Does fertilizer type and method of application cause significant differences in essential oil yield and composition in rosemary (Rosmarinus officinalis L.)?

Does fertilizer type and method of application cause significant differences in essential oil yield and composition in rosemary (*Rosmarinus officinalis* L.)?

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ABSTRACT

Organic fertilizers based on seaweed extract potentially have beneficial effects on many crop plants. Here we investigate the impact of organic fertilizer on *Rosmarinus officinalis* measured by both yield and oil 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 NMR. The chemical compositions of the plants were compared, and qualitative differences were found between fertilizer treatments and application methods. Thus sprayed seaweed fertilizer showed a significantly higher percentage of β-pinene, α-phellandrene, α-terpinene (monoterpenes) and 3-methylenecycloheptene than other treatments. Italicene, α-bisabolol (sesquiterpenes), α-thujene, and E-isocitral (monoterpenes) occurred in significantly higher percentages for plants watered with the seaweed extract. Each was significantly different to the inorganic fertilizer and to controls. 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.

Keywords: Seaweed, Essential oil, Rosemary, Organic fertilizer

1. Introduction

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. The oils are often found as droplets of fluids under the surfaces of leaves and bark in secretory cavities of plant-cell walls or in glandular hairs (Koul et al., 2008; Prins et al., 2010). The flora of the Mediterranean basin has a very high proportion of aromatic plants and thousands of hectares have been dedicated to their production in countries bordering the Mediterranean Sea. The amount of material produced is about 38 million tonnes per year with Turkey being the highest producer (Viuda-Martos et al., 2007). The genus *Rosmarinus* or Rosemary a member of the Lamiaceae family is one of the plants used for essential oil production comprises of three species; the most productive species, *Rosmarinus officinalis* L., has been widely cultivated since antiquity as herb and garden plant, and also for its essential oil (Port et al., 2000).

The essential oils of Rosemary are used in various industry sectors and are commercialised, since they show a range of properties, for example, they may be antibacterial, antioxidant, antifungal, anti-inflammatory and recently, they have been used in pest control products (Koul et al. 2008 & Derwich et al., 2011). Due to the importance of essential oils, many studies have been conducted to identified the wide variety of components, and how composition of oils vary as a result of external factors; such factors include climate and habitat conditions, planting, harvesting stages and methods, and internal conditions in the plant, such as genetics and plant age (Viuda-Martos et al., 2007; Jamshidi et al., 2009; Gurusaravanan et al., 2010; Derwich et al., 2011; Singh & Guleria, 2013). These studies have shown the essential oil components and yield can be varied in a number of ways. The origin of the variation of course is less clear cut. However, it is possible the conditions may directly influence secondary metabolite biosynthesis, or it may be that changes in other biological process have an indirect effect. This later 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).

Recently there have been several attempts to determine the impact of growing conditions on yield by controlling a range of factors and monitoring their impact on cultured plants. Fertilization is one of these factors, because plants respond in a distinct (and relatively well-understood) fashion to the application of nutrients, as well as the methods of application. A Fertilizer that has been widely used recently is one based on marine algae, (seaweed) which is commonly known as Seaweed Liquid Fertilizer (SLF) (Selvam & Sivakumar, 2014). Seaweed consists of large marine algae that anchor to the sea bed and are considered a good source of nutrients and natural growth regulators for plants; in addition, seaweeds are non-polluting, biodegradable, non-hazardous and non-toxic (Dhargalkar& Pereira , 2005; Gurusaravanan et al., 2010).

While there have been a number of investigations on the effect of inorganic fertilizer on the yields and oil composition of rosemary oil, there is a dearth of information available on the effect of organic fertilizer, and especially seaweed. The present study reports the growth, yield and oil composition of rosemary using a seaweed extract (organic) and compares this with a model system based solely on the mineral (inorganic) content of the seaweed fertilizer.

