Identifying drought tolerant short rotation coppice willows

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Abstract

Short rotation coppice (SRC) willows are of interest as they provide a source of renewable carbon for bioenergy and biofuels. One of the major challenges facing future supply of willow biomass is sustaining sufficient yields in drought challenged environments, with research in this area limited to date.

The effects of drought responses on *Salix* germplasm were studied in two pot experiments in a rain out shelter at Rothamsted Research using a split plot design. In both experiments, plants were subjected to two water treatments, drought stressed or well-watered. A temporary water stress was imposed by applying two cycles of drought within a growing season.

Experiment 1 aimed to screen 56 diverse *Salix* genotypes, including subsets of existing genetic mapping populations, to identify potentially informative germplasm for further study in a more focused second pot experiment. Experiment 2 contained 36 genotypes from two willow full-sibling genetic mapping populations, F and K8. Assessing the potential of mapping population progeny to segregate for drought traits of interest is an important aim of the study as it offers a potential route to the development of markers for drought tolerance trait selections within the Rothamsted Research willow breeding programme.

Phenotypic and final harvest yield measurements were taken on all plants. Primary results reveal; that pot experiments were effective in producing a useful response to drought stress, that genotypic diversity for drought tolerance exists in *Salix*, an early drought coinciding with the exponential growth phase has a more negative effect on yield than a drought that occurs later in the growing season, and that top and middle leaf lengths may potentially offer the breeder a high throughput method of assessing the impact of drought on germplasm.

Declaration

This thesis has been written by myself and describes the work carried out by myself unless otherwise stated. Information from other sources has been fully acknowledged and referenced in the text.

William Macalpine, December 2019

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Chapter 1. General introduction

1.1 Introduction

Short rotation coppice (SRC) willows are of interest as they provide a source of renewable carbon for bioenergy and biofuels. Biomass has the potential to become a major primary energy source in the future and agricultural crops are predicted to become the largest source of biomass for energy (Berndes et al., 2003). Cultivation of this low input perennial crop on light land, with low water holding capacity, where there are fewer profitable land use options, has the benefit of easing potential food versus fuel conflicts (Lovett et al., 2014; Weih et al., 2014). Future climate change predictions forecast that drought is likely to become more prevalent (Rahiz and New, 2013). These two factors combine to raise questions about the future sustainability of yield from current SRC willow varieties. Drought is a major limiting factor in agriculture and is considered the most important cause of yield reduction in crop plants (Boyer, 1982). SRC willow biomass production is limited by water availability, even in the cooler climate of northern Europe (Lindroth and Bath, 1999) and will be further limited in future climates when grown on lighter land.

The native environment of willows are often riparian zones, however, willows are not always synonymous with the wetlands. Examples of species associated with more arid sites include; *S. turnorii*, *S. silicicola*, *S. relli* and *S. planifolia*, which originate from the Athabasca sand dunes in northern Saskatchewan, Canada, and *S. psammophila* which originates from Mu-us, an arid sandy area of Inner Mongolia.

A number of pot experiments in *Salix* have been conducted by researchers. However, pot water deficit was controlled by maintaining a ‘fixed’ level of drought stress (Bonosi et al., 2010; McIvor, 2005; Rönnberg-Wästljung et al., 2005; Weih et al., 2011; Weih et al., 2006). This was achieved by calculating the water holding capacity of the pot and maintaining it at a fixed level e.g. 50%. Blum (2014) criticised this method of applying water stress treatment as it is not a true drought in terms of physiology as the plant undergoes short cycles of hydration and dehydration. These wetting cycles and their physiological consequences are likely to be unreal and unpredictable (Blum, 2014). A water deficit inflicted by stopping irrigation, so that the drought stress can progress slowly for at least a week until the first symptoms of the drought can be observed will be more favourable.

Although pot trials cannot mimic field conditions, this method of inducing drought stress is thought to be more realistic than inducing drought stress using the 'fixed' method.

A number of studies have worked with existing SRC willow varieties (Bonosi et al., 2010; Linderson et al., 2007; Toillon et al., 2013). However, much of the diversity within the genus *Salix* has not been assessed. Bonosi et al. (2010) assessed 15 genotypes response to a well-watered control and four levels of water shortage. Water deficit periods lasted; 4, 8, 12 and 30 days. This study represents the most diverse set of genotypes tested and it found that there was genetic variability in response to drought and that genotypes differed in their ability to respond to different water stress treatments. The study concluded that breeders should define what drought is relevant to them before choosing their selection criterion. Other studies contained fewer genotypes, four genotypes only were assessed in each of these studies; (McIvor, 2005; Wikberg and Ogren, 2004; Wikberg and Ögren, 2007). Successive drought and re-watering cycles were investigated in *Salix* pot experiments (Doffo et al., 2016; Zhivotovsky and Kuzovkina, 2010) and in *Populus* pot experiments (Marron et al., 2003). A similar water regime will be selected as it allows water deficit inflicted by stopping irrigation to be used more than once.

1.1.1 Climate change

It is now acknowledged that human influence on the climate system is real and that climate change has had widespread impacts on human and natural systems. It has also been concluded that the more we disrupt our climate the more we risk severe, pervasive and irreversible impacts. However, with substantial and sustained reductions in greenhouse gas emissions the risks of climate change can be limited (IPPC, 2014). Used as an alternative to fossil fuels, woody biomass produced by sustainably managed SRC willow plantations have a role to play in mitigating greenhouse gas (GHG) emissions.

To produce useful quantities of woody biomass, SRC plantations need to be productive in these future climates. In general, more frequent and prolonged summer droughts appear likely across northern latitudes. These predictions have raised questions about the future sustainability of yield from current SRC willow varieties. According to predictions, summer rainfall will decrease by as much as 20% in the East of England by 2020. With this backdrop, identifying drought tolerance and/or water use efficiency and their mechanisms in willows is crucial so

that they may be introduced into the Rothamsted Research willow breeding programme.

Although it is acknowledged that production of energy crops are not sufficient to reduce GHG emissions alone, energy crops, including SRC willow, form an important component in a portfolio of climate mitigation options to provide a sustainable energy resource to displace fossil fuels (Sims et al., 2006).

The Renewable Energy Directive was implemented in the EU to stimulate the uptake of renewable energy in Europe (EC, 2013). Renewable sources currently provide 14.1% of the European (EU28) energy supply (EC, 2014), although the overarching target is to generate 20% by 2020 (EC, 2009). It is envisaged that this will be met through adoption of a number of technologies, though woody biomass could contribute up to two thirds of the target (EC, 2007): equivalent to approximately 124 million tonne of oil equivalent (Mtoe) (Atanasiu, 2010). In 2006, the European Environment Agency forecast that by 2020, a total of 19.3 million ha (100 Mtoe) of agricultural land could be diverted to dedicated bioenergy production while complying with good agricultural practice, safeguarding sustainable production of biomass and without significantly affecting domestic food production (EC, 2005; EEA, 2006). However, although demand for woody biomass has increased continuously in recent years, new plantings have not materialised and the forestry sector is struggling to meet demand. The amount of forestry in the EU (1,039 million ha 27% of global forest land) does not provide the barrier to supply. Barriers come from complex government policies, landowners' attitudes, technical and economical accessibility, environmental considerations and market conditions, all which combine to limit the mobilisation of woody biomass from forests. The direct effects of climate change could also reduce output from forests with increased tree mortality and associated forest dieback being predicted to occur in many regions over the 21st century, due to increased temperatures and drought (IPPC, 2014).

Hartwich et al. (2014) suggest that due to SRC willow plantations' high water abstraction rate, the cultivation of SRC should not be applied in areas with a negative climatic water balance. However, this recommendation was made for existing SRC varieties which do not have improved drought tolerance traits. Oliver et al. (2009) predicted that the physiological response of C3 Salicaceae trees to elevated CO_2 may increase drought tolerance because of improved plant water

use and that consequently yields in temperate environments may remain high in the future. It should be acknowledged that this is only possible if adequate water is available and indeed that limited available water is the single most important factor that reduces global crop yields (Chaves, 1991).

The challenge presented by climate change is therefore to improve productivity under conditions where there will be periodic drought stress imposed on crops.

1.1.2 Land use issues and food vs fuel debate

Another reason for pursuing drought tolerance and water use efficient traits in SRC willows is that plantations are likely to be grown on sub-optimal land to reduce competition with food crops. Such land often has a poor water holding capacity, making it not economic to grow high input food crops. Deep rooted perennials, such as SRC willows, may be more economic than food crops on these so-called marginal lands or on agriculturally degraded and abandoned lands (Valentine et al., 2012). Dedicated lignocellulosic 'second generation' perennials are seen as being less controversial than first generation energy crops. These first-generation crops, such as wheat. maize and oil seed rape for producing bioenergy often exacerbate the land use conflict between food production and energy production.

1.2 SRC willow

1.2.1 The genus Salix

Willow (*Salix* spp.) is a very diverse group of catkin-bearing trees and shrubs. Willow belongs to the family Salicaceae, which also includes the *Populus* genus.

There are around 450 species of *Salix* worldwide (Argus, 1997). These are mainly distributed in the northern temperate and arctic regions with a small number of species being found in the tropics (Skvortsov, 1968). The centre of diversity is believed to be in Asia, with over 200 species in China. Around 120 species are found in the former Soviet Union, over 100 in North America, around 65 species in Europe, and one species is native to South America (Karp et al., 2011). Willows are dioecious, thus obligate outcrossers, and highly heterozygous.

1.2.2 Ploidy

The haploid chromosome number of *Salix* is 19 (Hanley and Karp, 2014). Around 40% of willow species are polyploid (Suda and Argus, 1968), ranging from triploids to the atypical dodecaploid *S. maxxaliana* with 2n=190 (Zsuffa et al., 1984). The

majority of current SRC willow varieties are diploid, although triploid, pentaploid and hexaploid hybrids are registered SRC varieties (Macalpine et al., 2008). Polyploid species of interest for bioenergy production include; tetraploid *S. miyabeana* and *S. rehderiana* and hexaploid *S. dasyclados.* Polyploid germplasm has not been used when developing current mapping populations. Diploid material has been used to produce mapping populations so difficulties in analysing complex polyploid data can be avoided.

As assessing the potential of mapping population progeny to segregate for drought traits of interest is a key aim of the study. This is important as it offers a potential route to the development of genetic markers for drought tolerance trait selections. To allow the potential for this output diploid germplasm only will form the basis of this study.

1.2.3 The domestication of SRC willow

Domestication of SRC willows has been comparatively recent with breeding programmes being established from the 1980s (Ahman and Larsson, 1994; Kopp et al., 2001b; Larsson, 1997; Lindegaard and Barker, 1997; Macalpine et al., 2008; Stott et al., 1981; Zsuffa, 1979). Parental selections have come from shrub species of willow are utilised for bioenergy production because they perform well in short-rotation coppicing systems and can maintain vigorous growth through multiple harvest cycles. Breeders have concentrated on; yield, pest and disease resistance and selecting a growth habit that facilitates mechanical harvesting (Macalpine et al., 2008). Leaf rust *Melampsora* spp. has been the major disease selection criteria and willow beetles (*Chrysomelidae*) the major pest consideration, with some breeding programmes paying attention to pests including; Terminalis midge (*Dasineura* spp.), giant willow aphid (*Tuberolachnus salignus*), and sawfly larvae (*Nematus pavidus*) (Larsson, 1997). To date drought tolerance and/or water use efficiency has not been included as selection criterion within breeding programmes. Research efforts have focused on screening existing varieties and genotypes for suitability in environments with a high risk of drought or where a temporary water stress is likely (Bonosi et al., 2010). Due to; future climate change predictions, cultivation on lighter land and unsuitable current varieties, there is a pressing need to assess the degree of genotypic diversity for drought tolerance traits so they can be included as criterion within breeding programmes.

1.2.4 Current state of play in commercial SRC willow breeding

Current market conditions in England see 200 – 300 ha of SRC willows being planted annually, with the area of SRC willow plantations totalling ~5,000 ha. These market conditions have led breeders to focus on exploiting their existing pipeline, which takes >10 years from crossing to variety (Macalpine et al., 2010), rather than actively crossing. The Rothamsted Research breeding programme was crossing actively from 2004-2013 for biomass SRC willows.

In Europe, there are 53 short-rotation coppice (SRC) biomass willow cultivars registered with the Community Plant Variety Office (CPVO) for plant breeders' rights (PBRs), of which ~25 are available commercially in the United Kingdom. There are eight patented cultivars commercially available in the US (Clifton-Brown et al., 2019).

Other active breeding programmes in Europe include: Swedish based Salixenergi Europa AB, European Willow Breeding AB, and a programme at the University of Warmia and Mazury in Olsztyn (Poland). Cultivars are also being marketed by the European Willow Breeding Programme (EWBP) (UK), which was actively breeding biomass varieties from 1996 to 2002. There is one active willow breeding programme based at Cornell University, in North America. Cultivars are protected by plant breeders' rights (PBRs) in Europe and by plant patents in the United States.

1.2.5 Cultivation of SRC willow

Willows are currently propagated commercially by planting winter-dormant stem cuttings in spring. SRC willow best practice is well established (AFBI, 2015). Commercial planting systems for willow use mechanical planters that cut and insert stem sections from whips (~2 m long one-year old stems) into a well-prepared soil with a step planter. When commercial plantations are established, the industry standard is to plant intimate mixtures of ~5 diverse rust (*Melampsora* spp.) resistant varieties (McCracken and Dawson, 1997).

After an establishment year, the perennial plantations are harvested on 2 – 4 year harvesting cycles and harvested mechanically with whole rod or direct cut and chip harvesters (Mitchell et al., 1999).

Currently there are between 2200 – 5500 ha of SRC willow being commercially grown in the UK (Lovett et al., 2014), but this could rise significantly in the future

with predictions an area of 1.4 Mha of UK land to second generation bioenergy crops (*Miscanthus*, SRC willow and short rotation forestry (SRF) by the 2050s (Evans, 2017).

1.2.6 End uses of SRC willow

The renewable woody biomass from these perennial plantations is a substitute for fossil fuels. Life cycle analyses indicate that large greenhouse gas reductions are achievable from heat and power generated from SRC willow (Whittaker et al., 2016).

SRC willow plantations also bring benefits to the environment. In comparison to conventional arable cropping, SRC willows require low agrochemical and fertiliser inputs and have great potential for carbon sequestration, bioremediation and enhancement of farmland biodiversity (Haughton et al., 2015). These advantages have led SRC willow to be among the sources of sustainable and renewable feedstocks for bioenergy and biofuel industries.

1.3 Controlling the water stress environment

Imposing drought stress on field grown SRC willow is challenging due to the crops physical size and its three-year harvest cycle. A three-year old SRC willow crop can measure over 5 m in height (Karp et al., 2011). In other crops researchers have controlled the water regime via irrigation alone by conducting field experiments in ‘desert’ environments, like those at International Maize and Wheat Improvement Centre’s (CIMMYT), Ciudad Obregon experimental station in NW Mexico (Monneveux et al., 2006) or by conducting experiments during the ‘dry season’. Such dry season or off-season approaches are suited to semi-arid tropics such as those conducted at International Rice Research Institute (IRRI) in the Philippines (Bernier et al., 2007). These approaches are not suited to UK conditions or the temperate climates where SRC willows are likely to be grown. Rainout shelters have the potential to induce water stress by intercepting precipitation. Stationary rainout shelters have been used successfully by Ober et al. (2004) in a study to assess genetic resources to improve drought tolerance in sugar beet (*Beta vulgaris* L.). The stationary rain out shelters allowed managed drought conditions to be inflicted on a diverse group of beet genotypes. Useful germplasm was identified to allow improvement in drought tolerance. A negative of this approach is that the stationary rainout shelter produced a significant effect on crop microclimate, a climate that was unlikely to occur in the control blocks. As

stationary rainout shelters are more cost-effective they are often preferred for larger screening as more genotypes can be tested in larger plots and with more replicates.

Moveable rainout shelters offer benefits over stationary shelters as when rain is not forecast plants can be exposed to ambient conditions, alleviating the climatic differences between water treatments seen in stationary rainout shelters. Movable rainout shelters have been successfully used in many studies including those at the International Crop Research Institute for the Semi-arid Tropics (ICRISAT), India (Kashiwagi et al., 2005) and the National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, China (Yue et al., 2006). The cost of movable rainout shelters is often prohibitive, making them more suitable for smaller trials. There is also the risk with movable rainout shelters that unexpected precipitation can reach the drought treatments.

However, due to the size and associated costs involved, rainout shelters are not appropriate for field grown SRC willow. Previous field grown experiments have simply used rain-fed and irrigated as their two water treatments (Linderson et al.; Monclus et al., 2006). This approach is feasible if grown at a site with light soil with a low water holding capacity, however it does leave the control of the water regime to the vagaries of the weather. For these reasons a pot experiment will be explored.

Drought stress has been inflicted in pots grown outside (Weih and Nordh, 2002; Wikberg and Ögren, 2007), however this is not an ideal approach as rain intercepted by the plants may run down the stem into the pot. If rain guards were too tight they may affect stem development and root/soil respiration. A promising methodology has been used at Ordos Sandland Ecological Station (OSES), China where seedlings were planted into ‘sand-pools’ (similar to lysimeter chambers) measuring 2.01 m^3 (Xiao et al., 2005). Movable rain-out shelters were used when precipitation was forecast. This methodology represents an interesting half way between field and pot conditions and avoids some of the compromises associated with growing plants in pots.

