FACTORS AFFECTING ESSENTIAL OIL PRODUCTION IN ROSEMARY

(*Rosmarinus officinalis* L.)

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Anas Muneer Tawfeeq

October 2016

Abstract

There are many factors shown to have beneficial effects on many crop plants. Here we investigate the impact of fertilizers and genetic variation on *Rosmarinus officinalis* L. measured by both oil yield and quality.

Plants grown in a temperature-controlled greenhouse with a natural photoperiod and a controlled irrigation system were treated with seaweed fertilizer and an inorganic fertilizer of matching mineral composition but with no organic content. Treatments were either by spraying on to the foliage or watering direct to the compost. The essential oil was extracted by hydro-distillation with a Clevenger apparatus and analysed by gas-chromatography mass-spectrometry (GC–MS) and nuclear magnetic resonance spectroscopy (NMR). The crop responded positively to the application of fertilizer when compared to the control (no fertilizer). The seaweed treatments caused a significant increase in oil amount and leaf area as compared with both inorganic treatments and the control regardless of application method. The application of cytokinin in seaweed form also had a positive role with plant growth and oil production. The chemical compositions of the plants were compared, and qualitative differences were found between fertilizer treatments, application methods, ages of the plant and different genotype. The difference in oil composition were influenced partly by applying seaweed fertilizer. A full chemical analysis of the essential oil was conducted in order to identify the main components. Nine compounds were determined. Eucalyptol and camphene were shown to make up more than half. The other compounds made up the remaining 30%. In general, oil yields are reduced in the older plants whether from those with applied fertilizer or the control. The different genotypes showed a highly significant difference in oil composition and yield compared with other factors affecting essential oil production showing that rosemary plants vary greatly and the correct cultivar should be chosen with reference to its intended final use.

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1. Chapter One: Introduction

The production of essential oils from plants is a multi-million pound industry (Munir and Hensel, 2009) yet opinion is divided on why plants produce these oils and many functions have been suggested from anti-herbivory to allelopathy to adaption to a fire-based ecology (Langenheim, 1994; Kennedy and Wightman, 2011). To understand and control essential oil production requires a basic knowledge of the natural environment of oil-producing plants and consideration of the taxonomic distribution of species containing oils.

The Earth is unlike every other planet in the solar system. It has liquid water on its surface and a distinctive atmosphere rich in oxygen different from any planet, and it is probably the only planet in the solar system that has life. The distribution of this life depends on the balance of resources in order to optimise growth. Temperature and sun light intensity are factors along with the fundamental characteristics of the planetary atmospheric environment. In terms of maintaining life, the Earth appears to have the appropriate amount of solar radiation and is at the ideal temperature.

Biodiversity is the variety of species present in the community of an ecosystem. The Earth holds a high number of species and its biodiversity in general species are affected by abiotic (non-living environmental) and biotic (interaction associated with living things) factors. High levels of crop production appear to disrupt the ecosystem. For example, the use of chemical fertilizers has been associated with the accumulation of high levels of nitrates and phosphates in the water supply. One major concern has been recently to decrease the use of chemical fertilizers in order to reduce pollution resulting from the demands of an increasing world population, which is expected to reach 9 billion by 2050 (Oliver *et al.*, 2013). Both the environment and the costs of production are concerns; therefore, they should be considered equally when increasing land productivity. For this purpose, a precise investigation of crop environment such as soil properties and micro-climate is required which can differ significantly in spatial and temporal scales (Blondel *et al.*, 2010).

1.1 Mediterranean plants

The Mediterranean Basin region is a global biodiversity hotspot (Cuttelod *et al.*, 2009). The total number of flowering plants belong to the Mediterranean regions is large and difficult to assess, this richness of the flora is due to the great variations in climate and habitat (Polunin and Huxley, 1965). The prevailing climate in this region is characterised by distinctive physical environments with mild to cool, wet winters and warm to hot, dry periods in summer, as well as by high inter-annual variability. The native vegetation of the Mediterranean climate lands is adapted to these environmental conditions and suits a wide range of different kinds of plants (Table 1.1).

Typical adaptations include deep-rooted evergreen sclerophyll shrubs and trees which maintain green leaves and tolerate water stress during the drought period, semi-deciduous shrubs which lose some of their leaves and annual, biennial and perennial herbs and geophytes which finish their annual cycle before summer to escape the drought period (Ehleringer and Mooney, 1983).

Table 1.1 Types of plants living and adapted to Mediterranean environment

Type of plant	Example
Evergreen trees	Pines, cypresses, and oaks
Deciduous trees	Sycamores, oaks, horse and sweet chestnut
Fruit trees	Olives, figs, citrus, walnuts and grapes
Shrubs	Bbay laurel, *ericas*, rosemary, thyme and lavender
Sub-shrubs	Sages, artemisias, *Echinospartum horridum*, *Salvia lavandulifolia* and *Linum suffruticosum*
Grasses	Grassland types such as *Themeda triandra*, *Eragrostis barrelieri, Schismus barbatus* and *Rostraria cristata*
Herbs	Chamomile, silene, calendula and *Narcissus obesus*

1.2 Plants producing essential oils

For more than 5000 years ago, the Egyptians used aromatic plants* for medicinal and cosmetic purposes, as well as for the embalming of the deceased, and this was probably one of the earliest ways of using aromatics (Sipos *et al.*, 2004).

***** Aromatic plants are a class of plants used for their aroma and flavour and term relates to the aroma. To avoid confusion with aromatic (chemistry) to indicate that a compound contains a benzene ring, this term will only be used in a botanical context.

In the 10th century, Arabic physician Avicenna studied and described 800 plants and their effects on the human body in addition to the development of the distillation process for essential oils for which he is credited. In the 12th century, the essential oils industry became important in Europe, and the first perfume derived from essential oil was made in Italy. Since that date the perfume industry grew as new aromatic materials were intredused. In England, the distillation of aromatic oil from lavender has begun after the Romans brought the perfume to British Isles. By 1750, the commercial production peppermint was started in Mitcham, Surrey (Rhind, 2012).

However, there are many plants used due to their volatile oil components for different purposes in food processing, pharmaceutical industries and the perfumery sector; such volatile components are often termed essential oils. There are many plants that are utilized as major sources of essential oils and different parts of the plants may contain this essential oil. Such parts include seeds, fruits, leaves, roots and flowers as shown in Table 1.2 each of which may provide the major source of the oil in specific plant. Essential oil production is described in terms of plant secondary metabolites and this has been a fertile area of chemical investigation (Zuzarte and Salgueiro, 2015). Based on this, several studies have been dedicated to the production of essential oil-containing plants in countries bordering the Mediterranean Sea (Mediterranean Basin) (Fig 1.1), which have attracted increasing interest from both the general population and the scientific community due to their essential oil content and their uses as alternative remedies, sources of natural aromas, and flavourings. These studies have shown the importance of the essential oils which are found and isolated from these many different plants (Friedman *et al.*, 2002; Tongnuanchan and Benjakul, 2014). From the commercial point of view, the production of aromatic plants in Mediterranean countries, is approximately 38 million tonnes per year, with Turkey being the highest producer (Rosmini *et al.*, 2000; Viuda-Martos *et al.*, 2007).

Table 1. 2 Plant material containing essential oils.

Parts	Plants
Leaves	Basil, bay leaf, cinnamon, common sage, eucalyptus, lemon grass, citronella, melaleuca, mint, oregano, patchouli, peppermint, pine, rosemary, spearmint, tea tree, thyme, wintergreen, kaffir lime, laurel, savory, tarragon, cajuput, lantana, lemon myrtle, lemon, teatree, niaouli, may chang, petitgrain, laurel, cypress
Seeds	Almond, anise, cardamom, caraway, carrot celery, coriander, cumin, nutmeg, parsley, fennel
Wood	Amyris, atlas cedarwood, himalayan cedarwood, camphor, rosewood, sandalwood, myrtle, guaiac wood
Bark	Cassia, cinnamon, sassafras, katrafay
Berries	Allspice, juniper
Resin	Frankincense, myrrh
Flowers	Blue tansy, chamomile, clary sage, clove, cumin, geranium, helichrysum hyssop, jasmine, lavender, manuka, marjoram, orange, rose, immortelle, neroli
Peel	Bergamot, grapefruit, kaffir lime, lemon, lime, orange, tangerine, mandarin
Root	Ginger, plai, turmeric, valerian, vetiver, spikenard, angelica
Fruits	Xanthoxylum, nutmeg, black pepper

(Tongnuanchan and Benjakul, 2014)

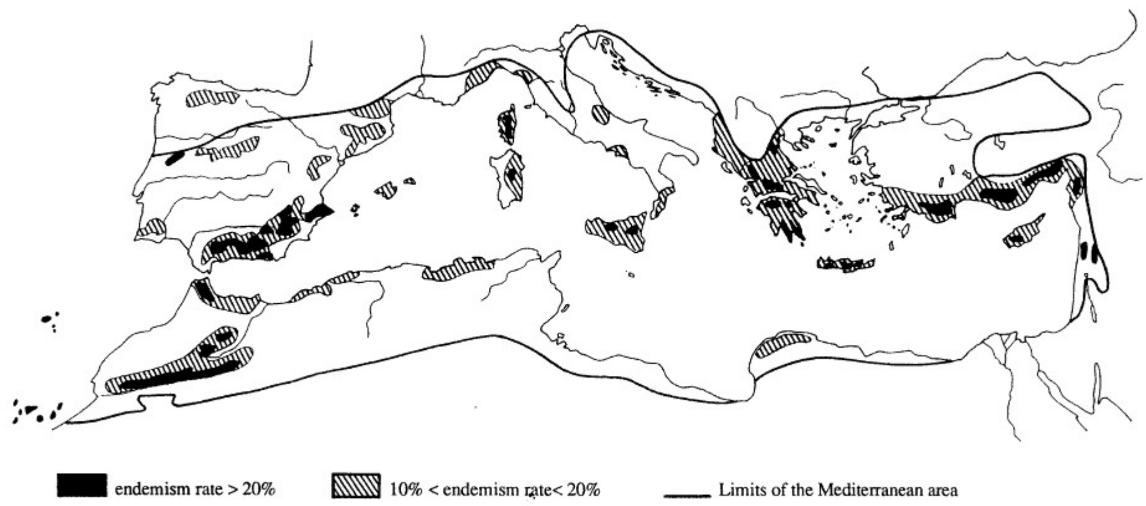


Figure 1. 1 Biogeographical sectors with high incidences of plant endemism in the Mediterranean Basin (Medail and Quezel, 1997)

Families and species

Many plant species produce essential oils and show variation in monoterpene production. These plants are distributed among many different families and species and can be found worldwide, such as oregano, peppermint, rosemary, sage, thyme, and garlic (Christaki *et al.*, 2012). Essential oils are produced commercially from about 400 species distributed between 67 plant families around the world (Bernáth, 2009). Nearly 49% of the world's aromatic plants occur in the Mediterranean Basin, including Lamiaceae, Asteraceae and Apiaceae which contain the majority of these plants (Allen, 2014). Sombrero (1992) reported that 49 families and 153 genera of plants bear essential oils, and most of them occur in Mediterranean-type environments around the world. In the Mediterranean basin alone there are approximately 90 genera that produce and accumulate essential oil in their cells (Ross and Sombrero, 1991). Bernáth (2009); Nurzyńska-Wierdak (2013) and Allen (2014) reported some of these species characteristic of the Mediterranean region such as: *Rosa damascena* (Rosaceae)*;* Myrtaceae: *Myrtus communis* (Myrtaceae); *Carum carvi, Pimpinella anisum*, *Foeniculum vulgare, Coriandrum sativum*, *Anethum graveolens, Angelica archangelica, Levisticum officinalis* (all Apiaceae)*; Mentha piperita*, *Mentha spicata, Salvia officinalis*, *Salvia sclarea, Rosmarinus Officinalis, Thymus vulgare*, *Lavandula* spp. (Lamiaceae); *Citrus aurantium* subsp. and Bergamia, *Citrus bergamia.*

1.3 Essential oils

Essential oils, also known as volatile oils, etheric oils, essences, or aetheroleum, are formed by natural products representing several volatile compounds (Sangwan *et al.*, 2001), obtained from plant raw material by several methods such as hydrodistillation, steam distillation or dry distillation. Essential oils are mixtures of volatile compounds, and vanish rapidly without leaving any stain, have a strong aroma, do not form a homogeneous mixture with water, but are soluble in organic solvent, and can be obtained from different parts of plants by distillation (Zuzarte and Salgueiro, 2015). This highly variable mixture contains terpenes as a predominant constituent besides other chemicals such as phenylpropanoids. There is indirect evidence apropos the various terpenoid types that are synthesized within the secretory cells (specific oil cells in and around specialized glands). Among the Lamiaceae, the primary secretory organ is the glandular trichome. The accumulation of oil is often found in a bulbous, sub-cuticular chamber, in droplets of fluids located under the surfaces of leaves, trichomes and in secretory cavities in bark or the secretory canals of plant-cell walls or in glandular hairs which are found on the upper leaf surface of the plant (Venkatachalam *et al.*, 1984; Abdelmajeed *et al.*, 2013).

1.3.1 Function of essential oil in plants

It has been shown that essential oils can play an important role in the interactions between plants and their environments. Essential oils serve and perform several functions and benefits in plant defence and communication (Kirby and Keasling, 2009). Many studies have demonstrated that essential oils have a role in most interactions between the plant and other plants, animals or micro-organisms; examples include the attraction of honey bees or protection against insect pests (Beker *et al.*, 1989; Harborne, 1991; Shaaya *et al.*, 1991). According to Goodwin and Mercer (1983), terpenoids are produced by plants in great variety (over 1,000) but the functions are poorly understood. Some of them have important function activities such as protecting against photodynamic sensitization, hormonal function as they contribute to the chemical structures of growth regulators like cytokinin, gibberellins, abscisic acid and xanthoxins; and polyprenyl pyrophosphates function in cell-wall formation in glycosylation. Also, terpenoids play an important role in plant metabolism and photosynthetic electron transport by a phytyl side chain (which is a terpenoid structure) through activating chlorophyll. Further, monoterpenes may be used as a source providing both carbon and energy under photosynthate deficiency situations inside the plant (Croteau, 1988).

It has been determined that 1,8-cineole (eucalyptol) is one of the main components of essential oils which is often responsible for the oil's effectiveness against some insects, such as the beetle *Rhyzopertha dominica* (Sombrero, 1992). Thus, essential oils can play a vital role to protect the plant; for example, from insects and thermal damage, and they may play an important role in the plant's fitness under extreme environmental conditions (Koul *et al.*, 2008; Llusià *et al.*, 2009; Prins *et al.*, 2010). Further, it has been stated that terpenoid constituents correlate with plant systematic and population patterns indicating a strong and conserved genetic basis (Naydenov *et al.*, 2006).

1.3.2 Use of essential oils

Plants producing essential oils have been used widely for maintaining human health as medicine and in food additives as flavour. About 25% of prescription medicines are derived directly or indirectly from 100 plant species, and aromatic plants constitute the largest proportion of these sources (Barboza *et al.*, 2009). However, essential oils are also used in various sectors as a natural source of additives for food, perfumes, cosmetics, soaps and other products. They can be used commercially as an alternative remedy for the treatment of several infectious diseases or as a purifier with properties which include anti-bacterial, anti-oxidant, spasmolytic, carminative (a drug that relieves flatulence), hepatoprotective, anti-viral, anti-fungal, anti-inflammatory, anticarcinogenic activities; more recently, the toxicity of essential oil has been applied to pest control products (Agunu *et al.*, 2005; Tongnuanchan and Benjakul, 2014; Szumny *et al.*, 2010; Jiang *et al.*, 2011; Derwich *et al.*, 2011).
Essential oils have been used throughout history in a wide variety of "wellness" applications. The Egyptians were some of the first people to use aromatic essential oils extensively in medical practice, beauty treatment, food preparation, and in religious ceremonies. The ancient civilizations of Rome, the Orient and Greece were familiar with these compounds and

oils and resin collected from the plants such as frankincense, sandalwood, myrrh and cinnamon were very valuable cargo and were sometimes exchanged for gold (Urdang, 1943). Borrowing from the Egyptians, the Greeks used essential oils for therapeutic massage and aromatherapy. The Romans also used aromatic oils to promote health and personal hygiene. In addition, influenced by the Greeks and Romans, as well as Chinese and Indian Ayurvedic (a system of medicine with historical roots) use of aromatic herbs, the Persians began to develop distillation methods for extracting essential oils from aromatic plants. Essential oil extracts were used throughout the dark ages in Europe for their anti-bacterial properties, and for their fragrance.

Aromatic plants have continued to be very popular, they have been used for the treatment of diseases and added to food to improve the flavour and organoleptic properties (Szumny *et al.*, 2010). Currently therapeutic uses include as a pain reliever, to treat anxiety, alertness and they are also used as a stimulator for hair growth and skin care (Oluwatuyi *et al.*, 2004). Furthermore, the interest in the essential oils industry has expanded rapidly during the 20^{th} century, especially during the 1990s, when culinary herbs, fresh or dried, started to attract attention as a source of natural anti-oxidants to provide an alternative to synthetic anti-oxidants; particular attention was paid to those extracted from rosemary oil, and these are used as natural additives in foods and in the food industry, (Munné-Bosch and Alegre, 2001).

1.3.3 Regulation of essential oil production

Essential oils variation in flavour and odour, and the quantity of oil produced has been reported to be associated with the early growth period of the shoot and depends on which plant they are extracted from, and the growth stage of that plant. In general, not much is known about regulation of production of essential oils. The variation in oil is closely tied in to the physiology of the plant and is highly dependent on the metabolic state and pre-determined developmental segregation programme of the synthesising tissue (Sangwan *et al.*, 2001). The variability in oil amount and composition is linked to intrinsic and extrinsic factors (Flamini *et al.*, 2002b).For example; the level of cinnamyl alcohol dehydrogenases enzyme has been shown to be well correlated with the citral: geraniol ratio in some plants, not only with a difference in species but also with developmental stages (Sangwan *et al.*, 1993). Hence significant increase in monoterpene synthesis has been reported at the time of flowering and a rapid decline at the full bloom stage (Ganjewala and Luthra, 2010). However, monoterpenes such as linalool, elemol, 1,8-cineole, and limonene are derived from geranyl pyrophosphate (GPP) after various secondary transformations. Monoterpene production in glandular trichomes, as determined by studies with radio-carbon dioxide ($^{14}CO_2$), is restricted to leaves 12 to 30 days of age (Ganjewala and Luthra, 2010). Moreover, monoterpene content and composition also changes considerably during leaf development.

1.3.4 Variation in essential oils

The complex mixture of an essential oil consists of compound types classified chemically on the basis of the arrangement of carbon and hydrogen atoms and their number; such as terpenes (monoterpenes and sesquiterpenes), terpenoids (isoprenoids) and aromatic compounds (alcohol, methoxy derivative, aldehyde and so on). Indeed, largest fraction of the contents of essential oil are monoterpenes including acyclic (geraniol) (Fig 1.2), monocyclic (limonene) (Fig 1.3) or bicyclic (α and β-pinene) (Fig 1.4). These compounds could be unsaturated hydrocarbons (limonene) or contain functional groups such as alcohol (menthol), and aldehydes or ketones (menthone, carvone) (Harborne, 1973). These compounds can be classified under two major groups: terpene hydrocarbons and oxygenated compounds (Bakkali *et al.*, 2008; Tongnuanchan and Benjakul, 2014).

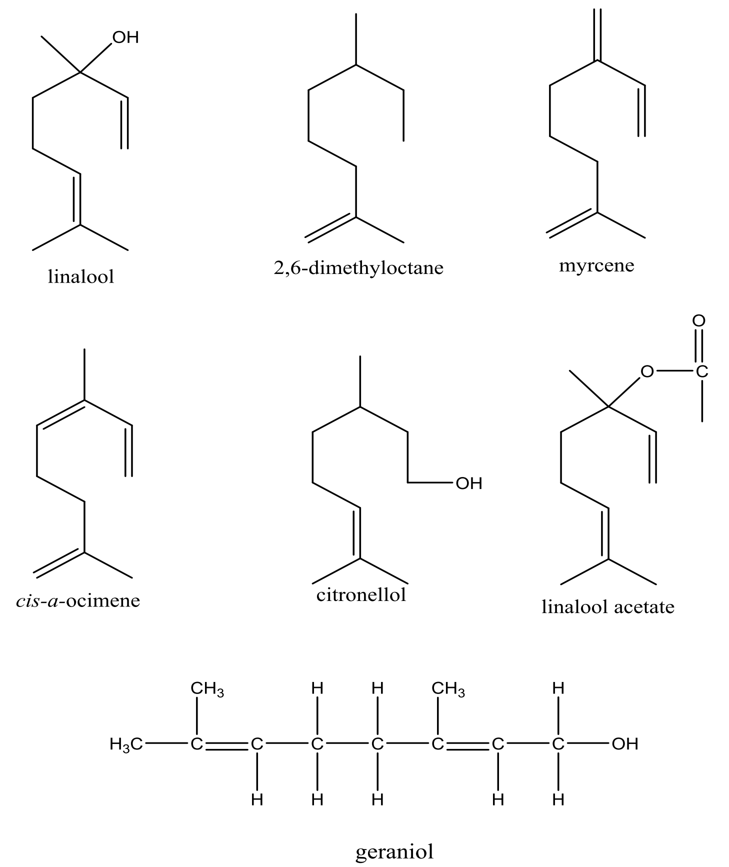


Figure 1. 2 Chemical structures of acyclic monoterpenes

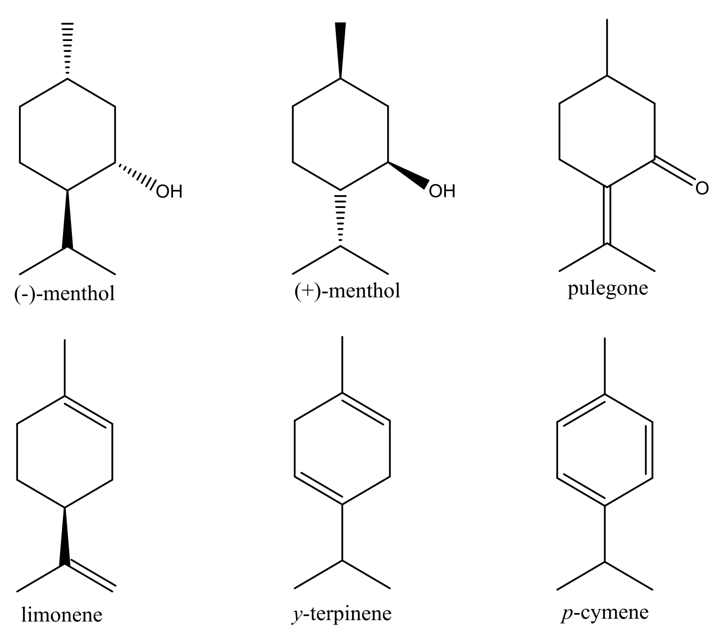


Figure 1. 3 Chemical structures of monocyclic monoterpenes

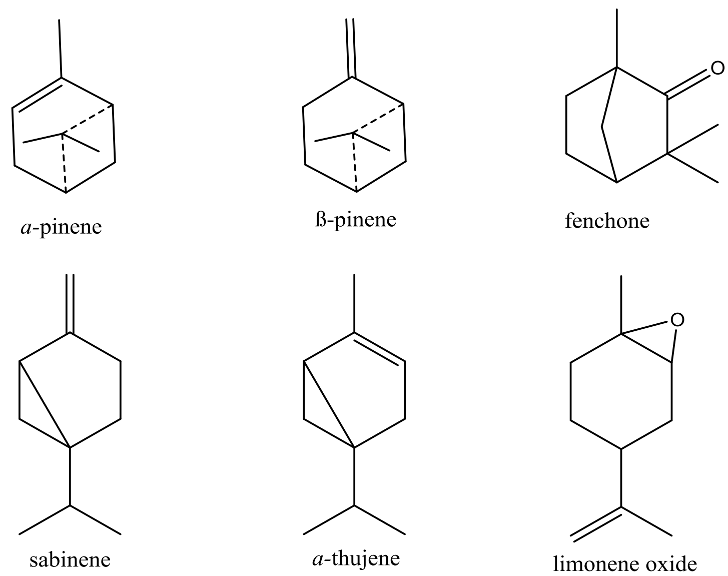


Figure 1. 4 Chemical structures of bicyclic monoterpenes

Terpene hydrocarbons

Terpene hydrocarbons are constituted of carbon atoms (with hydrogen) arranged in the form of chains. Terpenes are made from several five-carbon base units $(C_5H_8)_n$, based on isoprene[*n*] which is synthesised by the mevalonic acid pathway (Fig 1.5) to be either an aromatic or alicyclic occurring in essential oils as a principal hydrocarbons. To build up isopentenyl diphosphate (IPP) via the mevalonic acid pathway in the cytosol, initially two units of Acetyl coenzyme A (Ac-CoA) are condensed into aectoacetyl-CoA through a Claisentype reaction catalysed by acetoacetyl (AcAc)-CoA thiolase (AACT) (Hemmerlin *et al.*, 2012). The hydrocarbons differ in nomenclature according to the number of isoprene units comprising the molecules. Thus, terpenes are classified in terms of multiples of five carbons (Fig 1.6): monoterpenes are combinations of two isoprene units (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) and tetraterpenes (C_{40}) that exist at low concentrations (Chen *et al.*, 2011). Monoterpenes $C_{10}H_{16}$ (M_w 136 amu) and sesquiterpenes $C_{15}H_{24}$ (M_w 204 amu) constitute the major content of the essential oils both in terms of concentration and number of components, with the remainder being diterpenes, triterpenes and tetraterpenes although these larger molecules exist in essential oils at very low concentration (Bakkali *et al.*, 2008; Tongnuanchan and Benjakul, 2014). For example, rosemary essential oil contains 90-95% monoterpene with sesquiterpenes at 2-5% (Angioni *et al.*, 2004).

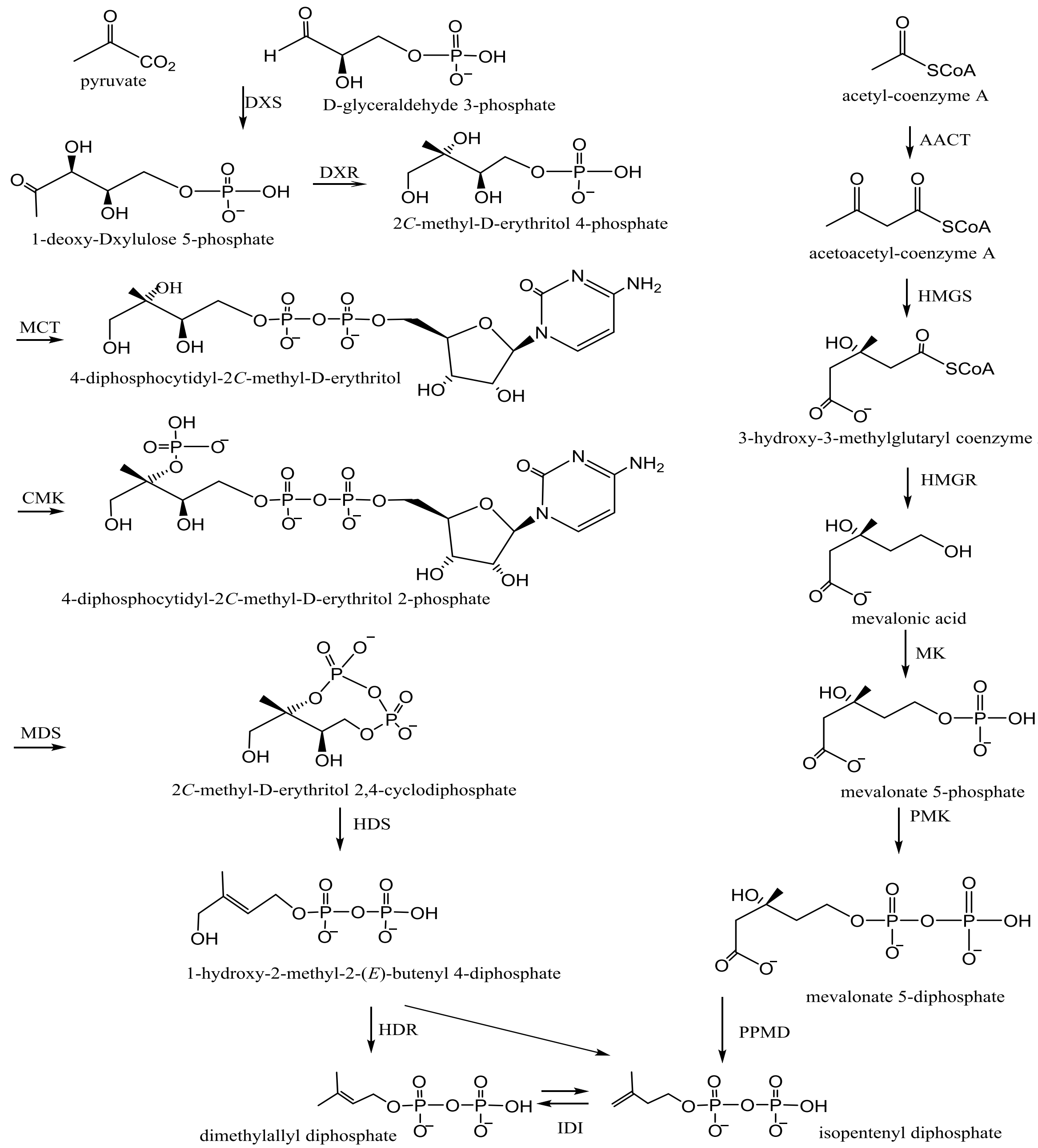


Figure 1. 5 Isopentenyl diphosphate synthesis via the 2*C*-methyl-D-erythritol 4-phosphate (MEP) or via the mevalonic acid (MVA) pathway. Enzymes of the MEP pathway are as follows: DXS, 1-deoxy-D-xyluloose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2*C*-methyl-D-erythritol 4-phosphate cytidyl transferase; CMK, 4-diphosphocytidyl-2*C*-methyl-D-erythritol kinase; MDS, 2*C*-methyl-D-erythritol 3,4-cyclodiphosphate synthase; GDS, 1-hydroxy-2-methyl-2(*E*)-butenyl 4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; Enzymes of the MVA pathway are as follows: AACT, acetoacetyl-coenzyme A thiolase; HMGS, 3-hydroxy-3-methylglutaryl coenzyme A synthase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PPMD, diphosphao-mevalonate decarboxylase (Hemmerlin *et al.*, 2012)

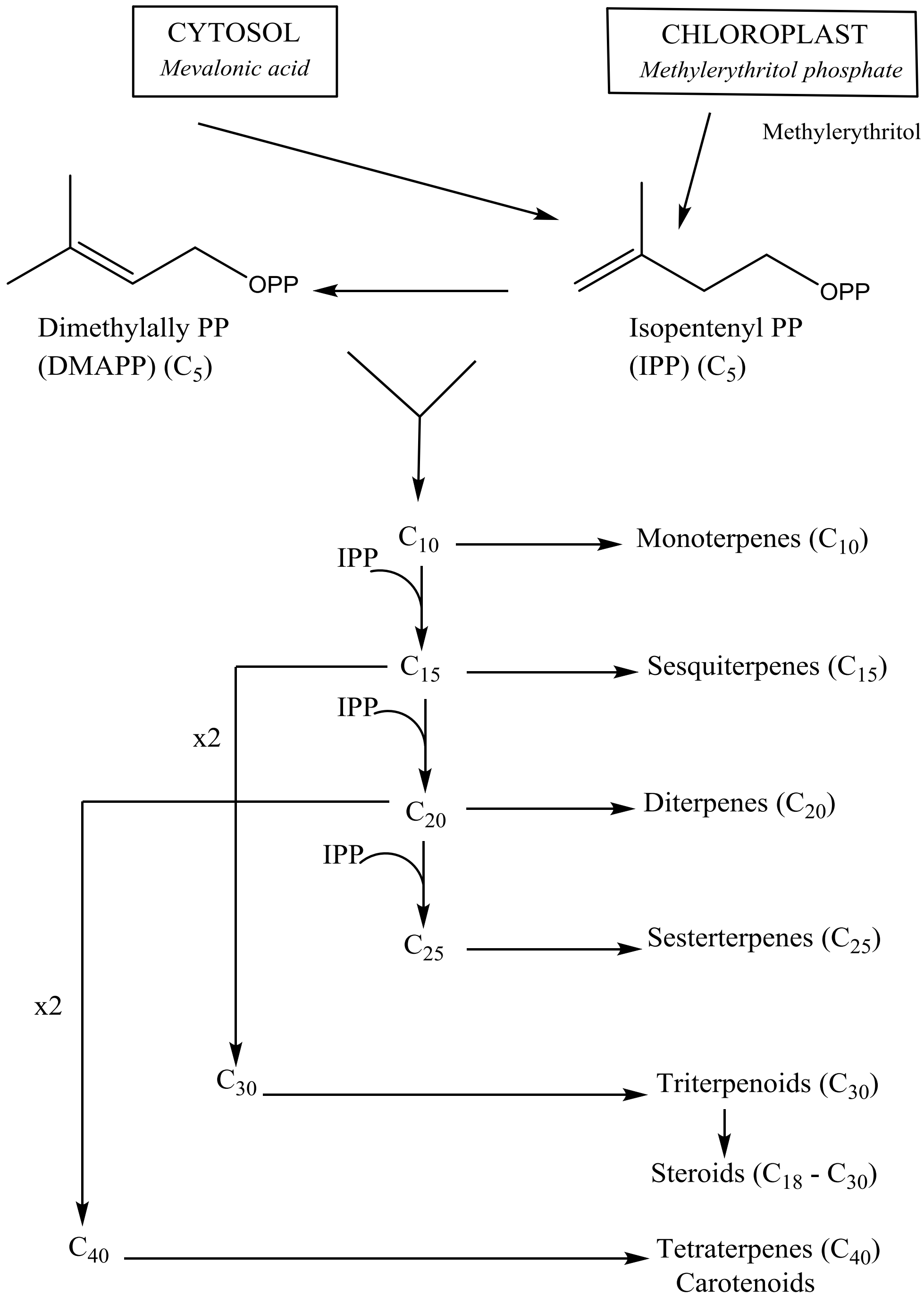


Figure 1.6 Simplified scheme of mevalonate and methylerythritol phosphate pathways for terpenoids biosynthesis (Dewick, 2009).

Oxygenated compounds

The oxygenated "terpenoids" are molecules that contain a combination of C, H and O. Terpenoids exist as a variety of compounds in essential oils. Table 1.3 lists some of oxygenated compounds which can be derived from terpenes and are widespread in plant essential oils:

Table 1. 3 Some of oxygenated compounds which can be derived from the terpenes

Phenols	Chavicol, thymol, eugenol, carvacrol
Alcohols: Monoterpene alcohol Sesquiterpene alcohol	 Borneol, isopulegol, lavandulol, α-terpineol Elemol, nerolidol, santalol, α-santalol
Aldehydes	Citral, myrtenal, cuminaldehyde, citronellal, cinnamaldehyde, benzaldehyde
Ketones	Carvone, menthone, pulegone, fenchone, camphor, thujone, verbenone
Esters	Bornyl acetate, linalyl acetate, citronellyl acetate, geranylacetate
Oxides	1,8-Cineole, bisabolone oxide, linalool oxide, sclareoloxide
Lactones	Bergaptene, nepetalactone, psoralen, aesculatine, citroptene
Ethers	1,8-Cineole, anethole, elemicin, myristicin

(Tongnuanchan and Benjakul, 2014)

The difference between essential oils in terms of smell or flavour is a consequence of the variation of the aforesaid constituents (Burt, 2004; Tongnuanchan and Benjakul, 2014).

1.3.4.1 Theories on effects of factors on production of essential oils

The many studies on essential oils have identified a wide variety of components, and there are discrepancies between studies of the same species that may result from external (ecological and environmental aspects) and internal (sexual, seasonal, ontogenetic, and genetic variations) factors impacting the plants. Oils are one of the outputs of the metabolic process. As secondary metabolites in the plant, their composition and yield will depend on climate and habitat conditions, planting and harvesting methods, in addition to genetics and plant age (Mulas and Mulas, 2005; Viuda-Martos *et al.*, 2007; Jamshidi *et al.*, 2009; Taiz and Zeiger, 2010; Derwich *et al.*, 2011).

Logically, there is expected to be a relationship between the phytochemistry of the plants and their ecological conditions of growth. Environmental variation, particularly fluctuating water availability and temperature are considered to be the main factors behind variations and have a significant effect on plant growth and yield through anatomical, morphological and biochemical adjustments (Boyer, 1982; Luković *et al.*, 2009). It has been suggested that resource limitation leads plants to change their allocation patterns in order to increase the efficiency with which they use the limiting resources (Chapin, 1989). Limitations on photosynthesis may be caused by reduction in carbon balance inside a plant as a result of stomatal closure or metabolic impairment, as well as the decrease in mesophyll density which reflect on the balance between respiration and photosynthesis (Flexas and Medrano, 2002; Chaves *et al.*, 2003; Flexas *et al.*, 2006). As a consequence of the climatic conditions prevailing in the Mediterranean basin region, the natural vegetation has developed an array of adaptations producing a high diversity of growth forms. Plants located in semi-arid areas of the Mediterranean environment have adapted to drought stress and excessive heat through the development of xeromorphic characters. This strategy leads to reduced leaf size and an increase in the thickness of cell walls inside the leaves to reduce water loss down to levels similar to wet-site species.

To further avoid the damage caused by the factors described above, there is another process found inside the leaf tissue which increases the development of palisade tissue within the mesophyll at the expense of the spongy tissue (Fig 1.7). This is accompanied by a reduced density of stomata and a dense vascular system with a decrease in cell enlargement (Bussotti and Gerosa, 2002; Bacelar *et al.*, 2006; Syros *et al.*, 2006; Trubat *et al.*, 2006; Abdelmajeed *et al.*, 2013).