2. Materials and methods

Approximately 10-15 cm shoot-tip cuttings were made on 20th November 2012 from a mature plant of Rosemary *(R. officinalis* L.), the new cuttings were grown inside a greenhouse at the Research Station of the School of the Biological Sciences at the University of Reading 51°26'12.0"N 0°56'34.1"W. The cuttings were placed in 1.0 L nursery containers (ten cuttings per pot) filled with 20% compost and 80% Seramis granules; a propagation medium for rooting, after removing the leaves from the lower third of each cutting; these were grown under confined environment conditions in a greenhouse with natural daylight and watered with equal amounts of water by a dripping irrigation system. Three months later, rooted cuttings were pruned by removing about 1-2 cm of the shoot tips; then plants were transplanted into 0.5 L separate plastic containers (one plant per pot) filled with 1:1 combination of horticultural grit and JI no.2 compost for another three months, when they were transferred to 3L pots with the same ratio of growth media.

The experiment was laid out in a Complete Randomized Design (CRD) as a factorial experiment with 7 replicates. Two kinds of fertilizers (organic and inorganic) and a control (water) were in two ways; directly to the leaves, and to the soil. Each treatment had 7 replications (pots) distributed randomly across experimental units as shown in (Table 1).

Table 1. Different treatments combinations and applied nutrient methods under different treatments

Treatments [a]	Water	Organic fertilizer	Inorganic fertilizer	Spray	Pour to soil
SC	Yes			Yes	
WC	Yes				Yes
SO		Yes		Yes	
WO		Yes			Yes
SI			Yes	Yes	
WI			Yes		Yes

[a] WI: watered inorganic, SI: Spray inorganic, WO: Watered organic, SO: Spray organic, WC: Watered control (water), SC: Spray control (water)

'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 species of wild algae *Ascophyllum nodosum* , *Sargassum* and *Laminaria*. This product was used in the experiment as a source of organic fertilizer due to the presence of macro and micro elements that are important for plants growth (Table 2).

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

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%

The Inorganic fertilizer was prepared using a process which produced the 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 in the required proportions 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; to avoid precipitation the materials present in the highest concentration were added first. The final solution's pH was 8.85 and this was stored in a refrigerator, there was no observable solid material.

Plant growth parameter measured in different ways depends to the measurement. Leaf area taken by using *WD3 - WinDIAS* Leaf Image Analysis System. For measuring biomass, the basic technique is weigh the leaves before and after drying in an oven to remove water. It's a percentage of dry weight compare to fresh weight of the leaves.

For the extraction process, the leaves and twigs with young and fresh branches were obtained from each treatment of cultivated plants at harvest (100 days after planting) and 100 g (Rahman et al., 2007; Jamshidi et al., 2009) were submitted to a Clevenger apparatus after cutting into small pieces using a blender (fresh weight were recorded in each plot).

The identification of the essential oil was performed using Gas Chromatography with Mass Spectrometry by the Thermo scientific system, and SLB-5 ms fused silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The carrier gas was Helium at 1 ml/min. The oven temperature was kept at 50˚C for 1 min, followed by 50-101˚C at a rate of 3˚C/min, then followed by 101-300 at a rate 15˚C/min. An example of the traces obtained is shown in Figure 1a.

