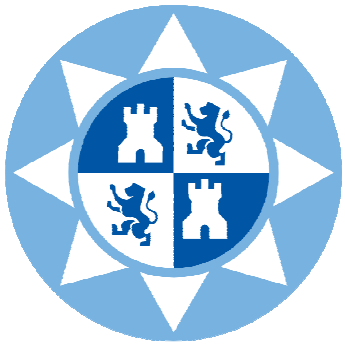


UNIVERSIDAD POLITÉCNICA DE CARTAGENA
DEPARTAMENTO DE CIENCIA Y TECNOLOGÍA AGRARIA

GENETIC TRANSFORMATION AND ELICITATION TO OBTAIN MEDICINAL COMPOUNDS IN GRAPEVINE (*Vitis vinifera* L.) AND IN *Bituminaria bituminosa* (L.) STIRT.

María Pazos Navarro

2012

UNIVERSIDAD POLITÉCNICA DE CARTAGENA
DEPARTAMENTO DE CIENCIA Y TECNOLOGÍA AGRARIA

GENETIC TRANSFORMATION AND ELICITATION TO OBTAIN MEDICINAL COMPOUNDS IN GRAPEVINE (*Vitis vinifera* L.) AND IN *Bituminaria bituminosa* (L.) STIRT.

María Pazos Navarro

Directora
Mercedes Dabauza Micó

2012

Universidad Politécnica de Cartagena

DT-12

CONFORMIDAD DE SOLICITUD DE AUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR LA DIRECTORA DE LA TESIS

Dª. Mercedes Dabauza Micó Directora de la Tesis Doctoral "Aplicación de la transformación genética y métodos de elicitación para la sobre-producción de compuestos de interés medicinal en vid (*Vitis vinifera* L.) y en *Bituminaria bituminosa* (L.) Stirt."

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por Dª. María Pazos Navarro, dando mi conformidad para que sea presentada ante la Comisión de Doctorado.

La rama de conocimiento por la que esta tesis ha sido desarrollada es:

- [x] Ciencias
- [ ] Ciencias Sociales y Jurídicas
- [ ] Ingeniería y Arquitectura

En Cartagena, a 6 de marzo de 2012

LA DIRECTORA DE LA TESIS

Mercedes Dabauza

Fdo.: Mercedes Dabauza Micó

COMISIÓN DE DOCTORADO

Universidad Politécnica de Cartagena

DT-12

CONFORMIDAD DE SOLICITUD DEAUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL TUTOR DE LA TESIS

D. Antonio A. Calderón García, Tutor de la Tesis doctoral Aplicación de la transformación genética y métodos de elicitación para la sobre producción de compuestos de interés medicinal en vid (*Vitis vinifera* L.) y en *Bituminaria bituminosa* (L.) Stirt.

INFORMA:

Que la referida Tesis Doctoral, ha sido realizada por Dª. María Pazos Navarro, dando mi conformidad para que sea presentada ante la Comisión de

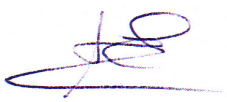
Doctorado.

La rama de conocimiento por la que esta tesis ha sido desarrollada es:

- [X] Ciencias
- [ ] Ciencias Sociales y Jurídicas
- [ ] Ingeniería y Arquitectura

En Cartagena, a 6 de marzo de 2012

TUTOR DE LA TESIS



Fdo.: Antonio Calderón

COMISIÓN DE DOCTORADO

Universidad Politécnica de Cartagena

DT-13

CONFORMIDAD DE DEPOSITO DE TESIS DOCTORAL POR LA COMISIÓN ACADÉMICA DEL PROGRAMA

D Francisco Artés Hernández, Presidente de la Comisión Académica del Programa Técnicas Avanzadas en Invstigación y Desarrollo Agrario y Alimentario.

INFORMA:

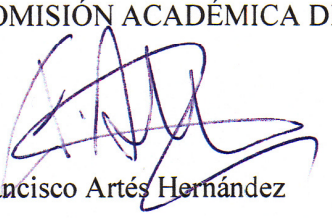
Que la Tesis Doctoral titulada, "Aplicación de la transformación genética y métodos de elicitación para la sobreproducción de compuestos de interés medicinal en vid (*Vitis vinifera* L.) y en *Bituminaria bituminosa* (L.) Stirt.", ha sido realizada por Dª. María Pazos Navarro, bajo la dirección y supervisión de la Dra. Mercedes dabauza Micó, dando su conformidad a la misma la Comisión Académica, con la finalidad de que sea presentada ante la Comisión de Doctorado.

La Rama de conocimiento por la que esta tesis ha sido desarrollada es:

☒Ciencias
☐Ciencias Sociales y Jurídicas
☐Ingeniería y Arquitectura

En Cartagena, a 6 de marzo de 2012

EL PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA



Fdo: Francisco Artés Hernández

COMISIÓN DE DOCTORADO

ACKNOWLEDGEMENTS

Me gustaría dar las gracias a todas aquellas personas que han tenido algo que ver en la realización de esta tesis, ya sea de manera directa o indirecta. Espero no olvidar mencionar a nadie…

Primero de todo, quiero agradecer a mi directora de tesis, la Dra. Mercedes Dabauza, su esfuerzo y paciencia durante la realización de esta tesis. Al final de todo seguimos llevándonos muy bien, y puedo decir que además de una gran directora de tesis, es una muy buena amiga. Muchas gracias por todo.

Elena, Domingo y Antonio muchas gracias por esos viajes a Cartagena a las clases del Master. Entre todos hacíamos menos aburridos esos viajes.

No puedo olvidarme del Equipo de Fruticultura del IMIDA; que puedo decir de ell@s: Pepe Cos y Antonio Carrillo, lo que me he reido y lo bien que me lo he pasado con vosotros emasculando flores; muchísimas gracias por esos buenos recuerdos, hacéis un buen tándem, seguid así. Marga, amiga mía, después de tantos años creo que nos lo hemos dicho casi todo; así que solo te digo que ¡dentro de poco te tocará a ti! Ten paciencia. Marcos, comencé siendo tu sombra en el laboratorio, y al final no he salido del todo mal (jejejeje…) gracias por ser buen amigo y compañero. Diego Frutos, Alfonso, Goyo y Belén muchas gracias por los buenos momentos que hemos pasado juntos.

Gracias a Adrián, Pascual y Vicente por facilitarnos el trabajo, con iniciativa y buenas ideas. Sois los mejores técnicos del IMIDA.

Me gustaría agradecer a David Walker su paciencia en la revisión de este manuscrito y su “buen” Inglés.

A Enrique Correal le agradezco su apoyo y predisposición para ayudarnos tanto en el desarrollo de esta tesis como en proyectos futuros. Es una suerte contar con usted.

José Antonio del Río y Ana Ortuño, muchísimas gracias por vuestra ayuda, por los análisis y por poner vuestro laboratorio a nuestra disposición. Licinio, a ti también muchas gracias por enseñarme a preparar las muestras.

Leonor muchas gracias por darme buenas ideas para que no me perdiese en el maravilloso mundo de los marcadores moleculares.

A Almudena le agradezco esos momentos en los que me encontraba aburrida y/o agobiada y podía ir a hablar con ella.

Carlos, no me olvido de ti, gracias por esos ratos de conversación en el laboratorio. Y por cierto, todavía no he cobrado los honorarios por ser tu secretaria.

A Las Pilares (Pilar Flores y Pilar Hellín), gracias por colaborar con nosotras y por vuestra dulzura.

No puedo olvidarme de mi tutor Antonio Calderón, muchísimas gracias por echarme una mano siempre que lo he necesitado.

A Marcos Egea muchas gracias por solucionarme todas las dudas en el Master y Doctorado. Siempre te has preocupado por conseguir lo que crees que es justo y beneficioso para tus alumnos.

Quiero agradecerle a Fco. Torrella su colaboración, valoro mucho su paciencia…hacía muchos años de sus clases de Microbiología.

Daniel Real y Matt Nelson, gracias a vosotros además de aprender mucho sobre genómica y genética pude disfrutar de una experiencia maravillosa en Australia. Es un lujo poder trabajar con vosotros.

Janine Croser, muchísimas gracias por abrirnos las puertas de tu casa, por organizar ese magnífico viaje a Margaret River, y hacer que ese último fin de semana en Australia fuese inolvidable.

Natasha Teakle, Caroline Snowball y Junko, muchas gracias por ayudarme en el laboratorio. Al principio nos costó comunicarnos pero siempre estabais allí para ayudarme.

Gracias al Servicio de Microscopía del SACE (Universidad de Murcia) y a la Colección Española de Cultivo Tipo (CECT) por su ayuda en la caracterización de los microorganismos estudiados en este tesis.

Gracias a la Universidad Politécnica de Cartagena por su gestión y buena organización.

Gracias al Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario y a la Consejería de Educación y Ciencia de la Región de Murcia (Proyecto BIO-AGR06/03-0006) por concederme la beca gracias a la cual ha sido posible la realización de esta tesis.

Por supuesto no puedo olvidarme de mi familia, gracias por vuestro esfuerzo, apoyo y paciencia.

Pensaba que serían unos agradecimientos cortos, pero me he tomado al pie de la letra ese refrán que dice “Es de bien nacidos, ser agradecidos”.

Muchísimas gracias a tod@s, si me he olvidado de alguien espero que no me lo tome en cuenta.

María Pazos Navarro

ABSTRACT

Plants have been used for centuries in traditional medicine due to the properties of their secondary metabolites (SMs) as pharmaceuticals, cosmetics, chemicals or nutraceuticals. For a long time, the production of SMs has only been achieved through the field cultivation of medicinal plants. However, in most cases the active natural product is present at low levels, or is accumulated only in a specific tissue and at a specific vegetative growth stage or under certain environmental conditions, which complicates its extraction. Furthermore, collecting material from the wild is not always feasible and the over-collection could provoke the habitat destruction. Plant Biotechnology is an alternative to produce SMs on a large-scale, and to ensure the conservation and multiplication of interesting plants with active molecules.

The phenolic compounds *trans*-resveratrol and furanocoumarins are among the relevant SMs reported as interesting for human health. Resveratrol exhibits antioxidant and anti-carcinogenic properties and prevents cardiovascular diseases. Furanocoumarins are used for the treatment of skin disorders (psoriasis and vitiligo), and show anti-mutagenic and anti-microbial activities. Two different natural sources of these compounds are known: Grapevine (*Vitis vinifera* L.) and *Bituminaria bituminosa* (L.) Stirt. (Fabaceae) as *trans*-resveratrol and furanocoumarin producers respectively. Genomic and plant tissue culture techniques have been developed for grapevine but not for *B. bituminosa.*

In the present thesis, biotechnological tools have been successfully developed for the production of those SMs: (1) genetic modification with the *stilbene synthase* (*Vst1*) gene and plant regeneration of grapevine (cv. Sugraone) for over-producing *trans*-resveratrol and (2) plant tissue culture and elicitation techniques for furanocoumarin over-production, and genetic tools for genetic variability studies in *B. bituminosa*.

INDEX

INTRODUCTION

INTRODUCTION

Plants produce a huge and diverse collection of organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances, traditionally referred to as secondary metabolites (SMs), have effect on ecological interactions between the plant and its environment, and often are differentially distributed among limited taxonomic groups within the plant kingdom. Each plant family, genus, and species produces a characteristic mix of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants. The primary metabolites (PMs), in contrast, such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids, are found in all plants and participate in nutrition and essential metabolic processes inside the plant (Wink 1999).

Based on their biosynthetic origins, plant natural products (PMs and SMs) can be divided into three major groups: (i) phenolic and polyphenolic compounds that are formed by either the shikimic acid pathway or the malonate/ acetate pathway; (ii) terpenoids (or isoprenoids), including PMs and more than 25,000 SMs; and (iii) nitrogen- (i.e. alkaloids) and sulphur-containing compounds that are biosynthesized principally from amino acids (Wink 1999).

SMs play an important role in plant defence. Plants in their natural environment are challenge by large number of pathogens (fungi, bacteria or virus) (Jeandet *et al* 2002), and even by other plants. Plants respond to infection by accumulating low-molecular-weight antimicrobial metabolites called phytoalexins. The phytoalexins are generally lipophilic substances, and they often accumulate at infection sites to concentrations which are inhibitory to the development of fungi and bacteria (Kúc and Rush 1985). Moreover, SMs may also be involved in attraction of animals for pollination or seed dispersal, and have other functions as antioxidants: (i) preventing the destruction of cell membranes, (ii) maintaining the operation of cellular organelles, (iii) increasing the chlorophyll content and (iv) delaying the plant senescence caused or induced by pathogens. In addition, some SMs exhibit physiological functions such as mobile-toxic nitrogen transport, compound storage, and phenolics like flavonoids may function as UV-protectants (Wink 1999).

Besides the ecological defence properties and their implications in plant fitness, SMs may be beneficial for human health as pharmaceuticals (Terryn *et al*. 2006). Rout *et al*. (2000) reported that in the order of 40% or more of the pharmaceuticals currently used, are derived, at least partially, derived from natural sources. This involves cultivation of plants, harvesting the desired parts, and extraction and purification of the desired product. This conventional method has a number of limitations such as plants are often not readily available because of geographical or governmental restrictions, requires huge area of land and it is a labour-intensive and time-consuming process. Moreover, plants are often difficult to cultivate, become endangered because of over-harvesting (Namdeo 2007) and the accumulation of SMs is often limited to certain tissues or cell types and regulated by environmental or developmental factors. By other hand, the production of SMs by total or partial chemical synthesis is not always economically feasible because of their highly complex structures and stereochemical requirements (Terryn *et al*. 2006).

Plant Biotechnology, in its broadest sense, offers multiple methods (including plant tissue culture, cell biology, and molecular biology) for conservation and commercial propagation of valuable plants for pharmaceutical industry (Wink 1999), and for the large-scale production of interesting SMs.

1. Plant tissue culture techniques applied to Secondary Metabolite production

Numerous factors influence the success of establishing *in vitro* plant cultures, such as plant growth regulators or the plant genotype. Different studies that involve screening of SM hyper-producing plants, establishment of the *in vitro* culture medium and conditions, and genetic stability analyses of cells and regenerated plants must be carried out. Moreover, it must be taken into account that *in vitro* cultures (calli, cell suspensions, organs and regenerated plants) could show somaclonal variation due to the spontaneous mutations that might occur during the sub-culture cycles and produce structural and physiological changes. So, finally, the evaluation of SM content in cells, calli and regenerated plants is necessary to determine the genetic stability of the culture.

Among all plant tissue culture techniques, the development of **micropropagation** protocols through rapid proliferation of shoot-tips and axillar buds (Figure 1A), yield plants genetically identical to the donor plants ensuring the conservation and commercial propagation of plants with interesting SMs (Rout *et al*. 2000). For instance, micropropagation protocols have been developed (Faisal and Anis 2005) to regenerate *Ruta graveolens* plants producing furanocoumarins (FCs). Even, an increase in FC production in micropropagated shoots has been achieved by optimizing the *in vitro* culture conditions (Ekiert *et al*. 2001; Massot *et al*. 2000).

When the elite genotype has been *in vitro* established, other biotechnological tools may be applied to produce SMs, such as the induction of undifferentiated cell growth (callus or cell suspensions), accomplished by the differential application of growth regulators and the control of conditions in the culture medium (Rout *et al*. 2000). Once calli are obtained, they can undergo somaclonal variation and SM production is often variable from one sub-cultured cycle to another, but after a period of time (from several weeks to several years) genetic stability occurs and each callus can be considered as homogeneous cell aggregate (Bourgaud *et al*. 2001). When genetic stability is reached, cell suspension cultures from selected calli, can be established for producing SMs (Bourgaud *et al*. 2001).

With the stimulus of endogenous growth substances or by the addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced (Rout *et al*. 2000). The development of plant regeneration protocols by organogenesis (Figure 1B) or somatic embryogenesis could ensure the conservation and commercial propagation of interesting SM producing plants and could be an alternative to cell cultures for the production of secondary products (Baskaran and Jayabalan 2009a, b, c; Ahmad *et al*. 2010). The organized cultures present a cellular organization level higher than that of calli or cell suspensions. Diwan and Malpathak (2010) observed an increase in the FC synthesis as a function of the cell organization level in *R. graveolens* cultures, maybe due to a development of specialised sites of FC accumulation. According to Subroto *et al*. (1996), syntheses of some SMs are specifically located in organs like roots or shoots. In all cases, the

production of SMs in regenerated organs or plants needs to be analysed (Baskaran *et al*. 2011).

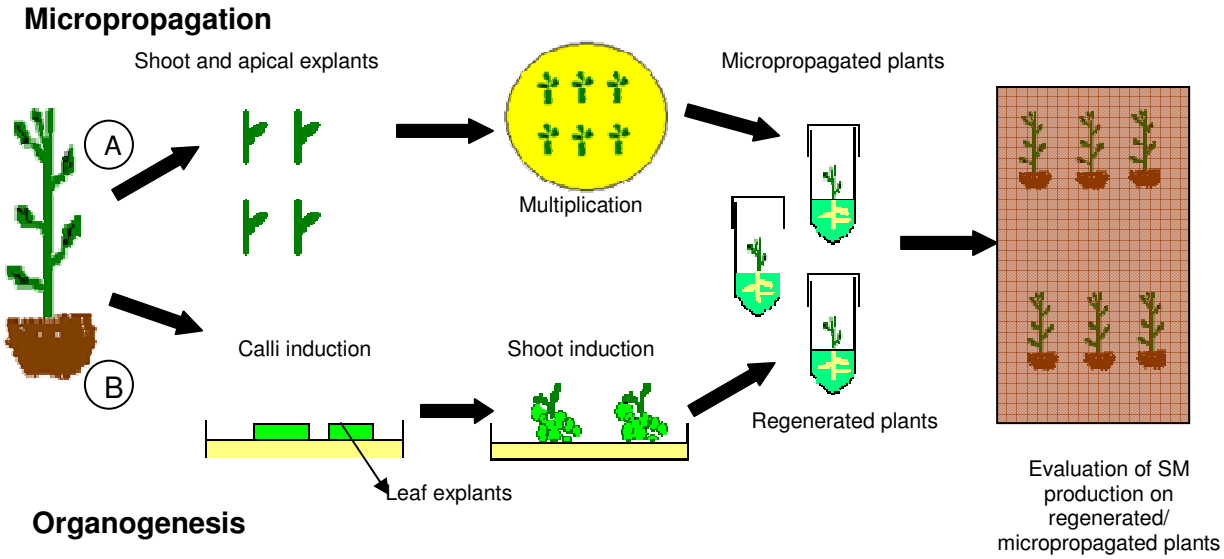


Figure 1. Plant cell tissue culture techniques for secondary metabolite (SM) production by (A) Micropropagation and (B) Organogenesis.

Hairy root cultures (Figure 2) could also represent a valuable system for the production of plant SMs (Zhou *et al*. 2011). These cultures are obtained after the successful transformation of a plant tissue with *Agrobacterium rhizogenes*. Their advantages include relatively-fast growth rate (in hormone-free media), genetic and biochemical stability, and capacity for organogenesis-associated synthesis of metabolites (Sevon and Oksman-Caldentey 2002). One problem is that not all natural products are able to accumulate in these cultures (Kim *et al*. 2002). This system has often been used, because it gives a lot of material easy to maintain, can grow in liquid media (Donnez *et al*. 2009), and offers a scalable and continuous product recovery platform for naturally-derived SM. Hairy root system for psoralen production has been achieved in *Psoralea corylifolia* (Baskaran and Jayabalan 2009d) and in *Ammi majus* (Królicka *et al*. 2001).

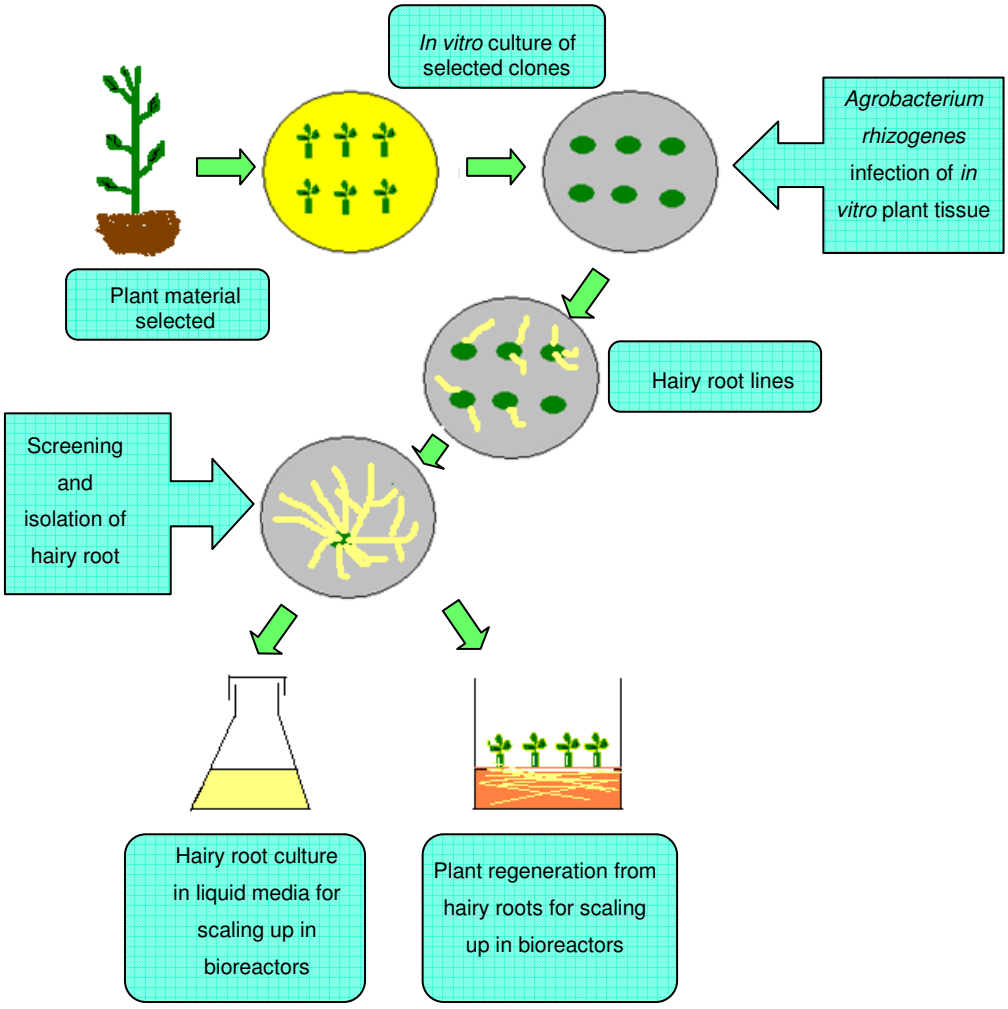


Figure 2. Scheme of hairy root induction and biotechnological applications

To enhance the production of SMs, elicitation methods may be used for the *in vitro* cultures. When plants are infected by pathogenic microorganisms, respond with rapid activation of various spatially and temporally regulated defence reactions inducing synthesis of SMs. Other stress factors like UV-irradiation, osmotic shock, fatty acids, inorganic salts or heavy metals also stimulate the production of SMs. Both biotic and abiotic stresses are termed as elicitors and induce product accumulation, not only in intact plants or plant organs, but also in plant cell cultures as a result of their defensive, protective or offensive reactions. This technique has been developed in different species to enhance SM production, for instance, the synthesis of *trans*-resveratrol in elicitated cell suspension cultures of *Vitis vinifera* (Belchí-Navarro *et al*. 2012; Delaunois *et al*. 2009), and the FC production in elicitated cell suspension

cultures of *R. graveolens* (Austin and Brown 1973) and *A. majus* (Hamerski and Matern 1988).

Plant genetic engineering has already been used for the production of SMs (Delaunois *et al.* 2009). Gene transfer enables the introduction of foreign genes (transgenes), specifically designed hybrid genes from the sexually-compatibility crop (intragenes), or native gene with its own promoter and terminator (cisgenes) into host plant genomes, creating novel varieties with specific characteristics, including resistance to pests, diseases, environmental stress, and production of bioactive substances (Couthos-Tévenot *et al*. 2001; Dhekney *et al*. 2011; Fan *et al*. 2007; Jacobsen and Schouten 2008; Lièvre *et al*. 2005; Rommens *et al*. 2007) (Figure 3).

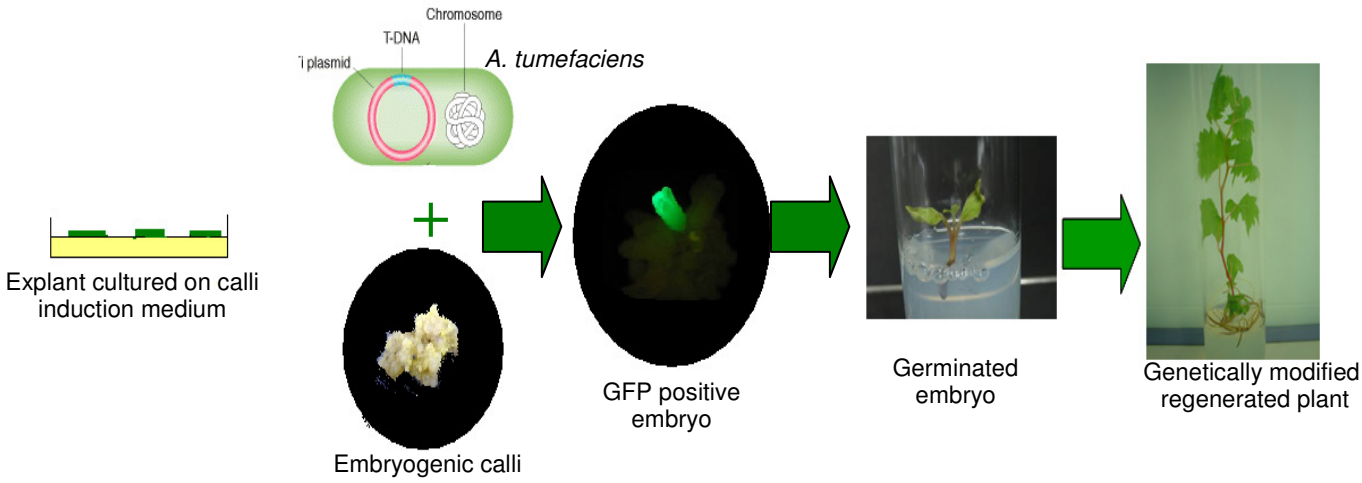


Figure 3. Scheme of a genetic transformation protocol for embryogenic calli, involving *Agrobacterium tumefaciens* and plant regeneration

The use of cisgenes is a real new alternative for traditional and for genetic transformation breeding, particularly in vegetative crops, and a realistic option that could involve a change in the public opinion about genetic modification (Rommens *et al*. 2007). Cisgenesis has been successful in some crops, such as potato and apple (Jacobsen and Schouten 2008). Dhekney *et al*. (2011) showed the first report of partially cisgenic *V. vinifera* modified with the thaumatin-like protein (*vvtl-1*) gene.

Genetic modification could help establishing new knowledge on the biosynthesis of selected SMs for pharmaceutical purposes. There is already considerable interest in manipulating SM plant biosynthetic pathways. Veronese *et al*. (2001) reported the modification of essential oil production in the trichomes of *Mentha* spp (mints) and an increased resistance of the plant to fungal infection and abiotic stresses by engineering modification of biosynthetic pathways. Gómez-Galera *et al*. (2007) reviewed the production of indole, tropane, nicotine, isoquinoline alkaloids, monoterpenoids such as menthol and related compounds, diterpenoids such as taxol, sequiterpenoids such as artemisinin and aromatic amino acids by genetic transformation of medicinal plants.

Clearly, Plant Biotechnology offers alternative methods for the production of SMs and may provide competitive metabolite production systems when compared to whole plant extraction. However, for molecular biology and biotechnological applications, there are still many hurdles: compound production is often species- or even genotype-specific, and their accumulation is limited to certain tissues or cell types and regulated by environmental or developmental factors. In addition, the function of most of the metabolites is not known, nor is it clear which of the secondary metabolites from the plant's metabolome are responsible for its medicinal activity (Terryn *et al*. 2006). Our limited knowledge of the biosynthesis of natural products and its regulation form a bottleneck for using tissue culture in combination with advanced tools such as metabolic-pathway engineering. This will be necessary in order to pursue a biotechnological approach whereby plant cell tissue cultures could be genetically engineered to produce profitably a natural product of choice (Oksman- Caldentey and Inzé 2004).

Between all interesting SMs for pharmaceutical purposes, the present thesis has focused on two phenolic compounds using two experimental systems: grapevine (*V. vinifera* L.) as a natural source of *trans*-resveratrol and *Bituminaria bituminosa* as a natural source of furanocoumarins.

V. vinifera L. is the sole cultivated European representative of the genus *Vitis*, a large member of the *Vitaceae* with around 60 species. Two-thirds of these are native to North America and one third is distributed over central and east Asia (Aradhya *et al.* 2003). Grapevine is one of the most cultivated crop in the world; and besides of its food interest, this species synthesises resveratrol and its derivates, viniferins, in response to biotic or abiotic factors (Langcake and Pryce 1976; 1977). The facts that grapevine's genome has been sequenced (Jaillon *et al*. 2007; Velasco *et al.* 2007), and many plant cell culture techniques have been developed for several purposes (i.e. molecular breeding, functional genomic analyses) allow us to set up a system for over-producing resveratrol from *V. vinifera* by genetic modification.

Bituminaria bituminosa (L.) C.H. Stirton (syn*. Psoralea bituminosa* L., *Fabaceae, Psoraleeae*; Stirton 1981) is a perennial legume widely distributed in the Mediterranean Basin and Macaronesia. In the Canary Islands, the plants (commonly known as "Tedera") are grazed by small herbivores, but are mainly used for hay to feed milking goats (Méndez 2000; Méndez and Fernandez 1990; Muñoz and Correal 1998). *B. bituminosa* plants are also a source of SMs of pharmaceutical and medicinal interest, such as FCs (Martínez *et al*. 2010) (for more information about this species, see Introduction in Chapter II: Micropropagation).

For pharmaceutical purposes, the industry prefers homogeneous samples with more or less constant levels of the active ingredient, which cannot be ensured with whole-plant extraction from random wild-sampling. So, as a first step, the selection of *B. bituminosa* individuals by conventional breeding, was carried out by our research team at the IMIDA for more than 30 years (Project RTA2007-00046-00-00). The domestication of these plants is a valuable alternative, giving rise to a more controlled environment, and thus more stable production of FCs.

On the other hand, this species has not yet been used extensively in research. Thus, there is no knowledge of, for example, its genome and no experience with its tissue culture and genetic engineering. So, *in vitro* culture and molecular biology methods must be developed for enhancing production of FCs from *B. bituminosa*.

2. A drawback of *in vitro* plant tissue culture: endogenous contamination

Contamination by microorganisms, especially bacteria is known to be one of the most serious problems in plant tissue culture, mainly for commercial and research purposes. Contamination is not always seen at early stage of cultures; some endophytic contaminants are discovered at later subcultures and they are difficult to eliminate. Detection at the culture establishment stage may aid in selecting bacteria-free cultures (Reed at al. 1995). Antibiotics or other treatments may be needed to eliminate persistent microbial contaminations (Reed *et al*. 1995, Tanprasert and Reed 1998).

Detection of bacterial contaminants in the first stage could be carried out by visual inspection of the medium at the base of the explants, but is not adequate for slow growing bacteria, endophytes or those bacteria which do not grow on plant tissue culture media (Leifert *et al*. 1989). Screening methods must be favourable to bacterial or fungal growth, and easily used and interpreted (Reed *et al.* 1995). Screening procedures (indexing tests) are available for identifying many contaminants (Viss *et al*. 1991). This indexing system involves serial stem slices inoculated into liquid or agar solidified media and incubated at 30°C. Initial growth of explants in a liquid culture system at pH 6.9 and later testing on 523-Bacterial Medium detected most contaminants from over 400 mint explants (Reed *et al*. 1995), so it could be an accurate procedure.

Those detected bacteria, not pathogenic under natural conditions, could become opportunistic and may fail to produce diseases symptoms in *in vitro* cultures. In the present thesis, internal contaminants have been observed in *in vitro* cultures of *B. bituminosa*, and different microbiological studies have been developed, such as physiological and phenotypical test, to characterise the endophytes.

3. Resveratrol

Resveratrol (3-5-4'-trihydroxy-*trans*-stilbene or *trans*-resveratrol) belongs to the family of stilbenes, which are low-molecular weight phenolics, occurring in different plant families, such as *Pinaceae, Myrtaceae, Fagaceae, Liliaceae, Moraceae, Papilionaceae* and *Vitaceae* (Bavaresco and Fregoni 2001). They

are synthesised by plants in response to biotic and abiotic factors, acting like phytoalexins (Bavaresco *et al*. 2001). In the family *Vitaceae*, *V. vinifera* L. soft tissues produce stilbenes only in response to pathogens like fungi, such as powdery mildew (*Uncinula necator*), anthracnose (*Elsinoe ampelina*), downy mildew (*Plasmopara viticola*) or gray mould (*Botrytis cinerea*), or to abiotic stresses, like UV irradiation or wounding. They are found in lignified tissues as constitutive compound, being involved in the mechanism of wood resistance to decay (Jeandet *et al*. 1991; Langcake and Pryce 1977; Bavaresco and Fregoni 2001). Pool *et al*. (1981) found that there is no correlation between the constitutive resveratrol content in lignified tissues and their resistance response to oidium, while a positive correlation with induced leaf production exists.

Apart of *trans*-resveratrol, other compounds such as oligomers of resveratrol, termed viniferins, have also been found in grapevine as a result of infection or stress. The major components of these appear to be ε-viniferin, a resveratrol dimer and α-viniferin, a cyclic resveratrol trimer; although other viniferins are present as β-viniferin (resveratrol tetramer) and γ-viniferin (complex oligomers). Simple stilbenes have been identified as well: *trans*-pterostilbene, a dimethylated resveratrol derivative (3,5-dimethoxy-4-hydroxystilbene), *trans*- and *cis*-piceid, a 3-*O*-*β*-D-glucoside of resveratrol, *trans*- and *cis*-astringin, a 3-*O*-β-D-glucoside of 3'-hydroxy-resveratrol and *trans*- and *cis*-resveratrol-oside, a 4'-*O*-*β*-D-glucoside of resveratrol (Jeandet *et al*. 2002). Jeandet *et al*. (1995) reported either pterostilbene or viniferins are more active inhibitors in response to fungi infections, while *trans*-resveratrol is the most abundant phytoalexin; so it is a good marker of disease resistance.

3.1. Biosynthesis of stilbenes

Stilbenes are derived from shikimic acid pathway (primary metabolism) via phenylalanine (phenylpropanoid pathway), as first proposed by Langcake and Pryce (1977). The central enzyme in phenylpropanoid metabolism that directs carbon from aromatic amino acids to the phenylpropanoids is phenylalanine ammonia-lyase (PAL), which forms cinnamic acid from phenylalanine. This step is regarded as the branch point between the primary metabolism (shikimate

pathway) and the secondary metabolism which leads to various natural products including coumarins, phenolic acid esters, lignins and flavonoids (Figure 4). While PAL is the first enzyme of phenylpropanoid metabolism, chalcone synthase (CHS) and stilbene synthase (*STS*) catalyse the first reaction of this metabolism using the same precursors (malonyl-CoA and coumaroyl-CoA) leading to the production of flavonoids and stilbenes, respectively. STS condenses three malonyl-CoA molecules with one molecule of coumaryl-CoA to produce resveratrol. This diphenol compound is metabolized, producing ε-viniferina (dimerization), pterostilbene (methylation) and piceid (resveratrol glucoside). Resveratrol accumulation decreases 16 weeks post-flowering (Bais *et al*. 2000). This decrease may be due to a limitation in substrate availability, which may result either from decreased synthesis of coumaroyl-CoA and malonyl-CoA or from competition between CHS and STS for the same substrates (Fischer *et al*. 1997) (Figure 4).

The *STS* gene was first purified from cell suspension cultures of *Arachis hypogea* (Schöeppner and Kindl, 1984). It is encoded by a multigenic family (Wiese *et al*. 1994) and in grapevine is composed by 21-43 different genes (Jaillon *et al*. 2007; Velasco *et al*. 2007). Some resveratrol forming *STS* genes from grapevine are: *pSV21*, *pSV25*, *pSV696* and *pSV368* (Melchior and Kindl 1991) *Vst1*, *Vst2*, *Vst3* and *StSy* (Sparvoli *et al.* 1994; Wiese *et al*. 1994). *STS* genes from pine forming pinosylvin are: *PST-1*, *PST-2*, *PST-3*, *PST-4* and *PST-5* (Preisig-Müller *et al*. 1999). Other *STS* genes were isolated from roots of *Pinus densiflora* (*pdsts1*, *pdsts2* and *pdsts3*, Kodan *et al*. 2001, 2002), from *Vitis riparia* cv. Gloire de Montpellier (Goodwin *et al*. 2000) and from *Vitis pseudoreticulata* (Fan *et al*. 2007). There is, at present, only one *STS* gene described in monocotyledonous plants, the *SbSTS1* gene isolated from sorghum (Yu *et al*. 2005).

CHS and STS have a common evolutionary origin: the former is expressed constitutively, while the latter is inducible by biotic and abiotic stresses. Thus, the infection by *B. cinerea* produces a shut off in general proteins and active STS and PAL (Bavaresco and Fregoni 2001), and elicitation of cell suspension cultures with ciclodextrins, MeJA or a combination of both, specifically activate

the PAL, cinnamate 4-hydroxylase (C4H), 4-cumarate-CoA ligase (4CL) and STS expression, independently of CHS (Almagro 2011).

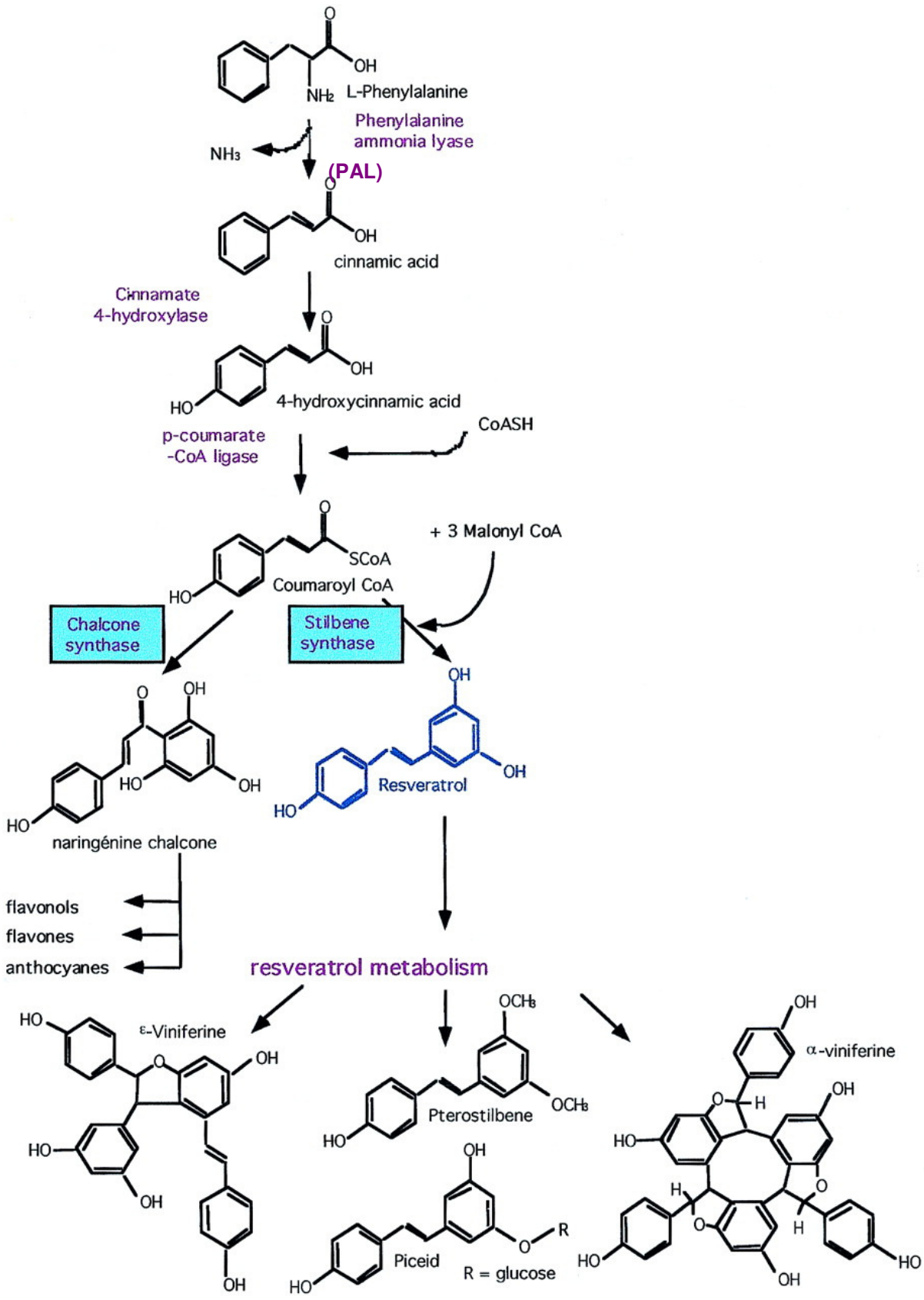


Figure 4. Stilbene and chalcone biosynthesis pathways in grape and major metabolites derived from resveratrol (Coutos-Thévenot *et al.* 2001).

3.2. Resveratrol applications on human health

Resveratrol was first isolated from *Veratrum grandiflorum* O. Loes (Takaoka 1940) and has also been found in knotweed (*Polygonum cuspidatum* syn. *Fallopia japonica*), grapevines and peanuts (Burns *et al*. 2002). This molecule is one of the best known plant SMs (Kiselev 2011), and thousands of articles have been published about metabolism and bioactive properties of *trans*-resveratrol and its derivates, concerning not only their biological properties but also the benefits to human health and the applications in pharmaceutical industries (Kiselev 2011). It helps preventing development of cardiovascular diseases (Bradamante *et al*. 2004; Stervo *et al*. 2007), prevents thrombocyte aggregation through regulation of the synthesis of eicosanoids (Olas *et al*. 2001), presents antioxidant properties by interfering with oxidative modifications of lipids (Leighton *et al*. 1999), has capacity to inhibit cellular proliferation and a cytotoxic effect on tumour cells (lymphoid and myeloid cancers; multiple myeloma; cancers of the breast, prostate, stomach, colon, pancreas, and thyroid; ovarian carcinoma; and cervical carcinoma) (Aggarwal *et al*. 2004; Shankar *et al*. 2007). Moreover, *trans*-resveratrol has a wide list of applications such as protection against diabetes and neurodegenerative diseases and it has been proposed to extend the life span of lower and higher organisms (Pearson *et al*. 2008; Sharma *et al*. 2007; Wang *et al*. 2006) through the sirtuin (silent information regulator enzymes, SIRT) pathway (Parker *et al*. 2005). However, other studies report that resveratrol does not directly active sirtuins involved in the regulation of critical metabolic pathways (Beher *et al*. 2009; Burnett *et al.* 2011; Pacholec *et al*. 2010).

Moreover, *trans*-resveratrol is consumed in the form of numerous biological additives so it is important to develop an effective method to obtain it commercially (Kiselev 2011). Currently, field cultures of *P. cuspidatum* are the main natural source of *trans*-resveratrol and several Chinese companies produce it for the derivation of biologically-active additives from root extracts with different degrees of purity. The use of biotechnological methods could be an alternative for large-scale production *of trans*-resveratrol, with a high and homogeneous degree of purity and preventing intensive cutting and decimation of the natural source.

3.3. The role of Plant Biotechnology in *trans*-resveratrol production

The use of Plant Biotechnology represents a reliable alternative, and powerful method for large-scale, low-cost *trans*-resveratrol production under controlled conditions. Employing different methods of plant tissue culture (cell suspensions, hairy roots, callus cultures, and genetic modification) and strategies (addition of precursors or chemical elicitors, environmental stresses, or pathogen attack), several groups have achieved resveratrol production in *in vitro* conditions (Delaunois *et al*. 2009; Kim *et al*. 2008; Kiselev 2011; Lijavetzky *et al*. 2008).

3.3.1. Cell suspension cultures

The main advantage of cell suspensions is that these cultures produce *trans*-resveratrol constitutively, or in response to stress, which can be exploited in *in vitro* conditions using elicitation to optimize the production (Donnez *et al*. 2009). A large variability of elicitors has been tested, i.e. methyljasmonate (MeJA), cyclodextrins, chitosan or L-alanine. For the production of *trans*-resveratrol from cell suspensions different plants have been used as cotton (Kouakou *et al.* 2006), *Vitis labrusca* cv. Concord (Chen *et al*. 2006) and *Vitis thunbergii* cv. Sieb. and Zucc. However, the main plant used for stilbene production is grapevine where a synergistic effect of MeJA and cyclodextrins is observed (Lijavetzky *et al*. 2008; Belchí-Navarro *et al*. 2012).

The amount of *trans*-resveratrol obtained vary in response to elicitors, plant species and culture conditions used (Bru *et al*. 2006; Chen *et al*. 2006; Medina-Bolivar *et al*. 2007; Belchí-Navarro *et al*. 2012). Table 1 shows resveratrol production in different *Vitis* cell cultures.

