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Morpho-anatomical changes in leaves of *Mentha* x *gracilis* Sole (Lamiaceae) submitted to different levels of shade

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Abstract

Mentha x *gracilis* Sole (Lamiaceae), known as gingermint, is a medicinal species of great importance, whose pharmacological properties are associated with the production of essential oil, with is rich in monoterpenes. This study aimed at evaluating the impact of different light regimes on a *M*. x *gracilis* clone. The plants were cultivated under shading (30% and 60%) and in full sunlight for 105 days. Subsequently, the leaf samples were submitted to morphometric analysis (leaf area, thickness, hardness, stomatal index and trichome density). The different conditions of light intensity promoted distinct changes in leaf morphology and anatomy. Plants cultivated in full sunlight were harder, thicker and presented a higher density of trichomes while plants grown under 30%-60% shade presented higher stomata density and leaf area.

Key words: Plant morphological and anatomy, Phenotypic plasticity, Photoperiod, Shade

Resumo

[Variações morfoanatômicas em folhas de *Mentha x gracilis* Sole (Lamiaceae) submetidas a diferentes níveis de sombreamento]. *Mentha x gracilis* Sole (Lamiaceae), conhecida como hortelã, é uma espécie medicinal de grande importância,

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cujas propriedades farmacológicas estão associadas à produção de óleo essencial rico em monoterpenóides. O presente estudo objetivou avaliar o impacto de diferentes regimes de luz em um clone de *M.* x *gracilis*. As plantas foram cultivadas por 105 dias sob sombreamento (30% e 60%) e sol pleno. Durante 90 dias de cultivo, o comprimento do caule foi avaliado quinzenalmente. Posteriormente, amostras de folhas foram submetidas a análises morfométricas (área, espessura, dureza, índice estomático e densidade de tricomas) e comprimento dos entrenós. As diferentes condições de intensidade luminosa promoveram alterações morfológicas e anatômicas. As plantas cultivadas em pleno sol apresentaram maior dureza, espessura e densidade de tricomas enquanto as plantas cultivadas a 30% e 60% de sombreamento obtiveram maiores índices estomáticos, área foliar e comprimentos caulinares e dos entrenós.
Palavras-chave: Morfologia e Anatomia vegetal, Sombreamento, Fotoperíodo, Plasticidade fenotípica.

Introduction

Plant leaves presents high phenotypic plasticity, which promotes wide adaptation to environmental conditions (Whatley & Whatley 1982; Sultan 2004). the changes can be morphological, physiological or even biochemical and can affect several traits such as: leaf area, size, thickness, hardness, pilosity and number of stomata per unit of area and per leaf-area as well as the production of secondary metabolites (Cutter 1986; Pegoraro et al. 2010).

Light intensity is one the most important factors able to cause such changes (Jurik et al. 1982; Vogelmann 1993; Vogelmann et al. 1996; Almeida-Cortez et al. 2004; Egbert et al. 2008). According to Milaneze-Gutierre et al. (2003), leaves developed under high light intensity ("sun leaves") are smaller and thicker than those developed under low light intensity ("shade leaves") although such pattern may vary among species. The epiderm is that part of the leaf most exposed to photoperiodic changes, with the adaxial epidermis more sensitive than the abaxial epidermis (Whatley & Whatley 1982; Vogelmann 1993; Vogelmann et al. 1996).

Higher of light intensity also promotes an increase in leaf-thickness by the elongation or addition of cells from the palisade parenchyma (Nobel et al. 1975). such changes have a substantial physiological impact, especially on the production of secondary metabolites (Bernath & Teténgyi 1980; Brown Júnior 1988; Li et al. 1996; Tebet et al. 1996; Castro et al. 2003).

Numerous studies (Silva & Anderson 1993; Atroch et al. 2001; Castro et al. 2003; Barp et al. 2006) have reported anatomical variations for plants exposed to different light regimens. Howewer, such studies on medicinal plants cropped in Brazil are incipient.

