6	DXR1	DXR2	DXR3	DXR4	DXR5
α-pinene	1.80 ± 0.21	1.55 ± 0.07	1.08 ± 0.04	1.02 ± 0.09	1.85 ± 0.09	1.40 ± 0.09	1.67 ± 0.11
camphene	1.26 ± 0.14	1.40 ± 0.05	0.80 ± 0.04	0.82 ± 0.17	1.00 ± 0.07	1.46 ± 0.06	0.95 ± 0.02
α- terpinene	0.08 ± 0.03	0.06 ± 0.03	0.04 ± 0.01	0.10 ± 0.03	0.05 ± 0.02	0.03 ± 0.00	0.08 ± 0.04
myrcene	0.04 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.02	0.02 ± 0.00	0.10 ± 0.02
limonene	1.38 ± 0.75	0.69 ± 0.32	0.54 ± 0.05	0.82 ± 0.09	0.88 ± 0.08	1.27 ± 0.07	0.73 ± 0.09
γ- terpinene	0.12 ± 0.01	0.08 ± 0.01	0.08 ± 0.02	0.14 ± 0.03	0.06 ± 0.02	0.10 ± 0.009	0.10 ± 0.01
terpinolene	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.11 ± 0.07	0.02 ± 0.00	0.06 ± 0.01
p-cymene	0.19 ± 0.00	0.24 ± 0.01	0.13 ± 0.01	0.17 ± 0.00	0.13 ± 0.03	0.19 ± 0.01	0.17 ± 0.00
Hidrocarbon monoterpenes	4.91 ± 0.40	4.05 ± 0.18	2.71 ± 0.06	3.14 ± 0.33	4.11 ± 0.24	4.50 ± 0.26	3.87 ± 0.07
1.8-cineol	31.36 ± 1.65	29.44 ± 1.29	36.37 ± 0.26	37.81 ± 2.72	31.39 ± 0.53	3.85 ± 0.14	41.46 ± 0.90
sabinene hidrate	1.21 ± 0.05	1.11 ± 0.07	1.66 ± 0.09	1.45 ± 0.16	1.61 ± 0.10	0.67 ± 0.57	1.77 ± 0.05
camphor	41.47 ± 1.21	46.12 ± 1.83	39.17 ± 0.98	41.31 ± 1.34	38.78 ± 0.49	60.47 ± 0.06	33.49 ± 0.18
linalool	0.28 ± 0.11	0.65 ± 0.59	0.21 ± 0.01	0.26 ± 0.02	0.24 ± 0.02	0.22 ± 0.01	0.26 ± 0.02
lavandulol acetate	0.03 ± 0.00	0.03 ± 0.00	0.05 ± 0.04	0.06 ± 0.06	0.03 ± 0.01	0.05 ± 0.00	0.02 ± 0.01
lavandulol	1.58 ± 0.04	1.27 ± 0.22	1.11 ± 0.08	1.01 ± 0.11	1.56 ± 0.07	1.27 ± 0.03	1.50 ± 0.03
α-terpineol	2.39 ± 0.10	1.75 ± 0.07	1.77 ± 0.22	1.70 ± 0.19	2.44 ± 0.06	3.08 ± 0.49	2.29 ± 0.09
borneol	3.87 ± 0.21	2.28 ± 0.14	1.90 ± 0.22	1.07 ± 0.15	3.26 ± 0.43	12.57 ± 0.51	1.81 ± 0.05
geranyl acetate	0.15 ± 0.00	0.08 ± 0.00	0.28 ± 0.03	0.13 ± 0.02	0.30 ± 0.01	0.21 ± 0.00	0.11 ± 0.02
myrtenol	0.41 ± 0.27	0.07 ± 0.01	0.12 ± 0.03	0.17 ± 0.12	0.15 ± 0.00	0.14 ± 0.04	0.10 ± 0.03
geraniol	0.26 ± 0.02	0.28 ± 0.00	0.24 ± 0.02	0.26 ± 0.03	0.25 ± 0.02	0.35 ± 0.07	0.24 ± 0.01
myrtenal	1.05 ± 0.04	0.84 ± 0.05	0.95 ± 0.05	2.78 ± 1.68	1.14 ± 0.02	0.60 ± 0.02	1.18 ± 0.07
Oxigenated monoterpenes	84.07 ± 0.44	83.93 ± 3.59	83.82 ± 0.22	88.02 ± 2.02	81.15 ± 0.64	83.48 ± 0.27	84.24 ± 0.52
methyl butyrate	0.05 ± 0.00	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.08 ± 0.01	0.05 ± 0.00	0.07 ± 0.00
octenol	0.48 ± 0.04	0.14 ± 0.00	0.09 ± 0.01	0.14 ± 0.00	0.76 ± 0.03	1.13 ± 0.01	0.32 ± 0.01
Others	0.53 ± 0.04	0.20 ± 0.01	0.14 ± 0.01	0.18 ± 0.01	0.83 ± 0.04	1.17 ± 0.01	0.39 ± 0.01
t- caryophylene	0.14 ± 0.00	0.13 ± 0.01	0.27 ± 0.23	0.11 ± 0.03	0.17 ± 0.08	0.14 ± 0.00	0.14 ± 0.02
caryophylene oxide	0.49 ± 0.03	0.60 ± 0.06	2.02 ± 0.23	0.68 ± 0.09	1.36 ± 0.17	0.91 ± 0.07	0.57 ± 0.18
Sesquiterpenes	0.62 ± 0.03	0.74 ± 0.06	2.28 ± 0.28	0.79 ± 0.12	1.53 ± 0.25	1.05 ± 0.07	0.71 ± 0.19
Total essential oil	90.14 ± 0.41	88.92 ± 3.74	88.96 ± 0.09	92.13 ± 1.61	87.63 ± 0.22	90.20 ± 0.38	89.21 ± 0.49

Table 5. Percentage of essential oil constituents in hydrodistilled flowers from control and transgenic T_0 DXR spike lavender plants transformed with the *Arabidopsis DXR* gene. Reported values represent the means ± SD of three measurements.

	Control	DXS6	DXR1	DXR2	DXR3	DXR4	DXR5
α-pinene	0.46 ± 0.05	0.98 ± 0.05	0.30 ± 0.01	0.73 ± 0.06	0.33 ± 0.02	0.37 ± 0.02	0.57 ± 0.05
camphene	0.68 ± 0.06	1.04 ± 0.03	0.42 ± 0.01	0.71 ± 0.05	0.36 ± 0.02	0.54 ± 0.03	0.55 ± 0.03
α- terpinene	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.03 ± 0.00
myrcene	0.11 ± 0.01	0.25 ± 0.01	0.13 ± 0.00	0.22 ± 0.02	0.12 ± 0.01	0.16 ± 0.01	0.20 ± 0.02
limonene	0.50 ± 0.12	0.84 ± 0.30	0.52 ± 0.01	1.09 ± 0.09	0.24 ± 0.02	0.73 ± 0.02	0.59 ± 0.04
γ- terpinene	0.07 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.06 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.05 ± 0.01
terpinolene	0.11 ± 0.01	0.19 ± 0.00	0.13 ± 0.00	0.11 ± 0.01	0.06 ± 0.00	0.19 ± 0.01	0.14 ± 0.01
p-cymene	0.15 ± 0.01	0.17 ± 0.01	0.09 ± 0.01	0.18 ± 0.01	0.07 ± 0.01	0.08 ± 0.00	0.13 ± 0.01
Hidrocarbon monoterpenes	2.11 ± 0.15	3.56 ± 0.39	1.63 ± 0.01	3.12 ± 0.22	1.23 ± 0.06	2.10 ± 0.7	2.27 ± 0.17
1.8-cineol	22.46 ± 1.17	15.58 ± 0.3	4.32 ± 0.03	21.20 ± 0.05	7.33 ± 0.14	0.80 ± 0.11	22.79 ± 1.46
sabinene hidrate	0.54 ± 0.03	0.62 ± 0.02	0.99 ± 0.02	0.75 ± 0.03	2.39 ± 0.25	1.25 ± 0.16	0.68 ± 0.02
camphor	32.80 ± 0.58	33.27 ± 1.33	29.29 ± 0.45	36.18 ± 0.67	20.27 ± 0.96	35.59 ± 1.86	28.46 ± 1.82
linalool	25.04 ± 1.49	31.06 ± 1.33	43.62 ± 0.60	19.86 ± 1.32	49.29 ± 2,20	38.71 ± 1.47	23.12 ± 1.41
lavandulol acetate	0.03 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.02 ± 0.01
lavandulol	0.65 ± 0.08	0.69 ± 0.03	0.48 ± 0.05	0.69 ± 0.03	0.84 ± 0.20	1.01 ± 0.76	0.74 ± 0.16
α-terpineol	1.43 ± 0.09	0.71 ± 0.03	1.09 ± 0.09	1.49 ± 0.08	1.25 ± 0.11	1.52 ± 0.30	1.69 ± 0.11
borneol	3.21 ± 0.16	4.91 ± 0.17	4.44 ± 0.26	3.46 ± 0.12	2.87 ± 0.49	4.81 ± 0.31	2.96 ± 0.25
geranyl acetate	0.18 ± 0.01	0.15 ± 0.01	0.46 ± 0.02	0.39 ± 0.33	0.22 ± 0.02	0.08 ± 0.01	0.11 ± 0.08
myrtenol	0.21 ± 0.01	0.21 ± 0.01	0.13 ± 0.00	0.26 ± 0.01	0.14 ± 0.02	0.17 ± 0.05	0.22 ± 0.26
geraniol	0.24 ± 0.00	0.25 ± 0.01	0.18 ± 0.01	0.23 ± 0.01	0.16 ± 0.01	0.21 ± 0.04	0.19 ± 0.01
myrtenal	0.42 ± 0.02	0.38 ± 0.03	0.24 ± 0.07	0.52 ± 0.02	0.45 ± 0.27	0.16 ± 0.09	0.47 ± 0.03
Oxigenated monoterpenes	87.21 ± 0.48	87.86 ± 3.30	85.28 ± 0.33	85.06 ± 0.70	85.23 ± 0.93	84.34 ± 3.27	81.45 ± 4.9
methyl butyrate	0.18 ± 0.02	0.12 ± 0.01	0.23 ± 0.02	0.07 ± 0.00	0.25 ± 0.01	0.18 ± 0.01	0.23 ± 0.02
octenol	0.14 ± 0.01	0.06 ± 0.01	0.03 ± 0.00	0.06 ± 0.01	0.14 ± 0.01	0.22 ± 0.02	0.17 ± 0.01
Others	0.32 ± 0.03	0.18 ± 0.02	0.26 ± 0.02	0.13 ±0.00	0.39 ± 0.01	0.40 ± 0.03	0.40 ± 0.02
t- caryophylene	0.31 ± 0.08	0.93 ± 0.06	0.76 ± 0.07	0.55 ± 0.01	0.35 ± 0.02	0.33 ± 0.04	1.08 ± 0.07
caryophylene oxide	0.94 ± 0.07	1.01 ± 0.03	2.29 ± 0.11	1.35 ± 0.08	1.89 ± 0.22	1.32 ± 0.70	1.90 ± 0.17
Sesquiterpenes	1.26 ± 0.12	1.93 ± 0.09	3.05 ± 0.19	1.90 ± 0.07	2.24 ± 0.19	1.65 ± 0.66	2.98 ± 0.15
Total essential oil	90.89 ± 0.55	93.53 ± 3.36	90.22 ± 0.40	90.21 ± 0.85	89.10 ± 0.78	88.49 ± 2.71	87.10 ± 5.18

Table 6. Essential oil yield (μg/g fresh weight) in leaves from the first+second (Set I) and third (Set II) whorl of control and transgenic T_0 DXR and DXS spike lavender plants. Reported values represent the means ± SD of 3 measurements. For each column, values followed by the same letter are not significantly different according to Tukey's test at $p \leq 0.05$.

Line	Oil yield				Monoterpenes							
					Hidrocarbon				Oxigenated			
	Set I		Set II		Set I		Set II		Set I		Set II	
Control	4,143 ± 726	d	3,031 ± 123	bc	504 ± 27	d	172 ± 3	bc	3,458 ± 632	d	2,479 ± 173	b
DXS6	11,424 ± 664	a	5,071 ± 194	a	1,509 ± 102	a	590 ± 155	a	9,872 ± 558	a	4,469 ± 1100	a
DXR1	2,974 ± 175	d	1,183 ± 47	d	206 ± 27	e	51 ± 13	c	2,720 ± 156	de	1,125 ± 64	c
DXR2	8,597 ± 318	b	3,987 ± 460	ab	1,056 ± 19	b	102 ± 14	bc	7,517 ± 325	b	3,868 ± 447	a
DXR3	3,685 ± 257	d	1,796 ± 377	cd	515 ± 38	d	209 ± 132	bc	2,740 ± 187	de	1,577 ± 264	bc
DXR4	3,029 ± 143	d	1,143 ± 149	d	312 ± 23	e	124 ± 15	bc	2,362 ± 122	e	1,014 ± 134	c
DXR5	6,791 ± 321	c	2,469 ± 194	cd	872 ± 87	c	339 ± 105	b	5,831 ± 236	c	2,119 ± 216	bc

ANOVA — Mean Squares

Source of variation	df	Essential oil content	Hidrocarbon Monoterpenes	Oxigenated Monoterpenes
Line	6	32,405,670.10 **	574,908.15 **	26,193,178.23 **
Set	1	103,365,530.32 **	2,457,094.19 **	68,283,340.20 **
AxB	8	5,735,653.85 **	165,855.60 **	4,361,323.46 **
Error	28	235,797.58	5,338.90	180,461.04
Total	42			

** significant at $p \leq 0.001$

Essential oil yield in adult and young leaves cannot be directly compared (values calculated on dry and fresh weight basis, respectively). Nevertheless, taking into account the water content of developing spike lavender leaves (about 80%), it is clear that these leaves are more productive than mature ones, which is in agreement with previous studies (Muñoz-Bertomeu et al., 2008) and could be due to the low DXR protein level found in these mature leaves (Figure 15). As expected, and irrespective of the line, flowers produced more essential oil than leaves (Muñoz-Bertomeu et al., 2007a). As compared to control, only flowers from transgenic DXR2 line produced significantly more essential oils (1.32-fold); both hydrocarbon and oxygenated terpenes contributed to the increased oil yield of this plant (Table 7). In the other transgenic DXR lines

essential oil yield did no differ (DXR1, DXR3 and DXR5) or was reduced (DXR4) as compared to control (Table 7).

As stated above, transgenic DXS6 line was analyzed as an internal control. Leaf essential oil content in this line was significantly higher than that of control and transgenic DXR lines. This also holds true for flower essential oil. Note however that there was not significant difference in flower essential oil content between DXS6 and the most productive DXR2 transgenic line (Table 7).

Table 7. Essential oil yield and monoterpene production (mg/g dried weight) in hydrodistilled leaves (4th to 10th whorls) and flowers of control and transgenic, DXS and DXR spike lavender plants. Reported values represent the means ± SD of 3 measurements. For each column, values followed by the same letter are not significantly different according to Tukey's test at p≤0.05.

Line	Oil Yield		Monoterpenes			
			Hidrocarbon		Oxigenated	
	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
Control	17.38 ± 0.37 b	53.99 ± 2.97 bc	2.69 ± 0.08 b	3.22 ± 0.37 d	14.60 ± 0.29 b	50.28 ± 2.54 c
DXS6	25.24 ± 1.94 a	76.43 ± 5.53 a	3.12 ± 0.06 a	5.67 ± 0.31 b	22.01 ± 1.90 a	70.19 ± 5.47 a
DXR1	16.38 ± 0.83 b	42.83 ± 5.14 cd	1.99 ± 0.05 c	3.34 ± 0.11 d	14.29 ± 0.79 b	38.83 ± 5.01 de
DXR2	19.05 ± 1.58 b	71.28 ± 3.91 a	2.45 ± 0.03 b	7.07 ± 0.48 a	16.54 ± 1.60 b	63.59 ± 3.42 ab
DXR3	12.81 ± 0.84 c	59.96 ± 4.01 b	2.19 ± 0.10 c	4.67 ± 0.32 c	10.51 ± 0.80 c	54.54 ± 3.65 bc
DXR4	19.22 ± 1.05 b	37.82 ± 2.66 d	2.55 ± 0.17 b	4.38 ± 0.21 c	16.53 ± 1.00 b	33.05 ± 2.42 e
DXR5	18.69 ± 0.92 b	53.63 ± 2.96 bc	2.67 ± 0.08 b	5.72 ± 0.50 b	15.92 ± 0.84 b	46.90 ± 2.47 cd

ANOVA

Source of variation	df	Mean Squares					
		Essential oil yield		Hidrocarbon Monoterpenes		Oxigenated Monoterpenes	
		Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
Line	6	42.2 **	588.81 **	0.41 **	5.78 **	35.65 **	513.45 **
Error	14	1.39	16.15	0.01	0.12	1.32	14.07

** significant at p≤0.001

Results and Discussion

Table 8. Percentage of essential oil constituents in hexane extracts from leaves (1st+2nd whorls) of control and transgenic DXS and DXR spike lavender plants. Reported values represent the means of 3 measurements ± SD.

	Control	DXS6	DXR1	DXR2	DXR3	DXR4	DXR5
α-pinene	5.60 ± 1.10	5.13 ± 0.41	1.99 ± 0.31	5.13 ± 1.09	5.49 ± 2.50	3.57 ± 0.54	6.15 ± 0.37
camphene	0.63 ± 0.18	0.66 ± 0.05	0.19 ± 0.05	0.40 ± 0.06	0.75 ± 0.01	0.66 ± 0.32	0.65 ± 0.02
myrcene	1.50 ± 0.11	1.62 ± 0.05	1.16 ± 0.11	2.27 ± 0.34	1.45 ± 0.31	1.65 ± 0.07	1.28 ± 0.05
limonene	1.31 ± 0.11	1.19 ± 0.15	0.71 ± 0.04	1.26 ± 0.25	29.38 ± 4.08	2.02 ± 0.12	0.93 ± 0.05
γ- terpinene	0.87 ± 0.16	1.92 ± 0.11	0.39 ± 0.05	1.27 ± 0.04	0.30 ± 0.07	0.87 ± 0.03	0.71 ± 0.03
terpinolene	0.45 ± 0.02	0.47 ± 0.01	0.31 ± 0.02	0.40 ± 0.03	0.63 ± 0.06	0.85 ± 0.04	0.41 ± 0.01
p-cymene	0.17 ± 0.03	0.66 ± 0.24	0.08 ± 0.01	0.19 ± 0.01	0.10 ± 0.07	0.19 ± 0.00	0.11 ± 0.01
Hidrocarbon monoterpenes	10.53 ± 1.69	11.64 ± 1.02	4.83 ± 0.59	10.91 ± 1.82	10.15 ± 3.31	9.81 ± 1.11	10.24 ± 0.54
1.8-cineol	34.00 ± 1.60	32.24 ± 0.34	35.09 ± 1.09	35.98 ± 1.24	29.38 ± 4.08	12.10 ± 1.14	37.14 ± 0.84
sabinene hidrate	1.77 ± 0.04	1.80 ± 0.02	2.05 ± 0.06	1.97 ± 0.11	1.66 ± 0.25	0.85 ± 0.04	2.21 ± 0.06
camphor	29.61 ± 1.95	32.55 ± 0.34	24.43 ± 1.24	27.10 ± 1.3	27.50 ± 3.44	43.81 ± 1.49	27.62 ± 0.44
linalool	0.09 ± 0.00	0.12 ± 0.00	0.11 ± 0.00	0.14 ± 0.01	0.10 ± 0.01	0.14 ± 0.01	0.11 ± 0.00
lavandulol acetate	0.04 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.74 ± 0.18	0.01 ± 0.01	0.03 ± 0.01	0.04 ± 0.03
lavandulol	0.59 ± 0.10	0.60 ± 0.06	1.00 ± 0.04	0.74 ± 0.18	0.91 ± 0.14	0.46 ± 0.04	1.07 ± 0.04
α-terpineol	3.43 ± 0.18	3.88 ± 0.32	3.93 ± 0.11	4.39 ± 0.56	3.02 ± 0.54	1.15 ± 0.08	4.34 ± 0.14
borneol	3.84 ± 0.11	3.77 ± 0.24	4.07 ± 0.11	3.74 ± 0.51	5.45 ± 0.47	11.75 ± 0.83	4.02 ± 0.73
geranyl acetate	0.23 ± 0.01	0.10 ± 0.04	0.85 ± 0.01	0.19 ± 0.02	0.75 ± 0.12	0.54 ± 0.02	0.19 ± 0.01
myrtenol	0.07 ± 0.03	0.02 ± 0.01	0.07 ± 0.05	0.02 ± 0.03	0.01 ± 0.01	0.05 ± 0.00	0.00 ± 0.00
geraniol	0.07 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.02 ± 0.02	0.05 ± 0.05	0.00 ± 0.00
myrtenal	0.07 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.01 ± 0.01
Oxigenated monoterpenes	73.80 ± 2.04	75.16 ± 0.87	71.68 ± 1.24	74.35 ± 1.65	68.82 ± 4.99	70.97 ± 1.41	76.78 ±
methyl butyrate	0.002 ± 0.002	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.003 ± 0.003	0.00 ± 0.00	0.00 ± 0.00
octenol	0.63 ± 0.10	0.04 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.35 ± 0.22	1.27 ± 0.08	0.23 ± 0.02
Others	0.64 ± 0.10	0.04 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.35 ± 0.22	1.27 ± 0.08	0.23 ± 0.02
t- caryophylene	0.13 ± 0.01	0.14 ± 0.02	0.69 ± 0.02	0.13 ± 0.02	0.56 ± 0.10	0.19 ± 0.00	0.14 ± 0.01
Sesquiterpenes	0.13 ± 0.01	0.14 ± 0.02	0.69 ± 0.02	0.13 ± 0.02	0.56 ± 0.10	0.19 ± 0.00	0.14 ± 0.01
Total essential oil	85.10 ± 2.23	86.98 ± 0.57	77.20 ± 1.75	85.40 ± 1.72	80.89 ± 5.66	82.24 ± 2.56	87.40 ± 1.76

Table 9. Percentage of essential oil constituents in hexane extracts from leaves (3rd whorl) of control and transgenic DXS and DXR spike lavender plants. Reported values represent the means of 3 measurements ± SD.

	Control	DXS6	DXR1	DXR2	DXR3	DXR4	DXR5
α-pinene	1.92 ± 0.22	3.48 ± 0.30	0.58 ± 0.50	0.00 ± 0.00	3.51 ± 3.03	1.92 ± 0.38	3.54 ± 0.25
camphene	0.28 ± 0.28	0.87 ± 0.05	0.05 ± 0.04	0.00 ± 0.00	0.63 ± 0.55	0.69 ± 0.26	0.47 ± 0.49
myrcene	1.08 ± 0.02	1.64 ±0.03	1.37 ± 0.44	0.73 ± 0.02	1.73 ± 0.45	1.61 ± 0.15	1.88 ± 0.09
limonene	1.26 ± 0.05	2.30 ± 0.41	1.01 ± 0.02	0.51 ± 0.05	1.81 ± 0.26	3.10 ± 0.30	1.98 ± 0.13
γ- terpinene	0.48 ± 0.09	1.59 ± 0.29	0.20 ± 0.02	0.74 ± 0.07	0.16 ± 0.14	0.62 ± 0.10	1.17 ± 0.06
terpinolene	0.31 ± 0.03	0.29 ± 0.02	0.31 ± 0.06	0.28 ± 0.01	0.80 ± 0.07	0.89 ± 0.17	0.54 ± 0.07
p-cymene	0.09 ± 0.01	0.20 ± 0.02	0.04 ± 0.02	0.09 ± 0.00	0.14 ± 0.12	0.23 ± 0.02	0.17 ± 0.01
Hidrocarbon monoterpenes	5.40 ± 0.70	10.38 ± 1.12	3.55 ± 1.11	2.36 ± 0.14	8.79 ± 4.61	9.05 ±1.38	9.76 ± 1.10
1.8-cineol	35.32 ± 0.52	33.56 ± 0.21	38.13 ± 0.85	39.35 ± 1.54	29.15 ± 2.42	13.49 ± 0.98	40.34 ± 0.84
sabinene hidrate	2.07 ± 0.06	1.94 ± 0.10	2.51 ± 0.14	2.48 ± 0.07	1.87 ± 0.32	0.84 ± 0.04	2.57 ± 0.07
camphor	30.98 ± 1.49	35.17 ± 0.17	28.50 ± 0.65	34.68 ± 1.55	28.64 ± 4.58	42.01 ± 0.97	24.44 ± 0.63
linalool	0.11 ± 0.00	0.12 ± 0.01	0.14 ± 0.06	0.12 ± 0.00	0.12 ± 0.03	0.13 ± 0.01	0.12 ± 0.01
lavandulol acetate	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.02	0.01 ± 0.00	0.09 ± 0.09	0.00 ± 0.00
lavandulol	0.25 ± 0.04	0.11 ± 0.01	0.41 ± 0.29	1.49 ± 0.03	1.18 ± 0.32	0.12 ± 0.04	1.51 ± 0.03
α-terpineol	4.74 ± 0.26	3.84 ± 0.25	5.29 ± 0.41	5.61 ± 0.06	4.05 ± 1.18	1.53 ± 0.13	5.62 ± 0.40
borneol	3.98 ± 0.42	3.05 ± 0.22	3.82 ± 0.49	3.36 ± 0.34	5.16 ± 1.86	13.01 ± 0.62	2.77 ± 0.12
geranyl acetate	0.72 ± 0.05	0.17 ± 0.09	1.58 ± 0.19	0.47 ± 0.05	1.08 ± 0.40	0.99 ± 0.09	0.64 ± 0.07
myrtenol	0.14 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
geraniol	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
myrtenal	0.14 ± 0.02	0.16 ± 0.02	0.06 ± 0.01	0.02 ± 0.00	0.06 ± 0.02	0.07 ± 0.01	0.10 ± 0.01
Oxigenated monoterpenes	78.47 ± 0.88	78.13 ± 1.03	80.45 ± 1.06	87.60 ± 1.73	71.30 ± 3.26	72.28 ± 1.44	78.10 ± 1.11
methyl butyrate	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
octenol	1.54 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Others	1.54 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
t- caryophylene	0.20 ± 0.01	0.12 ± 0.01	0.30 ± 0.20	0.21 ± 0.02	0.23 ± 0.01	0.18 ± 0.01	0.21 ± 0.01
Sesquiterpenes	0.20 ± 0.01	0.12 ± 0.01	0.30 ± 0.20	0.21 ± 0.02	0.23 ± 0.01	0.18 ± 0.01	0.21 ± 0.01
Total essential oil	85.62 ± 0.35	88.63 ± 0.12	84.31 ± 1.20	90.17 ± 3.00	80.32 ± 2.37	81.51 ± 2.13	88.07 ± 0.82

III. 1. 1. 2. 2. Photosynthetic pigments content

Chlorophyll and carotenoid content increased in parallel with the developmental stage of the leaf. The effect of *DXR* transgene expression on total chlorophylls was age dependent; thus, in the youngest leaves (Set I) chlorophyll content increased significantly as compared to controls in all transgenic plants except in DXR4, that was similar, and in DXR5 that decreased. In contrast, only one of the transgenic lines produced more chlorophyll than the controls in Set II (line DXR2) or Set III (Line DXR3) whorls (Table 10), whereas the rest of the lines did not show variation respect to the control. Transgenic DXS6 line

Table 10. Total chlorophyll content (μg/g fresh weight) in leaves from 1st+2nd (Set I), 3rd (Set II) and 4-10th (Set III) whorls of control and transgenic DXS and DXR spike lavender plants. Reported values represent the mean ± SD of at least 3 measurements. Mean values followed by the same letter are not significant different according to Tukey's test at $p \leq 0.05$.

Line	Whorls					
	Set I		Set II		Set III	
Control	365.90 ± 19.30	d	608.47 ± 43.31	b	1,088.56 ± 203.28	bc
DXS6	571.09 ± 12.58	a	805.54 ± 30.03	a	1,575.45 ± 186.15	ab
DXR1	441.91 ± 24.55	c	642.27 ± 14.14	b	768.81 ± 198.97	c
DXR2	523.79 ± 52.32	ab	808.51 ± 85.45	a	1,399.85 ± 221.83	ab
DXR3	482.01 ± 17.88	bc	654.05 ± 37.30	b	1,790.98 ± 195.24	a
DXR4	432.71 ± 22.25	cd	578.25 ± 84.01	b	1,286.04 ± 251.24	abc
DXR5	281.06 ± 28.88	e	517.61 ± 15.33	b	1,072.82 ± 146.81	bc

ANOVA

		Mean Squares		Mean Squares		Mean Squares
Source of variation	df	Set I	df	Set II	df	Set III
Line	6	28,611.92 **	6	36,599.72 **	6	368,385.80 **
Error	15	757.19	14	2,709.03	17	41,123.58

* significant at $p \leq 0.05$, ** significant at $p \leq 0.001$

accumulated more chlorophyll than the control in young leaves (Sets I and II) but did not significantly differ from the controls in adult leaves (Table 10). Chlorophyll content in young leaves from DXS6 was also higher than in

transgenic DXR lines except for DXR2 line that did not significantly differ (Table 10).

The effect of *DXR* transgene on carotenoid content followed the same pattern described above for chlorophylls, although the increased amount of this pigment in the young leaves (Sets I and II) from lines DXR1 and DXR3 or DXR2, respectively, were not significant in relation to their controls (Table 11). Note that the highest photosynthetic pigments content of adult leaves from DXR3 transgenic line correlated negatively with its essential oil yield (Table 6, 10 and 11). The youngest (Set I) leaves from DXS6 line also accumulated more carotenoids than the control and two of the transgenic DXR lines (DXR3 and DXR5).

Table 11. Carotenoid content (µg/g fresh weight) in leaves from 1st+2nd (Set I), 3rd (Set II) and 4-10th (Set III) whorls of control and transgenic DXS and DXR spike lavender plants. Reported values represent the mean ± SD of at least 3 measurements. Mean values followed by the same letter are not significant different according to Tukey's test at p≤0.05.

Line	Whorls					
	Set I		Set II		Set III	
Control	79.04 ± 5.25	c	128.40 ± 6.74	ab	225.74 ± 47.49	bc
DXS6	101.21 ± 3.21	a	135.69 ± 6.93	ab	324.53 ± 31.51	ab
DXR1	90.22 ± 6.66	abc	122.64 ± 3.41	ab	183.36 ± 46.31	c
DXR2	94.26 ± 6.64	ab	144.12 ± 16.89	a	271.44 ± 44.87	abc
DXR3	85.56 ± 2.58	bc	123.49 ± 11.67	ab	391.26 ± 41.95	a
DXR4	88.09 ± 4.63	abc	109.11 ± 21.33	b	282.66 ± 69.77	abc
DXR5	57.90 ± 5.89	d	110.29 ± 12.78	ab	226.36 ± 41.11	bc

ANOVA

		Mean Squares		Mean Squares		Mean Squares
Source of variation	df	Set I	df	Set II	df	Set III
Line	6	577.73 **	6	483.87 *	6	15,531.16 *
Error	15	25.67	14	163.53	17	2,247.80

* significant at p≤0.05, ** significant at p≤0.001

III. 1. 1. 3. Inheritance of the *DXR* gene

Self-pollination of transgenic DXR plants was performed for three consecutive years, but the quantity of seeds produced was low. Thus, seeds were only recovered from lines DXR2 (9 seeds), DXR4 (3 seeds) and DXR5 (12 seeds). Finally, 4 T_1 plants from DXR2 and 5 T_1 plants from DXR5 were produced. PCR analyses demonstrated the inheritance of the *DXR* transgene in all of the 5 DXR5 progenies and in 3 out of the 4 DXR2 progenies (Figure 16). After *in vitro* seed germination, seedlings were grown under controlled conditions for two months and transferred to the greenhouse. We were able to acclimatize the 5 plants from line DXR5 (named after DXR5-1 to DXR5-5) and 3 plants from line DXR2 (named after DXR2-1 to DXR2-3), from which two inherited the *DXR* transgene and one did not (Figures 16 and 17).

The number of transgene inserts in each of the five lines was determined by Southern blotting using both *npt*II and *DXR* probes. As shown in Figure 18 and 19, the number of inserts in the progenies were in accordance with those of the mother plants. Progenies of plant DXR5 had 1 insertion each (Figure 18); progenies of line DXR2 had 0, 2, or 4 insertions (Figure 19), proving the independent inheritance of the inserts.

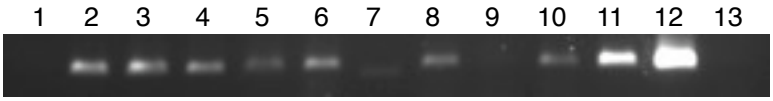


Figure 16. PCR analysis of the *DXR* gene in T_1 spike lavender plants. Lane 1, control; Lane 2 DXR 1; Lanes 3-6 and 8 DXR5 progeny; Lane 7 Marker; Lane 9-11 DXR2 progeny; Lane 12 plasmid; Lane 13 water.

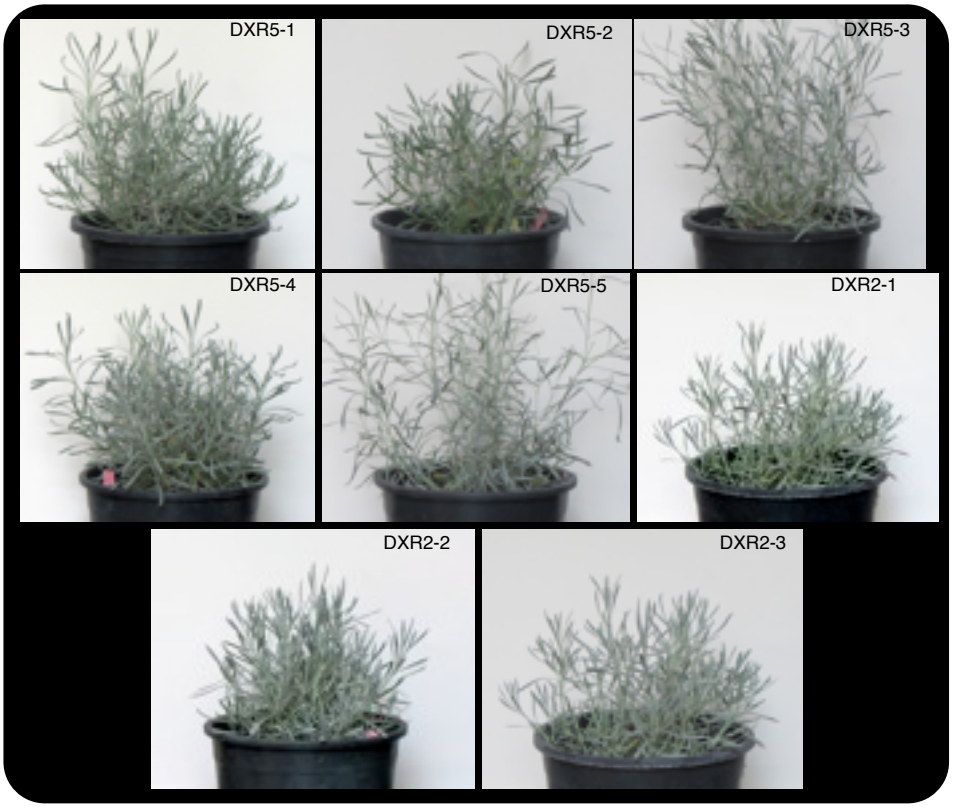


Figure 17. Progenies of the transgenic DXR5 and DXR2 in transgenic spike lavender plants grown in the greenhouse.

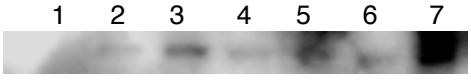


Figure 18. Southern Blot analysis of the *DXR* gene in transgenic T_1 spike lavender plants. Lane 1, control; Lane 2, DXR5; Lane 3-7, DXR5 progeny.

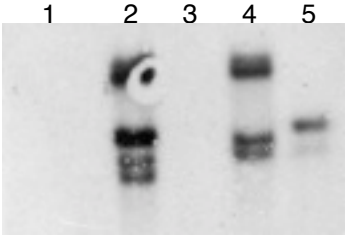


Figure 19. Southern Blot analysis of the *DXR* gene in transgenic T_1 spike lavender plants. Lane 1, negative control; Lane 2, DXR 2; Lane 3-5, DXR2 progeny.

III. 1. 1. 4. Discussion

The DXR enzyme catalyzes the first committed step of the MEP pathway (Carretero-Paulet et al., 2002). It was therefore expected that the up regulation of this enzyme in spike lavender leads to an increased yield in the essential oil, as was previously achieved in this species by overexpressing the *DXS* gene, coding for the first enzyme of the MEP pathway (Muñoz-Bertomeu et al., 2006). Nevertheless, this was not the general effect in most of the T_0 DXR transgenic spike lavender lines. In fact, when compared to controls, only the line DXR2 showed an increased essential oil content in leaves (2.0-fold for Set I and 1.3-fold for Set II) and flowers (1.3-fold). Line DXR5 also showed a high essential oil production than controls but only in the youngest leaves (1.6-fold). These results sharply contrast with those obtained in peppermint, another aromatic species (Mahmoud and Crouteau, 2001) where those transgenic lines overexpressing *DXR* gene had up to 44% increased oil yields when compared with wild type controls; this increased phenotype has been maintained, although reduced to 18%, over several growth seasons in field testing (Lange et al., 2011). Reasons for the differential effect of the *DXR* transgene between both species have not been investigated but it is clear that is not due to co-suppression effect since none of the DXR transgenic lines presented abnormal pigmentation as did some of the peppermint transgenic lines (Mahmoud and Crouteau, 2001). In fact, the role of DXR in the regulation of MEP pathway still remains unclear, since the production of MEP from DXP catalyzed by DXR might limit the biosynthesis of at least some plastidial isoprenoids in some, but not all, plants (see Rodríguez-Concepción et al., 2006 for review). Differences in the control of the metabolic flux through the MEP pathway between peppermint and spike lavender is also evident in the results obtained when the *DXS* gene was constitutively expressed in these species. Thus, while in spike lavender it led to the most highly increased essential oil yields reported thus far (up to 359% in leaves, and up to 74% in flowers; Muñoz-Bertomeu et al., 2006), did not result in significant increases in essential oil yield in peppermint (Lange et al., 2011). Corroborating this, none of the DXR overexpressing lines (DXR2 and DXR5) was as effective in leaf essential oil yield as the DXS6 line, used as a control in the present work.

In those species, where the DXR enzyme is rate-determining, the overexpression of the gene leads to a general increase in plastidial terpenes like chlorophylls and carotenoids (Estévez et al., 2001, Carretero-Paulet et al., 2002, 2006; Hasumuma et al., 2008; Xing et al., 2010). This also holds true in three out of the five spike lavender transgenic DXR plants, although the effect was particularly evident in the youngest leaves. The increased content in these photosynthetic pigments was also observed in young leaves from DXS6 line, an effect that was not previously observed when only adult leaves (4-10 whorl) were analyzed (Muñoz-Bertomeu et al., 2006).

In *Arabidopsis* Carretero-Paulet et al., (2006) demonstrated that overexpression of DXS always produced more plastidial terpenes than the DXR transgenic lines. In our experiments, however, no clear differences in chlorophyll and carotenoid contents were found between DXS6 and the only DXR line showing an increased essential oil phenotype (DXR2).

Metabolic control analysis demonstrate that changes in gene expression not always represent similar changes in protein level or, most importantly, in enzyme activity or metabolic production (Rodríguez-Concepción et al., 2006). In the present work, neither *DXR* transgene insert number nor *DXR* transcript abundance of the T_0 plants correlated clearly with the essential oil and photosynthetic content of transgenic plants. On the other hand, the protein content of DXR T_0 and DXS6 plants was higher in young leaves, suggesting that the overexpression of the *DXS* gene and/or protein may also lead to an overproduction of the DXR protein. In *Arabidopsis*, DXR protein is also modulated through development, in contrast to the constitutive DXR expression in tomato fruit ripening (Rodríguez-Concepción et al., 2001).

In conclusion, contrary to what was report in peppermint, overexpression of the *Arabidopsis thaliana DXR* gene in spike lavender, do not leads to a generalized increase in essential oil yield. These results also contrast to those previously reported in DXS overexpressing spike lavender plants (Muñoz-Bertomeu et al., 2006) that significantly enhanced essential oil yield. All these suggest that the control flux of the MEP pathway is primarily exerted by the DXS enzyme. Nevertheless, current studies with T_1 progenies of the DXR2 and

DXR5 transgenic lines will help to clarify the role of DXR enzyme controlling MEP pathway in spike lavender.

III. 1. 2. Generation of transgenic plants overexpressing the *LIS* gene

Spike lavender transformation was achieved after the Nebauer et al., (2000) protocol using *A. tumefaciens* strain C58 harboring the plasmid *pBILIS* containing the *Clarkia breweri LIS* and *npt*II genes. All kanamycin-resistant plants (26) were first screened by PCR for the presence of *neomycin phosphotransferase* II (*npt*II) and *LIS* genes (data not shown). All *npt*II+/*LIS*+ plants (14) were cloned, acclimatized to *ex vitro* conditions, and transferred to the greenhouse for further analyses. Eight putatively independent primary transformants (T_0), designed as LIS1 to LIS8, were obtained. All the transgenic lines were morphologically indistinguishable from the control plants (Figure 20).

Figure 20. Forty-eight weeks transgenic spike lavender plant overexpressing the *LIS* gene

III. 1. 2. 1 Molecular analyses of the LIS T_0 plants

The number of *LIS* transgene inserts in the 8 transgenic lines was determined by Southern Blot analysis. As shown in Figure 21, the eight transgenic lines displayed different hybridization patterns indicating independent transformation events. Specifically, lines had one (LIS1 and LIS4), two (LIS5 and LIS6), three (LIS8 and LIS7) or four (LIS3 and LIS2) inserts of the *LIS* transgene. Identical hybridization patterns were obtained for the *npt*II transgene (data not shown), corroborating the insertion of the complete T-DNA.

