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1 Altered stomatal dynamics of two Euramerican poplar genotypes
2 submitted to successive ozone exposure and water deficit

3

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10 **ABSTRACT**

11 The impact of ozone (O₃) pollution events on the plant drought response needs special
12 attention because spring O₃ episodes are often followed by summer drought. By causing
13 stomatal sluggishness, O₃ could affect the stomatal dynamic during a subsequent drought
14 event. In this context, we studied the impact of O₃ exposure and water deficit (in the presence
15 or in the absence of O₃ episode) on the stomatal closure/opening mechanisms relative to
16 irradiance or vapour pressure deficit (VPD) variation. Two genotypes of *Populus nigra* x
17 *deltoides* were exposed to various treatments for 21 days. Saplings were exposed to 80
18 ppb/day O₃ for 13 days, and then to moderate drought for 7 days. The curves of the stomatal
19 response to irradiance and VPD changes were determined after 13 days of O₃ exposure, and
20 after 21 days in the case of subsequent water deficit, and then fitted using a sigmoidal model.
21 The main responses under O₃ exposure were stomatal closure and sluggishness, but the two
22 genotypes showed contrasting responses. During stomatal closure induced by a change in
23 irradiance, closure was slower for both genotypes. Nonetheless, the genotypes differed in
24 stomatal opening under light. Carpaccio stomata opened more slowly than control stomata,
25 whereas Robusta stomata tended to open faster. These effects could be of particular interest,
26 as stomatal impairment was still present after O₃ exposure and could result from imperfect
27 recovery. Under water deficit alone, we observed slower stomatal closure in response to VPD
28 and irradiance, but faster stomatal opening in response to irradiance, more marked in
29 Carpaccio. Under the combined treatment, most of the parameters showed antagonistic
30 responses. Our results highlight that it is important to take genotype-specific responses and
31 interactive stress cross-talk into account to improve the prediction of stomatal conductance in
32 response to various environmental modifications.

33 Capsule : This study indicates that the possible multiple stomatal responses to environmental
34 stimuli must be taken into account in order to better define the parameters of the stomatal
35 conductance model.

36 Keywords: ozone, drought, stomata, VPD, light

37 1 Introduction

38 Forest health depends at least on our capacities to improve risk assessment. This
39 evaluation depends on our understanding of tree biological and physiological responses to
40 multiple environmental stressors (Sicard et al., 2016). Among abiotic stress factors,
41 tropospheric ozone (O₃) and drought are detrimental for tree growth and health (Allen et al.,
42 2010; Wittig et al., 2007). O₃ is a phytotoxic air pollutant that impairs gas exchanges and
43 reduces plant biomass (Dizengremel et al., 2013; Jolivet et al., 2016; Wittig et al., 2007). It is
44 a secondary pollutant resulting from a photochemical process in the troposphere, with an
45 annual cycle: higher daily O₃ concentrations are reported in spring and summer, lower ones in
46 autumn and winter. Recurrent spring maxima have been reported in the northern hemisphere
47 (Kalabokas et al., 2017; Monks, 2000; Parrish et al., 2013). On the other hand, drought events
48 are predicted to increase in the near future. Due to global warming, wet regions are becoming
49 wetter and dry regions drier (Liu and Allan, 2013). A reduction of primary growth was
50 observed following the 2003 drought and heat wave (Ciais et al., 2005). Stand mortality was
51 reported all over the world (Allen et al., 2010). Models integrating temperature, vapour
52 pressure deficit (VPD) and rainfall predict an increased probability of similar events in the
53 near future (IPCC, 2014; Lehner et al., 2006; Park Williams et al., 2013). Against this
54 environmental fluctuation, the main physiological responses of trees and more generally
55 plants are the control of transpiration by stomata. In the case of O₃, stomatal closure limits O₃
56 entrance. Under drought, stomatal closure prevents water loss. Nevertheless, there is a cost for
57 carbon assimilation under both constraints. Stomata exert a major control on both the water
58 and carbon cycles round the world (Hetherington and Woodward, 2003).

59 Meta-analyses of published data indicate that ambient O₃ reduces tree biomass
60 production (Li et al., 2017; Wittig et al., 2009), while an analysis of survey data showed how
61 O₃ reduces tree growth (Braun et al., 2014). Different metric indicators have been created to
62 assess critical levels of O₃ for plants. The simplest ones were only based on cumulative
63 exposure levels. The USA selected the SUM-index (sum of all hourly average concentrations
64 over *X* ppb), whereas the EU selected the AOT40 (Accumulated Ozone over a Threshold of
65 40 ppb) which takes into account hourly O₃ concentrations above 40 ppb *per* hour when
66 irradiance is 50 W.m⁻² minimum (Fuhrer et al., 1997; Musselman et al., 2006). The advantage
67 of these metrics is that they make it simple to determine exposure levels only based on O₃
68 concentration data. In the early 2000's, the biologically more relevant concept of flux-based
69 approach emerged, resulting in the scientific adoption of the Phytotoxic Ozone Dose over a

70 threshold of $Y \text{ nmol.m}^{-2}.\text{s}^{-1}$ (PODy) (Emberson et al., 2007; Hayes and Bangor, 2017;
71 Karlsson et al., 2000; Mills et al., 2011). This metric is available for risk assessment and takes
72 O_3 uptake by the leaves through stomata into account. The DO_3SE model (Deposition of O_3
73 for Stomatal Exchange) was developed to account for the variation in stomatal opening and
74 closure with climate, soil, and plant factors (Büker et al., 2012; Emberson et al., 2007); it is
75 based on the empirical Jarvis-type stomatal conductance model (Jarvis, 1976). This model
76 was developed using steady-state parameters, and the stomatal dynamic modification
77 specifically induced by O_3 was ignored (Hoshika et al., 2013). Tropospheric O_3 is known to
78 slow down the stomatal responses to environmental factors, named stomatal sluggishness.
79 Stomatal sluggishness has been reported in response to changes in light (Dumont et al., 2013;
80 Paoletti and Grulke, 2010), VPD (Dumont et al., 2013; Grulke et al., 2007), and soil water
81 stress (Durand et al., 2019; Gérardin et al., 2018; Hoshika et al., 2013). Hoshika et al. (2017)
82 showed that stomatal sluggishness and closure should be taken into account in stomatal
83 response modelling in a Jarvis-type model. O_3 -induced stomatal sluggishness potentially
84 increases transpiration (Hoshika et al., 2015). O_3 - and drought-induced loss of stomatal
85 function may enhance both leaf water loss and O_3 uptake (Hoshika et al., 2013). In natural
86 conditions, an O_3 spring episode is very likely before summer drought in Europe. Plants
87 subjected to O_3 stress in spring can be particularly sensitive to drought events in summer
88 (Pollastrini et al., 2014). O_3 could affect the stomatal dynamic under drought by causing
89 stomatal sluggishness. The present study aims to decipher the response of stomatal
90 closure/opening relative to light or vapour pressure deficit variation in O_3 and in water deficit
91 stress conditions (with or without previous O_3 stress). The two *Populus deltoides x nigra*
92 (Moench.) genotypes (“Carpaccio” and “Robusta”) were documented in our recent
93 experiments under 120 ppb O_3 as being impaired in radial growth and gas exchanges, and
94 exhibiting increased visible leaf injuries and senescence, and changes in detoxification
95 capacities (Dghim et al., 2013; Dumont et al., 2013, 2014a, 2014b; Dusart et al., 2018). Better
96 stomatal control has been observed under O_3 in the Carpaccio genotype as compared to
97 Robusta (Dumont et al., 2013), while both genotypes showed efficient stomatal closure under
98 soil water deficit (Durand et al., 2019; Dusart et al., 2019; Ridolfi and Dreyer, 1997). The
99 present paper addresses the following questions: i) are any of the differences in stomatal
100 dynamics linked to environmental variables (light, VPD) between the two poplar genotypes?,
101 ii) does 80 ppb O_3 or water deficit induce stomatal sluggishness in poplar?, iii) does O_3
102 treatment modify water-deficit-induced stomatal closure?, and iv) does stomatal dynamics
103 recover after 7 days in the absence of O_3 treatment?

