Components of transpiration efficiency in poplars: genetic diversity, stability with age and scaling from leaf to whole plant level

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Doctorat ParisTech

T H E S E

Presented to get the doctorate degree in

L'Institut des Sciences et Industries du Vivant et de l'Environnement

(AgroParisTech)

Spécialité: Biologie Végétale et Forestière

Presented and defended in public by

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28 August 2012

Components of transpiration efficiency in poplars: genetic diversity, stability with age and scaling from leaf to whole plant level

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Acknowledgements

I would like to present my high, respectful gratitude to my Supervisor Dr. Erwin DREYER for his guidance, supervision and help throughout my thesis. The time when I joined this laboratory I know nothing about the stable isotopes and never knew even about their existence. Under his guidance, I put my first steps in this field of research completely new for me. I can remember my first day when walked into this lab and had a meeting with Erwin, he tried to explain some basic principals concerning the stable isotopes and I was sitting and saying "yes" every now and then like I knew those things. I can remember the moments that I passed in his office, He use to say "you are doing an excellent job" but "we need to modify it altogether" and "I need the values up to three significant figures". Nevertheless, his knowledge in this field allowed me to learn a lot that I would never forget.

I would present my heartiest regards and respect to Dr. Didier LE THIEC, being there for me when ever I needed. I can safely say that he was the MR CONSTANT through out my thesis. He was there helping me during the experiments, organizing the protocol for gas exchange measurements and for wood and leaf anatomy measurements. We shared diverse moment together. We also had some interesting discussion where he used to say "Non, non, non je suis pas d'accord" and some time "mais fahad ……..!". His help, his concern and his presence was vital and I am sincerely thankful to him.

I am thankful to Beatrice RICHARD for helping me in the extraction of cellulose and leaf soluble sugars. I am thankful to Pierre MONTPIED for helping me in statistics. We shared some enthusiastic discussions on bird and a famous hiking trip during which I sweated like never before. Thanks to the dependable Claude and Christian for isotope analysis. They passed some long series of samples and provided the results in time.

Special thanks to Franck BRIGNOLAS, Eric Paillassa & Cécile VINCENT-BARBAROUX for their contribution in providing plant materials and especially organizing the age effect experiments.

Can not forget Pascal COURTOIS (Pascaldo) with whom I shared the office for four years. What an office it was "jam-packed". He use to say "tout est utile". We smoked and joked together during this time. Two thing which we shared together I would never forget "his protocols" and post lab trip to Besançon for Kebab "450km just to eat Kebab!" Big thanks to Cyril BURE for organising the field visits for the last experiment during which we spent a quality time together (dangerous but quality time!). Thanks to Frank RADNAI for providing physical support during my experiments and Bernard CLERC for reserving vehicles for field visits.

At the end I would like to thanks my parents and my sister for their support and prayers without which I would not had completed this big task. They always encouraged me especially at the beginning where I found myself as complete stranger in France. They were there till the end and whenever I needed them the most!

Last but not the least I am thankful to my supportive wife Claire for staying by my side in all situations.

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List of abbreviations

Variables	Description
α	Discrimination against $^{13}CO_2$ during diffusion through stomata
A, A_{sat}	Net CO_2 assimilation rate under ambient/saturating condition (μmol m^{-2} s^{-1})
(A/g_s)	Intrinsic transpiration efficiency over a diurnal cycle (μmol mol^{-1})
A_{sat}/g_{sat}	Intrinsic transpiration efficiency under saturating condition (μmol mol^{-1})
β	Discrimination against $^{13}CO_2$ during carboxylation
BM	Total plant biomass accumulated during the experiment (g)
C_i/C_a	Ratio of CO_2 concentration in the atmosphere and in the substomatal spaces
C_{lb}	% carbon in bulk leaf tissues
C_{ss}	% carbon % in the leaf soluble sugars
D	Diameter at stem base (mm)
g_m	Mesophyll conductance to CO_2
g_s, g_{sat}	Stomatal conductance to water vapour under ambient/saturating condition (mol m^{-2} s^{-1})
H	Stem height (cm)
LT	Leaf thickness (μm)
N_{lb}	% nitrogen in bulk leaf tissues
N_{ss}	% nitrogen in the leaf soluble sugars
$PPFD$	Photosynthetic photon flux density (μmol photons m^{-2} s^{-1})
SD	Stomatal density (mm^{-2})
TE	Whole plant transpiration efficiency = BM/WU (g l^{-1})
T_l	Leaf temperature (°C)
TLA	Whole plant leaf area (m^2)
LMA	Leaf mass to area ratio (g m^{-2})
VPD	Vapour pressure deficit (kPa)
WU	Cumulated water use (l)
$\delta^{13}C_{air}$	Carbon isotope composition of the air (‰)
$\delta^{13}C_{ss}$	Carbon isotope composition of leaf soluble sugars (‰)
$\delta^{13}C_{lb}$	Carbon isotope composition of bulk leaf matter (‰)
$\Delta^{13}C_{ss}$	Carbon isotope discrimination between atmosphere and leaf soluble sugars (‰)
$\Delta^{13}C_{lb}$	Carbon isotope discrimination between atmosphere and bulk leaf matter (‰)
$\delta^{13}C_{offset}$	Difference between $\delta^{13}C_{ss}$ and $\delta^{13}C_{lb}$ (‰)
$\Delta^{13}C_M$	Carbon isotope discrimination predicted by the simple discrimination model (‰)
$\Delta^{13}C_{dif}$	Carbon isotope discrimination difference between predicted and observed (‰)
$\delta^{18}O_{sw}$	Oxygen isotope composition of source water (‰)
$\delta^{18}O_{lb}$	Oxygen isotope composition of bulk leaf matter (‰)
$\delta^{18}O_{lw}$	Oxygen isotope composition of leaf water (‰)
$\Delta^{18}O_{lb}$	Oxygen isotope enrichment between source water and bulk leaf matter (‰)
$\Delta^{18}O_{lw}$	Oxygen isotope enrichment between source water and leaf water (‰)
Φ_c	Proportion of carbon lost through respiration not used during photosynthesis
Φ_w	Proportion of water lost during the day and night not used during photosynthesis
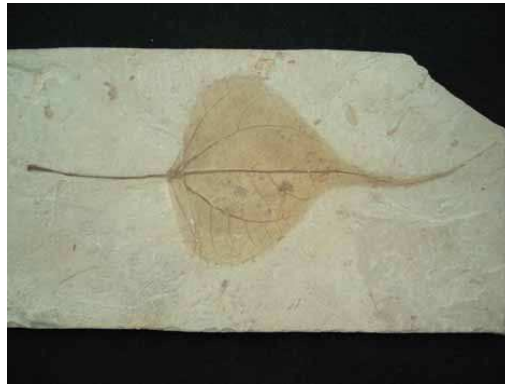
Figure 1. Leaf of *Populus wilmattae* (a.k.a. *Populus cinnamomoides*) in a fossil record (58 million years B.P.) Photo by D. Dickmann.

Figure 2. Natural range of *Populus nigra* in Eurasia and Africa. Redrawn from Vanden Broeck (2003).

Figure 3. Natural range of *Populus deltoides*. Redrawn from U.S. Geological Survey Earth Surface Processes http://esp.cr.usgs.gov/data/atlas/little/.

INTRODUCTION

Poplars have a long cultural history. They are a part of agro-forestry systems and are managed to supply timber, fuel wood, forage, and serve as windbreaks. During the period between 1830 and 1848, poplar has been planted in France as a symbol of liberty and ambassador of the people (*Populus*). Fossil traces of poplar leaves date from Paleocene (55-60 million years BP) for the section *Tacamahaca* and Miocene (10-15 million years BP) for the section *Aigeiros* (Fig. 1). *Populus nigra* and *Populus deltoides* seem to descend from *Populus latior* and *Populus glandulifera* respectively. Although *Populus nigra* cv. Italica has been present for a long time in the Mediterranean regions of Europe, it has spread in France from 1749 on the banks of the Briare canal via Italy. *Populus deltoides* was introduced to Europe from North America in the late 17th and 18th century as cuttings.

1.1. The genus *Populus*:

Poplars are deciduous or rarely semi-evergreen trees with a wide natural distribution in the Northern Hemisphere, from the equatorial tropics to the limits of tree distribution area. Taxonomically, poplars are divided into six sections (Table 1). *Populus deltoides* and *Populus nigra* belong to the section *Aigeiros* and are also known as eastern cottonwood and black poplar, respectively. Barriers to gene flow among *Populus* species in natural populations are frequently ineffective, so spontaneous hybrids are common. Interspecific hybridization is common within most *Populus* sections. Table. 2 illustrates some inter-specific and inter-sectional hybrids in the genus *Populus*, but inter-sectional hybrids between *Aigeiros* and *Tacamahaca* also occur in nature. Successful mating occurs with difficulty or not at all across sectional lines in *Abaso*, *Turanga*, *Leucoides*, and *Populus* (Zsuffa 1973; Stettler *et al.* 1980). The dominant poplar in southern France is *Populus nigra* (for the natural range see Figure 1). *Populus deltoides*, native to North America, has been introduced in Europe. These two poplars are parents to many cultivated clones (for the natural range see Figure 2).

The vegetative propagation of poplars uses 20 to 30 cm long sections of dormant, one-year-old woody shoots as planting stock. If these "cuttings" are planted in the spring, they quickly produce roots from existing primordia in the inner bark and new shoots from lateral buds. Due to their fast growth, planted cuttings grow from 1 to 4 meters tall during the first growing season, depending on genotype, local environment and site conditions. The ability to produce adventitious roots also allows the use of entire young, several meter long shoots (sets) to be used as planting stock.

Table 1. Taxonomic classification of the genus *Populus* (FAO/IPC Poplars and willow in the world 2008)

Section	Taxon	English common name	Notes and synonyms
Abaso	*P. mexicana* Wesmael	Yaqui cottonwood	Monotypic section
Turanga (Afro-Asian poplars)	*P. euphratica* Olivier	Euphrates poplar	Includes *P. diversifolia*
	P. ilicifolia (Engler) Rouleau	Kenyan poplar	Formerly synonymous with *P. euphratica*
	P. pruinosa Schrenk	Desert poplar	Formerly synonymous with *P. euphratica*
Leucoides (Swamp	*P. glauca* Haines	Asian swamp cottonwood	Formerly *P. wilsonii*
	P. heterophylla Linnaeus	Swamp cottonwood	
	P. lasiocarpa Oliver	Heart-leaf poplar	
Aigeiros (Cottonwoods black poplar)	*P. deltoides* Marshall	Eastern cottonwood	Includes *P. sargentii*, *P. palmeri*, and *P. wislizenii*
	P. fremontii S. Watson	Fremont cottonwood	Includes *P. arizonica*
	P. nigra Linnaeus	Black poplar	
Tacamahaca (Balsam poplars)	*P. angustifolia* James	Narrowleaf cottonwood	
	P. balsamifera Linnaeus	Balsam poplar	Formerly *P. tacamahaca*
	P. cathayana Rehder	Cathay poplar	May be synonymous with *P. suaveolens*; Heretofore in section Leucoides; the former
	P. ciliata Royle	Himalayan poplar	Likely synonymous with *P. suaveolens* or *P. maximowiczii*
	P. koreana Rehder	Korean poplar	May be synonymous with *P. suaveolens*; Includes *P. przewalskii* and *P. kangdingensis*
	P. laurifolia Ledebour	Laurel poplar	
	P. maximowiczii Henry	Japanese poplar	
			May be synonymous with *P.balsamifera*
	P. simonii Carrière	Simon poplar	
	P. suaveolens Fischer	Siberian poplar	
	P. szechuanica Schneider	Szechuan poplar	
	P. trichocarpa Torrey & Gray	Black cottonwood	
	P. yunnanensis Dode	Yunnan poplar	
Populus (White poplars and aspens	*P. alba* Linnaeus	White poplar	
	P. guzmanantlensis Vazquez & Cuevas	Manantlán poplar	May be synonymous with *P.simaroa*
	P. monticola Brandegee		
		Baja poplar	A.k.a. *P. brandegeei*; may be naturalized *P.*
	P. simaroa Rzedowski		
	P. adenopoda Maximowicz	Balsas poplar	
	P. gamblei Haines	Chinese aspen	
	P. grandidentata Michaux	Himalayan aspen	
	P. sieboldii Miquel	Bigtooth aspen	Includes *P. jesoensis*
	P. tremula Linnaeus	Japanese aspen	Includes *P. davidiana* and *P. rotundifolia*
		Common aspen	
	P. tremuloides Michaux	Quaking aspen	

Table 2. Some naturally occurring hybrids among taxa in the genus *Populus* (Eckenwalder 1996)

Hybrid parents	Hybrid binomial	English common name and notes
P. adenopoda × P. ×tomentosa	*P. × pseudotomentosa* Wang & Tung	Backcross hybrid
	P. × tomentosa Carrière	
P. alba × P. adenopoda		Peking poplar or Chinese white poplar; aspen parent may be *P. tremula* var. davidiana
	P. × rouleauiana Boivin	
P. alba × P. grandidentata	*P. × canescens* Smith	Gray poplar
P. alba × P. tremula	*P. × heimburgeri* Boivin	
P. alba × P. tremuloides	*P. × brayshawii* Boivin	Brayshaw's poplar
P. angustifolia × P. balsamifera	*P. × acuminata* Rydberg	Lanceleaf cottonwood; a.k.a. *P. × andrewsii*
P. angustifolia × P. deltoides	*P. × hinkeleyana* Correll	
P. angustifolia × P. fremontii	*P. × sennii* Boivin	
P. angustifolia × P. tremuloides	None	Trihybrid
P. angustifolia × P. balsamifera x P. deltoides	*P. × charbinensis* Wang & Skvortzov	Unverified trihybrid
P. ×berolinensis × P. simonii	*P. × rollandii*	
	P. × generosa Henry	
P. ×canadensis × P. balsamifera	*P. × jackii* Sargent	Interamerican poplar; a.k.a. *P. × interamericana*
P. deltoides × P. trichocarpa		Jack's hybrid poplar or heartleaf balsam poplar; a.k.a. *P. balsamifera* var. subcordata, *P. candicans*, or *P. × gileadensis*
P. deltoides × P. balsamifera		
	P. × dutillyi Lepage	Trihybrid
	P. × polygonifolia Bernard	
P. balsamifera × P. tremuloides		Euramerican poplar; a.k.a. *P. ×euramericana*
P. balsamifera × P. deltoides × P. tremuloides	*P. × canadensis* Moench	Bernard poplar; may actually be *P. × jackii*
	P. × bernardii Boivin	
P. deltoides × P. nigra	*P. × euramericana*	
P. deltoides × P. tremuloides	*P. × inopina* Eckenwalder	A.k.a. *P. × barnesii*
P. fremontii × P. deltoides	*P. × smithii* Boivin	Berlin or Russian poplar; a.k.a. *P. × rasumowskyana* or *P. × petrowskyana*
P. fremontii × P. nigra	*P. ×berolinensis* Dippel	Trihybrid
P. grandidentata × P. tremuloides		
P. laurifolia × P. nigra	None	Trihybrid
		Lesser black poplar; a.k.a. *P. × gansuensis* or *P. × xiaozhuanica*
(P. laurifolia × P. nigra) × P. balsamifera	None	Hebei poplar, a trihybrid
(P. laurifolia × P. nigra) × P. deltoides	*P. × xiaohei* Hwang & Liang	Parry cottonwood
P. nigra × P. simonii		
	P. × hopeiensis Hu & Chow	
P. tremula × P. ×tomentosa	*P. × parryi* Sargent	
P. trichocarpa × P. fremontii		

Table 3. Area (in ha) planted with wood producing poplars in 2003 and 2006 in France according to regions (http://www.peupliersdefrance.org).

Regions	2003	2006
Alsace	2000	2176
Aquitaine	17200	24974
Auvergne	1400	3329
Basse Normandie	4700	4586
Bourgogne	11900	14442
Bretagne	6100	8235
Centre	22600	22814
Champagne-Ardenne	21100	26864
Franche-Comté	2900	4134
Haute Normandie	1600	1890
Ile de France	2400	9576
Languedoc-Roussillon	900	1143
Limousin	900	329
Lorraine	3600	3540
Midi-Pyrénées	12500	14824
Nord Pas de Calais	9000	12863
Pays de la Loire	18900	22147
Picardie	23900	32108
Poitou-Charentes	13000	12177
Provence Alpes Côte d'azur	900	418
Rhône-Alpes	7700	10836
Total	**185100**	**233406**

Easy vegetative propagation from hardwood cuttings is a remarkable trait that has allowed the widespread and successful planting of selected pure species and hybrid clones, sspecially in the sections *Aigeiros* and *Tacamahaca*. On the other hand, Aspens (*Populus tremula*) cannot be propagated from hardwood cuttings, although they can from root cuttings, or leafy softwood cuttings. Thus, plantations of poplars outside these two sections (*Aigeiros* and *Tacamahaca*) are less common worldwide. During 2008, 52 million ha of poplars were growing all over the globe. Poplar cultivation is well developed in most countries of the northern hemisphere as compared to the southern hemisphere

1.2. Poplar hybrids

Spontaneous hybrids have been used in commercial culture for several decades. In addition to that, geneticists bred hybrids that were deployed in poplar cultivation throughout the world. The most common of these commercial hybrids belong to *Populus × euramericana,* which is the cross *Populus deltoides* (♀) × *Populus nigra* (♂). To date, tree breeders continue producing new hybrid cultivars using microculture and biotechnology techniques to cross mating barriers.

In France, apart from a few genotypes of *Populus deltoides, Populus trichocarpa* and *Populus alba*, poplar cultivation is dominated by (i) *euramericana* hybrids (*Populus deltoides × nigra* (ii) *interamericana* hybrids, stemming from the cross *Populus deltoides × trichocarpa*. *Interamericana* hybrids have been present in the French national catalogue since 1982. Over the past 20 years, the resistance of these genotypes to fungal attacks drove the relative share of these two types of hybrids in french plantations. *Interamericana* hybrids showed remarkable hybrid vigour. Thus, between 1987 and 1997, *interamericana* hybrids, represented mainly by *'Beaupré'* left very little room for *euramericana* cultivars. Nevertheless, these cultivars grown in dense plantations suffered severe rust attacks (*Melampsora larici-populina* Kleb.) after 1997 due to their low quantitative resistance (Dowkiw & Bastien 2007). They were less and less used thereafter. Until 1998, sales of *interamericana* hybrids increased meagerly but thereafter decreased sharply in favour of the e*uramericana* hybrids (Breton 2000; Paillassa 2001; Balzinger & Ginisty 2002). For almost 20 years, a strong increase in the relative proportion of *Euramericana* cultivars growing in France, especially for the clone '*I214*' since 2000 was detected by FCBA (Forêt, Cellulose, Bois-construction, Ameublement; Thivolle-Cazat 2002). This shows that after a fall in interest for *Euramericana* cultivars between 1987 and 1997, farmers are showing a renewed interest in these hybrid poplars (see Figure 4 for its distribution in 2003).
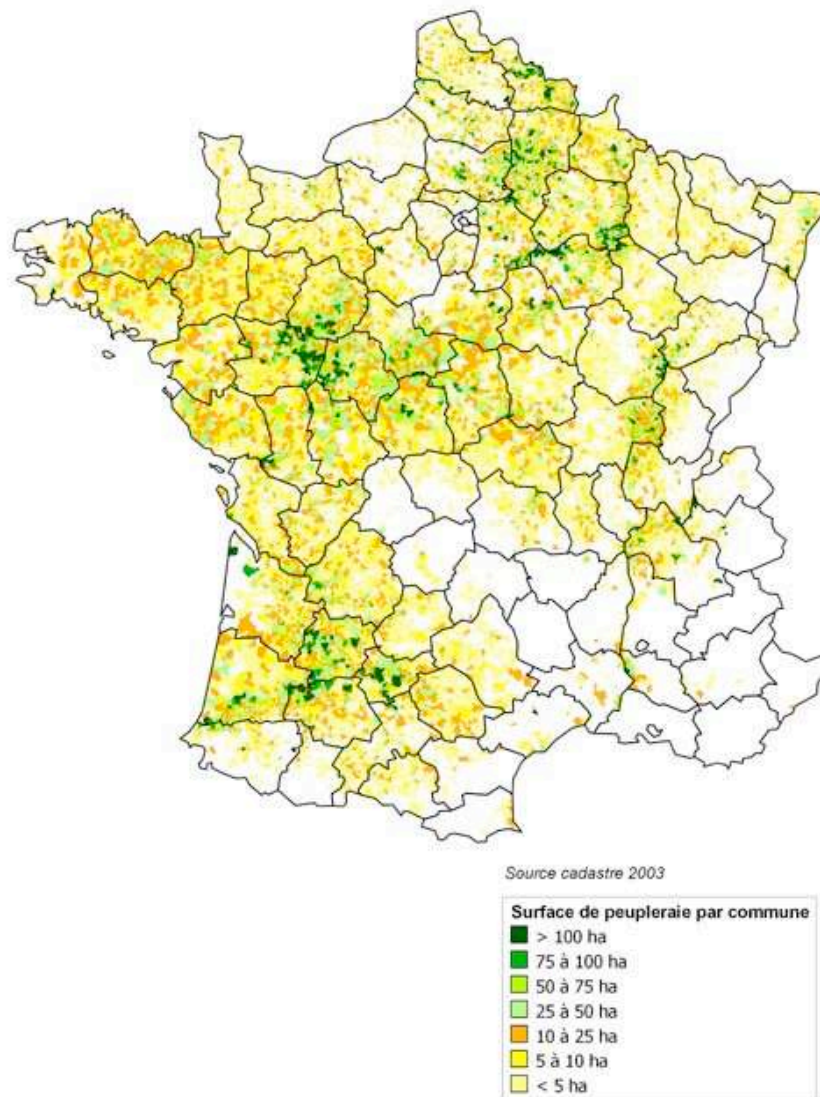
Figure 4. Distribution pattern of poplar cultivation in France (ha) in 2003 (Ministère de l'
Agriculture et de la Pêche 2004).

1.3. Poplar cultivation

Traditionally, poplar has been cultivated in France since the 18th century. Due to import ban against trembling aspen for matches and okoume for plywood, poplar cultivation has regained farmer's interest. Poplar cultivation was once again boosted after the creation of "The French Poplar Commission" in 1942, which became the "International Poplar Commission IPC" in 1947. It was a substantial initiative of France to promote poplar cultivation in collaboration with FAO (Food and Agriculture Organization of the United Nations). Since then, additional 28 countries have joined in and have enlarged the list of 8 pioneer members who responded to the call of attending the poplar week in April 1947. The tasks of IPC are multiple: to study the scientific, technical, socio-economical aspects of poplar culture; to promote the exchange of ideas and materials among researchers, producers and users; to create joint research programs and stimulate organization of meetings (Viart & Fugalli 1997; Viart 1999). In France, due to the long efforts of IPC and other forestry related organizations, the area under poplar cultivation increased from 100000 ha in 1939 to almost 185100 ha in 2003 (Table 3), which increased to 235000 ha in 2008.

Region wise, Picardie (32000 ha), Champagne-Ardenne (27000 ha), Aquitaine (25000 ha) and Centre along with Pays de la Loire (44000 ha) share most of the area under poplar cultivation (Table 3 & Figure 4). Such a large area makes poplar the third highest wood producing species in France after oaks and beech in 2003 and the second one in 2008 after oaks (1.5 million m^3 of wood per year, FAO 2008).

Poplar wood is very versatile and widely used. It is light in weight (specific gravity 0.3 to 0.4) because of its porous anatomy. The wood is soft, creamy white in colour (except for a dark, pale brown heartwood or wood core), straight-grained and uniform in texture. Some species readily produce tension wood characterized by a low cell wall lignification which results in a woolly aspect of the wood. Wood of many poplar species displays a low strength, stiffness, shock resistance, decay resistance and bending properties. However, the wood can be worked easily with hand or machine tools. In addition, poplar wood can be easily glued, screwed and nailed. Staining can be patchy but paints and varnish are easily applied. Poplar wood is widely used to manufacture pulp and paper, peeled and sliced veneer, composition boards (including oriented-strand board), sawn lumber, crates, boxes, matches, chopsticks, poles, furniture, cabin logs, and excelsior. In 2008 in France, 32% of the harvested poplar wood volume was used for light peeling, 7.9% for veneer plywood, 24.4% for export veneer, 24.7% for sewing palettes and 11% for sawing crates and bedding.
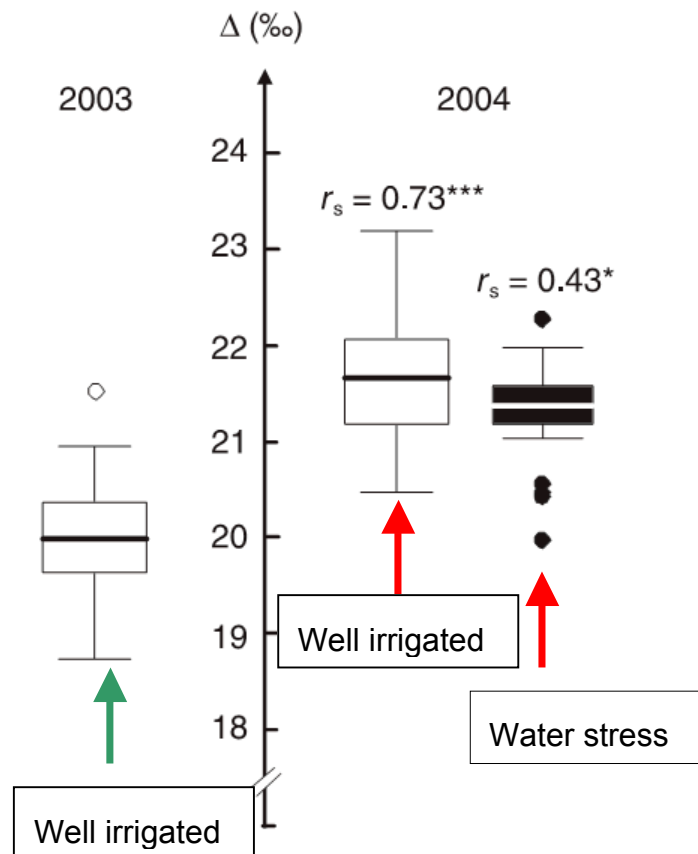
Figure 5. Boxplots for the values of the discrimination against ^{13}C (Δ^{13}C) recorded in the bulk leaf matter of a range of *Populus × euramericana* genotypes grown in a factorial experiment in the field at Orléans, during 2003 and 2004. During 2004, a moderate drought treatment was applied to half the individuals and induced a decline of Δ^{13}C. Values recorded during 2003 were influenced by the heat and drought stress that happened during that year. Based on Spearman's rank correlation (r_s), the genotypic ranking remained same as found in 2003. Modified from Monclus *et al.* 2006.

1.4. Research questions and thesis objectives

Poplars occupy an important place in the timber industry. To meet the currently increasing industrial demand, growers are bringing new areas under poplar cultivation such as in the Pays de la Loire and Garonne regions. As a result of this extension in addition to predicted climate change, an enhanced risk of drought events might have a negative influence on the productivity of poplar genotypes. In this scenario, the question of the effect of water supply on productivity is becoming more and more important. In this context, the concept of transpiration efficiency (total biomass accumulated with respect to water transpired) gains interest. More transpiration efficient poplar genotypes, selected on the basis of reduced water consumption (or stomatal conductance) could ensure efficient water use and a sustained production by enhancing conservation of soil water and allowing plants to remain physiologically active.

In this respect, the work done during this thesis fits the long chain of studies done on poplar genotypes during a collaboration between two laboratories, Forest Ecology and Ecophysiology (EEF, UMR 1137 INRA/Université de Lorraine) and Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC, UPRES-EA 1207, University of Orléans). The aim of these two labs is to identify the ecophysiological and molecular bases controlling the genetic and phenotypic variation of complex traits in trees. Traits of interest include water use efficiency (WUE) and drought tolerance. In this regard, their main focus is on the commercialized poplar cultivars like *Populus × euramericana* and wild type *Populus nigra*. The aim is to detect genetic material that is both water-use efficient and adapted to the different cultivation regions. In this context, the detection of genotypes, noticeably of *Populus × euramericana*, were the centre of interest (thesis dissertation of N. Marron 2000-2003, R. Monclus 2002-2006 and L. Bonhomme 2005-2009).

Poplar hybrids are known for their potential to produce high volumes of wood in short periods of time (Marron *et al.* 2005; Marron & Ceulemans 2006; Marron *et al.* 2007) but are generally sensitive to drought. Their productivity is very closely linked to water availability that can limit growth, wood quality and eventually favour the installation of pathogens (Marçais *et al.* 1993; Barigah *et al.* 1994; Pinon & Valadon 1997; Loustau *et al.* 2005). Therefore, it becomes a very important issue to detect genotypes that combine productivity, high wood quality and tolerance to biotic and abiotic stresses. In *Populus × euramericana* genotypes, previous studies have highlighted a wide range of genotypic variation for growth potential, transpiration efficiency at leaf level (estimated through ^{13}C discrimination; see Figure 5 b;
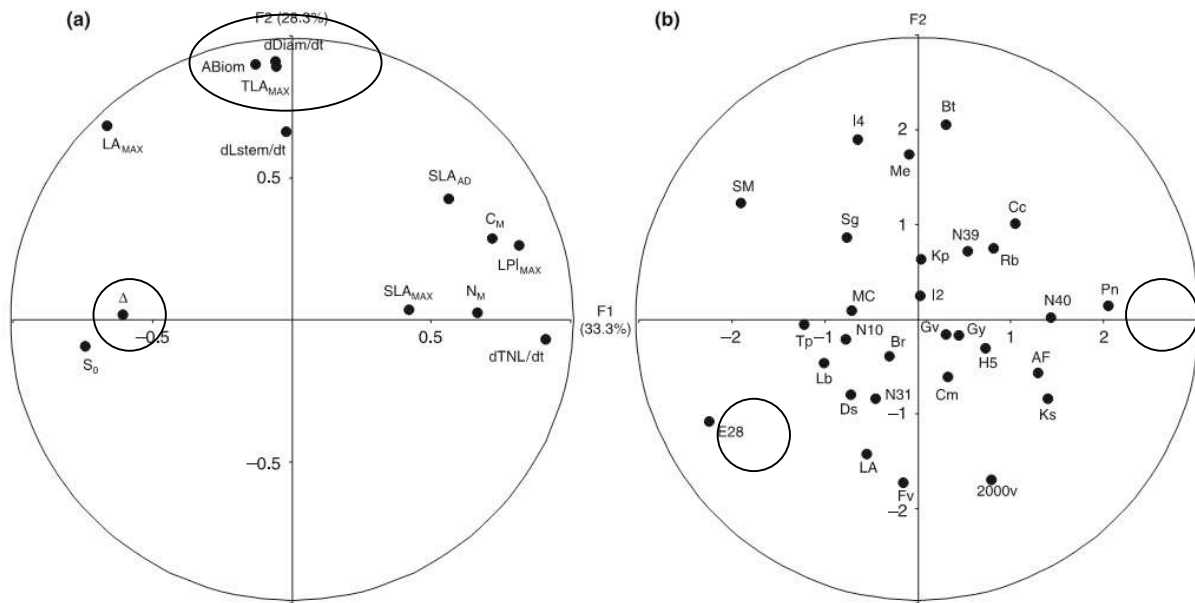
Figure 6. PCA from Monclus *et al.* (2005) showing (a) lack of correlation between istopic discrimination against [13]C (Δ) and productivity traits like above ground biomass (ABiom), stem diameter (dDiam) and maximum leaf area (TLA$_{max}$) as they are present on the opposite axis on the PCA (b) and large genotypic variability for productivity traits and Δ between tested 29 genotypes (*Populus deltoides × nigra*). Δ varied between 18.73‰ for Pannonia (Pn) and 21.51‰ for Eco-28 (E28). Reprinted from Monclus *et al.* 2005.

Marron *et al.* 2005; Dillen *et al.* 2008) and tolerance to moderate drought (Monclus *et al.* 2005; Monclus *et al.* 2006; Bonhomme *et al.* 2008). A large genotypic variability has also been found in the amplitude of the drop of productivity in response to water shortage among poplar cultivars (Gebre *et al.* 1994, 1998; Liu & Dickmann 1996; Chen *et al.* 1997; Harvey & van den Driessche 1997; Robison & Raffa 1998; Tschaplinski *et al.* 1998; Marron *et al.* 2003; Monclus *et al.* 2006).

These initial studies were based on the measurement of instant transpiration efficiency (*TE*) through leaf gas exchange measurements and of time-integrated *TE* using the natural abundance of stable carbon isotopes *(δ^{13}C)* in leaf tissues (Marron *et al.* 2005; Monclus *et al.* 2005, 2006; Bonhomme *et al.* 2008; Dillen *et al.* 2008, 2009). Along with the variability detected among genotypes in the discrimination against ^{13}C from the atmosphere to the leaf matter *(Δ^{13}C)*, morphological indicators for productivity were identified such as the maximum area of single leaves and total leaf area. Furthermore, no relation was evidenced between productivity and Δ^{13}C (Fig. 6 a, b) both in open field experiments and under controlled environment. Under moderate drought, Δ^{13}C decreased but the genotypic ranking remained intact, evidenced through Spearman's rank correlations (see Figure 5; Monclus *et al.* 2006). Along with this large genotypic variability for productivity and the stability of genotype ranking for Δ^{13}C, stomatal conductance was found to be responsible for genetic variation in Δ^{13}C (Monclus *et al.* 2006). More recently, Dillen *et al.* (2008) also evidenced the lack of direct relationship between stomatal traits and plant growth or Δ^{13}C in *Populus × euramericana* genotypes. Such results in various poplar genotypes (i.e., the absence or weak relationship between Δ^{13}C and growth) open the possibility of selecting genotypes combining high productivity and high transpiration efficiency.

In these studies, the genetic variability of *TE* was exclusively measured through Δ^{13}C (as an estimator of *TE*). Furthermore, experiments included very young individuals (up to 2 years) grown in a greenhouse or in open-field experiments. Extrapolating the conclusions obtained at leaf scale and concluding about stability of genotypic variability for whole plant *TE* can be tricky. Factors like Φ_c and Φ_w (carbon lost during respiration and water lost during night respectively) may vary between genotypes, thus the observed genotype variability in Δ^{13}C may not perfectly match the genotype variability in whole plant *TE*. Previous studies have found that variability in Δ^{13}C does not necessarily reflect the variability in whole plant *TE* (Sun *et al.* 1996; Picon *et al.* 1996a; Turner *et al.* 2007; Matzner *et al.* 2001). Thus, to select genotypes for lower Δ^{13}C, we need to be sure that observed genotype differences in Δ^{13}C

really reflect differences in whole plant *TE*. Another important question is the temporal stability of genotype ranking for $\Delta^{13}C$. $\Delta^{13}C$ decreases in many species with increasing age and the intensity of the decrease varies among species (Francey & Farquhar 1982; Bert *et al.* 1997*;* Duquesnay *et al.* 1998*;* McCarroll & Pawellek 2001). A 2.8‰ range of genotype values of $\Delta^{13}C$ has been detected across 29 *Populus × euramericana* genotypes. This range and the genotype ranking is susceptible to change with age. Thus, it becomes important to verify if the genotype ranking for $\Delta^{13}C$ remain stable till the end of a rotation. Around these two main objectives, four experiments were designed along two general objectives:

1) To check whether genotypic differences in $\Delta^{13}C$ reflect genotypic differences in whole plant *TE* (upscaling from leaf to whole plant).

- **under optimum growth conditions:** the upscaling approach was tested in six *Populus deltoides × nigra* genotypes showing contrasting $\Delta^{13}C$ (Monclus *et al.* 2006). Plants were grown under a controlled and stable humid environment where optimum light, temperature and soil moisture were provided. Water transpired, biomass produced, $\Delta^{13}C$ (leaf soluble sugars and bulk leaf matter), diurnal variations in net CO_2 assimilation (*A*) and stomatal conductance to water vapour (*g_s*) and $\Delta^{18}O$ (bulk leaf matter and leaf water) were measured (Chapter 1);

- **under different evaporative demands:** the upscaling approach was tested in six *Populus nigra* genotypes originating from natural populations along the Loire river with contrasting $\Delta^{13}C$ (Chamaillard *et al.* 2011). Plants were grown in two growth chambers under 75% and 45% relative humidity. Optimum light, temperature and soil moisture were provided. Water transpired, biomass produced, $\Delta^{13}C$ (leaf soluble sugars and bulk leaf matter), diurnal variations in *A* and *g_s* and $\Delta^{18}O$ (bulk leaf matter and leaf water) were measured (Chapter 2).

2) To check for the stability of genotype ranking for $\Delta^{13}C$ with age with:

- **a diachronic approach:** $\Delta^{13}C$ was recorded in annual rings along tree cores. Different genotypes of similar age and growing under a similar environment (common garden) were used. This approach was repeated on three different sites (Chapter 3);

- **a synchronic approach:** $\Delta^{13}C$ was recorded in the last annual ring (2009). Three *Populus deltoides × nigra* genotypes were tested and samples were taken from trees of different ages growing under different environments. Using this approach, the effects of long term environmental changes were minimized and tree age effect was maximized (Chapter 4).
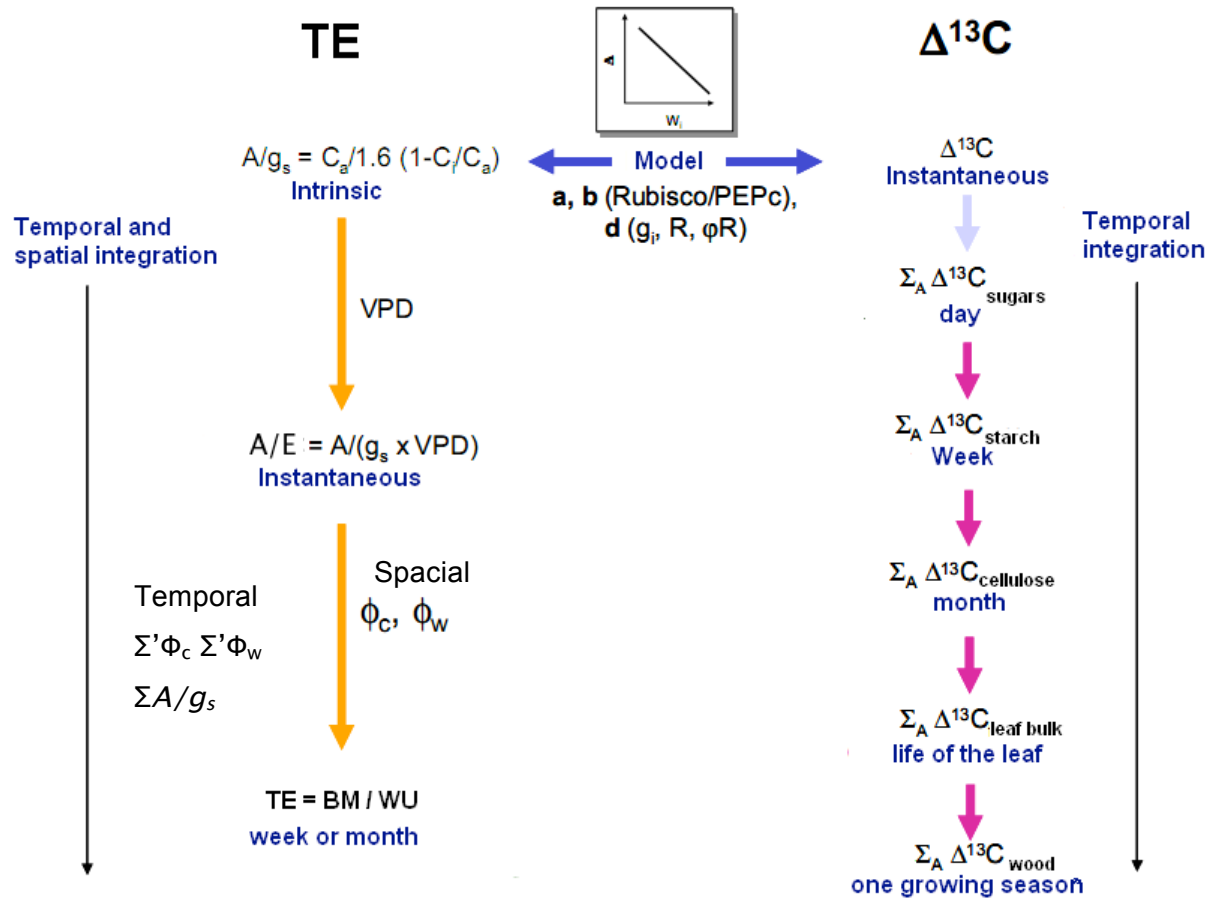
Figure 7. Theoretical relationship between transpiration efficiency measured at different integration levels. At leaf level through gas exchange, at whole plant level by estimating biomass accumulated and water transpired and through isotopic discrimination of ^{13}C in the organic matter with respect to source (Modified from Konate thesis dissertation 2010).

2. STATE OF THE ART

2.1. Water use efficiency at different integration scales

Water use efficiency has become a trait of interest in various fields: agronomy, forestry or ecology. In agronomy, improving water use efficiency in crop plants means to increase production under a given water supply (Richards *et al.* 2002). This can be done: (i) by reducing runoff and drainage, (ii) by reducing evapotranspiration, (iii) by improving transpiration efficiency *TE*, (iv) by improving the harvest index. Evapotranspiration in a crop can be reduced simply by increasing crop density. This will reduce the water loss directly from the soil surface and will increase the fraction water used by the plants. *TE* can be increased by acquiring more carbon in exchange of water transpired by the plants, and harvest index can be increased by increasing of the ratio harvestable product vs. the achieved biomass.

In forestry, *TE* is often studied at plant scale as well as at ecosystem scale (i.e. the gain of ecosystem carbon vs. actual evapotranspiration; Law *et al.* 2002). At plant scale, several definitions of *TE* coexist, allowing access to different spatial and temporal integration levels. At whole plant scale, *TE* is measured as the ratio between biomass accumulated and the amount of water transpired by the plant for a certain period of time.

2.1.1 At leaf level

At leaf level, *TE* can be defined as "intrinsic" or "instant" *TE* (Figure 7; A/g_s and A/E respectively). Intrinsic *TE* or A/g_s (µmol CO_2 mol^{-1} H_2O) is the ratio of net CO_2 assimilation (A, µmol CO_2 m^{-2} s^{-1}) and stomatal conductance to water vapour (g_s, mol H_2O m^{-2} s^{-1}). The influx of CO_2 (A) from atmosphere to the leaf interior is described as product of the mole fraction difference of CO_2 between the atmosphere and the intercellular spaces respectively (C_a & C_i, in µmol CO_2 mol^{-1} air) and stomatal conductance to CO_2 (g_c, mol CO_2 m^{-2} s^{-1}), which is the inverse of resistance, hence $A = g_c (C_a - C_i)$. As water vapour diffuses 1.6 times faster than CO_2, then: $g_s = 1.6 \, g_c$ thus,

$$\frac{A}{g_s} = \frac{C_a - C_i}{1.6} = \frac{C_a(1 - C_i/C_a)}{1.6} \tag{1}$$

Instant *TE*, i.e., *A/E*, depends on instrinsic *TE* and another environmental component, namely ν (the difference in water vapour mole fraction between the leaf and the atmosphere), which can be highly variable with time.

A/E (μmol CO_2 mol^{-1} H_2O) represents the ratio of influx of CO_2 entering the leaf and the flux of water vapour out of the leaf (or transpiration, denoted by E in mol H_2O m^{-2} s^{-1}) during photosynthesis. Transpiration is calculated using the product between g_s and the mole fraction gradient of water vapour (v, mmol H_2O mol air^{-1}) between the leaf (w_i, mmol H_2O mol air^{-1}) and atmosphere (w_a, mmol H_2O mol air^{-1}) ($v = w_i - w_a$), hence $E = g_s v$.

$$\frac{A}{E} = \frac{C_a(1 - C_i/C_a)}{1.6v} \tag{2}$$

2.1.2 At whole plant level

Whole plant *TE* is the integrated value of instant *TE* over time across the entire plant. Whole plant *TE* is usually recorded over several weeks or months and is based on gravitational measurements of accumulated dry biomass (*BM*) and water loss through transpiration accumulated over the entire period of the experiment (*WU*; Figure 7). Thus, whole plant *TE* is expressed in g *BM* g^{-1}H_2O.

The estimation of whole plant *TE* is laborious because it includes weighing potted plants at leastevery day to assess the total water transpired over a given period. On the other hand, measuring whole plant *TE* on a mature tree is very difficult. Olbrich *et al.* (1993) measured *TE* at whole tree level, which was measured from total biomass gain (including roots), which is not easy to determine. Intrinsic *TE* being much simpler to measure could be used to deduce whole plant *TE* based on the theoretical relationship between the two terms (Farquhar & Richards 1984):

$$TE = \frac{C_a(1 + \phi_c)(1 - C_i/C_a)}{1.6v(1 + \phi_w)} \tag{3}$$

where Φ_c is the fraction of carbon lost during respiration in non photosynthesising tissues and during night, and Φ_w is the fraction water lost during night and in non-photosynthesising tissues. This relationship between intrinsic *TE* and whole plant *TE* has been experimentally detected in different species, under varying environmental conditions, such as different water supply (Osorio & Pereira 1994; Matzner *et al.* 2001) or under different doses of nitrogen fertilization (Ripullone *et al*. 2004). Only a few studies have found correlations between these traits at intra-specific level, under non-limiting conditions like in *Eucalyptus* populations (Li 2000). At times, relating intrinsic and whole plant *TE* can be very tricky as both traits are very plastic under natural environment. Since intrinsic *TE* is measured at leaf scale, while whole plant *TE* is integrated to whole plant and over long periods of time, different environmental factors affecting both traits can interfere during this upscaling.

Influencing factors

Leaf morphology   Irradiance

$\delta^{13}C_{air}$

Air humidity

Root to leaf
distance   Temperature

Xylem hydraulic   Precipitation
conductivity   amount and
seasonality

Bark photosynthesis

Recycling of
respired CO_2 Snow-melt

Soil freezing

Fine root network

Rooting depth

Soil moisture status
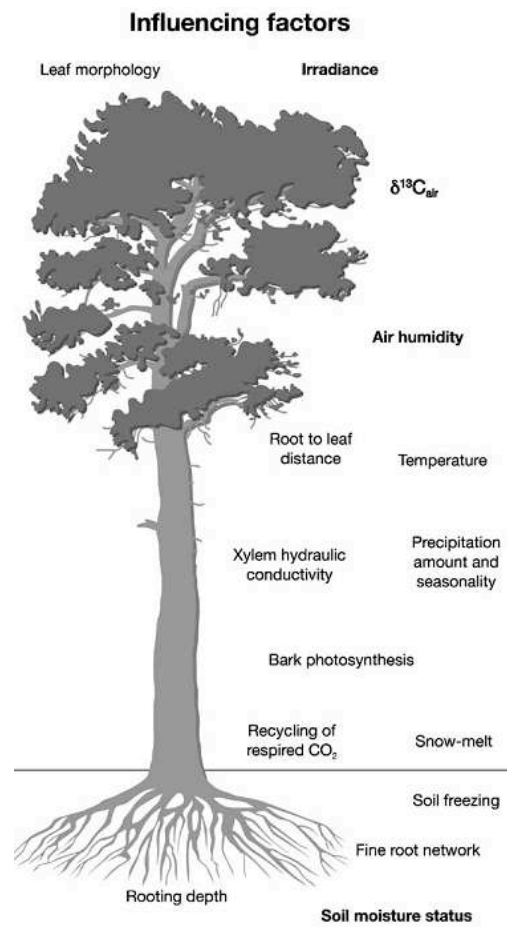
Figure 8. Diagram showing the main environmental factors influencing $\Delta^{13}C$ (reproduced from McCarroll & Loader 2004).

