Production of Phenolic Diterpenes in Tissue Culture of *Rosmarinus officinalis* L.

By

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ABSTRACT

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Rosemary (*Rosmarinus officinalis* L.) is an herb native to the Mediterranean that has been used to prevent the oxidation of fats and oils in foods and cosmetics. The leaves of rosemary are a powerful source of natural antioxidants; the potent antioxidant properties of its extracts are mainly due to the phenolic diterpenes, carnosol and carnosic acid. At present, field-grown plants are the sole source of carnosic acid and carnosol. Rosemary is relatively easy to grow in many parts of world; however the variation of seasonal stresses such as temperature, solar irradiation, and rainfall can significantly alter the level and quality of phenolic diterpenes. An alternative method with more consistent results to obtain phenolic diterpenes from rosemary is a biotechnological approach, specifically, plant tissue culture. In this research, different culture types (callus cultures, suspension cultures, and shoots from *in vitro* propagated plants) of rosemary were established, and the effects of temperature, light intensity, and UV radiation for enhancing the synthesis of carnosic acid, carnosol, and related phenolic diterpenes in these cultures were evaluated. MS medium supplemented with either 10 mg/L indole-3-acetic acid (IAA), 4 mg/L benzylaminopurine (BAP) and 1 mg/L 6-furfurylaminopurine (kinetin) or 0.05 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.5 mg/L BAP at 18 and 25°C with 67.5 to 81.0

µmol $m^{-2}s^{-1}$ proved to be the best medium and conditions for developing and sustaining rosemary callus cultures.

Shoots from *in vitro* regenerated plants of rosemary accumulated various amounts of carnosic acid and carnosol, which were also present in callus culture, but the yields of these compounds were considerably lower than in regenerated shoots or in dried field grown leaves. Only carnosol and rosmanol, not carnosic acid, were detected in cell aggregates in suspension cultures, whereas spent medium contained only rosmanol. Exposure of the callus cultures to several stress conditions by increasing temperatures and light intensities, and UV radiation treatments enhanced the level of the phenolic diterpenes, carnosic acid and carnosol.

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CHAPTER ONE

Introduction

For centuries, people have used plants as valuable sources of chemicals including drugs, flavors, pigments, and agrochemicals (Balandrin and Klocke, 1988). Antioxidants (natural products), especially from food plants, may be important for humans in order to combat reactive oxygen species that are thought to contribute to the onset of a variety of diseases. Many of the "modern" drugs sold today are of natural plant origin, and natural products continue to play an important role in drug discovery programs of the pharmaceutical industry (Baker *et al*. 1995; Cordell 1995).

During the twentieth century, the demand for large quantities of pharmaceuticals motivated researchers to chemically synthesize them rather than extracting them from plants. Many of these natural products, however, have very complex structures and cost limitations on the chemical synthesis of them make their synthesis uneconomical. Plants are, therefore, still a major source of natural products used as drugs (Raskin *et al*. 2002).

In 1982, E. J. Staba presented a summary of at least 30 products known to accumulate in plant cultures at the same or higher concentrations as these of the plant. However, only two products, shikonins and ginseng, are so far being manufactured commercially. The most recently discovered economically important pharmaceuticals derived from plants are taxol from *Taxus brevifolia*, vincristine and vinblastine from *Catharanthus roseus* L., and camptothecin from *Camptotheca acuminate* that have dramatically improved the effectiveness of chemotheraphy against some cancers (Raskin *et al*. 2002), and artemisinin from *Artemisia annua* that has been shown to be effective in

treating cancer and that possessed antiparasitic effect against *Plasmodium falciparum* (Jung *et al*. 2004; Sriram *et al*. 2004; Singh and Lai 2005).

The use of a plant tissue culture system to produce desirable medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenes, steroids, phenolics, flavonoids, and amino acids (Vanisree *et al*. 2004). Taxol, for example, is one of the most promising anticancer drugs, which was originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*). Taxol supply from this original source cannot meet the increasing demand for clinical use because of the scarcity and slow growth of *Taxus brevifolia*, and the costly synthetic process (Cragg *et al*. 1993). Tissue cultures of various *Taxus* species, therefore, have been studied extensively as an alternative route for production of taxol and other useful taxane compounds (Christen *et al*. 1991; Ellis *et al*. 1996; Srinivasan *et al*. 1996; Phisalaphong and Linden 1999; Wu and Lin 2003; Kim *et al*. 2004; Naill and Roberts 2005).

The advantages of a tissue culture system over the conventional cultivation of whole plants are: (1) useful compounds can be produced under controlled conditions by manipulating temperature, light intensity, and photoperiod, independent of climatic requirements or soil conditions; (2) cultured cells would be free of microbes and insects; (3) the cells of any plant could be manipulated to yield their specific metabolites; (4) cell suspension culture systems can be used to study the biosynthetic pathways of desired compounds just as well as in plants; (5) Compounds of interest are extractable from such cultures. DiCosmo and Misawa (1995) stated that manipulating the biosynthetic activity of cultured cells in order to obtain high yields of compounds of interest suitable for

commercial exploitation can be achieved by optimizing the cultural conditions, selecting high-producing strains, utilizing transformation methods, and immobilization techniques.

Rosmarinus officinalis L. (Lamiaceae) is widely cultivated all over the world as an ornamental and aromatic plant. Today, the interest in its cultivation is strongly rising, due to the antioxidant properties exerted by the phenolic diterpenes extracted from its leaves. Rosemary (**Figure 1.1**) is an herb native to the Mediterranean region of Europe (Kowalchik and Hylton 1987). It is an evergreen perennial shrub with needle-like leaves and pale blue flowers. Rosemary species are cultivated in the Mediterranean countries (Spain, Morocco and Tunisia), the United States, and the United Kingdom (Leung and Foster 1996), but *Rosmarinus officinalis* is the only species available in the United States. There are over 40 varieties of which Arp, Hill's Hardy that are frost tolerant, and *prostrates* which is not.

Rosemary is propagated by seeds, rooting cuttings, layering, and division of roots. Because of the poor germination of its seeds and the genetic diversity of seedlings, the most common method is rooting cuttings of non-flowering branches in early summer. Rosemary plants grow best in well-drained, fairly dry soil with a pH of 5-8 in full sun. Commercial production is from both cultivated and wild plants, and best in zones 9 and 8 (10° to 30° F) with yields as high as 1,500 pounds per acre of dry herb per year in a proper environment. Fields of rosemary are usually harvested once, or at most, twice each year, depending upon the geographical area and whether the harvest is for the plants *per se* or for the essential oil.

The name rosemary comes from the Latin *ros maris* or “dew of the sea”. Traditionally, the Romans used rosemary to strengthen memory functions; scholars wore garlands of rosemary during examinations to improve memory and concentration (Bown 1995 and Grieve 1979). Moreover, in Shakespeare’s Hamlet, Ophelia says “There’s rosemary, that’s for remembrance, pray you love, remember” (Rowse 1978). To encourage couples to remember their wedding vows, it was entwined into the bride’s head wreath, tied with ribbons and was presented to wedding guests. It has also become a funeral flower because it symbolizes the memories of loved ones. In ancient Egypt it was placed in tombs. These are some of the reasons the people have regarded rosemary as the herb of remembrance.

Since ancient times, people have taken essential oil internally and applied it externally against all manner of ills, including upset stomach, nervous disorders, poor appetite, headaches, baldness, arthritis, pain, strains, and bruises (Simon *et al*. 1984; Pitman 1994; Al-Sereiti *et al*. 1999).

Figure 1.1: *Rosmarinus officinalis* L. in a research bed at the Lehman College Greenhouse CUNY, Bronx, New York (2007).

Young shoots, leaves, and flowers of rosemary have been used fresh, dried, or extracted in solvents for various purposes. Rosemary contains many compounds including tannins, flavonoids, caffeic acid derivatives such as rosmarinic acid (**Figure 1.2 C**), phenolic diterpenes such as carnosic acid and carnosol (**Figure 1.2 A** and **B**), and essential oils. Essential oils from rosemary are widely used in cosmetics, perfumes, food, and by the pharmaceutical industry as a fragrant component of soaps, creams, deodorants, hair tonics, and shampoos (Hethelyi *et al*. 1989; Panizzi *et al.* 1993; Flamini *et al*. 2002).

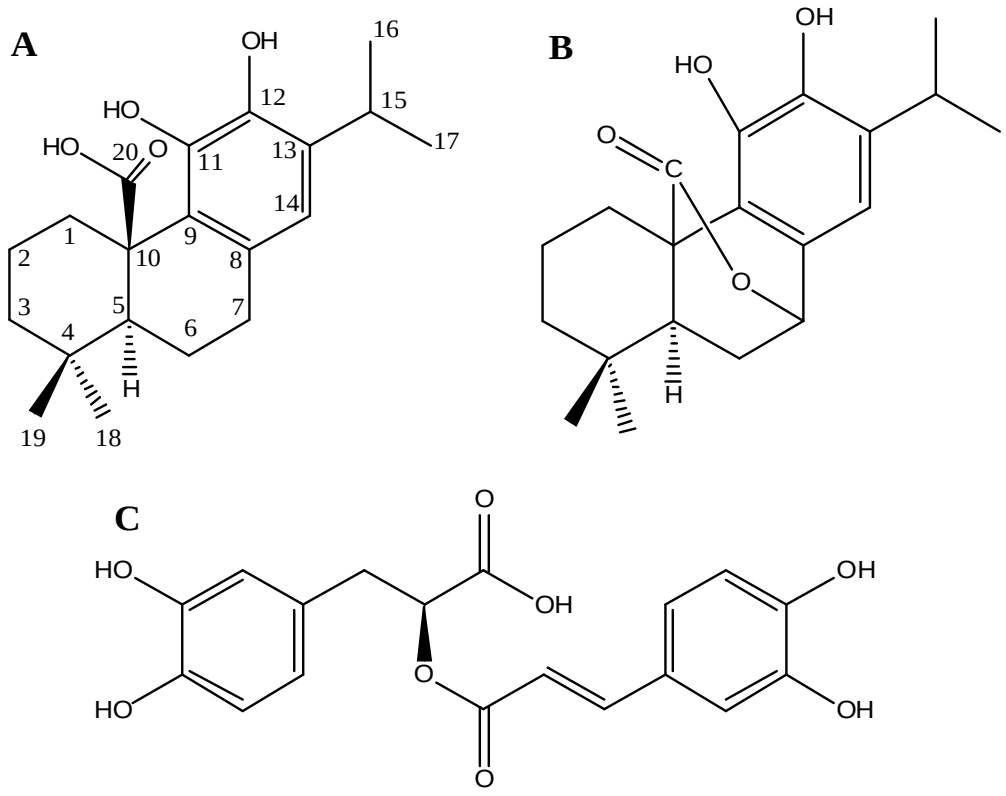


Figure 1.2: Chemical structures of carnosic acid (A), carnosol (B), and rosmarinic acid (C).

The antioxidant properties of rosemary have been known for centuries. Rosemary extracts are increasingly used commercially to preserve foods, in cosmetic products, to replace synthetic preservatives such as butylated hydroxyanisole (BHA) and butylated

hydroxytoluene (BHT) or to allow the use of synthetics in reduced quantities (Richheimer *et al*. 1999; Martinez-Tome *et al*. 2001; Etter 2004). The synthetic preservatives have come to be regarded as less safe than natural antioxidants; the phenolic compounds isolated from rosemary are more powerful than BHA and BHT and do not have the cytotoxic and carcinogenetic risks of synthetic antioxidants (Richheimer *et al*. 1999; Ito *et al*. 1983).

In addition to their use as food preservatives, rosemary antioxidants are also important for human health. The compounds mainly responsible for these antioxidant properties are the phenolic diterpenes, such as carnosic acid and carnosol (**Figure 1.2 A** and **B**), which have the highest antioxidant activity in rosemary extracts (Aruoma *et al*. 1992; Haraguchi *et al*. 1995; Frankel *et al*. 1996). Carnosic acid (**Figure 1.2 A**) is a phenolic abietane diterpene and is primarily responsible for the antioxidant activity of rosemary extracts (Schwarz and Ternes 1992b; Aeschbach and Philippossian 1993; Richheimer *et al*. 1999; Thorsen and Hildebrandt 2003). Carnosic acid has two hydroxyl groups in the molecule in the *ortho* positions at C_{11} and C_{12}, which show high antioxidant activity. Inatani *et al*. (1983) and Masuda *et al*. (2001) suggested that the hydroxyl group at C_{11} is more important for its antioxidant activity. This group is first oxidized by donating a hydrogen to a radical species such as a lipid peroxyl radical, and then forms a carnosate radical. Wenkert *et al*. (1965) was the first to discover carnosic acid in a plant extract of *Rosmarinus officinalis*, and this phenolic diterpene has been isolated from rosemary by other researchers (Aeschbach and Philippossian 1993; Paris *et al*. 1993; Richheimer *et al*. 1999) as well as from several species of *Salvia* (Gonzalez *et al*. 1987; Luis *et al*. 1992 and 1994; Aeschbach and Philippossian 1993). Other known phenolic

diterpenes isolated from rosemary are carnosol, epirosmanol, epiisorosmanol, isorosmanol, 7-methylepirosmanol, 12-methoxycarnosic acid, rosmadial, rosmanol (**Figure 1.2** and **1.3**). These compounds appear to arise from an oxidative degradation and rearrangement cascade, which start with carnosic acid (Wenkert *et al*. 1965; Aeschbach and Philippossian 1993; Gonzalez *et al*. 1992) (**Figure 1.4**). Richheimer *et al*. (1999) and Miura *et al*. (2002) reported that these carnosic acid degradents possess less antioxidant activity than carnosic acid, but they showed remarkably strong activity that may contribute to the overall antioxidant activity of rosemary extracts.

In addition to its use as an antioxidant in foods, nutritional supplements, and cosmetic products, carnosic acid, carnosol, and related diterpenes from rosemary have been shown to have great medicinal importance to human health as antioxidants, anti-inflammatories, antitumorigenics, and chemoprotectives. Many researchers report pharmacological studies of rosemary: Paris *et al*. (1993) showed that carnosic acid could act to inhibit HIV-1 protease activity and HIV-1 virus replication; Aruoma *et al*. (1996) also found that purified carnosol inhibited HIV infection at a low, non-cytotoxic concentration. Topical application of rosemary extracts, carnosol or ursolic acid to mouse skin inhibited the covalent binding of benzo (a) pyrene to epidermal DNA, tumor initiation by 7, 12-dimethylbenz [a] anthracen (DMBA), TPA-induced tumor promotion, ornithine decarboxylase activity and inflammation (Huang *et al*. 1994; Offord *et al*. 1997; Singletary *et al*. 1991and 1996). Chan *et al*. (1995) and Lo *et al*. (2002) reported that carnosol suppresses nitric oxide production and inducible nitric oxide synthase gene expression by inhibiting NF-kappaB activation in macrophages, which provides possible mechanisms for its anti-inflammatory and chemoprotective action. Moreover, carnosol

prevents adenoma formation in the C57BL/6J/Min/+ (Min/+) mouse by inhibiting β-catenin tyrosine phosphorylation (Moran *et al*. 2005). Moran's group found that dietary administration of 0.1% carnosol decreased intestinal tumor multiplicity by 45%.

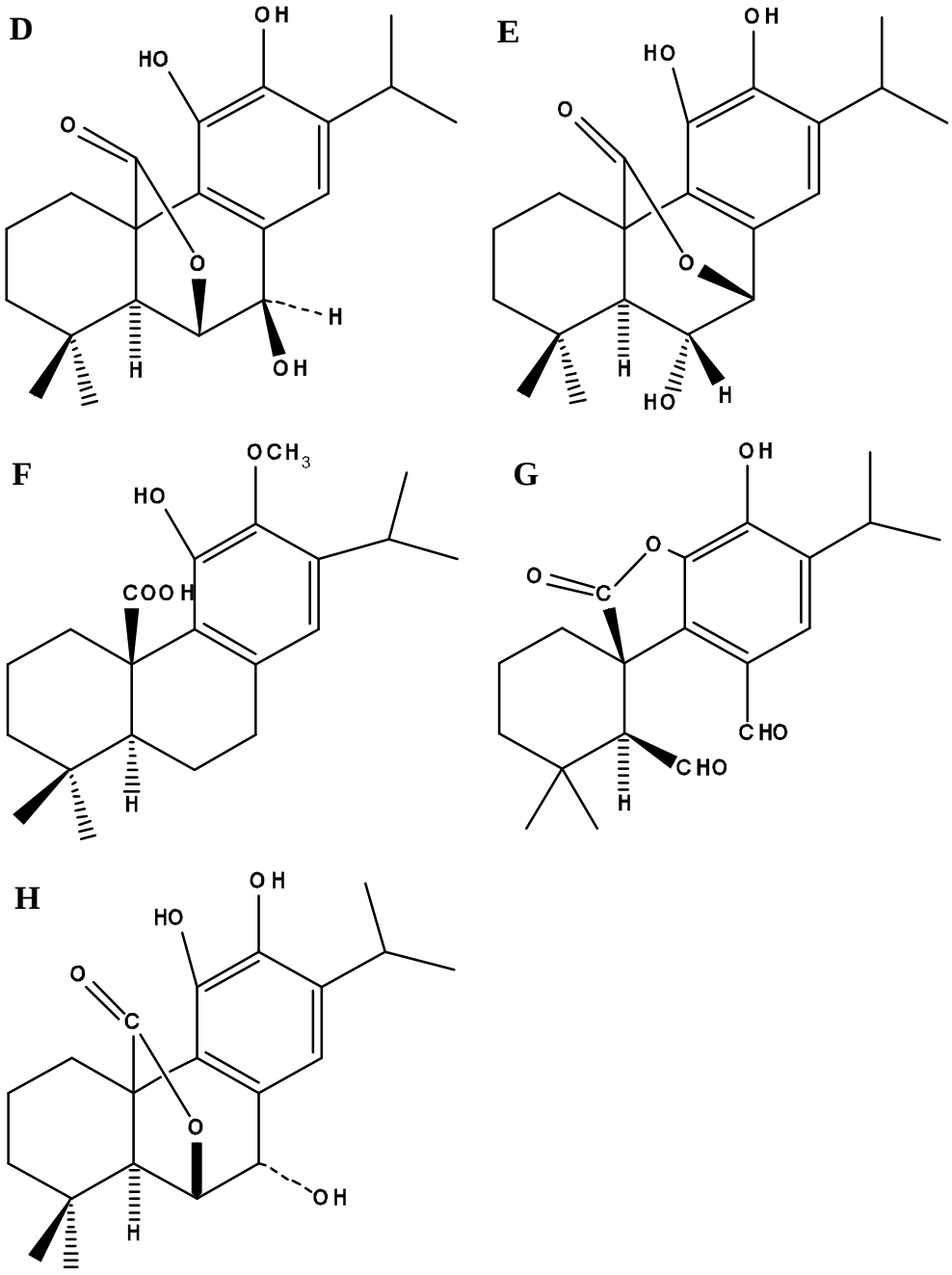


Figure 1.3: Chemical structures of related diterpenes from rosemary: epirosmanol (D), isorosmanol (E), 12-methoxycarnosic acid (F), rosmadial (G), and rosmanol (H).

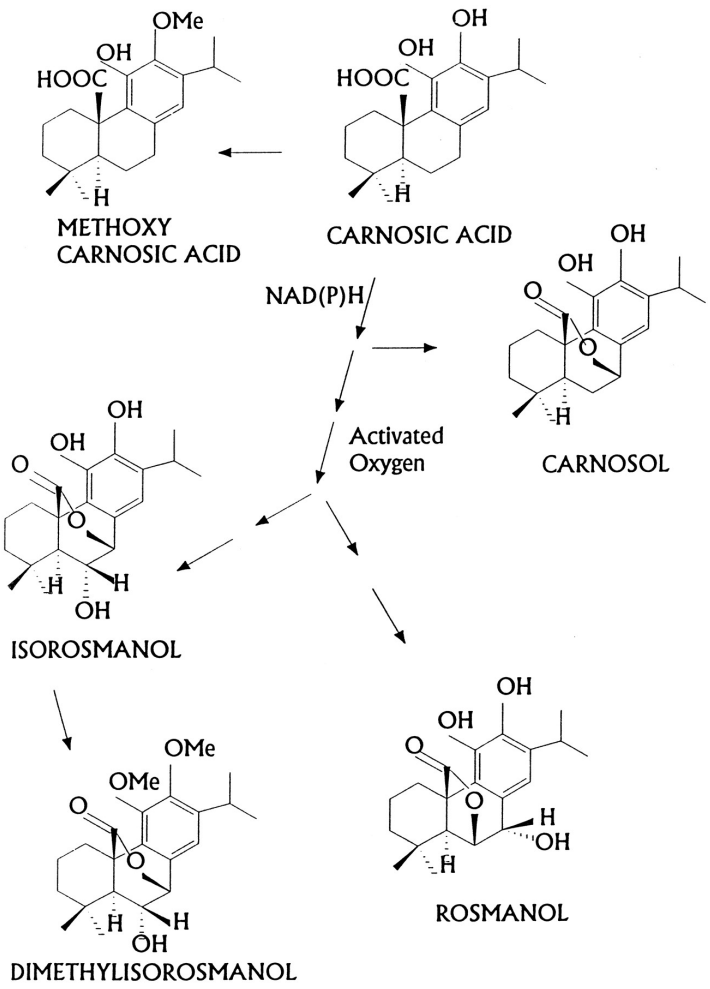


Figure 1.4: Formation of the highly oxidized diterpenes from carnosic acid in *Rosmarinus officinalis* plants (adapted from Luis *et al*., 1994).

