Impact of elevated CO$_2$ and O$_3$ on gas exchange parameters and epidermal characteristics in potato (Solanum tuberosum L.)

Tracy Lawson$^{1,4}$, Jim Craigon$^1$, Colin R. Black$^1$, Jeremy J. Colls$^2$, Geoff Landon$^1$ and Jonathan D.B. Weyers$^3$

$^1$School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK
$^2$School of Life and Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK
$^3$School of Life Sciences, University of Dundee, Dundee DD1 4HN, UK

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Abstract

Potato plants (Solanum tuberosum L. cv. Bintje) were grown in open-top chambers (OTCs) under three CO$_2$ levels (ambient and 24 h d$^{-1}$ seasonal mean concentrations of 550 and 680 $\mu$mol mol$^{-1}$) and two O$_3$ levels (ambient and a seasonal mean 8 h d$^{-1}$ concentration of 50 nmol mol$^{-1}$). The objectives were to determine the effects of season-long exposure to these key climate change gases on gas exchange, leaf thickness and epidermal characteristics. The experimental design also provided an ideal opportunity to examine within-leaf variation in epidermal characteristics at the whole-leaf level. Stomatal and epidermal cell density and stomatal index were measured at specific locations on the youngest fully expanded leaf (centre of lamina, mid-way between tip and base) and representative whole leaves from each treatment. Effects on leaf conductance, assimilation rate and instantaneous transpiration efficiency were determined by infrared gas analysis, while anatomical characteristics were examined using a combination of leaf impressions and thin sections. Exposure to elevated CO$_2$ or O$_3$ generally increased leaf thickness, leaf area, stomatal density, and assimilation rate, but reduced leaf conductance. The irregular stomatal distribution within leaves resulted from a combination of uneven differentiation and expansion of the epidermal cells. The results are discussed with reference to sampling protocols and the need to account for within-leaf variation when examining the impact of climate change or other environmental factors on epidermal characteristics.

Key words: CO$_2$, epidermal cell density, leaf conductance, O$_3$, open-top chambers, potato, Solanum tuberosum, stomatal density, stomatal index.

Introduction

Global atmospheric carbon dioxide (CO$_2$) concentration, currently c. 367 $\mu$mol mol$^{-1}$, has increased steadily since pre-industrial times and is predicted to range between 540–970 $\mu$mol mol$^{-1}$ by the end of the present century (IPCC, 2001). This increase has been accompanied by a concurrent rise in tropospheric ozone (O$_3$) levels; O$_3$ is not only an important ‘greenhouse gas’, but is widely regarded as the most important phytotoxic air pollutant (Ashmore and Bell, 1991). Both gases exert direct effects on the physiology, morphology and productivity of plants (Cure, 1985; Heagle, 1989).

$^4$Present address and to whom correspondence should be sent: Department of Biological Sciences, John Tabor Laboratories, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, UK. E-mail: tlawson@essex.ac.uk

Abbreviations: ECD, epidermal cell density; $g_s$, leaf conductance; ITE, instantaneous transpiration efficiency; OTC, open-top chamber; SD, stomatal density; SI, stomatal index.

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Elevated atmospheric CO₂ may affect plants at both the physiological (Long et al., 1996) and anatomical levels (Beerling et al., 1998). Stomatal responses to elevated CO₂ may be non-uniform across leaves and species-dependent (Taylor et al., 1994). Some species exhibit increases in both cell initiation and expansion, whereas others show increases in cell expansion in the absence of effects on cell numbers, or a combination of an increase in cell number and a decrease in cell size (Taylor et al., 1994). Stomatal density is influenced by various environmental stimuli including light, water and nutrient availability and atmospheric CO₂ concentration (Willmer and Fricker, 1996). Several studies suggest that stomatal density is reduced by elevated CO₂ (Woodward, 1987; Beerling et al., 1998), although others have found no change (Ceulemans et al., 1995; Poole et al., 2000) or suggest that stomatal density may even increase (Atkinson et al., 1997). The influence of elevated O₃ is less clear, although some studies suggest that stomatal density is increased (Paakkonen et al., 1997; Keutgen et al., 1999).