104 2 **Materials and methods**

105 2.1 *Plant material and exposure conditions*

106 Cuttings of the two Euramerican poplar genotypes “Carpaccio” and “Robusta” were
107 grown in growth chambers as already described in Dusart et al. (2019) with slight
108 modifications. Cuttings were planted in ten-liter pots filled with a sand/peat mixture (1/1, v/v)
109 and fertilised by adding 15 g of slow-release nutritive granules (Nutricot T-100) and 1 g.L⁻¹
110 CaMg(CO₃)₂. For both genotypes, forty-eight plants were randomly distributed in eight
111 phytotronic chambers, i.e. twenty-four plants in control chambers (charcoal-filtered air), and
112 twenty-four plants in chambers set for O₃ treatment (80 nmol.mol⁻¹ for 13 hours, from 09:00
113 to 22:00). For reasons of space in the culture chambers and length of measurement times, the
114 experiment was duplicated separately for each genotype. After a 7-day-long acclimation
115 period, the O₃ treatment started while control saplings were exposed to charcoal-filtered air
116 for 13 days. After 13 days (d) of fumigation, the total cumulative sum of O₃ flux (SUM00),
117 the cumulative O₃ dose above a threshold of 40 ppb (AOT 40), and the phytotoxic O₃ dose
118 above a threshold flux of 0 nmol.m⁻².s⁻¹ (POD₀) (based on measured stomatal conductance, see
119 Bagard et al., 2015) were determined (Table S1). At the end of the O₃ exposure period, half of
120 the saplings were submitted to a moderate water deficit for 7 d. Soil Water Content (SWC)
121 was determined with 24 wireless Time Domain Reflectometry (TDR) probes (CWS655E,
122 Campbell Scientific Ltd, Antony, France). A calibration between volumetric SWC measured
123 by TDR and pot weight was performed. The biological available water was expressed as
124 relative extractable water (REW), as described by Wildhagen et al. (2018) for the same soil.
125 Poplars were watered with a known volume of water several times a day to maintain the level
126 of REW stable. For the well-watered treatment, poplars were irrigated at 75 % (±10%) of
127 REW, whereas for the water deficit treatment, irrigation was set to 45% (± 2%) of REW until
128 the end of the experiment (Fig. S1). A cumulative sum of the amount of water added for each
129 treatment for 21 d is presented in Table S1.

130 2.2 *Plant growth*

131 The number of leaves and the diameter at the collar and height were recorded twice a
132 week until the end of the experiment for each individual. At the end of the experiment, leaves,
133 stems and roots were oven-dried at 60 °C until they reached a constant dry mass.

134 2.3 *Gas exchanges and photosynthetic pigment kinetics*

135 Gas exchanges (A_n , net CO₂ assimilation, and g_s , stomatal conductance to water vapour)
136 were measured using a Li-6200 (Li Cor, Lincoln, NE, USA) as described in Dusart et al.
137 (2019). Non-destructive determination of the chlorophyll pigment content was performed
138 with a Dualex (Force-A, Orsay, France). For all non-destructive leaf measurements, the same
139 leaf was used, i.e., the first fully expanded leaf (the 10th leaf from the apex) at the beginning
140 of the O₃ treatment.

141 2.4 Stomatal response to irradiance and vapour pressure deficit

142 2.4.1 Gas exchange measurements

143 Gas exchange measurements were performed with a Li-6400 system, as described in
144 Durand et al. (2019) with some minor modifications. Parameters of the leaf cuvette were for
145 light: PAR: 800 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ with 30 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ of blue irradiance and VPD: 0.8 kPa, until
146 g_s reached a steady state (g_0 , defined as a variation lower than 5% over 5 minutes). Then light
147 was turned off (as well as in the phytotronic chamber) until g_s got to a new steady state (g_1),
148 then turned on to 800 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ until stomatal conductance reached the last steady state
149 (g_2). The 800 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ value was chosen to avoid photoinhibition due to excess light
150 (Niinemets and Kull, 2001). A similar procedure was used to monitor g_s response to a change
151 of VPD: it was switched to 3 kPa instead of 0.8 kPa (for a fixed PAR: 800 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$). VPD
152 from leaf tissues to air was controlled with a dew point generator as described in Vialet-
153 Chabrand et al. (2013).

154 2.4.2 Modelling

155 The obtained stomatal response curves were fitted using the following sigmoidal
156 model (Vialet-Chabrand et al., 2013):

$$157 \quad g_s = g_0 + (G - g_0) e^{-e^{\left(\frac{\lambda-t}{\tau}\right)}}$$

158 where g_s is the fitted stomatal conductance, g_0 and G are the steady-state values of g_s ($\text{mol.m}^{-2}.\text{s}^{-1}$),
159 respectively at the start and at the end of the curve, τ is a time constant (s), λ is the lag
160 time (s), and t is time (s). The speed of the stomatal response was estimated by calculating the
161 maximum slope (SL_{max}), as follows:

$$162 \quad SL_{\text{max}} = \frac{G - g_0}{\tau \cdot e}$$

163 where $(G-g_0)$ represents the amplitude of the stomatal response and e is Euler's number ($e \approx$
164 2.718). Further information regarding the model parameters and fitting procedure can be
165 found in Gérardin et al. (2018) and Durand et al. (2019).

