Bots. J. Agric. Appl. Sci (2013) 9 (Issue 1) 82-89

BOJAAS

ORIGINAL RESEARCH

Responses of leaf parameters of *Arabidopsis thaliana* ecotypes to change in CO_2 concentrations

Selebatso, T.[1 2], Lake, J. A[3]. and Woodward, F. I.[1]

[1]University of Sheffield, Sheffield, S10 2TN, UK . [3]University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershine, LE12 5RD. UK.

ST; conceived idea, designed study, collected data, statistical analysis, prepared manuscript; LJA, conceived idea, designed study, major review of manuscript, WFI, conceived idea

ABSTRACT

An increase in anthropogenic generation of carbon dioxide (CO_2) is one of the characteristic features of global climate change. How plants respond to this change has critical implications for the performance of both natural ecosystems and agricultural systems. The aim of this study was to quantify the effects of increasing CO_2 on morphological features of ecotypes of *Arabidopsis thaliana*, as a primary model plant species, originating from different altitudes and geographical areas. Different altitude implies different growing conditions which would result in phenotypic variations between ecotypes including adaptation to different partial pressures of CO_2. Ecotypes of *A. thaliana* were grown in controlled environment chambers to assess genetic constraints on phenotypic plasticity across a wide range of atmospheric CO_2 concentrations. Results showed variation in stomatal density and epidermal density among ecotypes but this variation could not be explained by their altitude of origin. Most ecotypes showed a trend of increased stomatal density and epidermal cell density under elevated CO_2 (800 ppm) whereas in sub-ambient CO_2 there was no significant ($P > 0.05$) change. Leaf temperature was reduced under CO_2 enrichment compared to current ambient CO_2 (400 ppm) and sub-ACO_2. This was an indication of high transpiration rates which then could have led to increased stomatal and epidermal cell densities. Knowledge of responses of this model plant is useful for directing further studies for plants of potential evolutionary patterns in relation to climatic changes.

Keywords *Arabidopsis thaliana,* CO_2 enrichment, stomatal density, altitude, epidermal cell density.

[1]Correspondence Author: Tel: +2673650152, Email: tselebatso@bca.bw

[2]Present Address: Department of Basic Sciences, Botswana College of Agriculture, Private bag 0027 Gaborone, Botswana

Publisher: Botswana College of Agriculture

INTRODUCTION

An increase in global carbon dioxide is one of the characteristic features of global climate change. Future increases in global carbon dioxide and its impact on plants has been a subject of substantial research (Woodward and Bazzaz, 1988, Woodward and Kelly, 1995, Ward and Strain, 1997, Lake and Woodward, 2008). It is important to understand how plants respond to these atmospheric changes in order to enhance our ability to predict plant responses to future CO_2 changes (Hovenden and Schimanski, 2000). Studies on effects of low CO_2 concentrations will also help to understand how plants have adapted to low CO_2 concentrations in the past as well as in identifying specific plant traits that confer a selective advantage under changing CO_2 concentrations (Ward and Strain, 1997). At the interface between plant leaf and the atmosphere are the stomata which regulate gaseous exchange, mainly CO_2 and water humidity (Casson and Gray, 2008). More than 90% of water transpired by the plant is lost through stomatal pores (Raven *et al*., 1999). Hetherington and Woodward (2003) also reported that stomata exert a major control on both global water and carbon cycles. Although responses of stomata to changes in CO_2 concentration can be variable, most studies show that stomatal density has decreased with an increase in CO_2 (Woodward and Kelly, 1995; Woodward et al., 2002; Hetherington and Woodward, 2003). Reduced stomatal density under CO_2 enrichment is selectively advantageous to plants since it allows plants to retain more water when CO_2 is abundant (Miller-Rushing *et al*., 2009). Most studies (Woodward and Bazzaz, 1988, Woodward and Kelly, 1995) on the responses of stomata to changes in CO_2 usually deal with comparisons between plant species. Apart from interspecific comparisons there are also intraspecific comparisons of plant responses from the same geographical location (Hovenden and Schimanski, 2000; Woodward *et al*., 2002; Lake and Woodward, 2008) with different altitudes (Hovenden and Vander Schoor, 2003, 2005; Woodward, 1986). For most crop plants, comparisons of responses are between species or if are within species the members are from the same altitude (Sekiya and Yano, 2008; Kudoyarova *et al*., 2007). In addition, most studies on crop plants involves varieties/cultivars and not ecotypes (variety defined as a

taxonomic rank below a species, cultivar meaning cultivated variety). An ecotype in this context is a distinct race of a species genetically adapted to a particular habitat (or wild homozygous lines) (Alonso-Blanco and Koornneef, 2000).

