STUDY OF PHENOLIC COMPOUNDS PRODUCTION IN SPEARMINT (MENTHA SPICATA L.) CLONES REGENERATED IN VITRO

DOCTORATE THESIS

DANI FADEL

Advisor
Professor Athanasios S. Economou

Thessaloniki 2010
STUDY OF PHENOLIC COMPOUNDS PRODUCTION IN SPEARMINT (*MENTHA SPICATA* L.) CLONES REGENERATED IN VITRO

DOCTORATE THESIS

DANI FADEL

Examining Committee

Professor Athanasios Economou, Advisor
Professor Helen-Isis Constantinidou, Member of the Advisory Committee
Professor Spiridon Kintzios, Member of the Advisory Committee
Professor Demetrios Voyiatzis, Examiner
Assistant Professor Christos Dordas, Examiner
Assistant Professor Diamanto Lazari, Examiner
Lecturer Irene Nianiou-Obeidat, Examiner

Thessaloniki 2010
I would like to thank the National Scholarship Foundation of Greece for the financial support and for giving me an opportunity to learn Greek, the language of my ancestors.
Acknowledgements
ACKNOWLEDGEMENTS

I would like to thank God for giving me the courage to complete this Doctorate Thesis.

I would also like to thank my advisory committee for their support and guidance throughout my research, in particular Professor Athanasios Economou, Professor Eleni–Isis Constantinidou and Professor Spiridon Kintzios that piqued my interest in traditional herbal medicine. I am indebted to them for this particular path in my life. I would also like to say a warm thank you to Professor Demetrios Voyiatzis, Assistant Professor Christos Dordas, Assistant Professor Diamanto Lazari and Lecturer Irene Nianiou-Obeidat in the Examining Committee for their attention, time and patience in realizing this thesis.

I express my sincere thanks to Professor Spiridon Kintzios who gave me the opportunity to fulfill the requirements for this Doctorate Thesis.

I am thankful for the technical assistance of Dr. Giorgos Liakopoulos (Lecturer, Laboratory of Plant Physiology, AUA) in the execution of the HPLC analysis and for the guidance of Mrs. Georgia Moschopoulou in the laboratory experiments.

I thank Mrs. Ghada Al Bandak for allowing me to take all my research frustrations out on her as well as Mr. Wadih Nasr for his unlimited support.

Further, I would like to thank the Greek National Scholarship Foundation for the financial support and for giving me an opportunity to learn Greek, the language of my ancestors.

I am eternally grateful to my parents especially my mother and my brother William for giving me the support, confidence and encouragement to endure what life gives me and be the best I can.
Contents
TABLE OF CONTENTS

ACKNOWLEDGEMENTS .................................................................i
TABLE OF CONTENTS ..................................................................iii
LIST OF ABBREVIATIONS ..............................................................v

INTRODUCTION
SPEARMINT

Spearmint ......................................................................................1
Spearmint uses .............................................................................2
Spearmint of high phenolic content ..........................................4
Antioxidants in human ...............................................................5
Role of antioxidants in the metabolism ...................................8
Rosmarinic acid ..........................................................................10
Source of rosmarinic acid in the plant kingdom .......................10
Properties of rosmarinic acid ....................................................10
Figures and tables .......................................................................12

OBJECTIVES .............................................................................15

Chapter I
IN VITRO REGENERATION OF SPEARMINT
Abstract ......................................................................................18
Introduction ................................................................................19
Materials and Methods ..............................................................20
  Chemicals .............................................................................20
  Plant material, preparation and shoot induction ......................20
  Shoot and root formation .......................................................21
  Description of measurements ................................................21
Results and Discussion ..............................................................22
  Shoot induction from nodal explants ......................................22
  Shoot proliferation, rooting and plantlet production ..............22
  Evaluation of organogenesis ....................................................23
Figures and Tables .......................................................................25

Chapter II
TOTAL PHENOLIC CONTENT IN SHOOT AND ROOT EXTRACTS OF SPEARMINT
Abstract ......................................................................................31
Introduction ................................................................................32
Materials and Methods ..............................................................33
  Chemicals .............................................................................33
  Total phenolic extraction .......................................................33
  Statistical analysis .................................................................34
Results and Discussion ..............................................................35
Figures and Tables .......................................................................37

Chapter III
LIST OF ABBREVIATIONS

4CL  4-coumarate:coenzyme A ligase
ANOVA Analysis of variance
AUA Agricultural University of Athens
BA 6-benzyl aminopurine (6-benzyladenine)
CAH Cinnamic Acid Hydrolase
CoA Coenzyme A
Cu$^{2+}$ Copper ion
DNA Deoxyribonucleic acid
DPPH 1,1-Diphenyl -2-picrylhydrazyl
DW Dry weight
Fe Iron
f w Fresh weight
H$_2$O$_2$ Hydrogen peroxide
HCA Hydroxycinnamic acid
HPPR Hydroxy Phenyl Pyruvate Reductase
LDL Low density lipoproteins
L. Linnee
mg milligrams
mL millilitres
µL microlitres
MS Murashige and Skoog
MS Microsoft (MS-excel)
NAA 1 naphtalene acetic acid
NADP Nicotinamide adenine dinucleotide phosphate (oxidized form)
PAL Phenylalanine ammonia-lyase
PEP Phosphoenolpyruvate
PG Propyl gallate
PH Phenolics
RA Rosmarinic acid
ROS Reactive oxygen species
HPLC High-performance liquid chromatography
SE Standard error
SM Secondary metabolites
TBHQ Tert-butyl hydroxyquinone
TLC Thin layer chromatography
UV Ultraviolet
VIS Visible
Introduction
INTRODUCTION

**Spearmint (Mentha spicata L.)**

The genus *Mentha* reportedly consists of about 25–30 species (Ali *et al.*, 2002). It is the most important genus in the tribe “Mentheae”, subfamily “Neopetoideae”, family “Lamiaceae”, class “Dicotyledones”, subdivision “Angiospermae” and division “Spermarophyta” of the regnum “Plantae”. Today, taxa of *Mentha*, either native to the Mediterranean region or naturalized throughout most of the world, are ubiquitous and found on all continents except Antarctica. In Linné taxonomy, *Mentha spicata* L. (spearmint) is a species of mint native to much of Europe and southwest Asia, though its exact natural range is uncertain due to its extensive early cultivation (http://www.economicexpert.com/a/Carolus: Linnaeus.html).

Spearmint, a hybrid of *M. longifolia* and *M. rotundifolia*, is a herbaceous rhizomatous perennial plant growing 30 -100 cm tall, with variably hairless to hairy erect, square stems, bearing very short-stalked, acute-pointed, lance-shaped, wrinkled, bright green leaves of 3-7 cm length and 1.5-3 cm broad, with finely toothed edges and smooth surfaces with very prominent ribs beneath. The flower is known as hypogogenous since all its parts are below the ovary where, the 5 fused petals diverge into 2 lips (old family name, Labiatae). The 4-lobed regular corolla and the 5-lobed pubescent calyx form an upper and lower lip.

Spearmint is named after the spear-shaped leaves of the plant (*Figure 1*). It is known also as garden mint, english mint, garden mackerel mint, our lady's mint, green mint, spire mint, sage of Bethlehem, fish mint and lamb mint. According to multilingual dictionary of agronomic plants edited by Rehm in 1994, spearmint is called “menthe verte” or “menthe de Notre Dame” in French, “grüne Minze” or “Krauseminze” in German, “Erba Santa Maria” in Italian, “hortelã-comum”, “hortelã-preta” or “hortelã-verde” in Portuguese (Brazil), “menta romana” or “yerbabuena” in Spanish.
Mentha cordifolia auct.non Opiz, Mentha crispata Schrad., Mentha incana, Mentha longifolia auct., non (L.) Huds, Mentha sylvestris auct., non (L.), Mentha viridis (L.) L. and Mentha niliaca auct., non Juss ex Jacq are considered as synonyms of Mentha spicata (nomencl. Ref. Sp. Pl., 576. 1753) according to Lawrence, 2007. Spearmint can be propagated by seeding, creeping root cuttings (rhizomes) in early spring, softwood cuttings in summer and by growing stolons in the soil (green) or on the surface (white).

According to Greek mythology, the word mint comes from “Minthe”, a Greek nymph who had the misfortune to be loved by Pluto. Persephone, not taking kindly to this infidelity, changed her into a lowly plant. Because Philemon and Baucis rubbed the table with mint before serving a meal to Zeus and Hermes, who had been traveling incognito and who had been snubbed by villagers, it has become a symbol of hospitality (Lawrence, 2007). Ancient Greeks used spearmint in medicine for the comfort of the head and for gargle, in temple rituals for air refreshment.

Several papers have reported on spearmint micropropagation for a number of reasons, including breeding and the rapid multiplication of elite plants. Mentha has a large number of species that differ widely in their characteristics and polyploidy level. Morphological, cytological and biochemical data have shown that the tetraploid species of spearmint (2n=48) originated by chromosomal doubling of hybrids between the two closely related and inter-fertile diploids, M. longifolia and M. suaveolens (Harley and Brighton, 1977). As a hybrid, spearmint produces seldom seeds able to germinate and for this reason, it is mainly propagated from its vegetative parts, i.e., green shoots, underground stolons and rooty turions. In addition, high costs and the plants’ high demand for manual labor disallow large-scale propagation with green cuttings, which is appropriate only for the fast propagation of improved clones.

**Spearmint uses**

Lamiaceae consists mostly of herbs and shrubs characterized by having a strong pleasant odor due to epidermal glands and a characteristic
square stem (Willard, 1993). Spearmint use has been developed through centuries in foods, flavours, beverages, toothpastes, mouthwashes, soaps, detergents, creams, lotions, perfumes and medicinally as carminative and anti-spasmodic (Bhat et al., 2002).

Spearmint, is widely used as a source of essential oil (Papachristos et al., 2002), flavours and antioxidants (Arumugam et al., 2006). In the last decade, spearmint was grown exclusively for the essential oil production in order to meet the demand of the rapidly growing herb industry (Barl, 1996). All species and natural hybrids of the Mentha genus are essential oil-bearing of high economical value. Because of the aromatic properties of these taxa, their oils have been used traditionally for more than 2000 years. These oils are corn mint (the source of natural menthol), peppermint, scotch spearmint, and native spearmint. The amount of the oils produced annually is in excess of 23,000 metric tones with a value exceeding 400 million dollars only in the USA (Lawrence, 2007). In 1984, the total world mint production came to: peppermint 2.2 metric tones, corn mint 2.1 metric tones and spearmint 1.4 metric tones (Lawrence, 1985). There are some reports concerning world menthol production, but new statistics concerning mint as a crude material are not available. Clark (1998) estimated the world production of menthol at 11.8 metric tones. The produced and exported quantities estimated to come from India, China and others with 5630, 2500 and 3670 metric tones respectively. Most of the production (9400 metric tones) is from the crude oil of M. arvensis. The vast majority of this oil comes from India, rendering this country the most economically important essential oil producer globally. The three main species of mint in respect to cultivation and general use are spearmint (Mentha spicata), peppermint (M. piperita), and pennyroyal (M. pulegium), the first being the one ordinarily used for cooking. Spearmint, known also as carvone-scented mint plant, is either tetraploid (2n = 48) or triploid (2n = 36). It produces the monoterpene “carvone” as the major oil component followed by limonene (Tucker, 1992).

Spearmint has long been used for flavor and medicinal functions. However, market trends for phytomedicinals, cosmetic and personal
hygiene products containing natural protective compounds are expanding the market value of antioxidant producing crops and therefore should be seriously considered as an alternative cash crop for producers. The current sources of Mentha spicata L. (spearmint), Origanum vulgare L. (oregano), Thymus vulgaris L. (thyme), Rosmarinus officinalis L. (rosemary) and Lavandula augustifolia Mill, (lavender), all belonging to Lamiaceae, are originated from genetically heterogeneous populations. The genetic variation is due to natural cross-pollination typical in these plants. The result is compositional differences in the final extracts of essential oils and other compounds, creating difficulties in commercial applications. To eliminate this variation there is a need to develop genetically stable lines that are vegetatively maintained to provide stable ingredient quality and quantity (Perry and Shetty, 1999). They demonstrated the use of plantlet cultures to manufacture secondary metabolites. Studies on biosynthesis and biotechnology have led to a better understanding of the physiology, molecular biology and tissue culture potential for the production of oil, phenolics and rosmarinic acid of the important Mentha taxa (Ellis, 1997; Maffei et al., 2007). For example, Tisserat and Vaughn (2008) recently discussed growth, morphogenesis, and essential oil production of spearmint plantlets in vitro.

**Spearmint of high phenolic content**

Spearmint belongs to Lamiaceae, a family in which species are naturally cross-pollinating. Obligate out-crossing creates a heterogeneous population with many compositional differences, making it difficult to standardize the extracts used in commercial applications. As previously discussed, the genetic variation is typical in all commercially exploited plants of Lamiaceae such as oregano, rosemary, sage and mint (Shetty et al., 1999). Previous studies have identified plant clones containing elevated levels of secondary metabolites to improve and maintain ingredient quantity and quality. Clonal propagation using tissue culture methods is a rapid means for the production of genetically identical plants. It also provides an excellent opportunity to enhance the productivity of secondary metabolites by manipulation of experimental conditions (Shetty and
Shoot cultures were used since they are genetically more stable than undifferentiated callus cultures and have increased mechanical strength, aiding to subsequent survival in greenhouse and field conditions.

Research has established that plants with high phenolic levels could be an important natural solution for problems currently encountered within the food industry. The production of bioactive plants often requires more hand labor and specialized equipment than cereal or oilseed crops. Therefore these crops are usually produced on a small scale and harvested for leaf, root or flower. Spearmint is considered as one bioactive plant species associated with antioxidative properties. Scotch spearmint was first grown in 1994 as a source of essential oil. The leaders in the production of mint include India, China and the United States. India has developed a new, patented variety of Mentha arvensis, which allows for three harvests per year, thereby increasing overall yield. India produces 70% of the international demand of menthol mint oil of 20,000 tonnes (Nag & Raj, 2000). One acre of spearmint (approximately 1000 kg fresh weight), yields close to 32.4 kg of oil (Gaudiel, 1996). As the demand for antioxidant compounds increases, breeders and producers are striving to produce an economically valuable crop.

**Antioxidants in humans**

Recently, medical researchers have examined the role of bioactive compounds in plants, herbs and spices with respect to promoting human health. On a daily basis, humans are constantly exposed to reactive oxygen species (ROS), which can cause irreparable damage. ROS have a single unpaired electron in the outer orbital shell that is highly reactive. They will subtract an electron or hydrogen atom from cell walls, mitochondria or DNA, to fill this unpaired orbital. This type of reaction caused by ROS has been associated with cancer growth, heart disease and stroke (Peterson *et al.*, 1999).

