Universal Journal of Agricultural Research 10(6): 804-813, 2022 http://www.hrpub.org
DOI: 10.13189/ujar.2022.100618

Effect of Bio-nitrogen Fertilization, Plant Density and Irrigation on Physiological and Agronomic Characteristics of *Salvia officinalis* Organic Cultivation

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Received October 14, 2022; Revised December 1, 2022; Accepted December 23, 2022

Cite This Paper in the Following Citation Styles

(a): *[1] Elpiniki Skoufogianni, Aikaterini Molla, Anastasia Angelaki, Evangelia E. Golia , "Effect of Bio-nitrogen Fertilization, Plant Density and Irrigation on Physiological and Agronomic Characteristics of Salvia officinalis Organic Cultivation," Universal Journal of Agricultural Research, Vol. 10, No. 6, pp. 804 - 813, 2022. DOI: 10.13189/ujar.2022.100618.*

(b):*Elpiniki Skoufogianni, Aikaterini Molla, Anastasia Angelaki, Evangelia E. Golia (2022).Effect of Bio-nitrogen Fertilization, Plant Density and Irrigation on Physiological and Agronomic Characteristics of Salvia officinalis Organic Cultivation. Universal Journal of Agricultural Research, 10(6), 804 - 813. DOI: 10.13189/ujar.2022.100618.*

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Abstract Sage (*Salvia officinalis* L) is a widespread aromatic and medicinal plant with various positive affects in human diet and health. In order to investigate the interaction among bio-fertilization, plant density and irrigation regimes, in *Salvia officinalis* cultivation, an experimental field was established at the University Farm of Thessaly, in Velestino (Greece). The transplant was carried out in October 2016 and the experiment data concern the plant height, Leaf Area Index (LAI), Chlorophyll content and dry biomass of the 3rd and 4th growing years. A split-split-plot experimental design was used, including two irrigation regimes (IR0: no irrigation and IR1: irrigation), three bio - nitrogen fertilization systems (N0: 0 kg ha^{-1}, N1: 40 kg ha^{-1}, N2: 80 kg ha^{-1}) and two different plant density (D1: 10.000 and D2: 20.000 plants ha^{-1}). The use of the drip irrigation system ameliorated the plant height, the dry leaves yield and the LAI. The dry leaves yield in 3rd year was ranged between 166.3 – 522 kg ha^{-1} and 101.67 – 359 kg ha^{-1} in May and August, respectively. As it concerns the Chlorophyll index (CCI), the bio - nitrogen fertilization and the irrigation had provoked an increase to its amount during the studied growing year. Therefore, *Salvia officinalis* can be a promising perennial crop, cultivated under the Greek climatic conditions.

Keywords Sage, Leaf Area Index, Chlorophyll Index, Biomass, Greece

1. Introduction

In the past years, the cultivation of medicinal and aromatic plants has been ubiquitous by the view of the fact that these plants can be used not only for the treatment of various human diseases but also in human diet [1].

Sage (*Salvia officinalis* L.) belongs to the Lamiaceae family and is one of the most economically popular aromatic and medicinal herbs [2]. Sage is a perennial crop and it is found that the essential oil is higher in the first cutting (in the end of spring) while the leaf yield is lower when the cultivation takes place at the end of summer [3]. It is cultivated worldwide but mainly found in Mediterranean basis [4-6]. The edible parts of *Salvia officinalis* are used for pharmaceutical, cosmetic and aromatic purposes [6,7]. In addition, the leaves and the volatile oil of the *Salvia officinalis* are used in the food industry, as they have antioxidative, antibacterial, anti-inflammatory and antiviral effects [8–10].

The application of the chemical fertilizers is one of the most important causes for the contamination of water, soil, air, and agricultural production [11]. Compared with chemical fertilization, the use of the bio - fertilizers and the solid wastes leads to an improvement in growth and yield of various crops such as medicinal and aromatic plants [12–14]. Furthermore, the application of an organic fertilizer is significant for the protection of the nature and the soil ecosystem [14]. For these reasons, nowadays, there is a tendency for agricultural land producers to use more and more biological fertilizers instead of the chemical ones [4]. Additionally, the consumers pay attention to the product quality and they prefer to consume products which are cultivated under organic farming systems [15].

