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Seaweed extract enhances the biochemical and essential oil compounds in sage (*Salvia officinalis* L.)

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- ✓ GC-MS.

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Abstract

The effect of seaweed extract (SWE) of *Enteromorpha intestinalis* (E), *Fucus spiralis* (F) and *Ulva rigida* (U) on sage plants was studied. Plants were cultivated and treated with SWE by foliar spray. The results show an improvement in the number of leaves. Similarly, a significant increase in chlorophyll (a) and (b) was observed in plants treated with *Fucus spiralis* extract. The phenol content of treated plants is improved, but plants treated with 1% *Enteromorpha intestinalis*, causes a decrease in the total phenol content. Moreover, the treatment with seaweed extracts shows an improvement in some essential oil's compounds. Essential oil (EO) analyzes by gas chromatography coupled with mass spectroscopy (GC-MS) identified 37 compounds. Also, the plants treated with seaweed extracts has shown remarkable effects on the levels of certain compounds (α-pinene, 1,8-cineole and camphene). SWE treatment improves the physiology and the quality of essential oil.

1. Introduction

In agriculture, algae are considered an alternative organic fertilizer, a new generation of competitive fertilizers and growth promoters [1]. Some studies indicate that seaweed extracts may partially substitute fertilizers [2, 3,4] because they contain minor and major elements, as Phosphor, Potassium Calcium, Iron, Copper and Zinc [5]. Seaweed extract plays a role of plant biostimulants for the presence of auxins, cytokinins, polyamines, gibberellins, abscisic acid and brassinosteroids [6] The saccharides existing in seaweed extracts can act as elicitor of plant defensive mechanism [7]. Various authors reported that bioactive secondary metabolites, vitamins and vitamin precursors, Betaines, Polysaccharides, Phloroglucinol and eckol of seaweed has some important effects in plant growth [6, 8].

Sage (*Salvia officinalis* L.) is a perennial aromatic and medicinal plant of the family Lamiaceae, which is widely cultivated all over the world. It comprises about 900 species 30-60 cm high, stems forming upright, hairy quadrangular branches, oval and elongate leaves, greenish gray due to cottonous pubescence on the underside, and small blue-violet flowers have distinctive aromatic camphor smell that flourish in June or July [9]. Its dried leaves are mainly used as raw material in medicine, perfumery, food industry [10], and as herbal teas and food flavorings. Thus in the cosmetic and pharmaceutical field [11].

Sage is native to Mediterranean regions, prefers light and calcareous soils and cannot withstand winter cold and prolonged periods of drought [12,13]. It is grown today in the whole world in an extensive way.

The data show that essential oil composition of sage varies significantly according to the mineral soil fertilization, climatic and environmental conditions [13,14] and the organ [15]. For this variation, the essential oil composition does not have the same profile. According to ISO 9909 standard, the official composition profile of sage essential oil it is: cis-thujone (18-43%), Trans-thujone (3-8.5%), camphor (4.5-24.5%), 1,8-cineole (5.5-13%), α-humulene (0-12%) α-Pinene (1-6.5%), camphene (1.5-7%), limonene (0.5-3%), linalool, and bornyl acetate (2.5% maximum) and linalool + linalyl acetate (≤ 1.0%) [10]. Research that has studied the effect of seaweed extracts on the quality and quantity of essential oils remains rare [16]. Among the methods used to improve the morphological characteristics and composition of essential oils in aromatic plants is the use of seaweed extracts. Studies have shown that treatment with extracts of *Ascophyllum nodosum* (brown algae) improves the essential oils composition of basil [16].

The aim of this work is to test the impact of treatment with liquid seaweed extracts (*Fucus spiralis*, *Ulva rigida*, *Enteromorpha intestinalis*) on the phenol, proteins, chlorophyll and essential oils compounds of sage, these parameters are determined in order to estimate the beneficial effect of the treatments.