Numerous studies have been performed in pot experiments in *Salix* L. (Bonosi et al., 2010; McIvor, 2005; Rönnberg-Wästljung et al., 2005; Weih et al., 2011; Weih et al., 2006; Wikberg and Ogren, 2004), *Salix* L. and *Populus* L. combined studies

(Splunder et al., 1996) , Black Locust (*Robinia pseudoacacia* L.) (Mantovani et al., 2014) and apple (*Malus communis* L.) (Ferrara and Flore, 2003). This is unsurprising as pot experiments are probably the most common in plant research. Not all papers detail exact protocols in their methodologies, it is assumed that pot size, pot colour, potting media and stress treatment were the same. Pot size is a concern with fast growing perennial plants as root confinement in a container/pot can have a negative effect on plant growth, despite the plant being adequately watered (Ismail and Davies, 1998).

Sanchez (2013) warned that if experiments performed under controlled or semi controlled conditions do not properly model agronomic environments, then functional genomics of complex traits will likely serve no purpose in the advancement of biotechnology. Regardless of whether the goal of the study is gene discovery or not, the message serves as an excellent reminder about the importance of trial design. The relevance of results described in (Rönnberg-Wästljung et al., 2005) may not be applicable to the field situation. In this study a large number of plants, 1,920, were grown in small 1 l containers. Plants reached heights of over 130 cm in these pots. This small pot size, combined with the associated leaf area is likely to increase the drought effect, highlighting the importance of pot size choice in the experiment design phase. Plants in the drought treatment received *circa* 55% of the water given to the irrigated plants daily. This fixed level approach to applying the drought stress is discussed below. It should be acknowledged that conditions in (Rönnberg-Wästljung et al., 2005) are very different from that of the field and that results may be specific to the experiments environment.

A number of previous pot experiments in *Salix* control pot water deficit by maintaining a ‘fixed’ level of drought stress. This is done by calculating the water holding capacity of the pot and maintaining it at a set level e.g. 50%. Blum, (2014), questioned this method of applying the stress treatment saying, ‘*This is correct in terms of book-keeping but not in terms of physiology. The plant then undergoes short daily cycles of hydration and dehydration, the physiological consequences of which are unreal and unpredictable*’. Inflicting a water deficit by stopping irrigation is the preferred method ensuring that the pot size is large enough for the drought stress to progress slowly for at least a week until the first symptoms of the drought stress are observed.

1.4 Plants response to drought

In the context of a plant breeding programme drought is defined as an insufficient moisture supply which causes a reduction in plant production. An agricultural drought can cause a range of plant growth reductions from mild yield reductions up to a crop failure.

Drought is initiated when demand for water is not matched by supply. When this demand is not met a plant will enter a state of water deficit. Drought is a complex phenomenon in plants, restricting normal growth, disturbing water relations and reducing water-use efficiency.

In water stress conditions, the plants physiological processes are affected, and this is reflected in the plants phenotype. Depending on the severity, a water deficit may cause a reduction in cell size, reduced water use efficiency and a reduction in biomass production (Ashraf and Harris, 2005).

1.4.1 Mechanisms of drought stress resistance

Drought escape

Drought escape is an adaptive mechanism which involves rapid plant development to enable the completion of the full life-cycle prior to a drought event. Flowering time and crop duration are key factors for this adaptation (Blum, 2010). This mechanism of drought stress escape is not suitable for a perennial crop like SRC willow. This mechanism is more suitable for annual seed-based crops.

Drought avoidance

Drought avoidance is the ability of plants to maintain a (relatively) higher tissue water content despite reduced water content in the soil. Lowering stomatal conductance and transpiration are key mechanisms in drought avoidance (Basu et al., 2016). Adaptive traits involving drought avoidance can involve the minimization of water loss and/or the optimization of water uptake. Optimizing water uptake can achieve higher tissue water status by maintaining the water uptake through increased rooting, hydraulic conductance, etc. under drought stress. In contrast, minimizing water loss uses water effectively through reduced loss of water by reducing transpiration, transpiration area, radiation absorption, etc. under drought stress condtions (Basu et al., 2016).

Drought tolerance

Drought tolerance refers to the degree to which a plant is adapted to function under low plant water status. Plants in dry environments can be exposed to drought events where it is impossible for them to escape the adverse conditions. Drought tolerant plants have the ability to endure water stress through certain morphological or biochemical adaptations to avoid cell injury. The mechanism of drought tolerance involves the maintenance of turgor pressure through osmotic adjustment, increase in elasticity in cells, and decrease in cell size by protoplasmic resistance (Valliyodan and Nguyen, 2006)

Drought avoidance and drought tolerance traits carry the largest potential for developing in SRC willow plantations and perennial energy crops.

1.4.2 Phenotypic measurements to monitor plant water status and assess drought tolerance

Measurements that have a potential to be high throughput will be of great interest. High throughput measurement methods with the capacity to be used in large mapping populations and field trials will be of great value.

The measurements in Table 1.1 will be considered as possible approaches to assess and measure the effects of drought stress. In addition to the measurements listed, visual scores will be considered for use on experimental material. Dry matter yield, moisture content and portioning of the components of yield (including roots) will be considered at final harvest timepoints. Consideration will be given to throughput, availability of equipment and suitability to a pot experiment.

Table 1.1 List of possible phenotypic measurements to monitor plant water status and assess drought tolerance and water use efficiency

Trait or parameter	**Method**	**Measurement environment**	**Capacity/day**
Plant water status			
Leaf relative water content (RWC)	leaf disk	F,P,C	≤50
Leaf water potential (pre-dawn, mid-day)	pressure chamber or psychrometry	F,P	≤50

Physiology			
Air-canopy temperature differential	infrared thermometry	F,P	>100
Leaf chlorophyll content	SPAD meter	F,P,C	>100
Photosynthetic efficiency (FPSII)	chlorophyll fluorescence	F,P,C	>100
Leaf rolling/wilting	visual score	F,P,C	>100
Leaf senescence	visual score	F,P,C	>100
Carbon isotope discrimination ratio 13C/12C, D	mass spectrometry	F,P,C	50-75
Oxygen discrimination ratio 18O/16O	mass spectrometry	F,P,C	50-75
Drought stress 'injury'	TBARS, FOX,CMS assays	F,P,C	≤50
Transpiration rate	Sap flow gauge	F,P,C	<10
Xylem cavitation susceptibility	acoustic emission sensors	F,P,C	<10
Root and/or stem hydraulic conductivity	high pressure flow meter	L	<10
Leaf Morphology			
Leaf thickness	digital thickness gauge	F,P,C	>100
Stomatal density	leaf impressions	F,P,C	>100
Wax/glaucousness	visual score	F,P,C	>100
Specific leaf area	leaf area meter, balance	F,P,C	50-100
Leaf succulence index	leaf area meter, balance	F,P,C	50-100
Gas exchange			
Stomatal conductance	Porometer	F,P,C	>100
Maximum light-saturated photosynthetic rate	IRGA	F,P,C	<50
Intrinsic water use efficiency	IRGA	F,P,C	<50

Non-stomatal limitations to photosynthesis	IRGA (A/Ci curves)	F,P,C	<20
Excess energy dissipation and mesophyll conductance	IRGA + chlorophyll fluorescence	F,P,C	<20
Water use/rooting			
Total soil water extraction during drought	Capacitance-type soil moisture meter	F	50-100
Water use efficiency	Soil moisture meter, biomass harvest	F	50-100
Water use efficiency	Gravimetric	P	50-100
Depth of maximum root activity	Soil moisture meter	F	50-100
Root length density	soil cores	F	<50
Remote sensing			
Green canopy cover (NDVI)	spectral ratio meter	F	>100
Photochemical reflectance index (PRI)	spectral ratio meter	F	>100
Canopy temperature	thermal imaging	F,P	>100
Tree geometry, foliage:woody biomass	LIDAR	F,P	<20

Consideration is given to the type of environment in which the measurements can be made, and the maximum number of measurements that can be made (or number of samples that can be processed) per day. F, field; P, pot; C, controlled environment room; L, laboratory.

In willows this will be observed in reduced height and biomass yields. Morphological changes including reduced leaf size area, decreased shoot:root ratio and increased root distribution; depth and root length were observed in a pot experiment studying *S. alba*, *S. triandra* and *S. viminalis* seedlings (Splunder et al., 1996). Stem height, biomass yield and leaf size will therefore be monitored. Whole plant leaf chlorophyll mass from a *Salix* pot experiment was shown to predict performance in the same genotypes grown in field conditions (Weih and Nordh, 2005) making high throughput SPAD readings of interest. Leaf traits have been shown to significantly correlate with growth parameters (Robinson et al., 2004; Weih and Nordh, 2005), making them of great interest for further study. It is

proposed that; leaf counts, leaf measurements (lengths and width), leaf area, SPAD, sylleptic leaf counts and branch counts, and final harvest yield responses will be measured in experiment 1.

1.5 Drought tolerance indices

Different drought tolerance indices can be used for selecting genotypes response to drought stress. Gholinezhad et al. (2014) studied nine drought tolerance indices in an experiment exposing sunflower (*Helianthus anuus* L.) to; well-watered conditions, a mild, and severe drought stress. The study found in moderate drought conditions the indices; mean productivity (MP), stress tolerance index (STI), geometric mean productivity (GMP), harmonic mean (HARM) were suitable indicators for screening drought tolerant genotypes. In severe drought stress conditions, the following indices supported stable and high yield in both non-stress and stress treatments: mean productivity (MP), stress tolerance index (STI), geometric mean productivity (GMP), harmonic mean (HARM), stress non-stress production index (SNPI) and modified stress tolerance index in moderate and severe stress (MsSTI). Two further indices are also considered. Tolerance Against Stress (TOL) which Rosielle and Hamblin (1981) defined as the difference in yield between the stress and non-stress environments. A higher value of TOL indicates susceptibility of a given genotype to drought. Drought tolerance efficiency (DTE) (Fischer and Wood, 1981), provides a simple route to assess the difference in performance of each genotype in droughted and well-watered conditions.

DTE > 1, shows that the genotype performs relatively better in drought than the well-watered control.

DTE < 1, indicates that the genotype performs better in well-watered than in the drought treatment.

DTE = 1, suggests the genotype has a stable performance across the different treatments.

The suitability of drought tolerance indices will be assessed using data from the pot experiments

Table 1.2 Drought tolerance indices

Index Name	Equation	Reference
Drought tolerance efficiency (DTE)	$DTE = \left(\frac{Ys}{Yp}\right)$	(Fischer and Wood, 1981)
Yield Index (YI)	$YI = \frac{Ys}{\overline{Yp}}$	(Gavuzzi et al., 1997)
Mean Productivity (MP)	$MP = \frac{Ys + Yp}{2}$	(Rosielle and Hamblin, 1981)
Stress Tolerance Index (STI)	$STI = \frac{(Ys)(Yp)}{(\bar{Y}p)^2}$	(Fernandez, 1992)
Tolerance Against Stress (TOL)	$TOL = (Ypi - Ysi)$	(Fernandez, 1992)
Geometric Mean Productivity (GMP)	$GMP = \sqrt{(Ys)(Yp)}$	(Schneider et al., 1997)
Harmonic Mean (HARM)	$HARM = \frac{2(Yp \times Ys)}{Yp + Ys}$	(Jafari et al., 2012)
Stress Non-Stress Production Index (SNPI)	$SNPI = \left[\frac{Yp+Ys}{Yp-Ys}\right]^{\frac{1}{3}} \times [Yp \times Ys \times Ys]^{\frac{1}{3}}$	(Farshadfar and Sutka, 2002)
Modified Stress Tolerance Index in Moderate and Severe Stress (MsSTI).	$MsSTI = \frac{[(Y)]s^2}{[(\bar{Y})]s^2} \times STI$	(Farshadfar and Sutka, 2002)

Ys and Yp are stress and optimal (potential) yield of any given genotype.
$\bar{Y}s$ and $\overline{Y}p$ are average yield of all genotypes under stress and optimal conditions.

1.6 Considerations associated with the Salicacae

As a crop SRC willow falls between forestry and agriculture. Its woody and dioecious nature are uncommon in conventional agricultural crops and need some special consideration.

As the yield component of the crop is above ground biomass, it makes the formation of yield under drought less complicated when compared to other crops where the economic yield component is just a proportion of the total biomass (grain, fruit, tuber etc).

1.6.1 Xylem cavitation

Stems/trunks of tree species are particularly susceptible to xylem cavitation, generally with taller stems being more vulnerable. Willows and poplars are among the most vulnerable temperate tree to xylem cavitation (Cochard et al., 2007). Hydraulic conductance can decrease when water supply becomes limiting. It can also decrease when air enters conduits along the pressure gradient causing them to cavitate (embolise). Although xylem vessels can be refilled, their ability to do so can be lost with repeated cavitation. Stem xylem cavitation and the follow-on embolism can be a reason for reduced stem conductance. Xylem cavitation therefore will cause reduced leaf water status under drought stress. Cochard et al. (2007) found a negative correlation between cavitation resistance and above ground biomass production in *Populus* and *Salix*. Vulnerability to cavitation across clones was also found to correlate poorly with anatomical traits such as vessel diameter, vessel wall strength, and fibre wall thickness. Such anatomical traits were found to vary in a study of *Salix* species endemic to sand dunes in Saskatchewan, Canada using light and scanning electron microscopy (Cooper and Cass, 2001). Cochard et al. (2007) findings suggest that selection in a breeding programme for xylem cavitation resistance would lead to drought tolerant willows. However, this approach would also lead to a lower yielding biomass crop as the most resistant genotypes were also the lowest yielding. The Cavitron technique is also not currently high throughput and not capable of screening large populations. These results also agree with the findings of Wikberg and Ogren (2004). Results from these two studies go some way to explain an earlier study that hypothesised that fast-growing SRC cultivars would be more sensitive to water stress than a slower growing natural willow (Weih, 2001). This study found that the unimproved natural willow clone outperformed the bred variety when water was limited, and a low rate of fertilizer was applied and that the bred variety was only superior under optimum conditions. An interesting result, but it should be acknowledged these conclusions were based on juvenile growth from one season. A long-term field trial would be needed to validate results.

Xylem cavitation resistance has been correlated with the increasing density of the wood in *Salix* (Wikberg and Ogren, 2004; Wikberg and Ögren, 2007), *Salix*, *Populus* and a number of other angiosperms and conifers (Hacke et al., 2001). Wood density could be a suitable high throughput proxy measurement for xylem

cavitation. Sennerby-Forsse (1989) found that there was a linear decrease of wood density from the base towards the top of the stems so any sampling would need to follow a strict protocol to ensure homogeneity of sample. It is acknowledged that xylem cavitation is prevented by closing stomata, a response to drought stress. This trait could be assessed in parallel with xylem cavitation.

1.6.2 Sex and stress

Diocy is found in 7.5% of flowering genera (Renner and Ricklefs, 1995) including in *Salix* and *Populus*, sister genera within the *Salicaceae*. It is generally hypothesised that in dioecious plants, pistillate plants have to pay a higher reproductive cost than staminate plants. Differences between sexes in *Populus cathayana* have been found with pistillate individuals suffering greater negative effects than staminate individuals when grown in drought stressed conditions (Xu et al., 2008). Further work on *P. cathayana* supported this and indicated that males were better equipped to survive drought stress as photosynthesis, antioxidant enzyme activities, damage to the integrity of cellular membranes and electrolyte leakage were less severe in males under drought stress (Xiao et al., 2009; Zhang et al., 2012). The effects of drought stress between the sexes has not been specifically studied in detail in *Salix*. Despite being siblings, the female Tora and male Bjorn produced the most and least xylem vulnerability curves in a study consisting of two other unrelated genetically diverse genotypes (Cochard et al., 2007). This study was not designed to compare gender, but it is interesting that two genotypes with the same pedigree can segregate so clearly for susceptibility (Tora) and resistance (Bjorn) to vulnerability to xylem cavitation.

Although not drought stress, gender studies have been conducted on other abiotic and biotic stresses in *Salix*. In a study of gender in relation to growth, herbivory and disease in *Salix viminalis* L. populations (Ahman, 1997), no significant differences on growth rate or herbivory from lepidopterans (*Earias clorana*) and gall midges *Dasineura ingeris* and *D. marginemtorquens* between the sexes were found. However, there were some gender differences in susceptibility of *Melampsora* (an economically important foliar rust disease of SRC willow) depending on the reproductive stage. In another study, no differences in size were found between the two genders in native populations of *S. cinerea* in North Wales (Alliende and Harper, 1989). Yield assessments in this study were based on height only, the natural population studied consisted of plants of unknown ages, this does

not make ‘size assessment’ very robust. A study of *S. reinii* at varying altitudes in Japan contradicts the hypothesis that female plants have to pay a higher reproductive cost than males as females maintained higher reproductive biomass than males at all altitudes (Sakai et al., 2006). Interestingly the reproductive allocation for both sexes decreased at a similar rate as the abiotic stress increased (Kao et al., 1998). The author suggested that in response to this abiotic stress that female *S. reinii* will have a mechanism to compensate for the extra investment in reproduction irrespective of a changing environment. As shoot production did not change with altitude, it implies that *S. reinii* gave priority to vegetative investment at the cost of reproductive output at higher altitudes/ abiotic stress levels.

These findings have so far led *Salix* breeders to ignore gender when selecting yield and resistance to certain insect pests. The evidence from studies in poplar suggests that the relationship between gender and drought stress should be investigated in *Salix* but that responses could be species specific.

1.7 Rationale for the study

One of the major challenges now facing future supply of willow biomass is sustaining sufficient yields in challenged environments. This has come to the fore for two reasons: climate change and the increasing need to grow the crops on sub-optimal land to reduce competition with food production for the world’s growing population. This project aims to identify a route to obtaining sustainable yields on sites where water resources are limited. It is hoped that this will be achieved by advances in understanding the physiology and growth of willow, and in the underlying key traits.

Chapter 2. Broad-range genotype pot experiment

2.1 Introduction

Currently drought tolerance has not been included as a selection criterion within *Salix* breeding programmes as it is not easy to screen for. There is a pressing need to assess the degree of genotypic diversity for drought tolerance and consider how to use it in future breeding activity.

