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BIOMASS DETERMINANTS AND THEIR USE AS YIELD PREDICTORS IN SALICACEAE

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ABSTRACT

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Fast growing hybrids of Salix and Populus can be grown as coppice for short-rotation forestry as a source of renewable energy. Field trials of Salix genotypes of differing yields were assessed to identify anatomical and physiological differences, which determine biomass yield. The high yielding genotypes had the largest stem heights and diameters. Furthermore, high yielding genotypes tended to attain larger individual leaf areas, larger number of cells per leaf, and fast rates of leaf production and leaf extension. Large numbers of sylleptic branches were found on the highest yielding *Salix* genotype studied. Photosynthetic characterisation of genotypes and leaf area index investigations showed that light harvesting and leaf longevity may be more important to above ground biomass accumulation than measures of individual leaf photosynthesis.

Many traits hypothesised as yield determinants in Populus were measured in an F_2 pedigree of P. trichocarpa x P. deltoides hybrids, Family 331, originating from North

America. A replicated field trial was established and assessments were made of single stem and coppiced plants. Principal components analysis (PCA) and partial least squares regression showed that stem traits most important as determinants of yield were total basal stem area and stem height. The number of sylleptic branches influenced yield in coppice. The leaf traits most influential of yield were the number of leaves on the leading stem of the coppice stool and the area of a fully mature, recently expanded individual leaf. These are easily measured traits which can be used in assessments of phenotype for breeding novel genotypes for high yield. The strongest outliers in the PCA score plots may be those of interest to plant breeders, as they show extreme behaviour in traits related to yield. Broad-sense heritability was calculated and QTL analyses were conducted on the data set. The date of bud burst in the spring and stem height and diameter were highly heritable; these traits yielded QTL in addition to QTL for whole tree fresh weight, individual leaf area, stem extension increment and leaf extension rates.

The Family 331 pedigree was grown in open top glasshouse chambers in both ambient and elevated concentrations of CO_2. Leaf area and adaxial epidermal cell area were found to be larger in elevated CO_2. Adaxial cell number per leaf showed different responses at the two stages in the growing season studied. QTL were located for all three traits measured, several of which co-located on more than one linkage group.

The traits most important in determining yield have been identified and this knowledge can now be utilised in breeding programs or for directing the efforts of future research.

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List of abbreviations

A	Assimilation (photosynthetic rate)
aCO_2	ambient carbon dioxide concentration
A_{max}	light saturated rate of net photosynthesis
AFLP	amplified fragment length polymorphism
ANOVA	Analysis of variance
c_i	intercellular (substomatal) concentration of carbon dioxide
ca	ambient concentration of carbon dioxide
cm	centimetre
cM	centimorgan
CO_2	carbon dioxide
$\delta^{13}C$	carbon isotope composition, per mil
DAC	days after coppicing
DAP	days after planting
eCO_2	elevated carbon dioxide concentration
g	grams
g_s	stomatal conductance
H^2	broad-sense heritablility
ha	hectare
IRGA	infra red gas analyser
J_{max}	electron transport capacity of thylakoid

K	potassium
LAI	leaf area index
LARS	Long Ashton Research Station
LER	leaf extension rate
LOD	likelihood of odds
LPR	leaf production rate
LSD	least significant difference
m	metre
MAI	mean annual increment
MAS	marker assisted selection
mm	millimetre
N	nitrogen
ODT	oven-dried tonnes
OTC	open top chamber
P	phosphorus
Pa	Pascals
PAR	photosynthetically active radiation
PCA	principal components analysis
PFD	photon flux density
PLS	partial least squares regression
QE	apparent quantum efficiency
QTL	quantitative trait locus; quantitative trait loci
RuBP	ribulose-1,5-bisphosphate

s.d.	standard deviation
s.e.	standard error of the mean
SEI	stem extension increment
SRC	short-rotation coppice
SSR	short sequence repeat
$V_{c,max}$	maximum RuBP-saturated rate of carboxylation
VIP	variable importance plot
WUE	water-use efficiency
yr	year

Chapter 1

General Introduction

1.1 PROJECT OVERVIEW

This project investigates the physiology of yield in *Populus* and *Salix* grown in short rotation culture as energy crops. Both genera, particularly interspecific hybrids within each genus, exhibit characteristically fast growth which promotes the Salicaceae as a renewable energy resource. Poplars are also extensively grown for the pulping industry, but whatever the purpose, yield is the driving factor in the selective breeding of poplar and willow, particularly given the pressure of developing diseases such as *Melampsora* spp. (rusts). Yield is one of many traits of agronomic importance which is quantitative rather than qualitative and this study aims to elucidate the quantitative genetic and physiological background to some of the factors affecting above-ground biomass accumulation. The first step to achieving this aim was to identify the physiological basis of high yield. This has been widely studied in single stem poplars at the whole organ level but there is a paucity of such knowledge at the cellular and subcellular level and a limited understanding altogether in willow. Investigations of yield determinants in both genera grown in coppice will further the current knowledge. Thus a primary step in this project has been to compare willow genotypes of known, consistent yields to gain an understanding of traits important to yield and to draw the understanding of willow yield closer to that of poplar. The second aim of the work was to investigate these yield traits in a diverse family of F_2 hybrid poplar siblings, to identify the traits influencing yield. This would provide valuable information for gathering useful phenotype data in mapping populations for quantitative trait loci (QTL) analysis to understand the area of the chromosome(s) likely to be controlling each particular trait. Further to identifying QTL

in a given environment, it is of interest whether QTL are equally operative in different environmental conditions or treatments. To further this understanding, a poplar mapping population was exposed during growth to two differing carbon dioxide concentrations and physiological traits considered to be responsive to changes in CO_2 concentrations were measured and subsequently used for QTL analysis. Such knowledge of QTL positions provides a basis for future applications for tree breeding, especially in marker assisted selection for the early selection of seedlings for traits of interest.

1.2 RESOURCE CAPTURE, PROCESSING AND ALLOCATION

1.2.1 Energy from biomass

Plants exploit solar energy, water and carbon dioxide through the process of photosynthesis to manufacture glucose, which is essentially the precursor of all biomass energy. Glucose is converted to an array of carbohydrates including cellulose, sugars, oils, and starches. These natural organic compounds form the basis of the stored energy which is released on combustion of a bioenergy crop (El Bassam, 1998). On a global scale, biomass represents approximately 15% of primary energy production, although the potential of biomass crops as a carbon source is expected to exceed this level in the future, as fossil fuel reserves are depleted and reductions in atmospheric carbon dioxide concentrations are sought (Hall and House, 1995). Owing to the uptake of atmospheric carbon dioxide and subsequent carbon storage, biomass crops are considered carbon neutral, since their combustion releases no more carbon dioxide into the atmosphere than was accumulated during the lifetime of the plant (Hall and House, 1994). In addition to being carbon neutral, the combustion of biomass fuels emits lower concentrations of

sulphur and oxides of nitrogen than fossil fuels (Hall and House, 1995). The benefits and drawbacks of bioenergy plantations, harvest and processing of energy crops, land use and crop management have been discussed in a number of articles (Wright and Hughes, 1993; Christian *et al.*, 1994; Van den Broek *et al.*, 1997; Abrahamson *et al.*, 1998).

1.2.2 The potential of trees

Fuels of particular interest for use in power stations are those with a high cellulose and lignin content which can be processed as chips, pellets and briquettes amongst other forms (El Bassam, 1998). Plant species already found to make suitable solid biofuels include the C4 grass *Miscanthus* and a large number of woody species. The types of woody plants grown as solid biofuels inevitably depends on the local environmental conditions, and yields may vary in tandem with environment. In the tropics, multipurpose trees such as the nitrogen-fixing genus *Leucaena*, are grown as fuelwood. Such fast-growing tropical hardwoods managed as short-rotation coppice include *L. leucocephala* (Cunilo and Prine, 1995). Other potential agroforestry crops in tropical countries include *Erythrina burana* (Teketay 1990), *Gmelina arborea* (Fuwape and Akindele, 1997) and *Acacia* species (Ariga, 1997).

Growth forms

Traditionally, forestry practice has involved the growth of large trees with single stems over long periods of time: up to 25 years for pulpwood production and 70 years or more for timber. When grown as energy crops, either hardwood or softwoods can be grown as single stems, with a single and final harvest when the required yield has been attained.

New rootstocks are then planted after harvest for the next crop. An alternative practice for hardwoods is coppicing. This technique involves more than one harvest from the original rootstock. The practice of coppicing involves cutting stems back to the base after one growing season to encourage the regrowth of multiple stems in the next (Abrahamson *et al.*, 1998). Compared to single stem growth, the first harvest of trees grown under coppice management enables the grower to obtain some financial return on the initial investment early after planting (Gullberg, 1993).

Short rotation coppice

The practice of coppicing involves only fast-growing hardwoods, for a variety of purposes including basketry and other crafts, for panelling, for pulping and for energy production. Tree species grown as coppice for energy include *Eucalyptus*, poplar (*Populus*) and willow (*Salix*). Crops grown as coppice are used as a single stem until the first harvest and thereafter the resprouts are cropped at various time intervals depending on the vigour of resprout growth (Sims *et al.*, 1999). Herve and Ceulemans (1996) have defined short-rotation woody coppice to involve rotation periods shorter than 15 years. From an economic perspective, coppice management reduces replanting costs and reduces site disturbance (Harrington and Fownes, 1993). Over time, yields decrease due to root death, nutrient depletion, and disease infections at cut surfaces. Hansen (1991) suggested that experimental yields are greater than commercial yields owing to the shorter rotations reducing the risk of exposure to unfavourable conditions which would cause tree death. Coppice yields vary with species, planting density, and the age of the root stock, amongst other factors. The number of stems produced by a rootstock, or stool,

is variable. Studies of five genetically different hybrids of poplar, for example, produced between 3 and 8 stems per stool during the first coppice cycle (Herve and Ceulemans, 1996). The lifespan of a coppice tree stand is dependent on the stool's capacity to resprout after harvest, but equally importantly, the ability to survive constant disturbance to the plant's root: shoot ratio (Verwijst, 1996).

Genotypes selected for short-rotation coppice ideally need to grow with near equal vigour between each successive harvest (Sims *et al.*, 1999). Several willow species have been shown to exhibit such uniform vigour and the stools may be used for up to 20 years, within which coppice rotations span between 3 and 5 years (Gullberg, 1993). Growers of short rotation coppice hardwoods aim to make the greatest number of harvests possible from a given stool, as the main expense is the replanting of rootstocks. This requirement amounts to a high coppice regrowth vigour, and the ability to tolerate repeated cutback cycles. Sims *et al.* (2001) compared dry mass yields in trees grown for three years as single stems, followed by a three year period as coppice, which showed consistently higher yields as coppice in the willow, acacia, eucalypt and poplar genotypes studied. The comparison between single stem and coppice yields is not ideal in this experiment owing to the rootstock age and possible annual climatic differences, as the growth forms were studied sequentially and not in parallel, but is a strong pioneer study.

Self-pruning is a distinguishing feature of the Salicaceae and is a natural dispersal technique for species native to riparian habitats: living material shed into waterways will be washed up elsewhere and may propagate from these natural cuttings. It is mainly the

bases of young branches of willow that will break off and disperse, however, as typical dicotyledon secondary thickening occurs over the course of time to strengthen the branches (Raven, 1992). Self-thinning also occurs in managed short-rotation coppice crops over a growing season; vigorous stem growth early in a growing season leads to the production of a large total leaf area during that period, which in turn gives rise to early biomass gain (Ceulemans and Deraedt, 1999). Within-stand competition also gives rise to self-thinning of both shoots on a stool and stools in a plantation (Verwijst, 1996).

Spacing is, therefore, an important factor influencing the yield of short rotation woody crops. In an experiment comparing yield in three poplar genotypes grown at three sites in the U.K., consistently higher yields were attained from those grown at a 1.0 x 1.0 m spacing between plants than at a 2.0 x 2.0 m spacing (Armstrong *et al.,* 1999).
A further factor which affects yield in coppice crops is the duration of the cutting cycle. Productivity has been shown to be higher when poplars were grown in a single, 4-year rotation, than in two rotations of two years' duration (Armstrong *et al.,* 1999). A study of optimum rotation lengths of the poplar genotype 'Beaupré' (Auclair and Bouvarel, 1992) concluded that growth should continue for at least three years before each cutback.

In terms of coppice, a number of studies report tree height to be highly positively correlated to the diameter of the coppice shoots (Heilman and Xie, 1993; Pontailler *et al.,* 1997; Laureysens *et al.,* 2003). Tuskan and Rensema (1992), for example, calculated a correlation of 0.96 for these traits in poplar.

Short-rotation coppice mirrors agricultural practices rather than silviculture (Kenney *et al.,* 1990). This is due to the high intensity of cultivation, inputs of energy and chemical supplements, mechanised practices, and outputs measured in yield per unit land area. Conversely, conventional forestry focuses more on achieving desirable stem sizes and wood qualities for different purposes (Ceulemans, 1990). The highest short rotation coppice biomass yields tend not to be achieved in large commercial plantations, but in small, experimental plots where rotations are particularly short and maintenance is usually manual rather than mechanised. Large plots may also include greater soil heterogeneity including some adverse, or less than ideal, conditions, whereas small plots may avoid this and may be grown on well-selected ground (Hansen, 1991). Willow culture as an energy crop is already a large industry in Sweden, whilst in the U.S.A. poplar is grown in vast plantations as a crop for the pulpwood industry.

Poplar and willow

Both willow and poplar belong to the Salicaceae and therefore exhibit features in common. They have 19 chromosomes. They represent some of the fastest-growing temperate trees, which are mostly dioecious, producing wind-pollinated flowers before the leaves (Eckendwalder, 1996). This research project investigates yield physiology in both poplar and willow and also considers whether these genera have yield traits in common.

The genus *Salix* has been chosen as an energy crop because of its rapid growth and wood production in young shoots, which grow well following coppicing (Gullberg, 1993).

Willows are highly productive, reaching single stem heights of up to 20 metres (*S. alba*), gaining heights of up 4 metres in the first three year period when grown as coppice, and producing up to 20 ODT (oven-dried tonnes) ha^{-1} yr^{-1} (El Bassam, 1998). There are between 300 and 350 species of willow worldwide (Ledin, 1996). There are two sub-genera of *Salix*: *Vetrix* (medium-sized shrubs, 2 – 10 metres tall) and *Salix* (tree willows, 10 – 35 metres tall). Willow, like poplar, readily forms inter- and intraspecific hybrids, and vegetative propagation of willow is a useful property for use in research and commercial production. The sub-genus *Vetrix* is considered best for producing biomass (Barker *et al.*, 1999) and grows well on coppice cycles of 3 – 5 years (Ledin, 1996). In Sweden, biomass willows are a major crop in the energy plantations the government has promoted on former agricultural land (Gullberg, 1993). Owing to their riparian habitats in natural populations, cultivated willows are better than many agricultural crops at managing a fluctuating or constantly high ground water level (Ledin, 1996).

Like willow, short-rotations of poplar coppice can run for 3 to 4 years, with a yield of up to 15 ODT ha^{-1} yr^{-1} over a period of between 20 and 25 years (El Bassam, 1998) and experimental yields of up to 35 ODT ha^{-1} y^{-1} (Scarascia-Mugnozza *et al.*, 1997). Usual annual height increments in hybrid poplar in single stem plantations range from 3 to 5 metres (Bradshaw, 1998). Poplars may successfully form hybrids between and within species and sections of the *Populus* genus, and the majority of species will easily reproduce vegetatively (Dickmann and Keathley, 1996). The genus *Populus* is diverse, comprising 29 species across 6 taxonomic sections (Riemschneider *et al.*, 1996). Favourable interspecific hybrids have been obtained from crosses between *Populus*

sections *Tacamahaca* and *Aigeiros* in which the F_1 generations exhibit hybrid vigour owing to the heterozygosity of the parents (Stettler *et al.*, 1996; Wu *et al.*, 1991). The North American parents in the three generation poplar Family 331 pedigree belong to each of these sections. The maternal parent, *P. trichocarpa*, clone 93-968 of the native black cottonwood from western Washington State, was hybridised in 1981 with the male clone of the eastern cottonwood, *P. deltoides*, from Illinois (Bradshaw *et al.,* 1994). Two siblings of the resulting Family 53, the female 53-246 and male 53-242, were crossed in 1988, to produce Family 331, which has over 350 F_2 components (Bradshaw, 1996). This pedigree forms the basis of the research described in Chapters 3 and 4 of this thesis.

P. trichocarpa comprises much upland forest and riparian habitats in north-western U.S.A. and Canada (Dunlap and Stettler, 2001). The species is excellent for vegetative propagation, and tends to produce suckers from both roots and shoots (Critchfield, 1960). Early and late leaf forms have also been observed in this species, which differ in venation and stomatal patterning, whereby early leaves only have stomata at the leaf tip on the adaxial side, and late leaves have more stomata across the adaxial surface. Early and late leaf forms also differ in that early leaves are thinner and greener than late leaves, which exhibit white abaxial surfaces due to the presence of a thick mesophyll layer (Critchfield, 1960). *P. deltoides,* however, has large, deltoid leaves with short, thick petioles (Wu *et al.,* 1997). Natural stands of the hybrids of *P. trichocarpa* x *P. deltoides* exist in the valleys of south-east Washington state and Benton City (Dunlap and Stettler, 2001). The green abaxial surface of *P. deltoides* leaves, in addition to the adaxial surface, harvests light for photosynthesis (Ceulemans and Isebrands, 1996).

1.2.3 Yield physiology

Woody tissue

The Salicaceae form two types of branch from the main stem. Ceulemans (1990) summarises these. Sylleptic shoots are formed from a new lateral axis, the apical meristem of which has grown continuously without a period of dormancy. In contrast, proleptic shoots are new lateral shoots which develop following a period of dormancy in the apical meristem of that axis. Vast diversity in the phenology of branch development, the abundance of lateral branches and their presence or absence exists between poplar genotypes.

Studies have found that sylleptic branches in poplar may enhance the overall growth rate, although this has not been observed to be the case in willow (Rönnberg-Wästljung and Gullberg, 1999). Sylleptic branching has been found to positively influence stem volume growth in poplar (Bradshaw, 1998). Scarascia-Mugnozza *et al.,* (1999) found that sylleptic branches in *Populus* export carbon photosynthates to the lower stem and roots of the plant. In a study by Barigah *et al.* (1994), for example, the branchiest growth form in the study of several poplar genotypes produced the most biomass. Sylleptic branches respond to changes in the canopy as the crown of the tree gains height, and respond to the resulting shading by detaching from the stem. Sylleptic branches are usually smaller than proleptic branches but carry a larger proportion of the tree's leaves (Ceulemans and Isebrands, 1996).

There is great genetic diversity between poplar genotypes, resulting in many wood properties reflected by the different uses of poplar, including wood to bark ratio, specific gravity and moisture content. In the interests of the biofuel industry, lignocellulose is the product required in wood to make poplar a viable fuel (Tuskan and Rensema, 1992). Lignin is good for combustion as it has a high energy content, but reduces the pulp yield for pulping. Bark has a higher lignin content (16%) than wood (10%). The bark content is proportional to the number of stems on a coppice stool (Kenney *et al.,* 1990), which explains that in a study of 110 coppiced poplar genotypes that varied in stem production, there was significant variation in bark content of 1st year coppice trees. Ledin (1996) reported that willow wood harvested in winter has a typical moisture content of 50%.

Moisture content in stems has been found to vary throughout different height sections of growing poplar and willow stems. Telenius (1997a) calculated differences between height sections of leading stems to vary from 1% to 9% between sections of the leading part of the shoot. Moisture content was found to be higher in poplar than in willow, and between the willows studied, *Salix dasyclados* (a broadleaved willow genotype) yielded higher wood moisture contents than *S. viminalis*. Although the percentage moisture content differences were small in this study, it should perhaps be considered that fresh stems are often the basis for estimating the dry biomass of stems, and moisture content variability may need to be corrected for, for estimates to be as accurate as possible.

Measuring the yield of woody crops

Comparisons between genotypes in experimental trials usually calculate yield in oven-dried tonnes per hectare per year. These calculations often use the biomass figure gained from drying the harvested material, a destructive method. Many growth parameters are measured to gain an insight into biomass gain at the time of harvest, including wood volume, leaf area estimations, and wood dry weight (see articles by Verwijst and Telenius, 1999; Barigah *et al.,* 1994, for details of methods). Since stem height and stem diameter are strongly correlated in poplar and in willow, it can be easier to measure stem diameter than height, particularly in dense plantations where the top of the stem is distant and not visible (Verwijst and Telenius, 1999).

Another method is to calculate the energy yield by finding the calorific value of the whole tree, or a small wood sample, by combustion. Alternatively, many models have been developed to enable the standing biomass of poplar and willow crops to be estimated non-destructively, enabling the continuation of experiments into subsequent growing seasons. Some researchers have used multivariate statistical methods to assess the future growth of single stem trees based on particular growth traits, but this approach has rarely been used in coppiced plants as has been discussed in the work of Thakaran *et al.* (2001).

Leaf characteristics

i. Leaf growth

Leaf growth is an important factor in tree stem growth, since the leaf is the organ where carbon is fixed. Thus, in the processes determining leaf growth, both the environmental factors influencing leaf expansion rate and the internal regulation of cell expansion to promote leaf expansion are important features in the accumulation of stem biomass (Van Volkenburgh and Taylor, 1996). Equally, the vast diversity of leaf forms in the large number of existent poplar genotypes is due to underlying physiological processes, which in turn are controlled by both genes and the environment. The final leaf area of, for example, a poplar genotype will be determined by factors such as the rate of growth of an individual leaf, the rate of production of leaves on the plant, and the duration of growth throughout the season (Ridge *et al.,* 1988).

Both total leaf area and individual leaf area have been shown to be strongly correlated to total woody biomass production in poplar hybrids, and this may suggest that fast-growing, large leaves lead to increased stem biomass in these hybrids (Barigah *et al.,* 1994). The genotypes in the study with the highest biomass yields were also those with the largest individual leaf area and largest total leaf area per tree. Furthermore, Harrington *et al.,* (1997) found a high positive correlation between mature individual leaf area and the relative growth rates in poplar genotypes. Leaf area has also been related to stem biomass sufficiently for Harrington and Fownes (1993) to produce an equation to estimate total biomass and leaf area index from basal diameter measurements.

It has been observed (Ceulemans, 1990) that hybrids of *P. trichocarpa* and *P. deltoides* exhibit much greater overall rates of leaf growth than any European or American native *Populus* species or any Euramerican hybrids. Such hybrids have characteristically large individual leaf areas (Barigah *et al.*, 1994).

There are two main components in the process of increasing the total leaf area of a plant: the initiation of new leaf primordia and laminar expansion. The rates at which these processes occur are important indicators of plant growth (Ceulemans, 1990). The rate of production of new leaves can be calculated in plants using the plastochron index (P.I.). This index, developed by Erickson and Michelini (1957), is based on the principle that leaf growth is sigmoid and its log is exponential for the period when a leaf is expanding most quickly. It also depends on a constant pattern of leaf production within a plant. The P.I. of a plant can be measured as:

$$P.I. = n + \frac{(\log L_n - \log 20)}{(\log L_n - \log L_{n+1})}$$

Where n is the serial number of the leaf, L_n is the length of the index leaf and L_{n+1} is the length of the leaf immediately younger than the index leaf. Provided that leaves are being produced at a uniform rate, *P.I.* is effectively the rate of leaf initiation of a plant with a given number of leaves, which is a useful measure to compare like plants growing in different conditions (Larson and Isebrands, 1971).

A study by Ceulemans *et al.* (1988) found a trade-off between leaf extension rate and leaf production rate in poplar in that high leaf production rate values gave rise to low rates of leaf extension and therefore a slow rate of leaf maturation. In both *Populus* and *Salix* studies, different genotypes differ in their rates of leaf production (Porter *et al.,* 1993).

ii. Leaf cell area and number

Leaves attain their final, mature area through the processes of cell expansion and cell division which is led by the epidermis, since this is the determining factor in growth rates (van Volkenburgh, 1999). The area and number of epidermal cells in a leaf are evidence of the pattern of leaf growth. Several studies have found that under normal conditions, in the absence of flooding or drought, the area of individual epidermal cells changes very little, and thus differences in leaf area may be solely due to differences in the numbers of cells present on the leaf surface (Hinckley *et al.,* 1989). In *Populus trichocarpa,* for example, the cell areas are large, suggesting the final leaf areas are the result of much cell expansion, relative to those of *P. deltoides* which has many, small cells, with this magnitude of cells being the result of much cell division. It is suggested (Ridge *et al,* 1986) that the hybrids of these two species may display such heterosis due to inheriting the lack of restraints on both cell expansion (from the *P. trichocarpa* parent) and cell division (from the *P. deltoides* parent). Thus leaf growth control mechanisms are likely to be important when selecting poplars for overall yields.

iii. Leaf anatomy

The study of the structure of a leaf provides evidence about its physiology. *Populus trichocarpa*, for example, is the only poplar in which stomata occur infrequently on the adaxial leaf surface (Critchfield, 1960); all other poplar species are fully amphistomatous (Ceulemans, 1990). *Populus trichocarpa* is a native plant to the western U.S.A., often growing at higher altitudes than most poplars, and this climatic difference may explain its unique stomatal characteristics. Low numbers of stomata are a recognised drought response, which is also true of a thick mesophyll layer: *P. trichocarpa* is identifiable also by the white appearance of its abaxial surface of the thick leaves (Ridge *et al.,* 1988). The leaf whiteness is derived from a thick, loosely packed layer of spongy mesophyll cells which can be 200 μm or more thick; conversely, *P. deltoides* has a green abaxial appearance accounted for by bilateral palisade layers which are up to 20μm in thickness (Ceulemans *et al.,* 1984).

Leaf thickness, measured as specific leaf area ($mm^2\ g^{-1}$) varies at different elevations through the canopy; in the lower canopy, leaves have a higher area per weight (resulting in thinner leaves) whereas in the upper canopy, leaves have a higher weight per unit area (Verwijst and Telenius, 1999).

Petioles and veins have two roles: structural support and nutrient and fluid transport in leaves (Ninnemets and Fleck, 2002); petiole properties influence leaf angles to increase light interception. The structure of poplar petioles, by design, facilitates leaf flutter even in light winds (Roden, 2002). This is because poplar petioles are flattened, angled

perpendicular to the blade, and non-rigid. Leaf flutter affects the amount of light available to the lower canopy, making the light environment more dynamic and evenly distributed in the canopy (Roden, 2002). Post-illumination CO_2 fixation may enable nearly as much carbon to be fixed in fluttering sun leaves as in leaves at constant high photon flux densities (PFDs) (Roden and Pearcy, 1993). Leaf angle corresponds to petiole and lamina size and morphology, and petiole mechanics vary with irradiance, with more rigid petioles at high irradiance (Ninnemets and Fleck, 2002). It was found that thicker leaves were produced at higher irradiance in *Liriodendron tulipifera*, with more vascular tissue per unit area in these leaves to supply the demands of higher physiological activity. Ninnemets and Fleck (2002) observed that within-canopy shading was reduced owing to increases in petiole length, leaf length and internode length in this species at high irradiance, with longer petiole lengths also reducing the drag caused by the higher wind speeds present at higher elevations in the canopy. Compared to leaves at fixed angles and azimuths, Roden (2002), using a computer model, found that fluttering leaves intercepted light more uniformly than fixed leaves. Depending on the angle fixed, fixed leaves may not always intercept maximum PFDs, even in full sun. Regardless of leaf orientation and solar position, the random nature of leaf flutter was calculated to create uniform PFD of intercepted light (Roden, 2002). This is advantageous for carbon gain, especially in the upper canopy.

iv. Leaf phenology

It is essential to understand how leaves grow, export carbon, translocate carbon and senesce in order to interpret the reasons for a tree's yield and predict future growth

responses (Isebrands *et al.,* 1996). The proportion of the year throughout which leaves are photosynthetically active, from the breaking of winter dormancy when leaves flush until senescence in the autumn, should indicate the period of time over which a tree is accumulating carbon (Michael *et al.,* 1988; Frewen *et al.,* 2000). Also the Michael *et al.* (1990) observed the importance of photosynthetic activity late in the growing season. They found that, although poplar genotype 'Tristis' set bud the earliest, the genotype 'Eugenei' out-grew it because its terminal meristem was active for a greater number of days, thereby using the growing season for longer in terms of active leaf development and photosynthesis.

Poplars and willows lose their lower leaves first and keep only their uppermost leaves until the end of the growing season. Willow leaves grow more slowly than poplar leaves and retain leaves for longer (Ceulemans *et al.,* 1996a). Minimal individual leaf longevity in *Salix* genotypes was found to be similar between genotypes in a study by Porter *et al.* (1993), but the maximum longevity significantly differed. Cannell *et al.* (1987) reported that young, green willow stems are photosynthetically active prior to leaf emergence. Nardini (2002), linking final leaf area to maximum growth rate, found that the duration of individual leaf growth is related to plant hydraulic architecture (r = -0.922). This is owing to the influence of water transport efficiency to the leaves, affecting the turgor pressure influencing cell expansion, which itself drives leaf expansion.

Canopy light harvesting

Canopy architecture is important in water relations, gas exchange and nutrient exchange (Gower and Norman, 1991). The distribution of leaf angles in the canopy affects the amount of light interception and penetration. It also affects the redistribution of light at different layers in the canopy. The distribution of canopy leaf angles can be useful for working out the photosynthetic rate of the canopy. Furthermore, photosynthesis in the canopy can be considered to behave like a single leaf scaled up to a whole-stand level (Kull and Jarvis, 1995). Estimates of canopy photosynthesis are normally made on an individual leaf basis and then adjusted for the position in the canopy and time of measurement made on the leaf (Kull, 2002). Leaf area index (LAI) is the (dimensionless) ratio of total leaf area per unit land area. Indirect LAI measurements, using canopy analysis instruments, correlate total leaf area with the probability of light penetration through a canopy (Nackaerts *et al.,* 2000). When LAI is high, crops with erect leaves have a higher yield than crops with horizontal leaves. When LAI is low, however, leaf angle is less important to yield. Lawlor (2002) has indicated that because of the non-linear nature of LAI, after an LAI of 3, when light interception approaches 90%, further increases in LAI do not greatly enhance light interception. Additionally, when solar elevations are low, leaf angle does not make an especial impact on yield (Ceulemans, 1990). Stems contribute a very small proportion of light interception relative to leaves (Gower and Norman, 1991).

Leaf angle distribution further affects canopy photosynthesis by influencing leaf temperature and the distribution of leaf temperatures within the canopy, which in turn

affects the rate of photosynthesis. All of the gas exchange which takes place between the leaf and the environment has to pass through the boundary layer. This is a still layer of air surrounding the leaf surface. The shape and orientation of a leaf, in addition to the leaf area, affect the magnitude of the boundary layer and its conductance. While the boundary layer matters little to photosynthesis and transpiration rates, it does have and effect on heat transfer (Ceulemans, 1990). At low values of leaf area index, the leaf angle has little effect on light and overall carbon gain, but at LAI values greater than 3, it is more influential (Hopkins, 1995). The zenith angle is, in effect, an index of leaf position relative to the sun. More specifically, it is the angle between the normal of the leaf and the zenith, and is calculated from three measurements: the lamina angle (the amount of leaf tilt), midrib angle (the angle of the hanging leaf), and azimuth angle (the leaf angle relative to North).

The total leaf area of a poplar is often tightly correlated with the total accumulated biomass of that plant (Larsson and Isebrands, 1971). In a poplar experiment, the highest yielding of five biomass clones were those with the highest LAI values (Barigah *et al.,* 1994). It is important, however, in comparisons between species or genotypes, to consider the phenology of the canopy with respect to light harvesting and yield. Milne *et al.,* (1992), for example, report a comparative study of biomass production in three poplar clones: the genotype 'Robusta' achieved the highest solar radiation conversion ratio and highest leaf area index (approximately 25% higher than 'Beaupré' and 'Fritzi Pauli') but, owing to its canopy development later in the season, less biomass was

accumulated. Measurements of leaf area index were not taken in the Autumn when most leaves on each stem had senesced.

Individual leaf photosynthesis

Photosynthesis is essentially light capture followed by conversion of simple CO_2 and H_2O into more complex organic molecules (Ceulemans and Isebrands, 1996). Barigah *et al.* (1994) reviewed the positive correlation between photosynthetic activity and biomass production in loblolly pine (*Pinus taeda*), *Larix* hybrids and *Populus* hybrids, yet it is observed that in many studies there has been no correlation between these two factors.

There is a strong correlation between the nitrogen content of a leaf and its photosynthetic properties (Evans,1989; Reich *et al.,* 1994). This makes it possible for leaf nitrogen content to be a useful surrogate measure of leaf photosynthesis, since proteins have a high nitrogen content and the most abundant protein in plants is Rubisco. The main limitations on leaf carbon acquisition are leaf chlorophyll content and the maximum rate of photosynthesis in saturated CO_2 conditions (Kull and Niinemets, 1998).

Photosynthetic efficiency can be calculated through the study of rates of carbon dioxide use or light response. There is an almost linear response of photosynthetic assimilation rate to carboxylase activity, the slope of the curve of the rate of CO_2 assimilation to partial pressure of CO_2 in the chloroplast being directly proportional to the maximum activity (or amount, although the enzymes are not always active) of Rubisco in the leaf

(Farquhar and Sharkey, 1982). The efficiency of this activity can be measured by applying a range of carbon dioxide concentrations to the leaf surface and plotting a curve of assimilation against these different concentrations. The resulting curve can be used to find the gradient of two slopes. The initial slope, J_{max}, relates to the maximum rate of photosynthetic electron transport. The final slope of the curve, $V_{c,max}$, is the maximum rate of carboxylation of Rubsico (von Caemmerer and Farquhar, 1981).

Low temperatures can decrease the activity of Rubisco and reduce the capacity for electron transport; high temperatures, however, can both reduce electron transport capacity and increase the evolution rates of CO_2 from photorespiration, causing a decrease in assimilation rate (Farquhar and Sharkey, 1982).

Kull and Niinemets (1998) conducted typical gas exchange measurements on detached shoots excised under water and measured in a laboratory for photosynthetic response to CO_2 (A/C_i curves). They set a leaf temperature of 26 °C and used PAR at 1000 μmol m^{-2} s^{-1}, which avoided any photodamage in shade leaves and was enough to saturate photosynthesis at high concentrations of CO_2. The standard, stabilising CO_2 concentration was 32 Pa and levels ranged from 1 to 160 Pa with 5 minutes at each CO_2 concentration, after allowing 10-15 minutes to stabilise photosynthesis at 32 Pa between measurements (Sharkey, 1985). Von Caemmerer and Farquhar (1981)'s equations can be used to calculate the gas exchange parameters. The initial A/C_i curve slope in the study by Kull and Niinemets (1998) was between 1 and 6 Pa CO_2 and P_{max}, the maximum photosynthetic rate, was measured at 160 Pa CO_2. In this study, there was a dependence

on leaf nitrogen content of both A/C_i slope and P_{max} (the maximum rate of photosynthesis) indicating that these are proportional to nitrogen content.

In a study by Casella and Ceulemans (2002), using photosynthetic measurements to understand the productivity of a coppice stand of *Populus,* light response curves and A/C_i curves were constructed on overcast days only, again making measurements between 10:00 and 12:00 o'clock to avoid disturbing diurnal patterns of gas exchange, using PAR between 0 and 2000 $\mu mol\ m^{-2}s^{-1}$, C_i between 5 and 150 Pa, and a cuvette temperature in the IRGA at 25°C ±0.3°C.

Near the CO_2 compensation partial pressure, the slope of the A/C_i curve is proportional to V_{max}, the maximum rate of carboxylation. The photoynthesis value at high partial pressures of CO_2 supplies an estimate J_{max}, the electron transport capacity (von Caemmerer and Farquhar, 1981).

In many instances, Rubisco activity is highly correlated to the maximum photosynthetic rate (Wareing *et al.,* 1968). Since Rubisco, a protein with a high nitrogen content, constitutes the most part of the protein of a leaf, it is reasonable to use total leaf nitrogen content as an indicator of total photosynthetic activity. However, there are enzymes in the Calvin Cycle other than Rubisco that limit photosynthesis (Lawlor, 1995). Ericsson *et al.* (1996) argued that light capture and processing is more important to yield than the rate of CO_2 assimilation; crops and trees show strong relationships between light interception and dry matter production (Montieth, 1977). Because light provides the

energy for all metabolism, there is a linear relationship between radiation interception and total dry matter production (Lawlor, 2002). For maximum net photosynthesis and productivity to occur, conditions should be close to optimum. This is not possible in the U.K. in the current climate for C_3 plants, but at ambient CO_2 concentrations of 720 μmol mol^{-1}, it is estimated that crop productivity could increase by 30% (Lawlor, 2002). Although some experiments have found tight relationships between photosynthetic rate and crop yield (Faville *et al.*,1999), there are many factors limiting photosynthesis so the complex sequence of events that follow CO_2 uptake, make it unlikely that yield can be directly affected (Lawlor, 1995).

Carbon isotope discrimination

There are two natural, stable isotopes of carbon: ^{12}C, constituting 98.9% of all carbon, and ^{13}C, forming the remaining 1.1%. The distribution of these isotopes is not consistent and varies within different compounds, which, in biological as well as in other systems, provides evidence about the metabolic processes involving carbon (Farquhar *et al.*, 1989). One of these processes is photosynthesis. The photosynthetic pathway is one of a variety of factors, which cause plant carbon isotope ratios to vary in plants. The atmosphere itself varies in its ratio of $^{13}CO_2/^{12}CO_2$, and plants are, as a rule, depleted in ^{13}C relative to atmospheric CO_2 (Lloyd and Farquhar, 1994). This depletion, or selection against the uptake, of ^{13}C is known as discrimination.

The carbon isotope composition of plant tissue gives an indication of long-term physiological activity and is often used as an estimate of water use efficiency by the plant,

as described by Leffler and Evans (1999). When water availability is decreased, the stomata close, resulting in a decrease in the ratio of intercellular to atmospheric CO_2 concentrations (the C_i/C_a ratio). The C_i/C_a ratio in turn alters the relative contributions of the diffusional fractional term and the Rubisco fractional term to discriminate against ^{13}C during gas exchange in the leaf. The accumulated discrimination against ^{13}C therefore links back to plant water status. The stability of the carbon isotopes, in effect, supplies an average water use status over the lifetime of the tissue in question.

Carbon isotope discrimination can be measured in three main ways. Farquhar *et al.* (1989) estimate that all of these techniques provide the same results, and they outline the destructive method and the stirred cuvette method. The destructive method calculates the composition of CO_2 released on combustion of plant tissue relative to the atmosphere in which the plant was grown. This provides an average discrimination value for the time period over which the carbon was fixed by the plant. As mentioned above, the two isotopes of carbon are both stable, so, where there is no net gain or loss of carbon in the plant, the ratio of ^{13}C to ^{12}C will remain the same. The cuvette method developed by Farquhar *et al.* (1989) is non-destructive and measures the changes in the $^{13}CO_2/^{12}CO_2$ ratio in the air as it passes a leaf sealed in a cuvette of stirred air, as a measurement of discrimination by the gas exchange system of the whole leaf. The greater the discrimination against $^{13}CO_2$, the higher the concentration of $^{13}CO_2$ that will remain in the air leaving the cuvette relative to that entering the cuvette. One further method (O'Leary *et al.*, 1986) is non-destructive also. It involves sealing the plant into a bell jar containing a known CO_2 composition until the plant has consumed all the available CO_2.

Equations are then used to calculate the continuing isotopic enrichment of the remaining CO_2 to gain a discrimination value.

Smith and Epstein (1971) ranked the $^{13}C/^{12}C$ ratios for a range of monocotyledon and dicotyledon plants in decreasing order (towards more negative values). Plants fell into two groups, with most higher plants having low ratios of -23.2 to -34.3 ‰. Adaptations leading to higher $\delta^{13}C$ values tended to be responses to stressed habitats, for example xeric or aquatic environments. Carbon isotope discrimination is an indirect measure of transpiration efficiency, the ratio of dry matter production to water transpired (Merah *et al.,* 2001). Increased water use efficiency (WUE) has been noted to accompany reduced discrimination against ^{13}C (Condon *et al.,* 2002; Osorio and Pereira, 1994). For example, Farquhar and Richards (1984) found a relationship between the $\delta^{13}C$ of plant tissue and its water use efficiency in wheat.

1.2.4 Other factors affecting yield in Salicaceae

Further considerations regarding growing poplars and willows is their susceptibility to infection by rust such as *Melampsora* species (Cervera *et al.,* 1996; Dawson and McCracken, 1994) which is a serious impediment to leaf productivity. Researchers have identified links between the rates of water loss from leaves and infection with rusts in poplar leaves. The least susceptible of a range of poplar clones to *Melampsora larici-populina* infection were those which exhibited the lowest rates of water loss (Siwecki and Przybyl, 1981). Resistance genes are present in the Family 331 pedigree in the U.S.A. (Newcombe *et al.,* 1996) and these are yet to be identified for infections of rusts in Europe.

Hebivory, if unchecked, may have a considerable impact on the yield of, especially, young trees. Coley (1980) observed that old leaves tend to be well defended against herbivores and therefore receive less grazing, but younger, pioneer leaves are less defended and thus tend to be more readily consumed. The herbivore may also express preferences in the developmental stage of the leaf; Wait *et al.* (1998) found that *Chrysomela scripta,* feeding on *Populus deltoides,* preferred to consume leaves at 40 to 60 % of full expansion. The cottonwood leaf beetle, *Chrysomela scripta,* is a serious poplar defoliator in North America, with plantations being most susceptible to damage in the first three years from establishment. Adult beetles selected certain clones for feeding on, and these preferences were correlated with the parentage of hybrids; *Aigeros* parentage significantly affected the total amount of leaf consumed (Bingaman and Hart, 1992).

1.3 QTL MAPPING

1.3.1 Quantitative versus qualitative traits

Traits known to be qualitative are those controlled by a single gene, which has a large effect on the trait compared to the relatively small effect on the phenotype provided by the environment. Such single gene traits would give a discontinuous distribution of few discrete phenotypes in the F_2, such as the 1:2:1 ratios with which Mendel's work was concerned. These clear phenotypes representing qualitative traits are usually easily visually identified (Kearsey and Pooni, 1996). An example of this would be the flower colour of an F_2 generation of peas derived from a red-flowered parent and a white-flowered parent: the F_2 are only likely to have either red, pink or white flowers.

Quantitative genetics is concerned with the study of traits which are under the control of several genes (usually more than five), with each gene occurring as different alleles in a given population (Riemenschneider *et al.*, 1996). Quantitative trait loci were first considered in a study by Sax (1923) which used anaysis of variance to conclude that several genes were acting on seed traits to cause the observed phenotypic variation. Mather (1949) coined the term polygenes to refer to what we now call quantitative trait loci (QTL), the genes underlying quantitative traits (Gelderman, 1975). Each of the several genes will segregate in the population, and there will additionally be environmental effects on the phenotype, giving rise to a continuous distribution of phenotypes in the F_2. Equally, very slight allelic differences may occur in the genes which have further small effects on the phenotype. This gives rise to the normal

distribution range in the population which is characteristic of a quantitative trait. (Kearsey and Pooni, 1996). The heights of the F_2s in poplar Family 331, for example (Wu, 1998), were influenced by both the environment and several genes giving rise to a normal distribution of height phenotypes. The presence and activity of a QTL does not exclude the possibility of there also being qualitative alleles present for a character, but any single gene effect is likely to be hidden by the other variation (Kearsey, 1998). Markers are characteristics which identify a given trait. A phenotypic marker is an expressed physical attribute of the phenotype, such as leaf shape. This may give clues as to the genotype, but will also contain environmental effects. Protein markers are the products of gene activity and can be measured in tissues. Like phenotypic markers, however, the genes coding for the proteins may not be continuously expressed, so it is likely that only a small amount of the genetic variation for a trait is apparent. Molecular markers are unique in that they are constantly present. A vast array of techniques is now available to detect single site, genetic differences between individuals of different genotypes. These reliable markers of molecular variation exist across all the chromosomes of an individual and enable a molecular map to be constructed for a given population, indicating the positions of markers on each chromosome and the theoretical distances between them (Jones *et al.*, 1997; Burrow and Blake, 1998). The molecular map of an inbred population (ideally a backcross or F_2 generation, or doubled haploids or recombinant inbred lines) in tandem with phenotypic data for a trait measured in each individual of a population, can be statistically analysed to enable QTL to be detected and their likely positions identified (Kearsey and Pooni, 1996).

1.3.2 Principles of mapping quantitative traits

There are certain necessary requirements which have to be met in order to accomplish QTL mapping. Initially, a segregating population, such as an F_2 or a backcross, is required, in which every individual must display the variable trait of interest. The phenotype of this trait then needs to be scored for the analysis. Molecular markers must then be identified in the individuals and their recombination ratios amongst them used, to construct genetic linkage groups for the population on a molecular linkage map (Prioul *et al.,* 1997). The phenotyping and genotyping data together are essential for all current methods of QTL analysis.

Analysis of variance or a t-test can be primarily conducted to decide if there are differences between the mean of the known genotype and the mean for the phenotype, although this does not tell you where the QTL should be located (Kearsey, 1998). To properly identify the sites of QTL action, however, there are two major approaches to mapping quantitative trait loci, although it is thought that all QTL methods give essentially similar estimates of QTL position and gene effects (Kearsey and Farquhar, 1998).

Regression analysis

In a similar manner to the ANOVA approach, if the phenotypic value for the trait is regressed onto the marker genotype, the regression will be the greatest at the markers flanking the QTL, provided the QTL is on the same chromosome as the marker. If, however, the QTL and the marker are not linked on the chromosome, there will be no

regression. Kearsey and Hyne's (1994) model uses regression analysis to fit the marker data to a likely locus, to estimate the effect of the fit, and to state the significance of that value as well as a measure of confidence in the estimate.

Interval mapping

Interval mapping computer programs scan along the length of the molecular map at intervals of every two markers, and between each pair of markers along each chromosome it is attempted to fit the QTL in the position. The phenotype data is compared with the trait data at every interval and this comparison gives the probability of a QTL falling at locus between them (Lander and Botstein, 1989). The likelihood of the presence of a QTL at a given position, measured against the probability of the result arising by chance, gives the 'likelihood of the odds ratio,' which to $\log_{10}$ is given as the 'LOD' score. Over a given threshold for the population, a LOD score will suggest the presence of a QTL at the given position, and a degree of confidence is supplied. Thus, interval mapping localises the effect of a QTL between pairs of qualitative markers rather than locating it to a single, individual locus (Mansur *et al.,* 1993). A parallel method is used with a further regression approach, most suitable for outbreeding populations (Haley and Knott, 1992).

Heritability is the proportion of the total variation for a trait caused by genetic influences; for most characters of commercial interest, heritability is less than 50%, so at least half of the total variation is due to the environment. The lower the heritability of a trait, the less statistical confidence there can be for a suggested QTL position (Kearsey, 1998).

Bradshaw and Stettler (1995) outline two models for the genetic control of growth characters. The polygenic model, suggests that a large number of unlinked genes each contribute a small amount to the resulting phenotype. The alternative, oligogenic, model infers that very few loci may have large effects on phenotypic traits, and recognises that environmental factors may influence aspects of the phenotype not controlled by these loci. Their study found, however, that five traits affecting growth in a poplar population, Family 331, all exhibited continuous distribution of phenotypic traits in the F_2 generation, thus favouring the polygenic model. Wu (1998), relating leaf traits (total number and total area) and branch traits (number, average length, and angle of sylleptics and proleptics) in Family 331, found a total of 113 significant QTL for two- and three-year-old leaves, each QTL representing between 10 and 48.6% of the entire phenotypic variance for these features. The study showed that relatively few QTL accounted for a large proportion of the phenotypic variance in leaf forms, which contrasts with the polygenic model. Furthermore, Thoday (1961), in analysis of genetic data from *Drosophila* studies, calculated that a small number of genes were acting to produce the given phenotypes in the population, favouring the oligogenic argument. Kearsey (1998) has stated that with increasing number of QTL present in a population, there is increasing difficulty of detecting them. Thus, only the QTL with large effects are actually recognised, leading the false impression that there are only a few QTL with large effects. Paterson *et al.* (1988), using tomato as the experimental organism, acknowledged that yield, a complex trait, was controlled by a few QTL with large effects, and that the remaining variation was accounted for by many more genes each with smaller effects.

Wu (1998) reported the presence of QTL for height and basal stem area in the poplar F_2, but no QTL were found for stem volume, a parameter which is strongly mathematically related to height and basal diameter in poplar. It is suggested that where a QTL is rare or absent, larger sample sizes in the pedigree and denser linkage maps may show the QTL, since there may be action of many genes each with a small effect. Richer molecular maps may aid the detection of QTL, but to add markers to a map at densities higher than one every 10 cM, there is little enhancement to QTL detection and map length (Kearsey, 1998). Schneider *et al.* (2002), for example, found little advantage of adding 99 AFLP markers to their sugar beet molecular map, which increased the map length only from 417 to 446 cM. Darvasi *et al.* (1993) have more leniently calculated that a molecular map saturated with markers will not have an advantage in the resolution of QTL mapping after a density of approximately one marker every 20 cM, after which only increasing the progeny size in the population would assist the precision of QTL location. For confident QTL mapping, Prioul *et al.* (1997) recommend genome coverage by molecular markers totalling 100 to 150. These should be evenly spaced across the genome.

Some QTL may be specific to a certain environment (Bradshaw, 1996) so that, within one clone, the statistical significance of QTL from different environments may vary. This emphasises the importance of the experimental design of a study; the use of randomised complete block design, with several replicates of each clone, has been used in several published trials (e.g. Bradshaw and Stettler, 1995; Wu *et al.*, 1997) to eliminate any *genotype x environment* interactions. This operates by replicating the individual

genotypes from each family, the number of replications per family (blocks) and the number of locations in which a block is planted (Bradshaw and Stettler, 1995).

1.4 QTL AND PHYSIOLOGY

1.4.1 Agricultural crops

There has been considerable research to date advancing our awareness of QTL for physiological traits which influence yields in agricultural crops as well as in forestry.

Mansur *et al.*, (1993) studied soybean seed traits in hybrids of high-yielding *Glycine max* of similar phenotype (unlike the usual QTL mapping approach which uses hybrids of contrasting parents) called transgressive segregants, some of which exceeded the parental maximums for yield traits. The large ranges of values for phenotypic traits in the hybrids were typical of transgressive behaviour. Analysis revealed that leaf area was highly correlated with seed yield in the population. They also found co-located QTL for morphological traits including plant and canopy heights, and leaf area and carbon isotope discrimination, which were grouped into clusters associated with seed yield. QTL were identified for seed yield itself. Other complex traits such as sugar-related traits have been studied for QTL analysis in *Beta vulgaris*. Schneider *et al.* (2002), for example, detected QTL for sugar yield, beet yield and ion balance amongst other traits, and found a major beet yield QTL co-locating with a major QTL for sugar yield.

QTL have been identified in rice leaves, *Orzya sativa,* for the traits associated with nitrogen use efficiency, including Rubisco to leaf nitrogen ratios and total leaf nitrogen (Ishimaru *et al.,* 2001a). Ishimaru *et al.* (2001b) also detected QTL for stomatal frequencies in the leaves of *Oryza sativa*, finding that positions of QTL for yield and adaxial and abaxial stomatal frequencies overlapped. They also found some QTL for leaf rolling, a drought response, in rice leaves, emphasising the link between genetic and environmental control of plant characteristics.

Ferris *et al.* (2002), using the Family 331 poplar pedigree discussed in this thesis, also found QTL for stomatal and epidermal cell traits. These findings are considered further in Chapter 4 of this volume. Yin *et al.* (1999) Specific leaf area describes leaf thickness, and QTL for this trait were found at different developmental stages in barley, *Hordeum vulgare,* indicating that QTL are dynamic and not necessarily expressed at every stage in the lifetime of an organism. Other physiological traits in plants for which QTL have been found include carbon isotope discrimination (Thumma *et al.*, 2001; Teulat *et al.,* 2002) and flowering time in *Brassica oleracea* (Rae *et al.,* 1999). QTL have been found in tree species. Weng *et al.* (2002) for example, found QTL for tree height in pine seedlings.

The heritability of complex traits (e.g. yield) can be increased through the genetic linkage or pleiotropic effects of genes determining simpler, highly heritable traits (Mansur *et al.,* 1993). High heritabilities and genetic correlations between traits could be due to single pleiotropic genes with major effects (Mansur *et al.,* 1993). Loci with seemingly large effects may be the manifestation of blocks of genes with major effects.

1.4.2 Poplars

In poplar, pioneer QTL work was conducted using the Family 331 pedigree of *P. trichocarpa* x *P. deltoides*. Extensive studies in this pedigree on morphological variation in the hybrids have included measurements of propleptic and sylleptic branching, height and basal diameter ratios, and branch length and angle. Hybrid vigour was observed in growth-related traits in the F_1s (Wu and Stettler, 1994). Bradshaw and Stettler (1995) found that the timing of spring bud flush is highly heritable, with up to 98 % of the phenotypic variation for the trait being explained by genetic factors, and only 2 % by environmental factors. Five QTL accounted for 85 % of the genetic variation for the timing of spring bud flush. In Family 822, half-sibs to Family 331, multiple QTL were identified affecting bud set and bud flush (Frewen *et al.*, 2000). Wu (1998) detected two QTL for stem dry weight in Family 331. He found that both *P. trichocarpa,* which produces many sylleptic branches, and *P. deltoides*, in which prolepsis is prevalent, provided positive alleles for their branching patterns to the pedigree. The male and female parents of the pedigree provided positive alleles to the pedigree of height and basal area respectively (Wu, 1998).

