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Drought resistance of willow short rotation coppice genotypes

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ABSTRACT

This thesis reports on an investigation of drought resistance of willow SRC genotypes. Experiments were conducted at Silsoe, Bedfordshire, in pots and field trials in 2002 and in lysimeters in 2003 to evaluate the range of water use efficiency (WUE) of 50 willows varieties (*Salix sp.*) and isolate morpho-physiological traits related to WUE and drought resistance. Within the genotype pool tested there was a wide range of responses. The results depict the morpho-physiology of an ideal candidate that plant breeding could produce for drier area of UK, which are summarised below. Its cuttings do not develop calluses when stored in darkness at +4°C. After planting, the candidate does not grow rapidly but has an early exponential phase of stem elongation, after a year of growth it has few stems per stool (< 5). Its long, narrow ($R_{l/w} > 8$) hairless leaves are characterised by small adaxial epidermal cells (AECS < $330\mu m^2$). The ideal candidate prioritises less biomass to its root system (root/shoot < 0.8) mainly in the top 0.2 m. When grown under optimum condition, the large leaf area has high stomatal conductance and leaf temperature. As water stress progresses, the leaf area decreases leaving little time for leaves to senesce and few yellow leaves remain on the stems. The stomatal conductance decreases slowly and the leaf temperature is almost unaffected. If water stress occurs before August the candidate is able to recover faster the initial physiological state and grow new leaves when re-watered. The results indicate that the best parents to produce such candidate are *S. viminalis* and *S. schwerinii* or their related hybrids. Water use (WU) of high yielding willow short rotation coppice hybrids is similar which indicates that the opportunity to reduce WU is limited and that productivity can be only improved by increasing WUE to produce above ground biomass and drought resistance.

The current willow breeding programme has great chance to produce hybrids with high WUE however the production of a progeny population from high yielding hybrids that contrast widely in resistance to water stress is recommended. In theory, from such a population, valuable data on morpho-physiological traits related to drought resistance and high WUE can be collected and help genomics to develop quantitative trait loci to the condition that reference hybrids are grown along to quantify the level of water stress experienced by the planting.

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SYMBOLS AND ABBREVIATIONS

µm	Micrometre	
®	Registered	
°C	Degree Celsius	
A	Photosynthetic rate	(µmol CO_2 m^{-2} s^{-1})
ADAS	Agricultural Development and Advisory Service	
AECS	Leaf adaxial epidermal cell size	(µm^2)
A_{leaf}	Single leaf area	(mm^2)
ANOVA	Analysis of Variance	
A_{plant}	Total leaf area	(m^2)
ARBRE	Arable Biomass Renewable Energy	
BEGIN	Biomass for Energy Genetic Improvement Network	
B_{root}	Root oven dried biomass	(kg)
B_{stool}	Stool oven dried biomass	(kg)
C1	Component plan 1	
C2	Component plan 2	
C3	Three carbons photosynthetic pathway	
C4	Four carbons photosynthetic pathway	
CD	Compact Disc	
CI	95% confidence interval	
cm	Centimetre	
cm^3	Cubic centimetre	
Co	Company	
CO_2	Carbon dioxide	
D	Drainage	(mm)
D	Total dry matter produced	(kg)
d^{-1}	Per day	
DC 1, 2, 3 and 4	Drying Cycles	

DEFRA	Department for Environment Food and Rural Affairs	
DTI	Department of trade and Industry	
E	Evaporation from soil	(mm)
e^-	Electron	
e.g.	For example	
ET	Evapotranspiration	(mm $time^{-1}$)
ETo	Reference evapotranspiration	(mm $time^{-1}$)
ETR	Electron transport rate	
EWBP	European Willow Breeding Partnership	
F	Female	
F	Fluorescence intensity	
F'm	Maximal fluorescence (light)	
F'o	Minimal fluorescence (light)	
F'v	Variable fluorescence (light)	
Fm	Maximal fluorescence (dark)	
FMS 2	Fluorescence Monitoring System	
Fo	Minimal fluorescence (dark)	
Fs	Fluorescence at steady state (light)	
Fv	Variable fluorescence (dark)	
FW_f	Field stem biomass fresh weight	(kg)
$FW_{f'}$	Field sample stem biomass fresh weight	(kg)
FW_{od}	Field stem biomass oven dried weight	(kg)
$FW_{od'}$	Field sample stem biomass oven dried weight	(kg)
g	Gram	
g_s	Stomatal conductance	(mol H_2O m^{-2} s^{-1})
h^{-1}	Per hour	
H_2O	Water	
Ha^{-1}	Per hectare	
I	Intercepted water by aerial part of the plant	(mm)
i.e.	That is	
IARC	Institute of Arable Crop Research	

Inc	Corporation	
K	Potassium	
Kc	Crop coefficient	
kg	Kilogram	
l	Litre	
LAI	Leaf Area Index	(m^2 m^{-2})
LARS	Long Ashton Research Station	
L_{bare}	Length of bare stem	(m)
L_{green}	Length of stem bearing green leaves	(m)
L_{leaf}	Leaf maximum length	(mm)
L_{max}	Length of tallest shoot	(m)
$L_{max-harvest}$	Stem height of the highest shoot at harvest	(m)
LSA_{fresh}	Leaf specific area fresh	(m^2 g^{-1})
LSA_{od}	Leaf specific area oven dried	(m^2 g^{-1})
LSD	Least Significant difference	
Ltd	Limited	
L_{total}	Total length of stem bearing leaves	(m)
LW_{fresh}	Fresh leaf weight	(g)
LW_{od}	Oven dried leaf weight	(g)
L_{yellow}	Length of stem bearing yellow leaves	(m)
m	Meter	
M	Male	
m^2	Square metre	
m^{-3}	Per cubic metre	
mm	Millimetre	
N	North	
N	Nitrogen	
n	Number of observation	
na	Non applicable	
No	Number	
NP	Number of remaining plants	

NPQ	Non-photochemical quenching	
ns	Non significant	
O_2	Oxygen	
odt	Oven dried ton	
P	Phosphorus	
$p \leq 0.05$	Probability at 5% error	
p^+	photon	
Pa	Pascal	(Pa)
PAR	Photosynthetically active radiation	
PCCA	Principal Component and Classification Analysis	
ppm	Part per million	
PSII	Photosystem II	
PW_f	Pot stem biomass fresh weight	(kg)
PW_{od}	Pot stem biomass oven dried weight	(kg)
Q_N	Non-photochemical quenching	
Q_P	Photochemical quenching	
QTL	Quantitative trait loci	
r	Correlation coefficient	
r^2	Square correlation coefficient	
$R_{l/w}$	Leaf length width ratio	
R_{leaf}	Leaf stem ratio	(%)
RRA	Rothamsted Research Association	
RSBP	Relative Stem Biomass Production	(%)
RWR	Relative Water Retention	
S	Water storage in the plant	(l)
S.	*Salix*	
s^{-1}	Per second	
SB	Stem Biomass	(kg)
$SB_{harvest}$	Stem biomass at harvest	(kg)
SB_{t1}	Stem biomass on t1	(kg)
Sem	Standard error of the mean	

Sp.	Species	
SRC	Short Rotation Coppice	
Stdev	Standard deviation	
SWD	Soil water deficit	(mm m^{-1})
T	Transpiration of plant	(mm)
TM	Trade Mark	
UDL	Upper drainable limit	(mm m^{-1})
UK	United Kingdom	
V	Volume	(l)
W	West	
WD	Wood density	(kg m^{-3})
W_{leaf}	Leaf maximum width	(mm)
W_{od}	Sample cutting oven dried weight	(g)
WU	Water Use	(l)
WUE	Water Use Efficiency	(g kg^{-1})
WUEi	Instantaneous water use efficiency	(µmol H_2O mol CO_2^{-1})
WUE_{stem}	Water use efficiency to produce stem biomass	(g kg^{-1})
WUE_{total}	Water use efficiency to produce total biomass	(g kg^{-1})
X3	Magnification times 3	
yr^{-1}	Per year	
ΔC13	Isotope carbon 13 duration	
µmol	Micromole	
Φ_{PSII}	Quantum efficiency of PSII	mol e^- mol $p^{+\ -1}$

CHAPTER 1. Introduction

1.1. Background

Energy comes from a number of sources some of which are renewable and some are not. Non-renewable energies are fossil fuels: coal, oil and natural gas. Renewable energies are solar power, wind, waves, hydroelectricity and energy crops such as wood, which is used as a domestic fuel in many countries.

The combustion of fuel generates gases including carbon dioxide (CO_2). The industrial development of the last two centuries along with the heavy and increasing consumption of fossil fuels has generated more carbon dioxide than the global photosynthetic organisms can fix. Studies of the last 20 years have noticed a dramatic global change in climate due to the accumulation of carbon dioxide in the atmosphere (Green, 2000).

In the late 1980's, the concept of "global warming" became a worldwide concern and a conference took place in Kyoto (1992) to discuss the issue and try to tackle the problems generated by the need for fuels. The need for renewable energies was clear, but at the time of the conference, none of them could compete with the price of non-renewable energies. The 1997 Kyoto Protocole on Climate Change required countries consuming non-renewable resources to significantly reduce their green house gas emittion or to be taxed for the quantity of greenhouse gas they emitted, as a result some of them decided to use and develop renewable resources.

Consequently, methods of energy transformation resulting in no greenhouse gas emissions had to be promoted. Powlson *et al.* (2001) reported that "*if biofuel crops are used for electricity generation they are 'CO*$_2$ *neutral'; i.e. CO*$_2$ *absorbed from the atmosphere during the growth of the crop is released back to the atmosphere when it is burned, though in practice there will always be some expenditure of carbon in cultivation, transport and handling of the material*". But also a part of the carbon is stored in the ground by the roots (Matthews and Grogan, 2001). Therefore, the net balance of carbon dioxide emission is negative and for this reason many countries took a strong interest in energy crops.

As a consequence of the 1992 Kyoto Convention on Climate Change several developed countries allocated funds to increase their use of renewable energy in order to reduce carbon dioxide emissions. In the UK, the New and Renewable Energy Policy (DTI, 2003; Miller and Cuer, 2003) set a target of 20% of the electricity from energy crops by 2020 in England and Wales. Given the current energy requirement and the energy crops available, the establishment of 100,000 to 150,000 ha of energy crops by 2010 was suggested (Stephens et al., 2001b). This area was to be divided into units of production centred around power stations and their associated energy crop supplies. These units of production will be distributed around the country to meet the energy requirements of local communities, while the energy supplies would be close to the power stations to minimise the costs of transport. The Arable Biomass Renewable Energy (ARBRE) project is one example of this (Pitcher and Everard, 2001) but this project was not economically viable and went into liquidation in summer 2002 (Miller and Cuer, 2003). At present, there is some co-firing[1] of energy crops at power stations which are already burning waste, coal or oil. The DTI predicts that energy crops could represent 75% of the fuel used to produce electricity through co-firing by 2016 (Miller and Cuer, 2003).

The main energy crops found in Europe are coppiced willow (*Salix* sp.) and poplar (*Populus* sp.) as well as perennial rhizomatous C4 grasses such as miscanthus (*Miscanthus x giganteus*) and switchgrass (*Panicum virgatum*) or C3 seed propagated grasses such as reed canary grass (*Phalaris arundinacea*) and prairie cordgrass *(Spartina pectinata)* and some cereals such as wheat (*Triticum sp.*) which are grown for straw; all of these energy crops are grown for combustion. Some other conventional crops like oil seed rape (*Brassica napus*) and sunflower (*Helianthus annuus*) can be used as energy crops when their oil is used to produce methyl ester (biodiesel). To satisfy the energy production requirement of England and Wales, energy crops would need to be grown very widely and willow short rotation coppice was seen as one of the most promising way to achieve the governmental targets (DTI, 2003).

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[1] Co-firing is the simultaneous combustion of different fuels in the same boiler.

Salix is a predominantly riparian genus with a natural growth habit that requires large quantities of water. However, *Salix* has evolved into hundreds of species colonising a wide range of habitats (Polunin and Walters, 1985; Trybush et al., 2004). Raven (1992) reports the history of willow uses: over the years, clones of wild species of willow were selected to comply with human requirements. Willows were used for basket making (*Salix purpurea, S. viminalis and S. triandra*), cricket bats (*S. alba*), tool handles and for windbreaks or shelters. It is important to note that species identification is still on going and *S. dasyclados* and *S. burjatica* were identified to be close genetically and almost indistinguishable (Trybush et al., 2004). It is only recently that willow has re-emerged as a source of fuel because of its high biomass production potential. Plant breeders in response to grower and industry demands started to generate new hybrids of willow aiming for high yield and pest resistance with growth habits that facilitate mechanical harvesting (Åhman and Larsson, 1994; Lindegaard and Barker, 1997).

England and Wales comprise a wide range of agroclimatic zones (Figure 1-1). From the characteristics of a particular agroclimatic zone it is possible to estimate the possibility of growing a crop but more interestingly what level of productivity to expect from it (Stephens et al., 2001a).

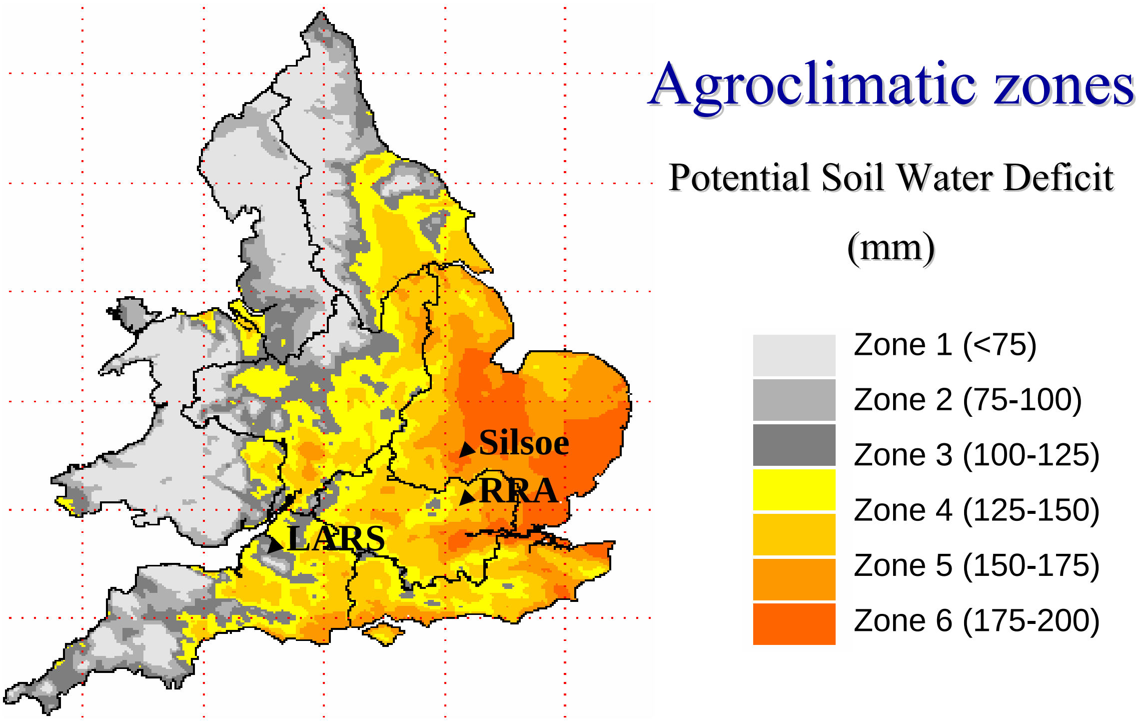

Figure 1-1. Map of England and Wales showing the agroclimatic zones defined as mean annual soil water deficit under permanent grassland (Knox and Weatherhead, 2000)

In large part of England and Wales, soil water deficits can develop from spring to autumn. The eastern part of the country is the driest with deficits above 200 mm during the summer months. Such soil water deficits would affect energy crop productivity and, in the case of willow short rotation coppice (SRC), Stephens et al., (2001b) calculated that in comparison to areas with little or no soil water deficit, a reduction of 4.5 to 10 odt ha^{-1} year^{-1} of the above ground biomass could be expected when using the willow cultivars currently available. They suggest increasing the water use efficiency (WUE) which is defined by the biomass produced per unit of water used, through selection and breeding. As a result, various energy crops improvement projects were initiated including the one reported here. This project at Cranfield University at Silsoe, Bedfordshire was named “water use efficiency of willow short rotation coppice”.

The feasibility of growing energy crops efficiently depends on the potential of the cultivation areas considered and the varieties grown. Anticipating the demand for new varieties of energy crops, breeding programmes were initiated in the early 90’s to produce biomass willow SRC cultivars.

Soon after new hybrids of willow SRC were planted, several issues were raised by growers. The willow SRC yields did not reach the expected level of yield and other issues such as the plant's susceptibility to pests and agricultural practices needed to be improved. This led to a range of studies from plant genetics through plant physiology, mode of resistance to pathogens, planting design and the mode of harvest to the size of the woodchips to be produced for efficient storage and combustion. Willow SRC breeding seems the appropriate way to improve varieties. In parallel, others were interested in the habitat created for wild life, the possibility for soil bioremediation or the general change in the environment due to the growth of willow SRC (Bullard et al., 2001).

The willow breeding programme requires many years to certify a commercial cultivar (Lindegaard, 2002). Most seeds of new varieties are obtained by cross pollinating a female parent with a male parent. These are then sown in trays and grown from spring to autumn. From that moment, a set of morphological traits is used to select an elite population of willow hybrids; for example stem length and lack of branching are used to select for high yield and rust infection on leaves and stems is used to assess pest resistance. During the first year, many hybrids are discarded because they do not fulfil the selection criteria. Few get through, and the elite population of new hybrids is propagated for field trials in the second year of the willow breeding programme. Willows are propagated via cuttings. After a year of growth, the stools are coppiced and left to re-grow for a willow coppice cycle of generally three years until the first harvest of above ground biomass takes place. During this first five years of the willow breeding programme, if a hybrid is judged to have a high yield and pest resistance potential, it can be propagated for more testing into further variety trials or propagated to produce cuttings for industrial planting. The minimum time for a cultivar to be released into the market from the original seed is 7 to 11 years.

Usually variety trials are carried out in different agroclimatic zones to enhance the selection of new cultivars which are best adapted to the location of plantation. For example: Lindegaard, *et al.,* (2001) set up an experiment in the UK testing a subset of numerous willow cultivars from the UK and Swedish breeding programmes, while Larsson (2001) was investigating the Swedish part of the subset in Sweden. The results showed that in the UK, the elite UK cultivar Ashton Stott achieved better yield than the elite Swedish cultivar Tora. However, at national level the number of sites is limited offering a small range of agroclimatic zones.

The results confirmed the importance of location of the breeding programme at a national level. In the UK, the willow breeding programme took place at Long Ashton Research Station (LARS) situated near Bristol. It was believed that the cultivars produced at LARS could be transplanted with low impact on productivity into the rest of England and Wales but this was not the case. Lindegaard *et al.* (2001) state that the hybrids bred at LARS produced on average 11.6 oven dry tonnes per hectare per year (odt ha^{-1} yr^{-1}) at LARS, 9.1 odt ha^{-1} yr^{-1} at North Molton (in Devon), 8.8 odt ha^{-1} yr^{-1} at Loughgall (Northern Ireland) and 7.9 odt ha^{-1} yr^{-1} at Markington (Yorkshire) and concluded that the ranking was probably due to abiotic factors such as soil and climate. They also stated that recent research set the economic threshold for growing willow SRC at 10-12 odt ha^{-1} yr^{-1} which depends on inputs, costs and the price paid for the woodchip. Soil water deficit is one of main abiotic factors to be taken in account because shortage of water or drought in certain part of England and Wales affects willow SRC yield (Stephens et al., 2001b) like it can for some other crop.

"*Water constitutes more than 80% of most plant cells and tissues in which there is active metabolism, rising in some cases to over 90%; a change in water content of 20-25% of the value at maximum hydration frequently resulting in a cessation of most growth processes*" (Slatyer, 1967). For a plant the supply of water to its tissue is crucial to biological processes as the entire plant biochemistry takes place in hydric environment and requires water as chemical compound (Ehlers and Goss, 2003). A plant that grows uses water to develop and maintain life and plant water requirements vary with life cycle changes. Plants adopted different processes of drought resistance in order to survive under drought stress. In agriculture, resistance is a reduced sensitivity to environmental stresses. Therefore, resistance is a quality and this quality can be improved by evolution or generation of more adapted progeny (plant breeding). Paleg and Aspinal (1981) described three main processes of drought resistance as:

- "Drought escape: the ability of a plant to complete its life cycle before a serious plant water deficit develops;
- Drought tolerance at high tissue water potential or drought avoidance: the ability

of a plant to endure period of rainfall deficit while keeping a high tissue water potential. Plants with these mechanisms do not avoid drought but avoid tissue dehydration; and

- Drought tolerance at low tissue water potential: the ability of a plant to endure rainfall deficits at low tissue water potential."

Jones (1992) reformulated these mechanisms and induced the notion of efficiency as part of the drought resistance process. He described the mechanisms as:

- "Avoidance of plant water deficits.
    - (a) *Drought escape* - short growth cycle, dormant period;
    - (b) *Water conservation* - small leaves, limited leaf area, stomatal closure, high cuticular resistance, limited radiation absorption; and
    - (c) *Effective water uptake* – extensive, deep or dense root systems.
- Tolerance of plant water deficits.
    - (a) *Turgor maintenance* – osmotic adaptation, low elastic modulus; and
    - *(b) Protective solutes, desiccation tolerant enzymes, etc.*
- Efficiency mechanisms.
    - *(a) Efficient use of available water; and.*
    - *(b) Maximal harvest index.*"

Some processes of drought resistance can be found in trees and these might take place in the genus *Salix* in different extent. In addition, drought resistance mechanisms can be long term factors such as the leaf morphology, medium term factors such as the leaf area or short term factors such as stomatal responses to drought.

These last observations orientated the next step in the willow breeding programme of which the Silsoe research project is part. New cultivars needed to be produced to maintain profitable productivity in all parts of the UK. The slow process of willow propagation prevents early dispatch of new hybrids into a wide range of agroclimates and slows down the certification that a new hybrid has reached the economic threshold for a particular agroclimatic zone. Therefore the willow breeding programme has to integrate a set of morpho-physiological traits that lead to the identification of the elite individuals exhibiting drought resistance characteristics.

1.2. Plant breeding

Plant breeding has been practiced for centuries and some of the theory that governs breeding is given below. Genetics theory led to a standardization of breeding methods that involve crossing of two fertile varieties or pure species which are referred as “parents”. In plant breeding parents are normally very different from each other (Poehlman, 1966) and most of the characteristics of the parents are quantifiable i.e. pest resistance. Breeding parents mixes genes randomly; as a result, quantitative characteristics are normally distributed over the offspring population. Figure 1-2 illustrates the theoretical result of plant breeding. In most cases, if a quantitative characteristic recorded in a parent population appears to be normally distributed, the offspring population issued from the crossing of these parents can result in different means and standard deviations. Plant breeders extract from the progeny population an elite population which expresses the desired characteristics. As a result, the selected elite population has a very different mean, standard deviation and distribution compared to the parent population and entire progeny population (Figure 1-2). In this figure hybrid vigour is suggested (Poehlman, 1966).

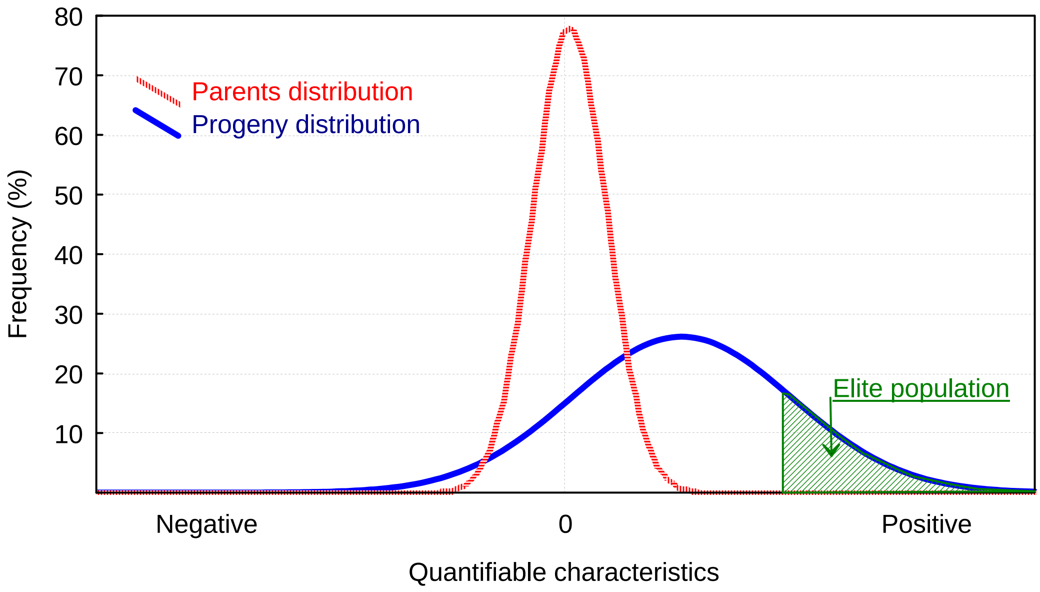

Figure 1-2. Theoretical distribution of progeny from breeding pure species and identification of an elite population with advantageous traits

The different steps in the selection of an elite population may cover their responses to stress. If stress occurs while a quantitative characteristic is recorded, the values in comparison to un-stressed condition may be altered (mean and standard deviation). This effect may or may not be proportional. A stress effect is proportional if the mean and standard deviation maintain the same ratio. Alternatively a stress is non-proportional if the ratio between the mean and standard deviation changes (see Figure 1-3).

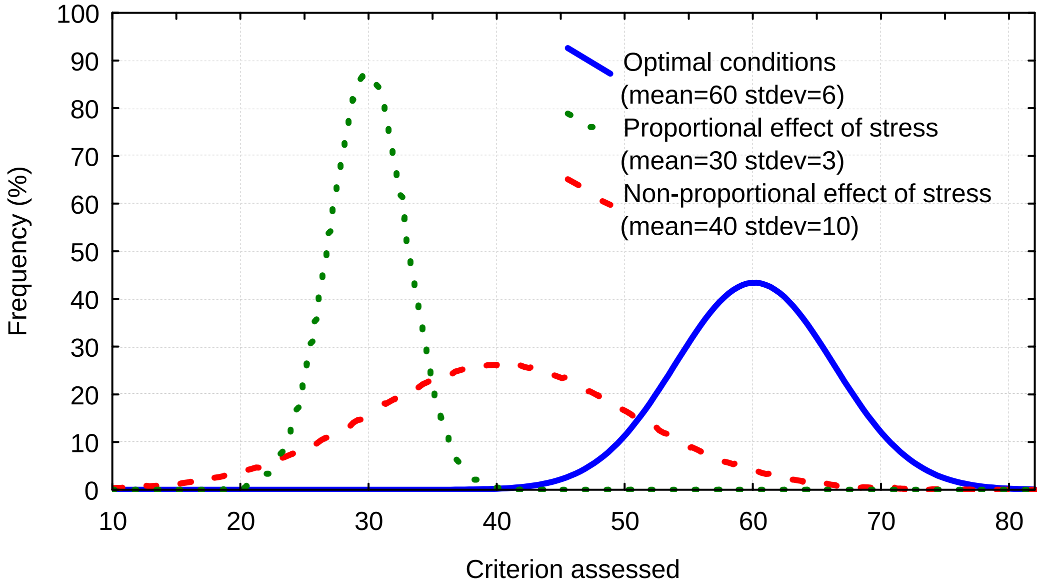

Figure 1-3. Theoretical proportional and non-proportional effects of stress on the mean and standard deviation (stdev) of a population

The hypotheses of an experiment where stressed and non-stressed conditions are compared in relation to a particular stress on a population are: if a proportional effect of stress on the population occurs, there is no expectation that one individual is more resistant or sensitive than another individual. In other words, there is no change in the ranking of varieties. If a non-proportional effect occurs, then one or more individuals are more or less resistant or sensitive, in which case changes in the ranking between varieties may occur. However, if no effect is observed, the criterion can still be used as an indicator, as the value and the derived ranking obtained for each individual can characterise the varieties studied.

An alternative method to determine the effect of stress on the performance of a population is to plot the performance under stress against the performance without stress. A proportional response within the population would result in a straight-line relationship compared to a less well-defined distribution for a non-proportional response (Figure 1-4). By exposing a large population of willow varieties to water stress the type of response could be identified and the likelihood of identifying elite varieties that exhibit higher water use efficiency evaluated.

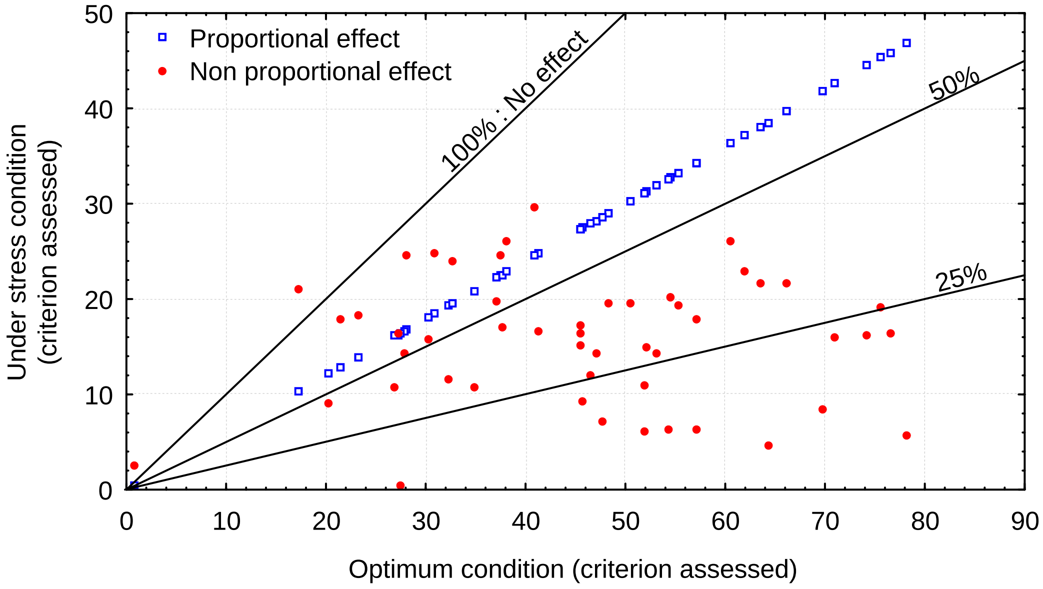

Figure 1-4. Hypothetical results of proportional and non-proportional relative responses to stress in a population. The solid lines indicate equal relative performances

1.3. Silsoe project

The project reported here is part of a DTI funded project with the European Willow Breeding Partnership (EWBP) in conjunction with ADAS and the Rothamsted Research Association (RRA) (formerly the Institute of Arable Crop Research (IARC)) willow breeding program at LARS. In 2003-2004, the breeding programme and the willow collection were relocated to RRA at Harpenden in Hertfordshire. EWBP collapsed at the end of 2002, and the DEFRA-funded Biomass for Energy Genetic Improvement Network (BEGIN) took over in spring 2004. The project reported here is now a part of BEGIN which aims to:

1. Determine the range of WUE and drought resistance of willow varieties, with the aim of identifying the potential for improving productivity by plant breeding;
2. Determine the patterns of water use, its role in determining crop yield, and the importance of irrigation in maximising yield and;

3. Evaluate simple techniques for the early screening of drought resistance in the breeding programme by detailed observations of crop physiology in order to develop an understanding of characteristics that lead to improved WUE and drought resistance.

The main project was split, allocated to different teams and started in December 2001. Willows cuttings were planted on two sites in spring 2002. 12 varieties were planted in a medium scale irrigation trial at ADAS at Gleadthorpe, Nottinghamshire whilst 50 varieties were selected for more detailed study at Cranfield University at Silsoe, Bedfordshire.

The UK willow SRC breeding programme took place until 2003, at Long Ashton Research Station (LARS) in collaboration with the European Willow Breeding Partnership (EWP). LARS is in Somerset just south of Bristol. LARS is in a relatively wet area in comparison to the rest of England. At the start of this project no clear physiological or morphological criteria had yet been identified which could be linked with particularly high WUE or water stress resistance abilities. Weih and Nordh, (2002) carried out experiments on the physiology of 14 willow varieties looking at nitrogen and water use (WU) efficiency. They concluded that some differences of efficiencies were significant between the varieties but without giving any indication of the physiology or morphology involved in the efficiency of nitrogen and WU. As a result, nothing was linked with the morpho-physiology of *Salix sp.* and its response to water stress. Consequently no physiological trait could be added to the set of criteria used in the breeding programme and the willow breeders at LARS were unable to guarantee the production of highly water use efficient or water stress tolerant willow varieties.

The detailed studies for the project outlined above were at Cranfield University at Silsoe, Bedfordshire: (52.005^{O} N; 0.428^{O} W; altitude 100 m). Silsoe was chosen as it is one of the driest parts of the UK (Weatherhead and Knox, 2000) and the reference evapotranspiration (ETo)[2] exceeds rainfall from April to August (Figure 1-5).

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[2] The FAO Penman-Monteith estimate of ETo is defined as "*the rate of evapotranspiration from an hypothetical crop with assumed height of 12 cm, a fixed canopy resistance of 70s m^{-1} and an albedo*

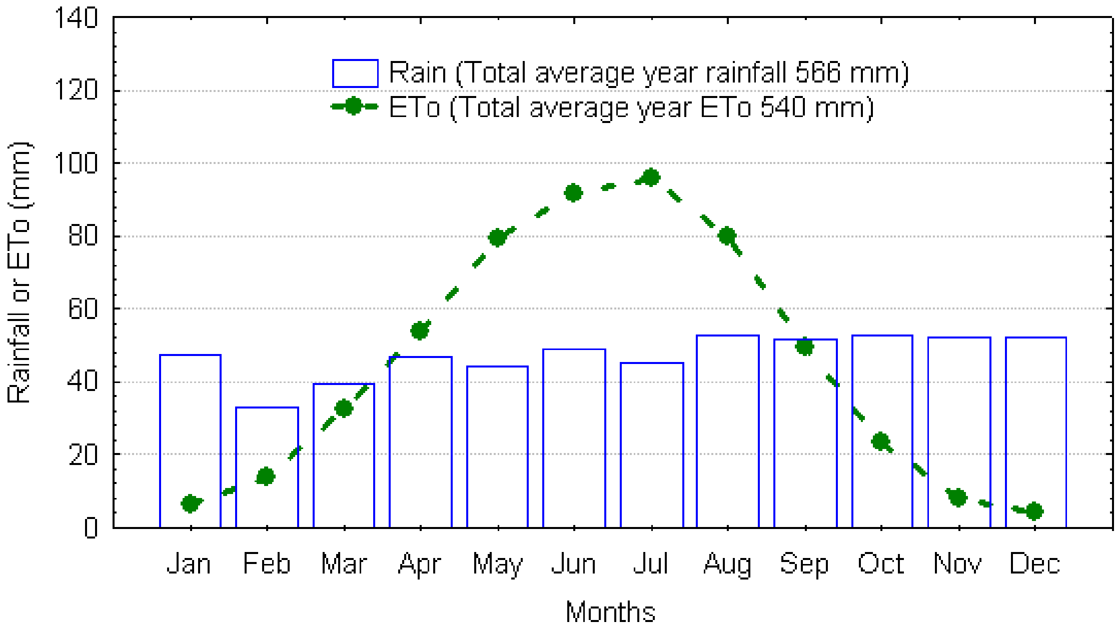

Figure 1-5. Mean monthly rainfall and evapotranspiration (ETo) at Silsoe, Bedfordshire from 1962 to 2000. Sources: Silsoe Research Institute and Cranfield University at Silsoe

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of 0.23. This is closely resembling the evapotranspiration from an extensive surface of green grass of uniform height, actively growing and completely shading the ground and not short of water"(Allen et al., 1994).

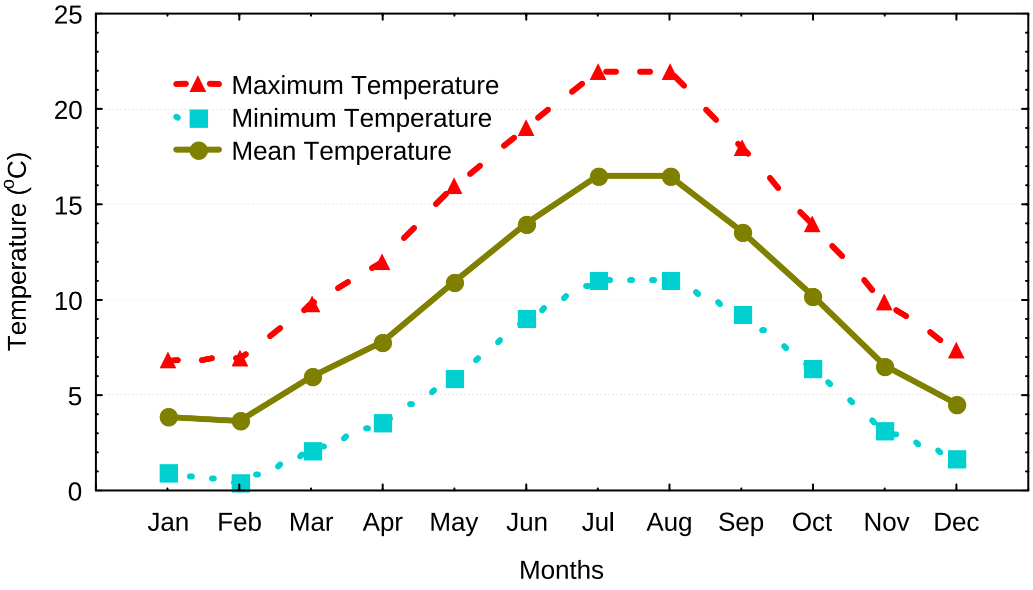

Figure 1-6. Monthly average of daily mean maximum and minimum temperature at Silsoe, Bedfordshire, from 1970 to 1995. Source: Cranfield University at Silsoe

The climate of Silsoe is characterised by an even distribution of rainfall all year round, a mild winter and a cool summer (Figure 1-5 and Figure 1-6). Soil water deficits typically develop from April to August resulting in water stress[3] for many crops.

1.4. Objectives

Although all three objectives of the overall project are linked the third objective was especially allocated to the Silsoe team. The work required to reach that objective was estimated to be quite substantial and the intermediate objectives and their corresponding research had to be issued and planned.

The objectives of the research are to:

1. screen a large population of willow hybrids to determine whether drought affects their yield and development in the same proportion;
2. evaluate the morphological traits related to drought resistance;
3. estimate the water use and water use efficiency of high yielding willow hybrids and the impact of drought on them;

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[3] Water stress in the whole thesis corresponds to plant stress induced by water shortage. Water shortage can be associated to a greater demand than the supply or availability over a time period.

4. evaluate morpho-physiological changes related to drought resistance;
5. determine the impact of different drought intensities on high yielding willow hybrids' leaf gas exchange and photosynthetic activity; and
6. examine the feasibility of using a set of morpho-physiological traits to enable the early characterisation of high WUE and drought resistance in the willow SRC breeding programme.

1.5. Thesis structure

The thesis comprises seven chapters. The first is this introduction, which encompasses the background of the research and defines the gap in the knowledge. The objectives of the project are also specified above.

The second and third chapters cover the broad scale studies conducted during the summer 2002 on 50 varieties. Chapter 2 reports on the development of willows and the biomass production when subjected to two growth conditions and rank the varieties according to their water stress resistance. Chapter 3 investigates the possibilities of combining morpho-physiological traits into a set and using it to anticipate the water stress resistance of a pool of varieties.

The next three chapters are concerned with more detailed studies of a subset of five varieties grown under two water regimes. These varieties were identified in chapter 2 and chapter 3 to rank the water stress resistance. Chapter 4 describes the experiments set up for the summer 2003 and the calculation of seasonal WUE. Chapter 5 analyses the morphology of willows, with a major focus on the leaf population role in WU, and also reports on the biomass partitioning between the varieties. Chapter 6 pinpoints the WU and WUE on a daily basis and describes the work on physiology at the leaf level of the five varieties under two water regimes.

Finally, in Chapter 7, discussion covers the information presented in the previous chapters and the feasibility of incorporating into the willow breeding programme a water stress resistance morpho-physiological set made of physiological and morphological assessments.

CHAPTER 2. The effects of water stress on the growth and biomass production of 50 varieties of *Salix*

2.1. Introduction

The tree genus *Salix* is bred to produce hybrids for growth as short rotation coppice (SRC), (Lindegaard and Barker, 1997). Until 2002 the European Willow Breeding Partnership (EWBP) coordinated links between the different European partners, especially relating to the strategy and techniques of willow breeding programmes. European willow breeders select hybrids with high yield potential, strong pest resistance, low moisture content in the wood and upright growth. Other criteria such as frost resistance, salt tolerance or tolerance of hot and dry weather necessitate trials in regions where these stresses encountered (Larsson, 2001). The assessment of these criteria is dependent on the time required to propagate new varieties for variety trials and for coppice rotation, as a result several years are needed before the release of a new cultivar onto the market (Larsson, 1998).

In England and Wales, willow SRC will be grown widely in the near future in order to satisfy demand for energy production from energy crops (DTI, 2000). Shortage of water, especially in the East of England, could result in a reduction of potential biomass production from SRC of up to 10 odt ha^{-1} yr^{-1} (Stephens et al., 2001a). The current willow breeding programmes do not categorise the hybrids according to their resistance to water stress as the mechanisms involved are not identified and have not been studied (Lindegaard et al., 2001). Therefore in order to guarantee that the cultivation of a hybrid reaches the economic threshold for biomass production criteria are required to identify water stress resistant varieties (Stephens et al., 2001a; Armstrong, 2002).

The knowledge of the physiology involved in the process of water stress resistance is limited. Recent work has compared willow varieties and concluded that varieties have different responses to water stress but did not try to identify the physiological traits specifically linked with water stress resistance or sensitivity (Weih, 2001; Weih and

Nordh, 2002). Other studies have identified some water stress resistance mechanisms specific to willow varieties but these were on only four clones of *Salix* (Wikberg and Ögren, 2004).

To summarise, previous studies have focused on a limited number of varieties and as a result the full range of drought resistance and the impact of drought on growth for the genus Salix is still unknown. Consequently, there are no criteria for categorising willow varieties according to their resistance to water stress and this is required to improve the UK willow breeding programme. The aims of this study were to:

1. screen a large population of willow hybrids to determine whether drought affects their yield and development in the same proportion and define the range of resistance observed amongst the *Salix* genus and;
2. examine the feasibility of using the data recorded on stem elongation to enable the early characterisation of drought resistance in the willow SRC breeding programme.

2.2. Material and methods

To fulfil the aim of the project, 50 varieties of willows were planted in two research trials; the first was a non water-stressed field trial and the second a water-stressed pot trial. The two trials were set up in parallel at Cranfield University at Silsoe, Bedfordshire: 52.005^{O} N. ; 0.428^{O} W.

2.2.1. Varieties and cuttings selection

The EWBP produced thousands of new seeds every year at Long Ashton Research Station (LARS) Somerset, each of which was potentially a unique variety; only small proportion were kept for the plant-breeding programme. In the first year, the selection criteria were: stem height; rust resistance; and lack of branching. This led to the selection of an elite population of potentially suitable varieties to be used in further cycles of the breeding programme (Lindegaard, 2002).

The guidelines for selecting the varieties were: to create a population of 40 promising willow hybrids and to add 10 pure species: the parents of the hybrids which are commonly used in the UK plant breeding programme (Table 2-1).

Table 2-1. List of 50 varieties used in the Silsoe trials. The commercial names of the varieties, the parents' pedigrees, regions of origin are indicated when possible. * indicates pure species. Bedfordshire; 2002-2003

LARS Variety code	**Name Sex**	**Parent pedigree *Salix***		**Parent Origin**	
		Female	**Male**	**Female**	**Male**
001/01*	M	*udensis*	*udensis*	Asia	Asia
003/01*	M	*aegyptica*	*aegyptica*	Middle East	Middle East
033/20*	?	*dasyclados*	*dasyclados*	Denmark	Denmark
034/10	Ashton Stott F	*viminalis*	*burjatica*	UK/Denmark	UK/Denmark
037/01*	M	*caprea*	*caprea*	UK	UK
055/01*	M	*discolor*	*discolor*	North America	North America
062/01*	F	*glaucophylloides*	*glaucophylloides*		
087/01*	M	*miyabeana*	*miyabeana*	Asia	Asia
102/01*	F	*rehderiana*	*rehderiana*	Asia	Asia
109/03*	F	*schwerinii*	*schwerinii*	Russia	Russia
115/34*	Bowles Hybrid F	*viminalis*	*viminalis*	UK	UK
127/02	Tora F	*viminalis*	*schwerinii*	Russia/Sweden	Russia/Sweden
LA940044	?	*viminalis* 115/34 'Bowles Hybrid'	*candida* 036/01	UK	UK
LA960048	F	*viminalis* 115/34 'Bowles Hybrid'	*bebbiana* 110/02	UK	North America
LA960326	Beagle F	*viminalis* 115/70 'Astrid'	*viminalis* unknown pollen source	Sweden	Sweden
LA970048	F	*schwerinii* 109/03	*viminalis* 115/27 'Readers Red'	Russia	UK
LA970136	F	*schwerinii* 109/03	*viminalis* 115/10 'Vigorous'	Russia	UK
LA970164	Endeavour F	*schwerinii* 109/03	*viminalis* 115/65 'Jorr'	Russia	Sweden
LA970184	M	*dasyclados* 033/17 'Aud'	*dasyclados* 075/01 'Kotens'	Poland	Denmark
LA970217	F	*schwerinii x viminalis* 127/02 'Tora'	*caprea* 037/01	Russia/Sweden	UK
LA970249	?	*viminalis* 115/71	*cordata* 052/01 'Purpurescens'	Sweden	North America
LA970416	F	*viminalis* 115/68 'Jorunn'	*burjatica* 109/07 'Lapin'	Sweden	Russia
LA970485	F	*dasyclados* 041/03	*discolor* 055/01	UK	North America
LA970504	M	*dasyclados* 041/03	*dasyclados* 033/18 'Loden'	UK	Poland
LA970523	?	*dasyclados* 041/03	*capreola* 024/02	UK	UK
LA970534	F	*viminalis* 115/11 'Romanin'	*caprea*	UK	UK
LA970573	F	mollissima	miyabeana	UK	Asia
LA970617	F	*viminalis* LA940147 (115/34 Bowles Hybrid x 112/18 Brunette Noire)	*aegyptiaca* 003/01	UK	Middle East
LA970654	F	*glaucophyloides* 062/01	*aegyptiaca* 003/01	North America	Middle East
LA970766	F	*glaucophyloides* 062/01	*caprea* 037/01	North America	UK
LA980024	Discovery F	*schwerinii* 109/03	*schwerinii x viminalis* 127/01 'Bjorn'	Russia	Russia/Sweden
LA980030	F	*schwerinii* 109/03	*dasyclados* 77056	Russia	Denmark
LA980038	M	*schwerinii x viminalis* 127/02 'Tora'	burjatica 109/07 'Lapin'	Russia/Sweden	Russia
LA980125	Nimrod F	*schwerinii x viminalis* 127/02 'Tora'	*udensis* 001/01 shrubby willow ex China	Russia/Sweden	Asia
LA980132	Terra Nova F	*viminalis* LA940140 (115/34 'Bowles Hybrid' x 112/18 'Brunette Noire')	*udensis* 001/01 shrubby willow ex 'China'	UK	Asia

LARS Variety code	**Name Sex**	**Parent pedigree *Salix***		**Parent Origin**	
		Female	**Male**	**Female**	**Male**
LA980172	M	*schwerinii x viminalis* 127/02 'Tora'	*dasyclados* 81090	Russia/Sweden	Sweden
LA980190	F	*schwerinii x viminalis* 127/02 'Tora'	*miyabeana* 087/01	Russia/Sweden	Asia
LA980200	F	*schwerinii* 109/03	*discolor* 055/01	Russia	North America
LA980221	F	*schwerinii* 109/03	*viminalis* 127/01 'Yellow Osier'	Russia	UK
LA980230	F	*viminalis* LA940140 (115/34 'Bowles Hybrid' x 112/18 'Brunette Noire')	*schwerinii x viminalis* 127/01 'Bjorn'	UK	Russia/Sweden
LA980266	F	125/05svs (*schwerinii x viminalis* x smith) V7535	*aegyptiaca* 003/01	Finland	Middle East
LA980279	M	*schwerinii x viminalis* 127/02 'Tora'	*discolor* 055/01	Russia/Sweden	North America
LA980289	F	125/01svs (*schwerinii x viminalis* x smith) V7535	*viminalis* 115/65 'Jorr'	Finland	Sweden
LA980309	M	*glaucophyloides* 062/01	*udensis* 001/01 shrubby willow ex 'China'	North America	Asia
LA980348	F	*viminalis* 115/34 'Bowles Hybrid'	*udensis* 001/01 shrubby willow ex 'China'	UK	Asia
LA980372	M	*viminalis* 115/34 'Bowles Hybrid'	*sachalensis* 106/1 'sekka'	UK	Asia
LA980402	F	125/05svs (*schwerinii x viminalis* x smith) V7535	*sachalensis* 106/1 'sekka'	Finland	Asia
LA980414	Resolution F	*viminalis x schwerinii x viminalis x (viminalis x schwerinii)* LA960231 SW900812	*viminalis* LA960231 'Quest'	Russia/Sweden/ Finland	Russia/Sweden
LA980442	Endurance F	*rehderiana* 102/01	*dasyclados* 77056	Asia	Denmark
LA980496	F	*dasyclados* 041/03	*viminalis* 115/10 'Vigorous'	UK	UK

M: male; F: female

Note that 115/34 Bowles hybrid was included in the pure species list. Bowles hybrid was the only pure S. viminalis to be included in the 50 varieties selected. Bowles hybrid is an old hybrid (Chittendon, 1956) and was used as the S. viminalis female parent for many hybrids.

The named hybrids have a good chance of being released commercially. Tora (127/02) and Ashton Stott (034/10) are well known commercial hybrids which are already planted on a large scale; they were used as reference hybrids as they have been included in some other trials (Lindegaard et al., 2001). Although a large range of genetic material was selected, it did not represent as wide genetic range of *Salix* varieties as listed by Rechinger, (1992). Therefore, when a criterion is assessed the combined results from the pure species and the hybrid populations might not be normally distributed but might form a bimodal distribution.

Willow SRC is usually propagated using cuttings 200 mm long, 10 to 20 mm in diameter and free of branches. 25 cuttings of 50 varieties were collected at LARS on 18/02/02. The cuttings were stored at +4 °C until the planting date: 11/04/02 for the pot trial; and 25/04/02 for the field trial. On 11/04/02 the cuttings were labelled and weighed individually. The pot trial comprised 4 randomised replications of 50 varieties and the field trial comprised 3 randomised replications of 50 varieties. One cutting was to be planted in each pot and four in each field plot; these were sorted according to their mass. Cuttings of a similar weight were chosen to start the pot trial with a homogenous population. The remaining cuttings were not homogeneous and 12 cuttings were required for the field trial. For these reasons three populations of four cuttings of similar mass (small, intermediate and big) were selected for each variety. It was necessary to create homogenous groups of cuttings (same mass), to avoid potential competition between the four cuttings at emergence.

Within each replicate the plot had to be as homogenous as possible. Therefore, for all varieties, replicate I was planted with small cuttings, replicate II with intermediate size cuttings and replicate III with large cuttings.

2.2.2. The field trial

Set-up

The field trial was a rectangular area of 20 x 30 m. It was sprayed with Roundup® (5 l ha^{-1}) to clear the weeds, then ploughed and cultivated with a power harrow. The soil type is a sandy loam, Cottenham series (King, 1969). The soil is dark brown, very friable with a few ironstone fragments and subangular flints; it has a weak, fine subangular blocky to fine crumb structure.

The varieties were planted at a square spacing of 0.5 x 0.5 m with a density of 40,000 cuttings ha^{-1}; each plot occupied an area of 1 m^2. The field trial was a randomized block design of 150 plots, divided into three replicates of 50 plots of four cuttings; these were selected as described above. The replicates were divided into five blocks of 10 plots surrounded by paths to facilitate access to the plots.

Rows of the willow variety Q683 were planted as guard rows to separate each plot and to surround each block of 10 plots. The cuttings of Q683 were harvested on the day prior to planting. The cuttings of Q683 were separated into three populations of small, intermediate and large cuttings and planted in replicates I, II and III respectively. The guard rows were planted on 24/04/02 and the varieties on the following day. The details of the layout of the field trial are in Appendix 1.

The density was double that used in commercial plantations (Lindegaard et al., 2001). Densities between 23700 and 63500 cuttings ha^{-1} have little impact on yield but higher density reduces the number of stems per stool and encourages a more erect habit (Bullard et al., 2002). Higher densities are an advantage for weed control in the first year and after cut back of a plantation leading to fewer herbicide applications (Proe et al., 2002). A rabbit fence was placed around the trial to prevent animal damage (Plate 1).

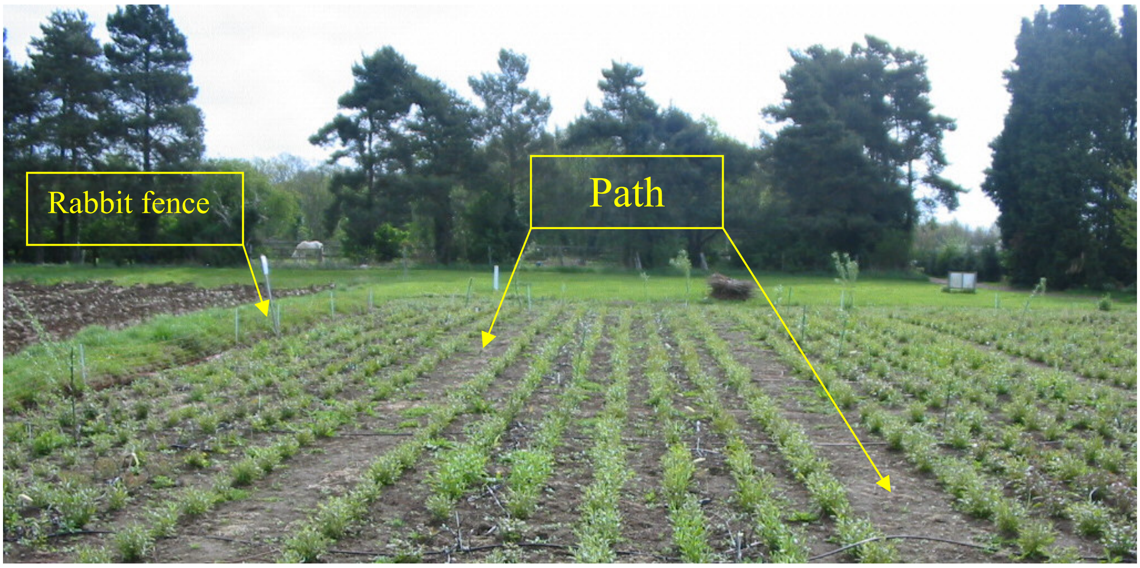

Plate 1. The field willow SRC variety trial at Silsoe; Bedfordshire; regenerating after coppicing. Spring 2003

Irrigation

The first irrigation was carried out on 25/04/02, when the planting was complete; 25 mm of water was applied using a set of sprayers mounted on an automatic spool. Later, two lines of three sprinklers were set up at a square spacing of 6 x 8 m on 25/06/02 and the field was irrigated with 15 mm of water. The line setting was changed after the second irrigation to 6 x 12 m to increase the irrigation uniformity. The field was irrigated twice a week. 30 mm per week were applied on average until

29/07/02. The irrigation was not maintained after this date as the jet of the sprinklers damaged some varieties.