The observed differences between studies in the effects of CO₂ and O₃ on stomatal characteristics may, to some extent, reflect variation in sampling procedures. Although numerous reports have described treatment effects on stomatal characteristics, most have relied on limited sampling procedures which may not have adequately described the full extent of within-leaf heterogeneity (Weyers and Lawson, 1997). The first systematic study of within-leaf heterogeneity used contour maps to illustrate spatial variation in stomatal density and aperture in Commelina communis L. (Smith et al., 1989). A similar approach was used to examine the influence of soil water availability on stomatal density in the same species (Weyers et al., 1997), while variation in stomatal density and stomatal index was investigated in the hypostomatus sun and shade leaves of Alnus glutinosa (Poole et al., 1996). Poole et al. have recently examined the impact of elevated CO₂ on stomatal characteristics in the same species, and observed considerable within-leaf variation under both ambient and elevated CO₂ (Poole et al., 2000); they also found that elevated CO₂ promoted an increase in SI but had no effect on SD. Factors responsible for local variation in stomatal characteristics are not always easily identifiable, and may involve three possible scenarios (Beerling and Chaloner, 1993; Poole et al., 1996): (a) uneven guard cell differentiation: ‘differentiation hypothesis’; (b) uneven expansion of epidermal cells following differentiation: ‘expansion hypothesis’; or (c) a combination of both: ‘mixed differentiation expansion hypothesis’. As stomatal differentiation occurs early in the ontogeny of leaves, stomatal density declines as leaves expand. The concept of stomatal index was introduced to describe the proportion of epidermal cells made up by stomata (Salisbury, 1928). Although Salisbury claimed this parameter was almost constant over the leaf surface, others have reported variation on a number of scales (Smith et al., 1989; Poole et al., 1996).

The objectives of the present study were: (1) to establish the interactive effects of season-long exposure to elevated CO₂ and/or O₃ on leaf conductance, assimilation rate and stomatal and epidermal cell density; (2) to examine the heterogeneity of stomatal patterning in potato (Solanum tuberosum L. cv. Bintje); and (3) to determine whether correlations between different cell types provide information concerning the possible source(s) of within-leaf variation. This is the first known study of stomatal characteristics at the whole leaf level in potato, and is important because failure to quantify spatial variation resulting from the use of inappropriate sampling procedures may impede detection of significant treatment effects on stomatal and gas exchange characteristics. Cv. Bintje is commercially important in Europe for the production of high value processed food products, and previous work by the authors has shown that season-long exposure to elevated CO₂ and/or O₃ may affect tuber yield and quality, leaf chlorophyll content and photosynthetic characteristics (Donnelly et al., 2001; Lawson et al., 2001a). The present study investigated whether effects on gas exchange were attributable to changes in epidermal characteristics and leaf anatomy.

Materials and methods

Site preparation and open-top chambers
The experimental site and layout are described in detail (Lawson et al., 2001b). Briefly, open-top chambers (3.1 m in diameter and 2.4 m in height; Heagle et al., 1973) were placed on 10 m centres to avoid mutual shading. These were covered with 200 µm PVC in three sections: air from a fan box (Model PSA 402/2, Jones and Attwood, Stourbridge, UK) was supplied through the lower section. The soil was a sandy loam of the Astley Hall series and was ploughed and harrowed twice before planting. The pre-emergence herbicide, Parable (Zeneca, Surrey, UK), was applied at a rate of 3 dm³ ha⁻¹ to control weeds.

Experimental design
A factorial design containing three CO₂ and two O₃ treatments in 18 OTCs, randomized in three blocks, was used. The six treatments comprised ambient air OTC control plots (chAA; 378 µmol mol⁻¹ CO₂), elevated CO₂ OTCs maintained at 550 or 680 µmol mol⁻¹ under ambient O₃ conditions (c550 and c680), and elevated O₃ OTC plots (target seasonal mean of 60 nmol mol⁻¹) grown under ambient (oz), 550 (oz550) and 680 µmol mol⁻¹ CO₂ (oz680).