The positions of poplar clones within an experiment can be designed to reduce competition between individuals within the plantation by placing the clones in rows of decreasing or increasing size, according to the previous season's growth increment (Wu *et al.*, 1998). Ramet position and spacing have been found to be important to competitive growth in Family 331 (Wu *et al.*, 1998) and close spacing may affect yield in *P. trichocarpa* x *P. deltoides* hybrids (Heilman and Stettler, 1990). Where spacing between

individuals is small, competition between the trees causes a higher carbon investment in growth to prevent out-competition of slower-growing genotypes. Genotypes likely to be out-competed will invest more in height growth at the expense of basal diameter (radial growth), whereas the more vigorous-growing genotypes are likely to allocate more resources to basal diameter (Wu *et al.,* 1998).

It is expected that, while some QTL will be influential in one environment, they may be unimportant in the same genotypes grown in a different environment. In the future it may be possible to detect precisely which genes play which roles in genotype x environment interactions. For example, one particular gene on one linkage group may only be functional in a certain environment (Bradshaw, 1996). While it can be advantageous to screen for forest tree traits at an early stage (by marker-aided selection), particularly owing to the long lifespan of trees (Bradshaw, 1996), nursery selection can be misleading in some characteristics.

1.4.3 Selecting genotypes for optimum biomass yield

In selecting a poplar genotype with the desirable characteristics of an energy crop, or an 'ideotype' (a genotype giving the optimum for one or more required characters in a given environment), it should be considered that many physiological factors affect yield (Dickmann and Keathley, 1996). It is unlikely that an ideotype is ever realised in a single genotype, but the ideotype provides a strong framework of the desired result around which to formulate research targets (Martin *et al.,* 2001).

The length of tree generations has hindered the progress of tree breeding by traditional methods, as seen in the limited current knowledge base of molecular biology of trees compared with agricultural crops (Tzfira *et al.,* 1998). The escalating demand for wood products, particularly as bioenergy crops, suggests a need to identify tree genotypes suitable for breeding for commercial use. The poplar Family 331, with its extensive molecular map, is an ideal candidate with scope for identifying genotypes for molecular selection. This family was unknown to the U.K. before experiments in open-top glass chambers were conducted in 1999. There is potential for investigating the F_2 Family 331 as both single stem trees and coppice in the field, focussing on finding QTL for physiological and morphological traits affecting yield.

Leaf growth is an important factor in tree stem growth, since the leaf is the organ where carbon is fixed. Thus the environmental factors influencing leaf expansion rate and the internal regulation of cell expansion to promote leaf expansion are important feature in the accumulation of stem biomass (Van Volkenburgh and Taylor, 1996). Leaf form, which varies across Family 331 (personal observation), may be the key to some yield traits.

The proportion of the year throughout which leaves are photosynthetically active, from the breaking of winter dormancy when leaves flush, until senescence in the autumn, should indicate the period of time over which a tree is accumulating carbon. Bradshaw and Stettler (1995) identified five QTL accounting for 85% of phenotypic variation in spring leaf burst in Family 331, since this trait is almost entirely (98%) controlled

genetically compared to environmentally (2%). Several QTL have also been identified in Family 822, half sibs to Family 331, influencing both autumnal bud development and spring bud flush (Frewen *et al.,* 2000).

Sylleptic branching is known to positively influence stem volume growth (Bradshaw, 1998). Sylleptic branches are formed from lateral buds which are not halted in their development (Wu and Stettler, 1994). Sylleptics respond to changes in the canopy as the crown of the tree gains height, and respond to the resulting shading by detaching from the stem. The parents of the pedigree are heterozygous for sylleptic branch production, which occurs frequently on *P. trichocarpa* (Bradshaw, 1998). Since sylleptic branches are considered a key to stem production, there should be an opportunity to investigate this both in the single stem and in coppice. There has been little investigated concerning the genetic variation of coppice productivity in *P. trichocarpa* hybrids. These hybrids have been shown to have a higher mean dry weight yield than the *P. trichocarpa* parent (Heilman and Stettler, 1990). Wu (1998) found that *P. trichocarpa* supplied positive alleles for stem height to the progeny of the pedigree, whereas the strength of *P. deltoides* was stem basal area.

Genetic analysis of this pedigree is complicated by inbreeding depression, due to the crossing of full sibs; however the large level of segregating variation in the F_2 generation suggests that QTL mapping is nevertheless possible even using the small progeny size that is available in the pedigree (Bradshaw, 1998).

Tree breeders investigating selected genotypes are interested in the economic characteristics of trees grown as short-rotation coppice (Wu, 1994), indicating that the qualities sought in Family 331 genotypes should include stem uniformity, stem height and diameter, and wood quality including yield in oven-dried tonnes. Knowledge of the genetic control of these traits would be of great value to future conventional tree breeding or genetic manipulation (Wu, 1994). Further considerations regarding growing poplars in the U.K. is their susceptibility to infection by rust such as *Melampsora* species (Cervera *et al.,* 1996) which is a serious impediment to leaf productivity. Resistance genes are present in the Family 331 pedigree in the U.S.A. (Newcombe *et al.,* 1996) and these are yet to be identified for infections of rusts in Europe.

1.5 APPLICATIONS OF PLANT PHYSIOLOGY AND GENETICS TO PLANT BREEDING

1.5.1 The ideotype concept

The length of tree generations has hindered the progress of tree breeding by traditional methods, as seen in the limited current knowledge base of molecular biology of trees compared with agricultural crops (Tzfira *et al.,* 1998). The escalating demand for wood products, particularly as bioenergy crops, suggests a need to identify tree genotypes suitable for breeding for commercial use.

Tree breeders investigating selected genotypes are interested in the economic characteristics of trees grown as short-rotation coppice. The 'ideotype' concept was originally developed by Donald (1968) who sought to construct a plant model which

would produce high yield and high quality when developed as a real genotype. An ideotype can include morphological, phenological, biochemical, silvicultural or other desired characteristics in order to tighten the focus for selecting tree genotypes (Martin *et al.*, 2001). Thus it is a framework for tree selection and breeding (Dickmann and Keathley, 1996).

1.5.2 Marker assisted selection

Marker assisted selection (MAS) is a tool which can enable selection of plant material at an early stage based on the presence of a known genetic marker at a particular locus. This is particularly useful in forest trees where the generation times may be very long for traditional breeding methods and MAS reduces the resource input required to conduct successful breeding programmes (Bradshaw and Foster, 1992). Unless a QTL has a large effect and the environmental variation for that trait has a small effect, the confidence interval for the QTL is unlikely to be less than approximately 10 cM (Kearsey, 1998). A confidence of 10cM is sufficiently accurate for MAS, even though much more accuracy would be required for positional cloning.

1.5.3 Where are we in poplar and willow?

Willow is the renewable energy crop nearest to full release in the U.K. (Bullard *et al.*, 2002). It is already grown widely on a commercial scale in Sweden, where land set aside from agricultural is being used for plantations. Willow is also being used to fuel several small-scale power plants in the U.K. (DTI, 1999). Both willow and poplar are already in use as feedstocks for energy production in the U.S.A. (Abrahamson *et al.*, 1998).

Work is currently underway to promote poplar Family 331 as a genetic standard for *Populus* research, including a number of published studies of genetic mapping of polygenic or 'quantitative' traits, controlling various aspects of *Populus* biology (e.g. Heilman and Stettler, 1990; Dunlap *et al.*, 1992; Wu, 1998).

In recent years, intensive plantations of *Populus trichocarpa* x *P. deltoides* hybrids have become widespread in the north-western United States, emphasising the strength of hybrid vigour as a characteristic of economic interest to foresters (Dunlap *et al.,* 1992). Equally, poplars grown at high densities (1000 to 10 000 stems/ha) have potential for energy production (Tabbush and Beaton, 1998). Characteristics of interest include tree height, stem diameter and total stem volume (Bradshaw and Foster, 1992).

1.5.4 Poplar as a model tree for research

Populus is increasingly becoming the tool for forestry research, or model tree (Taylor, 2002; Bradshaw *et al.,* 2000). Its geographical distribution spans the northern hemisphere and there are over 30 species of poplar (or more, depending upon how species are defined). This shows the diversity of environmental adaptations, and lends breeding potential to future research (Eckenwalder, 1996).

All members of the genus *Populus* are diploid, although one published exception exhibited triploidy: a Family 331 (F_2) individual, which is triploid and therefore sterile, having inherited the usual one allele from the male parent, but two alleles from the female. This F_2 individual had been omitted from most studies (Bradshaw and Stettler,

1993). All *Populus* species have the same chromosome number: 19. The nuclear genome is reasonably small. Its haploid genome complexity of 500 Mb is five times larger than *Arabidopsis*, around the same size as rice and tomato, six times smaller than maize and considerably smaller than most forest tree species. The genome of poplars is small relative to loblolly pine (*Pinus taeda*), a genetic model of a coniferous tree which has a genome size approximately forty times greater than *Populus* (Bradshaw, 1998). This shows the relative ease of studying *Populus* as a model for forest tree research and facilitates comprehensive genetic mapping (Bradshaw *et al.,* 1994). Molecular maps of poplar already exist for *P. trichocarpa* x *P. deltoides* hybrids (Bradshaw *et al.,*1994); *P. nigra* x *P. deltoides* (Cervera *et al.,* 1997); *P. deltoides* (Wu *et al.,* 2000a); and *P. trichocarpa, P. nigra,* and *P. deltoides* (Cervera *et al.,* 2001). Mapping of the physical sequence of poplar genome is currently in progress in the U.S.A. (Taylor, 2002).

Poplars from different sections of the genus can be easily hybridised; each pollination yields hundreds of seeds within approximately 6 to 8 weeks and this can be done on detached branches in the greenhouse (Stanton and Villar, 1996). Most interspecific hybrids are fertile, and these are usually the most important poplars commercially (Bradshaw *et al.,* 1994). Hybrids are usually fertile and their F_2 populations are normally segregating for most traits (Bradshaw, 1998). Most species and hybrids can be vegetatively reproduced. Vegetative propagation is straightforward and most species or hybrids root from cuttings (Dunlap *et al.,* 1992). This facilitates the indefinite maintenance of clonal material and is of paramount importance in poplar silviculture and in poplar genetic research.

Poplars can be easily genetically transformed and are amenable to transformation using *Agrobacterium,* direct DNA transfer, and electroporation. The first transformations were those involving genes to improve lignin/ cellulose balance in wood (Li *et al.,* 2003; Jouanin *et al.,* 2000), and in herbicide resistance (Meilan *et al.,* 2000).

Chapter 2

Leaf traits determining yield in short-rotation coppice *Salix*

2.1 INTRODUCTION

Short-rotation forestry can provide a renewable source of carbon-neutral energy (Hall and House, 1995). The genus *Salix* has been chosen as an energy crop because of its rapid growth and wood production in young shoots, which grow well following coppicing (Gullberg, 1993). Willow, in particular, is increasing in Europe as a biomass energy crop grown as coppice on a short-rotation basis (Rosenqvist *et al.*, 2000). Willows are highly productive, often providing 20 or 25 shoots from a single coppice stool (Sennerby-Forsse *et al.*,1994), reaching heights of up to 4 metres in the first three-year coppice cycle and capable of yielding up to 20 ODT ha^{-1} yr^{-1}(El Bassam, 1998). Commercial yields, however, are often considerably below this value (Taylor *et al.*, 2001). Genetic improvements are sought through breeding programmes to improve the yields of *Salix* genotypes to achieve the highest possible yields which are easily harvestable and free from disease pressures (Rönnberg-Wastlung and Gullberg, 1996; Lindegaard and Barker, 1997). It has been suggested that, owing to the slower breeding cycle in trees than in agricultural crops, it would be beneficial to find a means of early selection of genotypes using physiological traits correlated with high yield (Hansen, 1991). Such traits may then be linked to molecular markers and used in marker assisted selection, as has been the practise for crops such as maize (Mazur and Tingey, 1995).

A number of physiological traits which correspond to yield have been established in woody plants grown as short-rotation coppice. Much research has concentrated on poplar but, to date, there has been a paucity of work defining yield traits in *Salix.* Biomass estimation modelling has found that stem features such as basal diameter and height are

closely linked to total biomass yield (Verjwist and Telenius, 1999) and highly correlated to each other (Tuskan and Rensema, 1992). Willows usually produce small sylleptic branches which fall off at the end of the growing season. Sylleptic branches have been found in poplar to positively influence stem volume growth through the export of carbon photosynthates to the lower stems and roots of a plant (Scarascia-Mugnozza *et al.*, 1999). Barigah *et al.* (1994), for example, found that the most branching growth form in the study of several poplar genotypes produced the most biomass. However, this positive contribution to yield has not been consistently found in studies on willow (Rönnberg-Wastlung and Gullberg, 1996).

High positive correlations have been found between mature individual terminal leaf area and biomass productivity (Barigah *et al.,* 1994). The total leaf area of a plant is often tightly correlated with the total accumulated biomass of that plant (Larson and Isebrands, 1971). Studies have suggested that leaf area index is highly linked to, and may be estimated from, basal stem diameter measurements (Harrington and Fownes, 1993). Leaf area index is the ratio of single-surface leaf area per unit of land area (Nackaerts *et al.,* 2000).

Bullard *et al.* (2002) have reported a decrease in leaf area index through the season in relation to canopy closure and leaf loss. Rates of leaf maturation also affect leaf area index, suggesting that both individual leaf development and canopy development may be indicative of yield. A study by Ceulemans *et al.* (1988) found a trade-off between rates of leaf extension and leaf production, whereby high leaf production rates gave rise to low

leaf extension rates and therefore a slow rate of leaf maturation. Leaf epidermal cell area and the number of epidermal cells in a leaf provide some evidence of the means of final leaf area development (Van Volkenburgh, 1999). Epidermal cell number per leaf has been found to be strongly correlated with stemwood yield values in poplar (Taylor *et al.*, 2001).

Photosynthesis in the canopy can be considered to behave like a single leaf scaled-up to the whole-stand level (Kull and Niinemets, 1998). Leaf photosynthesis has been discussed as being positively correlated to biomass production in loblolly pine (*Pinus taeda*), *Larix* hybrids and *Populus* hybrids (Barigah *et al.*, 1994). In crop plants, the rate of photosynthesis has been found to be tightly correlated with plant yield such that it might be a predictor of yield (Faville *et al.*, 1999), although it is observed that in many studies there has been no correlation between these two factors (Ericsson *et al.*, 1996). CO_2 response efficiency can be measured by applying a range of carbon dioxide concentrations to the leaf surface and plotting an A/C_i curve of photosynthetic assimilation (A) against these different concentrations (C_i). The resulting curve can be used to find the gradient of two slopes. The initial slope, J_{max}, relates to the maximum rate of photosynthetic electron transport. The final slope of the curve, $V_{c,max}$, is the maximum rate of carboxylation of Rubisco (ribulose-1,5 bisphosphate carboxylase-oxygenase) (von Caemmerer and Farquhar, 1981). A further investigation of photosynthesis can be the construction of a light response curve to investigate the apparent quantum efficiency (QE) (Gill *et al.*, 1998). This can be investigated by applying differing PAR levels to the leaf surface. Taylor *et al.* (2001), using A/C_i curves

in a study of photosynthetic characteristics in five poplar genotypes, found little connection between photosynthesis and biomass yield, concluding that individual leaf-level rates of photosynthesis may not be a direct yield-defining characteristic in *Populus*.

This study seeks to establish basic photosynthetic and anatomical leaf characteristics, canopy development and stemwood traits to elucidate the physiological basis of high yield in coppice willows. A variety of genotypes has been chosen over a range of yield categories, and more detailed attention has been paid to one high- and one low-yielding genotype across four stages of coppice development.

2.2 MATERIALS AND METHODS

2.2.1 Study site and field trials

Two experimental field campaigns were undertaken in 2000 (July and September) and 2001 (monthly visits throughout the season). Measurements were made on coppiced willows at IACR-Long Ashton Research Station, University of Bristol (51°26′ N, 02°39′ W) to establish morphological and physiological bases of yield in *Salix*, with a view to enhancing the selection of willows for biomass fuels. On the basis of consistent yield data from previous research at IACR-Long Ashton (Lindegaard *et al.*, 2001), six willow genotypes (Table 2.1) were initially selected for investigation in a multi-clone study. These included reliably high, medium and low yielding varieties and, within this selection, two broadleaved cultivars. The leaves of these genotypes are illustrated in Figure 2.1. This initial study provided an overview of basic leaf and stem characteristics which were hypothesised to be correlated to yield. Following these measurements, two genotypes were selected for more detailed analysis: *S. viminalis* L. x *S. schwerinnii* E.Wolf 'Tora,' which has given consistently high yields, and *S. viminalis* L. 'L78183,' a wild genotype widely used as a reference standard in European field trials which has given consistently low yields. All trials were part of the Comparative Trials of Elite Swedish and UK Biomass Clones, established at IACR-Long Ashton between 1996 and 2000 studied as detailed in Table 2.1, using the design described by Lindegaard *et al.* (2001). The field trials had each been established from 20 cm cuttings and were maintained on a short-rotation basis.

Table 2.1 Comparative Trials of elite Swedish and U.K. Biomass clones studied at IACR-Long Ashton.

Trial (coppice stage in 2000)	Year planted	Genotypes	Yield category*
Multi-clone trial (1+2)	1996	*S. viminalis* L. 'L78183'	Low
		S. dasyclados auct. 'Aud'	Low
		S. viminalis L. 'Bowles Hybrid'	Medium
		S. dasyclados Wimmer 'Loden'	Medium
		S. schwerinii E.Wolf x *S. viminalis* L. 'Tora'	High
		S. burjatica Nas. x *S. viminalis* L. 'Aston Stott'	High
Second rotation Year 1 (1+1)	1997	*S. viminalis* L. 'L78183'	Low
		S. schwerinii E.Wolf x *S. viminalis* L. 'Tora'	High
First rotation Year 2 (0+2)	1998	*S. viminalis* L. 'L78183'	Low
		S. schwerinii E.Wolf x *S. viminalis* L. 'Tora'	High
First rotation Year 1 (0+1)	1999	*S. viminalis* L. 'L78183'	Low
		S. schwerinii E.Wolf x *S. viminalis* L. 'Tora'	High
Establishment Year (0+0)	2000	*S. viminalis* L. 'L78183'	Low
		S. schwerinii E.Wolf x *S. viminalis* L. 'Tora'	High

* Lindegaard *et al.*, 2001

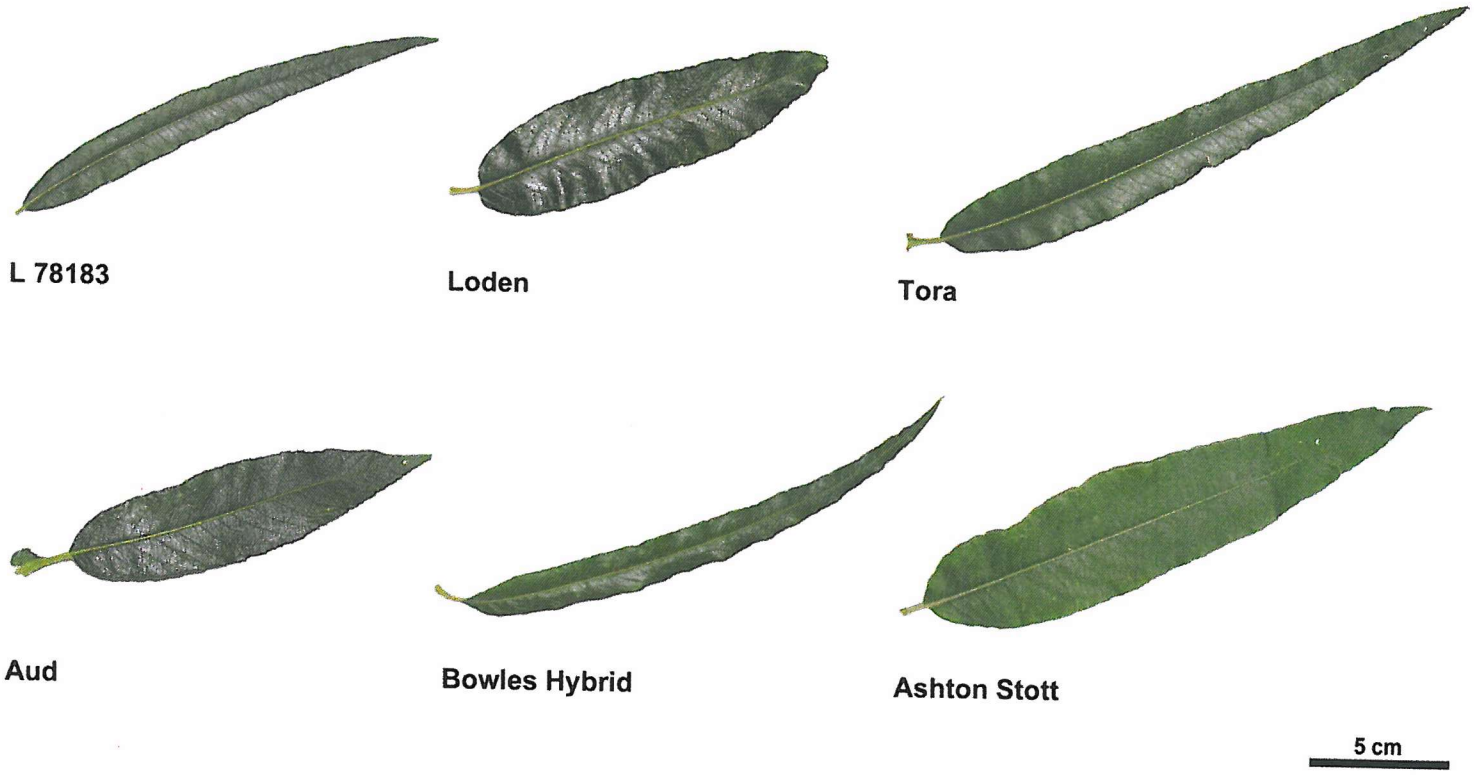


Figure 2.1 Leaf morphology of six *Salix* genotypes in the Multi-clone trial. Genotypes are arranged in yield category from low yield (left) to high yield (right). Genotype details are given in Table 2.1.

Plants were coppiced (1) after the establishment year, (2) after two further years of growth, and (3) after three further years of growth. All of the trials were polyclonal. The design of the multi-clone trial, and trials planted between 1999 and 2000, comprised of three replicate plots, each containing 52 cuttings in double rows in a 4 x 6 m area. Spacing of the central double row was 0.7 m, with 0.5 m between coppice stools within the row, and 1.3 m between double rows (Tabbush and Parfitt, 1999).The trial planted in 1997 included the same spacing between and within rows, but comprised of four replicate plots, each containing 40 cuttings in an area 3.5 x 6 m. The trial planted in 1998 included the same standard spacing but comprised of four replicate plots of 52 cuttings in a 4 x 6 m area. The developmental stages of the coppice studied ranged from an establishment year trial to the second year of the second coppice rotation.

2.2.2 Multi-clone trial

Stem measurements

Stem measurements were made in July 2000 on two stools in the central double row of each replicate genotype plot, totalling six stools per genotype. The tallest stem from each assessed stool was identified and the height of the stem recorded using a digital reading measure pole (Shenshin Industry Co., Japan). Measurements were taken from the base of the coppice stool. Basal stem diameters were measured on every stem on the stool with heights taller than 1 m using digital callipers. Maximum stem heights and diameters were calculated as a percentage of the low-yielding reference genotype, 'L78183.'

Leaf measurements

Leaves were measured in July 2000 across all six genotypes in the multi-clone trial on two stools in the central double row of each replicate plot, totalling six stools per genotype. The tallest two stems of each coppice stool were identified and from each, a representative, fully expanded leaf was collected, a total of 12 leaves per genotype. The leaf samples selected were as free as possible from damage from herbivory and mechanical damage. Petioles were removed and the leaf samples were scanned using a desk top scanner (Corel Photopaint, Corel Inc.) in TIFF (tagged information format file) format and the images analysed to obtain leaf area using Scion Image (release Beta 3b, Scion Corporation, Frederick, MD, U.S.A.). Epidermal imprints were taken using a method adapted from Ferris and Taylor (1994). At a distance of 1cm from the leaf base, an area 1.5 x 1.5 cm of the adaxial epidermis was coated with clear nail varnish and allowed to dry. Once dry, the coating was removed using clear Sellotape and transferred onto a glass microscope slide, leaving the leaf intact. Digital images were obtained of the epidermal impressions using a microscope (Axiophot 2 Universal Microscope, Carl Zeiss Jena, Germany) and digital imaging software, Metamorph (Metamorph Imaging System, Westchester, PA, U.S.A.). This software was used to obtain one digital image per slide of mature adaxial epidermal cells between the midrib and the major veins. The areas of ten randomly selected adaxial epidermal cells per slide were obtained from the digital image using Scion Image. An estimation of the number of adaxial epidermal cells per leaf was calculated for each genotype from the mean cell area and the individual leaf area. Leaf samples were dried for 48 h at 85°C and dry weights obtained. Each value for leaf area was divided by the dry weight to calculate specific leaf area.

2.2.3 Comparing a fast- and slow-growing willow: 'Tora' and 'L78183'

Stem measurements

Stem measurements were made in July 2000 on two stools in the central double row of each replicate genotype plot, totalling eight stools per genotype in the trials planted in 1997 and 1998, and totalling six stools per genotype in the trials planted in 1999 and 2000. Thus measurements were made in every plot of each trial, according to the trial design. Maximum stem height and stem diameter were measured as described for the multi-clone trial, above. The number of sylleptic branches on the leading stem of each stool was counted in June 2001 in the first year of the first coppice cycle (0+1), as these often abscise in later stages of development (Raven, 1992). Counts were made on 14 randomly selected stools in the central double row of each genotype plot, giving a total of 42 counts per genotype.

Leaf measurements

Leaves were measured in July 2000 on eight stools (16 leaves) per genotype of the trials planted in 1997 and 1998, and six stools (12 leaves) per genotype in the trials planted in 1999 and 2000. This sampling enabled measurements to be made in every plot of each trial. One leaf sample was taken from each of the two tallest stems on a coppice stool, and leaf area, adaxial epidermal cell area, estimated adaxial epidermal cell number per leaf and specific leaf area were measured as described for the multi-clone trial. Leaf extension rate was measured in September 2000 on the tallest two stems of each of eight stools per genotype in the trials planted in 1997 and 1998 and six stools per genotype in

the trials planted in 1999 and 2000. The youngest leaf with a petiole mature enough to attach a weatherproof tag to was flattened along a transparent acrylic ruler and the length (mm) measured from the leaf base to leaf tip along the midrib. This distance was measured again after a ten day period and the difference averaged over this period to give the extension rate in mm per day. This method was repeated in June 2001 in the first year of the first coppice cycle (0+1) using a larger sample size, measuring a total of 30 randomly selected young leaves across the three replicate plots. Leaf production rate was measured in June 2001 in the first year of the coppice cycle (0+1), measuring 30 randomly selected young leaves across the three replicate plots. A weatherproof tag was attached to a leaf on the leading stem of each stool near the stem apex. The number of existing leaves, excluding those which were unrolled, between the tag and the apex was counted. After seven days, the count was repeated and leaf production rates were calculated. The number of leaves on the tallest stem of the stool was counted in June 2001 in the first year of the first coppice cycle (0+1). Counts were made on 14 randomly selected stools in the central double row of each genotype plot, giving a total of 42 counts per genotype.

Leaf photosynthesis

Leaf photosynthesis was measured in September 2000 in the establishment year trial (0+0), and again in the same trial (0+1) in June 2001when A/C_i analysis was undertaken, and in August 2001 when light response curves were constructed. Measurements were made between 0800 and 1200 hours to minimise interference with diurnal patterns of photosynthesis. For the A/C_i curves, five newly expanded mature leaves from each

genotype were collected by cutting the base of the petiole from the stem and re-cutting the petioles under water. The petioles remained under water for the duration of the measurements. A known area of each leaf, 2 cm from the leaf base, was sealed into the chamber of an infra red gas analyser (LI-COR 6400 portable photosynthesis system, Lincoln, NE, U.S.A.) and exposed to varying concentrations of carbon dioxide at 20°C and 1500 μmol m^{-2} s^{-1} PAR from red and blue light emitting diodes. The data were analysed using Photosyn Assistant (Version 1.1, Dundee Scientific, Dundee, U.K.) to caluculate $V_{c,max}$ (an *in vivo* measurement of Rubsico activity, calculated as the maximum ribulose-1,5-bisphosphate (RuBP)-saturated rate of carboxylation) and J_{max} (the electron transport capacity of the thylakoid during regeneration of RuBP from Fructose 6-phosphate) as described by Von Caemmerer and Farquhar (1981). Using the infra-red gas analyser readings at ambient carbon dioxide concentrations (taken as 360 μmol mol^{-1}) stomatal conductance was recorded, and percentage water use efficiency (WUE) calculated using values of photosynthetic rate, *A* (μmol m^{-2} s^{-1}), and transpiration, *E* (μmol m^{-2} s^{-1}), thus

$$WUE = \frac{A}{E} \times 100.$$

Light response curves were measured *in situ* on three fully expanded, mature leaves per genotype, at 20 °C and using PAR from red and blue light emitting diodes starting at 1500 μmol m^{-2} s^{-1} and decreasing stepwise to 50 μmol m^{-2} s^{-1} as follows: 1500, 1350, 1200, 1000, 800, 600, 400, 200, 100, 50 μmol m^{-2} s^{-1}. The photosynthetic rate was plotted against each light level to calculate the light saturated rate of net photosynthesis

(A_{max}) and the apparent quantum efficiency (QE) from the initial slope of the light response curve, using Photosyn Assistant (Version 1.1, Dundee Scientific, Dundee, U.K.) based on the model by Prioul and Chartier (1977). The experiment was repeated using three leaves per genotype which had been excised and the petioles re-cut under water, as was the procedure for the A/C_i curves, to explore any differences in photosynthetic patterns between excised leaves and those *in situ*.

Leaf area index

Canopy analysis was conducted using an LAI 2000 Canopy Analyser (LI-COR Inc., Lincoln, NE, U.S.A.). The Canopy Analyser calculates leaf area index based on the speed of light attenuation as light passes through the canopy. Measurements are made at five angles from the zenith to elucidate the orientation of the foliage. Foliage is assumed to be black and randomly distributed to aid these calculations (LI-COR, 1992). *Salix* fits these assumptions as well as any natural plant system. Two measurements were made using the sensor: the first was above the canopy and the second was at ground level. Thus the foliar light interception was calculated based on the modelling described by Montieth (1977). Leaf area index was measured in March, April, June and August 2001 (30, 56, 103 and 176 days after 1st March 2001 respectively). Leaf area index was measured at three points along each double row of 'Tora' and each double row of 'L78183' in trials 0+2 (the second year of the first coppice rotation) and trial 1+2 (the second year of the second coppice rotation). This gave a total of nine LAI readings in trial 0+2 and 12 LAI readings in trial 1+2. To complement the LAI results obtained in the 2001 growing season, an investigation of leaf longevity was carried out in 2002. In the trial planted in

1998, which in the 2002 season was at coppice stage (1+2), 100 leaves per genotype of 'L78183' and 'Tora' were tagged on 31st May 2002 using the procedure for the leaf extension rate measurements. The tallest two coppice stems per stool were selected and, on each, the youngest leaf to which a thread could be attached was selected and tagged. A map was produced of the positions of the stools with tagged leaves. On 14th August 2002, a count was made of the number of tags remaining on the matured leaves. Where tags were absent, it was assumed that the leaves had abscised.

2.2.4 Statistical analysis

Statistical analysis was conducted using the software Minitab Release 13.1 (Minitab Inc. State College, PA, U.S.A.) and SPSS Version 11.0 (SPSS Inc.). All results were tested for normality using the Kolmogorov-Smirnov test. All results from the multi-clone trial were analysed using one-way analysis of variance. The Student-Newman-Keuls multiple comparison procedure was used to identify differences between groups. In the study comparing a fast- and slow-growing willow, results for count data were analysed using the Mann-Whitney U-test, gas exchange data, sylleptic branch number, leaf number and leaf extension and production rate data in June 2001 were analysed using one-way analysis of variance, and all other results analysed using two-way analysis of variance.

2.3. RESULTS

2.3.1 Multi-clone trial

Relative to the standard genotype, 'L78183,' maximum stem heights and mean stem diameters increased with yield category (Figure 2.2). 'Ashton Stott,' the highest-yielding genotype gained the greatest maximum stem height, whilst 'Aud,' which gave slightly lower yields than 'L78183,' was the smallest, although this limitation may have been offset slightly by greater stem diameters.

Leaf and cell characteristics in this trial (Table 2.2) reflected the varied leaf morphologies represented in Figure 2.1. Individual leaf area and mean epidermal cell area were variable between genotypes, with the broad-leaved *S. dasyclados* genotypes and 'Ashton Stott' attaining the largest leaf areas and the narrow-leaved *S. viminalis* genotypes, 'Bowles Hybrid' and 'L78183' reaching smaller final leaf areas. Specific leaf area was not significantly different between the genotypes in this trial and did not reflect the yield hierarchy in this study. The *S. dasyclados* genotypes gained the largest adaxial epidermal cell areas, producing relatively few cells per leaf, whereas 'Bowles Hybrid' and 'Tora' gained final smaller leaf areas through the production of many, small epidermal cells.

2.3.2 Comparing a fast- and slow-growing willow: 'Tora' and 'L78183'

The genotype 'L78183' was selected for this study as the lowest-yielding genotype and the standard used for many *Salix* yield investigations. The highest-yielding genotype in the multi-clone trial, 'Ashton Stott,' was not selected as the standard high-yielding genotype owing to its increasing susceptibility to infection by *Melampsora* spp. which reduces biomass yield performance; to date, 'Tora' has not been reported as rust-susceptible. The multi-clone stem diameter measurements are mirrored here, in the two-genotype comparison, particularly for maximum stem height, where values for 'Tora' were much greater than 'L78183' (Figure 2.3). In the establishment year (0+0) before the plants were coppiced, however, 'L78183' maximum stem heights were greater than those of 'Tora.' In the later coppice stages, mean stem diameters in 'Tora' were either equal to or greater than 'L78183' but the ANOVA identified no statistical difference between genotypes, only between trials. 'Tora' showed an abundance of sylleptic branches on the leading stem relative to 'L78183' which had much lower counts of sylleptics, shown in Figure 2.4.

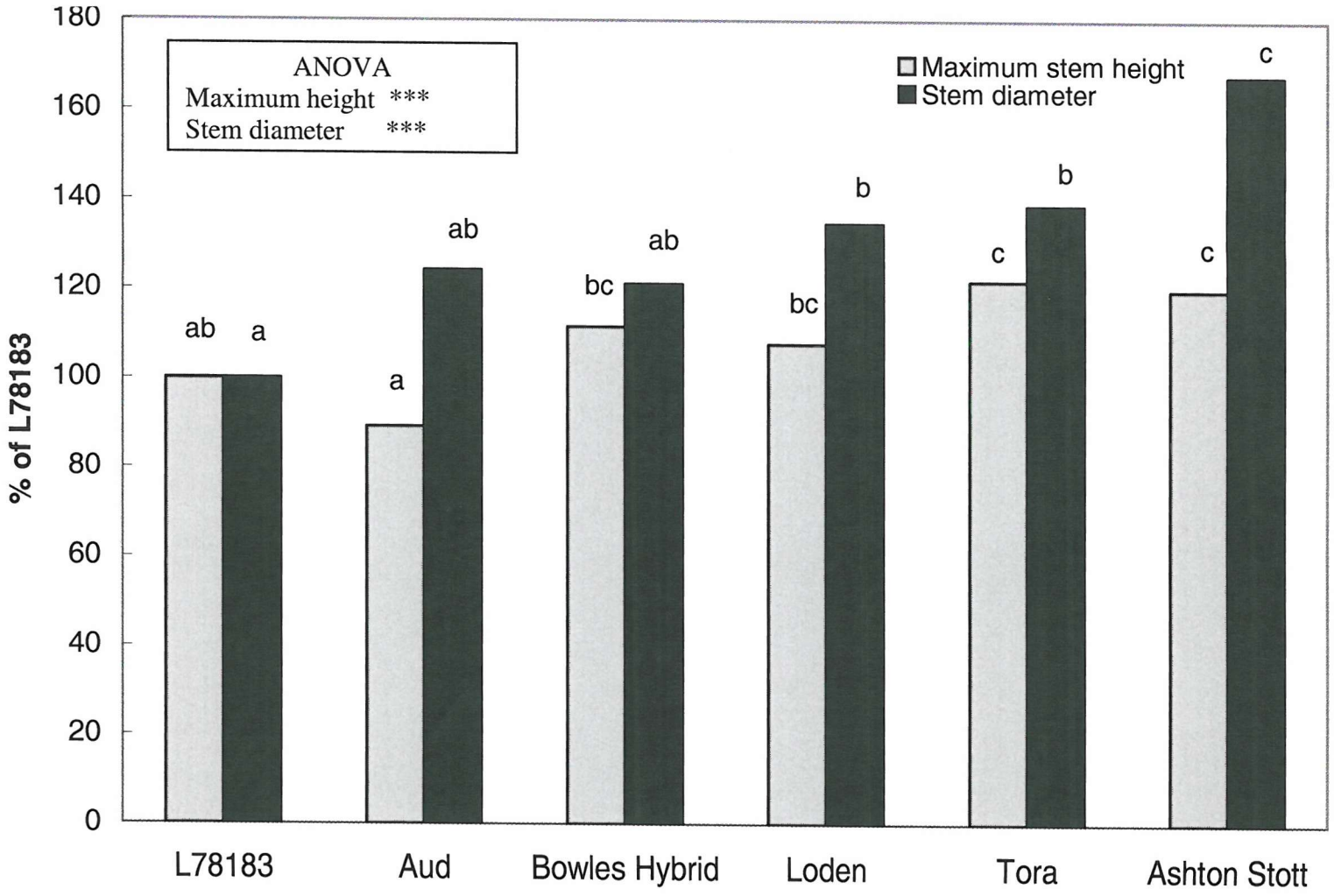
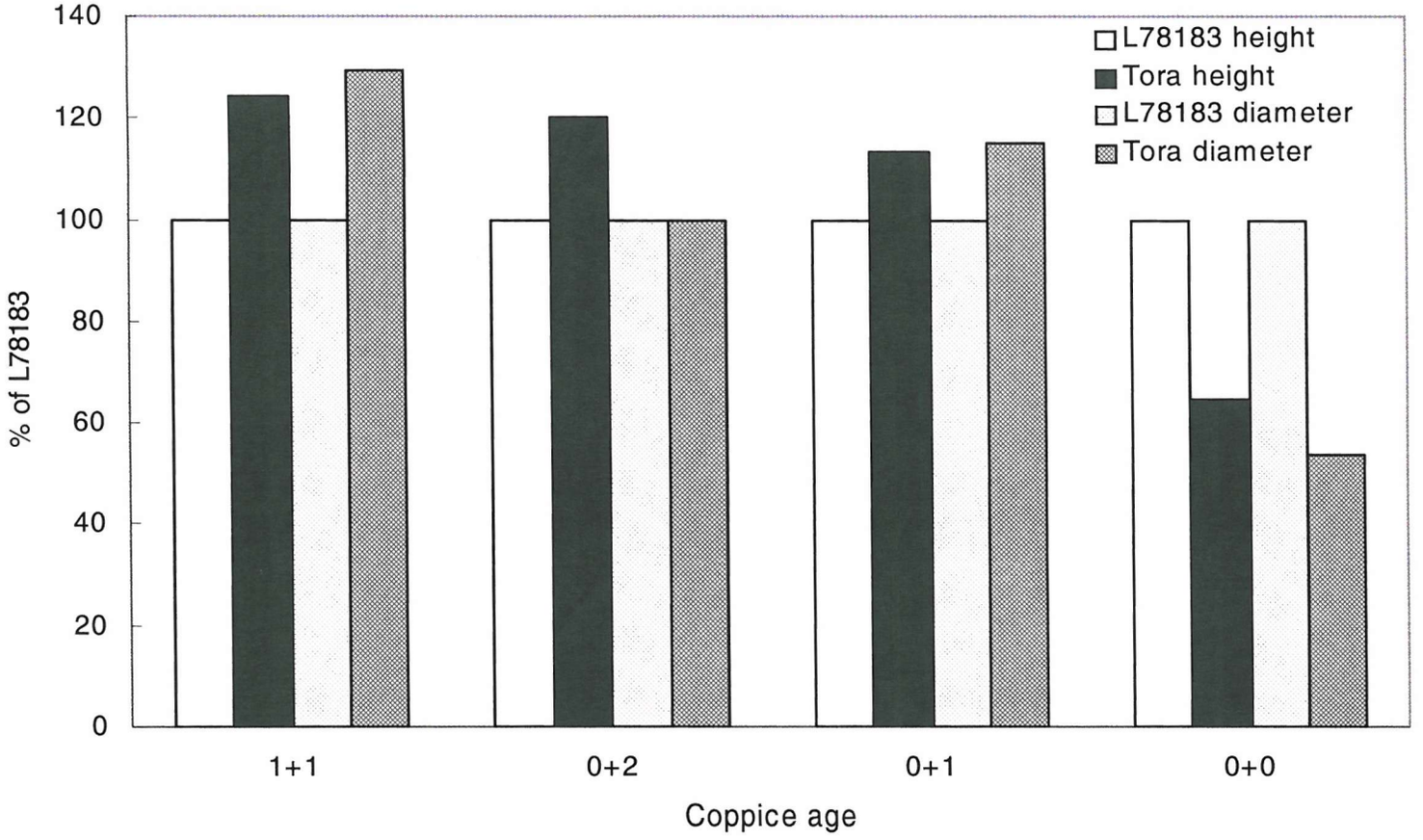


Figure 2.2 Mean maximum stem heights and mean stem diameters of six *Salix* genotypes in the multi-clone trial, expressed as a percentage of height and diameter of reference low-yielding genotype, *S. viminalis* 'L78183.' Measurements were made in July 2000 when the trial age was the second year of the second rotation (1+2). Each bar represents a mean of six coppice stools. Letters denote Student Newman-Keuls post-hoc groupings, with different letters showing a significant difference.

Table 2.2 Leaf characteristics of low, medium and high yielding willow genotypes and comparisons of 'Tora' (high yield) and 'L78183' (low yield). Standard error values are shown in parentheses. Analyses of variance are also shown. Letters represent Student-Newman-Keuls post-hoc groupings.

Trial	Genotype	Final individual leaf area mm^2	Specific leaf area $mm^2 g^{-1}$	Epidermal cell area μm^2	Epidermal cell number per leaf $x10^6$
Multi-clone trial	L78183	2146 (140) [a]	14 361 (367)	812.2 (122) [b]	2.942 (0.349) [a]
	Aud	4239 (312) [bc]	12 645 (598)	1389.0 (113) [c]	3.274 (0.401) [a]
	Bowles Hybrid	2060 (220) [a]	12 129 (226)	410.9 (27.0) [a]	5.308 (0.818) [b]
	Loden	3799 (331) [bc]	14 643 (1716)	1249.2 (57.9) [c]	3.056 (0.222) [a]
	Tora	3117 (336) [ab]	12 673 (1157)	585.1 (55.0) [a]	7.144 (0.833) [c]
	Ashton Stott	4573 (574) [c]	14 923 (1345)	905.8 (66.8) [b]	4.543 (0.564) [ab]
ANOVA		***	ns	***	***
Tora versus L78183					
Second rotation Year 1 (1+1)	L78183	2715 (105) [c]	14 763 (464) [ab]	683.3 (37.2) [b]	4.414 (0.340) [c]
	Tora	3856 (111) [d]	19 319 (491) [c]	696.9 (22.7) [b]	5.644 (0.279) [d]
First rotation Year 2 (0+2)	L78183	2274 (146) [b]	17 449 (2 107) [ab]	1044.4 (83.7) [c]	2.588 (0.335) [b]
	Tora	2916 (178) [c]	14 449 (634) [a]	553.6 (30.9) [a]	5.513 (0.478) [d]
First rotation Year 1 (0+1)	L78183	1996 (102) [b]	15 789 (247) [ab]	683.1 (32.9) [b]	3.061 (1.698) [b]
	Tora	3001 (98.3) [c]	18 478 (634) [bc]	574.9 (12.9) [a]	5.284 (1.967) [d]
Establishment Year (0+0)	L78183	1152 (80.4) [a]	14 082 (1 801) [a]	710.6 (35.9) [b]	1.558 (0.10) [a]
	Tora	1151 (130) [a]	17 257 (338) [ab]	762.4 (27.0) [b]	1.553 (0.199) [a]
ANOVA	Coppice age	***	ns	***	***
	Genotype	***	**	***	***
	Coppice age x genotype	***	**	***	***

Comparisons of leaf characteristics between 'Tora' and 'L78183' are shown in Table 2.2. Individual leaf areas were larger in 'Tora' than 'L78183' except in the establishment year trial (0+0) where the the genotypes did not differ. Epidermal cell areas were larger in 'L78183' than 'Tora' in both the first (0+1) and second (0+2) year of the first rotation. In the remaining two trials (1+1 and 0+0), cell areas for the two genotypes were in the same post-hoc groups, indicating that they did not differ significantly. Epidermal cell numbers per leaf were consistently higher in high-yielding 'Tora' than low-yielding 'L78183,' except in the establishment year coppice age 0+0, where the values for the two genotypes were very similar. This matches the Multi-clone trial result. Specific leaf area differed between the two genotypes, but not between trials, with 'Tora' producing thinner leaves than 'L78183.'



ANOVA			
Height	**	Diameter	ns
Stage of development	***	Stage of development	***
Height x stage of development	**	Diameter x stage of development	***

Figure 2.3 Mean maximum stem heights and mean stem diameters of *S. viminalis* 'L78183' (low yield) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield), expressed as a percentage of reference genotype *S. viminalis* 'L78183.' Measurements were made in July 2000 when the trials were in the first year of second rotation (1+1), the second year of first rotation (0+2), first year of first rotation (0+1) and the establishment year (0+0). Each bar represents a mean of at least 6 coppice stools.

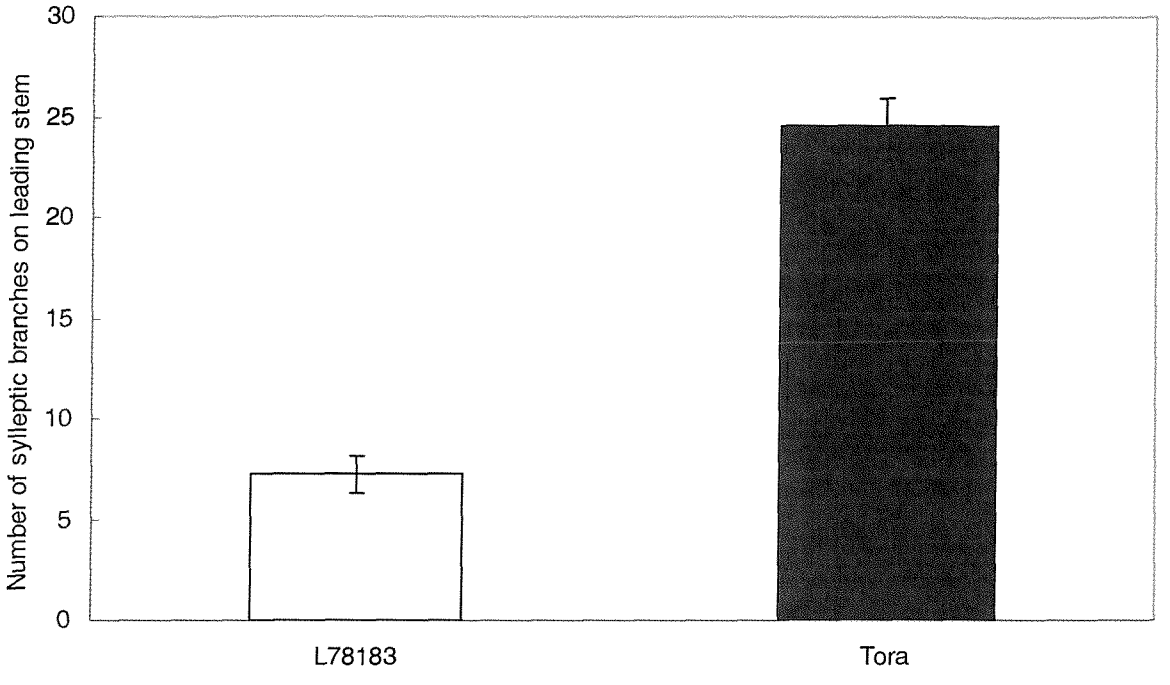


Figure 2.4 Number of sylleptic branches on the leading stem of *S. viminalis* 'L78183' (low yield) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield). Measurements were made in June 2001 in the first year of the first coppice rotation (0+1). Each bar represents a mean of 42 coppice stools. (Mann-Whitney U-test: U = 950.5, $P < 0.001$). Error bars represent s.e. values.

Leaf extension rates in September 2000 were highest for 'Tora' across all coppice ages (Figure 2.5), with the greatest difference observed between the two genotypes in the establishment year (0+0). This result was confirmed in June 2001 in the same trial following coppice (0+1), as shown in Figure 2.6. The rate of leaf production measured in June 2001 in the same trial (0+1) was also greatest in 'Tora.'

Canopy analysis provided leaf area index estimates in the second year of the second rotation (1+2) and the second year of the first rotation (0+2), illustrated in Figure 2.7. In both trials, 'Tora' produced a thinner canopy than 'L78183,' gaining maximum LAI values of 1.43 in June, after 103 d. After this point, the canopy continued to develop in 'L78183' to reach 2.06 at coppice age 1+2 in August, after 176 d, and 1.67 at coppice age 0+2. This continuing development contrasted with 'Tora' in which, over the same period, LAI increased only slowly in coppice age 0+2 and decreased in coppice age 1+2. The higher LAI values in 'L78183' are reflected in the leaf count in June 2001 (Figure 2.6), in which 'L78183' had the greater number of leaves on the leading stem. The preliminary observation of leaf longevity in 2002 found that over the period of investigation, 76% of the tagged leaves had abscised from 'Tora,' whereas only 33% of the tagged leaves had been lost from 'L78183.'

Photosynthesis measured in the establishment year (0+0) showed clear differences in photosynthetic efficiency between the two genotypes, wherein both light response curves showed the higheat A_{max} values for 'Tora' (Figure 2.8), and $V_{c,max}$ and J_{max} were higher in 'L78183' than in 'Tora' (Figure 2.9).

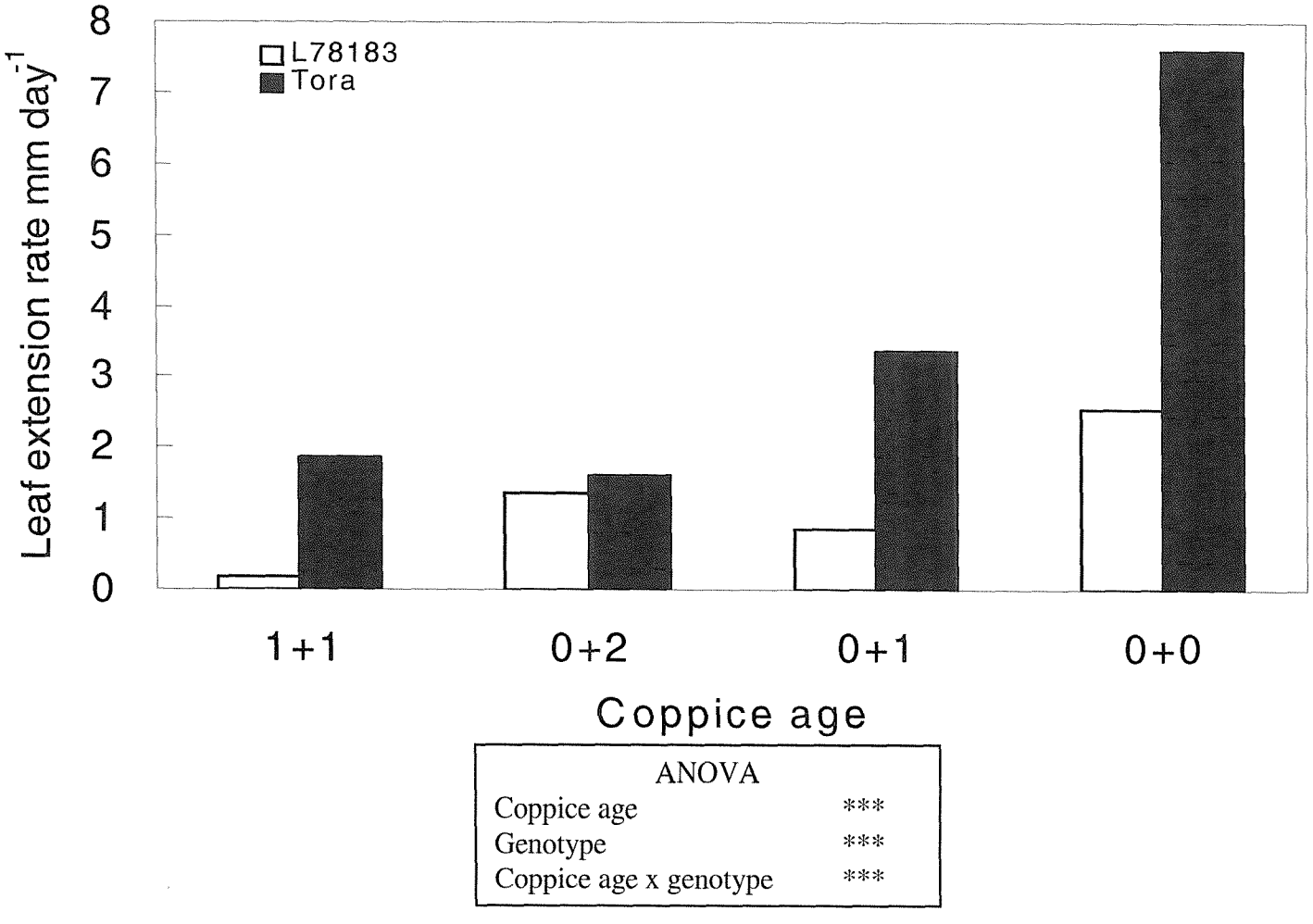


Figure 2.5 Leaf extension rates of *S. viminalis* 'L78183' (low yield) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield) measured in September 2000, when the trials were in the first year of second rotation (1+1), the second year of first rotation (0+2), first year of first rotation (0+1) and the establishment year (0+0). Measurements were made on the youngest leaf of the leading stem of each coppice stool. Each bar represents a mean of at least six coppice stools.