2. Methodology

2.1 Preparation of seaweed extract

Seaweed extracts are prepared from three species of macroalgae: *Ulva rigida*, *Enteromorpha intestinalis* (Chlorophyceae) and *Fucus spiralis* (Pheophyceae). Were collected from the coast of Sidi Abdellah to the region of El Jadida (33° 19' 88" North and 8° 59' 19" West). The algae were handpicked and washed by sea water to remove the sand particles stuck to the thalli. These are placed in coolers and transported to the laboratory. Algae are washed with tap water at ambient temperature to remove salinity from surface, after which they are dried in open area until total dehydration. Dry matter is crushed using an electric grinder. Seaweed extracts were prepared by maceration of algal dry matter for one hour in distilled water at 100°C.

2.2 Plant material preparation and treatments

Plant tested for this study is sage (*Salvia officinalis* L.). Propagation of seedlings is carried out by cuttings. Mothers plants issued of one plant, were grown in a nursery, 23 Km south-east of Marrakech. Fragments of stems of two axillary buds and four leaves are cut from the mother plants and transplanted at levels of plates filled with peat. After 25 days, the plants are transplanted into a greenhouse in plastic containers (1.5 liters) containing a mixture of agricultural soil and rinsed sand with a ratio of 1: 1 (w / w). After 30 days, the plants are grown in a land of 680 m² in rural commune Sidi Abdallah Ghiat, 27 km south of Marrakech, for a period of 5 months (Early March to late July).

After 45 days of culture, the seaweed extracts are applied by foliar spraying (8 mL per plant) at different concentrations every 6 days, for 108 days. The plants are split into 7 lots, each lot contain 150 plants:

Lot 1: Plants sprayed with distilled water (Control 0%)

Lot 2: Plants sprayed with *Fucus spiralis* extract at 1%

Lot 3: Plants sprayed with *Fucus spiralis* extract at 2%

Lot 4: Plants sprayed with *Ulva rigida* extract at 1%

Lot 5: Plants sprayed with Ulva rigida extract at 2%

Lot 6: Plants sprayed with *Enteromorpha intestinalis* extract at 1%

Lot 7: Plants sprayed with *Enteromorpha intestinalis* extract at 2%

2.3 Measuring leaves number

Plant growth is measured based on the number of leaves.

2.4 Chlorophyll and carotenoid content

The determination of chlorophyll (a), (b) and carotenoid was carried out by measuring the optical density at three wavelengths 647 nm, 663 nm and 452 nm according to the method of Arnon [17]. The leaves (50 mg) were ground in 6 mL of 80% acetone in the dark. After centrifugation of the mill at 5000 rpm for 10 minutes, we recovered the supernatant to measure the optical density at 647 nm, 664 nm and 452 nm. We used the following formulas for calculation of chlorophyll and carotenoid content:

Chlorophyll (a) = 12.7 × OD at 663 nm - 2.69 × OD at 647 nm

Chlorophyll (b) = 22.9 × OD at 647 nm - 4.68 × OD at 663 nm

Carotenoides = 4.2 × OD at 452- [(0.0264 × Chlorophyll (a)) + (0.426 × Chlorophyll (b))]

2.5 Total phenolic content (TPC)

Grinding of 50 mg of the leaves in 1 mL of 80% methanol at 4° C. The homogenate was centrifuged at 19000 g for 20 minutes. The supernatant was used for analysis of the phenol content. The phenolic compound in extract was estimated by the method of Taga et al. [18]. 100 µL of supernatant was mixed with 2 ml of 2% Na_2CO_3 and allowed to room temperature for 2 min. After incubation, 100 µL of 50% Folin Ciocalteu phenol reagent was added and then the reaction mixture was thoroughly mixed and allowed to stand for 30 min at room temperature in the dark. The absorbance of all sample solutions was measured at 720 nm using a spectrophotometer. Gallic acid was used as a standard with a concentration range of 10 to 200 mgL^{-1}, the phenolic content was expressed as equivalent gallic acid (EGA) per mg dry matter.