Dorrie *et al*. (2001) found that carnosol may be useful as a novel chemotherapeutic agent against cancers that expresses high levels of Bcl-2, a protective protein, which is effective in inducing apoptosis in *B*-lineage leukemia cells. Steiner *et al*. (2001) showed that carnosic acid inhibits proliferation of human leukemic cells without induction of apoptotic or necrotic cell death.

In 2003, Kosaka and Yokoi reported that carnosic acid and carnosol promote markedly enhanced synthesis of nerve growth factor (NGF), a factor vital for the growth and functional maintenance of nerve tissues, in T98G human glioblastoma cells. This

research may lead to development of anti-AD (Alzheimer disease) agents in future.

The many economic and potentially important therapeutic uses for carnosic acid, carnosol, and related compounds derived from the leaves of rosemary suggest that there will be ever increasing demand for rosemary extracts.

All terpenes including the abietane diterpene carnosic acid, that are produced from rosemary plants, are derived from the common branched five-carbon unit isopentenyl diphosphate (IPP) and its isomer dimethylally diphosphate (DMAPP) (Lichtenthaler 1999). The terpene biosynthesis in plants is a complicated process associated with two independent pathways (**Figure 1.5**): The mevalonic acid (MVA) pathway is located in the cytosol, and the methylerythritol phosphate (MEP) pathway, which proceeds via 1-deoxyxylulose-5-phosphate, is localized in the plastids. Diterpenes (C20, including gibberellins and the phytyl tail of tocopherols and chlorophylls) are synthesized via the MEP pathway in plastids. Munné-Bosch and Alegre (2001) demonstrated that carnosic acid was found only in chloroplasts, and the immediate precursor (copalyl pyrophosphate) of the abietane diterpene, carnosic acid, has also been found in these organelles, thus suggesting that carnosic acid is synthesized in the chloroplast.

In all photosynthetic organisms, carotenoids, zeaxanthin and tocopherols serve an important photoprotective role, either by dissipating excess excitation energy as heat or by scavenging reactive oxygen species and suppressing lipid peroxidation.

Phenolic diterpenes from rosemary plants are also known to inhibit lipid peroxidation and superoxide generation in isolated chloroplasts and microsomes, protecting biological membranes against chemically induced oxidative stresses

(Haraguchi *et al*. 1995; Haraguchi *et al*. 1998). Furthermore, carnosic acid cooperates with tocopherols and carotenoids in the protection of photosynthetic membranes against oxidative damage and can provide plants with an additional mechanism for photoprotection under adverse climate conditions (Munné-Bosch and Alegre 2003; Lichtenthaler 2007).

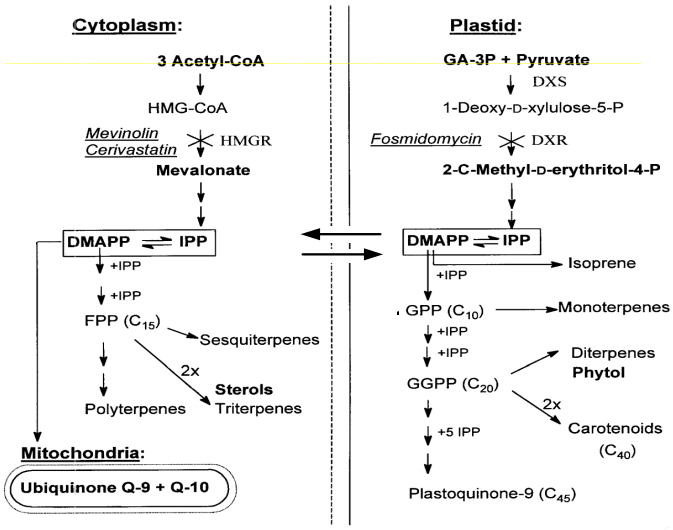


Figure 1.5: Compartmentation of the two pathways of IPP and isoprenoid biosynthesis within plant cells between cytosol (mevalonic acid pathway) and plastid (methylerythritol phosphate pathway). Adapted from Lichtenthaler, 1999.

Carnosic acid is the major phenolic diterpene present in the leaves of rosemary plants and is known as a potent antioxidant. It has been demonstrated that carnosic acid is oxidized into other phenolic diterpenes such as carnosol, rosmanol, isorosmanol, and dimethyl isorosmanol (**Figure 1.2** and **1.3**) by enzymatic degradation and the participation of active oxygen in rosemary plants (Masuda *et al*. 2001; Munné-Bosch *et al*. 1999, 2001) (**Figure 1.4**). Since carnosic acid is highly sensitive to oxidation the concentration of carnosic acid and related diterpenes in rosemary extracts varies depending not only on geographical origin and seasonal/environmental growth

conditions, but also on harvest time and extraction methods. Obtaining consistent and consistently high yields of carnosic acid is rather difficult because of the influences of these factors. Richheimer's group (1996) reported that variability of carnosic acid content in dried and wild rosemary was based on country of origin, with Albanian rosemary having the lowest at 1.7%, and with Moroccan rosemary the highest at 3.8%. Hidalgo *et al*. (1998) stated that greater variability of phenolic diterpene content was found among populations of wild rosemary than among populations of cultivated rosemary. This finding is supported by the results of Wellwood *et al*. (2004) "the variability of foliage carnosic acid concentrations within accessions was small enough for selection by accession to have a significant effect on yields of the compounds per plant."

Extraction processes, dried or fresh plant material, the polarity of the solvent, the extraction temperature also determine the level of phenolic diterpenes in extracts. The concentration of carnosic acid in rosemary leaves ranges from a low of 0.35 % (Wenkert *et al*. 1965) to a high of 8% (Troncoso *et al*. 2005) (**Table 1.1**). Carnosic acid has to be extracted in an apolar solvent, but it is not stable in methanol especially in the presence of active carbon when it degrades into carnosol and more highly oxidized compounds (Aeschbach and Philippossian 1993; Luis *et al*. 1994; Schwarz *et al*. 1992b, Del Bono *et al*. 2003). Raising temperature and light tend to promote the degradation of carnosic acid in extraction processes (Cuvelier *et al*. 1994; Ibanez *et al*. 1999).

Table 1.1: The concentration of carnosic acid (expressed as percentage) from leaves of *Rosmarinus officinalis* as reported in the literature.

Carnosic acid (%)	Extraction Method	Leaf Source	Reference
1.5-2.5	Various	Unspecified	Aeschbach and Philippossian 1993
1.2-0.4	Liquid solvent	Cultivated	Del Bono *et al*. 2003
3.1-5.3	SC-CO_2	Wild	Hidalgo *et al*. 1998
6.9-7.4	SC-CO_2	Cultivated	Hidalgo *et al*. 1998
1.10	Liquid solvent	Cultivated	Munné-Bosch *et al*. 2001
1.7-3.5	Liquid solvent	Unspecified	Richheimer *et al*. 1996
2-8	Liquid solvent	Cultivated	Troncoso *et al*. 2005

Some researchers demonstrated that the distribution of phenolic diterpenes changes during growth and vegetative development of rosemary plants; the highest accumulation of carnosic acid was observed in the early growth stage of leaves (Hidalgo *et al*. 1998; Munné-Bosch *et al*. 2000; Munné-Bosch *et al*. 2001; Del Bano *et al*. 2003). Del Bano's group (2003) also found that the level of carnosic acid decreased with increase of leaf age because young leaves are more sensitive to growing conditions. In addition, the combination of high solar radiation and drought causes the oxidation of carnosic acid which leads to an enhanced formation of highly oxidized diterpenes such as carnosol, rosmanol, isorosmanol (Okamura *et al*. 1994; Munné-Bosch *et al*. 1999b; Munné-Bosch *et al*. 2000; Munné-Bosch and Alegre 2000). Increase in the highly oxidized phenolic diterpenes reflects the fact that carnosic acid performs a photoprotective role against oxidative damage.

Munné-Bosch *et al*. (2000) stated that carnosic acid and carnosol concentrations were relatively high from October to February and low from May until the end of August. This is similar to the finding of Luis *et al*. (2007), where cold stressed plants (temperature between 6 to12 °C) of two accessions showed increases in carnosic acid concentrations.

On the other hand, Yesil-Celiktas *et al*. (2007) reported that the highest level of carnosic acid was observed in September. Hidalgo *et al*. (1998) stated that carnosic acid content increased gradually during the spring, peaked in the summer months and then dropped abruptly in September. The high variability in the content of carnosic acid from plant extracts (**Table 1.1**) depends on several factors such as the quality of the original plant, its geographic origin, the seasonal/environmental conditions, the harvesting times, and extraction method.

Until recently, carnosic acid and carnosol were not available commercially. Now the price range of carnosic acid sold in the market is $2.80 to $ 6.10 per milligram (A.G. Scientific, Inc. in CA; Alexis Biochemicals in Switzerland; Sigma-Aldrich in U.S.A.). Surprisingly, the price of carnosol is more variable than carnosic acid; Alexis Biochemicals and A.G. Scientific, Inc. sell carnosol for $37.00 to $60.00 per milligram.

There is an increasing interest in the use of rosemary antioxidants as food preservatives and in therapeutic and pharmaceutical applications. Rosemary is relatively easy to grow in many parts of world, particularly those areas with a Mediterranean climate, but the level of phenolic diterpenes in this plant is strongly influenced by seasonal conditions. Researchers have been examining methods to produce uniform and enhanced levels of carnosic acid. Biotechnological methods based on *in vitro* culture of tissues and plants are often used to detect and study the formation of secondary metabolites in plant material, independent of environmental factors (Murashige and Skoog 1962; Staba 1982). Moreover, the advantage of growing cultures in a laboratory is that culture conditions can be manipulated to enhance yield of desired compounds, which

could be an important alternative to the unstable and the unreliable supply of compounds from field grown plants.

So far the study of the natural antioxidants in tissue culture of rosemary has been mainly focused on the production of monoterpenes and rosmarinic acid (**Figure 1.2**). Webb *et al*. (1984) reported that regenerated shoots contained monoterpenes, and Tawfik (1992) stated that these compounds were also produced by rosemary callus. Rosmarinic acid was isolated from regenerated shoots and genetically transformed callus (Yang *et al*. 1997; Komall and Shetty 1998). Yesil-Celiktas *et al*. (2007) obtained rosmarinic acid from rosemary callus. Up to now, there are only a few researchers who have investigated the production of phenolic diterpenes in rosemary tissue culture including the yield of carnosic acid (Caruso *et al*. 2000; Nadosy 2002; Kuhlmann *et al*. 2006).

Nadosy (2002), in our lab, started the project to investigate the production of potent antioxidant phenolic diterpenes such as carnosic acid and carnosol from rosemary tissue cultures. She found that green calli and shoots regenerated from green callus of rosemary contained carnosic acid and carnosol, and that levels of illumination and temperature significantly affected the growth of rosemary in tissue culture. She was the first researcher to report not only the presence of phenolic diterpenes such as carnosic acid, carnosol, rosmanol, and 12-methoxy carnosic acid, but also the yield of these compounds in rosemary tissue grown *in vitro*. The carnosic acid content of regenerated shoots from green callus and green callus grown MS medium supplemented with IAA and BAP were 0.5 % and less than 0.7 to 1.1% respectively, whereas the presence of carnosic acid from callus grown on B5 medium and liquid medium was not detected. In

addition, she developed a method to extract these compounds from rosemary tissue grown *in vitro*.

The objective of the work for this dissertation was to expand on the research initiated by Nadosy (2002) with a focus on significantly increasing the amounts of carnosic acid and carnosol in the tissue cultures of *R. officinalis* in her seminal study. This study consists of two parts: (1) to investigate and compare the effects of media formulation, phytohormones, and different environmental factors (temperature, light intensity, duration, quality including UV light) on growth and development of rosemary callus and suspension cultures and (2) to extract and investigate the concentration of phenolic diterpenes in those tissues.

CHAPTER TWO

Effects of different physical factors on the yield of rosemary callus in *in vitro* cultures

SUMMARY

Single node stem segments of *Rosmarinus officinalis* were cultured on Murashige and Skoog (MS) medium supplemented with 30 g/L of sucrose, 45 g/L Gellan, and different combinations of auxin and cytokinin. MS medium supplemented with 10 mg/L indole-3-acetic acid (IAA), 4 mg/L benzylaminopurine (BAP) and 1 mg/L 6-furfurylaminopurine (kinetin) produced both green callus and shoots while media containing 0.05 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.5 mg/L BAP proved to be the best media for the production of green callus without shoot initiation. The growth and survival of rosemary callus when routinely subcultured was strongly affected by temperature and light intensity. Cultures grown at 18 and 25°C with 67.5 to 81.0 µmol $m^{-2}s^{-1}$ of light proved to be most suitable for sustaining rosemary callus cultures for extended periods**.** When these cultures were exposed to UV light for 2, 4, 6, or 8 hours at room temperature, the calli changed their chemical and morphological characteristics, without altering their increase in biomass over comparable incubation periods.

Suspension cultures were established by transferring callus to MS liquid medium of the same composition as used for cultures solidified with Gellan**.**

INTRODUCTION

At present, field-grown rosemary plants are the sole source of the most effective antioxidant compounds, carnosol and carnosic acid. Although rosemary is relatively easy to grow in many parts of world, particularly those areas with a Mediterranean climate, the variety of seasonal stresses such as variations in temperature, illumination, and rainfall ultimately determines the level and quality of secondary metabolite production. In fact, several authors have demonstrated that the distribution of phenolic diterpenes changes during growth and vegetative development of rosemary leaves (Hidalgo *et al*. 1998; Munné-Bosch *et al*. 2000; Munné-Bosch *et al*. 2001; Del Bano *et al*. 2003). The high variability in the content of carnosic acid from plant extracts depends not only on the environmental conditions and the harvesting date, but also on the characteristics of the plant, its geographic origin, and extraction method (Cuvelier *et al*. 1996).

Tissue culture techniques are often used to detect and study the formation of secondary metabolites in plant material (Murashige and Skoog 1962; Misawa 1996; Trigiano, R.N. and D.J. Gray 1996). The advantage of growing tissue cultures in the laboratory is that environmental conditions such as temperature, light, and humidity and also experimental conditions such as nutrient composition, phytohormones, and pH can be manipulated to improve growth rates of cells and/or yield of desirable products. Misra and Chaturvedi (1987 and 1991) and Basile *et al*. (1993) demonstrated that an effective approach to improve the production of secondary metabolites as well as the initiation of callus cultures is to manipulate the physical and chemical factors such as the nutrient-hormone regime governing callus development. D.V.Basile *et al*. (1993) and M.R. Basile *et al*. (1994) have shown that tissue cultured *Artemisia annua* can produce a greater yield

of artemisinin per dry weight than field grown *Artemisia annua* and the cultures can be "harvested" 26 times a year instead only once annually. Evidently, under the right conditions, the production of secondary metabolites by tissue culture could be an important alternative to the potentially unstable and the certainly unpredictable supply of phytochemicals presently obtained from field grown plants.

Kaul and Staba (1967) were one of the first groups to isolate visnagin from the cell culture of *Ammi visnaga* in larger quantities than those from field grown. In 1982, E. J. Staba presented a summary of at least 30 products known to accumulate in plant cultures at the same or higher concentrations than those of the plant. However, only two products, shikonins and ginseng, are so far being manufactured commercially.

The research reported herein was undertaken to determine whether culture conditions could be developed that would induce cultured rosemary tissue to produce carnosol and/or carnosic acid in quantities comparable to, or exceeding, those obtainable from field-grown plants.

Some groups used tissues cultures to investigate the effects of hormones on the production of monoterpenes or phenolic acids in regenerated shoots from rosemary tissue cultures and rosemary callus (Jain *et al*. 1991; Tawfik *et al*. 1992; Webb *et al*. 1984;Yesil-Celiktas *et al*. 2007).

Only a few researchers have investigated the production of phenolic diterpenes such as carnosic acid and carnosol using tissue-cultured rosemary (Caruso *et al*. 2000; Nadosy 2002; Kuhlmann *et al*. 2006). Experimental procedures, such as the manipulation of temperature, light quality and quantity (Nadosy, 2002), the addition of specific elicitors (DiCosmo *et al*. 1985; Wang *et al*. 2001), and the adjustments of the pH

level (Caruso *et al*. 2000) have been found effective in influencing the production of secondary metabolites. Light seems especially important for the production of diterpenes by rosemary tissues. Caruso *et al* (2000) and Nadosy (2002) reported that carnosic acid was produced only in green callus of *Rosmarinus officinalis*, as distinct from non-green callus.

The production of phenolic diterpenes, such as carnosic acid and carnosol are not restricted to *Rosmarinus officinalis*. They are also present in several species of *Salvia* (Lamiaceae) (Gonzalez *et al*. 1987, 1991, and 1992; Luis *et al*. 1992 and 1994; Aeschbach and Philippossian 1993). For instance, carnosic acid and carnosol were isolated from 7-day-old plantlets developed from axillary buds of *Salvia carnarienesis* (Luis *et al*. 1992). Santos-Gomes *et al*. (2002) demonstrated the production of phenolic diterpenes by *in vitro* shoots, calli and cell suspensions (Santos-Gomes *et al*. 2003), of sage (*Salvia officinalis* L.). It is now well established that phenolic diterpenes can be synthesized by sage callus and cell suspension cultures.

Experiments to increase the levels of phenolic diterpenes by tissue cultured *R. officinalis* have been limited. The research reported here was under taken to build on what has been achieved so far. Therefore, experiments were designed to evaluate the effects of media formulation, phytohormones, and different environmental factors (e.g. temperature, light intensity, duration, quality including UV light) on growth, development and phenolic diterpene production of rosemary callus and suspension cultured tissues.

MATERIALS AND METHODS

Plant material and sterilization

Rosemary plants (*Rosmarinus officinalis* L.) grown in the Lehman College Research Greenhouse and research beds were used in all experiments. Young shoot cuttings, measuring about 8 cm in length, were surface sterilized in 95 % ethanol for 30 sec, followed by immersion in 15 % (v/v) aqueous Clorox solution containing 0.1% (w/v) Alconox detergent for 15 min with occasional shaking, and rinsed 3 times for 1 minute in sterile-distilled water in a laminar flow hood. The stem segments were transferred to sterile petri dishes; single-node stem segments (5-6 mm) with the basal portion of the leaf blades attached were excised from the shoot cuttings. They were placed horizontally, either two per jar (100 mL glass baby food jar), containing 30 mL media covered with clear Magenta ® caps, or five or seven per disposable plastic petri dish containing 20 mL media for 6-8 weeks. The cultures were sealed with Parafilm (**Figure 2.1**).

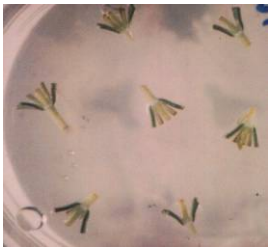


Figure 2.1: Rosemary (*Rosmarinus officinalis*) stem segments inoculated on solid medium in a Petri dish.

