

# Photosynthetic behavior, growth and essential oil production of *Melissa officinalis* L. cultivated under colored shade nets

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## ABSTRACT

The modulation of light is of importance during cultivation of medicinal plants to obtain desirable morphological and physiological changes associated with the maximum production of active principles. This study aimed to evaluate the effect of the light spectrum transmitted by colored shade nets on growth, essential oil production and photosynthetic behavior in plants of lemon balm (*Melissa officinalis* L.) Plants were cultivated in pots for 4-mo under black, red, and blue nets with 50% shading, and full sunlight exposure. Biometric and anatomical variables, essential oil yield, global solar radiation, photon flux density, chlorophyll content, and gas exchange parameters were measured in *M. officinalis* leaves. The results showed that despite being considered a partial shade plant, this species is able to adapt to full sunlight conditions without increasing biomass production. The spectral changes provided by colored shade nets did not caused any noticeable change in leaf anatomy of *M. officinalis*. However, the use of blue net resulted in increments of 116% in plant height, 168% in leaf area, 42% in chlorophyll content and 30% in yield of essential oil in lemon balm plants. These plant's qualities make the use of blue net a cultivation practice suitable for commercial use.

**Key words:** Medicinal plant, development, gas exchange, leaf anatomy.

## INTRODUCTION

Lemon balm (*Melissa officinalis* L., Lamiaceae) is a medicinal plant of great economic importance due to its proved therapeutic properties and their inclusion in the Pharmacopoeia of different countries (Weitzel and Petersen, 2010). In recent years, due to the new commercial interest in natural products, the increased demand for the production of plant extracts of this species has required the development of intensive cultivation techniques which might favor a faster growth and contribute to the productivity of plant-derived products.

The use of shade nets with different colors allows physical plant protection combined with promotion of the physiological responses regulated by light (Martins et al., 2008), since these nets are able to modify both the quantity and quality of solar radiation transmitted. Thus, photoselective nettings have been developed in different colors to alter the spectrum of transmitted light, with the blue and red nets exhibiting peaks of transmittance in the blue-green (400-540 nm) region and in the red region (590 nm), respectively (Oren-Shamir et al., 2001).

The use of colored nets has been related to the production of essential oil, growth and biomass allocation, pigments content, and leaf anatomy (Brant et al., 2009; 2010; 2011). However, there are few reports on the effect of the quantity and quality of light on the photosynthetic capacity in *M. officinalis*. Cassol et al. (2007) examined the absorption characteristics of light energy and photosynthetic parameters in lemon balm plants when cultivated under reduced light conditions. According to these authors, net photosynthesis was negatively influenced by shading, and the biochemical steps of photosynthesis proved to be more sensitive to the effects of light in this species.

Taking into consideration that leaves have high plasticity to adjust their anatomy and physiology in response to different light conditions that alter plant development and secondary metabolites production, the aim of this study was to analyze the changes in growth, leaf anatomy, photosynthetic behavior, and essential oil production of *Melissa officinalis* cultivated under different colored shade nets.

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## MATERIALS AND METHODS

### Study site and plant material

The experiment was conducted from May to December 2012 in Presidente Prudente (22°7'39" S, 51°23'8" W, 471 m a.s.l.), São Paulo, Brazil. According to Köppen classification, the regional climate is type Aw mesothermal with hot summers and dry winters (Brazil, 1992). Climatological data from Presidente Prudente are shown in Table 1.

Lemon balm seeds were planted in a nursery in a seed bed formed with a mixture of sand and commercial substrate Bioplant (Bioplant Agrícola) (1:1 v/v). The seedlings were grown under 50% shading for 60 d and then were transplanted to 5 L pots containing soil, sand, and cattle manure in a 2:1:1 ratio (v/v/v) for the shading experiment. Plants were maintained in the nursery, where they received full sunlight or were under shade. For the shade treatments, light was artificially reduced using red, blue, and black nets (ChromatiNet-Polysack Industries, Nir Yitzhak, Israel) allowing 50% shade. Plants were cut twice (10 cm above soil surface) during the growth period, the first cut at 50 d after transplanting (DAT) and the second after regrowth at 120 DAT. Irrigation was manually performed three times a week to maintain high soil humidity and a new fertilization was done with cattle manure after the first cut (125 g per pot).

