The production of phytochemicals due to biotic and abiotic factors are a result of the changes in growth parameters of the plant. Changes in the intensity and quality of light results in alterations of several biochemical and physiological processes of plants and manifests as changes in morphological and anatomical parameters. The relationship between phytochemicals production of peppermint and its growth responses under different photoperiods was determined. Photoperiod significantly affected the number and size of both stomata and capitate and peltate trichomes in leaves. This effect different photoperiod resulted in different numbers of peltate trichomes and different capitate trichome sizes between same surfaces of different leaves and between different surfaces of the same leaf. As a result, we found that the most suitable photoperiod (8 h light / 16 h dark), which improves the amount and content of phytochemicals with parameters changing coordinated with photoperiod change in phytochemical synthesis metabolism of Peppermint.

Introduction

Secondary metabolites called phytochemicals are formed as an intermediate or a final product of various physiological processes during growth and development stages of plants and stored in various forms in cellular structures. These substances play important roles in growth development and distribution of plants such as regulation of the interaction with environment (adaptation), formation of a defense mechanism against herbivores and pathogens (Junker et al., 2017). At the same time, the fact that phytochemicals have different contents among plants reveals their economic importance with their use in the food, pharmaceutical, perfumery and disinfectant industries (Santoro et al., 2013). For this reason, even though various researches conducted a lot of studies about increasing the quality and quantity of these products in plants, the morphological, anatomical and physiological changes that take place in the structures responsible...
for the biosynthesis of these chemicals under the influence of photoperiod still need to be answered.

Light has a direct effect on the development, differentiation, and synthesis of phytochemicals in plants (Fazal et al., 2016). Light intensity and quality changes cause changes in some biochemical and physiological processes in the plant, which is visible with its reflection on its morphological and anatomical parameters. Light is the fundamental energy source of photosynthetic carbon assimilation in plants and therefore is an important factor in the regulation and synthesis of phytochemicals (Khan et al., 2013). There are various studies suggesting that the amount and wavelength of light increases the biosynthesis of various phytochemicals (Taulavuori et al., 2016; Arena et al., 2016; Craver et al., 2017).

However, there is little knowledge on the morphological and anatomical structures, and the physiological processes which have a part in the biosynthesis and regulation of phytochemicals in peppermint under the influence of photoperiod.

Synthesis and accumulation of phytochemicals in plants is a result of complex biosynthesis mechanisms that take place in their specialized structures under very special conditions with considerable energy consumption (Gupta et al., 2017). Environmental factors, which are defined as special conditions, are the stress conditions that occur when the plant is under or over the optimum conditions (light, temperature, water, soil characteristics, etc.) that the plant needs. Stress induces plants to activate their defense mechanisms by synthesizing a number of phytochemicals and so plants adapt to stress conditions (Ventura-Aguilar et al., 2013). Peppermint (Mentha x piperita L.) is a medicinal plant of the Lamiaceae family. Phytochemical production occurs in glandular trichomes of peppermint. These structures are capitate hairs which consist of a circular head containing four cells and a stalk with various morphologies, and peltate hairs that are formed by the separation of cuticula from the cell membranes of about eight disk shaped cells (Turner et al., 2000). Their secretions accumulate in subcuticular chamber (Ascensão et al., 1998). Changes in the number and morphology of these structures that are originated from the epidermal system are proportional to internal and external influences which plants are exposed to during growth and differentiation. For this reason, we tried to explain the relationship between the essential oil content of peppermint and its morphological, anatomical and physiological parameters under different photoperiod applications. Therefore, the objectives of the present study were to determine the effects of photoperiod levels on (i) the growth, (ii) stomatal parameters, (iii) trichome morphology and density, (iv) total chlorophyll content, and (v) phytochemical amount and content of peppermint. In addition, this research will provide an important source for identifying the most suitable photoperiod that increases the quantity and quality of the phytochemicals contained in peppermint as it promotes its growth and development.