Establishment of *in vitro* callus cultures

Callus cultures were initiated and maintained on either Murashige and Skoog (MS) (Murashige and Skoog 1962) or Gamborg's B5 basal (B5) (Gamborg *et al*. 1968) media (**Appendix A**) to which different combinations of auxin and cytokinin were added. The culture media were adjusted to pH 5.8 ± 0.01 before adding 0.45% w/v Gellan (Research Organics, Inc., Cleveland, OH) and poured into glass baby food jars and

autoclaved at 250 °C at 10-20 pounds pressure for 20 min or autoclaved first and poured into 90 X 90 mm disposable plastic petri dishes. The cultures were incubated at 20°, 25°, or 30 °C in the dark, 24 hour light, or 16 hour light provided by Coolwhite ® fluorescent lights. After 6-8 weeks of incubation, calli were cut into small pieces of about 5-8 mm and transferred to media of the same composition. Thereafter, they were subcultured every 2-4 weeks. Periodically calli were assayed for the phenolic diterpene content. Those that exhibited the highest concentrations of the compounds of interest were used to prepare the suspension cultures. A list of experimental conditions for rosemary callus cultures used for initiation of calli culture and production of phenolic diterpenes is in Appendix B.

The effect of light and temperature on phenotype, biomass accumulation and phenolic diterpene production

To investigate the effects of light and temperature on the biomass accumulation and phenolic diterpene production, cultures were established on MS medium as described above and maintained at photoperiods of 16 hours or in the dark for 24 hours under three temperature regimes (18, 25, and 30 °C) and at different light intensities. Illumination was supplied by Coolwhite ® fluorescent lights. Five replicate culture dishes were used for each treatment. Earlier studies indicated that only green callus tissue produced phenolic diterpenes. Phenotypes of calli and the production of phenolic diterpenoids were analyzed after 8 and 12 weeks. These experiments were undertaken to determine if the relation of callus color (phenotype) and phenolic diterpene production could be improved by changing light and temperature conditions relative to those obtained solely by manipulating nutrient-hormone conditions (Nadosy, 2002).

The effects of UV light on callus growth and development, and phenolic diterpene production

To investigate the effects of UV radiation on both callus characteristics and concentration of phenolic diterpenes, calli grown for 7 to 8 weeks on the MS medium supplemented with 1.5 mg/L BAP, 0.05 mg/L 2,4-D, and 3% wt/v sucrose in a petri dishes at a photoperiod of 16 hours and light intensity of 62.1 µmol $m^{-2}s^{-1}$ at 18 °C were used for this experiment. Cultures were divided into two groups. One group was exposed to UV light for 2, 4, 6, or 8 hours while the other was exposed only to Coolwhite ® fluorescent lights. For the UV experiments, covers of the Petri dishes were removed and each culture was exposed to UV-C (13.5 µmol $m^{-2}s^{-1}$) light in sterilized transfer hoods. After the UV treatment, the cultures were returned to the original environmental conditions. The calli from each treatment were harvested after 2, 4, and 6 week incubation periods and their fresh weight taken before being freeze-dried and analyzed for phenolic diterpene content.

Establishing rosemary suspension cultures

Rosemary suspension cultures were established by transferring 1 g callus, grown on MS medium supplemented with 0.05 mg/L of 2,4-D and 1.5 mg/L of BAP, to 50 mL liquid MS medium in 250 mL long neck culture flasks containing the same supplements as those of solid media. Culture flasks were placed on a gyratory shaker (New Brunswick model G-10) set for 100 rpm in the dark. After two weeks of incubation, five to six cultures were randomly selected and independently filtered through Whatman #5 paper. Thereafter, cultures were harvested at intervals of seven days. The weight of tissues harvested from each flask was determined before and after freeze-drying. The

freeze-dried tissue and the filtered suspension medium were extracted separately and assayed for phenolic diterpene production in order to establish a baseline for eventual "optimization of conditions" for the growth of calli and accumulation of the compounds of interest in the suspension cultures.

In order to test the continuous secretion of phenolic diterpenes in the suspension medium, another set of rosemary suspension cultures was established. The cultures were initiated with 1 g calli grown on MS medium supplemented with BAP, 2, 4-D and 30 g/L sucrose. The flasks were placed in the dark on a gyratory shaker set for 100 rpm. After two weeks of incubation, liquid medium from these cultures was withdrawn every two weeks, and replaced by fresh medium. Harvested liquid medium was extracted to determine the presence of carnosol and carnosic acid in suspension cultures.

Statistical analysis

Experiments were all repeated at least twice and the data were analyzed statistically. All results are given as means ± standard error (SE). Differences between variables were tested for significance by the student's *t*-test ($p < 0.05$).

RESULTS

Calli cultures initiation

Calli of *Rosmarinus officinalis* were induced from single-node stem segments on MS or B5 basal nutrient medium supplemented with several combinations of auxins and cytokinins (**Appendix A and B**) to establish the cultures which support relatively fast callus growth and contain relatively high concentrations of the compounds of interest.

Callus was initiated from nodal explants on both MS and B5 media supplemented with 1.5 mg/L BAP and 0.05 mg/L 2,4-D at 25°C. The explants on MS medium became swollen three days after inoculation and callus was formed during the second week. More than 90 % of nodal explants produced green and hard calli within 40 days after which the calli were routinely subcultured. By contrast, the explants on the B5 medium started swelling and producing callus only after three weeks from inoculation, and then only 50 to 60 % of explants formed green, hard callus. Furthermore, survival of the tissue deteriorated gradually through several subcultures. In short, biomass accumulation was significantly greater on MS medium than on B5 medium during the three-month incubation period (**Figure 2.2**). Based on these findings, MS basal nutrient medium was chosen for further experiments in an attempt to determine conditions that would result in significantly increasing phenolic diterpene production by tissue-cultured rosemary. It should be noted that Caruso *et al* (2000) and Nadosy (2002) had previously settled on MS medium for establishing rosemary *in vitro* cultures.

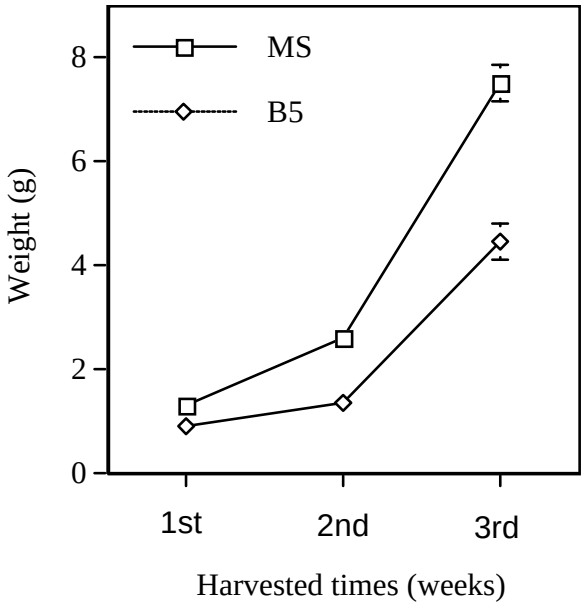


Figure 2.2: Time-course of growth (expressed as biomass fresh weight) of calli cultures of *Rosmarinus officinalis* on MS and B5 medium supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP at 25°C. Callus was harvested every 2 to 3 weeks. Points represent mean values of samples constituted by 6 cultures. Error bars represent mean ± SE.

Effects of hormone combinations on callus phenotype and growth rate

In the following experiments, the MS medium used in the forgoing experiments was modified by supplementing it with various combinations of auxins and cytokinins (**Appendix B**). It had been noted while conducting pilot experiments that calli developing on MS medium supplemented with the different concentrations of BAP or kinetin when combined with different concentrations of 2,4-D, NAA, or IAA displayed distinctly different phenotypes (**Table 2.1 and Figure 2.4**). It appeared that when 2,4-D, in any concentration ranging from 0.5 to 2.0 mg/L, was supplemented or combined with kinetin or BAP, in any concentration ranging from 0.025 to 1.0 mg/L , white-colored to cream-colored friable callus developed (**Figure 2.4: 3, 5L and 6L**). On the contrary, when the medium was supplemented with NAA, in any concentration ranging from 0.5 to

2.0 mg/L, when combined with Kinetin or BAP, in any concentration ranging from 0.025 to 1.0 mg/L, green, hard callus developed (**Figure 2.4: 5R and 6R**). Growth was slower on media supplemented with NAA compared to media supplemented with 2,4-D. More important, the tissue became necrotic after two to three months. Because of the unfavorable effects of NAA on the development of callus in these cultures, no further experiments were conducted using NAA.

The next experiments were conducted with MS nutrient medium supplemented with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin (the medium developed by Nadosy, 2002). As in Nadosy's experiments, the calli that developed were green, hard, and fast growing (**Figure 2.3**), with shoots forming within seventeen weeks (**Figure 2.4: 1, 8 and 9**). It was clear that the medium developed by Nadosy supported the development of green callus at both 18 and 25°C as well as higher biomass production than MS media with 0.05 mg/L 2,4-D and 1.5 mg/L BAP, 10 mg/L IAA, 4 mg/L BAP and 1mg/L kinetin, however, it had one disadvantage; it fostered the formation of shoots.

In parallel experiments in which the concentration of BAP was supplied at 1.5 mg/L or higher in combination with 2,4-D at 0.05 to 0.1 mg/L, there resulted satisfactorily growing green or greenish-white calli without development of shoots (**Figure 2.4: 2 and 4R**). Calli grown on a medium with 0.05 mg/L 2,4-D and 1.5 mg/L BAP maintained the above mentioned characteristics with routine subculture for longer intervals.

Table 2.1: Phenotypes of some of the callus cultures of *Rosmarinus officinalis* grown on MS medium supplemented with cytokinins, BAP or Kinetin combined with auxin, IAA, 2,4-D, or NAA.

Hormonal supplementation	Calli		shoots
	Color	Texture	
10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin	green	hard	++
0.05 mg/L 2,4-D + 1.5 mg/L BAP	green	very hard	−
0.5 mg/L 2,4-D + 0.075 mg/L Kinetin	white-brownish	friable	−
0.11 mg/L 2,4-D + 11.25 mg/L BAP	green-whitish	hard	+
0.5 mg/L 2,4-D + 0.025 mg/L BAP	white-brownish	friable	−
0.5 mg/L NAA + 0.025 mg/L BAP	green	hard	+
0.5 mg/L 2,4-D + 0.025 mg/L Kinetin	green	hard	+
0.5 mg/L NAA + 0.025 mg/L Kinetin	white-brownish	friable	−

Formation of shoots (+); Absence of shoots (-)

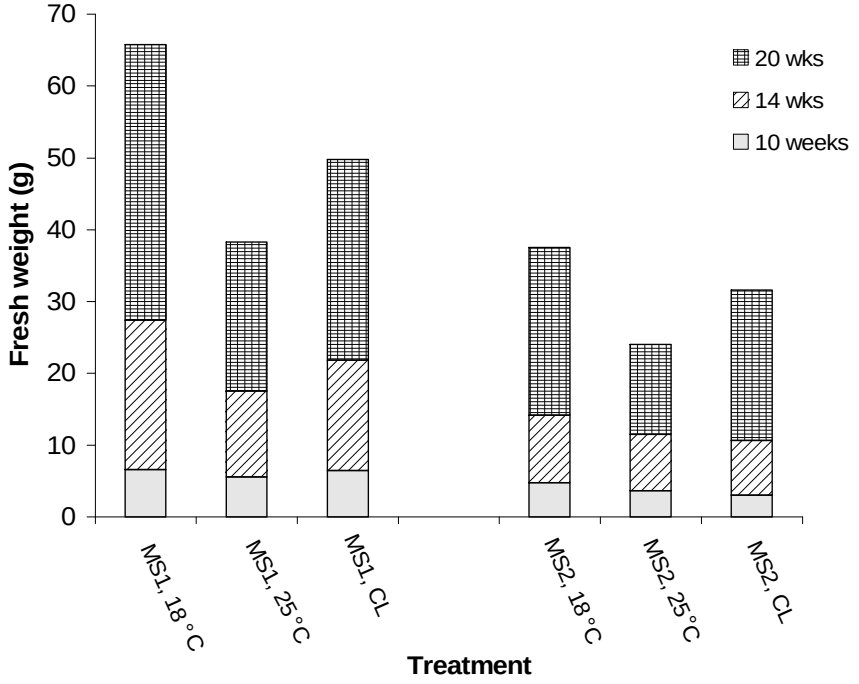


Figure 2.3: Rosemary cultures grown on MS medium supplemented with different combinations and concentrations of auxin and cytokinin under two temperature conditions (18 °C and 25 °C) with 16 hour photoperiod. Callus (expresses as biomass fresh weight) was harvested on 10, 14, and 20 weeks. MS1 (MS medium with 10 mg/L IAA, 4 mg/L BAP, 1 mg/L kinetin); MS2 (MS medium with0.05 mg/L 2,4-D and 1.5 mg/L BAP). CL: 24 hours light.

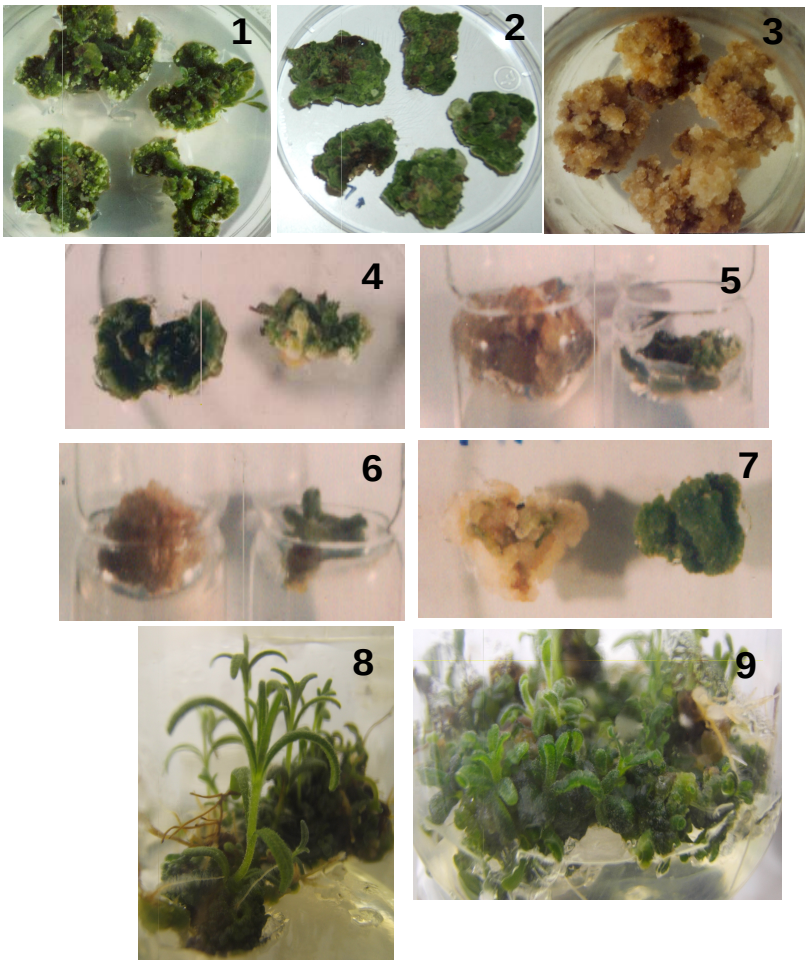


Figure 2.4: Callus proliferation from nodal section of *Rosmarinus officinalis* on MS basal nutrient medium with various combinations and concentrations of auxin and cytokinin. (L) left, (R) right.

1. 10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin (MS1)
2. 0.05 mg/L 2,4-D + 1.5 mg/L BAP (MS2)
3. 0.5 mg/L 2,4-D + 0.075 mg/L Kinetin (MS3)
4. (L) 10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin
   (R) 0.11 mg/L 2,4-D + 11.25 mg/L BAP
5. (L) 0.5 mg/L 2,4-D + 0.025 mg/L BAP
   (R) 0.5 mg/L NAA + 0.025 mg/L BAP
6. (L) 0.5 mg/L 2,4-D + 0.025 mg/L Kinetin
   (R) 0.5 mg/L NAA + 0.025 mg/L Kinetin
7. 10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin
   (L) in the dark, (R) in the light
8. Regenerated shoots with roots on MS medium supplemented with 10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin (average height was 2.0 cm, n=20), 24 weeks old
9. Emerging shoots growing out of the callus in MS with 10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin, 24 weeks old

Temperature and light effects on callus growth and survival

To investigate the effects of variations in light and temperature on the callus growth, survival, and phenolic diterpene accumulation, a series of experiments were conducted. The sets of cultures were incubated at 18°, 25°, or 30 °C, either in the dark or with 16 hours of light provided by Coolwhite ® fluorescent lamps.

In these experiments, callus was incubated on MS medium. The callus that developed under light was hard and very green at all three temperatures, but growth and fresh weight were best at 25°C. (**Figure 2.4: 7R and Figure 2.5**). Furthermore, calli that developed under light at 25°C and 18°C survived when subcultured over the following 6 months, but not those incubated at 30°C.

Callus grown in continuous darkness was hard and white to creamy. The increase of callus biomass was significantly correlated with an increase of temperature. Unfortunately, callus tissues maintained in continuous darkness could not be maintained beyond 22 weeks (**Figure 2.4: 7L and Figure 2.5**).

To further investigate the effect of light intensity on the callus growth and survival, callus cultures were initiated on the MS medium with the same hormonal combination tested above and incubated at 18°, 25°, or 30 °C under light intensities of 67.5 to 81.0 µmol $m^{-2}s^{-1}$ and 202.5 to 216.0 µmol $m^{-2}s^{-1}$.

During the first two weeks, callus growth was very slow. Nevertheless, cultures maintained at 25° and 30°C produced green calli faster than those maintained at18°C (**Figure 2.6**). At the end of 12 weeks however, cultures maintained under all the experimental conditions produced calli of comparable mass.

Unfortunately, shoots developed on calli incubated at 18 °C and 25 °C exposed to both 67.5 to 81.0 µmol $m^{-2}s^{-1}$ and 202.5 to 216.0 µmol $m^{-2}s^{-1}$, as well as those incubated at 30 °C under 67.5 to 81.0 µmol $m^{-2}s^{-1}$ (**Table 2.2**). Calli incubated at 30°C under both low and high light intensities were brownish and friable. Green to very green, hard calli were produced in cultures under 18 and 25°C (**Figure 2.7**). Increasing light intensity increased growth rate as well as development of friable callus, but necrosis on this callus occurred much faster than the tissue maintained under low light intensity (**Figure 2.7**). It was concluded from these experiments that callus incubated at 18 and 25°C under low light intensities were better suited for maintaining undifferentiated rosemary callus culture. These results corroborate previous studies by Nadosy (2002) where callus maintained under low light intensity e.g. 4.10 µmol $m^{-2}s^{-1}$ and a temperature of 25°C were the best conditions for undifferentiated callus production.

Table 2.2: Observation on 12 week old rosemary cultures incubated under two light intensities (67.5 to 81.0 µmol $m^{-2}s^{-1}$ and 202.5 to 216.0 µmol $m^{-2}s^{-1}$) and three temperatures (18, 25, and 30 °C): High: light intensity 202.5 to 216.0 µmol $m^{-2}s^{-1}$, Low: light intensity 67.5 to 81.0 µmol $m^{-2}s^{-1}$. Formation of shoots or roots from callus (+); Absence of shoots or roots (-).

