
Stomatal Density in Antirrhinum majus L.: Inheritance and Trends with Development

William J. Martin1 and Dennis P. Stimart2
Department of Horticulture, University of Wisconsin-Madison, 1575 Linden Drive, Madison, WI 53706

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Abstract. Stomatal density during plant development and inheritance of the trait were investigated with the goal of utilizing stomatal density as a correlated trait to cutflower postharvest longevity in Antirrhinum majus L. Inbred P1 (stomatal index = 0.2) was hybridized to inbred P2 (stomatal index = 0.3) to produce F1 (P1 × P2), which was backcrossed to each parent producing BCP1 (F1 × P1) and BCP2 (F1 × P2). P1, P2, F1, BCP1, and BCP2 were used to examine changes in stomatal density with plant development and early generation inheritance. An F2, (F1 self-pollinated), and F3, F4, and F5 families, derived by self-pollination and single seed descent, were used to obtain information on advanced generation inheritance. Stomatal density was stable over time and with development of leaves at individual nodes after seedlings reached two weeks of age. Therefore, stomatal density can be evaluated after two weeks of plant development from a leaf at any node. Stomatal density is quantitatively inherited with narrow sense heritabilities of h2

Stomatal function is responsive and sensitive to the environment (van Rensburg et al., 1999). Holding solution additives including hydroxyquinolone compounds (Larsen and Scholes, 1966) and sucrose (Marousky, 1969) increased PHL through stoma closure. Evaluation of cut flowers in the dark increased PHL in Antirrhinum majus L., snapdragon (Marousky and Raulston, 1970), and Rosa sp., rose, plant water regulation changed with photoperiod (Skootweg and van Meeteren, 1991) supporting a role for stoma in water balance. Stoma do not function normally in plants grown in vitro (Hartmann et al., 2002) or in greenhouses with high relative humidities (Mortensen and Fjeld, 1998). Nonfunctional stoma result in rose cut flowers with short PHL (Mortensen and Fjeld, 1998; Mortensen and Gislerød, 1999). Under recommended growing conditions, stoma on short-lived rose cut flowers remained open longer, implying an inability to respond to water stress (Mayak et al., 1974).

Stomatal densities have little effect upon rates of photosynthesis (Jarvis and Davies, 1998; Jones, 1998) as stomatal density failed to correlate to plant productivity in grass species (Bhagwat and Bhatia, 1993; Wilson, 1971; Yamashita et al., 1995) and in Rhododendron simsii Planch. azalea (Heursel et al., 1987). Stomatal densities have been related to tolerance of environmental stresses, including drought (Jarvis and Davies, 1997; van Rensburg et al., 1999) and temperature extremes (Kleinhenz et al., 1995; Nayeem, 1989), and to water use efficiency in grass species (Bhagwat and Bhatia, 1993; Wilson, 1971; Yamashita et al., 1995). Though plant productivity was deemed difficult to advance, breeding for more efficient water use by modification of stomatal density remained a viable option (Bhagwat and Bhatia, 1993; Liang et al., 1975; Miskin et al., 1972; Paul, 1992; Wilson, 1971) suggest breeding for decreased stomatal density can progress

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1Research associate.
2Professor and chair of department. To whom reprint requests should be addressed; e-mail dstimart@wisc.edu.

Fig. 1. Low (A) and high (B) extremes of stomatal density in an F2 population derived from a cross of inbreds P1 and P2 in Antirrhinum majus L; stomatal index = 0.15 and 0.38, respectively. Red and blue dots locate stoma and epidermal cells, respectively (130×).
Methods and Materials

Commercial inbred lines of *A. majus* were evaluated for cutflower postharvest longevity (PHL) in 1991 and 1992 at the University of Wisconsin-Madison (Stieve and Stimart, 1994). The two inbred lines selected represented extremes in PHL, P1 (white, 163 d PHL) and P2 (white, 3.0 d PHL) (Stieve and Stimart, 1994). Also, P1 and P2 vary for stomatal index, 0.2 and 0.3, respectively (Schroeder and Stimart, 1994). Also, P1 and P2 are self-pollinated for five generations and hybridized to produce an F1 (F1 × P1) that was backcrossed to each parent to form BCP1 (F1 × P1) and BCP2 (F1 × P2) lines. Hereafter, P1, P2, F1, BCP1, and BCP2 are termed foundation genotypes.