Northern Blot analyses were first performed in leaves from the first and second whorls of all T_0 transgenic and control plants (Figure 22). *LIS* mRNA

was detected in all lines but the control. Irrespective of the transgenic line, the level of transcripts drastically decreased in the second whorl in comparison with the first one. In this whorl the higher expressions were found in lines LIS2, LIS3, LIS7 and LIS8 (Figure 23).

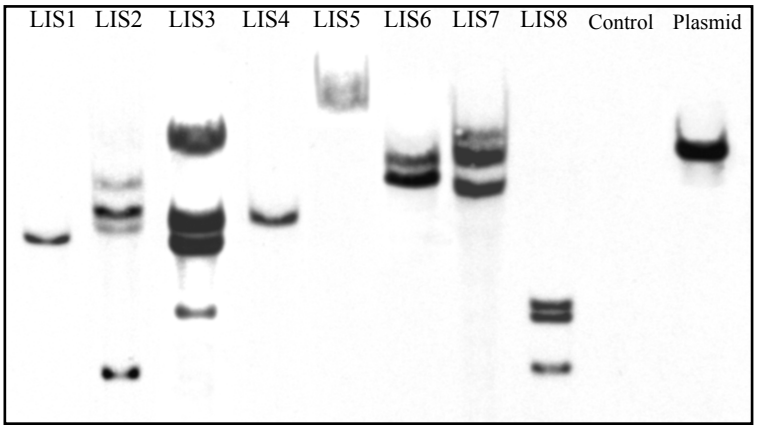


Figure 21. Southern Blot hybridization analysis of the *LIS* transgene in spike lavender T_0 plants (LIS1 to LIS8 lines, control plants and plasmid).

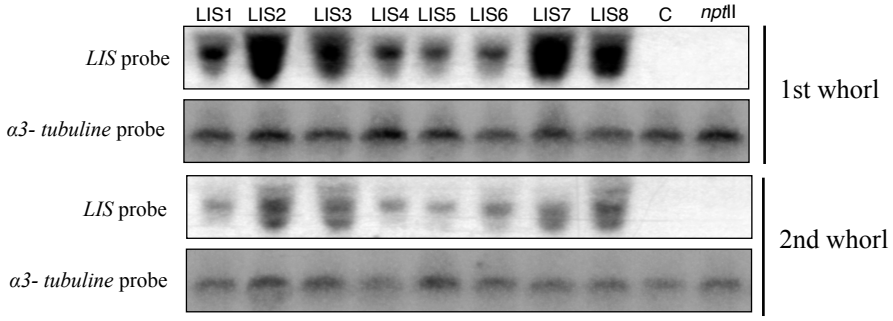


Figure 22. Expression analysis of the *LIS* transgene in leaves from the first and 2nd whorls of control (C) and the eight LIS transgenic T_0 spike lavender lines. *npt*II, transgenic *npt*II spike lavander plant. The expression of the *α3- tubuline* gene is shown to certify equal loading.

The expression of the *LIS* transgene was also determined in cohorts of leaves sampled at different developmental stages of lines LIS3 (with 4 inserts of

the transgene) and LIS4 (with 1 insert). Developmental leaf stages were: a) 1st whorl with fully open leaves; b) 2nd whorl; c) 3rd and 4th whorls; d) 5th-7th whorls; e) and 8th-10th whorls (see Figure 9). In both lines, transgene expression was dependent on the developmental stage of the leaves, with the youngest (a) showing the highest expression (Figure 23). No *LIS* gene signal was detected in controls at any of the developmental leaf stages tested (Figure 23).

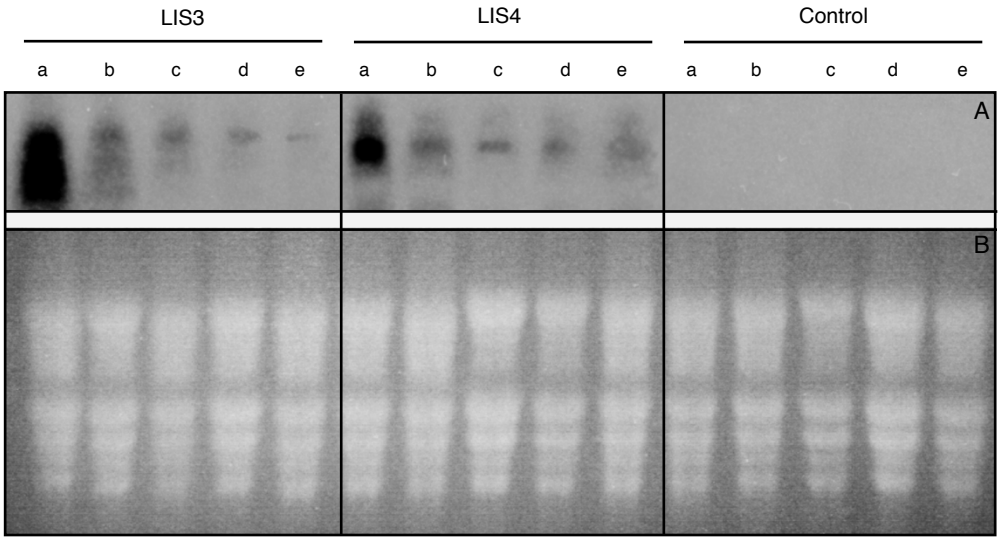


Figure 23. Expression analysis of the *LIS* gene during leaf development in transgenic T_0 spike lavender lines LIS3 and LIS4. A) Transcript acummulation in leaves at five developmental stages (a-e) from transgenic and control plants. (B) Gel loading control for the Northern Blot.

III. 1. 2. 2. Essential oil content in LIS T_0 plants

S-Linalool synthase catalyzes the conversion of geranyl diphosphate to linalool. This reaction has been shown to be the source of linalool in plants (Lücker et al., 2001). Nevertheless, in the bacteria *Castellaniella defragrans,* linalool can be also formed from other monoterpenes, such as myrcene and geraniol (Brodkorb et al., 2010). The possible metabolic pathways implicated in the biosynthesis of linalool are summarized in Figure 24. Because of this, to investigate whether *LIS* gene overexpression affected the terpene profile of spike lavender leaf essential oil, we quantified, along with linalool, myrcene and geraniol. Other quantitatively important monoterpenes such as cineol and

camphor as well as essential oil yield were also determined by direct extraction in hexane.

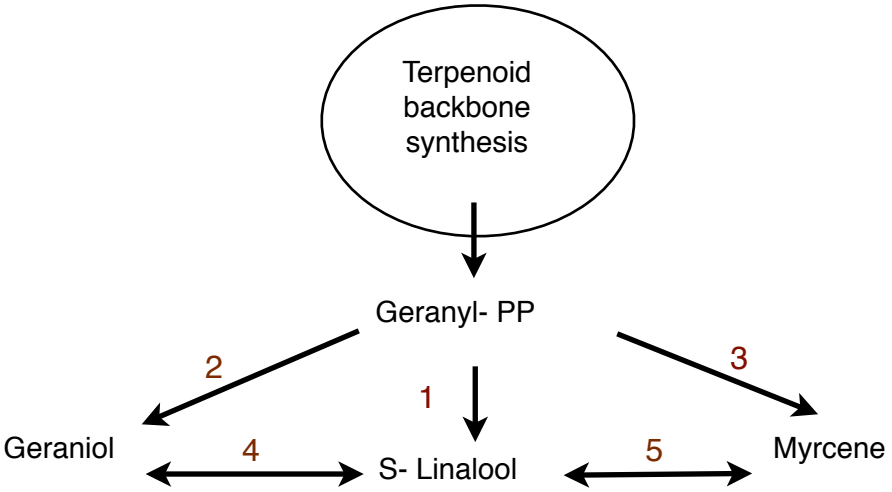


Figure 24: Scheme for the synthesis of linalool from geranyl-PP, myrcene or geraniol. Enzymes: (1) S-linalool synthase (2) monoterpenyl-pyrophosphatase (3) myrcene synthase (4) geraniol hydroxymutase (5) linalool dehydratase - hydrolyase.

In transgenic spike lavender plants overexpressing the limonene synthase gene, the high amount of limonene was detected in developing leaves (Muñoz-Bertomeu et al., 2008). Then, the first series of essential oil analyses were performed in leaves from the first and second whorls of the 8 LIS transgenic lines and controls. GC and GC/MS determinations allowed the identification of 19 constituents, accounting for 95% to 97% of the oil. In all transgenic and control lines, the content in linalool, myrcene and geraniol was higher in leaves from the first whorl, which positively correlate with the *LIS* mRNA levels in these leaves (Table 12 and Figure 20). Linalool production in leaf oils of spike lavender significantly increased (from 100 to 1,000%) in 7 out of the 8 transgenic lines as compared to controls; these increases were particularly striking for lines LIS3 (10.8- and 12.8-fold in the first and second whorl, respectively) and LIS4 (12.7- and 9.4-fold in the first and second whorl,

respectively). There was not a clear correlation between linalool and myrcene or geraniol contents, being difficult to establish whether these monoterpenes could contribute to linalool production in spike lavender (Table 12).

Table 12. Content (μg/g fresh weight) of Myrcene, Linalool, and Geraniol in hexane extracted essential oil from leaves (whorl 1 and 2) of control and transgenic T_0 spike lavender plants transformed with the *Clarkia breweri LIS* gene. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey's test at P≤0.05.

Line	Whorl	Myrcene	Linalool	Geraniol	Whorl	Myrcene	Linalool	Geraniol
LIS1	1	72.8 ± 0.2 d	8.7 ± 0.1 d	2.3 ± 0.0 cd	2	21.6 ± 0.4 ef	2.1 ± 0.0 de	0.7 ± 0.0 de
LIS2	1	79.9 ± 0.9 c	10.1 ± 0.1 c	2.9 ± 0.0 ab	2	38.8 ± 1.6 b	3.0 ± 0.1 de	1.2 ± 0.1 cd
LIS3	1	55.8 ± 0.5 f	57.1 ± 0.2 a	1.1 ± 0.1 e	2	19.3 ± 0.6 f	45.7 ± 0.2 b	0.4 ± 0.0 e
LIS4	1	116.1 ± 4.4 a	61.7 ± 1.7 b	3.1 ± 0.1 a	2	51.3 ± 1.2 a	33.4 ± 1.1 a	3.0 ± 0.1 b
LIS5	1	63.3 ± 1.1 e	6.3 ± 0.0 e	1.9 ± 0.2 d	2	12.5 ± 2.1 g	1.4 ± 0.0 e	0.6 ± 0.1 e
LIS6	1	37.8 ± 3.1 g	3.6 ± 0.0 g	0.7 ± 0.1 e	2	15.0 ± 0.4 g	3.0 ± 0.0 de	1.3 ± 0.0 c
LIS7	1	93.8 ± 0.2 b	5.1 ± 0.0 f	2.7 ± 0.3 bc	2	25.2 ± 0.6 de	2.9 ± 0.1 de	6.1 ± 0.5 a
LIS8	1	79.8 ± 0.6 c	6.7 ± 0.0 e	3.1 ± 0.2 a	2	33.5 ± 2.2 c	5.4 ± 0.1 c	5.7 ± 0.1 a
Control	1	61.2 ± 0.1 ef	4.9 ± 0.0 f	2.2 ± 0.0 d	2	26.5 ± 0.8 d	3.6 ± 0.0 d	1.6 ± 0.0 c

ANOVA

		Mean Squares					
		1st whorl			2nd whorl		
Source of Variation	df	Myrcene	Linalool	Geraniol	Myrcene	Linalool	Geraniol
Line	8	1,539.65**	981.41**	2.27**	456.89**	1,533.97**	14.38**
Error	17	3.70	0.16	0.02	1.69	0.33	0.03

** significant at p≤0.001

Based on the above results, essential oil content in the transgenic lines LIS3 and LIS4 was extended to older leaves. Specifically, we analyzed extracts from cohorts of leaves sampled at different positions in the stems (a-e; Figure 9). Along with linalool, myrcene and geraniol, we quantified camphor and cineol, the main contributors to the spike lavender leaf essential oil, as well as essential oil yield for each of the 5 leaf developmental stages (Tables 13 and 14).

Table 13.Effect of leaf developmental stage (LDS, see Figure 9) on the producction (μg/g fresh weight) of representative terpenes in hexane-extracted essential oil of transgenic T_0 spike lavender lines LIS3, LIS4 and Control. Essential oil yield is also shown. All reported values represent the mean ± SD of at least 3 measurements. Mean values followed by the same letter are not significant different according to Tukey's test at P≤0.05.

LSD	Line	Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Oil Yield
a	LIS3	55.77 ± 0.53 b	3,401.82 ±5.38 b	4,898.74 ± 4.59 b	57.13 ± 0.18 b	1.12 ± 0.12 c	9695.96 ± 5.81 c
	LIS4	116.11 ± 4.41 a	3,221.82 ± 4.34 c	5,883.03 ± 2.07 a	61.69 ± 1.66 a	3.18 ± 0.10 b	10671.89 ± 18.25 a
	control	61.86 ± 2.84 b	6,334.05 ± 7.98 a	1,705.60 ± 2.00 c	6.15 ± 0.17 c	3.73 ± 0.14 a	9284.74 ± 12.47 b
b	LIS3	19.30 ± 0.64 c	2,020.87 ± 0.83 c	3,172.85 ± 3.42 b	45.72 ± 0.20 a	0.38 ± 0.03 c	5985.21 ± 3.76 c
	LIS4	51.30 ± 1.20 a	2,748.97 ± 12.75 b	4,719.92 ± 2.94 a	33.41 ± 1.12 b	2.95 ± 0.05 b	8686.82 ± 23.78 b
	control	39.68 ± 0.63 b	7,042.11 ± 7.48 a	1,699.93 ± 0.42 c	6.19 ± 0.08 c	3.44 ± 0.00 a	10036.50 ± 3.36 a
c	LIS3	29.43 ± 0.53 a	584.73 ± 0.27 b	601.17 ± 0.81 a	16.89 ± 0.18 a	0.83 ± 0.03 b	1578.44 ± 2.69 b
	LIS4	4.13 ± 0.09 c	245.94 ± 0.30 c	337.36 ± 0.36 b	5.55 ± 0.19 b	2.17 ± 0.03 a	705.10 ± 0.69 c
	control	15.35 ± 0.37 b	1,552.12 ± 3.98 a	313.50 ± 0.62 c	1.51 ± 0.03 c	0.64 ± 0.01 c	2178.86 ± 4.11 a
d	LIS3	8.33 ± 0.77 b	177.74 ± 1.00 b	149.35 ± 0.20 b	14.25 ± 0.07 a	0.92 ± 0.04 b	469.76 ± 1.67 b
	LIS4	3.12 ± 0.02 c	146.01 ± 0.33 c	215.35 ± 1.31 a	1.18 ± 0.00 b	2.25 ± 0.12 a	427.80 ± 0.57 c
	control	10.44 ± 0.23 a	391.30 ± 1.18 a	38.40 ± 0.37 c	0.60 ± 0.01 c	0.61 ± 0.01 c	573.72 ± 0.80 a
e	LIS3	4.18 ± 0.31 b	74.86 ± 0.69 c	65.53 ± 0.41 b	8.32 ± 0.09 a	0.67 ± 0.00 b	210.25 ± 0.04 c
	LIS4	3.87 ± 0.13 b	190.03 ± 0.19 b	316.67 ± 2.02 a	1.91 ± 0.02 b	3.43 ± 0.09 a	571.07 ± 2.10 a
	control	6.19 ± 0.08 a	237.67 ± 0.02 a	16.22 ± 0.06 c	0.35 ± 0.01 c	0.49 ± 0.03 c	337.07 ± 0.34 b

Within each LSD and terpene, values followed by the same letter are not significantly different according to Tukey's test at P≤0.05.

Table 14. ANOVA of the data presented in Table 13.

			Mean Squares					
LDS		df	Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Oil Yield
a	Line	2	3,310.62 **	9,158,120.0 **	14,307,944.99 **	2,851.98 **	5.66 **	1,522,865.92 **
	Error	6	9.27	37.18	9.76	0.94	0.02	174.18
b	Line	2	787.06 **	22,087,041.25 **	6,841,609.34 **	1,227.70 **	8.07 **	12,766,610.75 **
	Error	6	0.75	73.12	6.85	0.44	0.001	196.64
c	Line	2	481.93 **	1,378,365.42 **	76,457.20 **	190.74 **	2.09 **	1,647,599.41 **
	Error	6	0.14	5.34	0.39	0.02	0.001	8.23
d	Line	2	42.66 **	53,389.30 **	23,989.12 **	178.81 **	2.28 **	16,929.58 **
	Error	6	0.22	0.83	0.63	0.002	0.005	1.25
e	Line	2	4.76 **	21,018.92 **	77,884.68 **	53.60 **	8.12 **	100,514.95 **
	Error	6	0.04	0.17	1.43	0.003	0.003	1.51

** significant at $p \leq 0.001$

In both transgenic and control lines, the essential oil yield and the amount of the analyzed monoterpenes, but geraniol in both transgenic lines, decreased with leaf development; this trait was more evident after the leaves reached 3 cm in length (stage c) (Tables 13, 14 and Figure 9).

A clear correlation between overexpression of *LIS* transgene and essential oil yield could not be established. In contrast, the effect of this transgene on linalool content was evident at all leaf developmental stages, although the highest amounts, as compared to control, were obtained in leaves at developmental stages a, b and c (Table 13). Furthermore Northern Blot analyses at the different developmental stages indicated that increases in the expression of the *Clarkia LIS* gene paralleled increased in the linalool content of the transgenic spike lavender LIS3 and LIS4 (Figure 21 y Table 13). The effect of *LIS* transgene expression on myrcene and geraniol was line-dependent; thus, and as stated before, our results do not support another biosynthetic pathways for linalool than the activity of the linalool synthase enzyme in spike lavender.

The two transgenic lines showed increased or reduced amounts in camphor and cineol, respectively, as compared to control. Since the content of these two main constituents of the spike lavender essential oil is genotype dependent (Harborne and Willians 2002; Muñoz-Bertomeu et al., 2007b), it is difficult, however, to attribute this pattern to a direct effect of the transgene.

III. 1. 2. 3. Inheritance of the *LIS* transgene

Self-pollination of transgenic T_0 LIS plants was performed for two consecutive years. Seeds produced from lines LIS3, LIS4, LIS6 and LIS8 were germinated *in vitro* (Table 15). Subsequently, seedlings were grown aseptically, analyzed for the inheritance of the transgene, acclimatized under controlled conditions for two months and transferred to the greenhouse.

Table 15. Seeds obtained and percentage of germinated seeds from self-pollinated transgenic T_0 LIS lines.

Line	Seeds obtained	% germinated seeds
LIS3	4	50.0
LIS 4	6	66.6
LIS 6	36	75.0
LIS 8	20	40.0

III. 1. 2. 3. 1. Molecular analyses of LIS T_0 progenies

The inheritance of the LIS transgene was analyzed by PCR. Although most of the analyzed seedlings inherited the transgene (Table 16 and Figure 25), only line LIS6 produced enough seedlings to accurately study the inheritance of the transgene. The chi-squared analysis (Table 16) demonstrated that although the LIS6 mother plant had two inserts of the LIS gene, showed a 3:1 segregation, suggesting that in this plant the two inserts were integrated in the same chromosome; thus LIS transgene behaves as typical dominant, linked gene. Southern Blot analysis showed that LIS^+ T_1 plants inherited the two copies of the transgene, corroborating this hypothesis (Figure 26). Twenty-seven plants were acclimatized and used for further analyses.

Table 16. Segregation of *LIS* gene in T_1 LIS6 plants from self-pollinated transgenic T_0 spike lavender plants. LIS^+, LIS^- = PCR-positive and PCR negative, respectively. A Chi-square value > 3,84 indicates a significant deviation from the expected ratio (P=0.05).

Ratio	Observed		Expected		Chi- square value
	LIS +	LIS -	LIS +	LIS -	3:1 (1 insert)
3:1	22	5	20.25	6.75	0.605
15:1	22	5	25.31	1.68	6.830

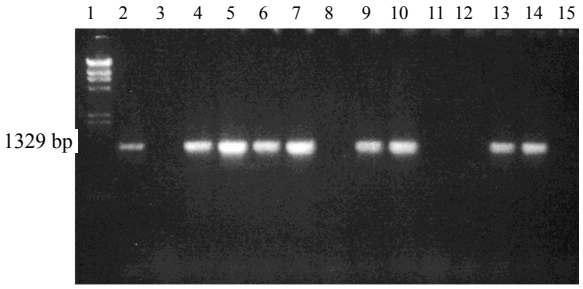


Figure 25. Electrophoresis agarose gel from PCR products of the progeny of the self-pollinated LIS6 of spike lavender using the primers for the gene *LIS*. Lane 1: *Hin*dIII Ladder; Lane 2, LIS plasmid; Lane 3, non-transgenic plant; Lane 4, LIS6; Lane 5-15, LIS6 progeny.

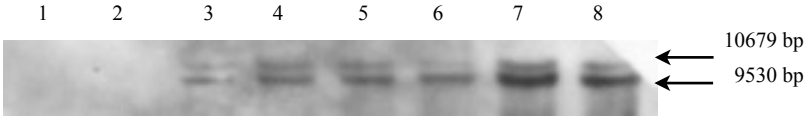


Figure 26. Southern Blot, of *LIS* gene in transgenic T_0 and T_1 spike lavander plants. Lane 1, control; Lane 2, LIS6-12 T_1 plant that did not inherit the *LIS* transgene; Lanes 3-7, selected progenies of LIS6 plant that inherited the *LIS* transgene; lane 8, parental T_0 LIS6.

III. 1. 2. 3. 2. Essential oil content in LIS T_1 plants

We first randomly chose five of the T_1 plants (LIS6-12, LIS6-14, LIS6-21, LIS6-28, and LIS6-30) to analyze, by direct hexane extraction, linalool content in leaves sampled at the five developmental stages detailed above (see Figure 9) , although those from stages a and b were sampled together. As shown in Figure 27, the temporal linalool accumulation pattern observed in leaves from the LIS6 T_0 mother plant was retained in those progenies that inherited the transgene. These plants also had elevated linalool phenotype as compared to their T_1 counterpart (LIS6-12) that did not inherit the *LIS* gene. The positive effect of *LIS* transgene on linalool production was more noticeable when the percentage of linalool content over the total essential oil was determined. In that case, all lines that inherited the *LIS* transgene showed an increased proportion of linalool at all leaf developmental stages (Figure 27B).

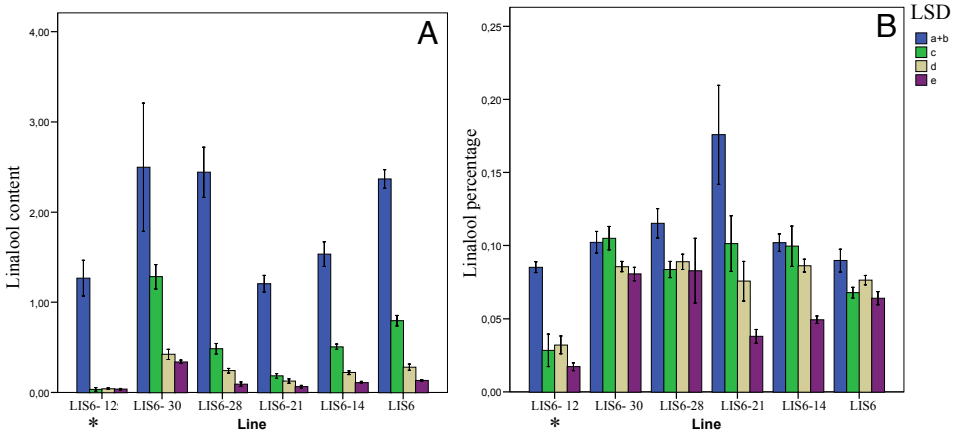


Figure 27. Effect of leaf developmental stage (LDS, see Figure 9) on the linalool content in hexane extracted essential oils from transgenic T_0 and T_1 plants. A) Linalool production (µg/g fresh weight); B) Linalool percentage. All reported values represent the mean ± SE of at least 3 measurements. * T_1 plant that did not inherit the *LIS* transgene.

Since the commercial spike lavender oil is obtained from hydrodistillation, essential oil analyses were also accomplished in hydrodistillates of pooled air-dried leaves (whorls 4th to 10th) and flowers from T_0 LIS6 and their progenies.

Data from hydrodistillated leaves (Tables 17 and 18) corroborated those previously obtained using the direct hexane extraction method (Figure 27 A and B) related to the increased linalool content in T_1 lines that inherited the transgene. Note that the essential oil yield as well as the content of the other analyzed terpenes (myrcene, cineol, geraniol and camphor) was line dependent and were not affected by the presence of the transgene.

As expected, flowers produced more essential oil than leaves (Tables 19 and 20). The effect of *LIS* transgene on essential oil composition was less evident in flowers since only two out of the 8 analyzed T_1 lines that inherited the transgene (LIS6-25 and LIS6-29) produced significantly more linalool than their counterparts that did not inherit the transgene (Table 19). Furthermore, only transgenic T_1 LIS6-29 line significantly increased their linalool percentage in the flower essential oil (Table 19).

Table 17. Essential oil yield and representation monoterpene production (µg/g dried weight) from hydrodistilled leaves (4th-10th whorls) of transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LIS6 line. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey's test at P≤0.05. # T_1 plant that did not inherit the *LIS* transgene.

Line	Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Oil yield
LIS6-12 #	1.20 ± 0.17 d	5,047 ± 179 c	3,891 ± 255 d	0.68 ± 0.04 d	1.59 ± 0.11 d	9,046 ± 321 d
LIS6-14	3.56 ± 0.23 b	6,786 ± 51 a	4,563 ± 231 c	1.24 ± 0.07 b	1.84 ± 0.21 c	11,513 ± 225 c
LIS6-21	0.43 ± 0.02 e	1,928 ± 114 d	3,446 ± 418 e	0.61 ± 0.06 d	1.42 ± 0.24 de	5,446 ± 540 e
LIS6-28	4.94 ± 0.77 a	6,927 ± 146 a	8,467 ± 307 a	1.60 ± 0.06 a	3.17 ± 0.15 a	15,622 ± 317 a
LIS6-30	0.52 ± 0.03 e	6,108 ± 186 b	2,735 ± 118 f	0.91 ± 0.04 c	1.19 ± 0.07 e	8,951 ± 306 d
LIS6	2.14 ± 0.64 c	6,758 ± 366 a	5,970 ± 256 b	1.19 ± 0.03 b	2.36 ± 0.15 b	12,923 ± 296 b

ANOVA

Source of Variation	df	Mean Squares					
		Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Oil yield
Line	5	29.32 **	33,403,003.00 **	39,215,273.00 **	1.27 **	4.77 **	113,663,660.03**
Error	48	0.18	39,589.38	77,727.13	0.003	0.03	120,112.71

** significant at p≤0.001

Table 18. Percentage of representative essential oil constituents from hydrodistilled leaves (4th-10th whorls) of transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LIS6 line. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey's test at $P \leq 0.05$. # T_1 plant that did not inherit the *LIS* transgene.

Line	Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Total
LIS6-12 #	1.20 ± 0.05 b	38.58 ± 0.93 b	28.92 ± 1.50 c	0.06 ± 0.00 e	0.16 ± 0.01 b	85.94 ± 0.86 a
LIS6-14	1.20 ± 0.05 a	37.72 ± 1.11 b	24.92 ± 0.80 d	0.08 ± 0.00 d	0.14 ± 0.01 c	85.02 ± 0.97 ab
LIS6-21	0.31 ± 0.01 c	24.56 ± 1.07 e	43.07 ± 1.01 a	0.09 ± 0.00 a	0.24 ± 0.02 a	86.47 ± 0.72 a
LIS6-28	1.11 ± 0.15 a	28.95 ± 0.87 d	34.15 ±0.88 b	0.08 ± 0.00 b	0.17 ± 0.01 b	83.73 ± 0.98 ab
LIS6-30	0.22 ± 0.01 c	45.81 ± 0.41 a	20.08 ± 0.26 e	0.08 ± 0.00 c	0.12 ± 0.01 d	83.03 ± 0.41 b
LIS6	0.65 ± 0.17 b	34.67 ± 2.23 c	29.59 ± 0.69 c	0.07 ± 0.00 d	0.16 ± 0.01 b	85.39 ± 4.51 ab

ANOVA

Source of Variation	df	Mean Squares					
		Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Total
Line	5	1.48 **	510.58 **	564.50 **	0.001 **	0.02 **	15.57 *
Error	48	0.01	1.52	0.87	0.00	0.00	3.94

** significant at $p \leq 0.001$; *significant at $p \leq 0.05$

Table 19. Essential oil yield and representation monoterpene production (µg/g dried weight) from hydrodistilled flowers of transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LIS6 line. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey's test at P≤0.05. Control: pooled flowers from LIS6-12 and LIS6-26 that did not inherit the *LIS* transgene.

Line	Myrcene	1.8-cineol	Camphor	Linalool	Geraniol	Oil Yield
Control	0.86 ± 0.26 bc	13.17 ± 1.60 ab	12.89 ± 2.17 cd	11.31 ± 1.46 bcd	0.09 ± 0.01 cd	47.62 ± 1.48 bcd
LIS6	0.59 ± 0.13 bcd	14.51 ± 1.40 a	12.67 ± 0.72 cd	8.82 ± 0.47 d	0.10 ± 0.02 bcd	44.76 ± 1.16 cd
LIS6-13	0.39 ± 0.02 cd	12.72 ± 0.96 ab	12.87 ± 0.36 cd	9.76 ± 0.73 cd	0.09 ± 0.01 cd	44.38 ± 1.37 cd
LIS6-14	0.40 ± 0.05 cd	13.07 ± 0.39 ab	10.60 ± 0.32 d	8.99 ± 0.39 d	0.09 ± 0.01 cd	41.71 ± 1.51 d
LIS6-21	0.80 ± 0.11 bcd	11.95 ± 0.39 abc	23.25 ± 0.43 a	14.17 ± 1.79 ab	0.13 ± 0.00 a	61.28 ± 0.79 a
LIS6-25	1.06 ± 0.05 ab	11.55 ± 0.19 bc	20.35 ± 0.54 a	15.82 ± 1.84 a	0.14 ± 0.01 ab	60.09 ± 2.48 a
LIS6-28	0.37 ± 0.06 d	8.02 ± 0.52 d	17.03 ± 1.23 b	13.43 ± 1.03 abc	0.11 ± 0.01 ab	49.06 ± 2.78 bc
LIS6-29	0.68 ± 0.15 bcd	9.15 ± 0.89 cd	09.90 ± 0.87 d	15.04 ±2.46 a	0.08 ± 0.02 d	42.66 ± 4.93 d
LIS6-34	1.49 ± 0.30 a	14.50 ± 1.19 a	14.02 ± 0.26 bc	11.55 ±0.47 bcd	0.09 ± 0.00 cd	51.65 ± 1.84 b

ANOVA

Source of Variation	df	Mean Squares					
		Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Oil Yield
Line	8	0.41 **	15.29 **	61.77 **	23.30 **	0.001 **	156.41 **
Error	21	0.03	1.16	1.46	1.92	0.00	5.06

** significant at p≤0.001; *significant at p≤0.05

Table 20. Percentage of representative essential oil constituents from hydrodistilled flowers of transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LIS6 line. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey's test at P≤0.05. Control line is a mixtured of the non PCR positive plants LIS6-12 and LIS6-26.

Line	Myrcene	1.8-cineol	Camphor	Linalool	Geraniol	Total
Control	0.31 ± 0.05 b	27.43 ±2.54 a	27.90 ± 5.36 bc	24.32 ± 2.40 bc	0.18 ± 0.02	91.30 ± 1.77 ab
LIS6	0.21 ± 0.04 bc	30.66 ± 1.84 a	27.69 ± 0.29 bc	19.28 ± 1.01 c	0.22 ± 0.06	89.85 ± 0.87 abc
LIS6-13	0.16 ± 0.01 c	27.47 ± 1.90 a	28.73 ± 0.42 bc	21.77 ± 1.56 bc	0.20 ± 0.02	89.42 ± 1.53 abc
LIS6-14	0.17 ± 0.01 c	29.43 ± 0.08 a	24.65 ± 0.28 c	20.91 ± 0.89 c	0.20 ± 0.04	87.43 ± 0.51 c
LIS6-21	0.22 ± 0.04 bc	19.21 ± 0.87 bc	38.61 ± 1.27 a	23.50 ± 2.67 bc	0.22 ± 0.01	92.61 ± 0.29 a
LIS6-25	0.27 ± 0.02 b	18.92 ± 0.79 bc	34.39 ± 0.63 ab	26.66 ± 1.93 b	0.22 ± 0.03	91.66 ± 0.06 ab
LIS6-28	0.13 ± 0.01 c	15.35 ± 0.57 c	33.64 ± 0.25 ab	26.54 ± 1.34 b	0.22 ± 0.00	88.51 ± 1.18 bc
LIS6-29	0.27 ± 0.02 b	20.96 ± 0.89 b	23.43 ± 0.68 c	37.53 ± 1.39 a	0.19 ± 0.05	91.94 ± 0.28 a
LIS6-34	0.04 ± 0.04 a	27.81 ± 1.26 a	27.82 ± 0.89 bc	22.94 ± 1.81 bc	0.17 ± 0.00	91.55 ± 1.42 ab

ANOVA

Source of Variation	df	Mean Squares					
		Myrcene	1,8-cineol	Camphor	Linalool	Geraniol	Total
Line	8	0.023 **	95.45 **	73.57 **	85.82 **	0.001 NS	9.44 **
Error	21	0.001	2.59	7.23	3.48	0.001	1.40

NS: non significant; ** significant at p≤0.001; *significant at p≤0.05

III. 1. 2. 4. Discussion

The monoterpene synthases catalyze the first committed step that leads to the synthesis of the different monoterpene families (Bohlman et al., 1998). Because of this, the manipulation of the gene expression of monoterpene synthases might be a fundamental tool to modify the profile of the essential oil. This strategy has been successfully used to provide the capability for the synthesis of monoterpenes to plants and/or tissues that do not naturally do so (Lewinsohn et al., 2001; Degenhardt et al., 2003; Aharoni et al., 2005; Wu et al., 2006). To date, however, this strategy has been employed with variable success in Lamiaceae; thus, whereas in mints the overexpression of the *Mentha spicata limonene synthase* (*MsLS*) gene did not produce substantive variations on oil composition (Krasnyanski et al., 1999; Diemer et al., 2001; Mahmoud et al., 2004), in spike lavender the overexpression of the *MsLS* gene lead to a significant increase of limonene (Muñoz-Bertomeu et al., 2008). Linalool is one of the monoterpenes that determines more directly the commercial value of the spike lavender essential oil. Therefore the availability of spike lavender plants with an enhanced production of this monoterpene does have a potential industrial interest.

Following the *Agrobacterium* transformation protocol described for the species (Nebauer et al., 2000), eight spike lavender lines overexpressing the *LIS* gene from *Clarkia breweri* were produced. Most of these T_0 plants showed a significant increase in linalool content as compared to control. The effect of *LIS* transgene was particularly striking in the youngest leaves of 2 of the lines, where a linalool increase up to a 1,000% was observed. *LIS* gene was driven by a constitutive promoter (CaMV35S), but their transcript abundance was dependent on the developmental stage of the leaf, reaching a maximum in the youngest leaves, which correlated with its higher linalool content. *LIS* transcripts and linalool content decreased along with the developmental stage of the leaf, but the increase in linalool content was maintained at all developmental stages in transgenic plants as compared to control. This high linalool producing phenotype was maintained in leaf essential oil of the progenies that inherited the transgene. This phenotype was, however, less evident in the essential oil from flowers of these progenies. The most important difference between leaf and

flower spike lavender oil is the amount of linalool (traces in leaves and more than 15% of the total oil in flowers), which suggest a strong spatial regulation of the LIS enzyme as reported for other monoterpene synthases and plant species (Dudareva et al., 2004; Thol, 2006; Irmisch et al., 2012). Thus, it seems that the overexpression of *LIS* transgene do not increase linalool content in organs with a high endogenous LIS activity as it does in those with less activity (ca. leaves), which is in accordance with previous studies in other model plants, like *Dianthus caryophyllus* (Lavy et al., 2002), *Solanum lycopersicum* (Lewinsohn et al., 2001) and *Petunia hybrida W115* (Lücker et al., 2001). In the case of *Dianthus caryophyllus,* the headspace GC/MS analyses showed emission of linalool and its derivatives, *cis*- and *trans*-linalool oxide. In contrast, the analysis of flowers extracts of the transgenic plants produced an enhanced amount of *trans*-linalool oxide but not linalool, revealing that the oxidation of linalool might be a way to store this compound (Lavy et al., 2002). Glycosylation of linalool, which converts it into a non-volatile form, was the reason for the lack of linalool emission in transgenic petunia overexpressing the *LIS* gene (Lücker et al., 2001). In the case of tomato fruits, not only linalool was to be found but also its derivative 8-hydroxylinalool (Lewinsohn et al., 2001). All of this data support the idea that linalool might be converted to another compound in order to store it or as a side effect of the normal metabolism of monoterpenes in plants. This might explain the differences observed between the leaves and flowers in spike lavender. A limitation due to the viability of IPP might also be the cause for it in the case of flowers.

As a conclusion, transgenic spike lavender plants overexpressing the linalool synthase gene from *Clarkia breweri* lead to an increased linalool content in leaves without modifying the total content of essential oil. As remarked, the quality and economical value of spike lavender essential oil depends highly on its percentage of linalool. Therefore, the achievement of plants with an enhancement of this monoterpene production in flowers is very desirable. We know that this *Clarkia breweri* gene is expressed and functional in spike lavender plants as shown by the results achieved in leaves. Thus, the next step in the production of plants of economical importance would be a new

transgenic approach using promoters and terminator specifically addressed to flowers.

III. 1. 3. Generation of double transgenic spike lavender plants overexpressing genes from the terpene synthesis pathways

Plants were obtained from seeds recovered after controlled crosses between different T_0 spike lavender lines. Specifically the following crosses were undertaken: DXS x HMGR and DXS x LIS. Plants resulting for these crosses were cloned *in vitro*, acclimatized to *ex vitro* conditions and transferred to the greenhouse.

III. 1. 3. 1. Molecular characterization of double transgenic lines

III. 1. 3. 1. 1. DXS x HMGR lines

Crosses between transgenic DXS6 and four HMGR (HMGR1; HMGR2; HMGR3 and HMGR4) produced 109 seeds, although only 10 of them yield viable offspring (Table 21). PCR analyses showed that only one plant (DXS6-HMGR1-4) inherited both DXS and HMGR genes (Figure 28 and Table 21). Further Southern Blot analysis (Figure 29) confirmed that DXS6-HMGR1-4 plant inherited all the inserts of both parental, that is the two inserts of the *DXS* gene and four inserts of the *HMG1* gene. This plant was used for inhibitor experiments (see III. 4. 3).

Table 21. Characterization by PCR of the progeny resulting from the cross of plants trangenic for the *HMGR* or *DXS* genes. The + or – signs stand for the presence or absence of the corresponding band.

♂	♀	Seeds	Plants	HMGR+ DXS +	DXS+	HMGR+	-/-
DXS6	HMGR3	26	0	0	0	0	0
DXS6	HMGR1	14	2	1	0	1	0
HMGR1 or HMGR3	DXS6	14	1	0	1	0	0
DXS6	HMGR4	7	4	0	0	2	2
DXS6	HMGR2	41	3	0	0	2	1
HMGR4 or HMGR2	DXS6	7	0	0	0	0	0

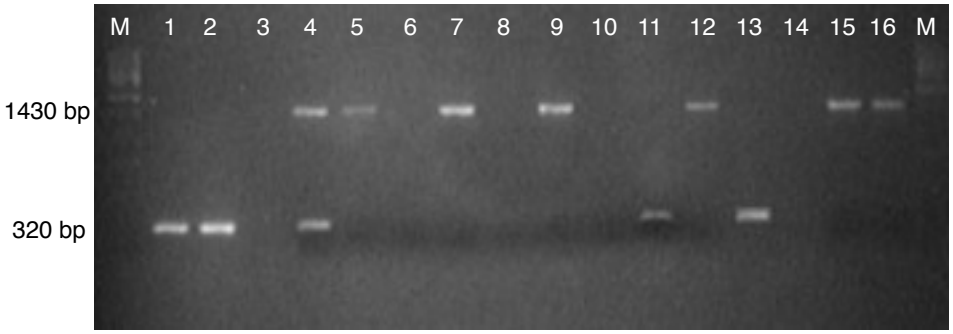


Figure 28. PCR analysis of the *DXS* and *HMG1* genes**.** M, molecular marker; lane 1, *DXS* plasmid; lane 2, Parental DXS plant; lane 3, WT plant; lanes 4-13: Progenies from DXS x HMGR1, HMGR4 and HMGR2; lane 14, WT plant; lane 15, Parental HMGR plant; Lane 16: *HMG1* plasmid.