The field was fully equipped with drippers in spring 2003, two per plot, for the season 2003 in order to irrigate the willow SRC varieties but not the guard rows. The discharge was 2 l h^{-1} for each dripper. Irrigation was maintained weekly for eight hours with a total discharge of 16 l per dripper or 32 l per plot. In total 32 mm of water were applied to each plot weekly.

Weed control

The field was hand hoed on 10-11/06/02 and repeated on 17/06/02 and on 17/07/02. Thereafter the willow canopy was sufficiently developed to inhibit weed propagation.
In 2003 the field was sprayed with Roundup® 5 l ha^{-1} soon after cut back. It was not necessary to hand weed afterwards.

Fertilizer

No fertilizers were applied in 2002 as the field had previously been under grass fallow.
In 2003, a fertilizer composed of 17% N, 17% K_2O, 17% P_2O_5 was used to fertilize the field. A final quantity of 60 kg ha^{-1} of each component was applied to the entire field, in line with applications on the LARS trials (Lindegaard et al., 2001).

Replacement of cuttings

On 18/06/02, cuttings that had not produced any shoots were replaced with fresh ones (Table 2-2).

Table 2-2. Codes and number of cuttings replaced on 18/06/02 in the field trial; Silsoe, Bedfordshire; 2002

Variety code	Number of cuttings replaced
109/03	1
LA980030	5

Note that none of the replacement grew like the other clones in the field and some of the cuttings of 109/03 died even after emergence of shoots during summer 2002.
At harvest in February 2003 the number surviving was recorded. The cuttings were replaced as indicated by Lindegaard, (2003) using 1 m long rods. The replaced cuttings are summarised in Table 2-3.

Table 2-3. Codes and number of cuttings replaced at harvest in January 2003 in the field trial; Silsoe, Bedfordshire

Variety code	Number of cuttings replaced
102/01	5
109/03	3
LA960048	1
LA980030	9

2.2.3. The pot trial

Set-up

The pot trial consisted of 200 pots of 20 l capacity (Plate 2). The soil used to fill the pots was a local sandy loam, Cottenham series (King, 1969) taken from a mixture of the top 0.2 m of a local field profile. It was mixed in a concrete mixer to homogenize the soil moisture and the aggregate size. 20 kg ± 10 g of soil were poured into each pot and compacted to a dry soil bulk density of 1160 kg m^{-3} ± 100 kg m^{-3}. The range of soil compaction used has been shown to have a minimal impact on the development of the willow (Remy, 1997).

The cuttings were planted as randomized block design with four replicates of 50 varieties on the evening of 11/04/02; the pots were fully watered as soon as the planting was finished and on the following morning to make sure that the soil reached the upper drainable limit[4]. See Appendix 2 for detail of the layout of the pot trial.

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[4] The upper drainable limit is the maximum quantity of water retained in a volume of soil after drainage.

Plate 2. The pot willow SRC variety trial at Silsoe, Bedfordshire, one day after planting: spring 2002

Irrigation

The pots were individually drip irrigated by gravity using a slave tank per replicate. These were irrigated daily for an establishment period of two and a half months. On 24/06/02, a second dripper per pot was added to increase the irrigation uniformity and discharge. The plants were irrigated for a two hour period each day. The volume of water was sufficient to maintain a good wetting of the pots as drainage was observed after each irrigation.

Each pot was placed on an upside down saucer to isolate the pot from the soil in order to prevent rooting into the ground and to allow drainage of excess water.

In order to generate water stress four drying cycles, where irrigation was stopped, were imposed on the crop: from 27/06/02 until 9/07/02; from 23/07/02 until 29/07/02; from 24/08/02 until 28/08/02 and from 30/08/02 until 6/09/02.

Fertilizer

Each pot was fertilised with 5 g of NPK 15-15-15 on 8/07/02.

Weed control

The pots were weeded by hand every two weeks from 16/05/02, until the population of weeds was low enough to be ignored.

Replacement of cuttings

On 27/05/02, the cutting variety LA980221 planted in the pot 36 of Rep. II, had not produced any shoots since the planting date and was replaced.

2.2.4. Growth monitoring

Stem length and stem elongation rate

Stem length is highly correlated to stem biomass (SB) in woody short rotation coppice (Tuskan and Rensema, 1992; Verwijst and Telenius, 1999; Robinson et al., 2004). Monitoring stem growth might therefore indicate how and when water stress affects the stem length of the willows and consequently the biomass accumulation.

The length of the tallest shoot (L_{max}) from the top of the planted cutting to the end of the tip of the stem was measured every week using a 1 m ruler. When shoots were longer than 1m, they were tagged every meter at each assessment. In the field, the stool in the bottom right hand corner of the plot from the path was assessed.

From the data of the stem height monitoring, it was possible to calculate the elongation rates in mm day^{-1}.

Stem Biomass (SB)

SB includes the main stem and any attached branches at harvest. SB from the trials set up in 2002 was harvested a second time in February 2004. In the pot trial, all the stems of each plant were harvested and weighed "fresh" (PW_f) and "oven dry" (PW_{od}).

In the field, firstly all the stems of each plot were harvested and weighed fresh (FW_f). Secondly, a sample composed of two complete stems representative of the population of stems (about 0.1 to 0.3 kg depending on the variety) was weighed fresh ($FW_{f'}$) and oven dried at 105°C for 48h and weighed ($FW_{od'}$). The total oven dry weight of each plot (FW_{od}) was calculated as follows.

Equation 1

$$FW_{od} = FW_f (FW_{od'} / FW_{f'})$$

SB was expressed in kg plant^{-1}. In the pot trial, SB equals PW_{od} while in the field the number of remaining plants (NP) per plot must be taken into account and was calculated as follows.

Equation 2

$$SB = \frac{FW_{od}}{NP}$$

Weather

The air temperature (maximum and minimum), rain, wind, solar radiation and relative humidity were recorded hourly using an automatic weather station (Skye instrument, Wales) sited 60 m from the pot trial and 1 km from the field. ETo was calculated on a daily basis using the modified Penman-Monteith equation (Allen et al., 1994).

2.2.5. Statistical analysis

All analyses were carried out using STATISTICA software (V6 AX, Statsoft Inc, 2001). Statistical analyses were required in all cases to determine whether any observed differences were statistically significant. Data were analyzed in a systematic way. Both trials were a randomized block design, with four replicates for the pot trial and three for the field. The total population made of 50 varieties divided into 10 pure species and 40 hybrids. To summarise, the degrees of freedom available for the analysis were, 3 and 2 for the replications in the pot and field trial respectively, 9 and 39 for the number of individuals for the pure species and hybrids population respectively.

The statistical analysis took place in eight steps:

1. an analysis of variance (ANOVA) was run to determine the significance of the differences of stem biomass and stem length observed between the pure species and hybrid populations for the different experiments (null-hypothesis: the two populations biomass and stem length were not different);
2. an ANOVA was run to determine the significance of any observed differences of stem biomass and stem length between individuals within each population (null-hypothesis: there were no differences between the individuals) and to determine the significance of any observed differences between sites (null-hypothesis: there was no differences between the sites);

3. a factorial ANOVA was run to test the significance of differences between the field and the pot trial (null-hypothesis: there were no differences and interactions between the sites and the individuals). A factorial ANOVA on the raw data was not appropriate as the errors of the trials were of different magnitudes because the variances are dependent on the means. Thus the raw data were transformed, using a square root transformation prior to the Factorial ANOVA;
4. the relative stem biomass production (RSBP) between the two trials run in 2002 was calculated for each variety. RSBP is the ratio between the biomass produced under water stress conditions and the biomass produced in irrigated conditions. The varieties were then classified according to their RSBP;
5. the relationship between stem length and stem biomass was tested by linear regression in both 2002 trials;
6. the stem elongation rates in 15 different periods were calculated and an ANOVA was run to test the significance of differences between the individuals in both 2002 trials (null-hypothesis: the individuals are not different). For the rate where significant differences were observed, factorial ANOVAs were run to test the significance of differences between individuals and sites (null-hypothesis: there are no differences and interaction between the sites and the individuals);
7. the relations between RSBP and the 15 stem elongation rate measured in the field were tested by linear regression; and
8. a principal component and classification analysis (PCCA) of the 15 stem elongation rates observed in the field was run to reduce the number of variables and determine whether or not it is possible to predict RSBP by looking at the elongation rate.

PCCA computes a wide range of associated statistics; in STATISTICA the PCCA module produces a wide range of results. The features used here are: principal component coordinates of variables and cases, contributions of variables, principal component scores, eigenvalues and descriptive statistics (StatSoft Inc., 2001).

Here, to determine which elongation rates in the field were most related to the RSBP the PCCA was used as follows. The coordinates for each variable included in the

PCCA were computed for each principal component. The variables used in the analysis were then projected into the first two components plan for visualisation of interrelated variables. The first two components explain the maximum proportion of variations between the cases. Interrelated variables tend to cluster so it was thus possible to reduce the number of variables by picking one variable per cluster to represent the others in the further steps of the analysis. The process was repeated until few variables are left and belonging distinctively to different clusters. Finally a PCCA was run with the last variables and the case coordinates were calculated and projected into the first two factors plan. It was therefore possible to check if the individuals cluster according to their RSBP value.

2.3. Results

2.3.1. Weather

The monthly rainfall and ETo at Silsoe in 2002 and 2003 were plotted in Figure 2-1 and the monthly mean, maximum, and minimum temperature at Silsoe in 2002 and 2003 were plotted in Figure 2-2.

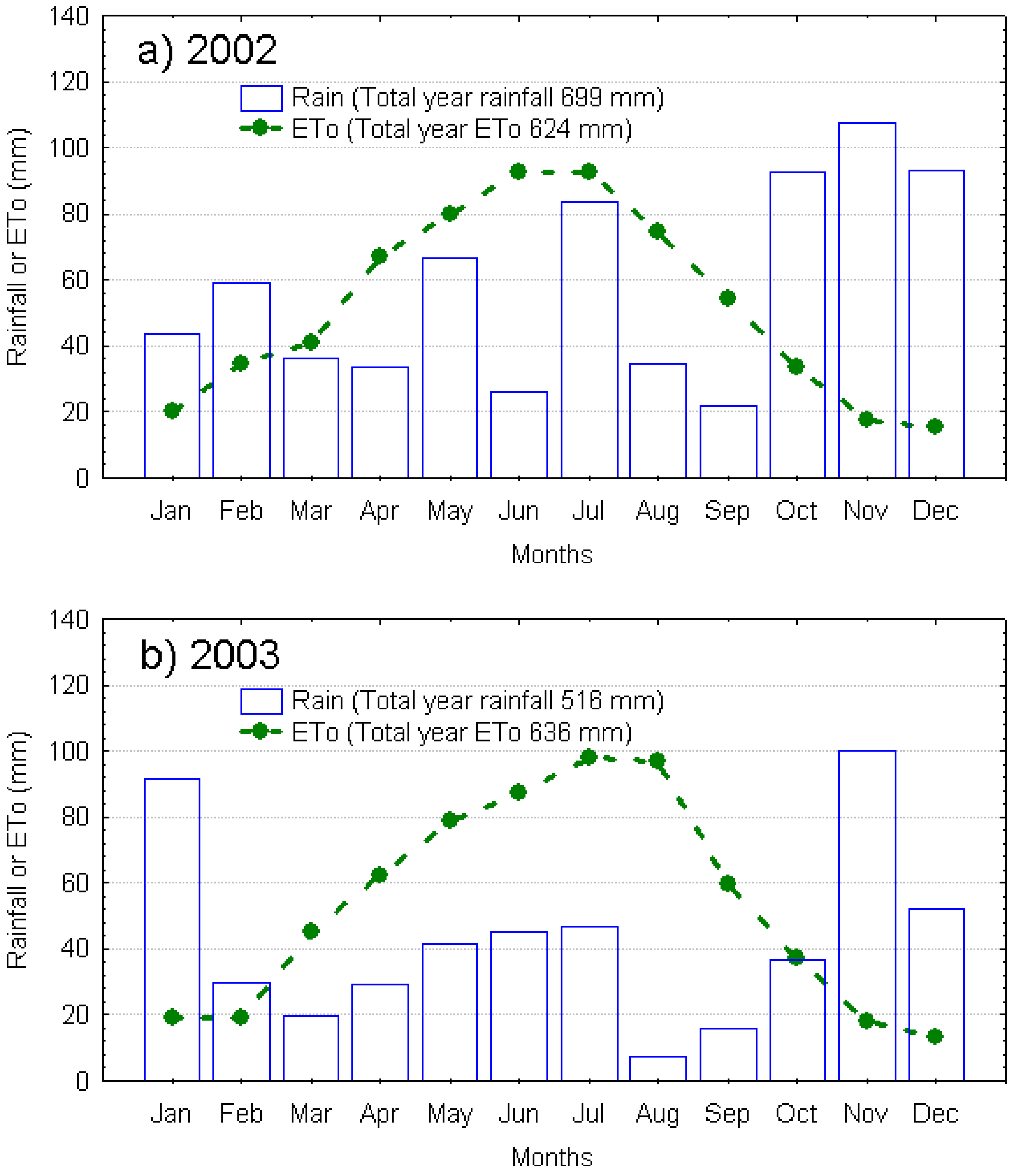


Figure 2-1. Monthly rainfall and reference evapotranspiration (ETo); Silsoe, Bedfordshire; in a) 2002 and b) 2003

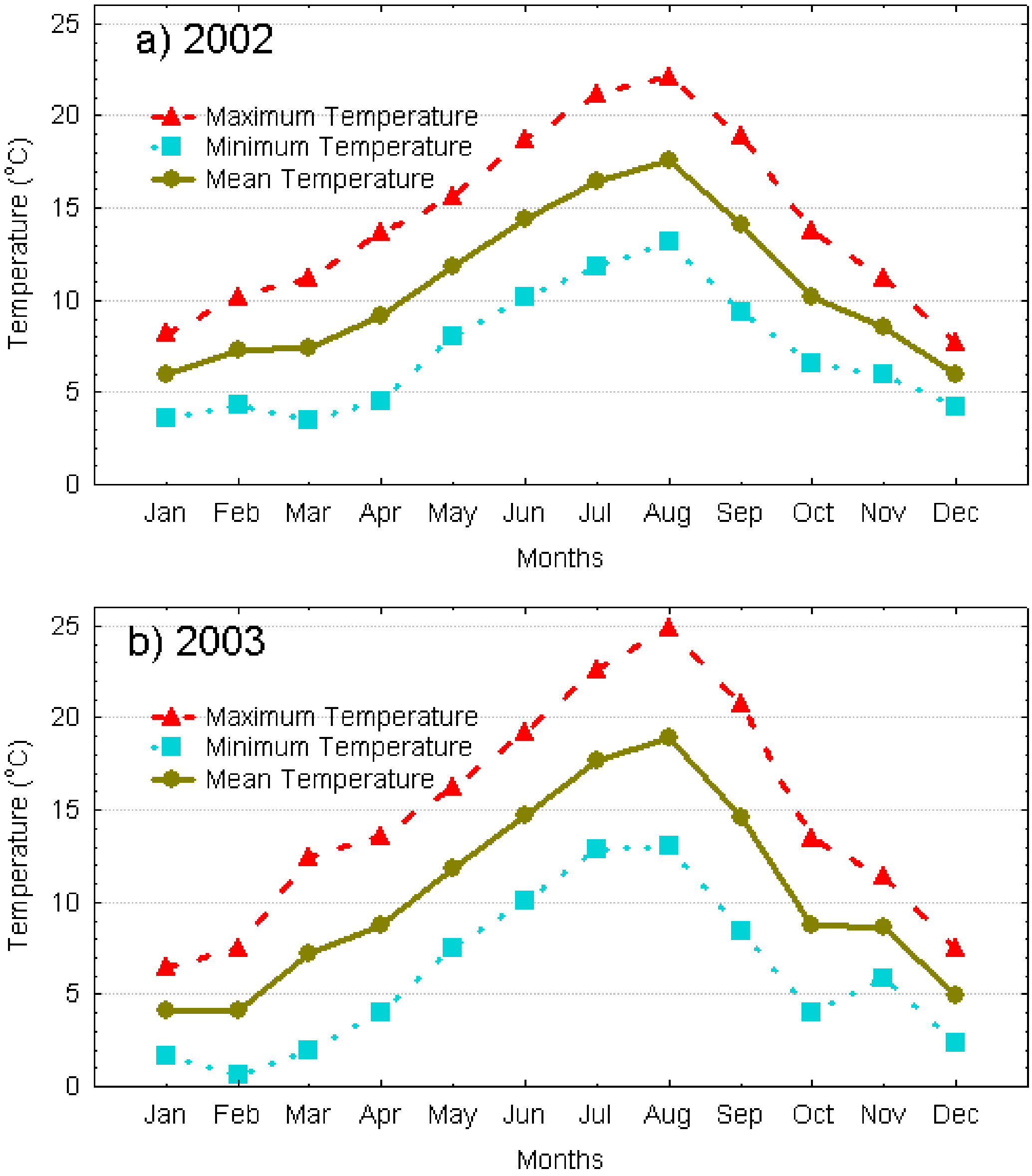


Figure 2-2. Monthly average of daily mean, maximum and minimum temperatures; Silsoe, Bedfordshire; in a) 2002 and b) 2003.

ETo exceeded rainfall from March to September inclusive in both 2002 and 2003 (Figure 2-1). Rainfall was not evenly distributed in 2002 and was 133 mm above the long term average due to heavy autumn rainfall events and storm events in the summer (Figure 1-5). In July 2002 there were two important rainfall events: 19 mm the 8th and 31 mm the 30th. Summer 2003 was characterised by rainfall close to the long term mean in June and July, but was exceptionally dry in August and September; although the autumn months were wetter than average, a dry spring and a dry summer resulted in total annual rainfall 50 mm below long term average. In both years, the summers were ideal to study water stress effects on plants in open-air trials as water stress developed in the absence of irrigation especially since ETo sums in 2002 and 2003 were higher than the long term mean. Note that 2003, was exceptionally dry with a deficit (rainfall – ETo) of 297 mm between 01/04/03 and 31/10/03.

2.3.2. Stem length and biomass

The missing plants recorded at harvest in 2003 and 2004 for the field were summarised in Table 2-3 and Table 2-4 respectively.

Table 2-4. Number of cuttings missing at harvest in the field trial; Silsoe, Bedfordshire; 2003

Variety Code	Number of cuttings replaced
001/01	2
102/01	3
109/03	1
LA97136	1
LA980030	5
LA980172	1
LA980442	1

LA980030 did not survive over the two years of trial in the field. Only 42% of the LA980030 cuttings survived in 2002 and again in 2003. The remaining plants performed poorly and all the stem wood had to be reused in 2002 to replace the missing plants. For this reason, LA980030 was excluded from the statistical analysis carried out on the field data sets for 2002. As a result the degree of freedom for the hybrid population was 38.

The first step in the statistical analysis was to determine if there were significant differences between the two populations in the trials. The results of the ANOVA run on the biomass measured in the field in 2002 are presented in Table 2-5.

Table 2-5. Analysis of variance of the stem biomass production of two populations of 39 hybrids and 10 pure species, grown in the field trial; Silsoe, Bedfordshire; 2002. The levels of significance are represented as: ns: non significant; *: significant at $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

	SS	Degrees Of freedom	MS	F	p
Population	0.110	1	0.109986	60.2139	***
Error	0.265	145	0.001827		

Table 2-5 shows that the pure species population and the hybrid population were significantly different. This suggests that the total population should be divided for further analyses.

Other ANOVAs were run on the other trials and also for the stem length (See attached CD). These are not presented as similar conclusions were drawn from these results.

Table 2-6 summarises the basic statistics of the data collected in the field and pot trial. The results show stem length and biomass at the end of the growing season. The number of surviving plants was taken into account in the calculation of stem biomass.

Table 2-6. Means and standard errors of the means (sem), of two growth variables at harvest (biomass and maximum stem length) for a population of 49 varieties of Salix (39 hybrids 10 pure species) grown in field in 2002 and 2003 and pot trial in 2002; Silsoe, Bedfordshire

	Field trial 2002 (a) pure species (b) hybrids	Field trial 2003 (a) pure species (b) hybrids	Pot trial 2002 (a) pure species (b) hybrids
Biomass (kg plant^{-1}) Mean (sem)	(a) 0.069 (0.0096) (b) 0.137 (0.0464)	(a) 0.138 (0.0182) (b) 0.306 (0.0105)	(a) 0.038 (0.0015) (b) 0.048 (0.0010)
Stem length (m) Mean (sem)	(a) 1.91 (0.007) (b) 2.39 (0.003)	(a) 2.38 (0.010) (b) 2.87 (0.004)	(a) 1.42 (0.005) (b) 1.64 (0.003)

Table 2-6 shows that the means of the biomass and stem length of each population decreased when grown in the pot trial compared to the field. In addition, stem height increased in the field in 2003 in comparison to 2002 by about 20% whilst biomass production was more than double that in 2002. This indicates that the conditions of growth in the pot trial were stressful in comparison to those imposed in the field and that established willow stools grew better than first year cuttings.

A factorial ANOVA was carried out to test the significance between the varieties and the field in 2002 and 2003 (Table 2-7). A separate ANOVA was run for the data collected from the pot trial as the magnitudes of the errors were very different to the field ones (Table 2-6). Table 2-8 presents the ANOVA of SB of the hybrid population grown in the pot trial in 2002. The other analyses are on the appended CD.

Table 2-7. Factorial analysis of variance of stem biomass at harvest for a population of 39 hybrids of Salix grown in field trial in 2002 and in 2003 after coppicing; Silsoe, Bedfordshire. The levels of significance are represented as: ns: non significant; *: significant at $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

	SS	Degree of freedom	MS	F	p
Hybrids	0.760	38	0.01998	4.705	***
Years	1.797	1	1.79672	423.079	***
Hybrids x Years	0.223	38	0.00586	1.380	ns
Error	0.663	156	0.00425		

Significant differences in stem biomass production were measured between the years and within the hybrid population (Table 2-7). However, no significant differences were observed for the interaction hybrids x years. This meant that the hybrids above ground biomass at harvest were in a similar order in both years. The same conclusions could be drawn from the analysis of the stem length (see appended CD).

For SB harvested from the pure species population significant differences were observed, between the two years and between the pure species and the years (details found on appended CD). This last point meant that the pure species did not rank in a similar order between the two years in terms of the biomass they produced. However, for the stem length of the pure species, significant differences were observed within the population and between the sites, but not for the interaction of both variables. This non significance of the interaction suggests that the rank of the pure species was not significantly different between the two years.

Table 2-8. Analysis of variance of the stem biomass production of 39 hybrids, grown in the field trial in 2002; Silsoe, Bedfordshire. The levels of significance are represented as: ns: non significant; *: significant at $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

	SS	Degree of freedom	MS	F	p
Hybrids	0.145	38	0.003821	4.097	***
Error	0.073	78	0.000933		

Significant differences were found within the hybrid population grown in the pot trial in 2002 (Table 2-8); the same results were obtained for the pure species population (see appended CD).

The average biomass, stem length and the ranking of each variety are presented in Appendix 3.

At this stage of the analysis, only 39 hybrids were considered for further analysis. Because the pure species seemed to have suffered from competition with the guard rows and the hybrids: their stem biomass production, length and elongation rates were not representative of their potential in field conditions. LA980030 was discarded from the analysis as all its field biomass was used to replace missing cuttings.

In the hybrid population no significant changes in rank were observed, except for the significance of the difference between the field trial in 2002 and 2003 (Table 2-7). This indicates that during the establishment of a willow coppice field, the biomass and stem length in the first year trial are good indicators of willow behaviour in the subsequent year, so the field data for 2003 are not considered further.

To compare, the effects of water stress on willow growth and development, the field and pot trial data gathered in 2002 were adressed. A factorial ANOVA of the square root of the data was used (null hypothesis: they are no differences in the rank within the hybrid population for stem biomass) (Table 2-9).

Table 2-9. Factorial analysis of variance of 39 hybrids grown in two sites in 2002 (irrigated field and the water stressed pot trial). Silsoe, Bedfordshire. The levels of significance are represented as: ns: non significant; *: significant at $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

	SS	**Degree of freedom**	**MS**	**F**	**p**
Hybrids	0.239	38	0.00629	6.90	***
Sites	1.441	1	1.44194	1582.10	***
Hybrids x sites	0.136	38	0.00359	3.94	***
Error	0.178	195	0.00091		

Water stress induced a significant decrease in stem biomass and length in almost all cases (Table 2-9, Appendix 3 and appended CD). The significant interaction between the hybrids and the sites also indicated that water stress had a significant non-proportional effect on the growth of the hybrid population. Consequently the rank of the hybrids within the population was altered and some individual varieties shifted positively and some negatively from the means of the field and the pot trial. By plotting biomass production in the pot trial against production in the field the relative stem biomass production could be seen, and varieties with a similar stress resistance or sensitivity to water stress identified (Figure 2-3).

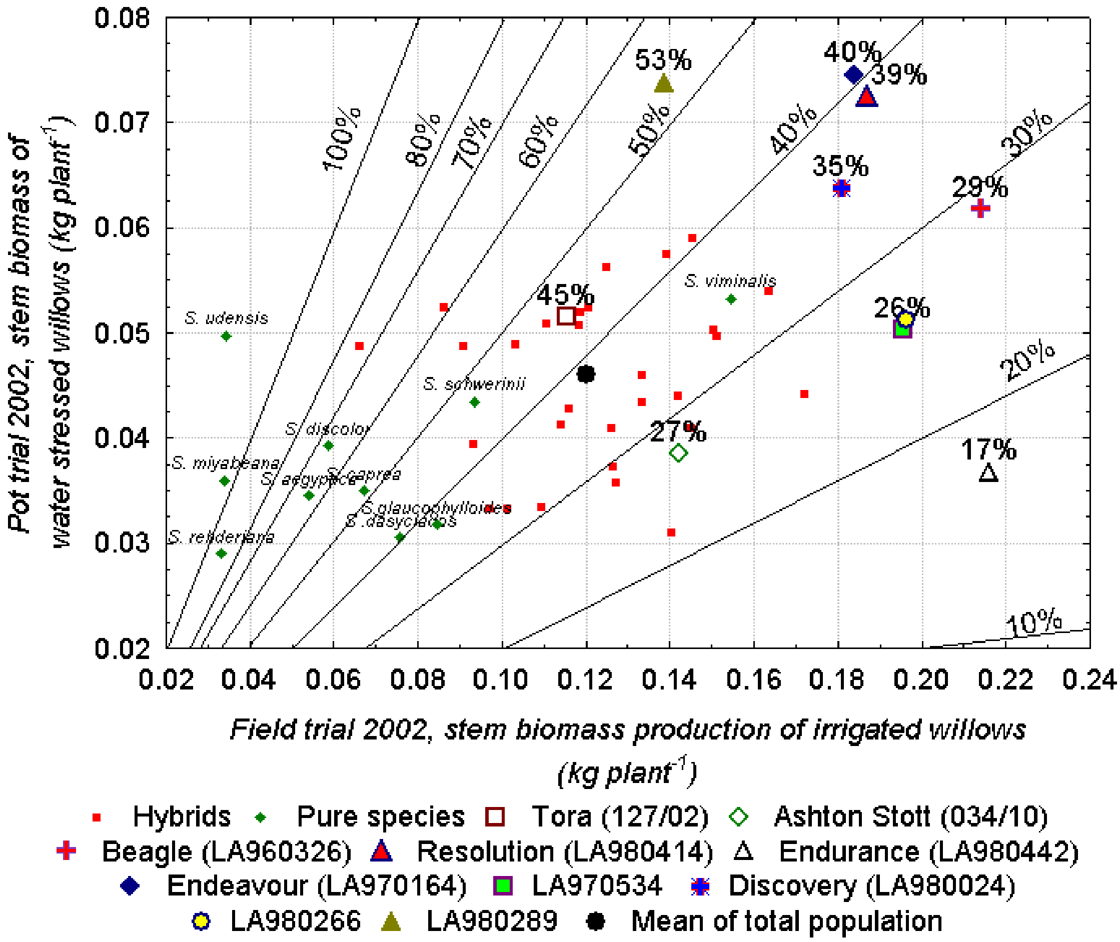


Figure 2-3. Relative stem biomass productions of 50 willow varieties grown under water stress in the pot (n=4) and with irrigation in the field (n=3) trial; Silsoe, Bedfordshire; 2002. The lines indicate boundaries between different relative stem biomass productions. Ten high yielding hybrids are highlighted

Figure 2-3 shows the range of biomass production of all the varieties in both trials and their resulting relative stem biomass production (RSBP). Ten of the hybrids are highlighted because they are high yielding in the field and represent the range of RSBP.

The stem lengths were assessed, as stem length is one component of biomass. Figure 2-4 shows, changes in ranking of stem length at harvest between the two trials.

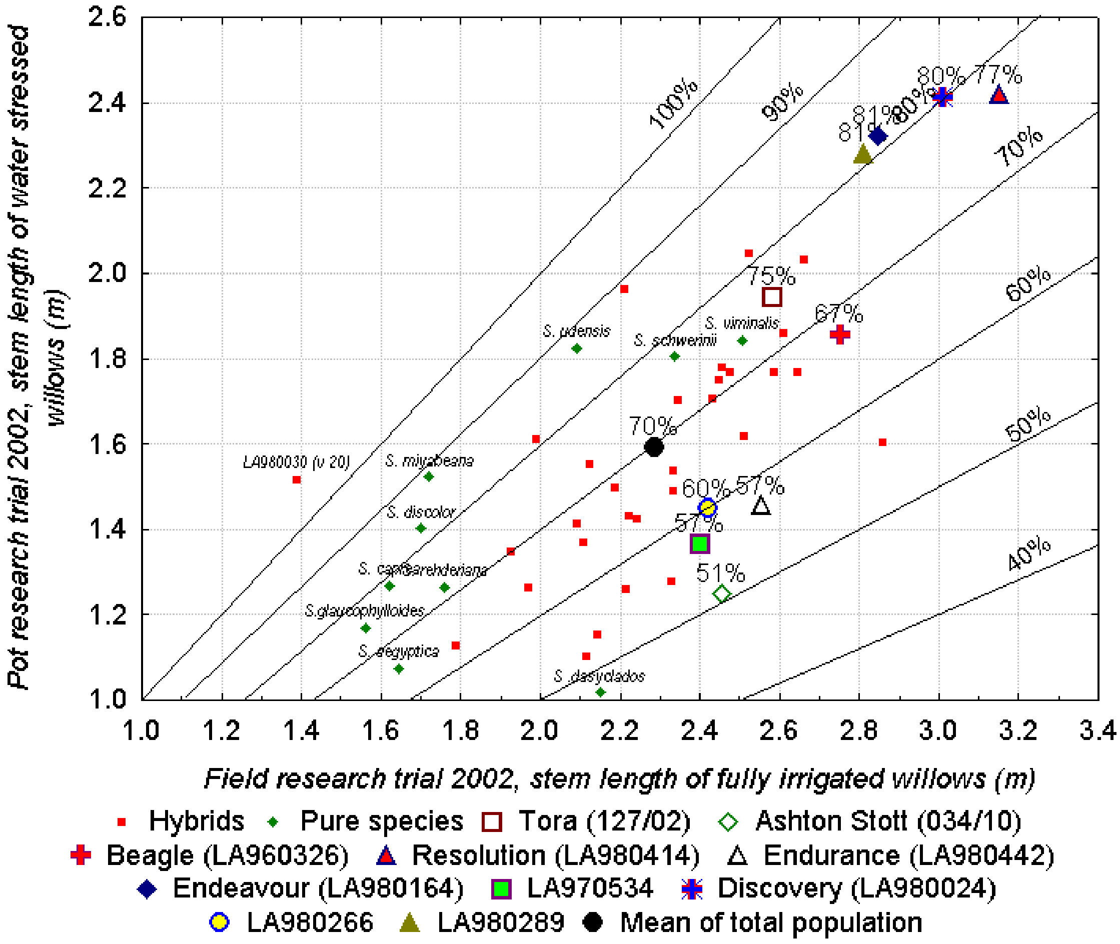


Figure 2-4. Relative stem lengths of 50 willow varieties grown under water stress in the pot (n=4) and with irrigation in the field (n=3) trial; Silsoe, Bedfordshire; 2002. The lines indicate boundaries between different relative stem lengths. Ten high yielding hybrids are highlighted

The stems were shorter in the pot trial than in the field with the exception of LA980030 which did not establish well in the field. Later, this hybrid was not able to compete for light with the guard rows and the other varieties but did not suffer in the pot trial to such a great extent. Thus, the field conditions were more stressful for this particular hybrid.

A similar effect might have occurred for most pure species that tend to be small and/or shrubby (*S. miyabeana, S. discolor,* and *S. aegyptica*). The relative stem length of the hybrid population (without LA980030) ranged from 89% for LA980221 to 51% for LA970617 and Ashton Stott. The ranks were rearranged between the two experiments for the pure species which reflects difference in plant morphology.

It appeared that hybrids of *S. burjatica*, *S. dasyclados* and *S. rehderiana* were more sensitive than those of *S. viminalis* and *S. schwerinii*, although when these two groups were hybridised the range of response was wider. Here, the hybrids must be considered individually regardless of their genetic composition, as no pure species could be statistically confirmed to be linked with drought resistance with such a small number of genotypes and the assortment of parents of the same species. The record of biomass and stem height at harvest does not explain the difference in ranking between the two trials, but suggest that varieties express a range of responses to water stress.

The hybrids used as indicator varieties in Figure 2-5 to Figure 2-7 were Tora and Ashton Stott which act as reference to commercial hybrids. Resolution, Endurance and LA980289 represent a new generation of hybrids. These five hybrids were selected because they are high yielding and were evenly distributed across the range of observed RSBP.

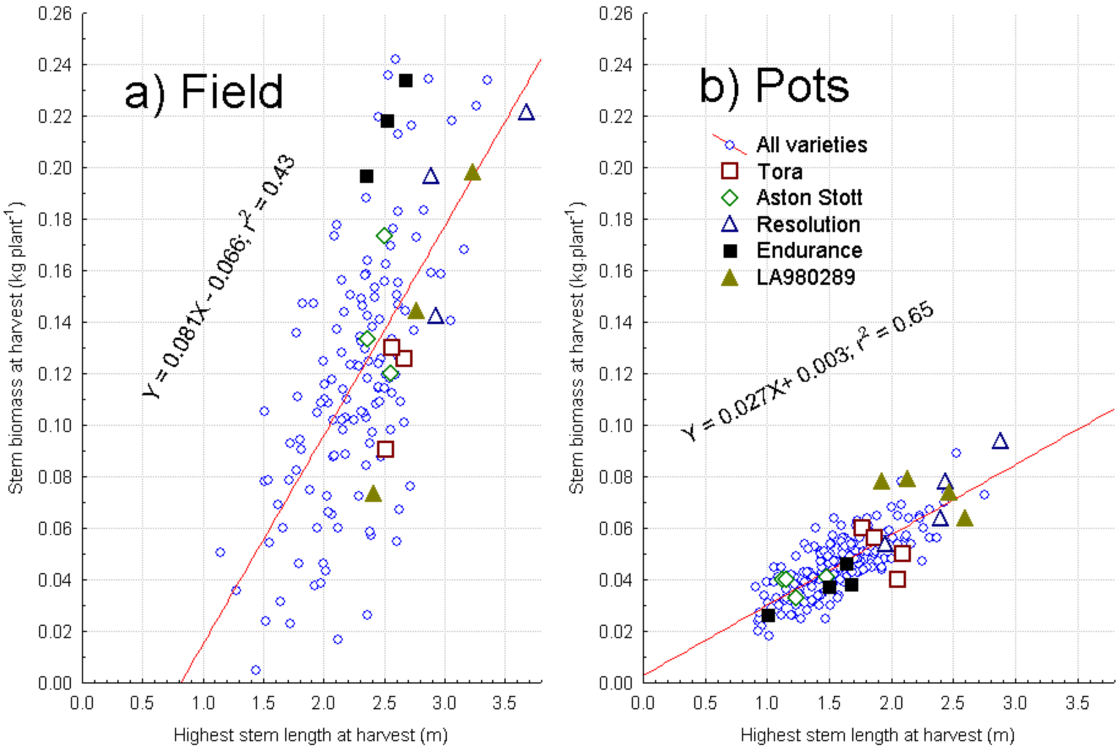


Figure 2-5. Linear regressions of stem length of highest shoot at harvest versus biomass for all plots grown in a) the field (n=150) and b) the pot (n=200) trial; Silsoe, Bedfordshire; 2002. n=3 in the field with n=4 in the pot trial for individual varieties

In the field, the regression line accounted for 43% of the observed variation. Under water stress the relationship was stronger, with 65% of variation accounted for (Figure 2-5).

Different relationships (biomass versus stem height) were observed for the highlighted hybrids in each trial. In the field, the biomass/stem length relationship of the five hybrids appeared to be linear and described a similar trend, as predicted by the model. In pot trial, it appeared to be more scattered and did not follow the model of the total population with the exception of Resolution.

There was considerable variability within varieties in the field, as illustrated by the reference varieties Tora, Ashton Stott as well as the other indicator varieties.

2.3.3. Stem elongation and elongation rate

At harvest, differences in stem length were observed between the field and pot trial. These differences became apparent at different times in the growing season (Figure 2-6).

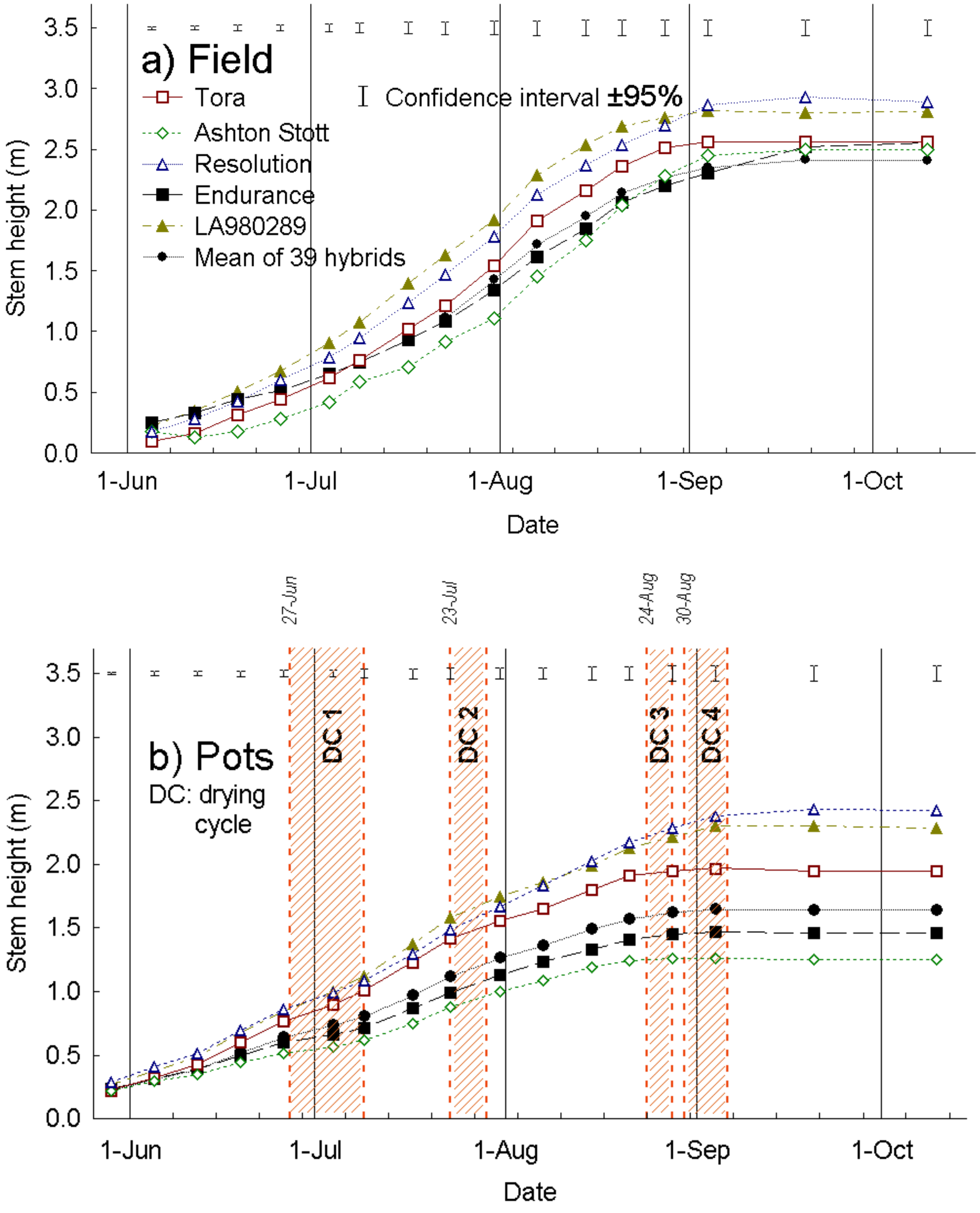


Figure 2-6. Means of the stem heights of five hybrids grown in a) the field (16 occasions) and b) the pot (17 occasions) trial; Silsoe, Bedfordshire; 2002. The mean of the total population of 39 hybrids is included for comparison. n=117 and n=3 in the field for total and hybrid population respectively. n=156 and n=4 in the pot trial for total and hybrid population respectively. The error bars indicate the 95% confidence interval. The dashed areas illustrate the drying cycles imposed on the plants

A sigmoid pattern of growth was observed in all varieties in both trials. In the field trial, all five indicator varieties reached a height above the mean of the 39 hybrids. This was expected as these were high yielding hybrids and stem height is a major component of biomass (Figure 2-5). In the pot trial, Ashton Stott and Endurance fell below the mean of the 39 hybrids while Tora, Resolution and LA980289 remained above. This meant that on average Ashton Stott and Endurance suffered more than Tora, Resolution and LA980289 from the conditions of growth in the pot trial and especially during the four drying cycles. To determine the effects of the four drying cycles on the willow development, the stem elongation rates were calculated. The rates of the indicator varieties are presented in Figure 2-7.

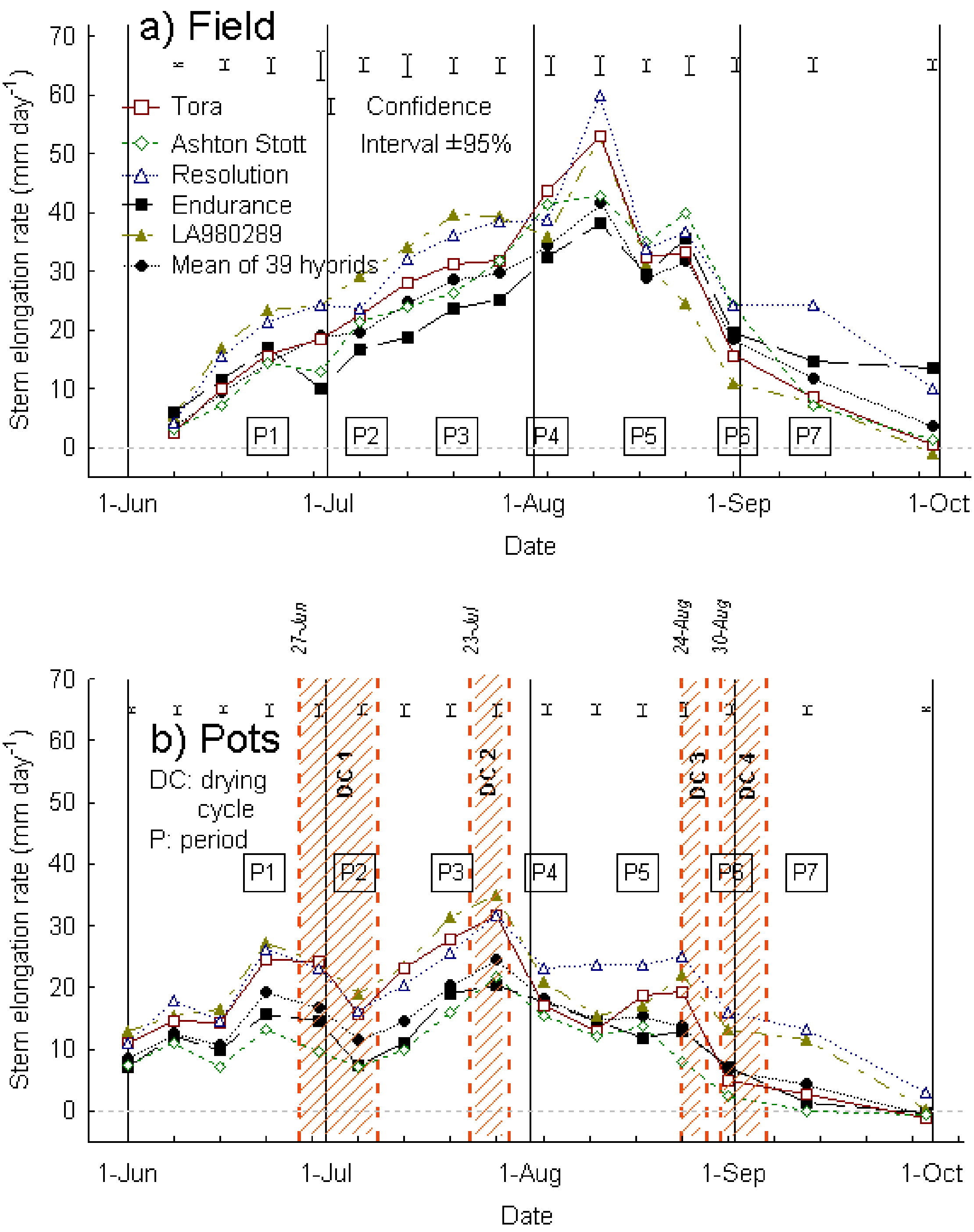


Figure 2-7. Means of the stem elongation rates of five hybrids in a) the field (15 intervals) and b) the pot (16 intervals) trial; Silsoe, Bedfordshire; 2002. The mean of the total population of 39 hybrids is included for comparison. n=117 and n=3 in the field trial for total and hybrid population respectively. n=156 and n=4 in the pot trial for total and hybrid population respectively. The error bars represent the confidence interval at 95%. The dashed areas illustrate the drying cycles imposed on the pot trial. Seven periods (P1-P7) are highlighted for further comments

On a few occasions at the end of the season growth rates were negative due to the death of the apex resulting in the reduction of stem length.

Comparison of stem elongation rates between the two trials illustrated firstly, the general effect of restricted root zone and four drying cycles on willow SRC; and secondly, highlights key dates where differences in varieties and trials were visible, but this needed to be confirmed by statistical analysis.

In the field (Figure 2-7a), the stem elongation rates increased until early August and then decreased abruptly, whereas in the pot trial the drying cycles (especially the first and second) induced a substantial decrease in the stem elongation rates. On average elongation rates reduced from the beginning of each drying cycle. After the first cycle (DC 1) a strong recovery was observed in the pot trial between 9/07 and 23/07.

The second drying cycle (DC 2) had a similar effect on stem elongation, with a decrease for all varieties. On average, between 29/07 and 24/08 the rate decreased. Resolution managed to maintain its rate during this period. Tora and LA980289 first decreased followed by a recovery nine days after the end of DC 2. Ashton Stott started to recover nine days after the end of DC 2, but the rate started to decrease again before the third drying cycle (DC 3). Endurance started to recover 16 days after the end of DC 2. The differences were however too small to be significant in the period between DC 2 and DC 3.

During DC 3 and DC 4 Resolution and LA980289 maintained above average stem elongation rates while Tora, Ashton Stott and Endurance were more affected: stem elongation ceased during DC 4.

Table 2-10 shows the key periods when differences between varieties were observed.

Table 2-10. Key periods (identified from Figure 2-7), where a general change in behaviour was observed in the pot trial in comparison to the field trial for most 39 hybrids; Silsoe, Bedfordshire; 2002

PERIOD (Interval)	Effect observed in pot trial	Stage
1 (19/06-26/06/02)	Peak rate before stress	Before DCs.
2 (4/07-9/07/02)	Decrease in rate due to water stress	Middle of DC 1.
3 (17/07-23/07/02)	Two weeks after end of DC 1 and maximum rate observed in the pot trial for all varieties.	Second peak of the elongation rate
4 (31/07-7/08/02)	Some varieties maintained stem elongation rate.	9 days after DC 2.
5 (15/08-21/08/02)	Recovery observed in some varieties.	Third peak of the elongation rate
6 (28/08-4/09/02)	Stem elongation maintenance.	In the middle of DC 4.
7 (4/09-20/09/02)	Delayed senescence	End of growing season for most of varieties.

DC: Drying Cycle.

The mean and standard errors of the total population was calculated for each period and to test the significance of the differences observed between the hybrids on those dates ANOVAs were computed. Those are presented in Table 2-11. To test the significance of the differences between the trials factorial ANOVAs were computed (Table 2-12).

Table 2-11. Means, standard errors of the mean (sem) and Analysis of variance of stem elongation rates (mm d^{-1}) recorded during seven periods for a population of 39 hybrids of Salix grown in the field (n=117) and pot (n= 156) trial; Silsoe, Bedfordshire; 2002. The levels of significance are represented as: ns: non significant; *: significant at $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

Period	Field trial 2002			Pot trial 2002		
	Mean	Sem	ANOVA	Mean	Sem	ANOVA
1	14.7	0.70	**	19.4	0.49	***
2	19.6	0.62	**	11.7	0.43	***
3	28.5	0.63	***	20.4	0.48	***
4	34.5	0.84	***	18.3	0.42	***
5	28.9	0.50	ns	15.4	0.46	***
6	18.5	0.57	***	6.7	0.39	***
7	11.9	0.56	***	4.35	0.36	***

Table 2-12. Factorial analyses of variance of the stem elongation rates during seven periods for a total population of 39 willow hybrids grown in field and pot trial; Silsoe, Bedfordshire; 2002. The levels of significance are represented as: ns: non significant; *: significant at $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$

		Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7
Hybrid (pot vs field 2002)	Hybrid	***	***	***	***	***	***	***
	Site	***	***	***	***	***	***	***
	Hybrid x site	ns	**	**	***	***	***	***

For more detail on the results see appended CD.

For all seven periods, significant difference of elongation rates were observed within the hybrid population and this in both trials except in the field on period 5 (Table 2-11). This meant that the elongation rates can be used to differentiate between some hybrids in different water regimes.

During Period 1 the elongation rates were significantly higher in the pot trial than in the field (Table 2-12); perhaps reflecting that the field was planted two weeks after the pot trial. However no significant interaction between the hybrids and the sites was recorded indicating that the rank of elongation rate of the 39 hybrids at establishment between the two trials was similar.

During periods 2 to 7 stem elongation rates in the field were significantly higher than in the pot trial (Table 2-12). This meant that the elongation rates were significantly reduced because stresses including the ones caused by water shortages. In addition, a significant interaction of hybrids within the sites was observed. Thus the rank of the hybrids in their population differs significantly between both trials. This means that water stress significantly affected the elongation rates of the hybrids in different proportion.

The next step of the analysis was to check if stem elongation rates could be used to predict RSBP

A multidimensional statistical analysis was used because the linear square correlation coefficients (r^2) between RSBP and the 15 stem elongation rates in the field were < 0.03. A PCCA was run to identify any key dates segregating with the principal components. The PCCA computed the elongation rates calculated at 13 intervals of time where ANOVA shown significant difference between hybrids at $p \leq 0.05$ (Appended CD). The elongation rates recorded on the intervals 26/06/02 - 4/07/02 and 15/08/02 – 21/08/02 were omitted from the PCCA as on those dates no significant differences were recorded within the hybrid population.

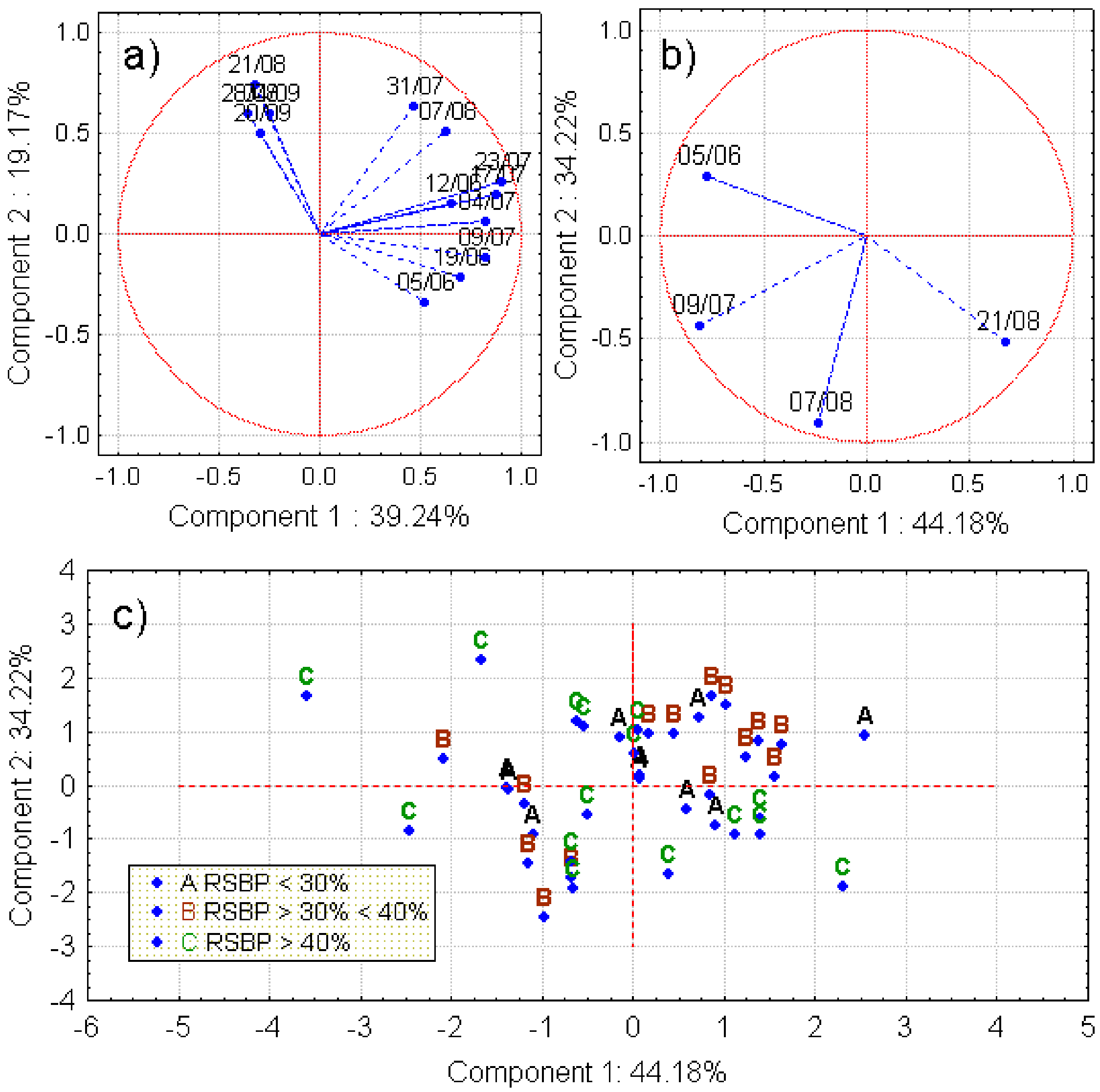


Figure 2-8. Principal Component and Classification Analysis (PCCA) of 13 elongation rates of 39 Salix Hybrids. Field trial; Silsoe, Bedfordshire; 2002. a) projection of 13 stem elongation rates on the components plan C1 x C2 calculated from the first PCCA; b) projection of four stem elongation rates on the components plan C1 x C2 calculated from the last PCCA; c) projection of 39 hybrids on the components plan C1 x C2 calculated from the last PCCA

The number of stem elongation rates integrated in the PCCA were reduced from 13 (Figure 2-8-a) to four after six analyses (Figure 2-8-b). At this stage, four stem elongation rates were extracted: 1) in early June, 2) in early July at the beginning of the stem elongation exponential phase, 3) in early August in the middle of exponential phase and 4) in late August at the end of the exponential phase. Unfortunately, the hybrids projected on the component plan C1 x C2 (Figure 2-8-c) did not clearly cluster. This meant that stem elongation rates alone cannot be used to segregate hybrids according to their RSBP.