This question becomes even more important when the objective is to use the upscaling approach among closely related poplar genotypes for testing if differences in intrinsic *TE* actually reflect the differences in whole plant *TE*. While scaling up from intrinsic to whole plant *TE,* the proportion of carbon fixed during photosynthesis and lost through respiration of roots and stems during the day and the roots, stems and leaves at night (Φ_c), and the proportion of water lost by the plant independently of photosynthetic processes (Φ_w) play an important role. On the other hand, intrinsic *TE* is highly reactive to the diurnal variation in irradiance, temperature, *VPD*, CO_2 concentration. Other influencing factors are summarized in Figure 8. Sometimes these factors become so important that we are unable to evidence any correlation between intrinsic and whole plant *TE* (e.g. Sun *et al.* 1996 for white spruce). Thus, such relationships must be verified. A first step was taken in this study by testing this upscaling approach under controlled environmental conditions and under different evaporative demands, i.e., different vapour pressure deficits (*VPD*).

2.2. ^{13}C discrimination as an estimator of intrinsic *TE*

In nature, there are two isotopes of carbon, the lighter isotope ^{12}C (98.9%) and the heavier isotope ^{13}C (1.1%). The composition in ^{13}C *($\delta^{13}C$)* is expressed with respect to a tandard, namely Pee Dee Belemnite.

$$\delta^{13}C = (R_{sample} / R_{standard} - 1)1000 \tag{4}$$

where R_{sample} and $R_{standard}$ are the ^{13}C/^{12}C ratio in a sample and in the standard (Pee Dee Belemnite), respectively. $\delta^{13}C$ values are negative as organic matter always contains less ^{13}C than the standard (Farquhar & Richards 1984). Isotopic discrimination (*$\Delta^{13}C$*) is defined as the isotopic composition of the plant material with respect to [th] of the air on which the plant has fed:

$$\Delta^{13}C = \left(\frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1000 + \delta^{13}C_{plant}} \right) \times 1000 \tag{5}$$

where $\delta^{13}C_{air}$ and $\delta^{13}C_{plant}$ are the isotopic composition of air and sample, respectively. The two traits (*$\delta^{13}C$* and *$\Delta^{13}C$*) are negatively related to each other. In C_3 plants, this discrimination is partly caused by the diffusion of CO_2 from the atmosphere into the intercellular spaces (*a*) and
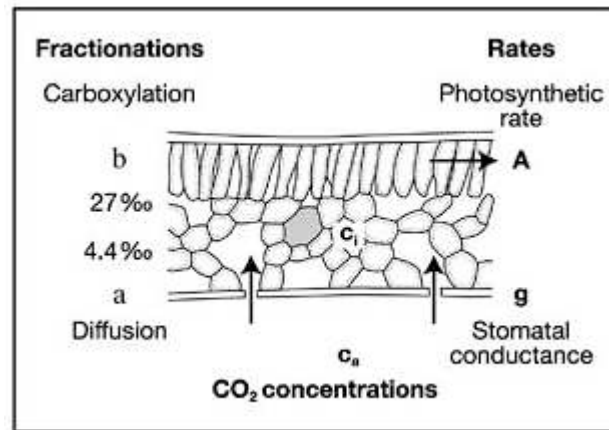
Figure 9. Diagram showing the main fractionation factors that contribute to the carbon isotope discrimination during photosynthesis (*a* and *b*); modified from McCarroll &Loader 2004).
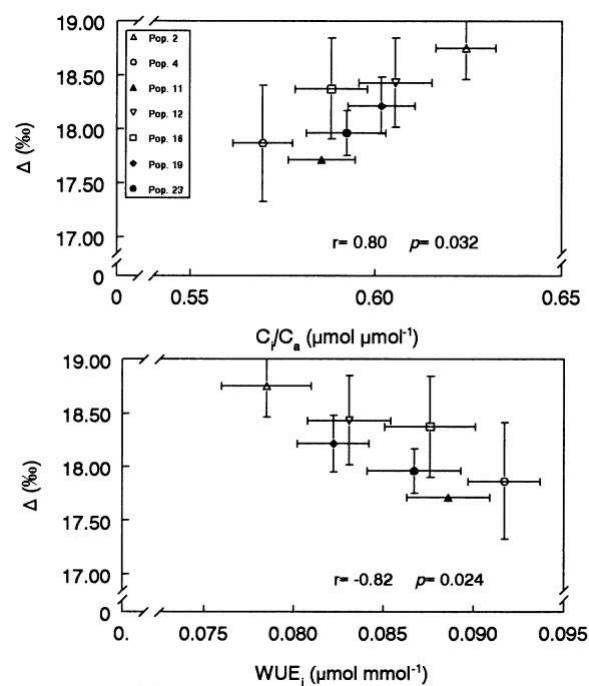
Figure 10. Carbon isotope discrimination (Δ) displays a positive correlation with the ratio of internal to ambient CO_2 concentration (C_i/C_a) and a negative one with intrinsic *TE* (WUE$_i$) in western red cedar. Changes in both traits were induced by changes in *VPD*. Reproduced from Grossnickle *et al.* 2005

the initial reaction of carboxylation (mainly ribulose-1,5-bisphosphate carboxylase (Rubisco, b). Other fractionation processes are also involved like the dissolution and the diffusion of CO_2 in the liquid phase, photorespiration and respiration during the day (Farquhar *et al.* 1988; Farquhar *et al.* 1989). Initially, the effect of these fractionation factors on $\Delta^{13}C$ values was considered to be small; but there is now a consensus that they are significant (Brugnoli & Farquhar 2000; Ghashghaie *et al.* 2003). Farquhar *et al.* (1982) established a discrimination model linking ^{13}C discrimination of CO_2 during photosynthesis and the C_i/C_a ratio (the mole fractions of CO_2 in the intercellular spaces and in the atmosphere), which is inversely related to A/g_s.

$$\Delta^{13}C = a + (b - a)\frac{C_i}{C_a} \qquad\qquad (6)$$

where a is the discrimination against $^{13}CO_2$ during diffusion through stomata (4.4‰) and b is the discrimination at the time of carboxylation; b varies between 27- 30‰; Warren 2006 (Figure 9).

Given the relationship between $\Delta^{13}C$ and C_i/C_a and between A/g_s and C_i/C_a (Equation 4 and 1 respectively), for C_3 plants, $\Delta^{13}C$ is related negatively to A/g_s and positively to C_i/C_a (Farquhar & Richards 1984). The theoretical relationship between A/g_s and $\Delta^{13}C$ was validated experimentally in trees (see Figure 10), in intra-specific and several other studies (Zhang *et al.* 1994; Lauteri *et al.* 1997; Roupsard *et al.* 1998; Cregg *et al.* 2000; Grossnickle *et al.* 2005). In oaks, Ponton *et al.* (2002) showed a negative relationship between A/g_s and $\Delta^{13}C$. However, some studies failed to obtain this relationship, such as in white spruce (Sun *et al.* 1996), in sessile oak (Picon *et al.* 1996) and in six lentil genotypes (Turner *et al.* 2007). The lack of relationship between $\Delta^{13}C$ and A/g_s is often due to (i) time scale integration; instantaneous measurements of gas exchange (A/g_s) being highly sensitive to variable environmental conditions; (ii) variation in the quantity and turn-over rate of different leaf metabolites; some metabolites being enriched and others depleted in ^{13}C (addressed in detail in the following discussion).

In many earlier studies, a shift was observed between predicted (using Equation 6) and observed values of discrimination. The magnitude of this shift differed between species as has been summarized in Figure 11. It is well established now that this under-estimation is due at least partly to limitations in the pathway of CO_2 diffusion from sub-stomatal cavities to the sites of carboxylation inside the chloroplast (often referred to as mesophyll conductance; g_m), which is not taken into account in the simple discrimination model. These limitations imply that $C_i >$ C_c. Several methods have been used to estimate g_m, which includes online
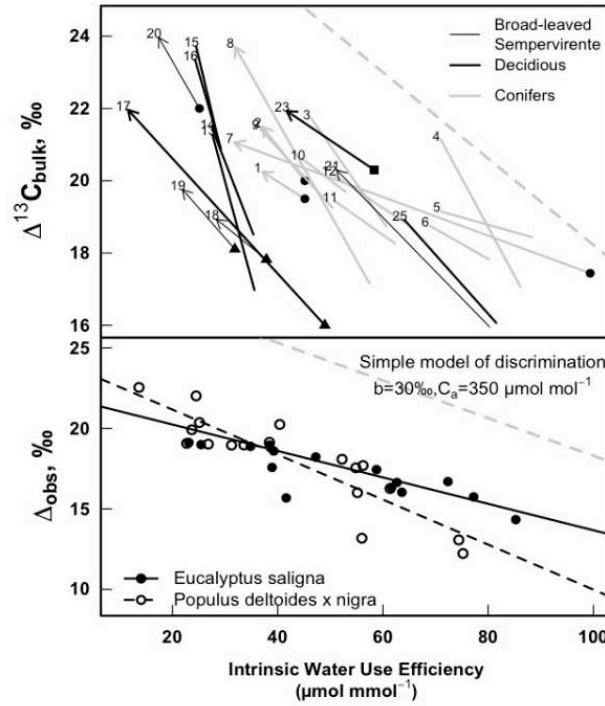
Figure 11. Upper panel: relationships between intrinsic *TE* calculated as A/g_s in μmol CO_2 mol^{-1} H_2O and $\Delta^{13}C$ (in ‰) measured on leaf bulk matter in different species. The dashed grey line is the relationship predicted by the simple Farquhar model of discrimination (Equation 6), with b=30‰ and C_a=400 μmol mol^{-1}

Lower panel: Relationship between $\Delta^{13}C$ measured with online discrimination and intrinsic *TE* (A/g_s) for *Eucalyptus saligna* leaves (filled circle, each is an average of $\Delta^{13}C$ and W_i recorded during 15 min) and *Populus deltoides* x *nigra* cuttings (empty circle, each is an average of $\Delta^{13}C$ and W_i recorded during 30-45 min). The dashed grey line is the relationship predicted by the simple model of discrimination, with b=30‰ and C_a=350 μmol mol^{-1}(i.e. the averaged C_a during the experiments). Reproduced from Cyril Douthe dissertation 2010).
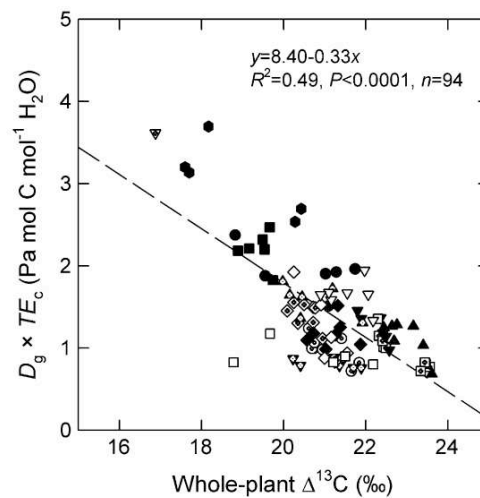
Figure 12. The product of whole plant *TE* of C gain (TE$_c$) and daytime vapour-pressure deficit of ambient air (D$_g$) plotted against whole-plant ^{13}C discrimination. Different symbols refer to different species. Reproduced from Cernusak *et al*. 2008.

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measurement of ^{13}C discrimination and A/g_s (from gas exchange measurements) or comparing A/g_s with the isotopic signatures recorded in the primary photosynthetic product (soluble sugars in leaves, Brugnoli *et al.* 1988). These limitations impose a significant slow-down to the diffusion of CO_2 inside the leaf. They are variable among genotypes (Barbour *et al.* 2010). By combining the $^{13}C/^{12}C$ fractionation associated to respiration and the diffusion of CO_2 through the mesophyll layers and assuming that these effects are either constant or negligible, Farquhar & Richards (1984) produced a complete description of $\Delta^{13}C$, which can be represented as follows:

$$\Delta^{13}C = a_b \frac{C_a - C_s}{C_a} + a \frac{C_s - C_i}{C_a} + (b_s + a_l)\frac{C_i - C_c}{C_a} + b\frac{C_c}{C_a} - f\frac{\Gamma^*}{C_a} - e'\frac{R_d}{A + R_d}\frac{C_c - \Gamma^*}{C_a} \quad (7)$$

where a_b (2.9‰) and a_l (0.7‰) are the fractionation associated with the diffusion through leaf boundary layer and leaf water. b_s is the fractionation as CO_2 moves in to the solution which is taken as 1.1‰ at 25°C. c_s and c_c are the CO_2 concentration at the leaf surface and at the site of carboxylation respectively. e' and f are the fractionation associated to mitochondrial respiration during the day and to photorespiration respectively. Γ^* is the CO_2 compensation point in the absence of non-photorespiratory respiration (R_d). Thus we can weight the fractionation during carboxylation as a ratio between CO_2 concentrations in the chloroplast to ambient air (C_c/C_i) rather CO_2 concentrations in the intercellular spaces to ambient air (C_i/C_a).

Instant *TE* at leaf level and $\Delta^{13}C$ can be extrapolated to the whole plant scale. Indeed, by combining equations 3 and 6, we obtain a theoretical relationship between $\Delta^{13}C$ and whole *TE*. Cernusak *et al.* (2008) found a negative correlation between $\Delta^{13}C$ and whole plant *TE* in tropical tree species (see Figure 12). The same way as for the relationship between instant and whole plant *TE*, factors (evaporative demand, Φ_c, Φ_w) may intervene at times and can result in lack of correlation between *TE* and $\Delta^{13}C$ (Guehl *et al.* 1995; Matzner *et al.* 2001; Ripullone *et al.* 2004; Turner *et al.* 2007). It is therefore important to verify whether variability of $\Delta^{13}C$ among poplar genotypes reflects the variability in whole plant *TE*.

2.3. Other sources of variation in ^{13}C signals: isotopic signature of different organic compounds

^{13}C isotope composition ($\delta^{13}C$) can be analysed for different compounds or organs. At both ends of time scales from instant values of A/g_s and the isotopic composition of total organic matter, there is a long chain of physiological and biochemical processes. Each of these processes is likely to add additional isotopic fractionation and thus may cause changes in the carbon isotope signature (for a review see Bowling *et al.* 2008 and figure 13).
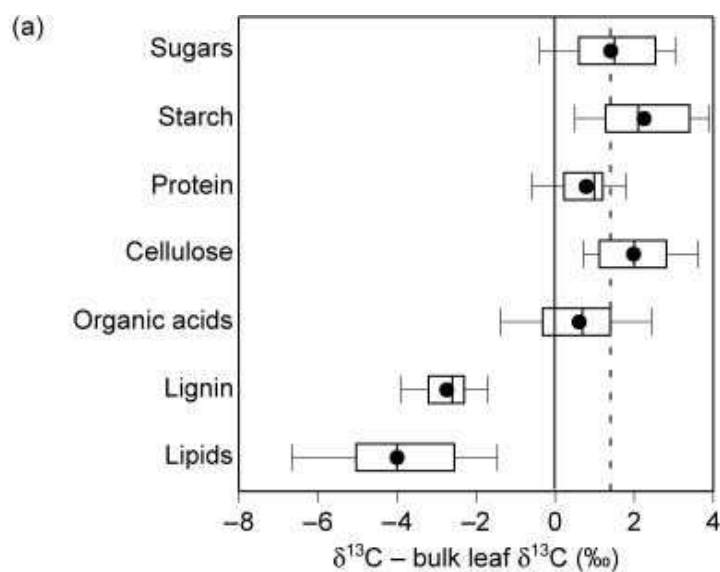
Figure 13. An update from Boutton (1996) comparing the isotopic composition of compounds isolated from leaves of C_3 plants, expressed relative to bulk leaf biomass. The boxes encompass the upper and lower quartiles of the data, the line shows the median, the symbol shows the arithmetic mean, and the error bars show the upper and lower 10th percentiles of the data. The vertical dashed line is included for easy reference to the ^{13}C of leaf sugars. Reproduced from Bowling *et al*. 2008

Plant organs consist of complex mixtures of molecules with different isotopic signatures. Leaves and stems are composed of cellulose, hemicellulose, lignin and many other compounds. These molecules form a stable pool of carbon accumulated over leaf growth. The second reservoir of carbon molecules displays a rapid turnover, and includes soluble sugars and starch. The isotopic composition of total organic matter is the sum of that of each of these pools. Any discrimination and the use of certain substrates enriched or depleted in ^{13}C during the process of mitochondrial respiration may contribute to alter the isotopic signature of leaf matter.

In addition, the isotopic composition of organic matter also depends on the discrimination involved in the respiratory and photorespiratory processes (for a review, Ghashghaie *et al*. 2003). During photorespiration, the CO_2 released is depleted in ^{13}C, and this affects the discrimination observed during measurements of gas exchange. The impact of photorespiration on the isotopic composition of the plant is considered to be small (Douthe *et al.* 2012). Some authors concluded that non-photorespiratory respiration does not significantly affect the isotopic composition of the leaf (Lin & Ehleringer 1997), as hypothesised by Farquhar *et al.* (1982, 1989), Farquhar & Richards (1984) and Farquhar & Lloyd (1993). However, studies with *Phaseolus vulgaris* L. (Duranceau *et al.* 2001), *Nicotiana sylvestris* and *Helianthus annuus* (Ghashghaie *et al.* 2001) invalidated the previous statements: the CO_2 released during non-photorespiratory respiration can be enriched with respect to organic matter by 6‰ as also shown by Xu *et al.* (2004). However this fractionation caused by non-photorespiratory respiration varies according to species, stage of plant development and with environmental conditions including drought (Ghashghaie *et al.* 2001; Xu *et al.* 2004).

Differences in the ^{13}C signature of different organs (Brugnoli & Farquhar 2000; MacFarlane *et al.* 2004; Peuke *et al.* 2006; Cernusak *et al.* 2007a,b), and changes during leaf development (Holtum & Winter, 2005) and along the growing season (Jaggi *et al.* 2002; Keitel *et al.* 2003) have also been observed. The isotopic discrimination between atmospheric CO_2 and leaf soluble sugars (glucose, fructose and especially sucrose), the first products of photosynthesis, reflect an integration of gas exchange over a few days (Brugnoli *et al.* 1988; Brugnoli & Farquhar 2000). ^{13}C signature of starch is an integrator in the medium term (approximately one week, Jaggi *et al.* 2002). The analysis of components with longer life spans, such as cellulose, leaves, helps to integrate isotopic discrimination during a much larger period (formation period of the leaf, or different types of wood). Moreover, post-photosynthetic fractionation results in changes of isotopic signals depending on compound family (Brugnoli & Farquhar 2000; Badeck *et al.* 2005, Bowling *et al.* 2008). Thus, the relative quantity of these

molecules present in bulk material can significantly influence the overall ^{13}C signal and deconnect it from the values expected from A/g_s. Thus, care must be taken while interpreting the results. One way of dealing with this potential discrepancy is the extraction of single components close to the photosynthetic process.

2.4. Use of oxygen isotopes as estimator of stomatal conductance

It is of importance to identify the relative contribution of A and of g_s to the variability of intrinsic TE ($=A/g_s$). The use of ^{13}C alone brings no clue to this question. Analysis of the ^{18}O isotopic composition ($\delta^{18}O$) of leaf water and leaf organic matter is another tool that might help overcome this limitation. It can indeed estimate g_s independently of A, and thus can be used to differentiate the changes in $\delta^{13}C$ due to A or to g_s.

2.5. ^{18}O enrichment in the leaf

There are three stable isotopes of oxygen: ^{16}O, ^{17}O and ^{18}O. ^{16}O is the most abundant (99.76%), followed by ^{18}O (0.20%). ^{18}O isotopic composition ($\delta^{18}O$) of a sample represents the $^{18}O/^{16}O$ ratio of the sample with respect to that of the international standard V-SMOW (Vienna Standard Mean Ocean Water).

$$\delta^{18}O = \left[\left(\frac{R_{sample}}{R_{s\tan dard}} \right) - 1 \right] \times 1000$$

$$(8)$$

where R_{sample} and $R_{standard}$ are the $^{18}O/^{16}O$ ratios in a sample and standard (Vienna-Standard Mean Ocean water). Isotopic discrimination in the plant material is then estimated as:

$$\Delta^{18}O_{sa} = \left(\frac{\delta^{18}O_{sw} - \delta^{18}O_{sa}}{1000 + \delta^{18}O_{sa}} \right) \times 1000 \qquad (9)$$

where $\Delta^{18}O_{sa}$ represents the enrichment of the plant material (leaf bulk or leaf water) with respect to the source water ($\delta^{18}O_{sw}$).

The enrichment of total leaf water ($\Delta^{18}O_{lw}$) with respect to source water depends on various factors. First, ^{18}O isotopic composition of soil water (or source) reflects the average ^{18}O composition of precipitation, and is influenced by the direct evaporation from the soil surface. No isotopic fractionation is expected as the water moves into the roots and from the roots towards the other parts of the plants (leaves). Thus, the isotopic composition of xylem water and the water flowing through the petiole is assumed to be the same than that of the soil water (Sternberg *et al.* 2003; Gan *et al.* 2002). Leaf water is enriched with respect to xylem water. This enrichment occurs at the sites of evaporation as $H_2^{16}O$ evaporate slightly faster than $H_2^{18}O$.

2.5.1. ^{18}O enrichment of leaf water at the sites of evaporation

The enrichment of water at the sites of evaporation ($\Delta^{18}O_e$) with respect to source water is described by following model (Craig & Gordon, 1965; Dongmann *et al.* 1974):

$$\Delta^{18}O_e = \varepsilon_k + \varepsilon^* + (\Delta^{18}O_v - \varepsilon_k)\frac{e_a}{e_i} \tag{10}$$

where $\Delta^{18}O_v$ is isotopic composition of water vapour ($\Delta^{18}O_v = \delta^{18}O_v - \delta^{18}O_{sw}$; $\delta^{18}O_{sw}$ is the isotopic composition of the source water).

ε_k is the kinetic fractionation as water vapour diffuses through the stomata and the boundary layer and is calculated by the following equation:

$$\varepsilon_k = \frac{32r_s + 21r_b}{r_s + r_b} \tag{11}$$

where r_s and r_b are stomatal and boundary layer resistance to water flux (= $1/g_s$ and $1/g_a$), respectively.

ε^* is the fractionation associated with phase change from liquid water to water vapour and is sensitive to temperature. ε^* (‰) is calculated following Majoube (1971):

$$\varepsilon^* = \exp\left(\frac{1.137}{T^2} - \frac{0.4156}{T} - 0.0020667\right) - 1 \tag{12}$$

where T is in K;

Computations show that ε^* is 9.1‰ at 25°C and 9.5‰ at 20°C.

e_i is the mole fraction of water vapour in the intercellular spaces (mmol H_2O mol^{-1} air)

$$e_i = 10*(6.1078*\exp^{(17.2694*\theta)/(237.2+\theta)})/p \tag{13}$$

where θ is leaf temperature in ° C and p, the atmospheric pressure (mbar)

e_a is the mole fraction of water vapour in the atmosphere (mmol H_2O mol^{-1} air)

where $e_a = e_i * 100/RH$ with RH, relative humidity (%)

given that v is the difference between e_i and e_a, e_a/e_i can be replaced with ($1-v/e_i$)

$$\Delta^{18}O_e = \varepsilon_k + \varepsilon^* + (\Delta^{18}O_v - \varepsilon_k)(1 - v/e_i) \tag{14}$$

thus $\Delta^{18}O_e$ is predicted to increased with increased v.

Enriched water is likely to flow back inside the leaf and dilute the expected ^{18}O signals of the leaf water. This process called "Péclet effect" (Farquhar & Lloyd 1993) was described as a continuous isotopic gradient in the leaf, with maximum enrichment at the sites of evaporation, and exponentially decreases until it reaches the isotopic signature of the source water ($\delta^{18}O_{sw}$) near the veins. The ^{18}O enrichment of mesophyll water ($\Delta^{18}O_L$) is also connected to $\Delta^{18}O_e$ (Farquhar & Lloyd 1993) by the following equation:

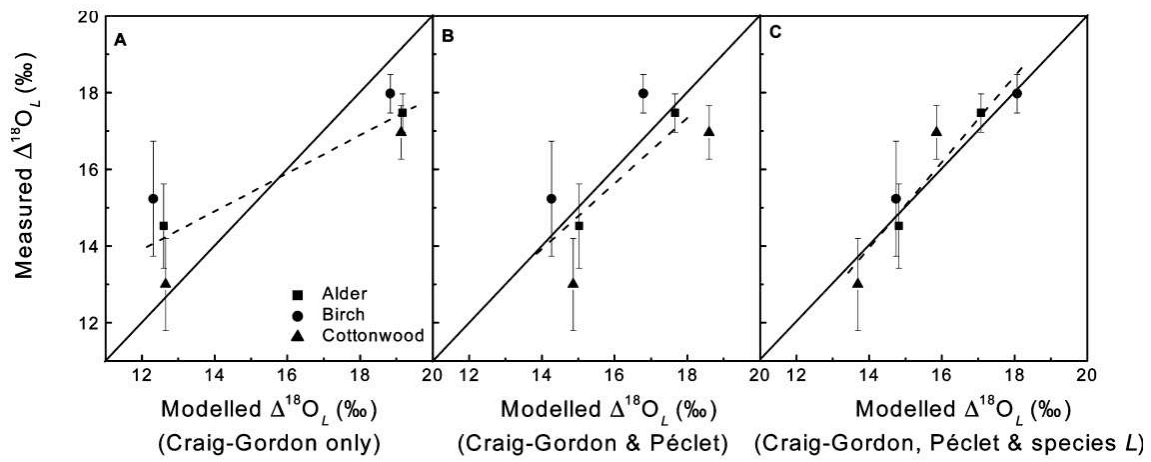**Figure 14**. The relationship between measured and modelled laminar mesophyll water enrichment (Δ_L) for tree species grown at high and low humidity: (A) the Craig-Gordon model, (B) the Péclet effect model using a single fitted effective length, and (C) the Péclet effect model using effective lengths for Alder, Birch and cottonwood. Reproduced from Barbour *et al.* 2004.

$$\Delta^{18}O_L = \Delta^{18}O_e(1-e^{-\wp})/\wp \qquad (12)$$

or $\Delta^{18}O_L = \delta^{18}O_L - \delta^{18}O_{sw}$, or $\delta^{18}O_L$ is the isotopic composition of water from the mesophyll (‰) and $\wp$ the Péclet number:

$$\wp = \frac{EL}{CD} \qquad (15)$$

where $E = g_s * \nu$ with ν the difference in mole fraction water vapour between the atmosphere and the intercellular spaces ($\nu = e_i - e_a$, in mmol H_2O mol air^{-1});

-L is the effective distance between the sites of evaporation and veins; L varies between 4 to 166 mm, after Wang *et al.* (1998);

-C is the molar concentration of water (55.5 mol m^{-3});

-D is the diffusivity of water $H_2^{18}O$ (2.66×10^{-9} m^2 s^{-1} ; Barbour *et al.* 2004).

Since leaf water is a mixture in varying proportions of depleted and enriched water from different pools, the enrichment of mesophyll water (or total leaf water, $\Delta^{18}O_L$) is lower than the enrichment observed at the sites of evaporation ($\Delta^{18}O_e$) (Farquhar & Lloyd 1993). This discrepancy was explained by the factor $\wp$, which depends on the effective distance between the sites of evaporation and veins (L; Figure 14). The actual distance between the veins and the stomata is about 100 μm for most of the leaves. L depends on the route by which water passes (symplasmic through the plasmodesmata, transcellular through aquaporins or apoplasm), but also of the site from which it is evaporated (intercellular spaces, sub-stomatal cavities or cells surrounding the cavities sub-stomatal and stomatal) (Barbour & Farquhar 2003). The factor $\wp$ is also determined by transpiration (E). However, E is defined as the product of stomatal conductance to water vapour (g_s) and the vapour pressure deficit between the atmosphere and the intercellular spaces (ν). When the changes in E are caused by changes in ν while g_s is constant, the relationship between $\Delta^{18}O_L$ and E (and hence ν) is positive. On the other hand, where ν is constant and that changes in g_s are a cause of changes in E, then the relationship between $\Delta^{18}O_L$ and E (and thus g_s) is negative (Farquhar *et al.* 2007). This negative relationship is caused by different processes: an increase in g_s has the effect of reducing the kinetic fractionation during diffusion of water vapour through the stomata and boundary layer (ε_k), which leads to a decrease in $\Delta^{18}O_e$. In addition, when the stomata close and transpiration decreases, leaf temperature increases and thus e_i, which also results in a decrease in $\Delta^{18}O_e$ and vice versa. The change in temperature can also influence the fractionation factor bounded to the equilibrium phase: changes from liquid water to water vapour (ε^*), but the impact on $\Delta^{18}O_e$ are low.

In addition to this heterogeneity, diurnal cycles of ^{18}O isotopic enrichment of leaf water ($\Delta^{18}O_L$) were detected in lupine ($\Delta^{18}O_L$ ranging from 0 to 25 ‰; Cernusak *et al.* 2002), eucalyptus ($\Delta^{18}O_L$ between 6 and 20 ‰; Cernusak *et al.* 2005) or Scots pine ($\Delta^{18}O_L$ ranging from 4 to 25 ‰; Barnard *et al.* 2007).

Studies that compared observed and theoretical values of $\Delta^{18}O_L$ found a good correspondence between the two (Cernusak *et al.* 2002; Cernusak *et al.* 2005; Barnard *et al.* 2007; Gessler *et al.* 2007). So, despite these variations in time and space, the isotopic signature of ^{18}O in leaf water can be used as a tool for estimating stomatal conductance if the other parameters of the equation are known (water vapour deficit between the leaf and atmosphere, path length from veins to evaporation sites). This model makes it possible to access an instantaneous measurement of g_s. The use of ^{18}O isotopic signature of organic matter can, in turn, access to a more time-integrated status of g_s (Barbour 2007).

2.5.2. ^{18}O enrichment of leaf organic matter

The oxygen present in leaf organic matter is derived from three sources: CO_2, O_2 from the air and leaf water (Schmidt *et al.* 2001). However, leaf water is the most important source of oxygen. Due to transpiration, ^{18}O of leaf water gets enriched. There is also a fractionation during biosynthetic reactions, resulting in the formation of various organic molecules. ^{18}O isotopic composition of organic compounds is variable since, during their biosynthesis, the oxygen atoms are introduced by different mechanisms that have different fractionation effects. Once these compounds are synthesized, some of their chemical functions are likely to exchange oxygen with water in which they are transported or stored. Organic matter has an ^{18}O signature, which reflects both the source ($\delta^{18}O_{sw}$), and that of leaf water enrichment that varies with transpiration, and in some cases, with stomatal conductance. The isotopic signature of leaf organic matter integrates the signals from molecules formed from various biosynthetic pathways. The ^{18}O enrichment of total leaf organic matter is therefore interesting as it integrates g_s over longer periods of time (leaf life-span).

Given the spatial and temporal heterogeneity of the composition of the source water ^{18}O, ^{18}O signature of leaf organic matter (soluble or total) is supposed to reflect these changes. Indeed, several studies show diurnal cycles of leaf soluble organic matter enrichment (taken from phloem) and total leaf (Cernusak *et al.* 2002; Gessler *et al.* 2007). However, these diurnal variations are smaller (4 ‰ difference between maximum and minimum) than those of leaf water (about 10-20 ‰). In some cases, no variation in $\Delta^{18}O_{lb}$ is observed during the day (Gessler *et al.* 2007). Variations in g_s caused by soil factors (water stress; Cernusak *et al.* 2003),
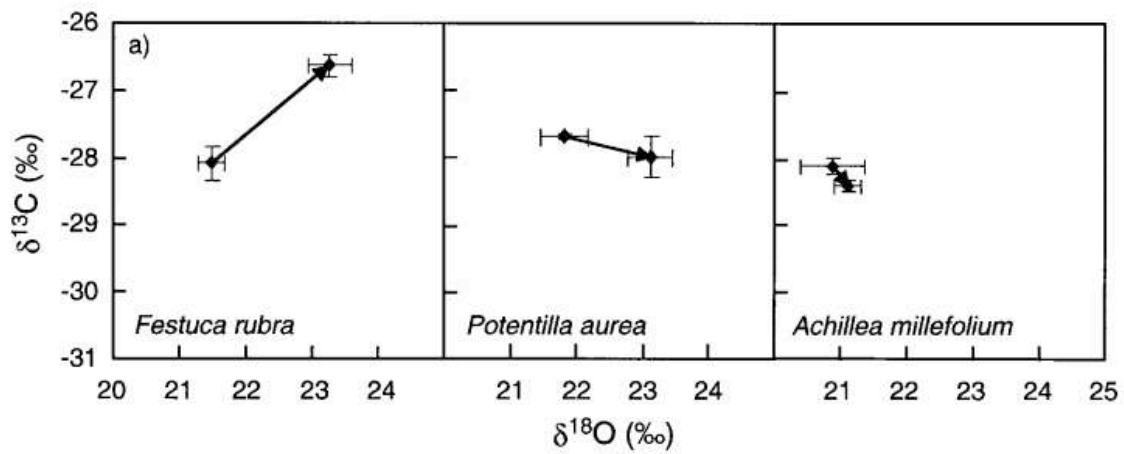
Figure 15. The $\delta^{18}O$-$\delta^{13}C$ relationship for the organic matter of *F. rubra, P. aurea* and *A.* (±SE, *n*=6). Reprinted from Scheidegger *et al.* 2000.

by addition of stomatal closure signals (abscisic acid, Barbour & Farquhar 2000) or due to genetic affects (Barbour *et al.* 2000) affect the ^{18}O isotopic signature of organic matter (Farquhar *et al.* 1998). Given that g_s varies with time, using the ^{18}O signature in the leaf water and leaf bulk is a tool that could prove to be very useful, since it could be used as an indicator of instant and time-integrated stomatal conductance.

2.6. Dual isotopic approach (^{13}C and ^{18}O)

The ^{18}O isotopic enrichment in leaf water and leaf organic material is a function of transpiration rate, which is strongly related to g_s when the leaves are under the same conditions of vapour pressure deficit between the atmosphere and the intercellular spaces (v). Thus, isotope variations of ^{18}O can be used to differentiate whether differences in intrinsic *TE* (A/g_s) are due to differences in g_s or in A (Roden & Farquhar 2012). Figure 15 recapitulates the three different kinds of relations that can be observed while using ^{18}O to differentiate the relative effect of g_s and A on ^{13}C (Scheidegger *et al.* 2000).

(I)   When the slope of the relationship between $\Delta^{13}C$ and $\Delta^{18}O_L$ is close to zero: the changes in $\Delta^{13}C$ are caused predominantly by the variation in A rather than g_s, since changes in A have no effect on ^{18}O enrichment, while on the other hand an increase in A would decrease $\Delta^{13}C$.

(II)   When $\Delta^{13}C$ and $\Delta^{18}O_L$ are negatively correlated with a sharp slope: the changes in $\Delta^{13}C$ are predominantly due to g_s, as an increase in g_s would result in decreased ^{18}O enrichment and increased $\Delta^{13}C$ mainly due to g_s.

(III)   When $\Delta^{13}C$ and $\Delta^{18}O_L$ are negatively correlated but with a shallow slope: in this case, changes in $\Delta^{13}C$ are caused by A and g_s simultaneously. As increasing g_s, would decrease ^{18}O enrichment but increase in $\Delta^{13}C$ would be both due to A and g_s and would result in higher variation on its axis.

This dual approach isotope can be very helpful in analysing the causes of variability in the water-use efficiency. However, to date only few studies have used the dual isotope approach through comparisons of genotypes in intra-specific and non-limiting conditions. Barbour & Farquhar (2000) studied variation in carbon and oxygen isotope ratio in cotton leaves induced due to different relative humidity and ABA application and found that the enrichment was
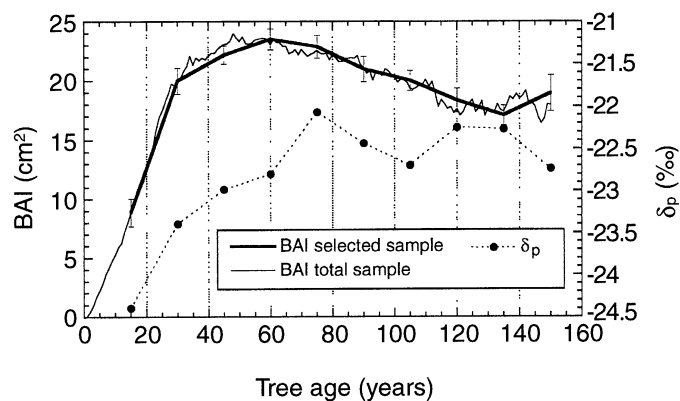
Figure 16. ^{13}C content in cellulose recorded in tree rings (δ^{13}C) and basal area increment recorded during the period from 1940-1988 in *Abies alba* showing the effect of tree age using synchronic approach. Reproduced from Bert *et al.* 1997
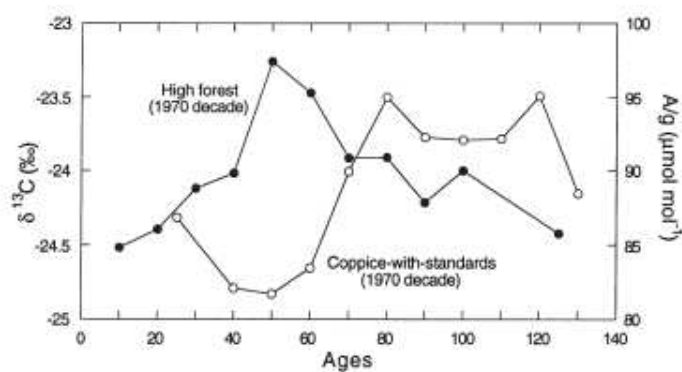
Figure 17. δ^{13}C recorded along with mean corresponding *A/g$_s$* showing an age effect. Two different silviculture systems were studied: high forest (closed circles) and coppice with standard (open circles). Reproduced from Duquesnay *et al.* 1997

negatively related to g_s due to increase in leaf temperature with in each treatment and increased under higher low relative humidity.

2.7. Age effect and potential causes

One of the key questions facing tree-ring isotopic research is whether there are age-related trends in the isotope ratios obtained from tree rings. Several studies using individual trees have reported age-related trends in the carbon isotope ratio (e.g. Bert *et al.* 1997; McCarroll & Pawellek 2001, Figure 16). Duquesnay *et al.* (1998) reported a clear age effect ($\delta^{13}C$ increasing with age) in high forest beech trees by sampling trees in different age classes for the same years (Figure 17). Several interpretations have been formulated to explain this age-related trend: a first interpretation refers to recycling of respired air, already depleted in ^{13}C by young trees growing close to the forest floor (Schleser & Jayasekera 1985). Decreasing contribution from bark photosynthesis was suggested as potentially responsible for this trend as photosynthates from the bark refix respired CO_2 (Cernusak *et al.* 2001). An alternative explanation relates this decreasing trend with age to changes in the hydraulic conductance of trees as they age (Ryan & Yoder 1997; McDowell *et al.* 2002). This decrease in hydraulic conductance was held responsible for the decrease in leaf water potential as the tree height increases. Thus, this decline in hydraulic conductance results in declines in leaf-water potential, and in lower stomatal conductance as trees grow higher, which would eventually have a marked effect on ^{13}C discrimination. Schafer *et al.* (2000) have detected a declining stomatal conductance in beech trees (*Fagus sylvatica*) with tree height. Monserud & Marshall (2001) correlated carbon discrimination with tree height and showed clear negative trends in two pine species (*Pinus moniticola* and *Pinus ponderosa*). However, no such trend was found for Douglas fir (*Pseudotsuga menziesii*), but for the same species, McDowell *et al.* (2002) have shown a clear trend of declining discrimination and stomatal conductance for three height classes. Indeed, hydraulic conductivity can be estimated from measurements of wood anatomy in both angiosperms and conifers (Ponton *et al.* 2001; McDowell *et al.* 2002, 2004), but it remains complicated to scale from the conductivity of stem tissues to whole tree conductance. Thus, potential for modelling changes in stomatal conductance as trees ages might be helpful.

To date, there is insufficient isotope data available from individual trees to determine whether this age effect is similar among species or not. This question becomes even more important in the case of poplar, which is a short rotation tree species and the cultivars that are commercially available are genetically closely related. Thus, any change in the age effect pattern among genotypes could result in a change in genotype ranking for $\Delta^{13}C$ and needs to be

investigated. Till now studies have suggested that age effect result in a negative trend in $\Delta^{13}C$ with tree age. More data are required from individual trees of varying age and longevity, before this question can be fully addressed.

2.8. Sampling strategies to detect potential age effect

Due to the recent advances in the tree ring isotopic research, physiological controls responsible for the variation in tree rings isotope ratios are reasonably well understood. Thus, a retrospective study of these isotopic ratios, noticeably of $\delta^{13}C$, can be used to check long-term stability of the genotypic ranking in poplar for *TE*. These studies offer the opportunity to scale up the greenhouse observation to forest level tree plantations. Tree-ring isotopic data are rich in information. They contain trends and variability related to climate, age and recent natural declines in in ^{13}C in atmospheric CO_2. The aim of data treatments should be to remove the extraneous 'noise' without weakening the desired signal. This was found surprisingly difficult to achieve, thus a wide range of dendrochronological approaches has been used to get rid of other signals and maximizing the desired signal. In dealing with long term changes in tree physiological functions as recorded in the tree rings, we need to take into consideration that there are two types of effects recorded in tree rings: one is related to 'age' often known as the 'segment length curse' and the other is 'environmental'.

In this context of making a distinction between the effects due to "age" and "environment", there are two possible approaches to study age effects: "synchronic" and "diachronic" approaches. A diachronic approach is suitable for trees growing in common gardens. For this approach, tree cores are sampled from bark to pith from trees growing in common gardens and $\delta^{13}C$ signals is recorded along the tree cores. This approach relies on the asumptions that trees growing in common garden share a common environment, and that long-term $\delta^{13}C$ signals recorded along the radial core represent age-related trends under the given environment while they may also be due to long term environmental trends. A synchronic approach requires a very large sampling with trees of different ages, sometimes growing in quite different environments. In this approach the age related trend is separated from any environment trend as the $\delta^{13}C$ signal is recorded in tree rings build during the same year in trees with different ages. In this way the long-term environmental signal is minimized and the age related trend is maximized. In this approach, possible site-effects are limited by pooling the ^{13}C signals from different sites with different environments.
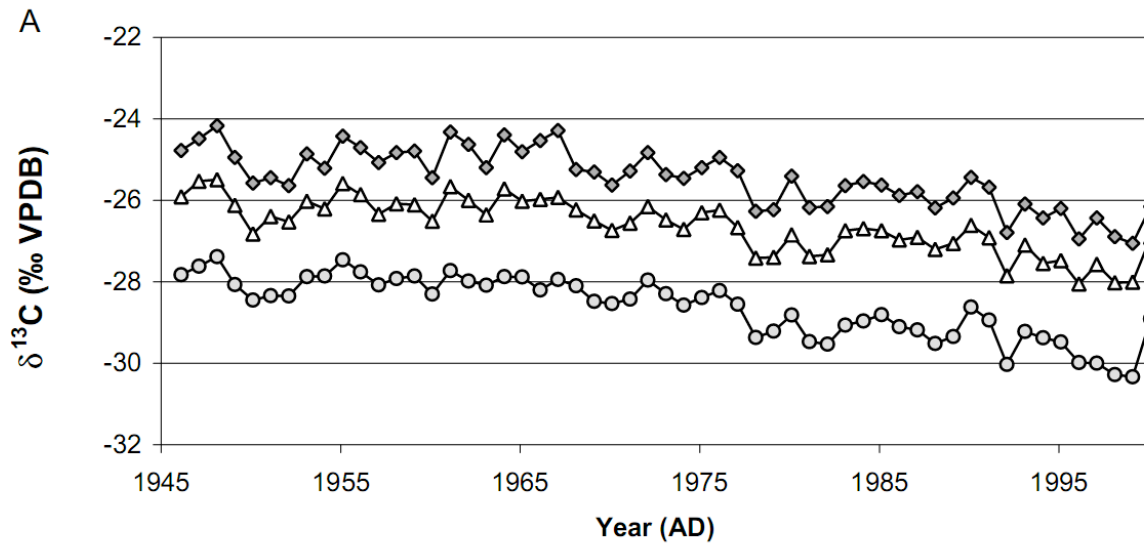
Figure 18. Carbon isotope series from a tree ring sequences in oak revealing a consistent cellulose - lignin offset of 3‰. ^{13}C composition in tree rings (δ^{13}C) as recorded from: cellulose (grey rhomboids), whole wood (white triangles) and lignin (grey circles). Reproduced from Loader *et al.* 2003.

2.9. Common problems in tree ring isotope studies

The study of stable isotopes in tree rings is facing other common problems apart of separating environmental from age effects. Presently, there are several rules that outline the basic principles of isotopic trends in tree rings.

2.9.1. Choice of a wood component for the isotope analysis

Early work on stable isotope ratios in tree rings used whole wood (Craig 1954; Farmer & Baxter 1974; Libby *et al.* 1976), but Wilson & Grinsted (1977) demonstrated that different components of wood differ isotopically (Figure 18). Wood is a complex material with a range of chemical components including cellulose, lignins, hemicelluloses, resins, tannins, etc. The biochemical processes implicated in the formation of each component from the initial photosynthates differ widely, and may include a diversity of metabolic steps inducing additional isotopic discrimination; as a consequence the stable isotope ratios of the resulting components may diverge significantly (Barbour *et al.* 2002; Loader *et al.* 2003). Presently, most studies concentrate on the analysis of cellulose, as the dominant and most easily isolated component of wood. The reasons for this shift away from wholewood towards α-cellulose are due to several important issues. Firstly, the cellulose of each annual increment ring is unambiguously linked to a specific growth period since it is cellulose that forms the primary cell walls in wood tissues. Secondly, extracting a single chemical component reduces problems associated with variability in the lignin:cellulose ratio that could occur between individuals or within a sequence through time. Third reason is the greater level of homogeneity that is attained by separating a single component from the others. Large isotopic variability has been demonstrated to occur within individual tree ring (Loader *et al.* 1995; Switsur *et al*. 1995; Schleser *et al.* 1999) consequently homogeneity of sample material remains an important consideration.

However, the extraction of cellulose is a limiting step due to the complex procedure required, so several authors have suggested considering again the use of wholewood (Leuenberger *et al.* 1998; Schleser *et al.* 1999; Barbour *et al.* 2001). Isolating a single chemical component of the wood removes the variability due to differences in the proportion of different components between rings and between trees, but this is not the main source of variability in isotopic ratios. On the contrary, there is clear evidence of a great deal of variability between trees (McCarroll & Pawellek 1998; Ponton *et al.* 2001). The $\delta^{13}C$ values obtained from lignin and cellulose are offset by about 3‰, with no apparent temporal trend (Loader *et al.* 2003;

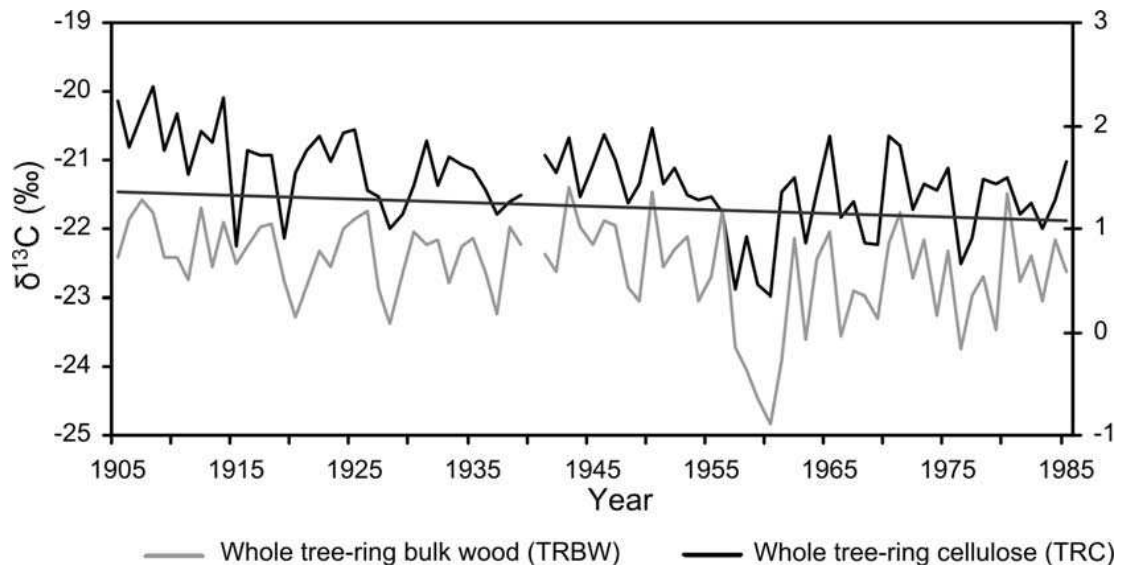**Figure 19**. Carbon isotope chronologies from tree-ring bulk wood and cellulose in *Pinus nigra spp* laricio. Grey line represents the offset between bulk wood and cellulose isotope values which decreased with time. Reproduced from Szymczak *et al.* 2011
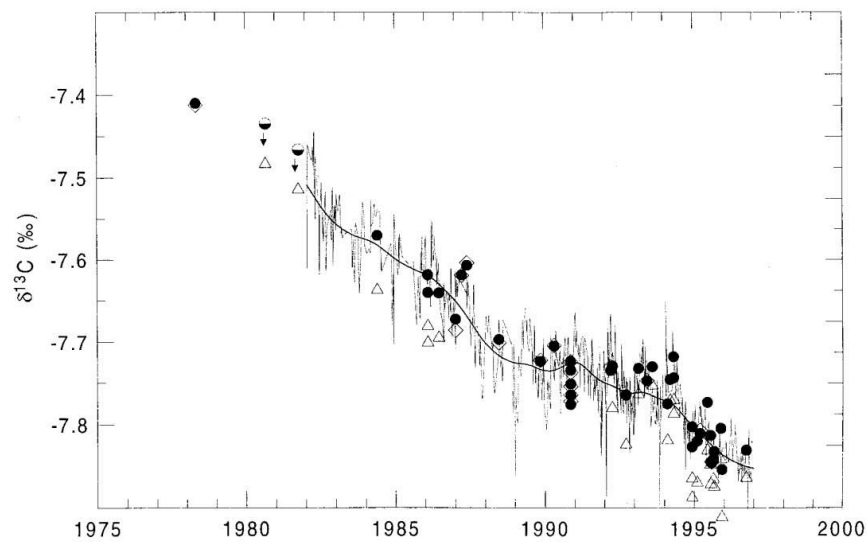
Figure 20. The $\delta^{13}C$ of CO_2 in the Cape Grim Air Archives (CGAA), which decreases with time. Reproduced from Francey *et al.* 1999.