166 2.5 Statistical analyses

167 Statistical analyses were performed using R 3.1.0 (R Development Core Team) open-
168 source software. Linear models created from the *nlme* package (Pinheiro et al., 2018) were
169 used to study growth parameters with ANOVA, including the effects of water deficit, O_3 and
170 genotype. The growth chamber was also tested and excluded from the models because the
171 effect was not significant for all the parameters tested throughout the whole experimental
172 period. Model parameters, G , g_0 , λ , τ , SL_{max} were explored in the same way. The *lme4*
173 package (Bates et al., 2015) was used to fit a linear mixed-effect model on gas exchange and
174 chlorophyll content data with fixed variables (water deficit and O_3 data) whereas biological
175 replicas were random variables. Residual plots of the model were used to assumed
176 heteroscedasticity and variance homogeneity. The *emmeans* package (Lenth, 2016) was used
177 to perform multiple comparisons. To determine if O_3 and water deficit had an additive,
178 synergistic or antagonistic impact on g_s , we compared the observed effects to the expected
179 additive effects for the saplings exposed to O_3 and then to water deficit (Methods 1 available
180 in Supplementary data).

181 3 Results

182 3.1 Effect on growth, chlorophyll contents and gas exchange kinetics

183 O_3 impacted only the stem biomass of the two genotypes. Water deficit only slightly
184 impacted the number of leaves of Robusta genotype, nevertheless leaf surface area decreased
185 in the Carpaccio genotype only (Table S2).

186 An increase in chlorophyll contents was observed in both genotypes under the WW:FA
187 modality throughout the 21 days of the experiment (Fig. 1A and B), and Robusta
188 systematically contained 1.5 times more chlorophyll than Carpaccio. Chlorophyll contents
189 were significantly lower under O_3 treatment in both genotypes; from 11 days, chlorophyll
190 levels remained lower than the control treatment (Fig. 1A and B). The chlorophyll content of
191 the water deficit treatment (D:FA) was significantly lower in Robusta as compared to the
192 WW:FA modality (Fig. 1A and B). D: O_3 impacted chlorophyll contents similarly to WW: O_3 .

193 Concomitantly, gas exchanges were recorded twice or three times a week (Fig. 1C and
194 D). The control treatment values decreased throughout the experiment because the leaves
195 received less light (because the upper leaves were still growing). Although net CO₂
196 assimilation (A_n) decreased over time, A_n values were 3 μmol CO₂.m⁻².s⁻¹ higher in the
197 Robusta genotype. ANOVA on A_n only showed a significant effect of O₃ on both genotypes
198 (Fig. 1C and D). O₃ decreased A_n, from 10 d in Carpaccio and 6 d in Robusta. After the O₃
199 treatment was switched off on d 13, Robusta photosynthesis reached the same levels as the
200 control, *i.e.*, 7.5 μmol.m⁻².s⁻¹.

201 Concerning stomatal conductance values, O₃ decreased g_s from 6 d in Carpaccio and 2 d
202 in Robusta, with a significant effect only on Carpaccio (Fig. 1E and F). At the end of the 13 d
203 of O₃ fumigation, g_s went back to the control level in both genotypes, *i.e.*, 400 mmol.m⁻².s⁻¹
204 and 300 mmol.m⁻².s⁻¹ in Robusta and Carpaccio, respectively. Water deficit decreased g_s
205 (around 230 mmol.m⁻².s⁻¹) in both genotypes (Fig. 1E and F). Carpaccio stomata displayed
206 similar conductance values under the D:FA and D:O₃ conditions. A difference was observed
207 in Robusta, *i.e.*, stomata under D:O₃ were less closed than under D:FA (330 mmol.m⁻².s⁻¹, Fig.
208 1F), resulting in a significant interaction between O₃ exposure and water deficit.

209 3.2 Effect of O₃ and recovery of stomatal behaviour

210 3.2.1 Responses to irradiance

211 At the end of the O₃ treatment (13 d), net CO₂ assimilation in steady state 0, *i.e.*, A₀,
212 was down from 19.6 to 7.9 μmol.m⁻².s⁻¹ in Carpaccio, and from 19.2 to 15.05 μmol.m⁻².s⁻¹ in
213 Robusta (Table S3). The steady states of g_s after stomatal closure (g₁) were similar for both
214 genotypes and treatments (around 100 mmol.mm⁻².s⁻¹). After stomatal closure, net CO₂
215 assimilation reached negative values (A₁) due to dark respiration (Table S3). The respiration
216 rates in FA conditions were higher in Robusta than in Carpaccio (-1.6 vs. -1.3 μmol.m⁻².s⁻¹).
217 Under WW:O₃ conditions, an increase of respiration was observed, *i.e.*, +30% and +18% in
218 Carpaccio and Robusta, respectively. Concerning the stomatal closure phase, the τ and λ
219 parameters were not modified by O₃ treatment in Carpaccio (Fig. 2A and E). In Robusta, τ
220 tended to increase under O₃ exposure (1.5 fold) (Fig. 2A) and λ was not modified (Fig. 2E).
221 Moreover, λ values differed between genotypes, with a higher value for Carpaccio (1.2-fold
222 higher) (Fig. 2E). SL_{max} values highlighted a trend for an O₃ effect: the stomatal closure speed
223 was reduced by 0.5 fold and 0.4 fold in Carpaccio and Robusta, respectively (Fig. 2I). As
224 regards the stomatal opening phase, λ was 6 min faster in Carpaccio than in Robusta (Fig.
225 2F). λ was not impacted by O₃ in Carpaccio, whereas it significantly decreased by 25% in

226 Robusta. SL_{max} values significantly differed between the two genotypes (Table S4). Stomatal
227 opening was slower in Carpaccio, with an SL_{max} 0.5 fold lower under O_3 , but changes were
228 not significant in Robusta (Fig. 2J). The steady states of conductance (g_2) after the opening
229 phase (Table S3) returned to the same levels as the first steady states in Carpaccio. In Robusta
230 under O_3 , g_2 was 30% lower than g_0 before stomatal closure (Table S3). Similarly,
231 assimilation went back to the same levels ($A_0=A_2$) in the control leaves (Table S5).