2.4. Discussion

Like most agricultural research, the trials conducted at Silsoe in 2002 were subject to uncontrollable factors such as the weather. In addition, the design of the trials may have influenced the growth of the willow varieties; some may have been advantaged in comparison to others. Water stress was not the only stress experienced by the varieties in the trials. These stresses are discussed below.

2.4.1. Climate and weather

The weather in 2002 (Figure 2-1 and Figure 2-2) compared to the climate of the last decades was characterised by particularly high minimum temperatures in the winter. The years 2002 and 2003 had an uneven distribution of rainfall and the summers were drier, but with heavy storms in July 2002.

These, two years were almost ideal to run outdoor experimentations on drought resistance. Since there were long dry spells during the summer.

2.4.2. Trials design

Some components of the trials created slight discrepancies in the conditions of growth from plot to plot. Small areas of crops surrounded by a smaller crop or bare soil are subjected to the "clothesline effect". This effect is reported to generate discrepancies in water consumption in comparison to larger fields where water consumption is supposedly homogeneous throughout the field. This phenomenon is because "*turbulent transport of sensible heat into the canopy and transport of vapour away* *from the canopy are increased by the broadsiding of wind horizontally into tall vegetation*" (Allen et al., 1998). In other words, at the edge of the field the heat exchange and the vapour differences are greater than in the middle of the field. In the field trial an edge effect appeared to exist, where plants grown at the edge were shorter than those in the middle.

An attempt to minimize this effect was made in the field trial with the establishment of a guard rows all round the plots. Stephens et al. (2001) reported that the clothesline effect occurred up to 20 - 50 m for vegetation between 2 and 5 m high. However, a small edge effect was observed up to the second row around the field (data not recorded) but no noticeable differences were recorded after the second row. Thus the guard rows should have been doubled around the field trial.

In the pot trial, no guard rows were used, but the plants situated at the edge did not appear to suffer more in comparison to their clones grown in the middle of the trial. This was probably due to the low density of the pot trial and the stress imposed which kept the plants short and consequently generated an increased evaporative demand across the pot trial.

Overall, the clothesline effect was greater in the field than in larger commercial planting.

The fitted relationship between stem length and biomass shown in Figure 2-5 passed through the origin in the pot trial but not in the field. Differences in plant spacing might have induced those results as plants grown at high density did not have enough room to express shrubby phenotype or to branch. These results are confirmed by the work of Bullard et al. (2002) who showed that willow SRC planted in high densities tended to have an erect habit. In addition, the high density in the field encouraged some stems to etiolate as they were competing for light. The close proximity of the plants also attenuated the wind force and long thin stems were not affected by wind which can result in shorter, thicker stems (Spatz and Bruechert , 2000). To summarise, high planting densities result in stems being long, erect and thin, and some of the stems count for little biomass.

In the pot trial, some plants remained shrubby and developed more branches. Consequently, the plant morphologies in the field were uniform while in the pot trial they were not.

The potential rooting volume was greater in the field than in the pot trial. The plants grown in the pot trial responded more rapidly to stress so that symptoms could be observed within a day. In the field the irrigation was maintained to supply the crop need while in the pot trial the irrigation was maintained with the same objectives, except for the drying cycles, but the water was not distributed evenly and a second dripper had to be installed. This may have caused an uneven distribution of the root activity in the pots during the early stage of the growing season and consequently added water stress to the plants. Also, the small volume of soil may have resulted in nutrient shortage even though fertilisers were added to the pots the frequent excess irrigations may have washed away a good proportion of nutrient e.g. nitrate. In addition, the pots were black (low albedo) and exposed to solar radiation; therefore, the soil contained in the pots heated rapidly causing extra stress to the plants especially during July and August.

Stem biomass production in the field in 2003 was higher than in 2002 for all the varieties, suggesting that the established stools performed better than first year cuttings. However, the difference between the trials run in 2002 was used as the main comparison because the field and pot trial in 2002 started with the same willow material, and were subject to the same weather. Furthermore, the field in 2003 was not perfectly irrigated, with discrepancies in irrigation amounts applied in the plots closest to and farthest from the pump. This was due to friction in the pipe and also to the slope of the field. As a result, although the willow achieved better biomass in the field, hybrids such as Resolution did not seem to have benefited as much as the others from the cut back. In the field, the plots of LA980289 and Endurance were closer to the pump in average than Tora, Ashton Stott and Resolution (Figure 2-9); this has slightly affected the conservation of ranking between the two years as no statistical differences were recorded.

For many experiments on willow SRC, the stem biomass of the first year prior to cut back is ignored, but breeders wish to reduce the time taken to breed new hybrids as much as possible. The consistency in yield rank between years in the field experiment suggests that biomass production in the establishment year can be used to predict production in at least the subsequent year.

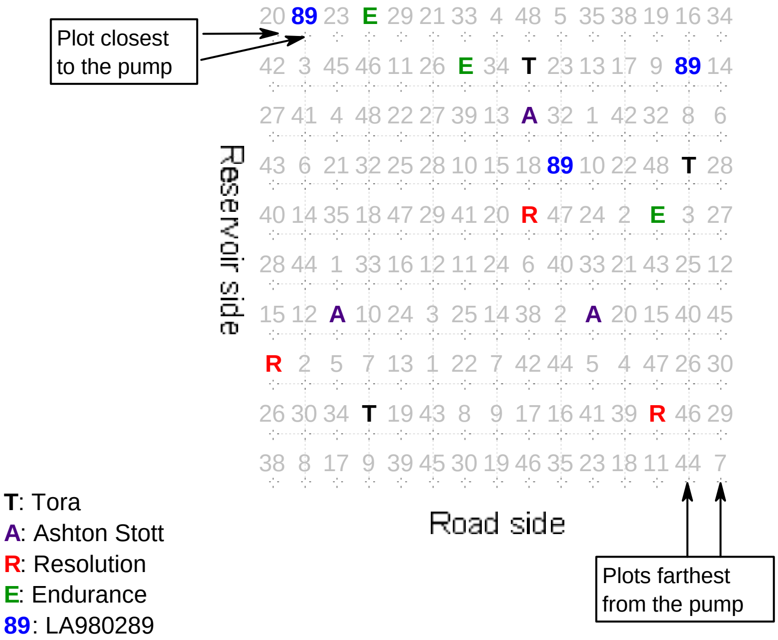


Figure 2-9. Map of the field trial; Silsoe, Bedfordshire; 2002-2003. The varieties are indicated by numbers and the five hybrids are highlighted, the road side is the highest side

The pure species were the most affected by the cropping conditions in the field as most of them were unable to compete with the high yielding hybrids. Consequently the values of RSBP of the pure species population should be treated with caution. A separate experiment including only pure species would have helped to link genotypes and traits linked with better biomass and stem length achievement under water stress. But this was not a constraint given that the prominent interest is in the high yielding hybrids.

2.4.3. Willows and water stress

The study of the hybrid population was good but the stress experienced in the pots of 20 l has included more components than water shortage. A field water stress experiment might have resulted in a different ranking.

There are very few data from variety trials in willow SRC, especially using promising hybrids. For example Lindegaard et al. (2001) did not present enough data to compare directly with this experiment. However in 2002, the yield of Tora and Ashton Stott are only half and one third respectively of the yields in established trials at LARS in 1992, 1993 and 1996. In 2003, yields were comparable but slightly lower. This is not surprising given that most experiment ignore the first year biomass. Also, biomass accumulation might be more important in year 2 and 3.

The growth under stress was very different between hybrids, with a broad range of water stress responses. This chapter concentrated only on the stem height and elongation rate. These two variables indicated that different groups or individual hybrids adopted different responses to stress. Drought tolerant varieties should be able to maintain growth under stress, recover faster and maintain growth longer than non-drought tolerant varieties.

Some experiments describe the natural processes of willow SRC growth, one recent study is the one of (Martin and Stephens, in press-c). In this study the leaf area was accurately measured. The leaf area appeared to increase until middle of July and decrease until complete defoliation this, independently of the water regimes and soil types suggesting that leaf population management is dependent on day length. In contrast, Figure 2-7-a presents a fall in stem elongation rate from the middle of August. The delay between the decrease of leaf area and stem elongation rate might be linked to the effectiveness of leaves to maintain growth. This particular aspect ought to be studied to increase the knowledge of biomass accumulation and might give information on water stress resistance processes.

Higher relative stem biomass production (RSBP) and apparent drought resistance had to be linked to field trials to be able to integrate physiological assessment that would tie in the current plant-breeding programme. The PCCA run on the field results in 2002 did not demonstrate any relationship between elongation rates and RSBP. However, for the high yielding hybrids used to illustrate the figures, it appeared that hybrids with high RSBP had an early start and finish of their stem elongation exponential phase while hybrids with lower RSBP tended to have a delayed exponential phase (Figure 2-7-a). Consequently, varieties with early growth benefit more from the long day length of June-July. They were also growing rapidly when other stresses (e.g. temperature and soil volume available for rooting) were less important.

It is important to note that the drying cycles applied to the pot trial at the end of June and end of July were prior to and in the middle of the exponential phase of stem elongation so these should not have disadvantaged any of the varieties by altering their seasonal timing of senescence.

The 40 hybrids were selected in order to represent a wide genetic range. However, some genotypes were more represented than others, because hybrids of certain species such as *S. viminalis* and *S. schwerinii* are preferred to other such as *S. caprea* or *S. rehderiana* by plant breeders. As a result most hybrids generated nowadays have *S. viminalis* or *S. schwerinii* hybrid parents. The poor representation of certain genotypes gives rise to some doubts about some parents transmitting resistance or sensitivity to water stress and further studies of genotypes should be carried out once indicators are clearly defined. Nevertheless, *S. dasyclados* or *S. burjatica* hybrids had on average lower RSBP than those without this parentage.

Stem length have been reported in numerous occasions in commercial planting to be linked with stem biomass (e.g. Tuskan and Rensema, 1992; Verwijst and Telenius, 1999) and is currently used in the plant breeding programme as a selective criteria for stem biomass (Shield and Macalpine, personal communication, 2004). However the architecture of a plant depends on the combination environment and genotype (Spatz and Bruechert , 2000). The results presented in Figure 2-3 and Figure 2-4 demonstrate that stem height and biomass of willow SRC varieties were not affected by water stress in the same proportion. This is explained because an upright architecture governed by environmental factor such as the high density, was expressed in the field while the lower density in the pot trial allowed some willow SRC to express their shrubby phenotypes resulting in different relationships between stem length and biomass in both trials (Figure 2-5).

A range of responses to water stress was observed in the 50 varieties. Five hybrids were used to illustrate the results in more detail: Tora, Ashton Stott, Resolution, Endurance and LA980289 covering the range of RSBP. Slight differences were observed between them in the field and highly significant differences were observed in the pot trial. Stem height and its elongation rate recorded during and shortly after water stress, appeared to give valid information on the sensitivity of a variety to water stress. However, RSBP cannot be part of a set of criteria for the willow breeding programme, as the trial use in the breeding programmes does not impose drying cycles yet. The field data in 2002 gives some information on a possible link between early growth and senescence with drought resistance although no significant correlation was found due to a restricted number of individuals.

To summarise, field trials set up in areas where little or no water deficit occurs will not differentiate between varieties that exhibit very different responses to water stress. In addition measurements of stem elongation at different time through the growing season are not sufficient to differentiate varieties with contrasting RSBP. As a result, additional information is required to identify criteria enabling to quantify the varieties' sensitivity to water stress in the early stage of the plant-breeding programme. Other physiological and morphological attributes need to be examined to see whether they can help differentiate between varieties with different response to water stress. The following chapter evaluates a range of morphological and physiological factors in their use as indicators of water stress resistance and WUE.

CHAPTER 3. Morpho-physiological traits linked with water stress resistance and stem biomass

3.1. Introduction

In the previous chapter, an experiment on 50 willow varieties grown under two water regimes was presented; the first in a field and irrigated on a regular basis, the second in pots and subjected to drying cycles. The relative stem biomass production (RSBP) is the ratio of the pot stem biomass (SB) to the field SB; this was used to quantify the ability of the varieties to develop a proportion of their SB potential under water stress. RSBP can be related to the growth pattern; in most cases the hybrids with the highest RSBP had an earlier exponential growth phase than the others. However, stem elongation monitoring alone was not sufficient to ascertain the drought resistance ability of a particular hybrid.

Paleg and Aspinal (1981) and Jones (1992) enumerated the main processes of drought resistance in plants and indicated that the morphology and physiology has a great importance in these processes. In an attempt to explain the differences in water stress resistance, the leaf and stem morphology and physiology of the 50 varieties was analysed and is reported in this chapter. According to Jones (1992) classification of drought resistance mechanisms, some morpho-physiological traits of leaves reported in this chapter refer to “avoidance of plant water deficits: *water conservation* by small leaves, limited leaf area, limited radiation absorption” and “tolerance of plant water deficit: *leaf turgor maintenance*”.

For some genus of fast growing herbaceous plants e.g. peas (*Pisum sativum*) (Khan et al., 2001) and wheat (*Triticum aestivum*) (Botwright et al., 2002); it has been reported that some morpho-physiological traits might be linked to drought resistance abilities and high yield. Botwright et al. (2002) compared three early vigour and three low vigour hybrids of wheat and concluded that early vigour can increase yield. No experiment of on early vigour of trees in relation to water stress resistance was reported.

To date, few of these traits have been studied in *Salix,* because economic interest in *Salix* is recent. As a result, a set of morpho-physiological criteria to detect drought tolerant varieties has never been compiled. For this study, the morpho-physiological traits used were adapted from other experiments on other genus or specially introduced.

In an experiment to detect drought resistance or high WUE among *Salix* varieties, Lindroth and Cienciala, (1996) concluded from their experiment on two clones that "*water availability will be a critical factor in SRC willow forestry despite the relatively high water use efficiency of the species*". Sennerby-Forsse (1995) reported that differences in leaf canopy dynamics for the genus *Salix* might have a role in growth under water stress, although no clear statistical evidence was presented.

In plants, the roots and the leaves are apparatus that govern the exchanges of water in liquid or vapour phases with the environment. These have been the subject of extended studies of drought influence on plant growth (e.g. Slatyer, 1967; Delvin, 1969; Jones, 1992; Sennerby-Forsse, 1995; Rytter, 2001; Ben Nouna et al., 2002; Brooks et al., 2002; Liu et al., 2002).

The aerial parts of plants are easily accessible and non-destructive experiments can be combined and repeated: as described in the second chapter e.g. the stem lengths were measured on a regular basis. The leaves are the aerial part of the plant where most water and gas are exchanged (Delvin, 1969). There have been many studies on leaves in the last century (e.g. Lichtenthaler, 1984; Niinemets and Kull, 1998; Yin et al., 2004).

Water and gas exchanges are driven by the environmental conditions: water, light and nutrients availability, wind and temperature, and equations using meteorological data to predict evapotranspiration (ET) have been developed (Smith et al., 1992). A reference evapotranspiration (ETo) that gives an indication on the evaporative demand was needed to facilitate comparison between environments (Allen et al., 1994). However, different ET rate have been observed for different crops grown in the same environment (Allen et al., 1998) suggesting that particular plant morpho-physiological attributes may enhance or reduce the amount of gas and water exchange.

Cullinan (1920) quoted by Delvin (1929), while pruning apple trees realized that the water consumption decreased because of the reduction in the canopy, while the water consumption increased per unit of leaf area. This observation suggested that apple tree water use was connected to its architecture as the remaining leaves were probably less shaded and less protected from the wind which indicates that plant morphology alters its relationship with the environment.

Willow leaves develop from the apex of the stem or branches as they extend (Ross and Ross, 1998), thus the leaves located at the top of the stem are younger than the ones located lower down. After a time the leaves senesce and drop. This phenomenon takes place generally in autumn for deciduous species. However, in willow SRC, this phenomenon takes place as the stand grows, leading to a dynamic leaf canopy balanced between the emergence and death of leaves. Sennerby-Forsse, (1995) produced a model of willow stand canopy, incorporating leaf longevity as one of the components. It has also been reported that drought may influence willow SRC leaf canopy duration for *S. dasyclados* (Liu et al., 2001).

Leaf morphology has been reported as one of the main factors in a plant’s ability to manage water loss. Cuticle, leaf hair, stomata and leaf pose are among the morpho-physiological features that regulate water loss through leaves (Jones, 1992). Khan et al. (2001) reported that leaf morphology was linked to greater yield under water stress for some varieties of *Pisum sativum*. Cameron et al. (2002) reported that differences in leaf cuticular wax were observed in three *Salix* species and might have a role in the water stress resistance.

Jones (1992) noted that “*leaf hairs have significance for radiation balance and for the balance between water loss and assimilation*”. Ehleringer, (1976) reported a decrease in the absorbance and subsequent photosynthesis of genus *Encelia* a woody perennial due to the leaf hairiness. Some willow varieties present leaf hairs which causes difficulties in measuring stomata density. This was predicted by Weyers and Meider

(1990) who observed that "*trichomes also make observation of the pores difficult*". As a result stomata density was not measured in this study.

Plant leaves can be of diverse colours. "*Leaves may contain up to 1 g of chlorophyll* m^{-2} *of surface area, but this varies with species*" (Lawlor, 1987). The chlorophyll content of the plant contributes to the colour of the leaf. The chlorophylls a and b are pigments that allow the plant to convert light into chemical energy and consequently drive the water consumption as the plant metabolizes. Therefore, the colour of the leaves might be an indicator of the WUE of the plant but to date no record of leaf colour linked with WUE has been reported.

The morphology of willow has been described and linked with yield (Verwijst, 1991). Preliminary observation of the 50 varieties revealed that their morphologies were very different. Plant morphology includes the leaves' location and space distribution and therefore the ability of the plant to intercept light and perform photosynthesis. The stems and branches are the main components of the harvestable biomass of willow SRC. Robinson et al. (2004) recorded a series of "basic" leaf and stem morpho-physiological traits in six *Salix* varieties and some of which significantly linked with yield potential. Unfortunately due to the absence of information on drought resistance abilities of their clones, no indication of morpho-physiological predisposition to high drought resistance was given.

To summarise, the above notes show that a range of morpho-physiological traits can be used or adapted to characterised willow SRC. To date, the studies on willows focused on traits linked with yield potentials. Those studies used a small number of varieties widely contrasting in their yield potential. Consequently, morpho-physiological traits that might be linked with drought resistance abilities are not identified for high yielding varieties.

This chapter examines a range of characteristics in 50 varieties selected from the Long Ashton willow collection. The objectives were to:

1. identify and set up methods to record a set of morpho-physiological traits in the 50 willow varieties;
2. identify morpho-physiological traits related to yield;

3. identify morpho-physiological traits predicting high RSBP;
4. propose a set of morpho-physiological traits enabling the grouping of hybrids with similar yield potential; and
5. propose a set of morpho-physiological traits enabling the grouping of hybrids with similar RSBP potential.

The morpho-physiological traits studied here relate to long term mechanisms of drought resistance of the *Salix* species or hybrids.

3.2. Material and methods

3.2.1. The field and pot trials

Details of both trials set up and husbandries were described in the previous chapter. To summarise, the trials were set up at Silsoe, Bedfordshire in spring 2002. 50 *Salix* varieties divided into 10 pure species and 40 hybrids were planted. The trials were randomised block design of three and four replicates for the field and the pots trial respectively. The field was the Wet regime and was irrigated, while the pot trial was the Dry regime and four drying cycles were schedule in the summer.

Note that the field regenerated in 2003 and some morpho-physiological assessments were added to those carried out during the summer 2002.

3.2.2. Willow cuttings early stages of growth

Willow cuttings conditions after storage

During storage, some cuttings initiated growth of differentiated and undifferentiated tissues. No reports have been found on the early development of tissue from willow cuttings being stored for several weeks. The development of tissue in the absence of light and at low temperature may be seen as a vigour characteristic of plant and early vigour might be of interest in this study (Botwright et al., 2002). The cuttings of the varieties were harvested at LARS on 18/02/02 and stored at +4°C in the dark, sealed in plastic bags until 11/04/02.

Three phenomena took place:

- Some buds burst;
- Some roots developed; and

- Some calluses developed from the stem parenchyma.

From preliminary observations it was clear that the varieties exhibited different stages of development during storage. To quantify these tissue developments, an index from zero to five was created for each type and used to score the stems as a whole for each variety (Table 3-1). These indices were created in order to score the morpho-physiological state of the cuttings in a quantitative way; this to enabled statistical analyses. The index was calibrated after observation of a large sample of varieties after storage.

Table 3-1. After storage indices, 0 to 5, of three morphological traits used to assess the morpho-physiological state of willow cuttings after eight weeks of storage at +4 °C; Silsoe, Bedfordshire; 2002

Index	Calluses per cutting (average)	Roots per cutting (average) short < 10 mm; long ≥ 10 mm	Buds and shoots (average)
0	0	0	Dormant
1	≤ 5	Few short	Buds swell
2	≤ 10	Few long	Buds burst
3	≤ 20	Many short	Emergence of flowers and/or leaves
4	≤ 30	Many long	Flowers and/or leave developed
5	> 30	Covered with	Branches developed

Note that only one score per variety was recorded. This limited the statistical applications as no errors could be calculated for individual varieties.

Growth index

To measure early vigour, after planting a growth index was developed to assess the morpho-physiological state of the willow during the first two weeks of development; this was used to correlate early vigour, plant biomass and water stress resistance.

The willow varieties selected for the experiment had a very similar development process even though the stem and leaf morphologies were different. To facilitate statistical analysis, an index was designed as acontinuum to track in regular steps the bud development into leaves and stems (Table 3-2). After two weeks, this index was not used any more; instead the stem length of the tallest shoot was measured in the fourth week (see previous chapter).

To calibrate the growth indices, an overview of the plantation aspect was carried out in order to define the range of growth in which the cuttings were observed.

Table 3-2. Willow SRC emergence growth index 0 to 10, used on willow SRC 1 to 2 weeks after planting; Silsoe, Bedfordshire; 2002

Index	Morpho-physiological state
0	No growth of the buds
1	The buds have started to swell or burst
2	The buds have burst and leaves start to develop
3	First two leaves able to perform photosynthesis
4	First four leaves able to perform photosynthesis
5	First six leaves able to perform photosynthesis, and elongation of the stems.
6	First eight leaves able to perform photosynthesis. Two leaves fully expanded
7	First ten leaves able to perform photosynthesis. Four leaves fully expanded.
8	First twelve leaves able to perform photosynthesis. Six leaves fully expanded.
9	First fourteen leaves able to perform photosynthesis. Eight leaves fully expanded.
10	First sixteen leaves able to perform photosynthesis. Ten leaves fully expanded.

3.2.3. Willow leaves

Leaf population

Jones (1992) defines leaf area index (LAI) as "*the total projected leaf area per unit of area of ground*" and argues that LAI is an important parameter of canopy structure because of "*the strong links to primary productivity, through light interception and evapotranspiration*". In most contemporary studies, LAI has been measured or calculated using a large canopy of several trees or by modelling a particular variety canopy development (e.g. Proe et al., 2002).

An attempt to use a SunScan (Delta-T Devices Ltd, Burwell, UK) in the field in 2002 resulted in unreliable data as the plots of four plants were too small for the equipment. Nor was the SunScan appropriate to measure LAI on individual potted plants (Potter et al., 1996).

Destructive measurements of leaf area are relatively simple but were not possible given the small number of replicates of each variety. To model a good estimation of the willow varieties LAI, requires allometry of each variety, in this instance morphologies of the varieties would have required massive data collection and computation. Therefore, a simplified method was created to monitor the leaf canopy: the leaf stem ratio (R_{leaf})

Maximum stem length (L_{max}) in the field and the length of stem from which the leaves had dropped were recorded (L_{bare}) on four dates: 31/07; 29/08; 5/09 and 20/09. Thus, it was possible to monitor the canopy on these stems and to calculate R_{leaf} for the field as below.

Equation 3

$$R_{leaf} = \frac{L_{total}}{L_{\max}}$$

Equation 4

$$L_{total} = L_{\max} - L_{bare}$$

R_{leaf} seemed more appropriate for one year old stems than the length in meters of stem bearing leaf as L_{total} was different from one stem to another among and between the varieties. Until the leaves started to senesce, R_{leaf} was 100%.

Leaf colour and hair density

No reports of leaf colour or hair density assessment of *Salix* have been reported in the literature. The leaf colour is believed to be dependent on the pigment types and content; mainly chlorophyll; their content depends on the age of the leaf and nutrient availability (Kawashima and Nakatani, 1998). Leaf colour could not be quantified accurately as the visual methods found in the literature where not accurate enough to cover the small range of colour observed in the 50 Salix varieties studied and more recent digital technology was not available at Silsoe.

Leaf hair has a clear role in the regulation of leaf evaporation (Jones, 1992) and this was easily assessed visually. An index was created to quantify the hair density (Table 3-3; Plate 3). Each variety was assessed using this index. The index was created as a continuum to be able to quantify the hair density from the absence of hair to the maximum density observed and run statistical analyses of the data collected. The index was calibrated after observation of the 50 willow varieties. Fully developed leaves from each plot of the three replicates of the field trial were assessed in early July 2002.

Table 3-3. Hair density index of willow leaves and stem apex; Silsoe, Bedfordshire; 2002

Index	*Characteristics*
0	No hair
1	Short hair only on leaves
2	Long hair only on leaves
3	Long and dense hair on leaves and short on stem apex
4	Long and very dense hair on leaves and stem apex

Short ≤1 mm, Long >1 mm.

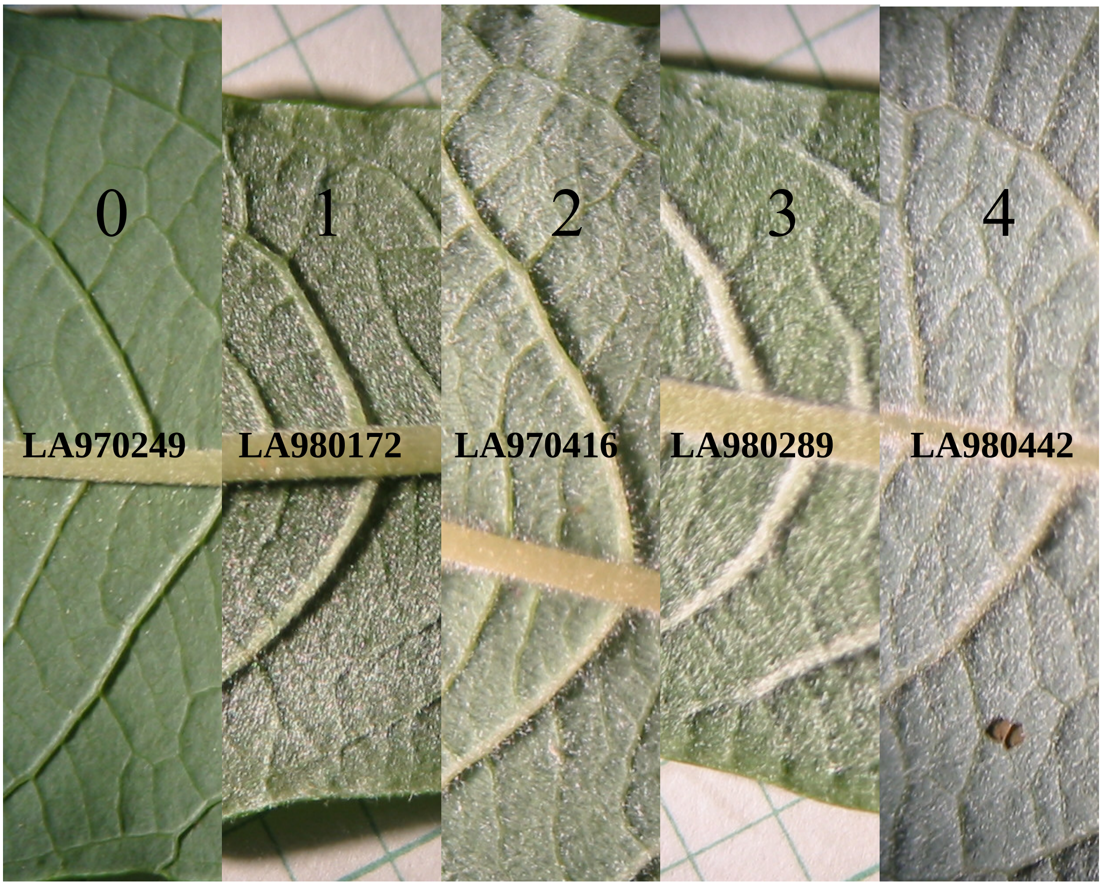


Plate 3. Leaf hair density observed on abaxial leaf surface of five Salix hybrids covering the range of index used to quantify leaf hair density of willow leaves (X3)

No variations in score were recorded within the same variety. This limited the statistical applications, as no standard errors could be computed.

Leaf shape and specific leaf area

For the study of leaf shape and specific area at Silsoe, the leaves were sampled from the plants grown in the field trial in August 2002. The leaves selected were fully developed, undamaged, green, located on a main stem and representative of the average size and shape of the population of leaves in the variety.

Three leaves per plot of replicate II were sampled, each from different plants in the plot. The leaves were sealed in plastic bags and brought to the laboratory. On the same day, the petioles were removed, the leaves were weighed fresh (LW_{fresh}), then placed on a leaf scanner (Delta T device ltd, Burwell, UK). The scans were analysed, to measure the maximum length (L_{leaf}') and width (W_{leaf}) and area (A_{leaf}) of each leaf. Then the leaves were oven dried for 48 hours at 105 °C and weighed again (LW_{od}).

A ratio $R_{l/w}$ (Equation 5) was calculated. Eversham (personal communication, 2002) suggested that $R_{l/w}$ is an indicator to rapidly differentiate willow and sallow families and/or species.

The fresh (LSA_{fresh}) and dried (LSA_{od}) leaf specific areas were calculated as shown in Equation 6 and Equation 7.

Equation 5

$$R_{l/w} = \frac{L_{leaf}}{W_{leaf}}$$

Equation 6

$$LSA_{fresh} = \frac{A_{leaf}}{LW_{fresh}}$$

Equation 7

$$LSA_{od} = \frac{A_{leaf}}{LW_{od}}$$

The field research trial was preferred to the pot research trial to sample the leaves as it was an unstressed trial and it was not yet clear if the leaves would be affected by water stress. On the pot trial, destructive methods of assessment were avoided when possible to conserve the canopy.

Relative water retention (RWR)

Bertholdsson (personal communication, 2002) indicated that he believed that RWR could be used to screen clones for increased drought resistance. The method that he indicated was as follows: "*Three to four leaves of a similar age are sampled from each willow tree. These are sealed in plastic bags until the fresh weights (W0) are measured. In a green house the leaves are left to dry on paper sheets for four hours and weighed afterwards (W4). Finally they are oven dried for 48 hours at 80^oC and weighed (W48).*" RWR is calculated as in Equation 8.

Equation 8

$$RWR = \frac{W0 - W4}{W0 - W48}$$

Bertholdsson found that RWR was correlated with stomatal conductance (g_s), ΔC13 discrimination and with WUE. He only used six clones grown in a greenhouse, but apparently these clones with high RWR performed relatively better than others during dry years. These results have not been published.

The method was applied once to each replicate in the field. Three leaves per plot were sampled. At first the method described by Bertholdsson (personal communication, 2002) was strictly followed (RWR 1); it was then slightly modified in an attempt to get uniformity in the first drying session. For the second attempt (RWR 2) the leaves were dried on a bench in the laboratory and finally for the third attempt (RWR 3) the leaves were dried in a fan oven at 25 ^oC. Leaves from the field research trial were used.

Leaf adaxial epidermal cell size (AECS)

Robinson et al. (2004) described a method to produce leaf prints to assess AECS. Although their results suggested that varieties bearing large leaves composed of a large number epidermal cells "*may be more indicative of yield than the individual epidermal cell area*"; it has to be taken in account that from AECS, Robinson et al. (2004) extrapolated the number of cells per leaf incorporating A_{leaf}. However A_{leaf} depends on environmental factors such as light (intensity at the leaf level, day length) as well as the age of the plant, position and orientation of the leaf. None of these components was presented for each individual leaf by Robinson et al. (2004) and results have therefore to be interpreted with caution. As an example of different leaf sizes, 11 fully developed and mature leaves of Ashton Stott harvested in August 2003 are presented in Plate 4. The two smaller ones were harvested on Type B branches while the others were harvested from stems.

Plate 4. Mature leaves of Ashton Stott grown in the field sampled in August 2003. From left to right, the first five sampled from main stems, the next four from secondary stems, the last two from Type B branches

To provide consistency in this experiment, the samples taken on 11/08/03 were located on the main stems and were the 10th fully developed leaf from the apex of a main stem.

A method to produce the leaf print is described as follow: "*at a distance of 1 cm 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 leaving the leaf intact*" (Robinson et al., 2004).

In this study, numerous *Salix* varieties do not bear big enough leaves to sample such a large area so an area of 1 x 1 cm was sampled in the middle of the leaf, where the width is almost constant.

In order to prevent the varnish cracking the leaf prints were stored at +5°C until observation. The leaf prints were observed and photographed with different equipment than that used by Robinson et al. (2004). Images were obtained using a

Nikon™ contrast phase microscope with a Canon™ digital camera (2.0 megapixels) mounted on it. The images were pictures of the area of adaxial epidermis magnified 400 times and randomly selected from the print. Robinson et al. (2004) described a method of analysis of the leaf pictures. "*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.*" In this experiment a different method was used: after preliminary observation of the first leaf prints, it was clear that the epidermal cells had a variety of shapes and sizes and the leaf surface was organized in structures composed of leaf veins, trichomes and simple epidermal cells (see Plate 5 and Plate 6). Therefore, to pick ten leaf cells randomly might lead to misjudgement. In this study the leaf print pictures were printed and an area of 100 x 100 µm was defined where no veins or trichomes were observed. In the defined area all cells were then counted. The average cell area was calculated as the defined area divided by the number of cells.

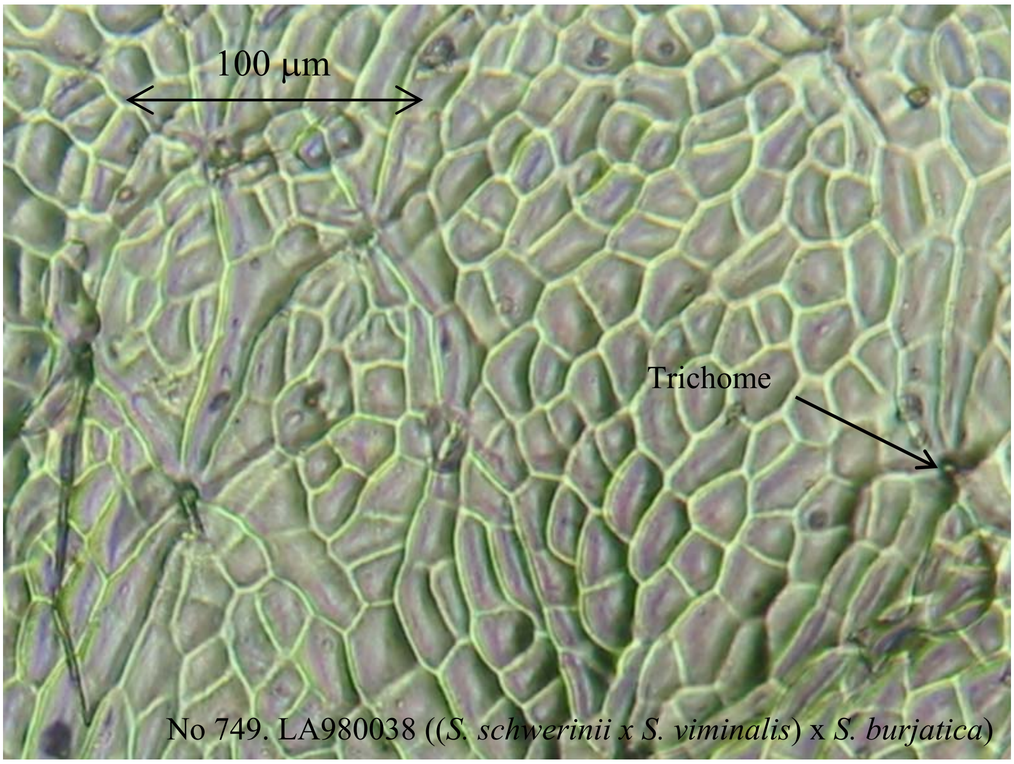


Plate 5. Leaf print of the adaxial epidermal cells of LA980038 (X400)

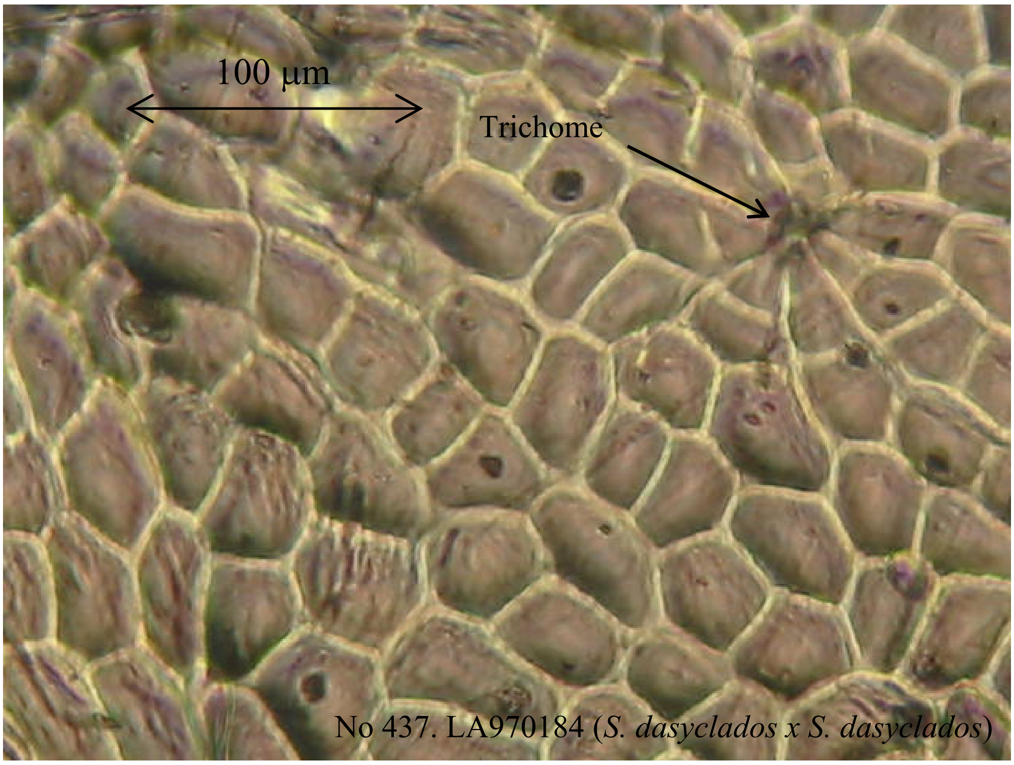


Plate 6. Leaf print of the adaxial epidermal cells of LA970184 (X400)

There are clear differences in cell sizes between the two plates, but also between the cells of the same plate (variety).

Cuticular waxes

Unfortunately, the cuticular waxes of the 50 varieties of *Salix* grown at Silsoe were not quantified because of time constraint and lack of equipment.

3.2.4. Willow morphology

Roots and stools

At Silsoe, the roots in the field trial were not harvested so as to conserve the trial intact for subsequent years. The roots in the pots were not harvested, as no control was available to compare the water stressed plants with. The study of roots required equipment and an experimental design different to those available at Silsoe.

Number of stems per stool

The number of stems per stool after cut back is a criterion used in plant breeding (Lindegaard, 2002) and is a component of biomass (Verwijst, 1991). The number of stems was recorded when the field trial was harvested in 2004

Stem diameter

The stem diameters of all stems of each stool on each plot in the field were measured at harvest in 2004 because this is a criterion commonly used for describing woody short rotation coppice.

Wood density

Wood density (WD) varies according to species and the rate of growth, but generally some are hardwoods like mahogany others softwoods like balsa. At harvest in 2004 the wood density (WD) was measured as follow. One 100 mm stem sample per plot with a diameter between 10 and 15 mm was taken from the wood harvested in the field in 2004 to estimate wood density (WD). It was oven dried, weighed (W_{od}) and its volume (V) assessed.

A 5 l beaker was filled with water and put on a precision scale. The precision scale was then tared. The wood sample was pinned with a needle on one extremity and forced into the water avoiding contact with the beaker until it was totally immersed. As a result, a volume of water equal to the sample volume V was displaced and its weight measured with the precision scale. 1 g of water equals 1 cm^3 therefore the weight of water displaced is equal to the volume V. WD was calculated as in Equation 9.

Equation 9

$$WD = \frac{W_{od}}{V}$$

Branching

The absence of branches is one of the first criteria used by plant-breeders to select high yielding varieties. Therefore, little difference in branching habit was expected amongst the hybrids included in this experiment since they had already been through a selection procedure. However, branching was observed in the pots in 2002 and recorded at harvest. The plants grown in the field were not assessed as the branching habit was inhibited by high density (Bullard et al., 2002).

To assess the branching two indices were created, one to characterise the type of branch and the second referring to the position of the branches on the plant at harvest

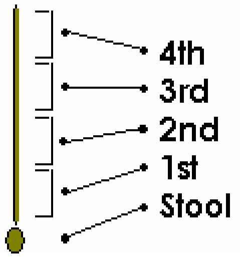
(Table 3-4 and Table 3-5). The branch indices were set up as a continuum to reflect a progression: the branch type index increased as the contribution to plant biomass increased; the branch position index increased in relation to the height at which the branches were positioned on the stems.

Table 3-4. Branch type index of willow SRC; Silsoe, Bedfordshire; 2002

Index	***Branch type***
0	No branches
1	Type A
2	Type A and Type B
3	Type B
4	Stem division above stool

Note: Type A implies that these most branches do not survive over the winter months and generally drop off as they defoliate, Type B implies that these branches survive the winter months and reshoot the following spring.

Table 3-5. Branch positions index of willow SRC. Silsoe, Bedfordshire; 2002

Index	***Branch position***	
0	No branches	
1	First quarter of the stem length from the stool	
2	Second quarter of the stem length from the stool	
3	Third quarter of the stem length from the stool	
4	Fourth quarter of the stem length from the stool	

3.2.5. Statistical analysis

As in the previous chapter, the set of data collected from the population of *Salix* were separated into the pure and hybrid populations, the mean and the standard error were then calculated. All sets of data of individual varieties are presented in the appended CD. All sets of data were analysed in the same way and the analysis took place in three steps.

1. The means and standard error of the mean were calculated; an analysis of variance (ANOVA) was run (null-hypothesis: there are no differences between individuals), the confidence intervals were calculated at 95% and the data were checked for normality by calculating the skewness.

Skewness is the deviation of distribution from symmetry *(StatSoft Inc., 2001)*. Here the distribution was considered normal if the skewness was under 1 (Bellamy, personal communication, 2004). The distribution is not essential to carry out further steps of the statistical analysis. Nevertheless, the closer the data to normality the easier it was to integrate into statistical analyses. Strictly speaking, if a set of data is extremely non-normal, it should be transformed for further analysis. Some sets of data collected showed non-normality so graphs were plotted (see appended CD) to judge the necessity and the type of transformation required to include the data set in the matrix prior to analysis.

2. A Kendal *tau* analysis on ranks was the most appropriate technique for the data set available. Statistics based on ranks were used to determine which variables correlated most with the relative stem biomass production (RSBP) and the biomass production in the field 2002. Three levels of confidence were used, $p \leq 0.01$; ≤ 0.05 and ≤ 0.10 to gradually isolate more variables for the next step of the analysis.

Since the morpho-physiological traits measured varied widely in units and range of the variables, they were all standardised by subtracting the mean value and dividing by the standard deviation (Scott and Clarke, 2000). Only the hybrid population was used for the analysis of ranking, because the pure species data were not representative of their SB and RSBP potential.

3. A cluster analysis K-means clustering was carried out to verify if the morpho-physiological traits identified in the Kendal *tau* analysis on ranks permitted the segregation of groups of varieties with similar SB and/or similar RSBP.

In other words, a cluster analysis was used to verify the validity of a potential set of morpho-physiological traits to predict high SB and RSBP potential. The variables extracted from the analysis on ranks at different probability levels were compiled into different cluster analyses. These were computed to create clusters of hybrids as distinct as possible from each other considering the variables extracted earlier. Five clusters for each analysis were created in order to obtain clusters with a reasonable number of hybrids. An unlimited number of iterations were used to compute the clusters. The differences between mean SB and RSBP of each cluster were compared using an analysis of variance and post-hoc test: Fisher least significant difference at $p \leq 0.05$ (LSD).

3.3. Results

The results are presented in two main sections. The first section covers the first step of the statistical analysis; it is divided into three sub-sections which group associated morpho-physiological features of the willow cuttings in the early stages of growth; the leaves and; the architecture of the willows.

The second section of results comprises the analyses on ranks and the cluster analyses.

3.3.1. Descriptive statistics

Willow cuttings in the early stages of growth

The analyis of morpho-physiological state after 52 days of storage at + 4 °C and during the first four weeks of growth are presented in Table 3-6.

Table 3-6. Mean, standard error of the mean (sem), analysis of variance (ANOVA), 95% confidence interval (CI) and skewness of seven variables measured on the early stages of development of cuttings of 50 willow varieties; Silsoe, Bedfordshire; 2002. The significance of ANOVA is represented as na: not applicable; ns non significant; * significant at $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; n=150 in the field, n=200 in the pots

Variable	Mean (sem) (a) total (b) pure species (c) hybrids	Anova & ±CI (a) total (b) pure species (c) hybrids	Skewness (a) total (b) pure species (c) hybrids
Calluses (index) *After 52 days storage at +4^{o}C*	(a) 0.70 (0.22) (b) 0.43 (0.43) (c) 0.74 (0.24)	(a) na (b) na (c) na	(a) 1.95 (b) 2.64 (c) 1.89
Roots (index) *After 52 days storage at +4^{o}C*	(a) 1.70 (0.27) (b) 2.14 (0.86) (c) 1.61 (0.28)	(a) na (b) na (c) na	(a) 0.66 (b) 0.35 (c) 0.72
Shoots (index) *After 52 days storage at +4^{o}C*	(a) 1.30 (0.26) (b) 2.29 (0.81) (c) 1.13 (0.26)	(a) na (b) na (c) na	(a) 1.07 (b) 0.34 (c) 1.25
Pot growth index at 1 week	(a) 2.7 (0.11) (b) 2.2 (0.23) (c) 2.9 (0.12)	(a) *** ±1.25 (b) *** ±1.03 (c) *** ±1.30	(a) -0.02 (b) 0.32 (c) -0.10
Pot growth index at 2 weeks	(a) 5.1 (0.18) (b) 3.5 (0.33) (c) 5.5(0.20)	(a) *** ±2.05 (b) *** ±1.68 (c) *** ±2.15	(a) -0.20 (b) -0.15 (c) -0.33
Field growth index at 1 week	(a) 1.9 (0.07) (b) 2.0 (0.17) (c) 1.9 (0.07)	(a) *** ±0.80 (b) ns (p= 0.19) ±1.03 (c) *** ±0.76	(a) 0.63 (b) 0.62 (c) 0.61
Field growth index at 2 weeks	(a) 6.0 (0.16) (b) 5.1 (0.30) (c) 6.3 (0.18)	(a) *** ±1.78 (b) *** ±1.44 (c) *** ±1.88	(a) -0.10 (b) 0.02 (c) -0.22

The complete set of data can be found on the appended CD.

During storage, on average the willow hybrids developed more calluses than the pure species. However, on average the pure species developed more roots and shoots than the hybrid species. This suggests that the pure species have a more vigorous habit of rooting and shooting under cold and dark conditions than the hybrids.

Conversely once transplanted in the field the hybrid population grew faster than the pure population in both trials. Note that in most cases the ANOVA performed on the growth index resulted in significant differences, which means that the methods could differentiate between the varieties.

These observations show that cuttings from hybrids can be conserved better and establish faster than those from pure species.

The skewness of the indices of calluses and shoot development after storage were above 1 because many of varieties did not develop any calluses or any shoots and scored zero. It was not appropriate to transform the data as the indices were made of very few steps (Pat Bellamy, personal communication 2004). For all growth indices significant differences were observed within the populations. Some varieties did not establish well and extreme values were recorded affecting the shewness. No transformations were required, as the rest of the data resembled normal distributions.

Willow leaves

The results of assessment of willow leaf population are presented in Table 3-7.

Table 3-7. Mean, standard error of the mean (sem), analysis of variance (ANOVA), 95% confidence interval (CI) and skewness of leaf population ratio on stem (R_{leaf}) on four dates measured on the leaves of 50 varieties of willows. Field trial; Silsoe, Bedfordshire; 2002. The significance of ANOVA is represented as * significant at $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ n=150

R_{leaf} (%)	Mean (sem) (a) total (b) pure species (c) hybrids	Anova & ±CI (a) total (b) pure species (c) hybrids	Skewness (a) total (b) pure species (c) hybrids
31/07/02	(a) 90.4 (0.76) (b) 93.0 (1.71) (c) 89.7 (0.84)	(a) *** ± 6.40 (b) ** ± 8.38 (c) *** ± 5.96	(a) -1.88 (b) -1.74 (c) -2.03
29/08/02	(a) 75.1 (1.06) (b) 80.8 (2.61) (c) 73.6 (1.13)	(a) *** ± 9.32 (b) ** ±12.54 (c) *** ± 8.58	(a) -0.31 (b) -0.77 (c) -0.31
4/09/02	(a) 67.8 (1.11) (b) 74.7 (3.18) (c) 66.1 (1.09)	(a) *** ±10.27 (b) ** ±13.78 (c) *** ± 9.45	(a) -0.06 (b) -0.94 (c) 0.04
20/09/02	(a) 58.7 (1.02) (b) 68.2 (3.18) (c) 57.1 (1.01)	(a) *** ± 9.57 (b) ** ±12.79 (c) *** ± 8.83	(a) 0.13 (b) -0.44 (c) 0.08

For R_{leaf} on all dates significant differences were observed within the populations. The set of data can be found in the appended CD.

On the four dates, the proportion of stem bearing leaves was higher for the pure species than the hybrids. The general trend was a decrease of R_{leaf} from the end of July to the end of September. The skewnesses of the set of data of R_{leaf} calculated on 31/07 were above 1. This was due to extremely low values recorded on varieties LA980132, LA970249, LA970416, *Salix miyabeana* and LA970485 in which leaf senescence started at a higher rate than the rest of the population. No transformations were required because the rest of the data appeared normally distributed.

The results of assessment on willow leaf phenotype are presented in Table 3-8.

Table 3-8. Mean, standard error of the mean (sem), analysis of variance (ANOVA), 95% confidence interval (CI) and skewness of eight variables measured on the leaves of 50 varieties of willows. Field trial; Silsoe, Bedfordshire; 2002-2003. The significance of ANOVA is represented as na: not applicable; ns non significant; * significant at $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Variable	Mean (sem) (a) total (b) pure species (c) hybrids	Anova & ±CI (a) total (b) pure species (c) hybrids	Skewness (a) total (b) pure species (c) hybrids
Leaf hair density (index)	(a) 2.4 (0.23) (b) 2.3 (0.63) (c) 2.4 (0.24)	(a) na (b) na (c) na	(a) - 0.58 (b) - 0.41 (c) - 0.67
Leaf shape ratio $R_{l/w}$	(a) 5.7 (0.22) (b) 5.2 (0.61) (c) 5.8 (0.23)	(a) *** ±0.93 (b) *** ±0.73 (c) *** ±0.97	(a) 0.49 (b) 0.70 (c) 0.51
LSA_{fresh} ($mm^2\ g^{-1}$)	(a) 4840 (67) (b) 4887 (93) (c) 4827 (81)	(a) *** ± 306 (b) ** ± 348 (c) *** ± 301	(a) 1.80 (b) 0.40 (c) 1.83
LSA_{od} ($mm^2\ g^{-1}$)	(a) 13554 (209) (b) 13343 (305) (c) 13607 (256)	(a) *** ±1294 (b) *** ±1422 (c) *** ±1282	(a) 2.20 (b) 0.10 (c) 2.21
RWR 1 %	(a) 51 (1.1) (b) 56 (3.4) (c) 49 (1.1)	(a) *** ± 7.75 (b) *** ± 8.27 (c) *** ± 7.75	(a) 1.01 (b) 0.62 (c) 0.93
RWR 2 %	(a) 51 (1.4) (b) 54 (3.3) (c) 50 (1.5)	(a) *** ± 12.3 (b) *** ± 15.5 (c) *** ± 12.2	(a) 0.24 (b) 0.16 (c) 0.24
RWR 3 %	(a) 94 (0.14) (b) 94 (0.54) (c) 94 (0.11)	(a) *** ± 1.3 (b) ** ± 2.7 (c) *** ± 0.8	(a) - 4.35 (b) - 4.26 (c) - 0.50
AECS (μm^2)	(a) 361 (3.5) (b) 372 (8.2) (c) 358 (3.9)	(a) *** ±36.4 (b) *** ±37.2 (c) *** ±36.2	(a) 1.95 (b) 1.32 (c) 2.14

For all morpho-physiological traits but leaf hair, significant differences were observed within the populations. The complete set of data can be found on the appended CD. The leaf hair density recorded on the pure species and the hybrids seemed similar and in all cases the data were normally distributed.

The leaf shape ratio $R_{l/w}$ was slightly higher for the hybrids than the pure species one. This may be due to the large representation of *S. viminalis* in the hybrid population ($R_{l/w}^{bowles\ hybrid} = 9.8$).

