ROSMARINIC ACID PRODUCTION FROM *LAVANDULA ANGUSTIFOLIA* HEMUS AND *LAVANDULA* X *INTERMEDIA* VAR. SUPER A SPECIES

by

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ROSMARINIC ACID PRODUCTION FROM *LAVANDULA ANGUSTIFOLIA* HEMUS AND *LAVANDULA* X *INTERMEDIA* VAR. SUPER A SPECIES

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ABSTRACT

ROSMARINIC ACID PRODUCTION FROM *LAVANDULA ANGUSTIFOLIA* HEMUS AND *LAVANDULA* X *INTERMEDIA* VAR. SUPER A SPECIES

Plants produce large amounts of secondary metabolites as a response to biotic and abiotic factors to create a defense mechanism. Today, they are used in many industries such as pharmaceuticals, pesticides, flavorings, fragrances, cosmetics, colorants and food additives. Rosmarinic acid (RA) is an important secondary metabolite especially for the pharmaceutical and cosmetic industry since RA is effective in many biological activities such as antimicrobial, antioxidant and antiviral. Its production has traditionally been based on harvesting plants from the field but it has many disadvantages such as low yield and seasonal variations. Therefore, plant cell suspension culture has been developed as an alternative method for the RA production regardless of the environmental changes. Secondary metabolite production can be further increased by applying different strategies such as selection of cell lines with higher metabolite production by using a selective agent. In this study, plant cell suspension cultures from two lavender species (*Lavandula angustifolia* Hemus and *Lavandula x intermedia* var. Super A) were established using 4 year-old callus cell lines in order to provide RA production, and increase the yield with resistant cell lines. Growth parameters were measured based on the fresh weight, pH of the media, and extraction of RA over time. RA extraction was optimized with different volumes (2 and 5 ml) of extraction solvent and resuspension solvent types (water and 70 per cent ethanol). Furthermore, a selection strategy was performed in order to obtain cell lines with higher RA production by using m-f-D,L-phenylalanine (MPHE) in different concentrations (0.4 mM, 0.6 mM, 0.8 mM). The effect of cell density (inoculation size) on cell growth and RA production was examined as well. Use of cell suspension culture for the selection was found to be the best method. The suspension cells which were resistant to MPHE demonstrated significantly higher RA production (0.399 g/L) compared to non-selected cells. In addition, the production of RA decreased when the resistant cells were maintained in the absence of MPHE. Finally, from the resistant cells of both lavender species, the expression of the *LaAT1* gene, a role in the formation of RA, was quantified by using qPCR. The resistant Hemus cell lines under 0.6 mM MPHE selection showed higher *LaAT1* gene expression levels. This study will be a base for the production of RA in Lavender species in further studies.

ÖZET

LAVANDULA ANGUSTIFOLIA HEMUS VE *LAVANDULA* X *INTERMEDIA* VAR. SUPER A TÜRLERİNDEN ROSMARİNİK ASİT ÜRETİMİ

Bitkiler, savunma mekanizması oluşturmak için biyotik ve abiyotik faktörlere yanıt olarak büyük miktarlarda sekonder metabolitler üretir. Günümüzde ilaç, pestisit, tatlandırıcılar, parfüm, kozmetik, renklendirici ve gıda katkı maddeleri gibi birçok endüstride kullanılmaktadırlar. Rosmarinik asit (RA), antimikrobiyal, antioksidan ve antiviral gibi birçok biyolojik aktivitede etkili olması nedeniyle özellikle ilaç ve kozmetik endüstrisi için önemli bir ikincil metabolittir. Üretimi geleneksel olarak tarladan bitki toplamaya dayalıdır, ancak düşük verim ve mevsimsel farklılıklar gibi birçok dezavantajı bulunmaktadır. Bu nedenle, çevresel değişikliklerden bağımsız olarak RA üretimi için alternatif bir yöntem olan bitki hücre süspansiyon kültürü geliştirilmiştir. Sekonder metabolit üretimi, seçici bir ajan kullanılarak daha yüksek metabolit üretimine sahip hücre dizilerinin seçilmesi gibi farklı stratejiler uygulanarak daha da artırılabilir. Bu çalışmada, RA üretimini sağlamak ve dirençli hücre hatları ile verimi artırmak amacıyla iki lavanta türünden (*Lavandula angustifolia* Hemus ve *Lavandula* x *intermedia* var. Super A) 4 yıllık kallus hücre hatları kullanılarak bitki hücre süspansiyon kültürleri oluşturuldu. Büyüme parametreleri, ortamın yaş ağırlığına, pH'ına ve zamana bağlı RA ekstraksiyonuna dayalı olarak ölçüldü. RA ekstraksiyonu, farklı hacimlerde (2 ve 5 ml) ekstraksiyon çözücüsü ve farklı çözücülerle (su ve yüzde 70 etanol) tekrardan seyreltilmesi ile optimize edildi. Ayrıca farklı konsantrasyonlarda (0.4 mM, 0.6 mM, 0.8 mM) m-f-D,L-fenilalanin (MPHE) kullanılarak, daha yüksek RA üretimine sahip hücre hatları elde etmek için seleksiyon stratejisi uygulandı. Hücre yoğunluğunun (inokülasyon miktarı), hücre büyümesi ve RA üretimi üzerindeki etkisi de incelendi. Hücre süspansiyon kültürünün kullanılması, seleksiyon için en iyi yöntem olarak bulundu. MPHE'ye dirençli süspansiyon hücreleri, seçilmemiş hücrelere kıyasla önemli ölçüde daha yüksek RA üretimi (0.399 g/L) gösterdi. Ek olarak, dirençli hücreler MPHE'nin yokluğunda muhafaza edildiğinde RA üretimi azaldı. Son olarak, her iki lavanta türünün dirençli hücrelerinden, RA oluşumunda rol oynayan *LaAT1* geninin ekspresyon seviyesi, qPCR kullanılarak ölçüldü. 0.6 mM MPHE seleksiyonu altındaki dirençli Hemus hücre hatlarında daha yüksek *LaAT1* gen ekspresyon seviyeleri ölçüldü. Bu çalışma, Lavanta türlerinde RA üretimine dayalı gelecekteki çalışmalara temel oluşturacaktır.

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2,4-D.	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of variance
cDNA	Complementary DNA
EtOH	Ethanol
FDA	Fluorescein diacetate
HPPR	Hydroxyphenylpyruvate reductase
LS	Linsmaier & Skoog medium
PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PHE	Phenylalanine
MPHE	m-F-D,L-phenylalanine
RA	Rosmarinic acid
RAS	Rosmarinic acid synthase
RS	Rosmarinic acid agar-solidified medium
RL	Rosmarinic acid suspension liquid medium
RPM	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SM	Secondary metabolite
TAT	Tyrosine aminotransferase
qPCR	Quantitative polymerase chain reaction

1. INTRODUCTION

1.1. PLANT METABOLITES

The plant kingdom is thought to produce between 200,000 to 1,000,000 distinct metabolites with a single plant producing between 5 to 25,000 molecules at any given moment. Plant-based molecules are used as additives in food and animal feedstocks, pigments, medicinal substances, polymers, scents, which makes plant metabolites crucial for human life. Using current technology, hundreds of metabolites can be detected in a plant extract. Depending on the metabolites' roles in plant survival, they are divided into two groups as primary or secondary [1].

1.1.1. Plant Primary Metabolites

Primary metabolites which are generally simple molecules are essential for plants basic cellular function [1,2]. Plant primary metabolism is involved in growth, development, photosynthesis, respiration, and also absorption, degradation and synthesis of molecules [3]. Proteins, carbohydrates, lipids, nucleic acids and pigments are the most common metabolites in primary metabolism [4]. Plant primary metabolites serve as precursors for secondary metabolite production [3].

1.1.2. Plant Secondary Metabolites

Plants contain an enormous variety of metabolites apart from primary metabolites, called secondary metabolites (SM). Plant SMs do not directly play a significant role in the maintenance of plant life processes [5]. However, these metabolites have roles for giving odors, tastes and colors to the plants. Plants produce SMs to defend and protect themselves against environmental stress which can be biotic or abiotic [2,6]. Since plants cannot move, they must rely on other ways to protect and defend themselves against external environmental conditions. In evolutionary terms, those that adapted to these external

conditions, defended themselves, and were stronger, survived by producing a few SMs that emerged through adaptation [7].

SMs are formed from primary metabolites as a result of various modifications such as glycosylation, methylation, and hydroxylation. These modifications give SMs a more complex structure compared to primary metabolites [8]. Since plants produce many SMs based on the purpose, the metabolites differ from each other in terms of chemical building units. Based on the biosynthetic pathways, there are three main SMs which are terpenes, phenolics and alkaloids [9].

Terpenes, which are the largest class of SMs, are derived from isoprene units [10]. Terpenes have a great effect on resistance to insects and pathogens [11]. Some terpenes act as signaling chemicals in pathways, and they also have a role in pollinator attraction [12].

Phenolic compounds are the SMs that helped plants evolve and adapt to land from aquatic environments. Plant phenolic compounds are generally defined as aromatic metabolites that have hydroxyl group(s) linked to phenyl ring [13]. Phenolic acids, tannins, anthocyanins, coumarins, flavonoids, etc. are the most common phenolic compounds of SMs. They can be simple or in polyphenolic form [14]. Most of them are synthesized through phenylpropanoid pathway. Phenolic compounds have various roles such as defending plants, giving certain flavors, establishing cell walls and flower color [13]. Their pharmaceutical potential is immense due to their anti-oxidant, anti-inflammatory and anti-carcinogenic effects [14].

Alkaloids are the most heterogeneous category of plant SMs. It is assumed that there are about 12.000 alkaloids from different plant species [15]. Plant alkaloids are pharmacologically active and nitrogen-containing compounds. Synthetically produced modern drugs have been inspired by plant alkaloids [13].

1.2. BIOTECHNOLOGICAL APPROACH TO THE PRODUCTION OF PLANT SECONDARY METABOLITES

Plants are a great resource for researching new products. The research and laboratory production of SMs started at the end of the 1960s [9]. SMs are now used in many countries in the world as medications, flavor and scents, coloring agents, pesticides, and dietary

supplements. Therefore, in recent years, the industrial value of SMs has generated a lot of interest. For example, it is estimated that the global market value of plant-derived drugs would reach $5 trillion in 2050 [16].

SMs production has been accomplished by traditional methods which extract SMs directly from whole plants [9]. However, this method has certain disadvantages including unstable product quality and yield due to the availability problems of fertilizer or nutrient, effects of pests, diseases and stress, environmental and seasonal conditions [17]. Therefore, the idea of using biotechnology to regulate SMs synthesis gained importance. Unlike the conventional methods, biotechnology serves an important function in the production and extraction of SMs at the commercial level. Producing SMs by using biotechnological techniques, particularly plant tissue culture techniques, show enormous potential over conventional methods, as follows: (I) production regardless of time and climatic conditions, (II) more stable, efficient, homogeneous, consistent and large volumes of production, (III) quick and efficient SMs extraction when compared to extraction from the entire plants, (IV) prevention of unwanted molecules that exist in field-grown plants [18].

After the emergence of *in vitro* technology, it has been widely attempted to use plant cells and tissues for the production of SMs due to the ability of cultured plant cells, tissues, and organs to produce and accumulate many of the same chemical compounds as the mother plant. Biotechnology enables cells, organs, or plantlets growing *in vitro* to produce SMs [19]. Hairy root culture is an example of organ culture. The main feature of hairy root culture is that they can produce SMs in parallel with their growth. Since hairy roots are continuously producing SMs, hairy root culture is a rather easy way of obtaining SMs [20]. However, hairy roots are only derived by the infection of *Agrobacterium rhizogenes*, which is the main drawback of this technique due to inconsistent infection efficiency in different species [21]. Organ cultures typically have larger and more consistent secondary metabolite outputs compared to cell cultures [19]. However, cell cultures can be easily scaled up, and it is also advantageous for the extraction of specific SMs since there are less impurities compared to organ cultures [22].

In 1970s, it was thought that plant cells could be used as a vehicle to produce SMs [23] since every cell in callus tissue which is group of unclassified plant cells has a potential to differentiate into any type of plant tissue, a property known as totipotency. For this reason,

plant cell suspension culture which is an example for plant cell culture can be used for producing SMs [24,25],[19,26]. The expression "suspension culture" refers to cultures that contain morphologically undifferentiated single plant cells (and also tiny aggregates) cultured in liquid media [27]. Previous studies showed that SMs production in cell suspension culture is higher than the production from the whole plant [28]. Moreover, it is shown that cells grow faster in liquid medium, therefore suspension cultures are more preferable than callus cultures [29].

1.3. LAVENDER (*LAVANDULA* SPP.) AND ITS SECONDARY METABOLITES

Lavender (*Lavandula* spp.) an important medical aromatic plant with several therapeutic effects, belongs to the Lamiaceae (also Labiatae) family and a member of the Nepetoideae subfamily. In literature, thirty-two lavender species have been recognized up until now. The classification of *Lavandula* species is done based on their morphological characteristics since every species has different morphological traits such as leaf shape, calyx, bracte and corolla. Lavender is a flowering shrub plant with spike flowers that are located at the tip of the flower stalk. The cymes are generally separated by bracts. Cymes can be a single flower without bracts or have 3-9 flowers with bracts. The bracts are usually small but some species like *Lavandula stoechas* and *Lavandula dentata* have big bracts. Both calyx and corolla are bilabiate. The color and shape of them changes based on the species [30].

Most *Lavandula* spp. are native to Mediterranean region. Since lavender is a plant which is resistant to drought, hot and cold weather, it can grow in almost any soil. Because of that some species are found in the Canary Islands, North Africa, South West Asia and Arabian Peninsula. Although lavender is a non-selective soil plant, it grows best in calcareous and dry soils. Both species and the soil that it grows affect the therapeutic actions of lavender because lavender essential oil composition or the oil amount changes based on species and soil [31]. According to that the aroma of the oil, its usage purpose and its market value change [32]. For millennia, lavender essential oil has been used for many therapeutic purposes due to its antimicrobial, relaxing, and anti-depressive properties. Moreover, it also has an important place in cosmetics [33].

Essential oils are the mixture of aromatic chemical constituents. For the chemical compositions analysis, a variety of procedures are available including gas chromatography with mass spectrometry (GC/MS) [33]. Linalool, linalyl acetate and camphor are three main constituents of *Lavandula* oil (Figure 1.1.) [32]. Linalool and camphor are antioxidants that have the ability to efficiently relieve pain and inflammation as well as muscular spasms and stress [34]. For the best scent in cosmetic and perfumery industries, the *Lavandula* essential oil should contain linalool, linalyl acetate and low level camphor [35].

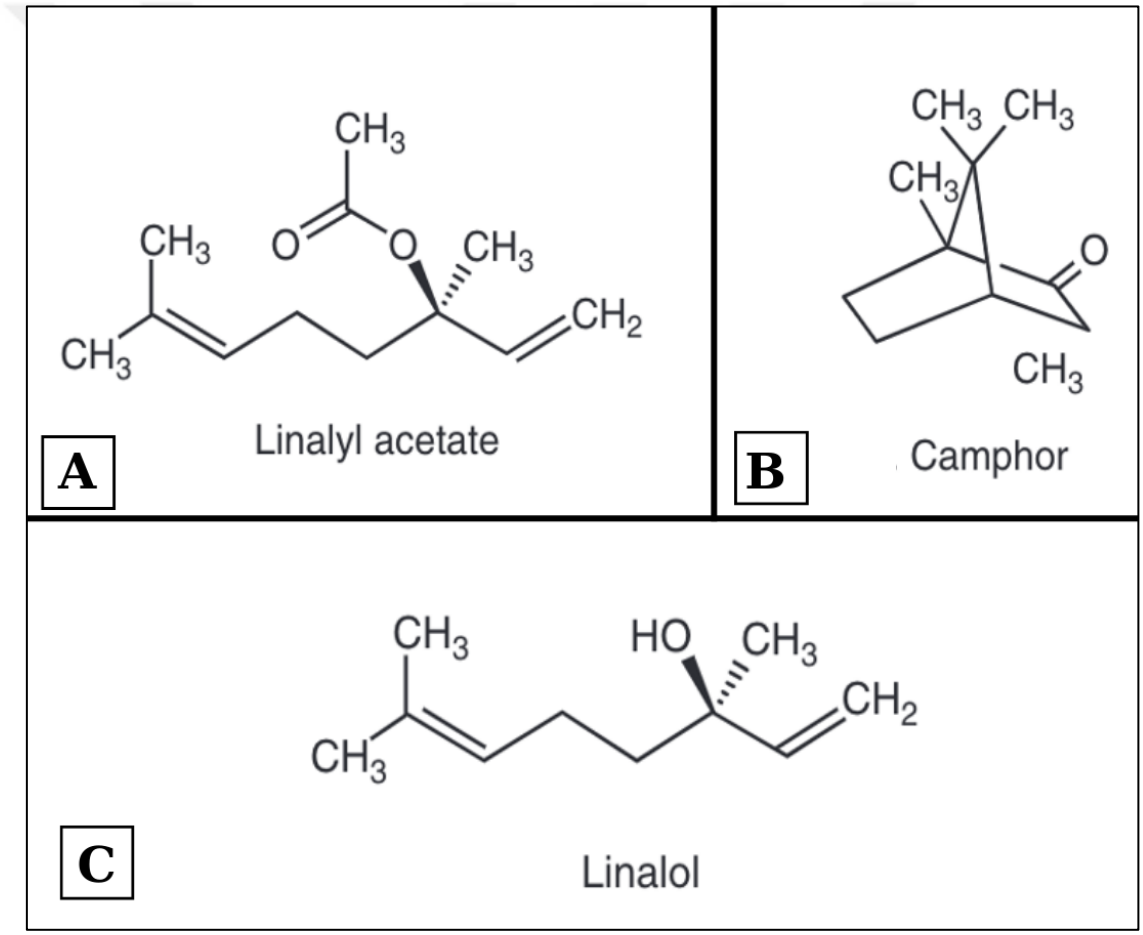


Figure 1.1. The main components of Lavandula essential oil which are linalyl acetate (A), camphor (B), linalol (C) [36].

The most popular *Lavandula* species are *Lavandula angustifolia*, *Lavandula latifolia*, and *Lavandula* x *intermedia* (*Lavandula hybrida* or Lavandin) which is a hybrid one

(a*ngustifolia* X *latifolia*) [30,36,37]. The essential oil of *L. angustifolia* (known as mainly British Lavender) has a higher quality than the other *Lavandula* spp. due to its lower camphor content which makes it more desirable for perfumery and cosmetic industries (Figure 1.2). The demand for good quality *L. angustifolia* essential oil is high; however, the price is even higher due to low plant production and low essential oil yield. *L. latifolia* is known as Spanish lavender and/or Portuguese lavender. *L. latifolia* produces approximately three times higher amounts of essential oil compared to *L. angustifolia*; however, the oil has poor quality due to its high camphor content [38].

