V.1. Introduction du chapitre

Dans les conditions naturelles, il est très probable d'observer un épisode de concentration d'O_3 relativement élevé au printemps juste avant une sécheresse estivale. L'O_3 pourrait alors affecter la réponse des arbres à la sécheresse, notamment à travers le ralentissement de la fermeture des stomates. En effet, l'O_3 est connu pour ralentir la fermeture des stomates en réponse à des facteurs environnementaux, modifiant ainsi la transpiration des végétaux (Hoshika et al., 2015). Or, la perturbation du fonctionnement des stomates par le déficit hydrique et l'O_3 peut également augmenter la perte en eau et le flux d'O_3 entrant (Hoshika et al., 2013). Il est donc pertinent de s'intéresser aux conséquences qu'aurait un ralentissement de la fermeture des stomates sur la réponse de la plante dans le cas d'une succession de contraintes O_3 et sécheresse.

Ce ralentissement des stomates, appelé « stomatal sluggishness », a été observé en réponse à des changements de lumière (Dumont et al., 2013; Paoletti & Grulke, 2010), au VPD (Dumont et al., 2013; Grulke et al., 2007) et au déficit hydrique du sol (Durand et al., 2019a; Gérardin et al., 2018; Hoshika et al., 2013).

Pour mieux comprendre le « stomatal sluggishness » et son impact sur la réponse de la plante, nous avons mis en place une expérimentation permettant de mettre en évidence les mécanismes d'ouverture/fermeture des stomates en réponse à des variations de la lumière et du déficit hydrique à la vapeur d'eau (VPD) sous différentes contraintes : O_3, déficit hydrique du sol et la succession des deux. Les deux génotypes Carpaccio et Robusta ont été exposés aux différents traitements pendant 21 jours (section II.1.5). Les arbres ont été soumis à une fumigation d'O_3 de 80 ppb/jour pendant 13 jours, puis à un stress hydrique modéré pendant 7 jours. L'objectif était de déterminer i) si l'O_3 et/ou le déficit hydrique du sol modifiaient la réponse des stomates à la lumière et au VPD ; ii) si l'O_3 modifiait la réponse des stomates à la sécheresse. Pour cela, des mesures de courbes de réponse des stomates à la lumière et au VPD ont été réalisées (section II.2.3). Cette première partie a été soumise à publication et est présentée ci-après sous forme d'article. Une seconde partie traitera de l'évolution des niveaux d'AsA et de GSH en réponse à la succession de traitements, l'objectif étant de valider la méthode de détermination par HPLC chez le peuplier et de déterminer la réponse de ces deux antioxydants majeurs à la succession de contraintes, O_3 puis sécheresse. Les teneurs en AsA et en GSH ont été déterminées par HPLC à la fin des 13 jours de traitement O_3 et à la fin de l'expérimentation, après 21 jours de traitement.

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

Les résultats ont été soumis dans la revue *Environnemental Pollution* :

Dusart N., Vaultier M.-N., Olry J.-C., Buré C., Gérard J., Jolivet Y., Le Thiec D., 2019, *Altered stomatal dynamics of two Euramerican poplar genotypes submitted to successive ozone exposure and water deficit.*

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Abstract

The impact of ozone (O_3) pollution events on the plant drought response needs special attention because spring O_3 episodes are often followed by summer drought. By causing stomatal sluggishness, O_3 could affect the stomatal dynamic during a subsequent drought event. In this context, we studied the impact of O_3 exposure and water deficit (in the presence or in the absence of O_3 episode) on the stomatal closure/opening mechanisms relative to irradiance or vapour pressure deficit (VPD) variation. Two genotypes of *Populus nigra* x *deltoides* were exposed to various treatments for 21 days. Saplings were exposed to 80 ppb/day O_3 for 13 days, and then to moderate drought for 7 days. The curves of the stomatal response to irradiance and VPD changes were determined after 13 days of O_3 exposure, and after 21 days in the case of subsequent water deficit, and then fitted using a sigmoidal model. The main responses under O_3 exposure were stomatal closure and sluggishness, but the two genotypes showed contrasting responses. During stomatal closure induced by a change in irradiance, closure was slower for both genotypes. Nonetheless, the genotypes differed in stomatal opening under light. Carpaccio stomata opened more slowly than control stomata, whereas Robusta stomata tended to open faster. These effects could be of particular interest, as stomatal impairment was still present after O_3 exposure and could result from imperfect recovery. Under water deficit alone, we observed slower stomatal closure in response to VPD and irradiance, but faster stomatal opening in response to irradiance, more marked in Carpaccio. Under the combined treatment, most of the parameters showed antagonistic responses. Our results highlight that it is important to take genotype-specific responses and interactive stress cross-talk into account to improve the prediction of stomatal conductance in response to various environmental modifications.

V.2.1. Introduction

Forest health depends at least on our capacities to improve risk assessment. This evaluation depends on our understanding of tree biological and physiological responses to multiple environmental stressors (Sicard et al., 2016a). Among abiotic stress factors, tropospheric ozone (O_3) and drought are detrimental for tree growth and health (Allen et al., 2010; Wittig et al., 2007). O_3 is a phytotoxic air pollutant that impairs gas exchanges and reduces plant biomass (Dizengremel et al., 2013; Jolivet et al., 2016; Wittig et al., 2007). It is a secondary pollutant resulting from a photochemical process in the troposphere, with an annual cycle: higher daily O_3 concentrations are reported in spring and summer, lower ones in autumn and winter. Recurrent spring maxima have been reported in the northern hemisphere (Kalabokas et al., 2017; Monks, 2000; Parrish et al., 2013). On the other hand, drought events are predicted to increase in the near future. Due to global warming, wet regions are becoming wetter and dry regions drier (Liu & Allan, 2013). A reduction of primary growth was observed following the 2003 drought and heat wave (Ciais et al., 2005). Stand mortality was reported all over the world (Allen et al., 2010). Models integrating temperature, vapour pressure deficit (VPD) and rainfall predict an increased probability of similar events in the near future (IPCC, 2014; Lehner et al., 2006; Park Williams et al., 2013). Against this environmental fluctuation, the main physiological responses of trees and more generally plants are the control of transpiration by stomata. In the case of O_3, stomatal closure limits O_3 entrance. Under drought, stomatal closure prevents water loss. Nevertheless, there is a cost for carbon assimilation under both constraints. Stomata exert a major control on both the water and carbon cycles round the world (Hetherington & Woodward, 2003).

Meta-analyses of published data indicate that ambient O_3 reduces tree biomass production (Li et al., 2017; Wittig et al., 2009), while an analysis of survey data showed how O_3 reduces tree growth (Braun et al., 2014). Different metric indicators have been created to assess critical levels of O_3 for plants. The simplest ones were only based on cumulative exposure levels. The USA selected the SUM-index (sum of all hourly average concentrations over *X* ppb), whereas the EU selected the AOT40 (Accumulated Ozone over a Threshold of 40 ppb) which takes into account hourly O_3 concentrations above 40 ppb *per* hour when irradiance is 50 $W.m^{-2}$ minimum (Fuhrer et al., 1997; Musselman et al., 2006). The advantage of these metrics is that they make it simple to determine exposure levels only based on O_3 concentration data. In the early 2000's, the biologically more relevant concept of flux-based approach emerged, resulting in the

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

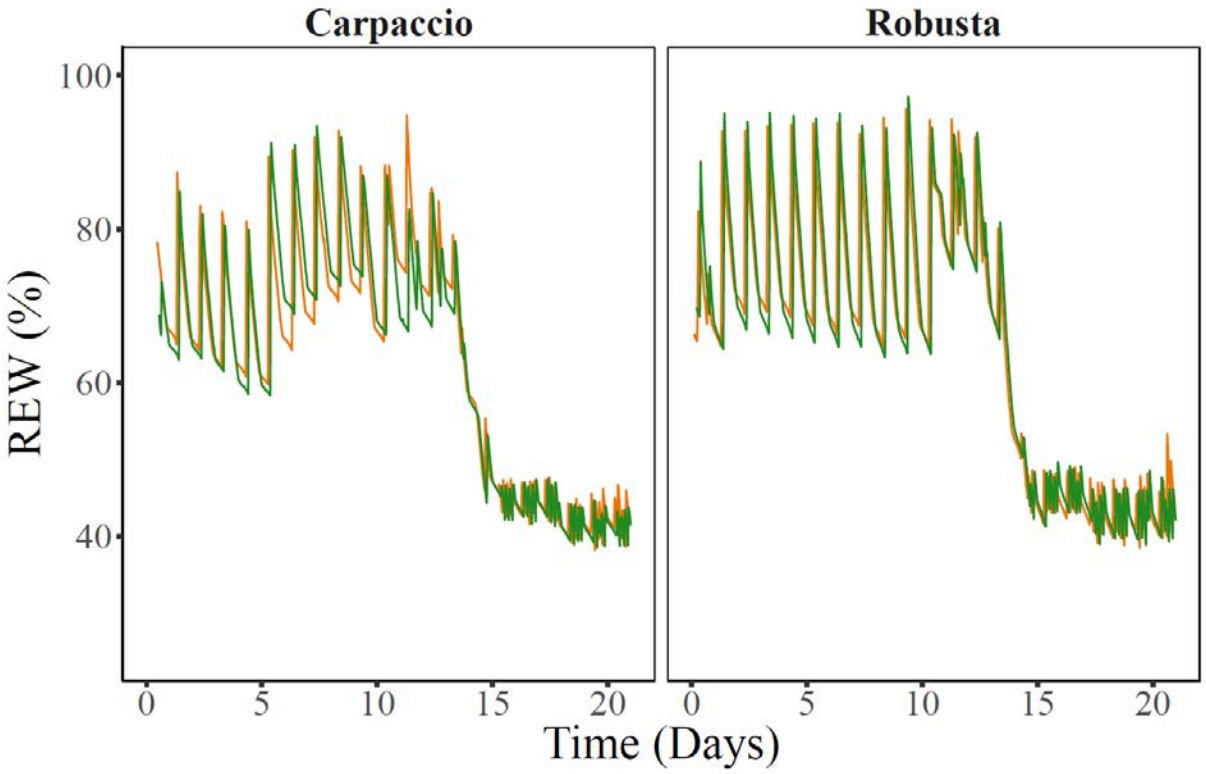

Figure 53 : Evolution of the soil relative water content (REW) calculated from soil water humidity for the Carpaccio and Robusta genotypes and for the water deficit treatments. Orange, D:FA; green, D:O_3.

Tableau 13 : Average O_3 exposure represented by SUM00 (sum of hourly O_3 concentrations) and AOT40 (Accumulated Ozone over a Threshold of 40 ppb) as well as metric dose (POD_0, Phytotoxic Ozone Dose above a threshold flux of 0 nmol m^{-2} s^{-1}) at the end of the fumigation period for Robusta and Carpaccio. Values are means of the four fumigation chambers. The "water used" values are the amounts of water consumed by each plant for 21 days, taking the cumulative sum of the amount of water added for each plant every day into account (n=6). FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone.