Materials and Methods

Seedling planting and light applications

Peppermints (Mentha x piperita L.) used in the study were obtained as seedlings. Five seedlings were planted to each pot which contained a mixture of peat, perlite and soil (1:1:1). Plants were grown in a growth chamber at controlled temperature (23/21°C ±2 light/dark), humidity (75/80%), and light intensity (120 μmol quanta m⁻² s⁻¹) for 3 months under different photoperiods: 4, 8 and 16 h. Plants were irrigated daily with Hoagland’s nutrient solution (1/2 strength). Experiments were setup in a completely randomized design with three replicates.

Measurements

Morphological parameters

Plant height and root length (15) were measured with the aid of a ruler and the average values were recorded. Approximately 30 leaf samples of each application were separated from the trunks to
determine leaf surface area. The leaves were laid on a graph paper, copied and weighed. 1 cm² of the same graph paper was also cut and weighed. The leaf area was calculated according to Pandey and Singh (2011) using the following equations;

\[ LA = \frac{x}{y} \]

Where

\[ x = \text{Graph paper weight of the leaf surface} \]
\[ y = \text{Similar graph paper weight of 1 cm² area} \]

The density of glandular trichomes (peltate and capitate) were counted on a stereo microscope in about 50 microscopic field at independent measurement (0.04 mm²), on both surfaces of peppermint. Trichome sizes were determined using an ocular micrometer under microscope, calibrated and determined as µ and both sides of each leaf pair from ten plants at a magnification of ×40.

To plant growth rate, fresh weights of the plants were measured. Then, plants were dried in an oven at 105°C for 48 h. The amount of obtained dry matter (%) was determined as the growth curve of the plants.

\[ \text{Amount of dry material} = \frac{\text{Weight of dry material}}{\text{Weight of fresh material}} \times 100 \]

Epidermal tissue stripped from superficial sections of leaves was used to determine the number and sizes of the stomata (s) and epidermis cells (e) of adaxial and abaxial surfaces. Stomata and epidermal cells in 50 microscopic fields were counted on each surface with independent measurements (1 mm²). Stomatal index (SI) of lower surfaces was calculated using the formula described below (Rengifo et al., 2002). Stomata sizes (length and width) were determined using an ocular micrometer under light microscope at a magnification of ×40 for lower surfaces of the leaves, calibrated and determined as µ.

\[ SI = \frac{s}{e + s} \times 100 \]

Where

\[ s = \text{stomata number} \]
\[ e = \text{epidermal cells number} \]

SEM (scanning electron microscopy) image analysis was used to examine the submicroscopic structures of trichomes and stomata. Samples were stored in 70% ethanol for SEM analysis. After passing through ethyl series samples were mounted on metallic stubs for imaging (LEO Stereoscan 360 SEM).

**Physiological analysis**

Phytochemical analysis; Supelco SPME fiber (polydimethylsiloxane (PDMS) 100 μm) was used for SPME method for the identification of the content of the Peppermint. Peppermint was prepared by blending 100 g plant for 3 min without water in a Waring blender. 1 g of sample was placed in a 4 mL glass septum vial and allowed to equilibrate for 15 min. The fiber was exposed to the headspace for 20 min in 60°C to extract volatile compounds from the peppermint. The SPME fiber was transferred to the injection port of the GC and the GC-MS instruments through the whole GC analysis time of 30 min for the desorption of flavor compounds.

Total chlorophyll content; 50 leaves from each application were used to determine the total chlorophyll contents in a chlorophyll meter (Minolta SPAD-502 Chlorophyll Meter, Minolta Co. Ltd., Japan).

**Statistical analysis**

All data were analysed using standart ANOVA. Significant differences between treatments were determined using the Post Hoc Duncan’s Multiple Range Test at \( p \leq 0.05 \) and \( p < 0.05 \) significance.
Figure 1. Effects of different photoperiod lengths on the growth parameters of peppermint.

Figure 2. Stomata parameters; stomata and epidermis density (a), stomata sizes (width and length).
levels. Data in the figures indicate mean values ± standard errors (SD) based on three replicates for each application.