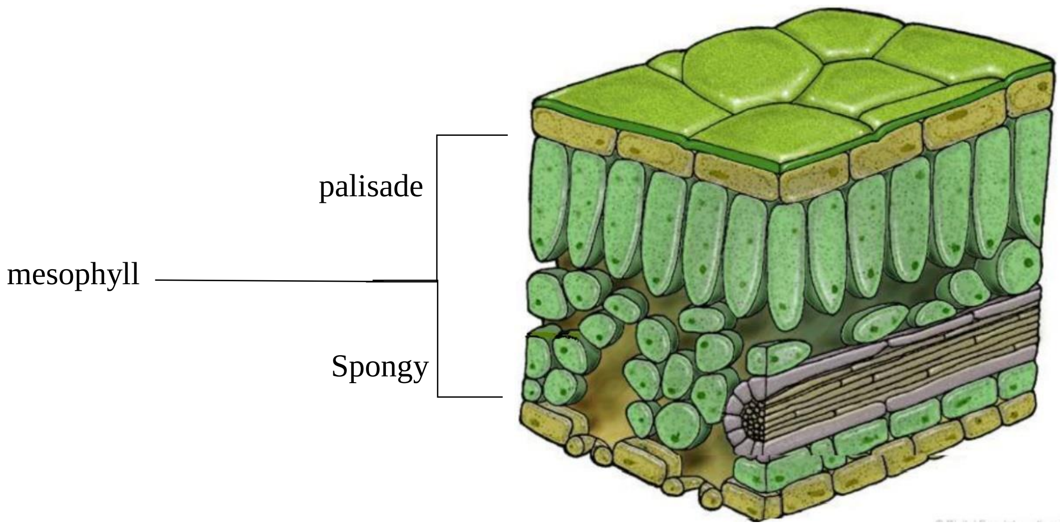


Figure 1. 7 Mesophyll and spongy tissue in leaf

(https://www.emaze.com/@AIFLRTWO/Presentation-Name)

1.4 Factors affecting plants in the wild

The variability in yield and composition of essential oils results from several factors which influence biosynthesis (Flamini *et al.*, 2002a). Environmental stresses are the most influential factors in crop production due to their huge effect on all plants functions (Abdelmajeed *et al.*, 2013). These factors affect the plant diversity in two ways; morpho-phenological form diversity and ecophysiological trait diversity (Galmés *et al.*, 2005).
The following is a review of the most important factors affecting the plant and the production of essential oils:

1.4.1 Climate (rainfall and temperature)

It has been found that the lack of rain and water scarcity is one of the strongest constraints on plants growth, flowering shoot development, and essential oil yield. It may present an evolutionary pressure leading to limitations on photosynthesis, affecting respiration, translocation, ion uptake, carbohydrate production, concentration of growth promoters and nutrient metabolism (Joffre *et al.*, 1999; Galmés *et al.*, 2007; Leithy *et al.*, 2006; Abdelmajeed *et al.*, 2013).

In aromatic plants, there is a significant change in terpene emission as a response to drought conditions in many Mediterranean species (Ormeno *et al.*, 2007; Lavoir *et al.*, 2009; Said *et al.*, 2011). For example, the species *Erica multiflora* and *Globularia alypum* from drier sites in Greece and Algeria show similar results with respect to the occurrence of terpenic components (Llusià *et al.*, 2009; Said *et al.*, 2011). The plants existing under this kind of climate (dry and hot) had a lower photosynthetic capacity and a higher leaf nitrogen (N) and phosphorous (P) contents (Wright and Cannon, 2001). Furthermore, the high leaf nitrogen concentration is linked with lower leaf toughness during photosynthesis in order to enhance water conservation, because the dry-mass economics of leaf construction (leaf lifespan) and (leaf-mass per area), is intrinsically linked with the economics of N and water use (Wright *et al.*, 2002). Confirming the above, Baghalian *et al.* (2011) found that drought stress decreased shoot weight, plant height, flower yield and apigenin contents in German chamomile (*Matricaria recutita* L.); but at the same time, oil composition was not effected significantly. However, this is not compatible with the theory that oil yield increases with sever water stress, as the plants tend to close their stomata under such conditions and this would lead to shortages in carbon in the leaves under these conditions.

1.4.2 Light

The photoperiod is the dominant factor influencing flowering, and hence growth habit throughout the period of flowering and maturity, adaptation and yield (Wallace *et al.*, 1993). Light quality affects both quality and quantity of essential oil as one of several environmental factors. Many researchers have demonstrated the influence of light on the physiology of the plant, they have described the correlation of leaf content with temperature, photoperiod or solar radiation, particularly with the relative water content of leaves. They acknowledged its importance as essential oils production dependent on physiology and development stat of the synthesizing tissue of the whole plant (Wallace *et al.*, 1993; Hidalgo *et al.*, 1998; Munné-

Bosch and Alegre, 2001). It has been concluded that there was a considerable correlation between essential oil content and the light intensity throughout the flowering time in caraway (*Carum carvi* L.). They determined the most productive photoperiod when the light is at a certain wavelength or certain intensity (certain duration) for an increased in essential oil production is from March to July (Toxopeus and Bouwmeester, 1992). Furthermore, the essential oil yield and quality of the oil composition of (*Carum carvi* L.) decreased significantly in a shaded treatment. Thus, carvone dropped from 1.16% to 0.69%; while the limonene content was not affected (Bouwmeester *et al.*, 1995). This difference in yield between hydrocarbons and oxygenated compounds in general, or between limonene and carvone specifically can be related to the different biosynthetic pathway for these two groups or components (Abdelmajeed *et al.*, 2013), as shown in (Fig. 1.8).

Pentose phosphate pathway here is a source of energy gives NADPH which is required for the generation.

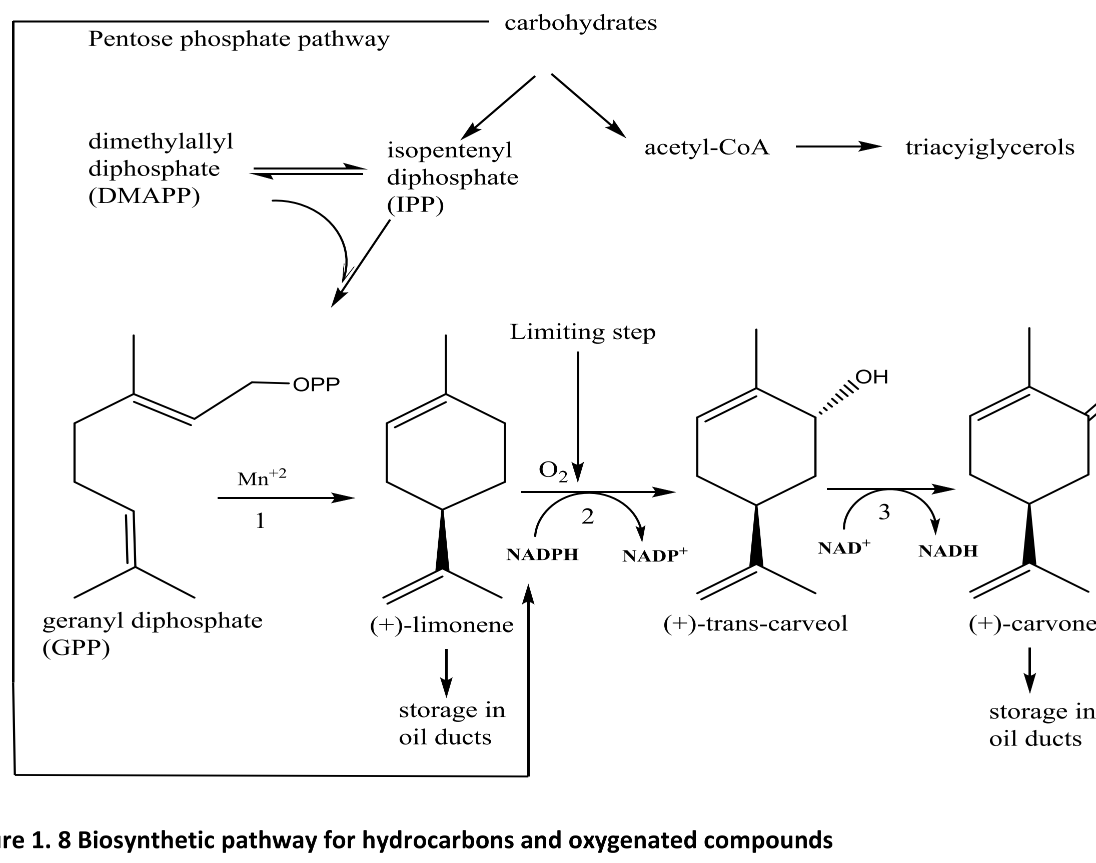


Figure 1. 8 Biosynthetic pathway for hydrocarbons and oxygenated compounds

Hence, photosynthesis which is producing essential oil as a secondary metabolite, has the key role in the formulation of the quality of the oil. This role becomes clear when photosynthesis stops; as a rule, this stopping leads to an increase in the oil content of terpenes hydrocarbons over more oxygenated compounds due to the shortage in energy supply provided by ATP and NADPH which are cofactors in the synthetic pathway for oxygenated compounds.

Accordingly, UV-B radiation caused a 50% increase in essential oil production in one of two different chemotypes plants of *Mentha spicata* (Karousou *et al.*, 1998). While, under field conditions, it found that UV-B radiation supplementation lead to considerably thicker cuticles in both *Laurus nobilis* L. and *Ceratonia siliqua* L. and slightly thicker leaf in *Laurus nobilis* L. (Grammatikopoulos *et al.*, 1998). In another case, when 22 different samples of essential oil extracted from sweet basil (*Ocimum basilicum*) were analysed to identify the effect of UV-B radiation, the results were mostly positive for oil glands development and this effect increased with older plants (Ioannidis *et al.*, 2002).

Rosemary (*Rosmarinus officinalis* L.) had a significant influence on essential oil production by increasing or decreasing the contents of specific chemical compounds as a result of end-of-day light treatments, involving red or far-red light. Thus, limonene production (8.7%) increased under red light treatment; while α-pinene (34.1%), camphene (4.9%), p-cymene (1.5%), α-terpinolene (0.8%) and geranyl acetate (0.7%) production also increased under far-red light treatment as compared with red-light treatments and control (not exposed to light treatments). On the other hand, there was an increase in plant height for each of the two treatments when compared with the control treatment. Therefore, these results confirm the supporting role of far-red light to involvement the phytochromes in the synthesis process of essential oil (Mulas *et al.*, 2006). The conclusion is that photoperiod is more effective than growing media or fertilizer application on production of essential oil. Photoperiod influences the level of the oil contents and buds or flower formation (Miguel *et al.*, 2007; Farahani *et al.*, 2009).

On other hand, it has been stated that UV-B radiation did not effect chlorophyll content, total stem length, or quality of the essential oil in aromatic plants. On the contrary, the low radiation can achieve an increase in the secondary metabolite production without any negative effect on growth or any visible damage. Accordingly, the UV-A or UV-C radiation in some cases could be more suitable for aromatic plants than UV-B (Grammatikopoulos *et al.*, 1998; Zhang and Björn, 2009; Abdelmajeed *et al.*, 2013).

1.4.3 Soil

Mediterranean plants are quite flexible in term of their ability to survive in different ecological conditions. One of these ecological conditions is the growing media or the soil which the plant is grows in. Thus, soil fertility, soil moisture, soil salinity, soil pH and so on are considered the main traits of the soil on which type and quantity of plant production relies. The importance of the soil is a result of the adoption of growth strongly on soil moisture, which is the nutrients source of the plant (Fernandez *et al.*, 1994). Many studies have linked the effect of drought stress in Mediterranean region directly with the level of soil fertility depletion, because soils in these regions are often classified as poor in nutrients, it can be divided into three types according to the presence of elements: the calcium rich soils tend to be deficient in phosphate; moderately to highly-leached and calcium dominated, soils suffer low nitrogen and phosphate (Specht and Moll, 1983; Aziz and Hendawy, 2008; Baghalian *et al.*, 2011).

Another possibility would be that water content is more important than mineral availability in determining Mediterranean vegetation patterns (Sombrero, 1992). It was noted that a thermophilous xerophyte *Pistacia atlantica* grows well on silty or clay soils, as well as in dry rocky or stony hill sides, snd thrives on calcareous rocks inside cracks, close to base of stone walls, roadsides and edges of field (Tzakou *et al.*, 2007). Hence, differences in the substrate reflected on the physiological activity and morphological appearance of the plants. This is in agreement with a study by Belhadj *et al.* (2007) whose results indicated significant difference in morphological data between populations through leaf epidermis analysis. On other hand, an excess of soluble salts in soil leads to a reduction in flower yield and oil content as well as reduced plant fresh and dry masses for both shoots and roots of *Hyoscyamus niger* and *Ammolei majus* (Ashraf and Orooj, 2006; Razmjoo *et al.*, 2008). This is due to osmotic stress which produces an ion imbalance and specific ion toxicity in the soil with consequential by lower essential oil yield (Rout and Shaw, 2001). Many researchers confirmed the effect of soil salinity on the composition of essential oils in a range of different plants (Rout and Shaw, 2001; Ozturk *et al.*, 2004; Shalan *et al.*, 2006; Razmjoo *et al.*, 2008; Taarit *et al.*, 2010). According to Said-Al Ahl and Hussein (2010), soil salinity at 1500 and 4500 mg/kg affected the components of basil *Ocimum basilicum* var. purpurascens through on increase in linalool content and a decrease in the quantity of eugenol. Soil pH is one of the factors that affects the quality of the soil and determines its suitability. Several mineral nutrients such as N, P, K, Fe, Mg, Ca, Zn are available in the pH range of 5.5 to 6.5.

1.5 Rosemary in the wild

Rosmarinus officinalis L. belongs to family Lamiaceae, and is an aromatic perennial long-living shrub with scaly bark; evergreen shiny leaves which are 5-40 mm long, green on top and whitish beneath, because of the very fine hairs. It produces small pale blue flowers that are plentiful everywhere in the plant in short axillary racemes. Rosemary has a long flowering season and blooms from winter through to spring and can grow up to about 2M tall (Porte *et al.*, 2000). This plant prefers low humidity, mild winters, moderate summers and well-drained soil for normal growth, it is endemic and grows heavily in the Mediterranean region's dry climate, particularly in areas which are mountainous, rocky, and especially along the coast (Domokos *et al.*, 1997). Portugal, Spain, France, Italy, Dalmatia, Greece, Turkey, Egypt and North Africa are the main producers of rosemary (Svoboda and Deans, 1992).

Rosemary's essential oil produced from plants that live naturally in the wild showed a high variation in chemical composition as a result to the effect of many environmental factors.

For example, many studies confirmed the wide variation in yield and composition of essential oils in wild rosemary plants grown in different regions (Tomei *et al.*, 1995; Viuda-Martos *et al.*, 2007; Jamshidi *et al.*, 2009; Derwich *et al.*, 2011). These variations are related to many different reasons, such as region (Verma *et al.*, 2011), time of harvest (Celiktas *et al.*, 2007), environmental and agronomic conditions (Moghtader and Afzali, 2009), stage of development (Ruberto and Baratta, 2000), method of extraction (Lopez *et al.*, 2005; Santoyo *et al.*, 2005; Okoh *et al.*, 2010) and genetic traits/diversity (Zaouali *et al.*, 2012).

1.5.1 Species

The genus *Rosmarinus* L. or rosemary, widely studied for the quality of its essential oils, is a well-known Mediterranean plant which includes five species. Its species exist extensively in this region and some of them have been cultivated since ancient times as an herb and garden plant (Upson, 2006) and spread to other places around the world. Practically, according to several researchers, the five species (Table 1.4) which belong to the genus *Rosmarinus* are: *R. officinalis, R. eriocalyx, R. tomentosus, R. laxiflorus* and *R. lavandulaceus*; all produce terpenes and exhibit high morphological variability. Compared to other species, *R. officinalis*, a diploid ($2n = 2x = 24$) allogamous species, is the most prevalent species in the Mediterranean and distributed wildly over many countries in the south of Europe and north of Africa down to Turkey in the east (Varela *et al.*, 2007; Mateu-Andrés *et al.*, 2013) (Fig 1.9). It has been considered an important aromatic plant due to the properties of its essential oil and biological properties (Pottier Alapetite, 1981; Porte *et al.*, 2000; Rozman and Jersek, 2009).

Table 1. 4 Rosemary species in the Mediterranean region

Species	Spain	Morocco	France	Tunisia	Libya	Greece	Turkey	Portugal	Italy	Algeria
R. officinalis	√	√	√	√	√	√	√	√	√	√
R. eriocalyx	√	√								
R. tomentosus	√									
Hybrid (*R. eriocalyx* X *R. officinalis*	√	√								
Rosmarinus X *mendizabalii* (*R. officinalis* X$_x$ *R. tomentosus*) Hybrid	√									

(Mateu-Andrés *et al.*, 2013; Morales *et al.*, 2010; Fennane *et al.*, 2007).

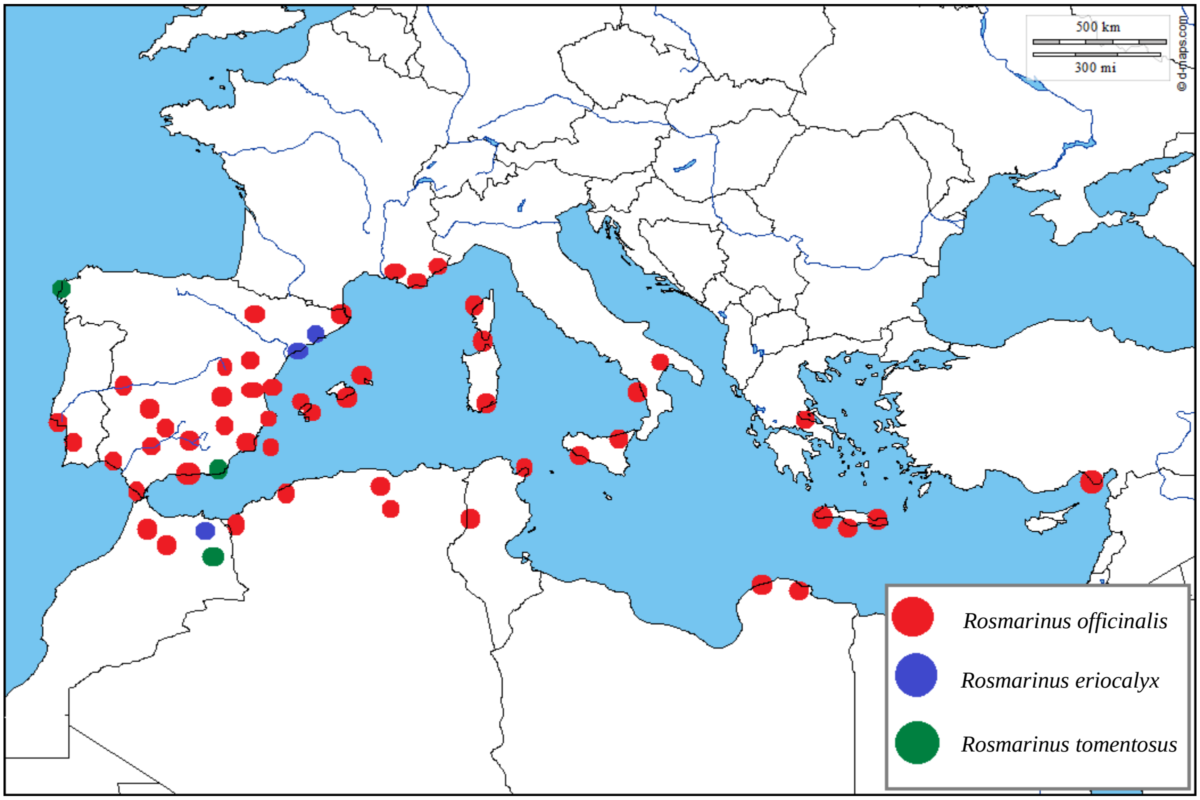


Figure 1. 9 Geographical distribution in the Mediterranean basin of rosemary species (Mateu-Andrés *et al.*, 2013; Morales *et al.*, 2010; Fennane *et al.*, 2007).

1.5.2 Geographical location (Comparison of the essential oils from rosemary plants of different origins)

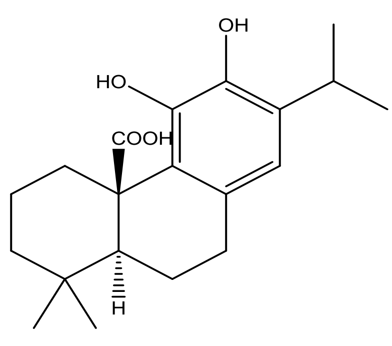
The variation in essential oil obtained from different regions has been subject to many studies. The essential oil of rosemary can be more complex and richer in flavour than other oils obtained from plants grown in different regions (Guillén and Cabo, 1996). The studies investigated the chemical composition of essential oils from several different places around the world, in order to establish the chromatographic fingerprint for each region and reported as a function of geographical distribution of species. In general, it has been pointed out previously that there are generally 2-3 kinds of rosemary oils, namely: (1) with high α-pinene and verbenone content from Corsica and Algeria; (2) with high cineole and camphor from Yugoslavia, Algeria France, Italy, Tunisia and Greece; and (3) with low cineole from Spain and some regions of Italy (Boelens, 1985). Accordingly, studies on essential oils of *Rosmarinus* *officinalis* L. (Table 1.5) confirmed these conclusions partly by investigation of oils from fresh leaves gathered from Egypt, Russia, Brazil, Turkey, Spain, Morocco Yugoslavia, Algeria France, Italy, Tunisia and Greece and stated that the oil yields and its composition were different depending on the region and genotype. Correspondingly, Itmad and Nisreen (2014) classified the essential oils depending on region to four chemotypes: **1-** α-pinene from Spain, France, Romania, Italy and Iran. **2-** 1,8 cineole from Morocco, Algeria and Austria. **3-** Camphor from India and Cuba. **4-** Myrcene from Portugal and Argentina. In addition, they added bornyl acetate as a new fifth chemotype found in Sudan, which had not been found as the highest concentration component in any region before.

Table 1. 5 The major constituents of rosemary essential oil from different regions

Country		Myrcene	Eucalyptol	α-Pinene	Camphor	Camphene	Borneol	*p*-Cymene	Linalool	Reference
Argentina		17.9	14.5	10.9	9	5.1				(Mizrahi *et al.*, 1991)
Italy			20.64	25.16	10.26	5.52	13.7			(Reverchon and Senatore, 1992)
Morocco	Rabat			37-40						(Elamrani *et al.*, 2000)
	Taforah				41.7-53					
	Elateuf		58.7-63.7							
Spain				24.7						(Chalchat *et al.*, 1993)
Morocco			47.44							
France				35.8						
Cuba			11	8.17	34.8	5.18	11.6			(Pino *et al.*, 1998)
Algeria			52.4	5.2	12.6					(Boutekedjiret *et al.*, 2003)
India		4.86	23.4	9.94	26.4					(Rahman *et al.*, 2007)
Spain			12.02	36.42	15.65					(Viuda-Martos *et al.*, 2007)
Portugal		30	12.8	16.5						(Miguel *et al.*, 2007)
Turkey			2.64	2.83				44.02	20.5	(Özcan and Chalchat, 2008)
Iran		3.9	11.1	46.1	5.3	9.6	3.4			(Jamshidi *et al.*, 2009)
Romania			7.06	62.18		11.08				(Socaci *et al.*, 2010)
Austria			41.6	9.9	17		4.85			(Tschiggerl and Bucar, 2010)
Iran			10.63	15.52	11.66					(Moghtader *et al.*, 2011)
India			31.6	15.6	35.8					(Verma *et al.*, 2011)

1.5.3 Genetic variation

Given the climatic conditions prevailing in the Mediterranean basin region, the natural vegetation has developed an array of adaptations and thus produced a high diversity of growth forms. The genetic cause of the divergence in essential oils composition of rosemary across the species has been demonstrated by numerous studies. The rosemary cultivars can be classified into groups according to the chemotype of their oil, each group was characterized by a high amount of one of the oil contents such as α-pinene, 1,8-cineole, camphor, camphene etc… These characteristics varied due to the genetic variation between varieties (Tucker and Maciarello, 1986). In addition, rosemary individuals and species have a huge variation in carnosic acid (Fig 1.10), morphological properties such as flower colour, size and shape of both plant and leaves, leaf colour depth and brightness, flower colour and growth circumstances (Hidalgo *et al.*, 1998; Mateu-Andrés *et al.*, 2013). However, it has been suggested that allozymes (which are variant forms of an enzyme that are coded by different alleles at the same locus) variability, correlated with structure and composition of essential oils dramatically (Zaouali and Boussaid, 2008). In the same way, the variation in the genetics of aromatic plants including rosemary showed the possibility of obtaining a similar yield and composition of essential oils from different varieties grown in the same region (Viuda-Martos *et al.*, 2007). They indicated that the concept of environmental and spatial factors is stronger than the genetic variation factor in its effect on oil composition by contribute to the content and quality of essential oil.



Carnosic acid

Figure 1. 10 Chemical structure of Carnosic acid

1.6 Rosemary in cultivation

Rosemary (*Rosmarinus officinalis* L.) grows freely in large areas of southern Europe and northern Africa and is cultivated worldwide, in fact it has been cultivated for a long time (Stefanovits-Bányai *et al.*, 2003). The plant is presence is no longer confined to one area, but it is now cultivated over almost the entire planet. Thus, there are many studies that have been conducted on cultivated rosemary around the world as has already been described in this chapter.

1.6.1 Time in cultivation

Because the plant is considered one of the plants belong to Mediterranean Basin region, so it prefers warm and sunny climate. For open-field cultivation, stem cuttings are usually taken in the spring to propagate new plants. The cuttings can be prepared in the greenhouse at any time and be transplanted to the field in spring to mid-summer.

The annual value of harvest depends on the internal and external factors influencing the plant, such as geographical area and whether the harvest is for plant material or essential oil. Rosemary is usually harvested once or twice a year. In general, 12 to 13 tons per hectare of fresh leaves (2.5 tons per ha per year of dried leaves) is the average of yield production in rosemary. Oil yield production ranges between 80 to 100 kg per ha (DAFF, 2012)

1.6.2 Uses of the yield

Different parts of the plants fresh or dried are using to obtain essential oils by several methods of extraction; the parts include flowers, leaves, seeds, roots, stems, bark and wood through secretionary (secretory) parts. It takes about 100 grams of plant material to produce approximately one gram of oil, depending on the type of plant and the growth conditions (Derwich *et al.*, 2011).

Essential oils of rosemary are used in various industry sectors and are commercialised as a source of anti-bacterial, anti-oxidant, anti-fungal and anti-inflammatory properties and for their toxicity. In insecticides, rosemary recently has been used in pest control products (Koul *et al.*, 2008; Derwich *et al.*, 2011; Jiang *et al.*, 2011).

Moreno *et al.* (2006) and Munné-Bosch and Alegre (2001) confirmed that the rosemary plant is a good supplier of phenolic compounds whose anti-bacterial properties are highly effective against the effects of both Gram-positive and Gram-negative bacteria. The inhibitory effect of rosemary extract increased dramatically by reducing the growth of microbial when the concentration of the extract was raised in the culture media, compared with lesser concentrations of the same extract (Özcan and Chalchat, 2008). Consequently, synthetic food preservatives such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (Khanuja *et al.*) have been replaced by some aromatic plants extracts as a food additive and carnosic acid identified as a major component exhibiting the highest anti-oxidant activity in the phenolic diterpenoid fraction from rosemary (Hidalgo *et al.*, 1998).

Historically, the use of rosemary for the treatment of diseases has been very popular since the time of the ancient Greeks and Romans. It is used in southern Europe extensively due to its pungent smell, as an additive in a traditional cuisine, due to its astringent taste, which complements a wide variety of foods as a flavour or to make a tisane. Rosemary has been used as a medicine to treat many disease states over the long term. It has been used to treat renal colic, as a pain reliever to relive symptoms caused by respiratory disorders, dysmenorrhea, a stimulator for hair growth, anxiety-related conditions and to raise alertness (Oluwatuyi *et al.*, 2004; Derwich *et al.*, 2011; Jiang *et al.*, 2011).

1.6.3 Current methods of growing the plant

Currently, attention is being given to the possibility of cultivation of aromatic and medicinal plants to diversify agricultural production. This interesting approach includes using new production methods which have the ability to increase yield and reduce costs with reduced use of fertilizers and irrigation water (Leithy *et al.*, 2006).

Seeds, cuttings, layering or division of roots are used for propagation in rosemary like most other plants. Propagation from seed is used on a very small scale due to very slow germination (25 days at 18°C) and because of the problem of cross-pollination, growing true-to-type plants from seed. However, seedbeds with a width of 1.2 m and row space of 40 to 50 cm are found to be effective with mechanised cutting.

Cuttings from actively-growing stem tips are a good way to propagate new plants efficiently. Approximately 10-15 cm shoot-tip cuttings are taken from a mature plant. The lower third of each cutting should be stripped from leaves. The cuttings are inserted in a proper growing medium, for half to two thirds of the it length. Full sun and well-drained, sandy soils high in organic matter, but not over-rich, are considered the ideal conditions for growth. Rooting hormones, mist bed with a heated floor and removing about 1-2 cm of the shoot tips can all be used to improve rooting and give the best results. Field spacing is also very important; it must usually be done according to the farming method applied (Westervelt, 2003; DAFF, 2012).

1.7 Factors affecting the cultivation of plants

1.7.1 Irrigation

Water is very important for the plants because of its presence in all of the physiological processes such as absorbing nutrients from the soil, photosynthesis, transpiration etc…. Water availability affects the quantity and quality of production in the plants and lack of water was identified to be the most harmful factor in terms of effect on yield. It has been found that the plants grown in a soil-based growing medium required less frequent irrigation than plants grown in soil-less mix because of the higher water-holding capacity in soil-based environments (Boyle *et al.*, 1991). Also, the prolongation of water irrigation intervals for rosemary causes a clear increase in plant height, number of branches, fresh and dry weights and an increase in essential oil content and percentages of some oil components, such as linalool, eucalyptol and camphor, especially in sandy soil as compared with clay soil, and under normal or irregular irrigation (Leithy *et al.*, 2006). The limitation of water affects negatively on rosemary growth and yield as a result to reduction in photosynthesis. This reduction in photosynthesis is due to lessened stomatal and mesophyll conductance which lead to low availability of CO_2 (Delfine *et al.*, 2005). Also, the effect of water shortage and lack of CO_2 in the plant leads to excessive excitability energy by chloroplasts. Alternatively, photosynthesis in leaves of rosemary plants remains unaffected by severe drought, which may indicate the plant retains small amounts of foliar water to below 35% for a long time (up to three months) with a decrease in the level of chlorophyll up to 85%; and that the plant returns to normal after autumn rainfalls (Munné-Bosch *et al.*, 2000). It has been suggested that it is possible to reduce the water use of some aromatic plants by using appropriate techniques such as the method and timing of irrigation (Rao *et al.*, 1998). Furthermore, the effect of different irrigation times and levels reflected on the physiological and morphological features of *Cuminum cyminum* and *Mentha piperita* L., the highest oil percentage was achieved under water stress conditions (Khorasaninejad *et al.*, 2011; Ahmadian *et al.*, 2011). In addition, water stress in the plant root zone of purple basil (*Ocimum basilicum* L.) impacted negatively on yields and plant height as vegetative growth indicators. In contrast, there was a positive effect on the essential oil rate of the plants (Ekren *et al.*, 2013).

1.7.2 Growing media

The growing media has a strong and effective influence on the state of the plants and the amount of oil production. The specifications of soil play an important role in the growth and yields of rosemary, as well as the amount of the components in the oils. It is the main supplier in determining the availability of water and nutrients for plants (Hidalgo *et al.*, 1998; Moretti *et al.*, 1998). Rosemary requires well-drained sandy to clay loam soil with a pH range of 5.5 to 8.0, although it can withstand a certain proportion of clay (could reach up to 30%).
This effect is due to the physical and chemical properties of soil such as bulk density, the soil's ability to hold water, pH, soluble salt content and cation exchange capacity (Boyle *et al.*, 1991). In contrast to the above, different soils results do not affect the essential oil production of rosemary (Leithy *et al.*, 2006). However, Miguel *et al.* (2007) found a high amount of 1,8-cineole (11.8 %) in the essential oil of rosemary grown in sandy soil compared with other plants grown in fertilized and non-fertilized peat growing materials, and confirmed the important effect of growth media on essential oil production of the plants.

1.7.3 Fertilizer

All plants need a balance of mineral resources in order to maintain optimal growth. The application of fertilizer whether containing macro- or micro-elements or both is one of the agricultural operational tasks that has a role in the determining nature of growth and production as well as in the chemical composition quality and quantity of essential oils.

The integrated supply of nutrients to plants through a variety of organic and inorganic sources has become one of the most important aspects of environmentally-sound agriculture (Singh and Guleria, 2013). Organic fertilizer obtained from seaweed is one of the important sources required to produce “clean” products which are free of harmful agrochemicals, in order to improve both human health and the environment (Lampkin, 1990). This is because in the past the use of chemical fertilizers has caused a lot of problems to the soil, groundwater, and the environment in general and also increases production costs. It reduces soil fertility, and causes environmental pollution, for example making groundwater become contaminated with a several chemicals, as well as ensuing the potential risk of those substances to human health.

Organic fertilizers derived from seaweed are considered one of the most popular types of fertilizers because of the availability of materials necessary for plant nutrients and hormones. Consequently, they are added to plants to obtain an effect especially in terms of vegetative growth because they contain substantial amount of nitrogen.

In general, the effect of fertilizer on essential oil is greater than that of the growing media , as fertilizer application can make a significant effects on growth, oil yield and their refractive index in rosemary plants (Boyle *et al.*, 1991). There is a strong relationship between micro-nutrients and plant extracts in medicinal plants and especially those containing phosphorus, including rosemary (Konieczynski and Wesolowski, 2007; Attememe and Al-Zahwan, 2011).

1.8 Motivation of this study

Nowadays, many researchers are keen to maximize and improve crop yields while simultaneously minimizing the inputs in order to reduce both the cost of production and the negative outcomes that result from the use of certain fertilizers or pesticides that may negatively affect the environment. These goals may partly be achieved by identification of the key factors controlling yield and growth of crops (Oliver *et al.*, 2013).

The essential oil of rosemary has a variety of use in many fields of daily life. There has been considerable interest in factors affecting rosemary oil production; fertilizer, irrigation, date of harvest, and growth medium are examples of these many factors (Boyle *et al.*, 1991; Moretti *et al.*, 1998; Munné-Bosch and Alegre, 2001; Leithy *et al.*, 2006). However, in order to identify and assess the results of application of these approaches on the plant, the variables in plant growth and production in addition to the factors that underlie this need to be determined systematically. Generally, the effect of each factor on rosemary separately, or in isolation from the other factors affecting the plant have not been identified (Lamb and Brown, 2001; Mondal and Al Mamun, 2011).

In order to identify and assess the results of varying one of the many factors which might affect essential oil production, it is necessary to determine the interacting variables in plant growth and production in addition to environmental factors.

On the basis of the results of many studies which have investigated the effect of factors such as geographical location and growing media, the response of rosemary in oil yield and composition to these factors has now been measured, but the effect of organic fertilizers derived from seaweed on rosemary are still uncertain. Nevertheless, much data relating to the crop’s response to some factors such as the availability of water, intensity of illumination, genetic variation or the location of the plant have not considered the interactions between these factors, and therefore, the correct interpretation may have been unclear without separation of these factors. Studying one factor without any variation in other factors can have great value for determining how to optimally exploit a plant. Thus, identifying the main factors causing significant variation and their correlation with the final yield and oil content is the major topic for study in this thesis.

An objective analysis of organic fertilizers by comparison with artificially-synthesised inorganic equivalents has not previously been undertaken and is demonstrated here. Abiotic factors such as irrigation, light and temperature will be considered to be limiting factors, so all these factors are standardized to allow differentiation between the response of species and variation to the fluctuation of the climatic condition in order to select the best production material.

1.9 Study objectives and hypotheses

This project investigates some of the factors that control oil quality (chemical composition) and oil quantity (total yield for gram fresh weight of plant). Using Rosemary (*Rosmarinus officinalis* L.) one of the many plants bearing essential oils, and a member of the mint family, which includes many other herbs, as a model system I have explored harvest time, mode and type of fertilizer and the effect of cultivar choice on oil yield and quality. The general aims of this project were to optimize the best production conditions, taking into account the reduction of the use of artificial fertilizers in order to provide information, which might be useful for production management of the rosemary crop. A subsidiary objective was to examine the application of seaweed fertilizer in rosemary based on the effect of fertilization factor on growth and production.

1.9.1 Objectives:

1- To know the extent of the differences in the response of rosemary plants to organic fertilizers and matching inorganic copies to better understand the impact of fertilizer choice on oil quality and quantity.
2- To examine the possibility of cultivar choice as a primary consideration when growing commercial herbs for oil production.
3- To assess the impact of differing harvest regimes on oil yield of rosemary plants.
4- To better understand the causes of variation in oil quality.

1.9.2 General hypothesis:

1- Mineral copies of organic fertilizers will cause the same effects on plant growth as their organic equivalent.

2- Plant growth substances (hormones) found in seaweed fertilizer have no impact on crop plant growth.

3- The mode of application of fertilizer does not influence the response of the plant.

4- Genotype has no impact on yield or quality of essential oil.

5- The age of a plant does not impact on the quality or quantity of oil yield.

6- The percentage harvest of rosemary does not impact on subsequent harvests.

2. Chapter two

2.1. Introduction

Due to the commercial importance of essential oils, many studies have been conducted to identify the wide variety of essential oil components found in plants, and to discover how yield and composition of oils may vary as a result of external factors such as: climate and habitat conditions, planting or cultivation method, using fertilizers, date of harvest, and internal conditions in the plant, such as genetics and plant age (Viuda-Martos *et al.*, 2007; Jamshidi *et al.*, 2009; Derwich *et al.*, 2011; Singh and Guleria, 2013; Nurzyńska-Wierdak, 2013). The studies have shown the essential oil components and yield can be substantially modified by these factors. The origin of the variation, of course, is less clear cut. However, it is possible that the conditions directly influence secondary metabolite biosynthesis, or it may be that changes in other biological processes have an indirect effect. This latter consideration is distinctly possible, as oil composition may be influenced by a range of factors including climate, pollution, and exposure to pests or diseases (Figueiredo *et al.*, 2008).