Genotype	Water treatment	Ozone treatment	SUM00 (ppm.h^{-1})	AOT40 (ppm.h^{-1})	POD_0 (mmol.m^{-2})	Water used (L)
Carpaccio	WW	FA				11.39
Carpaccio	WW	O_3	13.30	5,93	9.68	11.28
Carpaccio	D	FA				8.46
Carpaccio	D	O_3	13.30	5,93	9.74	8.36
Robusta	WW	FA				11.47
Robusta	WW	O_3	12.58	5.58	11.40	11.88
Robusta	D	FA				10.09
Robusta	D	O_3	12.58	5.58	11.60	9.54

V.2.2. Materials and methods*

V.2.2.1. *Plant material and exposure conditions*

Cuttings of the two Euramerican poplar genotypes "Carpaccio" and "Robusta" were grown in growth chambers as already described in Dusart et al. (2019b) with slight modifications. Cuttings were planted in ten-liter pots filled with a sand/peat mixture (1/1, v/v) and fertilised by adding 15 g of slow-release nutritive granules (Nutricot T-100) and 1 $g.L^{-1}$ $CaMg(CO_3)_2$. For both genotypes, forty-eight plants were randomly distributed in eight phytotronic chambers, i.e. twenty-four plants in control chambers (charcoal-filtered air), and twenty-four plants in chambers set for O_3 treatment (80 $nmol.mol^{-1}$ for 13 hours, from 09:00 to 22:00). For reasons of space in the culture chambers and length of measurement times, the experiment was duplicated separately for each genotype. After a 7-day-long acclimation period, the O_3 treatment started while control saplings were exposed to charcoal-filtered air for 13 days. After 13 days (d) of fumigation, the total cumulative sum of O_3 flux (SUM00), the cumulative O_3 dose above a threshold of 40 ppb (AOT 40), and the phytotoxic O_3 dose above a threshold flux of 0 $nmol.m^{-2}.s^{-1}$ (POD_0) (based on measured stomatal conductance, see Bagard et al., 2015) were determined (Tableau 13). At the end of the O_3 exposure period, half of the saplings were submitted to a moderate water deficit for 7 d. Soil Water Content (SWC) was determined with 24 wireless Time Domain Reflectometry (TDR) probes (CWS655E, Campbell Scientific Ltd, Antony, France). A calibration between volumetric SWC measured by TDR and pot weight was performed. The biological available water was expressed as relative extractable water (REW), as described by Wildhagen et al. (2018) for the same soil. Poplars were watered with a known volume of water several times a day to maintain the level of REW stable. For the well-watered treatment, poplars were irrigated at 75 % (±10%) of REW, whereas for the water deficit treatment, irrigation was set to 45% (± 2%) of REW until the end of the experiment (Figure 53). A cumulative sum of the amount of water added for each treatment for 21 d is presented in Tableau 13.

V.2.2.2. *Plant growth*

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

* N.D.A : Cette partie étant incluse dans l'article en anglais, elle est redondante avec le Chapitre IIMatériels et méthodes, excepté pour les informations plus précises concernant la mise en place de l'expérimentation (cf. Tableau 13 et Figure 53)

V.2.2.3. *Gas exchanges and photosynthetic pigment kinetics*

Gas exchanges (A_n, net CO_2 assimilation, and g_s, stomatal conductance to water vapour) were measured using a Li-6200 (Li Cor, Lincoln, NE, USA) as described in Dusart et al. (2019b). Non-destructive determination of the chlorophyll pigment content was performed with a Dualex (Force-A, Orsay, France). For all non-destructive leaf measurements, the same leaf was used, i.e., the first fully expanded leaf (the 10th leaf from the apex) at the beginning of the O_3 treatment.

V.2.2.4. *Stomatal response to irradiance and vapour pressure deficit*

V.2.2.4.a. Gas exchange measurements

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

V.2.2.4.b. Modelling

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

$$g_s = g_0 + (G - g_0)\, e^{-e^{(\frac{\lambda - t}{\tau})}}$$

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

$$SL_{max} = \frac{G - g_0}{\tau . e}$$

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

V.2.2.5. *Statistical analyses*

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

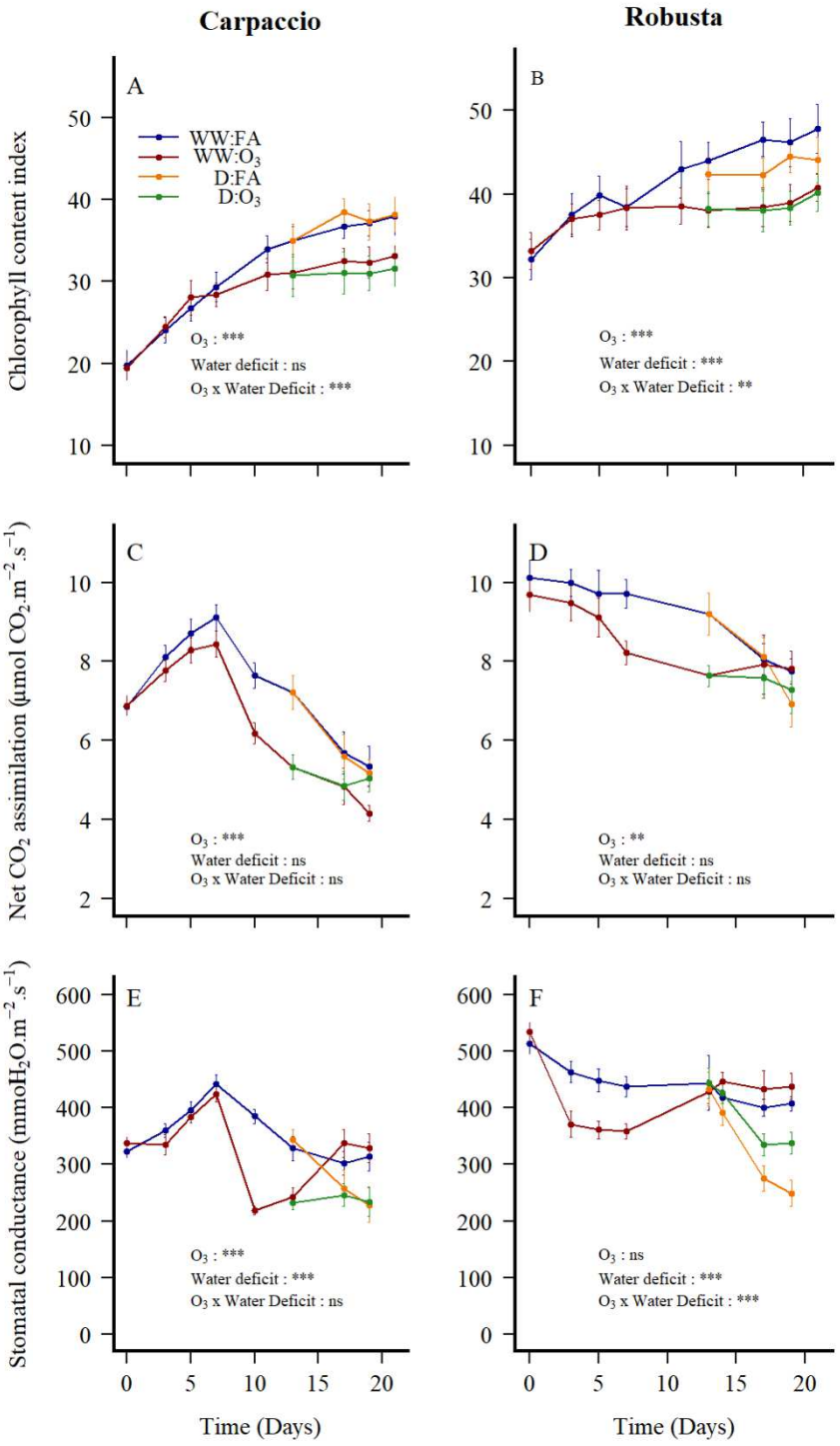

Figure 54 : Impact of O_3 or/and water deficit on total chlorophylls (A, B), net CO_2 assimilation (C, D) and stomatal conductance to water vapour (E, F). Measurements were conducted on leaves of the Carpaccio and Robusta genotypes two to three times a week. For chlorophyll contents, results are presented in arbitrary units obtained with Dualex. Means ± se, n ≥ 4. Blue, WW:FA; orange, D:FA; red, WW:O_3; green, D:O_3. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone. Asterisks indicate the significance of the factors or their interactions tested by a linear mixed-effect model: '***'P ≤ 0.001, '**'P ≤ 0.01, '*'P ≤ 0.05, 'ns' non-significant.

V.2.3. Results

V.2.3.1. *Effect on growth, chlorophyll contents and gas exchange kinetics*

O_3 impacted only the stem biomass of the two genotypes. Water deficit only slightly impacted the number of leaves of Robusta genotype, nevertheless leaf surface area decreased in the Carpaccio genotype only (Table S1).

An increase in chlorophyll contents was observed in both genotypes under the WW:FA modality throughout the 21 days of the experiment (Figure 54A and B), and Robusta systematically contained 1.5 times more chlorophyll than Carpaccio. Chlorophyll contents were significantly lower under O_3 treatment in both genotypes; from 11 days, chlorophyll levels remained lower than the control treatment (Figure 54A and B). The chlorophyll content of the water deficit treatment (D:FA) was significantly lower in Robusta as compared to the WW:FA modality (Figure 54A and B). D:O_3 impacted chlorophyll contents similarly to WW:O_3.

Concomitantly, gas exchanges were recorded twice or three times a week (Figure 54C and D). The control treatment values decreased throughout the experiment because the leaves received less light (because the upper leaves were still growing). Although net CO_2 assimilation (A_n) decreased over time, A_n values were 3 µmol $CO_2.m^{-2}.s^{-1}$ higher in the Robusta genotype . ANOVA on A_n only showed a significant effect of O_3 on both genotypes (Figure 54C and D). O_3 decreased A_n, from 10 d in Carpaccio and 6 d in Robusta. After the O_3 treatment was switched off on d 13, Robusta photosynthesis reached the same levels as the control, *i.e.*, 7.5 µmol.$m^{-2}.s^{-1}$.

