Evaluation of the Effect of Irrigation on Biometric Growth, Physiological Response, and Essential Oil of *Mentha spicata* (L.)

Stefano Marino [1,*]**, Uzair Ahmad** [1]**, Maria Isabel Ferreira** [2] **and Arturo Alvino** [1,*]

[1] Department of Agriculture, Environment, and Food Sciences, University of Molise, 86100 Campobasso, Italy; u.ahmad@studenti.unimol.it

[2] Department of Sciences and Engineering of Biosystems (DCEB) and Research Center Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa (ULisboa), Tapada da Ajuda, 1349-017 Lisboa, Portugal; isabelferreira@isa.ulisboa.pt

*   Correspondence: stefanomarino@unimol.it (S.M.); alvino@unimol.it (A.A.); Tel.: +39-0874-404709 (S.M.)

Abstract: A field experiment was performed on spearmint (*Mentha spicata* L.) under different irrigation regimes in a hilly area of Southern Italy. Objectives of the study include evaluating the physiological and biometrical response of mint from plant establishment up to its complete maturation, as well as the yield composition in essential oil at two different dates. Increasing levels of water stress affected later developing leaves and plant's water status and net photosynthesis (from the beginning of stress (DAT 63), while affecting negatively the biometric response very soon and significantly from 35 DAT. Photosynthesis limitation played a critical role from DAT 53 on, namely later, in the harvest period (DAT 35–70). Under severe water stress, crop restricted water losses by modulating stomatal closure and, at harvest, showing lowered mesophyll conductance. Irrigation treatments did not affect the concentration of organic compounds, while the yield of essential oils was negatively affected by water stress due to reduced crop growth, in terms of total and leaf biomass, leaf area index (LAI) and crop height.

Keywords: aromatic; balsamic; irrigation; photosynthesis; spearmint; water stress; water balance

1. Introduction

The medicinal and aromatic plants contain the chemical constituents which were first used by humans as medicines for various significant treatments, mind relaxing treatments for some patients, as flavoring agents for food and drink, and as mental stimulants for mystic interactions with super natural gods [1]. The global market for herbal products is continuously expanding and it is expected to reach 5 trillion dollars by the year 2050 [2], currently stands at over 60 billion US dollars annually and it is growing progressively [3].

Among many aromatic plants, spearmint (*Mentha spicata* L.) plays a crucial role, since international demand for mint essential oils increased in the past few years. Mint produces secondary metabolites applicable in different sectors, such as food, confectionery, and chocolate industries [4–6] food preservation [7], taste and aroma [6], cosmetic, confectionery, and pharmaceutical industry, as well as, for its essential oil. It is also used for its insect-repellent properties, antibacterial and antifungal activities and as antioxidant agent [8]. Besides, *Mentha spicata* is added in Italian cuisine, either in fresh or dried form, to fish and shellfish plates before or after cooking [9,10]. In terms of medical uses, *Mentha spicata* is considered an herbal medicine in folkloric remedies for treating cold and flu, respiratory tract problems, gastralgia, hemorrhoids, and digestive problems [9,11]. Others papers, instead, have reported the two most popular *Mentha spicata* flavors which are due to the constituents' menthol

and carvone, terpene alcohol with strong, intense and refreshing spearmint flavor, and a terpene ketone with a typical flavor of Maghrebi mint tea, respectively. The interest in studying *Mentha spicata* is majorly related to its phytosanitary characteristics. *Mentha spicata* phenols have also shown strong antioxidant, acetylcholinesterase (AChE), butyryl-cholinesterase (BChE) and histone deacetylase inhibitory effects [12–14]. The *Mentha spicata* from different origin contains variable percentages of menthol (20–54 %), menthone (5–43%), menthyl acetate (1–29%), and menthofuran (1–8%) [15].

Chrysargyris et al. [16] reported the role of aromatic plants in the Mediterranean environment, explaining the composition of essential oils, and the mixture of low-molecular-weight isoprenoid of specific fragrance. In particular, spearmint essential oils (EO) mainly contains monoterpenes (the C10 class of isoprenoids), which are produced and stored in the glandular trichomes of the leaf. [17] underlain the role of different factors affecting the formation and isolation of secondary metabolites in plants and that affect mint yield's quantity and quality includes: (i) geographical variations; (ii) environmental conditions; (iii) genetic factors and evolution (iv); physiological variations, (v) political/social conditions; (vi) amount of plant material/space and manual labor needs. In a given environmental condition and on a selected genotype, agronomic factors deeply affect plant growth and quality. Some years before, Yazdani and Jamshidi [18] pointed out that secondary metabolites are fundamentally produced by genetic pathways, although their biosynthesis is strongly influenced by environmental factors. This means that biotic and abiotic stress factors highly affect their growth parameter, essential oil yields and chemical composition. Among the abiotic environmental stress factors, salinity and drought have the most important effect on medicinal plants [19–21].

There are a few researches about irrigation of *Mentha spicata*, and some researchers report the data about impressions on spearmint irrigation. Mint requires frequent and adequate irrigation supplies; Thomas et al., [22] recommend at least thrice weekly. It is important to keep the soil constantly moist, although well drained. The crop has high water demands in summer due to the high temperatures [23]. Care has to be taken to prevent waterlogged soil, especially in winter, as this will influence growth [24]. Few studies have shown that spearmint and spearmint biomass production is highly sensitive to water stress [25]. In order to increase spearmint yield, furrow irrigation is preferred over sprinkler irrigation, which damages the leaf essential-oil glands and reduces marketable essential-oil yield production [26]. Mitchell and Yang [27] reported that the alternate furrow irrigation method is more advantageous over conventional furrow irrigation, as it allows the frequent application of small water amounts without reducing root volume. Research for water deficit stress in *Mentha spicata* reveals that the soil moisture treatments had significant effects on the survival rate of the plants. Trends in the numbers and lengths of branches and leaf numbers suggest that the number and lengths of stolons in spearmint were significantly increased for all treatments [28].

Reduction of photosynthesis in *Mentha spicata* due to the water stress may, therefore, reduce monoterpenes yield. Photosynthesis inhibition under water stress is often transitory as it reflects the limitation of CO_2 uptake because of stomata closure and increasing mesophyll resistances [29]. However, severe drought may cause oxidative stress due to the formation of reactive oxygen species and photo inhibitory damage especially under high solar radiation [30,31]. In chloroplasts, protection against oxidative damage is provided by both enzymatic and non-enzymatic antioxidants [30]. Antioxidant contents may, therefore, increase in water-stressed plants [32]. Despite their importance in plant protection and food science, the physiological mechanisms that regulate the production of monoterpenes in officinal plants have seldom been examined.

