Limited photosynthetic plasticity in the leaf-succulent CAM plant *Agave angustifolia* grown at different temperatures

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Abstract. In *Agave angustifolia* Haw., a leaf-succulent constitutive crassulacean acid metabolism (CAM) plant of tropical Panama, we tested whether nocturnal CO$_2$ uptake and growth were reduced at night temperatures above 20°C. Unlike some CAM model species from habitats with pronounced day–night temperature variations, in *A. angustifolia* temperature affected little the relative contributions of CAM and C\textsubscript{3} photosynthesis to growth. In plants grown under 12 h light/dark regimes of 25/17, 30/22 and 35/27°C, biomass increased with temperature. Maintaining day temperature at 35°C and reducing night temperature from 27 to 17°C markedly lowered growth, a reduction partially reversed when roots were heated to 27°C. Across all treatments, whole-shoot $\delta^{13}$C values ranged between −14.6 and −13.2‰, indicating a stable proportion of CO$_2$ was fixed at night, between 75 and 83%. Nocturnal acidification reflected growth, varying between 339 and 393 µmol H$^+$ g$^{-1}$ fresh mass and 63–87 µmol H$^+$ cm$^{-2}$. In outdoor open-top chambers, warming the air 3°C above ambient at night did not reduce biomass accumulation. The persistence of a high capacity for nocturnal CO$_2$ fixation at the expense of a limited capacity for switching between C\textsubscript{3} and CAM probably makes this *Agave*, and others like it, potential species for biomass production in seasonally-dry landscapes.

Additional keywords: biofuel, C$_3$ photosynthesis, climate change, crassulacean acid metabolism, open-top chamber.

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Introduction

Crassulacean acid metabolism (CAM) in terrestrial plants is a water-use efficient photosynthetic adaptation that assists plants to occupy habitats subject to periodic water stress (Osmond 1978; Winter 1985). CAM is water-use efficient because CO$_2$ is assimilated at night when temperatures and rates of evaporation are at daily minima (Winter et al. 2005).

In addition to fixing CO$_2$ during the night, many species with CAM can also incorporate atmospheric CO$_2$ during the light via C\textsubscript{3} photosynthesis, a pathway that is less water-use efficient than CAM. It is not uncommon for CAM species with a capacity for light fixation to adjust the proportions of carbon fixed at night and during the day in response to the availability of water (Winter 1985; Borland et al. 2011; Dodd et al. 2002) such that CO$_2$ fixation in the light increases when water is available and decreases when water is scarce. The ability to regulate CO$_2$ uptake during the dark and the light thus enables many CAM species to maintain some daily carbon gain as they reduce water-loss, whereas C\textsubscript{3} and C\textsubscript{4} plants may not be able to maintain a positive carbon balance under similar stressful conditions.

Evaporative water loss increases with the difference in vapour pressure between the leaf and the atmosphere, and thus responds to increasing temperature. It is therefore not surprising that in CAM species the proportion of carbon fixed at night and during the day responds to temperature (De Vries 1884; Kluge and Ting 1978). In CAM plants, the temperature responses of CO$_2$ fixation during the night and the day are not the same (Nobel 1988; Yamori et al. 2014), as might be expected when two biochemically distinct processes are involved. CO$_2$ uptake in the dark is enhanced when daytime temperature maxima are typically above 30°C, and night maxima are below 20°C. As a result, night temperatures significantly above 20°C are not only widely considered suboptimal for nocturnal CO$_2$ uptake (Neales 1973a, 1973b; Kluge and Ting 1978; Neales et al. 1980; Medina and Osmond 1981) but, by extrapolation, suboptimal for growth. The night-time temperature responses are at odds with the expression of pronounced CAM in the many species (both epiphytes and terrestrial plants) that inhabit the tropics (Wong and Hew 1976; Holtum and Winter 1999; Crayn et al. 2004; Silvera et al. 2005) where night temperatures are commonly around 20–25°C.