Concerning stomatal conductance values, O_3 decreased g_s from 6 d in Carpaccio and 2 d in Robusta, with a significant effect only on Carpaccio (Figure 54E and F). At the end of the 13 d of O_3 fumigation, g_s went back to the control level in both genotypes, *i.e.*, 400 mmol.$m^{-2}.s^{-1}$ and 300 mmol.$m^{-2}.s^{-1}$ in Robusta and Carpaccio, respectively. Water deficit decreased g_s (around 230 mmol.$m^{-2}.s^{-1}$) in both genotypes (Figure 54E and F). Carpaccio stomata displayed similar conductance values under the D:FA and D:O_3 conditions. A difference was observed in Robusta, *i.e.*, stomata under D:O_3 were less closed than under D:FA (330 mmol.$m^{-2}.s^{-1}$, Figure 54F), resulting in a significant interaction between O_3 exposure and water deficit.

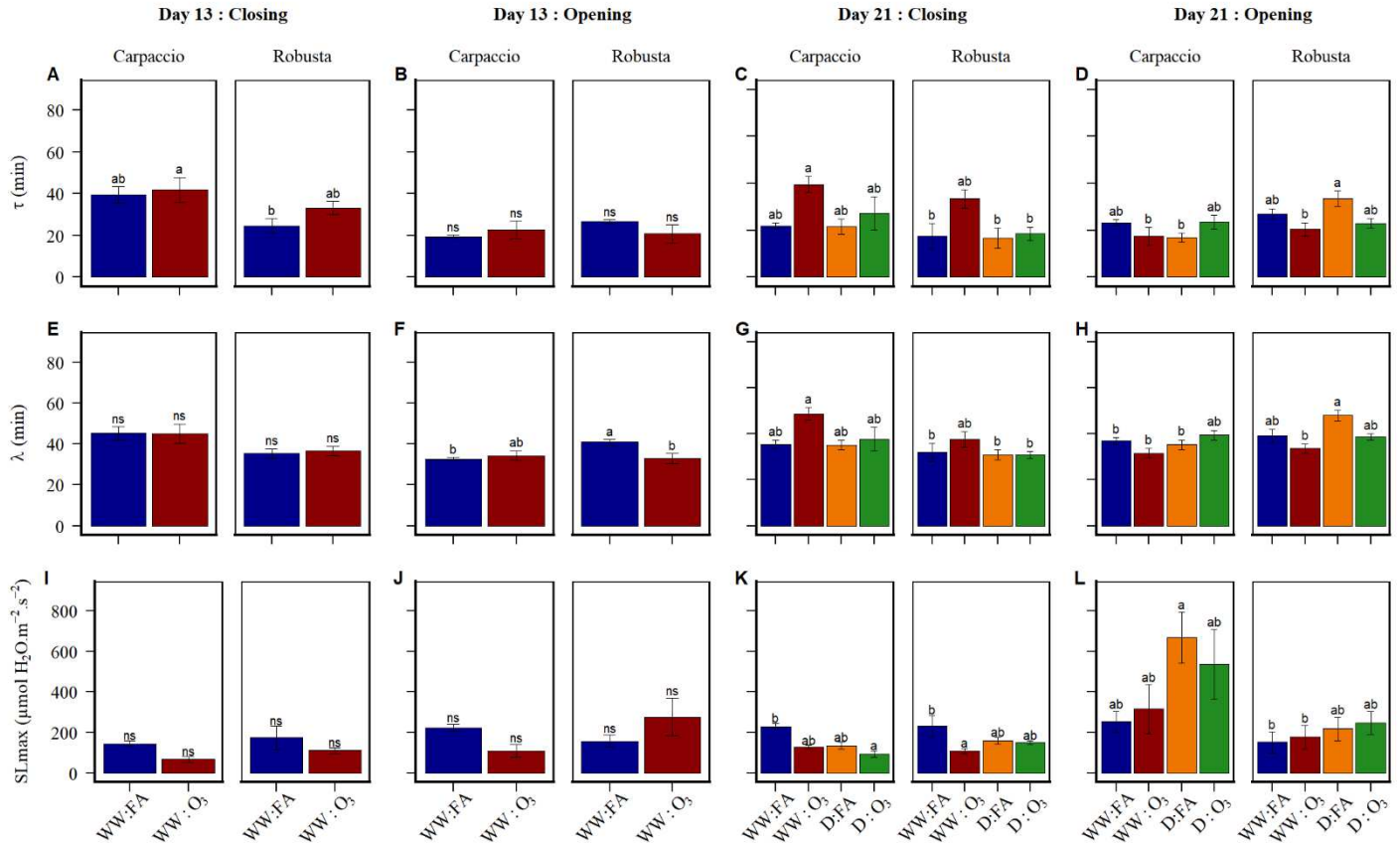

Figure 55 : Sigmoidal model parameters of stomatal dynamics in response to irradiance changes in the Carpaccio and Robusta poplar genotypes after 13 days (A, B, E, F, I, J), or 21 days (C, D, G, H, K, L) when submitted to 80 ppb of O_3 for 13 days and/or water deficit for an additional week. (A, B, C, D): τ, response time(s); (E, F, G, H): λ, lag time(s); (I, J, K, L): SL_{max}, maximum slope ($mmol.m^{-2}.s^{-2}$). Means ± se, n ≥ 4. Letters show the significance levels between treatments and genotype; ns, not significant. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone. ANOVA, P-values are available in Table S3.

V.2.3.2. *Effect of O_3 and recovery of stomatal behaviour*

V.2.3.2.a. Responses to irradiance

At the end of the O_3 treatment (13 d), net CO_2 assimilation in steady state 0, *i.e.*, A_0, 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 Robusta (Table S2). The steady states of g_s after stomatal closure (g_1) were similar for both genotypes and treatments (around 100 mmol.mm^{-2}.s^{-1}). After stomatal closure, net CO_2 assimilation reached negative values (A_1) due to dark respiration (Table S2). The respiration rates in FA conditions were higher in Robusta than in Carpaccio (-1.6 *vs.* -1.3 µmol.m^{-2}.s^{-1}). Under WW:O_3 conditions, an increase of respiration was observed, *i.e.*, +30% and +18% in Carpaccio and Robusta, respectively. Concerning the stomatal closure phase, the τ and λ parameters were not modified by O_3 treatment in Carpaccio (Figure 55A and E). In Robusta, τ tended to increase under O_3 exposure (1.5 fold) (Figure 55A) and λ was not modified (Figure 55E). Moreover, λ values differed between genotypes, with a higher value for Carpaccio (1.2-fold higher) (Figure 55E). SL_{max} values highlighted a trend for an O_3 effect: the stomatal closure speed was reduced by 0.5 fold and 0.4 fold in Carpaccio and Robusta, respectively (Figure 55I). As regards the stomatal opening phase, λ was 6 min faster in Carpaccio than in Robusta (Figure 55F). λ was not impacted by O_3 in Carpaccio, whereas it significantly decreased by 25% in Robusta. SL_{max} values significantly differed between the two genotypes (Table S4). Stomatal opening was slower in Carpaccio, with an SL_{max} 0.5 fold lower under O_3, but changes were not significant in Robusta (Figure 55J). The steady states of conductance (g_2) after the opening phase (Table S2) returned to the same levels as the first steady states in Carpaccio. In Robusta under O_3, g_2 was 30% lower than g_0 before stomatal closure (Table S2). Similarly, assimilation went back to the same levels (A_0=A_2) in the control leaves (Table S4).

After a week without O_3 exposure (21 d), net CO_2 assimilation (A_0) went back to the same level as the control in Carpaccio, whereas a 20% decrease in CO_2 uptake was observed in Robusta as compared to the WW:FA modality (Table S2). In parallel, g_0 was the same under the O_3 and control treatments in Carpaccio. As for Robusta, g_0 was higher under O_3, and stomata opened 31% more (Table S2). During stomatal closure, τ and λ values in the WW:O_3 treatments changed non significantly as compared to 13 d (Figure 55C and G). However, as compared to the WW:FA treatments, τ and λ values were 66% and 38% greater in Carpaccio, respectively, and 60% and 15% greater in Robusta, respectively (Figure 55C and G). SL_{max} decreased by 56% in Carpaccio and by 47% in Robusta (Figure 55K).

Regarding stomatal opening, g_1 was not significantly affected by O_3 in either genotype (Table S2). Final steady states were similar under the WW:O_3 and WW:FA treatments, *i.e.*, around

340 mmol.m^{-2}.s^{-1}. τ and λ non significantly decreased under O_3 treatment in both genotypes (Figure 55D and 2H). Stomatal opening tends to be faster in both genotypes under WW:O_3 (Figure 55L).

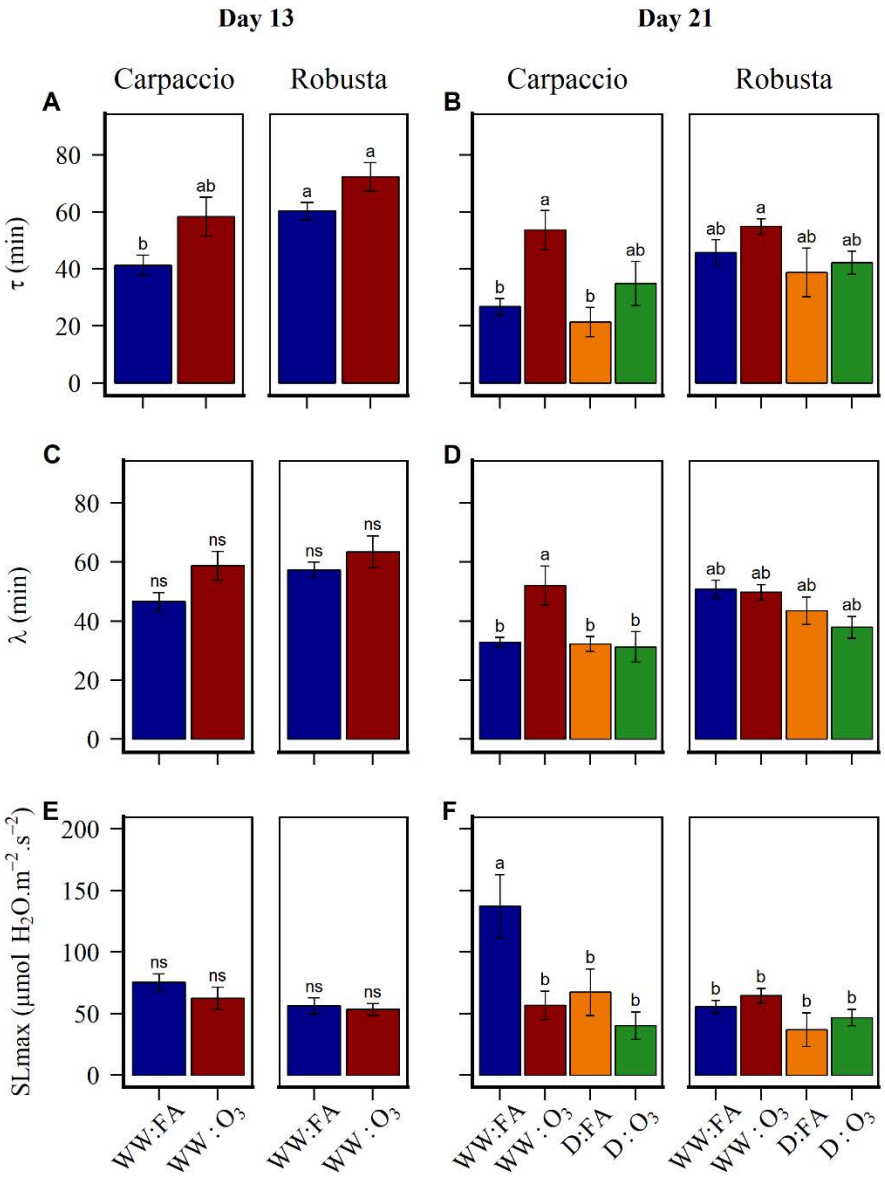

Figure 56 : Sigmoidal model parameters of stomatal dynamics in response to VPD changes in the Carpaccio and Robusta poplar genotypes after 13 days (A, C, E) or 21 days (B, D, F) when submitted to 80 ppb of O_3 for 13 days and/or water deficit for an additional week. (A, B): τ, response time(s); (C, D): λ, lag time(s); (E, F): SL_{max}, maximum slope (mmol.m^{-2}.s^{-2}). Means ± se, n ≥ 4. Letters show the significance levels between genotype and treatments ($p < 0.05$); ns, not significant. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone. ANOVA, P-values are available in Table S4.