F1 (P1 × P2) were self-pollinated to produce F2 populations. From the F2, 485 plants were selected randomly and self-pollinated to produce F3 families; 101 F3 families remained with 110 randomly selected for evaluation. Poor germination in the 110 families being evaluated resulted in 4% reduction of the population. In addition, 620 plants were selected randomly from the F3, and self-pollinated to produce F4 families; 101 F4 families were selected randomly for evaluation.

Foundation genotypes were grown in Winter 1998–99 in a completely random design for stomatal evaluations (described below). The 620 F4 were grown in Fall 1999 and the derived F5 were in Spring 2000 for stomatal evaluations. Individuals from each of P1, P2, F1, F2, and F3 were grown in Winter 2001–02 in a randomized complete block design (three blocks, one replication per genotype/block) for stomatal evaluations. All plants were grown in a polyhouse at the University of Wisconsin-Madison according to standard forcing procedures (Rogers, 1992). Briefly, seeds were germinated in a cell of a cell pack and seedlings individually transplanted to 96-cell (65-cm3) flats. Seedlings were transplanted to square plastic pots (1250 cm3) when the third to fourth set of true leaves appeared and grown through anthesis. Growing medium consisted of equal volumes soil, peat and perlite. Plant bench spacing was on 22-cm centers. Plants were fertilized every other week with 200 mg L–1 N using Peter’s 20N–8.7P–16.6K (Scott’s Sierra Horticultural Products Co., Marysville, Ohio) and provided supplemental light of 27 µmol·m–2·s–1 at bench level using 1,000-W high-pressure sodium lamps from 0600 through 2400 hr.

Stoma per area and stomatal index, \[
\frac{\text{[(number of stomata/number of stomata + number of epidermal cells) per area]}}{100}
\]
were assessed using abaxial leaf imprints created in super glue on glass microscope slides (Sampson, 1961). Fully expanded leaves from the third node above soil line were sampled destructively 8 weeks after seed sowing. Leaf samples were taken from plants per foundation genotype, single plants of 620 F4, and three plants per F5, F4, and F3 families. Three; two; and one image(s), 0.24 µm2, per leaf imprint were collected for stomatal evaluations. All plants were grown in a polyhouse at the University of Wisconsin-Madison (Stieve and Stimart, 1999). Stoma per area and stomatal index are being investigated as potential correlated traits to PHL that can be evaluated before anthesis and therefore, used in direct selection for PHL. A caveat to utilization of the relationship between stomatal density and PHL is that stomatal density can vary with node position and environmental conditions (Beerling and Chaloner, 1993; Cole and Dobrenz, 1970; Nayeem, 1989; Wild and Wolf, 1980). Leaf stomatal density is examined to verify proper evaluation of this environmentally sensitive trait and to provide inheritance information in early and advanced generations of *A. majus* with relevance to future work on PHL.
Results and Discussion

Stoma per area and stomatal index in the F₁ show normal distributions (Fig. 3). F₁ falls intermediate to P₁ and P₂, and BCP₁ and BCP₂ fall intermediate to F₁ and respective parents. All foundation genotype means are significantly different. F₁ stoma per area is intermediate to P₁ and P₂ with a significant bias towards P₁ from the expected midparent value (Fig. 3a, Table 1). Incomplete dominance for stomatal density in the F₁ toward low parent has been reported in corn (Zea mays L.) (Heichel, 1971) and toward high parent in tobacco (Nicotiana tabacum L.) (Tell, 1985). Significant specific combining ability, hence significant dominance variance, was reported in forage rape (Brassica napus L.) (Paul, 1992) though dominance direction was not reported, and mating-specific directional dominance was shown in sorghum (Sorghum bicolor (Moench.) (Liang et al., 1975) and mango (Mangifera indica L.) (Arora et al., 1978). F₁ stoma per area relative to P₁ and P₂ suggests inheritance is partially dominant in A. majus. However, failure of BCP₁ and BCP₂ to follow expected segregation patterns suggests stoma per area may be more complexly inherited or may reveal expected environmental sensitivity of the trait.