Altitudinal differences are important to plants because the partial pressure of CO_2 and temperature decline with increasing altitude whereas solar irradiation increases with altitude. The ecotypes involved in the present study originate from different altitudes and places (Table 1). This broad distribution encompasses different growing conditions hence phenotypic variation among ecotypes is expected to reflect the genetic variation that enables them to adapt to specific growing conditions (Alonso-Blanco and Koornneef, 2000). It is reported (Körner and Diemer, 1994) that plants originating from different altitudes remain true to their character when grown at different altitudes to that of their origins. There is little reported literature (Ward and Strain, 1997) on investigations that involve ecotypes from different altitudes of different geographical origins. This study by Ward and Strain, (1997) on *A. thaliana* measured biomass and reproduction of different ecotypes. *A. thaliana* has a rich source of natural genetic diversity. It is distributed widely, particularly in the moderate temperate zones of the world (Ward and Strain, 1997) and it provides an excellent model for determining plant responses to environmental change (Ward and Kelly, 2004).

Therefore, the aim of this study was to quantify the effects of changing CO_2 concentration on morphological characteristics of plants of ecotypes of *A.thaliana* with different geographical and altitude origins (USA, UK, Libya, USSR and Spain).

MATERIALS AND METHODS

Plant material and description

A number of ecotypes from different geographical and altitudinal origins of *A. thaliana* (Nottingham *Arabidopsis* stock centre, UK, http://www.nasc.nott.ac.uk/home.html) were used in these investigations (Table 1). The seeds of these ecotypes were a maximum of two generations old and therefore the risks of genetic drift were assumed minimal. *A. thaliana* was chosen for this study because it is easy to grow; from germination to seed set it takes approximately nine weeks.

Growing conditions

Seeds were stratified at 4 $\pm 1^0$C for two to three days, then germinated and grown in Bowyers multipurpose compost (Williamsons, Sheffield, UK) in controlled environment growth chambers (Conviron BDR16, Winnipeg, Canada; Fitotron SG970/C/FM/HQI and SGC2352/FM/HFL, Sanyo Gallenkamp,).

Table 1 Ecotypes of *Arabidopsis thaliana* and their place of origin, altitude and their description. (http://arabidopsis.info/)

Ecotype & Stork No	Country	Location	Altitude (m)	Brief description of ecotype*
Col-0 N1092	US	Columbia	1-100	Hairy leaves with serrate margins, H: 15-24cm
Ksk-1 N1634	UK	Keswick, UK	1-100	(no information)
Su-0 N1540	UK	Southport	1-100	Early leaves rounded, late leaves elongated, H: 24-32cm
Lan-0 N1304	UK	Lanack	100 -200	Height: 33-44cm, Large leaves, weakly hairy
Mt-0 N1384	Libya	Martuba/Acyrenaika	100 -200	Height: 16-26cm
Rsch-4 N1494	USSR	Rschew/Starize	100 -200	H: 34-42cm. Large rosette, single flowering stem, slightly serrated leaves
Ba-1 N952	UK	Blackmount	500 -600	H: 15-25cm. *Sinuate* leaves
Mc-0 N1362	UK	Mickles Fell	790	Bright green thin leaves with serrate margins. H: 28-38cm.
Can-0 N1064	Spain	Canary Islands	1260	H: 15-22cm. Rosette turns pink on maturing. Possibly late flowering

*H = height

Mean growth temperature was 22/18 °C day/night, with a photoperiod of 16:8 hr. night/day and relative humidity of 55%. Mean irradiance provided by PL-L 55W/840/4P ICT, and 60W tungsten filament at plant height was 200 µmol $m^{-2}s^{-1}$. The irradiance was checked every week with a light meter (Skye Instruments Ltd, UK). Two growth chambers were supplied with two CO_2 concentrations of 400 (ambient = ACO_2) and 800 (elevated = ECO_2) ppm. These concentrations correspond closely to the current CO_2 concentration in the world and the projected concentration at the end of the 21st century, respectively (Ziska, 2003). A second experiment was conducted with two chambers supplied with CO_2 of 400 and of 280 ppm (280 ppm being the sub-ambient (sub-ACO_2) the CO_2 concentration which existed at the beginning of the 20th century (Ziska, 2003)). When the seedlings had 2 to 4 leaves, which is between stages 1.02 and 1.04 (Boyes et al., 2001) they were transplanted into seedling trays. When the seedlings had produced 8 to 10 leaves (between 1.08 to 1.10 stages of growth) they were transplanted into pots of 8 cm diameter. Every week, the growing conditions and the plants were swapped between the chambers set at 400 and 800 ppm CO_2 to minimize chamber effects. The plants were also rotated within each cabinet. Pots were watered regularly from their base to maintain moist compost conditions at all times. The treatments were maintained over 24 hours for each growing season from germination to maturity.

Morphological traits

Leaf area was measured using the formula (length + width)/2. Measurements were taken when the plants were fully mature and had produced reproductive buds. Leaf length was measured with a ruler from the tip of the blade to the end of the petiole. The width was measured from the widest point of the leaf from margin to margin (maximum width of blade). Measurements were taken from three leaves *per* plant and five replicates *per* ecotype *per* treatment.