### Photosynthetic characteristics and chlorophyll content

The measurements of radiation were made under clear sky conditions during 3 consecutive days at 08:00, 13:00 and 18:00 h. Solar radiation ( $R_g$ ,  $W m^{-2}$ ) was determined by a pyranometer (Li-200R, LI-COR, Lincoln, Nebraska, USA). Measurements of photosynthetic photon flux density (PPFD,  $\mu mol photons m^{-2} s^{-1}$ ) were made using a quantum meter (LQ model 250A, LI-COR). The estimated amount of incident radiation reaching the top of the plants, which was probably used in photosynthesis realization, was calculated by the  $R_g/PPFD$  ratio.

Photosynthetic light-response curves (curves A/PPFD, where A corresponds to the net  $CO_2$  assimilation) were created by measuring the steady rates at 40 DAT using five

**Table 1. Climatological data of Presidente Prudente, São Paulo, during the experimental period (May-December 2012).**

Month	Temperature of the air (°C)			Precipitation mm
	Minimum	Maximum	Mean	
May	15.7	24.2	21.7	109.6
June	14.3	25.2	20.4	284.3
July	13.1	25.4	20.6	23.7
August	20.5	25.3	23.7	-
September	16.1	31.2	24.6	116.1
October	18.3	31.5	26.3	22.0
November	20.8	30.4	25.8	107.5
December	22.9	31.4	28.0	297.6

Source: Center for Weather and Climate Research Applied to Agriculture (CEPAGRI/UNICAMP).

plants per treatment (one leaf per plant). All A/PPFD curves were estimated from 09:00 to 13:00 h on subsequent days using measurements obtained from healthy, fully expanded leaves. Inside the leaf chamber of an infrared gas analyzer (Li-6400XTR, LI-COR) temperature was adjusted to 30 °C,  $CO_2$  concentration was 380  $\mu mol mol^{-1}$ , and vapor pressure deficit was maintained at 1.5 kPa with a dew point generator (model Li-610, LI-COR) connected to the leaf chamber. Light was provided by LEDs emitting in the blue-red spectrum connected to the Li-6400XTR sampling chamber. Variables obtained from A/PPFD curves were maximum  $CO_2$  assimilation ( $A_{max}$ ), light compensation ( $P_{com}$ ), and saturation ( $P_{sat}$ ) points calculated when A reaches 90%  $A_{max}$ , apparent quantum efficiency (AQE) corresponds to the slope of the initial linear region of the curve A/PPFD, transpiration (E), stomatal conductance ( $g_s$ ) obtained from the saturation point of photosynthesis at light, and leaf dark respiration ( $R_d$ ) when PPFD = 0  $\mu mol photons m^{-2} s^{-1}$ .

Chlorophyll content index (CCI) was estimated using a chlorophyll content meter (CCM-200 Opti-Sciences, Hudson, New Hampshire, USA) based on the absorbance of the intact leaf at two wavelengths (660 and 950 nm). Measurements were made on three randomly selected and completely expanded leaves per plant in each treatment at the time of the first cut (50 DAT).

### Plant growth and leaf anatomy

Total leaf area (LA), dry mass (DM) of leaves, stems, roots, and whole plant, root/shoot ratio, specific leaf mass, and leaf weight ratio (LWR) were evaluated in two periods (50 and 120 DAT) using five plants per treatment. Plant height was evaluated only at 120 DAT, when plants were standardized by the first cut. Leaf area was measured using a leaf area meter (LI-3000A, LI-COR). After measuring the plants, they were placed into a forced air circulation oven at 60 °C until constant mass. The leaf weight ratio was determined as leaf DM/total DM and specific leaf area (SLA) was determined as LA/leaf DM.