V.2.3.2.b. Responses to VPD

The study of the vapour pressure deficit response curves consisted in measuring stomatal closure under pressures ranging between 0.8 and 3 kPa. At the end of the O_3 treatment (13 d), the assimilation (A_0) and stomatal conductance (g_0) values of the initial steady states of Carpaccio and Robusta were almost the same as those of the light response curves (Tables S3 and S4). After stomatal closure, CO_2 assimilation (A_1) decreased by 16 to 30% as compared to A_0 depending on genotype or treatment (Table S4). The τ parameter in control conditions differed between genotypes (Figure 56A), as it took Robusta 19 min more than Carpaccio. O_3 increased τ by 17 min and 11 min in Carpaccio and Robusta, respectively (Figure 56A). The λ parameter also differed between the two genotypes in the control conditions: it took Robusta saplings 11 min more to reach the inflection point (Figure 56C). λ increased under O_3 treatment in both genotypes, *i.e.*, by 12 min and 5 min in Carpaccio and Robusta, respectively (Figure 56C).

After a week without O_3 treatment, stomatal dynamic responses to VPD still showed a few differences between the control and the O_3 treatment. In Carpaccio, O_3 increased τ and λ by 1.8 and 1.6 fold, respectively (Figure 56B and D). SL_{max} decreased by 70% in Carpaccio (Figure 56F). Robusta was impacted in a different way, as τ slightly increased by 1.1 fold under O_3 treatment, but λ was unaffected (Figure 56B and D).

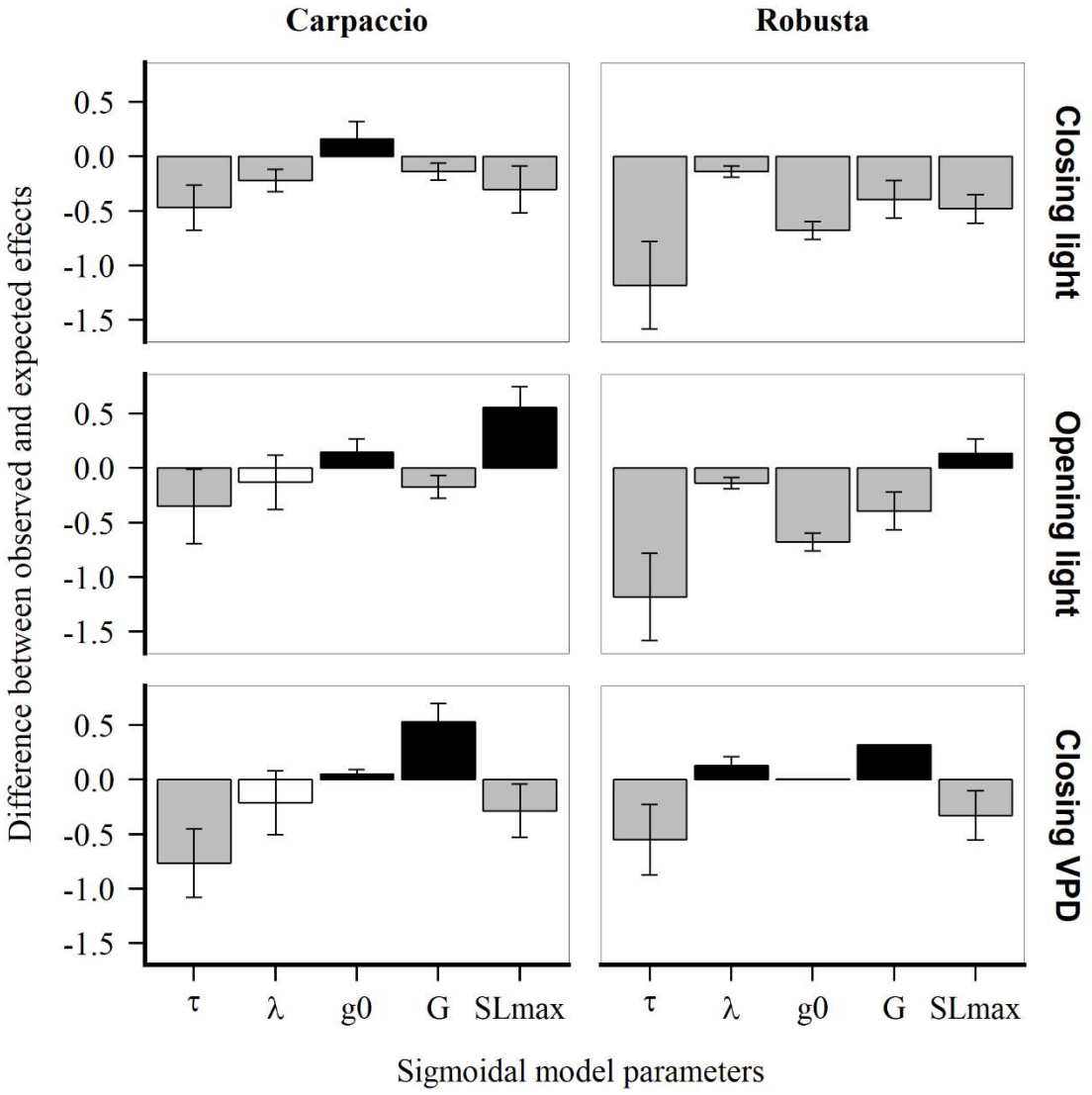

Figure 57 : Combined successive impacts of O_3 exposure and moderate water deficit on the parameters of the sigmoidal model for irradiance and VPD responses for the Carpaccio and Robusta genotypes. τ, response time; λ, lag time, and SL_{max}, maximum slope; g_0 and G, steady-state values of stomatal conductance at the beginning and the end of the experiment. Bars represent the mean difference (± 95% confidence interval) between the observed and expected additive effects of the combined two stressors. The zero line represents the expected additive effects of the combined stressors. Additive effects are in white; when the means were greater or lower than zero, they were considered as synergistic (black) or antagonistic (grey), respectively. (Bansal et al., 2013).

V.2.3.3. *Effect of water deficit on stomatal behaviour*

V.2.3.3.a. Response to irradiance

After 7 days of water deficit treatment, a significant water deficit effect was observed for some parameters of the model. Regarding stomatal closure under light, the initial steady state showed stomatal closure under water deficit conditions (Table S2). Water deficit induced

a 45% decrease of g_0 in both genotypes (Table S2). After closure, g_1 showed some marked differences with the control treatment, *i.e.*, an 84% decrease in Carpaccio and a 47% decrease in Robusta. Moreover, stomatal closure was greater in Carpaccio than in Robusta (88 *vs*. 122 $mmol.m^{-2}.s^{-1}$ for g_1, respectively). During the stomatal closure phase, τ and λ were unaffected by water deficit in either genotype (Figure 55C and 2G).

During the stomatal opening phase due to irradiance, the water deficit effect was significant on τ and SLmax (Table S4). τ decreased by 74% in Carpaccio but increased by 26% in Robusta as compared to the control (Figure 55D). λ was unaffected in Carpaccio but increased in Robusta (+ 21%) (Figure 55H). SL_{max} increased under water deficit almost 3 times faster in Carpaccio (Figure 55L). These increases differed significantly between the genotypes (Table S4).

V.2.3.3.b. Responses to VPD

After the VPD closing phase, g_1 were unaffected in Carpaccio, whereas it was 50 $mmol.m^{-2}.s^{-1}$ below the WW:FA values in Robusta (Table S4). A_0 and A_1 decreased under water deficit by the same amplitude (around 30%) in both genotypes (Table S4).

Water deficit affected the stomatal dynamics through a non significant decrease of the τ parameter in both genotypes, by 20% in Carpaccio *vs*. 15% in Robusta (Figure 56B). The λ parameter was not modified in Carpaccio but decreased in Robusta (-13%) (Figure 56D). Finally, a lower SL_{max} was observed: -51% and -33% in Carpaccio and Robusta, respectively, as compared to the control (Figure 56F).

V.2.3.4. *Effects of O_3 and water deficit on stomatal behaviour*

V.2.3.4.a. Responses to irradiance

After 7 days of water deficit treatment, most of the parameters showed an antagonistic response in the combined treatment (Figure 57), except the initial steady state g_0 in Carpaccio. In this genotype, stomatal closure was greater than under the water deficit treatment alone (Table S2). During stomatal closing under irradiance, τ slightly increased by 6 min in Carpaccio, in-between the values under water deficit and O_3 exposure, and was unaffected in Robusta as compared to the control (Figure 55C). λ showed the same trends as under the D:FA modality (Figure 55G). SL_{max} was significantly affected by the water deficit x O_3 interaction (p=0.05): in Carpaccio, SL_{max} was lower than under the D:FA and WW:O_3 modalities, by 23% as compared to the WW:FA modality. In Robusta, SL_{max} was in the same range as under D:FA conditions (Figure 55K). Stomatal opening as a result of irradiance under the combined treatment revealed no significant effect of the water deficit x O_3 interaction on any of the parameters of the models except τ (Table S4). In Carpaccio, SL_{max} values were intermediate between D:FA and WW:O_3 values, almost twice the WW:FA value. In Robusta, SL_{max} tended to increase as compared to the other treatments (Figure 55L), resulting in a synergistic effect (Figure 57).