Table 1. Resveratrol production in *Vitis* cell cultures. (Modified from Kiselev *et al*. 2011)

***Vitis* cultivar**	**Inducer/elicitor/precursor**	**Maximal quantity of *trans*-resveratrol**	**References**
V. lambrusca cv. "Washington Concord"	L-Alanine	0.22% DW[b], 0.011% FW[b], 2.2 mg l^{-1}[b]	Chen *et al*. (2006)
V. vinifera cv Barbera	MeJA	<0.01% DW[a], 0.001% FW[b], 0.11 mg l^{-1}[b]	Tassoni *et al*. (2005)
	Control	0.02% DW[b], 0.001% FW[b], 1.6 mg l^{-1}[b]	Ferri *et al*. (2009)
V. vinifera L. cv Gamay Freaux var. Teinturier	MeJA and sucrose	0.06% DW[b], 0.003% FW[a], 5.5. mg l^{-1}[b]	Belhadj *et al*. (2008)
V. vinifera L. cv Gamay	DIMEB (5 mM)	0.51% DW[b], 0.025% FW[b], 100.3 mg l^{-1}[a]	Morales *et al*. (1998)
	DIMEB and incubation with *Xylophilus ampelinus*	1.02% DW[b], 0.051% FW[b], 202.9 mg l^{-1}[a]	
V. vinifera L. cv Gamay rouge	DIMEB (50 mM)	15.3% DW[b], 0.765% FW[b], 3,060 mg l^{-1}[a]	Bru and Pedreño (2006)
	RAMEB (50 mM)	16.6% DW[b], 0.83% FW[b], 3,320 mg l^{-1}[a]	
V. vinifera L. cv Monastrell albino	DIMEB (50 mM)	23.4% DW[b], 1.17% FW[b], 4,680 mg l^{-1}[a]	
	RAMEB (50 mM)	25.2% DW[b], 1.26% FW[b], 5,027 mg l^{-1}[a]	
V. vinifera L. cv Monastrell albino	DIMEB (50 mM)	5.02% DW[b], 0.251% FW[a], 753 mg l^{-1}[a]	Lijavetzky *et al*. (2008)
	DIMEB and MeJA	36.51% DW[b], 1.823% FW[b], 3,651 mg l^{-1}[b]	
V. vinifera cv Barbera	Sucrose	0.01% DW[b], <0.001% FW[b], 6.7 mg l^{-1}[b]	Ferri *et al*. (2011)
V. vinifera L. cv Monastrell albino	CD (50 mM)	0.79 % DW[b], 0.39 % FW[b], 628.6 mg l^{-1}[b]	Belchí-Navarro *et al*. (2012)
	MeJA (100 μM)	0.03 % DW[b], 0.00019 % FW[b], 38 mg l^{-1}[b]	
	CD (50 mM) +UV (5 min)	1.5 % DW[b], 0.008 % FW[b], 1,519.7 mg l^{-1}[b]	
	CD(50 mM) +MeJA (100 μM)	3.4 % DW[b], 1.68 % FW[b], 3,363 mg l^{-1}[b]	
	CD(50 mM) +MeJA (100 μM)+UV (5 min)	3.82 % DW[b], 0.019 % FW[b], 3,819.7 mg l^{-1}[b]	
	Sucrose (10 g l^{-1})	2.34 % DW[b], 0.0117 % FW[b], 2.34 mg l^{-1}[b]	

DW= dry weight, UV= ultraviolet, MeJA= methyljasmonate, DIMEB= 2,6-di-O-methyl-b-cyclodextrin, CD= ciclodextrin

[a] Indicates the quantity of resveratrol obtained in the cited article

[b] Indicates the quantity of resveratrol calculated from data on resveratrol content in terms of fresh or dry biomass accumulation given that plant cells usually accumulate 10-20 g l^{-1} dry and 200-300 g l^{-1} fresh biomass of cultivated cells

3.3.2. Hairy root cultures

Growth of hairy roots and production of *trans*-resveratrol depend on *A. rhizogenes* strain and bacterial infection frequency (Kim *et al*. 2008). In peanut (*Arachis hypogea*), hairy roots induced by *A. rhizogenes* R1601 produced 1.5 mg g^{-1} DW. Medina-Bolivar *et al*. (2007) elicitated hairy root cultures of peanut with sodium acetate, achieving levels of 98 μg mg^{-1} DW.

3.3.3. Callus cultures

To produce *trans*-resveratrol, callus cultures from different species have been obtained. Donnez *et al*. (2009) reported that calli are formed by coalescence of dedifferentiated cells growing on a solid culture medium, and their production of stilbenes has been related to the stimulation of plant defence mechanisms. Peanut calli were able to produce 0.012 mg g^{-1} FW *trans*-resveratrol after UV irradiation (Lin *et al*. 2007) and calli of *V. amurensis* synthesised 0.15% DW *trans*-resveratrol after elicitation with sodium nitroprusside (Kiselev *et al*. 2007).

3.3.4. Genetic modification

Although numerous studies have been carried out for genetic modification with *STS* genes to enhance fungi resistance, to the best of our knowledge, no studies have been carried out for large-scale *trans*-resveratrol production, for pharmaceutical purposes.

Other authors have reported genetic modification with *STS* genes of rice (Stark-Lorenzen *et al*. 1997), tomato (Thomzik *et al*. 1997; Giovinazzo *et al*. 2005; Morelli *et al*. 2005; Nicoletti *et al*. 2007), alfalfa (Hipskind and Paiva 2000), kiwifruit (Kobayashi *et al*. 2000), apple (Szankowski *et al*. 2003; Rühmann *et al*. 2006), *Arabidopsis* (Yu *et al*. 2006), aspen (Seppänen *et al*. 2004), white poplar (Giorcelli *et al*. 2004), pea (Richter *et al*. 2006), hop (Schwekendiek *et al*. 2007), papaya (Zhu *et al*. 2004), lettuce (Liu *et al*. 2006), oilseed rape (Hüsken *et al*. 2005), barley and wheat (Leckband and Lörz 1998; Fettig and Hess 1999; Liang *et al*. 2000; Serazetdinova *et al*. 2005), banana

(Vishnevetsky *et al*. 2005), *Rehmannia* (Lim *et al*. 2005) and grapevine (Coutos-Thévenot *et al*. 2001; Fan *et al*. 2007) to enhance disease resistance (Table 2).

To increase the stilbene production the use of chimerical genes or the combination of two encoding genes (*Vst1* and *Vst2*) has been reported (Fischer *et al*. 1997). The most common promoter selected to drive the transgene is pCaMV35S (Fischer *et al*. 1997) that produces a strong and constitutive stilbene accumulation, and as consequence, triggers a drastic depletion of the endogenous pools of precursors (Delaunois *et al*. 2009). The promoter pVst1 from grapevine is induced by both biotic and abiotic factors, leads to stilbene accumulation without interfering with secondary biosynthetic pathways (Thomzik *et al*. 1997), is regulated by common transcriptional factors present in most plant species (Delaunois *et* al. 2009), and provides a rapid and strong accumulation of both STS transcripts and their products following inoculation with phytopathogenic fungi (Hain *et al*. 1990; Stark-Lorenzen *et al*. 1997; Thomzik *et al*. 1997; Zhu *et al*. 2004). The use of an inducible promoter appears to be a promising way to maintain precursor pools, while allowing strong stilbene accumulation at the infection site (Delaunois *et* al. 2009). Fischer *et al*. (1997) reported over-expression of *STS* in tobacco flowers using a tissue-specific promoter that permits the resveratrol accumulation only in a determined organ or tissue.

The effect of strong resveratrol accumulation on modified-plant development has been studied. In many cases, the expression of STS genes did not alter the normal growth of plants (Hain *et al*. 1993) but in other cases, accumulation of resveratrol modified the colour and morphology of the flowers and altered pollen maturation leading to male sterility because STS and CHS compete for the same precursors (malonyl-CoA and coumaroyl-CoA). Fischer *et al*. (1997) reported that the expression of an *STS* gene in tobacco anthers resulted in a decreased supply of the substrate for the endogenous CHS enzyme. It is possible that the constitutive resveratrol synthesis inhibits flavonol biosynthesis in the *tapetum* tissue (pollen), which eventually leads to a loss of pollen viability. Ingrosso *et al*. (2011) reported parthenocarpy and abnormal pollen development in tomato due to over-expression of a grapevine *StSy* (stilbene synthase) cDNA because of malonyl-CoA and coumaroyl-CoA are key

molecules in several other pathways, including sporopollenin or lignin biosynthesis, involved in pollen development. Therefore, the over-accumulation of resveratrol could produce changes in several other pathways with relevant functions and they must be analysed.

To the best of our knowledge, no studies involving genetic modification have been carried out regarding *trans*-resveratrol production for commercial purposes. A possible method to obtain this molecule commercially may be the combination of different approaches, for instance, genetic modification with *STS* genes, establishment of modified-cell suspension cultures and subsequently elicitation with MeJA and cyclodextrins. This thesis describes the *trans*-resveratrol over-production brought about by genetic modification of *Vitis vinifera* cv. Sugraone as a first step of this approach.

Table 2. Stilbene synthase genes and promoters used to genetically transform plants, and the resulting effects on stilbene levels, resitance to pathogens and antioxidant activities (Modified from Delaunois *et al*. (2009))

Plant species	**Gene**	**Promoter**	**Biochemical output**	**Stilbene concentration (µg/g FW)**	**Biological activity**	**References**
Nicotiana tabacum L.	*Arachis hypogaea STS gene*	Stress-induced promoter	*trans*-Resveratrol	-	-	Hain *et al*. (1990)
	Vst1 and Vst2	Vst1	*trans*-Resveratrol	400	Resistance to *Botritys cinerea*	Hain *et al*. (1993)
	Chimeric STS gene	CaMV35S	Resveratrol	50-290	Altered flower morphology, male sterility	Fischer *et al*. (1997)
Triticum aestivum L.	*Vst1*	Vst1 (+35S-4 fold)	-	-	Resistance to *Botritys cinerea*	Leckband and Lörz (1998)
						Liang *et al*. (2000)
	Chimeric STS gene	Maize ubiquitin promoter	Resveratrol	2	-	Fetting and Hess (1999)
	Vst1 and Vst2	Vst1 (+35S-4 fold)	Unknow derivative stilbene compounds	35-190	Resistance to *Puccinia recondita* and *Septoria nodorum*	Serazetdinova *et al*. (2005)
Hordeum vulgare L.	*Vst1*	Vst1 (+35S-4 fold)	-	-	Resistance to *Botritys cinerea*	Leckband and Lörz (1998)
Medicago sativa L.	*Arachis hypogaea STS gene (AhRS)*	CaMV35S	*trans*-Piceid	0.5-20	Resistance to *Phoma medicaginis*	Hipskind and Paiva (2000)
Arabidopsis thaliana L.	*SbSTS1*	CaMV35S	*trans*- and *cis*-Piceid	584	-	Yu *et al*. (2005, 2006)
Actinidia deliciosa	*pSV25*	CaMV35S	*trans*-Piceid	20-182	No resistance to *Botritys cinerea*	Kobayashi *et al*. (2000)
Vitis vinifera L.	*Vst1*	ms PR10.1	Resveratrol		In vitro resistance to *Botritys cinerea*	Coutos-Thévenot *et al*. (2001)
	Vitis pseudoreticulata STS gene	CaMV35S	Resveratrol	2.6	Under investigation	Fan *et al* (2007)
Malus domestica	*Vst1*	Vst1	Unknown resveratrol glycoside	-	-	Szankowski *et al*. (2003)
Borkh.	*Vst1*	Vst1	*trans*-Piceid	3-7 for non-UV-irradiated fruit; 23-62 for UV-irradiated fruit	No influence on other phenolic compounds	Rühmann *et al*.(2006)
Lycopersicon esculentum Mill.	*Vst1 and Vst2*	Vst1	Resveratrol	-	Resistance to *Phytophtora infestans*. No resistance to *Botritys cinerea* and *Alternaria solani*	Thomzik *et al*. (1997)
	Stsy	CaMV35S	*trans*-Resveratrol and *trans*-Piceid	4-53	Antioxidant primary metabolism and increase in total antioxidant activity	Giovinazzo *et al*. (2005)
	Stsy	CaMV35S	*trans*-Resveratrol and *trans*-Piceid	0.1-1.2	Enhancement of natural antiradical properties	Morelli *et al*. (2006)
	Stsy	CaMV35S	*trans-, cis*-Resveratrol and *trans-, cis*-Piceid	0.42-126 depending on the stage of ripening and fruit samples	Differences in rutin, naringenin and chlorogenic acid contents	Nicoletti *et al*. (2007)

Table 2. Continued

Plant species	Gene	Promoter	Biochemical output	Stilbene concentration (µg/g FW)	Biological activity	Reference
Lycopersicon esculentum Mill.	*Stsy*	CaMV35S	*trans*-Resveratrol and *trans*-Piceid	25-125 in red fruit peel in 35SS tomato, 25-200 in flowers	Effects on flavonoids biosynthesis, parthenocarpy and abnornal pollen development	Ingrosso *et al*. (2011)
		TomLoxB		in TomLoxB tomatoes, 20-fold lower than in 35SS, 8-10 times lower than in 35SS		
Oryza sativa L.	*Vst1*	Vst1	-	-	Resistance to *Pyricularia oryzae*?	Strak-Lorenzen *et al*. (1997)
Rehmannia glutinosa Libosch.	*AhRS3*	CaMV35S	Resveratrol and piceid	22-116; up to 650 with stress treatment	Antioxidant capabilities, resistance to *Fusarium oxysporum*	Lim *et al*. (2005)
Lactuca sativa L.	*Parthenocissus henryana STS*	CaMV35S	*trans*-Resveratrol	56.4	Effect on Hela cell morphology	Liu *et al*. (2006)
Pisum sativum L.	*Vst1*	Vst1	Ocurrence of two resveratrol glucoside compounds	0.53-5.2	-	Richter *et al*. (2006)
Populus alba L.	*Susy*	CaMV35S	*trans*- and *cis*-Piceid	309-615	*No in vitro resistance to Melampsora pulcherrima*	Giorcelli *et al*. (2004) Seppänen *et al*. (2004)
Carica papaya L.	*Vst1*	Vst1	Resveratrol glucoside	54	Resistance to *Phytophtora palmivora*	Zhu *et al*. (2004)
Brassica napus L.	*Vst1*	p-nap	Resveratrol glucoside	361-616	Food quality improvement: high piceid rate content an reduction of sinapate esters	Hüsken *et al*. (2005)
Humulus lupulus L.	*Vst1*	CaMV35S	*trans*- and *cis*-Piceid, unknown stilbene *cis*-isomer, *trans*-astringin, *trans*- and *cis*-Resveratrol	490-560	Higher amounts of flavonoids and acids	Schwekendiek *et al*. (2007)

4. Furanocoumarins

The furanocoumarins (FCs) are phenolic compounds within the coumarins group. Coumarins are lactones (1,2-benzopyrone) that according to their structure are classified as: i) simple coumarins, ii) furanocoumarins, iii) pyranocoumarins, and iv) pyron-ring sustituted coumarins and their hydroxylated, alkoxylated, and alkylated derivates and their glycosides (Murray *et al*. 1982).

The FCs are phytoalexins synthesised in response to fungal infection (Tiejten *et al*. 1983) and against insects (Berembaum 1983). They can be grouped into linear and angular types (Figure 5). Linear FCs (syn. psoralens) are distributed in *Apiaceae* (*Ammi majus* and *Apium graveolens*)*, Moraceae, Rutaceae* (*Ruta graveolens*) and *Leguminosae* (*Psoralea corylifolia* and *B. bituminosa*). The angular FCs (syn. angelicins) are less widely distributed and have only been found in *Apiaceae* and *Leguminosae* (reviewed by Bourgaud *et al*. 2006).

4.1. Biosynthesis of furanocoumarins

The biosynthesis of FCs in plants presents a double origin: the cumarinic nucleous derives from the shikimic pathway and is transformed into *trans*-cinnamic and finally into umbelliferone, and the furanic-ring derives from the mevalonate pathway (dimethyl-pyrophosphate –DAP-) (Figure 5a). Floss and Mothes (1964) proposed for the first time, that umbelliferone is the precursor of FC biosynthesis.

FCs can be grouped into the linear type, where a three carbon chain is attached at umbelliferone C(6) to get dimethylsuberosine, and the angular type, carrying the substitution at C(8) to get ostenol (Hamerski *et al*. 1990) (Figure 5b). Dimethylsuberosine is transformed into marmesin and latter into psoralen in two reactions catalised by P450s cytochromes: marmesin synthase and psoralen synthase (Hamerski and Matern 1988). The two enzymes formally catalyze very different reactions, the first forming the dihydrofuran-ring from the ortho-prenylated phenol (marmesin synthase) and the second catalyzing the oxidative carbon-carbon chain cleavage reaction (psoralen synthase). Psoralen

5-monooxygenase, characterized as a cytochrome P450 enzyme (Hamerski and Matern 1988), catalyzes the subsequent hydroxylation of psoralen to bergaptol. This compound is then o-methylated to bergapten by an *O*-methyltransferase (Hehmann *et al*. 2004). The enzyme is highly specific for bergaptol and does not accept xanthotoxol. On the other hand, to synthesise angular FCs (Figure 5b), ostenol is transformed into columbianetin and subsequently into angelicin, also in two reactions catalysed by columbianetin synthase and angelicin synthase. These reactions were assumed to be very similar to the steps leading to the conversion of demethylsuberosin to marmesin and psoralen (Figure 5b). This assumption is particularly convincing when the catalytic mechanisms of psoralen synthase and angelicin synthase are compared (Larbat *et al.* 2009), although, angular FC pathway has not been as well documented, especially at the level of enzyme activity.

The enzymes for angular FC biosynthesis may have emerged by evolutionary adaptation from the linear pathway. This would be consistent with the fact that angular FCs are less abundant in plants than the linear type and are always found concomitantly with linear FCs (Bourgaud *et al*. 2006).

The FC biosynthesis depends on organ, physiological stage and plant cycle and there is an interaction between temperature and FC accumulation (Walker *et al*. 2012; Zobel and Brown 1991).

The most abundant linear FCs are psoralen, xanthotoxin, bergapten and isopimpinellin, whereas the angular type is mostly represented by angelicin, sphondin, and pimpinellin (Figure 5b).

.

Figure 5. A: Phenylpropanoid pathway leading to coumarins. Pathways in grey have been unequivocally established. Glycosylated compounds are not shown for clarity. Enzymes assigned by a question mark are hypothetical. R = CO2H or CO-SCoA; C2H, cinnamic acid 2-hydroxylase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CO2H, 4-coumaric acid 2-hydroxylase; HCT, hydroxycinnamoyl-transferase; CAOMT, caffeic acid O-methyltransferase; CCoAOMT, caffeoyl CoA O-methyltransferase, CA2H, caffeic acid 2-hydroxylase; FA2H, ferulic acid 2-hydroxylase; MDCA2H, methylenedioxycinnamic acid 2-hydroxylase; O-MT, O-methyltransferase. B: Enzymes designated with a question mark are hypothetical. O-MT, O-methyltransferase (Modified from Bourgaud *et al*. 2006 and Larbat *et al.* 2009). (See next page)

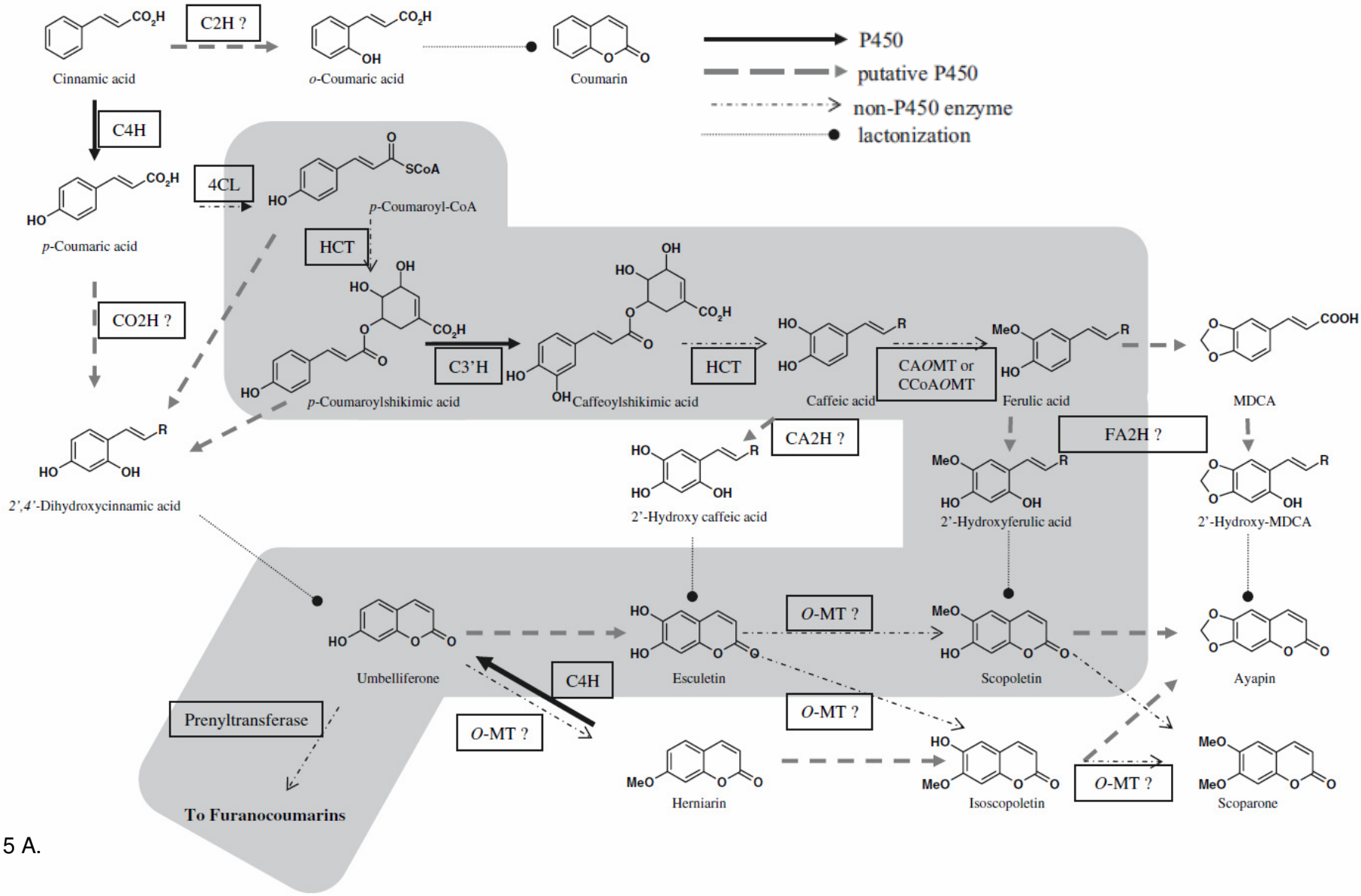

Cinnamic acid
C2H ?
o-Coumaric acid
Coumarin
P450
putative P450
non-P450 enzyme
lactonization
C4H
4CL
p-Coumaric acid
p-Coumaroyl-CoA
HCT
CO2H ?
C3'H
p-Coumaroylshikimic acid
Caffeoylshikimic acid
HCT
Caffeic acid
CAOMT or
CCoAOMT
Ferulic acid
MDCA
CA2H ?
2',4'-Dihydroxycinnamic acid
2'-Hydroxy caffeic acid
FA2H ?
2'-Hydroxyferulic acid
2'-Hydroxy-MDCA
Umbelliferone
O-MT ?
Esculetin
Scopoletin
Ayapin
C4H
O-MT ?
Prenyltransferase
O-MT ?
Herniarin
Isoscopoletin
O-MT ?
Scoparone
To Furanocoumarins

Figure 5 A.

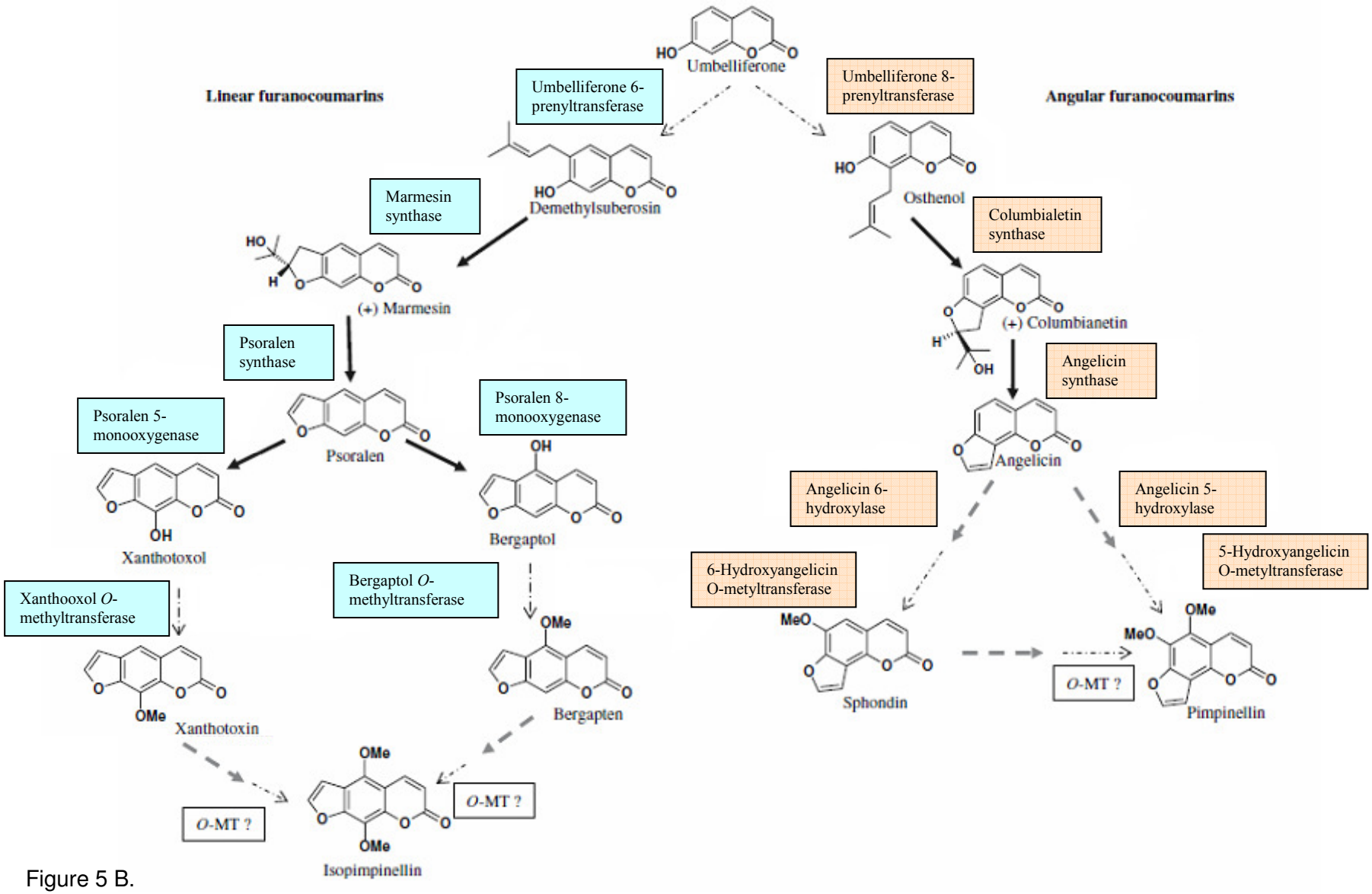

Linear furanocoumarins
Angular furanocoumarins
Umbelliferone
Umbelliferone 6-prenyltransferase
Umbelliferone 8-prenyltransferase
Demethylsuberosin
Osthenol
Marmesin synthase
Columbialetin synthase
(+) Marmesin
(+) Columbianetin
Psoralen synthase
Angelicin synthase
Psoralen 5-monooxygenase
Psoralen 8-monooxygenase
Psoralen
Angelicin
Xanthotoxol
Bergaptol
Angelicin 6-hydroxylase
Angelicin 5-hydroxylase
6-Hydroxyangelicin O-metyltransferase
5-Hydroxyangelicin O-metyltransferase
Xanthooxol O-methyltransferase
Bergaptol O-methyltransferase
Sphondin
Pimpinellin
Xanthotoxin
Bergapten
O-MT ?
O-MT ?
O-MT ?
Isopimpinellin

Figure 5 B.

4.1.1. The role of P450s in furanocoumarin biosynthesis

The cytochrome P450 monooxygenases (P450s) comprise a vast superfamily of heme-thiolate enzymes that catalyze the NADPH-associated reductive cleavage of oxygen to produce a functionalized product (Schuler 1996). Cytochrome P450 enzymes are pivotal enzymes of FC biosynthesis, i.e., the formation of xanthotoxin relies, at least, on four sequential P450 reactions catalyzed by C4H, marmesin synthase, psoralen synthase and psoralen 8-monooxygenase (Figure 5A, B).

Until the genetic studies on *A. majus*, the molecular genetics of FC formation has remained largely unresolved due, in part at least, to the labiality of the consecutively operating P450s. The first furanocoumarin-committed monooxygenase gene cloned and functionally characterised was the psoralen synthase from *A. majus* (*CYP71AJ1*). The enzyme revealed specificity for (+)-marmesin, whereas (+)-columbianetin, which is considered as the analogous precursor of the angular FC angelicin, acted as a competitive inhibitor (Larbat *et al*. 2007).

The genes encoding these enzymes constitute one of the largest known gene superfamilies. P450-catalyzed reactions are important for detoxification of exogenous compounds, such as drugs, and plant allelochemicals. Cytochrome P450 monooxygenases provide a case in point; these enzymes are involved in the biosynthesis of FCs, toxic allelochemicals in plants, as well as in their detoxification by lepidopterans (Schuler 1996).

Larbat *et al*. (2009) reported the cloning and functional expression of two new psoralen synthase orthologues of *A. majus*, from *A. graveolens* (celery *CYP71AJ2*) and *Pastinaca sativa* (parsnip *CYP71AJ3*). Additionally, the closely related *CYP71AJ4* was isolated from parsnip and annotated as angelicin synthase, the first P450 gene committed to angular FC biosynthesis. Several studies of the paralogues (*CYP71AJ3* and *CYP71AJ4*) revealed that the sequence identity dropped to only 40% in substrate recognition sites at an overall identity of 70%. Assays conducted with *CYP71AJ4* and *syn*-[3-D] columbianetin demonstrated a reaction mechanism analogous to that of psoralen synthase initiated by the abstraction of *syn*-C-3-hydrogen. These results demonstrate that the CYP71AJ subfamily is involved in the synthesis of both linear and angular FCs and suggest that *CYP71AJ3* and *CYP71AJ4* derived from a common ancestor gene.

Recently, Parast *et al*. (2011) reported the isolation, cloning and characterisation of the psoralen synthase gene of *P. corylifolia*. The sequence showed a significant degree of similarity 93% to the sequence of *A. majus* psoralen synthase (GeneBank ACC. No. AY532370).

4.2. Furanocoumarins and their application to human health

Psoralens and the methoxylated psoralens, bergapten and xanthotoxin, have numerous biological activities (reviewed by Martínez *et al*. 2010) and are the most relevant natural FCs in terms of their therapeutic potential (Hehmann *et al*. 2004). Psoralens exhibit photosensitising and anti-proliferative activities and were evaluated as photosensitising drugs for the treatment of psoriasis and vitiligo using oral psoralen in combination with UVA irradiation (PUVA therapy). They showed photosensitised lethal effect in bacteria (Scott *et al*. 1976), showed activity in darkness against *Candida albicans* and *Cryptococcus laurentii* (Camm *et al*. 1976) and exhibited anti-HIV effect (Shikishima *et al*. 2001). Moreover, psoralen presents antimutagenic activity that allows its use in medicine for cancer prevention (Martínez *et al*. 2010). It is able to produce cross-linking of DNA strands, leading to significant distortions in the DNA helix, creating sites that are recognised by DNA repair enzymes (Cheng *et al.* 1988); therefore, it is a useful tool in DNA structural analyses. Angelicin has potential therapeutic applications in haematology disorders, such as β-thalassemia (Patent US2006/0111433(A1)) and sickle cell anaemia (Lampronti *et al.* 2003). In addition, it shows calmative, sedative, anticonvulsant, anti-inflamatory, anti-pyretic activities and also shows high activity against some microorganisms (Backhouse *et al*. 2001; Santana *et al*. 2004).

In the pharmaceutical industry, the FCs have been extracted from bergamot oil (*Citrus bergamia*) which contains bergapten (Späth 1937), the psoralen derivative mainly used in medicine, but the bergamot production is rather low. Other natural source of FCs is *P. corylifolia,* which has been widely used in traditional medicine in China, although the psoralen contents are low. *A. majus* L. (*Apiaceae*) is also a natural source of linear coumarins and furanocoumarins (Królicka *et al*. 2001), and due to the photosensitizing activity of these compounds, the fruits has been used in the Mediterranean region in the treatment of leucoderma, psoriasis and vitiligo (Hamerski *et al*. 1990).

The economic importance of FCs, together with the fact that their chemical synthesis is very expensive, has stimulated an interest in looking for other natural sources to meet the increasing requirements of the pharmaceutical industry. In this context, *B. bituminosa* appears to be a good species for commercial production of psoralen and angelicin (Martínez *et al*. 2010).

The use of biotechnological methods could be an alternative for large-scale production of FCs, preventing intensive cutting and decimation of the natural sources. In the present thesis, *in vitro* cultures techniques and genetics studies have been developed, for first time, for *B. bituminosa* in order to establish the bases for large-scale production of FCs from this species, by applying a combination of different biotechnological tools.

4.3. The role of Plant Biotechnology in furanocoumarin production

The species reported as FC producers such as *P. corylifolia, R. graveolens, A. majus and A. graveolens* (Baskaran *et al* 2011; Hamerski and Matern 1988; Milesi *et al*. 2001; Trumble *et al*. 1990) have been widely studied in tissue culture for FC production, and also plant regeneration protocols have been developed (Ahmad *et al*. 2010; Baskaran and Jayabalan 2009a, b; Baskaran *et al* 2011; Pande *et al*. 2002). In *in vitro* cultures (calli, cell-suspensions and shoots), the FC content is tissue dependent and a function of the plant organization level (Baskaran *et al*. 2011; Diwan and Malpathak 2010).

4.3.1. Cell suspension cultures and elicitation

The production of FCs in plant cell suspension cultures has been studied by several authors in *A. majus* and *R. graveolens*. Staniszewska *et al*. (2003) reported that treatment of cell suspension of *A. majus* with dead cells of *Enterobacter sakazaki* strongly induced production of umbelliferone, and the treatment with BION® induced production of umbelliferone, dehydrogenjerin and bergapten. Other biotic elicitors have been tested for FC production in *A. majus,* such as *Alternaria carthami* and *Phytophthora megasperma*, increasing in its production (Hamerski and Matern 1988). Bohlmann *et al*. (1995) reported that elicitation of cell suspension cultures of *R. graveolens* with homogenate of the yeast *Rhodotorula rubra* showed enzyme

induction and an accumulation of FCs 40 h after treatment, although there was no strict correlation between them. Besides of these biotic elicitors, FC pathway precursors may be used. Austin and Brown (1973) observed an increase in FC production after elicitation by addition of marmesin to cell suspension cultures of *R. graveolens.*

4.3.2. Callus and shoot cultures

In different species, the FC production has been studied in callus and shoot cultures: in *P. corylifolia* , *R. graveolens*, and *A. majus* (Bourgaud *et al*. 1995; Ekiert 1993; Ekiert *et al.* 2001; Ekiert and Gomólka 2000; Ekiert *et al*. 2005; Massot *et al*. 2000; Milesi *et al*. 1998), the amount of FC produced in unorganized cell cultures (Bourgaud *et al*. 1995; Ekiert *et al*. 2005) was comparatively lower than the obtained in organized cultures (Ekiert *et al*. 2001; Diwan and Malphathak 2008; Massot *et al*. 2000). Diwan and Malphathak (2010) demonstrated that differentiation of cells into shoot and root primordia increased the capacity for synthesis and accumulation of FCs.

Recently, Parast *et al*. (2011) reported the synthesis of psoralen in callus from *P. corylifolia* and its increasing when calli were exposed to different organic elicitors (yeast extract, proline, inositol, casein hydrolyzate (CH), glycine, glutamine and sucrose) and precursors of psoralen (umbelliferone, cinnamic acid and NADPH).

4.3.3. Plant regeneration and micropropagation

In vitro regeneration and micropropagation protocols have been published for *P. corylifolia* (Baskaran and Jayabalan 2007; 2008; 2009a; Baskaran *et al*. 2011; Saxena *et al*. 1997), *R. graveolens* (Ahmad *et al*. 2010; Anis and Faisal 2005), and *A. majus* (Pande *et al*. 2002). Baskaran and Jayabalan (2011) evaluated the FC content of micropropagated plants and reported higher levels of psoralen than in plants grown under natural conditions. This increment could be due to *in vitro* stress (culture conditions) during the cultures and the addition of mineral salt solution (L2 medium) during acclimatisation. Massot *et al*. (2000) compared the FC content in different *R. graveolens* materials (cell suspensions, calli, micropropagated shoots and entire plants), and reported higher concentration in micropropagated shoots.

4.3.4. Hairy roots cultures

Due to its capacity of SM production this technique has been also used for FC production in *P. corylifolia, R. graveolens* and *A. majus.* Staniszewska *et al*. (2003) reported the presence of umbelliferone and bergapten in hairy roots of *A. majus*. Sidwa-Gorycka *et al*. (2003) observed a high level of bergapten and isopimpinelin, and the presence of other FCs such as psoralen or xanthotoxin in hairy roots of *R. graveolens*. Other authors have studied the root culture techniques, Baskaran and Jayabalan (2009d) reported higher level of FCs in suspension grown hairy roots than in solid media.

4.3.5. Genetic modification

To the best of our knowledge, no studies have been yet reported about genetic modification for FC production. Lièvre *et al*. (2005) reported an efficient *Agrobacterium*-mediated transformation method to obtain *R. graveolens* modified plants with a plasmid harboring neomycin phosphotransferase and β-glucuronidase encoding genes. Following this protocol, they obtained genetically-modified plants with a cinnamate-4-hydroxylase (C4H) gene under the control of the CaMV 35S promoter, although no data about FCs were reported.

Some genes involved in FC biosynthesis pathway have already been isolated and characterised (Larbat *et al*. 2007; 2009; Parast *et al*. 2011) however, the bottleneck is the development of genetic modification protocols for FC-producing species. In the future, the development of these protocols will allow controlling the synthesis of linear or angular FCs.

4.4. Genetic characterization by molecular markers

The *B. bituminosa* genome is currently uncharted and genome-wide markers have not yet been developed. Indeed, *B. bituminosa* could be considered a ‘genomic orphan’ species (Varshney *et al*. 2009) with almost no genomic resources available. New DNA sequencing technologies provide an opportunity to develop high quality molecular markers for such orphan species.

Breeding programmes in *B. bituminosa* have been developed (Project RTA2007-00046-00-00) and molecular markers would be a valuable support to these programmes (Collard *et al*. (2008). Markers provide the means to characterise genetic diversity within breeding programmes and help identifying new genetic diversity in the wild or in germplasm collections. Markers are valuable in determining or confirming pedigrees and for marker-assisted selection of traits that are difficult and/or expensive to measure. However, no genomic resources or high-quality codominant markers are available for genetic analysis in *B. bituminosa*. The few genomic resources readily available for molecular marker development for *B. bituminosa* consist largely of chloroplast gene sequences developed for phylogenetic studies (Doyle *et al.* 1997; Egan and Crandall 2008).

In the recent years, new high-throughput genome sequencing technologies have been developed and provide a relatively low-cost opportunity for rapid development of markers for a species like *B. bituminosa* that have few available genomic resources (for more information, see Introduction in Chapter II: Next generation DNA sequencing).

OBJECTIVES

OBJECTIVES

The general objective of this thesis is the application of Plant Biotechnology and Molecular Biology in order to over-produce secondary metabolites. This general objective is divided into two main objectives:

1. Genetic modification of *Vitis vinifera* L. cv. Sugraone with the *stilbene synthase 1* (*Vst1*) cisgene to over-produce *trans*-resveratrol.

2. Development of *in vitro* culture and elicitation techniques to over-produce furanocoumarins (FCs), and molecular biology tools for genetic variability studies in *Bituminaria bituminosa* (L.) Stirt.

a. Micropropagation and evaluation of the FC content in regenerated plants.
b. Plant regeneration from leaf explants and evaluation of the FC content in different plant materials.
c. Elicitation by ultraviolet radiation of calli and *in vitro* regenerated plants for FC over-production.
d. Detection and characterisation of endophytic bacterial contamination in *in vitro* cultures of *B. bituminosa*.
e. Development of a cDNA library and sampling of the leaf transcriptome using 454 GS-FLX pyrosequencing technology. Identification of simple sequence repeat (SSR) motifs to characterise a broad set of *B. bituminosa* accessions.

The thesis has been divided into two chapters, enclosing the studies carried out in *V. vinifera* (Chapter I) and in *B. bituminosa* (Chapter II). The organisation of chapters has followed a chronological development of objectives, and the results have been prepared for their publication in international scientific journals.

CHAPTER I: *TRANS*-RESVERATROL PRODUCTION BY ENGINEERED GRAPEVINES THAT OVER-EXPRESS THE CISGENIC *Vitis vinifera Stilbene Synthase 1* GENE.

CHAPTER II: DEVELOPMENT OF *IN VITRO* CULTURE, ELICITATION AND MOLECULAR BIOLOGY TECHNIQUES IN *Bituminaria bituminosa*.

1. Micropropagation from apical and nodal segments of *Bituminaria bituminosa* and the furanocoumarin content of propagated plants. J Hort Sci Biotech 87(1): 29–35, 2012.
2. Plant regeneration from leaf explants of *Bitumnaria bituminosa* and the furanocoumarin content of regenerated plants. Plant Growth Regulation (submitted).

   Elicitation by ultraviolet radiation of calli and *in vitro* plants for furanocoumarin over-production.
3. Detection and characterisation of endophytic bacteria from *B. bituminosa in vitro* cultures.
4. Next generation DNA sequencing technology delivers valuable genetic markers for the genomic orphan legume species, *Bituminaria bituminosa*. BMC Genetics 12: 104, 2011.

CHAPTER I

TRANS-RESVERATROL PRODUCTION BY ENGINEERED GRAPEVINES THAT OVER-EXPRESS THE CISGENIC *Vitis vinifera Stilbene Synthase 1* GENE

INTRODUCTION

Trans-resveratrol (syn. resveratrol) is a beneficial molecule to human health (Kiselev 2011). Resveratrol is a stilbene that prevents cardiovascular diseases and inhibits apoptotic cell death, thereby providing protection from myocardial ischemic reperfusion injury, atherosclerosis, ventricular arrhythmias (Fernández-Mar *et al*. 2012), and thrombocyte aggregation through regulation of the synthesis of eicosanoids (Olas *et al*. 2001). Resveratrol has an intrinsic antioxidant capacity that could be related to its chemo-protective effects (Fernández-Mar *et al*. 2012) and may also provide some protection against certain types of cancer (Aggarwal *et al*. 2004). The anti-carcinogenic effects of resveratrol reside in its capability to inhibit cellular proliferation and have a direct cytotoxic effect in tumour cells (Shankar *et al*. 2007). Moreover, it may play a role in the prevention of diabetes and diabetic complications (Harikumar and Aggarwal 2008), extends the lifespan of *S. cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Howith *et al*. 2003; Valenzano *et al*. 2006; Wood *et al*. 2004) and combats the neuronal dysfunction caused in Huntington's and Alzheimer's diseases, through the activation of sirtuin pathway (Parker *et al*. 2005). These silent information regulator enzymes or sirtuins (SIRTs family) have recently emerged as central players in the regulation of critical metabolic pathways such as insulin secretion and lipid mobilisation (Pacholec *et al*. 2010). Later studies have reported that resveratrol does not directly active SIRTs (Beher *et al*. 2009; Burnett at el. 2011; Pacholec *et al*. 2010) but SIRT1 activators and resveratrol interact with multiple unrelated targets including receptors, enzymes, ion channels and transporters (Pacholec *et al*. 2010).

In grapevine, the synthesis of resveratrol is controlled by a key enzyme, stilbene synthase, which belongs to a multigenic family composed by 21-43 genes (Jaillon *et al*. 2007; Velasco *et al*. 2007; Wiese *et al*. 1994), some of them have already been isolated and cloned (Sparvoli *et al.* 1994; Wiese *et al.* 1994)

The use of genetic engineering in grapevines to alter the stilbene biosynthesis pathway could be a powerful tool to establish a starting point for large-scale production of resveratrol. In fact, genetic transformation protocols using *Agrobacterium tumefaciens* are available for different *Vitis* sp. (Coutos-Thévenot *et al.* 2001; Fan *et al.* 2007; López-Pérez *et al*. 2008) and the transfer

of different *STS* genes into the *Vitis* genome has been possible (Coutos-Thévenot *et al.* 2001; Fan *et al.* 2007), obtaining modified plants with increased resveratrol levels, that produced an increase in the resistance to fungal infection.

In the present work, partially-cisgenic plants of Sugraone (Superior Seedless®) have been obtained, with a *Vst1* cisgene, a natural gene from grapevine. The modified plants exhibited resveratrol levels significantly higher than the control plants.

MATERIAL AND METHODS

Bacterial strains and plasmids

Agrobacterium tumefaciens EHA105 strain (Hood *et al.* 1993), a disarmed derivative of A281, was used for *in vitro* transformation of grapevine embryogenic calli.

The binary plasmid pKSTS706 was constructed in our laboratory using the Gateway cloning system (Invitrogen). RNA from Red Globe berries was extracted and cDNA from the stilbene synthase gene was amplified by RT-PCR using primers attB1full*Vst1*U1 (5' GGGACAAGTTTGTACAAAAAAGCAGGCTC TTCCTCAACTTAATCTTAGGCCT) and attB2polyT (5'GGGGACCACTTTGTA CAAGAAAGCTGGGT). Next, cloning of cDNA by Gateway BP recombination with plasmid pDONR221 (Invitrogen) was performed to obtain plasmid pEDSTS3105 that was used to transform *Escherichia coli* DH5α cells. Finally, after Gateway LR recombination between pEDSTS3105 and pK7WG2D (Karimi *et al.*, 2002, 2005), plasmid pKSTS706 was obtained and transferred by transformation into *E. coli* DH5α cells. Restriction and sequencing analyses were carried out to check that the construction was correct. Electroporation of *A. tumefaciens* EHA105 with this plasmid was carried out to obtain the strain EHA105-pKSTS706. The binary plasmid pKSTS706 contains the complete coding region for the *Vst1* cDNA under the CaMV35S promoter (p35S) and terminator and includes a kanamycin resistance gene under the control of the

nopaline synthetase gene promoter and terminator, as well as a GFP expression module provided by the *EgfpER* gene (Figure 3A).

