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Impact of elevated CO_2 and O_3 on gas exchange parameters and epidermal characteristics in potato (*Solanum tuberosum* L.)

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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 μmol 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 μmol mol^{-1}, has increased steadily since pre-industrial times and is predicted to range between 540–970 μmol 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).

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

Elevated atmospheric CO_2 may affect plants at both the physiological (Long *et al.*, 1996) and anatomical levels (Beerling *et al.*, 1998). Stomatal responses to elevated CO_2 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 CO_2 concentration (Willmer and Fricker, 1996). Several studies suggest that stomatal density is reduced by elevated CO_2 (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 O_3 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 CO_2 and O_3 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 hypostomatous sun and shade leaves of *Alnus glutinosa* (Poole *et al.*, 1996). Poole *et al.* have recently examined the impact of elevated CO_2 on stomatal characteristics in the same species, and observed considerable within-leaf variation under both ambient and elevated CO_2 (Poole *et al.*, 2000); they also found that elevated CO_2 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 CO_2 and/or O_3 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 CO_2 and/or O_3 may affect tuber yield and quality, leaf chlorophyll content and photosynthetic characteristics (Donnelly *et al.*, 2001; Lawson *et al.*, 2001*a*). 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.*, 2001*b*). 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 dm^3 ha^{-1} to control weeds.

Experimental design

A factorial design containing three CO_2 and two O_3 treatments in 18 OTCs, randomized in three blocks, was used. The six treatments comprised ambient air OTC control plots (chAA; 378 μmol mol^{-1} CO_2), elevated CO_2 OTCs maintained at 550 or 680 μmol mol^{-1} under ambient O_3 conditions (c550 and c680), and elevated O_3 OTC plots (target seasonal mean of 60 nmol mol^{-1}) grown under ambient (oz), 550 (oz550) and 680 μmol mol^{-1} CO_2 (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*., 2001*b*).

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*., 2001*b*).

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 ($\pm 10\%$) and allowed to stabilize for *c*. 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 $\times400$ 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 $3\,CO_2\times2\,O_3$ 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 1. *Seasonal mean values for incident short-wave radiation, air temperature, saturation vapour pressure deficit (SVPD) and vapour pressure (e_a) for the ambient field environment (Ambient) and open top chambers (OTCs)*

OTC values are means for all six treatments ±standard error of the mean.

Environmental variable	Ambient	OTC
Solar radiation (MJ m^{-2})		
Average hourly mean	0.81 ± 0.018	0.64 ± 0.014
Average daily total	15.0 ± 0.41	12.1 ± 0.51
Accumulated seasonal total	1274 ± 4.7	1031 ± 3.7
Air temperature (°C)		
Daily mean	13.9 ± 0.08	14.8 ± 0.09
Daily mean maximum	18.6 ± 0.34	20.5 ± 0.36
Daily mean minimum	8.9 ± 0.25	9.4 ± 0.24
Soil temperature (°C)		
Daily mean	15.0 ± 0.04	14.8 ± 0.50
SVPD (kPa)		
Daily mean	0.31 ± 0.02	0.36 ± 0.02
e_a (kPa)		
Daily mean	1.35 ± 0.021	1.38 ± 0.022

within 3% of the target values (563 and 673 μmol mol^{-1}, respectively). Seasonal mean O_3 concentrations during the 8 h d^{-1} exposure period were respectively 21.3 and 49.9 nmol mol^{-1} in the ambient and elevated O_3 treatments (Lawson *et al.*, 2001*b*).

Leaf conductance and assimilation

Figure 1 shows mean values for leaf conductance (g_s), 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. g_s was unaffected by elevated CO_2 in the c550 treatment or by elevated O_3 in the oz treatment, but was reduced by 41–55% relative to the ambient CO_2 treatment in the c680, oz550 and oz680 treatments (Fig. 1a; $P<0.01$). There was also a significant $CO_2 \times O_3$ interaction ($P<0.05$) as an O_3-induced reduction in g_s occurred only under 550 CO_2. A was significantly greater in the c550 elevated CO_2 treatment than in any other (Fig. 1b; $P<0.05$), but exposure to O_3 had no detectable effect. ITE increased progressively with increasing CO_2 concentration (Fig. 1c; $P<0.001$) under both ambient and elevated O_3.