Early studies proposed that the mechanistic basis for reduced nocturnal CO$_2$ uptake at higher night temperatures is a product of differing thermal optima of malate synthesising and consuming reactions and of increased respiration (Brandon 1967; Kaplan et al. 1976) but these ideas have not been further pursued experimentally. Similarly, there is little experimental evidence that the negative effects of high night temperatures on CO$_2$ uptake in the dark that have been observed by several authors ultimately adversely affect growth, even amongst the extensive
Agave and cactus research published by Nobel and his colleagues (see Nobel 1988 for a review).

Here we explored the relationship between growth, $\delta^{13}$C (as indicator of the relative contributions of light and dark fixation to carbon gain), and day and night temperatures in Agave angustifolia Haw., a constitutive strong-CAM species from the tropics and subtropics of Central America and Mexico. A. angustifolia is a putative wild ancestor of the domesticated agaves (Gentry 1982; Colunga-García Marín et al. 1999). Agave fourcroydies Lem. (henequen) which is used for fibre production, and Agave tequilana F.A.C.Weber, the source of tequila. Both A. fourcroydies and A. tequilana have been proposed as biofuel feedstock crops for seasonally dry landscapes in tropical northern Australia and elsewhere (Chambers and Holtum 2010; Holtum et al. 2011; Owen and Griffiths 2013). In a warming world containing roughly 400 million ha of abandoned agricultural land much of which is in the semiarid tropics and sub-tropics (Campbell et al. 2008), it could be expected that the area suitable for growing appropriate Agave species for biofuels might be expanding. However, if the expression of CAM in Agave is progressively reduced with increasing night temperature, and the expression of daytime CO$_2$ uptake is reduced as well, then growing CAM Agave for biomass may become a less attractive proposition.

Here we demonstrate that A. angustifolia accumulates biomass more rapidly at a relatively high day/night temperature regime of 37/27°C than it does at 25/17°C. At a high daytime temperature of 35°C, growth increased when night temperature was increased from 17 to 27°C. In addition, the proportional contribution of light and dark CO$_2$ uptake to net carbon gain, measured using $\delta^{13}$C values as a proxy (Winter and Holtum 2002), responded little to temperatures to which A. angustifolia might be exposed in its natural habitat. We suggest that this lack of photosynthetic plasticity may be a desirable trait when growing constitutive strong CAM plants for biomass production in seasonally-dry environments in the tropics and subtropics.

Materials and methods

Plant material

Agave angustifolia Haw. was collected from Playa Majagual, Panama (8°43’N, 79°45’E) and maintained outdoors in forest topsoil at the Smithsonian Tropical Research Institute, Santa Cruz Experimental Research Facility, Gamboa, Republic of Panama (9°07’N, 79°42’W).

Experiment using growth chambers

Bulbils of 0.68 ± 0.09 (s.e.) g DM with leaf areas of 33.04 ± 7.13 cm$^2$ were planted in 6 L pots (16 cm internal diameter) containing 80% forest topsoil, 20% sand and 2 g Osmocote Plus fertiliser (Scotts-Sierra Horticultural Products, OH, USA). The plants ($n = 6$ per treatment) were grown from 1/7/2012 until 24/9/2012 inside five controlled-environment chambers (Model GC15, Environmental Growth Chambers, OH, USA) operating under 12 h light/12 h dark cycles of 25/17°C, 30/22°C, 35/27°C, 35/17°C, and 35/17°C and maintaining soil temperature at ≥27°C (Table 1). Photon flux density was 500 µmol m$^{-2}$ s$^{-1}$ at the top of the plants.

Air temperatures inside growth chambers, measured using copper-constantan fine wire thermocouples, were essentially identical to chamber set-temperatures and assumed their new value within 10 min of light/dark and dark/light transitions.