Genetic Modification

For genetic modification, *A. tumefaciens* EHA105-pKSTS706 at OD_{600}=0.2 were co-cultured with embryogenic calli (0.08 g) from Sugraone for two days following the protocol described by Dabauza and Velasco (2012): calli were washed with MS (Murashige and Skoog 1962) + 900 mg l^{-1} cefotaxime for 20 minutes and cultured on ½MSAC (ANNEX I) with 300 mg l^{-1} cefotaxime. After ten days, calli were transferred to the same medium with 50 mg l^{-1} kanamycin to select modified somatic embryos. The cultures were maintained in darkness at 26±1 ºC and sub-cultured to fresh medium every 30 days. GFP-positive somatic embryos were transferred onto germination medium (ANNEX I) with cefotaxime (300 mg l^{-1}) and maintained at 25 ºC and 16:8 h photoperiod with a photon flux density of 45 µmol m^{-2} s^{-1} provided by Grolux fluorescent tubes (Sylvania). Germinated somatic embryos were cultured into test tubes on plant development medium (ANNEX I) with cefotaxime (300 mg l^{-1}) to regenerate plants. Plants were micropropagated on the same medium in 500-ml glass pots without cefotaxime.

Calli were examined periodically under a Leica MZ16F fluorescence stereomicroscope with a GFP-Plant filter that not blocks the red auto-fluorescence of chlorophyll. The light source was provided by an HBO 100W high-pressure mercury lamp. Photographs were taken using a Canon Power Shot S50 digital camera.

Eight calli (0.08 g fresh weight per callus) were used and the experiment was repeated twice. The number of genetically-modified somatic embryos was counted.

Acclimatisation of plants was carried out following the protocol described by López-Pérez *et al*. (2005). Well-rooted and elongated plants were transplanted into 10-cm pots with a mixture of 50% peat and 50% perlite, covered with a plastic bag and incubated in a chamber under constant conditions, 16:8 h photoperiod, 27± 1 ºC for 2 weeks. Then, the plastic bag was

gradually raised before being completely removed. For resveratrol analyses, plants were transferred to a greenhouse.

PCR analyses

Genomic DNA was isolated from young leaves according to Dellaporta *et al.* (1983). A standard PCR technique was used to determine the presence of the *EgfpER* and *nptII* genes, using specific primers that amplify the encoding sequences. The primer pairs used were 5´-ATGGTGAGCAAGGGCGAGGA-3' and 5'-GGACCATGTGATC GCGCTTC-3', that amplified a 600-bp fragment of the *EgfpER* gene and 5´-GACGAGGCAGCGCGGCTAT-3' and 5´-AAGAAGGCGATAGAAGGCGA-3', that amplified a 660-pb fragment of the *nptII* gene (Ghorbel *et al*. 1999). PCR reactions were performed in 25 µl containing 4 ng DNA, 0.4 mM dNTPs, 1.25 mM $MgCl_2$, 0.4 µM of each primer, 1x buffer and 0.5 U of GoTaq DNA polymerase (Promega). Reactions were subjected to 40 cycles of 30 s at 95ºC, 30 s at 55ºC and 1 min at 72ºC. Amplified DNA was detected by UV after electrophoresis on agarose gel (1% w/v) with ethidium bromide. In order to detect the introduced foreign *Vst1* gene and to discriminate from the endogenous *Vst1* gene, a region of 195 bp from the CaMV35S promoter was amplified using the primers 5´-GCTCCTACAAATGCCATCA-3' and 5´-GATAGTGGGATTGTGCGTCA-3' (Lipp *et al.* 1999) and PCR was subjected to 40 cycles in the same conditions described above.

Southern blot

Genomic DNA of the samples (20 µg) was digested with *Eco*RI, separated on 1% (w/v) agarose gels and blotted onto positively-charged nylon membranes (Hybond®, Roche, Basel, Switzerland) using a Bio-Rad 785 vacuum blotter system. Following the transfer, the DNA was UV cross-linked (0.120 Joules cm^{-2}, 254 nm) to the membrane prior to overnight hybridisation with DNA DIG-labelled probes (20 ng ml^{-1}) derived from the p35S region and the coding region of the *nptII* gene, obtained after a PCR with the above-described primers (Figure 3A). Detection of the hybridisation signals was carried out according to the manufacturer´s instructions (Roche).

Cisgene copy number determination

In addition to the Southern blot, quantitative real-time PCR was performed for cisgene copy number determination. For the analysis, individual samples were replicated three times in two independent experiments. All reactions were carried out in a 20 µl final volume containing 1 µl sample DNA (a total of 10 ng), 10 µl of 2× SYBR Green FAST qPCR Kit (KAPA Biosystems, Cape Town, South Africa), 2 µl of each primer (0.5 µM) and 5 µl sterile water. External controls representing 1–5 gene copies were prepared by diluting *Eco*RI-linearised plasmid pK7WG2D to a given concentration and adding the DNA to reaction mixtures that contained 10 ng of SS-C2 genomic DNA (control plant). The amount of plasmid DNA corresponding to a single copy of the gene per reaction was calculated as 10 ng (genomic DNA amount) × 1.28×10^4 bp (size of pK7WG2D)/4.75×10^8 bp (1C genome size of grape)=0.269 pg. The parameter used for cisgene copy number estimation was the crossing point (Cp) in qPCR analysis. The qPCR conditions were the following: 95°C for 3 min followed by 40 thermal cycles of 95°C for 15 s and 60°C for 45 s. The level of SYBR-specific fluorescence (483–533 nm) at the end of each cycle was measured and recorded via a CCD camera connected to the Bio-Rad iQ5 Thermal cycler, and was further analysed using the software Bio-Rad iQ5 System Software v. 2.1. Following the thermal cycling, melting-curve analysis was performed to verify amplicon sequence specificity. The cisgene indicator used for copy number estimation was the *35S* promoter.

RNA extraction and reverse transcription

For the RT-qPCR studies, the middle leaf of five plants per genetically-modified and control line was sampled in the month of August (15 individual samples in total). RNA was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich Chemie, GmbH) according to the manufacturer´s instructions and then immediately stored at -80°C until further use. To eliminate DNA contamination of RNA, DNase treatment was performed using RNase-free DNase I (Promega, Madison, WI). The RNA purity and yield were determined spectrophotometrically using a NanoDrop ND-1000 spectrophotometer

(NanoDrop Technologies, Wilmington, DE). RNA integrity was checked in 2 % agarose TBE gels. Five plants of each line were sampled along the period considered in this study. Reverse transcription (RT) was performed, in a 20 µl final volume, on one microgram of DNase-treated RNA using 20 U of MuMLV Reverse Transcriptase (Eurogentec, Seraing, Belgium) with 10 pmol of the reverse primers described in the next section.

Quantitative real-time PCR

Primers STS2-For (5'-ATCGAAGATCACCCACCTTG-3') and STS2-Rev (5'-CTTAGCGGTTCGAAGGACAG-3') used for *Vst1* expression analysis were designed with the Primer3 online tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Rozen *et al.* 1998). For the internal reference genes *Actin* and *NAD5,* we used the primer pairs described in Gutha *et al.* (2010).

By using the Bio-Rad iQ5 Thermal cycler, the real-time qPCR was done using the KAPA SYBR Green FAST qPCR Kit (KAPA Biosystems). The qPCR was performed in white 96-well PCR plates (Bioplastics RV, The Netherlands) with 100 ng of cDNA template, 10 µl of SYBR Green qPCR mix and 500 nM of each primer in a 20 µl reaction volume. Samples were subjected to the following conditions: 95°C for 3 min and 40 cycles of 95°C for 15 s, 60°C for 45 s. Product specificity for the *Vst1* gene was determined with the software package Bio-Rad Optical System Software v.2.1, by melting-curve analysis of 60 s at 95 °C, 60 s at 55 °C, followed by fluorescence reading at 0.5 °C increments from 55 to 95 °C (Bustin *et al*. 2009).

***Vst1* relative expression**

The relative expression of the *Vst1* gene, detected by real-time PCR, was compared as the means of five biological replicates and three technical replicates from each of the three lines studied (SS-E38, SS-E39 and SS-C2), based on the standard curve method as described by Pfaffl *et al*. (2006). Briefly, for *Vst1*, fold ratio determination was derived from the formula: ratio= E_{target} EXP(∆Cq(target)(control-sample))/E_{ref}EXP(∆Cq(ref)(control-sample)) where E_{target} refers to the amplification efficiency for the cisgene (*Vst1*) and E_{ref} is the

amplification efficiency for the reference genes (*Actin* or *NAD5*). The ΔCq values of samples were determined and normalised to the mean value of five plants of the control line SS-C2 included in each plate. For the calculus of the efficiency (E=10EXP[–1/slope]), the Cq of a dilution series of the positive control (plasmid) in three replicates ranging from 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were determined and the intercept and slope were calculated based on the linear regression. Similarly, the amplification efficiencies for the *Actin* and *NAD5* reference genes were obtained.

During the analyses, the *Vst1* expression effect was investigated. A total of 15 individual samples from two different genetically-modified lines (SS-E38 and SS-E39) and the control line SS-C2 were analysed, with three technical repetitions. Because the Cq data were not normally distributed, the values were log10-transformed for the calculations.

Resveratrol analyses

Frozen leaves of plants grown in a greenhouse were ground with a mortar and pestle in liquid nitrogen and a known concentration of keracyanin chloride was added as internal standard. Resveratrol was extracted with methanol, followed by centrifugation at 10,000 rpm (14,136xg) for 20 min to separate the insoluble residue. The extracts were evaporated to dryness under vacuum and dissolved in methanol/formic acid (97:3 v/v) before HPLC analysis. All procedures were carried out in a dark room under reduced light.

Samples of 20 µl of extract were analysed using an HPLC system (Hewlett-Packard, Germany) equipped with photodiode array UV/Vis and fluorescence detectors. Separation was performed on a 250 mm x 4 mm i.d., 5 µm reversed phase Lichrocart C_{18} column (Merck, Germany) with 6% acetic acid (A) and 5:30:65 acetic acid: acetonitrile: ultra pure water (B) as the mobile phase at a flow-rate of 0.4 ml min^{-1}. The chromatograms were recorded at 510 and 306 nm by a UV detector and, for fluorimetric detection the maximum excitation wavelength was measured at 270 nm and emission at 372 nm (Roggero and García-Parrilla 1995). Pure resveratrol (trans-3, 4´ 5-trihydroxystilbene) and keracyanin chloride were purchased from Sigma

Chemical Co. and used as external and internal standards, respectively. The peak assignment of resveratrol was based on the retention time of the standard and calibration curves were plotted versus known concentrations of commercial resveratrol. Resveratrol concentration was expressed as μg g^{-1} fresh weight (FW).

As resveratrol is induced by biotic and abiotic factors, to study the effect of environmental conditions, the resveratrol concentration was evaluated in two consecutives years, in five independent genetically-modified lines (SS-E24, SS-E27, SS-E28, SS-E38, SS-E39) and two control plants (SS-C1 and SS-C2). In the first year, sampling of leaves was performed at 0 (2008/09/19), 15 (2008/10/04) and 30 (2008/10/19) days after transfer of plants to greenhouse conditions. In the second year, plants were acclimatised in a greenhouse for 15 days (2009/06/16).

Statistical analyses

One-way analysis of variance was used to compare the means. When appropriate, the Student–Newman–Keuls (SNK) multiple range test was used to determine significant differences among groups. All effects were tested at the 5% significance level. For the statistical analysis, the SPSS v.13 (Chicago, IL, USA) statistical package was used.

RESULTS

Genetic transformation and plant regeneration

During the first 2 weeks of culture without selection, a high number of GFP spots were observed in embryogenic calli (Figure 1A-C). The culture with kanamycin significantly decreased the GFP expression. Two months after calli co-cultivation, independent somatic embryos of 5-8 mm with visible root primordia were isolated on medium with kanamycin. The number of GFP-somatic embryos recovered during the selection phase (1-2 months) was 247 (Figure 1C, D) and 33% of them were able to germinate in medium without

kanamycin. From germinated embryos, 26 independent partially-cisgenic plants (10.5%) were obtained (Figure 1E, F), micropropagated and acclimatised (Figure 1G) as described in Material and methods.

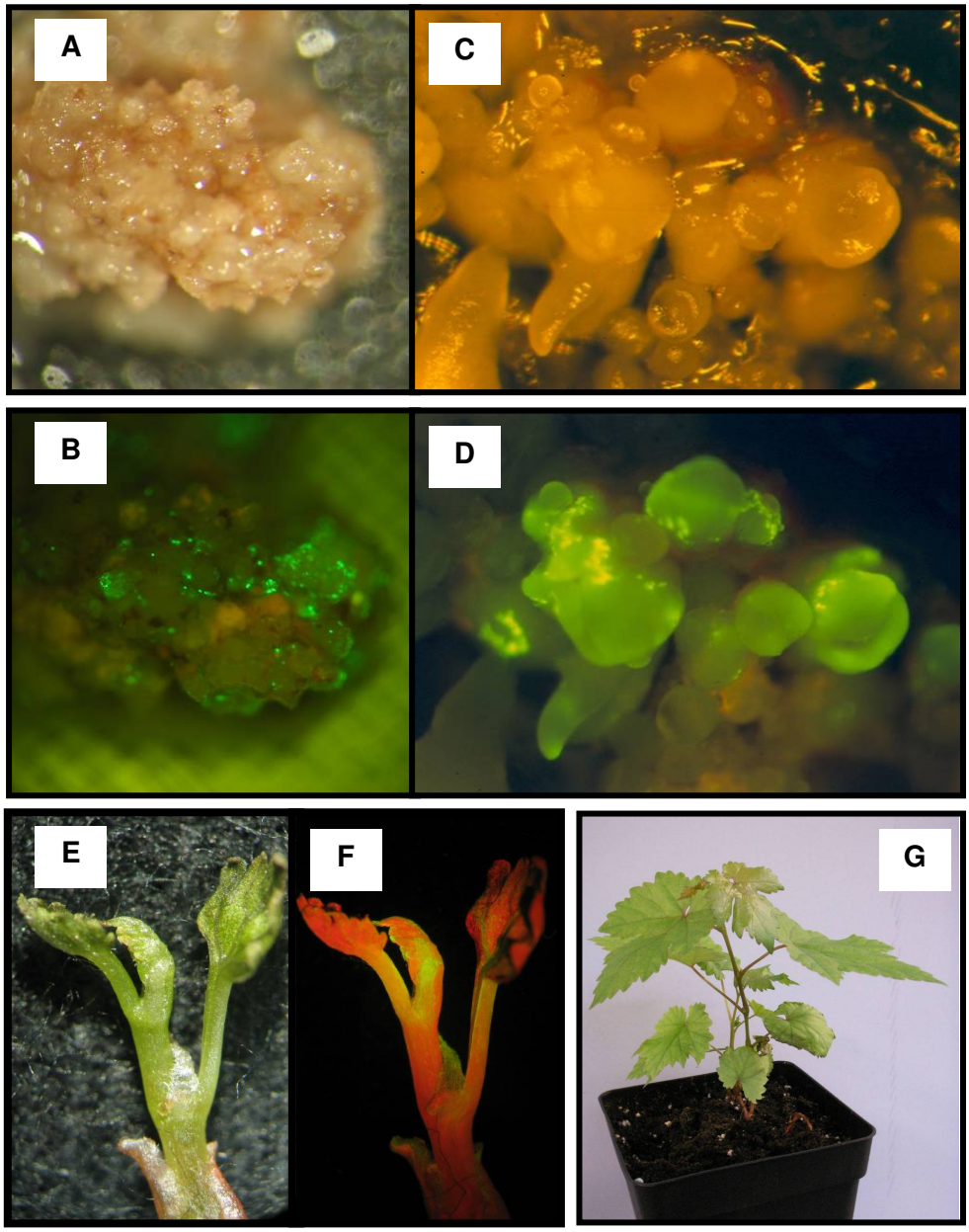
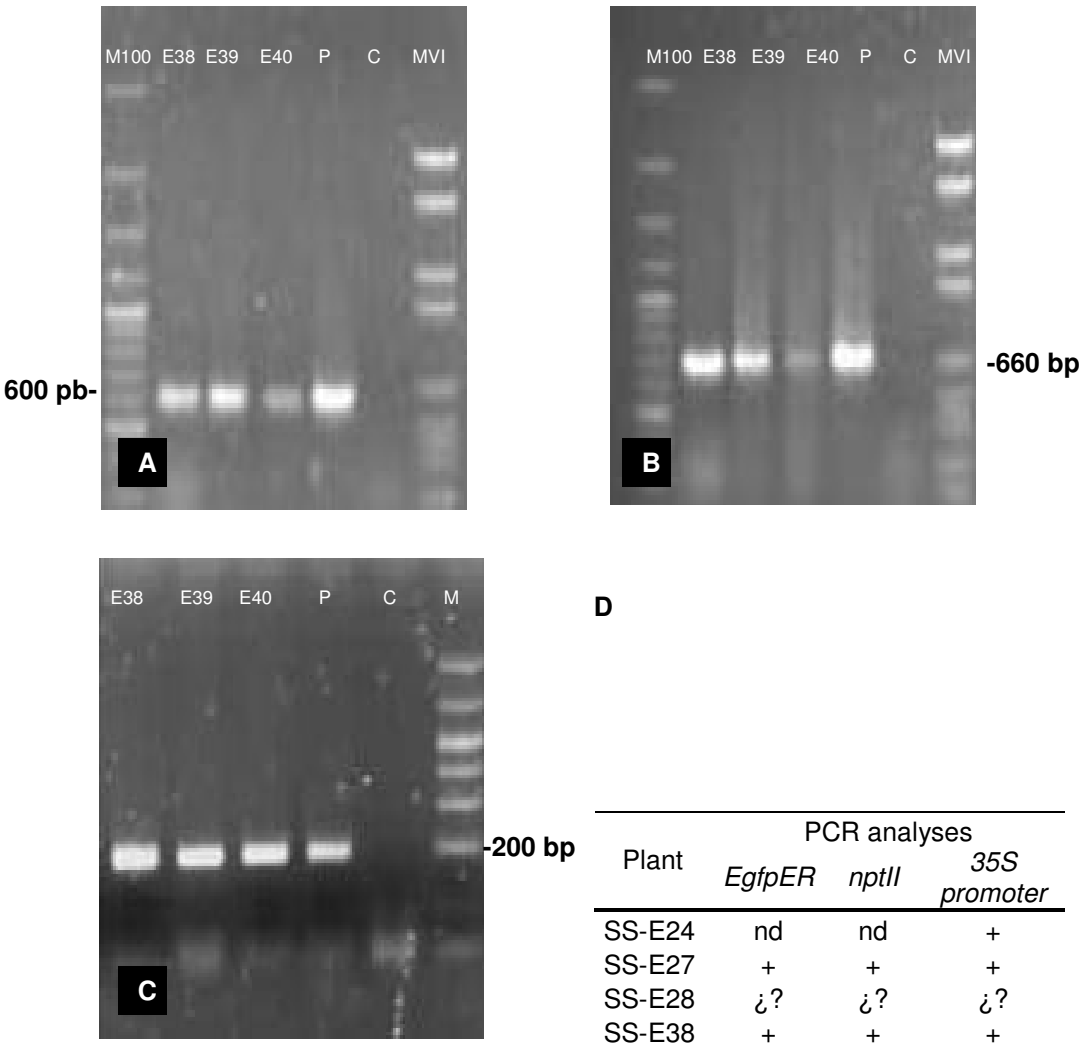


Figure 1. Different stages of development in genetically-modified Sugraone material (A-B) Transient GFP expression 5 d post co-cultivation. (C-D) Somatic embryos showing stable GFP expression at different stages of development. (E-F) Apical shoot showing stable GFP expression. (G) Partially-cisgenic plant over-expressing *Vst1* gene. A, C, E, and G: illuminated by white light; B, D, and F: illuminated by blue light.

PCR analyses

In grapevine, the stilbene synthase is encoded by a multigene family and several chalcone synthase genes have 70% homology with the stilbene synthase. This would make it difficult to analyse by PCR or Southern blot the integration of any grapevine *STS* gene. Thus, to analyse the Sugraone modified plants without any kind of doubts, PCR for the CaMV35S promoter and Southern blot with a fragment of this promoter as a probe were performed (Figure 3A).

The PCR analysis of some putative genetically-modified plants showed the amplification of the three transferred genes (Figure 2A-D): 600 bp for *EgfpER* (Figure 2A), 660 bp for *nptII* (Figure 2B) and 195bp for the CaMV35S promoter that reveals the integration of the *Vst1* gene (Figure 2C). Only in one modified line (SS-E28) were the results contradictory, as analyses carried out over time showed positive and negative amplifications, indicating that this line is possibly a chimera (Figure 2D)



Plant	PCR analyses		
	EgfpER	*nptII*	*35S promoter*
SS-E24	nd	nd	+
SS-E27	+	+	+
SS-E28	¿?	¿?	¿?
SS-E38	+	+	+
SS-E39	+	+	+
SS-E40	+	+	+

Figure 2. PCR products of the *EgfpER* gene (A), *nptII* (B) and *35S promoter* (C) in three regenerated genetically-modified Sugraone plants (E38, E39 and E40). P: Positive control (pKSTS706), C: negative control plant of Sugraone. M100, MVI and M: molecular weight markers. Results of three PCR products from the genetically-modified plants analysed for resveratrol production (D). nd: not determined, ¿?: ambiguous results

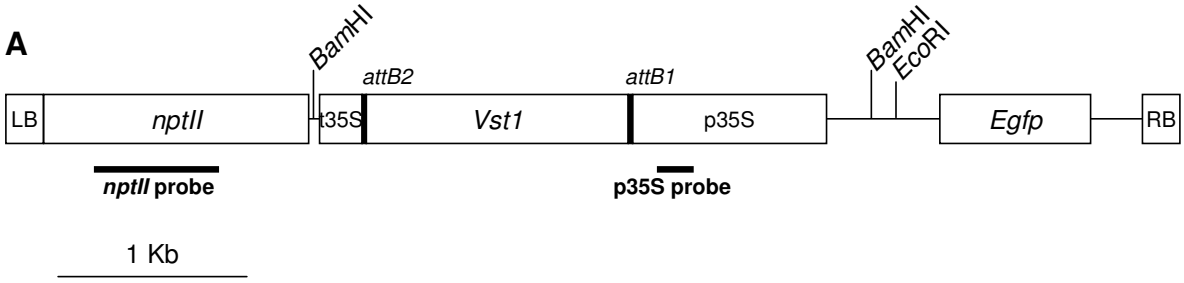
Southern blot

The stable insertion of the *nptII* gene and the CaMV35S promoter was confirmed by Southern Blot analysis (Figure 3). Digestion with *Eco*RI, which has a unique restriction site between the *Egfp* marker and the LB signal that comprises the p35S and the exogenous *Vst1* gene (Figure 3A), revealed one single, stable integration event in the two Sugraone SS-E38 and SS-E39

genetically-modified plants analysed for the *nptII* gene (Figure 3B) and for the p35S in the same plants (data not shown). No hybridisation signals were detected in non-modified control SS-C2 for either probe. The cisgene copy number in the genetically-modified plants was also estimated from quantitative real-time PCR experiments for the p35S. Cp values for the *p35S* in the genetically modified lines were compared with an equivalent copy number of plasmid dilutions. The results for the SS-E38 and SS-E39 indicated one single copy of the cisgene in both lines and none in the non-modified control (Table 1).

Amplification specificity of *Vst1*

A primer pair was designed for the reverse transcription and specific amplification of *Vst1* mRNA. The cDNA obtained was of the expected size (163 bp) and was cloned and sequenced, confirming the identity of the gene. Dissociation curve analysis done at the end of the RT-qPCR also demonstrated that the primer pairs amplified a single PCR product (data not shown).



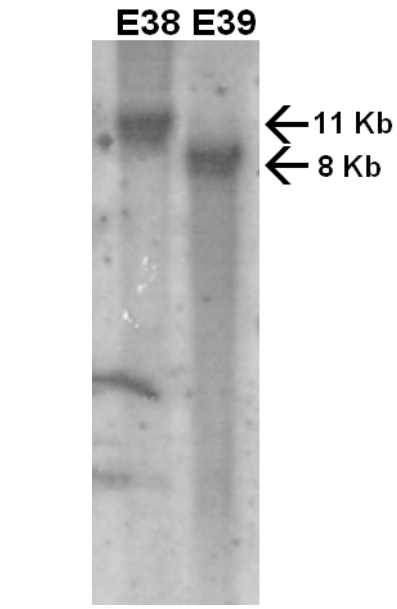


Figure 3. Southern blot hybridisation for the *nptII* gene. (A) Map of the T-DNA of pKSTS706 that includes the *Vst1* cDNA with the restriction site for *Eco*RI used to digest plant genomic DNA and the position of the *nptII* and p35S probes. (B) SS-E38 and E-39 cisgenic plants genomic digested DNA hybridised with the *nptII* probe

Table 1. Estimation of cisgene copy number in Sugraone plants using quantitative real-time PCR

Sample	Average Cp[a]	SD[b]	Estimated copy no.
SS-E38	26.02	0.29	1
SS-E39	25.51	0.42	1
SS-C2	31.61	0.47	0
Plasmid 1 copy	25.70	0.13	1
Plasmid 2 copies	24.80	0.40	2
Plasmid 3 copies	23.43	0.24	3
Plasmid 4 copies	23.19	0.44	4
Plasmid 5 copies	22.88	0.22	5

[a]Average values for p35S crossing points from three replicates in two independent experiments. [b]Standard deviation for the crossing points

Relative expression of *Vst1*

Quantification of molecular targets using real-time RT-qPCR requires a linear relationship between the threshold PCR cycle (C_T) defined during the exponential increase of the reporter's fluorescence and the logarithm of the initial target quantity in the corresponding sample. Serial dilutions in three replicas of target *Vst1* and the reference genes *Actin* and *NAD5* cloned DNAs were performed in order to obtain solutions containing from 10^0 to 10^{-5} copies of the target and internal controls sequences, in 1 µl. When such samples were tested by RT-qPCR assays, the standard curves showed a linear relationship between the amount of input DNA and the C_T values for the three templates over a range of six log units (Figure 4). Curves showed high correlation coefficients and were linear within the range of the standard and experimental DNA concentrations examined. Amplification efficiencies were calculated resulting in 2.10 for *Vst1*, 1.97 for *Actin* and 1.89 for *NAD5*.

The expression pattern of the *Vst1* gene was examined in the control line and in the two genetically-modified lines. Using two previously-described reference genes, *Actin* and *NAD5* (Gutha *et al*. 2010), Cq data from *Vst1* real-time PCR experiments were normalised and the relative expression levels are shown in Figure 5. *Vst1* gene expression was significantly higher in both genetically-modified lines with respect to the non-modified control. When the reference gene *Actin* was used, *Vst1* expression was, on average, eight-fold

higher for the SS-E38 than for the control. For the SS-E39, the fold increase was five times with respect to the control. When the reference gene considered is *NAD5*, *Vst1* expression in SS-E38 was 15-times higher with respect to the control while only 3.5-fold higher regarding SS-E39. In summary, *Vst1* expression was significantly higher in the genetically-modified plants than in the non-modified plants.

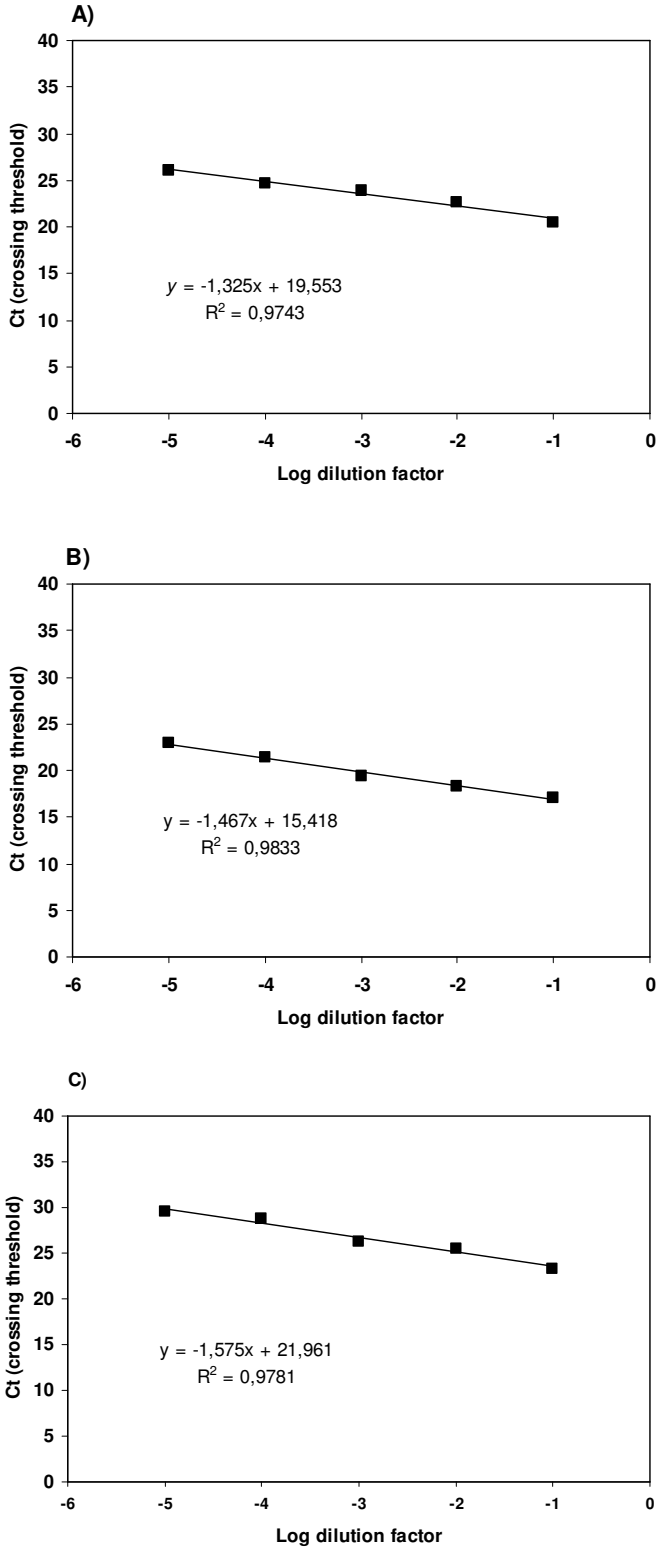


Figure 4. Evaluation of amplification efficiency and dynamic range of amplification of the target *Vst1* (A) and references *Actin* (B) and *NAD5* (C) specific DNA templates. Mean Ct values were obtained from serial log 10 dilutions of cloned sequences amplified by PCR. The figure inserts show the regression function and coefficient of determination.

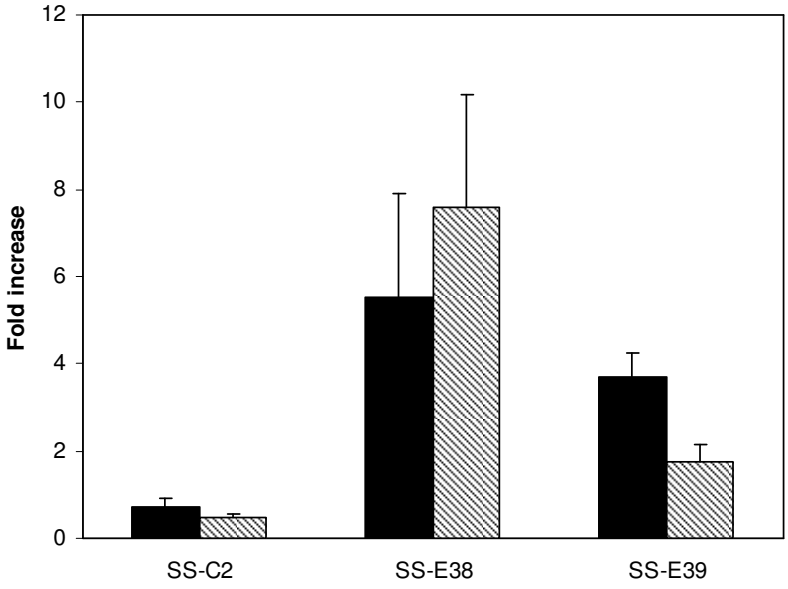


Figure 5. Relative expression of the *Vst1* gene in the control and genetically-modified lines. The relative expression levels for the *Vst1* gene are shown for the leaves of the non-modified control SS-C2 and the two genetically-modified lines studied, SS-E38 and SS-E39. Columns represent mean values from five biological replicates. Fold increase values were normalised to the reference genes *Actin* (black) or *NAD5* (dashed). Bars represent the standard error of the means. Significant differences in *Vst1* gene expression between the non-modified control and the genetically-modified lines were determined by one-way ANOVA, using the SPSS software v. 13 ($P < 0.05$).

Resveratrol analyses

As resveratrol is induced by biotic and abiotic factors, production of resveratrol was analysed by HPLC in two consecutive years, in five independent genetically-modified lines (SS-E24, SS-E27, SS-E28, SS-E38, SS-E39) and two control plants (SS-C1 and SS-C2). In the first year, sampling of leaves was performed at 0, 15 and 30 d after transfer of plants to greenhouse conditions, to analyse the effect of some stresses on the resveratrol production (Figure 6A). The second one was performed with the same plants one year later to test the production (Figure 6B).

The mean concentration of trans-resveratrol was higher in engineered plants than in the controls. The different modified plants showed different resveratrol production. At 15 days after transfer to the greenhouse (Figure 6A),

the resveratrol production in modified plants was increased up to 7-fold (7 μg g^{-1} FW SS-E38) over the controls (up to 1 μg g^{-1} FW), and was stabilised. One year later, the resveratrol was quantified again in the same plants but in June, when plants are in a better physiological stage, and each modified plant maintained the resveratrol levels (Figure 6B). In accordance with the usual tendency throughout the grapevine life cycle, in November resveratrol levels fell drastically, to below 0.5 μg g^{-1} FW, and in December were hardly detected (data not shown).

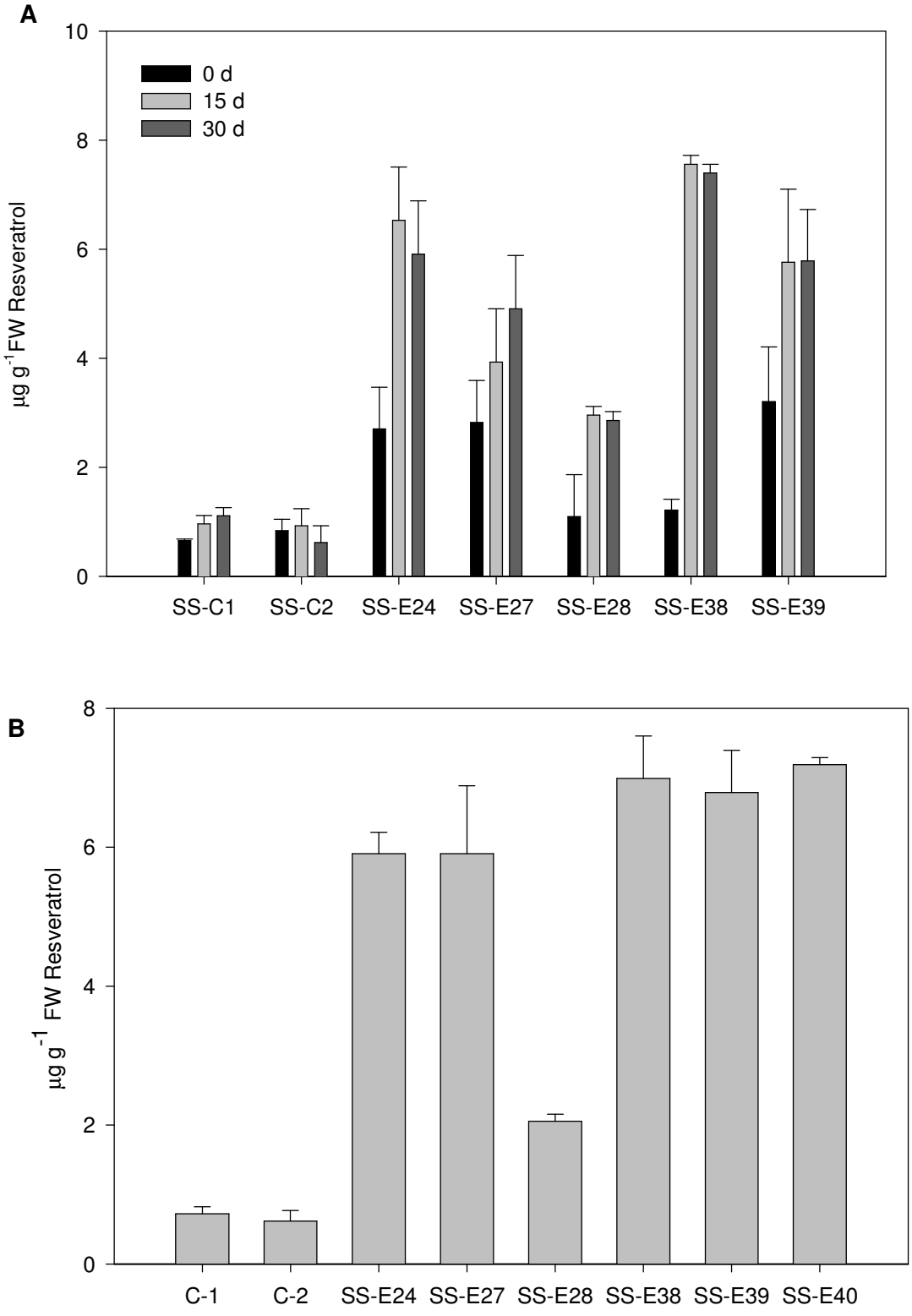


Figure 6. *Trans*-resveratrol content (µg g^{-1}) in fresh leaves of grapevine. (A) Production of resveratrol at 0, 15 and 30 days after transfer of plants to greenhouse conditions. Sampling of leaves was performed on 2008/09/19, 2008/10/04and 2008/10/19. (B) Production of resveratrol at 15 days after transfer of plants to greenhouse conditions. Sampling of leaves was performed on 2009/06/16.

DISCUSSION

In the present study, the over-production of resveratrol in genetically-modified grapevines with the pKSTS706 plasmid, that express the *Vst1* cisgene, was achieved. The partial cisgenic Sugraone plants also contain viral and bacterial genetic elements (used as promoters and terminators) along with a reporter marker gene. According to Dhekney *et al*. (2011), this methodology provides an avenue for the production of true cisgenic plants containing only *Vitis* sequences.

Genetic modification. After co-culture of Sugraone embryogenic calli with *A. tumefaciens*, many GFP spots were observed in the embryogenic calli during the first 2 weeks of culture without selection. After selection with kanamycin the GFP expression decreased significantly, as previously described by López-Pérez *et al*. (2008). Among 247 GFP-positive embryos recovered, 26 independent regenerated plants (10.5%) were obtained. Similar results were described by López-Pérez *et al*. (2008), where 15.5% of the Sugraone germinated embryos regenerated plants.

PCR analysis, Southern blot, amplification specificity of Vst1. In grapevine, the stilbene synthase is encoded by a multigene family, which makes difficult to analyse by PCR or Southern blot the integration of any grapevine *STS* genes. Different strategies have been adopted to analyse grapevine plants engineered with grape genes. Coutos-Thévenot *et al*. (2001) modified the 41B rootstock grapevine with the cDNA of the *Vst1* gene under the Pr10prom-Vst1 inducible-promoter and performed Southern blot with a probe of the the *nptII* gene, as an indirect approach to test transformation by the chimerical gene. Dhekney *et al*. (2011) used a specific forward primer at the junction region between the core promoter (duplicated CaMV35S enhancer) and the *vvtl-1* cisgene (thaumatin-like protein gene) start codon, and a reverse primer at the junction region between the stop codon of the cisgene and the terminator sequence (35S-T). Fan *et al*. (2007) transformed *V. vinifera* cv. Thompson Seedless with an STS gene from *V. pseudoreticulata* under the control of an enhancer CaMV35S promoter and used specific STS primers, and a DIG-labelled STS probe to analyse the transgenic plants by PCR and Southern blot. In our study, the expression of cDNA of the *Vst1* gene was

controlled by the constitutive CaMV35S promoter, and the use of specific primers for this promoter helped us to analyse, without any kind of doubts, the integration of the *Vst1* gene by PCR, Southern blot and RT-qPCR analyses. Moreover, a primer pair was designed for the reverse transcription and amplification of *Vst1* mRNA, and the cDNA obtained was of the expected size (163 bp) and was cloned and sequenced, confirming the identity of the gene.

Relative expression and copy number of Vst1. The cisgene copy number was estimated by Southern blot but also by quantitative real-time PCR, as described by Dutt *et al*. (2008) and Dhekney *et al*. (2009; 2011). The two modified Sugraone plants analysed by Southern blot and quantitative real time-PCR (SS-38 and SS-E39) showed only one copy of the cisgene. These plants were two of the higher resveratrol producers. The grapevine transgenic line tested by Fan *et al*. (2007) also carried a single copy of the STS gene. However, Coutos-Thévenot *et al*. (2001) refer to the expression (by RNA blot analysis) of the *Vst1* gene in modified 41B plants, after UV-induction, but do not refer to the copy number of the gene in the different grapevine modified lines obtained. Single gene insertion in transgenic plants is much more useful for eventual breeding purposes (Fan *et al*. 2007) although some studies showed no definite relationship between copy number of inserted genes and level of enzyme activity (Flavell 1994; Stam *et al*. 1997; Vaucheret *et al*. 1998; Zhu *et al*. 2004).

By using gene-specific RT-qPCR, this study presents evidence that the insertion of a cisgenic *Vst1* gene under the control of a CaMV*35S* promoter enhances the synthesis of specific mRNA. The increased levels of *Vst1* transcripts may explain the higher resveratrol production in the modified plants compared with the non-modified control.

Resveratrol analysis. As resveratrol production depends on both biotic and abiotic factors, including the physiological stage of the plant (Jeandet *et al*. 2002), leaves of partially-cisgenic plants were analysed from the day of transfer to greenhouse conditions (day 0) until several months after, and also in consecutive years. The production of resveratrol reached a maximum 15 days after transfer to the greenhouse, stabilised during the summer months until

October and then decreased dramatically from November, when the temperature decreases and plants start to die off.

The levels of synthesised resveratrol in the best seasonal conditions were from 2 to 7 μg g^{-1} FW (2 to 7-fold higher than the control) depending on the plant, SS-38 and SS-E39 being two of the best. In *in vitro* conditions resveratrol in leaves was undetectable.

The production of resveratrol in other modified grapevine plants has been evaluated. Coutos-Thévenot *et al*. (2001) achieved resveratrol over-production in eight of the 30 STS-modified plants of the 41B rootstock analysed, only after induction of the Pr10prom inducible-promoter (that controls the expression of the *Vst1* gene) by *Botrytis* infection of leaves from *in vitro* plants: 40-2000 μg g^{-1} DW (2-100-fold increase over the control). They estimated that 2000 μg g^{-1} DW from *in vitro* leaves correspond approximately to 200 μg g^{-1} FW. Without induction, the levels of resveratrol were undetectable. Fan *et al*. (2007) transformed the grapevine Thompson Seedless with an STS gene of *V. pseudoreticulata* under the control of an enhancer CaMV35S promoter, and leaves of *in vitro* modified plants were able to produce 2.5 μg g^{-1} FW resveratrol (5-fold greater than the control).

In the current work, the resveratrol levels obtained (2-7 fold greater than the control) were in the same range as these two other reports (2-100 fold greater), although comparisons between these different modified grapevine plants synthesising resveratrol are difficult because the promoters and genes were different and different types of leaves were used (greenhouse plants in the current work, *in vitro* plants in the other studies).

Using the *Vst1* gene, leaves of neither Sugraone nor 41B *in vitro* modified plants were able to synthesise resveratrol (this work and Coutos-Thévenot *et al*. 2001). But the expression of this gene was optimised by using the Pr10prom inducible-promoter, and so, 41B *in vitro* plants were able to produce resveratrol after induction by *Botrytis*-infection (Coutos-Thévenot *et al*. 2001). In the present work, the constitutive CaMV35S promoter was used, and only in growth chamber (data not shown) or greenhouse conditions were the cisgenic Sugraone plants able to produce resveratrol at a detectable level. Moreover, the

levels found were higher in greenhouse-grown plants than in chamber-grown plants (data not shown), and were higher in summer months than in cold months, showing an effect of environmental (abiotic and biotic) factors and the physiological state of the plant on resveratrol production.

The expression of *STS* genes in tobacco did not alter the normal growth of modified plants (Hain *et al*. 1993), but in other caused male sterility (Fisher *et al*. 1997; Ingrosso *et al*. 2011). In the current case, no morphological differences with respect to the control plants have been observed, although it will be necessary to wait for the first flowering period to test any putative abnormality in modified Sugraone plants.

In conclusion, partially-cisgenic Sugraone plants have been obtained, following the protocol established by López-Perez *et al.* (2008). The leaves of genetically-modified plants over-produced resveratrol at levels significantly higher than the control plants.

The resveratrol content was only examined in leaves, so evaluations in other parts of the plants should be done. Constitutive expression would be good for the control of pathogens that attack multiple sites on the plant, such as stems, leaves and berries of grapevine (Fan *et al*. 2007). In this sense, the resistance of the partially-cisgenic Sugraone plants to fungal diseases is under investigation.

As a future prospect, the establishment of cell suspensions obtained from leaf-calli and subsequently elicitation could be a good alternative to increase large-scale resveratrol production for commercial purposes.