2.6 Protein content

Grinding of 0.1 g of leaves in 1 mL of 50 mM phosphate buffer (pH 7.5) containing 5% insoluble polyvenylpirolidone (pvp). The extract was centrifuged at 4° C. for 20 minutes at 12 500 g. The supernatant was then used for the determination of total proteins. The reaction mixture contained 100 µL of distilled water, 100 µL of supernatant, 2 mL of the Bradford reagent. After incubation of the tubes for 5 min, the optical density is determined by spectrophotometer at 595 nm. Bovine serum albumin (BSA) was used as a standard with a concentration range of 10 to 100 mgL^{-1} [19].

2.7 Extraction of essential oils (EO)

The extraction is carried out by steam distillation apparatus. The plants are harvested, dried in the air under the shade, and weighed. Dried samples (stems and leaves) were extracted for 4 hours by steam distillation units as described previously in Cannon et al. [20], the oil and the herbal distillate are recovered. The residue of oil is extracted from the herbal distillate by hexane. After evaporation of solvent, the remaining oil is recovered.

2.8 Determination and quantification of terpene compounds by GC-MS

The GC-MS analyzes were performed using a gas chromatograph (Agilent 6890N) equipped with a column of 30 m × 0.25 mm, with a stationary film HP-5 ms of 0.25 µm thick (Agilent J & W) coupled to a mass selective detector having a electronic ionizer, and a quadruple analyzer (Agilent 5973). The temperature program used is 60 to 246 °C at 3 °C min^{-1}, then the temperature is maintained at 246 °C for 20 minutes. The other operating conditions are as follows: the carrier gas is helium (purity ≥ 99.9999%); the flow rate is 1 ml / min and the temperature of the injector is 250 °C. The injection of 1 µL of diluted sample (1: 100 in hexane, wt / wt) was performed at a division ratio of 1:20, using an autosampler (Agilent Model 7683B). The conditions of MS are as follows: the transfer line temperature MS is 240 °C; The temperature of the ion source is 200 °C with an ionization energy of 70 eV; and a quadrupole temperature of 150 °C with a scanning rate of 3.2 scans per second. The identification of the EO constituents was accomplished by comparison of their retention indices and their mass spectra with the literature data and the mass spectra databases [21].

2.9 Statistical analysis

All data is analyzed using the SPSS statistical software version 20.0. Analysis of the ANOVA two way variance followed by the post hoc multiple comparison test, which is performed to determine the homogeneous groups using the Student Newman-Keuls test with a significance threshold ($p < 0.05$).

3. Results and Discussion

3.1 Leaf number

Figure 1 shows an increase in the leaves number of plants in some treatment with seaweed extracts. Extracts from green algae (E and U) exhibit a significant ($p<0.05$) favorable effect on the number of leaves of the plants treated by 1% (32 and 28 respectively) or treated by 2% (29 and 26 respectively) compared to control (18 leaves). However, plant treated with 2% Fucus improved the number of leaves (26 leaves; $p<0.05$) compared to the control. This result is consistent with those obtained by Kulkarni et al. [22]. Indeed, several compounds of seaweed extracts lead to the improvement of growth parameters, such as polysaccharides, growth hormones and betaines [5]. In contrast, a wide range of growth responses is induced by seaweed extracts with the presence of hormones and promoters [22]. Cytokinins, a plant hormone have an effect on plant growth parameters, which are detected in seaweed extracts [23]. The cytokinins exhibit trans-zeatin, isopentenyladenine, and derivatives of these two forms [24]. Some polysaccharides contribute to the improvement of the morphological parameters of plants [25], as is the case for complex of sulfated polysaccharides which do not exist in terrestrial plants [26]. For example, *Fucus vesiculosus* contains polysaccharides such as laminaran and fucoidan [27]. Laminaran is a (1,3) - β-D-glucans with branching β - (1,6) [7,28]. The fucoidan in brown seaweeds consists mainly of sulfated fucoses linked in the α- (1,3) and α- (1,4) configuration [29].

