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Reduced plant water status under sub-ambient pCO_2 limits plant productivity in the wild progenitors of C_3 and C_4 cereals

Jennifer Cunniff[1*], Michael Charles[2], Glynis Jones[3], and Colin P. Osborne[1]

[1]Department of Animal and Plant Sciences, Alfred Denny Building, University of Sheffield, Western Bank, Sheffield, S10 2TN, UK. [2]Institute of Archaeology 36 Beaumont St, Oxford, OX1 2PG, [3]Department of Archaeology, Northgate House, University of Sheffield, West Street, Sheffield, S1 4ET, UK.

Corresponding author: Jennifer Cunniff

[*]Current address: CABI, Nosworthy Way, Wallingford, Oxfordshire, OX10 8DE, UK.

Tel: +44 (0)1491 832111

j.cunniff@cabi.org

Running title: sub-ambient pCO_2 and plant water status

ABSTRACT

- **Background and Aims** The reduction of plant productivity by low atmospheric CO_2 partial pressure (pCO_2) during the last glacial period is proposed as a limiting factor for the establishment of agriculture. Supporting this hypothesis, previous work has shown that glacial pCO_2 limits biomass in the wild progenitors of C_3 and C_4 founder crops, in part due to the direct effects of glacial pCO_2 on photosynthesis. Here, we investigate the indirect role of pCO_2 mediated via water status, hypothesising that faster soil water depletion at glacial (18 Pa) compared to post-glacial (27 Pa) pCO_2 due to greater stomatal conductance feeds back to limit photosynthesis during drying cycles.
- **Methods** We grew four wild progenitors of C_3 and C_4 crops at glacial and post-glacial pCO_2 and investigated physiological changes in gas exchange, canopy transpiration, soil water content, and water potential between regular watering events. Growth parameters including leaf area were measured.
- **Results** Initial transpiration rates were higher at glacial pCO_2 due to greater stomatal conductance. However, stomatal conductance declined more rapidly over the soil drying cycle in glacial pCO_2 and was associated with decreased intercellular pCO_2 and lower photosynthesis. Soil water content was similar between pCO_2 levels as larger leaf areas at post-glacial pCO_2 offset the slower depletion of water. Instead the feedback could be linked to reduced plant water status. Particularly in the C_4 plants, soil-leaf water potential gradients were greater at 18 Pa compared with 27 Pa pCO_2 suggesting an increased ratio of leaf evaporative demand to supply.
- **Conclusions** Reduced plant water status appeared to cause a negative feedback on stomatal aperture in plants at glacial pCO_2, thereby reducing photosynthesis. The effects were stronger in C_4 species, providing a mechanism for reduced biomass at 18 Pa. These results have added significance when set against the drier climate of the glacial period.

Key words: Setaria viridis, Panicum miliaceum var. ruderale, Hordeum spontaneum, Triticum boeoticum, sub-ambient pCO_2, origin of agriculture, C_4 photosynthesis, C_3 photosynthesis, water relations, crop progenitors.

INTRODUCTION

Deglaciation at the end of the Pleistocene period was coupled to a rapid rise in atmospheric pCO_2 from below 18 Pa to 27 Pa between 15,000 and 12,000 years ago (Petit et al., 1999, Jouzel et al., 1993). Humans began to cultivate plants in the Fertile Crescent of western Asia soon afterwards (Willcox et al., 2008) and, within five millennia, cultivation and subsequent domestication of plants had occurred in at least five primary centres across the globe (Purugganan and Fuller, 2009). This sequence of agricultural origins in widely separated regions of the world suggests the involvement of a global factor, and Sage (1995) proposed that pCO_2 during the last glacial period may have been too low to support the level of productivity required for the successful establishment of agriculture.

An experimental test of this hypothesis showed that the wild progenitors of C_3 and C_4 founder cereals from independent centres of origin displayed significant increases in vegetative biomass with a 50% increase in pCO_2 from 18 Pa to 27 Pa (2010, Cunniff et al., 2008). Biomass of the one C_3 species in this experiment near-doubled and measurements of leaf gas exchange revealed that photosynthesis (A) was strongly limited by glacial pCO_2 in this species, highlighting a direct mechanism for biomass limitation. Biomass in the five C_4 species showed a smaller, but still substantial, 40% increase (Cunniff et al., 2008). However, measurements of leaf gas exchange immediately after watering revealed that A was only significantly limited by glacial pCO_2 in two of these C_4 crop progenitors, a finding consistent with the known CO_2-concentrating mechanism of C_4 photosynthesis (Pearcy and Ehleringer, 1984). Therefore an alternative, indirect explanation for the biomass increase is required.

The elevation of pCO_2 from a glacial to postglacial level in this experiment was associated with large reductions in stomatal conductance (g_s) and transpiration (E), resulting in a decrease in the use of water at leaf and canopy scales in all of the C_4 species, and greatly improved water-use efficiency (WUE). These observations raise the possibility of an indirect

feedback on biomass accumulation at sub-ambient pCO_2 mediated via water relations because, although plants were not exposed to drought per se, they experienced a soil drying cycle between watering events on alternate days (Cunniff et al., 2008). This hypothesised mechanism is consistent with studies investigating the effects of elevated atmospheric pCO_2 (55-70 Pa) on the water relations of C_4 plants, where reduced g_s and E improve plant and soil water status, and extend the active period for photosynthesis and growth (Ghannoum et al., 2000). This indirect effect of elevated pCO_2 is particularly important when C_4 plants experience some kind of water deficit (Wall et al., 2001, Conley et al., 2001, Leakey et al., 2004, Leakey et al., 2006, Leakey, 2009). It seems likely that these feedbacks of water status on stomatal conductance, and hence photosynthesis, should be greater at sub-ambient than elevated pCO_2. This is because the response of A to intercellular pCO_2 (P_i) in C_4 plants is typically saturated at ambient pCO_2 levels, but shows a steep, near-linear response to pCO_2 at lower P_i (Pearcy and Ehleringer, 1984). Therefore, decreases in g_s under water deficits could lead to substantial reductions in A. This effect has been recognised in studies using maize grown at ambient and elevated pCO_2 (Samarakoon and Gifford, 1995, Samarakoon and Gifford, 1996).

It has been demonstrated previously that WUE improves linearly with CO_2 across sub-ambient pCO_2 gradients in both C_3 and C_4 species (Polley et al., 1993, Polley et al., 2002, Anderson et al., 2001, Polley et al., 1996). However, few studies have investigated the interacting effects of water limitation and sub-ambient pCO_2. Polley et al. (2002) showed that mid-day xylem potentials were depressed by sub-ambient pCO_2 in C_3 and C_4 species during naturally occurring seasonal droughts. Furthermore Ward et al. (1999) found that A was limited in the C_4 annual *Amaranthus retroflexus* by sub-ambient pCO_2 and, when a drought treatment was applied experimentally, the CO_2 limitation was stronger. Additionally, these plants showed the least recovery of leaf area and biomass upon re-watering at the end of the

drought period. These responses at sub-ambient pCO_2 were similar to those of C_3 annual Abutilon theophrasti and showed that C_3 and C_4 species were equally affected by drought at sub-ambient pCO_2, which is predicted from other studies (Polley et al., 1993, Polley et al., 1995).