Epidermal and stomatal counts

When the plants reached reproductive stages (shown by an appearance of a reproductive bud), rosette leaves that were fully expanded were harvested to measure stomatal traits. Three leaves were obtained from five different plants *per* treatment. Leaf impressions were made with high definition dental putty (Coltene Whaledent, Altstatten, Switzerland) and colourless nail vanish, a method used by Lake and Woodward (2008). Stomata and epidermal cells were then counted on both abaxial (below) and adaxial (upper) leaf surfaces under a light microscope (Olympus CX40RF200) mounted with a 249 by 249 µ m grid, calibrated with a stage micrometre at a magnification of X400. All the stomata within the grid were counted, avoiding the midrib and the area of the leaf with large veins. Counts were taken from 5 different fields of view *per* leaf. The number of stomata was then converted to number of stomata/mm^2 to obtain the stomatal density. The same procedure was applied to epidermal cells. Replication was 5 plants *per* ecotype, three leaves *per* plant and five fields of view on each leaf *per* treatment. Leaf temperature was measured using an infrared thermometer (Fluke model, 63/66/68, PN 2149032 4519; www.fluke.com) from 3 to 5 plants *per* ecotype. Only seven ecotypes were available at the time of temperature measurements. The fastest growing ecotypes had lost most of their leaves when temperature measurements were conducted.

Statistical analysis

Two-way ANOVA was used to assess the effects of CO_2 and ecotypes on the different variables and interactions between ecotypes and treatments (main effects) using Minitab statistical program version 16 (Minitab Ltd, USA). Correlation analysis was performed to determine the relationship between altitude and the different plant traits

RESULTS

Epidermal parameters

Stomatal density increased with CO_2 in most ecotypes (Figure 1 and 2). The increase was significant in Col-0, Su-0 ($P \leq 0.01$) and Rsch-4 ($P \leq 0.05$) (on the abaxial leaf surface) and Col-0 and Rsch-4 ($P \leq 0.05$) (on the adaxial leaf surface). For ecotypes with reduced stomatal density in elevated CO_2, the reduction was only significant in Ksk-1 on both leaf surfaces ($P \leq 0.01$).

Under sub-ACO_2, ecotypes Su-0 and Rsch-4 had significantly increased stomatal density and Lan-0 and Ba-1 had significantly reduced stomatal density ($P \leq 0.05$) (abaxial leaf surface). Sub-ACO_2 did not significantly affect adaxial stomatal density for most ecotypes except Su-0 which had a significantly increased stomatal density ($P \leq 0.01$).

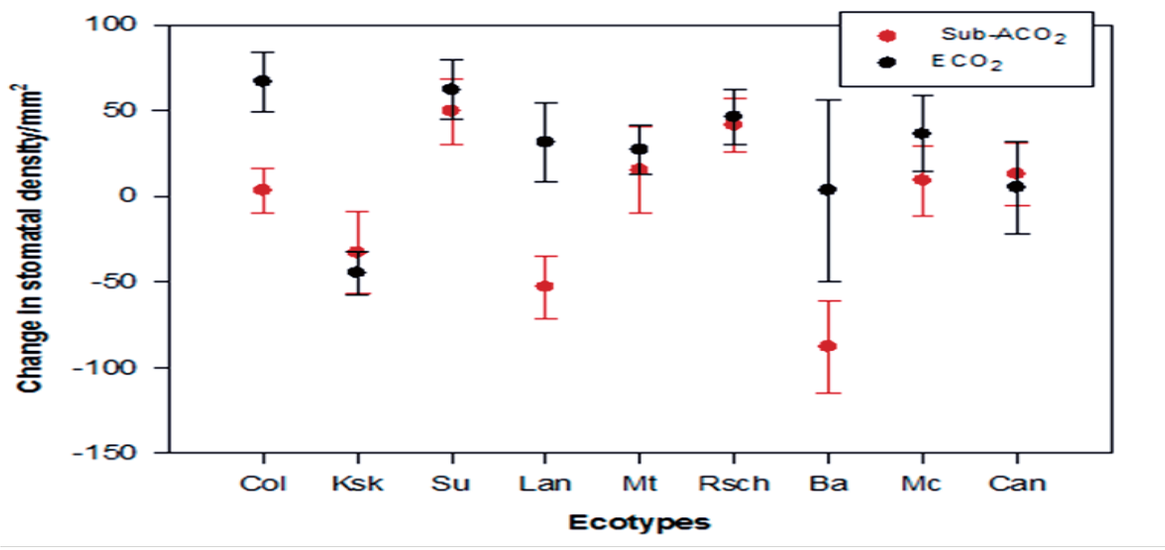

Fig. 1 Variation in abaxial stomatal density of ecotypes of *A.thaliana* grown under different CO_2 concentrations. Values = mean differences between ECO_2 and ACO_2, Sub-ACO_2 and ACO_2