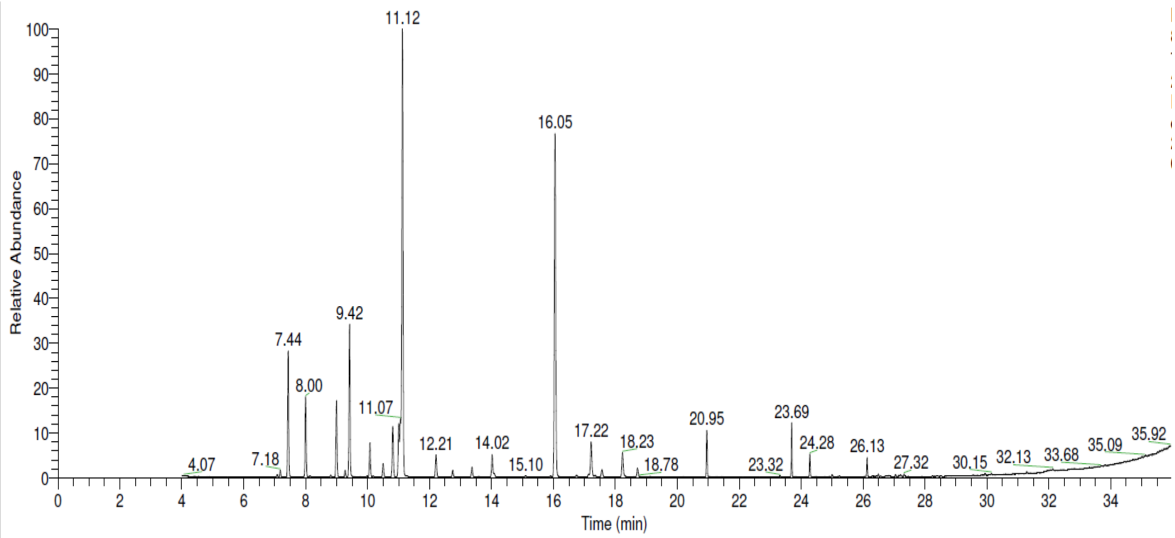


Figure 1a. GC mass spectrometry trace obtained from Rosemary extract (SI)

Meanwhile, Nuclear Magnetic Resonance spectroscopy (NMR) has been used as an alternative method to identify the major components of the essential oil. 20 mg of essential oil was dissolved in 1 ml of chloroform-d ($CDCl_3$) containing 0.5 mg of 1,4-dibromobenzene. The latter component allowed a direct quantification of the NMR spectrum by a comparison with the integrated signal from the 4 equivalent aromatic hydrogens. The data was processed using *Topspin* software from Bruker. A typical example of the spectra obtained is shown in Figure 1b.

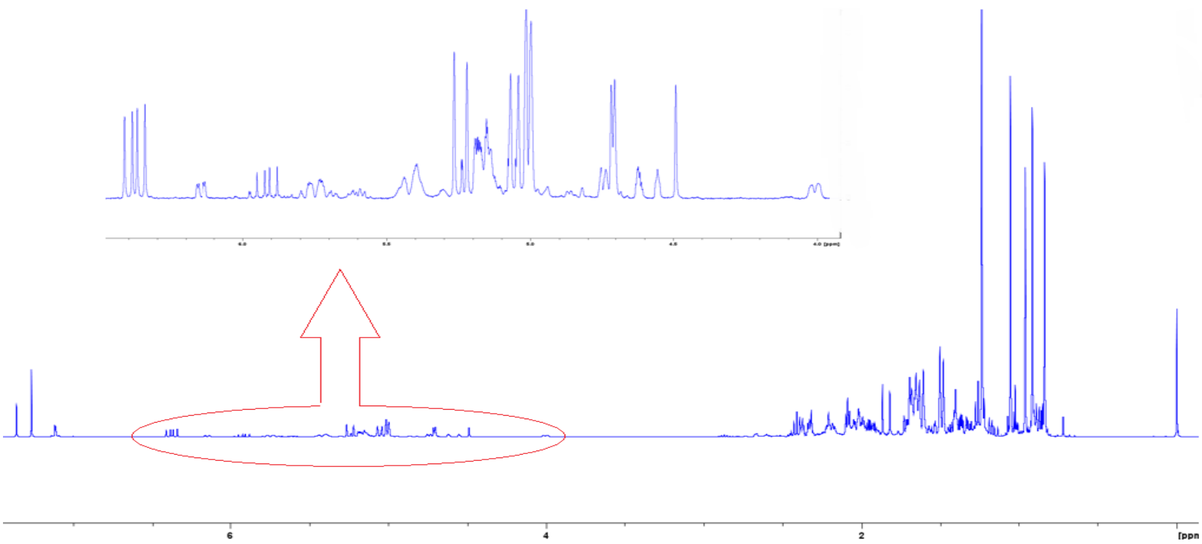


Figure 1b. NMR trace obtained from rosemary oil extract

In each analytical technique, the main oil components (pinene, camphor *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 mass spectrometry these were used to directly compare concentrations.

