**Additive effects of high growth rate and low transpiration rate drive differences in whole plant transpiration efficiency among black poplar genotypes**

Bogeat-Triboulot MB1, Buré C1, Gerardin T1, Chuste PA1, Le Thiec D1, Hummel I1, Durand M1, Wildhagen H2 \*, Smith HK3, Valdes-Fragoso PM3, Douthe C4, Molins A5, Galmès F4, Flexas J4, Polle A2, Taylor G2,6 and Brendel O1.

1 Université de Lorraine, INRA, AgroParisTech, UMR Silva, 54000 Nancy, France

2 Forest Botany and Tree Physiology, University of Goettinen, Büsgenweg 2, 37077 Göttingen, Germany

3 Biological Sciences, University of Southampton, Southampton, Hampshire, SO17 1BJ, UK.

4 Research group on plants biology under Mediterranean conditions – INAGEA. Universitat de les Illes Balears, Palma de Mallorca, 07122, Balearic Islands.

5 Universitat de Valencia, Instituto Cavanilles de Biodiversidad y Biología Evolutiva, Facultat CC de Biologia, 46100 Burjassot, Valencia.

6 Department of Plant Sciences, University of California, Davis, One Shields Avenue, Davis, CA. 95616, USA

\* Present address: HAWK University of Applied Sciences and Arts, Faculty of Resource Management, Büsgenweg 1A, 37077 Göttingen, Germany

**Corresponding author :**

Oliver Brendel

Centre INRA Grand Est-Nancy

UMR Silva

54280 Champenoux, France

Tel +33 (0)3.83.41.00

oliver.brendel@inra.fr

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**Abbreviations** :

A: net CO2 assimilation rate,

Amean: mean of net CO2 assimilation rate measured *in situ*,

Asat: net CO2 assimilation rate measured under light-saturated conditions,

Ci: CO2 internal concentration,

CumulT: cumulated water loss,

DMincr: total dry mass increment,

DTR: diurnal transpiration rate,

δ13C: carbon isotope composition,

FinalH: final stem height,

FinalD: final stem diameter,

g: stomatal conductance to water vapour,

gm: mesophyll conductance for CO2,

gmean: stomatal conductance to water vapour measured *in situ*,

gsat: stomatal conductance to water vapour measured under light-saturated conditions,

Jmax: maximum photosynthetic electron flux,

LA: total leaf area,

LeafDM: leaf dry mass,

LeafF: leaf fraction,

w: proportion of unproductive water loss to productive water loss,

NTR: nocturnal transpiration rate,

RootF: root fraction,

StemF: stem fraction,

TE: whole plant transpiration efficiency,

TotalDM: total dry mass,

TR: daily transpiration rate,

Vcmax: maximum CO2 carboxylation rate,

WUE: water use efficiency,

Wi: leaf intrinsic water use efficiency

Wisat: leaf intrinsic water use efficiency measured under light-saturated conditions,

Wimean: mean leaf intrinsic water use efficiency measured *in situ*.

**Abstract + graphical abstract**

Poplar plantations, widely used for the production of woody biomass, might be at high risk from the climate change-induced increase in the frequency of droughts. Therefore, selecting improved genotypes, which are highly productive but with a high water use efficiency (WUE), is becoming a major target. The use of automated weighing systems in controlled environments facilitates the estimation of cumulated water loss and whole plant transpiration efficiency (TE). Differences in TE and leaf level intrinsic WUE as well as the contribution of underlying ecophysiological traits were estimated in three contrasting *P. nigra* genotypes. Strong differences in TE among the selected genotypes were congruent with differences in leaf level intrinsic WUE. Our data showed that a high total leaf area was offset by a low per leaf area transpiration rate, leading to higher TE in highly productive genotypes originating from cool locations. Nocturnal water loss was relatively low but contributed to variations in TE among genotypes. In response to drought, leaf level WUE increased but not TE, suggesting that carbon losses due to whole plant respiration could offset the drought-induced increase in intrinsic WUE.

**Highlights (3 à 5, de 85 characters including space max)**

* The Mediterranean genotype was less water use efficient than northern genotypes
* Low WUE was linked to a high transpiration rate and a large root system
* A trade-off appeared between total leaf surface and per surface transpiration rate
* Intrinsic WUE was increased by drought but not transpiration efficiency

**1. Introduction**

To limit the increasing global temperature, there is an urgent need to reduce greenhouse gas emissions coming from fossil fuels. Biofuels which come from dedicated crops and tree plantations can contribute to meet this target (Sannigrahi et al., 2010) and poplar plantations are widely used for the production of woody biomass (Navarro et al., 2018). On the other hand, future climate change is projected to reduce the productivity of plantation forestry in the coming decades through changes in net primary production (Hanson and Weltzin, 2000). Moreover Domec et al. (2015) suggested that intensively managed plantations are more drought-sensitive than natural forests. Considering this, as well as the high vulnerability of poplars to drought-induced cavitation (Fichot et al., 2015), poplar plantations might be at high risk from the climate change-induced increase in the frequency of droughts. To meet the worldwide increasing demand of wood biomass in the context of climate change, selecting improved tree genotypes, which are highly productive but with a high water use efficiency (WUE), is becoming a major target.

