

1 Increase in leaf temperature opens stomata and decouples net photosynthesis from stomatal
2 conductance in *Pinus taeda* and *Populus deltoides x nigra*

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18 **Title:** Increase in leaf temperature opens stomata and decouples net photosynthesis from stomatal
19 conductance in *Pinus taeda* and *Populus deltoides x nigra*

20

21 **Highlight:** Stomatal conductance of two species (a broadleaf and a conifer) increased with
22 increasing temperature. This response was independent of carbon metabolism, plant water status,
23 or vapor pressure difference.

24

25 **Abstract:** The effect of temperature on stomatal conductance (g_s) and corresponding gas
26 exchange parameters was studied in two tree species with contrasting leaf anatomy and
27 ecophysiology – a broad-leaf angiosperm, *Populus deltoides x nigra* (poplar) and a needle-leaf
28 gymnosperm, *Pinus taeda*, (loblolly pine). Experiments were conducted in growth chambers
29 across a leaf temperature range of 19 to 48 °C. Manipulations of temperature were done in well-
30 watered and droughted soil conditions and under ambient and elevated air CO₂ concentrations
31 ([CO₂], 400 and 800 ppm, respectively). Increases in leaf temperature caused stomatal opening at
32 both ambient and elevated [CO₂]. The g_s increased by 42 % in poplar and by 40 % in loblolly
33 pine when leaf temperature increased from 30 °C to 40 °C at vapor pressure difference of 1 kPa.
34 Stomatal limitation to photosynthesis decreased in elevated temperature in loblolly pine but not in
35 poplar. The ratio of net photosynthesis to g_s depended on leaf temperature, especially at high
36 temperatures. Evaporative cooling of transpiring leaves resulted in reductions in leaf temperature
37 up to 9 °C in well-watered poplar but only 1 °C in drought stressed poplar and in loblolly pine.
38 As global mean temperatures rise and temperature extremes become more frequent and severe,
39 understanding the effect of temperature on stomatal conductance, and modelling that relationship,
40 will become increasingly important.

41

42 **Keywords:** Ball-Berry model; elevated temperature; evaporative cooling; global change; heat
43 waves; stomatal conductance

44

45 **Abbreviations:**

46 Ψ_P – prelight water potential (Pa)

47 A – light saturated net photosynthesis ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

48 C_a – atmospheric concentration of CO_2 ($\mu\text{mol mol}^{-1}$)

49 C_i – intercellular concentration of CO_2 ($\mu\text{mol mol}^{-1}$)

50 E – transpiration ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$)

51 g_m – mesophyll conductance ($\text{mol m}^{-2} \text{s}^{-1}$)

52 g_s – stomatal conductance ($\text{mol m}^{-2} \text{s}^{-1}$)

53 J_{max} – maximum rate of photosynthetic electron transport ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

54 L_s – stomatal limitation to photosynthesis (%) R_d^* - day respiration ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

55 T_a – air temperature ($^{\circ}\text{C}$)

56 T_l – leaf temperature ($^{\circ}\text{C}$)

57 V_{Cmax} - maximum rate of RuBisCO carboxylation ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

58 VPD – vapor pressure deficit (Pa)

59 V_{Tpu} - maximum rate of triose-phosphate utilization ($\mu\text{mol m}^{-2} \text{s}^{-1}$)

60

61 **Introduction**

62 Plant stomata play a key role in water and carbon cycles. On average, plant transpiration
63 accounts for 61 % of global evapotranspiration (Schlesinger and Jasechko, 2014). In other words,
64 most water moving from terrestrial ecosystems into the atmosphere passes through plants and the
65 precise amount is regulated by stomata. At the same time, stomatal conductance (g_s) is a key
66 factor determining the rate of net photosynthesis and, therefore, the global carbon cycle and plant
67 carbon metabolism. As a result, stomatal regulation is one of the main factors which determine
68 local growth and survival of plants and global cycles of mass and energy. Stomatal conductance
69 is so important that it has become central to many models on scales from the leaf (Ball *et al.*,
70 1987; Leuning *et al.*, 1995; Jarvis and Davies, 1998; Tuzet *et al.*, 2003), to the tree- and forest-
71 stand level (Mirfenderesgi *et al.*, 2016; Xu *et al.*, 2016), and even up to the global level (Niyogi
72 *et al.*, 2009; Berry, 2012; Verhoef and Egea, 2014). However, the conditions in which plants
73 grow are changing and we still do not know enough about plant stomatal regulation to predict
74 future stomatal responses of plant species and their effects at ecosystem and global scales (Lin *et*
75 *al.*, 2015).

76 Temperature is one of the most variable factors in the environment and it affects many
77 plant physiological processes, yet little is known about its effect on g_s , especially at high
78 temperatures (Teskey *et al.*, 2015). Historically, temperatures over 40 °C have been recorded in
79 many places in North America. It has been predicted that later in this century, mean maximum
80 summer temperatures will increase 5 °C in the eastern United States (Lynn *et al.*, 2007). Here, we
81 studied effects of temperature on the leaf gas exchange of two North American tree species,
82 *Pinus taeda*, (loblolly pine) and *Populus deltoides x nigra* (poplar). Loblolly pine is native to the
83 southeastern United States where the highest temperatures recorded among the 12 states in the
84 region range from 43 to 49 °C, with a mean maximum temperature for all 12 states of 45 °C
85 (National Climatic Data Center, 2016). Hybrid poplar is widely planted in the Northern Great
86 Plains, which includes the states of Nebraska, Wyoming, Montana, North Dakota and South
87 Dakota. The highest recorded temperatures in those states range from 46 to 49 °C with a mean of
88 48 °C. In addition to increases in mean air temperature, the frequency of extreme temperatures
89 and the severity of heat waves have also increased, and are likely to increase further (Meehl and
90 Tebaldi, 2004; Perkins *et al.*, 2012). Summertime extreme temperatures associated with
91 prolonged heat waves now impact approximately 10 % of land surfaces, up from 1 % in the

92 1960s (Hansen *et al.*, 2012). Over recent decades record-breaking monthly temperature extremes
93 have occurred five times more often than during the late 19th through the mid-20th century
94 (Coumou and Robinson, 2013). Heat waves are usually associated with low precipitation and soil
95 drought (Ciais *et al.*, 2005; Stéfanon *et al.*, 2014). However, the frequency of heat waves during
96 wet periods is also increasing. When temperature and precipitation were compared between the
97 periods of 1951 – 1977 and 1978 – 2004, it was apparent that both wet/hot and dry/hot conditions
98 were increasing substantially worldwide (Hao *et al.*, 2013). Effects of increasing frequency and
99 severity of extreme temperature events on g_s are largely unknown.

100 Results of experiments that examined the direct dependence of stomatal conductance on
101 temperature have not been consistent. Previous studies have reported a complete range of
102 responses to increased temperature, including stomatal opening (Schulze *et al.*, 1974; Freedman
103 and Sage, 1999; Lu *et al.*, 2000; Mott and Peak, 2010), no significant response (Teskey *et al.*,
104 1986; Sage and Sharkey, 1987; Cerasoli *et al.*, 2014; von Caemmerer and Evans, 2015), and
105 stomatal closure (Raven *et al.*, 2005; Weston and Bauerle, 2007; Lahr *et al.*, 2015). A peaked
106 response with maximum g_s at 20 °C (Way *et al.*, 2011) or more complex responses with one peak
107 between 20 and 30 °C and an additional increase at extremely high temperatures (Slot *et al.*,
108 2016) have also been described. One possible explanation for these inconsistent results is that to
109 isolate the direct effect of temperature on g_s requires a well-controlled environment, which is
110 often hard to achieve, particularly with respect to vapor pressure difference (*VPD*). In addition,
111 differences in sensitivity to heat are likely related to species, whether plants were grown in the
112 lab or in the field, and the range of measurement temperature (Slot *et al.*, 2016).