ACKNOWLEGEMENTS

We thank A. Palazón and M. López-Romero for their collaboration and excellent technical assistance and Dr. D. Walker for his critical review of the manuscript. This research was supported by IMIDA (PR06-002) and by a studentship provided by IMIDA to M. Pazos-Navarro. M. Dabauza, P. Flores and P. Hellín were co-supported by the European Social Fund and IMIDA.

CHAPTER II

DEVELOPMENT OF *IN VITRO* CULTURE, ELICITATION AND MOLECULAR TECHNIQUES IN *Bituminaria bituminosa*

1. MICROPROPAGATION FROM APICAL AND NODAL SEGMENTS OF *Bituminaria bituminosa* AND THE FURANOCOUMARIN CONTENT OF PROPAGATED PLANTS

M. Pazos-Navarro[1], J.A. Del Río[2], A. Ortuño[2], P. Romero-Espinar[3], E. Correal[3] and M. Dabauza[1*]

[1]Department of Biotechnology and Crop Protection, Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), C/ Mayor s/n, 30150 La Alberca, Murcia, Spain

[2]Department of Plant Biology, Faculty of Biology, University of Murcia, Espinardo, 30100 Murcia, Spain

[3]Department of Natural Resources, IMIDA, C/ Mayor s/n, 30150 La Alberca, Murcia, Spain

Journal of Horticultural Science & Biotechnology (2012) 87(1): 29-35

INTRODUCTION

Bituminaria bituminosa (L.) C.H. Stirton (syn*. Psoralea bituminosa* L., *Fabaceae, Psoraleeae*; Stirton 1981) is a perennial species that is widely distributed in the Mediterranean Basin and Macaronesia. In the Canary Islands, the plants are grazed by small herbivores, but are mainly used for hay to feed milking goats (Méndez, 2000; Méndez and Fernandez 1990; Muñoz and Correal 1998). *Bituminaria* plants are also a source of secondary metabolites of pharmaceutical and medicinal interest, such as the furanocoumarins (Martínez *et al*. 2010) and pterocarpans (Maurich *et al*. 2004; 2006).

In the Canary Islands three botanical varieties of *B. bituminosa* (L.) C.H. Stirt. are found in habitats ranging from coastal semi-arid regions (var. *albomarginata,* referred to as "Tedera"), to sub-humid mountainous areas (var. *crassiuscula*). The third variety, *bituminosa*, is more widely adapted and is also found throughout the Mediterranean Basin. The *B. bituminosa* variety *albomarginata*, remains green with abundant vegetative growth and a high protein content during the hot, dry Mediterranean Summer. The latter, may therefore be considered as a good candidate for more extensive cultivation in the low-rainfall areas of Spain and other regions with Mediterranean climates such as Western Australia. In Australia, *B. bituminosa* is currently the subject of a breeding programme to develop commercial cultivars to improve pasture production during the dry Summer period (Real *et al*. 2008).

Among the many biological compounds that this species synthesises, furanocoumarins such as psoralen and angelicin are of considerable pharmaceutical interest. Psoralens are widely used both in cosmetics and dermatology to treat human skin diseases such as psoriasis, mycosis fungoides, vitiligo, photodermatitis, and eczema (Innocenti *et al*. 1991), while angelicin has calmative, sedative, and anti-convulsant activities and is used for the treatment of thalassemia [US Patent No: US2006/0111433(A1)]. *B. bituminosa* also contains other secondary metabolites of interest such as pterocarpans, which have anti-proliferative, estrogenic, hepatic-protective, anti-allergy, anti-inflammatory, apoptotic, and anti-tumour activities (Maurich *et al*. 2004; 2006; Pistelli *et al*. 2003). In addition, this species has potential as an

ornamental plant (especially var. *albomarginata*), and for the phyto-stabilisation of former mining areas contaminated by heavy metals (Walker *et al*. 2007).

Several research teams from Spain, Italy, Israel, Greece, and Australia are co-operating on research into *B. bituminosa* as a potential Mediterranean pasture species and in particular, the Spanish and Australian teams are carrying out a breeding programme in which genetic variability in *B. bituminosa* is being studied (Martínez *et al.* 2010). As a result of this breeding programme, the Spanish team at Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) has selected hybrid and non-hybrid lines showing high levels of expression of, or a combination of desirable traits such as high forage quality, tolerance to biotic or abiotic stresses, or high contents of furanocoumarins (FCs). Therefore, the establishment of an *in vitro* culture system offers an attractive approach for the large-scale propagation and conservation of germplasm resources of these plants, especially in relation to their potential pharmaceutical use as sources of FCs and pterocarpans. Other species such as *Psoralea corylifolia* and *Ruta graveolens* are currently being used to obtain FCs and the FC contents of these micropropagated plants and shoot cultures were higher in micropropagated *ex vitro* plants than in *in vivo* plants (Ekiert *et al*. 2001; Baskaran and Jayabalan, 2008).

In this report, our aim was to develop an efficient, rapid, and reproducible protocol for the *in vitro* establishment and micropropagation of two accessions that synthesise more psoralen than angelicin [var. *albomarginata* ("albo-tedera") from accession 'Famara-Lanzarote' and hybrid var. *bituminosa* x var. *albomarginata* ("bitu-hybrid") from accession 'Bullas-La Perdiz'] and two accessions that have a high angelicin:psoralen ratio [var. *bituminosa* ("bitu-Calnegre") from accession 'Calnegre-Murcia' and hybrid var. *albomarginata* x var. *bituminosa* ("albo-hybrid") from accession 'Purias-Famara'], using apical and nodal explants cultured on a modified Murashige and Skoog (MS; 1962) medium with organic additives and plant growth regulators (PGRs). FC contents were later examined in micropropagated plants that had been established in the field and in non-micropropagated mother plants (i. e., mother plants). To our knowledge, this is the first report on a protocol for the

micropropagation of *B. bituminosa* and on the FC contents of *ex vitro* (micropropagated) plants.

MATERIALS AND METHODS

Plant material and establishment of aseptic shoot cultures

Four accessions of *B. bituminosa* (L.) C.H. Stirt. were used: var. *albomarginata* (hereafter called "albo-tedera") from accession 'Famara-Lanzarote'; var. *bituminosa* (hereafter called "bitu-Calnegre") from accession 'Calnegre-Murcia' and two hybrid lines, one from a cross between var. *albomarginata* x var. *bituminosa* (hereafter called "albo-hybrid") from a 'Purias-Famara' accession, and another from var. *bituminosa* x var. *albomarginata* (hereafter called "bitu-hybrid") from a 'Bullas-La Perdiz' accession.

Stems 9 - 12 cm-long were collected from the IMIDA experimental field at La Alberca, Murcia, Spain. The stems were washed in running tap water, followed by treatment in 1% (v/v) commercial anionic detergent (Clen vajillas; Reckit Benkiser S.L., Barcelona, Spain) for 30 min. To study the effect of disinfectant concentration, apical and nodal explants of "albo-tedera" and "bitu-Calnegre" were cut into 1.5 cm fragments and surface-sterilised in sodium troclosene dehydrated (CTX-200/GR®; CTX S.A.U., Barcelona, Spain) at various concentrations (1.0, 1.5, 2.0, 2.8 or 3.5 g l^{-1}) each containing 0.1% (v/v) Tween-20, with shaking for 2 h. The explants were then immediately transferred to test-tubes containing 10 ml MS (Murashige and Skoog, 1962) basal medium with 3% (w/v) sucrose and 0.8% (w/v) Noble agar (Difco™; Becton, Dickinson and Company, Sparks, MD 21152, USA). The experiments were carried out in different seasons of the year: Spring (May), Summer (July), Autumn (October), and Winter (January). Seven hundred explants were cultured per treatment, and the experiments were repeated three-times. The number of contaminated explants was scored after 20 d.

Shoot multiplication

Healthy apical and nodal explants were used to study shoot multiplication capacity. The effect of agar type was evaluated using 0.8% (w/v) American bacteriological agar (Pronadisa; Laboratorios Conda, Madrid, Spain), European bacteriological agar (Pronadisa), or Noble agar (Difco^{TM}) in T1 medium [MS microelements and 0.5x MS macroelements, supplemented with 0.01 mg l^{-1} biotin, 100 mg l^{-1} casein hydrolysate, 100 mg l^{-1} L-glutamine, 2% (w/v) sucrose, 400 mg l^{-1} cefotaxime, and 35 mg l^{-1} gentamicin]. The frequency of hyperhydrated explants was scored at 30 d.

For shoot multiplication, the effects of adding various plant growth regulators (PGRs) or 2.5 g l^{-1} activated charcoal (AC) to T1 medium were studied: T2 medium [T2 medium= T1 medium+ 10 µM indole acetic acid (IAA) + 1.0 µM gibberellic acid (GA_3)], T1+AC medium, or T2+AC medium (see ANNEX I). Two explants were cultured per glass bottle, and a total of 100 explants were cultured per treatment. The experiments were repeated three-times. After 4 weeks, explants were sub-cultured on the same fresh medium (T1, T1+AC, T2, or T2+AC). The frequency of explants that exhibited shoot multiplication and the average number of shoots $\geq$ 5mm per explant were evaluated after 8 weeks of culture.

Rooting of shoots

To test for rooting ability, elongated shoots (1.5 – 2.0 cm) were excised and cultured on three different media: T1+AC, T2+AC, or T3 [T3 medium = T1+AC supplemented with 10 µM indole butyric acid (IBA)] (see ANNEX I). At least 30 shoots were cultured per treatment, and the experiment was repeated three-times.

In all experiments, the pH of the various media was adjusted to 5.8 with 1.0 M KOH before autoclaving at 1.06 kg cm^{-2} and 121ºC for 15 min. For shoot multiplication and rooting, explants were cultured in 300 ml glass bottles containing 100 ml culture medium. All cultures were maintained in a culture chamber at 25º $\pm$ 2ºC with a 16 h-photoperiod at a photosynthetic photon flux density of 45 µmol m^{-2} s^{-1}.

Acclimatisation of micropropagated plantlets

Plantlets with well-developed roots were extracted from the various culture media and their roots were gently washed with tap water to remove all agar. The plantlets were transplanted into 250 ml plastic pots containing autoclaved 3:1 (v/v) mix of Kekkilä® substrate (Kekkilä Iberia S.L., Valencia, Spain, N-P-K 15-10-20 + micronutrients):perlite and covered with an inverted glass bottle to maintain high humidity. All pots were placed in a cultured chamber, at 27º $\pm$ 2ºC with a 16 h-photoperiod at a photosynthetic photon flux density of 70.74 µmol m^{-2} s^{-1}. The glass bottles were raised-up for increasingly long periods until the plants were completely acclimatised, at which point they were transferred to a greenhouse (*ex vitro* plants). After 1 month, 30 *ex vitro* plants of each accession were established in the experimental field at La Alberca, Murcia, Spain (37º56´N, 1º08´W).

Furanocoumarin contents of micropropagated plants

After 18 months, selected micropropagated plants (15 plants of each hybrid and no-hybrid accessions) from the experimental field were evaluated for their FC content (i.e., psoralen and angelicin) by HPLC, and the values obtained were compared with those obtained from the non-micropropagated mother plants. The aerial parts (10 g FW of leaves and stems) of the different plants described above were dried to constant weight at 50ºC immediately after collection. The aerial parts (0.8 g) were then ground and were shaken with 14 ml of a 1:1 (v/v) mix of 100% (v/v) methanol:water. Each extract was hydrolysed with 1 ml 1.0 M HCl prior to quantification. The solutions were filtered through a 0.45 µm nylon membrane before analysis. A Jasco liquid chromatograph fitted with an analytical column (LiChroCARTR C_{18}; Agilent, Santa Clara, California, USA), with an average particle size of 5 µm (250 mm x 4 mm i d) at 30ºC was used for HPLC. To separate the furanocoumarins, a 50:50 (v/v) mixture of acetonitrile:water was used as solvent. The identities of psoralen and angelicin were confirmed by HPLC-MS, using a HPLC Agilent 1100 with an Agilent model VL ion trap mass spectrometer equipped with ESI interface.

Statistical analysis

Each experiment was repeated three-times. Shoot multiplication and hyperhydricity values were statistically compared for each accession using the chi-square test ($P \leq 0.05$) and means separation was carried out by Duncan's multiple range test. To compare the average number of shoots $\geq$ 5 mm per explant, the F-test was used for one-factor ANOVA and means were compared using Tukey's test. For FC contents, data were analysed using ANOVA and means were compared using Tukey's test and LSD. The significant difference level was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Establishment of aseptic shoot cultures

The effect of disinfectant concentration was similar in *B. bituminosa* "albo-tedera" and "bitu-Calnegre". No superficial disinfectant treatment was able to completely eliminate fungi and bacterial contaminants, 25.6 and 34.0% uncontaminated explants were obtained for each accession (Table 1). Concentrations greater than 3.5 g l^{-1} of sodium troclosene solution were detrimental for explant viability (data not shown). A concentration of 3.5 g l^{-1} sodium troclosene was therefore adopted, as this was the most effective concentration to reduce bacterial contamination, and was tested with the "albo-hybrid" and "bitu-hybrid", resulting in 25.8% and 19.2% uncontaminated explants, respectively. Further experiments were carried out to study the effect of collecting season. The best results were obtained in Spring and Summer (71 - 93% of uncontaminated explants for "albo-tedera", and 65 - 87% for "bitu-Calnegre", respectively) while in Autumn and Winter the number of non-contaminated explants fell significantly (32 - 5% for "albo-tedera" and 24 - 3% for "bitu-Calnegre", respectively). The effect of collecting season on surface-sterilisation has been studied by Hohtola (1988), who reported that contamination was significantly higher from December to April for tissue cultures of Scots pine, likely due to better resistance of the tissue against

microbes during the active period and/or resistance of microbes to the disinfectants during the winter.

Table 1 Effect of treatment with sodium troclosene at 1.0, 1.5, 2.0, 2.8, or 3.5 g l^{-1}, on contamination of explants of *Bituminaria bituminosa* after 20 d in culture.

Plant accession	Disinfectant treatment (g l^{-1})	Contaminated explants (%)		Uncontaminated explants (%)
		Fungi	Bacteria	
"Albo-tedera"	1.0	40.7 b[†]	58.0 b	1.3 c
	1.5	44.8 b	55.2 b	0
	2.0	42.7 b	31.7 c	25.6 a
	2.8	15.8 c	72.3 a	11.9 b
	3.5	60.9 a	16.0 d	23.1 a
"Bitu-Calnegre"	1.0	65.4 a	34.6 b	0
	1.5	59.4 a	39.3 b	1.3 c
	2.0	40.8 b	38.6 b	20.6 b
	2.8	13.3 c	52.7 a	34.0 a
	3.5	51.5 a	16.6 c	31.9 a

[†]For each plant accession and column mean values (n = 700) followed by a different lower-case letter indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test.

Shoot multiplication

Table 2 shows the effect of three different types of agar on the frequency of hyperhydricity in shoot explants. American bacteriological agar [0.8% (w/v); Pronadisa] provided the best results (i.e., the lowest number of explants exhibiting hyperhydricity). This effect could be due to differences among agars in ash content [Noble (2%) < European (4.5%) < American (6.5%)] and gel strength [Noble (700 g cm^{-2}) < American (600 – 750 g cm^{-2}) < European (800 – 1,100 g cm^{-2})], being American bacteriological agar the best gelling agent for *B. bituminosa.* This agar was therefore adopted for further experiments. Other reports have studied the effect of the type and concentration of gelling agent on the proliferation frequency and quality of regenerated shoots. Ichi *et al.* (1986) demonstrated that shoot propagation of *in vitro* cultures of tobacco, cabbage

and cucumber was enhanced when gellan gum was used instead of agar. Abdoli *et al*. (2007) reported that a lowest frequency of hyperhydrated explants was achieved with 0.8% (w/v) agar-agar on *in vitro* sunflower cultures.

The results for shoot multiplication are shown in Table 3 and Figure 1. Regardless of the accession, the frequency of shoot multiplication and the average number of shoots per explant were highest with apical explants rather than nodal explants; this may be due to apical explants having more than one bud. These results are similar to those reported for *in vitro Quercus robur* L. cultures by Puddephat *et al*. (1997), who observed that the explants from basal and apical positions contained more than one bud. In contrast, Anis and Faisal (2005) obtained higher number of regenerated shoots with nodal segments of *P. corylifolia*. Shoot multiplication frequency of apical explants (Table 3) was 80.0 - 93.8% for "albo-tedera" (Figure 1A, B), 88.9 - 95.7% for "bitu-Calnegre" (Figure 1G, H), 90.4 - 100% for "bitu-hybrid", and 80.0 - 94.7% from "albo-hybrid". Although no differences were observed among the media, the addition of AC increased the quality and development of regenerated shoots for all four accessions, which appeared less hyperhydrated. This could be explained by the beneficial effects of activated charcoal on plant tissue cultures reported by Wang and Huang (1976), suggesting improved growth due to adsorption of toxic compounds. López-Pérez *et al.* (2005) reported that AC increased the somatic embryo differentiation in different cultivars of *Vitis vinifera*.

The average number of shoots per explant for "albo-tedera" and "albo-hybrid" were higher from apical explants cultured on T2 media (Table 3) possibly due to these two accessions having shorter internodes than "bitu-Calnegre" and "bitu-hybrid", and so GA_3 added to this medium could improve shoot elongation, as Wochoka and Sluisa (1980) observed in *in vitro* shoot cultures of *Atriplex canescens*. Although nodal explants developed lower number of shoots per explant, the response on the culture media was similar to that of apical explants. Moreover, as mentioned above, the addition of AC increased the quality of regenerated shoots. So, the T2+AC medium was adopted for "albo-tedera" and "albo-hybrid" micropropagation (7.8 - 7.6 shoots per apical explants, and 4.0 - 4.6 shoots per nodal explants, respectively, Table

3) and T1+AC for “bitu-Calnegre” and “bitu-hybrid” (5.9 - 5.3 shoots per apical explants, and 2.4 - 2.3 shoots per nodal explants, respectively, Table 3).

Table 2. Effect of agar type on the incidence of hyperhydricity in *Bituminaria bituminosa* shoots cultures.

Plant accession	Type of agar (8 g l^{-1})	Hyperhydricity (%)
“Albo-tedera”	Noble	85.0 a[†]
	European	64.0 b
	American	40.0 c
“Bitu-Calnegre”	Noble	88.0 a
	European	60.0 b
	American	35.0 c
“Albo-hybrid”	Noble	87.9 a
	European	61.3 b
	American	36.0 c
“Bitu-hybrid”	Noble	90.0 a
	European	59.0 b
	American	37.8 c

[†]For each plant accession mean values (n= 100) followed by a different lower-case letter indicate significant differences at $P \leq 0.05$ by Duncan’s multiple range test. Noble= Noble agar (Difco™), European= European bacteriological agar (Pronadisa), and American= American bacteriological agar (Pronadisa).

Rooting of shoots

Rooting frequency depended on the plant material used and the three treatments tested. For “albo-tedera” and “bitu-Calnegre” rooting of shoots was 100% in all treatments, although it occurred at different times. Shoots cultivated on T2+AC medium developed roots significantly faster than in the other treatments, and root induction was achieved within 4 weeks of culture (Figure 1C, I). Similar results were reported by Anis and Faisal (2005) for *P. corylifolia*,

achieving up to 100% rooting with 0.5 µM IBA, and up to 90% with 0.5µM IAA, after 4 weeks of culture. T3 medium induced rooting within 5 weeks, but also induced calli (Figure 1D, J). With the T1+AC medium, rooting was achieved within 6 - 7 weeks.

For hybrid plants, the rooting frequency was significantly lower. For “albo-hybrid” 18.3% rooting was achieved with T3 medium, and 0 - 5.6% with T1+AC and T2+AC, respectively. For “bitu-hybrid” the best rooting was achieved with T1+AC medium (40.7%), while with T2+AC and T3 media, 5.1% and 14% were achieved, respectively.

Table 3. Effect of culture medium on the frequency of shoot multiplication and the average number of shoots ≥ 5 mm per apical and nodal explants of *Bituminaria bituminosa.*

Plant accession	Culture medium	Type of explant	Shoot multiplication (%)	Average no. of shoots per explant
"Albo-tedera"	T1	Apical	90.1 a[†]	3.5 b[‡]
		Nodal	65.8 b,c	2.8 b
	T1+AC	Apical	85.5 a	3.1 b
		Nodal	48.9 c	2.2 b
	T2	Apical	93.8 a	6.2 a
		Nodal	34.7 d	3.0 b
	T2+AC	Apical	80.0 a,b	7.8 a
		Nodal	50.0 c	4.0 b
"Bitu-Calnegre"	T1	Apical	95.7 a	6.3 a
		Nodal	49.0 b	3.9 a,b
	T1+AC	Apical	88.9 a	5.9 a
		Nodal	53.0 b	2.4 b
	T2	Apical	93.4 a	5.8 a
		Nodal	40.2 b	2.3 b
	T2+AC	Apical	89.6 a	5.1 a
		Nodal	38.5 b	1.9 b
"Albo-hybrid"	T1	Apical	90.3 a	2.0 c
		Nodal	50.7 b	1.5 c
	T1+AC	Apical	80.0 a	2.1 c
		Nodal	48.9 b	1.1 c
	T2	Apical	91.3 a	6.5 a
		Nodal	64.6 b	3.3 b
	T2+AC	Apical	94.7 a	7.6 a
		Nodal	64.7 b	4.6 b
"Bitu-hybrid"	T1	Apical	100.0 a	1.7 c
		Nodal	51.9 b	0.7 c
	T1+AC	Apical	98.1 a	5.3 a
		Nodal	66.4 b	2.3 b
	T2	Apical	90.4 a	4.1 a
		Nodal	41.3 b	2.0 b
	T2+AC	Apical	93.1 a	3.3 b
		Nodal	40.1 b	0.3 c

[†]For each plant accession mean values (n= 100) followed by a different lower-case letter indicate significant differences at $P \leq 0.05$ by Duncan's multiple range test. [‡]For each plant accession mean values (n= 100) followed by a different lower-case letter indicate significant differences at $P \leq 0.05$ by Tukey's test and LSD.

Acclimatisation

Rooted plantlets were successfully acclimatised in a culture chamber, obtaining 81.4% survival for “albo-tedera”, 84.8% for “bitu-Calnegre” (Figure 1E, K), 70.0% for “albo-hybrid” and 79.9% for “bitu-hybrid”. After 6 weeks, 30 plants were transferred to a greenhouse for further acclimatisation and a substantial increase in plant height was observed (Figure 1F, L). After 1 month in the greenhouse, micropropagated plants were established successfully in the experimental field (Figure 1M), and 95% survival was achieved. There were no significant morphological variations between regenerated and non-micropropagated mother plants. Similar results were reported in *P. corylifolia* by Anis and Faisal (2005) and Baskaran and Jayabalan (2007) reaching between 85 - 100% successful acclimatisation.

Furanocoumarin contents of micropropagated plants

Among the species reported as sources of FCs, *B. bituminosa* is a good alternative, because the level of total FCs obtained in “bitu-Calnegre” non-micropropagated mother plants was up to 19.07 mg g^{-1} DW (Figure 2B), higher than 10.00 mg g^{-1} DW reported for *R. graveolens* plants (Milesi *et al.* 2001) and the 1.49 - 6.06 mg g^{-1} DW reported for *P. corylifolia* (Baskaran and Jayabalan, 2008).

The FC contents of micropropagated plants of *P. corylifolia* have also been studied by Baskaran and Jayabalan (2008). They reported levels of psoralen slightly higher than those of non-micropropagated mother plants. In our study, the results depended on the *B. bituminosa* accession tested. For “albo-tedera” (Figure 2A), psoralen contents of micropropagated field-grown plants were similar to that in non-micropropagated mother plants, but angelicin was higher. In micropropagated plants of “bitu-Calnegre” (Figure 2B), large, significant differences were observed; angelicin contents increased and psoralen decreased compared to non-micropropagated mother plants. For the two hybrid lines (Figure 2C, D), no significant differences were found between the micropropagated and non-micropropagated mother plants. When the total FC contents were studied, only micropropagated plants of “bitu-Calnegre”

showed higher levels of FCs (22.24 mg g^{-1} DW) compared to the non-micropropagated mother plants (19.07 mg g^{-1} DW), although no significant differences were observed.

Figure 1. Micropropagation of *B. bituminosa* accession "albo-tedera" (Panels A-F): Panel A, shoot multiplication on T2 medium. Panel B, shoot multiplication on T2+AC medium. Panel C, rooting of shoots on T2+AC medium. Panel D, rooting of shoots on T3 medium. Panel E, plants acclimatised to growth chamber conditions. Panel F, plants acclimatised to greenhouse conditions. Micropropagation of *B. bituminosa* accession "bitu-Calnegre" (Panels G-L): Panel G, shoot multiplication on T1 medium. Panel H, shoot multiplication on T1+AC medium. Panel I, rooting of shoots on T2+AC medium. Panel J, rooting of shoots on T3 medium. Panel K, plants acclimatised to growth chamber conditions. Panel L, plants acclimatised to greenhouse conditions. Panel M, "albo-tedera" and "bitu-Calnegre" plants established in an experimental field. Scale bars = 5mm (Panels A, B, G, H); 20 mm (Panel C); 10 mm (Panels D, I, J); 40 mm (Panel E); 70 mm (Panel K); 200 mm (Panels F, L, M).

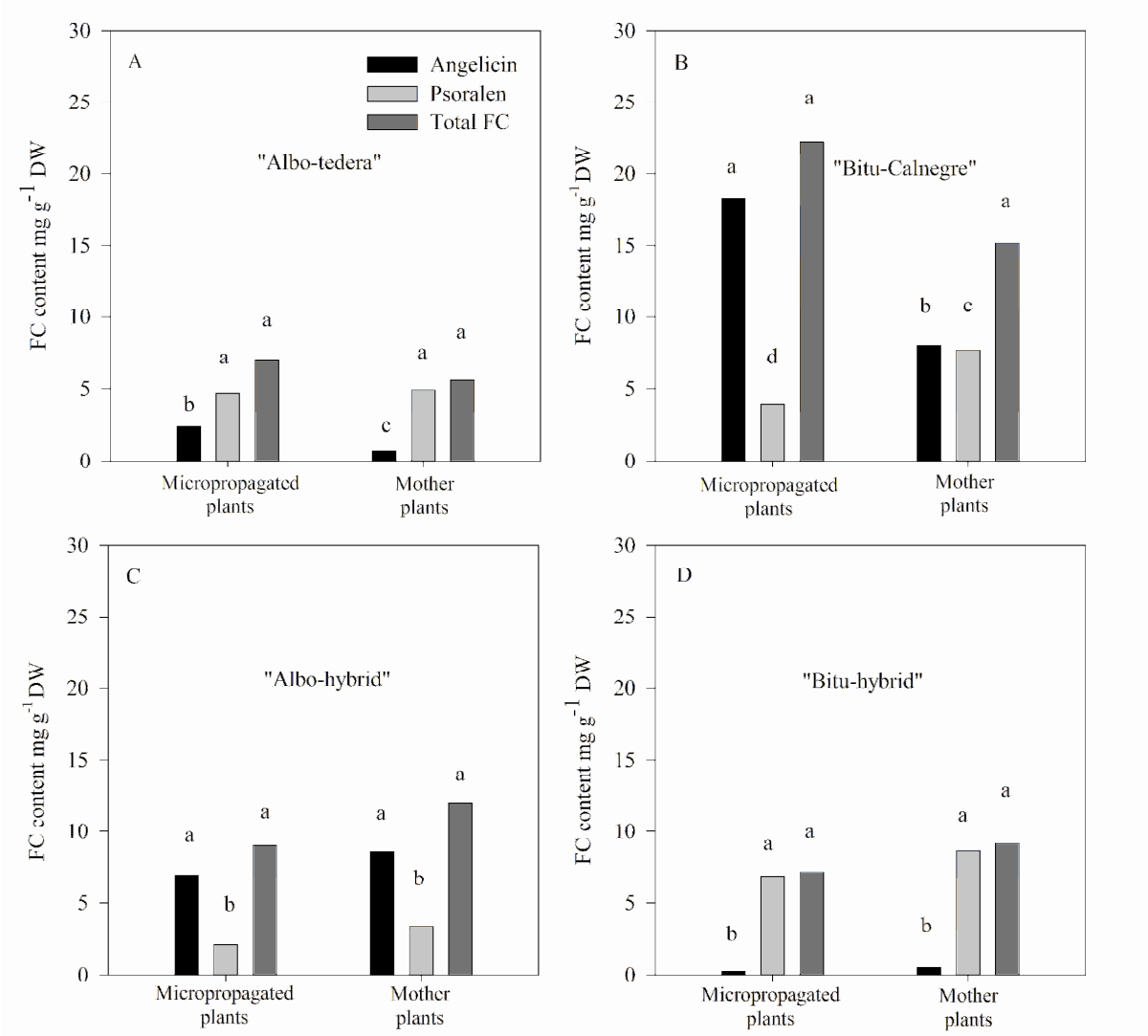


Figure 2. Total furanocoumarin (FC) contents in mg g^{-1} DW and levels of psoralen and angelicin in the aerial parts (i.e., leaves and stems) of the non-micropropagated mother plants ("Mother plants") and micropropagated plants established in the field ("Micropropagated plants") from "albo-tedera" (Panel A), "bitu-Calnegre" (Panel B), "albo-hybrid" (Panel C), and "bitu-hybrid" (Panel D). Columns in each Panel with a different lower-case letter indicate significant differences between means (n = 15) at $P \leq 0.05$ compared by the LSD and the Tukey's tests.

In conclusion, the present report describes a protocol for the large-scale micropropagation of *B. bituminosa* using apical and nodal explants from four plant accessions: *B. bituminosa* var. *albomarginata* ("albo-tedera") and *B. bituminosa* var. *albomarginata* x var. *bituminosa* ("albo-hybrid") cultured on T2+AC medium, and *B. bituminosa* var. *bituminosa* ("bitu-Calnegre") and *B. bituminosa* var. *bituminosa* x var. *albomarginata* ("bitu-hybrid") cultured on T1+AC medium. Micropropagated plants were established in the field and

contained FC levels equal to or even higher than those in the non-micropropagated mother plants. They also maintained the original plant characteristics of the non-micropropagated mother plants, showing that micropropagation of *B. bituminosa* offers a method to multiply plants selected for their high FC content, because they maintained the genetic identity of the non-micropropagated mother plants.

ACKNOWLEDMENTS

We thank Dr. J. Croser, Dr. D. Real, and Dr. D. Walker for their critical reviews of the manuscript, and A. Palazón, and V. Arnau for their collaboration and excellent technical assistance. This research was supported by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Project No. RTA2007-00046-00-00) and by a fellowship provided by IMIDA to M. Pazos-Navarro. M. Dabauza was co-supported by The European Social Fund and IMIDA.

2. PLANT REGENERATION FROM LEAF EXPLANTS OF *Bituminaria bituminosa*. UV-ELICITATION OF CALLI AND *IN VITRO* PLANTS FOR FURANOCOUMARIN OVER-PRODUCTION.

INTRODUCTION

In recent years, there has been increasing interest in the production and commercialisation of active biological molecules for use in human and animal healthcare. About 250,000 living plant species contain a much greater diversity of bioactive compounds than any chemical library made by humans (Raskin *et al*. 2002). Among the bioactive molecules, the furanocoumarins are reported as phytoalexins synthesised in the response to fungal infection (Tiejten *et al*. 1983) and against insects (Berembaum 1983), present pharmaceutical interest, and they are used in cosmetics, dermatology or haematology (Innocenti *et al.* 1991; Lampronti *et al.* 2003).

Plant regeneration protocols have been developed for FC producers as *Ruta graveolens*, *Ammi majus*, *Psoralea corylifolia* or *Apium* sp. (Ahmad *et al*. 2010; Baskaran and Jayabalan 2009a, b; Baskaran *et al* 2011; Pande *et al*. 2002). Baskaran *et al*. (2011) evaluated the FC content in regenerated plants acclimatised in the field and in natural field-grown plants of *P. corylifolia*, and reported that the accumulation of FCs was higher in natural field-grown plants and was tissue dependent, being higher in seeds than in leaves and roots. The FC content has also been reported from *in vitro* cultures (calli, cell-suspensions and shoots) of *R. graveolens* by Diwan and Malpathak (2010), who observed an increase in the FC synthesis as a function of the organization level in cultures.

These protocols would also allow the possibility of enhancing FC production by selecting somaclonal variants, obtaining diplo-haploid plants (Croser, personal communication), or elicitating *in vitro* cultures.

Several studies have demonstrated, under controlled conditions, the influence of different factors on the synthesis and accumulation of FCs, for instance ultraviolet (UV) radiation. *Apium graveolens* petioles, *Glehnia littoralis* roots*, Pastinaca sativa* seedlings and *R. graveolens* leaves treated with UV light showed an increase in linear FCs (Beier and Oertli 1983; Masuda *et al.* 1998; Zangerl and Berenbaum 1987; Zobel and Brown 1993). However, no effects on angular FCs of UV radiation or effects under *in vitro* conditions have been reported.

This work describes a high frequency of organogenesis and high *in vitro* plant regeneration using leaf explants of *B. bituminosa*. The linear, angular and total FC content (i.e., psoralen, angelicin and total content) was examined in organogenic calli, *in vitro* regenerated plants and acclimatised regenerated plants, and compared with plants grown under natural conditions. The induction of FC production by UV elicitation was evaluated in organogenic calli and regenerated *in vitro* plants.

MATERIALS AND METHODS

Plant material

Leaves and petioles of 30-day-old *in vitro* propagated plants of *B. bituminosa* var. *bituminosa* (accession 'Calnegre-Murcia', interesting for its high contents of FCs), monthly cultured in T1+AC (activated charcoal) propagation medium, were used (Pazos-Navarro *et al*. 2012).

Calli induction and shoot development

Leaflets were excised from *in vitro* plants, cut transversely to produce injury and placed with the abaxial side on the culture medium. Petioles were cut into three pieces, and the intersection between petioles and leaflets (hereafter called "cross explant") was cultured horizontally (Figure 1a). The three explant types were cultured on a modified Murashige and Skoog (1962) mineral solution (half strength macroelements, ½MS), supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} hydrolysed casein, 0.01 mg l^{-1} biotin, 100 mg l^{-1} L-glutamine and combinations of naphthalene acetic acid (NAA) or indole acetic acid (IAA; 5.0 or 0.5 μM) with benzylaminopurine (BA; 5.0, 10.0 or 20.0 μM) for calli induction. Calli were subcultured monthly to the same fresh medium.

Calli with buds and shoots were cultured on shoot development medium, with the same composition as above but with reduced plant growth regulators (PGRs; 5.0 μM BA and 0.5 μM NAA or IBA) for 30 days.

Rooting of shoots

Regenerated shoots (≥ 5 mm long) were excised and cultured on rooting medium containing ½MS supplemented with 20 g l^{-1} sucrose, 100 mg l^{-1} casein hydrolysate, 0.01 mg l^{-1} biotin, 100 mg l^{-1} L-glutamine, 100 mg l^{-1} myo-inositol, 2.5 g l^{-1} AC, 1 μM gibberellic acid (GA_3) and 10 μM of one auxin: NAA, IAA or IBA (indole butyric acid). The non-rooted shoots were subcultured monthly on the same medium, for 3 months.

Acclimatisation of plantlets

Rooted shoots were extracted from the culture medium and the roots were gently washed with tap water to remove agar. The plantlets were transplanted and acclimatised following the protocol described by Pazos-Navarro *et al*. (2012). After 1 month, 30 plants were established in the experimental field at La Alberca, Murcia, Spain (37º56´N, 1º08´W).

All the culture media were adjusted to pH 5.8 and solidified with 8 g l^{-1} American bacteriological agar (Pronadisa; Laboratorios Conda, Madrid, Spain) before autoclaving at 121ºC and 101 kPa for 15 min. For calli induction, explants (petiole, cross and leaflet) were cultured in 9 cm Petri dishes with 25 ml of medium. For shoot development and rooting, calli and shoots were cultured in 550 ml glass bottles with 100 ml of medium. Incubation was performed in a tissue culture chamber at 26 ± 1ºC under 16 h photoperiod and a photosynthetic photon radiation flux of 45 μmol m^{-2} s^{-1} provided by fluorescent tubes (Philips). At least 60 explants for each explant type were used per treatment and all experiments were repeated twice with similar results. The frequency of calli with shoots ≥ 5 mm and the number of shoots (≥ 5 mm) per explant were recorded after 4 months on calli induction media. The number of shoots (≥ 5 mm) per calli was recorded after 30 days on shoot development medium. The accumulative frequency of rooted plants was recorded over 3 months of culture on rooting medium.

Furanocoumarin content of different plant materials

Organogenic calli, *in vitro* regenerated plants, regenerated plants grown under greenhouse conditions and regenerated plants grown for 1 and 4 months under field conditions were evaluated for their FC content (i.e., psoralen and angelicin) by HPLC-MS, using an Agilent 1100 HPLC with an Agilent model VL ion trap mass spectrometer equipped with ESI interface, following the method described by Pazos-Navarro *et al*. (2012).

UV-elicitation of *in vitro* cultures for furanocoumarin production

For this experiment organogenic calli and *in vitro* regenerated plants were cultured in Steri vent containers (Duchefa Biochemie B. V., The Netherlands). After 30 days of culture, when plants were rooted and calli well grown, the lids were removed in a laminar flux cabin prior to irradiation. The UV radiation source consisted of PHILIPS TUV30W/G30T8 tubes ($8.6x10^{-3}$ W cm^{-2} UV-A and $18x10^{-3}$ W cm^{-2} UV-B). Three different irradiation times (5, 30 and 60 min) were tested on organogenic calli. Regenerated *in vitro* plants were exposed for 5 and 30 min. After UV irradiation, *in vitro* cultures were maintained in darkness for 2 hours before transferring to a growth chamber, as described above.

Samples from organogenic calli and regenerated *in vitro* plants were collected at 24, 48 and 72 hours after UV irradiation and the FC content was analysed following the methodology described above. Controls followed the same treatment except for the UV irradiation. For each treatment (irradiation time + collecting time after irradiation) two replicates of 12 calli or 12 *in vitro* plants were used and the average FC content was calculated. The results were represented as the FC increment between the UV irradiated material and its controls.

Statistical analysis

Each experiment was repeated twice. The frequencies of calli induction, calli with shoots ($\geq$ 5 mm) and rooting of shoots were compared statistically in a chi-square test and mean separation was carried out by Duncan's multiple

range test. To compare the average number of shoots per calli, the F-test was used for a one-factor ANOVA and means were compared using Tukey's test. For the FC content, the data were analysed using ANOVA and means were compared using Tukey's test. The significant difference level was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Calli induction and shoot development

In order to evaluate the ability of different explant types (Figure 1a) to induce calli, different combinations of PGRs at different concentrations were evaluated. Friable, greenish calli were initiated from the cut ends of the different explant types (Figure 1b) as Saxena *et al*. (1997) and Baskaran and Jayabalan (2009a, b) reported for *P. corylifolia*. Ahmad *et al*. (2010) reported that cell proliferation at the injured part of leaf explants of *R. graveolens* could be due to the accumulation of auxins at the point of injury, which stimulated cell proliferation in the presence of PGRs. After 4 weeks of culture, the aspect of calli was more compact and green (Figure 1c, d), and 100% of calli showed buds regardless of the PGR combination and explant type. Moreover, the development of shoots in calli induction media was observed (Table 1). The average number of shoots ($\geq$ 5mm) per explant depended on auxin and explant type, and was higher in medium with NAA than with IAA. The best explant type was cross, reaching up to 8.8 shoots per callus in medium with 0.5 µM NAA and 5 µM BA. Saxena *et al*. (1997), Baskaran and Jayabalan (2009a,b) and Ahmad *et al*. (2010) studied the effect of PGRs on plant regeneration in species such as *R. graveolens* and *Psoralea corylifolia*, and also reported that NAA and BA was the best combination for calli induction and shoot development from different explant types such as leaves, stems and hypocotyls.

Calli with buds were cultured on shoot development media, where the PGR concentrations were reduced (5 µM BA and 0.5 µM NAA or IAA). The average number of shoots ($\geq$ 5mm) per callus depended on the explant type and the previous calli induction medium (Table 2); although, in general, shoot

development media with NAA yielded better results. For leaflet and cross explants, plant regeneration was achieved by culturing them on 5 μM IAA + 10 μM BA (Figure 1e) followed by a second step on 0.5 μM IAA + 5 μM BA, obtaining 40 and 26 shoots per callus, respectively. For petiole explants the best combination was 0.5 μM NAA + 10 μM BA for calli induction and a second step on 0.5 μM NAA + 5 μM BA for shoot development, obtaining 21 shoots per callus.

Table 1. Effect of the plant growth regulator combination in the calli induction medium on the shoot development from three explant types of *B. bituminosa*. Average number of shoots ≥ 5mm per explant after 4 months of culture.

Calli induction medium: Plant Growth Regulator (μM)			Average number of shoots per explant		
NAA	IAA	BA	Petiole	Cross	Leaflet
0.5	-	5	1.7 b	8.8 a	0.8 c
0.5	-	10	1.8 b	7.7 a	0.7 c
0.5	-	20	2.4 b	7.8 a	0.4 c
5	-	5	1.5 b	8.0 a	0.5 c
5	-	10	1.0 c	0.0	0.0
5	-	20	2.2 b	0.5 c	0.0
-	0.5	5	0.0	2.0 b	0.0
-	0.5	10	1.2 c	0.5 c	0.0
-	0.5	20	0.0	0.0	0.0
-	5	5	0.0	1.0 c	0.0
-	5	10	0.3 c	3.0 b	0.7 c
-	5	20	0.0	0.0	0.0

Data represent average of two experiments, each with 60 explants for the three explant types. Means with the same letter were not significantly different according to Tukey's test ($P \leq 0.05$).

Shoot rooting and acclimatisation

In order to achieve rooting of shoots, three different auxins were evaluated at 10μM: NAA, IAA or IBA. Independent of the explant type used, the best results were achieved with NAA (Figure 1f and Figure 2), reaching up to 45% rooting along the 3 months of culture. With IAA, 25% rooting was achieved and no rooting was observed with IBA. Ahmad *et al*. (2010) and Saxena *et al*. (1997)

reported the effectiveness of various auxins and found that IBA was the best plant growth regulator for rooting *R. graveolens* and *P. corylifolia* shoots, in contrast with the results obtained in this report. Although the frequency of rooting was not very high, the acclimatisation was successful, achieving 96.4% survival in the greenhouse and 100% in the field (Figure 1g).

Table 2. Effect of auxin on shoot development of organogenic calli of *B. bituminosa* based on the calli induction medium and explant type used. Average number of shoots (≥ 5mm) per calli and frequency of calli with shoots (≥ 5mm).

Calli induction medium: Plant growth regulators (μM)			Shoot development medium: Plant growth regulators (μM)			Average number of shoots per calli (Frequency of calli with shoots)		
NAA	IAA	BA	NAA	IAA	BA	Petiole	Cross	Leaflet
0.5	-	5	0.5	-	5	16.8 b[†] (100% a)[‡]	29.3 a (75.0% b)	23.0 b (8.3% g)
0.5	-	10	0.5	-	5	21.0 b (100% a)	33.5 a (100% a)	1.7 d (100% a)
0.5	-	20	0.5	-	5	11.4 c (80.0% b)	21.0 b (100% a)	6.8 c (33.3% e)
5	-	5	0.5	-	5	7.5 c (33.3% e)	33.5 a (100% a)	4.7 d (16.7% f)
5	-	10	0.5	-	5	18.5 b (33.3% e)	27.3 a (100% a)	4.5 d (11.1% f)
5	-	20	0.5	-	5	15.0 b (11.1% f)	24.0 b (75.0% b)	18.0 b (16.7% f)
-	0.5	5	-	0.5	5	10.3 c (100% a)	10.5 c (100% a)	0.0
-	0.5	10	-	0.5	5	16.3 c (88.9% a,b)	8.8 c, d (50.0% d)	0.0
-	0.5	20	-	0.5	5	7.0 d (100% a)	3.0 e (50.0% d)	0.0
-	5	5	-	0.5	5	3.6 d (62.5% c)	3.3 e (66.7% c)	0.0
-	5	10	-	0.5	5	6.7 d (100% a)	40.0 a (100% a)	26.0 b (100% a)
-	5	20	-	0.5	5	3.1 e (100% a)	9.0 d (100% a)	1.2 e (66.7% c)

Data represent average of two experiments, each with 40 calli for the three explant types. [†]Means with the same letter were not significantly different according to Tukey's test ($P \leq 0.05$). [‡] Frequencies with the same letter were not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

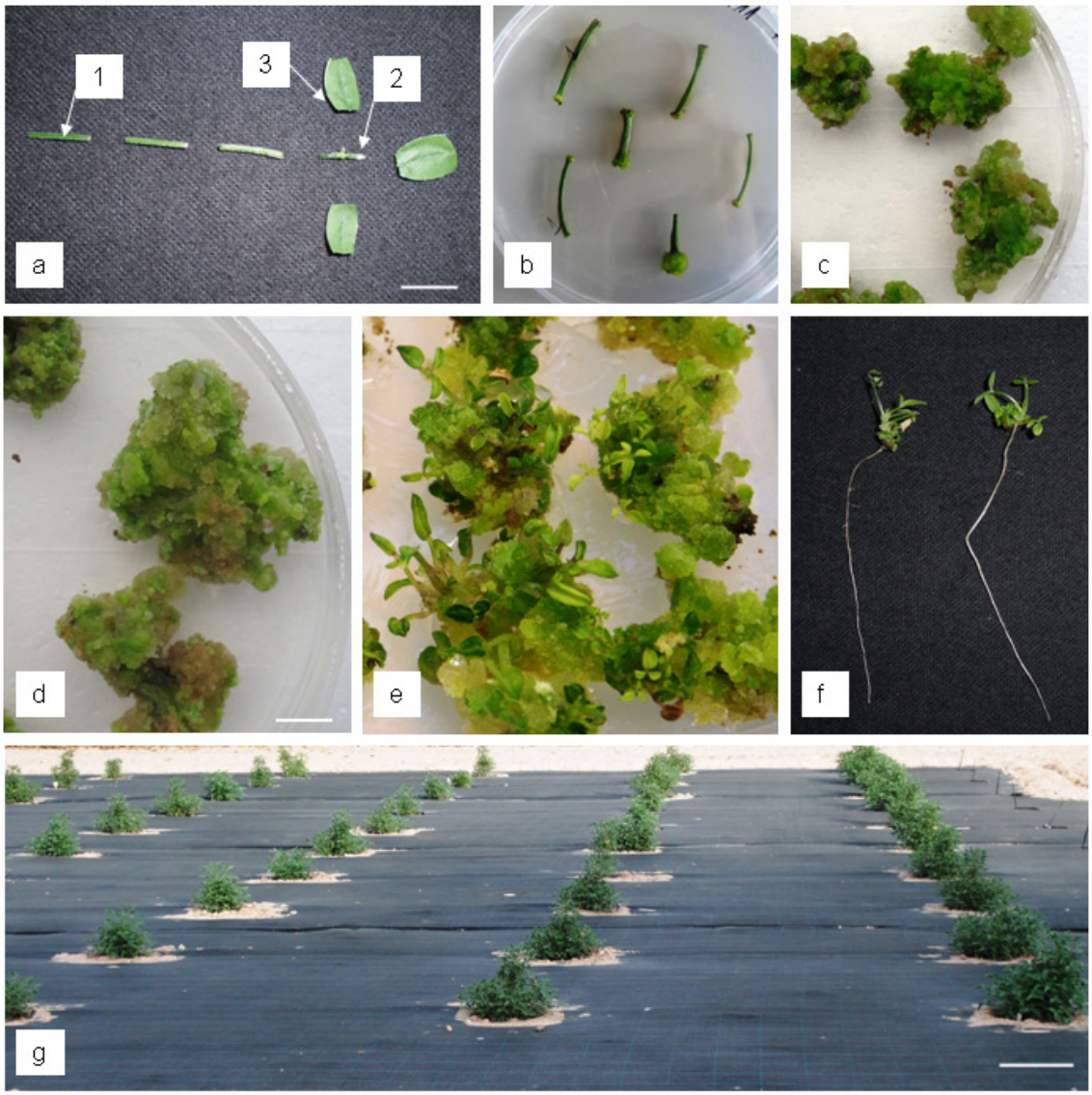


Figure 1. Different stages of the plant regeneration protocol in *B. bituminosa* (a-c and f, *bar* = 15 mm; d-e, *bar* = 10 mm; g, *bar* = 35 cm). **a** Different explant types used 1= petiole, 2= cross, 3= leaflet. **b** Calli formation at the cut injury of the petiole explants. **c** Calli and bud formation from cross explants on 0.5 □M NAA and 5 µM BA. **d** Calli and bud formation from cross explants on 5 µM IAA and 10 µM BA. **e** Shoot development on 0.5 µM IAA and 5 µM BA from cross calli obtained on 5 µM IAA and 10 µM BA. **f** Shoot rooting on 10 µM NAA and 1 µM GA_3. **g** Acclimatised, regenerated plants growing in the field for 4 months.