At the whole plant level, WUE is called transpiration efficiency (TE) and is defined as the ratio between the biomass accumulated and the water transpired over a defined period of time. At the leaf level, WUE is reflected by intrinsic WUE (Wi), the ratio between net CO2 assimilation rate (A) and stomatal conductance of water vapour (g). For any one plant, the relationship between A and g is curvilinear, approaching asymptotically a maximum A when stomata are fully open. Under optimal watering conditions, stomata are often more open than required to achieve a maximum A under the given atmospheric conditions resulting in a “luxurious” water consumption. During an increasing soil water deficit, this results in stomatal closure affecting A less than proportionally, thereby increasing Wi (see for example Suppl Fig 2 of Marguerit et al., 2014). In the case of large screenings for WUE (Kruse et al., 2012; Viger et al., 2013), an indirect estimation of Wi is often used by measuring the carbon stable isotope composition (δ13C) of organic material such as leaf, wood or extracted cellulose (Bussotti et al., 2015; Farquhar et al., 1982). However, even if δ13C is measured on wood or extracted cellulose, it still represents a spatio-temporal assimilation-weighted integration of leaf level processes during daytime (A and g). This trait does not include processes in other plant parts and those occurring during the night, which can contribute to variations in biomass accumulation and water loss, and thus TE. These processes relate to respiration of the whole plant during day and night (except leaves during the daytime as this is included in net CO2 assimilation), water losses from plant organs other than leaves and also water losses from leaves during the night (Cernusak et al., 2007). Thus choosing water efficient genotypes for tree plantations on the base of the whole plant transpiration efficiency could be more judicious than on the more widely used leaf level estimates (δ13C, Wi). However, the estimation of TE in adult trees in the field is challenging, because of the difficulties of estimating both the biomass increase, especially that of the root system, and the water use of a whole tree over long time periods. TE of a single tree can be estimated by an allometric estimation of aboveground biomass increase and direct sap flow measurements (Navarro et al., 2018). However, the root biomass increase is ignored and such measurements are not feasible on a large number of individuals. Biomass increments are more easily estimated in potted plants and the use of automated weighing systems facilitates the estimation of cumulated water use in controlled environments. Such systems are either based on multiple balances (Cirelli et al., 2012) or robotic systems (Buré et al., 2016; Granier et al., 2006) and allow many plants to be weighed at a high frequency, thus both controlling soil humidity and quantifying water loss. This in turn allows an accurate estimation of TE and underlying traits as well as comparisons with δ13C or Wi.

Commercial poplar genotypes have been selected primarily for high productivity or resistance to foliar rust but not for high WUE (Monclus et al., 2006). The lack of correlation between productivity and Wi across 29 *Populus* *× canadensis* hybrids suggested that it would be possible to select genotypes which combine high productivity and high WUE (Monclus et al., 2005). Conversely, a negative relationship between TE and productivity was found in the Asian species *P. davidiana* (Zhang et al., 2004), questioning the independence between productivity and WUE. European black poplar (*P. nigra*, L.) is a key pioneer tree species, essential for the dynamics of riparian habitats and for soil stabilisation. Further, it has an economic value as a parent pool for genetic breeding of *P. canadensis* cultivars (Chamaillard et al., 2011; Sow et al., 2018). *P. nigra* has a wide natural distribution with populations growing in different climatic conditions across Europe and showing significant genetic differentiation as well as phenotypic variation in growth rate, plant architecture and leaf size (DeWoody et al., 2015; Viger et al., 2016).

To improve our understanding of the determinants of TE and its response to drought, we determined TE in three contrasting *P. nigra* genotypes, which originate from different regions and which strongly differ in terms of growth and leaf morphology (DeWoody et al., 2015; Wildhagen et al., 2018), and we analysed underlying ecophysiological traits as well as leaf level estimators of WUE. Our first aim was to investigate which traits explained differences in TE among genotypes under optimal watering conditions. In particular, were these differences driven by differences in biomass accumulation rate or in transpiration rate? How much variation would be explained by leaf level WUE compared to unproductive water losses? A second aim was to determine if TE would be changed differently among genotypes in response to drought, and which underlying traits would drive this acclimation.