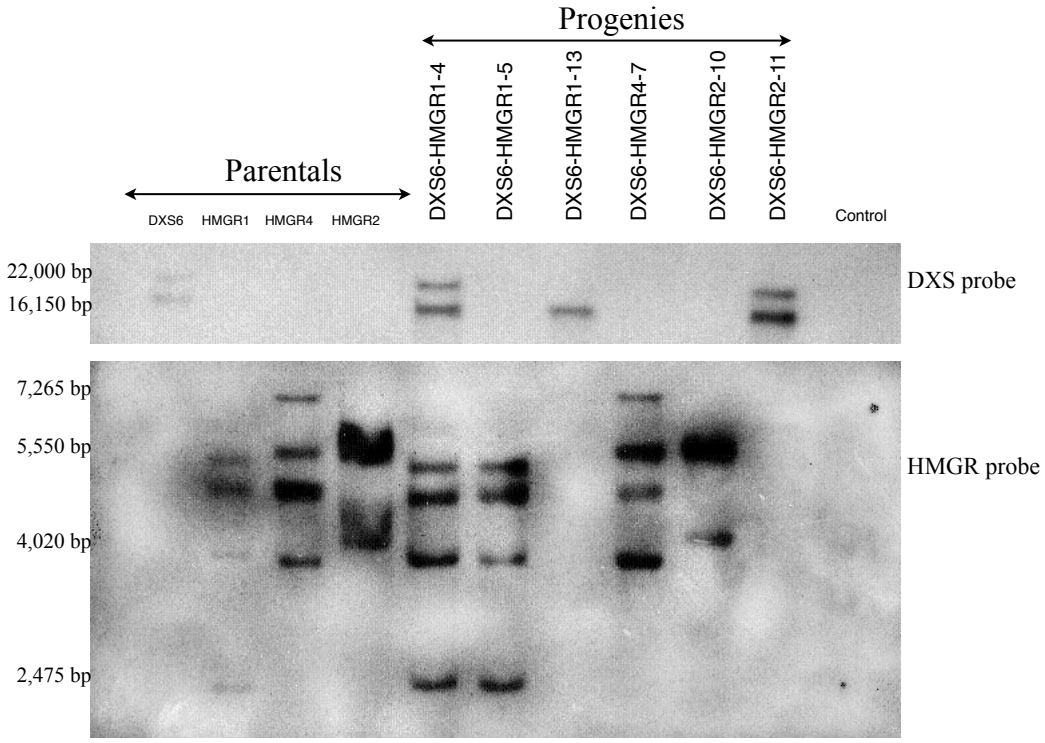


Figure 29. Southern Blot analysis of *DXS* and *HMGR* genes in spike lavender progenies (DXS x HMGR) and parental plants.

III. 1. 3. 1. 2. DXS x LIS lines

Crosses between transgenic DXS6 and three LIS plants (LIS1, LIS2 and LIS8) produced 20 seeds, although only 10 of them yielded viable offspring (Table 22). PCR analyses showed that 2 plants (DXS6-LIS8-1 and DXS6-LIS8-2) inherited both *DXS* and *LIS* genes (Figure 30 and Table 22). Further Southern Blot analysis (Figure 31) showed that DXS6-LIS8-1 plant inherited one of the two inserts of the *DXS* gene and the 3 inserts of the *LIS* gene, whereas DXS6-LIS8-2 plant inherited the two inserts of *DXS* gene and the insert of *LIS* gene (Figure 31). Line DXS6-LIS8-3 did not inherit any of the genes and consequently was used as internal control in further experiments.

Table 22. Characterization by PCR of the progeny resulting from the cross of plants transgenic for the *LIS* or *DXS* genes. The + or – signs stand for the presence or absence of the corresponding band.

♂	♀	Seeds	Plants	LIS+ DXS +	DXS+	LIS+	-/-
DXS6	LIS 1	3	3	0	3	0	0
DXS6	LIS 2	2	0	0	0	0	0
DXS6	LIS8	10	7	2	4	0	1
LIS 1 or LIS 2	DXS6	5	0	0	0	0	0

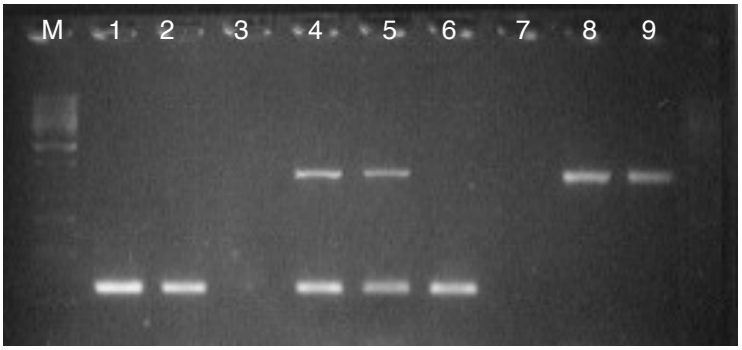


Figure 30. PCR analysis of the *DXS* and *LIS* genes**.** M, molecular marker; lane 1, *DXS* plasmid; lane 2, Parental DXS plant; lane 3, WT plant; lanes 4-6: Progenies from DXS x LIS; lane 7, WT plant; Lane 8, Parental LIS plant; Lane 9: *LIS* plasmid.

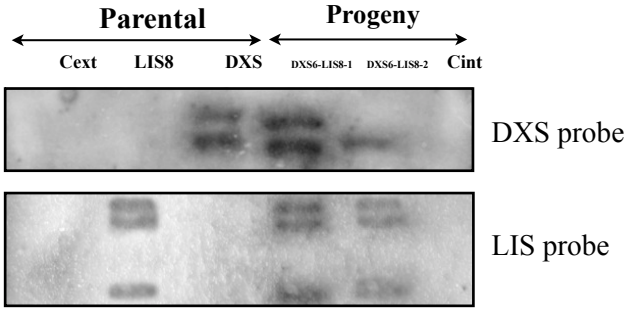


Figure 31. Southern Blot analysis of *DXS* and *LIS* genes in spike lavender progenies (DXS x LIS) and parental plants. Cint: progeny that did not inherit any of the genes. Cext: non-transgenic plant.

Expression levels of *DXS* and *LIS* transgenes from the double transgenic (DXS6-LIS8-1 and DXS6-LIS8-2) plants were determined by Northern Blot analyses. Figure 32 shows the *LIS* and *DXS* transcript levels in leaves from the 1^{st}-3^{rd} and 4^{th}-10^{th} whorls of all double transgenic, DXS6 and negative control plants. *DXS6* mRNA was detected in all lines but the controls; all double transgenic lines showed a lower level of *DXS* transcripts than the parental DXS6, specially in the older (4^{th}-10^{th}) whorls. This also holds true for the *LIS* gene, although in this case the transcript level reduction was even more dramatic as compared to the *LIS* expression level in T_0 LIS8 parental plant (Figure 22). These results suggest the occurrence of a generalized transcript suppression process affecting both *DXS* and *LIS* genes of these double transgenic plants.

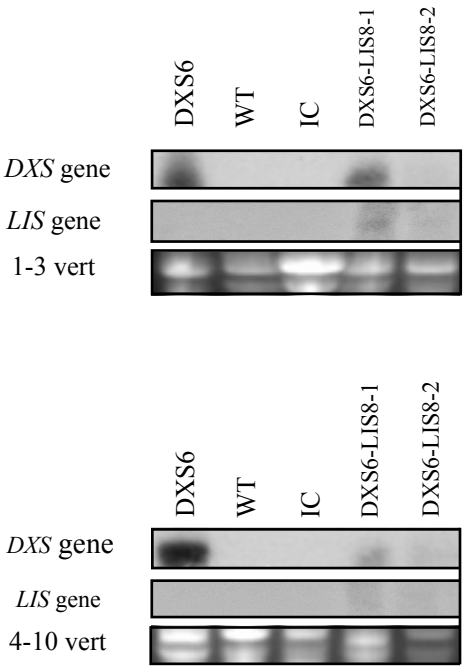


Figure 32. Expression level analysis of the *DXS* and *LIS* transgenes in leaves of the whorls 1-3 and 4-10 of lines DXS6-LIS8-1, DXS6-LIS8-2, DXS6 and both WT and internal negative (IC) controls. The gel loading control is also showed.

III. 1. 3. 2. Essential oil content in double transgenic (DXS x LIS) lines

Essential oil analyses by direct extraction in hexane were performed in cohorts of leaves sampled at the following developmental stages: a+b) 1st and 2nd whorls with fully open leaves; c) 3rd and 4th whorls, and d) 5th-7th whorls. In all cases, DXS6 parental and progenies that did not inherit any of the genes (internal control, IC) were used as controls.

As expected, and irrespective of the presence of the transgenes, leaves from the youngest whorls produced more essential oil and linalool. Transgenic plants containing one (*DXS*) or two (*DXS* and *LIS*) genes, produced more essential oil than non transgenic controls (Figure 33). Surprisingly, none of those plants that inherited both genes produced a specific linalool increase (Figure 34).

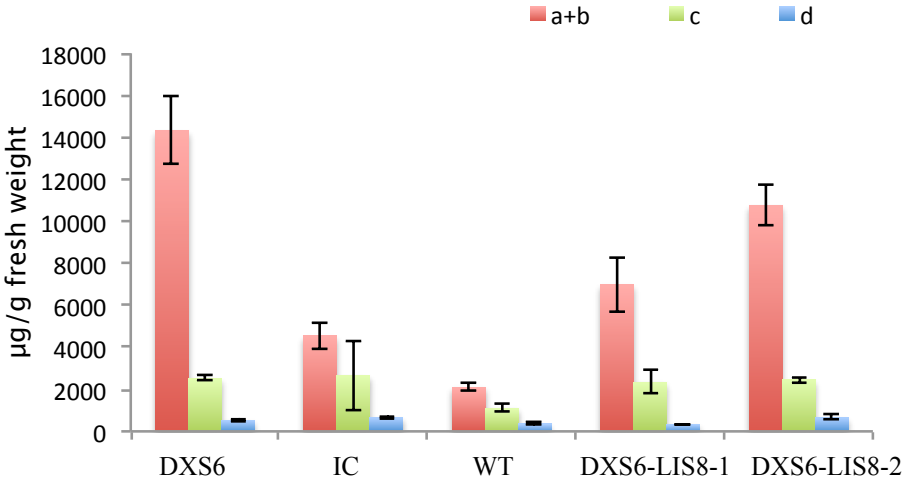


Figure 33: Essentiol oil yield (µg/g fresh weight) in leaves sampled at three developmental stages (a+b, c and d) from DXS6 parental and double transgenic DXS6-LIS8 plants. IC, internal control (progeny that did not inherit any of the genes). WT, non transgenic plant. Reported values represent the mean ± SE of three measurements.

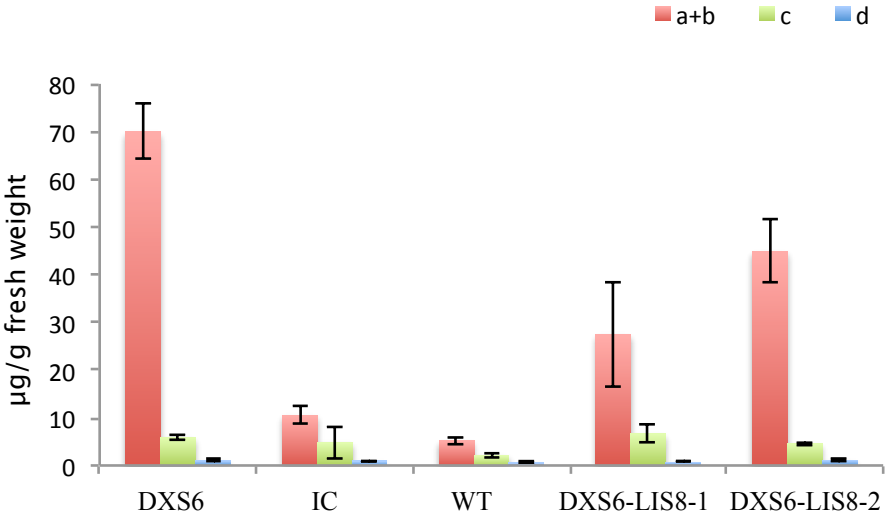


Figure 34: Linalol content (µg/g fresh weight) in leaves sampled at three developmental stages (a+b, c and d) from DXS6 parental and double transgenic DXS6-LIS8 plants. IC, internal control (progeny that did not inherit any of the genes). WT, non transgenic plant. Reported values represent the mean ± SE of three measurements.

III. 1. 3. 3 Discussion

Overexpression of *DXS* transgene in spike lavender lead to an increased linalool content in essential oil from flowers but not from leaves (Muñoz-Bertomeu et al., 2006). These results support the idea that, besides precursor availability, spatial regulation of enzyme activities are necessary for the production of linalool in spike lavender leaves. Based on this idea we hypothesized that the generation of spike lavender plants overexpressing both *DXS* and *LIS* genes would be an appropriate tool to increase linalool content in leaf essential oil. In the present work we report the generation of these double transgenic plants by controlled pollination. The obtained results show, however, that double transgenic plants did not increase linalool content in leaves and produced less essential oils than their DXS6 mother plant. Northern Blot analyses show a decrease in the level of transcripts of both genes. Then, transgene suppression processes, could explain the decreased amount of essential oil founded in double transgenic plants, as has been already observed in many plants. It is known, that transgenes can be silenced by the host plant RNA silencing machinery (Baulcombe, 2004; Vaucheret, 2006; Kalantidis et al., 2006; Kanazawa, 2008). The RNA silencing machinery evolved as a defense mechanism against transposable elements, viruses and other forms of foreign nucleic acids (Baulcombe, 2005; Wang and Metzlaff, 2005). Why transgenes activate this silencing machinery is not yet fully understood, although transcripts from genes like *LIS* and *DXS* transgenes, with no CAP structure or poly(A) tail, could have a trigger function (Gazzani et al., 2004; Wassenegger and Krczal, 2006; Luo and Chen, 2007). Also, the lack of introns in the transgene sequence might be a problem (Wassenegger and Krczal, 2006; Luo and Chen, 2007). An intro-less transcript might be processed into dsRNA by the RNA-dependent RNA polymerase 6 (RDR6) and at that point the dsRNA would trigger the RNA silencing machinery. Finally, *DXS* and *LIS* transgenes were under the control of a strong promoter like CaMV35S, whose activity may lead to an accumulation of RNAs that, when exceeds a certain threshold, will activate the plant defense mechanism (Wassenegger and Krczal, 2006). Transgenic *Nicotiana bethamiana* and *N. tabacum* (Dalakouras et al., 2011), expressing a transcriptional fusion of the green fluorescence protein (GFP)

cDNA and a 98-bp PSTVd cDNA fragment showed diverse response to spontaneous silencing. In *N. benthamiana*, the self-silencing process involved mRNA degradation and dense DNA methylation of the homologous coding region. In *N. tabacum*, silencing occurred without complete mRNA degradation and with low methylation of the gene region. This might indicate that in plants, siRNA-mediated spontaneous silencing might involve translational inhibition in addition to mRNA degradation (Dalakouras et al., 2011). Studies in transgenic *Arabidopsis* (Luo and Chen, 2007) expressing the β-glucuronidase gene in different copy number and arrangements, support the positive correlation among transgene copy number, expression, and RDR6 mediated RNA silencing. The transgenic mRNA probably trigger RDR6 by acting as templates for the RNA polymerase .

III. 2. Contribution of MVA and MEP pathways to monoterpene biosynthesis in spike lavender

Overexpression in spike lavender of the *DXS* gene, which encodes the first enzyme of the MEP pathway (1-deoxy-D-xylulose 5-phosphate (DXP) synthase), significantly increased the production of essential oil (Muñoz-Bertomeu et al., 2006). Overexpression of the gene *HMG1* which encodes the first MVA pathway enzyme (3-hydroxy-3-methylglutaryl-CoA reductase or HMGR) caused the same effect on some of the obtained transgenic lines (Muñoz-Bertomeu et al., 2007a). These results suggest that both pathways may be involved in the biosynthesis of monoterpenes in *Lavandula latifolia* Medicus.

The aim of this work was to validate this hypothesis by using two different approaches. First, the use of inhibitors of these pathways, Mevinoline (MEV) that inhibits the enzyme HMGR, and fosmidomycin (FSM), an inhibitor of the enzyme DXP reductoisomerase (DXR). Secondly, the use of labeling methods by using tracers, such as [U-$^{13}C_6$]-glucose, $^{13}CO_2$ and [1,2-$^{13}C_2$]-mevalonate.

III. 2. 1. Effects of MEV and FSM on spike lavender

MEV and FSM are inhibitors of the MVA and MEP pathways, respectively. MEV, a statin, is a competitive antagonist of the HMGR enzyme (Alberts, 1980) and therefore used, as other statins, to decrease the cholesterol blood content in the medical field since the end of the 1980's (Shigi, 1989). On the other hand, FSM is produced by *Streptomyces lavendulae,* and was discovered by researchers at Fujisawa Pharmaceutical Co. (Iguchi et al., 1980; Okuhara et al., 1980) in the late 1970s. FSM is an inhibitor of the DXR enzyme (Shigi, 1989) that catalyses the formation of DXP into MEP. FSM works as a mixed-type inhibitor (competitive with DXP and no-competitive with NADPH, which also is required for these reactions) (Koppisch et al., 2002; Kuntz et al., 2005; Possel et al., 2010). FSM shows low toxicity for animals and humans but demonstrates highly toxicity for most gram-negative bacteria (Murakawa et al., 1982; Tsuchiya et al., 1982; Kuemmerle et al., 1987) and plants (Zeidler et al.,

1997). That therefore makes FSM not only useful as a antibiotic but also as an herbicide (Patterson, 1987).

To test the effects of both inhibitors on spike lavender, a series of experiments were performed using wild type or transgenic plants. In some of them, the effect of MVA, the product of the reaction catalysed by the HMGR enzyme, on phenotype recovery of the inhibitor-treated plants was also tested.

Since there was not available information on the effect of MEV and FSM on spike lavender, first, it was necessary to determine concentrations of both inhibitors that produce significant phenotypic alterations in the species. To this end, seeds and shoot apices from *in vitro* grown plantlets were cultured *in vitro* in the presence of MEV or FSM.

MEV affected seed germination and further seedling development. The percentage of germinated seeds was significantly reduced at concentrations equal or higher than 1 µM MEV (Figure 35, Figure 36 and Table 23). All MEV concentrations tested negatively affected leaf development (Figure 35, Figure 36 and Table 23) and stem and root elongation (Figure 37 and Table 24). Roots were more sensitive to MEV than stems since the inhibitory effect was significantly evident at the lower MEV concentration tested (Figure 35, Figure 37 and Table 24). At the end of the experiment, chlorophyll and carotenoid content of the survival plantlets were determined. As shown in Figures 38, Figure 39 and Table 25, MEV did not significantly affect the chlorophyll and carotenoid content as compared to controls. All these results are in accordance with those reported in seedlings of other plant species including *Arabidopsis thaliana* (Bach and Lichtenthaler, 1983; Re et al, 1995; Rodríguez-Concepción, 2004), *Raphanus sativus* (Bach and Lichtenthaler, 1983) and *Triticum spp.* (Bach and Lichtenthaler, 1983).

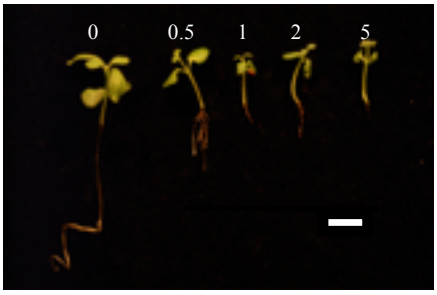


Figure 35. Spike lavender seedlings germinated on BM medium supplemented with 0, 0.5, 1, 2 and 5 µM MEV. Bar=1cm.

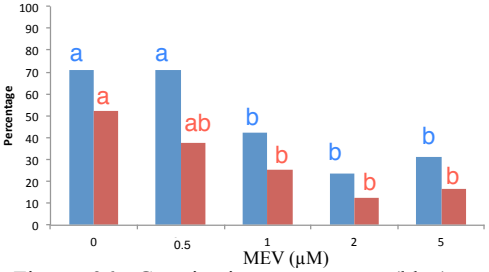


Figure 36. Germination percentage (blue) and percentage of seedlings with true leaves (red) on seeds germinated on BM medium supplemented with 0, 0.5, 1, 2 and 5 µM of MEV. For each parameter, data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 23. ANOVA for the data showed on Figure 36.

Source of variation	df	Mean Squares	
		Germination	Seedlings with true leaves
MEV	4	27,725.63**	9,838.13 **
Error	235	1,595.58	1,512.29

** significant at p≤0.001

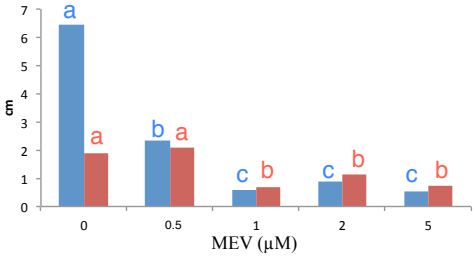


Figure 37. Root (blue) and stem (red) length of seedings germinated on BM medium supplemented with 0, 0.5, 1, 2 and 5 µM of MEV. For each parameter, data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 24. ANOVA for the data showed on Figure 37.

Source of variation	df	Mean Squares	
		Root lenght	Stem lenght
MEV	4	56.73**	3.88 **
Error	40	0.67	0.31

** significant at p≤0.001

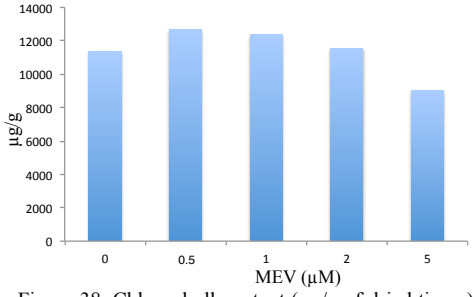


Figure 38. Chlorophyll content (µg/g of dried tissue) in seedings germinated on BM medium supplemented with 0, 0.5, 1, 2 and 5 µM of MEV.

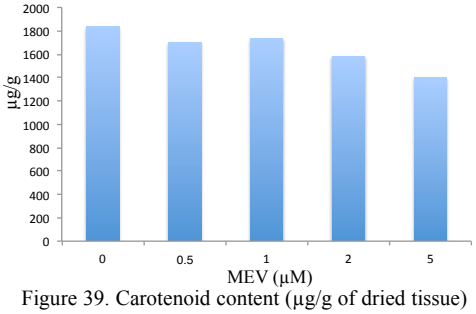


Figure 39. Carotenoid content (µg/g of dried tissue) in seedings germinated on BM medium supplemented with 0, 0.5, 1, 2 and 5 µM of MEV.

Table 25. ANOVA for the data showed on Figures 38 and 39.

Source of variation	df	Mean Squares	
		Chlorophyll content	Carotenoid content
MEV	4	7,538,035.4 NS	98,147.01 NS
Error	40	1,774,053.7	39,323.69

NS: non-significant

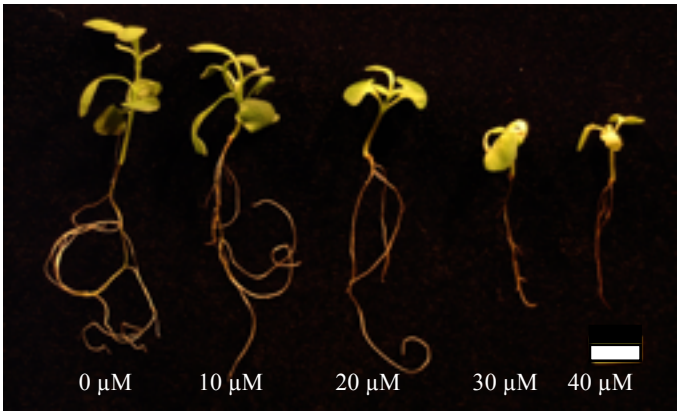


Figure 40. Spike lavender seedlings germinated on BM medium supplemented with 0, 10, 20, 30 and 40 µM FSM. Bar=1cm.

None of the FSM concentrations tested significantly affected seed germination (Figure 40, 41 and Table 26); in contrast, all FSM concentrations significantly decreased leaf development and stem and root elongation (Figures 42-44 and Tables 27 and 28). As expected (Laule et al., 2003; Rodríguez-Concepción et al., 2004) this inhibitor caused leaf chlorosis (Figure 40) that was due to a significant decrease in chlorophyll and carotenoid contents (Figures 43 and 44 and Table 28). In fact a significant negative correlation between chlorophyll or carotenoids and FSM concentrations was observed (R^2=0.876 and R^2=0.906, respectively).

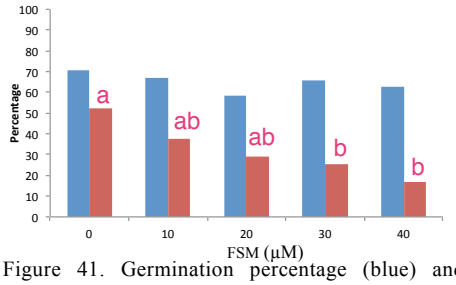


Figure 41. Germination percentage (blue) and percentage of seedlings with true leaves (red) on seeds germinated on BM medium supplemented with 0, 10, 20 and 30 µM of FSM. For each parameter data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 26. ANOVA for the data showed on Figure 41.

Source of variation	df	Mean Squares	
		Germination	Seedlings with true leaves
FSM	4	2,497.50 NS	6,868.13 *
Error	235	1,818.19	1,673.14

NS: non significant and **significant at p≤0.05

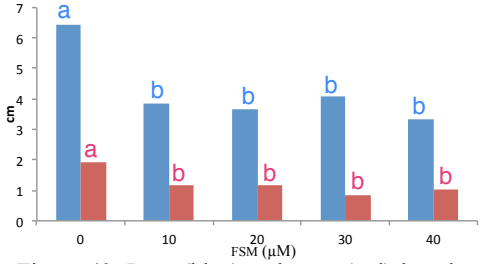


Figure 42. Root (blue) and stem (red) length of seedings germinated on BM medium supplemented with 0, 10, 20 and 30 µM of FSM. For each parameter, data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 27. ANOVA for the data showed on Figure 42.

Source of variation	df	Mean Squares	
		Root lenght	Stem lenght
FSM	4	13.92 *	1.50 **
Error	40	2.68	0.10

** significant at p≤0.001; * significant at p≤0.05

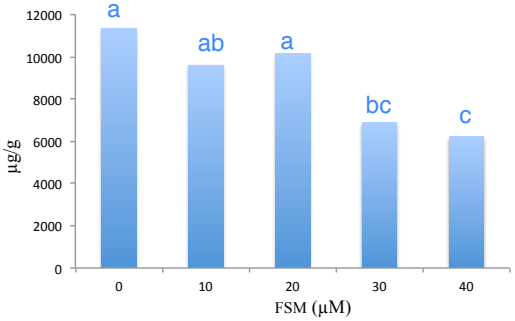


Figure 43. Chlorophyll content (mg/g of dried tissue) in seedings germinated on BM medium supplemented with 0, 10, 20 and 30 µM of FSM. Data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

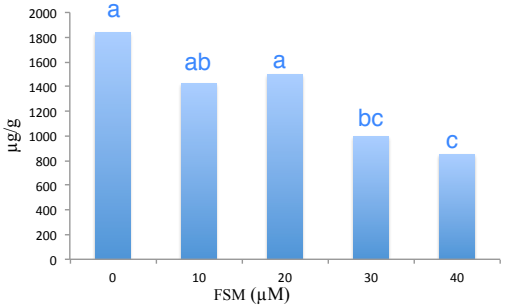


Figure 44. Carotenoid content (mg/g of dried tissue) in seedings germinated on BM medium supplemented with 0, 10, 20 and 30 µM of FSM. Data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 28. ANOVA for the data showed on Figures 43 and 44.

Source of variation	df	Mean Squares	
		Chlorophyll content	Carotenoid content
FSM	4	14,514,884.00 *	475,006.87 **
Error	40	1,458,378.5	32,529.81

** significant at $p \leq 0.001$; * significant at $p \leq 0.05$

Results from the experiments using shoot apices as initial explants reproduced those obtained with seeds. The presence of MEV or FSM in the culture medium affected further development of shoot apices. Generally, explants treated with either MEV or FSM showed short stems and a drastic inhibition of adventitious rooting (Figures 45 A and B).

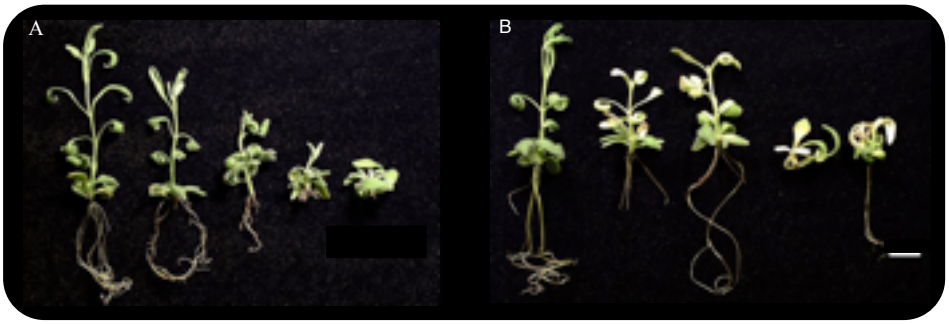


Figure 45. Seedlings germinated on BM medium supplemented with 0, 0.5, 1, 2, and 5 µM MEV (A) and 0, 10, 20, 30 and 40 µM FSM (B). Bar =1 cm.

MEV treatments reduced the stem and root length (Figure 46 and Table 29) and the formation of new stem whorls (Figure 47 and Table 30); in contrast, the synthesis of chlorophylls and carotenoids was not significantly altered (Figure 48 and Table 31).

The shoot apices grown in the presence of FSM showed shorter stems with a reduced number of developed new whorls than the controls, but no significant differences were observed regarding the length of adventitious roots (Figures 49 and 50 and Tables 32 and 33). Again, leaves were chlorotic due to a significant decrease in chlorophylls and carotenoids (Figure 45, Figure 51 and Table 34).

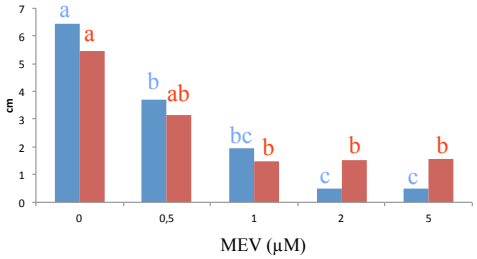


Figure 46. Root (blue) and stem (red) length of apexes cultivated on BM medium supplemented with 0, 0.5, 1, 2, and 5 µM MEV. For each parameter, data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 29. ANOVA for the data showed on Figure 46.

Source of variation	df	Mean Squares	
		Root lenght	Stem lenght
MEV	4	23.05 **	33.34 **
Error	67	3.31	0.60

** significant at p≤0.001

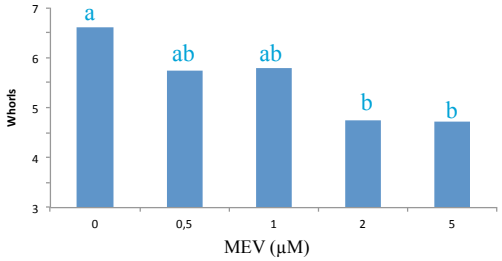


Figure 47. Mean whorl number of apexes cultivated on BM medium supplemented with 0, 0.5, 1, 2, and 5 µM MEV. Data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 30. ANOVA for the data showed on Figures 47.

Source of variation	df	Mean Squares
		Whorl mean number
MEV	4	8.50 **
Error	66	1.15

** significant at p≤0.001

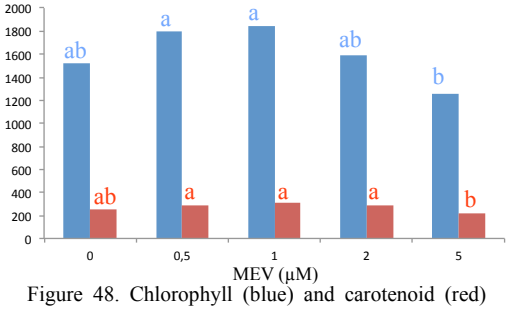


Figure 48. Chlorophyll (blue) and carotenoid (red) content (mg/g of fresh tissue) in apexes cultured on MS medium supplemented with 0, 0.5, 1, 2 and 5 µM of MEV. For each parameter, data followed by the same letter are not statically different according to Tukey's test at $P \leq 0.05$.

Table 31. ANOVA for the data showed on Figure 48.

Source of variation	df	Mean Squares	
		Chlorophyll content	Carotenoid content
MEV	4	166,583.68 *	4,136.53 *
Error	10	19,853.04	603.37

* significant at p≤0.05

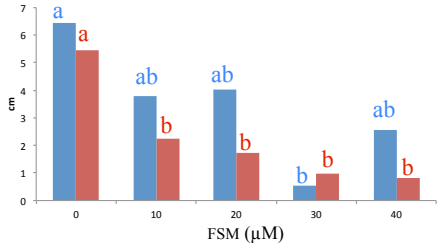


Figure 49. Root (blue) and stem (red) length on apexes treated with 0, 10, 20, 30 and 40 µM of FSM. For each parameter, data followed by the same letter are not statically different according to Tukey's test at P ≤ 0.05.

Table 32. ANOVA for the data showed on Figure 49.

Source of variation	df	Mean Squares	
		Root lenght	Stem lenght
FSM	4	28.40 *	21.93 *
Error	64	5.80	3.43

* significant at p≤0.05

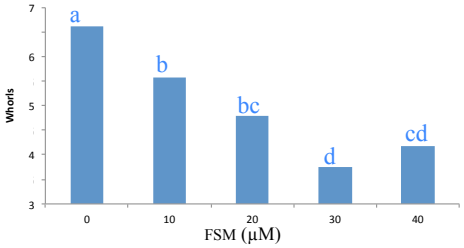


Figure 50. Whorl mean number of apexes treated with 0, 10, 20, 30 and 40 µM of FSM. Data followed by the same letter are not statically different according to Tukey's test at P ≤ 0.05.

Table 33. ANOVA for the data showed on Figure 50.

Source of variation	df	Mean Squares
		Whorl mean number
FSM	4	17.16 **
Error	64	0.89

** significant at p≤0.001

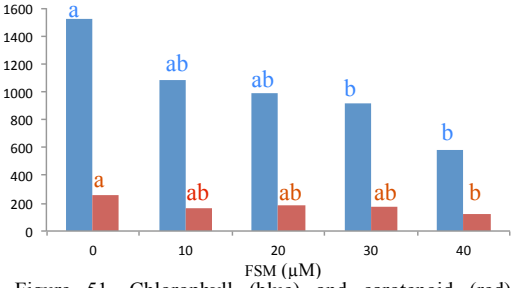


Figure 51. Chlorophyll (blue) and carotenoid (red) content (mg/g of fresh tissue) in apexes cutivated on MS medium supplemented with 0, 10, 20, 30 and 40 µM of FSM. For each parameter data followed by the same letter are not statically different according to Tukey's test at P ≤ 0.05.

Table 34. ANOVA for the data showed on Figure 51.

Source of variation	df	Mean Squares	
		Chlorophyll content	Carotenoid content
FSM	4	347,798.43 *	6,798.53 *
Error	10	48,533.78	1,208.93

* significant at p≤0.05

From these results it can be concluded that in spike lavender, as in other species (Rodríguez-Concepción, 2006; Vranová et al., 2012), the MEP pathway provides precursors for the synthesis of phytol and carotenoids, whereas MVA pathway gives precursors for the synthesis of terpenes that seems to be crucial for seedling development and root growth. Based on the above results we selected 1 μM MEV and 30 μM FSM as the most appropriate concentrations for further experiments.

III. 2. 2 Effect of MVA on FSM- or MEV-treated spike lavender shoot tips

In this experiment, we tested whether MVA, the substrate of the HMGR enzyme, overcomes the effect of 1 μM MEV or 30 μM FSM on shoot apices cultured *in vitro.*

First we tested the effect of mevalonate alone. Shoot tips grown in the presence of this compound developed normally as compared to controls (Figure 52). Nevertheless, the highest MVA concentration (3.5 mM) slightly reduced root length, whorl stem number, and fresh weight (Figure 52, Table 35).

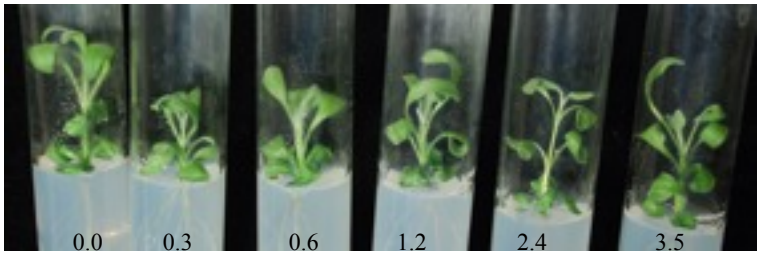


Figure 52. Phenotypic traits observed after 28 days treatment with increasing MVA concentrations.

Table 35. Phenotypic traits observed after 28 days of treatment with mevalonate (MVA) in spike lavender seedlings. For each column, values followed by the same letter are not significantly different according to Tukey´s test at $P \leq 0.05$.

MVA (mM)	Root lenght (cm)	Whorl mean number	Stem fresh weight (mg)
0.0	6.27 a	5.92 a	126.43 ab
0.3	5.48 ab	5.71 ab	112.37 bc
0.6	5.75 ab	6.04 a	142.56 a
1.2	5.51 ab	5.52 ab	116.37 bc
2.4	4.75 b	5.41 ab	110.21 bc
3.5	5.12 b	5.13 b	95.18 c

ANOVA		Mean Squares		
Source of Variation	df	Root lenght	Whorls mean number	Stem fresh weight
MVA	5	6.62*	2.84*	5,979.52 **
Error	137	1.71	0.75	847.27

** significant at $p \leq 0.001$; * significant at $p \leq 0.05$

On the other hand, MVA enhanced the photosynthetic pigment production in young leaves (Table 36 and Table 38), but reduced essential oil yield in both young and mature leaves (Table 37 and Table 38).

Table 36. Effect of mevalonate (MVA) on chlorophyll and carotenoid production (µg/g fresh weight) in young and mature leaves of *in vitro* grown spike lavender seedlings. For each mean, values followed by the same letter are not significantly different according to Tukey´s test at $P \leq 0.05$.

	Chlorophill a + b			Carotenoids		
MVA	Young leaves	Mature leaves	Mean	Young leaves	Mature leaves	Mean
0.0	1,778.68 c	1,559.70 ab	1,669.19 b	292.14 b	253.80 bc	272.97 c
0.3	1,903.63 bc	1,443.11 b	1,673.38 b	322.21 ab	242.91 c	282.56 bc
0.6	1,923.47 abc	1,604.35 ab	1,763.91 ab	308.25 b	254.06 bc	281.16 bc
1.2	2,051.74 ab	1,746.27 a	1,899.01 ab	321.14 ab	291.38 a	306.26 ab
2.4	1,997.86 abc	1,574.45 ab	1,786.16 a	325.04 ab	275.46 ab	300.25 abc
3.5	2,154.99 a	1,554.21 ab	1,854.60 a	367.13 a	266.34 bc	316.74 a
Mean	1,968.40 a	1,580.35 b		322.65 a	263.99 b	

Table 37. Effect of mevalonate (MVA) on essential oil production (µg/g fresh weight) in young and mature leaves of *in vitro* grown spike lavender seedlings. For each mean, values followed by the same letter are not significantly different according to Tukey´s test at P ≤ 0.05.

	MVA (mM)						
Leaves	0.0	0.3	0.6	1.2	2.4	3.5	Mean
Young	1,363.94	1,236.69	1,095.88	953.82	882.13	660.34	1,032.13 a
Mature	648.46	529.71	321.04	397.08	315.34	208.82	387.74 b
Mean	1,006.20 a	883.20 a	703.96 ab	675.45 ab	598.74 ab	434.58 b	

Table 38. ANOVA for the data showed on Tables 36 and 37.

		Mean Squares		
Source of Variation	df	Chlorophill a + b	Carotenoids	Essential oil
Age of verticil (A)	1	2,710,451.60 **	61,936.75 **	3,574,639.4 **
Mevalonate (B)	5	104,354.07 **	3,459.66 **	248,284.26 *
A x B	5	55,146.65 *	2,130.85 *	23,434.94 NS

NS non significant; **significant at p≤0.001; * significant at p≤0.05

Although the causes of this negative effect have not been determined, an accumulation of IPP could negatively affect normal metabolism of the trichome secretory cells as described for other systems (Martin et al., 2003). Based on the results obtained in this experiment, several MVA concentrations were subsequently employed to test whether this compound overcomes the effect of 1 µM MEV or 30 µM FSM on shoot apices cultured *in vitro*.

As previously reported, MEV affected shoot apices development. Specifically, this inhibitor decreased adventitious root length, stem whorl number, stem length and stem fresh weight but increased root number (Table 39, Figure 53).

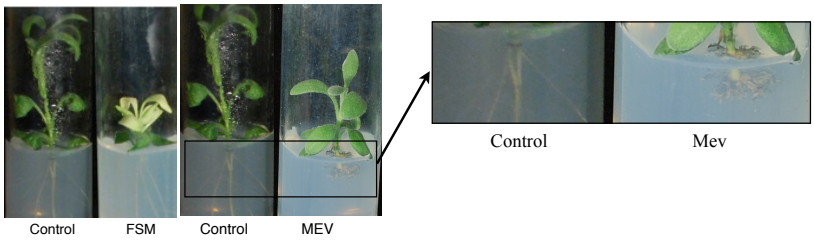


Figure 53. Phenotypic traits observed after 28 days treatment with 1µM Mev, 30µM FSM or Control (left). Effect of MEV on root development (right).

Table 39. Phenotypic traits observed in spike lavender seedlings grown *in vitro* for 28 days in the presence of 1µM Mev. For each trait, values followed by the same letter are not significantly different according to Tukey´s test at P ≤ 0.05.

Inhibitor	Number of roots	Root lenght (cm)	Whorl mean number	Stem Lenght (cm)	Stem Fresh weight (mg)
None	4.88 b	6.27 a	5.92 a	2.43 a	126.43 a
Mev	6.50 a	1.39 b	5.25 b	1.55 b	89.28 b

ANOVA		Mean Squares				
Source of Variation	df	Number of roots	Root lenght	Whorls mean number	Stem lenght	Stem fresh weight
Inhibitor	1	31.69 *	285.68 **	5.33 **	9.19 **	16,561.47 **
Error	46	4.06	0.81	0.27	0.34	541.02

**significant at p≤0.001; * significant at p≤0.05

In contrast, neither photosynthetic pigment content nor essential oil yield was significantly affected as compared with controls (Table 40).

Table 40. Effect of 1 µM Mev on photosynthetic pigments and essential oil content in leaves from *in vitro* grown spike lavender seedlings. For each trait, values followed by the same letter are not significantly different according to Tukey´s test at P ≤ 0.05.