This initial pot experiment aimed to screen diverse *Salix* germplasm to identify interesting families, populations and genotypes for further study. The inclusion of representative germplasm from selected mapping populations gives the potential to assess if mapping population progeny segregate for drought traits of interest. Potentially QTLs that underpin these key drought traits could be identified if segregating populations can be selected in conjunction with high throughput phenotyping techniques.

The central hypothesis of this MPhil is: 'It is possible to identify and select drought tolerant genotypes in short rotation coppice (SRC) willow'

Hypotheses to be investigated in this chapter are;

1. Useful genetic variation exists for drought tolerance traits in the genus *Salix* and can be identified in a pot experiment.
2. Effective methodologies for screening drought tolerant willows can be developed.
3. It is possible to identify and select for drought tolerant genotypes in SRC willow.
4. The inclusion of progeny from mapping populations will allow segregating progeny from defined populations to be identified and selected for further study.

2.2 Materials and Methods

2.2.1 Plant material

Rothamsted Research (RRes) maintains many unique germplasm resources including one of the largest willow collections in the world the UK National Willow Collection (NWC), twelve diploid mapping populations (mp) mp A – K and mp K8 and one association mapping population (Hanley and Karp, 2014; Trybush et al., 2008). All genotypes included in the pot experiment are maintained as coppiced collections either at Rothamsted Research, Harpenden, UK (51°48′30″N,

0°21′22″W) or in the case of the association mapping population, at Woburn Experimental Farm, Husbourne Crawley, UK (52°51.0"N, 0°35'33"W).

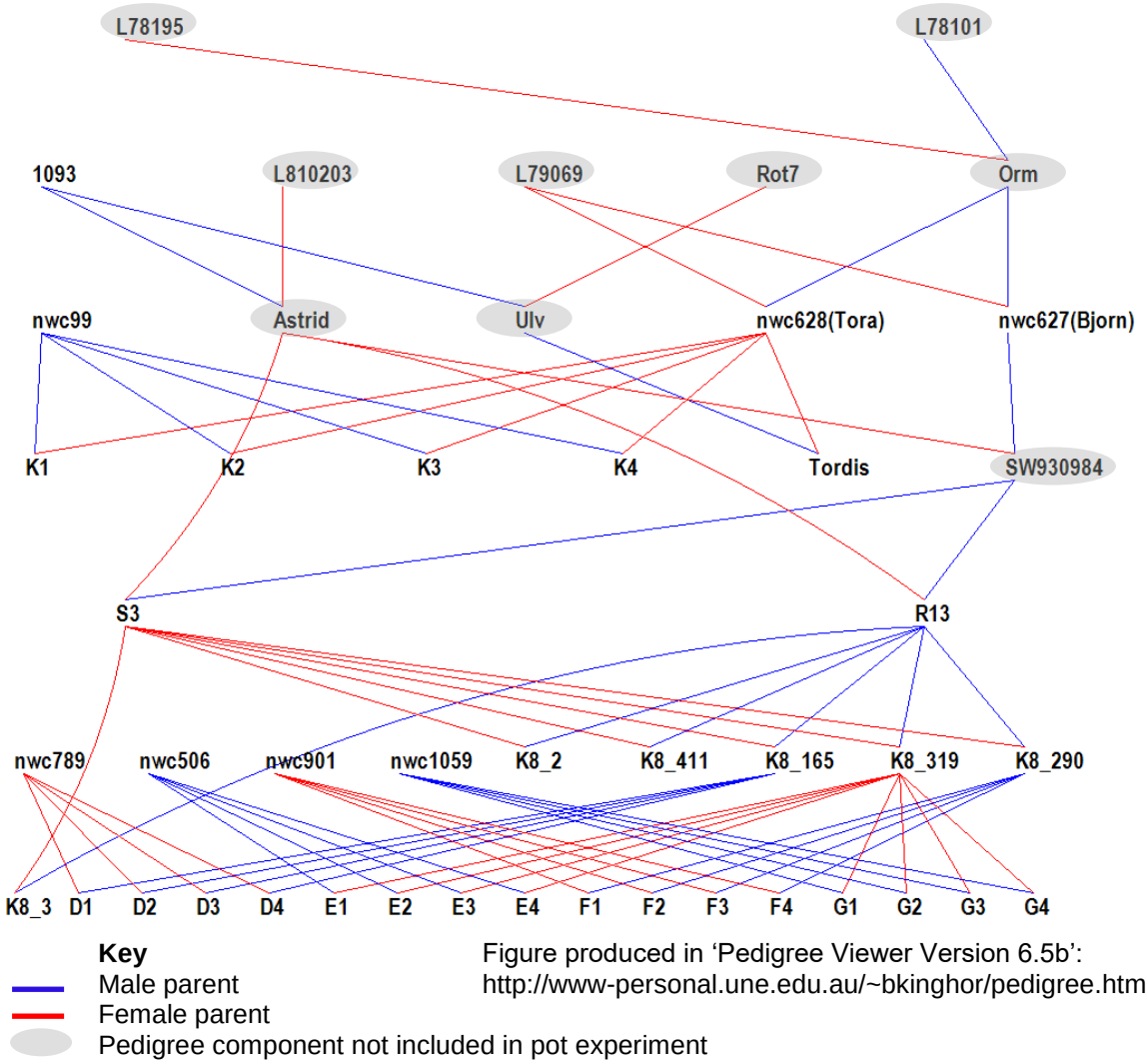

Figure 2.1 Pedigree plot.

Parents of all twelve mapping populations were included along with subsets of progeny from six of these mapping populations (Table 2.1). There were 14 members of an *S. viminalis* association mapping population also included. Fig. 2.1 shows the pedigree structure for 37 of the 54 test genotypes. Genotypes shaded grey in Fig. 2.1 were not included in the pot experiment. Only related genotypes are shown in the pedigree diagram; genotypes included in the pot experiment are listed in Table 2.1. Tora, Bjorn and Tordis are of note as they are commercial SRC varieties produced by Svalöv Weibull, Sweden and are currently marketed by SalixEnergi Europa AB. Tora and Bjorn have been subject to various water stress studies (Cochard et al., 2007; Weih, 2001; Weih and Nordh, 2002; Wikberg and Ogren, 2004). All germplasm to be studied is diploid.

Table 2.1 *Salix* germplasm used in 2014 pot experiment.

ID	Pedigree	Comment
nwc789	789 *S. purpurea* × *viminalis* 'Ulbrichtweide'	D female parent
K8 165	S3 × R13	B, C & D male parent
D1	789 *S. purpurea* × *viminalis* 'Ulbrichtweide' × K8165	
D2	789 *S. purpurea* × *viminalis* 'Ulbrichtweide' × K8165	
D3	789 *S. purpurea* × *viminalis* 'Ulbrichtweide' × K8165	
D4	789 *S. purpurea* × *viminalis* 'Ulbrichtweide' × K8165	
K8 319	S3 × R13	E & G female parent
nwc506	506 *S. caprea* × *cinerea* × *viminalis* 'Grandis'	E male parent
E1	K8319 × 506 *S. caprea* × *cinerea* × *viminalis* 'Grandis'	
E2	K8319 × 506 *S. caprea* × *cinerea* × *viminalis* 'Grandis'	
E3	K8319 × 506 *S. caprea* × *cinerea* × *viminalis* 'Grandis'	
E4	K8319 × 506 *S. caprea* × *cinerea* × *viminalis* 'Grandis'	
nwc901 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42'	F female parent
K8 290 *	S3 × R13	F & I male parent
F1 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F2 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F3 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F4 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
nwc1059	1059 *S. repens*	G male parent
G1	K8 319 × 1059 *S. repens*	
G2	K8 319 × 1059 *S. repens*	
G3	K8 319 × 1059 *S. repens*	
G4	K8 319 × 1059 *S. repens*	
nwc628	628 (*S. schwerinii* × (*S. vim* × *S. vim*) 'Tora'	K female parent, commercial SRC variety
nwc56	56 *S. triandra* 'Baldwin'	

K1	628 (*S. schwerinii* × (*S. vim* × *S. vim*) 'Tora' × 99 *S. triandra* 'Semperflorens'	
K2	628 (*S. schwerinii* × (*S. vim* × *S. vim*) 'Tora' × 99 *S. triandra* 'Semperflorens'	
K3	628 (*S. schwerinii* × (*S. vim* × *S. vim*) 'Tora' × 99 *S. triandra* 'Semperflorens'	
K4	628 (*S. schwerinii* × (*S. vim* × *S. vim*) 'Tora' × 99 *S. triandra* 'Semperflorens'	
S3 *	Astrid × SW930984	K8 female parent
R13 *	Astrid × SW930984	K8 male parent
K8 003	S3 × R13	
K8 002	S3 × R13	
nwc663	663 *S. viminalis* 'Pulchra Ruberrima'	Association mapping population (UK NWC), B female parent
nwc615	615 *S. schwerinii* 'K3 Hilliers'	J female parent
nwc627	627 (*S. schwerinii* × (*S. vim* × *vim*) 'Bjorn'	J male parent, commercial variety
nwc453	453 S. *aurita*	I female parent
nwc432	432 *S. daphnoides* 'Fastigiate'	H male parent
nwc844	844 *S. purpurea* 'Uralensis'	
Tordis	Tora × Ulv	Commercial variety
K8 411	S3 × R13	A female parent
nwc1093	*S. viminalis* 'L81102'	Association mapping population (UK NWC), K8 great grandparent
003_CZ	*S. viminalis*	Association mapping population (Czech natural population)
024_CZ	*S. viminalis*	Association mapping population (Czech natural population)
33_CZ	*S. viminalis*	Association mapping population (Czech natural population)
77_CZ	*S. viminalis*	Association mapping population (Czech natural population)
64_CZ	*S. viminalis*	Association mapping population (Czech natural population)
13_CZ	*S. viminalis*	Association mapping population (Czech natural population)
IA159	*S. viminalis*	Association mapping population (Swedish population)
S_Hallstad1	*S. viminalis*	Association mapping population (Swedish population)
IA136	*S. viminalis*	Association mapping population (Swedish population)
IA102	*S. viminalis*	Association mapping population (Swedish population)

IA162	*S. viminalis*	Association mapping population (Swedish population)
IA143	*S. viminalis*	Association mapping population (Swedish population)

* Genotype also present in 2014 pot experiment

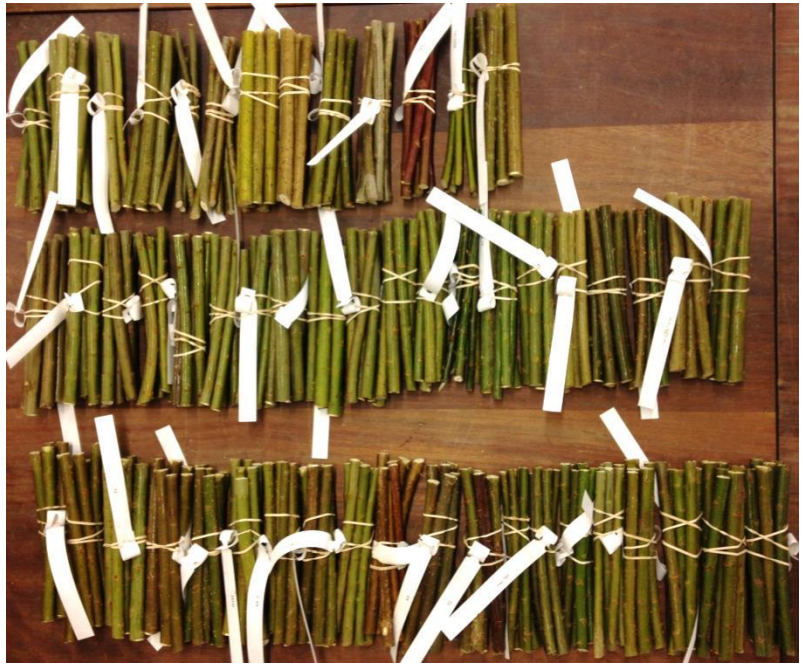

Figure 2.2 Dormant 15 cm woody cuttings.

The study material offers the opportunity of assessing a broad-range of *Salix* germplasm, but also allows the potential for interesting families and populations to be identified for further study. The progeny included from mapping populations, (D1, D2, D3, D4, E1, E2, E3, E4, F1, F2, F3, F4, G1, G2, G3, G4, K1, K2, K2, K3, K4, K8002 and K8003 were chosen at random as there was no prior knowledge to the performance of mapping population progeny in drought conditions. Including this progeny gives the potential to select mapping populations of interest for further study, with the ultimate goal of assessing if mapping population progeny segregate for drought traits of interest. Potentially QTLs that underpin these key drought traits could be identified if segregating populations can be selected in conjunction with high throughput phenotyping techniques.

Dormant woody cuttings were collected from field trials in January, cut to 15 cm lengths, labelled and wrapped in plastic and stored in a -4°C freezer until planting in May.

2.2.2 Experimental design

A split plot design with ± irrigation on main plots and genotype on split plots was used to conduct an experiment to assess the differences between the performance of 54 genotypes in 2014. The water treatment was applied on whole plots and genotype was applied on sub-plots. The 'Design' function in GenStat for Windows, 16th edition was used to plan the experiment. Appendix 1 details the experiment layout.

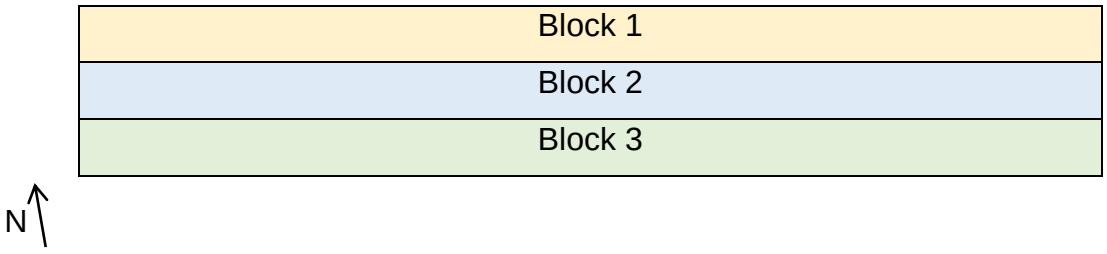

Figure 2.3 The blocking arrangement in a rain-out shelter (GH44).

2.2.3 Planting and growth conditions

Cuttings were removed from cold storage 24 hours before planting. Once defrosted, cuttings were soaked overnight in cold water to rehydrate before planting. Square black 11 l plant pots measuring 22 x 22 x 26 cm were placed in black polypropylene 25 cm diameter, 4 cm deep saucers. Pots were filled with a

homogeneous mix of 50:50 by volume of perlite and Rothamsted prescription mix (RPM) compost with added nutrients; (75% medium grade [L&P] peat, 12% screened sterilized loam, 3% medium grade vermiculite, 10% grit [5-mm screened, lime free], 3.5 kg “Osmocote Exact Standard 3-4M” per m^3 (Scotts UK Professional, Ipswich, Suffolk).], 0.5 kg PG mix/m3 [Hydro Agri (UK) Ltd., Bury St. Edmunds, Suffolk], lime [approximately 3 kg/m 3 to pH 5.5–6.0], Vitax Ultrawet [wetting agent: 200 ml/m^3]). Equal volumes of each growth medium were measured into a 90 l dustbin. The RPM and perlite were mixed thoroughly with a shovel until homogenous. A piece of paper towel was placed in the bottom of the pot to prevent growth medium from escaping. Pots were filled with 3 kg of growth medium. Each pot was filled with a large shovelful, tapped on the ground then the additional growth media to obtain 3 kg per pot (±5g) was added, before being tapped on the floor again to ensure a similar bulk density.

Figure 2.4 The open south facing side of GH44, the rain out shelter that housed the pot experiment.

The experiment was located at Rothamsted Research in a rain out shelter (GH44). The area was designed for work requiring ambient temperatures, but also requiring cover from rain. GH44 (Fig. 2.4) has open netted sides and a polyethene roof, providing cover from rain fall. The shelter, manufactured by Clovis Lande, was erected in 2001. The area of the cage is 160 m^2. The surface of the covered area is a concrete slab. The south facing side of the cage is open whilst the north and east facing sides are shaded by shrubs in the adjacent herbaceous borders. The higher light intensity from the southern edge appears to be the greatest

potential cause of heterogeneity. Fig. 2.3 details the blocking approach selected to mitigate this identified heterogeneity.

Pots were placed in GH44 using a twin row design, with a spacing of 26 cm between pots in twin rows and 65 cm between pairs of rows. Pots were spaced at 26 cm within each row, see Fig. 2.5. There was a 110 cm gap between the pots on the north and south edge of the experiment and the edge of GH44. Once arranged, pots were watered by filling to the brim individually with a hosepipe and lance fitted with a rose nozzle prior to planting. The pot experiment was planted on 16th May 2014. Cuttings were inserted into the centre of each pot. *Circa* 1 cm of the cutting was left above the growth medium surface. Pots were watered to field capacity after planting.

Pots were weeded as necessary. The insecticide Hallmark (100 g/l lambda-cyhalothrin) was applied on 16th July 2014 at the recommended rate to control Terminalis midge (*Dasineura* spp.), aphids, willow beetles (*Chrysomelidae*) and sawfly larvae (*Nematus pavidus*). Pots were fertilised after the first drought stress period on 7th August 2014 with 5g of Osmocote Exact Standard 3-4M per m^3 (Scotts UK Professional, Ipswich, Suffolk) to ensure plants had sufficient nutrients.