232 After a week without O_3 exposure (21 d), net CO_2 assimilation (A_0) went back to the
233 same level as the control in Carpaccio, whereas a 20% decrease in CO_2 uptake was observed
234 in Robusta as compared to the WW:FA modality (Table S3). In parallel, g_0 was the same
235 under the O_3 and control treatments in Carpaccio. As for Robusta, g_0 was higher under O_3 ,
236 and stomata opened 31% more (Table S3). During stomatal closure, τ and λ values in the
237 WW: O_3 treatments changed non significantly as compared to 13 d (Fig. 2C and G). However,
238 as compared to the WW:FA treatments, τ and λ values were 66% and 38% greater in
239 Carpaccio, respectively, and 60% and 15% greater in Robusta, respectively (Fig. 2C and G).
240 SL_{max} decreased by 56% in Carpaccio and by 47% in Robusta (Fig. 2K).

241 Regarding stomatal opening, g_1 was not significantly affected by O_3 in either genotype
242 (Table S3). Final steady states were similar under the WW: O_3 and WW:FA treatments, *i.e.*,
243 around 340 mmol.m⁻².s⁻¹. τ and λ non significantly decreased under O_3 treatment in both
244 genotypes (Fig. 2D and 2H). Stomatal opening tends to be faster in both genotypes under
245 WW: O_3 (Fig. 2L).

246 3.2.2 Responses to VPD

247 The study of the vapour pressure deficit response curves consisted in measuring
248 stomatal closure under pressures ranging between 0.8 and 3 kPa. At the end of the O_3
249 treatment (13 d), the assimilation (A_0) and stomatal conductance (g_0) values of the initial
250 steady states of Carpaccio and Robusta were almost the same as those of the light response
251 curves (Tables S3 and S4). After stomatal closure, CO_2 assimilation (A_1) decreased by 16 to
252 30% as compared to A_0 depending on genotype or treatment (Table S5). The τ parameter in
253 control conditions differed between genotypes (Fig. 3A), as it took Robusta 19 min more than
254 Carpaccio. O_3 increased τ by 17 min and 11 min in Carpaccio and Robusta, respectively (Fig.
255 3A). The λ parameter also differed between the two genotypes in the control conditions: it
256 took Robusta saplings 11 min more to reach the inflection point (Fig. 3C). λ increased under
257 O_3 treatment in both genotypes, *i.e.*, by 12 min and 5 min in Carpaccio and Robusta,
258 respectively (Fig. 3C).

259 After a week without O₃ treatment, stomatal dynamic responses to VPD still showed a
260 few differences between the control and the O₃ treatment. In Carpaccio, O₃ increased τ and λ
261 by 1.8 and 1.6 fold, respectively (Fig. 3B and D). SL_{max} decreased by 70% in Carpaccio (Fig.
262 3F). Robusta was impacted in a different way, as τ slightly increased by 1.1 fold under O₃
263 treatment, but λ was unaffected (Fig. 3B and D).

264 3.3 Effect of water deficit on stomatal behaviour

265 3.3.1 Response to irradiance

266 After 7 days of water deficit treatment, a significant water deficit effect was observed
267 for some parameters of the model. Regarding stomatal closure under light, the initial steady
268 state showed stomatal closure under water deficit conditions (Table S3). Water deficit
269 induced a 45% decrease of g_0 in both genotypes (Table S3). After closure, g_1 showed some
270 marked differences with the control treatment, *i.e.*, an 84% decrease in Carpaccio and a 47%
271 decrease in Robusta. Moreover, stomatal closure was greater in Carpaccio than in Robusta (88
272 *vs.* 122 mmol.m⁻².s⁻¹ for g_1 , respectively). During the stomatal closure phase, τ and λ were
273 unaffected by water deficit in either genotype (Fig. 2C and 2G).

274 During the stomatal opening phase due to irradiance, the water deficit effect was
275 significant on τ and SL_{max} (Table S4). τ decreased by 74% in Carpaccio but increased by
276 26% in Robusta as compared to the control (Fig. 2D). λ was unaffected in Carpaccio but
277 increased in Robusta (+ 21%) (Fig. 2H). SL_{max} increased under water deficit almost 3 times
278 faster in Carpaccio (Fig. 2L). These increases differed significantly between the genotypes
279 (Table S4).

280 3.3.2 Responses to VPD

281 After the VPD closing phase, g_1 were unaffected in Carpaccio, whereas it was 50
282 mmol.m⁻².s⁻¹ below the WW:FA values in Robusta (Table S5). A₀ and A₁ decreased under
283 water deficit by the same amplitude (around 30%) in both genotypes (Table S5).

284 Water deficit affected the stomatal dynamics through a non significant decrease of the τ
285 parameter in both genotypes, by 20% in Carpaccio *vs.* 15% in Robusta (Fig. 3B). The λ
286 parameter was not modified in Carpaccio but decreased in Robusta (-13%) (Fig. 3D). Finally,
287 a lower SL_{max} was observed: -51% and -33% in Carpaccio and Robusta, respectively, as
288 compared to the control (Fig. 3F).

289 3.4 Effects of O₃ and water deficit on stomatal behaviour

290 3.4.1 Responses to irradiance

291 After 7 days of water deficit treatment, most of the parameters showed an antagonistic
292 response in the combined treatment (Fig. 4), except the initial steady state g_0 in Carpaccio. In
293 this genotype, stomatal closure was greater than under the water deficit treatment alone (Table
294 S3). During stomatal closing under irradiance, τ slightly increased by 6 min in Carpaccio, in-
295 between the values under water deficit and O_3 exposure, and was unaffected in Robusta as
296 compared to the control (Fig. 2C). λ showed the same trends as under the D:FA modality
297 (Fig. 2G). SL_{max} was significantly affected by the water deficit \times O_3 interaction ($p=0.05$): in
298 Carpaccio, SL_{max} was lower than under the D:FA and WW: O_3 modalities, by 23% as
299 compared to the WW:FA modality. In Robusta, SL_{max} was in the same range as under D:FA
300 conditions (Fig. 2K). Stomatal opening as a result of irradiance under the combined treatment
301 revealed no significant effect of the water deficit \times O_3 interaction on any of the parameters of
302 the models except τ (Table S4). In Carpaccio, SL_{max} values were intermediate between D:FA
303 and WW: O_3 values, almost twice the WW:FA value. In Robusta, SL_{max} tended to increase as
304 compared to the other treatments (Fig. 2L), resulting in a synergistic effect (Fig. 4).