Temperature	Light intensity	Location of callus	Texture	Color	Shoot	Root
18°C	High	Over-all	Hard	Green, partial necrosis	+	-
18°C	Low	Over-all	Hard	Very green	+	-
25°C	High	Over-all	Hard	Green and light green	+	+
25°C	Low	Over-all	Hard	Green	+	-
30°C	High	Over-all	Friable	Brown	-	-
30°C	Low	Over-all	Friable	Light green to green	+	-

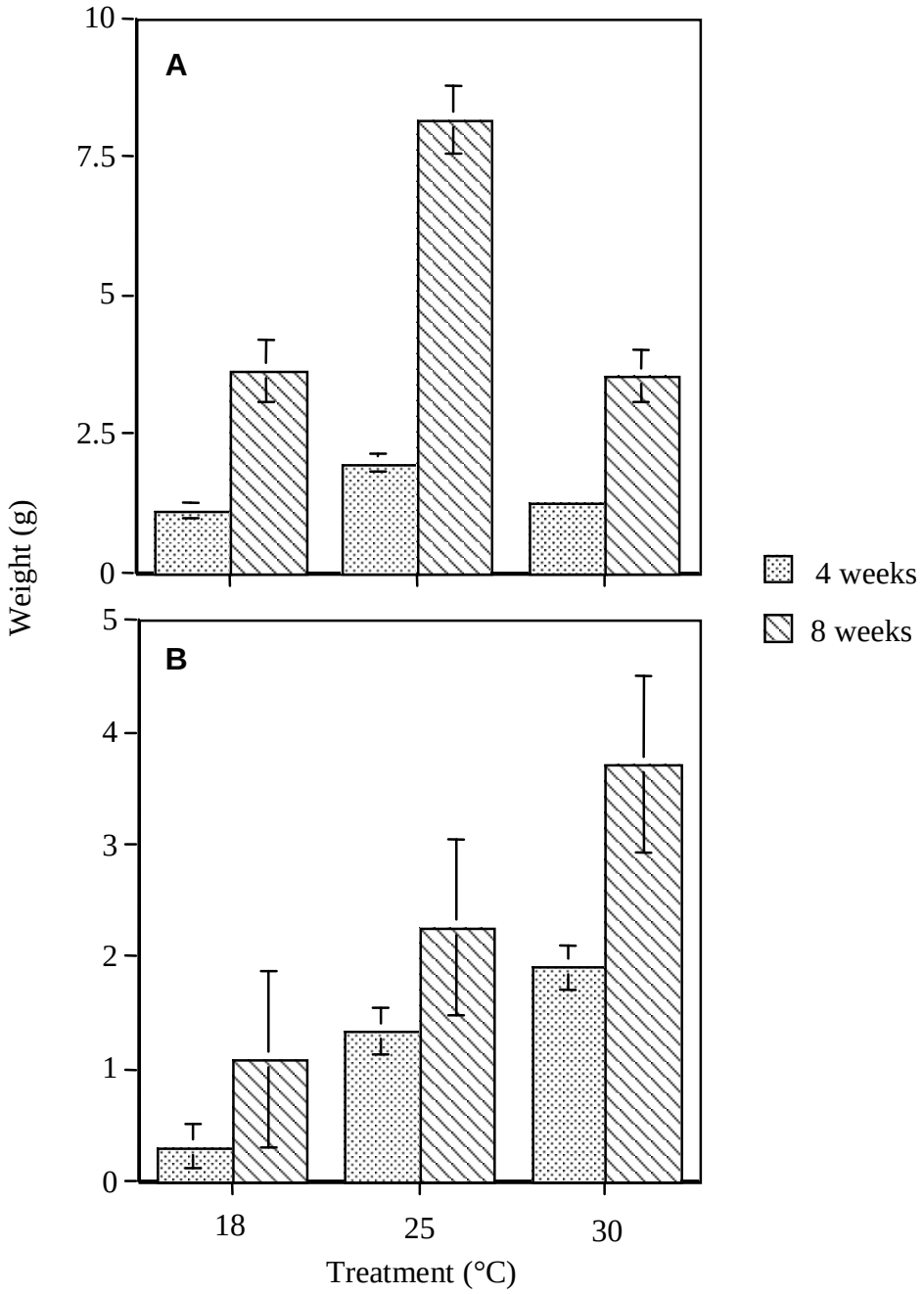


Figure 2.5: Rosemary cultures supplemented with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin grown under light (A) under 67.5 to 81.0 µmol $m^{-2}s^{-1}$ and dark (B) at 18, 25, and 30°C. Fresh weight (g) of callus (4 and 8 weeks old) was measured. Error bars represent mean ± SE.

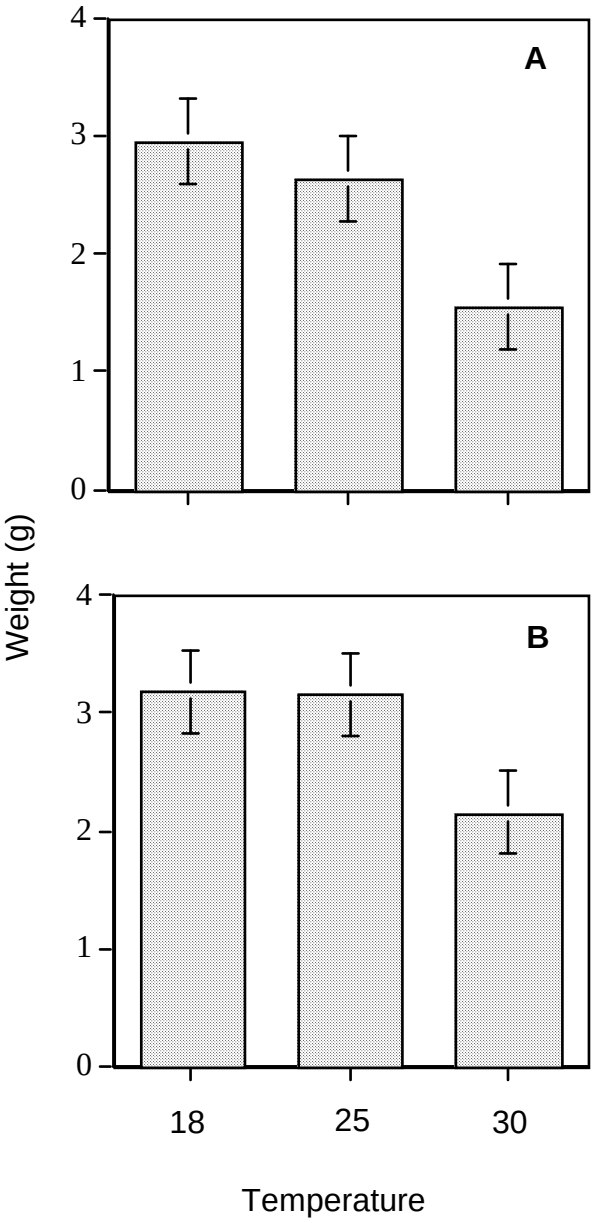


Figure 2.6: 6 week old rosemary callus (expressed as biomass fresh weight) grown under two light intensities (High and Low) and three temperatures (18, 25, and 30 °C). A: light intensity 202.5 to 216.0 µmol $m^{-2}s^{-1}$, B: light intensity 67.5 to 81.0 µmol $m^{-2}s^{-1}$. Error bars represent mean ± SE.

(1) (2) (3) (4) (5)

Figure 2.7: Callus grown under the different light and temperature regimens (12 week old). (1) 18 °C –H, (2) 18 °C –L, (3) 25 °C -H (4) 25 °C -L (5) 30 °C -L. H: 202.5 to 216.0 µmol $m^{-2}s^{-1}$ light intensity. L: 67.5 to 81.0 µmol $m^{-2}s^{-1}$. (30 °C – H is not available because of necrosis).

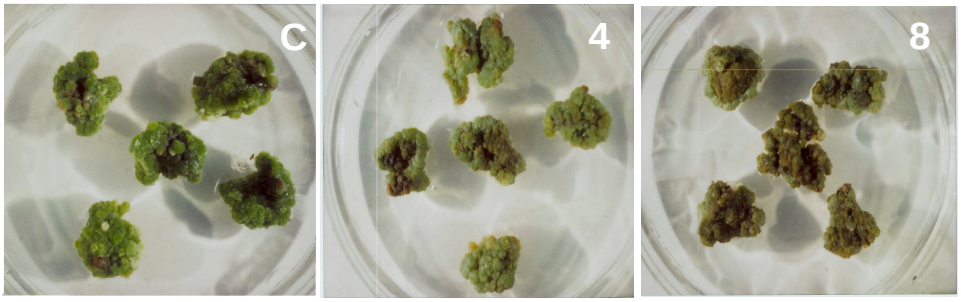


Figure 2.8: Callus treated with UV-C light. C: control (UV-untreated), 4: UV-treated for 4 hours, 8: UV-treated for 8 hours. These calli were photographed and harvested 2 weeks after the treatment.

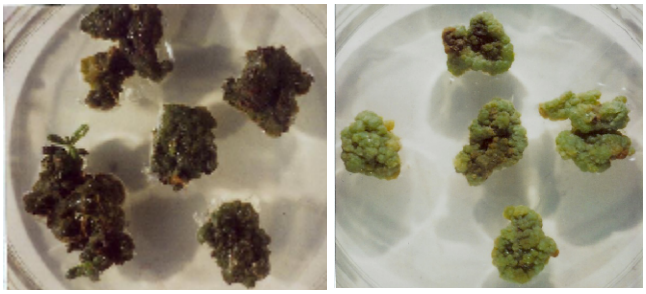


Figure 2.9: Callus grown on MS medium with 0.05 mg/L 2,4-D and 1.5 mg/L BAP (right) or 10 mg/L IAA, 4 mg/L BAP, 1 mg/L kinetin (left). Both cultures were treated with UV-C light for 4 hours. These calli were photographed and harvested 2 weeks after the treatment.

UV light experiments

Seven to eight week old green, hard calli maintained on MS medium supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP at 18°C and maintained under a 16 hour photoperiod were irradiated with UV light for 2, 4, 6, or 8 hours at room temperature. The cultures were returned to the original culture conditions and harvested after 2, 4 or 6 weeks. Exposing cultures to UV-C radiation changed their appearance. Two weeks after UV irradiation, the calli changed color from dark green to light brown; the degree of change depended on the duration of irradiation (**Figure 2.8**). In addition, the texture of the calli became more friable. However, there was no significant difference between the irradiated and (control) calli with respect to their fresh weight at 2, 4, or 6 weeks after the treatment (**Figure 2.10**). Although cultures irradiated with UV light continued growing for a while, by ten weeks after irradiation almost all cultures, except callus irradiated for only 2 hours, became necrotic with subsequent subcultures, and callus survival deteriorated gradually from one passage to another.

Phenotypical and morphological effects of UV irradiation were dependant on the characteristics of *in vitro* callus cultures. Irradiating callus with UV radiation for 4 hours changed their morphological and phenotypical characteristics. The callus grown on MS medium with 0.05 mg/L 2,4-D and 1.5 mg/L BAP was very hard and green, with some whitish regions on its surface without forming shoots did not change its phenotypical characteristic (**Figure 2.9**). Callus grown on MS medium with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin (MS1) was hard to semi-friable and very green, eventually forming some shoots; but it became severely affected by UV-C and necrotic overall which changed its color from dark green to light green (**Figure 2.9**).

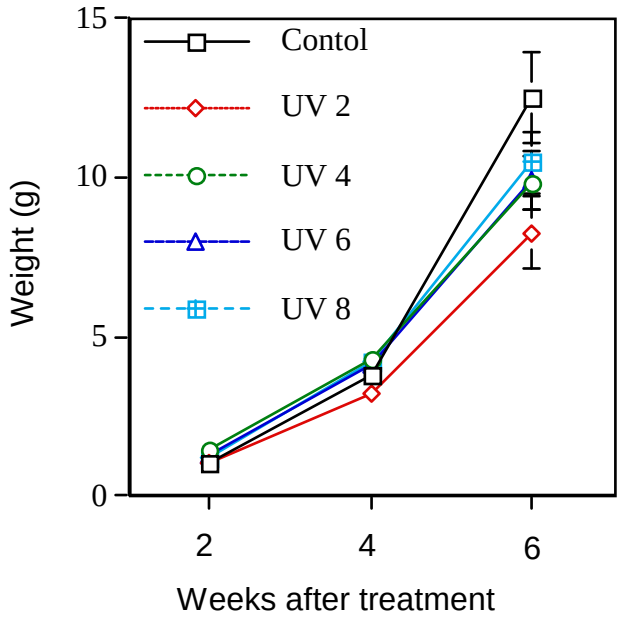


Figure 2.10: Callus on medium with 0.05 mg/L 2,4-D and 1.5 mg/L BAP treated UV light for 2 (UV2), 4 (UV4), 6 (UV6), or 8 (UV8) hours and harvested in 2, 4 and 6 weeks after the UV treatment (expressed as fresh weight). Error bars represent mean ± SE. Where error bars are not visible, the error is too small to be shown on the scale of the graph.

Suspension cultures

The suspension cultures were initiated and six replicates were harvested every week after a two week initial culture period. During the first 8 weeks of incubation, the calli and spent media were removed weekly and the tissue and spent medium extracted separately to assay for phenolic diterpenes. Growth of calli in the suspension cultures reached the stationary phase at week seven (**Figure 2.11**) and declined after 8 weeks. By observation, calli in these suspension cultures stayed green to light green for 6 weeks; within the next 3 weeks these calli became cream to light brown, and after 12 weeks calli reached the necrotic stage.

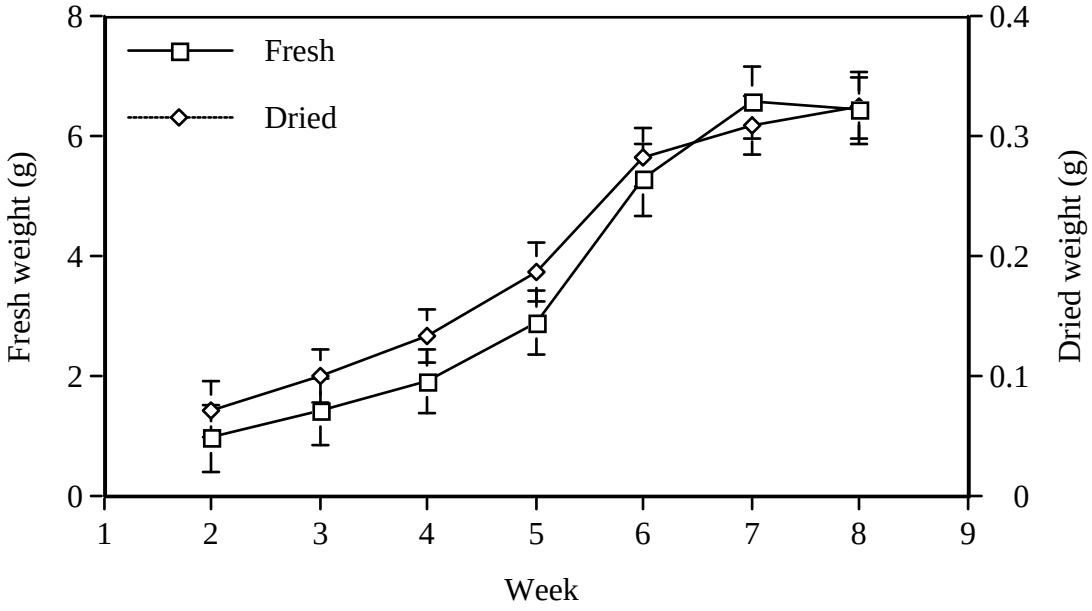


Figure 2.11: Fresh and dried weights of callus grown in MS Suspension medium supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP: Initial cultures were inoculated with 0.55 to 0.60 g callus in 30 mL of MS medium and incubated on shaker at 110 rpm in the dark. Cultures were harvested every week after two weeks incubation. Points represent mean values of samples consisting of 6 cultures.

DISCUSSION

The overall objective of this research was (1) to understand the effects of basal nutrient media and combinations of plant growth hormones on callus growth of *in vitro* grown rosemary cultures, (2) to determine if the effects of different physical factors can serve to increase, not only biomass, but also yields of phenolic diterpenes in the cultures.

The MS medium supplemented with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin proved to be the best combination of plant hormones for rapid production of green callus (fresh weight in rage of 38-65 g within 20 weeks), followed by the formation of shoots. Moreover, this culture medium had at least a 90% success rate for producing a desirable characteristic of callus in routine subcultures. Caruso *et al* (2000) and Nadosy (2002) demonstrated regenerated shoots of *Rosmarinus officinalis* contained carnosic acid; however, highly differentiated green calli were not ideal calli for establishing rosemary suspension cultures since regenerated shoots and habituated callus tended to reach a necrotic stage early in liquid medium. For this reason, this culture medium should be used for biomass accumulation and extracted for carnosic acid and carnosol. This project also demonstrated an effective hormone combination, for example 2,4-D at 0.05 mg/L and BAP at 1.5 mg/L, that allows for suitable callus growth (fresh weight in range of 27-37 g within 20 weeks) without formation of shoots. Callus established on this culture medium was used not only for extracting carnosic acid and carnosol, but also for establishing suspension cultures.

The data from this research demonstrated a significant influence of environmental factors on the biomass production. Callus grown in the dark produced more callus with the increase of temperature, but was white to cream and remained undifferentiated

throughout the culture period. Light was essential for growth, especially green to deep green callus. Caruso *et al* (2000) and Nadosy (2002) demonstrated that this type of callus was ideal for carnosic acid production. At an early stage, the cultures maintained under a light intensity of both 202.5 to 216.0 µmol $m^{-2}s^{-1}$ and 67.5 to 81.0 µmol $m^{-2}s^{-1}$ and at 18, 25, or 30°C showed no significant difference in callus growth. However, when the cultures were maintained more than 12 weeks under a light intensity of 202.5 to 216.0 µmol $m^{-2}s^{-1}$ and a temperature of 30°C, the callus became necrotic and did not survive subsequent subculturing. It was concluded that the cultures maintained at 18 and 25°C and also under low light intensity (67.5 to 81.0 µmol $m^{-2}s^{-1}$) were the best conditions for maintaining *in vitro* callus cultures.

The UV radiation experiments showed that treating rosemary tissue cultures with UV light for 4, 6 or 8 hours caused severe damage to the callus; the degree of damage was dependant on the morphological characteristics of callus (**Figure 2.9**). In numerous studies, the effects of UV have been reported to cause growth inhibition and an increase in antioxidant compounds (Cantos *et al* 2000, Xiong and Day 2001, Zagoskina *et al* 2003, Luis *et al* 2007). In the present study, however, UV-treated and UV-untreated callus grew continuously even 6 weeks after treatment; no significant difference on biomass among the treatments was observed (**Figure 2.10**). In addition, morphological and physiological effects of UV radiation varied among various plant species and tissues. The content of phenolic diterpenoids and chlorophylls are examined in Chapter 3.

CHAPTER THREE

Phenolic diterpenes from *Rosmarinus officinalis* in *in vitro* cultures

SUMMARY

In vitro cultures of rosemary (*Rosmarinus officinalis*) were analyzed for the phenolic diterpene profile under different environmental growth conditions and phytohormone treatments. Acetone extracts from undifferentiated cultures (callus), shoots of *in vitro* regenerated plants, and cells or cell aggregates harvested from suspension cultures, as well as from shoots of intact plants grown in the field were investigated for the concentration of phenolic diterpenes (carnosic acid and carnosol), total phenolic compounds, and total chlorophylls in each extract. Exposure of the callus cultures to several stress conditions by increasing or decreasing temperatures and light intensities, and UV radiation treatments, affected the level of phenolic diterpenes carnosic acid and carnosol.

INTRODUCTION

The antioxidants in rosemary (*Rosmarinus officinalis* L.) have been known as food preservatives for centuries. Extracts of rosemary are used to prevent the oxidation of fats and oils in foods and cosmetics to replace synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). As an antioxidant, rosemary has come to be regarded as potentially safer than synthetic antioxidants. Moreover, rosemary contains a large number of compounds such as phenolic acids, phenolic diterpenes, and triterpenes responsible for its antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, chemopreventive, antimicrobial, and antiviral activities (Singletary *et al*., 1991; Paris *et al*., 1993; Offord *et al*., 1995 and 1997; Aruoma *et al*., 1996; Hidalgo *et al*., 1998; Lo *et al*., 2002; Oluwatuyi *et al*., 2004). Kosaka and Yokoi (2003) also reported rosemary extract enhanced synthesis of NGF (nerve growth factor) in human glioblastoma cells.

The compounds mainly responsible for the antioxidant properties in rosemary are phenolic diterpenes. Carnosic acid (**Figure 3.1 A**) is the major phenolic diterpene in rosemary leaves. Two hydroxyl groups in this molecule in the *ortho* positions at C_{11} and C_{12} give higher antioxidant activity. Carnosic acid originates from a five-carbon unit isopentenyl diphosphate (IPP) via methylerythritol phosphate (MEP) and is localized in chloroplast and intracellular membranes where it cooperates with tocopherols and carotenoids to serve a significant photoprotective role under adverse climate condition (Munné-Bosch and Alegre, 2003). Several other phenolic diterpenes isolated from rosemary, such as carnosol, rosmanol, isorosmanol, and dimethyl isorosmanol, also have been identified as antioxidants. Carnosol (**Figure 3.1 B**) appears to be an artifact,

produced from air-oxidative degradation of carnosic acid (Aeschbach *et al*. 1993). Other diterpenes are formed from carnosic acid by enzymatic dehydrogenation and the action of activated oxygen (Wenkert *et* al. *1965*; Gonzalez *et* al. 1992; Luis *et al*. 1994). Figure 1.4 shows the formation of phenolic diterpenes in rosemary plants.