Despite the abundant scientific reports of *Mentha spicata*, knowledge about water stress effects is still scarce. Understanding how the mint crop prioritizes its biometric and physiological response under different water regimes is important for predicting the best agronomic approach, developing the total oil production, and/or researching the classes of organic compounds.

Therefore, the aim of the present paper is to evaluate the *Mentha spicata* behavior under water stress. Under three different irrigation regimes (I_0, I_{50} and I_{100}), *Mentha spicata* plants were evaluated for its qualitative and quantitative yield production and chemical compounds from plant establishment

stage to its complete maturation. This interdisciplinary approach is necessary due to the natural behavior of *Mentha spicata* depending on the water balance and allowing appreciating different aspects at the agronomic, eco-physiological, morphological level of the culture and the chemical components of the extracted essential oils, according to their classes.

2. Materials and Methods

2.1. Materials

The crop was transplanted in the autumn season to the definitive experimental plot located in Southern Italy, (41.53° N, 14.67° E), at an altitude of 517 meters above the sea level. According to the Soil Survey Staff, Natural Resources Conservation Service, USDA, the soil was a sandy clay loam (pH 7.9) with high values of cation exchange capacity (20 cmol kg^{-1}). The available water was estimated using the following values for water content at field capacity (FC) and permanent wilting point (PWP): 0.32 m^3/m^3 and 0.16 m^3/m^3, respectively, and root depth was estimated as increasing from transplantation to the end of the cycle, attaining 100 cm at its maximum. The allowable depletion factor (p) was considered at first as 0.5 (average value for crops shown, [33,34] which provides a maximum value (end of season) for readily available water (RAW) of 80 mm.

Plots were distributed according to a fully randomized experimental design, with six replicates. Each plot had 18 m^2 (6 m × 3 m), with plant density of 20 plants m^{-2}. During the preparatory work, weed weed removal was performed mechanical. Subsequently, plowing was carried out at a depth of 45 cm, followed by a rotary harrow to prepare the ground for transplantation of the culture and allowed an adequate deepening of the rooting system. In this process, the formation of plow pan was avoided in order to favor excess water by drainage.

Background mineral fertilization was performed with N, P and K, the equivalent of N (50 units ha^{-1}), P (100 units ha^{-1}), and K (120 units ha^{-1}). Fertilization with N was done at transplanting and then every 2 weeks throughout the experiment, while P and K were applied only at transplanting stages. The fertilizer was distributed on the tested plot, using granulated formulations of simple superphosphate (0-19-0), with sulfur (SO_3 28%), potassium sulphate (K_2O 30%), magnesium salt (MgO_2 10%), and urea (N 46%).

2.2. Irrigation Treatments and Water Balance

Local meteorological data (Figure 1) were collected by a standard agro-meteorological station (Skye Instruments Ltd., LIandrindod Wells, UK) located near the experimental field. Data included solar radiation, wind speed, air temperature, air relative humidity and precipitation. Data were collected every 60 seconds, with 30 min output (Figure 1).

Besides the rainfed treatment, two water regimes were scheduled starting from May 16 (DAT 0), based on the threshold value of water lost for evapotranspiration (10 mm), according to the practical experiences in the same environments. The plots with fully irrigated treatment (I_{100}, Control treatment) received 100% of the initially estimated water deficit taken, for simplification, as ETc minus total rainfall, assuming 1 for irrigation efficiency. The intermediate water stress treatment (I_{50}), received the half irrigation depth of the control treatment. Severe water-stressed treatment (I_0) received no irrigation. In the period ranging from transplanting date (October) to DAT 0, when experiment started, total rainfall was 673 mm and the mean daily temperature ranged from 9.5 °C in December 2014 to 2.5 °C in February 2015.

Reference evapotranspiration (ETo) was calculated by applying the FAO Penman-Monteith equation [34]. Along the crop cycle, crop evapotranspiration (ETc) was calculated with an initial value of Kc from 0.4 (transplant) that increases to 0.85 (up to plant establishment-May 16), and decline to 0.5 at harvest [35].

The water balance of the three treatments was done on ex-post basis calculating the water available (mm) in the root zone up to 100 cm, taking into consideration actual evapotranspiration (ETa) estimated

from ETc with crop coefficients as above (no water stress), where a stress coefficient (Ks) was applied as in Allen et al. [34] the other terms being effective rainfall, irrigation depths (I_{100} and I_{50}), and estimated drainage (when water stored would be above FC).
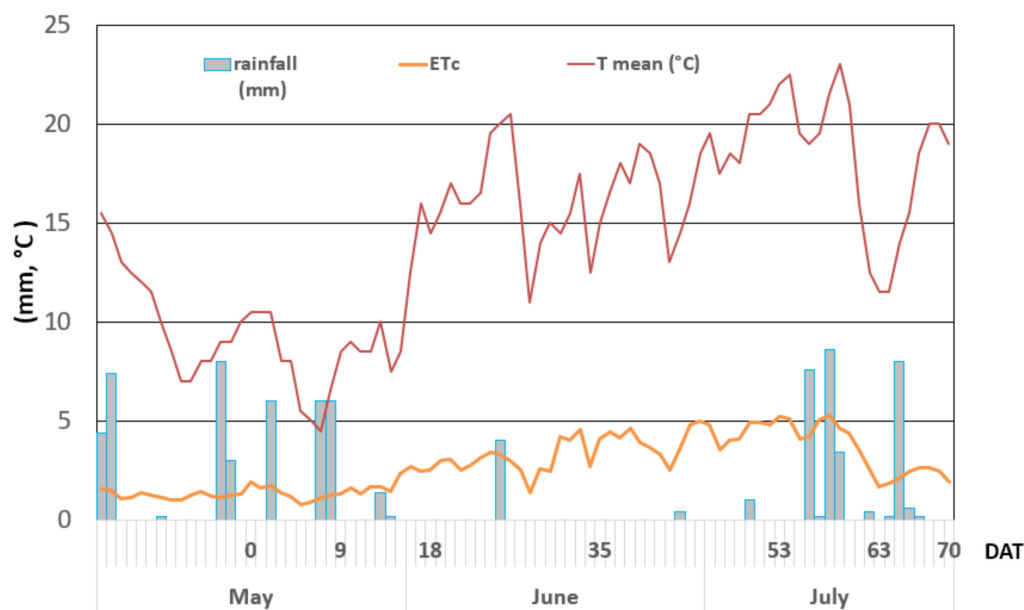

Figure 1. Meteorological data and ETc at the experimental site: mean daily temperatures (°C), daily rainfall and ETc (mm).