Figure 18) and little evidence that isolating the cellulose significantly reduces the variability due to different wood components. Loader *et al.* (2003) found in oak tree-rings that the ^{13}C content of cellulose was more tightly correlated with climate variables than that of lignin, but that the strongest correlations were obtained using bulk wood. Borella & Leuenberger (1998) found no difference in the climate signals of cellulose and wholewood δ^{13}C for oak and beech, concluding that cellulose extraction is unnecessary in such a case.

Even if there is some doubt about whether there is a slight loss of signal when using wholewood rather than cellulose, this must be taken into consideration. In palaeoclimate studies the aim is to maximize the precision of the estimate of the mean, not the precision of the individual measurements. The mean offset in δ^{13}C between cellulose and wholewood is 1‰ (Loader *et al.* 2003) and the initial cellulose/lignin ratio is 2:1; however, this latter ratio is susceptible to change with age. Szymczak *et al.* (2011) showed in *Pinus nigra* ssp. *laricio* that the offsets in ^{13}C of bulk wood and cellulose are not constant over time (Figure 19). Thus, the results imply that extraction of cellulose is still a pre-requisite for the reconstruction of high-resolution long-term chronologies from stable isotope series and selection of cellulose in place of whole wood remains a critical choice.

2.9.2. Correcting chronologies for trends in atmospheric ^{13}C

Since the beginnings of industrialization, the burning of fossile fuels (coal, oil and gas) depleted in ^{13}C has increased the concentration of CO_2 in the atmosphere, and lowered the δ^{13}C value of air by about 1.5‰. Since fractionation is additive, this trend is reflected in tree rings and evidenced in most tree- ring δ^{13}C series (February & Stock 1999; Treydte *et al.* 2001). We now have reasonable annual estimates of the δ^{13}C of the atmosphere: ice cores provide air samples to estimate the $\delta^{13}C_{air}$ from early and preindustrial period. Francey *et al.* (1999; Figure 20) compiled a high precision record of atmospheric δ^{13}C based on Antarctic ice cores, which, for the purposes of correcting tree-ring data, can be summarized by two straight segments between 1850 and 1961, with an annual decline of 0.0044‰ and between 1962 and 1980 and a steeper annual decline of 0.0281‰ later on (McCarroll & Loader 2004). If the latter is extrapolated to the end of the last century the estimated values fit well with those obtained from firn (compacted snow) in the same area. The estimated value for the year 2000 is –7.99‰. This can further be extrapolated (annual decline of 0.0281‰) to obtain the δ^{13}C value of the atmosphere of a given year. Thus these data are considered to be sufficiently precise to provide a standard method for extracting the atmospheric decline in δ^{13}C from tree-ring data.

CHAPTER 1

Upscaling from leaf level to whole plant level transpiration
efficiency in 6 *Populus deltoides × nigra* genotypes

Transition

Previous studies on *Populus deltoides × nigra* genotypes presented large genotype diversity for $\Delta^{13}C$ both controlled condition (Marron *et al.* 2005) and under field conditions (Monclus *et al.* 2005). This genotype diversity for $\Delta^{13}C$ was shown to remain stable under moderate drought evidenced using Spearman's correlation coefficient (Monclus *et al.* 2006). Extrapolation of these results based on $\Delta^{13}C$ to whole plant *TE* remained an open question. Up scaling from leaf level to whole plant level *TE* can be very important to verify due to the underlying important traits like genotypic differences in carbon lost during photo and dark respiration (Φ_c) and water lost through nocturnal transpiration (Φ_w). Genotypic differences in these traits could result in shuffling in the ranking made for $\Delta^{13}C$. In this context, 6 *Populus deltoides × nigra* genotypes were selected representing contrasting $\Delta^{13}C$ properties (Monclus *et al.* 2006) and were grown under controlled condition. $\Delta^{13}C$ was measured in both leaf bulk and leaf soluble sugars with respect to atmosphere, along with leaf level *TE* (A/g_s) and whole plant *TE* (biomass produced/ water transpired). ^{18}O enrichment was also evidenced in leaf bulk and leaf water with respect to source water as an indicator for g_s.

Genotype differences in ^{13}C discrimination between atmosphere and leaf matter match differences in transpiration efficiency at leaf and whole-plant level in hybrid *Populus deltoides x nigra*.

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Running Title: ^{13}C as an estimator of whole-plant transpiration efficiency in poplar.

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ABSTRACT

^{13}C discrimination between atmosphere and bulk leaf matter $(\Delta^{13}C_{lb})$ is frequently used as a proxy for transpiration efficiency. Nevertheless, its relevance is challenged due to: (i) potential deviations from the theoretical discrimination model, and (ii) complex time integration and upscaling from leaf to whole-plant. Six hybrid genotypes of *Populus deltoids x nigra* genotypes were grown in climate chambers and tested for whole-plant transpiration efficiency (i.e., accumulated biomass/water transpired). Net CO_2 assimilation rates (A) and stomatal conductance (g_s) were recorded in parallel to: (i) ^{13}C in leaf bulk material ($\delta^{13}C_{lb}$) and in soluble sugars ($\delta^{13}C_{ss}$) and (ii) ^{18}O in leaf water and bulk leaf material. Genotypic means of $\delta^{13}C_{lb}$ and $\delta^{13}C_{ss}$ were tightly correlated. Discrimination between atmosphere and soluble sugars was correlated with daily intrinsic transpiration efficiency at leaf level (daily mean A/g_s), and with whole-plant transpiration efficiency. Finally, g_s was positively correlated to ^{18}O enrichment of bulk matter or water of leaves at individual level, but not at genotype level. We conclude that $\Delta^{13}C_{lb}$ captures efficiently the genetic variability of whole-plant transpiration efficiency in poplar. Nevertheless, scaling from leaf level to whole-plant transpiration efficiency requires to take into account water losses and respiration independent of photosynthesis, that remain poorly documented.

Key-words: intrinsic water use efficiency, transpiration efficiency, carbon isotope composition, leaf soluble sugars, oxygen isotope composition, leaf anatomy.

INTRODUCTION

Transpiration efficiency (*TE*), the major component of the efficiency of water-use by plants, is a complex trait that attracted much attention over the past decades. In particular, a large number of investigations aimed at detecting and quantifying the genetic variability of this trait (in trees for instance, Brendel *et al.* 2002; Cernusak *et al.* 2008; Monclus *et al.* 2005). *TE* at whole-plant level is the ratio between accumulated biomass and transpired water (Richards *et al.* 2002). At leaf level, *TE* is approached by intrinsic *TE* (W_i), i.e., the ratio of net CO_2 assimilation rate *(A)* vs. stomatal conductance to water vapour *(g_s)* (Condon *et al.* 2002). An indirect method of assessing intrinsic *TE* at leaf level (i.e., A/g_s) was proposed by Farquhar, O'Leary & Berry (1982) and relates discrimination against ^{13}C during photosynthesis *($\Delta^{13}C$)* to the ratio of CO_2 mole fraction in the substomatal cavity and in air *(C_i/C_a)* and therefore to intrinsic *TE*. In turn, $\Delta^{13}C$ is often assessed from the difference between the isotopic composition of source CO_2 in the air *($\delta^{13}C_{air}$)* and of bulk leaf matter *($\delta^{13}C_{lb}$)*. $\Delta^{13}C_{lb}$ has been widely used to evaluate the plastic response of species to changing environments, and the occurrence of an intra-specific, genetic variability in crops (Condon *et al.* 2004; Rebetzke *et al.* 2006) and in trees (Guehl *et al.* 1996; Lauteri *et al.* 1997; Roupsard, Joly & Dreyer 1998 among many others).

The relationship between intrinsic *TE* and $\Delta^{13}C_{lb}$ was validated experimentally during several intra-specific studies in trees (Zhang & Marshall 1994; Lauteri *et al.* 1997; Roupsard, Joly & Dreyer 1998; Cregg, Olivas-Garcia & Hennessey 2000; Grossnickle, Fan & Russell 2005). However, some experiments failed to detect this relationship (Sun *et al.* 1996, in white spruce; Picon, Guehl & Ferhi 1996, in sessile oak). Such a lack of relationship can be ascribed to problems with time integration as intrinsic *TE* is highly variable due to fluctuating environment (VPD, temperature, irradiance…) and subjected to measurement uncertainties

(Flexas *et al.* 2007), while $\Delta^{13}C_{lb}$ integrates discrimination over the whole leaf life span. One of the approaches to reduce such discrepancies is to record the isotopic signal in the primary photosynthesis products, soluble sugars ($\Delta^{13}C_{ss}$, Brugnoli *et al.* 1988; Lauteri, Brugnoli & Spaccino 1993; Kodama *et al.* 2008; Merchant *et al.* 2011) which provide a signal integrated over a single or at most a few days.

The simple model that relates $\Delta^{13}C$ and C_i/C_a takes into account fractionation due to: (i) CO_2 diffusion to chloroplasts and (ii) carboxylation by Rubisco. In this approach, mesophyll conductance to CO_2 (g_m; Evans *et al.* 2009) is taken as infinite. It is now established that g_m has a major effect on ^{13}C discrimination independently of C_i/C_a (Warren & Adams 2006; Kodama *et al.* 2011; Douthe *et al.* 2011). In addition, fractionation due to photorespiration and respiration may contribute to the signal independently of C_i/C_a (see a recent synthesis by Tcherkez, Mahé & Hodges 2011).

Moreover, the use of $\Delta^{13}C_{lb}$ or even $\Delta^{13}C_{ss}$ as indicators of whole-plant *TE* needs a careful attention, as it requires to scale-up transpiration efficiency from instantaneous leaf level to whole-plant level during their life cycle (Condon *et al.* 2002). Direct estimates of whole-plant *TE* require a precise estimation of transpiration and biomass accumulation. The relationship between genetic variation of $\Delta^{13}C$ and whole-plant *TE* in tree species has only seldom been established (Guehl *et al.* 1996; Roupsard, Joly & Dreyer 1998; Cernusak *et al.* 2007b, 2008), while that between whole-plant and intrinsic leaf *TE* has been more frequently described (Osório, Chaves & Pereira 1998; Hobbie & Colpaert 2004, Cernusak *et al.* 2008). In the latter case, some studies again failed to detect any relationship (Matzner, Rice & Richards 2001; Ripullone *et al.* 2004). Although intrinsic *TE* is considered to be the primary source of variation in whole-plant *TE*, the fraction carbon lost during respiration overnight or in non-photosynthetic organs (Φ_c) and the water lost independently of carbon uptake (Φ_w)

may also have an important influence on whole-plant *TE* (Matzner, Rice & Richards 2001; Hobbie & Colpaert 2004). This shows the importance of calibrating $Δ^{13}C$ as an indicator of the genetic variability of whole-plant *TE* among genotypes.

Intrinsic *TE* and $Δ^{13}C$ depend on a number of environmental factors that affect g_s and A alone or in combination (Farquhar, O'Leary & Berry 1982). On the contrary, ^{18}O discrimination between source and leaf water *($Δ^{18}O_{lw}$)* or organic matter *($Δ^{18}O_{lb}$)* reflects the enrichment in ^{18}O due to transpiration and may therefore indicate differences in g_s and transpiration rates among genotypes (Barbour & Farquhar, 2000; Barbour 2007). ^{18}O enrichment is sometimes directly proportional to the transpiration rate (DeNiro & Epstein 1979; Sheshshayee *et al.* 2005) while in some cases a negative correlation may be found due to the Péclet effect (Saurer, Aellen & Siegwolf 1997; Ferrio *et al.* 2007). The combined use of $Δ^{13}C$ as an indicator of *TE* and $Δ^{18}O$ as an indicator of g_s, is a powerful tool to dissect intrinsi*c TE* into its components A and g_s (Farquhar, Ehleringer & Hubick 1989; Yakir & Israeli 1995; Barbour 2007) but was surprisingly little used to analyse the genetic variability in *TE* (Scheidegger *et al.* 2000; Cabrera-Bosquet *et al.* 2009).

Identifying poplar genotypes with high whole-plant *TE* may be very useful for the adaptation of poplar cultivation to areas with lower water availability (Braatne, Hinckley & Stettler 1992). In spite of the fact that productivity of poplar severely depends on water availability (Ceulemans & Deraedt 1999), a large variability in $Δ^{13}C_{lb}$ as indicator of intrinsic *TE* has been found among *Populus deltoides × nigra* genotypes (Monclus *et al.* 2005, 2006). Furthermore, productivity (i.e., biomass accumulation) was in many cases independent from $Δ^{13}C_{lb}$ both under controlled and open field conditions (Rae *et al.* 2004; Marron *et al.* 2002, 2005; Monclus *et al.* 2005, 2006). Such genetic differences of $Δ^{13}C$ in the young plants were maintained with age, as shown with $Δ^{13}C$ recorded from the cellulose in annual tree rings

(Rasheed *et al.* 2011).

Given the importance *TE* may gain for breeding poplar genotypes, and the sources of discrepancy between $\Delta^{13}C$ and *TE,* it was of importance to calibrate the use of $\Delta^{13}C$ as an indicator of the genetic diversity of *TE* at leaf and whole-plant scale in poplar. Thus, the main aims of the present research were to check whether:

- $\Delta^{13}C_{\text{lb}}$ and/or $\Delta^{13}C_{\text{ss}}$ are reliable estimators of intrinsic *TE* among poplar genotypes;

- the genetic variation found in $\Delta^{13}C_{\text{ss}}$ reflects the differences in whole-plant *TE* among genotypes;

- the observed differences of intrinsic *TE* could be ascribed to net CO_2 assimilation rate *(A)* or to stomatal conductance to water vapour *(g_s)*, and those of whole-plant *TE* to biomass accumulation or to transpiration;

- $\Delta^{18}O_{\text{lb}}$ and $\Delta^{18}O_{\text{lw}}$ differ among genotypes and indicate differences in stomatal conductance *(g_s)* as a driver of the genetic differences in whole-plant *TE*.

Six commercial *Populus deltoides × nigra* genotypes were grown under controlled environments and *TE* was measured (at instant and time integrated scales, and at leaf and whole-plant levels) and compared to the isotopic composition of ^{13}C and ^{18}O in leaves.

MATERIALS AND METHODS

Plant material and growth conditions

Six commercial genotypes of hybrid poplar *Populus deltoides × nigra, Agathe F (A),*
Cima (C), Flevo (F), I45/51 (I45), Pannonia (P) and *Robusta (R)* were selected on the basis of
contrasting values of $\Delta^{13}C_{lb}$ (Monclus *et al.* 2005, 2006). Ten liter pots were filled with a 1/1
v/v peat/sand mixture, heavily watered and extra water was left to drain overnight to reach
field capacity. The weight of filled pots was homogenized to 10000 g by adding substrate at
field capacity with a precision of 1%. Eight soil cores were sampled randomly and dried
(70°C) to a constant weight to assess water content at field capacity of the substrate, which
was estimated at 28.6%.

Shoot cuttings (16 copies of 6 genotypes) were planted during April 2009 and left to
grow in a greenhouse at INRA-Nancy (France), under natural daylight. After one month,
diameter at collar and height were recorded on all plants immediately before the transfer to
two climate chambers fitted with a rotating plate to homogenize the irradiance received by
each plant (Rotoplant, Strader, Angers). Six randomly selected individuals were harvested per
genotype, oven-dried at 60°C till constant weight to calibrate a unique relationship between
height (*H*), diameter (*D*) and biomass (*B*) across genotypes (see suppl. Figure 1):

$$B = 0.062 \, H^{0.98} \times D^{1.38} \; (r^2 = 0.988; P < 0.001) \hspace{2cm} (1)$$

This allometric equation was used to estimate the initial biomass of each remaining
individual in the experiment (recorded mean: 15 ± 4 g).

Five individuals per genotype were randomly distributed into each of the 2 growth
chambers. Microclimate in the chambers was: day/night, 16/8 h; air temperature, 25/18°C;
relative humidity, 85/45 %; irradiance at the top of the plants, 450 ± 15 μmol PPFD m^2 s^{-1}.

The light in the climate chambers was switched-on every day at 8:00 am.

Growth, daily water use and transpiration efficiency

Plant height *(H)* and diameter at collar *(D)* were measured twice a week. The soil surface of each pot was covered with a polyethane sheet to limit direct evaporation. Each pot was weighed daily (Sartorius-AG Göttingen, QC65EDE-D, Germany; accuracy: ± 0.1g) and watered back to the reference weight of 9428g (= 80% field capacity). The weight difference over 24h was assumed to represent daily water use. Cumulated water use (*WU*) was computed for each plant over the duration of the experiment.

After one month, plants were harvested, oven dried at 60°C till constant weight and dry mass of each compartment (leaves, stem and roots) recorded. Biomass increment (*BM*) was computed for each individual from the difference between harvested dry mass and biomass estimated at the start of the experiment. Whole-plant *TE* was computed per individual as biomass increment/total water use, and total leaf area (*TLA*) measured with an area meter (Li-Cor area meter, A1000 Li-Cor, Lincoln, NE, USA).

Leaf gas exchange and intrinsic transpiration efficiency

Intrinsic *TE* at leaf level was recorded on one leaf per individual: (i) under saturating irradiance as the ratio A_{sat}/g_{sat}, and (ii) under the standard irradiance available in the growth chamber (*A/g_s*). A_{sat} and g_{sat} were measured with a portable photosynthesis system (Li6400; Li-Cor, Lincoln, NE, USA) with a 6 cm^2 chamber and artificial irradiance provided by blue-red LEDs. Conditions were: PPFD, 1200 µmol m^2 s^{-1}; C_{inlet}, 380 µmol mol^{-1}; VPD, 1±0.2 kPa and leaf temperature, 25°C. *A* and g_s were monitored with a portable photosynthesis system (Li-6400) equipped with a 6 cm^2 chamber covered with a transparent lid. Measurements were repeated six times between 8:00 and 18:00 during the course of a single day. A fully expanded

and well-lit leaf of the same age was used on each individual. Microclimate in the photosynthesis chamber was close to ambient, i.e., irradiance: 450 ± 15 µmol m^2 s^{-1}, CO_2 in the chamber (C_a): 352 ± 20 µmol mol^{-1}, vapour pressure deficit, 0.5 ± 0.15 kPa and leaf temperature, 25°C. At the end of the measurement cycle, the leaf was clipped off, frozen in liquid nitrogen and stored at -80°C. No diurnal trend was detected for A and g_s (see suppl. Fig. 2). We therefore used the mean value of the ratio A/g_s as the value for diurnal, time-integrated, intrinsic *TE* at leaf level and the mean diurnal values of C_i/C_a was used to calculate the discrimination as predicted by the simple discrimination model (Farquhar & Richards 1984):

$$\Delta^{13}C = a + (b-a)\frac{C_i}{C_a} \tag{2}$$

with a = 4.4 ‰ and b = 27 ‰.

Leaf sugar extraction

Half of each stored leaf was freeze-dried at -196°C, ground and 60 mg of leaf powder was weighed in 2 ml microtubes. The protocol for soluble sugar extraction was modified from Wanek, Heinter and Richter (2001). 350µl of methanol-chloroform-water (MCW, 12:5:3, v/v/v) was added and the samples were placed in a water bath at 70°C for 30 min. After cooling, the microtubes were centrifuged at 11400g for 3 min. This step was repeated three times and the supernatant was collected into a new 2ml microtube. To induce phase separation, 200µl chloroform and 500µl deionised water were added to the supernatant and vigorously mixed. The samples were left for a few minutes, then centrifuged at 11400 g for 3 min. The aqueous phase was transferred to a new 2 ml microtube and complemented with 300 µl freshly hydrated Dowex 1X8, 200-400 mesh [Cl]$^-$ form resin, (Fluka France) converted to [HCO$_2$]$^-$ with sodium formate. The samples were agitated during 2 h at room temperature.

After centrifugation, the supernatant was transferred to a new 2 ml microtube complemented with 300 µl freshly hydrated Dowex 50W, 200-400 mesh [H]+ form, resin (Sigma Aldrich France). Samples were again agitated during 2 h at room temperature. After centrifugation, the supernatant was transferred to a pre-weighed microtube and dried to complete dryness on a rotary evaporator (HETO, DK3450, Allerød, Denmark). 1 mg bulk sugar was diluted with 60 µl water, transferred into tin capsule and freeze dried (FreeZone, Labconco, Kansas City, USA).

Carbon isotope discrimination between atmosphere and bulk leaf matter ($\Delta^{13}C_{lb}$) or soluble sugars ($\Delta^{13}C_{ss}$)

A fully mature leaf (below the one used for leaf gas exchange) was selected from each individual and was finely ground after drying at 60°C to a constant weight. 1 mg powder was weighed in tin capsules. ^{13}C content in both leaf bulk and leaf soluble sugars, as well as carbon and nitrogen content were analyzed with an elemental analyzer (Carlo Erba, NA 1500-NC, Milano, Italy) coupled to an isotope-ratio mass spectrometer (Finnigan, Delta-S, Bremen, Germany) with a precision of 0.1‰. ^{13}C discrimination between the atmosphere and leaf bulk matter or soluble sugars was calculated as:

$$\Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \dfrac{\delta^{13}C_{air}}{1000}} \tag{3}$$

where $\delta^{13}C_{air}$ is the isotopic composition of air measured in the two growth chambers. $\delta^{13}C_{air}$ was similar in the two chambers ($P = 0.548$, n = 46). $\delta^{13}C_{air}$ was -9.01‰ (SD ± 0.95‰) with small fluctuations during the diurnal cycles.

Leaf water extraction

The second half of each stored leaf was used for the extraction of leaf water, with a cryogenic vacuum distillation. Sealed tubes containing a frozen leaf were connected to the extraction apparatus with a collection tube at the other end. Air inside the whole apparatus was evacuated to remove any trace of external water vapour under approx. 8 Pa. The vial containing the sample was heated using a water bath at a constant temperature of 70°C and the collection tube placed in a Dewar containing liquid nitrogen in order to freeze the vapour emanating from the sample (see West, Patrickson & Ehleringer 2006). The extracted water was collected and used to measure ^{18}O composition.

Oxygen isotope discrimination between irrigation water and leaf bulk matter ($\Delta^{18}O_{lb}$) or leaf water ($\Delta^{18}O_{lw}$)

0.3-0.4 mg of leaf powder (the same than for ^{13}C analysis) and 0.4 μl of leaf extract were used to measure ^{18}O composition of leaf bulk matter ($\delta^{18}O_{lb}$) and of leaf water ($\delta^{18}O_{lw}$) respectively. Analyses were done with a high temperature elementar analyser (Pyrocube, Elementar, Hanau, Germany) coupled to a mass spectrometer (Isoprime, Manchester, UK). Samples were combusted and pyrolised at 1270°C. The oxygen isotope composition was determined with respect to the three laboratory standards. Laboratory standards were pre-calibrated against the international standard V-SMOW (Vienna- Standard Mean Ocean Water). Accuracy of the measurements was ± 0.3‰.

The ^{18}O content in irrigation water ($\delta^{18}O_{sw}$) was recorded from 9 samples collected at different dates during the experiment. The mean value was -7.58‰ (± 0.46‰) with no difference between the two chambers ($P = 0.780$, n = 9).

^{18}O discrimination between irrigation water and leaf bulk matter or water was computed

as:

$$\Delta^{18}O = \frac{\delta^{18}O_{plant} - \delta^{18}O_{SW}}{1 + \dfrac{\delta^{18}O_{SW}}{1000}} \qquad (4)$$

Leaf anatomy

Three 1cm^2 discs were harvested on one leaf per individual to record leaf thickness (*LT*) and stomatal density (*SD*) and were immediately frozen in liquid nitrogen and stored at − 80°C. As poplar leaves are amphistomatous, each disc was split for the separate analysis of adaxial and abaxial sides. Sample discs were stuck to aluminium stubs on a Peltier stage (– 50°C) before being examined under a controlled-pressure scanning electron microscope (model 1450VP, Leo, Cambridge, UK; 20–30 Pa inside the chamber, accelerating voltage 15 kV, working distance 12 mm). Nine microphotographs (×300) were taken on each disc and stomata were counted with an image analysis software (VISILOG, Noesis, France). Total stomatal density was calculated as the sum of adaxial and abaxial stomatal densities. On the other three discs, five semi-thin cryo-sections were photographed per disc to measure mean leaf thickness. Additional five discs of 1 cm^2 were sampled for *LMA* and weighed after oven drying for 24h. *LMA* (g m^{-2}) was determined as a ratio between dry weight and area.

Statistical analyses

All statistical analyses were made using STATISTICA (version 8.1, StatSoft, Maisons-Alfort, France). Normality and homoscedasticity of data were checked graphically with residues vs. predicted and normal quartile-to-quartile plots. Genetic effect, growth chamber effect and their interaction were assessed with two-way ANOVA and the following model:

$$Y_{ijk} = \mu + G_i + B_j + G_i \times B_j + \varepsilon_{ijk}$$

With: Y_{ijk}, response variable; μ, intercept; G_i, genotype effect; B_j, growth chamber effect; $G_i \times B_j$, interaction between genotype and growth chamber and ε_{ijk}, residue. A Tukey HSD test was used to evaluate pair-wise differences among genotypes. In the absence of growth chamber and interaction effects, data from both growth chambers were pooled. Correlations between the measured traits were tested at genotype level with a general linear regression model. All tests and correlations were taken as significant when $P < 0.05$. Means are expressed with their standard deviation (SD).

The variability of the recorded traits was assessed at individual level with a principal components analysis (PCA). Variables were represented on the main plane defined by the two main factors of the PCA (F1 and F2 axis); their coordinates were their linear correlation coefficients (r; Pearson's correlation coefficient) with these factors. Correlations were taken as significant when $P < 0.05$.

RESULTS

Table 1 presents a list of the recorded variables and Table 2 their genotype means. No climate-chamber effect was detected for any variable and there was no interaction between climate chamber and genotype effects on any variable. The presentation of the results therefore concentrates only on the genotype effects. Spearman correlations for the recorded traits at individual level (phenotypic correlation) are displayed in Table 3.

Plant height and stem diameter

Time courses of height (*H*), diameter (*D*) and daily water use (*WU*) displayed a continuous and gradual increase (Suppl. Fig. 3). At the end of the experiment, large genotypic differences were detected for *H* ($P < 0.001$) and to a lesser extent for *D* ($P = 0.034$). *Pannonia* displayed highest and *I45/51* lowest values of *H* and *D*.

$\Delta^{13}C$ between atmospheric CO_2 and bulk leaf matter $(\Delta^{13}C_{lb})$ or soluble sugars $(\Delta^{13}C_{ss})$

Leaf tissues contained about 3.1% N and 46.5% C as expected for poplar leaves, with no genotype effect for N and a very small one for C (Table 2). Significant genotypic differences were found for $\Delta^{13}C_{lb}$ (range: 21.5 to 22.5 ‰). *Pannonia* showed lowest and *I45/51* highest $\Delta^{13}C_{lb}$.

The purity of the soluble sugar fraction was tested from their N and C contents. N content was as expected very low (around 0.1%) with no genotype effect. C amounted 42% approx., which is very close to the 40% expected for pure carbohydrates. The soluble sugar extract contained sucrose, glucose, fructose and a sugar identified as mannose, representing between 45 and 55% of the extracts (not shown). A small genotype effect was detected for the

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C content, with *Agathe F* displaying slightly larger values than the other genotypes. The fraction of soluble sugars in the leaves was rather high (16.5 ± 0.36% of total dry matter) and stable across genotypes.

$\Delta^{13}C_{ss}$ also displayed a significant genotype effect ($P < 0.001$) and genotype means ranged between 22.5 and 24.3 ‰ (Table 2). A tight and positive correlation was found between $\Delta^{13}C_{ss}$ and $\Delta^{13}C_{lb}$, however $\Delta^{13}C_{lb}$ was always smaller than $\Delta^{13}C_{ss}$ (Fig. 1). The difference was not constant across genotypes and increased with $\Delta^{13}C_{lb}$: *I45/51* showed a larger offset than *Pannonia.*

Leaf gas exchange and intrinsic *TE* at leaf level

A genotype effect was evident for A_{sat}, g_{sat} and A_{sat}/g_{sat}. Overall means were 19.8 µmol m^{-2} s^{-1} for A_{sat} and 0.637 mol m^{-2} s^{-1} for g_{sat} (Table 2). No diurnal trend was evidenced in instant net CO_2 assimilation rate (A) or stomatal conductance to water vapour (g_s) recorded under ambient conditions (ANOVA with time as a random effect, $P = 0.748$ for A and $P = 0.239$ for g_s; Suppl. Fig. 2). Mean daily values of A, g_s and A/g_s were therefore used to address genotype differences and to correlate with $\Delta^{13}C_{ss}$. A very significant genotype effect was observed for A, g_s and A/g_s. A was around 10 µmol m^{-2} s^{-1}, i.e., approx. half the values of A_{sat} while g_s was very close to g_{sat}. Intrinsic *TE* ranged between 15.8 (*I45/51*) and 23.5 µmol mol^{-1} (*Pannonia*). Significant genotype differences were similarly found for C_i/C_a, which ranged between 0.871 (*Pannonia*) and 0.945 (*I45/51*).

Surprisingly, no relationship was evidenced between mean diurnal A/g_s and A_{sat}/g_{sat} (Fig. 2). Genotype values for $\Delta^{13}C_{ss}$ were negatively correlated to mean diurnal A/g_s (not shown) and positive correlations were evidenced between C_i/C_a and $\Delta^{13}C_{ss}$ and $\Delta^{13}C_{lb}$ as predicted by the simple discrimination model (Fig. 4, $P < 0.001$ and $P < 0.001$) Observed values of $\Delta^{13}C_{ss}$

were approx. 1.5‰ below predicted while those of $\Delta^{13}C_{lb}$ were 2.5‰ below predicted.

Genotype means of $\Delta^{13}C_{ss}$ were correlated to A but not to g_s (Fig. 4): differences in net CO_2 assimilation rates among genotypes had a much larger effect on intrinsic *TE* than stomatal conductance.

Whole-plant transpiration efficiency

Accumulated biomass (*BM*) differed among genotypes and ranged between 191 g (*Pannonia*) and 99.6 g (*I45/51*). Mean values of cumulated water use (*WU*) also differed among genotypes and ranged between 12.5 l (*Robusta*) and 9.39 l (*Flevo*). As a result, whole-plant *TE* varied among genotypes: *Pannonia* displayed highest (16.3 g l^{-1}) and *I45/51* lowest values (9.68 g l^{-1}, table 2). Genotype values of whole-plant *TE* were negatively correlated to $\Delta^{13}C_{ss}$ and positively to intrinsic *TE* (Fig. 5). At the same time, whole-plant *TE* was positively correlated to *BM* but not to *WU* (Table 3).

^{18}O discrimination between irrigation water and leaf bulk matter ($\Delta^{18}O_{lb}$) or leaf water ($\Delta^{18}O_{lw}$)

Genotype means were around 36.0‰ for $\Delta^{18}O_{lb}$ and 9.23‰ for $\Delta^{18}O_{lw}$ (Table 2). Due to relatively large intra genotype variations, no genotype effect was detected for $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$. Nevertheless, at individual level, $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$ were correlated to g_s and *WU* (Table 3).

Leaf mass to area ratio (*LMA*), leaf thickness (*LT*) and stomatal density (*SD*)

No genotypic variability was found for *LT* or for *LMA* that were close to 75 mm and 35 g m^{-2} (Table 2). *SD* differed among genotypes with *Pannonia* displaying highest and *I45/51*

lowest *SD*. $\Delta^{13}C_{ss}$ was negatively and *A* positively correlated to *SD* (Fig. 6).

Correlations among variables

A general PCA was performed with the 15 measured traits (Fig. 7). The main plane of the PCA (F1 × F2) explained 56.9% of the overall variability, with 29.1% for F1 and 27.8% for F2 axis. F1 was mostly correlated with traits defining water use efficiency at different scales like $\Delta^{13}C_{ss}$, $\Delta^{13}C_{lb}$, A/g_s, C_i/C_a, *A*, whole-plant *TE* that were also tightly correlated to *BM* and *SD*. The second axis was related to water use and oxygen isotope composition, with correlations to *TLA* and g_s.

Along the F1 axis A/g_s, *A*, whole-plant *TE* and *SD* were positively inter-correlated and negatively correlated to $\Delta^{13}C_{ss}$, $\Delta^{13}C_{lb}$ and C_i/C_a. Along the F2 axis *WU* scaled positively with *TLA* and g_s and negatively with $\Delta^{18}O_{lb}$. (see Table 3 for *r*- values of Pearson's correlation coefficients between traits). There was a clear grouping among individuals in the F1 × F2 planes with some overlaps between genotypes. *Pannonia* and *I45/51* were clearly discriminated along axis 2, the latter being much less productive and efficient with respect to water use than the former. Axis 1 was dominated by total leaf area and did less clearly discriminate the genotypes due to some variability in size within genotypes. This in particular explains why we were unable to detect any genotype difference in $\Delta^{18}O_{lb}$ or $\Delta^{18}O_{lw}$ despite large differences among individuals and a significant correlation with water use and stomatal conductance at individual level.

DISCUSSION

In this study the complex trait "transpiration efficiency" (*TE*) was recorded at whole-plant level over a month, and at leaf level during a day with gas exchange measurements and ^{13}C composition of soluble sugars. We used also ^{13}C composition of bulk leaf matter as an indicator integrating leaf life span. The different approaches provided convergent estimates for genotype differences in *TE*.

Discrimination against ^{13}C from the air to bulk leaf matter ($\Delta^{13}C_{lb}$) or to soluble sugars ($\Delta^{13}C_{ss}$)

The tight correlation observed between $\Delta^{13}C_{lb}$ and $\Delta^{13}C_{ss}$ at individual as well as at genotype level, confirms that the differences in the ^{13}C signal recorded in soluble sugars that display a rapid turn-over, were still visible in the bulk leaf matter built over several weeks. This is due to the stable conditions that prevailed in the climate chambers since the start of leaf expansion. It also confirms that post-photosynthetic ^{13}C discrimination had similar effects in all genotypes and did not modify the ranking of genotypes. A similar close relationship was described in a range of oak genotypes (Roussel *et al.* 2009a). Leaves were sampled from fast growing and very actively photosynthesizing poplar cuttings at the end of a 16 h illumination phase. They contained a large amount of soluble sugars (around 16% total dry matter), and the extracts displayed a high purity indicated by a very low N content (no contamination by amino acids) and a C content close to that of sucrose or of a mix of carbohydrates. In the forthcoming discussion we will focus on $\Delta^{13}C_{ss}$ as a potential index for intrinsic *TE*, as it is expected to closely reflect discrimination during photosynthesis.

TE at leaf level: correlation of $\Delta^{13}C_{ss}$ with *A/g$_s$*

The genotype means of $\Delta^{13}C_{ss}$ were negatively correlated to mean diurnal A/g_s over the whole illumination phase. In this respect, our data confirm that $\Delta^{13}C_{ss}$ is a relevant indicator for genetic differences in A/g_s as predicted by the model of Farquhar, Ehleringer & Hubick (1989), on-line with numerous earlier results (Brugnoli *et al*. 1988 for poplar).

To our surprise, $\Delta^{13}C_{ss}$ was larger than $\Delta^{13}C_{lb}$ while bulk leaf matter is usually depleted in ^{13}C with respect to carbohydrates, due to the presence of lipids and lignins (Bowling, Pataki & Randerson 2008; Monti *et al*. 2006). Such was the case for leaves of *Fagus sylvatica* (Gavrichkova *et al*. 2011) or of *Quercus robur* (Roussel *et al*. 2009a). We are not aware of any published result similar to ours. Several hypotheses may explain this discrepancy:

(i) a contribution to leaf structure of C stored before the transfer to the stable conditions of the climate chamber. This hypothesis is unlikely, as the leaves did expand after the transfer to the climate chamber, and as the biomass gain during the experiment was 9 times the initial biomass; the contribution of "old" C to the construction of new leaves was probably very minor;

(ii) diurnal changes have been recorded several times in $\Delta^{13}C_{ss}$ (for instance, Gavrichkova *et al.* 2011) and as a consequence in the ^{13}C signature of respired CO_2 (see Werner & Gessler 2011 for a synthesis). Such changes may partly be due to changes in the environment (irradiance, temperature, VPD), which did not occur here. They may be due also to switches between respiratory substrates with different isotopic signatures. Such switches mainly occur during day-night transitions and are associated for instance to post-illumination respiratory bursts (Gessler *et al*. 2009) and are unlikely to impact $\Delta^{13}C_{ss}$ recorded at the end of an illumination period;

(iii) the isotopic fractionation by aldolase results in a ~3-4 ‰ enrichment of the C-3

and C-4 atoms of fructose in the chloroplasts (Gleixner & Schmidt 1997), and in a small but significant enrichment for starch accumulated in chloroplasts, while in the meanwhile, glucose and sucrose in the cytosol and the vacuole are slightly depleted (Brugnoli *et al.* 1988; Badeck *et al.* 2005; Bowling, Pataki & Randerson 2008). This depletion remains visible in the sucrose exported to the phloem. This is no longer true during night, when the stored starch is hydrolysed into ^{13}C enriched sucrose.

The third hypothesis fits best with our situation as soluble sugars were sampled at the end of 16h illumination, with permanently high photosynthesis, and a large starch accumulation during the day (see Brugnoli *et al.* 1988 for an illustration of starch dynamics in poplar). This is likely to result in a depletion of soluble sugars with respect to the primary photosynthesis products (i.e., 3PGA), while leaf bulk matter contains large amounts of slightly enriched starch.

The tight and linear correlation between C_i/C_a and $\Delta^{13}C_{ss}$, paralleled the predictions of the simple form of the discrimination model of Farquhar & Richards (1984). The negative offset of 0.9 to 1.6‰, depending on the genotype, is partly due to the occurrence of a finite conductance to CO_2 transfer in the mesophyll, g_m (Evans & Von Caemmerer 1996; Evans *et al.* 2009; Flexas *et al.* 2012): this offset is the basis for indirect estimates of g_m.

There are a few small uncertainties around our estimates of $\Delta^{13}C_{ss}$ due for instance to our estimates of $\delta^{13}C$ in the chamber atmosphere. We used a mean value computed from records over several days. $\delta^{13}C$ in the atmosphere of the chamber may have undergone diurnal cycles due to the production of depleted CO_2 over night, and photosynthesis during the day, resulting in an increase of $^{13}CO_2$ in the air. As photosynthesis rates remained stable during the day, the use of a mean value of $\delta^{13}C$ in the atmosphere probably minimized this artefact.

There are still a number of uncertainties around the ^{13}C discrimination model (Douthe *et al*. 2012; Farquhar & Cernusak 2012). In particular, the values of parameter "b" vary between 27 and 30‰ in the literature (Warren 2006; Douthe *et al.* 2012). In our case, the use of a value of 30‰ instead of 27‰ would have amplified the offset. We therefore avoided a direct computation of g_m from our data set. Nevertheless, we may safely conclude that: (i) the different genotypes displayed as expected finite albeit probably large values of g_m and (ii) g_m probably differed significantly among genotypes, with smaller values (i.e., larger offsets) for *Agathe F* and *I45/51* with respect to the others. Nevertheless, these differences had only little impact on the ranking of the different genotypes as shown in Fig. 3.

Correlation of whole-plant *TE* with intrinsic *TE* and $\Delta^{13}C_{ss}$

Instant *TE* may be modulated by VPD and by genotype differences in intrinsic *TE*. For individuals grown under a common VPD, like here, genotypic differences in intrinsic *TE* are expected to be the main source of variation for whole-plant *TE* (Farquhar & Richards 1984, Hubick & Farquhar 1989). We indeed found a strong positive genotype correlation between whole-plant *TE* and intrinsic *TE* like in many species: *Larix occidentalis* (Zhang & Marshall, 1994), *Pinus pinaster* (Guehl *et al.* 1996), *Eucalyptus globulus* (Osório & Pereira, 1994), several tropical tree species (Cernusak *et al.* 2007b, 2008), *Quercus robur* genotypes (Roussel *et al.* 2009b). Nevertheless, whole-plant *TE* can be modulated by factors independent of intrinsic *TE*: (i) the fraction of carbon fixed during the day and lost through respiration over night in leaves, and in stems and roots over the whole day (Φ_c) and (ii) the fraction water loss not associated to photosynthesis (Φ_w, Farquhar, Ehleringer & Hubick 1989). Φ_c and/or Φ_w can be modulated by N (Hobbie & Colpaert 2004) or water availability (Osório, Chaves & Pereira 1998). Genotype difference in Φ_c and Φ_w could severely blur the relationship between intrinsic *TE* and whole-plant *TE*. The tight correlation between intrinsic and whole-plant *TE*

observed here suggests that Φ_c and Φ_w remained rather stable across the genotypes.

Nevertheless, the relative variation of intrinsic *TE* (27.6%) was much smaller than that of whole-plant *TE* (40.5%). This is a frequent occurrence: relative variations of intrinsic and whole-plant *TE* were 21% *vs.* 70% among tropical tree species (Cernusak *et al.* 2008), 44% *vs.* 55% among acacia species (Konaté 2010), and 44% *vs.* 14.5% among oak genotypes (Roussel *et al.* 2009b). This shows that in many cases, Φ_c and Φ_w have an impact on genotype or species-related differences of whole-plant *TE* in addition to that of intrinsic *TE* and that this impact still has to be unambiguously quantified through direct records of respiration of non photosynthetic tissues, or by records of nocturnal transpiration.

Whole-plant *TE* varied between 10 and 15 g kg^{-1}. Such values are unexpectedly high when compared to the current literature. A range of 3 - 4.5 g kg^{-1} was found in tropical tree species (Cernusak *et al.* 2007b, 2009a, 2009b), 4 - 5 g kg^{-1} in populations of *Faidherbia albida* (Roupsard, Joly & Dreyer 1998), 4.5 - 5.5 g kg^{-1} in *Quercus robur* genotypes (Roussel *et al.* 2009b), around 10 g kg^{-1} in *Pinus pinaster* and around 14 g kg^{-1} in *Quercus petraea* under elevated CO_2 and drought (Guehl *et al.* 1994). Hobbie & Colpaert (2004) did not cite any value above 11.8 g kg^{-1} in their review for trees or annual crops. The high values detected here for poplar were due to low VPD during the day, long days (16h per photoperiod) and moderate but constant irradiance and optimal irrigation and fertilisation. Moreover, our individuals were juveniles, displayed a very fast growth and low level of lignification. Under such conditions, Φ_w is likely minimal (short nights and therefore limited nocturnal transpiration) as is probably Φ_c (limited lignification which would be a source of respiration loss; small amount of lipid synthesis and heavy investment into photosynthesising tissues). This may only be a transitory phase, and we expect smaller values of *TE* at later developmental stages. Such a surprisingly high transpiration efficiency in a species that is

usually described as a water spender deserves further investigations.

Was the genotype ranking of *TE* similar to earlier studies?

Stability of genotype ranking among different environments is a very important question, which is only seldom addressed. There is currently no large-scale comparative study with the same poplar genotypes grown under different environments. A comparison of collections of genotypes grown in common plantations in France and Italy revealed very little similarity between the two sites (Dillen *et al.* 2011). Fortunately, the same set of 6 genotypes was used by several authors in field and greenhouse experiments. We computed the Spearman rank correlation coefficient for $\Delta^{13}C_{\text{lb}}$ and intrinsic *TE* between our results and earlier ones (see Table 4). The correlations were rather high in most cases except with the data-set from Marron *et al.* (2005) that differed from all others and was conducted in a greenhouse under very low irradiance. We can conclude from this rapid analysis that the ranking for $\Delta^{13}C_{\text{lb}}$ and intrinsic *TE* was rather stable in this set of genotypes under very different growth conditions.

Origin of the genotypic differences in intrinsic *TE* at leaf level.

Genotypic differences in intrinsic *TE* can be due to differences of net CO_2 assimilation rates *A* or stomatal conductance, g_s or a combination of both (Farquhar & Richards 1984; Condon *et al.* 2004). The positive correlation of *A* with intrinsic *TE* and the negative one with $\Delta^{13}C_{\text{ss}}$ show that larger *A* explained most of the variation of intrinsic *TE* and $\Delta^{13}C_{\text{ss}}$. Similar results were detected by Voltas *et al.* (2006) for *Populus × euramericana*. When we recorded A_{sat}, g_{ssat} under saturating conditions, we changed significantly the genotype ranking of intrinsic *TE*. Indeed, A_{sat} was much larger than *A* while g_{ssat} was very close to g_s: under ambient conditions, the stomata were already at maximal opening and were not limiting CO_2 assimilation. This observation also underlines that changes in the environment (irradiance but

also water availability, acting on the two components of intrinsic *TE*) may potentially change the ranking among genotypes.

This observation contradicts at least partly that of a rather tight correlation between stomatal density (*SD*) and $\Delta^{13}C_{ss}$ (and therefore also intrinsic *TE*). In our conditions, stomatal density was independent of stomatal conductance; Dillen *et al.* (2009) and Fichot *et al.* (2010) detected even a negative correlation between g_s and *SD*. Such a lack of correlation can be explained by interactive effects of pore depth, effective pore width and actual percentage of functional stomata (Aasamaa, Sober & Rahi 2001; Franks, Drake & Beerling 2009).

Correlation between *TE* and biomass accumulation (*BM*)

Whole-plant *TE* was tightly correlated to *BM*, but not at all to cumulated water use (*WU*). A positive correlation between whole-plant *TE* and *BM* confirms that changes in *A* had a larger effect than g_s (Condon, Richards & Farquhar 1987; Virgona *et al*. 1990). Both negative (Condon, Farquhar & Richards 1990; Martin & Thorstenson 1988; Ehleringer *et al.* 1990; White, Castillo & Ehleringer 1990) and positive (Hubick, Farquhar & Shorter 1986; Wright, Hubick & Farquhar 1988) correlations were found between whole-plant *TE* and *BM* in crop species. We suggest that whole-plant *TE* was rather controlled by *A* than by g_s under our conditions. This strengthens the hypothesis that breeding poplar genotypes for improved whole-plant *TE* would not necessarily come at the expense of productivity.