Agricultural and horticultural production of crops have developed over many centuries. However modern scientific method and the widespread availability of chemical fertilizers in the 1950s and 60s led to practices that turned out to be detrimental to the natural environment (Carson, 2002). Recent attention on the negative effects of chemical fertilizers and their negative long term impact on agriculture and on human beings (such as polluting groundwater, making plants more susceptible to the attack of diseases, destroying microorganisms and insects, and decreasing soil fertility) has resulted in a marked pressure for low input and organic approaches (Abdelaziz *et al.*, 2007). Choice of fertilizer, method of application and optimal harvest time have all been subjects of controlled experiments (Shanahan *et al.*, 2011). However, some lower input techniques have been shown to reduce the quantity or quality of the harvest, including negative impacts on essential oils (Singh and Guleria, 2013; Mechergui *et al.*, 2016).

Nevertheless, the integrated supply of nutrients to plants through a variety of organic and inorganic sources became one of the most important aspects of environmentally-sound agriculture (Singh and Guleria, 2013). Seaweed liquid fertilizer (SLF), based on marine macro-algae extracts, is one way to supply these nutrients. Recently, SLF has been widely used on a large range of crops (Selvam and Sivakumar, 2014). Seaweed generates low pollutant levels, is biodegradable, non-hazardous and non-toxic, is equipped with good plant nutrients such as macro- and micro-elements, and a high level of organic matter, fatty acids, vitamins, and natural growth regulators (Dhargalkar and Pereira, 2005; Gurusaravanan *et al.*, 2010; Neish and Bourgougnon, 2014). So, most of the responses obtained with seaweed extract were similar to those observed when hormones were applied to plants. Furthermore, bioactivity of organic compounds derived from seaweeds such as *Ascophyllum nodosum*, can be used to improve the rate of crop production in agricultural systems because it contains a high percentage of growth regulators which play an active role in promoting the vigour and vitality of the plant (Rayorath *et al.*, 2008). The first investigators who refer to the use of seaweed extracts in human and animal nutrition were the Chinese and the Japanese; in Britain, the origination of a commercial seaweed extract industry for agricultural uses as a fertilizer began around the 1950s, but on a limited scale. Now this approach is being used widely in various areas to feed and encourage growth and yield of plants, and the extract is prepared as a powder or liquid (Arioli *et al.*, 2015).

In general, fertilizer provides nutrients, which induce plants to grow faster through provision of essential physiological raw materials such as nitrogen (N), phosphate (P_2O_5) and potassium (potash, K_2O), regardless of whether it is delivered by organic or chemical fertilizers (Marschner, 2011).

In aromatic plants the effect of fertilizer is often stronger than the effect of other factors such as soil type (Boyle *et al.*, 1991). Consequently, plant growth is dependent on the ability to capture, store, and use carbon and nitrogen sources; and biochemical processes such as terpene biosynthesis and accumulation of essential oils are linked to growth (Gonzalez *et al.*, 2010). It has been reported that fertilizer application (organic or inorganic) correlates with an increase in chlorophyll content and metabolic rate, resulting in rapid growth and positive effect on number of branches, leaf area, fresh and dry weight, number of flowers, fruit yield and height of aromatic plants such as black gram and rosemary (Mahfouz and Sharaf-Eldin, 2007; Abdelaziz *et al.*, 2007; Boyle *et al.*, 1991; Selvam and Sivakumar, 2013; Vijayanand *et al.*, 2014). Moreover, the principal volatile compounds may be subject to change in the case of fertilizers added to plant crops. For example; higher nitrogen application decreases the percentage of linalool and increases methyl chavicol in the essential oil of some aromatic plant species. In contrast, a higher amount of potassium contributes to an increase in essential oil content and the percentage of linalool and 1,8-cineole in oil (Pino *et al.*, 1998; Rao *et al.*, 1998; Diaz-maroto *et al.*, 2007; Nurzyńska-Wierdak, 2013). Further, the yield and oil content of rosemary increased as a response to the use of fertilized compared with non-fertilized plants (Miguel *et al.*, 2007). Supplementary foliar addition of micro-nutrients alongside NPK in the form of organic or mineral fertilizer resulted in significant increase in both growth and the yield of essential oil of rosemary plants compared with control (no fertilizer). On the other hand, oil content was influenced as a result of using organic or inorganic fertilizer, but the use of both fertilizers together may give the plant the maximum benefit in terms of growth and production.(Attememe and Al-Zahwan, 2011; Singh and Guleria, 2013). Bio-fertilizers such as *Azotabacter vinelandii* liquid culture have made rosemary oil content and most of the growth characters improve, even within different irrigation periods and soil types, compared with a control with a standard NPK fertilizer (Leithy *et al.*, 2006). This difference in the range of oil yield between fertilizer treatment and control could be due to the different type or level of fertilizer or to the interaction with other factors like date of harvest, method of application of fertilizers and growing media. For instance, different ranges of soil nutrient concentrations do not enhance the plant to produce new terpenoids, but they could involve changes in the relative ratios among the different compounds (Ormeno and Fernandez, 2012).

While there have been several investigations on the effect of inorganic fertilizer on the yields and oil composition of rosemary, there is a dearth of information available on the effect of organic fertilizer, and especially seaweed on this crop.

This chapter reports the influence on growth, yield and oil composition of rosemary using a seaweed extract fertilizer (organic) and compares this with a model system based solely on the mineral (inorganic) equivalent content of the seaweed fertilizer. It tests the following hypotheses:

1) Seaweed fertilizer shows no benefit to growth and essential oil yield or composition compared with a mineral fertilizer equivalent.

2) The method of application of fertilizer, to the soil or direct to leaves, causes no difference in oil quantity or quality.

3) Oil quantity and quality do not vary with harvest date.

Type of fertilizer, method of application and date of harvest have all been shown to be influential factors in other crops.

2.2. Materials and methods

This section considers the general methodologies that have been used throughout the thesis to obtain the data used in most of the following chapters. However, methods particular to a specific chapter are described in more detail in that chapter.

2.2.1 Site and experimental design

This experiment has been conducted inside a greenhouse at the Research Station of the School of the Biological Sciences at the University of Reading at the geographic coordinates 51°26'12.0"N 0°56'34.1"W. The experiment was laid out in a Complete Randomized Design (CRD) as a factorial experiment. Two kinds of fertilizers, organic and inorganic as well as control (water) were applied in two ways; directly to the leaves (spray), and to the soil (watered). These treatments were divided into two different groups. One of them was harvested twice, after three and six months and the second group harvested one time after six months. Plants were harvested by cutting the fresh aerial parts of the plant and the material was wrapped in aluminium foil and frozen until needed. Each treatment had seven replicates (pots) distributed randomly across experimental units as explained below and shown in Table 2.1 and Fig 2.1:

Table 2. 1 Plants, relative agronomic treatments and harvest dates during the experimental period

Samples	Treatments						
	Water only	Organic fertilizer	Inorganic fertilizer	Spray	Pour to soil	Harvest 1 (after 6 month)	Harvest 2 (after 3 and 6 month)
Spray control	Yes			Yes		Yes	
	Yes			Yes			Yes
Watered control	Yes				Yes	Yes	
	Yes				Yes		Yes
Spray seaweed		Yes		Yes		Yes	
		Yes		Yes			Yes
Watered seaweed		Yes			Yes	Yes	
		Yes			Yes		Yes
Spray inorganic			Yes	Yes		Yes	
			Yes	Yes			Yes
Watered inorganic			Yes		Yes	Yes	
			Yes		Yes		Yes

OW2	IS1	OS2	CS1	IS2
CW1	OS1	CS1	IW1	OS1
CS2	CW2	IS2	OW2	CW1
IS1	OW2	OW1	IS1	CS2
CS1	IW2	CS2	CW2	IS1
IW2	CW1	IS1	OS2	IW2
CS2	OS2	OW2	IW1	OW1
CW1	IW1	CS2	OW1	CS1
OS1	CW2	OW1	IS1	OW1
IW1	IW1	IS2	OS2	IW2
IS2	CS1	OS1	IW2	OS2
CS1	IW2	IW1	OW2	CW2
IS1	CW1	OW2	CS2	IW1
OW2	OS1	IS2	OS2	CW2
CW2	CW1	CW2	IS2	OS1
CS2	IW1	IS1	CW1	IW2
IW1	IS2		OS2	CS1

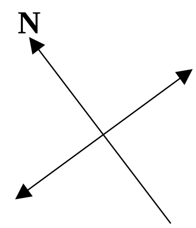


Figure 2. 1 Distribution of the treatments on the plants inside the greenhouse (experiment's map); O: seaweed fertilizer; I: inorganic fertilizer; C: control; S: spray method; W: watered method; 1: plants harvested one time after 6 months; 2: plants harvested two times after 3 and 6 months

2.2.2 Preparing the cuttings

Approximately 10-15 cm shoot-tip cuttings were made on 20th November 2012 by a sharp, clean knife from a mature plant of Rosemary *(Rosmarinus officinalis* L.). The cuttings were placed in 1 L nursery containers (10 cuttings per pot) filled with 20% compost and 80% Seramis granules; a propagation medium for rooting. The bottom two thirds of the stems were stripped of leaves; these cuttings were grown under confined environment conditions in a greenhouse with natural daylight, controlled temperatures (Table 2.2) and watered with equal amounts of water by a dripping irrigation system. Four months later, rooted cuttings were pruned by removing 1-2 cm of the shoot tips; then plants were transplanted into 0.5 L separate plastic containers (one plant per pot) filled with a 1:1 combination of horticultural grit and JI no.2 compost and grown on for three months. After four months, the plants were transferred to 1 L pots with the same ratio of growth media (1:1 horticultural grit to JI no.2 compost), then the plants were transplanted to 3 L pots with the same ratio of growth media (Fig 2.3). The process of preparing the plants took this amount of time because according to Boyle *et al.* (1991) the marketable size for potted plants can be reached in six months from propagation by taking cuttings. Plant material used in the experiments was propagated clonally to remove the impact of genetic variation from the experiment.

Figure 2. 2 Different size of pots used during the preparing of the rosemary cuttings

2.2.3 Irrigation system

The plants in this experiment were watered with equal amounts of water by a drip irrigation system (Fig 2.2) via 2 L/hr pressure-compensation button drippers connected to an automatic water controller. Plants were watered weekly by a programmed dripping system with about 340/ml of water for each pot (December 2013 – March 2014), raised to twice a week with 200 ml each time from the beginning of April, 2014 until the end of the experiment. There are many benefits of a drip system, including better disease control, lower risk of root diseases, flexibility in application timing, reduced labour costs and improved water efficiency (Reed, 1996).

Figure 2. 3 shows the distribution and irrigation system

Table 2. 2 Average temperatures inside the greenhouse (January - June 2014)

Month	January	February	March	April	May	June
minimum	13.7	13.51	14.54	16.31	18.06	17.96
maximum	18.58	23.03	25.77	26.48	27.24	32.03

2.2.4 Insect control

The plants were sprayed on 28th November 2013 with "Calypso," whose active ingredient is Thiacloprid at a concentration of 4 ml in 16 L water to kill the bugs such as Glasshouse leafhopper (*Hauptidia maroccana*); after only one day, the bugs were not observed.

2.2.5 Preparation of fertilizers

2.2.5.1 Organic fertilizer (seaweed)

'Bio magic' is a product of Leili Agrochemistry Co., Ltd. (England) and is used as an organic fertilizer that includes naturally occurring organic materials obtained from a seaweed source in a powder form extracted from three genera of wild algae: *Ascophyllum nodosum, Sargassum sp.* and *Laminaria sp*. This product was used in the experiment as a source of organic fertilizer due to the presence of both macro- and micro-elements that are important for plants (Table 2.3). It contains phosphate, one of the ingredients which are often absent or provided in very small quantities from soluble seaweed fertilizers. It was also chosen because most seaweed extracts are made from one type of alga, which contain the largest possible amount of trace elements rather than containing phosphorus with the omission of other elements. The ratio of dilution for this fertilizer was 1:3000 w/v and the solution's pH was 8.8. Correspondingly, 80 ml from this solution added to each plant every four weeks whether it is by spray or watered method.

Table 2. 3 Nutrient contents of seaweed extract (% w/w)

Nitrate Nitrogen N	Phosphorus P_2O_5	Potassium K_2O	Magnesium Mg	Calcium Ca	Iron Fe	Copper Cu	Sulphur S	Iodine I	Sodium Na
0.5~1.5%	6.00%	18~22%	0.4~0.6%	0.4~1.6%	0.15~0.3%	0.0025~0.0045%	1.5~2.5%	0.003~0.06%	2.2~3.2%

2.2.5.2 Inorganic fertilizer

There are many 'complete' fertilizer products on the market for use in liquid feeding programs. However, most of the single package dry or liquid concentrate formulations available are unable to supply all the fertilizers required to provide the same quantities as the organic fertilizer. Therefore, the inorganic fertilizer in this experiment was designed to be the closest possible match for the mineral components of the SLF used in the experiment. The nutrient composition of the SLF is given by the manufacturer. Stock solutions were made through the addition of macro (N, P, K, *etc.*) and micro (Fe, Cu, Mg *etc.*) elements in the required proportions (Table 2.4) in separate flasks with distilled water. The final solution was formed by adding the stock solutions to a known amount of water to produce the required volume in the desired concentrations, the ratio of dilution was 1:3000 w/w; in order to avoid precipitation the materials present in the highest concentration were added first. The final pH was 8.8. The mineral fertilizer was stored in a refrigerator after mixing, and there was no observable solid material. Similarly to seaweed fertilizer, the solution amount of inorganic fertilizer added for each plant every four weeks was 80 ml, equally with both methods of application

Table 2. 4 Composition (%) of nutrients solutions for in organic fertilizer

Chemical compound	Molecular weight	Contents %	Weight (gm) used	Amount of the ions supplied from these weights of chemical compounds										
				Nitrogen	Phosphorus	Potassium	S	Mg	Na	Fe	I	Cu	Ca	Other
$K_2HPO_4.3H_2O$	228.22	34.26 K, 13.57 P, 3.08 H, and 49.07 O	10.29		2.617	6.608								
K_2SO_4	174.25	44.87 K, 18.39 S and 36.726 O	10.00			4.487	1.839							
KNO_3	101.10	38.67 K, 13.85 N and 47.47 O	7.22	1.00		2.79								
K_2CO_3	138.20	56.58 K, 8.69 C and 34.73 O	4.44			2.15								0.385 (C)
KI	165.99	23.55K and 76.44 I	0.191			0.168				0.225	0.045			
$FeSO_4.7H_2O$	278.00	20.08 Fe, 11.53 S, 63.26 O and 5.07 H	1.117				0.1288							
NaOH	39.99	57.47 Na, 40.00 O and 2.52 H	4.69						2.70					
$CuSO_4.5H_2O$	249.67	25.98 Cu, 13.10 S, 63.26 O and 5.07 H	0.0134				0.0017					0.0035		
$Ca(OH)_2$	74.09	54.09 Ca, 43.16 O and 2.72 H	1.85										1.00	
$MgCl_2$	95.20	25.52 Mg and 74.47 Cl	2.00					0.51						1.489 (Cl)
Total			**41.811**	**1.00**	**2.617**	**16.203**	**1.9695**	**0.51**	**2.70**	**0.225**	**0.045**	**0.0035**	**1.00**	
Target totals% (the amounts in the seaweed fertilizer)				0.5 ~ 1.5	2.616	14.94 ~ 18.26	1.5 ~ 2.5	0.42 ~ 0.60	2.2 ~ 3.2	0.15 ~ 0.30	0.03 ~ 0.06	0.0024 ~ 0.0045	0.40 ~ 1.6	Alginic acid 10~12
Mid-range				1	2.616	16.6	2	0.51	2.7	0.225	0.045	0.0035	1	
Difference				0	0.001	-0.397	-0.0305	0	0	0	0	0	0	

2.2.6 Oil extraction (Hydrodistillation)

The oil extraction process can be done in several ways such as hydrodistillation, expression, enfleurage, solvent extraction and destructive distillation (Tongnuanchan and Benjakul, 2014). Amongst these methods, and with increasing interest in avoiding organic solvents in the extraction of compounds, hydrodistillation is considered the standard method of essential oil extraction and this method has been used widely for commercial production (Cassel and Vargas, 2006; Lira *et al.*, 2009).

In this study, aerial parts comprising leaves and twigs with young and fresh branches were obtained from each experimental treatment of cultivated plants at harvest (100 days after planting) and 50 g fresh weight (Rahman *et al.*, 2007; Jamshidi *et al.*, 2009; Szumny *et al.*, 2010) was chopped into small pieces by a blender in order to expose a large number of oil glands onside of leaves and increase the percentage of oil collected. This was then placed in a still with water and extracted in a Clevenger apparatus (Guenther, 1950) (Fig 2.5).

Fresh materials were used to avoid loss of quality by drying (Diaz-maroto *et al.*, 2007). The chopped material was completely immersed in water, which was boiled on an electric heater. After the hydrodistillation process was complete, the essential oil collected through the graduated distillate receiving tube in the low end of Dean stark trap (one of Clevenger apparatus pieces) (Fig 2.4). After cooling, the essential oil was dried using anhydrous magnesium sulphate. This is required because trace amounts of water will dissolve in the essential oil. The anhydrous magnesium sulphate absorbed the water and produced solid magnesium sulphate heptahydrate (Epsom salt).

$$MgSO_4 + 7H_2O \longrightarrow MgSO_4.7H_2O$$

All the essential oil samples were stored in dark glass vials with Teflon sealed caps at -18 °C in darkness.

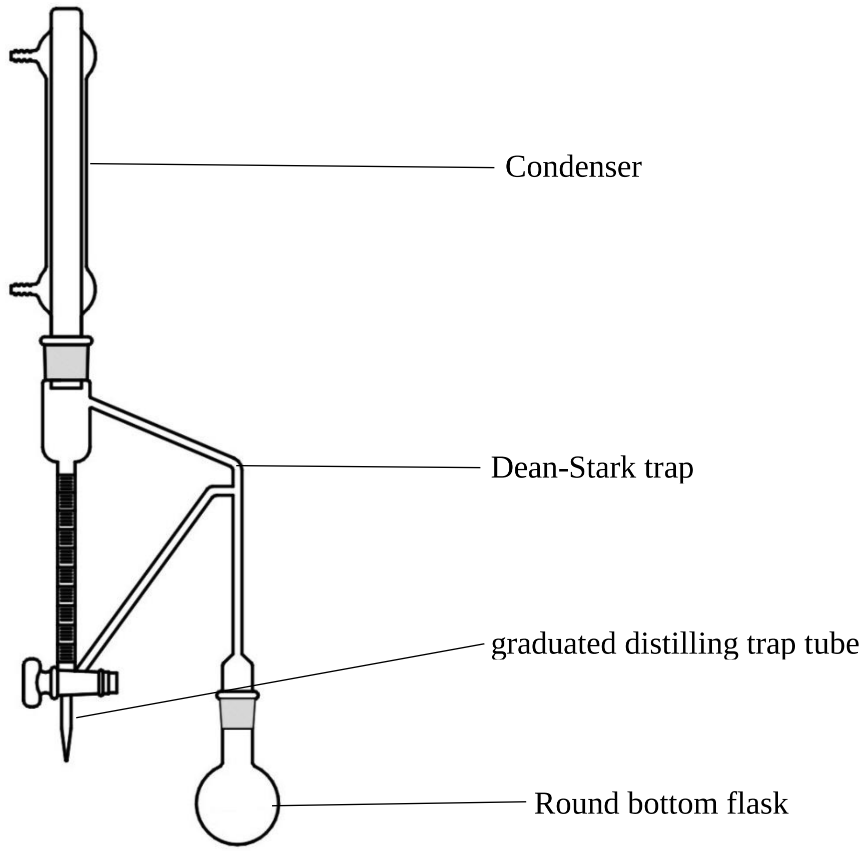


Figure 2. 4 Clevenger apparatus

(http://www.borosil.com/products/scientificindustrial/laboratory-glassware/distilling-apparatus/)

Figure 2. 5 Hydrodistillation by multiple Clevenger apparatuses

2.2.7 Measurements

2.2.7.1 Oil amount

In order to calculate the weight of the essential oil in leaves (g.100^{-1} fresh leaves), the fresh leaves were weighed before the start of extraction, and then the extracted oil weighed to identify how much oil per unit weight was present. The proportion of oil-to-plant fresh weight was calculated as the w/w ratio.

2.2.7.2 Identification of oil components

Typically, essential oils are highly complex mixtures of often hundreds of individual aroma compounds. Essential oils of rosemary are predominantly monoterpenes and their derivatives 95-98%; followed by sesquiterpenes 2-5%; most of these are oxygenated terpenes 75-80% (Rao *et al.*, 1998). Accordingly, oil analysis was conducted using two approaches. Gas chromatography combined with mass spectrometry (GC-MS), identifies fragments of oil molecules allowing the nature of even complex oil mixtures to be worked out. Nuclear magnetic resonance spectroscopy (NMR) gave further information on the structure and conformation of the oil components. In each analytical technique, the main oil components (camphor, myrcene *etc.*) were compared with commercial standards of high purity. In the case of NMR this allowed full identification of component peaks, in the case of GC-MS these were used to identity component peaks and directly compare concentrations.

2.2.7.2.1 GC-MS

The identification of the essential oils was performed using GC-MS on a Thermo Scientific system, Trace GC Ultra GC interfaced to an ITQ1100MS. The column was a Thames Restek RXI®-5HT fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The carrier gas was Helium at 1.2 ml/min. The oven temperature was kept at 50˚C for 1 min, followed by 50-85˚C at a rate of 3˚C/min, then followed by 85-140˚C at a rate 10˚C/min, followed by 140-300 ˚C at a rate of 20˚C/min. 1.5 µl were injected automatically into the system.

The main oil components (eucalyptol, camphor, α-pinene *etc.*) were compared with commercial standards for analysis of organic compound classes with high purity purchased from Sigma-Aldrich, UK. Six different mixtures of nine standard compounds were made in different concentrations (1, 5, 10, 25, 50 and 100 µg/ml) and used in each experiment. Additionally, two different quality controls (QC) were used in each run after each group of samples in each experiment. GCMS calculations were performed using the Quant component of Thermo Xcalibur (*Xcalibur™* Software-Thermo Scientific). In brief, a processing method was written which, using data obtained by the individually run standards, identified each essential oil on the basis of a retention time (RT) match window of 30 (±15) seconds. The electron impact (EI) spectrum of the samples needed to match that of the reference standards (Fig 2.6). The most prominent spectral peak, e.g. 91.1 in the below example of α-pinene (Fig 2.7), that was specific to that essential oil with the same retention time, was used for quantitation. The mass tolerance was set at 500 mmu.

This processing method was then used within the Xcalibur Quant software on all injections, standards, blanks, QCs and samples. The software automatically created extracted ion chromatograms (EICs) of the aforementioned prominent spectral peaks using its ICIS automatic peak integration algorithm. Every integration from every injection was manually interrogated and any that were deemed inadequate were manually adjusted taking care not to bias in any way. The internal standard (with a prominent 128.0 m/z ion at RT 16.2) was used to factor-in possible differences in injection volumes and instrument sensitivity over the course of the sample list run. The areas of the EICs were automatically generated, taking into account the internal standard data, and a standard curve was likewise automatically generated. The concentrations of each compound of the unknowns (samples, QCs) were automatically generated using these areas and the formula of the standard curve.

Each sample (essential oil, standard and QC) was analysed three times and the mean was used. The QCs were found to be within 10% of the theoretical concentration and the run was deemed valid and the $R^2 \geq 0.99$. Hexane was used as a solvent for all samples and standards with 15 µg/mL naphthalene as an internal standard. Essential oil samples made by dissolve 25 µg of essential oil in 1 ml of hexane containing 015 µg/mL naphthalene.

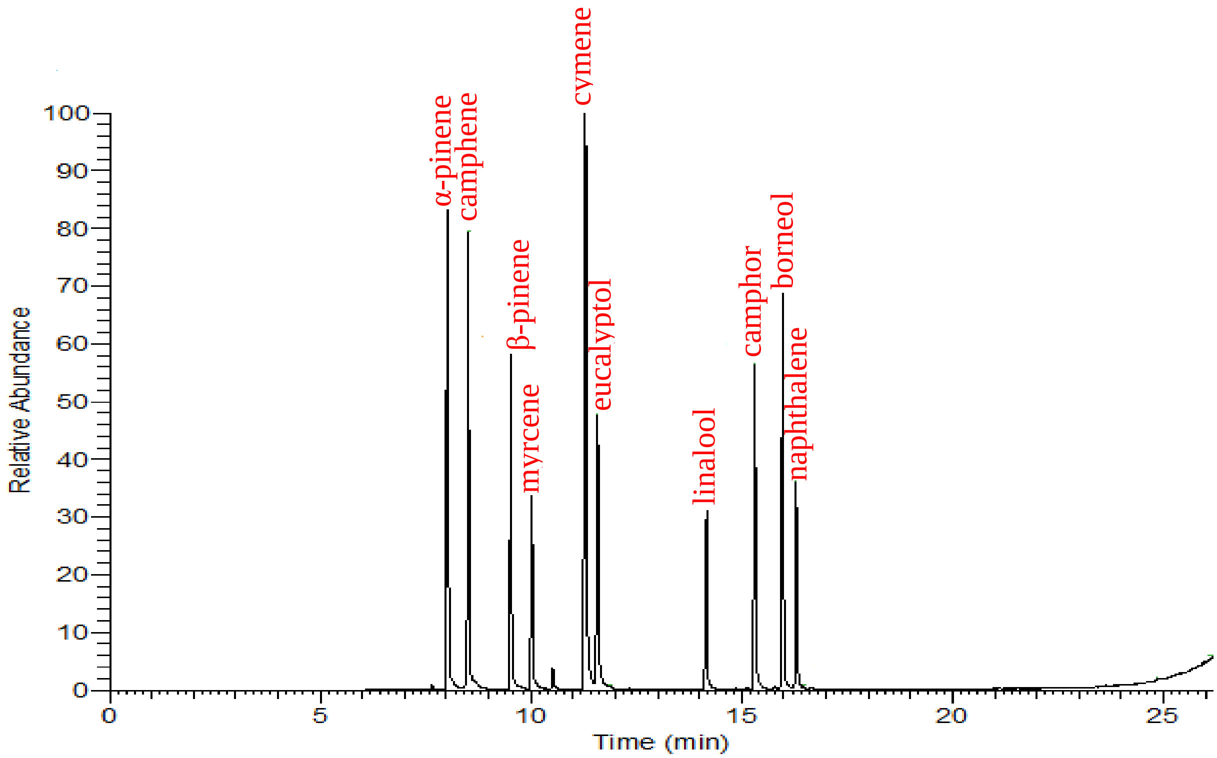


Figure 2. 6 GC chromatogram trace obtained from rosemary extract

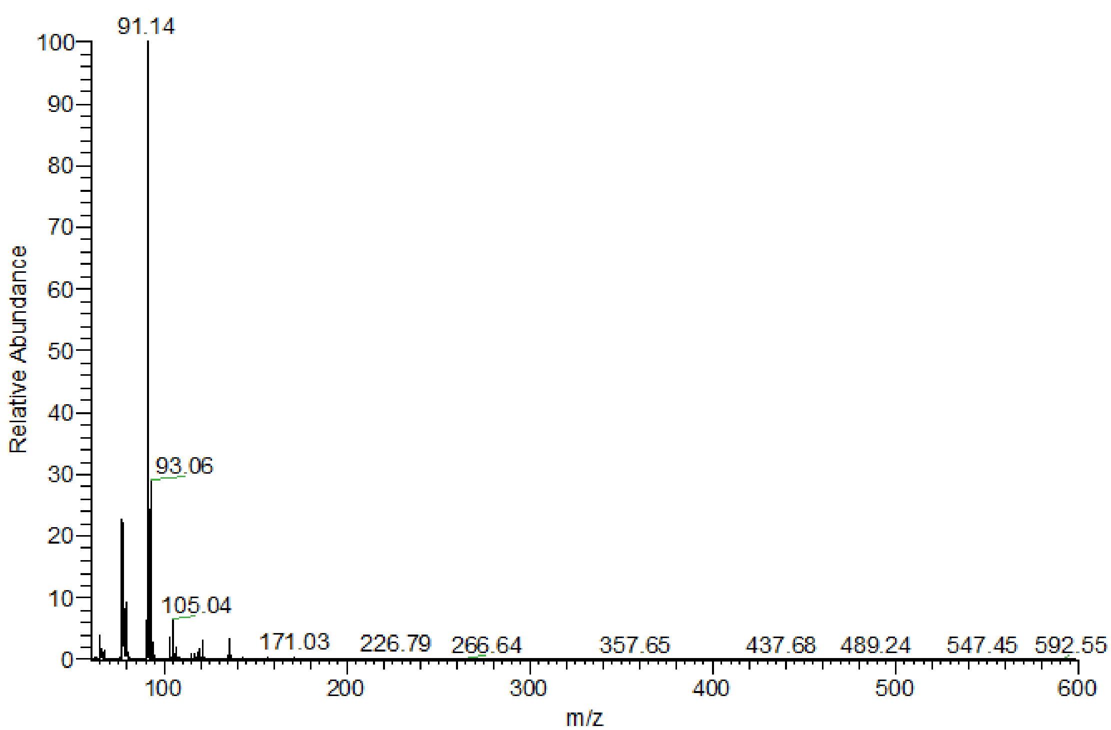


Figure 2. 7 GC mass spectrum trace for α-pinene

2.2.7.2 NMR

NMR has been used as an alternative method to identify the major components of the essential oil. NMR is an analytical chemistry technique used in quality control and research for determining the content and purity of an organic sample, as well as its molecular structure, by establishing the number and nature of hydrogen and carbon atoms.

Nine commercial standards of organic compound classes with high purity purchased from Sigma-Aldrich, UK were used for analysis. The nine standards have been used to allow full identification of major component peaks using data obtained by the individually run standards, identified each essential oil on the basis of the number and nature of hydrogen atoms. 20 mg of essential oil was dissolved in 1 ml of deuterated chloroform ($CDCl_3$) containing 0.5 mg of 1,4-dibromobenzene (internal standard) for comparison of the integrated peak areas of the ^{1}H NMR signals as the internal standard (Wang et al, 1996). The magnitude or intensity of NMR resonance signals is displayed along the Y axis of a spectrum, and is proportional to the molecular concentration of the sample. The latter component allowed a direct quantification of the NMR spectrum by a comparison with the integrated signal from the four equivalent aromatic hydrogens. The data was processed using *Topspin* software from Bruker. A typical example of the spectra obtained is shown in (Fig 2.7). The contents of oil compounds are calculated as follows:

$$amount\ of\ compound\ in\ 20\ mg\ of\ essential\ oil$$

$$= \frac{\text{number of H atoms are giving rise to that peak} * \text{integral of peak's signal} * 0.5 * \text{MW of compound}}{4 * \text{MW of dibromobenzene (235.9)}}$$

Then, the amount of each compound was calculated as a percentage:

$$\%\ amount\ of\ compound = \frac{amount\ of\ compound\ in\ 20\ mg\ of\ essential\ oil}{20} * 100$$

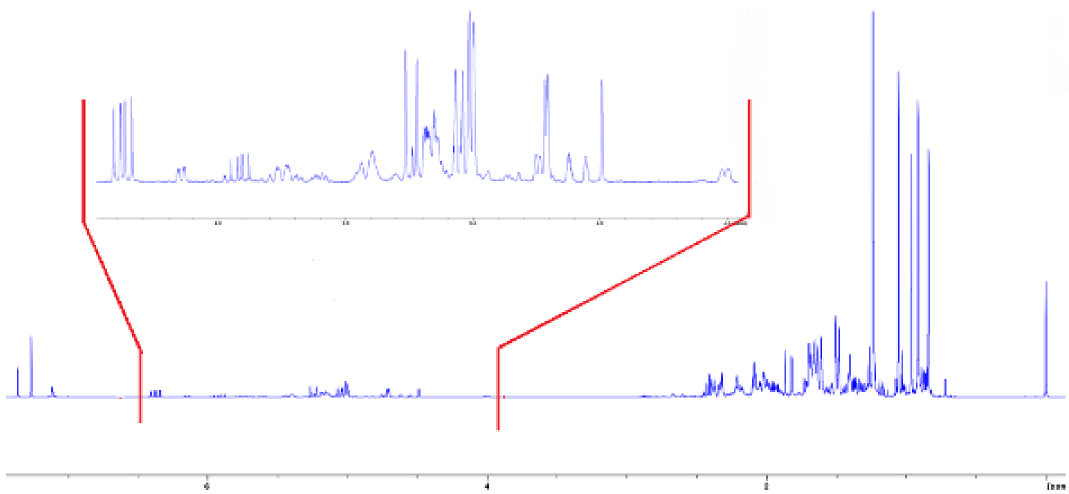


Figure 2. 8 NMR trace obtained from rosemary oil extract

2.2.8 Vegetative characters

Leaf area was measured using *WD3 - WinDIAS* Leaf Image Analysis System (Delta-T Devices Ltd, UK). Plant height was measured for each plant from the soil surface to the highest top tip of the plant using a tape measure. Percentage moisture in the leaf or root, was calculated by weight of fresh material followed by weight after oven-drying at 70°C for three days in a drying oven. Root volume was estimated by displacement of water in a measuring cylinder in cm^3.

2.2.9 Statistical analysis:

For statistical analysis; percentages were normalized by arcsine transformation as needed. In order to investigate the interrelationships between essential oil quantity, quality and crop growth parameters, the independent effects of each combination of variables was evaluated with ANOVA (Two-way statistical analysis) by using *Genstat* software (Payne *et al.*, 2009), considering each experimental condition as the "group variable". The analysis compared the effects of organic, inorganic, and control (no fertilizer) treatments and between sprayed and watered methods of application of the treatments in three different dates of harvest. The least significant difference (LSD) was used to account for variation between these factors.

The percentage composition of the essential oils was used to determine the relationship between the different treatments samples by principal components analysis (PCA) using the

Genstat software. PCA was employed based on GC-MS data to provide an overview of the capacity to distinguish essential oil components, or in other words to detect the distribution pattern of samples and to identify which chemical constituents can distinguish between these groups of individuals.

The dates of harvest have been named in this chapter H1, H2 and H3 (Table 2.5).

Table 2. 5 Abbreviations for dates of harvest names

H1	First date of harvest after three months from experiment starting
H2	Second date of harvest after six months from experiment starting and three months after first harvest
H3	Plants harvested one time after six months from experiment starting

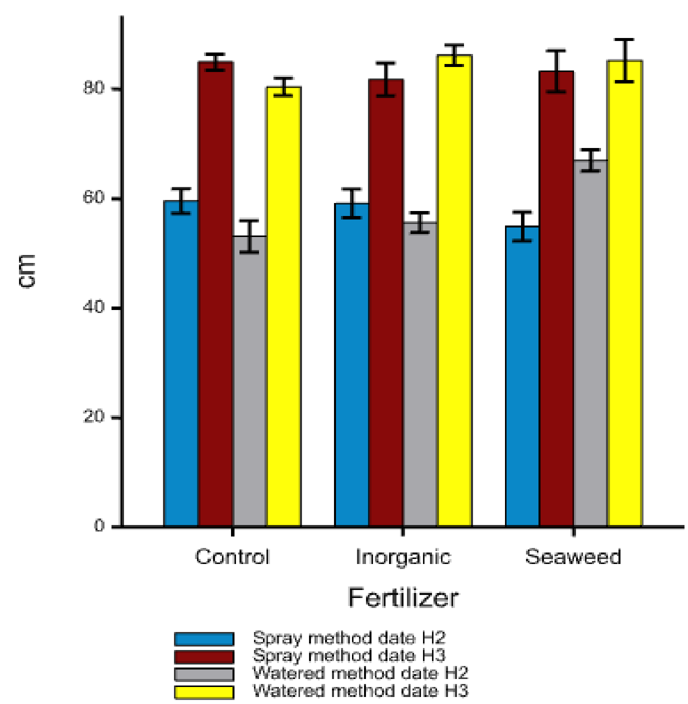
2.3 Results

2.3.1. Plant growth

2.3.1.1 Plant height

The effect of fertilizers, methods of application, and dates of harvest on the height of the *Rosmarinus officinalis* L. plants were recorded in an experiment to establish if there was any simple relationship with oil production. The measurement of plant height was taken at the end of this experiment for two of three dates of harvest, H2 and H3.

There are no significant differences in plant height between the three treatments, control, inorganic and seaweed in each date of harvest individually. At the same time, the difference between spray and watered methods of adding fertilizers did not differ significantly in H2 and H3 dates of harvest for all the treatments. The interaction between fertilizers, methods of application and dates of harvest shows a significant difference in plant height (Fig 2.8).