V.2.3.4.b. Responses to VPD

After 7 days of water deficit treatment on saplings previously submitted to O_3 treatment, no significant effect was observed (Table S4). There was a synergistic effect on G (g_1), under the D:O_3 modality, the final steady state tended to be “more closed stomata” in Carpaccio *vs*. “slightly more open stomata” in Robusta than under the WW:O_3 or D:FA modalities (Table S4). Otherwise, most of the parameters showed an antagonistic effect (Figure 57). In Robusta, the λ parameter decreased (by 26% as compared to the control) under the combined treatments (Figure 56D), more than under water deficit or O_3 alone, resulting in a synergistic effect (Figure 57). In Carpaccio, SL_{max} decreased in a similar way under all three modalities. (Figure 56F).

V.2.4. Discussion

V.2.4.1. *Sluggish stomatal response to O_3*

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

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

In addition, stomatal closure went along with decreased steady states. This stomatal response was reversible: when O_3 exposure was interrupted, saplings rapidly recovered the same steady states as in the control treatment. According to the dynamics parameters in response to irradiance, the lag time (λ) was not modified by O_3 during stomatal closure, but a few constitutive differences between the genotypes were visible. In Robusta, τ tended to increase under O_3 exposure. The main effect for both genotypes was a trend toward a lower SL_{max}, *i.e.*, the so-called O_3 sluggishness. In addition, steady states (g_1) in the dark were slightly higher. This might have consequences under natural conditions, when O_3 entrance during the night time could be significant (Hoshika et al., 2013) and could enhance water loss (Grulke et al., 2004). From our results, we can propose a hypothetical model of the stomatal daily course (Figure 58) in conditions of stable diurnal irradiance throughout the day, with maximum VPD at midday. This representation highlights the differences between treatments and genotypes and the potential impact on the O_3 flux, CO_2 assimilation (grey area), or used water (blue area). This theoretical representation was inspired by a hypothetical model of Dumont et al. (2013), the results of a Ball-Berry-type model from Tuzet et al. (2003), and daily conductance measurements from Durand et al. (2019). The differences in g_s values between treatments, genotypes, and irradiance variations were conserved. As for stomatal closure, the model parameters were also modified during stomatal opening.

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

day (Figure 58). Interestingly, after O_3 exposure was stopped, Robusta recovered the same assimilation rate as the control whereas Carpaccio photosynthesis remained impaired. Our results are consistent with stomatal recovery after O_3-induced stress observed in the literature. Similar recovery was reported in white clover (Francini et al., 2007). Nonetheless, this imperfect recovery due to carry-over or 'memory' effects could be particularly detrimental under repeated O_3 exposure (Oksanen, 2003; Oksanen & Saleem, 1999) combined with other biotic or abiotic stresses (Langebartels et al., 1998), *e.g.*, water deficit conditions. However, this 'memory' could also be responsible for a 'conditioning' mechanism through a hormetic response (Agathokleous et al., 2019) and/or cross-tolerance (Walter et al., 2013). In both cases, this effect could modify tree resilience and acclimation to a new disturbance.

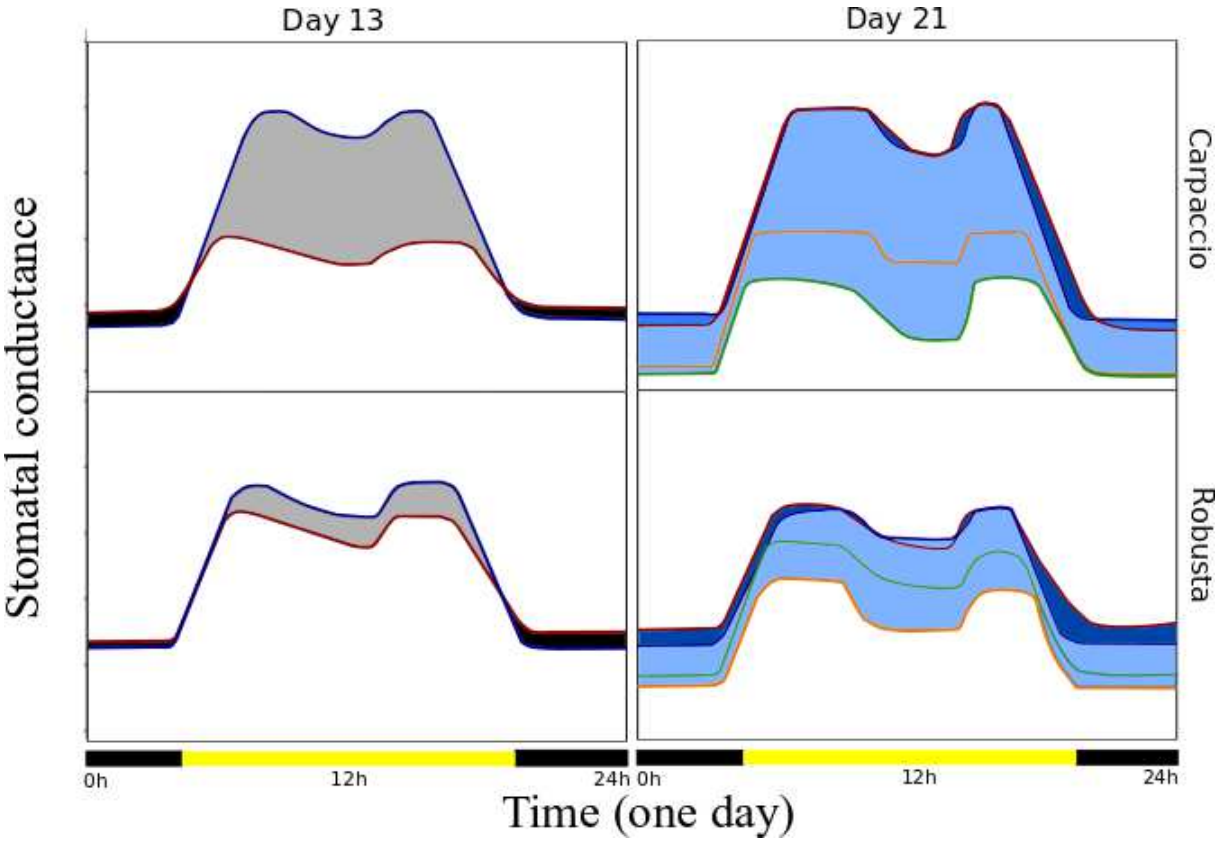

Figure 58 : Hypothetical stomatal daily courses in natural conditions for both genotypes after O_3 exposure (red line), water deficit treatment (green line), or the combined treatment (orange line). The O_3-induced effect is represented on d 13, with a decreased O_3 uptake in the daytime as the main effect with decreased steady states (grey area), but with an increased O_3 uptake at night (black area). Water consumption is represented on d 21 (blue area). Blue, WW:FA; orange, D:FA; red, WW:O_3; green, D:O_3. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone.

V.2.4.2. *Water deficit induces slower stomatal closure in response to light and VPD*

Water deficit reduced only the total leaf surface of Carpaccio genotype (Table S1) associated with an expected stomatal closure over time (Bogeat-Triboulot et al., 2007; Chaves et al., 2002; Dusart et al., 2019b). In response to darkness, the time response parameters were modified, with a few differences between the genotypes. Nevertheless, the lag time and closing speed were not impacted by water deficit in either genotype. Interestingly, in the dark both genotypes had their stomata more closed than the control saplings. Moreover, Robusta stomata were less closed than Carpaccio stomata. This could result in differences in water loss at night (Figure 58) (Caird et al., 2007). By contrast, stomatal opening following irradiance affected all the parameters of the models. The opening speed increased in both genotypes, especially in Carpaccio. Faster stomatal opening under water deficit has been reported in *Phaseolus vulgaris* (Barradas et al., 1994). Water loss might increase due to faster stomatal opening, but may also increase the leaf carbon assimilation (Barradas et al., 1994). In response to VPD, water deficit affected stomatal dynamics through a decrease of the τ parameter in both genotypes. The lag time parameter was not modified in Carpaccio and decreased in Robusta. There was great sluggishness in the response to VPD. Stomatal closure sluggishness in response to irradiance and/or VPD has been reported in different species, after leaf excision in *Populus trichocarpa* x *deltoides* (Reich & Lassoie, 1984), under soil water deficit conditions in *Phaseolus vulgaris* (Hoshika et al., 2013), *Nicotiana tabacum* (Gérardin et al., 2018), *Populus nigra,* and *Populus euramericana* (Durand et al., 2019a). Stomatal closure under soil water deficit or increased VPD was found closely related to ion transport and abscisic acid perception (McAdam & Brodribb, 2015; Pospíšilová, 2003). These mechanisms could be modified by O_3 through ABA synthesis (McAdam et al., 2017) or through other phytohormones such as ethylene (Wilkinson & Davies, 2010, 2009).

V.2.4.3. *Antagonistic effect of the successive exposure to O_3 and water deficit*

When O_3 exposure was followed by water deficit, we observed a combination of the effects of O_3 or water deficit alone. The impact on height and radial growth was similar to the effect of water deficit alone in both genotypes. Nevertheless, total biomass decreased more following the successive stresses (Table S1). As seen above, water deficit and O_3 separately modified stomatal behaviour in response to VPD or irradiance fluctuations. The interaction between the two stress factors may have modified stomatal responses (Hoshika et al., 2013; Wilkinson & Davies, 2009). Concerning gas exchanges, stomatal closure was observed in both genotypes, with a decreased steady state. Carpaccio stomata tended to be more closed than under O_3

exposure or water deficit alone, whereas Robusta stomata were slightly less closed than under water deficit alone (Figure 58). Under the combined treatment, most of the parameters showed an antagonistic response, *i.e.*, a weaker response than the expected additive effect (Bansal et al., 2013; Dusart et al., 2019b; Pellegrini et al., 2019). τ seemed to increase as compared to the control and drought treatment alone, but less than under O_3 exposure. The lag time and final steady state were modified in the same way as under water deficit alone. In Carpaccio, stomatal sluggishness increased as compared to the separate constraints, and this may have increased transpiration under water deficit. In Robusta, the slower closure was in the same range as under water deficit alone. Regarding stomatal opening due to irradiance, the response was the same as for water deficit alone on all the parameters of the models, without any significant interaction of O_3. In response to VPD, most of the parameters showed an antagonistic effect, except a synergistic effect on the lag time for Robusta. λ parameters indeed decreased under the combined treatment, more than under water deficit or O_3 exposure alone, as compared to the control. This effect could result from a better detection of environmental variation. The different stomatal behaviours under successive stresses in the two genotypes could result in different water losses (Figure 58) at the leaf scale. Robusta may have lost more water during the day when exposed to the combined stress than to water deficit alone. These differences in leaf scale between genotypes could be particularly detrimental for a water-deficit sensitive genotype. Nevertheless, in order to model physiological processes, it is important to take constitutive differences in total leaf area between genotypes into account, as this difference could impact extrapolation at the whole tree scale.