For leaf anatomical analysis, five leaves were randomly collected from plants in each treatment at 50 DAT. A sample of approximately 100  $mm^2$  was detached from the median region of the leaf under an optical microscope. The samples were fixed in formaldehyde-acetic acid (FAA) 50% (Johansen, 1940) and preserved in 70% ethanol. Semi-permanent slides were mounted from cross sections stained with toluidine blue and mounted with glycerol (O'Brien et al., 1965). The thickness of adaxial and abaxial epidermis, palisade, and spongy parenchyma were measured in cross-sections of the median region of leaves. The total number of xylem conduits and the diameter of the 10-15 widest conduits per section were evaluated in the leaf midrib using the ImageJ software (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). For venation density, sections from the leaves middle portion were collected, clarified following the procedure described by Fonseca et al. (2007) and then analyzed using light

microscopy. Images were taken and processed as above. The minor vein densities (4<sup>th</sup> and higher order) were calculated as the total length of the veins per unit of leaf area.

## Essential oil content

Ten grams of leaves dried in a forced air circulation oven at 35 °C were extracted in a Clevenger apparatus. Given the mass of oil obtained after removal of the salt (anhydrous magnesium sulfate) and evaporation of the solvent (dichloromethane), the percentage of essential oil content in leaves was determined by:  $T (\%) = \text{weight of oil (g)} / 10 \text{ g} \times 100$ . The oil yield was calculated by the formula (Brant et al., 2009):  $R = \text{total dry weight of leaves/plant (g)} \times \text{weight of oil obtained (g)} / 10 \text{ g}$ .

## Data analysis

The experimental design for the shading experiment was a completely randomized design with four replicates of five plants each (20 plants per treatment). ANOVA was performed on all experimental data using the statistical program Sisvar (Ferreira, 2000) and mean differences were assessed at a 5% level by Tukey's test.

## RESULTS AND DISCUSSION

The red, blue, and black shade nets as compared to full sunlight resulted in a reduction in global radiation ( $R_g$ ) of 36%, 60%, and 64%, respectively (Table 2). Likewise, photosynthetic photon flux density (PPFD) in the blue and black nets decreased approximately 65% when compared to full sunlight, while for red net the reduction was 59%. The black net presented the lowest value of the ratio  $R_g/\text{PPFD}$ , i.e. the amount of incident radiation, which is probably used in photosynthesis realization. In contrast, the red net showed the highest rate of this relationship, indicating a small proportion of photosynthetically active radiation (PAR) in relation to the amount of global radiation.

Regarding the gas exchange parameters, we found that the apparent quantum efficiency (AQE) did not differ between treatments despite the lower values of AQE for the red and blue nets compared to full sunlight, possibly due to the reduction in PPFD (Table 3).

Plants growing under intense radiation have higher C assimilation and dark respiration than plants in shaded conditions, either to maintain their structures of greater

**Table 2. Mean values of global radiation ( $R_g$ ) and photosynthetic photon flux density (PPFD) under different colored shade nets.**

	Full sun	Black	Blue	Red
$R_g$ , W m <sup>-2</sup>	436.34	156.80	174.16	275.94
PPFD, $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	786.70	271.96	238.33	317.37
$R_g/\text{PPFD}$	0.55	0.57	0.73	0.86

**Table 3. Photosynthetic traits derived from net CO<sub>2</sub> assimilation (A)/photosynthetic photon flux density (PPFD) curves of *Melissa officinalis* plants grown under different colored nets.**

	Full sun	Black	Blue	Red	CV (%)
AQE	0.07a	0.06a	0.05a	0.05a	13.00
$R_d$ , $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	1.80ab	2.29a	1.16ab	0.80b	42.15
$A_{max}$ , $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	16.70a	15.58ab	11.34b	11.93b	15.76
$P_{com}$ , $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	28.55a	24.16a	20.44a	15.55a	37.68
$P_{sat}$ , $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	589.51a	574.74a	601.57a	532.30a	28.49
$g_s$ , mmol H <sub>2</sub> O m <sup>-2</sup> s <sup>-1</sup>	0.25a	0.23a	0.15a	0.13a	34.91
E, mmol H <sub>2</sub> O m <sup>-2</sup> s <sup>-1</sup>	4.15ab	5.84a	4.17ab	2.33b	33.50
CCI	13.44b	15.90b	19.19a	14.06b	9.40

Distinct letters in the row indicate significant differences according to Tukey's test ( $P \leq 0.05$ ).