3.2 Chlorophyll (a) and (b)and carotenoids content

For chlorophyll (a) and (b) content was improved significantly ($p<0.05$) in plants treated by 1 and 2% of F up to (4.21 and 2.25 mg/g DW of chlorophyll a and b at 2%, respectively) compared to the control (1.32 and 0.61 mg/g DW of chlorophyll a and b respectively). Contrary, to treatment with U and E liquid

extract of reduced the chlorophyll (a) content in comparison with the control significantly (Figure 2a and 2b). On the other hand, was observed a decrease in the carotenoid content in the majority of plants treated with seaweed extracts (Figure 2c). This increase in chlorophyll content in some plants treated with seaweed extracts, which are consistent with those obtained by Selvam and Sivakumar [30].

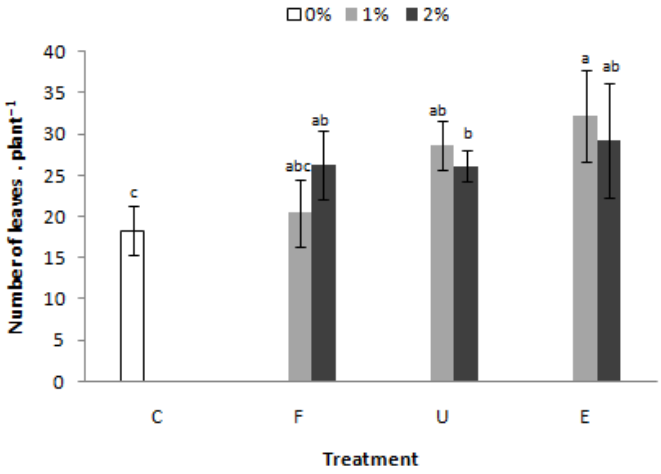

Figure 1. Effect of the liquid extracts of *Fucus spiralis* (F), *Ulva rigida* (U) and *Enteromorpha intestinalis* (E) on the number of leaves in sage (*Salvia officinalis* L.). C: Control. The results are the mean of 22 repeats ± standard deviation. The different lowercase letters mean the significant difference by using the Student Newman-Keuls test with a significance level of 5%.

They found an increase in chlorophyll content after treatment with the extracts. Moreover, the work of Ghatas et al. [31], who examine the effect of SWE on *Artemisia annua* L. plants, found an increase in the chlorophyll content of treated plants. Ördög et al. [32] confirmed that leaf spraying with seaweed extracts increases the chlorophyll content. At the same time, Karthik et al. [33] demonstrated that seaweed extracts improve the content of photosynthetic pigments such as chlorophyll (a) and (b) and carotenoids. These effects are due to the presence of natural hormones of algae in extracts [34,35]. Indeed, algae-based extracts are rich in various betaine and betaine-like compounds [36]. Betaines and the magnesium content in SWE plays vital role in organization of chlorophyll pigment and can improve the chlorophyll content [33,36]. Moreover, cytokinins of SWE inhibit the degradation in photosynthetic pigments [34]. According to Rouphael and Colla [37], betaines are used as a source of nitrogen by plant.

3.1 Protein content

Figure 3 shows the protein content of the leaves treated by the different concentrations of seaweed extracts. Treatment with F at 2% extract showed an improvement in protein content (3.58 µg/mg DW) relative to the control(3.08 µg/mg DW). In contrast, treatments with Enteromorpha at 1 and 2% and Ulva at 1% have no significant effect compared to the control. This increase in protein content can be explained by the stabilization of protein structures within plants using different betaine compounds that stabilize the protein structure and enzymatic activity [38]. Thus, the cytokinin content of the seaweed extracts increases cell division and causes an increase in the mass of the plants, which implies an increase in the protein content, without forgetting the existence of different sources of nitrogen in the extracts which are can be assimilated as foliar sprays [34,38]. In fact, treatment with seaweed extracts improves photosynthetic activity, which will influence protein metabolism.