**2. Material and methods**

*2.1. Plant material and growth conditions*

Three genotypes of *Populus nigra* L., originating from individual trees of natural populations in France (Drôme 6; FR-6), Italy (La Zelata; IT1) and Spain (Ebro 2; SP-2) (DeWoody et al., 2015) and showing different leaf morphology were studied in controlled conditions (Fig. 1). Growth, gas exchange and TE were measured on a subset of plants grown as part of the experiment described by Wildhagen et al. (2018), with six replicates per genotype x treatment. Briefly, woody cuttings were obtained from clonal propagation and were planted in 10 l plastic pots filled with a 1:1 (v/v) mixture of peat and sand, amended with a slow release fertiliser (4 g l-1 of Nutricote T100, 13:13:13 NPK and micronutrients; FERTIL S.A.S, Boulogne Billancourt, France) and 1 g l-1 CaMg(CO3)2. Plants were grown in two compartments of a glasshouse located at Champenoux, France (48°45’09.3”N, 6°20’27.6”E), under natural light conditions with daily maxima of irradiance ranging from 150 to 1000 µmol m-2 s-1 photosynthetically active radiation (PAR, Fig. 2). Environmental conditions in the greenhouse were affected by weather conditions, but the temperature was maintained between 15 and 26°C (Fig. 2). After planting, plants were watered 2–4 times a day –according to plant size and weather conditions – to 85% of field capacity with an automated weighing and watering system (Buré et al., 2016). The position of plants in the greenhouse was rotated at each weighing event.

*2.2. Control of water deficit*

After six weeks of growth, plants of each genotype were randomly assigned to either a control or a drought treatment for five weeks (day 0 was 21 May 2013). Control plants were watered to 85% soil relative extractable water content (REWsoil) by the automated system for the whole experiment. REWsoil of control plants oscillated between 65 and 85% (data not shown). For drought-treated plants, REWsoil was progressively decreased to reach 20% in two weeks and then maintained at this target level for the following three weeks (Fig. 2).

The control of the available soil water content (SWC) was based on pot weight, and a calibration between volumetric SWC measured by Time Domain Reflectometry (Trime Pico-32, IMKO) and pot weight. Target weights were defined individually for each pot, and were updated every day during the first two weeks to control the SWC decrease (Fig. 2) and were corrected for plant biomass increment using allometric relationships once a week. Each plant was thus submitted to the same stress level, irrespective of plant size and water consumption. Available water was expressed as soil relative extractable water content (REWsoil), which is defined as:

$$REW\_{soil}=\left(\frac{SWC-SWC\_{wiltingpoint}}{SWC\_{fieldcapacity}-SWC\_{wiltingpoint}}\right)×100\%,$$

with SWC at wilting point = 3%; SWC at field capacity = 32%.

*2.3. Growth, gas exchange, transpiration rate and transpiration efficiency*

*Height and diameter*

Plant height was measured from the soil surface to the shoot apex twice per week. The stem base was photographed with a ruler attached to the stem for scale calibration, twice per week. Stem diameter was measured from picture analysis with ImageJ (Schneider et al, 2012).

*Total leaf area*

For each genotype, a relationship between leaf area and maximal leaf width was built from a sample of approximately 80 leaves taken from the full range of leaf sizes. Regression coefficients were over 0.98 for each of the three genotypes. The width of all leaves of each plant was measured once a week and converted to area using the established relationships. Individual leaf areas were summed to calculate the total leaf area (LA) of each plant. Spline adjustment (interspline function, R) was used to estimate LA for dates in between days of measurement.

*Dry biomass*

At the end of the experiment, all plants were harvested. For each plant, the cutting, stem, roots and leaves were separated, dried at 70 °C for 48 h and weighed. Growth allocation was estimated through the calculation of root, leaf and stem biomass fractions (root biomass, leaf biomass and stem biomass over total biomass, respectively).

*Gas exchange and intrinsic water use efficiency*

Gas exchange was measured *in situ* in the greenhouse. Net CO2 assimilation (A) and stomatal conductance to water vapour (g) were measured using two inter-calibrated portable photosynthesis systems LI-COR 6200 (LI-COR® Inc, Lincoln, NE, USA). Measurements were performed on the youngest fully expanded mature leaves at the beginning of the experiment, corresponding to the 8th–10th leaf down from the first apical leaf, between 11:00–12:00 twice a week over the five week-experiment. Intrinsic water use efficiency at the leaf level (Wi) was calculated as the ratio of A/g. A, g and Wi were averaged over the five last measurement days corresponding to the steady drought period during the three last weeks (Amean, gmean and Wimean, respectively).

We also estimated the photosynthetic capacity by measuring gas exchange under light-saturated conditions, with calibrated Li-6400 XT portable gas analyzers (LI-COR® Inc, Lincoln, NE, USA). CO2 concentration was 400 µmol mol-1, light intensity (PAR) was 1500 µmol m-2 s-1 and block temperature was 25 ºC. All measurements were performed in the corridor next to the greenhouse compartments, on the same leaf used for *in situ* gas exchange. For each plant, the same procedure was followed. We waited for stomatal conductance to reach a steady state (typically after 20–30 min), then the A-Ci (Ci: CO2 internal concentration) curve was performed by changing the [CO2] entering the leaf chamber with the following steps: 400, 300, 250, 200, 150, 100, 50, 400, 400, 500, 600, 700, 800, 1000, 1200 and 1500 µmol mol-1, typically with 2–3 min between each step. Maximum carboxylation rate (Vcmax), maximum electron transport rate (Jmax) and mesophyll conductance (gm) were estimated with the method by Ethier and Livingston (2004) that fits A-Ci curves with a non-rectangular hyperbola version of Farquhar’s biochemical model of leaf photosynthesis (Farquhar et al., 1980). This is based on the hypothesis that gm reduces the curvature of the Rubisco-limited portion of an A-Ci response curve. The Rubisco kinetics and specificity were characterized *in vitro* as described previously (Galmes et al., 2014). The parameters values of the Michaelis-Menten constants for CO2 (Kc), and O2 (Ko) and the chloroplastic CO2 photocompensation point (\*) were measured at 15, 25 and 35 °C and adjusted to the measured temperature using the Arrhenius function (details on Rubisco kinetics and specificity in supplementary material and methods).