Here we report a controlled environment experiment investigating the interacting effects of sub-ambient pCO_2, plant and soil water status on the modern day representatives of C_3 and C_4 crop progenitors. We focus on two C_3 cereals from the Fertile Crescent and two C_4 millets from the Loess Plateau in China, all of which were among the earliest wild plants to be brought into cultivation. Our experiment tested three hypotheses: (i) Greater g_s at glacial pCO_2 increases the rate of soil water depletion and decreases leaf and plant water status compared with plants at post-glacial pCO_2, (ii) Faster soil water depletion and reduced plant water status increase the rate at which g_s and A decline in plants grown at glacial compared with post-glacial pCO_2, and (iii) Water deficits amplify the limiting effect of glacial pCO_2 on A to an equal extent in C_4 and C_3 crop progenitors. The aim was not to subject the crop progenitors to a drought treatment; rather, it was an investigation of how physiology changes between watering events in a controlled environment experiment.

MATERIALS AND METHODS

Growth conditions and plant material

The pCO_2 treatments were applied in controlled environment chambers (Conviron BDR16, Conviron, Winnipeg, Manitoba, Canada) at two levels throughout the full 24 days of plant growth: glacial (18 Pa) and postglacial (27 Pa). pCO_2 levels were maintained continuously throughout the day and night. The chambers were operated in a closed configuration, by connecting the outlet vent to the air inlet via a filter packed with a layer of activated charcoal and a layer of sodalime (Sofnolime 1.0 - 2.5mm granules, Molecular Products Ltd, Mill End,

Essex); activated charcoal was employed to filter the air and remove any trace gases which could be emitted by plants or soil, and have the potential to affect plant development. The pCO_2 level in each chamber was measured using a CO_2 sensor (CARBOCAP® Carbon Dioxide Probe GMP343, Vaisala, Finland) calibrated against a secondary standard. CO_2 control was achieved by linking the sensor to a feedback system regulating the circulation of chamber air through the soda-lime scrubber. The pCO_2 of air in the chambers was recorded every minute, giving overall mean values over the full growth period of 18.2 Pa (±SD 0.48) and 27.1 Pa (± SD 0.49). To maintain this tight control, the soda lime was changed as soon as pCO_2 started to drift above the target level, which was approximately every four weeks. Treatment and plants were exchanged between the two controlled environment chambers every week from germination to avoid confounding the effects of chamber and growth environment.

Seeds were obtained from germplasm holdings or commercial sources. They included two C_4 species, Setaria viridis (L.) P. Beauv [(green foxtail millet) (Herbiseed, Twyford, UK. Cat no. 9602)] and Panicum miliaceum var. ruderale (Kitag.) [(wild broomcorn millet) (Herbiseed, Cat no. 9507)] from North China, and two C_3 species, Hordeum spontaneum K. Koch [(wild barley) (Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, Accession number: HOR 13791)] and Triticum boeoticum Boiss. [(einkorn wheat) (IPK, Accession number: TRI 17111)] from western Asia. Batches of 20 seeds of each species were sown into trays containing a 1:1 sand: John Innes no. 2 compost mix (7 parts loam, 3 parts peat 2 parts sand N:P:K 20:10:10). This mix was chosen in an attempt to replicate an unimproved soil (Ivandic et al., 2000). Seeds were germinated under pCO_2 treatments in the controlled environment chambers at 25/15°C (day/night), with an 8 hour photoperiod and photosynthetic photon flux density (PPFD) of 300 μmol photons $m^{-2}s^{-1}$. Once established, eight even-sized seedlings of each species were selected and planted into 32

individual 2-litre pots [13.2cm (height) × 17cm (diameter)], containing the same growth media. The pots had 8 holes in the base and were free to drain. Seedlings were returned to the same controlled environment chambers; and in both chambers temperature was maintained at 25/15°C (day/night), photoperiod was increased to 14 hours with a maximum PPFD of 600 μmol $m^{-2}s^{-1}$, and vapour pressure deficit (VPD) had a minimum value of 0.49 kPa during the night and a maximum value of 0.96 kPa during the day.

Physiology

Physiological measurements of gas exchange, canopy transpiration and gravimetric water content, and leaf water potential were taken over three separate drying cycles of three day duration: photosynthesis (14 DAP-16 DAP), canopy transpiration and gravimetric water content (17 DAP – 19 DAP), and water potential (20 DAP – 22 DAP). In total, physiological measurements occurred over 9 days (14 DAP – 22 DAP). For each drying cycle, soil in the pots was watered to pot capacity before "dawn" (7am) on day 1 (D1), and left to dry down over a three-day cycle (D1-D3) before re-watering at the end of D3. We used a three-day watering cycle as opposed to the two-day cycle applied in our previous experiment (Cunniff et al., 2008) to exaggerate the hypothesized CO_2 × water interaction.

Gas exchange measurements

CO_2 and H_2O exchange were measured using a portable open gas exchange system (LI-6400P, LI-COR Biosciences, Lincoln, Nebraska, USA) with the 6400-02B LED Light Source chamber. Chamber conditions were set with the aim of approximating the growth environment: PPFD was 600 μmol $m^{-2}s^{-1}$, leaf temperature was 25°C, incoming air was maintained at a constant humidity to keep the leaf-air VPD at less than 1 kPa throughout all measurements, and pCO_2 was set at 18 or 27 Pa as appropriate.

Plants were watered to pot capacity before "dawn" on D1 (14 DAP), allowed to drain, and returned to the growth cabinets. Gas exchange was measured at nine time points: the beginning (8am), midpoint (2pm) and end of the day (8pm) from D1 to D3 (16 DAP). At each time point, measurements were commenced in a different cabinet, and were alternated between the two cabinets within each time point. This avoided confounding time with CO_2 treatment, within the approximately 90 min that it took to complete all measurements. On each occasion, the same, most recently expanded leaf was clamped in the leaf chamber and allowed to equilibrate with chamber conditions for ~90s, before measuring leaf gas exchange. The aim of these measurements was to obtain "snapshots" of photosynthesis (A), stomatal conductance (g_s) and intercellular pCO_2 (P_i) under growth conditions, and these were calculated using the equations of von Caemmerer and Farquhar (1981).

Transpiration and gravimetric soil water content

Transpiration and gravimetric soil water content (θ_g) were determined via lysimetry. Plants were watered to pot capacity before "dawn" on D1 17 DAP, allowed to drain, and then weighed (g). Pots were then returned to the controlled environment chambers and reweighed (g) at each of the nine time points detailed for gas exchange measurements. Alongside the plants, empty pots containing only the growth media were placed in the controlled environment chambers to estimate soil evaporation. These were treated in the same way as the plants, being weighed at saturation and the nine measurement occasions. At the end of D3 19 DAP, leaf area per plant was measured non-destructively using a ruler. Length × maximum width was measured for each leaf and used to estimate the total canopy area using an allometric relationship established before the experiment **[Supplementary Information - Table S1]**. The daily canopy transpiration of each plant (E_{plant}) was determined as:

$$E_{\text{plant}} = \frac{(\text{pot} + \text{plant weight}) - \text{empty pot weight}}{18}$$

(Equation 1),

where 18 is the molar mass of water. Similarly, the instantaneous rate of leaf transpiration (E_{leaf}) was calculated as:

$$E_{\text{leaf}} = \frac{\left(E_{\text{plant}} \div 50400\right)}{\text{Leaf area}} \times 1000$$

(Equation 2),

where 50400 is the number of seconds during the light period of 14 hours, and 1000 is the conversion from moles to mmoles.