Crop management
Seed tubers (Solanum tuberosum L. cv. Bintje) with single sprouts were planted at a depth of 5–10 cm at 20 cm intervals
in ridges 20 cm high and 25 cm apart, providing a density of 20 plants m$^{-2}$. Soil moisture was routinely monitored using septum tensiometers (Skye Instruments Ltd., Powys, Wales, UK) and maintained above 70% of field capacity by trickle irrigation. Standard procedures were used to control fungal and viral pathogens and insect pests (Lawson et al., 2001b).

Gas exposure and microclimatic conditions

The elevated CO$_2$ treatments were applied for 24 h d$^{-1}$ between emergence and final harvest. Ozone was supplied to the elevated O$_3$ treatment for 8 h d$^{-1}$ (09.00–17.00 h GMT) for 5 d week$^{-1}$ during the same period. Wet and dry bulb air temperature, soil temperature at a depth of 10 cm, wind speed, and incident, reflected and transmitted short-wave radiation were logged at 15 s intervals and used to calculate hourly means (Lawson et al., 2001b).

Gas exchange measurements

Instantaneous gas exchange measurements were made for leaf 15 (youngest fully expanded leaf) in two replicate plots of each treatment using a portable infrared gas analyser (CIRAS-1, PP Systems, Hitchin, Herts, UK). Measurements were made at weekly intervals between 46 d and 88 d after emergence (DAE) between 09.00–13.00 h to minimize the impact of diurnal variation in environmental conditions. A broad-leaf Parkinson cuvette (area 2.5 cm$^2$) and artificial light source supplying $>$1200 µmol photons m$^{-2}$ s$^{-1}$ were used. The IRGA was adjusted to match ambient CO$_2$ (±5 µmol mol$^{-1}$) and water vapour concentrations (±10%) and allowed to stabilize for 1.5 min before completing the measurement.

Stomatal measurements

Silicone rubber impressions of the entire abaxial surface of leaf 15 were made at 64 DAE for one randomly selected plant per treatment using Xantopren VL Plus dental impression material (Beyer Dental, Leverkusen, Germany; Weyers and Johansen, 1985); at this time, the leaves were fully expanded and well illuminated due to their position at the surface of the canopy. Spot impressions were made of the adaxial leaf surface at the centre of the left side of the lamina mid-way between the base and the tip for two other leaves of the same age in each treatment (one from each replicate chamber examined). The whole-leaf impressions were subdivided into 10×10 mm sampling squares and positive images were made using clear nail varnish. Stomatal and epidermal cell numbers were counted using a microscope and eye-piece graticule and the systematic sampling strategy described previously (Poole and Kürschner, 1999). Stomatal density and index were calculated and mapped using the bilinear interpolation option within the Unimap 2000 program (UNIRAS Ltd., Slough, UK; Lawson, 1997), in which the x and y spatial coordinates identify the centre of each sampling site, while the z value represents the site mean for the corresponding stomatal and cell characteristics. The two-dimensional maps obtained should be interpreted with care (Lawson and Weyers, 1999) as they involve interpolation based on an arbitrary algorithm. Nonetheless, previous studies have provided extensive validation of this approach (Lawson, 1997).

Leaf sections

The 10×10 mm leaf sections used to make impressions were excised and fixed overnight in gluteraldehyde (Agar Scientific, Stansted, UK) before being dehydrated by immersing them for 10 min each in a graded alcohol series (25, 50, 75, 80, 90, and 100% ethanol). Following a second rinse in 100% ethanol, the sections were placed in 1:1 ethanol:LR White resin (London Resin Co., London, UK) for 6 h, before allowing the ethanol to evaporate off overnight. The sections were placed in 100% LR White resin for several days, embedded in fresh resin in gelatin capsules and polymerized overnight at 60°C. Thin sections (10 µm) were cut using an ultramicrotome, placed on microscope slides and stained with a 0.1% aqueous solution of toluidine blue (BDH Ltd, Poole, UK). Leaf thickness was measured for three leaves from each treatment at a magnification of ×400 using an eye-piece graticule.