*Transpiration rates*

Daily transpiration rate (TR) was calculated on a daily basis as the ratio between the water loss over 24 h and LA on that day, and then averaged over the whole experimental period. Days 17, 18, 26, 27, 28, 29 were used to calculate a mean diurnal transpiration rate (DTR) and a mean nocturnal transpiration rate (NTR), using the ratio between water loss during the 05:00–22:00 period and the following 22:00–05:00 period, respectively, and LA. The 22:00–05:00 was chosen as a period of full darkness (astronomic sunset to sunrise). The proportion of unproductive water loss to productive water loss w (Farquhar et al., 1989) was estimated as w =NTR\*9/(DTR\*15) as the unproductive time (civil sunset to sunrise) was approximately 9 h during the experiment.

*Transpiration efficiency*

Transpiration efficiency (TE) was calculated as the ratio between the biomass gain (final total dry biomass – mean initial total dry biomass) and the cumulative water loss over the experiment period. For each genotype, the mean initial total dry biomass was estimated on a separate set of four plants harvested at day 0 (4.2 g, 6.2 g and 4.0 g for the French, Italian and Spanish genotypes, respectively)

*δ13C determination*

The first leaf that had completely developed during the drought stress (mature at the harvest time) was harvested for carbon isotope analysis; dried for 48 h in an oven at 70 °C and ground into a fine powder. Subsamples of 1 mg ± 0.1 mg were weighed into tin capsules. The carbon isotopic composition was measured with a coupled isotope ratio mass spectrometer (Thermo-Finnigan; Delta S, Bremen, Germany). δ13C was calculated according to the international standard (Vienna Pee Dee Belemnite, VPDB) using the following equation: δ13C = (Rs – Rstd)/Rstd x 1000, where Rs and Rstd are the isotopic ratios 13C/12C of the sample and the standard, respectively. The precision of spectrometric analysis (standard deviation of δ13C) was assessed with a calibrated, internal laboratory reference material with a matrix close to the measured samples (oak leaves, n = 16, SD = 0.05 ‰).

*2.4. Statistical analyses*

All statistical analyses were performed with R (R Core Team, 2018). All data-sets were tested for outliers using the generalized ESD test (Extreme Studentized Deviate, Rosner and Bernard, 1983). Only outliers for which evidence for analytical errors were found were actually removed from the analyses.

A two-way ANOVA model with interaction was run for traits in Tables 1 and 2, using genotype and treatment as factors and type III sum of squares (Anova function of the car library). As a large number of variables were tested, the model significance was adjusted using False Discovery Rate (p-adjust function with the "fdr" option"). Significant differences among factor levels were computed using Tukey’s Highest Significant Difference test (HSD.test function of the agricolae package).

Normality of the residuals was tested using Shapiro-Wilk test (shapiro.test function). Variables that showed a Shapiro-Wilk test with p<0.05 were transformed using the boxCox function (car package). Then the above described ANOVA was run again for all transformed variables and the significance levels were compared with those of untransformed variables. Only one result changed, the interaction for Wimean became significant (0.036 for transformed versus 0.060 for untransformed), therefore we presented the results of untransformed variables.

**3. Results**

*3.1. Genotype differences*

We tested the influence of genotype and drought on poplar traits by two-way ANOVA. We did not find significant genotype x drought interactions for 26 out of 28 variables tested. Therefore, differences between genotypes are presented based on overall means. After 11 weeks of growth, the development of the three genotypes differed significantly. The Spanish genotype was much smaller in height, stem diameter and biomass than the other two genotypes (Table 1). This difference in height was the result of a smaller growth rate of the Spanish genotype (2.5 cm day-1) compared to that of the French and Italian genotypes (3–3.5 cm day-1) (Fig supp 1). The differences in stem diameter growth rates between genotypes were smaller than those of height growth rates (Fig supp 1). In addition, the Spanish genotype had many branches (more than the French whereas the Italian had none, data not shown) and many leaves, but it showed the smallest total leaf area (LA) due to much smaller leaves (Table 1, Fig 1, Fig sup 2). The relative allocation of growth to the roots was another important difference between genotypes: the root fraction (RootF) of the Spanish genotype was higher than that of the French genotype, which was higher than that of the Italian genotype (Table 1).