2.3. Agronomic Samplings and Physiological Measurements

Samplings were performed to determine crop growth and quantity/quality of the extracted essential oils. Measurements of plant growth, physiological status were performed during the balsamic period (maximum qualitative/quantitative yield in essential oil), and monoterpenes yield were calculated at plant establishment period in different water regimes of the mint plant. Monitoring the health state of the crop was performed through non-destructive and eco-physiological sampling procedures. The plants were sampled five times during the test period at May 16 (DAT 0), May 25 (DAT 9), June 3 (DAT 18), June 19 (DAT 35), July 8 (DAT 53), July 18 (DAT 63) and the last at the harvest, July 25 (DAT 70). The harvest was performed at midday time from (12:00 to 14:00). The period from DAT 35 (full flowering) and DAT 70 (harvest) was considered balsamic. Within each treatment and replication, a crop strip, 20 cm long, was collected from the test site for measuring fresh biomass of leaves and culms. Of these, about one fourth of each plot was dried in a ventilated oven at 75 °C for 48 h. The dry mass of leaves and culms (above-ground dry mass) is reported as: fresh weight × dry mass (%) (measured in oven-dried samples). The leaf surface was measured with a LI-COR 3100c area meter [36]. Measured biometric parameters included above-ground dry mass, dry leaf weight, number of leaves plant^{-1}, green leaf surfaces, and plant height (cm). Measuring the number of leaves per plant and green leaf surfaces at harvest period was not possible due to lack of time.

Predawn and midday xylem water potentials were used under the field conditions as plant water stress indicators. They were measured with a Scholander type pressure chamber (Model 3005, Soilmoisture Equipment Corp., Santa Barbara, CA, USA) on five leaves of each plot. Measurements of photosynthetic parameters were performed as described in a previous research paper [23]. Net photosynthesis (P_n) and stomatal conductance (g_s) were measured on fully expanded spearmint leaves, with an infrared gas analyzer (LI-COR 6262 Inc., Lincoln, NE, USA) and a small leaf cuvette (4.9 cm^2). Measurements were performed on five plants for each treatment using the portable photosynthesis system equipped with CO_2 control module and light source consisting of blue-red light emitting diodes. Simultaneously to gas exchange determinations, the chlorophyll fluorescence

emissions of dark-adapted leaves were measured with the help of a portable pulse amplitude modulation fluorometer (PAM-2000, Walz, Effeltrich, Germany) for 30-min to estimate the effects of treatments on the efficiency of photo-system II (PSII). The ratio between fluorescence emission induced in illuminated leaves by actinic and saturating light was also measured to estimate the rate of photosynthetic electron transport rate (J_f), as described by [23].

At DAT 35 and 70, chemical analyses of essential oils (Table 1) were performed within each irrigation treatment at the University of Salerno, according to the protocols described in a previous experiment [23]. Results of the compound analysis were expressed in $mg\,m^{-2}$, whereas the concentration values were multiplied by the leaf dry weight of each irrigation treatment within each harvest data.

Table 1. List of tested essential oils, grouped in their own classes.

Classes	Tested Essential Oils
Alcohol	3-Octanol, 5-Methyl-3-hexanol, 1-Octen-3-ol
Aldehydes	2-Hexenal, Nonanal
Ester	2-Methylbutyl; 2-Methylbutyrate; *cis*-3 Hexene valerate
Ester Acetate	2 Methylbutyl 2-methylbutyrate
Monoterpene Hydrocarbons	DL-Limonene; β-Myrcene; Sabinene; α-Pinene; β-Pinene, β-*trans*-Ocimene; β-*cis*-Ocimene, Camphene; α-Thujene
Oxygenated Sesquiterpene	Germacrene D-4-ol, Spathulenol, Caryophyllene oxide, Cubenol, α-Muurolol
Sesquiterpene Hydrocarbons	β–Caryophyllene, Germacrene D, α–Bourbonene, β –Elemene, *t*–Elemene, Carvyl acetate, *cis*-Jasmone, α–Humulene, β–Cubebene
Oxygenated Monoterpenes	Carvone, 1,8-Cineole, *trans*-Sabinene hydrate, (+)-α-Terpineol, Eugenol, Terpinen-4-ol, *cis*-Carveol, α-Terpinolene, *cis*-Sabinene hydrate, Linalool, *cis*-Limonene oxide, *trans*-Limonene oxide, *cis*-Dihydrocarvone, (−)-α-Terpineol, Myrtenol/2-Pinen 10-ol, *trans*-(+)- Carveol, *trans*-Carvone oxide, Isopiperitenone, Perillaldehyde, *cis*-Carvone oxide, *p*-Mentha-1(7), 8(10)-dien-9-ol / (−)-Perillyl alcohol, Chrysanthenone, Limonene 10-yl-acetate

2.4. Statistical Analysis

The statistical package OriginPro-8 (Origin Lab Corporation, Northampton, MA, USA) was used to test the treatment effects. Normality test was performed with the two methods: Kolmogorov-Smirnov, and Shapiro-Wilk. Both methods were highly significant, so the null hypothesis that the data came from a normally distributed population was not rejected. The non-parametric Kruskal–Wallis test was used for comparing the two or more independent samples of equal or different sample sizes. It was used to compare organic compounds content of the three irrigation treatments (I_0, I_{50}, and I_{100}). The Mann Whitney-U test was used to compare the organic compounds content at the two harvest dates. Two-way ANOVA was adopted for oil yield amount for the main compounds and classes, and Tukey HSD Mean comparisons were done according to the Tukey test.

3. Results

The study evaluated qualitative and quantitative yield and chemical compounds of *Mentha spicata* plant, from plant establishment up to the end of the balsamic period. Experiments initiated at May 16 (DAT 0) in 2015, with all plots starting at same soil water content from the previous winter precipitation (673 mm). Cumulative ETo (DAT 0–70, 16th may to 25 July) was 252.4 mm, while cumulative ETc for the same period was 194.3 mm. Starting from DAT 0, the period was characterized by a thermal increasing trend with an average maximum temperature of 21.9 °C, and average minimum temperature of 10.1 °C; rainfall during the test reached 54.2 mm (Table 2). Plots with different irrigated treatments (I_{100} and I_{50}) received irrigation 13 times during the experimental period starting from DAT 0. I_{100} treatment received a total water amount of about 194.2 mm, about 28% from rainfall and 72% from artificial supply (130 mm), whereas the I_{50} treatment received 54.2 mm water from rainfall and 70 mm from irrigation.