	Chlorophill a + b (µg/g fresh weight)		Carotenoids (µg/g fresh weight)		Essential oil (µg/g fresh weight)	
Inhibitor	Young leaves	Mature leaves	Young leaves	Mature leaves	Young leaves	Mature leaves
None	1,778.68	1,569.08	292.14	253.80	1,363.94	648.46
Mev	1,568.08	1,592.46	272.83	262.98	1,163.66	531.77
				Mean	1,263.80 a	914.35 b

ANOVA		Mean Squares		
Source of Variation	df	Chlorophill a + b	Carotenoids	Essential oil
Whorl age (A)	1	28,694.52 NS	1,741.71 NS	1,361,541.00 *
MEV (B)	1	23,454.29 NS	76.96 NS	75,352.49 NS
A x B	1	44,051.35 NS	609.05 NS	5,242.14 NS

NS: non significant * significant at p≤0.05

FSM reduced root length, number of stem whorls, stem length and stem fresh weight (Figure 53 and Table 41). This inhibitor also caused a drastic reduction in photosynthetic pigments and essential oil production, particularly in young leaves (Table 42, Figure 53).

Table 41. Phenotypic traits observed in spike lavender seedlings grown *in vitro* for 28 days in the presence of 30µM FSM. For each trait, values followed by the same letter are not significantly different according to Tukey´s test at P ≤ 0.05.

Inhibitor	Root number	Root lenght (cm)	Whorl mean number	Stem Lenght (cm)	Stem fresh weight (mg)
None	4.88	6.27 a	5.92 a	2.43 a	126.43 a
FSM	4.42	5.12 b	5.38 b	1.55 b	96.20 b

ANOVA — Mean Squares

Source of Variation	df	Root number	Root lenght	Whorls mean number	Stem lenght	Stem fresh weight
FSM	1	2.52 NS	15.64 *	3.52 *	9.28 **	10,962.61 **
Error	46	2.14	1.37	0.29	0.34	529.96

NS: non significant; ** significant at p≤0.001; * significant at p≤0.05

Table 42. Effect of 30µM FSM on photosynthetic pigments and essential oil content in leaves from *in vitro* grown spike lavender seedlings. For each trait, values followed by the same letter are not significantly different according to Tukey's test at P ≤ 0.05.

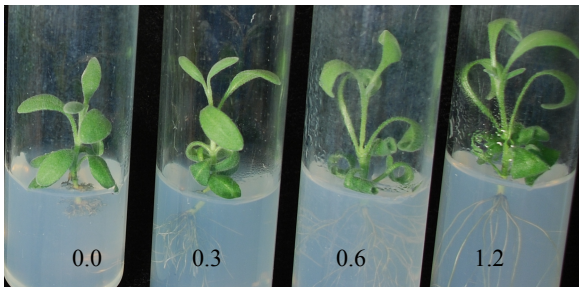
	Chlorophill a + b (µg/g fresh weight)		Carotenoids (µg/g fresh weight)		Essential oil (µg/g fresh weight)	
Inhibitor	Young leaves	Mature leaves	Young leaves	Mature leaves	Young leaves	Mature leaves
None	1,778.68 a	1,569.08 b	292.14 a	253.80 b	1,363.94 a	648.46 b
FSM	377.00 c	1,404.92 b	57.70 c	230.20 b	29.68 c	386.70 b

ANOVA — Mean Squares

Source of Variation	df	Chlorophill a + b	Carotenoids	Essential oil
Whorl age (A)	1	490,711.10 *	13,499.18 *	96,375.56 *
FSM (B)	1	1,816,762.40 **	49,938.48 **	1,910,467.90 **
A x B	1	1,165,938.80 **	33,342.24 **	862,686.83 **

**significant at p≤0.001; * significant at p≤0.05

MVA at 1.2 mM was able to recover the normal phenotype of MEV-treated plants, including a normal growth and development of roots (Figure 54 and Table 43) without affecting photosynthetic pigments and essential oil content (Table 44).



Mevalonate concentrations (mM)

Figure 54. Phenotype recovery with increasing concentrations of mevalonate in spike lavender seedlings grown in the presence of 1µM MEV.

Table 43. Phenotypic traits observed in spike lavender seedlings grown *in vitro* for 28 days in the presence of both different concentrations of MVA with or without 1 1µM Mev. For each trait, values followed by the same letter are not significantly different according to Tukey's test at $P \leq 0.05$.

MVA (mM)	Mev	Root mean number	Root Lenght	Whorl mean number	Stem lenght	Stem fresh weight
0.0	-	4.88	6.27 a	5.92 ab	2.43 a	126.43 abc
0.3	-	5.58	5.48 a	5.71 abc	2.55 a	112.37 cde
0.6	-	5.57	5.75 a	6.04 a	2.15 ab	142.56 a
1.2	-	5.13	5.51 a	5.52 bc	2.19 ab	116.37 bcd
0.0	+	6.50	1.39 d	5.25 c	1.55 b	89.28 e
0.3	+	6.41	2.79 c	5.50 bc	2.06 ab	102.21 de
0.6	+	5.75	3.88 b	5.58 bc	2.45 a	109.67 cde
1.2	+	5.91	5.64 a	5.70 abc	2.65 a	138.56 ab

ANOVA

Source of Variation	df	Mean Squares				
		Root mean number	Root lenght	Whorl mean number	Stem lenght	Stem fresh weight
MEV (A)	1	33.40 *	248.25 **	4.90 **	1.28 NS	9,763.96 **
MVA (B)	3	1.83 NS	28.05 **	0.94 *	1.67 NS	5,730.30 **
A x B	3	4.25 NS	50.65 **	1.76 *	4.57 **	8,577.86 **

NS non significant; **significant at p≤0.001; * significant at p≤0.05

Table 44. Essential oil, chlorophyll and carotenoid content (µg/g fresh weight) in spike lavender seedlings grown *in vitro* for 28 days in the presence of both different concentrations of mevalonate with or without 1µM Mev.

		Young leaves			Mature leaves		
MVA (mM)	Mev	Chlorophill a + b (µg/g fresh weigh)	Carotenoids (µg/g fresh weight)	Essential oil (µg/g fresh weigh	Chlorophill a + b (µg/g fresh weigh)	Carotenoids (µg/g fresh weight)	Essential oil (µg/g fresh weigh
0.0	-	1,778.68	292.14	1,363.94	1,569.08	253.80	648.46
0.3	-	1,903.64	322.21	1,236.69	1,761.17	272.83	529.71
0.6	-	1,923.47	308.25	1,095.88	1,865.86	292.62	312.04
1.2	-	2,051.74	321.14	953.82	2,028.12	304.71	397.08
0.0	+	1,559.7	272.83	1,163.657	1,592.46	262.98	531.77
0.3	+	1,443.11	292.62	903.68	1,683.37	288.45	182.6
0.6	+	1,604.35	304.71	538.75	1,430.9	231.73	189.71
1.2	+	1,746.27	350.09	603.56	1,473.87	271.30	511.56

ANOVA		Mean Squares		
Source of Variation	df	Chlorophill a + b	Carotenoids	Essential oil
Leaves age (A)	1	1,033,463.41 **	25,305.13 **	3,893,734.35 **
MEV (B)	1	68,890.84 NS	23.51 NS	685,679.07 *
Mevalonate (C)	3	82,268.26 *	3,487.51 *	344,630.96 *
A x B	1	12,721.52 NS	240.35 NS	176,070.93 NS
A x C	3	64,462.21 NS	875.50 NS	91,842.88 NS
B x C	3	22,501.43 NS	268.28 NS	41,510.08 NS
A x B x C	3	65,886.62 NS	2,223.36 NS	44,366.20 NS

NS non significant; **significant at p≤0.001; * significant at p≤0.05

In contrast, MVA only partially alleviated the toxic effects caused by FSM on spike lavender (Figure 55); thus, mevalonate slightly recovered photosynthetic pigment content and, to a lesser extent, essential oil production of the plantlets developed in the presence of FSM (Figure 55 and Table 45). The beneficial effect of MVA on the recovery of MEV- or FSM-treated plants has also been demonstrated in *Arabidopsis* (Nagata et al., 2002) and tobacco cell cultures (Hemmerlin et al., 2003).

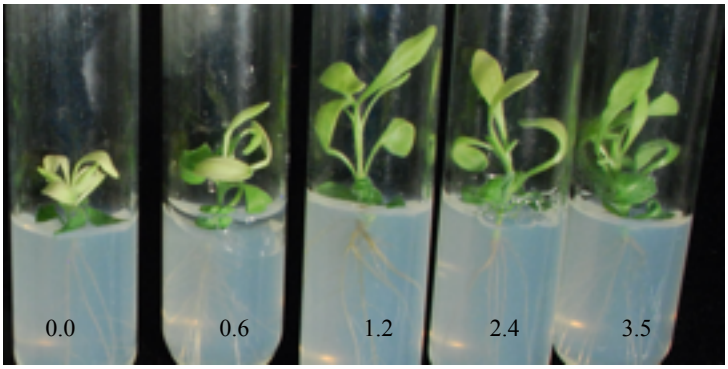


Figure 55. Phenotype recovery with increasing concentrations of mevalonate in spike lavender seedlings grown in the presence of 30 µM FSM.

Table 45. Production of photosynthetic pigments and essential oil in spike lavender seedlings grown in the presence of 30 µM FSM and increasing concentrations of mevalonate. For each column, values followed by the same letter are not significantly different according to Tukey´s test at $P \leq 0.05$.

	Young leaves			Mature leaves		
MVA (mM)	Chlorophill a + b (µg/g fresh weight)	Carotenoids (µg/g fresh weight)	Essential oil (µg/g fresh weight)	Chlorophill a + b (µg/g fresh weight)	Carotenoids (µg/g fresh weight)	Essential oil (µg/g fresh weight)
0.0	377.00 c	57.70 b	29.68	1,404.92	230.20	386.70
0.6	738.87 a	121.69 a	33.89	1,521.87	262.60	319.56
1.2	735.06 ab	109.24 a	83.03	1,526.33	267.01	280.91
2.4	516.53 cb	92.30 ab	69.77	1,374.76	217.76	543.51
3.5	649.41 ab	98.06 a	41.14	1,357.70	222.82	366.63

ANOVA		Mean Squares		
Source of Variation	df	Chlorophill a + b	Carotenoids	Essential oil
Whorl age (A)	1	794,116.52 **	30,204.24 **	308,690.06 *
FSM (B)	1	8,993,541.00 **	243,978.92 **	3,289,624.9 **
Mevalonate (C)	3	94,571.96 *	2,605.55 *	127,091.45 *
A x B	1	5,465,730.80 **	148,219.32 **	3,333,519.2 **
A x C	3	46,364.38 *	1,493.30 NS	22,199.17 NS
B x C	3	22,603.50 NS	2,036.31 *	150,242.58 *
A x B x C	3	7,636.12 NS	293.71 NS	19,747.68 NS

NS non significant; **significant at $p \leq 0.001$; * significant at $p \leq 0.05$

III. 2. 3. Effect of MEV and FSM on transgenic spike lavender lines

Results from MVA feeding experiments suggested that IPPs from the MVA pathway contribute to the synthesis of photosynthetic pigment and essential oils. To corroborate this hypothesis we undertook new experiments employing several transgenic spike lavender lines previously obtained by our group (Muñoz-Bertomeu et al., 2006 and 2007a).

In a first experiment, shoot apices from wild type (WT) plants and transgenic HMGR5 line overexpressing the *HMG1* gene from *Arabidopsis thaliana*, were cultured in the presence of 1 μM MEV or 30 μM FSM.

Regardless of the presence of either MEV or FSM, HMGR5 plants displayed higher stem and root growth, but with lower number of stem whorls as compared with WT (Figure 56A, B and C; Tables 46 and 47).

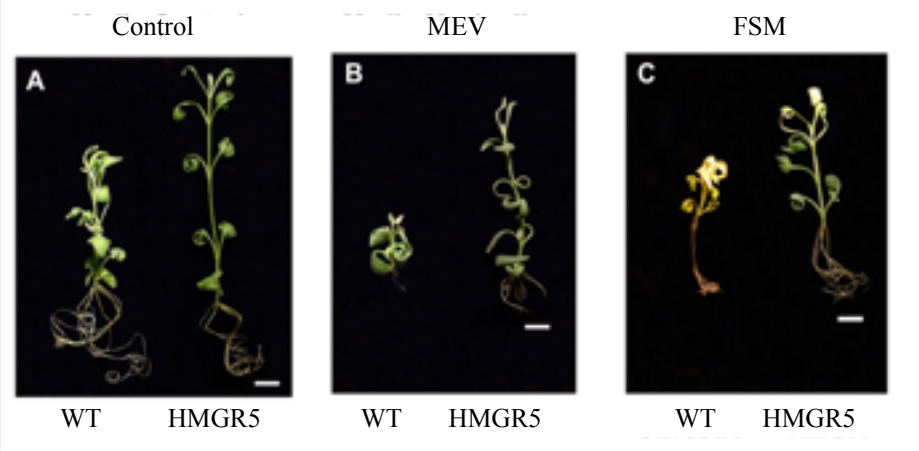


Figure 56. Effect of MEV and FSM on *in vitro* stems of lines HMGR5 and WT control. Bars=1cm

MEV inhibited the stem and root growth and the development of stem whorls in both transgenic and WT lines (Table 46 and Figures 56A and B). The chlorophyll and carotenoid content did not differ significantly between WT and HMGR5 line in untreated plantlets (Table 48). MEV reduced chlorophyll (-14%) and carotenoid (-3%) content in leaves of WT plants. In contrast,

inhibitor increased chlorophyll (20%) and carotenoid (36%) content in leaves from transgenic HMGR5 line ($p \leq 0.05$, Table 48).

Table 46. Effect of MEV (1 μM) on the root and stem lenght and the production of new whorls after 42 days of treatment. The percentage of change in each parameter related to control is shown in parenthesis. For each parameter, values followed by the same letter are not statistical different ($p \leq 0.05$) according to Tukey test.

Line	Root lenght (cm)			Stem lenght (cm)		Whorls		
	Control	Mev	Mean[y]	Control	Mev	Control	Mev	Mean[y]
WT	6.5	0.8	3.7 b	3.8 b	2.4 c	6.2	4.7	5.4 a
		(-88 %)			(-37 %)		(-24 %)	
HMGR5	7.7	2	4.8 a	7.3 a	4.0 b	5.3	4.4	4.9 b
		(-74%)			(-45%)		(-17%)	
Mean[z]	7.1 a	1.4 b				5.8 a	4.5 b	

ANOVA		Mean Squares		
Source of Variation	df	Root lenght	Stem lenght	Whorls
Line (A)	1	32.3 *	160.7 *	8.2 *
Treatment (B)	1	771.2 *	130.7 *	35.0 *
AxB	1	0.0 NS	22.8 *	1.5 NS

NS non significant; * significant at $p \leq 0.05$; y= effect of the line; z= effect of the treatment

Table 47. Effect of FSM (30μM) on the length of root and stem and new whorls development. Data after 42 days of treatment. The percentage of change in each parameter related to control is shown in parenthesis. For each row/column values followed by the same letter are not statistical different ($p \leq 0.05$) according to Tukey test.

Line	Root lenght (cm)			Stem lenght (cm)		Whorls		
	Control	FSM	Mean[y]	Control	FSM	Control	FSM	Mean[y]
WT	6.5	3.9	5.2b	3.8b	1.7c	6.2	5	5.6 a
		(-40%)			(-55%)		(-20%)	
HMGR5	7.7	5.3	6.5a	7.3a	2.7c	5.3	3.8	4.6 b
		(-30%)			(-63%)		(-28%)	
Mean[z]	7.1 a	4.6 b				5.75 a	4.4b	

ANOVA		Mean Squares		
Variation Source	df	Root lenght	Stem lenght	Whorls
Line (A)	1	39.6*	120.2*	23.0 *
Treatment (B)	1	143.8*	268.0*	44.0 *
AxB	1	0.4 NS	42.1*	0.5 NS

NS non significant; * significant at $p \leq 0.05$; y= effect of the line; z= effect of the treatment

FSM also inhibited root and stem growth as well as the development of new whorls in both transgenic and WT lines (Table 47). As expected, this MEP pathway inhibitor reduced significantly chlorophyll and carotenoids content in both lines (Table 49). Note however, that in transgenic HMGR5 line the decrease in these pigments was about half of that observed in the WT line (-26% chlorophyll and -22% carotenoid, vs. -43% chlorophyll and -42% carotenoid for HMGR5 and WT lines, respectively), which seems to corroborate the above mentioned flux of MEV-derived IPP from the cytosol to the plastid.

These last results prompted new experiments that employed potted stem cuttings from transgenic HMGR, DXS and HMGR-DXS lines obtained by our group (Muñoz-Bertomeu et al., 2006 and 2007a and this work) growing in the greenhouse. Stem cuttings from an *npt*II line were used as a control.

The irrigation of the pots with aqueous solutions of either 1 μM MEV or 30 μM FSM, every two days for two weeks did not further alter development of the cuttings and neither growth parameters recorded (root and stem length, fresh aerial weight, whorl mean number and number of axillary buds) nor photosynthetic pigments or essential oil contents were significantly affected as compared to controls (data no shown). Because of this, in a second assay the potted cuttings were irrigated twice a week for two months with the inhibitors. Results from this assay are summarized in Figures 57- 65 and Tables 50 and 51.

MEV did not significantly affect cutting development as measured by root (Figure 58) and stem length (Figure 59), stem whorls number (Figure 60) and the production of axillary shoots (Figure 61); this was also true for FSM treatment except in the double transgenic line where a significant (-43%) reduction in the production of axillary shoots was observed.

Table 48. Effect of MEV (1µM) on the chlorophyll and carotenoids content in HMGR and control plants. Data after 42 days of treatment. The percentage of change in each parameter related to control is shown in parenthesis. For each row/column values followed by the same letter are not statistical different (p≤0.05) acording to Tukey test.

Line	Chlorophyll (µg/g dried weight)		Carotenoids (µg/g dried weight)	
	Control	Mev	Control	Mev
WT	10,985 b	9,442 c (-14%)	1,860 b	1,803 b (-3 %)
HMGR5	11,465 b	13,819 a (+20%)	1,795 b	2,436 a (+36%)

ANOVA

Source of Variation	df	Mean Squares: Chlorophyll/ dried weight	Mean Squares: Carotenoids/ dried weight
Line (A)	1	17,693,776*	242,144*
Treatment (B)	1	492,067 NS	255,668*
AxB	1	11,388,454*	365,996*

NS non significant; **significant at p≤0.001; * significant at p≤0.05

Table 49. Effect of FSM (30µM) on the chlorophyll and carotenoids content of HMGR and wildtype stems treated. Data after 42 days of treatment. The percentage of change in each parameter related to control is shown in parenthesis. For each row/column values followed by the same letter are not statistical different (p≤0.05) according to Tukey test.

Line	Chlorophyll (µg/g dried weight)		Carotenoids (µg/g dried weight)	
	Control	FSM	Control	FSM
WT	10,986 a	6,263 c (-43%)	1,860 a	1,076 c (-42%)
HMGR5	11,466 a	8,517 b (-26%)	1,795 a	1,406 b (-22%)

ANOVA

Source of Variation	df	Mean Squares: Chlorophyll/ dried weight	Mean Squares: Carotenoids/ dried weight
Line (A)	1	5,608,938.8 *	52,539.9 NS
Treatment (B)	1	44,133,068.4 *	1,032,427.5 *
AxB	1	2,361,143.1 *	117,039.3 *

NS non significant; **significant at p≤0.001; * significant at p≤0.05

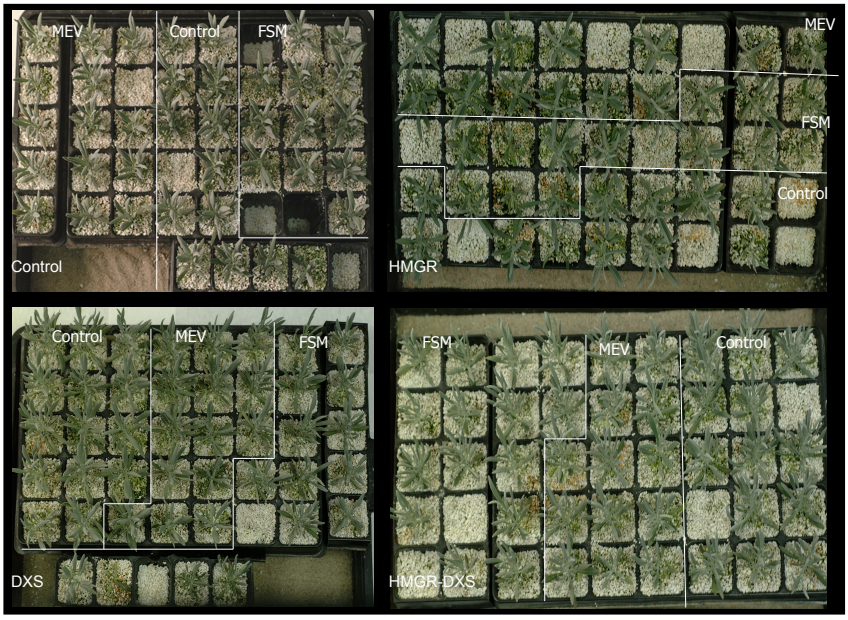


Figure 57. Phenotype of lines WT, HMGR, HMGR-DXS and DXS under the 3 treatments (Control 1 µM MEV and 30 µM FSM) after two months of treatment.

Generally, MEV did not affect the biochemical parameters studied (photosynthetic pigments and essential oil contents) in leaves from the 1st to 3th whorl. In contrast, FSM significantly decreased the chlorophyll and carotenoid contents in these leaves from all transgenic lines except in that overexpressing the *HMG1* gene (Figures 62-63). FSM also decreased essential oil content (Figures 64-65), especially in younger leaves (Figure 64) which can be explained by the fact that essential oil biosynthesis in spike lavender is higher in developing than in mature leaves (Muñoz-Bertomeu et al., 2008).

Selective inhibitors of either MVA or MEP pathways have been employed successfully in different plant systems (Rodríguez-Concepción et al., 2004 ; Roberts, 2007; Yoonram et al., 2008) to dilucidate cross-talk between both biosynthetic pathways. Our results with inhibitors-treated spike lavender explants, especially when MVA was also added or when *HMG1* transgenic plants were used, support that terpene precursors from the MVA pathway contribute to the synthesis of photosynthetic pigments.

In our experimental conditions the use of inhibitors seems not to be the

most appropriate approach to elucidate the metabolic origin (MVA or MEP pathway) of essential oil precursors.

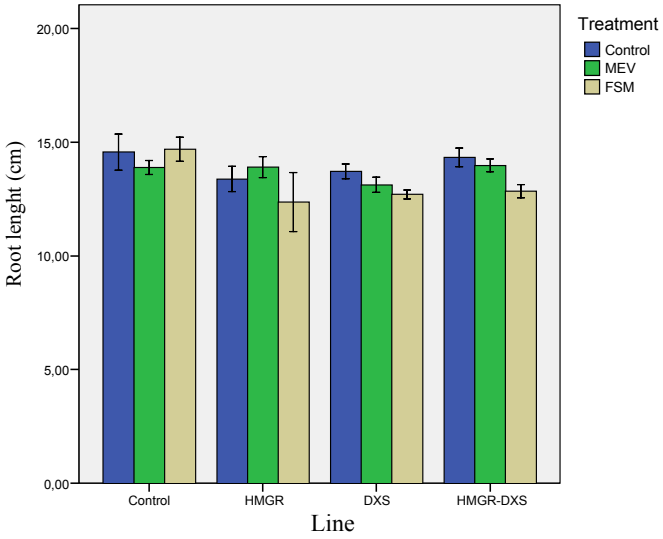


Figure 58. Root lenght (cm) of lines WT, HMGR, HMGR-DXS and DXS untreated (blue), treated with MEV (green) and FSM (yellow). Reported values are the mean ± SE of 3 measurements.

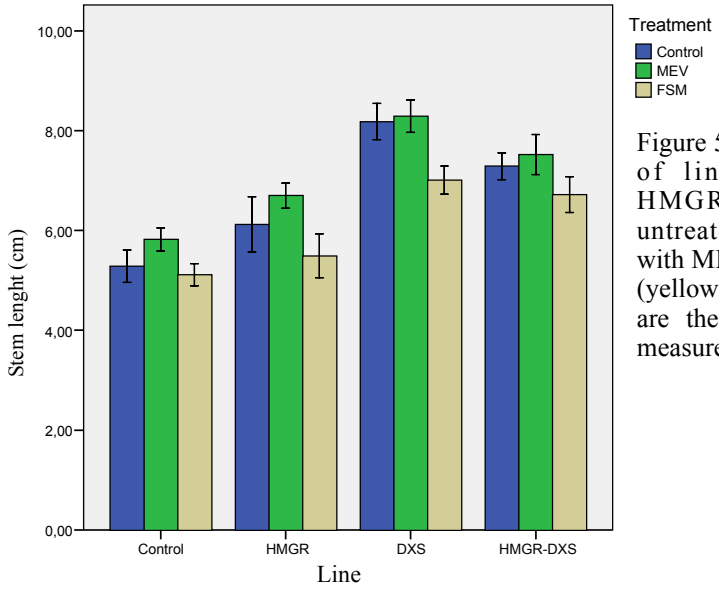


Figure 59. Stem lenght (cm) of lines WT, HMGR, HMGR-DXS and DXS untreated (blue), treated with MEV (green) and FSM (yellow). Reported values are the mean ± SE of 3 measurements.

Figure 60. Whorl number of lines WT, HMGR, HMGR-DXS and DXS untreated (blue), treated with MEV (green) and FSM (yellow). Reported values are the mean ± SE of 3 measurements.

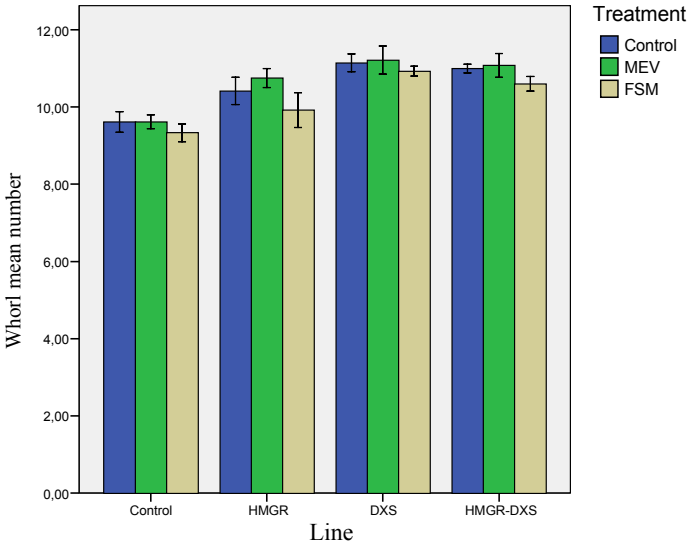


Figure 61. Auxillary bud number of lines WT, HMGR, HMGR-DXS and DXS untreated (blue), treated with MEV (green) and FSM (yellow). Reported values are the mean ± SE of 3 measurements.

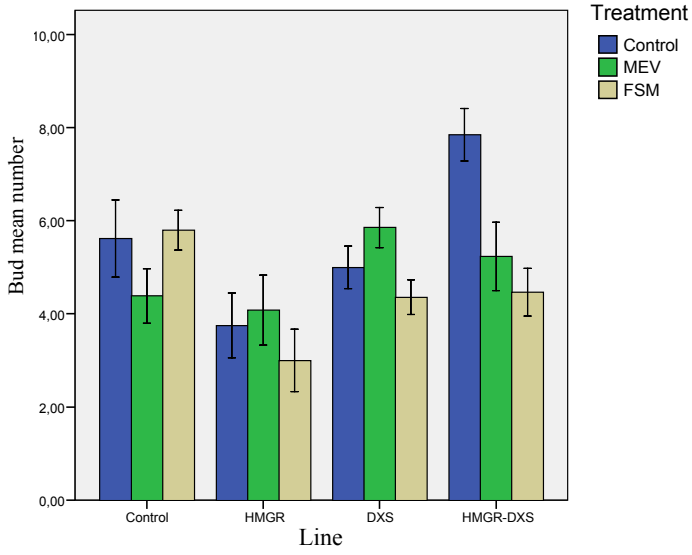
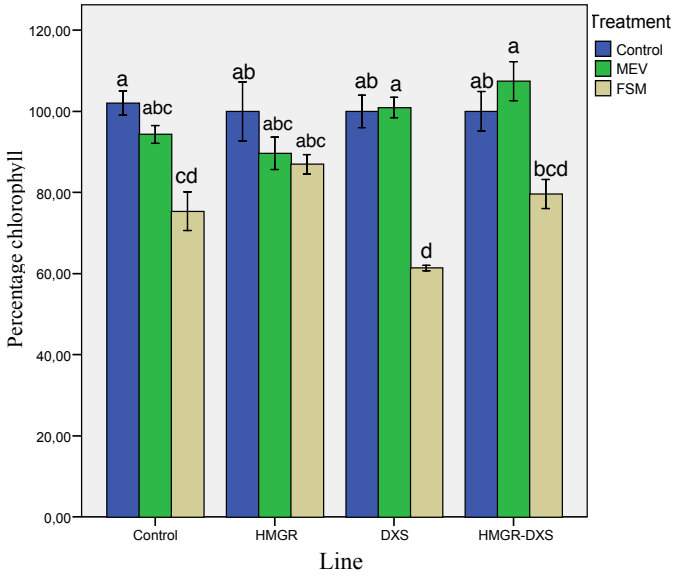


Table 50. ANOVA for the data showed on Figures 58, 59, 60 and 61.

Source of Variation	df	Mean Squares			
		Stem lenght	Root lenght	Whorl mean number	Bud mean number
Line (A)	3	47.82 **	12.58 *	20.28 **	33.96 **
Treatment (B)	2	13.87 **	10.13 NS	3.22 *	17.72 *
AxB	6	0.71 NS	4.10 NS	0.22 NS	14.92 *

NS non significant; **significant at p≤0.001; * significant at p≤0.05

Figure 62. Variation in chlorophyll content as compared to their control in the first 3 whorls in all lines untreated (blue), treated with MEV (green) and treated with FSM (yellow). The bars followed by the same letter are not significantly different (P ≤0.05) according to Tukey's test. Reported values are the mean ± SE of 3 measurements.



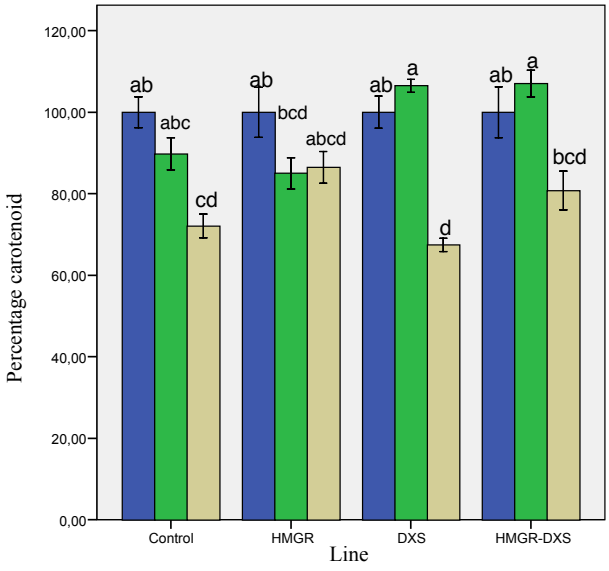
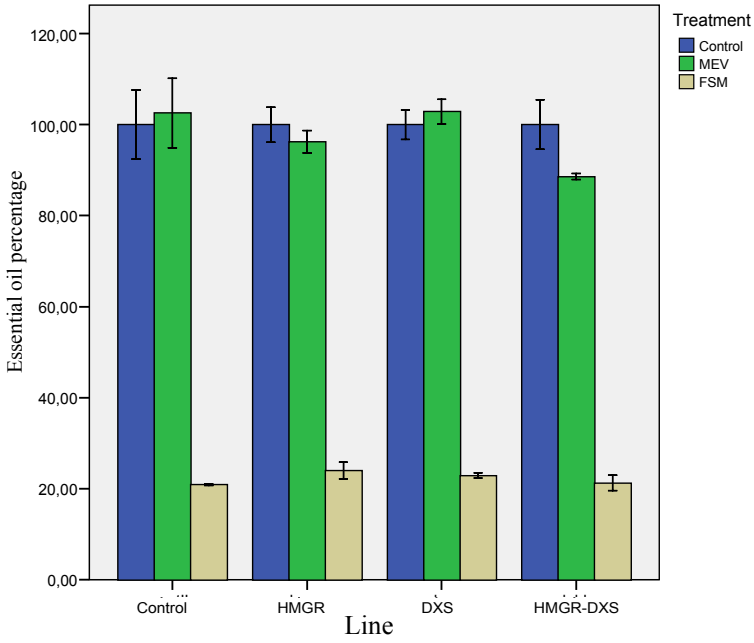


Figure 63. Variation in carotenoid content as compared to their control in the first 3 whorls in all lines untreated (blue), treated with MEV (green) and treated with FSM (yellow). The bars followed by the same letter are not significantly different (P ≤0.05) according to Tukey's test. Reported values are the mean ± SE of 3 measurements.

Figure 64. Variation in essential oil content obtained by direct extraction in the first 3 whorls as compared to their control of all lines untreated (blue), treated with MVE (green) and treated with FSM (yellow). Reported values are the mean ± SE of 3 measurements.



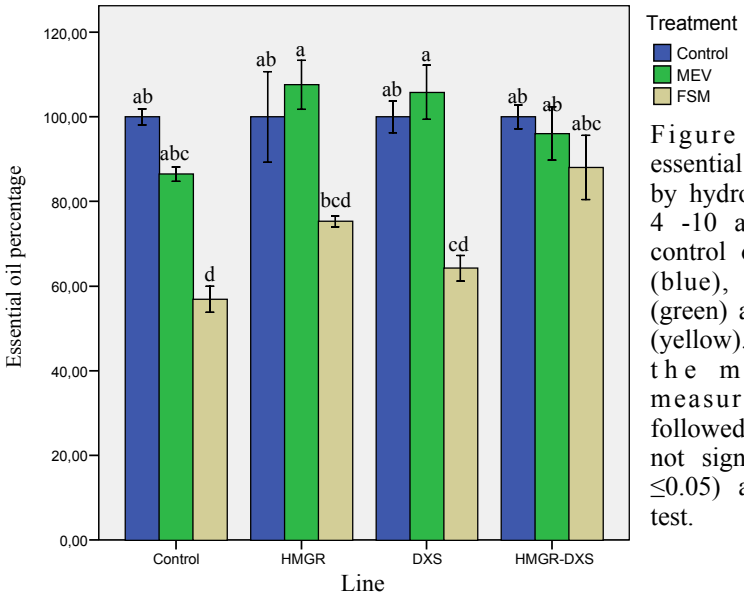


Figure 65. Variation in essential oil content obtained by hydrodistillation in whorls 4 -10 as compared to their control of all lines untreated (blue), treated with MEV (green) and treated with FSM (yellow). Reported values are the mean ± SE of 3 measurements. The bars followed by the same letter are not significantly different (P ≤0.05) according to Tukey's test.

Table 51. ANOVA for the data showed on Figures 62, 63, 64 and 65.

Source of Variation	df	Mean Squares			
		Chlorophyll content	Carotenoid content	Essential oil 1st-3rd whorls	Essentail oil 4th-10th whorls
Line (A)	3	106.13 NS	115.06 NS	49.47 NS	358.31 *
Treatment (B)	2	2,129.20 **	1,932.64 **	23,457.01 **	3,225.45 **
AxB	6	212.17 *	245.21 *	46.01 NS	240.61 *

NS non significant; **significant at p≤0.001; * significant at p≤0.05

This fact could be due to differences between secretory cells in glandular trichomes, where essential oil components are synthesized, and photosynthetically active mesophyl cells. Nevertheless, the fact that MVA partially restores the toxic effect of FSM on photosynthetic pigment content (Figure 54) and that the decrease of essential oil content produced by the application of FSM was lower in mature leaves of transgenic lines that overexpressed the HMGR enzyme (Figure 65), suggest that terpene precursors from the MVA pathway may contribute to the synthesis of all plastidial terpenes. To corroborate this, feeding experiments with labeled terpene precursors were also undertaken. Results of these experiments are presented below.

III. 2. 4. Labeling experiments

These experiments were carried out to identify the relative contribution of MEP and MVA pathways to the synthesis of monoterpenes, the main constituents of spike lavender essential oil. To this end, wild type and transgenic HMGR5 lines growing either in the greenhouse or *in vitro* were fed with $^{13}CO_2$, [U-$^{13}C_6$] glucose or [1,2-^{13}C] mevalonate. Subsequently, leaf essential oil was extracted with chloroform and its compounds and their isotope composition were analyzed by GC/MS and NMR spectroscopy.

Camphor and 1,8-cineol, the most abundant monoterpenes in the leaves of spike lavender essential oil, were chosen as models to determine the isotopologue distribution. The isotopologues are monitored via GC/MS by first

measuring a standard and then a sample (Römisch-Margl et al., 2007). The spectrum shows the m/z (mass-to-charge) values for all the molecules in the sample. According to their labeling, the m/z values for camphor and cineol can be assigned as M+0 for no labeling (no extra mass), M+1 for one C labeled, M +2 for two labeled and so on until M+10. The relative ^{13}C incorporation is determined in comparison to the external standard. The natural ^{13}C incorporation is then substracted from the measured values of the absolute incorporation. The remaining ^{13}C incorporation is referred to as the excess incorporation. When comparing the excess incorporations of different isotopomeres to each other, the ratio between different precursors of metabolic pathways can be determined.

III. 2. 4. 1. ^{13}C-Mevalonate labeling experiments

MVA labeled with ^{13}C in two specific positions (C1 and C2, Figure 66) was used in order to monitore the labeling pattern of camphor and 1,8-cineol in spike lavender seedling grown *in vitro*. The medium was supplemeted or not (control) with 1.2 mM [1,2-$^{13}C_2$] mevalonate, 30 μM FSM or a combination of both compounds.

In these experiments, we also evaluated the external phenotype by measuring the root length, whorl stem number, stem length and grow and stem fresh weight, and increased root number every week (Tables 52-55). In all parameters, control plants showed the highest values, followed by the MVA and far away both the FSM and FSM-MVA plants in a very similar manner.

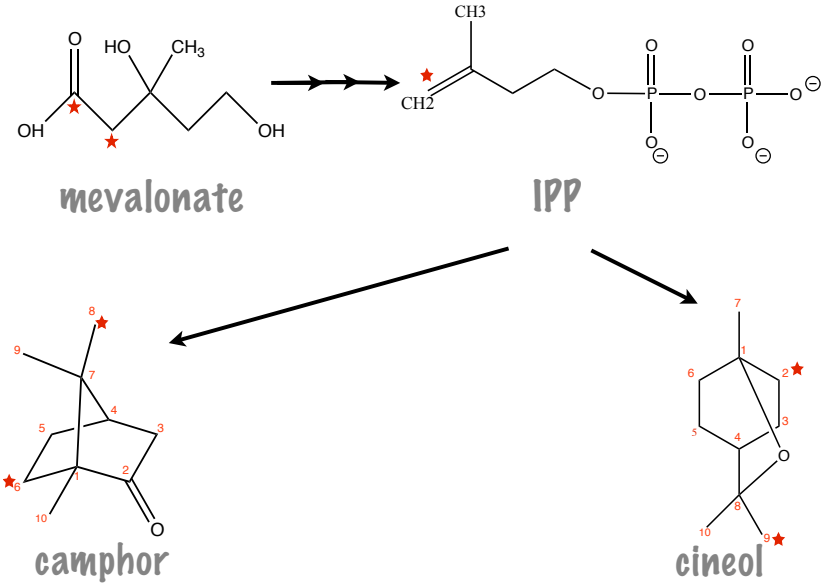


Figure 66. Labeling pattern of [1,2-$^{13}C_2$]mevalonate and expected labeling pattern of the IPP intermediate, camphor and cineol. For cineol and camphor, only one of the ^{13}C represented may be present in each resulting molecule.

Table 52. Effect of 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MVA+FSM added to the culture media on plant growth after 7 days of culture. For each parameter, values followed by the same letter are not significantly different according to Tukey´s test (α=0.05).

Treatment	Root number	Root length (cm)	Fresh weight (mg)	Stem length (cm)	Stem growth (cm/7 days)	Whorl number
Control	5.25 a	2.01 a	57.33 a	0.77	0.14 a	3.18
MVA	4.42 ab	1.34 b	51.33 ab	0.73	0.12 ab	3.11
FSM	4.70 a	0.81 c	50.81 ab	0.74	0.10 b	3.08
FSM+MVA	3.05 b	0.49 c	44.06 b	0.75	0.13 ab	3.13

ANOVA		Mean Squares				Mean Squares		
Source of Variation	df	Root number	Root length	Fresh weight	df	Stem length	Stem growth	Whorl number
Treatment	3	17.51 *	8.86 **	591.11 *	3	0.03 NS	0.02 *	0.14 NS
Error	75	2.93	0.18	182.49	316	0.04	0.01	0.11

NS: non significant; * significant at p≤0.05; ** significant at p≤0.001

Table 53. Effect of 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MVA+FSM added to the culture media on plant growth after 14 days of culture. For each parameter, values followed by the same letter are not significantly different according to Tukey´s test (α=0.05).

Treatment	Root number	Root length (cm)	Fresh weight (mg)	Stem length (cm)	Stem growth (cm/7 days)	Whorl number
Control	4.95	4.26 a	133.32 a	1.12 a	0.36 a	4.35 a
MVA	5.15	3.71 a	107.81 b	1.04 a	0.28 b	4.12 b
FSM	5.25	2.74 b	80.21 c	0.81 b	0.12 c	3.93 c
FSM+MVA	4.3	2.54 b	77.01 c	0.85 b	0.10 c	3.97 bc

ANOVA		Mean Squares				Mean Squares		
Source of Variation	df	Root number	Root length	Fresh weight	df	Stem length	Stem growth	Whorl number
Treatment	3	3.65 NS	13.20 **	13,941.35 **	3	1.32 **	1.01 **	2.16 **
Error	76	3.20	0.50	681.69	236	0.10	0.03	0.15

NS: non significant; * significant at p≤0.05; ** significant at p≤0.001

Table 54. Effect of 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MVA+FSM added to the culture media on plant growth after 21 days of culture. For each parameter, values followed by the same letter are not significantly different according to Tukey´s test (α=0.05).