Among the abiotic factors, water is believed to be one of the most significant, responsible factors for the crop yield decrease. Especially, in the medicinal and aromatic plants such as *Salvia officinalis*, the water deficiency can lead to the reduction of the essential oil production and affect negatively the physiological and biochemical characteristics of the herbs [16,17]. Experimental results from Corelli et al. [18] show that the average biomass of the Sage has decreased by more than 50% in water deficit conditions.

Leaf Area Index is the total leaf area per unit ground surface area. LAI is an index which indicates the ability of plant to photosynthesis and respire better, resulting in producing higher dry biomass [19,20]. In addition, Chlorophyll content plays a significant role in plant photosynthesis capacity and metabolic activities of the crops [21].

Although, worldwide, the Sage cultivation has been studied, in Greece, unfortunately, the researches on *Salvia officinalis* cultivation under bio – fertilizer are not extensive. This study is an effort to research the combined interaction between bio-fertilization, plant density and irrigation regimes, under the Greek climatic conditions.

Furthermore, the scope of this work was to investigate the impacts of different bio-nitrogen fertilization, plant density and irrigation regimes, on plant height, LAI, CCI, dry leaf biomass of *Salvia officinalis* L., cultivated under Mediterranean meteorological conditions (Greece).

2. Materials and Methods

2.1. Study Area and Plant Material

The field experiments were established in November 2016. In the present study, two growing periods of Sage, 2019 and 2020, are under research. The experiments took place at the farm of University of Thessaly (Greece). The *Salvia officinalis* plants were obtained from the Sporofyta Vasilikon Company. The studied area has a latitude of 39°33'55" and a longitude of 22°92'76" and is located at an altitude of 120m above sea level. Its climate is characterized as Mediterranean with hot and dry summers as well as cold and wet winters.

2.2. Soil Analysis

Two soil samples, from two depths (0-30 and 30-60cm), were collected using the proper soil sampler. Each soil sample consisted of six soil subsamples, from an area approximately with 1.5 m radius. The soil samples were immediately transported to the Soil Science laboratory of the University of Thessaly, air-dried and subjected to soil analyses according to Golia et al. [22], as follows: the soil samples after air drying were sieved through a 2-mm sieve. The percentages (%) of sand, clay, and slit were determined using the Bouyoukos method, and soil pH along with electrical conductivity values was determined using (1:1) (soil: water) ratio, organic matter with the Walkley–Black method, while the $CaCO_3$ content was measured using the calsimeter. For the determination of available soil phosphorus, the Olsen method was used, while for exchangeable K concentration the soil samples were extracted using CH_3COONH_4 solution 0.1M (pH=7). Finally, N_2 content was determined using Kjeldahl method.

2.3. Field Experiment

The experiment included two (2) irrigation regimes (IR0: no irrigation and IR1: irrigation), three (3) bio - nitrogen fertilization systems (N0: 0 kg ha^{-1}, N1: 40 kg ha^{-1}, N2: 80 kg ha^{-1}) and two (2) different plant density (D1: 10.000 and D2: 20.000 plants ha^{-1}). In total, the treatments were 12 with three replicates each. Finally, in the field 36 plots (2x3x2x3) were established. Every plot was 4m^2 in size (2 m width and 2 m length). A distance of 1.5 m was considered between the plots to eliminate the possible effects of the different treatments. The split-split-plot experimental design was implemented. The description of treatments is given in Table 1.

Table 1. Description of the experiment treatments

Treatment	Treatment description
IR0N0DI	no irrigation – no fertilization – 10.000 plants ha^{-1}
IR1N0DI	irrigation – no fertilization – 10.000 plants ha^{-1}
IR0N1DI	no irrigation – 40kg ha^{-1} bio fertilizer – 10.000 plants ha^{-1}
IR1N1DI	irrigation – 40kg ha^{-1} bio fertilizer – 10.000 plants ha^{-1}
IR0N2DI	no irrigation – 80kg ha^{-1} bio fertilizer – 10.000 plants ha^{-1}
IR1N2DI	irrigation – 80kg ha^{-1} bio fertilizer – 10.000 plants ha^{-1}
IR0N0D2	no irrigation – no fertilization – 20.000 plants ha^{-1}
IR1N0D2	irrigation – no fertilization – 20.000 plants ha^{-1}
IR0N1D2	no irrigation – 40kg ha^{-1} bio fertilizer – 20.000 plants ha^{-1}
IR1N1D2	irrigation – 40kg ha^{-1} bio fertilizer – 20.000 plants ha^{-1}
IR0N2D2	no irrigation – 80kg ha^{-1} bio fertilizer – 20.000 plants ha^{-1}
IR1N2DI	irrigation – 80kg ha^{-1} bio fertilizer – 20.000 plants ha^{-1}