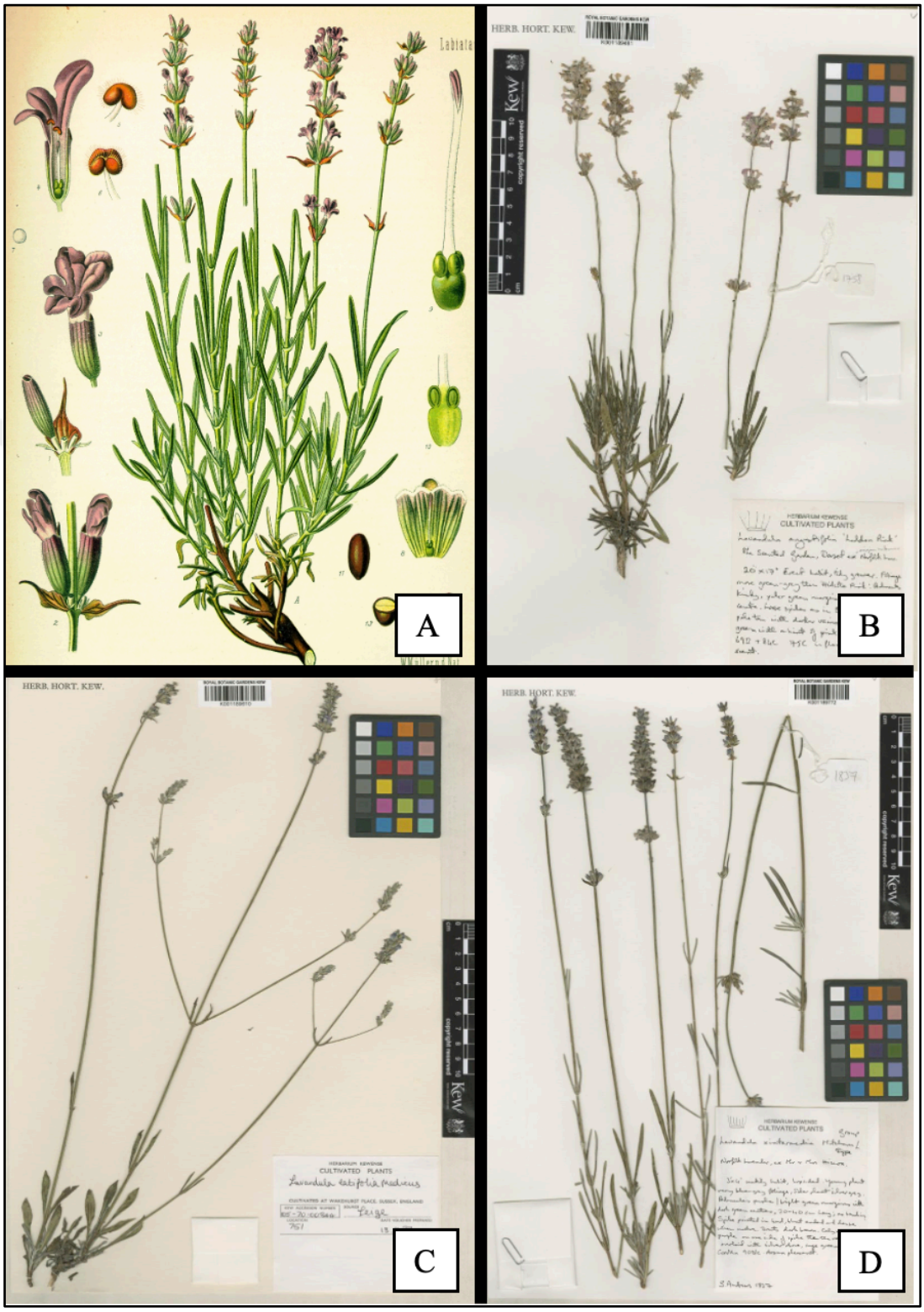


Figure 1.2. The images of (A-B) *L. angustifolia,* (C) *L. latifolia* and (D) *L.* x *intermedia* [39,40].

Lavandula x *intermedia* is a hybrid of *L. angustifolia* and *L. latifolia* and is often referred to as lavandin. Lavandin has high essential oil production; however, its oil is low in quality [41]. These three species have similar chemical constituents in their essential oil though they represent different therapeutic actions. For example, *L. latifolia* has an abortifacient effect while *L. angustifolia* has a diuretic effect [32]. While lavender oil has higher amount of linalyl acetate which makes it more preferable for perfume and pharmaceutical industries, lavandin oil has higher linalool levels [41,42]. These three *Lavandula* species have an important place in the world lavender essential oil market. In 2020, the lavender essential oil market was $530.5 million and is predicted to increase to $864.7 million by 2025 [43]. Lavender essential oil is among the top 15 essential oils traded in the world. Lavender essential oil is used in soap, cosmetics and perfume products due to its pleasant smell as well as in aromatherapy due to its insomnia and anxiety relief properties. Lavender flowers, on the other hand, are used as tea due to their sedative (calming) feature, and offer a visual feast in gardens and parks as ornamental plants. *L. angustifolia* and lavandin species are also used as ornamental plants [44].

1.3.1. Production and Cultivation of Lavender

Since lavender is native to Mediterranean region, its production is generally made in this region [30]. Lavender production can be carried out in three different ways. The first method is generative propagation through fertilization where the new plant is obtained from seed. Since it is practically impossible to produce genetically identical offsprings from the parental plants via generative propagation, it is not a preferable choice for industrial production [44,45]. Moreover, the small size of lavender seeds requires a lot of experience for sowing in the field so that yield loss is inevitable. Therefore, germinating the seeds in advance and planting saplings in the field is a more appropriate approach for this method. The second production method for lavender cultivation is vegetative propagation, which is done by cutting the trunk or branch part of the plant and then forming a whole plant from it. Although it is a simpler way of plant production, it may not always be possible to obtain a complete plant from its parts which prevents sustainable and effective production [45]. The third method for lavender production is micropropagation via plant tissue culture techniques. It is possible to produce genetically identical plants (clones) via micropropagation method in

laboratory conditions. Plant clones is of great importance for commercial production. Comparing to other methods, micropropagation has many advantages such as large scale production of pathogen-free and genetically identical plants. On the other hand, an expert is required for the optimization of variables such as temperature, humidity, culture media ingredients, pH for micropropagation protocol that is unique for each species [46].

1.3.2. Lavender Secondary Metabolites

The Labiatae family, to which lavender belongs, has several significant SMs and that makes it an important source for pharmaceutical industries since it has antiallergic, antimicrobial, antioxidant, anti-HIV and anti-inflammatory effects [14,47]. Phenolic chemicals such as rosmarinic acid and caffeic acid are primarily responsible for these effects [48]. Lavender has been studied for years in order to elucidate its SMs and increase their production efficiecy. The production of lavender SMs can be performed in cell suspension culture [49] as well as using shoots [50] and plantlets [51]. Researchers aimed to increase the production of different lavender SMs by changing factors such as sucrose, nitrogen, temperature and inoculum size [52]. For example, Georgiev *et al*. (2004) focused on the effects of temperature on the production of rosmarinic acid in *Lavandula vera* MM cell suspension culture [53]. Nitzsche *et al*. (2004) increased the production of cinnamic acid in *Lavandula officinalis* cell suspension culture after jasmonic acid treatment [22]. Ilieva and Pavlov (1997) changed sucrose concentrations in the media for increasing production of rosmarinic acid in *L. vera* MM cell suspension culture [54].

1.4. ROSMARINIC ACID

Rosmarinic acid (RA), which was first extracted from the *Rosmarinus officinalis* by Scarpati and Oriente in 1958, is a widely used phenolic acid. The structure of RA is an ester of caffeic acid and 3,4-dihydroxy-phenyllactic acid (Figure 1.3) [55].

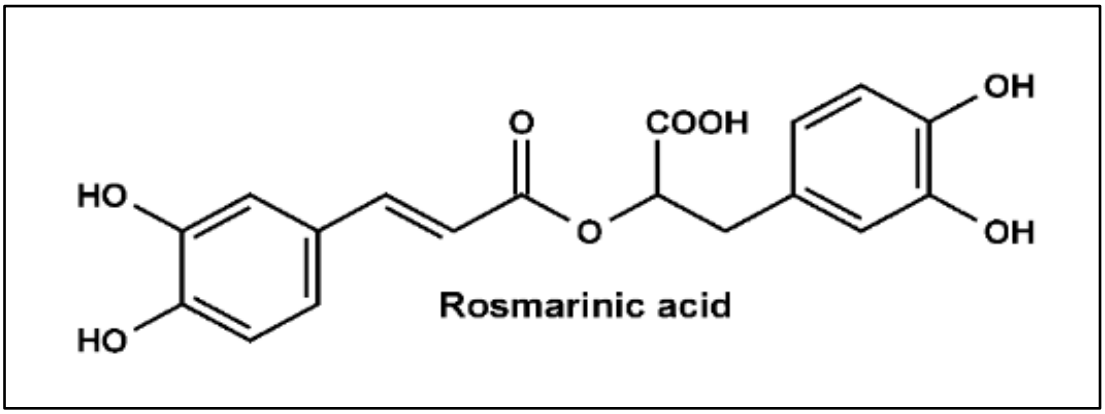


Figure 1.3. The structure of RA [56].

The biosynthesis of RA starts with phenylalanine and tyrosine as a precursor in two pathways (Figure 1.4). The caffeic acid part of RA comes from phenylalanine route while 3,4-dihydroxyphenyllactic acid part is formed via tyrosine route [57]. For phenylalanine pathway, phenylalanine is firstly deaminated to cinnamic acid by phenylalanine ammonia-lyase (PAL) enzyme. PAL is the important enzyme in the phenol production pathway and is considered the primary plant response against stresses [58]. After that 4-coumaric acid is produced, then 4-coumaroyl-CoA is formed. Meanwhile, in tyrosine pathway, tyrosine is transferred to 4-hydroxyphenylpyruvic acid by tyrosine aminotransferase (TAT) enzyme. Then, 4-hydroxyphenyllactic acid is formed. After that, 4-coumaroyl-Coa from phenylalanine pathway and 4-hydroxyphenyllactic acid from tyrosol pathway are linked to each other by Rosmarinic Acid Synthase (RAS) enzyme to form 4-coumaroyl-4-hydroxyphenyllactate. Finally, it is hydroxylated by the cytochrome P450 monooxygenase enzyme and RA is formed [56].

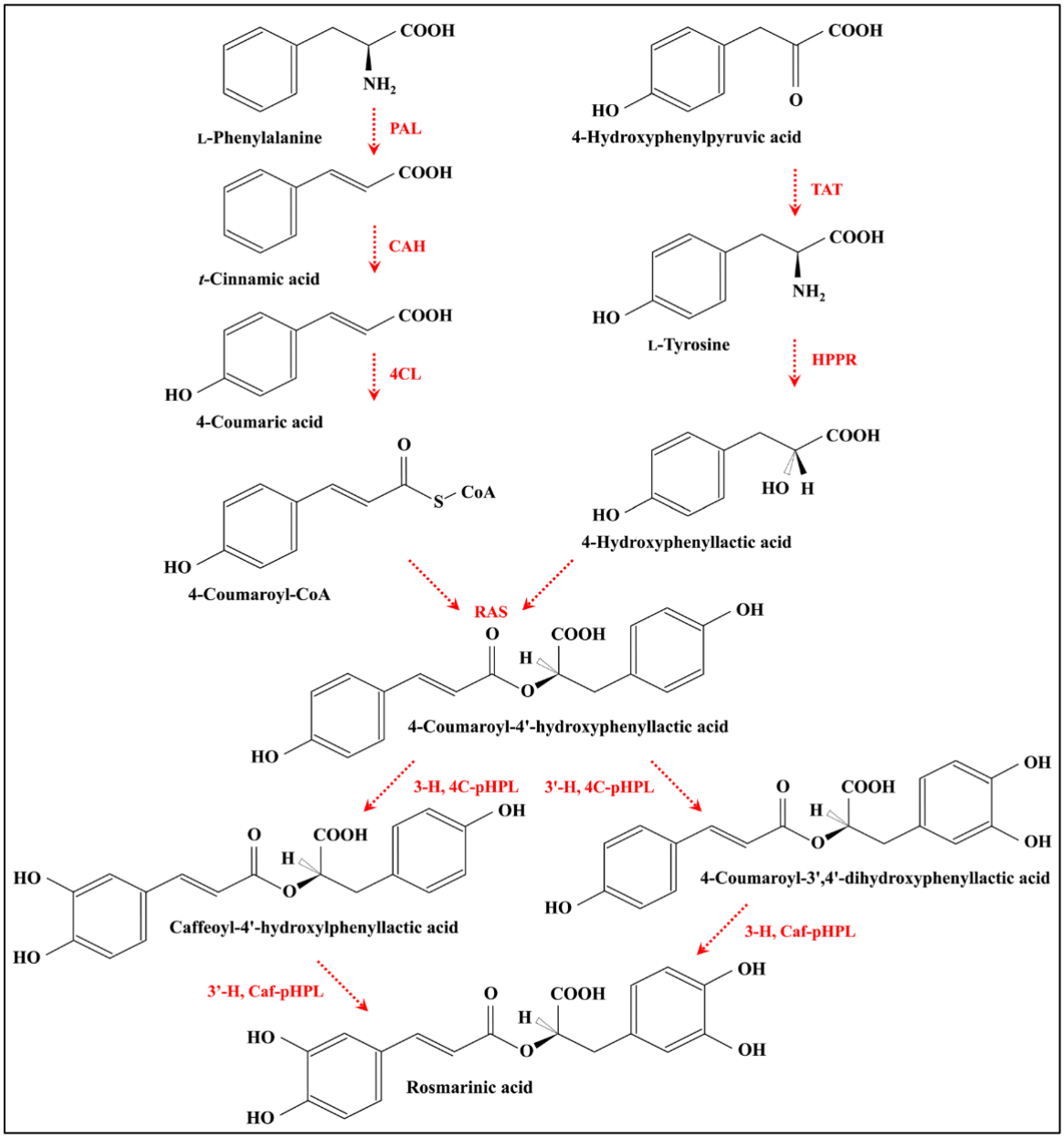


Figure 1.4. The biosynthesis pathway of RA [59].

RA is used in pharmaceutical and cosmetics industries due to its anti-bacterial, anti-inflammatory, anti-mutagenic and antioxidative effects [60]. Since it has high anti-viral activity, the recent studies were about the effects of RA on Herpes simplex virus [61]. In addition to that, there have been many studies for RA on the protection of Alzheimer's disease, preventing kidney disease, and preventing cancer chemotherapy effects [62].

1.4.1. The Effect of Culture Conditions on RA Production

Plant cell suspension cultures produce RA often at levels much greater than those found in the whole plant. In addition, plant cell suspension culture easily enables manipulation to increase the production of desired RA [28,56], [19]. In most of RA synthesis studies, Linsmaier–Skoog (LS) media was preferred to be used [49,53,54,63–66]. A few studies were used Gamborg's B5 media [67,68] or Murashige and Skoog media (1962) [69]. Ulbrich *et al*. (1985) compared the effects of different media and they obtained higher RA production LS medium than Gamborg's B5 and Murashige and Skoog media from *Coleus blumei* Benth [70].

The cell growth and RA production in time period from *L. vera* MM cell suspension culture were examined. It was determined that intensive RA production was achieved during the linear growth phase (6-8th days) [54]. The similar result was obtained from *Anchusa officinalis* cell suspension cultures that RA production was observed during the linear growth phase (6-12th days) [71].

The production of RA in cell suspension cultures of *Salvia officinalis* L. was analyzed by increasing sucrose level of the media since sucrose has a great effect on the SMs of the phenylpropanoid pathway, and it was founded that 5% sucrose level increases RA production compared to 3% sucrose level at the control group [69]. The similar result was also obtained from *C. blumei* cell suspension culture, the maximum RA production was observed with 5% sucrose supplementation of the growth medium [67].

Since phenylalanine which is a compound involving in RA biosynthesis pathway has the potential to significantly increase the production of RA, the effects of phenylalanine on RA production in *Satureja khuzistanica* cell suspension culture were investigated. They achieved higher RA production in phenylalanine medium [68]. The similar result was also obtained from *L. vera* MM cell suspension culture. After feeding with phenylalanine, the RA production increased when compared to control group (without phenylalanine) [49].

1.4.2. Investigation of RA Synthesis at Molecular Level

RAS gene is coding for rosmarinic acid synthase enzyme, which is thought to be a member of BADH superfamily of acetyltransferases, is the most studied enzyme in RA pathway since it is specific for RA synthesis. A full-length cDNA of RAS from *C. blumei* plant was synthesized and it is 1290 bp long, coding 430 amino acids [72]. Not only the gene of RAS enzyme but also two full-length cDNAs (*LaAT1* and *LaAT2*) coding acyltransferase enzymes were isolated from *L. angustifolia* L. It is also shown that *LaAT1* gene has 81% identity with the gene for RAS enzyme, except twenty nine amino acids coded by *LaAT1* gene. Due to the similarity, it is thought that *LaAT1* gene may take part in the pathway of RA synthesis [73].

Tyrosine aminotransferase (TAT) is the first enzyme in tyrosine route of RA biosynthesis. Full-length TAT cDNA from *Perilla frutescens* was synthesized and analyzed. Based on that it is 1,535 bp long and coding 411 amino acids. In addition, the expression level of TAT enzyme in different parts of *P. frutescens* (roots, leaves and stems) was analyzed with real-time PCR and the maximum expression level of TAT was found to be in leaves [74].

Phenylalanine ammonia-lyase (PAL) is the first enzyme in phenylalanine route of RA biosynthesis. The gene of PAL enzyme from two species of *Salvia* was analyzed [75]. Also, semi-quantitative RT-PCR analysis was used to investigate the relationship between PAL expression and RA accumulation. The results showed that inoculation of salicylic acid increased PAL expression. Higher salicylic acid concentrations applied to one species resulted in higher RA production, but the other species showed higher RA accumulation at lower salicylic acid concentrations. As a result of that there was no relationship between RA production and PAL expression rate for these species. The assumption that PAL is the rate-determining step in RA biosynthesis is thus not completely correct.

In green and purple basil (*O. basilicum* cv. ‘Cinnamon’ and ‘Redrubin’), the expression level of the genes involved in RA production, and also the amount of RA produced were investigated. As a result, the green basil leaves had the highest RA concentration with higher expression levels of the examined candidate genes of PAL, TAT, and HPPR (hydroxyphenylpyruvate reductase) which is the enzyme in the tyrosine route of RA biosynthesis [76].

1.4.3. Strategy for Selecting Cell Lines with Higher RA Production

RA production can be increased via different selection strategies. Various studies demonstrated 20-30 folds increase in SM production in cell suspension cultures [77]. The aim of selection strategies is to purposely kill wild type (sensitive) cells while promoting the survival of only the desired variants producing higher RA. A selective chemical is added into the media at a certain concentration that kills all wild type cells. By this way, only cell lines resistant to this chemical can live and produce a higher amount of SMs through the pathway and the sensitive ones are eliminated. This selective chemical can be an amino acid analog [27]. The selected cells which are resistant to selective agent have biochemical alterations in their corresponding metabolic pathway. The cells that are resistant to amino acid analog overproduce its natural amino acid [78]. For example, p-fluorophenylalanine (PFP) is an analog of L-phenylalanine which is naturally found in the cells [27] In the PFP resistant cells, the production of phenylalanine (PHE) increases since the activity of chorismate mutase enzyme, having a role in the PHE synthesizing pathway, increases or chorismate mutase loses the negative feedback controlled by PHE. Besides, overproduced PHE is not accumulated in the cells, it is used in the synthesis of phenolic acid and this indicates that amino acid analogue resistance cells have higher level of phenolic acids [78]. Georgiev *et al*. (2006) used two different analogues of PHE (*m*-F-D,L-phenylalanine and *p*-F-D,L-phenylalanine) in order to select the *L. vera* cell line that was resistant to PHE and produced high amounts of RA. They obtained the PHE resistant *L. vera* cell lines which produced 1.95 times higher amount of RA in *m*-F-D,L-PHE and 1.71 times higher in *p*-F-D,L-PHE [63].

Although PHE is naturally found in the pathway in L-phenylalanine form, higher amounts of L-amino acids can cause the growth inhibition of cultured cells [79,80]. If the cells can increase PAL production, they become resistant to natural amino acids and they survive [56]. Therefore, PHE resistant cell lines can be selected based on the growth inhibition. Berlin (1979) selected 4 out of 50 flasks as a PHE-resistant cell line of tobacco cell suspension cultures because the only growth was observed in these 4 flasks [78]. Palmer and Wildholm (1975) selected a few flasks which showed growth as a resistant cell line of tobacco cell suspension culture [81].

There are two other ways to select the resistance cells. One way is to extract RA from the cells and selecting the batch with the highest RA concentration [63]. The other way is the biochemical analysis. It has been demonstrated that the cell becomes resistant if there is a decrease in the uptake of the selective agent from the media [78,81].

In the selection method, callus culture which contains agar-solidified medium or suspension culture which contains liquid medium can be used [82]. However, the cells can contact the selective agent more in suspension culture than in callus culture [83].

1.4.4. Extraction and Quantification of RA from Plant Cell Suspension Culture

Although biotechnological approach has a great impact on the higher production of RA, finding the optimum RA extraction protocol is also as important. The first step of extraction is the treating the plant material [84]. In most of RA extraction studies, plant cell suspension was treated via homogenization [49,63,64,85]. After this pre-treatment, a few parameters such as solvent type and its concentration, extraction time and temperature should be optimized [86]. Dent *et al*. (2013) compared the effect of ethanol-water, acetone-water and only water on RA extraction from Sage (*S. officinalis*) and concluded that the most efficient solvent was found as ethanol [87]. Most researchers prefer using aqueous ethanol for RA extraction from plant cell suspension culture [53,63,66]. Water is added to increase the efficiency of RA extraction since water causes the plant tissue to swell, increasing the diffusion of phenols from the cells. This process helps to expand the extraction surface area of interaction between the solute and the solvent [88]. For the other parameters such as solvent concentration, extraction time and temperature, many researchers studied on RA production from *Lavandula* suspension cell culture used the extraction protocol which includes 50 per cent ethanol, treating three times for 20 minutes (totally 1 hour) and at 70°C [53,63,66].