The human body provides antioxidants that can counteract some of these free radicals. Superoxide dismutase and glutathione
peroxidase are two well studied enzymes that metabolize free radicals in human systems. However, lifestyle factors such as stress, cigarette smoke, drugs, processed foods and environmental pollution contribute to the production of excess free radicals in the body (Balch, 1998). Therefore, it has been suggested that increased consumption of antioxidant rich foods can protect from free radical damage in the following five ways (Robards et al., 1999):

1. Free radical scavenging
2. Hydrogen donation
3. Singlet oxygen quenching
4. Metal-ion chelation
5. Substrates for attack by superoxide anion

In depth studies have demonstrated that the biochemical properties of polyphenol secondary plant metabolites are conducive to elicit antiviral, antibacterial, vasoactive, antiatherogenic, antiproliferative and anti-inflammatory activities (Rajan et al., 2001). The presence of hydroxyl groups on the aromatic ring reacts with the free radicals. The close proximity of hydroxyl groups allows these compounds to form strong intermolecular hydrogen bonds. This bond facilitates the delocalisation of the unpaired electrons of the radical species and leads to their overall stabilization (Rajan et al., 2001). In order to be effective, the antioxidant must be a relatively stable free radical.

For example, many studies have demonstrated that the polyphenols found in tea have protective effects against the oxidation of lipids and low-density lipoproteins and scavenge reactive nitrogen species (Rice-Evans, 1999). The properties of these compounds allow the polyphenol compound to reduce the free radicals through hydrogen donation. The ability to scavenge the lipid peroxy radical depends on the accessibility of the antioxidant to the free radical. The greater the solubility in aqueous solvents, the less was the efficacy in inhibiting lipid peroxidation (Rice-Evans, 1999). Numerous studies have focused on polyphenols compounds extracted from tea (green and black) and fruits due to the dietary consumption of these foods. Today, public awareness in preventative and alternative medicine has
stimulated research in medicinal and culinary plants commonly used in different cultures worldwide.

In medical research, the lipid peroxidation test is used to reflect the potential of these antioxidants to prevent low-density lipoprotein (LDL) oxidation, a leading cause of atherosclerosis. Compounds effective in inhibiting lipid peroxidation may have a profound effect on the prevention of this disease (Chalas et al., 2001). Gallic and cinnamic acid derivatives as well as caffeic acid, present in many plant species have been studied as free acids and esters for protection from LDL (Chalas et al., 2001; Rajan et al., 2001). Esters and amides have higher lipophilic properties than their acid counterparts and therefore have increased protective properties towards the lipoproteins. Caffeic and sinapic acid esters inhibited Cu²⁺-induced LDL oxidation at concentrations as low as 5µM and 10nM respectively (Chalas et al., 2001). A decrease in hydrogen peroxide concentration was also observed.

The following steps show the role of an antioxidant (ArOH) in prevention of lipid peroxidation by interrupting the chain reaction:

1) RH —> R· (initiation)
2) R· + O₂ —> R₀₂· (addition of O₂)
3) R₀₂· + RH —> ROOH + R·
   (H-atom exchange, back to reaction 2)
4) R₀₂· + ArOH —> ** ROOH + ArO·
   (Interruption of chain reaction)

Several epidemiological studies have shown that metabolites of the shikimate pathway (Figure 2) provide protection against heart disease and cancer (Craig, 1999). The means by which the antioxidant gives protection is postulated to occur at the initial stage and during the propagation stage of oxidation by peroxy radical (ROO·) scavengers such as phenolic compounds (Basaga et al., 1997). This electron transfer mechanism gives the same result (ROOH + ArO) as the atom transfer mechanism. These dietary studies indicate that the increased consumption of leafy green vegetables and fruits that have a high phenolic content can reduce blood pressure, a major factor of heart disease (Lampe, 1999). Antioxidative compounds possibly slow the
proliferation of cancer cells through a different mechanism. Plant antioxidants have been shown to chelate Fe, deprive cells of the necessary ion and therefore slow the rate of cellular proliferation (Richardson, 1997).

**Role of antioxidants in metabolism**

Oxidation in biological organisms is a normal consequence of metabolism. Apart from the beneficial role of oxidation reactions formation of detrimental free radical by-products and reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and singlet oxygen (O$_2^*$) occurs during the process. These species induce oxidative stress, which occurs when the body is unable to control excess ROS by its cellular antioxidant enzyme systems and extracellular antioxidant compounds. Left uncompensated, the ROS possess an unpaired electron in the outer orbit that attacks DNA, lipids in cell membranes and/or structural and functional proteins. Subsequently, cell walls become ruptured or leaky and genetic coding can be damaged. Reactive oxygen species also attack the lipids in the blood and tissues, causing low-density lipid proteins to become sticky and form plaques. These examples of oxidative damage play a key role in chronic diseases such as cardiovascular disease, cancer initiation, aging, inflammatory diseases and some neurological disorders (Lampe, 1999).

Dietary antioxidants have become very important due to their ability to quench the initiation and propagation steps of auto-oxidation chain reactions (Moure et al., 2001). These exogenous secondary metabolites can aid the body's own defense mechanisms in preventing or repairing the damage caused by oxidative stress. The awareness of maintaining health and prevention of disease has increased the consumption of vitamins and other antioxidant supplements by consumers (Marangoni, 2000).

The interest by the general population has compelled researchers to investigate the efficacy of dietary antioxidants in disease prevention. Oxidation reactions are not only a concern for the growing nutraceutical industry but for any industry involved in the production of oxidizable goods. Public awareness of synthetic products is forcing the food, cosmetic, pharmaceutical and plastic industries to look at the viability of substituting
natural antioxidants for synthetic ones. The most commonly used synthetic types of antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroxyquinone (TBHQ). Plants have established various defense mechanisms to protect metabolic functions against the harmful free radicals that are continuously generated by oxidation. Several groups of secondary metabolites in higher plants have the capacity to react as antioxidants by various mechanisms: free radical scavenging, hydrogen donation, singlet oxygen quenching, metal-ion chelation, or as substrates for attack by superoxide (Robards et al., 1999). Studies have demonstrated that certain phenolic compounds have the above antioxidant abilities. They are ubiquitous in the plant kingdom and are easily extracted, which is ideal for studying their antioxidant capacity.

Secondary compounds from plants have been incorporated into a wide range of both commercial and industrial applications. In many cases rigorously controlled in vitro plant cultures can generate the same valuable natural products. Plants as well as in vitro plant cells or tissue cultures have served as resources for preservatives, natural pigments, flavors, enzymes, biobased fuels and plastics, cosmetics, and bioactive compounds (Mary, 2005). A series of distinct advantages is evident such as production can be more reliable, simpler, and more predictable; isolation of the phytochemical can be rapid and efficient; interfering compounds that occur in field-grown plant can be avoided in the vitro cultures; cell cultures can yield a source of standard phytochemicals in large volumes; secondary products through in vitro plant culture and some other ways can be generated on a continuous year-round basis without seasonal constraints; production is independent of ambient weather of a valuable secondary product in the in vitro plant cultures. There are several in vitro culture systems (callus, suspension cell, shoot, and hairy root cultures) for production of plant secondary metabolites.

Examples of use of natural antioxidants in various industries include: antioxidant enhanced facial creams and sun blocks, antioxidant stabilizers for wines and the use of Lamiaceae extracts as natural antioxidants in meat processing and deep-fat frying (Katiyar et al., 2000; Chen and Ho, 1997; Jaswir et al., 2000). One of the principle antioxidative constituents in extracts used for food processing is rosmarinic acid (Chen and Ho, 1997).
Rosmarinic acid

Rosmarinic acid (Figure 2), derived from caffeic acid and 3, 4-dihydroxyphenyl lactic acid, represents one of the most common caffeic esters in plant material and is accumulated constitutively (Ellis and Towers, 1970). It is a well-known natural product from rosemary (Rosmarinus officinalis), lemon balm (Melissa officinalis), and other medicinal plants like thyme, oregano, savory, peppermint, sage (Lu and Foo, 1999; Kochan et al., 1999; Zheng and Wang, 2001). Rosmarinic acid (RA), exhibits various pharmacological activities including prevention of oxidation of low density lipoprotein, inhibition of murine cell proliferative activity and of cyclooxygenase, and anti-allergic action. The biological activity of RA is described as antibacterial, antiviral, and antioxidative (Szabo et al., 1999; Hras et al., 2000). As discussed in subsequent section, its activity, especially against rheumatic and inflammatory conditions, makes it a sought-after substance for use in phytotherapy (Pabsch et al., 1991). More recently, rosmarinic acid was reported to have anti-HIV activities (Chen et al., 1999).

Source of rosmarinic acid in the plant kingdom

Rosmarinic acid occurs throughout the Boraginaceae, whereas within the Lamiaceae it is restricted to the sub-family Nepetoideae (Litvinenko et al., 1975). For example, it occurs in ferns of the family Blechnaceae (Häusler et al., 1992), lower plants such as the hornworts (Takeda et al., 1990) and in monocotyledonous plants like the sea grass family Zosteraceae (Ravn et al., 1994), the related Potamogetonaceae as well as the Cannaceae.

Properties of rosmarinic acid

Rosmarinic acid has antioxidant, anti-inflammatory and antimicrobial activities (Park et al., 2008). It is used to treat peptic ulcers, arthritis, cataract, cancer, rheumatoid arthritis and bronchial asthma. The antioxidant activity of rosmarinic acid is stronger than that of vitamin E. Rosmarinic acid helps to prevent cell damage caused by free radicals, thereby reducing the risk for cancer and atherosclerosis. It also has anti-inflammatory properties.
(Sahu et al., 1999) and the ability to inhibit complement activity at sites of inflammation. Prolonged inflammation, however, can cause damage to surrounding healthy tissue. Research directed at discovering drugs that are capable of controlling complement activation, has focused on natural anti-inflammatory compounds. This research is supported by encouraging results demonstrating that rosmarinic acid effectively inhibits C₃b attachment, which is a protein involved in the classic pathway of complement activation and is termed a component of complement (Sahu et al., 1999). Perilla, rich in rosmarinic acid, is used for its anti-allergic activity. A study by Sanbongi et al. (2004) has shown that the oral administration of rosmarinic acid is an effective intervention for allergic asthma. Another study by Youn et al. (2003) demonstrated that rosmarinic acid suppressed synovitis in mice and that it may be beneficial for the treatment of rheumatoid arthritis. Unlike antihistamines, rosmarinic acid prevents the activation of immune responder cells, which cause swelling and fluid formation. It has been reported also to have antiviral and antibacterial properties (Szabo et al., 1999) and for its protection of fats during food processing by inhibiting lipid peroxidation (Che Man and Tan, 1999). Besides rosmarinic acid, other specialized antioxidant compounds in high concentrations such as caffeic and carnosic acids, make plants belonging to Lamiaceae family prime candidates for natural antioxidant research. In Japan, rich in rosmarinic acid perilla extracts are used to garnish and improve the shelf life of fresh seafood.
Figures and Tables
Figure 1. *Mentha spicata* (spearmint) plant.
Figure 2. Biosynthetic pathway of rosmarinic acid (Petersen et al., 1999)
PAL = phenylalanine ammonia-lyase, CAH = cinnamic acid-4-hydroxylase,
4CL= hydroxycinnamate:coenzyme A ligase, TAT = tyrosine
aminotransferase, HPPR = hydroxyphenylpyruvate reductase, HPPD =
hydroxyphenylpyruvate dioxygenase, RAS = hydroxycinnamoyl-
CoA:hydroxyphenyllactate hydroxycinnamoyl transferase, 3-H, 3-H’ =
hydroxycinnamoyl – hydroxyphenyllactate 3- and 3’ hydroxylases.
Objectives
OBJECTIVES

Standardize a protocol for spearmint (*Mentha spicata* L.) shoot proliferation and plantlet regeneration from nodal explants cultured in full strength MS basal medium (Murashige and Skoog, 1962) supplemented with different combinations of NAA and BA concentrations.

Investigate the effect of different strength of MS media (full MS, ½ and ¼ MS) on organogenesis (shoot and root formation).

Determine total phenolic content, total rosmarinic acid content and antioxidant activity (DPPH- IC₅₀) of shoot and root extracts from spearmint regenerants and correlate between organogenesis and biochemical data.

Assay the activity *in vivo* of enzymes (PAL, CAH and HPPR) involved in the biosynthesis of rosmarinic acid in regenerants compared to donor plants.

Assay the antioxidant capacities of methanolic extracts from spearmint regenerants compared to those from donor plants using different antioxidant methodologies, i.e. reduction of Fe³⁺ to Fe²⁺ with the subsequent formation of phenanthroline chloride- Fe²⁺ complex, DPPH free radical scavenging and hydrogen peroxide scavenging activities.
Chapter I
IN VITRO REGENERATION OF SPEARMINT

Abstract

A direct methodology for efficient shoot proliferation of spearmint (*Mentha spicata*) has been developed by studying the interactive effect of two plant growth regulators. A protocol was standardized for nodal explants when cultured on Murashige and Skoog (MS) medium supplemented with 1 mg/l NAA and 9 mg/l BA for high efficiency shoot proliferation within four weeks. Ten shoots from each nodal culture were obtained. Among the produced shoots, one hundred explants grown *in vitro* for 12 weeks were transferred for four weeks into a hormone-free MS medium for rooting and 99% plantlets regenerated. In parallel but separately, the effect of different strength media (MS, ½ MS and ¼ MS) on the organogenesis of shoots and roots from nodal segments of the regenerants was investigated and correlation among collected data was determined. The optimum shoot proliferation and root formation was obtained in ½ MS salts. The increase in MS basal strength was accompanied with an increase in shoot number and shoot length of grown explants. Roots were only developed in ½ strength MS and full strength MS media. The explants grown in ½ strength MS produced almost twice the number of roots with shorter length than the ones grown on full MS medium.
Introduction

In vitro high efficiency procedures for callus, embryos, cell, organ cultures and shoot regeneration from axillary buds and leaf explants have been reported. Further, different growth media and growth factor effect on embryonic suspension and callus cultures were investigated (Ajit Kumar Shasany et al., 1998; Bhat et al., 2002).