2.2.4 Irrigation regime

All pots were watered by hand with a hosepipe until the beginning of June when a drip irrigation system was installed. Galcon 9001 irrigation controllers (Kfar Blum, Israel) were used to schedule watering. Water was delivered to each pot using a single Octa-Mitter adjustable stake dripper (Access Irrigation, Northampton, UK). From early June pots were watered once a day, two minutes per watering. In late June this was increased to twice a day. Irrigation was increased to three minutes, three times a day in late July. The drippers delivered approximately 0.4 l of water per minute to each pot. Irrigation was scheduled when the saucers were dry in an attempt to keep pots at pot capacity and avoid waterlogging.

Two periods of water stress were applied to the drought whole plots by stopping the irrigation. The first drought (D1) started for all plants on 21st July and ended between 24th July and 28th July (3-8 days), when droughted pots were watered again. The second drought (D2) was initiated on 4th September in 2014 and pots were returned to watering from 11th September to 25th September (7-21 days).

During the drought periods, pot soil moisture was monitored daily using a Delta-T SM200 Soil Moisture Sensor and HH2 logger (Delta-T Devices Ltd, Cambridge, UK). The sensor was calibrated as per the manufacturer's instructions. Three measurements were taken per pot and the mean of these measurements was used. Each replicate of each genotype, within the drought whole plot, was exposed to the same duration of water stress in 2014, with the three replicates of each droughted genotype being returned to watering when the mean of the three droughted pots' soil water content of 7 vol. % was reached. The first drought period lasted 3-8 days and the second drought period lasted for 7-21 days; the length of drought was dictated by the SM200 Soil Moisture Sensor readings.

2.2.5 Experiment monitoring

LogTag (Dorset, DT11 9EX, UK) temperature and humidity loggers were placed in each of the 6 whole plots (See Appendix 2 for LogTag locations). The sensors were suspended at 1 m above the ground from beams in GH44. The sensors were shielded from direct solar radiation by an 18 cm wide 8 cm deep cone covered in aluminium foil (See Fig 2.5). Sensors logged hourly temperature and humidity values from 23rd May 2014 until the end of the experiment. Meteorological data was provided by the Environmental Change Network (ECN) automatic weather station at Rothamsted Research. This is located 400 m south of GH44 and data were available for the duration of the experiment.

Figure 2.5 Suspended LogTag temperature loggers.

Log Tag temperature loggers are circled in white. They were suspended under a cone lined in aluminium foil to shade the temperature sensors.

2.2.6 Phenotypic measurements

Leaf emergence scores

Pots were assessed at three-day intervals post planting and scored using the 1-7 key presented in Fig. 2.6. Plants were considered fully emerged when they reached the score of 7 - fully unfolded leaves were observed, and stem extension has begun, stem >3cm.

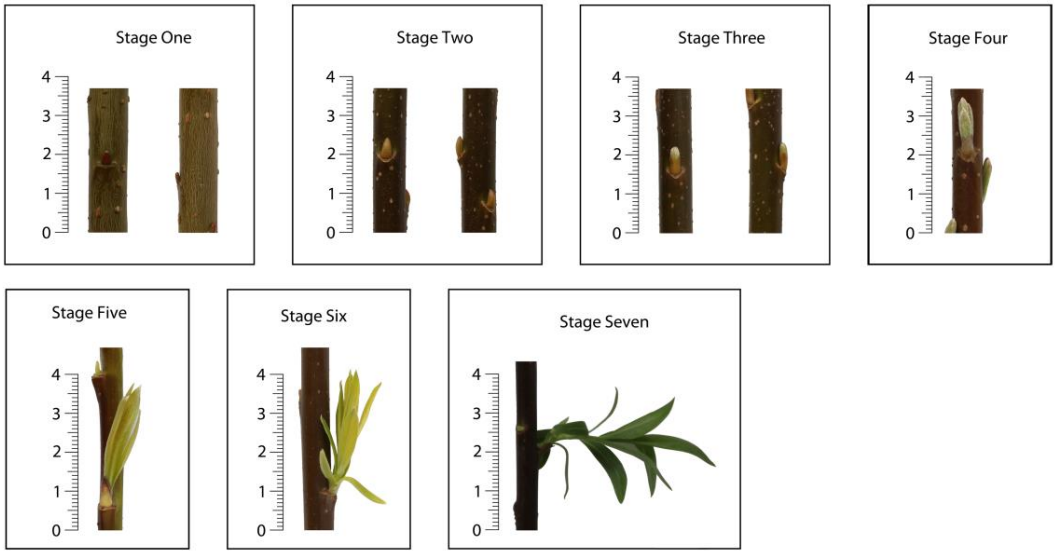

Figure 2.6 Shoot emergence score.

Source: Prepared by March Castle, Rothamsted Research
Developing buds were scored according to the above seven point scale adapted from (Weih, 2009): Stage one, No sign of bud growth, tip of bud tightly pressed to the shoot; Stage two, bud swollen (1-4 mm length) tip of bud bending away from the stem; Stage 3, bud burst stage 1: green leaf tips visible <5mm; stage four, bud burst stage 2: green leaf tips >5mm; stage five, elongating new leaves bending away from each other; stage 6 one or more leaves perpendicular to the shoot axis, some stem may be visible (<3cm); stage 7, numerous, fully unfolded leaves observed, stem extension has begun, stem >3cm.

Time points

The experiment was planted on 16th May 2014. Measurements were taken; before drought 1 (BD1), during drought 1 (D1), starting 21st July and lasting 3 – 8 days, after drought 1 (AD1), before drought 2 (BD2), during drought 2 (D2), starting 4th September and lasting 7 – 21 days, and after drought 2 (AD2).

Leaf counts

Leaves derived from the main proleptic stems were counted and recorded at three time points, BD1, 11th July, AD1, 30th July and BD2, 28th August. The dominant

lead stems were marked and counted first. Subsequent stems were counted in a clockwise order from the lead stem. Immature leaves that had not reflexed from the growing tip were not counted. Fig. 2.7 shows a cluster of unfolded immature leaves that would not be counted.

Figure 2.7 Leaves and growing tip.

Figure 2.8 Lanceolate willow leaf with petiole visible.

Leaf measurements

Leaf length and width measurements were taken on leaves on the lead stem at three time points, BD1, 15th July, AD1, 31th July and BD2, 1st September. Leaves were measured at three points on the stem at each time point; the top leaf (TL) ten leaves down from first reflexed leaf, the middle leaf (ML) the leaf attached at the mid-point of the stem, the bottom leaf (BL) ten leaves up from the lowest main leaf. Leaf lengths were taken from the tip of the lead to the point where the leaf blade ended, and the petiole started (See Fig. 2.8). Leaf widths were taken at the widest point of the leaf.

Leaf area calculations

A crude area was calculated by multiplying Length (L) and width (W) of leaves. Leaf area, adjusted was calculated to account for the shape of leaves using a non-linear regression developed in first year *S. viminalis* leaves (Verwijst and Wen, 1996):

$$\text{leaf area (cm}^2\text{)} = (b_0 + b_1\,\text{cat})LW^c$$

where; $b_0 = 0.906$, $b_1 = -0.036$, cat = 1 (leaves on proleptic shoots), LW = leaf length x leaf width and $c = 0.944$

The adjusted leaf area was then used to calculate the whole plant adjusted leaf area. This assumes that the TL, ML and BL are representative of a third of leaves respectively from the leaf counts.

Chlorophyll meter readings

On the second day of D1, 22nd July, a SPAD-502 meter, Konica Minolta Inc., was used to take non-destructive estimates of leaf chlorophyll concentrations. These were taken at the centre of TL, ML and BL, taking care to avoid the leaf midrib. These leaves were determined using the same leaf TL, ML and BL method as described above. An average value of TL, ML and BL was calculated and multiplied with the number of leaves on the leaf stem AD1 to give a lead stem total SPAD value.

Sylleptic branch and leaf counts

Leaves derived from the sylleptic branches were counted and recorded at three-time points, BD1, 11th July, AD1, 2nd August and BD2, 2nd September. The

sylleptic branches on the dominant, marked lead stem were counted first. Subsequent stems were counted in a clockwise order from the lead stem. Leaves on the lowest sylleptic branches were taken first, before working systematically up the stem to the top. Similar to main stem leaf counts, immature sylleptic leaves that had not reflexed from the growing tip were not counted. Sylleptic leaf counts were recorded per branch, so a sylleptic branch count per stem could be calculated.

Figure 2.9 Sylleptic branches protruding from the main proleptic stem on 29th July. (8 days after the start of D1).

2.2.7 Yield measurements

The experiment was harvested between 18th - 25th September in 2014. Genotypes were harvested at the end of their second drought period. All replicates of each genotype were harvested on the same day. Stems were cut at the soil surface using secateurs. Fresh and dry weight analysis was performed in 2014 on all above ground biomass (leaves were not stripped from the stems). Dry weights were taken after the stems were cut into *circa* 2 cm sections and the biomass was dried in aluminium trays at 80°C for 48 hours.

2.2.8 Statistical analyses

The following split-plot analysis of variance (ANOVA) was used:

*y ~ Genotype*Irrigation + Block/MainPlot/SplitPlot*

where *y* represents any particular response, *Genotype* is the fixed model term denoting the genotype, *Irrigation* is the fixed model term denoting the treatment effect (-Irrigation or +Irrigation), *Block* is the random model term denoting the *Block* The slash (/) indicates the nesting of model terms, and the star (*) indicates that main effects and interactions should be fitted. The statistical significance of fixed effects was tested using F-tests.

The predicted means for the relevant statistically significant model terms were output with standard error of the difference (SED), degrees of freedom and least significant difference (LSD) values at the 5% (p = 0.05) level of significance for their comparison.

The Genstat (2015, 18th edition, © VSN International Ltd, Hemel Hempstead, UK) statistics package was used for all analyses.

2.2.9 Drought tolerance indices

The drought tolerance indices defined in Table 2.2 will be calculated from the dry matter yield results. Ys are stress (drought treatment) and Yp represent optimal (potential or well-watered) yield of any given genotype. $\bar{Y}s$ and $\bar{Y}p$ are average yields of all genotypes under stress and optimal conditions.

Table 2.2 Drought tolerance indices

Index Name	Equation
Drought tolerance efficiency (DTE)	$DTE = \left(\frac{Ys}{Yp}\right)$
Yield Index (YI)	$YI = \frac{Ys}{\overline{Yp}}$
Mean Productivity (MP)	$MP = \frac{Ys + Yp}{2}$
Stress Tolerance Index (STI)	$STI = \frac{(Ys)(Yp)}{(\bar{Y}p)^2}$
Tolerance Against Stress (TOL)	$TOL = (Ypi - Ysi)$
Geometric Mean Productivity (GMP)	$GMP = \sqrt{(Ys)(Yp)}$
Harmonic Mean (HARM)	$HARM = \frac{2(Yp \times Ys)}{Yp + Ys}$

2.3 Pot experiment results 2014

2.3.1 Meteorological data

Fig. 2.10 shows the maximum and minimum daily temperatures inside the rainout shelter and from the Rothamsted Meteorological site. Whilst minimum temperatures were broadly similar to readings from the meteorological site (+0.58°C on average), maximum daily temperatures were elevated in the rain out shelter (+3.05°C on average). A maximum temperature in the rain out shelter of 32.5°C (29.9°C at the Met. site) was recorded on 18th July. Days recorded with a maximum temperature exceeding 30°C were 2 and days exceeding a maximum temperature of 25°C totalled 45, including the period during D1.

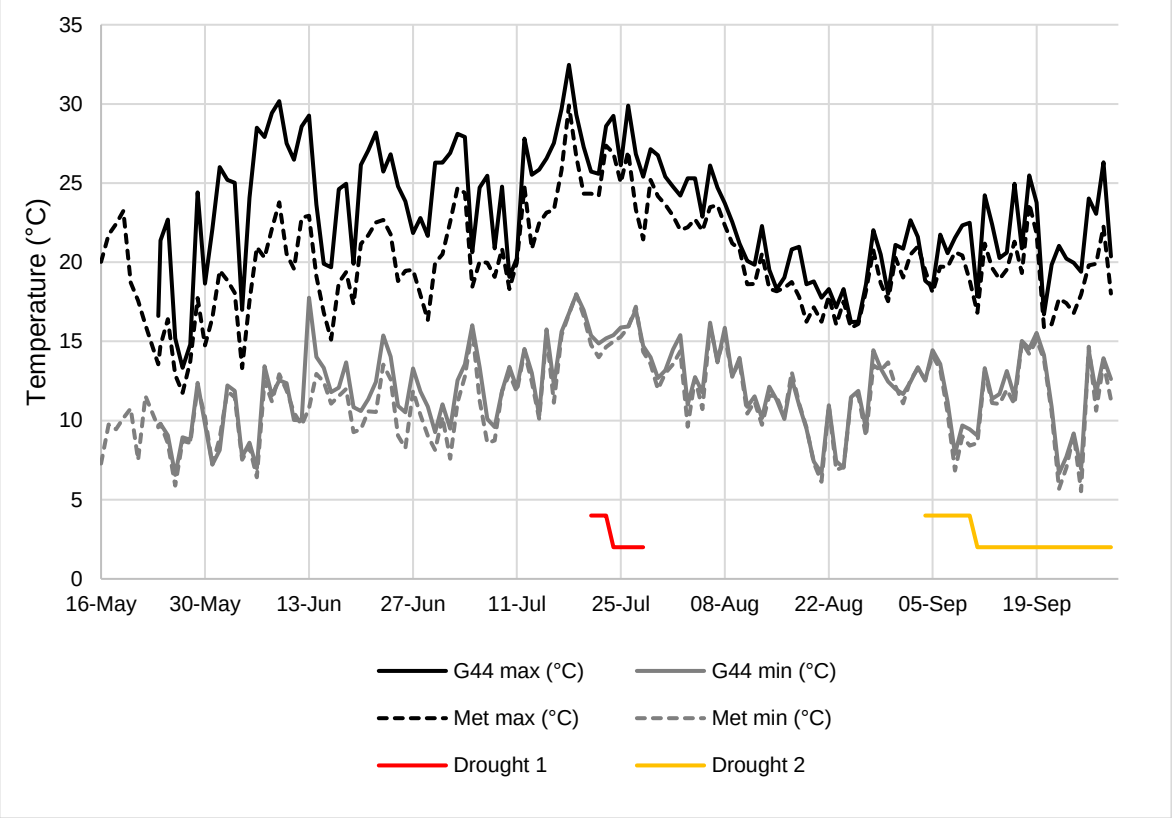

Figure 2.10 2014 maximum and minimum daily temperatures from Rothamsted Meteorological site and LogTag sensors in GH44.

Drought periods marked in a stepped line to represent drought time range, D1, 3 – 8 days, D2, 7 – 21 days.

The temperatures of >30°C observed during mid-July in Fig. 2.10 mean that symptoms could be attributed to a combination of heat and drought stress. Abiotic stresses including drought and heat stress induce a cascade of physiological and

molecular events resulting, in some cases, in similar responses. Heat stress is defined as the rise in soil and air temperature beyond a threshold level for a minimum amount of time such that permanent harm to plant growth and development occur (Lamaoui et al., 2018). A study of two desert willow species, *S. gordejevii* and *S. babylonica* showed no effect of temperature up to 35°C (Yang et al., 2004). However, the temperature threshold level of the desert species is likely to be higher than many of the genotypes in this study, which are unknown, meaning that heat stress could have been a factor during this period.

2.3.2 Results for key varieties in 2014 pot experiment

Table 2.3 ANOVA results for key varieties in 2014 pot experiment.

Timing	Variate	*Geno* F pr.	*Irrigation* F pr.	*Geno.Irrigation* F pr.
Leaf counts (leaf number)				
BD1	11/7/14 Lead stem	<.001	0.502	0.488
BD1	11/7/14 Whole plant	<.001	0.485	0.971
AD1	30/7/14 Lead stem	<.001	0.13	<.001
AD1	30/7/14 Whole plant	<.001	0.243	0.868
BD2	28/8/14 Lead stem	<.001	0.277	0.019
BD2	28/8/14 Whole plant	<.001	0.571	0.728
Leaf measurements (cm)				
BD1	15/7/14 Top leaf L	<.001	0.195	0.784
BD1	15/7/14 Top leaf W	<.001	0.56	0.844
BD1	15/7/14 Middle leaf L	<.001	0.874	0.672
BD1	15/7/14 Middle leaf W	<.001	0.777	0.22
BD1	15/7/14 Bottom leaf L	<.001	0.613	0.5
BD1	15/7/14 Bottom leaf W	<.001	0.246	0.743
AD1	31/7/14 Top leaf L	<.001	0.224	<.001
AD1	31/7/14 Top leaf W	<.001	0.459	<.001
AD1	31/7/14 Middle leaf L	<.001	0.302	0.34
AD1	31/7/14 Middle leaf W	<.001	0.429	0.431
AD1	31/7/14 Bottom leaf L	<.001	0.503	0.684
AD1	31/7/14 Bottom leaf W	<.001	0.266	0.071
BD2	01/9/14 Top leaf L	<.001	0.02	0.004
BD2	01/9/14 Top leaf W	<.001	0.987	0.06
BD2	01/9/14 Middle leaf L	<.001	0.031	0.051
BD2	01/9/14 Middle leaf W	<.001	0.556	0.518
BD2	01/9/14 Bottom leaf L	<.001	0.348	0.023
BD2	01/9/14 Bottom leaf W	<.001	0.496	<.001

Timing	Variate	*Geno* F pr.	*Irrigation* F pr.	*Geno.Irrigation* F pr.
Leaf area calculations				
AD1	31/7/14 Top leaf LxW	<.001	0.413	0.006
AD1	31/7/14 Top leaf LxW adj[a]	<.001	0.399	0.004
AD1	31/7/14 Whole plant adj[b] leaf area	<.001	0.276	0.003
Chlorophyll meter during first drought				
D1	22/7/14 Top leaf SPAD	<.001	0.148	0.439
D1	22/7/14 Middle leaf SPAD	0.365	0.567	0.178
D1	22/7/14 Bottom lead SPAD	<.001	0.24	0.967
D1	22/7/14 Mean SPAD	<.001	0.436	0.205
D1	22/7/14 Lead stem total leaf SPAD	<.001	0.161	0.002
Sylleptic branch and leaf counts				
BD1	11/7/14 Whole plant syll. branch	<.001	0.707	0.766
BD1	11/7/14 Whole plant syll. leaf	<.001	0.796	0.955
AD1	02/8/14 Whole plant syll. branch	<.001	0.4	0.06
AD1	02/8/14 Whole plant syll. leaf	<.001	0.613	0.064
BD2	02/9/14 Whole plant syll. Branch	<.001	0.203	0.052
BD2	02/9/14 Whole plant syll. Leaf	<.001	0.168	0.123
Final harvest yield responses				
AD2	Above ground biomass DW yield (g)	<.001	0.416	0.055
AD2	Above ground biomass FW yield (g)	<.001	0.112	<.001
AD2	Final harvest % DW	<.001	0.002	<.001

[a] Calculated using a non-linear regression (Verwijst and Wen, 1996)
[b] Adjusted leaf area (Verwijst and Wen, 1996) to whole plant level

Table 2.3 presents ANOVA results for varieties from different time points during the 2014 pot experiment. The residual plots indicated a random scatter with broadly homogeneous variability across the genotype by treatment combinations, so there was no need to transform the data.