These differences in development were accompanied by differences in ecophysiological traits. The Italian genotype had the lowest daily transpiration rate (TR, 1.70 kg m-2 day-1), diurnal transpiration rate (DTR, 126 g m-2 h-1) and nocturnal transpiration rate (NTR, 3.9 g m-2 h-1) and also the lowest proportion of unproductive water loss to productive water loss (w, 1.9 %) (Fig. 3, Table 1). The Spanish genotype showed a very high TR (2.66 kg m-2 day-1) and DTR (193 g m-2 h-1), in accordance with a significantly higher stomatal conductance (gmean, 0.88 mol m-2 s-1), and a very high NTR (13.5 g m-2 h-1) and w (4.2 %) (Fig. 2, Table 1). However, the Spanish genotype had a very small LA, resulting in a significantly lower cumulative water loss over the experiment (CumulT) than that of the two other genotypes (Table 1).

Traits related to gas exchange measured in optimal conditions (Vcmax, Jmax, Asat, gsat, gm, Ci, Wisat) or *in situ* (Amean, gmean, Wimean) were similar in the French and the Italian genotypes (Table 1). The Spanish genotype showed higher gm, gsat, gmean and Amean (and a tendency for higher Asat) compared to the two other genotypes (Table 1).

The three genotypes differed significantly in whole plant transpiration efficiency (TE), which was corroborated by the integrated leaf level intrinsic WUE as estimated by δ13C (Table 1). The Italian genotype had a higher TE and δ13C than the French, which had a much higher TE and δ13C than the Spanish. There was no significant difference among genotypes in instantaneous WUE (Wisat and Wimean), but the trait values showed a similar gradient across genotypes, confirming that the Spanish genotype had the lowest WUE.

*3.2 Drought effect*

The drought stress was applied for five weeks by reducing soil REW to 20%. Stress level was moderate so that droughted trees still grew but at a reduced rate (Table 1, Table 2, Fig Supp 1). Drought significantly reduced the growth rate in height of the French genotype as early as day 8, while this reduction in growth rate occurred later for the Spanish and the Italian genotypes (at day 11 and 15, respectively; Fig Supp 1). Stem diameter growth was also reduced but it seemed less sensitive than stem height growth in the French and Italian genotypes (-30 % for diameter growth rate versus -40% for height growth rate) and more sensitive for the Spanish genotype (-40% versus -30%) (Fig Supp 1). For all genotypes, the decrease of stem diameter growth became significant from day 15. As previously mentioned, almost no significant genotype x environment interaction effects were detected in the ANOVA. However, post-hoc Tukey’s HSD tests suggested some species specific drought responses. The total dry mass tended to be less reduced under drought in the Italian genotype (-20%), compared to that of the Spanish and French (-34 and -38%) (Table 2). Growth allocation was also differentially affected by drought among genotypes. In particular, the Italian genotype maintained allocation to roots during drought so that its root dry mass was not affected and its RootF increased (Table 2). The leaf fraction of the French genotype was reduced but not its RootF, whereas allocation was not changed in the Spanish genotype (Table 2). Drought reduced LA in all three genotypes, but the effect was most pronounced in the French genotype (Table 1, Fig Supp 2). Drought also reduced the total leaf number of the Spanish and French genotypes (Fig Supp 2). In the Italian genotype, drought reduced LA but not the number of leaves, indicating that leaf growth rate was more sensitive than leaf production rate by the meristem (Figure Supp 2).

The moderate drought level applied here did not significantly affect the following leaf traits: photosynthetic capacity (Vcmax, Jmax), mesophyll conductance to CO2 (gm) and net CO2 assimilation rate (Asat and Amean). By contrast, stomatal conductance decreased under drought as compared to well-watered conditions (gsat and gmean) (Table 1). TR, DTR, NTR and w (*p*=0.054) were also strongly decreased. Consequently, the cumulative water loss (CumulT) was lowered under drought. The estimates of intrinsic water use efficiency (Wisat, Wimean, δ13C) indicate a significant increase of WUE at the leaf level by 35%. By contrast, TE did not respond to drought as the biomass accumulation (DMincr) and CumulT were similarly affected within each genotype (Table 1, Table 2). However, DMincr and CumulT were more reduced by drought in the French and the Spanish genotypes (approximately -40%) compared to the Italian genotype (-22%).

*3.3 Correlations*

A correlation analysis based on individual data highlighted that, in both well-watered and drought conditions, TE strongly correlated with δ13C (R = 0.88 and 0.90 for control and drought, respectively, Fig. 4, Fig. 5A), but the relationship was weaker with Wisat (R = 0.26 and 0.62) and Wimean (R = 0.42 and 0.22) (Fig. 4). TE correlated more strongly with DMincr (R = 0.83 and 0.94) than with CumulT (R = 0.56 and 0.83) (Fig. 4). TE was also positively correlated with dry mass accumulation rate (DMinc/36 days, data not shown) and was related negatively with transpiration rates (daily, diurnal and nocturnal), and with gmean to a lesser extent (Fig 4, 5B and 5C). Traits related to photosynthetic capacity and assimilation rate were weakly related to TE and DMincr under control conditions, but they were slightly negatively related under drought. It is also noticeable that transpiration rates (TR, DTR and NTR) were highly negatively correlated to traits related to LA and overall biomass accumulation (DMincr), and positively correlated to the relative investment in roots (RootF) and to gmean (Fig 4). w and NTR were positively correlated to TR and negatively to TE (Fig 4 and 5D).