To calculate θ_g, a core of known size was removed from the pots at the end of D3 and this was oven dried at 100°C over 72 hours to a constant weight and the total dry weight of soil per pot was determined. The θ_g for each of the nine points was then calculated as:

$$\theta_g = \frac{M_{wet} - M_{dry}}{M_{dry}}$$

(Equation 3),

where M_{wet} is the soil fresh weight (g) at each time point, and M_{dry} is the soil dry weight, oven-dried at 100°C over 72 hours to a constant weight.

Leaf water potential

Leaf water potential (Ψ_{leaf}) was measured on detached leaves using a Scholander pressure chamber (Model 1000 Pressure Chamber, PMS Instruments, Corvallis, OR, USA), immediately following lysimetry measurements. Plants were watered to pot capacity before "dawn" on D1, 20 DAP, allowed to drain, and returned to the growth cabinets. Ψ_{leaf} was then measured at the end of D2 (8 pm) and before "dawn" (pre-dawn) on D3, 23 DAP. In the controlled environment chambers, conditions remained constant throughout the day, and we therefore expected the maximum plant water deficit to occur towards the end of the day. Pre-dawn Ψ_{leaf} measurements were used to provide an estimate of soil water potential as the plants

returned to equilibrium with soil water during the dark (Tardieu and Simonneau, 1998). Only two water potential measurements were taken, due to the destructive nature of the technique, and small size of the plants (20-40 leaves).

Biomass

Total biomass was determined by a destructive harvest immediately following the completion of water potential measurements (24 DAP). Plants were divided into roots and shoots, washed clean of the growth medium, dried at 70°C for 7 days, and weighed.

Experimental design and statistical analysis

Four species were used for the experiment, with four replicates at each pCO_2 treatment. Statistical analysis was carried out using the computing package R (version 3.0.1, The R Foundation for Statistical Computing), with P = 0.05 as the critical level of significance. Throughout the analysis the species were treated separately. ANOVA (aov) with repeated measure factors was used to analyse the data. For the parameters A, g_s, θ_g and $P_{i,}$ a three factor mixed design (pCO_2, time and day) with repeated measures on time and day, was used, and for the parameters E_{leaf} and Ψ_{leaf} a two factor design (pCO_2 and day) with repeated measures on day, was employed. The estimable function [(library(gmodels))] was used to apply a contrast matrix to the data, by computing a significance value between the levels of pCO_2 at each time point. The data collected at the final harvest were evaluated using Student's t-test to investigate the effect of pCO_2 on biomass and partitioning.

RESULTS

Stomatal conductance and photosynthesis

At the midpoint of D1 in P. miliaceum, g_s was 31% lower under post-glacial pCO_2 than glacial pCO_2 (Fig. 1A). This difference in g_s between pCO_2 levels was maintained for D2, but then declined in glacial pCO_2 and, by the midpoint of D3, was not significantly different from the value in post-glacial pCO_2 (Fig. 1A). This strong decline in g_s under 18 Pa pCO_2 is supported by a significant interaction between pCO_2 and day ($F_{2,62}$ = 8.8, <.001). Furthermore, g_s showed a steeper decline at the end of each day, at the 'pm' measurements, where, on all days, there was no significant difference in g_s between pCO_2 levels **[Fig. S1 – Supplementary information]**

Photosynthesis was impacted directly by the growth pCO_2 in P. miliaceum ($F_{1,64}$ = 59.8, <.001) but also indirectly via the faster decline in g_s at 18 Pa, which fed back to limit A (Fig. 1B). At the midpoint of D1, A was equal between the pCO_2 levels. However, by D2 A differed, and by the midpoint of D3 A was 46% greater at post-glacial than at glacial pCO_2 (Fig. 1B). This change led to a significant interaction between pCO_2 and day ($F_{1,64}$ = 5.7, <.001). P_i appeared to track g_s, decreasing from D1 to D3 at 18 Pa (Fig. 2A, $F_{2,66}$ = 3.9, <.05), with a minimum P_i always being reached at 'pm' on each day **[Fig. S2 – Supplementary information]**. This decline in P_i is not significantly greater in the glacial than post-glacial treatment, but P_i is lower overall at glacial pCO_2 ($F_{1,66}$ = 89.7, <.001), meaning that the same decline in P_i is more limiting for A in this environment than in plants grown at post-glacial pCO_2.

Values of g_s in the second C_4 species, S. viridis, showed a very similar pattern to those of P. miliaceum (Fig. 1C). At the midpoint of D1, g_s was lower at 27 Pa in comparison to 18 Pa pCO_2. However, g_s declined by a greater extent at glacial pCO_2 ($F_{2,64}$ = 6.6, <.01), and by D3 g_s was not significantly different between the two levels of pCO_2 (Fig.1C). Higher pCO_2

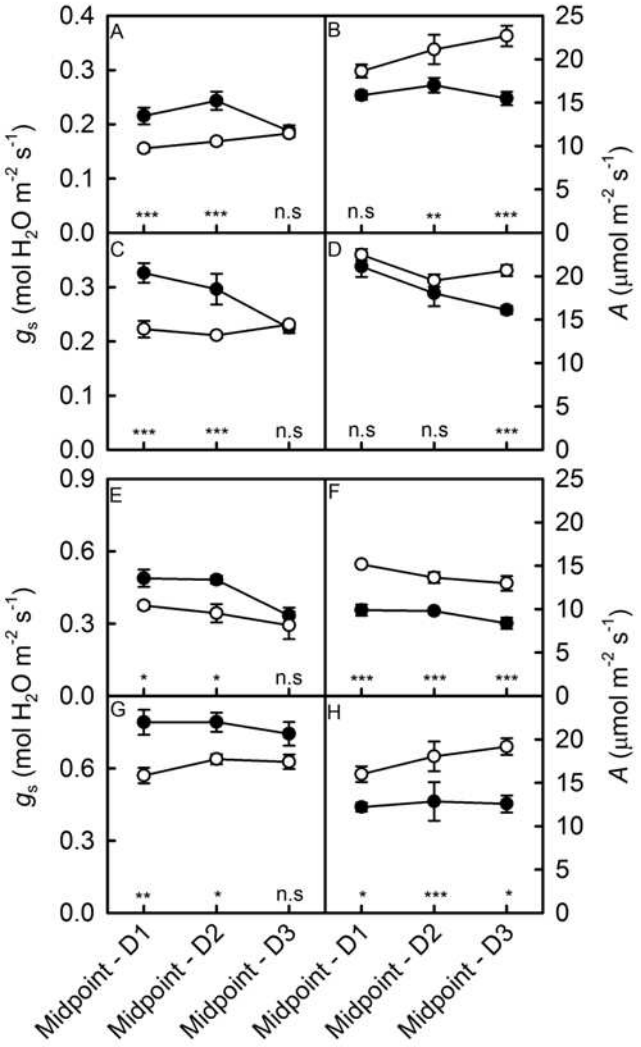

Figure 1. Changes in stomatal conductance [(g_s) A, C, E & G] and photosynthesis [(A) B, D, F & H] at the midpoint of each day over a three-day drying cycle (D1-D3) in two C_4 crop progenitors: (A & B) P. miliaceum and (C & D) S. viridis, and two C_3 crop progenitors: (E & F) H. spontaneum and (G & H) T. boeoticum grown at pCO_2 levels of 18 Pa (closed symbols) and 27 Pa (open symbols). Data are means ±SE of four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.0, n.s = not significant. Stomatal conductance is set to a different scale for the C_3 and C_4 species. See Supplementary Information **[Fig. S1 – Supplementary information]** for measurements taken at nine time points (am, midpoint and pm) over the three days.