Mentha x gracilis Sole (*M. arvensis* x *M. spicata* hybrid and synonym: *M.* x *gentilis* Auct.; *M*. x *cardiaca* J. Gerard ex. Baker), known commonly as gingermint, stands out among these species due to their economically importance species. Its medicinal properties are associated with the presence of carvone, linalol and limonene (Tucker 1992), acting as antifebrile, carminative and antiseptic agents. The essential oils are used mainly in perfume, foods and cosmetics, showing a worldwide production around 530 ton/year (Simões & Spitzer 2004).

Although it is widely consumed, mints are not commercially cultivated in Brazil, since most of the production is homegrown (Westphalen 1976). Studies carried out in Turkey with *Mentha piperita* L. showed that the biosynthesis and the quality of the essential oils are affected by the climatic (photoperiod and temperature); biological (ontogeny and age); agronomical (sowing and harvesting times, fertilization and irrigation) factors (Franz et al. 1984; Ozguven & Kirici 1999; Telci & Sahabaz 2005). According to Burbott & Loomis (1967) and Topalov & Zhelyazko (1991), in *Mentha* x *piperita*, the high production of essential oils is found during flowering in the summer. Thus, before exploiting plants from genus from medicinal purpose care should be taken about its behaviour in response to photoperiod level of shade, availability of mineral and water resources.

Therefore, present study is aimed at assessing the morpho-anatomical changes of leaves of a clone of *M. x gracilis* in response to different degrees of shading.

Material and Methods

The experiment was carried out in open air at an experimental area in the municipality of Xaxim, Western Santa Catarina State, Brazil. Seedlings from a clone of *Mentha x gracilis* Sole, sampled at natural environments, which were previously disinfected with 5% sodium hypochlorite and sowed as follows: 30cm between the plants and 40cm between the rows. The substrate was prepared with vermiculite, bovine manure, earth from subsoil (20:30:50) and 500g/m^2 of limestone. The plants were watered every two days with the same amount of water.

The experimental design was completely randomised and comprised the following treatments: full sunlight (0% shade), 30% and 60% shade with 30 plants per treatment and two seedlings per replication. Black screens made of nylon were used for shading. Identity of the plant was confirmed by Dr. Ray Harley from The Royal Botanic Garden, Kew, United Kingdom. The material was processed by routine herbarium technique and it was incorporated into the Herbarium of *Universidade do Contestado* – UnC, Campus Concórdia, Santa Catarina State, Brazil, under accession number HCB 0516 and Herbarium Flor of *Universidade Federal de SC* – UFSC ANDUR NUMBER 35852.

Regarding the morpho-metrical and anatomical analyses of the leaves, plants were divided into three segments (apical, median and basal) determined from the apical meristem and considering a segment every three inferior nodes.

Leaf thickness was determined from 100 to 105 days old plants by using hand made transversal sections at the middle region of fresh leaves of every segment per treatment. The measurements were carried out in the two opposed leaves of mean position of each segment, and mean value was obtained from the two readings. Ten readings were taken for every segment per treatment by using Brastec® optical microscope fitted with ocular micrometer.

Leaf hardness was determined indirectly as described by Barp et al. (2006). For each of five plants/treatment, two opposed leaves from each segment were sampled between crop days 100 and 105 and submitted to assessment by perforating the median region on the secondary leaf and recording the mean.

The area of the leaves was determined with the *Image tool*® software (Wilcox et al. 2002). Leaf selection followed the same criteria adopted for the assessment of leaf hardness.

For anatomical analysis, the branches from the apical, median and basal segments were sampled from 105 days old plants and were fixed in FAA (Johansen 1940). Leaf samples of $0.5cm^2$ were used from the median part (leaf of mean position of each segment) of five plants per treatment.