LSA_{fresh} and LSA_{od} were very close between the two populations.The skewnesses of LSA_{fresh} and LSA_{od} were close to 2. Among the hybrid population, LA970184 and

LA970136 had the lowest and the highest LSA_{fresh} and LSA_{od} respectively. The data collected on these two varieties were extreme values and resulted in a high value of skewness.

RWR 1 showed a greater difference between the pure and the hybrid populations than the second and third attempts.

The skewness of RWR 3 was equal to -4.26 for the pure species population; this was due to *Salix dasyclados* which had one leaf that lost a lot of water during the first drying cycle and generated an extreme value. The rest of the data appeared to be normally distributed and did not require transformation.

The epidermal cell areas were slightly bigger for the pure species population than the hybrid population.

The skewnesses of AECS were above 1. Most of the data were normally distributed but LA980030, *Salix dasyclados* and *Salix udensis* had extremely large cells in comparison to the other varieties, and created extreme values. No transformations were required as the rest of the data appeared to be normally distributed.

Willow morphology

The results of the assessment performed on willow morphology are presented in Table 3-9.

Table 3-9. Mean, standard error of the mean (sem), analysis of variance (ANOVA), 95% confidence interval (CI) and skewness of five variables measured from the willow of 50 varieties of willows. Field trial; Silsoe, Bedfordshire; 2002. The significance of ANOVA is represented as na: not applicable; ns non significant; * significant at p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001

Variable	Mean (sem) (a) total (b) pure species (c) hybrids	ANOVA & ±CI (a) total (b) pure species (c) hybrids	Skewness (a) total (b) pure species (c) hybrids
Number of stems per stool in field at harvest in 2004	(a) 5.4 (0.11) (b) 4.4 (0.25) (c) 5.7 (0.12)	(a) *** ±1.16 (b) *** ±1.01 (c) *** ±1.20	(a) 0.57 (b) 0.99 (c) 0.56
Stem diameter (mm)	(a) 11.6 (0.09) (b) 10.5 (0.25) (c) 11.8 (0.09)	(a) *** ±0.18 (b) *** ±0.49 (c) *** ±0.18	(a) 2.50 (b) 7.46 (c) 0.82
Wood density ($g\ cm^{-3}$)	(a) 0.52 (0.003) (b) 0.52 (0.007) (c) 0.52 (0.004)	(a) *** ±0.03 (b) ** ±0.04 (c) *** ±0.02	(a) 0.11 (b) 0.13 (c) -0.08
Branch type (Index)	(a) 1.6 (0.06) (b) 2.0 (0.15) (c) 1.5 (0.06)	(a) *** ±0.66 (b) *** ±0.67 (c) *** ±0.66	(a) 0.28 (b) -0.64 (c) 0.49
Branch position (Index)	(a) 2.4 (0.06) (b) 2.0 (0.15) (c) 2.6 (0.06)	(a) *** ±0.68 (b) *** ±0.68 (c) *** ±0.68	(a) -1.45 (b) -0.47 (c) -1.88

For all morphological traits significant differences were observed within the populations. The set of data can be found on the appended CD.

The number of stems per stool was higher for the hybrid population than for the pure one, and significant differences were observed between varieties in both populations. There were no differences in wood density between the populations although the ANOVAs were significant. Moreover, the number of stems per stool and their diameters appeared to be linked as the stem diameters decreased as the number of stems increased.

The hybrid population branches contributed less to their final biomass than the pure species. In addition, the branches occurred higher on the stem of the hybrids than the pure species.

The stem diameters were similar for the hybrid and the pure populations. This does not mean that the biomasses were similar as the hybrid population developed more stems per stool.

The skewness of the data relating to the branch position in the pot was equal to -1.88 for the hybrid population; this was because most hybrids developed branches (Type A or Type B) by the end of the season on the upper part of the stem, which was probably due to severe water stress which in some cases induced the death of the apex of the stems and development of branches when re-irrigated.

3.3.2. Further statistics

Further statistics were applied to the set of data. The analyses on ranks were performed at three different p levels: $p \leq 0.01$, 0.05 and 0.10. This generated different combinations of significantly related variables to SB and RSBP[5]. These combinations were then used to generate the group of varieties with K-clustering analyses.

The results of the Kendal *tau* analysis on ranks of SB and RSBP are presented in Table 3-10 and Table 3-11. The morpho-physiological traits recorded as the most correlated with SB and RSBP are ranked in order of importance.

Table 3-10. Significance and order of morpho-physiological traits most correlated with stem biomass production in the field in 2002. Data collected on 39 Salix hybrids grown in the field and the pot trials at Silsoe, Bedfordshire; 2002-2003

Kendal *tau*		Rank	Morpho-physiological traits correlated with stem biomass production.
p ≤	T		
0.01	0.30	1	Pot growth index 2
	0.29	2	Number of stems per stool in the field in 2003
0.05	-0.28	3	Branch type index
	0.28	4	Leaf hair density
	0.26	5	Field growth index 1
0.10	0.22	6	Field growth index 2
	-0.20	7	Leaf specific area oven dried (LSA_{od})

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[5] In the interpretation of the results, a high RSBP score was associated with a high water stress resistance potential and a low RSBP score with a low water stress resistance potential.

At $p \leq 0.01$, the growth index in the pots in the second week was most correlated with stem biomass production. For all growth indices, a positive correlation was observed which means that early vigour was a positive trait for high yield. The number of stems per stool was positively correlated, which means that hybrids with numerous shoots after coppicing were potentially high yielding.

At $p \leq 0.05$, the branch type index was negatively correlated with SB; thus absence or little branching was a positive trait for high yield. The hair density was positively correlated which indicates that hairiness is a positive trait for high yield.

Finally at $p \leq 0.10$ the dry leaf specific was negatively correlated. Therefore, low LSA_{od} is a positive trait for high yield potential.

Table 3-11. Significance and order of morpho-physiological traits the most correlated with relative stem biomass production. Data collected from 39 Salix hybrids grown in the field and in the pot trials at Silsoe, Bedfordshire; 2002-2003

Kendal *tau*		Rank	Morpho-physiological traits correlated with relative stem biomass production.
p ≤	T		
0.01	-0.35	1	Number of stem per stool in the field in 2003
	-0.30	2	Pot growth index 2
	-0.30	3	Field growth index 2
0.05	-0.27	4	Leaf hair density
	-0.26	5	Pot growth index 1
	-0.25	6	Callus index
	-0.24	7	R_{leaf} 20/09
	-0.23	8	$R_{l/w}$
0.10	-0.20	9	Adaxial epidermal cell size (AECS)
	-0.19	10	Field growth index 1

At $p \leq 0.01$, the number of stems per stool in the field after coppicing in 2003 was the most correlated with RSBP. The negative correlation suggests that the fewer stems developed on a coppice stool the greater the chance for the willow to be drought tolerant. The next variables to be highlighted within that probability were the growth indices in the Pots and in the Field on week 2 after planting. These indices were negatively correlated, which means that hybrids with high early vigour in the first two weeks are not a good indicator of drought resistance potential.

At $p \leq 0.05$, the hair density was highly negatively correlated. This indicates that the more leaf hair lesser the chance that were in the top range of *Salix* hybrid ability to resist water stress. The callus development during storage was negatively correlated and indicates that hybrids developing calluses during storage were likely to have susceptible poor resistance to water stress. The ratio R_{leaf} measured on 20/09/02 in the field was negatively correlated. This implied that a large proportion of stem bearing leaves at the end of the growing season was not a good trait for drought resistance. The ratio R, leaf length divided by leaf width was positively correlated which indicates that long narrow leaves are for drought resistance.

Finally, at $p \leq 0.10$, the AECS was negatively correlated, which suggests that the smaller the adaxial epidermal cells the more likely the hybrid to be drought tolerant.
The clusters of hybrids and the means and standard errors of the mean of SB and RSBP are presented in Table 3-12 and Table 3-13.

Table 3-12. Three K-means clustering analysis results, cluster members, mean stem biomass (kg plant $^{-1}$) and 95% confidence interval (CI). The variables used were extracted earlier in a set of Kendal tau analyses on ranks of the correlation of the variables with the stem biomass production Silsoe trials, Bedfordshire; 2002. Tora (50), Ashton Stott (49), Resolution (36), Endurance (37) and LA980289 (31) are highlighted as indicator hybrids. The letters represent Fisher least significant differences (LSD) post hoc grouping $p \leq 0.05$

Kendal *tau* analysis p value and variables	Cluster 1 *Members* Mean ± CI	Cluster 2 *Members* Mean ± CI	Cluster 3 *Members* Mean ± CI	Cluster 4 *Members* Mean ± CI	Cluster 5 *Members* Mean ± CI
1) $p \leq 0.01$ Pots growth index 2; Number of stem per stool.	*2; 6; 12; 13; 19; 22; 23; 28.*	*5; 11; 18; 21; 29; 33; 34.*	*3; 4; 7; 14; 38;* ***49****.*	*1; 8; 9; 15; 17; 26; 30; 35;* ***36****;* ***37****;* ***50****.*	*10; 25; 27;* ***31****; 32.*
	0.151 ± 0.015 (ab)	0.131 ± 0.017 (b)	0.163 ± 0.019 (a)	0.133 ± 0.014 (b)	0.103 ± 0.019 (c)
2) $p \leq 0.05$ Pots growth index 2; Number of stem per stool; Branches type index; leaf hair density; Field growth index 1.	*1; 2; 5; 11; 13; 14; 21; 28; 30; 35.*	*3; 4; 6; 7; 8; 9; 15; 23; 24; 33; 38.*	*12; 26;* ***36****;* ***37****;* ***49****;* ***50****.*	*10; 17; 18; 22; 25; 27;* ***31****; 32; 34.*	*16; 19; 29.*
	0.137 ± 0.015 (a)	0.148 ± 0.014 (a)	0.149 ± 0.019 (a)	0.107 ± 0.015 (b)	0.158 ± 0.027 (a)
3) $p \leq 0.10$ Pots growth index 2; Number of stem per stool; Branches type index; leaf hair density; Field growth index 1; Field growth index 1; LSA_{od}.	*16; 19; 26; 29;* ***36****;* ***50****.*	*3; 4; 6; 7; 8; 12;* ***37****; 38;* ***49****.*	*1; 2; 5; 11; 13; 14; 21; 28; 30; 35.*	*18; 25; 27;* ***31****.*	*9; 10; 15; 17; 22; 23; 24; 32; 33; 34.*
	0.145 ± 0.019 (ab)	0.163 ± 0.015 (a)	0.137 ± 0.014 (b)	0.100 ± 0.023 (c)	0.123 ± 0.014 (bc)

At three different probabilities, the number of variables included in the analyses to compute the clusters was different. As a result, the cluster members were different for the three probability levels.

At $p \leq 0.01$ the range of SB between the clusters was 0.060 kg plant $^{-1}$, with significant differences between the mean of cluster 5 the lowest mean SB and the other four clusters; as well as cluster 3 which has a significantly higher mean than the ones of clusters 4 and 2.

At $p \leq 0.05$ the range of SB between the clusters was 0.051 kg plant^{-1} but only cluster 4 had a significantly lower mean than the other four clusters.

At $p \leq 0.10$ the range of SB between the clusters was 0.063 kg plant^{-1}. Significant differences were recorded with the mean of cluster 4 lower than the means of clusters 1, 2 and 3; and the mean of clusters 3 and 5 lower than that of cluster 2.

The range observed in the third analysis is the highest of all three k-mean clustering analyses with 0.063 kg plant^{-1}. Tora, Ashton Stott, Resolution Endurance and LA980289 had an average SB of 0.115, 0.142, 0.187, 0.216 and 0.139 kg plant^{-1} respectively. At $p \leq 0.01$ these varieties were not segregated in cluster reflecting their SB; in addition at $p \leq 0.05$ the three varieties could not be segregated. At $p \leq 0.10$ Ashton Stott and Endurance regrouped in cluster 2, while Tora and Resolution were members of cluster 1 characterised by a lower mean, but clusters 1 and 2 are not statistically different. LA980289 one of the highest yielding hybrids and hybrids 25 and 27, which had the lowest SB were regrouped in the cluster with the lowest means in all three analyses.

Table 3-13. Three K-means clustering analysis results, cluster members, mean relative stem biomass production (%) and 95% confidence interval (CI). The variables used were extracted earlier in a set of Kendal tau analyses on ranks carried on the correlation of the variables with the relative stem biomass production; field trial; Silsoe, Bedfordshire; 2002. Tora (50), Ashton Stott (49), Resolution (36), Endurance (37) and LA980289 (31) are highlighted as indicator hybrids. The letters represent Fisher least significant differences (LSD) post hoc grouping $P \leq 0.05$

Kendal tau analysis p value and variable	Cluster 1 *Members* Mean ± CI	Cluster 2 *Members* Mean ± CI	Cluster 3 *Members* Mean ± CI	Cluster 4 *Members* Mean ± CI	Cluster 5 *Members* Mean ± CI
$p \leq 0.01$ Number of stem per stool in the field in 2003; pots growth index 2; Field growth index 2.	*10; 17; 19; 26; 30; 32; 35;* ***36; 50*.**	*16; 18; 25; 27;* ***31*.**	*1; 5; 11; 15; 21; 22; 24; 28; 29; 33; 34.*	*2; 3; 4; 6; 7; 12; 13; 14; 23; 38;* ***49*.**	*8; 9;* ***37*.**
	41.9 ± 6.1 (ab)	50.6 ± 8.1 (a)	36.6 ± 5.5 (bc)	30.1 ± 5.5 (c)	29.8 ± 10.5 (bc)
$p \leq 0.05$ Number of stem per stool in the field in 2003; pots growth index 2; Field growth index 2; leaf hair density; pots growth index 1; callus, Rleaf 20/09; R.	*5; 10; 18; 21; 24; 25; 27; 28; 29;* ***31****; 33; 34; 35.*	*9; 15; 17; 19; 22; 23; 32;* ***36; 50*.**	*3; 4; 6; 7.*	*1; 2; 11; 16.*	*8; 12; 13; 14; 26; 30;* ***37****; 38;* ***49*.**
	42.7 ± 5.9 (a)	38.9 ± 7.0 (ab)	30.8 ± 10.6 (ab)	34.9 ± 10.6 (ab)	31.8 ± 7.0 (b)
$p \leq 0.10$ Number of stem per stool in the field in 2003; pots growth index 2; Field growth index 2; leaf hair density; pots growth index 1; callus, Rleaf 20/09; R; AECS; field growth index 1.	*1; 10; 13; 15; 17; 18; 21; 22; 23; 24; 28; 30; 32; 33; 34; 35.*	*25; 27;* ***31*.**	*7;* ***37; 49*.**	*2; 5; 11; 12; 16; 19; 26; 29.*	*3; 4; 6; 8; 9; 14;* ***36****; 38;* ***50*.**
	38.5 ± 3.8 (b)	62.6 ± 8.7 (a)	24.0 ± 8.7 (c)	33.6 ± 5.3 (bc)	34.3 ± 5.0 (b)

At $p \leq 0.01$ the range between the cluster with the lowest (cluster 5) and highest (cluster 2) mean RSBP is 20.8%. Cluster 2 is significantly higher than clusters 3, 4 and 5. Cluster 1 is significantly higher than cluster 4.

At $p \leq 0.05$ only the maximum difference between cluster 1 and 5 is significantly different with a range of 11.9%.

At $p \leq 0.10$ the widest range of the three cluster analyses is observed with 38.6 % between cluster 3 and 2. Cluster 2 is significantly higher than the other four clusters. In addition, cluster 3 is significantly lower than cluster 1 and 5.

Tora, Ashton Stott, Resolution, Endurance and LA980289 are characterised by a RSBP of 44.6, 27.1, 32.6, 17.0 and 53.2% respectively. In all three cluster analyses they segregated to a greater or lesser extend while the clusters are in the same order as the hybrids. However, the third analysis presents the lowest error in comparison to the others, which is more accurate.

The objective of the study was to create set morpho-physiological traits to classify willow according to their potential yield and RSBP. The aim is to use the set of traits as soon as possible in the willow-breeding programme. In addition, an advantage of the set of traits would be its simplicity and ease of use for relatively unskilled personnel.

The analyses carried out above include the assessment of morpho-physiological traits that require time to become fully established in the coppice e.g. number of stems per stool. As a second step in the prediction of the potential for biomass production and resistance or sensitivity to water stress, only a limited number of morpho-physiological trait assessments were included in the K-means clustering analyses, in order to simulate the use of a set of traits as early as the second year of the willow breeding programme, making use of a limited number of morpho-physiological assessments.

From the Kendal *tau* analyses on ranks, a number of morpho-physiological assessments were identified as highly correlated with either SB or RSBP (see Table 3-10 and Table 3-11).

In the case of SB the morpho-physiological assessments selected were: the branch type index, the leaf hair density, the field growth index 2 and finally the LSA_{od}. The field growth index in the second week offered a wider range than in the first week and was therefore easier to use.

In the case of RSBP the morphological assessments selected were: the field growth index in the second week, the leaf hair density, the callus after storage index, R_{leaf} on the 20/09, the ratio $R_{l/w}$ and finally AECS.

A K-means clustering analysis was computed with the morphological features listed above for both SB and RSBP (Table 3-14).

Table 3-14. Two K-mean clustering analyses results, cluster members, means and 95% confidence interval (CI). The variables used were extracted earlier in a set of Kendal tau analyses on ranks carried on the correlation of the variables with the stem biomass and relative stem biomass production; Silsoe, Bedfordshire; 2002-2003. Tora (50), Ashton Stott (49), Resolution (36), Endurance (37) and LA980289 (31) are highlighted as indicator hybrids

Variable Morpho-physiological assessment used	Cluster 1 *Members* Mean ± CI	Cluster 2 *Members* Mean ± CI	Cluster 3 *Members* Mean ± CI	Cluster 4 *Members* Mean ± CI	Cluster 5 *Members* Mean ± CI
Stem biomass production Branch type index, leaf hair density, field growth index 2 and LSA_{od}.	*5.*	*1; 2; 11; 13; 14; 21; 27; 28; 30;* ***31****; 35.*	*3; 4; 6; 7; 8; 12; 16; 19; 26; 29;* ***36****;* ***37****; 38;* ***49****;* ***50.***	*9; 10; 15; 22; 23; 24; 33.*	*17; 18; 25; 32; 34.*
	0.103 ± 0.046 (bc)	0.136 ± 0.014 (b)	0.156 ± 0.012 (a)	0.128 ± 0.017 (bc)	0.102 ± 0.020 (c)
Relative stem biomass production Field growth index 2, leaf hair density, callus after storage index, Rleaf20/09, $R_{l/w}$ and AECS	*3; 4; 6; 10; 21; 27; 28;* ***31****; 35;* ***36****;* ***50****;*	*8; 12; 13; 14; 26; 30;* ***37****; 38;* ***49****.*	*1; 2; 7; 9; 11; 16.*	*5; 19; 29.*	*15; 17; 18; 22; 23; 24; 25; 32; 33; 34.*
	40.3 ± 6.7 (a)	31.8 ± 7.4 (a)	34.8 ± 9.0 (a)	36.3 ± 12.8 (a)	40.8 ± 7.0 (a)

The first K-means clustering analysis, carried out to segregate varieties according to their potential biomass production, created clusters that were statistically different ($p \leq 0.05$). The range observed was 0.054 kg plant^{-1} between clusters 3 and 5. Cluster 3 had a mean SB significantly higher than all four other clusters and cluster 2 was significantly higher than cluster 5. Unfortunately, the indicator hybrids chosen did not segregate. The second K-means clustering analysis, carried out to segregate the varieties according to RSBP did not create distinct clusters and no significant differences were recorded.

Finally, an attempt to combine both assessments was made. The matrix used for the K-means clustering analysis was composed of the same variables as Table 3-14 but only those that were significant with either SB or RSBP. These were: the branch type, the LSA_{od}, the callus development during storage, R_{leaf} at the end of September, the ratio $R_{l/w}$ and AECS. This was not successful as no clusters were significantly different from each other for both SB and RSBP (results not presented). This showed that SB and water stress resistance must be studied with a separate set of morpho-physiological traits.

3.4. Discussion

The relative stem biomass production (RSBP) is used as the main drought resistance indicator of the willow SRC hybrids in the Silsoe experiment. This indicator is also used to identify morpho-physiological traits related to high drought resistance.

The results indicate that there are various morpho-physiological traits that can be used to categorise *Salix* varieties. Nevertheless, most of these traits are interrelated e.g. the field growth index in week 1 with the one in week 2 or the stem lengths and diameters that were on many occasions highly correlated to each other and with aerial biomass (Tuskan and Rensema, 1992; Verwijst and Telenius, 1999; Robinson et al., 2004).

These interrelations are frequent but are not studied in detail in this report. The research focuses on the relationships between traits and stem biomass (SB) or RSBP. Kendal *tau* analyses on ranks indicate that some traits are closely related with SB and RSBP. The growth indices 1 and 2 were correlated positively with SB and negatively with RSBP. Which suggests that high yield and high water stress resistance are not compatible, corroborating the findings of Weih (2001). As such, a combination of indicators to select high yielding and high water stress resistant varieties appears to be complicated. To estimate willow development with these growth indices does not require specific equipment. However, it requires a synchronized planting in the same environment of all cuttings. If this is done growth indices can be used as early as the second year of the breeding programme.

The number of stems per stool, like the growth indices, is correlated positively with SB and negatively with RSBP. Many stems survive the first year but self thinning takes place in commercial planting in subsequent years. In comparison to dominant stems, secondary stems account for little biomass as they etiolate and may die due to lack of light (Verwijst, 1996; Ross and Ross, 1998). The production of secondary stems can be seen therefore, as a waste of water. It is important to note that a large number of stems per stool probably links with great vigour at emergence which is also positively correlated with SB but negatively with RSBP. Thus, early and rapid shoot development can mean that water resources are used rapidly and/or that those varieties with high vigour prioritise the assimilate allocation to shoots rather than roots which results in a reduced volume of soil explored, leading to a reduced ability to cope with water shortage.

The number of surviving stems per stool is highly dependent on the stool density of the plantation (Verwijst, 1996). At Silsoe, the assessment of the number of stems may be partially representative as self thinning occurred in 2003, encouraged by a density double that of most commercial plantings. The assessment of number of stems per stool requires the willow planting to be coppiced; as a result representative assessments of the variety potential can not be carried out before the third year of the breeding programme. Currently, the assessment of the number of stems per stool is conducted in the willow-breeding programme (Ian Shield and william Macalpine, personal communication, 2004) aiming to eliminate varieties with too many branches and those developing too many secondary stems. This is partially because industry requires varieties that produce wood chips of regular size for efficient co-firing (Hanley, 2004). Moreover, the branch type index revealed that too many branches are not a good indicator of high yield; in contrast, many branches is a good indicator for poplar coppice (Gail Taylor personal communication, 2004).

Leaf hair density was positively correlated with SB but negatively with RSBP. This is surprising since leaf hairs are often linked to water stress resistance as they tend to increase leaf albedo and stomatal resistance (Jones, 1992). Nevertheless, some succulent plants deprived of leaf hair are highly water stress resistant. Endurance fits these results, as its leaf hair index is high, it reaches high yield in the field but has a low RSBP. The assessment of leaf hair density can be carried out in the first year of the willow breeding programme.

The next morpho-physiological traits extracted from the Kendal *tau* analysis on ranks were specifically correlated to either SB or RSBP. This is particularly important as they are compatible with high yield potential and drought resistance.

Leaf specific area oven dried (LSA_{od}) is negatively correlated with SB. Low LSA_{od} area means that the leaves have a heavy structure. To the touch these leaves have a highly rigid structure in comparison to others. The assessment of LSA_{od} can be carried out in the first year of the breeding programme. The value of LSA_{fresh} and LSA_{od} were comparable to those of Robinson et al. (2004)

Calluses and roots developed on the cutting from the parenchyma under the bark at +4 ^{o}C in plastic bags in total dark for 50 days. Only the callus development appeared to have a high negative correlation with RSBP. These morpho-physiological traits can be assessed after storage of cuttings as described above; this experiment could be scheduled in the second year of the breeding programme.

The ratio of the length of stem bearing leaves to the stem length, the leaf stem ratio (R_{leaf}) at the end of September was negatively correlated with the RSBP. This indicates that the hybrids keeping leaves on a large proportion of one year old stems may be susceptible to water stress. However, the leaf population is sensitive to water stress (Martin and Stephens, in press-c) which could be different if water stress occurred prior to the measurement of R_{leaf}. R_{leaf} can be used in the first year of the breeding programme, but it is recommended to wait until the second year when more clones of the same variety are available.

The ratio of leaf length to leaf width (R) is positively correlated to RSBP. Long, slender leaves are characteristic of more drought tolerant varieties than those with round or oval leaves. $R_{l/w}$ can be measured non-destructively in the first year of the breeding programme.

Adaxial epidermal cell size (AECS) is the last morpho-physiological trait negatively correlated with RSBP. Varieties with small AECS on average are more drought tolerant than the ones with larger cells. Small AECS induces a dense leaf structure as seen in Plate 5 compared to Plate 6. Adaxial leaf prints can be taken in the first year of the breeding programme but this is a destructive method. It is important to note that LSA_{od} and AECS are negatively correlated to RSBP which could suggest the existence of relationship between these two traits. The absence of a relationship between SB and AECS in the Silsoe experiment may explain the difficulty encountered by Robinson et al. (2004).

In the second part of the statistical analysis, sets of morpho-physiological traits taken from those extracted by Kendal *tau* analyses on ranks were tested for their potential to predict RSBP. To do so K-means clustering analyses were computed and the mean SB and RSBP of the cluster members calculated. K-means clustering analyses using combinations of traits were able to separate out clusters of hybrids with similar yield and RSBP.

In further steps of the analyses the number of traits was reduced to those that can be assessed during the first steps of the breeding programme in order to check the viability of different sets of traits. The first trait selected was the growth index used in the field in the second week after planting. This trait presents a wide range of responses and correlates highly with both SB and RSBP. The second trait was the leaf hair density index which correlates to both SB and RSBP. The third and fourth traits were the branch type and LSA_{od} which correlate only to SB. Branch type was easy to assess and could be recorded in the second year of willow breeding programme. The fifth to eighth traits were leaf length to leaf width ratio ($R_{l/w}$), calluses after storage index, adaxial epidermal cell size (AECS) and leaf stem ratio (R_{leaf}) in September; these last four traits correlate only to RSBP.

The cluster analysis using the eight morpho-physiological traits mentioned above does not produce clusters with similar yield or RSBP potential (results not presented). Consequently, more traits are required to predict high yield aptitudes and drought resistance. Nevertheless, the results are encouraging, as some extended combinations of traits lead to cluster the varieties in groups with similar aptitudes (Table 3-12 and Table 3-13).

The records of morpho-physiological traits presented in this chapter can be assessed with basic tools and skills, which can be used in the first three years of the breeding programme. The yield potential and drought resistance ought to be studied alongside the breeding programme. In research and plant breeding trials, reference varieties must be included to set the baseline of observation. Reference hybrids that cover the range of drought resistance or high WUE must be identified but such a set should be flexible as new standards are reached in generation of willow SRC.

Unfortunately in the 2002 experiment no studies on roots were carried out because most reported methods are destructive and the field was to be left intact for the 2003 trials and the pot trial had no reference trial to be compared with. The roots develop into a network to explore the soil and to reach water and nutrients. Most of the exchanges occur at the level of the root hairs but these are fragile and do not last long making in vivo experiments hard to carry out (Slatyer, 1967; Delvin, 1969). Studies of the physiology of tree root systems in vivo are limited to root sap flow measurement such as the work of Brooks et al. (2002) or fine root development and biomass production estimation using minirhizotrons as undertaken by Rytter (2001). From pine tree stands subjected to drought, Brooks et al. (2002) observed a hydraulic redistribution of water into the soil via the root system. This mechanism is thought to be an adaptation to enhance seedling survival under the canopies of mature trees during drought. This phenomenon suggests that the root system of tree genera generate complex mechanisms of water redistribution in the soil. Other studies of root systems involved harvesting the roots to calculate root/shoot biomass ratio (Jones, 1992) or described the structure of the rooting system in different types of soil (Crow and Houston, 2004). Studies on roots can be considered in the design of further experiments on *Salix* response to drought.

To date some work is on going to determine the genetic linkages and distances between the different *Salix* species (Sviatlana Trybush et al., personal communication, 2004). Unfortunately, no such data is yet available for all the parent species of the breeding programme. Therefore, genetic linkage and distance between parent species could not be included in the statistics to pinpoint what parentage is likely to produce hybrids with high SB and RSBP potential.

Five indicator hybrids are highlighted in the second and third chapters. These were preferred to others because they range RSBP and a large set of morpho-physiological traits they are high yielding and represent different genotypes.

The first two hybrids selected are Tora and Ashton Stott, as they are already used as reference varieties in the plant breeding trials. The third is Endurance, which has very high yielding potential but has the lowest RSBP. The fourth is LA980289 as it is reasonably high yielding and reached the highest RSBP amongst the hybrid population. Finally, Resolution is selected as its RSBP is between those of Tora and Ashton Stott and it presents high yielding potential. Resolution is preferred to hybrids with similar aptitudes because its pedigree is characteristic of the new generation of hybrids in which the parents are hybrids themselves.

It is important to conclude that studies of morpho-physiological traits linked with particular potentials (yield, pest resistance, drought resistance) are of particular interest as they are part of the development of genomics. In genomics quantitative traits loci[6] (QTL) are subject to intense research in many genera including *Salix* (Hanley and Mallot, 2004). With QTL identified in willow SRC, scientists hope to speed up plant breeding programmes by screening a large number of seedlings as soon as tissues can be sampled. Most of the morpho-physiological traits described and are potentially related to corresponding QTL. But first, these traits need to be confirmed as indicators of high yield and drought resistance potential.

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[6] QTL: chromosome site containing alleles that influence a quantitative trait

CHAPTER 4. Water use, biomass production and water use efficiency of five *Salix* hybrids

4.1. Introduction

Water use (WU) refers to the quantity of water used by a cropping system during its growth (Equation 10), while water use efficiency (WUE) is the ratio of biomass produced to WU (Equation 11).

Equation 10

$$WU = E + T + S + I$$

Where E is the evaporation from the soil; T is the transpiration from the plant; S is the storage in the plant and; I is the water intercepted by aerial parts of the plant during rainfall which then evaporates.

Equation 11

$$WUE = \frac{Biomass}{WU}$$

Biomass can be the total biomass or a part of it such as above ground biomass or yield.

A conceptual model of the WU of a cropping system is presented in Figure 4-1.

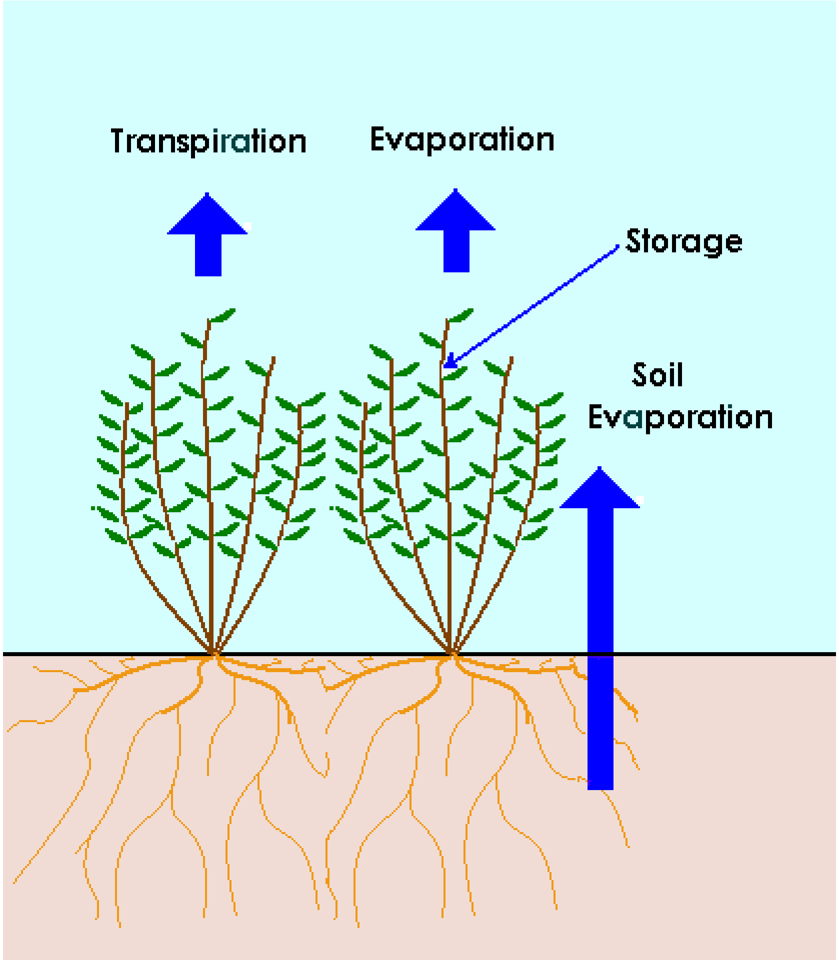

Figure 4-1. Conceptual model of water use in a cropping system

Agricultural and environmental sciences have a strong interest in WU and WUE as both depend on the water availability to the crop. As a result, WU and WUE are used to estimate the potential productivity of a crop when grown under a given climate (Vaux and Pruitt, 1983). The British government is encouraging the increase of commercial production of biomass crop such as willow SRC and *Miscanthus* to help meet targets for generation of electricity from renewable sources (DTI, 2003). To date, information on willow SRC WU and WUE is required as part of the development programme (Armstrong, 2002).

WU by any cropping system can be related to reference evapotranspiration (ETo) through a simple crop coefficient (Kc) (Equation 12).

Equation 12

$$WU = ETo \times Kc$$

Using this relationship, the water requirements of a crop in different environments can then be estimated from ETo (e.g. Vaux and Pruitt, 1983; Stephens et al., 2001a). As a result, WUE of a crop is a derivative of its yield and ETo. The water requirement can be calculated from the crop WUE and the yield required achieving profitability. The water requirement gives an indication of the suitability of a given environment for the profitable growth of a given crop.

WUE is an element that has been used to compare genus, species and varieties of crops. Jørgensen and Schelde, (2001) enumerate WUE of numerous energy crops aiming to model the feasibility of cultivation in different areas. They conclude that there is a wide spectrum of techniques to calculate WUE, which can cause confusion. For example it was reported that WUE ranges between 0.3 and 6.3 g kg^{-1} for willow SRC having widely varying yields. Consequently, few data are available on WUE of current commercial varieties.

Stephens et al., (2001b) report that the establishment of energy crops could affect ground water recharge in some areas of England and Wales where rainfall is limited. One of the current goals of water-saving farming is to seek a high crop WUE (Liu *et al.,* 2002).

There are several ways to express WUE. For example instantaneous WUE is calculated from gas exchange quantification assessed at leaf level where the biomass is the amount of carbon dioxide fixed and the WU is the amount of water transpired by a leaf sample. On other occasions total WUE is calculated with the biomass comprising the roots, stems and leaves and WU from a single or group of plants. This chapter focuses on WUE from the yield of current willow SRC varieties as this is required to estimate the profitability of willow SRC in a variety of areas (Jørgensen and Schelde, 2001; Stephens et al., 2001b). In this chapter WUE, defined as the ratio of harvestable biomass[7] (SB) to WU, is used. As the SB is predominantly composed of the stems, WUE was labelled WUE_{stem}.

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[7] SB is the biomass which includes the stems and the Type B branches.

In this project, the water use (WU) was not calculated in the first year of experimentation because the 50 varieties grown in the pot trial were too many to manage precise measurement or calculation of WU for individual plants. In the field trial, drainage, surface runoff and accurate quantification of the irrigation dose made it impossible to obtain accurate values of WUE at the plot level. As a result, a different experiment was required to quantify WU and subsequently WUE.

High WUE is a characteristic of drought resistance for crops such as the coffee (*Coffea canephora)* and wheat (*Triticum aestivum*) (DaMatta et al., 2003; Solomon and Labuschagne, 2003). However, in willow SRC, no morpho-physiological traits have been found with a specific link to high WUE which makes it impossible to relate high WUE and drought resistance in willow SRC.

In the second chapter of this thesis, the relative stem biomass production (RSBP) was formulated as an indicator of water stress sensitivity. RSBP was the ratio of SB produced in the pot to SB in the field. The main assumption is that water was not a factor limiting growth in the field but it was in the pot. In the third chapter, several willow morpho-physiological traits were assessed, some of which relate to RSBP.

Subsequent to the first year of experimentation, a detailed study of the water consumption of *Salix* hybrids was set up to obtain additional information on the physiological processes involved in the control of WU. One way to monitor WU was to grow the crop as a plant-environment system with a fully controlled water balance. This was done using lysimeters. A lysimeter is a "*laboratory column of selected representative soil or a protected monolith of undisturbed field soil with facilities for sampling and monitoring the movement of water and chemicals*" (Webnox Corp, 2000). The combination of lysimeter and precise irrigation scheduling allows full control of the water flows within the system. Two lysimeter types were used: a drainage lysimeter in which fluids can be measured only under water saturated conditions and a weighing lysimeter which is mounted on a precision scale to monitor input or output of fluid under non-saturated conditions. Grip et al., (1989); Rytter, (2001) and Ian Seymour, (personal communication, 2002) preferred lysimeters to field trials to conduct their experiments because it allows precise measurement of water use.

Information on willow WU and WUE was available for certain pure species i.e. (Dawson and Bliss, 1989; Liu et al., 2003) and a few hybrids (Lindroth and Cienciala, 1996; Weih and Nordh, 2002; Martin and Stephens, in press-c). Weih and Nordh, (2002) conclude that "*for biomass production on dry sites, productive clones that are characterized by high values of intrinsic WUE such as Jorr appear to be most promising*". Stig Larsson (personal communication, 2004) indicated that Jorr was not particularly drought tolerant, suggesting that the "*high values of intrinsic WUE*" reported by Weih and Nordh, (2002) have to be interpreted with caution.

The reports on WUE of tree genera are controversial and sometimes report WUE to be conservative which means that WUE is independent of the environment and is intrinsic to a given genotype or it can be elastic which means that a given phenotype may be characterised by different WUE when grown in different environments. Jørgensen and Schelde, (2001) had difficulty in reviewing the value of WUE as there are numerous ways of calculating WUE and the variability of conditions under which plants are grown; as a result, for some tree genera, a range of WUE was presented while for others only single values were available. Martin and Stephens, (in press-a) used a range of soil and water treatments and calculated WUE of total biomass between 1.36 and 5.06 g kg^{-1}. Their results suggest that willow SRC WUE is elastic, as higher levels of WUE are reached under water stress and as nutrient availability increases.

To summarise, WUE of current high yielding willow SRC hybrids is unknown which means that the link between drought resistance and high WUE for the genus *Salix* has never been identified. In addition, a wide range of willow SRC WUE can be found in previous work as a result of multiple calculation techniques but the potential for yield of these willow SRC also differ widely. Martin and Stephens, (in press-b) show that willow SRC WUE is affected by drought stress but this was quantified for only one commercial hybrid. As result, the range of current hybrid WUEs and their elasticity under water stress are both unknown.

In the light of the current information the objectives of this chapter were to:

1. quantify the differences of WU and WUE$_{stem}$ amongst a population of five willow SRC of contrasting genotypes and RSBP; and

2. check the individual elasticity of WU and WUE_{stem} of five willow SRC of contrasting genotypes and RSBP when subject to water stress.

This study was to check firstly if WU and WUE_{stem} were significantly different between high yielding varieties grown under irrigated conditions and secondly, to quantify the impact of water stress in WU and WUE_{stem} on the different hybrids and decide whether the WUE_{stem}s of these *Salix* hybrids were conservative or elastic.

4.2. Material and methods

4.2.1. Variety selection

In Chapter 2, the relative stem biomass production (RSBP) was identified as a way to classify the water stress resistance of 50 *Salix* varieties. For the second year of experimentation, a lysimeter research trial was set up to carry out precise studies of the willow physiology starting with accurate measurement of WU and WUE. It was decided to adapt the design of the trial for five hybrids. These hybrids were selected from the panel of 50 varieties described in the second chapter. In the two previous chapters, some reference hybrids illustrated the range of response observed in the trials, especially to range the water stress resistance using RSBP.

The study focused on the widest possible range of RSBP. Five hybrids were chosen among those with high yield potential. In addition, the genotypes of the varieties had to be as diverse as possible to create a subset of different phenotypes.

Tora and Ashton Stott were selected as reference varieties since they have different parents and, although they did not achieve particularly high yields in the field or in the pot trials in 2002, they have been used in many studies i.e. (Larsson, 2001; Lindegaard et al., 2001). Tora and Ashton Stott were bred in the late 80 early 90’s and are already grown in commercial plantings in the UK.

The range of RSBP amongst the high yielding hybrids can be seen in Figure 4-2.

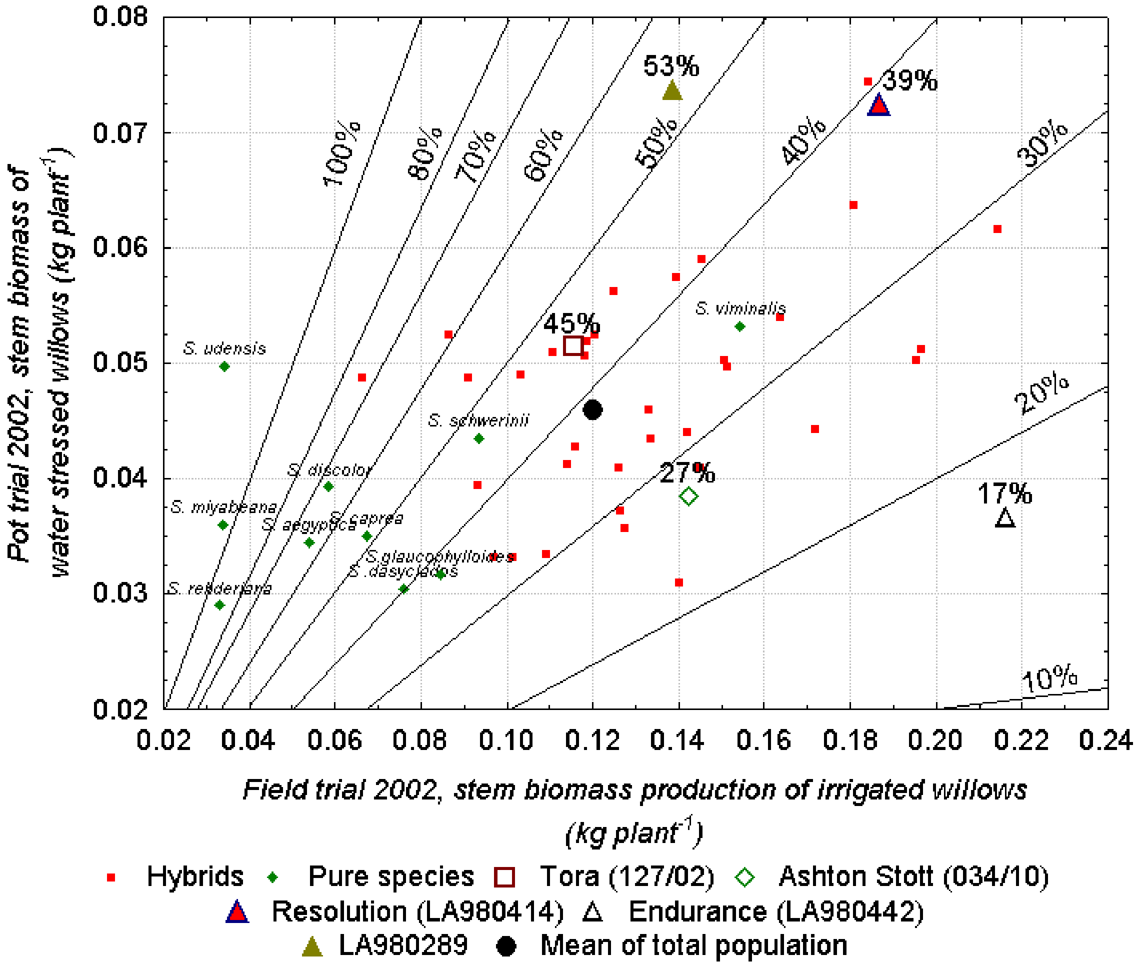

Figure 4-2. Relative stem biomass production of 50 willow varieties grown under water stress in the pot (n=4) and with irrigation in the filed (n=3) at Silsoe, Bedfordshire; 2002. The lines indicate boundaries between different relative stem biomass production. Five high yielding hybrids are highlighted

Endurance and LA980289 cover the range of RSBP as a result were used later as extreme references in sensitivity and resistance to water stress respectively.

Tora, Ashton Stott, Endurance and LA980289 show RSBP of 45, 27, 17, and 53 % respectively. The inclusion of Resolution (RSBP = 39%) in the panel of reference hybrids created a progressive range of RSBP between Ashton Stott and Tora. Resolution was preferred to LA980024 as it had slightly higher yields[8].

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[8] See second chapter for more details.

Finally a subset of five hybrids was created, these were: Tora, Ashton Stott, Resolution, Endurance and LA980289. Their parents are: *S. viminalis, S. schwerinii, S. burjatica, S. dasyclados, and S. rehderiana*. These are all from the genus *Salix*, subgenus *Caprisalix* and section *Viminale* and therefore very close genetically. *S. dasyclados* and *S. burjatica* are the closest genetically and almost indistinguishable (Trybush et al., 2004). It appears that the presence of *S. dasyclados, S. burjatica* and *S. rehderiana* as one of the parents of the hybrid, resulted in poorer RSBP than hybrids without one of these.

The pedigree of the five hybrids is presented in Table 4-1.

Table 4-1. List of five high yielding hybrid used in the Silsoe lysimeter trial. The commercial names of the varieties, the parents' pedigrees, regions of origin are indicated when possible; Silsoe, Bedfordshire; 2003

LARS Variety code	**Name Sex**	**Parent pedigree *Salix***		**Parent Origin**	
		Female	**Male**	**Female**	**Male**
127/02	Tora F	*viminalis*	*schwerinii*	Russia/Sweden	Russia/Sweden
034/10	Ashton Stott F	*viminalis*	*burjatica*	UK/Denmark	UK/Denmark
LA980414	Resolution F	*viminalis* x *schwerinii* x *viminalis* x (*viminalis* x *schwerinii)* LA960231 SW900812	*viminalis* LA960231 'Quest'	Russia/Sweden/ Finland	Russia/Sweden
LA980442	Endurance F	*rehderiana* 102/01	*dasyclados* 77056	Asia	Denmark
LA980289	F	125/01svs (*schwerinii* x *viminalis* x smith) V7535	*viminalis* 115/65 'Jorr'	Finland	Sweden

M: male; F: female

These hybrids were additionally segregated using a k-means cluster analysis from a matrix of standardized means of records of few physiological traits[9] which suggests that the five selected hybrids were physiologically different.

4.2.2. Lysimeter research trial

It was preferred to set up a lysimeter trial because it facilitates the measurement of WU and morpho-physiological assessment in comparison to field trials.

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[9] See Chapter 3 for more details.

Experiment design

In spring 2003, a lysimeter experiment was set up. Five varieties were selected from the analysis of the results gathered from the 2002 research trials (see section above). The availability of space and equipment determined the trial set up. The trial aimed to study the WU and WUE of five *Salix* hybrids in two irrigation regimes. The first treatment was called "Dry" and comprise several water stress cycles; the second was "Wet", where irrigation fully supplied the water demand of the plants.

The experiment was set up as a two-way factorial in a randomized block design with three replicates (Plate 7). The final design is shown in Appendix 4.

A total of 57 lysimeters were prepared comprising 26 guard row lysimeter set up to limit the clothesline effect (Allen et al., 1998), 30 treatment lysimeters and one bare soil lysimeter (lysimeter 31). The guard row lysimeters contained four year old, healthy willow plants var. "Jorr" that had been coppiced in February 2003.

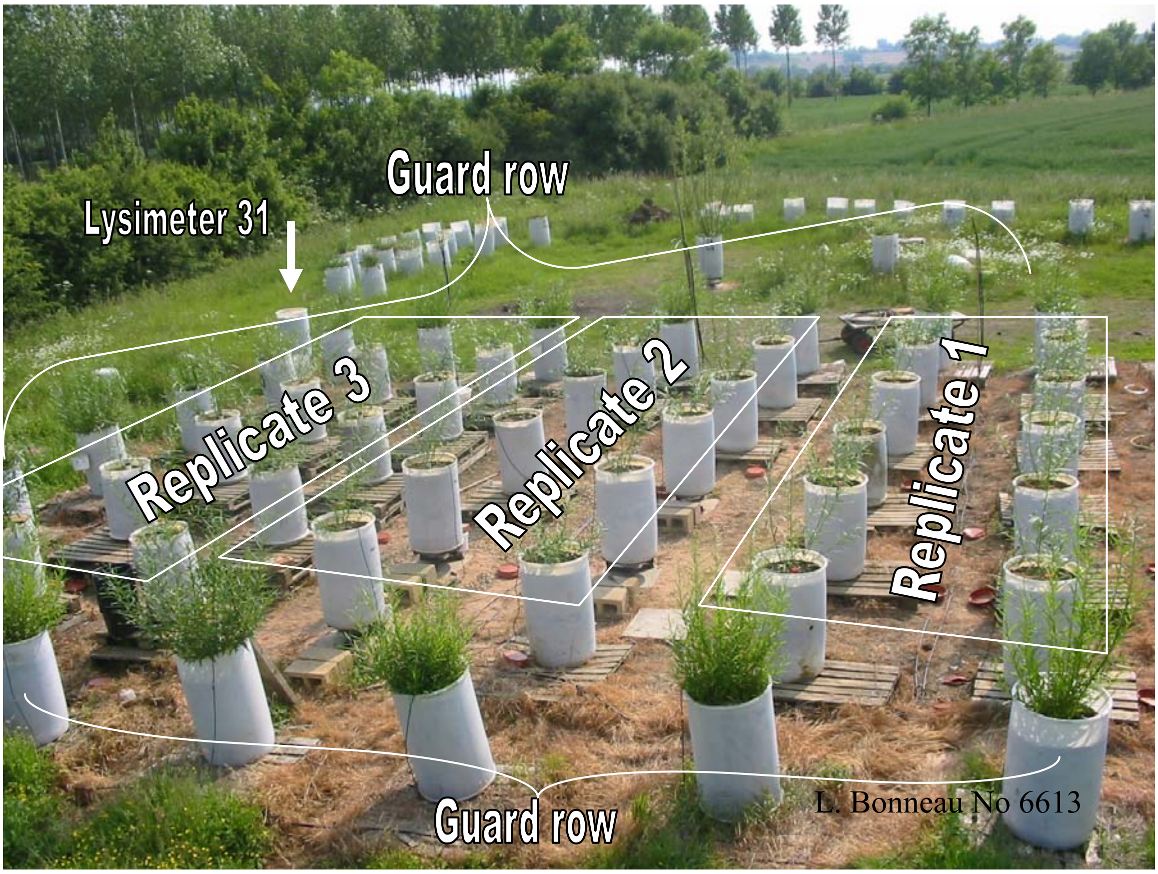

Plate 7. The lysimeter trial: with five willow SRC hybrids grown under two water regimes replicated three time, a guard row of 24 lysimeters and a bare soil lysimeter (lysimeter 31); Silsoe, Bedfordshire; June 2003

Experiment set-up

The lysimeters were 200 l polyethylene barrels. In January, the tops of the barrels were cut off. A 25 mm diameter hole was drilled at the base of each barrel and a tap was installed to allow control of the drainage. A 0.1 m layer gravel was poured into each lysimeter to allow good drainage and prevent upwards water movements by capillarity. They were filled with 0.7 m of sandy loam (Cottenham series, (King, 1969)), as previously used in the pots in 2002. The dry bulk density was 1.27 kg l^{-1}. The lysimeters were set up on palettes, transported to the site and were placed at two metres intervals. Figure 4-3 represents the typical design of lysimeters for the trial.

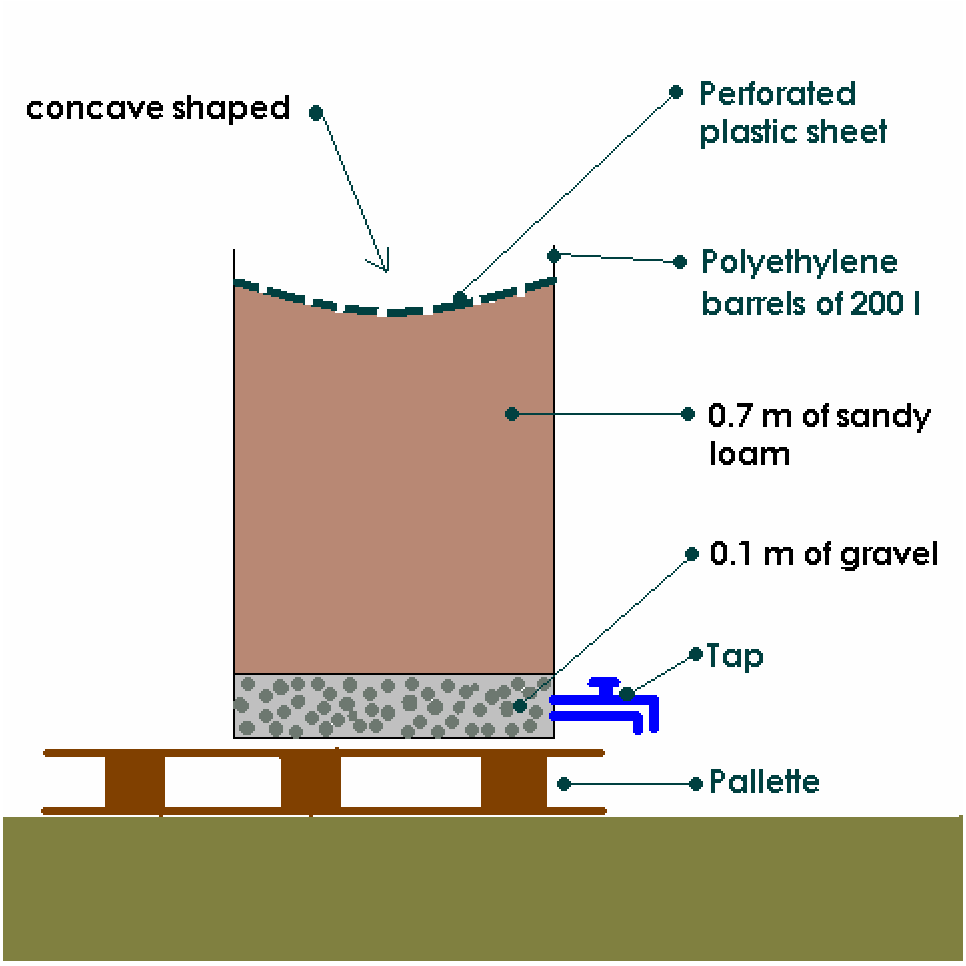

Figure 4-3. Schematic design of one lysimeter; Silsoe, Bedfordshire; 2003

The hybrids were transplanted from experiments run in the previous year. Some plants were replanted from the pot trial and others from the 2002 field trial. On 18/02/03, the plants from the pot research trial were replanted into the lysimeters in replicates I and II; keeping the soil and roots. On 19/02/03, the plants from the field trial were excavated with approximately 16 litres of soil and replanted in the lysimeters of replicate III.