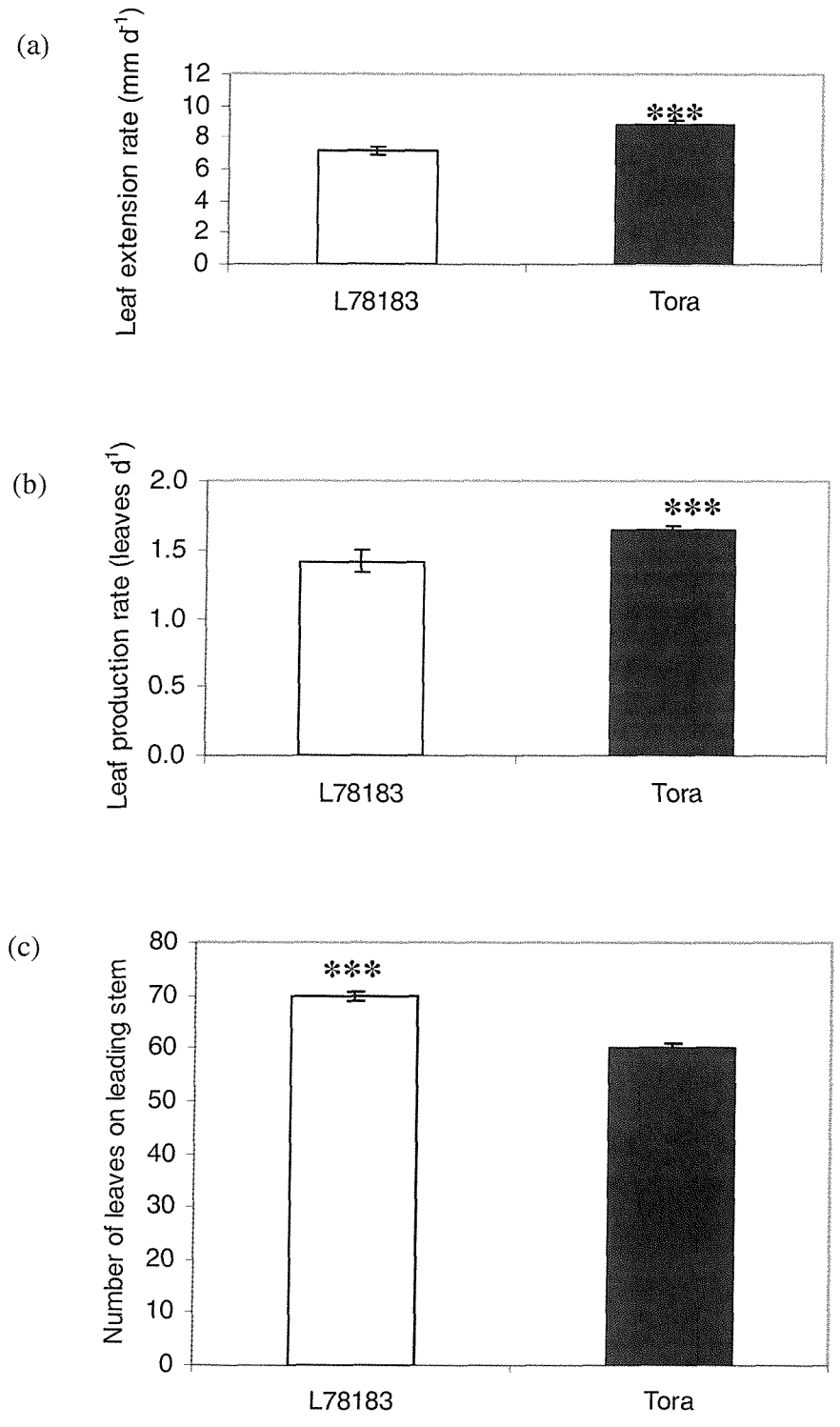
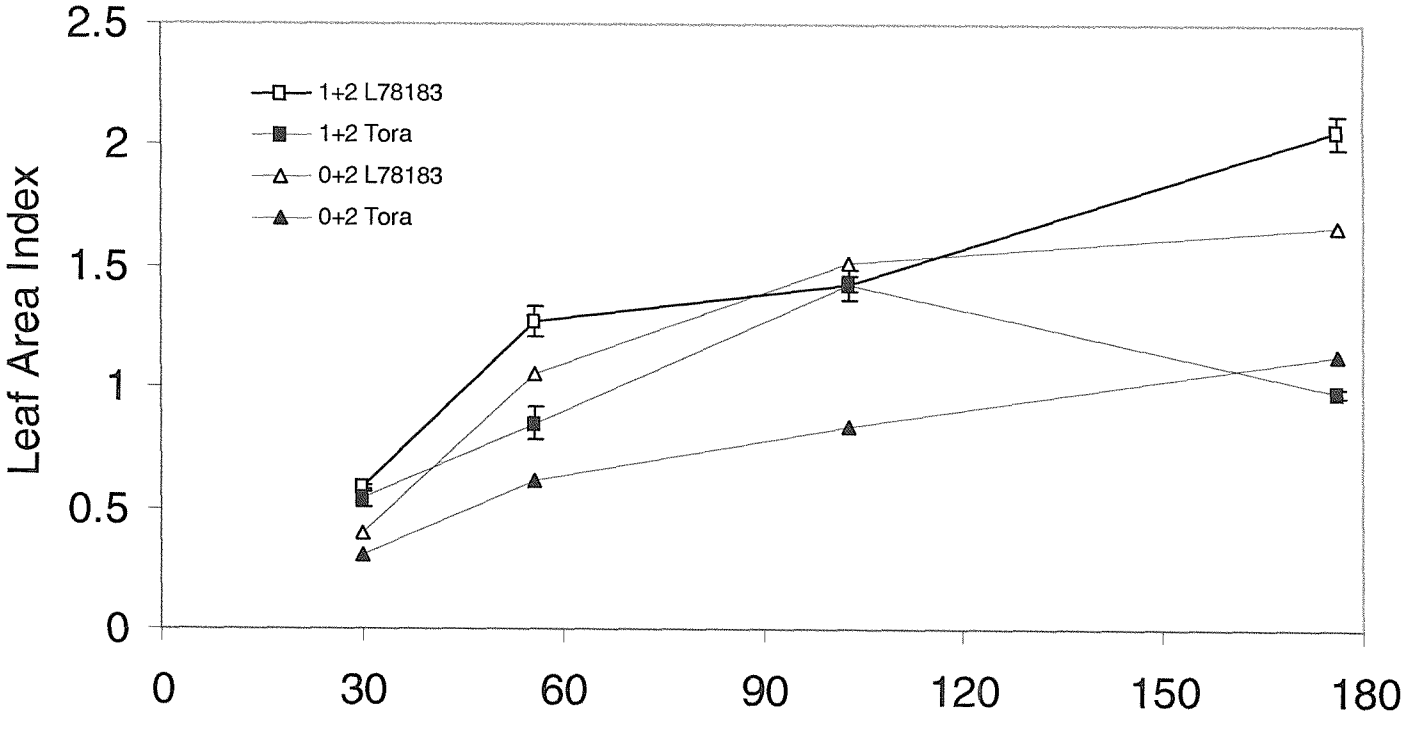


Figure 2.6 Leaf growth characteristics of *S. viminalis* 'L78183' (low yield) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield) measured in June 2001 in the first year of the first coppice rotation (0+1). (a) Leaf extension rate measurements of the youngest leaf of the leading stem of each coppice stool. Each bar represents a mean of at least 30 coppice stools. (b) Leaf production rate measurements on the leading stem of each coppice stool. Each bar represents a mean of 30 coppice stools. (c) Number of leaves on the leading stem. Each bar represents a mean of 42 coppice stools. (Mann-Whitney U-test: U = 2477, $P < 0.001$). Error bars represent se. values.



ANOVA (at 176 d)	
Coppice age	***
Genotoype	*
Coppice age x genotype	*

Figure 2.7 Development of leaf area index in *S. viminalis* 'L78183' (low yield) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield) measured at four time-points throughout the 2001 growing season in trials at two developmental coppice stages. Readings were taken in the second year of the second rotation (1+2) and the second year of the first rotation (1+2). Each bar represents a mean of at least 27 readings per genotype. Error bars represent s.e. values.

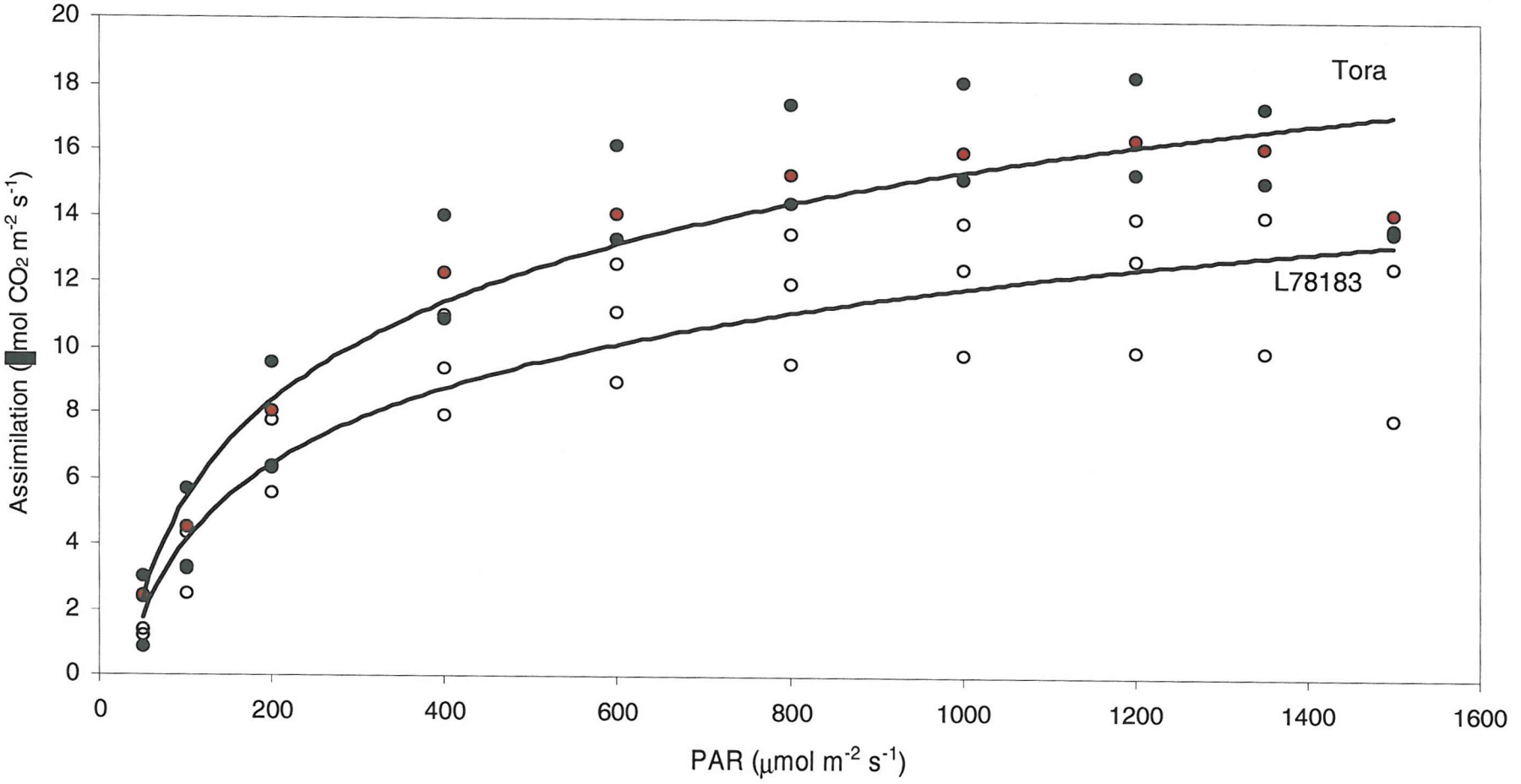


Figure2.8 Light response curves of *S. viminalis* 'L78183' (low yield, open symbols) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield, closed symbols) measured in August 2001 in the coppice age 0+1. Each curve represents a mean of three curves per genotype.

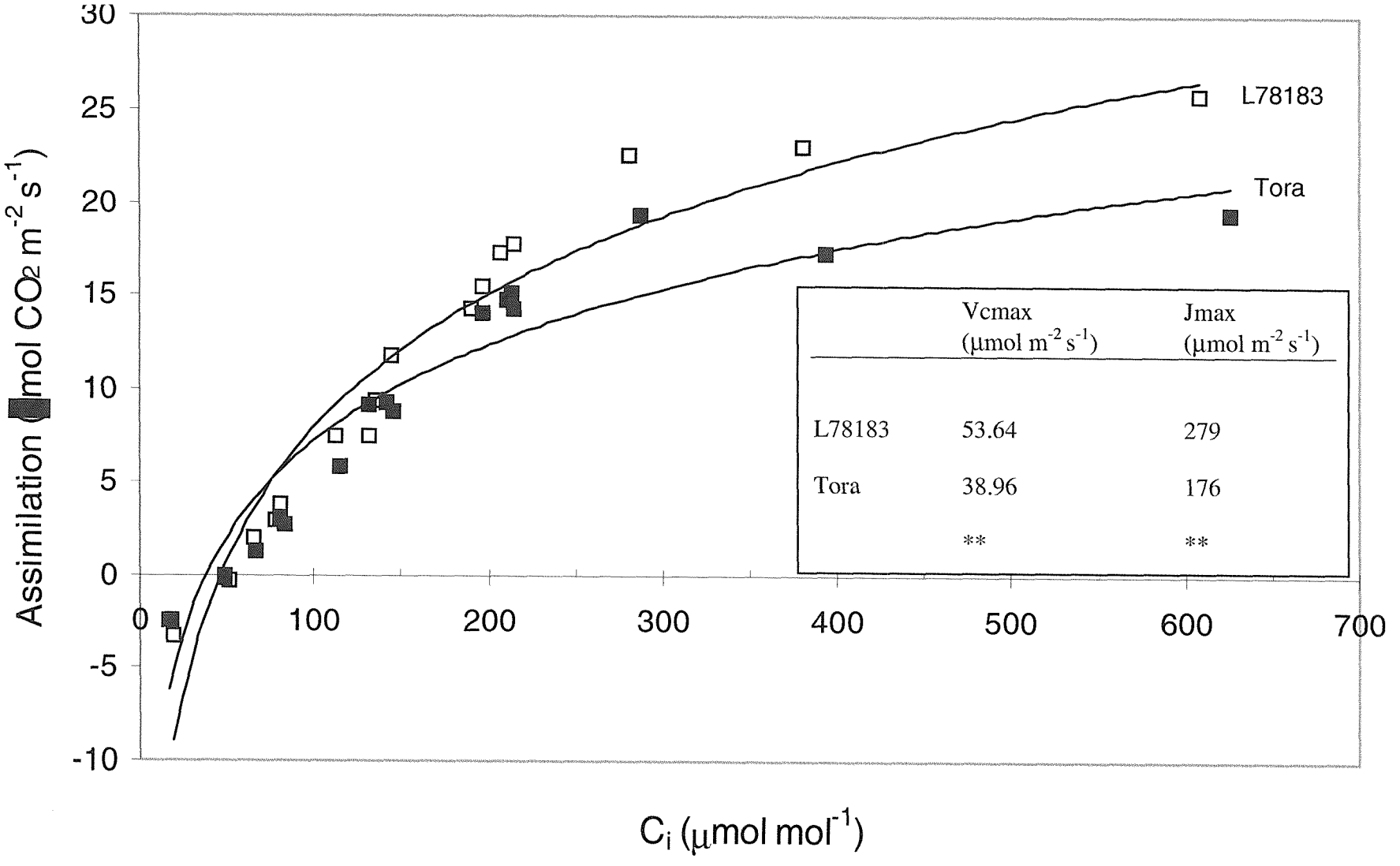


Figure 2.9 The relationship between photosynthesis, A, and intercellular carbon dioxide concentration, C_i, in a typical leaf of *S. viminalis* 'L78183' (low yield, open symbols) and *S. schwerinnii* x *S. viminalis* 'Tora' (high yield, closed symbols) measured in September 2000 in the establishment year (0+0). The inset shows values of the maximum RuBP-saturated rate of carboxylation *in vivo*, $V_{c,max}$, and the electron transport efficiency of the thylakoid, J_{max}, shown as a mean value of five measurements.

The gas exchange characteristics, assimilation rate (A), stomatal conductance (g_s), transpiration, and instantaneous water use efficiency (WUE) are summarised in Table 2.3. None of the parameters photosynthesis, transpiration, stomatal conductance (g_s), or WUE differed significantly between the two genotypes; WUE values were very similar between the two. The values of A_{max} were higher in 'Tora' than 'L78183' however the values of QE differed little. Although too few replicate measurements were made to allow t-tests on light response data, the curves for 'Tora' and 'L78173' were spatially different in the graph (Figure 2.8). This suggests that higher replication could show distinct differences between the two genotypes.

Table 2.3 Gas exchange measurements of *S. viminalis* 'L78183' (low yield) and *S. schwerinii* x *S. viminalis* 'Tora' (high yield) measured at IACR-Long Ashton in September 2000. Values represent the mean of five measurements, with the exception of A_{max} and QE values which are the mean of three measurements and which have too few replicates to qualify for ANOVA or t-tests.

	L78183	Tora	One-way ANOVA
Photosynthesis (μmol m^{-2} s^{-1})	20.94	20.6	ns
Transpiration (mmol)	6.69	6.33	ns
Stomatal conductance (mmol m^{-2} s^{-1})	609.2	511.6	ns
WUE (%)	32.5	31.5	ns
$V_{c,max}$ (μmol m^{-2} s^{-1})	53.64	38.96	**
J_{max} (μmol m^{-2} s^{-1})	279	176	**
A_{max} (μmol m^{-2} s^{-1})	13.29	16.27	-
QE (μmol CO_2 μmol^{-1} incident photon)	0.0361	0.0416	-

2.4 DISCUSSION

Commercial breeding programmes aim to produce biomass willows with large amounts of above ground biomass, in the form of few stems with a large diameter, or many stems with a small diameter (Lindegaard and Barker, 1997). Results here have confirmed that height and diameter are indicative of yield (Verjwist and Telenius, 1999). This result was confirmed for the trials comparing 'L78183' and 'Tora' across a variety of coppice ages. In this investigation, sylleptic branching was more abundant in 'Tora' than 'L78183,' supporting the notion that these branches are important for optimised above-ground productivity in *Salix.*

The leaf characteristics of the six genotypes studied (Table 2.2) show the diversity of leaf shapes and sizes present in this, a small cross-section of *Salix* species and hybrids grown for biomass. Although large leaf areas were found in broad-leaved *S. dasyclados* genotypes of low and medium yield, the *S. viminalis* x *S. burjatica* hybrid, 'Ashton Stott,' the highest yielding genotype (Lindegaard and Barker, 1997), had the largest individual leaf areas, further supporting that leaf area traits can be used to predict final stemwood yields. Final leaf area is obtained through the processes of cell division and cell expansion. The upper epidermis is the most appropriate tissue layer for these measurements because it is here that cell division ceases first during growth and because it is this layer of cells that 'limits' the expansion of the leaf (Dale, 1988). This study suggests that adaxial epidermal cell number per leaf may be more indicative of yield than the individual epidermal cell area, which has also been found in *Populus* (Taylor *et al.,* 2001). The *S. viminalis* x *S. schwerinii* hybrid, 'Tora' had larger individual leaf areas than 'L78183, ' which corresponds with other published results (Weih, 2001). If future willow breeding could concentrate on optimising such hybrid

vigour to combine traits of large cell area and many cells per leaf, larger leaf areas may result. The hybridisation of *Populus trichocarpa,* which produces few, large epidermal cells, and *P. deltoides,* which produces many, small cells, for example, has created F_1 genotypes superior in individual leaf area through the production of many, large epidermal cells (Ridge *et al,* 1986). If, indeed, large individual leaf area is linked to high yield in *Salix* as in other species (Barigah *et al.,* 1994; Larson and Isebrands, 1971), this could result in some higher-yielding clones for the biofuel industry. The achievement of this would be dependent on the new genotypes meeting the selection criteria of frost-tolerance, pest and disease-resistance, and the shape ideotype for mechanised harvesting (Larsson, 1998).

This study has found the leaf extension rates across the four coppice ages in the 2000 campaign and in the 2001 growing season, to be greater in 'Tora' than 'L78183.' The maximum length of 'Tora' leaves was 20 cm in this trial (K.M. Robinson, personal observation), which at extension rates of 7.6 mm d^{-1} from an initial measurement of between 3 and 4 cm suggests that 'Tora' leaves could reach maturity after an estimated five days from emergence. This approximation could be investigated in studies of leaf development. Leaf production rate measured in the 2001 campaign was also greater in 'Tora' than 'L78183,' which differs to the observation that fast extension rates accompany low production rates (Ceulemans *et al.,* 1988). These findings show that 'Tora' appears to be constrained in neither trait. This fast growth may give rise to a higher turnover of leaves in the canopy. At any one time, there may be between 30 and 50 expanding leaves in the expanding leaf zone on a *Salix* plant, and leaf area production is an established indicator of biomass production (Isebrands *et al.,* 1996). Genotypes of *Salix* have been found to have similar minimum life lengths whereas the

maximum leaf longevity and the rate of leaf production differ (Porter *et al.,* 1993). A typical willow leaf longevity is between two and three months. The Salicaceae lose lower leaves first, retaining only the upper leaves until the end of the growing season (Ceulemans *et al.,* 1996a). Likewise, Bullard *et al.* (2002) observed that the total leaf area of willow coppice was situated entirely in the top two metres of the canopy, with competition accounting for leaf loss at lower levels. This accounted for a decrease in LAI through the season following maximum canopy closure. In contrast to higher rates of leaf expansion and production, lower canopy leaf area indices of 'Tora,' compared to 'L78183, were found in this study (Figure 2.7). 'L78183' was also found to be the 'leafier' of the two genotypes (Figure 2.6). Leaf litter was also richer on the ground beneath the 'Tora' canopy than the 'L78183' canopy in the 2001 season (data not shown) suggesting that fast expansion and production of leaves in 'Tora' was associated with faster leaf loss. This suggestion was further explored in the 2002 season, when the leaf longevity investigation supported the idea that 'Tora' does have a greater rate of leaf loss. LAI results in this experiment were comparable to other leaf area index measurements in *Salix* at similar planting densities (Bullard *et al.,* 2002; Cannell *et al.,* 1987), so it seems likely that low LAIs in 'Tora' represent a faster leaf turnover in this species. Any differences in LAI may be affected by the genotypes studied or the coppice age and planting designs. Canopies of *Salix* are as efficient as most other C_3 crop plants at utilising intercepted solar radiation (Cannell *et al.,* 1987). Gower and Norman (1991), using the Plant Canopy Analyzer, detected slight over-estimations of LAI where defoliated branches were present, however coppice stems are unlikely to interfere with LAI readings since they intercept very little light relative to leaves. Given the design of the trials, with relatively narrow plots, the LAI readings may be slightly under-calculated owing to incoming light from the trial edges, as discussed by Casella

and Ceulemans (2002). However, short-rotation coppice willows exhibit similar temporal peaks in LAI in Sweden to those found in this study, with maximum values in August in mature trials, and July in young trials (Isebrands *et al.,* 1996).

Leaf-level photosynthetic efficiency values derived from A/C_i analysis did not appear to be the answer to differences in yield accumulation in these two genotypes, which follows the finding of Taylor *et al.* (2001). Values of J_{max} and $V_{c,max}$ were higher in the low yielding genotype than in the high yielding genotype. Although carbon fixation per unit of leaf area is a major factor of final dry matter production, with workers such as Faville *et al.* (1999) observing net photosynthesis measurements were tightly correlated with yield, it is often only weakly correlated with high yield in any plant breeding programmes (Lawlor, 1995). Ericsson *et al.* (1996) also suggested that photosynthesis per unit leaf area is no higher in high yielding crop varieties than in lower yielding predecessors and instead considers light havesting and subsequent within-plant carbon allocation to be the key to yield. These data would confirm this assertion. Light ultimately provides the energy for all metabolism in plant tissue, with the maximum amount of dry matter accumulation in the plant being closely associated with the amount of light intercepted by the foliage (Montieth, 1977). Isebrands *et al.* (1996) advocate this point, indicating that light harvesting is important in crop productivity and that light response curves are highly informative about the photosynthetic capacity of leaves. Increases in irradiance lead to increases in the potential rate of electron transport in photosynthesis (Wong *et al.,* 1979). In the light response curves constructed in this study, high-yielding 'Tora' achieved higher A_{max} values, with higher values of net photosynthesis than 'L78183' at saturating light levels, despite only a small difference between the genotypes in apparent quantum efficiency.

Where there is sufficient nitrogen for metabolism, senescence of leaves is reduced and slowed down, increasing individual leaf longevity (Lawlor, 2002). Weih (2001) found 'Tora' to have higher foliar nitrogen content than 'L78183.' It was also found (Weih, 2001) that although 'Tora' had a lower WUE, its use of nitrogen was not compromised. Furthermore, instantaneous leaf level WUE did not differ between the two genotypes and stomatal conductance did not differ significantly. Instantaneous WUE (A/E) may be important in short-rotation coppice willow plantations which are rarely irrigated (Lindroth and Cienciala, 1996), but neither genotype in this study appeared to be either outstanding or deficient in this capacity.

In conclusion, the findings in this study confirm that stem traits recognised as yield indicators still hold, but in addition the data suggest that more attention could be paid to resource allocation from sylleptic branches in *Salix*, if this branching growth form would be practical for mechanised maintenance and harvesting. The carboxylation efficiency of Rubisco and the maximum rate of electron transport in the thylakoid may be less important and useful as a yield indicator than the harvest and utilisation of light and simple morpho-physiological measurements of leaf production, extension and longevity. Furthermore, willow breeding for yield may benefit from crosses concentrating on leaf area characteristics involving the production of many, large epidermal cells.

Chapter 3

The genetic determination of yield in short-rotation coppice *Populus*

3.1 INTRODUCTION

Hardwood trees grown as short-rotation coppice crops have the potential for wider use as a source of carbon-neutral renewable energy (El Bassam, 1998). Fast-growing *Populus* hybrids are continually being developed as biofuels owing to their rapid growth accumulation and excellent coppice performance. Poplar biomass yields can reach 28 ODT ha^{-1} yr^{-1}. However, due to the slow process of tree breeding and the pressures of diseases such as rust (*Melampsora* spp.) and canker (*Xanthomonas*), there is demand for genetic material matching the coppice poplar 'ideotype' (Dickmann & Keathley, 1996). Overall biomass gain in woody coppice systems is a function of many internal plant processes and their interaction with the environment. These can be studied by focussing at several levels – at the biochemical, leaf, and canopy levels as well as considering the processes contributing to yield within a stand. Previous investigations into the determinants of poplar yields have involved detailed measurements on a small selection of clones (Ceulemans and Deraedt, 1999). Such work has highlighted easily-measured potential yield traits which can be tested for applicability across larger, more diverse populations.

The practice of coppicing involves cutting stems back to the base at the end of one year, to encourage the regrowth of multiple stems in the next, enabling more than one harvest from the original rootstock (Abrahamson *et al.*, 1998). Genotypes selected for short-rotation coppice should ideally grow with near equal vigour between each successive harvest (Sims *et al.*, 1999). Sims *et al.* (2001) compared dry mass yields in four tree genera grown for three years as single stems, followed by a three year period as coppice, in which poplar showed consistently higher yields as coppice.

Moisture content in stems has been found to vary throughout different height sections of growing poplar and willow stems. Telenius (1997a) calculated differences between height sections of leading stems to vary from 1% to 9% between sections of the leading part of the shoot. This has led stemwood harvests to be expressed in units of dry mass. Many growth parameters are measured to gain an insight into biomass gain at the time of harvest, including wood volume, leaf area estimations and wood dry weight (e.g. Verwijst and Telenius, 1999; Telenius, 1997b; Barigah *et al.,* 1994).

Tree stem height and stem diameter are strongly correlated in poplar. Close relationships have been established between basal area (or diamter) and tree weight (Heilmann and Xie, 1993). This relationship facilitates the measurement of basal stem diameter for biomass estimation rather than height, particularly in dense plantations where the top of the stem is distant and not visible (Verwijst and Telenius, 1999). Sylleptic branching has been found to positively influence stem volume growth (Bradshaw, 1998). Poplars form two types of branch from the main stem, as summarised by Ceulemans (1990). Sylleptic shoots are formed from a new lateral axis, the apical meristem which has grown continuously without a period of dormancy. In contrast, proleptic shoots are new lateral shoots which develop following a period of dormancy in the apical meristem of that axis. Vast diversity in the phenology of branch development, the abundance of lateral branches and their presence or absence exists between poplar genotypes. Scarrascia-Mugnozza *et al.,* (1999) found sylleptic (late season) branches in *Populus* to export carbon photosynthates to the lower stem and roots of the plant. In a study by Barigah *et al.* (1994), for example, the most branching growth form in the study of several poplar genotypes produced the most biomass. Sylleptic branches respond to changes in the canopy as the crown of the tree gains

height, and respond to the resulting shading by detaching from the stem. The number of stems produced by a rootstock, or stool, is variable. Studies of five genetically different hybrids of poplar, for example, produced between 3 and 8 stems per stool during the first coppice cycle (Herve and Ceulemans, 1996). Several studies report tree height to be highly positively correlated to the diameter of the coppice shoots. Tuskan and Rensema (1992), for example, calculated a correlation of 0.96 for these traits in poplar.

As the site of light interception and carbon fixation, leaf growth is an important factor in tree stem growth. Thus, in the processes determining leaf growth, both the environmental factors influencing leaf expansion rate and the internal regulation of cell expansion to promote leaf expansion are important features in the accumulation of final stem biomass (Van Volkenburgh and Taylor, 1996). Equally, the vast diversity of leaf forms across the *Populus* genus is due to underlying physiological processes, which in turn are controlled by both genes and the environment. Final leaf area will be determined by factors such as the rate of growth of an individual leaf, the rate of production of leaves on the plant, and the duration of growth throughout the season (Ridge *et al.,* 1988). The breaking of winter dormancy through to bud set and autumn senescence combine to indicate the period of time over which a tree is accumulating carbon (Frewen *et al.,* 2000). The proportion of the year throughout which leaves are photosynthetically active is closely related to stem biomass accumulation (Michael *et al.,* 1990). Work by Nelson (1985) suggested that mature leaves produce the most photosynthates for the production of woody biomass. This has led to the suggestion that, given an avoidance of damage by early frosts, long periods of leaf retention and photosynthetic activity in an extensive mature canopy may be an important selection criterion. Rapid rates of individual leaf maturation may give rise to a mature canopy

early in the growing season. A study by Ceulemans *et al.* (1988) found a trade-off between leaf extension rate and leaf production rate in poplar: high leaf production rate values gave rise to low rates of leaf extension and therefore a slow rate of leaf maturation. It has been observed (Ceulemans, 1990) that hybrids of *P. trichocarpa* and *P. deltoides* exhibit much greater overall rates of leaf growth than any European or American native *Populus* species or any Euramerican hybrids. Such hybrids also have characteristically large individual leaf areas (Barigah *et al.*, 1994).

Both total leaf area and individual leaf area have been shown to be strongly correlated to total biomass production in poplar hybrids. This suggests that fast-growing, large leaves lead to increased stem biomass (Barigah *et al.*, 1994). The genotypes in the study with the highest biomass yields were also those with the largest individual leaf area and largest leaf areas per tree. Tight positive correlations ($r = 0.91$) have been found between total leaf area and stem volume (Ridge *et al.*, 1986). Furthermore, Harrington *et al.*, (1997) found a high positive correlation between mature individual leaf area and the relative growth rates in poplar genotypes. Leaf area has also been related to biomass sufficiently for Harrington and Fownes (1993) to produce an equation to estimate total biomass and leaf area index from basal diameter measurements.

Final leaf area is obtained through the processes of cell division and cell expansion. The upper epidermis is the most appropriate tissue layer for these measurements because it is here that cell division ceases first during growth and because it is this layer of cells that 'limits' the expansion of the leaf (Dale, 1988; van Volkenburgh, 1999). The area and number of epidermal cells in a leaf are evidence of the pattern of leaf growth. In *P. trichocarpa*, the cell areas are large, suggesting the final leaf areas are the

result of much cell expansion. In contrast, *P. deltoides* which has many, small cells as a result of much cell division. Ridge *et al.* (1986) suggested that the hybrids of these two species attain leaf areas superior to either parent due to inheriting the lack of restraints on both cell expansion and cell division. Leaf growth control mechanisms are therefore likely to be important when selecting hybrids of *P. trichocarpa* and *P. deltoides* for overall yields (Hinckley *et al.,* 1989).

The study of the structure of a leaf provides evidence about its physiology. *Populus trichocarpa*, for example, is the only poplar in which stomata are usually absent from the adaxial leaf surface; all other poplar species are amphistomatous (Ceulemans, 1990). *Populus trichocarpa* is a native plant to the western U.S.A., often growing at higher altitudes than most poplars, and this climatic difference may explain its unique stomatal characteristics. Low numbers of stomata are a recognised drought response, which is also true of a thick mesophyll layer: *P. trichocarpa* is identifiable also by the white appearance of its abaxial surface of the thick leaves (Ridge *et al.,* 1988). The leaf whiteness is derived from a thick, loosely packed layer of spongy mesophyll cells which can be 200 μm or more thick; conversely, *P. deltoides* has a green abaxial appearance accounted for by bilateral palisade layers which are up to 20μm in thickness (Ceulemans *et al.,* 1984). These parental characteristics are reflected in the varied anatomies of their hybrids. The two speices also differ in their utilisation of atmospheric carbon isotopes (Herold, 1997).

There are two stable, natural isotopes of carbon in atmospheric carbon dioxide, and the atmosphere accordingly varies in its ratio of $^{13}CO_2/^{12}CO_2$. Likewise, plant $^{13}C/^{12}C$ ratios vary considerably with both environmental and genetic factors (Lloyd and

Farquhar, 1994). The exclusion of $^{13}CO_2$ relative to $^{12}CO_2$ during gas exchange in plants is termed carbon isotope discrimination, or $\delta^{13}C$. The carbon isotope composition of leaf tissue can give an estimate of water use efficiency. Stomatal closure is a response to low water availability, which subsequently decreases the ratio of intercellular to ambient CO_2 (C_i/C_a). The C_i/C_a ratio in turn affects the relative contribution of each fractional term of carbon against ^{13}C during gas exchange. This is caused by the lower reactivity of ^{13}C than ^{12}C and also the slower diffusion of the heavier isotope from the atmosphere to the carboxylation site in the chloroplast. Less negative $\delta^{13}C$ values give rise to a $^{13}C/^{12}C$ ratio closer to that of atmospheric CO_2, enabling the plant to fix CO_2 with a higher fractional limitation of photosynthesis by stomatal conductance. This reduces water loss in transpiration per unit of CO_2 fixed and therefore results in a higher water use efficiency. In this way, $\delta^{13}C$ gives a long-term average of water-use status in the tissue measured (Farqhuar *et al.*, 1989). A number of studies have confirmed the relationship in trees between $\delta^{13}C$ and water use efficiency (e.g. Osorio and Pereira, 1994; Leffler and Evans, 1999; Warren *et al.*, 2001) and transpiration efficiency (Thumma *et al.*, 2001).

AIM

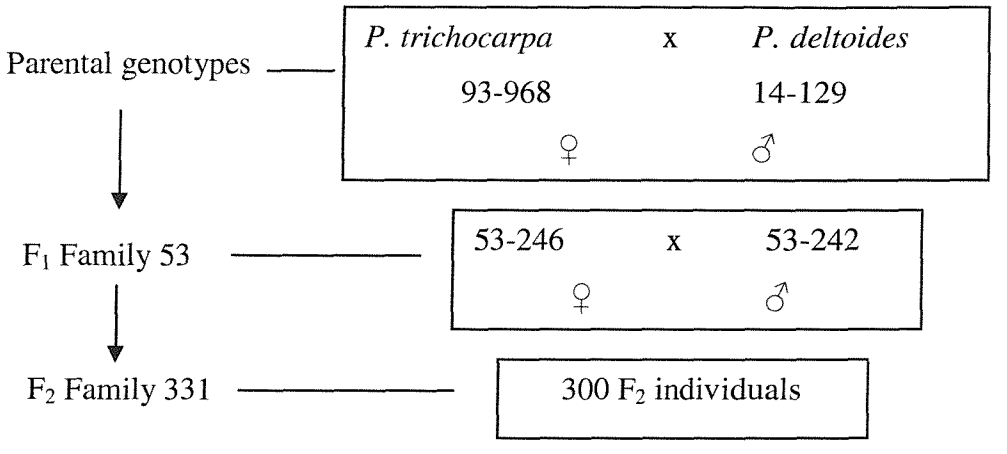
This study sought to elucidate the relative contributions of a number of anatomical, morphological and developmental leaf and stem properties to final tree yield, with particular emphasis on coppice for biomass trees. Traits were identified, which would be useful determinants of final biomass yield in poplar coppice, and a model was generated based on the weightings of the predictor traits. The pedigree used to investigate these traits was a morphologically diverse *P. trichocarpa* x *P. deltoides* F_2 population derived from parental material segregating for many leaf and stem traits.

Preliminary QTL analysis was used to identify traits that are heritable and may be useful for poplar breeding programmes for high yielding genotypes in the future.

3.2 MATERIALS AND METHODS

3.2.1 Establishment and maintenance of Family 331 field trial

Between January and May 2000, preparations were made for the establishment of a field trial. The field trial was designed to assess the pedigree derived from poplar Family 331, a pedigree derived from the hybridisation of *P. trichocarpa* Torr. & Gray, clone 93-968 of the black cottonwood originating from maritime Washington and *P. deltoides* Bartr. ex Marsh, clone 14-129 from continental mid-west U.S.A. (Bradshaw and Stettler, 1995). This pedigree is illustrated below:

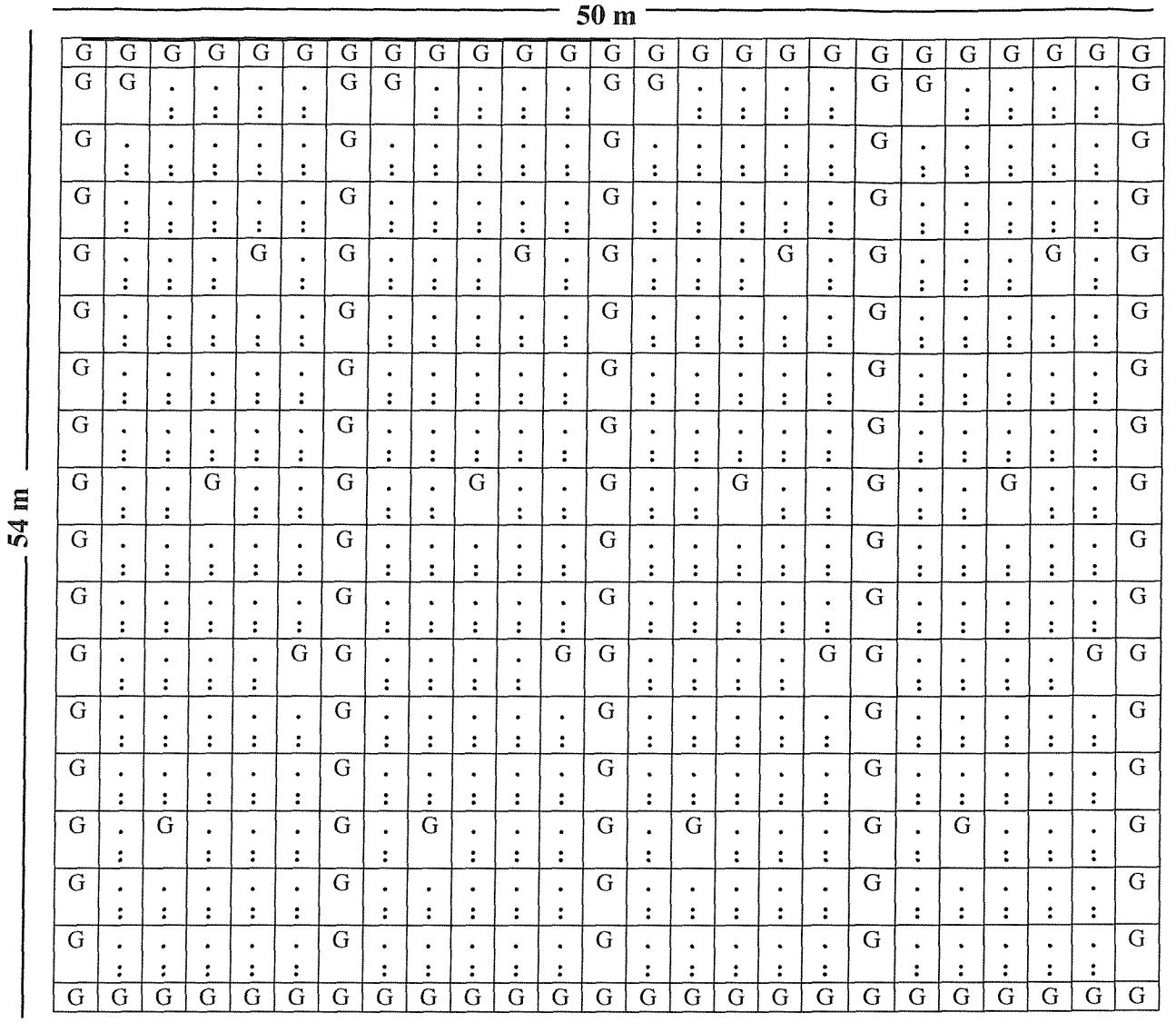


Two sites were assessed as possible locations for the poplar field trial. The first was at Selborne, Hampshire, U.K., which was measured accordingly but not used owing to the steep slope of the land and deep shade in certain areas of the site, which may have caused considerable inconsistency of growing conditions across the site. The second, and chosen, site was at the Forestry Commission nursery at Headley, Hampshire, U.K. (51°07′ N, 0°50′ W), which had available, flat land and a well drained, sandy soil suitable for the rooting of poplar cuttings. This site was not shaded. Two designs were

proposed for the layout of the Family 331 field trial at Headley Nursery. The first, based on an existing willow trial design implemented at Long Ashton Research Station, University of Bristol, included single plots of six replicates per genotype with a standard reference clone at regular spacings throughout the trial. The reference clone was intended as a standard for all measurements, to assess the homogeneity of the site. This design, originally designed for a trial in excess of 5 500 willow cuttings, was adapted for the 900 cuttings of poplar Family 331, as illustrated in Figure 3.1. An alternative design was recommended by the statistics department at Forest Research, encompassing all 900 poplar individuals as described below and illustrated in Figure 3.2. This second and final design was recommended owing to the smaller size of the site and limited number of poplar cuttings available (three per genotype) compared to the willow trial. It was advised that this design be used because it was deemed 'safer' to place the three replicate cuttings in separate positions in a trial rather than to group them; this would reduce the impact of any local damage within the trial, with a lower likelihood of mortality of an entire genotype if all three individuals of the genotype were separately positioned. On the basis of this advice, a field trial was established in May 2000 from 25 cm unrooted hardwood cuttings propagated from poplar Family 331 (a gift from Professor H.D. Bradshaw at the University of Washington). The cuttings were stored in a cold room at 0 to 5°C for the duration of preparation for the field trial. Cuttings were counted, cut to 25 cm lengths and sorted into genotype groups and given a code for each genotype to establish their random positions within the trial. The diameters of all cuttings for the field trial were measured using stainless steel precision manual callipers with the assistance of Fangzu Zhang. The cuttings were treated with fungicide prior to planting, in a solution of Tilt™ 250 EC (Ciba Geigy Agrochemicals, Whittlesford, Cambridge, U.K.), with the assistance of Michael Cotton. Planting

positions were organised prior to planting and the cuttings set out in trays to ease the planting process. The trays of cuttings were kept under cold, wet hessian during the planting process to prevent any desiccation and shrinkage prior to irrigation. Planting took place between 5th and 9th May 2000 at Headley Nursery, Hampshire (51°07′ N, 0°50′ W). The site was fed with mushroom compost prior to planting, prepared with a base dressing of N:P:K 0:24:24 prior to planting and treated with a residual pre-emergent herbicide each Spring of the experiment: Butisan (metazachlor at 2.5 litres per hectare, BASF AG, Leverkusen, Germany) and Stomp 440 Sc (pendimethalin at 6.0 litres per hectare, Cyanamid). Three cuttings of each genotype were planted at a depth of 20cm in a randomised design comprising 30 rows of 30 cuttings at 1 m spacing between cuttings and 1 m between rows. Ten rows (300 plants) comprised each of the three blocks. A double row of *P. deltoides* x *P. nigra* 'Gaver' (The Poplar Tree Company, Herefordshire) was planted around the entire trial at the same spacing to serve as a buffer (as illustrated in Figure 3.2). Planting was undertaken with the assistance of Graham Clarkson, Fangzu Zhang and Michael Cotton. Irrigation commenced at the close of the final day of planting, from overhead sprinklers and was maintained regularly and as necessary throughout the establishment of the trial and in the following growing season by the nursery staff. The trial was protected by a 1 m electric fence. The entire nursery was protected by anti-deer fencing. The trial was sprayed with permethrin insecticide (1 ml per litre) 'Ambush' (AstraZeneca, Macclesfield, U.K.) 68 days after planting (DAP) to avoid herbivory by chrysomelid beetles. Further treatment was applied annually after measurements of herbivory damage. All applications of insecticide were applied by the nursery staff. At 89 DAP, each plant was pruned back to the strongest shoot, removing all other shoots. Multiple stems only occurred where more than one bud on each cutting was present above

ground level at the time of planting. Where there was more than one strong shoot, the shoot nearest the top of the cutting was retained and the others removed. The 'Gaver' buffer rows were not pruned so as to maximise the physical protection of the trial. Where cuttings did not survive, replacement plants of the same genotype were raised from unrooted cuttings in a cool greenhouse and planted in the spaces in the trial to maintain the original random design and 1 m spacing throughout the trial. Replacement plants were planted in the final field positions at 142 DAP. These plants were measured as part of the field trial in subsequent growing seasons, with analyses of variance carried out on the data collected to test for effects of the later planting. These data were excluded from the final analysis because they differed significantly from the original neighbours in the field. During the second growing season, further replacements were added to the trials which had been pot-grown, as before. These replacements served only to maintain the original design without gaps and were not measured for use in the analysis.



Key	
G	triplet of 'Gaver' control trees
⋮	Triplet of one Family 331 genotype

Figure 3.1 Design of a poplar field trial based on a design for a willow trial at Long Ashton Research Station. Each of the 300 genotypes occurs in a plot of three individuals, with repeated plots of a standard clone, 'Gaver' (denoted G) spread evenly across the trial.

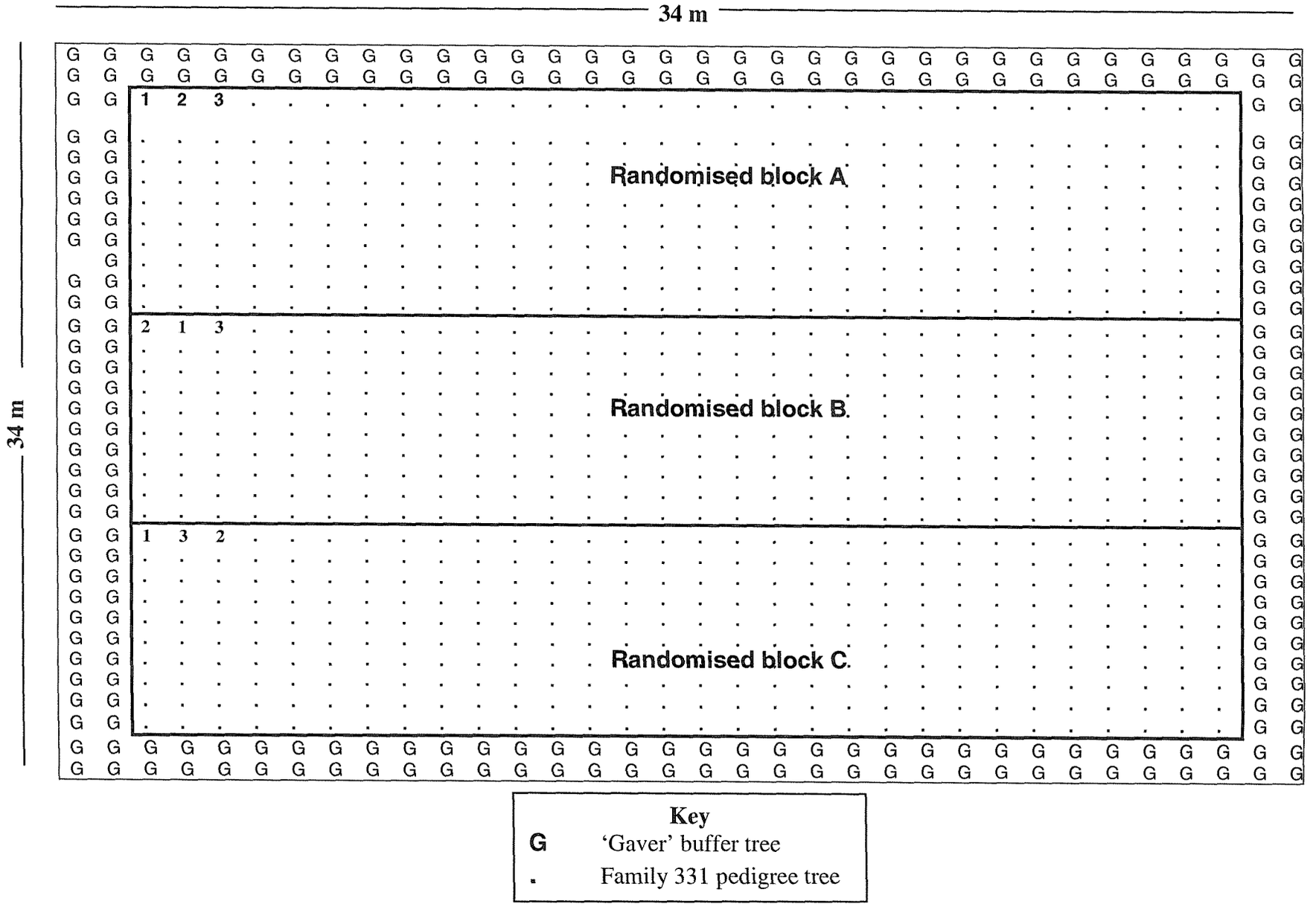


Figure 3.2 The final design of the poplar field trial, comprising three blocks of the 300 genotypes in single tree plots. The standard clone 'Gaver' (denoted G) is used as a buffer to surround and protect the trial, to remove edge effects.

Chronology and replication of all single stem and coppice measurements are shown in Table 3.1.

3.2.2 Single stem measurements

Basic measurements of tree architecture were made 203 DAP following senescence of all leaves. Maximum stem height was measured using a digital measure pole (Shenshin Industry Co. Ltd, Japan). Stem diameter was measured using digital callipers (DigiMax Measy 2000 Mecanic, Swiss Precision, Switzerland) at 10 cm from ground level. A count was made of the number of sylleptic branches on the main stem of each tree.

3.2.3 First year coppice measurements

The trial was coppiced on 11th January 2001 (= 0 DAC, days after coppice).

Bud burst

The degree of bud burst on the first leaf of the coppice stool in Family 331 was measured every 7 days between 69 and 133 DAC. Assessments were made visually on a 6 point scale as devised by Castellani *et al.* (1967):

Phase 0: Dormant buds, completely closed by scales.

Phase 1: Swelling buds with scales slightly open. Presence of one of more drops of sap.

Phase 2: Opening buds and clearly apparent apical part of the leaves.

Phase 3: Buds completely open. Leaves still closed and scales still present.

Phase 4: Separated bud buds with their laminae still wrapped. Scales present or absent.

Phase 5: Leaves completely open but not mature. Apparent elongation of the internodes. No scales.

The data were interpreted as the number of weeks for any given genotype to reach phase 5 from the first date of observation.

Table 3.1 Summary of chronology and replication of single stem and coppice traits measured in Family 331 over two growing seasons. DAP = days after planting (DAP 0 = 8th May 2000); DAC = days after coppice (DAC 0 = 11th January 2001).