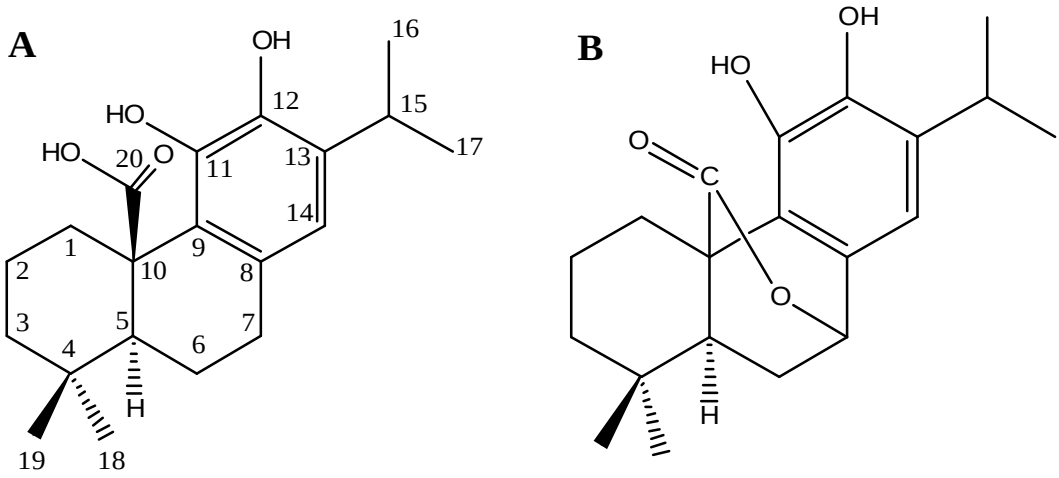


Figure 3.1: carnosic acid (A) and carnosol (B)

Carnosic acid is highly sensitive to oxidation and, rosemary extracts vary depending not only on geographical origin and environmental growth conditions such as temperature, light intensity, and drought, but also on distillation/extraction procedures. For example, carnosic acid has to be extracted in an apolar solvent, but it is not stable in methanol in which it is oxidized to lactone-containing diterpenes (Aeschbach *et al*. 1993; Luis *et al*. 1994; Schwarz *et al*. 1992b). Raising temperature and light tend to promote the degradation of carnosic acid in extraction processes (Cuvelier *et al*. 1994). Several authors have exhibited the effects of environmental growth conditions on the level of carnosic acid. The combination of high light and drought stresses causes the oxidation of carnosic acid, which leads to an enhanced formation of highly oxidized diterpenes (Okamura *et al*. 1994; Munné-Bosch *et al*. 1999). However, some rosemary varieties

showed an increase in carnosic acid concentration levels under cold stress condition (Luis *et al*. 2007). The most recent study done by Luis *et al*. (2007) demonstrated that UV-B radiation significantly increase the level of both carnosic and rosmarinic acids in rosemary plants.

The level of phenolic diterpenes in rosemary extracts showed great differences because of the variability of environmental factors. Therefore, *in vitro* cultures, where environmental and nutritional factors can be easily controlled may be the alternative to field-grown crops to chemically synthesize the production of desirable antioxidant compounds. However, production of phenolic diterpenes from *in vitro* rosemary cultures has not been sufficiently studied.

The goals of the research for this dissertation were to investigate and compare the effects of media formulation, phytohormones, and different environmental factors on phenolic diterpene production of rosemary callus and suspension culture tissues. Specific aims of the research were to:

- Determine the best nutrient and hormone combination for obtaining carnosic acid and related phenolic diterpenes in callus cultures.
- Determine the effects of physical factors such as temperature, light intensity, duration, and UV light on the level of carnosic acid and related phenolic diterpenes.
- Determine if carnosic acid and related phenolic diterpenes are present in the suspension cultures.
- Investigate the effects of elicitors on the level of carnosic acid and related phenolic diterpenes.

MATERIALS AND METHODS

Plant Material

Calli grown on solid culture media under different environmental growth conditions and phytohormone treatments, and cells or cell aggregates harvested from suspension cultures were rinsed with distilled water to remove Gellan or medium salt, and blotted gently to remove excess water. Finally, calli were weighed to obtain fresh weight, and then freeze dried, and ground using a mortar and a pestle.

The spent liquid medium from suspension cultures was collected filtered through Whatman filter paper #5 and extracted.

General Experimental Procedure

HPLC was done on a Waters 2690 using a Phenomenex Luna C_{18} column (4.60 x 250 mm, 5 μm) and monitored using a Water 996 Photodiode Array Detector scanning from 200 to 400 nm.

Extraction of phenolic diterpenes from rosemary *in vitro* cultures

For analysis of carnosic acid and carnosol, freeze-dried calli were extracted in acetone for 24 hours under argon on the G10 Gyrotary shaker at 125 rpm (**Appendix C**). Extracts were filtered by using #5 Whatman filter paper and calli were re-extracted in half volume of acetone for two hours. Combined acetone phases were evaporated, and then the residual extract was transferred to a separation funnel and was partitioned with equal volume of hexane. Hexane phases were then treated with anhydrous Na_2SO_4.

Extracts were transferred to an evaporating flask and dried completely. Dried extracts were reconstituted with acetone and dried under argon for further analysis.

Crude extracts from *in vitro* rosemary cultures: to minimize the loss of phenolic diterpenes during preparation of extracts from some *in vitro* rosemary cultures, freeze-dried calli were ground and extracted with acetone for 2 hours on the G10 Gyrotary shaker at 125 rpm. Extract was filtered by using Whatman #5 filter paper and calli were re-extracted in half volume of acetone for two hours. Combined acetone phases were transferred to the evaporating flask and evaporated completely. Dried extracts were reconstituted with acetone and dried under argon for further analysis.

Extraction of phenolic diterpenes from rosemary suspension culture media

The spent medium was first filtered through Whatman filter paper #5 into a shake flask and the filtrate was extracted for 24 hours in the equal volume of hexane under argon on the G-10 Gyrotary shaker at 125 rpm (**Appendix D**). The filtrate and the hexane mixture were transferred to a separatory funnel, and the hexane phase was collected in a flask. The hexane phase was then treated with anhydrous Na_2SO_4 and was transferred to an evaporating flask and dried completely in a rotary evaporator. The dried extract was reconstituted in acetone, dried under argon, and stored at –20 °C for further analysis. The suspension media were also extracted using a C_{18} SPE column (Alltech). Spent medium filtered through Whatman paper #5 was passed through the C_{18} SPE column to retain the terpenoids. The column was eluted with several solvents such as methanol, acetonitrile, acetone, or hexane.

Thin- layer chromatography (TLC) of extracts

Polygram® Sil G/UV plates (4 x 8cm) (Alltech) were used to detect the presence of phenolic diterpenes (carnosic acid and carnosol) in the extracts. The plates were spotted with 0.7 µl of each extract using a 10 µl pipetman, and then developed in hexane: ethyl acetate (7:3), sprayed with 10 % sulfuric acid in ethanol and heated at 110 °C for 5 min. Phenolic diterpenes showed a bluish color. Authentic standards, carnosic acid and carnosol (Alexis Corporation), were used as references.

Folin – Ciocalteu reagent for estimating total antioxidant phenolics

Folin – Ciocalteu reagent contained 2.5 % sodium molybdate, 10 % sodium tungstate, and 7.5 % sodium carbonate. Phenolic groups were oxidized with phosphomolybdic – phosphotungstic acid. The color yield was dependent on hydroxyl groups and their position in the molecule. The amount of total phenolics in extracts of rosemary calli or suspension cultures was determined according to the Folin – Ciocalteu procedure (Singleton and Rossi 1965). The samples (50 µl) were introduced in test tubes: 950 µl of distilled water, 250 µl of Folin – Ciocalteu reagent, 750 µl of sodium carbonate (20%), and 3 ml of distilled water were added. The mixture was allowed to stand for 30 minutes to 2 hours, and absorption at 750 nm was measured by spectrophotometer. The blank was prepared without a sample extract. The standard curve was prepared using serial dilutions of carnosic acid, and total phenolic content was expressed as carnosic acid equivalents. Instead of using the standard curve, the deduced equation of total phenol calculation is used:

% Total Phenols = {(Abs of sample x W. of standard) / (Abs of standard x W. of sample)} x 100
Abs : absorbance
W: weight

TLC-DPPH Spray reagent assay

The DPPH (2, 2-Diphenyl-1-picrylhydrazyl) assay was used to roughly determine the presence of antioxidant activity of a sample (Nia *et al*. 2004). Each extract and carnosic acid (standard) in acetone were spotted on the TLC plate and dried. The plates were developed with hexane: ethyl acetate (7:3) and dried, then the plate was dipped into the 400 µM ethanolic solution of DPPH (purple color). On the purple plate, the appearance of white spots indicated the presence of antioxidant compounds in the extract. Difference in the size of the spots gave an approximation of the relative concentration of the antioxidant in question.

Estimation of total chlorophylls

Calli grown on MS medium (MS1: 4 mg/L BAP, 10 mg /L IAA, and 1 mg/L of kinetin, MS2: 0.05 mg/L of 2,4-D and 1.5 mg/L of BAP, or MS3: 0.5 mg/L of 2,4-D and 0.075 mg/L of BAP) were used for determine total chlorophyll content (Al-Amier *et al*, 2005). Approximately 50 mg (fresh weight) of callus were placed in 3 ml of methanol contained in a 5 ml vial. Vials were covered with aluminum foil and incubated at 23 °C for 2 hours in darkness. Each sample was homogenized in a glass homogenizer and allowed to sit for 15 min in the dark. The methanol fraction was then decanted and the absorbance was measured at 650 nm and 665 nm. Total chlorophyll was expressed as (µg/ml methanol): Total chlorophyll = (25.8 X A650) + (4.0 X A665), A650 and A665 were the absorbencies of the methanol fraction at 650 and 665 nm, respectively.

Chlorophyll measurements were converted to the concentration of chlorophyll in the tissue using the formula: µg chlorophyll = (µg chlorophyll/ml methanol) X (3ml methanol/g of tissue).

HPLC analysis of carnosic acid and carnosol

Extracts were analyzed by HPLC using a Waters 2690 Separation Module (Milford, MA) equipped with a 996 Photodiode Array Detector and operated with Empower software. Samples were dissolved in methanol and separated on a Phenomenex Luna C_{18} column (4.60 x 250 mm, 5 µm) at ambient temperature with a flow rate of 1.0 ml/min with a 35:65 mixture of water with 0.5% phosphoric acid and acetonitrile. The 996 detector was used to scan between 200 and 400 nm. Identification of compounds was achieved by comparing the retention times and respective peak spectra with authentic standards (carnosic acid and carnosol, Alexis Biochemicals).

RESULTS

Acetone was chosen for primary extraction of phenolic diterpenoids because it has been shown that carnosic acid in acetone is relatively stable at room temperature compared to other polar solvents (Schwarz and Ternes, 1992; Okamura *et al*. 1994, Grzegorczyk *et al*. 2007). The presence of phenolic diterpenoids in extracts of *in vitro* callus and suspension cultures was analyzed by TLC, TLC-DPPH and HPLC. TLC and TLC-DPPH along with standard compound was used as a preliminary screen to check for the presence of carnosic acid and carnosol in callus grown on MS medium supplemented with several phytohormones and under various environmental conditions.

The HPLC run of standard compounds of carnosic acid and carnosol was performed under identical conditions to those employed for analysis of samples. To identify compounds in each extract, comparison of the retention times using standards and determination of maximum absorbance at different wavelengths for compounds' UV spectra using a photodiode array detector were developed. **Figure 3.2** shows the standard chromatograph for carnosic acid and carnosol. To quantify the carnosic acid and carnosol present in each extract, the individual peak areas obtained with HPLC chromatograms at 228 nm were compared with areas from standards of known concentrations. The carnosic acid standard curve concentration ranging from 0.01 to 0.4 µg was used to determine the contents of carnosic acid and carnosol in all extracts (**Appendix C.2**).

Extraction and analysis of phenolic diterpenes from *in vitro* cultures of rosemary

The freeze-dried calli grown on MS medium with different phytohormones (MS1, MS2, and MS3) and regenerated shoots were ground and extracted with acetone for 2 hours, then these extracts were screened by TLC and TLC-DPPH to check for the presence of carnosic acid and carnosol, while the extract of dried leaves obtained from the Lehman College greenhouse grown plants were used for comparison. TLC results (**Appendix D**) showed that regenerated shoots and MS2 callus contained spots at the R_f value of 0.43 identical to the standard of carnosic acid. Moreover the spots were visualized by spraying the TLC plate with the DPPH solution. The strong antioxidant activity was observed on the spot having the R_f value of 0.43 in the extracts of regenerated shoots and MS2 callus (**Appendix D, the plate B1**), whereas MS1 and MS3 did not show spots at the R_f value of 0.43 (**Appendix D, the plate A2**) on the TLC plate sprayed with sulfuric acid, but some antioxidant activity was observed at the identical spots with DPPH solution in Appendix D1, the plate B2. Additional spots were also visible in the TLC-DPPH plate at the R_f value of 0.78, which might represent other antioxidant compound in rosemary (**Appendix D, the plate B1 and B2**).

Figure 3.2 shows HPLC chromatographs of the standard compounds (carnosic acid and carnosol) and the samples. The presence of important compounds in each extract was identified based on retention time. The major component in all of the samples was phenolic diterpenes, however; especially in *in vitro* cultures such as regenerated shoots and calli contained rosmarinic acid (**Figure 3.3**). To confirm the identity of each compound, their UV spectra were obtained (**Figure 3.4**). A spectrum typical of carnosic acid was observed for the compound eluted at 16.8 min, while peaks

eluting at 10.1, 4.7 and 2.3 corresponded to the spectra of carnosol, rosmanol and rosmarinic acid, respectively (Nadosy 2002 and Moreno *et al* 2006).

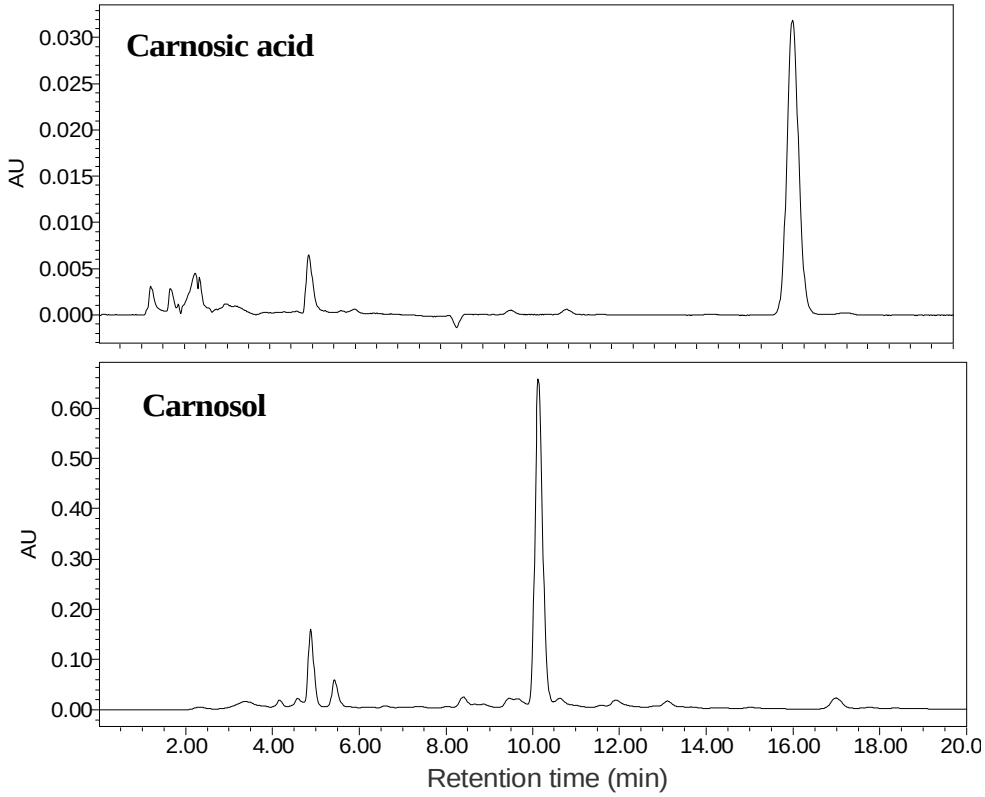


Figure 3.2: HPLC chromatograph of carnosic acid and carnosol, standards purchased from Alexis Biochemicals. Column Phenomenex Luna C_{18} 4.60 x 250 mm, 5µm; isocratic 35: 65 = water with 0.5% phosphoric acid: acetonitrile; flow rate 1 ml/min; UV detection at 228 nm. Carnosic acid and carnosol were detected at 16.8 min and at 10.1 min respectively.

The amount of total chlorophylls and total phenolic compounds, and amounts of carnosic acid and carnosol in all extracts (dried leaves, regenerated shoots, MS1, MS2 and MS3 calli) were determined (**Table 3.1**). The callus characteristic correlated with the amount of total chlorophylls in the callus extracts. MS1 and MS2 calli were green to very green, which contained greater amounts of chlorophylls than MS3 callus, which was

white. However, there was no direct correlation between the total chlorophyll and the total phenolic concentrations as well as the carnosic acid and carnosol contents. Surprisingly white and friable callus of MS3 contained a greater amount of total phenolic than MS1 and MS2 calli. For example, the amount of carnosic acid in MS3 was 0.40 mg/g DW of callus, whereas MS1 and MS2 calli contained 0.14 and 0.11 mg/g DW of callus respectively. Moreover, the extract of dried leaves contained lesser amounts of total chlorophylls and more carnosic acid and carnosol in comparison to the extract of regenerated shoots. However, the regenerated shoots contained significantly higher amount of total phenolics compared with the undifferentiated callus (MS1, MS2, and MS3). The amount of carnosic acid and carnosol together in dried leaves was highest among samples followed by regenerated shoots, MS1, MS2, and MS3.

Carnosol was the predominant compound in the extracts of MS1 and MS2 calli (**Figure 3.3**). The phenolic diterpene profile changed slightly according to the extraction processes. A crude extract of *in vitro* grown callus in acetone usually contained carnosic acid, carnosol, rosmanol, and rosmarinic acid as main compounds as well as several unknown compounds, whereas the metabolite profile from *in vitro* grown callus extracts using the extraction procedure in Appendix C (solvent-solvent partition with acetone and acidifying solvent during extraction) showed that either carnosic acid or carnosol was the only major compound. However, the large number of steps and complicated methods are labor intensive and probably cause the loss of phenolic diterpenes. Interestingly the crude extracts of *in vitro* grown callus as well as regenerated shoots contained rosmarinic acid, whereas dried leaves extracted with the same method contained no rosmarinic acid.

Table 3.1: The results of total chlorophylls, total phenolics, and carnosic acid and carnosol contents in various extracts of *R. officinalis*. The values are means ± SD.

