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Variation in Essential Oil Composition within Individual Leaves of Sweet Basil (*Ocimum basilicum* L.) Is More Affected by Leaf Position than by Leaf Age

Ravit Fischer,[†,||] Nadav Nitzan,[†,||] David Chaimovitsh,[†] Baruch Rubin,[‡] and Nativ Dudai*[,†]

[†]Division of Medicinal and Aromatic Plants, Agriculture Research Organization, Newe Yaa'r Research Center, P.O. Box 1021, Ramat Yishay, 30095 Israel

[‡]Faculty of Agriculture and Food Quality, The Hebrew University, P.O. Box 12, Rehovot, 76100, Israel

ABSTRACT: The aroma in sweet basil is a factor affecting the commercial value of the crop. In previous studies leaf age was considered to be a factor that influences the composition of essential oil (EO). In this study it was hypothesized that a single observation of the EO content in leaves from different positions on the main stem (young vs old) could predict the developmental changes in the plant during its life cycle. Plants harvested at week 16 demonstrated an exponential increase (R^2 = 0.92) in EO concentration in leaves on the main stem and lateral shoots, indicating higher EO concentrations in younger than in older leaves. Eugenol and methyleugenol predominated (28−77%) in the extract. Eugenol levels were higher in younger leaves (∼53%), and methyl-eugenol levels predominated in older leaves (∼68%). Linalool was lower in mature leaves than in younger leaves. This suggested that eugenol converted into methyleugenol and linalool decreased as leaf mature. However, in weekly monitored plants, the levels of these compounds in the EO had limited variation in the maturing leaf regardless of its position on the stem. This proposed that the EO composition in an individual leaf is mostly affected by the leaf position on the stem and not by its maturation process. Because leaf position is related to plant development, it is probable that the plant's physiological age at the time of leaf formation from the primordial tissue is the factor affecting the EO composition. It was concluded that interpretation of scientific observations should be carried out with caution and that hypotheses should be tested utilizing multifaceted approaches.

KEYWORDS: *Ocimum basilicum*, essential oil extract, leaf maturation, leaf position

INTRODUCTION

Sweet basil (*Ocimum basilicum* L., Lamiaceae) is an annual plant grown as a spice herb in Israel and in southern Europe.[1] It is a source of essential oils and oleoresin in the food and perfume industries.[2,3] The annual global basil essential oil production is estimated to be 100 tons.[4]

Sweet basil is divided into different chemotypes, which are subpopulations differing in their chemistry, chromosome number, plant morphology, and essential oil composition.[5] The biosynthesis of essential oil components takes place in the glandular trichomes, located predominantly on the surface of the leaves.[5−9] The essential oil (EO) is produced in the glandular trichomes, on young leaves, and as leaves age, the trichome density declines,[4,10] whereas at the same time the leaf gains weight. Therefore, the concentration and quantity of the EO change as leaves age. For an individual leaf, the total quantity of EO increases as the leaf ages; however, its concentration declines.[11−15] Generally, young leaves contain a lower quantity of EO than older leaves. However, due to its lower weight, a younger leaf has a higher EO concentration than an older leaf.

There are two documented main components of essential oils in basil; these are the terpenes (monoterpene and sesquiterpene) and phenylpropenes.[6,16,17] The major monoterpenes in the essential oils of sweet basil are (*R*)-linalool and 1,8-cineole. The major sesquiterpenes are germacrene D and α-bergamotene.[5,8] The main phenylpropenes are eugenol and methylchavicol. In the biosynthetic pathway of the EO, certain compounds are derived from each other. Methyleugenol and methylchavicol are the methylation products of eugenol and chavicol, respectively.[18,19] This conversion is carried out by the enzymes eugenol-*O*-methyltransferase (MT) and chavicol-*O*-methyltransferase (COMT), respectively. The level of activity of these enzymes in the different basil chemotypes is one of the major factors affecting the distinct phenylpropene composition in the EO.[9]

Leaves in different developmental stages (older vs younger leaves) have been recorded to have different EO profiles.[11,20,21] Comparing older leaves to younger leaves, Lange et al.[22] demonstrated that in older leaves the compounds, which were biosynthesized toward the end of the biosynthetic pathway, were present in higher proportions than compounds biosynthesized at early stages of the pathway. In younger leaves, the opposite trend was recorded, and compounds that were biosynthesized in the early stages of the pathway were the more common ones. This trend was characterized in *Mentha* × *piperita*, where younger leaves contained more menthone than menthol, whereas older leaves contained more menthol than menthone.[23−26] Similarly, in *Origanum syriacum*, the mature leaves mainly contained thymol, whereas its precursors *p*-cymene and γ-terpinene were predominant in the younger leaves.[27] Previous studies[9] in the sweet basil variety 'Perrie', a eugenol chemotype,[1] indicated that

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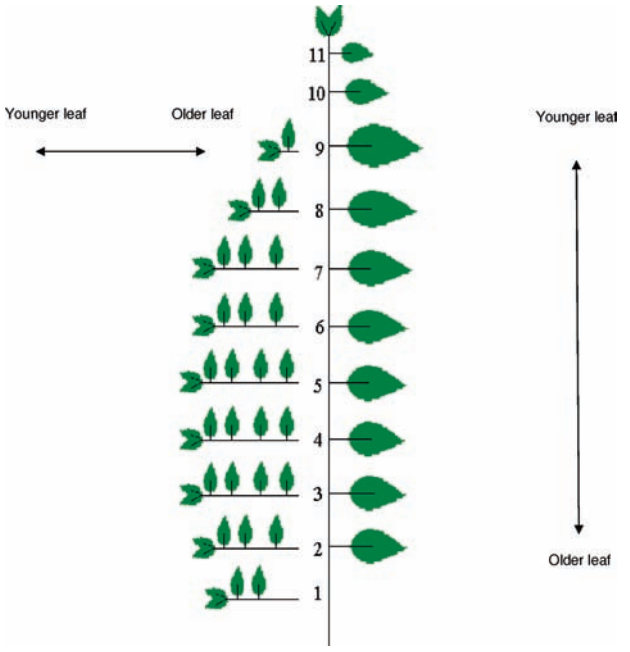