**4. Discussion**

***Transpiration efficiency differs strongly among genotypes***

Transpiration efficiency (TE) is a long term whole plant measure of WUE, estimated as the ratio between biomass accumulation and water loss over time. TE variations can originate from different processes, daytime leaf processes such carbon assimilation rate and stomatal conductance but also from unproductive water losses such as nocturnal transpiration, and from carbon losses such as respiration of non-photosynthetic organs. In this study, we measured TE and traits related to TE in three contrasting *P. nigra* genotypes. We found a strong genotype effect for TE, where the Italian genotype showed the highest value (5.2 g kg-1) and the Spanish genotype the lowest value (3.3 t g kg-1). This TE range was similar to that found in other *P. nigra* genotypes grown under high vapour pressure deficit (3.1 to 5.9 g kg-1) (Rasheed et al., 2015) and in the same French and Italian genotypes (4.9 and 5.4 g kg-1, respectively; Durand et al., 2019).

***Differences in transpiration rate and in the proportion of unproductive water loss explain the genotypic differences in TE***

The genotypic differences in TE were corroborated by the integrated measure of leaf level intrinsic WUE (δ13C), and by instantaneous measurements (Wisat, Wimean) although differences were not significant. Guet et al. (2015) tested genotypes from geographically close populations and also found higher WUE (δ13C) for Italian genotypes compared to French genotypes grown in a plantation with fertile soil and wet conditions. By contrast Viger et al. (2016) compared *P nigra* genotypes coming from close French, Italian and Spanish populations in a greenhouse experiment and did not find similar differences of carbon isotope discrimination among these populations.

The strong correlation between TE and δ13C, indicates that a significant part of the differences in TE among plants were driven by leaf level processes. Similarly strong correlations were found for P. *nigra* by Rasheed et al. (2015) and for *P. deltoides x nigra* crosses by Guo et al. (2011) and Rasheed et al. (2013). The weak difference in photosynthetic traits that we observed did not explain the genotypic differences in TE whereas the higher stomatal conductance of the Spanish genotype could clearly explain its low TE. Also all three transpiration rates, TR (day scale), DTR (diurnal) and NTR (nocturnal), correlated strongly and negatively with TE, indicating that the water efficient genotypes were transpiring less per leaf area, during the day as well as during the night. The French and Spanish genotypes with high NTR showed more negative values of predawn leaf water potential under control conditions (Supp Table 1), suggesting that the equilibration of the water potential between plant and soil was less complete for these genotypes than for the Italian one, which could be due to high NTR. The nocturnal transpiration represents an unproductive water loss (Farquhar et al., 1989) and therefore, in theory, impacts TE independently from leaf level WUE (Cernusak et al., 2007). Interestingly, the proportion of unproductive water loss to productive water loss (w) also correlated strongly and negatively with TE. Indeed the Italian genotype transpired proportionally less during the night than the other tested genotypes, and showed the highest TE. In our *P. nigra* experiment, w ranged from 1.9% to 4.2%, which is a similar range to estimations for three tropical tree species (1.2% to 5.2%; Cernusak et al., 2009) or for another plantation tree species such as *Eucalyptus grandis* (5%, Benyon, 1999). Higher w (9 to 30%) were found for other poplar species (Cirelli et al., 2016; Rohula et al., 2014), indicating that nocturnal transpiration was relatively low in *P. nigra*, and that a gain in TE due to reduced w would be small. However, it may be that introgressing *P. nigra* into other *Populus* species could reduce nocturnal water losses and increase TE. Differences in w were more closely related to differences in NTR than in DTR, suggesting that stomatal regulation during the night was partly independent from daytime regulation. Maintaining a significant nocturnal transpiration might enhance nutrient acquisition (Kupper et al., 2012), prevent a build-up of CO2 within the leaves (Marks and Lechowicz, 2007), or facilitate a fast increase of net photosynthesis during early morning (Dawson et al., 2007).