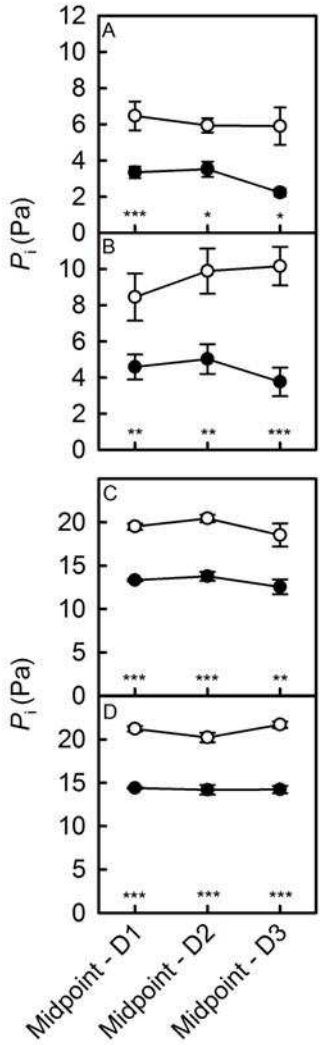

Figure 2. Changes in intercellular pCO_2 (P_i) at the midpoint of each day over a three-day drying cycle (D1-D3), in (A) P. miliaceum, (B) S. viridis, (C) H. spontaneum and (D) T. boeoticum grown at pCO_2 levels of 18 Pa (closed symbols) and 27 Pa (open symbols). Data are means ±SE of four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.0, n.s = not significant. Values of P_i are set to a different scale for the C_3 and C_4 species. See Supplementary Information **[Fig. S2 – Supplementary information]** for measurements taken at nine time points (am, midpoint and pm) over the three days.

led to greater A overall ($F_{1,62}$ = 26.9, <.001) but the effect was not seen on D1, and only became significant on D2 during the 'am' and 'pm' measurements (Fig. 1D, **Fig. S1 – Supplementary information**). Mirroring the response of g_s, the decrease in A was more pronounced at 18 Pa compared to 27 Pa pCO_2 ($F_{1,62}$ = 3.8, <.05). P_i was lower at 18 Pa pCO_2 compared to 27 Pa pCO_2 ($F_{1,66}$ = 99.8, <.001). Although P_i looked to decline at the midpoint of D3 at glacial pCO_2, there was no significant interaction between pCO_2×D.

Measurements of g_s were significantly greater at 18 Pa pCO_2 for the C_3 species, H. spontaneum (Fig. 1E, $F_{1,64}$ = 11.7, <.01). At the midpoint of D1, g_s was 23% lower at post-glacial pCO_2, g_s then declined from D1 to D3; with the decline being greater under glacial pCO_2 (Fig. 1E, $F_{1,64}$ = 5.8, <.01). There was a strong direct effect of pCO_2 on A (Fig. 1F, $F_{1,62}$ = 260.1, <.001), but the indirect effect mediated via the reduction in g_s was less clear. Photosynthesis declined marginally from D1 to D3 under both pCO_2 levels; and the decline was not significantly greater at glacial compared to post-glacial pCO_2 (Fig. 1F). Similarly, P_i was strongly affected by pCO_2 (Fig. 2C, $F_{1,64}$ = 361.4, <.001) and showed a small decline on D3, particularly at the 'pm' measurement **[Fig. S2 – Supplementary information]** however the reduction in P_i was no more rapid at 18 Pa pCO_2.

In the second C_3 species, T. boeoticum, g_s was 38% greater in glacial pCO_2 at the midpoint of D1, but by the midpoint of D3 the difference between pCO_2 levels had declined to 18% and was not significant (Fig. 1G). However, the decline of g_s at 18 Pa was not strong and the interaction between pCO_2 and day was not significant. The 18 Pa pCO_2 level did however, show an end-of-day decline at the 'pm' measurement which became stronger from D1 to D3 **[Fig. S1 – Supplementary information].** Photosynthesis was significantly lower at glacial pCO_2, and showed little change at each pCO_2 between D1 and D3 (Fig. 1H, $F_{1,68}$ = 70.3, <.001). At glacial pCO_2, A fluctuated marginally until the end of D3, when it dropped to 8.7 μmol $m^{-2}s^{-1}$, and this corresponded with the greatest decrease in g_s **[Fig. S1 –**

Supplementary information]. Consistent with these results, values of P_i showed no overall decline in either pCO_2 (Fig. 2D).

Transpiration

Independent measurements of in situ water-loss per unit leaf area, calculated using lysimetry, were consistent with the observed relationship between pCO_2 and g_s (Fig. 1 & Fig. 3). In P. miliaceum and S. viridis, E_{leaf} was lower at post glacial pCO_2 on D1 by 25% and 43% respectively, but a steeper decline in E_{leaf} at glacial pCO_2 meant that, by D3, there was no difference between the pCO_2 levels (Fig 3A & B). As a result, there was a significant interaction between pCO_2 and day for both P. miliaceum ($F_{2,18}$ = 6.1, <.01) and S. viridis ($F_{2,18}$ = 18.4, <.001). Similarly, in the C_3 species H. spontaneum, E_{leaf} was 25% lower at post-glacial pCO_2 on D1 yet, by D3, there was no significant difference between the pCO_2 levels and this was due to E_{leaf} declining more rapidly under glacial than post-glacial pCO_2 (Fig. 3C, $F_{2,18}$ = 12.8 <.001). For the second C_3 species T. boeoticum, although E_{leaf} was 41% lower at post-glacial than glacial pCO_2 on D1, and by D2 and D3 the difference had diminished, there was no significant interaction between pCO_2×D (Fig. 3D).

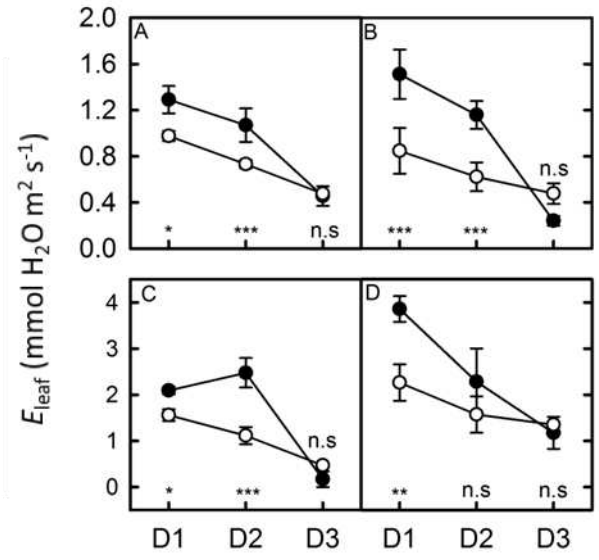

Figure 3. Changes in daily transpiration at the leaf scale (E_{leaf}) in the C_4 species (A) P. miliaceum and (B) S. viridis and the C_3 species (C) H. spontaneum and (D) T. boeoticum, over a three-day drying cycle (D1-D3). Plants were grown at pCO_2 of 18 Pa (closed symbols) and 27 Pa (open symbols). Data are means ±SE of four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.05, n.s = not significant. Values of E_{leaf} are set to a different scale for the C_3 and C_4 species.