The transplant was carried out in November 2016 in rows with a spacing of 20 cm and 40 cm, so that the final plant density was D1: 10.000 and D2: 20.000 plants ha^{-1}. In May of each cultivation year the weeds were treated by hand. No pesticides were applied. During March of each growing period a bio – fertilizer (6% total N, 0.5% P_2O_5, 0.3% K_2O and 85% organic matter) was applied. Specifically, for the NI: 40kg ha^{-1} and N2: 80kg ha^{-1} treatments 267g/plot and 540 g/plot were applied, respectively. Furthermore, 70 g of the same fertilizer was applied in every plot, so that the phosphorus needs to be fulfilled.

Two irrigation regimes were applied during the 3rd and 4th studied growing years. At 6 of the experimental treatments no irrigation was applied. On the other hand, the irrigation regime which was applied in the other 6 treatments was the drip irrigation system.

The experiment measurements took place in the 3rd and the 4th growing years (2018-2019 and 2019-2020). During the two growing years, three cuttings were conducted (May, July and August). The plant height of each plot was calculated from the average of five plants, using a measurement before each harvest. The plants were harvested by hand at 5cm above the soil surface and immediately were weighted to record the fresh weight, using a mobile balance. Then, the plants were transported to the Lab for further measurements. In the Lab the plants were separated into leaves, branches and stems and each edible part was weighted. Furthermore, plant issue of each plot was dried at 40°C until constant weight. Finally, the plant parts were weighted so that the dry weight to be calculated.

Field Leaf Area Index (LAI) and CCI measurements were performed during the three different cutting periods. LAI was calculated in the leaves which were removed from the five plants of every different plot. Chlorophyll index (CCI) in each plot was measured in the 3rd leaf of the five cultivated plants. The measurement of LAI and CCI was carried out using the LI-3100C Area Meter and CCM-200 (Chlorophyll Content Meter), respectively.

2.4. Hydraulic Properties

Soil samples were collected and tested in the laboratory using the pressure plate apparatus, in order to calculate field capacity, permanent wilting point, available soil moisture and critical soil moisture. The above data were used to calculate the critical soil moisture and the irrigation doses, along with meteorological data taken from the meteorological station settled at the University Farm, close to the experimental field. Field soil moisture was measured using Delta-T SM 150 device and when soil moisture reached the critical soil moisture, an irrigation event was planned. Measurement of plant height, Leaf Area Index (LAI), Chlorophyll and total leaf biomass.

2.5. Statistical Analysis

In the experimental data Analysis of Variance was performed, using the statistical analysis package Statgraphics plus 8.1, according to the LSD test about the level of significance 95% ($p<0.05$).

3. Results

3.1. Meteorological and Irrigation Data

Average Air temperature was 20°C, 27.6°C and 27.9°C during May, July and August of the 3rd cultivation year when the plant harvest was performed, while in the 4stcultivation year the average temperature was 20.3°C in

May, 24.9°C in July and 27.8°C in August. During the two studied cultivation years the total amount of the irrigation water applied was 350 mm and 320 mm, respectively while total seasonal precipitation was 108.2 mm and 108 mm respectively. Figure 2 (a, b) shows precipitation along with irrigation events for the years 2018-2019 and 2019-2020 respectively.

3.2. Soil Analysis

The soil was loam with pH 7.7. The physicochemical properties of the soil samples are presented in Table 2.