Statistical analysis

The values for leaf conductance, leaf thickness and the spot measurements of cell density and stomatal index were analysed as a replicated 3 CO$_2$×2 O$_3$ factorial experiment by analysis of variance (Genstat 5, Lawes Agricultural Trust). To test for trends induced by elevated CO$_2$, the effect of CO$_2$ was further partitioned into a linear component and the residual variation about the trend. In the whole leaf studies, linear correlation coefficients between specific epidermal characteristics were calculated using the data obtained for the numerous sampling locations within individual leaves.

Results

Microclimatic conditions and gas exposures

Seasonal daily mean short-wave radiation receipts in the open-top chamber (OTC) treatments were 19% lower than the ambient level ($P<0.001$; Table 1). Saturation vapour pressure deficit (SVPD) and mean, minimum and maximum air temperatures were slightly above ambient in the OTC treatments ($P<0.01$), while soil temperature and atmospheric vapour pressure ($e_a$) were comparable to the ambient values. Seasonal mean CO$_2$ concentrations in the 550 and 680 µmol mol$^{-1}$ treatments were

<table>
<thead>
<tr>
<th>Environmental variable</th>
<th>Ambient</th>
<th>OTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solar radiation (MJ m$^{-2}$)</td>
<td>0.81 ± 0.018</td>
<td>0.64 ± 0.014</td>
</tr>
<tr>
<td>Average hourly mean</td>
<td>15.0 ± 0.41</td>
<td>12.1 ± 0.51</td>
</tr>
<tr>
<td>Average daily total</td>
<td>1274 ± 4.7</td>
<td>1031 ± 3.7</td>
</tr>
<tr>
<td>Accumulated seasonal total</td>
<td>13.9 ± 0.08</td>
<td>14.8 ± 0.09</td>
</tr>
<tr>
<td>Air temperature (°C)</td>
<td>18.6 ± 0.34</td>
<td>20.5 ± 0.36</td>
</tr>
<tr>
<td>Daily mean maximum</td>
<td>8.9 ± 0.25</td>
<td>9.4 ± 0.24</td>
</tr>
<tr>
<td>Daily mean minimum</td>
<td>15.0 ± 0.04</td>
<td>14.8 ± 0.50</td>
</tr>
<tr>
<td>Soil temperature (°C)</td>
<td>0.31 ± 0.02</td>
<td>0.36 ± 0.02</td>
</tr>
<tr>
<td>SVPD (kPa)</td>
<td>1.35 ± 0.021</td>
<td>1.38 ± 0.022</td>
</tr>
</tbody>
</table>
within 3% of the target values (563 and 673 μmol mol⁻¹, respectively). Seasonal mean O₃ concentrations during the 8 h d⁻¹ exposure period were respectively 21.3 and 49.9 nmol mol⁻¹ in the ambient and elevated O₃ treatments (Lawson et al., 2001b).

Leaf conductance and assimilation

Figure 1 shows mean values for leaf conductance (gₛ), net assimilation rate (A) and instantaneous transpiration efficiency (ITE) for leaf 15 measured on six sampling dates between 46 d and 88 d after emergence (DAE) for all treatments. gₛ was unaffected by elevated CO₂ in the c550 treatment or by elevated O₃ in the oz treatment, but was reduced by 41–55% relative to the ambient CO₂ treatment in the c680, oz550 and oz680 treatments (Fig. 1a; P < 0.01). There was also a significant CO₂×O₃ interaction (P < 0.05) as an O₃-induced reduction in gₛ occurred only under 550 CO₂. A was significantly greater in the c550 elevated CO₂ treatment than in any other (Fig. 1b; P < 0.05), but exposure to O₃ had no detectable effect. ITE increased progressively with increasing CO₂ concentration (Fig. 1c; P < 0.001) under both ambient and elevated O₃.

Leaf thickness

Table 2 shows the effect of elevated CO₂ on total leaf thickness and the thickness of the palisade and spongy mesophyll layers; the upper and lower epidermes were regarded as part of the palisade and spongy mesophyll layers, respectively, for the purpose of these measurements. As responses to 550 or 680 μmol mol⁻¹ CO₂ were unaffected by O₃ level, the combined means for both O₃ treatments were used to improve the sensitivity of the statistical analysis when testing for CO₂ effects. Although no significant treatment effect was detected for the spongy mesophyll, there was a suggestion that elevated CO₂ increased the thickness of the palisade layer (P = 0.084), leading to a 12% increase in total leaf thickness (P < 0.05).