113 It has been well-established that plants regulate rates of transpiration and photosynthesis
114 in parallel, maintaining a balance between g_s and photosynthesis (Lawson *et al.*, 2011).
115 Therefore, the effect of temperature on stomata is often considered to be indirect, through *VPD*,
116 transpiration, leaf water potential, or the effect of temperature on photosynthesis or intercellular
117 CO₂ concentration (C_i). This parallel regulation results in the conservation of internal [CO₂] (C_i)
118 at a given atmospheric [CO₂] (C_a) and a close correspondence between g_s and net photosynthesis
119 (Wong *et al.*, 1979; Hetherington and Woodward, 2003). The latter relationship has been central
120 to several models of stomatal control of photosynthesis (Farquhar and Wong, 1984; Ball *et al.*,
121 1987; Leuning, 1995; Buckley *et al.*, 2003), which assume that the ratio of g_s correlates with net
122 photosynthesis over a wide range of environmental conditions. However, some studies indicated

123 that under extreme temperature during heat waves, this relationship was decoupled, such that net
124 photosynthesis decreased, but g_s did not. For example, during an imposed heat wave in which
125 daily maximum air temperature ranged from 47 to 53 °C and VPD ranged from 6 to 8 kPa, *Pinus*
126 *taeda* and *Quercus rubra* seedlings exhibited progressively lower net photosynthesis on each day
127 of the heat wave but almost no change in g_s (Ameye *et al.*, 2012). Similarly, stomatal
128 conductance of *Acer rubrum* changed very little across a temperature range of 35 to 48 °C
129 (Weston and Bauerle, 2007). In a study of five species, g_s either increased or did not decline as
130 air temperature increased from 20 to 40 °C, even though net photosynthesis initially increased
131 from 20 to 30 °C and then decreased (von Caemmerer and Evans, 2015). Collectively these
132 studies suggest that the mechanism modulating stomatal aperture may be independent of
133 photosynthesis at higher temperatures. However, because VPD varied with temperature in all of
134 these studies, it could not be determined to what degree the observed changes in g_s were due to a
135 change in VPD or in the rate of photosynthesis, or were a direct response to temperature.

136 In this study, we addressed the following questions: 1. What is the direct effect of
137 moderate to high temperature on g_s ? 2. Is the effect of moderate to high temperature on g_s altered
138 by water stress or atmospheric CO₂ concentration? 3. How does the response of g_s to temperature
139 link to other related factors such as photosynthesis, intercellular [CO₂] and water status
140 (transpiration, water potential), and how does the correlation between g_s and net photosynthesis,
141 which is crucial to many models, change with temperature? 4. What is the magnitude of
142 evaporative cooling under extreme temperatures? To answer these questions we performed leaf
143 gas exchange measurements on two contrasting tree species: poplar (*Populus deltoides x nigra*)
144 and loblolly pine (*Pinus taeda*) across a range of temperature and humidity and under well-
145 watered and drought conditions.

146

147 **Material and Methods**

148 *Growth chambers and tree material*

149 Trees were grown, and measurements conducted, in two walk-in growth chambers (EGC 36,
150 Environmental Growth Chambers, Chagrin Falls, OH, USA) at the University of Georgia campus
151 in Athens, Georgia USA. Prior to the start of experimental treatments, the trees were grown in the
152 chambers for 30 days at 26 °C/ 23 °C (Day/Night) air temperature (T_a), 1700 / 560 Pa
153 (Day/Night) air vapor pressure deficit, and a daily light period of 13 hours. Photosynthetically

154 active radiation (*PAR*) in the chambers was $520 \mu\text{mol m}^{-2} \text{s}^{-1}$. Air speed in each chamber was
155 maintained at 1 m s^{-1} . During the growth period the atmospheric concentration of CO_2 ($[\text{CO}_2]$)
156 was maintained above $400 \mu\text{mol mol}^{-1}$ as follows: A CO_2 sensor (GMM 220, Vaisala, Helsinki,
157 Finland) monitored $[\text{CO}_2]$ in each chamber and controlled a solenoid valve that released CO_2
158 from a compressed gas cylinder into the chamber whenever the $[\text{CO}_2]$ fell below the $400 \mu\text{mol}$
159 mol^{-1} setpoint. Although this procedure prevented $[\text{CO}_2]$ from decreasing below $400 \mu\text{mol mol}^{-1}$
160 during periods of active photosynthesis, it did not prevent increases above $400 \mu\text{mol mol}^{-1}$. To
161 mitigate buildup of CO_2 in the chambers, the exterior room windows were fully opened and a
162 large exhaust fan was placed in one window. We estimate that daytime ambient $[\text{CO}_2]$ in the
163 chambers was typically between 400 and $475 \mu\text{mol mol}^{-1}$.

164 Measurements were made on clones of two tree species: a poplar (*Populus deltoides x*
165 *nigra*) clone obtained as cuttings (OP-367, hybridpoplars.com, Glenmoore, PA, USA) and a
166 loblolly pine (*Pinus taeda*) clone from the South Carolina Coastal Plain (Arborgen, Ridgeville,
167 SC, USA). Two-year-old loblolly pine saplings, originally grown in 4-liter pots in a greenhouse
168 where they experienced natural temperature fluctuations with temperatures commonly reaching ~
169 40°C , and poplar cuttings were planted in March 2014 into 15 L pots in a potting medium
170 (Cofer's Nursery Mix, Cofer's, Athens, GA, USA). Each pot was fertilized with 40 g of 15-9-12
171 extended release fertilizer (Osmocote Plus #903286, Scotts-Sierra Horticultural Products,
172 Marysville, OH, USA) and 0.2 g of chelated iron (Sprint 138, Becker Underwood, Ames, IA,
173 USA). Trees were watered daily to full soil water capacity. At the beginning of the experiment, in
174 April 2014, mean stem height of the poplars was 1.05 m, and diameter 10 cm above soil was 9.2
175 mm. Mean height and diameter of the loblolly pines was 1.1 m and 13.9 mm, respectively.

176

177 *Gas exchange measurements*

178 Measurements of light-saturated net photosynthesis (*A*), stomatal conductance (g_s),
179 transpiration (*E*), and internal CO_2 concentration (C_i) were made with a portable photosynthesis
180 system equipped with a CO_2 mixer (LI-6400-20, LiCor Biosciences, Lincoln, NE, USA). Leaf
181 cuvette conditions were set as follows: block temperature was set at ambient (growth chamber)
182 temperature, $[\text{CO}_2]$ was set at either $400 \mu\text{mol mol}^{-1}$ or $800 \mu\text{mol mol}^{-1}$, equal to the
183 concentration in the growth chamber, relative humidity was maintained the same as in the growth
184 chamber, and *PAR* was set at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ where the photosynthesis was light saturated and

185 there was no decline as a result of photorespiration (see Fig. 2 in Ingwers *et al.* (2016) for the
186 photosynthetic light response curve of loblolly pine trees of the same clone measured in the same
187 growth chambers). Measurements of loblolly pine foliage were made on two fully developed
188 fascicles (six needles total) of the second flush attached to the main stem. The needles were
189 arranged in the cuvette on a flat plane with equal spacing between needles to maximize light
190 interception. After the gas exchange measurement, the widths of each of three sides of the
191 needles were measured with a scale lupe and used to calculate the foliage area in the cuvette. For
192 poplar, measurements were made on approximately the 30th leaf from the top of the plant. Gas
193 exchange measurements were performed on six trees of each species (n=6). Gas exchange results
194 were calculated on a total surface area basis for loblolly pine and a one-sided surface area basis
195 for poplar.