AQE: Apparent quantum efficiency,  $R_d$ : dark respiration,  $A_{max}$ : maximum CO<sub>2</sub> assimilation-,  $P_{com}$ : compensation (-),  $P_{sat}$ : saturation points of photosynthesis to light,  $g_s$ : stomatal conductance, E: leaf transpiration, CCI: chlorophyll content index.

energy cost for growth and biomass increment (Lichtenthaler et al., 1981; Lichtenthaler and Burkart, 1999). However, our results indicate that lemon balm plants under full sunlight had higher maximum CO<sub>2</sub> assimilation ( $A_{max}$ ), with no significant increase in  $R_d$  (Table 3) compared to the treatments under nets, which demonstrates its adaptation to a higher radiation condition, despite being considered a partial shade species.

Plants grown under black net had higher  $R_d$ , which is an indication that there was a greater expenditure of C for the process of respiration. Plants under the red and blue nets showed lower photosynthetic capacity as well as lower rates of dark respiration, suggesting that both the assimilation process and C consumption were affected by such treatments.

In the study of Cassol et al. (2007) with *M. officinalis* and *Mentha x piperita* L. a 50% reduction in luminance caused a decrease of 57% and 60% of CO<sub>2</sub> assimilation rate, respectively. According to the authors, this reduction in net photosynthesis was caused by a lower energy production by photochemical apparatus, since the biochemical efficiency of photosynthesis was more sensitive to the effect of light on the two species studied.

Here, we did not observe differences between treatments in relation to the compensation point and light saturation point of photosynthesis (Table 3). Valladares and Niinemets (2008) show the standard responses of light and shade on the plant physiology and suggest that a higher light compensation point and dark respiration in plants grown under full sunlight are common when compared to shaded plants. Although an increased  $R_d$  was observed for plants grown under the black net in relation to the red counterpart, no changes in  $P_{com}$  and  $P_{sat}$  indicate that the increase in C consumption by respiration is not necessarily accompanied by an increase in the amount of radiation.

Small changes (nonsignificant) in stomatal conductance ( $g_s$ ) and transpiration (E) were observed when comparing lemon balm plants grown under the colored nets in relation to full sunlight plants (Table 3). The control of gas exchange by the stomata is essential for the maintenance of photosynthetic activity and hydration of tissues (Oliver et al., 2009; Blum, 2009). Therefore, our results indicate that the photosynthetic decrease under colored net treatments was not due to stomatal

conductance, which could have reduced the CO<sub>2</sub> diffusion from atmosphere to inside the leaf, but most likely due to the lower radiation provided by the nets.

The highest content of chlorophyll in leaves (increment of 42% in relation to full sunlight plants) was found in plants grown under blue net (Table 3). The total chlorophyll content was also significantly higher in plants of *Mikania laevigata* grown under the blue net (Souza et al., 2011). This difference is probably more the effect of light quality than quantity, since in the black and red nets this same response was not detected. According to Poudel et al. (2008), blue light exerts a positive and coordinated influence during development of chloroplasts and synthesis of chlorophyll in plant cells, which may explain the chlorophyll increase under this net color in our experiment.

Few biometric parameters showed significant differences between treatments in the first cut at 50 DAT (Table 4). Plants in full sunlight showed similar development to those subjected to shading, highlighting again the potential for adaptation of *M. officinalis* to intense irradiance. Compared to full sunlight, black and blue nets positively influenced the values of stem DM. The reduction in the root/shoot ratio in plants subjected to black net indicates that there was less biomass allocation to roots in relation to the other treatments (Table 4).

In the second cut (120 DAT), dry mass of leaves and stems was higher in plants subjected to colored nets when compared with the treatment of full sunlight, but without differences among the nets (Table 5). Furthermore, we observed a significant increase of the values for leaf area (LA), leaf weight ratio (LWR) and plant height in plants subjected to blue net (168%, 12.5% and 116%, respectively) compared with plants grown under full sunlight (Table 4). The increase in LA caused by the blue net was similar to that observed in *Artemisia vulgaris* L. (Oliveira et al., 2009), *Ocimum gratissimum* L. (Martins et al., 2008) and *M. laevigata* (Souza et al., 2011). Increasing LA is a common strategy used by plants under lower levels of irradiance, as those produced by the blue net (lowest PPFD values, Table 2), to maximize the available area for capturing light energy. In addition, the increase of the photosynthetic surface can compensate the low