The amount of RA should be quantified following the extraction. To get the most accurate result for the analysis of RA, some studies used a high performance liquid chromatography (HPLC) [49,69,89]. However, this device is complex and has many operational steps. In previous studies, RA was detected by using a spectrophotometer at 327 nm [71]. Although this method is quick and easy, it can cause absorption of some unwanted metabolites rather

than RA. However, since RA has a metal-chelation property [90], RA in the extracts was reacted with Fe^{+2} ions and it gives the dark blue color. By this way, the spectrophotometer could only absorb RA, though it prolongs the time for analyzing RA [91]. Using Zr^{+4} ions instead sped the analysis up while being equally accurate [92].

1.5. AIM OF THE STUDY

The objectives of this study are;

1) To determine the growth characteristics of the cell suspension cultures from two different lavender species (*L. angustifolia* Hemus and *L.* x *intermedia* var. Super A),

2) To optimize the RA extraction protocol and determine RA concentration using spectrophotometric analysis,

3) To induce RA production in lavender cell suspension cultures using selection method via m-F-D,L-phenylalanine (MPHE),

4) To determine the expression levels of *LaAT1* gene in MPHE resistant and non-selected cells.

2. MATERIALS

2.1. GLASSWARE & CONSUMABLES

Aluminum Foil, Cling Film, Erlenmeyer Flasks (100 ml, 250 ml, 500ml, 1000ml), Eppendorf tubes (1.5mL and 2mL), Falcon Tubes (50mL), Forceps, Glass bottles (250mL, 500mL, 1000mL and 2000mL), Graduated Cylinder (100 ml, 1000 ml), Glass Beads, Filter Glass Side Arm, Glass Funnels Gooch with Sintered Glass Disc 125mL (pore 3), Microtube racks for 1.5mL and 2mL, Micropipettes (0.1-10µL, 20-200µL, 100-1000µL), Micropipette tips (10µL, 200µL, 1000µL), Petri dishes (9mm), Spectrophotometer Cuvettes, Syringe filters sterile, Syringe polypropylene sterile, Scalpel Handles, Scalpel Blade (no:10 and 11), qPCR 96-well plate, Teflon callus spoon, Weighing dishes

2.2. EQUIPMENTS

Autoclave (Wisd, MeXterile 60), Analytical scale (Shimadzu, Uni Block TW423L), BioSpec-Nano (Shimadzu Biotech), Class II biosafety cabinet (Esco, AC2-4E8), Horizontal gel electrophoresis (Bio-Rad, PowerPac Basic Power Supply1645050), Hot plate-magnetic stirrer (IKA Labortechnik), Incubator/ Drying oven (Wisd, Thermostable IR-150), Heat sterilizer (Swiss made, Steri 350), pH meter (Mettler Toledo, Seven compact), Plant growth room (Grotech, GR94), PCR Thermal Cycler (Bio-Rad, T100), Real-Time PCR Detection System (Bio-Rad, CFX96), Rotary evaporator (Heidolph, Laborata 4000), Spectrophotometer (Thermo Scientific, Genesys 10S), Orbital Shaker (Daihan Scientific, Thermostable IS-10RL), Water purification system (Merck, Milipore Milli-Q Gradient A10), Vortex (Wisd, WiseMix VM-10), Vacuum pump (KNF Neuberger, N022A_.18), Water bath (Grant, SUB Aqua 26 Plus), UV Translator (Vilber)

2.3. CHEMICALS

2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, Lot#D0911.0100), Agar plant tissue culture (Sigma Aldrich, Lot#BCBR3228V), Acetic acid (Sigma Aldrich, Lot#

STBG8116), Agarose (Prona Biomax), DNA Ladder (50 bp, GeneON, LoT# SE8), DNA Loading Dye (Thermo Scientific, LoT# 00129355), Ethanol (Duzey Lab, Cas#64-15-5), Ethidium Bromide (Sigma Aldrich, Lot# MKBP8953V), Ferrous Sulfate Heptahydrate (Sigma Aldrich, Lot# 013075.02), Hi-cDNA Synthesis Kit (HIMEDIA, LoT# 0000373009), Hydrochloric acid (Sigma Aldrich, Lot# SZBE2640V), Linsmaier and Skoog (LS) medium including vitamins (Duchefa Biochemie, Lot#P19417.01), m-F-D,L-phenylalanine (Sigma Aldrich, Lot# BCBW6158), NORGEN Total RNA extraction Kit, Rosmarinic acid (Sigma Aldrich, Lot# BCBZ7196), Sucrose (Duchefa Biochemie, Lot# 012100.06), Sodium hydroxide (EMD Millipore, Cas#1310-73-2), Sodium Acetate (Lot# OA63718), SYBR Green reaction mix, RedSafe (Intron, LoT# 90911751), Taq Polymerase (Thermo Fisher LoT# EP0401),

2.4. PLANT MATERIAL

Certified Lavandin (*Lavandula* x *intermedia* var. Super A) plant material was kindly supplied by Lavodor LTD ŞTI, Isparta, Turkey and certified *Lavandula angustifolia* Hemus plant material was kindly supplied by Trakya Agricultural Research Institute, Edirne, Turkey. *Lavandula angustifolia* Hemus and *Lavandula* x *intermedia* var. Super A callus cell lines that have been previously cultured in Plant Biotechnology Laboratory, Department of Genetics and Bioengineering, Yeditepe University were used for this study [93,94].

3. METHODS

3.1. ESTABLISHMENT OF LAVENDER CELL CULTURE

3.1.1. Maintenance of Lavender Plant Callus Culture

The callus culture for both Lavender species had already been established and maintained in Linsmaier & Skoog (LS) medium supplemented with 30 g/L sucrose, 0.2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) and 5.5 g/L agar (RS medium) [63]. pH of all media were adjusted to 5.7-5.8 and they were autoclaved before use. The calli (cell lines) had been subcultured every 21 days for almost 4 years [93,94].

3.1.2. Establishment and Maintenance of Lavender Cell Suspension Culture

1 g callus was chopped into small pieces and added into a 100 ml Erlenmeyer flask containing 20 ml RL medium (RS medium without agar). The suspension culture was grown in Erlenmeyer flasks with a 1/5 working volume on a 110 rad/s shaker at 25°C in darkness [63]. Cells were transferred to fresh media once a week.

3.2. CONSTRUCTION OF GROWTH CURVE FOR LAVENDER CELL SUSPENSION CULTURE

0.5 g of cells from 24 weeks-old cell suspension cultures of Hemus and Lavandin, and 1 g of cells from 22 weeks-old cell suspension cultures of Hemus were used to initiate the growth curves. The cells were inoculated in a 100 ml glass Erlenmeyer flask containing 20 ml RL medium. The experiment was conducted in fourteen replicates. Suspension cultures were maintained at 25°C with orbital shaking at 110 rpm in darkness. Calli were weighed every two days for 14 days.

The pH of the media used for the growth curve construction was measured every two days with a pH meter for 14 days.

Every two days for 14 days, RA was also extracted and analyzed at 327 nm using a spectrophotometer (Refer to Section 3.3.3).

3.3. ROSMARINIC ACID ANALYSIS

3.3.1. Construction of Standard Curve for Rosmarinic Acid Analysis

As a standard, pure RA (Sigma Aldrich) was used. Two different 1 mM standard stock RA solutions were prepared; dissolved either in distilled water or 70 per cent EtOH. The stock solutions were diluted in 0.1 M Na-acetate buffer (pH 6.0) to final concentrations of 10, 25, 50, 75, 100, 200, 300, 400, and 500 μM RA. $FeSO_4$ was added in each solution to a final concentration of 1mM [91]. Distilled water and 70 per cent EtOH without RA were used as a blank solution. All solutions were prepared in 3 replicates. The RA-iron reaction was carried out at room temperature (25°C) for an hour in darkness. The absorbance was read at 327 nm using a spectrophotometer.

3.3.2. Optimization of Rosmarinic Acid Extraction

0.5 g cells from Hemus suspension culture were collected, frozen with liquid nitrogen and crushed with mortar and pestle until they became powder as a pre-treatment [85]. For RA extraction, the samples were treated 3 times with 50 per cent ethanol in two different volumes (2 ml and 5 ml) (Table 3.1) at 70°C for 20 minutes each [63]. Each time, pellets were precipitated by centrifuging at 4.000 rpm and 4°C for 3 minutes. After combining all extracts, they were evaporated with a vacuum evaporator (Sartorius) with 40°C bath temperature, first at 175 mbar (for EtOH) and then at 75 mbar (for water) [95]. The extract was resuspended in 1 ml distilled water or 1 ml 70 per cent EtOH (Table 3.1). Finally, the amount of RA in the solutions were analyzed by using spectrophotometer. Statistical analysis was done using ANOVA and Student's *t*-test.

Table 3.1. The experimental set-up for the optimization of RA extraction.

Number	Volume of the Solvent (ml)	Resuspend (ml)
1	2 ml 50% EtOH	1ml Water
2	2 ml 50% EtOH	1ml 70% EtOH
3	5 ml 50% EtOH	1ml Water
4	5 ml 50% EtOH	1ml 70% EtOH

3.3.3. Rosmarinic Acid Extraction and Analysis

0.5 g of cells were pre-treated as described in the optimization protocol for RA extraction (Section 3.3.2). RA was extracted with 2 ml 50 per cent EtOH at 70°C water bath for an hour (3 times for 20 minutes). Each time, pellets were precipitated by centrifuging at 4.000 rpm and 4°C for 3 minutes. All supernatants were combined and evaporated at 175 mbar (for EtOH) and 75 mbar (for water). The extracts were resuspended in 1 ml water. Then, 0.5 ml extract was mixed with 0.25 ml 0.1 M Na-acetate buffer at pH 6.0, and also $FeSO_4$ was added to a final concentration of 1mM. The reaction was carried out at room temperature (25°C) for an hour. The absorbance was read at 327 nm. As a standard, pure RA (Sigma Aldrich) was used. Based on that, standard deviation was calculated and statistical analysis was performed via Student's *t*-test.

3.4. SELECTION OF RESISTANT CELL LINES

3.4.1. Preparation of Selection Media

The liquid selection media, named P4, P6, and P8, contain RL medium supplemented with 0.4, 0.6 and 0.8 mM m-F-D,L-phenylalanine (MPHE), respectively. RL medium without

MPHE was used as control. For the selection experiment with agar-solidified medium, MPHE was added to the RS media with a final concentration of 0.6 mM and it was kept overnight to absorb through the medium.

3.4.2. Optimization of Selection Procedure

3.4.2.1. Preliminary Work #1: Selection with Agar-Solidified Medium

For the selection of resistant cells, the protocols of Georgiev *et al.* (2006) and Duncan and Widholm (1990) were followed [63,83]. 0.25 g of 7-days old Lavandin and Hemus suspension cells were spread on the modified RS medium (supplemented with 0.6 mM MPHE) with a stainless steel cell spreader uniformly and incubated in dark at 25°C for 29 days without subculturing. The control group was incubated in the standard RS medium under the same conditions. After 29 days, the cells were transferred to a standard RS medium and incubated for another 28 days. The experiment was conducted in 3 replicates.

3.4.2.2. Preliminary Work #2: Selection with Liquid Medium

For the selection of resistant cells, the protocol of Georgiev *et al.* (2006) was followed [63]. 0.5 g of 7-days old Lavandin and Hemus suspension cells were incubated in a 100 ml Erlenmeyer flask containing 20 ml P6 medium for selection group and in RL medium for control group. The cultures were maintained in dark at 25°C on a shaking incubator (110 rpm) for 37 days without subculturing. After 37 days, the cells were harvested via vacuum filtration and weighed. All harvested cell were spread on a standard RS medium and incubated for another 28 days. The experiment was conducted in 3 replicates. Statistical analysis was done using ANOVA and Student's *t*-test.

3.4.2.3. Selection with Liquid Medium and Subculturing every 14 days for 1 g Suspension Cells

For the selection of resistant cells, the protocol of Duncan and Widholm (1990) was followed [83]. 1 g of 7-days old Hemus suspension cells were transferred into a 100 ml Erlenmeyer flask containing P6 medium for selection group and in RL medium for control group. The experiment was conducted in 10 replicates (labelled as #1S, #2S, #3S, #4S, #5S, #6S, #7S,

#8S, #9S, #10S) for the selection group and 5 replicates (#1C, #2C, #3C, #4C, #5C) for the control group. Suspension cultures were maintained in dark at 25°C on a shaking incubator (110 rpm) for 42 days. Every 14 days, only grown cells were transferred to P6 media during subculturing. After 42 days, the first 5 selection flasks (#1S, #2S, #3S, #4S, #5S) and the control group flasks were filtered, weighed and then kept for the extraction and analysis. The remaining 5 flasks of the selection group were transferred into standard RL medium for another 14 days before they were filtered and weighed [63]. Then, RA was extracted and analyzed at 327 nm. Statistical analysis was done using Student's *t*-test.

3.4.2.4. Selection with Liquid Medium and Subculturing every 14 days for 0.5 g Suspension Cells

For the selection of resistant cells, the protocol of Duncan and Widholm (1990) was followed [83]. 0.5 g of 7-days old Hemus and Lavandin suspension cells were transferred into a 100 ml Erlenmeyer flask containing 20 ml P4 and P8 media for selection group and in RL medium for control group. The experiment was conducted in 7 replicates for each treatment. 6 replicates were used subculturing and 1 replicate was used for viability test. The control group had 5 replicates, with 4 for subculturing and 1 for viability test. Suspension cultures were maintained in dark at 25°C on a shaking incubator (110 rpm) for 42 days. Every 14 days, only grown cells were transferred to the fresh medium including the same concentration of MPHE during subculturing. After 42 days, the cells were weighed. Statistical analysis was done using ANOVA and Student's *t*-test.

3.4.3. Viability Test via Fluorescent Microscope

A viability test was conducted by using fluorescein diacetate (FDA). For preparing the stock solution, 5 mg of FDA was dissolved in 1 mL acetone. 1 mL suspension culture sample was mixed with 950 µL fresh RL medium and 50 µL FDA solution. The sample was covered with aluminum foil and held for 5 minutes. Fluorescent illumination of the sample was examined using a fluorescent microscope equipped with a UV detector to observe the living cells.

3.5. GENE EXPRESSION ANALYSIS OF *LAAT1* GENE

3.5.1. RNA extraction and cDNA synthesis

For the gene expression analysis, resistant cells from the selection experiments with 1 g Hemus cells and 0.5 g Hemus and Lavandin cells as a starting material was used. The group with 1 g Hemus cells as starting material were divided into three: first selection group that the cells were incubated in the presence of MPHE for 42 days (now called as H6), the second selection group that the cells were incubated in the absence of MPHE for another 14 days (called H6-) and the control group cells (HC) without MPHE. The group with 0.5 g Hemus and Lavandin cells were incubated in P4 and P8 media containing different concentrations of MPHE. Hemus cells in P4 and P8 media are called H4 and H8, respectively. Lavandin cells in P4 and P8 media are called L4 and L8, respectively. The cells in the control groups are called HC and LC for Hemus and Lavandin, respectively. All these cells were frozen with liquid nitrogen, and then they were crushed with mortar and pestle until they turned into powder form. Total RNA extraction was done according to NORGEN manufacturer Total RNA Purification kit. Then, reverse transcription was performed according to Mol Gene manufacturer reverse transcriptase kit using 1 µg of RNA as a starting material for each sample.

3.5.2. Primer design and verification of primers via PCR

qPCR primers of the *L. angustifolia* Rosmarinic Acid Synthase (*LaAT1*) gene region (JF343521.1) were designed using NCBI Primer-BLAST. *LaAT1* was amplified by PCR using forward (5'-ACTCGCTCAAAGCCAAATGC-3') and reverse (5'-ACCTCGAACGTGCTGTATCG-3') primers. The PCR reaction was carried out using PCR Thermal Cycler (Bio-Rad, T100). The reaction was performed in 25 µl volume containing 1X Taq buffer (Thermo Scientific), 0.4 mM dNTPs, 2mM MgCl2, 1.25U Taq polymerase (Thermo Scientific), 500ng cDNA, 0.4 mM of each primer and molecular grade water. PCR reaction was performed under the following conditions: 95°C for 3 min, followed by 35 steps of 95°C for 30 s, 60°C for 30 s, 72°C for 15 s, then 72°C for 5 min. The PCR samples were analyzed via 1.5 per cent gel electrophoresis (Bio-Rad, PowerPac Basic Power

Supply1645050). For reference gene, housekeeping β-actin gene was selected. Finally, the primers for *L. angustifolia* β-actin gene were chosen from Guo *et al*. (2020) (Gene bank: KY596032.1) (Forward Primers: 5'- TGTGGATTGCCAAGGCAGAGT -3', Reverse Primer: 5'- AATGAGCAGGCAGCAACAGCA -3') [96] and amplified by PCR using the same conditions above.

3.5.3. Quantitative PCR analysis of *LaAT1* gene in Hemus and Lavandin

For gene expression analysis, qPCR amplification of *LaAT1* gene was carried out using β-actin as internal reference (Table 3.2). The reaction was adjusted to a final volume of 20 µl containing 5 µl cDNA (adjusted to 50 ng), 3 µl of molecular grade water, 1 µl of each primer (400 nM final concentration) and 10 µl SYBR Green Supermix (Bio-Rad). qPCR reaction was performed in duplicates under the following conditions: 95°C for 2 min, followed by 40 steps of 95°C for 30 s, 60°C for 30 s. Melting curve analysis was done using the default settings of the device (Bio-Rad). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression levels.

Table 3.2. qPCR plate design for Hemus and Lavandin species; NTC=no template control, STD=standard.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HC (1) LaAT1	HC (1) LaAT1	H4 (1) LaAT1	H4 (1) LaAT1	H6 (1) LaAT1	H6 (1) LaAT1	H6- (1) LaAT1	H6- (1) LaAT1	H8 (1) LaAT1	H8 (1) LaAT1	x	x
B	HC (2) LaAT1	HC (2) LaAT1	H4 (2) LaAT1	H4 (2) LaAT1	H6 (2) LaAT1	H6 (2) LaAT1	H6- (2) LaAT1	H6- (2) LaAT1	H8 (2) LaAT1	H8 (2) LaAT1	x	x
C	HC (1) Actin	HC (1) Actin	H4 (1) Actin	H4 (1) Actin	H6 (1) Actin	H6 (1) Actin	H6- (1) Actin	H6- (1) Actin	H8 (1) Actin	H8 (1) Actin	x	x
D	HC (2) Actin	HC (2) Actin	H4 (2) Actin	H4 (2) Actin	H6 (2) Actin	H6 (2) Actin	H6- (2) Actin	H6- (2) Actin	H8 (2) Actin	H8 (2) Actin	x	x
E	LC (1) LaAT1	LC (2) LaAT1	LC (3) LaAT1	L4 (1) LaAT1	L4 (2) LaAT1	L4 (3) LaAT1	L8 (1) LaAT1	L8 (2) LaAT1	L8 (3) LaAT1	NTC	NTC	NTC
F	LC (1) Actin	LC (2) Actin	LC (3) Actin	L4 (1) Actin	L4 (2) Actin	L4 (3) Actin	L8 (1) Actin	L8 (2) Actin	L8 (3) Actin	NTC	NTC	NTC
G	HC STD(1) Actin	HC STD(1) Actin	HC STD(2) Actin	HC STD(2) Actin	HC STD(3) Actin	HC STD(3) Actin	HC STD(4) Actin	HC STD(4) Actin	HC STD(5) Actin	HC STD(5) Actin	HC STD(6) Actin	HC STD(6) Actin
H	LC STD(1) Actin	LC STD(1) Actin	LC STD(2) Actin	LC STD(2) Actin	LC STD(3) Actin	LC STD(3) Actin	LC STD(4) Actin	LC STD(4) Actin	LC STD(5) Actin	LC STD(5) Actin	LC STD(6) Actin	LC STD(6) Actin

4. RESULTS & DISCUSSION

4.1. ESTABLISHMENT AND MAINTENANCE OF LAVENDER CELL CULTURES

L. x *intermedia* var. Super A callus cultures have been previously established and maintained for approximately 5 years [94]. *L. angustifolia* Hemus callus cell lines have been maintained for approximately 4 years. Both these two cultures were cultured in LS media supplemented with 0.2 mg/L 2,4-D and 5.5 g/L agar, called as RS media [63] (Figure 4.1). The callus cultures were subcultured into fresh media every 21 days.