[18]O enrichment between source and leaf water ($\Delta^{18}O_{lw}$) or leaf bulk matter ($\Delta^{18}O_{lb}$)

Leaf water is [18]O enriched with respect of the water source due to transpiration (Barbour 2007; Saurer, Aellen & Siegwolf 1997). If the transpiration rate is driven by different levels of g_s then a negative relationship is to be expected between g_s and $\Delta^{18}O_{lw}$ (Farquhar, Cernusak &

Barnes 2007). Due to a large intra genotypic variability, no genotypic effect was found here for $\Delta^{18}O_{\text{lw}}$ nor for $\Delta^{18}O_{\text{lb}}$. At individual level, both $\Delta^{18}O_{\text{lw}}$ and $\Delta^{18}O_{\text{lb}}$ were negatively correlated to WU and g_s. Similar negative correlations have been evidenced in cotton (Barbour *et al.* 2000), in a tropical tree, *Ficus insipida* (Cernusak *et al.* 2007a) and in durum wheat (Cabrera-Bosquet *et al.* 2009). $\Delta^{18}O_{\text{lw}}$ and $\Delta^{18}O_{\text{lb}}$ were 9.23‰ and 36.0‰ respectively; with a 26.8‰ difference. Such a difference is, under normal cellular temperature and pH, due to isotopic exchanges between water and the carbonyl groups of organic molecules and is usually close to 25 – 30‰ (Barbour 2007), close to the one we found here.

CONCLUSION

Transpiration efficiency recorded at different integration scales (from leaf to whole-plant) in *Populus × euramericana* genotypes displayed consistent genotype differences. $\Delta^{13}C$ recorded from soluble sugars was an efficient predictor of intrinsic *TE* at leaf level and of whole-plant *TE*. Nevertheless, the magnitude of genotype variability of *TE* was larger at whole-plant level than at leaf level, showing that either nocturnal transpiration or more likely differences in respiration from non-photosynthetic organs had a large impact on whole-plant transpiration efficiency and on its variability. Genotypic differences of *TE* were due to differences of net CO_2 assimilation rates (at leaf level) and of biomass production (at whole-plant level) rather than stomatal conductance or transpiration rates. Genotypes with large carbon assimilation and fast growth were also the most efficient, which shows again that breeding for improved *TE* does not come at the expense of productivity in *Populus × euramericana*.

ACKNOWLEDGEMENT

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TABLES

Table 1. List of variables and abbreviations used in the study

Variables	Description
D	Diameter at stem base (mm)
H	Stem height (cm)
$\delta^{13}C_{air}$	Carbon isotope composition of the air (‰)
$\delta^{13}C_{ss}$	Carbon isotope composition of soluble sugars in the leaf (‰)
$\delta^{13}C_{lb}$	Carbon isotope composition of bulk leaf matter (‰)
$\Delta^{13}C_{ss}$	Carbon isotope discrimination between atmosphere and soluble sugars in the leaf (‰)
$\Delta^{13}C_{lb}$	Carbon isotope discrimination between atmosphere and bulk leaf matter (‰)
$\delta^{13}C_{offset}$	Difference between $\delta^{13}C_{ss}$ and $\delta^{13}C_{lb}$ (‰)
N_{lb}, N_{ss}	N content in bulk leaf or in the soluble sugar fraction (%)
C_{lb}, C_{ss}	C content in bulk leaf or in the soluble sugar fraction (%)
A, A_{sat}	Net CO_2 assimilation rate under ambient/saturating condition (μmol m^{-2} s^{-1})
g_s, g_{sat}	Stomatal conductance under ambient/saturating condition (mol m^{-2} s^{-1})
A_{sat}/g_{sat}	Intrinsic transpiration efficiency under saturating condition (μmol mol^{-1})
A/g_s	Intrinsic transpiration efficiency of a leaf over a diurnal cycle (μmol mol^{-1})
C_i/C_a	Ratio of CO_2 concentration in the atmosphere and in the substomatal spaces
BM	Total plant biomass accumulated during the experiment (g)
WU	Cumulated water use (l)
TE	Whole-plant transpiration efficiency = BM/WU (g l^{-1})
$\delta^{18}O_{sw}$	Oxygen isotope composition of source water (‰)
$\delta^{18}O_{lb}$	Oxygen isotope composition of bulk leaf matter (‰)
$\delta^{18}O_{lw}$	Oxygen isotope composition of leaf water (‰)
$\Delta^{18}O_{lb}$	Oxygen isotope discrimination between source water and bulk leaf matter (‰)
$\Delta^{18}O_{lw}$	Oxygen isotope discrimination between source water and leaf water (‰)
LT	Leaf thickness (μm)
SD	Stomatal density (mm^{-2})
LMA	Leaf mass to area ratio (g m^{-2})
TLA	Whole-plant leaf area (m^2)

Table 2. Genotype means (SD) of traits recorded on 10 individuals from 6 *Populus x euramericana* genotypes grown in 2 climate chambers. A factorial ANOVA (df 5, 1 and 5) was used to test for the effects of genotype (*G*), climate chamber (*B*) and their interaction (*G x B*). Different letters represent significant differences among genotypes as tested by a *post hoc* Tukey test ($P < 0.05$). See Table 1 for the definition of variables.

	Agathe F	Cima	Flevo	I45/51	Pannonia	Robusta	G effect	B effect	G x B
D (mm)	10.5 (0.28) b	11.2 (0.28) ab	11.1 (0.50) ab	10.5 (0.46) b	11.9 (0.44) a	11.8 (0.11) a	**P=0.034**	P=0.938	P=0.306
H (cm)	85.9 (3.47) b	95.6 (3.47) a	78.6 (3.47) c	55.5 (3.65) d	93.4 (3.47) a	85.8 (3.65) bc	**P<0.001**	P=0.956	P=0.153
N_{lb} (%)	3.06 (0.26)	2.93 (0.30)	3.28 (0.29)	3.47 (0.21)	2.59 (0.34)	3.34 (0.11)	P=0.227	P=0.156	P=0.436
C_{lb} (%)	47.0 (0.23) a	46.5 (0.26) b	45.9 (0.45) b	47.1 (0.50) b	46.5 (0.47) b	47.5 (0.24) a	**P=0.047**	P=0.456	P=0.768
$\Delta^{13}C_{lb}$ (‰)	21.7 (0.15) ab	22.2 (0.12) bc	21.7 (0.17) ab	22.5 (0.25) c	21.4 (0.10) a	22.1 (0.08) bc	**P<0.001**	P=0.656	P=0.139
N_{ss} (%)	0.124 (0.01)	0.101 (0.01)	0.107 (0.01)	0.092 (0.01)	0.115 (0.01)	0.067 (0.01)	P=0.200	P=0.169	P=0.996
C_{ss} (%)	43.2 (0.33) a	41.0 (0.14) b	42.1 (0.23) b	41.1 (0.42) b	41.4 (0.26) b	42.1 (1.16) b	**P=0.021**	P=0.065	P=0.086
$\Delta^{13}C_{ss}$ (‰)	23.3 (0.13) b	23.2 (0.14) ab	23.0 (0.18) ab	24.3 (0.19) c	22.5 (0.17) a	23.3 (0.10) b	**P<0.001**	P=0.899	P=0.140
$\delta^{13}C_{offset}$ ($\delta^{13}C_{lb}$ - $\delta^{13}C_{ss}$)	1.47 (0.08) a	0.928 (0.15) b	1.20 (0.19) b	1.60 (0.18) a	1.01 (0.16) b	1.12 (0.16) b	**P=0.049**	P=0.799	P=0.743
Soluble sugar content in leaves	16.5 (0.16)	16.6 (0.32)	16.2 (0.41)	16.4 (0.39)	16.8 (0.27)	16.5 (0.58)	P=0.904	P=0.326	P=0.648
A_{sat} (µmol m^{-2}s^{-1})	19.1 (0.14) bc	20.1 (0.63) abc	21.1 (0.44) a	18.2 (0.64) c	20.1 (34) ab	20.4 (0.19) ab	**P<0.001**	P=0.362	P=0.848
g_{sat} (mol m^{-2}s^{-1})	0.518 (0.03) b	0.698 (0.03) a	0.630 (0.03) ab	0.572 (0.03) ab	0.742 (0.03) a	0.660 (0.06)	**P=0.003**	P=0.170	P=0.301
A_{sat}/g_{sat} (µmol mol^{-1})	38.4 (2.60) a	29.3 (1.59) b	34.1 (1.45) ab	32.2 (1.50) ab	27.8 (1.68) b	32.5 (2.26) ab	**P=0.004**	P=0.179	P=0.272
g_s (mol m^{-2}s^{-1})	0.479 (0.03) b	0.580 (0.03) ab	0.572 (0.02) b	0.572 (0.03) ab	0.587 (0.02) ab	0.656 (0.01) a	**P<0.001**	P=0.979	P=0.862
A (µmol m^{-2}s^{-1})	10.3 (0.68) ab	10.6 (0.40) ab	11.3 (0.47) a	8.80 (0.37) b	12.6 (0.44) a	11.8 (0.55) a	**P<0.001**	P=0.480	P=0.160
A/g_s (µmol mol^{-1})	21.5 (2.03) a	19.2 (1.16) ab	20.5 (1.69) ab	15.8 (1.08) b	21.9 (1.52) ab	17.9 (1.21) ab	**P=0.004**	P=0.605	P=0.603
C_i/C_a	0.882 (0.01) a	0.893 (0.01) ab	0.880 (0.01) a	0.934 (0.01) b	0.868 (0.01) a	0.913 (0.01)	**P=0.005**	P=0.468	P=0.624
BM (g)	148 (6.69) b	178 (9.57) ab	144 (12.5) b	99.6 (13.9) c	191 (9.18) a	151 (4.16) b	**P<0.001**	P=0.889	P=0.053
WU (l)	9.87 (0.52) ab	11.9 (0.65) ab	9.39 (0.72) b	10.0 (1.11) ab	12.3 (1.02) ab	12.5 (0.40) a	**P=0.009**	P=0.830	P=0.100
TE (g l^{-1})	15.3 (0.56) a	15.1 (0.42) a	15.6 (0.49) a	9.68 (0.37) c	16.3 (1.01) a	12.3 (0.18) b	**P<0.001**	P=0.837	P=0.996
$\Delta^{18}O_{lb}$ (‰)	36.2 (0.34)	35.9 (0.34)	35.7 (0.34)	36.0 (0.38)	36.3 (0.34)	35.7 (0.36)	P=0.775	P=0.854	P=0.803
$\Delta^{18}O_{lw}$ (‰)	9.46 (2.14)	7.70 (2.44)	10.6 (3.59)	9.13 (1.97)	8.36 (1.88)	9.13 (1.03)	P=0.406	P=0.874	P=0.502
LT (µm)	79.8 (2.67)	76.1 (2.66)	74.5 (2.17)	80.0 (3.31)	74.6 (3.35)	68.1 (3.28)	P=0.093	P=0.827	P=0.552
SD (mm^{-2})	188 (6.18) b	216 (2.25) ab	200 (4.31) b	134 (5.55) c	247 (14.8) a	203 (5.81) b	**P<0.001**	P=0.971	P=0.375
LMA (g m^{-2})	34.8 (0.805)	35.2 (0.402)	35.2 (0.982)	33.4 (1.87)	39.3 (2.11)	32.6 (0.643)	P=0.322	P=0.962	P=0.383
TLA (m^2)	0.379 (0.03) ab	0.418 (0.03) ab	0.320 (0.03) b	0.412 0.04) ab	0.405 (0.04) ab	0.483 (0.01) a	**P=0.017**	P=0.947	P=0.599

Table 3. Pearson's correlation table for the traits measured during the experiment. Correlations were computed at individual tree level. Degree of significance indicated as *, ** and *** indicating P<0.05, P<0.01 and P<0.001, respectively.

	$\Delta^{13}C_{lb}$	$\Delta^{13}C_{ss}$	A	g_s	A/g_s	C_i/C_a	BM	WU	TE	$\Delta^{18}O_{lb}$	$\Delta^{18}O_{lw}$	LT	SD	LMA	TLA
$\Delta^{13}C_{lb}$															
$\Delta^{13}C_{ss}$	**0.537*****														
A	**-0.397*****	**-0.336*****													
g_s	0.179	-0.242	0.096												
A/g_s	**-0.412*****	**-0.485*****	**0.579*****	-0.228											
C_i/C_a	**0.464*****	**0.453*****	**-0.726*****	0.224	**-0.916*****										
BM	**-0.530*****	**-0.570*****	**0.396*****	0.194	-0.009	-0.183									
WU	-0.166	-0.258	0.138	**0.361*****	-0.183	0.108	0.183								
TE	**-0.532*****	**-0.544*****	**0.283****	-0.197	**0.428*****	**-0.482*****	**0.431*****	-0.182							
$\Delta^{18}O_{lb}$	0.002	0.088	-0.119	**-0.288****	0.176	-0.091	-0.169	**-0.369*****	0.160						
$\Delta^{18}O_{lw}$	0.115	0.056	-0.069	**-0.257***	0.172	-0.082	-0.163	**-0.295****	0.082	**0.308****					
LT	-0.020	0.030	-0.150	-0.247	0.143	-0.034	-0.192	**-0.257***	0.112	0.178	-0.019				
SD	**-0.393*****	**-0.447*****	**0.387*****	0.100	**0.331****	**-0.378*****	**0.587*****	0.203	**0.401*****	-0.050	-0.151	-0.204			
LMA	-0.226	-0.227	0.132	0.082	0.071	-0.172	**0.298****	0.178	0.170	0.187	-0.132	-0.026	0.221		
TLA	0.050	-0.051	0.069	**0.470*****	-0.246	0.194	0.213	**0.386*****	-0.182	**-0.308****	**-0.283****	**-0.282****	0.219	0.035	

Table 4. Spearman rank correlation table (r) between genotype means for the isotopic discrimination between the atmosphere and bulk leaf matter ($\Delta^{13}C_{bl}$) and for the intrinsic transpiration efficiency at leaf level recorded for the 6 genotypes in different experiments including the present one. Data from the present paper (present), from Fichot *et al.* (2010, nursery grown rooted cuttings over 2 years), Marron *et al.* (2005, greenhouse grown cuttings after a few months) and Monclus *et al.* (2005, nursery grown rooted cuttings over 2 years). n=6.

	$\Delta^{13}C_{lb}$ Present	$\Delta^{13}C_{lb}$ Fichot	$\Delta^{13}C_{lb}$ Monclus	$\Delta^{13}C_{lb}$ Marron	A/g_s Present	A/g_s Fichot
$\Delta^{13}C_{lb}$ Present	1					
$\Delta^{13}C_{lb}$ Fichot	0.657	1				
$\Delta^{13}C_{lb}$ Monclus	0.714	0.714	1			
$\Delta^{13}C_{lb}$ Marron	-0.257	-0.143	0.371	1		
A/g_s Present	**-0.886**	-0.543	**-0.829**	0.0285	1	
A/g_s Fichot	-0.714	-0.714	-0.657	0.314	**0.829**	1

FIGURES CAPTIONS

Figure 1. Relationship between genotype means of the isotopic discrimination between atmospheric CO_2 and the C in bulk leaf matter $(\Delta^{13}C_{lb})$ or in soluble sugars in the leaf $(\Delta^{13}C_{ss})$. Letters indicate genotypes (*P, Pannonia; F, Flevo, I45, I45/51; A, Agathe F; C, Cima; R, Robusta*). Regression equation was: y = 1.34x - 6.30.

Figure 2. Relationship between genotype means of mean diurnal intrinsic transpiration efficiency A/g_s and of A_{sat}/g_{sat}. Letters indicate genotypes (*P, Pannonia; F, Flevo, I45, I45/51; A, Agathe F; C, Cima; R, Robusta*).

Figure 3. Relationship between genotypic means of the ratio of CO_2 concentrations in the intercellular spaces and in the atmosphere C_i/C_a with the isotopic discrimination between atmospheric CO_2 and the C in (i) bulk leaf matter $\Delta^{13}C_{lb}$ (closed circles, y = 18.0 x + 6.98) and (ii) soluble sugars extracted from the leaf $\Delta^{13}C_{ss}$ (open circles, y = 27.2 x + 0.620). The solid line represents the simple discrimination model (see text). Letters indicate genotypes (*P, Pannonia; F, Flevo, I45, I45/51; A, Agathe F; C, Cima; R, Robusta*).

Figure 4. Relationships between genotype means of the isotopic discrimination between atmospheric CO_2 and the C in soluble sugars $\Delta^{13}C_{ss}$, and (a) mean diurnal CO_2 assimilation rate (*A*), ($A = -2.00\ \Delta^{13}C_{ss} + 57.5$); or (b) mean diurnal stomatal conductance to water vapour (g_s). Letters indicate genotypes (*P, Pannonia; F, Flevo, I45, I45/51; A, Agathe F; C, Cima; R, Robusta*).

Figure 5. Relationships between genotype means of whole-plant transpiration efficiency (*TE*) and (a) the isotopic discrimination between atmospheric CO_2 and the C in leaf soluble sugars $\Delta^{13}C_{ss}$; y = -0.215x + 26.3; (b) the mean diurnal ratio of A/g_s; y = 0.872x + 7.19 (c) the biomass gained by the plants during the experiment (*BM*) y = 10.1x + 8.72 and (d) total water

use by the plants (*WU*) during the experiment. Letters indicate genotypes (*P*, *Pannonia*; *F*,

Flevo, *I45*, *I45/51*; *A*, *Agathe F*; *C*, *Cima*; *R*, *Robusta*).

Figure 6. Relationships between genotype means of stomatal density and (a) the isotopic

discrimination between atmospheric CO_2 and the C in soluble sugars extracted from the leaf

$\Delta^{13}C_{ss}$; y = -0.015 x + 26.3; (b) mean diurnal *A;* y = 0.032 x + 4.48. Letters indicate genotypes

(*P*, *Pannonia*; *F*, *Flevo*, *I45*, *I45/51*; *A*, *Agathe F*; *C*, *Cima*; *R*, *Robusta*).

Figure 7. Distribution of the 15 variables (a) and projection of the 6 *Populus x*

euramericana genotypes (b) in the factorial plane F1 × F2 of the PCA. Letters indicate the

names of the genotypes (*P*, *Pannonia*; *F*, *Flevo*, *I45*, *I45/51*; *A*, *Agathe F*; *C*, *Cima*; *R*,

Robusta). F1 and F2 are linear combinations of the 15 variables. See Table 1 for variable

codes.

Figure 1.
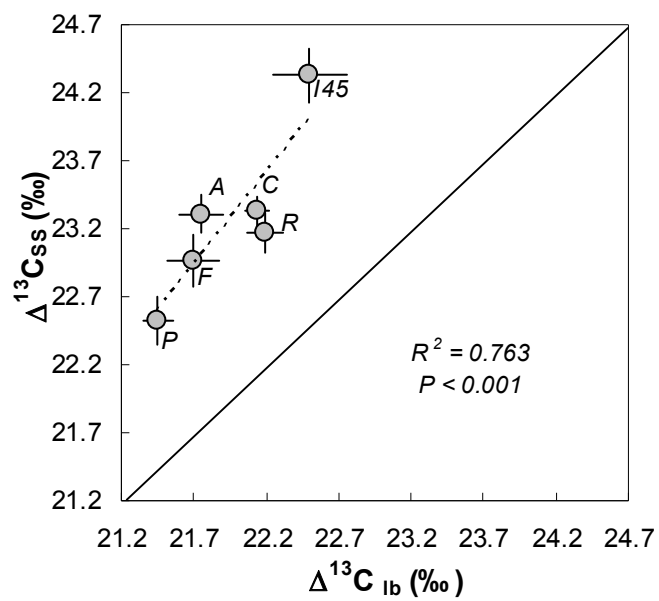
Figure 2.
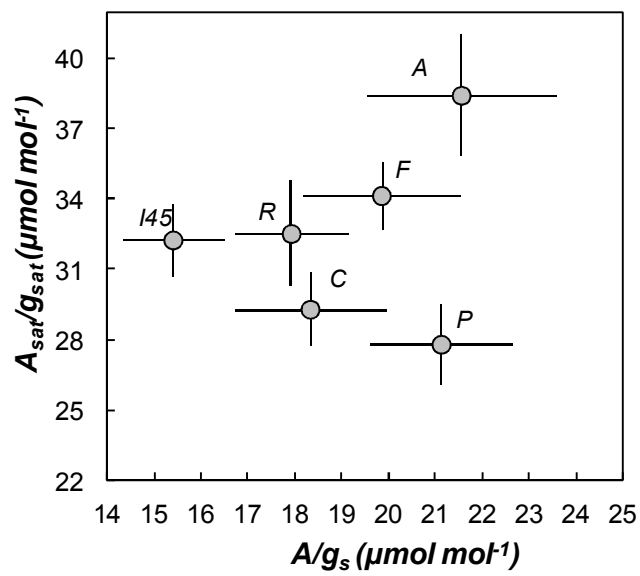
Figure 3.
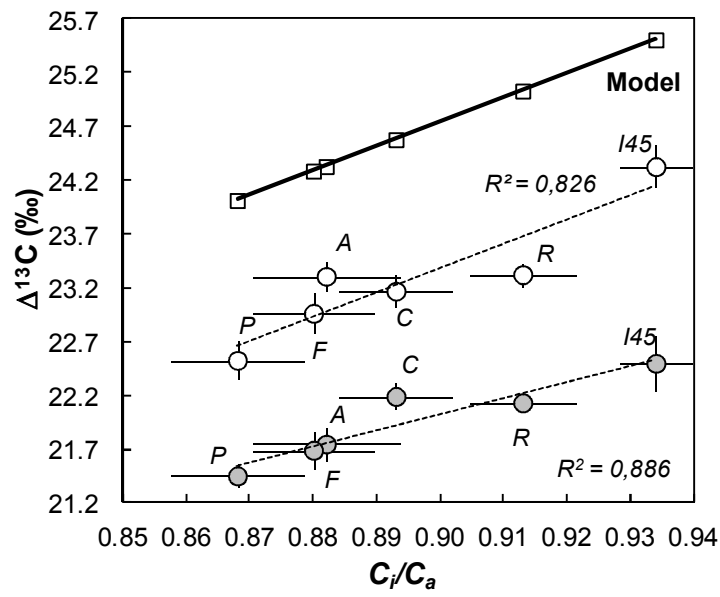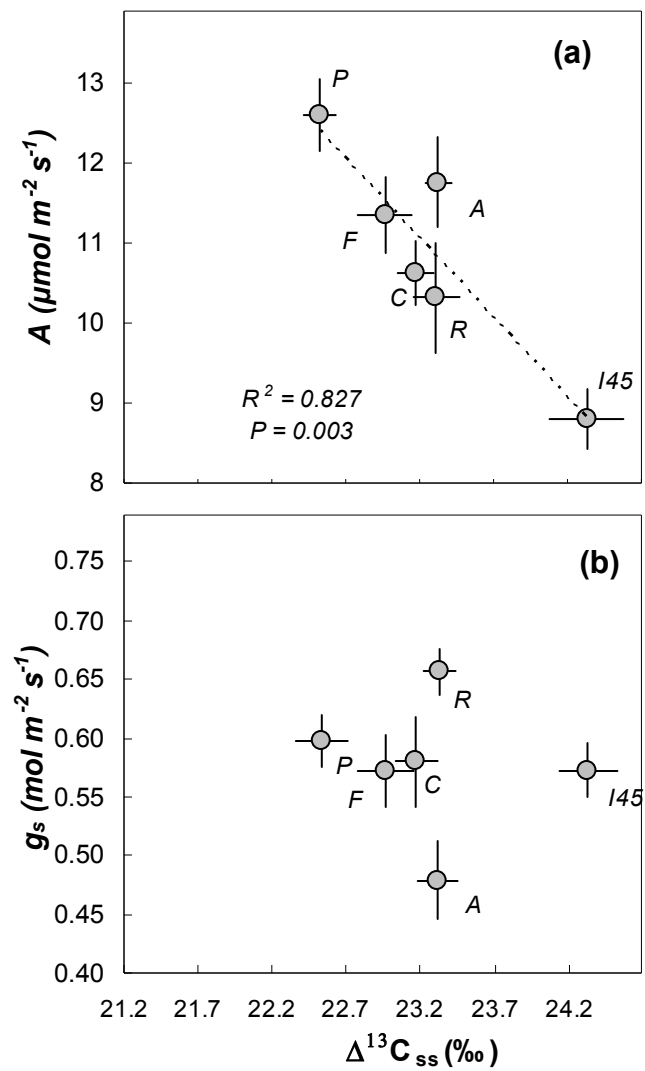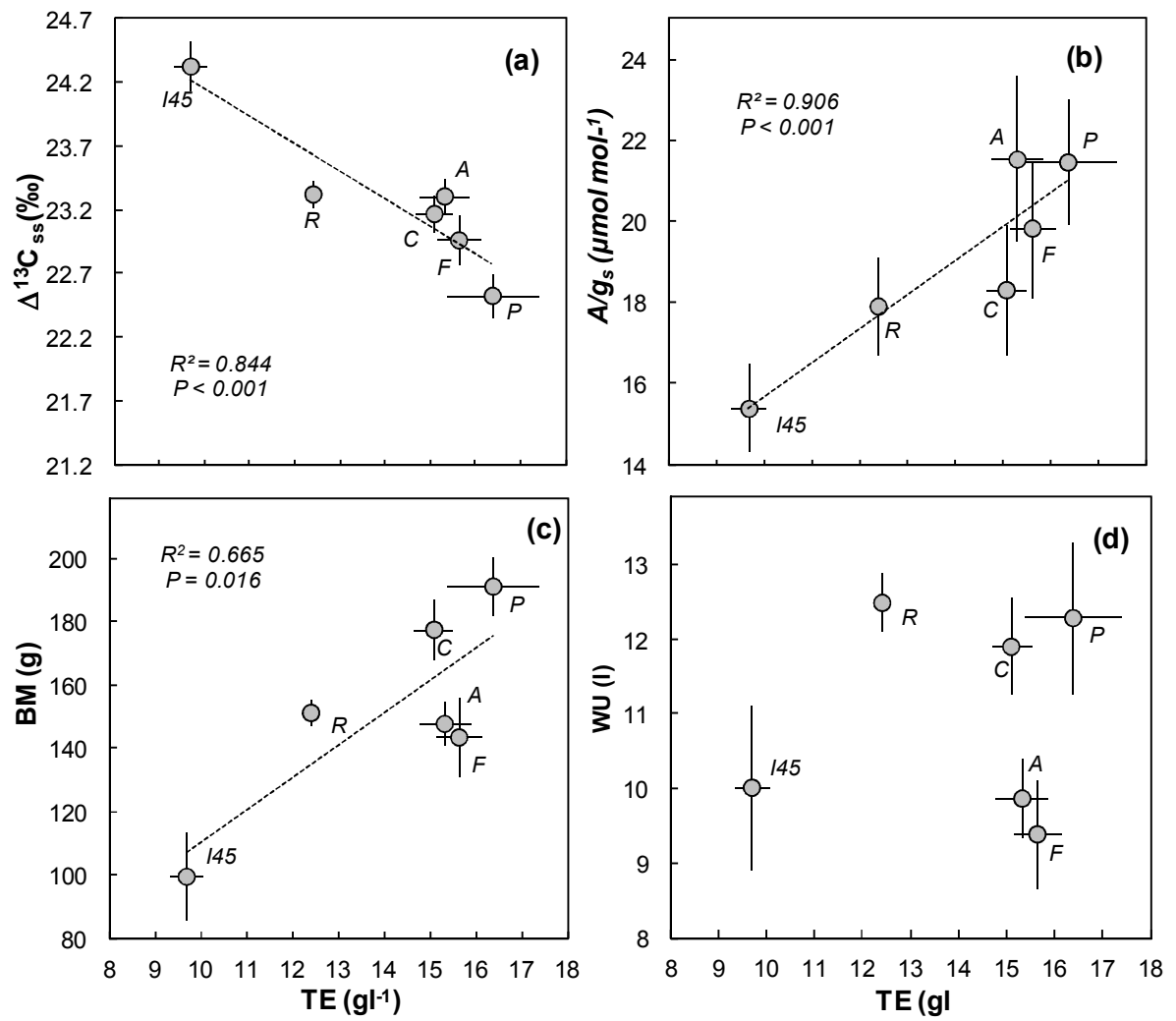
Figure 4.

Figure 5.

Figure 6.
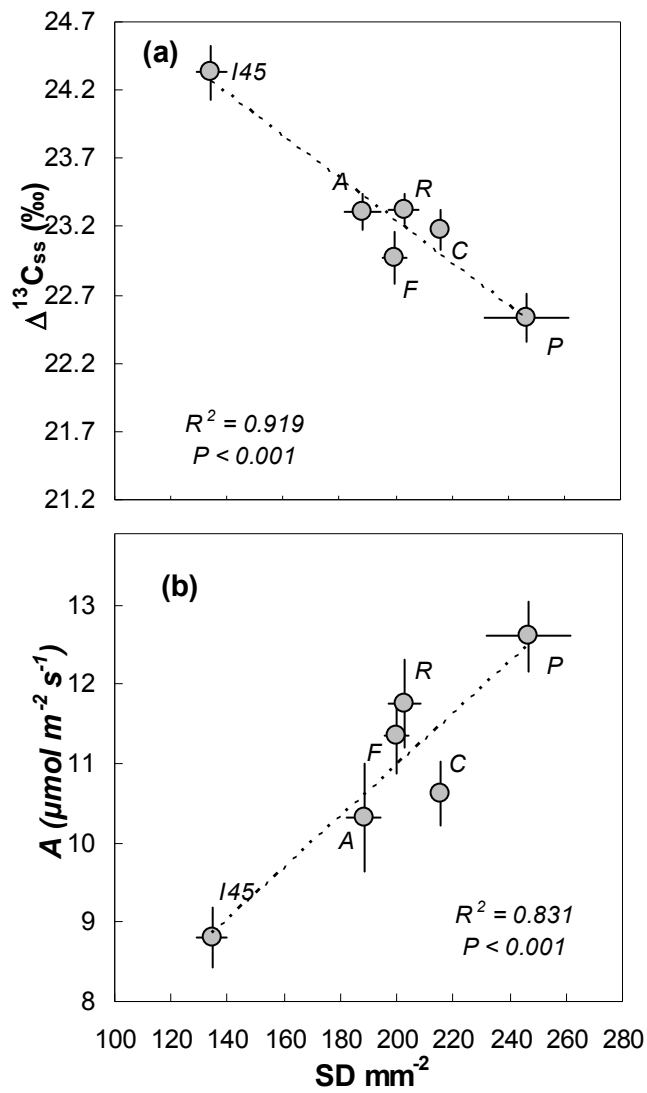
Figure 7.
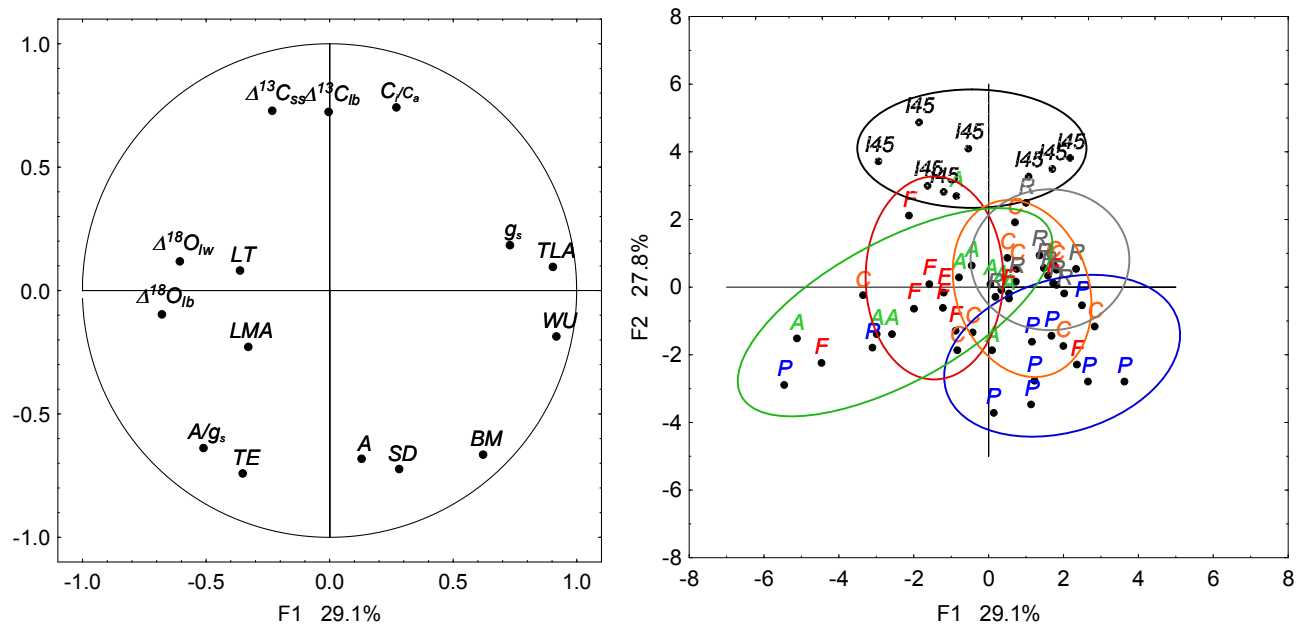
Supplementary Figure 1. Correlation between the product Height x Diameter and the biomass recorded on 6 individuals per genotype at the onset of the experiment. Each point represents data from a single individual. No genotype-specific relationship was detected.
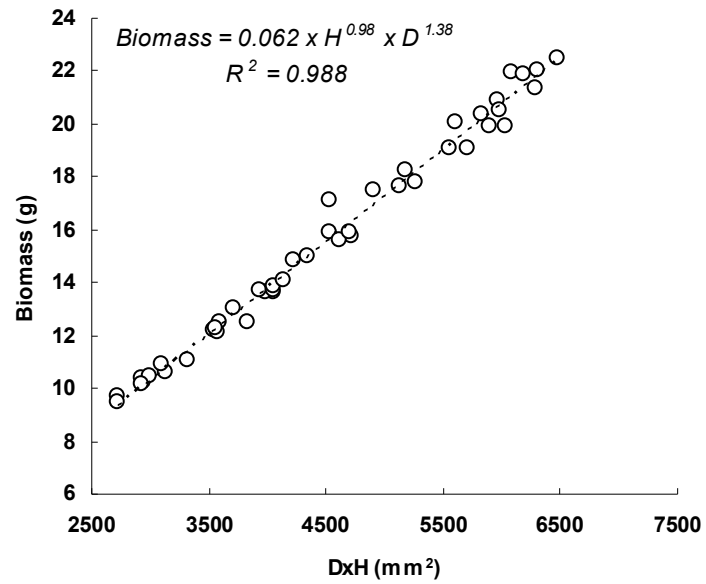
Supplementary Figure 2. Time course of stomatal conductance to water vapour g_s (a) and net CO_2 assimilation rate A (b). Means ($\pm$ SD) of ten plants per genotype along a single measurement day. Each point represents a mean value from ten individuals. No visible diurnal trend was identified in any of the genotypes.
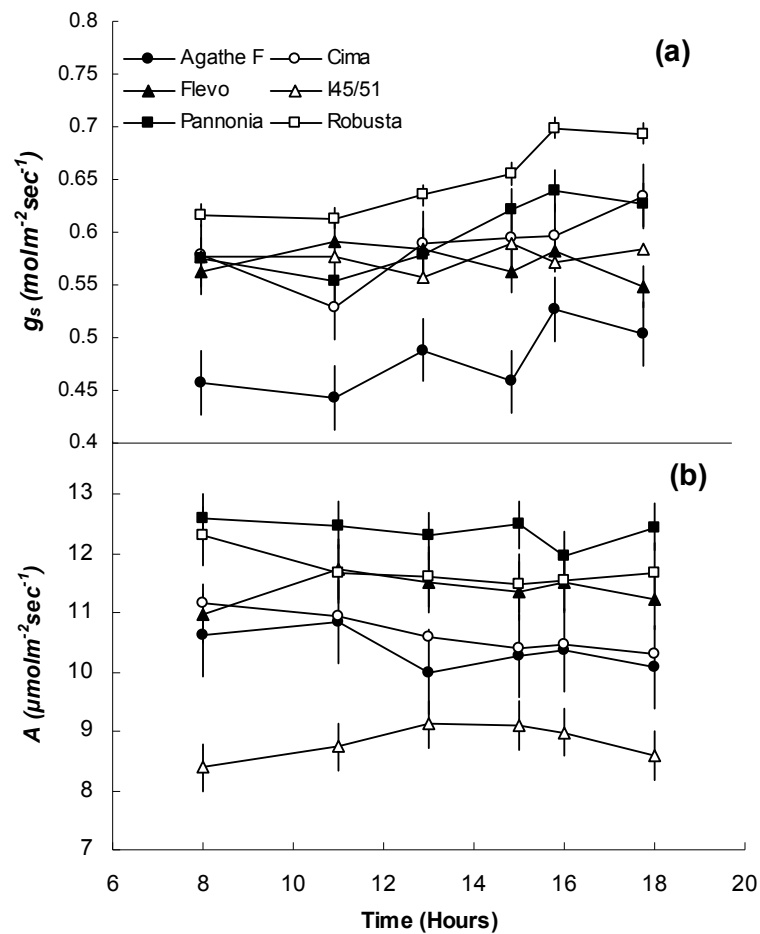
Supplementary Figure 3. Time course of genotypic means of plant height *(H)*, diameter at collar *(D)* and daily water use (*WU)* during the experiment in the climate chambers. Each point represents the mean value from ten individuals.
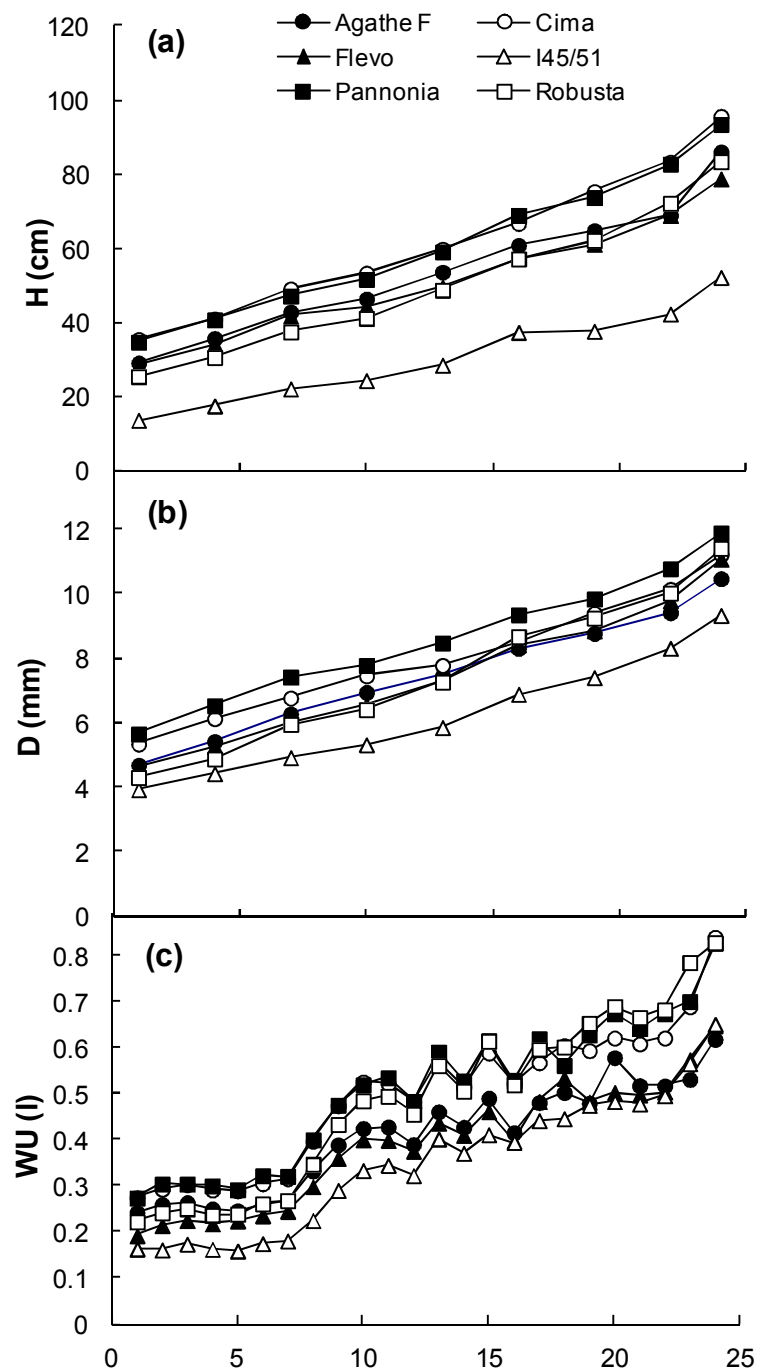
CHAPTER 2

Effects of *VPD* on upscaling from leaf level to whole plant level transpiration efficiency in 6 *Populus nigra* genotypes.

Transition

In the previous chapter we successfully showed that $\Delta^{13}C$ recorded in soluble sugars represented the genotypic differences in leaf and whole plant transpiration efficiency. As anticipated, the magnitude of genotypic variation intrinsic *TE* was much lower than that evidenced at whole plant level, which was attributed to carbon lost during photo and dark respiration (Φ_c) and water lost through nocturnal transpiration (Φ_w) that remained same across genotypes. Effects of *VPD* on g_s have been well documented, which decreases exponentially with increase in *VPD* (Oren *et al.* 1999), while increasing transpiration rate. However it might also effect variation of carbon lost during photo and dark respiration (Φ_c) and water lost through nocturnal transpiration (Φ_w) that could perturbate the up scaling approach. Thus, effect of *VPD* on upscaling from leaf to whole plant transpiration efficiency was evidenced in 6 *Populus nigra* genotypes grown under high and low condition of *VPD*. Genotypes selected show contrasting $\Delta^{13}C$ values and originated from the natural population along the Loire river (Chamaillard *et al.* 2011). $\Delta^{13}C$ was measured in both leaf bulk and leaf soluble sugars with respect to atmosphere, along with leaf level *TE* (A/g_s) and whole plant *TE* (biomass produced/ water transpired). ^{18}O enrichment was also evidenced in leaf bulk and leaf water with respect to source water as an indicator for g_s.

Vapour pressure deficit during growth does not affect the ranking of *Populus nigra* genotypes for transpiration efficiency at leaf as well as at whole tree level.

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Key words: leaf gas exchange, ^{13}C discrimination, ^{18}O enrichment during tranpiration mesophyll conductance, , stomatal conductance;. stomatal density, leaf thickness

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Total word count (total file): 12727; Word counts for Introduction: 1277; Word counts for Materials and Methods: 2001; Word counts for Results: 934; Word counts for Discussion: 2358; Word counts for Conclusion: 178; Word counts for Acknowledgements: 110

Number of figures: 8; Number of tables: 3; Number of supporting information: 2 Tables and 4

Figures

Summary

• Poplar genotypes differ in transpiration efficiency (*TE*) at leaf and whole plant level. Here, we test whether vapour pressure deficit (*VPD*) during growth affects the genotype ranking for *TE* at leaf and whole tree level.

• Six *Populus nigra* genotypes were grown under two *VPD*. We recorded: (i) ^{13}C content in soluble sugars; (ii) [18]O enrichment in leaf water vs. source water; (iii) leaf-level gas exchange and (iv) biomass accumulation and water use by whole plants.

• Enhanced *VPD* had no effect on biomass accumulation but increased water-use which results in reduced whole plant *TE*. However, it increased intrinsic *TE* due to reduced stomatal conductance (g_s). It also resulted in an enhanced enrichment of leaf water and leaf matter in [18]O. Whole plant and intrinsic leaf *TE* differed significantly among genotypes and *VPD* had no effect on genotype ranking. g_s had a larger contribution to the genotype variability of intrinsic *TE* than *A*.

• Finally, ^{13}C composition of leaf sugars differed significantly from the modeled one and this difference was modulated by genotype and was smaller under high *VPD*. This may reflect changes in mesophyll conductance to CO_2, which may increase under high *VPD*.

WORD COUNT = 195 words.

Introduction

Tree plantations in different regions of the world will experience an increased frequency and severity of drought episodes as a consequence of climate change (IPCC 2007), while biomass can only be produced at the expense of water use. One of the potential adaptations to such a situation could be the use of species or genotypes with enhanced water use-efficiency. Considerable gains in water-use efficiency have been made, noticeably in crops, by selecting plants with enhanced transpiration efficiency (*TE*), i.e., the ratio between accumulated biomass and transpired water (Rebetzke *et al.,* 2002, Richards *et al.,* 2002). *TE* at whole plant level is controlled by: (i) intrinsic *TE* (A/g_s) which is the ratio of net CO_2 assimilation rate A to stomatal conductance to water vapour g_s (Condon *et al.,* 2004); (ii.) atmospheric *VPD* which may increase directly transpiration but also decrease stomatal and (iii) scaling factors from leaf to whole plant, like respiratory carbon losses (Φ_c) and water losses (Φ_w) not associated to photosynthesis (Farquhar *et al.* 1989). Φ_c depends on the intensity of respiration from stems and roots, and of nocturnal respiration, while Φ_w depends on water losses by stems or fruits as well as nocturnal transpiration. *TE* also displays an importannt phenotypic plasticity in response to water (Hubick *et al.,* 1986; Zhang & Marshall, 1994; Sun *et al.,* 1996) and nutrient availabilities (Livingston *et al.,* 1999; Cernusak *et al.*, 2007a) and vapour pressure deficit (Lange *et al.,* 1971; Massman & Kaufmann, 1991; Oren *et al.,* 1999).

At leaf level, intrinsic *TE* is tightly correlated to the ratio between CO_2 mole fraction in the intercellular spaces (C_i) vs. in the atmosphere (C_a), assuming a lack of difference in atmospheric pressure (Farquhar *et al,* 1989). Cernusak *et al.* (2007b; 2008) confirmed that whole plant *TE* was correlated to C_i/C_a across a range of tropical tree species. Nevertheless, Φ_c and Φ_w remained important determinants of whole plant *TE* (Osorio *et al.,* 1998 and Hobbie & Colpaert, 2004). In *Populus × euramericana* genotypes, genotype values of C_i/C_a were tightly correlated to whole-plant *TE* (Rasheed *et al., 2012*) although the amplitude of

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variation was larger for whole-plant *TE* than for C_i/C_a, pointing to the importance of Φ_c and Φ_w. as drivers for some degree of genotype variability of whole plant *TE*.

The discrimination against ^{13}C between the atmosphere and leaf matter ($\Delta^{13}C$) has largely been used as an estimator of intrinsic *TE* (Farquhar & Richards, 1984; Farquhar *et al*., 1989). In particular, $\Delta^{13}C$ recorded from soluble sugars extracted from leaves, which are the primary products of photosynthesis, is closely correlated to mean diurnal A/g_s aand therefore C_i/C_a (Brugnoli *et al*., 1988; Rasheed *et al*., 2012). In the simple form of the discrimination model, fractionation during carboxylation is correlated to the ratio of CO_2 concentration in the intercellular spaces to ambient air (C_i/C_a) rather than to the ratio CO_2 concentration in the chloroplast to ambient (C_c/C_a) (Richards and Farquhar, 1984)

$$\Delta^{13}C = a + (b-a)\frac{C_i}{C_a} \qquad\qquad \text{Eqn 1}$$

This model is an oversimplification. C_c is significantly smaller than C_i due to the fact that mesophyll conductance (g_m) to CO_2 is not infinite (Evans & von Caemmerer, 1995; Flexas *et al.,* 2008). A genotypic variability of g_m may therefore modulate the relationship between $\Delta^{13}C$ and C_i/C_a i.e., intrinsic *TE* (Warren & Adams, 2006; Barbour *et al.,* 2010). Moreover, g_m varies in the short term and increases with irradiance and decreases under enhanced CO_2 concentration (Flexas *et al.,* 2009; Douthe *et al.,* 2011; 2012). Therefore, if $\Delta^{13}C$ is to be used as an indicator of intrinsic or whole plant *TE* it becomes very important to check the tightness of the relationship between $\Delta^{13}C$ and C_i/C_a and to refer to the developed model of $^{13}CO_2$ discrimination at leaf level that includes explicitly C_c and fractionation associated to respiration and photorespiration (Farquhar and Richards 1984):

$$\Delta^{13}C = a_b\frac{C_s - C_i}{C_a} + a\frac{C_s - C_i}{C_a} + (b_s + a_l)\frac{C_i - C_c}{C_a} + b\frac{C_c}{C_a} - f\frac{\Gamma^*}{C_a} - e'\frac{R_d}{A + R_d}\frac{C_c - \Gamma^*}{C_a} \qquad \text{Eqn 2}$$

Where: C_s is the CO_2 concentration at leaf surface,. e' and f are the fractionation constants associated to mitochondrial respiration during the day and photorespiration respectively. Γ^* is the CO_2 compensation point in the absence of mitochondrial respiration (R_d). The difference between the predictions of the simple model and recorded $\Delta^{13}C$ is the basis for indirect estimates of g_m (Evans & von Caemmerer, 1995; Flexas *et al.,* 2009).