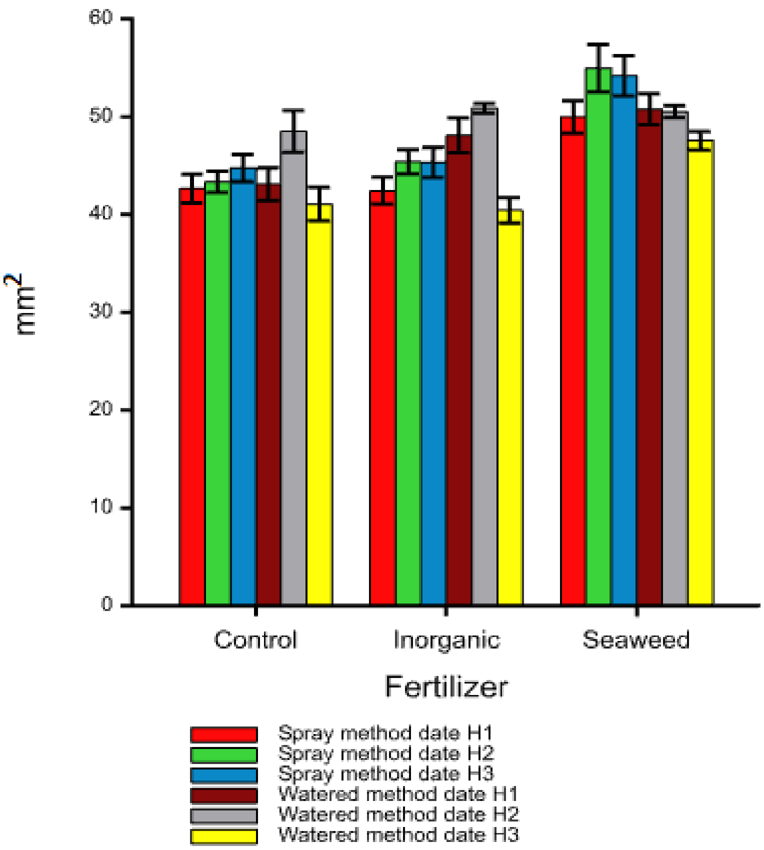


L.S.D $_{0.05}$ = 7.30

Figure 2. 9 Influence of fertilizers, methods of application and dates of harvest on height (cm) of rosemary plants

2.3.1.2 Leaf area

There is a variation between fertilizers effects on leaf area. In H1 plants, seaweed fertilizer discriminates significantly (49.9 mm^2 in spray method and 50.7 mm^2 in watered method) compared with control (42.6 mm^2) and inorganic copy of fertilizer (42.4 mm^2) in spray method and with control (43.1 mm^2) in watered method of application. H2 plants treated with spray seaweed had the higher significance leaf area (54.9 mm^2) compared with control (43.3 mm^2) and inorganic (45.4 mm^2), while watered method did not show any noticeable difference. H3 plants treated with spray and watered seaweed had the higher significance leaf area 54.1 and 47.5 mm^2 compared with control 44.7 and 41.0 mm^2 and inorganic 45.3 and 40.4 mm^2 respectively.



L.S.D $_{0.05}$ = 4.33

Figure 2. 10 Influence of fertilizers, methods of application and dates of harvest on leaf area (mm^2) of rosemary plants

There are significant differences in leaf area between methods of application fertilizers. Watered inorganic fertilizer treatment in both H1 and H2 gave highest leaf area 48.1 and 50.8 mm^2 respectively, compared with spray method. In H3, sprayed seaweed and inorganic fertilizer showed significantly highest leaf area 54.2 and 47.4 mm^2 respectively, compared with the watered method.

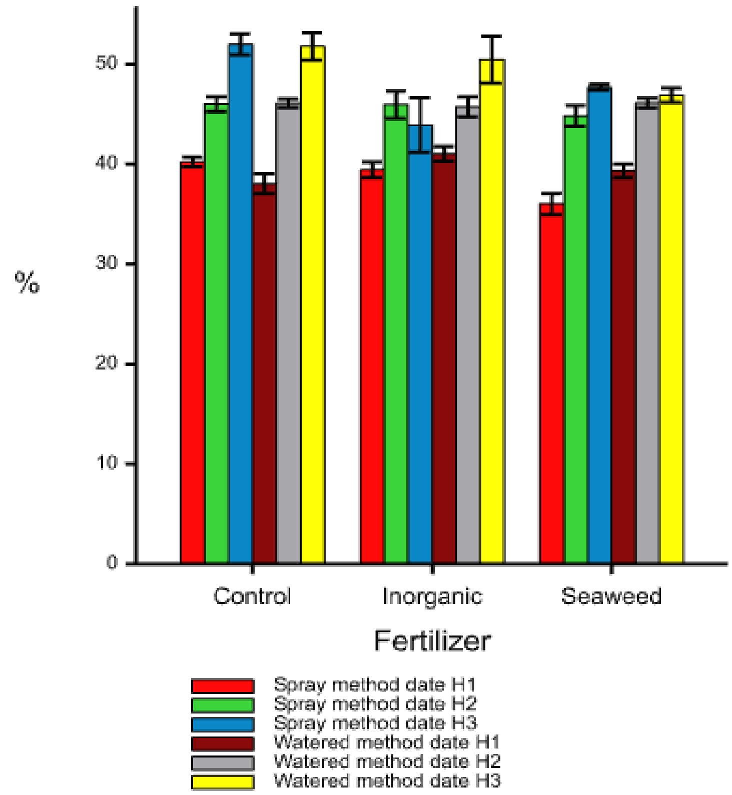
All other seaweed treatments did not give any significant differences between the two methods. The control treatment had no significant difference in leaf area between application methods. The interaction between fertilizers, methods of application and dates of harvest shows a significant difference in leaf area (Fig 2.9).

2.3.1.3 Percentages of dry material in leaves

In terms of leaf moisture, H1 and H3 spray control plants gave significantly higher amounts of dry material in leaves 40.2% and 51.9% as compared with seaweed (36.01 and 47.7% respectively) and inorganic fertilizers (43.9%) in H3 plants. However, there was no significant difference among the treatments sprayed on H2 plants.

In the watered method, H1 and H2 did not show any significant difference between all the treatments. In H3 plants, the control treatment had a significantly higher percentage of dry material in leaves, 51.7% compared with seaweed which was 46.9%. Leaf moisture of plants treated with Inorganic fertilizer 50.4% did not differ significantly from both other treatments.

The three dates of harvest with control and inorganic treatments did not appear to promote any significant differences between spray and watered methods of application. Seaweed treatment on H1 plants had a significant difference, the watered method was 39.3%, while spray method was the lowest at 36.0%. Both the methods in H2 and H3 did not differ significantly. The interaction between fertilizers, methods of application and dates of harvest shows a significant difference among the plants in percentages of dry material in leaves (Fig 2.10).



L.S.D $_{0.05}$ = 3.31

Figure 2. 11 Influence of fertilizers, methods of application and dates of harvest on percentage of dry material (%) in leaf of rosemary plants

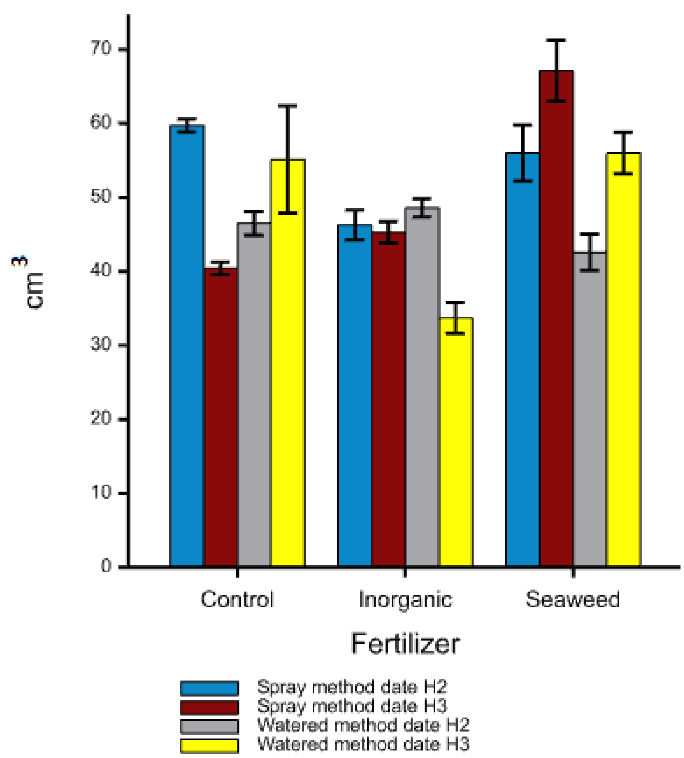
2.3.1.4 Root size

The role of roots in plants is to absorb soil solution including water and nutrients from the surrounding growing media, and usually, a healthy plant has a healthy root system, so the root-shoot ratio is a measure to help assess the overall health of the plants. Control group of plants will provide a "normal" root-shoot ratio, any changes from this normal level, whether positive or negative would be an indication of a change in the overall health of other plants. However, this measurement can explain the controlling variables for water and nutrient uptake.

The comparison of fertilizer indicated significant differences in the size of the roots between treatments. H2 plants treated with spray inorganic fertilizer had the lowest root size, (46.3

cm^3) and differed significantly from seaweed (56.0 cm^3 and control 59.7 cm^3) treatments, which did not differ considerably between themselves. On the contrary, spray seaweed fertilizer in H3 increased the root size, (67.1 cm^3) significantly compared with control (40.4 cm^3) and inorganic fertilizer (45.3 cm^3). Watered method also presented a significant difference between the treatments in H3 plants. The smallest size, 33.7 cm^3 was found with inorganic fertilizer treatment, while seaweed and control had no significant difference between them. H2 did not show any significant difference amongst the treatments (Fig. 2.14).

The comparison of methods of application of fertilizer showed that spray excelled over the watered method in H2 control, H3 inorganic, and H2 and H3 seaweed treatments. Only H3 control and H2 inorganic did not differ significantly. The interaction between fertilizers, methods of application and dates of harvest shows a significant difference among the plants (Fig 2.15).

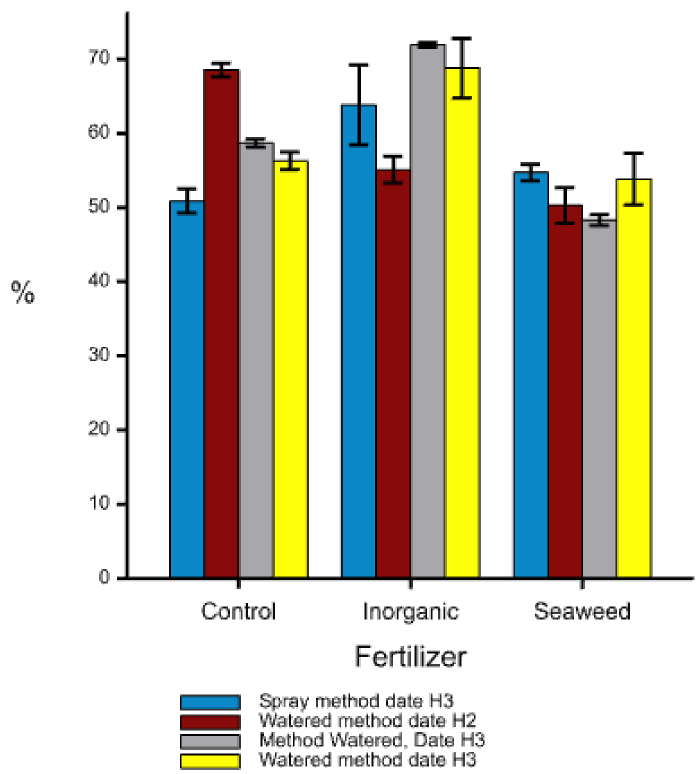


L.S.D $_{0.05}$ = 8.68

Figure 2. 12 Influence of fertilizers, methods of application and dates of harvest on root size (cm^3) of rosemary

2.3.1.5 Percentages of dry material in roots

The two different methods of application of fertilizers, even with different dates of harvest, had strong significant differences in percentages of dry material in roots. The H2 plants treated by foliar method showed a significant difference between inorganic, (63.80%) and control (50.87%) treatments, whilst seaweed (54.72%) did not differ significantly for either of them. In H3 plants, the control was (68.53%) which differed significantly when compared with inorganic and seaweed, which were 55.08% and 50.28%, respectively. Watered treatments showed that inorganic treatments in both H2 (71.91%) and H3 (68.77%) were significantly higher than control (58.68% and 56.28%) and seaweed (48.30% and 53.83%), respectively.



L.S.D $_{0.05}$ = 6.96

Figure 2. 13 Influence of fertilizers, methods of application and dates of harvest on percentage of dry material (%) in root of rosemary plants

When comparing between methods of application, H2 watered control 58.68% and H3 watered inorganic 68.77% showed significantly higher percentages of dry material in the root. H3 spray control 68.53% and H2 spray seaweed 50.87% were characterized by higher percentages. Otherwise, H2 inorganic and H3 seaweed did not show any significant difference between the methods. The interaction between the treatments shows a significant difference among the plants in percentages of dry material in roots (Fig 2.12).

2.3.2 Oil yield

The essential oil obtained from rosemary plants differs depending on type of fertilizer and method of application as well as date of harvest. Yield of essential oil in H1 plants with spray and watered methods showed no significant differences, although it is worth noting that the result of the spray seaweed was high (0.567 g/100g fresh leaves) compared with control (0.484 g/100g fresh leaves) and inorganic treatments (0.560 g/100g fresh leaves).

In H2, the oil amount of plants sprayed with control reduced significantly (0.287 g/100g fresh leaves) compared with inorganic and seaweed (0.433 and 0.425 g/100g fresh leaves respectively). In the same time, watered seaweed has high amount of oil (0.505 g/100g fresh leaves) and characterized significantly on control (0.384 g/100g fresh leaves) and inorganic (0.304 g/100g fresh leaves) treatments.

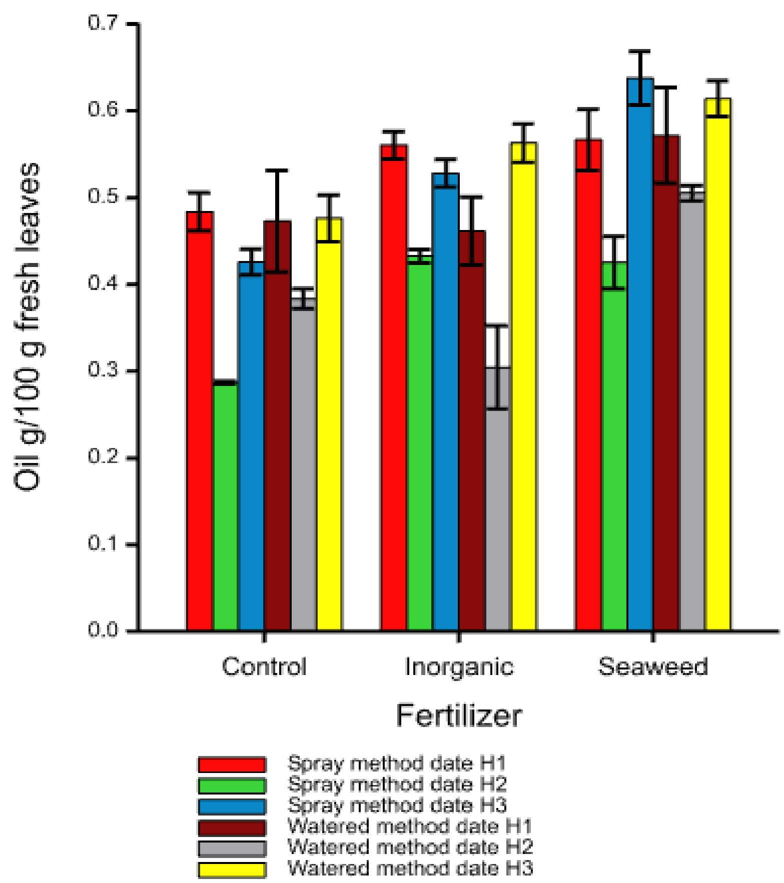
H3 plants with spray method contained a significant difference between the treatments. The highest amount of oil was with seaweed treatment, 0.638 g/100g fresh leaves compared with inorganic and control, 0.528 and 0.426 g/100g fresh leaves, respectively. On the other hand, watered control (0.476 g/100g fresh leaves) showed a significantly lower amount of oil compared with seaweed (0.614 g/100g fresh leaves) and inorganic fertilizer (0.563 g/100g fresh leaves).

For all the plants, the results of the seaweed treatments were higher than control and inorganic fertilizer, even if the difference was not significant.

Based on the summary statistics of the data which compared between the spray and watered method on oil amount, there was found to be a significant difference between spray (0.560

g/100g fresh leaves) and watered inorganic fertilizer (0.462 g/100g fresh leaves) in H1 plants, whilst control and seaweed did not show any difference between the two methods in this date of harvest.

H2 plants treated with control and seaweed showed the most significant excelling of the watered method (0.286 and 0.425 g/100g fresh leaves) over spray (0.383 and 0.505 g/100g fresh leaves) for these two treatments respectively. Within an inorganic fertilizer, there was the opposite: the spray method (0.433 g/100g fresh leaves) was higher than watered (0.304 g/100g fresh leaves). By contrast with H2, there was no significant difference in all the treatments among H3 plants. The interaction between fertilizers, methods of application and dates of harvest shows a significant difference among the plants in oil yield (Fig 2.13).



L.S.D $_{0.05}$ = 0.084

Figure 2. 14 Influence of fertilizers, methods of application and dates of harvest on oil yield (g/100g fresh leaves) of rosemary plants

2.3.3 Oil composition

The results of analysis variance performed on the essential oil composition data are shown in Table 2.5 & 2.6. These tables show the percentage composition of the nine major constituents of rosemary essential oil averaged for GC-MS and NMR analyses. Also, PCA was used to determine the relationship between the different treatment in essential oil composition.

2.3.3.1 GC-MS results

The differentiation between fertilizers applied by two different methods to the plants harvested on three different dates show major changes in the percentages of components.

H1 plants showed significant effects within the oil composition. Three out of nine compounds showed higher levels as a positive response to some treatments. However, β-pinene (3.96%) with the plants treated by watered inorganic fertilizer, eucalyptol (27.41%) and camphor (25.56%) with the plants treated by spray inorganic and spray seaweed respectively, appear to have higher percentages compared with other treatments. On other hand, α-pinene (3.83%) and camphene (2.86%) with spray control, myrcene (9.10%) with watered control, *p*-cymene (1.30%) and linalool (3.04%) with watered seaweed responded negatively by presented lower levels for these compounds compared with other treatments.

In H2 Plants, four compounds responded significantly to treatments. These compounds are: α-pinene (7.10%), *p*-cymene (3.22%) and linalool (6.95%) with spray control; and borneol (4.74%) with watered inorganic characterized by higher levels compared with all other treatments. Spray control has a clear effect on camphor by lowest level (4.74%) compared with other treatments.

H3 plants sprayed with seaweed fertilizer showed lowest percentages of β-pinene (2.27%), eucalyptol (20.77%) and borneol (3.00%) compared with other treatments. Watered seaweed increased the levels of camphene (4.72%) and myrcene (13.81%) significantly compared with all other treatments.

In general, type of fertilizer had a significant effect on β-pinene, myrcene, *p*-cymene, linalool, camphor and borneol. Method of application caused significant effects on *p*-cymene and linalool. Date of harvest affects significantly on all the nine compounds. The interaction between fertilizers, methods of application and dates of harvest shows significant differences among the plants (Table 2.6).

In order to explore the relationship between the samples from different treatments and their chemical constituents' relation to specific volatile compounds, the GC-MS data was subjected to Principle Component analysis (PCA). As a result, the analysis of chemical data by the technique of PCA permitted to group chemically the samples in three groups, in a way to express and evidence their similarities and differences. For H1 spray (Fig 2.14 A) and watered (Fig 2.14 B) methods of application , it was observed with the first main component and the second main one for each method individually, it was possible to describe 86.2% and 81.55% of the data respectively, being 66.92% and 46.58% of the total variance described by the first main component (PC-1); 19.28% and 34.97% as the second main component (PC-2) for both methods of application respectively according to their major volatile components. Camphor and eucalyptol were the main compounds in the essential oils in PC-1; eucalyptol and myrcene in were the major compounds in PC-2 for both methods of application.

PCA for H2 spray (Fig 2.15 A) and watered (Fig 2.15 B) methods explained 92.06% and 81.18% of the total variability with PC-1 76.33% and 61.1%; and PC2 for 15.73% and 20.08% of the total variability for both methods of application respectively. Camphor was the main compounds in the essential oils in PC-1; eucalyptol and myrcene in were the major compounds in PC-2 for both methods of application.

The H3 described 95.65% and 97.47% for spray (Fig 2.16 A) and watered (Fig 2.16 B) method respectively, the PC-1 accounts for 85.02% and 71.29% of total variance with significant amounts of camphor and eucalyptol, whereas PC-2 accounts 10.63% and 26.18% of total variance distinguishes with significant amounts of camphor, eucalyptol, α-pinene and myrcene for both methods of application respectively.

Table 2. 6 Influence of fertilizers, methods of application and dates of harvest on oil composition of rosemary plants analysed by GC-MS

Date of Harvest	Method of application	Fertilizer	α-Pinene %	Camphene %	β-Pinene %	Myrcene %	*p*-Cymene %	Eucalyptol %	Linalool %	Camphor %	Borneol %	Total %
After 3 months H1	Spray	Control	3.83	2.86	2.40	11.49	1.58	24.69	3.09	17.88	3.35	**71.21**
	Spray	Inorganic	4.42	3.46	3.81	11.55	1.48	27.41	3.45	23.55	3.84	**83.00**
	Spray	Seaweed	3.92	3.01	3.22	9.90	1.61	26.80	3.11	25.56	3.84	**81.02**
	Watered	Control	3.94	2.93	3.02	9.10	1.68	27.04	3.08	23.20	3.72	**77.74**
	Watered	Inorganic	4.44	3.52	3.96	11.49	1.37	26.69	3.27	24.23	4.07	**83.09**
	Watered	Seaweed	3.94	3.10	3.29	10.36	1.30	26.44	3.04	23.60	3.72	**78.80**
3 months after first harvest H2	Spray	Control	7.10	4.36	3.65	12.15	3.22	24.65	6.95	13.48	3.58	**79.19**
	Spray	Inorganic	6.05	4.37	3.15	13.71	1.65	24.45	4.81	24.43	4.58	**87.22**
	Spray	Seaweed	5.34	3.74	2.49	12.28	1.89	23.09	4.86	23.35	4.60	**81.67**
	Watered	Control	5.24	3.59	2.35	11.00	1.58	22.14	3.98	20.19	4.28	**74.39**
	Watered	Inorganic	5.89	4.05	2.76	12.42	1.48	21.52	4.74	22.21	4.74	**79.85**
	Watered	Seaweed	5.54	4.00	2.73	11.99	1.74	21.96	3.83	21.15	3.93	**76.92**
After 6 months H3	Spray	Control	4.49	3.47	2.42	10.96	1.44	23.03	3.78	21.28	3.87	**74.78**
	Spray	Inorganic	5.58	4.16	2.97	12.58	1.65	24.63	4.47	23.32	4.11	**83.50**
	Spray	Seaweed	4.62	3.45	2.27	9.63	1.43	20.77	2.98	18.87	3.00	**67.06**
	Watered	Control	5.53	4.12	2.76	11.22	1.57	23.10	3.27	19.14	3.49	**74.24**
	Watered	Inorganic	5.16	3.80	2.71	11.58	1.40	23.79	3.99	24.96	4.15	**81.58**
	Watered	Seaweed	6.17	4.72	3.12	13.81	1.91	24.39	3.24	19.34	3.30	**80.04**
	L.S.D		**0.849**	**0.574**	**0.418**	**1.382**	**0.249**	**2.876**	**0.598**	**2.637**	**0.588**	***d.f* 125**

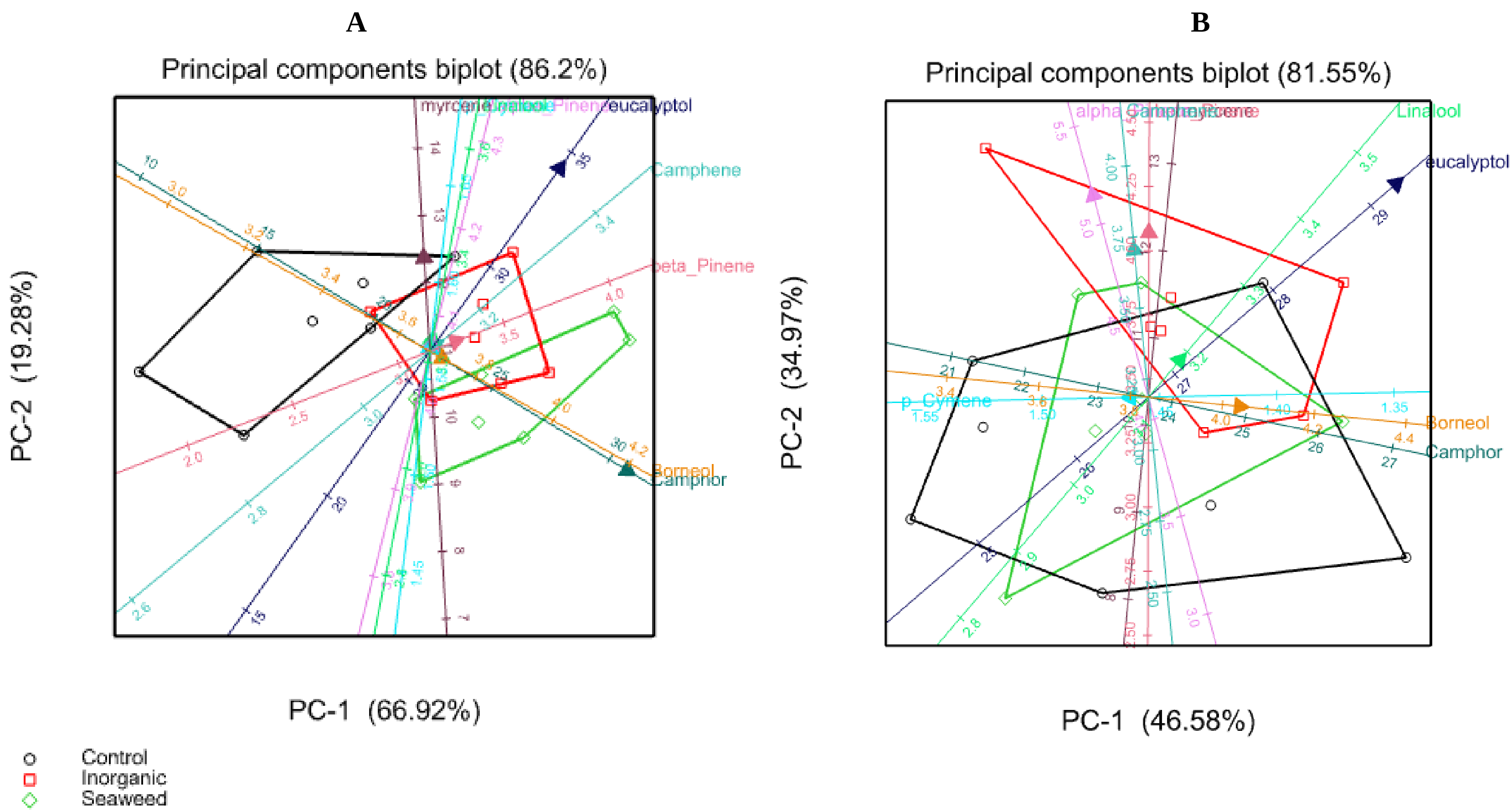


Figure 2. 15 Principle Components Analysis biplot distinguishing the effect of the three fertilizers (including control) with two different methods of application (A: spray and B: watered) for H1 plants into chemotypes using nine main volatile constituents.

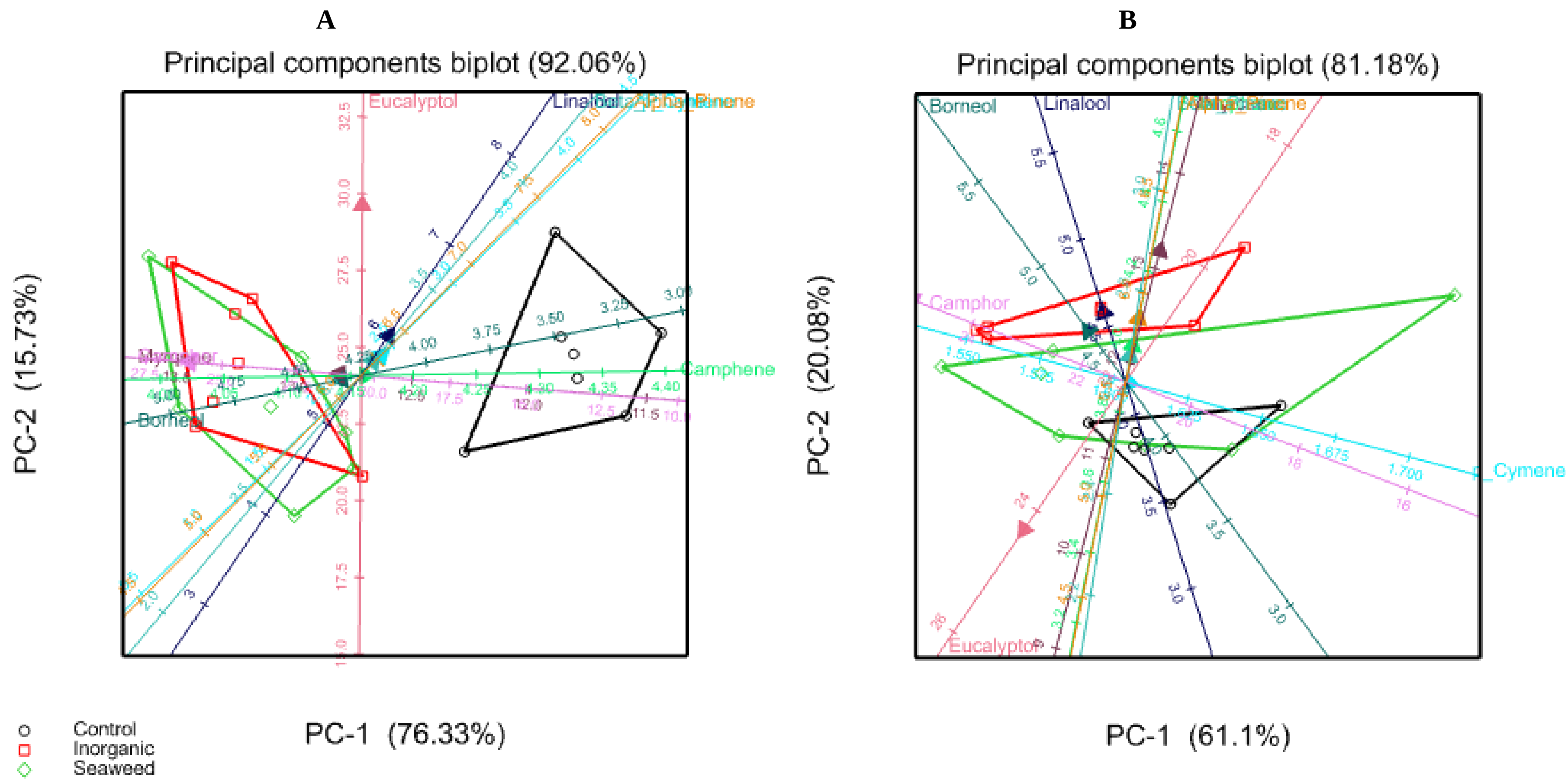


Figure 2. 16 Principle Components Analysis biplot distinguishing the effect of the three fertilizers (including control) with two different methods of application (A: spray and B: watered) for H2 plants into chemotypes using nine main volatile constituents.

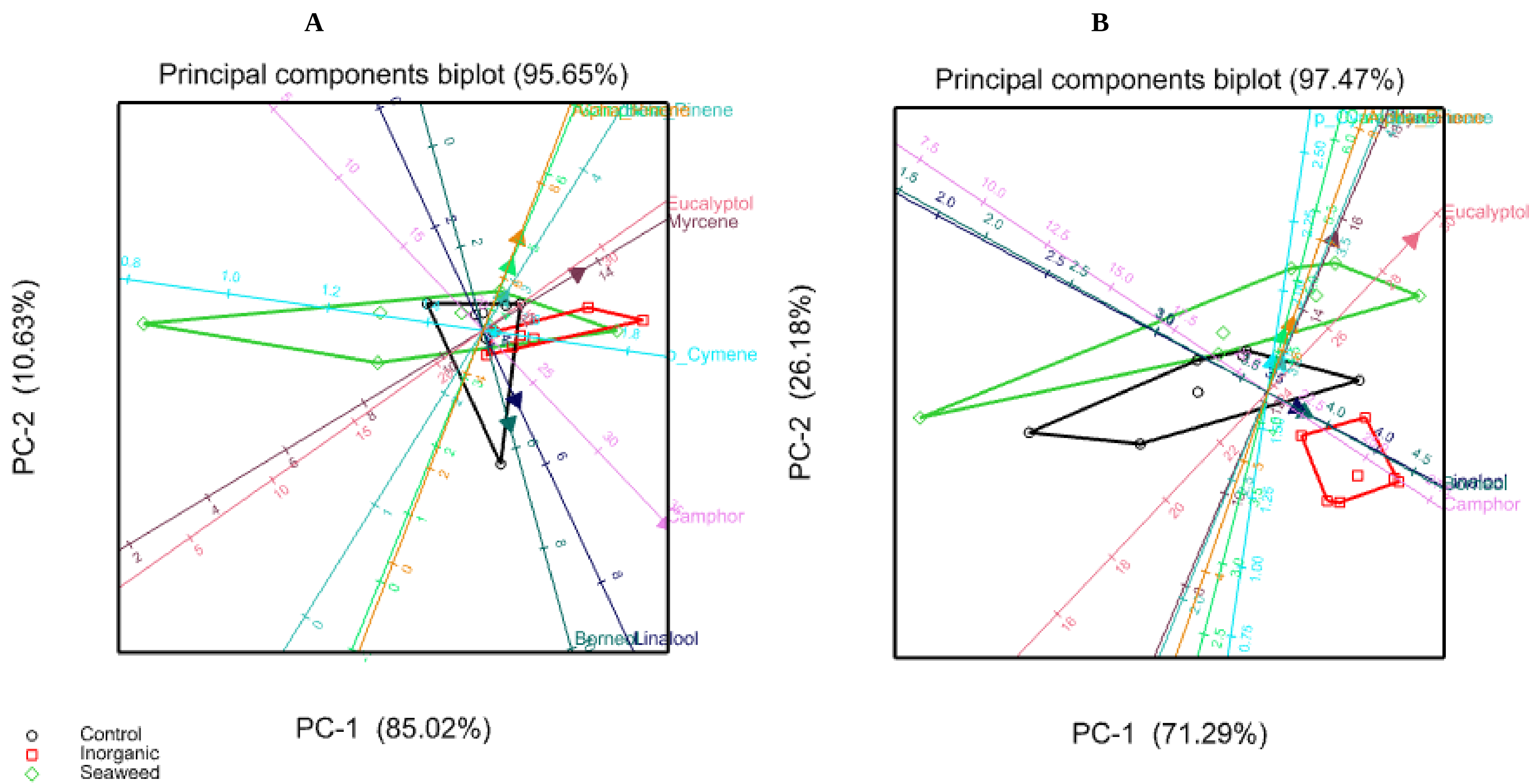


Figure 2. 17 Principle Components Analysis biplot distinguishing the effect of the three fertilizers (including control) with two different methods of application (A: spray and B: watered) for H3 plants into chemotypes using nine main volatile constituents.

2.3.3.2 ^{1}H NMR results

The ^{1}H NMR results were a little different when compared with GC-MS outcomes. Table 2.7 shows the result of oil composition analysed by this technique.

In H1 plants, there were significant effects on the plants treated with watered or spray control and with watered inorganic fertilizer on the concentrations of α-pinene, β-pinene, myrcene, eucalyptol and linalool. Control treatments gave higher levels of β-pinene (5.14%) and eucalyptol (21.1%) with spray and watered method of application respectively. Whilst watered inorganic fertilizer showed lowest level for α-pinene, myrcene and linalool (4.0%, 6.7% and 1.8% respectively) compared with other treatments.

H2 plants characterized significantly by higher level of linalool (3.1%) and borneol (3.1%) with watered control treatment. On other hand, plants treated with spray control showed lowest levels of *p*-cymene (0.6%) and borneol (1.6%). Also, plants treated with watered inorganic fertilizer under this date of harvest showed significantly lowest levels of β-pinene (2.7%), eucalyptol (12.4%) and camphor (13.3%).

Watered seaweed fertilizer on H3 plants showed significant increases in α-pinene, myrcene, *p*-cymene and camphor concentrations (7.0%, 10.2%, 1.4% and 21.7 respectively) compared with all other treatments.

In general, type of fertilizer had a significant effect on *p*-cymene only. Method of application did not show any significant difference in oil composition with NMR analysis. Date of harvest affects significantly on α-pinene, β-pinene, myrcene, *p*-cymene, eucalyptol, linalool and camphor. The interaction between fertilizers, methods of application and dates of harvest shows a significant difference among the plants. Camphene did not show any significant response for all treatments including the interactions (Table 2.7).