Figure 1. Schematic representation of a 16-week-old *Ocimum basilicum* var. 'Perrie' used in the present study. The plant has a main stem with up to 11 internodes. On the main stem two homologous leaves are attached to each internode, and lateral shoots extend sideways. By comparison, leaf age is related to its position on the stem. On the main stem, the closer the leaf is to the plant apex, the younger it is; and the closer it is to the roots, the older it is. On the lateral shoots, the closer the leaf is to the main stem, the older it is; and the farther it is from the main stem, the younger it is.

eugenol, the precursor of methyleugenol, was predominant in younger leaves, whereas methyleugenol predominated in the older leaves, and that this biosynthesis is the outcome of the enzyme eugenol *O*-methyltransferase activity. The information regarding the effect of leaf age on the essential oil characteristics is based on sampling leaves from an entire branch at the same time. Only a few studies were carried out in which individual leaves were sampled repeatedly as leaves matured.[28,29] These studies indicated that the decreasing levels of methylchavicol during leaf senescence correlated with a reduced activity of the enzymes EOMT and COMT.[29]

Israel produces and exports sweet basil to other countries year round. Sweet basil is the main herb, constituting 25% of the total fresh herbs exported with an income of €30 million in 2007.[30] The main parameter of quality in sweet basil, as well as other spices, is the aroma. Therefore, the breeding effort in sweet basil focuses on enhancement of the aroma's quality. To that end it is essential to extend our knowledge in regard to the genetic, physiological, and agro-technical factors that affect the quality of the aroma. The present study was aimed at understanding the dynamics of EO composition in individual leaves as influenced by leaf development and age, based on recording the kinetics in individual leaves.

MATERIALS AND METHODS

The sweet basil cultivar 'Perrie', a variety displaying homogeneous EO profiles, was used in all trials.[1] Figure 1 is a schematic representation of the morphology of a 16-week-old 'Perrie' basil plant. The plant has a main stem with up to 11 internodes. To each internode on the main stem are attached two homologous leaves, and lateral shoots extend from each internode. On the main stem, the closer the leaf is to the plant apex, the younger it is, and, vice versa, the closer it is to the roots, the older it is. On the lateral shoots, the closer the leaf is to the main stem, the older it is, and, vice versa, the farther it is from the main stem, the younger it is.

Phytotron and Greenhouse Experiment. Basil seeds were sown into 250 mL pots in Doron 6 commercial plant bedding mixture and grown in the greenhouse located on the Newe Ya'ar Research Center under ambient conditions. Five seeds were planted in each pot, and following germination, the seedlings were thinned to one uniform seedling per pot.

The greenhouse experiment was conducted at the Newe Ya'ar Research Center. The plants were grown in the greenhouse at 26−35 °C, relative humidity (RH) > 80%, and 16 h of daylight, for 16 weeks. Then three plants were harvested, removing all of the leaves from the main stem and lateral shoots. These were analyzed to determine the composition (concentration and quantity) of the essential oil.

The experiments in the phytotron were conducted on the campus of the Faculty of Agriculture of the Hebrew University of Jerusalem in Rehovot. Two hundred plants were divided equally and grown under two growth conditions. A batch of 100 plants was placed in a "warm" temperature regimen of 28/22 °C day/night, respectively, with 75% RH. The other 100 plants were placed in a "cool" temperature regimen of 16/10 °C day/night, respectively, with 70% RH. Individual plants within each chamber were considered to be a replication. All plants were subjected to a 16 h photoperiod (long day). The photoperiodic light was provided by 100 W incandescent bulbs. Plants were manually irrigated twice a day to field capacity. In the mornings, plants were watered with a standard nutritional solution (Schefer 1.34 mL/L, Koratin 0.5 mL/L, and Kalmagon 1 mL/L)m and in the afternoon, the plants were irrigated with regular tap water. Individual analogous leaves from each internode along the main stem were continuously sampled. At the "warm" temperature regimen all leaves were sampled on weekly intervals for 6 weeks. However, due to the plant's fast growth rate, after 6 weeks, the leaf at position 1 senesced and was no longer available. Therefore, only leaves at positions 3, 6, and 9 from the soil level were sampled. At the "cool" temperature due to the slow growth rate, the leaves were sampled on biweekly intervals for 14 weeks. Five plants (replications) were sampled per each interval per each temperature, a total of 10 leaves for each leaf position.

Extraction and Analysis of Essential Oil. GC-MS analysis was applied to determine the essential oil profile, quantity, and concentration in each sampled leaf. Fresh leaves were extracted individually with methyl *tert*-butyl ether (MTBE) 99.8% (Bio-Lab) containing 10 μg/mL isobutylbenzene 99% (Sigma-Aldrich catalog no. 113166) as an internal standard for quantitative calculations. The extraction was carried out in 20 mL scintillation vials with a plant material fresh weight to solvent volume ratio of 1 g/10 mL, respectively. The samples were shaken gently for 24 h at 25 °C, and the extract of each sampled leaf was filtered through Silica Gel 60 (230−400 mesh) (Merck & Co., Inc.) and sodium sulfate anhydrous (Na_2SO_4) (Merck & Co., Inc.) in Pasteur pipet columns to absorb water and polar and high-molecular-weight compounds. A volume of 1 μL of the extract was injected into a computerized gas chromatograph mass spectrometer (GC-MS, model GC-6890N) equipped with a mass selective (MS) 5973 Network (electron ionization 70 eV) detector (Agilent Technologies). The GC-MS apparatus was equipped with a 30 m × 0.25 mm fused-silica capillary column Rtx-5SIL MS (i.d. 0.25 μm) (Restek Corp.). Helium was used as a carrier gas at a constant rate of 1 mL/min. Samples were injected in splitless mode. The temperature of the injector was 250 °C, and the temperature of the transfer line and the detector was 280 °C. The temperature gradient program was 50 °C for 1 min, followed by an increase from 50 to 260 °C at a rate of 5 °C/min and a 10 min hold at 260 °C. Data were collected at a mass range of m/z 41−350. Identification of sample components was