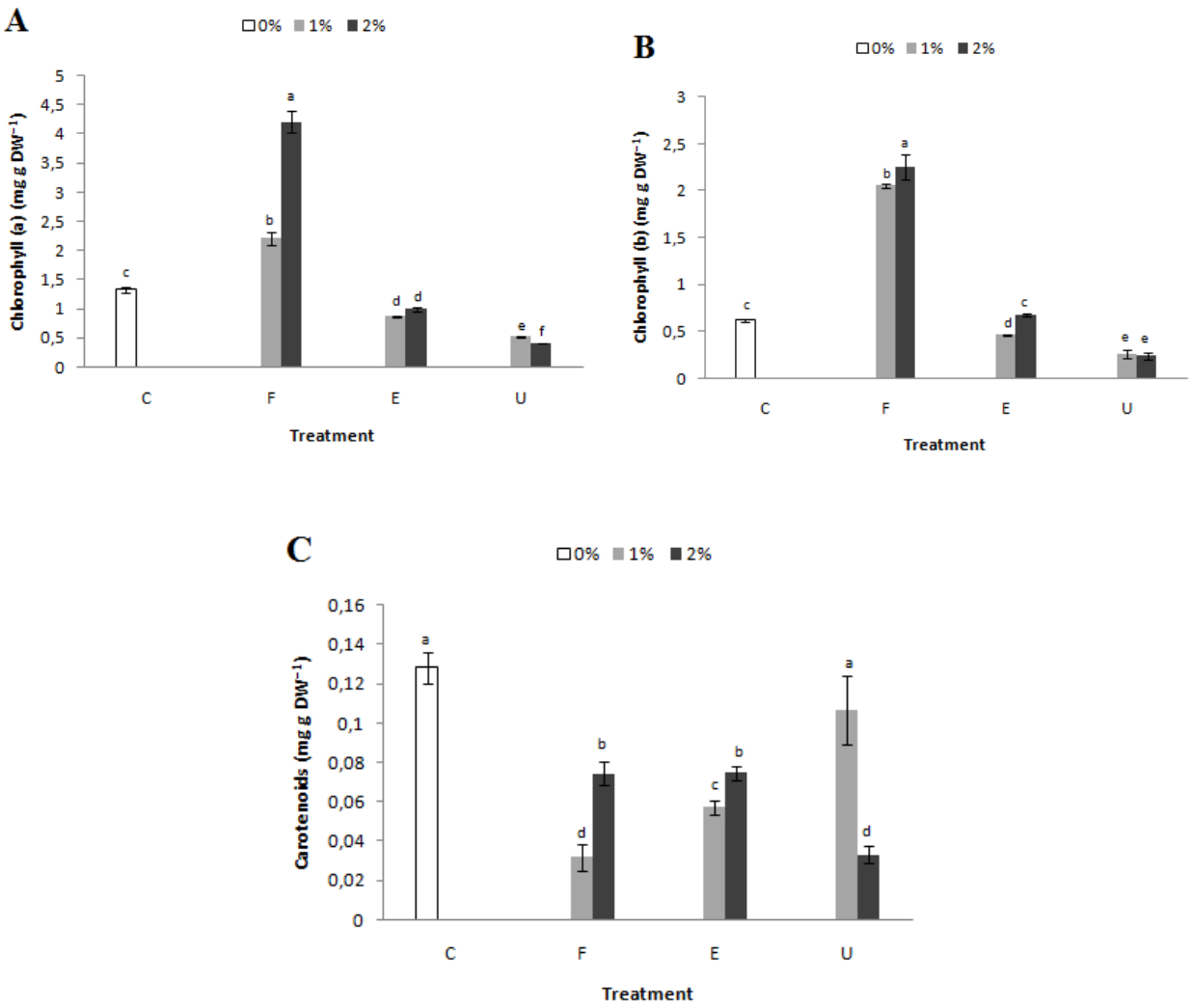

Figure 2. Effect of the liquid extracts of *Fucus spiralis* (F), *Ulva rigida* (U) and *Enteromorpha intestinalis* (E) on chlorophyll (a) (A), chlorophyll (b) (B) and carotenoids (C) content in sage (*Salvia officinalis* L.). C: Control. The results are the mean of 22 repeats ± standard deviation. The different lowercase letters mean the significant difference by using the Student Newman-Keuls test with a significance level of 5%.

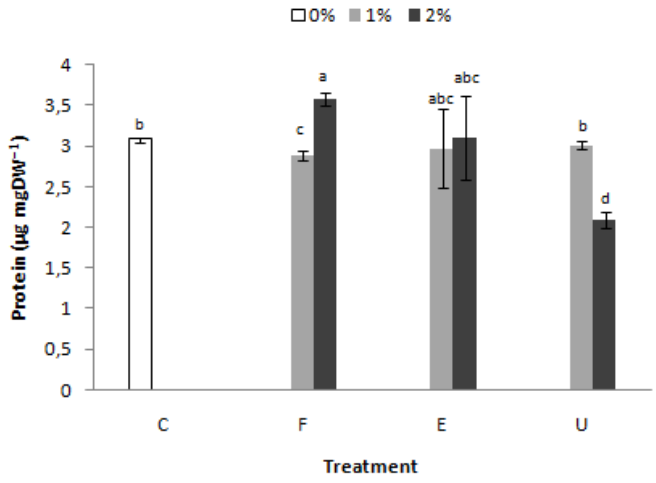

Figure 3. Effect of the liquid extracts of *Fucus spiralis* (F), *Ulva rigida* (U) and *Enteromorpha intestinalis* (E) on protein content in sage (*Salvia officinalis* L.). C: Control. The results are the mean of 22 repeats ± standard deviation. The different lowercase letters mean the significant difference by using the Student Newman-Keuls test with a significance level of 5%.

3.2 Total phenolic content (TPC)