Soil temperatures within all pots reflected the day/night air temperatures except for pronounced lags as they gradually heated or cooled following switchovers between temperature regimes (Fig. 1). For the treatment in which roots were heated, pots containing plants were placed in a water-bath equipped with a heating thermostat (Model Alpha, Lauda, NJ, USA) set to 27°C. Soil temperature was measured in each pot using two copper-constantan thermocouples embedded in silicone sealant.

Open-top chamber experiment

Agave angustifolia was grown outdoors in six open-top chambers between 1 November 2012 and 25 June 2013 at the Santa Cruz Experimental Research Facility. Each chamber

<table>
<thead>
<tr>
<th>Air temperature (°C)</th>
<th>Relative humidity (%)</th>
<th>Leaf temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>Dark</td>
<td>Light</td>
</tr>
<tr>
<td>25</td>
<td>17</td>
<td>65</td>
</tr>
<tr>
<td>30</td>
<td>22</td>
<td>70</td>
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<tr>
<td>35</td>
<td>27</td>
<td>79</td>
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<td>35</td>
<td>17</td>
<td>56</td>
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<tr>
<td>35 &amp;</td>
<td>77</td>
<td>80</td>
</tr>
</tbody>
</table>

*In this treatment, the roots were heated to achieve 27°C during the dark. Heating was maintained during the light.*
contained six 19 L containers each with one bulbil planted in forest soil. At the onset of the experiment, the average weight of bulbils was 0.99 ± 0.10 g DM and the average area of bulbil leaves was 41.38 ± 3.20 cm². The chambers and environmental control within the chambers are described by Cheesman and Winter (2013). In three chambers night temperature was maintained at 3°C above the ambient night temperature. Pots received ambient rainfall with additional daily watering as required to maintain soils at close to field capacity.

At harvest, soil was washed by hand from the roots. Leaf area was measured using a leaf area meter (Model LI-3100, Li-Cor, Lincoln, NE, USA). Roots, leaves and stems were oven-dried at 70°C.

Titratable acidity

From mature leaves of each plant, two discs, each of 0.9 cm diameter, were excised using a cork borer at the end of the light and dark periods and were frozen in liquid nitrogen. Organic acids were extracted by sequentially boiling samples in 50% methanol and in water for 5 min. Extracts were cooled to room temperature and titrated with 10 mM KOH to pH 6.5 (Holtum et al. 2004).

Stable isotope analysis

The δ¹³C values of finely-ground homogeneous powder from the pooled dried leaves of whole plants were measured using an isotope ratio mass spectrometer (Delta V; Thermo Fisher Scientific, Ottawa, ON, Canada) in the Stable Isotope Laboratory of the Smithsonian Tropical Research Institute (Cernusak et al. 2011; Winter et al. 2014).

Results

Whole-plant mean dry biomass recorded at the two higher day/night temperature regimes, 30/22 and 35/27°C, was significantly greater than that of plants grown at 25/17°C (Fig. 2a), with plants at 35/27°C, the warmest regime, having a mean biomass that was 27% greater than for plants at 25/17°C, the coolest regime.

Compared with 35/27°C, maintaining the day temperature at 35°C and reducing the night temperature to 17°C lowered mean biomass from 8.8 to 5.9 g plant⁻¹, a significant drop of 33%. In contrast, the mean biomass of plants at 25/17°C and 35/17°C was similar.

The response pattern of leaf area to temperature was qualitatively similar to the biomass pattern but overall amplified (Fig. 2b). Compared with the mean leaf area of plants at 25/17°C, leaf area was 25% greater at 30/22°C and 49% greater at 35/27°C. As with biomass, the mean leaf area of plants grown at 35/17°C was 32% less than for plants grown at 35/27°C. Leaf area ratio (LAR) increased with temperature (Fig. 2c) whereas leaf mass/area (LMA), a measure of leaf density, remained constant or decreased slightly (Fig. 2d).

Maintaining roots at 27°C during a 35/17°C air temperature regime increased leaf area and LAR significantly (Fig. 2b, c), whereas LMA decreased slightly (Fig. 2d). The heating of roots was accompanied by an associated 2.1°C increase in leaf temperature at night (Table 1), presumably the product of thermal dissipation from H₂O in the circulating bath used to heat the roots.