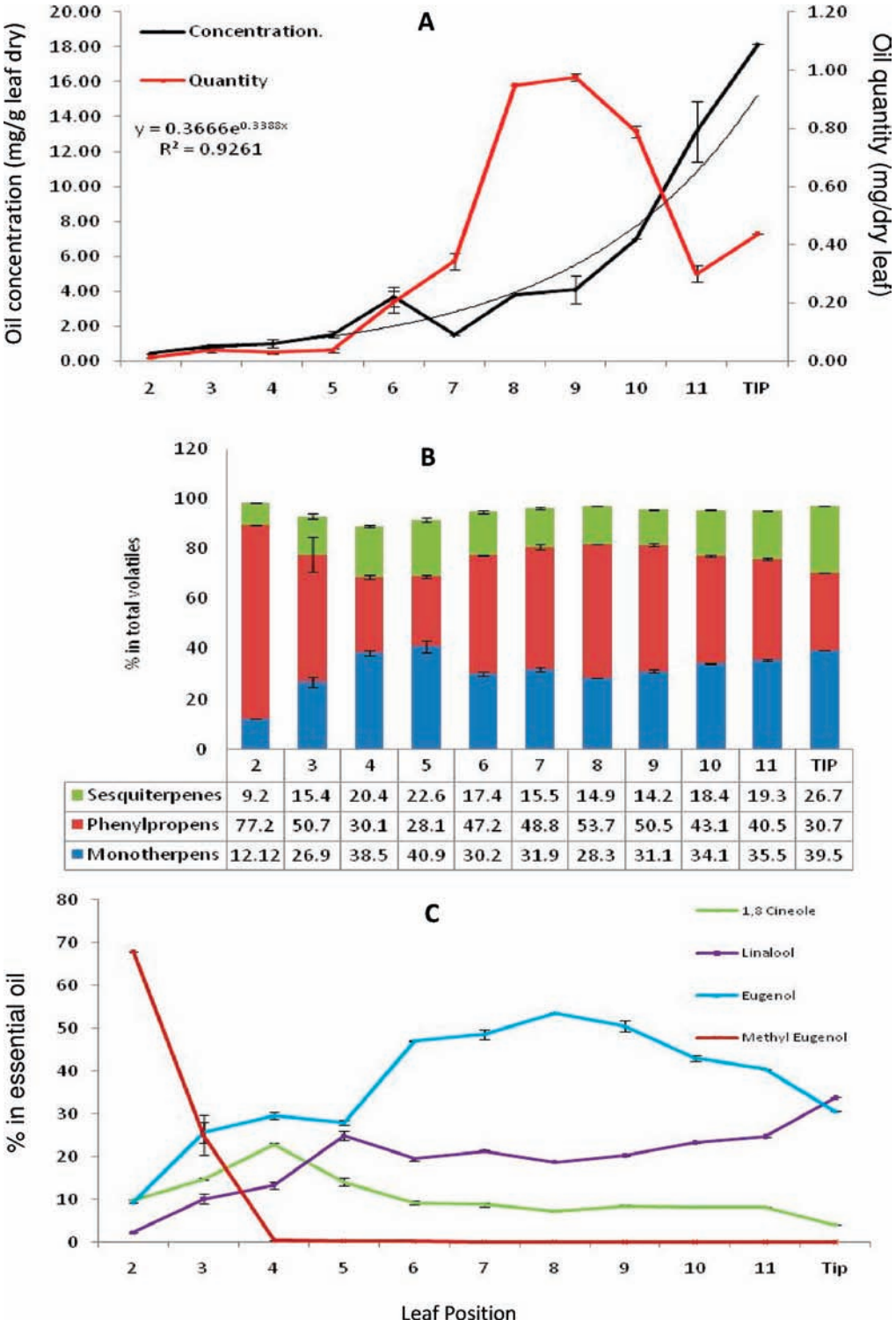

Figure 2. Variation in concentration and quantity (A), percent of monoterpenes, sesquiterpenes, and phenylpropens (B), and percent of eugenol, methyleugenol, linalool, and 1,8-cineole (C) in the essential oil extract of individual leaves along the main stem of *Ocimum basilicum* var. 'Perrie' plants that were grown in the greenhouse for 16 weeks until plant bloom. Each data point represents the mean ± standard deviation of six replicated leaves.

done by comparing their retention index (RI) to those of commercial standards and by comparing the electron ionization mass spectra of the samples' compounds to computerized GC-MS libraries (Adams 2001, NIST 98, and QuadLib 1697). Following extraction, the leaves were dried in an oven at 80 °C for 24 h and weighed.[11] The concentration C (μg/g) of a compound i in a sample j was calculated by using the formula

$$C_{ij} = (C_{IT} V_s S_i)/(W_j S_{IT})$$

where C_{IT} = 10 μg/g of internal standard, V_s = the volume (mL) of solvent used, S_i = the peak area of compound i, W^j = the dry weight (g) of