Treatment	Root number	Root length (cm)	Fresh weight (mg)	Stem length (cm)	Stem growth (cm/7 days)	Whorl number
Control	5.20	5.69 a	169.90 a	1.76 a	0.67 a	4.35 a
MVA	5.45	4.40 b	131.57 ab	1.54 a	0.54 a	4.12 b
FSM	4.60	3.77 b	124.17 b	0.97 b	0.15 b	3.93 c
FSM+MVA	3.95	3.53 b	101.55 b	1.04 b	0.15 b	3.97 bc

ANOVA		Mean Squares				Mean Squares		
Source of Variation	df	Root number	Root length	Fresh weight	df	Stem length	Stem growth	Whorl number
Treatment	3	8.97 NS	18.71 a	16,163.74 **	3	5.89 **	2.80 **	2.17 **
Error	76	4.00	1.76	2,554.72	156	0.25	0.06	0.27

NS: non significant; * significant at p≤0.05; ** significant at p≤0.001

Table 55. Effect of 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MVA+FSM added to the culture media on plant growth after 728days of culture. For each parameter, values followed by the same letter are not significantly different according to Tukey´s test (α=0.05).

Treatment	Root number	Root length (cm)	Fresh weight (mg)	Stem length (cm)	Stem growth (cm/7 days)	Whorl number
Control	5.60 a	6.25 a	169.90 a	2.48 a	0.62 a	4.35 a
MVA	5.20 a	6.62 a	131.57 ab	2.14 a	0.40 b	4.12 b
FSM	4.75 ab	5.19 ab	124.17 b	1.24 b	0.23 b	3.93 c
FSM+MVA	3.90 b	4.24 b	101.55 b	1.32 b	0.21 b	3.97 bc

ANOVA		Mean Squares					
Source of Variation	df	Root number	Root length	Fresh weight	Stem length	Stem growth	Whorl number
Treatment	3	10.65 *	14.33 **	45,178.10 **	7.49 **	0.73 **	3.02 **
Error	76	2.05	1.81	3,304.45	0.44	0.06	0.32

NS: non significant; * significant at p≤0.05; ** significant at p≤0.001

Table 56. Amount of 1,8-cineol (Cin.), camphor (Cam.) and coumarin (Cou.) for each sample and time pulse cultured in 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MVA+FSM or Control media. The pics were identified and normalize to the coumarin value.

Pulse days	Control			FSM			MVA			FSM+MVA		
	Cin	Cam	Cou	Cin	Cam	Cou	Cin	Cam	Cou	Cin	Cam	Cou
7	2.03	0.68	1.00	0.32	0.12	1.00	1.22	0.59	1.00	1.07	0.38	1.00
14	0.35	0.16	1.00	0.13	0.04	1.00	0.27	0.06	1.00	0.27	0.08	1.00
21	0.15	0.04	1.00	0.02	0.01	1.00	0.32	0.12	1.00	0.08	0.03	1.00
28	0.26	0.10	1.00	0.03	0.01	1.00	0.21	0.06	1.00	0.14	0.07	1.00

Although there was very little difference regarding the external phenotype between the FSM and the FSM-MVA samples (Figure 67), the variance in the monoterpene content among them was more evident. Thus, after 28 days, the FSM-MVA samples had a higher monoterpene percentage than those treated with FSM alone (0.21 and 0.04 %, respectively), that indicates a higher production of these two metabolites due to the availability of MVA in the culture medium. MVA samples showed slightly lower percentage of monoterpenes than the control samples (0.27 and 0.36 %, respectively), repeating what already observed in section III. 4. 2.

Table 57 shows the absolute ^{13}C abundance percentage of cineol and camphor in plantlets grown in the presence of 1,2-$^{13}C_2$MVA. Control samples without labeled MVA had ^{13}C values of 1.1 ± 0.2% for cineol and 1.1 ± 0.2% for camphor, which are coincident with the expected value of 1.1%, showing the accuracy of the method. The MVA samples showed no significant increase in neither cineol (1.1 ± 0.1%) and camphor (1.0 ± 0.1%), meaning that there was no notable ^{13}C incorporation into the compounds. Camphor in FSM samples showed significant deviation compared to expected values (1.7 ± 0.6%), which might be an artifact because camphor is present in very low quantities in these samples. This deviation was not observed in the case of cineol (1.1 ± 0.2%). The FSM+MVA samples also showed a deviation from the expected value for

camphor (1.7 ± 0.7%), whereas cineol exhibit an expected value of 1.1 ± 0.2%. Because camphor from FSM samples exhibit a higher ^{13}C abundance, the data obtained for FSM+MVA are made less reliable and cannot be atributed to the labeling strategy.

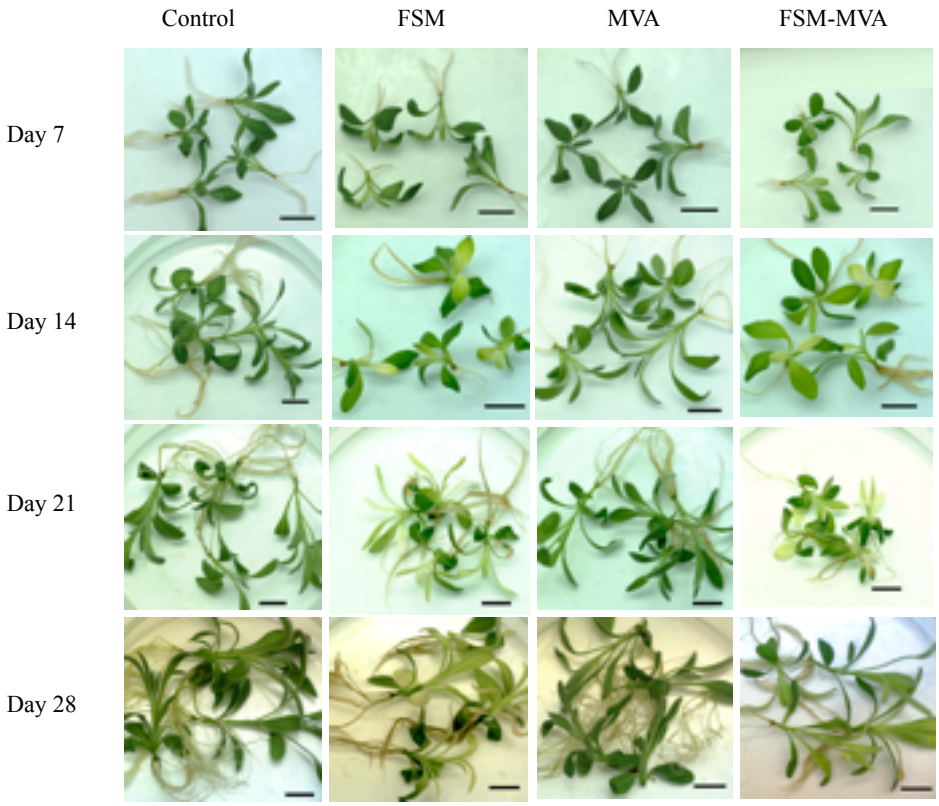


Figure 67. Evolution of the external phenotype of plantlets cultured on media with 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MVA+FSM and Control media. Note that after day 7 all plants treated with FSM exhibet chlorosis on their younger leaves.

The lack of labeled carbon atoms in cineol and camphor in the MVA samples may be due to the labeled mevalonate used, which has only two ^{13}C and may be not enough because of its dilution in the carbon pool; note that

culture medium has also a 3% sucrose concentration besides the 1.2 mM MVA. This means that every molecule of MVA has to compete with 72.9 molecules of sucrose, therefore diluting the ^{13}C carbons.

No conclusions may be drawn from the analysis of FSM and FSM+MVA treatments, since ^{13}C abundance increase in camphor could be an artefact of the method and no ^{13}C abundance increase was detected in cineol. In previous experiments (see section III. 2. 2) we proved that exogenous MVA is absorbed by the spike lavender cells, since MEV-treated plants regain their normal phenotype when cultured in the presence of 1.2 mM MVA (Figure 54). Furthermore, MVA increased chlorophylls and carotenoids content in young leaves from FSM-treated plants, suggesting that at least some of the IPP from the MVA pathway contributed to the synthesis of photosynthetic pigments (Table 45). These could also explain why FSM+MVA plants in this experiment showed a slightly less bleached phenotype than the FSM ones. Nevertheless, by analysing the ^{13}C total abundance data (Table 57), it seems that the IPP derived from the ^{13}C-mevalonate is not used to produce cineol and camphor.

Table 57. Absolut ^{13}C abundance percentage of cineol and camphor after 7, 14, 21 and 28 days of culture in 1,2-$^{13}C_2$MVA, FSM, 1,2-$^{13}C_2$MV+FSM media.

Pulse days	Control		FSM		MVA		FSM+MVA	
	Cineol	Camphor	Cineol	Camphor	Cineol	Camphor	Cineol	Camphor
7	1.02	1.35	1.08	1.10	1.14	1.04	1.01	1.13
14	1.35	1.19	1.33	1.95	1.03	1.00	0.96	2.03
21	0.89	0.88	1.01	2.41	0.92	0.94	1.15	2.60
28	0.94	0.87	1.04	1.22	1.09	1.06	1.34	1.22
Mean	1.05 ± 0.21	1.07 ± 0.24	1.12 ± 0.15	1.67 ± 0.62	1.05 ± 0.09	1.01 ± 0.05	1.12 ± 0.17	1.74 ± 0.70

III. 2. 4. 2. [U-$^{13}C_6$]-Glucose labeling experiments

The labeled glucose is expected to produce different labeling pattern in the cineol and camphor molecules produced depending on the pathway followed. Isotopomeres M+2 and M+3 are very important as they indicate the main biosynthetic pathway for monoterpenes (Römisch-Margl et al., 2007; Eisenreich et al., 2004). As M+3 includes all molecules carrying three ^{13}C atoms this isotopomere is a clear marker for the C3 precursor GAP and therefore point towards the building of these species via the MEP pathway (Eisenreich et al., 2004). M+2 isotopomeres can originate from the MVA pathway (Acetyl-CoA) as well as from the MEP pathway (pyruvate) (Eisenreich et al., 2004). This means that if [U-$^{13}C_6$]-Glucose follows the MEP pathway exclusively, blocks of two and three ^{13}C (M+2 and M+3) in a relation of 1:1 are expected. Meanwhile, if it follows the MVA pathway exclusively, blocks of 2C and 1C are expected in a ratio of 2:1. Any deviation of both situations will produce an intermediate ratio. Despite that, this will never happen, because of release and refixation of the $^{13}CO_2$ are expected as a result of the basal metabolism.

III. 2. 4. 2. 1. Solid medium experiments

In a first experiment, WT spike lavender seedlings grown *in vitro* were cultured on agar-solidified (Figure 68) BM medium containing 2 g/L [U-$^{13}C_6$]-glucose.

The percentages of the essential oil main components from seedlings cultured on solid medium are listed in Table 58. The excess ^{13}C abundance percentages for cineol and camphor ranged from 4.8 to 6.5 and from 5.3 to 6.1, respectively (Appendix II). As it is shown in Figure 69, camphor and 1,8-cineol of *in vitro* samples contain the isotopomeres M+1, M+2 and M+3 almost exclusively (>97%). As an example for this experiment, sample iv950 is discussed in detail below.

Figure 68. Phenotype of plantlets after 55 days of solid *in vitro* culture with BM medium supplemented with 2g/L of [U-$^{13}C_6$]glucose.

Table 58. Main components (%) of spike lavender essential oil from plantlets cultured *in vitro* for 55 days on solid BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose. Chloroform-d extracts determined by GC/MS analysis. The percentage is referred to the area of the 15 main peaks of each sample.

Sample	α-pinene	β-pinene	limonene	cineol	camphor	coumarine	total
IV2	8.5	0.6	2.3	45.1	34.3	0.0	90.8
IV3	3.8	0.5	0.1	17.2	14.43	59.1	95.2
IV4	1.6	0.0	0.4	10.1	6.1	76.2	94.5
IV14	14.8	1.1	4.2	42.1	27.1	0.0	89.2
IV15	8.9	7.5	4.2	40.4	26.8	0.0	87.6
IV800	3.5	2.9	1.8	24.7	15.8	25.2	73.9
IV950	4.5	3.5	2.6	28.4	18.7	26.3	84.0
IV1000	4.1	3.4	2.5	32.0	21.8	20.6	84.4
Mean	6.2	2.4	2.3	30.0	20.6	25.9	87.4

This sample was a mixture of 950 mg of plant material. A total percentage of 48.1% of cineol molecules were labeled. From them, more than 99% correspond to M+1, M+2 and M+3 isotopomeres. Many of the M+1 isotopomers, accounting for the 72.1% of all labeled molecules, could be attributed to the basal metabolism that produces $^{13}CO_2$ from the [U-^{13}C6]-glucose through respiration and subsequent refixation. Nevertheless, the absence of M+4, M+5, to M+10 isotopomeres suggest a reduced effect of the basal metabolism in the generation of $^{13}CO_2$ molecules and subsequent utilization in the build of the monoterpene camphor and cineol. Another source of the M+1 isotopomeres might be from the acetylCoA produced from the [U-$^{13}C_6$]-glucose through the MVA pathway. The presence of M+3 isotopomeres (accounting for 2.7% of the total isotopomeres) indicates that they are probably derived from GAP through the MEP pathway. Despite that, the M +2/M+3 and M+2/[(M+2) + (M+3)] ratios (Table 59) are much higher than expected (respectively, 1 and 0.5) if cineol was exclusively produced through the MEP pathway.

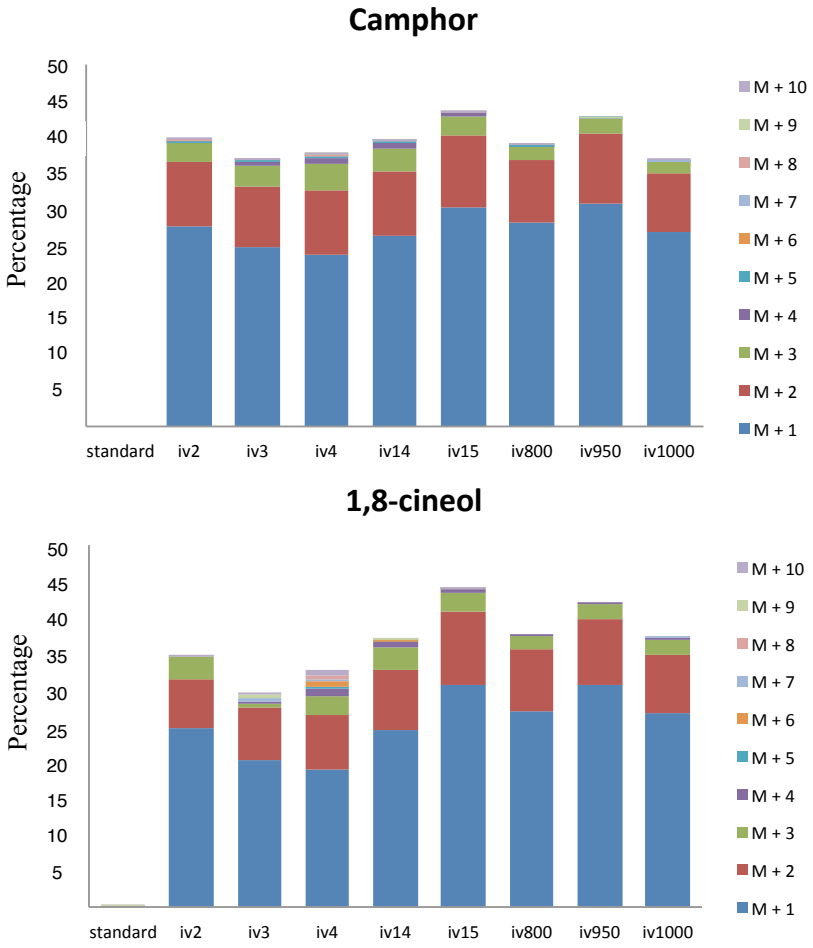


Figure 69. Isotopologue excess values and distribution of isotopomeres of camphor (up) and 1,8-cineol (down) of 8 independent samples of spike lavender essential oil from plantlets cultured *in vitro* for 55 days on solid BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose. Chloroform-d extracts determined by GC/MS analysis.

In the case of camphor, a total percentage of 48.9% molecules were labeled. From them, more than 99% correspond to M+1, M+2 and M+3 isotopomeres. The M+1 isotopomeres, accounting for the 71.6% of all labeled molecules, might come from the basal metabolism. However, and as in cineol, the absence of M+4…M+10 molecules points towards some restriction in that matter. The notable presence of M+3 isotopomeres (accounting for 5.7% of the total isotopomeres) indicate that the IPP are most probably derived through the MEP pathway. Despite that, the M+2/M+3 and M+2/[(M+2) + (M+3)] ratios

(Table 60) are also much higher than expected if camphor was exclusively produced through the MEP pathway.

Table 59. GC/MS excess data for M+2 and M+3 isotopomeres in cineol of spike lavender essential oil extractedwith chloroform-d and analyzed by GC/MS from WT *L. latifolia* seedlings at different labeling times with [U-$^{13}C_6$]glucose. (Mean ± SE)

Sample	Excess Values (%)		Ratios	
	M+2	M+3	M+2/M+3	M+2 / (M+2)+(M+3)
iv2	6.69	3.01	2.22	0.69
iv3	7.36	0.58	12.69	0.93
iv4	7.57	2.57	2.95	0.75
iv14	8.31	3.06	2.72	0.73
iv15	10.01	2.63	3.81	0.79
iv800	8.35	1.92	4.35	0.81
iv950	9.04	1.98	4.57	0.82
iv1000	7.97	2.22	3.59	0.78
Mean	8.16	2.25	4.61	0.79

Table 60. GC/MS excess data for M+2 and M+3 isotopomeres in camphor of spike lavender essential oil extractedwith chloroform-d and analyzed by GC/MS from WT *L. latifoli*a seedlings at different labeling times with U-$^{13}C_6$]glucose. (Mean ± SE)

Sample	Excess Values (%)		Ratios	
	M+2	M+3	M+2/M+3	M+2 / (M+2)+(M+3)
iv2	8.94	2.58	3.47	0.78
iv3	8.19	2.94	2.79	0.74
iv4	8.74	3.73	2.34	0.70
iv14	8.98	3.17	2.83	0.74
iv15	9.82	2.68	3.66	0.79
iv800	8.69	1.78	4.88	0.83
iv950	9.80	1.97	4.97	0.83
iv1000	8.11	1.71	4.74	0.83
Mean	8.91	2.57	3.71	0.78

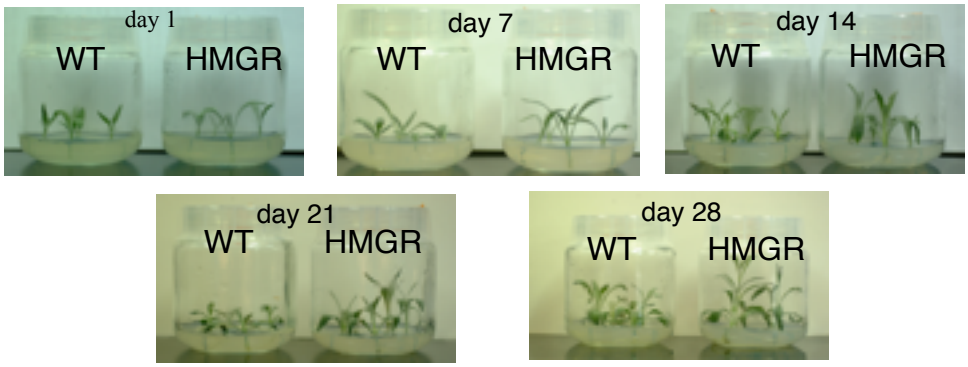


Figure 70. External phenotype of both HMGR5 and WT lines after 1, 7, 14, 21 and 28 days of culture in BM medium with 2 g/L of [U-$^{13}C_6$]glucose.

The labeling of M+3 isotopomeres provides strong evidence for the involvement of the MEP pathway in the biosynthesis of C5 units. M+2 alone does not provide sufficient evidence to implicate the MVA pathway in playing a role or not. Considering only the values from the transgenic line HMGR5, the MVA pathway was hypothesized to be involved, a subject that was investigated in the next experiment.

Transgenic HMGR5 and WT spike lavender plants were cultured *in vitro* on BM medium containing 2 g/L U-^{13}C-glucose for 7, 14, 21 or 28 days (Figure 70). After each culture period some growth parameters were recorded and leaf essential oil extracted and analyzed via GC/MS. Results are summarized in Figures 71 -73.

No differences in plant weight, root length and number of stem whorls were found between transgenic and WT plants. Nevertheless, stem length was significantly higher in HMGR5 plants than in WT plants (Figure 71); this may be due to an extra production of gibberelins, but it cannot be corroborated without further experiments.

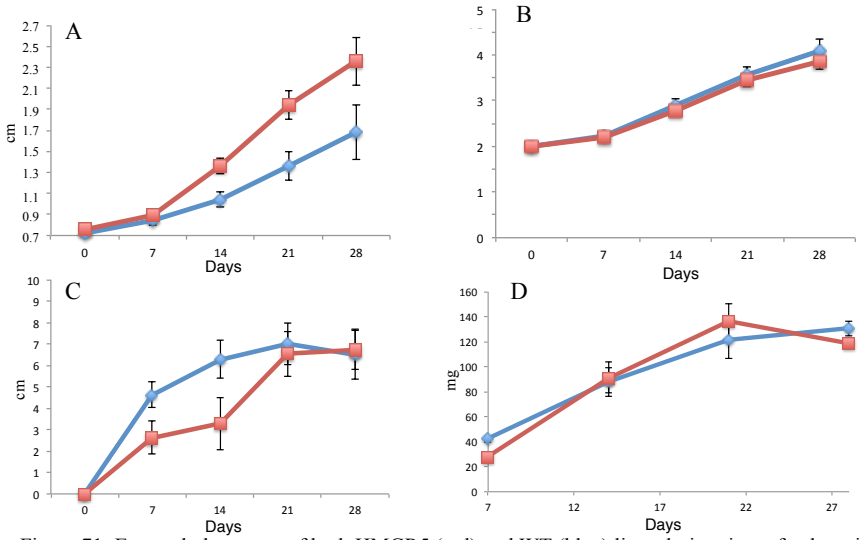


Figure 71: External phenotype of both HMGR5 (red) and WT (blue) lines during time of culture in BM with 2 g/L of [U-$^{13}C_6$]glucose . Means ± SE. A) Stem lenght B) Whorl mean number C) Root lenght D) Seedling weight.

The percentages of the main components of essential oil extracted at each sampled data, referred to the 15 main peaks of each chromatogram, are listed in Table 61. The isotopologue excess data for camphor and 1,8-cineol are displayed in Figures 72 and 73, and Tables 62 and 63. The number of ^{13}C excess labeled camphor and cineol molecules increased along with time in both transgenic and WT plants, being after day 21 always higher in HMGR5 plants (Figure 71). Also isotopomer M+2/M+3 and M+2/(M+2 + M+3) ratios for camphor and 1,8-cineol were higher in transgenic HMGR5 plants, which is in concordance to our hypothesis if the MVA is somehow involve in their synthesis, because this pathway is overexpressed in line HMGR5. A more detailed description of samples from the 28th day of experiment follows.

Table 61. Percentage of the essential oil content in all HMGR5 and WT samples cultured in BM medium with 2 g/L of [U-$^{13}C_6$]glucose for each time pulse. Essential oil was extracted with chloroform-d and analyzed by GC/MS. The percentage of α-pinene (A), β-pinene (B), 1,8-cineol (C), camphor (D) and coumarin (E) is referred to the area of the 15 main peaks of each sample.

Pulse days	HMGR5					WT				
	A	B	C	D	E	A	B	C	D	E
7	5.39	6.02	37.41	11.08	20.33	5.30	6.48	44.69	6.8	11.77
14	3.82	3.78	36.37	9.28	35.06	2.93	2.98	32.04	11.07	36.21
21	4.10	4.63	32.48	9.21	34.57	4.70	3.92	33.88	12.70	31.03
28	3.58	3.93	34.19	9.62	31.54	2.66	3.31	21.89	5.19	52.03

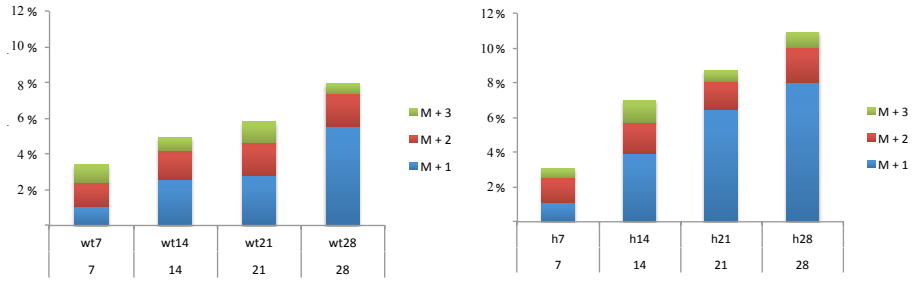


Figure 72. Isotopologue excess values and distribution of isotopomeres of camphor in both lines: WT (right) and HMGR5 (left). Plant material derived from spike lavender plants cultured for 7, 14, 21 and 28 days in liquid BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose. Essential oil extracted with chloroform-d and determined by GC/MS.

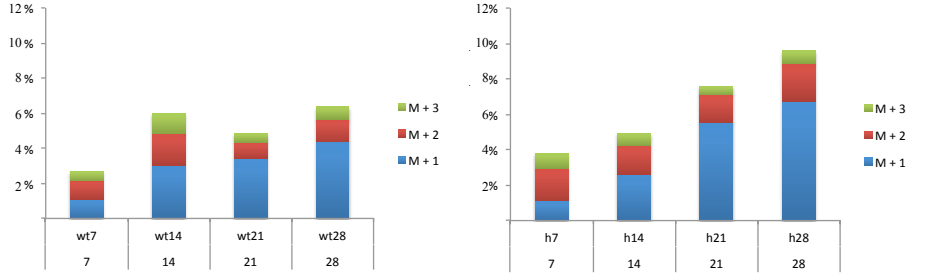


Figure 73. Isotopologue excess values and distribution of isotopomeres of cineol in both lines: WT (right) and HMGR5 (left). Plant material derived from spike lavender plants cultured for 7, 14, 21 and 28 days in liquid BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose. Essential oil extracted with chloroform-d and determined by GC/MS.

For camphor, the WT samples showed a total of 18.0 ± 1.4% labeled molecules. From them, 95.6% correspond to M+1, M+2 and M+3 isotopomeres, accounting for 66.7% of M+1 isotopomers. In the case of cineol, the samples showed a final amount of 16.5± 2.1% labeled molecules. From these, 95.6% corresponded to M+1, M+2 and M+3 isotopomers, accounting for 65.5% of M +1 isotopomers. Again the relative lack of M+4, M+5, to M+10 isotopomeres points towards a reduced effect of the basal metabolism in the generation of $^{13}CO_2$ molecules and subsequent utilization in the build of the monoterpene camphor and cineol.

In HMGR5 samples, a total of 20.6 ± 0.4% camphor molecules were labeled. This is an increase of almost 3% in comparison to the WT. The percentage of the M+1, M+2 and M+3 isotopomers was 96.9%, very similar to previous results, accounting for 71.1% of M+1 isotopomeres, which means almost 5% more than the WT equivalent.

These differences could be due to an extra IPP supply from the MVA pathway that is overexpressed in this line. In the case of cineol, 19.4 ± 0.3% of molecules were labeled, representing, an excess of 9.9%. The percentage of the M+1, M+2 and M+3 isotopomers was 96.8%, very similar to previous results, accounting for 68.1% of M+1 isotopomeres. This means almost 2.5% more than the WT equivalents. Again, this difference might be due to an extra IPP supply from the MVA pathway.

All these data support the interconnection between MEP and MVA pathways and suggest that MVA-derived precursors contributed to the synthesis of camphor and 1,8-cineol in *Lavandula latifolia.*

Table 62. GC/MS excess data from M+2 and M+3 isotopomeres in 1,8- cineol and camphor extracted with chloroform-d and analyzed by GC/MS from WT *L. latifolia* seedlings at different labeling times with [U-$^{13}C_6$]glucose. Mean ± SE.

WT	1,8 cineol				Camphor			
Pulse days	Excess Values %		Ratios		Excess Values %		Ratios	
	M+2	M+3	M+2 / M+3	M+2 / (M+2)+(M+3)	M+2	M+3	M+2 / M+3	M+2 / (M+2)+(M+3)
7	0.91	0.38	2.39	0.71	1.92	2.78	0.69	0.41
7	2.02	0.5	4.04	0.80	2.02	4.2	0.48	0.32
7	0.33	0.68	0.49	0.33	0.81	1.56	0.52	0.34
14	1.11	1.25	0.89	0.47	2.11	1.25	1.69	0.63
14	1.52	0.22	6.91	0.87	1.66	0.34	4.88	0.83
14	1.32	0.53	2.49	0.71	1.15	0.68	1.69	0.63
21	1.59	0.82	1.94	0.66	2.94	2.00	1.47	0.60
21	0.00	0.34	0.00	0.00	1.18	0.81	1.46	0.59
21	1.19	0.41	2.90	0.74	1.43	0.72	1.99	0.67
28	1.02	0.36	2.83	0.74	1.66	0.13	12.77	0.93
28	1.70	1.06	1.60	0.62	2.54	0.55	4.62	0.82
28	1.16	0.73	1.59	0.61	1.3	1.04	1.25	0.56
Mean	1.16	0.61	2.34	0.61±0.07	1.73	1.34	2.79	0.61±0.05

Table 63. GC/MS excess data from M+2 and M+3 isotopomeres in 1,8- cineol and camphor extracted with chloroform-d and analyzed by GC/MS from HMGR5 *L. latifolia* seedlings at different labeling times with [U-$^{13}C_6$]glucose. Mean ± SE.

HMGR5	1,8 cineol				Camphor			
Pulse days	Excess Values %		Ratios		Excess Values %		Ratios	
	M+2	M+3	$\frac{M+2}{M+3}$	$\frac{M+2}{(M+2)+(M+3)}$	M+2	M+3	$\frac{M+2}{M+3}$	$\frac{M+2}{(M+2)+(M+3)}$
7	1.48	0.50	2.96	0.75	1.91	0.38	5.03	0.83
7	2.68	1.45	1.85	0.65	0.51	1.23	0.41	0.29
7	1.34	0.56	2.39	0.71	0.08	0.00	-	1.00
14	2.91	1.96	1.48	0.60	0.80	0.00	-	1.00
14	1.89	0.77	2.45	0.71	3.02	3.51	0.86	0.46
14	1.68	0.77	2.18	0.69	1.55	0.30	5.17	0.84
21	1.67	0.57	2.93	0.75	1.78	0.95	1.87	0.65
21	1.30	0.43	3.02	0.75	1.19	0.09	13.22	0.93
21	1.81	0.41	4.41	0.82	1.91	0.78	2.45	0.71
28	2.23	0.94	2.37	0.70	1.70	1.05	1.62	0.62
28	2.19	0.85	2.58	0.72	2.31	0.74	3.12	0.76
28	2.01	0.33	6.09	0.86	2.14	0.77	2.78	0.74
Mean	1.93	0.84	2.89	0.72±0.02	1.52	0.82	3.65	0.74±0.06

III. 2. 4. 2. 2. Liquid medium experiments

In this experiment, WT spike lavender seedlings grown *in vitro* were cultured in liquid (Figure 74) BM medium containing 2 g/L [U-$^{13}C_6$]-glucose. Seedlings growing in liquid cultures turned the medium blue (Figure 74). Blue pigment accumulation seems to be an almost exclusive property of *in vitro* cultured *Lavandula* cells and is due to the secretion of (Z, E)-2-(3,4-dihydroxyphenyl)ethenyl ester of 3-(3,4-dihydroxyphenyl)-2-propenoic acid and is (E,E) isomer that produces this colour when it complexes with Fe^{2+} (Segura and Calvo, 1991; Trejo-Tapia, 2003). The percentages of the main components of essential oil extracted from seedlings cultured on liquid medium, referred to the 15 main peaks of each chromatogram, are listed in Table 64. It is worth noting that the oil contained high amounts of M-pyrol (up to 39.6 %), exo-fenchol (up to 9.4%) and m-toluyaldehyde (up to 22.5%), that were not found in potted plants nor in plants growing on *in vitro* solid medium.

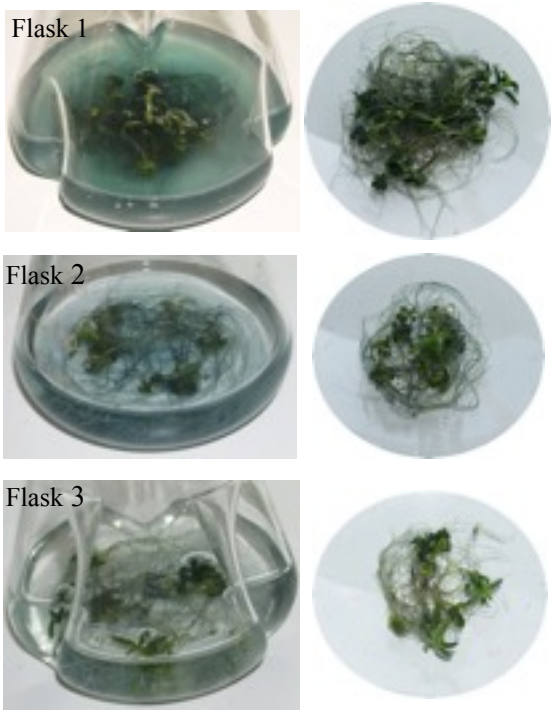


Figure 74. Phenotype of plantlets after 30 days of *in vitro* BM liquid medium culture supplemented with 2 g/L of [U-$^{13}C_6$]glucose. Note the obvious blue colour of the medium.

Plants growing in liquid cultures incorporated a higher amount of ^{13}C than potted plants treated with $^{13}CO_2$ (^{13}C excess abundance percentage of 13.3% vs. 2.1% for camphor and 10.6% vs. 1.8% for 1,8-cineol, respectively). As stated for plants cultured on solid medium, camphor and 1,8-cineol contained a high proportion (>69%) of the isotopomeres M+1, M+2 and M+3 (Figure 75).

The M+1 isotopomers,

accounting for 28.3% of all labeled excess molecules, can be attributed to the basal metabolism that produces $^{13}CO_2$ from the [U-$^{13}C_6$]-glucose.

In these samples, the presence of M+4, M+5, to M+10 isotopomeres is high, accounting for more than 18.0% of the molecules. The M+2/M+3 and M +2/[(M+2) + (M+3)] ratios (Table 65) are much more similar to the values expected if the synthesis of camphor was made exclusively through the MEP pathway, meaning 1 and 0.5 respectively.

For cineol, an excess of 35.9% molecules were labeled in comparison to the standard, a little lower value than that reported for camphor. From these, more than 86.2% corresponded to M+1, M+2 and M+3 isotopomeres. The M+1 isotopomers, accounting for 48.3% of all labeled excess molecules, can be attributed to the basal metabolism that produces $^{13}CO_2$ from the [U-$^{13}C_6$]-glucose. Again, the presence of M+4, M+5, to M+10 isotopomeres is preeminent, accounting for more than 13.8% of the molecules, supporting the idea that the basal metabolism might have an important role in the production and re-use of $^{13}CO_2$. The M+2/M+3 and M+2/[(M+2) + (M+3)] ratios (Table 66) are very similar to the values expected if the synthesis of camphor was made thorough the MEP pathway.

Data referring to the isotopologues M+2 and M+3 and to their ratios M +2/M+3 and M+2/(M+2 + M+3) for the monoterpenes cineol and camphor produced on the solid culture (Tables 59 and 60) and liquid culture (Tables 65 and 66) of *L. latifolia* seedlings are quite different. It is clear that the metabolism for the production of these two compounds is completely different in both situations, since in plants cultured in solid medium the involvement of the MVA seems to be more important than in the liquid medium.

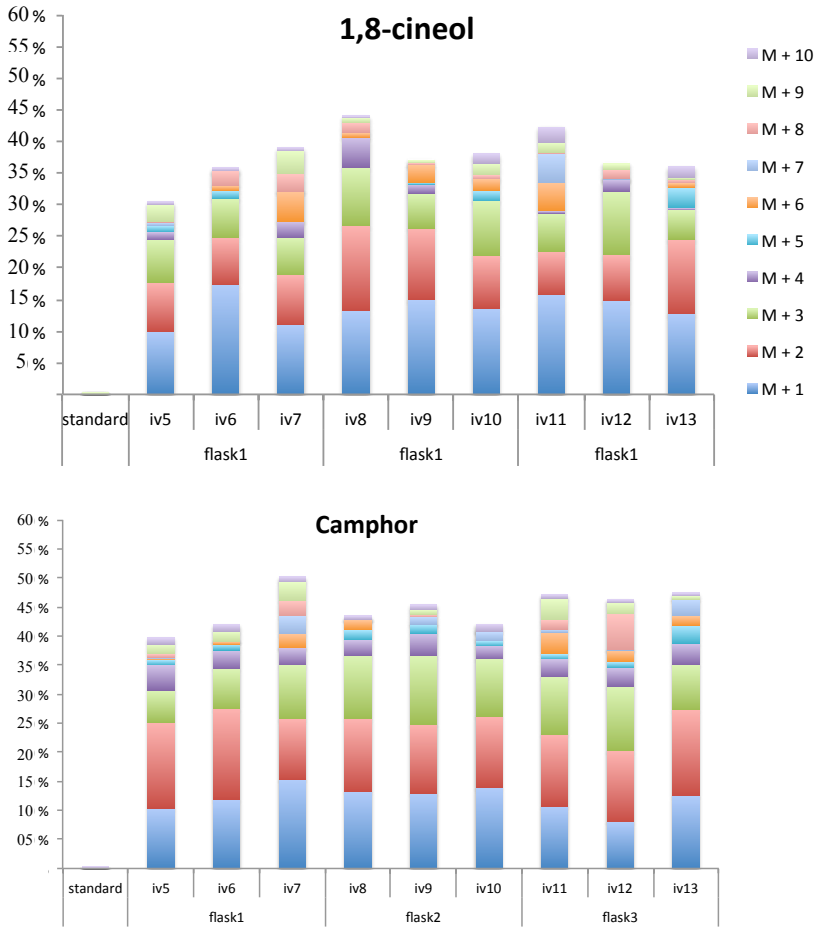


Figure 75. Isotopologue excess values and distribution of isotopomeres of cineol (up) and camphor (down) of 9 independent samples. Plant material derived from spike lavender seedlings cultured in *in vitro* liquid cultures after 30 days of culture with BM medium supplemented with 2 g/L of [U-$^{13}C_6$] glucose, extracted with CDCl3 and determined by GC/MS.

Table 64. Main components (%) of the spike lavender essential oil cultured *in vitro* for 30 days of culture in liquid BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose. Chloroform-d extracts determined by GC/MS analysis. The percentage is referred to the area of the 15 main peaks of each sample.

Flask	Sample	Cineol	M-Pyrol	m-Toluyaldehyde	exo-Fenchol	L-camphor	Total
1	IV5	19.2	39.6	6.3	5.1	7.2	77.4
1	IV6	19.1	9.3	20.8	6.4	9.8	65.4
1	IV7	24.3	1.6	22.5	9.4	12.9	70.7
2	IV8	42.9	0.0	7.6	3.6	12.3	66.4
2	IV9	44.4	0.0	13.5	2.8	12.0	72.7
2	IV10	22.6	28.9	7.7	3.8	10.2	73.2
3	IV11	36.9	0.0	12.1	7.7	13.4	70,1
3	IV12	21.4	37.0	12.2	4.2	8.0	82.8
3	IV13	42.4	0.0	10.7	3.2	13.7	70.0
	Mean	30.4	12.9	12.6	5.1	11.1	72.1

Table 65. GC/MS excess data for M+2 and M+3 isotopomeres in camphor, chloroform-d extraction, from apike lavender seedlings (leaves) cultured in liquid (30 days) BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose.

Camphor	excess values (%)		Ratios	
	M+2	M+3	M+2/M+3	$\frac{M+2}{(M+2)+(M+3)}$
iv5	14.76	5.64	2.62	0.72
iv6	15.57	6.95	2.24	0.69
iv7	10.43	9.30	1.12	0.53
iv8	12.50	10.92	1.14	0.53
iv9	12.35	9.85	1.25	0.56
iv10	11.85	11.87	1.00	0.50
iv11	12.35	9.85	1.25	0.56
iv12	12.40	9.95	1.25	0.55
iv13	12.20	11.15	1.09	0.52
Mean	12.71	9.50	1.44	0.57

Table 66. GC/MS excess data for M+2 and M+3 isotopomeres in cineol, chloroform-d extraction, from apike lavender seedlings (leaves) cultured in liquid (30 days) BM medium supplemented with 2 g/L of [U-$^{13}C_6$]glucose.

Cineol	excess values (%)		Ratios	
	M+2	M+3	M+2/M+3	M+2 / (M+2)+(M+3)
iv5	7.74	6.76	1.14	0.53
iv6	7.51	6.09	1.23	0.55
iv7	7.90	5.90	1.34	0.57
iv8	13.41	9.22	1.45	0.59
iv9	11.32	5.52	2.05	0.67
iv10	8.30	8.71	0.95	0.49
iv11	6.86	6.02	1.14	0.53
iv12	7.20	10.05	0.72	0.42
iv13	11.79	4.62	2.55	0.72
Mean	9.11	6.99	1.40	0.56

III. 2. 4. 3. $^{13}CO_2$ labeling experiments

III. 2. 4. 3. 1. Preliminary experiments

Individual WT potted plants were fed with $^{13}CO_2$ for a varying period of time (pulse) and sampled after different periods (chase) at standard greenhouse conditions (See Appendix I). Six different pulse periods were used beginning with 1 hour up to 8.8 hours. For each pulse period tested, the chase periods varied from 0 to 264 hours. Samples indicated with the same lower case letter were taken from the same plant. The denotation "EV" indicates that this sample was derived from an *ex vitro* plant labeled by $^{13}CO_2$.