All concentrations of F and U extract improved significantly ($p<0.05$) the total polyphenol content (56.71 and 57.65 µg/mg DW respectively for 1%, 95.15 and 37.24 µg/mg DW respectively for 2%) relative to the control (values). Similarly, treatment with Enteromorpha extract at 2% improved the polyphenol content (25.72 µg/mg DW) (Figure 4). The concentration of the extract plays a role on the polyphenol content. In plants treated with Fucus and Enteromorpha, the content increases as a function of the seaweed concentration. Plants treated with Ulva extract show a decrease in polyphenols as a function of the increase in the concentration of the extract.

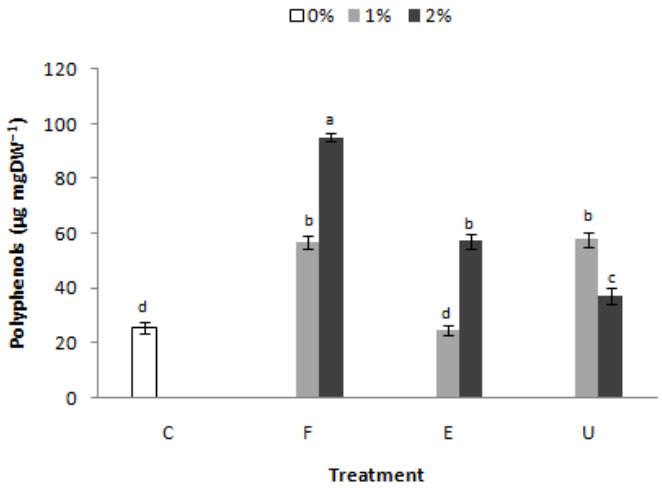

Figure 4. Effect of the liquid extracts of *Fucus spiralis* (F), *Ulva rigida* (U) and *Enteromorpha intestinalis* (E) on phenol content in sage (*Salvia officinalis* L.). C: Control. The results are the mean of 22 repeats ± standard deviation. The different lowercase letters mean the significant difference by using the Student Newman-Keuls test with a significance level of 5%.