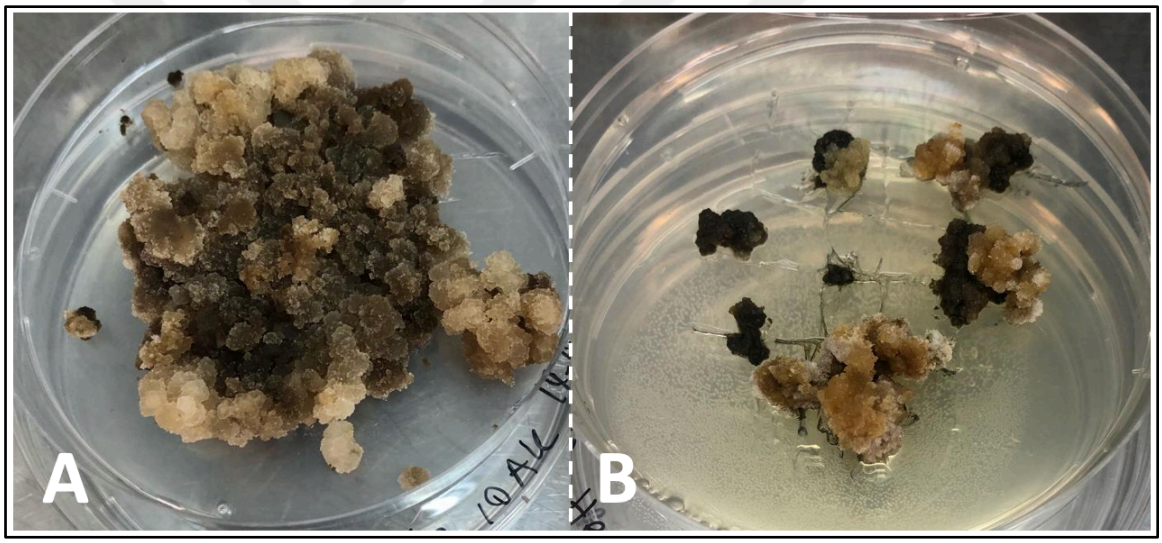


Figure 4.1. The images of callus cultures of (A) *L.* x *intermedia* var. Super A and (B) *L. angustifolia* Hemus.

The callus cells from these callus cultures were used to establish a cell suspension culture. They have been maintained in the modified liquid RS media, withour agar, called as RL media for approximately 1.5 years [63]. They were subcultured in fresh media every 7 days.

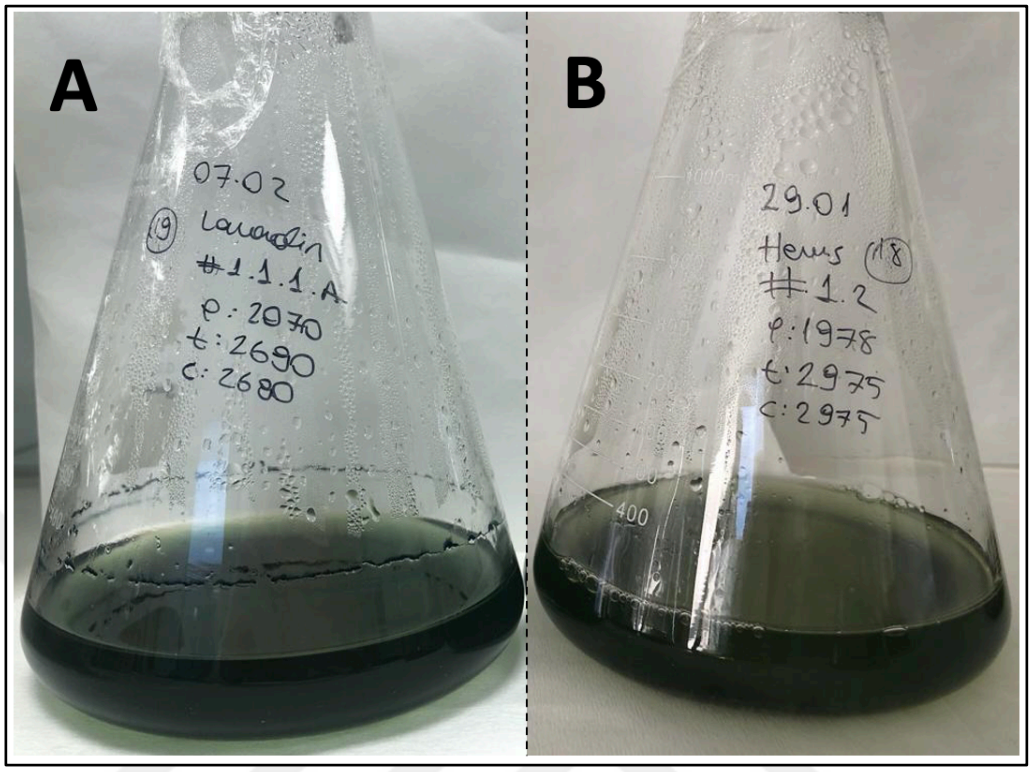


Figure 4.2. The images of cell suspension cultures of (A) *L.* x *intermedia* var. Super A and (B) *L. angustifolia* Hemus.

4.2. GROWTH CURVE ANALYSIS FOR LAVENDER CELL SUSPENSION CULTURE

4.2.1. Growth Curve Construction

For both species, the culture was initiated with 0.5 g of suspension cells in 20 ml RL media. The experiment for constructing the growth curve lasted for 14 days and conducted in duplicates (except day 0). The growth curve was constructed in two parts as first six days (0, 2, 4, 6) and last six days (8, 10, 12, 14) since the amount of cells was not sufficient to construct the growth curve at once; therefore, the second part of the growth curve was constructed one week after the first part and the data from both curves were combined. (The data of growth curve for Hemus and Lavandin is available in Appendix A.) One replicate of Lavandin was discarded at 10^{th} week due to contamination, thus, the standard deviation could not be calculated for 10^{th}, 12^{th} and 14^{th} weeks.

During the 14 days growth period, fresh weight of the cells was measured every other day. The cells for both species grew logarithmically until the last day, except for 10th day where Hemus cells decreased before they continued growing again on 12th day (Figure 4.3). The reason of this decrease on 10th day may be due to construction of growth curve in two parts. Except that day, all cells grew exponentially and did not enter to stationary phase (Figure 4.3). The reason of that might be the low amount of starting material. If the experiment had been initiated with 1 g cells with the same set up, then an earlier exponential phase could have been observed. Mustafa *et al*. (2011) reported that the lower inoculation amount resulted in longer lag phase whereas the higher inoculation amount caused an earlier exponential phase [97].

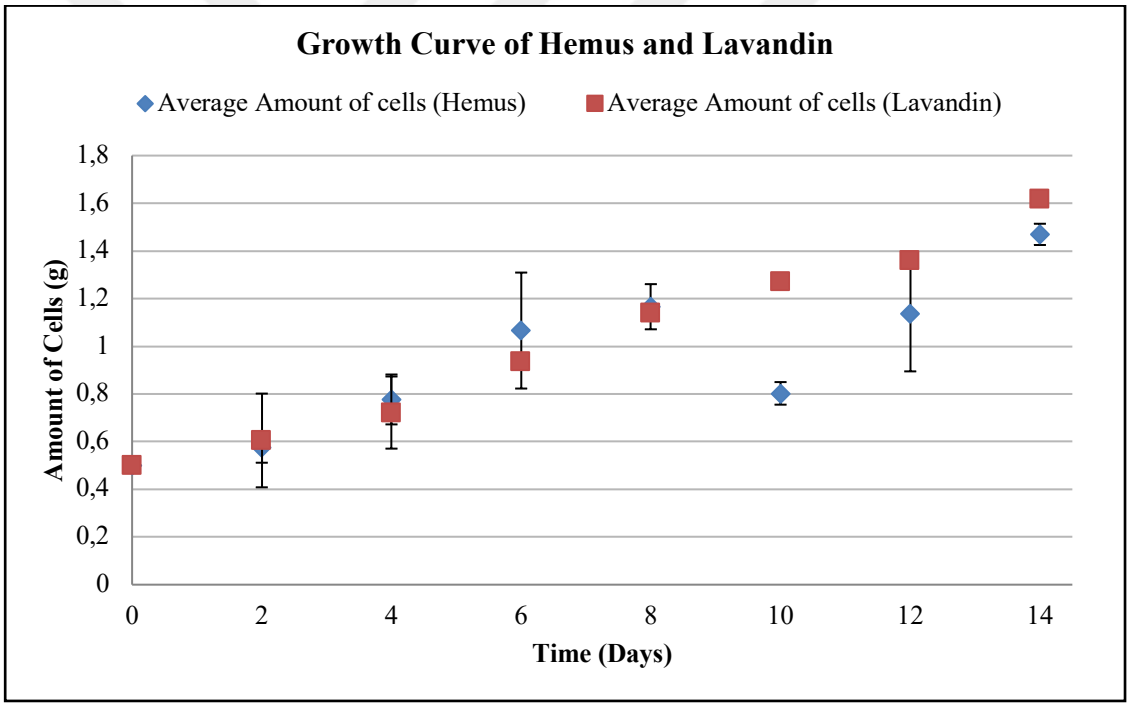


Figure 4.3. The growth curve of Hemus (blue) and Lavandin (red) cell suspension cultures initiated with 0.5 g cells.

In order to observe the stationary phase and obtain a complete growth curve, the experiment was repeated with 1 g of starting material. Due to low amount of cells in Lavandin suspension culture, the experiment was conducted only with Hemus. This time, the growth curve was constructed at once. (The data for the growth curve of Hemus starting with 1 g suspension cells are available in Appendix A.) On the 2nd day, the growth curve entered a

lag phase and between 4-6th day, it was the end of the log phase (Figure 4.4). The stationary phase was reached on 6-8th day. After that, the growth started to increase again. During the exponential growth phase, FW stays relatively steady and increases significantly only after the medium's sucrose is reduced [98], and also same result was obtained in the growth curve starting with 1 g suspension cells (Figure 4.4). The reason for this is actually an increase in the water content in the cell. In particular, the amount of water in the cells increases in the stationary phase, which increases the fresh weight [98]. Furthermore, the results of the growth curve starting with 1 g cells were similar to the results of the 0.5 g cells since the amount of cell at the end of growth curves were same. Inoculation size did not change the amount of cells on the 14th day because almost half amount of the cells reduced when they came to the 2nd day. While the inoculation size was 0.5 g, the cell continue to grow in the lag phase. On the other hand, the inoculation size increases up to 1 g, the amount of cells reduced. The reason for this reduction is that the large inoculum size can reduce the lag phase [99].

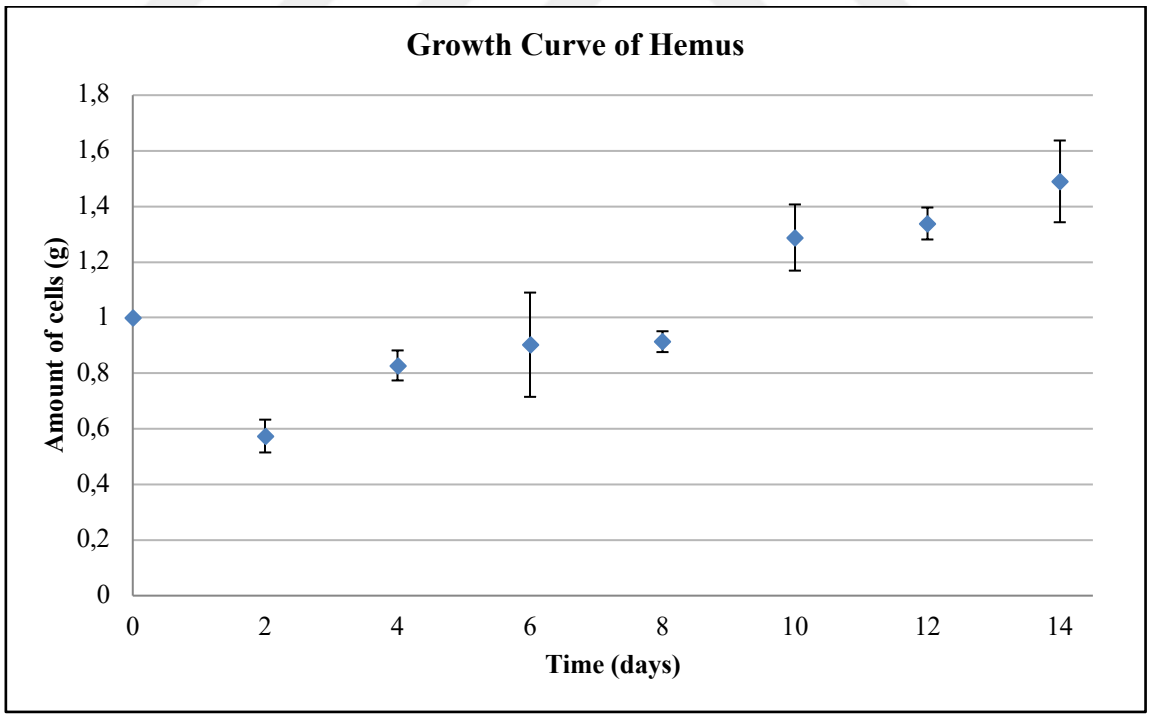


Figure 4.4. The growth curve of Hemus suspension culture initiated with 1g cells.

In most lavender suspension studies, the amount of starting material was not mentioned [22,49,53,63,89,100,101]. Although inoculation size was not stated, Ilieva and Pavlov

(1997) reported that the maximum growth of *L. vera* MM cell suspension culture in dry weight (DW) was observed on the 8th day [54]. In another research study, it was stated that maximum in biomass synthesis for *L. vera* MM in DW was reached on the 9th day without clarifying the inoculation size [89]. However, this experiment is not comparable with those in literature since the growth curve was constructed using fresh weight (FW) data instead of DW.

4.2.2. pH Analysis of the Cell Suspension Cultures

The pH of the media for all cultures were determined (Figure 4.5). For the cultures initiated with 0.5 g cells of Hemus and Lavandin, the pH was approximately around 5, throughout the experiment except at the 12th day where one replicate of Hemus was recorded as pH 6 and the other replicate as 9, which is considered as a measurement error. The pH values obtained in this study were consistent with the study of De-Eknamkul and Ellis (1984) performed on *Anchusa officinalis*. The pH was recorded between 4.9 and 5.7 (Appendix A) [71]. For the cultures initiated with 1 g cells of Hemus, the results of the pH analysis were similar with the cultures initiated with 0.5 g cells (Figure 4.6). The pH of the media was approximately 5, except for the 14th day where the pH was recorded as 8,63.

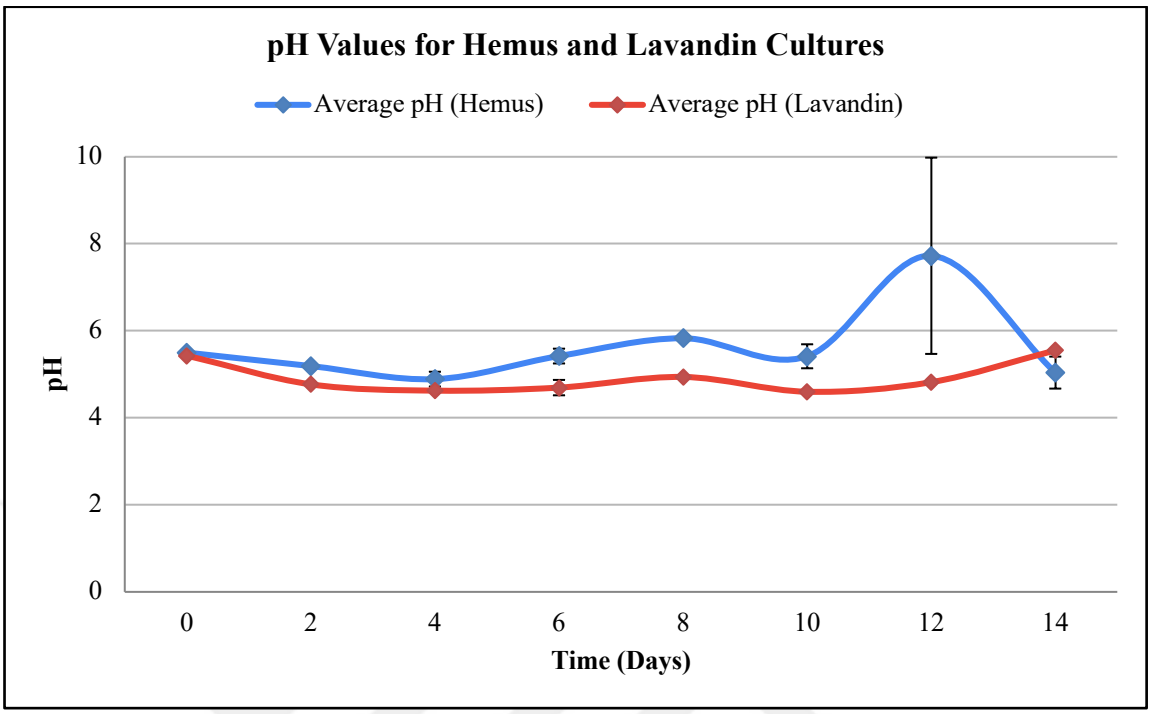


Figure 4.5. The pH of the media containing Hemus (blue) and Lavandin (red) suspension cultures initiated with 0.5 g cells.

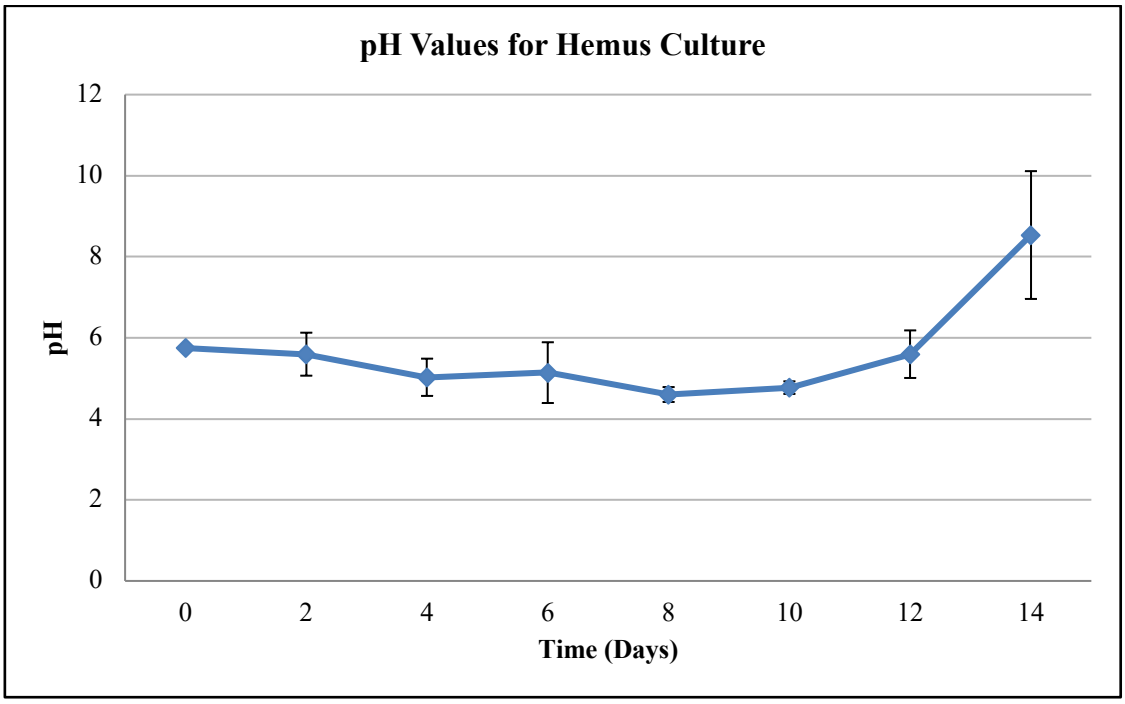


Figure 4.6. The pH of the media containing Hemus suspension culture initiated with 1 g cells.

4.2.3. The Efficiency of Rosmarinic Acid Production in Lavender Suspension Culture

The RA synthesis of suspension cells for each day was examined for 14 days (Appendix A). When initiated with 0.5 g cells, the RA production of two species was found to be significantly different ($p<0.05$), with Lavandin producing a higher amount of RA than Hemus in total. There could be the differences between species in terms of producing SMs [102]. The maximum RA production was obtained as 0.304 g/L RA on the 12th day for Hemus and 0.463 g/L on the 6th day for Lavandin (The data for RA concentions (μM/g FW) were available in Appendix A). During 14 days, there were two peaks in the RA production of both species (Figure 4.7). The reason for two peaks in this study may be due to the construction of the growth curve in two parts, because there is a decrease in the RA production on the 8th day when the second part was started. For the growth curve of Hemus starting with 1 g cells, RA production was decreasing during lag phases and then, the maximum peak was obtained on the 6th day, as 0.325 g/L RA (Figure 4.8). Besides, there is no significant differences for RA production between the growth curves starting with 0.5 g and 1 g Hemus suspension cells ($p>0.05$). De-Eknamkul and Ellis (1984) reported that the accumulation of RA from *Anchusa officinalis* cell suspension decreased during the lag and exponential growth phases [71]. Similar results were obtained in our study for Lavandin initiated with 0.5 g Lavandin cells. This can be explained by a "dilution effect" because the cell dry weight increased continuously during the same time period [71]. For Hemus initiated with 1 g cells, RA production also decreased during lag phase since the amount of cell was reduced during the same time. In addition, De-Eknamkul and Ellis (1984) also mentioned that the RA production was increasing between the 6-12th day of the cultivation which was expressed as linear phase coming after exponential phase and before the stationary phase, and they obtained maximum RA production on 12th day. Ilieva and Pavlov, 1997 reported that intensive RA production was obtained in the linear phase which was between 6-8th days and they obtained a maximum 68 mg/L RA (0.068 g/L) from *L. vera* MM cell suspension culture on 8th day.