196

197 *Experimental setup*

198 *Responses to changes in temperature and VPD under various [CO₂] and soil moisture*

199 To determine stomatal responses to temperature and VPD, T_a in the growth chamber was
200 controlled at 20, 30, 40, or 49 °C and relative humidity was changed from approximately 30 to
201 80 % at each temperature. The sequence of the temperature changes was chosen randomly and
202 individual trees were excluded from further measurements after they had been subjected to 49 °C.
203 Six trees were allowed to acclimate for at least 45 minutes after each change in environmental
204 conditions. At every measurement, g_s , A , E , and C_i were recorded. To insure high water
205 availability, during the measurement period the base of each pot was placed in a 5 cm tall
206 container that was kept full of water. Pre-light water potential (Ψ_P) and water potential at varying
207 T_a and VPD in the light were measured on foliage using a pressure chamber (model 700, PMS
208 Instrument, Albany, OR, USA). Mean Ψ_P was -0.28 ± 0.02 and -0.13 ± 0.02 MPa (mean \pm standard
209 error) for loblolly pine and poplar, respectively. Measurements were conducted under ambient
210 [CO₂] (400 $\mu\text{mol mol}^{-1}$) and elevated [CO₂] (800 $\mu\text{mol mol}^{-1}$). For measurements under elevated
211 [CO₂], the [CO₂] was increased in the growth chamber to 800 $\mu\text{mol mol}^{-1}$ as described above by
212 reprogramming the setpoint of the CO₂ sensor. The plants were allowed to equilibrate to elevated
213 [CO₂] for 24 hours prior to measurements.

214 In a subsequent experiment the effect of soil water deficit on the stomatal response to
215 temperature was investigated. After withholding water for 5 days, mean Ψ_P of the poplar plants

216 was -0.81 ± 0.10 MPa. After withholding water for 12 days, mean Ψ_P of the loblolly pine plants
217 was -0.97 ± 0.06 MPa. On those days, measurements were made using the same combinations of
218 temperature and humidity as in the first experiment. The effect of water deficit was studied only
219 at ambient $[\text{CO}_2]$. The first experiment and this experiment were conducted on different trees
220 ($n=6$ for each experiment).

221

222 *Effect of C_i on A at various temperatures (A/C_i curves)*

223 Under well-watered conditions, A/C_i curves were measured in the growth chamber on six
224 trees of each species. The VPD was held constant at 1.2 kPa at T_1 of 20 °C and 3.5 kPa at T_1 of 30
225 °C and 40 °C both in the growth chamber and the cuvette. Photosynthetically active radiation in
226 the cuvette was set at $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$. The concentration of CO_2 in the cuvette was
227 manipulated from 50 to 100 $\mu\text{mol mol}^{-1}$ and then in 100 $\mu\text{mol mol}^{-1}$ steps to 1800 $\mu\text{mol mol}^{-1}$.
228 The A/C_i Curve Fitting Utility, version 1.1 (Long and Bernacchi, 2003) was used to determine
229 maximum rate of RuBisCO carboxylation ($V_{c_{\text{max}}}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum rate of photosynthetic
230 electron transport (J_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum rate of triose-phosphate utilization (V_{TPI} , μmol
231 $\text{m}^{-2} \text{s}^{-1}$) and day respiration in the absence of mitochondrial respiration (R_d^* , $\mu\text{mol m}^{-2} \text{s}^{-1}$).

232 Stomatal limitation to photosynthesis (L_s) was estimated at $[\text{CO}_2]$ 400 $\mu\text{mol mol}^{-1}$ from
233 fitted curves using the equation

$$234 \quad L_s = \frac{A_0 - A}{A_0} \quad (1)$$

235 where A_0 is the photosynthesis rate that would occur at infinite stomatal conductance (Farquhar
236 and Sharkey, 1982).

237

238 *Cooling effect*

239 Under lighted conditions, the cooling effect of transpiration was estimated as the
240 difference between the temperature of normal transpiring foliage and foliage greased with
241 petroleum jelly to prevent transpiration (Jones, 2002) at the same position on the plant. Leaves
242 and needles were chosen for this comparison at a position on the plant close to the point where
243 gas exchange was measured. Leaf temperature (T_l) was measured with an infrared thermometer
244 (Model 561, Fluke, Everett, WA, USA) with emissivity set to 0.97.

245

246 *Statistical analysis*

247 Prior to the analyses, normality of data was determined using the Shapiro-Wilk test. We
248 used linear and non-linear multiregression analysis to describe the dependence of stomatal
249 conductance on external factors (i.e. T_1 , VPD). A least squares regression was used to fit the 3D
250 models to the data. Models used to fit data are listed in the supplementary Table S1. An F-test
251 was used to test significance of model parameters. Analysis of the generalized linear model
252 (GLZ) was used to test for differences among independent variables and a dependent variable
253 when VPD was a continuous predictor. Tests were performed at $\alpha = 0.05$. Most statistical
254 analyses were performed using SigmaPlot 12.5 software (Systat, San Jose, CA, USA) with the
255 exception of GLZ analysis which was done in Statistica 12 (StatSoft, Tulsa, OK, USA).

256

257 **Results**

258 *Responses of stomatal conductance, transpiration, and photosynthesis to T_1 and VPD*

259 The g_s increased with increasing T_1 and T_a in both species in all tested environmental
260 conditions (Figs. 1 and S1). Under unlimited soil water availability, when leaf temperature
261 increased from 30 °C to 40 °C, g_s increased by 42 % in poplar and by 40 % in loblolly pine, at a
262 VPD of 1 kPa and $[CO_2]$ of 400 $\mu\text{mol mol}^{-1}$ (Fig. 1a, d; Table S1; $p < 0.001$). The rate of increase
263 in g_s with temperature was linear in poplar, but g_s increased more at high than at low T_1 in
264 loblolly pine. Increasing the $[CO_2]$ from 400 to 800 $\mu\text{mol mol}^{-1}$ caused partial stomatal closure,
265 which was more pronounced in poplar (mean decrease of 21% at VPD 3.5 kPa, $p < 0.001$) than in
266 loblolly pine (mean decrease of 12 % at the same VPD , $p = 0.030$). However, similar to results in
267 ambient $[CO_2]$, g_s increased with increasing T_1 in both species under elevated $[CO_2]$ (Fig. 1b, e; p
268 < 0.001). Soil water deficit significantly reduced g_s in both species, but more so in poplar than
269 pine (Fig. 1c, f; $p < 0.001$). Even though g_s was reduced in drought, g_s of both species still
270 increased with increasing T_1 ($p = 0.040$ for poplar and $p < 0.001$ for loblolly pine).

271 Transpiration (E) increased significantly with increasing T_1 (and T_a) or VPD in both
272 species under unlimited soil water availability and ambient $[CO_2]$ (Figs. 2a, b and S2a, b).
273 However, the relationships between E and environmental variables differed substantially between
274 poplar and loblolly pine. Transpiration of poplar increased with VPD ($p < 0.001$) but not with T_1

275 ($p = 0.06$). Conversely, in loblolly pine, E increased only with T_l ($p < 0.001$) but not with VPD (p
276 $= 0.15$).

277 Under well-watered conditions, C_i increased with increasing temperature in both species
278 (Figs. 3a, c, $p < 0.001$ and S2c, d). A decrease in C_i with increasing VPD was observed in poplar
279 ($p < 0.001$) but not in loblolly pine ($p = 0.15$). In addition, the range of C_i was smaller in poplar
280 than in loblolly pine. Leaf (and air) temperature had an effect on net photosynthesis in both
281 species (Figs. 3b, d, $p < 0.001$ and S3). In both species, at a given T_l there was a specific
282 relationship between A and g_s . However, this relationship between A and g_s changed with leaf
283 temperature (Fig. 3 b, d, $p < 0.001$).

284 *A/C_i curves and stomatal limitations to A at various T_l*

285 Temperature had a large effect on the parameters of A/C_i curves in both poplar and
286 loblolly pine (Table 1). Stomata of poplar imposed a smaller limitation on diffusion of carbon
287 dioxide than stomata of loblolly pine. The relative stomatal limitations in poplar did not exceed
288 20 % while in loblolly pine they were between 23 and 78 %. Stomatal limitation was directly
289 comparable between 30 °C and 40 °C because it was measured at the same VPD . While stomatal
290 limitation in poplar did not change ($p = 0.21$) with a leaf temperature increase from 30 °C to
291 40 °C, stomatal limitation in loblolly pine declined under the same temperature increase ($p <$
292 0.001). The values of parameters related to biochemical processes of photosynthesis, i.e.
293 maximum rate of RuBisCO carboxylation ($V_{c_{max}}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum rate of photosynthetic
294 electron transport (J_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum rate of triose-phosphate utilization (V_{TPU} , μmol
295 $\text{m}^{-2} \text{s}^{-1}$) and day respiration (R_d^* , $\mu\text{mol m}^{-2} \text{s}^{-1}$), consistently increased with leaf temperature in
296 both species, with the exception of V_{TPU} in poplar.