GC/MS analysis of the essential oil fraction was performed: (i) to determine the composition of all samples; and (ii) to determine the ^{13}C enrichment and isotopologue profiles of the main components in each sample.

GC/MS analysis of chloroform extracts allowed detection of most of the components of the spike lavender essential oil (Woronuk et al., 2011; Muñoz-Bertomeu et al., 2007a) including monoterpenes, sesquiterpenes and, at longer retention times (over 21 minutes), coumarin, as previously reported (Pascual et al., 1983). Table 67 summarizes the percentage of the five main monoterpenes (1,8-cineol, camphor, limonene and α-/ β-pinene) in leaves from eigth individual plants (a to h) with 2-4 samples each. Table 68 summarizes the percentage of the five main monoterpenes (1,8-cineol, camphor, limonene and α-/ β-pinene) in leaves of 29 samples.

Table 67. Main components (%) of spike lavender leaf essential oil extracted with chloroform-d and determined by GC/MS. Data are referred to single plants. The percentage is referred to the area of the 15 main peaks of each sample. Means ± SD. rt: retention time.

Plant	Numer of Samples	α-pinene rt: 5.9	β-pinene rt: 7.2	limonene rt:9.0	cineol rt:9.2	camphor rt:14.3
a	3	5.7±0.5	3.7±0.3	19.3±1.8	21.1±1.8	41.6±2.1
b	4	8.5±1.9	3.9±1.2	17.0±3.2	0.0±0.0	53.5±3.8
c	2	13.7±0.5	6.0±4.2	3.5 ±0.1	40.0±0.2	27.3±3.2
d	2	6.0±3.5	4.7±2.8	24.0±14.2	27.2±16.0	27.6±16.0
e	3	13.3±3.8	4.4±1.7	14.4±3.5	38.7±8.2	14.9±8.5
f	3	11.3±4.4	4.2±1.6	13.7±0.9	35.0±2.7	21.2±10.9
g	3	7.3±1.0	3.1±1.9	13.9±12.2	14.0±2.2	26.0±2.4
h	2	10.3±0.6	2.5±1.5	9.8 ±3.2	27.5±0.1	11.3±2.1

Table 68. Main components (%) of spike lavender leaf essential oil extracted with chloroform-d and determined by GC/MS. The percentage is referred to the area of the 15 main peaks of each sample. rt: retention time.

Sample	Plant	α-pinene rt: 5.9	β-pinene rt: 7.2	limonene rt:9.0	cineol rt:9.2	camphor rt:14.3
EV30		10.94	6.47	12.32	22.51	41.77
EV31		8.69	0.11	1.71	32.4	44.7
EV32		12.3	7.60	6.00	33.2	34.2
EV33		10.75	5.97	20.42	22.74	31.46
EV34		11.98	1.64	5.55	27.62	43.22
EV35	a	6.32	3.99	21.25	19.27	39.23
EV36	b	11.21	5.58	21.46	0.00	48.16
EV37	b	8.09	2.87	15.99	0.00	53.32
EV38	a	5.57	3.59	18.60	21.14	43.01
EV39	b	7.27	3.45	16.76	0.00	56.26
EV40	a	5.30	3.50	18.05	22.84	42.45
EV41	b	7.28	3.39	13.75	0.00	56.33
EV42		22.36	8.81	13.03	33.73	10.22
EV43		13.18	9.58	15.55	34.90	16.63
EV44	c	13.28	8.92	3.65	39.77	25.01
EV45	d	6.25	5.26	27.00	24.27	26.05
EV46	c	13.99	2.95	3.43	40.15	29.53
EV47	d	5.57	4.05	21.04	30.07	29.20
EV48	e	14.99	3.13	11.93	29.59	24.74
EV49	f	16.18	4.34	13.13	36.97	8.61
EV50	e	16.04	3.67	12.75	41.33	9.41
EV51	f	10.03	2.63	14.69	31.90	26.88
EV52	e	9.00	6.33	18.37	45.32	10.51
EV53	f	7.64	5.75	13.21	36.12	28.08
EV54	g	8.53	5.23	27.89	14.32	25.08
EV55	h	10.68	3.54	11.97	27.63	9.78
EV56	g	6.71	1.75	8.29	11.62	24.08
EV57	h	9.83	1.39	7.55	27.37	12.69
EV58	g	6.79	2.38	5.37	15.98	28.66

Total excess of ^{13}C enrichment (isotopologue excess) and the relative contributions of the isotopologues (M+1, M+2,..., M+10) in camphor and 1,8-cineol are depicted in Table 69 and Figures 76 and 77.

As shown in Table 69, the proportion of the isotopomeres M+1, M+2 and M+3 for *ex vitro* (potted) plants, with a total enrichment over 5%, ranged from 38.1 to 76.1% (Mean 51.3 ± 22.3%). Material from *in vitro* cultures labeled with U-^{13}C-glucose incorporated a higher percentage of ^{13}C than potted plants treated with $^{13}CO_2$, specially in seedlings grown on solid medium (^{13}C overall excess percentages of 5.7% vs 2.1 % for camphor, and 5.4 % vs. 1.8% for cineol, respectively).

Figures 76, 77 and Table 69 clearly indicate that a pulse period below five hours did not lead to ^{13}C significant enrichments in both camphor and 1,8-cineol. Pulse periods above five hours showed similar levels for both ^{13}C enrichment and isotopologue distribution for camphor and 1,8- cineol (Figures 76 and 77). The data for camphor are presented in Figures 78A and B, where ^{13}C enrichment of samples from plants labeled with $^{13}CO_2$ for five hours are shown. Nine samples incorporated a low amount of ^{13}C (isotopologue excess values below 5%) and the remaining incorporated between 10 to 25%. There is a maximum of enrichment at 95 and 192 hours chase periods and a minimum with almost no ^{13}C incorporation at 119 and 140 hours chase periods. It can be concluded that the ^{13}C enrichment increases with higher chase periods (Figure 78B).

All these data demonstrate that both compounds show varying ^{13}C enrichments and isotopomere distribution that is directly related to pulse time, with enrichment being higher with longer pulses. The dilution with $^{12}CO_2$ cannot be excluded as the main problem with short-term pulses. Note, however, that there was a high variability in the extent of ^{13}C enrichment within a certain pulse or chase period as well as within samples taken from the same plant at different times. Some plants showed little ^{13}C incorporation into camphor and 1,8-cineol (plants a-g), whereas other incorporated ^{13}C very well (plant h). This means that not only the experimental settings may influence the ^{13}C enrichment achieved in the plants (pulse time, chase time, watering conditions), but also the genotype or the metabolic constraints of the plants. The uptake of CO_2 via the

stomata and its further processing is a very complex and regulated process. CO_2 uptake and photosynthesis rate are strongly influenced by the opening conditions of the stomata (Sitte et al., 2002).

Table 69. Total ^{13}C enrichment of camphor and 1,8-cineol for every sample in the $^{13}CO_2$ feeding experiment.

			Camphor		Cineol	
Sample and plant	Pulse (h)	Chase (h)	Total ^{13}C enrichment (%)	Percentage M+1- M+3	Total ^{13}C enrichment (%)	Percentage M+1- M+3
EV35 a	1	0	0.11	*	0.28	*
EV36 b	1	0	0.08	*	0.73	*
EV37 b	1	3	0.05	*	0.37	*
EV38 a	1	20.5	0.14	*	0.30	*
EV39 b	1	20.5	0.02	*	0.50	*
EV40 a	1	26.5	0.04	*	0.09	*
EV41 b	1	26.5	0.03	*	0.50	*
EV33	2	0	0.33	*	0.35	*
EV34	2	0	0.49	*	0.46	*
EV44 c	3.7	0	0.20	*	0.30	*
EV45 d	3.7	0	0.27	*	0.45	*
EV46 c	3.7	71	0.16	*	0.26	*
EV47 d	3.7	71	0.08	*	0.31	*
EV54 g	4.9	0	0.57	*	1.12	*
EV48 e	5.1	16	2.31	26.95	3.72	25.01
EV49 f	5.1	16	3.68	25.05	4.78	25.05
EV50 e	5.1	40	2.53	44.99	2.86	47.04
EV51 f	5.1	40	0.20	*	0.40	*
EV52 e	5.1	64	0.12	*	0.05	*
EV53 f	5.1	64	0.23	*	0.51	*
EV55 h	4.9	95	18.41	39.54	26.83	38.1
EV56 g	4.9	119	0.17	*	0.03	*
EV57 h	4.9	140	0.10	*	0.06	*
EV31	5	168	11.08	74.22	9.86	73.74
EV32	5.2	192	22.54	61.98	22.17	62.84
EV43	5.2	240	9.05	29.32	9.00	69.84
EV42	5	264	14.42	76.06	13.83	72.77
EV58 g	4.9	408	*	*	*	*
EV30	8.8	240	24.87	26.69	26.29	27.06

* not determined

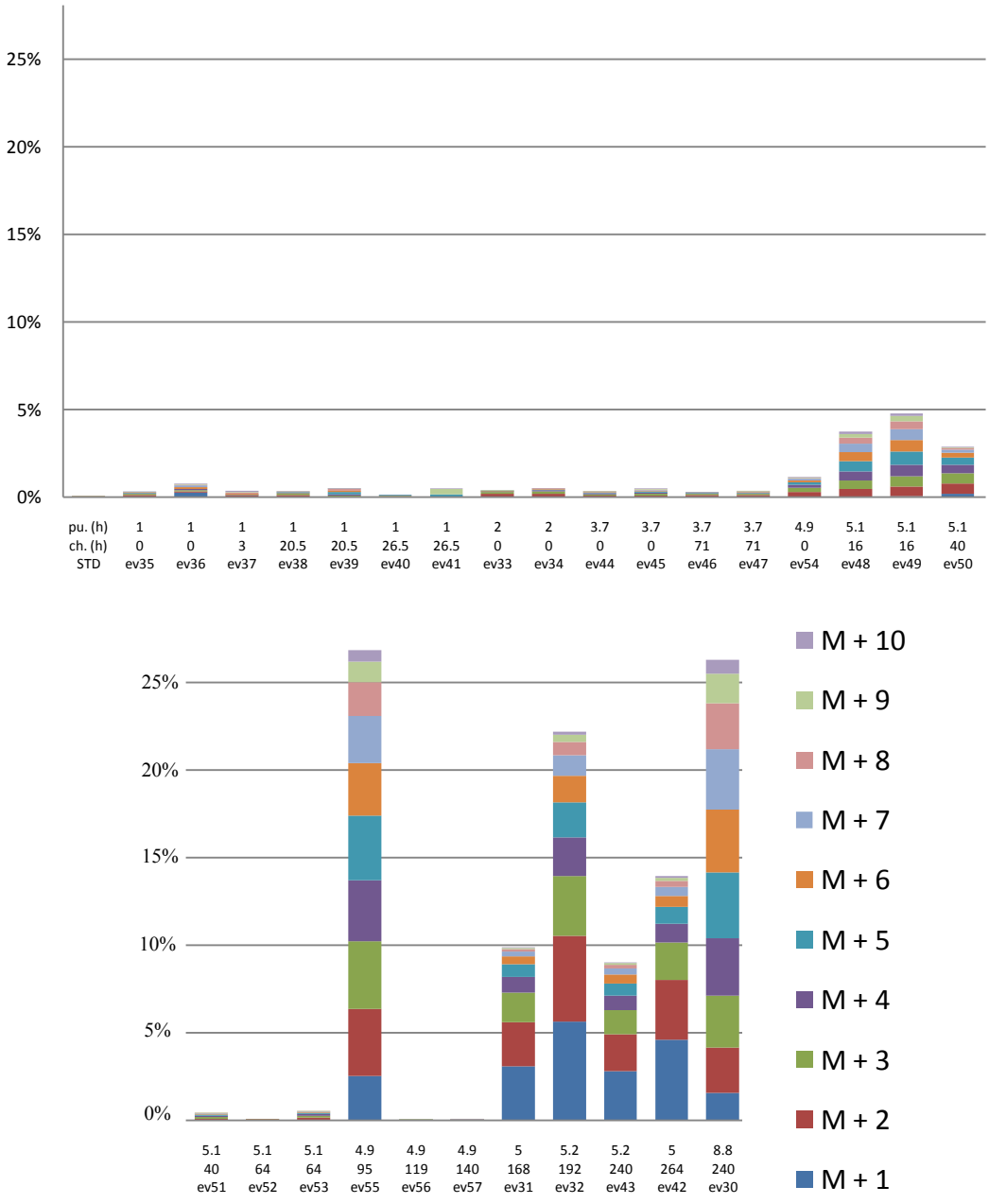


Figure 76. Isotopologue excess values and distribution of isotopomers of cineol in 51 $^{13}CO_2$ feeding experiments . All experiments are sort according to their chase time in ascending order. pu. (h): pulse time in hours. ch. (h): chase time in hours. STD: standard.

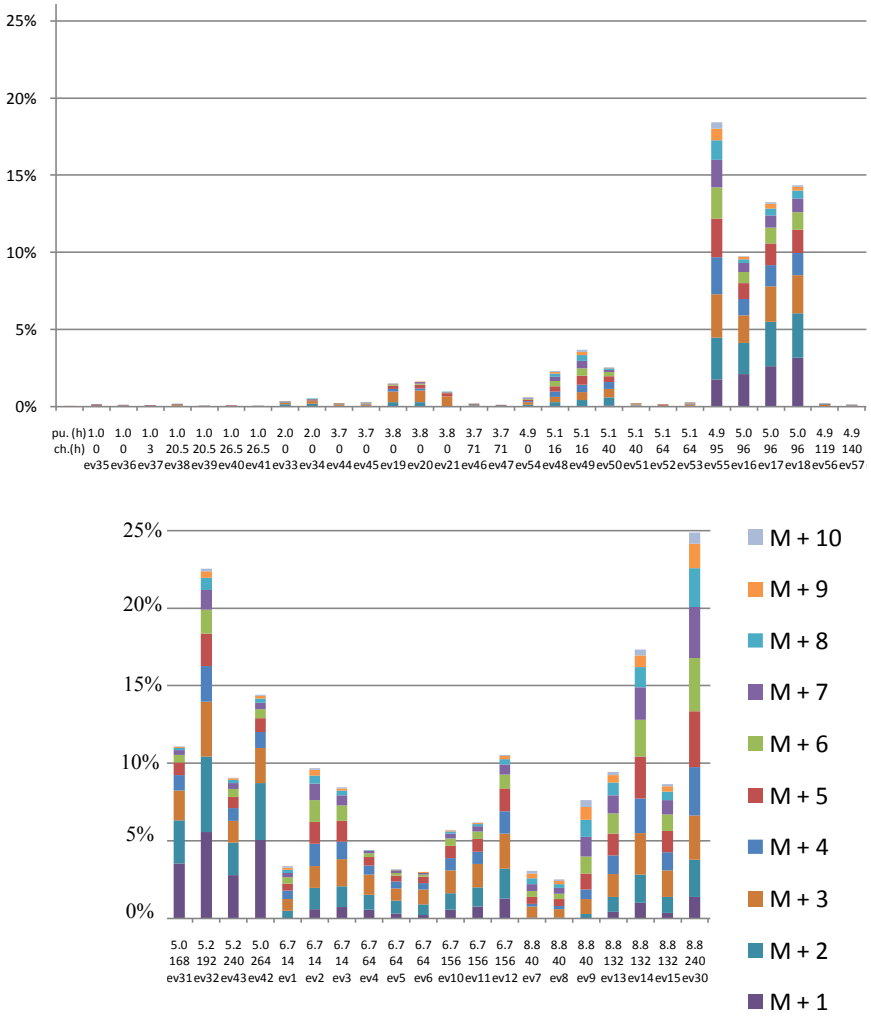


Figure 77. Isotopologue excess values and distribution of isotopomers of camphor in 51 $^{13}CO_2$ feeding experiments . All experiments are sort according to their chase time in ascending order. pu. (h): pulse time in hours. ch. (h): chase time in hours.

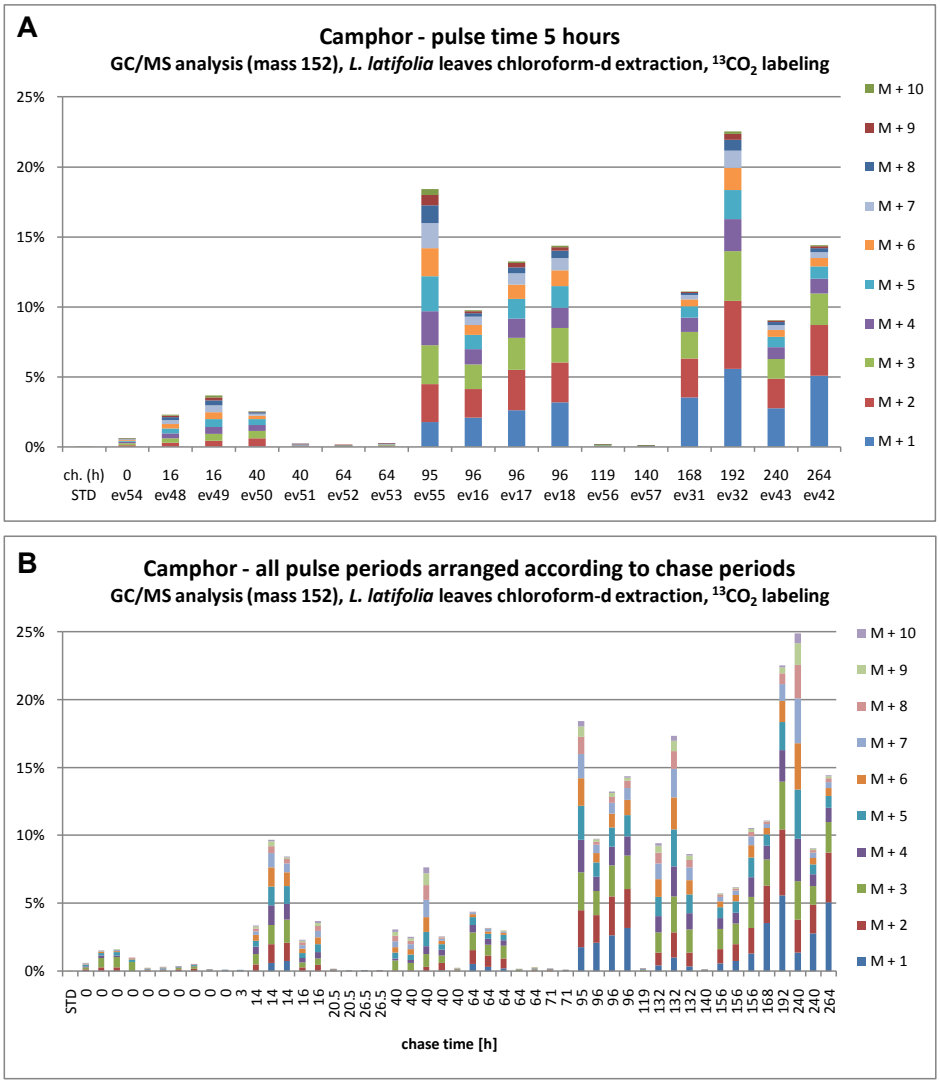


Figure 78. Isotopologue excess values and distribution of isotopomeres for camphor. A) Samples labeled with $^{13}CO_2$ for five hours, arranged according to chase phases. B) All experiments, arranged according to chase periods. ch. (h): chase time in hours. STD: standard.

Table 70. GC/MS excess data for M+2 and M+3 isotopomeres in 1,8-cineol and camphor extracted with chloroform-d and analyzed by GC/MS from spike lavender leaves at different points of time after the labeling pulse phase with $^{13}CO_2$.

				excess values (%)		Ratio	
	Chase time (h)	Pulse time (h)	Sample	M+2	M+3	M+2/ M+3	M+2 / (M+2)+(M+3)
Camphor	16	5.1	ev48	0.29	0.34	0.86	0.46
	16	5.1	ev49	0.44	0.48	0.90	0.47
	40	5.1	ev50	0.54	0.54	1.00	0.50
	95	4.9	ev55	2.72	2.80	0.97	0.49
	168	5.0	ev31	2.78	1.91	1.45	0.59
	192	5.2	ev32	4.86	3.53	1.38	0.58
	240	5.2	ev43	2.10	1.40	1.50	0.60
	264	5.0	ev42	3.65	2.25	1.63	0.62
	240	8.84	ev30	2.40	2.86	0.84	0.46
					Mean ± SD	1.17 ± 0.31	0.53 ± 0.07
Cineol	16	5.1	ev48	0.44	0.49	0.90	0.47
	16	5.1	ev49	0.55	0.60	0.92	0.48
	40	5.1	ev50	0.58	0.58	1.00	0.50
	168	5.0	ev31	2.52	1.66	1.52	0.60
	192	5.2	ev32	4.87	3.41	1.43	0.59
	240	5.2	ev43	2.13	1.37	1.55	0.61
	264	5.0	ev42	3.43	2.13	1.61	0.62
	240	8.84	ev30	2.61	2.96	0.88	0.47
					Mean ± SD	1.23 ± 0.33	0.54 ± 0.07

The first commited enzyme of the photosynthetic CO_2 metabolism, the ribulose-1,5-bisphosphate carboxylase oxygenase, has a low affinity to CO_2 and therefore CO_2 is assimilated slowly in normal conditions (Taiz and Zeiger, 2010). This fact may be also a reason for the minor incorporation rates of $^{13}CO_2$ at lower pulse phases along with the $^{12}CO_2$ dilution.

To discuss in further detail, the samples EV48, EV49, EV50, EV55, EV31, EV32, EV43, EV42 and EV30 were chosen. All these samples showed isotopologue excess values over 2.5% making the data more reliable. Values for the isotopomeres M+2 and M+3 and their ratios for both camphor and cineol are presented in Table 70. In all these samples the M+1 to M+3 fraction ranged from 25-73% of total labeled excess isotopologues. All these samples showed a quite stable M+2/M+3 ratio of 1.2 ± 0.3 for camphor and 1.2 ± 0.3 for cineol. Also, the M+2/(M+2 + M+3) ratio was very stable (0.5 ± 0.1 for camphor and 0.5 ± 0.1 for cineol). These values are the expected if these monoterpenes were exclusively produced through the MEP pathway (which are 1 and 0.5 respictevely for the M+2/M+3 and the M+2/(M+2 + M+3) ratios), which point towards a very small contribution, if any at all, of the MVA pathway in their biosynthesis in these culture conditions.

III. 2. 4. 3. 2. NMR data

In order to identify the signals of the camphor and 1,8-cineol in the complex NMR spectra of plant extracts, these monoterpenes were first measured as pure substances with its natural ^{13}C abundance of 1.1 % ("standards"). For this purpose, all ^{1}H and ^{13}C NMR signals of camphor and 1,8-cineol were assigned on the basis of two-dimensional experiments. This data are displayed in Tables 71 and 72.

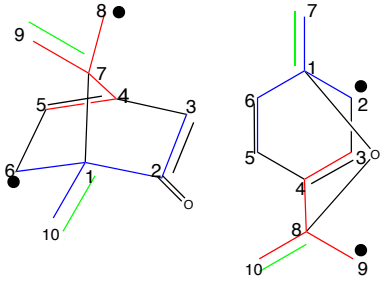


Figure 79. Numbering and biosynthetic origin of carbon atoms from camphor and 1,8-cineol synthesized via de MEP patchway. Carbon atoms derived from: DMAPP, IPP, Pyruvate, GAP.

Table 71. NMR Signal assigment for cineol

1,8-cineol carbon atom	Signal No.	Chemical shift (ppm)			Multiplicity	Integral	Coupling constant (Hz)	Correlation					
	^{1}H	^{1}H	^{13}C (HSQC, HMBC)					COSY	COSYph	TOCSY (long range)	HMBC	HSQC	NOESY
3/5	1	2.05	22.80	CH_2	Multiplet	2		2, 3, 4 (w)	2, 3, 4	2, 3, 5	1, 2, 3, 4, 5, 6	1, 3	
2/6	2	1.69	31.48	CH_2	Multiplet	2 (2.4)		1, 3	1, 3	1, 3, 4	1, 2, 3, 4, 6 (strong)	2, 3	
	3	1.52	22.80 31.48	CH_2	Multiplet	4		1, 2	1, 2	1, 2, 4			
4	4	1.43	32.91	CH	Multiplet	1		1 (weak)	1	1, 2, 3	1, 2, 3, 5 (s)	4	
9/10	5	1.26	28.87	CH_3	Singulet	6					4, 5 (strong)	5	
7	6	1.07	27.55	CH_3	Singulet	3					2 (weak), 3 (weak)	6	
1			69.92								1, 2, 3 (weak), 6 (strong)	-	
8			73.76								1, 2 (weak), 3, 5 (strong), 6 (weak)	-	

Table 72. NMR Signal assigment for camphor

camphor carbon atom	Signal No.	Chemical shift (ppm)			Multiplicity	Integral	Coupling constant (HZ)	Correlation					
	^{1}H	^{1}H	^{13}C (HSQC, HMBC)					COSY	COSYph	TOCSY (long range)	HMBC	HSQC	NOESY
3	1	2.36	43.32	CH_2	ddd	1	18.0 4.5 3.3	2, 4	2, 4, 7	2, 3	1, 3, 4, 5+6+7 (weak), 8, 10	1, 2, 4	
4	2	2.10	43.04	CH	Triplet	1	4.5	1	1	1, 3, 4, 5, 6, 7	1, 3, 4, 5+6+7 (weak), 8, 10	1, 2, 4	
5	3	1.96	27.04	CH_2	Multiplet	1		5, 7	5, 7	1, 2, 5, 6, 7	1, 2 (weak), 4, 5, 6	3, 7	
3	4	1.85	43.32	CH_2	Doublet	1	18.2	1	1	2	1, 3, 4, 5+6+7 (weak), 8, 10	1, 2, 4	
6	5	1.69	29.91	CH_2	dd	1	13.1 4.1	3, 6	3, 6	3, 6, 7	2, 3, 7, 9	5, 6	
6	6	1.41	29.91	CH_2	Multiplet	1		5, 7	5, 7	2, 3, 5, 7	2, 3, 7, 9	5, 6	
5	7	1.35	27.04	CH_2	Multiplet	1		3, 6	3, 6	2, 3, 5, 6	1, 2 (weak), 4, 5, 6	3, 7	
9	8	0.97	19.15	CH_3	Singulet	3					10	8	5, 3
10	9	0.92	9.27	CH_3	Singulet	3					5	9	5
8	10	0.85	19.80	CH_3	Singulet	3					2, 4, 8	10	1
2			220.06								1, 2, 4, 5, 6, 9	-	
1			57.76								2, 3+4 (weak), 5, 6, 7, 8, 9, 10	-	
7			46.84								4, 6, 7, 8, 9, 10		

Based on the numbering shown in Figure 79, carbon atoms from camphor and 1,8- cineol were attributed to NMR peaks. Chemical shifts and coupling constants for both monoterpenes are listed in Table 73.

Camphor and 1,8-cineol peaks were identified in the spectrum of EV32 due to the chemical shifts detected in the spectra of standard samples (see Tables 77 and 78). This sample clearly showed the expected satellites due to

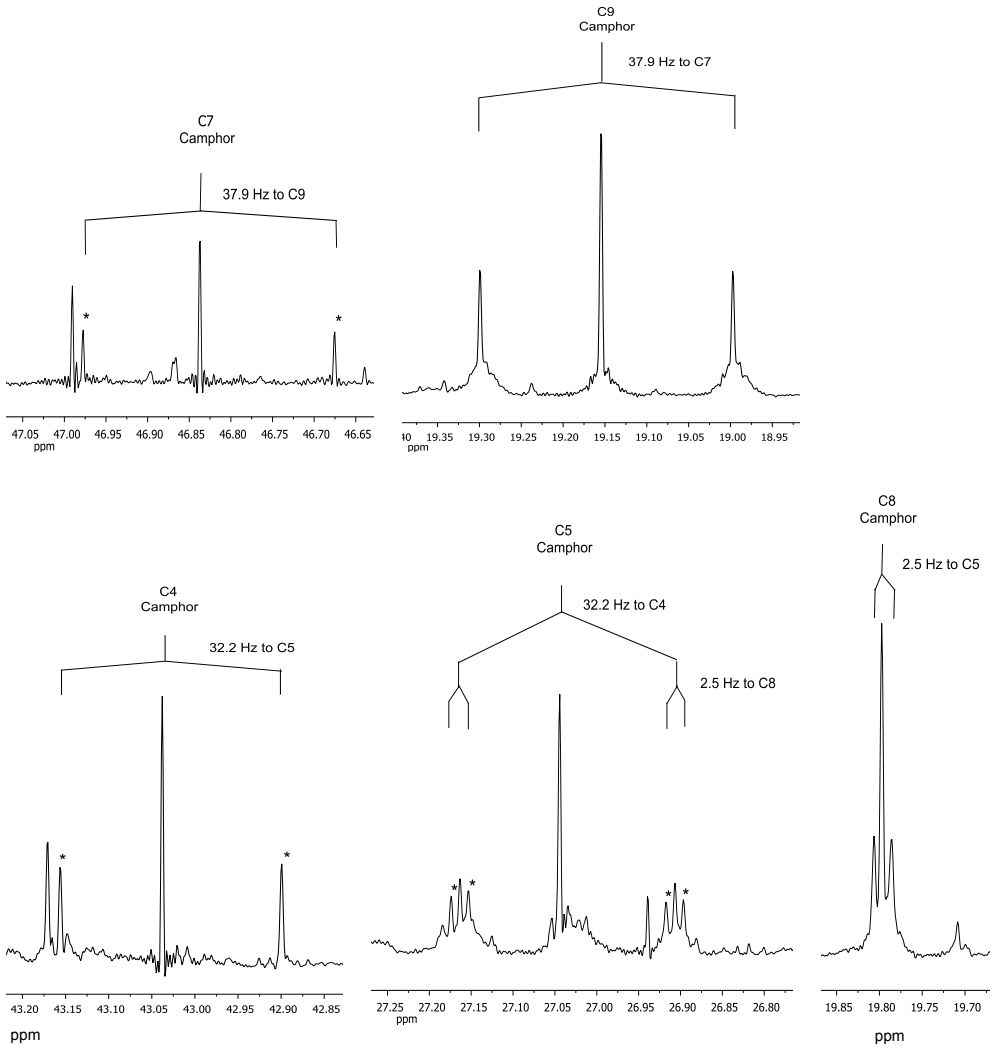


Figure 80. ^{13}C NMR camphor signals; chloroform-d leaf essentail oil extract from spike lavender after incorporation of $^{13}CO_2$ (pulse 5.2 hours, chase 192 hours). * indicate the satellite signals of the carbon atoms under study.

sufficient labeling with $^{13}CO_2$ in both the ^{13}C (for camphor and cineol) and INADEQUATE (for camphor) spectra (Table 73 and Figures 80, 81 and 82).

^{13}C NMR signals for all camphor carbon atoms are shown in Figure 80. If the monoterpenes under study were synthesized from MEP pathway precursors, a specific coupling pattern had to be detected as indicated in Figure 79. As expected, coupling between all carbon atoms generated from ^{13}C derived from a certain precursor, pyruvate or GAP, respectively, were detected. These findings are due to the labeling strategy that predominantly produces monoterpenes consisting of labeled blocks that are not directly together.

Table 73. NMR analysis of camphor and 1,8-cineol derived from chloroform-d extraction of spike lavender leaves pulse-labeled with $^{13}CO_2$ for 5.2 hours; chase time 192 hours.

Position carbon atoms	Chemical shift [ppm]	^{13}C Coupling	^{13}C-^{13}C coupling constant [Hz]
		Camphor	
10	9.27	to C1	41.1
9	19.15	to C7	37.9
8	19.8	to C5	2.5
5	27.04	to C4 to C8	32.2 2.5
6	29.91	to C2	2.3
4	43.04	to C5	32.2
3	43.32	to C2	34.3
7	46.84	to C9	37.9
1	57.76	to C10	41.1
2	220.06	to C3 to C6	34.4 2.3
		1,8-cineol	
3/5	22.8	C3 to C4	32.9
7	27.55	to C1	43.3
9/10	28.87	C10 to C8 C9 to C3/4	41.0 2.2
2/6	31.48		?
4	32.91	to C3	32.9
1	69.93	to C7	43.3
8	73.76	to C10	41

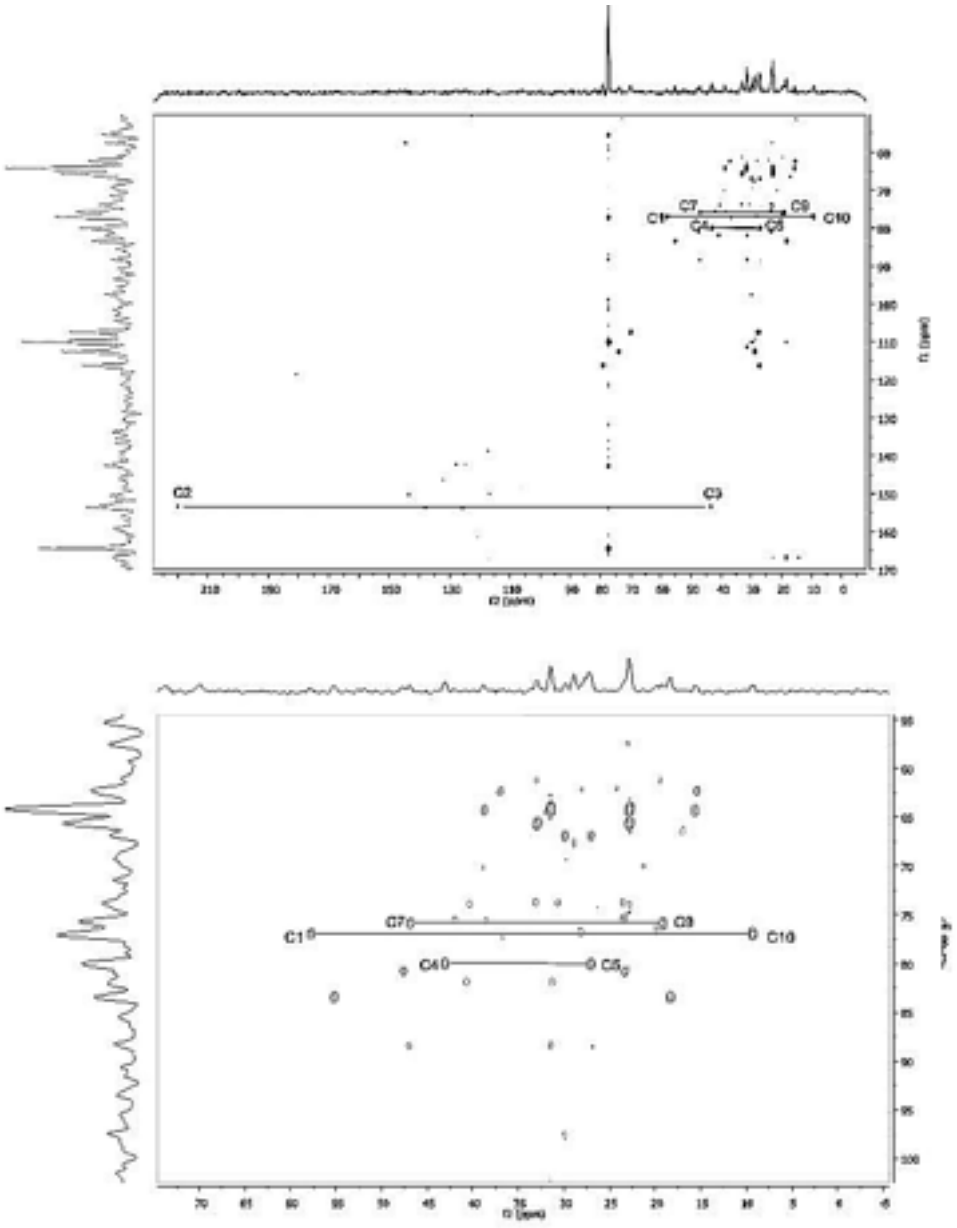


Figure 81. INADEQUATE ^{13}C NMR camphor signals; chloroform-d leaf essentail oil extract from spike lavender after incorporation of $^{13}CO_2$ (pulse 5.2 hours, chase 192 hours). (NMR parameter: pulse program: inadqf, TD1: 300, NS: 128, J(CC): 50 Hz, D1: 2 sec), measurement time: 22 hours.

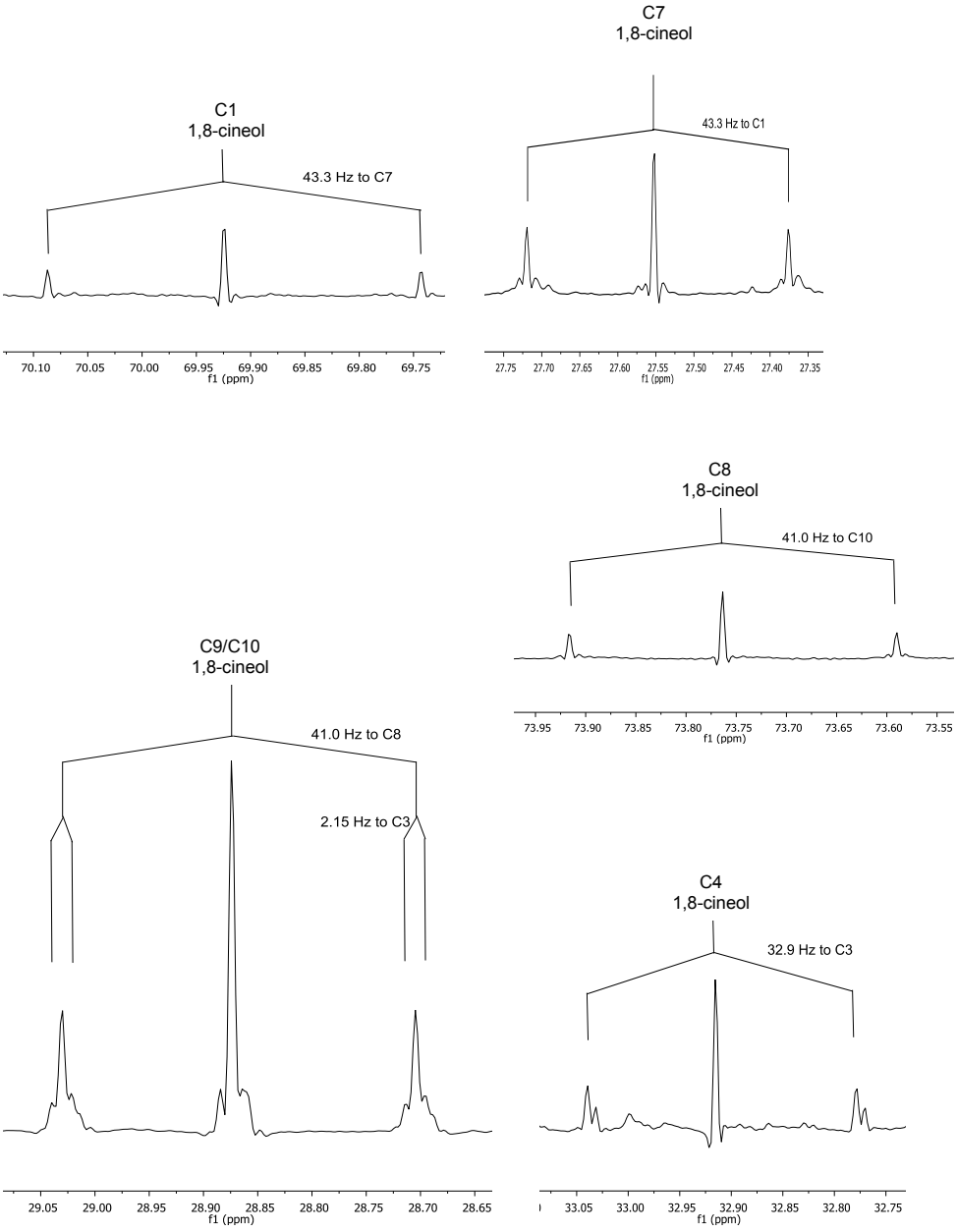


Figure 82. ^{13}C NMR 1,8-cineol signals; chloroform-d leaf essentail oil extract from spike lavender after incorporation of $^{13}CO_2$ (pulse 5.2 hours, chase 192 hours).

In the case of camphor, coupling constants of satellite pairs caused by C1, C10, C6, C2, C7, C9, C4 and C8 can be clearly attributed (see Figure 80). Also, the signal for carbon atom C5 shows a satellite pair caused by coupling to C4 (32.2 Hz) and an additional coupling to C8 (2.5 Hz), reflected by a splitting of the satellite pair. These signals were caused by ^{13}C atoms derived from GAP, which indicates the biosynthesis via the MEP pathway. Only the long range coupling of the carbonyl-C2 to C6 cannot be resolved in detail. These results were confirmed by the INADEQUATE experiment, which is shown in Figure 81. Clearly, Figure 79 shows all the couplings already described in Figure 80. Only the long-range coupling of the carbonyl-C2 to C6 cannot be resolved in the INADEQUATE spectrum as well.

In case of 1, 8-cineol, coupling constants of satellite pairs caused by C7, C1, C8, C4 and C10/C9 can be clearly attributed (see Figure 82).

A long-range coupling caused by C9 to C3 is detected, which can be ascribed to ^{13}C derived from GAP. This triple is only expected for a moiety where GAP serves as a precursor. Signals of C3 and C5, C6 and C2, and C9 and C10 respectively, cannot be distinguished because of the symmetry of cineol. Furthermore, it is possible that the signal of C3/C5 overlays with a signal of α-pinene. In Figure 82, signals caused by C2/C6 and C3/C5 are shown. The satellite pairs are multipletts and therefore cannot be clearly connected to a certain carbon atom. Nevertheless, this method proves itself powerful enough to provide evidence to support the hypothesis that the MEP pathway is responsible for the biosynthesis of 1, 8-cineol due to the C9 to C3 long-range coupling detected.