Table 2. 6 Influence of fertilizers, methods of application and dates of harvest on oil composition of rosemary plants analysed by ^{1}H NMR

Date of Harvest	Method of application	Fertilizer	α-Pinene %	Camphene %	β-Pinene %	Myrcene %	*p*-Cymene %	Eucalyptol %	Linalool %	Camphor %	Borneol %	Total %
After 3 months H1	Spray	Control	5.20	2.88	5.14	8.71	1.06	19.13	2.32	19.39	2.45	**66.3**
	Spray	Inorganic	4.83	2.65	4.65	8.14	1.07	19.85	2.23	19.74	2.54	**65.72**
	Spray	Seaweed	4.30	2.40	3.94	6.8	1.02	18.55	2.05	19.18	2.47	**60.74**
	Watered	Control	5.04	2.47	4.32	7.02	1.15	21.18	2.39	21.36	2.83	**67.799**
	Watered	Inorganic	4.03	2.26	4.21	6.76	0.76	15.65	1.85	15.68	2.46	**51.23**
	Watered	Seaweed	4.54	2.49	4.27	7.94	0.95	19.93	1.97	20.46	2.36	**64.96**
3 months after first harvest H2	Spray	Control	4.12	1.89	1.89	7.52	0.66	7.39	1.81	7.714	1.69	**34.70**
	Spray	Inorganic	6.92	3.10	3.27	8.52	1.04	16.31	2.74	16.60	2.69	**61.23**
	Spray	Seaweed	6.44	2.75	2.99	7.66	1.05	14.71	2.98	16.02	2.67	**57.31**
	Watered	Control	6.12	3.02	3.09	8.45	0.96	15.65	3.16	16.72	3.17	**60.39**
	Watered	Inorganic	5.67	2.86	2.71	7.51	0.83	12.40	2.52	13.38	2.52	**50.43**
	Watered	Seaweed	6.37	7.46	3.07	8.12	1.13	14.20	2.44	15.60	2.41	**60.83**
After 6 months H3	Spray	Control	5.61	2.78	3.11	8.53	0.85	16.50	3.05	17.36	2.99	**60.82**
	Spray	Inorganic	6.51	3.38	3.54	9.23	1.10	17.23	2.72	18.44	2.46	**64.64**
	Spray	Seaweed	5.54	2.64	2.88	7.01	1.00	17.55	2.38	19.60	2.39	**61.03**
	Watered	Control	6.83	3.81	3.91	9.41	1.10	17.80	2.65	18.36	2.902	**66.79**
	Watered	Inorganic	6.32	3.51	4.20	9.54	1.15	18.29	2.49	19.34	2.69	**67.56**
	Watered	Seaweed	7.05	4.03	4.03	10.24	1.43	19.09	2.22	21.71	2.39	**72.23**
	L.S.D		**1.152**	**3.096**	**0.807**	**1.639**	**0.259**	**1.820**	**0.249**	**4.232**	**0.558**	***d.f*** **125**

2.4 Discussion

Seaweed extract is a good source of nutrients for crop production. Many studies have detected that the applications of seaweed extract on plants improved crop efficiency and yield, as well as early seed germination and establishment, boosted resistance to biotic and abiotic stress, and enhanced post-harvest shelf life of perishable products (Sivasankari *et al.*, 2006; Khan *et al.*, 2009; Gurusaravanan *et al.*, 2010; Kumar and Sahoo, 2011). In addition, Vijayanand *et al.* (2014) pointed out that marine plant extracts lead to increased leaf area and increased chlorophyll content, and also leading to form healthy with many branched roots.

However, there is a shortage of knowledge available on the effect of organic fertilizer on the yields and composition of rosemary oil, particularly seaweed fertilizer. Based on our results, both fertilizers worked positively for rosemary plants compared with control.

There was an increase in growth and oil production with seaweed application, the leaf area, root size, and decrease in percentage of dry material in leaves, and roots compared with either the control or the inorganic fertilizer treatment.

This results are in agreement with AlMohammedi *et al.* (2014) who found that seaweed fertilizer improved plant height, fruit branch per plant, seed yield, capsules per plant, seeds per capsule, and 1000 seed weight of *Nigella sativa* . Rayorath *et al.* (2008) explained that the bioactivity of organic compounds derived from seaweed can be used to improve the rate of crop production in agricultural systems because it contains a percentage of hormones which play an active role in promoting the germination and vigour and vitality of the plant. Moreover, Jensen (2004) stated that the cause of improved fruit qualities was as a result of being sprayed with seaweed extract. This fertilizer is rich in necessary nutrients, particularly nitrogen, phosphorus, potassium, calcium, magnesium, sulphur and many amino acids, as well as auxins that stimulate cell division, increase leaf area and increase the photosynthetic process, thus improving the qualities of the fruit and increasing the content of the elements. Further, the effect of organic seaweed fertilizer, it has been suggested, is due to the fact that seaweeds contain many different polysaccharides, proteins, polyunsaturated fatty acids, pigments, polyphenols, minerals and plant growth hormones which are not found in inorganic fertilizer (Gollan and Wright, 2006; Chojnacka *et al.*, 2012). Furthermore, Chojnacka *et al.* (2012) reported that hormones are largely responsible for plant growth stimulation in terms of increased effectiveness of photosynthesis, by protecting chlorophyll from degradation and enhancing its content in leaves. Moreover, this effect is due to the positive relationship between NPK availability and terpenoid concentration in leaves. Higher photosynthetic rates permitted by ready availability of nutrients allow more rapid synthesis of isoprene which in turn allows greater terpenoid production. Phosphorus plays a key role in this because terpenoid precursors contain high-energy phosphate bonds in isopentenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Also phosphorus could be a factor limiting isoprene and terpenoid emission because it is required for terpenoid synthesis as a key component of ATP and NADPH (Ormeno and Fernandez, 2012).

On other hand, root size has not differed significantly between seaweed and control treatments under spray H2 and watered H3 plants. According to Mouat (1983) this is due to the nutrient deficiency which leads to increase relative root growth in order to react to nutrient deficiency by increasing exploration of the soil through increasing relative root growth.

In terms of the chemical constituents of the rosemary oils produced, GC-MS shows that there are up to 90 components. However, to provide a more accurate quantitative analysis we have concentrated on the components present in the highest concentrations. The major components tended to be those containing oxygen particularly camphor and eucalyptol although there is a significant amount of myrcene. Oil composition differs between treatments in percentages of components, but the chemotype of the oil does not change even with the different dates of harvest. Most of the changes in essential oil composition occur with supplying seaweed extract or inorganic fertilizer to the plants.

The quantitative investigation shows some variation in the concentration of the components but no treatment which produces any major change in the proportion of these components. The relationship between these observations and biosynthesis is discussed further in Chapter 6.

In the same way, these changes may be conjectures to some of the physiological processes that happen inside the plant and relate to photosynthesis, such as a decrease in the diffusion path between stomata and chloroplast (Parkhurst, 1994). It could also be due to increase a chlorophyll content and thereby increasing the carbohydrates (Thirumaran *et al.*, 2009). Eris *et al.* (1995) have suggested that selectively stimulating certain pathways of metabolism may be beneficial for variation in oil composition; this is due to concentration of some nutrient elements.

The review of results showed that the seaweed fertilizer either spray or watered, in terms of oil yield, show a significant effect for H2 and H3 compared with the control. Inorganic fertilizer differed significantly from control in some treatments. There is a dramatic increase in the percentage of moisture in leaves and roots for the plants sprayed with organic fertilizer compared with all other treatments, accompanied by increase in leaf area and root size.

This decrease may be due to quick cell division resulting in an increased leaf area, or due to the lack of need for protection measures because of the availability of ideal conditions for the plants. In particular, the percentage of moisture in leaves integrates both density and leaf thickness and is considered a measurement of the presence of sclerophylly (Grubb, 2002). The increase in density and thickness and sclerophylly presence is thought to be a protection for plants facing inappropriate conditions; it may extend the leaf longevity under conditions of limited resources or drought (Fonseca *et al.*, 2000). This method of protection in leaves works by diluting photosynthetic tissues with non-photosynthetic tissues and leads to a reduction in the rate of photosynthesis due to lower levels of light-capture (Wright and Cannon, 2001).

Methods of application have shown slight difference between treatments. Foliar fertilizer has raised the oil amount in H1 and H2 plants treated by inorganic fertilizer. This is due to a more rapid absorption of nutrients directly to the location of demand in the leaves (Mondal and Al Mamun, 2011), or due to the low level of nutrients supplied to the plants through the soil as a result of the decline in root length with this fertilizer.

Foliar fertilizers, as chelates, should be easily absorbed by the plants, rapidly transported, and should easily release their ions to affect the plant (LaRue, 1989). On the other hand, watered method shown increased in oil amount in H2 plants watered seaweed fertilizer. This increase could be due to the reduction in number of leaves in plants after harvest and therefore leads to lack of fertilizer absorption by leaves. H3 plants did not show a significant difference with all treatments, this could be dependant on leaf age and growth conditions because the age of the plants in this stage of growth had exceeded one year (Gonzalez *et al.*, 2010).

2.5 Conclusions

In the work described in this chapter we have developed techniques for extraction and analysis using NMR and GC-MS and related this to the growing methods. This study on fertilizer type and method of application with different dates of harvest showed significant differences in growth, essential oil yield and composition of rosemary. The quality and quantity of rosemary essential oil varied with the different fertilizers: organic and its inorganic equivalent but without affecting the chemotype of the oil. This means that fertilizer could affect the production of oil in quantity without changing the quality. Seaweed as an organic fertilizer applied to the plant showed clearly defined results in many aspects of growth and yield. Also inorganic fertilizer showed some effects in other aspects unlike the control. The spray and watered methods of application have shown some differences in the yield of oil and leaf area especially when inorganic fertilizer was used, this difference was very small compared to using seaweed.

3. Chapter Three: Effects of Cytokinin from seaweed extract on plant growth

3.1 Introduction

It has been stated frequently that crops usually respond to the application of low rates of organic fertilizers positively through increased growth, as low rates of application are able to cause a physiological response from the crops. The reason for this effect is due to the nutrients contained in the fertilizer as well as the other contents such as growth regulators, alginic acids and vitamins which stimulate plant growth and production (Pino *et al.*, 1998; Rao *et al.*, 1998; Diaz-maroto *et al.*, 2007; Selvam and Sivakumar, 2013; Neish and Bourgougnon, 2014; Selvam and Sivakumar, 2014). Seaweed can form the basis of organic fertilizers and releases a blend of minerals which are a good source of both nutrients and growth regulators such as auxins, gibberellins and cytokinins which are very important in plant growth and production (Vijayanand *et al.*, 2014). Most of the seaweed extracts cause responses similar to those observed by applying cytokinins on plants, due to the presence of cytokinins in several seaweed extracts (Sridhar and Rengasamy, 2010).

Hormones are organic compounds naturally biosynthesized at low levels which can inhibit or promote growth. Hormones are classified in five major recognized groups: auxins, gibberellins, cytokinins, ethylene and abscisic acid (Cleland, 1983; Moore, 2012; Li *et al.*, 2003; Peng *et al.*, 2014). Each hormone can cause a variety of physiological responses in the plant, and they commonly interact. The plant's response is a result of the balance between growth stimulus and inhibitors, such as the interaction between auxin and gibberellin to inhibit the activity of the IAA oxidase enzyme. In general, the phytohormones work in coordination with each other and any decrease or increase in the concentration of one of them will be reflected on the function of the other hormones. The effect of different concentrations of hormones called "physiological concentration". It means measuring the physiological effect of the stimulant or inhibitory hormones which occur effect on the plant cell under different levels and measure this effect by dynamic response. In this case, it can be concluded that the required concentration of cytokinin differs depending on the physiological status of the plant and the conditions around it as well as the presence and concentration of other hormones. In general, the hormone must be present in the correct quantity and in the correct location to avoid negative effects on plants. However, in some cases much higher concentrations of cytokinin could be required for growth, such as the absence of thiamine (Einset, 1977). As a rule, growth regulators affect plant growth and development, influencing physiological and biochemical processes, or even gene regulation. There are a great number of ways in which applications of those compounds could alter the essential oil production (Shukla and Farooqi, 1990). One of these ways is through effects upon plant growth by recruitment of leaf and flower production or a general increase in growth that can result positively in essential oil production. Hormones or growth regulators in plants stimulate growth and terpene biosynthesis in a wide number of aromatic plant species, which result in valuable changes in terpene quantity and quality (Prins *et al.*, 2010).

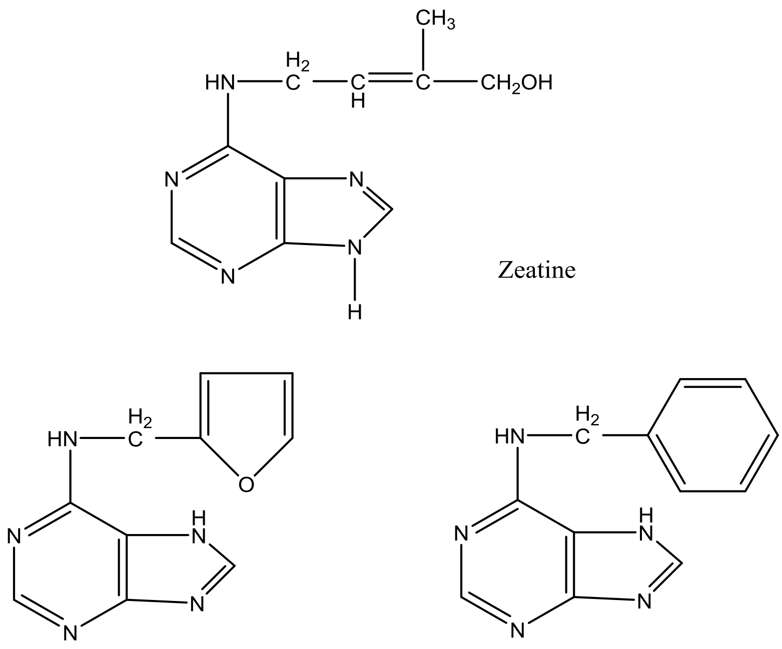
Cytokinin is one of the plant growth substances that stimulates cell division in plant shoots and roots. It is involved primarily in cell growth and differentiation, but it also affects axillary bud growth, apical dominance and leaf senescence. In general, the effects of cytokinin can be summarized in the following points:

1- Cytokinins such as kinetin, trans-zeatin, and benzyl adenine (Fig 3.1) promote cell division and this trait is a basis for proving the existence of cytokinins (Lambers *et al.*, 2008).

2- The presence of cytokinin delays senescence, and stops the dropping of leaves, flowers and fruits and prevents yellowing (Wingler *et al.*, 1998) by inhibition of enzymic activity for all the individual processes of aging (Berg *et al.*, 2002) and also helps to reduce the activity of the ribonuclease enzyme which is responsible for tissue in aging (Dickson *et al.*, 2005).

3- Cytokinins raise the contents of cytoplasmic rRNAs and stimulate endogenous RNA-polymerase-I activity and enhance the synthesis of RNA (Ananiev *et al.*, 1987).

4- Cytokinin play a role in the control of apical dominance through interaction with auxin by encouraging formation of side shoots in the plant in addition to its effects in breaking dormancy (Dun *et al.*, 2006; Saboora, 2009).



Kinetin (6-furfurylaminopurine) Benzyl adenine (6-Benzylaminopurine)

Figure 3. 1 Chemical structure of some types of cytokinin

The effects of cytokinins on essential oil production are very variable; they can cause changes to the yield and content of essential oil (El-keltawi and Croteau, 1987; Fraternale, 2003).

This chapter reports on the effects of components of the seaweed fertilizer other than the basic mineral composition and in particular the likely effect of plant growth substances. This chapter reports the influence on growth, yield and oil composition of rosemary using a seaweed extract fertilizer (organic) and compares this with a model system based on the mineral (inorganic) equivalent content of the seaweed fertilizer with different levels of cytokinin (growth regulator). It tests the following hypotheses:

1) Presence of cytokinin in mineral fertilizer shows no change to growth and oil yield and composition compared with seaweed fertilizer.
2) Presence of cytokinin in mineral fertilizer shows no benefit to growth and oil yield and composition compared with inorganic fertilizer without cytokinin.
3) The age of the plant causes no difference in growth and oil production.

3.2 Material and methods

3.2.1 Site and experimental design

This experiment was conducted inside a greenhouse at the Research Station of the School of the Biological Sciences at the University of Reading. The experiment was laid out in a Complete Randomized Design (CRD) as a factorial experiment. Two groups of plants, aged 6 and 26 months at the beginning of experiment were treated with five different fertilizers; seaweed extract (organic fertilizer), inorganic fertilizer of matching mineral composition but with no organic content (Cy_0), inorganic fertilizer of matching mineral composition and 0.5:1 matching cytokinin concentration in seaweed fertilizer (Cy_1), inorganic fertilizer of matching mineral composition and 1:1 matching cytokinin concentration (Cy_2), and inorganic fertilizer of matching mineral composition and 1.5:1 matching cytokinin concentration (Cy_3) (Table 3.1). Each treatment had seven replications (pots) distributed randomly across experimental units (Fig 3.2) and the plants were sprayed one time every four weeks. The plant ages were 12 months for young plants and 32 months for old plants at the end of the experiment.

Table 3. 1 Concentration of cytokinin in fertilizers treatments

Treatment	trans-zeatin-riboside (Zr) µg/l	isopentenyl adenosine (IPA) µg/l	trans-zeatin (Z) µg/l	isopentenyl adenine (IP) µg/l	total µg/l	rate to seaweed's cytokinin
Cy_0	0	0	0	0	0	0:1
Cy_1	3.5	1	0.35	8	12.85	0.5:1
Cy_2	7	2	0.7	16	25.7	1:1
Cy_3	10.5	3	1.05	24	38.55	1.5:1

All the treatments matching mineral composition of seaweed extract

Cy_0 6	**SW 26**	**Cy_0 26**	**Cy_3 6**	**SW 26**
Cy_1 26	**Cy_3 26**	**Cy_2 26**	**SW 6**	**Cy_1 6**
SW 6	**Cy_3 26**	**Cy_0 6**	**Cy_1 6**	**Cy_2 26**
Cy_3 6	**Cy_2 6**	**Cy_2 26**	**Cy_1 26**	**Cy_2 6**
Cy_0 26	**SW 26**	**Cy_2 6**	**Cy_0 6**	**Cy_3 6**
Cy_3 6	**Cy_1 6**	**Cy_2 6**	**SW 26**	**Cy_0 6**
Cy_1 6	**Cy_3 6**	**Cy_0 26**	**Cy_3 6**	**Cy_1 26**
Cy_3 26	**Cy_1 26**	**Cy_3 26**	**SW 26**	**Cy_3 26**
Cy_0 6	**SW 6**	**SW 6**	**Cy_1 6**	**SW 26**
Cy_2 26	**Cy_2 26**	**Cy_1 26**	**Cy_0 26**	**Cy_1 26**
SW 6	**Cy_0 6**	**Cy_3 26**	**Cy_1 6**	**SW 6**
Cy_0 26	**Cy_3 26**	**Cy_3 6**	**Cy_2 6**	**Cy_2 26**
Cy_2 6	**Cy_1 26**	**Cy_2 26**	**SW 6**	**Cy_0 26**
SW 26	**Cy_0 26**	**Cy_1 6**	**Cy_0 6**	**Cy_2 6**

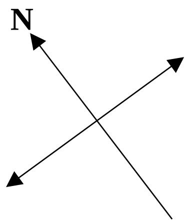


Figure 3. 2 Distribution of the treatments on the plants inside the greenhouse (experiments map)

The experimental set up was the same as that in chapter 2 except for the following changes.

3.2.2 Preparation of cuttings

As there were two groups of plants which differed in age, new cuttings were prepared at the end of September 2014 to be the youngest group of plants (6 month old) in this experiment. Meanwhile, some plants which had been prepared for the first experiment (Chapter 2) were used as an older age group (26 month old). Sprayed fertilizer treatment was started in 5th April 2015 and continued for 6 months at rate of dose (1ml fertilizer/3 l water), every four weeks. Harvest was started at the beginning of October 2015.

Table 3. 2 Average temperatures inside the greenhouse (April - September 2015)

Month	April	May	June	July	August	September
Minimum	16.36	17.83	18.76	19.16	18.12	17.7
Maximum	29.13	30.8	33.43	30.96	30.16	26.1

3.2.3 Irrigation system

The plants in this experiment were watered with equal amounts of water by a drip irrigation system via 2 L/hr pressure-compensation button drippers connected to an automatic water controller. Plants were watered twice weekly by a programmed dripping system with about 200/ml of water for each pot.

3.2.4 Preparation of fertilizer

Analysed seaweed extract was chosen to confirm its chemical components and a chemical copy of the fertilizer was compounded from inorganic components to allow comparison of the nutrient provision of the fertilizer independent of the organic seaweed source.

3.2.4.1 Organic fertilizer

'Seasol' is a product of Seasol International Pty Ltd. (Australia) which is used as an organic fertilizer in this experiment. This product is obtained from a seaweed source in a liquid form extracted from unique species of King Island (southern Australia) Bull Kelp *Durvillaea potatorum* that includes naturally occurring growth regulators, trace elements, carbohydrates, alginates and vitamins. This product was used in the experiment as a source of organic fertilizer due to the presence of many macro- and micro-elements (Table 3.3) as well as the presence of identified cytokinins: trans-zeatin-riboside (Zr) (7.0 µg/l), isopentenyl adenosine (IPA) (2.0 µg/l), trans-zeatin (Z) (0.7 µg/l), and isopentenyl adenine (IP) (16.0 µg/l). The rate of seaweed fertilizer was 1:500 (1 ml fertilizer/500 ml water).

Table 3. 3 Nutrient contents of seaweed extract (% w/w)

Nitrate Nitrogen N	Phosphorus P_2O_5	Potassium K_2O	Magnesium Mg	Calcium Ca	Iron Fe	Copper Cu	Sulphur S	Iodine I	Sodium Na	Chloride Cl	Boron B
0.22%	0.58%	4.3%	0.04%	0.098%	0.03%	0.000064%	0.2%	0.012%	0.9%	0.33	0.0013

Besides the elements listed in table 3.1, Seasol also contains trace amounts of cobalt, fluoride, magnesium, zinc, nickel, and molybdenum as well.

3.2.4.2 Inorganic fertilizer

The Inorganic fertilizer in this experiment was prepared using the process described in chapter one but taking into account the difference in the concentration of elements, which produced the inorganic fertilizer required in a final form which is similar to the organic fertilizer and was used as a parallel treatment. Stock solutions were made through the addition of macro- (N, P, K, *etc.*), and micro- (Fe, Cu, Mg *etc.*) elements and the four common types of cytokinin (Zr, IPA, Z and IP) in the required proportions (Table 3.4) in separate flasks with distilled water. The final solution was formed by adding the stock solutions to a known amount of water to produce the required volume in the desired concentrations (the ratio of final dilution was 1:500 v/v). To avoid precipitation, the materials present in the highest concentration were added first. The final solution was stored in a refrigerator, and there was no observable solid material.

3.2.5 Oil extraction (Hydrodistillation)

In this experiment, aerial parts comprising leaves and twigs with young and fresh branches were obtained from each experimental treatment of cultivated plants at harvest, 50 g fresh weight (Rahman *et al.*, 2007; Jamshidi *et al.*, 2009; Szumny *et al.*, 2010) was chopped into small pieces by a blender in order to expose a large number of oil glands and increase the percentage of oil collected. This was then placed in a still with water and extracted in a Clevenger apparatus (Guenther, 1950).

The chopped material was completely immersed in water, which was boiled on an electric heater. After the hydrodistillation process was complete, the essential oil collected through the graduated distillate receiving tube in the low end of Dean stark trap. After cooling, the essential oil was dried using anhydrous magnesium sulphate.

All the essential oil samples were stored in dark glass vials with Teflon sealed caps at -18 °C in darkness.

Table 3. 4 Composition (%) of nutrients solutions for inorganic fertilizer

Chemical compound	Molecular weight	Contents %	Weight (gm) used	Amount of the ions supplied from these weights of chemicals compounds														
				Nitrogen (N)	Phosphorus (P)	Potassium (K)	Sulphur (S)	Magnesium (Mg)	Sodium (Na)	Iron (Fe)	Iodine (I)	Copper (Cu)	Calcium (Ca)	Chloride (Cl)	Cobalt (Co)	Zink (Zn)	Boron (B)	Other
K_2HPO_4	174.2	44.88 K, 17.77 P, 0.57 H, and 36.73 O	3.26		0.58	1.46												
K_2SO_4	174.25	44.87 K, 18.39 S and 36.726 O	0.18			0.08	0.033											
KNO_3	101.10	38.67 K, 13.85 N and 47.47 O	1.58	0.22		0.614												
K_2CO_3	138.20	56.58 K, 8.69 C and 34.73 O	3.64			2.06												0.264 (C)
KI	165.99	23.55K and 76.45 I	0.157			0.037					0.12							
KF	58.09	67.29 K and 32.70 F	0.073			0.049												0.0239 (F)
$FeSO_4.7H_2O$	278.00	20.08 Fe, 11.53 S, 63.26 O and 5.07 H	1.49				0.17			0.3								
NaOH	39.99	57.47 Na, 40.00 O and 2.52 H	1.566						0.9									
$CuSO_4.5H_2O$	249.67	25.46 Cu, 12.84 S, 57.67 O and 4.03 H	0.00025				0.00003					0.000064						
$ZnCl_2$	136.28	47.97 Zn and 52.02 Cl	0.066											0.034		0.031		
$CaCl_2.6H_2O$	219.07	18.29 Ca, 32.36 Cl, 43.81O and 5.52 H	0.535										0.098	0.173				
$CoCl_2.6H_2O$	273.93	24.76 CO, 29.8 Cl, 5.08 H and 40.34 O	0.00157											0.00046	0.00039			
$MgCl_2$	95.21	25.52 Mg and 74.47 Cl_2	0.156					0.04						0.116				
H_3BO_3	61.83	17,48 B, 4.85 H and 77.63 O	0.0074														0.013	
$MnCl_2.4H_2O$	197.9	27.75 Mn, 35.82 Cl, 32.33O and 4.07 H	0.019											0.0068				0.0054 (Mn)
MoO_3	143.9	66.65 Mo and 33.34 O	0.00448															0.00299 (Mo)
$NiCl_2.6H_2O$	237.69	24.69 Ni, 29.83 Cl, 5.08 H and 40.38 O	0.008											0.0024				0.00199 (Ni)
Total			**12.4797**	**0.22**	**0.58**	**4.3**	**0.20**	**0.04**	**0.90**	**0.30**	**0.12**	**0.00064**	**0.098**	**0.33**	**0.00039**	**0.031**	**0.013**	Cytokinin: (Zr) 7.0, (Z) 0.7, (IPA) 2.0, and (IP) 16.0 µg per litre
Target totals% (the amounts in the seaweed fertilizer)				**0.22**	**0.58**	**4.3**	**0.20**	**0.04**	**0.90**	**0.30**	**0.12**	**0.00064**	**0.098**	**0.33**	**0.003**	**0.031**	**0.013**	

3.2.6 Measurements

The effect of fertilizers and age of plants on the growth of the *Rosmarinus officinalis* L. plants were recorded in an experiment to establish if there was any simple relationship with oil production. Plant growth and production measurements (Oil amount, identification of oil components by GC-MS and NMR and vegetative characters) were taken as described in Chapters 2, with the addition of a record of the number of essential oil glands on the surface of leaves. These measurements were taken using thermal printer paper that was placed on a tile or glass plate (hard and smooth surface), then a fresh leaf placed on it and pressed firmly using solid cylinder with a single rolling action. The expressed oils react immediately with the paper in an oxidative reaction to produce a distinct image of grey dots (Fig 3.3). Then, the number of grey dots was counted using a LEICA S6D microscope to take photographs through a stage micrometre (0.01mm scale: Graticules Pyser SGI Ltd) using transmitted light, then an enlarged copy printed to facilitate counting (Clery and Ross, 1992).

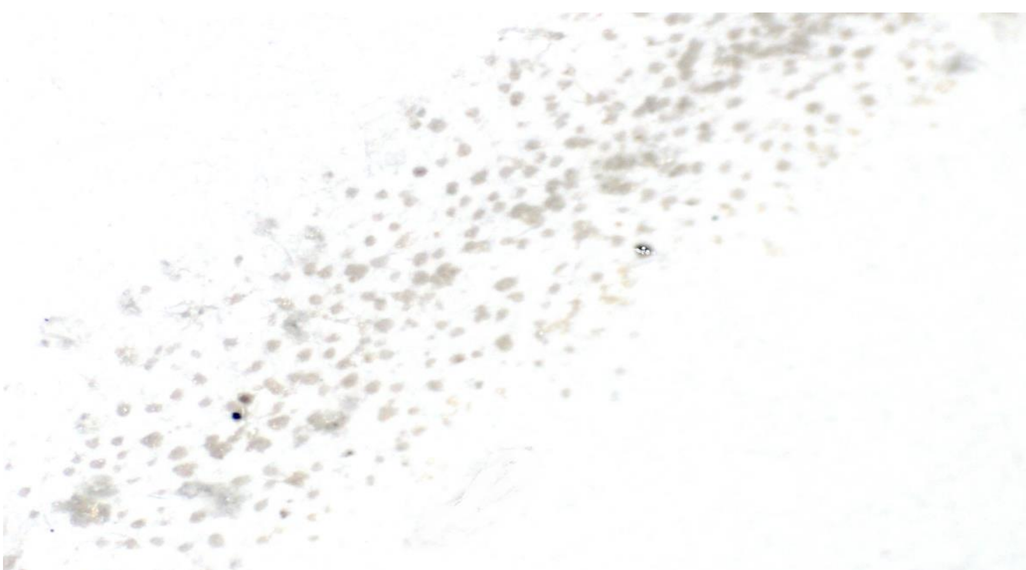


Figure 3. 3 Image for grey dots on thermal printer paper which represent oil glands on the upper surface of a rosemary leaf

3.2.7 Statistical analysis:

For statistical analysis; percentages were normalized by arcsine transformation as needed. In order to investigate the interrelationships between essential oil quantity, quality and crop growth parameters, the independent effects of each combination of variables was evaluated with ANOVA (Two-way statistical analysis) by using *Genstat* software (Payne *et al.*, 2009), considering each experimental condition as the "group variable". The analysis compared the effects of organic, inorganic, and control (no fertilizer) treatments and between sprayed and watered methods of application of the treatments in three different dates of harvest. The least significant difference (LSD) was used to account for variation between these factors.

The percentage composition of the essential oils was used to determine the relationship between the different treatments samples by principal components analysis (PCA) using the *Genstat* software. PCA was employed based on GC-MS data to provide an overview of the capacity to distinguish essential oil components, or in other words to detect the distribution pattern of samples and to identify which chemical constituents can distinguish between these groups of individuals.

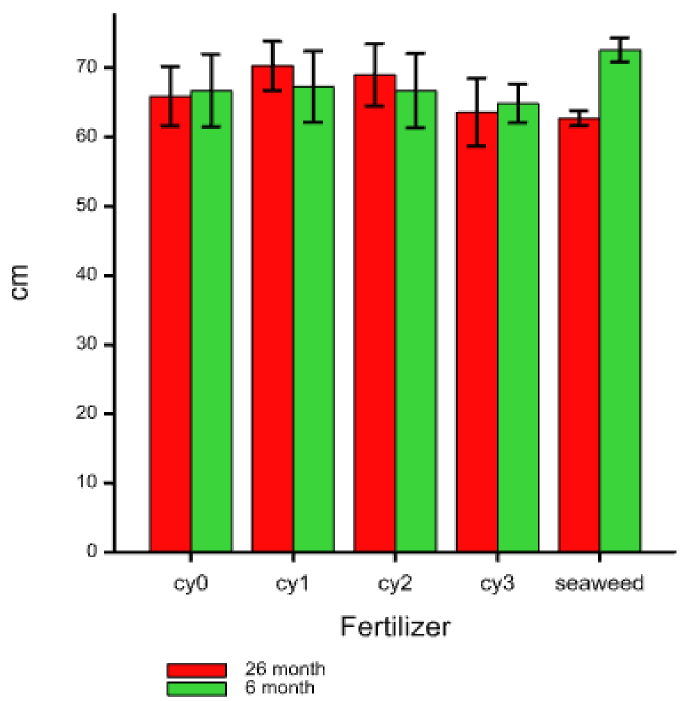
3.3 Results

3.3.1. Plant growth

3.3.1.1. Plant height

The presence of cytokinin in the fertilizer did not cause any significant increase in terms of the height of plants compared with Cy_0. The three different concentrations of cytokinin: Cy_1, Cy_2, Cy_3, and seaweed treatments did not differ significantly in the two groups of plant ages.

The comparison between ages of plants was conducted for Cy_0, Cy_1, Cy_2 and Cy_3 treatments, but did not show any significant difference on height of the plants. On the other hand, young plants treated with seaweed fertilizer showed significantly higher plant height (72.6 cm) compared with old plants (62.7 cm) which received the same fertilizer. The interaction between fertilizers and ages of the plants did not show a significant difference among the plants (Fig 3.4).



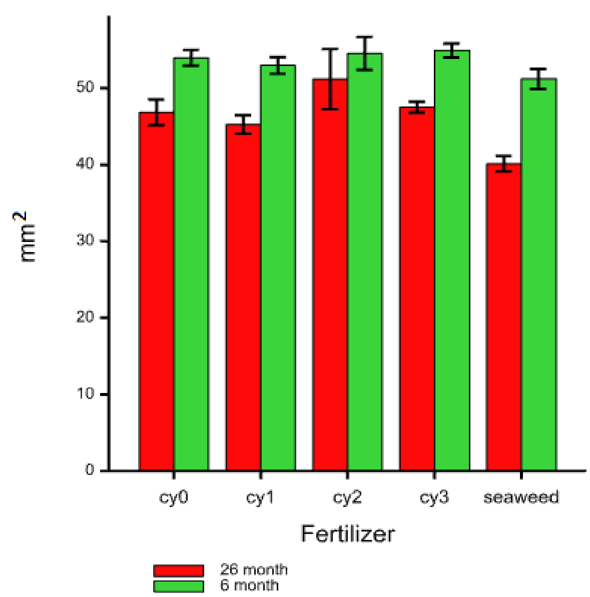
L.S.D $_{0.05}$ = 11.65

Figure 3. 4 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on height (cm) of two different age groups of rosemary plants

3.3.1.2. Leaf area

There was a significant difference between the effect of seaweed fertilizer and Cy_1, Cy_2 and Cy_3 on leaf area in old plants. Leaf area measurements show that the plants received seaweed fertilizer had the lowest area (40.1 mm^2) compared with Cy_0, Cy_1, Cy_2 and Cy_3 which were 46.7, 45.2, 51.2 and 47.5 respectively. There was no significant difference among the young plants. The presence of cytokinin at the 3 different levels used did not cause a significant change in leaf area for either age group compared with Cy_0.

There was variation in leaf area between young and old plants which were treated with different fertilizers. Significantly, the higher leaf area was found in young plants treated with Cy_0 (53.96 mm^2), Cy_1 (52.96 mm^2), Cy_3 (54.91 mm^2) and seaweed (51.20 mm^2) as compared with old plants, which were 46.80, 45.25, 47.51 and 40.13 mm^2 for Cy_0, $Cy_{1,}$ Cy_3 and seaweed respectively. There was no significant difference in leaf area under the Cy_2 treatment, although there was an increase in leaf area (54.5 mm^2) in young plants compared with older group (51.2mm^2). The interaction between fertilizers and dates of harvest shows a significant difference among the plants in leaf area (Fig 3.5).



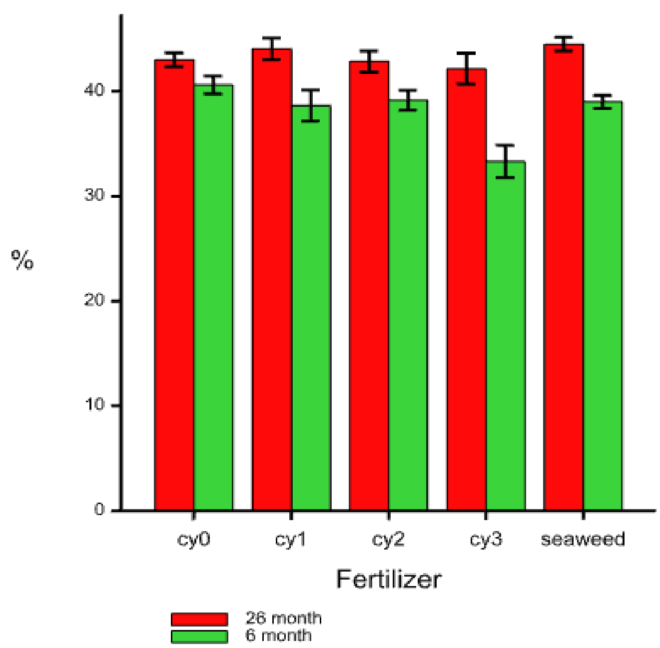
L.S.D $_{0.05}$ = 4.95

Figure 3. 5 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on leaf area (mm^2) of two different age groups of rosemary

3.3.1.3 Percentage of dry material in leaves

The old group of plants showed no significant response to any of the treatments. However, young plants given the highest cytokinin treatment (Cy_3) had a significantly lower percentage of dry material in leaves as compared with other treatments.

There was a significant difference in percentage of dry material in leaves between young and old plants with all treatments. The older group of plants had a consistently higher percentage dry weight as compared with the younger group with equivalent treatments Cy_0, Cy_1, Cy_2, Cy_3 and seaweed. The interaction between fertilizers and dates of harvest shows a significant difference among the plants (Fig 3.6).



L.S.D $_{0.05}$ = 3.05

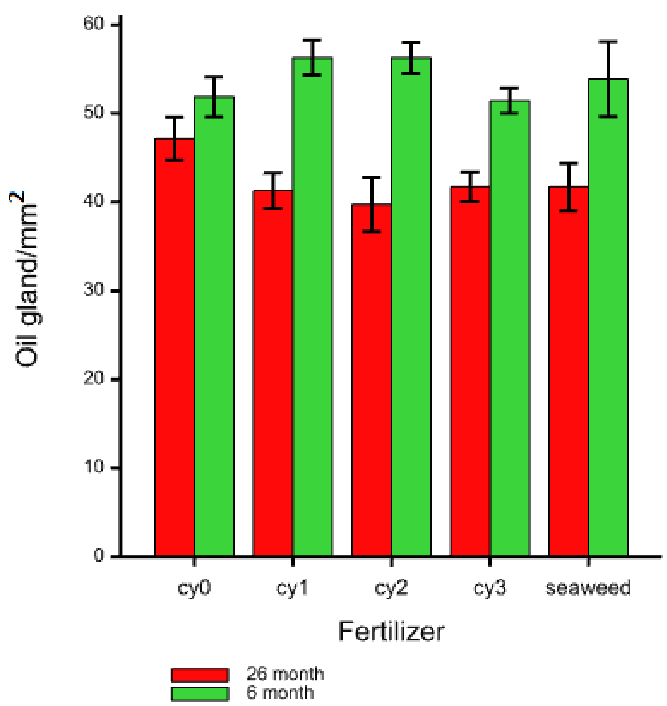
Figure 3. 6 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on percentage of dry material in leaves of two different age groups of rosemary plants

3.3.1.4 Density of oil glands

In terms of number of oil glands per square millimetre of fresh leaf, there were no significant differences between treatments in either age group.