Nocturnal acid accumulation (ΔH⁺) ranged between 339 and 393 μmol H⁺ g⁻¹ FM and 63–87 μmol H⁺ cm⁻² across treatments (Table 2). Acid levels were similar in plants grown at 25/17, 30/22 and 35/27°C, with the largest mean acid accumulation measured for plants at 30/22°C. In plants grown at 35°C during the light, mean acidification values were not significantly affected when the night temperature

<table>
<thead>
<tr>
<th>Night (°C)</th>
<th>17</th>
<th>22</th>
<th>27</th>
<th>17</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roots (°C)</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Fig. 2. Biomass (a), leaf area (b), leaf area ratio (leaf area per whole-plant dry mass) (c), leaf mass/area (leaf dry mass per unit leaf area) (d) and root mass ratio (root dry mass per whole-plant dry mass) (e) of Agave angustifolia plants exposed to 12 h day/night temperature regimes of 25/17°C, 30/22°C, 35/27°C, 35/17°C, and 35/17°C plus roots warmed to 27°C. Bars indicate 95% confidence levels of the means (n = 6 plants). Letters within columns indicate statistical significance, categories not sharing a letter differ significantly at P<0.05 (Tukey’s HSD).
Table 2. Nocturnal increases in leaf tissue acidity of *Agave angustifolia* grown under five 12 h light/12 h dark temperature regimes

Samples were taken from mature leaves at the end of the light and end of the dark periods. Values are means ± s.e. (n = 6 plants). Means followed by the same letter do not differ (Tukey’s HSD test, *P* < 0.05)

<table>
<thead>
<tr>
<th>Titratable acidity</th>
<th>Light/dark temperature (°C)</th>
<th>25/17</th>
<th>30/22</th>
<th>35/27</th>
<th>35/17</th>
<th>35/17 + root warmingA</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol g⁻¹ fresh mass</td>
<td>Light</td>
<td>34 ± 4</td>
<td>13 ± 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>399 ± 7</td>
<td>406 ± 10</td>
<td>371 ± 5</td>
<td>343 ± 8</td>
<td>340 ± 12</td>
</tr>
<tr>
<td>Δ</td>
<td>Light</td>
<td>364 ± 10ab</td>
<td>393 ± 10a</td>
<td>371 ± 5ab</td>
<td>343 ± 8b</td>
<td>339 ± 12b</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>8 ± 4</td>
<td>3 ± 5</td>
<td>0 ± 2</td>
<td>0 ± 3</td>
<td>0 ± 2</td>
</tr>
<tr>
<td>Δ</td>
<td>Light</td>
<td>88 ± 4</td>
<td>90 ± 5</td>
<td>75 ± 2</td>
<td>67 ± 3</td>
<td>63 ± 2</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>80 ± 4ab</td>
<td>87 ± 5a</td>
<td>75 ± 2abc</td>
<td>67 ± 3bc</td>
<td>63 ± 2c</td>
</tr>
</tbody>
</table>

AIn this treatment, the roots were heated to achieve 27°C during the dark. Heating was maintained during the light.

Table 3. δ¹³C values of pooled leaves of *Agave angustifolia* plants grown under five 12 h light/12 h dark temperature regimes

Values are means ± s.e. (n = 6 plants). Means followed by the same letter do not differ (Tukey’s HSD test, *P* < 0.05)

<table>
<thead>
<tr>
<th>δ¹³C (%e)</th>
<th>Light/dark temperature (°C)</th>
<th>25/17</th>
<th>30/22</th>
<th>35/27</th>
<th>35/17</th>
<th>35/17 + root warmingA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>−14.6 ± 0.1a</td>
<td>−14.5 ± 0.0a</td>
<td>−14.4 ± 0.1a</td>
<td>−13.2 ± 0.1b</td>
<td>−13.2 ± 0.1b</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>−80 ± 4ab</td>
<td>87 ± 5a</td>
<td>75 ± 2abc</td>
<td>67 ± 3bc</td>
<td>63 ± 2c</td>
</tr>
</tbody>
</table>

AIn this treatment, the roots were heated to achieve 27°C during the dark. Heating was maintained during the light.