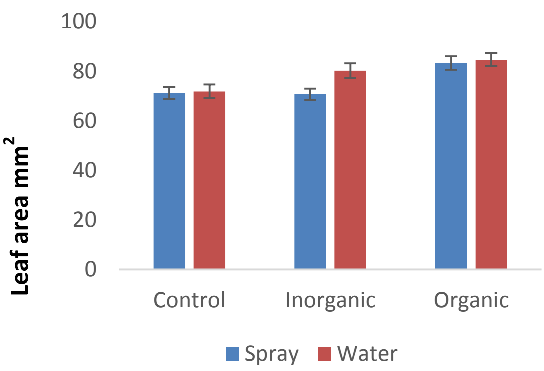
For statistical analysis; percentages were normalized by arcsine transformation as needed. In order to investigate the interrelationships between essential oil quantity, quality and crop growth parameters, the independent effects of each combination of variables was evaluated with ANOVA by using *Genstat* software (Payne et al., 2009), considering each experimental condition as the "group variable". The analysis compared the effects of organic, inorganic, and control treatments and between sprayed and watered methods of application. The LSD (least significant difference) was used to account for variation between these factors.

3. Results

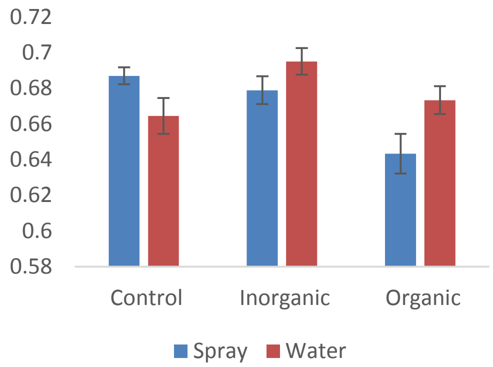
3.1. Plant growth

The effect of fertilizers on growth parameters (leaf area and leaf biomass) was recorded from *R.officinalis* L. plant in an experiment to establish if there was any simple relationship with oil production. Seaweed extracts (organic) and the model (Inorganic) were applied as a spray (S) over the whole plant or were applied directly to the soil (watered W). The results are shown in Figures 2a and 2b; Figure 2a shows that significant increases in leaf area were found for the spray/organic, watered/organic and watered/inorganic (83.3, 84.6, and 80.2 mm^2 respectively) as compared with the control (C) (71.4 and 71.8 mm^2 for spray and watered treatments respectively). Of all the samples, the spray inorganic showed the minimum leaf area (70.7 mm^2).

In terms of leaf biomass, as Figure 2b shows, the spray organic fertilizer gave significantly the lowest amounts (36.07%) compared with the watered inorganic fertilizer and spray water (control). The watered (control) showed a significantly lower weight of leaf biomass (38.06%) than the watered inorganic fertilizer and spray control. Inorganic fertilizer watered to the soil showed the highest value (41.20%) of biomass compared with other treatments. Whereas, spray inorganic fertilizer (39.44%), watered organic fertilizer (39.33%) and spray control did not show any significant difference with all treatments