The soil surface was made concave to minimize water infiltration down the sides of the lysimeter. An opaque plastic sheet was used to cover the soil for two reasons; firstly to prevent soil evaporation and secondly to prevent weed development. The plastic sheet was perforated to allow infiltration of water during irrigation and rainfall events.

Fertilisation

To prevent the plants suffering from nutrient deficiencies, all lysimeters were fertilised with 35.3 g of NPK 17%-17%-17%, for a total of 6 g N:P:K plant^{-1} on 27/05/03 and 12/08/03. At a typical density of 20,000 stools ha^{-1} this would be equivalent to 240 kg ha^{-1} year^{-1} of each element, four times the application rate used in the current plant breeding programme. Aronsson and Bergström, (2001) described a lysimeter grown willow experiment on a similar type of soil with fertilisation of 244 kg N ha^{-1} year^{-1} and observed nutrient leaching via the drained waters and concluded that willow coppice was able to absorb up to 190 kg N ha^{-1} year^{-1}; no nutrient toxicity was reported.

Water balance

The water use (WU) by individual plants was calculated as follows:

Equation 13

$$WU_{t1\rightarrow t2} = \Delta I + \Delta R - \Delta D - \Delta S$$

Where I is the irrigation (kg water), R is the rainfall (kg water), D is the drainage (kg water) S is the soil water storage (kg water) and $_{t1\rightarrow t2}$ is interval of time.
The minimum time interval to calculate WU with Equation 13 was the time between two irrigations or rainfall events and two records of drainage and/or two measurement of soil water storage.

Soil water storage

To monitor the water balance, an access tube of DIVINER® (Sentek Pty Ltd Adelaide, South Australia) was installed in each lysimeter. DIVINER is an hygrometric probe and daily measurement of soil moisture in each lysimeter were taken.

To monitor the water balance accurately over short time periods, six lysimeters were placed on load cells provided by Griffith Elder & Co Ltd (Bury St. Edmunds, Suffolk, Plate 8).

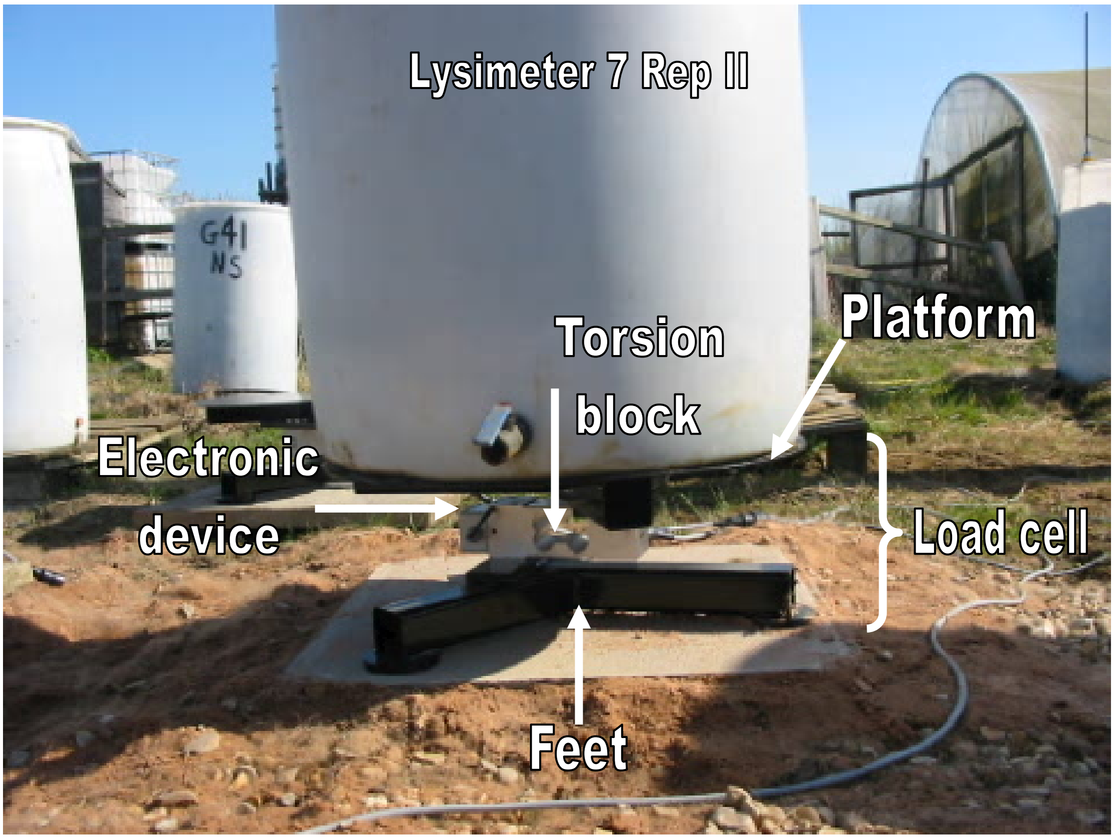

Plate 8. Lysimeter 7 of replicate II (Tora) mounted on a Griffith Elder & Co Ltd load cell, Silsoe, Bedfordshire; 2003

The load-cells were connected to each other and to a computer with DATAMAID® software installed to monitor the mass (max 500 kg ± 20g) of each lysimeter every 20 minutes. Technical problems during the summer prevented continuous data collection but records were obtained for the periods from 04/03/03 to 1/07/03 and 7/08/03 to 10/11/03.

All the dry treatment lysimeters in replicate II were placed on load cells. The sixth load cell was placed under an identical lysimeter (No.31) adjacent to the experiment (Plate 7) which was filled with soil but did not have a willow plant.

Irrigation

Under the Wet regime, the willows were irrigated by hand and the drainage collected daily if required from 25/04/03 when the willows exceeded 0.1 m on average; prior to this date the soil moisture was considered sufficient to supply the willow water demand. The irrigation was stopped on the 13/10/03 when the daily water use measured was negligible (<0.1 l d^{-1}). In case of a rainfall event the irrigation-drainage collection schedule was adjusted to maintain fully the water supply to the plants.

Under the Dry regime the same irrigation schedule as the Wet regime was applied during the establishment period from 25/04/03 until 10/06/03. The establishment period allowed the roots to explore the entire soil profile and reach a suitable size for physiological measurements of the leaves.

For the Dry regime, drying cycles and irrigation events took place alternatively. The irrigation events were required to keep the plants alive in order to carry out further physiological experiment on green leaves[10].

The first drying cycle (DC1) took place from 10/06/03 until 7/07/03 after which irrigation maintained soil moisture to the maximum upper drainable limit until the start of the second drying cycle (DC2) when irrigation was stopped completely from the 16/07/03 until 12/08/03. The third drying cycle (DC3) started immediately after the second. On 12/08/03 the plants were irrigated once with 10 l each, which was not enough to compensate for the soil water deficit. This cycle ended on 26/08/03. The fourth drying cycle (DC4) started immediately after the third but was run differently for each replicate. On 26/08/03 the lysimeters of the dry regime in replicate 1 were irrigated with 20 l, the plants of replicate 3 with 10 l and the plants of replicate 2 with 7 l spread over 5 days.

Not standardising the irrigation for each replicate led to a range of soil water deficit across the three replicates. This precaution was taken to increase the chance of reaching a desired combination of soil water deficit and plant physiological state when suitable weather was available to perform specific physiological measurements[11].

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[10] See Chapter 6.

[11] This was particularly applicable for the experiments run on leaf transpiration and chlorophyll fluorescence described in Chapter 6.

On 14/09/03 the plants were irrigated a final time back to the maximum upper drainable limit. No physiological assessments were made after that period and the plants were allowed to continue growing. This effectively imposed a further drying cycle until November but no further measurements were made because of the lack of suitable leaves.

Rainfall

Two weather stations (Skye Instruments, Ddole, Wales) recording rainfall, solar radiation, wind speed, temperature and air humidity were set up close to the lysimeter trial; the first 20 m to the North West of the experiment (weather station 20 was set to record at 20 minute intervals); and the second 60 m to the North East (weather station 60 was set to record hourly).

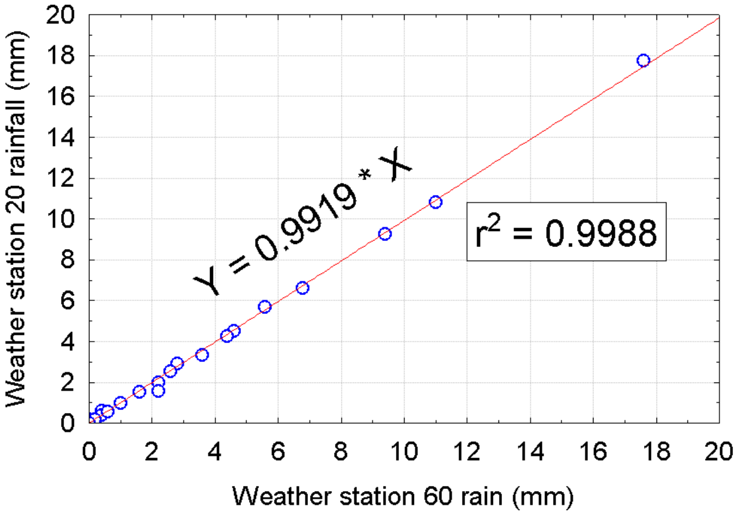

Figure 4-4. Correlation of records of rainfall recorded at Silsoe in May-June 2003 between two weather stations: Weather station 20 and weather station 60; Bedfordshire

Figure 4-4 shows that the records of rainfall were nearly identical in May-June 2003 between the two sites. Later in the summer, some technical difficulties were encountered with weather station 20 and data during July, August and September were not always available. Thus weather station 60 was used to sum the rainfall of the entire season.

The mass of lysimeter 31 was used to correlate the rainfall recorded by weather station 20 and the water caught in the lysimeters. Weather station 20 was chosen for the calibration because it logged data at the same interval as the load cells. Figure 4-5 illustrates this relationship.

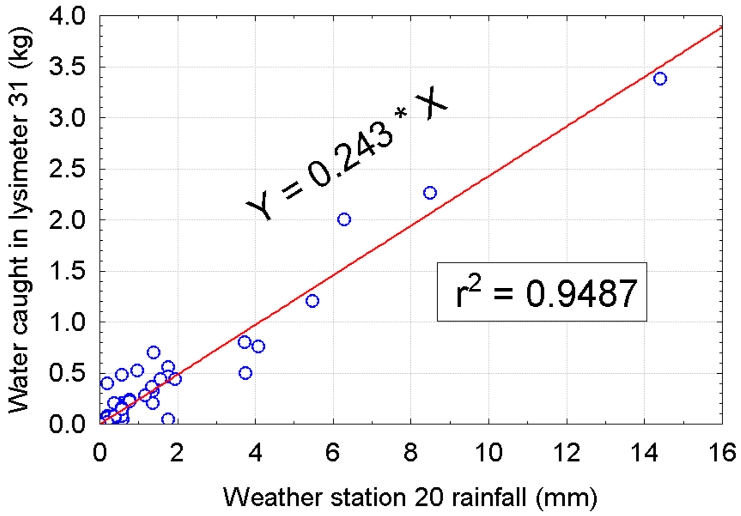

Figure 4-5. Correlation between the water caught in lysimeter 31 (bare soil) and weather station data during 52 rainfall events in 2003; Silsoe, Bedfordshire

There was a strong relationship between the two variables presented in Figure 4-5. The variations were probably due to evaporation that occurred during the rainfall event. An attempt to correct the data including the evaporation was made (result not presented). This was not conclusive as the amount of water lost via evaporation that occurred during the rainfall events was close to the limit of detection of the lysimeter; in addition, rainfall was often accompanied by windy conditions and the wind forces also altered the data above the limit of detection.

The development of the willow above the soil surface of the lysimeter might have influenced the amount of water caught during rainfall events. The combination, of variables influencing the amount of water caught in a lysimeter in which a plant was grown generated a complex model. For example the water intercepted by the foliage of willow stands has been reported to evaporate rapidly and not contribute effectively to the soil moisture (Stephens et al., 2001a); each of the 30 plants had different evolving morphologies; each rainfall was accompanied by a different wind and evaporative demand.

In order to quantify the difference in water caught during rainfall events between the lysimeter 31 and those onto which willow trees were grown, the data from the weighing lysimeters of replicate 2 were inspected. The data collected from the DIVINER were not accurate enough as some or all the water from the rainfall could be used between two assessments.

Over a period in which 113 mm of rain was recorded at weather station 60, the increase in mass recorded in lysimeter 31 was 27.8 kg compared to a maximum of 31.1 kg in lysimeter 17 (Tora). This difference represented < 2% of the total irrigation applied over the summer and therefore, for the purposes of the water balance calculations, all lysimeters were assumed to receive the same amount of water recorded in lysimeter 31 (Figure 4-5).

4.2.3. Stem biomass

Stem biomass (SB)

SB from the lysimeters was harvested in January 2004 and the total wood was oven dried for 48 h at 105 °C. Estimations of SB achieved at different times in the season were calculated at the end of the season by allometry.

During the growing season on 32 occasions at regular intervals, the stem length of the highest stem of each willow plant (L_{max}) was measured from the stool. At the end of the season at harvest, L_{max} and the fresh weight of each willow stem were recorded to determine the relationship between SB and L_{max} (Equation 14).

Equation 14

$$SB = f(L_{\max})$$

If a mathematical relation exists with only those two components, it should be possible to estimate SB at a particular time in the season with L_{max} records. For the next step, all the stems of a particular plant were considered to elongate with the same proportion as the largest one. This can be used to model biomass accumulation over time related to L_{max} at harvest (Equation 15)

Equation 15

$$SB_{t1} = \left(\frac{L_{\max - t1}}{L_{\max - harvest}} \right) \times SB_{harvest}$$

Where SB_{t1} is the stem biomass on t1, L_{max-t1} is the stem length of the highest shoot on t1; $L_{max-harvest}$ is the stem length at harvest and $SB_{harvest}$ is the stem biomass at harvest. The influence of the water regime and genotype on the relationship between stem biomass and length had to be checked for consistency across the range. In addition to the morphology, the water content of the fresh stems and the wood density were assessed to check if the same volumes of fresh wood for a particular hybrid grown in two water regimes resulted in the same dry weight, and alternatively if each clone was characterised by a different model.

4.2.4. Water use efficiency (WUE)

Boyer (1996), Jørgensen and Schelde (2001) and others defined WUE as the total dry matter produced D by plants per unit of water used (WU) (Equation 16).

Equation 16

$$WUE = \frac{D}{WU}$$

The calculation of WUE requires first the calculation of WU, which was carried out as Equation 13 and secondly the calculation or estimation of the biomass. This biomass can be total or partial which leads to a variety of expressions of WUE (Liu et al., 2002). Total biomass, including root system, stool, stem, branches and leaves, requires destructive measurement and is therefore unsuitable for intermediate observations during lysimeter experiments. Non-destructive methods can be used, such as: stem biomass estimation by allometry (Broad, 1998); leaf biomass via an estimation of the leaf area and the measurement of specific area; and root biomass by root development observation (Mackie-Dawson and Atkinson, 1991). Nevertheless, the ultimate method is destructive and almost all models of estimation should be validated by destructive methods when possible.

In this chapter, only the stem biomass (SB) was considered because willow coppice is harvested in winter when no leaves remain on the plants. As such the harvestable biomass was composed of the stems and the Type B branches[12].

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[12] Few Type A branches remained at harvest, but those were discarded, as there is no or little chance that they would be collected using the harvest method of commercial plantation.

4.2.5. Statistical analysis

Factorial analyses of variance (ANOVA) at $p \leq 0.05$ were used to compare the WU, stem biomass production and the WUE of the hybrids and water regimes in different combinations using STATISTICA (Statsoft) software. The 95% confidence intervals (CI) were extracted from the ANOVA and used to compare hybrids, treatments and combinations in pairs. All the tables, results, graphs and analyses are on the appended CD.

4.3. Results

4.3.1. Seasonal and monthly water use

The water used in each lysimeter was calculated and are presented in Figure 4-6 while Table 4-2 summarise the seasonal water use. During the growing season from 24/04/03 till 13/10/03 185.2 mm of rain was recorded, equivalent to 45.0 l of water caught in each lysimeter.

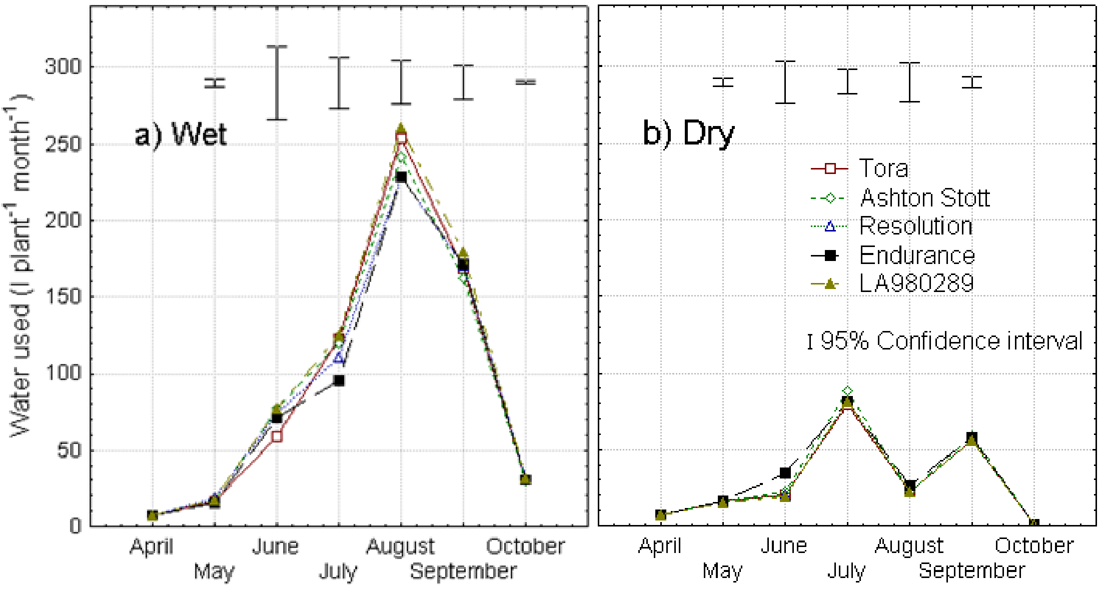

Figure 4-6. Monthly water use of five hybrids grown in lysimeters under two water regimes a) wet; b) dry; from April to October in 2003; Silsoe, Bedfordshire. The error bars represent 95% confidence intervals (n=3)

Table 4-2. Mean total water used (WU; l) by five Salix hybrids, grown in lysimeters between 18/02/03 and 1/11/03 under two irrigation regimes (dry and wet); Silsoe, Bedfordshire. CI indicates the 95% confidence interval. n=3

Water regime	Tora	Ashton Stott	Resolution	Endurance	LA980289	CI
Wet	656.7	653.4	639.6	619.8	696.7	91.22
Dry	202.6	215.6	204.6	223.3	202.0	49.96
Factorial						68.86

During April and May the willow plants were allowed to establish and no differences were observed between the two water regimes and the five hybrids.

From June to October, there were significant differences in WU between the two water regimes across all hybrids in every month except for Endurance in July.

In June, the first drying cycle was imposed on the plants in the dry treatment. During this period a significantly higher consumption was observed by Endurance than by the other hybrids except Ashton Stott; this occurred before the irrigation ceased at a point when the other hybrids had very similar WU.

In contrast, under the Wet regime Endurance had similar water consumption to the other hybrids except Tora. Tora had the lowest WU in June under the Wet regime; but this was not significant difference.

In July, under the Dry regime the WU was very similar among the five hybrids, although a difference of 9 l was observed between Ashton Stott and Tora, which had the highest and lowest WU respectively; this was a significant difference. Under the Wet regime the difference reached 29.3 l between LA980289 and Endurance, which had the highest and lowest WU respectively, but this difference was not significant.

In August, the dry regime plants received very little water. As a result the WU figures were almost identical across the five hybrids. Meanwhile, under the wet regime, the differences in WU increased between Endurance and Resolution on the one hand and Ashton Stott, Tora and LA980289 on the other. The difference in WU reached to

32.9 l between Endurance and LA980289 and was the only significant difference. In August, the ranking of WU was the same as in July, in increasing order: Endurance, Resolution, Ashton Stott, Tora and LA980289.

In September for both regimes there were no significant differences in WU.

Finally in October under the dry regime, the plants were damaged by the water stress imposed during the summer and barely used any water. However, under the wet regime, even though the WU were very small in comparison to the summer months, the WU of Ashton Stott was significantly smaller than that of LA980289 and Resolution.

Over the entire season (Table 4-2), the differences in WU between the two water regimes were significant. Under the dry regime, the differences between the WU of the hybrids were not significant. Nevertheless, Ashton Stott and Endurance consumed slightly more water than Resolution, Tora and LA980289. Under the Wet regime, no significant differences were measured. But the difference between the WU of Endurance and LA980289, the lowest and higher water consumers, reached 76.9 l. In order, LA980289 was the highest consumer of water over the growing period, followed by Tora and Ashton Stott, Resolution and Endurance.

4.3.2. Monthly and seasonal water use efficiency

Stem biomass (SB)

The mean stem biomass is presented in Table 4-3.

Table 4-3. Average stem biomass (kg plant^{-1}) of five Salix hybrids grown in lysimeter under two water regimes (Dry and Wet); 95% confidence interval (CI); n=3; Silsoe, Bedfordshire; 2003

Water regime	Tora	Ashton Stott	Resolution	Endurance	LA980289	CI
Wet	1.378	1.107	1.667	1.189	1.676	0.304
Dry	0.508	0.425	0.572	0.444	0.439	0.174
Factorial						0.228

In all varieties, SB was significantly lower in the Dry regime than in the wet (Table 4-3).

In the Dry treatment there were no significant differences between varieties. In the Wet treatment LA980289 and Resolution had significantly greater SB than Ashton Stott and Endurance.

The next step was the verification of the relationship between SB and $L_{max\text{-}harvest}$ (Figure 4-7). The water regimes had a non-significant impact on the water content of the wood but a significant impact on the wood density (result not presented here). In addition, the water contents and wood densities were significantly different between the hybrids at $p \leq 0.001$. Hence, each hybrid-water regime combination was characterised by a different model.

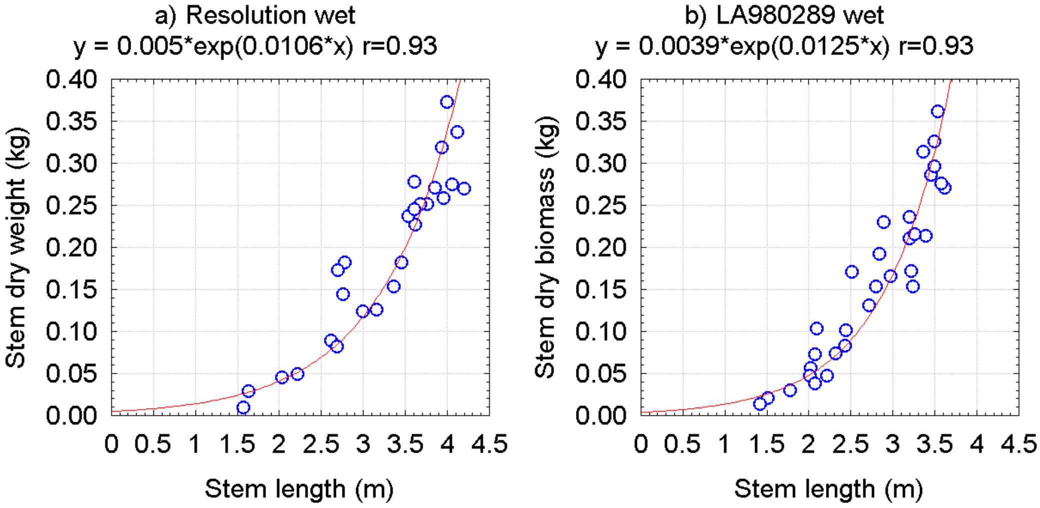

Figure 4-7. Two stem biomass prediction models (Stem biomass = f(Stem length) for two hybrids on a) Resolution and on b) LA980289 under the wet regime. Lysimeter trial; Silsoe, Bedfordshire; 2003

The models SB =f($L_{max\text{-}harvest}$) some of which are presented in Figure 4-7, were acceptable with correlation coefficients, r, between 0.68 and 0.93 for all hybrid-water regime combinations. From these it was possible to move to the next step and estimate SB accumulated during seven months from the records of stem length. The estimations calculated as described by Equation 15 and are presented in Figure 4-8.

As expected the sum of SB predicted (Figure 4-8) over the seven months and SB harvested (Table 4-3) were very close (Biomass harvested = 0.001+1.0069* biomass predicted; r^2=1); furthermore the confidence intervals were very similar.

In April and May, the method showed the first limits of use. In April and May no differences in water regime were imposed on the lysimeters, as a result no significant differences in L_{max} between the clones of the same hybrids were recorded. Because the methods described above (Equation 15) involve SB at harvest and SB was affected by water stress; the results of the establishment period wrongly reflect the effect of water stress. As a result the values obtained in April and May, are not reliable and therefore, not considered further.

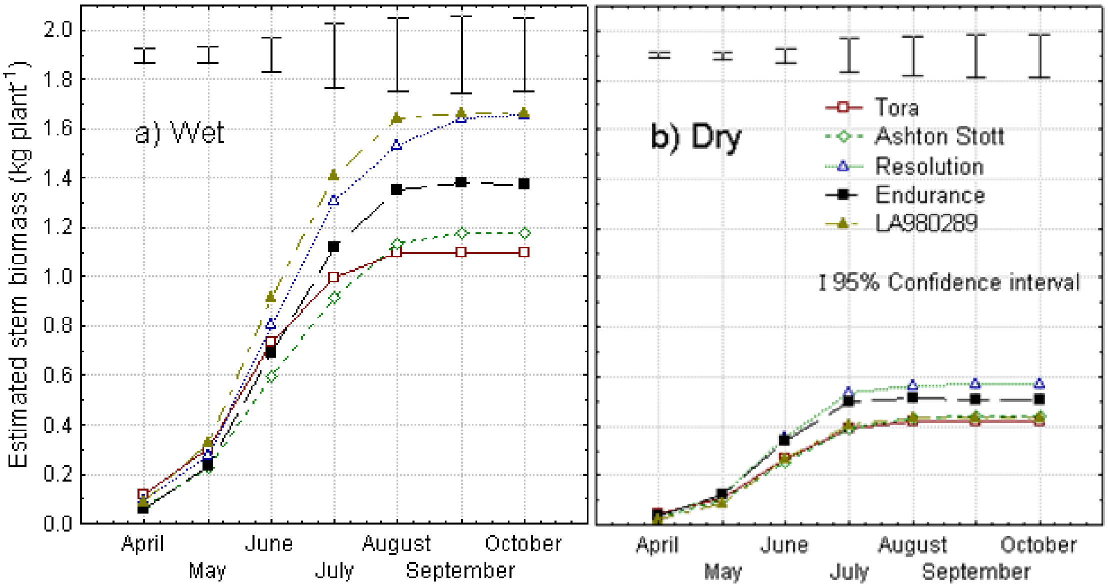

Figure 4-8. Estimated oven dry biomass accumulation of five hybrids grown in lysimeters under two water regimes a) wet; b) dry; from April to October in 2003; Silsoe, Bedfordshire. The error bars represent 95% confidence intervals (n=3)

In the wet regime from June to October, hybrids showed different trends of biomass accumulation.

June was the month during which SB appeared to accumulate the most for all five hybrids. Under the wet regime LA980289 was the hybrid that accumulated the most biomass, while under the dry regime Resolution was the most productive. Endurance produced the least SB under both water regimes.

In July under both water regimes Resolution was the most productive and Ashton Stott was the least. Resolution and LA980289 accumulated almost twice as much SB as Ashton Stott.

After July under the wet regime, Tora was the first to slow down and was closely followed by Endurance and LA980289. Ashton and Resolution maintained biomass accumulation for a longer period of time in comparison to the others.

After July under the dry regime, no significant biomass accumulation took place for any of the five hybrids.

Basal stem diameter has been shown to correlate with SB in numerous papers e.g. (Souch and Stephens, 1997; Robinson et al., 2004) but not enough records were available at Silsoe as only four assessments were performed during the season; the basal stem diameters appeared to have a linear relationship with L_{max} records (Results not presented).

Stem biomass water use efficiency (WUE_{stem})

The data collected were suspected to be not representative of the SB accumulation process over a season of growth. Finally, it was decided not to estimate WUE_{stem} during individual months as these would not have been representative.
The results of the seasonal WUE_{stem} were presented in Table 4-4.

Table 4-4. Mean seasonal stem biomass water use efficiency (WUE_{stem}) of five Salix hybrids grown under two water regimes in lysimeter; Silsoe, Bedfordshire; 2003. CI are the 95% confidence intervals CI; n=3

Water regimes	Tora	Ashton Stott	Resolution	Endurance	LA980289	CI
Wet	2.09	1.68	2.59	1.89	2.39	0.34
Dry	2.48	1.95	2.79	1.99	2.15	0.80
Factorial						0.58

Over the whole season the WUE were not significantly different between the two water regimes for all five hybrids. Nevertheless, a slightly higher WUE was observed for the Dry regime in comparison to the Wet one apart for LA980289.

In the Dry treatment, Resolution had the largest WUE, significantly greater than Ashton Stott and Endurance. Similarly, in the Wet regime, Resolution had a significantly higher WUE than Ashton Stott, Endurance and Tora. LA980289 was the second best and had a significantly higher WUE than Ashton Stott and Endurance. The differences between Tora and Ashton Stott were also significantly different.

4.4. Discussion

Silsoe is one of the warmest and driest areas in England and the experiment was conducted during one of the hottest summers on record, with less than 200 mm of rainfall between the middle of April and the middle of October compared with a 300 mm long term average for this period.

The rain caught in the lysimeters represents about 20% and 7% of the water supplied to the crop under the Dry and Wet treatments respectively. The rarity of rainfall events was definitely an advantage for this type of experiment as the irrigation represented most of the supply.

High temperatures have been reported to be stressful for numerous crops and to affect their productivity for example for rice and wheat (Lal et al., 1998). At Silsoe, the mean maximum temperatures in 2003 for August and September were more than 2.5 °C higher than the average for 1970 to 1995; there was a peak of 35.8 °C on 10/08/03 and five days when temperatures went above 30 ^{o}C in August. This may have influenced the physiology of the hybrids, their WU, biomass accumulation and consequently WUE_{stem}. In addition, the soil temperature in the lysimeters was likely to be greater than in the field: unfortunately soil temperature was not recorded. Summer temperatures for this region of Europe are predicted to increase as a result of global warming (Green, 2000). Hence, the results of this study may be relevant for the future.

The experiments in the previous season had suggested that LA980289 was the most drought tolerant hybrid and should therefore have produced the most biomass under the Dry regime. A microbial infection on the stems of this variety, however, led to a stem canker developing at the beginning of July 2003. As a result stem development was affected especially for the plants grown under the dry regime. Curiously, the canker was not observed in the first year of the project and did not develop in plants grown with no water stress. This canker was also reported on LA980289 and its progeny at Rothamsted Research, Harpenden, Hertfordshire, in the east of England in a non-irrigated field trial in 2003 (Steve Hanley personal communication, 2004). It was not observed during the summer 2004 but an identification of the canker should be programmed for the summer 2005.

In the wet treatment, Endurance performed relatively less well than it had in the first year of the field experiment as it produced significantly less stem biomass than Resolution and LA980289. There was no obvious reason for this reduction in relative performance, but root zone heat stress might have caused this change in ranking.

To quantify the impact of water stress in the trials 2002, the relative stem biomass production (RSBP) of the hybrids grown under the two water regimes was calculated to be used as an indicator of water shortage tolerance of the *Salix* hybrids. Using the data from the field trial 2002 presented in the first chapter (Appendix 3), it was possible to calculate a new RSBP from the lysimeter trial under both water regimes. As a result the ranking remained almost the same (Table 4-5).

Table 4-5. Relative Stem biomass production between the lysimeter trial 2003 (L03) and the field trial 2002 (F02) and rank for 5* Salix *hybrids; Silsoe, Bedfordshire

Water regime	Tora	Ashton Stott	Resolution	Endurance	LA980289
Wet (rank)	1198% (2)	779% (4)	891% (3)	550% (5)	1206% (1)
Dry (rank)	441% (1)	299% (4)	300% (3)	183% (5)	315% (2)

The stem biomass production from the five willow hybrids grown in both wet and dry lysimeters exceeded the biomass production of first year plants grown under irrigation in the field experiment by a factor of between 2 (Endurance – dry) and 12 (LA980289

– wet; Table 4-5). This is likely to be primarily related to the age of the plants and the closer spacing of the plants grown in the field. Despite the slight differences in ranking of RSBP observed in the pot and lysimeter trial, RSBP suggests that even the wet regime generated similar stresses as the one imposed in pot trial in 2002.

Table 4-6 shows RSBP as the ratio SB under dry regime to SB under wet regime.

Table 4-6. Relative Stem biomass production between the dry and the wet regimes for 5* Salix *hybrids. Lysimeter trial; Silsoe; Bedfordshire.

Tora	Ashton Stott	Resolution	Endurance	LA980289
36.9%	38.4%	34.3%	37.3%	26.2%

Curiously, the RSBP are very similar among the hybrids apart for LA980289 which suffered from a microbial infection. Significant differences in stem biomass production between the hybrids only occurred in the wet regime. Under the repeated water stress cycles imposed in the dry regime the differences were not significant in seasonal stem biomass production. This could suggest that the hybrids were restricted in different proportion when grown in dry conditions in comparison to the wet one but this was not the case. However, the only difference between the wet and dry regimes was the amount of water applied. Water stress should therefore have been the primary treatment difference but no difference of behaviour was observed among the hybrid which ranged RSBP calculated in 2002. Excluding LA980289, SB rankings are identical between hybrids in both water regimes and this is the reason for the hybrids to have very similar RSBP. Water use in each treatment was generally consistent between the varieties across the season with significant differences only occurring during August and October in the Wet regime. Seasonal water use is therefore more likely to be related to water supply coupled with climatic demand rather than the genotype. As a result differences of WUE_{stem} were observed between hybrids but water regime appeared not to affect the values which indicates that WUE_{stem} is specific to genotype with hybrids related to *S. burjatica* and *S dasyclados* (Ashton Stott and Endurance) having lower WUE_{stem} than those related to *S. viminalis* and *S. schwerinii* (Tora, Resolution and Endurance).

Growing plants in lysimeters imposes a range of stresses such as water shortages or excesses, nutrient shortages, air and soil heat and the restriction to the root expansion. Where the root system is constricted by the container, stressful growing conditions can sometimes result in a “bonsai effect” (Lesniewicz, 1985). Hormones are released by roots to signal to the rest of the plant about stresses, these might be linked to water, nutrient and mechanical attributes (Aiken and Smucker, 1996). This partially explains differences in ranking between a clone grown an irrigated field and an irrigated lysimeter amongst other varieties. The root system as it extended has encountered an impenetrable polyethylene layer of the lysimeter and may have signalled a stress and consequently plant growth was reduced. The ability of hormone signalling is linked with genes (Jackson, 1997) and this might be expressed to a different extent between the hybrids explaining the difference in ranking between the two trials. As such the hybrids related to *S. burjatica* and *S. dasyclados* appeared to have their biomass production much more affected by mechanical constraints due to the narrow shape of the lysimeter than those related to *S. viminalis* and *S. schwerinii*. Thus in 2002 the high temperature and the smaller pots might have had a greater influence on the growth of the varieties than water stress alone resulting in a RSBP in 2002 being very different to that in 2003.

Allometric relationships to estimate biomass are commonly used in forestry where destructive sampling is avoided. For example, allometry was used to estimate a regular interval the stem biomass over three year period in a similar size lysimeter trial (Martin and Stephens, in press-c).

The estimation of SB during the season using allometry related to maximum stem length (L_{max}) was subject to four sources of error.

Firstly, the relationship L_{max} : SB at harvest was subject to error due to branches, the orientation and the position of the stem on the stool, which led to varied stem morphology on the same stool.

Secondly, the relationship between biomass accumulation and harvested SB was affected by the repeated drought treatments which led to the death of the stem apex on some occasions. Following irrigation, several Type B branches developed and the ratio L_{max} : SB decreased. This may have affected the estimation of the biomass early in the season.

Thirdly, the models of SB production assumed that the stem wood densities were consistent throughout the season. Other allometric studies on willow coppice have been reported and similar assumptions made (Robinson et al., 2004; Martin and Stephens, in press-c). These assumptions were not valid especially at the beginning of the season on the first year of growth when the stem tissues would have been less dense.

Finally, the biomass estimation was calculated from the record of L_{max} of only one stem per stool, so the fractions of biomass described in Figure 4-8 were indicative only.

Over the season, WUE_{stem}, calculated on a monthly basis from allometric estimates of stem biomass decreased from about 8 g kg^{-1} to less than zero. Assuming that the photosynthesis and transpiration are closely related this indicates that partitioning of dry matter to the stems decreased progressively over the season. However the methods assumed that the stem density was consistent throughout the season while it should have increased as the lignin proportion increased in the stem tissue. This assumption generated an overestimation of SB in the early months of the growing season and underestimated the biomass accumulation later in the season. An experiment on change in wood density over the season should have been set up to enable the production to be modelled including this variable.

Evaporation from the cuticles of leaves was reported to have a significant impact on the total WU of plants (Allen et al., 1994). In willow coppice, as the stems elongate, the canopy increases and reaches a moment when some leaves start to age and become less efficient in terms of energy conversion while physical evaporation from these leaves is maintained. This process of premature senescence is a well known characteristic of willow coppice (Ross and Ross, 1998; Proe et al., 2002).

In the lysimeter trial, before these less efficient leaves senesced totally and dropped, the water used to conserve leaf tissue moisture could have had an impact on WUE_{stem}.

As a result for *Salix* hybrids, an aging leaf population became less water use efficient during a season.

Transpiration from leaves is governed by leaf temperature, the water deficit of the air and the boundary layer resistance to water vapour transfer to the bulk atmosphere. In the lysimeter trial, the plants were far apart in comparison to commercial stands, as such their coupling with the atmosphere was greater. Jarvis (1985) suggested that transpiration from crops poorly coupled with the atmosphere is likely to depend strongly on radiation receipt and to be rather insensitive to changes in stomatal conductance. Well-exposed, tall crops are generally well-coupled to the atmosphere; for these crops transpiration is likely to respond sensitively to small changes in stomatal conductance. As such stomatal conductance of those plants might be a good indication of the water saving mechanism ability of each hybrid.

The differences in WUE_{stem} between the hybrids were the focus of this study. Over the whole season WUE_{stem} of individual hybrids varied little with water regime suggesting that WUE_{stem} is independent of water stress in *Salix* hybrids. Similar observations were reported by Weih and Nordh (2002) who demonstrated that WUE might be intrinsic to varieties. On an experiment on soil type and nutrient availability, it was shown that for *S. viminalis,* Jorr grown in the same soil type as in the Silsoe lysimeter trial 2003 WUE_{stem} ranged between 2.33 and 3.37 $g\,kg^{-1}$ which is comparable to those observed for Resolution (Martin and Stephens, in press-a). However in their experiment they showed that soil density, water and nutrient shortage decreased WUE_{stem} value, primarily because soil evaporation was a larger component of WU when plants were small.

At Silsoe in 2003 significant differences of WUE_{stem} were observed between hybrids. For example, Resolution was the most efficient of the subset, using 8% less water to achieve the same stem biomass as LA980289 in the wet treatment and, where water was limited in the dry treatment, producing 23% more stem biomass from the same volume of water. The contrast is even more marked for the varieties with lower WUE_{stem}.

To summarise, it appears that the hybrids transpired a very similar amount of water which indicates that the cultivation of any high yielding willow SRC would result in the same water use. As a result water saving in willow SRC cultivation is unlikely to progress through willow breeding. However, productivity can be increased in areas where water is limiting as the hybrids characterised with the highest WUE_{stem} achieved the highest yield in both water regimes.

The observations made in the last three chapters are not sufficient to explain the differences between the hybrids and the method used sometime reach their limit and more information is needed. RSBP calculated in 2002 might not indicate drought resistance abilities but other mechanisms. The root system has to be quantified as root signalling may have contributed to a reduction in biomass and consequently altered the ranking observed in the lysimeter in comparison to the irrigated field. The harvest of the total biomass would allow to calculate WUE from the total biomass accumulated (WUE_{total}) and to evaluate the differences in biomass partitioning.

The quantification of the green and yellow leaves populations might contribute to the understanding on the efficiency of water saving mechanism of some hybrids. Finally, as the plants were very well coupled with the atmosphere, an investigation on the stomatal conductance (g_s) which must have had a critical role in the quantity of water transpired, might provide information on efficiency of the water saving mechanism at the individual leaf level.

CHAPTER 5. Leaf population, leaf area and biomass partitioning of five *Salix* hybrids grown in lysimeters under two water regimes

5.1. Introduction

A lysimeter trial on five *Salix* hybrids set up in 2003, showed that seasonal water use[13] (WU) was a function of water supply, conditions of growth which include the weather and the soil but that the willow hybrids grown had little influence. Stem biomass accumulation is driven by the climatic conditions i.e. solar radiation and evaporative demand and varietal characteristics (Cannell et al., 1988). However, seasonal harvestable biomass water use efficiency (WUE_{stem}), defined as the ratio of harvestable biomass to WU, appeared to be conservative within hybrids independently of the water regimes but varied significantly between hybrids. Other environmental factors influencing growth did not have a significant influence on WUE_{stem}. Similar conclusions were reported elsewhere (e.g. Tanner and Sinclair, 1983; Lindroth and Båth, 1999).

The studies presented in the previous chapters focused on the morphology of the hybrids and the leaves in order to associate particular features with potential drought resistance and high stem biomass. Some morpho-physiological features of *Salix* hybrids such as leaf hair, leaf shape and adaxial epidermial cell size were correlated with relative stem biomass production (RSBP) as calculated in the Silsoe 2002 trials. These features are inborn or the long term characteristics of drought resistance processes (Jones, 1992). A wide range of combinations of these features was observed in *Salix* hybrids which suggests that the plant's relationship with its environment (i.e. WUE) can be modified by breeding.

The WU and WUE_{stem} were calculated for five high yielding hybrids with a range of RSBP. It appeared that the plants with high RSBP had the higher WUE_{stem} and vice

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[13] The seasonal WU is the total mass of water evapotranspired from a cropping system during a season of growth, usually from March to November for willow SRC.

versa; although RSBP may be affected by factors such as heat stress and constriction of root growth. Only five hybrids were grown at Silsoe in 2003, the lysimeter trial was an appropriate setting to carry out physiological studies in more detail than in the 2002 trials. Information on the long term ability of willow species to cope with drought was gathered earlier but did not permit classification of high yielding hybrids according to their sensitivity to drought. However, they all appeared to be able to respond to water shortage. For example the hybrids were able to reduce WU without this leading to their death while WU could increase if the plant regained access to water. Consequently the short term ability of plants to achieve better yield in drought conditions or to show water use high efficiency needs to be studied.

Plants use water for several purposes including: maintaining the water balance of living tissues; the transport of nutrients and metabolites through the plant vessels; and within photosynthetic processes (Cutter, 1978). The roots and leaves are the interfaces between plant and environment, where active water uptake, transpiration and hormonal signalling take place (Slatyer, 1967; Jackson, 1997). Specific aspects of those interfaces that define plants' ability to control water movement have been studied widely in many genera. For example, Cameron et al. (2002) studied cuticular waxes of three *Salix* species. To date the studies of drought resistance or water saving mechanisms in *Salix* sp. have reported on the differences between wild species or individuals which differ widely in yield potential.

Drought also induces the plant morphology to be modified. Jones (1992) indicates for example that the root/shoot ratio increases with water stress because the roots are less sensitive to drought than aerial parts of the plants like the leaves. This suggests that a relative increase of the root/shoot ratio might be used as an indicator of drought resistance.

The leaves are specialized apparatus that governs biomass accumulation via photosynthesis (Lawlor, 1987). Thus the leaf and root populations must be closely linked with WUE. There have been numerous indoor and outdoor experiments on canopy and root development on a large spectrum of plants, including *Salix* (e.g. Choisel, 1997; Liu et al., 2001; Proe et al., 2002). Experiments on evapotranspiration have sometimes been combined with studies on root and leaf area, for example, on *Populus davidiana* (Zhang et al., 2004).

Comparative studies of willow are rare and mainly focus on *Salix* species which are very different to each other and not high biomass producers. The characteristics linked with drought resistance and WUE found in the genus *Salix* and other genera have not been studied for a range of high yielding willow hybrids. Theoretically, the differences in WUE between hybrids might be linked with different management of roots and leaf area, which govern WU. In this chapter the objectives were for five high yielding *Salix* hybrids grown in lysimeters:

1. determine how leaf area responds to water stress;
2. estimate the root biomass and root/shoot ratios and how they are affected by drought; and
3. examine to what extent these leaf areas and biomass partitioning are linked with variations in WUE.

5.2. Material and methods

5.2.1. Experimental layout

The lysimeter trial in 2003 was a two factorial randomised block design with three replicates. The factorial treatments were five *Salix* hybrids grown under two water regimes. The hybrids selected for this experiment, on the basis of their performance in the large scale variety experiments[14] in the previous year, were: Tora; Ashton Stott; Endurance; Resolution and LA980289.

Half the lysimeters were subjected to four drying episodes where irrigation was withdrawn whilst the other half were irrigated daily (except for a period between 18 and 22 July 2003). The plants were harvested in November 2003 to determine biomass production.

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[14] For more details on the origin, physiology and WUE_{stem} of the hybrids refer to Chapters 2, 3 and 4 respectively. For more details on the selection of the subset of hybrid varieties and the lysimeter trial set up refer to Chapter 4.

5.2.2. Leaf area

Plant leaf area (A_{plant}; m^2) and canopy leaf area are commonly monitored in plant physiological studies to quantify solar radiation interception and to relate to evapotranspiration. A wide range of tools is available to measure canopy leaf area, mostly by measuring light intensity above and below the canopy. This gives a direct measurement of light intensity from which the leaf area index (Jones, 1992) can be calculated based on assumptions about the canopy architecture. Unfortunately, these methods are not suitable for use with individual plants (Potter et al., 1996).

Alternatively, A_{plant} can be measured by integrating the leaf area of individual leaves or estimated using allometric methods (Chen et al., 1994). In willow SRC, A_{plant} has been estimated by relating leaf area to stem basal area (Martin et al., 2002) but this method is unreliable if the leaf area per stem fluctuates suddenly through time. Another method which combines the measurement of basal stem diameter of a minimum of six stems per stool and the measurement of the length and width of every twentieth leaf has been reported (Brierley et al., 2001) but the coefficients were specific to “Jorr”, a hybrid not included in this experiment.

A first approximation of leaf area can be obtained, by recording the length of stem where leaves are. On the tallest stem of each plant, the total stem length (L_{max}; m) and the length of stem bearing leaves (L_{total}; m) was recorded on 33 occasions from 19/03/03 until 3/11/03 at regular intervals. In addition, the length of stem bearing green leaves (L_{green}; m) and yellow leaves (L_{yellow}; m) was recorded at the same time. Leaves in which senescence had begun (i.e. were starting to turn yellow) were included in L_{yellow}. Consequently all the measurements were related using the following equations:

Equation 17 $$L_{bare} = L_{\max} - L_{total}$$

With L_{bare} the length of stem where no leaves were present, and:

Equation 18 $$L_{total} = L_{green} + L_{yellow}$$

Eversham (personal communication, 2002) suggested that willows can be characterised by the ratio of leaf length (L_{leaf}; mm) to width (W_{leaf}; mm). The relationship between L and W should be specific to each hybrid and consequently A_{leaf} can be determined by measuring L_{leaf}.

To measure A_{leaf} accurately, a destructive method using a leaf scanner can be used but this is not desirable in experiments on single plants. Destructive methods were avoided whenever possible in the trial. However, to define accurately the relationship between L, W and A_{leaf}, 15 to 20 fully developed and undamaged leaves per hybrid were sampled from the lysimeter trial and scanned in the laboratory using a leaf scanner (Delta-T Devices, Burwell, UK) to measure A_{leaf}, L_{leaf} and W_{leaf}.

The measurement of L was more accurate for two reasons: firstly because the two points that define L_{leaf} were easily distinguished on the leaf; and secondly, since L_{leaf} was always greater than W_{leaf}, the errors arising from measuring L_{leaf} with a ruler or callipers was proportionally less than for W_{leaf}.

To estimate A_{leaf}, the lengths of all the leaves were measured on each hybrid in the dry treatment of Replicate 2 on 23/06/03 and the leaf area calculated using the specific relationship A_{leaf}=f(L_{leaf}). These leaves matured prior to the imposition of drying cycles. In addition, L_{total} and the stem diameters at the height of the first leaf were measured. From these data, several models were tested to identify the most appropriate are to predict the plant leaf area.

Stem and branch diameters at the first leaf were measured four more times during the season on 27/06, 8/07, 4/08, 30/09. Unfortunately, after 27/06, the models did not provide a reliable estimate of A_{plant}. As a result, no model A_{plant}=f(L_{total}) could be computed as not enough data were available, so no further results of A_{plant} are presented.

The measurement of L_{leaf} could not be repeated on all leaves during the whole season of each plant because of time constraints. Therefore, stem and branch diameters at the level of the first leaf and the stem and branch lengths were measured at the same time. An allometric model was developed to relate A_{plant} to these more easily measured variables. Stem and branch diameters were measured regularly during the rest of the season and used to estimate A_{plant}.

5.2.3. Biomass partitioning

The estimation of total biomass production and partitioning took place in three steps. First the stems and branches were harvested and oven dried at 105 °C for 48 hours; this biomass was then weighed and referred later to the stem biomass (B_{stem}; see Chapter 4 for results). In the second step, the stools were extracted from the soil, cleared of roots and soil, oven dried at 105 °C for 48 h and weighed (B_{stool}). In the third step, a quarter of the soil of the lysimeter was sampled all the way down the lysimeter. Precautions were taken to distinguish four root zones in the lysimeter, 0-0.2; 0.2-0.4; 0.4-0.6 m and below 0.6 m including gravel (Figure 5-1).

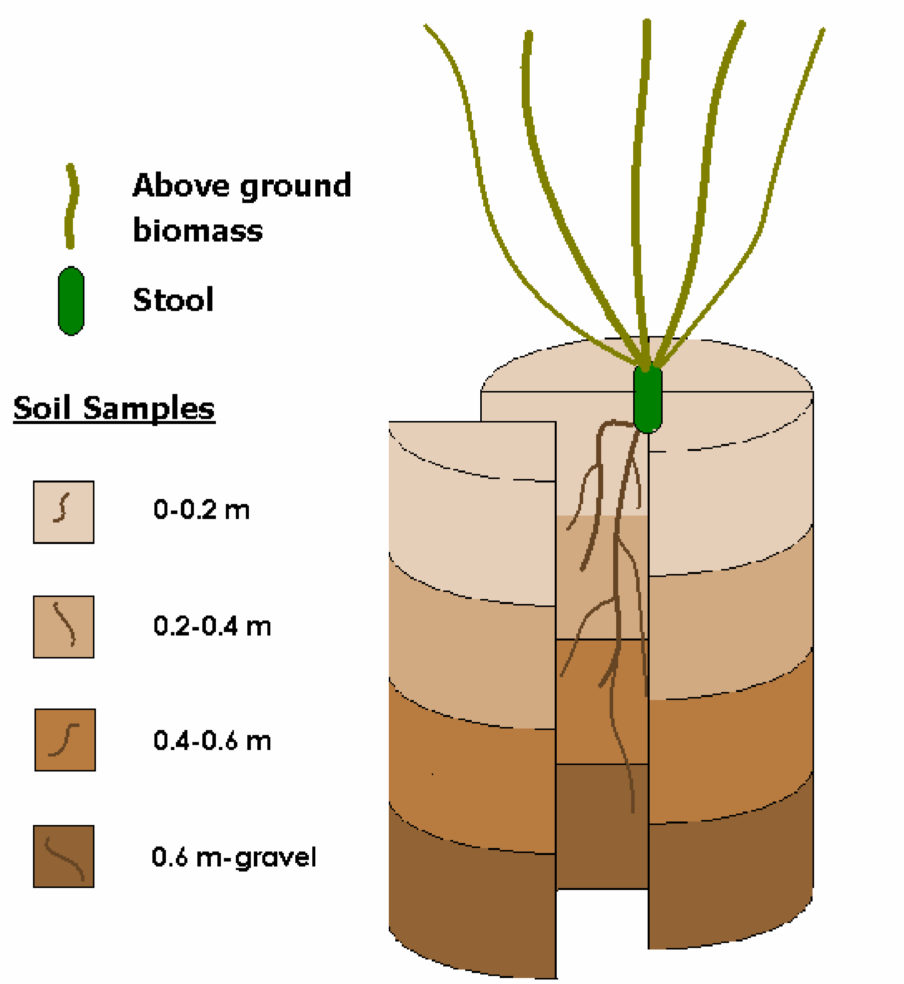


Figure 5-1. Stem, stool and roots biomass sampling from a lysimeter, 2003

The roots were washed to remove the soil and separated into fine (≤ 2 mm diameter) and coarse roots (> 2 mm diameter) before drying in the oven at 105 °C for 48 h. The roots were then weighed to separate the biomass into fine (B_{fine}) and coarse (B_{coarse}). B_{fine} and B_{coarse} were summed into the total root biomass B_{root}.

Finally, SB, B_{stool} and B_{root} were combined for each hybrid and the WUE was calculated to produce the total biomass (excluding leaves) (WUE_{total}).