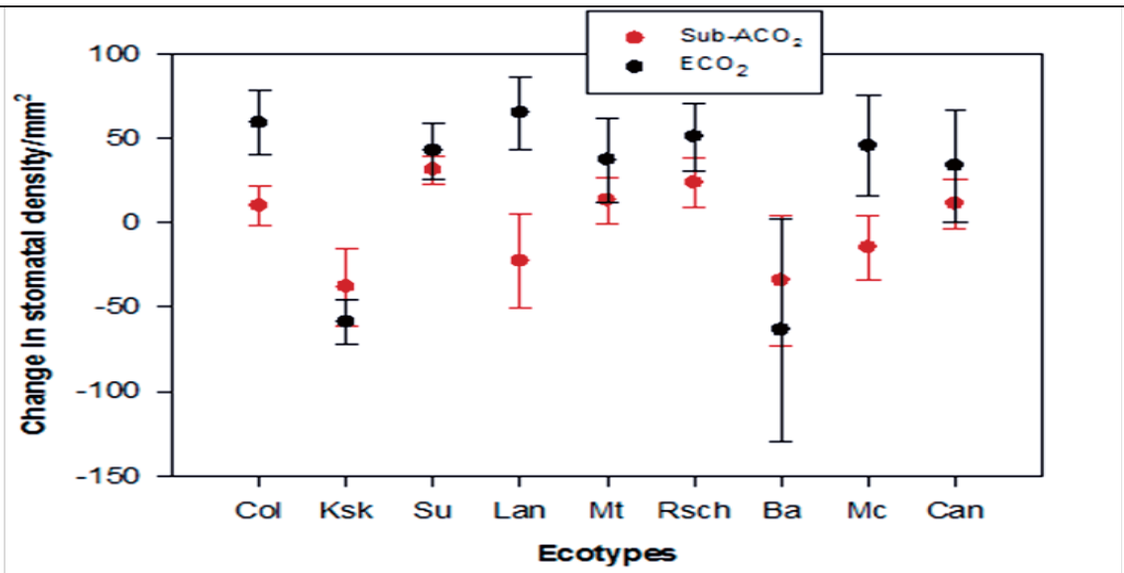

Fig. 2 Variation in adaxial stomatal density of ecotypes of *A.thaliana* grown under different CO_2 concentrations. Values = mean differences between ECO_2 and ACO_2, Sub-ACO_2 and ACO_2

The effect of increased CO_2 on the epidermal cell density mirrored that of the stomatal cell density (Figure 3 and 4) but only Su-0 ($P \leq 0.001$) and Rsch-4 ($P \leq 0.01$) (abaxial leaf surface) and Su-0 ($P \leq 0.01$) (adaxial leaf surface) had significant increases. Ksk-1 also had significantly reduced epidermal cell density on both leaf surfaces, ($P \leq 0.01$ abaxial) and ($P \leq 0.001$ adaxial).

Sub-ACO_2 significantly increased epidermal cell density in Rsch-4 ($P \leq 0.05$) and decreased density in Ba-1 ($P \leq 0.05$) on the abaxial surface of the leaf (Figure 3 and 4). Sub-ACO_2 had no significant effect on the adaxial leaf surface for all ecotypes. Altitude of origin showed no correlation with stomatal density and epidermal cell density in all treatments ($P > 0.05$). Leaf temperature was measured in addition to the above leaf parameters. Leaf temperature was found to be significantly lower ($P > 0.01$) under ECO_2 (mean of 20.63 ± 0.01^0C) compared to under ACO_2 (mean of 21.62 ± 1.40^0C) and sub-ACO_2 (mean of 22.05 ± 3.02^0C).

Leaf area responses

The leaf area for most ecotypes was not significantly affected by CO_2 variation (Figure 5). The trend was toward decreased leaf area for most ecotypes. Su-0 and Can-0 had significantly smaller leaves ($P \leq 0.01$) under CO_2 enrichment. The leaf area decreased significantly for Mt-0 $P \leq 0.05$) and Ba-1 ($P \leq 0.01$), Col-0 ($P \leq 0.05$) and Ksk-1 ($p \leq 0.01$) had significantly large leaves under sub-ACO_2.

DISCUSSION

The increased stomatal and epidermal cell densities in this study differ from the general trend observed for inter-specific responses showing a reduction of density with CO_2 enrichment (Woodward and Kelly, 1995). Woodward *et al*. (2002) reported an 11% mean reduction in stomatal density in response to elevated CO_2 for 48 accessions of *A. thaliana*. There are studies that have shown inconsistent responses with this general trend. For example, Lake and Woodward (2008) found that some accessions of *A. thaliana* showed no response to CO_2 while some showed increased and decreased stomatal density with CO_2 enrichment. Sekiya and Yano (2008) also reported an increase in stomatal density for cowpeas varieties. Driscoll *et al*. (2006) found no changes in stomatal density for maize varieties with elevated CO_2