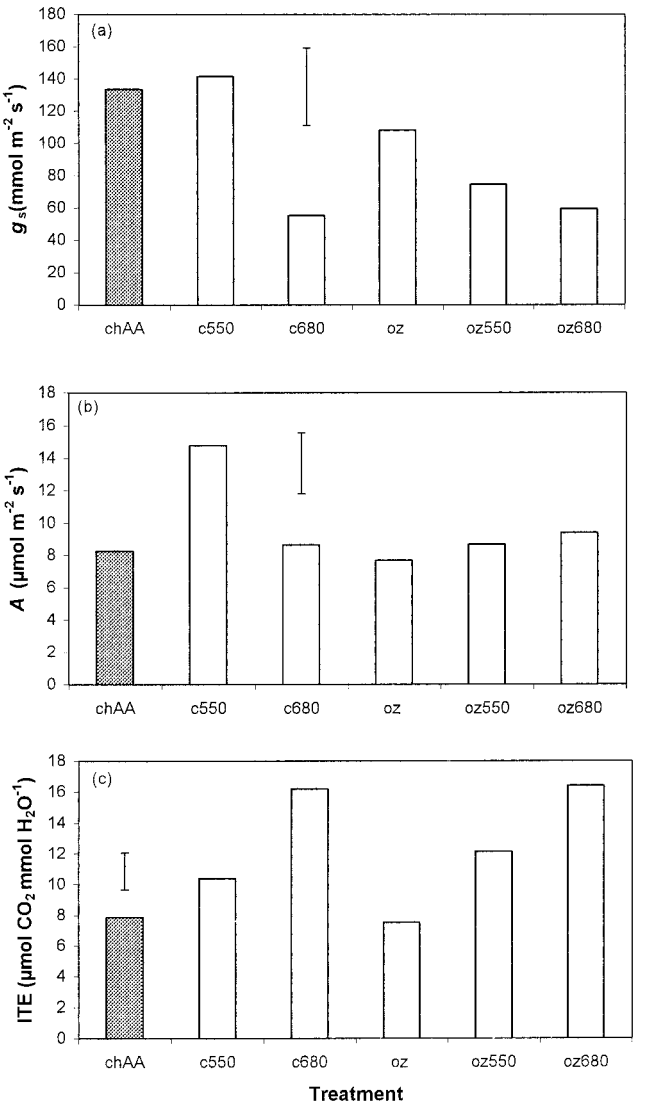

Fig. 1. Effect of elevated CO_2 and/or O_3 on: (a) seasonal mean leaf conductance (g_s); (b) assimilation rate (A); and (c) instantaneous transpiration efficiency (ITE) for leaf 15. chAA, c550 and c680 denote treatments receiving ambient, 550 or 680 μmol mol^{-1} CO_2 under ambient O_3 conditions; oz, oz550 and oz680, treatments receiving ambient, 550 or 680 μmol mol^{-1} CO_2 under elevated O_3 conditions. Bar shows the standard error of the difference for comparing treatments with 10 df.

Leaf thickness

Table 2 shows the effect of elevated CO_2 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} CO_2 were unaffected by O_3 level, the combined means for both O_3 treatments were used to improve the sensitivity of the statistical analysis when testing for CO_2 effects. Although no significant treatment effect was detected for the spongy mesophyll, there was a suggestion that elevated CO_2 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 CO_2 (Fig. 2a; $P<0.05$) except in the oz550 treatment. SD was also higher in the elevated O_3 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 CO_2 and O_3 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 O_3 treatments grown under 550 and 680 μmol mol^{-1} CO_2. SED, standard error of the difference; df, degrees of freedom.

Treatment	Palisade	Spongy mesophyll	Total
chAA	41.3	48.3	94.0
550	42.5	51.3	105.3
680	52.5	52.5	111.5
SED	4.94	8.20	6.17
df	5	5	5