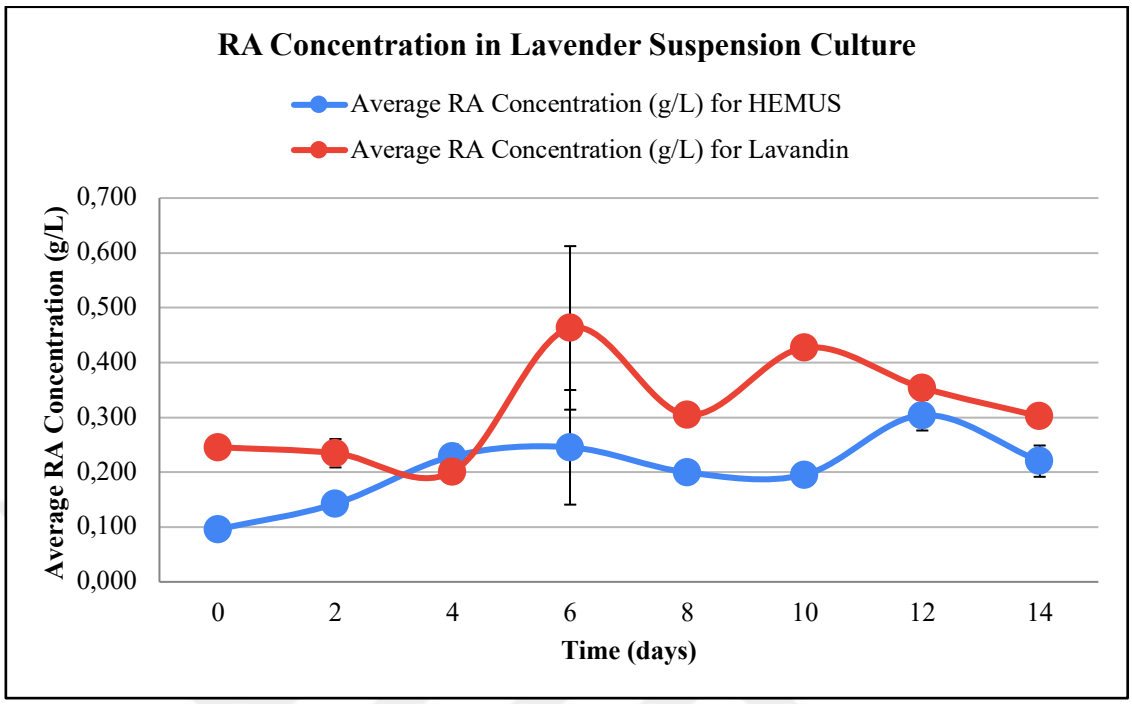


Figure 4.7. RA concentration (g/L) of Hemus (blue) and Lavandin (red) suspension cultures initiated with 0.5 g cells.

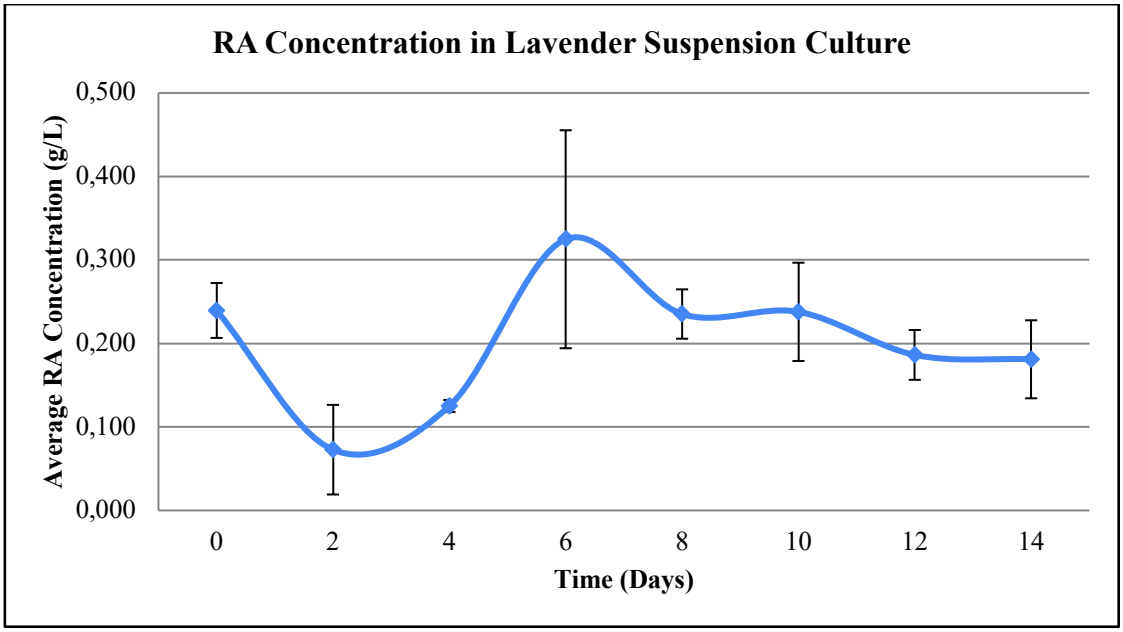


Figure 4.8. RA concentration (g/L) of Hemus suspension cultures initiated with 1 g cells.

4.3. QUANTIFICATION OF ROSMARINIC ACID

4.3.1. Optimization Studies for the Construction of Standard Curve

Two stock RA solutions prepared with water and 70 per ent EtOH were diluted in Na-acetate buffer (pH 6.0) for 10 µM to 500 µM concentration scale, and then $FeSO_4$ was added for the reaction with RA. The measurement was done with spectrophotometry at 327 nm. Blank solutions for each RA concentrations were prepared and measured, therefore, each RA concentration had its own blank solution (Appendix B). Based on the results, the absorbance of the blank solution for water decreased from 10 µM to 500 µM because the amount of water increased, and the solution was diluted. However, the differences between concentrations in water was not as expected as in ethanol. The color of blank solutions for water was almost the same (Figure 4.9-A). On the other hand, the color of ethanol darkened from 10 µM to 500 µM (Figure 4.9-B). Due to that the absorbance for ethanol increased in the same manner. The iron surface is adsorbed by ethanol [103]. In addition to that, when iron (II) reduced to iron (III), then its color changed to yellow [104]. For this reason, when the volume of ethanol in the blank increases, the color intensity increases and the absorbance value increases.

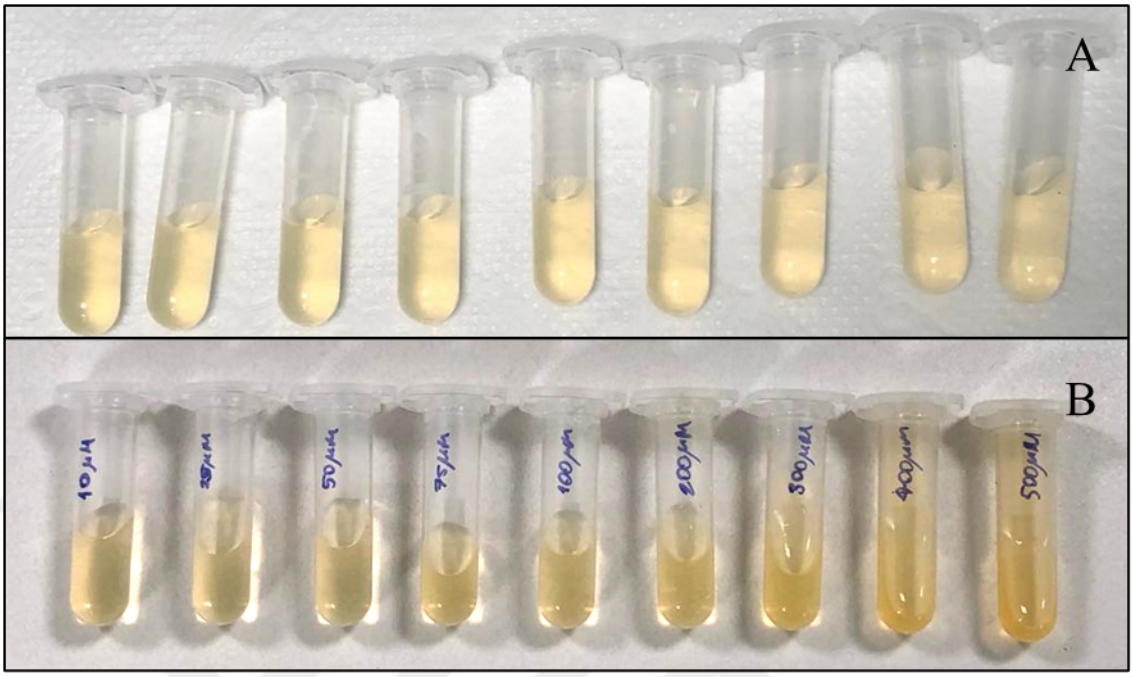


Figure 4.9. Blank solutions of RA dissolved in (A) water and (B) ethanol. Concentrations increase from left to right.

Two stocks of 1 mM standard RA solutions were prepared by dissolving one of them in distilled water and the other one in 70 percent EtOH. From these stock solutions, 10, 25, 50, 75, 100, 200, 300, 400, 500 µM RA was prepared in 0.1 M Na-acetate buffer (pH 6.0) and $FeSO_4$ to the final concentration of 1mM [91]. After the reaction, the color of RA solutions turned from colorless to grey or dark blue depending on the concentrations (Figure 4.10). The absorbance values were read at 327 nm (Refer to Appendix B).

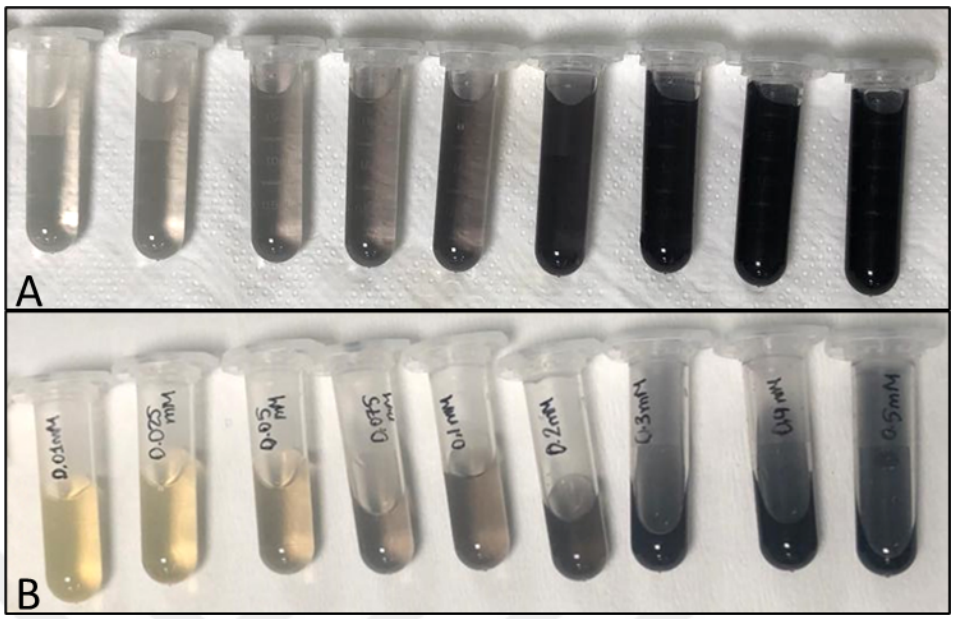


Figure 4.10. RA standard solutions dissolved in (A) water and (B) 70 percent EtOH after reacting with FeSO4 with increasing concentrations (from left to right, respectively) for an hour.

Based on the absorbance values, the absorbance-concentration graph was constructed and the trend line equation was obtained (Figure 4.11). For water, R^2 value was 0.994 and for EtOH it was 0.983. Without the absorbance values of blank solutions, the absorbance values of EtOH for each concentration was higher than that of water although there is no significant differences between them ($p>0.05$). On the other hand, with the absorbance values of blank solutions, the absorbance values of RA for both water and EtOH were almost same and there is no significant difference ($p>0.05$). As a result, dissolving in distilled water or in EtOH did not make any significant difference.

Zibetti *et al*. (2016) studied the solubility of pure RA in water, ethanol, methanol, ethanol-water and methanol-water solutions [105]. They showed that the solubility of RA is higher in aqueous ethanol or methanol solutions than that in only water when measuring RA concentration with a spectrophotometer at 328 nm which is not consistent with our results. The reason might be that they did not use any chemical that reacts with RA, and in this experiment, iron which reacts with EtOH and changes the color of the solution was used. The RA solubility result without any chemical could be reliable. However, if RA is wanted to be obtained from the plant sample and since there is unpurified RA in the plant

(contamination with other secondary metabolites), measuring RA concentration with a direct spectrophotometer without any chemicals causing a reaction with RA may not give the accurate result [92]. Because of that, $FeSO_4$ was used for analyzing RA in this experiment. However, the absorbance values of RA dissolving in EtOH were different from the previous studies since iron reacts with EtOH and changes the color of the solution.

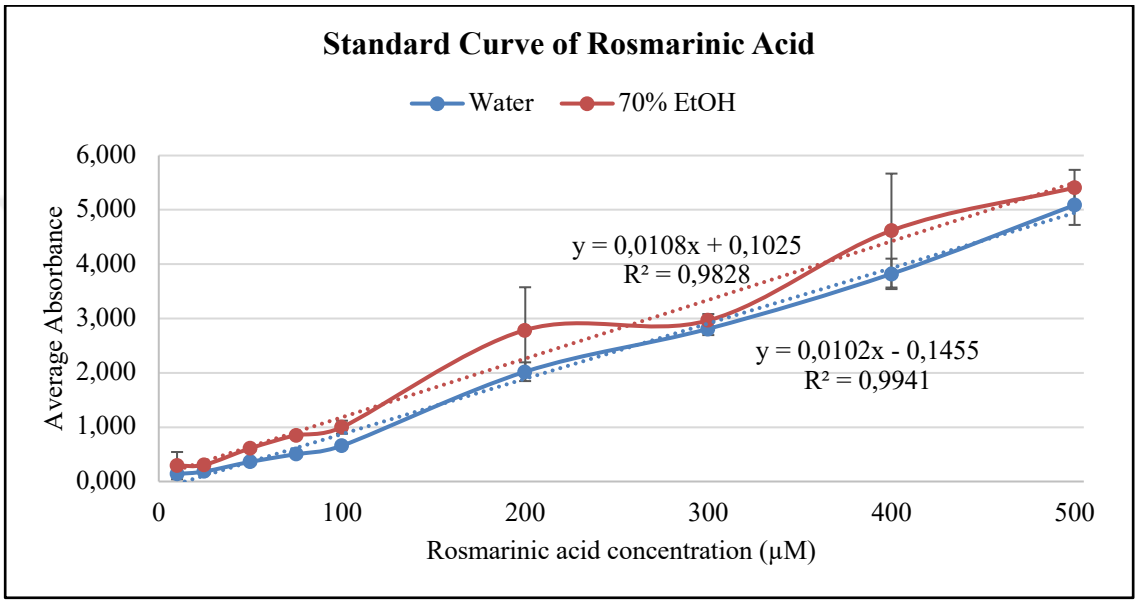


Figure 4.11. The standard curve for RA dissolved in water (blue) and 70 percent EtOH (red). The spectrophotometric analysis were performed at 327 nm.

4.3.2. Optimization of RA Extraction and Spectrophotometric Analysis

The extraction protocol was optimized using the methods for *Lavandula* suspension cells by Georgiev *et al*. (2006) and the methods for different plant suspension cells by Mizukami *et al*. (1992) and Morshed *et al*. (2011) [63,85,95]. For optimizing the extraction protocol of RA, different solvent volumes and types were tried. As a pre-treatment, the cells were frozen with liquid nitrogen and ground into powder form. Various studies used frozen suspension cells without explaining the method of freezing [63,106]. In this experiment, the pre-treatment method of Mizukami *et al*. (1992) was followed [95]. After that, RA were extracted in a 50 percent ethanol-water solution at a 70°C water bath three times for 20 minutes (3x20 minutes) as in other studies [63,66,69,89,106]. In this part of the extraction, solvent-sample ratio volume affects the extraction yield [107] though many studies did not

mention the solvent-sample ratio [63,65,66,89,100,101]. Mizukami *et al*. (1992) extracted RA from 0.5 g frozen *Lithospermum erythrorhizon* suspension cells in 5 ml EtOH-water for an hour [85]. However, it is not clear if 5 ml was used each time or in total. Various researchers applied the RA extraction procedure three times, each for 20 minutes, then combined the ethanolic extracts [63,100]. Therefore, in this experiment, 0.5 g frozen and grounded cells were mixed with 2 ml and 5 ml EtOH-water solutions three times for 20 minutes each. Then, all ethanolic extracts were combined and evaporated under vacuum pressure. The bath temperature of the evaporator was set to 40°C [108,109] and the pressure was initially set to 175 mbar for EtOH and then decreased to 75 mbar for water. By this way, the boiling temperature of the aqueous solution was adjusted and decreased gradually [95]. Then, the extracts were resuspended with 1 ml distilled water or 1 ml 70 percent EtOH-water. Only the extract resuspended in ethanol was stored for a day at -20°C [63]. For each trial, absorbance was measured at 327 nm after reaction with Fe^{+4} ion (Table 4.1), and their RA concentrations were calculated based on the equation of the standard RA curve (Figure 4.11).

There is a significant difference between four groups ($p<0.01$) (Table 4.1). However, the volume of the solvent did not have a significant effect on RA concentration whether the extracts were resuspended in water or ethanol ($p>0.05$). On the other hand, resuspending the extracts in water or EtOH affected RA concentration since the absorbances of blank concentrations of water and EtOH were different with EtOH having a higher absorbance comparing to water. Based on this, the RA concentration was higher when the cells were resuspended in water rather than in ethanol. The significant effect of dissolving RA in water or EtOH on RA concentration was observed for both solvent volumes ($p<0.05$). However, these results are not reliable due to the change of color caused by the reaction of EtOH and iron which affects the absorbance values.

Table 4.1. The absorbance values at 327 nm and RA concentrations (g/L) of the extracts using different solvent types and volumes during optimization of RA extraction.