Soil water content and leaf water potential

Measurements of θ_g and Ψ_{leaf} were made to investigate the potential mechanisms underpinning the reductions in g_s and E_{leaf}. The value of θ_g was marginally higher by D3 in post-glacial than glacial pCO_2 (Fig. 4). However, significant effects of pCO_2 were seen at only a few time points in each species and were due to a marginally faster decline in θ_g at glacial compared to post glacial pCO_2 (Fig. 4). This led to a significant interaction between pCO_2 and day in S. viridis ($F_{2,66} = 2.8 <.05$) and H. spontaneum ($F_{2,60} = 3.9 <.05$) only.

The end-of-day Ψ_{leaf} (D2-pm) was significantly affected by pCO_2, and this effect was strongest in the two C_4 species, where Ψ_{leaf} was 71% less negative in P. miliaceum and 41% less negative in S. viridis at the post-glacial pCO_2 level (Fig. 5A & B). In the C_3 species, only T. boeoticum showed a significant difference in Ψ_{leaf} (D2-pm), with a 27% less negative value in 27 Pa pCO_2 (Fig. 5C & D). The pre-dawn Ψ_{leaf} showed a small difference between glacial and post-glacial pCO_2 levels. Pre-dawn Ψ_{leaf} was 32% less negative in P. miliaceum and 24% less negative in S. viridis at post-glacial compared to glacial pCO_2, and the difference was only significant in P. miliaceum (Fig. 5A & B). In the C_3 species, only T. boeoticum showed a significant difference in pre-dawn Ψ_{leaf}, which was 23% less negative at the post-glacial pCO_2 level (Fig. 5C & D).

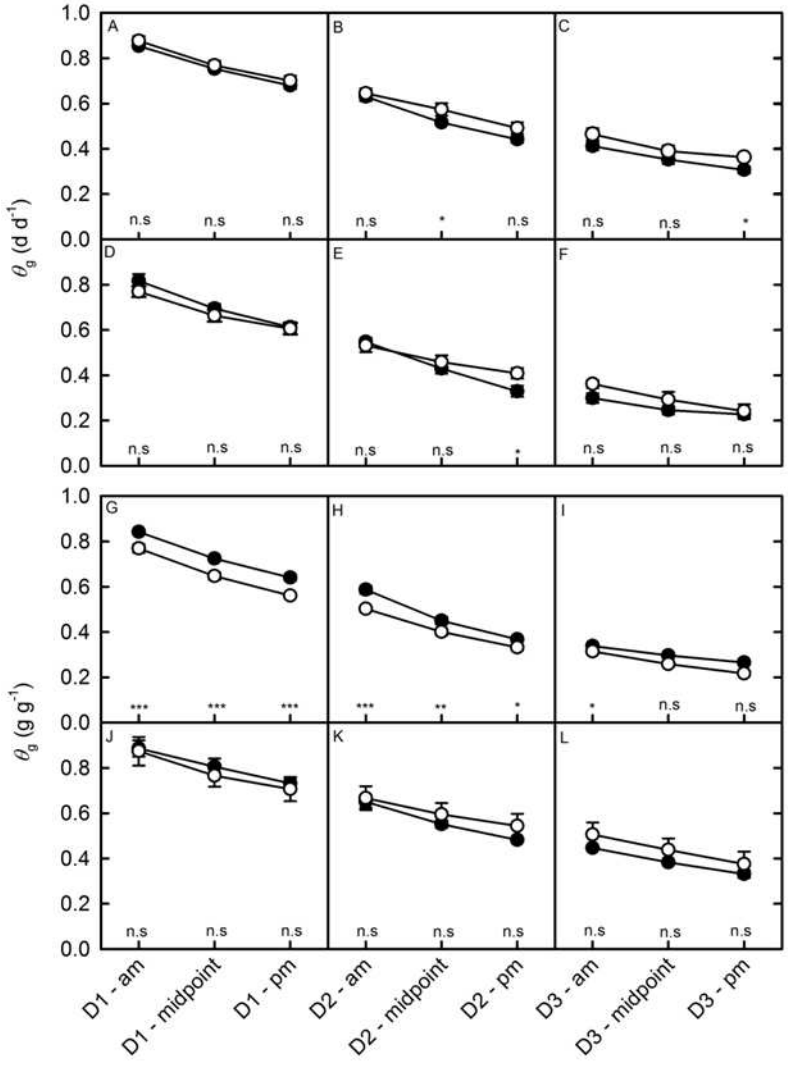

Figure 4. Changes in gravimetric soil water content (θ_g) over a three-day drying cycle (D1-D3) in two C_4 crop progenitors: (A-C) P. miliaceum and (D-F) S. viridis, and two C_3 crop progenitors: (G-I) H. spontaneum and (J-L) T. boeoticum grown at pCO_2 levels of 18 Pa (closed symbols) and 27 Pa (open symbols). Measurements were taken at 3 time points (am, midpoint and pm) over the 3 days. Data are means ±SE of four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.0, n.s = not significant. NB H. spontaneum likely had a significantly lower θ_g at the post-glacial level of pCO_2 on D1 because of a larger rooting. This may be introducing an error into the calculation of θ_g, since roots displace water but are less dense than soil.

The difference between daytime and pre-dawn leaf water potential (ΔΨ) provides an estimate of the soil-leaf water potential gradient, and the balance between the demand for H_2O by leaf transpiration and the supply of water from the soil. The two C_3 species showed no difference in ΔΨ between glacial and post-glacial pCO_2, as illustrated by the equal slope of the line joining the two water potential measurements (Fig. 5C & D). However, both P. miliaceum and S. viridis showed an increase in ΔΨ at 18 Pa, shown by the steeper slope in comparison to 27 Pa (Fig. 5C & D). This was supported by a significant interaction between pCO_2 and day in P. miliaceum ($F_{1,12}$ = 7.0 <.05; the p-value for the interaction was 0.08 for S. viridis).

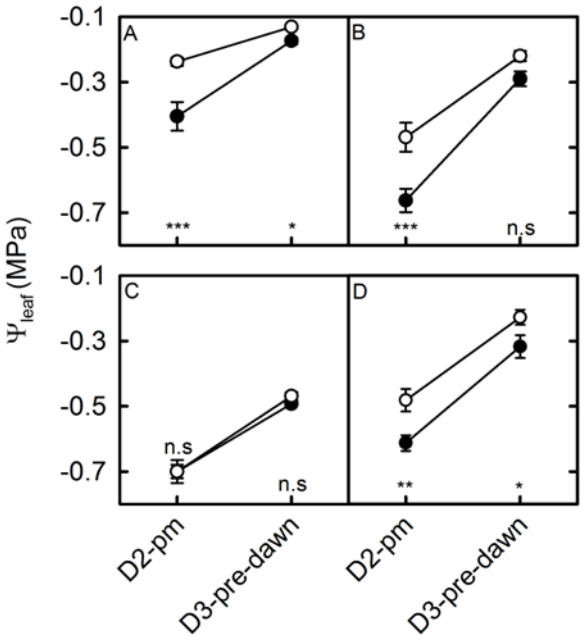

Figure 5. Changes in leaf water potential (Ψ_{leaf}) in the C_4 species (A) P. miliaceum and (B) S. viridis and the C_3 species (C) H. spontaneum and (D) T. boeoticum, over a three-day drying cycle (D1-D3), grown at pCO_2 of 18 Pa (closed symbols) and 27 Pa (open symbols). Due to the destructive nature of the method, measurements were taken only at two time points, at the end of day 2 (D2-pm) and before "dawn" on day 3 (D3-pre-dawn). Data are means ±SE of

four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.05, n.s = not significant.