Discussion

The growth response of the constitutive strong CAM plant *A. angustifolia* to a range of day-night temperature regimes contrasted with an often-cited generalisation that high day temperatures combined with low night temperatures favour CAM and enhance growth (Knipe and Ting 1978; Owen and Griffiths 2013). In *A. angustifolia*, biomass was greater for plants grown at day temperatures above 25°C and night temperatures above 17°C. Nocturnal accumulation of titratable acidity was similar for plants grown at night temperatures of 17 and 27°C.

Biomass accumulation, leaf area and LAR increased as day and night temperatures were increased from 25/17 to 35/27°C, whereas unchanged δ¹³C values indicated that the relative contribution of nocturnal CO₂ uptake to carbon gain remained around 75% (using the calibration of Winter and Holtum 2002). In general agreement with the CAM-type δ¹³C values, nocturnal acidification was substantial under the three treatments. As growth is driven by overall assimilation rate and LAR, and nocturnal CO₂ assimilation remained relatively constant, the principal contributor to increased growth between 25/17, 30/22 and 35/27°C appears to have been increased LAR.

Compared with the highest temperature regime, 35/27°C, maintaining the day temperature at 35°C and reducing the night temperature to 17°C markedly reduced biomass accumulation. As LAR was similar for both treatments it follows that the overall rates of CO₂ uptake were lower in plants under 17°C nights, a conclusion supported by marginally lower nocturnal acidification at 17°C. δ¹³C values suggest that over the life of the plants under 27 and 17°C nights, 76 and 83% of the carbon, that is, similar proportions, were obtained in the dark respectively.

Biomass accumulation was similar between plants subjected to 17°C nights and days of 25 or 35°C but LAR was greater at...
the higher day temperature. As was deduced following the comparison of plant growth at 35/27 and 35/17°C, it appears that the overall rate of CO₂ uptake per unit area was lower in the 35/17°C plants than in the 25/17°C plants, a deduction consistent with the lower nocturnal acidification in plants at 35/17°C.

In the 35/17°C treatment, warming the soil to 27°C increased total leaf area and LAR but biomass, acidification and δ¹³C values remained constant. Interpretation of the effects on growth of soil heating were complicated by an associated increase of nocturnal leaf temperature by 2.1°C (Table 1), as explained in ‘Materials and Methods’. Stimulation of growth following root heating has previously been reported for the C₃ species, cotton, possibly the consequence of temperature shifting source–sink relationships (Königer and Winter 1993).

Warming of A. angustifolia grown outdoors by 3°C during the night did not adversely affect growth. In fact, higher means for whole-plant dry mass (19% increase) and leaf area (24% increase) of heated plants, although not statistically different, suggest that a positive statistically significant correlation between night temperature and growth might well be observed in a more extensively replicated experiment. Significantly increased growth rates have been reported for the C₃ pioneer tree species, Ficus insipida and Ochroma pyramidale, maintained at 3°C above ambient at night (Cheesman and Winter 2013).

The perception that CAM activity in general is stimulated by pronounced differences in day and night temperature stems in the main from studies of species of Kalanchoë, Agave and Opuntia from habitats where cool nights and hot days predominate (Queiroz 1966; Neales 1973a; Szarek and Ting 1974; Kluge and Ting 1978; Nobel and McDaniel 1988), although broadly similar observations have been reported for Ananas comosus, a tropical species that has been modified by breeding for desirable agronomic characters (Neales 1973b; Neales et al. 1980; Zhu et al. 1999). One might expect any requirement for substantial day/night temperature differences to be less evident in species like A. angustifolia that are native to the humid tropics where nights are typically warm and day/night temperature fluctuations are less pronounced (Milburn et al. 1968; Wong and Hew 1976; Griffiths and Smith 1983; Winter 1985; Holtum and Winter 1999; Crayn et al. 2004; Silvera et al. 2005). Nobel and Hartsock (1978, 1981) demonstrated that CO₂ exchange by Agave species can rapidly acclimate to new temperature regimes but the implications of these short-term responses for growth are not clear.