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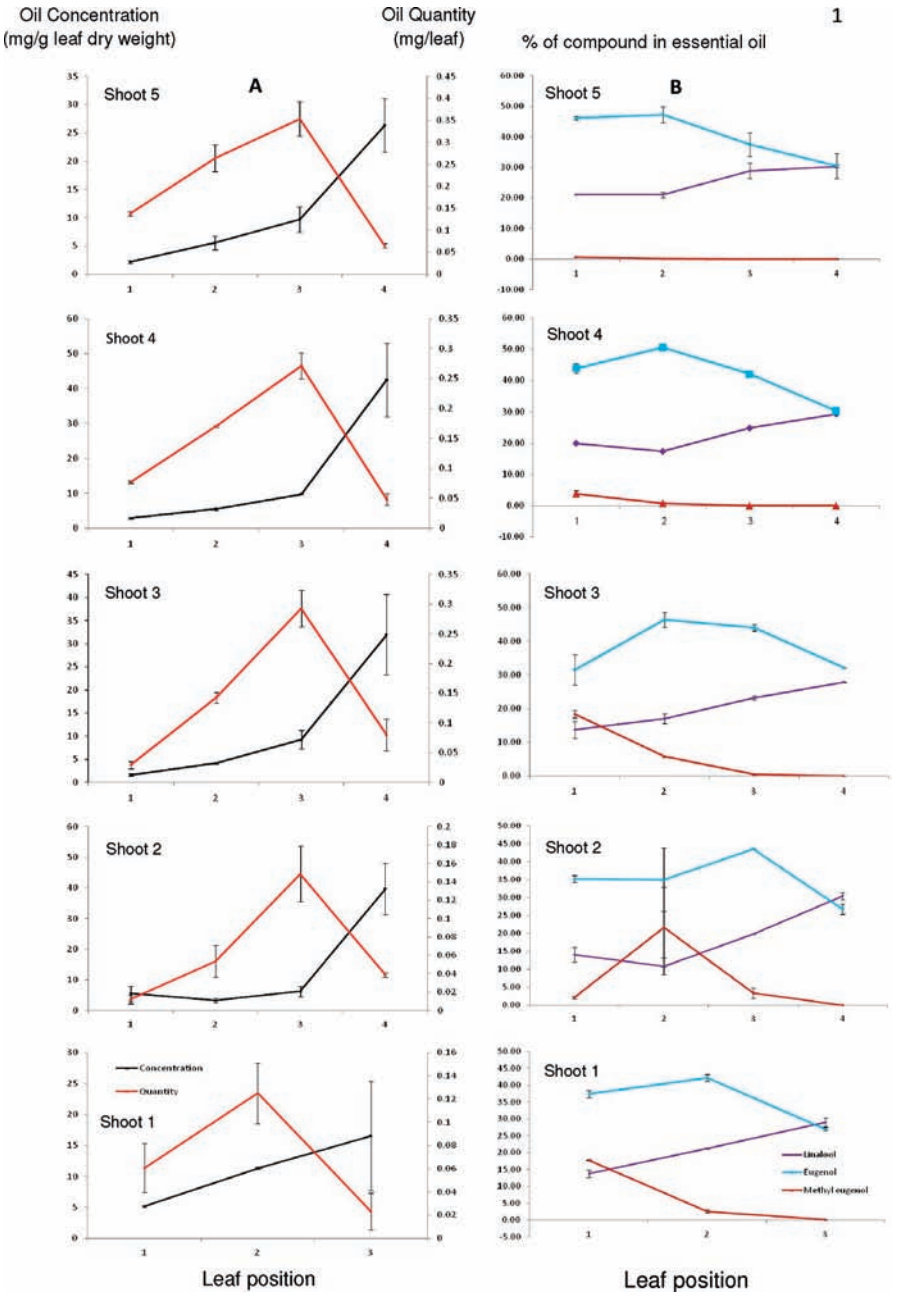

Figure 3. Variation in concentration and quantity of leaf essential oil (A) and percent of eugenol, methyleugenol, and linalool (B) in the essential oil of individual leaves along the lateral shoot of *Ocimum basilicum* var. 'Perrie' plants grown in a greenhouse for 16 weeks until plant bloom. Each data point represents the mean ± standard deviation of six replicated leaves.

sample *j*, and S_{IT} = the peak area of the internal standard. The percent of each compound in the essential oil was calculated on a dry weight basis of the sample.

RESULTS

Greenhouse Study. The concentration of the essential oil in the homologous leaves on the main stems of 16-week-old basil plants grown in the greenhouse demonstrated an acropetal increase with an exponential trend (R^2 = 0.92) (Figure 2A). Essential oil concentrations were higher for younger leaves and leaves positioned closer to the plant tip. The quantity of EO in the homologous leaves on the main stem also increased as leaves were younger and positioned higher on the stem, but reached its highest level at the leaf 9 and then declined (Figure 2A). On the lateral shoots, a similar pattern was recorded (Figure 3A). The EO concentration was higher in the younger leaves that were positioned closer to the tip of the lateral shoot and farther away from the main stem. The EO quantity also increased, reaching a peak at leaves 2 and 3, but then sharply declined (Figure 3A).

The EO profile of the leaves on the main stem was composed of 33 components (data not shown) including monoterpenes, sesquiterpenes, and phenylpropenoids, which comprised on average 88% of the essential oil (Figure 2B). The percent of monoterpenes within the EO extract ranged from 12.1 to 40.9% with the minimum in leaf 2 and the maximum in leaf 5. The main