Biomass and partitioning

Total vegetative biomass and leaf area were higher at post-glacial than glacial pCO_2, the effect size being greater in the C_3 than the C_4 species. Partitioning between roots and shoots was also altered by changes in pCO_2; at post-glacial levels more biomass was partitioned to roots in comparison to leaves. These data showed qualitative agreement with previous results from Cunniff et al. (2008) and are presented in Table 1.

			Variable ± SE		
Species	**Type**	**pCO_2**	**Total biomass (g)**	**Leaf area (m^2)**	**Root: shoot ratio**
P. miliaceum	C_4	18	24.5 ± 0.62 [a]	0.122 ± 0.0028 [a]	0.48 ± 0.019 [a]
		27	32.2 ± 1.41 [b]	0.147 ± 0.0044 [b]	0.86 ± 0.098 [b]
S. viridis	C_4	18	26.3 ± 0.92 [a]	0.094 ± 0.0025 [a]	0.26 ± 0.040 [a]
		27	34.1 ± 1.04 [b]	0.133 ± 0.0115 [b]	0.35 ± 0.011 [a]
H. spontaneum	C_3	18	12.6 ± 0.52 [a]	0.074 ± 0.0015 [a]	0.69 ± 0.028 [a]
		27	24.9 ± 1.25 [b]	0.111 ± 0.0057 [b]	0.91 ± 0.024 [b]
T. boeoticum	C_3	18	7.5 ± 0.19 [a]	0.026 ± 0.0004 [a]	0.62 ± 0.043 [a]
		27	13.9 ± 0.99 [b]	0.036 ± 0.0013 [b]	0.82 ± 0.085 [a]

Table 1. Values of total biomass, leaf area and root: shoot ratio for the C_4 and C_3 species. Values are means ±SE of four replicates. Different letters indicate statistically significant differences at $p < 0.01$.

DISCUSSION

Declining g_s at glacial pCO_2 limits carbon fixation

The data are consistent with a mechanism whereby higher g_s at the glacial than post-glacial pCO_2 led to a decrease in plant water status which limited A. In three of the species, a more pronounced decrease in g_s was associated with reduced plant water status at glacial pCO_2, and there was some evidence that this also restricted the diffusion of CO_2 into the leaf. The

feedback on A was evident both at the end of the day and towards the end of the drying cycle between watering events.

When mild water deficits develop, stomatal closure is one of the first leaf responses, followed by a decline in P_i and a down-regulation of photosynthetic capacity to match the available carbon substrate (Chaves et al., 2002, Brodribb, 1996, Lawlor and Cornic, 2002). In both C_4 species grown at glacial pCO_2, A declined alongside changes in g_s, suggesting that stomatal closure had brought P_i down to the steep initial slope of the A/P_i curve, where C_4 photosynthesis becomes highly sensitive to changes in pCO_2 (Samarakoon and Gifford, 1996, Samarakoon and Gifford, 1995). Values of P_i at glacial pCO_2 in P. miliaceum and S. viridis had declined to 2.2 Pa and 3.2 Pa respectively by the end of D3. These values represent only a small decline from those experienced on D1, but previous work with these species has shown that A is strongly limited by these values of P_i (Cunniff et al., 2008). Furthermore, measurements of A/P_i on well watered plants demonstrated that the operating point of A was on or below the inflexion point of the A/P_i curve so small changes in P_i (like those shown in this study) could cause significant changes in A in both C_4 species (Cunniff et al., 2008). Although P_i also declined through the drying cycle in the plants grown at post-glacial pCO_2, and often by a similar amount to plants grown at glacial pCO_2, values were still 5.5 Pa in P. miliaceum and 8 Pa in S. viridis at the end of D3 which are sufficient to achieve saturating levels of A in these species (Cunniff et al., 2008). This pattern of stomatal response was stronger in P. miliaceum than S. viridis, which is consistent with the previous observation that A saturates at much lower levels of pCO_2 in S. viridis (Cunniff et al., 2008).

The changes in P_i were not large in the C_4 species and changed little in the C_3 species over the three days in comparison to the strong interaction seen between g_s and pCO_2. Wong et al.(1979) showed that P_i remained constant whilst A and g_s varied together, and that stomatal aperture is determined by the capacity of the mesophyll tissue to fix carbon. Our

results could therefore be a reflection of direct effects of leaf water status on A mediated via non-stomatal (metabolic) limitations that are independent of P_i (Lawlor & Tezara, 2009). Furthermore, it has been demonstrated that C_4 plants are sensitive to non-stomatal limitations mediated via plant water status, and the effect was greater than in C_3 leaves (Ripley et al., 2007, Taylor et al., 2011). Alternatively the variance introduced into the calculation of P_i from both H_2O and CO_2 flux measurements could have diminished the power of statistical analyses for P_i.

In the C_3 species, evidence for a feedback on A mediated via plant water relations was more limited. Values of g_s and A did decrease more rapidly between D1 and D3 at glacial pCO_2. However, P_i showed little change until the end of D3 when it declined marginally to 9.9 Pa in H. spontaneum and 12 Pa in T. boeoticum under glacial pCO_2. The decreases in A expected to accompany these reductions in P_i are also much smaller in C_3 than C_4 species; since the initial slope of the photosynthetic response is shallower, and much larger decreases in P_i are required to produce comparable changes in A. Therefore, increases in biomass between 18 Pa and 27 Pa pCO_2 seen in these two C_3 species were more likely related to the direct effects of pCO_2 on A rather than the indirect effects of pCO_2 mediated via g_s.

Decreases in g_s at glacial pCO_2 may be related to reduced plant and soil water status.

The opening and closing of stomata are determined by the mechanical properties of the guard cells and the epidermal cells with which they interact (Franks et al., 1998). Models of stomatal function consider the hydration of epidermal cells an important control mechanism (e.g. Buckley et al., 2003, Gao et al., 2002, Dewar, 2002), and more recently Peak and Mott (2011) have linked stomatal movement to guard cell equilibration with water vapour in the air at the base of the stomatal pore. Plant hydraulic conductances and transpirational flux are

major components of these models and therefore represent an important component of the stomatal control mechanism, with considerable influence on A (Franks, 2003).

Our study provided evidence that plant water status may be controlling the response of stomata over the three-day interval between watering events. Values of Ψ_{leaf} were significantly more negative at glacial pCO_2 in both the C_4 species and in T. boeoticum (C_3), yet Ψ_{leaf} was restored at night, and pre-dawn Ψ_{leaf} showed only a small difference between pCO_2 levels. As a consequence, the inferred water potential gradient from root to shoot ($\Delta\Psi$) was larger under glacial pCO_2. These patterns suggest that the supply of water to the leaf was insufficient to meet the demands of transpiration, leading to lower daytime Ψ_{leaf} and then stomatal closure in the leaf, which fed back to limit A.