To observe stomata, the samples were brightened with 20% sodium hypochlorite for 4 hours at room temperature. Afterwards, they were washed in distilled water and immersed in alcohol. The abaxial and adaxial epidermis immersed in glycerol was obtained using a brush. With a drop the epiderm was stained with Safranin-Astra Blue (Bukatsch 1972, apud Kraus & Arduin 1997) and the slides were mounted in 50% glycerol. For stomatal frequency, five slides per segment of plant for each treatment were assessed. Using a Zeiss® microscope coupled with a bright chamber, we established, in two observation fields/slide, the equivalence of the number of stomata in μm^2 for $1mm^2$ – the stomatal density (Azevedo et al. 1990).

Trichomes were counted at the median portion of the abaxial epidermis of two opposed leaves from the median node by using a Tecnival WF® stereo microscope. Trichome density/frequency was determined in 1 cm^2 area of each leaf by a method as described by Barp et al. (2006).

Statistical analysis – Analysis of variance (ANOVA) followed by Tukey test, considering $p<0.05$, were employed. The results were compared with different treatments.

Results and Discussion

Median and basal leaves of plants grown in full sunlight were harder than the apical ones (Figure 1A), but no such difference was observed in leaf hardness was reported for median, basal or apical leaves of plants grown under 30 and 60% shade

(Figure 1B and 1C). Among all the three treatments, the hardness of apical leaves which exposed to total incidence of sunlight (mean + standard deviation of 15.72 + 0.58) is significantly lower than the samples treated for 30 and 60% shade (mean + standard deviation of 22.08 + 1.18 and 19.68 + 0.68), respectively, using ANOVA followed by Tukey test $p<0.05$, Figure 1).

Castro et al. (2003) and Raven et al. (2007) mentioned that shade plants, when exposed to sunlight and wind, will gradually develop more sclerenchyma with deposition and lignification of parenchymatic cell. The hardness and thickness of leaves may be also resulted due to the increase layers of palisade parenchyma and increased size of mesophyll cells (Esau 1967; Larcher 1986; Vogelman 1993). Basal and median leaves are the oldest and, generally, contain they present more sclerenchyma and wider central vascular bundles than younger leaves (Castro et al. 2003). However, in the present investigation reduction of solar exposition might have promoted changes in the leaves of *M. x gracilis* that are unrelated to the hardness seen with increased age, according to Figure 1.

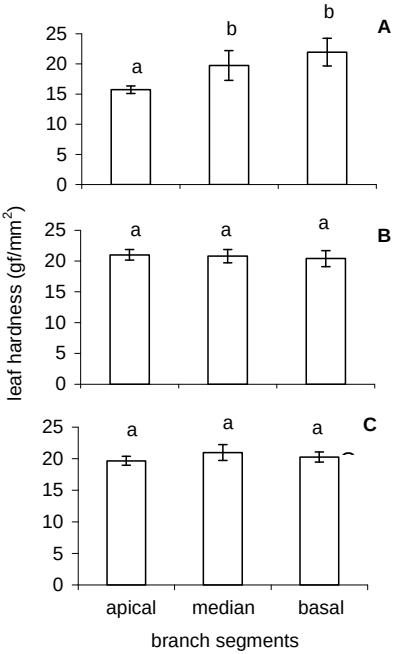

Figure 1. Leaf hardness (mean ± standard deviation) of *Mentha* x *gracilis* Sole grown under three different treatments: A, full sunlight; B, 30% shade; C, 60% shade in different branch segments. Distinct letters point to statistical differences (ANOVA followed by test of Tukey, $\alpha = 0.05$).

The leaves from median and basal segments of plants grown in full sunlight were thicker as compared to that of plants growing under shade (Figure 2). Similar results were obtained for *Plectranthus parviflorus* (Nobel et al. 1975), *Sphaeralcea incana* (Esau 1967), *Fragaria virginiana* (Jurik et al. 1982), Guaco (*Mikania glomerata*) (Castro et al. 2003) and *Passiflora suberosa* (Barp et al. 2006). According to available literature, an increase in the degree of light increases leaf thickness as a consequence of elongation and addition of palisade parenchyma cells (Esau 1967; Nobel et al. 1975; Jurik et al. 1982; Cutter 1986).