Spot measurements of stomatal characteristics

The spot measurements of stomatal and epidermal cell numbers showed that stomatal density (SD) increased substantially (c. 61%) under elevated CO₂ (Fig. 2a; P < 0.05) except in the oz550 treatment. SD was also higher in the elevated O₃ oz and oz680 treatments than in ambient air chAA control plants (P < 0.05); no equivalent increase was apparent in the oz550 treatment. There were no detectable treatment effects on epidermal cell density (ECD; Fig. 2b; P > 0.1), but stomatal index was lowest in chAA control plants (Fig. 2c; P < 0.01).

Table 2. Effect of elevated CO₂ and O₃ on the thickness of the palisade and mesophyll layers (μm) and total leaf thickness for leaf 15

The adaxial and abaxial epidermes were, respectively, included in the measurements of palisade and mesophyll thickness. chAA, ambient air control treatment; 550 and 680 represent the means for the ambient and elevated O₃ treatments grown under 550 and 680 μmol mol⁻¹ CO₂. SED, standard error of the difference; df, degrees of freedom.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Palisade</th>
<th>Spongy mesophyll</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>chAA</td>
<td>41.3</td>
<td>48.3</td>
<td>94.0</td>
</tr>
<tr>
<td>550</td>
<td>42.5</td>
<td>51.3</td>
<td>105.3</td>
</tr>
<tr>
<td>680</td>
<td>52.5</td>
<td>52.5</td>
<td>111.5</td>
</tr>
<tr>
<td>SED</td>
<td>4.94</td>
<td>8.20</td>
<td>6.17</td>
</tr>
<tr>
<td>df</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
Within-leaf variation in stomatal characters

Approximately 4000 stomata were counted for each leaf examined, although the number of sampling locations varied due to the differing area of individual leaves (Table 3). Leaf area and stomatal density (SD) were, respectively, 27–54% and 13–25% higher in plants grown under elevated CO2 and/or O3 than in chAA control plants, whereas epidermal cell density (ECD) was 7–38% lower; the only exception was the oz550 treatment, in which SD was unaffected. Inspection of the two-dimensional maps showing within-leaf variation in SD for all leaves examined (Fig. 3) reveals that the variation in SD was greatest in the elevated CO2 and O3 treatments (37–71% expressed as a percentage change relative to the lowest recorded value), with the exception of the oz550 treatment (Table 3). Leaves E and F showed a near 2-fold within-leaf variation in SD. Stomatal index (SI) also exhibited extensive within-leaf variation (30–67%) under both elevated CO2 and O3. No consistent patterning of SD or SI (data not shown) was apparent.

SI and SD were positively correlated in all leaves examined ($P<0.05$–$0.01$; Fig. 4; Table 4). SI and ECD were negatively correlated in leaves from the chAA ($P<0.001$), oz and c680 ($P<0.05$) treatments (leaves A, C and D, respectively), but not in leaves from the oz550 and oz680 treatments (leaves B and F). ECD and hence SI could not be determined for leaf E, from the c550 treatment, as the impressions were insufficiently clear for accurate analysis. ECD and SD were positively correlated in leaves from the oz550, c680 and oz680 treatments (leaves B, D, F; $P<0.05$; Fig. 4; Table 4).

Discussion

Effects on leaf thickness and gas exchange

The observation that elevated CO2 increased leaf thickness, mainly due to a thickening of the palisade layer, and reduced $g_s$ is consistent with previous studies (Thomas and Harvey, 1983; Arp, 1991; Mulholland et al., 1997). Although most reports suggest that leaf thickness is reduced by elevated O3 (Bennett et al., 1992) and that O3-sensitivity is lower in species with thicker leaves (Paakkonen et al., 1997), elevated O3 increased leaf thickness in the present study, but had little effect on photosynthetic characteristics or tuber yield (Lawson et al., 2001a, b). Elevated CO2 increased $A$ and reduced $g_s$, thereby increasing ITE. Such effects may have important beneficial implications for crop production under future climatic conditions, which are expected to be warmer and drier and involve increased atmospheric CO2 concentrations.