As a conclusion and based on the ^{13}C NMR spectra, clear evidence for the biosynthesis of camphor and 1,8-cineol predominantly via the MEP pathway in *Lavandula latifolia* is provided.

III. 2. 4. 3. 3. $^{13}CO_2$ labeling in HMGR5 plants

WT and HMGR5 plants were labeled with $^{13}CO_2$ for 5 hours and harvested at different chase times. The terpenes were extracted from leaves with chloroform-D and analysed by GC/MS.

The percentages of the 5 main components of the leaf essential oil (the monoterpenes cineol, camphor, α-pinene, β-pinene and limonene) were calculated in relation to the 15 main peaks of each sample and are presented in Table 74.

Table 74. Main components of spike lavender leaf essential oil from lines HMGR5 ans WT extracted with chloroform-d and determined by GC/MS. The percentage is referred to the area of the 15 main peaks of each sample. Rt: retention time

Sample and chase period	α-pinene rt: 5.9	β-pinene rt:7.2	cineol rt: 9.2	limonene rt: 9.0	camphor rt: 14.3	Total
HMGR5 plants						
4 days	8.15%	6.77%	53.78%	2.09%	16.83%	87.62%
4 days	2.79%	1.77%	53.87%	0.84%	33.01%	92.28%
4 days	5.33%	3.42%	49.91%	1.79%	30.49%	90.94%
7 days	3.63%	2.14%	37.56%	1.02%	28.88%	73.23%
7 days	2.37%	1.58%	52.94%	0.65%	31.96%	89.50%
10 days	4.87%	3.33%	48.44%	1.73%	27.94%	86.31%
10 days	5.02%	2.98%	50.99%	1.27%	30.42%	90.68%
10 days	4.22%	2.64%	52.82%	1.39%	33.47%	94.54%
Mean	4.55%	3.08%	50.04%	1.35%	29.13%	88.14%
WT plants						
4 days	4.8%	3.05%	49.83%	1.54%	29.23%	88.45%
11 days	5.89%	4.81%	44.54%	3.24%	19.43%	77.91%
11 days	5.54%	4.5%	42.8%	3.84%	12.13%	68.81%
11 days	7.08%	4.8%	32.6%	3.84%	29.27%	77.59%
Mean	5.83%	4.29%	42.44%	3.12%	22.52%	78.19%

These five molecules accounted for almost 88% of the total essential oil in the HMGR5 plants and 78% for the WT plants, revealing that apparently the HMGR5 plants had a higher proportition of monoterpenes in their essential oil. Coumarine was also detected in most samples (data no shown). The main compounds in all samples were camphor and 1,8-cineol.

Althought all plants were labeled for a pulse time of 5 hours, only poor excess ^{13}C incorporation was achieved, between 0.01% and 0.83% (See Appendix III). Still, some facts can be discussed. The M+2 and M+3 ratios for 1,8-cineol and camphor are displayed in Figures 83A and 84A respectively, and Table 75. These ratios could be determined for 8 transgenic plants and for 4 wild type plants. Three HMGR5 plants were harvested 4 days after the pulse time, two after 7 days and three after 10 days. The WT plants were harvested after 4 days (one plant) and 11 days (three plants).

Only harvested plants showing an excess of ^{13}C incorporation to 1,8-cineol are included in Figure 83A. Mean results for cineol revealed that HMGR5 and WT plants have very similar M+2/M+3 ratios (Figure 83B). Similar results were obtained in the M+2/M+3 ratios for camphor in WT plants (ratio mean value of 1.9 ± 0.1; Figure 84B). In contrast, HMGR5 plants showed a high deviation in the M+2/M+3 ratio within the same chase group (mean value for the ratios were 2.0± 0.4 and 1.8±0.4 for 4 and 10 chase periods, repectively; Figure 84C). In each of the three chase periods, one transgenic plant showed an increased M+2/M+3 ratio (mean ratio of 2.3 ± 0.2 vs. 1.9± 0.1 in transgenic and WT plants, respectively; Figure 84D). This would imply a high impact of the mevalonate pathway on the biosynthesis of camphor. It is not clear why some of the transgenic HMGR5 plants exhibit a WT-like phenotype. Since transgene integration was corroborated by PCR and Southern blot, further problems with gene silencing and or postranscriptional control could be involved. Thus, maybe, those plants do not overexpress the *HMG1* gene and therefore do not build an increased level of mevalonate, which could be used for monoterpene biosynthesis.

In summary, the M+2/M+3 ratios for camphor are within the same range except for one third (3 plants) of the HMGR5 plants that showed very increased values for their M+2/M+3 ratios (Figure 84D). Therefore, those *L. latifolia*

plants, grown under physiological conditions, producing more mevalonate seem to use the resulting isoprenoid moieties from the MVA pathway for the biosynthesis of the monoterpene camphor. The exchange of metabolites between the MVA and the MEP pathways has been previously suggested in transgenic *L. latifolia* (Muñoz-Bertomeu et al., 2007a). Results presented in this work confirm this suggestion. Nevertheless, for a final conclusion, NMR spectroscopy should be performed.

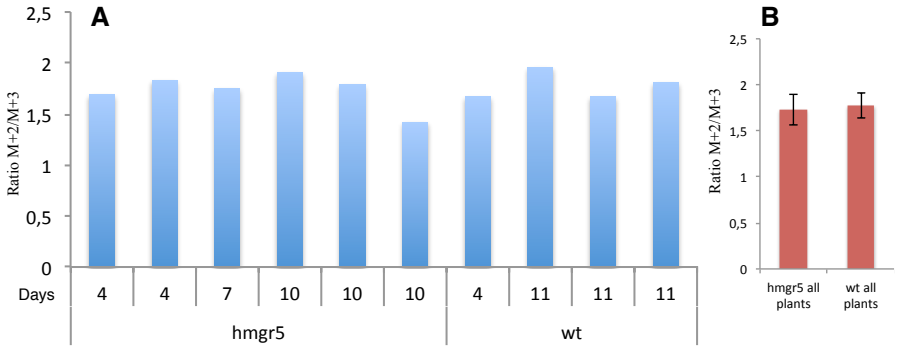


Figure 83. ^{13}C enrichment in 1,8-cineol. Excess values of M+2 and M+3 isotopomeres obtained from GC/MS analysis from spike lavender leaf essential oil (chloroform-d extracts) labeled with $^{13}CO_2$ for 5 hours. A) Ratios of M+2 and M+3 calculated for 6 HMGR5 transgenic plants and 4 wild type (WT) plants. B) Mean values of the M+2/M+3 ratios for all HMGR5 plants and all WT plants.

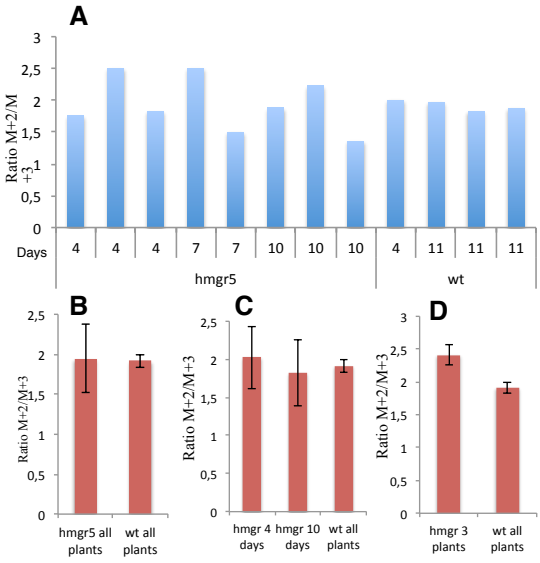


Figure 84. ^{13}C enrichment in camphor. Excess values of M+2 and M+3 isotopomeres obtained from GC/MS analysis from spike lavender leaf essential oil (chloroform-d extracts) labeled with $^{13}CO_2$ for 5 hours. A) Ratios of M+2 and M+3 calculated for 8 HMGR5 transgenic plants and 4 wild type (WT) plants. B) Mean ±SD values of the M+2/M+3 ratios for all HMGR5 plants and all WT plants. C) Mean ±SD values of the M+2/M+3 ratios for all HMGR5 plants harvested 4 and 10 days after labeling and all WT plants harvested 11 days after labeling. D) Mean values of the M+2/M+3 ratios for the three HMGR5 plants showing an increased M+2/M+3 ratio compared to the WT plants.

Table 75. GC/MS excess data for M+2 and M+3 isotopomeres in 1,8- cineol and camphor extracted with chloroform-d and analyzed by GC/MS from spike lavender leaves from lines HMGR5 and WT at different points of time after the labeling pulse phase with $^{13}CO_2$.

		Excess values (%)		Ratio	
		M+2	M+3	M+2/ M+3	M+2 / (M+2)+(M+3)
Camphor	HMGR5				
	4 days	0.30	0.17	1.76	0.64
	4 days	0.05	0.02	2.50	0.71
	4 days	0.20	0.11	1.82	0.65
	7 days	0.05	0.02	2.50	0.71
	7 days	0.03	0.02	1.50	0.60
	10 days	0.32	0.17	1.88	0.65
	10 days	0.29	0.13	2.23	0.69
	10 days	0.15	0.11	1.36	0.58
	WT				
	4 days	0.04	0.02	2.00	0.67
	11 days	0.65	0.33	1.97	0.66
	11 days	0.20	0.11	1.82	0.65
	11 days	0.86	0.46	1.87	0.65
1,8-cineol	HMGR5				
	4 days	0.27	0.16	1.69	0.63
	4 days	0.22	0.12	1.83	0.65
	7 days	0.07	0.04	1.75	0.64
	10 days	0.21	0.11	1.91	0.66
	10 days	0.32	0.14	1.79	0.70
	10 days	0.17	0.12	1.42	0.59
	WT				
	4 days	0.05	0.03	1.67	0.63
	11 days	0.53	0.27	1.96	0.66
	11 days	0.15	0.09	1.67	0.63
	11 days	0.87	0.48	1.81	0.64

III. 2. 5. Discussion

Irrespective of the experimental approach, we demonstrate that precursors for the biosynthesis of camphor and cineol in spike lavender are mainly provided by the MEP pathway. Thus, in potted plants feed with $^{13}CO_2$, this conclusion is not only highlighted by the NMR results from the EV32 sample (Table 73 and Figures 80, 81 and 82), but also by the M+2 and M+3 values and their ratios (Tables 70). In the *in vitro* experiments, the M+2 and M+3 values and their ratios support also the involvement of the MEP pathway in the synthesis of both monoterpenes (Table 59, 60, 62, 63, 65 and 66). This pathway is responsible for the production of monoterpenes, diterpenes and carotenoids (Lichtenthaler, 1999). Investigations in peppermint suggested that the MEP pathway might be the exclusive source of precursors for monoterpenes (Wildung and Croteau, 2005). Therefore, our results support previous research on this area.

In spite of all the above mentioned, the experiments with MEV and FSM, inhibitors of the MVA and MEP pathways, respectively, revealed not only that 1.2 mM MVA is enought to recover the normal phenotype of plantlets treated with MEV, but also that exogenous mevalonate partially restored the photosynthetic pigment content of plants treated with FSM. Experiments with mevalonate (both ^{13}C- labeled and non-labeled) highlighted the fact that the addition of this compound affect terpene content (Tables 36, 37 and 56). However, the experiment with ^{13}C-mevalonate emphasised the fact that this effect may not be due to IPP donated by MVA to MEP pathway, at least in the case of cineol and camphor, but rather to a somehow trigger effect of an excess MVA to “activate” the MEP pathway in the plastid.

Experiments with WT and HMGR5 lines *in vitro* showed that the transgenic line produced more monoterpenes that the WT, especially after 28 days (Table 61). Also, the study of WT and HMGR5 lines highlighted the fact that values of M+1, and M+2 and M+3 ratios are always higher in HMGR5 line (Tables 62, 63 and Figures 72 and 73), a feature demonstrated in the solid culture with $U^{13}C$-glucose experiments. As MVA pathway is overexpressed in HMGR5 lines this strongly implicates an active MVA pathway at some level. Still, it is not clear if this route is active in the production of cineol and camphor

also in the WT lines, although it is highlighted by the comparison of the two lines. Under physiological conditions, potted plant feed with $^{13}CO_2$, the mevalonate pathway apparently does not significantly contribute, with the exception of 1/3 of the HMGR5 plants.

The fact that more monoterpenes are produced in HMGR5 plants might not be necesary a consequence of more precursor units via the MVA pathway, but could reflects a more complex situation where intermediates or somewhat connected molecules of the MVA pathway stimulate the monotepene synthesis (i.e. not the C5 precursors but the formation of C-10 and its downstream reactions). A possibility is that with a glucose trigger, either the mevalonate pathway appears to be stimulated or the crosstalk of mevalonate-made C-5 into the plastids, where the monoterpenes are produce, somehow is increased. Suporting this suggestion, previous reports indicated that sucrose can modulate the carbon flux through the MEP pathway, possibly either as a carbon source or as an energy indicator (Hsieh and Goodman, 2005; Cordoba et al., 2009; Xing et al., 2010).

These results, achieved by the inhibitors experiments and with or without ^{13}C precursors and lines WT and HMGR5, show that the main source of monoterpenes and photosynthetic pigment is the MEP pathway; nevertheless, in some extent the MVA pathway might have a contribution in their biosynthesis. Also, that plant life (including terpene formation, regulation and crosstalk) is much more complex that anyone can currently imagine. Terpenes are plant essential metabolites whose formation is extremely robust with two different and quite independent sources (MVA and MEP) with a cryptic crosstalk and still obscure regulation with potential triggers (like glucose or mevalonate) from one pathway that regulates the other one.

Conclusions

IV. CONCLUSIONS

IV. 1. Overexpression of the *DXR* gene in transgenic spike lavender plants

- Transgenic spike lavender plants (T_0) overexpressing the *Arabidopsis thaliana DXR* gene, encoding the second enzyme of the MEP pathway, have been obtained using an *Agrobacterium tumefaciens* coculture technique. T_1 progenies were also obtained, by controlled selfing, from those T_0 lines that flourished after several years in the greenhouse.

- One of the transgenic T_0 lines (line DXR2) accumulates significantly more essential oil in the youngest leaves than the control. This increased amount in the essential oil is lower than that observed in the transgenic DXS6 line previously obtained in our laboratory, which overexpresses the *DXS* gene encoding the first enzyme of the MEP pathway.

- The chlorophyll and carotenoid contents in T_0 plants increase significantly in the younger leaves in all transgenic lines as compared with control, except for lines DXR4 and DXR5 that show no difference or a reduced pigment content, respectively.

- All transgenic lines have an identical phenotype as compared to control in terms of morphology, except line DXR1, which shows juvenile leaf traits.

- Our results suggest that synthesis of monoterpene precursors through the MEP pathway in spike lavender is mainly regulated by the DXS enzyme, which suggests a less relevant roll for the DXR enzyme.

IV. 2. Overexpression of the *DXR* gene in transgenic spike lavender plants

- Transgenic spike lavender plants (T_0) overexpressing the *Clarkia breweri LIS* gene, encoding linalool synthase enzyme responsible for the synthesis of linalool, have been obtained using an *Agrobacterium tumefaciens* coculture technique. T_1 progenies were also obtained, by controlled selfing, from those T_0 lines that flourished after several years in the greenhouse.

- The essential oil analyses of leaves at different developmental stages show that younger leaves accumulate more linalool than mature ones in both transgenic T_0 and control plants. These young leaves from T_0 transgenic plants accumulate more linalool than those from controls that correlates with their higher level of *LIS* gene transcripts.

- The phenotype of increased linalool content observed in young leaves is maintained in those T_1 progenies that inherit the *LIS* transgene.

IV. 3. Simultaneous overexpression of genes encoding enzymes of the terpene biosynthetic pathway in transgenic spike lavender plants

- Cross-pollination of transgenic spike lavender plants allows the generation of double transgenic plants co-expressing the *DXS* - *HMGR* and *DXS* -*LIS* genes.

- The leaf essential oil content in double *DXS*-*LIS* transgenic plants is lower than that of their T_0 DXS6 parental plant. The linalool content in these double transgenic plants is also lower, which could be due to co-suppression effects linked to the structures of the constructs used.

IV. 4. Contribution of MVA and MEP pathways to monoterpene biosynthesis in spike lavender

- MEV (1 μM) negatively affects seed germination and reduces the development of spike lavender plantlets, but does not alter both the photosynthetic pigment and essential oil content in mature leaves.

- FSM (30 μM) does not affect seed germination but reduces shoot development of spike lavender plantlets. This compound also reduces significantly the photosynthetic pigments and essential oil content, especially in young leaves.

- High concentration of MVA (3.5 mM) reduces root and shoot development in spike lavender plantlets. This concentration also diminishes the essential oil content but increases the photosynthetic pigments content in young leaves. Depending on the concentration used, MVA attenuates or reverts the toxic effects of fosmidomycin and mevinoline, respectively.

- The stems of both transgenic HMGR5 spike lavender and double transgenic DXS-HMGR plants are more tolerant than control to 30 μM FSM, especially in relation to their photosynthetic pigments content.

- Feeding culture medium with ^{13}C-mevalonate does not increase the percentage of ^{13}C in the monoterpenes cineol and camphor, but promotes an increased amount of these two monoterpenes, suggesting an activation of the MEP pathway at another level.

- The labeling experiment with [U-$^{13}C_6$]-Glucose in plants grown for 55 days *in vitro* on solid medium produces excess ^{13}C abundance percentages of between 4.8-6.5 and 5.3-6.1 for cineol y camphor, respectively. The ratios M+2/M+3 and M+2/[(M+2) + (M+3)] obtained are higher than those expected if the synthesis of cineol and camphor were achieved exclusively through the MEP pathway. After 28 days of *in vitro* culture on solid medium, the labeling of stems cultured with [U-$^{13}C_6$]-Glucose produces excess ^{13}C abundance percentages of 1.10 and 1.35 for cineol and camphor respectively in control plants, and of 1.53 and 1.63 in the transgenic line HMGR5. The ratios M+2/M

+3 and M+2/[(M+2) + (M+3)] in HMGR5 line are higher than the ones of the controls for both monoterpenes. These data support the existence of an interconnection between the MEP and MVA pathways, suggesting that MVA-derived precursors contribute to the synthesis of camphor and 1,8-cineol in spike lavender.

- The labeling of spike lavender seedlings with [U-$^{13}C_6$]-Glucose grown for 30 days *in vitro* in liquid medium produces excess ^{13}C abundance percentages of 10.6 and 13.3 for cineol and camphor respectively. The ratios M+2/M+3 and M +2/[(M+2) + (M+3)] obtained are exactly what expected if the synthesis of both monoterpenes were achieved exclusively through the MEP pathway, which indicated that in those conditions only MEP-derived precursors are involved in the camphor and 1,8-cineol biosynthesis.

- The optimal $^{13}CO_2$ labeling period to achieve significant ^{13}C incorporation into cineol and camphor in spike lavender plants should be higher than 5 hours. Nevertheless, ^{13}C incorporation is genotype dependent. The ratios M+2/M+3 and M+2/[(M+2) + (M+3)] obtained in control plants are very similar to that expected if the synthesis of cineol and camphor were achieved exclusively through the MEP pathway, which suggests a very small contribution of the MVA pathway to the biosynthesis of those monoterpenes when cultured in *ex vitro* conditions.

- NMR essential oil analyses of leaves from control spike lavender plants confirm that the major components of this oil, the monoterpenes cineol and camphor, are synthesized predominantly from the MEP-derived precursors. However, one third of the HMGR5 transgenic plants labeled with $^{13}CO_2$ produces M+2/M+3 and M+2/[(M+2) + (M+3)] ratios for cineol and camphor higher than those of control plants. These data support the existence of an interconnection between the MEP and MVA pathways in spike lavender.

References

V. REFERENCES

Aharoni, A., Jongsma, M.A., Bouwmeester, H.J. (2005) Volatile science? Metabolic engineering of terpenoids in plants. *Trends in Plant Science* 10: 594-602.

Alberts, A.W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Shonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., Springer, J. (1980) Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzymeA reductase and a cholesterol-lowering agent. *Proceedings of National Academy of Sciences of the United States of America* 77: 3957-3961.

Andrade, L.B., Echeverrigaray, S., Fracaro, F., Pauletti, G.F., Rota, L. (1999) The effect of growth regulators on shoot propagation and rooting of common lavender (*Lavandula vera* DC). *Plant Cell, Tissue and Organ Culture* 56: 79-83.

Angioni, A., Barra, A., Coroneo, V., Dessi, S., Cabras, P. (2006) Chemical composition, seasonal variability, and antifungal activity of *Lavandula stoechas* L. ssp. *stoechas* essential oils from stem/leaves and flowers. *Journal of Agricultural and Food Chemistry* 54: 4364-4370.

Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A., Zenk, M.H. (1997) Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. *Proceedings of National Academy of Sciences of the United States of America* 94: 10600-10605.

Bach, T.J., Lichtenthaler, H.K. (1983) Inhibition by mevinolin of plant growth, sterol formation and pigment accumulation. *Physiologia Plantarum* 59: 50-60.

Bacher, A., Rieder, C., Eichinger, D., Arigoni, D., Fuchs, G., Eisenreich, W. (1999) Elucidation of novel biosynthetic pathways and metabolic flux patters by retrobiosynthetic NMR analysis. FEMS Microbiology Reviews 22: 567-598.

Baldwin, I.T., Halitschke, R., Paschold, A., von Dahl, C.C., Preston, C.A. (2006) Volatile signalling in plant-plant interaction: “talking trees” in the genomics era. *Science* 311: 812-815.

Bassham, J., Shibita, K., Streenberg, K., Bourdon, L., Calvin, M. (1956)

Photosynthetic cycle and respiration: light-dark transients. *Journal of the American Chemical Society* 78: 4120-4124.

Baulcombe, D. (2004) RNA silencing in plants. *Nature* 431: 356-363.

Baulcombe, D. (2005) RNA silencing. *TRENDS in Biochemical Sciences* 30: 290-293.

Bede, J.C., Musser, R.O., Felton, G.W., Korth, K.L. (2006) Caterpillar herbivory and salivary enzymes decrease transcript levels of *Medicago truncatula* genes encoding early enzymes in terpenoid biosynthesis. *Plant Molecular Biology* 60: 519-531.

Berdonces i Serra, J.L. (2007) Gran enciclopedia de las plantas aromáticas y medicinales. Tikal Ediciones, Madrid (Spain).

Bohlman, J., Meyer-Gauen, G., Croteau, R. (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proceedings of National Academy of Sciences of the United States of America* 95: 4126-4133.

Botella-Pavía, P., Besumbes, O., Phillips, M.A., Carretero-Paulet, L., Boronat, A., Rodríguez-Concepción, M. (2004) Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. *The Plant Journal* 40: 188-199.

Böttcher, C., Dennis, E.G., Booker, G.W., Polyak, S.W., Boss, P.K., Davies, C. (2012) A novel tool for studying auxin-metabolism: the inhibition of grapevine indole-3-acetic acid-amido synthetases by a reaction intermediate analogue. *PLoS ONE* 7: 37632.

Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R.A., Camara, B. (1998) Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. *Plant Physiology* 117:1423-1431.

Bouvier, F., Rahier, A., Camara, B. (2005) Biogenesis, molecular regulation and function of plant isoprenoids. *Progress in Lipid Research* 44: 357-429.

Brauman, J.I. (1966) Least squares analysis and simplification of multi-isotope mass spectra. *Analytical Chemistry* 38: 607-610.

Brodkorb, D., Gottschall, M., Marmulla, R., Lüddeke, F., Harder, J. (2010) Linalool dehydratase-isomerase, a bifunctional enzyme in the anaerobic degradation of monoterpenes. *Journal of Biological Chemistry* 285: 30436-30442.

Buchbauer, G. (2002) Lavender oil and its therapeutic properties. In: *Lavender.*

The Genus Lavandula. Lis-Balchin, M. (Ed.), Taylor & Francis Inc., London, pp. 124-139.

Burlat, V., Oudin, A., Courtois, M., Rideau, M., St-Pierre, B. (2004) Co-expression of three MEP pathway genes and geraniol 10- hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. *The Plant Journal* 38: 131-141.

Calvo, M.C., Segura, J. (1988) In vitro morphogenesis from explants of *Lavandula latifolia* and *Lavandula stoechas* seedlings. *Scientia Horticulturae* 36: 131-137.

Calvo, M.C., Segura, J. (1989a) In vitro propagation of lavender. *American Society for Horticultural Science* 24: 375-376.

Calvo, M.C., Segura, J. (1989b) Plant regeneration from cultured leaves of *Lavandula latifolia* Medicus: Influence of growth regulators and illumination conditions. *Plant Cell, Tissue and Organ Culture* 19: 33-42.

Calvo, M.C., Segura, J. (1991) Lavandula spp. (Lavender): In Vitro Culture, Regeneration of Plants, and the Formation of Essential Oils and Pigments. *Biotechnology in Agriculture and Forestry* 15: 283-310.

Cardé, J.P. (1984) Leucoplasts: a distinct kind of organelles lacking typical 70S ribosomes and free thylakoids. *European Journal of Cell Biology* 34: 18-26.

Carretero-Paulet, L., Ahumanda, I., Cunillera, N., Rodríguez-Concepción, M., Ferrer, A., Boronat, A., Campos, N. (2002) Expression and molecular analysis of the *Arabidopsis DXR* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-C-methyl-D- erythritol-4- phosphate pathway. *Plant Physiology* 129: 1581-1591.

Carretero-Paulet, L., Cairó, A., Botella-Pavía, P., Besumbes, O., Campos, N., Boronat, A., Rodríguez-Concepción, M. (2006) Enhanced flux through the methylerythritol 4-phosphate pathway in *Arabidopsis* plants overexpressing deoxyxylulose 5-phosphate reductoisomerase. *Plant Molecular Biology* 62: 683-695.

Cavanagh, H.M., Wilkinson, J.M. (2002) Biological activities of lavender essential oils. *Phytotherapy Research* 16: 301-308.

Cordoba, E., Salmi, M., León, P. (2009) Unravelling the regulatory mechanisms

that modulate the MEP pathway in higher plants. *Journal of Experimental Botany* 60: 2933-2943.

Croteau, R. (1987) Biosynthesis and catabolism of monoterpenoids. *Chemical Reviews* 87: 929-954.

Cui, H., Zhang, S.T., Yang, H.J., Hao, J., Wang, X.J. (2011) Gene expression profile analysis of tobacco leaf trichomes. *BMC Plant Biology* 11: 76.

Chaded, K., Oudin, A., Guivarch, N., Hamdi, S., Chenieux, J.C., Ri- Deau, M., Clastre, M. (2000) 1-deoxy-D-xylulose 5-phosphate synthase, from periwinkle: cDNA identification and induced gene expression in terpenoid indole alkaloid-producing cells. *Plant Physiology and Biochemistry* 38: 559-566.

Chambon, C., Poupet, A., Beck, D., Bettachini, B., Touche, J. (1992) In vitro morphogenetic potential of various Lavandin and Lavander clones-preliminary observations on the agronomic value of the vitroplant. *Agronomie* 12: 173-181.

Cheng, A.X., Lou, Y.G., Mao, Y.B., Lu, S., Wang, L.J., Chen, X.Y. (2007) Plant terpenoids: biosynthesis and ecological functions. *Journal of Integrative Plant Biology* 49: 179-186.

Cheniclet, C., Cardé, J.P. (1985) Presence of leucoplasts in secretory cells and of monoterpenes in the essential oil: a correlative study. *Israel Journal of Botany* 34: 219-238.

Dalakouras, A., Moser, M., Boonrod, K., Krczal, G., Wassenegger, M. (2011) Diverse spontaneous silencing of a transgene among two *Nicotiana* species. *Planta* 234: 699-707.

Dale, S., Arró, M., Becerra, B., Morrice, N.G., Boronat, A., Hardie, A., Ferrer, A. (1995) Bacterial expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA reductase (isoform HMGR1) from *Arabidopsis thaliana*, and its inactivation by phosphorylation at Ser577 by *Brassica oleracea* 3-hydroxy-3-methylglutaryl-CoA reductase kinase. *European Journal of Biochemistry* 233: 506-513.

Damaj, M.I., Siu, E.C.K., Sellers, E.M., Tyndale, R.F., Martin, B.R. (2006) Inhibition of nicotine metabolism by methoxysalen: pharmacokinetic and pharmacological studies in mice. *Journal of Pharmacology and Experimental Therapeutics* 320: 250-257.

Degenhardt, J., Gershenzon, J., Baldwin, I.T., Kessler, A. (2003) Attracting friends to feast on foes: engineering terpene to make crop plants more

attractive to herbivore enemies. *Current Opinion in Biotechnology* 14: 169-176.

Dewick, P.M. (2002) Medicinal Natural Products, a Biosynthetic Approach, second ed. Wiley & Sons LTD, West Sussex, UK.

Dias, M.C., Almeida, R., Romano, A. (2002) Rapid clonal multiplication of Lavandula viridis L'Her through in vitro axillary shoot proliferation. *Plant Cell, Tissue and Organ Culture* 68: 99-102.

Diemer, F., Caissard, J.C., Moja, S., Chalcat, J.C., Jullien, F. (2001) Altered monoterpene composition in transgenic mint following the introduction of 4S-limonene synthase. *Plant Physiology and Biochemistry* 39: 603-614.

Disch, A., Hemmerlin, A., Bach, T.J., Rohmer, M. (1998) Mevalonate-derived isopentenyl diphosphate is the biosynthetic precursor of ubiquinone prenyl side chain in tobacco BY-2 cells. *Biochemical Journal* 331: 615-621.

Doyle, J.J., Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Dronne, S., Colson, M., Moja, S., Faure, O. (1999a) Plant regeneration and transient GUS expression in a range of lavandin (*Lavandula x intermedia* Emeric ex Loiseleur) cultivars. *Plant Cell, Tissue and Organ Culture* 55: 193-198.

Dronne, S., Jullien, F., Caissard, J.C., Faure, O. (1999b) A simple and efficient method for in vitro shoot regeneration from leaves of lavandin (*Lavandula x intermedia* Emeric ex Loiseleur). *Plant Cell Reports* 18: 429-433.

Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., Boland, W., Gershenzon, J. (2005) The non mevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proceedings of National Academy of Sciences of the United States of America* 102: 933-938.

Dudareva, N., Negre, F., Nagegowda, D.A., Orlova, I. (2006) Plant volatiles: Recent advances and future perspectives. *Critical Reviews in Plant Sciences* 25: 417- 440.

Dudareva, N., Pichersky, E., Gershenzon, J. (2004) Biochemistry of plant volatiles. *Plant Physiology* 135: 1893-1902.

Duke, S.O., Paul, R.N. (1993) Development and fine structure of the glandular trichomes of *Artemisia annua* L. *International Journal of Plant Sciences* 154: 107-118.

Echeverrigaray, S., Basso, R., Andrade, L.B. (2005) Micropropagation of *Lavandula dentata* from axillary buds of field-grown adult plants. *Biologia Plantarum* 49: 439-442.

Eisenreich, W., Bacher, A., Arigoni, D., Rohdich, F. (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cellular and Molecular Life Sciences* 61: 1401-1426.

Endo, A., Kuroda, M., Tsujita, Y. (1976) ML-236A, ML-236B, and ML-236C, new inhibitors of choles- terogenesis produced by *Penicillium citrinum*. *Journal of Antibiotics* 29: 1346-1348.

Enfissi, E.M.A., Fraser, P.D., Lois, L.M., Boronat, A., Schuch, W., Bramley, P.M. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnology Journal* 3: 17-28.

Estévez, J.M., Cantero, A., Romero, C., Kawaide, H., Jiménez, L.F., Kuzuyama, T., Seto, H., Kamiya, Y., León, P. (2000) Analysis of the expression of CLA1, a gene that encodes the 1-deoxy- xylulose 5-phosphate synthase of the 2-C-methyl-D-erythri- tol-4-phosphate pathway in *Arabidopsis*. *Plant Physiology* 124: 95-103.

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S., León, P. (2001) 1-deoxy-D-xylulose 5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *The Journal of Biological Chemistry* 25: 22901-22909.

Falk, L., Biswas, K., Boeckelmann, A., Lane, A., Mahmoud, S.S. (2009) An efficient method for the micropropagation of lavenders: regeneration of a unique mutant. *Journal* of *Essential Oil Research* 21: 225-228.

Ferhatoglu, Y., Barret, M. (2006) Studies of clomazone mode of action. *Pesticide Biochemistry and Physiology* 85: 7-14.

Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Scheffer, J.J.C. (2008) Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour* and *Fragrance Journal* 23: 213-226.

Fischer, E., Sauer, U. (2003) A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *Escherichia coli*. *Journal of*

Biological Chemistry 278: 46446-46451.

Flores-Perez, U., Perez-Gil, J., Closa, M., Wright, L.P., Botella-Pavia, P., Phillips, M.A., Ferrer, A., Gershenzon, J., Rodríguez-Concepción, M. (2010) PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) integrates the regulation of sugar responses with isoprenoid metabolism in *Arabidopsis*. *Molecular Plant* 3: 101-112.

Gazzani, S., Lawrenson, T., Woodward, C., Headon, D., Sablowski, R. (2004) A link between mRNA turnover and RNA interference in *Arabidopsis*. *Science* 306: 1046-1048.

George, EF. (1993) Plant propagation by tissue culture, part 1. Exegetics Ltd, Edington, Wilts, England.

Gershenzon, J., McConkey, M.E., Croteau, R.B. (2000) Regulation of monoterpene accumulation in leaves of peppermint. *Plant Physiology* 122: 205-213.

Gómez, M.P., Calvo, M.C., Cornejo, M.J., Segura, J. (1987a) Isolation and culture of *Lavandula stoechas* cells. *Plant Physiology* 6: 159-164.

Gómez, M.P., Calvo, M.C., Segura, J. (1987b) Callus formation from hypocotyls of *Lavandula stoechas* cells: Interaction between nutricional and hormonal factors. *Gartenbauwissenschaft* 52: 6-9.

Gonçalves, S., Romano, A. (2013) In vitro culture of lavenders (*Lavandula* spp.) and the production of secondary metabolites. *Biotechnology Advances* 31: 166-174.

Göpfert, J., Heil, N., Conrad, J., Spring, O. (2005) Cytological development and sesquiterpene lactone secretion in capitates glandular trichomes of sunflower. *Plant Biology* 7: 148-155.

Guillén, M.D., Cabo, N., Burillo, J. (1996) Characterisation of the essential oils of some cultivated aromatic plants of industrial interest. *Journal* of the *Science* of *Food* and *Agriculture* 70: 359-363.

Gunnarsson, N., Mortensen, U.H., Sosio, M., Nielsen, J. (2004) Identification of the Entner-Doudoroff pathway in an antibiotic-producing actinomycete species. *Molecular Microbiology* 52: 895-902.

Haig, T.J., Haig, T.J., Seal, A.N., Pratley, J.E., An, M., Wu, H. (2009) Lavender as a source of novel plant compounds for the development of a natural herbicide. *Journal of Chemical Ecology* 35, 1129-1136.

Hallahan, D.L. (2000) Monoterpenoid biosynthesis in glandular trichomes of

labiate plants. *Advances in Botanical Research* 31: 77120.

Hans, J., Hause, B., Strack, D., Walter, M.H. (2004) Cloning, characterization, and immunolocalization of a mycorrhiza inducible 1-deoxy-D-xylulose 5-phosphate reductoisomerase in arbuscule-containing cells of maize. *Plant Physiology* 134:614-624.

Harborne, J.B., Williams, C.A. (2002) Phytochemistry of the genus *Lavandula*. In: Lavender, Lis-Balchin M (ed), Taylor & Francis Inc, New York, pp. 86-99.

Harker, M., Bramley, P.M. (1999) Expression of prokaryotic 1-deoxy-D - xylulose-5-phosphatases in Escherichia coli increases carotenoid and ubiquinone biosynthesis. *FEBS letters* 448: 115-119.

Hart, S., Lis-Balchin, M. (2002) Pharmacology of *Lavandula* essential oils and extracts in vitro and in vivo. In: *Lavender. The Genus Lavandula.* Lis-Balchin, M. (Ed.), Taylor & Francis Inc., London, pp. 140-154.

Hasumuna, T., Takeno, S., Hayashi, S., Sendai, M., Bamba, T., Yoshimura, S., Tomizawa, K., Fukusaki, E., Miyake, C. (2008) Overexpression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase gene in chloroplast contributes to increment of isoprenoid production. *Journal of Bioscience and Bioengineering* 1005: 518-526.

Heinrich, G., Sawidis, T., Ingolic, E., Stabentheiner, E., Pfeifhofer, H.W. (2010) Ultrastructure of glandular hairs of *Sigesbeckia jorullensis* Kunth (Asteraceae). *Israel Journal of Plant Science* 58: 297-308.

Hemmerlin, A., Hoeffler, J.F., Meyer, O., Tritsch, D., Kagan, I.A., Grosdemange-Billiard, C., Rohmer, M., Bach, T.J. (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. *Journal of Biological Chemistry* 278: 26666-26676.

Herraiz-Peñalver, D., Cases, M.A., Varela, F., Navarrete, P., Sánchez-Vioque, R., Usano-Alemany, J. (2013) Chemical characterization of *Lavandula latifolia* Medik. essential oil from Spanish wild populations. *Biochemical Systematics and Ecology* 46: 59-68.

Hoagland, D.R., Arnon, D.I. (1950) The water culture method for growing plants without soil. *California Agricultural Experiment Station*, Circular 347.

Holsters, M., de Waele, D., Depicker, A., Messens, E., Van Montagu, M., Schell, J. (1978) *Molecular Genetics and Genomics* 163: 181-187.

Hsieh, M.H., Goodman, H.M. (2005) The *Arabidopsis* IspH homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. *Plant Physiology* 138: 641-653.

Huber, H., Gallenberger, M., Jahn, U., Eylert, E., Berg, I., Kockelkorn, D., Eisenreich, W., Fuchs, G. (2008) A dicarboxylate/4-hydroxybutyrate autotrophic carbonassimilation cycle in the hyperthermophilic *Archaeum Ignicoccus hospitalis*. *Proceedings of National Academy of Sciences of the United States of America* 105: 7851-7856.

Iguchi, E., Okuhara, M., Kohsaka, M., Aoki, H., Imanaka, H. (1980) Studies on new phosphonicacid antibiotics. II. Taxonomic studies on producing organisms of the phosphonic acid and related compounds. *The Journal of Antibiotics* 33:19-23.

Irmisch, S., Krause, S.T., Kunert, G., Gershenzon, J., Degenhardt, J., Köllner, T.B. (2012) The organ-specific expression of terpene synthase genes contributes to the terpene hydrocarbon composition of chamomile essential oils. *BMC Plant Biology* 12: 84.

Jahn, U., Huber, H., Eisenreich, W., Hugler, M., Fuchs, G. (2007) Insights into the autotrophic CO2 fixation pathway of the *Archaeon Ignicoccus hospitalis*: Comprehensive analysis of the central carbon metabolism. *Journal of Bacteriology* 189: 4108-4119.

Jordan, A.M., Calvo, M.C., Segura, J. (1990) Morphogenesis in callus and single-cell cultures of *Lavandula latifolia* Medicus. *The Journal of Horticultural Science and Biotechnology* 65: 49-53.

Jordan, A.M., Calvo, M.C., Segura, J. (1998) Micropropagation of adult *Lavandula dentata* plants. *The Journal of Horticultural Science and Biotechnology* 73: 93-96.

Kalantidis, K., Tsagris, M., Tabler, M. (2006) Spontaneous short-range silencing of a GFP transgene in *Nicotiana benthamianais* possibly mediated by small quantities of siRNA that do not trigger systemic silencing. *The Plant Journal* 45: 1006-1016.

Kanazawa, A. (2008) RNA silencing manifested as visibly altered phenotypes in plants. *Plant biotechnology* 25: 423-435.

Kavitha, K., Vidyavathi, M., Asha, S., Bindu, T.H. (2012) Microbial metabolism and inhibition studies of phenobarbital. *Tropical Journal of Pharmaceutical Research* 11: 62-68.

Koppisch, A.T., Fox, D.T., Blagg, B.S.J., Poulter, C.D. (2002) *E. coli* MEP

synthase: steady-state kinetic analysis and substrate binding. *Biochemistry* 41: 236-243.

Korzekwa, K., Howald, W.N., Trager, W.F. (1990) The use of Brauman's least squares approach for the quantification of deuterated chlorophenols. *Biological Mass Spectrometry* 19: 211-217.

Krasnyanski, S., May, R.A., Loskutov, A., Ball, T.M., Sink, K.C. (1999) Transformation of the limonene synthase gene into peppermint (*Mentha piperita* L.) and preliminary studies on the essential oil profiles of single transgenic plants. *Theoretical and Applied Genetics* 99: 676-682.

Kuemmerle, H.P., Murakawa, T., De Santis, F. (1987) Pharmacokinetic evaluation of fosmidomycin, a new phosphonic acid antibiotic. *Chemioterapia* 6: 113-119.

Kuntz, L., Tritsch, D., Grosdemange-Billiard, C., Hemmerlin, A., Willem, A., Bach, T.J., Rohmer, M. (2005) Isoprenoid biosynthesis as a target for antibacterial and antiparasitic drugs: phosphonohydroxamic acids as inhibitors of deoxyxylulose phosphate reducto-isomerase. *Biochemical Journal* 86: 127-135.

Kuzuyama, T., Shimizu, T., Takahashi, S., Seto, H. (1998) Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Letters* 39: 7913-7916.

Kuzuyama, T., Takahashi, S., Seto, H. (2000) Characterization of 1-deoxy-D-xylulose 5-phosphate reductoisomerase, an enzyme involved in isopentenyl diphosphate biosynthesis, and identification of its catalytic amino acid residues. *The Journal of Biological Chemistry* 275: 19928-19932.