**Results**

Root length of peppermint varied under different photoperiods. Root length increased from 0.73 cm before the experiment to 6.98, 6.71, 6.12 cm under 4, 8, 16 hours (h) of photoperiod, respectively. Significant changes were observed in stem length. Stem length increased from 6.4 cm to 15.4, 19.6 and 29.8 cm in parallel with increasing photoperiod length. The change in the growth rate was also in parallel with increasing photoperiod length. Growth rate was 8.66%, 11.5%, and 13.8% cm, under 4, 8, and 16 h of photoperiod, respectively (Figure 1).

**Figure 3.** SEM micrographs show the effects of photoperiod on stomata parameters on abaxial surfaces (A) and adaxial surfaces (B) of peppermint leaf. 1, 2 and 3 represent different treatments - 4, 8, 16-h photoperiod, respectively; ep: epidermis, st: stomata.
Figure 4. Leaf surface area and stomatal index parameters of peppermint under different lengths of photoperiod.

Figure 5. Trichomes on the lower surface of leaves; trichome size (a), trichome density (b).
Peppermint has hypostomatic type leaves. The number of stomata and epidermis on the lower surfaces of leaves were 2.72, 4.02, 3.06 and 8.94, 11.88, 10.14 under 4, 8, 16 h of photoperiod, respectively. Stomata index was 23.06, 25.27, and 23.18. Stomata sizes were similar after all applications. Stomata width and length were 17.56, 17.22, 19.28 µm and 28.05, 29.92, 27.5 µm, respectively (Figures. 2 - 3).

Initial value of 6.1 cm² of the leaf surface area increased to 8.8, 8.76, and 10.3 cm² under 4, 8 and 16 h of photoperiod, respectively (Figure 4).

Changes in the photoperiod affected the sizes and number of trichomes on both surfaces of leaves. On the lower surface of leaves, size of the capitate trichomes was 25.64, 24.34, 25.05 µm and peltate trichomes was 63.26, 68.03, 64.04 µm under 4, 8, 16 h photoperiod, respectively. Number of capitate trichomes on the same surface was 52, 58, and 62.
Peltate trichomes was 13, 23, and 16, under 4, 8, 16 h photoperiod, respectively (Figures 5 - 7).

On the upper surface of leaves, sizes of capitate and peltate trichomes were 19.95, 21.95, 19.4 µm and 64.88, 71.47, 63.5 µm under 4, 8, 16 h photoperiod, respectively. Numbers of capitate and peltate trichomes on the same surface were 26, 30, 45 and 6, 13, 3, respectively (Figures 6 - 7).

Initial total chlorophyll content was 6.85. Under 4, 8 and 16 h photoperiod applications total chlorophyll content increased to 10.3, 16.25 and 14.48, respectively (Figure 8).

About 62 compounds were identified in the phytochemical content of peppermint. Among them menthol, menthone, 1.8-cineole, menthofuran, limonene, β-myrcene, β-caryophyllene, pulegone,
Figure 8. Total chlorophyll content of peppermint under different photoperiods.

Table 1. The effect of photoperiod on phytochemical content of peppermint.

<table>
<thead>
<tr>
<th>Phytochemical content</th>
<th>Applications (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Menthol</td>
<td>13.08</td>
</tr>
<tr>
<td>Menthone</td>
<td>12.05</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td>1.35</td>
</tr>
<tr>
<td>Menthofuran</td>
<td>2.25</td>
</tr>
<tr>
<td>Limonene</td>
<td>5.98</td>
</tr>
<tr>
<td>β-myrcene</td>
<td>1.72</td>
</tr>
<tr>
<td>β-caryophyllene</td>
<td>0.36</td>
</tr>
<tr>
<td>Pulegone</td>
<td>0.74</td>
</tr>
<tr>
<td>Carvone</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Figure 9. Effects of photoperiod on the phytochemical amount of peppermint.
and carvone, which are the basic components of peppermint phytochemical, varied between different photoperiod applications (Table 1).

Menthol, menthol, and menthone compounds, which are factors determining the quality of peppermint in phytochemicals, were different in different photoperiod applications.