Younger plants had a significantly higher density of oil glands than the older plants for the Cy_1, Cy_2, Cy_3, and seaweed treatments. However, Cy_0 shows no significant difference and seaweed shows only a low level of significance between ages.

The interaction between fertilizers, methods of application and dates of harvest shows a significant difference among the plants (Fig 3.7).



L.S.D $_{0.05}$ = 6.98

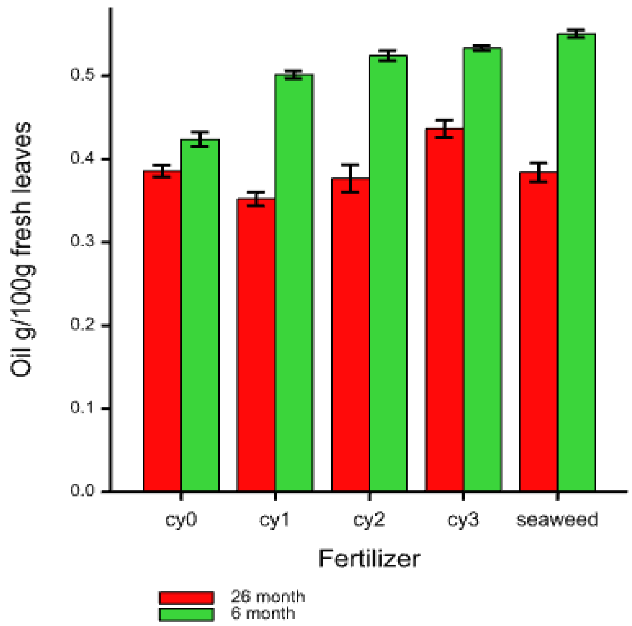
Figure 3. 7 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on density of oil glands in leaf (mm^2) of two different age groups of rosemary plants

3.3.2 Essential oil yield

The quantity of essential oil extracted per 100 g fresh weight from rosemary plants differs significantly in all treatments. The young plants that received seaweed fertilizer were characterized by a higher yield of oil (0.550 g/100 g fresh leaves) than those with Cy_0, Cy_1, Cy_2 and Cy_3 treatments (0.423, 0.501, 0.524 and 0.533 g/100 g fresh leaves respectively). Cy_0 differed significantly by lowest yield oil compared with all other treatments. Only Cy_2 and Cy_3 were not significantly different from one another. On the other hand, the older group showed a significantly higher yield of oil (0.436 g/100 g fresh leaves) for Cy_3 than Cy_0, Cy_1, Cy_3 and seaweed treatments (0.385, 0.352, 0.376 and 0.3840 g/100 g fresh leaves respectively).

Fig. 3. 8, illustrates the very highly significant differences between young and old plants in oil yield for all the treatments. The highest values of oil yield were obtained from young plants (0.423, 0.501, 0.524, 0.533 and 0.550 g/100 g plant leaves) compared with older group (0.385, 0.352, 0.376, 0.436, and 0.384 g/100 g fresh leaves) for Cy_0, Cy_1, Cy_2, Cy_3, and seaweed respectively.

The interaction between fertilizers and dates of harvest shows a significant difference among the plants in oil yield (Fig 3.8).



L.S.D $_{0.05}$ = 0.025

Figure 3. 8 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on yield amount (g/100g plant material) of two different age groups of rosemary plants

3.3.3 Oil composition

The results of analysis of variance performed on the essential oil composition data are shown in Tables 3.4, 3.5, 3.6 and 3.7. These tables show the percentage of the nine major constituents of rosemary oil averaged for GC-MS and ^{1}H NMR analyses for each of the two ages and the five different fertilizers (Cy_0, Cy_1, Cy_2, Cy_3 and seaweed) and the interaction between ages and fertilizers. As well, the GC-MS data was subjected to Principle Component analysis (PCA) to explore the relationship between the samples from different treatments and their chemical constituents' relation to specific volatile compounds.

3.3.3.1 GC-MS results

The young and old plants showed significant difference in oil content and composition. For the young plants: Cy_0 treatment showed significantly the highest levels of α-pinene (6.31%), camphene (4.44%) and β-pinene (2.17%); Cy_2 treatment showed the highest level of borneol (2.52%); Cy_3 treatment showed the highest levels of eucalyptol (29.02%) and camphor

(24.32%); and seaweed fertilizer showed the lowest percentages of α-pinene (4.37%), camphene (3.29%), myrcene (9.72%), *p*-cymene (1.45%) and eucalyptol (22.14%) compared with other treatments.

In old plants, Cy_0 and Cy_2 did not characterized significantly by higher or lowest level of any compound. However, Cy_1 which was characterized by highest levels of myrcene (14.49%) and *p*-cymene (2.38%), also characterized by lowest levels of both camphor (13.01%) and borneol (1.64%). The β-pinene (1.66%) level decreased significantly with Cy_3 treatment, while seaweed had the lowest level of eucalyptol (22.47%).

In general, type of fertilizer had a significant effect on α-pinene, camphene, β-pinene, myrcene, *p*-cymene and eucalyptol. Age of the plant shows significant effects on β-pinene, *p*-cymene, camphor and borneol. The interaction between fertilizers and age of the plants did not show any significant difference among the plants with all nine compounds. Linalool did not show any significant responc for all treatments (Table 3.5).

The results obtained from PCA described 95.02% for young plants (Fig 3.9 A) and 97.99% for old plants (Fig 3.9 B), the PC-1 accounts for young plants was 63.53% and 61.68% for old plants of total variance with significant amounts of eucalyptol and camphor, whereas PC-2 accounts for young plants was 31.48% and 36.31% for old plants of total variance distinguishes with significant amounts of camphor and eucalyptol for both methods of application respectively.

Table 3. 5 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on oil composition of two different age groups of rosemary plants analysed by GC-MS

Age	Treatments	α-Pinene %	Camphene %	β-Pinene %	Myrcene %	p-Cymene %	Eucalyptol %	Linalool %	Camphor %	Borneol %	Total %
Young plants	**Cy_0**	6.31	4.44	2.17	13.09	1.83	25.52	2.44	22.47	2.35	**80.65**
	Cy_1	5.71	4.42	2.06	12.46	1.90	26.13	2.20	23.52	2.45	**80.88**
	Cy_2	6.07	4.31	2.01	14.09	2.31	28.99	2.33	22.89	2.52	**85.57**
	Cy_3	5.31	3.95	1.87	11.63	1.97	29.02	2.41	24.32	2.47	**83.00**
	Seaweed	4.37	3.29	1.83	9.72	1.45	22.14	2.21	22.03	2.57	**69.66**
Old plants	**Cy_0**	5.74	3.89	1.84	13.32	2.06	26.44	2.38	20.11	2.21	**78.01**
	Cy_1	5.84	3.87	1.88	14.49	2.38	25.60	2.17	13.01	1.64	**70.91**
	Cy_2	5.88	4.17	1.96	13.93	2.34	27.44	2.28	17.37	2.08	**77.50**
	Cy_3	6.10	4.00	1.66	12.04	2.18	25.89	2.19	19.24	1.97	**75.31**
	Seaweed	4.88	3.30	1.68	11.16	1.62	22.47	2.24	19.47	2.22	**69.04**
L.S.D		**0.960**	**0.615**	**0.278**	**2.183**	**0.311**	**3.290**	**0.296**	**4.268**	**0.365**	***d.f*** **69**

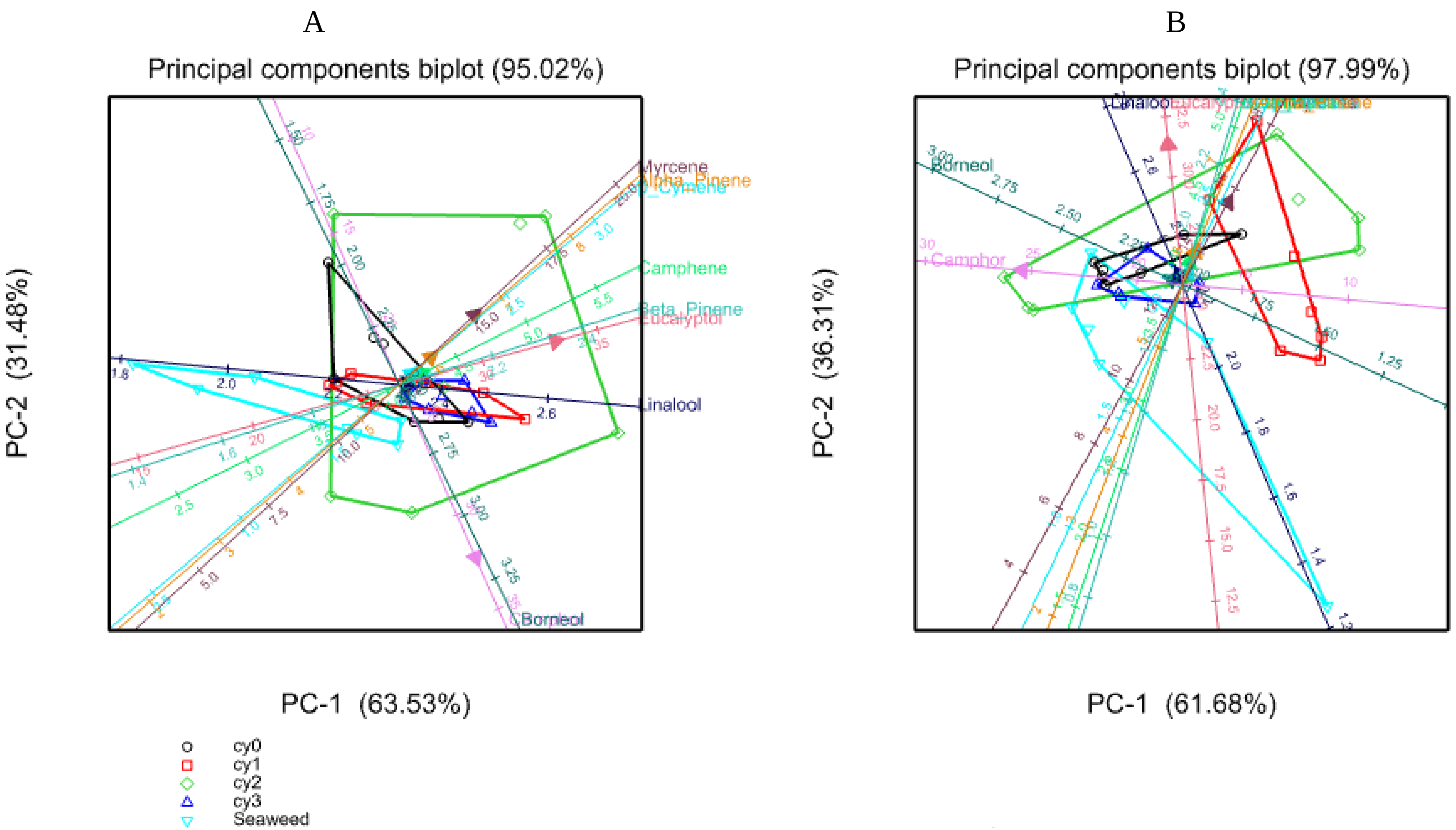


Figure 3. 9 Principle Components Analysis biplot distinguishing the effect of using seaweed, inorganic fertilizers and presence of cytokinin in fertilizer with two different plants' age into chemotypes using nine main volatile constituents.

3.3.3.2 ^{1}H NMR results

The Cy_0, Cy_1, Cy_2, Cy_3 and seaweed fertilizer treatments showed significant differences in percentages of oil contents when analysed using ^{1}H NMR. The Cy_0 treatment caused significant increase in percentages of camphene (5.27%), β-pinene (2.70%), camphor (24.14%) and borneol (2.70%) in young plants. While, Cy_0 treatment in old plants caused significant increase in percentages of α-pinene (8.853%), myrcene (3.43%), *p*-cymene (2.43%) and eucalyptol (25.78%). The Cy_1 treatment decreased significantly the concentrations of myrcene (8.96%), eucalyptol (19.34%), linalool (1.82%) with young plants, and borneol (1.73%) with old plants.

Cy_2 did not caused any significant effect for all compounds with both groups of plants' age compared with other treatments.

Lower levels of α-pinene (5.93%) and camphene (3.51%) with young plants, β-pinene (1.59%), and camphor (17.93%) with old plants were found under Cy_3 treatment; *p*-cymene (1.46%) under seaweed treatment with old plants. In old plants, seaweed fertilizer had significantly positive effect by increase the level of linalool (2.68%).

Based on statistical analysis, type of fertilizer had a significant effect on all the nine compounds. Age of the plant shows significant effects on α-pinene, myrcene, *p*-cymene and borneol. The interaction between fertilizers and age of the plants shows significant differences among the plants with all treatments (Table 3.6).

Table 3. 6 Effect of using seaweed, inorganic fertilizer and presence of cytokinin in fertilizer on oil composition of two different age groups of rosemary plants analysed by NMR

Age	Treatments	α-Pinene %	Camphene %	β-Pinene %	Myrcene %	p-Cymene %	Eucalyptol %	Linalool %	Camphor %	Borneol %	Total %
Young plants	**Cy_0**	8.82	5.27	2.70	12.37	1.78	24.60	2.55	24.14	2.70	**84.93**
	Cy_1	6.06	3.81	1.96	8.96	1.50	19.34	1.82	18.74	2.18	**64.37**
	Cy_2	6.78	3.93	1.90	10.99	2.12	21.54	2.33	19.27	2.57	**71.43**
	Cy_3	5.93	3.51	1.74	8.75	1.67	22.64	2.36	20.05	2.52	**69.17**
	Seaweed	6.15	3.59	1.98	9.35	1.46	19.88	2.06	19.91	2.24	**66.62**
Old plants	**Cy_0**	8.85	4.77	2.45	13.43	2.43	25.78	2.46	23.16	2.46	**85.79**
	Cy_1	7.20	3.86	2.00	11.98	2.25	20.46	2.05	18.40	1.73	**69.93**
	Cy_2	7.06	4.27	2.17	10.81	2.15	22.54	2.32	20.52	2.54	**74.38**
	Cy_3	6.91	3.71	1.59	9.33	1.90	19.36	2.12	17.93	2.01	**82.94**
	Seaweed	8.17	4.40	2.30	12.30	2.00	24.52	2.68	24.05	2.52	**82.94**
L.S.D		**0.733**	**0.425**	**0.239**	**1.286**	**0.228**	**1.240**	**0.272**	**0.694**	**0.385**	***d.f*** **69**

3.4 Discussion

It is known that plant growth and development are regulated by the action and balance of different groups of hormones, which promote or inhibit such processes. The application of growth regulators can influence both herbage and essential oil content as well as seed germination, nutrient mobilization, leaf senescence, the activity and formation of shoot meristems, apical dominance, and pathogen responses. Nevertheless, the effect of exogenous application of cytokinin is like that of an endogenous one in growth and development of plants (Reitz and Trumble, 1996).

Organic and the inorganic copy of the fertilizers were used to allow comparison of the nutrient provision of the fertilizer independent of the organic seaweed source. This analysis of the components and their application separately in the form of organic and inorganic fertilizer allowed the determination of the extent to which other micronutrients and plant growth substances contained in organic fertilizers influence growth. It has been confirmed that effect of growth regulators content in seaweed extract which is used as a fertilizer are similar to the action of cytokinin and auxin. They found an accumulation of cytokinin in some active parts and tissues depending on the age of that part of the plant (Stirk and Van Staden, 1996).

Based on results in this chapter, rosemary plants were demonstrated to be highly responsive to different organic and inorganic fertilizers with different level of cytokinin in terms of vegetative growth. An increase in leaf area occurred for old plants treated with exogenous cytokinin as an inorganic form. Moreover, there was a decrease in percentages of dry material in leaves treated with higher level of cytokinin comparing with all other treatments in younger group of plants. While, the effect of age was very clear with all the fertilizers through the superiority of younger plants by larger leaf area with lower dry mass. Hormones play an important role in vegetative growth of plants through their effect on processes of cell division and elongation, enhancing buds and new branches to grow and develop at the beginning of the growth phase to be effective in the production of twigs and leaves later. This may be achieved through stimulating plant branches by using industrial growth regulators. These manufactured hormones are taking part by increase the availability of photosynthesis products by greater amounts than those produced naturally by the plant without any external influence (Ruiliang, 1999). Lee (1971), Reitz and Trumble (1996) concluded that higher concentration of cytokinin has the ability to reduce indoleacetic acid (IAA) oxidase, particularly the anionic isoenzymes A5 and A6, promote cell division and delay senescence in addition to recovering leaves from herbivory or artificial damage. In addition, Lee (1971) found that a higher concentration of cytokinin (2 Mm cytokinin) decreased the total activity of IAA oxidase per callus two to three times more than with 0.2 Mm cytokinin. Stirk *et al.* (2011) suggested that this increase in cell number is a direct result of the increased concentration of exogenous cytokinin, in other words, there is a relationship between cell division and cytokinin concentrations. In addition, this affects carbohydrate and nitrogen metabolism and leads to increased pigment content (chlorophyll and carotenoids) (Piotrowska and Czerpak, 2009). This effect can be due to the production of an enlarged primordium as a result of the high concentration of cytokinin during the initiation at the meristem, or to a progressive increase in size resulting from a faster and a prolonged growth period. Final leaf size is the result of the action of two processes, cell division and cell expansion (Al Masoody and Stanica, 2015; Gonzalez *et al.*, 2010). Furthermore, Ghafour *et al.* (1999) stated that, contrary to most plant species that requires higher auxin levels than cytokinin, rosemary required for callus induction in light a higher cytokinin/auxin ration, while a balanced ratio was required for callus induction in dark and during maintenance. In the same way, the addition of cytokinin to the leaves, stems and buds shows little transition or it does not move from the site of adding. For example, if a small spot on a leaf is treated with cytokinin, that spot will remain green after the surrounding tissues on the same leaf begin to senesce, that is a phenomenon key for the so-called impact of the transition of cytokinin. The result of the addition of cytokinin to the leaf or a part of the leaf is to retards aging in this specific part and lead to the attraction of materials and ions from the other parts in the plant (Molnár and Ördög, 2011).

On the other hand, some of the traits were not affected significantly with different fertilizers. Plant height and number of oil glands in leaves were not affected by using different fertilizers. This agreed with Farooqi and Sharma (1988), and Baskaran and Jayabalan (2008) who stated that a higher leaf production associated with a decrease in plant size was the cause of the rise in essential oil yield in plants that received cytokinins and naphthalene acetic acid (NAA).

The result about oil glands in this chapter did not agree with Fraternale *et al.* (2003) which found cytokinin application at a concentration of 0.1 mg benzyladenine (BA) per litre in the medium culture of *Thimus mastichina* caused higher yield of essential oil and larger density of glandular hair in post secretory stage. They attributed this change to the influence of cytokinin in formation and development of essential oil biosynthesis and storage structures. This disagreement could be due to the genetic effects on distribution of oil glands on leaves (this has been confirmed in Chapter 4) as well as the genetic variation between *Rosmarinus officinalis* and *Thimus mastichina*. However, according to El-keltawi and Croteau (1987), the primary effect of cytokinins was a stimulus of monoterpene accumulation. The kinetin and diphenylurea (cytokines types) effects were higher than that attributed to the effects related to growth and developmental changes, or on gland formation and density, thus an effect on metabolism was suggested.

Application of seaweed and Cy_3 had a marked effect on the yield of essential oil of young and old plants that were examined respectively. This indicated that presence of cytokinin is biologically active and modifies the development of mature and immature tissues. It could be proposed that the primary influence of cytokinins is to stimulate the general increase of monoterpenes typical for the essential oil. It is known that the higher cytokinin content correlated with an increased number of proliferated buds and stimulating plant branches which taking part in availability of products of photosynthesis. In other words, cytokinin application stimulated essential oil accumulation, at least due to the direct effect on metabolism of monoterpenes (El-keltawi and Croteau, 1987; Ruiliang, 1999; Ghafour *et al.*, 1999). Povh and Ono (2007) detected higher essential oil content in sage *Salvia officinalis* treated by growth regulators compared to control plants (no growth regulators) as a result of an increase in leaf number. Furthermore, this simulative effect may be related to the good balance of nutrients and water in the root medium (Abdelaziz *et al.*, 2007). These increases might be related to the positive effect of compost and microorganisms in increasing the root surface area per unit of soil volume, water-use efficiency and photosynthetic activity, which directly affects the physiological processes and utilization of carbohydrates (Hammoda, 2001; El-Ghadban *et al.*, 2002).

There were a few significant differences between different treatments in terms of essential oil composition. On the other hand, the plants treated with seaweed had lower levels of all chemicals compared to plants treated by inorganic fertilizer with or without cytokinin. Likewise, these results agree with El-keltawi and Croteau (1987) who applied different concentrations (from 1 to 10 ppm) of cytokinin sources (diphenylurea, kinetin, benzylaminopurine, and zeatin) on species of Lamiaceae (*Mentha, suaveolens, M. spicata, M. piperita* and *Salvia officinalis*), and found that cytokinins did not significantly change essential oil composition of the studied species. There was an increase in the absolute levels of chemicals, nevertheless some compounds were reduced.

3.5 Conclusion

The application of cytokinin promotes vegetative growth in young and old rosemary plants increasing overall total leaf area and decreases the percentage weight of dry material. In this study, there was no significant change in plant height and the density of oil glands, however the total oil production increased. Growth of young plants increased with increase in artificial cytokinin concentration, however, the greatest growth was with seaweed fertilizer treatment. In old plants a different result was seen, with the greatest growth caused by the highest artificial cytokinin treatment while seaweed treatment gave similar results to lower cytokinin treatment and the control. Overall the results indicate that in young plants the effect of seaweed fertilizer may be in part due to natural cytokinin content, in addition to the mineral nutrients such fertilizers contain.

Generally, the majority of the principal oil constituents decreased with seaweed and increased in artificial cytokinin concentration in young plants. While in old plants the application of cytokinin raises the levels of most of these compounds.

The comparison between young and old groups of plants within all treatments shows clearly that the younger group was more active and differs significantly with older group in all of the plants growth and production characteristics. There was no significant change in oil composition, however, there were some significant changes with high level of cytokinin.

4. Chapter Four: Responses of Rosemary cultivars to fertilizer

4.1 Introduction

Rosemary plants have a wide range of variation in morphological and essential oil properties. The essential oil composition of wild and cultivated rosemary is well documented by many researchers who have been investigating what influences its production (Arnold *et al.*, 1997; Angioni *et al.*, 2004; Rahman *et al.*, 2007; Jamshidi *et al.*, 2009; Zaouali *et al.*, 2012; Li *et al.*, 2016). Variation in oil production has been attributed mostly to extrinsic factors such as geographical location, kind of soil and climate conditions. Therefore, the chemotype of the essential oils is classified depending on the region where the plant is cultivated (Li *et al.*, 2016).

The effect of intrinsic factors has mostly been attributed to difference and aging. Furthermore, the genetic variants produce the same quantity and quality of oil if grown together. This result exemplifies the concept that environmental and spatial factors are stronger than the genetic variation factor in its effect on plant production, both qualitative and quantitative (Viuda-Martos *et al.*, 2007)

Morphological features and molecular markers, both chemical and biochemical are very important to estimate the genetic diversity and genetic differentiation among rosemary plant populations (Hidalgo *et al.*, 1998; Zaouali *et al.*, 2012; Mateu-Andrés *et al.*, 2013). There are hundreds of *Rosmarinus officinalis* cultivars which are used for different purposes in several sectors, such as fragrances industry, medicines and drugs and as food additives depending on oil composition. These cultivars vary in many attributes such as the height of the plant, shoots size and habit, flower colour, leaf shape, leaf area, and the smell of the oil (Cervelli and Masselli, 2011) (Table 4.1).

Table 4. 1 The morphological difference between some rosemary cultivars

'Fota Blue'	40cm tall, frost hardy, evergreen perennial, striking small dark blue flowers in spring, short dark green needle-shaped aromatic leaves, arching prostrate habit
'Roseus'	80 cm tall, fully hardy evergreen perennial, small pale pink flowers in spring and summer, short dark green needle-shaped aromatic leaves, upright habit
'Haifa'	10 cm tall, frost hardy, evergreen perennial, small pale blue flowers in spring and summer, short green needle-shaped aromatic leaves, very prostrate habit virtually flat
'Tuscan Blue'	100 cm tall, frost hardy, evergreen perennial, small pale blue flowers in spring and summer, short, thick, dark green, needle-shaped, aromatic leaves, upright habit
'Primley Blue'	80 cm tall, fully hardy evergreen perennial, small blue flowers in spring and summer, short dark green needle-shaped aromatic leaves, bushy upright habit
'Benenden Blue'	80 cm tall, fully hardy evergreen perennial, small dark blue flowers in spring and summer, short fine dark green needle-shaped aromatic leaves, upright habit
'Aureus'	80 cm tall, fully hardy evergreen perennial, small pale blue flowers in spring, short dark green variegated with gold splashes, needle-shaped aromatic leaves, upright habit
'Green Ginger'	60 cm tall, frost hardy, evergreen perennial, small pale blue flowers in spring, short dark green, ginger scented, needle-shaped aromatic leaves, upright habit

https://www.jekkasherbfarm.com/plants?s=r

Wild rosemary has been classified into groups according to genotype and geographical location of the plants and the chemotype of their oil (Tucker and Maciarello, 1986). It has been stated that the spatial variability in chemotypes of essential oils may arise from the effects of genetic variability among the plants (Tigrine-Kordjani *et al.*, 2007).

It is believed that just different species can present variable responses to hormones application, the respond could be in different ways according to plant's development stage and number of hormone application (Prins *et al.*, 2010). However, fertilizer application has an important effect on the quality and quantity of oil. It causes different effects on the oil composition of the different cultivars, particularly on the percentage occurrence of alcohols and oxygenated compounds (Martinetti *et al.*, 2006). For example, N and K always reduced camphor content in 'Majorka pink' and enhanced it in 'Montfort form'. Also, in both the cultivars, N enhanced linalool, camphene and myrcene, P enhanced limonene, while K enhanced *p*-cymene and reduced eucalyptol. On other hand, the essential oil production was not affected by fertilizers.

The aim of this experiment was to understand the distribution of genetic variation between and within populations in a crop. Also, it tested the response of different genotype of rosemary plants to different types of fertilizers. It tests the following hypotheses:

1) Oil quantity and quality do not vary among different cultivars grown under the same conditions.

2) Seaweed fertilizer has the same impact on growth and oil production in all cultivars.

4.2 Materials and methods

Nine different named cultivars of rosemary were supplied by Gwynfor Growers nursery 'The Rosemary Specialist'; which has the largest collection of rosemary cultivars in the UK. Each cultivar is genetically different from the others but has been clonally propagated so as to be consistent within the cultivar. The plants were grown under homogeneous environmental conditions inside a greenhouse at the Research Station of the School of the Biological Sciences at the University of Reading. The experiment was laid out in a Complete Randomized Design (CRD) as a factorial experiment. Two kinds of fertilizers, organic (seaweed) and an inorganic copy which are used in the experiment in Chapter 2; were applied to the soil at the same ratio and same amount at monthly intervals. After eight months, the plants were harvested by cutting the fresh aerial parts and the essential oil was extracted by hydro-distillation, the composition of the oil analysed using GC-MS and ^{1}H NMR. Each treatment had seven replicates (pots) distributed randomly across experimental units.

Table 4. 2 Average temperatures inside the greenhouse (February - August 2015)

Month	February	March	April	May	June	July	August
Minimum	12.75	15.06	16.36	17.83	18.76	19.16	18.12
maximum	20.03	23.64	29.13	30.8	33.43	30.96	30.16

4.3 Results

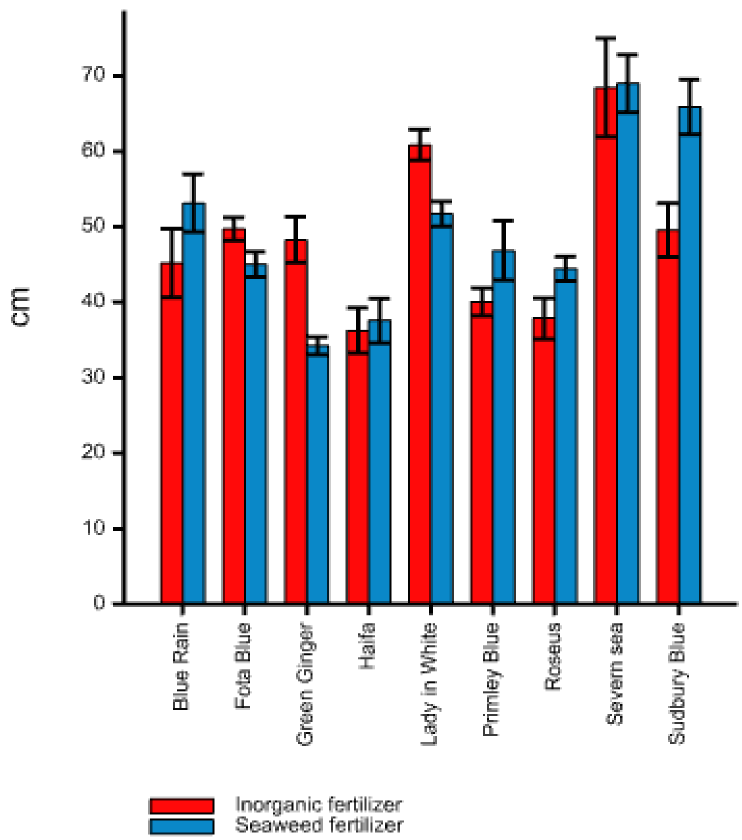
The variation in genotype leads to significant differences in the growth and production of rosemary plants.

4.3.1 Plant height

The height of plants varied among the different cultivars. 'Severn Sea' and 'Lady in White' were characterized by greater height among the plants (68.4 cm and 60.9 cm respectively) with inorganic fertilizer treatment. 'Haifa', 'Primly Blue' and 'Roseus' were the shorter plants under this treatment (36.3, 40.0 and 37.9 cm, respectively). Seaweed fertilizer affected plant height differently, the highest plants were 'Severn Sea' (69.0 cm) and

'Sudbury Blue' (65.86 cm), whereas 'Green Ginger' (34.29 cm) and 'Haifa' (37.57) cm were the shorter plants under this treatment.

Also, the response of rosemary cultivars to seaweed and the inorganic copy of the fertilizer individually was variable. Cultivars that were increased significantly by highest plant height under inorganic treatment were 'Green Ginger' and 'Lady in White'. Seaweed caused significant increase in plant height of 'Sudbury Blue' only, whereas other cultivars did not show any significant differences between the fertilizers. The interaction between genotype and fertilizers shows a significant difference among the plants in plant height (Fig 4.1).



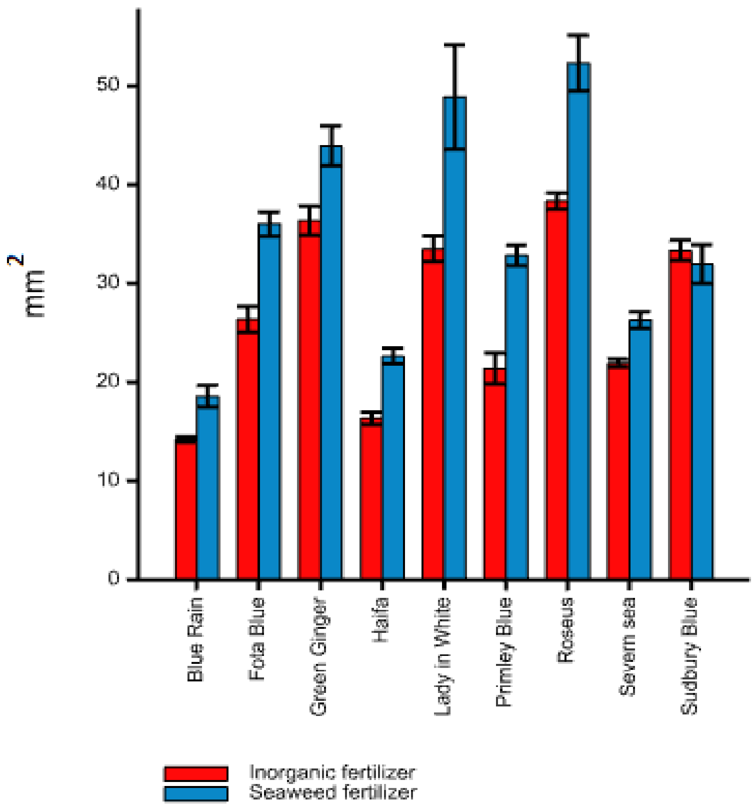
L.S.D $_{0.05}$ = 8.93

Figure 4. 1 Influence of genotype and fertilizers on plant height (cm) of rosemary plants

4.3.2 Leaf area

In terms of leaf area, there was a significant difference between the cultivars under each of inorganic and seaweed fertilizers. 'Roseus' (38.3, 52.2 $mm^{2})$, 'Lady in White' (33.2, 48.8mm^2) and 'Green ginger' (36.3, 43.9mm^2) characterized by highest leaf area and under the both treatments (inorganic and organic fertilizers respectively). On the other hand, 'Blue Rain' (14.2, 18.5 mm^2), and 'Haifa' (16.3, 22.6 mm^2) gave the lowest leaf area under inorganic and seaweed treatments respectively.

The difference between seaweed and the inorganic copy of mineral fertilizer showed the superiority of seaweed over the inorganic fertilizer for all cultivars except 'Severn Sea', which was not affected significantly as a result of different fertilizers. The interaction between genotype and fertilizers shows a significant difference among the plants in leaf area (Fig 4.2).



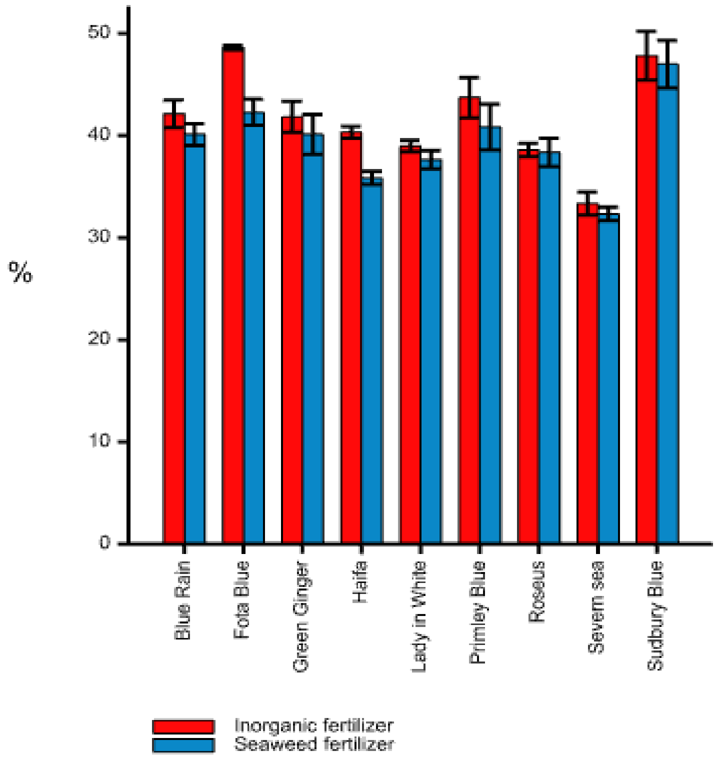
L.S.D $_{0.05}$ = 5.10

Figure 4. 2 Influence of genotype and fertilizers on leaf area (mm^2) of rosemary plants

4.3.3 Percentage of dry material in leaves

The percentage of dry material in leaves differed between the cultivars significantly treated with both seaweed and inorganic fertilizers. 'Sudbury Blue' was the cultivar with the higher percentage of dry material in leaves with both treatments (47.8, 47.0 %) compared with other cultivars, while 'Severn Sea' leaves contained the lowest percentage of dry material (33.3, 32.3%) for inorganic and seaweed treatments respectively.

The difference between seaweed and inorganic fertilizer for each cultivar expressed as a percentage of dry material in leaves was significant for 'Fota Blue' and 'Haifa' with characterized inorganic fertilizer by higher percentage of dry material in leaves (48.6, 40.3% respectively) compared with seaweed fertilizer (42.2, 35.8% respectively). The interaction between genotype and fertilizers shows a significant difference among the plants in percentage of dry material in leaves (Fig 4.3).



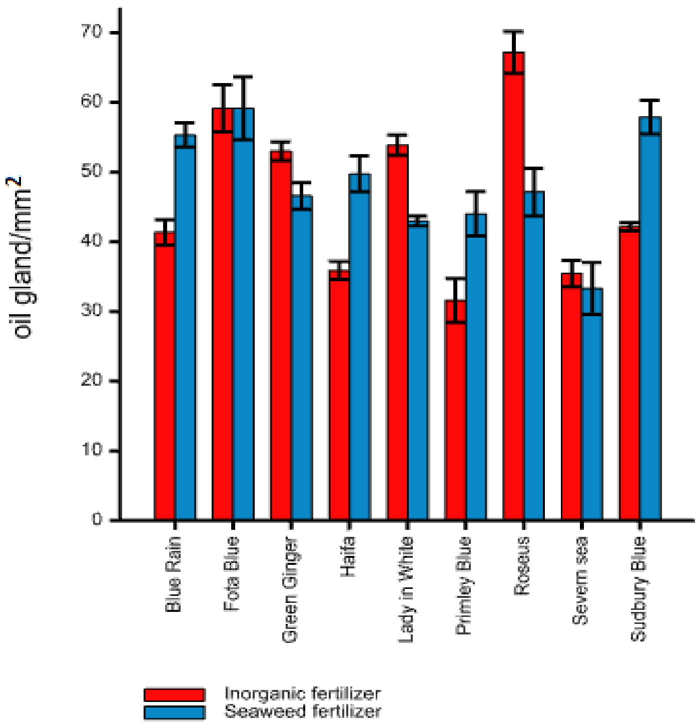
L.S.D $_{0.05}$ = 3.98

Figure 4. 3 Influence of genotype and fertilizers on percentages of dry material in leaves of rosemary plants

4.3.4 Density of oil glands

The density of oil glands in leaves of rosemary cultivars responded to the effect of fertilizer differently depending on genotype of the plants. The density of these glands increased significantly in 'Roseus' treated with inorganic fertilizer (67.14 oil gland/mm^2) as compared with all other cultivars. Under seaweed fertilizer treatment, 'Sudbury Blue', 'Fota Bleu 'and 'Blue Rain' differed significantly by a higher number of oil glands (57.8, 59.1 and 55.2 oil gland/mm^2 respectively) compared with other cultivars.

