HERITABLE VARIATION IN STOMATAL RESPONSES TO ELEVATED CO₂ IN WILD RADISH, RAPHANUS RAPANISTRUM (BRASSICACEAE)¹

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Rising atmospheric carbon dioxide may affect plant populations in the short term through effects on photosynthesis and carbon allocation, and over the long term as an agent of natural selection. To test for heritable effects of elevated CO₂ on stomatal responses and plant fecundity in *Raphanus raphanistrum*, we grew plants from 12 paternal families in outdoor open-top chambers at ambient (35 Pa) or elevated (67 Pa) CO₂. Contrary to results from a previous study of this species, total flower and fruit production were marginally lower under elevated CO₂. Across families, stomatal index and guard cell length showed little response to CO₂ enrichment, but these characters varied significantly among paternal families in both the direction and magnitude of their response to changing CO₂. Although these family-by-CO₂ interactions suggest that natural selection might affect stomatal characters when ambient CO₂ levels increase, we found no significant correlation between either character and flower or fruit production. Therefore, our data suggest that while heritable variation for stomatal index and guard cell length exists in this population, selection due to increasing CO₂ is not likely to act on these traits because they had no detectable effect on lifetime fecundity.

**Key words:** Brassicaceae; elevated CO₂; fitness; *Raphanus*; reproduction; stomata; stomatal index; stomatal length.

1 Atmospheric CO₂ is expected to double within the next 100 yr, possibly leading to large changes in the structure of many terrestrial ecosystems. Most research concerning ecological responses to elevated CO₂ has focused on short-term plant physiological changes (e.g., Mooney et al., 1991; Owensby et al., 1993) or on differential species-level responses (Hunt et al., 1991; Poorter, 1993). Less attention has been paid to intraspecific or intrapopulational responses, although variation in response to CO₂ at these levels could affect predictions of the ecological and evolutionary consequences of global change for communities and ecosystems (Bazzaz, 1990; Geber and Dawson, 1992). Genotype-specific CO₂ responses of fitness-related traits could favor selection of a new set of genotypes in a higher CO₂ environment, altering the course of natural selection within populations (Curtis et al., 1996).

One line of evidence that plants may respond evolutionarily to changes in atmospheric CO₂ levels comes from studies of the surface anatomy of herbarium specimens or fossil leaves. Change in the number, size, or distribution of stomata with changing atmospheric CO₂ levels could reflect adaptive responses to altered plant carbon and water relations (Robinson, 1994). For example, stomatal density (number of stomata per square millimetre) in *Olea europaea* declined ~40% over the past 3000 yr, showing a linear, negative response to increasing atmospheric CO₂ concentration (Beerling and Chaloner, 1993). A negative relationship between stomatal density and past atmospheric CO₂ levels has been demonstrated for a number of other woody and herbaceous species (Woodward, 1987; Penuelas and Matamala, 1990; Paolelli and Gellini, 1993). Van Der Burgh et al. (1993) suggested that the stomatal index (number of stomata/number of epidermal cells + number of stomata) × 100) of fossil *Quercus petraea* leaves could be used to predict paleoatmospheric CO₂ levels, with low stomatal indices expressed during periods of elevated CO₂ (>34 Pa) and high stomatal indices expressed during periods of reduced CO₂ (<30 Pa). Not all species appear to respond negatively to increased CO₂, however. Modern *Salix herbacea* leaves had significantly higher stomatal density than postglacial fossil S. herbacea leaves (Beerling et al., 1992) and, although Penuelas and Matamala (1990) found a reduction in stomatal density with increasing CO₂ in 14 Mediterranean species, they found no relationship between stomatal index and CO₂ for the same species. They suggested that variation in stomatal index may more accurately reflect altered patterns of stomate differentiation since stomatal density can vary solely as a function of changes in leaf area.