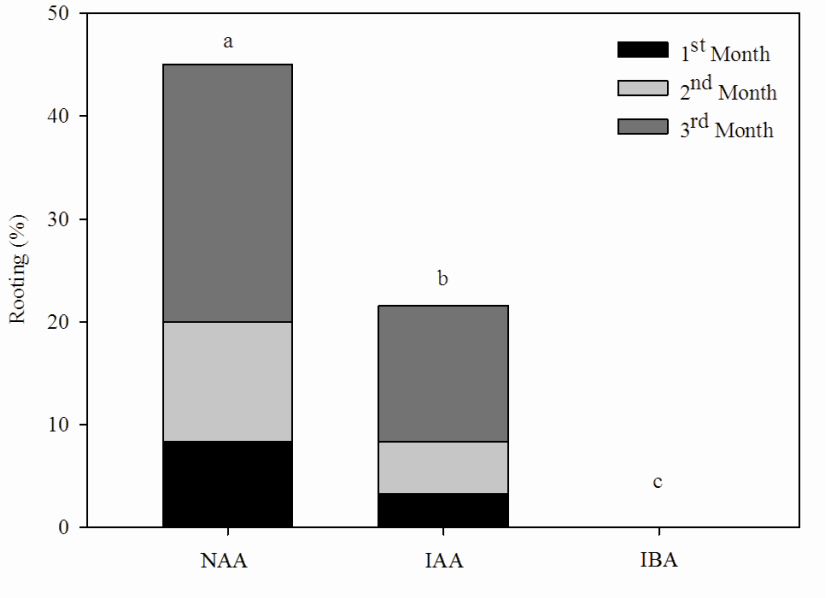


Figure 2. Effect of different auxins (10 µM NAA, IAA or IBA) in combination with 1 µM GA_3 on rooting of *B. bituminosa* shoots. Frequencies represent the accumulative rooting within 3 months of culture. Frequencies with the same letter were not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

Furanocoumarin content of different plant materials

Organogenic calli (i.e., "calli"), *in vitro* regenerated plants (i.e., "*in vitro* `plants"), regenerated plants grown under greenhouse conditions (i.e., "greenhouse plants") and regenerated plants grown under field conditions for 1 month (i.e., "field plants 1") or 4 months (i.e., "field plants 4") after their establishment were evaluated for their FC content (i.e., psoralen, angelicin and total content). The lowest amounts of FCs (Figure 3) were observed in "calli" (50 µg g^{-1} DW total FCs), and an increment in the FC production was observed in "*in vitro* plants" and in regenerated plants ("greenhouse plants", "field plants 1" and "field plants 4"). Diwan and Malpathak (2010) reported that the increased capacity for FC synthesis in organised cell cultures, in comparison with "calli", could be due to higher cellular differentiation and higher availability of sites for accumulation, which the unorganised cultures lacked.

Figure 3 shows that "field plants 1" synthesised approximately double the amount of FCs (7,810 µg g^{-1} DW total FC) of the "greenhouse plants" (3,448 µg

g^{-1} DW total FC), while "field plants 4" (9,824 μg g^{-1} DW total FC) had even higher levels. Baskaran *et al*. (2011) reported higher accumulation of psoralen in plants grown under natural conditions than in regenerated plants. In our case, the level of FCs found in plants of *B. bituminosa* grown under natural conditions was also higher (14,417 μg g^{-1} DW total FC). Pazos-Navarro *et al*. (2012) reported that micropropagated plants of *B. bituminosa* (accession "Calnegre"), grown for 18 months in the field, had FC levels equal to or higher than those of plants grown under natural conditions. This effect could be due to the fact that field plants suffered longer periods of biotic (i.e., bacteria and fungi) and abiotic (i.e., temperature, UV, water availability, etc) stresses and, like other phytoalexins, FC production could be incremented when a plant is attacked by herbivores (Reitz and Trumble 1997) or pathogens (Oginsky *et al*. 1959), or is subjected to adverse environmental factors (Martínez-Fernández *et al*. 2011). Therefore, it is possible that "field plants" could increase their FC synthesis over time.

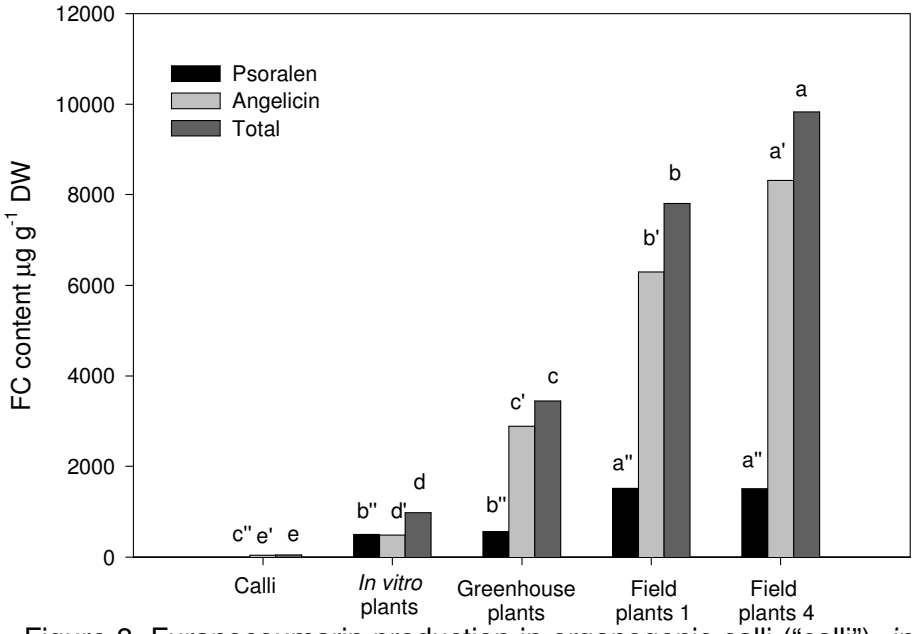


Figure 3. Furanocoumarin production in organogenic calli ("calli"), *in vitro* regenerated plants ("*in vitro* plants"), regenerated plants grown under greenhouse conditions ("greenhouse plants"), regenerated plants grown for 1 month under field conditions ("field plants 1") and regenerated plants grown for 4 months under field conditions ("field plants 4"). For each bar type, means with the same letter were not significantly different according to Tukey's test ($P \leq 0.05$).

UV-elicitation of *in vitro* cultures for furanocoumarin production

In general (Figure 4), the UV radiation produced a decrease in the FC content at 24 h after treatment, at 48 h no differences were observed, and at 72 h treated material produced more FCs than the control.

In organogenic calli (Figure 4 a, b), the highest increments in psoralen (linear), angelicin (angular) and total FC content were observed after 30 min of UV irradiation, reaching an increment of total FC content up to 24.58 μg g^{-1} DW at 72 h. A decrease in FC production was observed after 60 min of UV irradiation (data not shown), due to a great detrimental effect of UV radiation on the calli.

In *in vitro* plants (Figure 4 c), the best treatment was 5 min of UV irradiation, giving an increment of total FC content up to 207.33 mg g^{-1} DW over the control at 72 h. UV-irradiated *in vitro* plants for 30 min showed defoliation and a decrease of FC content (data not shown).

In other species the FC production in plants irradiated with UV was studied. In *A. graveolens* seedlings, Beier and Oertli (1983) showed an increase of 3-fold relative to the control, for linear FCs, after UV radiation. Zobel and Brown (1993) reported the same effect on *R. graveolens* leaves, increasing the linear FCs up to 20% over the control. In *P. sativa* seedlings grown in the field, Zangerl and Brown (1987) obtained an increase in linear FCs of 1.46-fold compared with the control. This increment of linear FCs may affect the parsnip resistance to herbivores, due to the UV-phototoxic effect of FCs on many organisms, including insects.

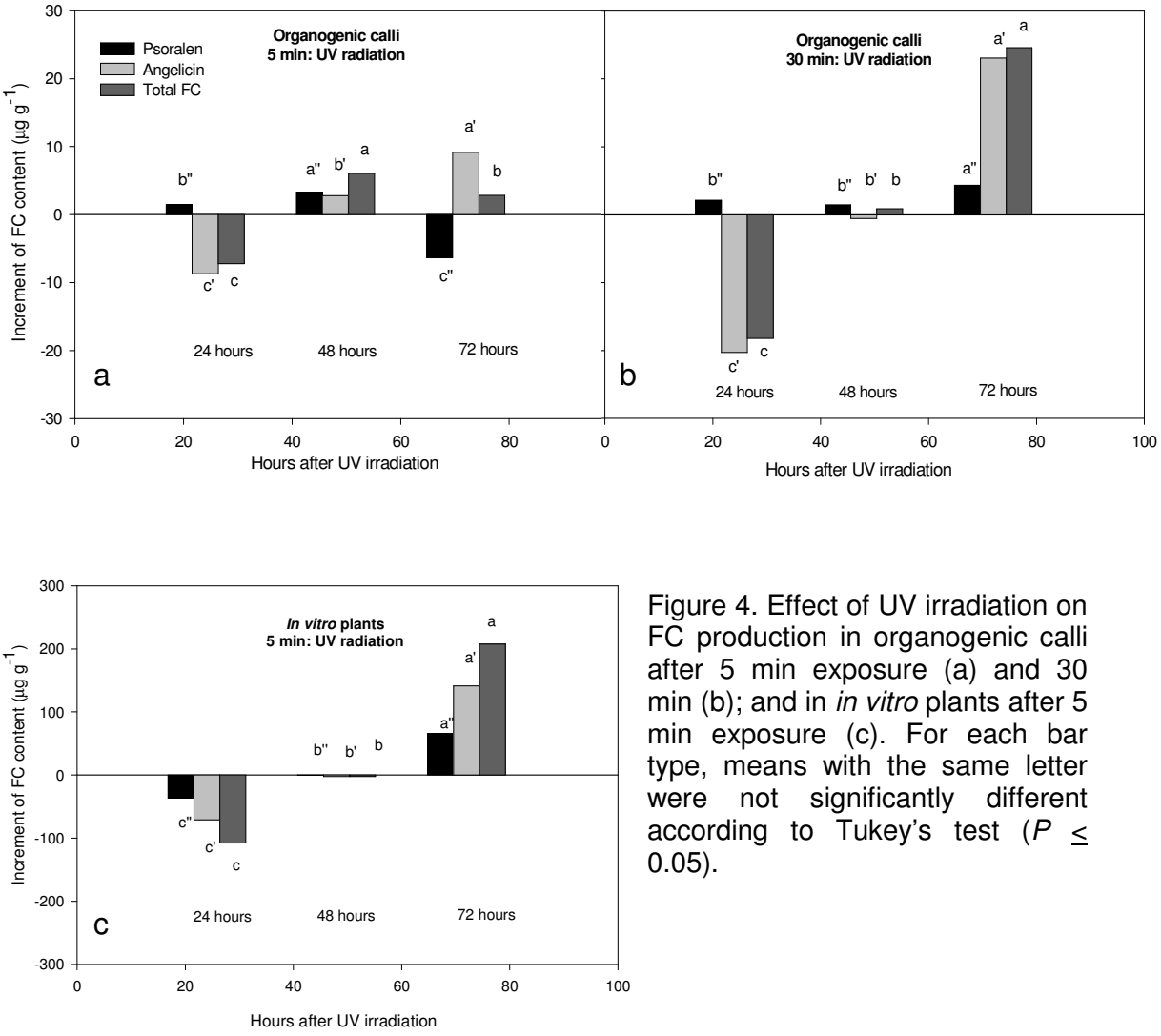


Figure 4. Effect of UV irradiation on FC production in organogenic calli after 5 min exposure (a) and 30 min (b); and in *in vitro* plants after 5 min exposure (c). For each bar type, means with the same letter were not significantly different according to Tukey's test ($P \leq 0.05$).

To the best of our knowledgem this is the first report about plant regeneration from leaf explants. For leaflet and cross explants, plant regeneration was achieved on 5 µM IAA + 10 µM BA for calli induction and on 0.5 µM IAA + 5 µM BA for shoot development. For petiole explants, the best combination was 0.5 µM NAA + 10 µM BA for calli induction followed by a culture on 0.5 µM NAA + 5 µM BA for shoot development. Shoots rooted on 10 µM NAA + 1 µM GA_3. The accumulation of FCs depended on the plant material evaluated, being higher in acclimatised, regenerated plants grown under field

conditions. UV radiation enhanced the angular and linear FC production under *in vitro* condition.

ACKNOWLEDGEMENTS

We thank A. Palazón and V. Arnau for their collaboration and excellent technical assistance. This research was supported by Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (RTA2007-00046-00-00), Ministerio de Ciencia e Innovación, (BFU2010-19599) and by a studentship provided by IMIDA to M. Pazos-Navarro. M. Dabauza was co-supported by the European Social Fund and IMIDA.

3. ENDOPHYTIC BACTERIA IN *Bituminaria bituminosa*: DETECTION AND CHARACTERISATION

INTRODUCTION

Plant regeneration protocols have been developed (Pazos-Navarro *et al*. 2012) to produce a high number of cloned plants as a source of biomass of *B. bituminosa*. During the establishment of 'aseptic' *in vitro* cultures, the presence of endophytic bacteria was observed associated with the plant material after several propagation cycles, even after several months of culture. Similar observations were made by Lata *et al*. (2006) in *in vitro* shoot cultures of *Echinacea*, where endophytic bacteria were noticed over a nine-month period. Hallmann *et al*. (1997) defined endophytic bacteria as microorganisms that do not visibly harm the host, live in the inner parts of the plants and can be isolated from tissues that have been previously surface-disinfected. Sometimes these bacteria are not known as pathogenic to plants growing *in vivo*, although they could act as vitropathogens in *in vitro* plant cultures (Leifert *et al*. 1994). Therefore, it is important to detect, identify and characterise plant-associated bacteria, to develop anti-bacterial therapies for plant tissue cultures (Leifert *et al*. 1991) and to eliminate persistent microbial contaminations (Reed *et al*. 1995; Tanprasert and Reed 1998).

On the other hand, endophytic bacteria may be beneficial, by improving plant stress tolerance (Nowak *et al*. 1998), and may induce or produce plant growth regulators that could modify the *in vitro* response of the tissue cultures (Holland 1997; Lata *et al*. 2006).

To the best of our knowledge, no search for endophytic bacteria has been carried out in *B. bituminosa*. Therefore, the aim of this study was to isolate, identify and characterise endophytic bacteria associated with *B. bituminosa*, and evaluate the resistance to antibiotics of bacteria and plant material. The potential biological characteristics are also presented.

MATERIAL AND METHODS

Plant material and *in vitro* culture techniques

In vitro cultures were established from four accessions of *B. bituminosa* (L.) C.H. Stirt.: var. *albomarginata* (hereafter called "albo-tedera") accession 'Famara-Lanzarote'; var. *bituminosa* (hereafter called "bitu-Calnegre") accession 'Calnegre-Murcia' and two hybrid lines, one a cross between var. *albomarginata* x var. *bituminosa* (hereafter called "albo-hybrid") 'Purias-Famara' accession, and another var. *bituminosa* x var. *albomarginata* (hereafter called "bitu-hybrid") 'Bullas-La Perdiz' accession, following the method described by Pazos-Navarro *et al*. (2012). Briefly, the method involves surface disinfection of the stems (9 - 12 cm-long), collected from the IMIDA experimental field at La Alberca, Murcia, (Spain) and, transferred immediately to test-tubes containing 10 ml MS (Murashige and Skoog 1962) basal medium with 3% (w/v) sucrose and 0.8% (w/v) American bacteriological agar (Pronadisa; Laboratorios Conda, Madrid, Spain). After 30 days of culture in a growth chamber at 25 $\pm$ 2ºC, with a 16-h photoperiod at a photosynthetic photon flux density of 45 µmol m^{-2} s^{-1}, non-contaminated sprouting buds were monthly sub-cultured on micropropagation medium (Pazos-Navarro *et al*. 2012).

Detection and isolation of bacteria

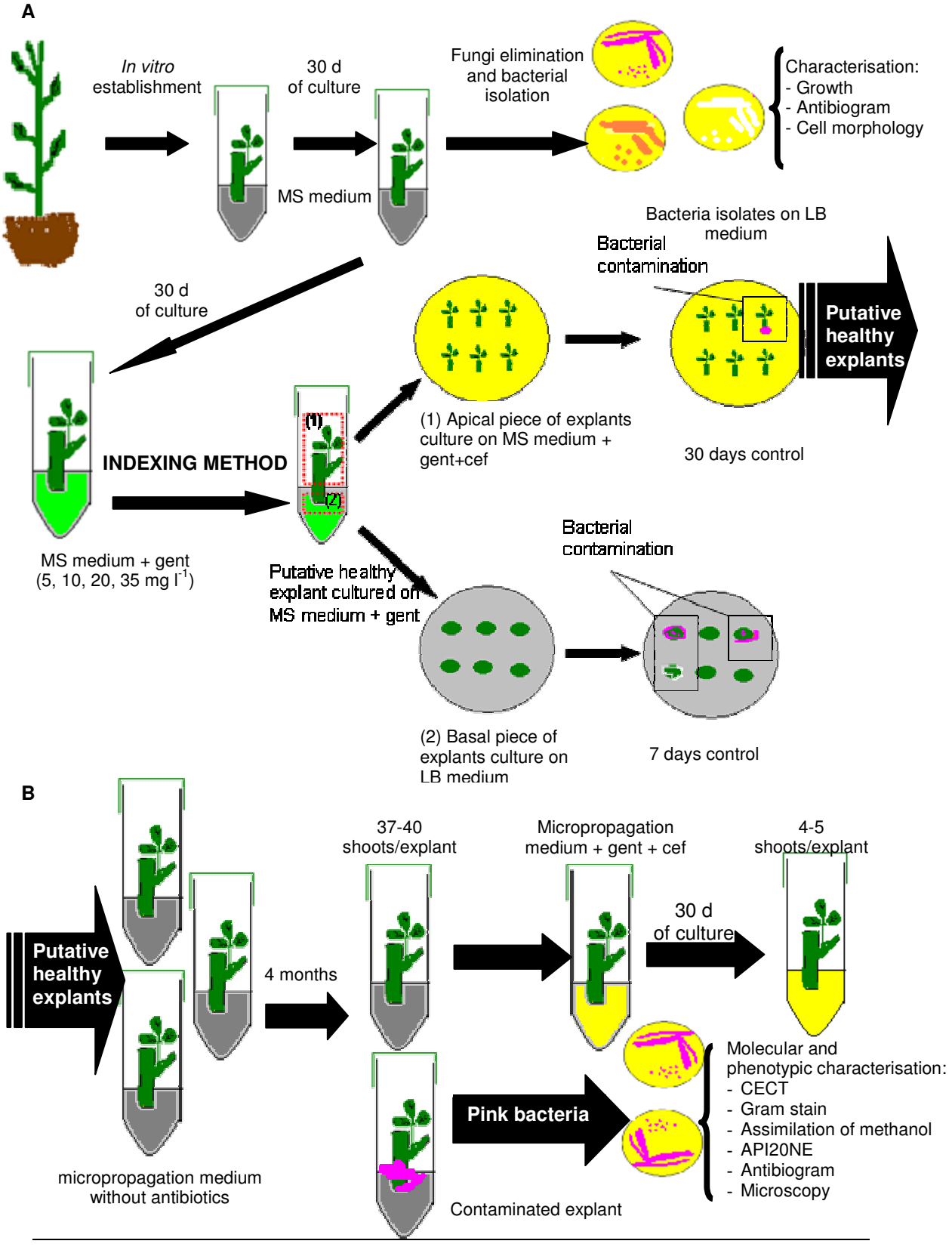
Bacterial isolates were obtained from contaminated explants after 30 days of culture on MS basal medium (Figure 1A). Bacteria were isolated and cultured in Luria-Bertani medium (LB: 10 g l^{-1} bacto-triptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, 15 g l^{-1} agar, pH 7) for 2 days at 28 ºC darkness. Isolates were purified by repetitive streaking and tentatively grouped according to their morphological and culture characteristics, including gram staining, distinctive colony colour, presence of diffusible pigments and morphology under phase contrast microscopy. Besides, bacterial isolates were also recovered from *in vitro* shoot cultures of "albo-tedera"

and “bitu-Calnegre” cultured for 6 months, and incubated on LB medium, following the same method .

Effect of culture media on bacterial growth

PYGA (Cornu and Michel 1987), MS (Murashige and Skoog 1962), LB and PDA media (Difco™; Becton, Dickinson and Co., Sparks, MD, USA) were used to study the growth capacity of the different bacterial isolates. Additionally, the following media were tested to further study two selected bacterial strains: Nutrient agar (Kato *et al*. 2005; Knief *et al*. 2008), R2A (Gallego *et al*. 2005), LM medium (Luedemann 1968), M72 medium (Sy *et al*. 2001), MMS medium (Guo and Lidstrom 2006), YMG medium (Salazar *et al*. 2006) and TYG medium (Kang *et al*. 2007) (For composition media see Annex II). In all cases, cultures were incubated at 30 ºC for 48 h darkness. Results were scored as percentage of bacterial growth on the Petri dish surface.

Figure 1. (A) Scheme of developed protocol to detect, characterise and eliminate possible endophytic contamination. (B) Scheme of micropropagation protocol and detection of endophytic bacteria. gent: gentamicin; cef: cefotaxime.



Bacterial resistance to antibiotics (antibiogram)

In order to control and eventually eliminate endophytic bacteria, the activity of 13 broad spectrum antibiotics (see Table 3 and Figure 1A) was tested at two different concentrations using a modified Kirby-Bauer disk diffusion method (Bauer *et al*. 1966). Briefly, bacteria were grown on Nutrient broth for 24 h darkness (30ºC and 240 rpm) and 0.5 ml of bacterial culture was surface inoculated on Petri dishes containing 25 ml of Nutrient agar. After the inoculum had dried, four cellulose discs without impregnation were placed aseptically on the agar surface and pressed down to ensure contact; afterwards 10 µl of antibiotics at two concentration (see Table 3) were added onto each disk. Stock solutionsof each antibiotic were previously prepared and filter-sterilised in the laboratory following the manufacturer's instructions. Plates were incubated for 48 h at 30 ºC and zone diameters (excluding the 6-mm disc, Figure 2) were measured. Bacterial response to an antibiotic was defined as follows, according to the diameter of the inhibition zone: resistant <4-6 mm; intermediate 6-12 mm; sensitive >12 mm.

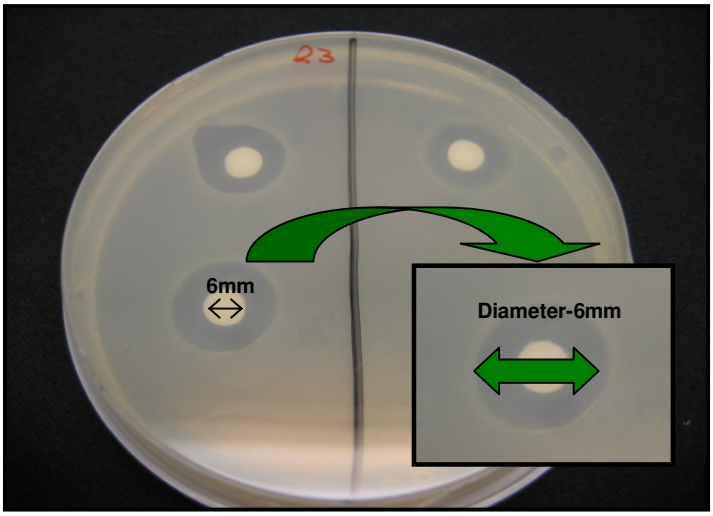


Figure 2. Image of an antibiogram plate with strain B and two antibiotics at two different concentrations. Zone diameter was measured as the diameter (green) minus the white disk (6 mm)

Effect of antibiotics on *in vitro* plant material (Indexing)

After 30 days of *in vitro* plant establishment, putative healthy explants were cultured on MS basal medium with the most-active antibiotics, gentamicin and cefotaxime, selected in the antibiogram (Figure 1A). Antibiotics were added aseptically to the culture medium after autoclaving. Twenty-four explants of "bitu-Calnegre" were cultured to test each treatment and the frequencies of contaminated and dead explants were scored after 30 d.

In a first assay, 4 different concentrations of gentamicin (5, 10, 20 and 35 mg l^{-1}) were evaluated. In the second experiment, an indexing method, developed by Viss *et al*. (1991) and modified in our laboratory, was applied and each putative healthy explant was cut into two pieces (Figure 1A):

1) The apical piece of the explant with sprouted shoots was sub-cultured for 30 days on MS basal medium with the best gentamicin concentration (35 mg l^{-1}) and 400 mg l^{-1} cefotaxime. The cefotaxime concentration was established following the experience, in our laboratory, of using this antibiotic in genetic transformation protocols for *Agrobaterium tumefaciens* elimination.

2) The basal piece of the explant (3 mm) was cultured on LB medium, to test the growth of any possible endophytic bacteria, and incubated at 30 ºC in darkness. After 7 days, the contamination of basal segments was recorded.

After 30 days of culture of each apical piece of explant, the presence/absence of bacteria in each explant was compared with results obtained at 7 days in LB medium for the respective basal piece of the explant, to determine the effectiveness of the antibiotics with regard to control of contamination and their effect on the plant material.

The putative healthy explants were sub-cultured on micropropagation medium (Pazos-Navarro *et al*. 2012) supplemented with 400 mg l^{-1} cefotaxime and 35 mg l^{-1} gentamicin (Figure 1B). The effect of the antibiotics on the average number of contaminated shoots per explant was evaluated after 4 months of culture.

Molecular identification and phenotypic characterisation of bacteria

Only two different pink bacteria were able to grow on micropropagation medium with 400 mg l^{-1} cefotaxime and 35 mg l^{-1} gentamicin (Figure 1B), from which they were isolated and sent to Colección Española de Cultivos Tipo, (CECT; Valencia, Spain) to be molecularly characterised by direct PCR amplification of the 16S rRNA gene, partial sequencing (both directions) and sequence BLAST analyses (Arahal *et al*. 2008) on the NCBI database (see ANNEX II for the methodology used by CECT). The identification was based on the amplified fragment, the percentage similarity being highest for the microorganism that presents the highest level of sequence identity with the database sequences.

Subsequent studies were performed only with these two bacteria, which were grown aerobically on Nutrient agar at 30 ºC for 48 h darkness and were subjected to different tests:

- *Gram stain according to Hucker´s method*, as described by Doetsch (1981).

- *Carbon source assimilation:* five organic compounds (maltose, glucose, malic acid, mannitol and trisodium acetate) at 0.15% w/v were used. Each compound was added to sterile basal saline medium (6 g l^{-1} NaCl, 0.075 g l^{-1} KCl, 0.1 g l^{-1} $CaCl_2$, 1.20 g l^{-1} K_2PO_4, 0.62 g l^{-1} KH_2PO_4, 2 g l^{-1} $MgSO_4*7H_2O$ and 0.1 g l^{-1} NH_4HCO_3) to evaluate its assimilation as the sole carbon and energy source.

- *Assimilation of methanol*. One of the isolates was a *Methylobacterium*, one of the pink-pigmented facultative methylotrophic bacteria (PPFMs) that assimilate C_1 compounds such as methanol (Omer *et al*. 2004b). The other isolate was also a pink-pigmented strain. The ability to use methanol was tested by adding it at different concentrations (0.25, 0.5 and 0.75% v/v) to the basal mineral solution of MMS medium (Guo and Lidstrom 2006).

- *API20NE test strips* (bioMérieux, France), which allow testing of 9 enzymes and assimilation of 12 carbon sources. The slow growth of the pink isolates meant that the final reading had to be made after one week of incubation.

- *Antibiogram.* Specific antibiograms were carried out to determine the

susceptibility or resistance of the pink bacteria, following the same methodology described above for “Bacterial resistance to antibiotics”.

- *Sensitivity to antibiotics.* Because of the slow growth of the pink isolates, and to avoid unclear results in the antibiogram, carbenicillin (500 mg l^{-1}), tetracycline (100 mg l^{-1}), ampicillin (100 mg l^{-1}), penicillin (100 mg l^{-1}) or cefotaxime (500 mg l^{-1}) were added to the Nutrient agar and broth, and results were scored at 7 days.

- *Optical microscopy.* A Nikon ECLIPSE 50i microscope equipped with phase contrast optics was used to study the morphology of the isolates. Cells were grown for 48 h darkness at 30 ºC on Nutrient agar. Cell suspensions for microscopic examination were prepared in sterile saline basal medium.

- *Electron microscopy*: the morphology and ultrastructure of the cells were studied with a Plips Tecnai 12 transmission electron microscope and scanning JEOL6.100 electron microscope. Cells were grown for 48 h at 30 ºC darkness on Nutrient broth. Cell fixation, block inclusion, ultrathin sectioning and post-staining were done by the University of Murcia Microscopy Service (SACE) according to their protocols used (see ANNEX II).

RESULTS AND DISCUSSION

Detection and isolation of bacteria

This is the first characterisation study of microorganisms associated with *B. bituminosa in vitro* cultures. The methodology described in other species such as *Prunus*, mint, hazelnut or strawberry has been used (Cornu and Michel 1987; Reed *et al*. 1995; Reed *et al*. 1998; Tanprasert and Reed 1998). Contamination by bacteria was observed in explants from four different plant accessions at 6 d of culture (Figure 3A). The types of bacteria were recorded at 30 days based on colour and colony morphology (white, yellow, pink and other; Table 1), white and pink being the most abundant. At this stage, it was not possible to determine if the

bacteria were endogenous or not (Figure 1A).

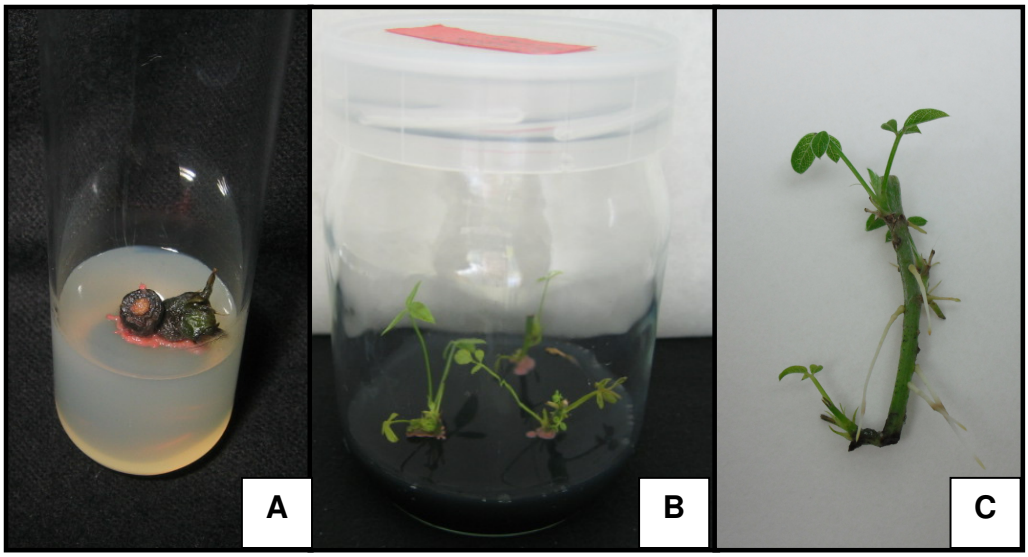


Figure 3. *Bituminaria bituminosa in vitro* plant material. A Contaminated explant cultured on MS basal medium without antibiotics. B Contaminated shoots on micropropagation medium 4 month after establishment. C Abnormal rooting after the addition of antibiotics in the culture medium.

The isolation of pure cultures of bacteria was performed by repetitive streaking in LB medium. Table 2 shows the characteristics of nine bacterial isolates (named A to I) that differ in growth rate. Colonies of fast growers were visible at 24 h of incubation. Slow growers produced visible colonies after 48-72 h of plate incubation. The colony colour and cell morphology of these strains are also shown in Table 2.

Table 1. Frequency of explants from four different accessions of *B. bituminosa* contaminated by different types of bacteria, based on colony colour, after 30 days of culture on MS basal medium.

Plant material	Frequency of explants contaminated by bacteria coloured colonies (%)			
	White	Yellow	Pink	Other
"Bitu-Calnegre"	7.8	3.6	78.6	10.0
"Bitu-hybrid"	81.0	10.0	9.0	-
"Albo-tedera"	23.7	13.4	45.4	17.6
"Albo-hybrid"	56.4	13.6	6.4	22.7

Effect of culture media on bacterial growth

In order to evaluate the effect of the culture media on bacterial growth, 4 media were tested (Table 2 and Figure 1A). Depending on the media used, differences in bacterial growth were observed. Isolates A, B, C, D, F, G and H showed 70-99% Petri dish surface growth on PYGA, LB and PDA, where the carbon source is either glucose or the carbohydrates of yeast extract. Isolates E and I showed 25-60% surface growth in all media.

Bacterial resistance to antibiotics (antibiogram)

Bacterial contamination is an important problem in plant tissue culture and it has been a subject of study in several species such as mint or strawberry (Reed *et al*. 1995; Tanprasert and Reed 1998). To eliminate bacteria from *B. bituminosa in vitro* cultures, 13 antibiotics at two different concentrations were tested (Table 3 and Figure 1A). Isolates E, F, G, H and I were not included in the experiment because it was not possible to obtain optimal liquid cultures from these isolates. No antibiotic was active against all four bacteria (A, B, C and D) at the same time. Bacterium A was sensitive to hygromycin and tetracycline. Bacterium B was sensitive to ampicillin, carbenicillin, nalidixic acid, cefotaxime and rifampicin. Bacteria C and D were not sensitive to any of the antibiotics at the concentration

tested. Therefore, taking into account the observed bacterial-sensitivity (full sensitivity or intermediate) towards the antibiotics ampicillin, carbenillicin, gentamicin, hygromycin, cefotaxime and tetracycline, these antibiotics could be good candidates for eliminating bacteria isolated from *in vitro B. bituminosa* cultures. In plant *in vitro* cultures, it must be considerd the fact that combinations of antibiotics at bactericidal concentrations are likely to be phytotoxic; and that the repeated use of single antibiotics may lead to bacterial resistance (Leifert *et al*. 1994). So, for further experiments two of those antibiotics were chosen, gentamicin and cefotaxime.

Table 2. Characteristics of different bacterial isolates from *B. bituminosa.* Effect of different culture media on the bacterial growth and cell morphology.

		Isolates								
		A	B	C	D	E	F	G	H	I
Growth rate[1]		24 h	24 h	24 h	24 h	48 h	48 h	48 h	48 h	48 h
Colony characteristics[2]		Yellow Diffusible pigment	Yellow Non-diffusible pigment	White Non-diffusible pigment	Yellow Non-diffusible pigment	Pink Non-diffusible pigment Mucous	White Non-diffusible pigment	White Non-diffusible pigment	Orange Non-diffusible pigment	Pink Non-diffusible pigment Dry surface
Cell morphology[3]		Motile rods	Motile rods	Non-motile, irregularly shaped rods	Non-motile, coccoid cells	Motile rod and coccoid cells	Non-motile, irregularly shape rods and cocoids	Motile rod cells	Motile, aggregates of rod cells in pairs	Motile rods
Culture media (Growth at 48h)[4]	PYGA (glucose)	95	99	90	99	25	80	80	70	30
	MS (sucrose)	25	30	25	40	25	35	30	25	25
	LB (yeast extract)	99	95	95	99	60	80	80	65	55
	PDA (glucose)	85	80	85	90	50	25	30	80	60

[1]Bacterial growth on LB medium at 24 or 48 hours. [2]Colony colour and type of pigment. [3]Cell morphology observed by phase contrast microscopy, 100x oil immersion objective. [4]Percentage of Petri dish surface growth after 48 h of culture

Table 3. Effect of different antibiotics on the growth of four bacterial isolates (A, B, C and D) cultured on Nutrient agar.

ANTIBIOTIC		Concentration	A		B		C		D	
		(mg l^{-1})	D†	INT‡	D	INT	D	INT	D	INT
β-Lactams	Ampicillin	50	7.35	I	17.85	S	10.65	I	10.85	I
		25	5.65	I	16.65	S	9.15	I	10.5	I
	Carbenicillin	250	6.35	I	12.85	S	11.5	I	11.5	I
		125	4.00	R	12.00	I	10.15	I	10.5	I
	Penicillin-G	50	5.00	I	10.00	I	4.35	I	9.50	I
		25	4.15	R	9.15	I	2.85	R	7.35	I
Aminoglycosides	Gentamicin	125	10.65	I	9.00	I	10.25	I	9.00	I
		62	9.35	I	7.35	I	9.35	I	8.10	I
	Hygromycin	75	13.15	S	10.5	I	6.35	I	6.65	I
		37	11.65	I	9.35	I	5.35	I	5.85	I
	Paromomycin	50	6.65	I	4.00	R	6.35	I	5.00	I
		25	8.15	I	5.15	I	8.00	I	6.65	I
	Streptomycin	8	9.85	I	7.65	I	3.65	R	6.35	I
		4	9.00	I	5.00	I	2.50	R	5.15	I
Macrolide	Erythromycin	16	6.15	I	3.65	R	3.50	R	4.80	I
		8	4.35	I	2.65	R	2.15	R	3.50	R
Glycopeptides	Vancomicin	20	0.85	R	1.85	R	0.15	R	0.00	R
		10	0.35	R	1.50	R	0.15	R	0.00	R
Quinolones	Nalidixic acid	25	4.00	R	16.35	S	8.35	I	5.85	I
		13	3.15	R	15.15	S	7.35	I	5.50	I
Cephalosporins	Cefotaxime	250	9.35	I	15.85	S	10.5	I	10.15	I
		125	7.85	I	14.85	S	9.00	I	10.65	I
Poliketides	Tetracycline-HCl	50	16.85	S	6.65	I	11.15	I	11.85	I
		25	14.35	S	6.50	I	11.65	I	11.3	I
Semisintetic rifamycin	Rifampicin	25	3.85	R	16.35	S	3.65	R	3.65	R
		13	2.85	R	15.35	S	2.85	R	2.35	R

†D= Growth inhibition diameter (mm), ‡INT= Author's Interpretation; R= Resistant, S=Sensitive, I= Intermediate

Effect of antibiotics on *in vitro* plant material

Gentamicin at different concentrations (5, 10, 20 and 35 mg l^{-1}) did not affect the visual quality of explants and the higher the concentration of the antibiotic, the lower the frequency of contaminated explants. At 35 mg l^{-1}, 23% of contaminated explants were still observed (data not shown).

After several subcultures of plant material with 35 mg l^{-1} gentamicin, bacterial contamination re-appeared (Figure 1A and 3B). So, cefotaxime at 400 mg l^{-1} was also added to the culture medium. Moreover, in order to study the effectiveness of antibiotics against contamination, and their effect on *in vitro* plant material, each putative healthy explant was cut into two pieces and an indexing test was carried out. The apical piece of the explant with sprouted shoots was cultured on MS basal medium with antibiotics and the basal piece of the explant was cultured on LB medium for bacterial growth (Figure 1A). Table 4 shows that the frequency of basal pieces of explants contaminated by bacteria, after 7 d of culture on LB medium, reached 55.2% in "bitu-Calnegre" explants. Pink bacteria were the most-abundant contaminant bacterial strain. When apical pieces of the explants were cultured with the combination of both antibiotics, no effect on the viability of explants was observed and the frequency of contamination went down to 5% (data not shown). This decrease indicates that antibiotics in the plant culture MS medium were effective in bacterial contamination control.

Apparently-healthy plant material was cultured on micropropagation medium without antibiotics (Figure 1B) but, at 4 months of culture, shoots stopped elongating resulting in gradual browning and defoliation of leaves and some signs of possible endophytic contamination were observed. Pink bacteria were observed at the base of the explants. To eliminate this contamination, 35 mg l^{-1} gentamicin and 400 mg l^{-1} cefotaxime were again added to the micropropagation medium and their effect was also evaluated on the average number of shoots per explant and on shoot rooting. In the presence of these antibiotics, the average number of shoots per explant was 4-5, while without antibiotics, the average number of shoots per explant was 37-40 (Pazos-Navarro *et al*. 2012). With antibiotics, shoot rooting was abnormal and the roots did not appear at the base of the shoot, but along the stem (Figure 3C).

Keskitalo *et al*. (1998) observed the same effects on *Tanacetum vulgare* cultures, where the number of shoots decreased linearly with increasing concentrations of gentamicin with cefotaxime, and root initiation was delayed with increasing concentrations of cefotaxime. These authors reported that combinations of antibiotics involving aminoglucosides and β-lactams are phytotoxic to plants at the concentrations that successfully eliminate bacterial contaminants. Such antibiotic combinations should therefore be used only for limited periods of time to eliminate known contaminants, whereas the prophylactic and long-term use of these combinations of antibiotics should be avoided (Leifert *et al*. 1992).

Combinations of aminoglucosides (gentamicin or streptomycin) with cephalosporin, as cefotaxime, or other β-lactamase inhibitors such as timentin have been reported in other species such as strawberry (Tanpraset and Reed 1998) or hazelnut (Reed *et al*. 1998), and they were effective at eliminating bacterial contamination, but these authors did not report any effect on micropropagation rate.

Cefotaxime is an antibiotic recommended for *in vitro* plant culture because of its broad spectrum and low phytotoxicity. Moreover, cephalosporin is reported to potentiate growth and morphogenesis and seems species-specific. Thus, Mathias and Mukasa (1987) showed that cefotaxime enhanced the frequency of embryogenic callus formation from immature embryos and activated tissue growth in wheat and regeneration of callus in *Hordeum vulgare*. In cultured sorghum tissues, cefotaxime prolonged the period of tissue capability for plant regeneration (Rao *et al*. 1995), whereas in maize culture, it accelerated callus differentiation (Danilova *et al*. 2004). In tobacco, cefotaxime weakly inhibited shoot formation from cotyledonary explants and rooting of regenerants from leaf discs (Nauerby *et al*. 1997).

In contrast, gentamicin is reported as an inhibitor of cotyledon differentiation and callus formation in artichoke (Dodds and Roberts 1981); thus several authors have examined its phytotoxic effect on plant tissue and cell cultures. Phytotoxicity of streptomycin and gentamicin (inhibition of growth, callus formation and cell division) has also been shown in *Cordyline, Datura, Delphinium, Ficus, Helianthus, Hemerocallis, Lactuca, Nicotiana, Philodendron*

and *Syngonium* (Dodds and Roberts 1981; Phillips *et al*. 1981; Bastiaens *et al*. 1983; Horsch and King 1983; Pollock *et al*. 1983; Fisse *et al*. 1987; Leifert *et al*. 1991). In our case, the combination of cefotaxime and gentamicine produced some phytotoxicity which led to a decrease in the micropropagation rate.

Tabla 4. Frequency of bacterial contamination of basal pieces of putative healthy explants of four different accessions of *B. bituminosa,* based on colony colour after 7 d of culture on LB medium.