	Chlorophyll (µg/g)	Phenolics (mg/g)	CA (mg/g)	CAR (mg/g)
Dried leaves	156.3 ± 2.08	28.4 ± 0.91	11.00	0.66
Regenerated shoots	2757 ± 9.82	18.2 ± 0.99	1.91	0.14
MS 1	1633 ± 11.1	5.66 ± 0.39	0.14	1.12
MS 2	2140 ± 15.6	8.12 ± 1.20	0.11	0.88
MS 3	24.67 ± 7.09	9.40 ± 0.43	0.40	0.05

Regenerated shoots: MS medium with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin;
MS 1: callus on MS medium with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin;
MS 2: callus on MS medium with 0.05 mg/L 2,4-D and 1.5 mg/L BAP;
MS 3: callus on MS medium with 0.5 mg/L 2,4-D and 0.075 mg/L Kinetin.
CA, carnosic acid; CAR, carnosol

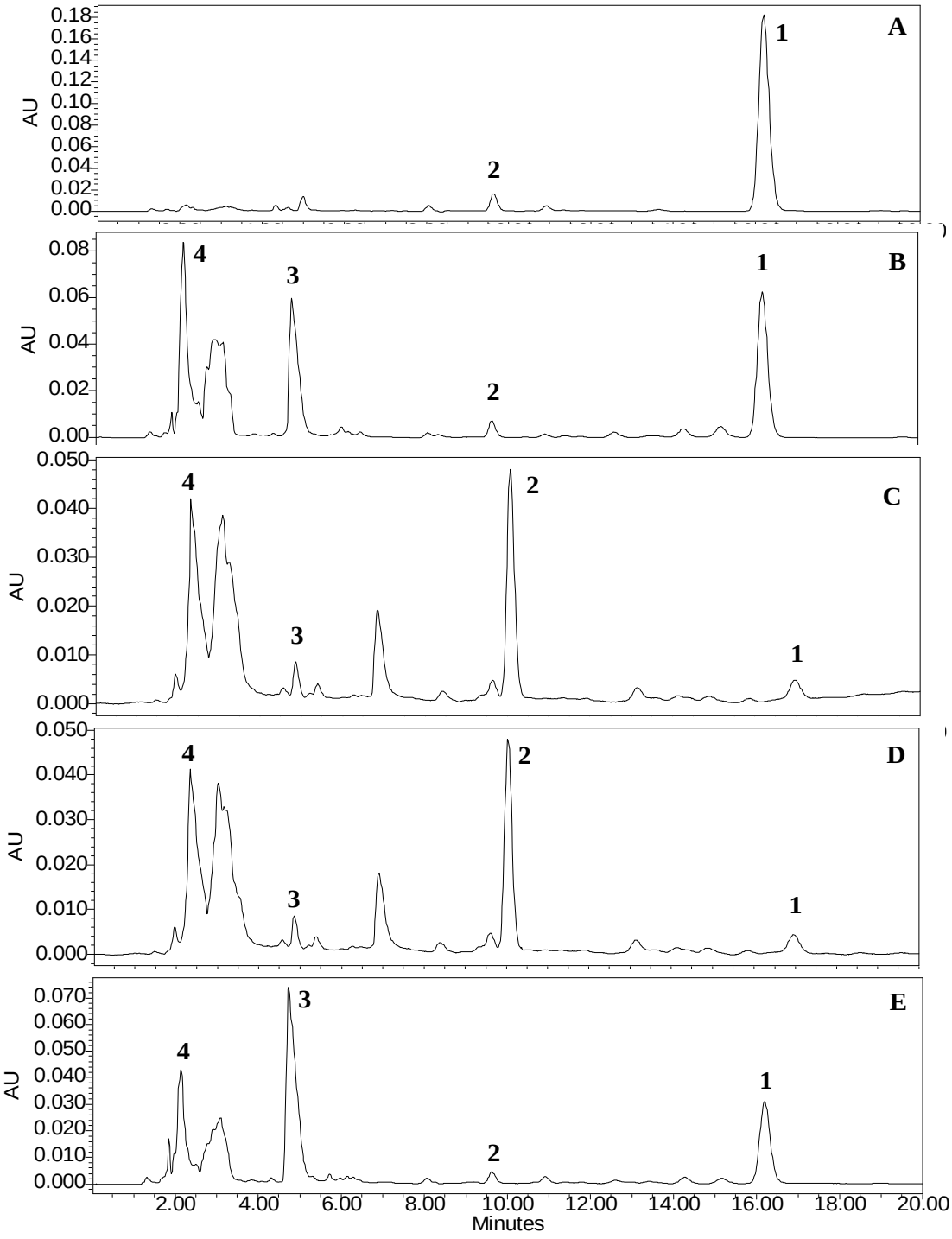
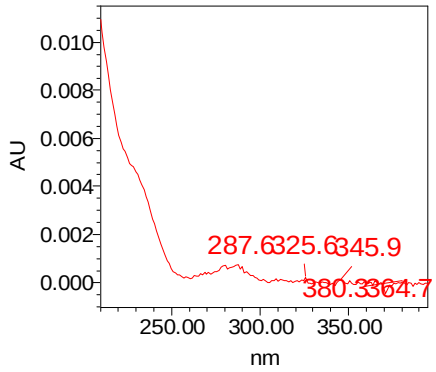
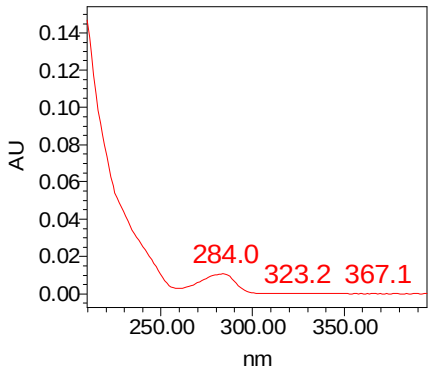


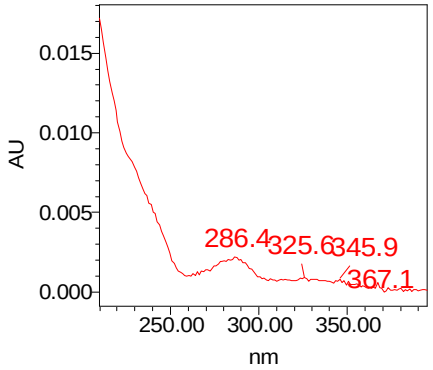
Figure 3.3: HPLC-PDA chromatograms at 228 nm of acetone extracts of (A) dried leaves, (B) regenerated shoots, (C) callus on MS medium with 10 mg/L IAA + 4 mg/L BAP + 1 mg/L kinetin (MS1), (D) callus on MS medium with 0.05 mg/L 2,4-D + 1.5 mg/L BAP (MS2), (E) callus on MS medium with 0.5 mg/L 2,4-D + 0.075 mg/L kinetin (MS3). 1, carnosic acid; 2, carnosol; 3, rosmanol; 4, rosmarinic acid.



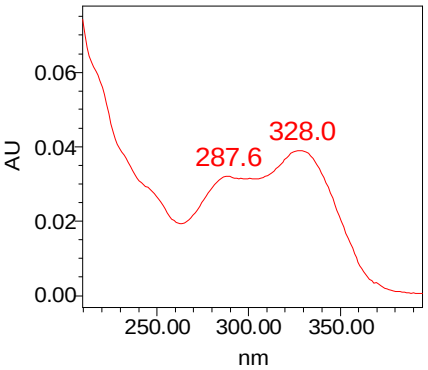
UV spectrum of carnosic acid (1)



UV spectrum of carnosol (2)



UV spectrum of rosmanol (3)



UV spectrum of rosmarinic acid (4)

Figure 3.4: UV spectra (detected at 228 nm) of carnosic acid, carnosol, rosmanol, and rosmarinic acid identified in acetone extracts of rosemary tissue grown *in vitro*.

Identification of compounds from *in vitro* grown callus under different environmental conditions

Calli grown under different physiological factors (temperature, light intensity, duration, quality including UV light) that might serve to increase yields of phenolic diterpenes over those obtained by manipulating nutrient-hormone were compared. Calli grown on MS medium supplemented with either with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin (MS1) or 0.05 mg/L 2,4-D and 1.5 mg/L BAP (MS2) with various environmental conditions were extracted in acetone, and evaluated for the presence of carnosic acid, carnosol, and related compounds.

The influence of temperature on the formation of phenolic diterpenes such as carnosic acid and carnosol are shown in Table 3.2. In MS1 calli, total chlorophyll level at 18°C was found at a higher concentration than calli at 25°C, whereas total phenolic level showed a similar response under both conditions. In contrast, in MS2 calli, total chlorophyll level at 25°C was higher than at 18°C, whereas total phenolic level showed no significant differences under both conditions. Results from HPLC analysis (**Table 3.2** and **Figure 3.5**) showed carnosic acid and carnosol in MS1 calli at 18°C were 0.132 mg/g DW and 0.036 mg/g DW respectively, found at highest concentrations among the treatments. Carnosic acid and carnosol were the predominant compounds in the extracts of MS1 calli grown at 18°C, whereas in the extracts of MS2 calli at both 18 and 25°C, rosmanol was the predominant compound (**Figure 3.5**). Production of carnosic acid and carnosol did not always follow the pattern for production of total phenolics.

The concentration of carnosic acid and carnosol increased with increasing light intensity (**Table 3.3**). The highest amount of carnosic acid and carnosol was observed in calli grown under 67.5 µmol $m^{-2}s^{-1}$. However rosmarinic acid and rosmanol were the predominant compounds in these cultures (**Figure 3.6**).

Table 3.2: The results of total chlorophylls, total phenolics, and carnosic acid and carnosol contents in various extracts of calli grown on MS medium either with 10 mg/L IAA, 4 mg/L BAP and 1 mg/L kinetin (MS1) or 0.05 mg/L 2,4-D and 1.5 mg/L BAP (MS2) under various environmental conditions (at 18 and 25 °C with 16 hours light). The values are means ± SD. CA, carnosic acid; CAR, carnosol.

	Chlorophyll (µg/g)	Phenolics (mg/g)	CA (mg/g)	CAR (mg/g)
MS 1				
18°C	1097 ± 47.5	3.93 ± 0.24	0.132 ± 0.008	0.036 ± 0.006
25°C	972 ± 47.5	1.77 ± 0.24	0.041 ± 0.004	0.031 ± 0.003
MS 2				
18°C	993 ± 42.0	2.80 ± 0.10	0.039 ± 0.006	0.027 ± 0.005
25°C	1223 ± 42.0	2.53 ± 0.10	0.042 ± 0.008	0.020 ± 0.005

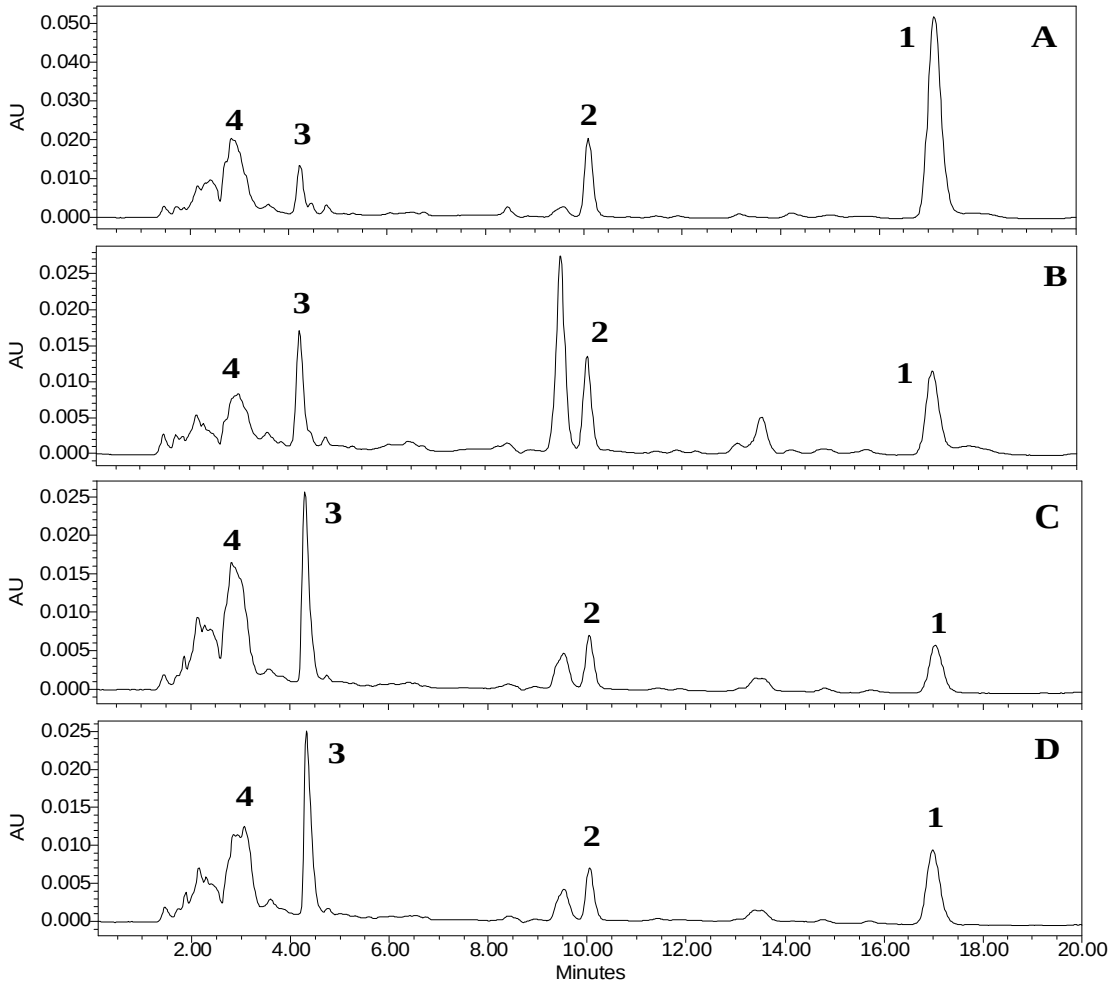


Figure 3.5: HPLC chromatograms at 228 nm of acetone extracts of calli grown at different environmental conditions: A, MS1 at 18°C; B, MS1 at 25°C with 16 hours light; C, MS2 at 18°C with 16 hours light; D, MS2 at 25°C with 16 hours light. 1, carnosic acid; 2, carnosol; 3, rosmanol; 4, rosmarinic acid.

Table 3.3: The results of total chlorophylls, total phenolics, and carnosic acid and carnosol contents in various extracts of calli grown on MS medium with 10 mg/L IAA, 4 mg/L BAP and 1 mg/L kinetin (MS1) at 18°C under different light intensities such as 54.0 µmol $m^{-2}s^{-1}$, 62.1 µmol $m^{-2}s^{-1}$, or 67.5 µmol $m^{-2}s^{-1}$. CA, carnosic acid; CAR, carnosol.

	Chlorophyll (µg/g)	Phenolics (mg/g)	CA (mg/g)	CAR (mg/g)
MS1 at 18°C				
54.0 µmol $m^{-2}s^{-1}$	876 ± 23.8	3.92 ± 0.22	0.043 ± 0.007	0.025 ± 0.003
62.1 µmol $m^{-2}s^{-1}$	1120 ± 23.8	4.27 ± 0.22	0.048 ± 0.002	0.025 ± 0.0002
67.5 µmol $m^{-2}s^{-1}$	1027 ± 23.8	5.20 ± 0.22	0.109 ± 0.004	0.040 ± 0.003

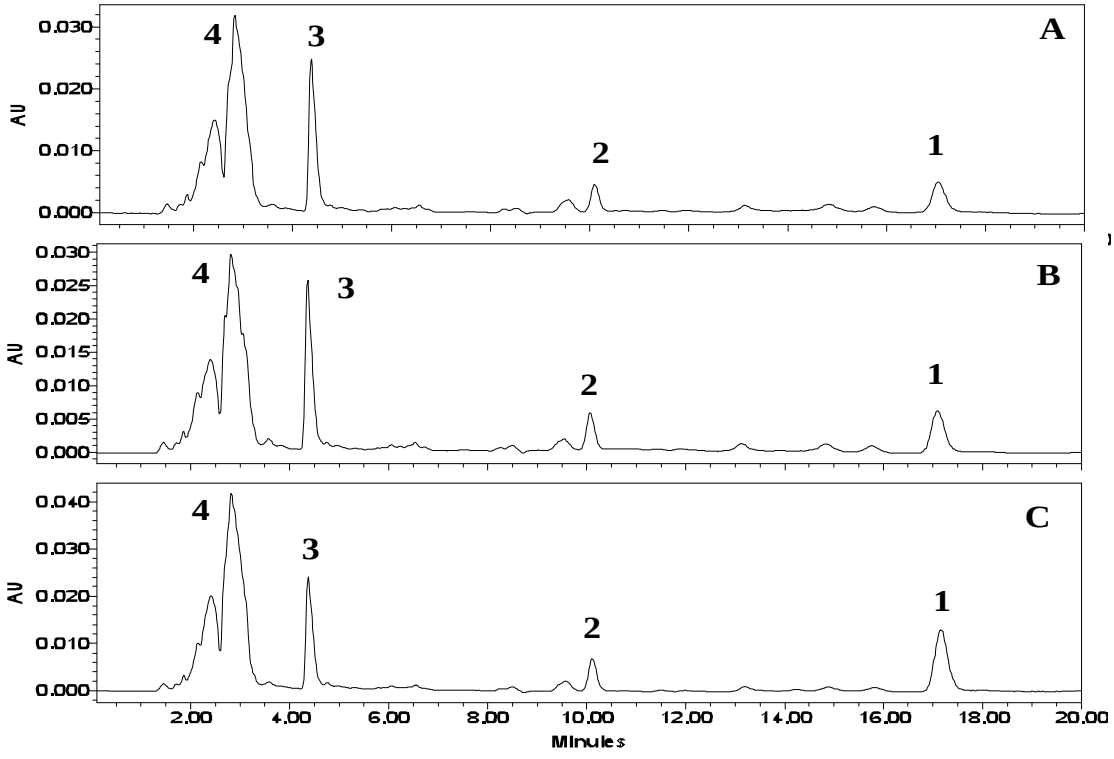


Figure 3.6: The results of total chlorophylls, total phenolics, and carnosic acid and carnosol contents in various extracts of calli grown on MS medium either with 10 mg/L IAA, 4 mg/L BAP and 1 mg/L kinetin (MS1) at 18°C under different light intensities such as (A) 54.0, (B) 62.1, or (C) 67.5 µmol $m^{-2}s^{-1}$. 1, carnosic acid; 2, carnosol; 3, rosmanol; 4, rosmarinic acid.

UV light experiment

Seven to eight weeks old green and hard calli maintained on MS medium supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP at 18°C was irradiated with UV-C (producing primarily 254 nm radiation) light of 62.1 $\mu mol\, m^{-2}s^{-1}$ for 2, 4, 6, or 8 hours at room temperature in a sterile hood. The cultures were then put back into the original condition and harvested after 2, 4 or 6 weeks.

Treating callus with UV-C radiation changed their morphological and physiological characteristics depending on the duration of irradiation; however, there was a continuous increase in callus biomass in all treatments (Chapter 2).

The content of chlorophyll in callus exposed to UV-C for 4 hours and harvested 2 weeks after the treatment was significantly higher than UV-untreated (control) callus, while callus irradiated for 2 hours had significantly lower chlorophyll concentration than UV-untreated callus (**Figure 3.6 A**). Moreover, the level of chlorophyll in UV-treated callus for 4, 6, and 8 hours continuously decreased and reached the lowest point at week 6, whereas callus treated with 2 hours of UV irradiation showed the recovery of chlorophyll content within 6 weeks despite the fact that the level was lower than UV-untreated callus.

The level of total phenolics in the UV-treated callus cultures, harvested 2 weeks after the treatment was significantly higher than UV-untreated callus, and no differences were found among treatments (**Figure 3.7 B**). However, the concentration of total phenolics started decreasing at week 2, and at week 4 it reached almost the same level as those in the UV-untreated callus, and at week 6 the level of total phenolics in UV-irradiated callus became slightly lower than in the UV-untreated callus (**Figure 3.7 B**).

