Stomatal distribution, stomatal density and daily leaf movement in *Acacia aroma* (Leguminosae)

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**Summary:** *Acacia aroma* Gillies ex Hook. & Arn. grows in the Chacoan and Yungas Biogeographic Provinces, Argentina. It has numerous medicinal applications, sweet and edible fruits, and it may be used as forage. The objective of the present contribution was to analyse the stomatal distribution and stomatal density on the secondary leaflet surfaces, in different parts of the leaf, and at different tree crown levels, establishing the leaf movement and environmental condition relationships. The work was performed with fresh material and herbarium specimens, using conventional anatomical techniques. Stomatal distribution on the secondary leaflet surfaces was established, and differences in stomatal density among basal, medium and apical leaflets were found. A decrease in stomatal density from the lower level to the upper level of the tree crown would be connected with that. The stomatal distribution and density appear related to the secondary leaflet shape and its position on the secondary rachis, interacting with the daily secondary leaflets and leaf movement, and the weather conditions. It is interesting that the medium value of stomata density were found in the middle part of the leaf and at the middle level of the tree crown. Original illustrations are given.

**Key words:** *Acacia aroma*, leaf movement, Leguminosae, stomata.

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Resumen: *Acacia aroma* crece en las Provincias Biogeográficas Chaqueña y de las Yungas, Argentina. Este árbol posee numerosas aplicaciones en medicina popular, sus frutos son comestibles y puede ser usado como forraje. Los objetivos de la presente contribución fueron: establecer la distribución y densidad de los estomas en el folíolo secundario, en distintos folíolos secundarios de la misma hoja y en los folíolos secundarios de las hojas de la parte basal, media y superior de la copa del árbol, estableciendo relaciones con el movimiento diario de las hojas y condiciones ambientales. Para el estudio se utilizó material fresco y ejemplares de herbario empleando técnicas de anatomía convencionales. Se estableció la distribución de los estomas sobre las superficies adaxial y abaxial del folíolo secundario. Se encontraron diferencias en la densidad de estomas entre los folíolos secundarios de la parte basal, media y apical de la hoja que están relacionadas a la posición de éstas en la copa del árbol. Dentro de la copa del árbol se encontró que la densidad de estomas decrece desde la parte basal hasta la parte superior. La distribución y densidad estomática estarían relacionadas a la forma del folíolo secundario y posición de éstos sobre el raquis, al movimiento diario de los folíolos secundarios y de la hoja interactuando con los factores ambientales. Cabe destacar que el valor medio de densidad de estomas se halló en la parte media de la hoja y en la parte media de la copa del árbol. El trabajo se acompaña con ilustraciones originales.

**Palabras clave:** *Acacia aroma*, Leguminosae, movimiento de las hojas, estomas.
The genus *Acacia* Miller (Leguminosae, Mimosoideae, Acacieae), with 1200 species, occurs in the tropical and subtropical regions of America, Africa, Asia and Australia (Vassal 1981). In Argentina, it is represented by 21 species distributed in the northern and central provinces up to latitude 30° S (Cialdella, 1984, 1997; Zuloaga et al., 2008). *Acacia aroma* Gillies ex Hook. & Arn. grows in the Chacoan and Yungas Biogeographic Provinces; xerophilous forests are its typical habitat (Digilio & Legname, 1966). This tree is up to 4-6(-9) m tall; its leaves are pinnately compound, with thorn-like stipules. The petiole has a nectariferous sessile gland and other glands are present on the primary rachis on which pairs of pinnae are attached to. Each pinna is made up of the secondary rachis and the secondary leaflets with pulvini which facilitate nastic movements (Strasburger et al., 1994). The secondary leaflets are oblong and the presence of a submarginal midvein divides the leaf blade into one smaller acrosopic half and one larger basiscopic half; these halves tend to even out in the apical secondary leaflet (Cialdella, 1984). The flowers are in yellow heads, and the fruit is a moniliform legume (Burkart, 1952; Gunn, 1984; Digilio & Legname, 1996; Cialdella, 1997). This tree has numerous applications; its wood is used for rough structures, and also for fuel and coal production (del Valle Perea et al. 2007). In traditional medicine, twig and leaf infusion is used for gastrointestinal, liver and stomach disorders, and as a digestive aid (Carrizo et al., 2005); its leaves are used as antiseptics, to treat canker sore, skin infections, conjunctivitis, and sore throat (Martínez Crovetto, 1981; Cialdella, 1984; Alonso & Desmarchelier, 2005; del Valle Perea et al., 2007). They may have anti-syphilitic powers (Hieronymus, 1882; Rojas Acosta, 1907; Toursarkissian, 1980), and the dry powdered leaves are used as drying agents to enhance wound scar formation. The root has antiseptic and anti-inflammatory properties (Barboza et al., 2006). The bark is antifungal (Martínez Crovetto, 1981). The oil from the flowers is used in cosmetics and, as infusions, for asthma and high blood pressure (Burkart, 1952; Peña-Choccaro et al., 2006). Its juice is used as a painkiller for earache (Martínez Crovetto, 1981) and the flue (Alonso & Desmarchelier, 2005). The fruits are sweet and edible (Demaio et al., 2002), and the juice is astringent, though it should be used carefully as it has cyanogenetic compounds (Alonso & Desmarchelier, 2005). As regards its cultivation, it adapts to silvopastoral production systems since it can be used for winter forage with rapid growth (Demaio et al., 2002; del Valle Perea et al., 2007).