Level	Trait	Date	Replication (blocks)
Stem and canopy	Maximum stem height (single stem)	203 DAP	A, B, C
	Maximum stem height (coppice)	138 DAC; 145 DAC	A, B, C
	Maximum stem height (harvest)	359 DAC	A, B, C
	Number of sylleptic branches	203 DAP	A, B, C
	Sylleptic branch presence	359 DAC	A, B, C
	Basal stem diameter	203 DAP	A, B, C
	Basal stem diameters	359 DAC	A, B, C
	Spring bud burst (week no.)	69 – 133 DAC	A, B, C
	Stem extension increment	138 – 145 DAC	A, B, C
	Stem number on stool	137 DAC	A, B, C
	Stem number on stool	359 DAC	A, B, C
	Whole tree fresh weight	366 DAC	A, B, C
	Whole tree dry weight	359 DAC	20 genotypes
Leaf	Leaf number on leader	140 DAC	A,B,C
	Leaf number on leader	181 DAC	A,B,C
	Plastochron index	140 DAC	A
	Plastochron index	181 DAC	A,B,C
	Leaf extension rate	137 – 144 DAC	A
	Leaf extension rate	174 – 181 DAC	A,B,C
	Leaf production rate	137 – 144 DAC	A, B, C
	Leaf production rate	174 – 181 DAC	A,B,C
	Individual leaf area	165 DAC	A, B, C
	Specific leaf area	165 DAC	A, B, C
	Petiole length	214 DAC	A, B, C
Cell	Adaxial epidermal imprints for cell area and number per leaf	172 – 174 DAC	A,B,C
Biochemical and disease	Carbon isotope analysis	224 DAC	A,B
	Herbivory damage	132 DAC	A,B,C
	Rust	256 DAC	A, B, C

Stem growth

The number of live coppice stems on each stool was counted.

Stem height (cm) was measured on the tallest stem, extended to its full length and its length and then re-measured after a 7 day period to give the extension increment.

Leaf growth

The tallest stem of each coppice stool was identified and from each, a single representative leaf was obtained. Only fully expanded, recently mature leaves were chosen. The leaf samples selected were as free as possible from damage by rust pustules, necrosis, herbivory and mechanical damage. Samples were kept cool and humidity was maintained during harvest and transportation. Leaf samples were photocopied and scanned into Corel Photopaint (Corel Inc.) in TIFF (tagged information format file) format and the images analysed to obtain leaf areas (excluding petiole area) using Scion Image (release Beta 3b, Scion Corporation, Frederick, MD, U.S.A.). Leaf samples were dried for a minimum of 48 hours at 85°C with petioles removed, and dry weights obtained using a top pan balance. Each value for leaf area was divided by the leaf dry weight to calculate specific area ($mm^2\ g^{-1}$).

Prior to photocopying and drying, epidermal imprints were made of the mature leaves in order to measure cell areas and estimate the number of cells per leaf as described by Ferris *et al.* (2002). At a distance of 2cm from the leaf base and in the second interveinal section from the leaf base, an area 1.5 x 1.5cm of the adaxial epidermis was coated with clear nail varnish and allowed to dry. Once dry, the coating was removed using Sellotape and transferred onto a glass microscope slide, leaving the leaf intact. Digital images were captured of the epidermal impressions using a microscope (Axiophot 2 Universal Microscope, Carl Zeiss Jena, Germany) and accompanying digital imaging software, Metamorph Imaging System (Westchester, Philadelphia, U.S.A.). This software was used to obtain one digital image per slide of mature adaxial epidermal cells taken between the midrib and the major veins. The areas of ten adaxial epidermal cells per slide were obtained randomly from the digital image using Metamorph. An

estimation of the number of adaxial epidermal cells per leaf was calculated for each poplar genotype from the mean cell area and the area of the leaf.

The petiole length of one recently mature, fully expanded leaf was taken from the upper canopy of the leading coppice stem from each of the selected stools. Only intact, undamaged leaves were measured. The length in mm, from the foliar base to the junction with the stem, of each fully extended petiole was measured using a paper ruler made from graph paper.

Plastochron index (P.I.), the rate at which leaves are being produced, was calculated based on the formula of Erickson and Michelini (1957):

$$\text{P.I.} = n + \frac{\log L_n - \log 20}{\log L_n - \log L_{n+1}}$$

where n is the leaf serial number on the plant, Ln is the length (mm) of the index leaf nearest to 20 mm, and $Ln+1$ is the length (mm) of the next youngest leaf to Ln which is less than 20 mm long.

Leaf production rate was measured over a seven day period. A weatherproof tag (colourfast embroidery silk) was loosely knotted on to the apical stem of the leading stem of each sample stool. The number of existing visible individual leaves (including those which were unrolled) between the tag and the tip of the stem was noted. After seven days, the number of leaves between the tag and the tip of the stem was counted again. The difference between the number of leaves on day one and the number on day seven was taken and converted to leaf production rate to give number of leaves produced day^{-1}.

Leaf extension rate was measured at 137-144 DAC and 174-181 DAC. The leaf selected was on the leading coppice stem and was chosen as the youngest unfolded leaf with a petiole mature enough to attach a weatherproof tag (colourfast embroidery silk) to, and with a lamina length as near to 20 mm as possible. The leaf length was measured using graph paper. This distance was measured again after a seven day period, and the extension rate calculated as mm

day $^{-1}$. This measurement was denoted Leaf Extension Rate 1 (LER 1). The extension rate was also measured of the next youngest leaf to that closest to 20 mm in length. This measurement was denoted LER 2.

The number of leaves on the leading stem on a coppice stool was counted as the number of whole leaves, including the distinct leaf scars where lower leaves had senesced. Only individual, visible leaves were included in the count.

One single, recently fully expanded leaf was taken from the leading stem of each stool and harvested into paper bags and dried in an oven at 85 °C until constant weight was reached (ca. 72 h). Of these, the genotypes which had two dried leaves available, and which were not replacement trees in the field, 600 leaves, approximately 2 per genotype, were selected and ground using a leaf grinder. The samples chosen were taken from field trial blocks A and B where possible, as these blocks had the highest tree survival rates. The grinder used was a Pulverisette 14 rotor-speed mill (Fritsch, Idar-Obenstein, Germany) using a 0.5mm mesh rotary blade. The midrib and major veins were excluded from the material ground to obtain a homogeneous powder that was stored in sealed glass vials prior to weighing. A 1 mg aliquot of powdered leaf tissue was weighed into standard weight tin capsules for elemental analysis using a milligram balance and maintaining a level of ± 5%. The samples were sealed into 96-well microtitre plates. Analysis was conducted by the Biomedical Mass Spectrometry Unit, Dental School, University of Newcastle. Elemental analysis was conducted against a reference plant flour standard belonging to the Unit, calibrated against IAEA standards. For every 40 foliar samples analysed, 18 one mg aliquots of plant reference flour were used for calibration.

Beetle and sawfly larva damage

Leaf damage caused by herbivory by both sawfly larvae and chrysomelid beetles was assessed at 132 DAC. The worst affected leaf on each plant was scored. A binary scoring system was used where 0 was damage to less than 20% of the leaf surface and 1 was damage to 20% or more of the leaf surface.

Rust

Severity of leaf infection by *Melampsora larici-populina* rust was assessed at 256 DAC. Visual assessments were made on a scale provided (personal communication from D. Lonsdale, Forest Research, Farnham, U.K.), where rust pustule density on the abaxial leaf surface was observed on a scale from 0 (no pustules) to 5 (surface pustule saturation) on a random leaf taken from mid-height of the main stem.

Final harvest

At 359 DAC, stem height and the number and diameter (at10 cm from ground level) of all stems on the coppice stool were measured. An assessment was also made of the presence or absence of sylleptic branches on each individual in the population. The entire trial was harvested and the fresh weight of each individual taken using a top pan balance. From the total population, a sample of 20 individual plants, representing a range of sizes and growth forms, was selected and harvested. Each entire plant was oven dried at 80 °C until constant weight was reached. Percentage moisture content by mass was then calculated.

3.2.4 Statistical analysis

Descriptive statistical analyses were conducted using Minitab Release 13.1 (Minitab Inc., State College, PA, U.S.A.). All data were plotted and tested for normality using the Anderson-Darling test.

Unit variance scaling was used on data prepared for analysis in the principal components analysis (PCA) and partial least squares regression (PLS). PLS is the projection to latent structures by partial least squares analysis (Eriksson *et al.*, 2001). The data were then mean centred. PCA is an unsupervised method of analysis; it treats all variables in the same manner for descriptive purposes. PLS provides a powerful dimension reduction strategy to relate a set

of response variables, *y*, to a set of predictor variables, *x*. It is the compromise between the PCA and the canonical correlation analysis of *x* and *y* (Pérez-Enciso and Tenenhaus, 2003). Multiple regression was used as an analytical tool for preliminary analysis of this data set, giving a numerical prediction of harvested biomass, as described in Rae *et al.* (2003). Further investigation of available methods of analysing multivariate data identified partial least squares regression (PLS), as a potentially improved method of analysis. One possible criticism of the use of multiple regression is that the results obtained may be influenced by the presence of multicollinearity. PLS is able to handle large amounts of missing data, and multicollinearity where the numbers of predictors are high in relation to the number of observations that occurs in such a data set owing to the many growth traits and genetically related individuals measured (Van Eeuwijk *et al.*, 2002; Zar, 1999). Both multiple regression and PLS indicated that the same traits are strongly influential to biomass. This statistically robust outcome (Sokal and Rolf, 1995) is intuitive considering the overwhelming importance of the traits influencing biomass. Both PCA and PLS analyses were conducted using Simca-P 10.1 (Umetrics AB, Umeå, Sweden).

Broad sense heritablility

Broad sense heritability was estimated for all traits, for which there were three replicates per genotype, as $V_G/(V_E+V_G)$. Where V_G is the genetic variation and V_E is the environmental variation. V_G was calculated from an analysis of variance as (mean square between genotypes – mean square within genotypes)/ number of replicates. The within-genotypes mean-square was taken as V_E (Falconer and Mackay, 1996).

QTL analysis

Preliminary QTL analysis was carried out on a subset of 90 F_2 genotypes in the Family 331 population, using the molecular map published by Bradshaw *et al.* (1994). This genetic linkage map, based on 90 F_2 individuals and constructed from STS, RFLP and RAPD markers, was

used for QTL mapping. The current Family 331 map has around 250 RFLP markers and 100 RAPD markers and covers approximately two thirds of the approximate 2600 cM length of the *Populus* genome (Bradshaw *et al.,* 1994). The data were analysed for QTL using MAPMAKER/QTL 1.1 (Lander and Botstein, 1989). Although LOD (logarithm of the odds) score 2.9 is recommended as a threshold for QTL presence based on a *P* significance of 0.05 for the false assignment of a QTL in a genome-wide search (Lander and Botstein, 1989; Bradshaw and Stettler, 1995; Bradshaw, 1996), given the small F_2 sample size used in this study, a LOD score of 2.6 was used to detetermine QTL presence, thus detecting QTL with smaller effect and reducing the Type II (false negative) errors (Wu *et al.*,1997).

3.3 RESULTS

3.3.1 Height and diameter, establishment year

The values for stem traits in the single stem and coppice habits are given in Table 3.2. Although *P. trichocarpa* exceeded *P. deltoides* in all first year stem traits, it was by a negligible margin of 1 cm in stem height, with *P. trichocarpa* attaining 174 cm and *P. deltoides* attaining 173 cm in height. The male and female F_1 plants were respectively shorter (170 cm) and taller (194 cm) than both of the parent plants.

The F_2 individual 331-1939 was 143% the height of the higher parent, *P. trichocarpa*, at the end of the first year. Figure 3.3 shows the distribution of maximum stem heights in the pedigree. The values for parental and F_1 genotypes in this figure are the means from all three replicates of each genotype. The distribution is normal across the population. The modal frequency in the population was 75 to 85 cm. Two genotypes, indicated in the minimum *x* axis category attained very limited heights in the establishment year. The distribution of basal diameters prior to coppice was also approximately normal (Figure 3.4), with the modal frequency 11 to 13 mm and the maximum F_2 individual for basal area being 331-1680 at 35 mm. The distribution of parental and F_1 values was similar to that of stem height at this time point, with the mean diameter of the female F_1 exceeding both parental means and the male F_1 between the two parents.

3.3.2 Sylleptic branch production, establishment year

The female parent, *P. trichocarpa,* produces large numbers of sylleptic branches, a maximum of 27 on one plant, as shown in Table 3.2, whereas the *P. deltoides* replicate with the highest number of branches yielded 10 branches. The other two replicates of *P. deltoides,* however, produced no sylleptic branches, which brought the mean number of sylleptic branches for the

genotype to 3.33, as indicated in the population distribution for this trait, Figure 3.5. The F_1s are intermediate to the parental plants for sylleptic branch production, and there is little spread around the mean for these individuals, since the maximums and means for these traits are similar. The F_2 individual 331-1103 produced 35 sylleptic branches prior to coppice, exceeding the maximum of 27 branches in *P. trichocarpa*.

Table 3.2 Stemwood traits of Family 331 parents, F_1's and the F_2 mean at three temporal stages. Maximum values for each trait are shown for each genotype. Maximum F_2 values represent the greatest of all replicates of between 274 and 280 F_2 genotypes. The F_2 genotype bearing the maximum value is stated.

	P. trichocarpa 93-968	*P. deltoides* 14-129	F_1 male 53-242	F_1 female 53-246	F_2 maximum Family 331	(F_2 genotype)
Single stem (203 DAP)						
Maximum stem height (cm)	174	173	170	194	249	331-1939
Stem diameter (mm)	23.2	19.4	19.2	23.1	30.8	331-1680
Number of sylleptic branches	27	10	18	18	35	331-1103
Coppice, summer (87-145 DAC)						
Maximum stem height (cm)	86	77	89	83	94	331-1689
Stem extension increment (cm)	14	13	15	13	34	331-1846
Total number of stems	18	8	6	7	30	331-1674
Coppice, winter (359 DAC)						
Maximum stem height (cm)	390	356	390	374	429	331-1918
Total stem diameter (mm)	234	137	114	120	268	331-1674
Number of live stems	15	6	6	5	22	331-1731
Sylleptics (0 = absent, 1 = present)	1	1	1	1	1	109 genotypes

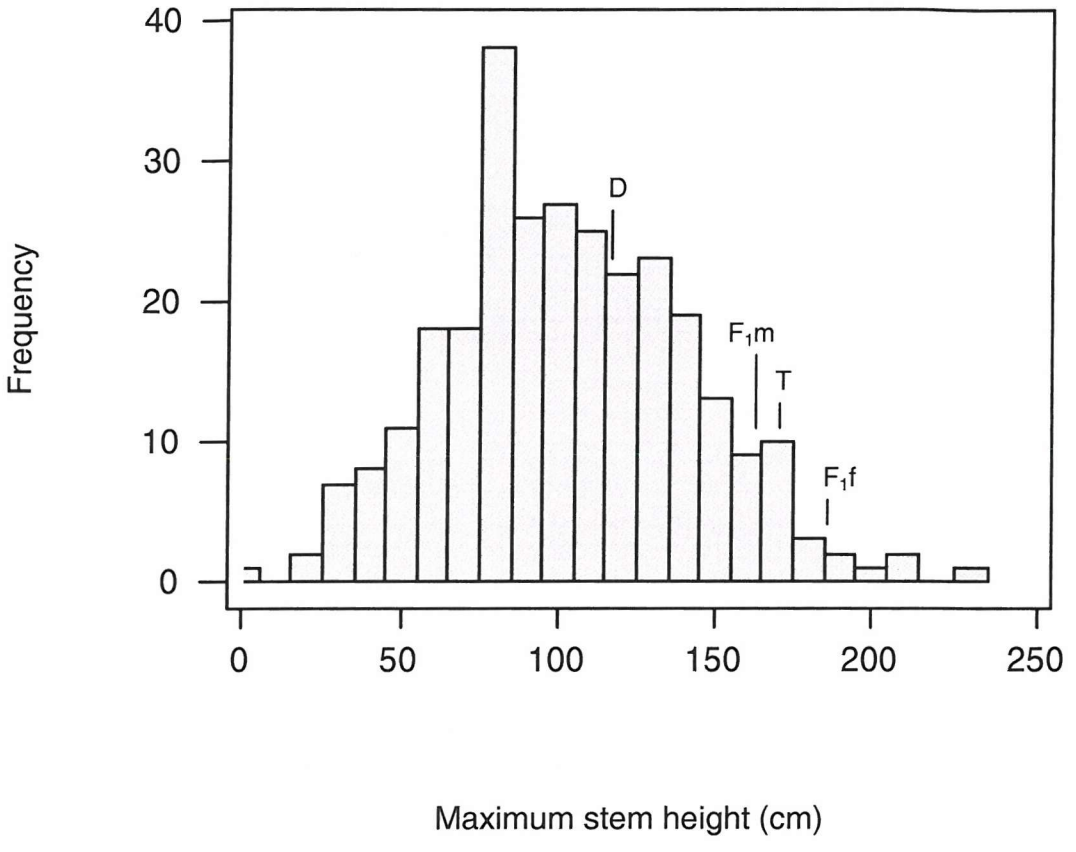


Figure 3.3 Distribution of single stem heights in the Family 331 pedigree, at 203 DAP, prior to the first coppice. Replicates from all blocks in the field are included, representing 281 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

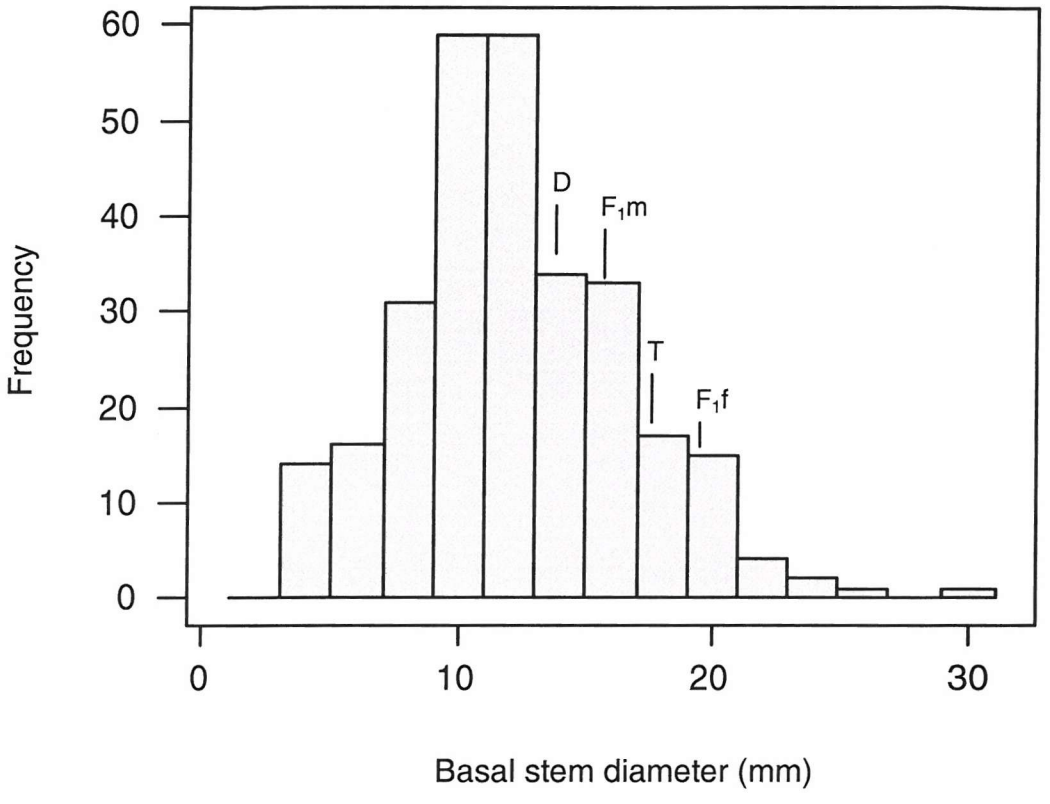


Figure 3.4 Distribution of basal single stem diameters in the Family 331 pedigree, measured at 203 DAP, prior to the first coppice. Replicates from all blocks in the field are included, representing 281 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

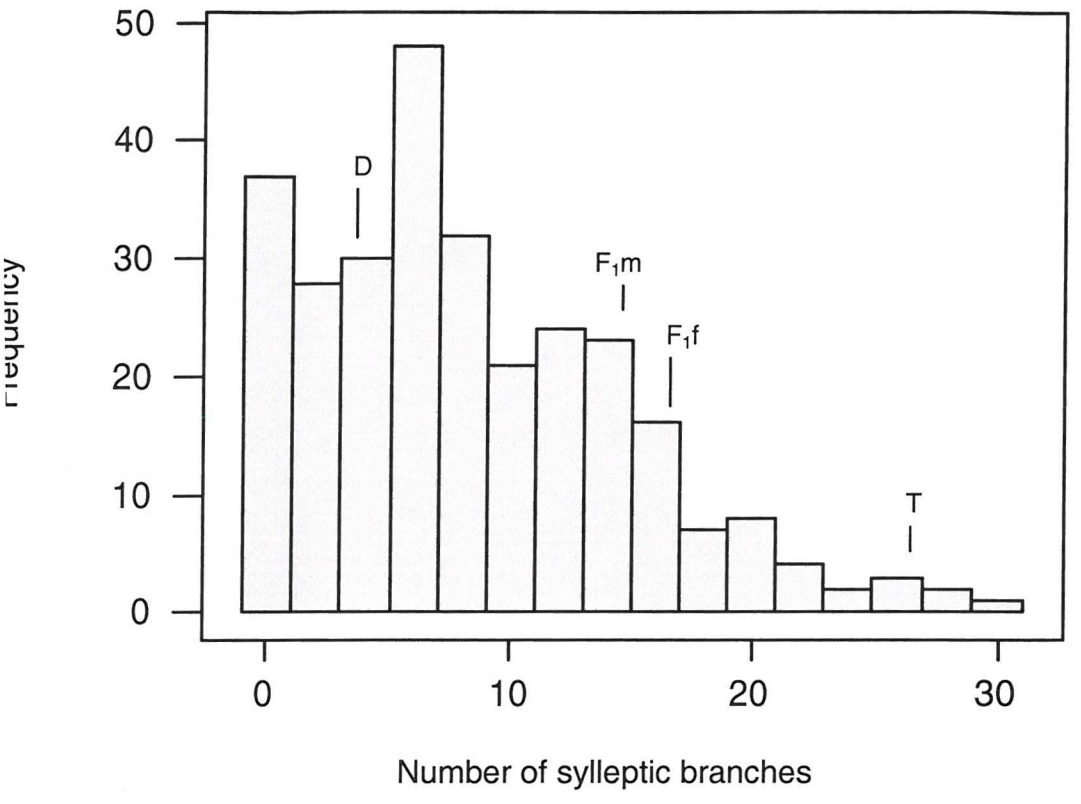


Figure 3.5 Distribution of numbers of sylleptic branches in the Family 331 pedigree, measured at 203 DAP, in the single stem habit, prior to the first coppice. Replicates from all blocks in the field are included, representing 281 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

3.3.3 Height, growth and stem production

During the second growing season, at 145 DAC, the coppiced population showed an approximately normal distribution for maximum stem height, as shown in Figure 3.6, with a modal frequency of 47.5 to 52.5 cm. The individual in the pedigree with the greatest stem height was 331-1689, for which the tallest individual measured 94 cm, as shown in Table 3.2. The parental and F_1 material were amongst the tallest plants in the field, with maximum individuals at 86 cm for *P. trichocarpa* and 77 cm for *P. deltoides,* with an intermediate value between the parents in the female F_2 of 83 cm. In contrast to the single stem measurements prior to coppice, when the female F_1 exceeded the male F_1 in maximum height, at 145 DAC the male F_1 was the taller, attaining a maximum of 89 cm. However, the mean heights across all three replicates in the male and female F_1s respectively were 78 and 82 cm. A large number of F_2 genotypes attained low height values (as a mean of all the replicates), with 30 genotypes exhibiting heights below 28 cm at 145 DAC (Figure 3.6).

Stem extension increment of the leading stem on the coppice stool at 137 to 145 DAC showed a normal distribution in the population, as shown in Figure 3.7. The parents and F_1s showed similar maximum rates of stem growth, in the region of 13 to 15 cm over the 7 day period (Table 3.2). The maximum and mean values for the parental and F_1 genotypes were similar, with the means for all four genotypes falling within the same frequency group of 11 to 13 cm. The F_2 genotype with the maximum stem extension increment was 331-1846, which grew 34 cm during the 7 day period.
A small number of F_2 genotypes achieved, on average, negligible growth increments over the period measured, as seen in the distribution, Figure 3.7. The modal frequency for this trait was 7 to 9 cm.

A large number of stems was produced in *P. trichocarpa* following coppicing: a maximum of 18 stems were produced in this genotype, with a mean number of 16 stems (Table 3.2, Figure 3.8). This was exceeded by two F_2 genotypes whose mean number of stems was between 24 and 25. The genotype 331-1674 yielded the highest number of stems in the population at 137 DAC, with 30 stems. This is the same genotype that yielded the greatest total coppice stem diameter in the population at harvest, 359 DAC, although it did not yield the highest number of live stems over 1cm diameter at that time. The distribution of stem numbers across the population is illustrated in Figure 3.8, where the modal frequency in the family is 4.5 to 5.5 cm. The means of both F_1 genotypes fall into this category. *P. deltoides* produced a maximum of 8 stems per stool at 137 DAC, which is consistent with the mean for this genotype. No genotypes produced a mean of between 18 and 24 stems per stool. Only 3 genotypes on average responded to coppice with by producing only one stem, as seen in the distribution (Figure 3.8).

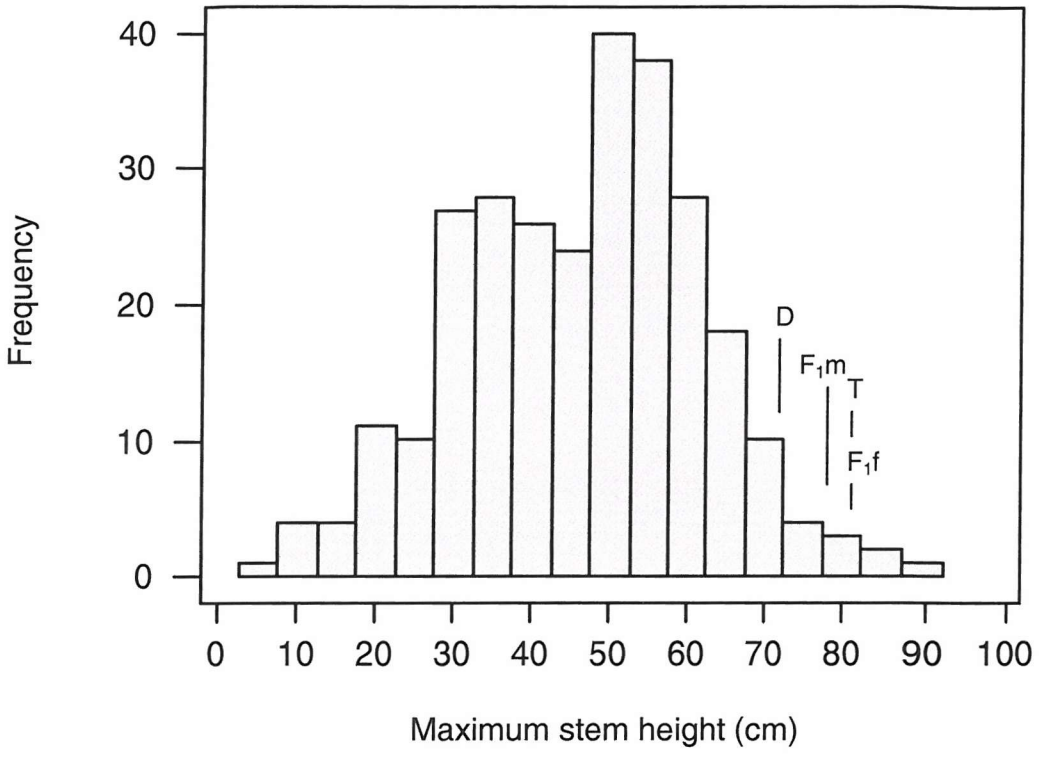


Figure 3.6 Distribution of maximum stem heights in the Family 331 pedigree, measured at 145 DAC. Replicates from all blocks in the field are included, representing 274 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

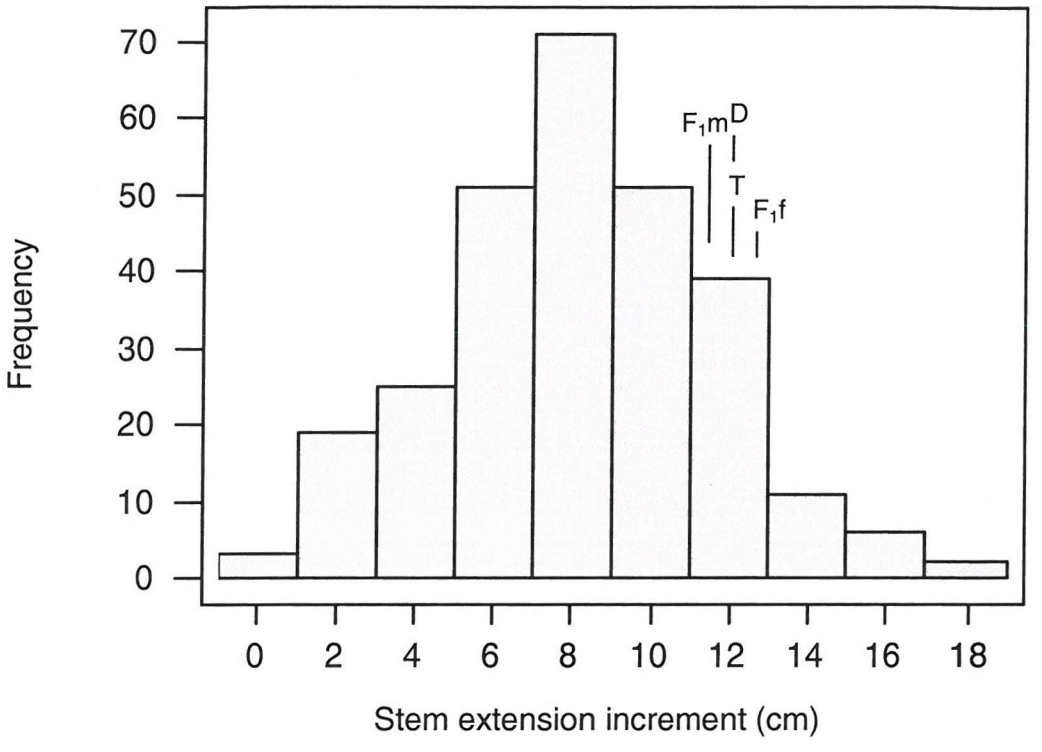


Figure 3.7 Distribution of stem extension increment of the leading stem on the coppice stool in the Family 331 pedigree, measured over the period 137 to 145 DAC. Replicates from all blocks in the field are included, representing 273 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

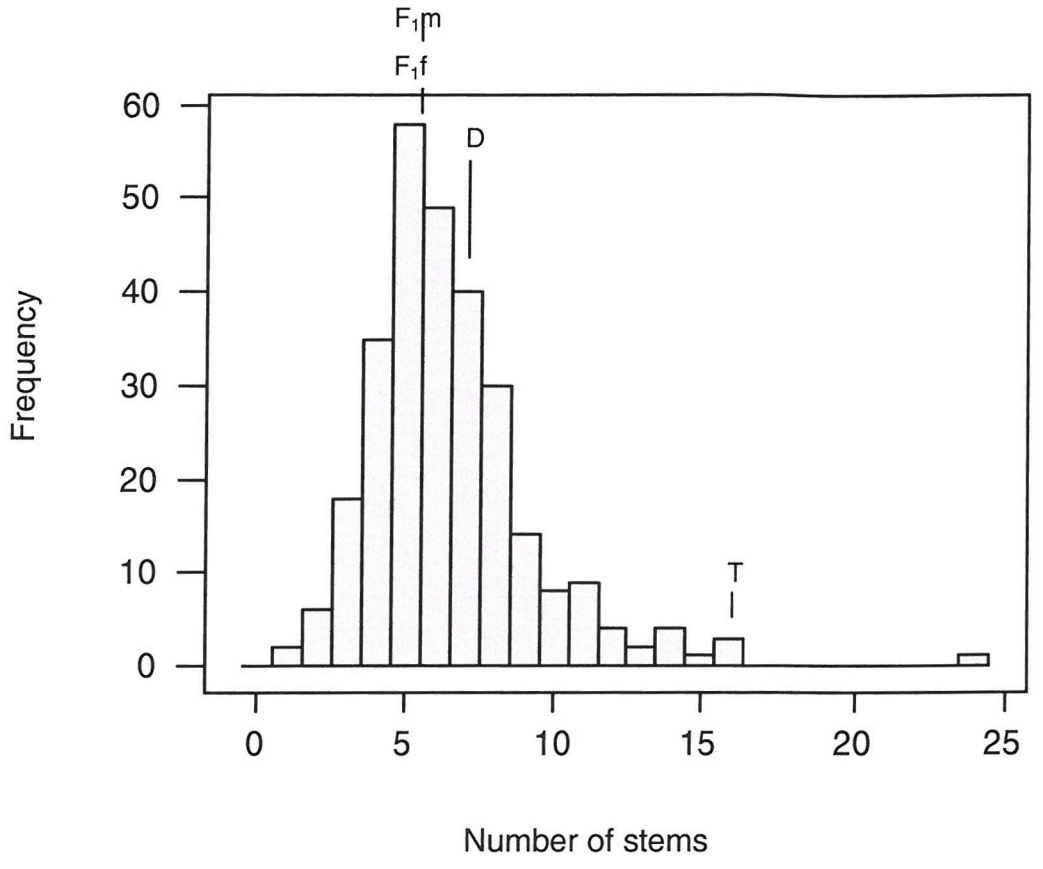


Figure 3.8 Distribution of stem number on the coppice stool in the Family 331 pedigree, measured at 137 DAC. Replicates from all blocks in the field are included, representing 273 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

3.3.4 Height, diameter and stem numbers at harvest

Table 3.2 indicates that *P. trichocarpa* and the male F_1 attained equal maximum stem heights at harvest, 359 DAC, of 390 cm. These were followed by the female F_1 with a maximum of 374 cm and *P. deltoides* with a maximum of 356 cm. The tallest tree in the field was F_2 genotype 331-1918, which reached 429 cm in height. The distribution of maximum stem heights, showing means for each genotype, is illustrated in Figure 3.9. Here, the mean values for both F_1 genotypes are intermediate to the parental means. These four genotypes fall into the upper quartile of the population distribution, indicating that the majority of F_2 genotypes are inferior to the parental and F_1 genotypes in the maximum coppice stem height attained. The modal frequency for this trait in the population is 201 to 220 cm.

The maximums for the total diameter across all of the stems on a coppice stool, and the number of live stems (> 1 cm diameter), at harvest, are shown in Table 3.2. The maternal parent, *P. trichocarpa,* possessed 15 stems with a total diameter of 234 mm. This was one of the greatest total diameters in the field trial and was exceeded by an F_2 genotype, 331-1674 with 12 live stems (data not shown) providing a total basal diameter of 268 mm, as discussed previously. The F_2 individual with the highest maximum stem number was 331-1731, with 22 live stems.

Figure 3.10 shows the stem diameter distributions in the pedigree, expressed firstly (Figure 3.10a) as the total basal diameter on a coppice stool, and secondly (Figure 3.10b) as the average basal diameter on each stool. The means of the genotypes are shown in these figures. The modal frequency for total basal diameter is 45 to 54 mm

and the modal average stem diameter, 12.5 to 13.4 mm. The figure indicates that the large mean total basal stem diameter in *P. trichocarpa*, 193 mm, was comprised of a mean of 16 stems. In contrast, *P. deltoides* produced a mean total diameter of 96 mm comprised of a mean of only 4 stems, resulting in the higher average stem diameter of 21 mm (Figure 3.11).

Syllepsis was present in both parental and both F_1 genotypes. In 109 of the F_2 genotypes, syllepsis was observed in two or three of the three replicates.

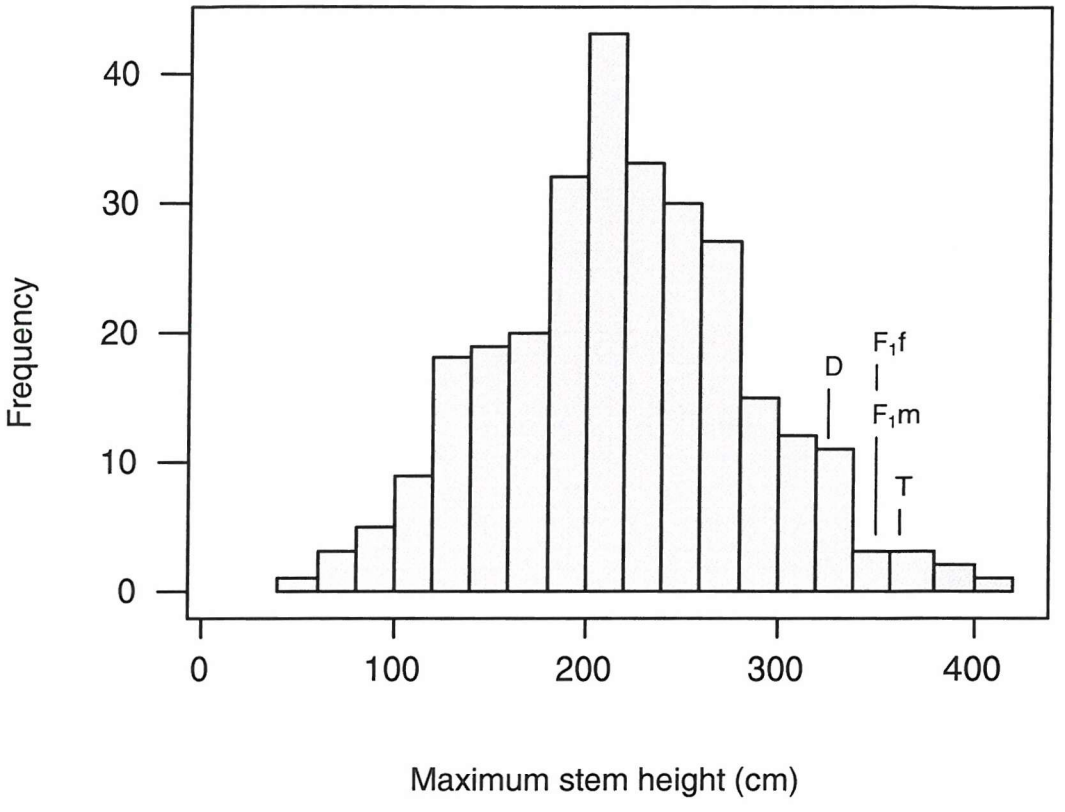
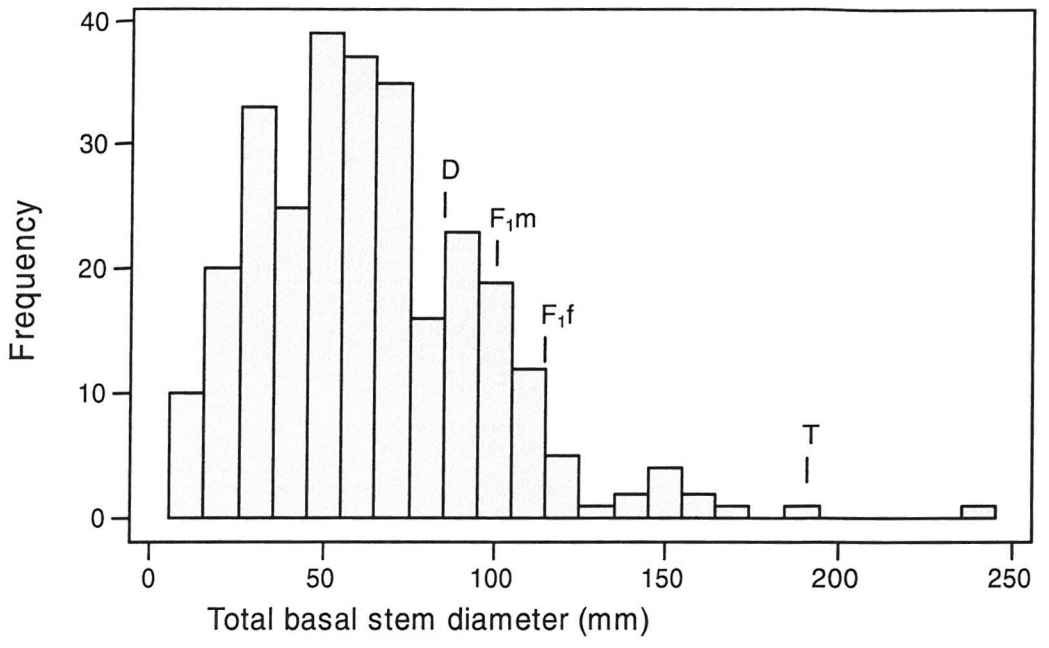


Figure 3.9 Distribution of maximum stem heights in the Family 331 pedigree, measured at 359 DAC, prior to harvest. Replicates from all blocks in the field are included, representing 282 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

(a)



(b)

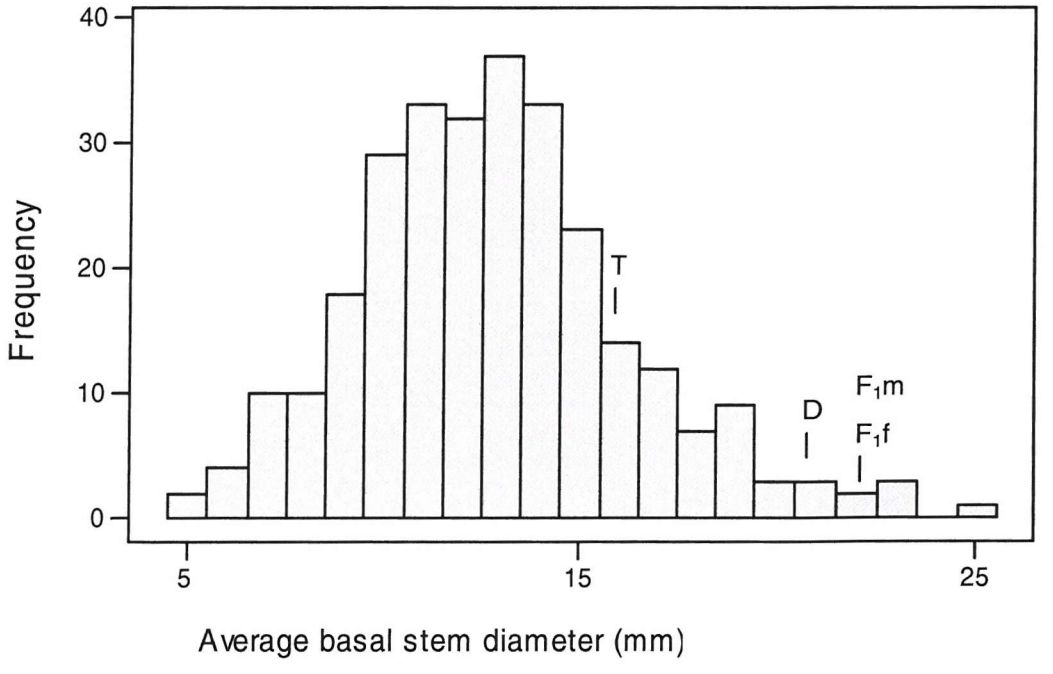


Figure 3.10 Distribution of (a) total and (b) average basal stem diameters in the Family 331 pedigree, measured on all live stems on the coppice stool, at time of harvest (359 DAC). Replicates from all blocks in the field are included, representing 281 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

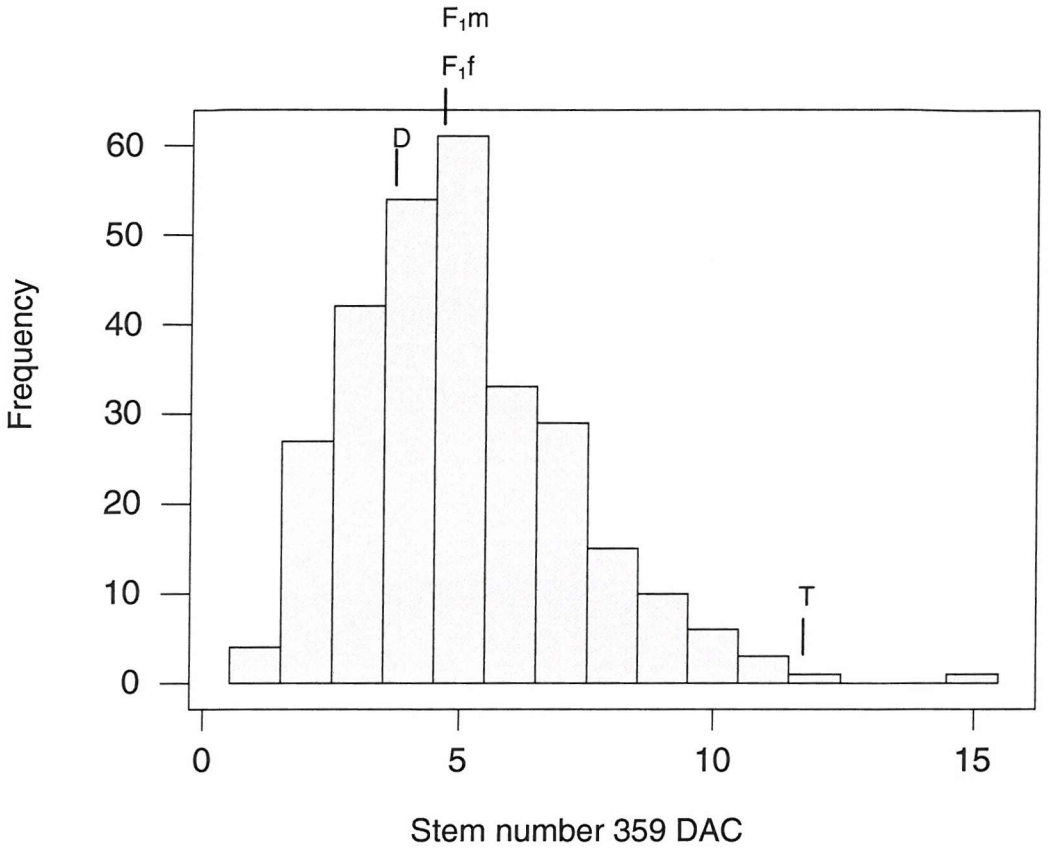


Figure 3.11 Distribution of stem number on the coppice stool in the Family 331 pedigree, measured at 359 DAC, prior to harvest. Replicates from all blocks in the field are included, representing 281 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

Table 3.3 Leaf anatomy traits of Family 331 parents, F_1's and the F_2 mean at 165–214 DAC. Maximum values for each trait are shown for each genotype. Maximum F_2 values represent the greatest of all replicates of between 221 and 279 F_2 genotypes. The genotype bearing the maximum F_2 value is stated.

	P. trichocarpa 93-968	*P. deltoides* 14-129	F_1 male 53-242	F_1 female 53-246	F_2 maximum Family 331	F_2 Genotype
Individual leaf area (cm^2)	149.42	160.12	268.42	256.64	339.24	1689
Specific leaf area ($mm^2\ g^{-1}$) (minimum)	7 527	9 010	7 215	7 859	1 495	1165
Adaxial epidermal cell area (μm^2)	489.64	330.53	368.96	389.72	911.85	1732
Estimated adaxial epidermal cell number per leaf (x 10^6)	30.51	35.27	65.77	63.27	115.26	1689
Petiole length (mm)	49	126	85	105	156	1884

3.3.5 Leaf anatomy traits

There was a great diversity of leaf shapes, colours, thickness, serrations and other traits that could be readily observed across the field trial. Table 3.3 contains maximum values of parental, F_1 and F_2 leaf anatomy traits. Maximum individual leaf areas are shown in Table 3.3 and distributions of means for the genotypes in the population are illustrated in Figure 3.12. The male parent, *P. deltoides,* attained a maximum individual leaf area of 160.12cm^2 and a mean area of 145.00 cm^2. The largest individual leaf of the female parent, *P. trichocarpa,* had an area of 149.42 cm^2 and across the three replicates this genotype had a mean leaf area of 116.83 cm^2. Both F_1 genotypes exhibited superior leaf areas to the parental genotypes; the male F_1 with a maximum individual leaf area of 268.42 cm^2 and the female F_1 with a similar maximum of 256.64 cm^2. The mean individual leaf areas of the male and female F_1s respectively were 156.85 cm^2 and 157.22 cm^2, lower than the maximum but very similar within the F_1. The largest F_1 leaves were grown on plants in block A of the field trial. There were few F_2 genotypes with mean individual leaf areas exceeding those of *P. deltoides* and the F_1 genotypes, as shown in the distribution of the population in Figure 3.12. However, the largest individual leaf on the leading stem that was measured belonged to genotype 331-1689 and measured 339.24 cm^2. The modal frequency was 35 to 74 cm^2.

The distribution of specific leaf area (SLA) in the population is illustrated in Figure 3.13. Large values of SLA indicate thinner leaves, since a larger area of tissue accounts for each gram of leaf tissue. *P. deltoides* had a larger maximum SLA, at 9 010 mm^{2}g^{-1}, than *P. trichocarpa* with thicker leaves at a maximum of 7 527 mm^{2}g^{-1}. The male and female F_1s were both closer in maximum SLA to the *P. trichocarpa* maximum, with values of 7 215 mm^{2}g^{-1} and 7 859 mm^{2}g^{-1} respectively. The thickest F_2 individual leaf reached 1 495 mm^{2}g^{-1} in the genotype 331-1165. Figure 3.13 shows a reasonably normal distribution of SLA in the population, with the mean F_1 SLA falling within the modal frequency of 10 500 to 11 499 mm^{2}g^{-1}. The mean values of both parents and the F_1s were lower than the maximums; nevertheless, *P. trichocarapa* (10 518

mm^2g^{-1})still had a lower mean SLA value than *P. deltoides* (12 646 mm^2g^{-1}). Unlike several other trait distributions from this field experiment, the parental and F_1 genotype means were close to the modal frequency of the F_2, rather than at one or the other tail of the distribution.

A typical image of the adaxial epidermis of an F_2 genotype is illustrated in Figure 3.14. Similar images were used to measure epidermal cell area. The maximum cell area values for the parental genotypes are shown in Table 3.3. *P. trichocarpa* produced reasonably large cells, with a maximum of 489.64 μm^2. *P. deltoides* produced smaller cells, at a maximum of 330.53 μm^2. The male and female F_1 genotypes were intermediate to the parents, at 368 .96 and 389.72 μm^2 respectively. The F_2 genotype, 331-1732 produced very large cells relative to the parents and other F_2s, at a maximum of 911.82 μm^2. Figure 3.15 is the distribution of epidermal cell areas per genotype across the population. The value for each genotype is a mean of 10 epidermal cells per epidermal impression; however there are no mean values as, owing to poorly-preserved slides there was no replication available in blocks B and C. The distribution is almost normal, and the modal frequency is 325 to 374 μm^2.

Estimation of adaxial epidermal cell number per leaf, using leaf area and mean cell area, are shown in Table 3.3 and the distribution through block A of the field trial is shown in Figure 3.16. The parental genotypes, *P. trichocarpa* and *P. deltoides,* produced 30.51 and 35.27 x 10^6 cells respectively. The F_1 generation cell numbers per leaf were greater, at 65.77 x 10^6 cells per leaf and 63.27 x 10^6 cells per leaf for the male and female F_1s respectively. The F_2 genotype with the largest maximum individual leaf area, 331-1689, was also the F_2 individual with the greatest number of cells per leaf, calculated as 115.26 x 10^6 cells per leaf. However, the mean epidermal cell area of 331-1689 was small relative to the mode for the population, at 294.33 μm^2, giving the impression that a great number of small cells account for the large leaf area in this genotype.

The maximum petiole length of *P. trichocarpa* was 49 mm, compared to 126 mm in *P. deltoides*. This shows a marked contrast between the anatomy of the original parents of this pedigree. Both F_1 genotypes were intermediate for petiole length relative to their parents, with the male F_1 maximum at 85 mm and the female F_1 maximum at 105 mm. The longest F_2 individual petiole length was 156 mm, observed in F_2 individual 331-1884. Figure 3.17 is the distribution of mean petiole lengths in the F_2 population with the mean values for the parental material indicated. The distribution is normal with a modal frequency of 45 to 54 mm, with the parental means, having little error, very similar to the maximum values.