Stoma per area is reported commonly in the literature; however, this value has the detriment of being environmentally sensitive whereas stomatal index is a more robust measure of stomatal density (Salisbury, 1927 as referenced by Lea et al., 1977). The environment affects the number of guard cell initials early in leaf development, which thereby affects stomatal index (Beerling and Chaloner, 1993; Pappas et al., 1988). Therefore, stomatal index should be unaffected by environmental conditions that affect growth (Walton, 1980), whereas stoma per area may. Environmental conditions known to affect stomatal density include light quality (Miskin and Rasmusson, 1970; Rawson and Craven, 1975; Wild and Wolf, 1980), moisture (Nayeem, 1989), and carbon dioxide concentration and temperature (Beerling and Chaloner, 1993). Growing foundation genotypes simultaneously for evaluation should standardize environmental impact upon stomatal density. The F₁ stomatal index value is not significantly different from expected midparent value.

Fig. 3. Stomatal densities (A) and indices (B) in an F₁ population derived from a cross of inbreds P₁ and P₂ in Antirrhinum majus L., n = 620. (P₁, P₂, F₁ (P₁ × P₂), and backcrosses BCP₁ (F₁ × P₁) and BCP₂ (F₁ × P₂) mean values located by arrows. Letters represent mean separation of P₁, P₂, F₁, BCP₁, and BCP₂ by LSD<sub>0.05</sub> (A) = 1.6 stoma and (B) = 0.6.

Table 1. Observed and expected, simple-inheritance segregation of stomatal index and stoma per area in inbreds P₁ and P₂, F₁ (P₁ × P₂), and backcrosses BCP₁ (F₁ × P₁) and BCP₂ (F₁ × P₂) of Antirrhinum majus L.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed stomatal index</th>
<th>σ²</th>
<th>Expected stomatal index&lt;sup&gt;2&lt;/sup&gt;</th>
<th>t value&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Significance</th>
</tr>
</thead>
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<tr>
<td>P₁</td>
<td>19.7</td>
<td>4.61</td>
<td>22.4</td>
<td>1.66</td>
<td>NS</td>
</tr>
<tr>
<td>BCP₁</td>
<td>23.2</td>
<td>9.11</td>
<td>22.4</td>
<td>1.66</td>
<td>NS</td>
</tr>
<tr>
<td>F₁</td>
<td>25.0</td>
<td>5.92</td>
<td>24.0</td>
<td>2.10</td>
<td>NS</td>
</tr>
<tr>
<td>BCP₂</td>
<td>25.7</td>
<td>5.26</td>
<td>26.7</td>
<td>–2.22</td>
<td>NS</td>
</tr>
<tr>
<td>P₂</td>
<td>28.3</td>
<td>9.42</td>
<td>26.7</td>
<td>–2.22</td>
<td>NS</td>
</tr>
</tbody>
</table>

Stoma per area<sup>3</sup>

<table>
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<tr>
<th>Genotype</th>
<th>Observed stomata per area</th>
<th>σ²</th>
<th>Expected stomata per area&lt;sup&gt;2&lt;/sup&gt;</th>
<th>t value&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Significance</th>
</tr>
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<tr>
<td>P₁</td>
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<td>23.69</td>
<td>28.5</td>
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<td>NS</td>
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<tr>
<td>BCP₁</td>
<td>27.4</td>
<td>36.32</td>
<td>28.5</td>
<td>–1.07</td>
<td>NS</td>
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<tr>
<td>F₁</td>
<td>32.0</td>
<td>25.59</td>
<td>37.3</td>
<td>–3.10</td>
<td>*</td>
</tr>
<tr>
<td>BCP₂</td>
<td>46.6</td>
<td>153.51</td>
<td>40.8</td>
<td>4.71</td>
<td>*</td>
</tr>
<tr>
<td>P₂</td>
<td>49.6</td>
<td>77.28</td>
<td>40.8</td>
<td>4.71</td>
<td>*</td>
</tr>
</tbody>
</table>

<sup>1</sup>F₁ = midparent value, BCP₁ and BCP₂ = (midparent value – respective inbred parent value).
<sup>2</sup>Derived by pairwise t test.
<sup>3</sup>Significant t value at p ≤ 0.05.