Effects of CO2 and O3 on stomatal characteristics

Although previous reports suggest that SD is reduced by elevated CO2 (Woodward, 1987), SD and SI were increased by elevated CO2 and O3 in the present study. Increases in SD and/or ECD were also found when oak and faba bean plants were grown under elevated CO2 (Atkinson et al., 1997; Visser et al., 1997). By contrast, SD was unaffected in Tradescantia (Besford et al., 1990) and Populus (Ceulemans et al., 1995). Current evidence therefore suggests that SD is affected by elevated CO2 in some (Woodward, 1987; Woodward and Bazzaz, 1988) but not all species (Taylor et al., 1994; Knapp et al., 1994).
Table 3. Leaf area, stomatal density, stomatal index and epidermal cell density for individual leaves sampled from all treatments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Leaf A (chAA)</th>
<th>Leaf B (oz)</th>
<th>Leaf C (c550)</th>
<th>Leaf D (oz550)</th>
<th>Leaf E (c680)</th>
<th>Leaf F (oz680)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf area (mm²)</td>
<td>4016</td>
<td>5091</td>
<td>5789</td>
<td>6196</td>
<td>5166</td>
<td>6083</td>
</tr>
<tr>
<td>Value relative to leaf A (%)</td>
<td>100</td>
<td>127</td>
<td>144</td>
<td>154</td>
<td>129</td>
<td>151</td>
</tr>
<tr>
<td>Number of sampling locations</td>
<td>38</td>
<td>52</td>
<td>52</td>
<td>42</td>
<td>43</td>
<td>52</td>
</tr>
<tr>
<td>Stomatal density (stomata mm⁻²)</td>
<td>Mean</td>
<td>84</td>
<td>103</td>
<td>105</td>
<td>95</td>
<td>75</td>
</tr>
<tr>
<td>Value relative to leaf A (%)</td>
<td>100</td>
<td>123</td>
<td>125</td>
<td>113</td>
<td>89</td>
<td>114</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>12.1</td>
<td>12.8</td>
<td>13.6</td>
<td>15.3</td>
<td>14.6</td>
<td>16.9</td>
</tr>
<tr>
<td>Epidermal cell density (cells mm⁻²)</td>
<td>Mean</td>
<td>357</td>
<td>328</td>
<td>N/A</td>
<td>332</td>
<td>221</td>
</tr>
<tr>
<td>Value relative to leaf A (%)</td>
<td>100</td>
<td>92</td>
<td>N/A</td>
<td>93</td>
<td>62</td>
<td>83</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>14.9</td>
<td>11.5</td>
<td>N/A</td>
<td>10.9</td>
<td>11.6</td>
<td>13.1</td>
</tr>
<tr>
<td>Stomatal index (%)</td>
<td>Mean</td>
<td>19</td>
<td>24</td>
<td>N/A</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>13.2</td>
<td>17.3</td>
<td>N/A</td>
<td>8.9</td>
<td>9.4</td>
<td>17.0</td>
</tr>
</tbody>
</table>

As already noted (Morison, 1998), few studies have attempted to correlate effects of environmental variables on SD with changes in gs. Observations that elevated O₃ increased SD but reduced gs in potato (present study) and O₃-sensitive clones of birch (Paakkonen et al., 1997, 1998) suggest that reductions in stomatal aperture may negate the stimulatory influence of increased SD on gas exchange. A similar situation may apply for CO₂, as concurrent increases in SD and decreases in gs under elevated CO₂ under ambient O₃ conditions. N/A, data not available.

Within-leaf variation

To our knowledge, this is the first study of stomatal patterning in potato. The extent of the variation in SD and ECD is comparable to other species (Weyers et al., 1997; Poole et al., 1996, 2000). However, although SD showed substantial spatial variation, the patterns contrast with other dicotyledonous species in which SD increased towards the leaf margins and tip (Salisbury, 1928; Smith et al., 1989). A similar absence of consistent spatial patterning was found in Alnus glutinosa (Poole et al., 1996, 2000).