305 3.4.2 Responses to VPD

306 After 7 days of water deficit treatment on saplings previously submitted to O_3 treatment,
307 no significant effect was observed (Table S4). There was a synergistic effect on G (g_1), under
308 the D: O_3 modality, the final steady state tended to be “more closed stomata” in Carpaccio vs.
309 “slightly more open stomata” in Robusta than under the WW: O_3 or D:FA modalities (Table
310 S5). Otherwise, most of the parameters showed an antagonistic effect (Fig. 4). In Robusta, the
311 λ parameter decreased (by 26% as compared to the control) under the combined treatments
312 (Fig. 3D), more than under water deficit or O_3 alone, resulting in a synergistic effect (Fig. 4).
313 In Carpaccio, SL_{max} decreased in a similar way under all three modalities. (Fig. 3F).

314 4 Discussion

315 4.1 Sluggish stomatal response to O_3

316 In our experiment, the first symptom of the daily exposure to 80 ppb O_3 was visible on
317 g_s : after one week of exposure, both genotypes closed their stomata in response to O_3 . This
318 stomatal closure was associated with decreased net CO_2 assimilation and chlorophyll
319 contents. Impairment of gas exchanges under O_3 exposure is well documented (Wittig et al.,
320 2007). The decrease in net CO_2 assimilation by the two poplar genotypes under O_3 exposure

321 had been mentioned previously and mainly linked to the modification of Rubisco activity and
322 chlorophyll degradation (Dghim et al., 2013; Guidi et al., 2001). This impact on
323 photosynthesis was clearly visible for each steady state at $800 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of PAR.

324 In addition, stomatal closure went along with decreased steady states. This stomatal
325 response was reversible: when O_3 exposure was interrupted, saplings rapidly recovered the
326 same steady states as in the control treatment. According to the dynamics parameters in
327 response to irradiance, the lag time (λ) was not modified by O_3 during stomatal closure, but a
328 few constitutive differences between the genotypes were visible. In Robusta, τ tended to
329 increase under O_3 exposure. The main effect for both genotypes was a trend toward a lower
330 SL_{max} , *i.e.*, the so-called O_3 sluggishness. In addition, steady states (g_1) in the dark were
331 slightly higher. This might have consequences under natural conditions, when O_3 entrance
332 during the night time could be significant (Hoshika et al., 2013) and could enhance water loss
333 (Grulke et al., 2004). From our results, we can propose a hypothetical model of the stomatal
334 daily course (Fig. S2) in conditions of stable diurnal irradiance throughout the day, with
335 maximum VPD at midday. This representation highlights the differences between treatments
336 and genotypes and the potential impact on the O_3 flux, CO_2 assimilation (grey area), or used
337 water (blue area). This theoretical representation was inspired by a hypothetical model of
338 Dumont et al. (2013), the results of a Ball-Berry-type model from Tuzet et al. (2003), and
339 daily conductance measurements from Durand et al. (2019). The differences in g_s values
340 between treatments, genotypes, and irradiance variations were conserved. As for stomatal
341 closure, the model parameters were also modified during stomatal opening.

342 The main atmospheric determinant of stomatal opening at midday is VPD (McAdam
343 and Brodribb, 2015). Higher VPD differently affected stomatal behaviour among genotypes
344 in the control. Firstly, the λ parameter differed between the genotypes; Robusta was less
345 responsive to VPD and needed more time to reach the inflection point. This could be linked to
346 constitutive differences between the genotypes. Previous works showed that Robusta stomata
347 were less responsive to VPD variation (Dumont et al., 2013). These genotypic differences
348 could be as determining as differences between species. Differences between species were
349 taken into account in the O_3 flux model (Hayes and Bangor, 2017). It is obvious that the
350 genotypic specificity could also affect the calculation of the species-specific stomatal O_3 flux;
351 moreover, O_3 -induced stomatal closure and sluggishness could affect water use efficiency and
352 transpiration calculations (Dumont et al., 2013; Hoshika et al., 2015; Paoletti and Grulke,
353 2005). These effects could be of particular interest, as stomatal sluggishness was still present

354 after O₃ exposure and could be responsible for a greater water use in the middle and at the end
355 of the day (Fig. S2). Interestingly, after O₃ exposure was stopped, Robusta recovered the same
356 assimilation rate as the control whereas Carpaccio photosynthesis remained impaired. Our
357 results are consistent with stomatal recovery after O₃-induced stress observed in the literature.
358 Similar recovery was reported in white clover (Francini et al., 2007). Nonetheless, this
359 imperfect recovery due to carry-over or ‘memory’ effects could be particularly detrimental
360 under repeated O₃ exposure (Oksanen, 2003; Oksanen and Saleem, 1999) combined with
361 other biotic or abiotic stresses (Langebartels et al., 1998), *e.g.*, water deficit conditions.
362 However, this ‘memory’ could also be responsible for a ‘conditioning’ mechanism through a
363 hormetic response (Agathokleous et al., 2019) and/or cross-tolerance (Walter et al., 2013). In
364 both cases, this effect could modify tree resilience and acclimation to a new disturbance.

365 4.2 *Water deficit induces slower stomatal closure in response to light and VPD*

366 Water deficit reduced only the total leaf surface of Carpaccio genotype (Table S2)
367 associated with an expected stomatal closure over time (Bogeat-Triboulot et al., 2007; Chaves
368 et al., 2002; Dusart et al., 2019). In response to darkness, the time response parameters were
369 modified, with a few differences between the genotypes. Nevertheless, the lag time and
370 closing speed were not impacted by water deficit in either genotype. Interestingly, in the dark
371 both genotypes had their stomata more closed than the control saplings. Moreover, Robusta
372 stomata were less closed than Carpaccio stomata. This could result in differences in water loss
373 at night (Fig. S2) (Caird et al., 2007). By contrast, stomatal opening following irradiance
374 affected all the parameters of the models. The opening speed increased in both genotypes,
375 especially in Carpaccio. Faster stomatal opening under water deficit has been reported in
376 *Phaseolus vulgaris* (Barradas et al., 1994). Water loss might increase due to faster stomatal
377 opening, but may also increase the leaf carbon assimilation (Barradas et al., 1994). In
378 response to VPD, water deficit affected stomatal dynamics through a decrease of the τ
379 parameter in both genotypes. The lag time parameter was not modified in Carpaccio and
380 decreased in Robusta. There was great sluggishness in the response to VPD. Stomatal closure
381 sluggishness in response to irradiance and/or VPD has been reported in different species, after
382 leaf excision in *Populus trichocarpa* x *deltoides* (Reich and Lassoie, 1984), under soil water
383 deficit conditions in *Phaseolus vulgaris* (Hoshika et al., 2013), *Nicotiana tabacum* (Gérardin
384 et al., 2018), *Populus nigra*, and *Populus euramericana* (Durand et al., 2019). Stomatal
385 closure under soil water deficit or increased VPD was found closely related to ion transport
386 and abscisic acid perception (McAdam and Brodribb, 2015; Pospíšilová, 2003). These

387 mechanisms could be modified by O₃ through ABA synthesis (McAdam et al., 2017) or
388 through other phytohormones such as ethylene (Wilkinson and Davies, 2010, 2009).