Plant material	Frequency of explants contaminated by bacteria (%)				
	White	Yellow	Pink	Other	Healthy
"Bitu-Calnegre"	-	-	55.2	-	44.8
"Bitu-hybrid"	-	-	13.6	-	86.4
"Albo-tedera"	5.9	-	20.6	-	79.4
"Albo-hybrid"	-	-	7.5	-	92.5

Molecular identification and phenotypic characterisation of endophytic bacteria

Pink bacteria isolated from 4-month-old *in vitro* cultures were considered as endophytes and "*vitropathogens*" (Leifert *et al*. 1995). The two bacteria differ in forming either mucous or dry pink colonies, and were sent to the Colección Española de Cultivos Tipo (CECT, Valencia, Spain) in order to identify them. The result of the CECT taxonomical analysis was:

- *Blastococcus* sp (Isolate E: pink mucous colony, see Table 2): presents a similarity of 873/904pb (96.6%) with the DQ200983 sequence (*Blastococcus jejuensis* strain KST3-10). Because strains with similarity values between 16S rRNA sequences lower than 97% are considered different species, this isolate might be provisionally considered as a new species within the genus *Blastococcus,* (hereafter *Blastococcus* strain E).

- *Methylobacterium extorquens* (Isolate I: pink dry colony, see Table 2): presents a similarity of 928/932pb (99.6%) with the AB175632 sequence (strain IAM 12631). Since strains with similarity values

between 16S rRNA sequences higher than 97% are considered members of the same species, this isolate may be considered a member of the mentioned taxon, (hereafter *M. extorquens* strain I).

Table 5.1 and Table 5.2 show the results of the phenotypic characterisation of *Blastococcus* sp. and *M. extorquens*. Although both bacteria are pink pigmented and present a slow growth rate, *Blastococcus* strain E is a Gram-positive motile rod with coccoid cells and the colonies are mucous while *M. extorquens* strain I is a Gram-negative motile rod and its colonies show a dry surface. As the growth on PYGA, MS, LB and PDA was similar and slow for both bacteria (Table 2, isolates E and I), seven more media were tested in order to find a better growth medium for each one (Table 5.1). Nutrient agar (NUT) gave a 95% Petri dish surface growth surface for both bacteria and LM medium 90-95% for *M. extorquens* strain I and *Blastococcus* strain E, respectively. R2A medium was good for *M. extorquens* strain I but not for *Blastococcus* strain E; this may be due to the fact that *Blastococcus* sp. is reported to be negative for starch hydrolysis and for D-glucose assimilation (Normand 2006) and these compounds are the main carbon sources in R2A medium. In order to evaluate the methanol assimilation capacity, three different concentrations of methanol (0.25, 0.5 and 0.75% v/v) were tested onbasal mineral solution of MMS medium (Table 5.1). Only *M. extorquens* strain I was able to growth at 0.5% (v/v) methanol while *Blastococcus* strain E did not grow with methanol as carbon source.

The assimilation of other different carbon sources (maltose, glucose, malic acid, mannitol and trisodium acetate at 0.15% w/v) added to saline basal medium (Table 5.1) was also evaluated. No growth of either bacterium was observed in media supplemented with maltose, glucose or mannitol. *Methylobacterium* grew on media supplemented with trisodium acetate better than *Blastococcus* and both bacteria grew with malic acid.

Table 5.1. Differentiation of the two isolates *Blastococcus* strain E and *Methylobacterium extorquens* strain I, according to their growth characteristics.

		Blastococcus strain E[1]	*M. extorquens* strain I[1]
Gram test		+	-
Culture media (Main carbon and energy source)	NUT (yeast extract)	95	95
	R2A (D-glucose and starch)	25	80
	LM (D-glucose and malt extract)	95	90
	M72 (Tri-sodium citrate)	60	60
	YMG (D-Glucose and malt extract)	50	70
	TYG (D-Glucose)	40	40
Effect of methanol concentration (% v/v) in MMS medium	0.25	0	0
	0.5	0	50
	0.75	0	0
Carbon source (0.15% w/v)	Maltose	0	0
	Glucose	0	0
	Malic acid	60	60
	Mannitol	0	0
	Trisodium acetate	10	30
	Control without Carbon source	0	0

[1] Percentage of Petri dish surface growth after 48 h of culture

API20NE tests (Table 5.2) revealed other differences between the two bacteria. *M. extorquens* strain I was positive for glucose assimilation and capric acid assimilation while *Blastococcus* strain E was negative. The rest of the tests did not show any differences.

As these two bacteria were possible endophytes and both damaged *in vitro* cultures dramatically, new antibiograms tests were carried out, adding to Nutrient broth the same antibiotics and concentrations as in Table 3. Both bacteria were sensitive to ampicillin, carbenicillin, cefotaxime and penicillin and resistant to erythromicin, streptomicin, gentamicin, hygromycin, nalidixic acid, paromomycin, rifampicin and vancomicin at the concentration tested (data not shown). Among the antibiotics tested, tetracycline (50 and 25 mg l^{-1}) was the

only one able to differentiate between *Blastococcus* strain E (resistant) and *M. extorquens* strain I (sensitive).

Table 5.2. Phenotypic characterisation of the two isolates *Blastococcus* strain E and *Methylobacterium extorquens* strain I.

		Blastococcus strain E	*M. extorquens* strain
API20NE	Reduction of nitrates to nitrites	+	+
	Reduction of nitrates to nitrogen	-	-
	Indol production	-	-
	Glucose fermentation	-	-
	Arginine hydrolase	+	+
	Urease	+	+
	Esculin hydrolysis (B-glucosidase)	-	-
	Gelatin hydrolysis (protease)	-	-
	β-galactosidase	+	+
	Glucose assimilation	-	+
	Arabinose assimilation	-	-
	Manose assimilation	-	-
	Mannitol assimilation	-	-
	N-acetyl-glucosamine assimilation	-	-
	Maltose assimilation	-	-
	Potassium gluconate assimilation	-	-
	Capric acid assimilation	-	+
	Adipic acid assimilation	-	-
	Malate assimilation	+	+
	Trisodium citrate assimilation	-	-
	Phenylacetic acid assimilation	-	-
	Cytochrome oxidase	+	+

-= negative test and + = positive test after a week of incubation at 30 ºC

To test the sensitivity to antibiotics at those concentrations used in *in vitro* plant culture, carbenicillin (500 mg l^{-1}), tetracycline (100 mg l^{-1}), ampicillin (100 mg l^{-1}), penicillin (100 mg l^{-1}) or cefotaxime (500 mg l^{-1}) were added to Nutrient agar and broth media. No differences were found on liquid and solid media. Carbenicillin, ampicillin, penicillin and cefotaxime were effective against both bacteria and no growth was observed. Tetracycline was only effective against

M. extorquens strain I. The last result is very interesting because tetracycline added to Nutrient culture medium could be used to select between the two bacteria.

Morphology of the strains

- *M. extorquens* strain I is a Gram-negative rod measuring 0.75-1.0 μm x 2.0-2.5 μm, occurring singly or in pairs (Figure 4A, B, Figure 5A and Figure 6). Cells are motile and non-spore-forming, with a possible micro-capsule formation. Colonies are smooth, dry and pink-pigmented. Figure 5A shows cell morphology by scanning electron microscopy. Transmission electron microscopy (Figure 6) shows that the bacteria present a thin exo-polysaccharide layer and granules of poly-β-hydroxybutyrate (PHB), or some other hydroxyl-alkanoate, and dark granules with another type of reserve substance, possibly poly-phosphate.

- *Blastococcus* strain E is Gram-positive, with cocci and rods measuring 0.5-1.0 μm x 0.8-1.0 μm (Figure 4C, D; Figure 5B and Figure 7). Cells are motile and non-spore-forming. Colonies are mucous and pink-pigmented. Figure 5B shows the cell morphology by scanning electron microscopy. Transmission electron microscopy (Figure 7) shows ultra-thin sections of this bacterium with granules of reserve substances, probably poly-phosphates and poly-hydroxy-alkanoates.

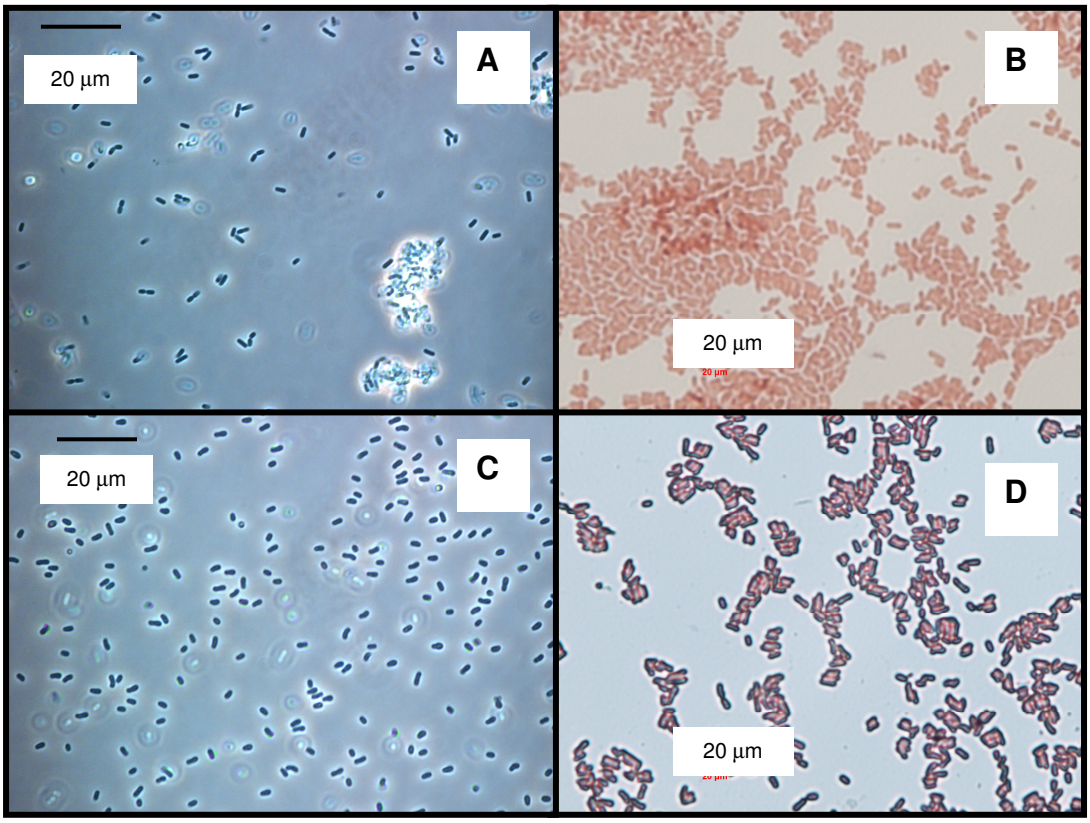


Figure 4. Cell morphology under contrast phase (A and C) and Gram stain (B and D) under bright field microscopy. A y B *M. extorquens* strain I; C y D: *Blastococcus* strain E.

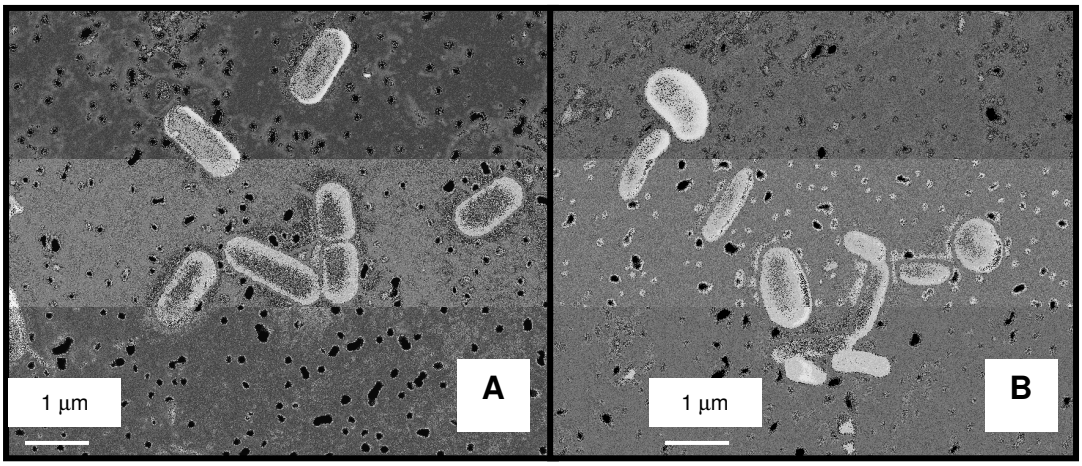


Figure 5. Scanning electron microscopy images of *M. extorquens* strain I (A) and *Blastococcus* strain E (B).

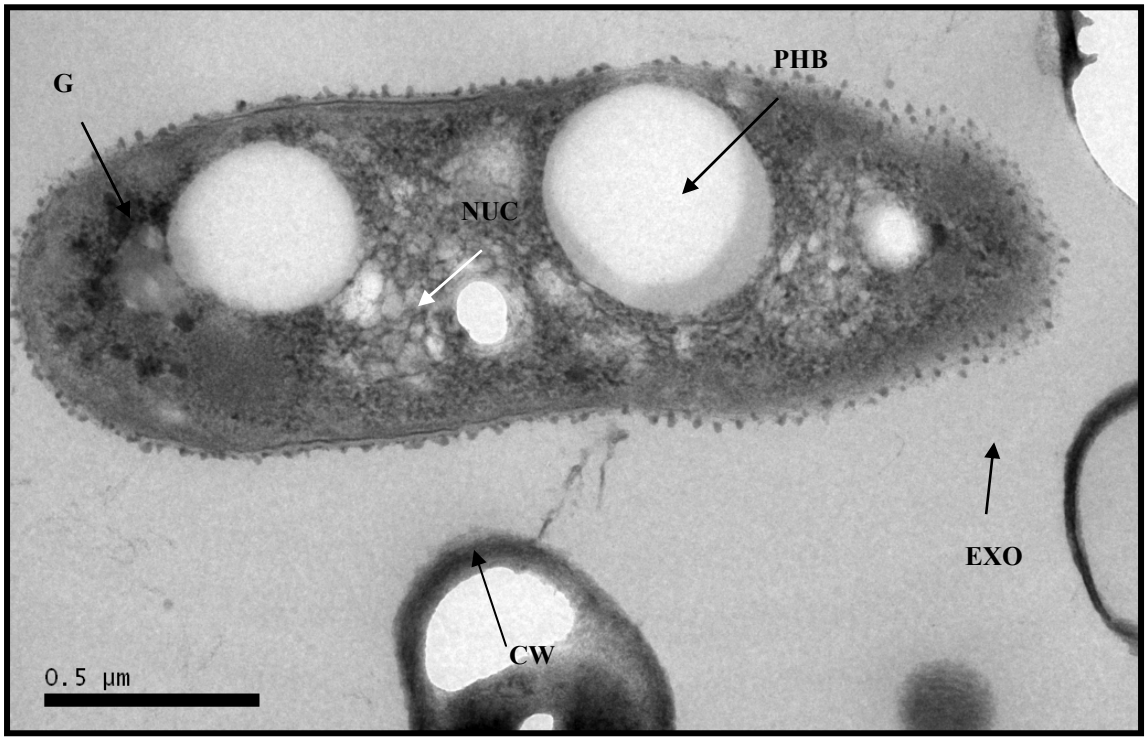


Figure 6. Transmission electron microscopy images of ultra-thin sections of *M. extorquens* strain I. General view of the rod morphology, G= small black granules, possibly poly-phosphate; NUC= nucleoid; PHB= poly-β-hydroxybutyrate granule; EXO= exo-cellular polymeric substance, possibly a microcapsule; CW: cell wall (Gram-negative type).

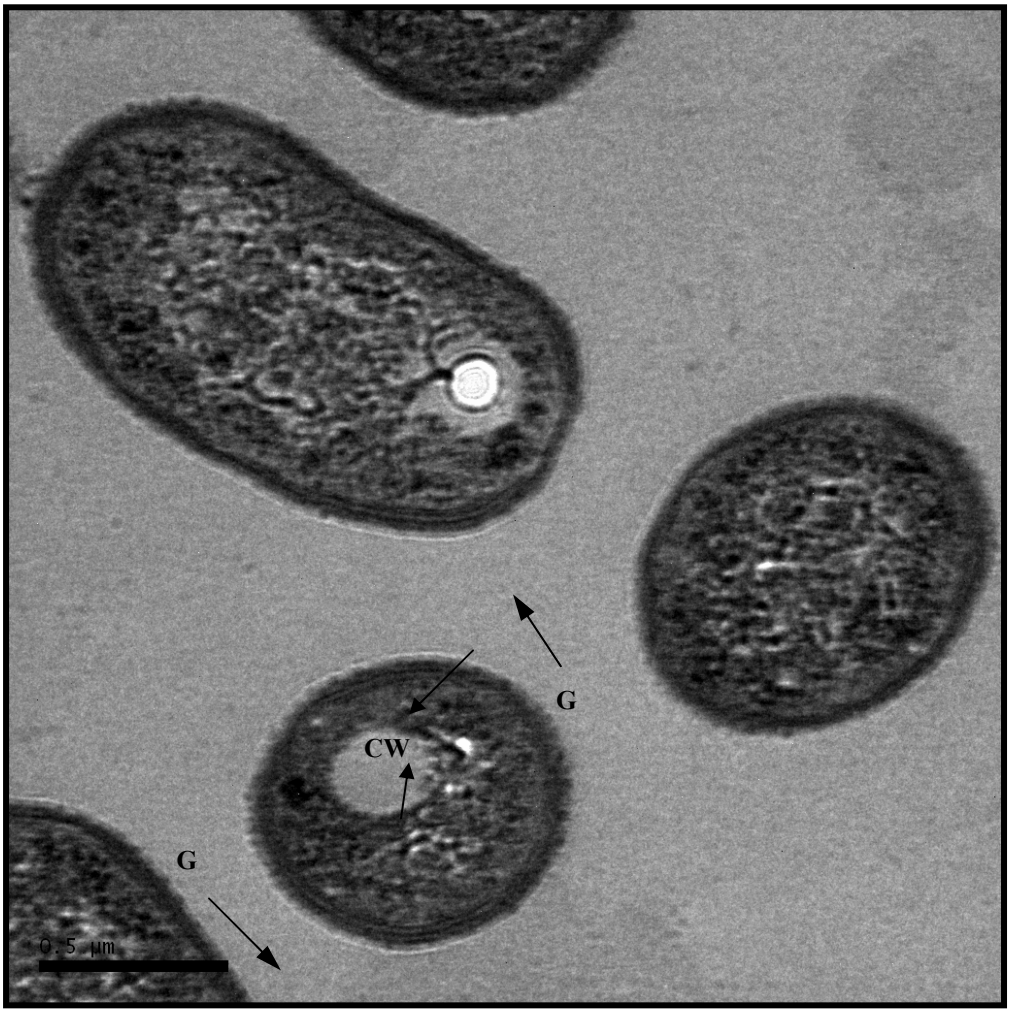


Figure 7. Transmission electron microscopy images of ultra-thin sections of *Blastococcus* strain E. A: rod and coccus morphology; G= granule; CW: cell wall (Gram-positive type).

In conclusion, this is the first study about the microorganisms associated with *in vitro* cultures of *B. bituminosa*. Of the nine different bacteria isolated, based on colony colours, the pink bacteria (*Blastococcus* strain E and *M. extorquens* strain I) were the most noteworthy, due to their persistence in *in vitro* tissue cultures and their possible endophytic character.

The elimination of bacteria can be achieved by addition of gentamicin (35 mg l^{-1}) and cefotaxime (400 mg l^{-1}) to the plant *in vitro* culture media, although at these concentrations the effect of the antibiotic cocktail was bacteriostatic and not bactericidal.

M. extorquens strain I isolated from *B. bituminosa in vitro* cultures is a rod-shaped bacterium, strictly aerobic, oxidase-positive, urease-positive, able to reduce nitrate to nitrite, arginine-hydrolase positive and β-galactosidase positive. Methanol and acetate are utilised as sole carbon source. It does not grow in the presence of maltose, mannitol or glucose at 0.15% (w/v). It oxidises malate, capric acid and D-glucose as sole carbon and energy sources in the API20NE test strips, and synthesises poly-hydroxyl-alkanoates, probably poly-β-hydroxybutyrate granules and accumulates small granules of poly-phosphates (Tables 5.1 and 5.2 and Figures 4, 5 and 6). It is sensitive to tetracycline at concentrations tested.

Blastococcus strain E isolated from *B. bituminosa* cultures is a strictly-aerobic, Gram-positive, coccus-shaped bacterium, oxidase-positive, urease-positive, able to reduce nitrate to nitrite, arginine-hydrolase positive, and β-galactosidase positive. Acetate is utilised as sole carbon source. It does not grow in the presence of methanol nor maltose, mannitol or glucose at 0.15% (w/v). It oxidises malate as sole carbon and energy sources (Tables 5.1 and 5.2 and Figures 4, 5 and 7). It is resistant to tetracycline.

What could be the role of these bacteria in plant physiology?

M. extorquens, which belongs to the *α-Proteobacteria*, is described as a pink-pigmented, facultatively-methylotrophic bacterium (PPFMs) whose strains are epiphytes on the leaf surface of plants; however, there is evidence that methylobacteria can also penetrate into the intercellular space through the stomata, attracted by the methanol emitted by sunflower (Kutchera 2007). It is known that certain isolates of PPFMs produce phyto-hormones (auxins and cytokinins) and vitamin B_{12} (Basile *et al.* 1985) and interact with plant nitrogen metabolism via their bacterial urease or through the induction of nitrogen-fixing root nodules, but the molecular basis for the growth-promoting activity is unknown (Jourand *et al*. 2004, Omer *et al*. 2004a; Lee 2006). As a consequence, several hypotheses have been postulated to explain the possible implication of this genus in promotion of plant growth. Another remarkable *Methylobacterium* characteristic is its capability for reducing metal uptake in

roots and shoots of tomato, and so these bacteria might prevent the biosorption and transport of such metals by the plant (Madhaiyan *et al*. 2007).

Blastococcus are actinobacteria that belong to the family *Geodermatophilaceae* (Lee 2006). As reviewed by Qin *et al*. (2009), in general, these endophytic organisms seem to have important roles in plant development and health, such as improving and promoting the growth of host plants as well as reducing disease symptoms caused by plant pathogens through various mechanisms, including the production of secondary metabolites, changes in host physiology or the induction of systemic acquired resistance in plants. In addition, they can act as antagonists against phyto-pathogens through the production of antibiotics, nutrient competition and induction of systemic disease resistance (Kunoh 2002; Hasegawa *et al*. 2006; Meguro *et al.* 2006; Conn 2008; Qin *et al*. 2009). Qin *et al*. (2009) reported the isolation of *Blastococcus* sp. from the interior of tropical medicinal plants, and suggested that these strains might represent a valuable source of new species and biologically-active compounds with antimicrobial activity and genes for their biosynthesis.

Taking into account these plant-bacteria interactions referred to by other authors, it is possible that these endophytes found in *B. bituminosa* have an effect on the FC synthesis by some type of plant-bacteria interaction. It would be interesting to carry out further research in this field. Moreover, in 2007, Walker *et al*. reported that *B. bituminosa* possesses a degree of (constitutive) resistance to heavy metals, manifested as restricted Zn transport to the shoot. Could it be possible that *Methylobacterium* is involved in this effect?

ACKNOWLEDGEMENTS

We thank Dr. F^{co} Torrella, Mr. E. Correal, Dr. J.A. del Río and Dr. D. Walker for their critical reviews of the manuscript. This research was supported by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (Project No. RTA2007-00046-00-00), the Ministerio de Ciencia e Innovación (Project BFU2010-19599) and by a fellowship provided by the IMIDA to M. Pazos-Navarro. M. Dabauza was co-supported by the European Social Fund and the IMIDA.

4. Next generation DNA sequencing technology delivers valuable genetic markers for the genomic orphan legume species, *Bituminaria bituminosa*

María Pazos-Navarro[1], Mercedes Dabauza[1], Enrique Correal[2], Kelly Hanson[3], Natasha Teakle[4,5], Daniel Real[5,6,7], Matthew N. Nelson[5]

[1]Departamento de Biotecnología y Protección de cultivos, Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA), C/ Mayor s/n, 30150-La Alberca, Murcia, Spain.

[2]Departamento de Recursos Naturales, IMIDA. C/ Mayor s/n, 30150-La Alberca, Murcia, Spain.

[3]Australian Genome Research Facility, Level 5 Gehrmann Laboratories, Research Road, University of Queensland, St Lucia, QLD 4072, Australia. [4]Centre for Ecohydrology, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.

[5]School of Plant Biology, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.

[6]Department of Agriculture and Food, Western Australia, South Perth, WA 6151, Australia.

[7]Future Farm Industries Cooperative Research Centre, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia.

BMC Genetics (2011) 12:104

INTRODUCTION

Bituminaria bituminosa (L.) C.H. Stirt., commonly known as Tedera in the Canary Islands, is a perennial legume species widely distributed in the Mediterranean Basin and Macaronesia. It is a self-pollinated diploid species (2n=20) with DNA content estimated to be between 0.998 and 1.094 pg DNA per diploid nucleus (Juan *et al*. 2004; Stirton 1981; Walker *et al*. 2006). *B. bituminosa* shows particularly high diversity in the Canary Islands, with three recognised botanical varieties: (i) var. *albomarginata*: native to semi-arid habitats in coastal areas of Lanzarote island and a few other niches in Fuerteventura, Tenerife and Gran Canaria, with an annual rainfall of 150 mm to 300 mm; (ii) var*. crassiuscula*: native to high altitude sub-humid areas in Tenerife island with up to 500 mm rainfall per year; and (iii) var. *bituminosa* widely distributed in all islands across varying altitudes and rainfall levels (Méndez and Fernández 1990; Méndez *et al*. 1990). In the Mediterranean basin, only var. *bituminosa* is found (Muñoz and Correal 1998). These botanical varieties were identified using morphological characteristics and were largely supported by preliminary molecular analyses using arbitrary DNA markers (Juan *et al*. 2005; Muñoz *et al*. 2000). However, Juan *et al*. (2005) found that accessions of var. *bituminosa* from the Mediterranean region formed a cluster distinct from a Canary Islands cluster that contained all three botanic varieties. Therefore, there is some ambiguity in botanical variety definitions that warrants further investigation.

In recent years, there has been growing international interest in *B. bituminosa* as a potential source of pharmaceutical compounds and also as a drought tolerant pasture species. The plant contains secondary compounds such as pterocarpans with antitumor activity against leukaemia and colon cancer (Maurich *et al*. 2006; 2004; Pistelli *et al*. 2003), antioxidants (Rosa *et al*. 2005) and furanocoumarins such as psoralen and angelicin, which are used in the treatment of skin diseases (psoriasis, vitiligo, melanoma) (Innocenti *et al*. 1991; 1997; Martínez *et al*. 2010). As a forage crop it is well adapted to high temperature and low rainfall. An important attribute of this species is that, unlike lucerne (*Medicago sativa* L.), it has a high retention of leaves when moisture stressed, therefore providing valuable feed over summer (Real *et al*. 2011; Real

and Verbyla 2010). Traditionally, the profitability and sustainability of livestock industries in southern Australia and in other regions with Mediterranean-like climates is severely constrained by the quantity and quality of forage available over summer and autumn. Therefore, there is strong demand for breeding drought-tolerant and productive forage legumes as well as improved understanding of the genetic basis of key agronomic traits.

Molecular markers contribute valuable support to breeding programmes (Collard and Mackill 2008). Markers provide the means to characterise genetic diversity within breeding programmes and help identify new genetic diversity in the wild or in germplasm collections. Markers are valuable in determining or confirming pedigrees and for marker-assisted selection of traits that are difficult and/or expensive to measure. However, *B. bituminosa* can be considered a true 'genomic orphan' (Varshney *et al*. 2009) in that there are almost no genomic resources or high-quality codominant markers available for genetic analysis. The few genomic resources readily available for molecular marker development for *B. bituminosa* consist largely of chloroplast gene sequences developed for phylogenetic studies within the tribe Psoraleeae and more broadly among phaseoloid legumes (Egan and Crandall 2008; Stefanović *et al*. 2009). However, *B. bituminosa* has a rich cousin in the genomic resources sense: soybean (*Glycine max* (L.) Merr.) that belongs to the neighbouring subtribe *Glycininae* (Stefanović *et al*. 2009). The complete genome sequence of soybean was recently determined (Schmutz *et al*. 2010), which could act as a useful reference genome for *B. bituminosa*. However, the taxonomic divide between these species is sufficiently wide to make marker transfer between soybean and *B. bituminosa* rather inefficient. Fortunately, the advent of new high-throughput genome sequencing technologies provides a relatively low cost opportunity for rapid development of locus-specific markers for a species like *B. bituminosa* that has little available genomic resources.

This study reports the generation of a cDNA library developed from leaf mRNA from a single *B. bituminosa* plant and sampling of the leaf transcriptome using 454 GS-FLX pyrosequencing technology. Simple sequence repeat (SSR) motifs were identified, primers designed and a subset of these markers were used to characterise a broad set of *B. bituminosa* accessions to assist in the

correct choice of parents in breeding programmes, and which could be used to provide guidance in managing and conserving germplasm collections. These SSR markers along with the first catalogue of expressed genes provide valuable resources for *B. bituminosa* genetic analysis and breeding.

MATERIAL AND METHODS

Selection of a *B. bituminosa* accession for transcriptome sequencing

In June 2008, one plant from each of 22 accessions was analyzed for psoralen and angelicin content (Ewald Sweeny, Chem Centre, Western Australia; unpublished data). The plant A13.1 (from accession A13) had the highest total content of furanocoumarins (highest in angelicin and third highest in psoralen) out of the 22 plants evaluated. This accession is from an original population collected from the Teno region of the Canary Islands. Plant A13.1 was cloned by propagating cuttings which were then kept at the Department of Agriculture and Food Western Australia (DAFWA) in a naturally lit glasshouse at a constant temperature of 25°C. One such clone was used for transcriptome sampling.

mRNA extraction and cDNA preparation

Young leaves were harvested from A13.1 and immediately frozen in liquid nitrogen. Leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted using RNeasy kit (Qiagen) and mRNA isolated using Oligotex mRNA kit (Qiagen). mRNA was purified using RNeasy Minelute kit (Qiagen) and quality checked at the Australian Genome Research Facility (AGRF; Brisbane, Australia) using an RNA6000 Pico chip (Agilent) run on a BioAnalyzer 2100 (Agilent). A cDNA library was constructed by AGRF following the standard Roche Diagnostics protocol ("cDNA Rapid Library Preparation Method Manual - GS FLX Titanium Series", October 2009 (Rev. Jan 2010)).

Sampling the *B. bituminosa* transcriptome by Roche 454 sequencing

Sequencing of the cDNA library was carried out at AGRF using the GS-FLX System (Roche Diagnostics) with Titanium sequencing chemistry on one half of a two-region gasket PicoTitre Plate; for full details, consult the Roche Diagnostics "Sequencing Method Manual - GS FLX Titanium Series", October 2009 (Rev. Jan 2010). The GS De Novo Assembler software (Roche Diagnostics) was used to assemble the sequencing output into contigs, using default parameters.

De novo assembly of *B. bituminosa* leaf transcriptome

The GS De Novo Assembler (version 2.3, Roche Diagnostics) software programs "newAssembly" (with "cdna" parameter) and "runProject" were used to align and assemble the sequencing output from Standard Flowgram Format into contigs and isotigs, using default parameters as described by the manufacturer.

Functional analysis

Gene ontology (GO) classification was conducted with the aid of Blast2GO software (Götz *et al.* 2008) using GenBank database version 173. Isotig and remaining singleton contigs > 100 bp were included in the analysis. Matches with significance values <1e-6 were allocated to three GO categories (Biological Process, Molecular Function, and Cellular Component) in 1 to 11 levels of hierarchical structure. For ease of visualisation, results are presented at levels 2 or 3 (Figure 1).

SSR detection and primer design

The raw GS-FLX sequencing output in FASTA format was submitted as input to the QDD program (Meglécz *et al.* 2010) for detection of SSR markers and primer design. The QDD program was run from the command line on a Linux system using the default parameters as described in the QDD user manual. For comparison, a manual search for repeat motifs among the

isotig/contig sequences was performed and primers flanking a subset of repeats were designed with the aid of Geneious 5.3 (Biomatters Ltd).

SSR marker amplification and fragment analysis

Genomic DNA was extracted from 50 *B. bituminosa* accessions provided by the Future Farm Industries Cooperative Research Centre (FFI CRC) at the Department of Agriculture and Food Western Australia (South Perth, Australia) and 29 *B. bituminosa* accessions provided by the Spanish Breeding programme at Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (Murcia, Spain) (Table 2), using Illustra Nucleon Phytopure Genomic DNA Extraction Kits (GE Healthcare). PCR reactions were carried out in a MasterCycler programmable thermal cycler (Eppendorf) in 20 uL volumes containing the following components: 2.5 ng/uL genomic DNA, 1x PCR buffer (comprising 50 mM KCl, 10 mM Tris HCl (pH 9.0) and 0.1% Triton-X), 2 mM $MgCl_2$, 200 µM dNTPs, 0.04 U/uL Taq DNA polymerase and 0.2 µM each of forward and reverse primers. Amplification conditions consisted of denaturation at 94ºC for 5 min followed by 35 cycles of denaturation at 94ºC for 45 sec, primer annealing at 55ºC for 45 sec and extension at 72ºC for 90 sec, followed by a final extension step of 72ºC for 7 min.

Initial screening of 96 SSR primer pairs for amplification efficiency was carried out using genomic DNA from plant A13.1 that had been used for transcriptome sequencing. Primer pairs that successfully amplified fragments in A13.1 (assessed using conventional TBE agarose electrophoresis) were then used to screen eight diverse *B. bituminosa* accessions (individual plants: A13.1, A27.2, A37.2, A42.3, A43.2, A48.2, A51.2 and S2b) for polymorphism. For those markers identified as polymorphic on 2% TBE agarose gels, fluorescently-labelled forward primers were synthesised and used to amplify fluorescently labelled amplicons in 79 lines. Fragment size analysis was performed relative to a Genescan LIZ500 internal size standard (Applied Biosystems) using an AB3730xl capillary DNA sequencer (Applied Biosystems) with the resulting electropherograms analysed using GeneMarker software (SoftGenetics) as described in detail by Nelson *et al.* 2009. Each marker allele

was recorded as estimated base pair length. For genetic distance estimates, alleles were scored as present (1), absent (0) or unknown (999).

Genetic diversity analyses

Pairwise Euclidean distances among 79 *B. bituminosa* accessions (Table 2) were calculated using NTSYSpc 2.21i (Applied Biostatistics Inc.). Pairwise distances were subjected to hierarchical cluster analysis using group averages and multidimensional scaling (MDS) using Kruskal fit scheme 1 with 100 restarts in Primer 6.1.6 software (Primer-E Ltd). Analysis of Molecular Variance (AMOVA) of variation within and among original populations (n=26, excluding var. *crassiuscula* as there was only one accession represented) and allele frequencies (n=27) were calculated using GenAlEx 6.4 (Peakall *et al.* 2006). Polymorphism information content (PIC) was calculated using the formula described by Pradhan *et al*. (2011).

RESULTS

Sampling the *B. bituminosa* leaf transcriptome by Roche 454 sequencing

Sequencing of the *B. bituminosa* leaf-derived cDNA library on the GS-FLX System resulted in 432,306 sequence reads with an average length of 345 bp (149.1 Mbp).

These sequence reads can be found in the Sequence Read Archive (SRA) public database of NCBI (accession number SRP006905). GS De Novo Assembler software assembled 266,461 (61.6%) of the reads into 4,542 contigs that were ≥100 bp in length. It then grouped contigs into 2,929 “isogroups” (analogous to genes) and 3,798 “isotigs” (analogous to transcripts) with an average isotig length of 707 base pairs. 3657 isotigs and remaining singleton contig sequences that were ≥200 bp in length are available at NCBI Transcriptome Shotgun Assembly (TSA) database under accessions: JL856153 - JL859809.

Functional characterisation of expressed gene sequences

3,838 isotigs and remaining singleton contig sequences ≥100 bp were subjected to *in silico* functional characterisation using Blast2GO software. Gene ontology (GO) terms were identified for 3,419 sequences (89.1%; see CD ANNEX III: Additional file 1). Figure 1 provides a summary of the main GO terms defined according to the cellular component, biological process and molecular function associated with these 3,419 sequences. The species that received the greatest proportion of BLAST hits was *Glycine max* (3,013 hits, or 88.1%), and *G. max* was also the species that was most frequently the top hit (for 1,454 sequences; 42.5%). The other main model legume species only rarely provided the most significant matches: *Medicago truncatula* was the top hit for 185 sequences (5.4%) and *Lotus japonicus* was the top hit for 14 sequences (0.4%). Together, these results indicate that *G. max* will serve as the most representative reference genome for *B. bituminosa*.

SSR primer design and testing

With the aid of QDD software, primer pairs for 186 'perfect' SSRs and 54 'compound' SSRs were designed (see CD ANNEX III: Additional file 2). Of these, 49 primer pairs for perfect SSRs and 38 primers pairs for compound SSRs were selected in descending order of SSR motif length along with a further 7 primer pairs were designed manually with the aid of Geneious software using contigs containing SSR motifs (see CD ANNEX III: Additional file 3). In total, 94 primer pairs were synthesised and screened for amplification efficiency using genomic DNA from *B. bituminosa* plant A13.1 as template. Without any specific optimisation, 82 out of 94 primer pairs (87.2%) amplified one or more bands (visualised on low-resolution 1% agarose gels), with one band being more common (78 primer pairs, 83.0%) than ≥2 bands (4 primer pairs, 4.3%) (see CD ANNEX III: Additional file 3).

The 82 primer pairs that gave amplification products were screened for gross-level polymorphism in eight diverse *B. bituminosa* accessions (one plant per accession) by electrophoresis using 2% agarose gels. Of the 82 primer pairs tested in this way, 21 showed clear band size polymorphism and were

selected for synthesis of fluorescently labelled forward primers (Table 1). These 21 fluorescently labelled primer pairs were used to genotype 79 *B. bituminosa* accessions (one plant per accession). These accessions were classed as 'original populations' (collected from wild or from traditional pasture lands; n=27) or 'breeding lines' (having undergone selection and possible uncontrolled cross-pollination within the Spanish breeding programme; n=52) (Table 2).

Fragment analysis using GeneMarker software revealed that 20 primer pairs gave clear peaks; the remaining primer pair gave variable amplification strength and was consequently omitted from subsequent analysis. Of the 20 high-quality SSR primer pairs, 19 appeared to detect single loci (1 to 2 alleles per primer pair) while one primer pair appeared to detect two loci (2 to 4 alleles for primer pair '*Bbit*-SSR079'). In total, 130 alleles were detected at 21 high-quality marker loci, an average of 6.19 alleles per locus indicating that these markers were generally highly polymorphic. The 19 single locus SSR markers detected between 3 to 11 alleles per marker, with polymorphic index content (PIC) values ranging from 0.13 to 0.76 (average = 0.407) (see CD ANNEX III: Additional file 4).

Table 1. *Bituminaria bituminosa* simple sequence repeat (*Bbit*-SSR) primer pairs selected for characterisation of *B. bituminosa* germplasm.

Marker name	Forward primer sequence	Reverse primer sequence
Bbit-SSR004	ACCACCCGCAGTTACTTTCACCT	CCTTGTGCTGGTTTCACGCAACG
Bbit-SSR005	ACCAAGTCAGGCTGGAACCCCA	GTCCTGGCCCACTGAACGCC
Bbit-SSR008	CATTGACATCCCTAAGCATAATGT	TCGTTAATAGCGGTCTTGGG
Bbit-SSR010	GCAGGCTTTCCTGAACTGAC	GTCTCCACCAGCAATACCGT
Bbit-SSR012	TCATCCCTTCTCTTCCTACTCG	CGGTTTCTTCGAATACACAGTA
Bbit-SSR013	GAAGGCAAGTGAAAAGCCAG	TCAGACACCAGTGGCTCAAC
Bbit-SSR015	GACTGCACGGTCTTCTCGAC	ATGTGCAGAGGCATTTGTTG
Bbit-SSR034	CAATCCCATTTTCCGCTTTA	TGCCCTCTTCCTTCATAGGTT
Bbit-SSR035	ATATCCACCACCTTCCGTGA	GTAGGATAGGGTCCGGTGGT
Bbit-SSR040	TAACCACTTGGAACTGGGGT	AATTGCAACAGCAGCAACAG
Bbit-SSR055	AGCATCACTACGACCATCCC	GGTGACAACAGAGTGGTCTGA
Bbit-SSR056	TCATCCCTTCTCTTCCTACTCG	CGGTTTCTTCGAATACACAGTA
Bbit-SSR059	CATTGACATCCCTAAGCATAATGT	TCGTTAATAGCGGTCTTGGG
Bbit-SSR064	TTGCTTCTGCGTAACTGTGG	AAAAGTCCACGTCAGCATCC
Bbit-SSR066	GGTCGTCCCATTTATCGAAG	GGAAGAACGGTCAATGGAGA
Bbit-SSR067	TCACCTTCCTCACAAACTACCA	TGAAATGCCTCAATGAGCTAAA
Bbit-SSR070	TGTCGAACTGTTGGATTGTGA	AATTGCAACAGCAGCAACAG
Bbit-SSR073	TTTGCTTGTGTCCTGTCCAA	CCTTCCCTTACCCACCAAGT
Bbit-SSR076	AGAAGGCAAGTGAAAAGCCA	TCAGACACCAGTGGCTCAAC
Bbit-SSR079	GAGCTTCGGAGGGAGTTCTT	CCAAAATCCATCACCTTCCA
Bbit-SSR090	CCCTAACATTGGTAACAGCCA	GAGGCTGGCATCAAGTCAAC

Validating SSR markers by genetic diversity analysis in 27 original populations

To determine if the SSR markers were suitable for inferring genetic relationships among breeding lines, a pairwise Euclidean distance matrix for 27 accessions collected from the wild or from traditional pasture-lands (termed 'original populations'; Table 2) was analysed by hierarchical clustering and MDS analysis (Figure 2). In both analyses, accessions grouped together according to botanical variety indicating that allelic variation at these markers reflected well-established botanical varieties. Interestingly, var. *bituminosa* accessions were sub-divided into two clear groups according to geographical origin (Canary Islands and Mediterranean region). Cluster analysis revealed that some

accessions were genetically so similar that they could not be distinguished using allele information at 21 SSR loci (Figure 2a). Heterozygosity of the original populations ranged from 0 - 62% (mean = 16.4%; Table 2). An analysis of molecular variance (AMOVA) confirmed that there was significant differentiation between populations accounting for 35% of the total allelic variance observed (Table 3).

Table 2. *Bituminaria bituminosa* plants used in this study. Single plants were taken from 79 accessions from original populations (n=27) or breeding lines (n=52). Accession names prefixed by 'A' were provided by the Australian breeding programme, while accession names prefixed by 'S' were provided by the Spanish breeding programme. The botanical variety of each accession, the original geographical origins and the level of marker-defined heterozygosity are shown. H_e= Heterozigosity

Accession code	Botanical variety	Geographical origin	Population type	H_e
A1.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	24%
A2.1	*albomarginata*	Canary Islands	Breeding line	24%
A3.2	*albomarginata*	Canary Islands	Breeding line	25%
A4.2	*albomarginata*	Canary Islands	Breeding line	5%
A5.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	10%
A6.2	*bituminosa*	Canary Islands (Teno, Tenerife)	Breeding line	33%
A7.2	*bituminosa*	Canary Islands (Teno, Tenerife)	Breeding line	24%
A8.1	*albomarginata*	Canary Islands	Breeding line	15%
A9.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	5%
A10.2	*albomarginata*	Canary Islands	Breeding line	0%
A11.17	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	14%
A12.3	*bituminosa*	Canary Islands (Teno, Tenerife)	Original population	0%
A13.1	*bituminosa*	Canary Islands (Teno, Tenerife)	Breeding line	29%
A14.1	*albomarginata*	Canary Islands (Malpaso, Lanzarote)	Original population	19%
A15.4	*albomarginata*	Canary Islands (Famara, Lanzarote)	Original population	10%
A16.2	*crassiuscula*	Canary Islands (Vilaflor, Tenerife)	Breeding line	10%
A17.2	*crassiuscula*	Canary Islands (Cañadas del Teide, Tenerife)	Breeding line	12%
A18.8	*albomarginata*	Canary Islands (Vinamar, Fuerteventura)	Original population	0%
A19.3	*crassiuscula*	Canary Islands (Tenerife)	Breeding line	24%
A20.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	19%
A21.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	38%
A22.2	*albomarginata*	Canary Islands (Famara, Lanzarote)	Original population	0%
A23.2	*bituminosa*	Canary Islands (Teno, Tenerife)	Original population	28%
A24.3	*bituminosa*	Canary Islands (Teno, Tenerife)	Original population	0%

A26.1	*bituminosa*	Canary Islands (Tenerife)	Original population	25%
A27.2	*crassiuscula*	Canary Islands (Teide, Tenerife)	Original population	33%
A29.2	*albomarginata*	Canary Islands (Famara, Lanzarote)	Original population	6%
A36.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Original population	27%
A37.2	*albomarginata*	Canary Islands (Malpaso, Lanzarote)	Original population	27%
A38.3	*bituminosa*	Canary Islands (Tefia, Fuerteventura)	Original population	14%
A39.2	*albomarginata*	Canary Islands (Vinamar, Fuerteventura)	Original population	0%
A40.1	*bituminosa*	Canary Islands (Bentacuria, Fuerteventura)	Original population	21%
A41.2	*bituminosa*	Canary Islands (Güimar, Tenerife)	Original population	5%
A43.2	*bituminosa*	Canary Islands (Tenerife)	Breeding line	38%
A44.1	*albomarginata*	Canary Islands	Breeding line	10%
A46.1	*albomarginata*	Canary Islands	Breeding line	10%
A48.2	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	52%
A49.3	*crassiuscula*	Canary Islands	Breeding line	52%
A50.1	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	15%
A51.2	*crassiuscula*	Canary Islands	Breeding line	19%
A52.1	*albomarginata*	Canary Islands	Breeding line	20%
A53.2	*albomarginata*	Canary Islands	Breeding line	38%
A54.1	*albomarginata*	Canary Islands	Breeding line	29%
A55.2	*albomarginata*	Canary Islands	Breeding line	35%
A56.2	*bituminosa*	Canary Islands (Teno, Tenerife)	Breeding line	37%
A58.2	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	52%
A62.1	*albomarginata*	Canary Islands	Breeding line	43%
A63.1	*crassiuscula*	Canary Islands (Vilaflor, Tenerife)	Breeding line	10%
A64.2	*bituminosa*	Canary Islands	Breeding line	43%
A65.1	*bituminosa*	Mediterranean region (Calnegre, Murcia, Spain)	Breeding line	0%
S1b	*bituminosa*	Mediterranean region (Llano del Beal, Murcia, Spain)	Breeding line	95%
S3c	*bituminosa*	Canary Islands (Tahonilla, Tenerife)	Original population	29%

S4a	*bituminosa*	Canary Islands (Tahonilla, Tenerife)	Original population	42%
S6c	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	48%
S8a	*albomarginata*	Canary Islands (Famara, Lanzarote)	Original population	62%
S9a	*bituminosa*	Mediterranean region (Calnegre, Murcia, Spain)	Breeding line	68%
S10b	*bituminosa*	Mediterranean region (Sardinia, Italy)	Original population	24%
S11b	*bituminosa*	Mediterranean region (La Unión, Murcia, Spain)	Breeding line	62%
S13c	*bituminosa*	Mediterranean region (Calnegre, Murcia, Spain)	Breeding line	30%
S14b	*bituminosa*	Mediterranean region (Israel)	Original population	33%
S17b	*bituminosa*	Mediterranean region (La Unión, Murcia, Spain)	Original population	0%
S18b	*bituminosa*	Mediterranean region (La Unión, Murcia, Spain)	Original population	5%
S19b	*bituminosa*	Mediterranean region (La Unión, Murcia, Spain)	Original population	5%
S20b	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	48%
S21c	*albomarginata*	Canary Islands (Famara, Lanzarote)	Breeding line	67%
S23b	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	95%
S29b	*bituminosa*	Canary Islands (Tenerife)	Breeding line	15%
S30b	*bituminosa*	Canary Islands (Tenerife)	Breeding line	0%
S31b	*bituminosa*	Mediterranean region (Spain)	Breeding line	50%
S32c	*bituminosa*	Canary Islands (Tenerife)	Breeding line	29%
S33b	*albomarginata*	Canary Islands	Breeding line	24%
S34c	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	38%
S35c	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	63%
S36a	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	35%
S37c	*bituminosa*	Mediterranean region (La Perdiz, Murcia, Spain)	Breeding line	55%
S38d	*bituminosa*	Mediterranean region (Greece)	Original population	11%
S39c	*bituminosa*	Mediterranean region (Greece)	Original population	5%
S40d	*bituminosa*	Mediterranean region (Greece)	Original population	11%
S41c	*bituminosa*	Canary Islands (La Palma)	Breeding line	28%

Table 3. Analysis of molecular variance (AMOVA) in single plants sampled from 26 original populations of *Bituminaria bituminosa*. Three populations were defined as var. *albomarginata* (n=9), var. *bituminosa* from the Canary Islands (n=9) and var. *bituminosa* from the Mediterranean region (n=8). Botanical variety *crassiuscula* was not included because there was only one accession used in this study.