On examining the anatomy of tree leaves from the Yungas Biogeographic Province (Arambarri et al., 2009), we have observed that in *Acacia aroma* the stomata were unevenly distributed both among the adaxial and abaxial sides of the blade and in the acrosopic and basiscopic semi-blades in the secondary leaflets. It is known that stomatal density is affected by the stage of leaf development, and it is different in several parts of the blade, and also among different leaves of the same plant (Stace, 1965; Poole et al., 1996). Stomatal density is also influenced by the plant species and environmental factors such as light brightness (Gay & Hurd, 1975), water availability, temperature and sun radiation (Ma et al., 2004), relative humidity (Bravo & Grau, 1992), drought and high salinity concentrations (Salas et al., 2001), nutrient availability and atmospheric carbon dioxide concentration (Woodward, 1987; Woodward & Bazzaz, 1988; Royer, 2001; Ma et al., 2004). Our first hypothesis was that the uneven stomatal distribution is a result of the light differential impact on secondary leaflets. We propose to study the stomatal distribution and stomatal density: (1) on both surfaces of the secondary leaflet; (2) in pinnae located in different parts of the leaf; (3) in leaves from different tree crown levels. We also studied the daily leaf movement in order to establish a possible relationship with the stomata distribution and density.

**Material and Methods**

*Plant materials studied*

The study was performed by using adult leaves of *Acacia aroma* collected from trees located in the area of the Arboretum and Botanical Garden “C. Spegazzini”, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de la Plata, situated at 21 m a.s.l.; the reference material was deposited at LPG herbarium. Samples from the herbarium (LP), Facultad de Ciencias Naturales y Museo de La Plata, with the purpose to corroborate the stomatal distribution were also examined. ARGENTINA. Buenos Aires province: Pdo. La Plata, La Plata,
Techniques

The fresh material of adult leaves was collected from the lower, middle and upper crown level and above the middle part of branches facing north, where they were exposed to ample sun. Leaf samples were fixed in formalin aceto-alcohol solution (FAA) for further study. The herbarium specimen leaves were reconstituted by immersion in water with a drop of detergent in an oven at 30-35°C, for 48 hours, previous to its FAA fixation. Semi-permanent preparations were made from the basal, medium and apical pinnae and also from the lower, middle and upper tree crown level. In order to achieve transparent leaves two clarification techniques were applied. The first technique of Dizeo de Strittmatter (1973) and the second technique is called cold clarification, which we are testing in the laboratory of Morfología Vegetal, at Facultad de Ciencias Agrarias y Forestales, UNLP, where the work has been carried out. This last technique involved placing a mixture of equal parts of 5% sodium hydroxide and 5% sodium hypochlorite in a glass container, where the pinnae were soaked for 4-5 days, the material was removed, washed and bleached with 50% sodium hypochlorite, and then washed and immersed in choral hydrate for 24 hours. The advantage of the cold method was that it allowed to mount the whole pinna and observe the secondary leaflets from the basal, medium and apical parts of the pinna. The cleared pinnae were stained with 80% alcohol-safranin and mounted in gelatin-glycerin. Stomatal density values are averages of 10 counts on each semi-blade surface, and are expressed in stomata number/mm$^2$. In order to examine the leaf movements, photographs were taken with a Kodak Easyshare digital camera, m1033, 10 Mpx, every two hours since before dawn until it was already dark, when they remained closed. To that effect, a camera tripod was set up and focused on the leaves located on the branches facing to north and at the middle crown level. The leaf morphology was analysed and photographs were taken with a stereo-scope (Wild M8). A light microscope (Leitz SM Lux) with a camera lucida was used in order to study the anatomy and to draw the stomatal distribution diagrams. A color PAL CCD camera attached to a microscope Gemalux allowed to capture the images digitalized by means of Hyper Media Center software. The leaf movement was photographed on 01-6-2009. The pictures were interpreted in light of the data recorded by the weather station located at 200 m from the Botanical Garden and provided by the staff of the Weather Information Office at