Lange, B.M., Mahmoud, S.S., Wildung, M.R., Turner, G.W., Davis, E.M., Lange, I., Baker, R.C., Boydston., R.A., Croteau, R.B. (2011) Improving peppermint essential oil yield and composition by metabolic engineering. *Proceedings of National Academy of Sciences of the United States of America* 108: 16944-16949.

Lange, B.M., Ahkami, A. (2013) Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes - current status and future opportunities. *Plant Biotechnology Journal* 11: 169-196.

Lange, B.M., Turner, G.W. (2013) Terpenoid biosynthesis in trichomes-current status and future opportunities. *Plant Biotechnology Journal* 11: 2-22.

Lange, B.M., Wildung, M.R., McCaskill, D., Croteau, R. (1998) A family of

transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. *Proceedings of National Academy of Sciences of the United States of America* 95: 2100-2104.

Langenheim, J.H. (1994) Higher plant terpenoids: a phytocentric overview of their ecological roles. *Journal of Chemical Ecology* 20: 1223- 280.

Laule, O., Fürholz, A., Chang, H.S., Zhu, T., Wang, X., Heifetz, P.B., Gruissem, W., Lange, B.M. (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proceedings of National Academy of Sciences of the United States of America* 100: 6866-6871.

Lavy, M., Zuker, A., Lewinsohn, E., Larkov, O., Ravid, U., Vainstain, A., Weiss, D. (2002) Linalool and linalool oxide production in transgenic carnation flowers expressing the *Clarkia breweri* linalool synthase gene. *Molecular Breeding* 9: 103-111.

Learned, R.M. (1996) Light suppresses *3-hydroxy-3-methylglutaryl- coenzyme A reductase* gene expression in *Arabidopsis thaliana*. *Plant Physiology* 110: 645-655.

Lee, W.P., Byerley, L.O., Bergner, E.A., Edmond, J. (1991) Mass isotopomer analysis: theoretical and practical considerations. *Biological Mass Spectrometry* 20: 451-458.

Lewinsohn, E., Schalechet, F., Wilkinson, J., Matsui, K., Tadmor, Y., Nam, K.H., Amar, O., Lastochkin, E., Larkov, O., Ravid, U., Hiatt, W., Gepstein, S., Pichersky, E. (2001) Enhanced levels of the aroma and flavor compound S-linalool by metabolic engineering of the terpenoid pathway in tomato fruits. *Plant Physiology* 127: 1256-1265.

Lichtenthaler, H.K., Rohmer, M., Schwender, J., Disch, A., Seemann, M. (1997b) A novel mevalonate-independent pathway for the biosynthesis of carotenoids, phytol and prenyl chain of plastoquinone-9 in green algae and higher plants. In: *Physiology, Biochemistry and Molecular Biology of Plant Lipids*. Williams, J.P, Khan, M.U, Lem, N.W. Kluwer Academis Publishers, Netherlands. pp: 177-79

Lichtenthaler, H.K. (1987) Chlorophylls and caratenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology* 148: 350-382.

Lichtenthaler, H.K. (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. *Annual Reviez of Plant Physiology and Plant Molecular Biology* 50: 47-65.

Lichtenthaler, H.K., Rohmer, M., Schwender, J. (1997a) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiologia Plantarum* 101: 643-652.

Lichtenthaler, H.K., Schwender, J., Disch, A., Rohmer, M. (1997c) Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate independent pathway. *FEBS Letters* 400: 271-274.

Lichtenthaler, H.K., Schwender, J., Seemann, M., Rohmer, M. (1995) Carotenoid biosynthesis in green algae proceeds via a novel biosynthetic pathway. In: *Photosynthesis: from Light to Biosphere.* Mathis. Kluver Academic Publishers, Amsterdam, pp. 115-118.

Liu, Y., Wang, H., Ye, H-C., Li, G-F. (2005) Advances in the plant isoprenoid biosíntesis pathway and its metabolic engineering. *Journal of Integrative Plant Biology* 47: 769-782.

Lois, L.M., Rodríguez-Concepción, M., Gallego, F., Campos, N., Boronat, A. (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *The Plant Journal* 22: 503-513.

Lubbe, A., Verpoorte, R. (2011) Cultivation of medicinal and aromatic plants for specialty industrial materials. *Industrial Crops and Products* 34: 785-801.

Lücker, J., Bouwmeester, H.J., Schwab, W., Blaas, J., van der Plas, L.H.W., Verhoeven, H.A. (2001) Expression of *Clarkia* S-linalool synthase in transgenic petunia plants results in the accumulation of S-linalyl-β-D-glucopyranoside. *The Plant Journal* 27: 315-324.

Luo, Z., Chen, Z. (2007) Improperly terminated, unpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in *Arabidopsis*. *The Plant Cell* 19: 943-958.

Mahmoud, S., Williams, M., Croteau, R. (2004) Cosuppression of limonene-3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil. *Phytochemistry* 65: 547-554.

Mahmoud, S.S., Croteau, R.B. (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proceedings of National Academy of Sciences of the United States of America* 98: 8915-8920.

Mahmoud, S.S., Croteau, R.B. (2002) Strategies for transgenic manipulation of

monoterpene biosynthesis in plants. *Trends in Plant Science* 7: 366-373.

Mandel, M., Higa, A. (1970) Calcium- dependent bacteriophage DNA infection. *Journal of Molecular Biology* 53: 159- 162.

Mandel, M.A., Feldman, K.A., Herrera-Estrella, L., Rocha-Sosa, M., León, P. (1996) CLA1, a novel gene required for chloroplast development, is highly conserved in evolution. *The Plant Journal* 9: 649-658.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) *Molecular Cloning - A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Plainview, New York, 545 pages.

Manzano, D., Fernandez-Busquets, X., Schaller, H., González, V. (2004) The metabolic imbalance underlying lesion formation in *Arabidopsis thaliana* overexpressing farnesyl diphosphate synthase (isoform 1S) leads to oxidative stress and is triggered by the developmental decline of endogenous HMGR activity. *Planta* 219: 982-992.

Martin, V., Pitera, D.J., Withers, S., Newman, J., Keasling, J. (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology* 21: 796-802.

Masotti, V., Juteau, F., Bessière, J.M., Viano, J. (2003) Seasonal and phenological variations of the essential oil from the narrow endemic species Artemisia molinieri and its biological activities. *Journal of Agricultural and Food Chemistry* 51: 7115-7121.

Mayrhofer, S., Teuber, M., Zimmer, I., Louis, S., Fischbach, R.J., Schnitzler, J.P. (2005) Diurnal and seasonal variation of isoprene biosynthesis-related genes in grey poplar leaves. *Plant Physiology* 139: 474-484.

McConkey, M.E., Gershenzon, J., Croteau, R.B. (2000) Developmental regulation of monoterpene biosynthesis in the glandular trichomes of peppermint. *Plant Physiology* 122: 215-223.

McNaught, A.D., Wilkinson, A. (1997) *IUPAC. Compendium of Chemical Terminology*, 2nd ed. Blackwell Scientific Publications, Oxford.

Mensualisodi, A., Panizza, M., Tognoni, F. (1995) Endogenous ethylene requirements for adventitious root induction and growth in tomato cotyledons and lavandin microcuttings in vitro. *Plant Growth Regulation* 17: 205-212.

Miller, B., Heuser, T., Zimmer, W. (2000) Functional involvement of a deoxy-D-xylulose 5-phosphate reductoisomerase gene harboring locus of

Synechococcus leopoliensis in isoprenoid biosynthesis. *FEBS Letters* 481: 221-226.

Miller, B., Madilao, L.L., Ralph, S., Bohlmann, J. (2005) Insect-induced conifer defense: White pine weevil and methyl jasmonate induce traumatic resinosis, *de novo* formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcript in Sitka spruce. *Plant Physiology* 137: 369-382.

Mishiba, K.I., Ishikawa, K., Tsujii, O., Mii, M. (2000) Effiecient transformation of lavender (*Lavandula latifolia* Medicus) mediated by *Agrobacterium*. *The Journal of Horticultural Science and Biotechnology* 75: 287-292.

Mitchell, D.I., Cowan, A.K. (2003) Mevastatin-induced inhibition of cell growth in avocado suspension cultures and reversal by isoprenoid compounds. *African Journal of Biotechnology* 2: 264-270.

Muñoz-Bertomeu, J. (2007) Ingeniería metabólica de la biosíntesis de monoterpenos en *Lavandula latifolia* PhD Thesis. PhD Thesis, University of Valencia.

Muñoz-Bertomeu, J., Sales, E., Ros, R., Arrillaga, I., Segura, J. (2007a) Up-regulation of an N-terminal truncated 3-hydroxy-3-methylglutaryl CoA reductase enhances production of essential oils and sterols in transgenic *Lavandula latifolia*. *Plant Biotechnology Journal* 5: 746-758.

Muñoz-Bertomeu, J., Arrillaga, I., Ros, R., Segura, J. (2006) Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. *Plant physiology* 142: 890-900.

Muñoz-Bertomeu, J., Arrillaga, I., Segura, J. (2007b) Essential oil variation within and among natural populations of *Lavandula latifolia* and its relation to their ecological áreas. *Biochemical Systematics and Ecology* 35: 479-488.

Muñoz-Bertomeu, J., Ros, R., Arrillaga, I., Segura, J. (2008) Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves. *Metabolic Engineering* 10: 166-177.

Murakawa, T., Sakamoto, H., Fukada, S., Konishi, T., Nishida, M. (1982) Pharmacokinetics of fosmidomycin, a new phosphonic acid antibiotic. *Antimicrobial Agents and Chemotherapy* 21: 224-230.

Murashige, T., Skoog, F. (1962) A revised medium for rapid groth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*15: 473-479.

Nagata, N., Suzuki, M., Yoshida, S., Muranaka, T. (2002) Mevalonic acid partially restores chloroplast and etioplast development in *Arabidopsis* lacking the non-mevalonate pathway. *Planta* 216: 345-350.

Nebauer, S.G., Arrillaga, I., Castillo-Agudo, L., Segura, J. (2000) *Agrobacterium tumefaciens*-mediated transformation of the aromatic shrub *Lavandula latifolia*. *Molecular Breeding* 6: 539-552.

Newman, J.D., Chappell, J. (1997) Isoprenoid biosynthesis in plants: carbon partitioning within the cytoplasmic pathway. In: *Biochemistry and function of sterols.* Parish EJ, Nes WD, eds. Boca Raton, FL: CRC Press. pp:123-134.

Nielsen, M.T., Akers, C.P., Ja ̈rlfors, U.E., Wagner, G.J., Berger, S. (1991) Comparative ultrastructural features of secreting and nonsecreting glandular trichomes of two genotypes of *Nicotiana tabacum* L. *Botanical gazette* 152: 13-22.

Nobre, J. (1996) *In vitro* cloning and micropropagation of *Lavandula stoechas* from field-grown plants. *Plant Cell, Tissue and Organ Culture* 46: 151-155.

Ohara, K., Matsunaga, E., Nanto, K., Yamamoto, K., Sasaki, K., Ebinuma, H., Yazaki, K. (2010) Monoterpene engineering in a woody plant *Eucalyptus camaldulensis* using a limonene synthase cDNA. *Plant Biotechnology Journal* 8: 28-37.

Okada, K., Kasahara, H., Yamaguchi, S., Kawaide, H., Kamiya, Y., Nojiri, H., Yamane, H. (2008) Genetic evidence for the role of isopentenyl diphosphate isomerases in the mevalonate pathway and plant development in *Arabidopsis*. *Plant and Cell Physiology* 49: 604-616.

Okuhara, M., Kuroda, Y., Goto, T., Okamoto, M., Terano, H., Kohsaka. M., Aoki, H., Imanaka, H. (1980) Studies on new phosphonic acid antibiotics. III. Isolation and characterization of FR-31564, FR-32863 and FR-33289.*The Journal of Antibiotics* 33: 24-28.

Panizza, M., Mensualisodi, A., Tognoni, F. (1993) Role of ethylene in axillary shoot proliferation on Lavandin-interaction with benzyladenine and polyamines. *Journal of Experimental Botany* 44: 387-394.

Panizza, M., Mensualisodi, A., Tognoni, F. (1997) Morphological differentiation in callus cultures of Lavandin: a role for ethylene. *Biologia Plantarum* 39: 481-489.

Panizza, M., Tognoni, F. (1988) Clonal propagation, callus formation and plant

regeneration of Lavandin. *Scientia Horticulturae* 37: 157-163.

Pascual, J., Caballero, E., Caballero, C., Machin, G. (1983) Constituents of the essential oil of *Lavandula latifolia. Phytochemistry* 22: 1033-1034.

Patterson, D.R. (1987) Rohm and Haas Co. US Patent 4693742.

Petersen, S., Graaf, A., Eggeling, L., Mollney, M., Wiechert, W., Sahm, H. (2000) *In vivo* quantification of parallel and bidirectional fluxes in the anaplerosis of corynebacterium glutamicum. *Journal of Biological Chemistry* 275: 35932-35941.

Phillips, M.A., Croteau, R.B. (1999) Resin-based defenses in conifers. *Trends in Plant Science* 4: 184-190.

Phillips, M.A., D'Auria, J.C., Gershenzon, J., Pichersky, E. (2008) The *Arabidopsis thaliana* type I isopentenyl diphosphate isomerases are targeted to multiple subcellular compartments and have overlapping functions in isoprenoid biosynthesis. *The Plant Cell* 20: 677-696.

Pickup, J.F., McPherson, K. (1976) Theoretical considerations in stable isotope dilution mass spectrometry for organic analysis. *Analytical Chemistry* 48: 1885-1890.

Pichersky, E., Gershenzon, J. (2002) The formation and function of plant volátiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology* 5: 237-243.

Possell, M., Ryan, A., Vickers, C.E., Mullineaux, P.M., Hewitt, C.N. (2010) Effects of fosmidomycin on plant photosynthesis as measured by gas exchange and chlorophyll fluorescence. *Photosynthesis Research* 104: 49-59.

Quazi, M.H. (1980) *In vitro* multiplication of *Lavandula* spp. *Annals of Botany* 45: 361-363.

Rauschenbach, P., Schmidt, H. L., Simon, H., Tykva, R., Wenzel, M. (1974) Messung von radioktiven und stabilen Isotopen. Springer Verlag, Berlin.

Re, E.B., Jones, D., Learned, R.M. (1995) Co-expression of native and introduced genes reveals cryptic regulation of HMG-CoA reductase expression in *Arabidopsis. The Plant Journal* 7: 771-784.

Renaud, E.N.C., Charles, D.J., Simon, J.E. (2001) Essential oil quantity and composition from 10 cultivars of organically grown Lavender and Lavandin. *Journal of Essential Oil Research* 13: 269-273.

Roberts, S. (2007) Production and engineering of terpenoids in plant cell culture. *Nature Chemical Biology* 3: 388-395.

Rodríguez-Concepción, M. (2006) Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochemistry Reviews* 5: 1-15.

Rodríguez-Concepción, M., Ahumada, I., Diez-Juez, E., Sauret-Güeto, S., Lois, L.M., Gallego, F., Carretero-Paulet, L., Campos, N., Boronat, A. (2001) 1-Deoxy-d-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening. *The Plant Journal* 27: 213-222.

Rodríguez-Concepción, M., Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiology* 130: 1079-1089.

Rodríguez-Concepción, M., Fores, O., Martinez-García, J.F., Gonzalez, V., Phillips, M.A., Ferrer, A., Boronat, A. (2004) Distinct light-mediated pathways regulate the biosíntesis and exchange of isoprenoid precursors during *Arabidopsis* seedling development. *The Plant Cell* 16: 144-156.

Römisch-Margl, W., Schramek, N., Radykewicz, T., Ettenhuber, C., Eylert, E., Huber, C., Römisch-Margl, L., Schwarz, C., Dobner, M., Demmel, N., Winzenhörlein, B., Bacher, A., Eisenreich, W. (2007) $^{13}CO_2$ as a universal metabolic tracer in isotopologue perturbation experiments. *Phytochemistry* 68: 2273-2289.

Salido, S., Altarejos, J., Nogueras, M., Sánchez, A., Luque, P. (2004) Chemical composition and seasonal variations of spike lavender oil from Southern Spain. *Journal of Essential Oil Research* 16: 206-210.

Sanbrook, J., Russel, D.W. (2001) *Molecular cloning: a laboratory manual*, 3rd ED., Volume 3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sánchez-Gras, M.C., Calvo, M.C. (1996) Micropropagation of *Lavandula latifolia* through nodal bud culture of mature plants. *Plant Cell, Tissue and Organ Culture* 45: 259-261.

Sangwan, N.S., Farooqi, A.H.A., Shabih, F., Sangwan, R.S. (2001) Regulation of essential oil production in plants. *Plant Growth Regulation* 34: 3-21.

Santana, O., Cabrera, R., Giménez, C., González-Coloma, A., Sánchez-Vioque, R., de los Mozos-Pascual, M., Rodríguez-Conde, M.F., Laserna-Ruiz, I., Usano-Alemany, J., Herraiz, D. (2012) Perfil químico y biológico de

aceites esenciales de plantas aromáticas de interés agro-industrial en Castilla-La Mancha (España). *Grasas y Aceites* 63: 214-222.

Sapir-Mir, M., Mett, A., Belausov, E., Tal-Meshulam, T., Frydman, A., Gidoni, D., Eyal, Y. (2008) Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. *Plant Physiology* 148: 1219-1228.

Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., Fisher, E. (2004) The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. *Journal of Biological Chemistry* 279: 6613-6619.

Sauer, U., Hatzimanikatis, V., Bailey, J.E., Hochuli, M., Szyperski, T., Würhrich, K. (1997) Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. *Nature Biotechnology* 15: 448-452.

Schäfer, S., Gotz, M., Eisenreich, W., Bacher, A., Fuchs, G. (1989) ^{13}C-NMR study of autotrophic CO_2 fixation in Thermoproteus neutrophilus. *European Journal of Biochemistry* 184: 151-156.

Schönheimer, R,. Rittenberg, D. (1935) Deuterium as an indicator in the study of intermediary metabolim. *Science* 82: 156-157.

Schuhr, C.A., Radykewicz, T., Sagner, S., Latzel, C., Zenk, M.H., Arigoni, D., Bacher, A., Rohdich, F., Eisenreich, W. (2003) Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. *Phytochemistry Reviews* 2: 3-16.

Schwab, W., Davidovich-Rikanati, R., Lewinsohn, E. (2008) Biosynthesis of plant-derived flavor compounds. *The Plant Journal* 54: 712-732.

Schwender, J. (1995) Untersuchungen zur Biosynthese der Isoprenoide bei der Grünalge *Scenedesmus obliquus* mittels ^{13}C-Isotopenmarkierung. PhD Thesis. Karlsr. Contrib. Plant Physiol. 31:1-85.

Schwender J, Lichtenthaler H.K. (1998) Biosynthesis of lycopene in tomato fruits proceeds via the non-mevalonate isoprenoid pathway. In: *Advances in Plant Lipid Research*. Univ. Sevilla: Secretariado Publ., pp. 429-32.

Schwender J, Zeidler J, Gröner R, Müller C, Lichtenthaler HK (1997b) Incorporation of 1-deoxy-D-xylulose into iso- prene and phytol by higher plants and algae. *FEBS Letters* 414: 129-34.

Schwender, J., Lichtenthaler, H.K., Disch, A., Rohmer, M. (1997a) Biosynthesis

of sterols in green algae (*Scenedesmus, Chlorella*) according to a novel, mevalonate-independent pathway. *Physiology, Biochemistry and Molecular Biology of Plant Lipids* 74: 180-182.

Schwender, J., Lichtenthaler, H.K., Seemann, M., Rohmer, M. (1995) Biosynthesis of isoprenoid chains of chlorophylls and plastoquinone in *Scenedesmus* by a novel pathway. In: Mathis P (ed) *Photosynthesis: From Light to Biosphere.* Kluwer, Amsterdam, pp. 1001-1004

Schwender, J., Seemann, M., Lichtenthaler, H.K., Rohmer, M. (1996) Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophyll and plastoquinone) via a novel pyruvate/glyceralde- hyde-3-phosphate non-mevalonate path- way in the green alga *Scenedesmus*. *Biochemical Journal* 316: 73-80.

Segura, J., Calvo, C. (1991) *Lavandula* spp. (Lavender): *in vitro* culture, regeneration of plants, and the formation of essential oils and pigments. *Biotechnology in Agriculture and Forestry* 15: 283-310.

Shao, M., Ye, Z., Chen, M., Gu, F. (2011) Tissue culture of *Lavandula pinnata* L. *Modern Agricultural Science and Technology* 22.

Shigi, Y. (1989) Inhibition of bacterial isoprenoid synthesis by fosmidomycin, a phosphonic acid-containing antibiotic. *Journal of Antimicrobial Chemotherapy* 24:131-145.

Simon, H., Floss, H. (1967) Anwendung von isotope in der organischen Chemie und Biochemie, Bestimmung der Isotopenverteilung in markierten Verbindungen. Vol I, Springer Verlag, Berlin.

Sitte, P., Weiler, E.W., Kadereir, J.W., Bresinsky, A., Körner, C. (2002) Strasburger. Lehrbuch der Botanik für Hochschulen, 35th edn. Spektrum Akademischer Verlag, Heidelberg.

Strauss, G., Eisenreich, W., Bacher, A., Fuchs, G. (1992) ^{13}C-NMR study of autotrophic CO_2 fixation pathways in the sulfur-reducing *Archaebacterium Thermoproteus neutrophilus* and in the phototrophic *Eubacterium Chloroflexus aurantiacus*. *European Journal of Biochemistry* 205: 853-866.

Sudriá, C., Piñol, M.T., Palazón, J., Cusidó, R.M., Vila, R., Morales, C., Bonfill, M., Cañigueral, S. (1999) Influence of plant growth regulators on the growth and essential oil content of cultured *Lavandula dentata* plantlets. *Plant Cell, Tissue and Organ Culture* 58:177-184.

Suzuki, M., Muranaka, T. (2007) Molecular genetics of plant sterol backbone

syhnthesis. *Lipids* 42: 47-54.

Suzuki, M., Nakagawa, S., Kamide, Y., Ohyama, K., Hashinokuchi, H., Kiuchi, R., Saito, K., Muranaka, T., Nagata, N. (2009) Complete blockage of the mevalonate pathway results in male gametophyte lethality. *Journal of Experimental Botany* 60: 2055-2064.

Suzuki, S., Kamide, Y., Nagata, N., Seki, H., Ohyama, K., Kato, H., Masuda, K., Sato, S., Kato, T., Tabata, S., Yoshida, S., Muranaka, T. (2004) Loss of function of 3-hydroxy-3-methylglutaryl coenzyme A reductase 1 (HMG1) in *Arabidopsis* leads to dwar®ng, early senescence and male sterility, and reduced sterol levels. *The Plant Journal* 37: 750-761.

Taiz, L., Zeiger, E. (2010) *Plant Physiology*, Fourth Edition. Sinauer Associates. Sunderland, MA.

Tang, J., Kobayashi, K., Suzuki, M., Matsumoto, S., Muranaka, T. (2010) The mitochondrial PPR protein LOVASTATIN INSENSITIVE 1 plays regulatory roles in cytosolic and plastidial isoprenoid biosynthesis through RNA editing. *The Plant Journal* 61: 456-466.

Tholl, D. (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Current Opinion in Plant Biology* 9: 297-304.

Trejo-Tapia, G., Castro, C.A., Mendiola, M.R. (2003) Influence of the culture medium constituents and inoculum size on the accumulation of blue pigment and cell growth of *Lavandula spica*. *Plant Cell, Tissue and Organ Culture* 72: 7-12.

Tsuchiya, T., Ishibashi, K., Terakawa, M., Nishiyama, M., Itoh, N., Noguchi, H. (1982) Pharmacokinetics and metabolism of fosmidomycin, a new phosphonic acid, in rats and dogs. *European Journal of Drug Metabolism and Pharmacokinetics* 7: 59-64.

Tsuro, M., Koda, M., Inoue, M. (2000) Efficient plant regeneration from multiple shoot formed in the leaf-derived callus of *Lavandula vera*, using the “open culture system”. *Scientia Horticulturae* 86: 81-88.

Tukey J.W. (1953) Some selected quick and easy methods of statistical analysis. *Transactions of the New York Academy of Sciences* 16: 88-97.

Varona, S., Kareth, S., Martín, A., Cocero, M.J. (2010) Formulation of lavandin essential oil with biopolymers by PGSS for application as biocide in ecological agriculture. *Journal of Supercritical Fluids* 54: 369-377.

Vaucheret, H. (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes & Development* 20: 759-771.

Veau, B., Courtois, M., Oudin, A., Chenieux, J., Rideau, M., Clastre, M. (2000) Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in *Catharanthus roseus*. *Biochimica et Biophysica Acta* 1517: 159-163.

Vercauteren, D., Vandenbroucke, R.E., Jones, A.T., Rejman, J., Demeester, J., DeSmedt, S.C., Sanders, N.N., Braekmans, K. (2009) The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Molecular Therapy* 18: 561-569.

Verlet, N. (1993) Comercial aspects of essencial oil production. In: Volatile Oil Crops: Their Biology, Biochemistry and Production. Hay, R.K.M., Waterman, P.G. (Eds), Longman Scientific and Technical, Essex, pp. 137-174.

Vranová, E., Coman, D., Gruissem, W. (2012) Structure and dynamics of the isoprenoid pathway network. *Molecular Plant* 5: 318-333.

Walter, M.H., Fester, T., Strack, D. (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. *The Plant Journal* 21:571-578.

Walter, M.H., Hans, J., Strack, D. (2002) Two distantly related genes encoding 1-deoxy-D-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. *The Plant Journal* 31:243-254.

Wang, M.B., Metzlaff, M. (2005) RNA silencing and antiviral defense in plants. *Current Opinion in Plant Biology* 8: 216-222.

Wang, X.J., Jin, L.A., Li, M., Zhao, M., Zhao, H., Xu, Y. (2007) Bioreactor culture and plant regeneration from cell clusters of the aromatic plant, *Lavandula angustifolia* 'Munstead'. *Journal of Horticultural Science and Biotechnology* 82: 781-785.

Wassenegger, M., Krczal, G. (2006) Nomenclature and functions of RNA-directed RNA polymerases. *Trends in Plant Science* 11: 142-151.

Webb, J.K., Banthorpe, D.V., Watson, D.G. (1984) Monoterpene synthesis in shoot regenerated from callus cultures. *Phytochemistry* 23: 903-904.

Werner, I., Bacher, A., Eisenreich, W. (1997) Retrobiosynthetic NMR studies

with ^{13}C-labeled glucose formation of gallic acid in plants and fungi. *The Journal of Biological Chemistry* 272: 25474-25482.

Wildung, M.R., Croteau, R.B. (2005) Genetic engineering of peppermint for improved essential oil composition and yield. *Transgenic Research* 14: 365-372.

Wise, M.L., Croteau, R. (1999) Monoterpene biosynthesis. Comprehensive Natural Products Chemistry: Isoprenoids. Oxford, UK: Elsevier Science; pp, 97-153.

Woronuk, G., Demissie, Z., Rheault, M., Mahmoud, S. (2011) Biosynthesis and therapeutic properties of *Lavandula essential* oil constituents. *Planta Medica* 77: 7- 15.

Wu, S., Schalk, M., Clark, A., Miles, R.B., Coates, R., Chappel, J. (2006) Redirecting of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. *Nature Biotechnology* 24: 1441-1447.

Xing, S., Miao, J., Li, S., Qin, G., Tang, S., Li, H., Gu, H., Qu, L. (2010) Disruption of the *1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)* gene results in albino, dwarf and defects in trichome initiation and stomata closure in *Arabidopsis*. *Cell Research* 20: 688-700.

Yoonram, K., Takahashi, S., Rattanapittayaporn, A., Koyama, T., Wititsuwannakul, D., Wititsuwannakul, D. (2008) cDNA, from Hevea brasiliensis latex, encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase. *Plant Science* 175: 694-700.

Zar, J.H. (1996) Biostatistical analysis. Prentice-Hall, New Jersey, USA.

Zeidler, J., Lichtenthaler, H.K., May, H.U., Lichtenthaler, F.W. (1997) Is isoprene emitted by plants synthesized via the novel isopentenyl pyrophosphate pathway? *Zeitschrift für Naturforschung* 52: 15-23.

Zuzarte, M.R., Dinis, A.M., Cavaleiro, C., Salgueiro, L.R., Canhoto, J.M. (2010) Trichomes, essential oils and in vitro propagation of *Lavandula pedunculata* (Lamiaceae). *Industrial Crops and Products* 32:580-587.

Appendix

Appendix I: Experimental overview *ex vitro* culture, preliminary experiments.

Sample	Plant material	Pulse period (hours)	Chase period (hours)	$^{13}CO_2$ (ml)	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
EV1	Pots, fresh leaves	6.73	14	200	128.8	1.58	-
EV2	Pots, fresh leaves	6.73	14	200	126.9	4.47	-
EV3	Pots, fresh leaves	6.73	14	200	130.3	3.47	-
EV4	Pots, fresh leaves	6.73	64	200	124	1.40	-
EV5	Pots, fresh leaves	6.73	64	200	124.8	1.07	-
EV6	Pots, fresh leaves	6.73	64	200	121.9	1.04	-
EV7	Pots, fresh leaves	8.84	40	300	131.8	1.81	-
EV8	Pots, fresh leaves	8.84	40	300	121.7	1.42	-
EV9	Pots, fresh leaves	8.84	40	300	156.4	4.73	-
EV10	Pots, fresh leaves	6.73	156	200	173.1	2.13	-
EV11	Pots, fresh leaves	6.73	156	200	159.7	2.23	-
EV12	Pots, fresh leaves	6.73	156	200	180.8	4.03	-
EV13	Pots, fresh leaves	8.84	132	300	184.5	4.74	-
EV14	Pots, fresh leaves	8.84	132	300	139.4	8.49	-
EV15	Pots, fresh leaves	8.84	132	300	152.6	4.07	-
EV16	Pots, fresh leaves	5.00	96	?	121.3	3.33	-

Appendix I: Experimental overview *ex vitro* culture, preliminary experiments.

Sample	Plant material	Pulse period (hours)	Chase period (hours)	$^{13}CO_2$ (ml)	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
EV17	Pots, fresh leaves	5.00	96	?	138.3	4.67	-
EV18	Pots, fresh leaves	5.00	96	?	160.8	5.01	-
EV19	Pots, fresh leaves	3.79	0	?	142.6	0.53	-
EV20	Pots, fresh leaves	3.79	0	?	152.3	0.58	-
EV21	Pots, fresh leaves	3.79	0	?	132	0.38	-
EV30	Pots, fresh leaves	8.84	240	300	500	13.02	13.73
EV31	Pots, fresh leaves	5.00	168	225	400	3.00	2.69
EV32	Pots, fresh leaves	5.21	192	161	400	7.54	7.32
EV33	Pots, fresh leaves	2.00	0	?	400	0.11	0.1
EV34	Pots, fresh leaves	2.00	0	?	400	0.16	0.15
EV35	Pots, fresh leaves	1.00	0	?	400	0.04	0.09
EV36	Pots, fresh leaves	1.00	0	?	400	0.02	0.28
EV37	Pots, fresh leaves	1.00	3	?	380	0.02	0.24
EV38	Pots, fresh leaves	1.00	20.5	?	400	0.05	0.09
EV39	Pots, fresh leaves	1.00	20.5	?	400	0.01	0.28
EV40	Pots, fresh leaves	1.00	26.5	?	400	0.01	0.03

Appendix I: Experimental overview *ex vitro* culture, preliminary experiments.

Sample	Plant material	Pulse period (hours)	Chase period (hours)	$^{13}CO_2$ (ml)	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
EV41	Pots, fresh leaves	1	26.5	?	400	0.01	0.39
EV42	Pots, fresh leaves	5	264	225	403	3.86	3.95
EV43	Pots, fresh leaves	5.21	240	161	403	2.66	2.63
EV44	Pots, fresh leaves	3.74	0	127	397	0.08	0.11
EV45	Pots, fresh leaves	3.74	0	127	400	0.13	0.21
EV46	Pots, fresh leaves	3.74	71	127	393	0.05	0.08
EV47	Pots, fresh leaves	3.74	71	127	393	0.03	0.11
EV48	Pots, fresh leaves	5.1	16	167.5	410	1.20	1.97
EV49	Pots, fresh leaves	5.1	16	167.5	417	1.97	2.55
EV50	Pots, fresh leaves	5.1	40	167.5	410	1.04	1.14
EV51	Pots, fresh leaves	5.1	40	167.5	417	0.09	0.17
EV52	Pots, fresh leaves	5.1	64	167.5	400	0.05	0.02
EV53	Pots, fresh leaves	5.1	64	167.5	400	0.09	0.20
EV54	Pots, fresh leaves	4.92	0	162.5	413	0.24	0.47
EV55	Pots, fresh leaves	4.92	95	162.5	413	8.33	12.35
EV55-2	Pots, fresh leaves	4.92	95	162.5	1090	-	-
EV56	Pots, fresh leaves	4.92	119	162.5	403	0.07	0.01
EV57	Pots, fresh leaves	4.92	139.5	162.5	403	0.05	0.04
EV58	Pots, fresh leaves	4.92	408	162.5	393	-	-
EV59	Pots, fresh leaves	4.92	408	162.5	1100	-	-

Appendix II : Experimental overview *in vitro* cultures.

Sample	Plant material	Pulse period (days)	Culture	Line	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
IV2	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	348.1	5.84	4.83
IV3	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	360.3	5.48	4.57
IV4	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	356	6.02	6.53
IV5	Whole Seedlings, pulver	22	Liquid, U-$^{13}C_6$-glucose	WT	316.9	11.18	8.90
IV6	Whole Seedlings, pulver	22	Liquid, U-$^{13}C_6$-glucose	WT	256.7	11.21	8.55
IV7	Whole Seedlings, pulver	22	Liquid, U-$^{13}C_6$-glucose	WT	350.6	17.08	14.32
IV8	Whole Seedlings	22	Liquid, U-$^{13}C_6$-glucose	WT	278.4	10.75	11.09
IV9	Whole Seedlings	22	Liquid, U-$^{13}C_6$-glucose	WT	253.6	12.3	8.37
IV10	Whole Seedlings	22	Liquid, U-$^{13}C_6$-glucose	WT	255.9	10.53	11.19
IV11	Whole Seedlings	22	Liquid, U-$^{13}C_6$-glucose	WT	215.1	16.04	14.77
IV12	Whole Seedlings	22	Liquid, U-$^{13}C_6$-glucose	WT	265.3	16.82	8.70
IV13	Whole Seedlings	22	Liquid, U-$^{13}C_6$-glucose	WT	263.4	13.59	9.62
IV14	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	420	5.76	5.56
IV15	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	727	6.05	5.02
IV800	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	800	5.34	5.41
IV950	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	950	5.85	6.10
IV1000	Whole Seedlings	55	Solid, U-$^{13}C_6$-glucose	WT	1000	4.93	5.05

Appendix II : Experimental overview *in vitro* cultures.

Sample	Plant material	Pulse period (days)	Culture	Line	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
W7	Whole Plantlets	7	Solid, U-$^{13}C_6$-glucose	WT	499	2.28	2.98
W7	Whole Plantlets	7	Solid, U-$^{13}C_6$-glucose	WT	98	3.38	1.30
W7	Whole Plantlets	7	Solid, U-$^{13}C_6$-glucose	WT	64	4.58	0.93
H7	Whole Plantlets	7	Solid, U-$^{13}C_6$-glucose	HMGR5	353	0.96	1.04
H7	Whole Plantlets	7	Solid, U-$^{13}C_6$-glucose	HMGR5	69	2.44	2.48
H7	Whole Plantlets	7	Solid, U-$^{13}C_6$-glucose	HMGR5	88	1.07	3.89
W14	Whole Plantlets	14	Solid, U-$^{13}C_6$-glucose	WT	928	0.72	0.73
W14	Whole Plantlets	14	Solid, U-$^{13}C_6$-glucose	WT	258	2.47	1.50
W14	Whole Plantlets	14	Solid, U-$^{13}C_6$-glucose	WT	181	2.11	3.93
H14	Whole Plantlets	14	Solid, U-$^{13}C_6$-glucose	HMGR5	986	0.86	1.03
H14	Whole Plantlets	14	Solid, U-$^{13}C_6$-glucose	HMGR5	213	5.89	1.79
H14	Whole Plantlets	14	Solid, U-$^{13}C_6$-glucose	HMGR5	183	1.80	2.13

Appendix II : Experimental overview *in vitro* cultures.

Sample	Plant material	Pulse period (days)	Culture	Line	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
W21	Whole Plantlets	21	Solid, U-$^{13}C_6$-glucose	WT	958	0.92	0.69
W21	Whole Plantlets	21	Solid, U-$^{13}C_6$-glucose	WT	302	3.41	2.32
W21	Whole Plantlets	21	Solid, U-$^{13}C_6$-glucose	WT	442	1.45	2.23
H21	Whole Plantlets	21	Solid, U-$^{13}C_6$-glucose	HMGR5	1167	1.35	1.09
H21	Whole Plantlets	21	Solid, U-$^{13}C_6$-glucose	HMGR5	351	0.96	0.92
H21	Whole Plantlets	21	Solid, U-$^{13}C_6$-glucose	HMGR5	394	1.50	1.05
W28	Whole Plantlets	28	Solid, U-$^{13}C_6$-glucose	WT	1624	1.82	0.97
W28	Whole Plantlets	28	Solid, U-$^{13}C_6$-glucose	WT	478	1.34	1.61
W28	Whole Plantlets	28	Solid, U-$^{13}C_6$-glucose	WT	420	0.89	0.72
H28	Whole Plantlets	28	Solid, U-$^{13}C_6$-glucose	HMGR5	378	1.73	1.53
H28	Whole Plantlets	28	Solid, U-$^{13}C_6$-glucose	HMGR5	494	1.59	1.66
H28	Whole Plantlets	28	Solid, U-$^{13}C_6$-glucose	HMGR5	1297	1.58	1.41

Appendix II : Experimental overview *in vitro* cultures.

Sample	Plant material	Pulse period (days)	Culture	Line	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
C7	Whole Plantlets	7	Solid, Control	WT	512	1.34	0.11
C7	Whole Plantlets	7	Solid, Control	WT	457	0.04	0.16
C7	Whole Plantlets	7	Solid, Control	WT	177	0.00	0.01
M7	Whole Plantlets	7	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	663	0.11	0.24
M7	Whole Plantlets	7	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	115	0.05	0.18
M7	Whole Plantlets	7	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	202	0.1	0.17
F7	Whole Plantlets	7	Solid, FSM	WT	225	0.21	0.3
F7	Whole Plantlets	7	Solid, FSM	WT	243	0.19	0.00
F7	Whole Plantlets	7	Solid, FSM	WT	546	0.29	0.16
FM7	Whole Plantlets	7	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	547	0.29	0.17
FM7	Whole Plantlets	7	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	153	0.19	0.05
FM7	Whole Plantlets	7	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	180	0.34	0.18

Appendix II : Experimental overview *in vitro* cultures.

Sample	Plant material	Pulse period (days)	Culture	Line	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
C14	Whole Plantlets	14	Solid, Control	WT	414	0.03	0.07
C14	Whole Plantlets	14	Solid, Control	WT	485	0.00	0.03
C14	Whole Plantlets	14	Solid, Control	WT	1763	0.91	1.34
M14	Whole Plantlets	14	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	414	0.01	0.04
M14	Whole Plantlets	14	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	462	0.11	0.2
M14	Whole Plantlets	14	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	1279	0.13	0.14
F14	Whole Plantlets	14	Solid, FSM	WT	183	1.65	0.09
F14	Whole Plantlets	14	Solid, FSM	WT	347	0.34	0
F14	Whole Plantlets	14	Solid, FSM	WT	1049	-	1.11
FM14	Whole Plantlets	14	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	286	1.36	0.05
FM14	Whole Plantlets	14	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	321	0.93	0.04
FM14	Whole Plantlets	14	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	936	0.88	0.06

Appendix II : Experimental overview *in vitro* cultures.

Sample	Plant material	Pulse period (days)	Culture	Line	mg material	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
C21	Whole Plantlets	21	Solid, Control	WT	713	0.00	0.01
C21	Whole Plantlets	21	Solid, Control	WT	774	0.05	0.1
C21	Whole Plantlets	21	Solid, Control	WT	1909	0.00	0.02
M21	Whole Plantlets	21	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	412	0.01	0.01
M21	Whole Plantlets	21	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	440	0.00	0.00
M21	Whole Plantlets	21	Solid, C_4C_5-$^{13}C_2$-mevalonate	WT	1779	0.00	0.00
F21	Whole Plantlets	21	Solid, FSM	WT	402	1.23	0.21
F21	Whole Plantlets	21	Solid, FSM	WT	516	1.36	0.02
F21	Whole Plantlets	21	Solid, FSM	WT	1124	1.71	0.17
FM21	Whole Plantlets	21	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	327	1.74	0.09
FM21	Whole Plantlets	21	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	425	1.27	0.52
FM21	Whole Plantlets	21	Solid, FSM + C_4C_5-$^{13}C_2$-mevalonate	WT	935	1.87	0.06

Appendix III: Experimental overview *ex vitro* WT and HMGR5 cultures.

Line	Plant material	Pulse period (hours)	Chase period (days)	Camphor excess ^{13}C abundance percentage	Cineol excess ^{13}C abundance percentage
HMGR5	Pots, fresh leaves	5	4	0.20	0.18
HMGR5	Pots, fresh leaves	5	4	0.02	0.02
HMGR5	Pots, fresh leaves	5	4	0.11	0.12
HMGR5	Pots, fresh leaves	5	7	0.02	0.04
HMGR5	Pots, fresh leaves	5	7	0.02	0.01
HMGR5	Pots, fresh leaves	5	10	0.30	0.19
HMGR5	Pots, fresh leaves	5	10	0.14	0.15
HMGR5	Pots, fresh leaves	5	10	0.15	0.16
WT	Pots, fresh leaves	5	4	0.03	0.04
WT	Pots, fresh leaves	5	11	0.44	0.37
WT	Pots, fresh leaves	5	11	0.15	0.10
WT	Pots, fresh leaves	5	11	0.83	0.93