Source	df	SS	MS	Est. Var.	%
Between Pops	2	180.425	90.213	8.549*	35%
Within Pops	23	373.306	16.231	16.231*	65%
Total	25	553.731		24.780	100%

*Significant at $P<0.001$

Using markers to characterise *B. bituminosa* breeding lines

Having established that the usefulness of the new SSR markers, genotype information from an additional 52 *B. bituminosa* breeding lines was subjected to pairwise distance analysis, along with plants from the 27 original populations (see CD ANNEX III: Additional file 5). This distance matrix was analysed by MDS and hierarchical clustering techniques (Figure 3 and see CD ANNEX III: Additional file 6). Adding these lines to the analyses resulted in an increased complexity of inter-relationships as evidenced by an increase in the two-dimensional MDS stress from 0.16 (27 original populations) to 0.23 (all 79 accessions). However, a similar pattern of groupings among the original populations was observed (compare Figure 2b and Figure 3).

Figure 3 a-d shows the placement of breeding lines of three botanical varieties from the Canary Islands and Mediterranean region relative to the original populations. In general, breeding lines described as var. *albomarginata* and var. *bituminosa* (from the Canary Islands) were placed within or near their respective clusters of original populations with one notable exception: breeding line S41c (Figure 3b) was described as Canary Island *bituminosa* but had much greater affinity to Mediterranean *bituminosa* according to SSR marker genotyping. Breeding lines described as Mediterranean *bituminosa* and var. *crassiuscula* had very wide distribution in the MDS plots (Figure 3 c and d). The average heterozygosity of breeding lines was substantially higher (32.0%) than

for original populations (16.4%), and had a greater range of values (0-95%; Table 2).

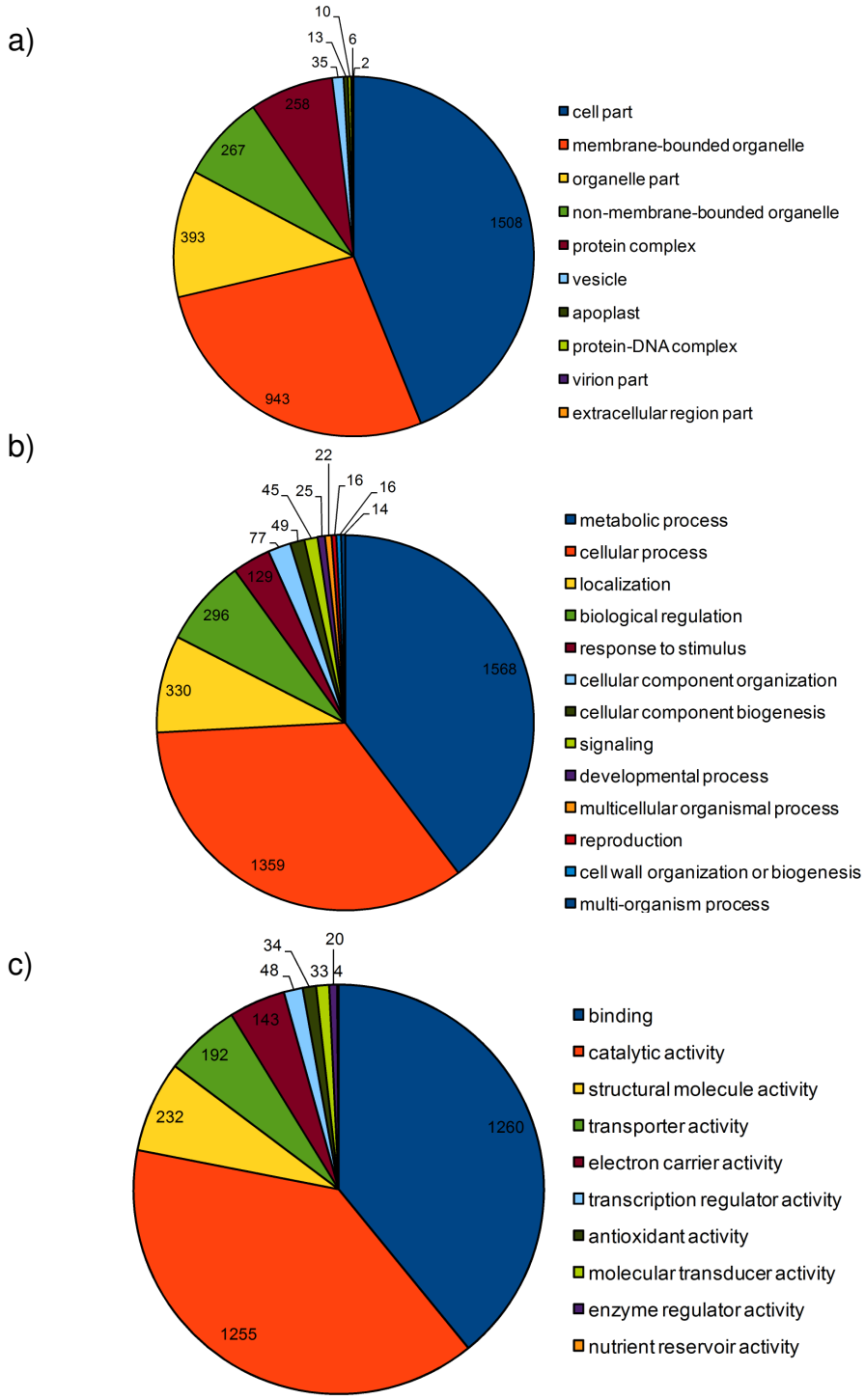
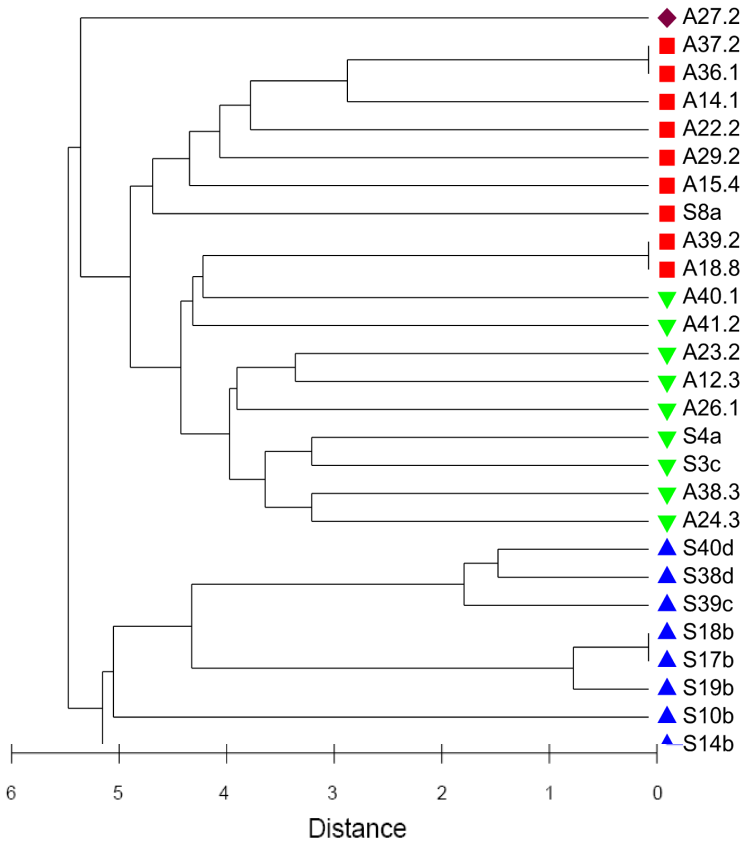


Figure 1. Gene ontology (GO) terms for 3,419 expressed gene sequences obtained by Roche 454 sequencing of leaf mRNA of *Bituminaria bituminosa* plant 13.1. GO descriptors are categorised according to: a) cellular components (level 3 terms); b) biological process (level 2 terms with >10 sequences); and c) molecular function (level 2 terms).

a)



b)

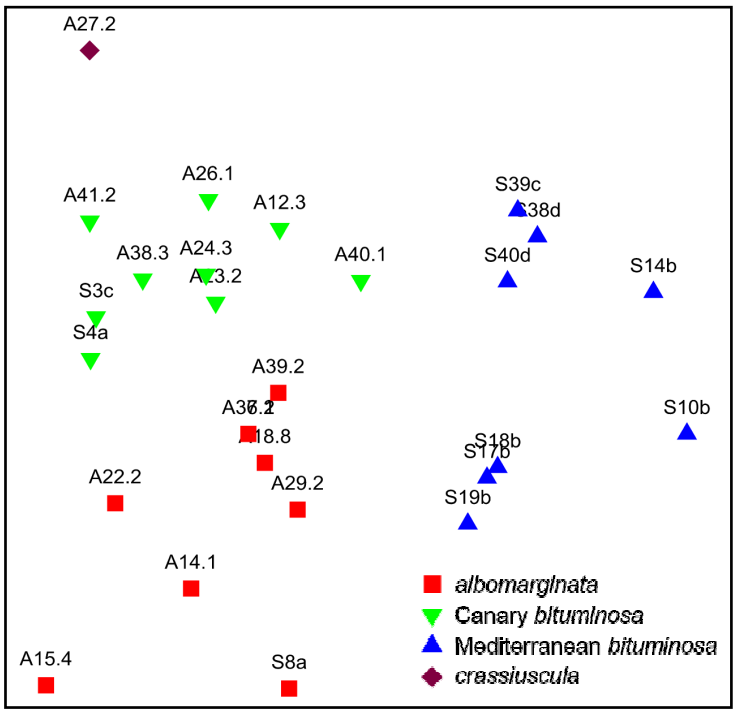


Figure 2. Genetic relationships among 27 *Bituminaria bituminosa* accessions from three botanical varieties collected from the Canary Islands and the Mediterranean region. One plant from each accession was assessed using 21 simple sequence repeat loci with the resulting 130 alleles used to calculate Euclidean pairwise distances. These distances are presented in: a) Dendrogram produced by hierarchical clustering analysis; b) Multi-dimensional scaling (MDS) plot (2D stress = 0.16).

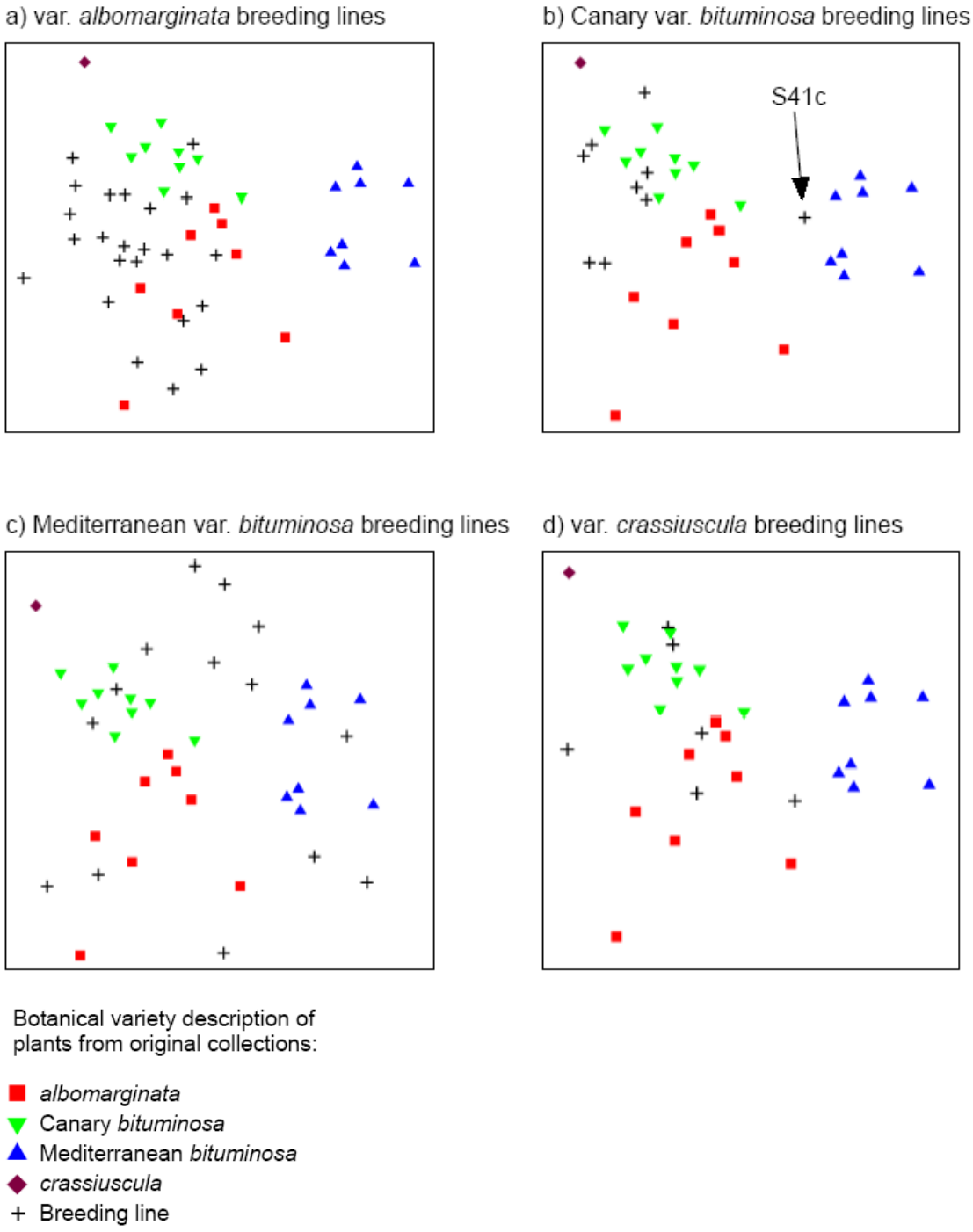


Figure 3. Multidimensional scaling (MDS) plots of 79 *Bituminaria bituminosa* accessions from three botanical varieties from the Canary Islands and the Mediterranean region. Each MDS plot was generated from pairwise Euclidean distances of all 79 accessions (2D stress = 0.23) with each plot showing the position of additional breeding lines (denoted by "+" signs) from botanical varieties: a) var. *albomarginata*; b) var. *bituminosa* (Canary Islands); c) var. *bituminosa* (Mediterranean region); and d) var. *crassiuscula*

DISCUSSION

New high-throughput DNA sequencing technologies offer many research opportunities for both model and crop species alike (Martínez *et al*. 2010). Arguably, minor grain and pasture crop species (also known as “orphan” crop species, Varshney *et al*. 2009) stand to gain the most since they are starting from a very low baseline of genomic resources. This paper describes a method for developing codominant and polymorphic genic SSR markers from a modest sampling of the leaf transcriptome derived from a single plant of *B. bituminosa* by Roche 454 pyrosequencing. This method was highly effective with 75 out of 87 (86.2%) automatically designed primer pairs successfully amplifying PCR products (see CD ANNEX III: Additional file 3). Of the 21 markers selected for high- resolution fragment analysis in plants from 79 *B. bituminosa* accessions, 20 gave consistently strong amplification products and were highly polymorphic (6.19 alleles per marker locus). The methods presented in this study could be used by researchers for other genomic orphan species for rapid development of high-quality codominant markers, although the extent of marker polymorphism will vary between species and between populations within species.

The usefulness of the SSR markers was demonstrated by a clear delineation of groups according to botanical variety and geographical location in plants sampled from 27 original populations. These markers advanced our understanding of genetic diversity in *B. bituminosa* in that we could clearly distinguish var. *bituminosa* types according to their geographical origin (Canary Islands and the Mediterranean region; Figure 2). This differentiation was of similar magnitude to that which distinguished botanical varieties and extends the observation by Juan *et al*. (2004) who found that Mediterranean var. *bituminosa* formed its own grouping while all botanical varieties from the Canary Islands formed another group. Therefore, there is a *prima facie* case for dividing var. *bituminosa* into two botanical varieties according to geographic origin (Canary Island or Mediterranean region), each distinct from var. *albomarginata* and var. *crassiuscula*. There are some differences in trait characteristics that support this division. For example, Mediterranean var. *bituminosa* are usually biennial whereas Canary Island var. *bituminosa* are perennial.

These SSR markers provided some useful insights into *B. bituminosa* breeding lines. Historically, the *B. bituminosa* breeding programme has practiced uncontrolled open pollination. While most pollination in *B. bituminosa* is considered to be by self-pollination, a certain proportion of out-crossing does occur (Stefanović *et al*. 2009) but until now there has been little direct evidence to support this assumption. In this study, two lines of evidence were found to support the occurrence of out-crossing in the *B. bituminosa* breeding programme. First, the average level of heterozygosity increased approximately two-fold in breeding lines (32.0%) versus original populations (16.4%) (Table 2). Second, cross-pollination between *B. bituminosa* plants from different botanical varieties and geographical locations is the most likely explanation of the wider distribution of breeding lines compared to original populations in MDS plots (Figure 3). Interestingly, this increased distribution was most pronounced in Mediterranean var. *bituminosa*. This may in part be explained by breeder observations in Murcia (Spain) that populations of var. *bituminosa* presented a higher percentage of fruit set compared to populations of var. *albomarginata*, which was taken to be indirect evidence of higher cross-pollination frequencies in var. *bituminosa* (Schmutz *et al*. 2010). The same observation was made for var. *crassiuscula*, though analysis of additional accessions is necessary to confirm this preliminary conclusion.

An advantage of using the transcriptome sequencing approach compared to more conventional genomic SSR approaches is that markers should be more transferable across species since they are based on gene sequences that are relatively well conserved in evolution compared to non-genic regions (Hecht *et al*. 2005). For example, these genetic markers may prove useful in other Psoraleeae species that remain genomic orphans, such as *Cullen australasicum,* an Australian native perennial legume species that shows promise as a drought-tolerant pasture species (Larbat *et al*. 2007).

In addition to generating useful SSR markers, this study provides a repertoire of many thousands of expressed gene sequences for potential follow-up experiments. Gene ontology analysis using Blast2GO was highly effective in identifying putative cellular location, biological process and molecular function in 3,419 out of 3,838 (89.1%) assembled mRNA sequences. Examples of follow-

up experiments may include investigating genes associated with flowering time regulation [e.g. *CONSTANS*-like a (Hecht *et al*. 2005); isotig02726, see CD ANNEX III: Additional file 1] or furanocoumarin biosynthesis genes [e.g. cytochrome p450 monooxygenases (Larbat *et al*. 2007; 2009); e.g. isotig00288, see CD ANNEX III: Additional file 1]. The species showing closest homology to *B. bituminosa* in 3,013 out of 3,419 (88.1%) mRNA sequences with significant hits was *G.max*, indicating that *G. max* will serve as a useful reference genome for future gene discovery and characterisation in *B. bituminosa*.

This work describes an efficient method for developing valuable SSR markers for *B. bituminosa*, a species that could previously be described as a genomic orphan. These markers gave new insight into genetic variation in *B. bituminosa*, providing evidence that a division of the botanical variety *bituminosa* may be appropriate. Evidence of cross pollination was found between botanical varieties in the *B. bituminosa* breeding programme. The expressed gene repertoire discovered in this experiment may be useful for follow-up experiments targeting biochemical pathways and/or important agronomic traits.

ADDITIONAL INFORMATION (see ANNEX III, CD)

Additional file 1: Gene ontology (GO) terms for 3,419 *B. bituminosa* mRNA sequences. GO terms with associated descriptions generated using Blast2GO software.

Additional file 2: 240 primer pairs targeting simple sequence repeat motifs. Primer pairs designed using Primer3 based on Roche 454 sequences identified as containing simple sequence repeats using QDD software.

Additional file 3: Primer pairs selected for testing. Details of 94 primer pairs targeting simple sequence repeat (SSR) motifs in transcribed genes of *B. bituminosa* leaves sampled by Roche 454 transcriptome sequencing.

Additional file 4: Allele frequencies in *B. bituminosa* plants. A total of 118 simple sequence repeat (SSR) marker alleles were detected using 19 single-locus markers. Marker Bbit-SSR079 detected two loci and so was not included here. Polymorphic information content (PIC) and allele frequencies were calculated based on the whole population of 79 *B. bituminosa* plants.

Allele frequencies are also presented for four original populations of botanical varieties albomarginata, bituminosa (from the Canary Islands and Mediterranean region) and crassiuscula that together comprised 27 plants.

Additional file 5: Euclidean distance matrix. 79 *B. bituminosa* plants were genotyped at 21 simple sequence repeat loci. The resulting 130 alleles were used to calculate pairwise Euclidean distances.

Additional file 6: Cluster tree of 79 *B. bituminosa* plants. Based on Euclidean distances estimated using 130 simple sequence repeat marker alleles. Symbols indicate botanical variety and type of line (original collection or breeding line).

ACKNOWLEDGEMENTS

We thank Future Farm Industries CRC, ICIA and IMIDA for providing the germplasm of Tedera. We also thank the Instituto Nacional de Investigación y Tecnología Agraria y Alimetaria (Project RTA 2007-00046-00-00) for funding the Spanish breeding programme and The Ministrario de Ciencia e Innovación (Project BFU2010-19599) for providing support for publication costs. MPN thanks IMIDA and Caja Murcia for funding travel to UWA, UWA for hosting the visit and Dr Leonor Ruiz for advice on SSR analyses. MD thanks European Social Fund. We thank Caroline Snowball for preparing fluorescent PCR samples for fragment analysis. MNN thanks James Hane for advice on genomic data analysis and sequence submission to NCBI.

CONCLUSIONS AND FUTURE PROSPECTS

CONCLUSIONS

CHAPTER I:

I. Partial cisgenic grapevines have been obtained with the *Vst1* gene from *Vitis vinifera*. The genetically-modified plants over-produce resveratrol and the levels were 2-7 fold greater than in the control plants.

II. This system of genetic modification is a good alternative to obtain true cisgenic plants for producing secondary metabolites, and avoids the problem of the negative public opinion concerning genetically-modified plants.

CHAPTER II:

I. This is the first time that *in vitro* plant tissue culture techniques for *B. bituminosa* have been developed. Micropropagation protocols using apical and nodal explants have been established from four plant accessions. The addition of activated charcoal improves the quality and development of regenerated shoots. GA_3 added to the culture medium improves shoot elongation in variety *albomarginata* because of having shorter internodes than var. *bituminosa.*

II. Micropropagated plants established in the field contained FC levels equal to or even higher than those in the non-micropropagated mother plants, showing that this protocol offers a good method to multiply plants selected for their high FC content.

III. A plant regeneration protocol from different leaf explants (leaflet, petiole and the intersection between petioles and leaflets) of *B. bituminosa* has been developed. An interaction between explant type and plant growth regulators used in the culture medium was observed. The best explant type was the intersection between petioles and leaflets (cross).

IV. The production of FCs has been evaluated in different plant material: organogenic calli, *in vitro* plants, *ex vitro* greenhouse-plants and *ex vitro* field-plants. The accumulation of FCs depended on the level of cell organization, plant development and environmental stress, being higher in *ex vitro* field-plants.

V. It has been demonstrated, for the first time, that elicitation of *in vitro* cultures (organogenic calli and *in vitro* plants) by ultraviolet radiation enhances the linear and angular FC production.

VI. *In vitro* cultures of *B. bituminosa* show contamination by two pink endophytic bacteria identified as *Blastococcus* sp (*Blastococcus* strain E) and *Methylobacterium extorquens* (*M. extorquens* strain I). *Blastococcus* strain E presents resistance to tetracycline and is not able to assimilate methanol, while *M. extorquens* strain I is sensitive to tetracycline and assimilates methanol as an energy and carbon source.

VII. The elimination of endophytic bacteria is achieved by the addition of gentamicin (35 mg l^{-1}) and cefotaxime (400 mg l^{-1}) to the plant *in vitro* culture media, although at these concentrations the effect of the antibiotic combination is bacteriostatic and not bactericidal.

VIII. An efficient method for developing valuable SSR markers for *B. bituminosa* has been developed. These markers have given new insights into the genetic variation in *B. bituminosa*, providing evidence that a division of the botanical variety *bituminosa* from the Canary Islands and from the Mediterranean region may be appropriate.

IX. Evidence of cross pollination was found between botanical varieties (*albomarginata, bituminosa* and *crassiuscula*) in the *B. bituminosa* breeding programme.

FUTURE PROSPECTS

I. The establishment of cell suspensions obtained from calli of leaves of Vst1-cisgenic grapevine plants and subsequent elicitation could be a good approach to increase large-scale resveratrol production for commercial purposes.

II. Plant regeneration from leaf explants of *B. bituminosa* is a useful tool for developing a genetic modification protocol that allows control of the synthesis pathway of linear or angular FCs.

III. The FC biosynthesis could be increased by using elicitors or precursors involved in the biosynthetic pathway.

IV. To develop a linkage map of the *B. bituminosa* genome and quantitative trait locus (QTL) mapping of genes controlling FC biosynthesis.

REFERENCES

Abdoli M, Moieni A, Dehghani H (2007) Effects of cultivar and agar concentration on *in vitro* shoot organogenesis and hyperhydricity in sunflower (*Helianthus annuus* L.). Pak J Bot 39: 31-35

Ahmad N, Faisal M, Anis M, Aref IM (2010) *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens.* South Afr J Bot 76: 597-600

Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y (2004) Role of resveratrol in prevention and therapy of cancer: Preclinical and clinical studies. Anticancer Res 24: 2783-2840

Almagro L (2011) Análisis funcional de la producción de compuestos con actividad antitumoral a partir de cultivos celulares sometidos a elicitación. Tesis Doctoral, Universidad de Murcia

Anis M, Faisal M (2005) *In vitro* regeneration and mass multiplication of *Psoralea corylifolia* - An endangered medicinal plant. Indian J Biotechnol 4: 261-264

Arahal DR, Sánchez E, Macián MC, Garay E (2008) Value of recN sequences for species identification and as a phylogenetic marker within the family "Leuconostocaceae". Int Microb 11: 33-39

Aradhya MK, Dangl GS, Prins BH, Boursiquot JM, Walker MA, Meredith CP, Simon CJ (2003) Genetic structure and differentiation in cultivated grape, Vitis vinifera L. Genet Res 81: 179–192

Austin DJ, Brown SA (1973) Furanocoumarin biosynthesis in *Ruta graveolens* cell cultures. Phytochem 12: 1657-1667

Backhouse CN, Delporte CL, Negrete RE (2001) Active constituents isolated from *Psoralea grandulosa* L. with anti-inflammatory and antipyretic activities. J Ethno Pharmacol 78: 27-31

Bais AJ, Murphy PJ, Dry IB (2000) The molecular regulation of stilbene phytoalexin biosynthesis in *Vitis vinifera* during grape berry development. Aust J Plant Physiol 27: 425-433

Basile DV, Basile MR, Li QY, Corpe WA (1985) Vitamin B12-stimulated growth and development of Jungermannia leiantha Grolle and Gymnocolea inflata (Huds.) Dum. (Hepaticae). Bryol 88: 77-81

Baskaran P, Jayabalan N (2007) Rapid micropropagation of *Psoralea corylifolia* L. using nodal explants cultured in organic additive-supplemented medium. J Hortic Sci Biotechnol 82: 908-913

Baskaran P, Jayabalan N (2008) Effect of growth regulator on rapid micropropagation and psoralen production in *Psoralea corylifolia* L. Acta Physiol Plant 30: 345-351

Baskaran P, Jayabalan N (2009a) *In vitro* regeneration of *Psoralea corylifolia* L. through callus cultures. Plant Biotechnol 26: 333-336

Baskaran P, Jayabalan N (2009b) An improve protocol for adventitious shoot regeneration and plant formation in *Psoralea corylifolia* L. Sci Hort 123: 283-286

Baskaran P, Jayabalan N (2009c) In vitro propagation of Psoralea corylifolia L. by somatic embryogenesis in suspension culture. Acta Physiol Plant 31: 1119-1127

Baskaran P, Jayabalan N (2009d) Psoralen production in hairy roots and adventitious roots cultures of Psoralea coryfolia. Biotechnol Lett 31: 1073-1077

Baskaran P, Jayabalan N, Van Staden J (2011) Production of psoralen by *in vitro* regenerated plants from callus cultures of *Psoralea corylifolia* L. Plant Growth Regul 65: 47-54

Bastiaens L, Maene L, Harboui Y, Sumere C, Casteele KL, Debergh PC (1983) The influence of antibacterial products on plant tissue culture: a review. Med Fac Landbouww Rijksuniv Gent 48: 13-24

Bauer AW, Kirby WMM, Sherris JC, Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45: 493–496

Bavaresco L and Fregoni C (2001) Physiological role and molecular aspects of grapevine stilbenic compounds. In: Molecular Biology and Biotechnology

of the grapevine. (KA Roubelakis-Angelakis. Ed Kluver academic Publishers). 153-182

Bavaresco L, Pezzuto S, Ragga A, Ferrari F, Trevisan M (2001) Effect of nitrogen supply on *trans*-resveratrol concentration in berries of *Vitis vinifera* L. cv. Cabernet Suavignon. Vitis 40: 229-230

Beher D, Wu J, Cumine S, Kim KW, Lu S, Atagan L, Wang M (2009) Resveratrol is not a direct activator of SIRT1 enzyme activity. Chem Biol Drug Des 74: 619–624

Beier RC, Oertli EH (1983) Psoralen and other linear furocoumarins as phytoalexins in celery. Phytochem 22: 2595-2597

Belchí-Navarro S, Almagro L, Lijavetzky D, Bru R, Pedreño MA (2012) Enhanced extracellular production of trans-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and methyljasmonate. Plant Cell Rep 31: 81-89

Belhadj A, Telef N, Saigne C, Cluzet S, Barrieu F, Hamdi S, Mérillon JM (2008) Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. Plant Physiol Biochem 46: 493-499

Berenbaum M (1983) Coumarins and caterpillars: a case of evolution. Evol 37: 163-179

Bohlmann J, Gibraltarskaya E, Eilert U (1995) Elicitor induction of furanocoumarin biosynthetic pathway in cell cultures of Ruta graveolens. Plant Cell Tiss Org Cult 43: 155-161

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: a historical perspective. Plant Sci 161: 839-851

Bourgaud F, Hehn A, Larbat R, Doerper S, Gontier E, Kellner S, Matern U (2006) Biosynthesis of coumarins in plants: a major pathway still to be unravelled for cytochrome P450 enzymes. Phytochem Rev 5: 293-308

Bourgaud F, Nguyen C, Guckert A (1995) XXII *Psoralea* species: *In vitro* culture and production of furanocoumarins and other secondary metabolism. In:

Biotechnology in Agriculture and Forestry, vol 33 Medicinal and Aromatic Plants VIII (Bajaj YPS ed). Springer-Verlag Berlin Heidelberg

Bradamante S, Barenghi L, Villa A. 2004. Cardiovascular protective effects of resveratrol. Cardiovasc Drug Rev 22: 169-188

Bru MR, Pedreno GMLDE (2006) Method for the production of resveratrol in cell cultures. US 2006/0205049 A1

Bru R, Selles S, Casado-Vela J, Belchi-Navarro S, Pedreño MA (2006) Modified cyclodextrins are chemically defined glucan inducers of defense responses in grapevine cell cultures. J Agr Food Chem 54: 65–71

Burnett C, Valentini S, Cabreiro F, Goss M, Somogyvári M, Piper MD, Hoddinott M, Sutphin GL *et al*. (2011) Absence of effects of Sir2 overexpression on lifespan in *C. elegans* and *Drosophila.* Nat 477: 482-486

Burns J, Yokota T, Ashihara H, Lean MEJ, Crizier A (2002) Plant foods and herbal sources of resveratrol. J Agric Food Chem 50:3337-3340

Camm EL, Wat CK, Towers GHN (1976) An assessment of the roles of furanocoumarins in *Heracleum lanatum*. Can J Bot 54: 2562-2566

Chen J, Hall DE, Murata J, De Luca V (2006) L-Alanine induces programmed cell death in *V. lambrusca* cell suspension cultures. Plant Sci 171: 734-744

Cheng S, Van Houten B, Gamper HB, Sancar A, Hearst JE (1988) Use of psoralen-modified oligonucleotides to trap three-stranded RecA-DNA complexes and repair of those cross-linked complexes by ABC exinuclease. J Biol Chem 263: 15110-15117

Collard BCY, Mackill DJ (2008) Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Phil Trans R Soc B: Biol Sci 363: 557-572

Conn VM, Walker AR, Franco CM (2008) Endophytic actinobacteria induce defense pathways in Arabidopsis thaliana. Mol Plant Microbe Interact 21: 208-218

Cornu D, Michel MF (1987) Bacteria contaminants in shoot cultures of Prunus avium L. choice and phytotoxicity antibiotics. Acta Hortic 212: 83-86

Coutos-Thévenot P, Poinssot B, Bonomelli A, Yean H, Breda C, Buffars D, Esnault R, Hain R, Boulay M. 2001. *In vitro* tolerance to *Botrytis cinerea* of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase *Vst1* gene under the control of a pathogen-inducible PR 10 promoter. J Exp Bot 52:901-910

Danilova SA, Dolgikh YI (2004) The stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture. Plant Physiol 51: 559-562

Dabauza M, Velasco L (2012) Development of highly efficient genetic transformation protocols for table grape Sugraone and Crimson Seedless. In: Transgenic Plants: Methods and Protocols, Methods in Molecular Biology, vol. 847 (Jim M. Dunwell and Andy C. Wetten eds.), Springer Science+Business Media, LLC. 227-235

Delaunoise B, Cordelier S, Conreux A, Clément C, Jeandet P (2009) Molecular engineering of resveratrol in plants. Plant Biotechnol J 7: 2-12

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Molec Biol Rep 1:19-21

Dhekney SA, Li ZT, Gray DJ (2011) Grapevines engineered to express cisgenic *Vitis vinifera* thaumatin-like protein exhibit fungal disease resistance. *In Vitro* Cell Dev Biol-Plant 47: 458-466

Dhekney SA, Li ZT, Zimmerman TW, Gray DJ (2009) Factors influencing genetic transformation and plant regeneration of *Vitis.* Am J Enol Vitic 60: 285-292

Diwan R, Malpathak N (2008) Novel technique for scaling up of micropropagated *Ruta graveolens* shoots using liquid culture systems: A step towards commercialization. New Biotechol 25: 85-91

Diwan R, Malpathak N (2010) Histochemical localization in *Ruta graveolens* cell cultures: elucidating the relationship between cellular differentiation and furanocoumarin production. *In vitro* Cell Dev Biol-Plant 46:108-116

Dodds JH, Roberts LW (1981) Some inhibitory effects on gentamicin on plant tissue cultures. *In vitro* 17:467-470

Doetsch RN (1981) Determinative methods of light microscopy. In: Manual of methods for general microbiology (Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB ed.). American Society for Microbiology, Washington, DC

Donnez D, Jeandet P, Clement C, Courot E (2009) Bioproduction of resveratrol ans stilbene derivates by plant cells and microorganism. Trends Biotechnol 27: 706-713

Doyle J, Doyle J, Ballenger J, Dickson E, KajitaT and Ohashi H (1997) A phylogeny of the chloroplast gene rbcL in the Leguminosae: taxonomic correlations and insights into the evolution of nodulation. Am J Bot 84: 541-554

Dutt M, Li ZT, Dhekney SA, Gray DJ (2008) A co-transformation system to produce transgenic grapevines free of marker genes. Plant Sci 175: 423-430

Egan AN, Crandall KA (2008) Incorporating gaps as phylogenetic characters across eight DNA regions: Ramifications for North American *Psoraleeae* (*Leguminosae*). Mol Phylogenet Evol 46: 532-546

Ekiert H (1993) Ammi majus L (Bishop's Weed): *in vitro* culture and production of coumarin compounds. Biotechnology in agriculture and forestry, vol. 21 medicinal and aromatic plants IV. Springer, Berlin. 1–17

Ekiert H (2001) Medical plant biotechnology: the *Apiaceae* family as the example of rapid development. Pharm 55: 561-567

Ekiert H, Abou-Mandour AA, Czygan FCh (2005) Accumulation of biologically active furanocoumarins in *Ruta graveolens* ssp. *divaricata* (Tenore) Gams *in vitro* culture. Pharm 60: 66-68

Ekiert H, Choloniewska M, Gomólka E (2001) Accumulation of furanocoumarins in *Ruta graveolens* L. shoot culture. Biotechnol Lett 23: 543-545

Ekiert H, Glomolka E (2000) Coumarin compounds in Ammi majus callus cultures. Pharm 55: 684-687

Faisal M, Anis M (2005) *In vitro* regeneration and mass propagation of *Ruta graveolens* L.- A multipurpose shrub. Hortsci 40: 1478-1480

Fan C, Pu N, Wang X, Wang Y, Fang L, Xu W, Zhang J (2007) *Agrobacterium*-mediated genetic transformation of grapevine (*Vitis vinifera* L.) with a novel stilbene synthase gene from Chinese wild *Vitis pseudoreticulata*. Plant Cell Tiss Organ Cult 92: 197-206

Fernández-Mar MI, Mateos R, García-Parrilla MC, Puertas B, Cantos-Villar E (2012) Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. Food Chem 130: 797-813

Ferri M, Tassoni A, Franceschetti M, Righetti L, Naldrett MJ, Bagni N (2009) Chitosan treatment induces changes of protein expression profile and stilbene distribution in *Vitis vinifera* cell suspensions. Proteomics 9: 610-624

Ferri M, Righetti L, Tassoni A (2011) Increasing sucrose concentrations promote phenylpropanoid biosynthesis in grapevine cell cultures. J Plant Physiol 168: 189-195

Fettig S, Hess D (1999) Expression of a chimeric stilbene synthase gene in transgenic wheat lines. Transgenic Res 8: 179-189

Fischer R, Budde I, Hain R (1997) Stilbene synthase gene expression causes changes in flower colour and male sterility in tobacco. Plant J 11: 489-498

Fisse J, Batalle A, Pera J (1987) Endogenous bacteria elimination in ornamental plants. Acta Hortic 212: 87-90

Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. P Natl Acad Sci USA 91: 3490-3496

Floss HG, Mothes U (1964) On the biochemistry of furocoumarin in *Pimpinella magna*. Z Naturforsch B 19: 770-771

Gallego V, García MT, Ventosa A (2005) *Methylobacterium isbiliense* sp. nov., isolated from the drinking water system of Sevilla, Spain. Int J Syst Evolut Microbiol 55: 2333-2337

Giorcelli A, Sparvoli F, Mattivi F, Tava A, Balestrazzi A, Vrhovsek U, Calligari P, Bollini R, Confalonieri M (2004) Expression of the stilbene synthase (*StSy*) gene from grapevine in transgenic white poplar results in high

accumulation of the antioxidant resveratrol glucosides. Transgenic Res 13: 203-214

Giovinazzo G, d'Amico L, Paradisio A, Bollini R, Sparvoli F, DeGara L (2005) Antioxidant metabolite profiles in tomato fruit constitutively expressing the grapevine stilbene synthase gene. Plant Biotechnol J 3: 57-69

Gómez-Galera S, Pelacho AM, Gené A, Capell T, Christou P (2007) The genetic manipulation of medicinal and aromatic plants. Plant Cell Rep 26: 1689-1715

Goodwin, P.H., Hsiang, T. and Erickson, L. (2000) A comparison of stilbene and chalcone synthases including a new stilbene synthase gene from *Vitis riparia* cv. Gloire de Montpellier. Plant Sci 151: 1-8

Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talón M, Dopazo J, Conesa A (2008) High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res 36: 3420-3435

Guo X, Lidstrom ME (2006) Physiological analysis of *Methylobacterium extorquens* AM1 grown in continuous and batch cultures. Arch Microbiol 186: 139-149

Gutha LR, Casassa LF, Harbertson JF, Naidu RA (2010) Modulation of flavonoid biosynthetic pathway genes and anthocyanins due to virus infection in grapevine (*Vitis vinifera* L.) leaves. BMC Plant Biol 10: 187

Hain R, Bieseler B, Kindl H, Schröder G, Stöcker R (1990) Expression of a stilbene synthase gene in *Nicotiana tabacum* results in synthesis of the phytoalexin resveratrol. Plant Mol Biol 15: 325-335

Hain R, Reif HJ, Krause E, Langebartels R, Kindl H, Vornam B, Wiese W, Schmelzer E, Schreier PH, Stöcker RH, Stenzel K (1993) Disease resistance results from foreign phytoalexin expression in a novel plant. Nat 361: 153 -156

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43: 895-914

Hamerski D, Matern U (1988) Elicitor-induced biosynthesis of psoralens in *Ammi majus* L. suspension cultures: Microsomal conversion of demethylsuberosin into (+)-marmesin and psoralen. Eur J Biochem 171: 369-375

Hamerski D, Beier RC, Kneusel RE, Matern U, Himmelspach K (1990) Accumulation of coumarins in elicitor-treated cell suspension cultures of *Ammi majus*. Phytochem 4: 1137-1142

Harikumar KB, Aggarwal BB (2008) Resveratrol: A multitargeted agent for age-associated chronic diseases. Cell Cycle 7: 1020-1035

Hasegawa S, Meguro A, Shimizu M, Nishimura T, Kunoh H (2006) Endophytic actinomycetes and their interactions with host plants. Actinomycetolog 20: 72-81

Hecht V, Foucher F, Ferrandiz C, Macknight R, Navarro C, Morin J, Vardy ME, Ellis N, Beltran JP, Rameau C *et al* (2005) Conservation of Arabidopsis flowering genes in model legumes. Plant Physiol 137: 1420-1434

Hehmann M, Lukacin R, Ekiert H, Matern U (2004) Furanocoumarin biosynthesis in *Ammi majus* L. Cloning of bergaptol *O*-methyltransferase. Eur J Biochem 271: 932-940

Hipskind JD, Paiva, NL (2000) Constitutive accumulation of a resveratrol glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis.* Mol Plant–Microbe Interact 13: 551-562.