The reduced plant water status found in the plants growing at sub-ambient pCO_2 has analogies with the physiological changes seen in plants growing under conditions of raised VPD (e.g. Franks and Farquhar, 1999, Brodribb and Jordan, 2008, Bunce, 2006). When the rate of water loss is increased by raising VPD, leaf turgor and stomatal aperture both decline. This response limits transpiration rates and also CO_2 supply to the mesophyll which, in turn, can reduce A (Bunce, 2006). Photosynthesis is also very sensitive to the direct effects of leaf water deficits measured as more negative Ψ_{leaf} (Lawlor and Tezara, 2009). Therefore, improvements in Ψ_{leaf} at post-glacial compared to glacial pCO_2 in this study indicate that plant water status was more favourable for carbon assimilation, and are consistent with studies finding that leaf Ψ_{leaf} and A are improved under elevated pCO_2 in both C_3 and C_4 plants under water deficits (Wall et al., 2001, Wall, 2001, Robredo et al., 2006, LeCain et al., 2003).

High pCO_2 can either increase, decrease, or have no effect on soil water status, depending on the relative strength of leaf area and stomatal responses to high pCO_2 in the species involved (Samarakoon and Gifford, 1995). Although the depletion of soil water in our

study was generally slower at post-glacial pCO_2, significant differences were only seen at a few time points, suggesting only a modest effect; this interpretation was supported by pre-dawn Ψ_{leaf}, which showed minor differences between pCO_2 levels. These small observed effects on soil moisture may have been due partially to the large CO_2-induced increases observed in leaf area, especially in the C_3 species, which counterbalanced reduced E_{leaf} (Chaudhuri et al., 1986). Leaf area increased by 21-50 % under the post-glacial regime for the four species tested, providing a substantial area for extra water loss. However, the θ_g provides an explanation for the limited evidence of a hydraulic feedback in T. boeoticum. T. boeoticum had a much smaller mass and leaf area than the other species in this experiment, and θ_g therefore declined by a lesser amount. Soil water may therefore have been sufficient at both pCO_2 levels to maintain g_s over the full three-day drying cycle.

The results of this study showed important points of difference with our three hypotheses. In agreement with hypothesis one, the g_s was greater at glacial pCO_2. However, this was linked to reduced plant water status, with only marginal differences in soil water content. Furthermore, although g_s and A declined more rapidly over the three-day soil drying cycle at glacial compared to post-glacial pCO_2, as predicted in hypothesis two, effects appeared to be more related to reduced plant water status than declining soil water content. The substantial increase in leaf area found in plants grown at post-glacial pCO_2 could be responsible for the small differences between SWC in the two pCO_2 regimes. Hypothesis three suggested that water deficits would affect A to a similar extent in C_3 and C_4 crop plants as predicted in other studies. However, our work showed some evidence of stronger effects in the C_4 species, which we attribute to the steeper response of A to P_i at low pCO_2 levels or a result of non-stomatal, metabolic limitations of plant water status on A.

These conclusions must be tempered by two caveats. First, logistic constraints meant that we investigated leaf gas exchange, plant transpiration, and water relations during

successive drying cycles. This means that we cannot exclude the possibility of carry-over effects from one cycle to the next, or ontogenetic effects as the plants developed (Harb et al., 2010, Walter et al., 2011). However, the patterns observed from one cycle to the next were internally consistent overall, and consistent with mechanisms of CO_2 x water interactions observed previously (Wall et al., 2001, Conley et al., 2001, Leakey et al., 2004, Leakey et al., 2006, Leakey, 2009). Secondly, since our interest was in how physiological characteristics vary over a short experimental watering cycle, we investigated changes over time and did not use a control on a shorter watering cycle.

Implications for the origin of agriculture

Large improvements in the water status of the wild progenitors of cereal crops caused by rising atmospheric pCO_2 levels during deglaciation would be particularly beneficial in the climatic regions where these plants were first cultivated. The C_4 species S. viridis and P. miliaceum were domesticated in North China, where the climate was arid, winters dry and cold, and summer rains unreliable (Yu et al., 2000). The C_3 species were also domesticated in dry climates. T. boeoticum currently colonises regions in western Asia which receive only 300-350 mm of rain per year (Willcox, 2005), and H. spontaneum is a major component of the semi-arid grasslands of the Middle East (Grünzweig and Körner, 2000), and found in areas with just 200-250 mm of yearly rainfall (Willcox, 2005). A detailed appraisal of climate change in western Asia from 25,000 to 5,000 years ago by Robinson et al. (2006) using data from both general circulation models and numerous geological climate proxies shows that, in general, the sources are in agreement. The last glacial maximum (LGM) [23,000-19,000 calendar years before present (cal yrs BP)] was colder (in some records predicted to be 5°C less) and more arid (with some sources predicting 50% less rainfall). Apart from a brief

climatic reversal at 12,700-11,500 cal yrs BP (the Younger Dryas) conditions began to ameliorate from 15,000 cal yrs BP onwards, becoming both warmer and wetter.

Precipitation was not only regionally but globally lower, as evidenced by higher dust fluxes in ice cores during glacial maxima compared to interglacials. It is predicted that the strength of the hydrological cycle in the late Pleistocene was about half of that at present (Yung et al., 1996). Deglaciation is therefore likely to have improved the water status of wild plants, including crop progenitors, via two routes. First, through an intensification of the hydrological cycle and an increase in the frequency and amount of rainfall. Secondly, via the indirect mechanism outlined in this paper, whereby rising pCO_2 caused a reduction in stomatal aperture and alleviated plant water deficits.

Enhanced productivity due to both the direct and indirect effects of pCO_2 would have increased both the yield of crop progenitors, and its interannual reliability. Following from this, the increasingly stable food base and reliable climate are hypothesized to have increased the carrying capacity of the environment, and promoted sedentism, leading to population growth (Cohen, 1977). More intensive exploitation of local resources could, in-turn, have led to specialisation on a limited number of preferred plant species and the development of cultivation practices (Sage, 1995). The isolation of a limited number of species from their natural environment via cultivation would have allowed the selection of attributes favourable to human use (both consciously and unconsciously), leading to their eventual domestication.

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APPENDIX

A, net leaf photosynthetic CO_2-assimilation rate (µmol CO_2 m^{-2} s^{-1}); Cal yrs BP, calendar years before present; DAP, days after planting; E, instantaneous rate of leaf transpiration (mmol H_2O m^{-2} s^{-1}); E_{leaf}, leaf transpiration (mmol H_2O m^{-2} s^{-1}); E_{plant}, daily canopy transpiration (mol H_2O $plant^{-1}$ d^{-1}); g_s, leaf stomatal conductance to H_2O vapour (mol m^{-2} s^{-1}); m_{dry}, soil dry weight (g); m_{wet}, soil fresh weight (g) pCO_2, atmospheric partial pressure of CO_2 (Pa); P_i, intercellular pCO_2 (Pa); PPFD, photosynthetic photon flux density (µmol m^{-2} s^{-1}); SLA, specific leaf area (m^2 g^{-1}); VPD, vapour pressure deficit (kPa); Ψ_{leaf}, leaf water potential (MPa); θ_g, gravimetric soil water content (g g^{-1}).

Supplementary information

Table S1. Regression and R^2 values for the allometric relationship between leaf length × maximum leaf width for H. spontaneum, T. boeoticum, P. miliaceum, and S. viridis.