Table 2. Crop evapotranspiration (ETc, mm), rainfall (mm), seasonal irrigation (mm), number of irrigation events and irrigation depths of each (mm) for the three irrigation treatments (I_0, I_{50}, I_{100}) starting from DAT 0 (May 16).

Irrigation Treatments	ET_c	Rainfall (mm)	Seasonal Irrigation (mm)	Irrigation Events (number)	Irrigation Depth (mm)
I_0	194.3	54.2	0	0	0
I_{50}	194.3	54.2	70	14	5
I_{100}	194.3	54.2	140	14	10

3.1. Physiological Measurements

The control treatment (I_{100}) showed approximately constant values of predawn leaf water potential (−1.5 MP) throughout the study period (Figure 2, Figure A1). All treatments behaved similarly until the middle of balsamic period (53 DAT). In the last days of the experimental period, stressed treatments (I_0 and I_{50}) started to differ from control (Figure 2a).
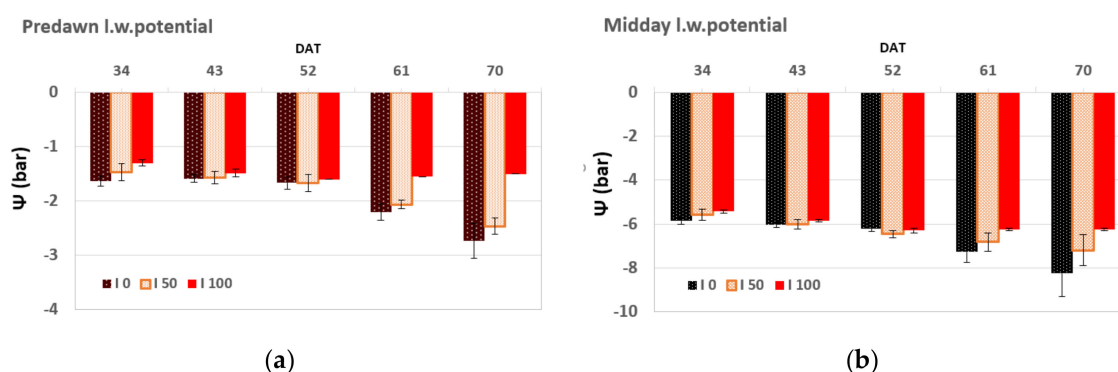

(**a**)   (**b**)

Figure 2. (**a**) Predawn and (**b**) midday water potentials of fully irrigated (I_{100}), moderately water-stressed (I_{50}) and severely water-stressed (I_0) spearmint plants along the growth cycle. Values are means ± 1 S.E.

Figure 2b shows the midday water potentials of the three treatments, that were similar at 34, 43 and 52 DAT. At 61 DAT, midday leaf water potential (l.w. potential) of I_0 was already 20% lower than the correspondent value for the control treatment. At predawn differences appeared higher (30%). At the end of the balsamic period (70 DAT), difference in the predawn condition increased by 45% for severe stress treatment, and 39% for the moderated stress treatment (I_{50}). Water potential values measured in the present experiment are lower than those measured in a previous experiment conducted on potted plants [23].

Figure 3 shows the photosynthetic and transpiration variables measured, including: net photosynthesis (P_n), stomatal conductance (g_s), mesophyll conductance (gm), and electron transport through PSII (J_f). At 70 DAT, differences in P_n were significant for both stress treatments, severe for (I_0) (−26%) and light (−8%) for moderate stress treatment (I_{50}). In severe stress treatment (I_0), limitations to photosynthesis were caused by a reduction in stomatal conductance (−40%), mesophyll conductance (−16%); the same percent were record as increment (+16%) in photosynthetic electron transport rate (J_f/P_n). The measurement of linear electron transport through PSII (J_f) and net photosynthetic rate (P_n) confirms that in drought-stressed leaves, the reduction of CO_2 assimilation [37] reduces the electron consumption by photosynthesis [38]. Results are comparable to a previous experiment [23], although the significant differences between the severe stressed treatment and the well-watered control are lower. Moderate stress treatment (I_{50}) showed close values with control in P_n, g_m and J_f/P_n, and intermediate value when dealing with g_s.

Severe water stress treatment started to maintain the turgor level (53 DAT) to support P_n. Ten days later (63 DAT), P_n of the treatments I_0 and I_{50} was as high as control, although some resistances at leaf level were evident (reduced g_s and g_m). At 70 DAT, growth regulation was not enough to sustain

a good physiological behavior of stress plants, so the electron transport of I_0 was impaired (+31%), as well as P_n (−38%), g_s (−55%), and g_m (−41%).
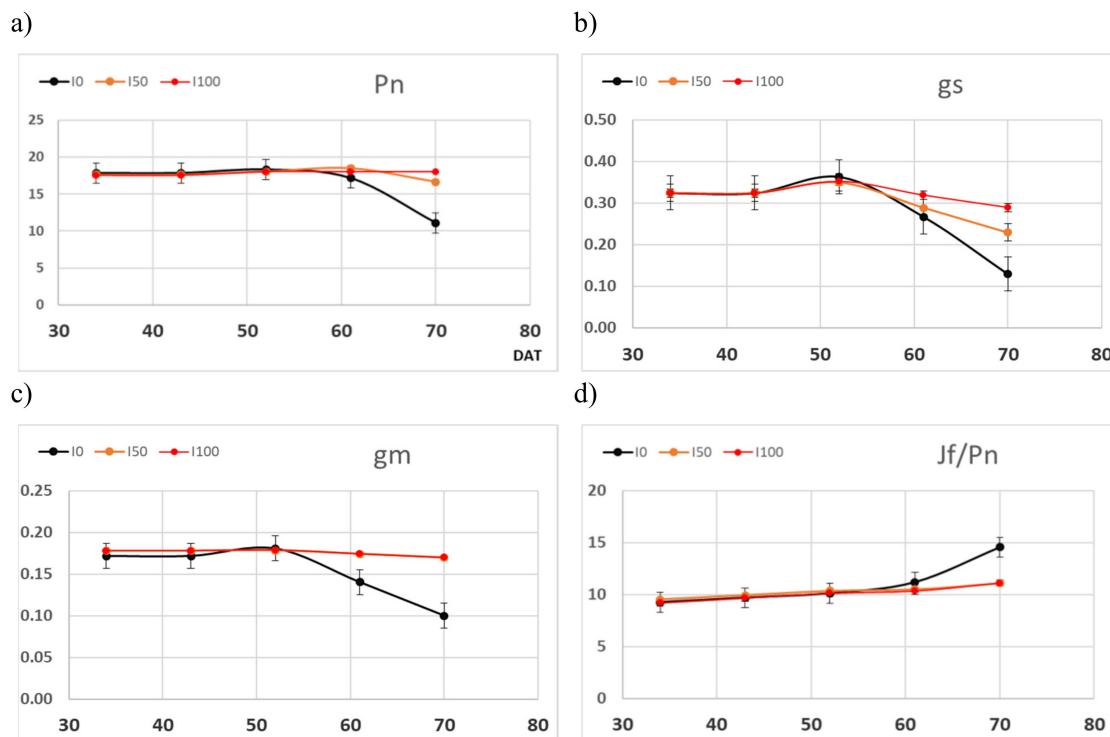
a)