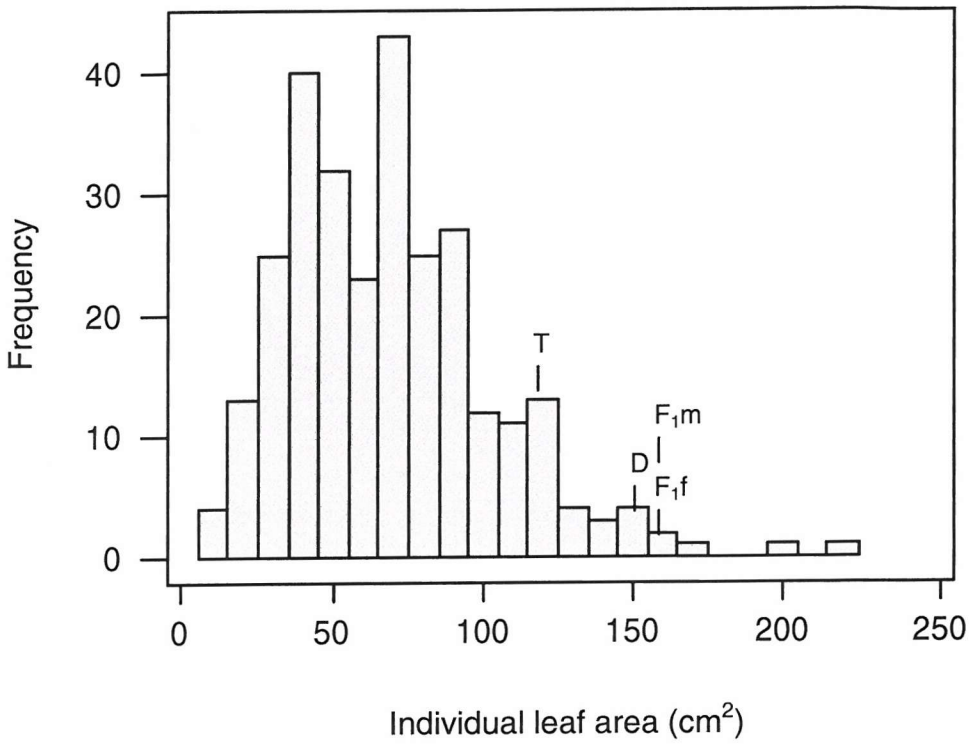


Figure 3.12 Distribution of leaf area phenotypes in the Family 331 pedigree, at 165 DAC. Replicates from all blocks in the field are included, representing 279 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

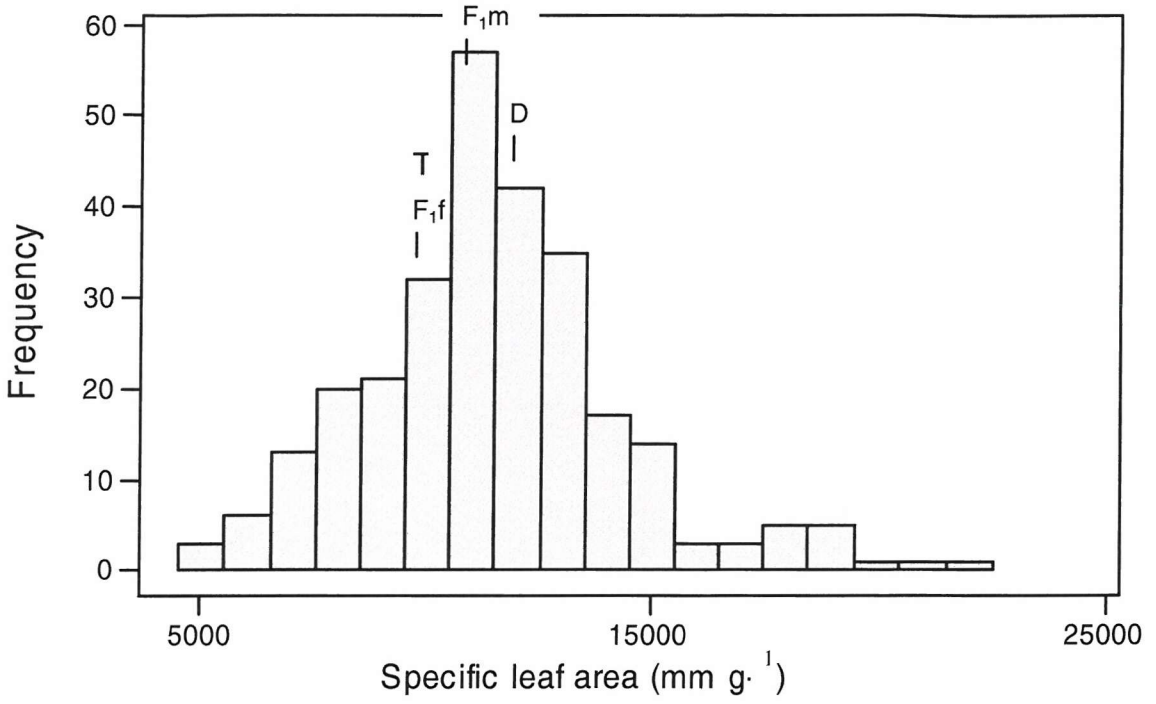


Figure 3.13 Distribution of specific leaf area phenotypes in the Family 331 pedigree (165 DAC). Replicates from all blocks in the field are included, representing 274 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

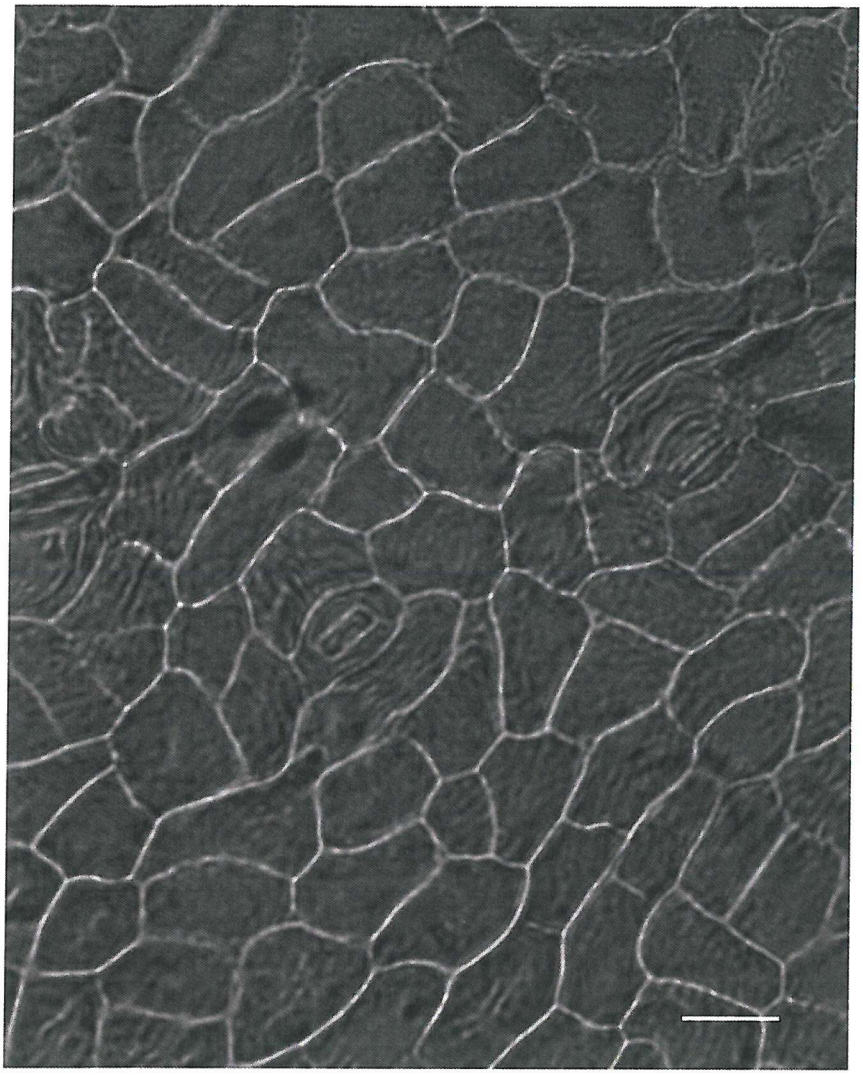


Figure 3.14 Digital image of a typical adaxial epidermal imprint from a Family331 (F_2) individual. Scale bar represents 50 μm.

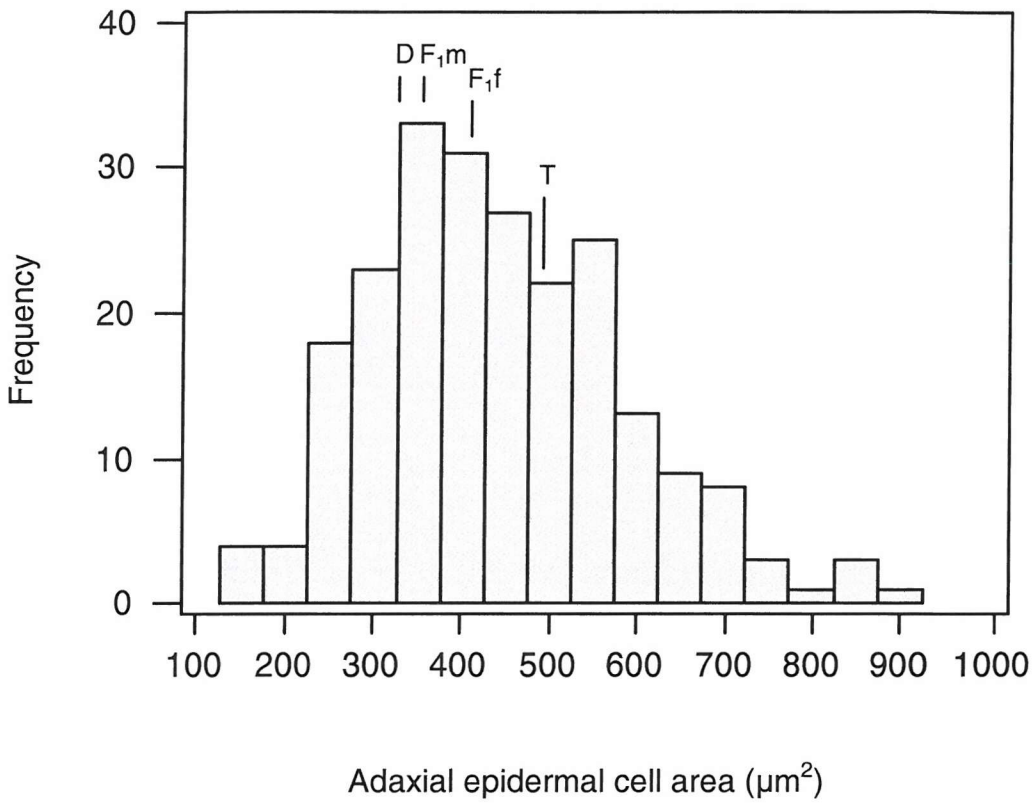


Figure 3.15 Distribution of adaxial leaf epidermal cell area phenotypes in the Family 331 pedigree, at 172-174 DAC. Replicates from block A in the field are included, representing 221 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

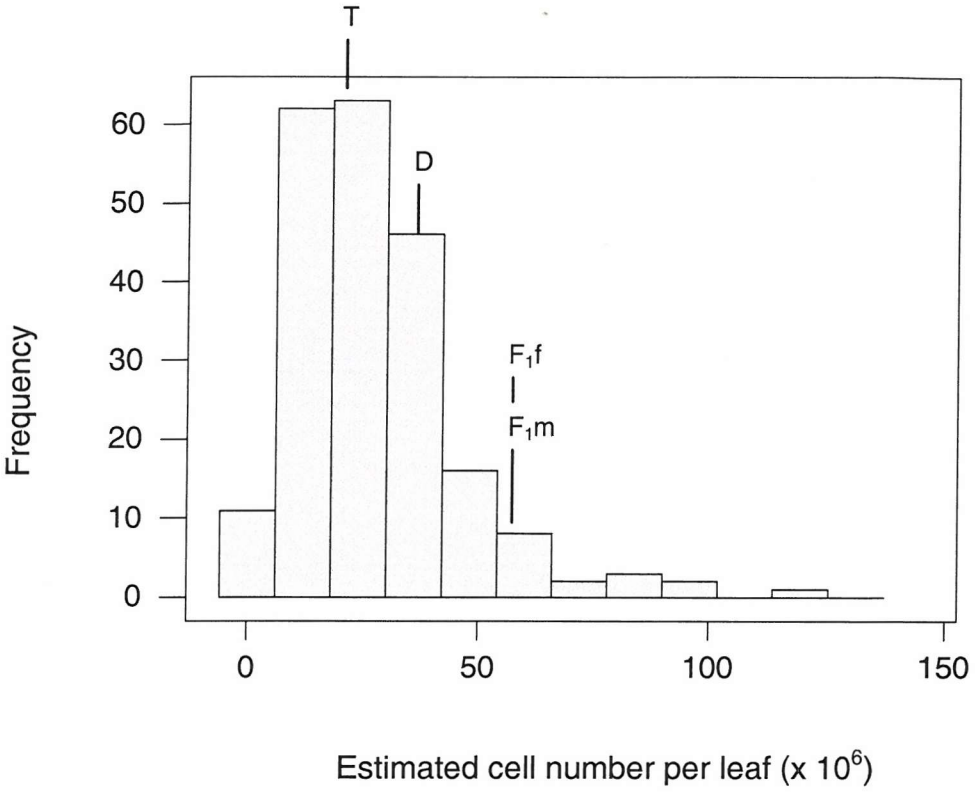


Figure 3.16 Distribution of estimated adaxial epidermal cell numbers per leaf phenotypes in the Family 331 pedigree, at 172-174 DAC. Replicates from block A in the field are included, representing 221 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

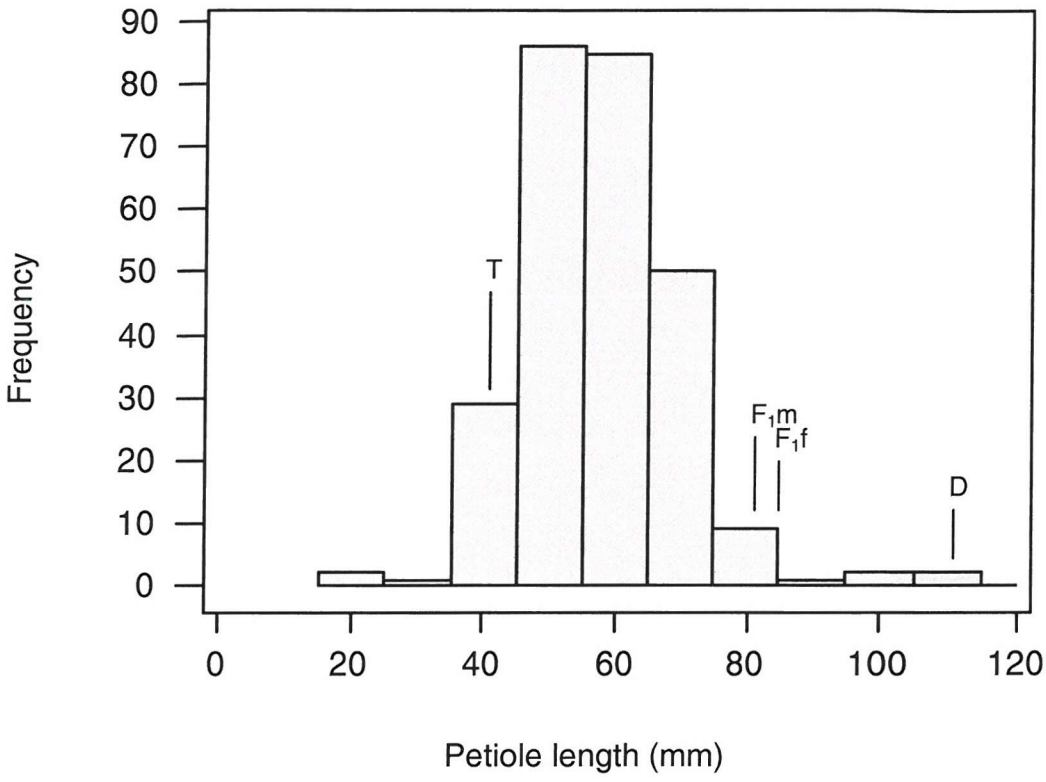


Figure 3.17 Distribution of petiole length phenotypes in the Family 331 pedigree, at 214 DAC. Replicates from all blocks in the field are included, representing 262 genotypes. Maximum parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

3.3.6 Leaf growth rates

The earliest dates of Spring bud burst are shown in Table 3.4. Of the parental plants, the earliest *P. trichocarpa* plant to flush was at week 5, whereas in *P. deltoides,* the earliest plant flushed at week 7. The male F_1 first flushed at week 5, and the female F_1 at week 6. The earliest F_2 individual to flush was genotype 331-1621 which burst leaf at week 3. The distribution of bud burst dates in the mapping population is shown in Figure 3.18. It can be seen that maximum and mean bud burst dates in each of the original parents and F_1s are very similar. The modal category for the population was week 7. The final week of measurement, when all live buds had burst into leaf, was week 10.

At 140 DAC the maximum number of leaves on the leading stem of *P. trichocarpa, P. deltoides,* and the male and female F_1s were all very similar (Table 3.4). The maximum was much greater in the F_2 population, however, with F_2 individual 331-1677 attaining the largest number of leaves. Figure 3.19a shows the distribution of leaf numbers in the population with a modal frequency of 14 leaves, with the mean leaf numbers of both parents and both F_1s exceeding this number. The smallest number of leaves on the leading stem was two. At this time point the maximum Plastochron index of *P. deltoides* was 17.5, higher than *P. trichocarpa*, which was 14.0. The F_1s exceeded both parents with values of 19.0 and 18.9, and in turn the F_2 was superior to the F_1s, with the highest index observed in 331-1677 with a value of 34. The distribution of this trait was approximately normal (Figure 3.19b), as with the number of leaves on the leading stem at this time point. The slowest Plastochron indices were between 5 and 6. The maximum leaf production rate was slower in *P. trichocarpa* (0.43 leaves d^{-1}) than in *P. deltoides* (0.57 leaves d^{-1}). The male F_1 mirrored *P. deltoides*, but the female F_1 was higher (0.71 leaves d^{-1}). These rates were exceeded in the F_2 by 331-1944, which had a maximum leaf production rate of 2.29 leaves d^{-1}. Leaf extension rates were higher on the first, compared to the second leaf measured on each plant. Again, *P. deltoides* (4.29 mm d^{-1}) exceeded *P. trichocarpa* (2.14 mmd^{-1}), with the male F_1 intermediate between the parent species at 3.86 mm d^{-1} and the

female F_1 exceeding *P. deltoides* slightly at 4.86 mm d^{-1}. The maximum F_2 individual leaf extension rate was 7.57 mm d^{-1} in two genotypes, 331-1093 and 331-1918. The second leaf extension rates mirrored the pattern of the first in these six genotypes. The distribution of leaf extension rate phenotypes is shown in Figure 3.20a and 3.20b, where the consistent hierarchy of parental species and F_1s is clear. The modal frequency for LER 1 is 3.25 to 3.74 and for LER 2 is 2.00 to 2.49.

Table 3.4 Leaf development traits of Family 331 parents, F_1's and the F_2 mean at 137-144 DAC and 174-181 DAC. Maximum values for each trait are shown for each genotype. Maximum F_2 values represent the greatest of all individuals of between 248 and 282 F_2 individuals. The genotype bearing the F_2 maximum value is stated.

	P. trichocarpa 93-968	*P. deltoides* 14-129	F_1 male 53-242	F_1 female 53-246	F_2 maximum Family 331	F_2 Genotype(s)
Week of Spring bud flush (earliest)	5	7	5	6	3	331-1621
137-144 DAC						
Leaf number on leading stem	20	21	21	21	38	331-1677
Plastochron index	14.0	17.5	19.0	18.9	34	331-1677
Leaf production rate (leaves d^{-1})	0.43	0.57	0.57	0.71	2.29	331-1944
Leaf extension rate 1 (mm d^{-1})	3.29	4.29	3.86	4.86	7.57	331-1093 331 -1918
Leaf extension rate 2 (mm d^{-1})	2.14	3.29	2.57	3.57	5.43	331-1615
174-181 DAC						
Leaf number on leading stem	29	33	27	30	42	331-1939
Plastochron index	32.2	32.4	28.3	29.0	42	331-1939
Leaf production rate (leaves d^{-1})	3	3	3	2	1.81	331-1122 331-1701 331-1809
Leaf extension rate 1 (mm d^{-1})	10.42	6.14	7.71	6.43	4.22	331-1642 331-1645
Leaf extension rate 2 (mm d^{-1})	6.86	4.71	5.71	4.23	3.18	331-1686

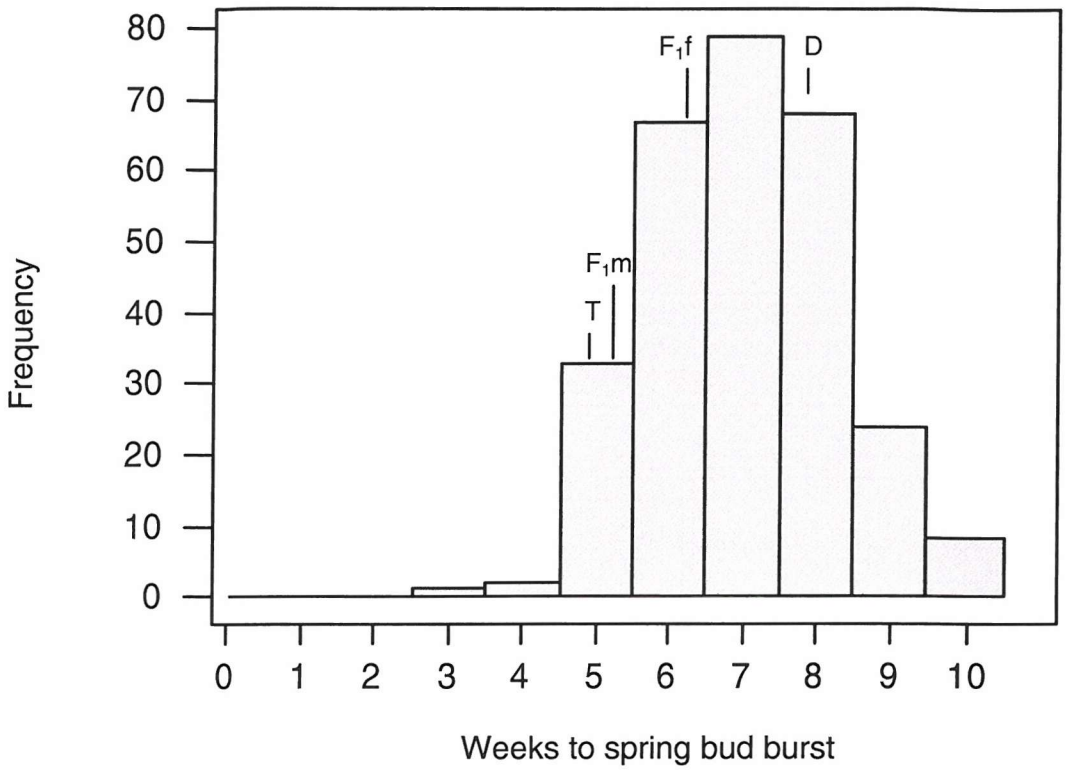


Figure 3.18 Distribution of numbers of weeks to spring bud burst in the Family 331 pedigree from the first week of measurement. Plants were scored between 69 and 133 DAC. Replicates from all blocks in the field are included, representing 277 genotypes. Mean parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

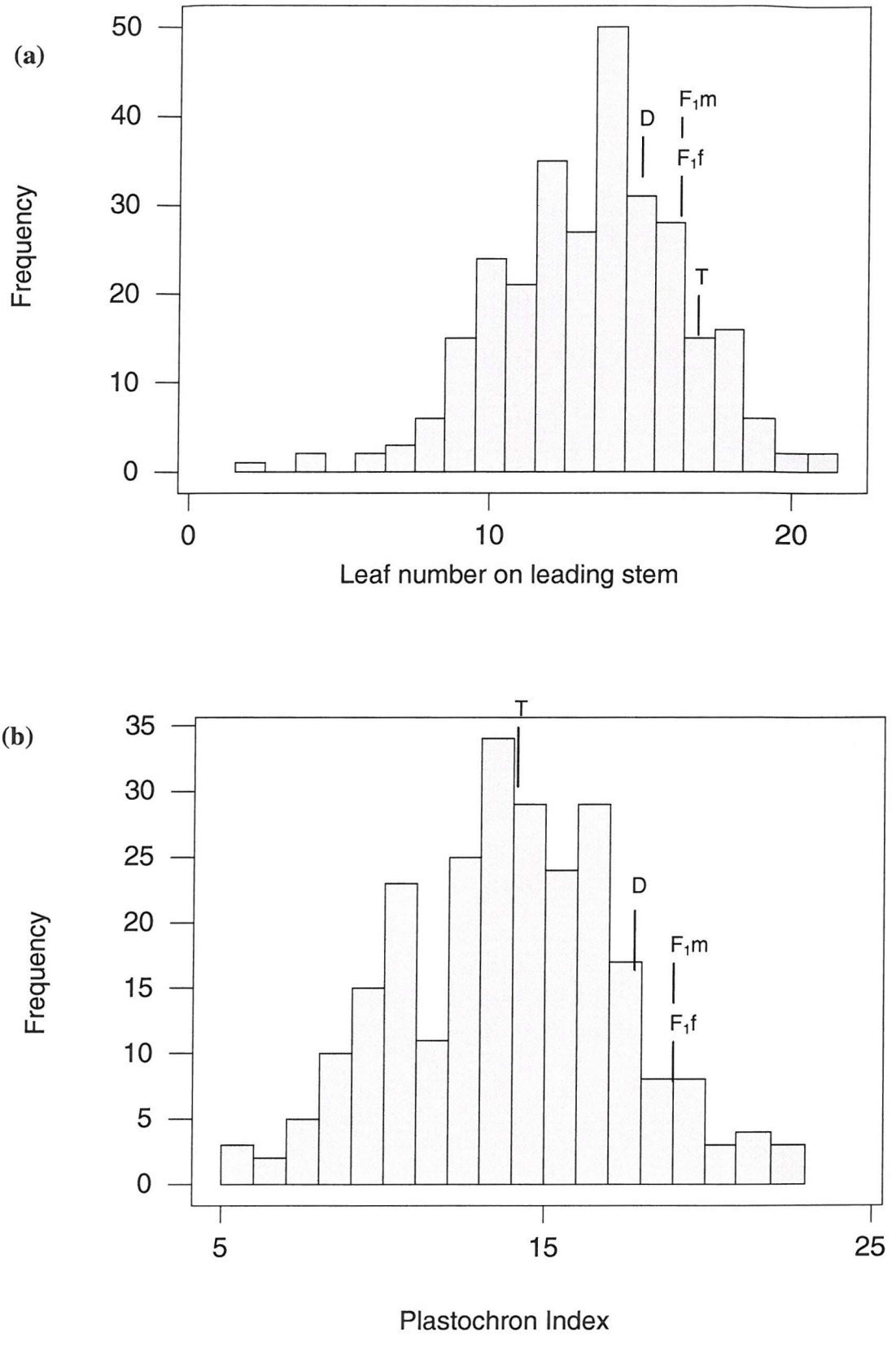


Figure 3.19 Distribution of (a) leaf number leading stem of the coppice stool and (b) Plastochron Index phenotypes in the Family 331 pedigree, measured at 140 DAC. Replicates from all blocks in the field are included in (a), representing 281 genotypes, and 248 genotypes in Block A are represented in (b). Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

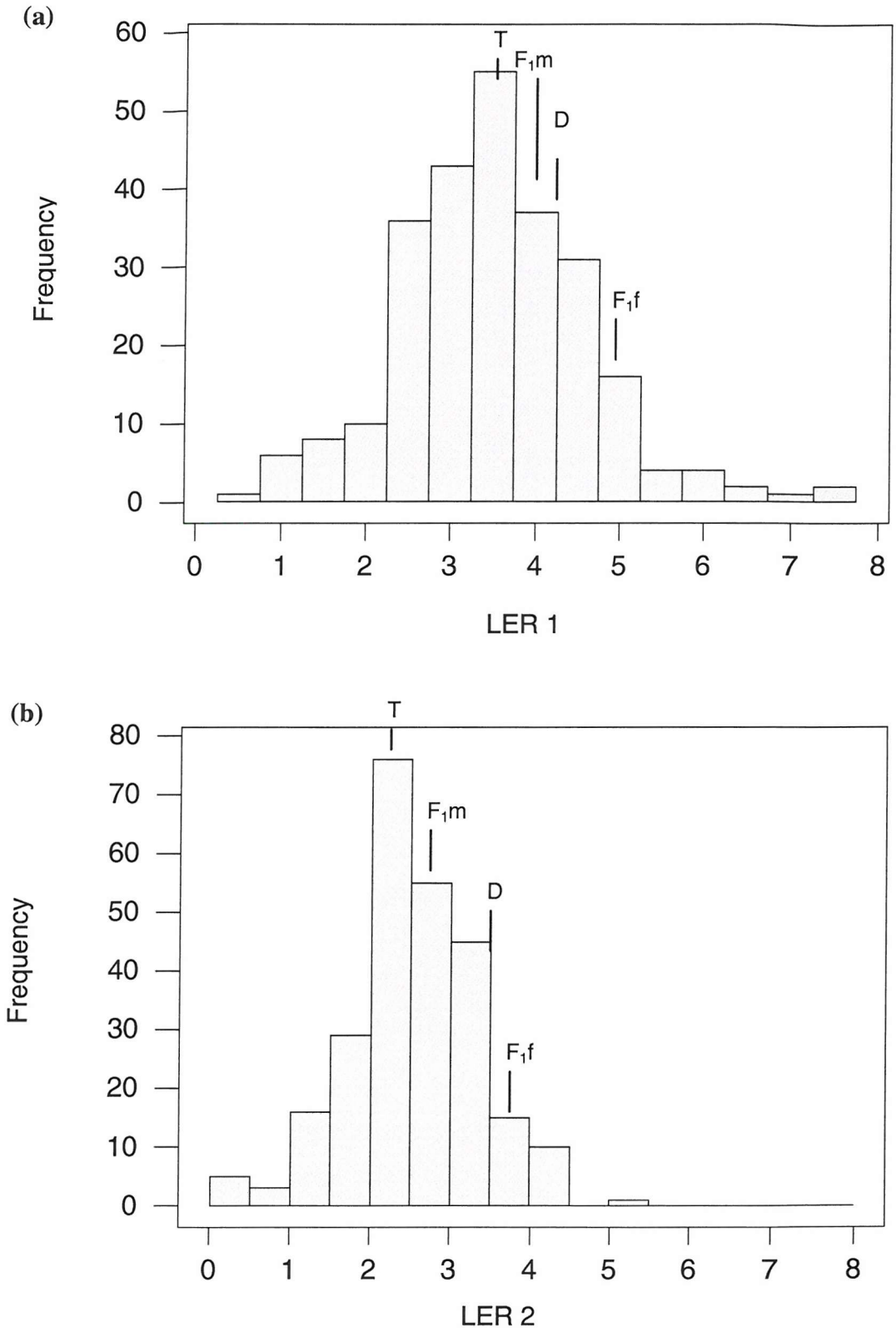


Figure 3.20 Distribution of leaf extension rate phenotypes, (a) LER 1 and (b) LER 2, on the leading stem of the coppice stool in the Family 331 pedigree, measured at 137-144 DAC. Replicates from all blocks in the field are included, representing 252 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

Later in the growing season, at 174-181 DAC, the leaf growth rates were invariably higher than at 137-144 DAC. At 181 DAC, a slight increase in maximum leaf number on the leading stem was observed in *P. deltoides* relative to *P. trichocarpa,* a difference of 4 leaves, as shown in Table 3.4. The maximum leaf number on the female F_1 was 30 compared to 27 in the male F_1. The maximum leaf number on the leading stem in the F_2 population was observed in 331-1939, which had 42 leaves. The distribution of mean leaf numbers (across all replicates of each genotype) is shown in Figure 3.21a. The modal frequency in the F_2 is between 22 and 23 leaves. At this stage in the season, the lowest mean number of leaves on the leading stem was 7 and 8. At 181 DAC the Plastochron indices of the parents were the same to 2 significant figures in *P. trichocarpa* and *P. deltoides,* at 32.2 and 32.4. The male and female F_1 indices were close to the parents, at 28.3 and 29.0 respectively (Table 3.4). The maximum F_2 Plastochron index was 42, in genotype 331-1939, the individual with the greatest leaf number. The distribution of phenotypes for this trait was approximately normal, with a modal frequency of 22.0 to 23.9 (Figure 3.21b). Leaf production rates were identical (3 leaves d^{-1}) in *P. trichocarpa, P. deltoides* and the male F_1, and lower (2 leaves d^{-1}) in the female F_1. The highest value was shared by three F_2 individuals, 331-1122, -1701 and -1809, at 6 leaves d^{-1}. The maximum first leaf extension rate at 174-181 DAC in *P. trichocarpa* was 10.42 mm d^{-1}, a rate much greater than that of *P. deltoides* (6.14 mm d^{-1}). None of the F_2 individuals exceeded *P. trichocarpa* in maximum first leaf extension rate, with the fastest rates in genotypes 331-1642 and -1645; even the maximum F_2 331-1686 did no exceed *P. trichocarpa* in maximum second leaf extension rate with a value of 9.71 mm d^{-1} compared to the 6.86 mm d^{-1} in the maternal parent. The distributions of these traits in the population are shown in Figure 3.22a, where the modal frequency is 4.00 to 4.49 mm d^{-1}, and Figure 3.22b, where the modal frequency is correspondingly lower for the second, younger leaf, at 3.00 to 3.49 mm d^{-1}.

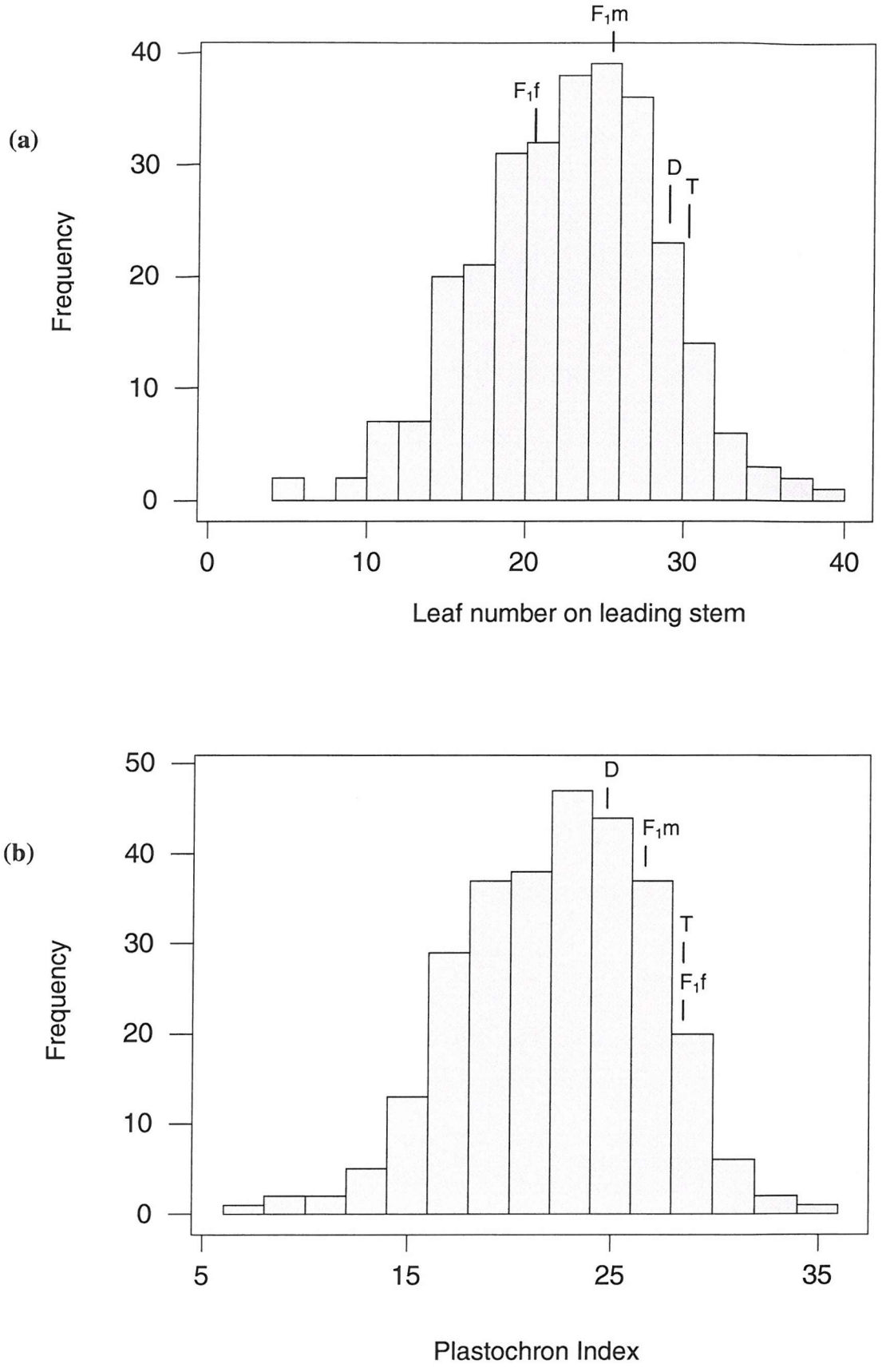


Figure 3.21 Distribution of (a) leaf number on the leading stem of the coppice stool and (b) Plastochron Index phenotypes in the Family 331 pedigree, measured at 181 DAC. Replicates from all blocks in the field are included, representing 279 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

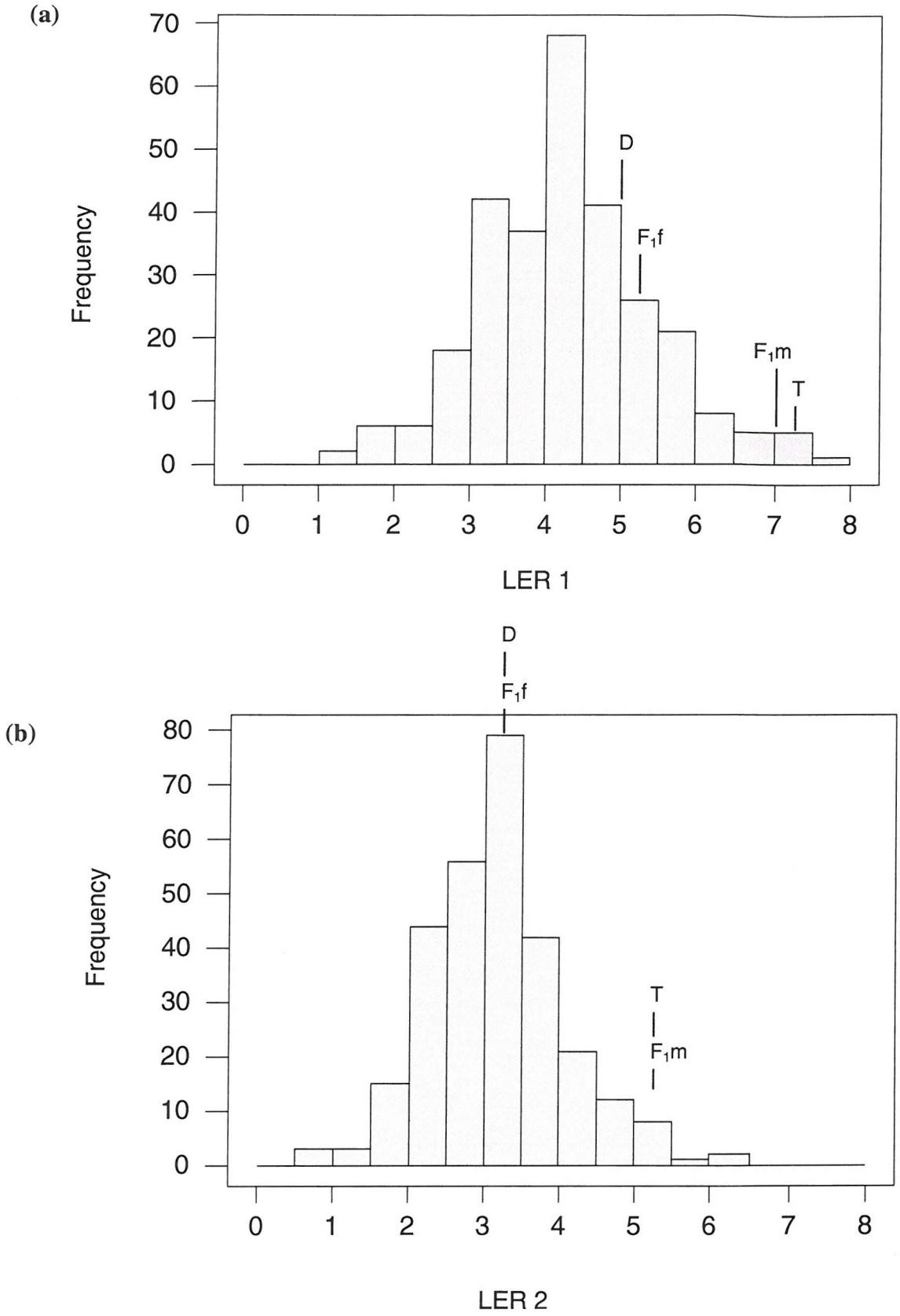


Figure 3.22 Distribution of leaf extension rate phenotypes, (a) LER 1 and (b) LER 2, on the leading stem of the coppice stool in the Family 331 pedigree, measured at 174-181 DAC. Replicates from all blocks in the field are included, representing 281 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

Carbon isotope analysis revealed the contrasting patterns of discrimination against ^{13}C in the parental genotypes, *P. trichocarpa* and *P.deltoides.* Their mean values are indicated in Table 3.5 and the frequency distribution of this trait in the pedigree is shown in Figure 3.23.The two replicates were found to be similar enough for the values to be mean for the purpose of analysis.

3.3.7 Biomass

Family 331 genotypes varied greatly in their biomass yield at the time of harvest (359 DAP). There was a consistent relationship between dry and fresh weight across the 20 genotypes, of different sizes, which were selected for measurements for drying (Figure 3.24). The mean moisture content of the wood was calculated as 53.06% (s.d. 15.33, n = 20). The regression of fresh weight and dry weight showed a significant, tight positive relationship, $R^2 = 0.9959$ ($F_{1,18} = 4403$, $P< 0.001$).

The distribution of fresh weights of the whole trees at harvest (359 DAC) is illustrated in Figure 3.25. The skew of this distribution shows the tendency towards smaller trees in the F_2 population than in the parental genotypes.

3.3.8 Other traits

Traits measured on non-continuous (or nominal) scales are shown in Table 3.6. Data are shown for Herbivory damage, Rust at mid height and the presence or absence of sylleptic branches at harvest. The parental and F_1 means are shown in addition to the F_2 mean and error.

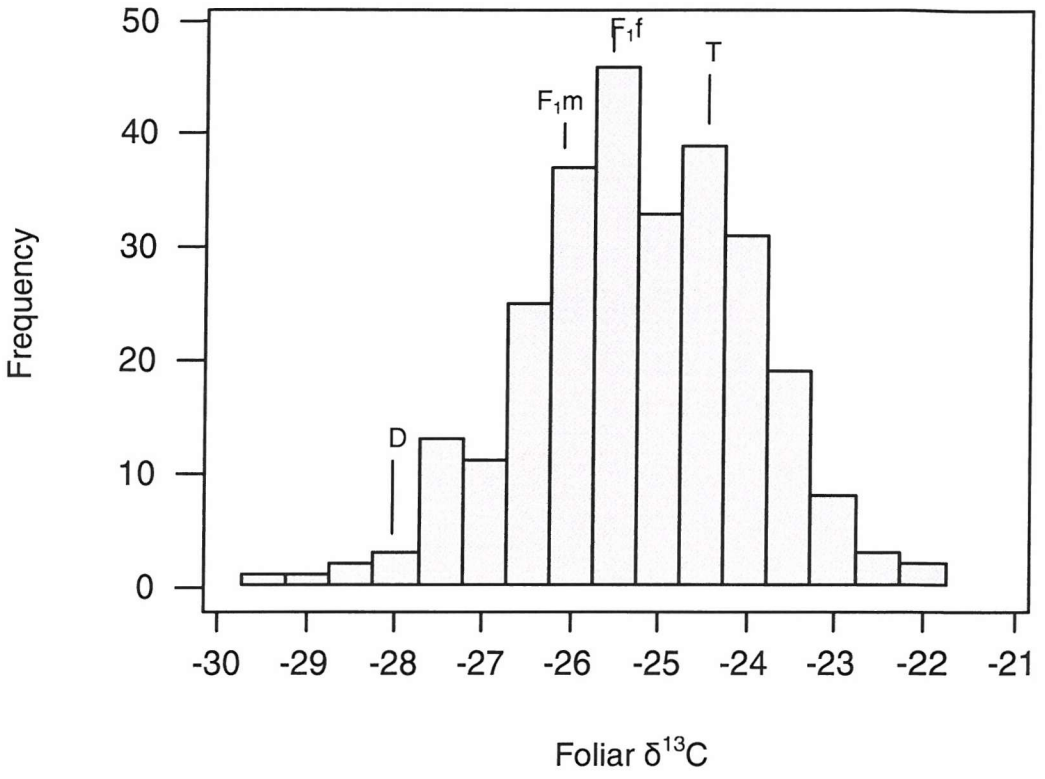


Figure 3.23 Distribution of foliar $\delta^{13}C$ phenotypes in the Family 331 pedigree, at 224 DAC. Two replicates of each genotype from the field are included, representing 269 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

Table 3.5 Values of foliar $\delta^{13}C$ for Family 331 parents, F_1's and the F_2 mean at 224 DAC. Mean values of two replicates for each trait are shown for each genotype. The mean F_2 (± standard deviation) value represents 277 F_2 genotypes.

P. trichocarpa 93-968	*P. deltoides* 14-129	F_1 male 53-242	F_1 female 53-246	F_2 mean (± SD) Family 331
-25.27	-28.29	-26.34	-25.86	-25.17 (1.46)

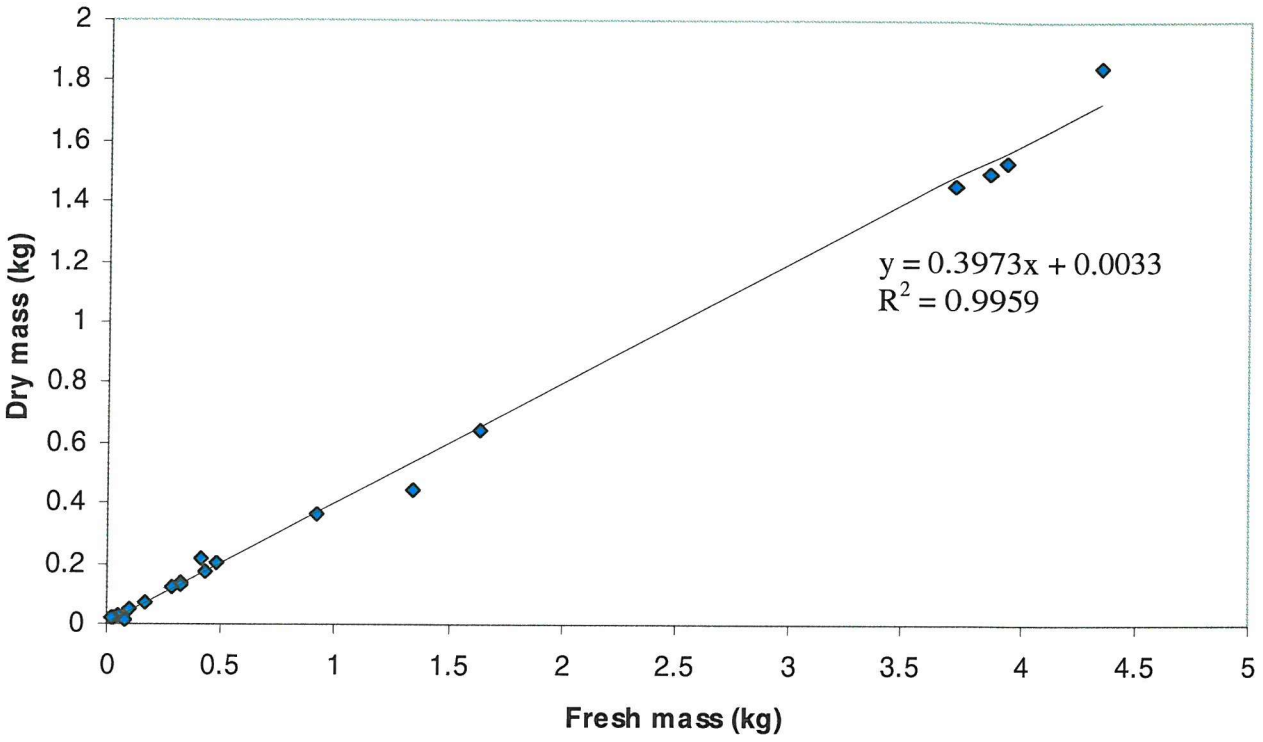


Figure 3.24 Relationship of fresh mass to oven dried mass for a sub-sample of 20 individuals from the Family 331 field trial harvested at 359 DAC. Regression: $F_{1,18} = 4403$, $R^2 = 0.9959$, $P < 0.001$

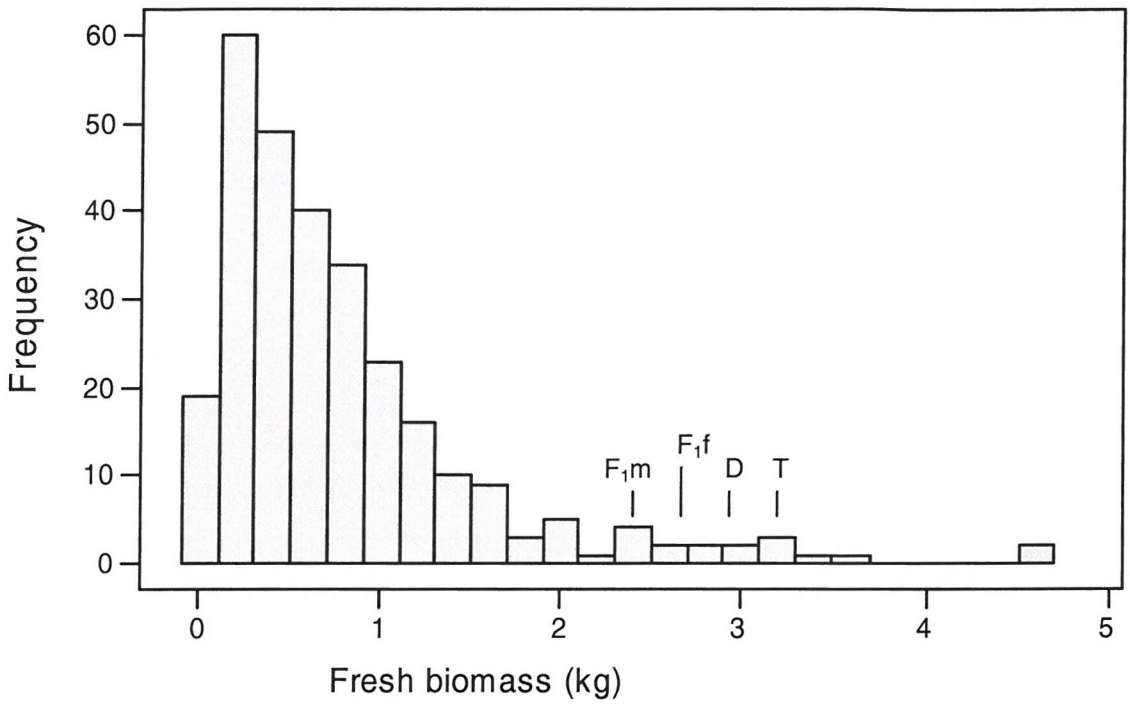


Figure 3.25 Distribution of whole tree above-ground fresh weights in the Family 331 pedigree at time of harvest (366 DAC). Replicates from all blocks in the field are included, representing 281 genotypes. Parental and F_1 values are indicated as T (*P. trichocarpa,* female parent) and D (*P. deltoides,* male parent) and F_1m (male F_1) and F_1f (female F_1).

Table 3.6 Phenotypes scored in coppiced Family 331 parents, F_1's and the F_2 mean on nominal data scales. Median values are shown for each parental and F_1 genotype. The F_2 maximums represent all live replicates of 278 (herbivory damage), 281 (sylleptic branch presence) and 282 (rust) F_2 genotypes. Standard errors of F_2 values are shown in parentheses.

Trait	*P. trichocarpa* 93-968	*P.deltoides* 14-129	F_1 male 53-242	F_1 female 53-246	F_2 mean mean (s.e.)
Herbivory damage (0 or 1)	0	1	1	1	0.57 (0.020)
Sylleptic branch presence (0 or 1)	1	1	1	1	0.58 (0.024)
Rust at mid-height (0 to 5)	0	0	1	2	1.11 (0.046)

3.3.9 Principal Components Analysis

The results of principal components analysis (PCA) are shown in Figures 3.26, 3.27 and 3.28. The score plot for all replicates of all genotypes grown in the field trial are shown in Figure 3.26. The ellipse represents the 95 %tolerance boundary for the data conforming the model using the Hotelling's T^2 test, a multivariate equivalent of the Student t-test. The individuals outside the boundary are considered to be biological outliers.

Figure 3.27 shows the first analysis of PCA using the rust scores as a "qualitative" variable in the Simca-P program. This is a detail of the main loading plot for the first two components, showing the positions of the scores relative to each other. There was no apparent link between the scores attained and each component. The data were therefore re-analysed in Simca-P, this time entering the trait as a standard "*x*" variable to eliminate this confusion. The final loadings plot is shown in Figure 3.28. This shows the loadings for the two components used. Syllepsis (0), bud burst and herbivory negatively affected the first component (PC1). Rates of leaf and stem growth clustered in the lower, right quartile, with a slight positive influence on PC1 but a negative influence on PC2. The most influential traits on both components were the number of stems on the coppice stool and measurements of sylleptic branching; both of these characteristics pulled the first component closer to biomass. Stem heights measured at different developmental stages positioned close to harvested biomass in the first component and individual leaf area and cutting diameter had a similar, although weaker effect.

Consideration of the score plot and loading plots together show the positions of the outliers in the score plot relative to the traits for which they exhibit extreme character in the loadings plot. Examples of this are 331-1918 for stem height (359 DAC) and 331-1674 for stem number (359 DAC).

Traits not shown in the PCA (and also PLS analysis) in Simca-P either showed too great a variation for the trait or too high a proportion of the data missing, and were eliminated from the analysis. These traits were leaf adaxial epidermal cell area, adaxial cell number per leaf, and $\delta^{13}C$.