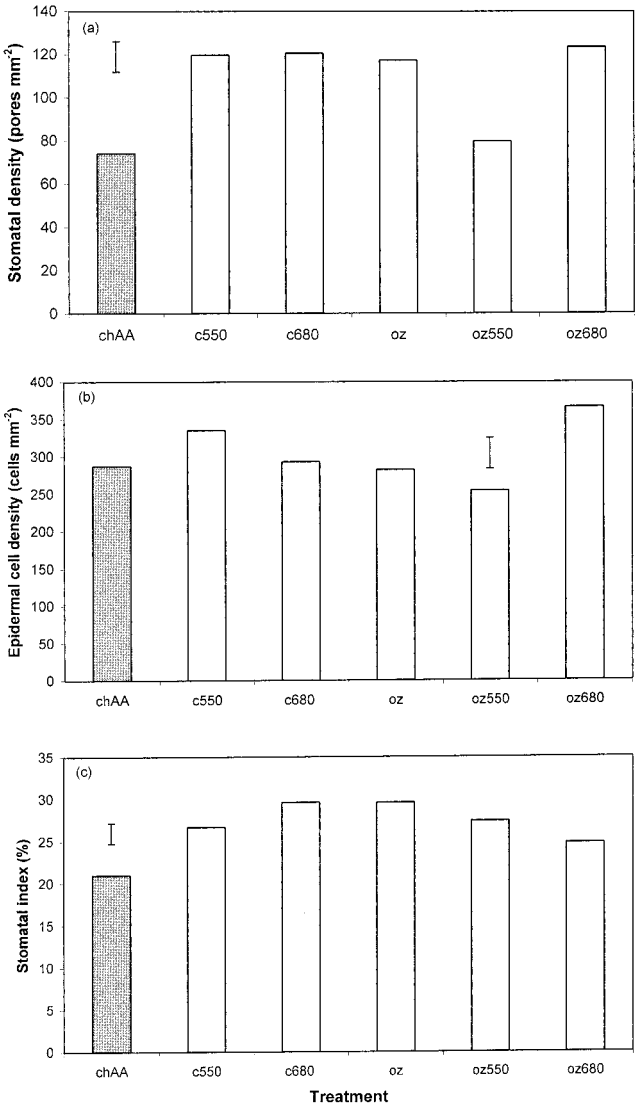

Fig. 2. Effect of elevated CO_2 and/or O_3 on stomatal density (SD), epidermal cell density (ECD) and stomatal index (SI) calculated from spot measurements made for leaf 15; a randomly selected leaf was sampled from all replicates of each treatment ($n = 3$). chAA, c550 and c680 denote treatments receiving ambient, 550 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. Bar shows the standard error of the difference for comparing treatments with 10 df.

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 CO_2 and/or O_3 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 CO_2 and O_3 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 CO_2 and O_3. 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 CO_2 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 O_3 (Bennett *et al.*, 1992) and that O_3-sensitivity is lower in species with thicker leaves (Paakkonen *et al.*, 1997), elevated O_3 increased leaf thickness in the present study, but had little effect on photosynthetic characteristics or tuber yield (Lawson *et al.*, 2001*a*, *b*). Elevated CO_2 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 CO_2 concentrations.

Effects of CO_2 and O_3 on stomatal characteristics

Although previous reports suggest that SD is reduced by elevated CO_2 (Woodward, 1987), SD and SI were increased by elevated CO_2 and O_3 in the present study. Increases in SD and/or ECD were also found when oak and faba bean plants were grown under elevated CO_2 (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 CO_2 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*

chAA, c550 and c680 denote treatments receiving ambient, 550 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. N/A, data not available.

Parameter	Leaf A (chAA)	Leaf B (oz)	Leaf C (c550)	Leaf D (oz550)	Leaf E (c680)	Leaf F (oz680)
Leaf area (mm^2)	4016	5091	5789	6196	5166	6083
Value relative to leaf A (%)	100	127	144	154	129	151
Number of sampling locations	38	52	52	42	43	52
Value relative to leaf A (%)	100	136	136	111	113	136
Stomatal density (stomata mm^{-2})						
Mean	84	103	105	95	75	96
Value relative to leaf A (%)	100	123	125	113	89	114
Coefficient of variation	12.1	12.8	13.6	15.3	14.6	16.9
Epidermal cell density (cells mm^{-3})						
Mean	357	328	N/A	332	221	296
Value relative to leaf A (%)	100	92	N/A	93	62	83
Coefficient of variation	14.9	11.5	N/A	10.9	11.6	13.1
Stomatal index (%)						
Mean	19	24	N/A	22	24	24
Value relative to leaf A (%)	100	126	N/A	116	126	126
Coefficient of variation	13.2	17.3	N/A	8.9	9.4	17.0