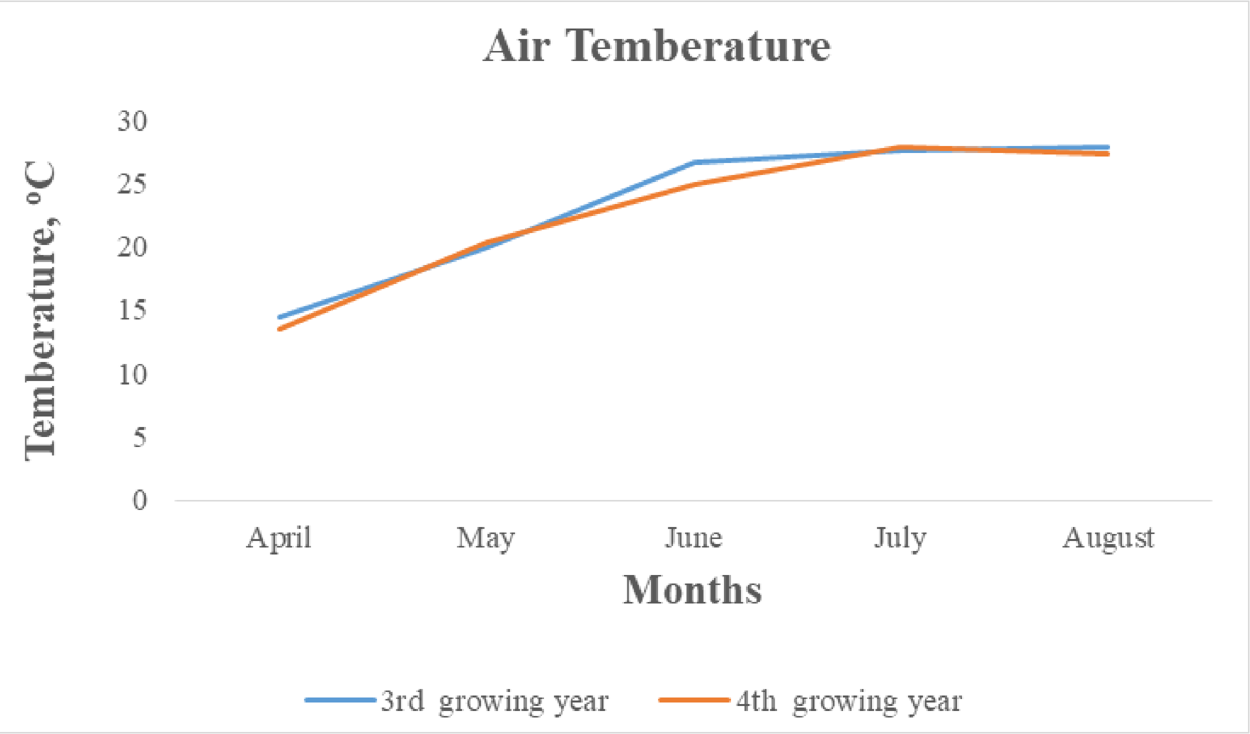
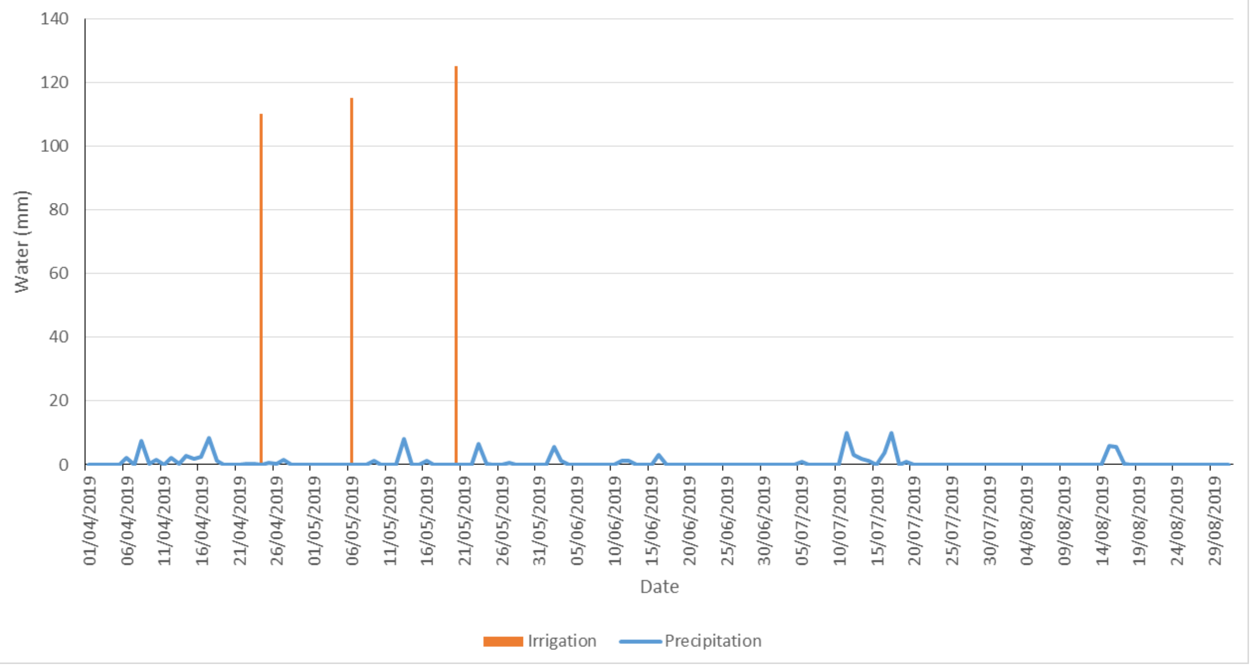