The difference in effect of inorganic and seaweed fertilizer was not significant in terms of the density of oil glands for 'Fota Blue' and 'Severn Sea' cultivars. Otherwise, 'Bleu Rain', 'Haifa', 'Primly Blue' and 'Sudbury Blue' characterized significantly by high density of oil glands in leaves with seaweed fertilizer, while inorganic fertilizer affected significantly both 'Green Ginger' and 'Lady in White'. The interaction between genotype and fertilizers shows a significant difference among the plants in density of oil glands in leaves (Fig 4.4).



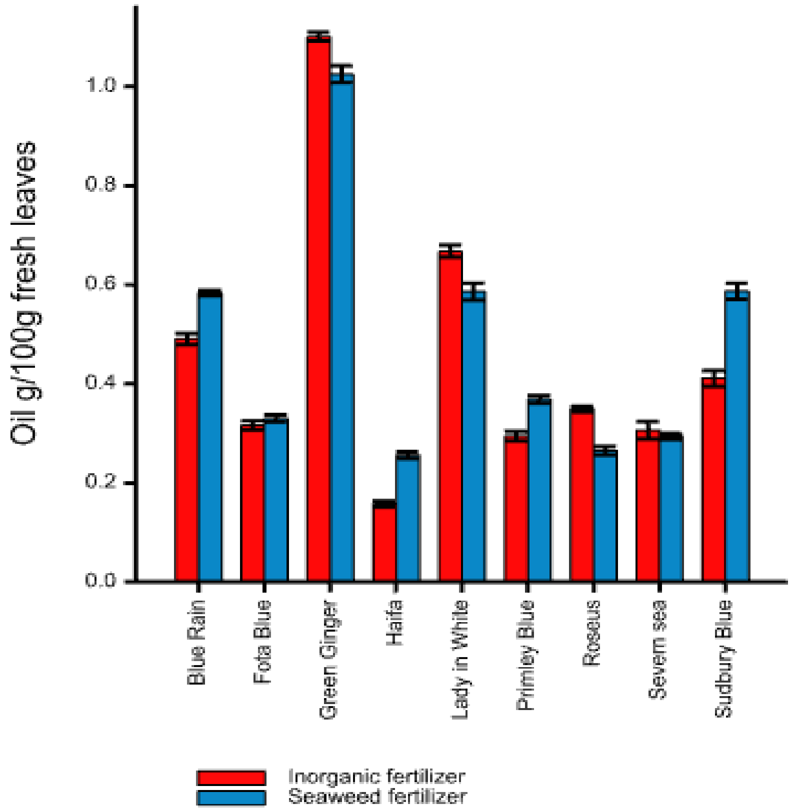
L.S.D $_{0.05}$ = 7.21

Figure 4. 4 Influence of genotype and fertilizers on the density of oil glands in leaves (mm^2) of rosemary

4.3.5 Oil yield

'Green Ginger' was identified as the cultivar that produced the highest amount of oil compared with other cultivars in this experiment, whether with inorganic or seaweed fertilizer (1.100 and 1.025 g/100g fresh leaves), followed by 'Sudbury Blue', 'Blue Rain' and 'Lady in White' with both fertilizers. The lowest amount of oil produced by cultivar 'Haifa' in both treatment (0.157 and 0.256 g/100g fresh leaves for inorganic and seaweed fertilizer respectively).

The oil amount differed significantly for most of the cultivars as a result of using two types of fertilizers. Seaweed treatment enhanced 'Blue Rain', 'Haifa', 'Primley Blue' and 'Sudbury Blue' positively to produce higher amount of oil compared with inorganic fertilizer. 'Green Ginger', 'Lady in White' and 'Roseus' were positively impacted by using inorganic fertilizer. On the other hand, 'Fota Blue' and 'Severn Sea' did not show any significant difference between the two fertilizers. The interaction between genotype and fertilizers shows a significant difference among the plants in percentage of dry material in leaves (Fig 4.5)



L.S.D $_{0.05}$ = 0.031

Figure 4. 5 Influence of genotype and fertilizer on oil amount (g/100g fresh leaves) of rosemary plants

4.3.6 Oil composition

4.3.6.1 GC-MS results

There is a great variability in the chemical composition of the essential oils obtained from different *Rosmarinus officinalis* cultivars. The results of GC-MS analysis showed that rosemary cultivars exhibited a significant difference in each oil constituent under both treatments of fertilizer. 'Severn Sea' and 'Sudbury Blue' were characterized by higher concentration of α-pinene; 'Fota Blue' and 'Primley Blue' in camphene; 'Haifa' in β-pinene, eucalyptol and camphor; 'Lady in White' in myrcene and linalool; 'Roseus' in *p*-cymene; and 'Blue Rain' in borneol.

There were significant differences between the effects of fertilizers on the composition of essential oil among rosemary cultivars. Inorganic fertilizer caused a significant increase in camphene (9.17%) in 'Primley Blue', β-pinene (1.03%) and eucalyptol (26.63%) in 'Haifa' and *p*-cymene (2.73%) in 'Roseus'. Also, this fertilizer caused a significant decrease in concentrations of α-pinene (3.96%), β-pinene (0.82%) and borneol (0.28%) with 'Lady in White', camphene (3.27%) with 'Roseus', myrcene (0.84%) with 'Green Ginger', eucalyptol (1.092%) with 'Sudbury Blue' and linalool (0.82%) with 'Fota Blue'.

On other hand, seaweed fertilizer had higher concentration of α-pinene (51.11%) in 'Severn Sea', myrcene (40.20%) in 'Lady in White', linalool (2.64%) in 'Sudbury Blue', camphor (24.29%) and borneol (6.60%) in 'Blue Rain'. While, 'Primley Blue' and 'Roseus'showed lowest level of *p*-cymene (0.59%) and camphor (9.65%) respectively.

However, type of fertilizer had a significant effect on camphene. The genotype of the plant shows significant effects on all the nine compounds. The interaction between fertilizers and genotype of the plants shows significant differences among the plants with all compounds except myrcene and camphor (Table 4.3).

The nine cultivars were subjected to a principal component analysis (PCA). The rate of accumulation of the previous nine major compounds reached 91.96% for seaweed fertlizer and 92.5% for inorganic fertilizer. The scatter plots obtained by PCA showed different groups of individuals (nine cultivars), along axis PC-1 (62.47% for seaweed fertilizer,61.23% for inorganic fertilizer) and PC-2 (29.49% for seaweed fertilizer,31.27% for inorganic fertilizer), according to their major volatile components. α-pinene and myrcene were the main compounds in the essential oils from the cultivars in PC-1 and PC-2 for both seaweed and inorganic fertilizers (Fig 4.6).

Table 4. 3 Influence of genotype and fertilizer on oil composition of rosemary plants analysed by GC-MS

Fertilizer	Cultivar	α-Pinene %	Camphene %	β-Pinene %	Myrcene %	*p*-Cymene %	Eucalyptol %	Linalool %	Camphor %	Borneol %	Total %
Inorganic	Blue Rain	8.94	4.12	3.66	5.71	2.56	25.16	1.30	23.88	5.94	**81.30**
	Fota Blue	12.88	8.12	3.41	1.41	0.97	21.13	0.82	16.35	2.43	**67.56**
	Green Ginger	10.91	3.43	1.74	0.84	0.84	11.47	1.00	18.36	2.79	**51.43**
	Haifa	14.30	7.24	10.35	2.17	1.51	26.63	1.66	23.99	2.07	**89.94**
	Lady in White	3.96	4.02	0.82	39.19	0.97	12.62	2.24	10.09	0.28	**74.23**
	Primley Blue	15.95	9.17	2.63	1.80	0.62	17.23	1.14	18.87	2.84	**70.28**
	Roseus	9.94	3.27	1.48	32.44	2.73	11.58	1.50	11.03	0.30	**74.31**
	Severn Sea	48.07	3.90	2.54	1.39	1.46	14.34	1.64	12.98	2.44	**88.79**
	Sudbury Blue	49.15	4.33	2.11	2.09	1.45	10.92	2.10	8.08	2.97	**83.23**
Seaweed	Blue Rain	10.14	3.99	2.75	5.75	2.27	20.19	1.42	24.29	6.60	**77.44**
	Fota Blue	15.81	7.29	2.71	3.34	1.20	20.86	0.96	15.81	2.26	**70.26**
	Green Ginger	11.77	3.55	1.87	0.86	0.83	12.11	0.88	19.30	2.38	**53.56**
	Haifa	9.24	4.51	6.41	1.39	1.37	24.25	1.34	21.32	1.52	**71.38**
	Lady in White	4.12	4.19	0.89	40.02	0.88	13.04	2.15	9.96	0.43	**75.72**
	Primley Blue	12.32	6.70	2.68	1.45	0.59	14.23	0.89	12.56	2.28	**53.73**
	Roseus	9.76	3.29	1.41	33.9	2.66	12.16	1.60	9.65	0.58	**75.08**
	Severn Sea	51.11	3.47	2.33	1.35	1.23	16.66	2.40	13.22	2.57	**94.37**
	Sudbury Blue	46.20	4.67	2.16	2.16	1.60	15.14	2.64	9.97	3.89	**88.46**
L.S.D		**3.558**	**0.970**	**0.586**	**1.521**	**0.221**	**1.777**	**0.276**	**4.085**	**0.616**	***d.f*** **125**

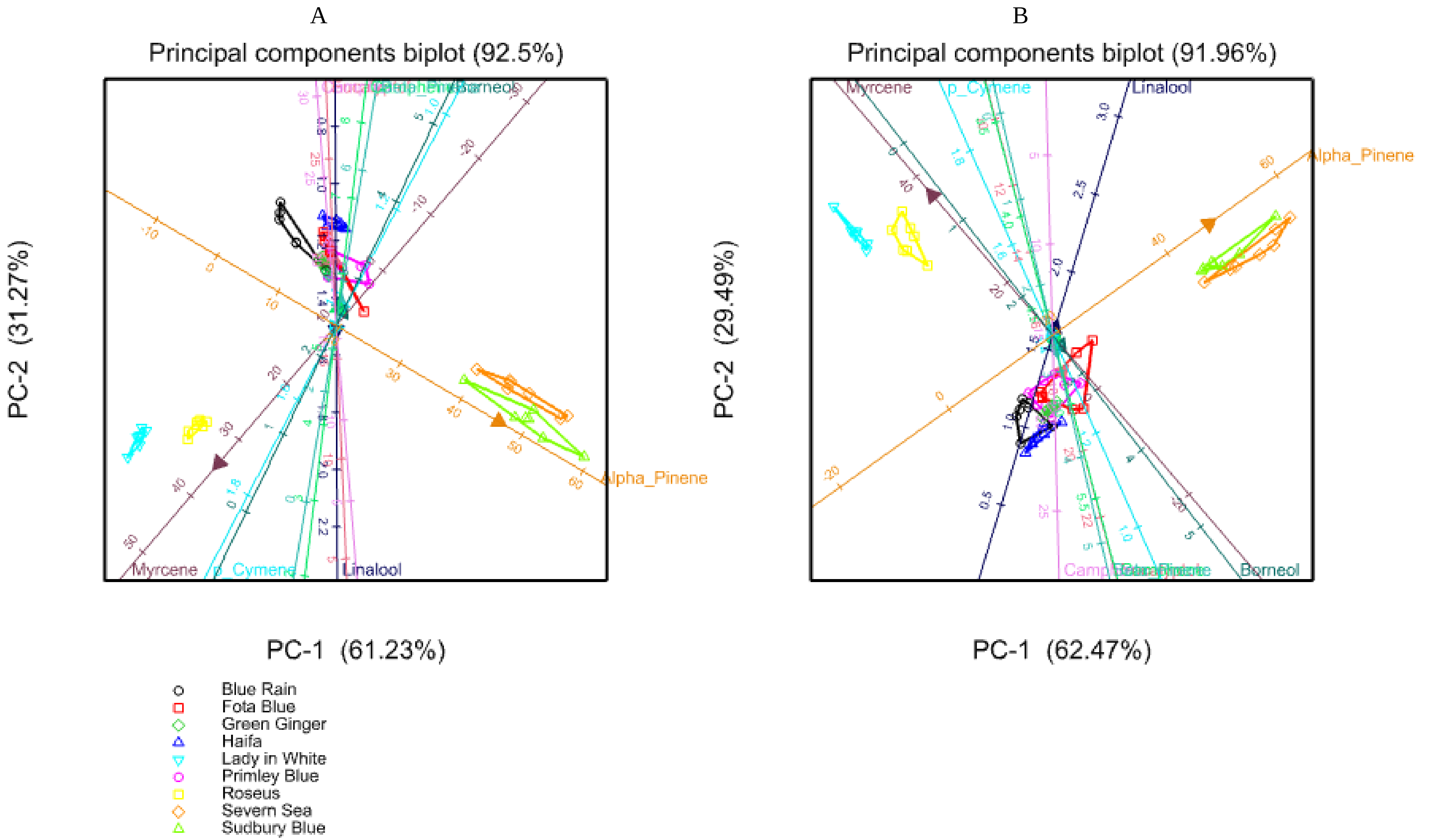


Figure 4. 6 Principle Components Analysis biplot distinguishing the effect of using seaweed and inorganic fertilizer with nine different cultivars of rosemary using nine main volatile constituents.

4.3.6.2 ^{1}H NMR results

The differentiation between cultivars and their responses to fertilizers in oil composition was further analysed using ^{1}H NMR. The rosemary cultivars that were characterized by a higher percentage of α-pinene were ‘Sudbury Blue’ and ‘Severn Sea’; camphene was highest in ‘Fota Blue’; β-pinene, eucalyptol, and camphor in ‘Haifa’; myrcene and linalool in ‘Lady in White’; *p*-cymene and borneol in ‘Blue Rain’ for both fertilizers.

Under inorganic fertilizer, ‘Fota Blue’ showed significant increase in camphene (9.39%); ‘Blue Rain’ in *p*-cymene (1.93%); ‘Haifa’ in β-pinene (8.09%), eucalyptol (23.99%) and camphor (23.16%); ‘Lady in White’ in myrcene (34.15%) and linalool (2.58%); and ‘Severn Sea’ in α-pinene (43.68%) compared with other cultivars and seaweed fertilizer. While the seaweed fertilizer characterized significantly by higher percentage of borneol (5.80%) only. The same fertilizer (seaweed), leads to significant decrease in α-pinene (3.35%), camphene (2.18%), β-pinene (0.57%) and camphor (7.65%) in ‘Lady in White’; linalool (0.61%) in ‘Fota Blue’; myrcene in ‘Green Ginger’; *p*-cymene (0.43%) in ‘Primley Blue’; and eucalyptol (8.83%) in ‘Roseus’. While the lowest percentages of borneol was with ‘Roseus’ but under inorganic fertilizer.

and camphor in ‘Haifa’; and eucalyptol, linalool and camphor in. On other hand, seaweed fertilizer raised the concentration of myrcene in; camphene in ‘Green Ginger’; eucalyptol and camphor in ‘Severn Sea’; and linalool, eucalyptol and camphor in ‘Sudbury Blue’.

and camphene, myrcene

However, type of fertilizer had a significant effect on α-pinene, camphene β-pinene, *p*-cymene, eucalyptol, camphor and borneol. The genotype of the plant shows significant effects on all the nine compounds. The interaction between fertilizers and genotype of the plants shows significant differences among the plants with camphene, β-pinene, *p*-cymene, eucalyptol, linalool and camphor (Table 4.4).

Table 4. 4 Influence of genotype and fertilizer on oil composition of rosemary plants analysed by ^{1}H NMR

Fertilizer	Cultivar	α-Pinene %	Camphene %	β-Pinene %	Myrcene %	*p*-Cymene %	Eucalyptol %	Linalool %	Camphor %	Borneol %	Total %
Inorganic	Blue Rain	12.08	4.16	3.27	5.53	1.93	19.48	1.22	19.94	5.55	**73.21**
	Fota Blue	16.72	9.39	3.12	1.34	0.86	19.78	0.68	21.73	1.79	**75.45**
	Green Ginger	10.73	3.42	1.87	0.69	0.57	11.19	0.77	18.33	2.13	**49.74**
	Haifa	12.33	5.23	8.09	1.69	1.23	23.99	1.33	23.16	1.93	**89.02**
	Lady in White	3.89	2.45	0.60	34.15	0.97	9.69	2.58	9.11	0.33	**63.80**
	Primley Blue	16.12	7.56	2.85	1.14	0.46	15.73	0.73	19.54	1.84	**66.00**
	Roseus	11.81	3.29	1.29	27.28	0.97	8.38	0.96	8.69	0.20	**62.92**
	Severn sea	43.68	4.11	2.19	1.57	1.21	10.93	1.97	7.52	2.02	**75.25**
	Sudbury Blue	43.90	4.34	1.91	1.37	1.29	10.11	2.21	8.80	3.40	**77.36**
Seaweed	Blue Rain	10.90	3.66	2.93	4.93	1.59	19.16	1.24	18.87	5.80	**69.13**
	Fota Blue	13.28	7.20	2.49	2.50	0.71	16.18	0.61	17.12	1.57	**61.69**
	Green Ginger	10.49	3.64	1.85	0.63	0.55	10.81	0.73	17.91	2.13	**48.79**
	Haifa	9.81	3.84	5.86	1.32	0.97	22.75	1.09	22.69	1.41	**69.77**
	Lady in White	3.35	2.18	0.57	29.26	0.80	9.41	2.05	7.65	0.25	**55.56**
	Primley Blue	14.93	7.42	2.80	1.02	0.43	12.78	0.49	15.29	1.37	**56.57**
	Roseus	11.77	3.35	1.31	28.03	1.08	8.83	1.03	9.10	0.39	**64.92**
	Severn sea	43.32	3.81	2.14	1.62	1.18	13.05	2.04	10.61	1.54	**79.35**
	Sudbury Blue	40.43	3.93	1.79	1.27	1.28	12.87	2.53	9.74	3.47	**77.34**
L.S.D		**3.187**	**0.842**	**0.500**	**2.491**	**0.197**	**2.363**	**0.324**	**2.176**	**0.553**	***d.f*** **125**

4.4 Discussion

Most of the variability in the qualitative and quantitative composition of the essential oils are due to intrinsic features such as genetics and plant age (Socaci *et al.*, 2007). It has been reported that the variation in oil yield and properties among different cultivars of many crops was found to be greater than the influence of any other factor (Li *et al.*, 2016). Martinetti *et al.* (2006) confirmed that variations in between cultivars have a significant effect on plant height, shape and many other morphological characteristics as well as oil production. They stated that different cultivars respond differently for their genotypic characters, input requirement, growth process and the prevailing environment during the growing season. Said-al Ahl Hussein *et al.* (2016) reported that there are significant differences between the cultivars of *Anethum Graveolens* (Dill) in plant height, branches number, fresh weight (g/plant), and amount of essential oil.

Angioni *et al.* (2004) reported the important role of genotype in *Rosmarinus officinalis* L plants. Rosemary cultivars varied in their performance, as they can be classified as having upright or prostrate growth habits (Warnock and Voigt, 2005). Also, the cultivars varied in many attributes of growth and production, such as the height of the plant, shoot size, flower colour, leaf shape and the smell or the composition of the oil (Cervelli and Masselli, 2011). Said-al Ahl Hussein *et al.* (2016) reported that the main differences in minor compounds is less than 10% in the essential oil of different dill cultivars. While, Tucker and Maciarello (1986) summarized the disparity between twenty-three cultivars of *Rosmarinus officinalis* L. by grouping them into six chemotypes according to the composition of their essential oil. Each group was characterized by a major constituent such as α-pinene, 1,8-cineole, camphor, camphene and so on. Mulas and Mulas (2005) stated that the composition of the essential oil was variable among six rosemary cultivars, while it is constant within the same cultivar for the two different periods of harvest. Some cultivars have shown that their essential oils composition characterized by one or more compounds; such as essential oil extremely rich in camphor, while other cultivars characterized by a high percentage of α-pinene in their essential oils. Socaci *et al.* (2007) stated that in all cases, the composition of essential oil was almost the same within the same cultivar.

The results presented here revealed that there were a wide range of differences among the nine cultivars of rosemary in height, leaf area, percentage of dry material in leaves, number of oil glands in the leaf, as well as oil yield and composition. There is a wide variation in oils due to the genetic variability between the plants, and this can be exploited in the development of commercial plantings (Zaouali *et al.*, 2012). These results are in agreement with the literature (Tucker and Maciarello, 1986; Mulas and Mulas, 2005; Martinetti *et al.*, 2006; Socaci *et al.*, 2007) in which is observed a similar trend of a higher degree of genetic variation within populations. At the same time, the results were partly in disagreement with Socaci *et al.* (2007) regarding eucalyptol level. These authors reported that the content of eucalyptol from rosemary can be variable depending on the environment of the plant. However, Elamrani *et al.* (2000) have stated that no differences were found between different taxa of rosemary.

The results of this study show that the response of rosemary cultivars to the fertilizer was not uniform. Previous studies have reported increases in leaf number and leaf area, dry weight and plant height in response to the addition of fertilizer on many horticultural crops (Singh *et al.*, 2002; Anwar *et al.*, 2005; Sotiropoulou and Karamanos, 2010; Chrysargyris *et al.*, 2016) including rosemary (Miguel *et al.*, 2007). According to the results of Chapter 2, the yield and oil content of rosemary increased as a response to the use of fertilizers compared with non-fertilized plants. It has been reported that higher nitrogen application decreases the percentage of linalool and increases methyl chavicol in the essential oil of some aromatic plant species. In contrast, a higher amount of potassium contributes to an increase in essential oil content and the percentage of linalool, and 1,8-cineole in oil (Pino *et al.*, 1998; Rao *et al.*, 1998; Diaz-maroto *et al.*, 2007; Nurzyńska-Wierdak, 2013). Nonetheless, only a few studies on the effect of fertilizer on different rosemary cultivars have been published. Martinetti *et al.* (2006) found that fertilizer concentration decidedly influenced plant growth, yield and nutrient uptakes in both the cultivars used in the experiment. Likewise, the two cultivars presented dissimilar oil composition and yield: ‘Majorka pink’ had the lowest oil content and was mainly rich in camphor (ketones), while 'Montfort form' was rich in α-pinene, borneol and eucalyptol (hydrocarbons and alcohols). In general, the concentrations of the main constituents were reduced by increasing the concentration of fertilizer. Salanta *et al.* (2015) noted that it is difficult to see much of a pattern in the distribution of metabolites between varieties of aromatic plants, due to dominance by one compound like eucalyptol or camphor. These results are in line with the outcomes found in this chapter. In most cases, seaweed had the best effect on growth and production of oil for most of the cultivars. There was a decrease in levels of most oil contents which coincided with using seaweed fertilizer.

The genetic mechanisms that in phase the development of volatiles formation in plants include gene repetition; convergent evolution; evolution of an existing gene; and loss of enzymatic activity. In all of these cases, these changes lead to variations in gene expression. Also, functional enzymatic range can arise with very few fluctuations in the enzyme structure and can be increased with the enzymes being unprotected to variable environments as a result of rapid changes in enzyme structure (Figueiredo *et al.*, 2008).

4.5 Conclusions

The effect of fertilizers on the different components of the essential oil of the nine cultivars was slightly different. Fertilizing, mainly with seaweed extract, often reduced the percentage occurrence of some oil components, but in some cases other components were enhanced.
Measured variables varied significantly across the rosemary cultivars. The variation in growth and yield was dependent on genetic variation among the plants. Genotype greatly influenced the composition of the essential oil. The percentage content of the volatile oil in all cultivars investigated was quite different qualitatively as well as quantitatively. Based on the results, it is clear that the composition of essential oils is under genetic control. In all cases, choice of cultivar should be a primary consideration in order to decide the destination of the product, when growing commercial herbs for oil production. It is species-specific and has to be determined according to the most favourable combination of oil composition and yield.

5. Chapter five: The influence of harvest regime and oil analysis on quality of rosemary oils

5.1 Introduction

The variability in the chemical composition of essential oils depends on several factors including climate, season, geographical location, genotype and age of the plant (Viuda-Martos *et al.*, 2007). Date and methods of harvest, extraction of the oil, and the methods of analysis for the chemical composition of essential oil, are all factors leading to variation in essential oil composition and could alter the commercial usefulness it (Tongnuanchan and Benjakul, 2014). Most of researchers have investigated the effect of main factors such as climate, soil, geographical location on oil yield and chemical composition without taking into consideration the effects of some factors such as method of extract and analysis that may occur after growth period and harvest.

Rosemary plants usually yield more material from frequent regrowth after being harvested. More often harvesting is carried out with mechanical harvesting in which case the plants will yield more material from frequent regrowth. In general, harvest can be prepared either at an interval of 4 to 6 months, or annually (two to three harvests) depending on the growing conditions (DAFF, 2012). The number of harvests a year can be increased or reduced annually as the climate becomes more temperate (Dempsey, 1975). The quality of rosemary extract is highly correlated to the time of harvest (Yesil-Celiktas *et al.*, 2007). Furthermore, the effect of the harvest date on essential oil content of rosemary has been studied, the highest percentage of the component changed depending on the time of harvest, the best oil yield being achieved with a rise in temperature and longer photoperiod (Miguel *et al.*, 2007). Aromatic plants at the pre-blossoming phase provide the optimal compromise between production and essential oil quality (Said-al Ahl Hussein *et al.*, 2016). The components are variable in leaves of rosemary depending on collection time (before, after and during blooming) (Emadi *et al.*, 2007). On the other hand, cultivated rosemary grown under the same conditions, but in different years or of different ages, will lead the plants to produce different oils composition (Atti-Santos *et al.*, 2005; Socaci and Socaciu, 2008).

After harvest and extraction, the essential oil is usually subject to analysis in order to know the chemotype of oil. Several techniques have been used to analysis essential oils from aromatic plants, such as TLC, HPLC, GC and GC-MS (Li *et al.*, 2007; Said *et al.*, 2011). In addition, there are different variations of each technique depending on the type of oil and the number of compounds. ^{1}H NMR is one of the methods which is used to identify the contents of organic compounds. The advantage of ^{1}H NMR technology is that analysis is moderately cheap, fast and the analytical technique that can be easily applied for a routine screening. On the other hand compared with GC-MS, ^{1}H NMR has limited sensitivity and dynamic range in chromatographic separation due to a large overlap of resonances making the identification of compounds difficult and not very accurate (Sieber, 2009).

This chapter reports on the effects of harvest regime in order to fully determine the best time of plant harvest in terms of oil composition and yield. It tests the following hypotheses:

1) Oil quantity and quality do not vary within plants harvested in different times.
2) Different percentages of harvest have no impact on growth and oil production in all cultivars of rosemary.
3) GC-MS and NMR techniques present similar results for essential oil analysis.

5.2 Materials and methods

This experiment was conducted inside a greenhouse (May-November 2014) and laid out in a Complete Randomized Design (CRD) with one-way ANOVA. The aim of this study was to show the effect of harvest (harvest ratio) on plant production, in terms of quantity of plant material harvested relative to the total plant weight. Different percentages of harvest (0%, 10%, 30%, 50% and 70% weight) were suggested as treatments. The percentages are representing the difference in amount of plant material harvested from the plant.

Among more than 300 plants planted and grown at the same time under the same conditions inside the greenhouse (same plants which prepared for the experiment in Chapter two), 35 plants were selected to form this experiment. These plants were similar and matched in shape, height (60 cm), weight (60 g) and number of branches (3 main branches) in general. Then, 0, 6, 18, 30 and 42 gm from the whole plant weight (plant weight is 60g) have been harvested, these weights represent 0%, 10%, 30%, 50% and 70% from the plant weight, as a treatment simulate the different in amount of plant material taken for harvest. All the plants grown under the same conditions inside the greenhouse (Table 5.1) and received a dose of seaweed fertilizer (the same dose and fertilizer used in experiments in Chapters two and four) watered to the soil every four weeks. Each treatment had seven replications (pots) distributed randomly across experimental units and the harvesting of leaves for extraction began at 6 months after the experiment started in November 2014.

Table 5. 1 Average temperatures inside greenhouse (May - October 2014)

Month	May	June	July	August	September	October
Minimum	18.06	17.96	18.83	17.16	18.56	17.80
Maximum	27.24	32.03	35.96	32.22	30.36	24.00

Plant growth and production measurements were taken as described in Chapter 2 with the addition of a record of the whole weight of the plant at the end of the experiment. The whole weight of the plants was measured by remove the plant above the soil surface and then weigh them using the balance.

5.3 Results

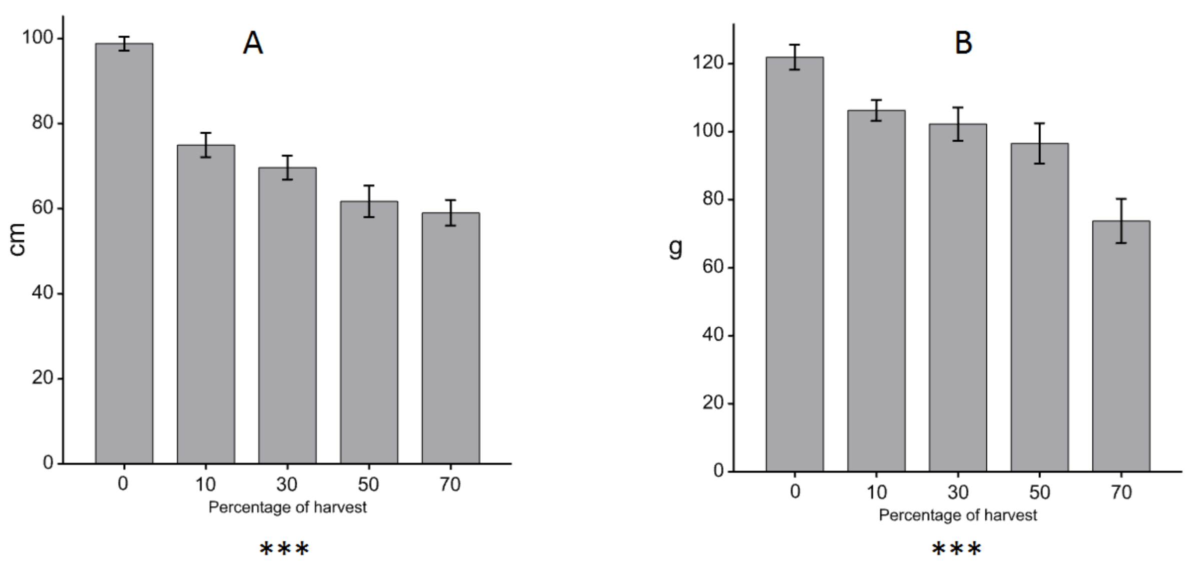
5.3.1 Different percentages of harvest

5.3.1.1 Plant height

There was a high significant difference amongst the plants in term of plant height. The plants treated with 0% harvest were the taller plants (98.8 cm) compared with other treatments. While, the plants treated with 50% and 70% harvest showed shorter height (61.7 cm and 59.0 cm respectively) compared with other treatments (Fig 5.1 A).

5.3.1.2 Plant weight

Plant weights were not quite matched with plants heights results: 0% was the treatment with higher weight (121.9 g) of plants compared with other treatments. The plants treated by 70% harvest, gave significantly lowest weight of plant (73.7 g) compared with all other treatments. 10%, 30% and 50% did not differ significantly amongst themselves (Fig 5.1 B).



L.S.D $_{0.05}$ = 8.32   L.S.D $_{0.05}$ = 14.40

Figure 5. 1 Effect of different percentages of harvest on (A) height (cm) and (B) weight (g) of rosemary plants

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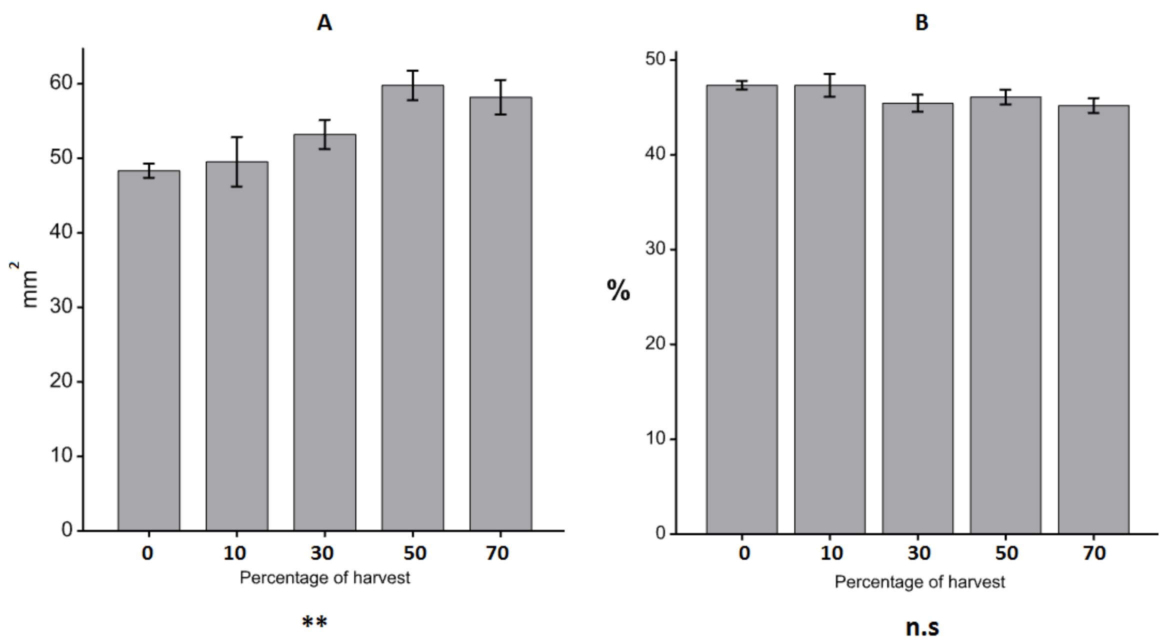
*****: *P* value ≤ 0.05; ******: *P* value ≤ 0.01; *******: p value ≤ 0.001; **n.s**: *P* value ≥ 0.05 (not significant)

5.3.1.3 Leaf area

The plants showed a positive response to the treatments of harvest. Plants treated by 50% and 70% of harvest showed the highest leaf area (59.7 and 58.1 mm^2 respectively) compared with 0, 10 and 30% treatments which had lower leaf area (48.3, 49.5 and 53.1 mm^2 respectively) (Fig 5.2 A).

5.3.1.4 Percentage of dry material in leaves

In terms of percentage of dry material in leaves, there was no significant difference between plants which were treated by different rates of harvest (Fig 5.2 B).



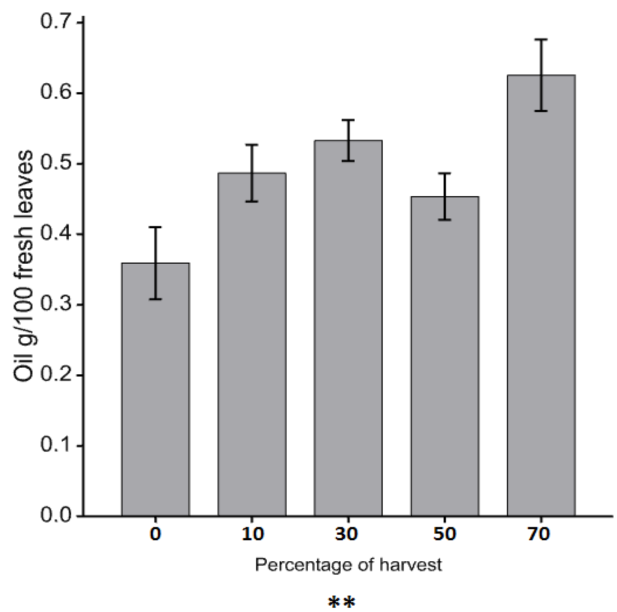
L.S.D $_{0.05}$ = 6.47

L.S.D $_{0.05}$ = 2.47

Figure 5. 2 Effect of different percentages of harvest on (A) leaf area (mm^2) and (B) percentage of dry material in leaves of rosemary plants

5.3.1.5 Oil amount

The five different percentage of harvest caused a significant difference between the plants. The higher level of oil was in plants harvested in rate of 70% (0.626 g 100 g^{-1} fresh leaves), followed by plants harvested in rate of 30% (0.533 g 100 g^{-1} fresh leaves). The lowest amount of oil (0.359 g 100 g^{-1} fresh leaves) was produced by control 0% (plants which were not harvested) (Fig 5.3).



L.S.D $_{0.05}$ = 0.12

Figure 5. 3 Effect of different percentages of harvest on oil yield production of rosemary plants

5.3.1.6 Oil composition

Oil composition was not very different between the plants that treated by different percentages of harvest. There were significant differences for the levels of β-pinene and borneol only. Control plants (0%) had the higher level of β-pinene (2.2%), and the 50% treatments caused a significant increase in borneol levels (4.0%), compared with other treatments (Table 5.2).

PCA for (Fig 5.4) explained 96.94% of the total variability with PC-1 86.76%; and PC2 for 10.18% of the total variability for different percentages of harvest. Camphor and eucalyptol were the main compounds in the essential oils in PC-1 and PC-2.