Increased *VPD* in the atmosphere is known to impact severely *TE* (i) directly by increasing transpiration and (ii) indirectly by inducing a decrease of stomatal conductance (Oren *et al.,* 1999). The first component is physical (it acts directly on evaporation) and contributes to Φ_w. The latter may differ significantly from air temperature as a function of stomatal conductance. Enhanced *VPD* therefore is expected to result in decreased *TE*. The second component relates to the sensitivity of stomata to *VPD*: enhanced *VPD* results in stomatal closure (Monteith, 1995). The short-term effect of increased *VPD* is therefore to increase intrinsic *TE*, and consequently to decrease $\Delta^{13}C$. The relationship between $\Delta^{13}C$ and intrinsic *TE* on the one hand, and whole plant *TE* on the other, is therefore likely to be severely impacted by *VPD*. Moreover, the sensitivity of stomata to *VPD* may display some degree of genotypic variability (Vadez *et al.*, 2011), although this remains poorly documented. It is therefore important to investigate the long term effects of changes in *VPD* on whole plant *TE*.

For genotypes growing under a common environment, discrimination against [18]O between source water and organic matter ($\Delta^{18}O_{lb}$) or leaf water ($\Delta^{18}O_{lw}$) reflects the enrichment due to evaporation in the intercellular spaces and is modulated by g_s (Craig & Gordon, 1965; Barbour *et al.,* 2000a; Barbour & Farquhar, 2000; Barbour, 2007). Increased *VPD* is expected to enhance that enrichment due to enhanced transpiration. Thus, Scheidegger *et al.* (2000) and Cabrera-Bosquet *et al.* (2009) used [18]O enrichment in leaves as an indicator of g_s to identify whether the genetic variability of intrinsic *TE* and $\Delta^{13}C$ was related to a variability of stomatal

control of transpiration or to a variability of assimilation rates. Rasheed *et al.* (2012) found a significant phenotypic correlation between g_s and ^{18}O enrichment in leaves of *Populus × euramericana* hybrids but no genotypic variability. The effect of *VPD*, which decrease g_s while increasing transpiration rate, needs be checked for potential genotype effects and for the interactions with $\Delta^{13}C$ to help identify the physiological basis of genotype variation in intrinsic as well as whole plant *TE* (Barbour, 2007; Grams *et al.,* 2007).

Although poplars require large amounts of water to sustain their productivity, a large variability has been found for $\Delta^{13}C$ among genotypes of a range of species including *Populus nigra* native to riverbanks in Western Europe (Chamaillard *et al.,* 2011). Moreover, $\Delta^{13}C$ was confirmed as an efficient estimator for the genotype variation in intrinsic *TE* and whole plant *TE* of *P. euramericana* genotypes (Rasheed *et al.,* 2012) under optimal growth conditions. In the present study we address the effects of environment (*VPD*) on $\Delta^{13}C$, intrinsic and whole plant *TE* for 6 genotypes of *Populus nigra* known to differ in $\Delta^{13}C$. Following questions were specifically addressed:

- Does $\Delta^{13}C_{ss}$ remain a reliable indicator for intrinsic *TE* (A/g_s) at leaf level in *Populus nigra* genotypes under low or high *VPD*)? This will be tested by comparing the ranking of genotypes for $\Delta^{13}C_{ss}$ and intrinsic *TE* under high and low VPD.

- Do genotypic differences for $\Delta^{13}C_{ss}$ and intrinsic *TE* reflect the differences in whole plant *TE* under different *VPD* ?

- Is there an offset between discrimination values predicted by the simple discrimination model and observed values, and is it affected by genotype and/or *VPD* during growth?

MATERIAL AND METHODS

Plant material and growth conditions

We selected six genotypes of *Populus nigra* L. from a natural population sampled along the Loire river. The genotypes displayed contrasting values of $\Delta^{13}C_{lb}$ (Chamaillard *et al.,* 2011). Ten liter pots were filled with a 1/1 v/v peat/sand mixture, heavily watered and extra water was left to drain overnight to reach field capacity. The weight of filled pots was homogenized to 9000 g by adding substrate at field capacity with a precision of 1%. Eight soil cores were sampled randomly and dried (70°C) to a constant weight; mean soil water content at field capacity was 312 g kg^{-1}..

Shoot cuttings (16 copies from 6 genotypes) were planted during April 2010 and left to grow for one month in a greenhouse at INRA-Nancy (France), under natural daylight. After this time, diameter at collar and height were recorded on all plants immediately before the transfer to two climate chambers (Rotoplant, Strader, Angers, France). Six randomly selected individuals per genotype were harvested, oven dried at 70°C till constant weight to calibrate a relationship between the product height-diameter and biomass that was common across genotypes (Fig. S3):

Biomass = 0.0042 x Height$^{0.63}$ x Diameter$^{2.68}$ (r^2 = 0.900; *P* < 0.001)   Eqn 3

This allometric equation was used to estimate the initial biomass of each of the remaining individuals (mean: 17 ± 4g).

Five individuals per genotype were randomly distributed in each of the two growth chambers under either low or high VPD. Chambers were equipped with a rotating plate to homogenize the irradiance intercepted by plants. Microclimate in the chambers was: day/night, 16/8 h; air temperature day/night, 30/18°C; *VPD* day/night, High: 2.3/0.8 kPa and

Low: 1.1/0.8 kPa; irradiance at the top of the plants, 450 ± 15 µmol PPFD m^2 s^{-1}. The light in the climate chambers was switched on at 8:00 am.

Growth, daily water use and transpiration efficiency *TE*

Plant height *(H)* and diameter at collar *(D)* were measured twice a week till the end of the experiment. Soil surface of each pot was covered with a polyethylene sheet to limit direct soil evaporation. Each pot was weighted daily (Sartorius-AG Göttingen, QC65EDE-D, Germany; accuracy: $\pm$ 0.1g) and watered back to the reference weight of 8370 g (corresponding to 80% field capacity). Weight difference over 24h was assumed to represent daily water use by the plant. Cumulated water use (*WU*) was computed for each plant over the duration of the experiment.

After one month, plants were harvested; oven dried at 70°C till constant weight and dry mass of each compartment (leaves, stem and roots) recorded. Biomass increment (*BM*) was computed for each individual from the difference between harvested and initial dry mass. Whole plant *TE* was computed per individual as the ratio biomass increment/cumulated water use. Total leaf area (*TLA*) of each individual was measured with an area meter (Li-Cor area meter, A1000 Li-Cor, Lincoln, NE, USA). Due to a problem during the last phase of the experiment, 11 plants (2 per genotype in 5 genotypes and 1 in the sixth) were lost in the high VPD chamber; as a result, means values of BM, WU and whole plant TE where computed with a smaller sample while all other data were recorded earlier on all individuals.

Leaf gas exchange and intrinsic *TE*

All measurements were recorded on fully expanded leaves that had grown in the climate chamber under the local environment. Intrinsic *TE* at leaf level was recorded on one leaf per

individual: (i) under saturating irradiance as the ratio A_{sat} /g_{sat}, and (ii) under the standard irradiance available in the growth chamber (A/g_s).

A_{sat} and g_{sat} were measured by placing a fully mature leaf for 15-20 min into the measurement chamber of a portable photosynthesis system (Li6400; Li-Cor, Lincoln, NE, USA). A 6 cm^2 chamber was used and artificial irradiance was provided by blue-red LEDs. Conditions were: PPFD, 1200 µmol m^2 s^{-1}, CO_{2inlet}, 380 µmol mol^{-2}, *VPD*, 1 ± 0.2 kPa and leaf temperature, 25°C.

A, g_s and T_l (leaf temperature) were monitored with a portable photosynthesis system (Li-6400; Li-Cor, Lincoln NE, USA) equipped with a transparent lid. Measurements were made during a diurnal cycle, five times between 8:00 and 18:00 pm on a preselected fully expanded and well-lit leaf of same age from each individual. Each measurement lasted 2-3 min for stabilization of gas exchange in the chamber. Microclimate in the measurement chamber was very close to that in the climate chamber: ambient irradiance, 450 ± 15 µmol m^2 s^{-1}, CO_2 in the chamber, C_a, 405 ± 30 mmol mol^{-1} of CO_2, VPD Low, 1.2 ± 0.2; High, 2.07 ± 0.2 kPa and leaf temperature, 30 ± 0.6°C estimated through the gas exchange system. *VPD* in the measurement chamber was very close to that in the climate chamber with no genotype effects (see Table 2). C_a did not differ among genotypes under the two treatments during the measurement (P = 0.527 and P = 0.326 respectively, data not shown). A significant diurnal fluctuation was evident for actual net CO_2 assimilation (A) and stomatal conductance to water vapour (g_s) recorded in the chambers (ANOVA with time as a random effect, $P < 0.001$ for A and $P < 0.001$ for g_s see Fig. 1a,b).

At the end of the measurement cycle, the leaf was clipped off, frozen in liquid nitrogen and stored at -80°C. As a diurnal decline was evidenced in A (Fig. 1a), a daily mean value of A/g_s weighted by A was calculated as:

$$(A/g_s)_{mean} = \frac{\sum_{t=1}^{n}(A^2/g_s)}{n\Sigma A} \qquad \text{Eqn 4}$$

Extraction of soluble sugars from leaves

Half of each stored leaves was freeze-dried at -35°C, grinded and 60 mg of leaf powder was weighted in 2 ml microtubes. 350 µl of methanol-chloroform-water (MCW, 12/5/3, v/v/v) was added and the samples were placed in a water bath at 70°C for 30 min. After cooling, the microtubes were centrifuged at 11400g for 3 min. This step was repeated three times and the supernatant was collected into a new 2 ml microtube.

To induce phase separation, 200µl chloroform and 500µl deionised water were added to the supernatant and vigorously mixed. The samples were left for a few minutes and then centrifuged at 11400 g for 3 min. The aqueous phase was transferred to a new 2 ml microtube and complemented with 300 µl freshly hydrated Dowex 1X8, 200-400 mesh $[Cl]^-$ form resin, (Fluka France) converted to $[HCO_2]^-$ with sodium formate The samples were agitated for 2 h at room temperature. Following centrifugation, the supernatant was transferred to a new 2 ml microtube to which 300 µl of freshly hydrated Dowex 50W, 200-400 mesh (cation exchange, H^+ form; Fluka, France) resin was added. Samples were again agitated for 2 h at room temperature. Following centrifugation, the supernatant was transferred to a pre-weighted microtube and dried to complete dryness on a rotary evaporator (HETO, DK3450, Allerød, Denmark). After being weighted, 60 µl of water per mg of bulk sugar was added to move 1 mg of bulk sugars into tin capsule and freeze-dried (FreeZone, Labconco, Kansas city, USA) and stored for isotopic analysis.

Carbon isotope discrimination between the atmosphere and bulk leaf matter $(\Delta^{13}C_{lb})$ or soluble sugars $(\Delta^{13}C_{ss})$

1 mg dry leaf powder was weighed into tin capsules and used to measure ^{13}C composition in bulk leaf matter. A similar procedure was used for the soluble sugar pool. ^{13}C content was analysed relative to PDB using an elemental analyser (Carlo Erba, NA 1500-NC, Milano, Italy) coupled to an isotope-ratio mass spectrometer (Finnigan, Delta-S, Bremen, Germany) with a precision of 0.1 ‰. ^{13}C discrimination between the atmosphere and leaf bulk matter or soluble sugars was calculated as:

$$\Delta^{13}C = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \dfrac{\delta^{13}C_{air}}{1000}} \qquad \text{Eqn 5}$$

where $\delta^{13}C_{air}$ and $\delta^{13}C_{plant}$ are the isotopic composition of air and plants measured in the two growth chambers. Air samples were collected in 8 ml flasks during 4 days (n = 36) and analyzed in a CF-IRMS (Finnigan, Delta-S, Bremen, Germany) with an accuracy of 0.1‰. A small but significant diurnal increase was recorded in $\delta^{13}C_{air}$ in parallel to the decrease in A (Fig. S4). For the computation of a mean daily discrimination, we used a daily weighted mean for $\delta^{13}C_{air}$ to take into account that $\delta^{13}C_{air}$ changed with time in correlation with the values of A:

$$\delta^{13}C_{air\,mean} = \frac{\sum\limits_{t=1}^{n}(\delta^{13}C_{air}^2 \times A)}{n\Sigma A} \qquad \text{Eqn 6}$$

No chamber effect was detected for $\delta^{13}C_{air}$ ($P = 0.356$, n = 72) and mean values were -10.0 ± 0.41 ‰ (Low *VPD*) and -10.3 ± 0.43 ‰ (High *VPD*). Carbon isotope discrimination predicted by the model $(\Delta^{13}C_M)$ was calculated using the simple discrimination equation:

$$\Delta^{13}C_M = a + (b - a)\frac{C_i}{C_a} \qquad \text{Eqn 7}$$

Where *a* was taken as 4.4‰ and *b* as 29 ‰ (O'Leary 1981; Farquhar *et al.* 1998; Warren 2006).

Leaf water extraction

The second half of the stored leaf was used for leaf water extraction with a cryogenic vacuum distillation. Sealed tubes with a frozen leaf were connected to the extraction apparatus with a collection tube at the other end. Air inside the whole apparatus was then evacuated to remove any trace of external water vapor under approx. 8 Pa. The vial containing the sample was heated using a water bath at a constant temperature of 70°C and the collection tube was placed in a Dewar containing liquid nitrogen in order to freeze vapors emanating from the sample (West *et al.*, 2006). The extracted water from each leaf sample was collected and used to measure ^{18}O composition.

Oxygen isotope discrimination between irrigation water and bulk leaf matter ($\Delta^{18}O_{lb}$) or leaf water ($\Delta^{18}O_{lw}$)

0.3 - 0.4 mg of leaf powder (the same than for ^{13}C analysis) and 0.4 µl of leaf extract were used to measure ^{18}O composition of leaf bulk matter ($\delta^{18}O_{lb}$) and ^{18}O composition of leaf water ($\delta^{18}O_{lw}$) respectively. Analyses were done using a high temperature elementar analyser (Pyrocube, Elementar, Hanau, Germany) coupled to a mass spectrometer (Isoprime, Manchester, UK). Samples were combusted and pyrolised at 1270°C. The oxygen isotope composition was determined with respect to the three laboratory standards (ATL1, U Prye, Snow D). Laboratory standards were pre-calibrated against the international standard V-SMOW (Vienna- Standard Mean Ocean Water). Accuracy of the measurements was ± 0.3‰.

The ^{18}O content in irrigation water ($\delta^{18}O_{ws}$) was recorded from 9 samples collected during different days. The mean value was -6.28‰ (0.38‰) with no difference between the two chambers ($P = 0.824$, n = 12).

^{18}O discrimination between irrigation water and bulk leaf matter or leaf water was computed as:

$$\Delta^{18}O = \frac{\delta^{18}O_{plant} - \delta^{18}O_{SW}}{1 + \dfrac{\delta^{18}O_{SW}}{1000}} \qquad \text{Eqn 8}$$

where $\delta^{18}O_{plant}$ and $\delta^{18}O_{sw}$ are the isotopic composition of the plant material and source water.

Leaf anatomy

Three discs of 1 cm^2 were harvested on 1 leaf per individual to record leaf thickness (*LT*) and stomatal density (*SD*) and were immediately frozen in liquid nitrogen and stored at $-80°C$. Because poplar leaves are amphistomatous, each disc was split for the separate analysis of adaxial and abaxial sides. Sample discs were then stuck to aluminium stubs on a Peltier stage ($-50°C$) before being examined under a controlled-pressure scanning electron microscope (model 1450VP, Leo, Cambridge, UK); 20–30 Pa inside the chamber, accelerating voltage 15 kV, working distance 12 mm). Nine microphotographs at ×300 were taken on each disc and stomata were counted using an image analysis software (VISILOG, Noesis, France). Total stomatal density was calculated as the sum of adaxial and abaxial stomatal densities. On the other two discs, five semi-thin cryo sections were photographed to record mean leaf thickness. Additional five discs of 1cm^2 were taken for *LMA* and were weighted after oven drying until constant weight. *LMA* (g m^{-2}) was computed as dry weight per unit area.

Statistical analysis

All statistical analyses were done using STATISTICA software (version 8.1, StatSoft, Maisons-Alfort, France). Normality and homoscedasticity of data were checked graphically with residual *vs*. predicted and normal quartile-to-quartile plots. Genetic effect, treatment effect and their interaction were evaluated using a two-way ANOVA with the following model:

$$Y_{ijk} = \mu + G_i + T_j + G_i \times T_j + \varepsilon_{ijk}$$

With: Y_{ijk}, response variable; μ, intercept; G_i, genotype effect; T_j, *VPD* effect; $G_i \times T_j$, interaction and ε_{ijk}, residue. A *post-hoc* Tukey HSD test was used to evaluate pair-wise differences among genotypes. Correlations between the measured traits were tested at genotype level in each *VPD* treatment with a general linear regression model. Spearman correlation coefficients were used to check the stability of genotype ranking between the two treatments. All tests and correlations were taken as significant when *P* < 0.05. Means are expressed with their standard deviation (SD).

Results

Table 1 displays a list of the recorded variables and Table 2 the overall means of the different variables in the two treatments along with their standard deviations and the probability of the different effects (*VPD*, genotype and their interaction). Interaction effects were never significant except for stomatal conductance (g_s): in the following, we will therefore concentrate on the main effects. Detailed mean values of the different genotypes are displayed in Table S1. Spearman correlations at individual level for the recorded traits are displayed in Table S2.

Plant height (*H*), stem diameter (*D*) and leaf traits (table 2).

Time courses of *H* and *D* displayed a continuous and gradual increase (see Fig. S1, S2). A significant genotype effect was found for final *H*, whole plant leaf area (*TLA*), leaf mass-to-area ratio (*LMA*) and leaf thickness (*LT*) while *VPD* had no effect on these variables. *VPD* effects were found in addition to genotype effects: final *D* was smaller and stomatal density *(SD)* higher under high *VPD*. LMA was rather large (around 100 g m^{-2}). Leaf N (N_{lb}) was very small (below 1%), differed among genotypes (from 0.736 to 1.01) and remained unaffected by *VPD*. Leaf *C* was close to 43.5% and remained stable among genotypes and treatments.

$Δ^{13}C$ between the atmosphere and bulk leaf matter ($Δ^{13}C_{lb}$) or soluble sugars ($Δ^{13}C_{ss}$)

The fraction of soluble sugars in the leaves was rather high (8.5 ± 0.28% of total dry matter) and stable across treatments but differed among genotypes. Purity of the soluble sugar extract was assessed by recording N and C contents. N_{ss} remained very small (0.16%) with no genotype nor *VPD* effect (Table 2), while C_{ss} was stable among genotypes but differed

slightly across *VPD* treatments but remained close to the 42% as expected for a fraction probably dominated by sucrose.

Significant genotype and *VPD* effects were found for $\Delta^{13}C_{lb}$. High *VPD* resulted in smaller values with a shift around 1‰. Significant genotype and *VPD* effects were similarly evident for $\Delta^{13}C_{ss}$ which declined also by 1.3‰ under high *VPD*. A tight and positive correlation was found between $\Delta^{13}C_{lb}$ and $\Delta^{13}C_{ss}$ in the two treatments, and the two regressions did not differ. $\Delta^{13}C_{ss}$ was larger than $\Delta^{13}C_{lb}$ by about 1.5 to 1.8‰ and this offset was affected by genotype but not by *VPD* (Fig. 2 and Table 2).

Genotype and *VPD* effects on leaf gas exchange and intrinsic transpiration efficiency at leaf level (A/g_s)

Under saturating conditions, A_{sat}, g_{sat} and A_{sat}/g_{sat} significantly differed among genotypes but were not affected by *VPD* during growth. The overall mean for A_{sat} was 14.8 µmol m^{-2} s^{-1} and for g_{sat} 0.382 mol m^{-2} s^{-1} (Table 2). The situation was quite different under ambient conditions. Daily courses of *A* displayed significant decreases (figure 1a) but were not affected by *VPD*, while those of g_s displayed constant values but large *VPD*-induced differences. Diurnal means of *A* were not affected by genotype, treatment or interaction effect, while diurnal mean g_s varied significantly among genotypes and was much smaller under high *VPD*. An interaction effect was also evident for mean g_s as the genotype effect observed under low *VPD* was completely absent under high under high *VPD*.

Tight positive correlations were evident between assimilation weighted mean diurnal A/g_s and instant A_{sat}/g_{sat}, but the correlations differed between *VPD* levels (Fig. 3). This was due to the fact that the differences between *VPD* treatments recorded *in situ* were alleviated when gas exchange was recorded under similar and optimal conditions after sufficient induction

time; in particular, stomatal conductance of leaves from the high *VPD* treatment increased rapidly during the induction for the g_{smax} records.

Genotype means of $\Delta^{13}C_{ss}$ were negatively correlated to mean diurnal A/g_s under both *VPD* treatments. Genotype means of A/g_s and $\Delta^{13}C_{ss}$ were correlated to g_s under low *VPD*, but no longer under high *VPD*, and never to *A* (Fig. 4 a,b). This shows that differences in g_s had a major effect on A/g_s and $\Delta^{13}C_{ss}$ as compared to *A*.

A tight correlation was detected between genotype means of the isotopic discrimination predicted with the simple discrimination model from the recorded C_i/C_a ($\Delta^{13}C_M$) and the observed $\Delta^{13}C_{ss}$ in each of the two treatments (Fig. 4). The difference between predicted and observed discrimination ($\Delta^{13}C_{dif}$) differed significantly among treatments and was 3.5 ‰ under low and 2.3‰ under high *VPD*. It was also significantly different among genotypes, with values ranging from 2.1 to 5.3‰ under low *VPD*. However, the genotype ranking of $\Delta^{13}C_{dif}$ remained stable across both treatments (Table 3). $\Delta^{13}C_{dif}$ was positively correlated to *LMA* under the two treatments (Fig. 5a). It was also negatively correlated to leaf thickness under high *VPD* (Fig. 5b).

$\Delta^{13}C_{ss}$ was not correlated to A in any treatment, and only to g_s under low VPD (fig. 6).

Scaling from leaf to whole plant transpiration efficiency (*TE*).

Produced biomass (*BM*) was similar across the treatments but differed among genotypes (Table 2). Cumulative water use (*WU*) was different both across treatments and among genotypes. Consequently, whole plant *TE* displayed very significant genotype and *VPD* effects. Mean values were 6.6 g l^{-1} under low and only 4.6 g l^{-1} under high *VPD*. Genotype means of *TE* were negatively correlated to $\Delta^{13}C_{ss}$ and positively to A/g_s with two different but

parallel regressions in the two cases (Fig. 7 a,b). At the same time, no correlation was evident between genotype means of *TE* and *WU* or *BM*.

Discrimination against ^{18}O between source water and leaf water ($\Delta^{18}O_{lw}$) and bulk matter ($\Delta^{18}O_{lb}$)

Leaf water and bulk matter were as expected significantly enriched in ^{18}O with respect to the source water by approx 8 and 26‰. Strong genotype and *VPD* effects were found for this enrichment, with a range of about 2 ‰ among genotypes for $\Delta^{18}O_{lw}$, and a difference of almost 8 ‰ between the two treatments (Table2). As expected, leaf water enrichment in ^{18}O was much larger under high *VPD*. The results were very similar for $\Delta^{18}O_{lb}$ in bulk leaf matter with a difference of 9 ‰ between the two *VPD*. Leaf temperature was larger under low than high VPD, as a result of a larger transpiration related cooling in the latter case; as a result, the actual difference in water vapour pressure between mesophyll tissues and the atmosphere was larger than the recorded atmospheric *VPD* under low *VPD*.

Genotype means of $\Delta^{18}O_{lb}$ were negatively correlated to g_s under low *VPD* and to $\Delta^{13}C_{ss}$ under both low and high *VPD* (Fig. 8 a,b). The lack of correlation under high *VPD* was due to the small variability observed in g_s. $\Delta^{18}O_{lw}$ was also negatively correlated to g_s and $\Delta^{13}C_{ss}$ only under low *VPD*, which confirmed that variation in *TE* and A/g_s was controlled by g_s rather than by *A*.

Discussion

^{13}C discrimination as recorded in soluble sugars $(\Delta^{13}C_{ss})$ and bulk leaf matter $(\Delta^{13}C_{lb})$

The tight positive correlation between genotype means of $\Delta^{13}C_{ss}$ and $\Delta^{13}C_{lb}$ under the two *VPD* treatments confirms that the effects of the isotopic discrimination during photosynthesis were not wiped out by post photosynthetic discrimination in leaves. $\Delta^{13}C_{ss}$ was larger than $\Delta^{13}C_{lb}$ while the reverse was expected from our knowledge of the isotopic composition of leaf products (with depleted lipids and lignins in the bulk matter). A similar result was found in *Populus deltoids × nigra* genotypes grown under controlled conditions (Rasheed *et al.* 2012). Causes for this apparent discrepancy were probably very similar to those already discussed by Rasheed *et al.,* (2012): (i) a pollution of the soluble sugar fractions by proteins or lipids can be excluded based on the N_{ss} and C_{ss} contents in the soluble sugar fraction; (ii) an effect of reallocation of carbon stored prior to the start of the experiment (i.e., in the greenhouse) cannot be excluded, as the final biomass was only twice the initial one due to the relatively slow growth of the plants (Lauteri *et al.,* 1993); (iii) substantial diurnal variation in the amount of starch might be one of the causes. Poplar leaves are rich in starch, and starch synthesis results in a 3‰ enrichment with respect to soluble sugars (Brugnoli *et al.,* 1988). The amount of starch reaches a maximum late in the afternoon, which results in parallel in a depletion of ^{13}C in soluble sugars; during the night, starch is hydrolised into sucrose which is exported to the phloem; this results in complex diurnal dynamics of ^{13}C as recorded in soluble sugars (Tcherkez *et al.* 2011). We collected leaves probably at the peak starch concentration, which resulted in a temporary ^{13}C impoverishment of the soluble sugar pool. This effect was nevertheless smaller in the *Populus nigra* genotypes used here than in *Populus deltoides x*

nigra genotypes, given the lower amount of soluble sugars, lower photosynthesis and slower growth in the former (Rasheed *et al.* 2012).

Correlation between ^{13}C discrimination recorded in soluble sugars ($\Delta^{13}C_{ss}$) and mean daily intrinsic transpiration efficiency (A/g_s)

The negative correlation found between $\Delta^{13}C_{ss}$ and assimilation weighted diurnal mean TE (A/g_s) under the two *VPD* treatments confirms that $\Delta^{13}C_{ss}$ is a reliable indicator of A/g_s as predicted by the model of Farquhar *et al.* (1989) and in agreement with Brugnoli *et al.* (1988), Lauteri *et al.*, (1993). Surprisingly, while we expected a unique relationship across the two *VPD* treatments, we obtained two separate and parallel regressions with a very stable genotype ranking as shown in Table 3. Some clues about this discrepancy were given by analysing the difference $(\Delta^{13}C_{dif})$ between the expected values of discrimination obtained with the simple model (Equ 1) and the observed data in soluble sugars.

Variation in $\Delta^{13}C_{dif}$ can be due to the additional terms in the complete discrimination model like the mesophyll conductance to CO_2 (g_m) or respiration (R_d, Equ 2), or to variation of the primary parameters of the model like *b* (see Douthe *et al.,* 2012). For instance, differences in mitochondrial respiration (R_d) could contribute to the observed differences, but the real weight of R_d in the discrimination model remains small (Douthe *et al.,* 2011, 2012). Usually this difference is attributed to the fact that the simple model assumes that $C_i = C_c$, i.e., that implies that mesophyll conductance g_m is infinite, which is obviously not the case (Evans & von Caemmerer, 1995). g_m is inversely proportional to $\Delta^{13}C_{dif}$ (Pons *et al.,* 2009; Flexas *et al.,* 2008), and is considered to be the main driver for changes in $\Delta^{13}C_{dif}$. (Douthe *et al.* 2012). In *Populus deltoides× nigra*, values of $\Delta^{13}C_{dif}$ were similar among genotypes (Rasheed *et al.* 2012). Genotype differences in g_m have been recently detected in barley (Barbour *et al.* 2010). These differences in g_m can result from variations in a number of leaf traits including leaf

mass to area ratio *LMA,* leaf thickness*,* surface area of chloroplasts exposed to the intercellular spaces (S_c), mesophyll cell wall thickness, permeability of the plasma and chloroplast membrane and (Evans *et al.,* 2009, Tholen *et al.,* 2011). The positive correlation between *LMA* and $\Delta^{13}C_{\text{dif}}$ within each VPD treatment and the negative one with leaf thickness would then be interpreted as meaning that denser leaves (low *LT* and high *LMA*) display smaller g_m. This fits to some extent with recent observations (Tholen *et al.,* 2011).

Short-term responses of g_m to irradiance and atmospheric CO_2 have been evidenced many times in different species (Flexas *et al.,* 2007; Douthe *et al*., 2011, 2012). Soil water depletion frequently results in decreased g_m (Roupsard *et al.* 1998; Flexas *et al.* 2008). Long-term acclimation of g_m to *VPD* has never been described before. Warren (2009) detected a stomatal closure and no change in g_m in response to increasing *VPD*. Our observation would mean some compensation of stomatal closure by higher mesophyll conductance decreasing C_i.

Integration from intrinsic leaf *TE* to whole plant *TE*

Whole plant *TE* varied between 5.56 and 8.28 g kg^{-1} under low *VPD* and between 3.06 and 5.93 g kg^{-1} under high *VPD*. These values are high when compared to the published values for other species. Very high values of whole plant *TE* (up to 15 g kg^{-1}) have been evidenced in *Populus deltoides x nigra* genotypes (Rasheed *et al.* 2012). This shows again that poplar can be highly efficient in terms of biomass production per Liter water transpired while these species are expected to be water spenders.

Variation in whole plant *TE* between the plants grown in the common environment can occur due variation in intrinsic leaf *TE* or *VPD* or a combination of both. As *VPD* was controlled within in each treatment, intrinsic leaf *TE* can be considered as a major source of genotypic variation in whole plant *TE*. Positive correlations between whole plant and intrinsic *TE* under the two environmental conditions confirm this hypothesis. The results are in agreement with

Rasheed *et al.,* (2012) in *Populus × euramericana* under controlled environment. However, the genotypes growing under high *VPD* displayed higher intrinsic *TE* but lower whole plant *TE* as compared to the genotypes under low *VPD*. This was obviously due to VPD induced stomatal closure (Lange *et al.,* 1971; McCaughey & Iacobelli, 1994) while transpiration rate increased. A close examination showed that the relative magnitude of variation in whole plant *TE* was smaller than that of intrinsic *TE* under low *VPD* (32.9% and 54% respectively). Under high VPD however, it became larger under higher *VPD* (48.3% and 29% respectively). In *Populus deltoides x nigra* genotypes Rasheed *et al.* (2012) found 27.6% relative variation in *A/g$_s$* vs 40.7% variation in whole plant *TE*. As we move from leaf level to whole plant level, inter genetic variation in *TE* can be affected due to variation of relative proportion of carbon fixed during photosynthesis which is subsequently lost though respiration (Φ_c; Osório *et al.,* 1998) and the proportion of water loss not associated to photosynthesis across genotypes (Φ_w; Hobbie & Colpaert, 2004). Our results suggest that Φ_c and Φ_w were higher under high *VPD*.

Correlation between whole plant transpiration efficiency (*TE*) and biomass production (*BM*)

The lack of correlation between whole plant *TE* and *BM* opens an opportunity to select genotypes for simultaneously enhanced *TE* and *BM*. Theory explains that this lack of correlation might be due to significant changes in stomatal conductance *(g$_s$)* that had a greater effect on *TE* than net CO_2 assimilation *(A)* (Ehleringer *et al.* 1990; Condon *et al.* 1987). Among the *populus nigra* genotypes used here, the variation of intrinsic *TE* was mainly explained by variation in stomatal conductance *(g$_s$)* rather than in net CO_2 assimilation rate *(A)*. These results are in contrast to Rasheed *et al.* (2012) where intrinsic *TE* was controlled by *A* under optimum growth conditions. The lack of differences in productivity (*BM*) between the genotypes strengthens the hypothesis that selecting genotypes for higher *TE* would be possible without sacrificing productivity.

$\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$ in relation to genotype variability of g_s

The transpiration-induced enhanced 18O in leaves (either water or bulk matter) can be used to access the variability of stomatal conductance (Barbour, 2007; Saurer *et al.,* 1997). Thus, oxygen isotopes can be used as a tool to assess the genetic diversity of stomatal conductance (Barbour *et al.,* 2000a). Our results show that within each treatment, ^{18}O enrichment in both $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$ was higher under lower g_s. These results are in accordance with Barbour & Farquhar (2000) and under controlled condition in *Populus × euramericanna* (Rasheed *et al.,* 2012) at individual level. Within each treatment decrease in g_s increased T_l that might have decreased the ratio between ambient and inter cellular vapour pressure and resulted in increased enrichment. In addition, $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$ increased under high *VPD*, in agreement with Craig & Gordon (1965) and Barbour *et al.* (2000a). This increased enrichment can be attributed to the decrease in leaf temperature (T_l) due to higher transpiration rate despite the decrease of g_s under high *VPD* (Farquhar & Lloyd, 1993; Barbour *et al.,* 2000a). Sometimes the actual enrichment of the leaf water is much less than predicted (Farquhar & Lloyd, 1993). Barbour *et al.,* (2000b) explained this discrepancy as mixing of enriched water due to high transpiration with the unfractionated xylem water (Péclet effect). Within each treatment, even if Péclet effect is in practice, low g_s accompanied by lower transpiration rate (*WU*) would have decreased the $\wp$, thus reinforcing the increase in enrichment. Thus, based on our results we can safely conclude that ^{18}O enrichment in the leaf water and bulk can be used as an indicator of g_s.

Interpreting the relationship between $\Delta^{13}C_{ss}$ and $\Delta^{18}O_{lb}$:

Farquhar *et al.,* (1994) proposed that measuring both $\Delta^{13}C$ and $\Delta^{18}O$ may help separating the effect of g_s and A on intrinsic transpiration efficiency, because if a negative relationship exist between the $\Delta^{13}C$ and $\Delta^{18}O$, a part of the variation in $\Delta^{13}C$ would be due to the variation of g_s

as A has no effect of the ^{18}O enrichment of the plant material. Our results show a negative correlation between $\Delta^{13}C_{ss}$ and $\Delta^{18}O_{lb}$ within both treatments, which could be interpreted as positive correlation between $\delta^{13}C_{ss}$ and $\delta^{18}O_{lb}$. These results are in line with positive correlation found between $\delta^{13}C_{ss}$ and $\delta^{18}O_{lb}$ in stem cellulose of three tree species (Saurer *et al.* 1997) and in leaf cellulose taken from different heights within a tropical forest (Sternberg *et al.* 1989). However Barbour & Farquhar (2000) found positive correlation between the two traits under glasshouse conditions which are inline with our results. The relationship between these two physiological traits must be interpreted with caution while deducing the results concerning the relative proportion of g_s and A on $\delta^{13}C$. It is clear from the Fig. 6 b that genotypic variation in $\Delta^{18}O_{lb}$ does not fully account for the variation in $\Delta^{13}C_{ss}$ (shallow slope), thus the correlation between the two traits can be interpreted as follows (i) the sensitivity of $\Delta^{18}O_{lb}$ to the genotypic changes in g_s were low (ii) a part of variation in $\Delta^{13}C_{ss}$ also reflects small variations in A. However, on the bases of the results, we are able to show that variation $\Delta^{13}C_{ss}$ is largely due to the variation in g_s rather than A under both treatments.

Conclusion

Effect of *VPD* on g_s and eventually on transpiration efficiency was measured at different time scale (from leaf level to whole plant level) in six *Populus nigra* genotypes. The results show that genotype differences for $\Delta^{13}C_{ss}$ matched genotype difference for $\Delta^{13}C_{lb}$ under both treatments. Genotype diversity for $\Delta^{13}C_{ss}$ also matched closely to two independent estimates of water use efficiency; intrinsic transpiration efficiency (A/g_s) and whole plant transpiration efficiency (*TE*) under both treatments. However, present results suggest that there was perturbation of the signals as they were transferred from one integration level to another. $\Delta^{13}C_{ss}$ and A/g_s were correlated to stomatal conduction (g_s) rather than assimilation (*A*). Significant genotypic and treatment effect of $\Delta^{13}C_{dif}$ (difference between the predicted ($\Delta^{13}C_M$) and observed ($\Delta^{13}C_{ss}$) discrimination) showed that part form other factors discussed, mesophyll conductance (g_m) could be the principle factor explaining the genotypic and treatment effect on $\Delta^{13}C_{dif}$. No correlation was found between whole plant *TE* and *BM*. Results shows that ^{18}O enrichment in both leaf bulk and leaf water can be used as an index for genotype variability in g_s.

Acknowledgement

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Tables

Table 1. Abbreviations and descriptions of the variables used in the study

Variables	Description
VPD	Vapour pressure deficit (kPa)
H	Stem height (cm)
D	Diameter at stem base (mm)
TLA	Whole plant leaf area (m^2)
LMA	Leaf mass to area ratio ($g\ m^{-2}$)
SD	Stomatal density (mm^{-2})
LT	Leaf thickness (μm)
$\delta^{13}C_{air}$	Carbon isotope composition of the air (‰)
N_{ss} , N_{lb} (%)	Nitrogen contents in the leaf bulk and soluble sugars(%)
C_{ss} , C_{lb} (%)	Carbon contents in leaf bulk oluble sugars (%)
$\delta^{13}C_{ss}$	Carbon isotope composition of leaf soluble sugars (‰)
$\Delta^{13}C_{ss}$	Carbon isotope discrimination between atmosphere and leaf soluble sugars (‰)
$\delta^{13}C_{lb}$	Carbon isotope composition of bulk leaf matter (‰)
$\Delta^{13}C_{lb}$	Carbon isotope discrimination between atmosphere and bulk leaf matter (‰)
$\delta^{13}C_{offset}$	Difference between $\delta^{13}C_{ss}$ and $\delta^{13}C_{lb}$ (‰)
A, A_{sat}	Net CO_2 assimilation rate under ambient/saturating condition ($\mu mol\ m^{-2}\ s^{-1}$)
g_s, g_{sat}	Stomatal conductance under saturating condition ($mol\ m^{-2}\ s^{-1}$)
A_{sat}/g_{sat}	Intrinsic transpiration efficiency under saturating condition ($\mu mol\ mol^{-1}$)
A/g_s	Intrinsic transpiration efficiency over a diurnal cycle ($\mu mol\ mol^{-1}$)
C_i/C_a	Ratio of CO_2 concentration in the atmosphere and in the substomatal spaces
$\Delta^{13}C_M$	Carbon isotope discrimination predicted by the simple discrimination model (‰)
$\Delta^{13}C_{dif}$(‰)	Difference between predicted and observed C isotope discrimination ($\Delta^{13}C_{ss}$) (‰)
BM	Total plant biomass accumulated during the experiment (g)
WU	Cumulated water use (l)
TE	Transpiration efficiency
$\delta^{18}O_{sw}$	Oxygen isotope composition of source water (‰)
$\delta^{18}O_{lb}$	Oxygen isotope composition of bulk leaf matter (‰)
$\delta^{18}O_{lw}$	Oxygen isotope composition of leaf water (‰)
$\Delta^{18}O_{lb}$	Oxygen isotope enrichment between source water and leaf bulk matter (‰)
$\Delta^{18}O_{lw}$	Oxygen isotope enrichment between source water and leaf water (‰)
T_l	Leaf temperature (°C)

Table 2: Means (SD) of morphological and physiological traits recorded on 6 *Populus nigra* genotypes under low and high *VPD*. A factorial 2 way ANOVA was used to test for the effects of genotype (*G*), treatment (*VPD*) and their interactions (*G* x *VPD*) for each trait and probability is taken as significant at P < 0.05 (bold fonts).

	Low VPD	*High VPD*	*VPD*	*G*	*G ×VPD*
VPD (kPa)	1.02 (0.242)	2.07 (0.141)	**P < 0.001**	P = 0.156	P = 0.267
H (cm)	89.4 (3.87)	88.1 (4.14)	P = 0.584	**P < 0.001**	P = 0.518
D (mm)	8.67 (0.35)	8.06 (0.32)	**P = 0.004**	**P < 0.001**	P = 0.887
TLA (m^2)	0.0852 (0.06)	0.0956 (0.07)	P = 0.242	**P = 0.009**	P = 0.908
LMA (g m^{-2})	96.5 (2.54)	96.9 (3.96)	P = 0.257	**P < 0.001**	P = 0.387
SD (mm^{-2})	168 (7.00)	183 (10.9)	**P = 0.015**	**P < 0.001**	P = 0.223
LT (μm)	219 (3.36)	227 (5.18)	**P = 0.047**	**P < 0.001**	P = 0.501
N_{ss} (%)	0.163 (0.048)	0.165 (0.062)	P = 0.142	P = 0.899	P = 0.930
C_{ss} (%)	41.2 (1.10)	41.6 (1.13)	**P = 0.036**	P = 0.221	**P = 0.038**
$\Delta^{13}C_{ss}$ (‰)	22.6 (0.27)	21.3 (0.24)	**P < 0.001**	**P < 0.001**	P = 0.399
N_{lb} (%)	0.817 (0.121)	0.929 (0.208)	P = 0.135	**P = 0.025**	P = 0.431
C_{lb} (%)	43.5 (0.780)	43.6 (0.737)	P = 0.103	P = 0.593	P = 0.155
$\Delta^{13}C_{lb}$ (‰)	20.7 (0.19)	19.7 (0.18)	**P < 0.001**	**P < 0.001**	P = 0.059
SS contents (%)	8.62 (0.30)	8.45 (0.23)	P = 0.572	**P = 0.002**	P = 0.318
$\delta^{13}C_{offset}$ (‰)	1.79 (0.26)	1.52 (0.19)	P = 0.064	**P < 0.001**	P = 0.691
A_{sat} (μmol m^{-2} s^{-1})	14.6 (1.17)	15.1 (1.16)	P = 0.489	**P = 0.021**	P = 0.395
g_{sat} (mol m^{-2} s^{-1})	0.374 (0.05)	0.390 (0.05)	P = 0.630	**P < 0.001**	P = 0.226
A_{sat}/g_{sat} (μmol mol^{-1})	43.6 (5.94)	45.9 (5.96)	P = 0.529	**P = 0.002**	P = 0.856
mean daily *A* (μmol m^{-2})	7.16 (0.39)	7.29 (0.43)	P = 0.642	P = 0.144	P = 0.096
mean daily g_s (mol m^{-2})	0.352 (0.02)	0.156 (0.01)	**P < 0.001**	**P < 0.001**	**P < 0.001**
mean daily A/g_s (μmol)	25.8 (2.12)	47.3 (2.16)	**P < 0.001**	**P < 0.001**	P = 0.505
mean daily C_i/C_a	0.884 (0.01)	0.782 (0.01)	**P < 0.001**	**P < 0.001**	P = 0.503
$\Delta^{13}C_M$ (‰)	26.1 (0.26)	23.6 (0.33)	**P < 0.001**	**P < 0.001**	P = 0.470
$\Delta^{13}C_{dif}$ (‰)	3.48 (0.35)	2.32 (0.39)	**P < 0.001**	**P < 0.001**	P = 0.227
BM (g)	34.2 (4.32)	37.2 (4.66)	P = 0.315	**P = 0.024**	P = 0.803
WU (l)	5.21 (0.56)	8.25 (0.99)	**P < 0.001**	**P = 0.008**	P = 0.669
whole plant *TE* (g l^{-1})	6.64 (0.28)	4.61 (0.17)	**P < 0.001**	**P < 0.001**	P = 0.333
$\Delta^{18}O_{lb}$ (‰)	22.6 (0.18)	30.4 (0.19)	**P < 0.001**	**P < 0.001**	P = 0.062
$\Delta^{18}O_{lw}$ (‰)	3.73 (0.22)	12.6 (0.36)	**P < 0.001**	**P < 0.001**	P = 0.290
T_l (°C)	31.4 (0.36)	29.4 (0.33)	**P < 0.001**	**P = 0.012**	P = 0.475

Table 3: Pearson's correlation coefficient between the two *VPD* treatments for genotype means of different proxies for transpiration efficiency. All correlations were highly significant (P<0.001).

Traits	r - values
$\Delta^{13}C_{lb}$	0.943***
$\Delta^{13}C_{ss}$	0.829***
A/g_s	0.829***
Whole plant TE	0.943***
$\Delta^{18}O_{lb}$	0.943***
$\Delta^{13}O_{dif}$	0.829***

FIGURES CAPTIONS

Figure 1: Time course of net CO_2 assimilation rate A, stomatal conductance to water vapour g_s and intrinsic transpiration efficiency A/g_s along a diurnal cycle. Each point represents a mean from six genotypes. Visible diurnal trends were evident in A under both *VPD* and only under low *VPD* in g_s and A/g_s.

Figure 2: Relationship between genotype means of isotopic discrimination between atmospheric CO_2 and C in bulk leaf-matter ($\Delta^{13}C_{lb}$) and soluble sugars in the leaf ($\Delta^{13}C_{ss}$) Numbers refer to genotypes. Open circles represent Low and closed circles High *VPD* treatment. Low, $\Delta^{13}C_{ss} = 1.15 \, \Delta^{13}C_{lb} - 1.41$; High, $\Delta^{13}C_{ss} = 1.12 \, \Delta^{13}C_{lb} - 0.841$). The diagonal line in panel is the 1:1 relationship.

Figure 3: Relationships between genotype means of diurnal mean intrinsic transpiration-efficiency (A/g_s) and of A_{sat}/g_{sat} (Low, $A/g_s = 0.989 \, A_{sat}/g_{sat} + 20.053$; High, $A/g_s = 1.59 \, A_{sat}/g_{sat}$ x - 31.9). Open circles represent Low and closed disks represent High *VPD*. The diagonal line in panel is the 1 : 1 relationship.

Figure 4: Relationship between genotype means of isotopic discrimination $(\Delta^{13}C_M)$ predicted from the simple discrimination model and recorded from soluble sugars in leaves $\Delta^{13}C_{ss}$ (Low, $\Delta^{13}C_{ss} = 1.86\Delta^{13}C_M - 26.0$; High $\Delta^{13}C_{ss} = 1.98\Delta^{13}C_M - 25.5$). Open circles represent Low and closed disks represent High *VPD* treatments. Solid regression line represents the 1:1 relationship. $\Delta^{13}C_M$ was computed as in Equ 1 using $a = 4.4‰$ and $b = 29‰$. The diagonal line in panel is the 1 : 1 relationship.

Figure 5: Relationship between the difference predicted $(\Delta^{13}C_M)$ vs. observed $(\Delta^{13}C_{ss})$ discrimination $\Delta^{13}C_{dif}$ and: (a) leaf mass to area ratio (*LMA*); Low *VPD, LMA* $= 0.888\Delta^{13}C_{dif} + 11.7$; High *VPD, LMA* $= 0.808\Delta^{13}C_{dif} + 13.0$. (b) leaf thickness (*LT*); High *VPD, LT* $= -17.4\Delta^{13}C_{dif} + 267.4$. Open circles represent Low and closed circles High *VPD* treatment.

Figure 6: Relationships between genotype means of the isotopic discrimination between atmospheric CO_2 and C in leaf soluble sugars $\Delta^{13}C_{ss}$, and (a) mean diurnal stomatal conductance (g_s), (Low, $g_s = 0.051\Delta^{13}C_{ss} - 0.823$; High, $g_s = 0.006\Delta^{13}C_{ss} + 0.017$). (b) mean diurnal net CO_2 assimilation rate (A). Open circles represent Low and closed circles High *VPD* treatment.