Mechanisms contributing to spatial variation in SD include (1) uneven differentiation of stomatal and/or epidermal cells, causing local variation in cell numbers; (2) uneven expansion of epidermal cells, leading to uneven spacing of stomata; or (3) a combination of both (Poole et al., 1996). The presence or absence of correlations between SI, SD and ECD may be used to distinguish between the hypotheses. Table 5 shows the relationships between cell characteristics expected if the cell expansion, cell differentiation or combined cell differentiation/expansion hypotheses apply. The close correlation between SD and SI in all leaves suggests that the heterogeneity in stomatal characteristics resulted from spatial variation in stomatal differentiation. However, the significant correlation between SI and ECD in leaves A, C and F (Table 4), suggests that a combination of uneven guard and epidermal cell differentiation was also involved. The absence of any correlation between SD and ECD in leaves A and C implies that local variation in epidermal cell expansion did not contribute to stomatal patterning. By contrast, the significant positive correlation between SD and ECD in leaves B, D and F indicates that local variation in epidermal cell expansion contributed to within-leaf variation, suggesting a role for the mixed differentiation and expansion hypothesis. The lack of correlation between SI and ECD in leaves B and D implies that the differentiation component of the mixed hypothesis resulted largely from differences in stomatal differentiation, whereas the significant correlation between these variables in leaf F suggests that the differentiation component originated from effects on epidermal cell differentiation.

The results for leaves A and C therefore suggest that within-leaf variation in stomatal characteristics resulted from local variation in stomatal and epidermal cell differentiation. By contrast, the significant correlation between SD and ECD in all other leaves implies that local
variation in differentiation and expansion were important determinants of spatial variability. However, the existence of correlations between specific cell characteristics does not conclusively demonstrate causal relationships due to the difficulty of distinguishing between variation arising from local differences in guard and epidermal cell differentiation and that resulting from mixed cell differentiation and expansion (Table 5). Variation resulting from local differences in leaf expansion or guard or epidermal cell differentiation may be distinguished more readily.

**Importance of heterogeneity**

The present study suggests that the source of extensive within-leaf variation in SD and SI may differ between leaves. The substantial spatial variation in SD suggests that artefacts may arise if inappropriate sampling procedures are used to assess the impact of factors influencing stomatal characteristics. Many studies have attempted to account for spatial variation by defining specific sampling areas (e.g. mid-way between the base and tip and the midrib and margin of the leaf; Weyers and Lawson, 1997). However, as shown here, some species exhibit inconsistent stomatal patterning, suggesting that observations made at specific locations may not provide representative estimates of mean treatment effects at the whole-leaf level. However, it should be noted that, if within-leaf patterning for the characteristic under study is random, spot measurements made at the same location

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**Fig. 3.** Spatial variation in stomatal density (SD, stomata mm$^{-2}$) on the abaxial surface of leaf 15. Leaves were sampled from the chAA (leaf A), oz (leaf B), c550 (leaf C), oz550 (leaf D), c680 (leaf E), and oz680 (leaf F) treatments.

**Fig. 4.** Relationship between: (a) stomatal index and stomatal density; (b) stomatal index and epidermal cell density; and (c) epidermal cell density and stomatal density for leaf 15. chAA and c680 denote treatments receiving ambient or 680 μmol mol$^{-1}$ CO$_2$ under ambient O$_3$ conditions; oz, oz550 and oz680, treatments receiving ambient, 550 or 680 μmol mol$^{-1}$ CO$_2$ under elevated O$_3$ conditions.