389 4.3 *Antagonistic effect of the successive exposure to O₃ and water deficit*

390 When O₃ exposure was followed by water deficit, we observed a combination of the
391 effects of O₃ or water deficit alone. The impact on height and radial growth was similar to the
392 effect of water deficit alone in both genotypes. Nevertheless, total biomass decreased more
393 following the successive stresses (Table S2). As seen above, water deficit and O₃ separately
394 modified stomatal behaviour in response to VPD or irradiance fluctuations. The interaction
395 between the two stress factors may have modified stomatal responses (Hoshika et al., 2013;
396 Wilkinson and Davies, 2009). Concerning gas exchanges, stomatal closure was observed in
397 both genotypes, with a decreased steady state. Carpaccio stomata tended to be more closed
398 than under O₃ exposure or water deficit alone, whereas Robusta stomata were slightly less
399 closed than under water deficit alone (Fig. S2). Under the combined treatment, most of the
400 parameters showed an antagonistic response, *i.e.*, a weaker response than the expected
401 additive effect (Bansal et al., 2013; Dusart et al., 2019; Pellegrini et al., 2019). τ seemed to
402 increase as compared to the control and drought treatment alone, but less than under O₃
403 exposure. The lag time and final steady state were modified in the same way as under water
404 deficit alone. In Carpaccio, stomatal sluggishness increased as compared to the separate
405 constraints, and this may have increased transpiration under water deficit. In Robusta, the
406 slower closure was in the same range as under water deficit alone. Regarding stomatal
407 opening due to irradiance, the response was the same as for water deficit alone on all the
408 parameters of the models, without any significant interaction of O₃. In response to VPD, most
409 of the parameters showed an antagonistic effect, except a synergistic effect on the lag time for
410 Robusta. λ parameters indeed decreased under the combined treatment, more than under water
411 deficit or O₃ exposure alone, as compared to the control. This effect could result from a better
412 detection of environmental variation. The different stomatal behaviours under successive
413 stresses in the two genotypes could result in different water losses (Fig. S2) at the leaf scale.
414 Robusta may have lost more water during the day when exposed to the combined stress than
415 to water deficit alone. These differences in leaf scale between genotypes could be particularly
416 detrimental for a water-deficit sensitive genotype. Nevertheless, in order to model
417 physiological processes, it is important to take constitutive differences in total leaf area
418 between genotypes into account, as this difference could impact extrapolation at the whole
419 tree scale.

420 4.4 Consequences for modelling

421 Modelling g_s is an important issue to use flux-based metrics for forest O_3 risk assessment
422 (Fares et al., 2013). Scientific literature about g_s models at the leaf level is abundant (see
423 Damour et al., 2010 for a review). Most current models used for O_3 , such as the DO_3SE model
424 (Emberson et al., 2000), are based on Jarvis multiplicative model (Jarvis, 1976) and do not
425 take O_3 -induced stomatal sluggishness into account (Hayes and Bangor, 2017). Hoshika et al.
426 (2017) proposed to take stomatal sluggishness into account through a single parameter (s) and
427 directly on steady states through an O_3 function (f_{O_3}). The main competitor of the Jarvis-type
428 model is based on the Ball-Woodrow-Berry model (BWB) (Ball et al., 1987). This model
429 integrates a more physiological approach and considers the relationship between g_s and
430 photosynthesis. Its modified version should be preferred for O_3 - induced decoupling between
431 photosynthesis and stomatal conductance (Cailleret et al., 2018; Lombardozzi et al., 2012).
432 Moreover, this model requires adding a soil water function. Contrasting results have been
433 found between the Jarvis or BWB models (Hoshika et al., 2017a). If these models are
434 properly parameterised, they can both accurately predict g_s in complex ecosystems (Fares et
435 al., 2013). This will depend on available data for model parameterisation and calibration
436 (Fares et al., 2013), but could be crucial for studying O_3 -sensitive tree species. Moreover, our
437 results highlight that it is important to take genotype-specific responses into account, *e.g.*
438 Carpaccio stomata closed faster than Robusta stomata under VPD, and opened faster under
439 irradiance. Nonetheless, these observations at the leaf scale might not be easily extrapolated at
440 the tree scale because of multiple obstacles: i) scaling up from leaf to canopy is tricky
441 (Ollinger et al., 1997; Zhou et al., 2017), ii) the impact of phenology, aging, and enhanced
442 leaf senescence should be taken into account (Anav et al., 2018), iii) leaf stomatal density is
443 modified in newly formed leaves (Durand et al., 2019; Pääkkönen et al., 1997), iv)
444 detoxification and repair processes occur (Tuzet et al., 2011), and v) conditioning
445 (Agathokleous et al., 2019) or cross-tolerance processes (Tausz et al., 2007) occur too. All
446 these points could challenge the implementation of larger models and the scaling up to whole
447 tree or forest ecosystems.

448 5 Conclusion

449 Despite constitutive differences between genotypes, O_3 and water deficit induced
450 stomatal closure and closing sluggishness. 80 ppb O_3 exposure followed by water deficit
451 modified stomatal closure differently between the two genotypes: Carpaccio stomata closed
452 more than under water deficit alone, whereas Robusta stomata closed less than under water

453 deficit alone. These modifications could have a non-negligible effect on O₃ uptake, carbon
454 storage, and water use efficiency. The underlying mechanisms still need investigations into
455 the active or passive physiological regulation induced by environmental fluctuations
456 (irradiance, VPD, O₃, soil water deficit, etc.). Previous studies suggested different stomatal
457 closure/opening mechanisms, *i.e.*, i) passive hydric regulation of guard cells (Buckley and
458 Mott, 2002), ii) mediation by a cross-talk between phytohormones (Daszkowska-Golec and
459 Szarejko, 2013), with a particular implication of abscisic acid or ethylene (McAdam and
460 Brodribb, 2015; Wilkinson and Davies, 2010, 2009), iii) O₃ and/or drought-induced stomatal
461 closure through modification of guard cell homeostasis *via* direct modulation of K⁺ channels
462 (Geiger et al., 2009; Vahisalu et al., 2010), alteration of Ca²⁺/H⁺ vacuolar antiporters (Dumont
463 et al., 2014a), and production of reactive oxygen species such as H₂O₂ (Damour et al., 2010).
464 Understanding these mechanisms and the cross-talks between the O₃ and water deficit
465 responses (as successive stresses or in combination) could allow for a better prediction of g_s
466 in response to various environmental modifications.