297 *Effect of E on leaf temperature*

298 The temperature of transpiring leaves was lower than the temperature of foliage that did
299 not transpire (Fig. 4). The magnitude of the temperature difference in poplars in wet soil reached
300 up to 9.0 °C and scaled with VPD ($p < 0.001$) but not with air temperature (Fig. 4a). Transpiring
301 leaves of poplar in dry soil were an average of 1.1 °C cooler than non-transpiring leaves ($p =$
302 0.02) and the magnitude of the cooling effect depended neither on temperature nor on VPD (Fig.
303 4b). In loblolly pine, transpiring needles were an average of 0.9 °C cooler than those that did not

304 transpire ($p = 0.002$). There was no effect of soil water availability and the magnitude of the
305 cooling did not depend on temperature or *VPD* (Fig. 4c).

306 *Leaf water potential*

307 Leaf water potential decreased with increasing T_1 and *VPD* in both species when the soil
308 was wet (Fig. 5a, c). When soil was dry, leaf water potential scaled with both T_1 and *VPD* in
309 poplar, but in loblolly pine only *VPD* had an effect on water potential (Fig. 5b, d). At the same T_1
310 and *VPD*, poplar maintained higher water potential than loblolly pine.

311

312 **Discussion**

313 *Stomatal conductance, stomatal limitations and photosynthesis*

314 Stomata play a key role in regulating fluxes of water and carbon dioxide between plant
315 and atmosphere. They regulate both plant growth and cycles of mass and energy. Therefore,
316 much attention has been focused on principles of stomatal regulation by and several regulatory
317 mechanisms have been identified. Most research has centered on the stomatal responses to
318 various indices of water status and carbon balance (Farquhar and Sharkey, 1982; Jones, 1998;
319 Buckley *et al.*, 2003). Surprisingly little attention has been paid to the responses of stomatal
320 conductance to temperature, even though it is one of the most variable environmental factors. A
321 few previous studies suggested a dependence of g_s on temperature. However, these studies have
322 often provided conflicting results. While some evidence suggested that g_s increased with
323 increasing temperature (Schulze *et al.*, 1974; Lu *et al.*, 2000; Mott and Peak, 2010), other studies
324 found that temperature had no effect on stomata (Teskey *et al.*, 1986; Sage and Sharkey, 1987;
325 Cerasoli *et al.*, 2014; von Caemmerer and Evans, 2015), or that increased temperature triggered
326 stomatal closure (Weston and Bauerle, 2007; Lahr *et al.*, 2015). One explanation for the
327 conflicting results across these studies might be that the experiments were often conducted in
328 uncontrolled environmental conditions in the field. The design of our experiment, where the
329 response of g_s to T_1 was separated from the effect of *VPD* and all measurements were made under
330 constant illumination, allowed us to separate the effect of temperature from the effects of other
331 factors.

332 Our results conclusively demonstrated that there is strong direct positive response of g_s to
333 increasing leaf temperature in two tree species. In well watered trees, temperature and VPD had
334 major effects on g_s , as suggested by Freeden and Sage (1999). Elevated atmospheric $[CO_2]$
335 caused a decline in g_s but did not fully mitigate increased stomatal opening in response to
336 increased temperature. The increase in g_s with increased T_l was found in both species despite
337 large differences in leaf morphology, xylem structure, and physiology. However, because of
338 these differences, the magnitude of stomatal opening in response to T_l and closing in response to
339 elevated $[CO_2]$, along with the effects on associated physiological processes (such as
340 transpiration and photosynthesis), differed between the two species. The interplay between
341 elevated T_l , which increased g_s , and elevated $[CO_2]$, which decreased g_s , differed between the two
342 species, suggesting that it could contribute to differences in behavior among species in the
343 predicted future climate.

344 The two experimental species stand at opposite ends of the range of mechanisms for
345 stomatal adjustment of water loss. Transpiration in poplar continuously increased with increasing
346 VPD , while transpiration of loblolly pine remained the same over a large range of VPD within a
347 given T_l and increased with increases in leaf temperature (Figs. 2, S2). These results suggest that
348 stomatal conductance is regulated by more complex mechanisms than simply transpiration rate
349 (Mott and Parkhurst, 1991), and that temperature changes affect the relationship between
350 transpiration and g_s .

351 Leaf water potential declined with both increased temperature and increased VPD in both
352 species (Fig. 5). Typically, g_s declines with a decline in water potential across a wide range of
353 both iso- and anisohydric species (Klein, 2014). But in our study, despite a decline in water
354 potential, g_s increased with temperature. The answer to why stomata opened with increasing
355 temperature may be, in part, changes in hydraulic conductivity. When temperature increases,
356 viscosity of water declines and mesophyll conductance increases which may improve the supply
357 of water to sites of evaporation and thus increase stomatal aperture (Cochard *et al.*, 2000; von
358 Caemmerer and Evans, 2015). However, this increase was not great enough to prevent a decline
359 in leaf water potential. Therefore, it was proposed that resistance to water vapor and heat transfer
360 among sites of evaporation and guard cells, which induce differences in temperature and VPD at
361 these sites, may also regulate stomatal opening in response to transpiration and T_l (Mott and
362 Peak, 2013). The general increase in overall tree hydraulic conductance due to water viscosity

363 may be further modified by temperature-dependent variability in tree xylem hydraulic
364 conductance which, due to differences in vascular traits, may contribute to differences in the
365 responses of conifers and angiosperm trees (Wolf *et al.*, 2016). Changes in leaf mesophyll
366 conductance may be further paired with xylem resistance to embolism and the safety margin
367 against cavitation which is higher in conifers than in angiosperms (Choat *et al.*, 2012). Trees
368 adjust their stomatal conductance to maximize CO₂ uptake (resulting in higher transpiration) but
369 still protect xylem against excessive cavitation (Brodribb *et al.*, 2016). Loblolly pine strictly
370 regulated transpiration such that it did not change with variation in VPD, thus protecting xylem
371 against cavitation and maintaining a broad safety margin. However, when temperature increased,
372 loblolly pine was not able to maintain this strict control over water loss, so transpiration
373 increased. This result may suggest that in the pine, overall resistance of the hydraulic pathway
374 (including xylem and mesophyll resistance) significantly contributed to regulation of
375 transpiration and that stomatal regulation was at least partly independent of the rate of
376 transpiration. In contrast, the broadleaf poplar exerted the same degree of stomatal control on
377 transpiration at all temperatures. The inability of loblolly pine to regulate transpiration when
378 temperature increases may negatively impact survival with climate change and may contribute to
379 succession by angiosperm tree species (Carnicer *et al.*, 2013).

380 Apart from plant water status, other mechanisms known to regulate g_s are related to
381 photosynthesis, to which stomata often present a large limitation. Stomatal limitation in loblolly
382 pine is usually lower than 65%. Higher L_s may occur but it is usually attributed to low soil water
383 potential or low temperature (Teskey *et al.*, 1986; Sasek and Richardson, 1989; Ellsworth, 2000).
384 In this study when VPD was high, stomatal limitation of 78 % was observed at 30 °C (Table 1),
385 indicating strong stomatal control of carbon gain in the range of temperature which is optimal for
386 photosynthesis. With increasing T_1 , stomatal limitation declined. Therefore, photosynthesis of
387 loblolly pine may partly benefit from the decline in L_s at increased temperature, even though the
388 extremely high temperature will set biochemical limits to A and the resulting A may be the same
389 or lower. In contrast to loblolly pine, L_s in poplar was unaffected by T_1 and was generally lower
390 than 20 %. Low L_s in poplar in this study corresponded to low L_s in poplar observed previously;
391 for example, L_s averaged 10 % in two clones of *Populus* (Noormets and Sober, 2001). The lower
392 stomatal limitation in poplar compared with loblolly pine may have been related to the ratio of g_s
393 to mesophyll conductance (g_m). Although we did not measure g_m , it is generally lower in conifers

394 than in angiosperm trees (Flexas *et al.*, 2012), suggesting L_s should also be lower. However,
395 because L_s was not lower, we speculate that the ratio of g_s to g_m also differed between the species.
396 The high rate of photosynthesis in poplar might be related to high g_s/g_m , which could support
397 increased photosynthesis by increasing C_i and keeping CO_2 concentration at chloroplasts high. It
398 could also increase nutrient acquisition through increased transpiration, which would enhance
399 photosynthetic capacity. Mesophyll conductance also increases with temperature in a wide range
400 of species (von Caemmerer and Evans, 2015). However, this mechanism does not explain the
401 increase in g_s at supra-optimal temperatures at which photosynthesis becomes low or negative.