**Table 4. Dry mass parameters and essential oil from the first cut (50 DAT) of *Melissa officinalis* plants under different colored nets.**

	Full sun	Black	Blue	Red	CV (%)
LA, dm <sup>2</sup>	16.98a	18.60a	24.06a	16.69a	19.16
Leaf DM, g	4.71a	4.67a	5.85a	5.01a	19.12
Stem DM, g	2.13c	3.79ab	4.18a	2.68bc	21.60
Root DM, g	8.71ab	4.06c	10.55a	7.12bc	19.14
Total DM, g	15.55ab	12.53b	20.60a	14.82b	16.15
LWR, g g <sup>-1</sup>	0.30b	0.37a	0.28b	0.34ab	8.33
Root/Shoot, g g <sup>-1</sup>	1.30a	0.48b	1.07a	0.94a	20.37
SLM, g dm <sup>-2</sup>	0.28a	0.25a	0.24a	0.23a	10.45
Essential oil content, %	0.27a	0.21a	0.39a	0.40a	25.13
OY, g plant <sup>-1</sup>	0.012a	0.011a	0.021a	0.018a	28.21

Distinct letters in the row indicate significant differences according to Tukey's test ( $P \leq 0.05$ ).

DAT: Days after transplanting, LA: total leaf area, LWR: leaf weight ratio, SLM: specific leaf mass, OY: oil yield.

**Table 5. Dry mass parameters and essential oil from the second cut (120 DAT) of *Melissa officinalis* plants under different colored nets.**

	Full sun	Black	Blue	Red	CV (%)
LA, dm <sup>2</sup>	16.80c	32.93b	45.03a	32.70b	14.92
Leaf DM, g	8.49b	10.24ab	11.87a	11.37ab	14.09
Stem DM, g	5.29b	8.78a	9.19a	10.50a	15.74
Root DM, g	20.59b	23.49b	21.88b	33.16a	16.78
Total DM, g	34.37b	42.53ab	42.94ab	55.04a	14.56
LWR, g g <sup>-1</sup>	0.24b	0.24b	0.27a	0.20c	6.02
Root/Shoot, g g <sup>-1</sup>	1.50ab	1.23ab	1.03b	1.51a	11.51
SLM, g dm <sup>-2</sup>	0.51a	0.30b	0.26b	0.34b	11.95
Height, cm	20.2c	37.2b	43.8a	34.2b	7.86
Essential oil content, %	0.52a	0.39b	0.41ab	0.15c	20.33
OY, g plant <sup>-1</sup>	0.030b	0.039ab	0.052a	0.010c	21.82

Distinct letters in the row indicate significant differences according to Tukey's test ( $P \leq 0.05$ ).

DAT: Days after transplanting, LA: total leaf area, LWR: leaf weight ratio, SLM: specific leaf mass, OY: oil yield.

photosynthesis rate per unit of LA, which is a characteristic of shade leaves (Souza et al., 2011; Meira et al., 2012).

All lemon balm plants grown under colored nets showed higher total biomass in the second cut compared to plants grown in full sunlight, with the red net presenting the most improved value (Table 5). Better plant growth using red net was also observed in *Catharanthus roseus* (L.) G. Don (Melo and Alvarenga, 2009). The leaves of plants grown under red net may have benefited by the greater availability of red radiation (above 590 nm), which is more efficient for the performance of photochemical step of photosynthesis (Henrique et al., 2011). However, this pattern of response cannot be generalized, since the effect of radiation is dependent on the plant species (Braga et al., 2009). For example, plants of *M. x piperita* showed no difference in total biomass between full sunlight and red net growth conditions (Costa et al., 2012).

The higher photosynthetic capacity ( $A_{max}$ ) in lemon balm plants under full sunlight was not converted into higher dry mass, possibly because the energy content may have been stored as starch in the leaves, as suggested by Brant et al. (2011). In addition, plants subjected to the full sunlight treatment must develop mechanisms to avoid cell damage under high irradiance condition (Gonçalves et al., 2012), which may result in greater energy expenditure for maintenance of cellular structures, increased carbohydrate consumption and, as consequence, decreased dry mass accumulation.