Equation 5-19

$$WUE_{total} = \frac{SB + B_{stool} + B_{root}}{WU}$$

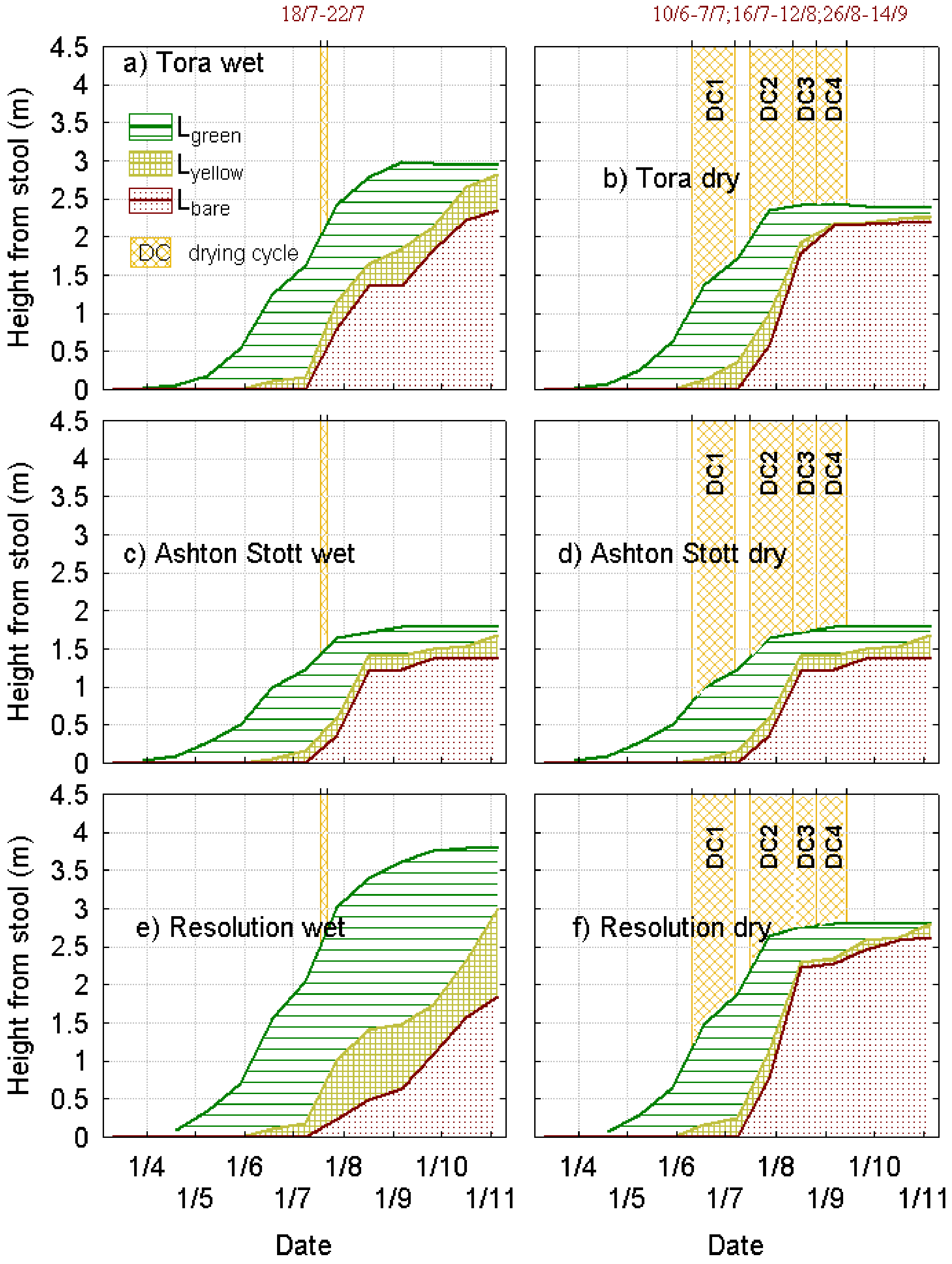
5.2.4. Statistical analysis

Analyses of variance were carried out using STATISICA software to identify whether significant differences existed between hybrids and water treatments and whether there were any significant interactions between treatments. WUE_{stem} and WUE_{total} were then correlated with factors where the hybrids segregated significantly (null hypothesis: there was no influence of the dry regime on the leaf population management of the hybrids and the hybrids did not manage their leaf populations differently). All the statistical analyses can be found on the appended CD. The chosen level of significance was 5%; the 95% confidence intervals are given to facilitate comparison between values.

5.3. Results

5.3.1. Length of leaf bearing stem

The contrasting development of L_{green}, L_{yellow} and L_{bare} over the growing season under the two water regimes illustrates the differing responses of the five willow hybrids to water stress (*Figure 5-2*).


18/7-22/7
10/6-7/7;16/7-12/8;26/8-14/9
a) Tora wet
b) Tora dry
c) Ashton Stott wet
d) Ashton Stott dry
e) Resolution wet
f) Resolution dry
L green
L yellow
L bare
DC drying cycle
DC1
DC2
DC3
DC4
Height from stool (m)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
1/4
1/5
1/6
1/7
1/8
1/9
1/10
1/11
Date

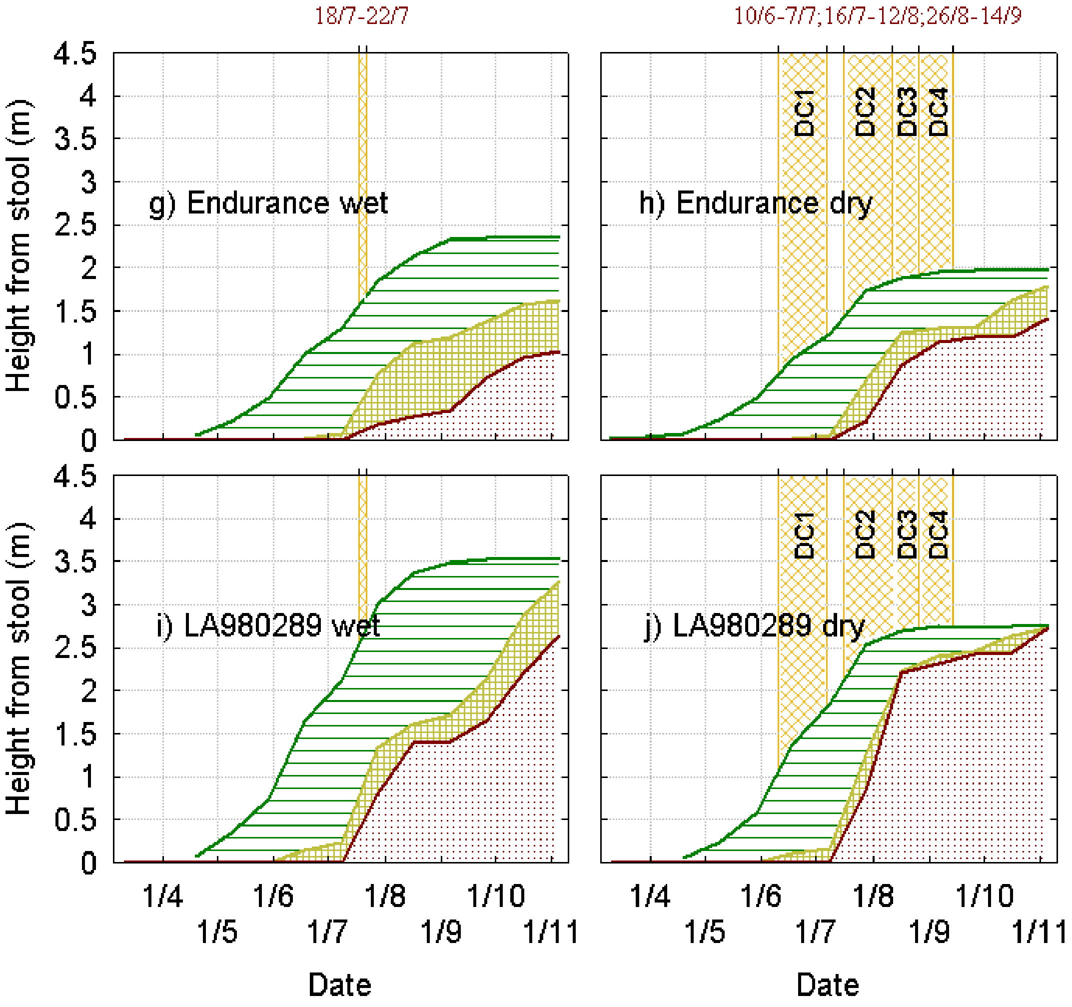


Figure 5-2. Seasonal pattern of mean length of stem bearing green (L_{green}) and yellow (L_{yellow}) leaves and the length of bare stem (L_{bare}) for five hybrids grown under two water regimes (Dry and Wet) with Tora (a and b); Ashton Stott (c and d); Resolution (e and f); Endurance (g and h) and LA9890289 (i and j). The four drying cycles (DC) imposed under the Dry regime are represented by the shaded areas. Lysimeter trial; Silsoe, Bedfordshire; 2003

Under the wet regime, L_{green} increased until 26/07/03 for all the hybrids, by which time significant differences ($p \leq 0.05$) had developed between Resolution on the one hand and Ashton Stott and Endurance on the other. At this point, Resolution had the longest L_{green} with 2.5 m, followed by LA980289 with 2.1 m, then Tora with 1.9 m, and finally Ashton Stott and Endurance with 1.5 m.

The unplanned break in irrigation in the wet regime between 18/07 and 22/07 had a major impact on the green leaf population and L_{yellow} increased for all hybrids by 0.20 to 0.55 m, though there were no significant differences between the hybrids. As the irrigation restarted L_{green} dropped suddenly but then stabilised or increased slightly until about mid-September in all hybrids except Ashton Stott. During August, Endurance and Resolution had a larger population of yellow leaves than Tora and LA980289 ($p \leq 0.001$).

Senescence began at the end of August in Ashton Stott, at the beginning of September in Endurance and Resolution, in the middle of September in Tora and at the end of September in LA980289. Once senescence commenced, the length of stem with green leaves decreased almost linearly until the end of the experiment in November. At this point, only Resolution and Endurance had more than 0.5 m of stem with green leaves remaining. Interestingly, L_{yellow} remained fairly constant, indicating that leaves were abscising at about the same rate as they were turning yellow.

For all five hybrids, the effects of the periods of water stress imposed in the “Dry” regime, were: first, a reduction in stem elongation rates; secondly, an increase in L_{yellow} and L_{bare} coupled with a reduction in L_{green}.

The first period without irrigation (DC1) led in some cases to a significant reduction in L_{green} compared with plants in the wet regime over the same period. L_{green} of Tora had the greatest reduction with 0.30 m on average followed by Resolution and Ashton Stott with 0.21 and 0.15 m respectively, while no significant changes were observed in LA980289 and Endurance. There were no bare stems during and soon after that period under both treatments for all hybrids. No interactions between the hybrids and treatments were recorded as significant, which meant that the hybrids behaved similarly during DC1.

DC2 had more effect than DC1. L_{green} decreased for all five hybrids in comparison with the wet regime ($p \leq 0.001$). Significant differences ($p \leq 0.001$) within the hybrid population as well as an interaction between hybrids and treatments were observed. This meant that although L_{green} decreased for all hybrids, the rate of the decrease varied between the hybrids.

In detail, LA980289 had the greatest reduction L_{green} with 1.27 m loss on average, which was seven times more than in the wet regime. Resolution had the second greatest reduction in L_{green} with 1.17 m on average while under the wet regime L_{green}

increased. Tora and Endurance doubled their decrease of L_{green} in comparison to their clone grown under the wet regime with 0.82 and 0.57 m respectively. Finally the Ashton Stott L_{green} reduced by 0.83 m while it increased under the wet regime. No significant differences in L_{yellow} between the hybrids were observed at the end of DC2 although DC2 had a significant and variable effect on L_{yellow} evolution across the hybrid population (the interaction between hybrids and treatments was significant at $p \leq 0.05$).

L_{yellow} decreased on average under the dry regime for Tora, Resolution and LA980289 while it increased for Endurance and Ashton Stott. Under the wet regime during the same period, L_{yellow} increased for all but LA980289. L_{bare} was initiated during DC2 for all five hybrids. Significant differences were observed in the hybrids population, between the treatments and their interactions. This meant that the reactions to DC2 were specific to each hybrid. Besides having the greatest drop of leaves (increase of L_{bare}), Resolution lost 4.7 times more leaves in the dry than in the wet regime; the leaves of Endurance and Ashton Stott dropped in similar proportion to Resolution. For Tora and LA980289 the leaves dropped only 1.5 times more than in the wet regime although this represented the loss of leaves on a similar length of stem as Resolution.

DC3 and consequently DC4 were not initiated in the same soil moisture conditions for all three replicates. During DC3, Ashton Stott had a significantly smaller L_{green} than Endurance. Endurance had a significantly ($p \leq 0.01$) longer L_{yellow} than LA980289 and Resolution. L_{bare} was significantly ($p \leq 0.001$) longer for Tora, Resolution and LA980289 than Endurance and Ashton Stott. No differences in behaviour were recorded during DC3 for all five hybrids concerning their leaf population management.

The last of the four drying cycles, DC4, had no significant effect on L_{green} and L_{yellow}, however, significant differences within the hybrid population were observed for L_{green}. At the end of DC4 Endurance kept the longest L_{green} of 0.64 m on average while the four other hybrids' L_{green} ranged between 0.21 and 0.27 m on average. Under the wet regime at the same date Resolution and LA980289 had the longest L_{green} with 2.17 and 1.54 m respectively. DC4 had a significant effect on L_{bare} ($p \leq 0.05$). The same significant differences as during DC3 were observed within the hybrid population

($p \leq 0.001$). Tora and Endurance were the hybrids on which L_{bare} progressed the most during the period while it developed the least in Ashton Stott and LA980289.

To summarise, during August and September under the wet regime, Tora and LA980289 had the longest bare stems. Ashton Stott and Endurance had the least bare stems and Resolution fell in between the two groups.

Under the dry regime, the leaf populations were significantly lower than in the wet treatment. All L_{green} followed the same sequence over the whole season determined by the four drying cycles. However differences were distinguishable and the five hybrids separated into groups at different moments.

The hybrids all reached maximum L_{green} in the middle of DC2 except LA980289, which reached its maximum at the end of DC1. LA980289, Tora and Resolution achieved longer L_{green} than Endurance and Ashton Stott. DC2 accentuated the effect of DC1 and provoked the drop of L_{green}. Towards the end of DC2 and the beginning of DC3 and throughout DC4 Endurance and Ashton Stott kept longer L_{yellow} than Resolution, Tora and LA980289. During the autumn Endurance maintained the highest L_{green} while the others continuously decreased their leaf populations by a process of natural senescence.

5.3.2. Model of Leaf Area

Relations between L and A_{leaf}

Over the range of leaf lengths sampled a quadratic relationship between length and area gives the best fit for all hybrids ($p \leq 0.001$); (Figure 5-3).

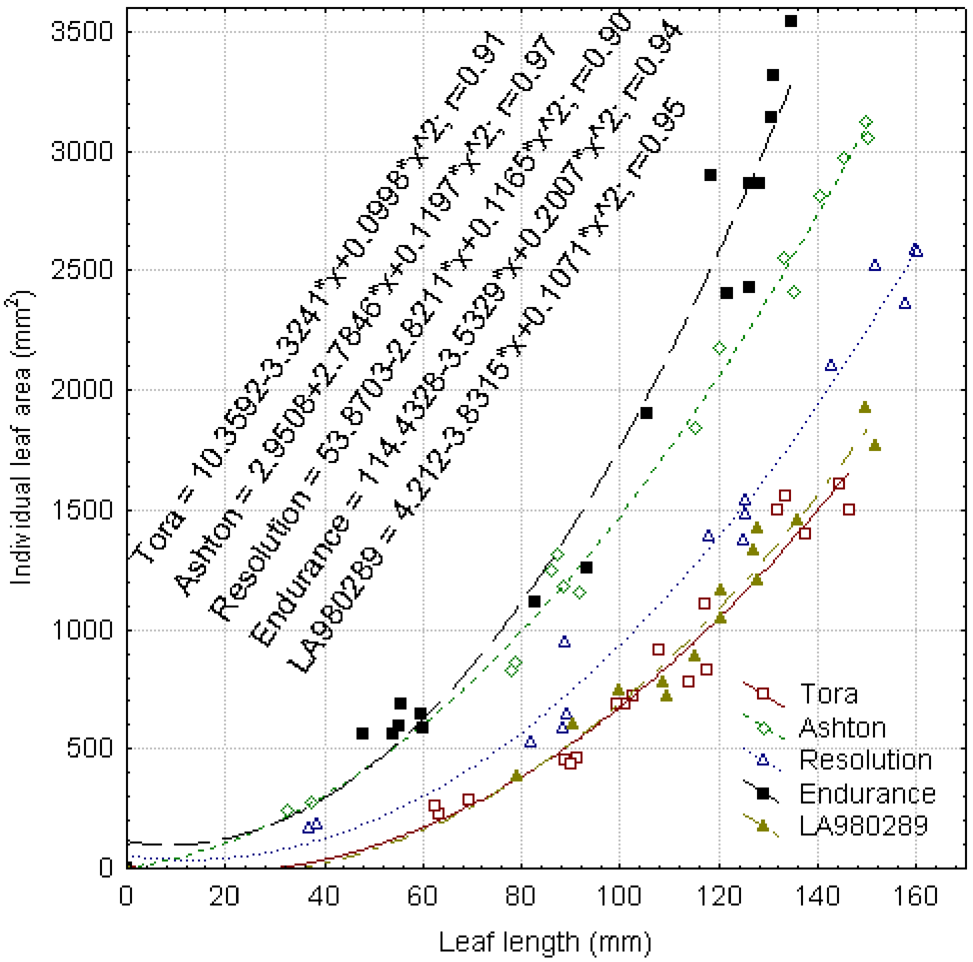


Figure 5-3. Relationship between leaf length and the individual leaf area, for five Salix hybrids grown in lysimeters; Silsoe, Bedfordshire; 2003

The hybrids were differentiated from each other, with the exception of Tora and LA980289. Ashton Stott and Endurance with *S. dasyclados* or *S. burjatica* parents had the roundest leaves and the steepest relationship between leaf length and area.

Prediction of plant leaf area

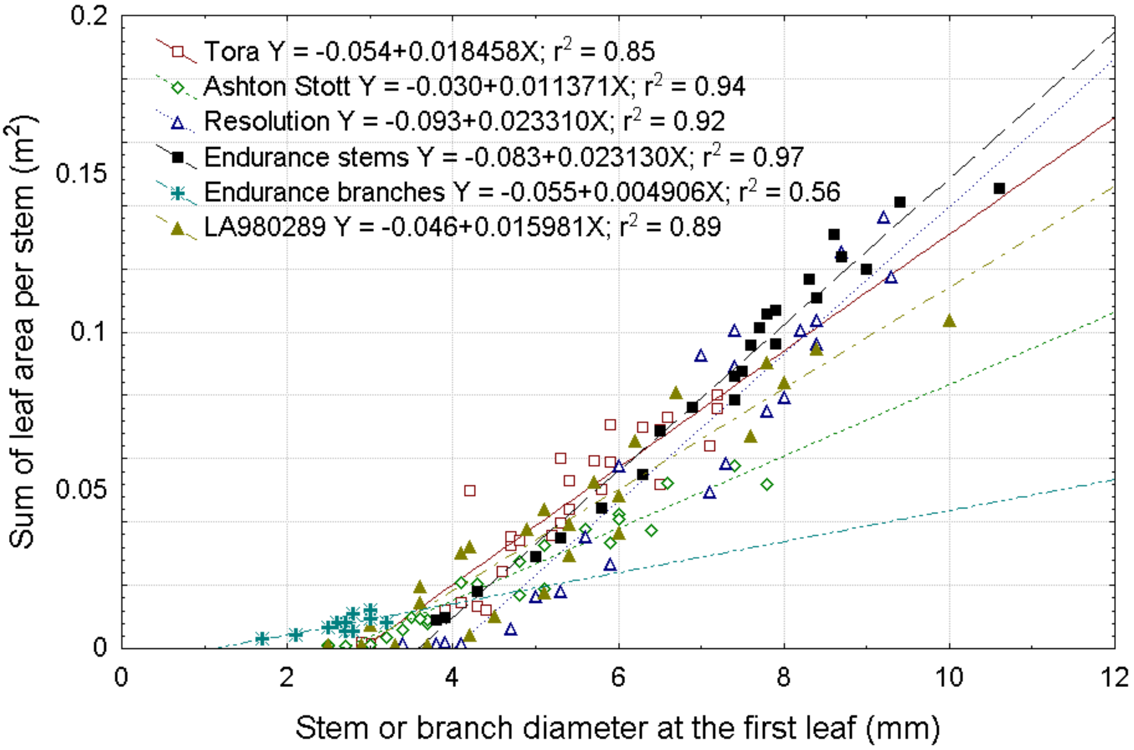


Figure 5-4. Relationship between stem or branch diameters at first leaf borne on stem or branch and the sum of the areas of all leaves borne on the stem for five Salix hybrids grown in lysimeter at Silsoe in June 2003

Six relationships are presented in Figure 5-4. Endurance required two equations to estimate the leaf area, the first for its stems and the second for its branches while for the others no branches developed below the first leaves on the stems, thus one equation per hybrid was sufficient. The correlations were good in the case of the stem diameters at the first leaf as they accounted for more than 85% of the observed variation.

5.3.3. Biomass partitioning and WUE

The mean root, stool and stem biomass harvested in November 2003 are presented in Figure 5-5. ANOVA and factorial ANOVA were carried out and the detailed results of each analysis are presented in the appended CD.

Biomass

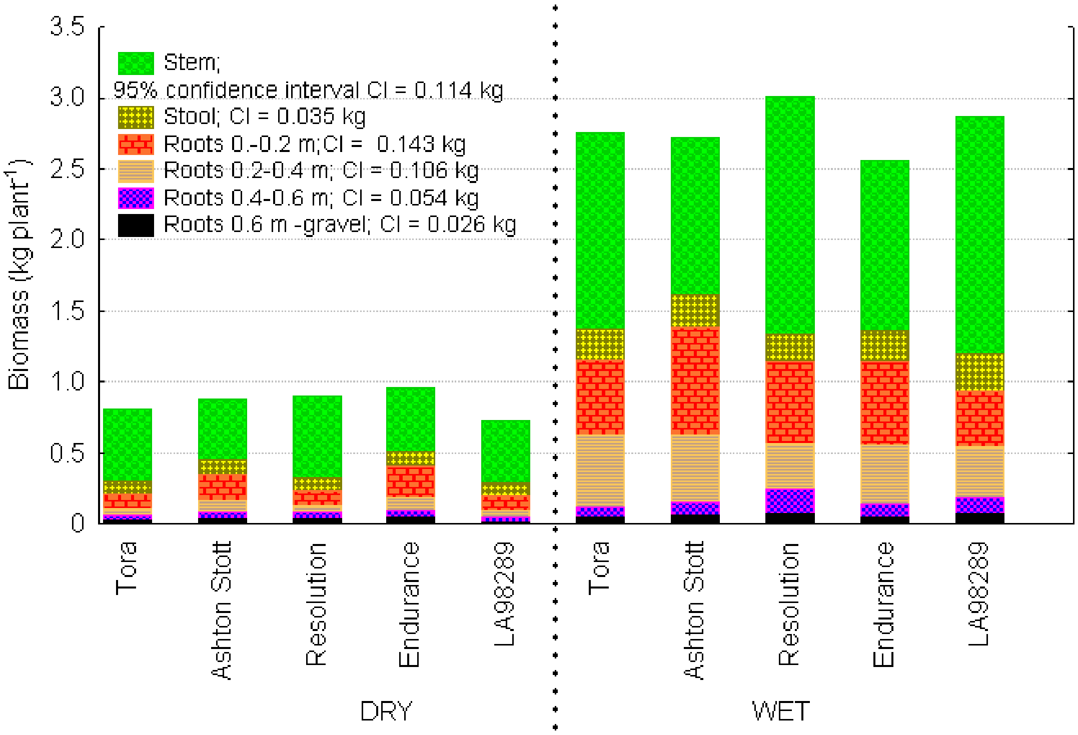


Figure 5-5. Biomass partitioning of five Salix hybrids grown in Lysimeters under two water regimes; Silsoe, Bedfordshire; 2003. CI represents the 95% confidence intervals (n=3). The proportion of fine roots is indicated

Under the wet regime Ashton Stott produced the most root biomass and LA980289 produced the least root biomass, the other three hybrids had very similar root biomass production. Subjected to drought, the root biomass was significantly reduced ($p \leq 0.001$). Ashton Stott had the second largest root biomass production after Endurance while LA980289 produced least root biomass; Tora and Resolution had very similar root production. The root partitioning of Ashton Stott and LA980289 are shown in Figure 5-6 to illustrate the data.

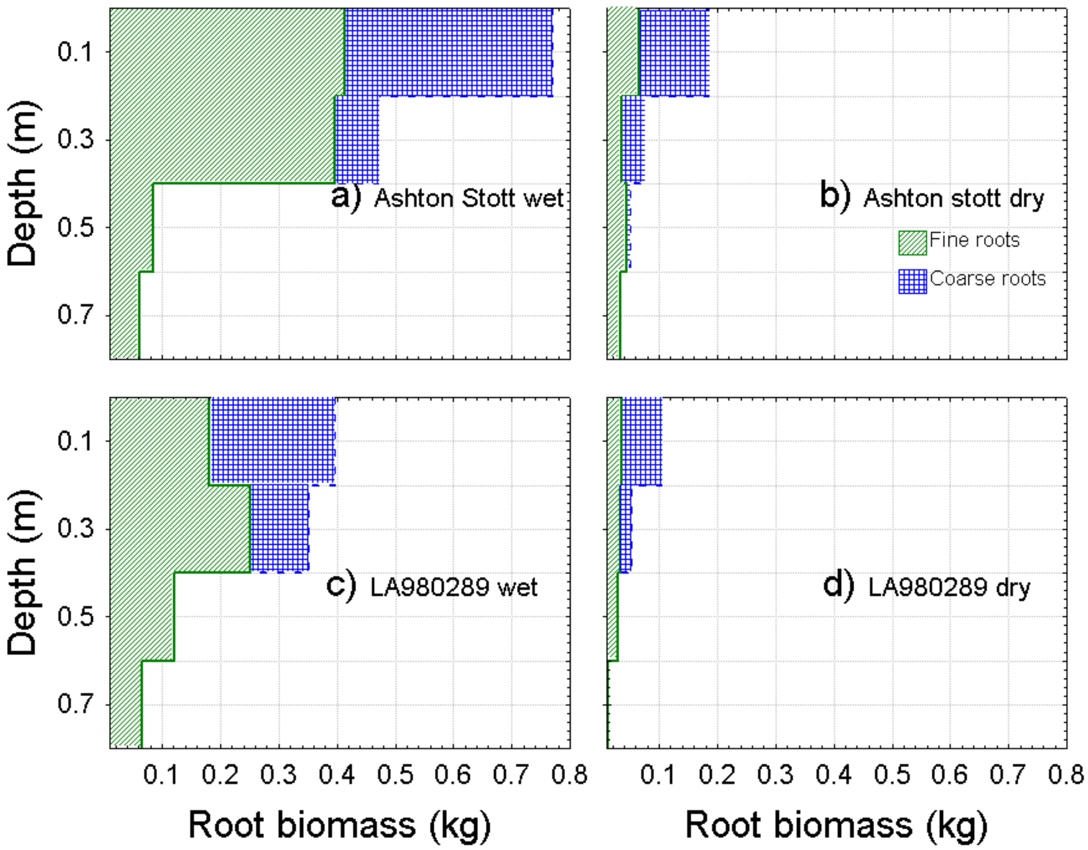


Figure 5-6. Average fine and coarse root biomass and distribution of two Salix hybrids (Ashton Stott a and b; LA980289 c and d) grown in lysimeter under two water regimes (wet a and c; dry b and d); Silsoe, Bedfordshire; 2003

In both water regimes, fine roots developed all the way down the profile for all varieties but coarse roots reached only 0.4 m except for Ashton Stott and Endurance under the dry regime. Under the wet regime LA980289 produced more fine roots below 0.4 m and more coarse roots below 0.2 m than Ashton Stott. The same observation can be done comparing *S. viminalis – S. schwerinii* hybrids compared to *S. dasyclados – S. burjatica* related hybrids.

For all hybrids, the development of the fine root biomass was significantly reduced ($p \leq 0.001$) by the water shortage imposed under the dry regime in comparison to the wet one. No significant differences were recorded within the hybrid population. There was a significant difference within the root depths ($p \leq 0.001$) : in the top 0.2 m a greater root biomass developed than in the 0.2-0.4 m zone. Below 0.4 m the fine root biomass was significantly lower than in the upper sections. No significant interactions between hybrids and treatments were recorded but significant interactions occurred between hybrids-root depths, treatments-root depths and hybrids-treatments-root depths. This meant that the hybrids rooted differently and that the dry regime affected their rooting habits.

Under the dry regime, all hybrids produced significantly less coarse root biomass than under the wet one. As the depth increased, the biomass of coarse roots decreased significantly more than fine root biomass.

In the top 0.2 m of the profile, significant differences ($p \leq 0.01$) in fine and total root biomass were observed between the five hybrids but for all other depths no significant differences were observed. In the top 0.2 m under the wet regime, Ashton Stott produced more than twice as many fine roots (414g) as LA982089 and Resolution (181 and 208 g respectively) (see attached CD).

The development of the coarse roots at 0-0.2 and 0.2-0.4 m was significantly reduced ($p \leq 0.001$) under the dry regime compared to the wet one for all five hybrids. No significant differences in coarse root production were recorded within the five hybrids. As a result, the total root production was significantly affected by water stress at all depths ($p \leq 0.01$) but significant differences of total root biomass were only recorded in the top 0.2 m of the profile within the hybrid population. Across the whole profile the dry regime significantly ($p \leq 0.001$) reduced the total root biomass but no differences within the hybrid population were observed.

Plants in the dry treatment also had significantly reduced ($p \leq 0.001$) biomass stored in their stools, but no differences were recorded between hybrids.

As seen in Chapter 3, stem biomass production was reduced significantly ($p \leq 0.001$) by the dry regime and significant differences ($p \leq 0.001$) were observed within the hybrid population.

Finally a significant interaction between hybrids and treatments ($p \leq 0.001$), indicated that the hybrids were not affected in the same proportion by the dry regime. This was recorded only for the fine root production in the top 0.2 m of the profile and the stem biomass production. Consequently, the dry regime significantly ($p \leq 0.001$) reduced the total biomass production of the *Salix* hybrids but no significant interactions between the five hybrids and the two treatments were recorded.

Root biomass/stem biomass ratio

From the data presented in Figure 5-5 and the WU presented in the previous chapter, the root/shoot ratio and the total WUE_{total} were calculated.

Table 5-1. Mean stem/root ratio and water use efficiency of total biomass (without leaves) WUE_{total} of five hybrids grown in lysimeters under two water regimes; Silsoe, Bedfordshire; 2003. CI represents the 95% confidence interval (n=3)

Variables	Hybrid	Tora		Ashton Stott		Resolution		Endurance		LA980289		CI
	Water regimes	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	
Root/shoot ratio		0.418	0.841	0.805	1.270	0.407	0.688	0.890	0.971	0.468	0.557	0.235
$(root/stem^{dry})/(root/stem^{wet})$ %		50		63		59		92		84		Na
WUE_{total} (g kg^{-1})		3.99	4.19	4.04	4.16	4.39	4.71	4.24	4.10	3.56	4.12	0.39

Significant differences ($p \leq 0.001$) in the root/shoot ratio were observed between the hybrids, with Ashton Stott and Endurance having a significantly higher ratio than LA980289 and Resolution. In addition, the dry regime significantly ($p \leq 0.01$) increased the ratio for all hybrids (Table 5-1); however, for LA980289 and Endurance the ratio was most consistent under both water regimes.

Total WUE (WUE_{total})

Overall, Resolution had a significantly higher WUE_{total} than LA980289 but there was no siginificant difference between the water treatments. The same results were found for the WUE_{stem} (See chapter 4). WUE_{stem} was positively correlated with WUE_{total} but accounted only for 17% of the variance.

5.3.4. Correlation between WUE and other variables

Where significant differences were observed between the hybrids, correlation coefficients were calculated for WUE_{stem} and WUE_{total} and the leaf area and biomass variables reported above. The data were separated into dry and wet regimes as, when combined, the data appeared for most variables as a bimodal distribution. The correlation coefficients (r) and the probability levels (p) are presented for each regression in Table 5-2.

Table 5-2. Correlation coefficients (r) and probability levels (p) between WUE_{stem} and WUE_{total} with 18 variables collected from five Salix hybrids grown in lysimeters under water regime (Wet and Dry). Significant correlations are highlighted. Lysimeter trial; Silsoe, Bedfordshire; 2003

Water regimes	Dry				Wet			
Variables	WUE_{stem}		WUE_{total}		WUE_{stem}		WUE_{total}	
	r	p	r	p	r	p	r	p
L_{green} 06/06	**0.59**	**.020**	-0.05	.854	**0.72**	**.003**	0.14	.616
L_{green} 07/07	0.48	.071	-0.11	.693	**0.86**	**.000**	0.36	.183
L_{green} 30/07	**0.74**	**.002**	0.14	.618	**0.54**	**.039**	**0.56**	**.030**
L_{yellow} 30/07	0.32	.241	0.44	.097	0.34	.210	0.17	.536
L_{bare} 30/07	-0.15	.589	**-0.73**	**.002**	0.31	.254	-0.13	.637
L_{green} 11/08	-0.02	.950	0.15	.582	**0.77**	**.001**	**0.54**	**.036**
L_{bare} 11/08	**0.63**	**.012**	-0.17	.552	0.37	.170	-0.21	.448
L_{green} 25/08	-0.20	.469	0.29	.300	**0.90**	**.000**	**0.59**	**.020**
L_{yellow} 25/08	-0.37	.171	0.13	.640	0.05	.860	0.36	.186
L_{bare} 25/08	**0.63**	**.011**	-0.25	.378	0.38	.163	-0.20	.470
L_{green} 06/09	-0.22	.431	0.36	.185	**0.89**	**.000**	**0.59**	**.020**
L_{yellow} 06/09	**-0.55**	**.032**	-0.19	.500	0.12	.676	0.39	.146
L_{green} 17/09	-0.34	.219	0.32	.243	**0.82**	**.000**	**0.63**	**.012**
L_{yellow} 17/09	0.06	.825	0.26	.347	0.34	.215	0.30	.278
L_{bare} 17/09	**0.60**	**.019**	-0.28	.312	0.29	.287	-0.22	.428
0-20 cm fine roots	-0.49	.061	0.42	.120	**-0.72**	**.003**	0.11	.709
0-20 cm total roots	**-0.61**	**.015**	0.27	.327	-0.47	.075	0.31	.260
Roots/stems ratio	**-0.74**	**.002**	0.19	.499	**-0.81**	**.000**	0.08	.775

Prior to the start of the water stress treatments and in the wet treatment there were significant positive correlations between WUE_{stem} and L_{green} on most dates, suggesting that plants with longer stems with green leaves had higher WUE_{stem}. There were similar correlations between WUE_{total} and L_{green} in the wet treatment but no correlations were found under the dry regime.

A negative correlation between WUE_{stem} and L_{yellow} was significant only on one occasion on 6/09 under the dry regime; this meant that the shorter the L_{yellow} after the three drying cycles the higher the WUE_{stem}.

WUE_{stem} was positively correlated with L_{bare} under the dry regime on 11/08, 25/08 and 17/09. This meant that the longer the L_{bare} after DC2 and DC3 the higher the WUE_{stem}. WUE_{total} was negatively correlated with L_{bare} on 30/07 under the dry regime. This meant that the shorter was L_{bare} in the middle of DC2 the higher was WUE_{total}.

The fine root biomass was negatively correlated with WUE_{stem} under the wet regime which meant that the larger quantity of fine roots grown by the hybrid the smaller the WUE_{stem}. Similar correlations were observed for the total root biomass harvested in the top 0.2 m of the profile and WUE_{stem} under the dry regime.

Finally, the root/shoot biomass ratio was negatively correlated with WUE_{stem} under both water regimes.

5.4. Discussion

Specific responses and resistance ability to water stress have frequently been related to the morphology and the physiology of plants (Brown et al., 1976; Jones, 1992). Within the same crop genera and even the same species, some hybrids have been identified as having different responses to water stress; this was demonstrated for two *Salix* species (Weih, 2001) where above ground biomass and leaf area were affected in different proportions by water stress of similar intensities.

The values of WUE_{stem} and WUE_{total} in the wet regime were comparable to those calculated for hybrid "Jorr" from a lysimeter trial carried out at Silsoe using the same lysimeters and the same soil (Martin and Stephens, in press-a).

In the experiment reported here, the close monitoring of the green and yellow leaf populations of the five hybrids (Tora, Ashton Stott, Resolution, Endurance and LA980289) revealed specific leaf management strategies related to WUE. It appeared that green leaf populations (L_{green}) were positively correlated with the harvestable biomass WUE (WUE_{stem}). This suggested that hybrids grown under adequate water supply and developing large canopies are more water use efficient. Close studies of photosynthetically active leaf physiology may reveal specific and inbuilt abilities of hybrids to use water more efficiently.

The leaf population of the five hybrids and consequently the total leaf area of the plants were significantly reduced by water stress, this was reported for other *Salix* hybrids elsewhere (e.g. Choisel, 1997; Xiao, 2001). In the Silsoe lysimeter trial, interactions between the hybrids and the treatments were recorded. Although the growing conditions in lysimeters are different from those in the field, these results indicated that hybrids differed in the way they managed their leaf populations under well irrigated and under drier conditions and that these related to WUE.

In the lysimeter trial, under the wet regime, irrigation was accidentally stopped for four days during a period when water demand was very high. This short drying cycle, shows that even a mild stress can generate an acute response. On the fourth day of the drying cycle, no significant changes were observed. When the plants were re-watered, however, a large number of green leaves turned yellow and many abscised, increasing the length of bare stem. As the soil dries, abscisic acid (ABA) is produced by the roots (Perks et al., 2002). Besides inducing stomatal closure (Liang et al., 1997b), ABA can induce defoliation and the level of expression of the ABA linked genes has been shown to be higher in drought adapted *Populus* species (Zhang et al., 2004). In the wet regime the four-day drying cycle, may have resulted in a rapid rise in ABA production inducing premature leaf senescence and abscission. The leaf population in both water regimes suffered from water stress and two phenomena were observed.

However, prior to this four days of no irrigation the plant suffered from striking change of weather conditions; from 12th to 15th of July the maximum temperature raised from an average below 20 °C over first two weeks of July to an average over 28 °C with the highest average daily radiation recorded in 2003 over a 4 days period (>24 MJ m^{-2} d^{-1}). From 23rd to 26th of July the temperature decreased abruptly below 20 °C paired with lower average solar radiations (<11 MJ m^{-2} d^{-1}). These abrupt changes in temperature and radiation can be the origin of additional stress which explains the difference between the little changes in L_{green} caused by DC1 in the dry regime compared to the four days without irrigation.

DC1 shown that Tora and Ashton Stott were the most affected by drought and L_{green} dropped or stopped increasing, while for Resolution, Endurance and LA980289 the rate of increase was slowed down. DC2 and the short water shortage that occurred in the wet regime both occurred in July and generated the loss of a large proportion of L_{green}. After these water shortages L_{green} was not recovered for all hybrids. This suggests that drought before July has a lower impact on leaf area than a drought occurring in July and after which may result in a considerable loss of leaf area. This loss cannot be recovered for all hybrids if the stress was too great, while subjected to a moderate stress Ashton Stott was not able to recover, Tora and Endurance had an insignificant increase in L_{green} but, Resolution and LA980289 experienced a significant increase in L_{green}.

ABA is produced by the roots when the soil surrounding them is drying, this was observed for *Salix dasyclados* (Liu et al., 2001), *Pinus sylvestris* L. (Perks et al., 2002). ABA induced leaf loss for *Populus sp.* (Yin et al., 2004). Under the dry regime, repeated water stress must have induced a repeated ABA production. Consequently, L_{bare} increased in steps as the plants got rewetted and ABA flushed into the aerial parts of the plants. L_{bare} under the dry regime was positively correlated with WUE_{stem} suggesting that hybrids able to drop their leaves at a higher rate subsequent to water stresses are characterised by higher WUE_{stem}. Hence an increasing ABA sap concentration may resulted in an increasing defoliation, therefore ABA production of a range of hybrids under similar water stress might be positively correlated with the WUE_{stem} and consequently with drought resistance, as has been suggested for *Populus davidiana* (Zhang et al., 2004). ABA was found to decrease stomatal conductance in *Salix dasyclados* (Liu et al., 2001) and might have a progressive effect on stomatal closure, as such stomatal conductance level might be related in some extend to the ABA sap concentration.

The quantification of leaf area in m^2 was not successful as many methods such as the measurement of intercepted solar radiation require large canopies (e.g. Potter et al., 1996; Proe et al., 2002) and are therefore not appropriate for lysimeter trials. Several authors have developed allometric models to predict leaf area. The level of complexity varies from elaborate computer programs able to anticipate canopy structure development by reducing the amount of allometry needed as developed for *Populus sp.* (Chen et al., 1994) to simple correlation when frequent allometry measurements could be carried out (Martin et al., 2002). In all cases, leaf area models have to be specific to each hybrid and are not highly accurate.

A method developed for the *Salix* hybrid "Jorr" grown in lysimeters (Martin et al., 2002), was possible because the number of treatments was small. Adapting the method to cope with the ten treatment combinations (five hybrids x two treatments) was difficult, particularly because the branch morphology changed in the dry treatments. The models were not accurate enough, partially because the design presented only three replications per treatment, and even if basal stem diameter and stem length were positively correlated with harvestable biomass (Robinson et al., 2004), the relationship between stem length and stem diameter was not consistent along the stem and evolved during the season as biomass accumulated. Accurate measurement of leaf area in such an experiment would require the models to be recalibrated at each assessment.

A quarter of the total soil volume was collected from each lysimeter for root biomass assessments. This represented a significant proportion of the total root biomass and as such the data set was considered reliable. However, the absolute value of root biomass were less than those observed elsewhere (Martin and Stephens, in press-c), but when root biomass was calculated as the production per year, there were comparable in the wet treatment and greatly inferior in the dry regime which produced only 25% on average as much of the one observed in their experiment. The study of root systems grown in containers has the advantage that a large proportion of the total root biomass can be sampled accurately, but has the major disadvantage of restricting the root zone and altering the natural rooting habit expressed in the field. Nevertheless, the root system study indicated specific behaviour which correlated with WUE_{stem}.

From the Silsoe lysimeter trial 2003, the hybrids with the least drought resistance and the smallest WUE_{stem} allocated a greater proportion of their biomass production to roots and had the largest root/shoot ratio, independent of the water regime. These findings contrast with those found for two *Populus* of the same species in wet and dry climates respectively (Zhang et al., 2004). However, in other plants like *Camellia sinensis*, drought had no effects on root dry matter but had an increasing effect by reducing the above ground biomass of hybrids (Burgess, 1992). Thus the root/shoot ratio in tea clones was also negatively correlated to drought resistance. Furthermore, in natural habitats under drier conditions, indigenous species developed more fine roots than in wetter conditions (Joslin and Devereux - Wolfe, 1998; Xiao, 2001), suggesting that plants submitted to mild water stress will explore greater soil volume to cope with water demand. The soil volume being restricted in the lysimeter such a habit could not be observed but other phenomenon might have take place.

The turnover rate of fine roots and especially root hair biomass production would be underestimated by the fine root dry matter harvested in the winter because most of this root biomass might have died and decayed prior to harvest. Nevertheless, in the top 0.2 m of the lysimeter profile Endurance and Ashton Stott developed a greater root system than the other three hybrids, indicating a habit of enhanced soil by root exploration by hybrids sensitive to water stress and showing lower WUE_{stem}. In addition for the root production below 0.2 m was smaller for Ashton Stott and Endurance than the other hybrids which suggest a reduced ability of the root system to explore the soil at greater depth.

The root biomass harvested indicate other growth mechanisms suspected to take place as well in pot trial 2002 which reduced above ground biomass. Ashton Stott and Endurance produce the more coarse roots on the upper layer of the profile under both water regimes. *S. burjatica - S. dasyclados* related hybrids orientate their biomass production toward the roots much sooner than the other parentages. This supports the theory (suggested in Chapter 4): that their root system reaches the impermeable layer of the container sooner than other and can signal the upper part of the plant to reduce growth while staying physiologically healthy. The consequences are that the above ground biomass produced in narrow container of the *S. burjatica - S dasyclados* related hybrids even under no water restrictions is not representative of their field yield potentials.

Finally, continuous monitoring of water extraction by roots might be a better indicator of the ability of hybrids to resist water stress. A recent experiment by Wikberg and Ögren, (2004) on the rate of soil exploration and water extraction by the root system of four *Salix* hybrids suggested that the soil water potential at which the varieties reached permanent wilting point is characterised by their drought resistance. A similar approach could be useful for modern high-yielding varieties.

5.5. Conclusions

The leaf population and the root population management are specific hybrids traits which induce specific WUE_{stem}.

S. viminalis – S. schwerinii related hybrids are able to produce leaf area at greater rate than *S. burjatica - S. dasyclados* related hybrids which induce a greater rate of stem biomass accumulation. In addition *S. viminalis – S. schwerinii* related hybrids are able to grow new leaf area at greater rate than *S. burjatica - S. dasyclados* related hybrids when subjected to mild water stress. When subject to strong water stress, *S. viminalis - S. schwerinii* related hybrids express a water saving mechanism encountered in many other genera (Jones, 1992); they dropped a greater proportion of their leaf area keeping a leaf area dominated by green leaves and only few yellow leaves compared to *S. burjatica - S. dasyclados* related hybrids which were not able to drop as much leaf area and kept a large proportion of it as yellow leaves. Consequently, as the plants were strongly coupled with the atmosphere, a greater transpiration rate took place for *S. viminalis - S. schwerinii* related hybrids when water is available while it took place for *S. burjatica - S. dasyclados* related hybrids when water is scarce as they had the greater stomatal conductance in these conditions respectively.

S. viminalis – S. schwerinii related hybrids are able to prioritise biomass allocation to above ground woody structure rather than those below ground in comparison to *S. burjatica - S. dasyclados* related hybrids. They are therefore, economically able to make efficient use of the water available. Those are also best at coping with water shortage as their root system is their proportion of root biomass is greater below 0.2 m; this phenomenon qualifies as a drought avoidance process (Jones, 1992).

Further studies are required to determine the physiology of water use efficiency, water saving and drought avoidance mechanisms related to the root and leaves interactions with the environment and the efficiency of those populations. In the next chapter, detailed studies are presented on varietal differences. A close look at photosynthesis and instantaneous water use efficiency at the leaf level is presented. The root system development and ability during the whole season is also examined.

As it was not carried at Silsoe, it is recommended that studies are undertaken to check on the ABA level produced by the hybrids during water stress events. More importantly, with recent genomics techniques, the genetic ability of the hybrids to produce ABA ought to be quantified by first identifying ABA coding and related genes as qualitative trait loci and use these in the plant breeding programme.

CHAPTER 6. Transpiration and photosynthesis of five *Salix* hybrids

6.1. Introduction

To date, the results of field, pot and lysimeter trials have failed to indicate the level of importance of particular physiological mechanisms in drought resistance. Nevertheless, many authors agree that there are differences in drought resistance and WUE between *Salix* species and that some morpho-physiological traits are responsible for these differences (e.g. Grip et al., 1989; Weih and Nordh, 2002; Wikberg and Ögren, 2004). In their study of relationships between water use and growth traits Wikberg and Ögren (2004) concluded that, there is considerable variation between interbreeding willows but their results did not offer much hope for any improvement without affecting biomass.

It appears logical that WUE is driven by biochemical processes such as photosynthesis, and physical ones such as leaf cuticular resistance which are themselves dependent on plant morphology and physiology. Consequently, there is a large number of combinations of mechanisms involved in the plant water relationship including drought resistance and water use efficiency which can not be characterised by a single morphological or physiological feature. A quick alternative way to identify drought tolerant individuals might be to measure the physiological response to drought over short time period or instant water use efficiency (WUEi) which are the outcome of given morpho-physiological.

While the plant physiology drives the degree of efficiency of water use and biomass production under a given environment, water uptake, transpiration and biomass accumulation are directly driven by environmental factors. In the leaves, light is converted into chemical energy of metabolites used for biomass production. These mechanisms were described extensively in the last century (e.g. Delvin, 1969; Lawlor, 1987; Jones, 1992). The parts of the plant involved in the relationship with the environment are mainly the leaves, which transpire and generate water demand, and the roots, which are supposed to meet this demand by abstracting water from the soil.

The ability of the plant to supply its tissue with water is crucial to life maintenance as the entire plant biochemistry takes place in a hydric environment (Ehlers and Goss, 2003).

One aspect of plants which are not in a dormant phase is the need to supply water to all their living tissues. Water is extracted from the soil via the roots; the extraction capability in a given environment is specific to each variety and its stage of growth (Allen et al., 1998). As such varieties of the same genus are able to extract water at different soil water potentials, leaving some varieties with the ability to retard the instant of stress in comparison to others when grown under the same conditions. Wikberg and Ögren, (2004) studying two *Salix* species and two hybrids used "*the level of soil water depletion that plants could sustain without wilting*[15]" to classify the willow varieties according to their drought resistance; differences ranged between 24% and 44% of soil relative water retention before wilting. Therefore, the rate at which the soil water deficit increases and the level that is reached before irreversible physiological damage occurs might be used to classify hybrids' drought resistance ability. It is important to note that their experiment was done on newly established cuttings, and therefore could reflect also the ability of the different phenotype to allocate biomass to the root system at greater rate than others.

The roots explore the soil growing into the wetter zones where water is more readily available. The root growth rate, the dynamics of root soil exploration and the rate of water abstraction have been observed for many genera in field and in lysimeters by using hygrometric probes (Bonneau, 2001) or minirhizotrons (Joslin and Devereux-Wolfe, 1998; Liedgens et al., 2000). These observations have shown that it might be possible to assess whether plants contrasting in drought resistance have different dynamics of root soil exploration.

In plants, water is transpired mainly by the leaves which are porous and permeable. Each pore, each tubular structure and each membrane presents a physical resistance to water fluxes. Physical movement of water can be calculated with *Darcy's law* which

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[15] Wilting is the phenomena when plant apex bends downwards and leaves curl. This is induced by the by loss of water which results in the loss of turgidity.

comprises the hydrostatic pressure[16] gradient and the hydraulic conductivity coefficient[17] at the pore or membrane level (Jones, 1992). The leaf opposes resistance to water effluxes from the inside to the outside. Evaporation at the leaf level is driven by the difference between the partial pressure of water vapour in the leaf and the atmosphere, and the leaf conductance[18] (Weyers and Meider, 1990). Stomatal conductance (g_s) appears to be the main component of leaf conductance in most higher plant genera. In addition, transpiration creates a water potential gradient between the leaves and the roots that plants use to transport water and nutrients and permits the leaves to cool off which protect the physiological activities from heat stress (Jones, 1992).

"*Stomates provide paths for carbon dioxide intake while minimising the unavoidable efflux of water vapour*" (Weyers and Meider, 1990). A range of stomata types exists and stomatal density varies: they are mainly found on the abaxial leaf surface but can occur on the adaxial leaf surface. Plants in their natural habitat have a greater stomatal conductance if they are from wet habitats than if they are from drier ones, which is mainly due to stomatal density and stomatal control ability. Stomatal control depends on the physiological state of the plant (water potential) and the environment (climatic demand). Soil water deficit has been reported to depress day time g_s (Davies et al., 1981) which reflects stomatal closure, a short term drought resistance mechanism (Jones, 1992). Variations in the ability to reduce respiration by stomatal control are encountered, with the plants from drier habitats having a greater control than plants from wetter habitats. Stomatal closure limits water losses and therefore has an influence on the water use of the plant. It was reported for several genera that trees may experience a midday partial stomatal closure even in very wet conditions (Zweifel et al., 2002). This midday stomatal closure might be a water conservation mechanism at the moment when the climatic demand is the greatest.

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[16] The hydrostatic pressure sums cell solute osmotic pressure and cell membrane turgor pressure.

[17] Hydraulic conductivity coefficient or diffusion coefficients which depend on environmental conditions.

[18] conductance is the inverse of the sum of all pore resistance to water fluxes.

The genus *Salix* can be found in a range of habitats so it might be characterised by a range of stomatal control abilities. However it is mainly found in riparian areas and is reported to transpire large quantities of water in comparison to other genus of trees (Hall et al., 1998). As such stomatal control is expected to be low in the genus *Salix*.

Stomatal aperture and consequently g_s are affected directly or indirectly by environmental factors, particularly solar irradiance, temperature, carbon dioxide supply, saturation deficit of the air, wind, nutrient supply and water supply of the plant (Weyers and Meider, 1990). Water deficit in plant tissues has been reported to induce loss of leaf turgor (Davies et al., 1981) and the production of plant hormones such as abscisic acid (ABA) (Liang et al., 1997a), induced by this phenomena the stomata close and g_s decreases.

g_s has been measured to determine differences between three wild *Salix* species and the maximum g_s were around 0.2 mol m^{-2} s^{-1} (Liu et al., 2003). Moreover, maximum g_s was observed in a range of four *Salix* hybrids ranging from 0.3 and 0.7 mol m^{-2} s^{-1} which is large in comparison with g_s of 0.07 mol m^{-2} s^{-1} for grape *Vitis vinifera* L. (Flexas et al., 2002) and a desert shrub *Larrea tridentate* (Hamerlynck et al., 2000). Comparison between two *Salix* hybrids (Tora and Jorr) with biomass potential and two pure species (*S. viminalis* and *S. purpurea*) resulted in the conclusion that maximal g_s was negatively correlated with drought resistance but positively with yield and shoot growth rate (Wikberg and Ögren, 2004). In this experiment, they did not give any indications of short term mechanisms of drought resistance such as the dynamics of stomatal closure as most of the physiological assessments were restricted to one set of conditions.

Photosynthesis is the process of converting inorganic substances, such as carbon dioxide (CO_2), nitrate ions (NO_3^-) and water (H_2O), into organic substances (biomass) such as carbohydrates and amino acids, using the sun's energy (Lawlor, 1987). As a result there is a straight correlation between the net amount of CO_2 used (i.e. the amount of biomass fixed) and the amount of water use. The ratio of CO_2 to H_2O is the instantaneous water use efficiency (WUEi) and is a characteristic of plant physiological capability that can be measured by gas exchanges assessment techniques. One by-product of photosynthesis is oxygen (O_2) which is generated by the breaking down of water and carbon dioxide molecules. CO_2, O_2 and H_2O (vapour phase) use the stomata as main pathway and as result if a drought occurs stomata may close as short term water saving mechanism and prevent the entry of CO_2 and the readily available water for photosynthesis might also be limited; this can significantly affect photosynthetic rates. Therefore monitoring photosynthesis is also a preferred method to monitor plant physiological state.

Promising techniques of assessment of photosynthesis performance were developed including chlorophyll a fluorescence which was reported as able to "*check the vitality of plant and document the stress effect on the photosynthetic apparatus*" (Lichtenthaler et al., 1986). Chlorophyll fluorescence assessment is a non invasive technique and can be repeated throughout long periods of time on the same leaf sample. Generally, the techniques employed involve the use of indicators derived from the observation made by Kautsky and Frank, (1943) quoted by Lichtenthaler et al (1986) which define the relationship between chlorophyll fluorescence and photosynthetic activity. There are two separate photochemical steps during photosynthesis which are associated with different group of pigments; these groups have become known as two distinct photosystems: PSI and PSII (Lawlor, 1987). Commonly, the kinetics of photochemical quenching allows quantifying photosynthesis efficiency by measuring chlorophyll fluorescence when the photosystems are illuminated with different intensities of light. In practice, 95% of the chlorophyll fluorescence signal observed under physiological temperature is emitted from pigments of PSII; hence chlorophyll fluorescence assessments reflect the efficiency with which absorbed light is used for PSII photochemistry (Govindjee, 1995).

Numerous articles have been published on the application of chlorophyll fluorescence assessments to study the effect of stresses (e.g. Lichtenthaler and Rinderle, 1988; Shabala et al., 1998; Flexas et al., 2000). The applications are wide as ultimately many stresses such as heat, leaf damage, light, drought, nutrient deficiency were reported to affect photosynthesis (Lichtenthaler, 1988; Bolhàr-Nordenkampf and Öquist, 1993) and could be potentially quantified via assessment of chlorophyll fluorescence. The effects of stresses on the photosynthetic apparatus were generally call photoinhibition.