V.2.4.4. *Consequences for modelling*

Modelling g_s is an important issue to use flux-based metrics for forest O_3 risk assessment (Fares et al., 2013). Scientific literature about g_s models at the leaf level is abundant (see Damour et al., 2010 for a review). Most current models used for O_3, such as the DO_3SE model (Emberson et al., 2000), are based on Jarvis multiplicative model (Jarvis, 1976) and do not take O_3-induced stomatal sluggishness into account (Hayes & Bangor, 2017). Hoshika et al. (2017) proposed to take stomatal sluggishness into account through a single parameter (s) and directly on steady states through an O_3 function (f_{O3}). The main competitor of the Jarvis-type model is based on the Ball-Woodrow-Berry model (BWB) (Ball et al., 1987). This model integrates a more physiological approach and considers the relationship between g_s and photosynthesis. Its modified version should be preferred for O_3- induced decoupling between photosynthesis and stomatal conductance (Cailleret et al., 2018; Lombardozzi et al., 2012). Moreover, this model

requires adding a soil water function. Contrasting results have been found between the Jarvis or BWB models (Hoshika et al., 2017a). If these models are properly parameterised, they can both accurately predict g_s in complex ecosystems (Fares et al., 2013). This will depend on available data for model parameterisation and calibration (Fares et al., 2013), but could be crucial for studying O_3-sensitive tree species. Moreover, our results highlight that it is important to take genotype-specific responses into account, *e.g.* Carpaccio stomata closed faster than Robusta stomata under VPD, and opened faster under irradiance. Nonetheless, these observations at the leaf scale might not be easily extrapolated at the tree scale because of multiple obstacles: i) scaling up from leaf to canopy is tricky (Ollinger et al., 1997; Zhou et al., 2017), ii) the impact of phenology, aging, and enhanced leaf senescence should be taken into account (Anav et al., 2018), iii) leaf stomatal density is modified in newly formed leaves (Durand et al., 2019a; Pääkkönen et al., 1997), iv) detoxification and repair processes occur (Tuzet et al., 2011), and v) conditioning (Agathokleous et al., 2019) or cross-tolerance processes (Tausz et al., 2007) occur too. All these points could challenge the implementation of larger models and the scaling up to whole tree or forest ecosystems.

V.2.5. Conclusion

Despite constitutive differences between genotypes, O_3 and water deficit induced stomatal closure and closing sluggishness. 80 ppb O_3 exposure followed by water deficit modified stomatal closure differently between the two genotypes: Carpaccio stomata closed more than under water deficit alone, whereas Robusta stomata closed less than under water deficit alone. These modifications could have a non-negligible effect on O_3 uptake, carbon storage, and water use efficiency. The underlying mechanisms still need investigations into the active or passive physiological regulation induced by environmental fluctuations (irradiance, VPD, O_3, soil water deficit, etc.). Previous studies suggested different stomatal closure/opening mechanisms, *i.e.*, i) passive hydric regulation of guard cells (Buckley & Mott, 2002), ii) mediation by a cross-talk between phytohormones (Daszkowska-Golec & Szarejko, 2013), with a particular implication of abscisic acid or ethylene (McAdam & Brodribb, 2015; Wilkinson & Davies, 2010, 2009), iii) O_3 and/or drought-induced stomatal closure through modification of guard cell homoeostasis *via* direct modulation of K^+ channels (Geiger et al., 2009; Vahisalu et al., 2010), alteration of Ca^{2+}/H^+ vacuolar antiporters (Dumont et al., 2014a), and production of reactive oxygen species such as H_2O_2 (Damour et al., 2010). Understanding these mechanisms and the cross-talks between the O_3 and water deficit responses (as successive stresses or in

combination) could allow for a better prediction of g_s in response to various environmental modifications.

V.2.6. Supplementary data

Methods 1

The method of Bansal et al., (2013) was used to determine if O_3 and water deficit had an additive, synergistic or antagonistic impact. On one hand, the expected additive effects were determined by adding the independent effects of O_3 alone (*i.e.*, the observed differences between WW:O_3 and WW:AF) and of water deficit alone (*i.e.*, the observed differences between D:AF and WW:AF) minus their product to avoid over-inflated response estimates (Darling et al., 2010). On the other hand, the observed effect for the D:O_3 treatment was determined from the differences between D:O_3 and WW:AF. The expected additive effects following separate treatments were compared to the observed combined effects. When the difference between the two effects was positive and the lower 95% confidence interval boundary was greater than zero, the impact of the combined stressors was classified as synergistic. Similarly, when the difference was negative and the upper 95% confidence interval boundary was below zero, the combined impact was classified as antagonistic.

Table S1: Impact of O_3 or/and water deficit on growth parameters of the Carpaccio and Robusta genotypes. Means ± se, n= 16. The significance of the factors or their interaction was tested by ANOVA. p-values *(P* ≤ 0.05) are in bold. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone.

Genotype	Water treatment	Ozone treatment	Height growth (cm)		Radial growth (cm)		Number of leaves	Total surface (m^2)	Leaves (g DW)	Stem (g DW)	Root (g DW)	Total biomass (g DW)	Root/shoot
			0d	21d	0d	21d							
Carpaccio	WW	FA	51.0 ± 1.3	121.8 ± 1.8	5.3 ± 0.2	9.2 ± 0.3	42 ± 1	0.51 ± 0.01	23.6 ± 0.6	15.8 ± 0.6	7.9 ± 0.4	47.2 ± 1.6	0.20 ± 0.01
		O_3	51.7 ± 0.8	118.6 ± 2.7	5.3 ± 0.1	8.8 ± 0.2	42 ± 1	0.51 ± 0.01	23.5 ± 0.6	14.3 ± 0.4	6.9 ± 0.4	44.7 ± 1.2	0.18 ± 0.01
	D	FA	48.6 ± 1.0	117.7 ± 1.5	5.0 ± 0.1	8.8 ± 0.2	41 ± 1	0.46 ± 0.01	22.8 ± 0.6	14.8 ± 0.6	6.6 ± 0.4	44.2 ± 1.3	0.18 ± 0.01
		O_3	52.2 ± 1.1	115.8 ± 1.9	5.3 ± 0.1	8.0 ± 0.2	38 ± 1	0.44 ± 0.01	21.2 ± 0.6	13.0 ± 0.4	6.0 ± 0.2	40.2 ± 1.6	0.18 ± 0.01
Robusta	WW	FA	55.1 ± 2.2	82.1 ± 0.7	6.7 ± 0.1	8.8 ± 0.1	40 ± 0	0.38 ± 0.01	24.1± 0.5	14.5 ± 0.4	18.9 ± 0.9	57.5 ± 1.1	0.50 ± 0.03
		O_3	53.3 ± 1.3	80.0 ± 1.0	6.6 ± 0.2	8.6 ± 0.1	36 ± 1	0.39 ± 0.01	23.1 ± 0.5	13.6 ± 0.4	18.2 ± 1.3	55.0 ± 1.9	0.49 ± 0.03
	D	FA	53.2 ± 0.8	82.1 ± 1.7	6.9 ± 0.1	9.3 ± 0.1	36 ± 1	0.38 ± 0.01	24.6 ± 0.5	15.3 ± 0.4	16.3 ± 1.1	56.2 ± 1.2	0.41 ± 0.03
		O_3	52.5 ± 1.2	81.8 ± 0.4	6.4 ± 0.2	8.5 ± 0.0	33 ± 1	0.37 ± 0.00	22.3 ± 0.5	13.6 ± 0.4	13.6 ± 0.8	49.5 ± 1.1	0.38 ± 0.03
Genotype			0.037	**<.001**	**<.001**	0.144	**0.007**	**<.001**	0.550	**0.049**	**<.001**	**<.001**	**<.001**
O_3			0.663	0.180	0.980	0.122	0.543	0.966	0.911	**0.028**	0.448	0.202	0.619
WD			0.180	0.086	0.266	0.125	0.299	**<.001**	0.306	0.129	0.576	0.123	0.481
Genotype x O_3			0.361	0.736	0.873	0.619	**0.034**	0.895	0.468	0.482	0.403	0.990	0.760
Genotype x WD			0.855	0.234	0.217	**0.033**	0.100	0.025	0.256	**0.048**	0.406	0.527	0.151
O_3 x WD			0.248	0.705	0.466	0.344	0.096	0.221	0.202	0.751	0.86	0.611	0.750
Genotype x O_3 x WD			0.606	0.897	0.254	0.757	0.240	0.882	0.922	0.652	0.582	0.485	0.509

Table S2: Stomatal closure/opening in response to irradiance. Gas exchanges steady state measurements for irradiance sigmoidal model response for the two Carpaccio and Robusta poplar genotypes submitted to 80 ppb of O_3 for 13 days and/or moderate water deficit for 7 days. g_0, steady state before stomatal closure (PAR: 800 µmol.m^{-2}.s^{-1}); g_1, steady state after closure before opening; g_2, steady state after opening (mmol.m^{-2}.s^{-1}); A_0 assimilation (µmol.m^{-2}.s^{-1}) before closing (PAR: 800 µmol.m^{-2}.s^{-1}), A_1 assimilation after closing and before opening in the dark (PAR: 0 µmol.m^{-2}.s^{-1}), A_2 assimilation after opening (PAR: 800 µmol.m^{-2}.s^{-1}). Water use efficiency (A/g) was calculated for open stomata. Means ± se, n ≥ 4. The significance of the factors or their interaction was tested by ANOVA. p-values *(P* ≤ 0.05) are in bold. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone.