Royer (2001) in a compressive review pointed out that situations where stomatal density increase with CO_2 enrichment are uncommon compared to where these variables decrease or show no change. The review (Royer, 2001) identified an inverse relationship between CO_2 and stomatal density in 50% of the literature reviewed. In the present study, although the direction of stomatal density changes under ECO_2 was similar for most ecotypes the magnitude of change differed between ecotypes. For example the range of change from ACO_2 to ECO_2 was from 2 to 33% on the abaxial surface of the leaf and from 13 to 37% on adaxial leaf surface, suggesting that the adaxial surface was more responsive to change in CO_2

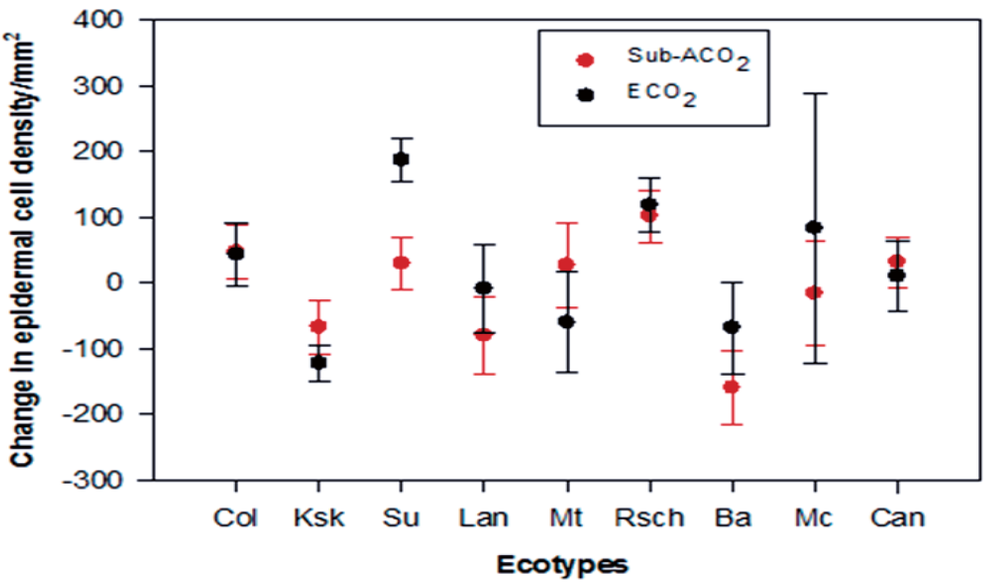

Fig. 3 Variation in abaxial epidermal density of ecotypes of *A.thaliana* grown under different CO_2 concentrations. Values = mean differences between ECO_2 and ACO_2, Sub-ACO_2 and ACO_2

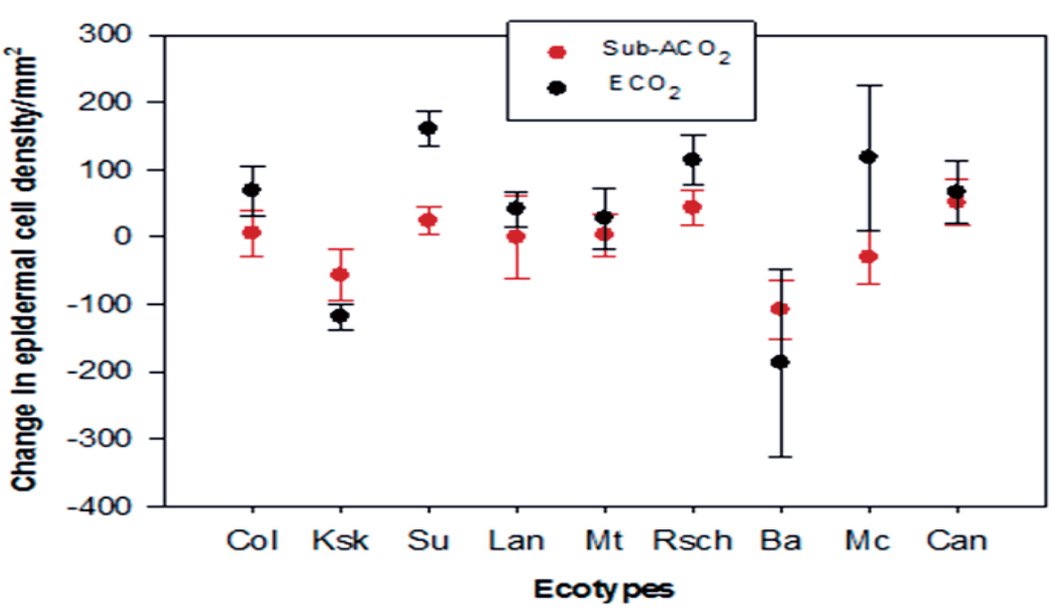

Fig. 4 Variation in adaxial epidermal density of ecotypes of *A.thaliana* grown under different CO_2 concentrations. Values = mean differences between ECO_2 and ACO_2, Sub-ACO_2 and ACO_2