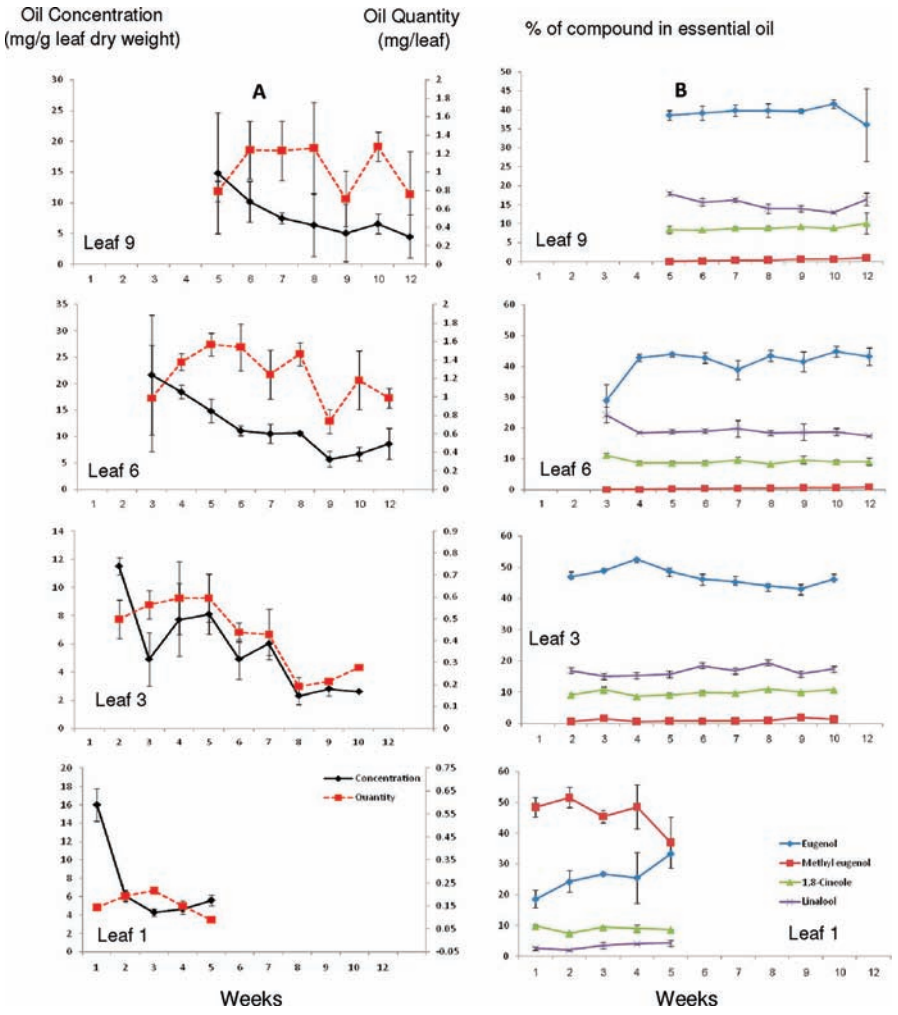

Figure 4. Variation in concentration and quantity of leaf essential oil (A) and percent of eugenol, methyleugenol, 1,8-cineole, and linalool (B) in the essential oil of individual leaves along the main stem of *Ocimum basilicum* var. 'Perrie' plants grown in the phytotron under a "warm" temperature regimen (22/28 °C). The plants were monitored and repeatedly sampled over 12 weeks. Each data point represents the mean ± standard deviation of 10 replicated leaves.

monoterpenes were linalool and 1,8-cineole (Figure 2C). The percent of sesquiterpenes in the EO extracts ranged from 9.2 to 26.7% with the minimum in leaf 2 and the maximum in the tip. The main sesquiterpenes were germacrene D and τ-cadinol. The phenylpropenes eugenol and methyleugenol were the dominant components of the essential oil extract in 8 of 11 leaves, excluding leaves 4 and 5 and the tip. Their levels within the EO extract ranged between a minimum of 28.1% in leaf 5 and a maximum of 77.2% in leaf 2. The levels of eugenol in the extract ranged from 9.3% in the older leaf at position 2 to a maximum of 53.6% in the younger leaf at position 8 (Figure 2C). In contrast, the level of methyleugenol in the extract ranged from a maximum 67.9% in the older leaf at position 2 to <0.5% in leaves 4−8. Traces (<0.02%) of methyleugenol were recorded in the youngest leaves at position 9 to the tip. Generally, among the phenylpropenes, eugenol was the dominate component in most leaves, excluding leaves at positions 2 and 3 (Figure 2C).

On the lateral shoots, the percent of eugenol in the EO extracts was higher than the percent of methyleugenol in all leaves and on all lateral shoots, excluding leaf 2 on the second lateral shoot (Figure 3B). Methyleugenol's levels decreased to traces (<0.02%) as the leaves on each lateral shoots were younger. The levels of methyleugenol remained this way from position 5 to the plant's apex, as the lateral shoots were younger.

Phytotron Study. Essential oil concentration per individual leaf of plants grown under "warm" (22/28 °C) conditions was highest in the early stages of leaf development, when each leaf was young, declining between 50 and 83% as the leaves matured (Figure 4A). The same was observed in leaves at positions 1−3 of plants grown under "cool" (10/16 °C) conditions with a decline between 80 and 96%, but not in the leaf at position 4 (Figure 5A). Comparing EO concentration in the oldest leaf (position 1) to that in the youngest leaf (position 9) of plants under "warm" conditions indicated that there were higher concentrations in the younger leaf (14.8 ± 6.9 mg/g) than in the older leaf (5.6 ± 0.4 mg/g) at week 5. This was not recorded in the "cool" temperature regimen. Essential oil quantity demonstrated an increase in its levels in the first 2−4 weeks of leaf maturation, at both temperature regimens. Under "warm" conditions, this increase was followed by a decline in leaves at positions 1 and 3 and by fluctuations in leaves at positions 6 and 9 (Figure 4A). Under "cool" conditions, this increase was followed by a decline in the leaves at positions 1, 2, and 4, but not in leaves at position 3.