402 Low stomatal limitation in poplar was linked to high stomatal conductance, which results
403 in low water use efficiency of photosynthesis. The advantage of low L_s , which favors fast-
404 growing species under unlimited soil water supply, may jeopardize their existence during heat
405 waves when high transpiration depletes available soil water, resulting in increased drought stress,
406 especially under initial conditions of low soil moisture. The effect of variable stomatal limitation
407 was further demonstrated by the alteration of C_i in loblolly pine. Normally the ratio of $C_i:C_a$ is
408 highly conserved (Liu and Teskey, 1995), as was observed in poplar where C_i consistently
409 remained at $\sim 300 \mu\text{mol mol}^{-1}$ at all temperatures (Fig. 3a). However, C_i in loblolly pine was
410 highly variable, ranging from about $165 \mu\text{mol mol}^{-1}$ to about $240 \mu\text{mol mol}^{-1}$ at temperatures of
411 20 and 40 °C (Fig. 3c), respectively, which corresponded with prior observations of high
412 variability in C_i with changing environmental conditions in this species (Green and Mitchell,
413 1992).

414 415 *Evaporative cooling*

416 Evaporation of water from the leaf surface can significantly lower leaf temperature
417 (Monteith, 1981; Jones, 1999). As long as stomata remain open, evaporative cooling can mitigate
418 the negative effect of supra-optimal air temperature on A during heat waves and can positively
419 affect photosynthesis, yield, and plant survival (Lu *et al.*, 1994; Ameye *et al.*, 2012). Maintaining
420 leaf temperature through regulation of transpiration to minimize stress at high air temperature
421 was theoretically suggested (Mahan and Upchurch, 1988) and observations in *Arabidopsis*
422 indicated that plants regulate water loss and even adjust their architecture to achieve the best
423 cooling effect (Crawford *et al.*, 2012). The magnitude of the cooling effect is often several
424 degrees (Jones, 1999; Feller, 2006). In our study the maximum cooling, 9 °C, was observed in

425 poplar at high T_a and high VPD (Fig. 4). This rate of cooling lowered T_l from 49 °C to 40 °C and
426 positive photosynthesis was observed at this extreme air temperature. In contrast to poplar, g_s of
427 loblolly pine was roughly ten times lower and therefore the maximum cooling effect was only 0.9
428 °C. Consequently, at T_a of 49 °C, poplar had positive photosynthesis and loblolly pine did not.
429 The cooling effect due to stomatal opening at high temperature (under well-watered conditions) is
430 likely to be much more beneficial in species with high g_s than those with low g_s .

431 Evaporative cooling may help plants survive heat waves, especially when the air is dry.
432 However, this mechanism requires sufficient soil water supply, which relies on high soil water
433 capacity and sufficient hydraulic conductivity. With a long-duration heat wave, high transpiration
434 may result in depletion of soil water storage and plants will no longer be able to utilize this
435 mechanism to minimize heat stress. This effect was observed in our study: only a very small
436 cooling effect (1.1 °C) was observed in drought stressed trees (Fig. 4b). Nevertheless,
437 evaporative cooling proved to have a significant effect on photosynthesis and may play an
438 important role in diurnal regulation of leaf temperature during short-duration heat waves. In
439 addition to soil water availability, elevated $[CO_2]$ affects g_s . Stomatal closure resulting from
440 elevated $[CO_2]$ will to some degree counteract the opening effect of elevated temperature. Results
441 of this study, demonstrating that stomata of poplar are more sensitive to $[CO_2]$ than stomata of
442 loblolly pine, were similar to previous findings on broad-leaf and conifer species in general
443 (Medlyn *et al.*, 2001). Therefore, if stomata in broad-leaf species close in response to future
444 predicted increases in $[CO_2]$, the difference in the rate of evaporative cooling between broad-leaf
445 and conifer species is may shrink.

446

447 *Relationships among g_s , C_i , and A*

448 In both species we found that the positive relationship between A and g_s observed at lower
449 temperatures was not present at extremely high temperatures. The most obvious impairment
450 occurred at $T_l > 40$ °C, when A became negative and yet the stomata remained open (Fig. 3). C_i at
451 this temperature increased and approached the ambient $[CO_2]$ of 400 $\mu\text{mol mol}^{-1}$. Under these
452 conditions a reduction in g_s would be expected (Hashimoto *et al.*, 2006), but instead the stomata
453 opened even more. These results do not imply that stomata do not react to C_i . Rather, it appeared
454 that there was a direct stomatal response to supra-optimal temperature that overrode the response
455 to C_i .

456 Many models of g_s assume a fixed relationship between A and g_s regardless of
457 temperature (Ball *et al.*, 1987; Leuning, 1995; Buckley *et al.*, 2003). These models have been
458 widely used and, in a comparison with other models of g_s provided the best results (Way *et al.*,
459 2011). Our study also provided evidence of a stable relationship between A and g_s at low
460 temperatures (Fig. 3). However that stability did not hold true at high temperature. As an extreme
461 example, when A became negative at temperatures over ~ 40 °C, the ratio $A:C_i$ also became
462 negative in both species. In such a case, the Ball-Berry-Leuning model, which uses that ratio to
463 predict g_s , would provide negative values of g_s . Correctly predicting g_s from photosynthesis, and
464 vice versa, especially at extreme temperatures during heat waves will require detailed study of the
465 interplay among A , C_i , VPD , T_1 and possibly other factors driving stomatal regulation, which,
466 when applied simultaneously can have complex effects (Merilo *et al.*, 2014).

467

468 *Conclusions*

469 We conclude that T_1 has a direct effect on stomatal opening in the two tree species we
470 examined. For accurate predictions of g_s and plant water use this temperature dependency should
471 be taken into account, especially at high temperatures. Elevated $[CO_2]$ reduced g_s of both species
472 but general trends of increasing g_s with increasing T_1 remained similar regardless of $[CO_2]$. Along
473 with changes in g_s , T_1 also affected stomatal limitation to photosynthesis, C_i , and corresponding
474 A . Net photosynthesis became negative in both species at extremely high T_1 . However, the effect
475 of evaporative cooling, which lowered T_1 in the rapidly transpiring poplar, significantly increased
476 photosynthesis. Stomatal conductance was decoupled from A at high T_1 in both species, which is
477 an indication that substantial changes are likely in gas exchange physiology at high temperatures.
478 Further research should focus on verifying results of this lab study in the field, as well as
479 discovering the principles of temperature dependency of stomatal regulation and implementing
480 temperature functions into the models of stomatal conductance.

481

482 **Supplementary data**

483 Figure S1. Stomatal conductance (g_s) of poplar and loblolly pine and its dependence on air
484 temperature and vapor pressure deficit.

485 Figure S2. Transpiration and intercellular $[CO_2]$ (C_i) of poplar and loblolly pine and their
486 dependence on air temperature and vapor pressure deficit (VPD).

487 Figure S3. Photosynthesis of poplar and loblolly pine and its dependence on stomatal
488 conductance (g_s) at air temperatures 20 – 49 °C.