b)

c)

d)

Figure 3. (**a**) Net photosynthesis (P_n µmol m^{-2}s^{-1}), (**b**) stomatal conductance (g_s mol m^{-2}s^{-1}), (**c**) mesophyll conductance (g_m µmol m^{-2}s^{-1}), and (**d**) $J_{f/}P_n$ (relative) of non-water-stressed (I_{100}-red), moderately water-stressed (I_{50}-orange), and severely water-stressed (I_0-black) spearmint plants along with the growth cycle are presented as above. Values with ± 1 S.E are given as means.

These results suggest that the spearmint plants are experiencing a slightly isohydric behavior [39] having maintained midday leaf water potential in I_{100} and also I_{50} (relative comfort), not too different from I_0 (rainfed), by decreasing stomatal conductance as a necessary strategy to reduce the transpiration rate. Therefore, this research on the physiological response of mint to drying soils and subsequent water stress classifies its behavior, in the circumstances of the study as relatively isohydric, and not anisohydric. Limpus [40] claims that isohydric responses occur when receptors in and around stomatal guard cells react to both these chemical and hydraulic signals to close stomata, and maintain leaf water potential as shown by the relatively constant result for noon leaf water potential, but declining stomatal conductance. Consequently, the leaf water potential should be measured at predawn in such case. Studies about stomatal behavior and assimilation rates in response to drought stress concerning isohydric behavior reveals medium level gas exchanges in plants [41], whereas some other studies [42] reported higher gas exchange rates under well-irrigated conditions. Further, the present study proved that the slight tendency for isohydric behavior in mint plants revealed minimum assimilation rates under well-watered conditions (maximum soil moisture, minimum VPD, and medium light/temperatures), but constant higher assimilation rates were found when drought stress occurred.

3.2. Soil Water Balance

The available water (AW, mm) stored in the soil profile (using root depth increasing up to a maximum of 1 m for all treatments) was later estimated with the soil and plant parameters indicated above (2.1), ETa and other terms of a simplified water balance estimated as described in 2.2. Assuming those parameters and variables, throughout the irrigation season AW stayed always within the interval assumed for RAW, for the I_{100} plot (Figure 4a). Conversely, for I_{50}, it showed a constant decrease after

DAT 20, overpassing the lower edge of RAW, therefore being under stress with consequent progressive reduction on ET, estimated as 10% by the end of the period under consideration (Ks = 0.9). The I_0 treatment showed also a continuous loss of available water starting soon after the 20th day of the experiment (DAT 20), with a significant water stress at harvest period. A decreasing stress coefficient (reduction of ETc) was applied to the rainfed treatment (I_0) from DAT 40 onwards, achieving estimated Ks = 0.5 at the end of season.
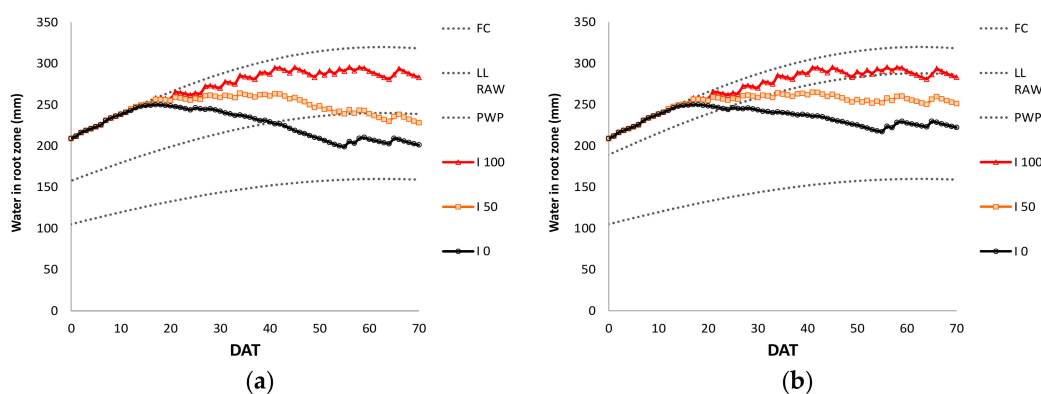

Figure 4. Total soil water in root zone during the irrigation season (0–70 DAT) for the three irrigation treatments (I_0, I_{50} and I_{100}), estimated from water balance (ex-post basis), considering different values for the depletion factor to estimate Ks: (**a**) p = 0.5, (**b**) p = 0.2, and assuming equal values for Kc in all treatments (see text).

These results apparently are consistent with the ones relating to the so-called physiological measurements (Figures 2 and 3), mainly for I_{50}, in that the water stress only is apparent when the lines for the three treatments (Figure 4a) stay below the lower limit of RAW (LL RAW), which happens after DAT 40 and 60 onwards, for I_0 and I_{50} respectively. However, there is uncertainty in several aspects, including in the critical value of p. In order to illustrate the impact of changing this parameter to a lower value, typical of plants where water use is very sensitive to soil water depletion, p = 0.2 was taken, obtaining outputs (Figure 4b) very different, where stress effects on evapotranspiration are expected from DAT 25 (I_0) and 30 (I_{50}) onwards. Unfortunately, to our knowledge, there are no dedicated experimental studies to get this parameter for this crop regarding stress function studies [43], so the uncertainty remains.