As already noted (Morison, 1998), few studies have attempted to correlate effects of environmental variables on SD with changes in g_s. Observations that elevated O_3 increased SD but reduced g_s in potato (present study) and O_3-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 CO_2, as concurrent increases in SD and decreases in g_s were found in the present study. There may therefore be no physiological need for SD to alter in response to increased atmospheric CO_2 or O_3 as changes in stomatal aperture may provide effective regulation of gas exchange over a wide range of ambient concentrations. The concurrent increase in SD and decrease in g_s under elevated CO_2 in the present study were accompanied by increases in A, ITE and biomass production during the early stages of the season (Lawson *et al.*, 2001*a*, *b*), emphasizing the importance of stomatal aperture in maintaining assimilation whilst regulating water loss.

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 these 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

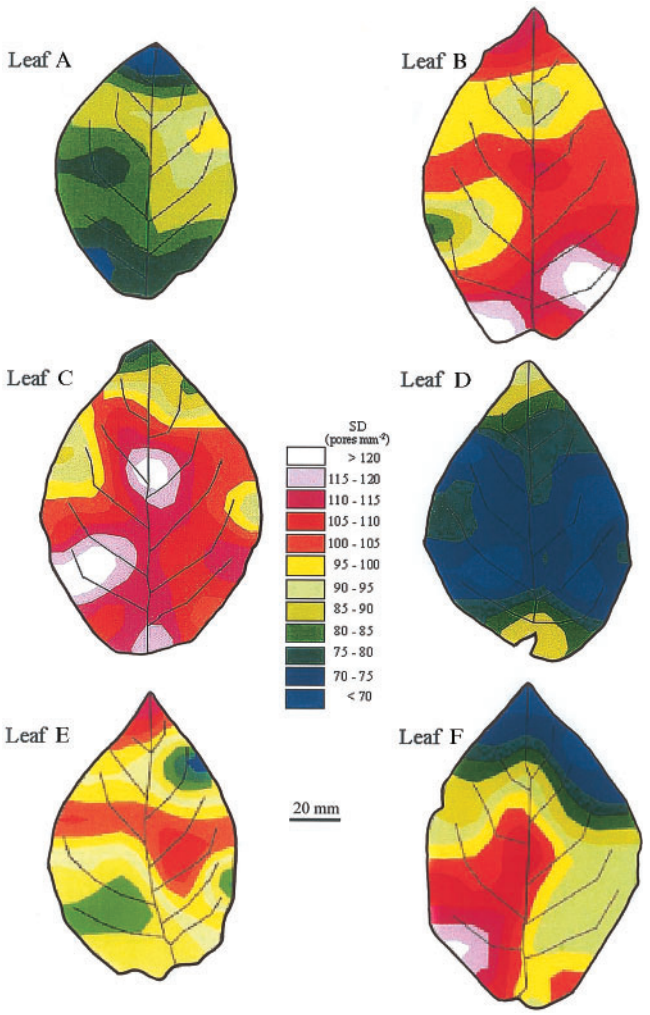

Fig. 3. Spatial variation in stomatal density (SD, stomata mm^{-2}) on the abaxial surface of leaf 15. Leaves were sampled from the chAA (leaf A), oz (leaf B), c550 (leaf C), oz550 (leaf D), c680 (leaf E), and oz680 (leaf F) treatments.