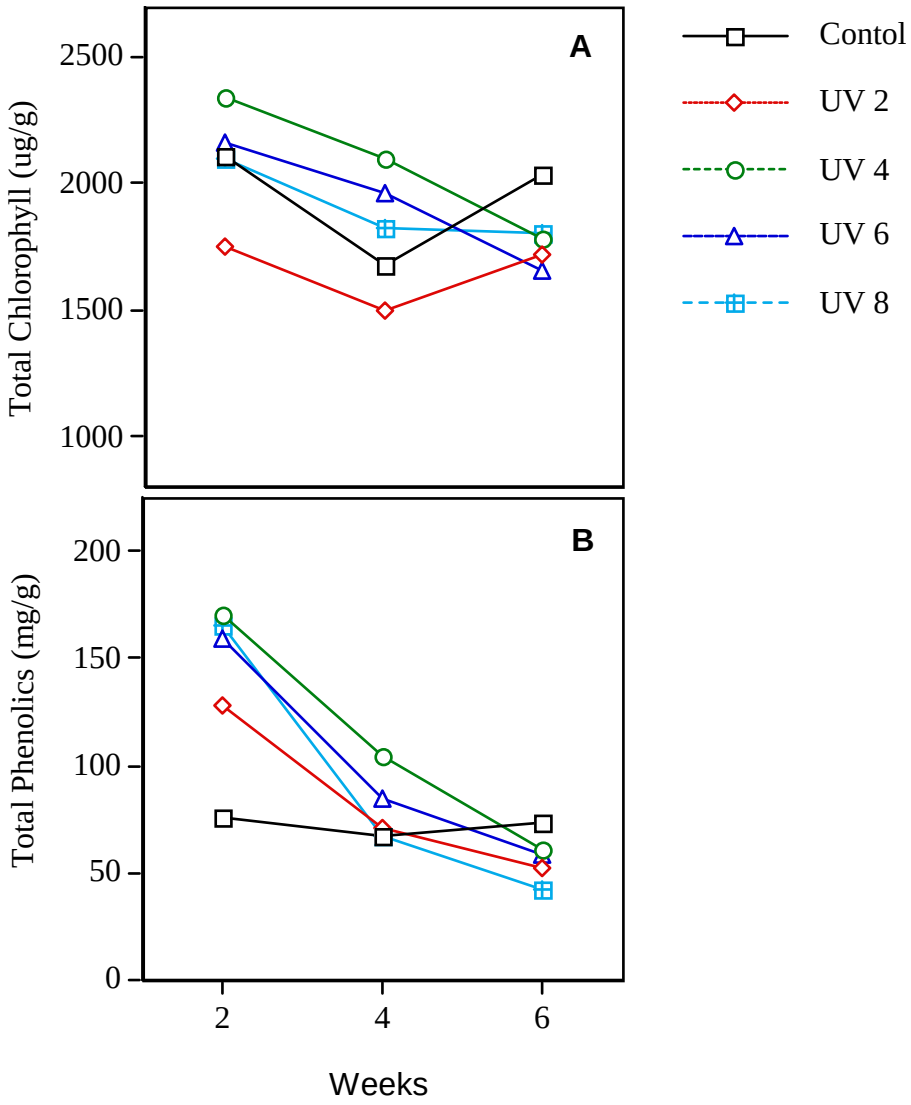


Figure 3.7: Total chlorophylls (µg/g) and phenolics (mg/g) in the extracts of rosemary callus. Callus treated with UV light for 2, 4, 6, and 8 hours and harvested at 2, 4 and 6 weeks after the UV treatment. Points represent mean values of samples with 5 cultures. Treatment time: UV2, 2 hours; UV4, 4 hours; UV6, 6 hours; UV8, 8 hours.

Analysis of phenolic diterpenes in suspension cultures of *R. officinalis*

Figure 3.8 shows the amount of total phenolics in the suspension cultures in MS liquid medium supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP (MS2), which were grown under light or dark. Spent media were tested for the presence of phenolics every week and were harvested on the 3rd and 6th weeks and replaced with fresh medium. The cultures under light kept producing increasing amounts of total phenolics after the 1st harvest, whereas cultures under dark never reached the original level (2nd week) of phenolics after the 1st harvest and calli became brown or died after the 2nd harvest. Therefore, suspension cultures in future experiments were grown under light.

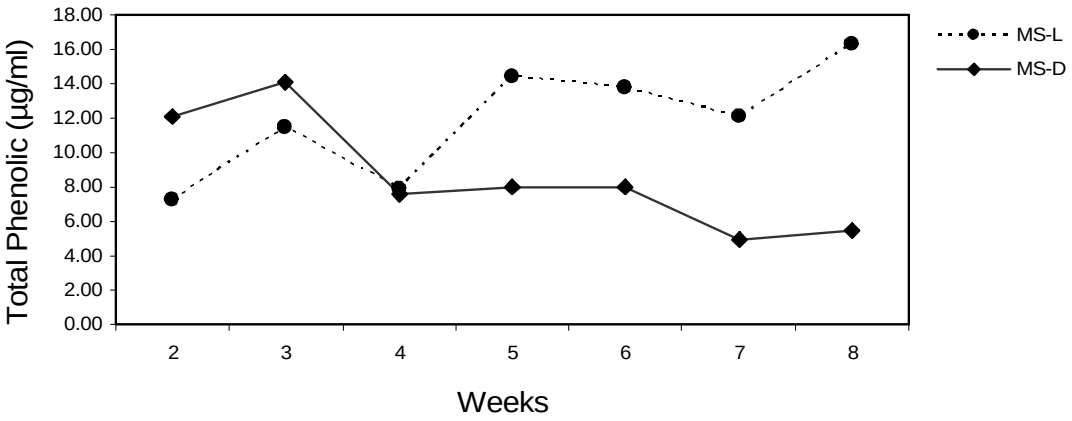


Figure 3.8: Suspension cultures (MS medium) supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP (MS2) were grown under dark and light. The level of total phenolics in spent liquid medium was tested every week.

The suspension culture in MS liquid medium supplemented with 0.05 mg/L 2,4-D and 1.5 mg/L BAP, originated from the compact calli grown on solid medium of the same composition. The growth rate of this culture reached a plateau at week seven (Chapter 2, **Figure 2.11**) and declined after eight weeks. **Figure 3.9** shows the amount of total phenolics in callus aggregate and spent liquid medium, which was sampled every

week after two weeks of incubation. The total phenolics in spent liquid medium increased steadily from week 4 and reached the maximum at week 8; in contrast, the phenolic level in callus aggregate increased during first 4 week-period, then gradually decreased and was significantly lower than the spent liquid medium at week 8.

TLC was used to screen the presence of carnosic acid and carnosol (phenolic diterpenes) in callus aggregate and spent liquid medium on the basis of R_f values with authentic standards. The TLC analysis showed that the presence of carnosic acid or carnosol in callus aggregates from the suspension cultures was detected at R_f 0.52 or 0.45, respectively. HPLC results confirmed (**Figure 3.10**) that carnosic acid, carnosol and rosmanol could be detected in callus aggregates, and the spent liquid medium contained neither carnosic acid nor carnosol, but did contain rosmanol. This provides strong evidence that all carnosic acid and carnosol are sequestered within the cells. One possible reason is that carnosic acid is a lipophilic compound; it is not getting excreted in the aqueous extracellular medium.

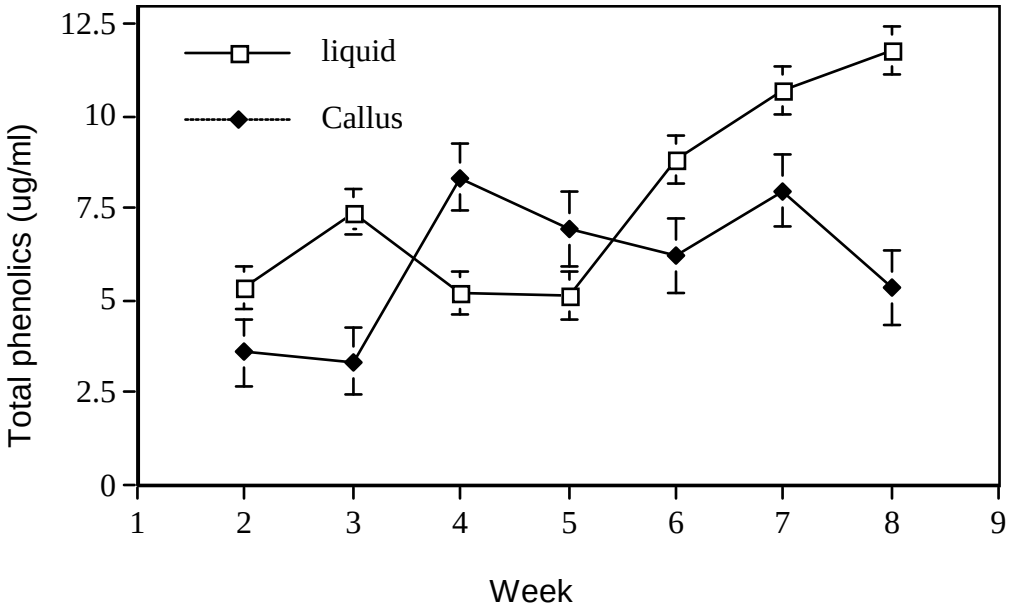


Figure 3.9: Suspension cultures (MS medium) supplemented with 0.05 mg/L of 2,4-D and 1.5 mg/L of BAP (MS2). The level of total phenolics in callus aggregate and spent liquid medium was tested every week.

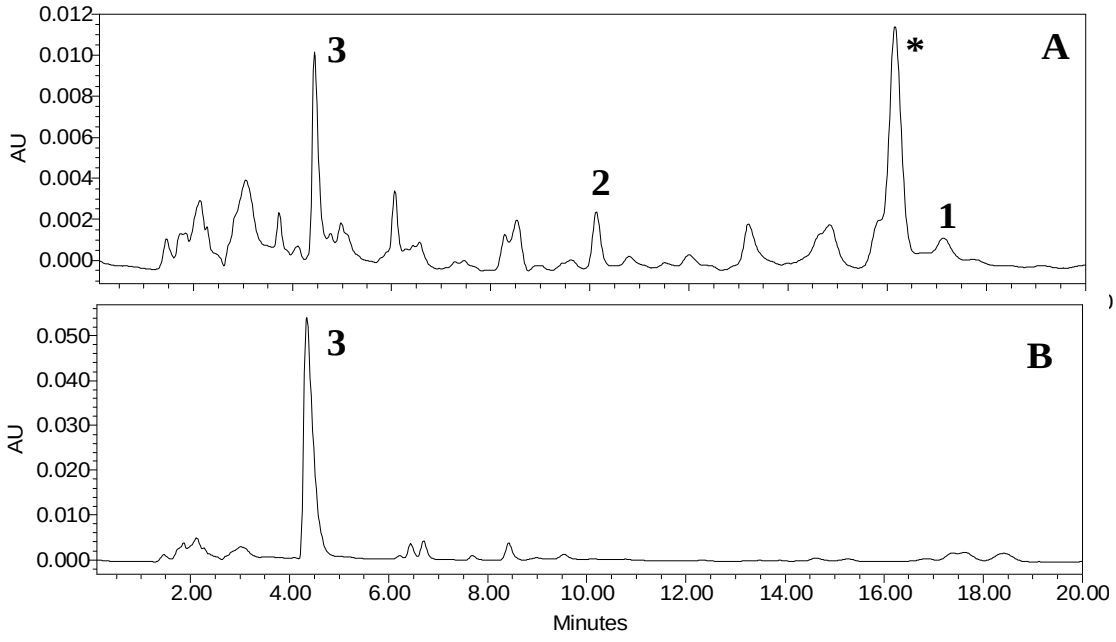


Figure 3.10: HPLC chromatograph of the extracts from rosemary cell aggregate (A) and spent medium (B). 1, carnosic acid; 2, carnosol; 3, rosmanol; *, unknown compound. Suspension cultures (MS medium) supplemented with 0.05 mg/L of 2,4-D and 1.5 mg/L of BAP (MS2).

DISCUSSION

The overall objective of this study was to investigate the effects of exogenously applied phytohormones and physiological factors such as temperature, light intensity, and UV light on phenolic diterpene production in *in vitro* cultures of *Rosmarinus officinalis*, and to develop suspension cultures for carnosic acid and carnosol production.

The concentration of carnosic acid and carnosol in cultivated rosemary plants varies in the range of 0.1% to 6% of dry weight according to climate conditions, agricultural factors, and geographical origin (Hidalgo *et al*., 1998; Munné-Bosch *et al*., 2000; Munné-Bosch and Alegre, 2001and 2003). Moreover, there are only few studies reported regarding the *in vitro* production of carnosic acid and carnosol from *Rosmarinus officinalis* (Caruso *et al*., 2000; Nadosy 2002; Kuhlmann and Rohl, 2006). Caruso *et al*. (2000) reported the presence of carnosic acid in regenerated shoots of rosemary and in green callus, however quantitative data was not provided. Nadosy (2002) showed that the concentration of carnosic acid in regenerated shoots and in green callus was 0.7% to 1.1% and less than 0.5% of dry weight respectively.

This was the first study to show the effects of several experimental conditions on the level of carnosic acid and carnosol production in *in vitro* rosemary cultures. The typical condition for growing callus has been at 25°C. However, the level of carnosic acid in callus culture (MS1) grown at 18°C was about two-fold higher than at 25°C. In a previous report by Luis *et al*. (2007), cold treated rosemary plants exhibited higher concentration levels of carnosic acid, which were negatively correlated with the total chlorophyll. Moreover, the highest concentration of the major diterpenes carnosic acid and carnosol were found during the Mediterranean winter (Munné-Bosch *et al*., 2000).

MS2 callus culture, however, showed no significant difference in the level of carnosic acid at 18 and 25°C.

With increasing light intensity, the level of total phenolics, carnosic acid, and carnosol in MS1 callus cultures increased, whereas no significant decrease in total chlorophyll content in these cultures was observed. However, these results were not in agreement with observations of Munné-Bosch *et al*. (2000 and 2003), who reported the concentration of carnosic acid decreased with increasing solar radiation and temperature under drought conditions. Since the humidity level of *in vitro* cultures was stable and quite high compared to field conditions, increasing the level of light intensity used in this study showed the positive influence of temperature on diterpene concentration.

Exposing rosemary callus cultures to ultraviolet radiation (UV-C) with several durations showed an increase in total phenolic levels, especially with callus harvested 2 weeks after the radiation treatment. These results agreed with the study reported by Luis *et al*. (2007) where UV-B radiation significantly increased the foliar concentration of carnosic and rosmarinic acids in rosemary plants. The level of accumulation of phenolic compounds plays an important role in the cell survival under the condition of UV-B radiation. Phenolic compounds are known to absorb light in the 200-320 nm range and to protect cells against oxidative stress by interaction with reactive oxygen species. UV-C radiation enhanced the accumulation of phenolic compounds in rosemary callus cultures. This study suggests an important role of phenolic compounds in the protection of cells against the effects of UV radiation.

CHAPTER FOUR

Conclusion and Future Research

The overall objective of this research was to investigate and compare the effects of basal nutrient media, phytohormones, and environmental factors such as light, temperatures, or photoperiods on growth and phenolic diterpene (carnosic acid and carnosol) production in *in vitro* rosemary (*R. officinalis*) culture systems. Due to increasing interest in the benefits of carnosic acid and carnosol for human health, the high price of isolated compounds, and great variability in their concentration due to individual origin, climate conditions, and agricultural factors, tissue culture should be investigated for alternative means of obtaining these compounds.

The content of carnosic acid, carnosol, and related diterpenes in extracts depended on the level of differentiation of culture (callus culture, shoot culture, and suspension culture). Rosmarinic acid, a phenolic acid, is not greatly influenced by the differentiation level. Shoots regenerated from green callus of rosemary accumulated carnosic acid and carnosol at the value of 2.05 mg/g of biomass dry weight. These compounds were also present in callus culture, but as about 2- to 4-fold lower levels than in regenerated shoots. The total content of carnosic acid and carnosol in callus culture varied from 0.45 to 1.26 mg/g of biomass dry weight. MS1 callus cultures (MS medium with 10 mg/L IAA, 4 mg/L BAP and 1 mg/L kinetin) were able to produce shoots emerging from callus in a relatively short period (**Figure 2.4, 8 and 9**). This, however, was not the ideal type of callus for establishing rosemary suspension cultures since regenerated shoots or habituated callus tended to reach a necrotic stage early in liquid medium. Therefore, this

medium formulation can be used to produce a large volume of regenerated shoots, which contain constant levels of carnosic acid and carnosol for extraction.

The MS1 and MS2 callus cultures can be harvested within 4 to 8 weeks, and the dry weight of callus is in a range of 0.20 to 0.80 g. The level of carnosic acid and carnosol is from 0.04 to 0.13 mg/g DW and 0.02 to 1.12 mg/g DW respectively.

According to Munné-Bosch's group, carnosic acid is highly unstable, depending on factors such as water stress, temperature, light, and oxygen contact. Those callus cultures which produced green callus were maintained under several stress conditions. The concentration of carnosic acid and carnosol in the MS1 callus culture (MS medium with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin) grown at 18°C under light intensity of 67.5 µmol $m^{-2}s^{-1}$ was the highest among experimental conditions tested (Chapter 3). UV-C radiation treatment enhanced the level of total phenolics in callus cultures. This result showed that tissue culture was able to provide an excellent research system to investigate and establish the best environmental condition for producing higher concentrations of carnosic acid and carnosol.

In callus aggregates from suspension cultures, only carnosol was detected, but no carnosic acid, and spent medium contained neither carnosic acid nor carnosol. This provides strong evidence that all carnosic acid and carnosol are sequestered within the cells. One possible reason is that carnosic acid, a lipophilic compound, is not getting secreted into the aqueous extracellular medium. Treating suspension cultures with an elicitor such as Pectinase showed a slight increase of total phenolics in the spent medium, but the yield was substantially lower than that detected in callus cultures (data not included). It would be worthwhile to continue testing different elicitors (e.g. jasmonic

acid, methyl jasmonate, and salicylic acid) as well as metabolic inhibitors of biosynthetic pathways that can divert precursors from those that lead to the synthesis of carnosic acid, carnosol, and related phenolic diterpenes. Furthermore, the accumulation of carnosic acid was observed in regenerated roots of rosemary (Caruso *et al*., 2000) and of *Salvia officinalis* (Grzegorczyk *et al*., 2007). Establishing photosynthetic hairy root cultures or *agrobacterium rhizogenes*-transformed hairy root cultures of rosemary would be another alternative technique for obtaining of carnosic acid, carnosol, and related diterpenes, since hairy root cultures are genetically stable and have relatively rapid growth rate, and are suitable for bioreactor systems.

The chemical profile of regenerated shoot and callus extracts was similar to those extracts of cultivated shoots. Carnosic acid, carnosol, and rosmanol as well as rosmarinic acid (phenolic acid) were present in these extracts. Qualifying and quantifying phenolic diterpenes by HPLC is time consuming and requires liquid extraction with an organic solvent. Moreover, callus contains significantly large amount of oil and water compared to shoots, which interfere the extraction processes. Therefore, development of an extraction protocol to minimize the loss of phenolic diterpenes and of fast, reliable, and inexpensive quantification procedure should be necessary.

Appendix

Appendix A

Media formulations of Murashige & Skoog medium (MS) by Murashige and Skoog (1962) and Gamborg's B-5 medium (B5) by Gamborg, Miller and Ojima (1968).

Media		MS		B5
Units		mg/L		mg/L
Macronutrients	$(NH_4)NO_3$	1650	$(NH_4)NO_3$	134
	KNO_3	1900	KNO_3	2500
	$CaCl_2$ (anhydrous)	333	$CaCl_2$ 2H2O	150
	$MgSO_4$ (anhydrous)	181	$MgSO_4$ 7H2O	250
	KH_2PO_4	170		
Micronutrients	H_3BO_3	6.2	H_3BO_3	3
	$CoCl_2$ $6H_2O$	0.025	$CoCl_2$ $6H_2O$	0.025
	$CuSO_4$ $5H_2O$	0.025	$CuSO_4$ $5H_2O$	0.025
	$MnSO_4$ H_2O	16.9	$MnSO_4$ H_2O	10
	KI	0.83	KI	0.75
	Na_2MoO_4 $2H_2O$	0.25	Na_2MoO_4 $2H_2O$	0.25
			NaH_2PO_4 H_2O	150
	$ZnSO_4$ $7H_2O$	8.6	$ZnSO_4$ $7H_2O$	2
Iron compounds			Na_2 EDTA	37.3
	FeNaEDTA	36.7	FeNaEDTA	27.8
	$FeSO_4$ $7H_2O$		$FeSO_4$ $7H_2O$	27.8
Vitamins	Glycine	2		
	Nicotinic acid	0.5	Nicotinic acid	1
	Pyridoxin HCl	0.5	Pyridoxin HCl	1
	Thiamin HCl	0.1	Thiamin HCl	10
	Myo-Inositol	100	Myo-Inositol	100
Units		g/L		g/L
Gellen		4.5		4.5
Sucrose		30		20
pH		5.7 to 5.8		5.7 to 5.8

Appendix B

The list of experimental conditions of rosemary callus cultures that are used in this project for initiation of callus and production of phenolic diterpenes.

Abbreviations: MS: Murashige and Skoog's basal medium, B5: Gamborg's B5 basal medium, IAA: indole acetic acid, 2, 4-D: 2, 4-dichlorophenoxyacetic acid, NAA: 1-naphthaleneacetic acid, BAP: benzylaminopurine, Kinetin: 6-furfurylaminopurine, Temp.: temperature (°C), Photo: photoperiod, Lux: light intensity (µmol $m^{-2}s^{-1}$).

(A) Experimental cultures treated with different temperature (°C) and light intensity (µmol $m^{-2}s^{-1}$).