***Genotypic differences in TE are related to origin and biomass allocation***

The three studied genotypes were chosen as representative of contrasting populations in terms of individual leaf size and of location in Europe: the Italian genotype had the largest leaves and the Spanish genotype the smallest leaves and a smaller LA than the two other genotypes. The observed strong correlation between TE and DMincr was mainly due to the smaller plant size and the lower TE of the Spanish genotype. The observed difference in plant size is in accordance with Viger (2011), who showed higher growth and larger leaves for *P. nigra* from central Europe compared to trees from regions with hot and dry Mediterranean summers. Also in hybrid poplars, individual leaf area was a good predictor of growth rate and productivity (Marron et al., 2007). Our data suggest a trade-off where the genotypes with a high growth rate, a high individual leaf area and a high total LA showed a much lower per leaf area transpiration rate, reducing total water loss, and resulting in a higher TE. The variation of TE, TR and growth rate among the genotypes appears coherent with the climatic gradient across their region of provenance. A high transpiration rate is expected to lead to a strong leaf cooling effect, which could be advantageous for plants growing in hot climates and having access to water. The constitutively higher investment into roots as compared to leaves by the Spanish genotype (higher RootF) is in agreement with an increased need in water supply and support this hypothesis. Overall this would result in the observed strong negative correlation between TE and RootF. Similarly it was shown for different provenances of *Castanea sativa,* that ecotypes from regions with low precipitation and higher mean temperature had lower intrinsic WUE (Lauteri et al., 1997) and a deeper rooting pattern (personal comm. M. Lauteri). Other populations from drought prone sites also showed lower intrinsic WUE, and lower growth and total biomass (Lauteri et al., 2004; Pliura and Eriksson, 2002). Also in maritime pine the ecotype originating from a dry and hot location in Morocco (Tamjoute) had a lower growth rate and a lower intrinsic WUE than the ecotypes from wet and cooler locations in France (Landes, Porto-Vecchio) (Guehl et al., 1995). Overall, the leaf cooling effect of transpiration and the high carbon allocation to the root system might be an adaptive strategy resulting in lower TE for ecotypes from hot environments where deep water is available. Using neutral markers and phenotypic measurements, DeWoody et al. (2015) showed that isolation by distance played a major role in the differentiation among the western European *P. nigra* populations. In addition, they showed that adaptive differentiation also occurred for small-leaf populations from the Mediterranean area, supporting the idea that the Spanish specificities could result from local adaptation.

***Drought increased intrinsic WUE but not TE***

Globally, the three genotypes responded similarly to drought and we found only two significant genotype drought interactions in the statistical model (δ13C and NTR). As expected, biomass of all compartments decreased, or tended to decrease, under drought. Growth was clearly more sensitive than assimilation rate and growth limitation was independent from carbon supply, as already found in other poplar species (Bogeat-Triboulot et al., 2007; Cohen et al., 2010). However, growth allocation showed some genotype specific patterns. The French genotype decreased mainly the leaf fraction, as found in a previous study (Durand et al., 2019). The Italian genotype, which showed the least reduction in biomass under drought, decreased mainly the stem fraction. These responses differ from those recorded in the same genotype in previous experiments (Durand et al., 2019; Viger et al., 2016), highlighting that the way the water deficit is applied affects the growth and physiological responses (Puertolas et al., 2017).

The drought-induced increase in leaf level WUE (Wimean, δ13C) was most likely due to a decrease in stomatal conductance, mainly observable in the *in situ* measurements, whereas neither photosynthetic capacity nor assimilation rates were significantly changed. Increased δ13C and thus intrinsic WUE under drought due to stomatal closure is a classical response in plants showing luxurious water consumption in well watered conditions, as shown for different poplar species (Monclus et al., 2006; Viger et al., 2016). In addition, changes in intrinsic WUE seemed genotype dependent: genotype x drought interaction was significant for δ13C and almost significant for Wimean (pint = 0.060). The French genotype showed a significant and greater increase in leaf level WUE, linked to a relatively stronger reduction in gmean than in the two other genotypes. These results suggest differences in stomatal regulation among these genotypes, as already found in a shorter drought experiment (Durand et al., 2019).

Surprisingly TE was not increased under drought in any genotype, indicating that there were other factors apart from leaf level processes, which negated the effects of improved intrinsic WUE on TE. One such process could be the nocturnal water loss, which decouples leaf level from whole plant water use efficiency (Cernusak et al., 2007). However the observed reduction in NTR and w under drought should have had a positive effect on TE and did therefore not offset the increased leaf intrinsic WUE. Another factor decoupling whole plant from leaf level WUE could be carbon losses other than day respiration by leaves (Cernusak et al., 2007). An increased whole plant respiration under drought would decrease TE and therefore would offset the increase of leaf intrinsic WUE. The observed decrease in leaf fraction under drought, which was stronger in the French genotype, implies an increase in the fraction of only respiring organs, which should in turn increase the whole plant respiration and contribute to offset the drought-induced increase of intrinsic WUE. This hypothesis is also congruent with the highest TE of the Italian genotype, which had the highest LA and should therefore have lower carbon losses by whole plant respiration relative to its size.

**Conclusions**

Strong differences in TE among the selected genotypes were congruent with differences in WUE at the leaf level. Our data suggest that a high total leaf area is offset by a low per leaf area transpiration rate, leading to higher TE in highly productive genotypes from cool locations. Nocturnal water loss contribute to variations in TE but are relatively low in *P. nigra*, reducing the possibility to improve TE in this species by selecting genotypes with low w. However, w has been shown to be much higher for other poplar species, and introgression of black poplar might provide a gain in nocturnal water losses. Our data also suggest that carbon losses due to whole plant respiration might contribute to the TE differences among genotypes and could offset the drought-induced increase in intrinsic WUE. Future studies should include measurements of respiratory carbon losses of different plant organs.

**Conflict of interest statement**

The authors have no conflicts of interest to declare.