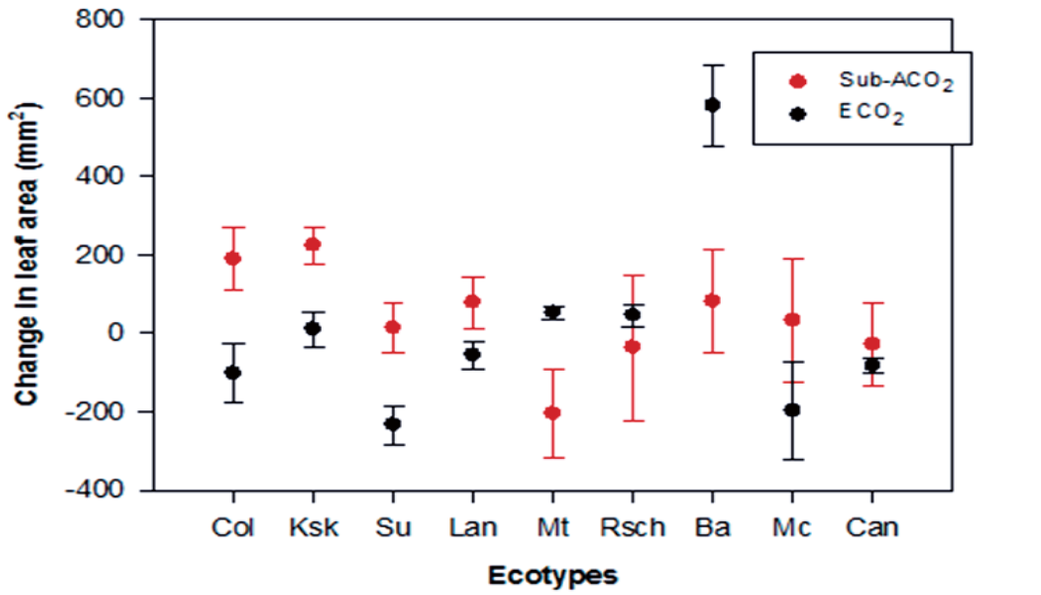

Fig. 5 Variation in leaf area of ecotypes of *A.thaliana* grown in different CO_2 concentrations. Values = mean differences between $ECO_2 - ACO_2$ and Sub-ACO_2 – ACO_2 and standard error difference.

Generally for most ecotypes the response to sub-ACO_2 was not significant. This response contradicts the expected trend in which stomatal density and epidermal cell density increase under low CO_2 concentrations as reported by Woodward and Bazzaz (1988). However, Rowland-Bamford *et al*. (1990) reported a decrease of stomatal density under low CO_2 concentration for rice. Results shown here agree with Royer (2001) conclusions that in many experiments there might be no responses to change in CO_2 concentration. The author (Royer 2001) suggested that an increase in these traits with a decline in CO_2 was a common occurrence on sub-fossil plants as these plants were exposed for a longer period of time. Therefore young plants (in evolutionary time scale) such as those used in the present experiment would show no change since they have not been exposed for a long time. These results showed that the direction and magnitude of responses of plants to changing climate conditions might not be easy to predict from experiments performed within growth chambers. Also the abrupt increases in CO_2 concentrations in growth chambers as opposed to gradual increase in CO_2 that occur in nature might cause different responses in plant species.

Changes in stomatal density and epidermal cell density in response to different CO_2 concentrations did not show any significant relationship with altitude of origin. This indicates genetic constraint on how much the leaf anatomy can change. High altitude ecotypes are hypothesized to have higher stomatal density and epidermal cell density than of low altitude ecotypes. High stomatal density in high altitude suggests a need for greater acquisition of carbon to enable plants to survive under low partial pressure of CO_2 at such high altitudes. Haworth *et al.* (2010) reported no significant correlation between stomatal density with altitude for conifer species. Stenglein *et a*l. (2005) also reported an inverse relationship between stomatal density with altitude for accessions of *Phaseolus vulgaris* var. aborigineous. As yet there is no comprehensive evidence as to why these ecotypes responded in this way. In order to find an explanation for these stomatal responses leaf temperature was measured. The mean leaf temperature was found to be lower under ECO_2 compared to under ACO_2 and sub-ACO_2. This suggests that transpiration might have been higher; causing increased evaporative cooling of leaves and hence reduced leaf temperature in ECO_2. Therefore increased stomatal density could be a result of increased transpiration under conditions in the growth chambers. This is consistent with Waldron and Terry (1987) who found that as transpiration in *Beta vulgaris* increased the leaf temperature declined steadily. The present results also agree with Mott and Parkhurst (1991) explanation that stomatal density might increase as a result of increased transpiration. Lake and Woodward (2008) in their studies of *A. thaliana* accessions also reported increased stomatal density with transpiration rates for Col-0 and they concluded that the transpiration rate of the whole plant could be one of the factors that might drive a change in the stomatal density of developing leaves. This current study also showed a decrease in leaf size which might suggest