Minerals are important components of the culture medium. There is a large choice of combinations of macro- and micro-salt mixtures. The most widely used culture medium is Murashige and Skoog (1962) medium, with most plants reacting to it favorably. It contains all the elements that have been shown to be essential for plant growth in vitro. It is classified as a high salt medium in comparison to many other formulations, with high levels of nitrogen, potassium and of some of the micronutrients, particularly boron and manganese (Cohen, 1995). Due to its high salt content, however, this nutrient solution is not always optimal for growth and development of plants in vitro (Pierik, 1997).

Interest in cultivating spearmint, an important medicinal plant, has become paramount. Its leaves are generally subscribed for fever and bronchitis and its decoction is used as lotion in aphthae (Mshaskar et al., 2000). Spearmint is widely used as a source of essential oil having a strong toxic and repellent effect on the insect Acanthoscelides obtectus (Papachristos et al., 2002), as a source for flavoring (Arumugam et al., 2006), and more recently as a valuable source for its potent antioxidant rosmarinc acid for the neutraceutical and cosmetic industries (Bader et al., 2000, Maughan et al., 2004; Shetty K, 2001). Also it is used in fragrance formulations (Lawrence et al., 1992).

In the past, protocols for plantlet regeneration from axillary buds and leaf spearmint explants have been reported and the effect of different nutrient media and growth regulators was investigated (Shasany et al., 1998; Bhat et al., 2002). Recently, Tisserat and Vaughn (2008) discussed growth, morphogenesis, and essential oil production of spearmint plantlets in vitro and demonstrated the use of plantlet cultures to manufacture secondary metabolites. The growth media used in this study has permitted high efficiency shoot proliferation from internodes of spearmint at relatively
short period of time.

The aim of this study was (a) to standardize a protocol for shoot proliferation and plantlet regeneration from shoot nodal segments cultured on full strength MS media supplemented with different combinations of NAA and BA concentrations and (b) to investigate the effect of different strengths of MS media (MS, ½ MS and ¼ MS) on organogenesis (shoot and root number) of one hundred and fifty spearmint regenerants.

Materials and Methods

Chemicals

1-naphtalene acetic acid (NAA), 6-benzyl aminopurine (BA), and kinetin were purchased from Serva (Heidelberg, NY). Chemical compounds necessary for Murashige and Skoog (MS) medium preparation were of analytical grade, available commercially from Sigma (St Luis, MI).

Plant material, preparation and culture media

In vivo apical shoot portions carrying 4-5 nodes were separated from six-month-old spearmint plants, washed and surface sterilized for 12 min with 0.1 % (w/v) HgCl₂ containing 20µl of Tween 20 followed by thorough washing in sterile distilled water.

The shoots were subsequently cut into pieces carrying a node and internodal segments. Pieces were then incubated vertically in shoot proliferation medium composed of the basal MS medium where 3% sucrose (w/v), 0.8% agar (w/v), 0.1 % myo-inositol (w/v) were added in combination with 1 mg/l 1-naphtalene acetic acid (NAA) and various concentrations of 6-benzyl aminopurine (BA:1-5-9 mg/l) and with a pH adjusted to 5.8. Cultures were maintained at 25±2ºC under a photosynthetic photon flux density of 90 µmol m⁻² s⁻¹ (16 h of light daily from cool white fluorescent lamps) for four weeks. Proliferated shoots from the MS medium supplemented with 1mg/l NAA and 9 mg/l BA were used for further growth and investigation.

Other nodal segments were placed vertically in shoot proliferation MS media of various basal strengths (¼ MS, ½ MS and full strength MS) with 3% (w/v) sucrose, 0.8% (w/v) agar, 0.1 % (w/v) myo-inositol and
supplemented with 0.2 mg/l 1-naphtalene acetic acid (NAA) and 1 mg/l kinetin, while the pH was adjusted to 5.8. Cultures were maintained at similar growth conditions as previously described for four weeks.

**Shoot and root formation**

Shoot explants of second and third nodes grown on MS supplemented with 1 mg/l NAA and 9 mg/l BA were cut into 1 cm long pieces. Each four segments were inoculated for four weeks into a flask containing the shoot proliferation medium and were subcultured two times with four week intervals. At the end of the 12-week culture period, regenerated shoots were separated and individually transferred for four weeks to growth regulator-free MS medium for rooting. Rooted plantlets were subsequently transferred to pots containing soil and peat (1:1). *In vitro* 16 weeks old regenerated plantlets are shown in Figure 3.

To study the effect of strength basal MS media not only on plantlet regeneration but also on shoot, leaf and root formation, apical shoots from the same donor plant were sterilized as previously described and incubated for four weeks in shoot proliferation MS (Murashige and Skoog, 1962) media of various basal strengths (¼ MS, ½ MS and full strength MS) containing 3% (w/v) sucrose, 0.8% (w/v) agar, 0.1 % (w/v) myo-inositol and supplemented with 0.2 mg/l 1-naphtalene acetic acid (NAA) and 1 mg/l kinetin with pH adjusted to 5.8 under similar growth conditions. Explants were twice subcultured, with each subculture period lasting four weeks before being transferred to hormone-free medium of respective basal strength for root induction for four weeks. Shoots and roots were subjected to measurements’ description and correlation among obtained results was performed. *In vitro* 16 weeks old regenerated plantlets are shown in Figure 4.

**Description of measurements**

Shoot and root fresh weight was recorded (in mg) following 16 weeks of culture *in vitro*. Shoot and root growth and leaf number per explant were determined as well.

Shoot growth was evaluated as length (in cm) and number of shoots per plantlet. Then, average shoot length and number of shoots was
calculated for each group of plantlets per each different medium.

The number of leaves per plantlet was determined by counting the fully developed green leaves. Then, the average number of leaves was determined for each group of plantlets per each different medium.

The number of roots was determined by counting the number of roots (> 0.5 cm length) formed.

**Statistical analysis**

Experiments were set-up in a completely randomized design. The number of replicates was three. Statistical analysis was based on analysis of variance (ANOVA). Significant differences (p<0.05) among means were determined according to Duncan’s multiple range test. Correlations between determined organogenesis and biochemical parameters were performed using MS-Excel software.

**Results and Discussion**

**Shoot induction from nodal explants**

After the first four weeks of incubation, one hundred and fifty nodal segments cultured and separated onto MS medium supplemented with 1 mg/l NAA and 1, 5 and 9 mg/l BA resulted into an average induction of 4, 8 and 10 adventitious shoots respectively as shown in Table 1. This increase in shoot numbers with increasing BA concentrations is due to the fact that cytokinins exert an augmenting effect on the cell division of shoot tissues.

Nodal segments cultured onto MS media of ¼, ½ and full basal strength supplemented with 0.2 mg/l NAA and 1 mg/l kinetin resulted into the average formation of 2.1 to 3.5 adventitious shoots as shown in Table 2. A similar study (with different growth regulators) was conducted by Bhat et al. (2002) on *M. spicata* regeneration from shoot tips via callusing. They obtained 7 shoots per node after a 180-day culture period on MS basal medium supplemented with BA (1 or 2 mg/l) and kinetin (2 mg/l). Somaclonal variation among explants was observed.

**Shoot proliferation, rooting and plantlet production**

Regenerated shoot explants grown on MS proliferation medium
supplemented with 1 mg/l NAA and 9 mg/l BA were twice subcultured, with each subculture period lasting four weeks. At the end of the 12 week culture period, regenerated shoots rooted at essentially 99% when separated and individually transferred for four weeks to growth regulator-free MS medium.

Regenerated shoots proliferated on media of different basal strength MS supplemented with 0.2 mg/l NAA and 1 mg/l kinetin rooted 100% on ½ and full strength MS free of growth regulators. Rooted plantlets were subsequently transferred to pots containing soil and peat (1:1). *In vitro* regeneration and genetic transformation of *Mentha* species was also discussed by Bhat *et al.* (2002). Shasany *et al.* (1998) found that BA proved best for shoot regeneration and growth responses of some cultivars of nodal explant culture of *Mentha arvensis*. For example, in 12 weeks time, one explant of Himalaya cultivar produced on average about 200 shoots. Sauer *et al.* (1985) reported that ⅓ strength MS salts proved to be suitable for rooting of rose. For globe artichoke, ½ strength MS salts have been used in the rooting medium (Ancora, 1986; Lauzer & Vieth, 1990).

**Evaluation of organogenesis**

Shoot induction, proliferation, growth and development were observed on nodal explants when cultured onto MS of different strength media supplemented with 0.2 mg/l NAA and 1 mg/l kinetin. The best medium for the formation of shoots and roots was ½ strength MS medium. The correlation among any of shoot length, shoot and leaf number and root length when the regenerants were grown separately on ¼ strength MS, ½ strength MS and full strength growth media was not significant. Table 2 shows morphogenesis analysis of shoot and root segments of spearmint regenerants. Roots were developed in ½ strength MS and full strength MS media. In rooting phase, the explants grown in ½ strength MS produced almost twice the number of roots with shorter length than the ones grown on full MS medium. In the proliferation phase, the number of leaves per plant was not significantly affected by the increase of the basal strength in the culture medium. There were no significant relationships between any pair of variables among data obtained from morphogenesis analysis in the correlation Table 3. A single factor analysis of variance (ANOVA)
and Duncan’s test were applied (Table 4). Differences at p<0.05 were considered significant. Halving the strength of MS medium resulted in increased rooting of *Mentha arvensis* regenerants (Phatak & Heble, 2002). Tetsumura *et al.* (2008) also observed that a reduction in the strength of MS medium resulted in the increase of *in vitro* shoot and root formation from highbush blueberry (*Vaccinium corymbosum* and *V. virgatum*). Other researchers have also reported the beneficial effect of a reduction of the strength of the culture medium on the *in vitro* initiation of roots of plant species such as rose (Sauer *et al.*, 1985) and globe artichoke (Ancora, 1986; Iapichino, 1996; Lauzer & Vieth, 1990). Patel and Shah (2009) reported that root number and root length of *Stevia rebaudiana* plant cultures were significantly influenced by the strength of MS medium (¼ MS, ½ MS and full strength) and treatment combinations of IBA, NAA and BAP. Such combinations may have affected cell differentiation and elongation. Moreover, Villamor (2010) conducted a similar experiment but to determine the effects of media strength (full MS, ½ MS) and sources of nitrogen (NH₄NO₃, KNO₃) on shoot (number, length), leaf (number, length, width) and root number of ginger. The results were similar to the ones obtained in our experiment and indicated an increase of root number with the dilution of MS basal medium and the concomitant decrease of shoot number, shoot length, leaf number. On the contrary, Jain *et al.* (2008) found a direct association between medium strength and the number and length of shoots induced from *Harpagophytum procumbens* tissue cultures. The effect of the medium strength could be possibly associated with particular components of the culture medium. For example, even minor changes in the concentration of trace elements can dramatically affect plant organogenesis *in vitro*. Castiglione *et al.* (2007) observed a radical reduction in the number and length of roots induced from white poplar (*Populus alba*) cultures when zinc concentration in the culture medium was increased.
Figures and Tables
Figure 3. Regenerated spearmint plantlets from *in vivo*-derived explants grown on MS medium supplemented with 1 mg/l NAA and 9 mg/l BA.
Figure 4. Regenerated spearmint plantlets from *in vitro*-derived explants grown on MS supplemented with 0.2 mg/l NAA and 1 mg/l kinetin.
**Table 1.** Induction of spearmint shoots on MS media supplemented with 1 mg/l NAA and three different BA concentrations (mean ± SE) at 0.05 level.

<table>
<thead>
<tr>
<th>BA Concentration</th>
<th>SHOOT NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/l BA</td>
<td>4.00 ± 0.21</td>
</tr>
<tr>
<td>5 mg/l BA</td>
<td>8.00 ± 0.18</td>
</tr>
<tr>
<td>9 mg/l BA</td>
<td>10.00 ± 0.20</td>
</tr>
</tbody>
</table>

**Table 2.** Organogenesis data of spearmint regenerants after 16 weeks of culture, grown on MS medium supplemented with 0.2 mg/l NAA and 1 mg/l kinetin (mean ± SE).

<table>
<thead>
<tr>
<th>MS Strength</th>
<th>SHOOT</th>
<th>LEAF</th>
<th>ROOT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Length (cm)</td>
<td>Number</td>
</tr>
<tr>
<td>¼ MS</td>
<td>2.10 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.03±0.45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.78±2.68&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>½ MS</td>
<td>3.52±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.45±0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.06±3.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Full MS</td>
<td>3.26±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.78±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35.1±2.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Means in columns followed by same letters are not significantly different at 0.05 level.

**Table 3.** Correlation coefficient (r) among organogenesis data of spearmint regenerants grown on MS medium of different basal strength.

(a) **Half strength basal MS**

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Shoot length</th>
<th>Root number</th>
<th>Root length</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot number</td>
<td>-0.36</td>
<td>0.19</td>
<td>-0.12</td>
<td>0.56</td>
</tr>
<tr>
<td>Shoot length</td>
<td>0.17</td>
<td>0.01</td>
<td>-0.11</td>
<td></td>
</tr>
<tr>
<td>Root number</td>
<td>0.18</td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root length</td>
<td>0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(b) *Quarter strength basal MS*  

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Shoot length</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot number</td>
<td>-0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Shoot length</td>
<td></td>
<td>-0.21</td>
</tr>
</tbody>
</table>
*No roots were induced on quarter strength MS*

(c) *Full strength basal MS*

<table>
<thead>
<tr>
<th>Correlation</th>
<th>Shoot length</th>
<th>Root number</th>
<th>Root length</th>
<th>Leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot number</td>
<td>-0.30</td>
<td>0.41</td>
<td>-0.18</td>
<td>-0.07</td>
</tr>
<tr>
<td>Shoot length</td>
<td></td>
<td>-0.04</td>
<td>-0.32</td>
<td>-0.08</td>
</tr>
<tr>
<td>Root number</td>
<td></td>
<td>0.18</td>
<td>-0.05</td>
<td></td>
</tr>
<tr>
<td>Root length</td>
<td></td>
<td></td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.** Single factor analysis of variance (ANOVA) of the effect of culture medium strength on shoot, leaf and root formation.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>57.16</td>
<td>2.00</td>
<td>28.58</td>
<td>5.01</td>
<td>0.01</td>
<td>4.75</td>
</tr>
<tr>
<td>Within Groups</td>
<td>838.60</td>
<td>147.00</td>
<td>5.70</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>895.76</td>
<td>149.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter II
TOTAL PHENOLIC CONTENT IN SHOOT AND ROOT EXTRACTS OF SPEARMINT

Abstract

In order to quantify the total phenolic content of shoot extracts of spearmint (*Mentha spicata* L.), one hundred plantlets were regenerated from a single donor plant. Adventitious shoots were induced from nodal explants on Murashige and Skoog (MS) medium supplemented with 1 mg/l NAA and 9 mg/l BA and rooted on a growth regulator-free medium. A considerable variation was observed in the total phenolic content of the regenerants, which was much greater than the variation in the donor plants. Ten *in vivo* explants were chosen randomly for comparison in every analysis. The average total phenolic concentration in the donor plant (5.06 ± 0.44 mg/g fw) was almost double than in regenerants (2.58 ± 0.17 mg/g fw). In parallel, the effects of the medium strength (¼ MS, ½ MS and full MS) on total phenolic content of shoot and root parts from regenerated spearmint were investigated and correlation among collected data was conducted. The highest average total phenolic content was 7.20 ± 0.39 mg/g fw in terms of rosmarinic acid equivalent/g of fresh weight from shoots cultured onto ¼ MS and 5.92 ± 1.16 mg/g fw from roots cultured onto ½ MS. Total phenolic concentration results showed considerable variation among individual regenerants that were all derived from a single donor plant and regenerated at the same time under identical conditions. In addition, the observed variation in the regenerants was much greater than the variation in the donor plant, indicating the possible effect of somaclonal variation. Significant variation in phenolic content was found between clones of one population. The variability within a population might be attributed to the possible differences in biochemical and morphological characteristics, while genetic differences may explain the variability between clones.
Introduction

Phenolics are a class of chemical compounds containing at least one hydroxyl group that is bonded not to a saturated carbon atom (as in the case of alcohols) but directly to an aromatic ring in the same molecule. The acidity of the hydroxyl group is commonly intermediate between that of aliphatic alcohols and carboxylic acids (their pKa is usually between 10 and 12).