Days	Genotype	Water treatment	Ozone treatment	g_0	g_1	g_2	A_0	A_1	A_2
13	Carpaccio	WW	FA	469 ± 29	88 ± 10	436 ± 19	19.63 ± 0.23	-1.33 ± 0.03	18.81 ± 0.22
			O_3	245 ± 22	107 ± 11	207 ± 20	7.94 ± 1.13	-1.72 ± 0.09	11.18 ± 0.45
	Robusta	WW	FA	370 ± 33	123 ± 8	375 ± 13	19.29 ± 0.78	-1.60 ± 0.07	19.74 ± 0.48
			O_3	486 ± 25	143 ± 10	326 ± 20	15.05 ± 0.52	-1.88 ± 0.06	13.80 ± 0.33
Genotype				**0.001**	**0.001**	0.131	**0.001**	**0.020**	**0.001**
O_3				0.086	0.068	**<.001**	**<.001**	**<.001**	**<.001**
Genotype x O_3				0.383	0.986	**<.001**	**<.001**	0.540	0.104
21	Carpaccio	WW	FA	458 ± 53	95 ± 38	415 ± 43	14.36 ± 1.78	-1.16 ± 0.06	12.23 ± 2.14
		D	FA	267 ± 43	16 ± 4	223 ± 33	14.15 ± 0.49	-1.23 ± 0.11	13.15 ± 0.63
		WW	O_3	439 ± 35	75 ± 14	339 ± 28	15.34 ± 0.53	-1.14 ± 0.07	12.85 ± 0.83
		D	O_3	181 ± 22	12 ± 4	155 ± 15	11.78 ± 0.47	-1.07 ± 0.06	10.88 ± 0.68
	Robusta	WW	FA	380 ± 28	134 ± 12	337 ± 22	17.78 ± 0.62	-1.42 ± 0.09	17.44 ± 0.67
		D	FA	211 ± 14	71 ± 7	234 ± 26	14.97 ± 0.80	-1.62 ± 0.09	14.35 ± 0.49
		WW	O_3	494 ± 36	161 ± 13	338 ± 12	14.36 ± 0.41	-1.28 ± 0.09	14.92 ± 0.50
		D	O_3	352 ± 49	87 ± 12	290 ± 30	13.68 ± 0.57	-1.65 ± 0.12	12.82 ± 0.55
Genotype				**<.001**	0.399	**<.001**	**<.001**	**0.006**	**<.001**
O_3				0.687	0.275	0.684	0.061	0.091	0.104
WD				**<.001**	**<.001**	**<.001**	0.490	0.123	0.397
Genotype x O_3				0.189	**0.014**	0.174	0.099	0.258	0.098
Genotype x WD				0.940	**0.006**	0.912	0.759	0.508	0.848
O_3 x WD				0.848	0.422	0.925	0.100	0.602	0.928
Genotype x O_3 x WD				0.597	0.542	0.564	**0.035**	0.288	0.363

Table S3: ANOVA P-values of the effects of ozone (O_3), water deficit, and genotype on τ, λ and SL_{max} on d 13 (the end of O_3 fumigation) and d 21 (the end of the experiment). Asterisks indicate the significance of the factors or their interactions tested by a linear mixed-effect model: '***'P ≤ 0.001, '**'P ≤ 0.01, '*'P ≤ 0.05, 'ns' non-significant.

		Closing irradiance			**Opening irradiance**			**Closing VPD**		
Days		τ	λ	SL_{max}	τ	λ	SL_{max}	τ	λ	SL_{max}
13	Genotype	0.009 **	0.955 ns	0.234 ns	0.392 ns	0.319 ns	0.348 ns	0.002 **	<.001 ***	0.051 ns
	O_3	0.204 ns	0.148 ns	0.026 *	0.663 ns	0.69 ns	0.963 ns	0.005 **	0.022 *	0.261 ns
	Genotype x Ozone	0.465 ns	0.046 *	0.811 ns	0.15 ns	0.619 ns	0.038 *	0.613 ns	<.001 ***	0.474 ns
21	Genotype	0.053 ns	0.131 ns	0.343 ns	0.005 **	0.061 ns	<.001 ***	0.009 **	0.105 ns	0.03 *
	O_3	0.002 *	<.001 ***	<.001 ***	0.038 *	0.787 ns	0.959 ns	0.003 **	<.001 ***	0.046 *
	Water deficit	0.025 *	0.023 *	0.025 *	0.258 ns	0.002 **	0.007 **	0.011 *	<.001 ***	0.007 **
	Genotype x O_3	0.633 ns	0.196 ns	0.849 ns	0.025 *	0.255 ns	0.651 ns	0.094 ns	0.424 ns	0.006 **
	Genotype x Water deficit	0.797 ns	0.182 ns	0.151 ns	0.221 ns	0.312 ns	0.074 ns	0.789 ns	0.302 ns	0.253 ns
	O_3x Water deficit	0.037 *	0.028 *	0.014 *	0.277 ns	0.645 ns	0.487 ns	0.248 ns	0.558 ns	0.217 ns
	Genotype x O_3xWater deficit	0.864 ns	0.979 ns	0.447 ns	0.036 *	0.719 ns	0.472 ns	0.64 ns	0.223 ns	0.23 ns

Table S4: Stomatal closure in response to VPD. Gas exchanges steady state measurements of the vapour pressure deficit (VPD) sigmoidal model response for the two Carpaccio and Robusta poplar genotypes submitted to 80 ppb of O_3 for 13 days and/or moderate water deficit for 7 days. g_0, steady state before stomatal closure; g_1, steady state after stomatal closure ($mmol.m^{-2}.s^{-1}$); A_0, assimilation before closure, A_1 assimilation after closure ($\mu mol.m^{-2}.s^{-1}$), A_2, assimilation before opening. Water use efficiency (A/g) was calculated for both steady states. Means ± se, n ≥ 4. The significance of the factors/interaction test by ANOVA *(P* ≤ 0.05) is in bold. FA: filtered air; WW, well-watered; D, water deficit; O_3, ozone.

	Days	Genotype	Water treatment	Ozone treatment	g_0	g_1	A_0	A_1
	13	Carpaccio	WW	FA	448 ± 27	182 ± 20	18.52 ± 0.49	15.51 ± 0.53
				O_3	262 ± 22	104 ± 17	12.15 ± 0.23	8.04 ± 1.00
		Robusta	WW	FA	406 ± 33	138 ± 12	20.26 ± 0.60	14.54 ± 0.81
				O_3	537 ± 25	160 ± 15	13.85 ± 0.84	11.10 ± 0.43
Genotype					**<.001**	0.716	**0.004**	0.167
O_3					0.393	0.11	**<.001**	**<.001**
Genotype x O_3					**<.001**	**0.005**	0.977	0.01
	21	Carpaccio	WW	FA	546 ± 64	122 ± 17	21,48 ± 3.46	12,66 ± 0.85
			D	FA	407 ± 53	123 ± 15	12,73 ± 0.36	9,50 ± 0.86
			WW	O_3	182 ± 34	79 ± 12	16,42 ± 1.31	10,05 ± 1.37
			D	O_3	207 ± 35	68 ± 6	10,04 ± 1.99	7,71 ± 0.38
		Robusta	WW	FA	323 ± 26	163 ± 8	17,66 ± 0.59	14,81 ± 0.34
			D	FA	487 ± 36	114 ± 25	12,45 ± 1.62	9,68 ± 1.07
			WW	O_3	167 ± 10	102 ± 4	17,38 ± 4.11	11,37 ± 0.95
			D	O_3	319 ± 24	124 ± 6	17,90 ± 6.49	10,82 ± 0.42
Genotype					0.684	**0.011**	0.342	**0.016**
O_3					0.090	0.383	0.086	**<.001**
WD					**<.001**	**0.001**	0.880	**0.017**
Genotype x O_3					**<.001**	0.658	0.119	0.943
Genotype x WD					**0.045**	0.266	0.069	0.436
O_3 x WD					0.201	0.160	0.189	**0.050**
Genotype x O_3 x WD					0.139	**0.050**	0.422	0.166

V.3. Implication de l'ascorbate et du glutathion

Lors de l'expérimentation exposant les plants à l'O_3 puis à une sécheresse modérée, un dosage de l'ascorbate et du glutathion par méthode HPLC a été réalisé à deux dates :

- À la fin du traitement O_3 : 13^e jour
- À la fin d'une période de déficit hydrique de 7 jours : 21^e jour

V.3.1. Résultats

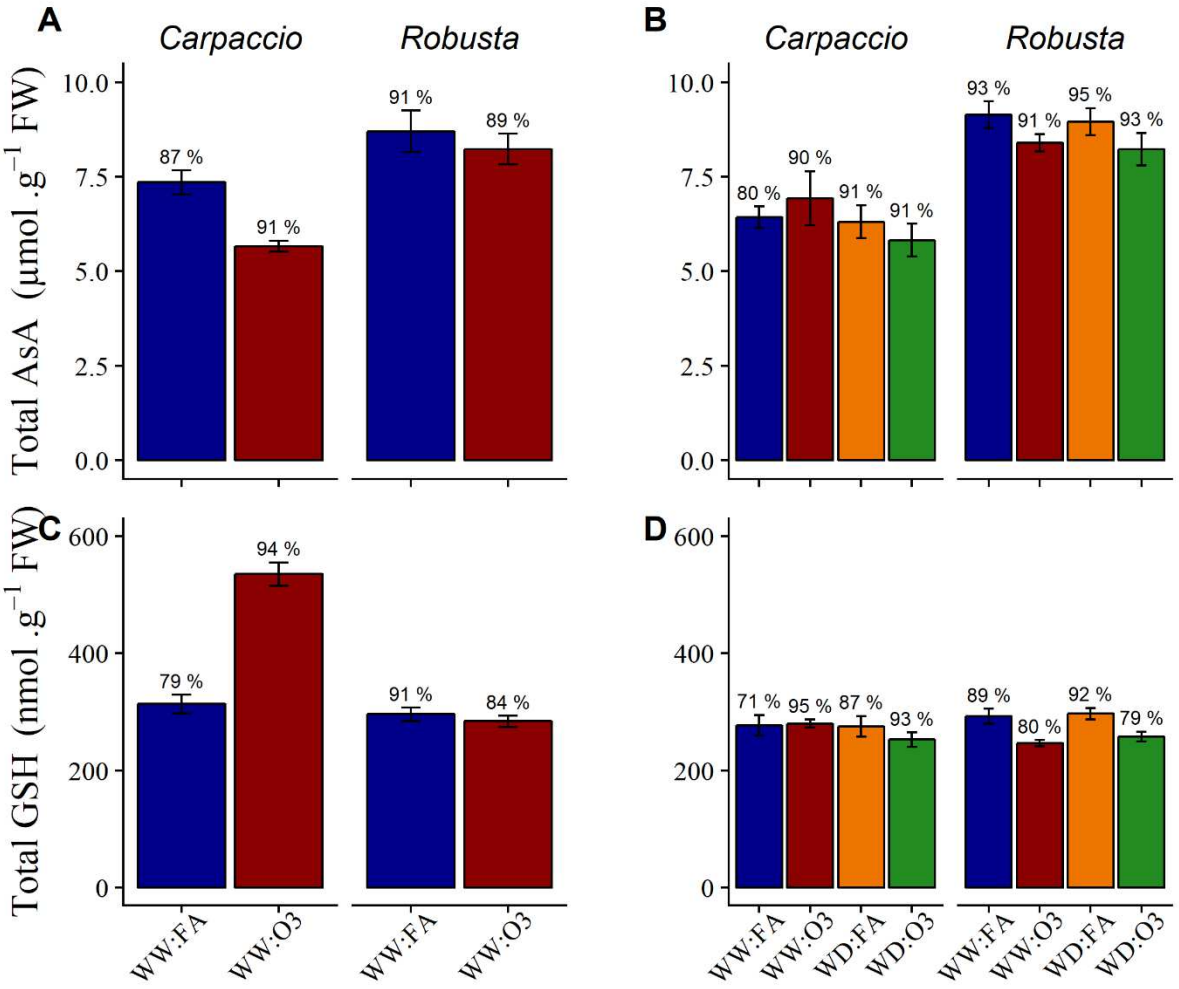

Figure 59 : Quantification du contenu total en ascorbate (A et B) en µmol.g^{-1} de matière fraîche (FW), et du contenu total en glutathion (GSH) (C et D), exprimé en µmol.g^{-1} de matière fraîche (FW), dans les feuilles de Carpaccio et Robusta exposées à l'O_3 pendant 13 jours, puis à un déficit hydrique avec un retour en condition air filtré. WW:FA (contrôle) : bleu, D:FA (déficit hydrique et air filtré) : orange; WW:O_3 (bien hydraté et ozone) : rouge; D:O_3 (déficit hydrique et ozone) : vert. Moyenne (n = 8) ± erreur standard. Les pourcentages correspondent au niveau de réduction.