Number	Volume of EtOH (ml)	Resuspend in	Replication	Blank	Absorbance at 327 nm	RA (g/L)	Average RA Concentration (g/L)	Standard deviation
1	2 ml 50% EtOH	Water	1.1	0,848	4,316	0,157	0,152	0,0073
			1.2	0,848	4,022	0,147		
2	2 ml 50% EtOH	70% EtOH	2.1	2,637	1,324	0,041	0,048	0,0098
			2.2	2,359	1,745	0,055		
3	5 ml 50% EtOH	Water	3.1	0,726	4,108	0,150	0,135	0,0218
			3.2	0,726	3,232	0,119		
4	5ml 50% EtOH	70% EtOH	4.1	2,404	1,603	0,050	0,047	0,0045
			4.2	2,654	1,408	0,044		

4.4. SELECTION OF MPHE RESISTANT CELL LINES

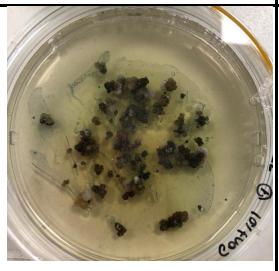
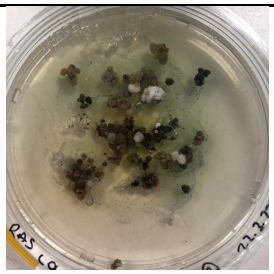
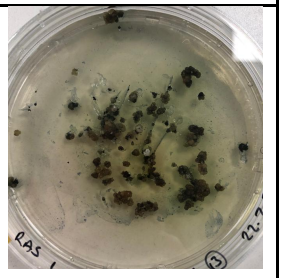
4.4.1. Preliminary Work #1: Selection of Cells in Agar-Solidified Medium

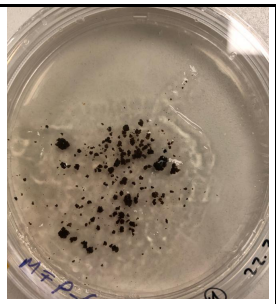
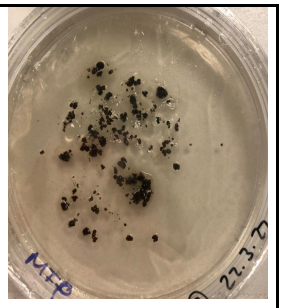
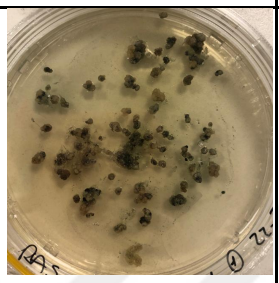
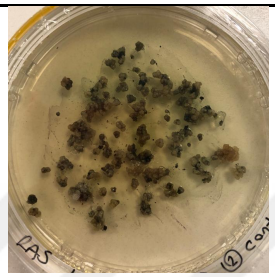
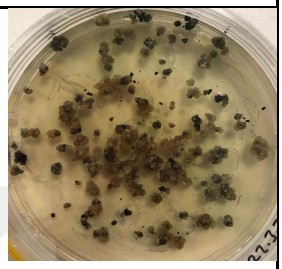
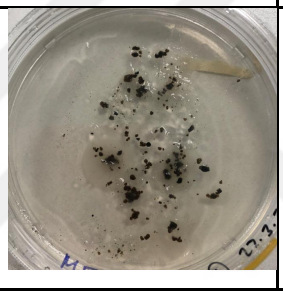
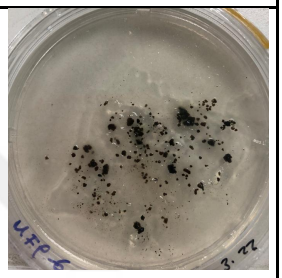
MPHE (m-F-D,L-phenylalanine) is a selective agent which kills the wild type cells. If the cells are able to produce more PAL, they become more resistant and survive. This allows for the selection of MPHE resistant cell lines based on the level of growth inhibition [110]. If a cell lives, then it can grow and develop [111], and produce fresh white cells [112]. The dead cells are distinguished with their dark-brown color and decreased weight [113]. In this experiment, 0.25 g Lavandin and Hemus cells were spread on the standard solid RS medium as a control group and the solid RS medium containing 0.6 mM MPHE as a selection group

[83]. After 29 days of dark incubation, all cells in control groups lived but all cells in selection groups had dark brown color which indicates they might die, except one tiny cell clump in Lavandin selection #2 Petri (Table 4.2). There may be two reasons why the cells in the selection group died and only this cell cluster survived. First, MPHE could not be uniformly dispersed in solid media and this small cluster of cells may have grown without MPHE. For the selection experiment with agar-solidified culture, the selective agent should be finely dispersed and also the callus cells should be uniformly distributed. Otherwise, there can be wild-type cells which are not exposed to selective agents [27]. Secondly, the cell may become resistant to MPHE, but it should have required more than 10-15 replicates to fully see this second possibility [83].

In addition to that Duncan and Widholm (1990) stated that the minimum incubation time should be 8 weeks for obtaining resistant cell type in the selection experiment [83]. In the study of Georgiev *et al*. (2006), suspension cells from *L. vera* were incubated for the selection experiment for 42 days (6 weeks) [63]. Berlin and Sasse (2005) mentioned that the resistance cell lines could be chosen after 3-8 weeks [27]. In this experiment, the cells could have been incubated for 4 weeks or more. On the other hand, the cells in the selection group did not grow so probably they would not grow if they would have been incubated more than 4 weeks. The possible reason why selection group cells did not grow on agar medium could be the application of the selective agent. They could be exposed to only selective agent and did not have a contact with nutrients which caused growth inhibition. In this experiment, the cells could not be weighed because the callus cells could not be separated from agar media resulting in inaccurate weight measurements.

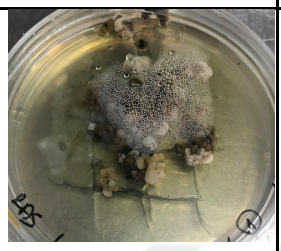
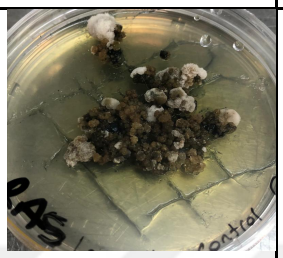
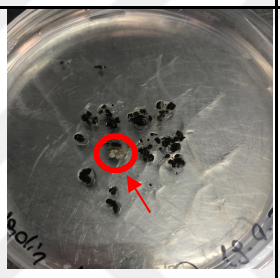
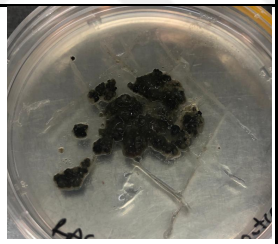
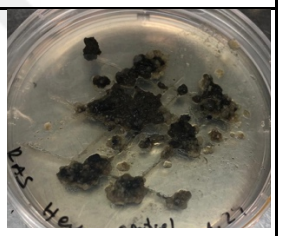
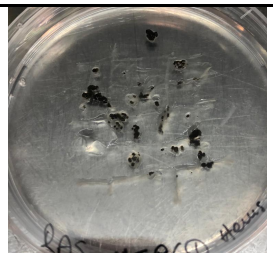
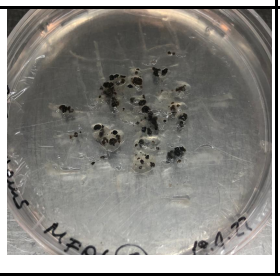
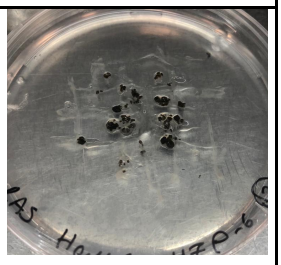
Table 4.2. The images of Lavender cells of control and selection groups after 29 days.

Lavender Species	Groups	#1 petri	#2 petri	#3 petri
	Control			

Lavandin	Selection			
Hemus	Control			
	Selection			

The cells were transferred to a standard RS solid medium without MPHE and incubated for 28 days [63]. In the absence of the selective agent, the resistant cells can live and transmit the new stable trait to the next generation. However, if the cells only adapt to selective agents, then new trait is only maintained in the presence of the selective agent [27]. After 28 days, the cells in the selection group did not grow or produce fresh white-beige cells, confirming their death, except Lavandin #2 (Table 4.3) [113]. For the control group, routine subculture process was applied. In the selection experiment conducted by Georgiev *et al.* (2006), cells were incubated in the standard solid media following the incubation in MPHE medium [63]. They observed the growth in the absence of MPHE. They measured cell growth based on the number of grown clusters obtained and rated them from 0 (no growth) to 5 (perfect growth). For this experiment, on the other hand, the grown cells in the standard medium could not be obtained since they die in the first incubation in MPHE media so that they could not be rated.

Table 4.3. The images of Lavender cells of control and selection groups after 28 days in the standard RS medium.

Lavender Species	Groups	#1 petri	#2 Petri	#3 petri
Lavandin	Control			
	Selection			
Hemus	Control			
	Selection			

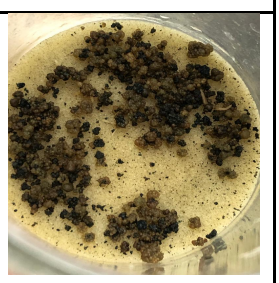
4.4.2. Preliminary Work #2: Selection of Cells in Liquid Medium

0.5 g Hemus and Lavandin suspension cells were incubated without subculturing for 37 days in RL liquid medium as a control group and in P6 medium for selection group. The color of the cells turning brown is an indication of decreased vitality, weakened growth and even death. Therefore, the color of cells is important for observing the cell viability at first [113]. After the incubation, the cells in control groups for both Lavandin and Hemus species turmed

into black-dark brown in color (Table 4.4) [113]. In selection groups, #1 and #2 flasks for Lavandin species, the cells were dark brown in color. However, the cells in Lavandin selection group #3 flask had both dark brown and light brown colored cells. In selection groups of Hemus, only #1 and #2 flasks had both dark brown and light brown colored cells.

Based on the color of cells, all control cells for both species had fully dark-brown color and seemed like cell death occurred. The reason of the different color between selection and control group can be that all cells in the control group tried to grow for 37 days without any subculture since there was no selective agent causing cell death. However, since this growth increased the cell density in the medium, it may have stressed the cells and thus, their color may have turned into dark brown. In addition, there could be competition between the cells for nutrients for growth and maintenance [113–115]. On the other hand, since the media in the selection group had a selective agent, only resistant cells survived, and therefore, the cell density in the media was not as high as in the control group. This might have enabled the cells in selection group to produce more whitish and fresh cells.

Table 4.4. The images of Lavender cells of control and selection groups grown is suspension cultures.

Lavender Species	Groups	#1 flask	#2 flask	#3 flask
Lavandin	Control			
	Selection			

Hemus	Control			
	Selection			

The cells were weighed at the end of 37 days and there was a significant difference between the groups ($p<0.01$) (Table 4.5). The differences between species for control groups were also significant ($p<0.01$) and the control cells of Hemus was higher in weight than Lavandin. There might be difference between species in terms of cell growth [116]. On the other hand, the differences between species for selection groups were not significant ($p>0.05$). The growth differences between control group and selection group for Hemus were significant ($p<0.01$) which showed that MPHE caused growth inhibition in Hemus. However, for Lavandin species, the weight of cells in control and selection groups were not statistically significant ($p>0.05$) since the Lavandin cells in the selection group could grow and produce fresh cells.

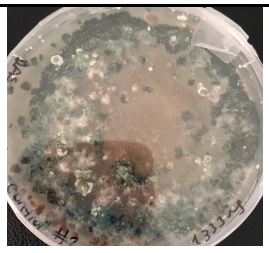
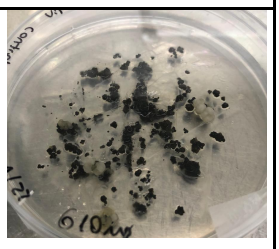
Table 4.5. The amount of cells (mg) of control and selection groups for each lavender species after 37 days in liquid culture medium.

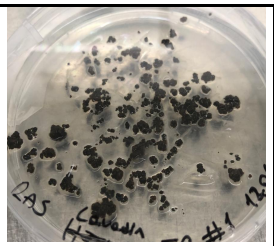
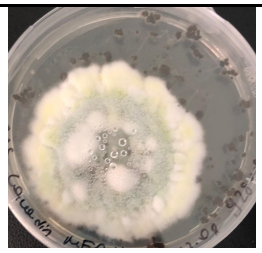
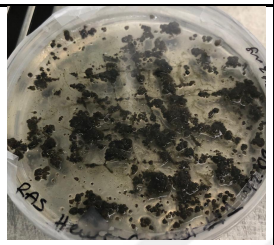
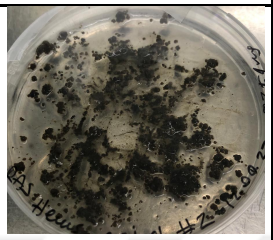
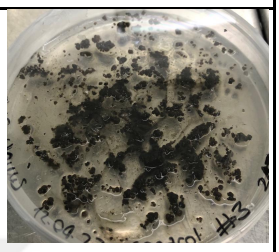
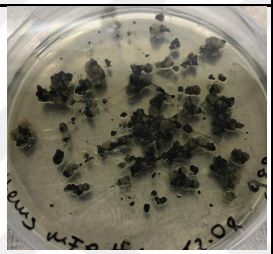
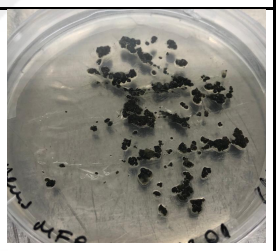
Lavender Species	Groups	#1 flask	#2 flask	#3 flask	Average Weight (mg)	Standard Deviation
Lavandin	Control	1291	1333	610	1078	405,8

	Selection	881	928	1016	942	68,5
Hemus	Control	2543	2254	2170	2322	195,7
	Selection	528	988	646	721	238,9

After 37 days incubation in the presence of MPHE media, the cells were transferred to a standard RS solid medium and incubated for 28 days (Table 4.6). After 28 days, one replicate from each control and selection groups of Lavandin were discarded due to fungal contamination. The cells in control groups of both species had still dark color in RS solid medium, except Lavandin control group #3 flask. Some cells in #3 flask produced fresh white cells. There were living cells in selection groups of Lavandin #3 flask and Hemus #2 flask, and they produced fresh cells. In the selection experiment conducted by Georgiev *et al*. (2006) cells which were incubated in the MPHE medium for 42 days were transferred to the standard solid medium which did not contain MPHE and they incubated them for another 21 days. They observed growth in the standard solid medium. While the maximum growth was observed in the control group, the cells which were incubated in the medium supplemented with 0.6 mM MPHE grew slightly. On the other hand, in this experiment, the results were the opposite of their results because the control cells in this experiment died due to lack of nutrient and high cell density. Therefore, subculture in the selection experiment should have been done biweekly [83] although Georgiev *et al*. (2006) did not give any information about whether they subcultured or not.

Table 4.6. The images of Lavender cells of control and selection groups after 28 days on standard RS medium.

Lavender Species	Groups	#1 flask	#2 flask	#3 flask
Lavandin	Control			

	Selection			
Hemus	Control			
	Selection			

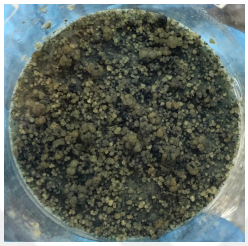
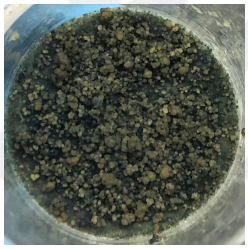
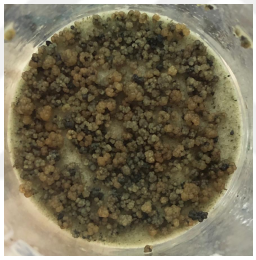
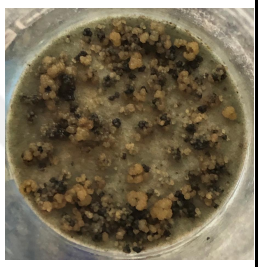
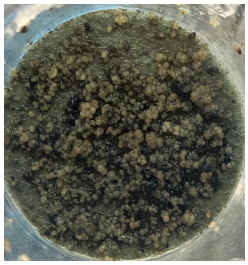
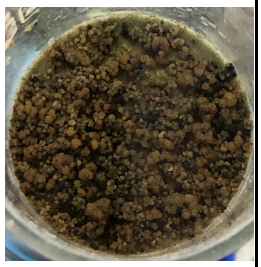
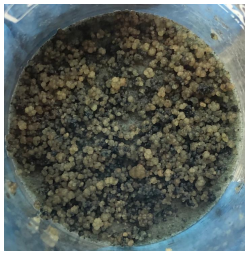
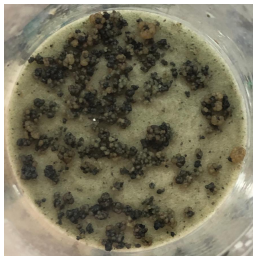
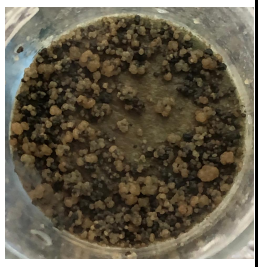
When comparing two selection methods, using liquid medium is more appropriate than using agar-solidified medium because all cells can equally be affected by the selective agent. By this way, only resistance cells can survive and the wild-type ones may be eliminated. In agar medium, wild-type cells can grow since all cells are not uniformly exposed to selective agent [27]. The other advantage of using liquid medium for selection is to easily weigh the resistant cells by the prevention of inaccurate weight measurements since there is no agar which the cells are stuck in.

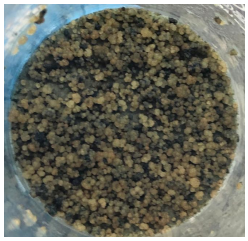
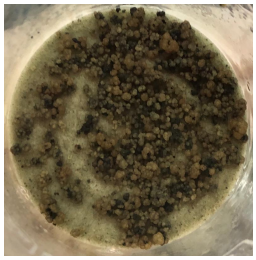
4.4.3. Selection using Liquid Medium and Subculturing every 14 days for 1 g Suspension cells

1 g Hemus suspension cells were incubated in P6 media for the selection group and in RL media for control group for 42 days (6 weeks). While the cells in preliminary work #2 were not subcultured for 37 days, during this part of the study the cells were subcultured biweekly into the same medium as suggested by Duncan and Widholm (1990) [83]. By this way, almost all cells in both groups could produce fresh cells unlike the cells in the preliminary

work #2. Visually, the main difference between control and selection group was that the control group had a large number of cells after 42 days (Table 4.7).

Table 4.7. The images Hemus cells in selection and control groups after 42 days.

Flasks	Control Group	Flasks	Selection Group	Flasks	Selection Group
#1C		#1S		#6S	
#2C		#2S		#7S	
#3C		#3S		#8S	
#4C		#4S		#9S	

#5C		#5S		#10S	

After 42 days with biweekly subculturing, 10 flasks of selection group were divided into two. The first five flasks (named as the first selection group) were weighed along with the control group (Table 4.8). The average weight was 1.153 g for the first selection group while the control group had 1.728 g. Although MPHE causes growth inhibition [110], the difference in cell weight between two groups was statistically insignificant ($p>0.05$), in consistency with the results of *Nicotiana tabacum* suspension cells resistant to MPHE [110].

The second five flasks of the selection group (named as the second selection group) were inoculated in the standard RL liquid medium without MPHE and incubated for another 14 days to check whether the cells produce RA stably in the absence of MPHE or not [27]. The cells in this group were weighed and they had an average weight of 1.146 g, which was close to the first selection group (Table 4.8). The difference between the first and second selection groups was not significant ($p>0.05$).

Table 4.8. The weight (g) of Hemus cells in the selection and control groups after 42 days.

Groups	Flasks	Weight (g)	Average weight (g)	Standard Deviation
Selection	#1S	1.468	1.153	0.6
	#2S	1.841		
	#3S	0.308		

	#4S	0.763		
	#5S	1.385		
	#6S	0.087	1.146	1.1
	#7S	1.107		
	#8S	2.203		
	#9S	2.218		
	#10S	0.116		
Control	#1C	2.207	1.728	0.3
	#2C	2.083		
	#3C	1.914		
	#4C	1.520		
	#5C	0.917		

RA was extracted from the cultures having more than 0.5 g cells (Table 4.9). The data for RA concentrations (μM/g FW) were available in Appendix C. The control group produced the lowest amount of RA with a concentration of 0.204 g/L while the first selection group produced the highest amount of RA with an average concentration of 0.399 g/L. The difference between the groups in RA production was statistically significant ($p<0.05$). As a result, the cells being resistant to MPHE produced higher RA, which was expected. This result was consistent with the results of *L. vera* MM cell lines which were incubated in two PHE analogs [63].

The second selection group produced 0.280 g/L RA which was the amount of production between control and first selection group. The difference between second selection group

and control group was not significant ($p>0.05$) which indicated that the resistant cells were only adapted to MPHE and higher RA producing cells were maintained in only presence of MPHE [110]. On the other hand, the difference between first and second selection groups was not also significant ($p>0.05$) which showed that the cells produced stably RA in the absence of MPHE. While the two statistical results seem contradictory, in fact this indicates that RA production will decrease as cells continue to be subcultured in the absence of MPHE media because there was numerically a decrease in RA production when first subcultured to MPHE-free media, although there was no statistically significant difference between first and second selection groups.

Table 4.9. The concentration of RA (g/L) production from Hemus suspension cells in the selection and control groups.

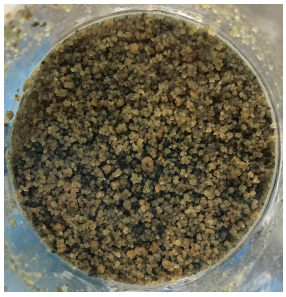
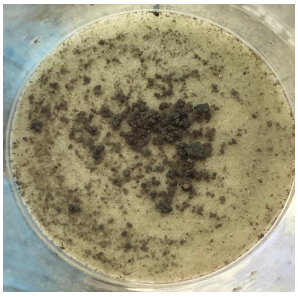
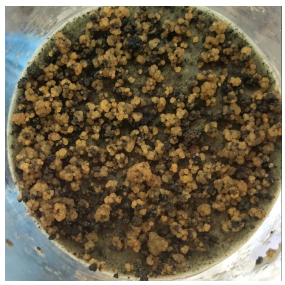
Groups	Flasks	Absorbance at 327 nm	R.A. Concentration (g/L)	Average	Standard Deviation
Selection	#1S	6.7	0.241	0.399	0.1227
	#2S	11.38	0.406		
	#4S	15.224	0.541		
	#5S	11.384	0.406		
	#7S	8.44	0.302	0.280	0.0908
	#8S	4.978	0.181		
	#9S	10.024	0.358		
Control	#1C	7.16	0.257	0.204	0.0772
	#2C	7.432	0.267		
	#3C	5.314	0.192		
	#4C	NA			

	#5C	2.67	0.099		

4.4.4. Selection with Liquid Culture and Subculturing every 14 days for 0.5 g Suspension Cells

0.5 g cells of Lavandin and Hemus cell suspension cultures were incubated in P4 and P8 media for the selection group and in RL media for control group for 42 days. The cells were subcultured biweekly. After 42 days, although the control group cells grew well as beige in color, for both species, all cells of the selection groups seemed to die since there was a major reduction in cell count and all remaining cells were dark brown in color (Table 4.10). For Lavandin, there were light brown colored cells in only one flask from P4 media and one flask from P8 media. On the other hand, for Hemus, there were light brown colored cells in 5 flasks of P4 media and 6 flasks of P8 media.