The percentages of menthol, menthofuran, and methone was 33%, 5.82%, and 31.21% at 4h photoperiod application; 37.68%, 2.83%, and 32.71 at 8h photoperiod application; and 14.41%, 3.93%, and 15.77% at 16h photoperiod application, respectively (Figure 9).

**Discussion**

Changes in the quality and the quantity of light influences the yield and nutrient quality of plants by regulating plant’s growth and development (Bian et al., 2015). Increasing photoperiod length decreased the root length of peppermint and longest root lengths (6.98 cm) were observed at 4 hour (h) photoperiod application. Tallest (29.8 cm) stems were observed at 16h photoperiod application. Relative increase in stem length was 59%, 68%, and 79%, under 4, 8, and 16 h photoperiod application.

Changes in root and stem lengths occur in opposite directions. As the length of illumination period increased, root lengths decreased but stem lengths increased \((p≤0.05)\). On the other hand, the fact that growth rate increased with photoperiod application and it was the highest at 16 h photoperiod shows that the stem capacity is maximum at the longest photoperiod. The relationship between light duration and growth is proportional to the reactivation of C reserves in the dark, such as starch, which accumulates in the light and allows growth. Therefore, the amount of C accumulated in the light has a limiting effect on growth (Greenham and McClung, 2015) and this determines the linear relationship between the light period and growth (Ohara and Satake, 2017). On the other hand, a low amount of C reserve caused by a short light period will stress the plant because the plant will have to catabolize its structural components to obtain the energy required for biochemical reactions (Ishihara et al., 2015).

The light contributes to plant growth and development by affecting stomata's movements, which are involved in the plant's biological processes by carrying out carbon uptake and water loss events (Robertson et al., 2009). Starch is the primary carbon storage compound that accumulates as the result of photosynthesis. Therefore, the presence of a strong correlation between stomata movement and starch accumulation establishes a linear relationship between stomata movement and photosynthesis (Horrier et al., 2016). Photosynthesis rate is very sensitive to light conditions (Ma et al., 2015) and the difference in biomass accumulation between plants that are grown under different light conditions is closely related to their photosynthetic rates (Yang et al., 2017). Stomata regulate photosynthesis and changes in their density and size will indirectly affect biosynthesis of phytochemicals. Because, biosynthesis and accumulation of phytochemicals are correlated to photosynthesis (Jung et al., 2013). Changes in the stomatal parameters due to photoperiod length alter the photosynthetic mechanism because of the effect of light and changes in the structures that provide gas exchange. Peppermint leaves are hipostomatic (Moon et al., 2009). Lower surface of the leaves gave different responses according to photoperiod application. While stomata numbers were similar between 4 and 16 h photoperiod applications \((p≤0.05)\), highest number of stomata was observed at 8 h photoperiod application \((p≤0.05)\). Similar results were observed for epidermis counts and stomata index. The value of stomata index was highest (25.27) at 8 h photoperiod application.

Stomata sizes (width and length) increased only at 8 h of photoperiod application \((p≤0.05)\). The difference between stomata sizes at 4 and 16 h of photoperiod application was not significant \((P≥0.05)\). Changes in the morphology and density of stomata give important clues as to the quality and
quantity of the phytochemicals which a plant will produce. Because these substances are originated from photosynthetic carbon and therefore produced at the rate of photosynthesis (Búfalo et al., 2016). Stomata regulate CO₂ uptake for photosynthesis. Thus, changes in stomata morphology and density on leaf surface affect photosynthesis mechanism (McAusland et al., 2016). Our study shows that with proper photoperiod it is possible to increase the photosynthetic capacity (Cioć et al., 2017) by changing stomatal parameters, and therefore optimizing gas exchange.