For all varieties investigated there was no interaction expected or observed between genotype and irrigation at the BD1 timepoint.

Final harvest yield responses

There were no main effects of the irrigation treatment for fresh or dry weight above ground biomass yield. There was a main effect of irrigation on the final harvest dry matter % ($p = 0.002$, F-test). ANOVA revealed a borderline interaction ($p = 0.055$,

F-test) for above ground biomass (stem and leaves) dry matter and a significant interaction for fresh weight above ground biomass and dry matter % (p = <0.001, F-test) between genotype and drought treatment in 2014. Differences between genotypes were significant (p < 0.001, F-test) for all final harvest yield responses.

Control plants yielded 10.50% higher than the drought treatment. Maximum dry matter yields for control and drought treatment plants were 572.6 g and 368.6 g respectively, whilst minimum yields were 3.8 g for control plants and 6.78 g for drought treatment plants.

Fig. 2.11 and 2.13 to 2.24 were sorted by family then within family performance for the well-watered control. Fig 2.11, 2.12 and 2.15 - 2.17 show final harvest yield responses. Fig. 2.13 and 2.14 and Table 2.3 show drought indices results.

Fig. 2.11 shows that mpF's parents, 901 and K8 290 (male parent K8 290 is grouped with other mpK8 family members) segregating at opposing ends of the yield range. Genotypes of mpK8 segregate for yield, however their parents S3 and R13 both rank above their progeny.

Including *Family* in the fixed term model for ANOVA of dry weight above ground biomass yield:

$$y \sim (Family/Genotype)*Irri + Block/MainPlot/SplitPlot$$

Revealed no main effect of *Irrigation* (0.416, F-test) but interaction for *Family* (p = <.001, F-test), *Genotype* (p = <.001, F-test) and a *Family.Irrigation* interaction (p = 0.019, F-test), but no interaction between G*enotype* and *Irrigation* (p = 0.837, F-test). Means for the families are plotted in Fig. 2.15.

Drought tolerance indices

Table 2.4 reveals that drought tolerance indices Geometric Mean Productivity (GMP), Mean Productivity (MP) and Harmonic Mean (HARM) produce the same results. These three indices highly correlate with Yield Index (YI) (r = 0.97) and the Stress Tolerance Index (STI) (r = 0.97). The Drought tolerance efficiency (DTE) and Tolerance Against Stress (TOL) have a strong negative relationship. Figs 2.13 (DTE) and 2.14 (HARM) show results from each of these two broad classes of drought tolerance indices that can be used for selecting genotypes response to drought stress.

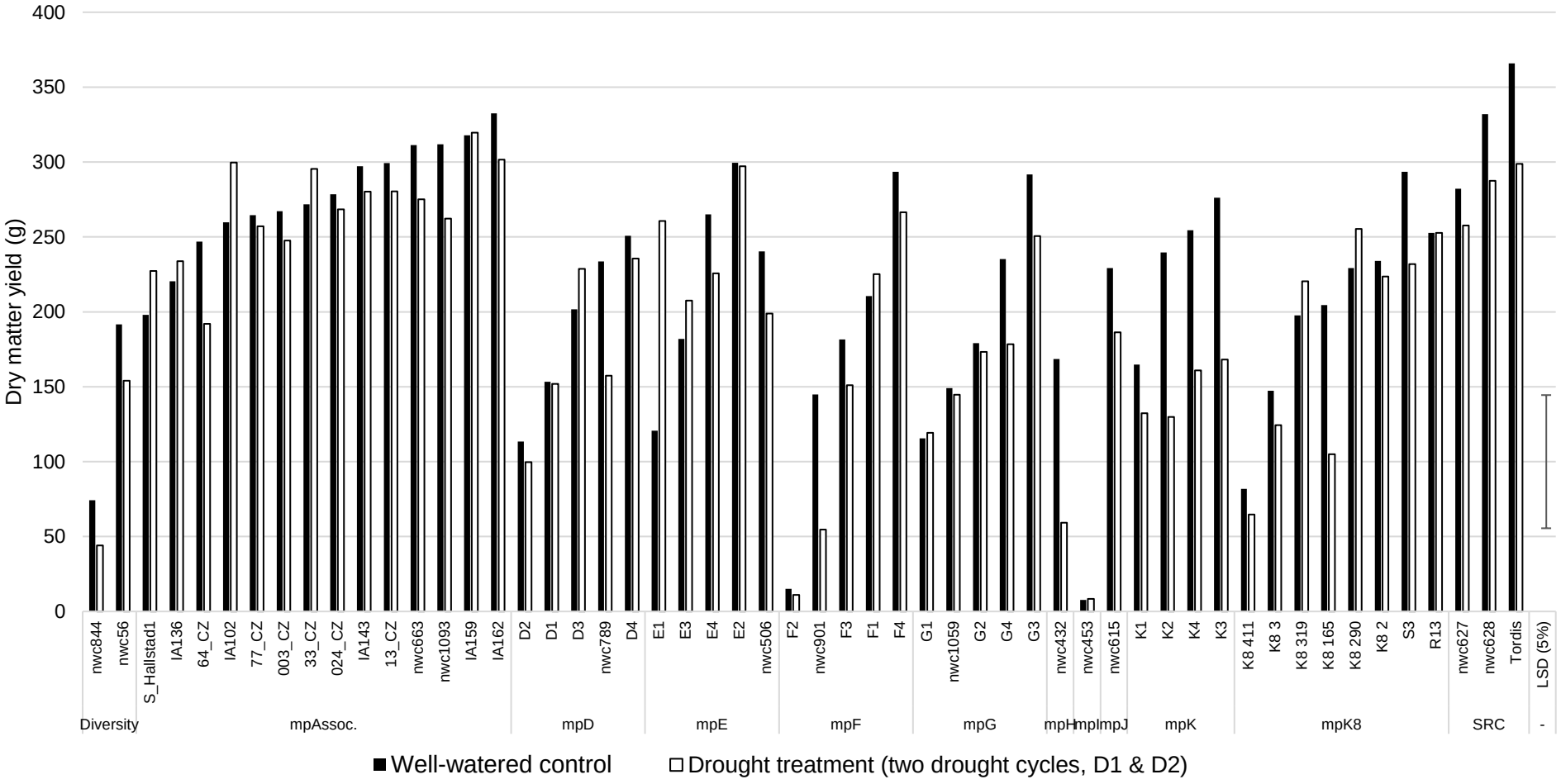

Figure 2.11 2014 Final harvest above ground biomass dry matter yield.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

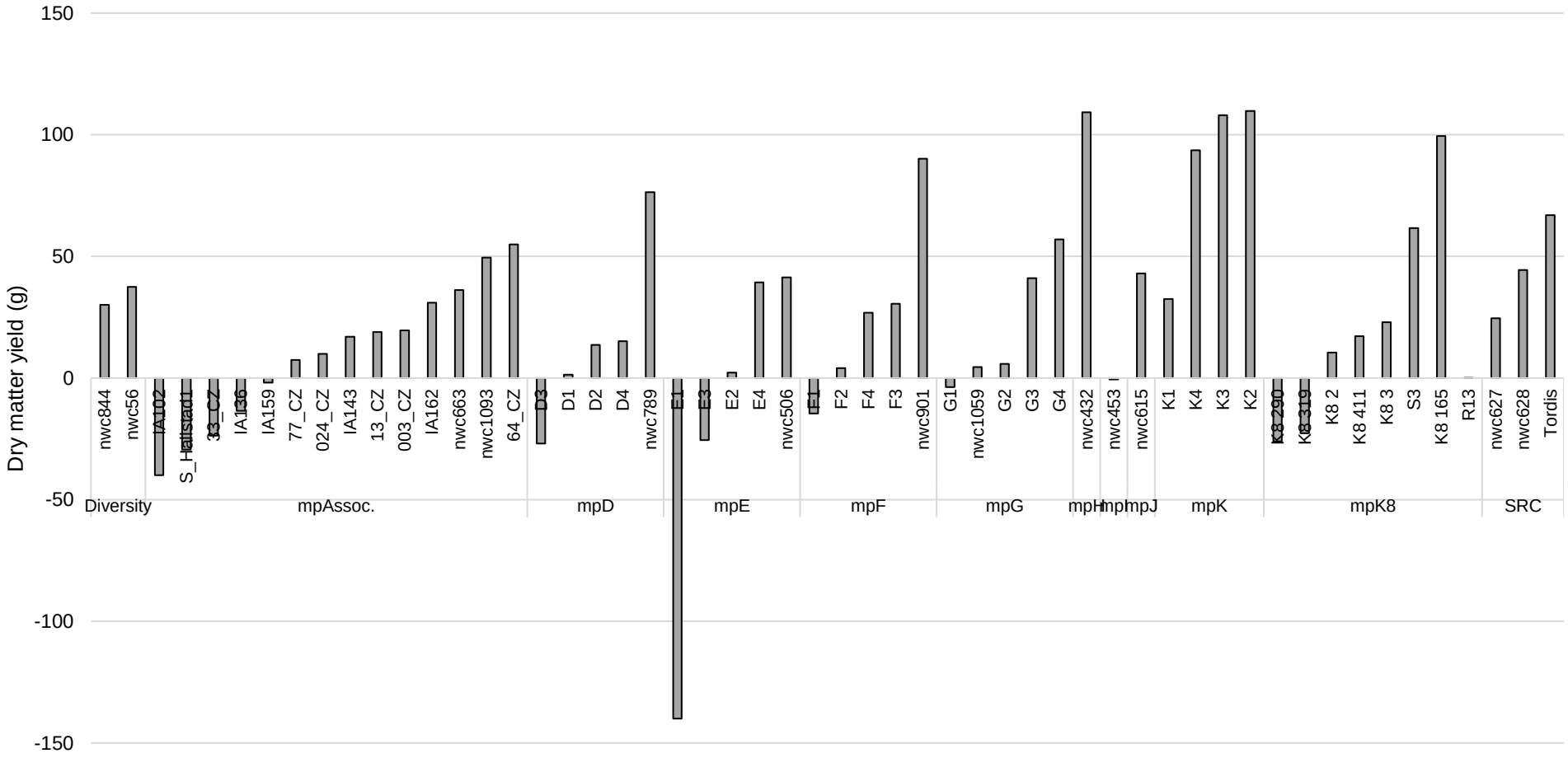

Figure 2.12 2014 Difference between (control – drought) in final harvest above ground biomass dry matter yield.

Table 2.4 Correlation coefficients between drought tolerance indices and 2014 final harvest dry matter yield

	DTE	YI	MP	STI	TOL	GMP	HARM	Well-watered control DM Yield (Yp)	Drought treatment DM Yield (Ys)
DTE	-								
YI	0.46	-							
MP	0.22	0.96	-						
STI	0.20	0.93	0.97	-					
TOL	-0.92	-0.32	-0.05	-0.06	-				
GMP	0.22	0.97	1.00	0.97	-0.06	-			
HARM	0.23	0.97	1.00	0.97	-0.07	1.00	-		
Well-watered Control DM yield (Yp)	-0.05	0.85	0.96	0.92	0.23	0.96	0.95	-	
Drought treatment DM yield (Ys)	0.46	1.00	0.96	0.93	-0.32	0.97	0.97	0.85	-

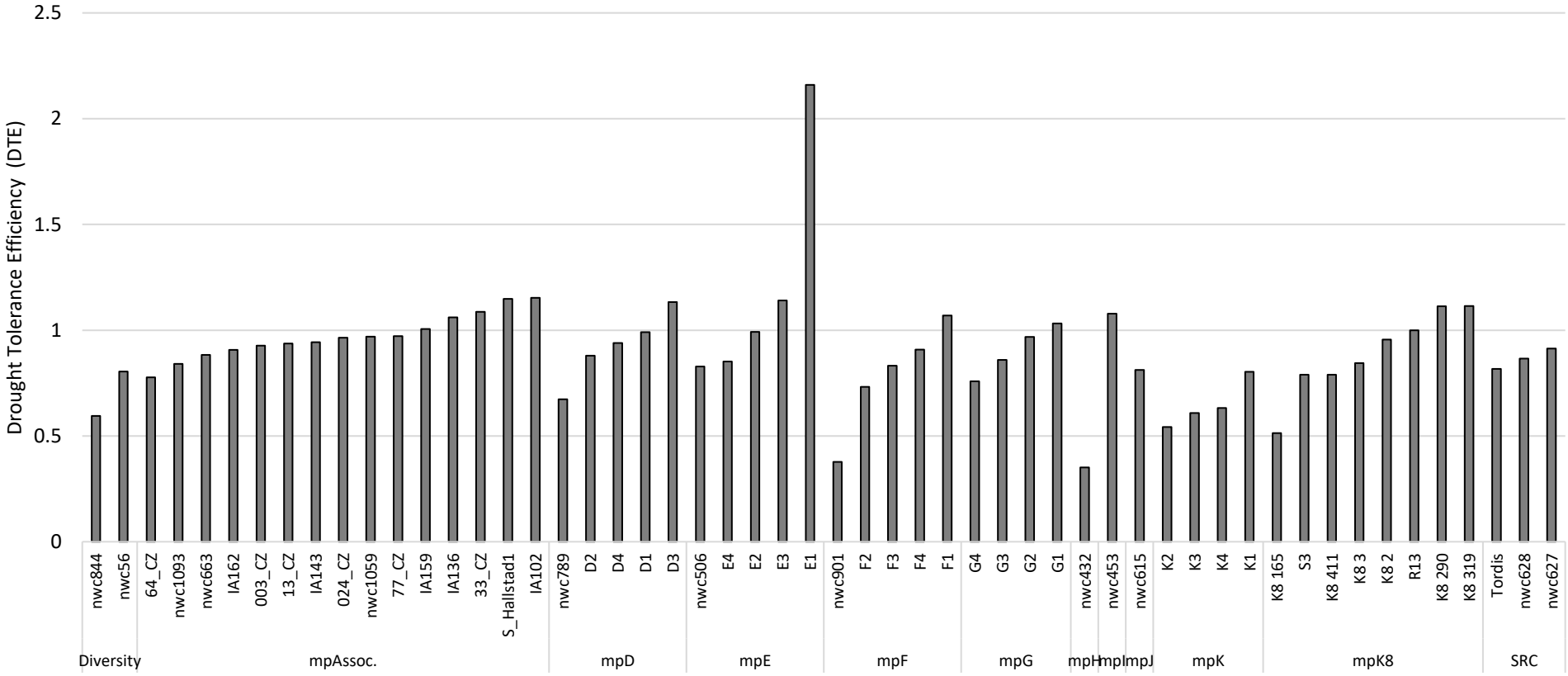

Figure 2.13 2014 Drought tolerance efficiency (DTE) index for final harvest above ground biomass dry matter yield

Drought tolerance efficiency (DTE) formula $DTE = \left(\frac{Ys}{Yp}\right)$

Plotted maintaining family order on the *x*-axis from Fig. 2.11.