If directional change in stomatal characteristics over time is indeed causally related to atmospheric CO₂ levels, this change must be due to some combination of (a) the effects (direct or indirect) of CO₂ on stomatal stem cell differentiation, and (b) the results of natural selection among individuals differing genetically in stomatal numbers or in their sensitivity to CO₂. Results from short-term CO₂ enrichment or depletion studies have shown that CO₂ can alter the pattern of stomatal and epidermal cell development, although the direction and magnitude of these effects vary considerably among species (e.g.,

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Malone et al., 1993; Ferris and Taylor, 1994; Beerling and Woodward, 1995) and the mechanism(s) of the CO2 response remain unknown. Genotypic variation in stomatal frequency and guard cell length also occurs in ambient CO2 regimes (Jones, 1987), indicating the possibility of evolutionary change in these characters. Radin et al. (1994) were able to select genetic lines of *Gossypium barbadense* for high stomatal conductance within a single generation, and found that conductance was positively associated with reproduction under high temperatures. If the inverse relationship between atmospheric CO2 concentration and stomatal index is the result of natural selection, we would predict that the fitness of genotypes with low stomatal indices would be greater than that of genotypes with high stomatal indices when both are exposed to elevated CO2 conditions.

In this study, we investigated genetic variation among 12 paternal families of *Raphanus raphanistrum* (wild radish) in the effects of elevated CO2 on stomatal index, density, and guard cell length. We also monitored vegetative growth and flower and fruit production at ambient and elevated CO2 in these families. Curtis, Snow, and Miller (1994) found genetic variation among five paternal half-sib families of *R. raphanistrum* in the degree of response to elevated CO2 for number of flowers and number of seeds per plant. Overall, seed production increased 13% at high CO2, but in three families there was no significant CO2 response and in one family lifetime fecundity increased by >50%. Here, our objectives were to further characterize levels of genetic variation in CO2 responses within this population of *R. raphanistrum* and to examine whether CO2 effects on stomatal development were correlated with CO2 effects on growth and reproduction. We were primarily interested in testing two hypotheses: (1) that *R. raphanistrum* genotypes differed significantly in their morphological and growth responses to CO2 enrichment (i.e., significant genotype × CO2 interactions existed), and (2) that genotypes with the lowest stomatal index at high CO2 would have the greatest reproductive output.

**MATERIALS AND METHODS**

*Raphanus raphanistrum* (Brassicaceae), wild radish, is a weedy, cosmopolitan annual found in disturbed habitats throughout northeastern North America. Plants produce a basal rosette of leaves and a raceme bearing hermaphroditic flowers. The species is self-incompatible and pollen from several randomly chosen plants using tissue-covered forcepts. Hand-pollinations were necessary since *R. raphanistrum* is self-incompatible. Days to bolting, leaf area at bolting, total number of flowers, and total number of fruits were recorded for each plant. Leaf area was estimated by measuring the length of each leaf (L) and calculating leaf area (A) using the equation: \( A = 0.53 + 0.4(L)^2 \) (\( r^2 = 0.98, N = 31 \)), determined by destructive harvest of a separate set of plants (Curtis, Snow, and Miller, 1994).

**Reproductive characters**—Plants flowered continuously for several weeks after bolting. To achieve maximum levels of fruit set, each flower was hand-pollinated 1–2 d after opening. Stigmas were covered with pollen from several randomly chosen plants using tissue-covered forcepts. Hand-pollinations were necessary since *R. raphanistrum* is self-incompatible. Days to bolting, leaf area at bolting, total number of flowers, and total number of fruits were recorded for each plant. Leaf area was estimated by measuring the length of each leaf (L) and calculating leaf area (A) using the equation: \( A = 0.53 + 0.4(L)^2 \) (\( r^2 = 0.98, N = 31 \)), determined by destructive harvest of a separate set of plants (Curtis, Snow, and Miller, 1994).

**Statistical analysis**—Effects of CO2 and maternal and paternal family on stomatal and reproductive characters were analyzed by analysis of variance for a randomized block, split-plot design. The main-plot CO2 effect mean square (1 df) was tested over the block × CO2 mean square (main-plot error, \( E_m \), with 2 df), while all subplot effects (e.g., paternal family mean square with 11 df) were tested over the overall error mean square (subplot error, \( E_s \), with 140 df).