If exposure to light causes biochemical and physiological changes in a plant, this can only be explained by the changes in morphological and anatomical structures of its leaves, especially anatomical components (Puglielli et al., 2017). Compared to the initial values, leaf surface area increased by 35%, 31%, and 42% at 4, 8, and 16 h photoperiod application, respectively. These changes in the leaf surface area were significant ($p \leq 0.05$). Leaf surface area was biggest at 16 h photoperiod application and smallest at 8 h photoperiod application. It was expected that increase in leaf surface area would be parallel to the increase in stomata numbers and sizes. However, the leaf surface area was found to be the highest value for 16 h photoperiod application. Because, increase in exposure to light causes changes in leaf anatomy and tissue density and this is positively reflected on leaf surface area (Baird et al., 2017). However, while the increase in stomata numbers and sizes were highest at 8 h photoperiod application, the increase in leaf surface area was highest at 16 h of photoperiod application. This suggests that hairs, which are the other complements of the epidermal system, could take stomata’s place at 16 h of photoperiod application. Trichome density is in proportion to the number of trichomes on upper and lower surfaces of leaves and the surface area of leaves (Mucciarelli et al., 2003). As a matter of fact, trichome counts and sizes in our study support this notion. Especially at 16 h of photoperiod application, while capitate hair counts of both surfaces of leaves were in direct proportion to leaf surface area, they were inversely proportional to stomata index. Increase in leaf surface area due to light (Zheng and Van Labeke, 2017) means leaves will catch more light (Lacube et al., 2017). While it can be thought that this will increase photosynthesis, it is not necessarily true as there is no direct relationship between leaf surface area and the photosynthesis mechanism. Because, while the leaf surface area was maximum under 16 h photoperiod, stomatal parameters per unit area were densest under 8 h photoperiod. Lack of parallelism between leaf surface area and stomatal parameters shows that the photosynthetic mechanism is not directly related to leaf surface area.

Capitate trichome size of lower leaf surfaces were affected more by the increase in photoperiod length. The hairs on the lower surfaces were 23%, 10%, and 22% longer than the hairs on the upper surfaces under 4, 8, and 16 h photoperiod, respectively. On the other hand, capitate trichomes showed almost similar values between different photoperiods on the lower surface of the leaves ($p \geq 0.05$). On the upper surface the highest value was observed at 8 h photoperiod application ($p \leq 0.05$). The difference between 4 and 16 h photoperiod applications was not significant ($p \geq 0.05$). Peltate trichomes responded different to photoperiod length on both surfaces of the leaves. Basal widths of peltate trichomes on lower surfaces of the leaves were significantly different between photoperiod applications ($p \leq 0.05$), especially at 8 h photoperiod application. Similar to lower surfaces, basal widths on upper surfaces of the leaves showed significant differences between photoperiod applications ($p \leq 0.05$), and the highest value was observed at 8 h photoperiod application. Basal widths of peltate trichomes were maximum at 8 h photoperiod application on both surfaces of the leaves. Changes in photoperiod length affected the number of trichomes on both surfaces of the leaves ($p \leq 0.05$). Capitate and peltate trichome numbers were higher on lower surfaces. On the other hand, while capit...
increase in photoperiod length on both surfaces of the leaves; peltate trichome number was highest at 8 h photoperiod. Capitate trichomes are responsible for the production, accumulation and secretion of defensive proteins (Sallaud et al., 2012) and compounds that used in the defense mechanism against pests (Gao et al., 2017). Peltate trichomes are responsible for the production, accumulation and secretion of semi-volatile organic compounds that protect plants from biotic and abiotic stress (Wagner et al., 2004). Increase in the number of capitate trichomes in parallel with leaf surface area and photoperiod results in a significant reduction of phytochemical quality. Because, longer photoperiods increase the number of capitate trichomes more than the number of peltate trichomes and this results in a decrease in the amount of menthol in phytochemical content of plants (Souza et al., 2016). In our study, the number of peltate trichomes and the amount of menthol decreased, but capitate trichome density and the amount of pulegone (a toxic substance for humans) increased with increasing photoperiod length. Variation in the amount of menthol, which is an important compound in determining the phytochemical quality of peppermint, together with peltate trichomes under the influence of photoperiod gives important clues as to the phytochemical quality.