The LWR at 120 DAT was superior in plants grown under blue net in relation to the other treatments (Table 5), indicating that DM accumulation was relatively higher in leaves than other parts of the plant. The use of colored nets did not affected the root/shoot ratio in comparison to plants grown in full sunlight (Table 5). However, it appears that the quality of light can change the allocation of biomass, as there was an increase in the DM distribution to the roots in plants grown under red net compared to plants under blue net. Plants in full sunlight showed the highest value of specific leaf mass (SLM) (Table 5), which may be indicative of thicker leaves, resulting in higher DM per leaf area.

The regrowth of the plants after the first cut was more pronounced in plants under blue net than the plants in full sunlight as the former treatment resulted in greater height at 120 DAT (Table 5). Similar result was observed in *O. gratissimum* (Martins et al., 2008) and *M. laevigata* (Souza et al., 2011) grown under blue net. As phytochrome and other photoreceptors absorb light in the blue region of the spectrum, which in turn causes differential responses in phototropism, growth and morphogenesis (Smith, 1982), the increased height in lemon balm plants may be due to higher blue/red ratio provided by the blue net.

There was nonsignificant effect of the colored nets on the production of essential oil in leaves in the first cut of lemon balm (Table 4). However, in the second cut, after 70 d of plant regrowth, a significant reduction in the content and yield of essential oil was observed in the leaves of plants grown under red net (Table 5), which corroborates the results presented by Brant et al. (2009). According to these authors, the change in the incident spectrum promoted by red net would cause the lack of another wavelength that is not absorbed by photosynthetic pigments and provokes cancellation of any major route for the synthesis of terpenes interfering in the production of essential oil. On the other hand, the use of blue net resulted in a 30% higher essential oil yield (Table 5), which is consistent with the highest values of plant height and LA seen in this treatment. Our results are in agreement with Martins et al. (2008), who showed that *O. gratissimum* plants under blue net were taller and had higher essential oil content.

Changes in the amount and quality of light imposed by colored nets did not result in noticeable anatomical differences in lemon balm plants compared to the full sunlight grown plants. Only a slight, but nonsignificant increase was detected in the values of the anatomical parameters in leaves of plants under or the blue net (Table 6).

## CONCLUSION

*Melissa officinalis* plants grown under shade from blue net showed the highest essential oil yield as a result of superior plant height and leaf area at 120 DAT. On the other hand, the

**Table 6. Leaf anatomy characteristics of *Melissa officinalis* plants under different colored nets.**

Treatment	Full sun	Black	Blue	Red	CV (%)
ET <sub>ad</sub> , $\mu\text{m}$	23.17a	22.59a	30.12a	26.06a	22.53
ET <sub>ab</sub> , $\mu\text{m}$	15.71a	13.70a	16.11a	14.72a	24.37
PPT, $\mu\text{m}$	53.46a	44.33a	42.59a	49.10a	17.49
SPT, $\mu\text{m}$	53.29a	53.45a	62.93a	47.70a	27.42
LT, $\mu\text{m}$	144.97a	132.87a	151.16a	136.00a	13.82
VD, mm mm <sup>-2</sup>	5.53a	5.66a	5.26a	4.87a	14.99
N <sup>o</sup> CM	63.75a	76.25a	84.00a	76.00a	25.27

Distinct letters in the row indicate significant differences according to Tukey's test ( $P \leq 0.05$ ).

ET<sub>ad</sub> and ET<sub>ab</sub>: Adaxial and abaxial epidermis thickness, PPT: palisade parenchyma thickness, SPT: spongy parenchyma thickness, LT: leaf thickness, VD: venation density, N<sup>o</sup>CM: total number of conduits of the midrib.

use of red net resulted in a reduction in the yield of essential oil in leaves. In addition, no anatomical changes were detectable in response to modifications in the quantity and quality of light energy that reaches the plants of *M. officinalis*. The comparatively low biomass produced by plants cultivated under full sunlight, and consequently low essential oil content, suggests that assimilates might have been directed to the maintenance and repair mechanisms necessary to support high irradiance conditions. Finally, our data showed that the photo-selective shade provided by the blue net could be incorporated into the protected cultivation practices currently used for producing *M. officinalis* essential oil.

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