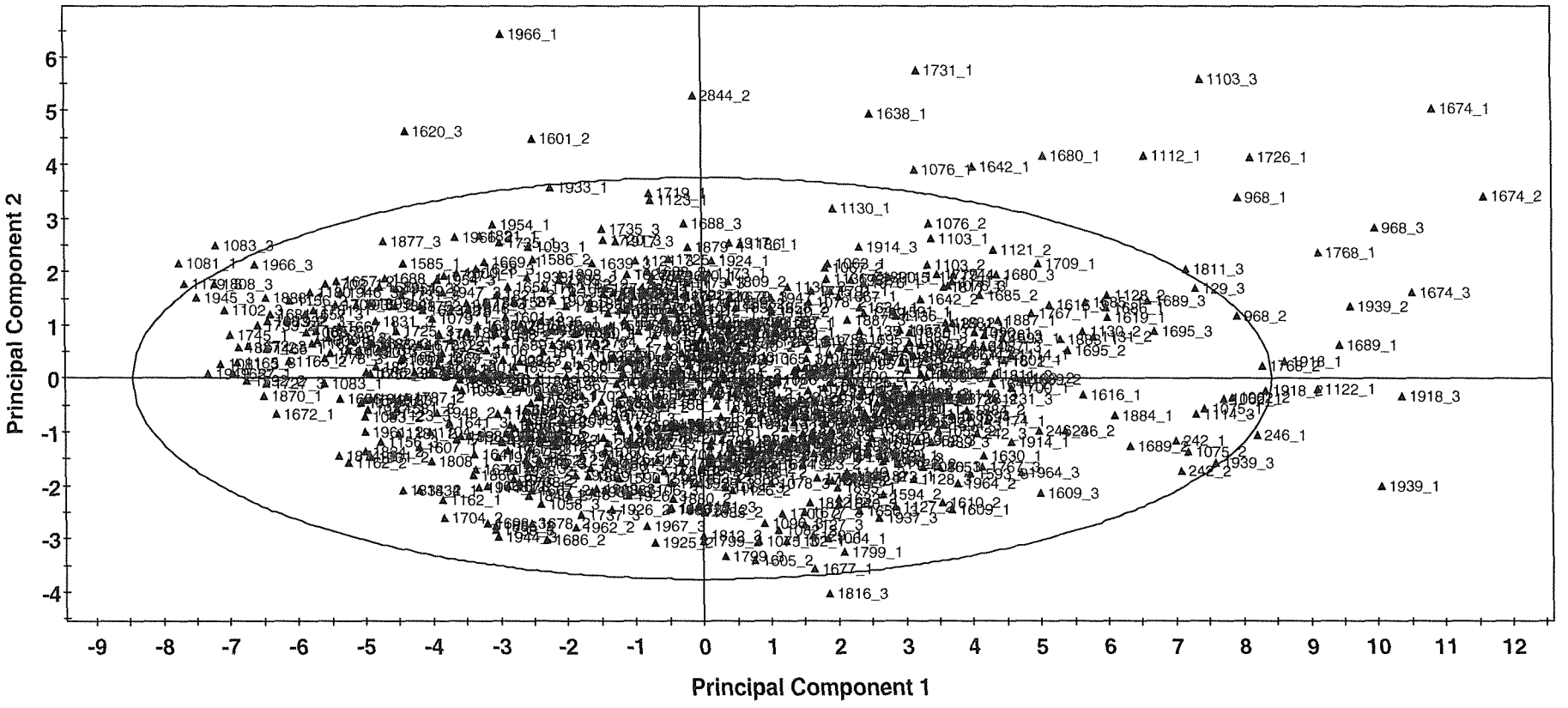


Figure 3.26 Score plot of the first and second principal components for all individuals in the field trial. The ellipse represents the 95% tolerance boundary of the Hotelling's T^2 test.

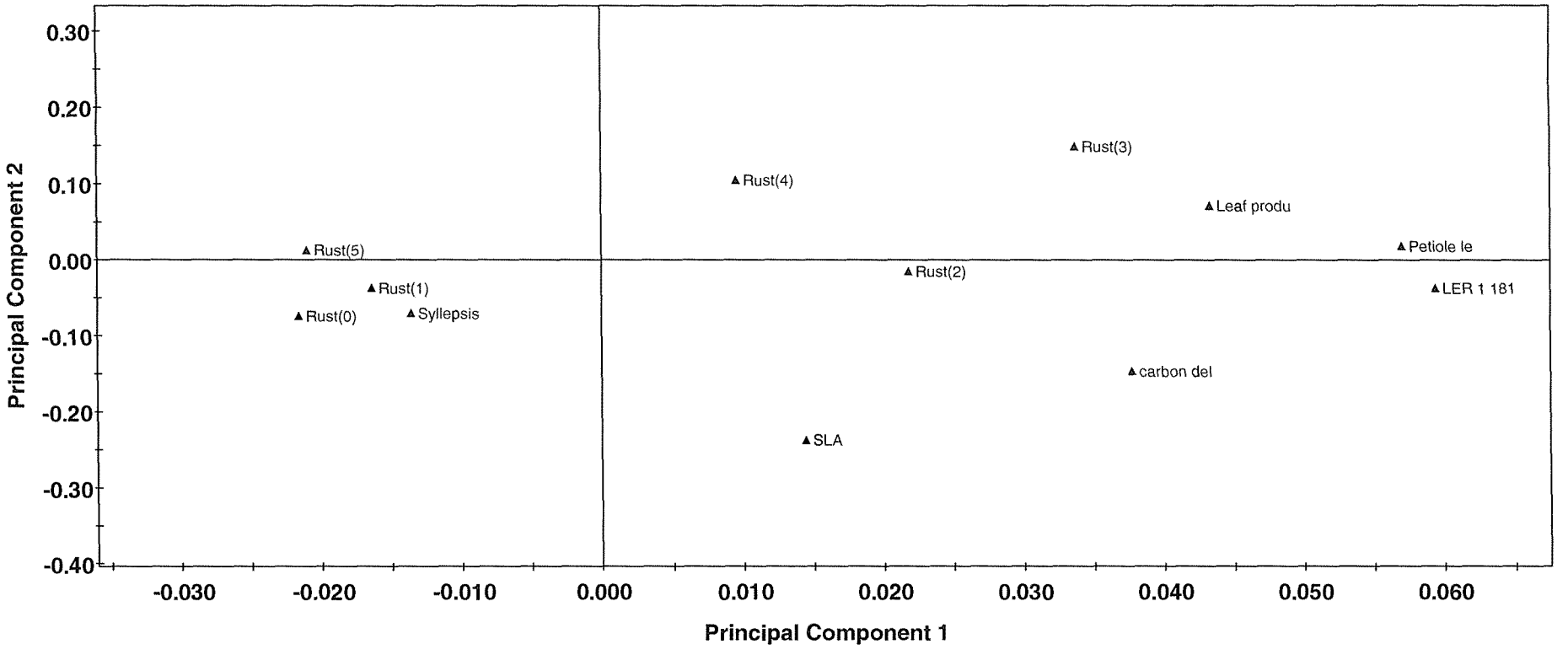


Figure 3.27 Detail of principal components analysis loading plot where rust scores were set as a qualitative trait. The relative positions of the five rust scores, (1) to (5), are shown. The varying influence of the different rust scores on the PCA model, for both components 1 and 2, is apparent.

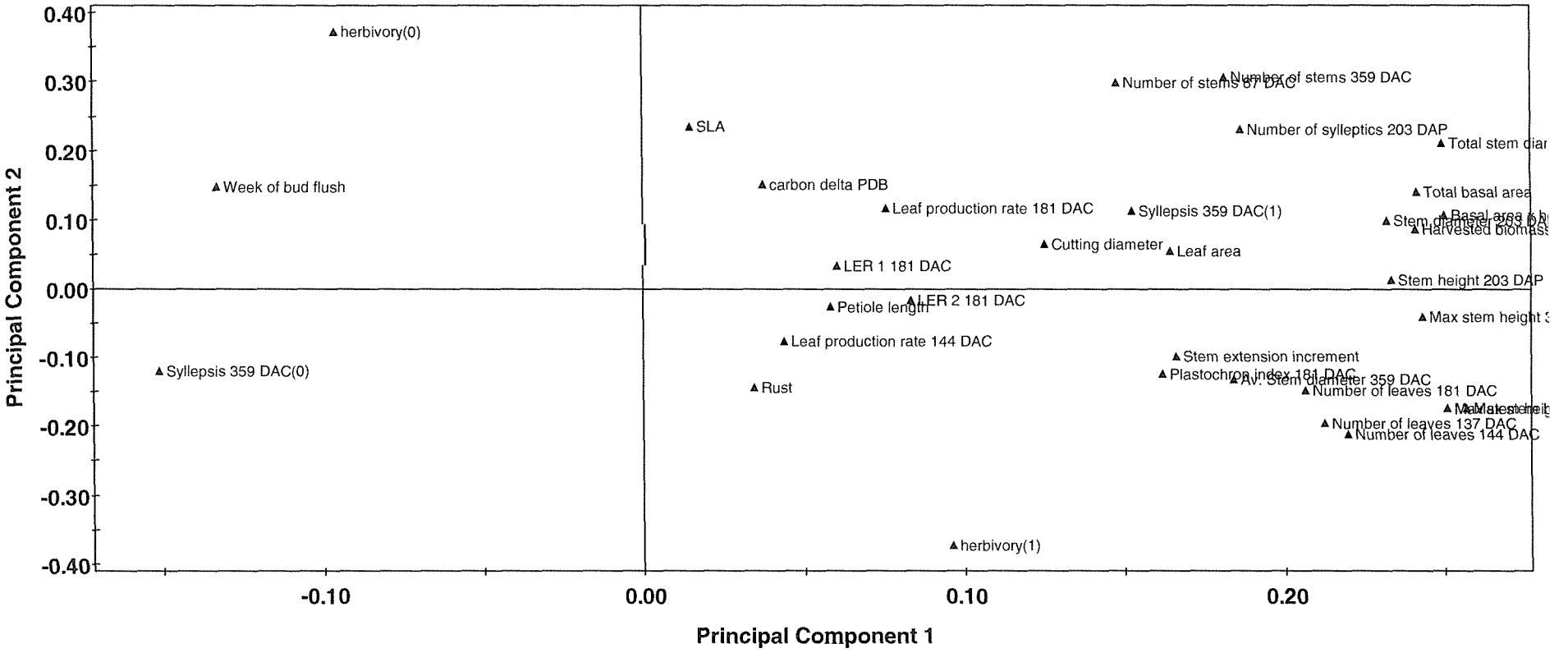


Figure 3.28 Principal components analysis loading plot of all replicated traits in the field trial. All traits were set as qualitative *x* variables with the exception of herbivory and syllepsis (359 DAC), which were set as qualitative traits.

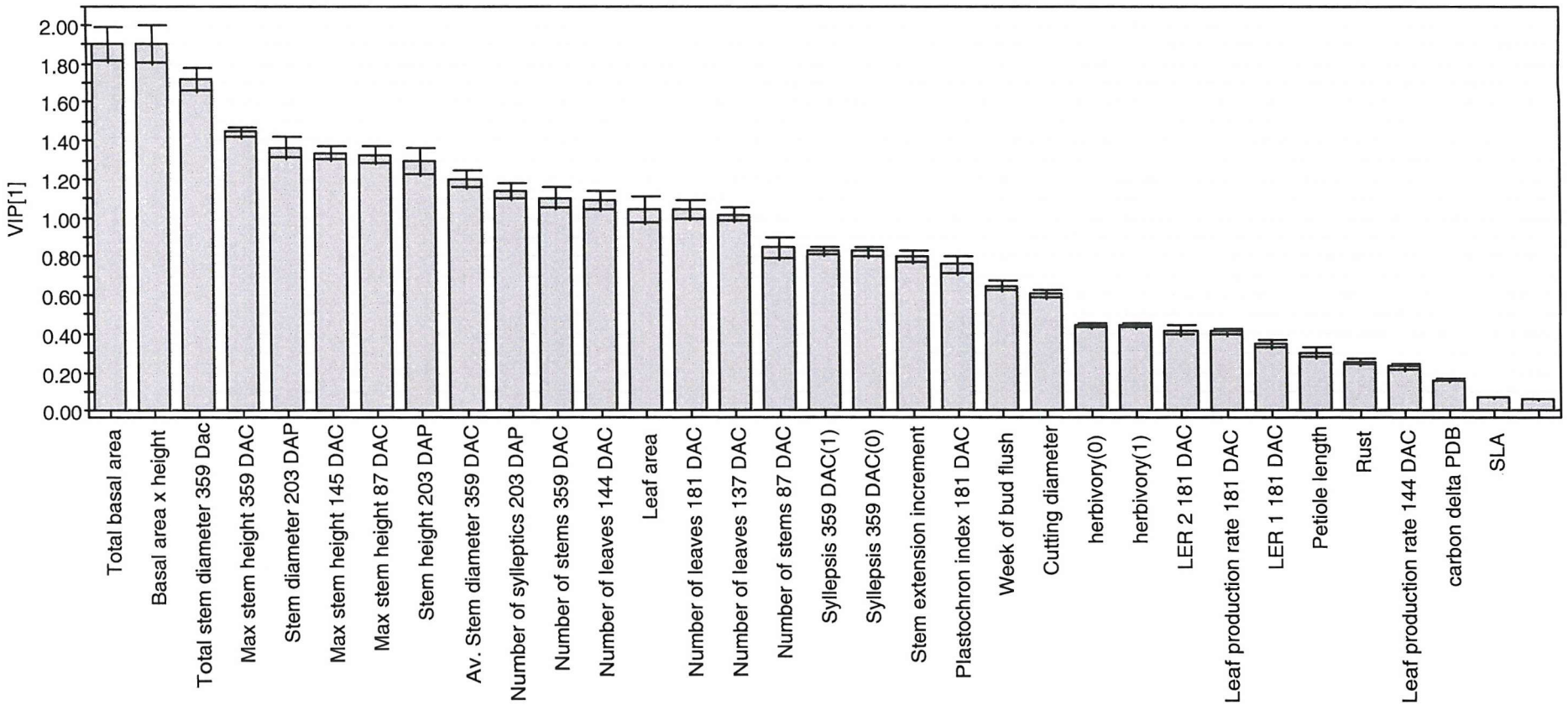


Figure 3.29 Variable importance plot (VIP) of traits in the first PLS component [1], where $R^2 = 0.925$, $Q^2 = 0.914$. The model was validated with 500 permutations and 3 components, where the the R^2 *y*-intercept, 0.0077and Q^2 *y*-intercept was -0.115. Error bars represent confidence intervals derived from jack-knifing.

3.3.10 Partial Least Square Regression (PLS)

A PLS model was constructed using 33 traits, explained by two components. Figure 3.29 shows the Variable Importance Plot (VIP), explaining the model, of traits in the first PLS component [1], where $R^2 = 0.925$, $Q^2 = 0.914$.The model was validated with 500 permutations and 3 components, where the the R^2 *y*-intercept, 0.925and Q^2 *y*-intercept was 0.914, which exceeded the significance thresholds of 0.00767 and 0.05 for R^2 and Q^2 respectively.

The VIP plot shows that stem traits were the *x* variables mostly responsible for the explaination of the *y* variable, harvested biomass. The variables, or traits, with the highest importance as biomass indicators were total basal area and basal area x height. These traits were equal in their importance, so height did not appear to contribute further to the importance to biomass of basal area. The most important leaf traits to the model of biomass were leaf number on the leading stem and individual leaf area. Traits with a variable importance below 1.0 were unlikely to have had any significant role in influence on biomass production in the population as a whole. These data describe the population, however it is possible that some individual genotypes may influence their own biomass through high or low activity of other organs or characteristics.

3.3.11 Broad-sense heritability

Several of the traits measured in the field trial showed high broad-sense heritability (H^2) values, over 0.5. All broad-sense heritability values that could be calculated are shown in Table 3.7. The most highly heritable traits were coppice stem traits These traits were total basal stem area and maximum stem height, with H^2 values 0.799 and 0.753 respectively. The third most heritable trait was the number of leaves on the leading stem (140 DAC),

with a heritability of 0.639. The date of Spring bud burst was also highly heritable, with a value of 0.549. Some of the traits measured in single stems were also highly heritable: maximum stem height (203 DAP) with an H^2 value of 0.565 and number of sylleptic branches (203 DAP) with an H^2 value of 0.558. Basal stem diameters showed values of H^2 over 0.5, in both single stem (0.526) and coppice (0.504) prior to harvest.

3.3.12 Preliminary QTL analysis

Table 3.8 shows the results of preliminary QTL analysis. QTL are shown with LOD scores above 2.6. One QTL for fresh weight (harvested biomass) on linkage group B explained nearly half of the variation for this trait. No other QTL mapped to this linkage group. Two leaf traits were located on linkage group C, cell area and Spring bud burst. These QTL had overlapping confidence intervals and were located reasonably close to one another. The QTL for cell area explained over half of the genetic variation for this trait. Another estimated 25.9 % of the genetic variation for cell area was explained by a cell area QTL on linkage group E, and one more was found on linkage group M with a LOD score of 3.53 explaining 32.8 % of the variation. Both were found to be close to QTL for maximum stem height. Cell number was found to have one QTL on linkage group D, with large confidence intervals. One individual leaf area QTL was mapped to linkage group I, explaining 28.1 % of the variation. Other leaf traits for which putative QTL were located were leaf number on the leading coppice stem on linkage group O, close to a QTL for stem height, and three QTL explaining LERs, on linkage gropus J, P and R. The QTL for the leaf extension rates explained between 16 and 23 % of the varation for these traits. A total of four QTL were identified in this analysis for maximum stem height at 359 DAC, and two cases these co-located with QTL for the same trait measured at 145 DAC. On linkage group Y, one QTL was identified for stem height prior to coppice, at 203 DAP. Two QTL were found for stem extension incremement: on linkage group J, explaining 39.3 % of the variation, and on

linkage group Y, explaining 32.2 % of the variation for the trait. One QTL was located for stem number, but none were found for stem diameter.

Table 3.7 Broad-sense heritabilities of all replicated traits in the field trial, with traits ranked from highest to lowest heritability. Broad-sense heritability calculated as V_G (genetic variance)/V_P (total variance).

Trait	Heritablility
Total basal stem area (359 DAC)	0.799
Maximum stem height (138-145 DAC)	0.753
Leaf number on leader (140 DAC)	0.639
Fresh weight (at harvest)	0.613
Maximum stem height (359 DAC)	0.602
Leaf number on leader (181 DAC)	0.586
Maximum stem height (203 DAP)	0.565
Number of sylleptic branches (203 DAP)	0.558
Date of Spring bud burst	0.549
Basal stem diameter (203 DAP)	0.526
Total basal stem diameter (359 DAC)	0.504
Stem number on stool (137 DAC)	0.406
$\delta^{13}C$	0.386
Sylleptic branch presence	0.372
Rust	0.371
Stem number on stool (359 DAC)	0.341
Plastochron index (181 DAC)	0.334
Stem extension increment	0.287
Individual leaf area	0.158
Leaf production rate (137-144 DAC)	0.142
Leaf production rate (174-181 DAC)	0.137
Leaf extension rate (174-181 DAC)	0.092
Herbivory damage	0.035

Table 3.8 Linkage group positions, confidence intervals, LOD scores and percentage variation explained by the QTL for the traits studied in the field trial. QTL were determined to be present above the LOD threshold of 2.6. Confidence intervals marked as "off end(s)" indicates that they extend beyond the linkage group end(s).

Linkage Group	Position	Confidence Interval	LOD	% Variance explained	Trait
B	4.0	off end – 14.0	3.07	45.5	Fresh weight
C	63.6	46.0 – 82.3	5.00	53.8	Adaxial leaf epidermal cell area
	87.2	59.6 – 106.2	3.30	37.6	Week of Spring bud burst
D	72.5	7.8 – off end	2.74	37.9	Adaxial cell number per leaf
E	81.6	49.4 – 81.5	2.65	25.9	Adaxial leaf epidermal cell area
	46.0	off end – 85.6	2.61	16.7	Maximum stem height 359 DAC
I	88.1	71.0 – off end	2.73	28.1	Individual leaf area
J	17.4	off end – 23.3	3.63	39.5	Maximum stem height 138 DAC
	17.4	off end – 23.3	3.56	39.6	Maximum stem height 145 DAC
	17.4	off end – 23.3	2.84	39.3	Stem extension increment
	21.3	off end – 45.4	3.79	36.4	Maximum stem height 359 DAC
	21.3	off end – 59.2	2.91	22.1	LER 1, 137 – 144 DAC
M	43.4	14.0 – 61.2	2.93	25.3	Maximum stem height 359 DAC
	47.4	14.0 – 77.5	2.62	26.1	Maximum stem height 145 DAC
	73.5	49.4 – 81.5	3.53	32.8	Adaxial leaf epidermal cell area
O	46.0	22.7 – off end	2.94	29.9	Leaf no. on leader 140 DAC
	55.8	36.0 – end	3.60	61.1	Maximum stem height 359 DAC
P	28.9	off ends	3.20	23.8	LER 2, 137 – 144 DAC
Q	24.2	off ends	2.63	23.3	Stem number 359 DAC
R	8.7	off ends	2.80	16.3	LER 2, 174 – 181 DAC
Y	2.0	off ends	3.00	35.1	Stem extension increment
	17.8	off ends	3.56	32.2	Stem height 203 DAP

3.4 DISCUSSION

Many environmental factors affect plant growth and therefore biomass production. Of particular importance to short rotation coppice crops, in addition to cultural practice, are humidity, day length, solar radiation, temperature and wind. More detail of these effects is given in Isebrands *et al.* (1996). The emphasis of this study has not been on environmental factors, but to observe the performance of the Family 331 pedigree in the coppice habit. In so doing, this experiment has identified the leaf and stem measurements most indicative of yield and investigated the genetic control of the traits measured. This has lead to suggestions being made for future tree breeding for the biomass industry.

The experimental design incorporated a buffer of 'Gaver' and this is a good attribute in a small field trial because, in small research trials, edge effects can be significant. A study by Cannell (1988) illustrated the impact of edge effects in the poplar genotype 'Tristis' grown in small plots, where the yield was 10 ODT ha^{-1} yr^{-1}, and in large plots at the same site where yields were between 25 and 30 ODT ha^{-1} yr^{-1}.

Ordination techniques, such as PCA, take a set of interdependent variables to extract major patterns amongst them. For PCA analysis, variables should, ideally, be either all categorical or all continuous variables, and each sampling entity (in this case, each tree) should be sampled using the same set of variables. True outliers, in the sense of biological outliers, should be eliminated from analysis, as they pull the component axis in a direction unrepresentative of the data cloud. However, extreme observations in the data set are in fact valid and informative, and should therefore be included (McGarigal

et al.,2000). Here, the extreme outliers provide much information about the genotypes in the field trial relative to the traits measured.

Eriksson *et al.* (2001) describe the principles of PCA. The first principal component (PC1) is the line in multi-dimensional space that best fits the data cloud in a least squares sense. It goes through the average point in the data set. Each observation, projected onto this line, receives a new co-ordinate value in a new co-ordinate system. It is this value that is the score. PC1 is not normally enough to model the variation in a data set sufficiently; thus PC2 is introduced, orthogonal to PC1, and this also crosses through the average point, to improve the approximation of the data set as much as possible. Both components define a model plane, such as that illustrated in Figure 3.28. The variables contributing similar information are grouped together. In this figure, negatively correlated values appear in opposite quadrants. In this instance, opposite traits are the absence (score 0) of sylleptic branches at 359 DAC and total basal diameter (Figure 3.28). Loadings, interpreted in this way that they affect the direction of the model plane in multivariate space, influence the direction of PC1 relative to the original variables (prior to the assignment of scores). The second set of loadings explains the direction of PC2. Any further PCs are required to again cross the average point in the data cloud and be orthogonal to the previous components. (Eriksson *et al.*, 2001; McGarigal *et al.*, 2000). Loadings give meaning to the scores, so they together provide information to interpret.

Although they are extreme in nature, strong outliers conform to the overall correlation occurring in PCA; they help an overview to be made of relationships between traits. Most of the extreme outliers are the biggest trees, especially in first component. This is

evident in the trait distributions also, where a small number of the genotypes appear in the highest frequency-group. Of the approximately 900 individual plants grown in the field, only 30 are extreme outliers in Figure 3.26, which emphasises the conformity of most genotypes to the 95 % tolerance ellipse. This would suggest that to incorporate new traits into the next generation, the best candidates for breeding would be the strong outliers. It is clear from the largest F_2 genotypes for traits in Tables 3.2 to 3.4 that they are clearly outliers in a positive direction for first two components.

Using univariate statistics to test each possible combination of an independent variable with a dependent variable increases the probability of a Type I (false positive) error occurring due to chance variation (McGarigal *et al.*,2000). For this reason, the correlation of each of the 33 traits measured in the field trial study against yield (fresh weight) would infer that two of them could be a false positive at $P = 0.05$. For the same reason, this data set is not appropriate for correlating each of the 33 traits against biomass.

Partial least squares to latent structures (PLS) modelling is designed for use with many – often correlated – x values in order to understand how they affect the y value (Eriksson *et al.*, 2001). The score vectors calculated for each of x and y are model coefficients for the variables; these are weights indicating the importance of each x variable (the trait measured) and how it relates to the modelling of y (biomass at harvest), relative to the other traits. PLS is thus a regression extension of PCA, used for interconnecting the information on x and y variables. The advantages of PLS are its ability to handle many variables, to analyse noisy data, to deal with multicollinearity, and process incomplete variables (handle missing data) (Van Eeuwijk *et al.*, 2002). The

larger the number of *x* variables, the better the outcome of a PLS model. First developed by Wold (1978), PLS was further developed by Wold *et al.* (1980) to facilitate the analysis of complex data sets, especially where regression was not applicable or violated. It has been extensively used for the analysis of chemical data but is becoming recognised as a tool to analyse biological data e.g. dimension reduction prior to QTL analysis (Lan *et al.*,2003) and the analysis of seed traits in forest trees (Tigabu, 2003). Vargas *et al.* (1998) used PLS regression to determine the relative performance of wheat cultivates to environmental variables. Other biological systems have also used a PLS regression approach to understand biomass yield (De la Vega *et al.* 2001).

An F_2 population is highly suitable for PLS because of the divergence of segregating traits. It is also appropriate for this approach because within the F_2 the observations for a trait are not truly independent; such multi-collinearity is handled by PLS but such data is less suitable for multiple and stepwise regression (Eriksson *et al.*, 2001; Zar, 1999), hence the PLS approach here is an extension of the preliminary, multiple regression approach by Rae *et al.* (2003). Contrary to tolerating multicollinearity, an assumption of PLS is that *x* and *y* variables are multicollinear, making the process more realistic than conventional regression. PLS accepts nearly any shape of data matrix, in terms of numbers of columns and rows. Unlike the multiple regression approach, the results of PLS do not only indicate the extent to which traits contribute to biomass, but how to adjust the process, in this case through genotypic modification (using whatever means) to adjust the process to achieve the desired effect.

A high degree of confidence can be expected in the model chosen, after verification using 500 permutations and 3 components, and the *y* intercepts of Q^2 and R^2 being below threshold for significance (Tigabu, 2003).The model can be further validated using a measurement of a sub-sample of the population and testing the fit of the same data set to the model obtained for the 2000 and 2001 growing seasons. The model could further be investigated at the next harvest, although it is possible that in a field trial of a different age, different traits may have different weightings on their influence on biomass.

The variable importance plot (Figure 3.29) shows that stem traits and leaf number and leaf area are the traits contributing most strongly to biomass in the PLS model. This would appear intuitive given that harvested biomass is a direct measurement (the fresh weight) of a combination of stem diameter, stem number and stem height. The next most important trait is the count of sylleptic branches at the end of the establishment year, which has a less direct influence on the biomass measurement at the end of the first coppice rotation. The leaf traits that ranked highest are leaf number on the leading stem and individual leaf area, which are the only leaf traits that have variable importance scores in excess of 1.0. These traits would be worthy of more detailed investigation in future coppice seasons.

Moisture contents of the selected genotypes in this study were similar to the average moisture content for poplar (at harvest) of 60%. This is comparable to moisture content of most SRC poplar and willow, which usually varies between 54% and 61% (Kauter *et al.,* 2003). Fresh weight and dry weight were strongly correlated in the sub-sample

of the population selected for drying (Figure 3.24), providing a high degree of confidence in the use of dry weight for comparisons with other studies.

Studies of poplar biomass productivity are not always easy to compare owing to some variation in the expression of yield. Some papers quote incremental growth (mean annual increment, MAI), others the percentage of accumulated biomass, others again state the distribution amongst biomass components (Tuskan and Rensema, 1992). Different sites around the world are studied with varying soil types and fertiliser inputs, contrasting planting densities and coppice rotations, in addition to the differing genotypes under study. For this reason, it is possible to adopt process models to predict growth and choose the correct genotype for a particular site (Isebrands *et al.,* 1996).

Cuttings should ideally be 1 cm or more in diameter to ensure rooting success and also to survive in commercial trials where weeding is not always viable and competition with weeds is a real concern (Kauter *et al.,* 2003). Cutting diameter was not of noteworthy influence in the PLS model (Figure 3.29), ranking 22nd of the 33 traits given a VIP score. In the PCA loading plot, cutting diameter was a moderate influence in PC2, but in PC1 was slightly more influential on harvested biomass, with a loading of 0.125. Thus, to increase cutting diameter could increase biomass yield. It is uncertain as to whether SRC trees of large biomass yield produce cuttings of large diameter, or whether it is the cutting size that does influence the final yield of the coppice stool. There may, therefore, be genetic reasons for the production of thick cuttings, or for cutting quality. Cutting traits have been investigated at the U.K. Forest Research Agency (personal communication from A. Armstrong, Forest Research, 2001).

In the coppiced field trial, the stem height and diameter traits positively influenced both PC1 and PC2 (Figure 3.28), falling very close to harvested biomass. Indeed, Tuskan and Rensema (1992) observed a positive correlation of 0.96 between tree height and coppice sprout diameter. Total stem diameter x height, maximum stem height and total basal diameter at harvest (359 DAP), and single stem height and single stem diameter at 203 DAP, all strongly affect biomass in PC1. The number of coppice stems and the presence of sylleptics (359 DAC) and the number of sylleptics (203 DAP) are stronger influences on PC2 than on PC1. This interpretation of these results are expanded in the PLS regression analysis (Figure 3.29), which shows the strong influences of the stem measurements in determining the harvested biomass. Total basal area was ranked as having the highest importance. No further influence on harvested biomass was obtained by multiplying total basal area by maximum stem height, as these two traits have an identical influence on the PLS model. This reflects other studies in trees: in formulating equations for use in estimating tree biomass, stem diameter can explain a high proportion of the variation for in biomass, and adding stem height into the equation provided little extra explanation in terms of biomass yield. Furthermore, in high density plantations, height can be difficult to measure (Onyekwelu, 2003; Fuwape *et al.,* 2001; Wang *et al.,* 2000; Verwijst and Telenius, 1999). Some researchers advocate that equations should be based on diameter at breast height alone (Wang *et al.,* 2000). The next most important trait was the total stem diameter in the coppice stool, a function of total basal area. Maximum stem height was ranked as the fourth most important trait. Stem diameter, stem height and the number of sylleptics at 203 DAP were ranked with a higher variable importance in the PLS analysis than any of the leaf traits, indicating that first year measurements in the single stem habit, just prior to coppice, were

influential in the final harvest biomass at the end of the first coppice growing season. Cross-sectional basal stem area, the most influential trait measured on harvested biomass, can be measured easily in a field trial and the data can be recorded using digital callipers (Telenius, 1997b). At this coppice stage, it was a strong indicator of yield. Future coppice harvests may or may not reflect this finding, since over several rotations, the stools may suffer mortality due to pests and diseases, lack of re-sprouting vigour and other fatalities.

The ability for SRC crops to re-sprout is of paramount importance; furthermore, the best genotypes should survive the disturbance of a continuous cycle of cutting back, and accordingly re-sprout with vigour (Sims *et al.,* 1999; Verwijst, 1996; Sennerby-Forsse *et al.,* 1994). There was a 16.4 % mortality rate in the field trial in the establishment year. There are a number of possible reasons for this. Some cuttings did not sprout following planting, either due to lack of reserves in the cutting material to enable sprouting, or the poor rooting ability of certain genotypes. A review by Ceulemans *et al.* (1996a) summarised that, on average, coppiced poplars produce between five and eight stems on the coppice stool, although sometimes more are produced. In the Family 331 field trial some coppice stools have yielded very large numbers of stems in the first coppice season, up to 30 live stems in mid growing season (137 DAC) by the F_2 genotype 331-1674, which appeared to mirror, and exceed, *P. trichocarpa* in its production of large stem numbers. *P. trichocarpa* is indeed recognised as producing many suckers from both its roots and its shoots (Critchfield, 1960). The genotypes producing the greatest numbers of stems or stem diameters stems were positioned as outliers in the PCA score plot in the upper right quartile (Figure 3.26) with high scores for PC2. Their influence on their respective traits can be seen by

comparing their positions on the score plot with those in corresponding position on the PCA loading plot (Figure 3.28). These individuals were genotypes 331-1103 and 331-1731 (which produced the greatest stem number at 359 DAP), 331-1674 (which produced the greatest total basal diameter at 359 DAP) and 331-1731 (which produced the largest number of stems at 359 DAP).

In addition to stem diameter influencing harvested biomass, the genotypes in the pedigree with the tallest maximum stem heights also strongly influenced PC1 as seen in both the PCA score plot and loadings plot (Figures 3.26 and 3.28). Those with the greatest stem heights, 331-1918 (the tallest F_2 at 359 DAC), 331-1674 (the tallest F_2 at 145 DAC) and 331-1939 (the tallest F_2 at 203 DAP) are positioned close to the loading for the stem height measurements on PC1. One point to consider is the usefulness of very large stems, if they are produced, to mechanised harvesting. If the basal diameter of a coppice stem exceeds 10 cm, it is not possible to use automated harvesting methods for short-rotation coppice trees; instead traditional felling methods have to be deployed, thus increasing the cost of harvest (Kauter *et al.,* 2003).

In studies over several growing seasons, the height extension in a tree stem is more widely expressed as the mean annual increment or an increment for a whole growing season (Bradsaw and Stettler, 1995). However in the one year observation of coppice in Family 331, stem extension increment has provided an indication of stem growth rate and its relation to harvested biomass. Although its influence on PC2 was negative, it was clustered with other growth characteristics such as leaf number on the leading stem and plastochron index.

Syllepsis has been found to be linked to increased stem wood production or growth vigour in several studies of *Populus* (Scarascia-Mugnozza *et al.*, 1999; Barigah *et al.*, 1994). In the experience of Ceulemans and Isebrands (1996), some poplar genotypes produce sylleptics early in the season, others later in the season. They have observed that genotypes with acute branch angles and high leaf area density are ideal as short-rotation coppice poplars, since they intercept the most light and do not take up too much space in the field. It has been recognised that *P. trichocarpa* produces predominantly sylleptic branches, *P. deltoides,* predominantly proletpics (Wu, 1998). In the PCA loadings plot (Figure 3.28), the score for no sylleptics (syllepsis 359 DAC (0)) was weighted near to –0.05 PC1, whereas the presence of sylleptics (syllepsis 359 DAC (1)) had a loading close to +0.15, thus positively influencing PC1 towards the loading for harvested biomass. Leaf positions on *Populus* branches affect the resources contributed to wood and stem production. Carbon isotope studies of *Populus* have shown that the contribution of sylleptic branches to plant growth is important during the establishment year (Scarascia-Mugnozza *et al.,* 1999). The presence of sylleptics provides an advantage to the tree early in the season, demonstrated when branchy genotypes export a high contribution of ^{14}C to the upper stem and new branches; later in the season, this contribution was diverted towards the lower stem and roots (Scarascia-Mugnozza *et al.*, 1999). Leaf positions on *Populus* branches affect the resources contributed to wood and stem production.

Isebrands *et al.* (1996) stressed the importance of understanding the processes occurring within the leaf in order to predict growth responses in the whole plant. In particular they drew attention to the export of carbon from leaves and the translocation of nutrients. Individual leaf areas in the field trial exhibit heterosis in the F_1 genotypes,

with F_1 leaves superior over the larger-leaved parent, and highly segregating behaviour in the F_2. This was found in the coppice field trial in the U.K., but further investigations of Family 331 leaf morphology have been made by Wu *et al.* (1997), who noticed segregation not only for individual leaf area but also leaf shape, serration and colouring. The number of leaves on the leading stem (137-144 DAC) and individual leaf area are the leaf traits in the PLS model with the greatest importance as *x* variables influencing harvested biomass, the *y* variable. This is not surprising, since both the plant canopy and the individual leaf are the organs where light capture and harvesting take place. Several studies report tight positive correlations between individual leaf area and accumulated woody biomass (Bunn *et al.,* 2003; Harrington *et al.,* 1997; Barigah *et al.,* 1994; Harrington and Fownes, 1993). Leaf area was found to be strongly correlated to seed yield in *Glycine max* (Mansur *et al.,* 1993). Furthermore, the tilt and hang of the leaf affect light interception. Although most light is intercepted by the adaxial leaf surface, as leaves become more vertical the abaxial surface contributes more to the total light interception of the leaf, up to 30 % in *Liriodendron tulipifera* (Ninnemets and Fleck, 2002). *P. deltoides* produces characteristically green abaxial leaf surfaces, owing to photosynthetic activity on this side of the leaf (Wu *et al.,* 1997; Ceulemans and Isebrands, 1996). When final leaf area was studied in six different tree species, individual leaf area was also strongly correlated with the maximum growth rate of the leaf (Nardini, 2002). The F_2 genotype 331-1689 was the leaf with the greatest maximum individual leaf area in the trial. This genotype also attained the greatest stem height at 145 DAC.

Specific leaf area (SLA) ranked very low in the variable importance plot in the PLS model. SLA describes leaf thickness (Yin *et al.,* 1999). In a study of *Salix* genotypes,

the highest yielding willow exhibited the highest SLA, but there was no correlation observed between SLA and growth rate (Weih, 2001). However, in the same study, leaf area productivity was positively correlated with relative growth rate. Thumma *et al.* (2001) found a strong negative correlation between SLA and biomass yield in *Stylosanthes scabra.* They interpreted this such that thicker leaves (with low SLA values) posses a thicker mesophyll to facilitate higher CO_2 assimilation rates. However, the PCA analysis shows that SLA has a very low loading for PC1 relative to harvested biomass, so would to detract from biomass, and a high loading in PC2, as shown in Figure 3.28. Using the interpretation of workers such as Thumma *et al.* (2001), the higher values of SLA indicate thinner leaves, and, as can be seen in the PCA loadings, increasing the values of SLA would detract further from the harvested biomass. Thus the work in Family 331 would appear to reflect that of previous findings.

Critchfield (1960) described the stages of bud burst in *P. trichocarpa.* During the initial phase, leaves protrude from the bud apex, followed by the emergence and unrolling of the leaves. Following this, the petioles reflex and the scales and stipules are shed. This pattern of bud burst, quantified into five stages of activity by Castellani *et al.* (1967) were clearly identified in Family 331 in the field trial. The dates of first bud burst across the genotypes of the population varied from 3 to 10 weeks from first investigation and followed an approximately normal distribution within the F_2 population (Figure 3.18), showing a large amount of genetic control over this trait. The parents differed in their average weeks of bud burst; this was earlier in *P. trichocarpa* averaging 5 weeks, than in *P. deltoides,* where the average was later, at 8 weeks. This result is similar to that of Bradshaw and Stetter (1995) who observed in the same population, grown in the U.S.A., bud burst in *P. trichocarpa* approximately 13 days

earlier than *P. deltoides*. The broad-sense heritability estimated for this trait is high, at 0.549. Frewen *et al.,* (2000) mapped QTL for bud burst in Family 822, half-sibs to family 331, and nominated five putative candidate genes involved in the regulation of bud dormancy. It has been pointed out as intuitive (Isebrands *et al.* 1996) that the length of leaf area duration through the growing season is related to overall plant growth. However, the PLS model shows that the timing of Spring bud burst alone does not have a high importance as a variable indicating harvested biomass. However, it can be seen from the more descriptive, PCA loadings plot (Figure 3.28) that PC1 is strongly pulled towards bud burst. Thus, to increase the number of weeks taken until Spring bud burst would further influence the model away from harvested biomass; to decrease the time taken until bud burst may be beneficial to achieving higher biomass yields. If, however, bud burst is too early in the Spring, late frosts could kill tissues, which is a disadvantage to development (Frewen *et al.,* 2000).

Interspecific hybrids of *P. trichocarpa* x *P. deltoides* are known to produce large numbers of leaves at a rapid pace (Ceulemans *et al.*, 1996a), and several genotypes in the Family 331 pedigree display this characteristic. An increase in total leaf area is a means of increasing the light interception of the whole canopy (Ninnemets and Fleck, 2002). A similar study of growth rates to the field study in the U.K. was one conducted by Ridge *et al.* (1986). Using *P. trichocarpa* x *P. deltoides* F_1 hybrids, they found leaf production rates to average 3.75 leaves $week^{-1}$, which translates to 0.536 leaves d^{-1}. This compares to a mean of 0.429 leaves d^{-1} at 137-144 DAC, and 2 leaves d^{-1} at 174-181 DAC, in the F_1s of this population (all replicates included). This raises the question of the optimum time in the season to make measurements of leaf traits most useful for the prediction of harvest biomass.

The Plastochron Index is essentially the rate of leaf initiation on a shoot (Erickson and Michelini, 1957). Different shoot apices with the same P.I. will be at similar stages of morphological development (Larson and Isebrands, 1971). Ceulemans *et al.* (1988) found that greater rates of leaf production were observed in tandem with lower rates of leaf extension in poplar, which caused late maturation of leaves. In coppice, neither leaf production rate, leaf extension rate, nor P.I. ranked highly in the PLS model, contributing little as direct indicators of harvested biomass. As seen in the PCA loading plot (Figure 3.28), the leaf extension rates at 174-181 DAC qualified for PCA (unlike those at 137-144 DAC which were excluded due to the proportion of missing data (Eriksson *et al.,* 2001) but this trait was not particularly influential in either component. Leaf production rates did not significantly influence PC1. In the second component, leaf production rate at 174-181 DAC was a positive influence with a loading of over 0.10, but leaf production rate at 137-144 DAC had a negative effect on the PCA model. P.I. at 174-181 DAC appears to be a more useful biomass indicator. In the loading plot for PC1 it appears to be positively linked to harvested biomass, and has no effect on PC2. In the PLS model it received a variable importance below 1.0, which is higher than those for leaf production and leaf extension, but not a variable of great use in the population for indicating harvested biomass at this stool age. Although the rates of early leaf production and expansion will influence the pace of canopy closure and maturity, it is important also to consider the duration of growth in the season. Michael *et al.* (1990) observed in one poplar genotype, 'Tristis,' a cessation of individual leaf production and expansion after budset, whereas another genotype, 'Eugenei,' continued these processes until leaf abscission commenced. Thus, 'Eugenei' outgrew 'Trisitis' owing to its continuing terminal meristem activity until later in the season.

Petiole length displayed a normal distribution in the F_2 population (Figure 3.17). The *P. deltoides* parent attained one of the longest petiole lengths, longer than *P. trichocarpa.* Wu *et al.* (1997) also studied other characteristics of leaf traits in Family 331 in single stems. Although petiole angles were not measured in the field trial, Wu *et al.* (1997) observed contrasting values between the parental genotypes of midrib angle (the hang of the leaf blade) and leaf angle (its tilt). It is known that the distribution of leaf angles in a tree canopy affects the extent of light interception and penetration, in addition to the manner in which light is redistributed at different levels of the canopy (Ceulemans, 1990). Leaf angles further affect canopy photosynthesis by influencing the temperature of the individual leaf and the distribution of temperature in the canopy. The petiole structure of poplars characteristically enhances leaf flutter even in slight air movements. Poplar petioles tend to be flattened, flexible and orientated perpendicular to the blade, causing a random fluttering motion to create a uniform PFD for light interception. This is advantageous for optimal carbon gain at the top of the canopy (Roden, 2002).

In the field trial, the range of foliar carbon isotope ratios ($\delta^{13}C$) was highly comparable to one other study of the same genotypes of *P. trichocarpa* x *P. deltoides* and their hybrids as studied in this experiment (Herold, 1997) and values fell within the range expected for poplar (Smith and Epstein, 1971). Water use efficiency is effectively the ratio of dry matter production to total evaporation, with instantaneous WUE the ratio of net CO_2 flux to net H_2O flux (Lindroth and Cienciala, 1996). WUE increases with decreasing discrimination against ^{13}C (Osorio and Pereira, 1994). Owing to the high costs of analysis two, rather than all three, replicates of each genotype in the population were analysed for $\delta^{13}C$, and as a result, this trait was excluded from the PCA and PLS

models; although these analyses can handle missing data, there are limits to the proportion of missing variables that can be tolerated while maintaining a reliable model (Eriksson, 2001). Leffler and Evans (1999) suggest that $\delta^{13}C$ is an average picture of the water status of a plant over the lifetime of the particular tissue measured. Studies of *Populus* in wet and dry field sites have suggested that there is less discrimination against ^{13}C, inferring greater water use efficiency, in wet plots than in dry plots (Ceulemans and Isebrands, 1996). Significant positive correlations been reported (Praslova *et al.,* 2000) between carbon isotope composition and stem height and diameter in pine in wet conditions, but the correlations were negative in dry plots. Isebrands *et al.* (1996) have considered that the water relations of a coppice stand compared to single stem plots may strongly influence biomass productivity owing to the root to leaf area ratio in coppice plants. Weih (2001) found a trade-off between fast growth and WUE in *Salix*, whereby the faster growing genotype of the two studied was more sensitive to water stress than slower growing genotype. Similarly, Osorio and Pereira (1994) in a study of *Eucalyptus globulus* observed increased WUE with decreased growth rate. In some environments, the efficiency of dry matter production may be inferred from $\delta^{13}C$; most plants with reasonably high values of $\delta^{13}C$ appear to have responses to stressed habitats, for example xeric conditions (Smith and Epstein, 1971).

Adaxial epidermal cell area was larger in *P. trichocarpa* than *P. deltoides,* but not to the extent as was found in the study in OTCs at Headley in 1999 (Chapter 4 of this thesis; Ferris *et al.,* 2002). Since this field experiment deals with different environmental conditions to the OTC study, it is possible that abiotic factors are governing leaf development processes in the field more than in the glasshouses. Like

Ridge *et al.* (1986), the adaxial cell number per leaf is an estimation serving to understand the cellular reasons for differences in leaf area between genotypes. As had been found by Ridge *et al.* (1986), the F_1 genotypes produced many more cells per leaf than either parent, with F_1 values in the region of 63 to 66 x 10^6 cells per leaf compared to 35 x 10^6 cells per leaf in *P. deltoides* and 30 x 10^6 in *P. trichocarpa.* A study of *P. trichocarpa* by Dunlap and Stettler (2001) reported adaxial epidermal cell numbers per leaf, estimated in a similar method to that used in this chapter, in the range of 74 to 211 x 10^5, several fold fewer than the range shown in Figure 3.16. The cellular characteristics of genotypes in the pedigree are further discussed in Chapter 4 of this thesis.

Melampsora larici-populina (rust) appears to have been an inadequate scoring system based on Figure 3.27. This figure of the PCA loading plot shows the inconsistency of influence of the ranked rust scores upon the first and second components. In both components, the scores were spatially confused: in PC1, the lowest and highest scores 0, 1 and 5 were clustered, with scores 4, 2 and 3 increasing in loading values respectively. Likewise in PC2, scores 3 and 5 appeared to be unranked in their loadings. This outcome suggests that the scoring should be reviewed, and the data set be re-analysed with rust as a qualitative variable (as in Figure 3.28). Often, infections in poplar may vary with season, as shown by Newcombe and Bradshaw (1996) who studied the infection of the Family 331 pedigree with, *Septoria populicola,* the cause of leaf spot. Using a presence or absence scoring system over two seasons, they found that only 69 % of Family 331 F_2s were rated identically in both years. Cervera *et al.* (1996) conducted rust experiments where the score for each genotype was calculated from an average of ten cuttings. This is a greater replication than used in this field trial.

Although the field trial of Family 331 has not shown this in the 2001 growing season, rusts in poplar and willow have been found to negatively influence stem height growth (Verwijst, 1993).

Siwecki and Przybyl (1981) explored the causal mechanisms governing the resistance of rust infection in poplar. Transpiration has been considered important for rust infection of *Populus* leaves. Differences between resistant and susceptible genotypes were observed in the functioning of stomata rather than the frequency of stomata on the leaf surface. In susceptible genotypes, the stomata were open for longer periods, whereas in resistant genotypes, stomata closed 15 minutes prior to transpiration stopping. *Melampsora larici-populina* spores penetrate poplar leaves through the stomata, and a strong positive correlation was observed between stomatal closure and rust resistance (Siwecki and Przybyl, 1981). Current work is being undertaken on stomatal opening in the Family 331 pedigree in relation to drought (personal communication, N. Street, University of Southampton), which may be considered in parallel with rust infection.

Sawfly larvae and Chrysomelid beetles were caught feeding on the leaves of some genotypes in the field trial. There is a paucity of information in the literature about the feeding preferences of the sawfly larvae, but there are some reported feeding preferences of Chrysomelid beetles. One such species is the cottonwood leaf beetle, *Chrysomela scripta,* a very serious pest defoliating poplars in North America, reducing the final yield and possibly initiating infection by pathogens. Tree architecture can be affected if the apical stem is consumed (Bingaman and Hart, 1992). Poplars are most susceptible during establishment (years 1 to 3), when they produce succulent, nutrient-

rich leaf tissues. In a study of six poplar genotypes including hybrids, adult beetles discriminated strongly between genotypes, but once the beetle was feeding on a certain leaf, genotype did not influence the proportion of the leaf consumed. *Populus* hybrids derived from parents belonging to the section of the genus, *Aigeiros* suffered more damage from beetles than those of parental genotypes from the section *Tacamahaca* (balsam poplar) (Bingaman and Hart, 1992). *Chrysomela scripta* feeding on poplars preferred leaves at 40 to 60 % of full expansion (Wait *et al.,* 1998). In the Family 331 field trial, the herbivory score, 0, negatively influenced the harvested biomass in PC1 (Figure 3.28) and the score for up to 20 % defoliation of a leaf, 1, had a positive loading of nearly 0.1, seemingly improving harvested biomass, although not by a significant amount. These two scores appear to be polar in their influence on PC2. In the PLS model, the two herbivory scores held equal rank, of little importance to harvested biomass. This can not be easily interpreted without further investigation in a field trial without treatment. A further observation from fieldwork was the difficulty of visually separating the damage caused by sawfly larvae from that of the beetles. It was also observed that of the leaves damaged, few showed more than about 25 % consumption of the leaf, so further scoring categories were not created. This would suggest that preference of genotypes by the invertebrates is a qualitative trait. Neither parent showed consistent damage or avoidance by the feeders in all replicates, but both F_1 genotypes were fed upon in all replicates. Beetles and sawfly larvae were eliminated using pesticides from the field trial after scoring so it is not known what effect these pests may have had on the health of the field trial in the 2001 growing season.

Heritability

Using estimates of broad sense heritability, some traits were found to be highly heritable, with H^2 values of over 0.50 (Table 3.7). Certain leaf growth rates showed much lower heritabilites, however growth rates can be strongly environmentally driven, based on factors such as temperature and soil moisture content. Height, diameter (or area), harvested biomasss, and leaf number strongly heritable; it can further be noted that these traits received amongst the highest weightings in the PLS regression. In *Salix viminalis,* Rönnberg-Wästljung and Gullberg (1996; 1994) reported that stem height and stem diameter had the highest heritabilities of the traits measured. Where heritability is low, however, they mentioned that phenotype is influenced more strongly by the environment, so the phenotypic correlations between traits are less indicative of the genetic relationships between traits. Bradshaw and Stettler (1995) calculated H^2 for Spring bud burst, of 0.980 compared to 0.549 in the coppice field trial studied here. Although there was insufficient replication for epidermal cell traits in the Family 331 field trial, other workers have calculated the heritability of these traits. Dunlap and Stettler (2001) estimated broad sense heritability by the same method as this study (Falconer and Mackay, 1996) in *P. trichocarpa* genotypes growing in different environments. They attained values of 0.870 for cell number per leaf, and 0.710 for cell diameter (an expression of cell area), on the adaxial leaf surface.

QTL

QTL with LOD scores in excess of 2.6 were found on 12 of the 19 linkage groups on the molecular map (Table 3.8), and although Wu *et al.* (1997) also found QTL on 12 linkage groups, these differed slightly from those in these results. QTL of large effect are most likely to be detected and indeed they often lie very close to major gene loci,

and in practice there may be very few QTL (Kearsey, 1997). Mansur *et al.* (1993) showed QTL of large effects were found to be clustered and to have reasonably high heritabilites. They also found these developmental traits to be highly correlated, suggesting further that the loci with reasonably large effects could be due to many genes segregating at a single locus, or the presence of a major gene with pleiotropic effects (Mansur *et al.,* 1993). This has been seen in some of the QTL found here. Of the five of the QTL were on linkage group J, three stem traits, stem extension rate and stem height at 145 and 359 DAC co-located at 17.3 cM, with a further peak for stem height (359 DAC) at 21.3 cM. These all overlap on this linkage group with leaf extension rate 1 (137-144 DAC), positioned at 21.3 cM also. However, when there is more than one peak on the same linkage group for the same trait, they would not normally be considered independent unless separated by at least a 40 cM distance (Paterson *et al.,* 1991). The confidence intervals for all of the QTL on linkage group J, however, were all large, extending beyond the linkage group end.

Wu *et al.* (1997), using the same poplar pedigree, reported that, although the QTL likelihood peaks were very close for leaf dimensions, 1st year height increment and basal area increment in Family 331, which could infer pleiotropic QTL, no QTL were actually detected as common to all of these traits.

QTL for maximum stem height at 145 and 359 DAC also mapped to close positions on linkage group M, each explaining approximately a quarter of the variance for the trait. These mapped to the same linkage group as a QTL for adaxial leaf cell area, which had a significant LOD score of 3.53 and explained a third of the variation in for the trait. Weng *et al.* (2002) observed that early height growth in *Pinus* hybrids is a quantitative trait controlled by a small number of genes with large effect. This observation was

mirrored in investigations using *Populus* (Bradshaw and Stettler, 1995) and young *Eucalyptus* plants (Byrne *et al.*,1997).