reduced leaf expansion. However, CO_2 enrichment is reported to promote leaf expansion and cell division (Bolin *et al*., 1989 and Riikonen *et a*l., 2010). In the current study leaves were harvested for stomatal analysis as soon as the plants showed a reproductive bud. Since most of the plants growing under ECO_2 reached the reproductive stages earlier, the length of time required for full leaf expansion might have been reduced. Various authors report different responses of leaf area to CO_2 enrichment, from no change in *Betula papyrifera* (Riikonen *et al.,* 2010), to increases in rice cultivars (Uprety *et al*., 2002), and *Sorghum bicolor* (Bolin *et al*., 1989). The high density of stomata under ECO_2 in the present study could also be attributed to the lack of expansion of these leaves. Uprety *et al*. (2002) explained that an increase in leaf size will generally affects the stomatal density by differential spreading of stomatal. The plants grown under elevated CO_2 were bushier than in ambient conditions, which could be attributed to increase in the number of leaves. Perhaps the total leaf area *per* plant rather than area *per* leaf would have been a better measurement to evaluate leaf area responses to changing CO_2 concentration. CO_2 enrichment is reported to increase the total leaf area of plants (Eamus, 1991). Radoglou and Jarvis (1990) observed an increase in the total leaf area *per* plant for the four clones of poplar they studied. They explained that the increase in leaf area was brought about by increase in leaf number rather than the leaf size. Garbutt *et a*l. (1990) reported a decline in leaf area under elevated conditions for some annual plants. Leaves of plants grown under ambient and sub-ambient conditions did not differ significantly in leaf size. These results correspond with those found by Hovenden and Schimanski (2000) in their studies of genotypes of *Nothofagus cunninghamii*. This lack of response of leaf area might explain the absence of stomatal responses to sub-ACO_2 in the present study.

Although the ecotypes varied significantly between each other in the sizes of their leaf, the variation did not correlate significantly with altitude of origin. This corresponds to Hovenden and Vander Schoor (2003) pilot study with cuttings of *Nothofagus* from different altitudes. The authors (Hovenden and Vander 2003) found that altitude of origin did not have any influence on leaf area or size when the cuttings were grown in a glass house.

CONCLUSION

This study showed a trend in increased stomatal density and epidermal cell density with CO_2 enrichment. This response to elevated CO_2 seemed to have been influenced by other conditions like plant transpiration. It is recommended that any future research conducted should be accompanied by a close monitoring of transpiration rates of the plants. Sub-ACO_2 exerted no significant effects on most of the leaf parameters.This study also showed no correlation between altitude of origin and the responses of stomatal density, epidermal cell density and leaf area, implying that altitudinal responses might not be universal in plants. These results indicate that it will be inappropriate to generalize plant responses to future carbon dioxide changes using particular plants. Or use present plant genera to learn about past CO_2 conditions. However, the knowledge of responses of this model plant, *A*. *thaliana* might be useful for directing further studies for plants of potential evolutionary patterns related to CO_2 changes.

Acknowledgements The Botswana College of Agriculture is acknowledged for providing funding for the study.

Conflict of interest None

REFERENCES:

Alonso-Blanco, C. and Koornneef, M. (2000) Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. *Trends in Plant Science* 5:22-29.

Bolin, B. Doos, B.O.R., Jager, J. and Warrick, R. A. (1989) The greenhouse effect, climatic change and ecosystems (Scope 29). New York: John Wiley and sons.

Boyes, D. C., Zayed, A. M. and Ascenz, R. McCaskill, A. J., Hoffman, N. E. , Davis, K. R. and Gorlach, J. (2001) Growth stage- based phenotypic analysis of *Arabidopsis*: A model for high throughput functional genomics in plants. *Plant Cell 13:1499-1510.*

Casson, S. and Gray, J. E. (2008) Influence of environmental factors on stomatal development. *New Phytologist* 178:9-23.

Driscoll, S. P., Prins, A., Olmos. E., Kunert, K. J. and Foyer, C. H. (2006) Specification of adaxial and abaxial stomata, epidermal structure and photosynthesis to CO_2 enrichment in maize leaves. *Journal of Experimental Botany* 57:381-390.

Eamus, D. (1991) The interaction of rising CO_2 and temperature with water use efficiency. *Plant, Cell & Environment* 14:843-852.

Garbutt, K., Williams, W. E. and Bazzaz, F. A. (1990) Analysis of the differential response of five annuals to elevated CO_2 during growth. *Ecology* 71:1185–1194.

Haworth, M., Heath, J. and McElwain, J. C. (2010) Differences in the response sensitivity of stomatal index to atmospheric CO_2 among four genera of Cupressaceae conifers. *Annals of Botany* 105:411-418.

Hetherington, A. M. and Woodward, F. I. (2003) The role of stomata in sensing and driving environmental change. *Nature* 424:901–908.