Figure 7: Relationships between genotype means of whole plant *TE* and (a) the isotopic discrimination between atmospheric CO_2 and the C in leaf soluble sugars $\Delta^{13}C_{ss}$; Low, $\Delta^{13}C_{ss} = -1.28TE + 31.1$; High, $\Delta^{13}C_{ss} = -1.56TE + 28.5$; (b) mean diurnal A/g_s; Low, $A/g_s = 5.08TE - 7.95$; High, $A/g_s = 5.45TE + 22.2$. Open circles represent Low and closed circles High *VPD* treatment.

Figure 8: Relationships between genotype means of ^{18}O enrichment in bulk leaf matter $\Delta^{18}O_{lb}$ (with respect to the source water) and: (a) the mean diurnal stomatal conductance (g_s); Low, $g_s = -0.120\Delta^{18}O_{lb} + 3.09$; (b) the isotopic discrimination between atmospheric CO_2 and the C in leaf soluble sugars $\Delta^{13}C_{ss}$ (Low, $\Delta^{13}C_{ss} = -2.04\Delta^{18}O_{lb} + 69.0$; High $\Delta^{13}C_{ss} = -2.10\Delta^{18}O_{lb} + 85.1$. Open circles represent Low and closed circles High VPD treatment.
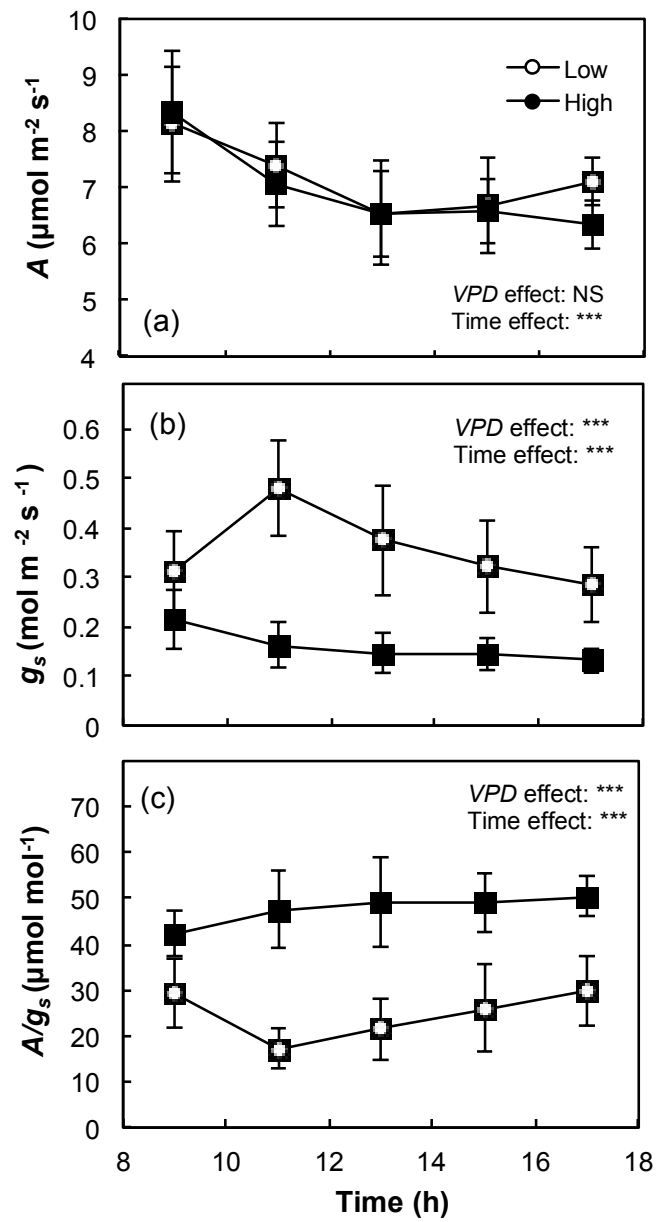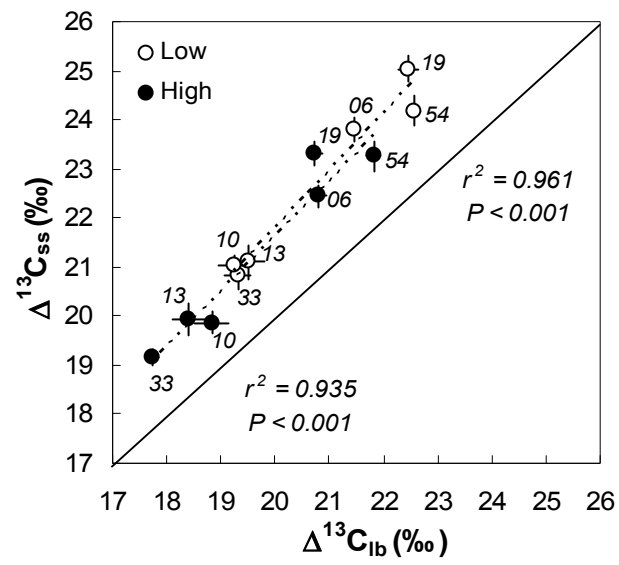
Figures

Fig. 1

Fig. 2
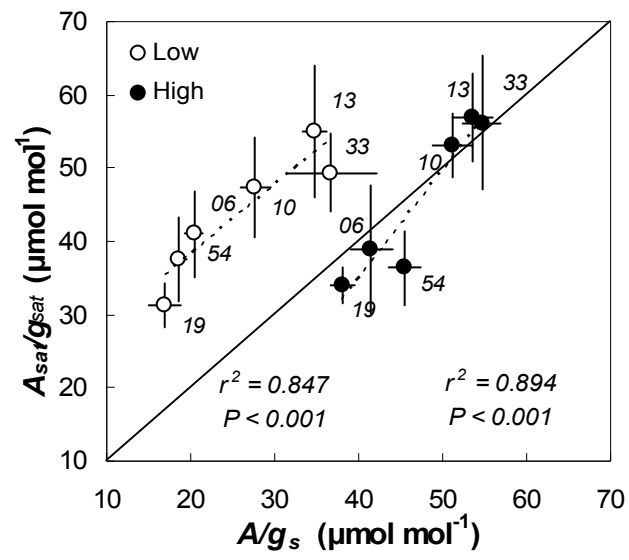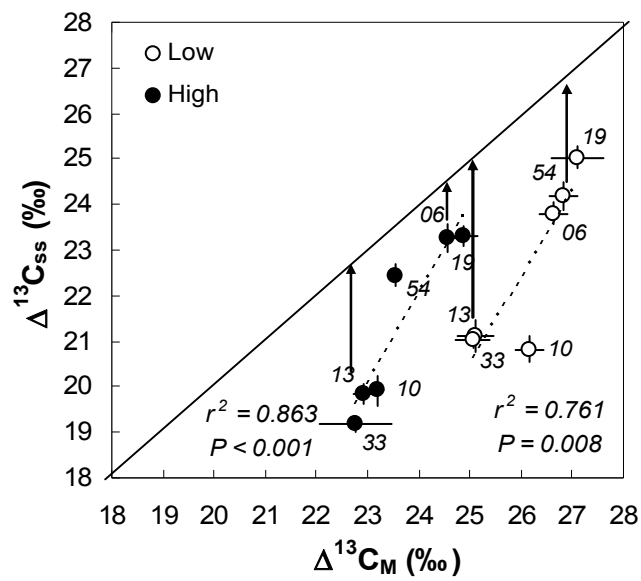
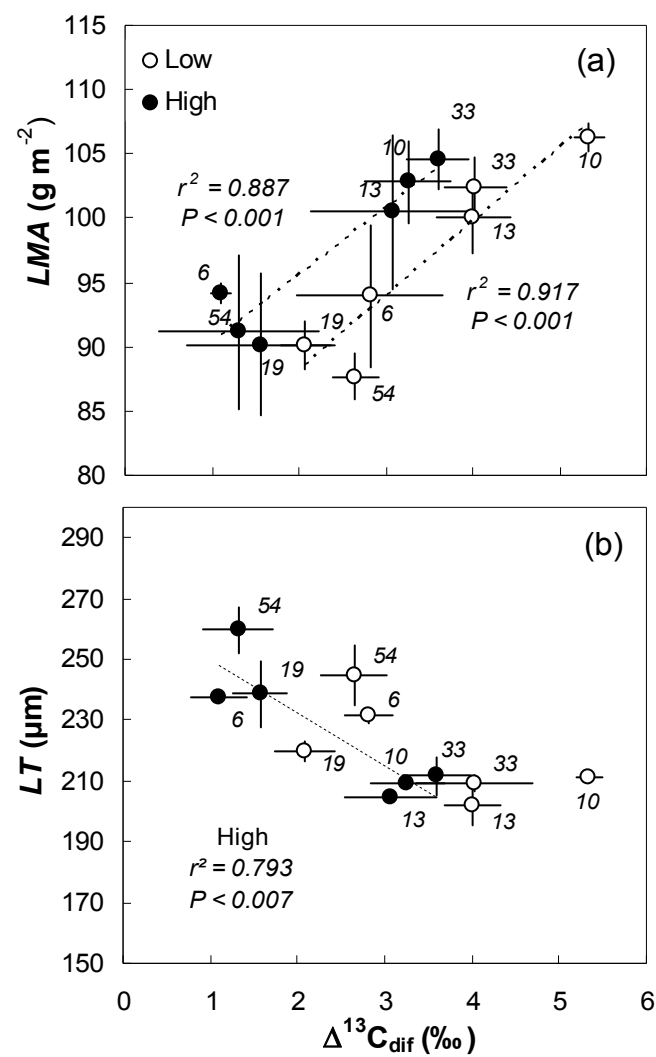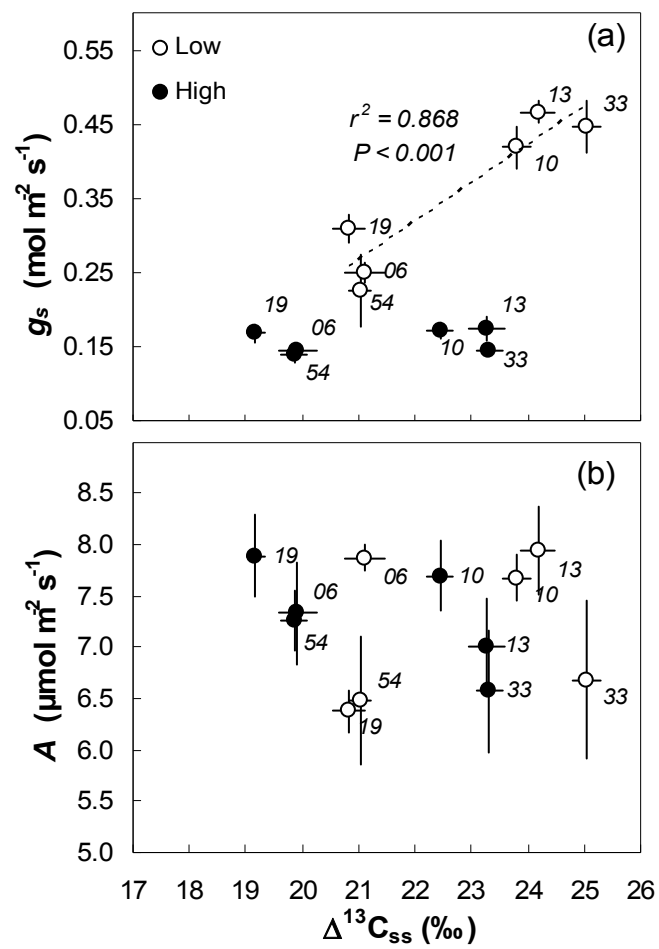**Fig. 3**
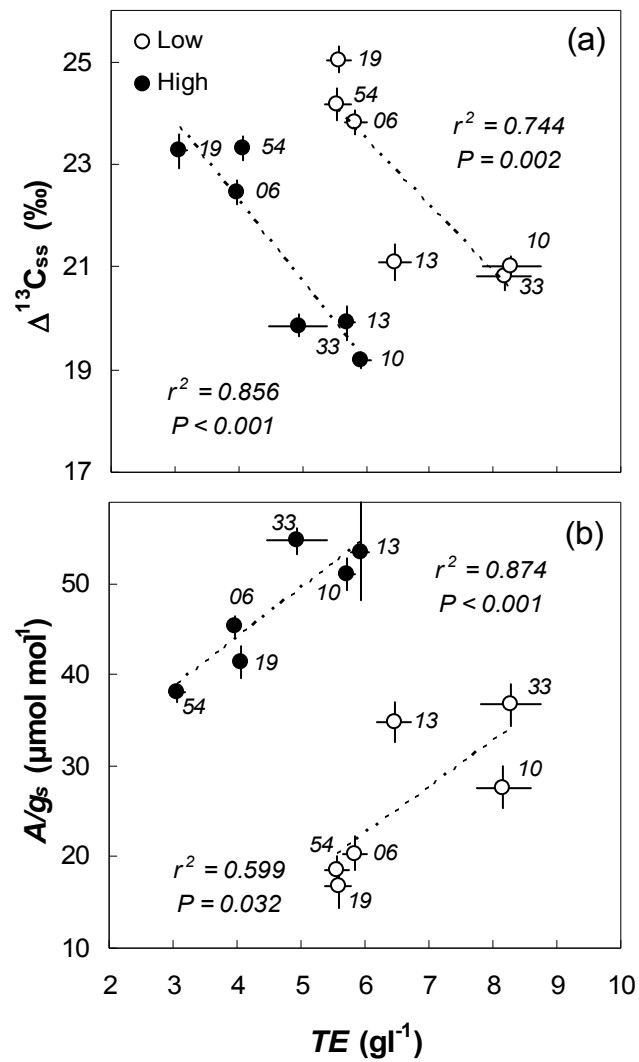
Fig. 4

Fig 5.
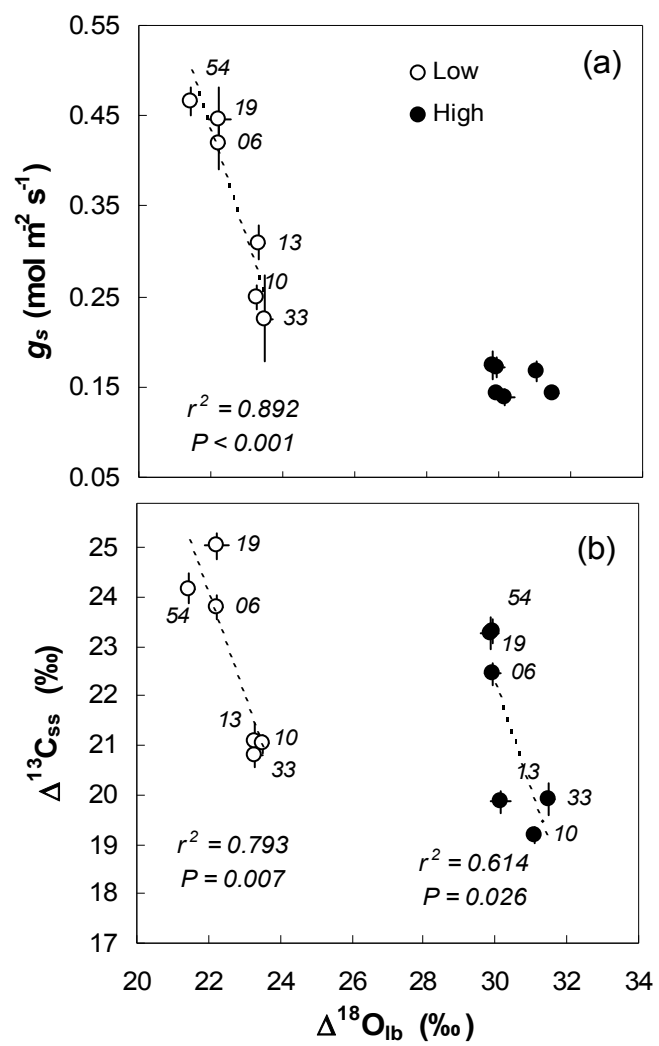
Fig. 6

Fig. 7

Fig. 8

Table S1: Genotype means (SD) of morphological and physiological traits recorded on 6 *Populus nigra* genotypes under two VPD treatments. A factorial 2 way ANOVA was used to test for the effects of genotype, treatment and their interaction. Letters represent significant differences among genotypes tested by a post-hoc Tukey test (P < 0.05). Interaction effect for all the variables is given in the last column. See table 1 for the definition and unites of variables.

	UNDER LOW VPD						UNDER HIGH VPD					
	Spm 06	*Spm 10*	*Spm 13*	*Spm 19*	*Spm 33*	*Spm 54*	*Spm 06*	*Spm 10*	*Spm 13*	*Spm 19*	*Spm 33*	*Spm 54*
VPD	1.09 (0.061)	1.08 (0,089)	1.39 (0,609)	1.07 (0.135)	1.53 (0.632)	1.03 (0.030)	2.07 (0.091)	2.12 (0.088)	2.18 (0.092)	1.96 (0.107)	2.14 (0.113)	1.95 (0.195)
H	75.3 (4.0)	97.7 (4.03)	70.5 (3.82)	96.3 (2.53)	119 (6.16)	77.8 (2.41)	80.5 (3.05)	97.1 (7.16)	67.7 (4.20)	85.2 (3.38)	120 (3.59)	78.3 (3.47)
D	8.51 (0.40)	8.56 (0.43)	7.47 (0.42)	9.45 (0.22)	9.27 (0.51)	8.76 (0.18)	8.18 (0.25)	7.90 (0.41)	7.13 (0.31)	8.35 (0.38)	8.80 (0.25)	8.02 (0.33)
TLA	0.065 (0.01)	0.083 (0.01)	0.059 (0.01)	0.118 (0.01)	0.094 (0.01)	0.089 (0.01)	0.088 (0.01)	0.091 (0.02)	0.071 (0.01)	0.116 (0.03)	0.111 (0.01)	0.086 (0.01)
LMA	93.3 (5.49)	106 (1.11)	100 (2.80)	90.1 (1.87)	102 (2.32)	87.6 (1.73)	94.1 (0.75)	102 (3.18)	100 (6.02)	90.1 (5.50)	104 (2.34)	91.1 (5.98)
SD	144 (4.29)	180 (7.66)	131 (4.73)	171 (5.41)	174 (9.43)	209 (10.5)	162 (3.35)	189 (7.69)	152 (15.9)	204 (18.6)	196 (8.85)	194 (11.2)
LT	231 (2.27)	210 (1.95)	202 (7.00)	219 (3.01)	209 (2.91)	244 (9.93)	237 (1.65)	209 (2.25)	204 (1.50)	238 (10.7)	211 (6.44)	259 (7.41)
N_{lb}	0.776 (0.158)	0.741 '0.154)	1.01 (0.075)	0.736 (0.035)	0.870 (0.158)	0.764 (0.164)	1.03 (0.239)	0.806 (0.149)	0.946 (0.154)	0.858 (0.317)	0.962 (0.133)	0.968 (0.255)
C_{lb}	43.7 (0.822)	43.8 (0.862)	43.4 (0.757)	43.5 (0.770)	44.1 (0.865)	42.4 (0.807)	43.1 (0.373)	43.7 (0.359)	43.2 (0.679)	43.9 (1.28)	44.2 (0.902)	43.7 (0.824)
$\Delta^{13}C_{lb}$	21.4 (0.06)	19.3 (0.24)	19.5 (0.29)	22.4 (0.18)	19.2 (0.28)	22.5 (0.09)	20.8 (0.12)	18.4 (0.29)	18.8 (0.31)	20.7 (0.13)	17.7 (0.12)	21.8 (0.14)
N_{ss}	0.208 (0.061)	0.182 (0.056)	0.161 (0.040)	0.139 (0.027)	0.135 (0.052)	0.156 (0.055)	0.202 (0.101)	0.176 (0.076)	0.182 (0.040)	0.160 (0.035)	0.145 (0.068)	0.128 (0.050)
C_{ss}	40.3 (0.824)	42.3 (0.457)	42.1 (1.09)	40.7 (1.43)	41.5 (1.23)	40.5 (1.57)	40.7 (0.782)	41.2 (1.36)	41.8 (1.16)	41.3 (1.53)	41.3 (1.35)	41.2 (0.581)
$\Delta^{13}C_{ss}$	23.8 (0.24)	20.8 (0.27)	21.1 (0.35)	25.0 (0.25)	21.0 (0.20)	24.1 (0.31)	22.4 (0.23)	19.9 (0.33)	19.8 (0.22)	23.3 (0.23)	19.1 (0.17)	23.2 (0.32)
SS contents	8.13 (0.33)	8.10 (0.55)	10.2 (0.32)	8.20 (0.42)	9.83 (0.92)	7.20 (0.58)	8.00 (0.63	9.04 (0.64)	9.46 (0.38)	8.25 (0.28)	8.11 (0.74)	7.70 (0.47)
A_{sat}	16.3 (1.59)	13.9 (1.11)	13.4 (0.84)	16.3 (0.52)	13.5 (1.34)	13.9 (1.66)	18.9 (0.77)	13.1 (1.16)	13.5 (2.05)	14.0 (1.74)	14.9 (0.91)	16.0 (0.34)
g_{sat}	0.435 (0.07)	0.312 (0.04)	0.264 (0.04)	0.541 (0.05)	0.288 (0.04)	0.407 (0.08)	0.562 (0.08)	0.252 (0.02)	0.341 (0.03)	0.421 (0.09)	0.280 (0.04)	0.484 (0.04)
A_{sat}/g_{sat}	41.0 (5.87)	47.4 (6.82)	55.0 (9.05)	31.1 (2.99)	49.3 (5.28)	37.6 (5.66)	36.3 (5.11)	53.0 (4.46)	56.1 (9.12)	38.9 (8.63)	56.9 (6.03)	34.0 (2.46)
A	7.67 (0.22)	6.37 (0.20)	7.86 (0.13)	6.68 (0.76)	6.48 (0.62)	7.94 (0.43)	7.69 (0.35)	7.32 (0.50)	7.25 (0.29)	6.56 (0.60)	7.88 (0.40)	7.00 (0.46)
g_s	0.418 (0.03)	0.309 (0.02)	0.248 (0.01)	0.446 (0.04)	0.225 (0.05)	0.466 (0.02)	0.171 (0.01)	0.143 (0.01)	0.138 (0.01)	0.143 (0.01)	0.137 (0.01)	0.174 (0.02)
A/g_s	20.3 (1.01)	27.6 (1.77)	34.8 (1.46)	16.8 (1.81)	36.7 (7.36)	18.6 (0.94)	45.3 (1.91)	51.1 (2.38)	54.7 (2.28)	41.8 (2.53)	53.5 (2.73)	38.0 (1.42)
C_i/C_a	0.904 (0.01)	0.885 (0.01)	0.842 (0.01)	0.923 (0.01)	0.840 (0.03)	0.912 (0.01)	0.778 (0.01)	0.768 (0.01)	0.753 (0.02)	0.825 (0.02)	0.750 (0.01)	0.820 (0.01)
$\Delta^{13}C_M$	26.6 (0.10)	26.1 (0.15)	25.1 (0.20)	27.1 (0.27)	25.0 (0.70)	26.8 (0.14)	23.5 (0.27)	23.1 (0.28)	22.9 (0.34)	24.9 (0.51)	22.8 (0.34)	24.6 (0.29)
$\Delta^{13}C_{dif}$	2.82 (0.27)	5.34 (0.14)	3.01 (0.31)	2.08 (0.34)	4.03 (0.65)	2.65 (0.38)	1.01 (0.31)	3.26 (0.42)	3.07 (0.52)	1.57 (0.31)	3.06 (0.39)	1.32 (0.40)

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BM	27.1 (3.72)	38.3 (5.96)	27.2 (3.01)	40.8 (3.93)	41.0 (6.28)	30.5 (3.04)	37.8 (1.48)	41.6 (7.26)	31.6 (3.11)	39.9 (7.36)	45.3 (4.66)	26.9 (4.13)
WU	4.63 (0.61)	4.65 (0.58)	4.24 (0.45)	7.29 (0.56)	4.95 (0.68)	5.50 (0.49)	9.53 (0.16)	7.32 (1.39)	6.49 (0.76)	9.78 (1.74)	7.65 (0.90)	8.72 (1.03)
TE	5.83 (0.18)	8.17 (0.42)	6.45 (0.25)	5.57 (0.19)	8.28 (0.46)	5.56 (0.20)	3.96 (0.09)	5.71 (0.13)	4.93 (0.46)	4.06 (0.10)	5.93 (0.14)	3.06 (0.12)
$\Delta^{18}O_{lb}$	28.5 (0.16)	29.6 (0.18)	28.5 (0.10)	28.5 (0.33)	29.7 (0.19)	27.7 (0.17)	36.2 (0.24)	37.7 (0.17)	36.4 (0.26)	36.2 (0.14)	37.3 (0.10)	36.1 (0.25)
$\Delta^{18}O_{lw}$	9.94 (0.31)	10.2 (0.19)	9.78 (0.14)	9.36 (0.23)	11.2 (0.28)	9.54 (0.18)	18.6 (0.19)	19.4 (0.17)	18.4 (0.32)	18.9 (0.62)	19.3 (0.33)	18.5 (0.54)
T_l	31.4 (0.011)	31.6 (0.08)	31.7 (0.11)	31.1 (0.14)	31.6 (0.26)	31.5 (0.12)	29.7 (0.09)	29.6 (0.07)	29.5 (0.11)	29.1 (0.17)	29.5 (0.10)	29.2 (0.23)

Table S2: Spearman correlation table for 16 traits measured during the experiment. Phenotypic correlations were computed at individual plant level in each treatment (white columns represents Low VPD and grey columns High VPD). Correlations are taken as significant at P < 0.05. Significant correlations are set in bold with (*) representing significance levels.

	$\Delta^{13}C_{lb}$ (‰)	$\Delta^{13}C_{ss}$ (‰)	$\Delta^{13}C_{dif}$ (‰)	A (umol mol⁻¹ m⁻² s⁻¹)	g_s (mmol m⁻² s⁻¹)	A/g_s (mmol m⁻² s⁻¹)	C_i/C_a	$\Delta^{18}O_{lb}$ (‰)	$\Delta^{18}O_{lw}$ (‰)	WU (l)	ΔBM (g)	TE (g l⁻¹)	TLA (m²)	SD (mm⁻²)	LMA (g m⁻²)	LT (um)
$\Delta^{13}C_{lb}$ (‰)	*1*	**0.867*****	**-0.827*****	0.006	**0.658*****	**-0.757*****	**0.562*****	**-0.678*****	**-0.402****	0.288	-0.384	**-0.863*****	-0.056	0.011	**-0.572*****	**0.679*****
$\Delta^{13}C_{ss}$ (‰)	**0.864*****	*1*	**-0.817*****	-0.157	**0.502*****	**-0.761*****	**0.675*****	**-0.552*****	-0.251	0.237	-0.321	**-0.719*****	-0.088	0.049	**-0.627*****	**0.560*****
$\Delta^{13}C_{dif}$ (‰)	**-0.710*****	**-0.834*****	*1*	-0.133	**-0.522*****	**0.474*****	-0.220	**0.532*****	0.352	-0.311	0.353	**0.882*****	0.105	0.060	**0.527*****	**-0.572*****
A (μmol mol⁻¹ m⁻² s⁻¹)	0.252	0.168	**-0.490*****	*1*	**0.620*****	0.314	**-0.434****	0.290	**-0.435****	0.281	0.049	-0.219	0.137	-0.062	0.335	0,125***
g_s (mmol m⁻² s⁻¹)	**0.808*****	**0.797*****	**-0.543*****	0.179	*1*	**-0.504*****	0.176	-0.280	**-0.525*****	0.370	-0.181	**-0.646*****	0.042	-0.082	-0.150	**0.514*****
A/g_s (mmol mol⁻¹)	**-0.630*****	**-0.665*****	0.255	0.281	**-0.853*****	*1*	**-0.731*****	**0.535*****	0.062	-0.016	0.377	**0.518*****	0.211	-0.135	**0.493*****	-0.396
C_i/C_a	**0.592*****	**0.618*****	-0.156	**-0.422****	**0.721*****	**-0.920*****	*1*	**-0.423****	-0.005	0.204	-0.167	-0.419	0.051	0.199	**-0.531*****	0.346
$\Delta^{18}O_{lb}$ (‰)	**-0.655*****	**-0.625*****	**0.604*****	**-0.556*****	**-0.669*****	**0.394****	-0.248	*1*	0.312	-0.126	0.389	**0.674*****	0.232	0.191	**0.611*****	-0.381
$\Delta^{18}O_{lw}$ (‰)	**-0.665*****	**-0.606*****	**0.644*****	**-0.460*****	**-0.624*****	0.356	-0.180	**0.684*****	*1*	**-0.647*****	-0.268	0.381	-0.412	0.356	-0.177	-0.282
WU (l)	**0.534*****	**0.548*****	**-0.604*****	0.273	**0.407****	-0.222	0.148	-0.164	**-0.538*****	*1*	**0.644*****	-0.293	**0.804*****	-0.076	0.166	**0.577*****
BM (g)	0.008	0.064	-0.190	0.009	-0.021	0.088	-0.124	0.304	-0.154	**0.806*****	*1*	**0.468*****	**0.825*****	-0.032	**0.571*****	0.072
TE (g l⁻¹)	**-0.829*****	**-0.693*****	**0.581*****	**-0.390****	**-0.687*****	**0.456*****	**-0.444*****	**0.700*****	**0.554*****	-0.297	0.257	*1*	0.063	0.022	**0.605*****	**-0.618*****
TLA (m²)	0.336	**0.377****	**-0.464*****	0.123	0.272	-0.177	0.070	-0.018	**-0.381****	**0.823*****	**0.798*****	-0.038	*1*	0.157	0.409	0.384
SD (mm⁻²)	**0.368****	0.329	-0.165	-0.285	**0.404****	**-0.476*****	**0.407****	-0.085	-0.103	0.361	**0.377****	0.049	**0.534*****	*1*	0.194	0.273
LMA (g m⁻²)	**-0.745*****	**-0.695*****	**0.556****	-0.068	-0.351	0.287	-0.253	0.482	0.356	-0.136	0.178	**0.568*****	-0.160	-0.324	*1*	-0.218
LT (μm)	**0.706*****	**0.564*****	**-0.560*****	0.321	**0.611*****	**-0.355****	0.312	**-0.493*****	**-0.553*****	0.283	-0.019	**-0.521*****	0.156	0.271	**-0.545*****	*1*

Fig. S1: Time course of genotypic means of plant height and diameter at collar under low (a, c) or high (b, d) *VPD*. Each point is the mean from five individuals per genotype. Numbers in the inlet represent the different genotypes.

Fig. S2: Time course of genotypic means of daily water use (WU_d) during the experiment under low and high *VPD*. Each point is the mean from five individuals per genotype. Numbers in the inlet represent the different genotypes.

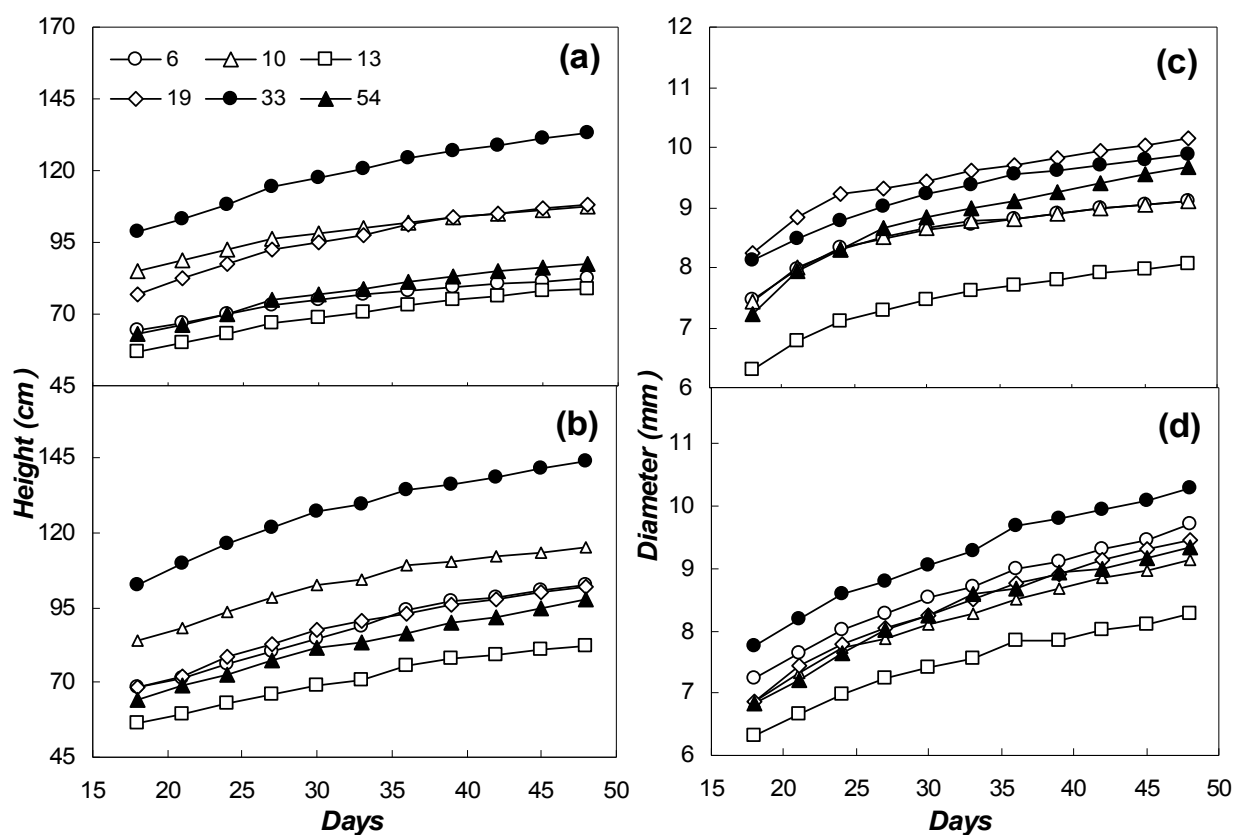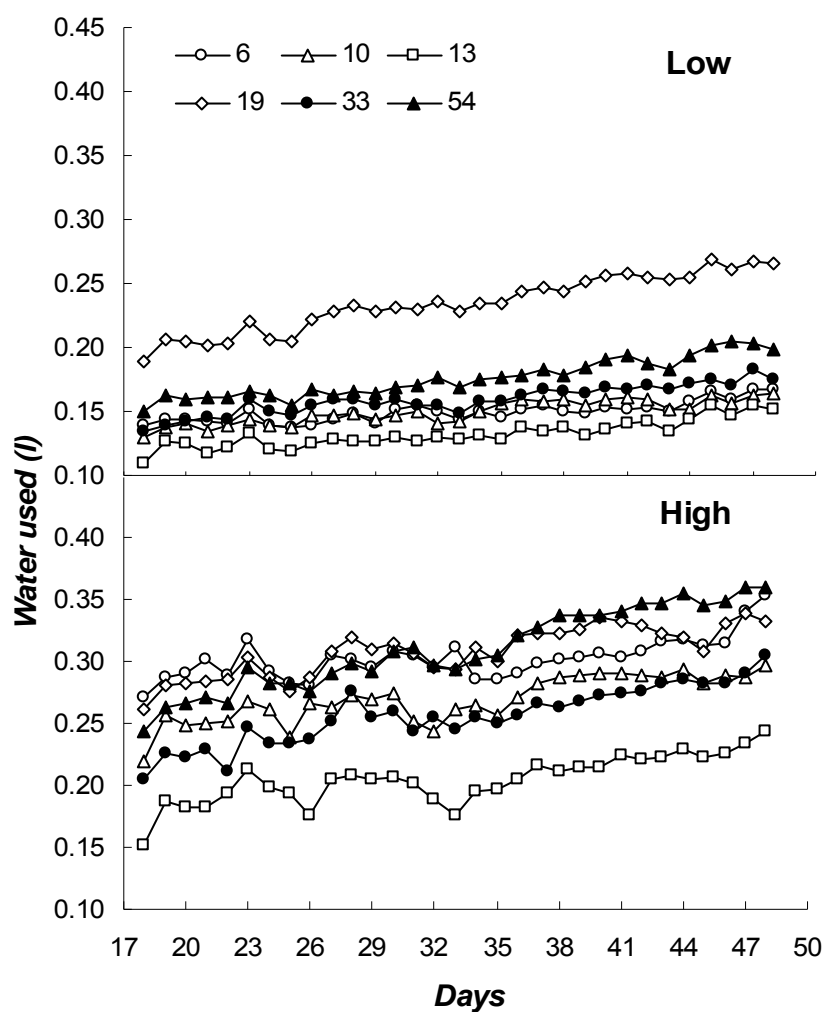**Fig. S3:** Correlation between the product Height x Diameter and the biomass recorded on 6 individuals per genotype at the onset of the experiment. Each point represents data from a single individual. No genotype specific relationship was detected.
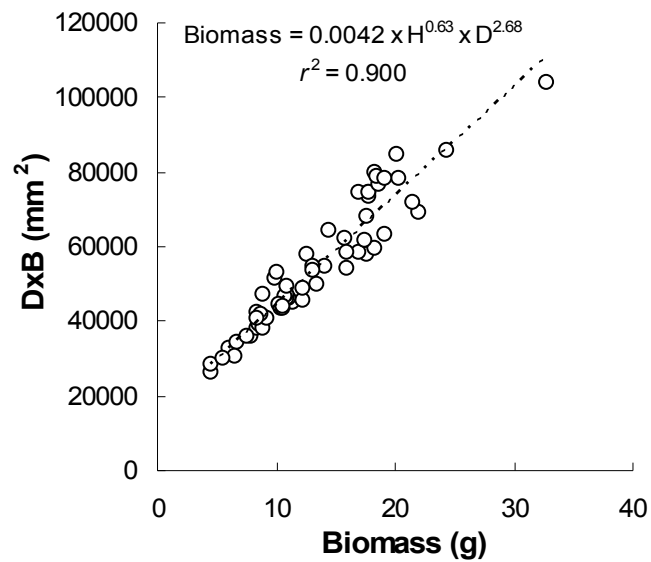
Fig. S4: Daily time course for the $\delta^{13}C_{air}$ over four days during the experiment. Each curve represents the diurnal evolution of over the day. Open shapes represents measurements in low VPD and closed shapes in high VPD. X-axis represents the time in hours, where each point represents a mean of three values. Numbers in the legend represents the sampling date during the experiment under High (open) or Low (close) VPD.
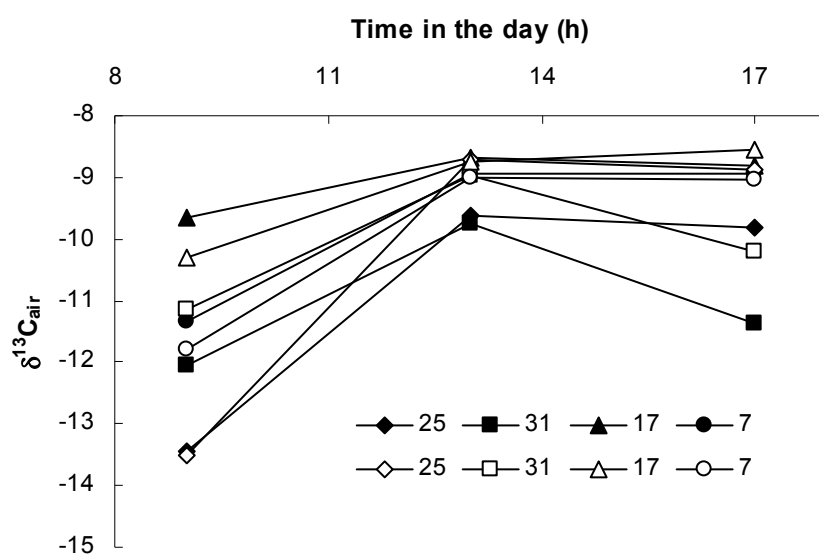
CHAPTER 3

Stability of genotype ranking for $\Delta^{13}C$ with age: A diachronic

approach

Transition

Studies on poplar (*Populus × euramericana* genotypes) presented large genotype diversity for $\Delta^{13}C$ both controlled condition (Marron *et al.* 2005) and under field conditions (Monclus *et al.* 2005). This genotype diversity for $\Delta^{13}C$ was shown to remain stable under moderate drought evidenced using Spearman's correlation coefficient (Monclus *et al.* 2006). Further more it was shown that the genotype differences for $\Delta^{13}C$ reflects the differences of *TE* both at intrinsic and whole plant level under both high humidity and different *VPD* treatments (Chapter 1 and Chapter 2). In this pursuit, stability of genotype ranking for $\Delta^{13}C$. time remained unchecked. Previous studies regarding other tree species, $\Delta^{13}C$ has been shown to vary with age depending upon species and environment (Bert *et al.* 1997; Duquesnay *et al.* 1998). Genotype difference of 2.78‰ was detected between high and low $\Delta^{13}C$ in 29 *Populus × euramericana* genotypes, which makes them closely related in terms of $\Delta^{13}C$ (Monclus *et al.* 2006). Thus, it becomes important to verify the stability genotype ranking with age for $\Delta^{13}C$. In this context, using dichromic approach, $\Delta^{13}C$ was measured along the tree cores over the period of ten years from 5-15 yeas and stability of genotype ranking was verified on three study sites.

Research paper

Time course of $\delta^{13}C$ in poplar wood: genotype ranking remains stable over the life cycle in plantations despite some differences between cellulose and bulk wood

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Genetic differences in $\delta^{13}C$ (isotopic composition of dry matter carbon) have been evidenced among poplar genotypes at juvenile stages. To check whether such differences were maintained with age in trees growing in plantations, we investigated the time course of $\delta^{13}C$ as recorded in annual tree rings from different genotypes growing at three sites in southwestern France and felled at ~15–17 years. Wood cores were cut from tree discs to record the time course of annual basal area increment (BAI). The isotopic ratio $\delta^{13}C$ was recorded in bulk wood and in extracted cellulose from the annual rings corresponding to the period 1996–2005. Discrimination against ^{13}C between atmosphere and tissues ($\Delta^{13}C$) was computed by taking into account the inter-annual time course of $\delta^{13}C$ in the atmosphere. Annual BAI increased steadily and stabilized at about 8 years. An offset in $\delta^{13}C$ of ~1‰ was recorded between extracted cellulose and bulk wood. It was relatively stable among genotypes within sites but varied among sites and increased slightly with age. Site effects as well as genotype differences were detected in $\Delta^{13}C$ recorded from the cellulose fraction. Absolute values as well as the genotype ranking of $\Delta^{13}C$ remained stable with age in the three sites. Genotype means of $\Delta^{13}C$ were not correlated to annual BAI. We conclude that genotypic differences of $\Delta^{13}C$ occur in older poplar trees in plantations, and that the differences as well as the genotype ranking remain stable while trees age until harvest.

Keywords: age, **Populus×euramericana**, tree rings, water-use efficiency.

Introduction

Fast-growing *Populus×euramericana* hybrids are frequently used in poplar cultivation (Ceulemans et al. 1992). These genotypes have been bred for high productivity, resistance to rust and wood quality. In the context of the predicted climate change, enhanced risk of drought events might have a negative influence on the productivity of such highly bred genotypes. Genotypes displaying larger transpiration efficiency (TE) might become more interesting if they are able to maintain their productivity even under decreasing soil water availability. Transpiration efficiency is the ratio between biomass production and water transpired by the trees (see Condon et al. (2004) for a review). Transpiration efficiency is to a large extent controlled by vapour pressure deficit in the atmosphere and by intrinsic water-use efficiency (W_i), i.e., the ratio between net CO_2 assimilation rate and stomatal conductance to water vapour. Discrimination against ^{13}C occurs during

photosynthesis, and $\delta^{13}C$ is smaller in leaves and stems than in the atmosphere. This difference ($\Delta^{13}C$) is frequently used as a proxy of W_i (Farquhar and Richards 1984), although some limits of the use of this proxy have been highlighted (Warren and Adams 2006, Seibt et al. 2008).

A large variability in $\Delta^{13}C$ of bulk leaf material has been found among poplar genotypes belonging to various genetic backgrounds (Marron et al. 2005, Monclus et al. 2005, 2006, 2009, Bonhomme et al. 2008). Moreover, no trade-off has been detected among genotypes between $\Delta^{13}C$ and productivity, which opens the prospects of selecting genotypes with a high water-use efficiency without affecting overall productivity.

This genetic variability of $\Delta^{13}C$ was usually recorded on very young individuals (up to 2 years) grown in greenhouses or open field experiments. Care must be taken when trying to extrapolate such results to adult tree plantations as $\Delta^{13}C$ might change with age, which could affect the genotype ranking over time. Recording ^{13}C content in cellulose extracted from successive tree rings is a suitable technique to study age-related changes in $\Delta^{13}C$. Several studies reported the occurrence of an age-related trend in $\Delta^{13}C$: larger values are usually recorded in the young stages as compared with older ones (Francey and Farquhar 1982, Bert et al. 1997, Duquesnay et al. 1998, McCarroll and Pawellek 2001). Juvenility effects are known to affect a whole range of traits, and genotype ranking for such traits can change to a large extent with tree age. Juvenility effects that might change the genotype ranking of $\Delta^{13}C$ among poplars at an older age have never been documented to our knowledge. This study therefore assessed the time course of $\Delta^{13}C$ in bulk wood and cellulose of annual rings of different poplar genotypes, with the aim of testing whether the genotype ranking for $\Delta^{13}C$ remains stable over 10 years (from 5 to 15 years) in various environments.

Bulk wood is typically depleted in ^{13}C by 0.5–2.0‰ in comparison to cellulose due to the presence of lignins (Tans and Mook 1980, Leavitt and Long 1991, Marshall and Monserud 1996, Loader et al. 2003). The fraction of cellulose, lignins and soluble material can be highly variable in wood, even within a ring (Helle and Schleser 2004), which might affect the ^{13}C signal of bulk wood with time. We therefore assessed ^{13}C in cellulose and in bulk wood as well as the cellulose content of wood and their changes in time, with the aim of testing whether the offset of $\delta^{13}C$ between extracted cellulose and bulk wood was stable with age, sites and genotypes.

The following questions were addressed:

(i)   Is the offset of $\delta^{13}C$ between bulk wood and extracted cellulose stable with genotype, site and age?

(ii)   Is there a genetic variability of $\Delta^{13}C$ in plantation-grown genotypes and is it stable with the age of the trees?

(iii)   Are there interactions between $\Delta^{13}C$ and basal area increment (BAI) among genotypes?

This approach requires the use of common garden plantations of mature poplar trees, which are rather uncommon in Europe. We used the opportunity of comparative tests developed by the 'Institut pour le Développement Forestier', in which trees were harvested to analyse wood quality at three different sites in southwestern France: Begaar, Migron and Sérignac. Stem disks were used to separate annual growth rings, and to assess BAI, $\delta^{13}C$ in bulk wood and extracted cellulose.

Materials and methods

Site description

Three study sites (Begaar 43°48′N, 0°52′W, Migron 45°48′N, 0°24′W and Sérignac 44°13′N, 0°28′E) were selected in southwestern France. The three sites have an oceanic climate with mean annual temperatures of 12, 13 and 14 °C, and annual rainfall values of 1130, 800 and 700 mm, respectively. At Begaar, soils are clayey down to a depth of 40 cm and silty-clayey from 40 to 200 cm. Soil pH is 5.7 and Fe/Mg is present in the second horizon. The soils of Migron and Sérignac are silty-clayey between 0 and 110 cm and sandy below 110–120 cm, with a pH of 7.5. Single genotypes were planted in plots of 25 individuals each, at a distance of 7×7 m, i.e., at a density of 204 stems ha^{-1}. Trees were growing freely at Begaar and Sérignac and were pruned during 1998 in Migron. They were felled at the age of 15–17 years.

Sampling

A total of 41 tree disks were sampled from nine genotypes. The genotypes were unevenly distributed among the three sites (Table 1) and only two, *Flevo* and *I214*, were present at

Table 1. Distribution of the poplar genotypes among the three study sites (Begaar, Sérignac, Migron) and number of individual trees sampled per genotype.