The experimental design was a randomized block, split-plot, with CO2 as the main-plot and family as the subplot. The six chambers were distributed among three blocks, each block having one elevated and one ambient chamber. Within each chamber were two replicate individuals of each of the 36 families (= 432 plants total). Procedures for monitoring and controlling partial pressures of CO2 were as described in Curtis and Teeri (1992). Daytime (0700–1900) CO2 partial pressure was 35.4 ± 0.3 Pa and 67.3 ± 0.3 Pa in ambient and elevated chambers, respectively. At night, CO2 partial pressure increased slightly to 38.3 ± 0.7 Pa in ambient chambers and 69.1 ± 0.7 Pa in elevated chambers. The plants were watered generously until 15 July, after which they received ~75 mL per day. On 17 July, 24 July, and 3 August, we added 23.1 mg of ammonium nitrate as fertilizer to each pot. On 15 and 30 July, pots were rotated among chambers within blocks to minimize potential chamber or microsite effects (CO2 delivery lines were also rotated, keeping treatments constant). After 30 July, plants were too large to continue rotations among chambers.

**Stomatal characters**—One individual from each family per chamber was selected for measurement of stomatal index (= 216 individuals). A 79-mm² diameter disc was removed from two basal, fully expanded leaves of each plant during 24–28 July. Stomatal density and epidermal cell density (number of epidermal cells per square millimeter) were calculated for each plant. On leaf samples from two of the three chamber blocks the guard cell length of one stome per field was measured to the nearest 3.5 μm (= 144 individuals).

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**Stomatal characters**—One individual from each family per chamber was selected for measurement of stomatal index (= 216 individuals). A 79-mm² diameter disc was removed from two basal, fully expanded leaves of each plant during 24–28 July. To equalize possible effects of leaf tissue removal on reproductive characters, discs were also removed from plants not chosen for stomatal measurement. Two 0.175-mm² grids on the abaxial surface of each disc were viewed at 400x magnification (four fields per plant) and number of stomata and epidermal cells per field were counted. We were able to observe these features in unclear tissue mounted in water. Stomatal index, stomatal density, and epidermal cell density (number of epidermal cells per square millimeter) were calculated for each plant. On leaf samples from two of the three chamber blocks the guard cell length of one stome per field was measured to the nearest 3.5 μm (= 144 individuals).

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RESULTS

Carbon dioxide treatment had no significant effect on either stomatal index or guard cell length (Table 1). Considered across all families, leaf surface characteristics were essentially unchanged in elevated compared to ambient CO2 plants (Table 2). However, stomatal index and guard cell length did vary significantly among paternal families (Table 1, Fig. 1). Under ambient CO2 there was a 29% difference between those paternal families with the largest and the smallest stomatal indices (family 11, \(X = 27.4\) vs family 3, \(X = 21.2\), Fig. 1A) and a 43% difference in guard cell length (family 2, \(\bar{X} = 23.3\ \mu m\) vs. family 12, \(\bar{X} = 16.3\ \mu m\), Fig. 1B). Paternal families also varied in the directions and magnitude of their response to changing CO2 (Fig. 1). Change in stomatal index due to CO2 enrichment ranged from a 13% decrease at elevated compared to ambient CO2 in family 1 to a 30% increase in family 5. Carbon dioxide effects on guard cell length ranged from a 10% decrease at elevated compared to ambient CO2 in family 1 to a 29% difference between those paternal families with the two CO2 treatments (Fig. 1).

In general, reproductive characters were strongly affected by one or both parental genotypes, but growth at elevated CO2 resulted in marginally significant decreases (\(P < 0.09\)) in numbers of flowers and fruits per plant, contrary to expectations (Tables 1, 2). Leaf area at bolting also appeared to be negatively affected by elevated CO2, although the magnitude of the effect was not statistically significant.