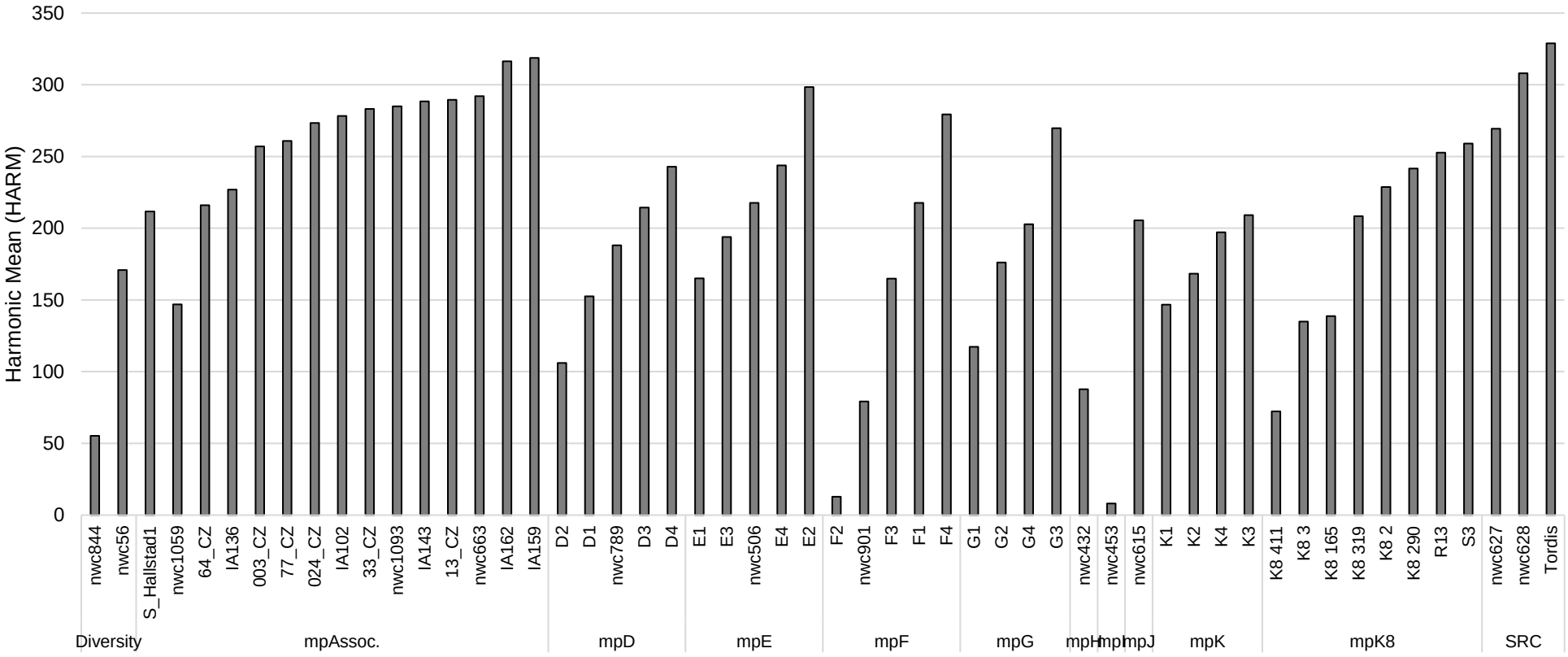

Figure 2.14 2014 Harmonic Mean (HARM) index for final harvest above ground biomass dry matter yield

Harmonic Mean (HARM) formula $HARM = \frac{2(Yp \times Ys)}{Yp+Ys}$

Plotted maintaining family order on the *x*-axis from Fig. 2.11.

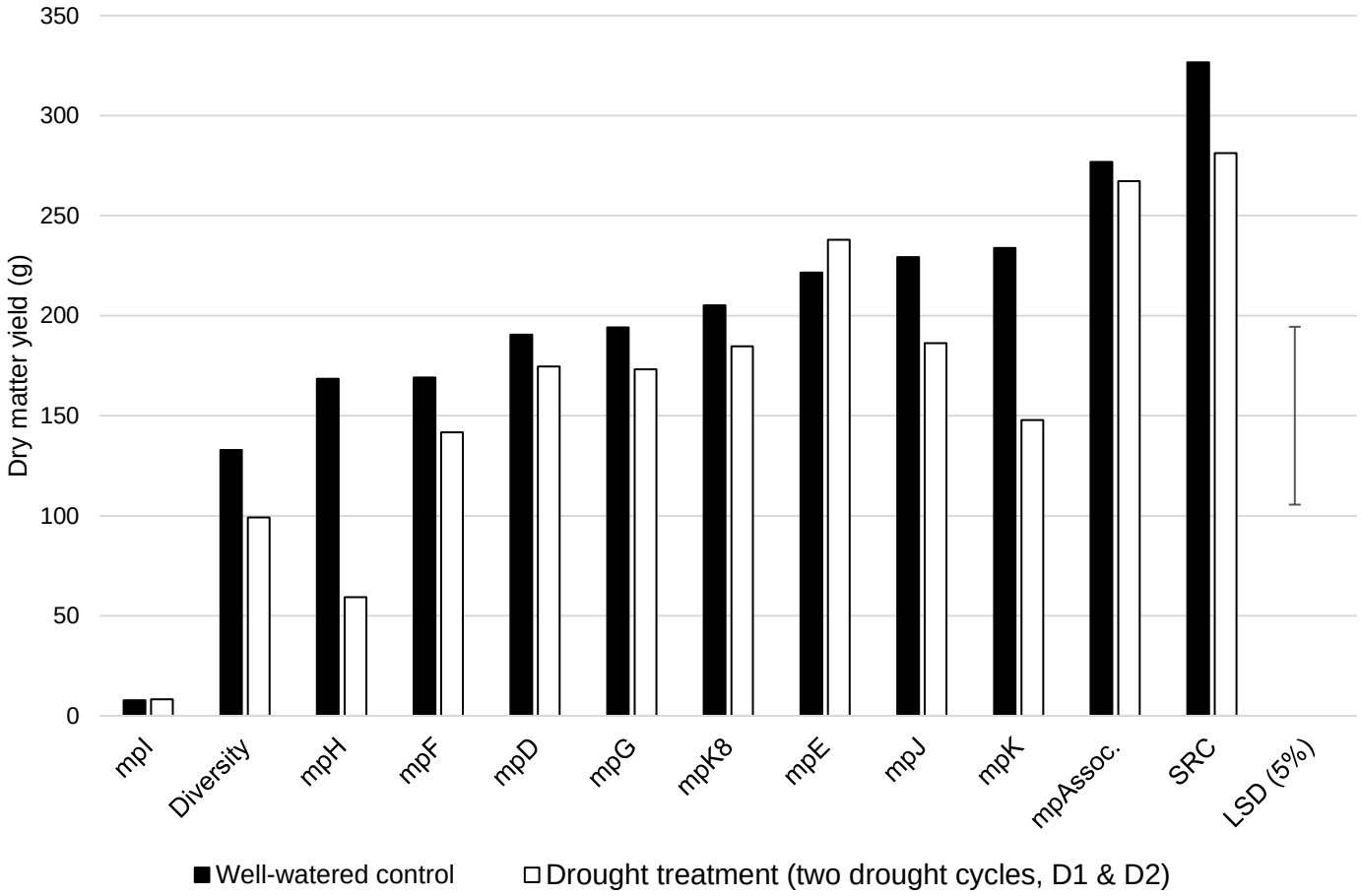

Figure 2.15 2014 Final harvest above ground biomass dry matter yield by family.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

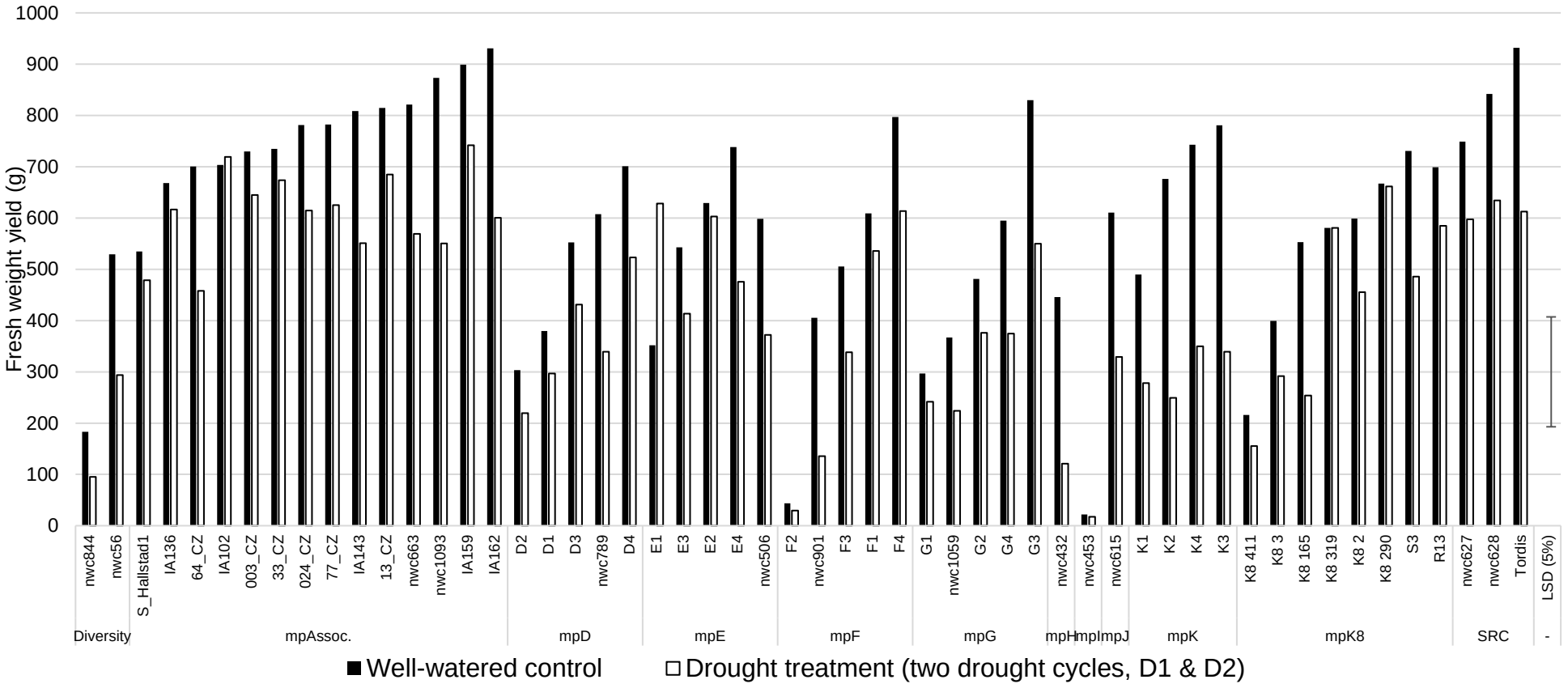

Figure 2.16 2014 Final harvest above ground biomass fresh weight yield.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

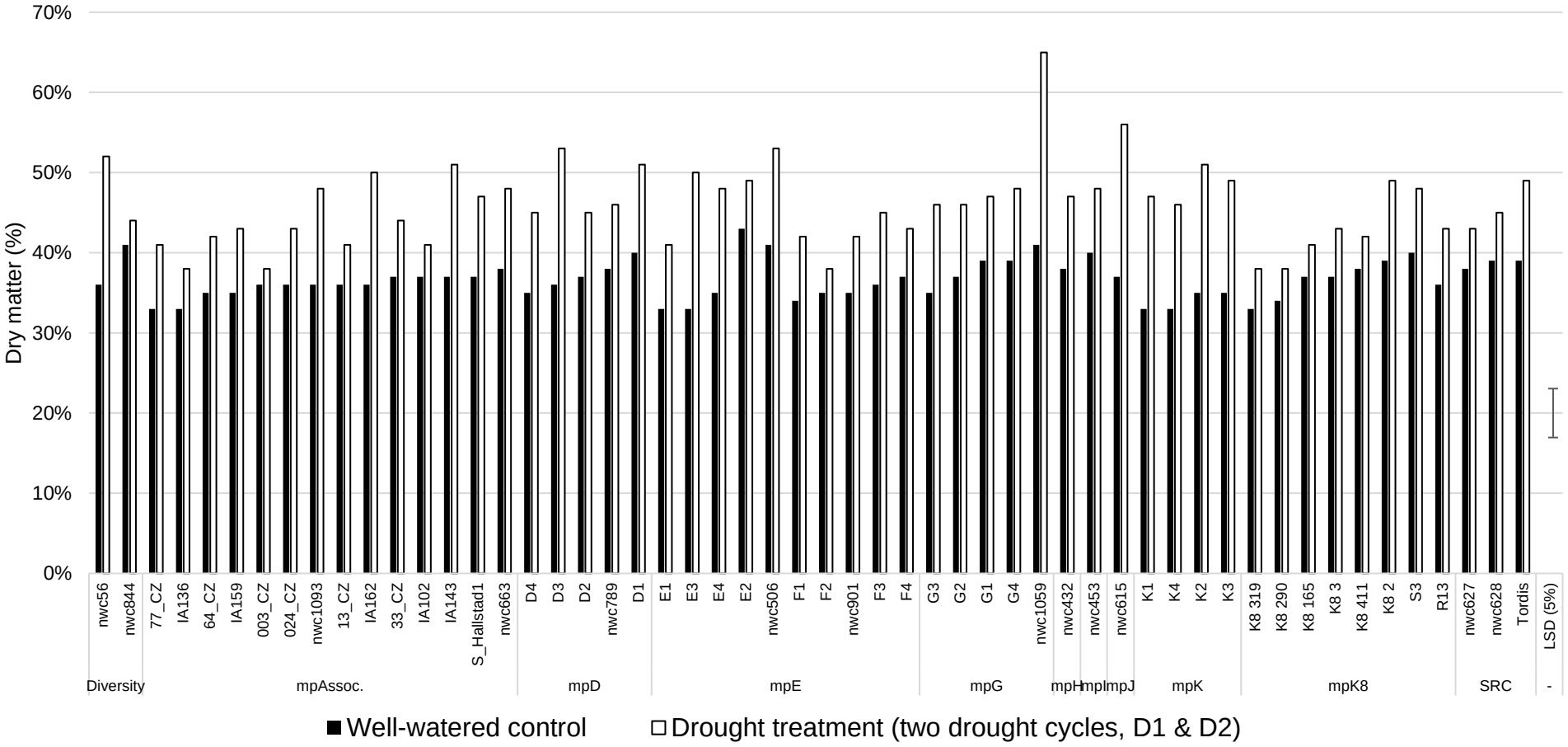

Figure 2.17 2014 Final harvest % dry matter.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

Leaf counts

The number of leaves on the lead stem (proleptic stem) leaves AD1 (p = 0.13, F-test), Fig. 2.20, shows potential to be an informative measurement, but there are no significant main effects for irrigation. There was a significant interaction between genotype and irrigation for the number of leaves on the lead stem at times AD1 p=<.001, F-test) and BD2 (p = 0.019, F-test). Differences between genotypes were significant (p < 0.001, F-test) for all whole plant and lead stem leaf counts.

Leaf measurements

There was a significant main effect of irrigation at the time point BD2 on the top leaf length measurement (p = 0.02, F-test), Fig. 2.22, and middle leaf length measurement (p = 0.031, F-test), Fig. 2.23. There were useful interactions between genotype and irrigation for the top leaf length and width p=<.001, F-test) at time point AD1, and at time point BD2 top leaf length (p = 0.004, F-test) and bottom leaf length (p = 0.023, F-test), Fig. 2.23, and width p=<.001, F-test). Differences between genotypes were significant (p < 0.001, F-test) for all leaf length and widths at all time points.

Leaf area calculations

There was no main effect of irrigation for leaf area calculations. Adjusting leaf area on the whole plant basis provided the most significant genotype and irrigation interaction (p = 0.003, F-test). Differences between genotypes were significant (p < 0.001, F-test) for all leaf area calculations.

Chlorophyll meter during first drought

There was no main effect of irrigation for SPAD meter readings during D1. Summing SPAD leaf readings for the lead stem provided genotype and irrigation

interaction ($p = 0.002$, F-test). Differences between genotypes were significant ($p < 0.001$, F-test) for all leaves and calculations apart from the middle leaf.

Sylleptic branch and leaf counts

There was no main effect of irrigation for sylleptic branch or leaf counts at any time point. There were also no interactions between genotype and irrigation. Differences between genotypes were significant ($p < 0.001$, F-test) at all time points for sylleptic branch and leaf counts.

Correlation matrix of traits measured and dry matter harvest yield

Apart from expected correlations between the same measurement at different timepoints, Fig. 2.25 shows whole plant leaf count as the most promising trait to predict yield and that this is more useful than lead stem leaf count only. However, correlations are weak due to associated problems with drought infliction methodology, so firm conclusions cannot be made.