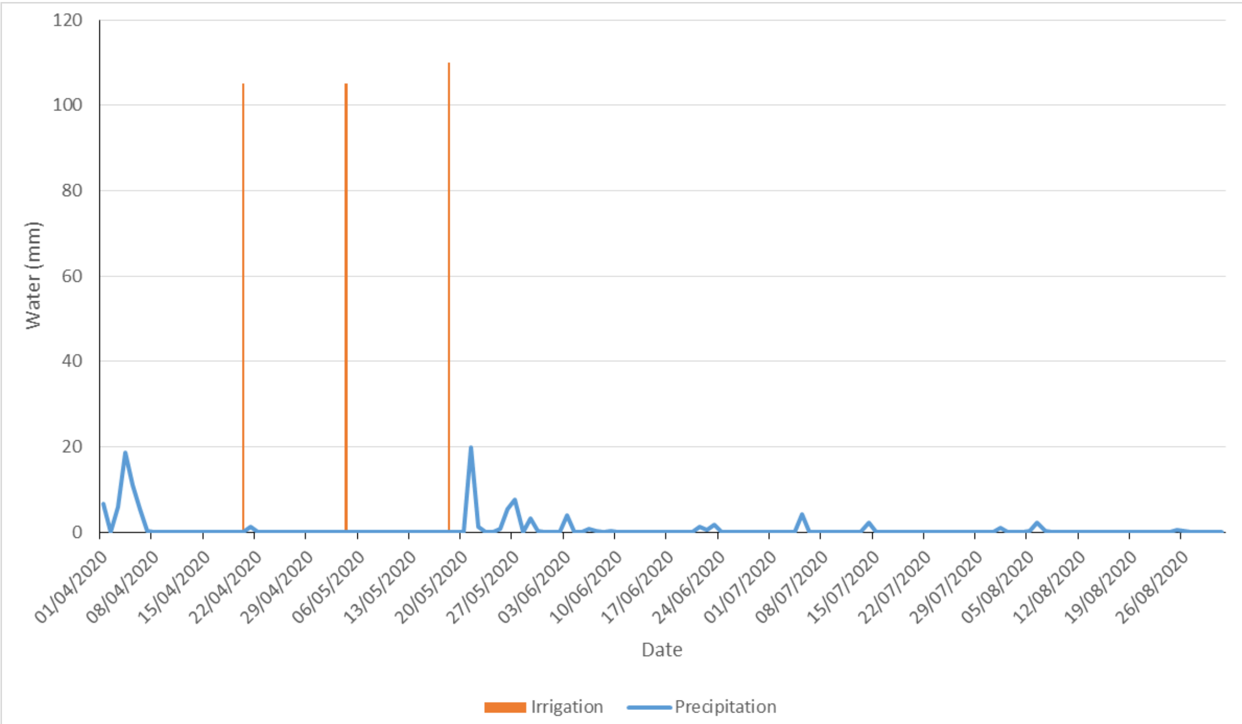