Table 1. Effects of maternal family (Fem.), paternal family (Male), and CO2 treatment on stomatal, growth, and reproductive characters. All effects were considered fixed in the analysis of variance, where CO2 was considered a main-plot effect and Male and Fem. were considered subplot effects.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Stomatal index</th>
<th>Guard cell length</th>
<th>Bolt date*</th>
<th>Bolt area*</th>
<th>Flowers per plant</th>
<th>Fruits per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO2</td>
<td></td>
<td>0.61</td>
<td>0.43</td>
<td>0.33</td>
<td>1.63</td>
<td>10.32*</td>
<td>9.77†</td>
</tr>
<tr>
<td>Block</td>
<td>2*</td>
<td>0.58</td>
<td>0.23</td>
<td>4.02*</td>
<td>1.92</td>
<td>8.22</td>
<td>12.95†</td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td>2.33*</td>
<td>2.25*</td>
<td>9.46***</td>
<td>6.08***</td>
<td>1.84†</td>
<td>1.9*</td>
</tr>
<tr>
<td>Male (\times) CO2</td>
<td>11</td>
<td>1.94*</td>
<td>2.18*</td>
<td>0.53</td>
<td>1.56</td>
<td>1.07</td>
<td>0.85</td>
</tr>
<tr>
<td>Fem.</td>
<td>2</td>
<td>0.04</td>
<td>2.72</td>
<td>18.6***</td>
<td>2.28</td>
<td>9.12***</td>
<td>1.75</td>
</tr>
<tr>
<td>Fem. (\times) CO2</td>
<td>2</td>
<td>0.02</td>
<td>0.38</td>
<td>0.20</td>
<td>0.56</td>
<td>1.25</td>
<td>0.86</td>
</tr>
<tr>
<td>Fem. (\times) Male</td>
<td>22</td>
<td>0.077</td>
<td>0.78</td>
<td>0.79</td>
<td>0.85</td>
<td>1.24</td>
<td>1.33</td>
</tr>
<tr>
<td>Fem. (\times) Male (\times) CO2</td>
<td>22</td>
<td>0.77</td>
<td>0.78</td>
<td>0.79</td>
<td>0.85</td>
<td>1.24</td>
<td>1.33</td>
</tr>
</tbody>
</table>

a Days after planting.

b Leaf area at bolting.

c \(P < 0.09\), * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\).

Table 2. Leaf surface characteristics (A) and reproductive characteristics (B) in Raphanus raphanistrum grown under ambient and elevated CO2 and their percentage change due to CO2 treatment (\(\Delta\%\)). Mean ± (1 SE), where the unit of replication was the chamber. Therefore, \(N = 3\) for all measures except for guard cell length where \(N = 2\). Thirty-six individuals per chamber were measured for leaf surface characteristics and 72 per chamber for reproductive characteristics.

- **A) Leaf surface characteristics**
  - Stomatal index: 24.3 (0.2) vs 24.6 (0.3), 1.2%
  - Guard cell length (\(\mu m\)): 18.4 (1.5) vs 19.7 (0.5), 7.1%
  - Stomatal density (no./mm\(^2\)): 50.9 (2.4) vs 50.7 (2.7), 0%
  - Epidermal cell density (no./mm\(^2\)): 155.4 (7.3) vs 152.7 (7.8), -1.7%

- **B) Reproductive characteristics**
  - Days to bolting: 30 (0.4) vs 30 (0.3), 0%
  - Leaf area at bolting (\(cm^2\)): 77 (3.5) vs 70 (5.8), -9.1%
  - Flowers per plant: 98 (9.7) vs 80 (8.0), -18.4%
  - Fruits per plant: 52 (5.6) vs 44 (3.3), -15.4%

† \(P < 0.09\), see Table 1.

![Fig. 1. Stomatal index (A) and guard cell length (B) in 12 paternal families of Raphanus raphanistrum grown under ambient and elevated CO2.](image)
significant. Days to bolting was unaffected by CO2 treatment.