<table>
<thead>
<tr>
<th>WEATHER CONDITIONS</th>
<th>at 9 am</th>
<th>at 3 pm</th>
<th>at 9 pm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>27.6°C</td>
<td>33°C</td>
<td>27.4°C</td>
</tr>
<tr>
<td>Humidity</td>
<td>50%</td>
<td>32%</td>
<td>51%</td>
</tr>
<tr>
<td>Pressure</td>
<td>1009.7 Hp</td>
<td>1605.0 Hp</td>
<td>1005.3 Hp</td>
</tr>
<tr>
<td>Visibility</td>
<td>10-20 km</td>
<td>10-20 km</td>
<td>10-20 km</td>
</tr>
<tr>
<td>Cloud cover</td>
<td>cumulus 1:3/10</td>
<td>cirrus 1:9/10</td>
<td>cumulus 1:7/10</td>
</tr>
<tr>
<td>Wind</td>
<td>NW 5 km/h</td>
<td>N 14 km/h</td>
<td>NNW 8 km/h</td>
</tr>
</tbody>
</table>
Facultad de Ciencias Astronómicas y Geofísicas, Universidad Nacional de La Plata (Chart 1).

Statistical analysis

The data obtained were averaged and processed according to the analysis of variance (ANOVA) in order to determine the level of statistical significance of the differences in stomatal density among lower, middle and upper crown. Tests of media comparison according to Tukey were carried out, at 5% level of probability ($P < 0.05$). The significant differences detected in stomatal density for the three crown levels were studied by means of the multiple range test. The analyses were performed by using Statistics 7.0 software for windows (Tables 1-4 and Graphs 1-4).

RESULTS

Morphology of the leaf

As it is observed in Fig. 1 A, the leaf is bipinnately compound; two rows of 10-25 pairs of opposite pinnae are located on the primary rachis. In Fig. 1 B, it is observed that every pinna has a secondary rachis with an adaxial rib where the bending pulvini, which give movement to the secondary leaflets, are attached to small cavities or pits. This arrangement of petiolules on the adaxial surface of the secondary rachis makes secondary leaflets close upwards. Each secondary leaflet is asymmetric with a smaller acroscopic semi-blade and a larger basiscopic semi-blade.

**Fig. 1.** *Acacia aroma*. Leaf morphology. A, leaf showing a pinna formed by secondary leaflets attached to the secondary rachis. B, detail of adaxial view of the secondary rachis with the two rows of secondary leaflets: *ac*, acroscopic semi-blade of the secondary leaflet; *ba*, basiscopic semi-blade of the secondary leaflet; *lea*, secondary leaflet; *pi*, pit where the pulvinus of the secondary leaflet is attached to; *ri*, adaxial rib of the secondary rachis; *pu*, pulvini. Simple eglandular trichomes may be seen on the secondary leaflet margin. Scale bar: A, 1 cm; B, 1 mm.

Stomatal distribution and density in the secondary leaflet, foliule or pinnule

Figure 2 Adx, represents the adaxial surface of the secondary leaflet: on the acroscopic semi-blade (ac), a few stomata are present in the basal part and the number increases gradually in the medium part, achieving the highest density in its apical end. On the basiscopic semi-blade (ba), stomata are nearly absent in the basal part. Also, the number of stomata in the medium part is very limited; while the number of stomata increases towards the apical end. Figure 2 Abx, corresponds to the abaxial surface of the secondary leaflet: on the acroscopic semi-blade (ac), the stomatal density is similar in the basal and the apical part of it, but the largest number is located in the medium part. On the basiscopic semi-blade (ba), the largest stomatal concentration is located in the basal part of the secondary leaflet and reduces gradually to the apical end.
Stomatal density per unit area (s n° / mm^-2) in the surfaces of the secondary leaflet located in the basal, medium and apical pinna of the leaf, and in the leaves situated at lower, middle and upper tree crown level.

On the adaxial surface the highest stomatal density was found in the medium pinna of the leaf, in every tree crown level. On the abaxial surface stomatal density varies according to the location of the leaf in the crown. Thus, at lower crown level (LL), the leaf medium pinna has the highest stomatal density; at middle crown level (ML), the stomatal density is higher in the apical pinna, and at the upper crown level (UL), the highest stomatal density corresponds to the basal pinna (Chart 2).