The study of chlorophyll a fluorescence has been intensive on several genera of plants (e.g. Basu et al., 1998; Flexas et al., 2000). Most of those studies focused on the effect of a stress onto the physiology of one species. Chlorophyll fluorescence was recently introduced in the study of *Salix* physiology (Lennartsson and Ögren, 2002; Liu et al., 2003). So far a single experiment was reported to use the technique to study a natural stress on *Salix* hybrids; this was to study cold hardening effect among six *Salix* hybrids categorised according to their latitudes of origin (Lennartsson and Ögren, 2002). In their study Lennartsson and Ögren assessed the stem chlorophyll fluorescence over a range of cold temperature before and after cold hardening. They concluded that period of cold hardening lowered the temperature at which frost damaged the stem photosynthetic apparatus. However no significantly different behaviour among their hybrids population were reported. At time of this report being written, no studies were able to differentiate stress responses among a population of closely related hybrids of higher plants via chlorophyll fluorescence.

The environmental factors and specific morphological attributes are partially responsible for WUE variations between varieties of the same genus (DaMatta et al., 2003; Solomon and Labuschagne, 2003). Differences in WUE between *Salix* varieties are identified in this study and others (Jørgensen and Schelde, 2001; Weih and Nordh, 2002). The roots and leaves are the plant-environment interfaces and the study of their physiology is a way forward to contribute to the understanding of the process involved in WUE. The trials where the physiology of *Salix* was closely studied are extremely rare since interest in improving this tree genus is relatively recent. The few reports available were unable to provide explanation of the total morphological and physiological traits responsible for a specific WUE. Consequently, to date no set of indicator hybrids were clearly identified as drought tolerant as it is used in willow SRC biomass trial (Lindegaard et al., 2001).

The morpho-physiological features combine into a plant ability to resist drought or to have high WUE. A closer look at the short term or instant processes involved in water use might be an alternative to differentiate accurately willow hybrids. This type of assessment would provide the end result of the combinations of features that characterise each hybrid. A set of experiments on the roots abilities to abstract water, the short term and instant processes of water use took place on the Silsoe lysimeter trial described in the Chapter 4. The lysimeter trial was set up to monitor the effect of water stress among a population of five high yielding *Salix* hybrids of contrasting performance under stress condition in the 2002 Silsoe trial[19]. The objectives were to:

1. evaluate the development of water deficit over as drought stress increases;
2. characterise the dynamic of root growth in lysimeters;
3. examine the daily water use pattern at different soil moisture levels and;
4. evaluate the leaf transpiration and photosynthetic activity over a progressive water stress.

6.2. Material and methods

The research was carried out outdoors, at Cranfield University, Silsoe, Bedfordshire during the summer 2003.

6.2.1. Treatments

Details of the lysimeter experiment and of the treatments imposed are presented in Chapter 4. The treatments are repeated here for ease of reference.

Five hybrids were selected for inclusion: Tora and Ashton Stott as reference varieties, and three new hybrids Resolution, Endurance and LA980289. These represent the major genotypes included in the breeding programme.

Half of the lysimeters were irrigated throughout the summer (wet regime); the others were submitted to periods of water stress (dry regime). Water stress was induced by cessation of irrigation for periods (drying cycles) sufficient enough to allow records of physiological changes. A total of four drying cycles were imposed to the plants of the dry regime (Table 6-1[20]).

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[19] See Chapter 2 and 3.

[20] See Chapter 4 for the exact schedule of irrigation.

Table 6-1. Summary table of the four drying cycles imposed to the plants grown under the dry regime. Lysimeter trial; Silsoe, Bedfordshire; 2003

Drying cycle	Period
DC1	10/06/03 – 7/07/03
DC2	16/07/03 – 12/08/03
DC3	12/08/03 – 26/08/03
DC4	26/08/03 – 14/09/03

6.2.2. Soil water and water use

The soil water content was monitored to calculate daily water use and the capacity of the hybrids to abstract water. Two methods were used to monitor: i) differences in water content through the profile; and ii) variations in the mass balance of the lysimeters.

Profile soil water was monitored using a capacitance probe (Sentek Pty Ltd, Adelaide, South Australia), which measures the soil dielectric constant around the sensor and relates those measurements to soil water content. The water dielectric constant is greater than the dielectric of the soil particles matrix and air. As such soil (soil particles matrix + air + water) dielectric is dominated by the water fraction (Fares and Alva, 1999). The DIVINER2000® has been widely used including in similar lysimeters at Silsoe (Martin and Stephens, in press-a; Martin and Stephens, in press-c).

In each lysimeter a DIVINER2000® access tube was placed at 0.15 m to the north-east of the stool allowing soil water content to be recorded at 0.1 m intervals from 0.1 m to 0.7 m depth. The soil moisture was measured daily usually in the evening before irrigation.

Any excess water from rainfall or irrigation drained out of the lysimeter and was collected, thus water logging did not occur in the lysimeters.

The soil water content was maintained close to the upper drainable limit (UDL) during the establishment period. The UDL of individual lysimeter were calculated as the mean soil water content between 10/04/03 and 06/06/03.

As the same probe was used to monitor all the lysimeters, differences between UDL (mm 0.7 m^{-1}) were assumed to be mainly due to the preparation of the lysimeters which may have induced slight variations of soil compaction throughout the profile or to the accuracy of installation of the access tubes. Therefore, to facilitate comparison between individuals, the data were recalculated as soil water deficit (SWD) from UDL.

The mass balance of the dry treatment imposed on lysimeters in one replicate was montitored using the five load cells (Griffith Elder, Bury St Edmunds, UK) described in Chapter 4, which allowed the water use to be calculated at 20 minute intervals.

Difficulties were experienced with DATAMAID® software (Griffith Elder & co ltd, Bury St Edmunds, UK) which was unable to record data between 1/07/03 and 7/08/03. Some random errors also occurred while logging in addition to errors caused by wind pressure on the lysimeters. The most common error was that the software truncated one or two decimal places giving errors of up to 1 kg. Consequently the data recorded by DATAMAID® software had to be filtered before they were analysed. However, little errors close to the limit of detection of the equipment (0.02 kg) must have remained in the data set.

The load cells were mainly used to visualise and compare daily patterns of water use at different soil moisture contents. To compare the five hybrids, the water use rates were calculated every 20 minutes and were regrouped per period of time for analysis. Due to software problems numerous data were missing and some were erroneous and therefore had to be discarded; as a result the number of observations were not constant for each load cell.

Since all the lysimeters were not the same mass at the beginning of the experiment, the data were analysed as variation of mass per unit of time. The variation of mass within the time scale used (20 minutes) was predominantly due to the addition or abstraction of water from the lysimeter, the gain in biomass from the plant being negligible in comparison to the water used from it during that time span.

These variations were, however, close to the limit of detection of the equipment (±20 g) so a 2 h running mean was used to reduce errors. Finally, from the data, a slope of water use over a 2 hour period was calculated every 20 minutes.

6.2.3. Stomatal conductance (g_s), Photosynthetic rate (A), instantaneous water use efficiency (WUEi) and leaf temperature

CO_2, O_2 and H_2O transit between the air and the leaf (Weyers and Meider, 1990), their fluxes can be monitored continuously (Lawlor, 1987) and the results can be used to characterise the transpiration and photosynthetic activity of the leaf tissue assessed. Gas exchange assessments (H_2O, CO_2) were carried out in vivo on fresh leaves as it is extensively done on similar experiment performed on other crops (e.g. Cerovic et al., 1996; Basu et al., 1998; Osmond et al., 1999). The assessments were performed on all plants of the Replicate II, on three leaves chosen being the 10th, 11th and 12th leaf from the apex of the tallest shoot if not damaged. Those leaves were chosen because across the five hybrids used, these were fresh leaves but mature, fully developed and barely shaded.

The gas exchange and leaf temperature were recorded using PP Systems CIRAS-1 Portable photosynthesis system (PP_Systems, Arlesey, UK); (PP_Systems, 2003). The instrument was set with a narrow leaf cuvette PCL4 50 mm x 25 mm. CIRAS-1 calculates a series of variables characterising the photosynthetic activity of the leaf sample entrapped in the leaf cuvette from the gases exchanges from it. Plate 9 illustrates the use of the equipment in the lysimeter trial at Silsoe in 2003.

Plate 9. Using the CIRAS-1 on willow leaves, Lysimeter trial; Silsoe; Bedfordshire; 2003.

CIRAS-1 is a mass flow system which measures the change in water vapour and CO_2 concentration and the volume of gas moving through the cuvette. From those measurements, CIRAS-1 calculates the rate of CO_2 uptake and the rate of H_2O transpired. It measures the gases using a system of detection using an infra red source; the gases with di-atomic molecules such as CO_2 and H_2O absorb strongly photons in the infra-red range. The CIRAS-1 supplies the leaf sample with a continuous flow of standard dehumidified air and is able to measure the quantity of CO_2 in and out of the leaf cuvette and the quantity of H_20 released from the leaf sample. The resolution of the apparatus for CO_2 is 0.2 μmol mol^{-1} at 0 ppm and 0.7 μmol mol^{-1} at 2,000 ppm; and for H_2O is 0.03 Pa at 0 Pa and 0.06 Pa at 75 Pa partial vapour pressure.

Note that the typical CO_2 atmospheric concentration is 360 ppm. While for this experiment, the CO_2 was supplied above atmospheric condition, at 600 ppm in order that CO_2 supply does not become a limiting factor to photosynthetic activity. Indeed, after preliminary experiments to finalise the setup of the CIRAS-1, it appeared that the CO_2 supply would not stabilise around the atmospheric concentration but that reasonable stabilisation was obtained at 600 ppm.

Photosynthesis is enhanced by elevated CO_2 (Karnosky, 2003) and it was reported that it had an impact on the photosynthetic rate and g_s of four *Brassica* species when submitted to water stress (Mishra et al., 1999) and also for *Eucalyptus cladocalyx (Palanisamy, 1999)*. However the measure did not alter the interpretation of the water stress effect, as the elevated CO_2 concentration has just amplified the signal and resulted in unaffected relative changes. As such it was assumed that elevated CO_2 would not alter the outcome of study on water stress on willow photosynthesis seeking to classify hybrids according to their drought resistance.

Similarly, a light source providing at 900 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) was used to standardise the assessment from leaf to leaf and avoid the errors due to the weather, time of the day and possible shading from leaves and stems during the assessment. These have been used in other experiments to study water stress over periods longer than a day e.g. (Parsons et al., 1997; Yin et al., 2004).

From mass flow measurements CIRAS-1 calculates: the stomatal conductance (g_s) in mol H_2O m^{-2} s^{-1}; the photosynthetic rate (A) in µmol CO_2 m^{-2} s^{-1}; and the instantaneous water use efficiency (WUEi) as the ratio of A/g_s. It also measures the leaf temperature.

In order to establish a diurnal pattern of photosynthetic activity, these variables were measured at hourly intervals from sunrise to sunset on nine occasions at the most (weather permitting) between 01/09/03 and 16/09/03 on the 10 plants of the second replicate of the lysimeter trial. The results of this experiment were used to specify the period during which the measurements of photosynthetic activity of willow coppice through gas exchange analysis were fairly constant.

Measurements were carried out daily, weather permitting, from 10:00 to 15:00 between 08/08/03 and 29/08/03 on the same plants. This schedule was set for two reasons. The first to measure the photosynthetic activity at a time of the day when it is supposed to reach its maximum as the solar radiations are the greatest. Secondly because during those three weeks of experiment the plants under the dry regime experienced water stress, then were irrigated with 10 l on 12/08/03 and left to dry until 26/08/03 when the plant where irrigated again with 2 l.

6.2.4. Chlorophyll fluorescence

Most authors use the same nomenclature to characterise photosynthesis measured by chlorophyll fluorescence. Most of it was described by Van Kooten and Snel, (1990) and some other parameters were added later (See Table 6-2). The parameters divide into two sections: “fluorescence intensity indicators” and “fluorescence quenching parameters”.

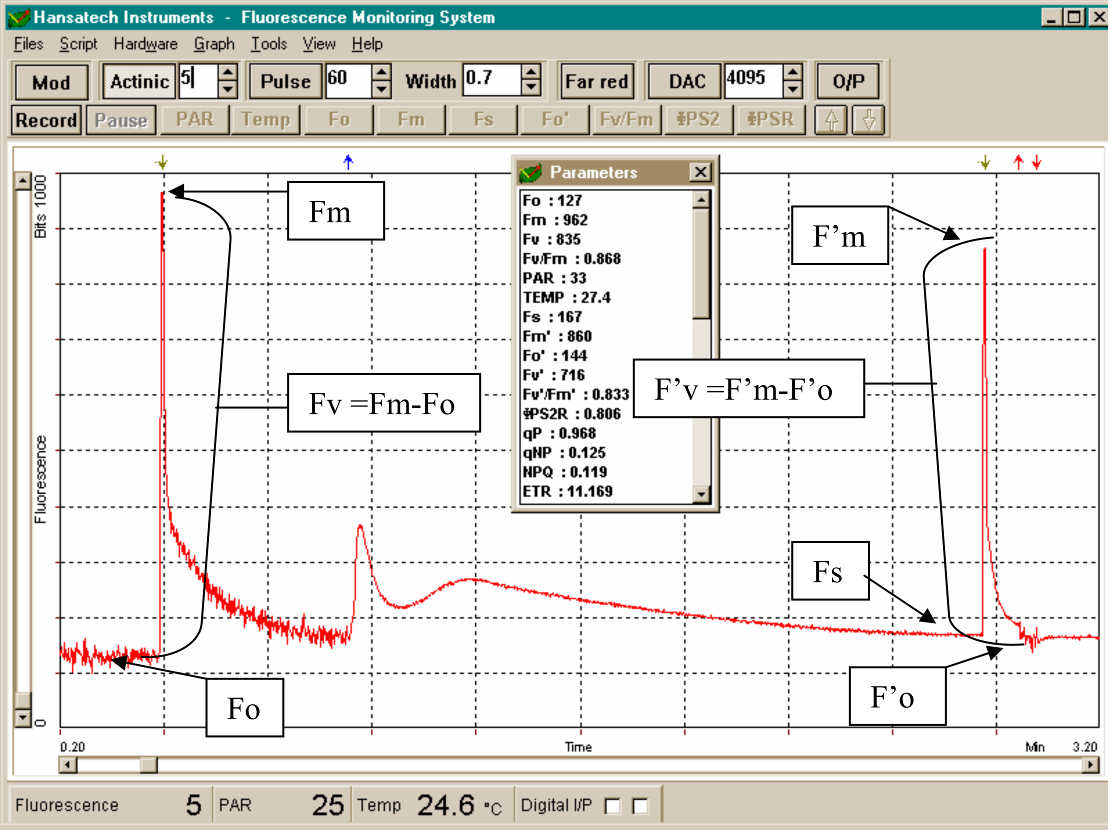
Table 6-2. Parameters used to study fluorescence and definition of chlorophyll fluorescence nomenclature adapted from Van Kooten and Snel, (1990)

A: Fluorescence intensity indicator		Description	Units
F	Fluorescence intensity	Actual fluorescence at any time	Bits
Fo	Minimal fluorescence (dark)	Fluorescence intensity with all PSII reactions centre open while the photosynthetic membrane is in a non-energised state, i.e., dark or low light adapted $Q_P = 1$ and $Q_N = 0$.	Bits
Fm	Maximal fluorescence (dark)	Fluorescence intensity with all PSII reactions centres closed (i.e., $Q_P = 0$) all non-photochemical quenching processes are at a minimum (i.e., $Q_N = 0$). This is the classical maximum fluorescence level in the dark or low light adapted state	Bits
Fv	Variable fluorescence (dark)	Maximum variable fluorescence in the state when all non-photochemical processes are at minimum, i.e., (Fm-Fo)	Bits
Fs	Fluorescence at steady state (light)	Steady state is defined by the author as a period within which the fluorescence intensity does not change while the external circumstances remain constant	Bits
F'o	Minimal fluorescence (light)	Fluorescence intensity with all PSII reactions centres open in any light adapted state, i.e., $Q_P = 1$ and $Q_N \geq 0$.	Bits
F'm	Maximal fluorescence (light)	Fluorescence intensity with all PSII reactions centres closed at any light adapted state, i.e., $Q_P = 0$ and $Q_N \geq 0$.	Bits
F'v	Variable fluorescence (light)	Maximum variable fluorescence in any light adapted state, i.e., (F'm-F'o)	Bits
B: Fluorescence quenching parameter			
Q_P	Photochemical quenching	(F'm-F)/(F'm-F'o) or (F'm-Fs)/(Fm'-Fo)	No unit
Q_N	Non-photochemical quenching	1-(F'm-F'o)/(Fm-Fo) or (Fm-F'm)/(Fm-Fo)	No unit
	Potential quantum yield	Fv/Fm	No unit
	Open PSII energy capture efficiency	F'v/F'm (Colom and Vazzana, 2003)	No unit
NPQ	Non-photochemical quenching	(Fm-F'm)/F'm	No unit
Φ_{PSII}	Quantum efficiency of PSII	(F'm-Fs)/F'm (Genty et al., 1989)	mol e^- mol $p^{+\ -1}$
ETR	Electron transport rate	PAR*0.5*Φ_{PSII}*0.84	No unit

Note: Bits are used as relative units and are specific to the setting of the assessing equipment; PSII is photosystem II; e^- are electrons, p^+ are photons absorbed.

In Figure 6-1, the fluorescence indicators, described in Table 6-2, are annotated on a light response curve of an ornamental tree *Ficus benjamina*. This tree was used to carry out preliminary experiment on the use of a portable modulated Fluorescence Monitoring System (FMS 2) (Hansatech Instruments Ltd, King's Lynn, UK) and because it needs the FMS 2 to be connected to a computer while recording to provide illustration (Figure 6-1).

The leaf sample was dark-adapted for 40 minutes, and successively, Fo and Fm were measured. The leaf sample was then light adapted with actinic light to measure Fv and F'm. F'o was measured after infrared illumination. Arrows on the figure indicates the sequence of illumination.



Actinic 5
Pulse 60
Width 0.7
DAC 4095

```
Fo  : 127
Fm  : 962
Fv  : 835
Fv/Fm  : 0.868
PAR  : 33
TEMP  : 27.4
Fs  : 167
Fm'  : 860
Fo'  : 144
Fv'  : 716
Fv'/Fm'  : 0.833
ΦPS2R  : 0.806
qP  : 0.968
qNP  : 0.125
NPQ  : 0.119
ETR  : 11.169
```

Fluorescence 5 PAR 25 Temp 24.6 °C

Figure 6-1. Two phases light response curve on Ficus benjamina, from the dark-adapted state, to light-adapted state. Light adaptation at 300 µmol m^{-2} s^{-1}. Indicators measured in bits and time in minutes; indoor experiment; Silsoe, Bedfordshire; 2002

Chlorophyll fluorescence was measured using FMS 2 on the same leaves selected as in the above experiment on gas exchange analysis. FMS 2 can measure instantaneous measurements of chlorophyll fluorescence and all fluorescence intensity indicators and fluorescence quenching parameters summarised in Table 6-2 can be assessed and calculated. FMS 2 is equipped with actinic (blue) and infra-red light sources to perform the different assessments.

Light-adapted experiments required standardisation of the photosynthetically active radiation (PAR) to be able to compare fluorescence indicators. For example, Fs was defined as the fluorescence measured during a period within which the fluorescence intensity does not change while the external circumstances remain constant (Van Kooten and Snel, 1990). Under natural environment, sunlight and therefore PAR changed inducing unsuitability of the measurements. Experiment on light adapted leaf samples required standardisation of illuminations.

It was decided after preliminary experiments to omit measurements of dark adapted fluorescence intensity indicators (Fo, Fm, Fv) for two reasons. Firstly, the leaf clips provided by Hansatech Instruments Ltd to dark adapt leaf samples were not uniformly manufactured and could possibly generate errors of the readings those being hard to quantify. Secondly, the method required a minimum of 30 minutes darkness (Bolhàr-Nordenkampf and Öquist, 1993) which was complex to schedule in case of numerous and repetitive assessments as it can be required by breeding programmes.

Finally from the measurement of light adapted fluorescence indicators (Fs and F'm), the open PSII energy capture efficiency (F'v/F'm) and the effective quantum efficiency of PSII (Φ_{PSII}) could be calculated (Table 6-2). All these fluorescence parameters measured in similar experiments on other genera subjected to water stress were reported to be indicators of the physiological state under water stress sensitivity or state.

A brief account of the use of the chlorophyll technique used here and in different experiment is given below to review the current use of chlorophyll fluorescence in plant physiology.

Flexas et al. (2002) reported that the ratio Fs/Fo could be used to quantify water stress from grapevine leaves, although Fo was not measured it did not appear necessary as Cerovic et al. (1996) reported from an experiment on sugar beet, maize and a the succulent plant *Kalanchoë sp* that "*Fo was remarkably stable among individuals and* *plant species, either in the presence or absence of stress*". Therefore Fo variations between hybrids and between water regime were assumed to be negligible and Fs was measured and analysed as a single factor.

F'm was not reported to be used as a single parameter to quantify of plant physiology in higher plants, but F'm is widely use to calculate other photosynthesis parameters (Table 6-2). Nevertheless in a recent study, F'm was used as a stress indicator; Altamirano et al. (2004) manufactured a biosensor using chlorophyll fluorescence to detect a chemical using a wild (sensitive to the chemical) and a mutant (resistant) strain of algae (*Dictyosphaerium chlorelloides*). They concluded that F'm of both strains of algae were reduced in different proportion when submitted to the same chemical stress; the sensitive strain having the greatest decrease of F'm in comparison to the resistant one.

Chlorophyll fluorescence was measured on light adapted potato leaves (*Solanum tuberosum* L. cv. Kufri Sindhuri) while the plant was submitted to water stress (Basu et al., 1998). In this article, the authors reported that Φ_{PSII} exhibited a decline at high irradiance (>500 µmol m^{-2} s^{-1} PAR) when the plants experienced water stress.

In a more recent study on two cultivars of the C4 grass, *Eragostis Curvula*, one being water stress sensitive the other being resistant (Colom and Vazzana, 2003), it was demonstrated that after a period of two weeks of water stress, the photosynthetic activity measured under light adapted condition via chlorophyll fluorescence (F'v/F'm and Φ_{PSII}) and gas exchange analysis under saturated CO_2 environment (g_s and A), were significantly reduced for the sensitive cultivar while it was less affected for the resistant one. In addition, Colom and Vazzana, (2003) suggested that the drought resistance of the resistant cultivar could be attributed to a higher WUEi.

In practice, the leaf clip for light adapted measurements was placed in the middle of the lamina, the extremity of the optic fiber used as light source and light receptor, positioned at 10 mm from the leaf surface. This was close enough to record accurately chlorophyll fluorescence and far enough to prevent tissue damage from the saturating pulse. FMS2 was programmed to measure Fs after an actinic illumination of 600 µmol m^{-2} s^{-1} provided by a light emitting diode (LED) for 60 s, F'm after an halogen illumination of 10,800 μmol m^{-2} s^{-1} for 0.7 s and F'o after an infra-red illumination of 7 μmol m^{-2} s^{-1} at 735 nm provided by a LED for 5 s.

The measurement took place concurrently with the CIRAS-1 between 08/08/03 and 29/08/03 to check on the evolution of the photosynthetic activity over an evolving water stress as stated in the previous sections. Only one attempt of an all day measurement was carried on 5/09/03 but this was not conclusive and did not add any information (results not presented). In addition a combination of both gas analysis and chlorophyll fluorescence results in difficulty of scheduling.

6.2.5. Statistical analysis

As the lysimeter trial design was a randomised block design one-way and factorial analyses of variance at $p < 0.05$ (ANOVA) were used to compare the hybrids, the water regimes their combinations and the time and or date of assessment. 95% confidence intervals were extracted from the ANOVA and used to compare pairs of hybrids, treatments or their combinations. In cases where different numbers of observations were made, e.g. because of errors in measuring water use rates; the 95% confidence intervals and least significant differences were computed using *post hoc* tests at $p < 0.05$ (Fisher LSD test). Treatments that were not significantly different from each other were identified using the same subscripts.

6.3. Results

6.3.1. Soil water

An example of soil water content in lysimeters in which Ashton Stott was grown illustrates the different UDL values in individual lysimeters (Figure 6-2). Soil water status was therefore standardised to give the soil water deficit (SWD).

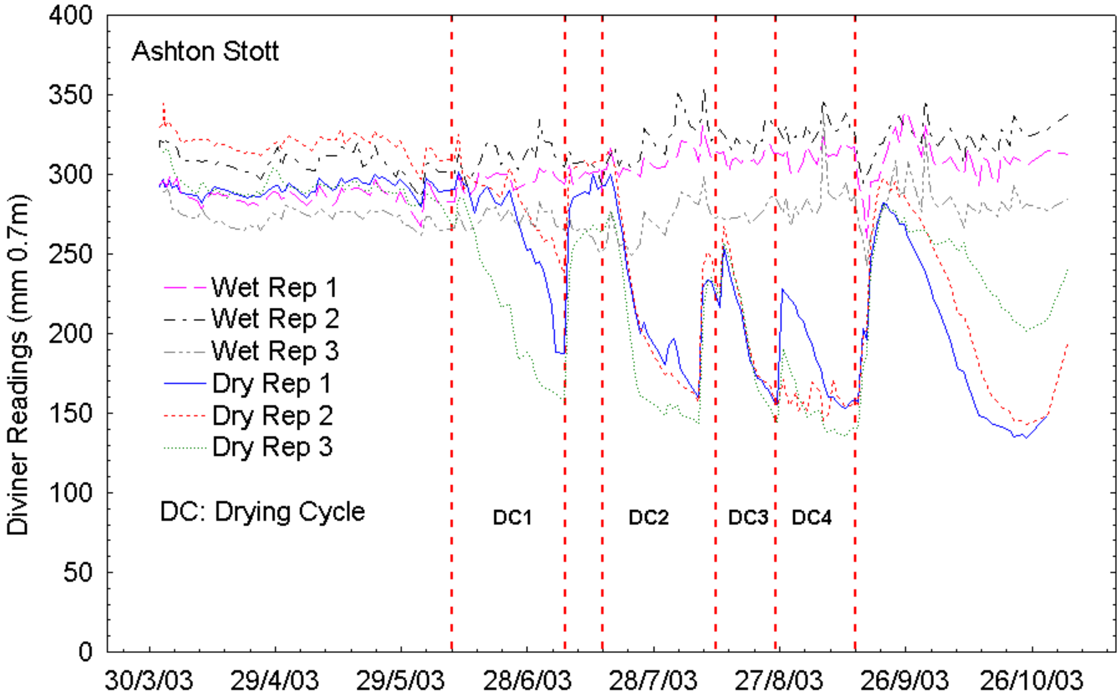


Figure 6-2. DIVINER2000® readings from 6 lysimeters in which Ashton Stott was grown under the wet and the dry regime between 1/04/03 and 3/11/03. Lysimeter trial; Silsoe, Bedfordshire. The drying cycles are indicated as DC 1 2 3 and 4

A factorial analysis of variance of SWD over 164 days on which soil water record were recorded, revealed significant differences between the water regime, the hybrids and the replicates. The first replicate under the dry regime generated a significantly smaller soil water deficit than the second and third for Tora and Endurance. For the three other hybrids, the deficit in replicate 1 was the smallest but not significantly different. The difference between replicate 1 and the other two was due to the difference of irrigation doses during DC 4. Therefore, the origin of stool[21] had no significant effect on the water deficit observed in the lysimeter experiment.

The average soil moisture deficit was pooled for all varieties in the wet treatment but is presented individually for each hybrid grown under the dry regime in Figure 6-3.

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[21] From the pot experiment 2002 for replicate 1 and 2, from the field experiment 2002 for replicate 3.

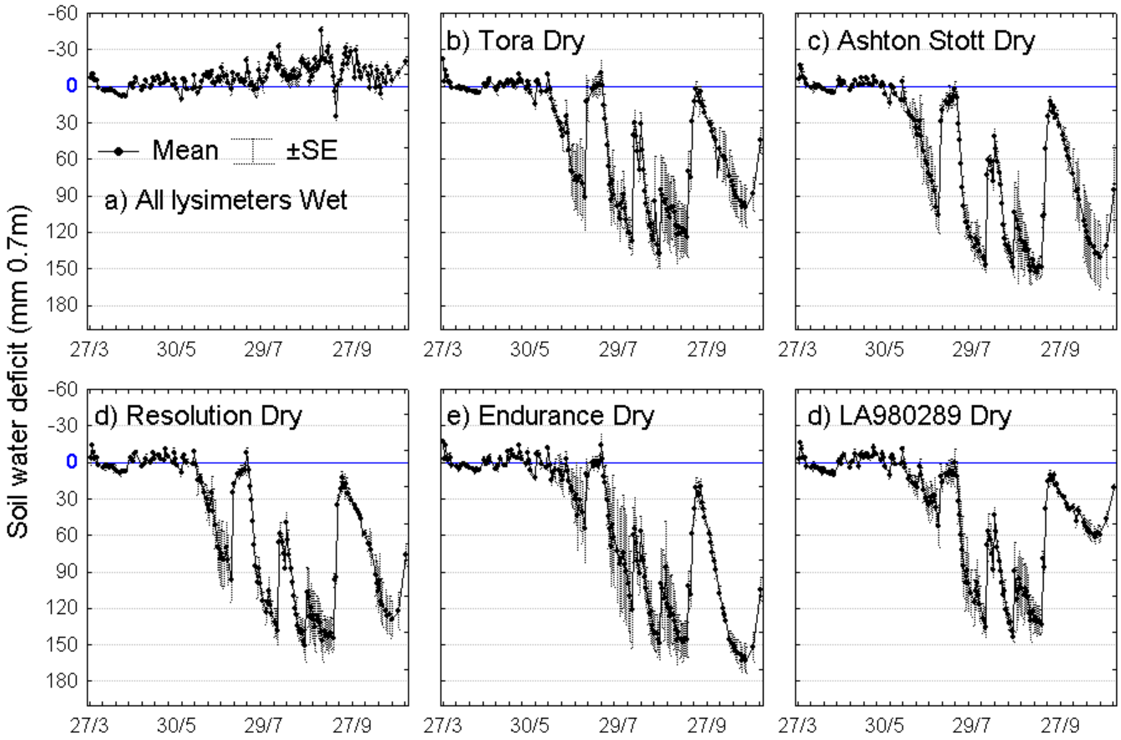

Figure 6-3. Average soil water deficit of a) 15 willow trees grown in lysimeters under the wet regime and three willow trees grown under the dry regime for b) Tora, c) Ashton Stott, d) Resolution and e) LA980289 between 1/04/03 and 3/11/03; Silsoe, Bedfordshire

For all lysimeters under the wet regime the deficit was between -50 and +29 mm 0.7 m^{-1} (Figure 6-3-a). It appeared that gradually over the season SWD decreased and tended to be negative, meaning that the soil water or UDL increased. Two main factors may have induced an increase of the dielectric of the soil at UDL; the first was the development of roots around the access tube and the second was probably caused by the soil filling of air gaps created when the access tubes were installed. During the small drying cycle that the plants of the wet regime experienced between 18/07/03 and 22/07/03, no records of the soil moisture were carried out and therefore the soil water deficit could not be quantified for that period. Overall, the soil water content was maintained at or above UDL for all willow trees of the wet regime.

Under the dry regime, SWD was close to 0 during the establishment period (Figure 6-3-b-c-d-e-f), For all hybrids, during DC 1 SWD developed at a constant rate and no significant differences between the varieties were recorded on 6/06/03, even if it is clear that Tora, Ashton Stott and Resolution reach a greater deficit than Endurance and LA980289.

During DC 2, DC 3 and DC 4 SWD increased at constant rate, until soil water became harder to extract and the plants experienced water stress.

By the end of DC 2, Tora, Ashton Stott, Resolution, Endurance and LA980289 had reached an average SWD 127, 147, 138, 121 and 135 mm 0.7 m^{-1} respectively; but no significant differences were recorded between the hybrids.

In DC 3 the SWD started at 30 to 60 mm after two irrigations and increased to between 138 and 151 mm 0.7 m^{-1} though no significant differences were observed. Similarly, in DC 4 the SWD was between 90 and 110 mm 0.7 m^{-1} at the beginning of the period. The SWD increased slowly in comparison to previous drying cycles, probably because of the smaller total leaf area[22]. SWD stabilised after 12 days in all cases and for the remaining eight days, no significant increases of SWD were recorded. Over the last eight days of DC4, the SWD of 113 and 123 mm 0.7 m^{-1} for Tora and LA980289 respectively were reached and these significantly smaller than the ones of 136, 140 and 144 mm 0.7 m^{-1} for Resolution, Endurance and Ashton Stott respectively. It is important to note that the *S. burjatica* and *S. dasyclados* related hybrids extracted the most water from the soil.

Following DC 4, the lysimeter were irrigated to return the soil water content close to the UDL. Except for LA980289, the hybrids were able to use most of the available water, until it rained for nine days starting on 27/09/03. Thereafter the apparent reduction in the rate of water use is related to the increasing water inputs from rain.

The SWD at 0.6 m during DC 1 indicated the differential ability of hybrids to explore the soil profile in the lysimeters (Figure 6-4).

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[22] See Chapter 5 result section for more details.

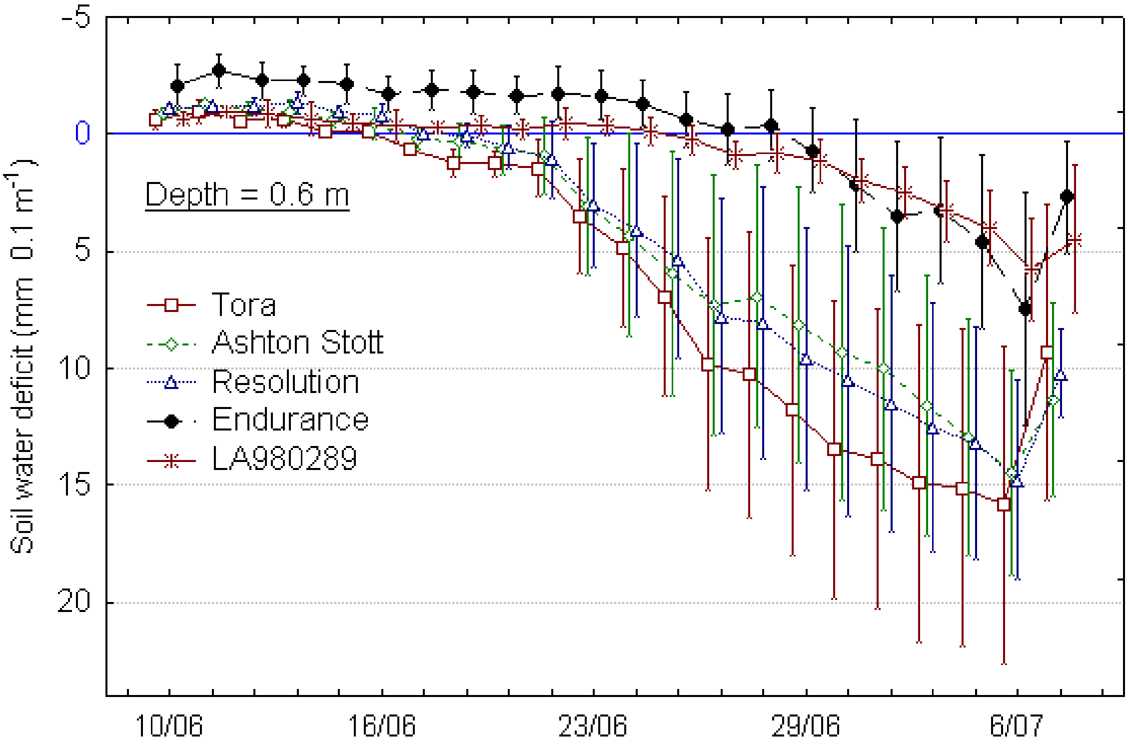

Figure 6-4. Average soil water deficit progress at 60 cm for five hybrids grown in lysimeter under the dry regime during DC 1 (10/06/03-7/07/03); Silsoe, Bedfordshire 2003. The errors bars represent the standard errors of the mean

Until 16/06 no water uptake from below 0.6 m was recorded for any of the hybrids but the hybrids then divided into two groups, with Tora, Ashton Stott and Resolution rapidly increasing the SWD from 17/06/03 and the Endurance and LA980289 beginning to abstract water 8 days later and at a slower rate.

By DC 2, the whole profile had been explored in all lysimeters. The increase in maximum SWD from one drying cycle to the next, however, might be explained by an increased root density and consequently soil exploration and ability to extract water at the different depths of the profile.

6.3.2. Daily water use pattern over progressive water stress

Figure 6-5 demonstrates the mass balance for Ashton Stott grown in Lysimeter 13 under the dry regime and illustrates the periods when no data were recorded.

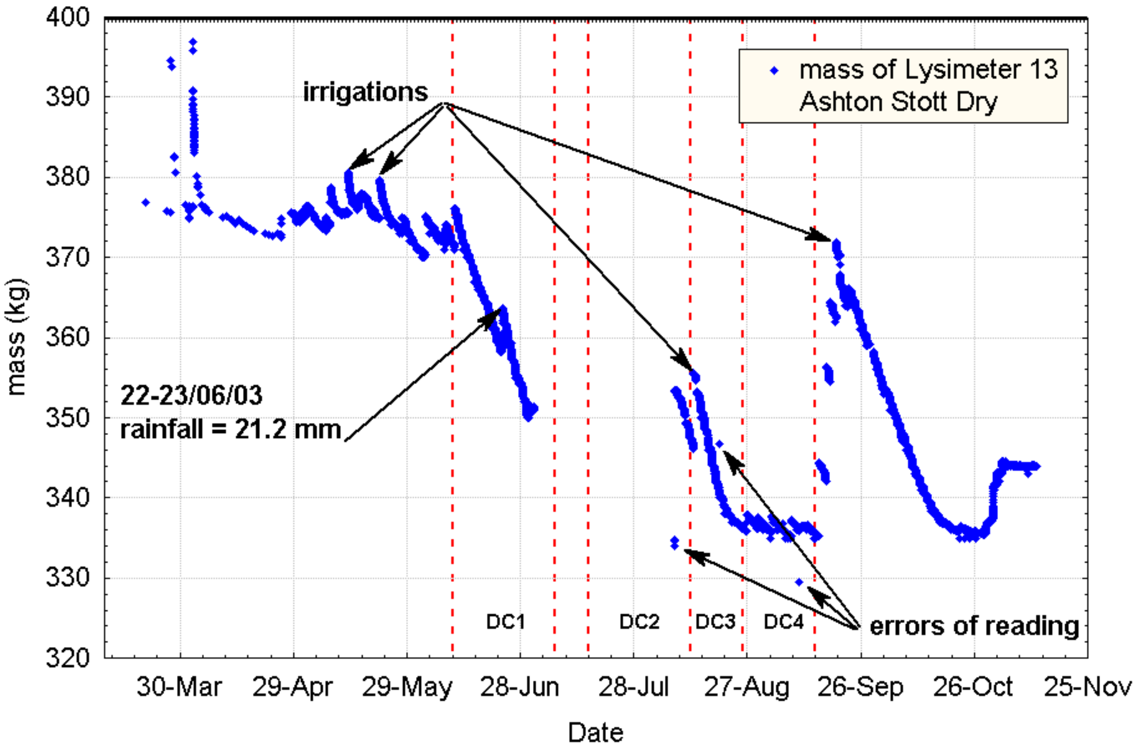

Figure 6-5. Load cells records from lysimeter 13, Ashton Stott dry regime; Lysimeter trial; Silsoe, Bedfordshire; 2003. The drying cycles are indicated as DC 1 2 3 and 4, some examples of irrigations are pointed as well as some examples of the errors recorded for unknown reasons. Rainfall of 21.2 mm that spread over 22nd and 23rd of June is also indicated

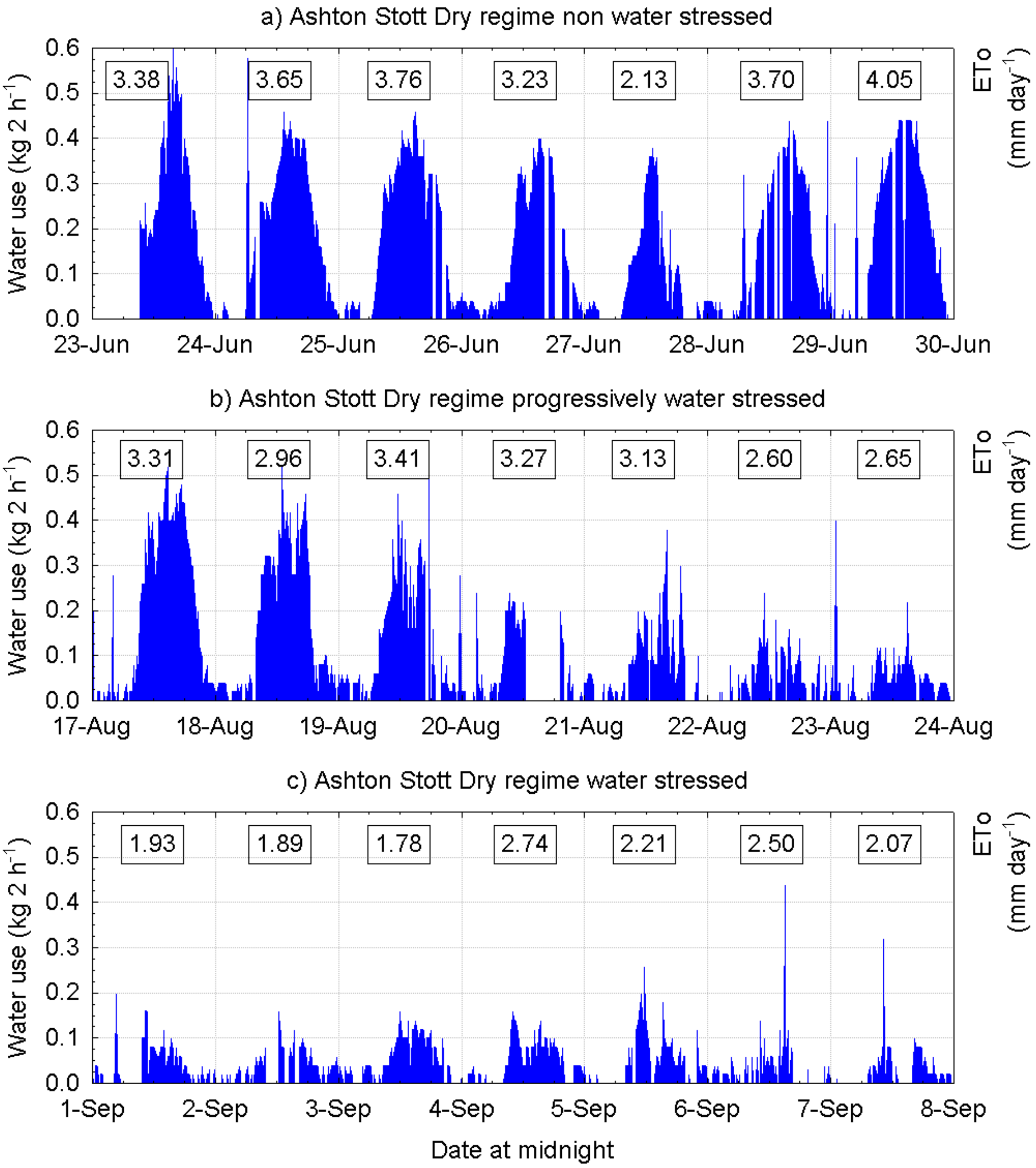

Figure 6-6. Moving average of water use rate of a Salix hybrid (Ashton Stott, lysimeter 12) at three different stage during the growing season, on a) non water stress; b) progressively water stressed; c) water stressed; Lysimeter trial; Silsoe, Bedfordshire; 2003. Daily ETo are indicated as extra information to quantify the environmental conditions

In Figure 6-6 water use is illustrated as a rate (mass per unit of time). As a result of the malfunction of DATAMAID® software and data filtering some gaps appeared in the data set; i.e in Figure 6-6-b on 20/08/03 data was missing from 12:15 until 17:15. The sharp peaks might be smaller errors that were not filtered and left in the data set.

Nevertheless, the diurnal pattern of water use rate was clearly identified, with little or no water use at night, a progressive increase to reach the maximum rate around 14:00 (when not stressed, Figure 6-6-a) and a decrease towards the end of the day.

In Figure 6-6-a, the water use of Ashton Stott grown in lysimeter 13 under the dry regime over a period when the plant was not water stressed (middle of DC 1) showed that the water use during the days described very similar patterns in shape and magnitude, the differences can be attributed to the environmental conditions.

In Figure 6-6-b, water use by the same plant recorded on the last few days of DC 3 showed a progressive decrease in the rate of water use in the early afternoon. In Figure 6-6-c, the plant was maintained under water stress (middle of DC 4). The diurnal pattern of water use rate can still be seen, but was greatly reduced.

In order to compare the hybrids, the rate of water use between 13:00 and 15:00 were analysed for three periods. Post hoc analyses were used to compare hybrids pair-wise.

Table 6-3. Mean water use rate of five Salix hybrids between 13:00 and 15:00 over three different periods characterised by different levels of water stress. Lysimeter trial; Silsoe, Bedfordshire; 2003. Values with the same letter appended were not significantly different using Fisher least significant differences (LSD) post hoc grouping

Period (water status)	Tora WU kg 2 h^{-1}	Ashton Stott WU kg 2 h^{-1}	Resolution WU kg 2 h^{-1}	Endurance WU kg 2 h^{-1}	LA980289 WU kg 2 h^{-1}
10/06/03-01/07/03 (unstressed)	0.317^{a}	0.285^{a}	0.288^{a}	0.299^{a}	0.308^{a}
17/08/03-24/08/03 (progressively stressed)	0.119^{a}	0.105^{a}	0.245^{bc}	0.197^{b}	0.277^{c}
1/09/03-8/09/03 (stressed)	0.046^{a}	0.075^{b}	0.109^{c}	0.105^{c}	0.095^{bc}

In unstressed conditions, no differences between the hybrids were observed for the water use rate between 13:00 and 15:00. As the water stress developed, however, the water use by hybrids decreased differentially with water stress. Tora and Ashton Stott had significantly lower rates of water use than the three other hybrids during late August. LA980289 maintained the highest water use rate similar to that in June and had a significantly greater rate than Endurance. Resolution had a rate between Endurance and LA980289 but was significantly different from them.

In the middle of DC 4, the willows in the dry treatment were water stressed. At that time, Tora had the lowest water use rate which was significantly lower from the four other hybrids. Ashton Stott had the second lowest rate that was significantly lower than the two highest rates observed among the selection, the ones of Resolution and Endurance. LA980289 rate fell in between but was not significantly different than the ones of Ashton Stott on the one side and Resolution and Endurance on the other.

6.3.3. Stomatal conductance (g_s), photosynthetic rate (A) and instantaneous water use efficiency (WUEi)

Between 1/09/03 and 16/09/03, not all days were appropriate to measure the diurnal gas exchanges of the five *Salix* hybrids, due to rainfall or wind some measurements sessions had to be stopped. Only three days, 5/09/03, 13/09/03 and 16/09/03 were suitable for all-day-long sessions gas exchange measurements. The measurements recorded on 16/09/03 were used to illustrate the results of gas exchange; this date was preferred to the other ones because there was no dew in the morning to interfere with early measurements. In addition, the plants of the dry regime were just irrigated and the physiological activity at the green leaf level was partially recovered.

On that day, nine rounds of gas exchange measurements on all five hybrids grown under the two water regimes were carried out between 6h30 and 19h00. The diurnal patterns for stomatal conductance (g_s), photosynthetic rate (A), instantaneous water use efficiency (WUEi) and leaf temperature for each hybrid grown under the two water regimes are presented in Figure 6-7.

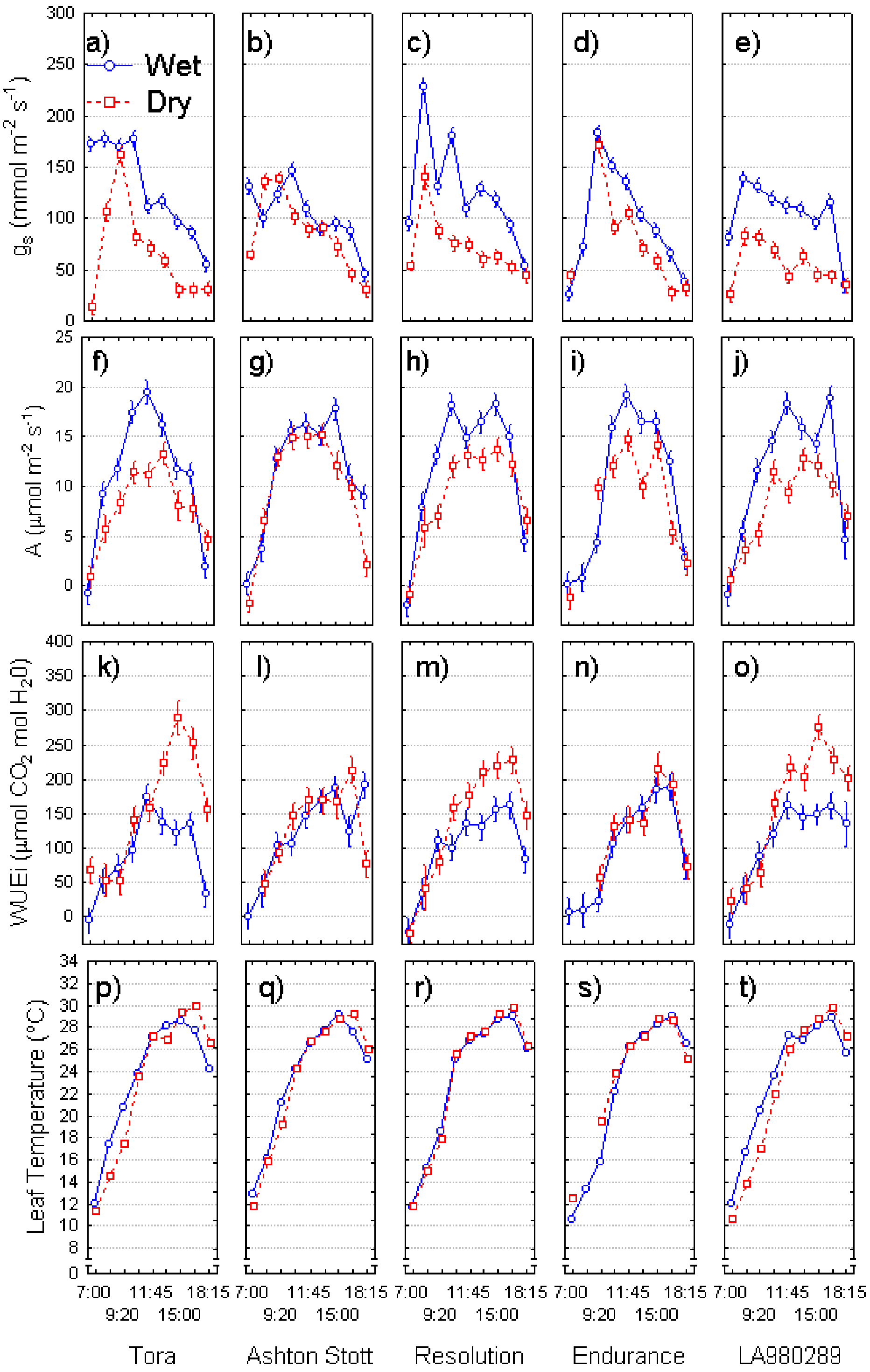

Figure 6-7. Average stomatal conductance (a to e), photosynthetic rates (f to j), instantaneous water use efficiencies (k to j) and leaf temperatures (p to t) of five Salix hybrids grown under two water regimes wet and dry at different times on 16/09/03, with Tora (a, f, k and p); Ashton Stott (b, g, l and q); Resolution (c, h, m and r); Endurance (d, i, n, and s) and LA980289 (e, j, o and t); Lysimeter trial; Silsoe, Bedfordshire. The error bars represent the 95% confidence intervals

Under both water regimes g_s, A, WUEi and leaf temperature increased as the sun rose in the morning, they reached a peak between 9h00 and 10h00 for g_s, between 11h30 and 15h00 for A, around 16h30 for WUEi and around 15h30 for the leaf temperature. Afterwards, a gradual decrease was observed for each parameter. However, a short period of time during which the average results observed were in most occasions not significantly different took place between 11h00 and 14h00.

The photosynthetic activity recorded through the three parameters g_s, A and WUEi showed that a similar patterns of photosynthetic activity were observed under both water regimes although g_s and A were reduced and WUEi increased under the dry regime. Between hybrids the activity differed widely especially in the early morning. Later, the photosynthetic activity of the hybrids decreased in slightly different pattern until they reached similar value as the sun set. This showed that diurnal photosynthetic activity was specific to each hybrid and that in order to compare photosynthetic activities of different hybrids grown under different conditions early and late measurements ought to be avoided. This latest observation confirmed the choice of sampling time used earlier in the season.

The photosynthetic activity of the five hybrids grown under wet and dry treatments was monitored between 8/08/03 and 29/08/03 (Figure 6-8), corresponding to the end of DC2 and the whole of DC3. the means over this period are presented in Table 6-4.

Table 6-4 Mean stomatal conductance (g_s), Photosynthetic rate (A), instantaneous water use efficiency (WUEi) and leaf temperature of five Salix hybrids grown under two water regimes wet and dry between 8/08/03 and 29/08/03; Lysimeter trial; Silsoe, Bedfordshire. The letters represent Fisher least significant differences (LSD) post hoc grouping

Hybrids	Tora		Ashton Stott		Resolution		Endurance		LA980289	
Treatments.	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
g_s (mmol m^{-2} s^{-1})	333^{a}	70^{hi}	256^{b}	147^{f}	224^{d}	75^{h}	244^{c}	60^{i}	170^{e}	106^{g}
A (μmol m^{-2} s^{-1})	23.3^{a}	9.0^{g}	21.5^{b}	14.5^{d}	19.3^{c}	11.1^{f}	22.6^{a}	9.9^{g}	18.9^{c}	12.9^{e}
WUEi (mmol CO_2 mol H_2O)	76^{g}	146^{c}	92^{f}	129^{d}	94^{f}	155^{b}	97^{f}	169^{a}	115^{e}	132^{d}
Leaf temperature (°C)	27.4^{de}	28.7^{ab}	27.4^{de}	28.4^{bc}	27.9^{cd}	28.6^{ab}	26.9^{e}	29.1^{a}	28.2^{bc}	28.1^{bc}

The results of the factorial ANOVA showed that the differences were significant between hybrids, between treatments, between dates and all interactions were also significant. This meant that hybrids, besides having quantifiable differences under both regimes, behaved differently according to the water stress level.