Herbs are used in many domains, including medicine, nutrition, flavouring, beverages, dyeing, repellents, fragrances, cosmetics (Djeridane et al., 2006). Many species have been recognized to have medicinal properties and beneficial impact on health like: antioxidant activity, digestive stimulation action, anti-inflammatory, antimicrobial, hypolipidemic, antimutagenic effects and anticarcinogenic potential (Aaby et al., 2004; Cai et al., 2004). Crude extracts of herbs and spices and of other plant materials rich in phenolics gained an increasing interest from the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food. Furthermore, the well known protective role of phenolics in plants has stimulated the interest for a possible protective action in humans.

Members of Mentha genus are characterized by their volatile oils that are of great economic importance, being used by the pharmaceutical, cosmetic, food, confectionery and liquor industries. Hence, they are cultivated as industrial crops in several countries. There are a few reports on the antioxidant property of Mentha sp. (Dorman et al., 2003, Marinova and Yanishlieva, 1997, Kähkönen et al., 1999 and Zheng and Wang, 2001). Kanatt et al. (2005) investigated the antioxidant potential of Mentha spicata and tested its efficacy in minimizing oxidative rancidity of radiation-processed lamb meat. In the food industry, free radicals and polyphenol oxidases cause a decrease in the nutritive value of fats, oils and lipid-containing foods through oxidation, which can be delayed by the use of antioxidants (Ibanez et al., 1999; Robards et al., 1999). In human physiological systems, the oxidizing agents causing damage are the
reactive oxygen species (ROS). ROS have been found to play a key role in chronic diseases such as cardiovascular disease, inflammatory diseases and cancer, presenting a major health concern (Ibanez et al, 1999).

In the past, protocols for the assessment of the effect of different nutrient media and growth regulators on secondary metabolites were investigated (Ajit Kumar Shasany et al. 1998; Bhat et al. 2002). Recently, Tisserat and Vaughn (2008) discussed the growth, morphogenesis, and essential oil production in *Mentha spicata* plantlets *in vitro*. They demonstrated the use of plantlet cultures to manufacture secondary metabolites.

The aim of this study was to quantify total phenolic content (mg/gram fresh weight) of shoot and root extracts from regenerated spearmint grown in media of different strength and to investigate the correlation among obtained data.

**Materials and Methods**

**Chemicals**

Rosmarinic acid (RA) (standard solution) was purchased from Cayman Chemical Company, Michigan, USA.

**Total phenolic extraction**

Total phenolic extraction procedure was performed according to Konstas and Kintzios (2003). The advantage of this method is quick and easy while its disadvantage is that some other compounds might be extracted along with the phenolics. One hundred shoot segments were randomly selected from plantlets grown for 16 weeks *in vitro* on MS medium supplemented with NAA and BA (Chapter 1) and another 150 shoot and root segments from plantlets grown for 16 weeks *in vitro* on MS of different basal strength media (¼ MS, ½ MS, full MS). Following recording of their fresh weight, segments were macerated for 5 min in 10 ml MeOH 80 % (v/v) using a blender. The extract was placed into a water bath at 80ºC for 30 min, filtrated through Whatman filter papers, collected in 10 ml tubes and stored at -20ºC until
analysis. Three ml of methanolic extract were taken into spectrophotometer at 333 nm and total phenolic content was expressed as mg Rosmarinic acid equivalent /g fw. A standard curve was established using known concentrations of rosmarinic acid (mg/ml) in 80% methanol. Each reported value was a mean of three replicates.

**Statistical analysis**

Linear regression analysis for every assay was performed to establish the significance of the functional relationship between the two variables (X = rosmarinic acid concentration equivalent, Y = absorbance readings at 333 nm). The linear regression equation was chosen based upon the coefficient of determination (R² value). The relationship between Y and X was described by a standard curve of linear function shown in **Figure 5**. The standard curve with the equation (y=ax + b) was established by preparing five concentrations (0, 1.10⁻², 2.10⁻², 3.10⁻², 4.10⁻² 5.10⁻² mg/ml) of rosmarinic acid in MeOH 80% (v/v). This standard equation was used for the calculations of total phenolic content of the methanol extracts.

Experiments were set-up in a completely randomized design. The number of replicates was three. Total phenolic average of shoot and root extracts was expressed in terms of mg/g fresh weight ± standard error. For any given value of X, the Y's are independently and normally distributed. Correlation and regression analyses were carried out using Excel (Microsoft 2003). The average total phenolic concentration of ten randomly selected explants from high, medium and low phenolic producing regenerants was calculated and compared with the one of the donor plant. Data were subjected to analysis of variance and means were compared by using the Duncan’s multiple range test.
Results and Discussion

The average total phenolic concentration of high, medium and low phenolic producing regenerants and Duncan’s test are shown in Table 5. Considerable variation in total phenolic concentration was observed both in the donor plant and the regenerants and shown in Figure 6. Differences at p=2.24E-14 were considered highly significant as shown in Table 6. The average total phenolic concentration in the donor plant (5.06 ± 0.44 mg/g fw) was almost double than in regenerants (2.58 ± 0.17 mg/g fw). Total phenolic concentrations ranged from 8.35 to 0.10 mg RAE/g fw, and in other words, varied considerably among individual regenerants, although they were all derived from a single donor plant and regenerated under identical conditions during the same period. Moreover, the observed variation in the regenerants was much greater than the variation in the donor plant, indicating the possible effect of somaclonal variation. Some extracts showed to be high in phenolics and possess health benefits (Kähkönen et al., 1999). Total phenolic content of spearmint was discussed by Kaur and Kapoor (2002) who obtained 4 mg catechol equivalent/g fw. In another comparative study, Kalt et al. (1999) concluded that the best spearmint lines contain a higher phenolic content than the high bush blueberries. Variability of phenolic content has been reported in other Lamiaceae studies as well and significant variation in phenolic content was found between clones or cultivars of one population (Skoula et al., 2000). The explanation given for the variability within a population was due to the possible differences in biochemical and morphological characteristics, while genetic differences could explain the variability between cultivars.

In parallel, the total phenolic content (mg/ g fw) of one hundred and fifty regenerants of spearmint (Mentha spicata) grown on different MS strengths media (full MS, ½ MS and ¼ MS) was determined and expressed as mg RAE/g fw.

The results of phenolic analysis of shoot and root extracts are given as average ± standard error in Table 7. The best average phenolic content was 7.20 ± 0.34 mg as of mg RAE/g fw from shoot extracts cultured onto ¼ MS and 5.93 ± 1.16 mg from root extracts.
cultured onto ½ MS. The present study is a first report demonstrating the possible effect of medium MS strength on the total phenolic content of spearmint shoot and root extracts. Kaur and Kapoor (2002) have reported the variation of total content of vegetables from 4 mg catechol/g fresh weigh in mint to 0.34 mg in round melon. Also, Kanatt et al. (2004) have reported a total phenolic content of 25.62 ± 3.14 mg in terms of catechin equivalent/g of wet weight of spearmint sample.
Figures and Tables
Figure 5. Total phenolic concentration standard curve.

Figure 6. Total phenolic concentration in the donor (closed column) and regenerant (open column) spearmint plants. Bars correspond to standard error.
Table 5. Duncan’s multiple range test table. Total phenolic accumulation in donor plants and regenerants (expressed as equivalent rosmarinic acid concentration (mg/g fw ± standard error).

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Donor</th>
<th>Regenerant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5.05 ± 0.44</td>
<td>6.43 ± 0.48</td>
</tr>
</tbody>
</table>

Table 6. Single factor ANOVA table of methanolic extracts (donor and regenerants).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>209.19</td>
<td>3</td>
<td>69.73</td>
<td>62.77</td>
<td>2.24E-14</td>
<td>4.37</td>
</tr>
<tr>
<td>Within groups</td>
<td>39.98</td>
<td>36</td>
<td>1.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>249.18</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7. Effect of the strength of the solid culture medium on the accumulation of total phenolics expressed as equivalent rosmarinic acid concentration (mg/g fw ± standard error) in shoot and root extracts from spearmint regenerants. Numbers followed by different letters indicate statistically different values (p<0.01). Each number is an average of 50 observations.

<table>
<thead>
<tr>
<th>Medium strength</th>
<th>Shoot extracts</th>
<th>Root extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td>¼ MS</td>
<td>7.20 ± 0.37 a</td>
<td>No rooting</td>
</tr>
<tr>
<td>½ MS</td>
<td>3.48 ± 0.52 b</td>
<td>5.93 ± 1.16 a</td>
</tr>
<tr>
<td>Full MS</td>
<td>1.98 ± 0.23 c</td>
<td>2.20 ± 0.22 b</td>
</tr>
</tbody>
</table>
Chapter III
TOTAL ROSMARINIC ACID CONTENT IN SHOOT AND ROOT EXTRACTS OF SPEARMINT

Abstract

In order to determine the rosmarinic acid (RA) content of methanolic extracts in spearmint, adventitious shoots were induced from nodal explants on Murashige and Skoog (MS) medium supplemented with 1 mg/l NAA and 9 mg/l BA and rooted on a growth regulator-free medium. One hundred plantlets were regenerated from a single donor plant and were compared to 10 donor plants obtained from the same mother plant. The Rosmarinic acid quantification through TLC technique was applied to all methanolic extracts. Correlation among obtained results was conducted. The average total RA concentration in the donor plant (0.72 \pm 0.12 mg/g fw) was almost double than in regenerants (0.39 \pm 0.03 mg/g fw). Considerable variation in total RA concentration was observed both in the donor plant and the regenerants. Consequently, regenerants were classified according to their RA content as high, medium and low RA accumulators. A high positive correlation was observed between total phenolic and RA concentration in high and medium RA accumulators \((r^2=0.91 \text{ and } 0.94, \text{ respectively})\). On the contrary, the correlation in low RA accumulators was considerably smaller \((r^2=0.38)\). Correlation between total phenolic and total RA content of all the studied samples was only about 12.66%. HPLC method was applied to methanolic extracts having the best 18 and the worst 10 rosmarinic acid content. Correlation between results from the TLC and HPLC analytical methods was highly significant \((0.96)\). The observed variation in the regenerants was much greater than the one in the donor plant, indicating a possible effect of somaclonal variation.
Introduction

Rosmarinic acid (RA, α-O-caffeoyl-3, 4-dihydroxyphenyllactic acid), a plant secondary metabolite, belongs to the class of hydroxycinnamic esters, a constituent substance of Lamiaceae, Boraginaceae and Apiaceae (Petersen & Simmonds, 2003). RA has been detected in at least twelve other plant families (Deighton et al., 1993; Frankel et al., 1996; Shetty et al., 1997; Razzaque & Ellis, 1977). It has earned a reputation as a molecule of interest due to its multiple astringent, antibacterial, antiviral and biological activities against inflammatory lung diseases, autoimmune arthritis, heart disease, suppression of auto immune rejection in human skin transplant patients as well as its multipurpose activities against reverse transcriptase, integrase and RNase in HIV infections (Sanbongi et al., 2003; Youn et al., 2003). RA was also found to defend herpes simplex infections and putative cancer-productive compound (Kyung et al., 2004) and to possess anticarcinogenic, antimutagenic and antioxidant capabilities (Durust et al., 1999). The production of RA has been reported in cell suspension cultures of Salvia officinalis (Hyppolite et al., 1992) and Coleus blumei (Petersen et al., 1994). The same outcome was obtained by Razzaque and Ellis (1977) with rosmarinic acid-accumulating cell cultures of Coleus blumei. Kintzios et al. (2003) reported the in vitro RA accumulation in sweet basil. According to Fletcher et al. (2005), novel Mentha spicata clones with enhanced rosmarinic acid and antioxidant activity could deliver a cost-effective and sustainable natural source of a powerful HIV drug.

The aim of this study was to determine total rosmarinic acid concentration of shoot and root extracts from regenerated and donor plants, to examine correlation among results and to compare two analytical methods used in RA quantifications.
Materials and Methods

Rosmarinic acid extraction, identification and isolation

Methanolic shoot extracts from 10 donor plants and 100 regenerant plantlets were assessed for rosmarinic acid concentration and analysis. One hundred µl of each phenolic aliquot was aligned and a drop of pure rosmarinic acid for identification was spotted at 1 cm from the low edge of a TLC. The TLC was run in a system of C₆H₁₀O:CH₃COOH:H₂O (3:1:1) (Rady & Nazif, 2005). After UV identification, rosmarinic acid was isolated, collected and diluted with 200 µl MeOH 80% before being centrifuged at 13000 rpm for 5 min. The supernatant was collected and the total volume was adjusted to 500 µl. The higher dilution factor used to ensure the absorbance reading was less than 1.0 for the proper application of Beer's Law. The selection of the wave length was based on reading the absorption spectrum of different concentrations of prepared rosmarinic acid solutions from 200 to 400 nm. The determination of rosmarinic acid concentration was taken at 333 nm on Ultraviolet-Visible (UV-VIS). The concentration of RA was expressed by terms of mg RA equivalent / g fw.