Paternal genotype had significant effects on bolt date, bolt area, and number of fruits per plant, while maternal genotype affected bolt date and number of flowers per plant (Table 1). There were significant paternal × maternal genotype interactions for bolt date, number of flowers per plant, and number of fruits per plant. Unlike our results for stomatal characters, we found no evidence for differential responses to CO2 in growth or reproductive characters among paternal families (Tables 1, 3). That is, there were no reversals in the direction of the response, but rather responses varied from either no CO2 effect to a consistent effect of the same sign across families.

There was no correlation between stomatal index and reproduction across the 12 paternal families (Table 4). Stomatal index was weakly negatively correlated with stomatal length at elevated CO2 and positively correlated with leaf area at bolting under ambient CO2. There was a weak positive correlation between guard cell length and number of flowers at ambient CO2. As expected, there were positive correlations between number of flowers and number of fruits and number of flowers and leaf area at bolting at both CO2 concentrations.

**DISCUSSION**

Carbon dioxide can act as a selective agent when plants exhibit heritable variation in fitness-related traits and there are paternal family × CO2 interactions for these traits. Studies of anthropogenic environmental perturbations such as heavy metal, ozone, and herbicide pollution have shown that rapid evolutionary responses are possible within natural populations given even low levels of genetic variation in tolerance to these stresses (Bradshaw, 1991). However, not all populations or species possess the requisite genetic variation. Clearly, where heritable variation is absent, no adaptive change is possible. We found heritable variation in both stomatal index and stomatal length among 12 paternal genotypes of *R. raphanistrum* selected from a single population. In addition, these characters showed differential responses among genotypes to growth at elevated CO2 levels. These data indicate the potential for a shift in the distribution of genotypes in this population based on selection for stomatal characters at high CO2 assuming that these characters influence plant fitness.

Although variation among paternal genotypes in stomatal responses to CO2 enrichment was found, there was no phenotypic correlation between stomatal index and our two measures of fitness, flower or fruit number, at either CO2 treatment level. This suggests that, at least under the conditions of this experiment, genotypes with either higher or lower, stomatal indices did not differ in fitness in a high CO2 environment. If these phenotypic correlations reflect patterns of genetic covariance, then the action of natural selection on stomata will not result in corresponding changes in fitness as atmospheric CO2 levels rise. The lack of a correlation between stomatal index and fitness could be due to the inverse relationship between stomatal index and guard cell length at high CO2, with the net effect of little variation in total pore area per leaf area among genotypes. A larger sample of genotypes might include some in which the linkage between stomatal index and size was weaker than in those used here, with the possibility of greater among-genotype variation in leaf conductance to CO2 and water vapor. Also, under conditions of water stress or interplant com-
petition for resources (both of which were negligible in the present experiment), variation in carbon gain and/or water loss could have an important effect on fitness in this annual species.

An inverse relationship between stomatal index (or frequency) and stomatal pore size has been observed in other species (i.e., Rajendra, Mujeeb, and Bates, 1978; Pallardy and Kozlowski, 1979) and results in a relatively stable total pore area per unit leaf area across different genotypes and environments (Jones, 1987). This homeostatic developmental behavior favors the maintenance of a uniform gradient between ambient CO$_2$ ($C_a$) and leaf internal CO$_2$ ($C_i$) partial pressures during maximal stomatal opening. For many species (Sage, 1994), including $R$. raphanistrum (P. Curtis, unpublished data), this gradient is $\sim 30\%$ under well-watered conditions (i.e., $C_i/C_a = 0.70$). Given a constant ratio between $C_i$ and $C_a$, as $C_i$ increases, $C_a$ increases proportionately with attendant increases in photosynthetic rate. Water loss per unit leaf area, however, remains unchanged. Such a linkage between stomatal index and guard cell size suggests a developmental response to rising CO$_2$, whereby increased photosynthetic carbon assimilation (high $C_i/C_a$) is favored over reduced water loss (low $C_i/C_a$). To the extent that this linkage can be broken, natural selection might act to either increase or decrease the maximum $C_a/C_i$ ratio. Clearly, the availability of other resources in addition to CO$_2$, particularly water and light, would be important in determining the ultimate effect of CO$_2$-induced changes in stomatal characters on plant growth, survival, and reproduction.