According to Larcher (1986), plants exposed to more intense radiation develop an efficient axial system for water transport, denser bundles and leaves with a greater number of mesophyll cell, hence, increased leaf thickness.

On the basis of analysis of the three segments (apical, median and basal), plants grown in full sunlight presented a smaller leaf area than those grown in 30 and 60% shade (Figure 3). Similar data were found for *Garcinia mangostana* (Wiebel et al. 1994), *Mikania glomerata* (Castro et al. 2003) and *Passiflora suberosa* (Barp et al. 2006).

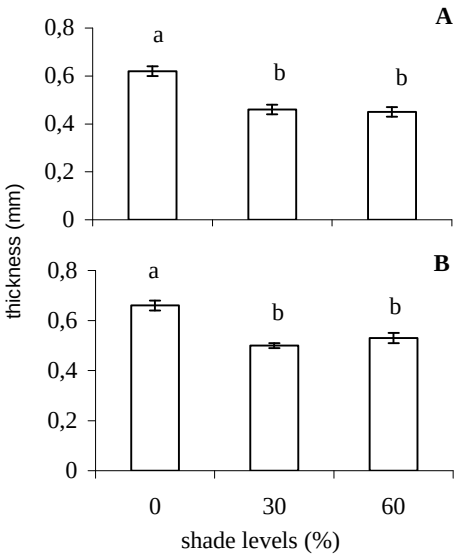

Figure 2. Leaf thickness (mean ± standard deviation) of *Mentha* x *gracilis* Sole grown under three different treatments: A, median segment; B, basal segment. Distinct letters evidence statistical differences (ANOVA followed by Tukey test, $\alpha = 0.05$).

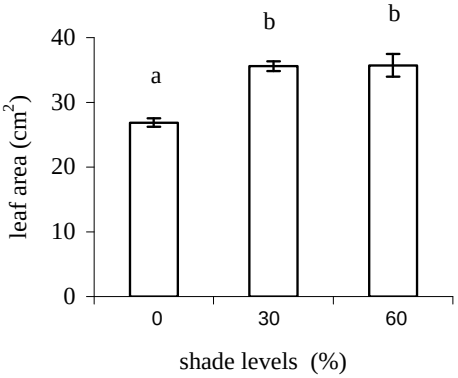

Figure 3. Leaf area of *Mentha* x *gracilis* Sole submitted to three different treatments: full sunlight, 30 and 60% shades. Distinct letters point to statistical differences (ANOVA followed by Tukey test, $\alpha = 0.05$).

Also, this information is corroborated by the study of Pegoraro et al. (2010) for M.x piperita which affirms that high radiation and low fertilization cause reduction of the leaf area.

Regarding the thickness and leaf-area, the results points to significant correlations for both variables, especially in the treatments in which plants were grown in full sunlight and 60% shade (α=0.05; p=0.023 and 0.024, respectively).

The leaf area of plants under 30 and 60% shade didn't shown significant difference. This behaviour can be explained by the plant adaptation to the principle of maximum efficiency, whereby the plant seeks maximal leaf expansion in order to increase the light absorption and photosynthetic efficiency (Taiz & Zeiger 2004). Thus, while sunlight reduction is a key in increasing the dimension of shade leaves, so too, is water availability (Gaba & Black 1983; Espírito Santo & Pugialli 1999). Similar result was observed by Pegoraro et al. (2010) where plants of M. x piperita under radiation below 30% didn't show significant difference in leaf area.

Results points to significant correlations for both thickness and leaf area, especially for treatments in which plants were grown in full sunlight and 60% shade (α=0.05; p=0.023 and 0.024, respectively).