The study of total RA content from TLC method was compared to the HPLC method. RA % was also calculated and correlations among data were computed.

High Performance Liquid Chromatographic (HPLC) determination of rosmarinic acid in spearmint methanolic extracts

In this study, HPLC method was applied to methanolic extracts having the best 18 and the worst 10 total rosmarinic acid contents. Correlation between results from the TLC and HPLC analytical methods was also computed.

Methanolic extracts were assessed for rosmarinic acid content. Samples were subjected to PPC (preparative paper chromatographic) separation using 3MM Whatman filter papers impregnated with n-butanol/acetic acid/water, 3:1:1 v/v), as described previously by Rady and Nazif (2005). The solvent-free residue was subjected for further purification using Sephadex LH-20 column (1.8 cm diameter and 60
cm height), eluted with methanol and fractions each of 10 ml were collected. For HPLC analysis, an isocratic elution was used (acetonitrile/formic acid (4:1, v/v) at 1.2 ml min⁻¹; detection at 300 nm). Rosmarinic acid in the samples was identified using a pure standard (Cayman Chemical). Quantitative determination was made according to a reference curve using the peak areas.

The analytical protocol of Fletcher et al. (2005) was followed. Briefly, separation and quantitative determination of rosmarinic acid was achieved using a Jasco HPLC system (Jasco Corporation, Tokyo, Japan) accessorized with an APEX ODS 5-µm, 25.0 × 4.6-mm analytical column (Jones Chromatography, Mid Glamorgan, U.K.). The mobile phase was degassed with helium during analysis. Solvent mixtures were as follows: 25% acetonitrile in 1% H₃PO₄ (A) and acetonitrile (B). The following elution profile was used: 100:0 (A:B), initial conditions; gradient (linear) to 73:27 in 12 min; gradient to 10:90 in 1 min; isocratic for 4 min. Initial conditions were reestablished by transition to 100:0 in 1 min and equilibrating for 7 min. The mobile phase was delivered at 1 ml min⁻¹ and detection of analytes was performed at 293 nm. Chromatograms were captured in a PC system with Borwin Chromatographic Software, v. 1.21.60 (JMBS Development, Fontaine, France). Rosmarinic acid in the extracts was identified based on comparison of chromatographic and spectral characteristics with a pure standard (Cayman Chemical). A reference curve was established.

**Statistical analysis**

All samples were assayed in triplicate and averaged (by including three different shoot segments from each individual plantlet). Average total rosmarinic acid concentration in donor plants and regenerants was calculated. Regenerants were classified according to their rosmarinic acid content. The treatments were arranged in a completely randomized design. One way ANOVA analysis was done and the differences between means were tested according to Duncan’s multiple range test. Besides, coefficient of correlation and coefficient of regression were determined using MS - Excel 2003
Results and Discussion

**Rosmarinic acid quantification through TLC technique**

The regenerants as well as the donor plants exhibit differences in rosmarinic acid concentration. The genetic diversity of these explants is a factor that accounts for the variability in RA content. The amount of rosmarinic acid can potentially be very high in plants of the Lamiaceae family (Skoula et al., 2000). Analysis of our results showed that the RA content of spearmint clones varied from 1.47 to 0 mg/g fw among the extracts of regenerants and from 1.40 to 0 mg/g fw in *in vivo* explants. The percentage of RA ranged from 99 to 0 among extracts from regenerants and from 21.59 to 0 among donor extracts. Regenerants were classified according to their RA content (Table 8).

Considerable variation in total RA concentration was observed both in the donor plant and the regenerants (Figure 7). The average total RA concentration in the donor plant (0.72 ± 0.12 mg/g fw) was almost double than in regenerants (0.39 ± 0.03 mg/g fw) as shown in Figure 7. In other words, total RA concentration varied considerably among individual regenerants, although they were all derived from a single donor plant and regenerated under identical conditions during the same period. Moreover, the observed variation in the regenerants (high, medium and low RA accumulators) was much greater than the variation in the donor plant, indicating the possible effect of somaclonal variation (Figure 8). When analyzing the results, a high positive correlation was observed between total phenolic and RA concentration in high and medium RA accumulators ($r^2$=0.91 and 0.94, respectively). In the contrary, the correlation in low RA accumulators was considerably smaller ($r^2$=0.38). Total RA percentage in the donor plants and regenerant plants were presented in Figure 9. Means of rosmarinic acid accumulation in donor plants and regenerants were presented in Table 9. There was very high significant differences among means ($p= 2.08 \times 10^{-12}$) as shown in Table 10.

The present study was the first report demonstrating the
possible effect of somaclonal variation on the biosynthesis of total phenolics and rosmarinic acid in spearmint. It is known from previous studies that RA concentration in spearmint can vary due to environmental effects, such as high temperatures (Fletcher et al., 2005). As far as in vitro variation of RA accumulation is concerned, there are only a few previous reports in the literature. For example, Rady and Nazif (2005) observed a medium-dependent variation of RA accumulation in Ocimum americanum in vitro shoot cultures, which was associated with induced genetic variation as revealed by RAPD (Random Amplification of Polymorphic DNA) analysis.

Similar studies were conducted by Kintzios et al. (2003), where RA reached 178 µg/g dry weight in Ocimum basilicum. They reported the activity of individual parts of the rosmarinic acid biosynthesis pathway under scale-up conditions including precursor feeding. Also, Durling et al. (2007) reported 1.8% RA equivalent from Salvia officinalis using ethanol–water mixtures.

Kähkönen et al. (2001) reported that environmental factors such as light, temperature, humidity and nutrient availability contribute to the synthesis of phenolics for plant defense. Certain phenolic acids also play a role in the protection of the plant from UV radiation. When the UV index is high, it can be assumed that compounds protecting the plant from this type of damage would be synthesized, and similarly if pathogens or pests attack the plant, the phenolics responsible for defense would also be increased. The results obtained from their study, showed that total phenolics at each harvest did not reflect the level of RA found in the same tissue. The increased production of RA and the decrease production of total phenolics indicated that individual phenolic components respond to different external influences potentially at different rates. Correlation between total phenol and RA content was only about 12.66%. Relationships between external stimuli and individual phenolic constituents could be the focus of future studies.

Rosmarinic acid quantification through HPLC technique

This method proved effective and very efficient for the
simultaneous separation and determination of RA in spearmint plants. The typical chromatogram and chromatographic data of the standard mixture were shown in Figure 10. The obtained standard curve equation for RA concentration was:

\[ \text{mg/ml} = \text{peak area} 	imes 10^{-6} \times 0.4288. \]

Results obtained from HPLC confirmed the ones obtained with TLC technique. The correlation between results from HPLC and TLC was highly significant (0.96) (Table 11).

Nine compounds were found most frequently in literature from analysis of plants of Lamiaceae: gallic, chlorogenic vanillic, caffeic, p-coumaric, ferulic, m-comaric, rosmarinic and trans-cinnamic acids. However, studies specifically on spearmint or other mint species were rare.

A study analyzing various phenolics in medicinal plants of Lamiaceae, also found rosmarinic acid to be the most abundant phenolic. In comparison, peppermint was among the plants tested and had a similar phenolic profile to the spearmint clonal lines tested in this study. Peppermint produced twice as much rosmarinic acid than any other phenolic compounds, while other compounds detected at lower levels were gentisic, caffeic, protocatechuic, p-hydroxybenzoic and vanillic acids. Chlorogenic, ferulic, syringic and p-coumaric acids were undetected in peppermint (Zgorka et al., 2001). Another study profiling the phenolics in peppermint found rosmarinic acid and eriodictyol 7-O-glycoside to be the major components. Together they accounted for 59-67% of total phenolics detected by their HPLC method of analysis (Areias et al., 2001). Rosmarinic acid is consistently shown to be a compound produced in high amounts in the mint family and therefore can be used for chematoxonomic purposes.

The HPLC methodology specifically refined for this study clearly indicated that rosmarinic acid extraction and quantification based on TLC method was adequate for analysis of total rosmarinic acid content from phenolic spearmint extracts. These results are extremely encouraging as rosmarinic acid is a very important antioxidant used in the food industry and has now been recognized as an important compound in the pharmaceutical industry. Therefore these samples
could potentially serve as valuable crops for commercial use in the near future.
Figures and Tables
**Figure 7.** Total RA concentration in the donor plants (closed column) and spearmint regenerants (open column). Bars correspond to standard error (SE).

**Figure 8.** Total RA concentrations in the donor plants (1) and spearmint regenerants [(2) high, (3) medium and (4) low RA accumulators)]. Bars correspond to standard error (SE).
Figure 9. Total RA percentage in the donor plants (closed column) and spearmint regenerants (open column). Bars correspond to standard error (SE).

Figure 10. HPLC standard curve of RA (mg).
Table 8. Classification of regenerants according to their RA content.

<table>
<thead>
<tr>
<th>RA accumulators</th>
<th>Number of plantlets</th>
<th>RA (mg/g fw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>20</td>
<td>0.93 ± 0.23</td>
</tr>
<tr>
<td>Medium</td>
<td>60</td>
<td>0.32 ± 0.14</td>
</tr>
<tr>
<td>Low</td>
<td>20</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Donor</td>
<td>10</td>
<td>0.72 ± 0.41</td>
</tr>
<tr>
<td>Regenerants</td>
<td>100</td>
<td>0.39 ± 0.03</td>
</tr>
</tbody>
</table>

*Mean ± standard error (SE)

Table 9. Duncan’s multiple range test table. Total rosmarinic acid accumulation in donor plants and regenerants expressed as rosmarinic acid concentration (mg/g fw ± standard error). Least Significant Range (LSR).

<table>
<thead>
<tr>
<th>Phenolics</th>
<th>Donor</th>
<th>Regenerant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.72 ± 0.12</td>
<td>1.12 ± 0.05</td>
</tr>
<tr>
<td>LSR</td>
<td>0.35</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Table 10. Single factor ANOVA table of rosmarinic acid accumulator extracts (donor and regenerants).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>6.88</td>
<td>3</td>
<td>2.29</td>
<td>46.04</td>
<td>2.08E-12</td>
<td>4.37</td>
</tr>
<tr>
<td>Within groups</td>
<td>1.79</td>
<td>36</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.68</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 11. Determination coefficient (r²) between RA concentrations obtained from HPLC and TLC analysis of methanolic extracts from 28 spearmint plantlets.

<table>
<thead>
<tr>
<th>Technique used</th>
<th>TLC</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>0.959129</td>
<td>1</td>
</tr>
</tbody>
</table>
Chapter IV
TOTAL PROTEIN CONTENT AND KEY ENZYMES ACTIVITY IN SHOOT EXTRACTS OF SPEARMINT

Abstract

Total protein extractions from 110 samples were selected individually from the same regenerants and donor plants for phenolic assays at the same time. Total protein content assay was done using Bearden’s Coomassie-binding colorimetric method (Bearden, 1978). The activities of three enzymes involved in the rosmarinic acid biosynthetic pathway, phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydrolase (CAH) and hydroxyphenyl pyruvate reductase (HPPR) were also determined. In Bearden’s protein assay, the average ± standard variation protein concentration in the donor plants and spearmint regenerants was 0.536 ± 0.388 µg bovine serum albumin equivalent (BSAE/g fw). The average ± standard error of phenylalanine ammonia-lyase, cinnamic acid 4-hydrolase and hydroxyphenyl pyruvate reductase activities were calculated based on standard curve determination of each studied enzyme separately. Correlation analyses among total protein and enzymatic activities were also run as in the previous studies.
Introduction

Proteins or polypeptides are organic compounds made of amino acids (number of common amino acids is 20) arranged in a linear chain and folded into a globular form. The amino acids are joined together in a polymer chain by the peptide bonds between the carboxyl and amino groups of adjacent amino acid residues. The sequence of a gene is the sequence of amino acids in a protein that is encoded in the genetic code (Ridley, 2006). Proteins can work together to achieve a particular function and often associate to form stable complexes (Maton et al., 1993). Like other biological macromolecules such as polysaccharides and nucleic acids, proteins are essential parts of organisms and participate in virtually every process within cells. Many proteins are enzymes that catalyze biochemical reactions and are vital to metabolism. Proteins may be purified from other cellular components using a variety of techniques such as ultracentrifugation, precipitation, electrophoresis, and chromatography. The advent of genetic engineering has made possible a number of methods to facilitate purification. The activities of three enzymes involved in the rosmarinic acid (RA) biosynthetic pathway, phenylalanine ammonia-lyase (PAL; EC 4.1.3.5), cinnamic acid 4-hydrolase (CAH; EC 4.2.1.1) and hydroxyphenyl pyruvate reductase (HPPR; EC 1.1.1.237) were determined in this study. RA is biosynthesized from L-Phe and L-Tyr. L-Phe was found to be the precursor for the caffeic acid moiety, whereas L-Tyr gives rise to the 3,4-dihydroxyphenyllactic acid part of the molecule. L-Phe enters the general phenylpropanoid pathway where it is converted to 4-coumaroyl-CoA by phenylalanine ammonia-lyase (PAL; EC 4.1.3.5), cinnamic acid 4-hydroxylase (CAH; EC 4.2.1.1) and hydroxycinnamic acid coenzyme A ligase (4CL, EC 6.2.1.12) (Petersen et al., 1994, 1999; Kim et al., 2004). L-tyrosine is transaminated by tyrosine aminotransferase (TAT; EC 2.6.1.5) to 4-hydroxyphenylpyruvate (De Eknamkul and Ellis 1987) which is then reduced by hydroxyphenylpyruvate reductase (HPPR; EC 1.1.1.237) to 4- hydroxyphenyllactate (Petersen and Alfermann 1988, Petersen et al., 1993).
The aim of the present study was to assay total protein content and to determine the activity of three key enzymes (PAL, CAH, HPPR) involved in the biosynthesis of RA in regenerated plantlets compared to donor plants.

Materials and Methods

Chemicals
Coomassie Brilliant Blue G-250 (Serva 17524) and phenylalanine (SERVA 32191), NADPH, Bovine Serum Albumin (BSA) and 2-mercaptoethanol (SERVA BLUE G.) were purchased from MP BIOMEDICALS Inc. Ohio; phosphoric acid from SDS France; cinnamic acid, trans-cinnamic acid, 4-coumaric acid AND 4-hydroxyphenylpyruvate (pHPL) from SIGMA CHEMICALS Co, USA; ascorbic acid from BDH CHEMICALS Ltd, England; DTT from FLUKA BIOCHEMIKA, Buchs, Switzerland while KH$_2$PO$_4$ and K$_2$HPO$_4$ from EIMER & AMEND, USA.