On the basis that nocturnal CO₂ uptake is reflected in growth, minimum night-time temperatures have been used to form the temperature index components of environmental productivity indexes (EPI) designed to predict the productivities of various Agave and Opuntia in the field (Nobel 1984, 1985, 1988, 1989, 1991; Nobel and Meyer 1985; Nobel and Quero 1986; Nobel and Valenzuela 1987; Pimienta-Barrios et al. 2001). Similarly, a model that integrates EPI estimates with soil water retention characters and GIS methodology to predict potential productivity of Agave species in Australia, apportioned to minimum night-time temperatures 95% of the effects of temperature on productivity in A. fourcroydes (Owen and Griffiths 2013).

The observations on the growth of A. angustifolia reported here suggest that EPI modelling based on minimum temperatures may be a too simplistic approach, particularly for tropical CAM species.

The responses of growth in A. angustifolia to day and night temperatures will not only reflect the effects of temperature on the CAM cycle but also the effects of temperature on other cell processes. Diet patterns of leaf and cladode expansion have been observed in CAM M. crystallinum, and in CAM perennials such as Opuntia engelmannii, O. oricola and Kalanchoë beharensis (Gouws et al. 2005) but the interaction between the demands of CAM cells for carbon skeletons and the demands by the rest of the plant for carbon skeletons and energy are not well understood, even for long-studied models such as the annual Mesembryanthemum crystallinum and perennial Kalanchoë spp. (Borland and Dodd 2002; Antony and Borland 2009). It has been proposed that the substantial requirements for carbon skeletons in CAM cells limits the export of carbohydrates for growth during acidification at night, and during deacidification in the light (Gouws et al. 2005; Haider et al. 2012). Growth could be particularly sensitive to temperature when carbon export for growth is expected to be most evident – during the first and last phases of the light, and perhaps late at night when the accumulation of malic acid is close to completion and turgor pressures are high.

Photosynthetic plasticity, which is the ability to switch between C₃ and CAM photosynthesis, contributes to the ecological success of some CAM species, particularly annually, trees and hemi-epiphytes in seasonally-dry habitats (Zotz and Winter 1994a, 1994b; Winter and Holtum 2014). These photosynthetically highly flexible species often exhibit reduced rates of growth when CAM is the predominate photosynthetic pathway. In contrast, many open-field CAM perennials such as A. angustifolia and its close relatives, A. fourcroydes and A. tequilana, combine strong CAM with substantial growth rates. In these species, the persistence of CAM throughout large parts of the dry season can enable appreciable rates of growth in concert with high water-use efficiency (Nobel 1985, 1988, 1991; Nobel and Valenzuela 1987). It is this ability to exhibit year-round water-use efficient growth in seasonally-dry conditions that enables agaves to accumulate biomass at sizeable annualised rates and has resulted in them being considered as biofuel feedstock crops in regions where growth of traditional food and bioenergy crops is suboptimal (Nobel 1988; Borland et al. 2009; Davis et al. 2011; Holtum et al. 2011).

In conclusion, consistent with its tropical occurrence, A. angustifolia successfully grew at high day/night temperatures, and low night temperatures combined with high day temperatures adversely affected growth. In all situations, CAM was the principal contributor to carbon gain, suggesting limited photosynthetic plasticity in this leaf-succulent constitutive CAM species.

There is substantial literature on the effects of temperature on daily CO₂ exchange patterns of CAM plants. What is clearly needed is research that extends these studies to temperature effects on growth, particularly of species native to habitats with contrasting temperature regimes.
Acknowledgements

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