Effects of CO$_2$ and O$_3$ on epidermal characteristics
Table 4. Correlation coefficients for the relationships between stomatal density (SD), stomatal index (SI) and epidermal cell density (ECD) for individual leaves sampled from all treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SD versus SI Coefficient</th>
<th>Significance</th>
<th>SD versus ECD Coefficient</th>
<th>Significance</th>
<th>SI versus ECD Coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf A (c550)</td>
<td>0.606</td>
<td>P&lt;0.01</td>
<td>0.111</td>
<td>n.s.</td>
<td>-0.685</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Leaf B (oz)</td>
<td>0.756</td>
<td>P&lt;0.01</td>
<td>0.160</td>
<td>n.s.</td>
<td>-0.454</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Leaf C (oz550)</td>
<td>N/A</td>
<td></td>
<td>N/A</td>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Leaf D (oz680)</td>
<td>0.406</td>
<td>P&lt;0.01</td>
<td>0.556</td>
<td>P&lt;0.01</td>
<td>-0.319</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leaf E (c680)</td>
<td>0.601</td>
<td>P&lt;0.01</td>
<td>0.631</td>
<td>P&lt;0.01</td>
<td>-0.159</td>
<td>n.s.</td>
</tr>
<tr>
<td>Leaf F (oz680)</td>
<td>0.446</td>
<td>P&lt;0.05</td>
<td>0.431</td>
<td>P&lt;0.05</td>
<td>-0.501</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

Table 5. Likelihood of expected observations and correlations being obtained depending upon the hypothetical source of variation in stomatal density (SD) and stomatal index (SI)

<table>
<thead>
<tr>
<th>Observation or correlation</th>
<th>Differentiation hypothesis (1)</th>
<th>Expansion hypothesis (2)</th>
<th>Combined differentiation and expansion hypothesis (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local variation in SD</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Local variation in SI</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Positive correlation between local values for SD and SI</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Negative correlation between local values for EPC and SI</td>
<td>Low</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Positive correlation between local values for SD and EPC</td>
<td>Low</td>
<td>Low</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

Natural heterogeneity of stomatal characteristics must therefore be considered, not only when choosing appropriate sampling strategies to assess responses to previous climatic conditions, but also when scaling from gas exchange measurements made at the stomatal or leaf level to the canopy level, or when modelling stomatal and gas exchange characteristics (Weyers et al., 1997). For example, some predictions of water use efficiency and carbon balance have relied on estimates of SD and pore length to derive maximal stomatal conductance (Beerling and Woodward, 1997). Although SD may be closely correlated with stomatal conductance in some species (Woodward and Bazzaz, 1988), the stomatal sensitivity model developed for Commelina communis (Weyers and Lawson, 1997) showed that SD was approximately one-third as important as stomatal aperture in determining $g_s$ and hence gas exchange. The present study provides further evidence that $g_s$ and SD are not always closely correlated, and that $g_s$ is regulated primarily through changes in stomatal aperture rather than numbers.

Where concurrent measurements of the responses of $g_s$ and SD or SI to elevated CO$_2$ are available, agreement is usually poor (Morison, 1998), perhaps due to within-leaf variation in stomatal or epidermal cell characteristics or physiological responses (Lawson and Weyers, 1999). Stomatal aperture may also change in the opposite direction to that anticipated; for instance, exposure to elevated CO$_2$ may increase stomatal numbers but reduce $g_s$ (Morison, 1998). Variation in stomatal characteristics merits further investigation as these may be either beneficial or detrimental for individual plants and have important implications for crop productivity (Mansfield et al., 1990). Potentially beneficial aspects such as changes in SD have been recognized as valuable traits in breeding programmes for drought resistance (Jones, 1977, 1987).
However, without detailed knowledge of the causes and consequences of stomatal heterogeneity, a full understanding of stomatal function is impossible.

Conclusions

This study has shown that, although long-term exposure to elevated CO2 affected gs, A and ITE, these responses resulted primarily from reductions in stomatal aperture rather than stomatal numbers. No anatomical changes were detected which might account for the observed effects of elevated CO2 on these gas exchange parameters. The results showed considerable heterogeneity of SD and ECD at the whole leaf level in potato. The sources of this heterogeneity ranged from effects on epidermal cell differentiation to a combination of effects on cell differentiation and leaf expansion. Such within-leaf variation must be considered when examining the effects of factors such as elevated CO2 on stomatal characteristics or gas exchange parameters, or when attempting to predict past climatic conditions or the impact of future climatic change.

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