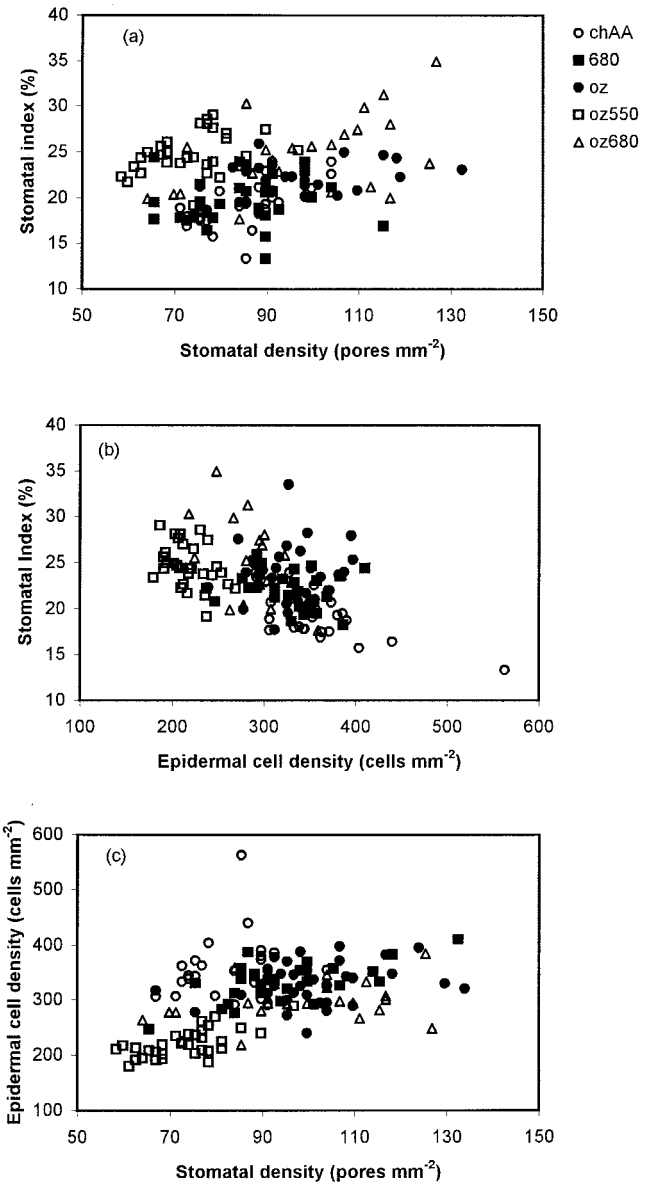

Fig. 4. Relationship between: (a) stomatal index and stomatal density; (b) stomatal index and epidermal cell density; and (c) epidermal cell density and stomatal density for leaf 15. chAA and c680 denote treatments receiving ambient or 680 μmol mol^{-1} CO_2 under ambient O_3 conditions; oz, oz550 and oz680, treatments receiving ambient, 550 or 680 μmol mol^{-1} CO_2 under elevated O_3 conditions.

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

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*

chAA, c550 and c680 denote treatments receiving ambient, 550 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. N/A, data not available. *P* values indicate the level of significance.

Treatment	SD versus SI Coefficient	Significance	SD versus ECD Coefficient	Significance	SI versus ECD Coefficient	Significance
Leaf A (chAA)	0.606	$P<0.01$	0.111	n.s.	−0.685	$P<0.01$
Leaf B (oz)	0.756	$P<0.01$	0.160	n.s.	−0.454	$P<0.05$
Leaf C (c550)	N/A		N/A		N/A	
Leaf D (oz550)	0.406	$P<0.01$	0.556	$P<0.01$	−0.319	n.s.
Leaf E (c680)	0.601	$P<0.01$	0.631	$P<0.01$	−0.159	n.s.
Leaf F (oz680)	0.446	$P<0.05$	0.431	$P<0.05$	−0.501	$P<0.05$

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)*

The differentiation hypothesis is subdivided into three possible outcomes where: A represents a scenario where local variation in differentiation occurs only for guard cells, and epidermal cell density remains unchanged; B is where local variation in differentiation occurs for epidermal cells but stomatal density remains unchanged; and C is where there is local variation in both epidermal and guard cell differentiation.

Observation or correlation	Differentiation hypothesis (1)			Expansion hypothesis (2)	Combined differentiation and expansion hypothesis (3)
	A	B	C		
Local variation in SD	High	Low	High	High	High
Local variation in SI	High	High	High	Low	High
Positive correlation between local values for SD and SI	High	Low	High	Low	High
Negative correlation between local values for EPC and SI	Low	High	High	Low	Moderate
Positive correlation between local values for SD and EPC	Low	Low	Moderate	High	High

on different leaves effectively provide a random sampling strategy. Improved sampling protocols would therefore involve fewer observations for a larger number of leaves. Poole *et al*. concluded that the differences in SD used to predict past CO_2 concentrations in paleoclimatic reconstructions were of similar magnitude to those found in individual leaves (Poole *et al*., 1996). Spot sampling approaches may therefore lead to conclusions regarding treatment effects which are not applicable at the whole leaf level. A possible solution would be to adopt a stratified sampling strategy involving measurements of sufficient replicate leaves to account for within-leaf variation (Lawson *et al*., 1998).

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 CO_2 affected g_s, 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 CO_2 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 CO_2 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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