The increase in the total polyphenol content of the plants after treatment with the seaweed extracts in the present study is consistent with the results obtained by Elansari et al. [16]. Phenolic compounds are major secondary metabolites that play an important role in the plant. These secondary metabolites have strong antioxidant activity [39]. The increase in phenolic compounds following treatment with seaweed extract is still found in several studies [40,41,7]. In addition, they found a significant increase in antioxidant activities associated with polyphenols. Also, Arokia rajan et al. [42] found an increase in the phenolic compound content of treated *Capsicum annuum* with *Padina gymnospora*, *Gracilaria edulis* and *Ulva fasciata* aqueous extracts. According to Xu and Leskovar [43], foliar spraying by seaweed extracts by certain doses, is more effective in improving the antioxidant properties of plants. Seaweed extracts may not be considered as biostimulants only but also as an enhancement of the medicinal value of sage.

3.3 Effect of seaweed extracts on the composition of essential oils (EO)

The table 1 represents the seaweed extracts effect on the qualitative and quantitative composition of EO of sage. GC-MS analysis yielded 46 EO compounds of which 37 were identified. Among these 37 compounds, we found that 9 of them are the most dominant (α-pinene, camphene, 1,8-cineole, cis-thujone, transthujone, camphor, β-caryophyllene, α-humulene and viridiflorol). The treatment of plants with the seaweed extracts studied has shown remarkable effects on the levels of certain compounds. We

noted a strong increase in α-pinene levels (4.9% in control plants became 14.7%, 15.8%, 15.6% and 15.6% in treated plants respectively by 1% and 2% Ulva, 1% Fucus and 1% Enteromorpha).

Table. Essential oils (EO) composition in % rate in sage treated or not by seaweed extract; N.I. : Not identified ; tr : traces. - : absent

	Control	Ulva 1%	Ulva 2%	Fucus 1%	Enteromorpha 1%
cis-Salvene	-	0,2	0,2	0,2	0,1
Tricyclene	tr.	0,3	0,3	0,3	0,3
α-Thujene	tr.	0,2	0,3	0,3	0,2
α-Pinene	4,9	14,7	15,8	15,6	15,5
Camphene	2,9	8,1	8,5	8,1	8,0
Sabinene	tr.	0,1	0,1	0,1	0,1
β-Pinene	1,3	2,7	2,9	2,8	2,6
Myrcene	0,8	1,5	1,6	1,6	1,6
α-Phellandrene	tr.	0,1	0,1	0,1	0,1
α-Terpinene	0,3	0,3	0,5	0,4	0,4
o-Cymene	0,6	0,7	0,8	0,8	0,7
Limonene	1,5	2,2	2,5	2,5	2,4
1,8-Cineole	6,8	14,5	10,6	10,5	14,1
γ-Terpinene	0,5	0,6	0,7	0,6	0,6
N.I.	-	0,1	tr.	-	tr.
Terpinolene	0,5	0,5	0,7	0,6	0,6
cis-Thujone	3,2	3,2	3,0	3,0	3,1
trans-Thujone	22,0	19,4	18,9	18,9	20,4
Camphor	15,4	16,0	11,3	10,6	15,8
Borneol	1,0	0,8	0,5	0,5	0,7
Terpinen-4-ol	tr.	0,3	0,2	0,2	0,3
Acetate de bornyl	0,9	0,4	0,6	0,6	0,4
N.I.	tr.	tr.	0,1	0,1	tr.
α-Copaene	tr.	tr.	0,1	0,1	tr.
β-Caryophyllene	14,6	5,6	7,7	7,5	4,8
Aromadendrene	0,5	0,2	0,2	0,2	0,1
α-Humulene	10,0	3,6	5,2	4,9	3,0
allo-Aromadendrene	tr.	0,1	0,2	0,2	tr.
N.I.	-	-	-	0,2	-
γ-Muurolene	0,5	0,2	0,3	0,2	0,1
N.I.	-	-	-	0,1	-
Viridiflorene	0,5	0,2	0,3	0,3	0,1
α-Muurolene	tr.	tr.	tr.	0,1	tr.
γ-Cadinene	tr.	tr.	0,1	0,1	tr.
δ-Cadinene	0,8	0,3	0,4	1,8	0,2
α-Calacorene	-	-	tr.	0,1	
Caryophyllene Oxyde	1,5	0,4	0,6	0,6	0,5
Gleenol	-	-	-	0,1	-
Viridiflorol (diterpene)	6,3	1,7	3,0	2,8	2,0
N.I.	tr.	tr.	0,1	0,1	tr.
N.I.	1,3	0,4	0,5	0,5	0,4
1-epi-Cubenol	-	-	tr.	0,4	tr.
N.I.	tr.	tr.	tr.	0,1	tr.
N.I.	-	-	-	0,3	-
N.I.	tr.	tr.	0,1	tr.	tr.
Manool	1,6	0,4	1,0	0,8	0,6