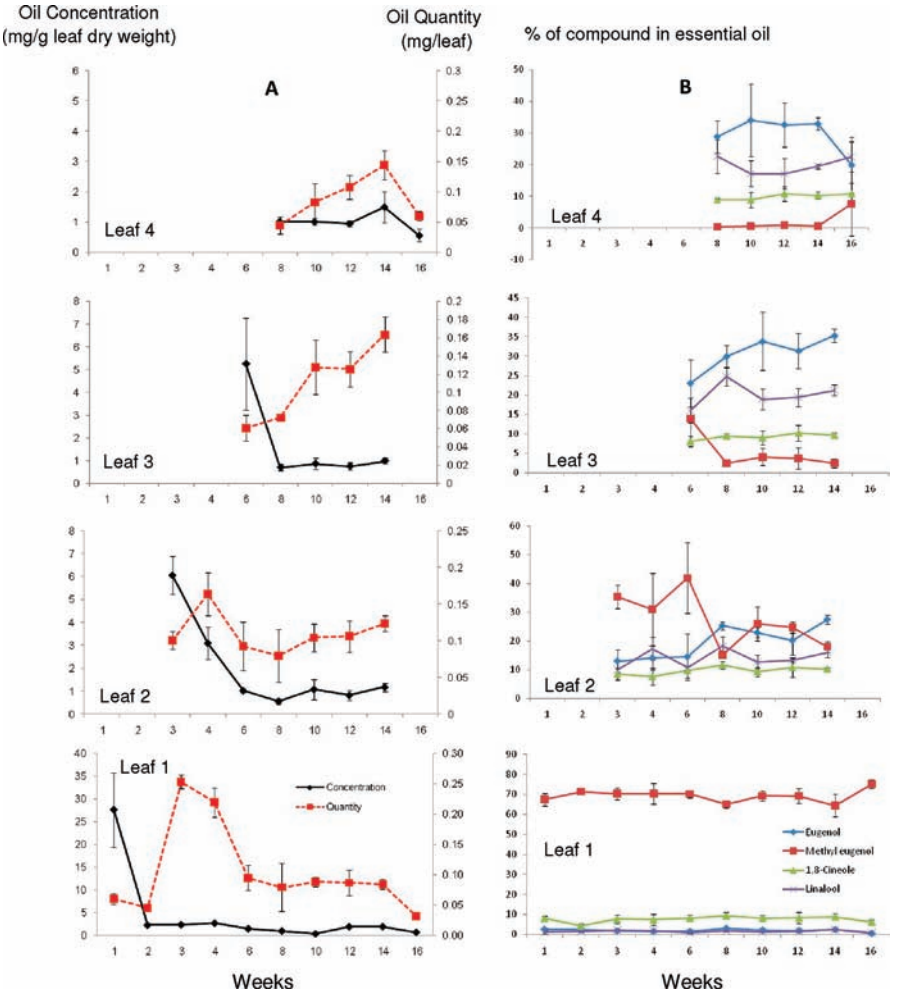

Figure 5. Variation in concentration and quantity of leaf essential oil (A) and percent of eugenol, methyleugenol, 1,8-cineole, and linalool (B) in the essential oil of individual leaves along the main stem of *Ocimum basilicum* var. 'Perrie' plants grown in a phytotron under a "cool" temperature regimen (10/16 °C). The plants were monitored and repeatedly sampled over 12 weeks. Each data point represents the mean ± standard deviation of 10 replicated leaves.

Figures 4B and 5B summarize the dynamics of eugenol, methyleugenol, 1,8-cineole, and linalool, and Figures 6 and 7 summarize in tabulated form the outcome of the analysis of variance for the same data set. A significant interaction ($P < 0.05$) between leaf position and time of observation postemergence (week) was recorded for eugenol and methyleugenol at both temperature regimens and for linalool and 1,8-cineole at the "warm" conditions (Figures 6 and 7). Methyleugenol was the main phenylpropene in leaf position 1 in both temperature regimens (Figures 4B and 5B) across the 12 weeks of the trial, but its levels declined significantly ($P < 0.05$) to traces in the younger leaves (Figures 6A and 7A). In contrast, the levels of eugenol were lowest in leaf 1, but increased significantly ($P < 0.05$) in the younger leaves (Figures 6B and 7B). Such variation was also recorded for linalool under "warm" conditions (Figure 6D), but not under "cool" conditions. Contrary to the differences recorded among leaves at different positions, within each leaf the levels of the different compounds remained relatively constant, presenting limited variation as the leaf matured (Figures 6 and 7). For example, the levels of methyleugenol under "warm" temperature differed in leaf 1 only between weeks 1 and 5, and under "cool" conditions, its levels in leaf position 1 were 69% on average for the entire duration of the study. The limited variation in compound levels within each leaf was observed for all compounds at both temperature regimens.

DISCUSSION

The consensus in the literature is that biosynthesis of chemicals and essential oil in sweet basil takes place in the trichomes located predominantly on the leaves.[5−9] As leaves age, the glandular trichome density declines,[4,10] while at the same time the leaf gains weight. Therefore, the concentration and quantity of the EO change during leaf maturation. Generally, a younger leaf, positioned higher on the main stem, contains a lower quantity of EO than an older leaf positioned lower on the stem. However, due to its lower weight a younger leaf tends to have a higher EO concentration than an older leaf. At the same time, because EO evaporates, its levels decrease as the leaf becomes older. Consequently, within an individual leaf, the quantity of EO decreases with time. The results of the greenhouse study presented a snapshot of the status of the EO components within each leaf 16 weeks postemergence (Figure 2A) and were in agreement with this consensus. Furthermore, the same pattern also was observed in the leaves on the lateral shoots.