Table 4.10. The images of Lavender cells of control, P4 and P8 groups after 42 days.

Species	Control	P4	P8
Hemus			
Lavandin			

The cells were weighed after 42 days (Table 4.11). For the control group, Hemus had more cells than Lavandin had, with an average of 2.217 g Hemus cells and 1.896 g Lavandin cells. In the selection group of Hemus, there were significant differences between control groups and selection groups ($p<0.01$). The amount of control cells was statistically higher than that of both P4 and P8 cells ($p<0.01$). However, there was no significant difference between selection groups ($p>0.05$). In the selection group of Hemus, the cells in P4 media had an average weight of 0.096 g, and the cells in P8 had average weight of 0.070 g. For Lavandin, the cells in P4 and P8 media weighed as 0.059 g and 0.088 g respectively. However, there was only one flask of cells remained viable from both of these selection groups for Lavandin. The possible reason for major growth inhibition may be the inoculation size. 0.5 g cells were probably insufficient as inoculation density for resistance to MPHE to produce fresh cells. Since MPHE causes growth inhibition, the amount of the remaining cells could be below the cell density threshold which is needed for cells to grow [117]. Although 0.5 g cells were enough to construct a growth curve, it was not enough for the selection experiment because the cells did not survive. Therefore, 1 g suspension cells in 20 ml media can be minimum effective cell density for selection experiments. Inoculum size is crucial and might affect SMs synthesis [118]. The reason for increasing the cell growth and the production of SMs by increasing inoculum size could be cell-to-cell communication [119]. Moreover, if the plant cell suspension is subcultured below a crucial minimum inoculum size, the cell growth will often fail [118]. In addition, the growth inhibition differences between species is also related with cell density since Hemus species tends to produce more fresh cells than Lavandin did as shown in the weight measurements of control groups (Table 4.11). Thus, there was only one flaks left for Lavandin.

It was also expected that the cells would grow more in P4 media than compared to P6 and P8 media because due to the lowest MPHE content of P4. Although a higher concentration of MPHE (0.6 mM) was used in the preliminary work #2, better growth was observed for both species because 1 g of cells was used in that experiment. On the other hand, although the lower concentration of MPHE (0.4 mM) was used in this experiment, the cells did not survive because the amount of inoculum was small. Georgiev *et al*. (2006) also obtained more cells in lower concentrations of MPHE in the media for *L. vera* MM species but they

did not mention the inoculation size or cell density [63]. Besides, Berlin (1979) conducted a selection experiment for *N. tabacum* species with 50 replicates and observed growth in only 3-6 flasks [78]. Since the selective agents can cause growth inhibition [110], the selection experiment should have been done with many replicates a s possible.

Table 4.11. The weight (g) of Hemus and Lavandin cells in the P4, P8 and control groups after 42 days.

Species	Groups	Replicate	Weight (g)	Average weight (g)	Standard Deviation
Hemus	Control	1	1.996	2.217	0.67
		2	2.350		
		3	3.060		
		4	1.464		
	P4	1	-	0.096	0.02
		2	0.095		
		3	0.086		
		4	0.074		
		5	0.106		
		6	0.120		
	P8	1	0.050	0.070	0.02
		2	0.070		
		3	0.095		
		4	0.075		
		5	0.053		
		6	0.080		
Lavandin	Control	1	1.848	1.896	0.36
		2	2.28		
		3	-		
		4	1.562		
	P4	1	-	0.059	-
		2	0.059		
		3	-		
		4	-		

		5	-		
		6	-		
	P8	1	-	0.088	-
		2	0.088		
		3	-		
		4	-		
		5	-		
		6	-		

4.4.4.1. Viability Analysis

Fluorescein diacetate (FDA) is used to observe living cells under fluorescence microscope by dying them [120]. Fluorescein esters enter cells where they are hydrolysed by esterases to produce fluorescein. Only alive and intact cells accumulate fluorescein to fluoresce brightly [121]. While viable cells are observed as bright green, nonviable cells are not visible [122]. In this experiment, the cells in control, P4 and P8 media for both species were used for viability test. The cells after incubating for first 14 days were dyed with FDA to check their viability (Figure 4.12 and 4.13). Only part of the cells grown in P4 medium were bright green as expected, indicating that only a fraction of the cells were viable. However, control cells were not any more brighter than selection group cells, although the cell count was much higher. The reason of that can be cell explosion since the cells were frozen and thawed two times.

Although it is recommended to check the viability of selected cells [83], none of the previous studies that conducted the selection experiment gave any information about using the viability test [78,110,123,124]. Besides, while the viability test with FDA helps to observe living cells [120], Evan's Blue assay is used for the quantity measurement of the dead cells with spectrophotometry rather than visual observation [125]. Li *et al*. 2016, also used Evan's Blue assay but they also used 2,3,5-triphenyltetrazolium chloride (TTC) for obtaining the quantitative cell viability data from *G. inflata* cell suspension culture via spectrophotometer [113].

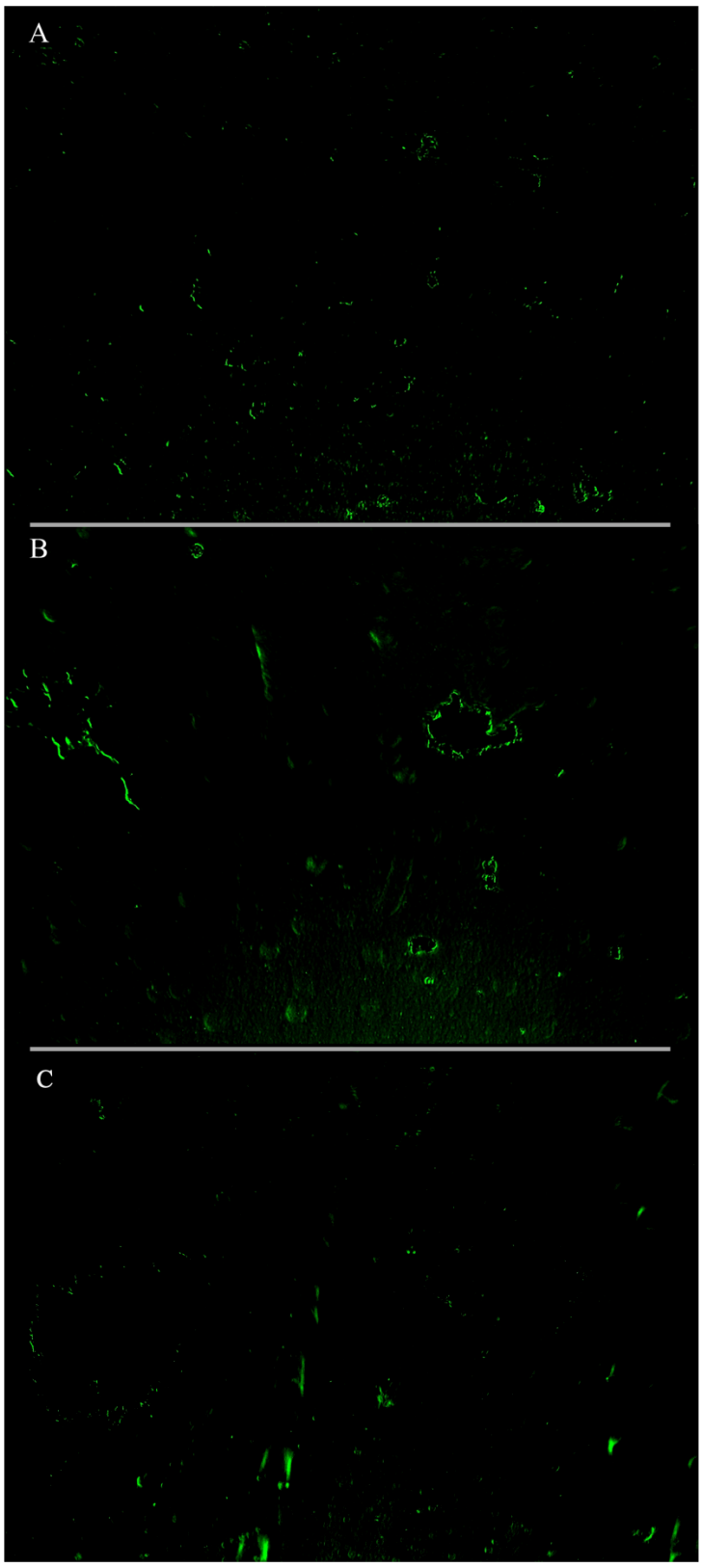


Figure 4.12. FITC images of (A) control, (B) selection-P4 and (C) selection-P8 groups of Lavandin species, which FDA test was applied to under 5X magnification.

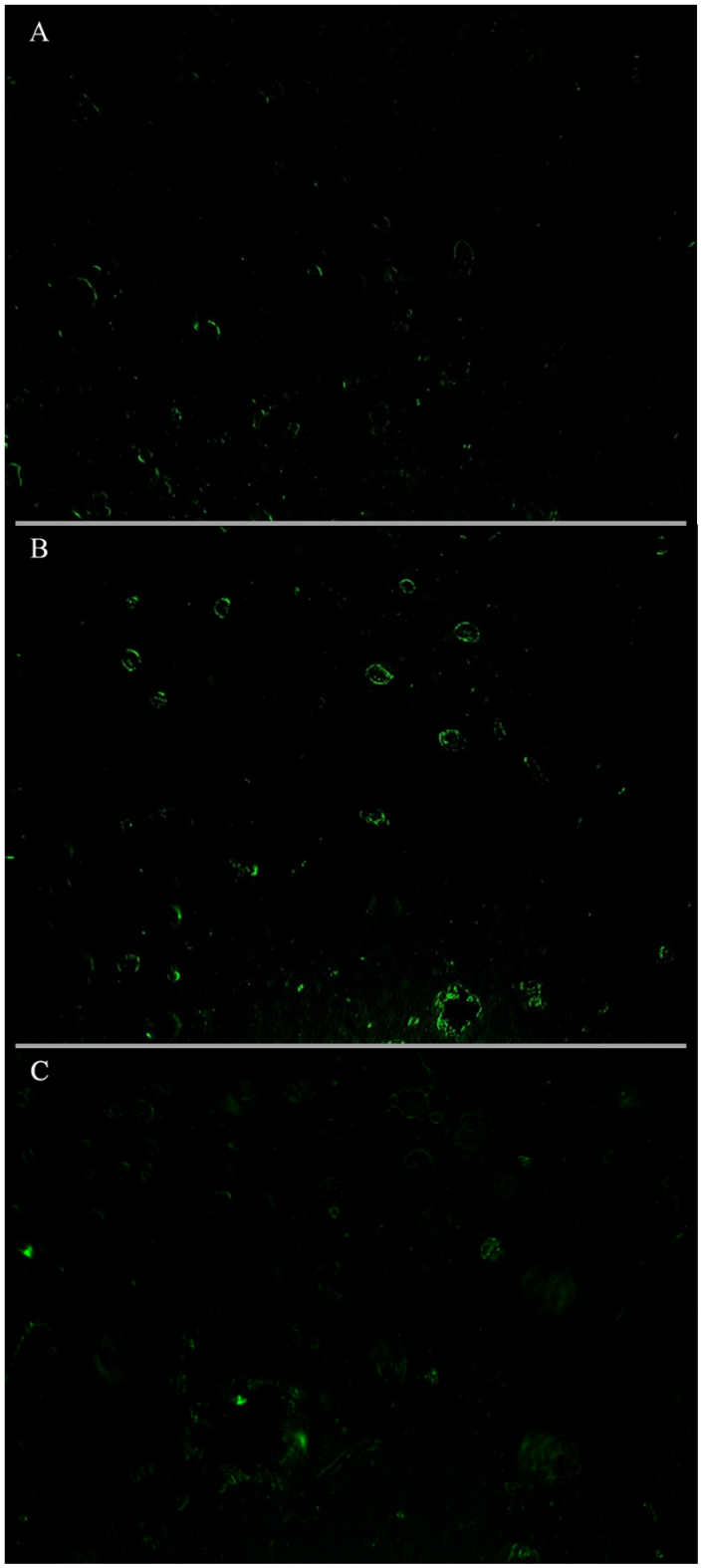


Figure 4.13. FITC images of (A) control, (B) selection-P4 and (C) selection-P8 groups of Hemus species, which FDA test was applied to under 5X magnification.

4.5. EXPRESSION OF LAAT1 GENE

4.5.1. Verification of *LaAT1* and β-actin primers

Total RNA of the selected and control cells were extracted and then converted to cDNA. qPCR primers designed for *LaAT1* and β-actin genes were verified by PCR. *LaAT1* primers yielded 160 bp product and β-actin yielded 118 bp product which were visualized by agarose gel electrophoresis (Figure 4.14 and 4.15, respectively).

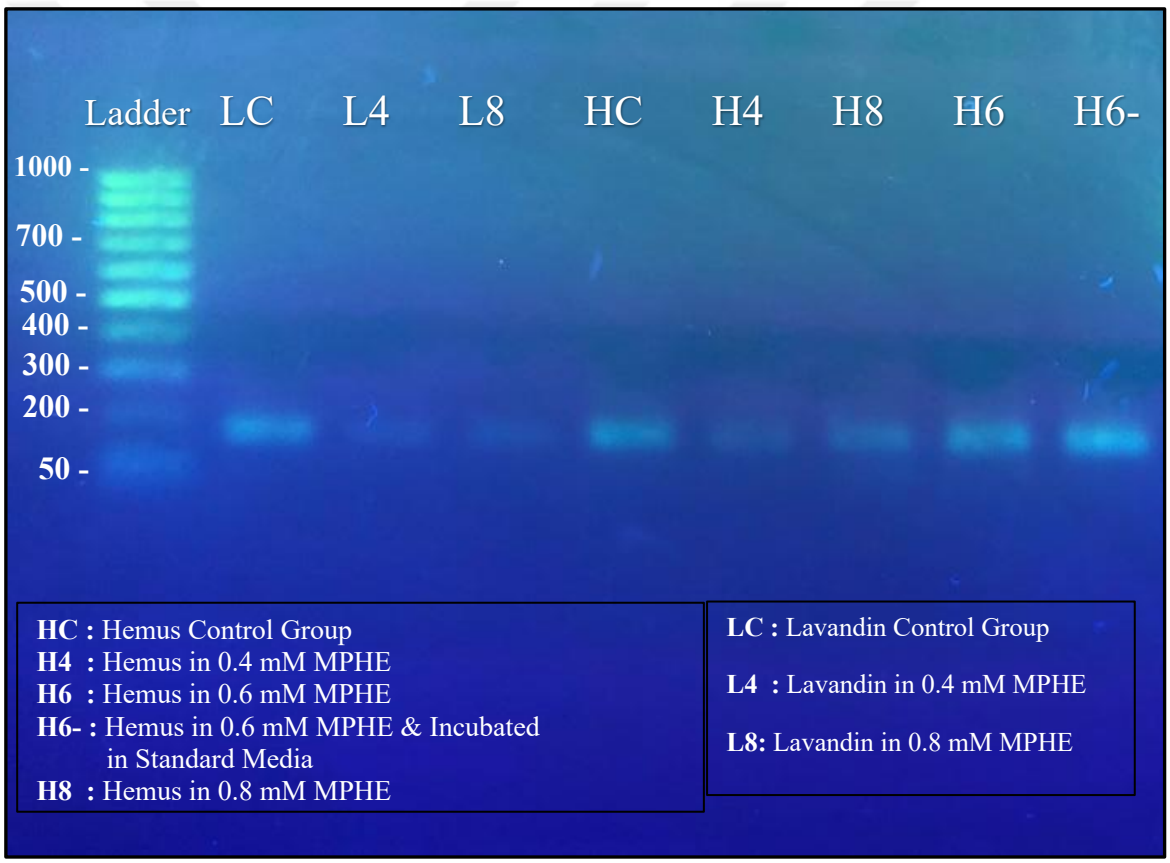


Figure 4.14. PCR amplification of *LaAT1* gene in different treatments of Lavender samples, visualized with 1.5 per cent agarose gel and with 50 bp DNA Ladder.

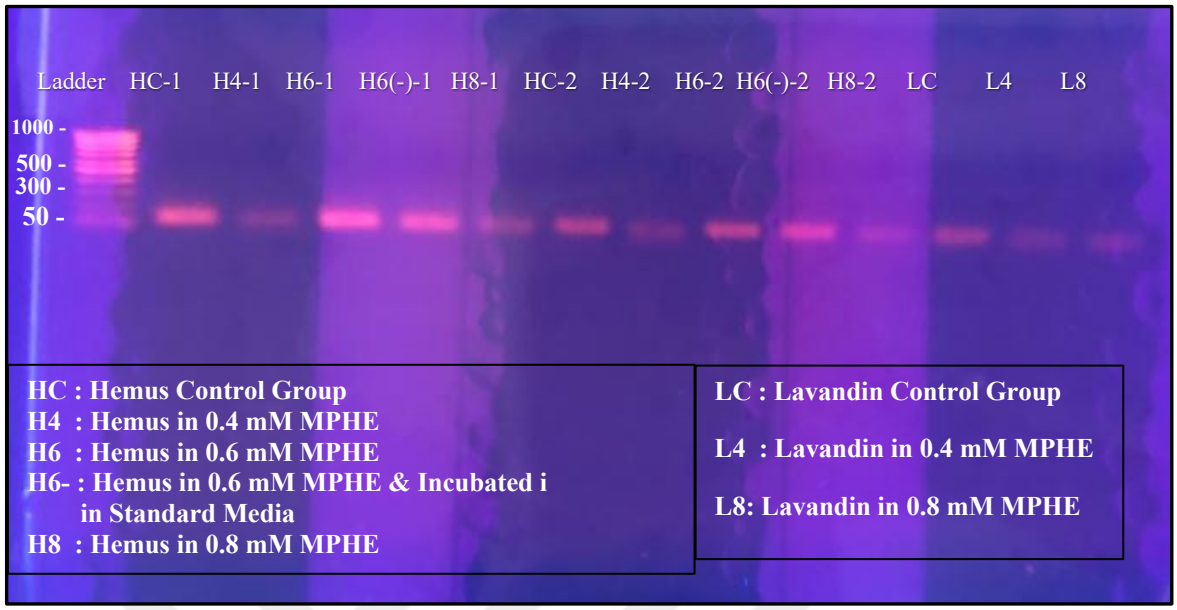


Figure 4.15. PCR amplification of β-actin housekeeping gene in different treatments of Lavender samples, visualized with 1.5 per cent agarose gel and with 50 bp DNA Ladder.

4.5.2. Analysis of qPCR Reaction

To our knowledge, the analysis of *LaAT1* gene expression after treating lavender suspension cells with MPHE is the first study in the literature. qPCR reaction for all *LaAT1* and β-actin genes were carried out by quantitative PCR (qPCR) (Appendix D). Compared to control group of Hemus species, the *LaAT1* gene expression level was higher in H6 and H6- groups (Figure 4.16). While H6 group represents the group of 1 g Hemus suspension cells incubated in P6 media, H6- represents the cells which incubated in the standard media for another 14 days after incubating in P6 media. *LaAT1* gene for H6 and H6- groups was expressed more than approximately two times compared the control groups, which they have 2,42 and 1,81 fold change value, respectively. The results of *LaAT1* gene expression level and RA production level were consistent for these groups. Thus, the cells acquired the resistant to MPHE, and that caused to increase the expression level of *LaAT1* gene so the production of RA increased compared to control group. There were also differences in the expression level between these groups. The expression level of H6 compared to H6- was 1,33 ddCT value (Appendix D). In other words, the expression level of H6 was higher than H6-. This result is also consistent with RA production result. Furthermore, H4 and H8 groups represent 0.5

g Hemus suspension cells incubated in P4 and P8 media, respectively. The expression level for H8 was expressed 1,47 times more than the control group while the expression of *LaAT1* gene was suppressed in H4 group, which had 0,49 fold change. Since P8 media have more MPHE, it may affect more the RA biosynthesis pathway [78] so the expression level in H8 was higher than in H4. On the other hand, the expression level in H6 and H6- was still higher than H8. The cell density in the culture affects the production of SMs due to cell signaling [118] so it might affect the expression level of *LaAT1*.