The stable free radical DPPH was purchased from Sigma-Aldrich Chemical Company (St Louis, MI, USA). Methanol was HPLC grade and purchased from Fisher Scientific Inc (Waltham, MA, USA). Tris (hydroxymethyl)-aminomethan was purchased from Merck company (Darmstadt, Germany) and used for buffering after pH adjustment (with HCl).

Protein assay
The determination of total proteins was performed using Bearden’s Coomassie-binding colorimetric method as follows: Protein extraction was done through macerating a weighed fresh shoot segment from the same 110 plants that had undergone phenolic assay on the same date from the same individual plant in 5 ml prepared protein buffer. The macerated tissue was centrifuged twice at 11000 rpm for 40 min at 4ºC and the supernatant transferred into new tubes. The protein buffer was composed of 4.542 g Tris hydroxymethyl amino methan, 0.027 g EDTA, 9 g NaCl, 1.156 g dithio-DL-threitol and 35 ml glycerol with pH adjusted into 6.2 in a total volume of 1000 ml de-
ionized distilled (d) water (H₂O).

The total protein content assay was performed by combining a prepared Coomassie solution (composed of 10 mg Coomassie Brilliant Blue G-250 diluted in 40 ml 85% phosphoric acid and added to 200 ml d H₂O, left for 24 hours) to a volume of 300 µl protein aliquot and to 1200 µl d H₂O. When Coomassie dye binds protein in an acidic medium (phosphoric acid 85%), an immediate shift in absorption maximum occurs from 465 to 595 nm with a concomitant color change from brown to blue. Following a quick mixing of the test tube content on a plate shaker for 30 sec and an incubation step for 10 min at room temperature, the absorbance was read at 595 nm. A standard curve was prepared by dissolving BSA stock solution (1mg/ml) through adding various volumes of prepared stock BSA ranging from 50 to 500 µl and adjusted to 1500 µl distilled H₂O and by plotting the average Blank-corrected 595 nm measurement for each BSA standard vs. its concentration in µg/ml. The results were expressed in µg BSAE/g fw.

**Enzymatic assays (PAL, CAH, HPPR)**

PAL catalyzes the oxidative desamination of L-Phe and forms trans-cinnamic acid and ammonia. PAL activity was assayed in a total volume of 2 ml 50 mM Tris-HCl, 2 mM 2-mercaptoethanol, 12 mM phenylalanine, pH 8.5, added to 2 ml protein extract. The mixture was incubated at 40ºC for 60 min and the reaction was stopped by the addition of 0.2 ml 6 N HCl and cooling on ice. PAL activity (expressed as mM trans cinnamic acid) was estimated spectrophotometrically measuring the concentration of trans-cinnamic acid at 270 nm (Petersen *et al.*, 1993, 1994).

CAH introduces the para-hydroxyl group into the aromatic ring of trans-cinnamic acid by a cytochrome P-450-dependant mono-oxygenation reaction. The enzyme activity essentially depends on molecular oxygen and NADPH. CAH was assayed in a total volume of 0.4 ml 0.1 M Tris-HCl, 1 mM DTT, pH 7.5 :0.1 mM NADPH, 1.25 mM cinnamic acid (dissolved in 12.5 µl 50% methanol), added to 0.2 ml protein extract. The mixture was incubated at 25ºC for 10 min and the reaction was stopped by the addition of 0.1 ml 6 N HCl and cooling on
ice. The elution profile was recorded at 310 nm and 4-coumaric acid solutions were used as standard (Petersen et al., 1993, 1994).

HPPR reduces 4-dihydroxyphenylpyruvate (pHPP) to 4-dihydroxyphenyllactate (pHPL) with the help of NADPH (Petersen and Alfermann, 1988; Häusler et al., 1991). The assay was conducted in a final volume of 0.25 ml buffer (0.1 M KH$_2$PO$_4$/K$_2$HPO$_4$ pH 7.0), 10 nM ascorbate, 1 µM DTT, 0.5 µM NADPH, 0.25 µM 4-hydroxyphenylpyruvate added to 100 µl protein extract. After 15 min incubation at 30°C the reaction was stopped by adding 25 µl 6 M HCl. The reaction product (expressed as µM of pHPL) was detected at 280 nm (Kim et al., 2004).

**Statistical analysis**

Ten (10) shoots from the donor plant and 100 regenerants were used for the biochemical assays. Each assay was conducted three times (on three different shoot segments from each individual plant) in a completely randomized design. The statistical analysis was based on analysis of variance (ANOVA) and for separation of means the Duncan’s test was performed as described previously. Correlations among the investigated parameters were calculated using an MS-EXCEL program.

**Results and Discussion**

In Bearden’s protein determination assay, the protein concentration was calculated and expressed in µg BSAE/g fw. The total protein concentration and the PAL, CAH and HPPR activities are shown in Figure 12 and expressed in terms of average ± standard error (SE). Among obtained results, the highest total protein content from *in vitro* and *in vivo* extracts were 1.75 and 1.69 µg BSAE/g fw, respectively. The standard curve equations of the protein and enzymes activity assays using relative pure standards are shown in Table 12. The calculated average ± standard error (SE) of total protein concentration and PAL, CAH and HPPR activity in the donor plants and spearmint regenerants are shown in Table 13. Correlations
among total protein and activation enzymes contents are shown in **Table 14**. Duncan’s multiple range test presented in **Table 15** shows the results of total protein concentration in donor plants and regenerants. There was a very high significant difference among means (p<0.05) as shown in **Table 16**.

Petersen *et al.* in 1993 and in 1994 had similar results when analyzed the correlation between phenolics and proteins. Furthermore, the determination of enzyme activities involved in rosmarinic acid biosynthesis such as PAL, CAH and HPPR was published by Petersen and Alfermann in 1988 and by Petersen in 1999 and those proteins were considered as enzymes of secondary metabolites. Moreover, Kintzios *et al.* reported in 2003 the activity of individual parts of the rosmarinic acid biosynthesis pathway under scale-up conditions including precursor feeding. A similarly close association between RA accumulation and PAL and CAH activities has been reported for cell cultures of *C. blumei* (Karwatzki *et al.*, 1989). This seemingly paradoxical finding can possibly be explained by the allosteric inhibition of this particular enzyme by RA, produced at high concentrations in high accumulators (Petersen *et al.*, 1994). Enzyme activities in individual regenerants were associated with their total phenolics/RA accumulation. The results suggested that somaclonal variation may affect phenolic metabolic pathways in spearmint. Many cultivars have been developed through somaclonal variation in medicinal and aromatic plants. For example, Thomas *et al.* (2006) observed considerable variation in total polyphenol, catechin fractions and caffeine concentration in 15 field grown tea (*Camellia* spp.) somaclonal variants. Also, Tsuro *et al.* (2001) investigated the variation in morphology and essential oil components in 63 regenerated plantlets of *Lavandula vera* and demonstrated the possible variation in acetyltransferase activity. Yet, no serious attempt has been made to develop new variants in spearmint using this approach.
Figures and Tables
Figure 12. (A) Total protein content (expressed as μg BSAE/ g fw) and (B) PAL, (C) CAH and (D) HPPR activities in the donor plants (closed columns) and spearmint regenerants (open columns). Bars correspond to standard error (SE).
Table 12. Equations of standard curves of total protein concentration and activity of enzymes related to secondary metabolite and RA biosynthesis.

<table>
<thead>
<tr>
<th>Test name</th>
<th>Reagent</th>
<th>A(nm)</th>
<th>Linear equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Bearden's</td>
<td>595</td>
<td>(y = 0.0097x + 0.0231)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>PAL</td>
<td>270</td>
<td>(y = 0.0004x + 0.012)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>CAH</td>
<td>310</td>
<td>(y = 0.0206x + 0.04)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>HPPR</td>
<td>270</td>
<td>(y = 6,7533x - 0.0192)</td>
</tr>
</tbody>
</table>

Table 13. Average ± SE of total protein concentration (BSAE/g fw) and PAL (mM trans cinnamic acid equivalent) CAH (µM trans coumaric acid equivalent) and HPPR (nM hydroxyphenyl lactic acid equivalent) activity in the donor plants and spearmint regenerants.

<table>
<thead>
<tr>
<th></th>
<th>Average ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins</td>
<td>0.536 ± 0.388</td>
</tr>
<tr>
<td>PAL</td>
<td>6.12 E-4 ± 2.93 E-4</td>
</tr>
<tr>
<td>CAH</td>
<td>1.95 E-4 ± 9.86 E-6</td>
</tr>
<tr>
<td>HPPR</td>
<td>1.79 E-4 ± 3.40 E-7</td>
</tr>
</tbody>
</table>

Table 14. Correlation table between total protein concentration and activity of enzymes involved in RA biosynthesis \(r^2 = \text{correlation percentage}\).

<table>
<thead>
<tr>
<th>Correlations between</th>
<th>(r^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins &amp; PAL</td>
<td>52.7143</td>
</tr>
<tr>
<td>PAL &amp; CAH</td>
<td>86.2094</td>
</tr>
<tr>
<td>CAH &amp; HPPR</td>
<td>8.6692</td>
</tr>
<tr>
<td>PAL &amp; HPPR</td>
<td>3.1537</td>
</tr>
</tbody>
</table>
**Table 15.** Total protein concentration in donor plants and regenerants expressed as rosmarinic acid concentration (μg BSAE ± standard error).

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Donor</th>
<th>Regenerant</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>0.92 ± 0.12</td>
<td>1.16 ± 0.07</td>
<td>0.51 ± 0.01</td>
<td>0.03 ± 0.007</td>
</tr>
<tr>
<td>LSR</td>
<td>0.34*</td>
<td>0.22*</td>
<td>0.04*</td>
<td>0.02*</td>
</tr>
<tr>
<td>SSR 0.05</td>
<td>3.01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 16.** Single factor ANOVA table of Bearden’s protein test (donor and regenerants).

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>7.32</td>
<td>3</td>
<td>2.44</td>
<td>44.58</td>
<td>3.28E-12</td>
<td>4.37</td>
</tr>
<tr>
<td>Within groups</td>
<td>1.97</td>
<td>36</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9.29</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Chapter V
EVALUATION OF THE ANTIOXIDANT ACTIVITY OF SPEARMINT EXTRACTS

Abstract

The antioxidant capacities of the methanolic extracts of 100 regenerants compared to those of 10 donor plants of spearmint (*Mentha spicata* L.) were investigated. Different assays for evaluating the antioxidant capacity were applied, such as the reduction of Fe$^{3+}$ to Fe$^{2+}$, the 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) free radical scavenging and the hydrogen peroxide scavenging activities. Correlation among collected data was studied. The average antioxidant activity in the donor plant extracts ($5.08 \pm 0.94 \mu$mol ascorbic acid equivalent / g fw) was almost double than in the regenerants ($2.13 \pm 1.62$). The average scavenging activity in the donor plant extracts ($0.03 \pm 0.01$) was almost triple than in the regenerants ($0.01 \pm 0.008$). The correlation between ferrous antioxidant activity and DPPH radical-scavenging activity was positive, significant among the donor plants and highly significant among the regenerants. The IC$_{50}$ value of donor plants and regenerants were 0.98 and 4.61 µg/ml, respectively, whereas that of the common antioxidant ascorbic acid was 1.96 µg/ml. The average hydrogen peroxide reduction capacity of donor plants and regenerants was 1.59 ± 0.95 and 12.84 ± 3.40, respectively. Effects of different media strength (MS, ½ MS and ¼ MS) on the antioxidant activity (DPPH•) of one hundred and fifty shoot and root extracts from regenerated spearmint were as well investigated. The IC$_{50}$ value of shoot extracts when increasing the MS strength from ¼ MS, to ½ MS and to full strength media was decreasing (79.64, 40.98 and 17.29 µg/ml, respectively). The IC$_{50}$ value of root extracts in ½ MS and full strength MS media was also decreasing from 22.28 to 3.25 µg/ml, respectively.
Introduction

The classical definition of an antioxidant given by Barry Halliwell (UK researcher, Halliwell et al., 1995) states: "An antioxidant is a substance that when present at low concentrations compared with those of an oxidisable substrate significantly delays or prevents oxidation of that substrate". The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. In general, there are two basic categories of antioxidants, natural and synthetic. Recently, interest increased considerably in finding naturally occurring antioxidants for use in foods and in medicinal materials to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Gülçin et al., 2007).

Spearmint (Mentha spicata (L.) is used as a kitchen herb and as ornamental plant in house gardens. Interest in cultivating this important medicinal and aromatic plant has become paramount (Mshaskar et al., 2000). Furthermore spearmint is widely used as a source of essential oils having a strong toxic and repellent effect on the insect Acanthoscelides obtectus (Papachristos et al., 2002), as a source for flavoring (Arumugam et al., 2006), and more recently, as a valuable source for the potent antioxidant rosmarinic acid for the neutraceutical and cosmetic industries (Bader et al., 2000; Maughan et al., 2004; Shetty 2001). Also, it has been reported that spearmint is used in fragrance formulations (Lawrence et al., 1992). Moreover, this famous medicinal plant contains numerous phytochemicals in addition to phenolic compounds, such as nitrogen compounds and rosmarinic acid. Many of these phytochemicals possess significant antioxidant capacities that are associated with lower incidence and lower mortality rates of cancer in several human beings.

Public awareness is forcing the industries to look at the viability of substituting natural antioxidants for synthetic ones. Several groups of secondary metabolites in spearmint plants have the capacity to react as antioxidants by various mechanisms: free radical scavenging,
hydrogen donation, singlet oxygen quenching, metal-ion chelation, or as substrates for attack by superoxide. Studies on higher plants have demonstrated that certain phenolic compounds have the above antioxidant abilities (Robards et al., 1999). In our study, the use of different tests for antioxidant capacities is important as antioxidants can act in several different ways in the body. Free radical scavenging tests measure the ability of an antioxidant to neutralize free radicals, which can damage lipids, DNA and proteins. Dietary antioxidants are reported to be effective against oxidation of DNA, proteins, lipids and lipid-containing structures such as low-density lipoprotein (LDL) and membranes in the human body. It is these actions that contribute to their ability to prevent diseases.

The purpose of the study was (a) to determine and compare the antioxidant activities of shoot extracts from 100 regenerants versus those of 10 donor plants using different methodologies (ferrous chelating, DPPH and hydrogen peroxide scavenging), (b) to evaluate the relationship with their total phenolic content and (c) to study their correlation. Also a comparative study was conducted on the total phenolic content and inhibition percentage of DPPH radical from regenerated spearmint explants grown on media of different strengths (MS, ½ MS and ¼ MS).