In addition, the rate of 1,8-cineole increased significantly, from 6.8% in the control plants to 14.5%, 10.6%, 10.5% and 14.1% in plants treated respectively with Ulva at 1% and 2%, Fucus at 1% and Enteromorpha at 1%. The rate of camphene also increased significantly from 2.9% in control plants to values of about 8 to 8.5% in plants treated with seaweed extracts. The tricyclene, α-thujene, α-terpinene, β-pinene, myrcene, terpinolene, α-terpinene, o-cymene and limonene levels of the treated sage oils remained slightly higher compared to the control. Thus, camphor levels increased from 15.4% to 16% in plants treated with Ulva at 1%. On the other hand, the treatment with seaweed extracts allowed the appearance of cis-salvene not detected in control plants.

Our results follow the essential oil profiles of sage [10]. These values are in function of several conditions. Indeed, environmental conditions such as temperature, day length and light, plant date, cutting-time, edaphic factors influence the compositions of EO [44,45]. In sage, the main monoterpenes, 1,8-cineole, camphor and both thujone show a variability of the content during a vegetative cycle [46]. According to Grausgruber-Gröger et al. [47] 1,8-cineole decreases gradually during the vegetative period, with a decrease in camphor in the middle of this period, and the gradual increase of thujone.

Our work also demonstrated that the application of extracts greatly increased the levels of α-pinene, 1,8-cineole and camphore that exceed the maximum rate in a sage EO. This increase takes place despite the vegetative period of sage in our present study (harvest in late July), which confirms a significant effect of seaweed extracts on the EO composition. The same effects have been reported by Elansary et al. [16] who noticed an increase in the EO content following the application of *Ascophyllum nodosum* liquid extract. This positive shuffling of the EO compounds could be due to the presence of the secondary metabolites in the seaweed extract, which play the role as elicitors of the plant mechanisms defense, thus promoting the production of EO. The studies of Chrysargyris et al. [48] have confirmed that the presence of nitrogen and boron in the extracts help in the EO production, and assist in improving the quality of the EO compounds These elements also help in the production of EO, and help in improving the quality of the oil.

Conclusion

In this study, seaweed extracts (SWE) have more influence in the different morphological and biochemical parameters of the treated sage (*Salvia officinalis* L.), either positively (improving) or negative (decreasing). The concentration of seaweed extract affects significantly the parameters which are studied (Chlorophyll, Phenols, Proteins). However, the treatment with the SWE had a positive influence on essential oil composition variability in sage, with the ameliorations in the EO compounds.

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Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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