The leaves of *M.* x *gracilis* are amphistomatic with fewer stomata on adaxial epidermis. According to Alquini et al. (2003), amphistomatic leaves with such trait may be still classified as amphihypostomatic. The presence of stomata on both surfaces is common among Lamiaceae species (Metcalfe & Chalk 1979) as the trait has been considered as an adaptive mechanism to maximize the CO_2 conductance when light and water are key factors (Kramer 1969). In Kudzu (*Pueraria lobata*),

Pereira-Neto et al. (1999) suggested that the amphistomatic characteristic might be associated with the ability to accumulate photosynthates through a more potent photosynthetic rate. According to Kramer (1969), when high intensity of light strikes the adaxial epidermis a smaller stomatal density will lead to greater efficiency in the use of water through the reduction of water.

Similar to the in Argentinean species of *Mentha*, as previously observed by Bonzani et al. (2007), the stomata of *M.* x *gracilis* are diacytic, thus corroborating the description of Metcalfe & Chalk (1979) for Lamiaceae.

High stomatal density was observed in at full light and in the apical segment of the leaf if compared with median and basal segment under the same radiation (Figure 4).

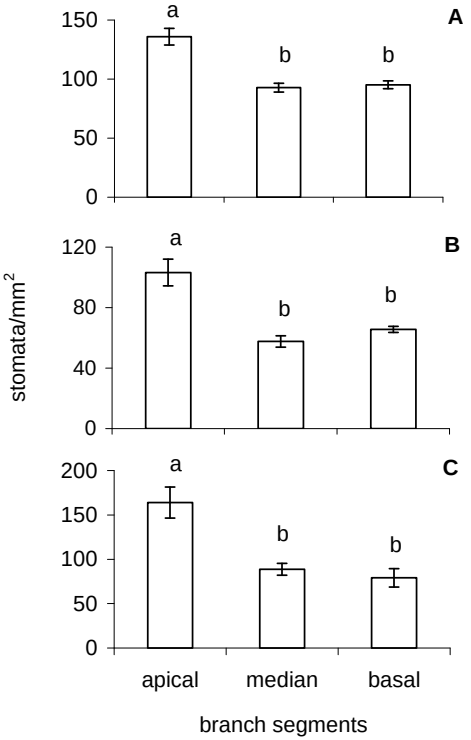

Figure 4. Stomatal density of abaxial surface of leaves (mean ± standard deviation) of *Mentha* x *gracilis* Sole submitted to three different treatments: A, full sunlight; B, 30% shade; C, 60% shade. Distinct letters point to statistical differences (ANOVA followed by Tukey test, α = 0.05).

In *Phaseolus vulgaris* (Silva & Anderson 1993) and in *M. piperita* (Pegoraro 2007) it was observed that the number of stomata/mm^2 increased as the light increases, wherever the total number of stomata for leaves continue the same.

Castro et al. (2003) observed a greater number of stomata/mm^2/leaf area of *Mikania glomerata* grown at 50% shade. However, Knetcht & O'Leary (1972) and Atroch et al. (2001) demonstrated that stomatal density and light intensity are not always correlated positively in all species.

Glover (2000) reports that the distribution pattern of stomata in the leaf is not random, but is, instead, dependent on the size and shape of the cells and the shape and size of the stomata sub-chamber, resulting in an optimized pattern that may be modified through the interactions among stomata, epidermis and environment. For *Arabidopsis thaliana* and *Antirrhinum majus*, this author found that excessive increase in the number of trichomes, as a result of environmental modifications.

In agreement with the descriptions of Metcalfe & Chalk (1979), Turner et al. (2000) and Martins (2002) for the species of *Mentha*, the leaves of *M. x gracilis* analysed presented tector trichome uni and pluricellular and peltate and capitate glandular trichomes (Figure 5).