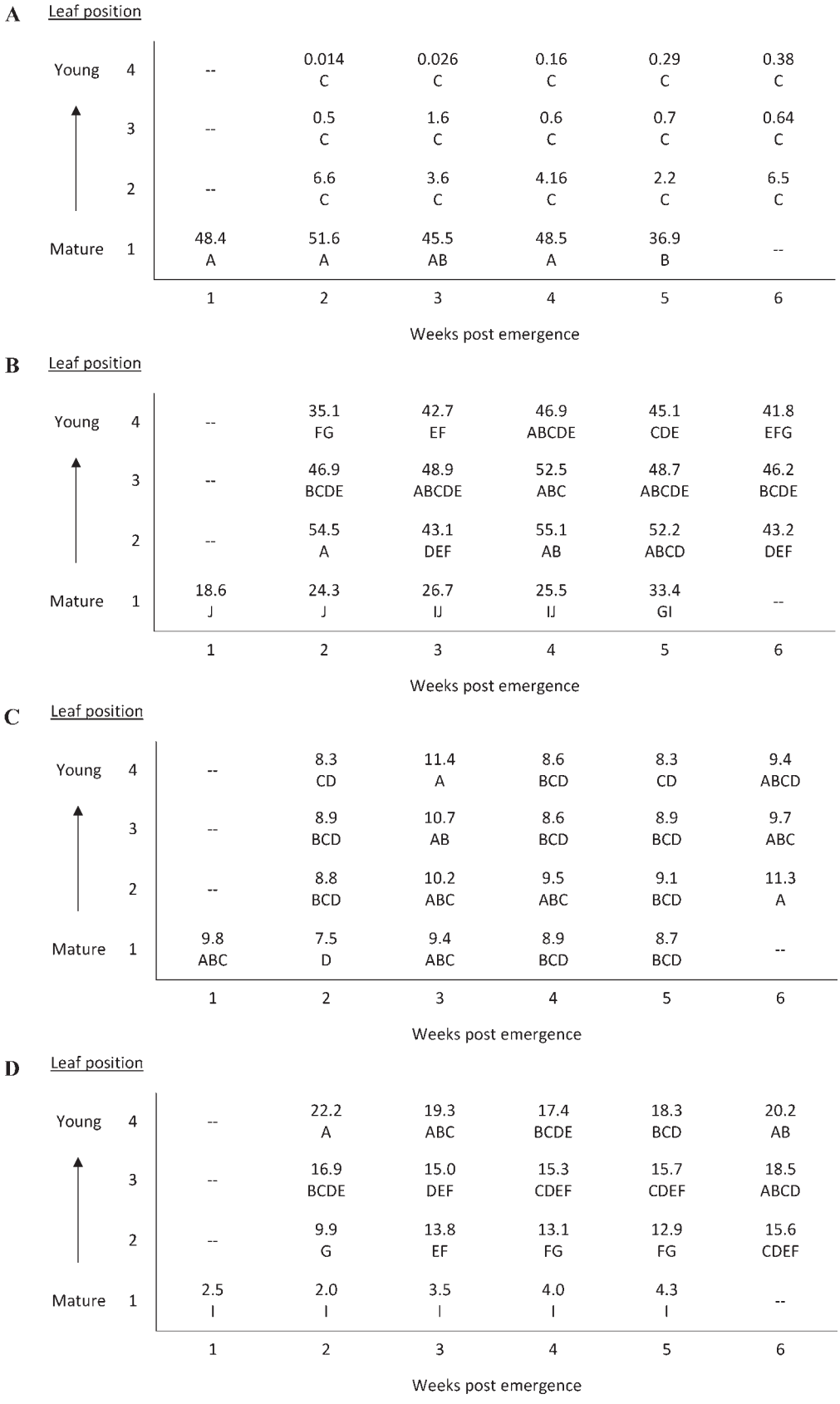

Figure 6. Percent of methyleugenol (A), eugenol (B), 1,8-cineole (C), and linalool (D) in the essential oil of sweet basil cv. 'Perrie' leaves at positions 1−4 across 6 weeks following plant emergence in the phytotron at 22−28 °C. Two-way ANOVA indicated an interaction between week and leaf position (A, $P < 0.0019$; B, $P < 0.0001$; C, $P = 0.0118$; D, $P < 0.0001$). Mean comparison was carried out using Tukey's HSD test. Different capital letters indicate statistically significant differences. Values of leaves 1 and 3 correspond with Figure 4B.

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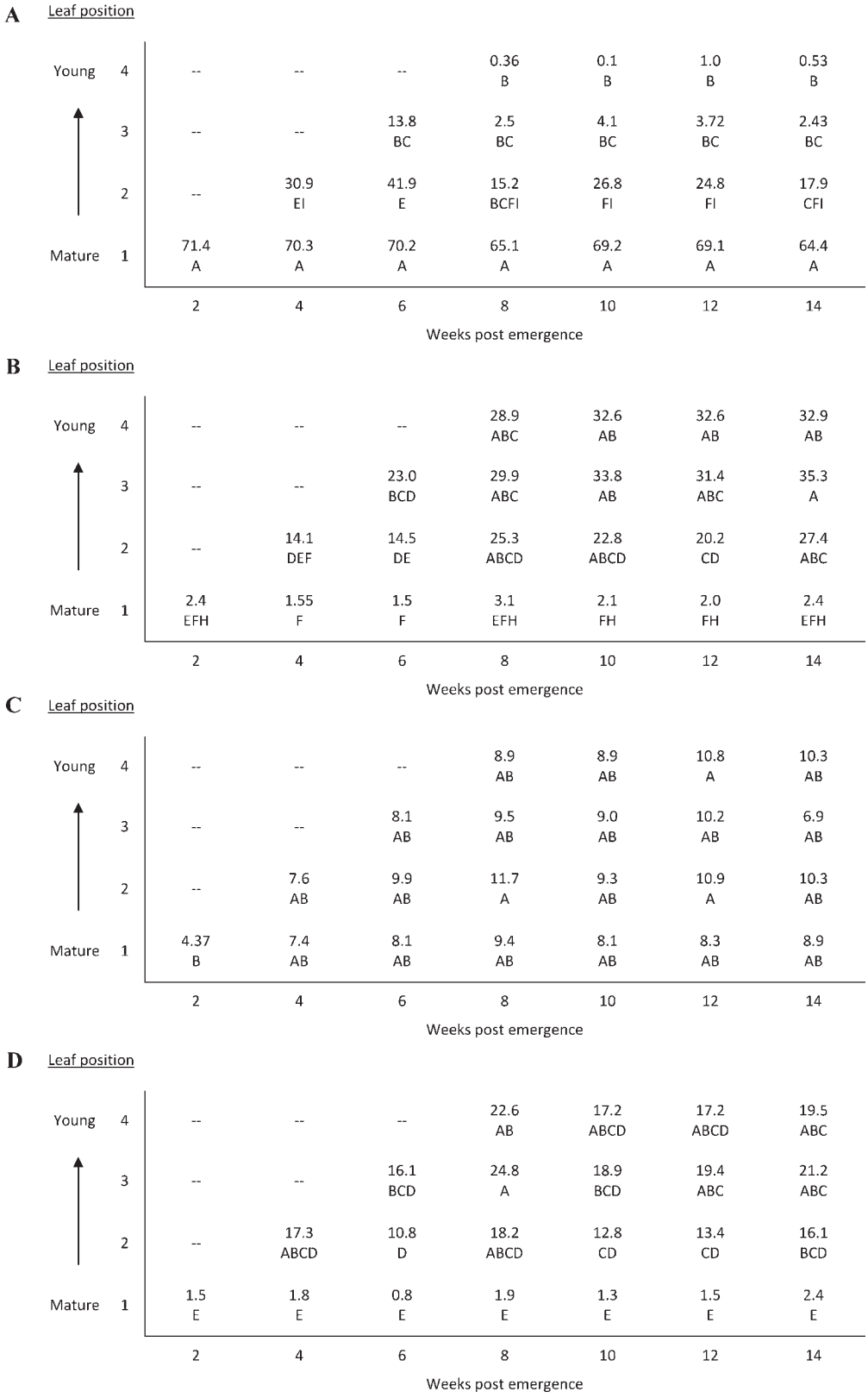