3.3. Biometric Data

Figure 5 reveals biometric results for the three irrigation treatments (I_0, I_{50} and I_{100}). The data recorded non-significant differences in the period 9–18 DAT. For all the parameters (except leaf ratio dry mass^{-1}), differences among treatments appeared significant from DAT 35. At the harvest, plant dry mass was −28% for I_{50} and −40% for I_0 compared to I_{100} treatment. The leaf dry mass was 20% lower and 31% lower than I_{100}, respectively for I_{50} and I_0.

These early detected differences are apparently not consistent with the late detection of stress by water status or other physiological measurements, as well as from estimated soil water status as in Figure 4a. The combined results could suggest that these plants present very high values, on average, for the yield response factor (Ky) [44], still remaining not clear from this study the mechanism for such reduction in growth. The reduction in yield compared to the reduction in water use usually ranges from 0.1 to 1.5 [45], being generally higher in early stages and other specific ones such as flowering. However, in this case, only late stress was identified, considering the parameters used in Figure 4a. Conversely, if the values obtained for water balance outputs of Figure 4b are considered, they seem consistent with the results shown in Figure 5.

Furthermore, the water balance outputs could also be adjusted considering possible significant variations in ETc, due to reduced Kc derived from long-term stress impacts in vegetation indexes,

as suggested by results discussed further on (Figure 5). An adjustment in Kc would provide intermediate results between Figures 4a and 4b, considering the onset of stress (not shown).
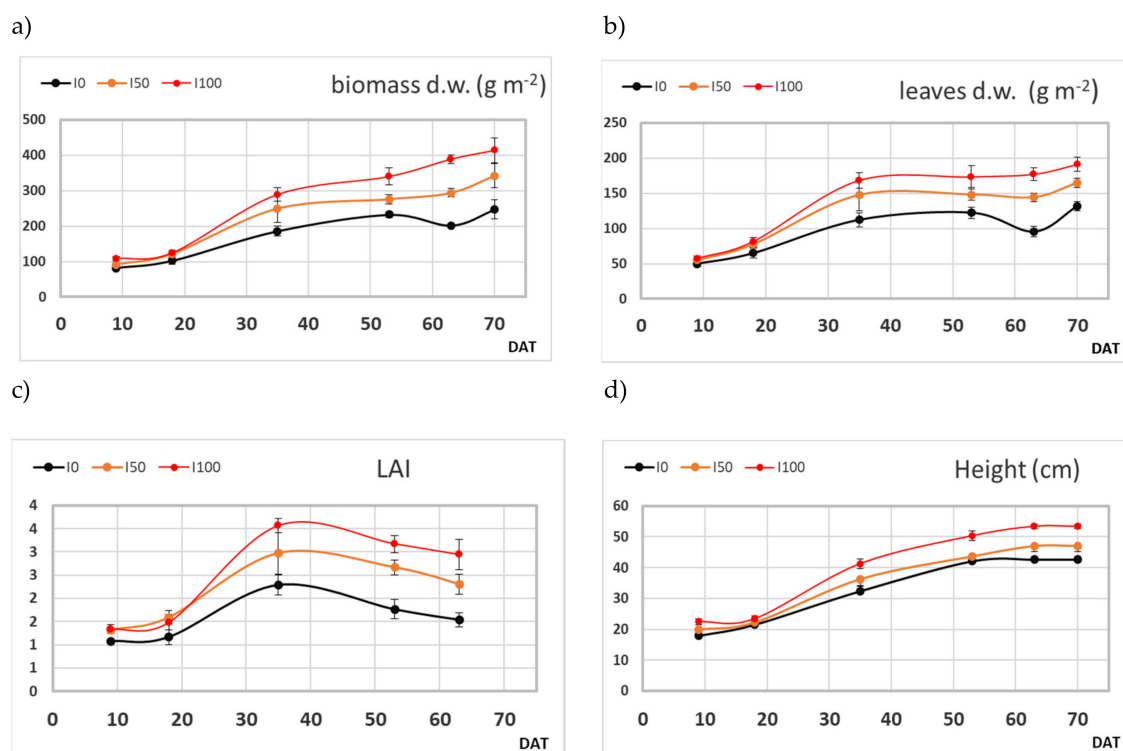
a)

b)

c)

d)

Figure 5. (**a**) Dry biomass (g plant^{-1}), (**b**) dry leaf (g plant^{-1}), (**c**) LAI, and (**d**) plant height (cm) of non-water-stressed (I$_{100}$), moderately water-stressed (I$_{50}$), and severely water-stressed (I$_0$) spearmint plants along the growth cycle are presented as above. Values with ± 1 S.E are given as means.

It should be also stressed that the same value for gs corresponds to lower values for bulk crop conductance (gc) if leaf area is reduced. Consequently, the results in Figure 3 could include an adjustment considering the results shown in Figure 5, and, consequentially, much earlier reduction in gc.

Yield data of fully irrigated treatment (I$_{100}$) are in parallel with data from Rolhoff [46], and comparable with results of Kizil et al. [47], who obtained a dry herbage yield (t ha^{-1}) at full flowering of a first-year crop of mint. Mitchell et al. [48] reported lower levels of yield, significantly reduced with the reduction in irrigation regimes. Rahimi et al. [49] reported the reduction in leaves area (cm^2), leaves number, leaves fresh weight and leaves dry weight. This significant decline occurred at moderate stress treated, named by those authors as 0.75 FC.

Similarly, decreasing biomass with increasing deficit irrigation was recorded by other researchers [24]. Meskelu et al. [50] reported that deficit irrigation had a significant effect on fresh biomass yield per hectare, while the highest fresh biomass was recorded at 100% ETc conditions. Alves et al. [51] indicated that the plant biomass (in total) were significantly affected in deficit irrigation conditions, and reported that suppressing irrigation for 12-days (before harvesting) has no effects on plant developmental stages. The study of García-Caparrós et al. [52] showed a significant decrease in drought stress regimes of *Mentha piperita* plant fresh weight. Chiappero et al. [53] reported that after increasing level of drought stress, a total reduction in plant growth, fresh weight, leaf number and leaf area is produced. The negative impact of drought was mitigated in plants exposed to PGPR (Plant Growth Promoting Rhizobacteria) inoculation, ultimately leads to a significant reduction in different growth traits of the spearmint plants those un-treated with PGPR. Chiappero et al. [53] also revealed that shoot fresh weight, leaf area, number of leaves and root dry weight were reduced with the increase of water stress. The same authors also reported the positive effect of PGPR on the behavior of stressed plants, ameliorating their physiological and biometric responses.