Tableau 14 : Effet du génotype, du traitement O_3 , du déficit hydrique (WD) et de leur interaction à 14 et 21 jours sur le contenu en ascorbate total (Total AsA), en glutathion total (Total GSH) et sur le pourcentage de réduction des deux métabolites. La significativité de l'ANOVA de type II à 2 ou 3 facteurs est représentée en gras (p>0.05).

Days		Total AsA	% AsA reduced	Total GSH	% GSH form
14	Genotype	**<0.001**	0.832	**<0.001**	0.873
	O_3	**0.005**	0.680	**<0.001**	0.368
	Genotype x O_3	0.110	0.428	**<0.001**	0.064
21	Genotype	**<0.001**	0.161	0.747	0.637
	O_3	0.184	0.717	**0.002**	0.797
	WD	0.192	0.287	0.720	0.278
	Genotype x O_3	0.229	0.337	0.064	**0.001**
	Genotype x WD	0.467	0.580	0.195	0.418
	O_3 x WD	0.431	0.497	0.625	0.192
	Genotype x O_3 x WD	0.402	0.507	0.344	0.321

En condition contrôle, les deux génotypes montrent des différences de contenu en AsA. Robusta présente les niveaux les plus élevés, à 9 µmol.g^{-1} DW contre 7 µmol.g^{-1} DW pour Carpaccio. Les teneurs sont relativement stables en condition contrôle entre les deux temps de mesure (Figure 59, A et B). Quant au glutathion, les teneurs sont équivalentes entre les deux génotypes et stables entre les deux temps de mesure (Figure 59, C et D).

Après 14 jours de fumigation à l'O_3, l'ANOVA révèle un effet significatif de l'O_3 sur les teneurs en AsA total, mais pas d'effet sur le pourcentage de réduction (Tableau 14). La tendance est à la diminution pour les deux génotypes. Le plus marqué est Carpaccio, à 5,6 µmol.g^{-1} DW contre 7 µmol.g^{-1} FW en condition contrôle (Figure 59, A). Par ailleurs, le contenu en glutathion est impacté par l'O_3 seulement pour Carpaccio. En effet, le contenu en GSH total double sous O_3 pour ce même génotype (Figure 59, C).

Après 21 jours de traitement, le retour en condition contrôle ou la sécheresse ne modifie pas les teneurs en AsA (Figure 59, B et Tableau 14). Pour Carpaccio, le niveau de glutathion des arbres soumis à l'O_3 retourne au même niveau que le contrôle. Néanmoins, le pourcentage de réduction du glutathion est plus élevé comparé à celui du contrôle (+ 24 %) (Figure 59, D). Pour Robusta, les teneurs totales en AsA et GSH tendent à être plus faibles pour les arbres soumis à l'O_3 (non significatif) (Figure 59, B et D). De plus, le pourcentage de réduction du glutathion est plus faible de 10 % dans les traitements ayant reçu de l'O_3 (Figure 59, D).

V.3.2. Discussion

Cette expérimentation a permis de vérifier l'utilisation de l'HPLC pour la détermination des contenus en AsA et GSH chez le peuplier. En effet, les valeurs obtenues sont du même ordre de grandeur que les résultats précédemment présentés lors de la première expérimentation à 120 ppb (Dusart et al., 2019b). Cependant, la méthode par HPLC semble plus précise (réduction de la variance) et plus facilement reproductible. La fumigation d'O_3 à 80 ppb pendant 13 jours induit un doublement du contenu en GSH pour le génotype Carpaccio. Cette augmentation ne semble pas liée à un flux entrant équivalent entre 11 jours à 120 ppb et 13 jours à 80 ppb. En effet, lors de l'expérimentation à 120 ppb présentée dans le chapitre 4, Carpaccio révèle un doublement du contenu en GSH pour un POD_0 de15 $mmol.m^{-2}$ après 11 jour d'O_3. Lors de cette expérimentation à 80 ppb, le POD_0 était de 9.7 $mmol.m^{-2}$ après 13 jour d'O_3. Il est néanmoins délicat de comparer la dose d'O_3 entrante entre les deux expérimentations, notamment à cause de différences d'âge et de sélection de la feuille. Lors de cette expérimentation à 80 ppb pour compenser les différences d'éclairement de la feuille suivie entre les deux génotypes, le génotype Carpaccio a été exposé à l'O_3 après 5 semaines de culture contre 6 semaines pour Robusta. Il en résulte une différence de maturation de la feuille, notamment visible sur les teneurs en anthocyanes (Figure 60). Cet aspect sera discuté plus en détails dans le Chapitre VI :Discussion générale.

Pour Robusta, ni les teneurs en AsA et en GSH ni leur statut redox ne sont impactés par la fumigation à 80 ppb d'O_3. Robusta semble être capable de maintenir la régénération des deux antioxydants via les enzymes du cycle HAF.

À la fin des 21 jours d'expérimentation, le déficit hydrique ne modifie pas les pools d'antioxydants foliaires pour aucun des génotypes. Ce résultat est cohérent avec l'expérimentation publiée dans Dusart et al. (2019b). En effet, si les pools foliaires d'AsA et GSH ne sont pas modifiés par nos conditions expérimentales de déficit hydrique, alors que la littérature rapporte des modifications des augmentations d'AsA et GSH (Laxa et al., 2019; Wujeska et al., 2013), nous l'avions expliqué par des conditions d'éclairement des chambres de cultures insuffisantes pour induire un important stress photo-oxydant associé au déficit hydrique.

Les 7 jours de récupération après l'O_3 entraînent un retour au niveau de base des teneurs des deux métabolites pour les deux génotypes. Néanmoins, pour le génotype Carpaccio, les pourcentages de réduction pour l'AsA et le GSH tendent à être plus élevés. Cette observation pourrait être le résultat d'un conditionnement à l'O_3, qui entraînerait une meilleure réponse dans

le cas d'une nouvelle période d'exposition (Agathokleous et al., 2019). À l'opposé, pour Robusta, le pourcentage de réduction des deux métabolites tend à diminuer. Ces modifications de l'équilibre redox pourraient être responsables de modifications de la physiologie de la feuille à plus long terme.

L'exposition successive à l'O_3 et à un déficit hydrique ne semble pas affecter différemment les pools d'AsA et GSH. En l'absence d'informations plus complètes concernant le cycle HAF, notamment concernant les activités enzymatiques, il faut rester prudent quant à un possible effet combiné. Dans nos conditions expérimentales, l'arrêt de l'O_3 semble permettre une récupération des deux peupliers, qu'ils soient ensuite soumis à la sécheresse ou non. Dans des conditions naturelles, où l'O_3 et le déficit hydrique pourraient être associés à un stress photo-oxydant fort, l'impact pourrait être délétère.

De plus amples recherches sont donc nécessaires pour mieux appréhender l'impact que peut avoir la succession de contraintes sur la plante et la résilience de celle-ci face aux stress, notamment sur les modifications de l'équilibre redox, l'impact sur la signalisation phytohormonale et, par conséquent, sur le « cross-talk » responsable des mécanismes de régulation du fonctionnement des stomates.

V.3.3. Figures suplémentaires

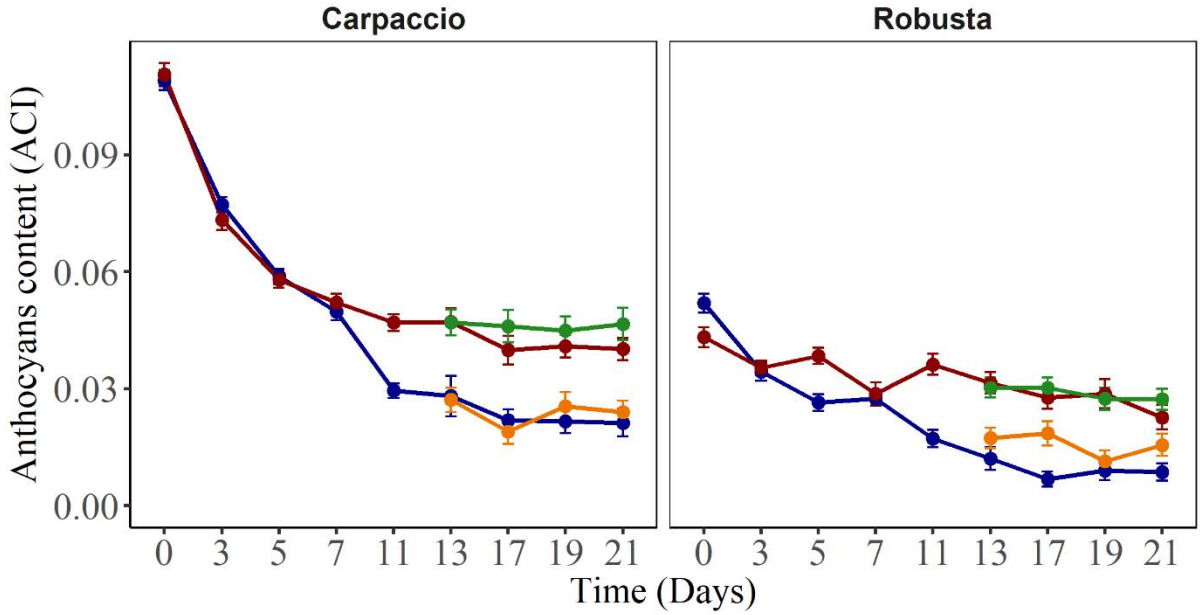

Figure 60 : Cinétique de réponse de des teneurs en anthocyane chez Carpaccio et Robusta soumis à l'O_3 puis au déficit hydrique. Moyenne ± se, n= 4. WW:FA (contrôle) : bleu, D:FA (déficit hydrique et air filtré) : orange; WW:O_3 (bien hydraté et ozone) : rouge; D:O_3 (ozone puis déficit hydrique) : vert. L'unité est celle du dualex (Anthocyans content index, ACI).

Chapitre VI. Discussion générale