Table 1. Observed, simple-inheritance segregation of stomatal index and stoma per area in inbreds P₁ and P₂, F₁ (P₁ × P₂), and backcrosses BCP₁ (F₁ × P₁) and BCP₂ (F₁ × P₂) of Antirrhinum majus L.
Stomatal index in *A. majus* appears to be inherited simply, as $F_1$ falls intermediate to $P_1$ and $P_2$ and backcross populations fall intermediate between $F_1$ and respective parents (Poehlman and Sleper, 1995). Inheritance of stomatal index was reported as partially dominant in *Solanum* sp., potato, though backcrosses did not follow expected segregation (Kleinhenz et al., 1995). Lack of agreement between inheritance of stoma per area and stomatal index can be explained by sensitivity of stoma per area estimates to varying growth rates. As shown above (Fig. 2), foundation genotypes reach anthesis at different times after seed sowing; therefore during sampling, plants are at different developmental stages. Stoma per area may be sensitive to this variation between genotypes, whereas stomatal index is robust against variation generated by growth rates. Inheritance of stomatal density is quantitative and stomatal index is best for genotype comparisons due to reduced environmental sensitivity.

Transgressive segregation in the $F_2$ is observed for stoma per area and stomatal index (Fig. 3). Since $P_1$ and $P_2$ were selected as extremes for PHL and vary for stoma per area, transgressive segregation can be expected and suggests presence of genetic variation for the trait (Falconer and Mackay, 1996). Similarly, high parent (Arora et al., 1978; Heichel, 1971) and bidirectional (Bhagwat and Bhatia, 1993) transgressive segregation of stoma per area have been reported when parents were selected to differ for the trait. Stomatal density has limited impacts on net carbon dioxide assimilation, but affects water use efficiency (Jones, 1998; Wild and Wolf, 1980). Therefore, transgressive segregation in stoma per area or stomatal index may best be utilized in a program to breed for reduction in transpiration rates as proposed in wheat (*Triticum aestivum* L.) (Bhagwat and Bhatia, 1993).

Stomatal index for $F_1$, BCP1, and BCP2 is low in cotyledons, shows an increase through the third set of true leaves, falls slightly at the fifth node, and then is stable acropetally (Fig. 4A). $P_1$ and $P_2$ vary slightly from this pattern. $P_1$ stomatal index varies with a decrease at node 11 and $P_2$ stomatal index starts high, shows an increase in the first set of true leaves and then follows a pattern similar to other genotypes (Fig. 4A). Stomatal density decreases from the plant apex in alfalfa (*Medicago sativa* L.) (Cole and Dobrenz, 1970), *Triticum* sp. (Nayeem, 1989), and *Z. mays* (Heichel, 1971), to peak at midplant in soybean (*Glycine max* L.) (Lugg and Sinclair, 1979), and be uniform across leaf position in sunflower (*Helianthus annuus* L.) and *N. tabacum* (Rawson and Craven, 1975).

In addition, variability in stomatal density with leaf position is cautioned against for sampling purposes in *Brassica napus* L. (Paul, 1992), bromegrass (*Bromus inermis* Leyss) (Walton, 1980), and rice (*Oryza sativa* L.) (Yamashita et al., 1995). An extensive study in *A. majus* (Harte and Hansen, 1971) using nine cultivars reported stomatal index stability in evaluated whorls in seven cultivars and an increase and decrease in the remaining two, respectively. Stability of stomatal index with leaf position in *A. majus* appears to be inherited simply, as $F_1$ falls intermediate to $P_1$ and $P_2$ and backcross populations fall intermediate between $F_1$ and respective parents (Poehlman and Sleper, 1995). Inheritance of stomatal index was reported as partially dominant in *Solanum* sp., potato, though backcrosses did not follow expected segregation (Kleinhenz et al., 1995). Lack of agreement between inheritance of stoma per area and stomatal index can be explained by sensitivity of stoma per area estimates to varying growth rates. As shown above (Fig. 2), foundation genotypes reach anthesis at different times after seed sowing; therefore during sampling, plants are at different developmental stages. Stoma per area may be sensitive to this variation between genotypes, whereas stomatal index is robust against variation generated by growth rates. Inheritance of stomatal density is quantitative and stomatal index is best for genotype comparisons due to reduced environmental sensitivity.

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Fig. 5. Nonsynchronized stomatal development on a cotyledon of *Antirrhinum majus* L. Arrows designate comparatively juvenile stoma (130×).
reported here agrees with previous work in *A. majus*.