Species	Regression	R^2
H. spontaneum	y = 0.6824x+1.4316	0.9867
T. boeoticum	y = 0.8362x-0.1681	0.9787
P. miliaceum	y = 0.7492x-0.2826	0.9941
S. viridis	y = 0.0426x+13148	0.9756

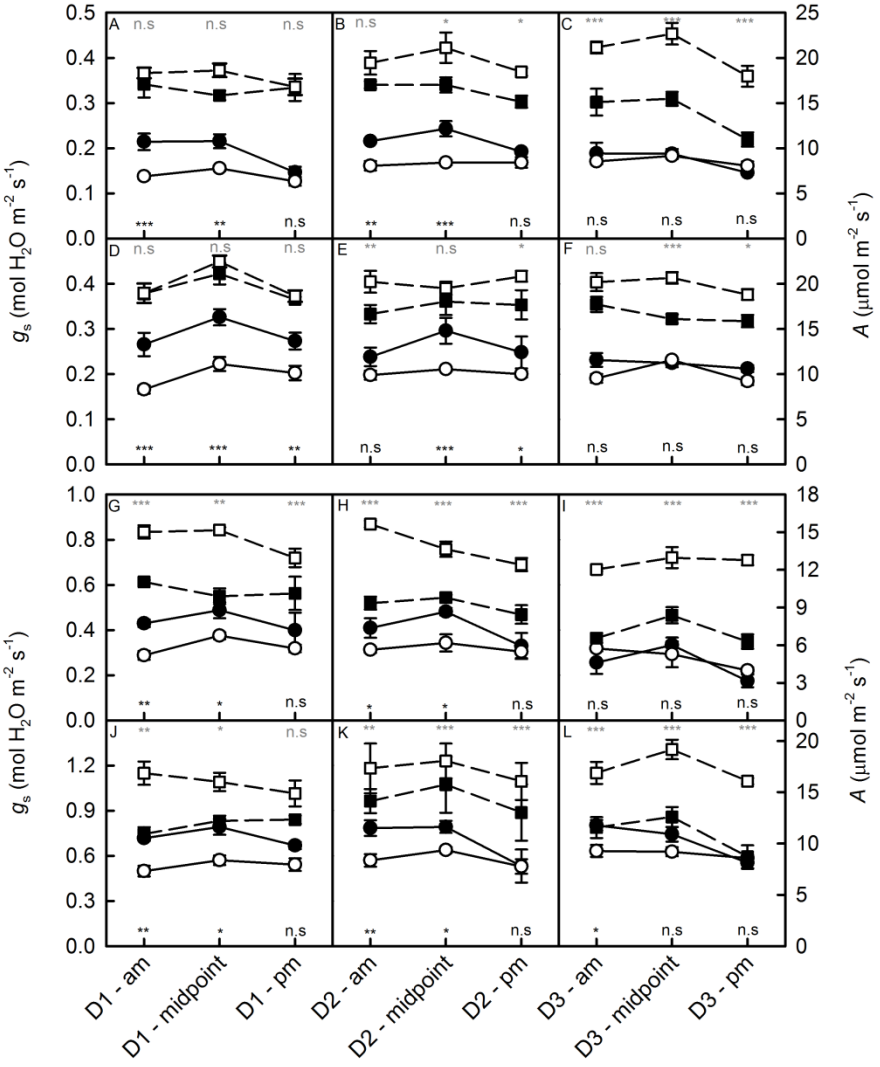

Figure S1. Changes in stomatal conductance ($g_{s;}$ circles) and photosynthesis (A; squares) over a three-day drying cycle (D1-D3) in two C_4 crop progenitors: (A-C) P. miliaceum and (D-F) S. viridis, and two C_3 crop progenitors: (G-I) H. spontaneum and (J-L) T. boeoticum grown at pCO_2 levels of 18 Pa (closed symbols) and 27 Pa (open symbols). Measurements were taken at 3 time points (am, midpoint and pm) over the 3 days. Data are means ±SE of four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.0, n.s = not significant and are black for g_s and grey for A. Values of A and g_s are set to a different scale for each species.

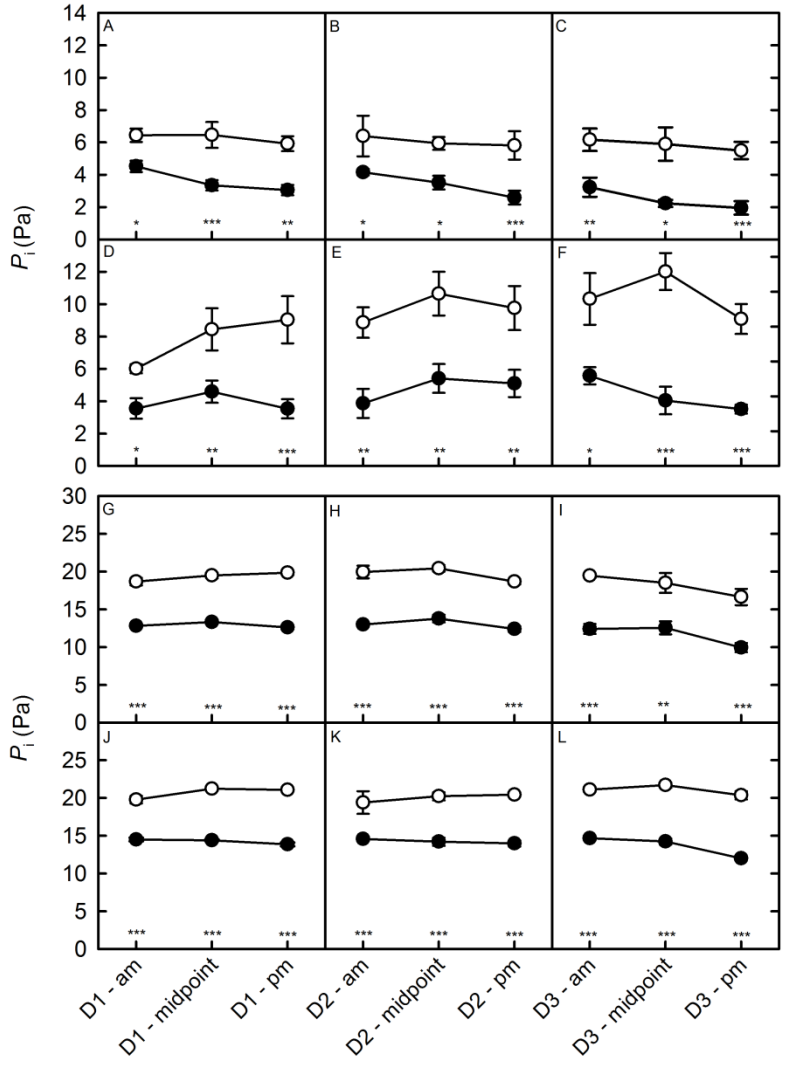

Figure S2. Changes in intercellular pCO_2 (P_i) over a three-day drying cycle (D1-D3), in (A-C) P. miliaceum, (D-F) S. viridis, (G-I) H. spontaneum and (J-L) T. boeoticum grown at pCO_2 levels of 18 Pa (closed symbols) and 27 Pa (open symbols). Measurements were taken at 3 time points (am, midpoint and pm) over the 3 days. Data are means ±SE of four replicates. Significance codes are ***=<0.001, **=<0.01 and *=<0.0, n.s = not significant. Values of P_i are set to a different scale for the C_3 and C_4 species.