489 Table S1. Regression equations and parameters of models used in Figures 1 - 5.

490

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Tables

Table 1. Maximum rate of RuBisCO carboxylation ($V_{C_{\max}}$, $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum rate of photosynthetic electron transport (J_{\max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum rate of triose-phosphate utilization (V_{TPU} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), day respiration (R_d^* , $\mu\text{mol m}^{-2} \text{s}^{-1}$) and stomatal limitation (L_s , nondimensional) of poplar and loblolly pine plants measured at three leaf temperatures (T_l). Significant differences between measurements at different temperatures indicated by $p < 0.05$.

Species	T_l ($^{\circ}\text{C}$)	$V_{C_{\max}}$	J_{\max}	V_{TPU}	R_d^*	L_s
Poplar	20	66	132	10.05	2.10	0.19
	30	165	151	11.11	1.9	0.16
	40	301	165	11.46	3.25	0.2
<i>p</i> - value		<0.001	<0.001	0.07	<0.001	0.21
Loblolly pine	20	21	45	3.62	1.55	0.41
	30	67	71	4.57	2.73	0.78
	40	163	75	4.99	6.52	0.23
<i>p</i> - value		<0.000	<0.001	<0.001	0.011	<0.001

Figures

Fig. 1. Stomatal conductance (g_s) of poplar (left panels) and loblolly pine (right panels) and its dependence on leaf temperature and vapor pressure difference (VPD). Plants were measured in high soil moisture conditions and ambient $[CO_2]$ (panels a and d) or elevated $[CO_2]$ (panels b and e). Panels c and f show measurements made on drought stressed trees at ambient $[CO_2]$. Linear regression was used to fit the data for poplar and non-linear regression was used for loblolly pine. Asterisks at the z-axis label indicate overall significance of the model; asterisks at x and y axes indicate significance of the respective parameters (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 2. Response of transpiration to vapor pressure difference (VPD) in poplar (left panels) and loblolly pine (right panels) at varying leaf temperature and vapor pressure difference (VPD). Asterisks at the z-axis label indicate overall significance of the model; asterisks at x and y axes indicate significance of the respective parameters (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 3. Relationship between intercellular concentration of CO_2 (C_i), leaf temperature and vapor pressure difference (VPD) (panels a, c) for poplar (left panels) and loblolly pine (right panels). Relationship between net photosynthesis, leaf temperature and stomatal conductance (g_s) (panels b, d). Asterisks at the z-axis label indicate overall significance of the model; asterisks at x and y axes indicate significance of the respective parameters (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 4. Evaporative cooling effect (temperature difference) of transpiration on well-watered poplar (panel a), drought stressed poplar (panel b) and loblolly pine (panel c) at varying air temperature and vapor pressure difference (VPD). Asterisks at the z-axis label indicate overall significance of the model; asterisks at x and y axes indicate significance of the respective parameters (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 5. Leaf water potential of poplar (left panels, a, b) and loblolly pine (right panels, c, d) in wet soil (top panels, a, c) and dry soil (bottom panels, b, d). Asterisks at the z-axis label indicate overall significance of the model; asterisks at x and y axes indicate significance of the respective parameters (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 1