Hohtola A (1988) Seasonal changes in explant viability and contamination of tissue cultures from mature Scots pine. Plant Cell Tiss Org Cult 15: 211-222

Holland MA (1997) Are cytokinins produced by plant?. Plant Physiol 115: 865-868

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgen Res 2: 208-218

Horsch RB, King J (1983) A covert contamination of cultured plant cells: elimination of a *Hyphomicrobium* spp. from cultures of *Datura innoxia* (Mill.). Plant Cell Tiss Org Cult 2: 21-28

Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG *et al*. (2003) Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nat 425: 191-196

Hüsken A, Baumert A, Milkowski C, Becker HC, Strack D, Möllers C (2005) Resveratrol glucoside (piceid) synthesis in seeds of transgenic oilseed rape (*Brassica napus* L.). Theor Appl Genet 111: 1553-1562

Ichi T, Koda T, Asai, I, Hatanaka A (1986) Effects of gelling agents on *in vitro* culture of plant tissues. Agric Biol Chem 50: 2397-2399

Ingrosso I, Bonsegna S, De Domenico S, Laddomada B, Blando F, Santino A, Giovinazzo G (2011) Over-expression of a grape stilbene synthase gene in tomato induces parthenocarpy and causes abnormal pollen development. Plant Physiol Biochem 49: 1092-1099

Innocenti G, Cappelletti E, Caporale G (1991) Furanocoumarin contents in the vegetative organs of cultivated *Psoralea* species. Int J Pharmacogn 29: 141-146

Innocenti G, Piovan A, Filippini R, Caniato R, Cappelletti E (1997) Quantitative recovery of furanocoumarins from *Psoralea bituminosa*. Phytochem Anal 8: 84-86

Jacobsen E, Schouten HJ (2008) Cisgenesis, a new tool for traditional plant breeding, should be exempted from the regulation on genetically modified organisms in a step by step approach. Potato Res 51: 75-88

Jaillon *et al*. (The French–Italian Public Consortium for Grapevine Genome Characterization) (2007) The grapevine genome sequence suggests ancestralhexaploidization in major angiosperm phyla. Nat 449: 463-469

Jeandet P, Bessis R, Gautheron B (1991) The production of resveratrol (3,5,4'-trihydroxystilbene) by grape berries in different developmental stages. Am J Enol Vitic *42*: 41-46

Jeandet P, Bessis R, Sbaghi M, Meunier P (1995) Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attacks in the vineyard. J. Phytopathol 143: 135-139

Jeandet P, Douillet AC, Debord S, Sbaghi M, Bessis R, Adrian M (2002) Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism. J Agric Food Chem 50: 2731–2741

Jourand P, Giraud E, Béna G, Sy A, Willems A, Gillis M, Dreyfus B, Lajudie P (2004) *Methylobacterium nodulans* sp. nov., for a group of aerobic, facultatively methylotrophic, legume root-nodule-forming and nitrogen-fixing bacteria. Int J Syst Evolut Microbiol 54:2269–2273

Juan A, Coca B, Crespo MB, Rios S, Correal E (2005) Datos preliminares sobre variabilidad molecular poblacional en el agregado de *Bituminaria bituminosa* (Leguminosae). Producciones agroganaderas: Gestión eficiente y conservación del medio natural Vol. II: 971-977

Juan A, Moñino I, Correal E, Méndez P, Crespo MB (2004) Comparación de las tasas de fructificación de *Bituminaria bituminosa* (Leguminosae) bajo condiciones de cultivo en Canarias y la Península Ibérica. In: Pastos y Ganadería Extensiva Actas XLIV Reunion Cientifica de la SEEP. Salamanca, Spain. Sociedad Espanola para el Estudio de los Pastos: 111-115

Kang Y, Kim J, Shin H, Nam Y, Bae J, Jeon CO, Park W (2007) *Methylobacterium platani* sp. nov., isolated from a leaf of the tree *Platanus orientalis*. Int J Syst Evolut Microbiol 57:2849–2853

Karimi IA, Srinivasan R, Han PL (2002) Unlock supply chain improvements through effective logistics. Chem Eng Prog 5: 32-38

Karimi M, De Meyer B, Hilson P (2005) Modular cloning and expression of tagged fluorescent protein in plant cells. Trends Plant Sci 10: 103-105

Kato Y, Asahara M, Arai D, Goto K, Yokota A (2005) Reclassification of *Methylobacterium chloromethanicum* and *Methylobacterium dichloromethanicum* as later subjective synonyms of *Methylobacterium extorquens* and of *Methylobacterium lusitanum* as a later subjective synonym of *Methylobacterium rhodesianum.* J Gen App Microbiol 5: 287-299

Keskitalo M, Pohto A, Savela M, Valkonen JPT, Simon J, Pehu E (1998) Alterations in growth of tissue-cultured tansy *(Tanacetum vulgare* L.) treated with antibiotics. Ann Appl Biol 133:281-296

Kim JS, Lee SY, Park SU (2008) Resveratrol production in hairy root culture of peanut, *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains. Af J Biotechnol 7: 3788-3790

Kim Y, Wyslouzil BE, Weathers P (2002) Secondary metabolism of hairy root cultures in bioreactors. *In Vitro* Cell Dev Biol-Plant 38: 1-10

Kiselev KV (2011) Perspectives for production and application of resveratrol. Appl Microbiol Biotechnol 90: 417-425

Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN (2007) The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. J Biotechnol 128: 681–692

Knief C, Frances L, Cantet F, Vorholt JA (2008) Cultivation-independent characterization of *Methylobacterium* populations in the plant phyllosphere by automated ribosomal intergenic spacer analysis. Appl Environ Microbiol 74: 2218-2228

Kobayashi S, Ding CK, Nakamura Y, Nakajima I, Matsumoto R (2000) Kiwifruits (*Actinidia deliciosa*) transformed with a *Vitis* stilbene synthase gene produce piceid (resveratrol-glucoside). Plant Cell Rep 19: 904-910

Kodan A, Kuroda H, Sakai F (2001) Simultaneous expression of stilbene synthase genes in Japanese red pine (*Pinus densiflora*). J Wood Sci 47: 58-62

Kodan A, Kuroda H, Sakai F (2002) A stilbene synthase from Japanese red pine (*Pinus densiflora*): implications for phytoalexine accumulation and down-regulation of flavonoid biosynthesis. Proc Natl Acad Sci USA 99: 3335-3339

Kouakou TH, Téguo PW, Valls J, Kouadio YJ, Decendit A, Mérillo J (2006) First evidence of trans- resveratrol production in cell suspension cultures of cotton (*Gossypium hirsutum* L.). Plant Cell Tiss Org Cult 86: 405-409

Królicka A, Kartanowicz R, Wosínski SA, Szpitter A, Kamínski M, Lojkowska E (2006) Induction of secondary metabolite production in transformed callus of *Ammi majus* L. grown after electromagnetic treatment of the culture medium. Enzyme Microb Technol 39: 1386-1391

Królicka A, Staniszewska I, Bielawski K, Malinski E, Szafranek J, Lojkowska E (2001) Establishment of hairy root cultures of *Ammi majus*. Plant Sci 160: 259-264

Kúc J, Rush JS (1985) Phytoalexins. Arch Biochem Biophys 236: 455-72

Kunoh H (2002) Endophytic actinomycetes: Attractive biocontrol agents. J Gen Plant Pathol 68: 249-252

Kutchera U (2007) Plant-associated methylobacteria as co-evolved phytosymbionts. A hypothesis. Plant Signal Behav 2: 74-78

Lampronti I, Bianchi N, Borgatti M, Fibach E, Prus E, Gambari R (2003) Accumulation of gamma-globin mRNA in human erythroid cells treated with angelicin. Eur J Haematol 71: 189-195

Langcake P, Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Physiol Plant Pathol 9: 77-86

Langcake P, Pryce RJ (1977) The production of resveratrol and the viniferins by grapevines in response to ultraviolet irradiation. Plant Physiol Biochem 16: 1193-1196

Larbat R, Hehn A, Hans J, Schneider S, Jugdé H, Schneider B, Matern U, Bourgaud F (2009) Isolation and functional characterization of *CYP71AJ4* encoding for the first P450 monooxygenase of angular furanocoumarin biosynthesis. J Biol Chem 284: 4776-4785

Larbat R, Kellner S, Specker S, Hehn A, Gontier E, Hans J, Bourgaud F, Matern U (2007) Molecular cloning and functional characterization of psoralen synthase, the first committed monooxygenase of furanocoumarin biosynthesis. J Biol Chem 282: 542-554

Lata H, Li XC, Silva B, Moraes RM, Halda-Alija L (2006) Identification of IAA-producing endophytic bacteria from micropropagated *Echinacea* plants using 16S rRNA sequencing. Plant Cell Tiss Org Cult 85: 353–359

Leckband G, Lörz H (1998) Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance. Theor Appl Genet 96: 1004-1012

Lee SD (2006) *Blastococcus jejuensis* sp. nov., an actinomycete from beach sediment, and emended description of the genus *Blastococcus* Ahrens and Moll 1970. Int J Syst Evolut Microbiol 56: 2391-2396

Leifert C, Camotta H, Waites WM (1992) Effect of combinations of antibiotics on micropropagated *Clematis, Delphinium, Hosta, Iris* and *Photinia.* Plant Cell Tiss Org Cult 29: 153-160

Leifert C, Morris CE, Waites WM (1994) Ecology of microbial saprophytes and pathogens in tissue culture and field-grown plants: Reasons for contamination problems *in vitro*. Crit Rev Plant Sci 13: 139-183

Leifert C, Camotta H, Wright SM, Waites B, Cheyne VA, Waites WM (1991) Elimination of bacteria from micropropagated plant cultures using antibiotics. J Appl Bacteriol 71:307-330

Leifert C, Waites WM, Nicholas JR (1989) Bacterial contaminants of micropropagated plant cultures. J Appl Bacteriol 67: 353-361

Leighton F, Cuevas A, Guasch V, Perez DD, Straobel P, San Martin A, Urzua U, Diez MS, Foncea R, Castillo O, Mizon C, Espinoza MA, Urquiaga I, Rozowski J, Maiz A, Germain A (1999) Plasma polyphenols and antioxidants, oxidative DNA damage and endotelial function in a diet and wine intervention study in humans. Drug Exp Clin Res 25: 133-141

Liang H, Zheng J, Shuange JIA, Wang D, Ouyang J, Li J, Li L, Tian W, Jia X, Duan X, Sheng B, Hain R (2000) A transgenic wheat with a stilbene synthase gene resistant to powdery mildew obtained by biolistic method. Chin Sci Bull 45: 634-638

Lièvre K, Hehn A, Tran TLM, Gravot A, Thomasset B, Bourgaud F, Gontier E (2005) Genetic transformation of the medicinal plant *Ruta graveolens* L. by an Agrobacterium tumefaciens-mediated method. Plant Sci 168: 883-888

Lijavetzky D, Almagro L, Belchí-Navarro S, Martínez-Zapater JM, Bru R, Pedreño MA (2008) Synergistic effect of methyljasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures. BMC Res Notes 1: 132

Lim JD, Yun SJ, Chung IM, Yu CY (2005) Resveratrol synthase transgene expression and accumulation of resveratrol glycoside in *Rehmannia glutinosa*. Mol Breed 16: 219-233

Lin LL, Lien CY, Cheng YC, Ku KL (2007) An effective sample preparation approach for screening the anticancer compound piceatannol using HPLC coupled with UV and fluorescence detection. J Chromatogr B 853: 175-182

Lipp M, Brodmann P, Pietsch K, Pauwels J, Anklam E (1999) IUAPC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. J AOAC Int 82: 923-928

Liu S, Hu Y, Wang X, Zhong J, Lin Z (2006) High content of resveratrol in lettuce transformed with a stilbene synthase gene of *Parthenocissus henryana*. J Agric Food Chem 54: 8082-8085

López-Pérez AJ, Carreño J, Martínez-Cutillas A, Dabauza M (2005) High embryogenic ability and plant regeneration of table grapevine (*Vitis vinifera* L.) induced by activated charcoal. Vitis 44: 79-85

López-Pérez AJ, Velasco L, Pazos-Navarro M, Dabauza M (2008) Development of highly efficient genetic transformation protocols for table grape Sugraone and Crimson Seedless at low *Agrobacterium* density. Plant Cell Tiss Org Cult 94: 189-199

Luedemann GM (1968) *Geodermatophilus*, a new genus of the *Dermatophilaceae* (*Actinomycetales*). J Bacteriol 96: 1848-1858

Madhaiyan M, Poonguzhali S, Sa T (2007) Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). Chemosphere 69: 220–228

Martínez S, Correal E, Real D, Ortuño A, Del Río JA (2010) *Bituminaria bituminosa*: A source of furanocoumarins of pharmaceutical interest. In: *Encyclopedia of Medicinal Plants. Drug Plants I.* (Awaad A S, Govil J N, Singh V K, Eds.). Texas, Houston, USA. 307-322

Martínez-Fernández D, Walker DJ, Romero-Espinar P, Flores P, del Río JA (2011) Physiological responses of *Bituminaria bituminosa* to heavy metals. J Plant Physiol 168: 2206-2211

Massot B, Milesi E, Gontier F, Bourgaud F, Guckert A (2000) Optimized culture conditions for the production of furanocoumarins by micropropagated shoots of *Ruta graveolens.* Plant Cell Tiss Org Cult 62: 11-19

Masuda T, Takasugi M, Anetai M (1998) Psoralen and other linear furanocoumarins as phytoalexins in *Glehnia littoralis*. Phytochem 47: 13-16

Mathias RJ, Mukasa C (1987) The effect of cefotaxime on the growth and regeneration of callus from four varieties of barley *(Hordeum vulgate* L.). Plant Cell Rep 6: 454-457

Maurich T, Iorio M, Chimenti D, Turchi G (2006) Erybraedin C and bitucarpin A, two structurally related pterocarpans purified from *Bituminaria bituminosa*, induced apoptosis in human colon adenocarcinoma cell lines MMR- and p53-proficient and -deficient in a dose-, time-, and structure-dependent fashion. Chem-Biol Interact 159: 104-116

Maurich T, Pistelli L, Turchi G (2004) Anti-clastogenic activity of two structurally related pterocarpans purified from *Bituminaria bituminosa* in cultured human lymphocytes. Mut Res 561: 75-81

Medina-Bolivar F, Condori J, Rimando AM, Hubstenberger J, Shelton K, O'Keefe SF, Bennett S, Dolan MC (2007) Production and secretion of resveratrol in hairy root cultures of peanut. Phytochem 68: 1992-2003

Meglécz E, Costedoat C, Dubut V, Gilles A, Malausa T, Pech N, Martin J (2010) QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinforma 26: 403-404

Meguro A, Ohmura Y, Hasegawa S, Shimizu M, Nishimura T, Kunoh H (2006) An Endophytic actinomycete, *Streptomyces* sp. MBR-52, that accelerates emergence and elongation of plant adventitious roots. Actinomycetolog 20: 1-9

Melchior F, Kindl H (1991) Coordinate- and elicitor-dependent expression of stilbene synthase and phenylalanine ammonia lyase genes in *Vitis* cv. Optima. Arch. Biochem Biophys 288: 552–557

Méndez P (2000) El heno de tedera (*Bituminaria bituminosa*): un forraje apetecible para el caprino. In: III Reunión Ibérica de Pastos y Forraxes, Galicia, Spain, 412-414

Méndez P, Fernández M (1990) Interés forrajero de las variedades de *Bituminaria bituminosa* (L.) Stirton ("tedera") de Canarias. In: XXX Reunión Científica de la Sociedad Española para el Estudio de los Pastos. Donostia-San Sebastian, Spain, 264-272

Méndez P, Fernández M, Santos A (1990) Variedades de *Bituminaria bituminosa* (L.) Stirton (Leguminosae) en el archipiélago canario. Pastos 20: 157-166

Milesi S, Massot B, Gontier F, Bourgaud F (2001) *Ruta graveolens* L.: a promising species for the production of furanocoumarins. Plant Sci 161: 189-199

Morales M, Bru R, Garcia-Carmona F, Barcelo AR, Pedreno MA (1998) Effect of dimethyl-b-cyclodextrins on resveratrol metabolism in Gamay grapevine cell cultures before and after inoculation with *Xylophilus ampelinus*. Plant Cell Tissue Org Cult 53: 179-187

Morelli R, Das S, Betelli A, Lo Scalzo R, Das DK, Falchi M (2005) The introduction of the stilbene synthase gene enhances the natural antiradical activity of *Lycopersicon esculentum* mill. Mol Cell Biochem 282: 65-73

Muñoz A, Correal E (1998) *Bituminaria bituminosa* L. Stirton, leguminosa de interés forrajero en la cuenca mediterránea: I. Situación taxonómica, distribución y autoecología. Actas XXXVIII Reunión Científica de la Sociedad Española para el Estudio de los Pastos. (Universidad de Valladolid Ed.) Soria, Spain, 87-91

Muñoz A, Ortiz-Dorda J, Correal E (2000) Morphological and molecular characterization of *Bituminaria bituminosa* accessions from South-East Spain and the Canary Islands. Cahiers Options Mediter 45:103-107

Murashige T Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473-497

Murray RDH, Mendez J, Brown SA (1982) The Natural Coumarins: Ocurrence, Chemistry and Biochemistry. Phytochem 22: 803-804

Namdeo AG (2007) Plant cell elicitation for production of secondary metabolites: A review. Pharmacog Rev 1: 69-79

Nauerby B, Billing K, Wyndaele R (1997) Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. Plant Sci 123:169-177

Nelson M, Mason A, Castello MC, Thomson L, Yan G, Cowling W (2009) Microspore culture preferentially selects unreduced (2n) gametes from an interspecific hybrid of *Brassica napus* L. × *Brassica carinata* Braun. Theor Appl Genet 119: 497-505

Nicoletti I, De Rossi A, Giovinazzo G, Corradini D (2007) Identification and quantification of stilbenes in fruits of transgenic tomato plants (*Lycopersicon esculentum* Mill.) by reversed phase HPLC with photodiode array and mass spectrometry detection. J Agric Food Chem 55*:* 3304-3311

Normand P (2006) *Geodermatophilaceae* fam. nov., a formal description. Int J Syst Evolut Microbiol 56: 2277-2278

Nowak J (1998) Review: Benefits of *in vitro* “Biotization” of plant tissue cultures with microbial inoculants. *In Vitro* Cell Dev Biol-Plant 34: 122-130

Oginsky EL, Green GS, Griffith DG, Fowlks WL (1959) Lethal photosensitization of bacteria with 8-methoxypsoralen to long wave length ultraviolet radiation. J Bacteriol 78: 821-833

Oksman-Calendentey KM, Inzé D (2004) Plant cell factories in the post-genomic era: new ways to produce designer secondary metabolites. Trends in Plant Sci 9: 433-440

Olas B, Wachowicz B, Szewczuk J, Saluk-Juszczak J, Kaca W 2001 The effect of resveratrol on the platelet secretory process induced by endotoxin and thrombin. Microbios 105: 7-13

Omer ZS, Tombolini R, Broberg A, Gerhardson B (2004a) Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. Plant Growth Regul 43: 93-96

Omer ZS, Tombolini R, Gerhardson B (2004b) Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). FEMS Microbiol Ecol 47: 319-326

Pacholec M, Bleasdale JE, Chrunyk B, Cunningham D, Flynn D, Garofalo R, Griffith D, Loulakis P, Pabst B, Qiu X, Stockman B, Thanabal V, Varghese A, Ward J, Withka J, Ahn K (2010) SRT1720, SRT2183, SRT1460, and Resveratrol are not direct activators of SIRT1. J Biol Chem 285: 8340-8351

Pande D, Purohit M, Srivastave PS (2002) Variation in xanthotoxin content in *Ammi majus* L. cultures during *in vitro* flowering and fruiting. Plant Sci 162: 583-587

Parast B, Chetri SK, Sharma K, Agrawal (2011) *In vitro* isolation, elicitation of psoralen in callus cultures of *Psoralea corylifolia* and cloning of psoralen synthase gene. Plant Physiol Biochem 49: 1138-1146

Parker JA, Arango M, Adderrahmane S, Lambert E, Tourette C, Catoire H *et al*. (2005) Resveratrol rescues mutant polyglutamine cytotoxicity in nematode and mammalian neurons. Nat Genet 37: 349-350

Pazos-Navarro M, Del Río JA, Ortuño A, Romero-Espinar P, Correal E, Dabauza M (2012) Micropropagation from apical and nodal segments of

Bituminaria bituminosa and the furanocoumarin content of propagated plants. J Hort Sci Biotechnol 87: 29-35

Peakall R, Smouse P (2006) Genalex6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6: 288-295

Pearson KJ, Baur JA, Lewis KN, Peshkin L, Price NL, Labinskyy N, Swindell WR, Kamara D, Minor RK, Perez E, Jamieson HA, Zhang Y, Dunn SR, Sharma K, Pleshko N, Woollett LA, Csiszar A, Ikeno Y, Couteur DL, Elliott PJ, Becker KG, Navas P, Ingram DK, Wolf NS, Ungvari Z, Sinclair DA, Cabo R (2008) Resveratrol delays age-related deterioration and mimics transcriptional aspects of dietary restriction without extending life span. Cell Metab 8: 157-168

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45

Phillips R, Arnott SN, Kaplan SE (1981) Antibiotics in plant tissue culture: rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of Helianthus tuberosus. Plant Sci Lett 21: 235-240

Pistelli L, Noccioli C, Appendina G, Bianchi F, Sterner O, Ballero M (2003) Pterocarpans from *Bituminaria morisiana* and *Bituminaria bituminosa*. Phytochem 64: 595-598

Pollock K, Barfield DG, Shields R (1983) The Toxicity of Antibiotics to Plant Cell Cultures. Plant Cell Rep 2: 36-39

Pool RM, Creasy LL, Frackelton AS (1981) Resveratrol and the viniferins, their application to screening for disease resistance in grape breeding programs. Vitis 20: 136-145

Pradhan A, Nelson MN, Plummer JA, Cowling WA, Yan G (2011) Characterization of *Brassica nigra* collections using simple sequence repeat markers reveals distinct groups associated with geographical location, and frequent mislabelling of species identity. Genome 54: 50-63

Preisig-Müller, R., Schwekendiek, A., Brehm, I., Reif, H.J. and Kindl, H. (1999) Characterization of a pine multigene family containing elicitor-responsive stilbene synthase genes. Plant Mol Biol 39: 221-229

Puddephat IJ, Alderson PG, Wright NA (1997) Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quercus robur* L. *in vitro.* J Exp Bot 48: 951-962

Qin S, Li J, Chen HH, Zhao GZ, Zhu WY, Jiang CL, Xu LH, Li WJ (2009) Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forest in Xishuangbanna, China. Appl Environ Microbiol 75: 6176-6186

Rao AM, Sree KP, Kishor PBK (1995) Enhanced plant regeneration in grain and sweet sorghum by asparagine, proline and cefotaxime. Plant Cell Rep 15: 72-75

Raskin I, Ribnicky DM, Komarnytsky S, Ilic N, Poulev A, Borisjuk N, Brinker A, Moreno DA, Ripoll C, Yakoby N, O'Neal JM, Cornwell T, Pastor I, Fridlender B (2002) Plants and human health in the twenty-first century. Trends Biotechnol 20: 522-531

Real D, Albersten T, Snowball R, Howieson J, Revell C, Ewing M, Correal E, Méndez P, Ríos S (2008) *Bituminaria bituminosa* var. *albomarginata* (Lancelot trefoil), a novel perennial forage legume for low-rainfall Mediterranean environments in Western Australia. In: XXI International Grassland Congress and VIII International Rangeland Congress. Hohhot, P.R. China. Vol II. 452

Real D, Li GD, Clark S, Albertsen TO, Hayes RC, Denton MD, D'Antuono MF, Dear BS (2011) Evaluation of perennial forage legumes and herbs in six Mediterranean environments. Chilean Journal of Agricultural Research 71(in press)

Real D, Verbyla A (2010) Maximizing genetic gains using a "plant" model in the Tedera (*Bituminaria bituminosa* var. *albomarginata* and var. *crassiuscula*) breeding program in Australia. Options Méditerranéennes 92: 87-96

Reed BM, Buckley PM, DeWilde TN (1995) Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro* Cell Dev Biol-Plant 31: 53-57

Reed BM, Mentzer J, Tanprasert P, Yu X (1998) Internal bacterial contamination of micropropagated hazelnut: identification and antibiotic treatment. Plant Cell Tiss Org Cult 52: 67-70

Reitz SR, Trumble JT (1997) Effects of linear furanocoumarins on the herbivore *Spodoptera exigua* and the parasitoid *Archytas marmoratus*: host quality and parasitoid success. Entomol Exp Appl 84: 9-16

Richter A, de Kathen A, de Lorenzo G, Briviba K, Hain R, Ramsay G, Jacobsen, HJ, Kiesecker H (2006) Transgenic peas (*Pisum sativum*) expressing polygalacturonase inhibiting protein from raspberry (*Rubus idaeus*) and stilbene synthase from grape (*Vitis vinifera*). Plant Cell Rep 25: 1166-1173

Roggero JP, García-Parrilla C (1995) Effects of ultraviolet irradiation on resveratrol and changes in resveratrol and various of its derivatives in the skins of ripening grapes. Sci Aliments 15: 411-422

Rommens CM, Hairing MA, Sword K, Davies HV, Belknap WR (2007) The intragenic approach as a new extension to traditional plant breeding. Trends Plant Sci 12: 397-403

Rosa A, Deiana M, Corona G, Atzeri A, Incani A, Appendin G, Dessì MA (2005) Antioxidant properties of extracts and compounds from *Psoralea morisiana*. Eur J Lipid Sci Technol 107: 521-529

Rout GR, Samantaray S, Das P (2000) *In vitro* manipulation and micropropagation of medicinal plants. Biotechnol Adv 18: 91-120

Rozen S, Skaletsky HJ. 1998. Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html

Rühmann S, Treutter D, Fritsche S, Briviba K, Szankowski I (2006) Piceid (resveratrol glucoside) synthesis in stilbene synthase transgenic apple fruit. J Agric Food Chem 54: 4633-4640

Salazar O, Valverde A, Genilloud O (2006) Real-Time PCR for the Detection and Quantification of *Geodermatophilaceae* from Stone Samples and

Identification of New Members of the Genus *Blastococcus*. Appl environ microbiol 72: 346–352

Santana L, Uriarte E, Roleira F, Milhazes N, Borges F (2004) Furanocoumarins in medicinal chemistry. Synthesis, naural occurrence and biological activity. Current Med Chem 11: 3239-3261

Saxena C, Palai SK, Samantaray S, Rout GR, Das P (1997) Plant regeneration from callus of *Psoralea corylifolia* Linn. Plant Growth Regul 22: 13-17

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J *et al* (2010): Genome sequence of the palaeopolyploid soybean. Nat 463:178-183

Schöeppner A, KindlH (1984) Purification and properties of a stilbene synthase from induced cell suspension cultures of peanut. J Biol Chem 259: 6806-6811

Schuler MA (1996) The role of cytochrome P450 monooxygenases in plant-insect interactions. Plant Physiol 112: 1411-1419

Schwekendiek A, Spring O, Heyerick A, Pickel B, Pitsch NT, Peschke F, de Keukeleire D, Weber G (2007) Constitutive expression of a grapevine stilbene synthase gene in transgenic hop (*Humulus lupulus* L.) yields resveratrol and its derivatives in substantial quantities. J Agric Food Chem 55: 7002-7009

Scott BR, Pathak MA, Mohn GR (1976) Molecular and genetic basis of furanocoumarin reactions. Mut Res 39: 29-74

Seppänen SK, Syrjaälä L, Weissenberg K, Teeri TH, Paajanen L, Pappinen A (2004) Antifungal activity of stilbenes in in vitro bioassays and in transgenic *Populus* expressing a gene encoding pinosylvin synthase. Plan Cell Rep 22: 584-593

Serazetdinova L, Oldach K, Lörz H (2005) Expression of transgenic stilbene synthases in wheat causes the accumulation of unknown stilbene derivatives with antifungal activity. J Plant Physiol 162: 985-1002

Sevon N, Oksman-Caldentey KS (2002) *Agrobacterium rhizogenes*- mediated transformation: Root cultures as a source of alkaloids. Planta Medica 68: 859-868

Shankar S, Singh G, Srivastava RK (2007) Chemoprevention by resveratrol: molecular mechanisms and therapeutic potential. Front Biosci 12: 4839-4854

Sharma S, Kulkarni SK, Chopra K. 2007. Effect of resveratrol, a polyphenolic phytoalexin, on thermal hyperalgesia in amouse model of diabetic neuropathic pain. Fundam Clin Pharmacol 21: 89-94

Shikishima Y, Takaishi Y, Honda G, Ito M, Takeda Y, Kodzhimatov OK, Ashurmetov O, Lee KH (2001) Chemical constituents of *Prangos tschimganica*; structure elucidation and absolute configuration of coumarin and furanocoumarin derivatives with anti-HIV activity. Chem Pharm Bull 49: 877-880

Sidwa-Gorycka M, Królicka A, Kozyra M, Głowniak K, Bourgaud F, Łojkowska E (2003) Establishment of a co-culture of *Ammi majus* L. and *Ruta graveolens* L. for the synthesis of furanocoumarins. Plant Sci 165: 1315-1319

Sparvoli F, Martin C, Scienza A, Gavazzi G, Tonelli C (1994) Cloning and molecular analysis of structural genes involved in flavonoid and stilbene biosynthesis in grape (*Vitis vinifera* L.) Plant Mol Biol 24: 743-755

Späth E (1937) Die natürlichen cumarine. Chem Ber 70(A): 83-117

Stam M, Mol JNM, Kooter JM (1997) The silence of genes in transgenic plants. Ann Bot-Lond 79: 3-12

Staniszewska I, Królicka A, Malinski E, Łojkowska E, Szafranek J (2003) Elicitation of secondary metabolites in in vitro cultures of *Ammi majus* L. Enzym Microb Technol 33: 565–568

Stark-Lorenzen P, Nelke B, Hänbler G, Mühlbach HP, Thomzik JE (1997) Transfer of a grapevine stilbene synthase gene to rice (*Oryza sativa* L.). Plant Cell Rep 16: 668-673

Stefanović S, Pfeil BE, Palmer JD, Doyle JJ (2009) Relationships among phaseoloid legumes based on sequences from eight chloroplast regions. Syst Bot 34: 115-128

Stervo U, Vang O, Bonnense C (2007) A review of the contento f the putative chempreventive phytoalexine resveratrol in red wine. Food Chem 101: 449-457

Stirton CH (1981) Tribe 11. *Psoraleeae* (Benth.) Rydb. (1919). In: *Advances in Legume Systematics. Part 1.* (Polhill, R. M. and Raven, P. H., Eds.). Royal Botanic Gardens. Kew, London, UK. 337-343

Subroto MA, Kwok KH, Hamill JD, Doran PM (1996) Coculture of genetically transformed roots and shoots for synthesis, translocation, and biotransformation of secondary metabolites. Biotechnol Bioeng 49: 481-494

Sy A, Giraud E, Jourand P, Garcia N, Willems A, De Lajudie P, Prin Y, Neyra M, Gillis M, Boivin-Masson C, Dreyfus B (2001) Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacteriol 183: 214-220

Szankowski I, Briviba, K, Fleschhut J, Schönherr J, Jacobsen HJ, Kiesecker H (2003) Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera* L.) and a PGIP gene from kiwi (*Actinidia deliciosa*). Plant Cell Rep 22: 141-149

Takaoka MJ (1940) Of the phenolic substances of white hellebore (*Veratrum grandiflora* Loes. Fil.). J Fac Sci Hokkaido Imp Univ 3: 1-16

Tanprasert P, Reed BM (1998) Detection and identification of bacterial contaminants of strawberry runner explants. Plant Cell Tiss Org Cult 52: 53-55

Tassoni A, Fornale S, Franceschetti M, Musiani F, Michael AJ, Perry B, Bagni N (2005) Jasmonates and Na-orthovanadate promote resveratrol production in *Vitis vinifera* cv. Barbera cell cultures. New Phytol 166: 895–905

Terryn N, Van Montagu M, Inzé D, Goossens A (2006) Functional genomic approaches to study and engineer secondary metabolismo in plant cell

cultures. In: Medicinal and Aromatic Plants (Bogers RJ, Craker LE, Lange D Eds.) Springer. 291-300

Thomzik JE, Stenzel K, Stöcker R, Schreier PH, Hain R, Stahl DJ (1997) Synthesis of a grapevine phytoalexin in transgenic tomatoes (*Lycopersicon esculentum* Mill.) conditions resistance against *Phytophthora infestans.* Physiol Mol Plant Pathol 51: 265-278

Tiejten KG, Hunkler D, Matern U (1983) Differential response of cultured parsley cells to elicitors from two non-pathogenic strains of fungi 1. Identification of induced products as coumarin derivatives. Eur J Biochem 131: 401-407

Trumble JT, Dercks W, Quiros CF, Beier RC (1990) Host plant resistance and linear furanocoumarin content of *Apium* accessions. J Econ Entomol 83: 519-525

Valenzano DR, Tezibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A (2006) Resveratrol prolongs lifespan and retards the onset of age-realted markers in a short-lived vertebrate. Current Biol 16: 296-300

Varshney RK, Close TJ, Singh NK, Hoisington DA, Cook DR (2009): Orphan legume crops enter the genomics era!. Current Opin Plant Biol 12: 202-210

Vaucheret H, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene induced gene silencing in plants. Plant J 16: 651-659

Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A, *et al*. (2007) A High Quality Draft Consensus Sequence of the Genome of a Heterozygous Grapevine Variety. PLoS ONE 2: e1326

Veronese P, Li X, Niu X, Weller SC, Bressan RA, Hasegawa PM (2001) Bioengineering mint crop improvement. Plant Cell Tissue Organ Cult. 64: 133-144

Vishnevetsky, J., Flaishman, M., Cohen, Y., Elad, I., Velchea, M., Hanania, U. and Perl, A. (2005) Transgenic disease resistant banana. World Patent WO2005/047515

Viss PR, Brooks EM, Driver JA (1991) A simplified method for the control of bacterial contamination in woody plant tissue culture. *In vitro* Cell Dev Biol 27P: 42

Walker DJ, Bernal MP, Correal E (2007) The influence of heavy metals and mineral nutrient supply on *Bituminaria bituminosa.* Water Air Soil Poll 184: 335-345

Walker D, Moñino I, Correal E (2006) Genome size in *Bituminaria bituminosa* (L.) CH Stirton (Fabaceae) populations: separation of “true” differences from environmental effects on DNA determination. Environ Exp Bot 55: 258-265

Walker DJ, Martínez-Fernández D, Correal E, Romero-Espinar P (2012) Accumulation of furanocouamrins by Bituminaria bituminosa in relation to plant development and environmental stress. Plant Physiol Biochem 54: 133-139

Wang J, Ho L, Zhao Z, Seror I, Humala N, Dickstein DL, Thiyagarajan M, Percival SS, Talcott ST, Pasinetti GM (2006) Moderate consumption of Cabernet Sauvignon attenuates Abeta neuropathology in a mouse model of Alzheimer's disease. FASEB J 20: 2313-2320

Wang PJ, Huang LC (1976) Beneficial effects of activated charcoal on plant tissue and organ cultures. *In Vitro* Cell Dev Biol- Plant 12: 260-262

Wiese W, Vornam B, Krause E, Kindl H (1994) Structural organization and differential expression of three stilbene synthase genes located on a 13 kb grapevine DNA fragment. Plant Mol Biol 26: 667-677

Wink M (1999) Function of plant secondary metabolites and their exploitation in biotechnology. Sheffield Academic Press and CRC Press (Wink M Ed), Annual Plant Reviews, Vol. 3, 362 pp

Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M *et al*. (2004) Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nat 430: 686-689

Wochoka ZS, Sluisa CJ (1980) Gibberellic acid promotes *Atriplex* shoot multiplication and elongation. Plant Sci Lett 17: 363-369

Yu CKY, Lam CNW, Springob K, Schmidt J, Chu IK, Lo C (2006) Constitutive accumulation of cis-piceid in transgenic *Arabidopsis* overexpressing a *Sorghum* stilbene synthase gene. Plant Cell Physiol 47: 1017-1021

Yu CKY, Springob K, Schmidt J, Nicholson RL, Chu IK, Yip WK, Lo C (2005) A stilbene synthase gene (*SbSTS1)* is involved in host and non host defense responses in *Sorghum.* Plant Physiol 138: 393-401

Zangerl AR, Berenbaum MR (1987) Furanocoumarins in wild parsnip: effects of photosynthetically active radiation, ultraviolet light, and nutrients. Ecol 68: 516-520

Zhou ML, Zhu XM, Shao JR, Tang YX, Wu YM (2011) Production and metabolic engineering of bioactive substances in plant hairy root culture. Appl Microbiol Biotechnol 90: 1229-1239

Zhu YJ, Agbayani R, Jazckson MC, Tang CS, Moore PH (2004) Expression of the grapevine stilbene synthase gene *VST1* in papaya provides increased resistance against diseases caused by *Phytophthora palmivora*. Planta 12: 807-812

Zobel A, Brown SA (1991) Psoralens in senescing leaves of *Ruta graveolens*. J Chem Ecol 17: 1801-1810

Zobel A, Brown SA (1993) Influence of low-intensity ultraviolet radiation on extrusion of furanocoumarins to the leaf surface. J Chem Ecol 19: 939-952

ANNEX I

Plant culture media

Table 1. Media used for genetic modification and plant regeneration of *Vitis vinifera* L. cv. Superior Seedless.

MEDIA	½ MSAC	½ MSAC	Germination media	Plant development
MACRONUTRIENTS	½ MS	½ MS	½ MS	½ MS
MICRONUTRIENTS	MS	MS	MS	MS
VITAMINS (mg/l)				
Myo-Inositol	100	100	100	100
Nicotinic Acid	-	-	0.5	0.5
Pyridoxine-HCl	-	-	0.5	0.5
Thiamine-HCl	-	-	0.1	0.1
Biotin	-	-	0.01	0.01
PLANT GROWTH REGULATORS (µM)				
IAA	-	-	10	-
GA3	-	-	1	-
AMINOACIDS (mg/l)				
L-Glycine	-	-	2	2
Casein Hydrolysate	-	-	100	100
L-Glutamine	-	-	100	100
OTHERS (mg/l)				
Activated Charcoal	2500	2500	2500	-
Sucrose	20000	20000	20000	20000
Noble Agar (Difco)	8000	8000	8000	8000
Cefotaxime	300	300	300	300
Kanamicin	-	50	-	-

MS: Murashige and Skoog (1962)

Table 2. Media used for *Bituminaria bituminosa* in vitro culture: Micropropagation

MEDIA	T1	T1+AC	T2	T2+AC	T3
MACRONUTRIENTS	½ MS	½ MS	½ MS	½ MS	½ MS
MICRONUTRIENTS	MS	MS	MS	MS	MS
VITAMINS (mg/l)					
Myo-Inositol	100	100	100	100	100
Nicotinic Acid	0.5	0.5	0.5	0.5	0.5
Pyridoxine-HCl	0.5	0.5	0.5	0.5	0.5
Thiamine-HCl	0.1	0.1	0.1	0.1	0.1
Biotin	0.01	0.01	0.01	0.01	0.01
AMINOACIDS (mg/l)					
L-Glycine	2	2	2	2	2
Casein Hydrolysate	100	100	100	100	100
L-Glutamine	100	100	100	100	100
PLANT GROWTH REGULATORS (μM)					
IAA	-	-	10	10	-
GA3	-	-	1	1	-
IBA	-	-	-	-	10
OTHERS (mg/l)					
Activated Charcoal	-	2500	-	2500	2500
Sucrose	20000	20000	20000	20000	20000
American Bacteriological Agar	8000	8000	8000	8000	8000
Cefotaxime	400	400	400	400	400
Gentamicin	35	35	35	35	35

MS: Murashige and Skoog (1962)

Organogenesis:

For calli induction and shoot development, the medium used was T1 without antibiotics and supplemented with plant growth regulators: NAA or IAA (0.5 and 5 μM) and BA (5.0, 10.0 or 30 μM).

For rooting of shoots, the medium used was T1+AC without antibiotics and supplemented with plant growth regulators: NAA, IAA or IBA (10 μM) and 1 μM GA_3 .

ANNEX II

Bacterial characterisation

BACTERIAL COMPOSITION MEDIA

Composition Media (g l^{-1})[1]	LM	R2A	M72	TYG	MMS	Nutrient	YMG
Yeast extract	5	0.5	-	2.5	-	2	4
Malt extract	15	-	-	-	-	-	10
Starch	10	0.5	-	-	-	-	-
Sucrose	10	-	-	-	-	-	-
CaCO3	2	-	-	-	-	-	-
Peptone	-	0.5	-	-	-	5	-
Casein hydrolysed	-	0.5	-	-	-	-	-
D-Glucose	-	0.5	-	1	-	-	4
Sodium citrate	-	-	3	-	-	-	-
Soduim piruvate	-	0.3	-	-	-	-	-
Triptone	-	-	-	5	-	-	-
Beef extract	-	-	-	-	-	1	-
NaCl	-	-	0.64	-	0.1	5	-
KH_2PO	-	-	0.3	-	0.62	-	-
K_2HPO4	-	0.3	0.7	-	1.20	-	-
$(NH_4)_2SO_4$	-		2.6	-	0.005	-	-
$MgSO_4 * 7H_2O$	-	0.05	0.2	-	0.2	-	-
$CaCl_2 * 2H2O$	-	-	-	-	0.03	-	-
$FeCl_3 * 6H_2O$	-	-	-	-	0.001	-	-
$MnSO_4 * 1H_2O$	-	-	-	-	0.007	-	-
$Na_2MoO_4 *2H_2O$	-	-	-	-	0.0129	-	-
H_3BO_3	-	-	-	-	0.01	-	-
$ZnSO_4 * 7H_2O$	-	-	-	-	0.07	-	-
$CuSO_4 * 5H_2O$	-	-	-	-	0.005	-	-
$CoCl_2 * 6H_2O$	-	-	-	-	0.005	-	-
pyridoxal hydrochloride	-	-	-	-	0.002	-	-
Folic acid	-	-	-	-	0.00001	-	-
Rivoflavine	-	-	-	-	0.005	-	-
Biotine	-	-	-	-	0.000001	-	-
Pantotenate calcic	-	-	-	-	0.005	-	-
Nicotinic acid	-	-	-	-	0.005	-	-
Tiamine	-	-	-	-	0.005	-	-
Methanol (%v/v)	-	-	-	-	0.5	-	-
American Bacteriological Agar	15	15	15	15	15	15	15

[1]Nutrient agar (Kato *et al*. 2005; Knief *et al*. 2008), R2A (Gallego *et al*. 2005), LM medium (Luedemann 1968), M72 medium (Sy *et al*. 2001), MMS medium (Guo and Lidstrom 2006), YMG medium (Salazar *et al*. 2006) and TYG medium (Kang *et al*. 2007)

COLECCIÓN ESPAÑOLA DE CULTIVOS TIPO (CECT) METHODOLOGY

16S rRNA gene partial sequencing

- DNA was extracted using a microbial DNA isolation kit (Ultra Clean, MoBioLaboratories, Inc.). The DNA was checked for purity, using standard methods (Sambrook *et al.*1989).

- DNA templates were amplified by the polymerase chain reaction (PCR) on a Genius thermocycler (Techne), using universal primers amplifying a 1,000 bp region of the 16S rRNA gene, 616V: 5´- AGA GTT TGA TYM TGG CTC AG -3´, 699R: 5´- RGG GTT GCG CTC GTT -3´, obtained from Invitrogen (Life Technologies). The amplification mixture (100 µl) comprised 2 µl (50 pmol/µl) each of 616V and 699R primers, 0.5 µl (2U μl^{-1}) of Taq DNA Polymerase (Finnzymes), 10 µl of 10× reaction buffer (Finnzymes), 10 µl of dNTP mixture (containing 1 mM each of dATP, dGTP, dCTP and dTTP, Roche), 70 µl of sterile filtered water (Milli-Q purification system, Millipore) and 5,5 µl of DNA template. The DNA templates were amplified by initial denaturation at 94º C for 10 min, followed by 40 cycles of denaturation at 94º C for 1 min, annealing at 55º C for 1 min, extension at 72º C for 1 min, and a final extension at 72º C for 10 min. Controls, devoid of DNA, were simultaneously included in the amplification process. The integrity of PCR products was assayed by the development of single bands following electrophoresis for 1 h at 100 V in 2% (w/v) agarose gels in tris-borate EDTA buffer.

Sequencing of PCR products:

- Amplicons were purified using an UltraClean PCR clean-up kit (MoBio Laboratories, Inc.), and subsequent sequencing reactions were performed on an Abi Prism 3730 automated sequencer using the Big Dye Terminator v3.1 cycle sequencing kit, premixed format. Sequencing primers were the same ones used in the amplification reaction but diluted ten times (5 pmol).

- The resulting sequences were automatically aligned and then inspected by eye. The resulting 16S rRNA gene sequences were compared in a BLAST search with those in the National Library of Medicine (Bethesda, MD, USA) database (Altschul *et al*. 1997).

References

- Stephen A, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402

- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory

EPON TECHNIQUE: TRANSMISSION ELECTRON MICROSCOPY

1. Fix in 0.1 M cacodylate buffer + 3% glutaraldehyde at 4 ºC……3-5 h
2. 0.1 cacodylate buffer + sucrose (washing liquid)……overnight
3. It post-fix in 1% osmic acid at 4 ºC…….2 h 30 min
4. Washed in cacodylate buffer + sucrose (washing liquid)……...12 h
5. Stained in uranyl acetate veronal at 4 ºC……2 h
6. Alcohol at room temperature: 30, 50, 70, 90 % 10 min in each one
7. Absolute alcohol + copper sulphate……..10 min
8. Absolute alcohol + copper sulphate……..10 min
9. Propylene oxide……15 min
10. Propylene oxide……..15 min
11. Epon + propylene oxide ratio 1 :2.....45 min
12. Epon + propylene oxide ratio 1 :1.....2 h
13. Epon + propylene oxide ratio 2 :1.....2 h
14. Pure Epon……..overnight
15. Capsules are made and left in the oven at 70 ºC…….48 h

The sections are cut in the ultramicrotome Leica UC6, contrasted with uranyl acetate and plumb citrate (Reynolds 1961) and observed in the microscope Plips Tecnai 12 with an acceleration of 80 kV. The imaging was performed with a digital camera Megawiev III.

SCANNING ELECTRON MICROSCOPY TECHNIQUE

1. Wash the material before fixing
2. Fix in 3% glutaraldehyde + 0.1 M cacodyalte buffer……3-5 h
3. Wash in 0.1 M cacodylate buffer + sucrose……...overnight
4. Post-fix in 1% osmium……..1-2 h
5. Wash in 0.1 M cacodylate buffer + sucrose……...overnight
6. Dehydration in acetone 30%, 50%, 70%, 90%, 100% 10 min in each one
7. Critical point with 100% acetone and liquid CO_2
8. Gold coating with BIO-RAD metallizer POLARON DIVISION CP 0500
9. Observation with JEAOL6.100 Scanning Eletron Microscope with a system of Oxford Instrument INCA