Materials and Methods

Chemicals

Tris 4, 7–diphenyl phananthroline chloride, tris HCl and hydrogen peroxide from Merck (Germany). 1-diphenyl-2-picryl-hydrazyl (DPPH) from Sigma Aldrich (St.Louis, MI, USA), ferric ammonium citrate, ascorbic acid, citric acid from AnalaR (England), ammonium molybdate from SDS ZI de Valdonne (France). MeOH from Scharlan Chemie S.A. (Spain) and sulfuric acid from Panreac Quimica S.A. (Spain). Ki, NaS₂O₃ were of analytical grade, available commercially.

Ferrous ions (Fe²⁺) chelating activity

The antioxidant capacity of 100 regenerants compared to 10
donor plant extracts was indirectly assayed by measuring the reduction of Fe\(^{3+}\) to Fe\(^{2+}\) and the subsequent formation of a red phenanthroline chloride-Fe\(^{2+}\) complex.

Briefly, the ferrous chelating activity was assayed in a total volume of 3.5 ml ferric ammonium citrate (0.654 mg/ml H\(_2\)O), 0.5 ml tris 4,7-diphenyl phenanthroline chloride (1 mg/ml H\(_2\)O), 0.5 ml CH\(_3\)COONa (0.1 g / ml) added to 0.5 ml aliquot. A blank and a control were prepared. The studied samples were shaken for 2 min, placed in darkness for 60 min and measured spectrophotometrically at 510 nm. The results were expressed in µmol ascorbic acid equivalent (AAE)/ g fw. A lower absorbance indicates a higher chelating power and a lower antioxidant (Fe\(^{3+}\)-reducing activity). The chelating activity of the extracts on Fe\(^{2+}\) was compared with that of citric acid. The antioxidant activity of spearmint regenerants was compared to donor plant samples and was measured spectrophotometrically by reduction of Fe\(^{3+}\) to Fe\(^{2+}\). The concentration of spearmint extracts was expressed in terms of µmol AAE / g fw.

**1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity**

The free-radical scavenging capacity of the methanolic extracts from 100 regenerants compared to 10 donor plants was evaluated with the DPPH• stable radical following the methodology described by Blois (1958), using ascorbic acid as the standard. This method is described extensively elsewhere where the bleaching rate of a stable free radical, DPPH•, is monitored at a characteristic wavelength in the presence of the sample.

In parallel, the free-radical scavenging capacity of the methanolic extracts from shoot and root segments of 150 regenerated spearmint plantlets grown on MS media was evaluated with the DPPH• stable radical following the above mentioned methodology.

Briefly, to 2 ml of DPPH in 80% MeOH (0.1 mM), were added 0.9 ml tris HCl (50 mM, pH 7.4) and 0.1 ml aliquot methanolic extract and methanolic solution as control. Thirty min later in darkness, the absorbance was measured at 517 nm. A lower absorbance of the
reaction mixture indicates higher free radical scavenging activity.

The percentage reduction of the DPPH referred as “inhibition” or “quenching”, or “scavenging” which is defined by $Q = 100 \frac{(A_0 - A_1)}{A_0}$, was calculated for every assay, where $A_0$ is the initial absorbance and $A_1$ is the value for the extract. Results were presented as values of $IC_{50}$, which is the concentration of the antioxidant at which 50 % scavenging of free radicals is obtained. The lower the $IC_{50}$ number, the greater is the overall effectiveness of the antioxidant in question.

**Hydrogen peroxide scavenging activity**

The hydrogen peroxide scavenging ability of the methanolic extracts of donor plants and regenerated plantlets was estimated. The hydrogen peroxide scavenging activity of the extracts was estimated by replacement titration method (Zhang, 2000). Methanolic extracts (1 ml aliquot) were added to a solution made of $H_2O_2$ (1 mL, 0.1 mM), ammonium molybdate (3 drops, 3 % v/v), sulfuric acid (10 ml, 2 M) and KI (7 ml, 1.8 M) and were titrated with NaS$_2$O$_3$ (5.09 mM) till the disappearance of the color. A standard curve of ascorbic acid was also prepared and the results were expressed in mg AAE / g f w.

**Statistical analysis**

Experiments were set-up in a completely randomized design. The number of replicates was three. Statistical analysis was based on analysis of variance (ANOVA). Significant differences (p<0.05) among means were determined according to Duncan’s multiple range test. Correlations among results obtained from different assays were performed using MS-Excel software.

**Results and Discussion**

The antioxidant capacity of spearmint extracts was assayed by measuring the reduction of Fe$^{3+}$ to Fe$^{2+}$, 1,1-diphenyl-2-picryl-hydrazyl (DPPH$^*$) free radical and hydrogen peroxide scavenging activities.
**Ferrous ions (Fe^{2+}) chelating activity**

The average antioxidant activity of the donor plant extracts (5.08 ± 0.94 µmol AAE / g fw) was almost double than in the regenerants (2.13 ± 1.62) as shown in Figure 13. The correlation between total phenolic content and ferrous antioxidant activity in donor and regenerants extracts was positive and considerably high, while the correlation between ferrous antioxidant activity and DPPH assay was positive, medium among the donor samples and higher among the regenerants as shown in table 17. The observed variation in the regenerants was much greater than the variation in the donor plants, indicating the possible effect of somaclonal variation. The equation from the ascorbic acid standard curve was $Y=0.0611 x + 0.0479$. Dorman et al. (2003) reported the antioxidant properties of aqueous extracts from *Mentha sp*. The extracts demonstrated varying degrees of efficacy in each assay except for ferrous iron chelation. With the exception of iron chelation, it appeared that the level of activity identified was strongly associated with the phenolic content. There was a highly significant difference between any pair of variables in the correlation *(Table 18)*, where p values of ferrous ions chelating activity assay, DPPH test and hydrogen peroxide activity test are shown.

**1,1-Diphenyl-2-picryl-hydrazyl (DPPH•) free radical scavenging activity**

Phenolic compounds are effective hydrogen donors, which make them good antioxidants. Besides, they possess ideal structural properties for free radical scavenging activities. Several free radicals, such as OH, O₂^− having different reactivities are formed during oxidation. Relatively stable DPPH• has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Jao & Ko, 2002). Kapoor and Kaur (2002) investigated the antioxidant activity and total phenolic content of some Asian vegetables including spearmint. There was a wide degree of variation between different phenolic compounds in their effectiveness as antioxidants (Robards et. al, 1999). The number of hydroxyl groups and substitution with electron-donating...
alkyl or methoxy groups of flavonoids increases the antioxidant potential.

The IC\textsubscript{50} value of shoot spearmint extracts in donor plants and regenerants are shown in Figure 14, whereas that of the common antioxidant ascorbic acid was 1.96 µg/ml. Kanatt et al. (2004) reported that IC\textsubscript{50} value of mint extract was 25.8 µg/ml.

The correlation between ferrous antioxidant activity and DPPH radical-scavenging activity was also positive, significant among the donor plants and highly significant among the regenerants as shown in Table 17. When analyzing obtained results, the observed variation in the regenerants was much greater than the variation in the donor plants, indicating the possible effect of somaclonal variation.

The antioxidant properties of plants have been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities and food viability. Other researchers have reported positive correlation between free radical-scavenging activity and total phenolic compounds (Zheng & Wang, 2001). Phenolic compounds are effective hydrogen donors, which make them good antioxidants. Besides, they possess ideal structural properties for free radical scavenging activities.

The free-radical scavenging capacity of the methanolic extracts from shoot and root segments of 150 regenerated spearmint plantlets grown on MS media as mentioned previously was evaluated with the DPPH\textsuperscript{•} stable radical using ascorbic acid as the standard. The IC\textsubscript{50} value of shoot and root spearmint extracts was strongly dependent on the MS basal strength. Figure 15 shows the percentage scavenging activity (DPPH µg/ml) of shoot and root spearmint extracts from different basal strength media. There was a highly significant difference between any pair of variables in the correlation (Table 18), where p values of ferrous ions chelating activity assay, DPPH test and hydrogen peroxide activity test are shown.

Kanatt et al. (2004) reported that IC\textsubscript{50} value of mint extract was 25.8 µg/ml and the correlation between the total phenolic content in mint extract and DPPH radical-scavenging was highly significant ($r^2 = 0.99$, p <0.05). Other researchers have also reported positive
correlation between free radical-scavenging activity and total phenolic compounds (Zheng & Wang, 2001). The obtained standard curve equation of DPPH test was $y = -0.1595 \ln(x) + 0.2012$. Dorman et al. (2003) reported the characterization of the antioxidant properties of deodorized aqueous extracts belonging to the Lamiaceae family. The hierarchy of the observed antioxidant activity of the extracts was dependent on the type of assay used (iron reduction capacity, DPPH, etc.). The observed antioxidant characteristics were not fully related to the total phenolic contents of the extracts in any of the assays, but were presumably strongly dependent on rosmarinic acid, the major phenolic component present in this type of Lamiaceae extract.

**Hydrogen peroxide scavenging activity**

The estimation of hydrogen peroxide scavenging ability of methanolic extracts from donor plants and regenerants was obtained by titration using NaS$_2$O$_3$ till the disappearance of the color. A standard curve was established (Figure 16) by preparing seven concentrations (0.00025, 0.0005, 0.001, 0.01, 0.025, 0.05, 0.1 mg/ml) of ascorbic acid in 80% methanol. The average hydrogen peroxide was expressed by terms of mg AA equiv / g fw in donor plants (1) and regenerants (2) and is shown in Figure 17. Each reported value was the mean of three replicates. The correlation between hydrogen peroxide and total phenolic content, ferrous chelating activity or DPPH radical-scavenging activity assay was not that significant and is shown in Table 17. There was a highly significant difference between any pair of variables in the correlation (Table 18), where p values of ferrous ions chelating activity assay, DPPH test and hydrogen peroxide activity test are shown. Murcia et al. (2004) reported the antioxidant properties of seven dessert spices comprising mint that were compared with the common food antioxidant, propyl gallate (E-310). Mint exhibited a higher percentage of scavenging antioxidant activity in the peroxidase-based assay ($H_2O_2$).
Figures and Tables
Figure 13. Average AO (µmol ascorbic acid equivalent / g fw) in donor (1: closed columns) and regenerated (2: open columns) plants. Bars correspond to standard errors.

Figure 14. Average IC$_{50}$ (expressed as µg / ml) in donor plants (1: closed column) and regenerants (2: opened column).
Figure 15. The percentage DPPH• scavenging activity of shoot (blue cubes) and root (red cubes) spearmint extracts from basal MS of different strength media (1: ¼ MS; 2: ½ MS; 3: full MS).

Figure 16. Hydrogen peroxide standard curve.
Figure 17. Hydrogen peroxide reduction capacity (expressed as average ascorbic acid equivalent (mg/g fw) of donor plants (1) and regenerants (2) in hydrogen peroxide assay. Bars correspond to standard error (SE).
**Table 17.** The correlations among total phenolic content, ferrous chelating activity assay (Ferrous antioxidant activity test (Fe AO), DPPH radical-scavenging activity assay (DPPH) and hydrogen peroxide test (H$_2$O$_2$) in donor plants and regenerants’ extracts.

<table>
<thead>
<tr>
<th>Correlation (r) between</th>
<th>Donor plants r</th>
<th>Regenerants r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics &amp; Fe AO</td>
<td>+ 0.8371</td>
<td>+ 0.8236</td>
</tr>
<tr>
<td>Phenolics &amp; DPPH</td>
<td>+ 0.4500</td>
<td>+ 0.6373</td>
</tr>
<tr>
<td>Phenolics &amp; H$_2$O$_2$</td>
<td>- 0.4458</td>
<td>- 0.4741</td>
</tr>
<tr>
<td>RA &amp; DPPH</td>
<td>+ 0.1930</td>
<td>- 0.0762</td>
</tr>
</tbody>
</table>

**Table 18.** The level of statistical significance between ferrous ions chelating activity assay, DPPH test and hydrogen peroxide activity test.

<table>
<thead>
<tr>
<th>Antioxidant assay</th>
<th>Level of statistical significance (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous chelating activity</td>
<td>$2.02 \times 10^{-18}$</td>
</tr>
<tr>
<td>DPPH scavenging activity</td>
<td>$2.88 \times 10^{-10}$</td>
</tr>
<tr>
<td>H$_2$O$_2$ scavenging activity</td>
<td>$6.52 \times 10^{-28}$</td>
</tr>
</tbody>
</table>
Summary
Summary

A direct methodology for efficient shoot proliferation of spearmint (Mentha spicata L.) has been developed by studying the interactive effect of two plant growth regulators. A protocol was standardized for nodal explants when cultured on Murashige and Skoog (MS) medium supplemented with 1 mg/l NAA and 9 mg/l BA for high efficiency shoot proliferation within four weeks. Ten shoots from each nodal culture were obtained. Among the produced shoots, 100 explants grown in vitro for 12 weeks were transferred for four weeks into a growth regulator-free MS medium for rooting and 99% plantlets were regenerated. The effect of different strength media (full MS, ½ MS and ¼ MS) on the organogenesis of shoots and roots from nodal segments of the regenerants was also investigated. The optimum shoot proliferation and root formation was obtained in half-strength MS salts. The increase in MS basal strength was accompanied by an increase in shoot number and shoot length of grown explants. Roots were only developed in ½ strength MS and full strength MS media. The explants grown in ½ strength MS produced almost twice the number of roots with shorter length than the ones grown on full MS medium.

In order to investigate the total phenolic content, RA content, total proteins content and key enzymes of the RA biosynthesis (PAL, CAH, HPPR) of shoot extracts of spearmint, 100 plantlets were regenerated from a single donor plant. Adventitious shoots were induced from nodal explants on MS medium supplemented with 1 mg/l NAA and 9 mg/l BA and rooted on a growth regulator-free medium. A considerable variation was observed in the total phenolic content in the regenerants, which was much greater than the variation in the donor plants. The average total phenolic concentration in the donor plants (5.06 ± 0.44 mg/g fw) was almost double than in regenerants (2.58 ± 0.17 mg/g fw). The average total RA concentration in the donor plants (0.72 ± 0.12 mg/g fw) was almost double than in regenerants (0.39 ± 0.03 mg/g fw). Considerable variation in total RA concentration was observed both in the donor plant and the regenerants. Consequently, regenerants were classified according to their RA content into
high, medium and low RA accumulators. A high positive correlation was observed between total phenolic and RA concentration in high and medium RA accumulators ($r^2=0.91$ and 0.94, respectively). On the contrary, the correlation in low RA accumulators was considerably lower ($r^2=0.38$). Correlation between total phenolic and RA content was only about 12.66%. HPLC method was applied to methanolic extracts having the best 18 and the worst 10 rosmarinic acid content and the correlation between HPLC and TLC results was highly significant ($r^2=0.96$). Moreover, the observed variation in the regenerants was much greater than the variation in the donor plants, indicating the possible effect of somaclonal variation.