Genotypes	Begaar n	Sérignac n	Migron n	Total n
Flevo	3	3	3	9
I214	2	2	3	7
Dorskamp	2	3		5
I45/51		3	3	6
Robusta	3			3
Ghoy			3	3
Beaupré		3		3
Blanc du Poitou			3	3
Fritzi Pauley			2	2
Total	10	14	17	41

the three sites. Stem cross-sections comprising 10 year-rings (1996–2005) were dried and polished progressively with finer grades of sandpaper to enhance ring boundaries (Tarhule and Hughes 2002). Three radii at right angles to each other were selected per disk for growth measurements. Wood splits and dead knots were avoided. Annual rings were cross-dated and ring width was measured using LINTAB 1.08 © 2002 at a ×12.5 magnification (Leica MZ 6; Tokyo, Japan). The accuracy of measurements was ±1 μm.

Mean annual ring width (of three radii) was used to compute radial increment (RI) and BAI. Basal area increment was derived from RI under the hypothesis of a cylindrical stem.

Annual ring separation and grinding

Three wood cores (width = 3 cm) were excised from each disk at right angles, to take into account a potential circumferential variability of δ¹³C (Leavitt and Long 1984). The outer 10 annual rings were separated (1996–2005) using a sharp razor blade and a hammer. As the date of felling was known and the annual rings were reasonably large and distinct, cross-dating of year rings was easy. Annual rings were further sliced (rings being large) to grind them into fine homogeneous wood powder using a ring grinder (SODEMI; CEP Industries Department, Cergy-Pontoise, France). In each genotype, an individual was selected and the annual ring formed during year 1997 was further divided into three sections to record within-ring changes in δ¹³C at Begaar and Migron.

Cellulose extraction

A total of 410 annual rings were analysed (10 year series, 41 individual trees). Following the procedure of Leavitt and Danzer (1993) adapted by Ponton et al. (2001), extraction of α-cellulose was carried out using 60 mg of wood powder. Organic soluble extractives were removed in two strokes (double extraction) in an extractor type Soxhlet. During the first stroke, samples were successively washed three times with a mix of 95% toluene and 95% ethanol, 2/1 (v/v). In the second stroke, samples were successively washed again three times with absolute ethanol (100%). Water-soluble extractives were removed by boiling for 6 h in de-ionized water. Each sample was de-lignified using a sodium chlorite (NaClO₂) solution at 70 °C. Samples were placed in 1000 ml distilled water in an ultrasound stirrer. Sodium chlorite (2.46 g NaClO₂/g dry weight) was added five times and the solution pH was maintained at 3.8 using acetic acid after each sodium chlorite addition. To reduce the proportion of hemi-celluloses, the samples were washed in NaOH (0.1 M) in an ultrasonic bath at room temperature for 45 min. The samples were then rinsed in distilled water and neutralized in HCl 0.06 N at room temperature for 30 min. The samples

were thoroughly rinsed and agitated in distilled water overnight. The next day, the samples were treated with ethanol in an ultrasound stirrer and were left to dry in air. Cellulose fraction was calculated as the percentage of cellulose present in the 60 mg of wood powder. Subsamples (~1 mg) of α-cellulose were then micro-weighed and used for δ¹³C measurements.

Carbon isotope analysis

Samples were combusted to CO_2 at 1050 °C in sealed evacuated quartz tubes containing pre-combusted cobalt oxide and chromium oxide as the oxygen source in an elemental analyser (Carlo Erba, NA 1500-NC, Milano, Italy). Combustion products were separated by gas chromatography and the CO_2 was delivered to an isotope ratio mass spectrometer (Finnigan, Delta-S, Bremen, Germany) for isotopic analysis. The stable carbon isotope composition of a naturally occurring material can be expressed as δ¹³C, which is the relative deviation from the internationally accepted Vienna-PDB standard.

The accuracy of the measurements was ±0.1‰. As a result of human activities, the ratio ¹³C/¹²C has decreased in the atmosphere. We corrected for this effect and used the published values (from −7.88 to −8.12‰ with a regular decrease of 0.0281‰ per year; Francey et al. 1999, McCarroll and Loader 2004). Carbon isotope discrimination was calculated as

$$\Delta^{13}C = \left(\frac{\delta_a - \delta_p}{1000 + \delta_p} \right) \times 1000$$

(1)

where δ_a and δ_p are the values of δ¹³C in the atmosphere and in the plant, respectively.

Statistical analysis

The data set covering 10 years (1996–2005) was analysed. Site differences were detected using one-way analysis of variance (ANOVA) with data from the genotypes *Flevo* and *I214* present in the three sites. Afterwards, each site was tested separately for genotype differences, temporal trends and the time stability of the genotype ranking. All the tests were performed using the statistical package R (R version 2.5.1, 2007, The R foundation for statistical computing, http://www.r-project.org/foundation/).

In order to test the temporal trend in Δ¹³C and the offset between cellulose and wood, we fitted a one-way analysis of covariance (ANCOVA) mixed model. We used year (centred values over 10 years 1996–2005) as a fixed regressor and genotype as a fixed factor. We introduced a random tree effect on intercept and slope as each tree represents a time series. Years were centred to de-correlate the random effects on slopes and intercepts. When no genotype × year

interaction was detected, the genotype effect was tested using a one-way ANOVA of 10-year means within each site. Pairwise comparisons of genotypes within site were done using a multi-comparison test.

The relationship between the response variable and the predictor variable was described using a linear mixed model:

$$Y_{cta} = (\alpha + \alpha_c + \alpha_{ct}) + (\beta + \beta_c + \beta_{ct}) \times \gamma_a + \varepsilon_{cta}$$

where Y_{cta} is the response variable, $\alpha + \alpha_c$ refers to the fixed effects of genotype and time, α_{ct} is the random effect of trees, $\beta + \beta_c$ is the fixed interaction effect, β_{ct} is the random interaction effect of trees, γ_a is the centred year and ε_{cta} is the residual. Normality and homoskedasticity were tested graphically with residual vs. predicted and normal quantile to quantile plots. Correlation between the parameters was tested for each genotype within each site separately in order to avoid the potential genotype effect on the correlation. Tests were considered to be significant when $P < 0.05$.

Results

Basal area increment

Mean annual BAI displayed significant genotypic differences within sites (Table 2). At Begaar, BAI was lower in *Robusta* than in *Flevo* ($P < 0.001$), *I214* ($P < 0.001$) and *Dorskamp* ($P = 0.005$). *I214* displayed highest BAI at Migron, as compared with *Flevo* ($P = 0.02$), *Ghoy* ($P = 0.001$), *Blanc du Poitou* ($P < 0.001$) and *Fritzi Pauley* ($P < 0.001$). *I214* and

Dorskamp displayed highest and *I45/51* lowest BAI at Sérignac. Basal area increment was much smaller during 2003 at Begaar and during 2002 at Sérignac (Figure 1). No such deficit was evident at Migron. In general, year 2003, which was marked by a severe drought and heat wave in northern France, had only little impact at these sites from southwestern France.

At Begaar and Sérignac, BAI increased rapidly towards a peak and then remained stable or decreased slightly (Figure 1). The largest BAI was recorded in almost all genotypes during years 1998–2001 (~8 years after plantation). At Migron, BAI displayed a more variable pattern among genotypes, probably due to pruning.

A significant site effect was evidenced for BAI in the two common genotypes: at Sérignac, BAI was smaller for *Flevo* as compared with Begaar ($P < 0.001$) and Migron ($P < 0.001$) and for *I214* as compared with Migron ($P < 0.001$).

Cellulose fraction in bulk wood

Genotypic differences of cellulose fraction in bulk wood were evidenced within sites, with values ranging from 51.4 to 57.6% (Table 2). *Flevo* displayed higher values than *I214* in the three sites. Over the 10-year period, no genotype × year interaction and no time-related trend were detected in any site or genotype (Supplementary Table 1 available as Supplementary Data at *Tree Physiology* Online). This demonstrates the stability of the cellulose fraction and of the genotype ranking with time. The two common genotypes displayed smaller values at Begaar and Migron than at Sérignac ($P < 0.001$, Table 2).

Table 2. Means (± SE) over 10 years (1996–2005) of annual BAI, cellulose fraction in bulk wood, isotopic discrimination recorded from wood and cellulose ($\Delta^{13}C$), offset between wood and cellulose ($\delta^{13}C$), density of vessels in a year ring (n_v), and average vessel cross-section (AVSA) of poplar genotypes grown at three sites. Each value represents a 10-year mean of 2–3 individuals. Different letters within columns and sites indicate significant differences ($P < 0.05$).

| Genotypes	BAI (cm²)	Cellulose (%)	$\Delta^{13}C$ wood (‰)	$\Delta^{13}C$ cellulose (‰)	$\delta^{13}C$ offset (‰)	n_v (mm⁻²)	AVSA (µm²)
Begaar							
Flevo	102 ± 3.99 a	56.2 ± 0.57 a	20.6 ± 0.06 b	19.4 ± 0.09 b	1.15 ± 0.06 a	55.4 ± 2.02 a	5780 ± 117 ab
Dorskamp	85.8 ± 4.88 a	55.2 ± 0.70 ab	21.2 ± 0.07 a	19.9 ± 0.12 a	1.21 ± 0.08 a	51.5 ± 2.47 a	6160 ± 142 a
Robusta	64.6 ± 3.99 b	54.5 ± 0.57 ab	20.6 ± 0.06 b	19.4 ± 0.09 b	1.07 ± 0.07 a	53.1 ± 2.02 a	5170 ± 117 c
I214	94.1 ± 3.99 a	53.6 ± 0.57 b	20.6 ± 0.06 b	19.4 ± 0.09 b	1.18 ± 0.07 a	45.0 ± 2.02 b	5440 ± 117 bc
Sérignac							
Flevo	74.7 ± 3.96 ab	57.6 ± 0.51 a	20.3 ± 0.06 a	19.3 ± 0.09 a	0.96 ± 0.05 a	59.2 ± 4.14 a	4990 ± 155 b
Dorskamp	84.8 ± 3.96 a	56.4 ± 0.51 ab	20.4 ± 0.06 a	19.4 ± 0.09 a	1.02 ± 0.05 a	51.9 ± 3.65 a	5370 ± 136 ab
I214	83.8 ± 4.85 a	55.6 ± 0.63 b	20.6 ± 0.07 a	19.6 ± 0.12 a	0.91 ± 0.05 a	57.9 ± 4.14 a	5410 ± 155 ab
I45/51	61.3 ± 3.96 b	57.0 ± 0.51 ab	19.5 ± 0.06 b	18.5 ± 0.09 b	0.97 ± 0.05 a	47.8 ± 3.38 a	5740 ± 126 a
Beaupré	79.5 ± 3.96 a	56.9 ± 0.51 ab	19.6 ± 0.06 b	18.5 ± 0.09 b	0.94 ± 0.06 a	56.6 ± 3.38 a	5580 ± 126 a
Migron							
Flevo	106 ± 4.66 b	54.9 ± 0.55 b	20.2 ± 0.06 a	19.1 ± 0.09 a	1.11 ± 0.08 a	52.9 ± 5.29 b	5400 ± 118 c
I214	129 ± 5.70 a	52.7 ± 0.67 bc	19.9 ± 0.07 a	19.0 ± 0.11 a	0.91 ± 0.09 ab	39.0 ± 6.09 b	6260 ± 134 b
I45/51	112 ± 4.66 ab	54.9 ± 0.55 b	19.2 ± 0.06 b	18.5 ± 0.09 b	0.64 ± 0.08 b	39.1 ± 4.97 b	6800 ± 110 a
Ghoy	101 ± 4.66 b	53.5 ± 0.55 bc	19.5 ± 0.06 b	18.5 ± 0.09 b	0.96 ± 0.08 ab	44.7 ± 4.97 b	5870 ± 110 b
Blanc du Poitou	98.9 ± 4.66 b	57.5 ± 0.55 a	19.9 ± 0.06 a	19.0 ± 0.09 a	0.84 ± 0.08 ab	78.3 ± 6.09 a	6060 ± 134 b
Fritzi Pauley	67.6 ± 5.70 c	51.4 ± 0.67 c	18.4 ± 0.07 c	17.5 ± 0.11 c	0.83 ± 0.09 ab	51.0 ± 6.09 b	4870 ± 134 d
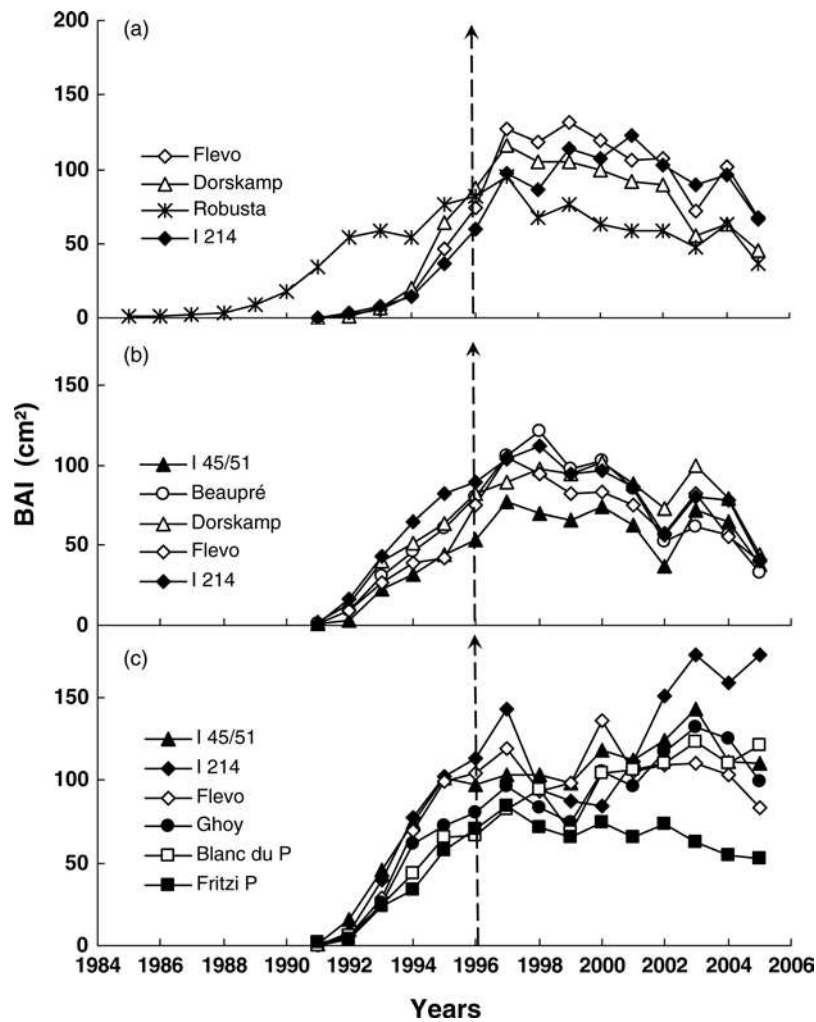
Figure 1. Time course of mean annual BAI for the genotypes grown at Begaar (a), Sérignac (b) and Migron (c). The effects of pruning, done only at Migron, are clearly visible after year 1998. The arrow on each graph points to 1996, when isotopic analyses were started. *Robusta* was older than the other genotypes at Begaar.

Intra-annual pattern of Δ¹³C

We analysed the intra-annual pattern of Δ¹³C in cellulose during year 1997, selected because of large rings enabling the cutting of three similar segments, and the lack of severe environmental constraints on growth. The segments were compared with a mixed linear model with Δ¹³C as fixed and segments as random factor for differences among them (see Figure 2). The third (late season) segment displayed a smaller value of Δ¹³C than the first ($P < 0.0001$) and second ones ($P = 0.00021$) while the latter two did not differ.

Offset between δ¹³C of α-cellulose and bulk wood

As expected, cellulose displayed higher values of δ¹³C than bulk wood (Table 2, Figure 3). The offset between bulk wood and extracted cellulose varied between 0.83 and 1.21‰, and the correlation between δ¹³C of bulk wood and cellulose was very tight once outlier values were excluded from the analysis (Figure 3, $r^2 = 0.87$). We checked that these outliers were nei-

ther site-, nor genotype- or year-specific, but occurred randomly. We therefore ascribed them to random deviations due to a technical variation during cellulose extraction.

At Begaar, no genotype effect but a small interaction effect (genotype × year) was detected for the δ¹³C offset ($P = 0.582$ and 0.037, respectively). At Sérignac, neither genotype nor interaction effects were detected ($P = 0.730$ and 0.710, respectively). At Migron, both genotype and interaction effects were significant ($P = 0.004$ and 0.042, respectively). A temporal increasing trend was evidenced in the δ¹³C offset (Figure 4); it was significant at Begaar and Migron, but not at Sérignac. A detailed analysis revealed that some genotypes (*Flevo* in two sites out of three, *I45/51* and *Fritzi-Pauley*) displayed such a temporal increase while others were particularly stable (*I214* in the three sites for instance, Table 3). A small site effect was detected in *I214* (the offset was larger at Begaar than at Sérignac, $P = 0.001$, and Migron, $P = 0.001$) but none in *Flevo*. There was no detectable cor-
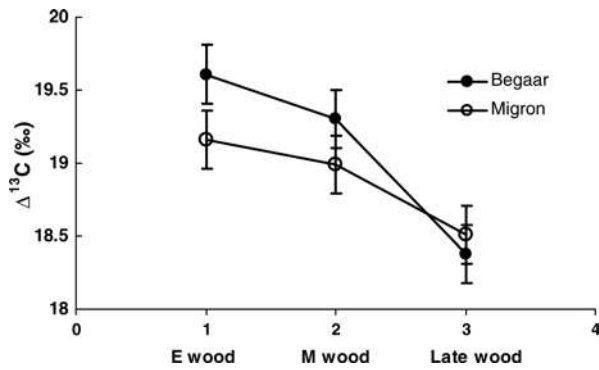
Figure 2. Variation of $\Delta^{13}C$ within annual tree rings recorded in all poplar genotypes at two sites (Migron and Begaar) for the ring from year 1997. Early, mid-season and latewood were analysed separately.
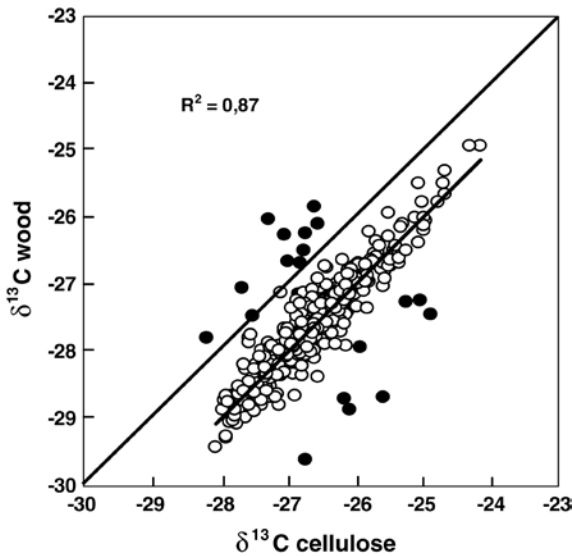

Figure 3. Correlation between $\delta^{13}C$ of cellulose and of bulk wood for all tree-ring samples from all poplar genotypes at the tree sites. Black: outlier values; white: values included in the correlation. The outliers were neither site- nor genotype-dependent but occurred randomly (see the text for details).

Figure 4. Time course of the mean offset between $\delta^{13}C$ of cellulose and of bulk wood in poplar genotypes grown at three sites (Begaar, a; Sérignac, b; and Migron, c). Genotypes were bulked. Means ± SE. There was a temporal increase in the offset at Begaar and Migron but not at Sérignac.

relation between $\delta^{13}C$ offset and cellulose fraction in the wood ($r^2 = 0.015$ and $P = 0.821$).

Due to the temporal increase in the offset found in some genotypes and to homogenize our samples, we decided to concentrate on the isotopic signals of tree-ring cellulose to assess genotypic differences and temporal evolution of $\Delta^{13}C$.

Genotype differences for $\Delta^{13}C$ recorded in tree-ring cellulose

Genotype means ranged from 18.4 to 21.2‰ (*Fritzi Pauley* to *Dorskamp*, Table 2, Figure 5). Genotypic differences for $\Delta^{13}C$ were evidenced at Begaar ($P = 0.022$), Sérignac ($P < 0.001$) and Migron ($P < 0.001$). Highest $\Delta^{13}C$ was exhibited by *Dorskamp*, which differed from all the other genotypes (*Flevo*, $P = 0.016$; *I214*, $P = 0.003$; and *Robusta*, $P = 0.016$) at Begaar.

Two groups were detected at Sérignac: *Beaupré and I45/51* differed from *Dorskamp* ($P < 0.001$), *Flevo* ($P < 0.001$) and *I214* ($P < 0.001$). At Migron, *Fritzi Pauley* displayed a lower mean $\Delta^{13}C$ than all the other genotypes. *I45/51* and *Ghoy* were also significantly different from *I214* ($P = 0.024$), *Blanc du Poitou* ($P = 0.005$) and *Flevo* ($P < 0.001$).

Site effects were also evidenced, as $\Delta^{13}C$ was smaller by ~0.5‰ at Migron than at the other two sites for *Flevo* (Begaar $P = 0.014$ and Sérignac $P = 0.001$) and for *I214* ($P = 0.023$ and $P < 0.001$).

Time course of $\Delta^{13}C$ over 10 years

No interaction (genotype × year) was detected at Begaar ($P = 0.163$) or at Sérignac ($P = 0.208$), which shows the stability of the genotype ranking with time. No time-related trend was evidenced for any of the genotypes within the two sites (Supplementary Table 2 available as Supplementary Data at *Tree Physiology* Online). At Migron, significant interaction effects ($P < 0.001$) were detected, showing some changes in the genotype ranking. This was due to the significant decreas-

Table 3. Regression estimates for the offset between $\delta^{13}C$ of cellulose and bulk wood vs. time over 10 years in the genotypes at three sites. Significant values in bold ($P < 0.05$).

Genotypes	Begaar		Sérignac		Migron	
	Estimates	P values	Estimates	P values	Estimates	P values
Flevo	**0.096**	**0.009**	0.032	0.100	**0.067**	**0.009**
I214	0.049	0.180	−0.005	0.821	0.012	0.691
Dorskamp	0.035	0.427	0.036	0.063		
Robusta	0.007	0.824				
Beaupré			0.027	0.157		
I45/51			0.028	0.152	**0.119**	**< 0.001**
Blanc du Poitou					0.011	0.667
Fritzi Pauley					**0.074**	**0.019**
Ghoy					0.041	0.111

ing trend displayed by *Ghoy* (−0.142, $P < 0.001$) and *I45/51* (−0.089, $P = 0.001$, Figure 5) while the other four genotypes remained stable.

Correlations between BAI and Δ¹³C

Inter-annual variability: Over the period of 10 years at Begaar and Migron, no correlation was detected between BAI (as a surrogate of productivity) and $\Delta^{13}C$ within any genotype. This

was not the case at Sérignac where a significant positive correlation was detected between BAI and $\Delta^{13}C$ for all genotypes except *Flevo* (see Table 4).

Inter-genotype variability: Within each site, no correlation was found between mean BAI and $\Delta^{13}C$ (Begaar $r^2 = 0.006$, $P = 0.92$, $n = 4$; Migron $r^2 = 0.595$, $P = 0.06$, $n = 6$; Sérignac $r^2 = 0.446$, $P = 217$, $n = 5$). Taking all three sites together, there was again no correlation between genotypic mean BAI and $\Delta^{13}C$ ($r^2 = 0.012$, $P = 0.693$, $n = 15$).

Discussion

Variability in annual BAI

The poplar genotypes grown at the three locations showed a consistent increase in annual BAI during the early growth period (years 1–8) followed by a slow decrease. This pattern was similar to that of cottonwood and other species (see Bert et al. 1997, Poage and Tappeiner 2002, Willms et al. 2006). However, maximum annual BAI was reached at the age of 8 years vs. 15–20 years in cottonwood. This probably reflects differences in plantation density leading to different ages for
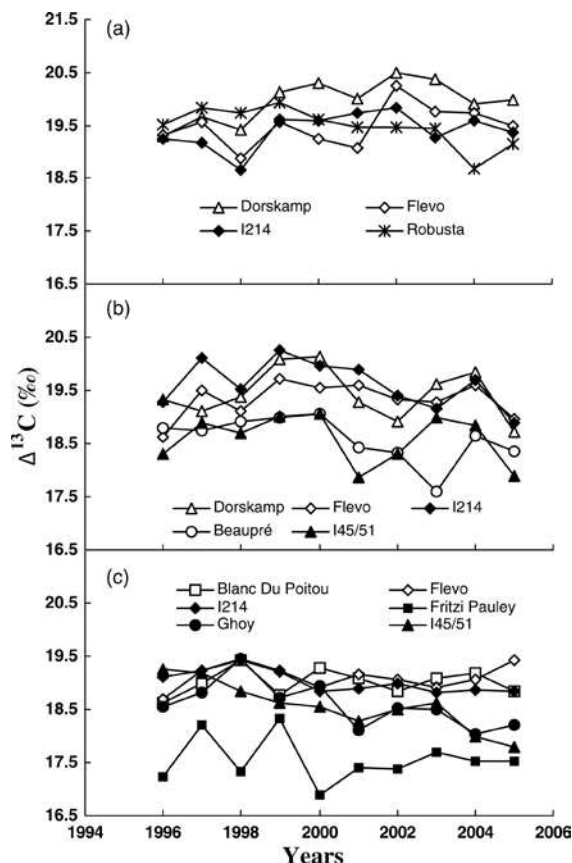
Figure 5. Time course of genotype values of $\Delta^{13}C$ (‰) of cellulose extracted from wood over 10 years at three sites (Begaar, a; Sérignac, b; and Migron, c). Large inter-annual variations, but no time-related trend, were recorded, except for a small negative trend in *Ghoy* and *I45/51* at Migron.

Table 4. Correlation over 10 years between yearly BAI and $\Delta^{13}C$ recorded in the cellulose extracted from wood in poplar genotypes at three sites. Significant values in bold ($P < 0.05$).

Genotypes	Begaar		Sérignac		Migron	
	r^2	P values	r^2	P values	r^2	P values
Flevo	0.017	0.712	0.065	0.532	0.299	0.101
I214	0.279	0.116	0.425	**0.041**	0.236	0.154
Dorskamp	0.061	0.488	0.497	**0.023**		
Robusta	0.284	0.112				
Beaupré			0.407	**0.047**		
I45/51			0.514	**0.019**	0.083	0.417
Blanc du Poitou					0.159	0.252
Fritzi Pauley					0.003	0.878
Ghoy					0.188	0.210

canopy closure. At the site where trees were pruned during 1998, BAI remained stable till harvest, except for *I214* which displayed a consistent increase. Cutting of the lower branches probably reduced the size of competing sinks and resulted in a larger allocation of carbon and biomass to trunks. *I214* was found to be most productive at all three sites followed by *I45/51* at Migron. *I45/51*, which was productive at Migron, showed a significant decrease in BAI over 10 years at Serignac: this could be attributed to low soil fertility, low water availability or a combination of both at this site as compared with the other two site. *I214* remained more productive than *Flevo* at Serignac and Migron. These results are consistent with those found by Marron et al. (2003) for young individuals.

$\delta^{13}C$ offset between bulk wood and α-cellulose

Cellulose extraction and the use of purified holo-cellulose is usually considered a prerequisite to establish reliable long-term trends in $\delta^{13}C$ in tree-ring chronologies for two reasons: (i) the isotopic composition of holo-cellulose is supposed to be close to that of the primary products of photosynthesis with little post-photosynthetic discrimination, and (ii) cellulose is deposited during the year of ring formation while lignin can still be deposited several years later: as a consequence, bulk-wood signals may not reflect year-to-year chronologies.

Our estimates of the offset between bulk wood and α-cellulose varied from 0.8 to 1.2‰, which is consistent with the range found previously (Tans and Mook 1980, Leavitt and Long 1991, Loader et al. 2003, Cullen and MacFarlane 2005). Due to the presence of lignins (3‰ more depleted than cellulose, Loader et al. 2003), bulk tissue is typically more depleted in $\delta^{13}C$ in comparison to α-cellulose. This offset between bulk wood and holo-cellulose is generally considered to be stable without any temporal change (Loader et al. 2003, McCarroll and Loader 2004, Cullen and MacFarlane 2005): if this was the case, measuring $\delta^{13}C$ in bulk wood would provide a consistent signal for inter-annual and inter-genotype variability of δ^{13} without the need for a tedious holo-cellulose extraction. Unfortunately, we evidenced some significant temporal trends in the offset in several genotypes in each site over 10 years (in *Flevo* at Begaar and in *Flevo, Fritzi Pauley* and *I45/51* at Migron in particular, with positive regression slopes). Meanwhile, there was no temporal trend in the isotopic composition of cellulose. Increases of lignin content with ring age (due to continuing lignin deposition in older rings) could be a potential cause for the increase in the offset. We have only little data to discuss this point. The cellulose fraction in year rings remained constant with time. We therefore cannot draw conclusions about the causes of the changes in this offset. A detailed analysis of the three major components of wood (holo-cellulose, hemi-cellulose and lignins) would be required to reach a firm conclusion, and this was beyond the objectives of the present research.

Intra-annual variability of δ^{13} in tree rings

The isotopic composition of holo-cellulose changed as expected within year-rings (Schleser et al. 1999). Earlier observations suggested that the intra-annual variability of $\delta^{13}C$ may reach 1–2‰ and in some exceptional cases up to 4‰ (Li et al. 2005). Our results evidenced 1–2‰ variation of $\delta^{13}C$ within rings for all genotypes. Such intra-annual changes can be due to the influence of micro-climate on stomatal conductance and photosynthesis: higher air temperature, lower precipitation and moderate water deficit are likely to result in larger TE during summer and therefore in smaller $\Delta^{13}C$ (Walcroft et al. 1997, Nguyen-Queyrens et al. 1998). Similarly, Leavitt and Danzer (1993) and Porté and Loustau (2001) found that discrimination was negatively correlated to summer temperatures.

Inter-annual variation of $\Delta^{13}C$ and related traits

Inter-annual variations of $\Delta^{13}C$ were around 1–2‰, which falls within the range reported by many authors (Duquesnay et al. 1998, Nguyen-Queyrens et al. 1998, Porté and Loustau 2001, Tarhule and Leavitt 2004, Li et al. 2005). Inter-annual variation in $\Delta^{13}C$ is usually attributed to variable soil water availability and vapour pressure deficit associated with changes in tree height, canopy closure and stand level competition (Leavitt and Long 1991). Positive correlations between $\Delta^{13}C$ and annual precipitation have been documented in conifers (Walcroft et al. 1997, Korol et al. 1999) or in broadleaved trees (Saurer et al. 1995).

Correlations between annual values of BAI and $\Delta^{13}C$ were only detected in the site where growth was apparently limited by overall fertility (Sérignac): a positive correlation was found between BAI and $\Delta^{13}C$ in three among four genotypes. In these cases, $\Delta^{13}C$ was smaller (i.e., water-use efficiency was larger) during the years of reduced growth. This explanation fits with the concept that under moderate water deficits, instant water-use efficiency is enhanced (Osorio and Pereira 1994, Korol et al. 1999). A similar positive correlation between BAI and $\Delta^{13}C$ was found in *Pinus radiata* by Rowell et al. (2008). The positive correlation between $\Delta^{13}C$ and BAI suggests that the inter-annual variation of $\Delta^{13}C$ is controlled to a larger extent by stomatal conductance than by photosynthetic capacity (Johnsen et al. 1999, Xu et al. 2000). In poplar, stomatal conductance rather than photosynthetic capacity seems to control carbon gain and thus growth (Ceulemans et al. 1987).

Genotypic means of $\Delta^{13}C$ and of BAI were not correlated at all. This again shows the independence of these two traits at genetic level. These results are in agreement with those of Monclus et al. (2005, 2006), where no correlation was found between productivity traits and $\Delta^{13}C$ among the poplar clones tested in several experiments under both well-watered and water stress conditions.

Inter-annual time course of $\Delta^{13}C$

Over the period of 10 years (ages 5–15 years), our results did not evidence any age- or time-related trend on $\Delta^{13}C$. These results are in contrast with the 2‰ decrease till the age of 70 years in *Abies alba* (Bert et al. 1997) and the 1‰ decrease till the age of 50 years in *Fagus sylvatica* L. (Duquesnay et al. 1998). Similar results were also reported for *Pseudotsuga menziesii* and *Tsuga heterophylla* (Fessenden and Ehleringer 2002). Age-related trends are usually attributed to three groups of factors: (i) the impact of the $\delta^{13}C$ of soil respiration, which contributes as a source to photosynthesis and therefore the $\delta^{13}C$ of young seedlings (Duquesnay et al. 1998, Fessenden and Ehleringer 2002); this was unlikely to happen in the case of poplars planted as woody cuttings and displaying a very rapid height growth; (ii) the impact of increasing hydraulic constraints on stomatal conductance with tree height; this is likely to happen in taller trees at a more advanced age (Delzon et al. 2004) and rather unlikely in young and highly productive poplars; (iii) canopy closure and the resulting competition for light and the increasing contribution of shaded leaves to the overall water-use efficiency and $\Delta^{13}C$. Canopy closure is likely to occur early in the case of poplar plantations and much later in conifer plantations or in broadleaved species. Taken together, these three specificities of poplar with respect to the other tree species explain why there was no visible time-related trend in the recorded $\Delta^{13}C$ over the 10 years of measurements.

Stability of genotype ranking for $\Delta^{13}C$

Genotype ranking for $\Delta^{13}C$ within the three sites may be compared with that found on the young plants in the open field (Monclus et al. 2005), although such a direct comparison is made difficult by (i) the fact that only a few genotypes are common to the two experiments, and (ii) the small number of replicate trees in our case. A quick comparison showed that *I45/51* displayed low values in the two experiments as compared with others, while *Flevo* displayed low values in Monclus et al. (2005) and was among the genotypes with rather high values in our case. This shows that any direct comparison with earlier experiments is unlikely to produce conclusive observations.

To the best of our knowledge, there have been only very few attempts to test for the age-related stability of genotype ranking for $\Delta^{13}C$. Most genotype comparisons were made at juvenile stages, with (in a few cases) the attempt to check for the stability of ranking over a few successive years (usually <3). Using this procedure, Brendel et al. (2008) and Monclus et al. (2005, 2006) found a reasonable stability of the ranking of numerous genotypes of oaks and poplar. Tree-ring analyses of $\Delta^{13}C$ were seldom used for testing genetic ranking. Nguyen-Queyrens et al. (1998) compared two provenances of *Pinus pinaster* and found that the difference recorded at juvenile stages was conserved in older trees. Ponton et al. (2001) compared two co-occurring oak species and found that the

1‰ difference was maintained over almost a century. We are not aware of any other attempt.

The data set presented here shows that, over the rather short life span of a poplar plantation (15 years in the present case), the genetic differences among genotypes were maintained despite some significant inter-annual variability. The result is rather unambiguous. Can we generalize this observation to any other species? Probably not at this stage, as our sample was rather small and unbalanced, due to the lack of large-scale common garden plantations of poplar genotypes in Europe that could be used for a large-scale study. Moreover, the use of a diachronic approach based on successive tree rings has several shortcomings as it may confound long-term changes in $\Delta^{13}C$ due to climate change and age-related effects. We minimized this risk by showing that there was no visible trend over the 10 years investigated, and by concentrating on the genotype ranking and not on absolute values of $\Delta^{13}C$. If there was a small drift due to climate change, it did not affect the genetic ranking.

The data we presented here are a first attempt to tackle this difficult question, and there is a need to continue similar analyses despite the lack of long established common garden plantations of genotypes in trees. A synchronic approach (i.e., sampling last year rings of trees of different ages) is another solution to these difficulties, and such an attempt is currently in progress in our team.

Conclusions

Genotype ranking of the proxy for intrinsic water-use efficiency, $\Delta^{13}C$ recorded in tree rings, was found to be stable over 10 years among poplar genotypes in plantations. Moreover, over this short time scale there was no increasing or decreasing trend in $\Delta^{13}C$ except for two genotypes in a unique site. This lack of age-related change of $\Delta^{13}C$ at young ages differentiates poplar from many other tree species. The expected difference in $\Delta^{13}C$ between cellulose and bulk wood was ~1‰, but increased slightly with age. This increase could be due to changes in the content of lignins with tree-ring ageing. No correlation was found between genotypic means of BAI (an estimator of productivity) and $\Delta^{13}C$. These results show that genotypic differences observed at juvenile stages are likely to be maintained over the (rather short) life cycle of a plantation.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Acknowledgments

analysis and growth measurement, Catherine Bastien (INRA Orléans) who helped construct the project, and Christian Hossann and Claude Bréchet (Plateau Technique d'Ecologie Fonctionnelle, INRA Nancy) for spending much time doing all isotope analyses. The authors warmly thank Pierre Montpied (INRA Nancy) for his help with statistical analyses.

Funding

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CHAPTER 4

Age effect and stability of genotype ranking for $\Delta^{13}C$: A synchronic

approach

Transition

In the previous chapters, we were able to show that in *Populus deltoides × nigra* genotype, ranking for $\Delta^{13}C$ made on young individuals reflects the differences in whole plan transpiration efficiency under controlled condition. Furthermore, we also showed that the genotype ranking for $\Delta^{13}C$ remained stable with age using diachronic approach, chapter 3. Diachronic approach implies the measurement of $\Delta^{13}C$ along the tree core present in a common garden, thus distinction between the effects of tree age and the effect of environmental variations on $\Delta^{13}C$ is difficult. In this context, in the following chapter, we used synchronic approach in which we compared $\Delta^{13}C$ recorded in tree ring with the same formation date (2009) but from trees of different ages. In this way the long-term environmental signal was minimized and age effect was maximized. $\Delta^{13}C$ was evidenced on whole wood rather than cellulose. Effect of lignin : cellulose ratio was minimizes by taking samples of same cambial age (last annual ring).

Tree age-related trends do not affect the ranking for in $\Delta^{13}C$ among 3 *Populus deltoides × nigra* genotypes: A synchronic approach in plantations across France.

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Running Title: Genotypic differences in $\delta^{13}C$ in ageing poplar

Keywords: transpiration efficiency, age, wood, tree ring, *Populus × euramericana*

Abstract

A large variability of transpiration efficiency (as recorded from its proxy $\Delta^{13}C$, the discrimination against ^{13}C between the atmosphere and wood in annual tree rings) occurs among poplar genotypes. Genotypic differences for $\Delta^{13}C$ are shown to be stable with tree age using diachronic approach. However using this approach it is difficult to separate the age effect from environment effect. Thus to minimize the environmental effect and to support this earlier conclusion, we used a synchronic approach to test for age-related effects and stability of genotype ranking in three *Populus deltoides* × *nigra* genotypes. $\Delta^{13}C$ was measured in the bulk wood of the last tree ring built during 2009 and taken from trees grown in different plantations across France, aged between 4 and 20 years. We found a significant increase of $\Delta^{13}C$ with age form 4 to 6 years that may be translated into a decrease of transpiration efficiency with tree age. This trend of $\Delta^{13}C$ was opposite to the one usually described in the literature. Genotypic ranking was stable over the tested period and conform to that found during earlier surveys with young plants. These results based on a novel and large scale survey confirm that genotypic differences detected in the young age in poplars are maintained until tree harvest.

Introduction

Poplar genotypes are known for their large variation in growth performance and biomass production (Ceulemans *et al.,* 1987). At whole plant level, the relationship between productivity and water use (i.e., transpiration efficiency, *TE*) has been a subject of interest, especially when water availability is limiting. Intrinsic transpiration efficiency (A/g_s) at leaf level, that is the ratio between net CO_2 assimilation rate and stomatal conductance to water vapour displays also a large variability parallel to the genotypic variability of *TE* (Rasheed *et al.*, 2012). Enhanced A/g_s may be a desirable trait to improve productivity under water limited conditions (Condon *et al.,* 2002, 2004). Isotopic discrimination during photosynthesis against ^{13}C ($\Delta^{13}C$) is an indirect method of estimating A/g_s (Farquhar & Richards, 1984). It is negatively related to A/g_s (Farquhar & Richards, 1984; Farquhar *et al.,* 1989) and is found largely heritable in pines and oaks (Brendel *et al.,* 2002; 2008).

A large genotypic variability of $\Delta^{13}C$ was found among *Populus deltoides × nigra* genotypes under controlled environment (Marron *et al.,* 2005), during open field experiments (Monclus *et al.,* 2005) and under moderate drought stress (Monclus *et al.,* 2006). Interestingly, no correlation was found between productivity and $\Delta^{13}C$, thus showing potential of selecting the genotypes for high water use efficiency without affecting productivity. Furthermore, it was found that $\Delta^{13}C$ reflects differences of *TE* among polar genotypes (*Populus deltoides × nigra*, Rasheed *et al.,* 2012). All these studies were conducted with young (1-2 years old) individuals.

It has been repeatedly shown that $\Delta^{13}C$ recorded in wood formed at young stages is much larger than the one of rings formed at later stages of tree life (Francey & Farquhar 1982). This is often referred as "age effect" or "juvenility effect". Similar effects have been observed in *Abies alba* (Bert *et al.,* 1997) and in *Fagus sylvatica* (Duquesnay *et al.,* 1998).

We have only little information about such age effects in poplar genotypes displaying different values of $\Delta^{13}C$ in the young age. A stability of genotype ranking for $\Delta^{13}C$ with ageing of the trees is a prerequisite for any attempt to select genotypes for improved A/g_s and *TE*. Two sampling approaches can be used to evidence such age effects: a diachronic and a synchronic approach. Using a diachronic approach with successive tree rings, Rasheed *et al.*, (2011) found no age effect and no change in genotype ranking for $\Delta^{13}C$ in *Populus deltoides* × *nigra* genotypes. Unfortunately, the influence of long term environmental variability and annual effects related to rainfall cannot be completely excluded in this approach. Using a synchronic approach, investigating $\Delta^{13}C$ in tree rings built during a unique year, from trees of different ages growing under similar environments might be helpful to minimize the environmental effect in $\Delta^{13}C$ signals.

Early studies used whole wood for the isotopic analysis on tree rings, however the isotopic composition of individual wood components differs largely (Wilson and Grinsted, 1977). Loader *et al.*, (2003) demonstrated a consistent and stable offset between isotopic signatures of cellulose and whole wood. However, Rasheed *et al.*, (2011) found a temporal increase in this offset in *Populus deltoides* × *nigra* genotypes. This temporal increase in offset is attributed to temporal changes in cellulose vs. lignin ratios along the tree core. Evidencing last annual ring for $\Delta^{13}C$ with same cambial age might reduce risk of changes in cellulose vs. lignin ratios and its effects on $\Delta^{13}C$ signals.

In the present study we assessed tree-age related changes in $\Delta^{13}C$ of three different genotypes of *Populus deltoides* × *nigra* using $\delta^{13}C$ recorded from bulk wood of the last annual tree ring, produced during 2009. Given that there was no common garden plantation available with the three genotypes at different ages, we sampled trees from a large array of

sites and experiments across France in order to reach a balanced experimental design with trees from same ages in different environments.

Material and methods

Study sites and ring sampling

There is no experimental design available in France and to our knowledge in the world comparing a number of poplar genotypes at different tree ages in a common factorial design. To obtain a satisfying sample of trees of different ages, we therefore had to sample across a large number of plantations in France. Plantations were established by Centre National de la Propriété Forestière-Insitut pour le Développement Forestier to compare the local performance of genotypes in small plantations with private owners. Forty-one sites all over France were selected (See table 1 for their names and coordinates). Sites covered a large range of climates with mean annual temperature from 10 to 14°C, and annual rainfall from 700 to 1130 mm. genotypes were planted in plots of 25 trees at a distance of 7 x 7 m, i.e., at a density of 204 stems ha^{-1}. We concentrated on the three genotypes of *Populus deltoides × nigra*: *Koster*, *I214* and *Dorskamp* that were present in a large number of the surveyed plantations and are known display contrasting $\Delta^{13}C$ at leaf level (Monclus *et al.* 2005). The last annual ring with same cambial age (corresponding to year 2009) was sampled in February 2010. A total of 375 trees were sampled using an increment borer (0.5cm^2). 1 sample tree^{-1} and 5 trees site^{-1} genotype^{-1} were sampled

Seven age classes were defined: with tree age of 4, 7, 9, 11, 13, 15 and 18 years, and the sampled trees assigned to one of these classes. The 2009 ring was carefully separated from the adjacent one (2008) with a sharp razor blade. After drying at 70°C for 48h, each ring was grinded separately into fine homogeneous wood powder using a ring grinder (SODEMI; CEP industries department, Cergy-Pontoise, France). 1 mg of wood powder was micro weighted for $\delta^{13}C$ analysis.

Carbon isotope analysis

Wood powder samples were combusted to CO_2 at 1050°C in sealed evacuated quartz tubes containing pre-combusted cobalt oxide and chromium oxide as the oxygen source and C and N were measured in an elemental analyser (Carlo Erba, NA 1500-NC, Milano, Italy). Combustion products were separated by gas chromatography and the CO_2 was delivered to an isotope ratio mass spectrometer (Finnigan, Delta-S, Bremen, Germany) for isotopic analysis. Stable carbon isotope composition was expressed as $\delta^{13}C$:

$$\delta^{13}C = (R_{sample}/R_{standard}-1)1000 \qquad (1)$$

where R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratios in a sample and the standard (Pee Dee Belemnite), respectively. Accuracy of the measurements was ± 0.1‰. Carbon isotope discrimination was calculated as:

$$\Delta^{13}C = \left(\frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1000 + \delta^{13}C_{plant}} \right) \times 1000 \qquad (2)$$

where δ_{air} is the ^{13}C composition of CO_2 in the atmosphere and δ_{plant} is ^{13}C composition of the wood powder. Given that δ_a was -8.07‰ during 2003 and an annual decrease of 0.0281‰ (McCarroll & Loader 2004) δ_{air} was estimated at -8.24‰ for 2009. We assumed that the mean value of δ_a was similar across sites and did only marginally change with the season.

Statistical analysis

The data set covering the seven age classes was analyzed using linear mixed models, fitted with genotype, age and their interaction as fixed effects and site as a random effect: Multiple comparison tests were used to evaluate pair-wise differences between age classes within each genotype. All tests were performed with R (R Development Core Team, 2012)

and R packages *nlme* (Pinheiro *et al.,* 2012) and *multcomp* (Hothorn *et al.*, 2008). Tests were declared significant at $P < 0.05$. Normality and homoscedasticity of data were checked graphically with residual *vs.* predicted and normal quantile-to-quantile plots.

Results

The general results of the mixed linear model are displayed in table 2. Carbon content of bulk wood differed among genotypes, was higher for *Koster* and *I 214* (around 47.6%) with respect to Dorskamp (46.8%, Fig. 1). There was no significant age or interaction effect in C contents (Fig. 2). On the contrary, N content in bulkwood was not affected by any genotype or interaction effect, but decreased gradually with tree age from 0.2 down to 0.11% (Fig. 3). Genotype, age and interaction effects were detected for $\Delta^{13}C$. *Koster* had the lowest mean $\Delta^{13}C$ across ages (19.4‰) and *Dorskamp* the highest (20.3‰, see Fig. 4). The age-effect was very clearly visible, with in the three genotypes (Fig. 5), with a very significant increase of $\Delta^{13}C$ from age 4 to 10 in *Dorskamp* and *Koster* by about 2‰. The trend is less visible in *I 214*, as values from that genotype were above *Dorskamp* at young ages and very close to those of *Koster* at 18. This time course resulted in the interaction effect detected between genotype and age effects.

Multi comparison test for $\Delta^{13}C$ and N contents showed that first age class (4th year) were significantly different from the other age classes in $\Delta^{13}C$ and first two age classes (4 and 7 years) were significant different from all the other age classes with higher N contents.

Discussion

Age effect

Whole wood consists of complex mixtures of molecules like cellulose, hemicellulose and lignin with variable isotopic signatures. Age related changes in the relative proportion of these wood components may have an impact on $\Delta^{13}C$ independently of *TE* (Rasheed *et al.,* 2011). In this study, we used last annual ring with same cambial age for all genotypes and age classes. In this way, changes related to the proportion of these wood components could largely be reduce. Further more carbon contents differed significantly between genotypes but remained stable with age. This absence of age related trend in the carbon contents might be due to consistent lignin contents, which were genotype specific. Thus it can be deduced that the observed increase in $\Delta^{13}C$ was not due to the wood composition effect.