Further QTL were located for stem traits on linkage group Y. This is a short linkage group, only 18.2 cM long, and so the confidence intervals span its length. These QTL explain 35.1 and 32.2 % of the variation for single stem height and stem extension rate respectively.

QTL for stem height were also found on the same linkage group as one for leaf number on the leading stem, on LG O. QTL have been found in *Oryza sativa* have been found for total leaf number (Ishimaru *et al.* 2001a). Where QTL occur in the same location for more than one trait, it is circumstantial evidence that two traits are causally related, however it is not until the genes for these QTL are finally resolved that evidence becomes clear for a causal relationship (Thumma *et al.*, 2001).

Bud burst QTL on LG C explained 37.6% of variation. Frewen *et al.* (2000) found five QTL for Spring bud burst accounting for between 28 and 52 % of the variation, mirroring the results of Bradshaw and Stettler (1995). A QTL in the coppiced trial for bud burst was located on linkage group C, accounting for 37.6 % of the variation in this trait, but reports of QTL for bud burst on this linkage group have not been previously published.

No QTL were found for specific leaf area. However, in other organisms, this has been found to be a quantitative trait. For example, Yin *et al.* (1999) found QTL for SLA at six developmental stages in *Hordeum vulgare*. However, they point out that such

developmental traits are dynamic and detection of QTL for these traits varies at different times in the growing season. Mansur *et al.* (1993) found QTL for carbon isotope ratio in soybean (*Glycine max*). Teulat *et al.* (2002) also located QTL for carbon isotope discrimination in *Hordeum vulgare* L. In the Family 331 field trial a QTL was found accounting for 14 % of the genetic variation but the LOD score was 2.14, below the threshold in this instance. However, Araus *et al.* (2003) found little advantage of breeding to raise water-use efficiency, and therefore yield, in wheat, unless the environmental conditions were relatively wet.

There were no QTL found on linkage group B that co-located with the one found here for fresh weight at harvest. However, Ferris *et al.* (2002) in the OTC study, reported QTL for stomatal density and stomatal index on this linkage group. In the study of this pedigree by Wu (1998), two QTL collectively accounted for 24.1 % of the variance in stem dry weight on linkage groups E and O, no QTL were found for stem volume, and for harvest index approximately 50 % of phenotypic variance was accounted for by QTL on linkage groups J and O. Other researchers have found QTL for yield in other plant species. For example, Mokrani *et al.* (2002) found QTL for grain weight and grain oil yield in *Helianthus annuus* L., where each QTL explained between 2.6 % and 70.9 % of the variation.

Although total stem diameter has been found to be a trait strongly influencing biomass yield, QTL for stem diameter were not found, although several for stem height were. Likewise, in experiments in the U.S.A., no QTL were found for basal area increment or volume (Wu, 1998). It could be, like all the other traits for which there were no QTL located in this study, that the trait is not quantitative (Lynch and Walsh, 1998).

Occasionally, the most intuitive QTLs to co-locate due to causal relationships are not even located on the same linkage group. A prominent QTL for grain protein content in *Hordeum vulgare*, for example, did not co-localise with the QTL for nitrogen remobilisation (Mickleson *et al.*, 2003).

For some traits, no QTL were located in this study. This does not necessarily mean that these traits are not controlled by QTL; it is likely that they were not detected given the current marker coverage on the linkage map, that there should be genotype information for more individuals in the population. Hyne *et al.* (1995) explained that there is no evidence that dense maps are required for intial QTL location; it is more important to collect data for a large population and have a few (between 5 and 10 are sufficient) markers per chromosome. QTL mapping in other studies usually uses maps based on more progeny than were genotyped on the poplar map constructed by Bradshaw *et al.* (1994). It should also be considered that not all QTL are detectable through associations with molecular markers; the detection of a QTL also depends on the size of its effect on the trait, the heritability of the trait, the selection of parental genotypes, and the size of the mapping population (Cervera *et al.*, 1997).

Marker assisted selection (MAS) is concerned with the use of any genetic markers which are suitable and sufficiently recognisable to enable the selection of linked genes that control useful traits (Kearsey, 1997). A primary advantage of this technique in forest research is that many genes are only expressed in the mature plant, and MAS enables screening for markers in juvenile plant tissue.

For marker-assisted gene transfer to take place, the region transferred in the target tissue must be large enough to be certain of the gene being contained within it (Wu *et al.*, 2000). It also has to be small enough so as not to transfer any linked, but undesirable, alleles. Traditional breeding involves repeatedly crossing the original F_1 plant with the original parent cultivar, to introduce the allele of interest. Even after ten or more generations of backcrossing, linkage drag (large areas of the genome surrounding the desired allele) remains. This number of backcross generations can be decreased to two or three whilst reducing linkage drag through MAS (Kearsey, 1997).

The long-lived nature of forest trees, usually grown for their vegetative rather than reproductive parts, is different to that of most food crops, which are usually grown as annuals and grown for fruit or grain structures. Since many commercially planted trees are still strongly related to native species, GM trees could pose more of a contamination threat than would highly domesticated food crops (Bradshaw and Strauss, 2001). Furthermore, many trees do not flower in the early years of growth, so their wood products may safely be harvested from coppiced, GM trees prior to the onset of flowering.

For MAS to take place, QTL need to be detected accurately, for example to within $P < 0.01$ and a high LOD score to remove the number of false positives occurring. This would suggest locating QTL with more stringency and interpreting them as "subject to further analysis" (Thumma *et al.*, 2001). Conventional selection, when used properly, continues to have great potential for breeding using both quantitative and qualitative traits (Kearsey, 1997). The selection and breeding of poplar and willow genotypes for biomass is discussed further in Chapter 5 of this thesis.

Conclusions

In 1990, Michael *et al.* suggested that, owing to the complexity of morphological and physiological factors affecting yield, none in particular can be a sole yield indicator. This chapter has found otherwise. In an investigation of the stem and leaf traits affecting harvested biomass in an intersectional poplar pedigree, it has been shown that total basal stem diameter of a 1st year coppice stool, the stem height of the leading coppice shoot (at different stages in the season) and the number of sylleptic branches on the single stem in the establishment year, are primary indicators of harvested biomass. Of the leaf traits measured in this study, the most indicative of yield have been the number of leaves on the leading stem of the coppice stool, and the individual leaf area of a mature, fully expanded leaf sampled from the leading stem. Leaf and stem growth rates or increments were not found to contribute greatly to the biomass accrued. Early dates of spring bud burst may be beneficial to early season development and increases in biomass yield through canopy duration. Yields appear to be greater in genotypes where sylleptic branches are present rather than in those which do not produce sylleptics.

A noteworthy result of this study has been that rust infection, specific leaf area, carbon isotope ratios, and leaf production rates are not useful yield indicators at this stage in the coppice cycle in this population. Many of the traits studied have shown high values of broad sense heritability, especially stem height and the number of leaves on the leading coppice stem. These traits are both quantitatively controlled by several gene loci. QTL have also been detected for other traits; it is anticipated that the resolution of mapping will be improved and confidence intervals decreased, when QTL analysis is performed on the expanded Family 331 map currently in preparation (personal

communication, A.M.Rae, University of Southampton). Analysis using a greater number of genotyped F_2 individuals would facilitate the detection of QTL of small effect in addition to the QTL of large effect already found (Lynch and Walsh, 1998).

This, like most studies, looks at first and second rotations; rarely are the later cutting cycles investigated. It will be interesting to track the progress of further work on this population as coppice age increases. It should also be considered that many trials have also investigated fuel quality from biomass, which can be as important as fuel quantity for energy production.

Chapter 4

The Genetic Determinants of Leaf Response to Elevated CO_2

4.1 INTRODUCTION

Carbon dioxide, light and water are the essential raw materials required for leaf growth as well as total plant growth (Dale, 1988). Since plants acquire carbon and lose water mainly through the leaves, leaf growth and anatomy is a very important aspect of studying plant responses to elevated CO_2. Ambient CO_2 at present is approximately 350 μmol mol^{-1} (I.P.C.C., 2001). Plants growing in habitats at different elevations have adapted to various ambient concentrations of CO_2. These concentrations vary naturally with resistances to diffusion into a leaf and the photosynthetic pathway (Mott, 1990). The increasing concentration of CO_2 in the Earth's atmosphere, owing largely to increased world population size and fossil fuel consumption, may have a future impact on individual plant growth and plant population structure. To gain a greater understanding of the physiological and genetic processes of carbon acquisition and allocation within plants, which may prove helpful to understanding biomass accumulation in trees, plants may be grown in concentrations of CO_2 which have been elevated above that of ambient. Changes in the biomass components of poplar, as well as many other species, have been found to be responsive to elevated CO_2 concentrations, as discussed below.

Wolfe *et al.* (1998) define a CO_2 response as an adjustment in the system of CO_2 acquisition. Plants are nearly always larger under elevated than ambient CO_2 (Pritchard *et al.,* 1999), because ambient CO_2 is often limiting to optimal photosynthesis. Ceulemans *et al.* (1996b) conducted experiments on two poplar hybrids, *Populus trichocarpa* x *P. deltoides* 'Beaupré' and *P. deltoides* x *P. nigra* 'Robusta', finding that both genotypes increased in stem volume in elevated CO_2. The increased height in

elevated CO_2 was due to increased internode distances. However, carbon dioxide concentrations did not affect bud phenology (which could potentially influence the seasonal duration of photosynthetic carbon acquisition). In another study, involving the poplar genotypes 'Beaupré,' 'Robusta,' and *P. trichocarpa* 'Columbia River,' elevated CO_2 made no effect on the height, internode length or stem volume growth of 'Robusta' or 'Columbia River.' It did, however, positively influence the growth of 'Beaupré,' in which the biomass at the end of the first year of growth was 25% higher than ambient (Ceulemans *et al.,* 1994).

Other plant species vary in their responses to CO_2, mirroring the diverse responses across the genus *Populus.* Wheat (*Triticum aestivum*), for example, can be responsive to elevated CO_2 concentrations. An experiment by Masle (2000) used elevated CO_2 to manipulate the sugar concentrations supplied to expanding organs of wheat seedlings. Those exposed to 900 μmol m^{-2} s^{-1} CO_2 grew more during the same period than those at ambient, producing an increase in total dry weight at the end of the experiment between 52 and 93% greater in elevated CO_2 (Masle, 2000). In addition to changes in leaf growth, root to shoot ratios usually go up or down in elevated CO_2 but rarely stay the same (Pritchard *et al.,* 1999) and plant dry weight nearly always increases under elevated CO_2 (Mott, 1990).

There are several major short-term and long-term effects of increased CO_2 on photosynthesis. Mott (1990) summarises some short term effects, including the observation that, in general, exposure to elevated CO_2 leads to stomatal closure, if photosynthetic conditions permit. The photosynthetic rate depends on the activity of the chloroplast. The presence of more CO_2 triggers the activity of Rubisco in the

chloroplast to conduct more carboxylase activity and less oxygenase activity. This starts a feedback loop which reduces the level of RuBP to decrease the proportion of active Rubsico sites. In response to elevated CO_2 concentrations, the sucrose synthesis pathway is rapidly affected through the accumulation of phosphorylated sugar intermediates, which restrict the inorganic phosphate availability for ATP synthesis and RuBP regeneration. This process reduces the effect of elevated CO_2 on carbon assimilation. In the long term, however, plants usually adjust this process. Furthermore, Rubisco activity, shown to reduce initially in the short term, has been found to increase in longer term studies (Wolfe *et al.,* 1998).

As a further, longer term response, most plants may reduce the photosynthetic rate per unit of leaf area. Also over longer periods of time, changes take place in leaf stomatal density in response to elevated CO_2 (Woodward, 1987; Jarvis *et al.,* 1999). Decreased stomatal opening may cause increased water potential and turgor pressure which may enhance growth. Mott (1990) suggested that many CO_2 effects are due to changes in C_i and photosynthetic rates. The stimulation of photosynthesis by increased CO_2 concentrations causes increases in the production of glucose and sucrose. Carbon storage and partitioning also need to adjust (Wolfe *et al.,* 1998). A study by Morison (1985) found that increased concentrations of CO_2 caused decreased stomatal conductance. Gravimetrically measured transpiration decreased in elevated CO_2. The factors influencing increased overall growth in elevated CO_2 are the increased carbon availability from greater photosynthetic activity, higher water potential and turgor pressure owing the closure of stomata, and modified rates of cell division and expansion. The improved supply of carbon causes more efficient osmoregulation under water stress (Mott, 1990).

In the long term, plants either up- or down-regulate the activity of Rubisco and other photosynthetic enzymes (Lawlor, 2002). The increase in CO_2 concentration also causes decreases in foliar nitrogen concentration, through the 'dilution' effects of increased starch and sugar levels (Wolfe *et al.,* 1998). In experiments with *Quercus alba* L., Norby and O'Neill (1989) also found that the overall growth response to elevated CO_2 was an increase in photosynthesis per unit leaf area.

The response of total and individual leaf area to elevated CO_2 has been widely studied. For example, the three *Populus* species studied in a free air CO_2 exposure (FACE) experiment all appeared to be sensitive to elevated CO_2 (Ferris *et al.,* 2001) with the conclusion that the increased leaf area drives the increased biomass production. Other *Populus* experiments have shown similar results; for example, poplar plants grown in greenhouse chambers in elevated CO_2 produced 55% or more total leaf biomass than in ambient CO_2 (Ceulemans *et al.,* 1996b). Other tree genera have sown different responses. Studies of *Quercus alba* concluded that there was no response of individual leaf area to elevated CO_2 in this species (Norby and O'Neill, 1989). Like *Liriodendron tuliperfa* L. (Norby and O'Neill, 1991), the sweet chestnut, *Castanea sativa* L. reduced its leaf area in elevated CO_2, possibly due to early cessation of growth in the terminal bud, which did not occur in the control CO_2 concentration (Mousseau and Enoch, 1989).

Leaf thickness, or specific leaf area (SLA), is commonly responsive to CO_2 concentrations. Leaf area tends to increase disproportionately to leaf weight in elevated CO_2 so SLA is reduced accordingly. Mesophyll and palisade cell volumes increase

providing a greater surface area for CO_2 absorption (Wolfe *et al.,* 1998). Norby and O'Neill (1991) noticed trade-off whereby leaf area decreased and leaves became heavier in elevated CO_2; neither result was significant, but the two results combined resulted in a significant decrease in SLA in elevated CO_2. The SLA of mature *Populus* leaves, however, was not affected by CO_2 in the FACE experiment by Ferris *et al.* (2001).

Radaglou and Jarvis (1990a) studied leaf growth with respect to anatomical features in poplar. Their findings suggested that it was increased cell expansion driving increased leaf area in elevated CO_2. The size of a leaf cell is variable according to ontogengy and environmental factors, and it is leaf expansion that enables plant growth, however the largest leaves usually have the greatest number of epidermal cells, which is a function of cell division (Dale, 1988). The leaf epidermis is the *restraint* on leaf tissue enlargement. It does not *drive* leaf enlargement (Dale, 1988). As expanding cells reach a size threshold, they reach a limit where they can no longer be controlled by the nucleus, so the cell divides, making new cell walls. Therefore, cell expansion drives mitosis. Hormones also control apical meristem division (Pritchard *et al.,* 1999). During the process of leaf growth, cell division stops first, then cell expansion takes place. During lamina unfolding, much cell expansion occurs and also during lamina unfolding 99% of cell divisions take place, so both processes are busy at this stage. Occasionally, cell division may continue until 95% of the final leaf area has been achieved. Thus, cell division and cell expansion are by no means temporally exclusive. There is normally a final period of growth, however, which consists entirely of cell expansion (Dale, 1988). Increased organ size is dictated by expansion and to some

extent division, but they are highly interlinked processes and it is not easy to distinguish which is responding to elevated CO_2 (Pritchard *et al.,* 1999).

Different leaf tissues grow at different rates. Cell division ceases first at the tip of the leaf and continues for the longest period at the leaf base (Granier and Tardieu, 1998). Cell division stops in the adaxial epidermis first, followed by the parenchyma and the abaxial epidermis (Roggatz *et al.,* 1999). Relative growth rates tend to be greatest at the centre of leaf, adjacent to the main veins (Dale, 1988).

At different leaf positions on a plant, the epidermal cell density may vary (Ceulemans *et al.,* 1995). In one experiment using both mature and recently-mature leaves, epidermal cell density neither differed on the adaxial or the abaxial surface between ambient and elevated CO_2 treatments. These leaves had between 8 000 and 10 000 cells per mm^2 on one surface. 'Beaupré' had more adaxial epidermal cells per leaf in elevated carbon dioxide than in ambient carbon dioxide: 2 824 x 10^6 and 2 936 x 10^6. 'Robusta' had less cells in elevated (1 802 x10^6) than ambient (2 069 x 10^6). It appears that the CO_2 effect of more cells per leaf may be because of larger leaf areas i.e. more cells per unit area. The study found fewer cells per leaf on the adaxial than the abaxial surface. They suggest that this may be due to the different light environments between the two sufaces (Ceulemans *et al.,* 1995). Masle (2000) found that elevated CO_2 reduced the time taken for the epidermal cells to undergo cell division. Much of the large influence of elevated CO_2 on leaf area development in wheat (*Triticum aestivum* L.) leaves is caused by an increased supply of photoassimilates which influences the length of epidermal cells. This also increased the number of cells in the leaf (Masle, 2000).

In a study of 'Beaupré' and 'Robusta,' Chen *et al.* (1997) found that leaf area index (LAI) was greater in elevated than ambient CO_2. Using a model which varied leaf size, canopy light interception was manipulated. The differences in above ground biomass production after two years, using this model, were largely a result of increased photosynthesis owing to larger leaf areas in elevated CO_2 (Chen *et al.*, 1997).

Branching and tillering is often responsive to elevated CO_2 in plants. Branches form due to partitioning of the apical meristems at the shoot axis (Pritchard *et al.*, 1999). All of the *Populus* genotypes in the study by Ceulemans *et al.* (1994) produced fewer branches and smaller branches in ambient than elevated CO_2. In wheat, elevated CO_2 caused the production of more secondary and tertiary tillers which behave as carbon sinks in the plant. In the same plants, a higher carbon content per unit of leaf area was observed (Masle, 2000).

The largest response to increased CO_2 in *Castanea sativa* was an increase in root growth (Mousseau and Enoch, 1989). Other observations of increased root growth were made in *Liriodendron tulipifera* by Norby and O'Neill (1991), who did not observe any effect of elevated CO_2 until 24 weeks of growth, when it was observed that both fertilised and unfertilised plants increased in total dry weight in elevated CO_2. This result was due entirely to increases in root dry weight (increased root: shoot ratio) as no increase was observed in above ground biomass. Norby and O'Neill (1991) also observed that the ratio of leaf area to fine root biomass decreased in elevated CO_2, indicating an adjustment in the balance of water-absorbing and water-losing organs. This result would suggest a greater drought resistance in yellow poplar in elevated CO_2.

The use of growth chambers and greenhouses minimises environmental variation to enable the identification of real treatment effects more easily than in the field. The field, of course, receives more rapidly changing and extreme fluctuations in all environmental conditions Nilson and Ehlers (1984).

This chapter describes a glasshouse experiment of pot-grown poplars grown over one season in each of ambient and elevated carbon dioxide concentrations, to investigate the responses of leaf growth and to identify QTL for anatomical leaf features in both the CO_2 treatment and the control. The population used is *Populus trichocarpa* x *Populus deltoides* F_2 Family 331, introduced in Chapter 3, which naturally exhibits much variation in leaf anatomy owing to the hybridisation of distinct parental leaf characteristics. The aim of this work is to interpret the leaf response to CO_2 in terms of both physiology and QTL.

4.2 MATERIALS AND METHODS

Experimental design and implementation

The experiment was conducted at the Forestry Commission open top chamber (OTC) facility at Headley, Hampshire, U.K. (51°07′ N, 0°50′ W). The facility consisted of 16 chambers arranged in a 4 x 4 square formation. The OTC frames were aluminium (Waytogrow, Essex, U.K.), 3 m in diameter and 2.5m high, constructed from aluminium sections (Waytogrow PLC, Essex, UK) and glazed with 3mm horticultural glass, chosen in preference to plastic due to robustness and retention of transparency (Gardner *et al.,* 1995). Light transmittance into the chambers was approximately 85% of ambient light. The OTCs were ventilated by a 1 hp centrifugal pump, providing air through a circular polythene tube held against the walls of each chamber. Each polythene tube had approximately 280 holes, 25 mm in diameter, distributed evenly along its lengths, providing ventilation rate of $75m^3\ min^{-1}$ so that a complete change of air was provided every 15 seconds (Gardner *et al.,* 1995).

Plant material was imported from the University of Washington, U.S.A., a gift from Prof. H.D. Bradshaw, as unrooted hardwood cuttings of a three-generation poplar pedigree, Family 331. Two cuttings were grown of each parent and F_1 genotype and of 285 F_2 genotypes. The male parent was clone 14-129 of *P. deltoides* Bartr. ex Marsh, the eastern cottonwood from central Illinois. The female parent was 93-968 of *P. trichocarpa* Torr. & Gray, the black cottonwood, found west of the Rocky Mountains. These parents were crossed to give F_1 Family 53, a male (53-242) and female (53-246) of which were successively crossed to give F_2 Family 331. The pedigree is described in

more detail in Chapter 3 of this thesis. Planting took place on 13th and 14th May 1999. The experiment was designed and planted by Gail Taylor, Rachel Ferris and Stephen Bunn. Cuttings were treated with fungicide (Tilt 250 EC, Ciba-Geigy PLC Agrochemicals, Cambridge, U.K.) at a dilution of 5 ml 3 l^{-1} prior to planting. One cutting was planted in each of 589 custom built pots, 50 cm high and 20 cm in diameter, in John Innes No. 2 (lime free) compost. At 13 days after planting (DAP), the floors of the OTCs were treated with a pre-emergent herbicide, Stomp 400 SC (MAFF No. 04183, BASF PLC, Cheadle Hulme, U.K.) applied with a sprayer (Gloira-Werke, Wadersloh, Germany) at a dilution of 20 ml l^{-1}. Plants were provided with a slow-release fertiliser, Osmocote (Grace-Sierra, Nottingham, U.K.) at 5 g per pot at 67 DAP. An insecticide, 'Ambush' (AstraZeneca, Macclesfield, U.K.) was applied to all chambers at 21 and 92 DAP. Plants were staked and tied for support at 68 DAP. A further application of Ambush was applied at 92 DAP. A drip irrigation system supplied water to each individual pot commencing on 14th May 1999 (0 DAP). Irrigation rate was adjusted according to seasonal changes in precipitation and humidity. Pot bases were sunken 10 cm deep into the ground for stability.

Plant material was exposed to either ambient CO_2 (target concentration 350 – 400 μmol mol^{-1}) or elevated CO_2 (target concentration 600 μmol mol^{-1}). Eight OTC's were maintained at ambient CO_2 concentrations and eight chambers at elevated. Concentrations of CO_2 were monitored continuously for the duration of the experiment. The genotypes were assigned to the ambient OTC's in a randomised complete block design, with 36 individuals assigned to each OTC at a spacing of 25 cm between pots. The second plant of each genotype was grown in an adjacent OTC at elevated CO_2

concentrations. Each chamber housed 36 genotypes that were placed in a circular pattern with a distance of 25cm between plants.

The mean CO_2 concentration (± s.d.) recorded between throughout the experiment (May – September 1999) in the elevated chambers was very consistent, ranging between 588.29 (± 63.35) and 595.30(± 83.52) μmol mol^{-1} CO_2. In the ambient chambers, the CO_2 concentration ranged between 400.81 (± 39.00) and 408.08 (± 38.84) μmol mol^{-1} CO_2. Mean monthly air temperatures (± s.d.) outside and inside the chambers respectively were as follows: 13.2 (4.0), 16.4 (5.7) °C; June: 14.3 (4.4), 17.6 (6.1) °C; July: 18.1 (5.1), 21.3 (6.9) °C; August: 16.3 (4.5), 19.0 (6.0) °C; and September: 15. 3 (4.3), 17.4 (5.6) °C.

Leaf anatomy measurements

At 80-82 and 130-134 DAP, one fully expanded mature leaf of each the 578 individuals was harvested. Leaves were placed into clear, labelled plastic bags and returned to the laboratory for preparation of epidermal imprints. Leaves were stored in cool boxes and maintained at 0 – 4 °C to prevent shrinkage during measurement and epidermal imprinting. Epidermal impressions were made following the technique of Ferris *et al.* (1994), applying clear lacquer spray (Halfords, Redditch, U.K.) at 80-82 DAP and clear nail varnish at 130-134 DAP, to a 1.5 cm^2 area of adaxial epidermis close to the midrib at maximum leaf width. When dry, the lacquer was removed using Sellotape and the peel transferred directly onto a glass microscope slide. Adaxial epidermal impressions were viewed using a Zeiss Axiophot 2 Universal Microscope (Carl Zeiss, Jena, Germany) and images saved in TIFF (tagged information file

format). Epidermal cell areas were measured using imaging software, Metamorph Imaging System (Metamorph Inc., Westchester, PA, U.S.A.). This software was used to obtain one digital image per slide of mature adaxial epidermal cells taken between the midrib and the major veins. The areas of ten cells per slide were obtained randomly.

After epidermal impressions were made, every leaf was photocopied and photocopies used to obtain leaf areas using an image analyser (Delta-T Devices Ltd, Cambridge, U.K.). Louise Long prepared the epidermal impressions and obtained the leaf area data for this study. The number of adaxial epidermal cells per leaf was estimated using the mean adaxial epidermal cell area and the leaf area at each corresponding time point (80-82 DAP and 130-134 DAP).

Statistical analyses and QTL mapping

Statistical analysis was conducted using Minitab release 13.1 (Minitab Inc. State College, PA, U.S.A.). All data were tested for normality using the Anderson-Darling test. Count data were square root transformed before analysis (Sokhal and Rolf, 1995). The genetic linkage map based on 90 F_2 individuals and constructed from STS, RFLP and RAPD markers reported in Bradshaw *et al.* (1994) was used for QTL mapping. The data were analysed for QTL using MAPMAKER/QTL 1.1 (Lander and Botstein, 1989). A LOD (logarithm of the odds) score 2.9 was used as a threshold for QTL presence based on a *P* significance of 0.05 for the whole genome (Lander and Botstein, 1989; Bradshaw and Stettler, 1995; Bradshaw, 1996). Those QTL with LOD scores close to 2.9 were also considered suggestive of a QTL.

4.3 RESULTS

Parental characteristics

The contrasting leaf characteristics of the parents of the Family 331 pedigree, *Populus trichocarpa* and *Populus deltoides*, grown in aCO_2, are illustrated in Figure 4.1. The leaves of *P. trichocarpa* are elongate relative to those of *P. deltoides,* with a long petiole, and large epidermal cells. Stomata occur only infrequently on the adaxial leaf surface of *P. trichocarpa.* In contrast, *P. deltoides* has larger leaves, deltoid in shape, comprised of small epidermal cells and the presence of stomata on the adaxial surface. Table 4.1 (a) describes these leaf attributes, measured at two time points, 80- 82 DAP and 130-134 DAP, in each of aCO_2 and eCO_2. Table 4.1 (b) shows the percentage change in the leaf traits in eCO_2 at each time point. The individual leaf area of *P. trichocarpa* is very sensitive to eCO_2, responding with a 303.5% increase at 80-82 DAP, and a 136.8% increase at 130-134 DAP. This level of sensitivity was not expressed in *P. deltoides,* which maintained similar leaf areas in each of aCO_2 and eCO_2 at 80-82 DAP, and which responded with a moderate 24% increase from 293.41 to 363.69 cm^2 at 130-134 DAP. The leaf areas of both species were larger later in the growing season. In contrast, epidermal cell areas in aCO_2 were consistent in both parent species between the two time points, varying by only 5.2 μm^2 in *P. trichocarpa* and 25 μm^2 in *P. deltoides.* The epidermal cell areas in *P. trichocarpa* increased moderately in eCO_2 by 15.6% and 10.8% at 80-82 and 130-134 DAP respectively. In accordance with increases in the leaf area of *P. trichocarpa*, the cell number per leaf increased, and in greater proportions than the gain in cell area. This shows cell number per leaf to be highly sensitive to eCO_2 in this species. The response of *P. deltoides,* however, was more varied. A 21.4% decrease in cell area was observed in eCO_2 at 80-

82 DAP but a 43.1% increase occurred at 130-134 DAP in the same trait. In accordance with these changes in cell area, cell number per leaf increased in tandem with the cell area reduction at 80-82 DAP, and the opposite response occurred at 130-134 DAP whereby a decrease in cell number accompanied an increase in cell area.

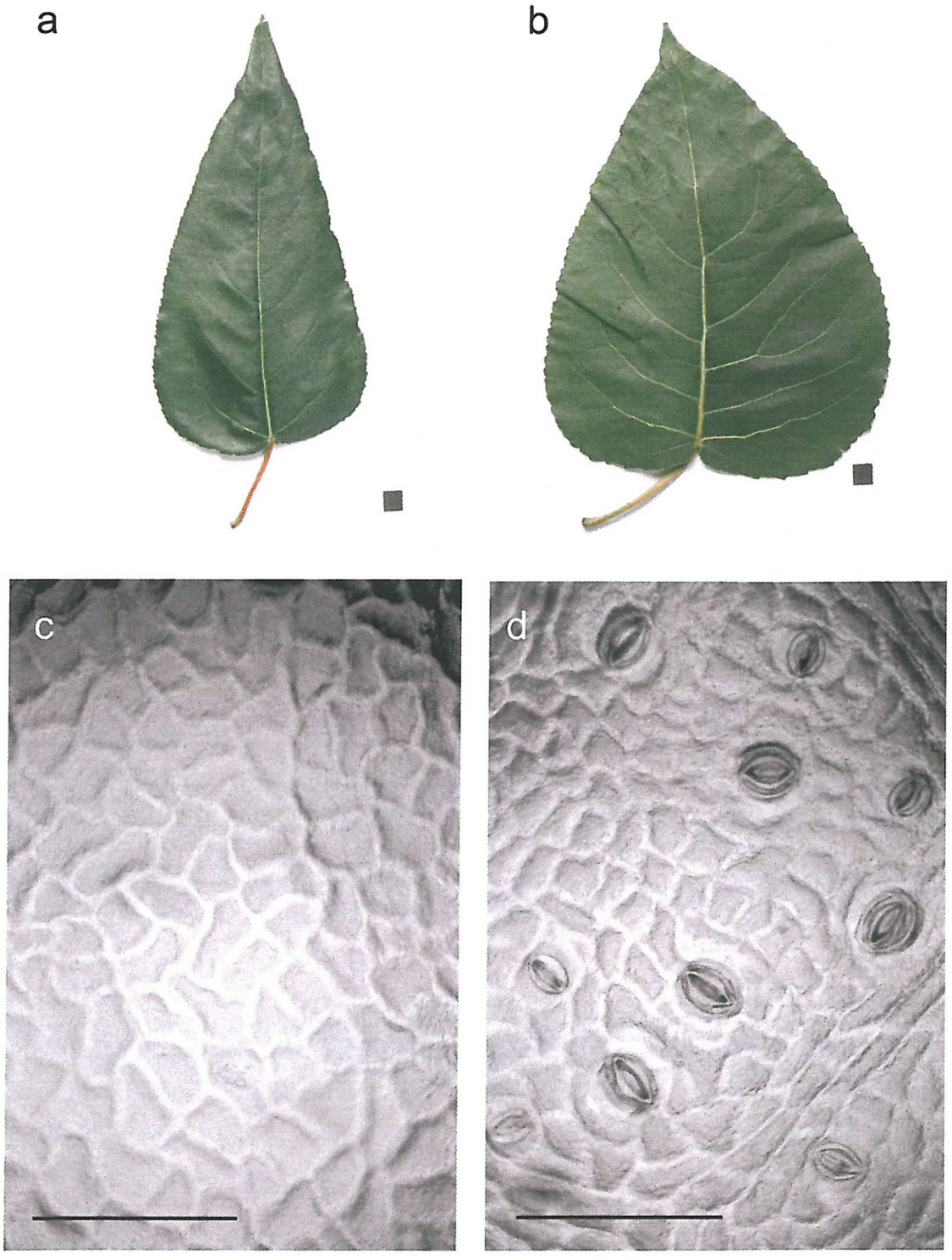


Figure 4.1 Mature leaf and image of adaxial epidermal cells of parents of the Family 331 pedigree, (a,c) *Populus trichocarpa* and (b,d) *Populus deltoides,* both grown in ambient CO_2. The scale square represents 1 cm^2 and the scale bar in the light microscope photograph represents 100 µm.

Table 4.1 (a) Leaf characteristics of the parents of the Family 331 pedigree grown in the OTC's in each of ambient or elevated CO_2. Data are shown for measurements made in August (80-82 DAP) and September (130-134 DAP).

	Parental characteristics			
	P. trichocarpa		*P. deltoides*	
	aCO_2	eCO_2	aCO_2	eCO_2
Trait				
80-82 DAP				
Leaf area (cm^2)	20.03	80.82	162.34	154.44
Adaxial epidermal cell area (μm^2)	648.0	749.0	426.8	335.6
Estimated cell number per leaf (x 10^6)	3.40	10.79	38.04	46.02
130-134 DAP				
Leaf area (cm^2)	68.09	161.26	293.41	363.69
Adaxial epidermal cell area (μm^2)	642.8	712.4	401.8	574.8
Estimated cell number per leaf (x 10^6)	10.59	22.64	73.02	63.27

Table 4.1 (b) Leaf trait responses of the Family 331 pedigree parents to the elevated CO_2 treatment.

	Parental responses to elevated CO_2	
	P. trichocarpa	*P. deltoides*
Trait		
80-82 DAP		
Leaf area	+ 303.5 %	- 4.9 %
Adaxial epidermal cell area	+ 15.6 %	- 21.4 %
Estimated cell number per leaf	+ 217.4 %	+ 21.0 %
130-134 DAP		
Leaf area	+ 136 %	+ 24.0 %
Adaxial epidermal cell area	+ 10.8 %	+ 43.1 %
Estimated cell number per leaf	+ 113.8 %	- 13.4 %

Individual leaf area

Leaf traits and their response to eCO_2 in the F_2 population are described in Table 4.2. As observed for the parent species, mean individual leaf areas for the population were smaller in August at 80-82 DAP than in September, at 130-134 DAP. Estimated adaxial cell number per leaf was also greater in September, owing to the consistency of cell area between both time points: in aCO_2, the F_2 mean differed by only 0.2 μm^2 between August and September. The responses of leaf area, cell area and cell number per leaf were more variable at the different seasonal stages and best examined using the phenotype distributions in Figures 4.2 to 4.7.

Figure 4.2 illustrates the distribution of leaf area phenotypes in the pedigree at 80-82 DAP. *P. deltoides* had larger leaves in aCO_2 than either F_1 genotype. The F_1 genotypes differed in leaf area. The female F_1 (F_1f) responded to eCO_2 with an increase in leaf area, whereas the male F_1 (F_1m) was not sensitive to the treatment. For the F_2 population, the distribution of phenotypes is similar in eCO_2 to aCO_2, with no statistically significant difference between the treatments ($P = 0.668$), as shown in Table 4.2. At 80-82 DAP the largest leaf area in the pedigree was that of *P. deltoides,* with no F_2 leaf areas greater than 240 cm^2. At 130-134 DAP, shown in Figure 4.2, the range of leaf areas in the pedigree was more extensive, with some F_2 leaves attaining areas of up to 320 cm^2, and with *P. deltoides* the only genotype in the highest frequency bar. The figure clearly shows the greater leaf area response to eCO_2 of *P. trichocarpa* over that of *P. deltoides.* The F_1m was more sensitive to eCO_2 here than at 80-82 DAP, and the F_1f attained larger leaf areas in eCO_2 at 130-134 than at 80-82 DAP. In aCO_2 the modal frequency was 100-120 cm^2 and in eCO_2 it was 80-100 cm^2,

although, unlike leaf area at 80-82 DAP, there was a significant difference between the treatments ($P = 0.002$).

Adaxial epidermal cell area

The distribution of cell area phenotypes for the pedigree at 80-82 DAP is shown in Figure 4.4. This distribution shows the smaller epidermal cell areas of *P. deltoides* than either F_1 genotype, and confirms the greater response of *P. trichocarpa* to eCO_2 than *P. deltoides.* The F_1 cell area values fell between the parental values, but neither F_1 was as responsive to eCO_2 as *P. trichocarpa.* In the distribution of F_2 phenotypes in aCO_2, the modal frequency co-located with the F_2 mean at 525-575 μm^2. Likewise the mode and mean fell at 625-975 μm^2 in eCO_2. This was a clear shift towards the production of larger cells in eCO_2 and was statistically significant ($P < 0.001$). The percentage change in the mean F_2 population cell area in eCO_2 was an increase of 6.03%. Figure 4.5 shows that, at 130-134 DAP, *P. deltoides* again had the smallest, and *P. trichocarpa* the largest, cell areas compared to the F_1s. The mean of the F_2 cell areas in eCO_2 was 757.8 μm_2 compared with 671.0 mm^2 (Table 4.2), showing the increased effect of eCO_2 later in the growing season. The greater range of cell area values in September (Figure 4.5) is shown in the *x* axis range extending to 1225 μm^2, compared to a maximum of 975 μm^2 in August (Figure 4.4). In aCO_2, there were two modal frequencies, at 525-575 and 625-675 μm^2, with the F_2 mean falling in the latter category. In eCO_2 there was a significant shift towards 19.79% larger cells ($P < 0.001$), with the mean and mode falling at 725-775 μm^2.

Table 4.2 Leaf characteristics of the F_2 of the Family 331 pedigree grown in the OTC's in each of ambient and elevated CO_2. 285 genotypes were grown in each treatment. Values are means (standard error) of the F_2 population. Percentage change represents the effect of elevated carbon dioxide on the trait, calculated as 100 (($eCO_2 - aCO_2$)/aCO_2). One-way ANOVA's are shown comparing the treatments for each trait. Data are shown for measurements made in August (80-82 DAP) and September (130-134 DAP).

	F_2 population			
	Family 331 mean (s.e.)		% change	One-Way ANOVA
	aCO_2	eCO_2		
Trait				
80-82 DAP				
Leaf area (cm^2)	71.98 (1.92)	70.58 (1.86)	- 1.94	df = 545, $F = 0.18$, $P = 0.668$
Adaxial epidermal cell area (μm^2)	632.8 (6.86)	671.0 (6.55)	+ 6.03	df = 524, $F = 16.08$, $P < 0.001$
Estimated cell number per leaf (x 10^6)	11.68 (0.33)	10.91 (0.32)	- 6.57	df = 502, $F = 2.14$, $P = 0.144$
130-134 DAP				
Leaf area (cm^2)	128.87 (3.06)	143.28 (3.58)	+ 11.18	df = 561, $F = 9.56$, $P = 0.002$
Adaxial epidermal cell area (μm^2)	632.6 (6.86)	757.8 (7.35)	+ 19.79	df = 542, $F = 168.22$, $P < 0.001$
Estimated cell number per leaf (x 10^6)	20.78 (0.53)	19.19 (0.52)	- 7.64	df = 540, $F = 4.36$, $P = 0.03$

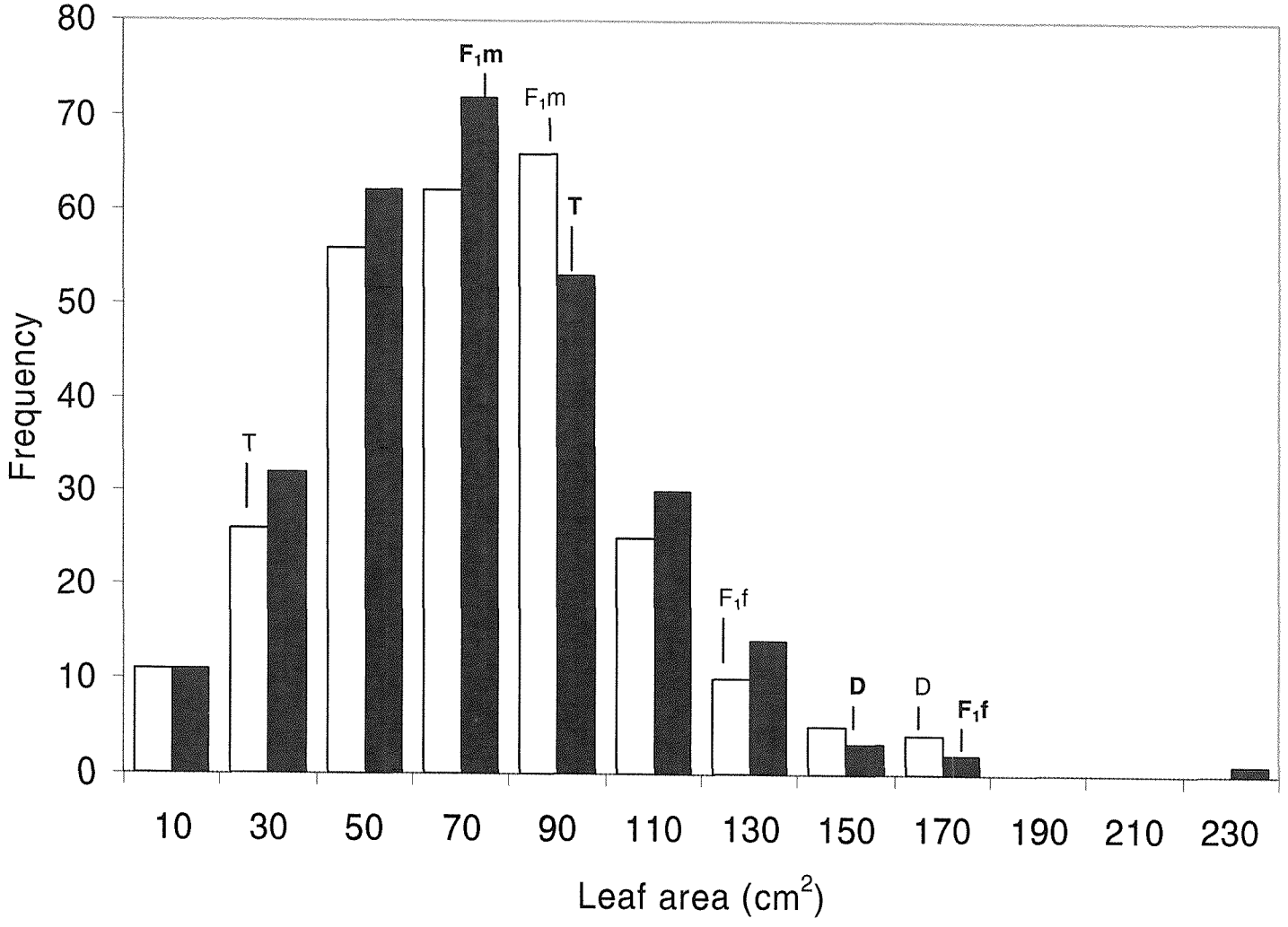


Figure 4.2 Distribution of individual leaf area phenotypes in the Family 331 pedigree in August (80-82 DAP), grown in ambient CO_2 (open bars) and in elevated CO_2 (solid bars). Parental and F_1 values, in regular text (ambient CO_2) and bold text (elevated CO_2), are indicated as T (*Populus trichocarpa,* female parent), D (*Populus deltoides,* male parent), F_1m (male F_1) and F_1f (female F_1).

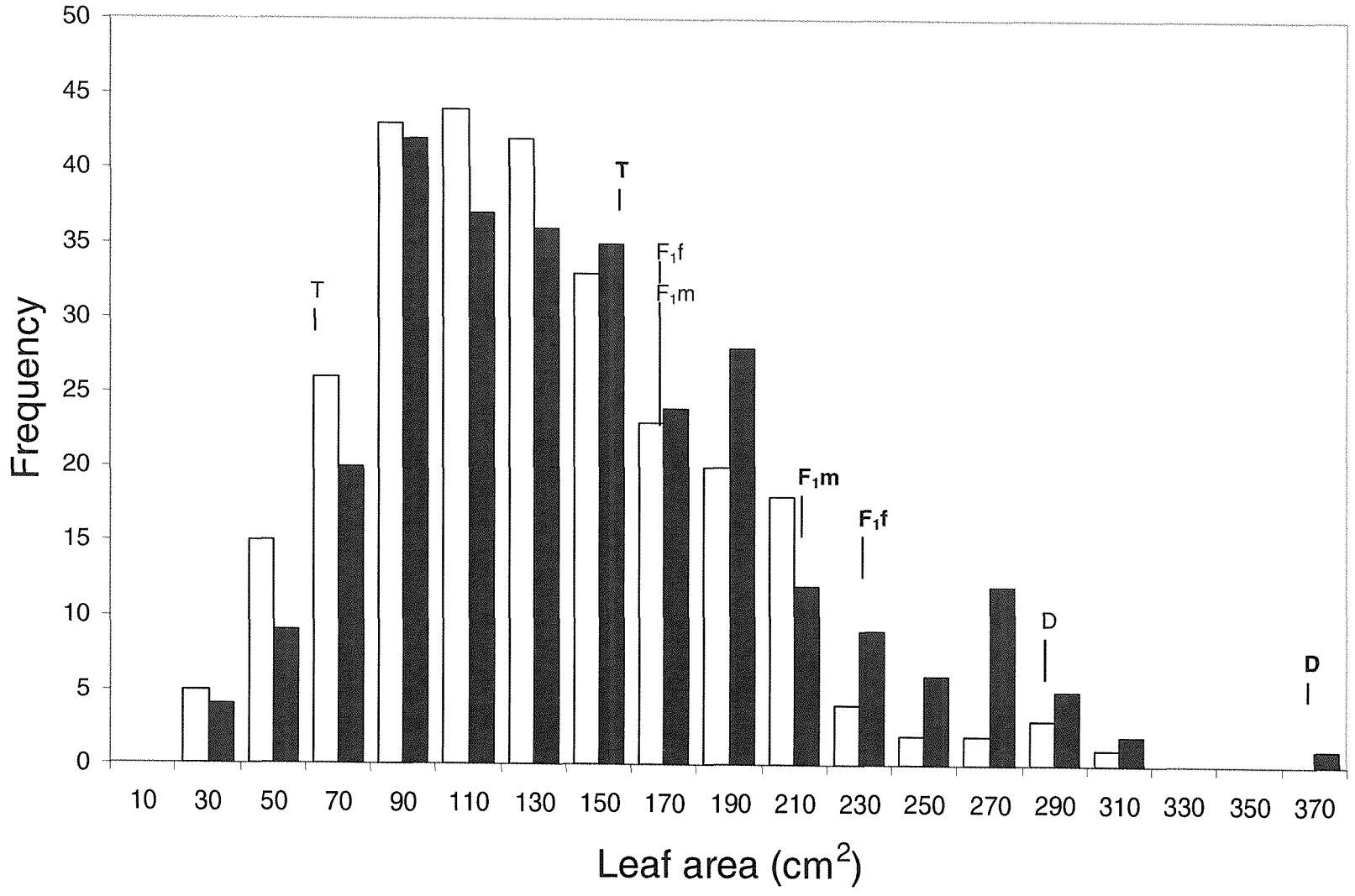


Figure 4.3 Distribution of individual leaf area phenotypes in the Family 331 pedigree in September (130-134 DAP), grown in ambient CO_2 (open bars) and in elevated CO_2 (solid bars). Parental and F_1 values, in regular text (ambient CO_2) and bold text (elevated CO_2), are indicated as T (*Populus trichocarpa,* female parent), D (*Populus deltoides,* male parent), F_1m (male F_1) and F_1f (female F_1).

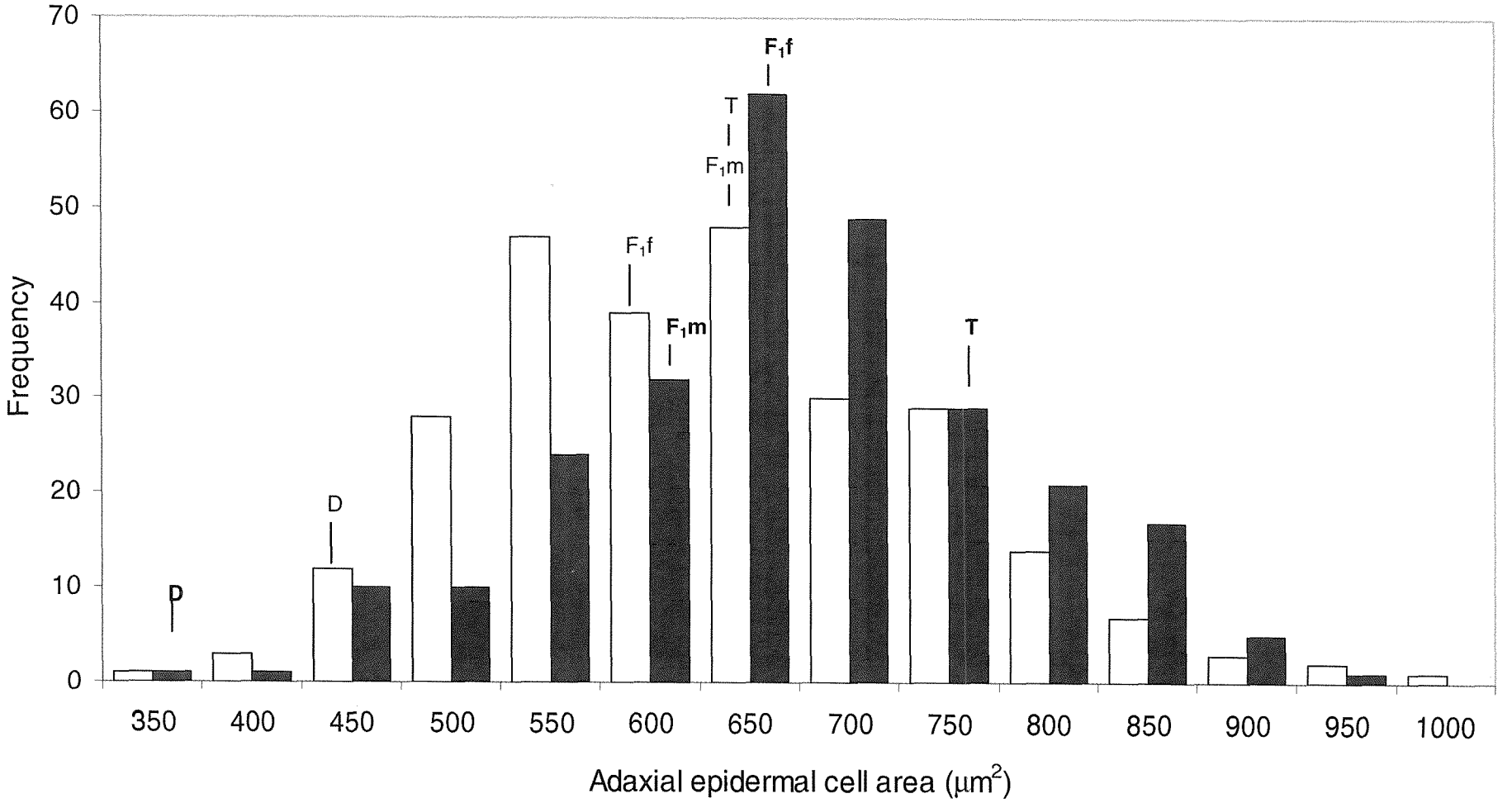


Figure 4.4 Distribution of adaxial epidermal area phenotypes in the Family 331 pedigree in August (80 - 82 DAP), grown in ambient CO_2 (open bars) and in elevated CO_2 (solid bars). Parental and F_1 values, in regular text (ambient CO_2) and bold text (elevated CO_2), are indicated as T (*Populus trichocarpa,* female parent), D (*Populus deltoides,* male parent), F_1m (male F_1) and F_1f (female F_1). Each genotype represents a mean of ten epidermal cell areas from one epidermal imprint.