Biomass %



P<0.05 L.S.D= 2.306

P<0.001 L.S.D= 7.6

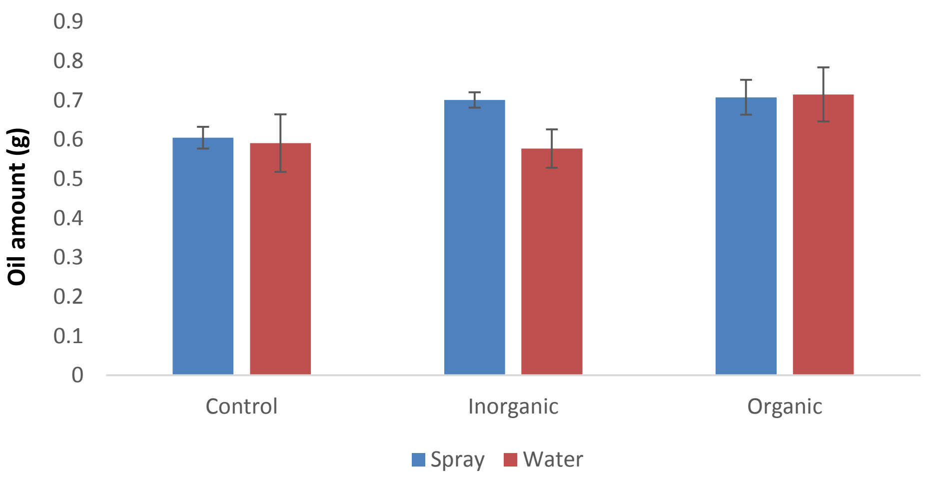
Figure 2a. Influence of seaweed and inorganic fertilizer and different methods of application on leaf area of *Rosmarinus officinalis* L.

Figure 2b. Influence of organic (seaweed) and inorganic fertilizer and different methods of application on % biomass of *Rosmarinus officinalis* L.

WI: watered inorganic, SI: Spray inorganic, WO: Watered organic, SO: Spray organic, WC: Watered control (water), SC: Spray control (water)

3.2. Oil yield

The volume of essential oil obtained from rosemary plant differs, depending on the type of fertilizer and, for a particular fertilizer, the method of application as shown in Figure 3. Both treatments of seaweed fertilizer showed significantly higher yields of essential oil (measured in terms of grams per 100g fresh material) than the watered inorganic treatment. The Supply of seaweed extract as an organic fertilizer increased the oil yields from rosemary plants slightly (0.723 Spray and 0.716 watered g per 100 g fresh material) as compared with other treatments, although the only significant comparison is with the watered inorganic fertilizer (0.577 g per 100 g fresh material). The control treatments (0.605 spray, 0.591 watered g per 100 g fresh material) and spray inorganic fertilizer (0.701 g per 100 g fresh material) did not differ significantly compared to other treatments.



P<0.005 L.S.D= 0.1248

Figure 3. Influence of organic (seaweed) and inorganic fertilizer and different methods of application on % oil amount (g per 100 g fresh material) of *Rosmarinus officinalis* L.

The results showed vary in differences among the treatments on the characteristics of the plants. As some of the treatments affected positively on some of the qualities; it affected negatively on other, such as the different between spray and watered inorganic fertilizer on leaf area and oil amount.

3.3. Oil composition

Treatment by spraying seaweed fertilizer produced statistically significantly higher percentages of β-pinene (7.95%), α-phellandrene (2.39%), α-terpinene (1.01%) (monoterpene) and 3-methylenecycloheptene (0.189%) than the control, inorganic and watered organic fertilizer. Italicene (0.224%), α-bisabolol (0.335%) (sesquiterpene), α-thujene (0.702%), and E-isocitral (0.233%) (monoterpene) were significantly distinguished by higher a percentage under the treatment of watered seaweed compared to the control, inorganic and spray organic fertilizer (Table 3). On the other hand, watered inorganic fertilizer showed a significantly higher percentage of δ-2-carene (1.394%) (monoterpene) than the control, organic fertilizer and spray inorganic fertilizer. The Spray inorganic fertilizer showed a significantly higher percentage of 10.10-dimethyl-2,6-dimethylenebicyclo[7.0.2]undecan-5 β-ol (0.162%) (sesquiterpene) than the control, organic fertilizer and watered inorganic fertilizer treatments. At the same time the spray water as control showed a significantly higher percentage of α-pinene (6.596%), α-terpineol (2.027%), *p*-cymene (1.005 %) (monoterpene) and (E)-caryophyllene (1.69%) (sesquiterpene) than the organic, inorganic and watered water (control) treatments. The full results are shown in Table 3.