Hovenden M J, Schimanski L. J. (2000) Genotypic differences in growth and stomatal morphology of southern Beech, *Nothofagus cunninghamii*, exposed to depleted CO_2 concentration. *Australian Journal of Plant Physiol*ogy, 27:281-287.

Hovenden, M. J. and Vander Schoor, J. K. (2003) Nature vs nurture in the leaf morphology of southern beech. *Nothofagus cunninghamii* (Nothofagaceae). *New Phytologist* 161:585-594.

Hovenden, M. J. and Vander Schoor, J. K. (2005) The response of leaf morphology to irradiance

depends on altitude of origin in *Nothofagus cunninghamii*. *New Phytologist* 169:291-297.

Korner, C. H. and Diemer, M. (1994) Evidence that plants from high altitude retains their greater photosynthetic efficiency under elevated CO_2. *Functional Ecology* 8:58-68.

Kudoyarova, G. R., Veselov, D. S., Faizov, R. G., Veselova, S. V., Ivanov, E.A. and Farkhutdinov, R. G. (2007) Stomata responses to changes in temperature and humidity in wheat cultivars grown under contrasting climatic conditions. *Russian Journal of Plant Physiology* 54:46–49.

Lake, J. A. and Woodward, F. I. (2008) Response of stomatal numbers to CO_2 and humidity: control by transpiration rate and abscisic acid. *New Phytologist* 179: 397-404.

Miller-Rushing, A. J. and Inouye, D. W. (2009) Variation in the impact of climate change on flowering phenology and abundance: An examination of two pairs of closely related wildflower species. *American Journal of Botany* 96: 1821-1829.

Mott, K. A. and Parkhurst, D. F. (1991) Stomatal responses to humidity in air and helox. *Plant, Cell & Environment*.14:509–515.

Radoglou K. M. and Jarvis, P. G. (1990) Effects of CO_2 enrichment on four poplar clones. 1. Growth and leaf anatomy. *Annals of Botany* 65:617-626.

Riikonen, J., Percy, K. E., Kivimäenpää, M., Kubiske, M. K., Nelson, N. D., Vapaavuori and Karnosky, D. F. (2010) Leaf size and surface characteristics of *Betula papyrifera* exposed to elevated CO_2 and O_3. *Environ. Pollution* 158:1029-1035.

Raven, P. H., Evert, R. F. and Eichhorn, S. E. (1999) Biology of plants. W. H. Freeman and company. Worth Publishers, NY, USA.

Rowland-Bamford A. J., Nordenbrock, C., Baker, J. T., Bowes G., Hartwell, L. Allen, J. R. (1990) Changes in stomatal density in rice grown under various CO_2 regimes with natural solar irradiance. *Environment and Experimental Botany* 30:175–180.

Royer, D. L. (2001) Stomatal density and stomatal index as indicators of paleoatmospheric **CO_2** concentration. *Review of Palaeobotany and Palynology* 114:1–28.

Sekiya, N. and Yano K. (2008) Stomatal density of cowpea correlates with carbon isotope discrimination in different phosphorus, water and CO_2 environments. *New Phytologist* 179:799–807.

Stenglein S. A., Arambarri, A. M., Sellivano, M. M. and Balatti, P. A. (2005) Leaf epidermal characters related with plant's passive resistance to pathogens vary among accessions of wild bean *Phaseolus vulgaris* var. Aborigineus (Leguminosae- Phaseoleae). *Flora* 200:285–295.

Waldron, L. J. and Terry, N. (1987) The influence of atmospheric humidity on leaf expansion in *Beta vulgaris* L. *Planta* 170:336–342.

Ward, J. K. and Kelly, J. K. (2004) Scaling up evolutionary responses to elevated CO_2: lessons from *Arabidopsis*. *Ecology letters,* 7:427-440.

Ward, J. K. and Strain, B. R. (1997) Effects of low and elevated CO_2 partial pressure on grown and reproduction of *Arabidopsis thaliana* from different elevations. *Plant, Cell & Environment* 20:254-260.

Woodward, I. F. (1986) Ecophysiological studies on the shrub *Vaccinium myrtillus* taken from a wide altitudinal range. *Oecologia:* 70:580-586.

Woodward, F. I. and Bazzaz, F. A. (1988) The response of stomatal density to CO_2 partial pressure. *Journal Experimental Botany* 39:1771-1781.

Woodward, F. I. and Kelly, C. K. (1995) The influence of CO_2 concentration on stomatal **density**. *New Phytologist* 13:311-327.

Woodward, F. I., Lake, J. A. and Quick, W. P. (2002) Stomatal development and CO_2: ecological consequences. *New Phytologist* 153:477–484.

Uprety, D. C., Dwivedi, N., Jain, V. and Mohan, R. (2002) Effects of elevated CO_2 concentration on the stomatal parameters of rice cultivars. *Photosynthetica* 40:315–319.

Ziska, L.H. (2003) Evaluation of the growth response of six invasive species to past, present and future atmospheric carbon dioxide. *Journal of Experimental Botany* 54:395–4049.