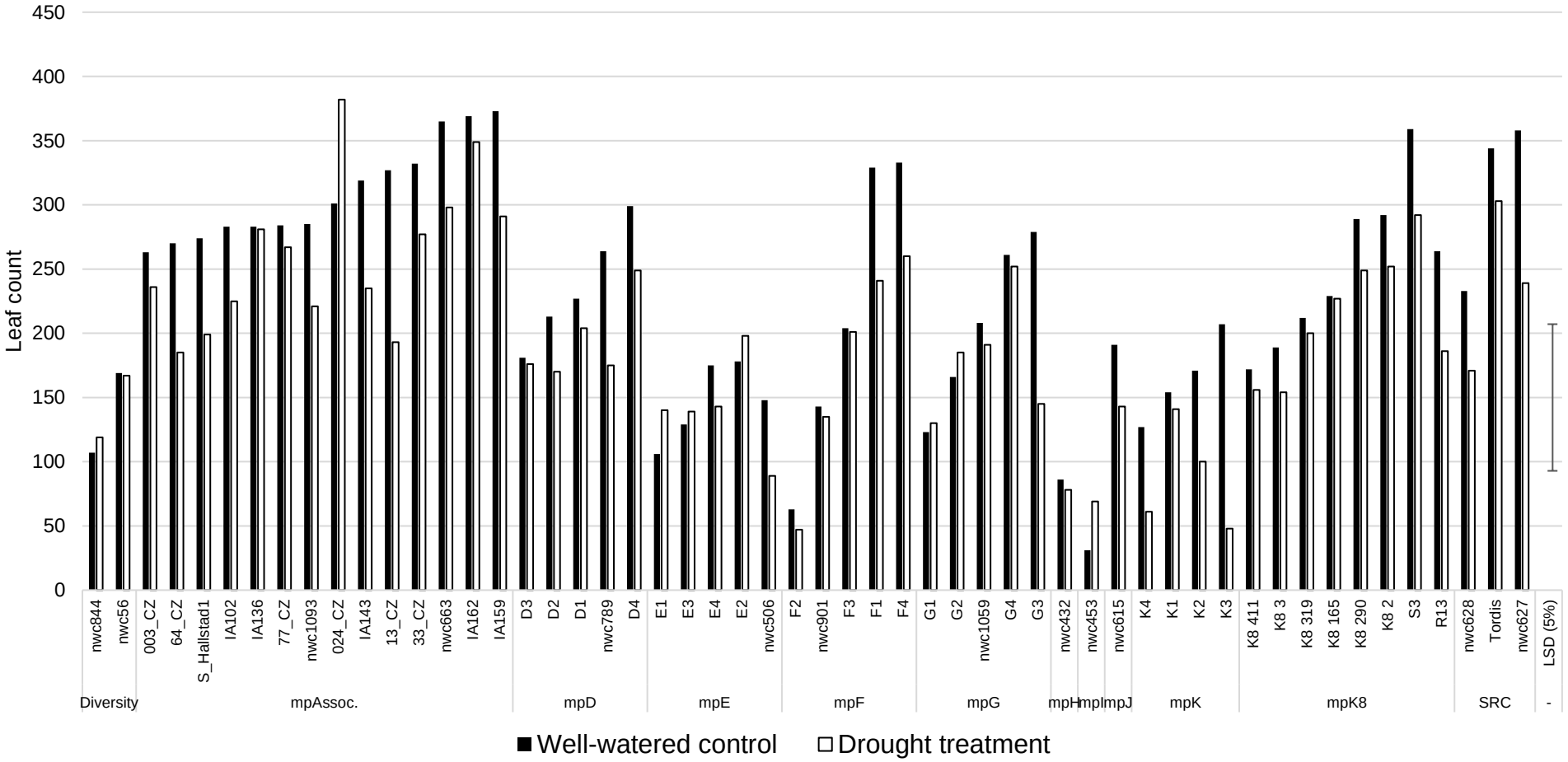

Figure 2.18 2014 After drought 1 (AD1) whole plant leaf count.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

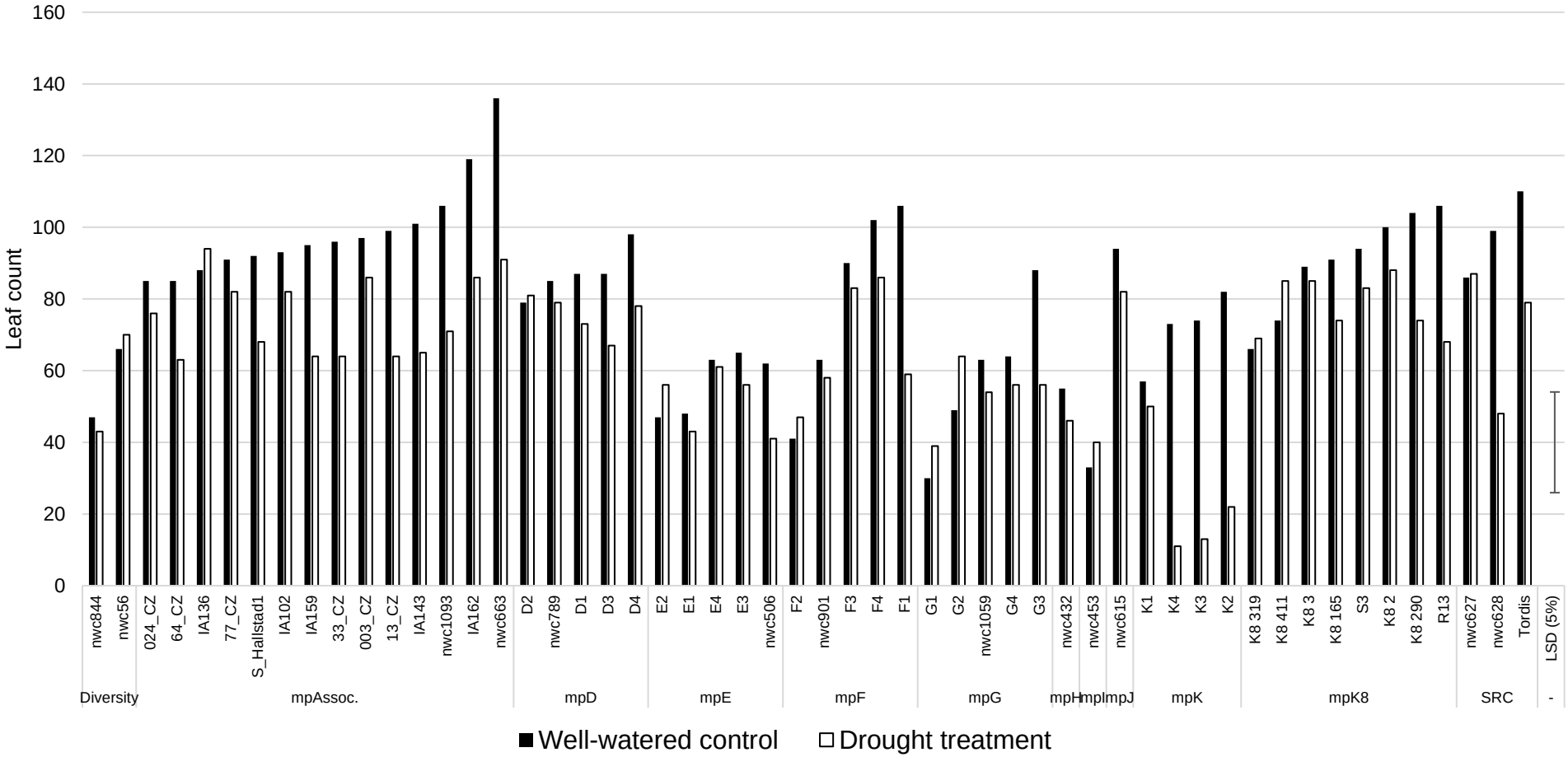

Figure 2.19 2014 After drought 1 (AD1) lead stem leaf count.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

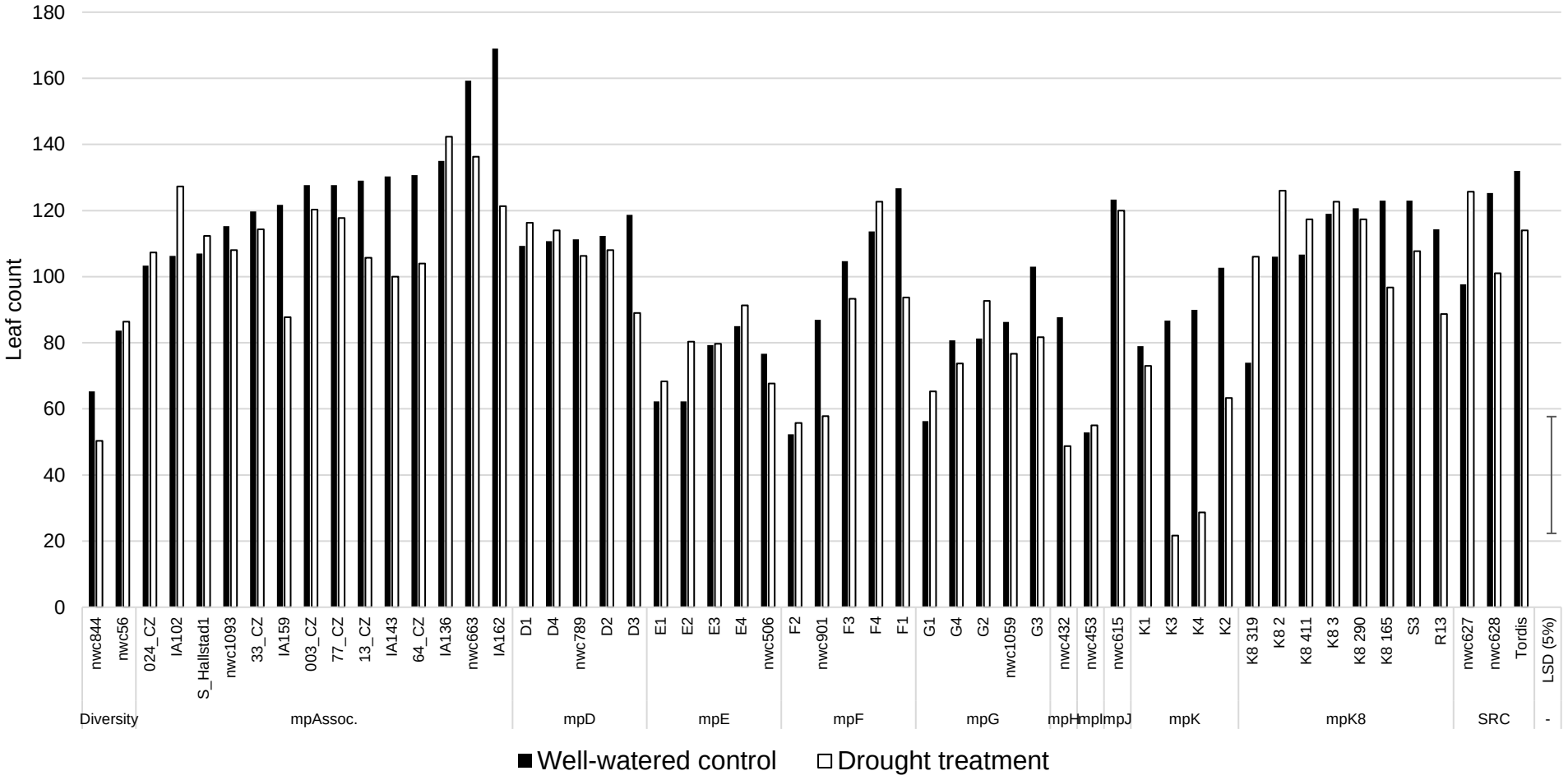

Figure 2.20 2014 Before drought 2 (BD2) lead stem leaf count.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

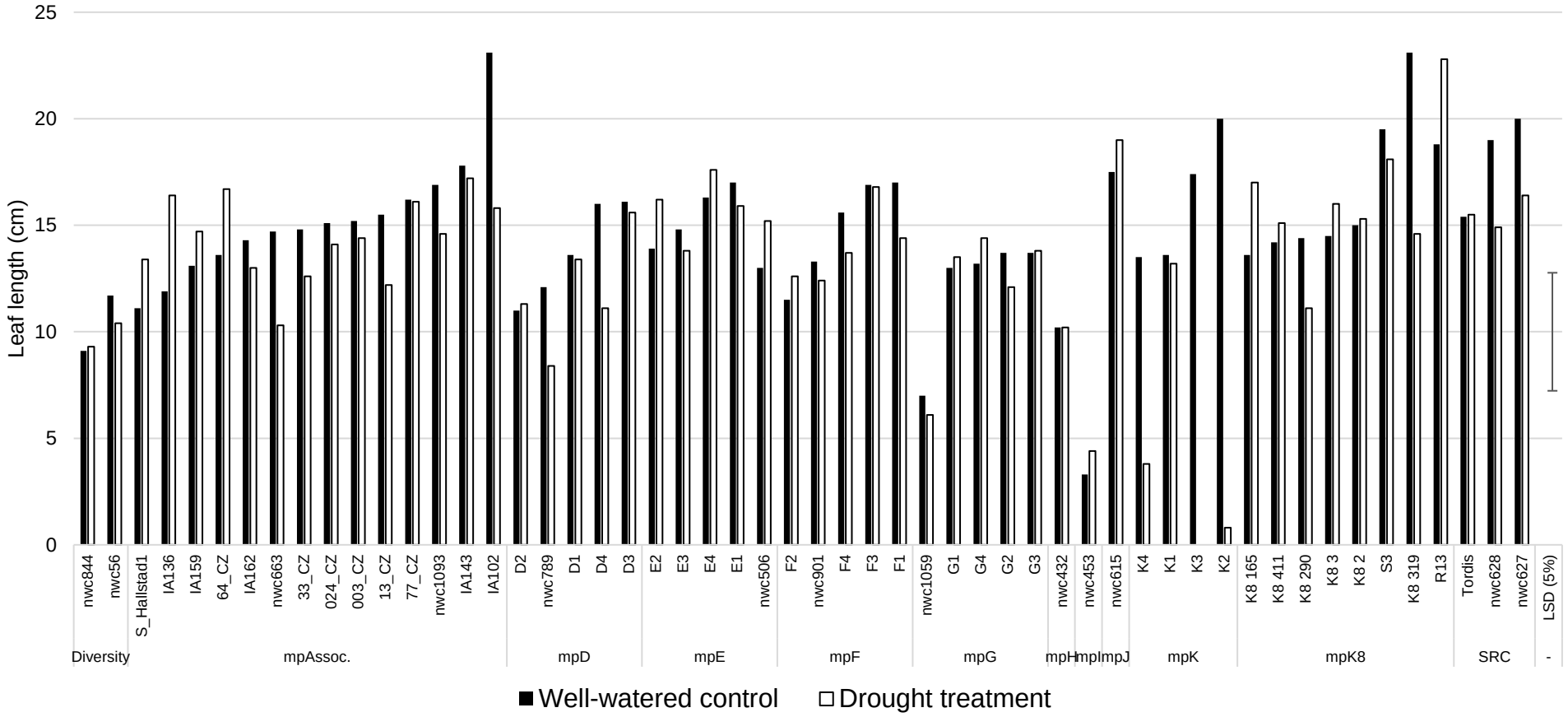

Figure 2.21 2014 after drought 1 (AD1) 1top leaf length.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

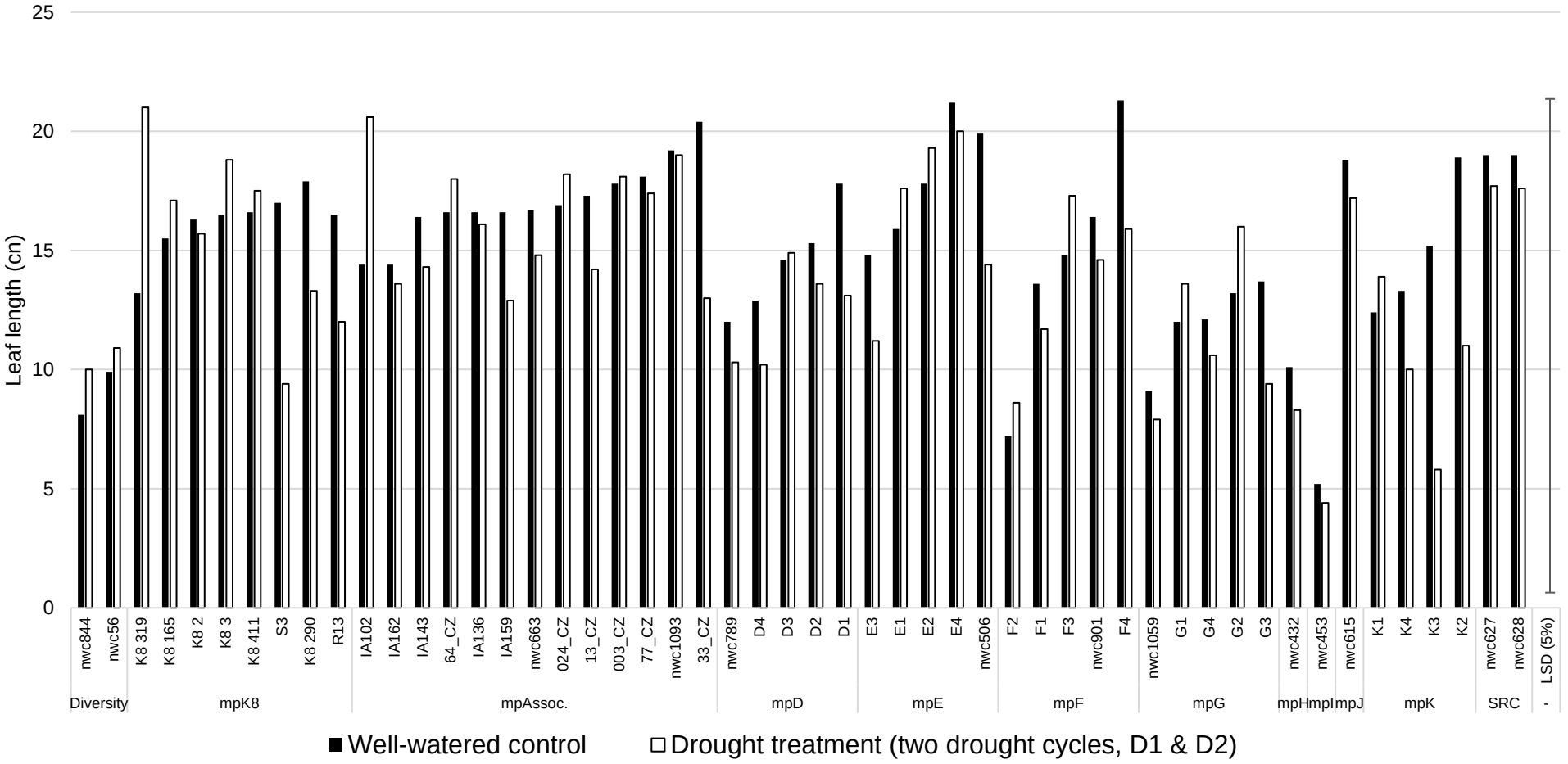

Figure 2.22 2014 Before drought 2 (BD2) top leaf length.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