Figure 1. Average monthly air temperature, during the growing periods (3rd and 4th growing years)



(a)



(b)

Figure 2. Precipitation and irrigation frequency during the growing periods 2018-2019 (a) and 2019-2020 (b)

Table 2. Physicochemical properties of the soil in two depths

Depth	Sand (%)	Clay (%)	Silt (%)	pH	E.C. (μS cm^{-1})	$CaCO_3$ (%)	Organic matter (%)	Olsen P (mg kg^{-1})	Exchangeable K (mg kg^{-1})	Total N(%)
0-30 cm	39	14	47	7.7	302	12.5	2.5	42	1118	0.08
30-60 cm	42	12	46	7.7	378	13.0	2.1	35	1077	0.11

3.3. Plant Height Results

The results of plant height are summarized in Table 3. Generally, the plant height was affected positively by the irrigation application. In the 3rd growing year the highest plant height was implemented in IR1N2D1 treatment (irrigation, 80kg ha^{-1} bio-fertilizer, 10.000 plants ha^{-1}). Analysis of variance indicated that in general, the plant height had not statistically significant differences among the treatments, except in the case between the treatments IR0N2D1and IR1N2D2 in the first cutting which was performed in May of the 3rd growing year. Furthermore, the plants were taller in the 4rd growing year compared to the 3rd cultivation year in all the treatments. The highest plant height was observed in the IR1N2D2 plots. In total, the D2: 20.000 kg ha^{-1} had a more positive effecton plant height in harvest in May as well in August, both in the two studied cultivation years. It is remarkable that in the 4^{th} cultivation year compared to the 3^{rd} year the height plant had a significant increase, ranging from 47.45% to 65.30% in May and from 27.36% to 49.74% inAugust. This is due to the fact that the biomass was lower in the 4^{th} year in comparison to the 3rd growing year. Moreover, the bio – fertilizer released at a slower rate compared to a chemical one.

3.4. Leaf Area Index (LAI)

The Leaf Area Index results are shown in Table 4. During the 3^{rd} year the LAI varied between 0.37 and 1.10 in May, between 1.85 and 5.04 in June and between 1.66 and 4.34 in August. Both in two cultivation years the highest LAI was obtain in IR1N2D1 treatment in July and in August. In May LAI was higher in IR1N0D1 and in IR1N1D1 plots during the 3^{rd} and 4^{th} year of cultivation, respectively. In general, the LAI was ameliorated by the irrigation of plants during the cutting period. Furthermore, the LAI was higher when the plant density D1:10.000 kg ha^{-1} was applied. In most of the treatments the LAI was ameliorated by the bio – nitrogen fertilization use in the 2^{nd} and 3^{rd} measurement (July, August).

3.5. Chlorophyll Index (CCI)

The Chlorophyll content results are presented in Table 5. In the two studied years, the average mean of CCI was increased when increasing the amount of the bio - nitrogen fertilization. Moreover, the irrigation regime had acted beneficial to the chlorophyll amount. Both in two growing years, the CCI was higher in the treatment IR1N2D2. The analysis of variance indicated that the increase of chlorophyll content due to the irrigation and the bio – fertilization application was only numerically and not statistically.

Table 3. Height of the *Salvia officinalis* plants in the different treatments

Plant height, cm				
	2018-2019	**2019-2020**	**2018-2019**	**2019-2020**
Treatment	**May**		**August**	
IR0N0D1	26.60 ab	65.40 a	41.50 a	68.17 a
IR1N0D1	29.75 ab	70.43 a	48.37 a	74.43 a
IR0N1D1	28.00 ab	61.10 a	46.17 a	81.80 a
IR1N1D1	29.15 ab	68.58 a	53.90 a	84.20 a
IR0N2D1	24.95 a	71.08 a	39.20 a	69.63 a
IR1N2D1	30.60 ab	72.92 a	58.47 a	74.20 a
IR0N0D2	27.07 ab	76.70 a	48.97 a	80.73 a
IR1N0D2	36.53 ab	78.00 a	46.97 a	81.17 a
IR0N1D2	29.15 ab	71.93 a	49.60 a	80.30 a
IR1N1D2	33.50 ab	73.23 a	55.23 a	84.52 a
IR0N2D2	30.17 ab	73.33 a	47.40 a	80.70 a
IR1N2D2	38.53 b	75.67 a	54.73 a	85.23 a
CV %	22.95	12.76	41.08	12.00