**Author contributions**

MBBT, HW, CD, DLT, HKS, IH, JF, AP, GT, and OB conceived the original screening and research plans. MBBT, CB, CD, JF, HW, PAC, TG, DLT, OB, HKS, PMVF, AM and FG performed the greenhouse experiment and the analytical measurements. MBBT, HW, HKS, CD, IH, DLT, MD, AM, FG and OB analysed the data; MBBT and OB wrote the article with contributions of all authors. All authors approved the final version of the manuscript.

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**Figure legends:**

**Figure 1**: A) general view of the plants in the greenhouse on day seven. B, C and D) Pictures of typical plants of the Italian, French and Spanish genotypes, respectively, on day 21. Mean individual leaf area was calculated as the ratio between total leaf area and leaf number on day 28 (mean  s.e., n=6).

**Figure 2**: A. Minimum and maximum temperature in the greenhouse (dotted black and plain black lines, respectively), mean PAR radiation over 8:00 to 19:00 (red line) and mean soil relative extractable water in the drought-subjected plants (blue line) over the 5 week-experiment.

**Figure 3**: Daily transpiration rate of the three genotypes under well-watered conditions (black circle) and under drought (white circle) over the 5 week-experiment. Mean PAR radiation over 8:00 to 19:00 (red dotted line). Mean ± s.e, n=6.

**Figure 4**: Correlations between traits in control plants (upper part) and in drought-subjected plants (lower parts) (n=18 for each subplot). Only significant correlations were displayed (P-value<0.05).

Amean: mean of net CO2 assimilation rate measured *in situ*, Asat: net CO2 assimilation rate measured under light-saturated conditions, Ci: CO2 internal concentration, CumulT: cumulated water loss, DMincr: total dry mass increment, DTR: diurnal transpiration rate, δ13C: carbon isotope composition, FinalH: final stem height, FinalD: final stem diameter, gm: mesophyll conductance for CO2, gmean: stomatal conductance to water vapour measured *in situ*, gsat: stomatal conductance to water vapour measured under light-saturated conditions, Jmax: maximum photosynthetic electron flux, LA: total leaf area, LeafF: leaf fraction, Φw: proportion of unproductive water loss to productive water loss, NTR: nocturnal transpiration rate, RootF: root fraction, TE: whole plant transpiration efficiency, TotalDM: total dry mass, TR: daily transpiration rate, Vcmax: maximum CO2 carboxylation rate, Wisat: leaf intrinsic water use efficiency measured under light-saturated conditions, Wimean: mean leaf intrinsic water use efficiency measured *in situ*.

**Figure 5**: Correlation between whole plant transpiration efficiency (TE) and A) carbon isotope composition (δ13C), B) daily transpiration rate (TR), C) biomass increment (DMincr) and D) proportion of unproductive water loss to productive water loss (w). Each point corresponds to a plant. The blue, green and red symbols denote the French, the Italian and the Spanish genotypes, respectively. Closed and open symbols denotes control and drought treatments, respectively. Internal whiskers represent s.e., external whiskers represent 95% confidence interval.

**Table1**:Results of Two-way ANOVA for different traits. Significance and adjusted correlation coefficient of the model, significance of the factors (genotype and drought) and of the interaction. Marginal mean ± s.e. are given for the three genotypes and for the treatments. Different letters denote significant differences between groups according to Tukey post-hoc tests.

Amean: mean of net CO2 assimilation rate measured *in situ*, Asat: net CO2 assimilation rate measured under light-saturated conditions, Ci: CO2 internal concentration, CumulT: cumulated water loss, DMincr: total dry mass increment, DTR: diurnal transpiration rate, δ13C: carbon isotope composition, FinalH: final stem height, FinalD: final stem diameter, gm: mesophyll conductance for CO2, gmean: stomatal conductance to water vapour measured *in situ*, gsat: stomatal conductance to water vapour measured under light-saturated conditions, Jmax: maximum photosynthetic electron flux, LA: total leaf area, LeafF: leaf fraction, Φw: proportion of unproductive water loss to productive water loss, NTR: nocturnal transpiration rate, RootF: root fraction, TE: whole plant transpiration efficiency, TotalDM: total dry mass, TR: daily transpiration rate, Vcmax: maximum CO2 carboxylation rate, Wisat: leaf intrinsic water use efficiency measured under light-saturated conditions, Wimean: mean leaf intrinsic water use efficiency measured *in situ*.

**Table 2:** Complement of Table 1. Mean ± s.e. of different traits within each genotype x treatment group (n=4 - 6). Different letters denote significant difference between groups according to Tukey post-hoc tests. Acronyms are identical to those in Table 1.

**Supplementary material**

**Supplementary material and methods:** Rubisco kinetics and specificity characterisation

**Supp Table 1:** Predawn leaf water potential (MPa) of the three poplar genotypes

**Supp Figure 1:** Growth rate in height and in stem diameter of the three genotypes over the 5-week experiment.

**Supp Figure 2:** Leaf number and total leaf surface area of the three genotypes over the 5-week experiment.

**Supp Figure 3:** Net CO2 assimilation rate, stomatal conductance and intrinsic water use efficiency over the 5-week experiment.