4. Discussion

Seaweed extract can be good sources of nutrients for crop production. It works as an intrinsic factor affecting the production of plants. Thus, this treatment has grown in popularity and led to development and production of a large number of crops. The increase in oil production under the treatment of seaweed extract application, as compared with either the control or the inorganic fertilizer treatment as observed in this study suggests there are different effects of fertilizers in this plant.

The production of essential oil is dependent on the physiology of the whole plant, particularly the development state of synthesizing tissue and metabolic processes. The effect of organic seaweed fertilizer is, it has been suggested, 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). These contents will positively affect cellular metabolism and beneficially condition the plants following root elongation and root formation. This has the consequence of improving bud and cell division to give larger vegetative growth and increasing the number of glands. 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. In addition, leaves which contain less chlorophyll and less developed chloroplasts lead to effective stomatal closure, which restricts gas exchange in the photosynthetic process.

Table3. Influence of organic (seaweed) and inorganic fertilizer and different methods of application on essential oil composition (expressed as a percentage of total oil extracted) of *Rosmarinus officinalis* L.

Compounds	**WI**	**SI**	**WO**	**SO**	**WC**	**SC**	***P* value**
Camphor	23.203 **a**	24.178 **a**	15.557 **b**	24.1 **a**	27.191 **a**	23.122 **a**	<0.05
1,8-Cineole	27.171	23.263	22.311	23.791	24.63	21.284	>0.05
Myrcene	9.547	9.466	10.2005	8.7085	8.59	11.031	>0.05
β-Pinene	4.339 **b**	5.7 **ab**	5.98 **ab**	7.952 **a**	6.442 **ab**	5.51 **ab**	<0.05
α-Pinene	5.105 **b**	3.915 **c**	4.147 **c**	5.094 **b**	3.41 **c**	6.596 **a**	<0.001
Camphene	2.5	3.6	4.01	4.15	2.65	4.25	>0.05
Borneol	3.228 **b**	4.097 **a**	2.505 **b**	3.813 **a**	4.232 **a**	2.698 **b**	<0.05
Linalool	2.664 **b**	2.996 **ab**	2.592 **b**	3.010 **a**	3.244 **a**	2.667 **b**	<0.05
γ-Terpinene	1.735	2.837	4.884	1.366	1.394	1.357	>0.05
Bornyl acetate	2.522	2.643	2.552	2.2165	2.065	1.25	>0.05
α-Phellandrene	1.771 **ab**	1.288 **b**	1.896 **ab**	2.39 **a**	1.493 **b**	2.034 **ab**	<0.05
α-Terpineol	1.731 **ab**	1.48 **ab**	1.126 **bc**	0.793 **c**	1.883 **ab**	2.027 **a**	<0.05
Terpinen-4-ol	0.452 **b**	1.5175 **ab**	0.991 **b**	1.617 **ab**	1.751 **a**	1.452 **ab**	<0.05
(E)-caryophyllene	0.83 **b**	0.89 **b**	1.15 **ab**	0.613 **b**	0.69 **b**	1.69 **a**	<0.05
δ-2-Carene	1.394 **a**	1.233 **ab**	1.065 **ab**	0.777 **ab**	0.340 **b**	0.107 **b**	<0.05
Verbenone	0.44 **b**	0.71 **a**	0.3 **b**	0.725 **a**	0.918 **a**	0.673 **a**	<0.05
(Z)-Caryophyllene	0.725 **ab**	0 **c**	0.995 **a**	0.592 **b**	0.585 **b**	0 **c**	<0.05
α-Thujene	0.323 **b**	0.365 **b**	0.702 **a**	0.389 **b**	0.269 **b**	0.361 **b**	<0.05
α-Terpinene	0.153 **b**	0.09 **b**	0.435 **b**	1.01 **a**	0.295 **b**	0.103 **b**	<0.05
Sabinene	0.211 **bc**	0.316 **bc**	0.117 **bc**	0.033 **c**	0.749 **a**	0.535 **ab**	<0.05
p-Cymene	0.274 **b**	0 **c**	0.296 **b**	0 **c**	0 **c**	1.005 **a**	<0.05