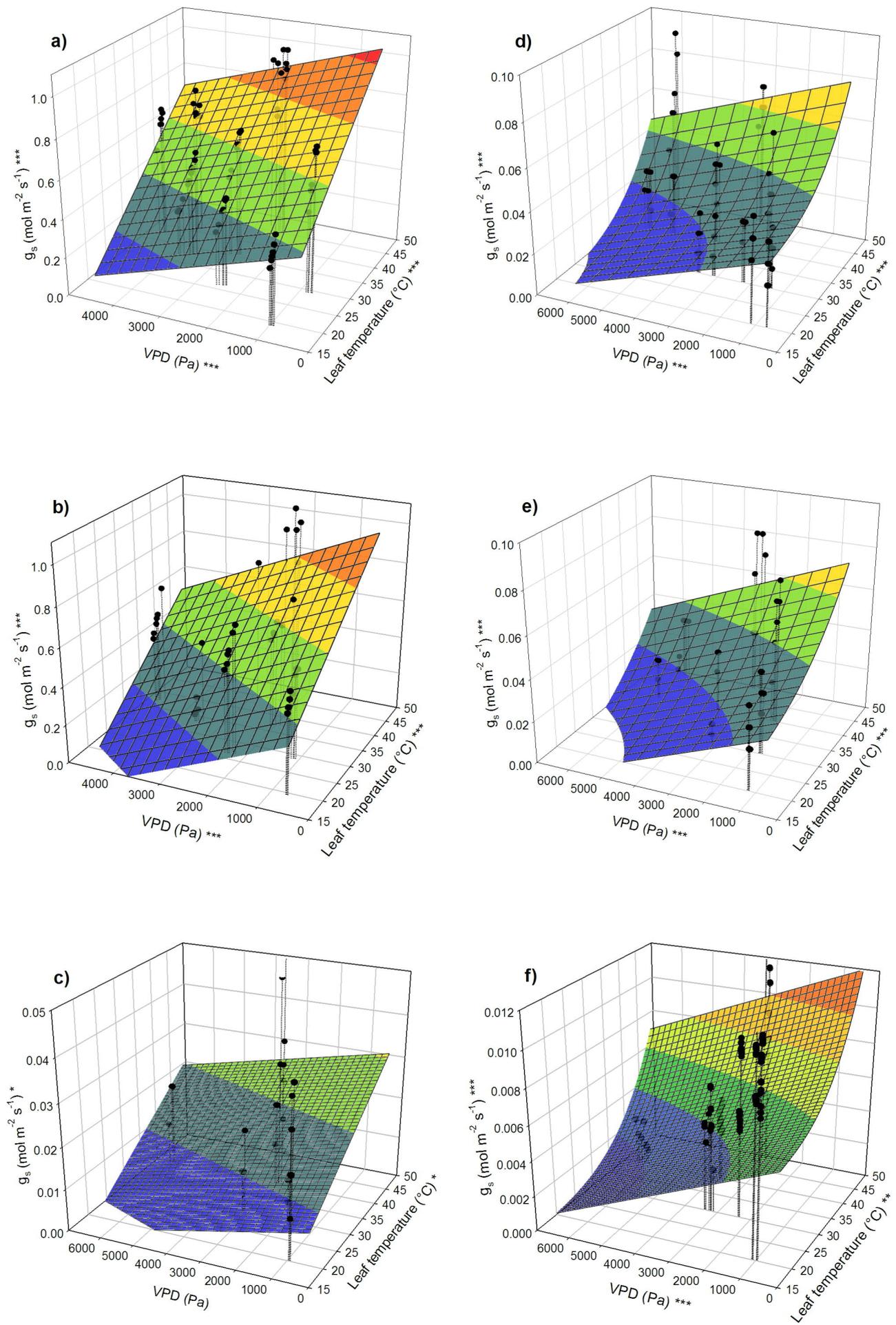


Figure 2

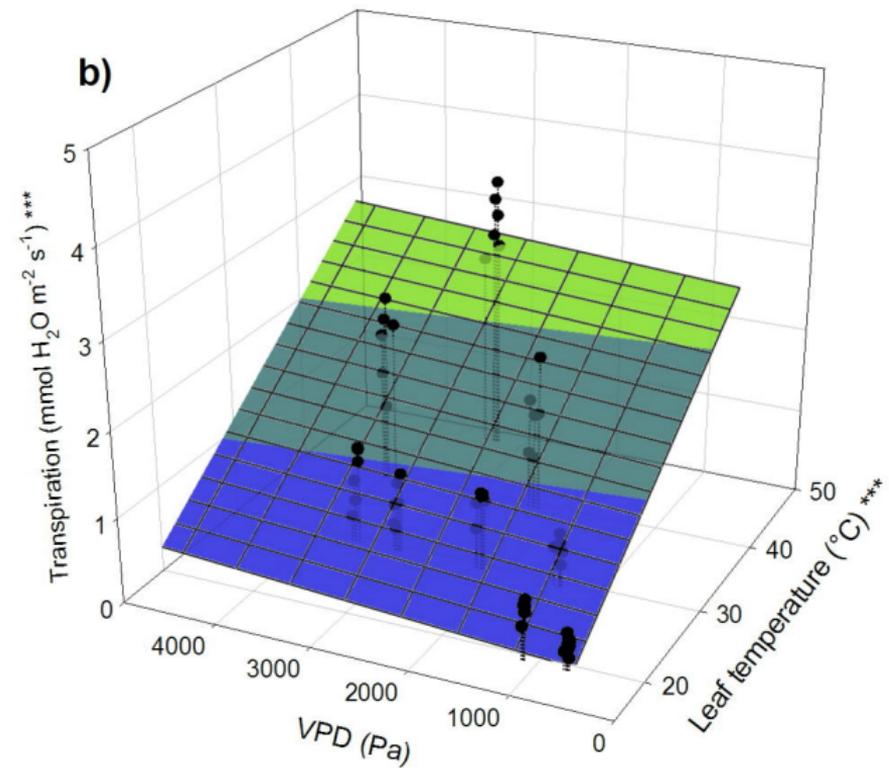
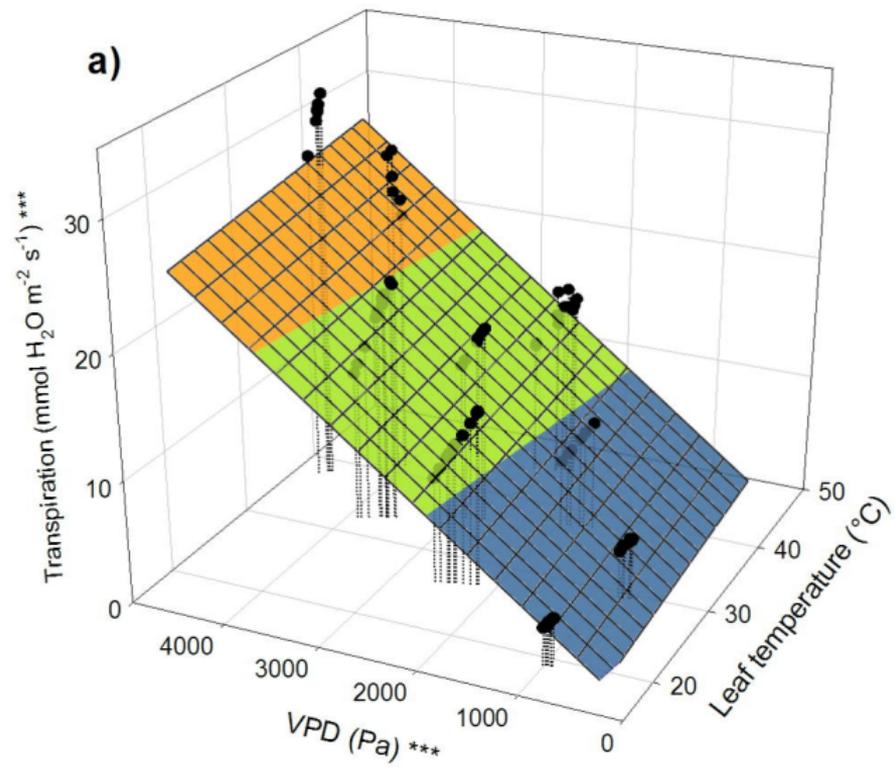


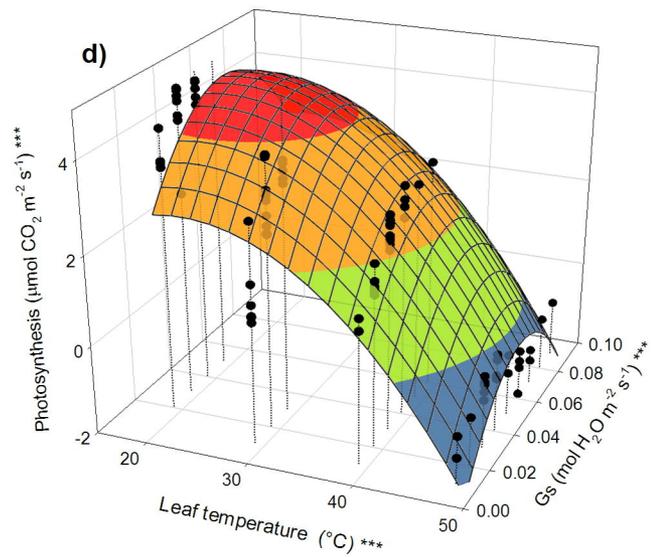
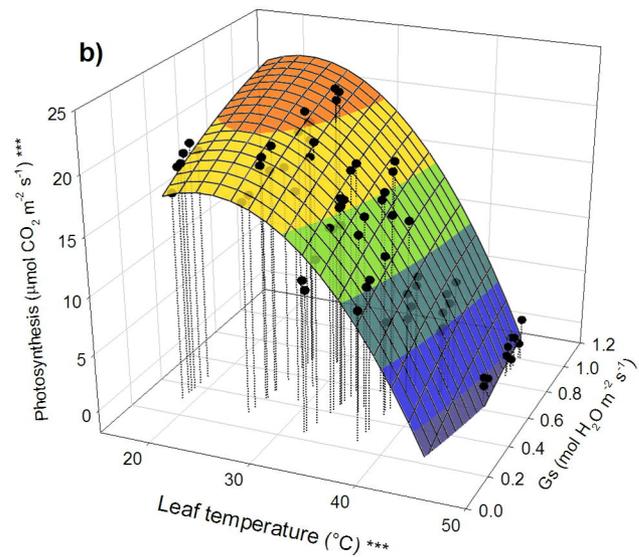
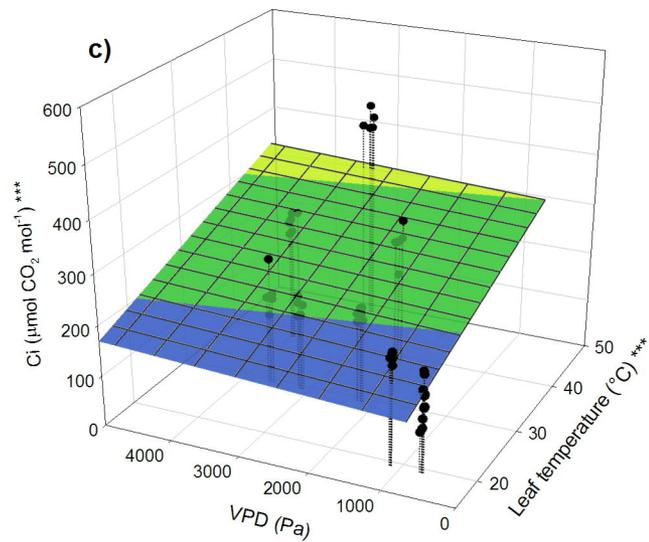
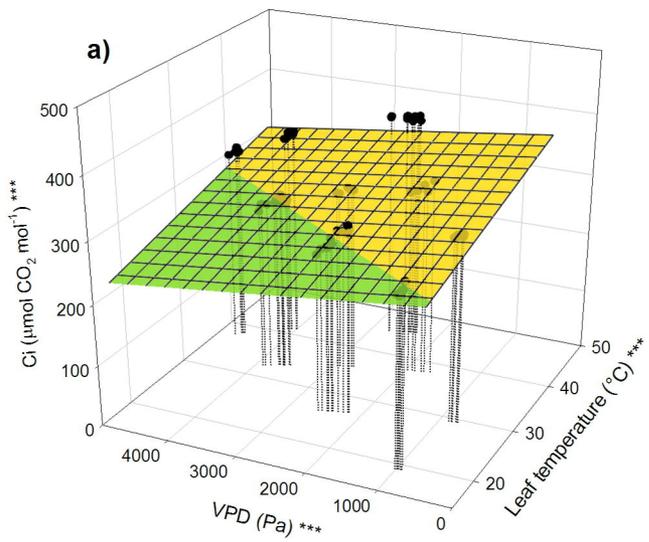
Figure 3