Figure 7. Percent of methyleugenol (A), eugenol (B), 1,8-cineole (C), and linalool (D) in the essential oil of sweet basil cv. 'Perrie' leaves at positions 1−4 across 14 weeks following plant emergence in the phytotron at 10−16 °C. Two-way ANOVA indicated interaction between week and leaf position (A, $P = 0.0428$; B, $P = 0.0648$; C, $P = 0.001$; D, $P = 0.411$). Mean comparison was carried out using Tukey's HSD test. This presentation corresponds with Figure 5B.

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Figure 2C summarizes the composition of the oil in the leaves on the main stem 16 weeks postemergence, indicating that eugenol was the principal component in younger leaves, whereas methyleugenol predominated in older leaves and declined to traces in younger leaves. Similar behavior, with minor variations, was recorded on the side shoots (Figure 3B). It is documented that eugenol is the precursor of methyleugenol;[9] hence, the hypothesis that during leaf maturation eugenol is converted into methyleugenol makes sense. However, the dynamics of these compounds recorded via repeated sampling of individual leaves in the phytotron indicated that this relationship is not so simple. In leaf 1 on the main stem, at both temperatures in the phytotron, methyleugenol levels predominated, and later in the younger leaves it was eugenol that dominated (Figures 4B and 5B). Nevertheless, when each leaf position was examined individually, the levels of these compounds had a limited variation as leaves matured (Figures 6 and 7). This observation questions the accuracy of the above hypothesis, suggesting that eugenol was not converted into methyleugenol. We therefore assume that leaf position has a greater role in determining the composition of the EO extract in a leaf rather than the leaf's maturation process.

Leaf position is related to plant age and therefore may be affected by the physiological processes that take place in the plant at the time of leaf birth. In this study we expected to record certain equilibrium between eugenol and methyleugenol, in other words, to record a reduction in eugenol levels and an increase in methyleugenol levels as the leaf matured. However, that was not the case. To elucidate our novel finding we plan to study the dynamics of the enzyme eugenol-*O*-methyltransferase, which is responsible for the conversion of eugenol into methyleugenol.[9] To do so we will use eugenol chemotype sweet basil plants and record the enzyme's activity in preselected leaves at different plant ages. In addition, we will examine hormonal status in the plants to correlate it with eugenol/methyleugenol levels. We assume that this approach will explain the phenomenon recorded here.

In sweet basil the aroma is the factor sought by consumers. In recent years claims have been made that the aroma of the Israeli-grown basil differs from that of the Italian-grown. Because the aroma of sweet basil is determined by its EO composition, the results presented here explain why the aroma of sweet basil plants grown in Italy differs from the aroma of Israeli exported plants. In Italy, sweet basil is marketed to the consumer when the plants are small, with approximately three homologous leaves at positions 1−3 on the main stem, without any lateral shoots. Israel exports fresh basil year round, and leaves are harvested from higher positions on the main stem from mature basil plants. Assuming that the EO concentrations associated with plant maturation are similar in the Italian-grown and Israeli-grown sweet basil, then the Italian sweet basil should have a higher percentage of methyleugenol when harvested, whereas the Israeli basil should have a higher percentage of eugenol. To export sweet basil with an aroma similar to that of the Italian, Israeli growers would need to change their crop management and harvest and market sweet basil plants at leaf position 3, when plants are approximately 3 weeks old. Such a change in agro-technique will have to be evaluated economically.

In summary, the present study reported the dynamics of EO in sweet basil using whole plants as well as individual leaves during their maturation. The findings are novel, indicating that the aroma is significantly affected by the position of the leaf on the stem rather than by the leaf age or maturation process. Furthermore, these results also point out that interpretation of scientific observations needs be carried out with caution and that hypotheses should be tested more than once and with multifaceted approaches. The outcome of this study should assist in developing new agronomic and horticultural strategies and techniques that will allow control and manipulation of aroma according to the customer's preference through the development of agro-techniques as well as breeding of new sweet basil varieties.

AUTHOR INFORMATION

Corresponding Author

*E-mail: nativdud@agri.gov.il or nativdud@gmail.com. Phone: +972-4-9539556.

Author Contributions

‖The first and second authors made equal contributions to this paper.

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