**Chart 2.** Stomatal density per mm^-2 on the adaxial and abaxial surfaces of the secondary leaflet, located at the basal, medium and apical pinna of the leaf and in the leaves at the lower, middle and upper tree crown level.

<table>
<thead>
<tr>
<th>TREE CROWN</th>
<th>Lower crown level (LL)</th>
<th>Middle crown level (ML)</th>
<th>Upper crown level (UL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEAF SURFACES</td>
<td>Adaxial</td>
<td>Abaxial</td>
<td>Adaxial (stomata / mm^-2)</td>
</tr>
<tr>
<td>PINNA LOCATION</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>187.87</td>
<td>1136.36</td>
<td>101.51</td>
</tr>
<tr>
<td>Medium</td>
<td>257.57</td>
<td>1389.39</td>
<td>107.57</td>
</tr>
<tr>
<td>Apical</td>
<td>175.75</td>
<td>984.84</td>
<td>48.48</td>
</tr>
</tbody>
</table>
Statistical analysis of the stomatal density per unit of area (s n° / mm²), in the semi-blade surfaces per tree crown level (LL = lower level; ML = middle level; UL = upper level).

1. Stomatal density of the acroscopic Adaxial semi-blade surface (SdacAdx) per tree crown level.

Table 1. Multiple range test for mean stomatal density (s n° / mm²) per tree crown level.

<table>
<thead>
<tr>
<th>Tree crown level</th>
<th>Mean value</th>
<th>Homogeneous groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL</td>
<td>66.16</td>
<td>x</td>
</tr>
<tr>
<td>ML</td>
<td>83.84</td>
<td>x</td>
</tr>
<tr>
<td>LL</td>
<td>195</td>
<td>x</td>
</tr>
</tbody>
</table>

Graph 1. Mean stomatal density (s n° / mm²) per tree crown level. It is clearly observed that mean stomatal density value decreases while the crown level increases. No significant differences between the mean values obtained for the middle (ML) and upper (UL) levels were found, but they were significantly different from the lower level (LL) (Table 1, Graph 1).
2- Stomatal density of the basiscopic Adaxial semi-blade surface (SdbaAdx) per tree crown level.

<table>
<thead>
<tr>
<th>Tree crown level</th>
<th>Mean value</th>
<th>Homogeneous groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML</td>
<td>2.02</td>
<td>x</td>
</tr>
<tr>
<td>UL</td>
<td>4.04</td>
<td>..........x.........x</td>
</tr>
<tr>
<td>LL</td>
<td>11</td>
<td>...... x</td>
</tr>
</tbody>
</table>

*Graph 2.* Mean stomatal density (s n° / mm^-2) per tree crown level. It is clearly observed that mean stomatal density values decrease from lower level (LL) to upper level (UL) and then middle level (ML). Although the lowest mean stomatal density value corresponds to the middle level, this value is larger at the upper level even increasing more at the lower level, the mean stomatal density value at (LL) obtained is not significantly different from the values found at (ML) and (UL), at the same time there is a significant difference between the values found at (LL) and (ML) (Table 2, Graph 2). The lowest stomatal density at the middle crown level is due to the fact that the basiscopic adaxial semi-blade is characterized by the almost absence of stomata (See Fig. 2 Adx, ba).
3 – Stomatal density of the acroscopic Abaxial semi-blade surface (SdacAbx) per tree crown level.

Table 3. Multiple range test for mean stomatal density (s n° / mm-2) per tree crown level.

<table>
<thead>
<tr>
<th>Tree crown level</th>
<th>Mean value</th>
<th>Homogeneous groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL</td>
<td>326,74</td>
<td>………………x</td>
</tr>
<tr>
<td>ML</td>
<td>434,85</td>
<td>x</td>
</tr>
<tr>
<td>LL</td>
<td>474</td>
<td>x</td>
</tr>
</tbody>
</table>

Graph 3. Mean stomatal density (s n° / mm²) per tree crown level. It is clearly observed that the mean stomatal density value decrease while the crown level increase. No significant differences between the mean values obtained for the lower (LL) and middle levels (ML) were found, but they were significantly different from the upper level (UL) (Table 3, Graph 3).
4 – Stomatal density of the basiscopic Abaxial semi-blade surface ($S_{dbaAbx}$) per tree crown level.