Contrary to our positive trend in $\Delta^{13}C$ with age, several studies using individual trees and diachronic approach (McCarroll and Pawellek 2001) and synchronic approach (e.g. Bert *et al.,* 1997; Duquesnay *et al.,* 1998) have reported age-related decreases in $\Delta^{13}C$. The interpretation of this age effect includes: (i) assimilation of respired CO_2 which is already depleted in ^{13}C (Schleser and Jayasekera 1985); (ii) Re-fixation of respired CO_2 (depleted in $\delta^{13}C$) from bark (Cernusak *et al.*, 2001) and (iii) decreasing hydraulic conductance in tall trees resulting in lower stomatal conductance and enhanced A/g_s and thus reduced $\Delta^{13}C$ (McDowell *et al.,* 2002).

The hypothesis of assimilating respired CO_2 depleted in ^{13}C is probably unlikely as poplar genotypes displays a fast growth and reaches a height of a few meters in two months. Furthermore, age-trend has been identified even in Alpine tree-line situations, where tree cover is sparse and slopes are open and windy. Thus recycling of respired air is unlikely to be

wholly responsible. Re-fixation of respired CO_2 (depleted in $\delta^{13}C$) from bark with age would result in a decrease in $\Delta^{13}C$ with age (Cernusak *et al.*, 2001), which is contrary to our results. Decline in hydraulic conductance as leaf water potential decreases with tree height or age (Ryan and Yoder 1997; Schafer *et al.,* 2000; McDowell *et al.,* 2002) would result decreased $\Delta^{13}C$ with age, which is neither the case in our study.

$\Delta^{13}C$ has been expected to be an estimator of water use efficiency. Several studies have shown a clear negative correlation between $\Delta^{13}C$ and *A/g_s* (intrinsic transpiration efficiency) and *TE* (whole plant transpiration efficiency; Rasheed *et al.,* 2012). Thus, increase in $\Delta^{13}C$ with age in our study reflects a decrease in whole plant transpiration efficiency with age. According to Farquhar *et al.,* 1989, increase in *$\Delta^{13}C$* can be explained by the decrease in ratio of *A/g_s*. This decrease in *A/g_s* can either be due to decrease of assimilation *(A)* or due to increase in stomatal conductance *(g_s)* (Farquhar and Richards 1984; Condon *et al.,* 2004). Hence, long term changes in *A* and *g_s* with age at canopy level need to be discussed in detail. Decrease in photosynthesis with increase in tree height (or age) has been evidenced in studies like Ryan and Waring (1992) and Yoder *et al.,* (1994). In Lodgepole pine, net carbon flux was shown to be 210 g m^{-2} yr^{-1} in a 40-yr-old stand, which decreased to 46 g m^{-2} yr^{-1} in 245 years old stand (Ryan and Waring 1992). Net photosynthesis per unit area in old *P. contorta* and *P. ponderosa* is found 14-30% lower than evidenced in same-aged foliage from younger trees by Yoder *et al.,* (1994). Hydraulic conductance can be estimated from measurements of wood anatomy in both angiosperms and conifers that could give an indication of *g_s* in old trees (Ponton *et al.,* 2001; McDowell *et al.,* 2002). In *Populus deltoides × nigra*, no temporal trend was evidenced in average vessel surface area in different genotypes at three different study sites (Rasheed unpublished data). Results of Barnad and Ryan (2003) showed the maintenance of water flux and average crown conductance in 1 and 5 years old *Eucalyptus saligna*. Thus decrease photosynthesis per unit area with increase in age and maintenance of

consistent hydraulic conductance could result in increase in $\Delta^{13}C$ with age. In this regard, within canopy variations in $\delta^{13}C$ that affects the over all signal integrated in the tree ring has been evidenced previously. Barnad and Ryan (2003) found within canopy variation of 1% in 1-year old and almost 1.5% in 5-year old in *Eucalyptus saligna* trees. Leaves taken from lower canopy were found more discriminate than from canopy top due to canopy closure. This implies that a consistent hydraulic conductance and a gradual decrease in average crown photosynthesis due to canopy closure can result in increased canopy discrimination with age.

Stability of the genotypic ranking

Age-related conservation of ranking for $\Delta^{13}C$ along with a high biomass production has been one of criteria of central importance for selecting genotypes for higher water use efficiency. In this pursuit, genotype ranking in *Populus deltoides × nigra* for $\Delta^{13}C$ was tested among the young plant grown under open field conditions with those under controlled condition (Monclus *et al.,* 2005). Subsequently, genotype ranking under well-irrigated field condition was compared with those under water stress condition under field condition (Monclus *et al.,* 2006). In both these studies genotypic ranking remained stable and no correlation was found between $\Delta^{13}C$ and productivity traits. The author concluded that lack of correlation between $\Delta^{13}C$ and traits defining productivity can be interesting in selection genotypes for higher $\Delta^{13}C$ and higher productivity at the same time. As $\Delta^{13}C$ displays age effect, ranking found in *Populus deltoides × nigra* genotypes are susceptible to change with age. Thus, stability of these genotype ranking was tested with age using diachronic approach. Rasheed *et al.,* 2011 evidence insignificant slopes in $\Delta^{13}C$ with tree age (from 5-15 years) under three environmental conditions and concludes that ranking remained stable over the tested period. In this study we used synchronic approach with the focus to maximize the age effect by large sampling across several trails and under different environments. Genotype ranking was

compared with previous studies having common genotypes to detect their stability. Genotypic ranking for $\Delta^{13}C$ averaged over the tested period matched with that found by Marron *et al.,* 2005, where *Koste*r had the lowest $\Delta^{13}C$ and *Dorskamp* had the highest. *Koster* displayed the lowest $\Delta^{13}C$ values in all the previous studies like Marron *et al.,* (2005) and Monclus *et al.,* (2005). Ranking was similar with that found by Monclus *et al.,* 2005 in the first age class (4th year), where *I 214* displayed the highest $\Delta^{13}C$ values and *Koster* the lowest but genotypic raking changes in this study over age as *I 214* was ranked second at the age of 18 years. In this study we are able to compare only 3 genotypes because of not finding other genotypes with same age classes and growing under variable environmental conditions.

Conclusion

$\Delta^{13}C$ in the last tree ring increased with tree age in the three genotypes followed here, which would indicate a decrease in transpiration efficiency at whole tree level. Significant genotypic effect was found as *Koster* displayed lowest $\Delta^{13}C$ over the period of 12 years. The genotypic ranking was only partly maintained in the 3 *Populus deltoides × nigra* genotypes. Ranking for $\Delta^{13}C$ averaged over tested period was consistent with that found by Marron *et al.,* 2005 and with Monclus *et al.,* 2005 for the first age class (4 year class).

Acknowledgements

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Table

Table 1. List of the 42 sampling sites along with their GPS coordinates and the different genotypes (*D, Dorskamp; I, I 214 and K, Koster*) and age classes present.

Region	Sites	GPS co-ordinates	Genotypes	Age
Centre, Pays de la Loire	Lizeray	01° 52' 12.8" E 46° 58' 39.5" N	*D, I*	7
	Buzancais	01° 25' 15.6" E 46° 53' 10.5" N	*D, I & K*	9
	Seuilly	00° 08' 33.3" E 47° 08' 35.1" N	*D, I*	4
	La Flèche	00° 03' 24.1" W 47° 41' 37.8" N	*D*	9
	Laigne enbelin	00° 14' 61.6" E 47° 52' 58.5" N	*D, I*	4
	Marçon	00° 29' 44.0" E 47° 42' 33.9" N	*D*	9
Midi-Pyrénées, Aquitaine	Deyme	01° 32' 26.8" E 43° 29' 18.5" N	*D, I& K*	15
	Verdun-sur -Garonne	01° 14' 15.5" E 43° 52' 6.31" N	*D, I*	9
	Begaar	00° 52' 5.05" E 43° 48' 31.3" N	*K*	7
	La Bastide d'Armagnac 2	00° 07' 22.7" W 43° 56' 54.4" N	*I, K*	11
	Argenton	00° 5' 41.2" E 44° 22' 8.22" N	*D, K*	7
	Montpouillan	00° 7' 1.46" E 44° 28' 24.6" N	*D, I*	18
	Sainte-Bazeille 1	00° 5' 36.7" E 44° 31' 28.6" N	*I, K*	7
	Saint-Pierre-sur-Dropt	00° 11' 48.9"E 44° 39' 8.55" N	*D, I & K*	11
	Rimons	00° 00' 5.41" W 44° 40' 6.12" N	*D, I & K*	11
	Siorac-en-Périgord	00° 56' 33.1"E 44° 50' 7.28" N	*D, I & K*	13
	Prigonrieux	00° 23' 32.6" E 44° 51' 16.2" N	*D, K*	9
Périgord, Poitou, Vendée	Arveyres	00° 15' 55.8" W 44° 54' 6.98" N	*D*	7
	Saint-Germain-de-la-rivière	00° 19' 55.8" W 44° 56' 29.7" N	*I, K*	9
	Saint-André-de-Lidon	00° 43' 59.5" W 45° 35' 0.210" N	*D, I & K*	11
	Saint-Pierre-de-l'ile	00° 26' 24.1" W 46° 01' 11.5" N	*D, I*	18
	Bioussac	00° 16' 47.2" E 46° 01' 59.4" N	*D, I*	15
	Saint-Christophe-du-ligneron	01° 45' 53.0" W 46° 48' 25.7" N	*D, I*	18
	Sainte-Hermine	01° 03' 0.366" W 46° 33' 29.2" N	*I*	15
Nord, Pas de Calais, Picardie	Lillers	02° 31' 39.2" E 50° 33' 55.1" N	*D, I & K*	18
	Havrincourt	03° 03' 38.3" E 50° 05' 25.1" N	*K*	9
	Onnaing	03° 34' 37.9" E 50° 24' 03.5" N	*D*	18
	Houdain lèz Bavay	03° 47' 46.3" E 50° 19' 23.6" N	*K*	4
	Gommegnies	03° 43' 22.3" E 50° 14' 57.2" N	*D, K*	9
	Coincy 1	03° 27' 32.1" E 49° 09' 39.0" N	*I*	7
	Coincy 2	03° 28' 44.9" E 49° 09' 34.1" N	*I*	4
	Allemant	03° 28' 58.4" E 49° 27' 20.2" N	*I, K*	11
	Flavy le Martel	03° 09' 44.9" E 49° 40' 57.5" N	*K*	11
	Manicamp	03° 10' 20.3" E 49° 34' 34.9" N	*I, K*	11
	St Paul aux Bois	03° 13' 05.4" E 49° 33' 34.5" N	*D, K*	4
Champagne-Ardennes	Esternay	03° 33' 31.3" E 48° 43' 41.5" N	*I, K*	9
	Norrois	04° 36' 53.1" E 48° 40' 24.6" N	*K*	15
	Ste Livière	04° 49' 29.1" E 48° 35' 17.5" N	*I, K*	9
Franche-Comté, Bourgogne	Melincourt	06° 08' 50.8" E 47° 53' 33.7" N	*I*	11
	Fontaine-française	05° 22' 2.10" E 47° 31' 0.76" N	*D, I & K*	13
	Vielverge	05° 28' 4.43" E 47° 16' 39.3" N	*D, I & K*	9
	Flammerans 2	05° 25' 15.9" E 47° 15' 1.52" N	*D, I & K*	7

Table 2: Statistical results of linear mixed model for Carbon and Nitrogen content and $\Delta^{13}C$ recorded on the bulk wood from the last tree rings, for the three genotypes of *Populus × euramericana* with Genotype, Age and interaction effects. Degrees of freedom were 2, 6 and 12 respectively.

	Genotype		Age		Interaction	
	F-values	P-values	F-values	P-values	F-values	P-values
C contents	10.7	P < 0.001	2.1	P = 0.051	1.2	P = 0.305
N contents	1.397	P = 0.248	8.171	P < 0.001	0.718	P = 0.734
$\Delta^{13}C$	55.7	P < 0.001	36.9	P < 0.001	6.6	P < 0.001

Table 3: Mean values of carbon and nitrogen content and $\Delta^{13}C$ recorded on the bulk wood from the last tree rings, for the three genotypes of *Populus × euramericana*). In each column, different letters represent significantly different values among age classes after a multi-comparison test using post-hoc test. Significance level: P < 0.005.

Age Classes	Dorskamp N %	Dorskamp $\Delta^{13}C$	I 214 N %	I 214 $\Delta^{13}C$	Koster N %	Koster $\Delta^{13}C$
4	0.195 (0.063) cd	18.9 (0.156) a	0.172 (0.033) cd	19.3 (0.107) a	0.200 (0.047) cd	18.1 (0.132) a
7	0.175 (0.043) d	20.3 (0.179) bc	0.140 (0.053) d	20.0 (0.172) bc	0.213 (0.095) d	18.7 (0.179) ab
9	0.164 (0.056) bcd	20.1 (0.103) b	0.138 (0.049) bcd	20.3 (0.079) c	0.175 (0.048) bcd	19.5 (0.096) c
11	0.146 (0.050) bc	20.9 (0.191) d	0.134 (0.047) bc	19.9 (0.095) bc	0.159 (0.054) bc	19.8 (0.136) cd
13	0.115 (0.024) ab	20.8 (0.095) bcd	0.146 (0.126) ab	20.3 (0.175) bc	0.102 (0.019) ab	20.3 (0.150) d
15	0.107 (0.027) a	20.6 (0.161) bcd	0.106 (0.039) a	20.1 (0.077) bc	0.102 (0.037) a	19.8 (0.103) cd
18	0.148 (0.039) ab	20.8 (0.092) cd	0.106 (0.029) ab	19.7 (0.151) ab	0.149 (0.054) ab	19.6 (0.443) bcd

Figure captions

Figure 1. Mean C content in the bulk wood of the last annual tree ring (2009) sampled during winter 2010. from three *Populus × euramericanna* genotypes. Each bar represents the mean value of 5 trees times 42 sites and 7 age classes (from 4 to 14 years). Error bars represent the SD for each genotype.

Figure 2. Age-related changes in the C content in the bulk wood of the last annual tree ring (2009) sampled during winter 2010. in three *Populus × euramericanna* genotypes over seven age classes. Error bars represents SD.

Figure 3. Age-related changes in the mean N content in the bulk wood of the last annual tree ring (2009) sampled during winter 2010. in three *Populus × euramericanna* genotypes over seven age classes. Error bars represents SD.

Figure 4. Mean isotopic discrimination between atmosphere and bulk wood in the 2009 year-ring (Δ^{13}C) for three *Populus × euramericana* genotypes. Isotopic composition of the atmospheric CO_2 was taken as -8.24 ‰. Each bar represents the mean value over 42 sites and 7 age classes (from 4 to 14 years). Error bars represent the SD for each genotype. Different letters indicate significant differences at p<0.05.

Figure 5 Age related changes in isotopic discrimination between atmosphere and bulk wood in the 2009 year-ring (Δ^{13}C) for three *Populus × euramericanna* genotypes. Ages were grouped over seven age classes. Each point represents a mean value from several sites. Error bars represents SD.
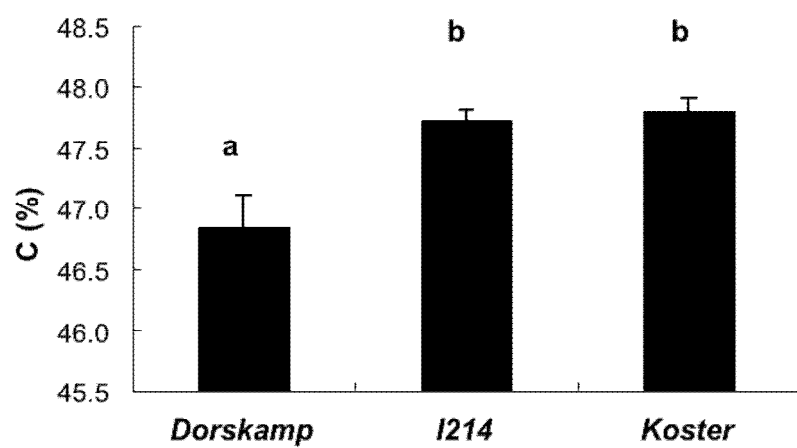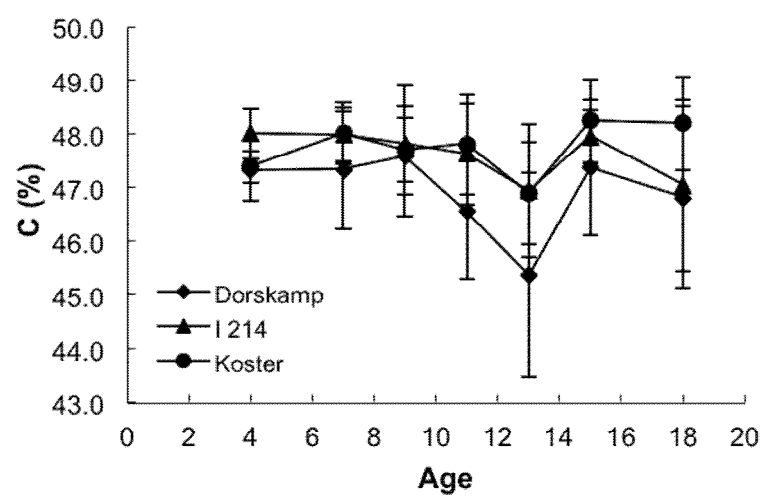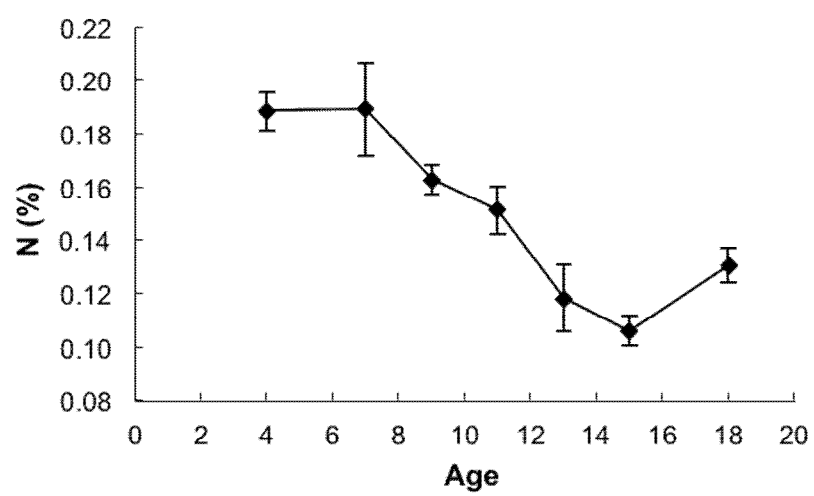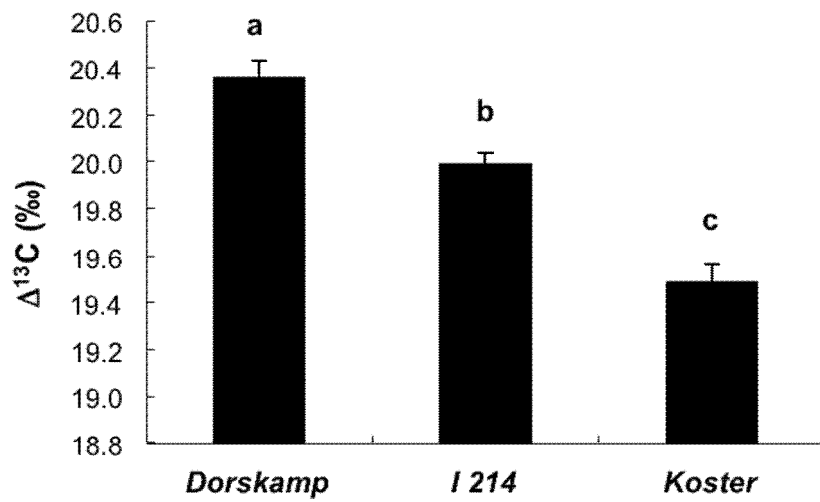
Figure 1.

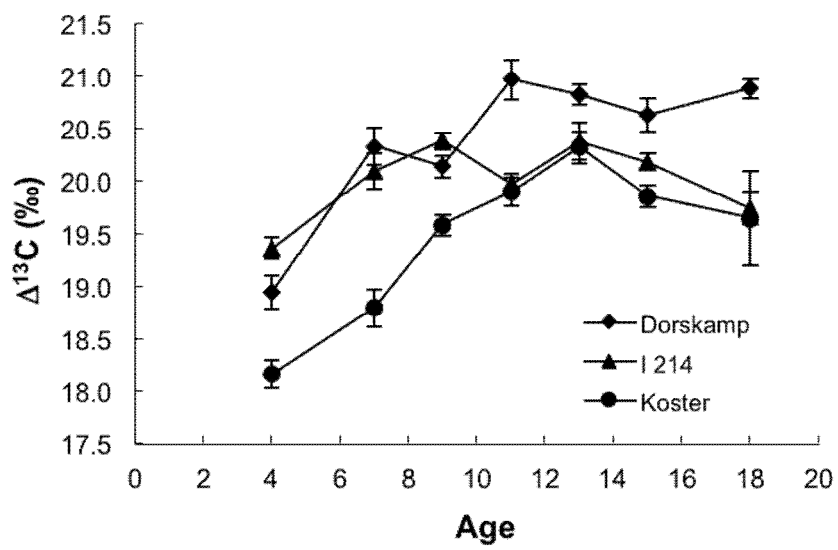**Figure 2.**

Figure 3.

Figure 4.

Figure 5.

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

Up-scaling approach

We used an up-scaling approach to investigate transpiration efficiency at leaf and whole plant level in different poplar genotypes. At leaf level, we compared leaf gas exchange data and discrimination against ^{13}C as recorded in bulk leaf matter or in soluble sugars.

At leaf level: correlation between $\Delta^{13}C_{ss}$ and $\Delta^{13}C_{lb}$:

Our work allowed us to show that genotype variations in $\Delta^{13}C_{ss}$ (isotopic discrimination in the leaf soluble sugars) were faithfully recorded as variations in $\Delta^{13}C_{lb}$ (isotopic discrimination recorded in bulk leaf matter) both in commercial genotypes of *Populus deltoides × nigra* (Chapter 1) and in genotypes selected from riparian populations of *Populus nigra* (Chapter 2). These results confirm that post-photosynthetic discrimination had no genotype-specific effect on the discrimination recorded during photosynthesis (as seen from the soluble sugars extracted from the leaves). In *Populus deltoides × nigra* (Chapter 1), $\Delta^{13}C_{ss}$ explained 78% of the variability in $\Delta^{13}C_{lb}$ and in *Populus nigra* genotypes, it accounted for 87% (under low *VPD*) and 93% (under high *VPD*). However in both experiments $\Delta^{13}C_{ss}$ was larger than $\Delta^{13}C_{lb}$; this was a surprise as we expected smaller values. We are not aware of any published result reporting such a difference. We have presented several hypotheses to account for this discrepancy mainly highlighting the contribution of other metabolites in the leaf bulk, and in particular the relative fraction of starch that might be responsible for this discrepancy.

Starch is the major storage metabolite in many plants. During rapid growth, starch accumulates in leaves during the day, and is degraded during night into sucrose which is loaded into the phloem and exported from the leaf, such that it is almost exhausted at dawn (Smith & Stitt 2007; Graf & Smith 2011). This pattern is retained in a wide range of conditions, including different irradiances, day lengths, CO_2 concentrations, nutrient and water supply (Gibon *et al.* 2009; Tschoep *et al.* 2009). During synthesis of both starch and soluble sugars, carbon assimilated via the Calvin cycle is partitioned with a fraction exported to the cytosol for sucrose synthesis and a fraction retained in the chloroplast for starch synthesis (Smith & Stitt 2007). During the light period, due to Aldolase activity that favours ^{13}C, chloroplastic fructose-6-phosphate and glucose-6-phosphate tend to be enriched in ^{13}C

compared to dihydroxyacetone phosphate, thereby enriching starch in ^{13}C by ≈3-4‰ and depleting the remaining triose-phosphate molecules. Triose-phosphates are exported to the cytosol to produce sucrose with little additional fractionation, so that during the day, sucrose is depleted in ^{13}C (see Tcherkez *et al.* 2011 for further details). During the dark, the reverse happens as starch accumulated during the day is hydrolysed to fructose and glucose.
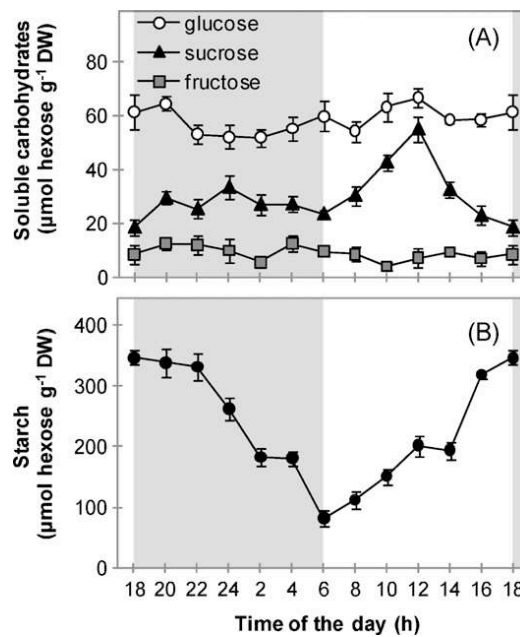
Figure 21. Diagram for starch and soluble sugar synthesis during the day and the hydrolysis of starch during the night in *T. pohliana* leaves. Figure from Freschi *et al.* (2010).

Furthermore, Brugnoli *et al.* (1988) measured starch and soluble sugar contents in poplar leaves during the day. Poplar leaves were found especially rich in starch (683 mmol C m^{-2}) as compared to cotton or bean leaves (454 and 373 mmol C m^{-2} respectively). Starch content was also found particularly high after 19 hours irradiance. In this study the leaves were clipped off during the photoperiod, thus the proportion of starches could have been sufficiently higher to affect the overall isotopic signature of leaf bulk. Diurnal variations of starch contents has been reported in many species However their potential effect on leaf bulk isotopic signature remains undocumented especially in fast growing specie like poplar with high average starch turn over rate.

At leaf level: temporal integration between $\Delta^{13}C_{ss}$ and intrinsic *TE* (A/g_s)

Based on the results from Chapter 1 and Chapter 2, we can safely state that $\Delta^{13}C_{ss}$ is a reliable indicator of intrinsic *TE* in both *Populus deltoides × nigra* and *Populus nigra*

genotypes. $\Delta^{13}C_{ss}$ accounted for 82% genotype variability in A/g_s among *Populus deltoides × nigra* genotypes (Chapter 1) and for 83% (under low *VPD*) and 70% (under high *VPD*) genotype variability among *Populus nigra* genotypes (Chapter 2). Importantly, the genotype ranking in *Populus deltoides × nigra* was very stable with respect to the previous studies (Monclus *et al.* 2005, 2006 and Fichot *et al.* 2010; with very high Spearman's rank correlations).

Our study also focussed to the question whether the simple discrimination model proposed by Farquhar *et al.* (1982) predicts efficiently the observed discrimination. This simple discrimination model has been widely used; but it was repeatedly observed that there is a large offset $(\Delta^{13}C_{dif})$ between observed and predicted discrimination, which can be species or genotype-specific. Our results showed that the simple model predicts efficiently the genotype differences, but not the observed discrimination. A systematic offset $(\Delta^{13}C_{dif})$ was observed in both experiments. In *Populus deltoides × nigra*, $\Delta^{13}C_{dif}$ was not genotype-specific (around 1.4 ‰), but in *Populus nigra*, it was both genotype- and VPD-specific (3.48 ‰ under low *VPD* and 2.32 ‰ under high *VPD*). The inter-genotype difference in $\Delta^{13}C_{dif}$ was partly correlated to a variability of leaf mass-to-area ratio (*LMA*) and leaf thickness (*LT*); increases in *LMA* and decrease in *LT* lead to increased $\Delta^{13}C_{dif}$.

Genotype effects on $\Delta^{13}C_{dif}$ can be explained refereeing to the following major factors (i) uncertainty around fractionation factor during carboxylation (*b*) in the simple model (ii) the effect of a finite mesophyll conductance (g_m) on ^{13}C discrimination.
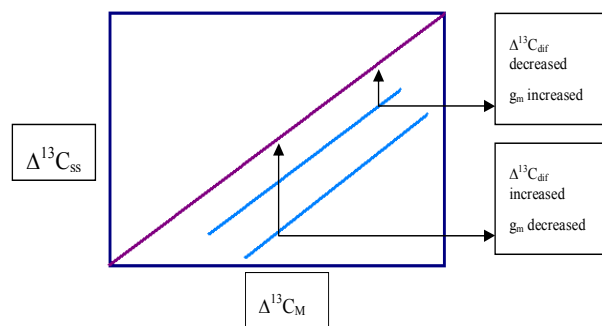
Figure 22. Schematic diagram to illustrate the relationship between $\Delta^{13}C_{M,}$ and $\Delta^{13}C_{ss}$ resulting in different $\Delta^{13}C_{dif}$ and the interpretation as different values of g_m

This offset $(\Delta^{13}C_{dif})$ indeed can be interpreted as reflecting the impact of finite g_m on ^{13}C fractionation during photosynthesis: a larger $\Delta^{13}C_{dif}$ means a smaller g_m (Figure 22). In this study, we never actually quantified g_m due to the numerous uncertainties around several

parameters of the detailed discrimination model and our measurements (R_d mitochondrial respiration, b carboxylation by Rubisco and PEPc). Through online measurements of leaf gas exchange and fractionation against ^{13}C during photosynthesis using TDL-AS (tuneable diode laser absorption spectrometry fro ^{13}CO$_2$ records), the occurrence of inter and intra-specific variability of g_m has been highlighted several times (for instance in barley genotypes; Barbour *et al.* 2010, see also the recent review by Flexas *et al.* 2012).

Fractionation during carboxylation (b) can be computed by following Farquhar & Richards (1984) as:

$$b = \beta b_4 + (1-\beta)b_3 \qquad\qquad (16)$$

where b_3 is the fractionation by Rubisco ($\approx$ 28.5 - 30‰, McNevin *et al.* 2007), b_4 is the fractionation by PEPc (-5.7‰, Farquhar & Richards 1984) and β is the fraction of carbon fixed by PEPc vs. Total carbon fixed by the leaf ($\approx$ 0 - 0.15, Vu *et al.* 1985 and Saurer *et al.* 1995). The combination of such values yields a range of b values between 23 -30‰. A dramatic effect of changes in b on the computed values of g_m has been highlighted by Douthe *et al.* (2011, 2012). Thus, calculating g_m without knowing the precise value of β may lead to unrealistic results. In this study, we have not computed the fractionation factor related to parameter b, which could have varied between *Populus nigra* genotypes along with other factors like R_d (mitochondrial photo respiration).

To our surprise, we found that expansion of the leaves under different *VPD* also resulted in different $\Delta^{13}C_{dif}$, which was smaller under high *VPD*. This implies that enhanced *VPD* in an increased g_m paralleling a large scale stomatal closure. This could mean that g_s limits transpiration under enhanced *VPD* while the increase of g_m could compensate partly for the stomatal closure effect on CO$_2$ diffusion into the leaf. Effects of *VPD* on g_s are a well known occurrence, but we have very little data about the impact of short-term changes of *VPD* on g_m, and even less on that of long term acclimation to different *VPD*. Warren (2009) found that short term changes in *VPD* resulted in stomatal closure but did not change g_m. We are not aware of any published data on acclimation of g_m to long term differences in *VPD*.

In this study, leaf anatomy (*LMA* and *LT*) was used to explain the treatment effects on $\Delta^{13}C_{dif}$. As *LMA* was similar across genotypes and *VPD*, slight but significant increase in *LT* could mean a larger porosity in the mesophyll tissues and impact g_m. g_m has two components: a gaseous diffusion through the intercellular airspaces and a liquid diffusion pathway from the cell wall to the sites of carboxylation. Gaseous diffusion through the intercellular airspaces

may be related to mesophyll porosity. The techniques available for assessing the internal drawdown from C_i to C_c, cannot separate these two components without the additional effort of making measurements in two different gas backgrounds, which alter the diffusivity of CO_2. For example, comparing leaf gas exchange in helox (80% helium, 20% oxygen) with those under actual atmosphere can help assess the magnitude of the drawdown in the intercellular airspaces, because the diffusivity of CO_2 is 2.3 times larger in helox than in air. Parkhurst & Mott (1990) found that helox increased photosynthetic rates of amphistomatous and hypostomatous leaves by 2 and 12%, respectively, meaning that gas phase diffusion in the leaves was a small bu significant component of the resistance to CO_2 influx.

Diffusion in the liquid phase is restricted by the permeability of membranes and the thickness of cell wall, cytoplasm and chloroplasts. These restrictions are likely to be shared by all chloroplasts in a given leaf. Liquid phase conductance is proportional to the surface area of chloroplasts exposed to intercellular airspace per unit leaf area and can also be affected by *LT*. The maximum value that can be reached for a given leaf is set by the mesophyll anatomy, which defines the surface area of mesophyll cells exposed to intercellular airspace per unit leaf area. Evans *et al.* (1994) clearly showed that internal conductance was related to exposed chloroplast surface area. Remarkably, the relationship between internal conductance and exposed chloroplast surface area found for tobacco also fits the data available for three other species: wheat, peach and citrus (Syvertsen *et al.* 1995; Evans & Vellen 1996; Evans 1998b; Evans & Loreto 1999).

Apart from these factors, diffusion of CO_2 can also be altered by the nature of exchange surface. It is being suggested that apart of their function in facilitating water diffusion across the membranes, aquaporin constitute a key means of regulating CO_2 diffusion through membranes (Nakhoul *et al.* 1998). Altered expression of aquaporins has been shown to result in changes the membrane permeability to CO_2 in plants (Heckwolf *et al.* 2011). Thus, profound analysis around the factors described could certainly increase the insight concerning this increase in g_m (or decrease in $\Delta^{13}C_{dif}$) under high *VPD*.

At whole plant level: spatial integration from intrinsic to whole plant *TE*

We showed that genotype differences in intrinsic *TE* reflect the differences in *TE* at whole plant level in *Populus deltoides × nigra* and in *P. nigra*. At the same time, the relative magnitude of genotype diversity for *TE* at whole plant level was higher (40.7%) than for intrinsic *TE* (27.6%) in *Populus deltoides ×nigra*. This difference became even higher under

high *VPD* (48.3% versus 29%, respectively) in *P. nigra*. These results highlight the role of carbon lost during photo and dark respiration (Φ_c) and water lost during night transpiration (Φ_w) that could play an important role in this increased genotypic variation in whole plant *TE* with respect to that at intrinsic level. In addition to that as the plant grow larger, Φ_c values may increase with tree grotwh as the ratio leaf vs. whole-plant biomass decreases. Higher Φ_c values have also been evidenced in slow growing genotypes because they generally display higher tissue construction costs (Vertregt & Penning de Vries 1987; Poorter 1994). Thus, quantification of these two parameters and their variation between poplar genotypes is a very important issues that deserves further attention.

Studies quantifying these two traits (Φ_c and Φ_w) are not common. Cernusak *et al.* (2008) estimated Φ_w in several species of gymnosperm and angiosperm trees, and angiosperm lianas grown under tropical field conditions. The parameter was estimated using the model relating *TE* to ^{13}C discrimination (Farquhar *et al.* 1982; Hubick & Farquhar 1989), taking Φ_c as constant :

$$TE = \frac{C_a(1-\phi_c)(b-\Delta^{13}C)}{1.6v(1+\phi_w)(b-a)} \tag{17}$$

where *TE* is transpiration efficiency, Φ_w, water lost during night transpiration measured over a given period and $\Delta^{13}C$ is the ^{13}C discrimination of whole plant organic matter.

However, to our knowledge, a direct quantification of these parameters at whole plant level has not been attempted. In some studies with trees, Φ_c has been suggested to be an important determinant of whole plant *TE* (Guehl *et al.* 1994; Osório *et al.* 1998; Matzner *et al.* 2001); and in one case Φ_w was suggested to have influenced *TE* significantly (Hobbie & Colpaert 2004).

Water lost during night transpiration (Φ_w) can be measured in plants grown in pots by taking differences in weight over night. In this regard, at INRA Nancy, a precise automated system of weighing the potted plants has been installed. This system is capable of measuring accurately the weight lost during a given time period (e.g., during night). Direct measurements of Φ_c are not easy on whole plant (different plant compartments, stem, leaves and roots), however can be estimated by using equation above, all other parameters being known.

Using ^{18}O enrichment in leaves with respect to source water as an estimator for differences in g_s:

Based on our results, we conclude that the discrimination against ^{18}O from source water to leaf water is an efficient indirect estimator of stomatal conductance (g_s) among poplar genotypes. We found no genotype variability for the ^{18}O signals in bulk leaf matter or in leaf water ($\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$) in *Populus deltoides × nigra* genotypes due to a high intra-genotype variability. This variability may be due to the high stomatal conductance resulting in unstable isotopic state due rapid influx of ^{18}O-depleted xylem water entering the leaf (Farquhar & Cernusak 2005). However, $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$ were found negatively correlated to g_s at phenotype level. In *Populus nigra*, within each treatment low g_s resulted in higher $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$. Across the two treatments, low g_s was accompanied by increased $\Delta^{18}O_{lb}$ and $\Delta^{18}O_{lw}$ as overall transpiration rate increased and leaf temperature decreased.

Age effect and stability of genotype ranking:

Based on our results, we can conclude that genotype ranking for $\Delta^{13}C$ is stable with age in *Populus deltoides × nigra*, as evidenced using both diachronic and synchronic approaches (Chapter 3, 4). These results have strong implications: in particular, we might exclude the occurrence of large juvenility effects on $\Delta^{13}C$; breeding for more water-use efficient genotypes at juvenile stages should not be counteracted by changes in genotype ranking with age.

Increases in $\delta^{13}C_{offset}$ (difference $\delta^{13}C$ wood and $\delta^{13}C$ cellulose) were evidenced along the tree core, which was attributed to cellulose vs. lignin ratio imbalance with ageing wood (diachronic appraoch, Chapter 3). Such effects of lignin deposition on the isotopic signal were minimized by sampling rings of same age but from trees of different ages (synchronic approach, Chapter 4). Furthermore, no age effect was detected between the age of 5-15 years in the two studies; however, $\Delta^{13}C$ was significantly lower in younger trees (below 5).

$\Delta^{13}C$ recorded in the tree rings represents the overall isotopic signal recorded in the tree canopy. At leaf level, increase in $\Delta^{13}C$ can be explained by the decrease in ratio A/g_s (Farquhar *et al.* 1989). Decreases in photosynthesis with increases in tree height (or age) have been reported by Ryan & Waring (1992) and Yoder *et al.* (1994) and explained by decreases in hydraulic conductance of trees with increasing dimensions that directly act on leaf water potential and stomatal conductance. Hydraulic conductivity of stem and branch segments in

old trees can be assessed indirectly through wood anatomy in both angiosperms and conifers (Ponton *et al.* 2001; McDowell *et al.* 2002). In *P. deltoides × nigra*, the number of vessels per year ring and average vessel surface area per ring were measured from 5 – 15 years. Neither increase nor decrease was evidenced in the two parameters over the tested period (see table below for regression slope analysis, Rasheed, unpublished data).

Table 4. p-values for of regression slope for Number of vessels year ring^{-1} (nv, mm^{-2}yr^{-1}) and Average vessel surface area year ring^{-1} (AVSA, m^2 yr^{-1}) over ten years in *P. deltoides × nigra* grown at three sites. Differences are considered significant when p<0.05.

	Begaar		Serignac		Migron	
Cultivars	*n*v (mm^{-2})	AVSA (µm^2)	*n*v (mm^{-2})	AVSA (µm^2)	*n*v (mm^{-2}) P-	AVSA (µm^2)
Flevo	0.612 (NS)	0.793 (NS)	0.364 (NS)	0.122 (NS)	0.968 (NS)	0.592 (NS)
I214	0.649 (NS)	0.935 (NS)	**0.049 (*)**	0.973 (NS)	0.812 (NS)	0.158 (NS)
Dorskamp	**0.019 (**)**	0.188 (NS)	0.589 (NS)	0.528 (NS)		
Robusta	**<0.001 (***)**	0.102 (NS)				
Beaupré			0.228 (NS)	0.870 (NS)		
I45/51			0.809 (NS)	0.658 (NS)	0.748 (NS)	0.282 (NS)
Blanc du Poitou					**<0.001 (***)**	0.596 (NS)
Fritzi Pauley					0.779 (NS)	0.593 (NS)
Ghoy					**<0.001 (***)**	0.242 (NS)

According to Poiseuille's law, xylem hydraulic conductivity is a function of conduit dimensions, and thus a function of AVSA. Variations in $\Delta^{13}C$, and in intrinsic WUE, are due to differences in assimilation and stomatal conductance, where stomatal conductance may be linked with differences in xylem hydraulic conductivity. Thus consistent AVSA over the period of ten year could result in a consistent water supply to the leaves. Different mechanisms directly coupling stomatal conductance to root-to-leaf conductance have been proposed (Sperry 2000; Nardini & Salleo 2000). However, on the other hand due to canopy closure with age in tree plantations could result in reduction in overall canopy assimilation. Thus, increase in $\Delta^{13}C$ with age could probably be due to canopy closure, resulting in decrease in over all canopy assimilation with age (low light penetration in the lower canopy) and maintenance of consistent hydraulic conductivity resulting in consistent stomatal conductance with age. However, this hypothesis needs to be confirmed in poplar genotypes.

Breeding for higher transpiration efficiency

In the context of increasing area under poplar cultivation and in order to cope with the potential risk of decreased water availability without loosing productivity, INRA Nancy UMR 1137, Forest ecology and Ecophysiology (EEF) and Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC-EA) of University Orléans have been working together to detect cultivars that can produce larger amounts of biomass per unit water used (cultivars with a larger transpiration efficiency). Selecting cultivars with higher *TE* (producing more biomass per unit of water consumed) can have considerable advantages. The major challenge was to identify specific traits leading to increased *TE* and to identify genetic variation among genotypes for these traits that might be further exploited in breeding programs. Mainly focussing on the commercially available poplar cultivars (*Populus deltoides × nigra* hybrids), they found a large genotypic variability in growth potential, *TE* (estimated through $\Delta^{13}C$) and drought tolerance both under controlled and field condition (Monclus *et al.* 2005, 2006; Marron *et al.* 2005; Bonhomme *et al.* 2008). Along with the stability of genotype ranking for productivity and $\Delta^{13}C$ were reported to be independent from each other and the hypothesis of selecting genotypes for enhancede *TE* without sacrificing on productivity was put forward. In the previous studies, measuring *TE* was confined at leaf level. Thus the present study was focussed to check stability of the genotype ranking made at leaf level (i) to whole plant level and (ii) with age in the mature tree plantation. Based on the results, we can conclude that genotypic variation in $\Delta^{13}C$ at leaf level reflect the differences in whole plant *TE* both in *Populus deltoides × nigra* and *Populus nigra* genotypes (Chapter 1 and 2). Furthermore, genotype ranking for $\Delta^{13}C$ was found to be stable with age (Chapter 3 and 4).

Selecting for lower $\Delta^{13}C$ with higher or lower productivity depends upon which traits like stomatal conduction and assimilation, account for the variability of $\Delta^{13}C$ for a given species or genotype. Selection for low $\Delta^{13}C$ may result in the selection of trees with reduced or higher productivity according to water availability and to the predominant trait (i.e. stomatal conductance or CO_2 assimilation) that accounts for the variability in $\Delta^{13}C$ (Farquhar *et al.* 1989; Condon *et al.* 2002). In this study, we found that assimilation (*A*) was responsible for the genotypic variability of $\Delta^{13}C$ and *TE* at leaf and whole plant level rather than stomatal conductance (g_s) among *Populus deltoides × nigra* genotypes (Chapter 1), which is in contradiction to earlier studies (Marron *et al.* 2005; Monclus *et al.* 2005, 2006; Dillen *et al.* 2008). In *Populus nigra*, g_s was responsible for the genotypic variability of $\Delta^{13}C$ and *TE* at

leaf and whole plant level (Chapter 2). A major cause of this discrepancy could be the humid growth conditions for *Populus deltoides × nigra* (Chapter 1). As a consequence, genotype differences for A/g_s under ambient environment were not inline with that under saturated condition. This also implies that genotype ranking might be susceptible to change under low humidity and higher light intensity.

In order to breed for increased whole plant *TE*, it is also necessary to examine specific traits defining *TE* at both intrinsic and whole plant scale and use variability of these specific traits in the breeding program. In the mean time, selection of genotypes for high *TE* under well defined or measured environmental conditions can be employed as a very useful initial screen of a large number of genotypes. However, prospects of improving *TE* along with increasing biomass production might become limited, although some improvements of *TE* and biomass production can be made under certain conditions.

If increasing *TE* is only a concern under water-limited conditions, and the objective is to increase the effective use of water for transpiration when water is limiting, then specific traits that enhance the effective use of water become critical. In this case, the two water-conservation traits could be, i.e. increased sensitivity in stomata conductance to vapour pressure deficit and to soil water deficit, both can contribute to increasing effective use of water. These traits not only can enhance *TE* but can also contribute to soil water conservation, which is important under drought. This could allow plants to remain physiologically active longer following the onset of soil water deficit conditions. In this way, breeding efforts may be more effective if specific traits are examined that are likely to allow plants to be effective in use of water.

PERSPECTIVES

Up scaling from leaf to whole plant *TE*

In this study *TE* was measured at different integration levels in growth chambers under controlled environment. Hence, the results are confined to the controlled environment, and following question remains unaswered : does the genotype variation of $\Delta^{13}C$ reflect the variation in *TE* under field environment and under drought stress.

Upscaling from intrinsic *TE* to whole plant *TE* among genotypes under soil water stress can be important because of possible genetic differences in the threshold where stomatal conductance declines in response to drought. If no other potentially confounding factor is involved, whole plant *TE* on dry soils is likely to be greater for the genotypes with a higher threshold for decreasing its transpiration rate. Reaching the point of non-availability of water for transpiration would be delayed in the genotypes having a high threshold of decreasing transpiration rate.

Genotype stability with age for $\Delta^{13}C$

The study presents nice but insufficient results regarding the stability of genotype ranking for $\Delta^{13}C$ with age, due to unbalanced data set and the small number of genotypes used. In this context, future research on this aspect should include larger sets of similar genotypes growing in different common gardens. In this study, there was a lack of explanatory traits to explain this positive age effect in $\Delta^{13}C$. Thus for the future experiments, all the traits highlighted in the discussion portion should be measured in order to explain the trends in $\Delta^{13}C$. Question of long term relationship of $\Delta^{13}C$ and productivity should also be checked.

GENERAL CONCLUSION

Up scaling approach was developed to check whether genotype differences in $\Delta^{13}C$ represent the differences in transpiration efficiency at leaf and whole plant scale. We also verified the stability genotype difference in $\Delta^{13}C$ with age. We found that genotype differences in $\Delta^{13}C$ were as such translated as the difference in transpiration efficiency at both leaf and whole plant scale. Magnitude of variability of whole plant transpiration efficiency was higher as compared to at leaf level highlighting the role of carbon lost during photo and dark respiration (Φ_c) and water lost during night transpiration (Φ_w). Genotype productivity increased with increase in transpiration efficiency in *Populus deltoides × nigra* genotypes but remained unchanged in *Populus nigra* genotypes. Values of whole plant transpiration efficiency were found higher as compared to other species. ^{18}O enrichment in both leaf bulk ($\Delta^{18}O_{lb}$) and leaf water ($\Delta^{18}O_{lw}$) reflected variation in stomatal conductance (g_s) either at individual level or at genotypic level. In *Populus deltoides × nigra* genotypes, no increase or decreasing trend was found in $\Delta^{13}C$ between the age of 5-15 years and genotypic ranking was conserved over the tested period. However, significantly lower $\Delta^{13}C$ values were evidenced between the age of 4-7, which increased with age. We conclude that in poplar, $\Delta^{13}C$ is a reliable estimator of whole plant transpiration efficiency and the ranking made in young plant for $\Delta^{13}C$ remain stable with age.

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