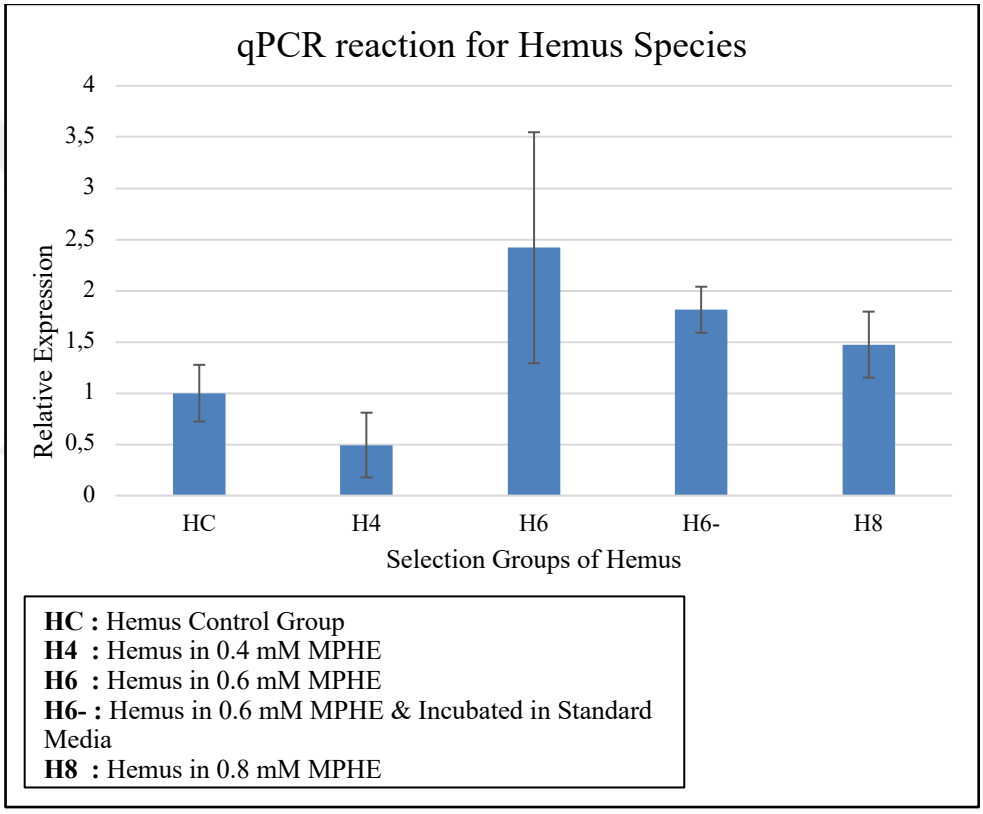


Figure 4.16. The expression levels of *LaAT1* gene for the selection groups of Hemus species.

The expression levels of *LaAT1* gene for Lavandin species was suppressed in L4 and L8 groups which represented 0.5 g Lavandin suspension cells incubated in P4 and P8 media, respectively (Figure 4.17). This result was expected because 0.5 g cell inoculation size was not enough for the selection experiment. The growth inhibition decreases the cell density which is needed for producing SMs [117,118]. Thus, the expression levels of *LaAT1* gene for L4 and L8 groups was lower than that of control group.

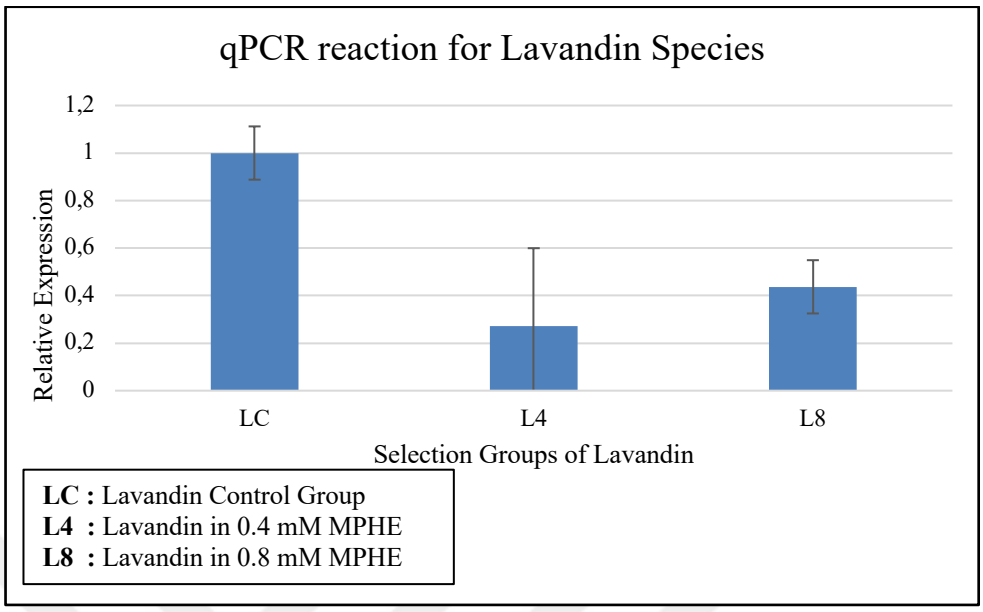


Figure 4.17. The expression levels of *LaAT1* gene for the selection groups of Lavandin species.

5. CONCLUSION AND FUTURE PROSPECTS

Two growth curves were constructed from *L. angustifolia* Hemus and *L. x intermedia* var. Super A cell suspension cultures. One was initiated with 0.5 g cells for both lavender species, while the other was started with only Hemus and 1 g cells. As a result, at the end of day 14, both growth curves which started with 0.5 g and 1 g cells resulted in the same amount of cell. In addition, RA was extracted and Lavandin produced higher RA than Hemus did. The intense RA production was on 6^{th} day for both species.

The standard curve was constructed for RA analysis. RA was dissolved in two different solvents (water and 70 percent EtOH) and measured with a spectrophotometer at 327 nm. Based on the results, there is no significant difference between the solubility of water and ethanol.

To optimize RA extraction, different volumes (2 ml and 5 ml) of extraction solvent and two different solvents (water and 70 percent EtOH) for resuspension were used. According to this, there was no significant difference between the volume of the solvent. On the other hand, there was a significant difference between the resuspension solvents. The RA concentration was higher when it was resuspended in water. However, since the absorbance of the blank of ethanol was higher, that causes a low absorbance of the sample which was resuspended in ethanol, and it tuns out be like water dissolves RA better.

For the selection of high producing RA cell lines, the lavender suspension cells were incubated in the agar-solidified medium and liquid medium containing MPHE. As a result, the liquid medium was more appropriate for the cells to be exposed to selective agents uniformly.

1 g Hemus cells were incubated in the medium containing MPHE and subcultured biweekly for 42 days in order to obtain higher RA producing cell lines. The cells in the PHE medium produced significantly higher RA (0.399 g/L) than the control cells did (0.204 g/L). Some cells were also incubated in the standard medium for another 14 days to determine the stable RA production in the absence of MPHE. The cells produced higher RA than the control cells did but less than the first selection group of cells.

From Lavandin and Hemus suspension cultures, 0.5 g cells were incubated in two different MPHE concentration media for 42 days and subcultured biweekly. As a conclusion, Hemus cells were light brown in color and could produce fewer fresh cells than Lavandin cells did. Additionally, the inoculation size might not be enough for the selection experiment since MPHE causes growth inhibition.

The expression level of *LaAT1* gene of the resistant cells for Hemus and Lavandin was analyzed by using qPCR. The resistant Hemus cells which are H6 and H6- had higher *LaAT1* gene expression level, which shows that these cells were resistant to MPHE and produced more RA than that of control group. On the other hand, *LaAT1* gene was suppressed in the selection group H4 of Hemus and L4 and L8 selection groups of Lavandin. The reason for the suppression might be the low cell density in the culture, which affects the SMs production.

In future studies, the RA extract can also be analyzed by using high performance liquid chromatography (HPLC) in order to determine the appropriate solvent for resuspending RA based on the exact amount of RA extracted.

The selection strategy should be performed with many replicates as possible (at least 15 replicates) for obtaining more accurate results. Due to the slow growth of the Lavender callus cells, new cell lines should be initiated regularly more often than routine tissue culture experiments. In addition, though the viability test could be performed via FDA, Evan's Blue assay can also be used for the quantity measurement of the dead cells.

APPENDIX A:

Table A.1. The amount of cells used for the growth curve construction and media pH data of Hemus and Lavandin starting with 500 mg cells.

Hemus							
Days	**Replicate**	**Amount of cells (mg)**	**Average Amount of cells**	**Standard Deviation**	**pH**	**Average pH**	**Standard Deviation**
0	0.1	500	500.0	-	5.50	5.50	-
	-	-			-		
2	2.1	530	574.5	62.93	5.19	5.19	0.000
	2.2	619			5.19		
4	4.1	703	777.0	104.65	5.01	4.89	0.170
	4.2	851			4.77		
6	6.1	1,238	1,066.0	243.24	5.54	5.42	0.170
	6.2	894			5.30		
8	8.1	1,099	1,166.0	94.75	5.82	5.83	0.014
	8.2	1,233			5.84		
10	10.1	769	802.5	47.38	5.22	5.42	0.276
	10.2	836			5.61		
12	12.1	1,310	1,138.0	243.24	9.32	7.73	2.256
	12.2	966			6.13		
14	14.1	1,438	1,469.5	44.55	5.30	5.04	0.368
	14.2	1,501			4.78		

LAVANDIN							
Days	**Replicate**	**Amount of cells (mg)**	**Average Amount of cells**	**Standard Deviation**	**pH**	**Average pH**	**Standard Deviation**
0	0.1	500.00	500.0	-	5.43	5.43	-
	-	-			-		
2	2.1	466	605.0	196.58	4.77	4.77	0.000
	2.2	744			4.77		
4	4.1	829	722.0	151.32	4.64	4.63	0.021
	4.2	615			4.61		
6	6.1	948	934.5	19.09	4.82	4.70	0.177
	6.2	921			4.57		
8	8.1	1121	1,140.5	-	4.93	4.94	0.014
	8.2	1160			4.95		
10	10.1	1271	1271	-	4.60	4.60	-
	10.2	-			-		
12	12.1	1359	1359	-	4.82	4.82	-
	12.2	-			-		
14	14.1	1618	1618	-	5.55	5.55	-
	14.2	-			-		

Table A.2. RA concentrations (g/L) of Hemus suspension cells during 14-day growth starting with 500 mg cells.

The days (with replication)	Absorbance at 327nm	R.A. Concentration (g/L)	Average	Standard Deviation
0	2.576	0.096	0.096	0.0000
2.1	4.174	0.152	0.143	0.0139
2.2	3.616	0.133		
4.1	5.934	0.214	0.229	0.0211
4.2	6.784	0.244		
6.1	8.920	0.319	0.245	0.1045
6.2	4.718	0.171		
8.1	5.402	0.196	0.199	0.0055
8.2	5.624	0.203		
10.1	5.364	0.194	0.196	0.0019
10.2	5.442	0.197		
12.1	9.048	0.324	0.304	0.0280
12.2	7.924	0.284		
14.1	6.676	0.240	0.220	0.0286
14.2	5.528	0.200		

Table A.3. RA concentrations (µM / g FW) of Hemus suspension cells during 14-day growth starting with 500 mg cells.

The days with replication	**R.A. Concentration µM / g FW**	**Average**	**Standard Deviation**
0	534	0,1	0,00
2.1	846	791,1	77,04
2.2	737		
4.1	1189	1272,3	117,36
4.2	1355		
6.1	1772	1362,1	580,18
6.2	952		
8.1	1085	1107,1	30,65
8.2	1129		
10.1	1078	1085,6	10,77
10.2	1093		
12.1	1797	1687,6	155,19
12.2	1578		
14.1	1334	1222,1	158,51
14.2	1110		

Table A.4. RA concentrations of Lavandin suspension cells during 14-day growth starting with 500 mg cells.

The days (with replication)	Absorbance at 327nm	R.A. Concentration (g/L)	Average	Standard Deviation
0.1	6.824	0.246	0.246	-
2.1	7.032	0.253	0.235	0.0259
2.2	5.992	0.216		
4.1	5.52	0.200	0.201	0.0019
4.2	5.598	0.202		
6.1	10.012	0.358	0.463	0.1491
6.2	16.008	0.569		
8.1	8.316	0.298	0.304	0.0089
8.2	8.672	0.311		
10.1	11.988	0.427	0.427	-
12.1	9.912	0.354	0.354	-
14.1	8.424	0.302	0.302	-

Table A.5. RA concentrations (µM / g FW) of Lavandin suspension cells during 14-day growth starting with 500 mg cells.

The days with replication	RA Concentration µM / g FW	Average	Standard Deviation
0,1	1363	1363,1	0
2.1	1404	1302,1	143,59
2.2	1201		
4.1	1108	1116,1	10,77

4.2	1124		
6.1	1986	2571,0	827,88
6.2	3156		
8.1	1654	1689,2	49,15
8.2	1724		
10.1	2371	2371,4	0
12.1	1966	1966,0	0
14.1	1675	1675,5	0

Table A.6. The amount of cells used for the growth curve construction and media pH data of Hemus starting with 1000 mg cells.

Days	Replicate	Amount of cells (mg)	Average Amount of cells	Standard Deviation	pH	Average pH	Standard Deviation
0	1.1	1,000	1,000	0	5.75	5.75	0.000
	1.2	1,000			5.75		
2	2.1	515	574	84.439	5.97	5.60	0.530
	2.2	633			5.22		
4	4.1	882	828	76.368	5.35	5.03	0.460
	4.2	774			4.70		
6	6.1	715	903	265.165	5.67	5.14	0.750
	6.2	1,090			4.61		
8	8.1	876	914	53.033	4.47	4.60	0.184
	8.2	951			4.73		
10	10.1	1,407	1,288	168.291	4.88	4.77	0.156
	10.2	1,169			4.66		
12	12.1	1,396	1,339	81.317	6.01	5.60	0.587
	12.2	1,281			5.18		

14	14.1	1,343	1,490	207.889	9.65	8.54	1.577
	14.2	1,637			7.42		

Table A.7. RA concentrations of Hemus suspension cells during 14-day growth starting with 1 g cells.

The days with replication	Absorbance at 327nm	R.A. Concentration (g/L)	Average RA Concentration	Standard Deviation
0.1	5.990	0.216	0.239	0.0329
0.2	7.312	0.263		
2.1	2.990	0.111	0.073	0.0537
2.2	0.833	0.035		
4.1	3.254	0.120	0.125	0.0072
4.2	3.544	0.130		
6.1	11.7	0.417	0.325	0.1305
6.2	6.452	0.232		
8.1	7.124	0.256	0.235	0.0296
8.2	5.936	0.214		
10.1	7.788	0.279	0.238	0.0589
10.2	5.422	0.196		
12.1	4.538	0.165	0.186	0.0298
12.2	5.738	0.207		
14.1	5.928	0.214	0.181	0.0467
14.2	4.050	0.148		

Table A.8. RA concentrations of Hemus suspension cells during 14-day growth starting with 1 g cells.

The days with replication	**RA Concentration (µM) / g FW cells**	**Average RA Concentration**	**Standard Deviation**
0.1	1200	1329,3	182,53
0.2	1458		
2.1	614	403,8	297,82
2.2	193		
4.1	666	694,3	40,04
4.2	723		
6.1	2315	1802,8	724,60
6.2	1290		
8.1	1422	1305,7	164,03
8.2	1190		
10.1	1551	1320,3	326,68
10.2	1089		
12.1	917	1033,9	165,69
12.2	1151		
14.1	1188	1004,8	259,30
14.2	821		

APPENDIX B:

Table B.1. The absorbance value of blank solutions for water and EtOH.

Rosmarinic acid concentration (μM)	Replication	Solvent			
		Water		70% EtOH	
		Absorbance at 327nm	Average Absorbance	Absorbance at 327nm	Average Absorbance
10	#1	1.127	1.146	0.944	0.971
	#2	1.16		0.985	
	#3	1.15		0.983	
25	#1	1.089	1.164	0.97	1.004
	#2	1.146		1.008	
	#3	1.258		1.033	
50	#1	1.054	1.411	1.048	1.083
	#2	1.228		1.091	
	#3	1.95		1.111	
75	#1	1.037	1.061	1.27	1.212
	#2	1.085		1.188	
	#3	1.062		1.178	
100	#1	1.016	1.048	1.304	1.297
	#2	1.077		1.319	

	#3	1.05		1.268	
200	#1	0.917	0.938	1.758	1.749
	#2	0.961		1.799	
	#3	0.935		1.689	
300	#1	0.848	0.879	1.968	2.215
	#2	0.86		2.284	
	#3	0.93		2.393	
400	#1	0.774	0.788	2.385	2.456
	#2	0.81		2.524	
	#3	0.779		2.458	
500	#1	0.702	0.726	2.699	2.674
	#2	0.759		2.668	
	#3	0.716		2.654	

Table B.2. The absorbance values of different concentrations of standard RA dissolved in water.

Rosmarinic acid concentration (μM)	Replication	Blank	Absorbance at 327nm	Average Absorbance	Standard Deviation
10	#1	1.145	0.156	0.139	0.0247
	#2	1.035	0.121		
25	#1	1.004	0.154	0.185	0.0438
	#2	1.026	0.216		

50	#1	1.064	0.333	0.363	0.0424
	#2	1.033	0.393		
75	#1	1.120	0.466	0.504	0.0537
	#2	1.040	0.542		
100	#1	1.296	0.641	0.659	0.0255
	#2	1.046	0.677		
200	#1	0.989	1.899	2.021	0.1725
	#2	0.917	2.143		
300	#1	1.125	2.729	2.811	0.1160
	#2	0.843	2.893		
400	#1	0.803	3.624	3.822	0.2800
	#2	0.782	4.020		
500	#1	0.700	5.348	5.089	0.3663
	#2	0.697	4.830		

Table B.3. The absorbance values of different concentrations of standard RA dissolved in 70 percent EtOH.

Rosmarinic acid concentration (μM)	Replication	Blank	Absorbance at 327nm	Average Absorbance	Standard Deviation
10	#1	0.944	0.600	0.292	0.2732
	#2	0.985	0.197		
	#3	0.983	0.079		
25	#1	0.97	0.374	0.309	0.0578
	#2	1.008	0.288		
	#3	1.033	0.264		
50	#1	1.048	0.596	0.612	0.0178

	#2	1.091	0.608		
	#3	1.111	0.631		
	#1	1.270	0.818		
	#2	1.188	0.868		
75	#3	1.178	0.874	0.853	0.0307
	#1	1.304	1.125		
	#2	1.319	0.946		
100	#3	1.268	0.928	1.000	0.1089
	#1	1.758	3.676		
	#2	1.799	2.417		
200	#3	1.689	2.271	2.788	0.7725
	#1	1.968	3.098		
	#2	2.284	2.890		
300	#3	2.393	2.907	2.965	0.1155
	#1	2.385	3.715		
	#2	2.524	5.660		
400	#3	2.458	4.482	4.619	0.9797
	#1	2.699	5.177		
	#2	2.668	5.792		
500	#3	2.654	5.258	5.409	0.3342

APPENDIX C:

Table C.1. The concentration of RA (μM/g FW) production from Hemus suspension cells in the selection and control groups.

Groups	Flasks	RA Concentration (μM /g FW)	Average	Standard Deviation
Selection	#1S	1339	2212,1	681,14
	#2S	2253		
	#4S	3003		
	#5S	2253		
	#7S	1679	1556,4	503,89
	#8S	1003		
	#9S	1988		
Control	#1C	1429	1132,7	428,52
	#2C	1482		
	#3C	1068		
	#5C	552		

APPENDIX D:

Table D.1. The dCT, ddCT and fold change values of *LaAT1* gene expression of Hemus and Lavandin species compared to their control groups.

Groups	dCT Value	ddCT Value	Fold Change
HC	1,81	0	1
H4	2,83	1,02	0,49
H6	0,54	-1,27	2,42
H6-	0,95	-0,86	1,81
H8	1,25	-0,56	1,47
LC	2,26	1	1
L4	4,13	1,87	0,27
L8	3,45	1,19	0,44

Table D.2. The dCT, ddCT and fold change values of *LaAT1* gene expression of H6 group, compared to H6- group.

Groups	dCT Value	ddCT Value	Fold Change
H6-	0,95	0,00	1
H6	0,54	-0,42	1,33

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