<table>
<thead>
<tr>
<th>Tree crown level</th>
<th>Mean value</th>
<th>Homogeneous groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL</td>
<td>530.30</td>
<td>.....................x</td>
</tr>
<tr>
<td>ML</td>
<td>664.65</td>
<td>x</td>
</tr>
<tr>
<td>LL</td>
<td>692</td>
<td>x</td>
</tr>
</tbody>
</table>

**Graph 4.** Mean stomatal density ($s$ nº / mm$^2$) per tree crown level. It is clearly observed that the mean stomatal density value decreases while the crown level increases. No significant differences between the mean values obtained for the lower (LL) and middle levels (ML) were found but they were significantly different from the upper level (UL) (Table 4, Graph 4).

**Leaf movement**

While there is darkness (Fig. 3 A), the leaflets are closed upwards, leaving only the apical end of the abaxial surface exposed. When day brightness increases, the pinnae are separated from the primary rachis and the two opposing secondary leaflets rows also separate from the secondary rachis. This happens while the light is increasing until the two secondary leaflets rows form an angle nearly to 180° with complete brightness (Fig. 3 B). This means that these move to a flat level. Between 11 am and 3 pm (Fig. 3 C and D), in coincidence with the highest temperature and level of brightness, more intense winds, low relative humidity and high atmospheric pressure, and all dehydrating elements (Chart 1), the pinnae are oriented
towards the leaf distal end. At the same time, the two secondary leaflets rows reduce their opening angle, forming a right angle first, and then an acute angle, with a slight movement towards the pinna apical end. They start becoming imbricated. This movement exhibit only the adaxial basiscopic semi-blade surface exposed, where the stomata are just absent (Fig. 2 Adx, ba). It has been observed that in this species the secondary leaflets are easily covered one another, and it is due to the fact that the distance between the pulvini over the secondary rachis is 0.5-0.8 mm in length, being the secondary leaflets very close to each other. If the above mentioned environmental conditions are maintained, the two leaflets rows keep forming an acute angle between them (Fig. 3 E) until these close completely at dusk when these adopt again the “circadian rhythm” position (Fig. 3 F).

Fig. 3. *Acacia aroma*. Leaves showing the pinnae and the parallel rows of secondary leaflets movement: A, at 7 am; B, at 9 am; C, at 11 am; D, at 3 pm; E, at 7 pm; F, at 9 pm. Scale bar: 1 cm.
**Discussion**

The tree of *Acacia aroma* has xeromorphic characteristics such as small leaves, tiny secondary leaflets, with glabrous adaxial surface and thorny-like stipules. The bicomound leaf has a ventral rib on the secondary rachis which only allows an upwards secondary leaflet movement when these are closed. According to the results, to the alternation of day and night which would correspond to the autonomous turgor movement (Strasburger *et al.*, 1994), the secondary leaflets movements (and leaf pinnae) shall be added along the day and with weather conditions, produced by the pulvini reaction which would be thermal and photonastically sensitive, performing opening and closure movements, which are called nictinastic (Esau, 1982; Strasburger *et al.*, 1994). *Acacia aroma* leaves are amphistomatic, the epidermal cells have periclinal walls with a cuticle covered by remarkable epicuticular waxes, and slightly curved anticlinal cell walls (Arambarri *et al.*, 2009). Thus, this would be adapted to high brightness levels in coincidence with what has been exposed by (Gay & Hurd, 1975; González, 1992). The stomata are anomocytic and paracytic types in coincidence with Metcalfe & Chalk (1979). The presence of more than one type of stomata is frequent in the Leguminosae (Stenglein *et al.*, 2003, 2005; Arambarri *et al.*, 2006). The stomatal density on the secondary leaflet surface is high on the abaxial and reduced on the adaxial sides, and they are infrequent on the adaxial basiscopic semi-blade surface. The stomatal distribution and frequency has been constant in every analyzed specimens. This distribution would be connected to secondary leaflets shape and position on the secondary rachis, interacting with daily leaf movement and environmental factors. The leaf movement interacting with the stomatal distribution and density would be contributing to reduce the water loss.

The variability found in the stomatal density among the basal, medium and apical pinnae of the leaves in relation to the placement at different levels of the tree crown show us that at lower and upper levels of tree crown where the leaves are more exposed to the environmental factors, the highest density of stomata is found in the basal and medium parts of the pinnae, whereas at the middle level of the tree crown where the leaves are more protected, the highest stomata density is found in the apical pinnae. As regards the placement in the tree crown, the stomatal density is high at the lower level of the crown reduced towards the upper level of it, in coincidence with the variability mentioned in the stomatal density at different crown levels by Salisbury (1927), and more recently, by the results obtained in leaves from different parts of the stem by Barrientos Priego *et al.* (2003). However, it shall be emphasised that stomatal density mean values are always in the secondary leaflet middle part and in the middle part of the tree crown.

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**Bibliography**


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