3.4. Organic Compounds

The essential oil produced (L ha^{-1}) is presented in Table 3, as the product of oil concentration x the leaf dry mass within each treatment at the two harvest dates (35 and 70 DAT). Data are presented as the most relevant compounds and classes.

Table 3. The essential oil yield of spearmint plants (L ha^{-1}) under different irrigation treatments (I_{100}, I_{50} and I_0) at two dates (DAT 35 and 70). Statistical analysis was performed by two-way ANOVA and Tukey test for mean differences. P-value *** means significant at 0.001; ** means significant at 0.01; * means significant at 0.05. Means followed by the same letter are not significatively different at 5% level.

	Irrigation Treatments (I)				DAT (D)			I x D
Classes (L ha^{-1})	I_0	I_{50}	I_{100}	p-value	35	70	p-value	p-value
Oxygenated Monoterpenes	14.5 b	16.8 b	22.3 a	***	15.5 b	21.2 a	***	n.s.
Monoterpene hydrocarbons	2.39 b	4.713 a	5.41 a	***	2.52 b	6.49 a	***	***
Sesquiterpene hydrocarbons	1.79 b	3.33 a	1.91 b	***	2.11 b	2.67 a	*	n.s.
Aldehydes	0.132 bc	0.161 b	0.172 a	***	0.110 b	0.217 a	***	***
Alcohol	0.093 a	0.061 b	0.064 b	***	0.058 b	0.093 a	***	***
Compounds (L ha^{-1})	I_0	I_{50}	I_{100}	p-value	35	70	p-value	p-value
Carvone	12.3 c	14.3 bc	17.6 a	***	12.6 b	17.7 a	***	n.s.
DL-Limonene	1.92 b	3.90 a	4.44 a	***	1.98 b	5.43 a	***	***
1,8-Cineole	1.31 b	1.41 b	3.26 a	***	1.74 b	2.36 a	**	***
trans-Sabinene hydrate	0.745 b	0.892 b	1.21 a	***	0.893	1.030	n.s.	**
β-Caryophylline	0.478 b	0.869 a	0.531 b	***	0.512 b	0.775 a	***	n.s.
Germacrene-D	0.485 b	0.995 a	0.543 b	***	0.641	0.721	n.s.	n.s.
β-Myrcene	0.233 b	0.345 a	0.397 a	***	0.221 b	0.428 a	***	n.s.
α-Bourbonene	0.424 b	0.766 a	0.456 b	***	0.482 b	0.641 a	**	*
Sabinene	0.076 c	0.128 b	0.205 a	***	0.08 b	0.216 a	***	***
2-Hexanal	0.132 b	0.161 a	0.172 a	***	0.110 b	0.217 a	***	***
β-Elemene	0.263 b	0.493 a	0.257 b	***	0.307 b	0.382 a	*	*
α-Pinene	0.078 b	0.156 a	0.133 a	***	0.089 b	0.170 a	***	***
β-*trans*-Ocimene	0.072 b	0.137 a	0.138 a	***	0.102 b	0.136 a	***	n.s.
Camphene	0.009 b	0.015 b	0.067 a	***	0.016 b	0.050 a	***	0
α-Terpenoel	0.096 b	0.139 b	0.198 a	***	0.198 a	0.069 b	***	n.s.
t-Elemene	0.135 b	0.213 a	0.120 b	***	0.159	0.151	n.s.	n.s.
3-Octanol	0.093 a	0.061 b	0.064 b	***	0.058 b	0.093 a	***	***
Eugenol	0.184 c	0.252 b	0.100 a	***	0.152 b	0.216 a	***	***
β-*cis*-Ocimene	0.024 b	0.051 a	0.055 a	***	0.035 b	0.054 a	***	n.s.
Tarpenene-4 ol	0.086 a	0.037 b	0.033 b	***	0.012 b	0.108 a	***	0

It is difficult to compare the results of oil yield (classes and compounds) since it depends on many factors as described by Figueiredo et al. [17] and briefed in the Introduction section. In the present study, nonsignificant differences were found among the three irrigation treatments and between the two harvest dates for organic compound concentrations in relation to different chemical classes (data not shown) The reason may be that the rainfed treatment did not reached a high stress condition as imposed in the paper by Coban et al. [54] on *Foeniculum vulgare* or by Rahimi et al. [49] that found different results since there was an increase in essential oil concentration with increasing water stress in mint.

In the present study increased irrigation levels augmented the level of oxygenate monoterpenes, monoterpenes hydrocarbons and aldehydes and lowered alcohol produced. The moderate stress (I_{50}) increased the sesquiterpene hydrocarbons. At 70 DAT all classes were significantly higher than those at 35 DAT. All differences were linked to hay yield differences at different irrigation regimes. According to Chrysargyris et al. [16] the spearmint essential oil (EO) mainly contains monoterpenes, while produced and stored in the glandular trichomes of the leaf.

In the present study, EO yield significantly increased in the irrigation treatments I_{100} and I_{50}, being in accordance with the increased EO content in basil through the increased irrigation levels [55]. Abdi et al. [56] revealed that different water regimes significantly affected the quality and quantity of EO, and they emphasize that peppermint plants cannot be grown successfully under water stress

regimes. The 70 DAT harvest showed the higher amount of EO than 35 DAT in all compounds except *trans*-sabinene hydrate, germacrene-D and β-elemene.

In the present study the major spearmint EO components were carvone, limonene, 1.8-cineole (eucalyptol), *trans*-sabinene hydrate, β-caryophylline and germacrene-D with accounted for the 90% of total oil yield (90%, I_0; 88%, I_{50} and 92 %, I_{100}).

Main compounds (concentration higher than 1.0 percent, calculated as percentage of total oil yield) were found to be: carvone (64% I_0; 57% I_{50}; 59% I_{100}), limonene (10% I_0; 15% I_{50}; 15% I_{100}), 1,8-cineole (6.8% I_0; 4.5% I_{50}; 10.8% I_{100}) and *trans*-sabinene hydrate (3.9% I_0; 3.6% I_{50}; 4%; I_{100}); βb-caryophyllene, germacrene-D, β-myrcene, α-bourbonene, β-elemene with lower oil amount.

The compounds analyses of the main compounds of *Mentha spicata* conducted by Jirovetz et al. [57] found the same main components with differences in the total amount of each compounds.