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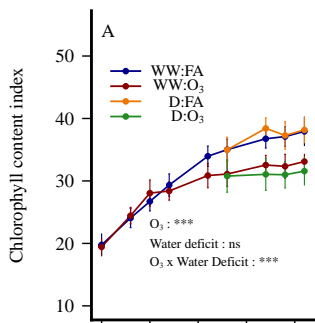
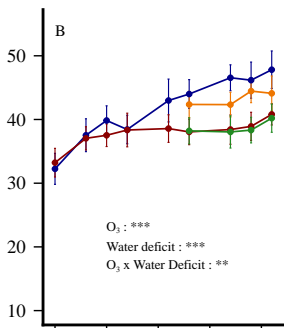
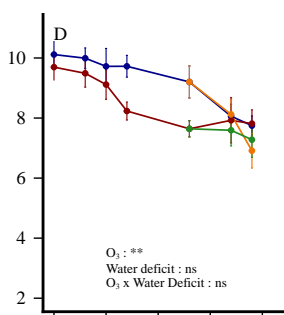
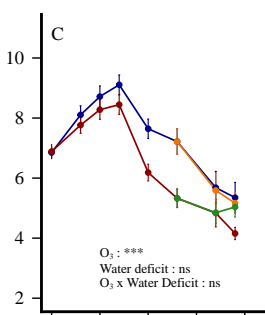
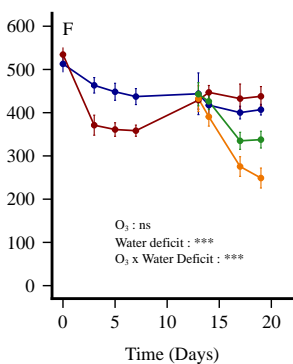
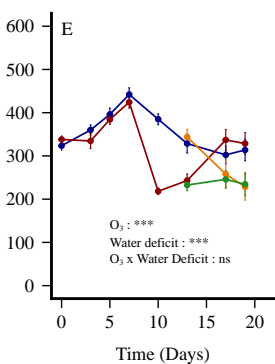
783 **Figure legends**

784 Figure 1: Impact of O₃ or/and water deficit on total chlorophylls (A, B), net CO₂ assimilation
785 (C, D) and stomatal conductance to water vapour (E, F). Measurements were conducted on
786 leaves of the Carpaccio and Robusta genotypes two to three times a week. For chlorophyll
787 contents, results are presented in arbitrary units obtained with Dualex. Means ± se, n ≥ 4.
788 Blue, WW:FA; orange, D:FA; red, WW:O₃; green, D:O₃. FA: filtered air; WW, well-watered;
789 D, water deficit; O₃, ozone. Asterisks indicate the significance of the factors or their
790 interactions tested by a linear mixed-effect model: '****'P ≤ 0.001, '**'P ≤ 0.01, '*'P ≤ 0.05,
791 'ns' non-significant.

792 Figure 2: Sigmoidal model parameters of stomatal dynamics in response to irradiance changes
793 in the Carpaccio and Robusta poplar genotypes after 13 days (A, B, E, F, I, J), or 21 days (C,
794 D, G, H, K, L) when submitted to 80 ppb of O₃ for 13 days and/or water deficit for an
795 additional week. (A, B, C, D): τ, response time(s); (E, F, G, H): λ, lag time(s); (I, J, K, L):
796 SL_{max}, maximum slope (mmol.m⁻².s⁻²). Means ± se, n ≥ 4. Letters show the significance levels
797 between treatments and genotype; ns, not significant. FA: filtered air; WW, well-watered; D,
798 water deficit; O₃, ozone. ANOVA, P-values are available in Table S4.

799 Figure 3: Sigmoidal model parameters of stomatal dynamics in response to VPD changes in
800 the Carpaccio and Robusta poplar genotypes after 13 days (A, C, E) or 21 days (B, D, F)
801 when submitted to 80 ppb of O₃ for 13 days and/or water deficit for an additional week. (A,
802 B): τ, response time(s); (C, D): λ, lag time(s); (E, F): SL_{max}, maximum slope (mmol.m⁻².s⁻²).
803 Means ± se, n ≥ 4. Letters show the significance levels between genotype and treatments (p <
804 0.05); ns, not significant. FA: filtered air; WW, well-watered; D, water deficit; O₃, ozone.
805 ANOVA, P-values are available in Table S4.

806 Figure 4: Combined successive impacts of O₃ exposure and moderate water deficit on the
807 parameters of the sigmoidal model for irradiance and VPD responses for the Carpaccio and
808 Robusta genotypes. τ, response time; λ, lag time, and SL_{max}, maximum slope; g₀ and G,
809 steady-state values of stomatal conductance at the beginning and the end of the experiment.
810 Bars represent the mean difference (± 95% confidence interval) between the observed and
811 expected additive effects of the combined two stressors. The zero line represents the expected
812 additive effects of the combined stressors. Additive effects are in white; when the means were
813 greater or lower than zero, they were considered as synergistic (black) or antagonistic (grey),
814 respectively. (Bansal et al., 2013).