Table 4. Interaction between irrigation regimes, bio-fertilization and plant density on Leaf Area Index of *Salvia officinalis* plants

LAI, m^2 m^{-2}						
	2018-2019	**2019-2020**	**2018-2019**	**2019-2020**	**2018-2019**	**2019-2020**
Treatment	**May**		**July**		**August**	
IR0N0D1	0.64 a	0.50 a	3.23 abc	2.73 abc	2.98 a	2.48 a
IR1N0D1	1.10 a	0.92 ab	4.79 bc	4.29 bc	3.76 a	3.26 a
IR0N1D1	0.87 a	0.83 ab	3.14 abc	2.64 abc	1.91 a	1.41 a
IR1N1D1	1.06 a	1.02 b	3.40 abc	2.90 abc	4.21 a	3.71 a
IR0N2D1	0.86 a	0.83 ab	3.53 abc	3.03 abc	2.77 a	2.27 a
IR1N2D1	0.91 a	0.87 ab	5.04 c	4.54 c	4.34 a	3.84 a
IR0N0D2	0.37 a	0.33 a	2.07 abc	1.57 a	3.29 a	2.79 a
IR1N0D2	0.69 a	0.65 ab	1.85 abc	1.35 a	2.11 a	1.61 a
IR0N1D2	0.49 a	0.44 ab	2.49 abc	1.99 ab	2.00 a	1.50 a
IR1N1D2	0.39 a	0.33 a	1.90 abc	1.40 a	2.37 a	1.87 a
IR0N2D2	0.37 a	0.33 a	2.69 abc	2.19 abc	1.66 a	1.16 a
IR1N2D2	0.62 a	0.57 ab	3.20 abc	2.70 abc	3.38 a	2.88a
CV %	38.36	35.4	33.44	42.39	33.19	12.76

3.6. Dry Drogue

The results of the average dry leaves weight are reported in Table 6. The yield in both studied years was higher in IR1N2D2 treatment (irrigation, 80 kg ha^{-1} bio-fertilizer, 20.000 plant ha^{-1}). Specifically, the highest biomass in IR1N2D2 plots was 7358 and 1573 kg ha^{-1} in the 3rd and 4th cultivation year, respectively. The dry biomass was lower in treatments with no irrigation compared to plots with irrigation. In 2018-2019 (the 3rd growing year) there was statistically significant differencebetween the irrigation and no irrigation treatments. The plant density provoked positive effects when the interaction ofbio – fertilizer and irrigation was applied. It has to be mentioned that in the 4th year there was a decrease in the total drogue yield. The reduction ranged from 75.1 (IR0N1D1) to 89.2% (IR1N0D2).

Table 5. Interaction between irrigation regimes, bio-fertilization and plant density on Chlorophyll index of *Salvia officinalis* plants

CCI						
	2018-2019	**2019-2020**	**2018-2019**	**2019-2020**	**2018-2019**	**2019-2020**
Treatment	**May**		**July**		**August**	
IR0N0D1	18.40 a	37.57 a	20.60 a	29.33 a	22.87 a	24.43 a
IR1N0D1	21.37 ab	39.17 ab	21.20 a	32.00 ab	21.21 a	25.37 a
IR0N1D1	19.83 ab	51.23 ab	19.75 a	37.33 ab	21.03 a	29.43 a
IR1N1D1	21.30 ab	58.03 ab	21.80 a	42.20 ab	23.80 a	33.47 a
IR0N2D1	20.37 ab	47.87 ab	21.10 a	45.13 ab	23.11 a	35.40 a
IR1N2D1	21.57 ab	61.77 b	23.27 ab	46.03 b	25.16 a	36.83 a
IR0N0D2	21.13 ab	38.53 ab	22.30 ab	31.90 ab	20.71 a	27.73 a
IR1N0D2	23.77 ab	46.33 ab	22.56 ab	39.60 ab	22.02 a	35.50 a
IR0N1D2	18.70 a	50.87 ab	19.77 a	39.37 ab	20.20 a	28.50 a
IR1N1D2	24.73 ab	54.83 ab	23.42 ab	42.27 ab	22.13 a	36.70 a
IR0N2D2	23.53 ab	52.90 ab	20.90 a	39.50 ab	22.97 a	31.97 a
IR1N2D2	29.33 b	91.47 c	26.65 b	63.37 c	24.54 a	37.60 a
CV %	19.90	24.43	11.78	21.69	18.15	25.14