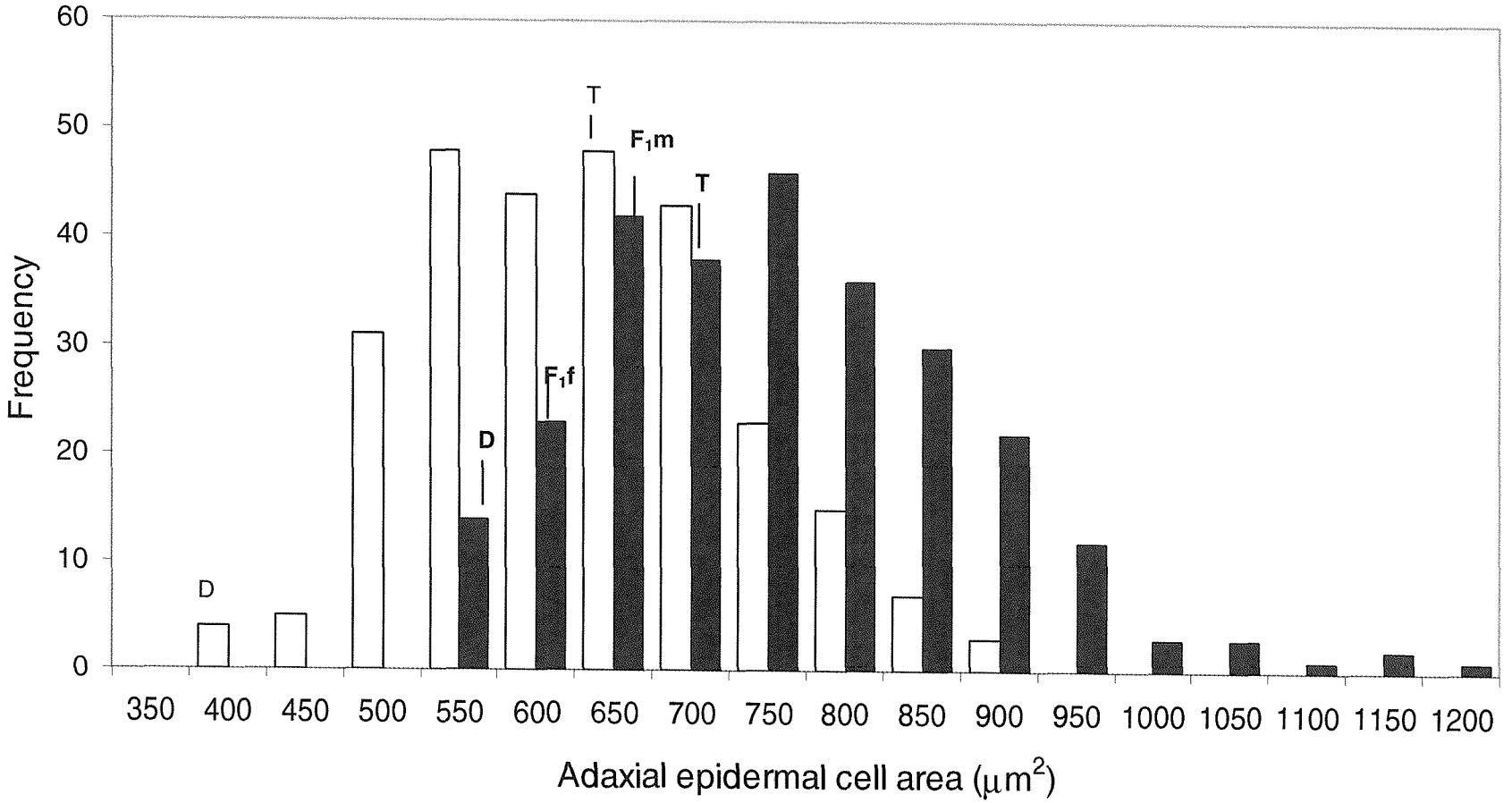


Figure 4.5 Distribution of adaxial epidermal area phenotypes in the Family 331 pedigree in September (130 - 134 DAP), grown in ambient CO_2 (open bars) and in elevated CO_2 (solid bars). Parental and F_1 values, in regular text (ambient CO_2) and bold text (elevated CO_2), are indicated as T (*Populus trichocarpa,* female parent), D (*Populus deltoides,* male parent), F_1m (male F_1) and F_1f (female F_1). Each genotype represents a mean of ten epidermal cell areas from one epidermal imprint.

Estimated cell number per leaf

The estimated number of adaxial epidermal cells per leaf at 80-82 DAP is illustrated in Figure 4.6. In both treatments, it is clear that *P. deltoides* attained the largest numbers of cells per leaf, exceeding any F_1 or F_2 genotype. In aCO_2, *P. trichocarpa* had the smallest number of cells per leaf. The F_1f was the most stable of the parents and F_1s in its cell numbers between aCO_2 and eCO_2, but the F_1m had considerably less cells per leaf in eCO_2, although this trend was observed not observed in either parent. The population modal frequency was the same in both aCO_2 and eCO_2, with no significant CO_2 response.

In Figure 4.7, which shows cell number phenotype distributions at 130-134 DAP, it can be seen that *P. trichocarpa* again produced fewer cells than *P. deltoides* or either F_1 genotype. *P. deltoides,* mirrored by the F_1f, had fewer cells per leaf in eCO_2 than aCO_2, but the F_1m behaved like *P. trichocarpa* in the production of more cells per leaf in eCO_2. The population modal frequency in aCO2 was 21.25—23.75 $x10^6$ cells per leaf, and in eCO2 was 16.25-18.75 x 10^6 cells per leaf. There was a significant ($P = 0.037$) 7.64% reduction in cell numbers in eCO_2.

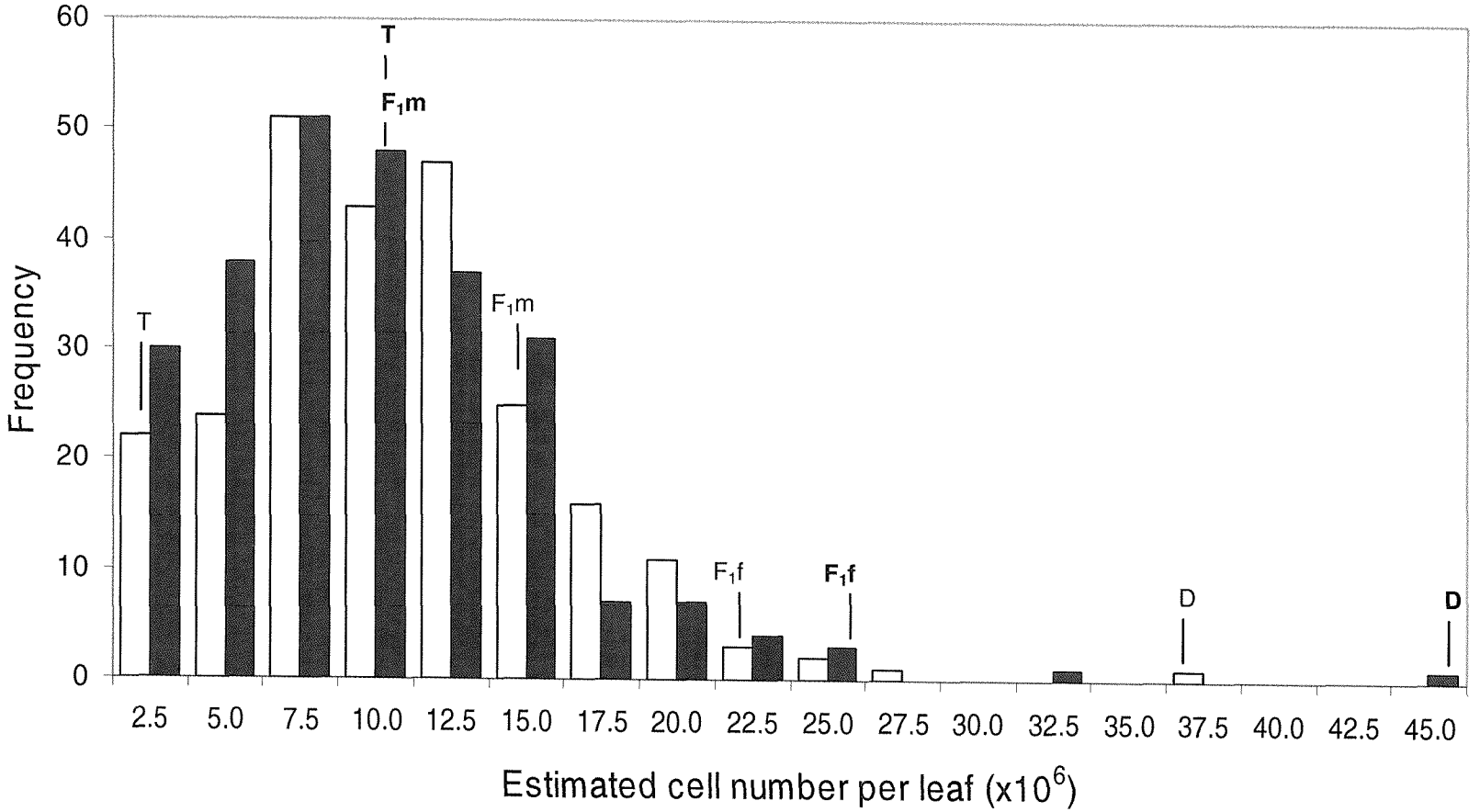


Figure 4.6 Distribution of leaf cell number phenotypes in the Family 331 pedigree in August (80 - 82 DAP), grown in ambient CO_2 (open bars) and in elevated CO_2 (solid bars). Parental and F_1 values, in regular text (ambient CO_2) and bold text (elevated CO_2), are indicated as T (*Populus trichocarpa,* female parent), D (*Populus deltoides,* male parent), F_1m (male F_1) and F_1f (female F_1).

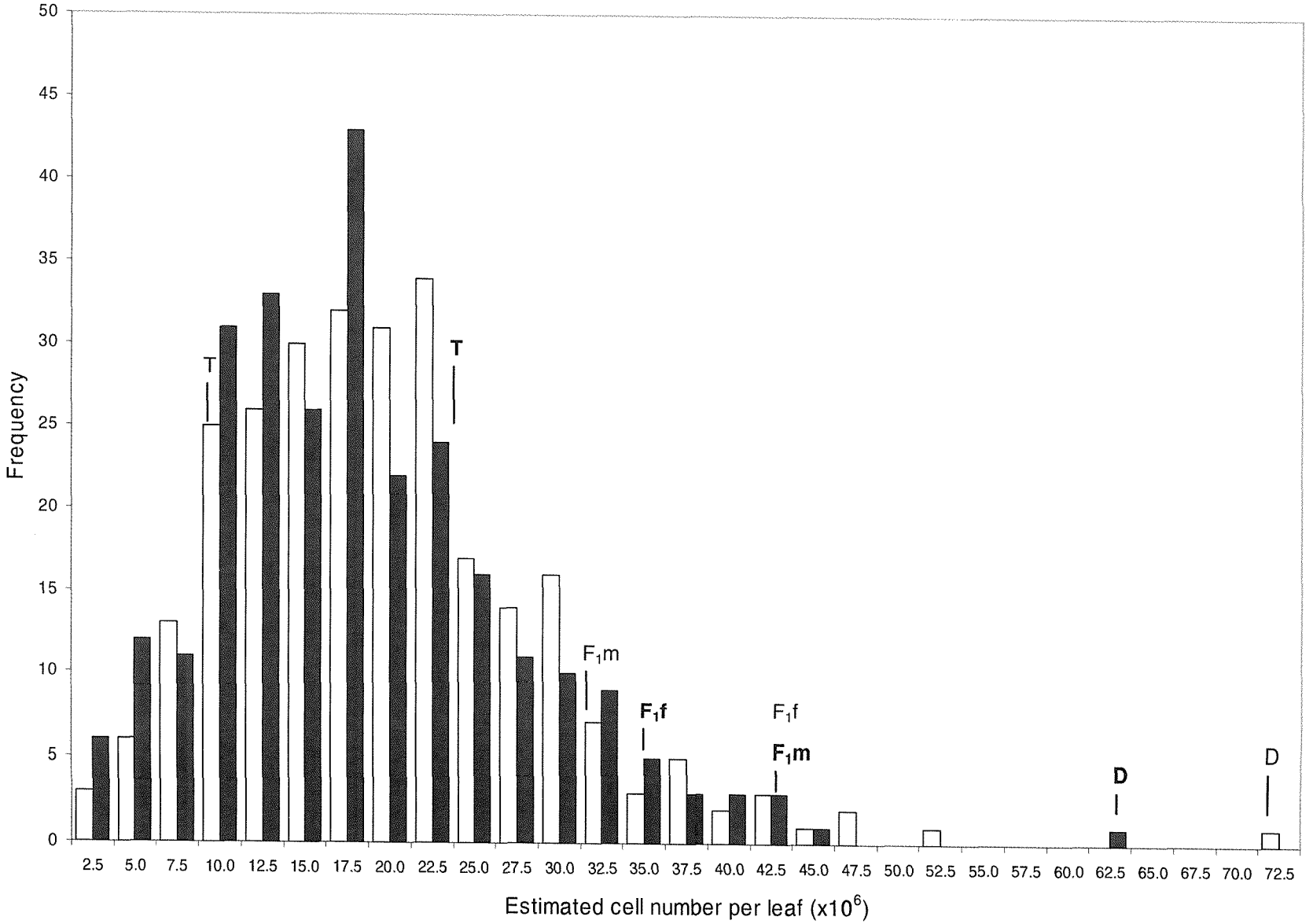


Figure 4.7 Distribution of leaf cell number phenotypes in the Family 331 pedigree in September (130 - 134 DAP), grown in ambient CO_2 (open bars) and in elevated CO_2 (solid bars). Parental and F_1 values, in regular text (ambient CO_2) and bold text (elevated CO_2), are indicated as T (*Populus trichocarpa,* female parent), D (*Populus deltoides,* male parent), F_1m (male F_1) and F_1f (female F_1).

QTL location

Putative QTL have been found for all three leaf traits measured in both aCO_2 and eCO_2, using a LOD threshold of 2.9. The linkage groups on the genetic linkage map to which these QTL have been mapped are A, B, C, D, E, F and G, and on four of these linkage groups, more than one QTL has been located. Five loci mapped to linkage group A. These loci controlled the traits: leaf area in aCO_2 (at 80-82 DAP); cell area in aCO_2 (at 80-82 and 130-134 DAP); cell area in eCO_2 (80-82 DAP); and cell number per leaf in aCO_2 (at 80-82 DAP). The QTL found include the three leaf traits measured at 80-82 DAP, in addition to some representation of both CO_2 treatments and the two stages in the season. These QTL mapped to proximate areas of linkage group A, with cell area (eCO2) at 88.6 cM, leaf area and cell number at 89.2 cM, and cell area (aCO_2, 130-134 DAP) at 95.6 cM. The QTL for cell area in aCO_2 at 80-82 DAP was mapped nearby, to 116.6 cM, and this was the QTL with the largest confidence interval. The confidence intervals of all of these loci overlapped.

Leaf area in eCO_2 (80-82 DAP) was also mapped to linkage group B, at 6.0 cM, and this was close to the cell area QTL in aCO_2 (130-134 DAP) at 12.0 cM. Two other cell area QTL were identified in eCO_2 on linkage groups C and D: at 80-82 DAP and at 130-134 DAP respectively. There were three more QTL found for leaf area. One of these was on linkage group F, for leaf area in aCO_2 at 80-82 DAP. This was the only QTL mapped to linkage group F. Leaf area QTL were also identified on linkage groups E and G, and in both of these instances, leaf area and cell number QTL co-located. On linkage group E, leaf area and cell number were located at 14.1 and 14.0 cM respectively. Both of these QTL were for eCO_2 measured at 130-134 DAP. The same

two traits (leaf area and cell number in eCO2 at 130-134 DAP) were also found to co-locate on linkage group G at 32.9 cM.

Although loci have been located for each of leaf area, cell area and cell number in aCO_2 and eCO_2, QTL were not identified for every trait in each treatment *and* stage in the season. QTL were not found for: leaf area in aCO_2 at 80-82 DAP; cell area in eCO_2 at 130-134 DAP; or cell number in aCO_2 at 130-134 and in eCO_2 at 80-82.

Table 4.3 Linkage group positions, confidence intervals, LOD scores and percentage variation explained by the QTL of the leaf traits studied in ambient and elevated CO_2.

Trait	Treatment	DAP	Linkage Group	Position	Confidence Interval	LOD	% Variance explained
Leaf area	aCO_2	80-82	A	89.2	74.1 – 110.4	3.32	28.8
			F	76.2	16.3 – 96.1	4.13	37.3
	eCO_2	80-82	B	6.0	0.0 – 30.0	2.72	42.6
		130-134	G	32.9	0.0 – 44.9	2.93	27.7
			E	14.1	0.00 – 42.0	2.72	27.1
Adaxial epidermal cell area	aCO_2	80-82	A	116.6	54.5 – 138.9	2.68	38.1
		130-134	A	95.6	77.0 – 126.6	2.84	33.2
			B	12.0	0.0 – 25.5	3.52	49.1
	eCO_2	80-82	A	88.6	62.4 – 124.6	3.29	31.8
			C	84.3	55.6 – 124.3	4.09	62.0
		130-134	D	78.5	0.0 – 130.1	5.54	50.5
Estimated cell number per leaf	aCO_2	80-82	A	89.2	72.1 – 114.4	3.29	29.8
	eCO2	130 – 134	E	14.0	0.0 – 40.0	3.21	36.5
			G	32.9	0.0 – 46.9	3.01	29

4.4 DISCUSSION

Natural stands of *P. trichocarpa* are adapted to upland forest and riparian habitats in Western U.S.A. and Canada. There is natural environmental variability between genotypes growing in different habitats (Hinckley *et al.,* 1989); furthest west, for example, *P. trichocarpa* produces large leaves with few, large epidermal cells. In the East, however, *P. trichocarpa* shows more xerophytic adaptations including small leaves that maintain high photosynthetic rates in high light concentrations (Dunlap and Stettler, 2001). Likewise, there is natural variation in the response of *P. deltoides* to the environment. The differences in leaf anatomy between the parent species indicate that genes for these traits are likely to segregate in the F_2.

The size and structure of leaves in the parent plants of the pedigree, *P. trichocarpa* and *P. deltoides,* are contrasting, as has been shown in other studies (Ridge *et al.,* 1986), with the leaves of *P. deltoides* larger than *P. trichocarpa* and the F_1s. In contrast to Ridge *et al.*'s (1986) findings, however, the leaf area of the F_1s in this experiment differed to each other and did not exceed that of the larger parent.

In aCO_2, both parents grew larger leaves at 130-134 DAP than at 80-82 DAP, which may be associated with accelerated growth due to accumulated biomass throughout the season. The range of F_2 leaf areas increased from 180 cm^2 in August (80-82 DAP) to 320cm^2 in September (130-134 DAP). Other work on trees has found, like in this study at 80-82 DAP, no effect of the CO_2 treatment on individual leaf area (Norby and O'Neill, 1989; Mousseau and Enoch, 1989; Norby and O'Neill, 1991). However, at

130-134 DAP, there was an increase in leaf area in the F_2 population by 11.18%, which reflects the findings of many other researchers for individual leaf area in eCO_2 (Radoglou and Jarvis, 1990a; Ceulemans *et al.,* 1995; Ferris *et al.,* 2001). The parent most responsive to eCO_2 in leaf area was *P. trichocarpa,* which attained increases of 303.5% and 136.8% in August and September respectively. *P. deltoides* did not demonstrate such sensitivity to eCO_2, with only a small leaf area change in August and a moderate leaf area increase of 24% in September. A useful consideration of larger organ size in eCO_2 is the scaling of the organ relative to the whole plant. Wolfe *et al.* (1998) have observed that large leaf areas observed in eCO_2 may not so much be due to a direct influence of eCO_2 on the leaves, but sometimes due to faster overall plant development. Results from this experiment do indicate that the parental plants were larger overall in eCO_2 (R. Ferris, University of Southampton, pers. comm.), but a direct scaling effect could not explain the considerable increases in the leaf area of *P. trichocarpa* in eCO_2.

Work on *Populus* has often considered the total leaf area of the plant as well as the individual leaf area in studying responses to eCO_2. A study of 'Beaupré' and 'Robusta,' Chen *et al.* (1997), for example, found that leaf area index (LAI) was greater in elevated than ambient CO_2. Radoglou and Jarvis (1990a) noted an increase in total plant leaf area in eCO_2, but did not find individual leaf area to be responsive. Other studies have reported increases in both individual and total leaf area (Ceulemans *et al.,* 1996b).

The results of cell area measurements in both aCO_2 and eCO_2 reflected the results in Chapter 3 of this thesis, and are also described in Ferris *et al.* (2002). *P. deltoides* had smaller epidermal cells than both F_1 genotypes, as also described by Hinckley *et al.* (1989) and Ridge *et al.* (1986). The F_1 cell area values fell between those of the parents. In the F_2 population, significantly larger cells in eCO_2 were found in both August and September, giving further confidence in the result, and showing that the response of cell area increase in eCO_2 is consistent over two points in the growing season. Radaglou and Jarvis (1990b) also studied leaf growth with respect to anatomical features in poplar. Their findings suggested that it was increased cell expansion driving increased leaf area in elevated CO_2. Furthermore, the parental cell areas were very consistent over the two stages in the season, confirming the technique of cell measurement as accurate and confirming that mature cell size in the parents is has a high heritability and is minimally controlled by the environment (Hinckley *et al.,* 1989; Dunlap and Stettler, 2001).This also mirrors the finding of Radoglou and Jarvis (1990b), that differences in epidermal cell density between genotypes reflected their parentage. The increase in cell area in *P. trichocarpa* in eCO_2 at the two seasonal stages is also reasonably consistent, with area increases of 15.6% and 10.8% in August and September respectively. In contrast, the response of *P. deltoides* altered between the two time points, with a decrease in cell area (and an increase in cell number) in August and an increase in cell area (and accompanying decrease in cell number) in September. This finding supports observations that cell expansion and cell division are intrinsically linked, making it difficult to identify which process is responding to eCO_2 (Pritchard *et al.,* 1999). However, as in the case of *P. deltoides* in aCO_2, the largest

leaves usually have the greatest number of epidermal cells, which is a function of cell division (Dale, 1988; Ferris *et al.,* 2002). The association between leaf cell area and number is inevitably adjusted to give the final leaf area.

Dunlap and Stettler (2001) calculated a maximum heritability of 0.71 for adaxial epidermal cell diameter in *P. trichocarpa*, and a maximum heritability of 0.87 for the number of adaxial epidermal cells per leaf. They found that there was considerable genetic control over some traits in this species, ranging from 0.6 to 0.9. Radoglou and Jarvis (1990b) observed that differences in cell characteristics between clones, including hybrids of *P. trichocarpa* and *P. deltoides* ('Beaupre' and 'Raspalje'), *P. trichocarpa* ('Columbia River') and *P.x euramericana* ('Robusta'), appeared to be affected by parentage. Thus it could be inferred that heritability for leaf cell traits is high in *Populus.* This is further suggested by the co-locating putative QTL for different leaf traits shown in Table 4.3..

Cell area was significantly different between CO_2 treatments in the F_2 at both stages in the growing season ($P < 0.001$ in both instances), whereas cell number per leaf was not different between treatments in August ($P < 0.144$) and less obviously different between treatments in September ($P = 0.037$). In the F_2 population, eCO_2 had a larger effect later in the growing season, with cell areas 19.79% larger than aCO_2 in Sept compared with a 6.03% increase in eCO_2 over aCO_2 in August.
Indeed, cell expansion is often sensitive to CO_2 concentration (Radoglou and Jarvis, 1990a; Ferris *et al.,* 2001; Taylor *et al.,* 2001).

In August, *P. deltoides* attained higher numbers of cells per leaf than any other genotype in the pedigree, and *P. trichocarpa* fell into the category with the fewest cells per leaf. Although the population response of cell number to eCO_2 was not significant in August, there was a small but significant (at $\alpha = 0.05$) reduction in cell number in September. Ridge *et al.* (1986) estimated the number of cells per leaf to gain an understanding of the cellular reasons for variation in leaf area. They found that *P. trichocarpa* x *P. deltoides* hybrids produced more cells per leaf than either parent. The results of this experiment show that *P. deltoides* had more cells per leaf than any other genotype in the pedigree (Figures 4.6 and 4.7). These results differ from those of Ridge *et al.* (1986) in that the F_1s do not show heterosis over the parents, but appear as intermediates between the two species. Using the same technique in studies of *P. trichocarpa,* Dunlap and Stettler (2001) calculated adaxial epidermal cell numbers per leaf to be in the region of 74 to 211 x 10^5 in ambient field conditions.

In one experiment using both mature and recently-mature leaves (Ceulemans *et al.,* 1995), epidermal cell density on either the adaxial or abaxial surface did not differ between ambient and elevated CO_2 treatments. These leaves had between 8 000 and 10 000 cells per mm^2 on one surface. The *P. trichocarpa* x *P. deltoides* hybrid, 'Beaupré' had more adaxial epidermal cells per leaf in elevated carbon dioxide than in ambient carbon dioxide: 2 824 x 10^6 and 2 936 x 10^6. 'Robusta' had less cells in elevated (1 802 x10^6) than ambient (2 069 x 10^6). It appears that the CO_2 effect of more cells per leaf may be because of larger individual leaf areas. Comparisons with

previous work thus show the results of this OTC experiment to have found the cell number per leaf estimates are within the similar orders of magnitude for the given leaf areas in this study, and the cell number per leaf response to eCO_2 is likewise sensitive, but not to the extent of the response of cell area.

Radoglou and Jarvis (1990a) found no effect of eCO_2 on the number of cells per unit leaf area or per leaf – their result was due to a large total leaf area per plant rather than there being more cells in each leaf. This may be worthy of measurement in Family 331; in the results we see only the group effect of CO_2, through the comparison of population means for aCO_2 and eCO_2, but some F_2 individuals may be simply producing more leaves on the plant and have not been discussed in this experiment. However, this work does show that cell area is highly heritable and responsive to eCO_2 in *P. trichocarpa* and the F_2s, and cell number is also influenced to a certain extent.

Much of the large influence of elevated CO_2 on leaf area development in wheat (*Triticum aestivum* L.) leaves is caused by an increased supply of photoassimilates which influences the length of epidermal cells. This also increased the number of cells in the leaf (Masle, 2000). Radoglou and Jarvis (1990b) also concluded that leaf cell size is the cellular basis of differences in leaf area.

Leaf characteristics do not always respond in the same way to eCO_2 in different genotypes or species (Ferris and Taylor, 1994; Woodward and Kelly, 1995). Ferris *et al.* (1996), for example, found both increases and decreases in stomatal density in

eCO_2. They observed that stomatal traits are dependent on varying amounts of cell expansion, which indicates that epidermal cell area is an important factor influencing a number of leaf traits, including stomatal density and index and final leaf area.

QTL is about mapping individual genes for complex traits. They are rarely, if ever, identified from the phenotype (Kearsey, 1997), because many genotypes could give the same, or a similar, phenotype. This study has identified putative QTL for individual leaf area, epidermal cell area and cell number per leaf in Family 331. This work compares with the results of Wu *et al.* (1997) who also found QTL for several leaf traits including single (individual) leaf area, as well as length-width ratio. Ferris *et al.* (2002) have reported the other Family 331 leaf traits under study in this experiment. These include measurements of the associated traits of epidermal cell density. QTL have also been found for adaxial and abaxial stomatal density in *Oryza sativa* and co-locate with yield on one linkage group (Ishimaru *et al.*, 2001b). Leaf area QTL have also been found in other genera including leaf length and width QTL identified by Rae (2000) in *Brassica.*

Mansur *et al.* (1993) also identified QTL for leaf area. Furthermore, they found seed yield and leaf area to be highly correlated. They also found clusters of QTL for yield traits and developmental traits (including flowering, maturity and height). They could be representing single, pleiotropic genes with major effects, or equally, a group of genes that segregates as a single locus. In a discussion of Family 331 leaf QTL, Wu *et al.* (1997) attributed the nearby QTL accounting for a large proportion of the variance

for each trait to be pleitropic effects. Work on sugar beet (Schneider *et al.,* 2002) found that the QTL found for different yield traits often overlapped in map position, as is the case in the results shown in this chapter. As well as attributing this to possible pleitropic effects, it was proposed that the lack of resolution between a number of tightly-linked genes of small effect, each controlling a single trait, might be identified as a single QTL. An alternative suggestion is that, because some complex traits occur as a result of other, 'primary' traits, the correlation of the two associated traits may be seen as co-locating QTL, when in fact the expression of the primary trait has triggered the expression of the other, 'secondary,' trait. Equally, it may simply be that a single gene has two different fuctions (Ishimaru *et al.,* 2001a). This could be investigated in *Populus* leaf studies, whereby the number of cells in a leaf and the individual leaf area are functions of one another, and the QTL have been found to closely co-locate. Locating QTL for two traits to the same position on a linkage group is circumstantial evidence that there is a causal relationship between the traits, although the only certain evidence for this would be to find the actual genes for these QTL (Thumma *et al.,* 2001). Putative pleiotropic effects of a gene have also been detected in QTL for Rubisco and soluble protein overlapping on a rice linkage group (Ishimaru *et al.,* 2001a).

This experiment has identified QTL for leaf traits at two stages in the growing season. Traits from both August (80-82 DAP) and September (130-134 DAP) measurements were mapped to the same area, on each of linkage group A and linkage group B (Table 4.3). Yin *et al.* (1999), however, found different QTL for specific leaf area at six

developmental stages. They observed that QTL related to developmental traits may be dynamic, varying according to the developmental stage. This has also been found with flowering time in *Brassica oleracea* (Rae *et al.,* 1999). It is possible that further studies of leaf development in Family 331 could identify more QTL for developmental traits, as have already been found by Frewen *et al.* (2000) for bud burst and bud set in *Populus* hybrids related to this pedigree.

Although MAPMAKER/ QTL is suitable for leaf traits in the Family 331 pedigree owing to the distinct parental characteristics of the parents (H.D. Bradshaw, University of Washington, pers. comm.), it is possible that the resolution of QTL location may further be improved by using alternative QTL mapping software for outbreeding species (Prioul *et al.,* 1997). However, it has been shown that all QTL mapping methods give essentially similar estimates of QTL position and gene effects (Kearsey and Farquhar, 1998), but population size and genome marker coverage are the main limitations on detecting and resolving QTL positions. However, there is no evidence that dense linkage maps are required for initial QTL location Hyne *et al.* (1995). The QTL identified here each represent between 27% and 62% of the genetic variation for the trait. The calculation of the genetic variance may be improved using larger sample size for QTL mapping or increasing the number of generations in the pedigree. It is therefore expected that with more the more extensive genome coverage and progeny size included in the update of the map by Bradshaw *et al.* (1994), currently in preparation, the QTL identified in this study will be mapped with more precision and smaller confidence intervals. The applications of finding QTL are of use to tree

breeders and can be applied in several ways, either using molecular techniques such as marker assisted selection or map-based cloning (Wu, 2001; Mazur and Tingey, 1995; Kearsey, 1997), or enhancing traditional breeding techniques using the principal of ideotypes (Dickmann and Keathley, 1996).

Chapter 5

General Discussion

5.1. BIOMASS FOR ENERGY PRODUCTION

The aim of this research thesis was to identify the anatomical, physiological and morphological traits most highly linked to yield in poplar and willow genotypes. The results of this work have shown that some key leaf and stem traits can be used as biomass determinants for future breeding programmes or for directing future research in short-rotation coppice.

Short-rotation coppice trees used as a renewable energy source have to compete with fossil fuels in order to be economical and political equals in the energy market (Kauter *et al.,* 2003). Therefore it is imperative that costs are reduced and efficient energy conversion takes place in the growing plants. Biomass energy also has to compete with other renewable energy sources, which further affects the potential of biomass as an energy supply. Berndes *et al.* (2003) evaluated several published forecasts of the contribution of biomass to future energy supply. Most studies predicted that energy crop production will be able to use land involved in set-aside schemes, to restore the productivity of such land. Developing countries may be expected to contribute most of the share of global bioenergy supply; moreover, they may be expected to export products such as methanol to industrialised countries.

Biomass has advantages over fossil fuels of being carbon neutral: unlike fossil fuels biomass only returns the amount of CO_2 assimilated in the plant's lifetime to the atmosphere (Hall and House, 1995). Furthermore, biomass sources tend to be lower in their emissions of nitrogen and sulphur compounds than fossil fuels, particularly coal.

Coal is typically 1.77 % N by weight; poplar is 0.42 % (Kauter *et al.,* 2003). Ideal biofuels need to produce high biomass yields as well as low moisture content, low concentrations of nitrogen, ash and alkali metals, and high concentrations of lignin and cellulose (Thakaran, 2003; Lemus *et al.,* 2002). In continents other than Europe and North America, where poplar and willow prevail as trees for bioenergy, hardwood trees, such as *Gmelina arborea* (Onyekwelu, 2003) exhibit vigorous growth from cuttings and coppice easily, and produce high biomass yields suitable for use as an energy crop. Perennial, herbaceous grass crops used for biomass energy include *Miscanthus*, which can be used either directly, for example, in co-firing in power stations with coal, or indirectly by fermentation to ethanol (Lemus *et al.,* 2002). Moisture content affects the energy conversion of a fuel. The higher the moisture content, the higher the cost of energy required to vaporise the water (Kauter *et al.,* 2003). Higher moisture content also increases transport costs. Kenney *et al.* (1990) have suggested that feedstocks with high moisture content are preferable for use in biochemical conversion process, whereas those with lower moisture content are more suitable for direct combustion. Most wood as an energy source is used for direct combustion.

While it is easy to focus on the yield potential of biomass crops in terms of physiology and genetics, it should be remembered that the theoretical extent of bioenergy plantations may be limited in practice by restrictions of land use in the interests of biodiversity, soil conservation and carbon sequestration in the future (Berndes *et al.,* 2003). If short-rotation coppice systems can be widely implemented, there is a wealth

of evidence showing the potential of trees as bioenergy crops. Heilman and Stettler (1990), for example, have recorded hybrids of *P. trichocarpa* and *P. deltoides* crosses to attain large dry weight yields in the coppice form. One example of this is the hybrid genotype 11-11 with a yield of 43.5 ODT ha yr^{-1}, and one genotype of pure *P. trichocarpa* parentage yielding 32.6 ODT ha yr^{-1}. Further potential for energy production could be the use of roots at the end of the final coppice rotation. This compares with an estimated maximum yield in the field trial of Family 331 (Chapter 3) where the highest yielding genotype, 331-1918, attained an equivalent of 19.5 ODT ha^{-1} yr^{-1}, based on the 1 m spacing and first coppice growing season. This was above-ground yield, but roots are also important factors to include in considerations of whole plant biomass gain. In *Salix,* for example, root biomass can be equal or greater in volume than the above ground biomass (Isebrands *et al.,* 1996). However, spent rootstocks may themselves be of use for carbon sequestration (Matthews, 2001).

There remain large discrepancies between the actual and potential yields in silviculture and agriculture for energy crops, especially in new crops where, so far, less effort has been invested in breeding and cultivation practices (Berndes *et al.,* 2003). Low yields on energy plantations may not be caused so much by inferior growing conditions, so much as by a poor match between the species and site characteristics (El Bassam, 1998). Most studies of the contribution of biomass to future renewable energy demand predict that forest plantations will be the major source of biomass. Field trials have been used to investigate the potential of different genotypes in different growing

conditions and coppice regimes, to identify good matches between genotype and site for optimum yield (Thakaran *et al.,* 2001).

5.2 THE IMPACT OF ELEVATED CO_2 ON BIOMASS

At 360 μmol mol^{-1}, atmospheric CO_2 is sub-optimal for plant growth and is effectively a stress. In the U.K., it is predicted that photosynthesis and crop yields would increase by approximately 30 % if the concentration of CO_2 in the atmosphere were increased to 720 μmol mol^{-1} (Lawlor, 2002). This is not an unrealistic projection, since the IPCC moderate scenario for future atmospheric CO_2 concentrations is 550 μmol mol^{-1} by 2050 (IPCC, 2001). This would affect the yield of woody perennials, such as wood yields from short-rotation coppice willow and poplar. The OTC study has demonstrated the effect of elevated CO_2 on leaf biomass, as shown in the shift towards larger individual leaf areas in the F_2 population in elevated CO_2. There was also a shift towards greater stem height and diameters, amounting to higher overall yield, in the Family 331 population grown in the OTC experiment (Bunn, 2001). Elevated CO_2 does not always affect biomass; in a free air carbon dioxide enrichment study by DeLucia *et al.* (2002), the height and diameter calculation of aboveground biomass allocation was not affected by elevated CO_2 in *Pinus taeda* L.. On many other occasions, however, stem biomass has been seen to positively increase in elevated CO_2, particularly in *Populus* (Chen *et al.,* 1997; Ceulemans *et al.,* 1994; Norby and O'Neill, 1991). Furthermore, increased temperatures linked to global warming are likely to influence plant productivity. Given sufficient water, many temperate crop plants have maximum CO_2 assimilation rates when the temperature is between 20 and 30°C

(Montieth, 1977). It is therefore useful to study the responses of poplar to predicted future atmospheric conditions to gain an understanding of the response of Salicaceae to these changes. This should enable genotype selection and breeding for this current, and future, source of renewable energy. In poplar Family 331 F_2 genotypes grown in OTC conditions, leaf area and adaxial epidermal cell area were increased by elevated CO_2 by up to 11.18 % and 19.79 % respectively, but the number of adaxial epidermal cell numbers per leaf showed less sensitivity to elevated CO_2. The overall stem biomass increased in the population in elevated CO_2 (Bunn, 2001).

There may be some disadvantages to enhanced biomass production in elevated CO_2 environments. For example, the production of increased leaf biomass in elevated CO_2 concentrations provides more tissue for consumption by herbivores. Insect performance on leaves has been shown to interact with elevated CO_2 (McDonald *et al.*, 1999). Thus it is important to develop genotypes likely to display characteristics of disease and pest resistance, as well as efficient resource capture and assimilation leading to high biomass yield.

5.3 BIOMASS DETERMINANTS

Willow and poplar are early successional plants; their fast growth suppresses competitors by shading and contributes to their early success (Kauter *et al.*, 2003). The use of certain Salicaceae species in short-rotation coppice forestry exploits the fast growth of pioneer species.

Of the genotypes of differing yields studied in *Salix* coppice, the highest yielding genotypes attained the largest estimated numbers of epidermal cells per leaf, and in more detailed analysis, the genotype with higher yield showed the greatest rates of leaf extension and production. Some high yielding genotypes in poplar Family 331 in the field trial also mirrored this result, especially the F_2 genotype 331-1689, which was a strong, high-yielding outlier in the statistical analysis. The OTC study in both ambient and elevated concentrations of CO_2 showed the patterns of epidermal cell area (indicating expansion) and number (evidence of overall production) in the poplar pedigree. These results together suggest that individual leaf development, through the production of many, large epidermal cells, may be important for light capture in the individual leaf and leaf area index development to increase leaf, and therefore stem, biomass in future atmospheres. Both light capture and processing may indeed be more important in determining plant biomass yield than photosynthesis per unit leaf area (Ericsson *et al.*, 1996).This rationale reflects earlier work by Montieth (1977), indicating that the maximum accumulation of dry matter in a plant system is highly positively related to the amount of radiation intercepted by its foliage. This was confirmed in the detailed analysis of the two willow genotypes in the willow experiment. Although positive correlations have been recorded between rates of individual leaf photosynthesis and crop yield (e.g. Faville *et al.*, 1999), no direct relationships were associated in the two willow genotypes studied. Wu (1998) suggests that a larger poplar canopy gives rise to larger total leaf area which would intercept more light and make more stem growth than would a smaller canopy. Leaf area index estimates found less canopy thickness in the highest yielding of the two *Salix*

genotypes studied, however the individual leaf area, leaf longevity, and rates of leaf extension and production were greater in the highest yielding *Salix* genotype. In the Family 331 field trial, large individual leaf area and high numbers of leaves on the leading coppice stem were also highly influential of high biomass yield. These factors appear to be more important in the determination of harvested biomass than LAI *per se.* However, Montieth (1977) compared four crops (potato, apple, barley and sugar beet) to show that the relationship gradient between intercepted radiation and dry matter production is very similar across most crops. In non-limiting nutrient conditions and sufficient irrigation, biomass yield of a crop is related, directly, to availability of PAR, the amount of the PAR intercepted by the stand canopy, and the efficiency of light use (Montieth, 1977; Casella and Ceulemans, 2002), which was highest in the high-yielding willow, 'Tora.'. Moreover, it is argued that light provides the energy for all metabolism, which explains the linear relationship between radiation interception and the production of dry matter (Lawlor, 2002), so it may be that light interception is the preferential factor determining yield, as has been shown in *Populus* (Bunn *et al.,* 2003; Taylor *et al.,* 2001). The preliminary light response curves in the high- and low-yielding *Salix* genotypes have shown that light harvesting may indeed be of great importance to biomass. The findings here could be extended to investigate the role of leaf angles in the canopy in light interception in all of the genotypes studied in this thesis. In Family 331, these traits have already been shown to be controlled by QTL (Wu *et al.,*1997). It is possible that smaller, more erectophile leaves on sylleptic branches may increase photosynthetic efficiency through improved temperature regulation at high light intensities (Wu, 1994). The most influential leaf traits in the

Family 331 poplar model accounting for harvested biomass in first year coppice plants were the number of leaves on the leading stem and the individual leaf area. The highest yielding F_2 genotypes achieved the greatest values in both of these traits, especially the highest yielding F_2 genotype, 331-1918. This was also true of the six genotypes investigated in the *Salix* field trial. The poplar and willow field trials have shown the importance of basal stem diameter, which can be used to calculate basal stem area, as indicator of harvested biomass (Telenius, 1997b). This work has also shown that in both willow and poplar, sylleptic branching is linked with biomass yield. There are few references in the literature to sylleptic branching in *Salix.*

The leaf growth rate traits in *Populus,* with the exception of spring bud burst, have reasonably low broad-sense heritability owing to a large proportion of environmental variation. As a result, few QTL were found for these traits and their influence on the poplar yield model was negligible. In the study of two willow genotypes, it was clearly seen that leaf growth rates were higher in 'Tora', the highest yielding genotype. The traits with the highest broad-sense heritabilities were mostly stem traits, including stem height and diameter. Preliminary QTL analysis has identified loci for several yield-related traits, particularly stem height and several leaf characteristics including individual leaf area and the number of leaves on the leading stem, which were strong determinants of biomass yield. These findings should be built upon for use in molecular breeding for optimum biomass yield.

5.4 SELECTING GENOTYPES FOR HIGH BIOMASS YIELD

It is expected that demand will increase for wood products in the future. Forestry is less advanced than agriculture, where molecular breeding has become standard. The size of trees, their long harvest cycles and the long intervals taken to reach maturity, have held back progress of genetic improvement for wood and fibre qualities, amongst other traits, by traditional approaches (Tuskan *et al.,* 2001; Tzfira *et al.,* 1998). There are currently approximately 10 to 12 *Salix* genotypes available for short-rotation coppice culture, compared to 4 or 5 recommended genotypes of *Populus* (Taylor *et al.,* 2001). More are being developed in *Salix* but less work is being done for commercial release in *Populus.* At present, *Salix* is the genus nearest to full release as a biomass energy crop in the U.K. (Bullard *et al.,* 2002). Since poplar, in particular, hybridises easily and species occur naturally in a range of environments across the Northern Hemisphere (Eckenwalder, 1996), there is great potential for breeding in traits of importance for short-rotation coppice as a renewable energy source. Most selective tree breeding has been done without considering the physiology of the plant (Michael *et al.,* 1990), but the importance of this is becoming more widely recognised (Martin *et al.,* 2000; Dickmann and Keathley, 1996). Rather than looking for individual traits, it is often more useful to look for a "multi-trait ideotype" as a conceptual guideline for research, incorporating, for example, several desirable physiological attributes. An ideotype could be viewed as a single, quantitative trait, with selections based on how well the genotype assessed fits to the ideotype as a whole (Dickmann and Keathley, 1996). Ideotypes can be continually improved, and used as a framework for a research programme (Martin *et al.,* 2000). Where breeding has been steered by clonal

evaluation, the certainty that the trait in question is based on a common genetic background can be increased by the identification of QTL, thus giving more confidence that elite performance of a genotype has a genetic basis (Dickmann *et al.*, 1994). Studies of a three generation pedigree of poplar in single stem, coppice and elevated CO_2 has shown morphological and physiological traits as yield determinants. Further understanding has been gained through the characterisation of two *Salix* genotypes with regard to yield. These results will contribute to the knowledge base of yield-related features of the Salicaceae for breeding, whether through the production of ideotypes, through traditional selection, or using molecular approaches.

Interspecific hybridisation in *Populus* is usually very easy (Dickmann *et al.*, 1996). There are huge advantages in crossing members of the different taxonomic sections of the *Populus* genus, especially the sections *Aigerios* (e.g. *P. deltoides*) and *Tacamahaca* (e.g. *P. simonii; P. trichocarpa*) because of their heterozygosity, leading to hybrid vigour (Wu *et al.*, 1991). This has been observed in the Family 331 pedigree, derived from *Aigerios* and *Tacamahaca* parentage; there were often several F_2 genotypes superior to the parents in many of the traits measured. In the *Salix* field trials, the two highest yielding genotypes, 'Ashton Stott' and 'Tora,' were indeed hybrids. To ease selection further, poplars have a straightforward silvicultural system. Dunlap *et al.* (1992) note that cloning is a useful tool in forest research, and is especially helpful in forest operations, and preserving genotypes for long periods of time. All of the trials studied in this thesis were established from hardwood cuttings. In addition, there is a wealth of genetic information on *Populus* and *Salix* including molecular maps and a

current research programme sequencing the entire poplar genome (Taylor, 2002; Hanley *et al.,* 2002). The knowledge base of poplar is more scientific than that of willow, with willow breeding having traditionally been focussed on traditional selection from field trials (Taylor *et al.,* 2001). Likewise, phenotypic and genetic correlations have been studied in *Populus* and *Eucalyptus*, but less work has been done in *Salix* (Rönnberg-Wästljung and Gullberg, 1996).

The domestication of plants makes them useful for human needs, removes the pressures of the natural environment, and decreases the ability of the cultivar to survive in its original, wild state (Bradshaw and Strauss, 2001). Although some trees producing fruits, such as apples, cherries and olives, have been domesticated for horticultural use, wild trees such as poplar and willow have been maintained in their undomesticated state to produce wood, a vegetative (as opposed to reproductive) product. Domestication can lead to unrecognisable changes in a species. Doebley and Stec (1991) compared the inedible teosinte and its domesticated descendant, maize. They reported that from at least 50 000 different genes, maize was developed from teosinte - nearly beyond recognition - from mutations in only five major genes. In forest trees, economically important traits are usually polygenic instead of involving simple Mendelian inheritance. Such quantitative traits include tree height, stem diameter and total stem volume. These may not be apparent until several years of growth have elapsed; furthermore, heritability is often low in desirable traits. This, as well as the outbreeding nature of forest trees and the large size and great longevity,

means that domestication is not as advanced in forest tree research as it is in annual crops (Bradshaw and Foster, 1992).

Domesticated trees would be expected to invest resources into stem diameter instead of height, as would be ideal for mechanised harvest and crop management. The ultimate goal of intercepting light, via tall growth, is to produce seed. However, domesticated trees grown for wood would not require this as a product, so investment could be channelled into stem diameter production. High yielding outliers identified in the Family 331 field trials have large stem diameters and stem numbers, including the F_2 genotype 331-1674. Such plants could be involved in future breeding programmes. The pioneer nature of poplars and willows, means that they rapidly produce many seeds. Seed production would have to be curtailed, or harvest occur prior to gamete production, in order to channel resources into wood formation (Bradshaw and Strauss, 2001). This is currently being researched in order to accelerate poplar breeding and modification (Brunner *et al.*, 2002). Bradshaw and Strauss (2001) further discuss that the random approach to traditional breeding, which involves finding and crossing mutants of interest, and continually cross-pollinating such interesting individuals to incorporate the desired allele, is difficult in trees. Trees have long generation spans and are often out-breeding (dioecious) and as result, recessive mutations are rare and hard to find. Furthermore, if these could be found, inbreeding results in low vigour due to high genetic load in forest trees, and could take many years to achieve.

QTL mapping is often the interface between genetics and breeding. Paterson (1998a) suggested that QTL mapping can identify genes which influence genetic variation in phenotypes of agricultural (or silvicultural) importance and this can be used as a starting point for using MAS in plant improvement. In Family 331, grown as single stem and coppiced plants and in elevated CO_2, QTL have been found for traits potentially linked to stem or leaf biomass. QTL for leaf extension rate and leaf number on the leading coppice stem, in addition to previously confirmed QTL for individual leaf area (Wu *et al.,* 1997) and spring bud burst (Frewen *et al.,*2000), should prove useful for future fine mapping. Multiple QTL have been found for stem height and these have been confirmed in different stages of the growing season. Stem extension rate and harvested biomass have also been found to be controlled by QTL. QTL for epidermal cell area and number per leaf have been found in the field trial as well as in the OTC study in two CO_2 regimes.

A complete genetic map usually requires a DNA marker every 5cM or approximately 5 % recombination (Paterson, 1998). Unless the effect of a QTL is large and the environmental variation is very much reduced by replication, it is difficult to decrease the confidence interval of a QTL position to below 10 cM for the purpose of map-based cloning (Kearsey, 1998). Raising the number of generations in a pedigree is believed to improve the resolution of QTL mapping. Alternatives to this are to conduct extensive crossing of genotypes prior to selecting the parents of a pedigree, or to increase the number of F_2 genotypes in a population (Lynch and Walsh, 1998). Bradshaw and Foster (1992) mention the volume and expense of work involving

making a linkage map from molecular markers and collecting phenotype data. Indirect measurements of yield should be easy, and therefore cheap, to measure if they are to be used in for selection in a breeding programme. They should be highly genetically correlated with the trait selected for and should have high heritability (Thumma *et al.*, 2001). These prerequisites have been met in the research reported here on Family 331. Stem diameter and stem height have high heritability; furthermore, the stem and leaf characteristics identified here as most influential of biomass yield are easy to measure and can be evaluated early in the growing season. Genetic causes of correlation between traits are pleiotropic effects, linkage between genes, or both of these factors (Rönnberg-Wästljung and Gullberg, 1996). When heritability is low, tree improvement involving selection based on phenotype is very difficult; this shows the importance of MAS which involves selection of seedlings for traits that are only shown at maturity in the phenotype (Bradshaw and Foster, 1992). Potentially, MAS can take place at the seedling stage; this is faster than growing the plant to maturity and then collecting phenotype data (Bradshaw, 1998). However, despite its clear potential and demonstration in agricultural crops, such as the use of MAS to introduce rust-resistance genes into barley (Toojinda *et al.*, 1998), this tool has yet to be developed in trees (Taylor, 2001). MAS is straightforward for single-gene transfer and has been proven in agricultural crops. Surprisingly, the process is still usually largely based on field observations. DNA markers from wild plants have been introduced into related agricultural crops. The fact that most agronomically important traits are quantitative increases the challenge of selection. It is relatively easy to incorporate a single gene into a recipient region of the genome. However, quantitative traits, controlled by

several genes, prove to be more difficult. MAS has always been a proposed use for the results of QTL location, as a purpose for QTL mapping (Ribaut and Hoisington, 1998). Studies of Douglas fir (*Pseduotsuga menziesii* (Mirb.) Franco) have suggested that MAS would not be economic in this species, owing to long lifecycles, lack of evidence for genetic control by QTL of major effects, and lack of use of clonal propogation (Johnson *et al.,* 2000). Although we await success stories of MAS for quantitative traits (Ribaut and Hoisington, 1998), with the current work sequencing the physical genome of poplar, the future is brighter for molecular breeding in *Populus.* The QTL found in this thesis will contribute to the scope of MAS once the technology is ready.

One further approach to improving the Salicaceae for yield is the use of transformants. Transformation is useful in that new genes can be inserted without too much effect on the existing genes. In contrast, traditional breeding requires a complete re-shuffle of the genotype and is restricted to the genes already existent in the genotype (Bradshaw and Strauss, 2001). The paucity of novel genes in the germplasm and problems in large-scale screening make traditional breeding problematic. Gene transfer techniques have decreased the time needed to improve genotypes (Rishi *et al.,* 2001). This can be achieved by either by introducing novel genes from other organisms or manipulating existing gene patterns. This is of especial importance for advances in tree breeding. Candidate genes from other crops can be used in transformed poplars and the effects studied to understand the gene's function. Transgenes are inserted into the genome at random, so the study of a family of transgenics would reveal the genetic and phenotypic variations produced, and eventually the gene may be tailored to produce

the desired expression in poplar (Bradshaw and Strauss, 2001). Poplar can be transformed either by direct gene transfer or using *Agrobacterium*-mediation. Poplar has been improved in several ways including stress tolerance, insect and herbicide resistance, phytoremediation ability, and lowering lignin content for the paper industry (Rishi *et al.,* 2001). The first transgenic poplars have been those which are insect resistant and herbicide tolerant. Transgenic poplars have been produced with reduced lignin concentrations and increased cellulose production, and have been tested in field trials, with transformants out-performing the controls (Dinus, 2000). Two genes with independent effects have been co-transferred into *Populus tremuloides* using an *Agrobacterium* vector, simultaneously reducing lignin content by up to 52 % and increasing cellulose by up to 30 % (Li *et al.,* 2003). The action of these genes was independent but additive. Concerns exist that transgenic members of Salicaceae may harm wild species. This shows the importance of incorporating sterility into transgenic trees so as not to reduce the viability of wild stands. Sterile, triploid hybrids have been found in the Family 331 pedigree, although these have been excluded from the field trial. This is not uncommon in *P. trichocarpa* x *P. deltoides* hybrids. Such sterile hybrids have the potential to be used for genetic modification, since transgenes would not be released into the environment in pollen or ovules (Bradshaw and Stettler, 1993). One further consideration is that poplars modified for accelerated flowering could help poplar selection by reducing the time to produce a pedigree (Brunner *et al.,* 2002; Rishi *et al.,* 2001) and this could take place in controlled conditions.

5.5 CONCLUSIONS

Although there is still work to be done to apply the most influential characteristics of high yield, such as those reported in this work, to tree breeding, the results presented here have identified several yield traits in plants derived from two sections of the *Populus* genus and four parent *Salix* species. MAS is currently being developed, while transgenic poplars are being researched to incorporate genes to improve productivity (Taylor, 2002; Rishi *et al.*, 2001). Given the often weak correlations between juvenile and mature traits (Thakaran *et al.*, 2001), either or both approaches to improvement of the Salicaceae for renewable energy production is potentially advantageous.

Chapter 6

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Chapter 7

Publications arising from this work

1. Robinson, K.M., Karp, A. and Taylor, G. (2004) Defining leaf traits linked to yield in short-rotation coppice Salix. *Biomass and Bioenergy* **26:** 417-431

2. Rae, A.M., Robinson, K.M. and Taylor, G. (2003) Morphological, physiological and biochemical traits influencing biomass productivity in short rotation coppice poplar. *Canadian Journal of Forest Research.* Submitted.

3. Ferris, R., Long, L., Bunn, S.M., Robinson, K.M., Bradshaw, H.D., Rae, A.M. and Taylor, G. (2002) Leaf stomatal and cell development: the identification of putative QTL in relation to elevated CO_2 in poplar. *Tree Physiology* **22:** 633-640

4. Taylor, G., Beckett, K.P., Robinson, K.M., Stiles, K. and Rae A.M. (2001) Identifying QTL for yield in biomass poplar. *Aspects of Applied Biology* **65**, *Biomass and energy crops* II pp. 173-182