Table 5. 2 The effect of percentage of harvest on oil composition of rosemary plants analysed by GC-MS

Percentage of harvest	0%	10%	30%	50%	70%	L.S.D	*P*-value
α-Pinene	5.7	5.2	4.8	5.8	4.8	0.033	**>0.05**
Camphene	3.7	3.3	3.0	3.5	2.8	0.026	**>0.05**
β-Pinene	2.2	1.6	1.5	1.9	1.6	0.019	**<0.05**
Myrcene	10.6	9.5	9.0	10.8	9.1	0.044	**>0.05**
p-Cymene	1.6	1.8	1.8	1.9	1.6	0.014	**>0.05**
Eucalyptol	21.6	22.2	19.5	23.9	20.5	0.062	**>0.05**
Linalool	3.7	3.1	3.3	4.1	3.6	0.022	**>0.05**
Camphor	23.3	21.1	21.5	25.7	22.7	0.068	**>0.05**
Borneol	3.2	2.6	3.2	4.0	3.8	0.022	**<0.05**
Total	**76.221**	**70.801**	**67.926**	**82.075**	**70.896**	***d.f* = 34**	

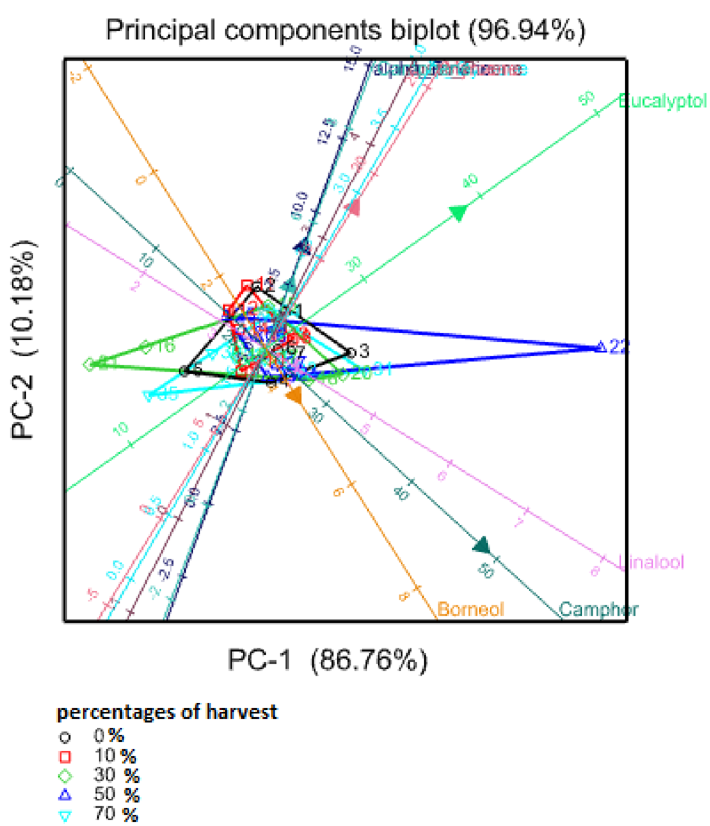


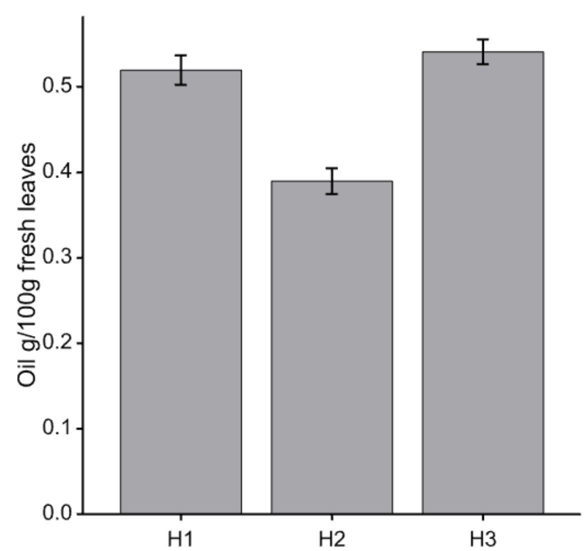
Figure 5. 4 Principle Components Analysis biplot distinguishing the effect of different percentages of harvest

5.3.2 Harvest time

In Chapter 2, there were three different dates of harvest. Here, we compared in general between these dates in order to show the effect of date of harvest on plant production (quantity and quality of the oil).

5.3.2.1 Oil amount

In terms of oil amount, there was a significant difference between the three different dates of harvest. The plants that were harvested after three months (H1) and plants harvested once after six months (H3) showed a higher amount of oil (0.519 and 0.540 g/100g fresh leaves) compared with plants harvested twice after six months (H2) (0.389 g/100g fresh leaves) (Fig 5.4).



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L.S.D $_{0.05}$ = 0.0436

Figure 5. 5 Oil amount (g/100 g fresh leaves) of rosemary plants harvested at three different dates

5.3.2.2 Oil composition

The contents of essential oil differed significantly with the different time of harvest. The H1 plants showed the lowest level of α-pinene (4.08%) and camphene (3.15%) compared with H2 plants (5.86% and 4.02%) and H3 plants (5.26% and 3.95%) for the same compounds, respectively. While, β-pinene 3.28%, eucalyptol 26.51% and camphor 23.0% were significantly higher than the H2 and H3 levels of these compounds. The H2 plants characterized significantly by highest level of myrcene (12.26%), *p*-cymene (1.93%), linalool (4.86%) and borneol (4.28%) compared with the plants harvested in H1 and H3. The H3 plants did not show any characterized either with a higher or lower level for each compound compared with H1 and H2 plants (Table 5.3).

Table 5. 3 The difference in percentages of oil composition of rosemary plants harvested in different dates

Compound	**Date of harvest**			**L.S.D**	***P*-value**
	H1	**H2**	**H3**		
α-Pinene	4.08	5.86	5.26	0.394	<0.001
Camphene	3.15	4.02	3.95	0.264	<0.001
β-Pinene	3.28	2.86	2.71	0.244	<0.001
Myrcene	10.65	12.26	11.36	0.696	<0.001
p-Cymene	1.50	1.93	1.57	0.185	<0.001
Eucalyptol	26.52	22.97	23.29	0.1.205	<0.001
Linalool	3.17	4.86	3.62	0.369	<0.001
Camphor	23.01	20.81	21.16	1.589	<0.01
Borneol	3.75	4.28	3.65	0.275	<0.001
Total	**79.11**	**79.85**	**76.57**	***d.f* = 125**	

5.3.3 The difference between GC-MS and ^{1}H NMR analysis

In order to create a comparison between GC-MS and NMR analysis for essential oils, we used the data from Chapters 1, 2 and 3. The NMR results divided on GC-MS results to get the ratio of the different between them.

Tables 5.4, 5.5 and 5.6 show the ratio of the difference between GC-MS and NMR results for the previous chapters. It clear that ratio for chapter two results is between 0.66 and 1.52 (NMR results to GC-MS results). While, the ratio was smaller for chapter three results (0.86 – 1.10 NMR to GC-MS). Using the results of chapter 3, the analysis of different cultivars essential oils yielded a ratio between 0.75 -1.37. In general, β-pinene had higher ratio of NMR to GC-MS for all three chapters.

Table 5. 4 The ratio of difference between the results of NMR to GC-MS analysis for Chapter 2

Date of harvest	Method of application fertilizer	Fertilizer	α-Pinene	Camphene	β-Pinene	Myrcene	*p*-Cymene	Eucalyptol	Linalool	Camphor	Borneol
After 3 month H1	Spray	Control	1.36	1.01	2.14	0.76	0.67	0.77	0.75	1.08	0.73
		Inorganic	1.09	0.77	1.22	0.70	0.72	0.72	0.65	0.84	0.66
		Seaweed	1.10	0.80	1.22	0.69	0.64	0.69	0.66	0.75	0.64
	Watered	Control	1.28	0.84	1.43	0.77	0.69	0.78	0.78	0.92	0.76
		Inorganic	0.91	0.64	1.06	0.59	0.56	0.59	0.57	0.65	0.59
		Seaweed	1.15	0.81	1.30	0.77	0.73	0.75	0.65	0.87	0.64
3 month after first harvest H2	Spray	Control	0.58	0.43	0.52	0.62	0.20	0.30	0.26	0.57	0.47
		Inorganic	1.14	0.71	1.04	0.62	0.63	0.67	0.57	0.68	0.59
		Seaweed	1.21	0.74	1.20	0.62	0.56	0.64	0.61	0.69	0.58
	Watered	Control	1.17	0.84	1.31	0.77	0.61	0.71	0.80	0.83	0.74
		Inorganic	0.96	0.71	0.98	0.60	0.56	0.58	0.53	0.60	0.53
		Seaweed	1.15	1.86	1.12	0.68	0.65	0.65	0.64	0.74	0.61
After 6 month H3	Spray	Control	1.25	0.80	1.28	0.78	0.59	0.72	0.81	0.82	0.77
		Inorganic	1.17	0.81	1.19	0.73	0.67	0.70	0.61	0.79	0.60
		Seaweed	1.20	0.76	1.27	0.73	0.70	0.84	0.80	1.04	0.80
	Watered	Control	1.23	0.93	1.42	0.84	0.70	0.77	0.81	0.96	0.83
		Inorganic	1.23	0.92	1.55	0.82	0.82	0.77	0.62	0.77	0.65
		Seaweed	1.14	0.85	1.29	0.74	0.75	0.78	0.69	1.12	0.72
Average of difference			**1.13**	**0.85**	**1.25**	**0.71**	**0.64**	**0.69**	**0.66**	**0.82**	**0.66**

Table 5. 5 The ratio of difference between the results of NMR to GC-MS analysis for Chapter 3

Treatments	Age	α-Pinene	Camphene	β-Pinene	Myrcene	p-Cymene	Eucalyptol	Linalool	Camphor	Borneol
Cy_0	Young plants	1.40	1.19	1.24	0.94	0.97	0.96	1.05	1.07	1.15
Cy_1		1.06	0.86	0.95	0.72	0.79	0.74	0.83	0.80	0.89
Cy_2		1.12	0.91	0.95	0.78	0.92	0.74	1.00	0.84	1.02
Cy_3		1.12	0.89	0.93	0.75	0.85	0.78	0.98	0.82	1.02
Seaweed		1.41	1.09	1.08	0.96	1.01	0.90	0.93	0.90	0.87
Cy_0	Old plants	1.54	1.23	1.33	1.01	1.18	0.98	1.03	1.15	1.11
Cy_1		1.23	1.00	1.06	0.83	0.95	0.80	0.94	1.41	1.05
Cy_2		1.20	1.02	1.11	0.78	0.92	0.82	1.02	1.18	1.22
Cy_3		1.13	0.93	0.96	0.77	0.87	0.75	0.97	0.93	1.02
Seaweed		1.67	1.34	1.37	1.10	1.23	1.09	1.20	1.24	1.14
Average of difference		**1.29**	**1.04**	**1.10**	**0.86**	**0.97**	**0.86**	**0.99**	**1.04**	**1.05**

Table 5. 6 The ratio of difference between the results of NMR to GC-MS analysis for Chapter 4

Cultivar	Fertilizer	α-Pinene	Camphene	β-Pinene	Myrcene	*p*-Cymene	Eucalyptol	Linalool	Camphor	Borneol
Blue Rain	Inorganic	1.35	1.01	0.90	0.97	0.76	0.77	0.94	0.83	0.94
Fota Blue		1.30	1.16	0.91	0.95	0.89	0.94	0.82	1.33	0.74
Green Ginger		0.98	1.00	1.07	0.81	0.68	0.98	0.77	1.00	0.76
Haifa		0.86	0.72	0.78	0.78	0.82	0.90	0.80	0.97	0.93
Lady in White		0.98	0.61	0.73	0.87	0.99	0.77	1.15	0.90	1.16
Primley Blue		1.01	0.82	1.08	0.64	0.75	0.91	0.64	1.04	0.65
Roseus		1.19	1.01	0.88	0.84	0.36	0.72	0.64	0.79	0.66
Severn sea		0.91	1.05	0.86	1.13	0.83	0.76	1.20	0.58	0.83
Sudbury Blue		0.89	1.00	0.91	0.66	0.89	0.93	1.05	1.09	1.14
Blue Rain	Seaweed	1.08	0.92	1.07	0.86	0.70	0.95	0.88	0.78	0.88
Lady in White		0.21	0.30	0.21	8.74	0.66	0.45	2.13	0.48	0.11
Fota Blue		1.13	2.03	1.33	2.93	0.85	1.34	0.69	0.89	0.66
Green Ginger		1.14	0.81	0.29	0.46	0.41	0.45	0.54	0.84	1.40
Haifa		2.38	0.92	6.56	0.03	1.10	1.74	0.51	2.28	3.24
Primley Blue		1.21	1.11	1.04	0.70	0.74	0.90	0.55	1.22	0.60
Roseus		1.21	1.02	0.93	0.83	0.41	0.73	0.64	0.94	0.67
Severn sea		0.85	1.10	0.92	1.20	0.96	0.78	0.85	0.80	0.60
Sudbury Blue		0.88	0.84	0.83	0.59	0.80	0.85	0.96	0.98	0.89
Average of difference		**1.09**	**0.97**	**1.18**	**1.33**	**0.75**	**0.88**	**0.88**	**0.98**	**0.94**

5.4 Discussion

Rosemary plants usually yield more material from frequent regrowth after being harvested for leaves or essential oil. Harvest is once or twice a year depending on the geographical area, method of harvest, and the purpose of harvest i.e. if it is for plant material or essential oil (DAFF, 2012). In general, harvest can be performed at an interval of 3 to 4 months, or annually giving three to four harvests. As a result, the different stages of the plant life cycle and harvest time significantly affect the chemical composition and yield of essential oil in rosemary. This effect is represented in the highest oil yields during the fruiting period (summer), with different concentrations of many compounds, such as camphor and α-pinene compared with winter. Further, the largest number of rosemary leaves fall just before autumn during August and September, and determined the existence of a strong relationship between the seasonal change of carnosic acid concentration and both air temperature and photoperiod which change according to the seasons of the year (Hidalgo *et al*., 1998; Munné-Bosch *et al*., 2000). Moreover, the effect of the harvesting date on essential oil content of rosemary was identified, the highest percentage of the component changed depending on the time of harvest, the best oil yield being achieved with a rise in temperature and longer photoperiod in summer (Leithy *et a*l., 2006; Miguel *et al*., 2007). The components are variable in leaves of rosemary depending on collection time (before, after and during blooming) (Emadi *et al*., 2007).

Based on the above results, rosemary plants were demonstrated to be highly responsive to different times and percentage of harvest. The harvest regime shown that older leaves gave the lowest yield of oil compared with younger leaves. The frequent harvest leads the plant to produce new leaves. So, the oil yield differed significantly among plants harvested in different times as a result to the age of the leaves. The different stages of the plant life cycle affect the chemical composition of essential oil in rosemary. While, the percentage of harvest leads to tiny different between the plants.

These results were similar to these of Leithy *et al.* (2006) who got different results depending on the harvest time (March, September and February), especially when this was “interacted” with other treatments. Also it agrees partly with Santos Atti-Santos *et al.*

(2005). Socaci and Socaciu (2008) stated that cultivated rosemary under the same conditions, but in different ages, will lead the plants to produce different oil compositions.

There are many plants that are used for their essential oil and extracts in for example food processing, the pharmaceutical industry and the perfumery sector. The liquids produced are sources of natural aromas and flavourings (Friedman *et al.*, 2002) and may have medicinal properties. Due to this importance of oil, it needs to be analysed and identified carefully in order to obtain the right identification compounds. The analysis methods and techniques which are used for identification of compounds can impact the chemical composition of the plants. For example, temperature program, column, solvents etc..., all these could change in oil composition in terms of GC-MS (Socaci and Socaciu, 2008).

Our results show that there is a difference between the two techniques. The most marked difference between GC-MS and NMR analysis is in the undetermined compounds (other compounds which are not included in the analysis) which overlapped with the main compounds in NMR method. These results in agree with Chatham *et al.* (2003) who stated the same reason for the difference between the two techniques.

5.5 Conclusion

This experiment shows the effect of harvest process on oil production. It links between the method of harvest and how many times harvest is performed in terms of their impact on the quantity and quality of essential oil.

The principal effect of the harvesting date on essential oil content of rosemary was identified, as being that the highest percentage of the component changed (Fig 5.5), depending on the time of harvest. The components are variable in leaves of rosemary depending on collection time.

In all cases, the harvesting time is species-specific and has to be determined according to the most favourable combination of oil composition and yield, from a commercial point of view. The percentage harvest of rosemary does not impact on subsequent harvests.

6. Chapter six: General Discussion

Previous studies have focused on single or few major components when comparing essential oils in rosemary (Boelens, 1985; Mulas and Mulas, 2005; Socaci *et al*., 2007; Itmad and Niserrn, 2014). However, the analysis of rosemary oil here revealed nine major components (and many others in smaller quantities). In general, for the genotypes used here the major components were eucalyptol, camphor and myrcene. In an attempt to further devise a relationship between oil production and treatment types, the data from the oil composition studies in terms of two groups of compounds was studied. The oxygenated group consists of linalool, eucalyptol, camphor and borneol. There is also a hydrocarbon group containing α-pinene, β-pinene, camphene, myrcene and cymene. This could be due to possible metabolic routes to the formation of these compounds although there may be more than one route to particular components for example myrcene could arise from dehydration of linalool, or directly from linalyl prophosphate as shown in Fig 6.1. In addition, there is evidence that *p*-cymene is produced from γ-terpinene (Poulose and Croteau, 1978, Mann, 1987 Zhang and Tiefenbacher, 2015) as shown in Fig 6.2, suggesting a longer biosynthetic route.

Fig 6.3 shows the relative quantity of oxygenated and non-oxygenated monoterpenes. In addition, the relative yields of oil produced are indicated (multiplied by a factor of 100 to keep on the same scale). As inspection of the figures show there is little systematic variation across the various treatments, but there is clear evidence that yield is increased by the seaweed fertilizer particularly for date of harvest after six months (H3). In terms of composition it is difficult to draw any great conclusions but is clear that the oxygenated components are present in larger amounts at least for the genotype studied in Chapter two.

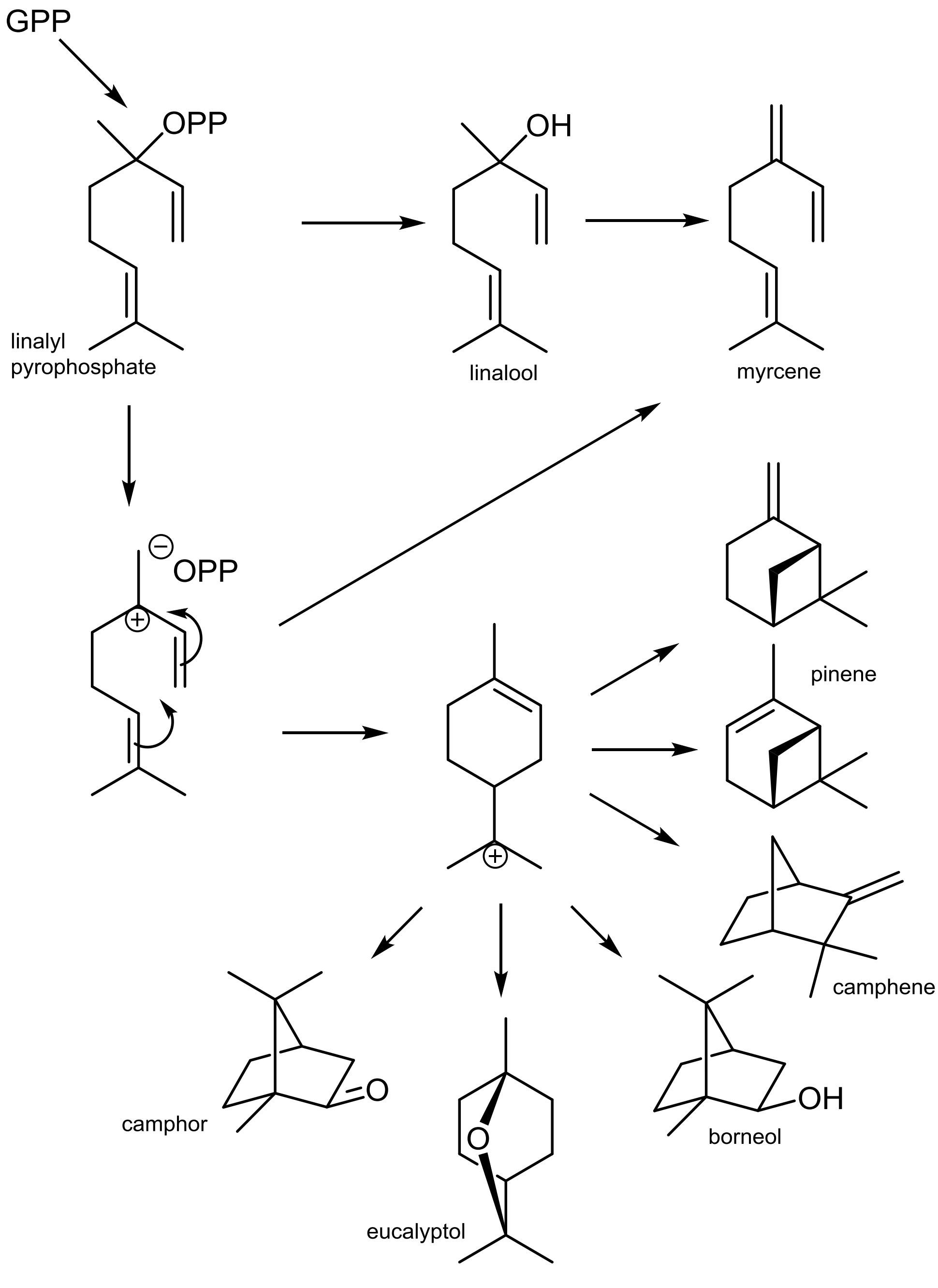

Figure 6. 1 myrcene arises from dehydration of linalool, or directly from linalyl pyrophosphate

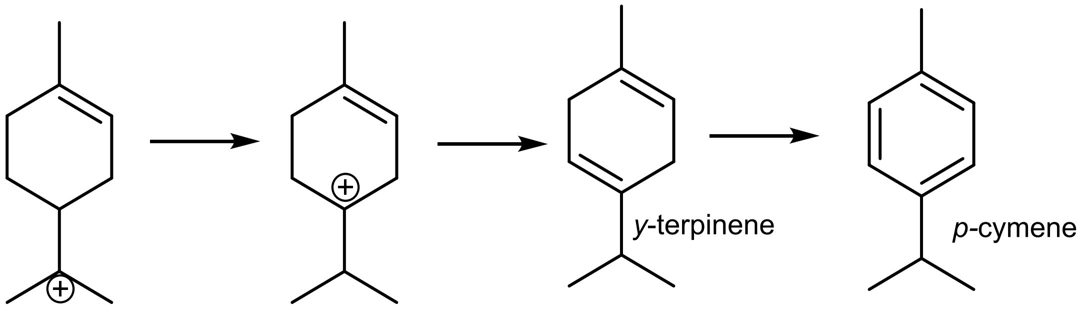

Figure 6. 2 *p*-cymene synthesis

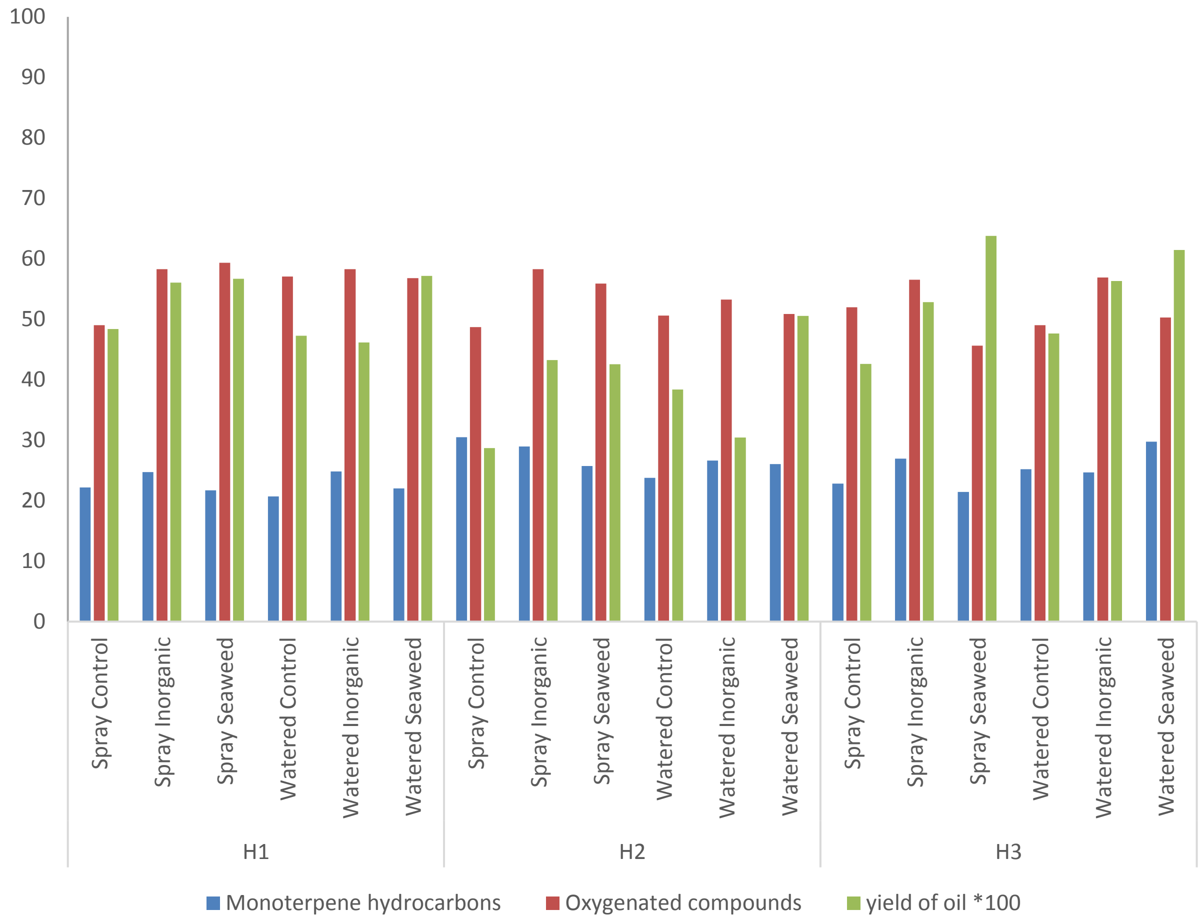

Figure 6. 3 the relative amount of oxygenated, non-oxygenated monoterpenes and yield of oil of experiment in Chapter 2

The question remains why yields can be affected by conditions and yet the chemical components of the essential oils change but in an unremarkable fashion. The possible reason for this lies in the potential routes for biosynthesis of the individual compounds shown in Fig. 6.1 Linalyl pyrophosphate may be rapidly converted into most of the major components found in these studies. The formation of the α-terpinyl cation will readily convert to α-pinene, β-pinene, camphene and by hydration to eucalyptol. Thus, all these compounds have the potential to be formed. Any slight differences in the trends revealed may reflect the formation of more of the many minor compounds which makeup the remaining 20-25% of the essential oil samples (90 different compounds were found).

As with the results described in Chapter 2, the data from Chapter 3, which shows a range of properties of the plant in response to the addition of different cytokinins, indicated some small variations in terms of the essential oil yields and composition. That being said it would seem that the plants response to seaweed fertilizer as opposed to the inorganic mixtures with variations cytokinins showing the same pattern. It was particularly noticeable that for the seaweed system, myrcene and eucalyptol appeared to be lower than observed in the cytokinin-containing systems. The analysis applied earlier to essential oil composition, namely considering hydrocarbons and oxygenated hydrocarbon monoterpenes was extended to data on page 92 (Table 3.5). The results of our analysis are shown in Fig 6.4.

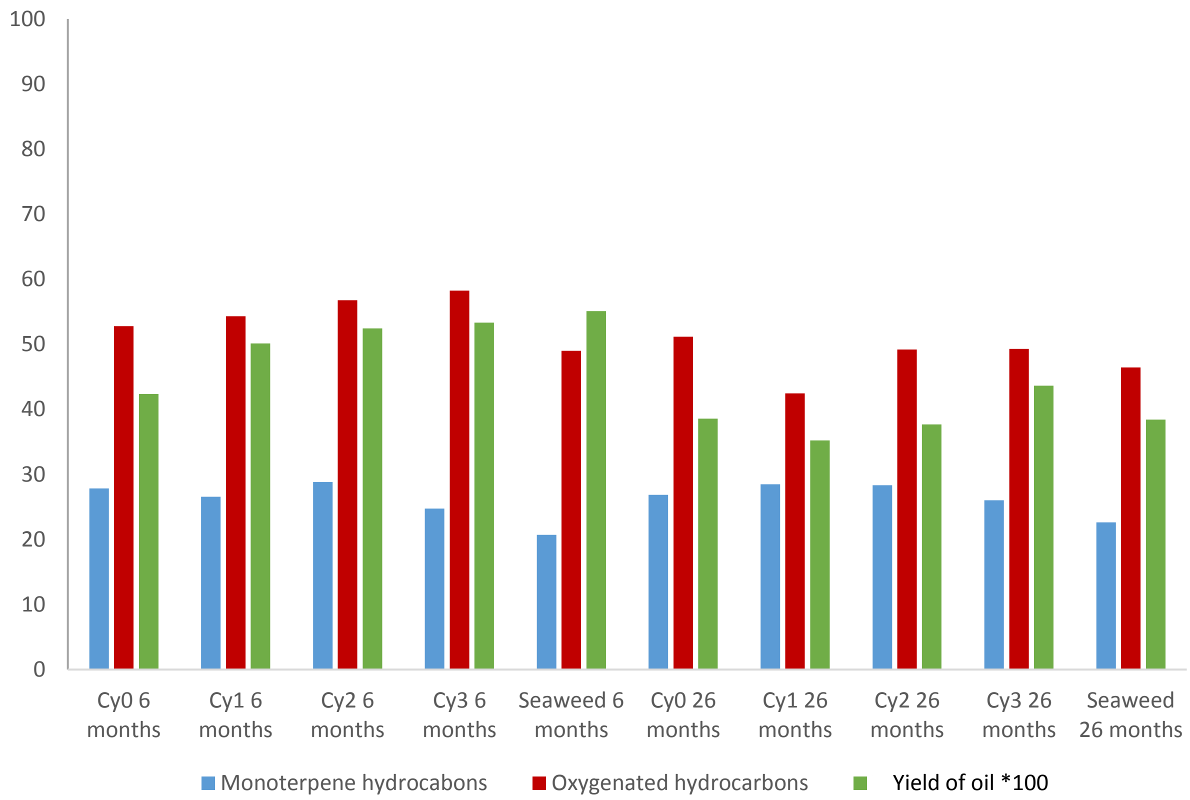

Figure 6. 4 the relative amount of oxygenated, non-oxygenated monoterpenes and yield of oil of experiment in Chapter 3

Fig 6.4 shows that in general oil yields are reduced in the older plants. This is reflected in a general decrease in both the hydrocarbon and the oxygenated monoterpenes, but for the seaweed system changes in eucalyptol and myrcene content appear to deliver a lower ratio of hydrocarbons monoterpenes to oxygenated monoterpenes. Once again referral to the potential biosynthesis of these materials suggests that the results for oil composition are driven largely by the relative similarity of all the secondary metabolic produced here. However, it would seem that conditions can be changed to alter yields.

This obviously means that from a commercial point of view it could be possible to increase yields in an economically significant way, without changing oil quality.

In Chapter 4, we looked at the effect of fertilizer on different cultivars. Here it was particularly noticeable that extreme differences there was as in terms of essential oil content for the different cultivars. Using the analysis applied earlier comparing “oxygenated” to non-oxygenated monoterpenes (Fig 6.5) some show more oxygenated components while others show more non-oxygenated. For ‘Severn Sea’ and ‘Sudbury Blue’ the major component, by a long way was α-pinene. Clearly, whatever the nature of the oxidation process, it is less effective in oil composition in these two cultivars. In terms of the different fertilizers, effects were noted but are in no way comparable to the changes correlating to genotypic differences among cultivars. Oil yields can be improved by changing the way the plant is grown. This has the useful consequence that by selecting the genotype to provide the desired oil composition, intervention in terms of fertilizer can improve yields without significant changes to oil quality.

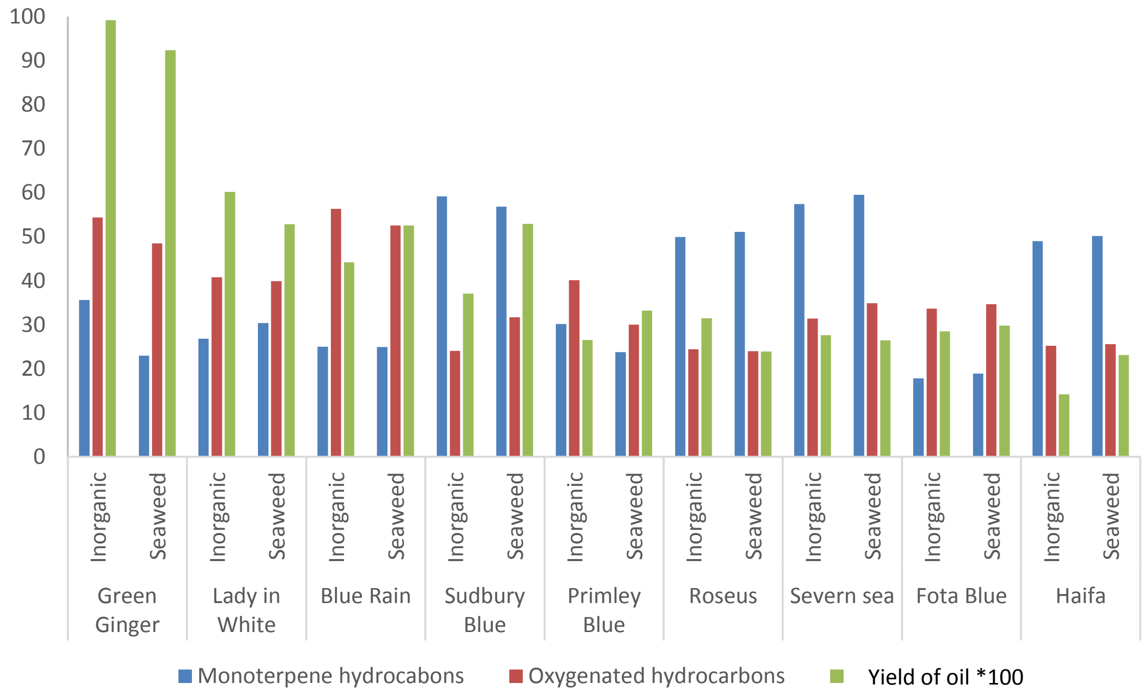

Figure 6. 5 the relative amount of oxygenated and non-oxygenated monoterpenes and yield of oil of experiment in Chapter 4

Comparison of NMR data and that from GC-mass spectrometry showed significant differences in the data for Chapter two, where instrument availability led to delays in GC-MS analysis. The data shown in Table 5.2 shows the ratio of GC-MS values to NMR in terms of percentage oil content. The table shows significant variation between the two techniques. This may in part because NMR has been less sensitive than GC-MS in characterisation, but in view of the improved agreement for the aged samples, we suspect that there may substantial changes to the oil content during storage. Table 5.3 also shows data for Chapter three where better agreement between the two techniques is apparent. We believe GC-MS to be the more precise methodology with errors only in the region of 10%. For NMR there are problems with baseline drift, overlapping peaks and other instrumental problems that restrict the accuracy to at best 10% and some components appeared to be more difficult to quantify accurately than others (possibly reflecting the type of proton monitored). However, NMR does allow immediate identification of components and can be performed very rapidly.

Percentage of harvest can be an influential factor in crop production. It works as an intrinsic factor affecting the production of plants. Thus, different percentages of harvest shown a significant effect on production of plants. This increase in yield is due to the new leaves produced by the plant after harvest.

6.1 Conclusion

This project has developed novel techniques for analysis of essential oils utilising NMR and GC-MS. Rosemary was used as a model system to examine the effects of fertilizer type and the application method upon essential oil yield. It is important to know the factors that influence essential oil production in order to fully understand the response of the plant to these factors in terms of oil composition and yield. Different rosemary genotypes were also analysed in order to examine the variation of oil composition amongst Rosemary cultivars.

The application of fertilizer promotes vegetative growth and production in all rosemary plants. It was shown here that there is a difference in the response of rosemary to different types of fertilizer. Seaweed fertilizers and matching inorganic alternatives developed the growth and yield significantly. In general, the application of seaweed increased the growth and yield of oil more than inorganic fertilizer, however both increased yield more than the water-only control. On the other hand, fertilizer effects do not have sufficient capacity to change the chemotype of oil. Plant growth substances (hormones) found in seaweed fertilizer had a significant impact on plant growth and production. The method of applying the fertilizer influenced the response of leaf area and subsequent oil yield depending upon the type of fertilizer, date of harvest and age of the plant (Tawfeeq *et al.*, 2016). The spray method was more effective for inorganic application when harvested at three monthly intervals compared with harvesting after six months. The percentage of plant material harvested shows a difference in quantity of oil production only. Cytokinin application improves the production and growth in both young and old plants. Although age has an impact on the quantity of oil, ageing plants had lower oil production compared with young plants.

The genotype effected the composition and yield of essential oil. It was shown that plants with different genotypes (cultivars) show variation in chemotype and different responses to the application of fertilizer. Cultivar choice should be a primary consideration when growing commercial herbs for oil production. The techniques developed throughout this project will allow commercial growers to select cultivars in order to gain higher yields of particular compounds in the future.

6.2 Suggested work for future research

1- A field trial using the same fertilizers could confirm the extent to which other environmental factors (such as higher UV) might influence oil production.

2- The other contents of seaweed extract such as the other types of growth regulators and alganic acids could be tested in order to further identify the active contents in this type of fertilizer.

3- The effect of storage time and conditions on the oil mixture would help users understand how best to market these oils for particular purposes.

4- The effect of other growth regulators on oil quantity and quality in a range of genotypes of rosemary could further aid decision making in commercial crop production.

7. Chapter seven: References

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