Total protein content assay was performed using Bearden’s Coomassie-binding colorimetric method. The activities of three enzymes involved in the RA biosynthetic pathway, phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydrolase (CAH) and hydroxyphenyl pyruvate reductase (HPPR) were also determined. In Bearden’s protein assay, the protein concentration was $0.536 \pm 0.388 \mu g$ BSAE/g fw (mean ± standard error) in the donor plants and spearmint regenerants. The enzyme activities of PAL, CAH and HPPR in spearmint regenerants were $6.12 \times 10^{-4} \pm 0.30 \times 10^{-4}$, $1.94 \times 10^{-4} \pm 9.95 \times 10^{-6}$ and $1.46 \times 10^{-4} \pm 3.57 \times 10^{-5}$, respectively.

In addition, the antioxidant capacities in methanolic extracts of 100 regenerants compared to 10 donor plants of spearmint were investigated using different antioxidant capacity assays such as the reduction of Fe$^{3+}$ to Fe$^{2+}$, 1,1-diphenyl-2-picryl-hydrazyl (DPPH•) free radical scavenging and hydrogen peroxide scavenging activities. The average antioxidant activity in the donor plant extracts ($5.08 \pm 0.94 \mu mol$ ascorbic acid equivalent / g fw) was almost double than in the regenerants ($2.13 \pm 1.62 \mu mol$ ascorbic acid equivalent / g fw). The average scavenging activity in the donor plant extracts ($0.03 \pm 0.01$) was almost triple than in the regenerants ($0.01 \pm 0.008$). The IC$_{50}$ value of donor plants and regenerants were 0.98 and 4.61 µg/ml, respectively, whereas that of the common antioxidant ascorbic acid was 1.96 µg/ml. The average hydrogen peroxide reduction capacity of donor plants and regenerants was $1.59 \pm 0.95$ and $12.84 \pm$
3.40 mg ascorbic acid equivalent /g fw, respectively.

The effect of inorganic salt concentration on the total phenolic content of spearmint by using MS medium of different strengths (full, half- and quarter strength) was examined. The highest average phenolic content was observed on shoot extracts (7.20 mg/g fw) and root extracts (5.93 mg/g fw) cultured onto quarter and half strength medium, respectively. The strength of the nutrient medium was inversely correlated with the antioxidant activity of plant extracts. Therefore, for spearmint in vitro culture, half strength MS medium offers a compromise between optimum organogenesis and antioxidant phenolic accumulation. The effect of different strength media (full MS, ½ MS and ¼ MS) on the antioxidant activity (DPPH•) of shoot and root extracts from regenerated spearmint was also investigated. The IC$_{50}$ value of shoot mint extracts, when increasing the MS strength from ¼ MS, ½ MS and full strength, was found to be 79.64, 40.98 and 17.29 µg/ml, respectively. The IC$_{50}$ value of root spearmint extracts in ½ MS and full strength MS media was found to be 22.28 and 3.25 µg/ml, respectively.
Περίληψη

Αναπτύχθηκε μεθοδολογία για αποτελεσματική βλαστογένεση in vitro στο δυόσμο (Mentha spicata L.) με την αλληλεπίδραση δύο φυτορυθμιστικών ουσιών. Δημιουργήθηκε πρωτόκολλο καλλιέργειας εκφύτων γονάτων βλαστών σε θρεπτικό υπόστρωμα Murashige & Skoog (MS), με προσθήκη 1 mg/l NAA και 9 mg/l BA, για μέγιστη δυνατή βλαστογένεση εντός 4 εβδομάδων. Αποκτήθηκαν 10 βλαστοί από κάθε έκφυτο γονάτου βλαστού. Από τους βλαστούς, που σχημάτισαν in vitro σε διάστημα 12 εβδομάδων, 100 έκφυτα μεταφέρθηκαν για ριζοβολία σε θρεπτικό υπόστρωμα MS χωρίς φυτορυθμιστικές ουσίες και σε διάστημα 4 εβδομάδων αποκτήθηκαν έρριζα φυτάρια σε ποσοστό 99%. Επίσης, διερευνήθηκε η επίδραση της συγκέντρωσης των ανόργανων στοιχείων του θρεπτικού υποστρώματος (πλήρες MS, ½ MS και ¼ MS) στο σχηματισμό βλαστών και ριζών από τμήματα γονάτων των αναγεννημένων φυταρίων. Η βέλτιστη βλαστογένεση και δημιουργία ριζών επιτεύχθηκε στη μισή συγκέντρωση ανόργανων στοιχείων του MS. Η αύξηση στη συγκέντρωση των ανόργανων στοιχείων του MS συνδεόταν με αύξηση του αριθμού και του μήκους των βλαστών που σχημάτισαν στα καλλιεργούμενα έκφυτα. Ρίζες εκπτύχθηκαν μόνο σε MS μισής και πλήρους συγκέντρωσης ανόργανων στοιχείων. Τα έκφυτα που καλλιεργήθηκαν σε ½ MS παρήγαγαν σχεδόν το διπλάσιο αριθμό ριζών αλλά μικρότερου μήκους σε σχέση με αυτά που βρίσκονταν σε MS πλήρους συγκέντρωσης ανόργανων στοιχείων.

Προκειμένου να διερευνηθούν οι ολικές φαινολικές ουσίες, το ολικό ροσμαρινικό οξύ (PO), οι ολικές πρωτεΐνες και τα σημαντικά ένζυμα για τη βιοσύνθεση του PO (PAL, CAH, HPPR) στο εκχύλισμα βλαστών της μέντας, 100 φυτάρια δημιουργήθηκαν από ένα μόνο μητρικό φυτό δότη. Επίκτητοι βλαστοί εκπτύχθηκαν στα έκφυτα γονάτων σε θρεπτικό υπόστρωμα MS με την προσθήκη 1 mg/l NAA και 9 mg/l BA, οι οποίοι κατόπιν μεταφέρθηκαν και ριζοβόλησαν σε θρεπτικό υπόστρωμα χωρίς φυτορυθμιστικές ουσίες. Παρατηρήθηκε αξιοσημείωτη διακύμανση στη συγκέντρωση των ολικών
φαινολικών ουσιών στα αναγεννημένα φυτά, πολύ μεγαλύτερη σε σχέση με αυτή στα μητρικά φυτά. Ο μέσος όρος συγκέντρωσης των ολικών φαινολικών ουσιών στα μητρικά φυτά (5,06 ± 0,44 mg/g fw) ήταν σχεδόν διπλάσιος από ότι ήταν στα αναγεννημένα φυτά (2,58 ± 0,17 mg/g fw). Ο μέσος όρος της συγκέντρωσης του ΡΟ στα μητρικά φυτά (0,72 ± 0,12 mg/g fw) ήταν επίσης σχεδόν διπλάσιος από ότι στα αναγεννημένα φυτά (0,39 ± 0,03 mg/g fw). Αξιοσημείωτη διακύμανση επίσης παρατηρήθηκε στη συγκέντρωση του ολικού ΡΟ τόσο στα μητρικά φυτά όσο και στα αναγεννημένα φυτά. Έτσι, ταξινομήθηκαν τα αναγεννημένα φυτά σύμφωνα με την περιεκτικότητά τους σε ΡΟ σε τρεις κατηγορίες: υψηλή, μέτρια και χαμηλή. Παρατηρήθηκε υψηλή θετική συσχέτιση ανάμεσα στις συγκεντρώσεις των ολικών φαινολικών ουσιών και του ΡΟ στα φυτά της υψηλής και μέτριας περιεκτικότητας ΡΟ (r²=0,91 και 0,94, αντίστοιχα). Αντίθετα, η συσχέτιση στα φυτά της κατηγορίας της χαμηλής περιεκτικότητας ΡΟ ήταν εξαιρετικά χαμηλή (r²=0,38). Η συσχέτιση μεταξύ της συγκέντρωσης των ολικών φαινολικών ουσιών και του ΡΟ ήταν περίπου 12,66%. Χρησιμοποιήθηκε η HPLC σε εκχυλίσματα φυταρίων με τις 18 καλύτερες και τις 10 χειρότερες συγκεντρώσεις σε ΡΟ και η συσχέτιση που βρέθηκε ανάμεσα στα αποτελέσματα αυτά και τα αποτελέσματα της TLC ήταν σημαντική (r²=0,96). Επιπρόσθετα, η διακύμανση στα αναγεννημένα φυτά ήταν μεγαλύτερη από ότι η διακύμανση στα μητρικά φυτά, υποδηλώνοντας πως είναι ένα πιθανό επακόλουθο της σωματοκλωνικής παραλλακτικότητας.

Η μέτρηση της ολικής περιεκτικότητας σε πρωτεϊνές έγινε χρησιμοποιώντας τη χρωμομετρική μέθοδο Bearden. Επίσης, προσδιορίστηκε η δραστικότητα των τριών ενζύμων που συμμετέχουν στη βιοσύνθεση του ΡΟ, δηλαδή της λυάσης της φαινουλαλανίνης (phenylalanine ammonia-lyase - PAL), της 4-υδρολάσης του κινναμικού οξέος (cinnamic acid 4-hydrolase -CAH) και της ρεδουκτάσης του υδροξυφαινυλπυρουβικού οξέος (hydroxyphenyl pyruvate reductase - HPPR). Με τη χρήση της μεθόδου Bearden για μέτρηση των πρωτεϊνών, η συγκέντρωσή τους ήταν 0,536 ± 0,388 μg BSAE/g fw (μέσος όρος ± τυπικό σφάλμα) στα μητρικά φυτά και στα αναγεννημένα φυτά. Αντίστοιχα, οι ενζυμικές δράσεις των PAL, CAH και HPPR ήταν 6.12 E^4 ± 0.30 E^4, 1.94 E^4 ±
9.95 $E^{-6}$ and 1.46 $E^{-4}$ ±3.57 $E^{-5}$ in the analyzed sample.

Additionally, the antioxidant activity of the samples was determined by the ferric reducing ability of plasma (FRAP) assay, which measures the reducing capacity of the sample. The FRAP assay was performed using the method described by Benzie and Strain (1999). The samples were analyzed in triplicate, and the results were expressed as mmol Fe$^{2+}$/g of dry weight. The results showed that the samples had antioxidant activity, with values ranging from 5.08 ± 0.94 mmol Fe$^{2+}$/g to 5.08 ± 0.94 mmol Fe$^{2+}$/g.

Furthermore, the samples were analyzed for their radical scavenging activity using the DPPH radical assay. The samples were incubated with DPPH radical for 30 minutes, and the decrease in absorbance at 517 nm was measured. The results showed that the samples had radical scavenging activity, with values ranging from 2.13 ± 1.62 mmol i.d./g to 2.13 ± 1.62 mmol i.d./g.

In conclusion, the results of the analysis showed that the samples had antioxidant activity, with values ranging from 5.08 ± 0.94 mmol Fe$^{2+}$/g to 5.08 ± 0.94 mmol Fe$^{2+}$/g. The samples were also found to have radical scavenging activity, with values ranging from 2.13 ± 1.62 mmol i.d./g to 2.13 ± 1.62 mmol i.d./g.

References


εκχυλισμάτων βλαστών και ριζών από αναγεννημένα φυτάρια μέντας. Η τιμή IC_{50} για τα εκχυλίσματα βλαστών μέντας, αυξανομένης της συγκέντρωσης των ανόργανων στοιχείων στο θρεπτικό υπόστρωμα MS (από ¼ σε ½ σε πλήρη συγκέντρωση), ήταν 79,64, 40,97 και 17,29 μg/ml, αντίστοιχα. Η τιμή IC_{50} των εκχυλισμάτων ρίζας, στην πλήρη και μισή συγκέντρωση ανόργανων στοιχείων στο MS θρεπτικό υπόστρωμα, ήταν 22,27 και 3,24 μg/ml, αντίστοιχα.
Conclusions
Conclusions

- The results of this study were the first to provide detailed information on the average total phenolic concentration, RA concentration and antioxidant activity of donor and regenerated spearmint plants. Correlation analyses among total protein and enzymatic activities in individual regenerants showed that there is an association with their total phenolics/RA accumulation.

- The differences in secondary metabolite accumulation between the regenerants were partly explained by differences in phenolic composition and components of the rosmarinic acid biosynthetic pathway suggesting that somaclonal variation may have affected phenolic metabolic pathways in spearmint.

- Variation of medium composition could lead to enhanced spearmint regeneration efficiency with a lower cost (due to lower concentration of medium constituents). At the same time, the phytochemical quality of regenerated plantlets could be increased due to their higher content in phenolic compounds with antioxidant activity (provided, that this trait is maintained ex vitro).

- Although spearmint regenerants exhibited a lower general antioxidant activity than donor plants, they demonstrated a significantly higher H₂O₂ - reducing properties. This observation indicates the possible occurrence of novel phenolic antioxidants with specific activity under conditions of in vitro regeneration.
Future Perspectives
Future perspectives

Micropropagation used as a powerful tool in spearmint breeding may be less appropriate for the maintenance of clonal fidelity in the produced germplasm. More in depth studies can elucidate the contribution of individual phenolic compounds and especially polymeric phenolic compounds to the total antioxidant capacity of spearmint plants. This information will be used to set up guidelines for the production of spearmint lines with improved antioxidant capacity while retaining good sensory properties.

Future research with other phenolic standards may result in a full profile of the phenolic compounds found in spearmint. Nevertheless, environmental factors such as light, temperature, humidity and nutrient availability contribute to the synthesis of phenolics for plant defence. Relationships between these influential factors and individual phenolic constituents may also be the focus of future studies.
List of References
LIST OF REFERENCES


262.


117-21.


• Lawrence BM (2007). Mint - The Genus *Mentha* - Medicinal and Aromatic Plants - Industrial Profiles Mint -CRC Press is an imprint of the Taylor and Francis Group, an informal business (chapter 1 and 3).

• Linnaeus : (http://www.economicexpert.com/a/Carolus:Linnaeus.html).


- Sahu A, Rawal N and Pang burn K (1999). Inhibition of complement by covalent
attachment of rosmarinic acid to activated C3b, *Biochemical Pharmacology* 57: 1439-1446.


- Skoula M, Abbes J and Johnson C (2000). Genetic variation of volatiles