If the available light exceeds the amount required for photosynthesis the photoprotection mechanism actives (Ort et al., 2015) and the plant adapts to extreme light condition (Gu et al., 2017). Total chlorophyll content increased by 30%, 64%, and 60% at 4, 8, and 16 h of photoperiod application, respectively, compared to initial values ($p<0.05$). The reason for the lower increase rate at 4 h of photoperiod is the lack of adequate light for the synthesis of the chlorophyll molecule. And the reason for the increase rate for 16 h of photoperiod application is lower than that of 8 h of photoperiod application is pigment degradation due to over exposure to light (Shao et al., 2014). Because, light regulates the transmission of some signals from plastids to nucleus for the synthesis of chlorophyll molecule during greening process. This transmission will be repressed and chlorophyll won’t be synthesized if there isn’t sufficient light (Zhang et al., 2016). On the other hand, shorter photoperiods active some hormones (such as etilen, ABA, JA) and cause chlorophyll degradation (Zhu et al., 2017). The fact that total chlorophyll content (16.25) was the highest at 8 h photoperiod shows that 8 h of photoperiod is optimum for chlorophyll synthesis. There is a positive correlation between chlorophyll content and photosynthetic effect (Slattery et al., 2017). Stomatal parameters were densest at the same photoperiod application. This shows that 8 h of photoperiod is also optimum for photosynthesis. On the other hand, reduction of menthone to menthol increases the amount of menthol and triggers chlorophyll biosynthesis (Voirin and Bayet, 1996). This is also supported by our study. The amount of menthol increased in parallel with chlorophyll biosynthesis with highest values observed at 8 h of photoperiod application.

Since changes in leaf morphology and anatomical parameters affect its physiological function under various environmental stresses, morphology and phytochemical contents of plants can be regulated in a controlled environment (Miyagi et al., 2017). These factors regulate plant growth and directly affect the biochemical processes which affect the metabolism of secondary structures (Holopainen et al., 2018). Light is one of the most important factors which increases phytochemicals and especially regulates monoterpeno oil composition when compared to the other factor (temperature) (Choi et al., 2017). Particularly, light is an external signal which influences the expression of genes which are responsible for the production of phytochemicals (Liu et al., 2006; Taulavuori et al., 2018). On the other hand, this factor (light) acts upon the enzymes which regulate the synthesis of precursor substances of essential oils (Amoozgar et al., 2017). Therefore lack of sufficient light or extreme light negatively affects phytochemical synthesis. For example, over exposure to light had adverse effects on the production of phytochemicals in Linum sp.
cultures (Anjum et al., 2017). In our study, periodic exposure to light had positive effects on phytochemical concentration. Phytochemical content of peppermint varied between different applications of photoperiod. Menthol and methone concentrations were different between all three applications. However their concentrations were higher than all other compounds in all cases. For example, 33% menthol and 31.21% menthone were observed under 4 h of photoperiod. 37.68% menthol and 32.71% menthone were observed under 8 h of photoperiod. Lowest menthol and menthone concentrations were measured at 16 h photoperiod application (14.41% and 15.77%, respectively). Menthol and menthone is important in determining the essential oil quality of peppermint (Rohloff, 1999). The fact that the concentration of these substances decreased under 16 h of photoperiod may be an indication of long photoperiod decreasing the quality of essential oils. In that study phytochemical quantity was highest at 10/14 h light/dark photoperiod.

Conclusion

Phytochemicals activate defense mechanism of plants under stressful conditions. It is usually thought that the variation in phytochemical content and quantity inversely correlated to plant growth and development. However, plants minimize their growth and development while under stress to battle with it. This is interpreted as plants using all of their energy to alleviate stress. It is possible for light to have positive effects on both plant growth and phytochemicals if applied periodically (Lu and Bernardo, 2017). It was determined that the optimal photoperiod for peppermint is 8 h light / 16 h dark since positive effects on morphological, anatomical and physiological parameters were observed and phytochemical content was the highest at this photoperiod.

References


Choi, H. G., Moon, B. Y., Kang N. J., 2017. Correlation between strawberry (Fragaria ananassa Duch.) productivity and photosynthesis-related parameters