The present study confirms the dominant presence of carvone (varied from 68.1–74.3%), in agreement with the studies of Kokkini et al. [58], who identified carvone content at 68.4% in Mediterranean area (Greece), while others [7,59] found it at 76.6% and 67.1% in India and Brazil, respectively.

As expected, the few studies on mint irrigation report that plant growth and essential oil yield and composition were influenced by different levels of irrigation regimes, following various water deficit stress levels [56]. Okwany et al. [24] reported unchanged yield across apparently different irrigation treatments at the first cutting but a decrease at the driest plots during the second harvest due to the loss of leaf area index, as the biomass (hay) production is highly sensitive to water stress. Our data confirm their results on an increasing effect of irrigation on hay and oil yield per hectare, while our findings are different for the harvest dates, lower in our case for the second harvest. Our data also confirm the results of Nakawuka et al. [60] at Washington State University's Irrigated Agriculture Research and Extension Center (IAREC), although hay and oil produced are lower here than in the IAREC study. Our results are also parallel to the results obtained by Khorasaninejad et al. [61], who indicated that drought stress motivated a significant reduction in all of the growth parameters and essential oil yield and percentage. The highest values of growth parameters and essential oil percent and yield were observed under full irrigation. In contrast, Hassanpour et al. [62] claims that the seedlings at moderate stress (treatment named 75 % FC) showed maximum growth and it was the threshold of drought-initiated negative effects on seedling growth. Oil yields of different *M. longifolia* L. genotypes were higher when mint was fully irrigated, in comparison with the stress treatment, named 60% FC.

Results of Polanski et al. [63] were similar to ours, indicating that drought stress caused a significant reduction in all growth parameters and essential oil yield and percentage. Our results are consistent with the ones from Rhaimi et al [49], which stated that the drought stress had a negative impact on the total phenol content (TPC) and flavonoid content and cause a reduction from 72.5 to 68.1% in control and 0.25 in full irrigation treatments. The study also suggests that moderate water deficit in spearmint plants will be appropriate to enhance its biological properties. Hassanpour et al. [64] showed that drought stress decreased the growth and productivity parameters of the plants. The EO yield increased by about 1.6 times under drought stress, and the highest amount of EO was obtained in drought-stressed with penconazole (PEN).

Okwany et al [25] indicated that the carvone EO decreased after increasing irrigation levels, while limonene increased. This suggests an early maturity of the spearmint plants following higher irrigation stress conditions. These results are in parallel to the study of other researchers [23,65,66]. Okwany et al. [67] reported a reduction in carvone (7.5%) and an increment in limonene (5%) content after an increase in the deficit irrigation regimes.

Taking into consideration that carvone is formed by the limonene (via the intermediate the trans-carveol), it can be stated that the higher irrigation regime (I_{100} and I_{50}) caused carvone increase, which actually defines the advancement in the metabolic process of oil synthesis. Other studies said that carvone improvements might be achieved by optimizing the fertigation of spearmint plants [68].

This finding recorded by Rhaimi et al. [49] states a light and significant increase in essential oil concentration with the increase of water stress. The percent of essential oil was affected by the water

deficit into the study, while a significant higher quantity of essential oil percentage, was recorded in the fully irrigated treatment. No significant differences were found in the percentage of essential oil between normal irrigation and what authors call 0.75 FC treatment. The oil yield of the present study is lower than reported in Nakawuka et al. study [60], who recorded higher fresh hay yield than our study. Our EO data agrees with the study performed by Okwany et al. [24].

4. Conclusions

I_0 and I_{50} treatments underwent progressive water stress; I_0 as well as other treatments faced a continuous reduction of soil available water, interrupted three times for rainfall (20 mm in 14 days). Until 52 DAT, the leaf water potential, P_n, g_s, g_m, and linear electron transport rate remained similar among all treatments and started to differ after 52 DAT. Comparison with water stored in soil, or its meaning in terms of AW, suggests a strong impact of the uncertainties related with the lack of knowledge on stress functions for this crop, namely the allowed depletion factor, a parameter of the stress function equation.

Severely water-stressed *Mentha spicata* (I_0) was able to moderate crop growth, reducing aboveground biomass accumulation, and then lowering the number of leaves (since 50 DAT), and their surface (since 63 DAT), and at harvest (data extrapolated). Apparently, the reduced lowered behavior and biometric shape allowed *Mentha spicata* to maintain high physiological performances until 50 DAT. From this point on, reduced crop growth was not preventing physiological changes to cope with water stress. At this time, water stressed treatment, in particular I_0, restricted water losses by modulating stomatal closure. At 63 and 70 DAT, mesophyll metabolism was impaired (gm and linear electron transport rate). Differences between treatments at plant establishment (DAT 0) were never significant, though consistent at balsamic period.

Our results in hilly environment showed that *Mentha spicata* can adopt a conservative behavior, diminishing plant growth to maintain—as long as possible—a good physiological response in terms of leaf water potential and photosynthesis. When *Mentha spicata* plants were unable to prevent decrease in leaf water potential even at predawn, dehydration started diminishing, reaching the maximum at harvest (70 DAT). The essential oil production per hectare was strongly related to the crop hay accumulated.

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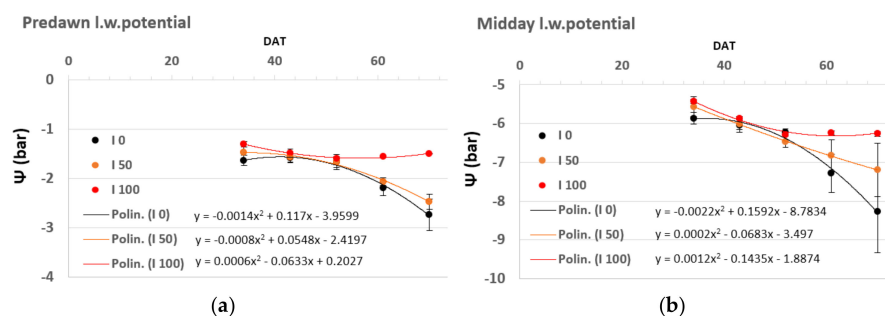
Appendix A

Figure A1. Statistical elaboration of data presented in Figure 2 (**a**) Predawn and (**b**) midday water potentials of fully irrigated (I_{100}), moderately water-stressed (I_{50}) and severely water-stressed (I_0) spearmint plants along the growth cycle. Values are means ± 1 S.E. In the figures are reported the equation that governs the predawn and midday water potentials variation

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