Stomatal index over equalized time of development is stable for all genotypes (Fig. 4B). Stomatal ontogeny is set early in leaf development and spreading of stoma due to growth of epidermal cells occurs with leaf expansion (Pappas et al., 1988). Therefore, stomatal ontogeny has been described as synchronous (Pappas et al., 1988); however in some dicotyledons, additional stoma may be added during leaf expansion though their relative number is low (Larkin et al., 1997). In both scenarios, stoma at various stages of development may be seen side by side (Pappas et al., 1988; Zhao and Sack, 1999) with distinguishable stoma proximal to cells that later develop into stoma. This variable rate of stomatal development is observed in *A. majus* (Fig. 5) though stomatal ontogeny is not known.

Stomatal index is stable with leaf position in *A. majus* and theoretically stable during leaf expansion. In the absence of significant *de novo* stoma formation during leaf expansion, it follows that stomatal index would be stable with plant development; our results agree. Therefore, sampling for stomatal index can be done anywhere on the plant yet consistency of node, maturity of leaf, and timing of sampling are advised to optimize effort.

Parent-offspring regressions demonstrate moderate narrow sense heritability ($h^2$) for stoma per area and stomatal index (Figs. 6 and 7). Heritability estimates of stomatal index include $h^2_{F_2} = 0.49$, $h^2_{F_3:F_4} = 0.37 \pm 0.06$ and $h^2_{F_4:F_5} = 0.50 \pm 0.07$, and for stoma per area: $h^2_{F_2} = 0.47$, $h^2_{F_3:F_4} = 0.60 \pm 0.07$, and $h^2_{F_4:F_5} = 0.47 \pm 0.07$. Standard error estimates for $h^2_{F_2}$, $h^2_{F_3:F_4}$ and $h^2_{F_4:F_5}$ are nonestimable due to $F_2$, $F_3$, and $F_4$ population being grown at different times. $F_2:F_3$ regressions show greater distributions than others, especially for stomatal index, due to $F_2$ estimates being an average of measurements from only one plant. Agreement between $h^2$ for the two traits is expected in that both represent measures of stomatal density and are presented for literature comparisons. Published $h^2$ estimates for stomatal density include 0.82 to 0.85 in *B. napus* (Paul, 1992), 0.27 ± 0.06 to 0.74 ± 0.15 in barley (*Hordeum vulgare* L.) (Miskin et al., 1972), 0.52 ± 0.06 in grassland ruawai (*Lolium perenne* L.) (Wilson, 1971), 1.02 ± 0.24 in *S. bicolor* (Liang et al., 1975), $h^2_{F_2:F_3} = 0.42$ and $h^2_{F_3:F_4} = 0.81$ in *T. aestivum* (Bhagwat and Bhattia, 1993), and broad sense heritability of 0.85 in *Silene nutans* Wallp. (Hazra et al., 1996). In addition, using parents that deviated for stomatal frequency, simple quantitative inheritance was demonstrated in *N. tabacum* (Tell, 1985) and *Z. mays* (Heichel, 1971).

Heritability estimates can be affected by environment, experimental design, species, population, sampling techniques, and complex interactions of these factors (Falconer and Mackay, 1996). High $h^2$ for stomatal frequency in several species suggests adequate control of these factors, although standard error estimates are not always presented. Heritability estimates for stomatal index are likely, due to nonindependent data, to be very similar to published results for stoma per area within a given study. In addition, comparison of stomatal index $h^2$ to published results for stoma per area $h^2$ is supported by mathematical nonindependence of data and agreement between $h^2$ estimates for the two traits in *A. majus*. Estimates of moderate $h^2$ for stomatal index and stoma per area in *A. majus* agree with published results for other species, though increased sampling in *A. majus* may improve $h^2$.

The nature of stomatal density was investigated due to previous knowledge of the inverse relationship between total stoma per cutflower and PHL in *A. majus*. Stomatal index was stable over leaf position though standardized sampling is advised. Stomatal index, being an environmentally robust measure of stomatal density, is a quantitative trait with simple inheritance in *A. majus*. The bidirectional transgressive segregation and moderate narrow sense heritability estimates for stoma per area and stomatal index suggest selection for these traits is feasible. With this knowledge in hand, the physiological influence of stomatal density on PHL can be elucidated through further work.

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Fig. 7. Regression of $F_3$ on $F_2$ (A), $F_3$ on $F_1$ (B) and $F_4$ on $F_3$ (C) stomata number in *Antirrhinum majus* L. $n = 101$ (A) and 105 (B and C). "Significant at $P \leq 0.01$.