Table 6. Dry drogue of the *Salvia officinalis* plants in 2018-2019 and 2019-2020

Dry drogue, kg ha^{-1}		
Treatment	**2018-2019**	**2019-2020**
IR0N0D1	3762 a	723 ab
IR1N0D1	7087 b	782 ab
IR0N1D1	3890 a	970 abc
IR1N1D1	6473 b	1115 bcd
IR0N2D1	3693 a	840 abc
IR1N2D1	7123 b	1110 bcd
IR0N0D2	3848 a	555 a
IR1N0D2	6153 b	662 ab
IR0N1D2	3522 a	843 abc
IR1N1D2	6765a b	1347 d
IR0N2D2	3232 a	747 ab
IR1N2D2	7358 b	1573 cd
CV %	21.6	29.5

4. Discussion

In this experiment, the tallest plants were observed in irrigated and in bio-fertilized plots. In agreement with our findings, the increase in plant height was also observed from other researches [23,24]. Sonmez and Bayram [25] noticed an increase in height of the plant which was affected by the irrigation and nitrogen application. According to Golia et al. [13] the key parameter that determines the healthy plant growth along with the increase of its biomass in an alkaline soil is the simultaneous application of irrigation water with high nitrogen levels and organic carbon as well. Furthermore, the dense sowing density had a positive effect on plant height. These results were noticed by Giannoulis et al. [5], too, although a micro-plus fertilization was applied.

Generally, the irrigation system and the bio – fertilization ameliorate the Leaf Area Index (LAI). Similar results were observed by other researches. They noticed that the nitrogen application increased the Leaf Area Index of the tomato, maize and wheat plants [26–28].

The results showed that the Chlorophyll amount had increasedby the water and the bio-fertilizer application. According to literature, studies found that there were positive effects to the amount of chlorophyll under certain water conditions and by the bio – fertilizer use [29–31].

Finally, the dry drogue was higher in IR1N2D2 treatment (irrigation, 80 kg ha^{-1} bio-fertilizer, 20.000 plant ha^{-1}) in both cultivation years. Previous studies reported that the dry biomass of *Salvia officinalis* can be improved under irrigation conditions [16,17,32]. Furthermore, in accordance with our findings, Corell et al. [18], also, found a significant increase in sage dry weight using the drip irrigation system. Our results are in disagreement with the Nadjafi et al. [33], due to the fact that our experiments showed that the bio – fertilizer ameliorated the total biomass of Sage in comparison with the control plots (not fertilized).

5. Conclusions

Main scope of the present research was to investigate the impact of the combination of different irrigation regimes, bio-nitrogen fertilization and plant density on Sage characteristics, under the Greek meteorological conditions. Results indicated that the irrigation of plants can cause a significant increase in plant height and in dry drogue. Specifically, in dry drogue biomass among the irrigation and the no irrigation plots, there was statistically significant difference according to the analysis of variance. Furthermore, the LAI had been ameliorated by the irrigation of plants during the cutting period. The D2: 20.000 plants ha^{-1} showed more positive effects in plant height in the two studied cultivation years. Moreover, the highest dry drogue was obtained when the interaction of irrigation, 80kg ha^{-1} bio – fertilizer and 20.000 plants ha^{-1} plant density was applied. Finally, the bio - nitrogen fertilization and the drip irrigation system had a positive effect on the Chlorophyll amount during the two studied years. According to our results, the best interaction for the Sage cultivation is the use of an irrigation regime, in our case the drip irrigation system, a dose of 80 kg ha^{-1} bio – fertilizer with a plant density of 20.000 plants ha^{-1}. To sum up, the cultivation of *Salvia officinalis* can be a promising crop for Greece's climate. This thesis could be a motivation for further studies concerning the cultivation of medicinal and aromatic plants.

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