Pinocarvone	0.249	0.267	0.219	0.123	0.288	0.245	>0.05
α-Bisabolol	0.181 **b**	0.199 **b**	0.335 **a**	0.137 **b**	0.095 **b**	0.232 **a**	<0.05
Limonene	1.063 **a**	0 **b**	0 **b**	0 **b**	0 **b**	0 **b**	<0.05
Italicene	0.115 **b**	0.074 **bc**	0.224 **a**	0.058 **c**	0.05 **c**	0 **c**	<0.05
E-Isocitral	0 **c**	0.049 **b**	0.233 **a**	0.0285 **b**	0 **c**	0 **c**	<0.05
3-Methylenecycloheptene	0.090 **b**	0 **c**	0.0295 **b**	0.189 **a**	0 **c**	0 **c**	<0.05
10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0] undecan-5β-ol	0 **c**	0.162 **a**	0 **c**	0 **c**	0 **c**	0.038 **b**	<0.05
Total	**87.6105**	**91.8675**	**86.011**	**94.3805**	**93.9995**	**90.2305**	

**Different letters within each row indicate significantly different means among treatments according to the ANOVA test at $p < 0.05$. **WI: watered inorganic, SI: Spray inorganic, WO: Watered organic, SO: Spray organic, WC: Watered control (water), SC: Spray control (water). **The percentage retention is calculated as considering as 100% the percentage of each compound in the fresh samples.

The watered inorganic fertilizer, in terms of oil amount, did not show any significant effect compared with control treatments, on the contrary, spray and watered organic fertilizer and spray inorganic fertilizer were different significantly with control and with watered inorganic treatments. This is may be due to the low level of nutrients supplied to the plants through this method of application using inorganic fertilizer which affected on biosynthesis in the plants activity (Miguel et al., 2007).

There is a dramatic decrease in the leaf biomass for the plants sprayed with organic fertilize compared with all other treatments. Leaf biomass of the plant integrates density and leaf thickness and is considered a measurement of the presence of sclerophylly (Grubb, 2002). 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. Leaf biomass 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 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 et al., 2001). Most of the changes in essential oil composition occur during the supply to the plants of seaweed extract. These changes are due 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), increasing chlorophyll content and thus increasing the carbohydrates (Thirumaran et al., 2009). Numerous studies have demonstrated the role of light in changes to the composition of essential oil. It has been stated that essential oil produced in young leaves which have less leaf mass is affected directly by photoperiodic modulation of the monoterpene synthesis pathway. Eris et al (1995) have suggested that selectively stimulating certain pathways of metabolism may be beneficial for variation in oil composition; this due to concentration of some nutrient elements. This can also be as a result of the involvement of phytochrome (a pigment that plants use to detect light) in the process of manufacturing essential oils and exposure of the plant to a specific wavelength will lead to increased production (Mulas et al., 2006), because light quality has the greatest impact on the oil synthesis from exogenous primary precursors, and the composition within the plant (Sangwan et al., 2001).

Methods of application have shown slight difference between treatments. Foliar fertilizer has raised oil amount due to a more rapid absorption of nutrients directly to the location of demand in the leaves (Mondal & Mamun, 2011). Foliar fertilizers as chelates should be easily absorbed by the plants, rapidly transported, and should easily release their ions to affect the plant (Larue & Johnson, 1989).

5. Conclusion

This study on fertilizer type and method of application showed significant differences in essential oil yield and composition in rosemary (*R. officinalis* L.). The quality and quantity of rosemary essential oil varied with the different fertilizers: organic and its inorganic equivalent. Seaweed as an organic fertilizer applied to the plant showed clearly defined results in all aspects of growth and yield unlike the inorganic fertilizer. 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.

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