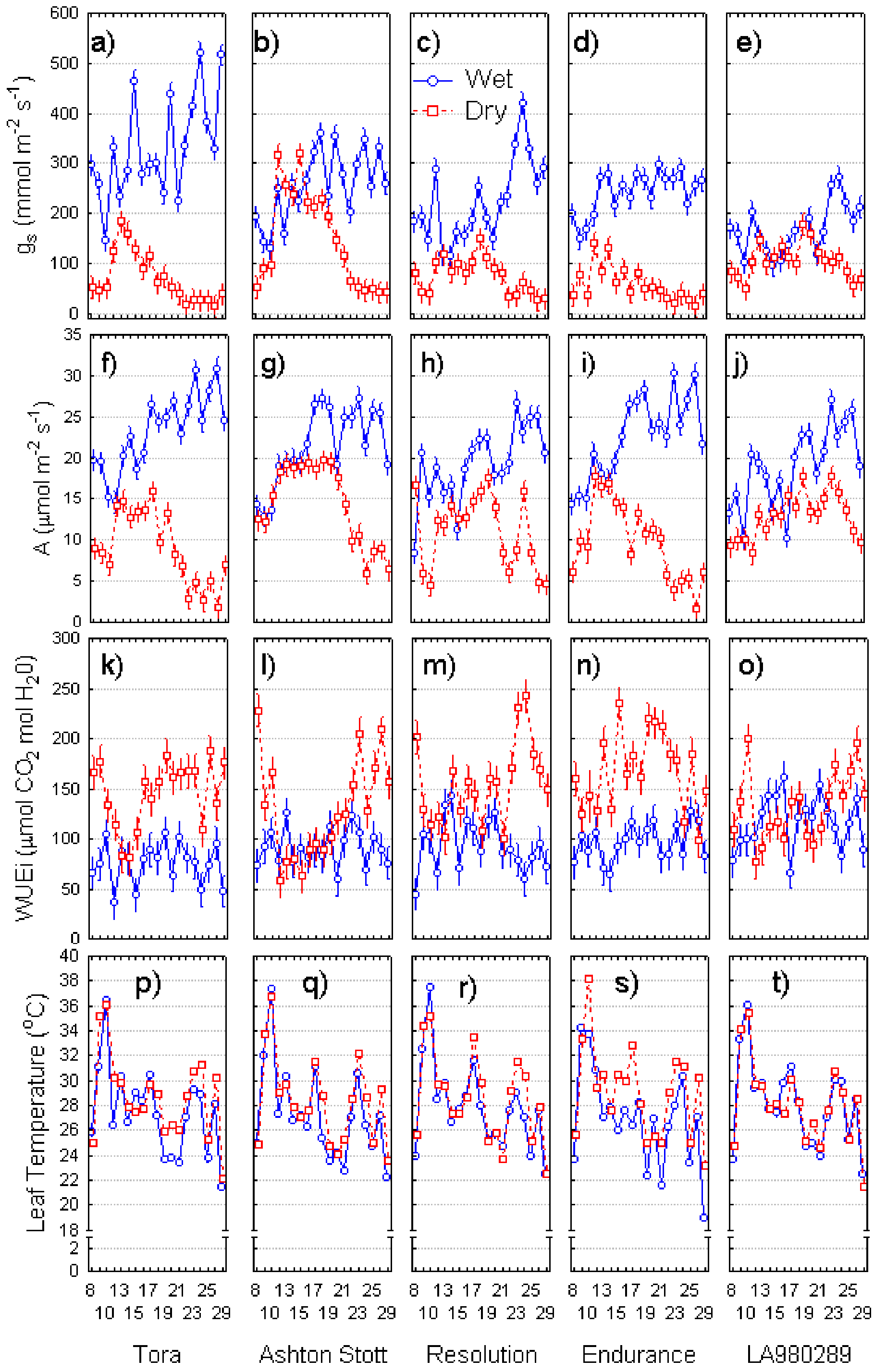

Figure 6-8. Average stomatal conductance (a to e), photosynthetic rates (f to j), instantaneous water use efficiencies (k to o) and leaf temperatures (p to t) of five Salix hybrids grown under two water regimes wet and dry between 10h00 and 15h00 in August 2003; with Tora (a, f, k and p); Ashton Stott (b, g, l and q); Resolution (c, h, m and r); Endurance (d, i, n, s) and LA980289 (e, j, o and t); Lysimeter trial; Silsoe, Bedfordshire. The error bars represent the 95% confidence intervals

Between 8/08/03 and 29/08/03 and under the wet regime the five hybrids had g_s significantly different from each other. Tora had the highest average g_s, Ashton the second highest, Endurance the third highest, Resolution the fourth highest and LA980289 the least. During that same period under the dry regime, Ashton Stott had the highest average g_s and had a significantly higher g_s than the ones of the other four hybrids.

On a daily basis, g_s of the plant grown under the wet regime, seemed to fluctuate more in proportion than the ones of the plant grown under the dry regime. However under the wet regime, g_s of Tora, Ashton Stott and Resolution fluctuated in greater proportion than the ones of Endurance and LA980289.

Furthermore under the dry regime in comparison to the wet regime, at the end of DC2 until 12/08/03, g_s was lower. As the plant got irrigated on 12/08/03 g_s fully recovered for Ashton Stott, Resolution and LA980289. Then g_s decreased and the reduction was much more accentuated for Tora and Endurance in comparison to Ashton Stott and LA980289 which were able to maintain a similar level of g_s for seven and nine days respectively. Resolution fell in between the two groups. By the end of DC3 between 20th and 24th August, g_s was reduced by 70-90% for Tora, Ashton Stott, Resolution and Endurance while it was only 50% for LA980289. All hybrids reached their minimum gs on 23/08/03 but LA980289 which reached it two days later.

On a daily basis between 8/08/03 and 29/08/03 under both water regimes and for the five hybrids, A showed a similar pattern to g_s and the same comments as before for g_s can be made. However the variations of the daily means of A had relatively reduced amplitude in comparison to the ones of g_s for Tora, Ashton Stott and Resolution and increased relative amplitude of the variations of the means for Endurance and LA980289. Between 8/08/03 and 29/08/03 and under the wet regime Tora and Endurance had the highest average A and Endurance and Tora the lowest.

Between 8/08/03 and 29/08/03 WUEi was significantly higher under the dry regime than under the wet one for all five hybrids. Under the wet regime, LA980289 had the highest WUEi which was significantly higher than the ones of the other four hybrids; the one of Tora was the least. Under the dry regime, Endurance had the highest WUEi which was significantly higher than the other four hybrids. LA980289 and Ashton Stott had the lowest WUEi.

On a daily basis, under the wet regime, WUEi of each hybrid was fairly consistent from one day to another. However WUEi of all five varieties appeared to have small variations which described a similar pattern, those might be due to the environmental and incidentally physiological conditions at the time of assessment but there were no obvious correlation with leaf temperature.

Under the dry regime WUEi was high for all five hybrids at the beginning of the experiment. When irrigated, WUEi fell for all hybrids and except for Endurance, those reached level of WUEi comparable to those measured on their clones grown under the wet regime. As the water was used by the plants, Tora and Endurance were the first to have an increase in WUEi only three days after 12/08/03 irrigation. In the same time Resolution had a slightly higher WUEi than its clone under the wet regime, but maintained it fairly constant for 10 days after irrigation. Ashton Stott and LA980289 described very similar pattern of WUEi evolution over the 21 days of the experiment.

6.3.4. Chlorophyll fluorescence

The photosynthetic activity of the five hybrids grown under both water regimes was measured between 8/08/03 and 29/08/03 using the chlorofluorometer FMS2. Those measurements were carried out alongside those of gas exchanges. From the measurements carried on over that period the means and daily means of Fs, F'm, F'v/F'm and Φ_{PSII} were calculated, those are presented Table 6-5 and Figure 6-9 respectively.

Table 6-5. Mean steady fluorescence (Fs), maximum light adapted fluorescence (F’m), open PSII energy capture efficiency (F’v/F’m) and Quantum efficiency of PSII (Φ_{PSII}) of five Salix hybrids grown under two water regimes between 8/08/03 and 29/08/03. Lysimeter trial; Silsoe, Bedfordshire. The letters represent Fisher least significant differences (LSD) post hoc grouping

Hybrids	Tora		Ashton Stott		Resolution		Endurance		LA980289	
Treatments.	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
Fs	276^{ab}	277^{ab}	258^{bc}	218^{d}	254^{bc}	212^{d}	291^{a}	278^{ab}	263^{abc}	239^{cd}
F’m	636^{a}	572^{bc}	613^{ab}	535^{cd}	644^{a}	481^{d}	665^{a}	572^{bc}	622^{ab}	571^{bc}
F’v/F’m	0.61^{ab}	0.60^{ab}	0.61^{ab}	0.62^{a}	0.63^{a}	0.61^{ab}	0.59^{ab}	0.58^{b}	0.63^{a}	0.63^{a}
Φ_{PSII}	0.54^{abc}	0.49^{bc}	0.55^{abc}	0.56^{a}	0.57^{a}	0.52^{abc}	0.53^{abc}	0.49^{c}	0.55^{ab}	0.56^{a}

The results of the factorial ANOVA showed that the differences between hybrids and between treatments were significant for Fs and F’m but not for F’v/F’m and Φ_{PSII}. However, the interactions hybrids - treatments were not significant for Fs and F’m. This meant that beside quantifiable difference between hybrids and treatments using the chlorophyll fluorescence parameters, the hybrids did not behave differently when subject to increasing water stress.

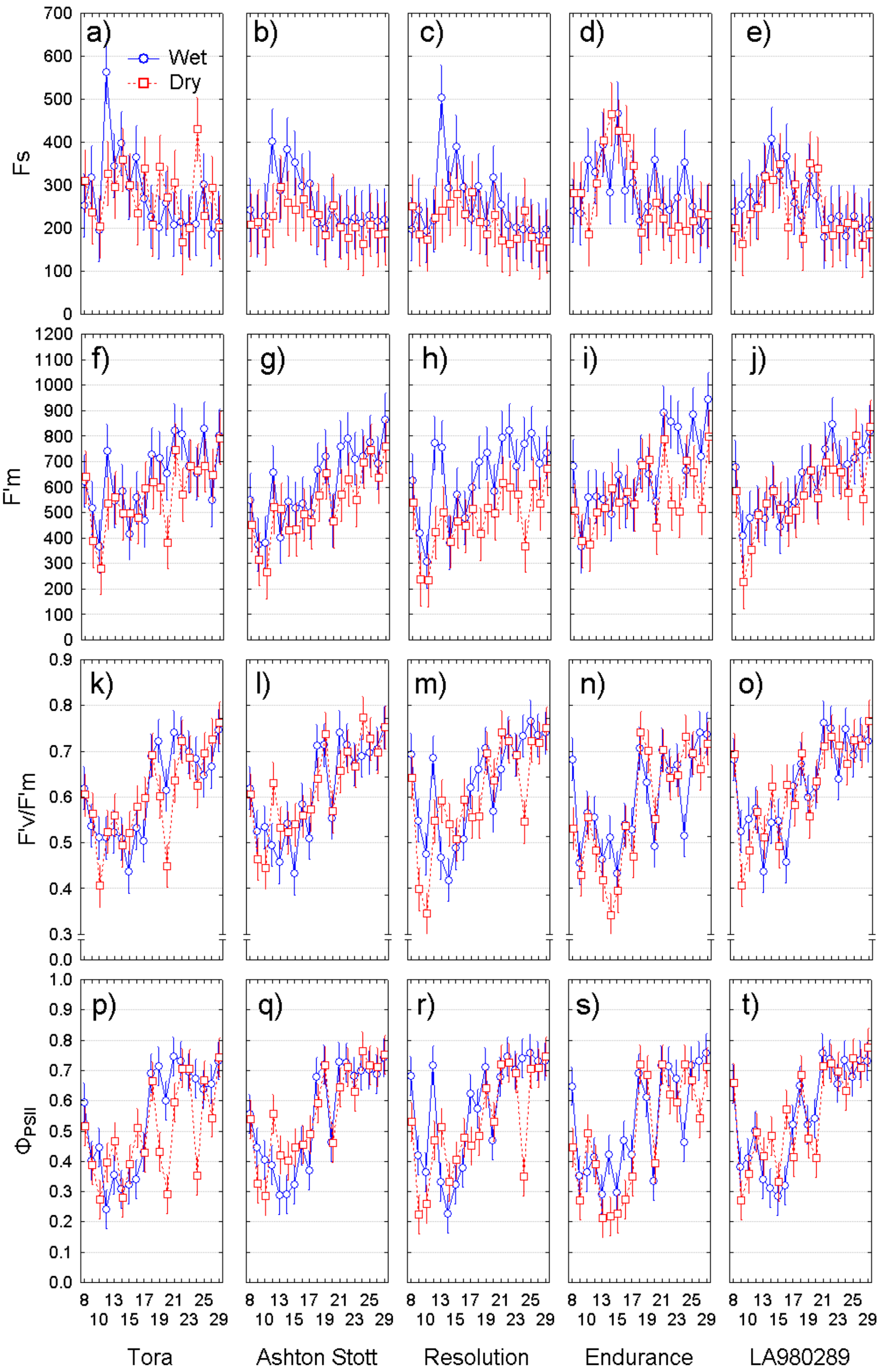

Figure 6-9. Average steady fluorescence (a to e), maximum fluorescence light adapted (f to j), open PSII energy capture efficiency (k to o) and quantum efficiency of PSII (p to t) of five Salix hybrids grown under two water regimes wet and dry with Tora (a, f, k and p); Ashton Stott (b, g, l and q); Resolution (c, h, m and r); Endurance (d, i, n, s) and LA980289 (e, j, o and t). Lysimeter trial; Silsoe, Bedfordshire; August 2003. The error bars represent the 95% confidence intervals

Under the wet regime very few differences were observed among the group of hybrids studied looking at the four parameters (Table 6-5). Endurance had on average the highest Fs which was not significantly different than the ones of Tora and LA980289. Ashton Stott had the smallest Fs but this was not significantly different than the one of Resolution and LA980289. Under the dry regime, only Ashton Stott and Resolution experienced a significant reduction of Fs. No extra information about the evolution of Fs could be extracted looking at the daily record in comparison to the means (Figure 6-9 and Table 6-5).

Under the wet regime no differences of F’m were observed between the hybrids. When subjected to drought, on average all hybrids except LA980289 experienced a significant reduction of F’m. Resolution had in proportion the greatest reduction of F’m with a decrease above 25% on average. In the meantime, for Tora, Ashton Stott and Endurance these reductions were 10, 13 and 14% respectively. On a daily basis, the decreases were clearly visible only for Resolution and Endurance as the water stress strengthened.

On average no significant differences between the hybrids and water regime were observed for F’v/F’m and Φ_{PSII}. On a daily basis no significant difference were observed neither. From 13th to 30th of September F’m, F’v/F’m and Φ_{PSII} all increased steadily, perhaps as leaf temperature decreased.

6.4. Discussion

Willows grown in containers are exposed to unrealistic conditions of growth in comparison to commercial plantations. Studies on the physiological responses to a particular stress suffer from interference from other stresses directly induced by the conditions of growth. In the lysimeter experiment run at Silsoe in 2003, the first interference came from the size of the container which restricted the root system and might have induced a bonsai effect (Lesniewicz, 1985) which may have also affected the pot trial 2002. However the first year stem biomass was comparable per unit area in the lysimeters than the one produced in the field which suggest that willow grown in isolated containers could grow larger than individuals in dense stands where they compete for light water and nutrients. In a three year experiment in the same lysimeters Martin and Stephens, (in press-b) observed stem biomass per plant comparable to dense stands which suggests that the lysimeter size is not a limiting factor within three years of growth. Secondly the weather, as mentioned in the previous chapters, was exceptionally hot and dry. This was beneficial for the experimental programme as little rain interfered with the schedule of work but on the other hand, heat stress may have developed alongside drought stress.

Heat stress has been reported to be responsible for change of the mechanism involved in the photosynthesis for many plants (e.g. Kuznetsov et al., 1997; Hamerlynck et al., 2000; Sinsawat et al., 2004). In addition, the temperature being relatively high, the soil temperature inside the containers and around the canopy might have being higher than the one that could occur in field conditions. However, the willow species used to breed the hybrids present in this study were not reported to be either particularly heat sensitive or psychrophilic. Whilst high temperatures during July and August do need to be taken into account, this would have affected all the lysimeters in a similar manner and water stress therefore remained the strongest external variable in the experiment.

The irrigation scheduling adopted under the dry regime paired with the characteristics of the willow hybrid grown in the lysimeter governed the intensity of the soil water deficit (SWD). The first common point between the hybrids was the increase of SWD from one drying cycle to the next at least until DC 3. As such the water use (WU) rate appeared to have increased for all hybrids, this was the result of an increase size of the plants including the root system which induced an improved capacity of the plant to extract water from the soil.

The cases of the first three drying cycles (DC1, DC2 and DC3) are the most relevant as for the fourth drying cycle (DC 4) the plant had suffered severe drought damage[23], the climatic demand was very reduced and the irrigation scheduling were not alike for each replicate of the experiment. Consequently, during DC 4 differences of SWD were observed within the three replicates of the dry regime of each hybrid.

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[23] See Chapter 5 for details.

According to their drought sensitivity identified in the Silsoe 2002 experiments[24] the hybrids were classified using the relative stem biomass production (RSBP). From the selection produced for the Silsoe lysimeter trial 2003, LA980289 ranked first and appeared the most tolerant to drought, Tora second, Resolution third, Ashton Stott fourth and Endurance fifth and appeared the least tolerant to drought of the selection. The details of the water use efficiency of the harvestable ground biomass (WUE_{stem}) measured on the Silsoe lysimeter trial 2003 revealed that the hybrids could be classified into two distinct groups[25]; Resolution, Tora and LA980289 with WUE_{stem} above 2 g kg^{-1} and Ashton Stott and Endurance with WUE_{stem} under 2 g kg^{-1} regardless of the water treatment, those two groups are the *S. viminalis - S. schwerinii* related hybrids and *S. burjatica - S. dasyclados* related hybrids respectively.

The record of SWD at the end of each drying cycle contrasted with those expected (Figure 6-3 and Figure 6-4). Wikberg and Ögren, (2004) assigned drought resistance capabilities to its selection hybrid according to the relative soil water content at wilting. With the same logic, it was expected that over the drying cycle, the hybrids would segregate; the most drought tolerant (high RSBP, high WUE_{stem}) would reach higher SWD than more sensitive ones. However, Wikberg and Ögren, (2004) used wilting which is not a refined method because appreciation of wilting might induce large errors when comparing leaves of different morphologies. During DC 1 all hybrids root systems colonised the whole depth of the profile, but LA980289 did not reached lower depth as fast as Resolution and Tora while Ashton Stott did. Later no significant difference of SWD at the end of DC 2 and DC 3 were observed among the five hybrids selection. This meant that water extraction abilities that characterised the willow hybrids had no links with WUE_{stem}. Notwithstanding that Martin and Stephens, (in press-a) showed that soil conditions affect WUE_{stem} independently of the water availability, though these differences were largely related to the relative contribution of soil evaporation to water use.

In Chapter 4 it was revealed that for the wet and dry regimes separately no significant differences of water use (WU) were detected between the five hybrids. In addition,

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[24] See Chapter 2 for details.

[25] See Chapter 4 for details.

WU rate recorded from the willow grown into the weighing lysimeter indicated that when unstressed no differences were apparent among the five hybrids (Table 6-3). However, in the middle of DC3 over a progressive period of water stress the hybrids segregated into two groups according to WU rate. The first composed of the old hybrids Tora and Ashton Stott and the second composed of the new hybrids Resolution, Endurance and LA980289; LA980289 having a significantly greater WU rate than the others. The differences of WU rate between hybrids appeared not to be linked with the leaf area as only Endurance had kept a significantly greater L_{leaf} in comparison to the other hybrids[26].

Between the unstressed and progressively stressed conditions WU rate was comparable for the new hybrids, but they had a significantly reduced leaf area in the middle of August in comparison to the last three weeks of June during which unstressed conditions were maintained[27]. This suggest that the new hybrids may use an increased amount of water per unit area of leaf, similar to that reported for apple trees (Cullinan, 1920) while this phenomenon was not clear for Tora and Ashton Stott. Almost the same distribution was observed when the hybrids were subjected to a severe drought stress, Tora in that case having the lowest WU rate which was significantly lower than the other four hybrids.

The ranking of the hybrids differed when looking at WU rate and WUE_{stem}. As a result the WU rate did not appear to indicate any predisposition of high WUE_{stem}.

In Figure 6-6 night time WU were recorded, it seemed unusual as no radiation drove the water evaporation. Three explanations were formulated for the "use" of 0.1 l of water in some nights: the first might be caused by errors of recording from the DATAMAID software, although stomata ought to be closed the second could be evaporation from the cuticle of the leaves and the last as the lysimeter accumulated heat during the day it is possible that water evaporated from the soil of the lysimeter as it cooled down by night. It is important to note that nighttimes WU were negligible in comparison to daytime WU.

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[26] See Chapter 5 for details

[27] See Chapter 5 for details.

Numerous authors have reported that water stress altered metabolism and particularly photosynthesis of higher plants (e.g. Sánchez-Rodriguez et al., 1997; Xiao, 2001; Colom and Vazzana, 2003). This phenomenon was observed in the five *Salix* hybrids studied. Throughout the measurements of gas exchanges, the metabolism of each hybrid was affected by drought and both stomatal conductance (g_s) and the photosynthetic rate (A) were reduced. The diurnal pattern indicated differences in behaviour between hybrids. The maximal conductance was observed between 9h30 and 11h30 for all hybrids this occurred at the same time for three *Populus sp.* hybrids (Souch, 1996) suggesting that midday stomatal closure, as it could take place, (Jones, 1992) is not occurring for *Salix sp.* and *Populus sp.* However, LA980289 showed lower maximal g_s than the other four and g_s remained at a fairly constant rate during the day until daylight declines. This suggests that LA980289 exert greater stomatal control than the other hybrids. It cannot be attributed to a reduced leaf population[28] as similar diurnal pattern of g_s and A were observed for both water regimes while leaf area were very reduced under the dry regime.

There were contrasting responses to water stress between the hybrids and the leaf metabolism was affected in different proportions, though throughout the development of SWD experienced by the plant similar behaviours were observed (Figure 6-8).

In a recent study of on four willow hybrids revealed that maximal g_s was negatively correlated with drought resistance (Wikberg and Ögren, 2004); in their study authors characterised drought resistance according to relative soil water content at wilting. In the Silsoe lysimeter trial, a different approach was taken to characterise the hybrids capacity to resist drought with RSBP and WUE_{stem}. It was unclear that using one or the other parameter would lead to rank the hybrids according to their drought resistance however, two groups were distinct, the first with Tora, Resolution and LA980289 the *S. viminalis - S. schwerinii* related hybrids, having high RSBP and WUE_{stem} the second with Ashton Stott and Endurance the *S. burjatica - S. dasyclados* related hybrids, having lower RSBP and WUE_{stem}. The maximal g_s observed on non stressed plants over a diurnal pattern and over progressive water stress did not

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[28] See Chapter 5.

correspond to any sort of correlations with RSBP, WUE_{stem} or SWD at the end of DC 2 or DC 3. In addition, the pattern of g_s and A over a progressive water stress seemed to be independent of WUE_{stem} and SWD at the end of DC2 and DC3.

WUEi seemed to be dependent on the weather condition as for each hybrid grown under the wet regime WUEi described the same evolution on a daily basis. This phenomenon seemed logical as carbon accumulation is driven by irradiance (Lawlor, 1987) and WU is driven by a total climatic demand which includes temperature (Allen et al., 1994). From the protocol followed to calculate WUEi from the parameters measured with CIRAS-1, temperature was the only uncontrolled factor. In Figure 6-8 it is clear that under the wet regime WUEi followed accurately the variations of leaf temperature.

Under water stress the hybrids experienced an increase of WUEi and this matched with the observations made earlier for WUE_{stem} which also increase in the dry regime; similar observations were made in other experiments i.e. for tea C*amellia sinensis* (Stephens et al., 1994). Differences in amplitude were observed under severe drought and this particularly for LA980289 which had its WUEi less affected than the other four hybrids but LA980289 had no differences of leaf temperature between the two water regimes and this might have a link with the little difference of WUEi between the two water regimes; this is perhaps due to the greater stomatal control seen in this hybrid. The ranking of the five hybrids obtain from WUEi in both water regimes did not match any of the others from RSBP, WUE stem or SWD.

There is no obvious relationship because WUEi was measured from leaf samples while the other indictors comprise the whole plant in other words the leaf area and the efficiency of each leaf. But the values are logically related and accurate measurement of the leaf area (m^2) could help to establish a clear relationship. Nevertheless, under the wet regime WUEi are very similar between hybrids which suggest that plants with relatively larger leaf area are able to store biomass faster than those with smaller leaf area.

As the water stress increased, WUEi increased for all five hybrids but the increase took place at different moment which suggest that water conservation mechanism were activated at different moments while no significant difference of SWD were observed. Tora and Endurance were the first, followed by Ashton Stott, Resolution and LA980289. A proportionally smaller increase of WUEi was observed for LA980289 in comparison to the other four hybrids.

Under the wet regime, the leaf temperatures were very similar even if LA980289 had significantly the highest temperature and Endurance significantly the lowest. When under water stress, leaf temperature increased for all hybrids in comparison to the unstressed conditions but LA980289. Leaf temperature increased when the plants experienced stomatal closure which can be related to the water status of the leaf tissue i.e. (Jones et al., 2002). Endurance was the hybrid that experienced the largest relative increase of leaf temperature and as such it was characterised by the coolest leaf temperature under the wet regime and the hottest leaf temperature under the dry regime. Endurance and LA980289 having the lowest and highest RSBP respectively had the highest and nil increase in leaf temperature as the plant experienced water stress. This indicated that an increase in leaf temperature during water stress could be used as an indicator of drought resistance. The increase on leaf temperature was suggested to be a good tool for the detection of drought stress (Jones et al., 2002). Varieties that appear to exert stomatal control even in wet conditions (e.g. LA980289) may, however, appear stressed due to higher leaf temperatures even when growing strongly.

Fluorescence has been developed as it was believed to be a tool with immense potential for studies on plant physiology (Lichtenthaler et al., 1986; Lichtenthaler and Rinderle, 1988; Lichtenthaler, 1988). Portable fluorometers were made available and commercialised under several trademarks some of them being dedicated especially to study plant physiology. To date drought stress of plant was ascertained to be perceptible through this technique especially through the fluorescence emitted by photosystem II (PSII). The steady fluorescence (Fs) of the hybrids was slightly different between hybrids and only Ashton Stott and Resolution experienced a significant decrease when under water stress. No difference of F'm between the hybrids but a significant decrease of F'm was observed when the plant were under water stress except in LA980289. Unfortunately, the study revealed that the use of two conventional indicators: the quantum efficiency of PSII (Φ_{PSII}) and the open PSII energy capture (F’v/F’m) did not establish clear differences between the water regimes and even less between the hybrids. Finally the hybrids did not rank according to their chlorophyll fluorescence in the same order as if other indicators.

From the daily measurements, no additional information on the effects of water stress was extracted in comparison to the averages. However the high temperature experienced at the beginning of the experiment between 10 and 17 august 2003 (Figure 6-8 p to t) did have a significant effect on the photochemical quenching of all plant (Figure 6-9). Fs rose, F’m, Φ_{PSII} and F’v/F’m decreased but in similar proportion, not revealing any particular aptitude of heat stress tolerance of the photosynthetic apparatus.

Although the phenotypes of the plants are very different, the hybrids are genetically very close and sometime with the same parentage. The physiological responses of the hybrids demonstrated that similar behaviours were adopted. However, the morpho-physiological traits reported to be involved in drought resistance mechanisms are numerous and were found to be expressed over a range of intensities. Individually, a morpho-physiological trait cannot be used as indicator of drought resistance. This demonstrated the complexity of identification of drought tolerant and high yielding willows according to a set of few criteria having uneven influences. Nevertheless the potential from hybridisation and selection of hybrids that express features positively correlated with drought resistance and high yielding is huge. This gives prospects for new set of criteria to be used and compiled as a very large number of combinations coupled with the level of expressions of those features is possible.

CHAPTER 7. Conclusions and recommendations

This chapter summarises the findings of the research reported in this thesis; from these, recommendations for the willow breeding programme and further research are suggested. In brief: *Salix* sp. possesses various mechanisms related to drought resistance and enhanced water use efficiency; breeding should aim to group those advantageous traits into a progeny which could present the required potential. The processes of drought resistance were classified into three categories: drought avoidance, drought resistance and water use efficiency (Jones, 1992). In each of these categories, the mechanisms of drought resistance are either: long, medium or short term.

7.1. Outcomes

7.1.1. Drought effects on yield and development

In 2002, the stem biomass and length of the 50 willow varieties were used to rank the hybrids according to yield and stem length and to calculate two ratios, the first the relative stem biomass production (RSBP) and second the relative stem length as the ratio of the values measured in pot trial to the ones in the field. Later it was emphasised that RSBP not only reflected drought stress, but some other stress such as heat stress and stress due to constriction of the root system in container which may have generated signals to reduce above ground biomass.

The ratios indicated that within a high yielding population of willow differing in parentage and therefore genotype, the response to the stresses imposed by the pots varied widely. A majority of the hybrids bred from *S. burjatica, S. dasyclados* and *S. rehderiana* appeared to be more affected by stress than those bred from *S. viminalis* and *S. schwerinii*. However, the number of varieties represented a small number of the *Salix* genotypes, and when available from the same species different clones (genotype) were used as parents. The pure species were in most cases slow growers in comparison to the high yielding hybrids and were not able to compete for light in the field trial, leading to unrepresentative biomass harvested in comparison to their potential without competition. This did not produce reliable data to quantify the heredity of drought resistance.

7.1.2. Morphological traits related to drought resistance

Morphological traits were identified and measured on the 50 willow varieties of the collection planted at Silsoe in 2002. Many traits can be used to categorise willows, some of them appeared to be related to RSBP and are summarised in Table 7-1.Water stress was the main stress imposed to the plants which suggests that these morphological traits are also indicators of enhanced WUE.

Table 7-1. Effect, classification and conditions for assessment of Salix morpho-physiological traits associated to drought resistance

Morphological traits	**Indicator of resistance** **+ positive** **- negative**	**Classification of drought mechanism**	**Conditions for assessment**
Long term drought resistance mechanism			
Number of stems	-	Avoidance: escape.	On second year of the coppice prior to self-thinning.
Emergence after planting (index or m)	-	Avoidance: escape.	After synchronised planting.
early exponential phase of growth	+	Avoidance: escape.	Stem elongation measured at weekly intervals in June July.
Leaf hair on abaxial surface (index)	-	Avoidance: water conservation.	On fully developed leaves.
Leaf shape (ratio length to width)	+	Avoidance water conservation.	On fully developed leaves.
Adaxial epidermial cell size (μm^2)	-	Tolerance: turgor maintenance.	On fully developed leaves.
Medium term drought resistance mechanism			
Callus development after storage (index)	-	unclassified	On cuttings after 50 days of storage in plastic bags, at +4°C in darkness.
Ratio stem length bearing leaf to total stem length	-	Avoidance: water conservation.	In first year planting in the middle of September.

These morphological traits can be assessed from willow collections generated by breeding programmes. Their use could help to identify candidates with a greater probability of enhanced stress resistance including drought.

7.1.3. Water use and water use efficiency

Following accurate measurement of WU and biomass in the lysimeter trial in 2003 WUE_{stem} and WUE_{total} were calculated. The WU were very similar across phenotypes in both water regimes. In regions where the water is scarce, willow SRC could use all the available water during the growing season, having a negative effect on hydrology as previously indicated by Stephens et al. (2001).

WUE_{stem} appeared to be specific to each *Salix* hybrid in given conditions of growth. The influence of growing conditions on WUE_{stem}, particularly soil type and nutrient availability, has been reported in other experiments (e.g. Martin and Stephens, 2004a). This can help to explain the range of WUE values encountered in other experiments (e.g. Lindroth et al., 1994; Choisel, 1997; Weih and Nordh, 2002). Significant differences were observed between hybrids: those related to *S. dasyclados* and *S. burjatica* had lower WUE_{stem} than those related to *S. viminalis* and *S. Schwerinii*. Grown in lysimeter, Resolution and LA980289 were the most efficient with in the wet regime with more than 30% more stem biomass produced than Ashton Stott and Endurance from similar amount water. One significant differences in WUE_{total} was observed between the hybrids, although on average Resolution remained the most efficient. This suggests that genotypic differences in biomass partitioning to root and shoot production can have a large effect on WUE_{stem}. There are interrelated morpho-physiological traits; in other words water use and saving mechanisms are interdependent.

7.1.4. Morpho-physiological changes

As the water stress increased in the dry treatment lysimeters, some morpho-physiological changes in the willow plants were observed related to high WUE_{stem} (Table 7-2).

Table 7-2. Effect, classification and conditions for assessment of Salix morpho-physiological traits associated to high WUE$_{stem}$

Morpho-physiological traits	**Indicator enhanced WUE$_{stem}$ + positive - negative**	**Classification of drought mechanism**	**Conditions for assessment**
Long term drought resistance mechanism			
Root/shoot ratio	-	Avoidance: effective water uptake.	At harvest.
Roots between 0 and 0.2 m (kg)	-	Avoidance: effective water uptake.	At harvest
Medium term drought resistance mechanism			
Green leaf population	+	Efficiency mechanism: efficient use of water available.	No water shortage in the middle of June
Green leaf population	-	Avoidance: water conservation	After water shortage inducing stress in July or August.
Yellow leaf population	-	Avoidance: water conservation.	After water shortage inducing stress in July or August.
Length of bare stem	+	Avoidance: water conservation.	After water shortage inducing stress in July or August.
Increase in leaf area	+	Efficiency mechanism: efficient use of water available.	After water shortage inducing stress and significant rewetting in July or August.

Changes in morpho-physiological traits are problematical to assess in plant breeding as it requires a trial design which involved different water regimes and possibility to assess under ground biomass. As such, because of the lack of cuttings in the first two years of breeding programme; the study of theses changes ought to take place in later years on elite hybrids only.

7.1.5. Leaf physiology

The hybrids used in the lysimeter trial represented an insight into the possible adaptations to use water efficiently at the leaf level and maintain physiological activity as drought progressed. The measurements of stomatal conductance and leaf temperature were the most promising technique to categorise hybrids in different conditions (Table 7-3).

Table 7-3. Effect, classification and conditions for assessment of leaf physiological traits as indicators of drought resistance of Salix

Physiological traits	**Indicator of enhanced WUE_{stem} + positive - negative**	**Classification of drought mechanism**	**Conditions for assessment**
Short term drought resistance mechanism			
Stomatal conductance (mmol H_2O m^{-2} s^{-1})	+	Efficiency mechanism: efficient use of water available.	No water shortage between 10h and 14h on sunny days. With mass flow gas analyser from June to September.
Stomatal conductance (mmol H_2O m^{-2} s^{-1})	-	Avoidance: water conservation.	Subject to mild water stress no wilting, between 10h and 14h on sunny days. With mass flow gas analyser from June to September.
Leaf temperature (°C)	+	Efficiency mechanism: efficient use of water available.	No water shortage between 10h and 14h on sunny days. With thermocouple or remote sensing from June to September.
Leaf temperature (°C)	-	Avoidance: water conservation	Subject to water stress between 10h and 14h on sunny days. With thermocouple or remote sensing from June to September.

Leaf temperature reported here contrasts with the observations made elsewhere (Jones et al., 2003). Leaf temperature is a physical response to incident solar radiation (related to leaf area index and leaf pose) and transpiration rate. However, in the subset studied in 2003 at Silsoe, leaves with high albedo (hairless) reached higher temperature when illuminated in the absence of heat and water stresses which can enhance physiological activity. In the case of water stress and probably also when combined with heat stress, the leaves with lowest stomatal control, experienced instant closure and were not able to evacuate heat via the release of vapour;

generating an increase in leaf temperature. Therefore, leaf temperature can be used as a selective trait if the stress level of the leaf is known.

7.1.6. Use of morpho-physiological traits characterising high WUE_{stem} and drought resistance

Using the morpho-physiological traits listed above (Table 7-1, Table 7-2 and Table 7-3), a description of an ideal hybrid can be made. Drought resistance comes from not one but multiple features which are interrelated. These features can be expressed over a wide range which leads to numerous combinations with a wide range of efficiency to cope with drought stress. The very large number of possible combination of traits gives good prospects of improvement through plant breeding.

7.2. Recommendations

In the UK, the willow breeding programme has moved to Hertfordshire in Harpenden which is a much drier area than Somerset. The selected hybrids will experience the shortage of water commonly encountered in the East of England, so potentially high water use efficient hybrids will be identified more easily than in the previous location.

The natural habitats of *S. rehderiana* and *S. burjatica* are alpine and riparian respectively (Sviatlana Trybush personal communication, 2004). The alpine habitat suggests that *S. rehderiana* and its progeny could be psychrophylic and therefore could suffer most from heat stress. A riparian habitat suggests greater water availability during growth, *S. burjatica* and its progeny are not normally found in drier places, suggesting a possible sensitivity to water stress. Thus the specific characteristics of the parents' natural habitat should be taken into account in orientating the willow SRC breeding programme to produce hybrids for particular agroclimatic zones.

The willow breeding programme focused on the selection of high yield and pest resistant hybrids. Overall, this selection is a good method to select drought resistant hybrids as the high biomass potential is sometimes linked to high WUE which gives

the hybrid relatively good drought resistance abilities. A typical example from this study is the elite hybrid Resolution.
However with the current procedure, extreme varieties can be missed out or not excluded from the selection programme depending on the growth conditions during the selection processes. Two examples are Endurance, which had one of the highest yield potentials in irrigated conditions but achieved poor yield once water stressed, and LA980289, which produced yield just above the average in the irrigated field but was above Resolution in the irrigated lysimeters and the best in the pots.

Mixed varietal commercial planting is common practice as it was found that monoclonal planting tended to suffer more from pest and diseases (e.g. Hunter and Peacock, 2001; McCracken and Dawson, 2001; McCracken et al., 2001). In the absence of major pest infestations, a scenario of mixed varietal planting with only Resolution, Endurance and LA980289 could result in yields far below the potential expected in average climatic conditions. Over an extremely wet period, Resolution and Endurance would grow much bigger than LA980289 which could risk etiolation and eventually death. Over an extremely dry period, Resolution and LA980289 could grow much bigger, leaving Endurance likely to disappear. Because such a planting ought to be established for several rotations, a succession of dry and wet extreme periods of two to three years could leave Resolution dominant with a possible reduction of up to 2/3rd of the initial density. The consequence is that productivity would be greatly affected.

The willow breeding programme ought, therefore, to carry on selecting high yield willows but should consider the morpho-physiological traits highlighted in this study. This would permit the breeder to orientate a varietal mix to a given agroclimatic zone. In the meantime extreme varieties like Endurance (very sensitive) and LA980289 (very resistant) should be included in breeding trials along with a more average high yield hybrid such as Resolution. The comparison would allow the breeder to characterise the water stress experience in the trial by comparing the yield of the three reference hybrids as follows: Endurance > Resolution > LA980289 = no water stress; LA980289 > Resolution > Endurance = acute water stress and finally Resolution > Endurance and LA980289 = mild water stress. The knowledge of the water stress experienced in the selection trial should be used to quantify the potential of each hybrid when grown in a single trial.

The planting of 12 hybrids grown under two water regimes (irrigated and non-irrigated) at ADAS Gleadthorpe should be used to produce RSBP scores to verify if the scores are significantly different. RSBP could be also calculated from yield harvested in field trials characterised by different soil water deficits in the summer months.

The best strategy for the plant breeder is to select for drought resistance during the first year as the seedlings are started in pots. Long-term drought resistant mechanisms expressed by the aerial parts of the plants can be observed. In addition, it is possible to restrict irrigation in the middle of summer and observe medium and possible short term mechanisms of drought resistance. A complete assessment would enable the breeder to discard those that do not express the required characteristics. In addition, in the first year it would be advisable to use larger pots than the trays currently used, which require constant irrigation in summer months. A larger volume of soil would allow the water stress not to develop too fast and rapidly damage the plants, thus not leaving time for the operator to make assessments.

7.3. Further work

This thesis has largely met the objectives defined in Chapter 1. However, prospects for more research on water use and saving mechanisms and their efficiencies remains as only a fraction was investigated in this research due to lack of time and equipment.
The first issue is to identified and study morpho-physiological traits such as cuticle wax thickness, stomatal density, ABA sap concentration and fine root turnover as those are related to drought resistance in Salix and other genera (e.g Liang et al., 1997; Joslin and Devereux - Wolfe, 1998; Xiao, 2001; Cameron et al., 2002).

The second issue is to assess a much larger population than that used in the Silsoe experiment and to analyse the traits specifically linked to drought resistance mechanism using advanced statistical methods. Multidimensional statistics as used in chapters 2 and 3 (principal components and cluster analysis) give the prospect of analysis of large matrices such as those generated by breeding programmes.

Genomics is the third issue to be investigated. The theory of breeding dictates that two widely different parents covering a range of quantifiable traits should produce a progeny covering a wider range of the same trait. The crossing of high yielding extreme varieties (sensitive with resistant) should produce progeny populations from different parentages. In theory the populations should cover a wide range of responses to drought.

The best prospect for genomics is to run such an experiment with the largest number possible of individuals along with three reference varieties (as suggested earlier: resistant, sensitive, average) to evaluate the stress experienced by the planting. Numerous traits should be measured and trait loci identified from such populations. This could be analysed statistically to identify quantitative trait loci (QTL) to be used in other populations. So far the technique available from genomics only allows rapid assessment of the presence, absence or predisposition of a trait of interest without indicating the exact potential of the variety; this is because no techniques are yet available to quantify the importance of the morpho-physiological traits and their interactions which means that genetic modification has not yet been considered. To date traditional breeding and field trials remain the most efficient techniques to produce elite willow hybrids; since the genetics mechanisms observed in *Salix* and other plants need to be linked to morpho-physiological traits involve in drought resistance to be fully understand.

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APPENDICES

Appendix 1. Silsoe 2002-2003 field trial design; Bedfordshire

Variety code	**Silsoe code**	Position in Replicate 1	Position in Replicate 2	Position in Replicate 3
LA940044	**1**	36	15	23
LA960048	**2**	27	20	14
LA960326	**3**	29	16	44
LA970048	**4**	14	48	38
LA970136	**5**	15	50	13
LA970164	**6**	40	22	34
LA970184	**7**	1	13	12
LA970217	**8**	39	7	4
LA970249	**9**	43	8	2
LA970416	**10**	35	34	19
LA970485	**11**	3	24	41
LA970504	**12**	21	25	17
LA970523	**13**	45	38	11
LA970534	**14**	41	18	27
LA970573	**15**	18	33	16
LA970617	**16**	49	10	21
LA970654	**17**	44	9	3
LA970766	**18**	4	32	29
LA980024	**19**	48	3	10
LA980030	**20**	17	28	46
LA980038	**21**	24	46	33
LA980125	**22**	34	14	40
LA980132	**23**	5	41	48
LA980172	**24**	26	23	20
LA980190	**25**	22	17	31
LA980200	**26**	12	45	6
LA980221	**27**	30	36	36
LA980230	**28**	31	35	25
LA980266	**29**	10	26	50
LA980279	**30**	11	4	7

Variety code	**Silsoe code**	Position in Replicate 1	Position in Replicate 2	Position in Replicate 3
LA980289	**31**	42	31	47
LA980309	**32**	38	40	32
LA980348	**33**	25	47	22
LA980372	**34**	50	43	8
LA980402	**35**	46	1	28
LA980414	**36**	8	29	15
LA980442	**37**	28	44	49
LA980496	**38**	47	19	5
033/20	**39**	7	37	1
001/01	**40**	19	21	26
003/01	**41**	6	27	37
037/01	**42**	37	12	45
055/01	**43**	23	6	35
062/01	**44**	2	11	24
087/01	**45**	20	5	43
102/01	**46**	9	2	42
109/03	**47**	13	30	30
115/34	**48**	33	49	39
034/10	**49**	16	39	18
127/02	**50**	32	42	9

Appendix 2. Silsoe 2002 pot trial design; Bedfordshire

Variety code	**Silsoe code**	Position in Replicate 1	Position in Replicate 2	Position in Replicate 3	Position in Replicate 4
LA940044	**1**	25	15	19	23
LA960048	**2**	21	20	26	14
LA960326	**3**	36	16	8	44
LA970048	**4**	9	48	25	38
LA970136	**5**	22	50	13	13
LA970164	**6**	29	22	34	34
LA970184	**7**	42	13	29	12
LA970217	**8**	28	7	7	4
LA970249	**9**	19	8	48	2
LA970416	**10**	26	34	35	19
LA970485	**11**	11	24	27	41
LA970504	**12**	34	25	20	17
LA970523	**13**	24	38	5	11
LA970534	**14**	38	18	42	27
LA970573	**15**	46	33	23	16
LA970617	**16**	15	10	40	21
LA970654	**17**	13	9	32	3
LA970766	**18**	12	32	44	29
LA980024	**19**	5	3	1	10
LA980030	**20**	31	28	11	46
LA980038	**21**	8	46	4	33
LA980125	**22**	33	14	9	40
LA980132	**23**	18	41	18	48
LA980172	**24**	47	23	10	20
LA980190	**25**	27	17	2	31
LA980200	**26**	6	45	33	6
LA980221	**27**	40	36	38	36
LA980230	**28**	43	35	31	25
LA980266	**29**	35	26	46	50
LA980279	**30**	44	4	6	7
LA980289	**31**	4	31	15	47

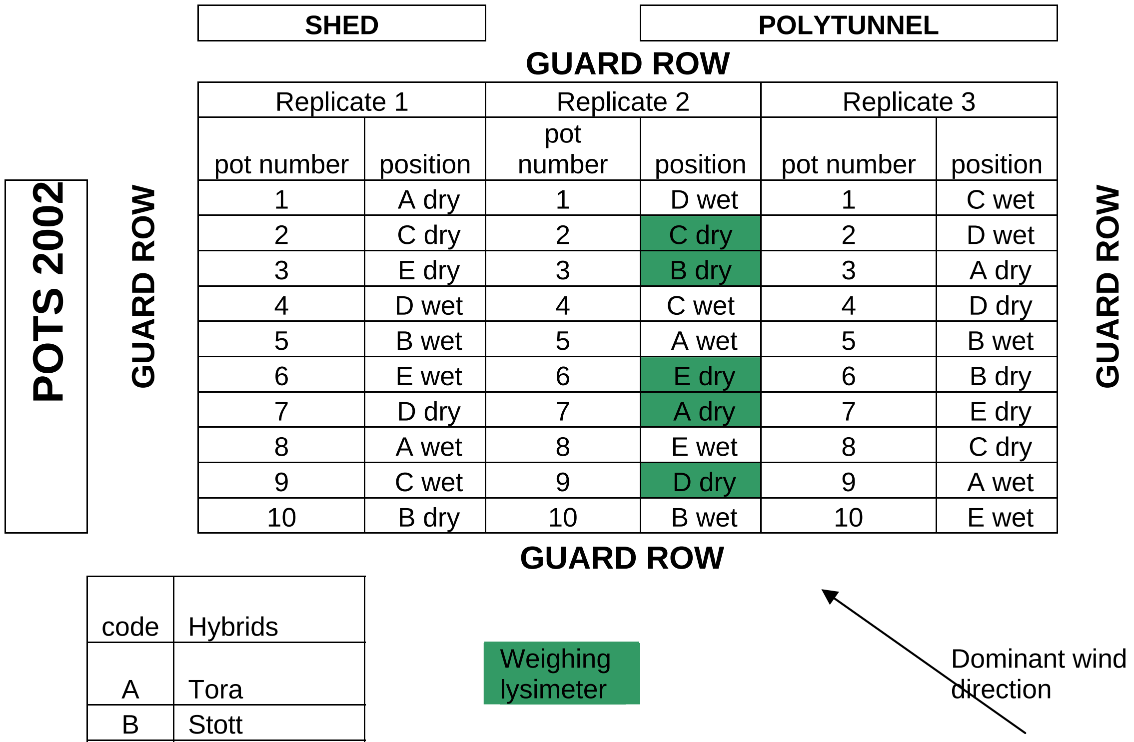
Variety code	**Silsoe code**	Position in Replicate 1	Position in Replicate 2	Position in Replicate 3	Position in Replicate 4
LA980309	**32**	45	40	39	32
LA980348	**33**	7	47	28	22
LA980372	**34**	50	43	22	8
LA980402	**35**	2	1	14	28
LA980414	**36**	1	29	43	15
LA980442	**37**	41	44	50	49
LA980496	**38**	17	19	30	5
033/20	**39**	23	37	12	1
001/01	**40**	10	21	45	26
003/01	**41**	14	27	24	37
037/01	**42**	16	12	36	45
055/01	**43**	3	6	21	35
062/01	**44**	48	11	16	24
087/01	**45**	37	5	17	43
102/01	**46**	32	2	3	42
109/03	**47**	20	30	47	30
115/34	**48**	39	49	37	39
034/10	**49**	49	39	49	18
127/02	**50**	30	42	41	9

Appendix 3. Summary of average biomass and stem length at harvest and corresponding rank of 50 willow varieties grown in field (with irrigation) in 2002 and 2003 and in pots (water stressed) in 2002. Varieties are ranked according to the biomass harvested in the field trial, 2002. The 10 varieties marked with * compose the pure species population.

Variable	Biomass (kg.plant^{-1})						Stem length (m)					
Sites	**Field 2002**		**Field 2003**		**Pot 2002**		**Field 2002**		**Field 2003**		**Pot 2002**	
Varieties	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**
LA980442	0.216	1	0.511	1	0.037	39	2.52	14	3.43	2	1.46	30
LA960326	0.214	2	0.492	2	0.062	5	2.76	6	3.20	12	1.86	9
LA980266	0.196	3	0.389	7	0.051	15	2.40	22	2.85	25	1.45	31
LA970534	0.195	4	0.489	3	0.050	19	2.54	12	2.77	28	1.38	36
LA980414	0.187	5	0.289	25	0.073	3	3.17	1	3.39	4	2.43	1
LA970164	0.184	6	0.393	6	0.075	1	2.85	4	3.27	10	2.33	3
LA980024	0.181	7	0.397	5	0.064	4	2.99	2	3.43	1	2.42	2
LA970048	0.172	8	0.451	4	0.044	26	2.47	18	3.01	16	1.78	16
LA980230	0.164	9	0.332	16	0.054	9	2.68	7	3.21	11	2.04	6
115/34*	0.154	10	0.353	10	0.053	10	2.50	15	3.31	7	1.84	11
LA960048	0.151	11	0.283	27	0.050	20	2.48	17	3.00	17	1.78	14
LA980132	0.150	12	0.384	8	0.050	18	2.44	21	3.32	6	1.71	19
LA980402	0.145	13	0.304	22	0.059	6	2.45	20	2.47	37	1.78	17
LA970485	0.145	14	0.272	28	0.041	32	2.23	29	2.62	32	1.43	32
034/10	0.142	15	0.345	13	0.039	36	2.47	19	2.90	20	1.25	44
LA970217	0.142	16	0.217	35	0.044	27	2.19	32	2.51	35	1.51	28
LA970504	0.140	17	0.344	14	0.031	48	2.14	34	2.49	36	1.16	46
LA970249	0.139	18	0.289	26	0.058	7	2.58	11	3.07	15	1.77	18
LA980289	0.139	19	0.242	32	0.074	2	2.80	5	2.91	19	2.30	4
LA980172	0.133	20	0.314	19	0.044	28	2.87	3	2.89	23	1.60	23
LA970523	0.133	21	0.349	11	0.046	25	2.49	16	3.17	14	1.62	22
LA970184	0.127	22	0.313	20	0.036	41	2.34	27	2.93	18	1.28	39
LA980038	0.126	23	0.207	38	0.037	38	2.23	30	2.36	41	1.25	43
LA980496	0.126	24	0.332	17	0.041	33	2.38	23	2.79	27	1.54	25

Variable	Biomass (kg.plant^{-1})						Stem length (m)					
Sites	**Field 2002**		**Field 2003**		**Pot 2002**		**Field 2002**		**Field 2003**		**Pot 2002**	
Varieties	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**	**Mean**	**Rank**
LA980348	0.125	25	0.340	15	0.056	8	2.53	13	3.29	8	2.05	5
LA980125	0.120	26	0.367	9	0.053	11	2.64	9	3.27	9	1.84	10
LA940044	0.119	27	0.214	36	0.052	13	2.00	41	2.27	45	1.62	21
LA980309	0.118	28	0.262	29	0.051	17	2.11	37	2.88	24	1.38	37
LA970573	0.116	29	0.307	21	0.043	30	2.13	35	2.71	31	1.55	24
127/02	0.115	30	0.241	33	0.052	14	2.58	10	3.34	5	1.95	8
LA980372	0.114	31	0.315	18	0.041	31	2.00	40	2.90	21	1.28	40
LA970416	0.111	32	0.289	24	0.051	16	2.35	25	3.18	13	1.70	20
LA970766	0.109	33	0.200	40	0.034	44	1.95	42	2.34	43	1.34	38
LA970136	0.103	34	0.301	23	0.049	22	2.34	26	2.89	22	1.50	29
LA970654	0.101	35	0.208	37	0.033	46	1.79	43	2.51	34	1.14	47
LA970617	0.097	36	0.202	39	0.033	45	2.11	36	2.37	40	1.08	49
109/03*	0.093	37	0.172	41	0.044	29	2.35	24	2.84	26	1.81	13
LA980200	0.093	38	0.347	12	0.040	34	2.24	28	2.28	44	1.41	35
LA980279	0.091	39	0.256	30	0.049	24	2.10	38	2.77	29	1.41	33
LA980221	0.086	40	0.249	31	0.053	12	2.21	31	3.43	3	1.97	7
062/01*	0.084	41	0.157	42	0.032	47	1.56	49	2.22	46	1.18	45
033/20*	0.076	42	0.240	34	0.031	49	2.15	33	2.36	42	1.02	50
037/01*	0.067	43	0.085	48	0.035	42	1.63	48	1.82	48	1.27	41
LA980190	0.066	44	0.131	43	0.049	23	2.65	8	2.76	30	1.78	15
055/01*	0.059	45	0.089	47	0.039	35	1.69	46	1.79	49	1.41	34
003/01*	0.054	46	0.098	44	0.035	43	1.66	47	2.39	38	1.08	48
001/01*	0.034	47	0.091	46	0.050	21	2.09	39	2.53	33	1.83	12
087/01*	0.034	48	0.067	49	0.036	40	1.72	45	2.38	39	1.53	26
102/01*	0.033	49	0.035	50	0.029	50	1.76	44	2.21	47	1.27	42
LA980030	0.000	50	0.092	45	0.038	37	1.39	50	1.77	50	1.52	27
Sed	0.033		0.092		0.008		0.30		0.43		0.20	

Appendix 4. Silsoe 2003 Lysimeter trial design; Bedfordshire



Replicate 1		Replicate 2		Replicate 3	
pot number	position	pot number	position	pot number	position
1	A dry	1	D wet	1	C wet
2	C dry	2	🟩 C dry	2	D wet
3	E dry	3	🟩 B dry	3	A dry
4	D wet	4	C wet	4	D dry
5	B wet	5	A wet	5	B wet
6	E wet	6	🟩 E dry	6	B dry
7	D dry	7	🟩 A dry	7	E dry
8	A wet	8	E wet	8	C dry
9	C wet	9	🟩 D dry	9	A wet
10	B dry	10	B wet	10	E wet

🟩 Weighing lysimeter

code	Hybrids
A	Tora
B	Stott
C	Resolution
D	Endurance
E	LA980289