Figure 4

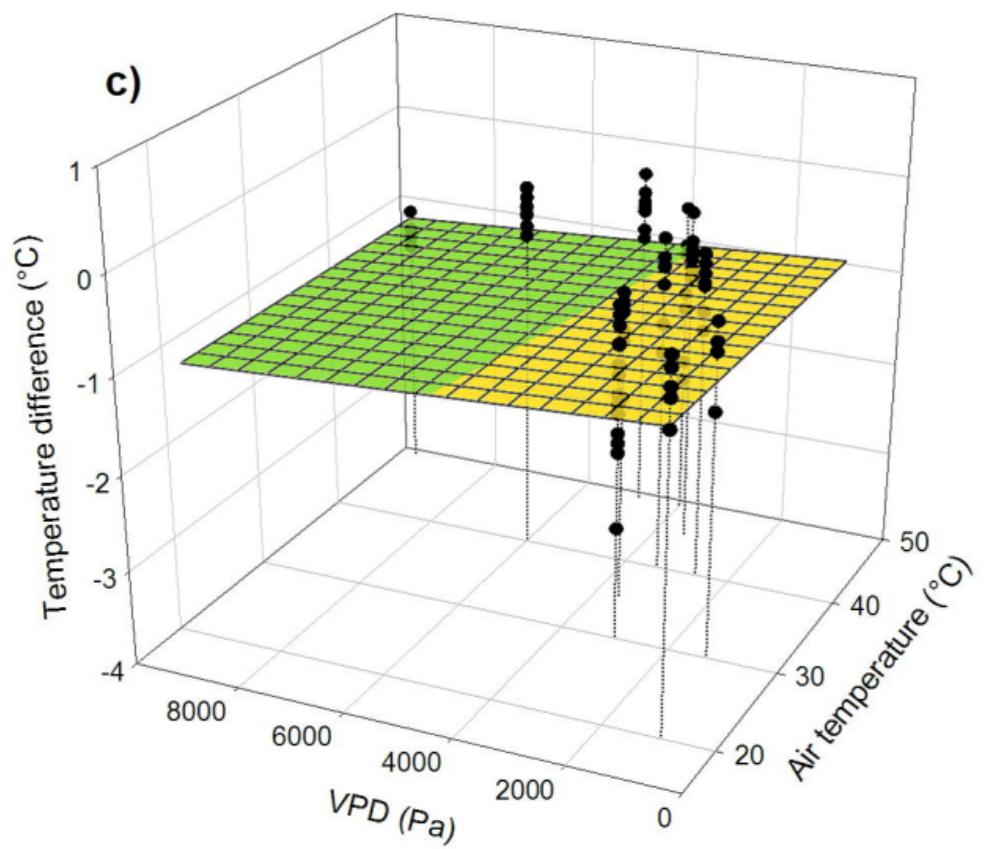
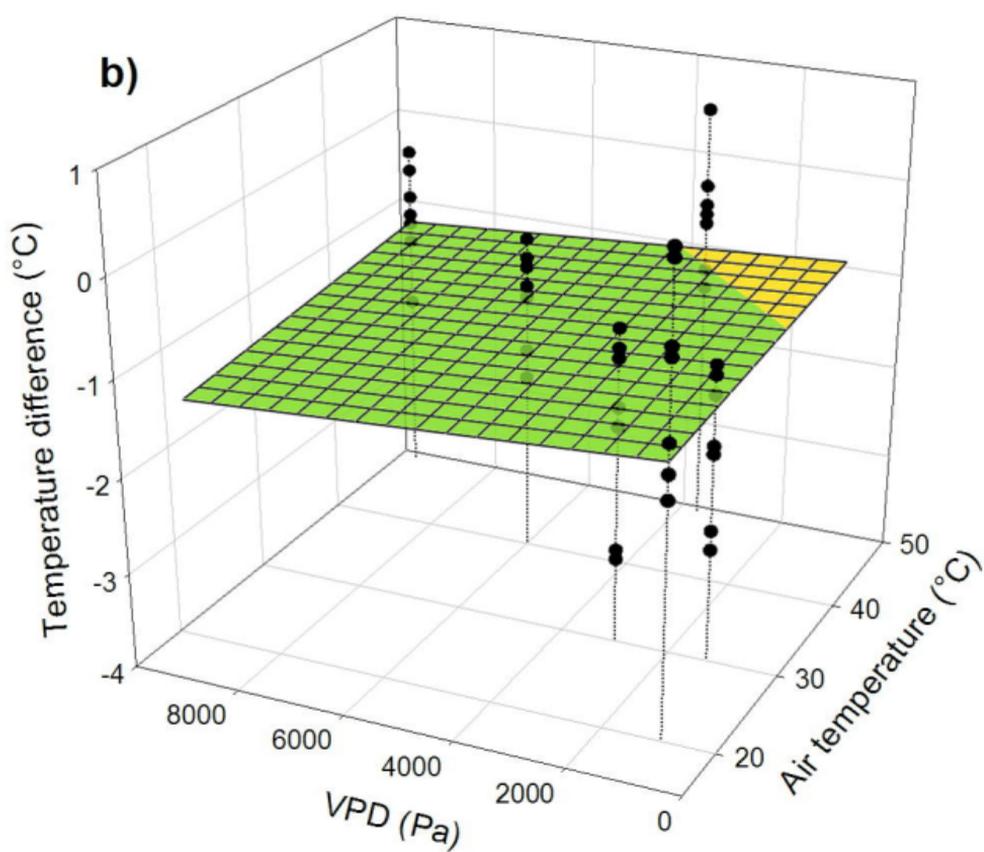
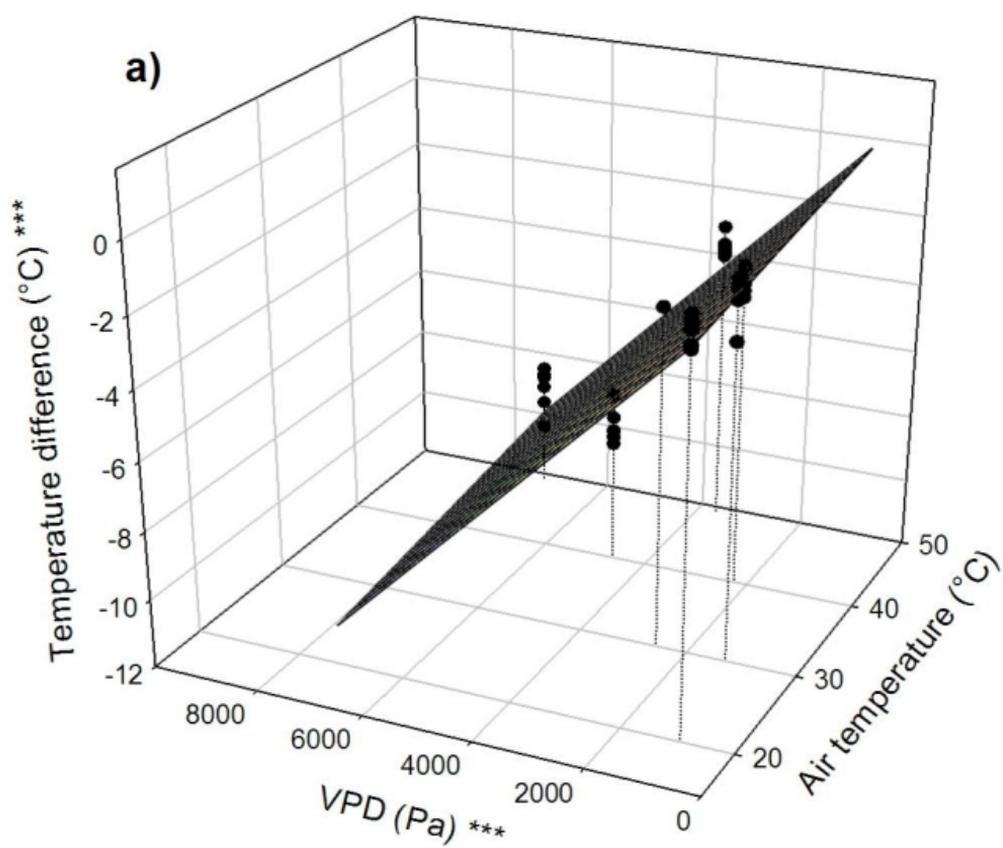


Figure 5