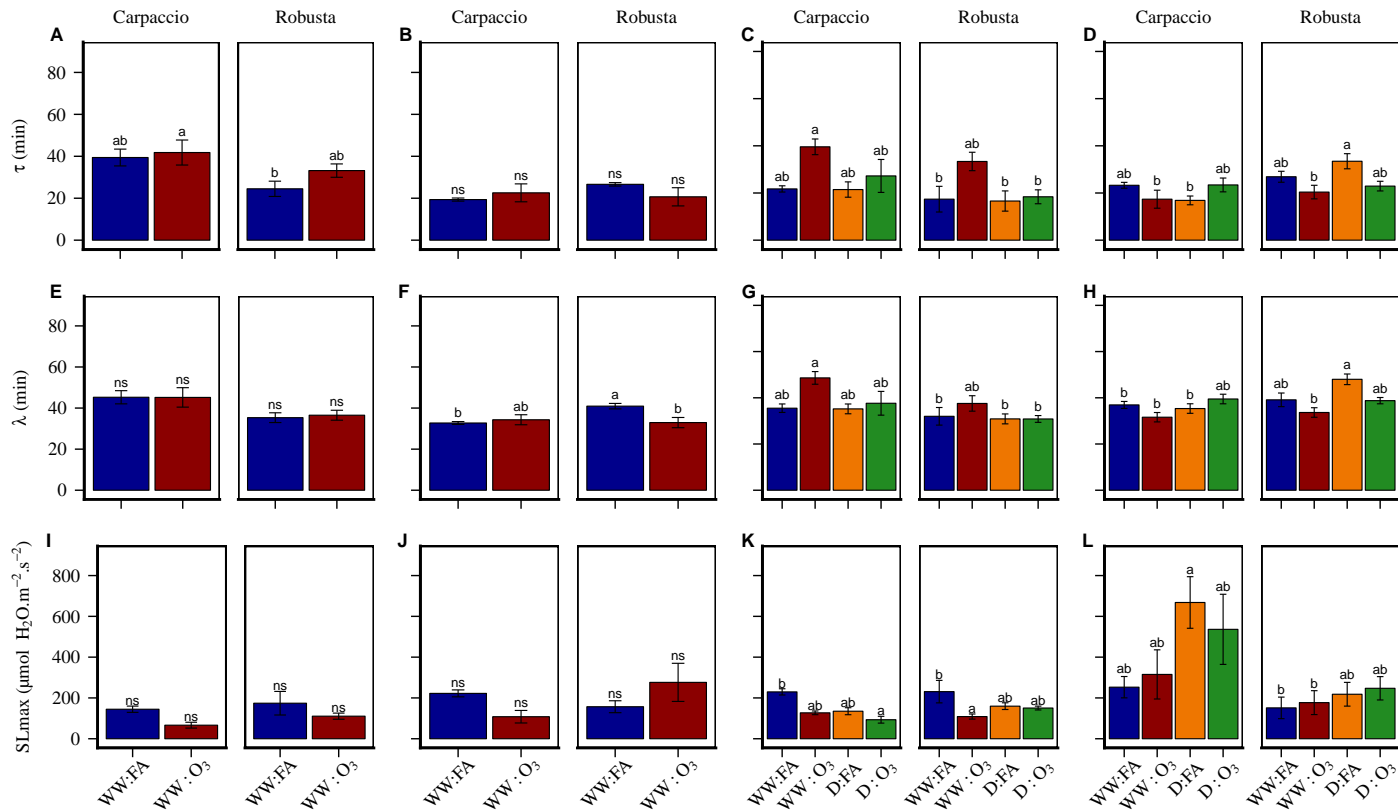
Carpaccio**Robusta**Net CO₂ assimilation ($\mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)Stomatal conductance ($\text{mmol H}_2\text{O} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$)

Day 13 : Closing

Day 13 : Opening

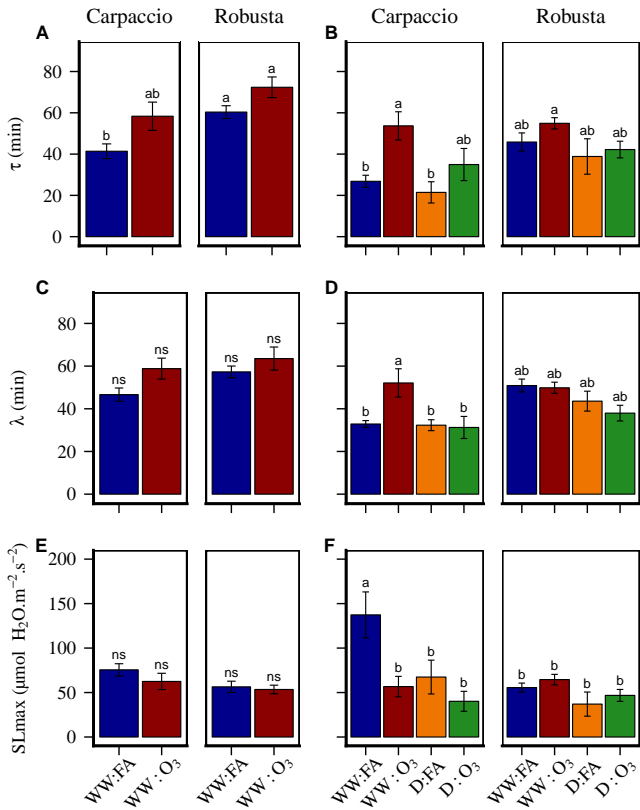
Day 21 : Closing

Day 21 : Opening



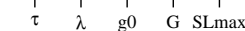
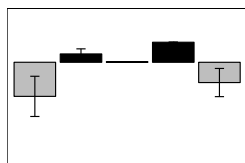
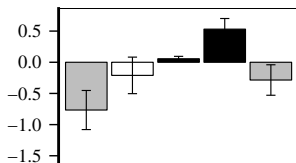
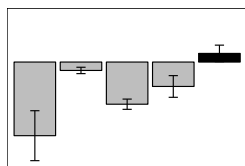
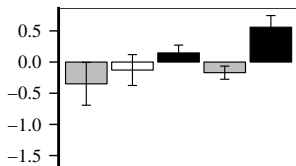
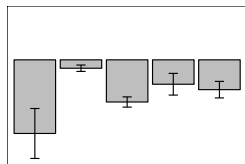
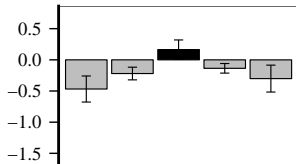
Day 13

Day 21



Carpaccio**Robusta**

Difference between observed and expected effects



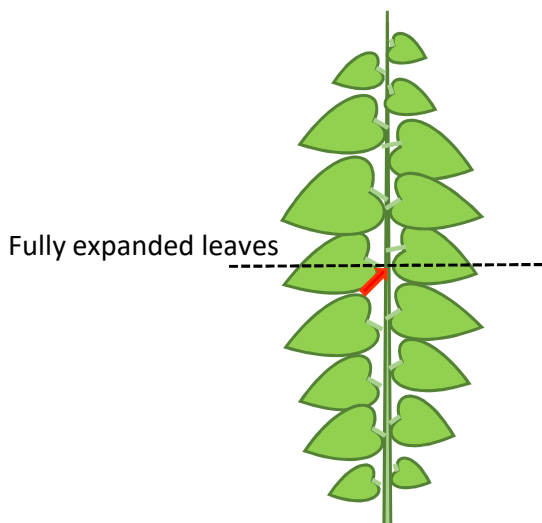
Closing light

Opening light

Closing VPD

Sigmoidal model parameters

Populus euramericana



Carpaccio Robusta

Stomatal dynamics

Well Watered & Filtered Air (WW:FA)

WW : FA Water Deficit : FA

WW : 80 ppb O₃ WW : FA

WW : 80 ppb O₃ Water Deficit : FA

13th day:
End of O₃ fumigation and beginning of drought