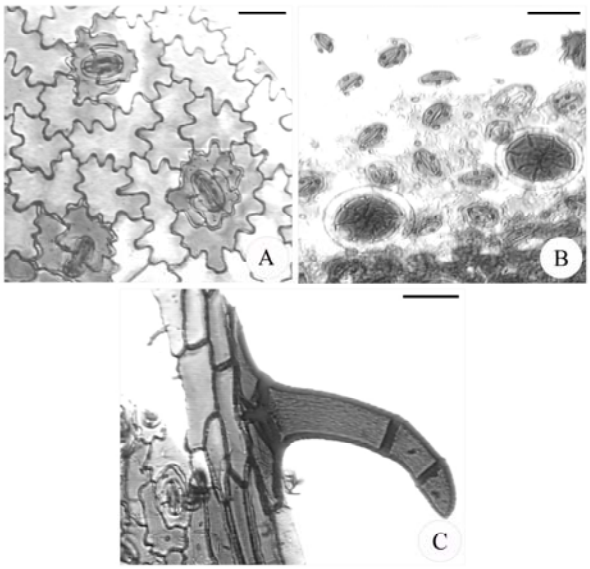

Figure 5. Stomata and trichomes from the leaf epidermis of *Mentha* x *gracilis* Sole: (A) diacytic stomata, (B) peltate glandular trichome and (C) tector trichome with three cell. The bar represents 50μm.

A plant grown in full sunlight and apical leaves of plants from other treatments present high numbers of such tector trichomes (Figure 6). Significant differences were found among apical and basal leaves, showing this last least number of basal leaves.

Among all characteristics analysed in this study, trichomes are structures that most directly influence essential oil production, since, according to Martins (2002), the biosynthesis and the accumulation of monoterpenes in *Mentha* occurs specifically in glandular trichomes and are carried out by leucoplastids from highly specialized secretory cells. Telci & Sahbaz (2005) have also found that the essential oil production in *Mentha* is higher for plants exposed to high sunlight incidence and high temperature.

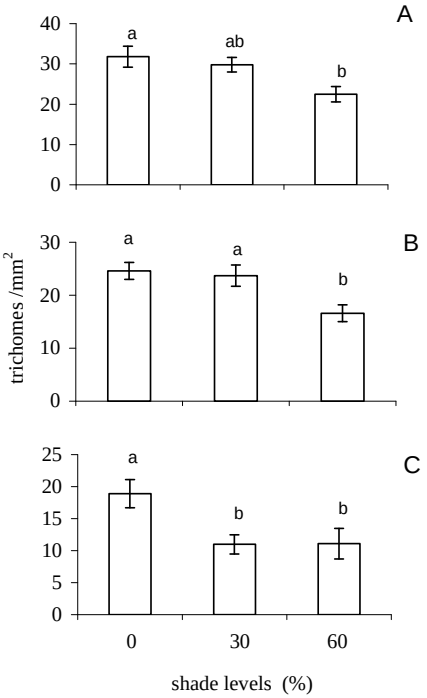

Figure 6. Number of tector trichomes of abaxial surface of leaf epidermis of *Mentha* x *gracilis* Sole submitted to three treatments: full sunlight, 30% and 60% shades: A, apical segment; B, median segment; C, basal segment. Distinct letters point to statistical differences (ANOVA followed by Tukey test, $\alpha = 0.05$).

Tector trichomes act as an evapotranspiration barrier (Jeffree 1986) and reflector of visible and infrared light spectra (Larcher 1986). In experiments carried out by Woodmann & Fernandes (1991) with *Verbascum thapus*, mature and less pubescent leaves are attacked more often by herbivores, while young leaves and those with more trichomes are to a greater degree, protected, indicating that tector trichomes function as specific-differential mechanical protection against herbivorous insects.

Different light regimens imposed on plants of *M.* x *gracilis* promoted correspondingly different morphological and anatomical variations in leaves. Such variations confirmed the plasticity and the capability of the plants, specially the leaves of this species, to acclimatize to different environments, adapting the photosynthetic machinery for better use of light.

Under such culture conditions, this species has produced more leaf biomass under shade conditions. Plants grown in full sunlight are harder, thicker and present a higher density of trichomes and stomatal density, while plants grown under less light intensity presented and wider leaf area. Since this is a plant whose essential oils have economic value, further studies regarding environmental and nutritional factors and plant age are necessary to understand the production of secondary metabolites.

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