	Medium	Sucrose	Auxin (mg/L)			Cytokinin (mg/L)		pH	Gellen	Temp.	Light	
A		(g/L)	IAA	2.4-D	NAA	BAP	Kinetin		(g/L)	°C	Photo	µmol $m^{-2}s^{-1}$
1	MS	30	10			4	1	5.8±0.1	4.5	18	dark	
2	MS	30	10			4	1	5.8±0.1	4.5	18	16	67.5 - 81.0
3	MS	30	10			4	1	5.8±0.1	4.5	18	16	202.5 - 216.0
4	MS	30	10			4	1	5.8±0.1	4.5	25	dark	
5	MS	30	10			4	1	5.8±0.1	4.5	25	16	67.5 - 81.0
6	MS	30	10			4	1	5.8±0.1	4.5	25	16	202.5 - 216.0
7	MS	30	10			4	1	5.8±0.1	4.5	30	dark	
8	MS	30	10			4	1	5.8±0.1	4.5	30	16	67.5 - 81.0
9	MS	30	10			4	1	5.8±0.1	4.5	30	16	202.5 - 216.0
10	MS	30	10			4	1	5.8±0.1	4.5	25	16	202.5
11	MS	30	10			4	1	5.8±0.1	4.5	25	16	270.0
12	MS	30	10			4	1	5.8±0.1	4.5	25	16	405.0
13	MS	30	10			4	1	5.8±0.1	4.5	25	16	675.0
14	MS	30	10			4	1	5.8±0.1	4.5	18	16	540.0
15	MS	30	10			4	1	5.8±0.1	4.5	18	16	621.0
16	MS	30	10			4	1	5.8±0.1	4.5	18	16	675.0
17	MS	30	10			4	1	5.8±0.1	4.5	18	16	688.5
18	MS	30	10			4	1	5.8±0.1	4.5	25	16	472.5
19	MS	30	10			4	1	5.8±0.1	4.5	30	16	297.0
20	MS	30		0.05		1.5		5.8±0.1	4.5	18	16	67.5 - 81.0
21	MS	30		0.05		1.5		5.8±0.1	4.5	25	16	67.5 - 81.0
22	MS	30		0.05		1.5		5.8±0.1	4.5	30	16	67.5 - 81.0
23	MS	30		0.05		1.5		5.8±0.1	4.5	25	24	67.5 - 81.0

(B) Latin square experiment: Murashige and Skoog basal medium with several combinations of auxin and cytokinin.

	Medium	Sucrose	Auxin (mg/L)			Cytokinin (mg/L)		pH	Gellen	Temp.	Light	
B		(g/L)	IAA	2.4-D	NAA	BAP	Kinetin		(g/L)	°C	Photo	µmol $m^{-2}s^{-1}$
24	MS	30		0.5		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
25				0.5		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
26				0.5		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
27				0.5		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
28	MS	30			0.5		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
29					0.5		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
30					0.5		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
31					0.5		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0
32	MS	30		1.0		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
33				1.0		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
34				1.0		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
35				1.0		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
36	MS	30			1.0		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
37					1.0		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
38					1.0		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
39					1.0		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0
40	MS	30		1.5		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
41				1.5		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
42				1.5		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
43				1.5		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
44	MS	30			1.5		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
45					1.5		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
46					1.5		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
47					1.5		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0
48	MS	30		2.0		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
49				2.0		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
50				2.0		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
51				2.0		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
52	MS	30			2.0		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
53					2.0		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
54					2.0		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
55					2.0		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0

(C) Latin square experiment: Gamborg B5 basal medium with several combinations of auxin and cytokinin.

C	Medium	Sucrose	Auxin (mg/L)			Cytokinin (mg/L)		pH	Gellen	Temp.	Light	
		(g/L)	IAA	2.4-D	NAA	BAP	Kinetin		(g/L)	°C	Photo	µmol $m^{-2}s^{-1}$
56	B5	20		0.5		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
57				0.5		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
58				0.5		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
59				0.5		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
60	B5	20			0.5		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
61					0.5		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
62					0.5		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
63					0.5		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0
64	B5	20		1.0		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
65				1.0		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
66				1.0		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
67				1.0		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
68	B5	20			1.0		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
69					1.0		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
70					1.0		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
71					1.0		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0
72	B5	20		1.5		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
73				1.5		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
74				1.5		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
75				1.5		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
76	B5	20			1.5		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
77					1.5		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
78					1.5		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
79					1.5		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0
80	B5	20		2.0		0.025		5.8±0.1	4.5	25	16	67.5 - 81.0
81				2.0		0.05		5.8±0.1	4.5	25	16	67.5 - 81.0
82				2.0		0.075		5.8±0.1	4.5	25	16	67.5 - 81.0
83				2.0		0.1		5.8±0.1	4.5	25	16	67.5 - 81.0
84	B5	20			2.0		0.025	5.8±0.1	4.5	25	16	67.5 - 81.0
85					2.0		0.05	5.8±0.1	4.5	25	16	67.5 - 81.0
86					2.0		0.075	5.8±0.1	4.5	25	16	67.5 - 81.0
87					2.0		0.1	5.8±0.1	4.5	25	16	67.5 - 81.0

Appendix C

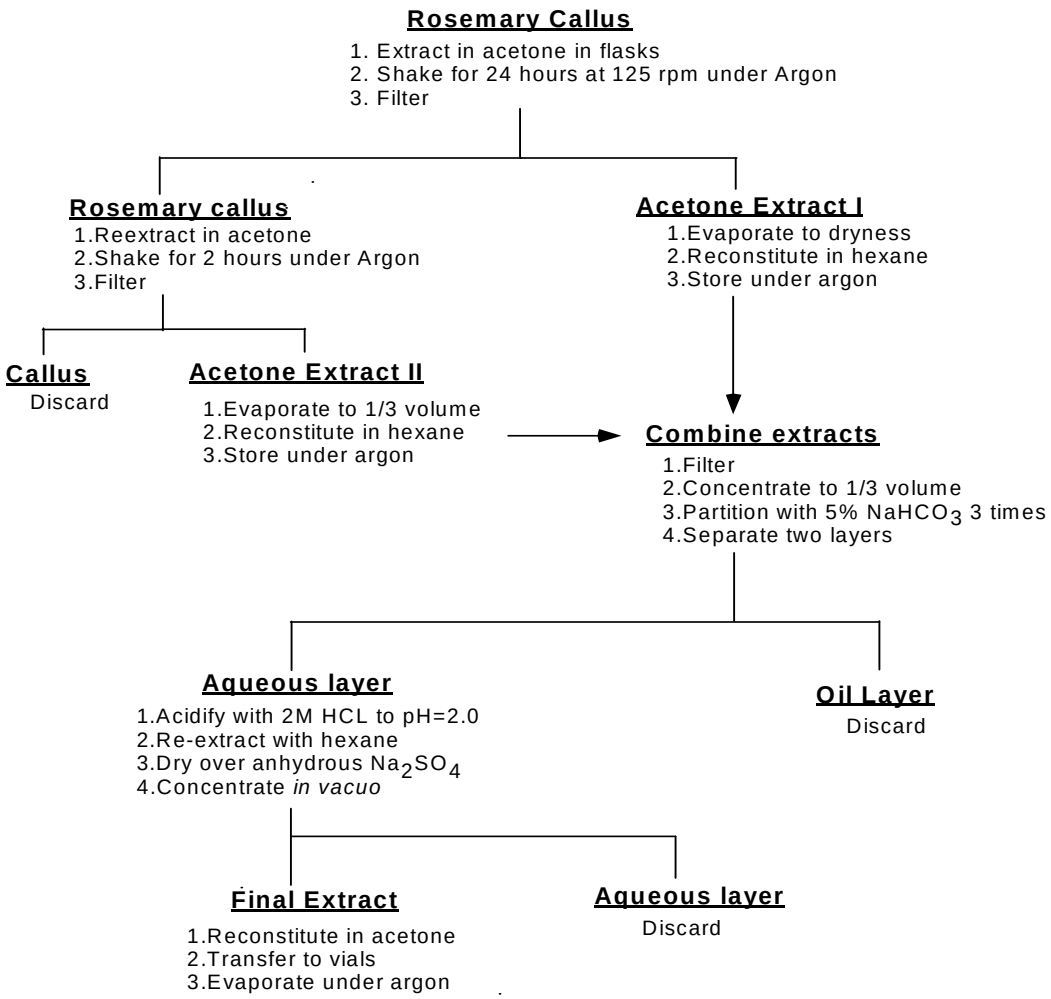


Figure C.1: Flow chart of phenolic diterpene extraction of rosemary calli established by Nadosy, 2002.

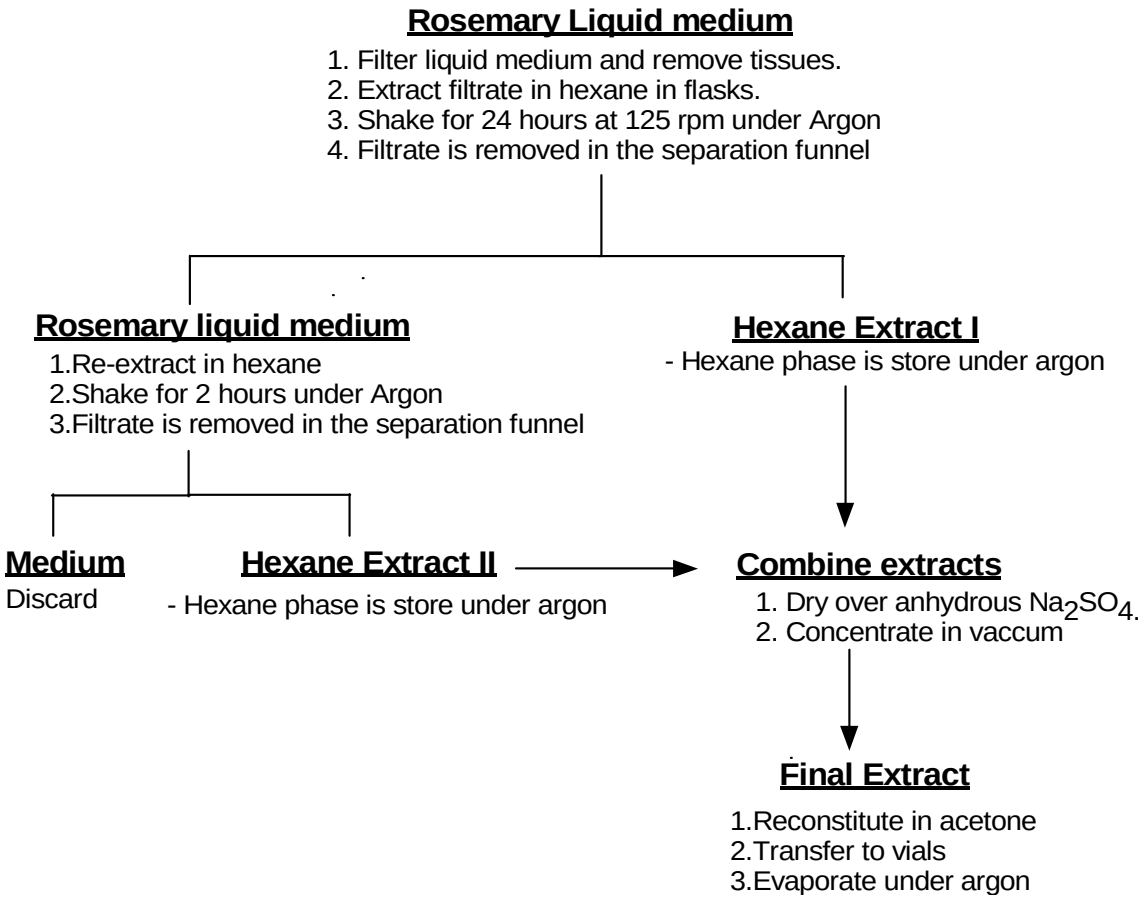


Figure C.2: Flow chart of rosemary liquid media extraction for phenolic diterpenes.

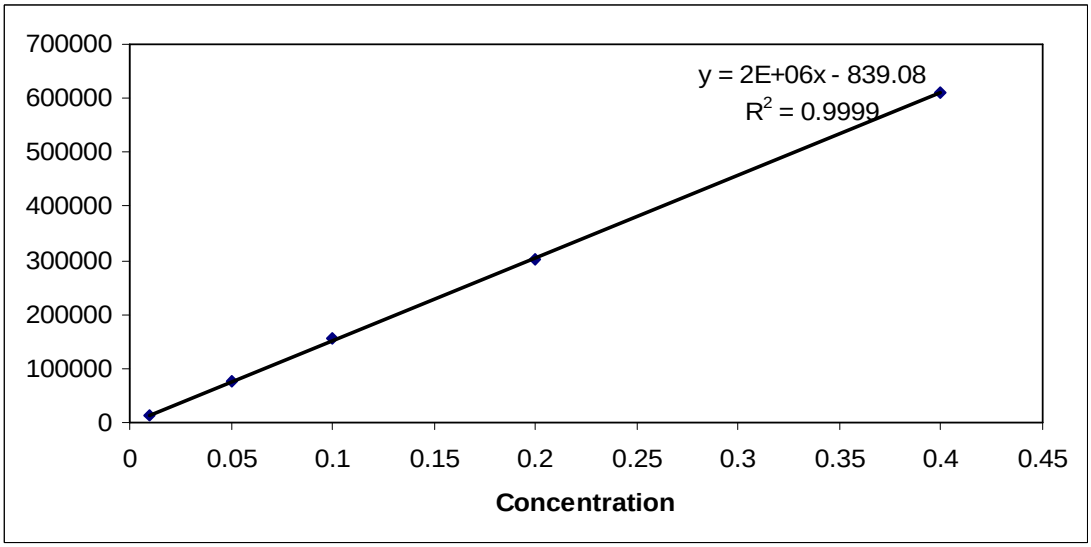


Figure C.3: Carnosic acid standard curve for HPLC.

Appendix D

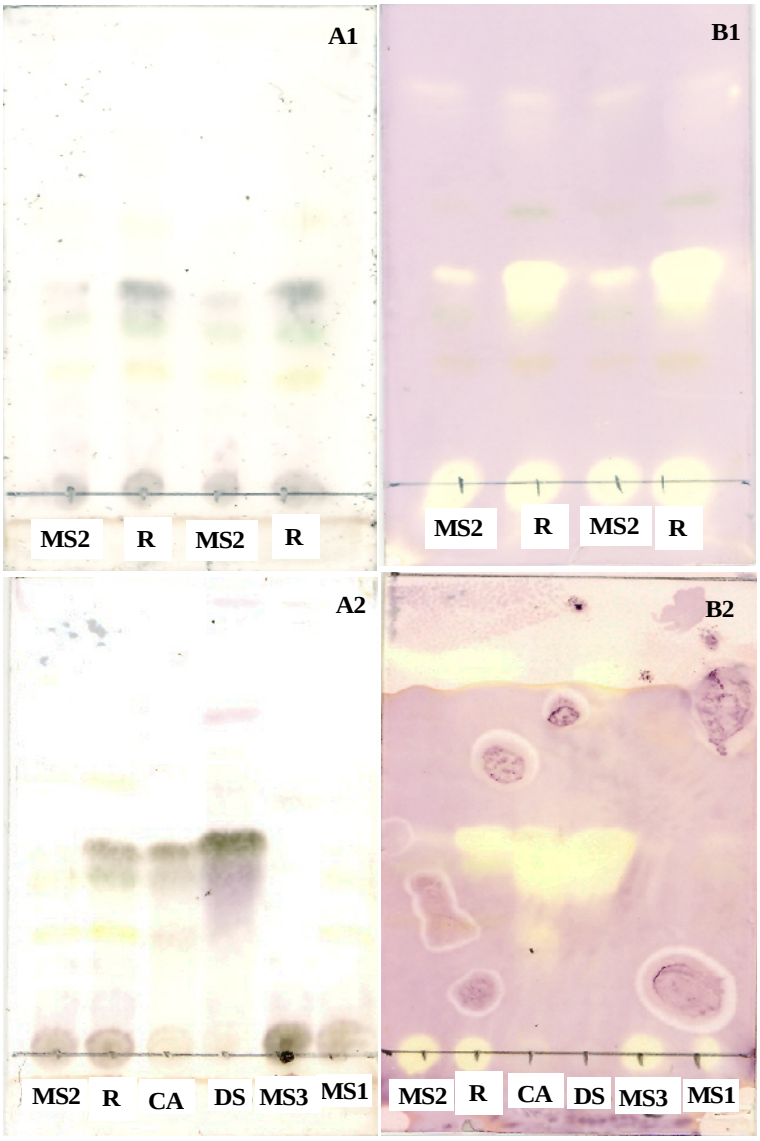


Figure D.1: The results of extracts screened by TLC and TLC-DPPH for the presence of carnosic acid and carnosol.
A1 and A2: TLC plate sprayed with 10% sulfuric acid;
B1 and B2: TLC-DPPH plate dipped in 400µM DPPH solution
MS1: Callus on MS medium with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin;
MS2: Callus on MS medium with 0.05 mg/L 2,4-D and 1.5 mg/L BAP;
MS3: Callus on MS medium with 0.5 mg/L 2,4-D and 0.075 mg/L Kinetin;
R: Regenerated shoots on MS medium with 10 mg/L IAA, 4 mg/L BAP, and 1 mg/L kinetin;
DS: Dried leaves of rosemary plants from Lehman college greenhouse;
CA: carnosic acid (standard)

Appendix E

Latin square experiment: Murashige and Skoog and Gamborg B5 basal media with several combinations of auxin and cytokinin (Concentration expressed in morality µM).

A	Medium	Auxin (µM)			Cytokinin (µM)	
		IAA	2.4-D	NAA	BAP	Kinetin
1	MS	1.752			0.901	0.215
2	MS	1.752			0.901	0.215
3	MS	1.752			0.901	0.215
4	MS	1.752			0.901	0.215
5	MS	1.752			0.901	0.215
6	MS	1.752			0.901	0.215
7	MS	1.752			0.901	0.215
8	MS	1.752			0.901	0.215
9	MS	1.752			0.901	0.215
10	MS	1.752			0.901	0.215
11	MS	1.752			0.901	0.215
12	MS	1.752			0.901	0.215
13	MS	1.752			0.901	0.215
14	MS	1.752			0.901	0.215
15	MS	1.752			0.901	0.215
16	MS	1.752			0.901	0.215
17	MS	1.752			0.901	0.215
18	MS	1.752			0.901	0.215
19	MS	1.752			0.901	0.215
20	MS		0.011		0.338	
21	MS		0.011		0.338	
22	MS		0.011		0.338	
23	MS		0.011		0.338	

B	Medium	Auxin (µM)			Cytokinin (µM)	
		IAA	2.4-D	NAA	BAP	Kinetin
24	MS		0.111		0.006	
25			0.111		0.011	
26			0.111		0.017	
27			0.111		0.023	
28	MS			0.093		0.005
29				0.093		0.011
30				0.093		0.016
31				0.093		0.022
32	MS		0.221		0.006	
33			0.221		0.011	
34			0.221		0.017	
35			0.221		0.023	
36	MS			0.186		0.005
37				0.186		0.011
38				0.186		0.016
39				0.186		0.022
40	MS		0.332		0.006	
41			0.332		0.011	
42			0.332		0.017	
43			0.332		0.023	
44	MS			0.279		0.005
45				0.279		0.011
46				0.279		0.016
47				0.279		0.022
48	MS		0.442		0.006	
49			0.442		0.011	
50			0.442		0.017	
51			0.442		0.023	
52	MS			0.372		0.005
53				0.372		0.011
54				0.372		0.016
55				0.372		0.022

C	Medium	Auxin (µM)			Cytokinin (µM)	
		IAA	2.4-D	NAA	BAP	Kinetin
56	B5		0.500		0.006	
57			0.500		0.011	
58			0.500		0.017	
59			0.500		0.023	
60	B5			0.500		0.005
61				0.500		0.011
62				0.500		0.016
63				0.500		0.022
64	B5		1.000		0.006	
65			1.000		0.011	
66			1.000		0.017	
67			1.000		0.023	
68	B5			1.000		0.005
69				1.000		0.011
70				1.000		0.016
71				1.000		0.022
72	B5		1.500		0.006	
73			1.500		0.011	
74			1.500		0.017	
75			1.500		0.023	
76	B5			1.500		0.005
77				1.500		0.011
78				1.500		0.016
79				1.500		0.022
80	B5		2.000		0.006	
81			2.000		0.011	
82			2.000		0.017	
83			2.000		0.023	
84	B5			2.000		0.005
85				2.000		0.011
86				2.000		0.016
87				2.000		0.022

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