Under conditions of subambient CO$_2$ (<35 Pa), there appears to be a general pattern of decreasing stomatal density with increasing $C_a$ supported by both paleontological (Woodward, 1987; Paolletti and Gellini, 1993; Beerling and Woodward, 1996) and experimental growth studies (Woodward and Bazzaz, 1988; Malone et al., 1993; Beerling and Woodward, 1995, but see Rowland-Bamford et al., 1990). Comparison of paleontological material across high (>100 Pa) to relatively low (<60 Pa) CO$_2$ periods is difficult due to a lack of extant species and the possibility of changes in ploidy, but Beerling and Woodward (1996) cite stomatal density data from studies of Silurian (high CO$_2$) and Carboniferous (lower CO$_2$) plants and from Miocene (high CO$_2$) and present (lower CO$_2$) plants, which also support a pattern of decreased stomatal density at high CO$_2$. Results from CO$_2$-enrichment studies, however, are considerably more variable. Increased (Ferris and Taylor, 1994), decreased (Ferris and Taylor, 1994), and no change (Mousseau and Enoch, 1989; Radoglou and Jarvis, 1990; Ryle and Stanley, 1992; this study) in stomatal density and/or stomatal index have been reported in plants grown at twice ambient CO$_2$.

Should one expect similar responses in stomatal index across an atmospheric $C_a$ range of $\sim 15$ Pa to $>100$ Pa? At low $C_a$ (<20 Pa) photosynthesis is drastically reduced and selection should favor high $C_i$; $C_i$ ratios (i.e., high stomatal index and high potential conductance to CO$_2$) over reduced water loss (low stomatal index). As $C_a$ increases, photosynthesis becomes less CO$_2$ limited, and the relative benefit of restricting water loss by decreasing the $C_i/C_a$ ratio becomes greater. At high $C_a$ (>70 Pa), both CO$_2$ assimilation rate and water use efficiency are high and further optimization of C gain vs. water loss may be largely driven by local patterns of resource availability. Thus, what may have been a general response among angiosperms to changing CO$_2$ over the past 30 $000$ yr may not be an accurate predictor of responses to future CO$_2$ increase.

Population-level consequences of CO$_2$-mediated changes in gas exchange physiology and morphology will depend on whether these changes translate into altered lifetime fecundity. In our study, CO$_2$ treatment had a marginally significant effect on reproduction in $R$. raphanistrum, with elevated CO$_2$ plants producing fewer flowers and fruits than ambient CO$_2$ plants. Although negative effects of elevated CO$_2$ on plant reproduction have been found in Abutilon theophrasti (Garbutt and Bazzaz, 1984; Garbutt, Williams, and Bazzaz, 1990), Plantago lanceolata (Fager, Bowers, and Bazzaz, 1991) and Tricium aestivum (Mitchell et al., 1993), our results differ from those of a 1992 study with a different set of genotypes taken from the same $R$. raphanistrum population (Curtis, Snow, and Miller, 1994). In that experiment, growth at elevated CO$_2$ resulted in an increase in flower and seed production but no change in leaf area at bolting.

Several factors may account for the differences in CO$_2$ effects on growth in the two studies. A relatively small number of paternal families were examined in each case (six in 1992, 12 in 1993), and there may have been intrinsic differences in the CO$_2$ responsiveness of the families tested. There is also the possibility of interactions between CO$_2$ treatment, other aspects of the growth environment, and patterns of carbon allocation. The 1992 growing season (early July through late September) was exceptionally cloudy and cool in northern lower Michigan (3-mo mean temperature 2.3°C below the 10-yr average, UMBS Climatological Station records), while 1993 was more characteristically sunny and warm (3-mo mean temperature 0.4°C below the 10-yr average). Time to bolting was faster and leaf area at bolting was greater in both CO$_2$ treatments in 1993 vs. 1992, but total number of flowers produced was either equivalent (ambient CO$_2$) or less (elevated CO$_2$) in 1993 relative to 1992. This suggests a shift in carbon allocation away from flower production in 1993, a process perhaps amplified by high CO$_2$ conditions.

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