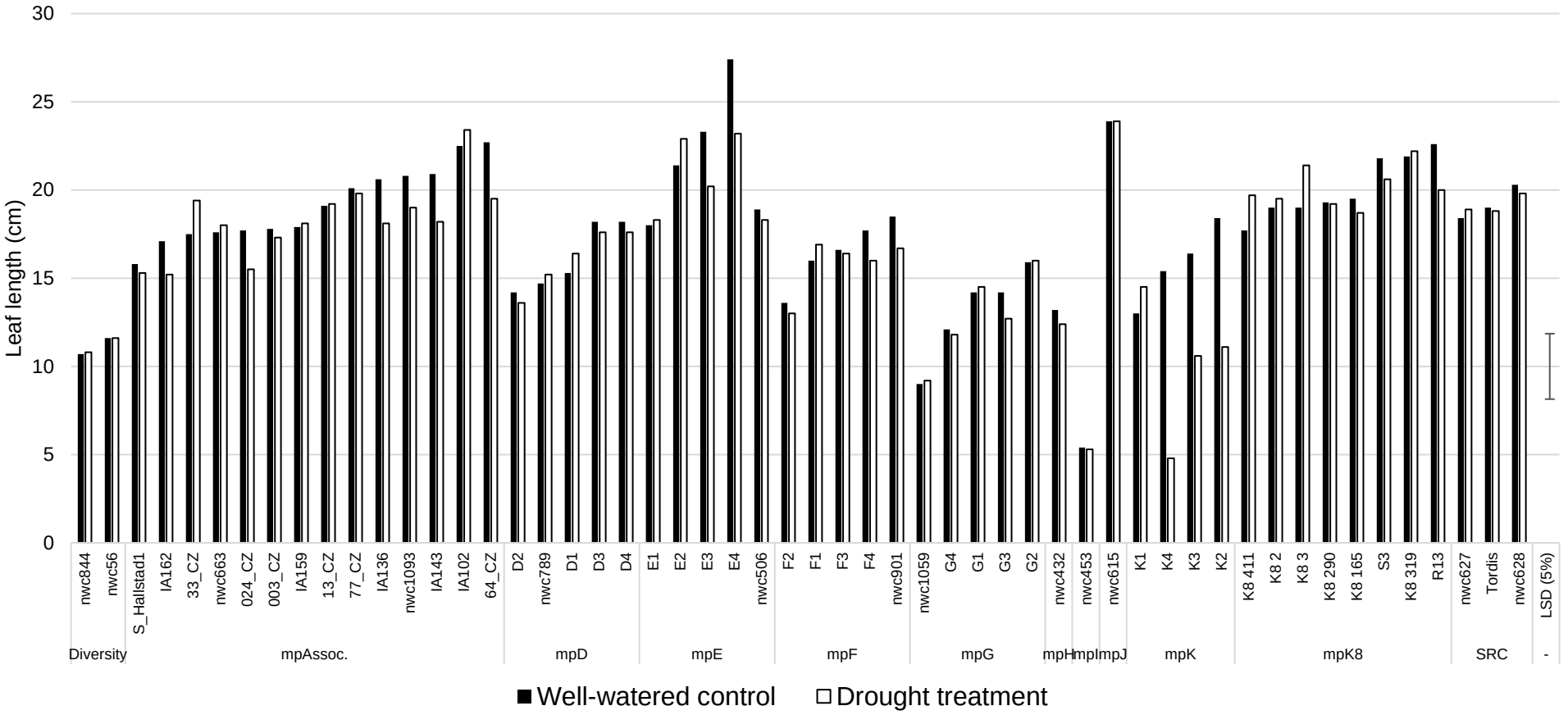

Figure 2.23 2014 Before drought 2 (BD2) middle leaf length.

Plotted maintaining family order on the *x*-axis from Fig. 2.11.
With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

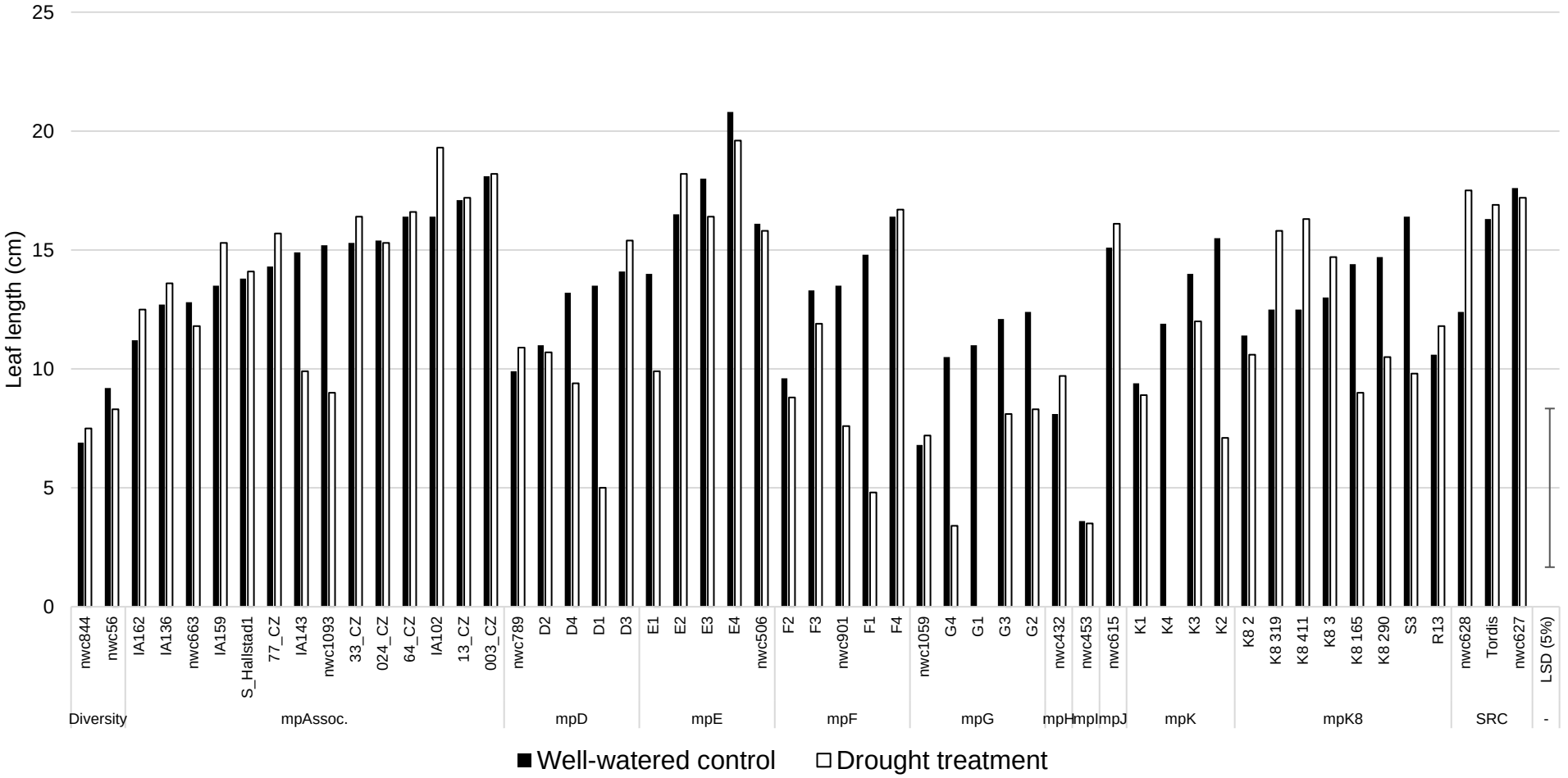

Figure 2.24 2014 Before drought 2 (BD2) bottom leaf length.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

		Yield DM (g)	Days to emerge	11 Jul Lead stem leaf count	11 Jul whole plant leaf count	30 Jul Lead stem leaf count	30 Jul whole plant leaf count	28 Aug Lead stem leaf count	28 Aug whole plant leaf count	31 Jul TL Length (cm)	31 Jul ML Length (cm)	BL Length (cm)	1 Sept TL Length (cm)	1 Sept ML Length (cm)	1 Sept BL Length (cm)	15 Jul ML Length (cm)	15 Jul BL Length (cm)
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Yield DM (g)	1	-															
Days to emerge	2	-0.38	-														
11 Jul Lead stem leaf count	3	0.61	-0.46	-													
11 Jul whole plant leaf count	4	0.65	-0.39	0.74	-												
30 Jul Lead stem leaf count	5	0.44	-0.29	0.63	0.47	-											
30 Jul whole plant leaf count	6	0.60	-0.31	0.61	**0.83**	0.69	-										
28 Aug Lead stem leaf count	7	0.48	-0.27	0.65	0.48	**0.85**	0.61	-									
28 Aug whole plant leaf count	8	0.65	-0.31	0.63	**0.86**	0.56	**0.88**	0.66	-								
31 Jul TL Length (cm)	9	0.32	-0.29	0.31	0.26	0.42	0.31	0.31	0.22	-							
31 Jul ML Length (cm)	10	0.42	-0.34	0.41	0.29	0.43	0.27	0.45	0.27	0.56	-						
31 Jul BL Length (cm)	11	0.43	-0.32	0.35	0.35	0.32	0.33	0.32	0.32	0.36	0.55	-					
1 Sept TL Length (cm)	12	0.35	-0.31	0.33	0.26	0.46	0.32	0.50	0.28	0.42	0.59	0.38	-				
1 Sept ML Length (cm)	13	0.42	-0.36	0.40	0.28	0.41	0.25	0.44	0.26	0.52	**0.84**	0.52	0.60				
1 Sept BL Length (cm)	14	0.39	-0.27	0.29	0.17	0.35	0.24	0.40	0.24	0.34	0.54	0.46	0.46	0.59	-		
15 Jul ML Length (cm)	15	0.56	-0.48	0.60	0.43	0.42	0.34	0.46	0.34	0.49	0.79	0.50	0.57	0.77	0.55	-	
15 Jul BL Length (cm)	16	0.46	-0.41	0.35	0.35	0.18	0.28	0.20	0.27	0.33	0.48	0.51	0.38	0.43	0.39	0.56	-

Figure 2.25 Correlation matrix of traits measured and dry matter harvest yield

2.4 Discussion

The drought periods were timed after the exponential growth phase of the logistic curve willow growth follows (Kopp et al., 2001a) in common with most plants. D1 was initiated on 21st July and ended 3 – 8 days later, D2 started on 4th September and ended 7 – 21 days later. Inducing drought stress during the exponential growth phase is likely to have a greater impact on yield than droughts occurring later in the season when the growth rates are lower. The timing of D1 and D2, later than the exponential growth phase, whilst legitimate timings for summer droughts in the UK, are likely to explain the limited main effect of the drought treatment.

In a pot experiment conducted by Doffo et al., (2016) in Argentina, responses of two willow genotypes, *S. matsudana* × *alba* and *S. alba,* dry matter yields were significantly reduced (Kruskal-Wallis test ($p \leq 0.05$). Both genotypes compared to controls to a cyclic drought consisting of; a two-week duration, followed by a two-week recovery at field capacity, followed by a final two-week drought. In this study pot water deficit was controlled by maintaining a 'fixed' level of drought stress by watering 4.5 l sized pots with 50 ml of water every second day. Despite this experiment having a similar time from planting to first drought, their D1 occurred in October and November, southern hemisphere, (equivalent of April and May) when days were rapidly lengthening, and plants were growing exponentially. However, limiting water deficits are rare in April and May in the UK in field conditions.

Leaf measurements

ANOVA for top leaf length revealed a significant interaction ($p = <.001$, F-test) between genotype and drought treatment after drought 1. The bottom leaf length shows a weak interaction ($p = 0.071$, F-test) immediately after drought 1, but this was stronger before drought 2 ($p = 0.023$, F-test) implying a less immediate response to the drought.

Savage and Cavender-Bares (2011), found the timing of drought-induced responses, such as drought-induced leaf senescence, varied significantly among the six diverse American native *Salix* species studied. The timing of measurements to capture the responses is therefore essential.

The significant genotype and drought treatment interaction ($p = <.001$, F-test) BD2 for bottom leaf width suggest drought-induced leaf senescence is an important

mechanism that helps the plant avoid losses through transpiration, so contributes to the maintenance of a favourable water balance. There was no genotype.irrigation interaction at AD1 timepoint, but the result suggest senescence has had time to be induced by BD2, one month after the end of D1. This concurs with findings that drought-induced leaf senescence occurs gradually and is characterised by specific macroscopic, cellular, biochemical and molecular changes (Munné-Bosch and Alegre, 2004). This study suggests that drought-induced leaf senescence contributes to nutrient emobilisation during stress. So, allows the rest of the plant (i.e. the youngest organs) to benefit from the nutrients accumulated during the life span of the leaf.

Calculating the leaf area adjusting top, middle and bottom leaf areas using: (Verwijst and Wen, 1996) then incorporating the whole plant leaf count revealed interaction ($p = 0.003$, F-test) between genotype and drought treatment after drought 1 shows an increased interaction from ($p = 0.006$, F-test) without using (Verwijst and Wen, 1996) adjustment, to ($p = 0.004$, F-test) with it for the top leaf only, increasing to ($p = 0.003$, F-test) at the whole plant level.

The predictive power of whole plant leaf chlorophyll mass from a *Salix* pot experiment on field biomass production has been highlighted (Weih and Nordh, 2005). It has also been shown as an important predictor of yield in a field experiment containing 12 diverse bioenergy hybrids and pure species (Bouman and Sylliboy, 2012). Our results concur with this. Leaf chlorophyll meter readings alone were not of use. However, when the SPAD readings were adjusted using the lead stem leaf count taken at the end of drought one (third of leaves x TL SPAD, one third of leaves x ML SPAD, third of leaves x BL SPAD), the ANOVA analysis revealed a significant interaction ($p=0.003$, F-test) between genotype and drought treatment. This metric needs further validation but has the potential to predict yield in pot and field conditions (Weih and Nordh, 2005).

Planting density

Although the pot experiment has a higher plant density than commercial SRC bioenergy plantations, the plant density used in the pot experiment is still viable. The twin row design, which facilitated access to the pot plants, is similar to the design of SRC plantations. However, the density of plants in the rain out shelter equates to 84,700 plants/ha^{-1}, a higher density than the 15,000 plants/ha^{-1}

recommended by SRC willow best practice guidelines (AFBI, 2015), and commonly adopted by the industry. The SRC planting density has been optimised for a three-year harvest cycle (Bullard et al., 2002). The experiment is not dissimilar to planting densities when willow plantations are grown for annual harvest. Whilst uneconomic, when grown for combustion markets at high densities, due to the prohibitive cutting costs, Bullard et al. (2002) found that *S. viminalis* could respond to higher densities of up to 111,000 plants/ha^{-1}. A study using *S. dasyclados* 'SV1' also found that a 0.3 m x 0.3 m planting density (111,000 plants/ha^{-1}) were productive producing 13 oven dried tonnes (Heckrodt) ha^{-1} year^{-1} (Kopp et al., 1997). Historically, annually harvested basket willow plantations containing *S. triandra* were planted at densities of 100,000 plants/ha^{-1} to 135,000 plants/ha^{-1} (Macalpine, 2018). The high planting density would likely cause competition issues if the experiment were to continue over subsequent years, but the planting density used is appropriate for a one-year pot experiment.

If adjusted to a 15,000 plants/ha^{-1} stocking density, yields from SRC varieties in the pot experiment are comparable to establishment year field yields, SRC family means equate to ~4 odt ha^{-1} for droughted plants and ~5 odt ha^{-1} for the well-watered control.

Yield penalty

Control plants yielded 10.50% higher than the drought treatment plants. If the genotypes that had a negative difference between control and drought treatment (Fig. 2.12) are excluded, this value increased to a yield reduction of 18.7%. This is still a relatively low drought response. Researchers observed the yield reductions over the controls in the following studies: 35 – 60% in a pot experiment (Wikberg and Ögren, 2007), 92.04% nwc627 Bjorn, 86.74% nwc628 Tora and 84.97% Tordis yield reductions in SRC varieties where pot plants received ~82% less water for the entire season in a fixed drought, control yields were similar between studies (Weih and Nordh, 2002). In a field experiment in Sweden where the water treatments of rain fed and reduced water recharge (plastic sheeting installed between rows of plants) were implemented from 5th July until the end of the growing season, yield reductions of 35.89% were observed for nwc628 Tora and average yield reductions of 42.84% for the five other SRC willow varieties included, none of these genotypes were in common with this experiment.

The effect of two drought periods were shown by Zhivotovsky and Kuzovkina, (2010) who conducted a glass house experiment using two genotypes, biomass variety *S. miyabeana* 'SX64' and *S. cinerea* '2007-10' collected from a native population. After three weeks of growth, three water treatments were applied: control, no drought, one six-day drought, two six-day droughts with four days recovery. Stem dry matter results revealed average yield reductions of 17.57% for *S. cinerea* and 32.03% for *S. miyabeana* over the control when exposed to one drought period, and a 36.49% yield reduction over the control for *S. cinerea and* 43.29% for the *S. miyabeana* over the control when two droughts were applied. The 11 – 19% yield penalty by adding the second drought is noted. Destructive yield assessments were not made in 2014, but stem height data would allow for analysis of the effects of repeated droughts on growth.

Negative difference between control and drought treatment

In a long-term pot experiment investigating flooding (Cerrillo et al., 2013), of four *Salix* genotypes; *S. nigra* 'AN4', *S. babylonica* × *alba* '131-27', *S. matsudana* × *alba* '13-44' and *S. babylonica* × *alba* '395', only one genotype, *S. matsudana* × *alba* '13-44' displayed a statistically significant reduction in growth when exposed to a three month summer flood (10 cm above soil level of pot). Fig. 2.16 shows mpE drought treatment out yielding the well-watered control. mpE could be a population sensitive to hypoxic or anoxic conditions associated with saturated soil. Although control plants were not submerged, when large 11 l pots contained small plants, there were occasions where plants could be considered over-watered. Although inundation is considered less stressful to willow plants than drought (Doffo et al., 2016) genotypes differ in their tolerance to inundation stress. mpE could be sensitive to over watering.

The large negative difference between control and drought treatment shown Fig. 2.12 for E2 can be explained by looking at the raw data.

Table 2.4 E2 raw yield data and lowest soil moisture from D1.

	+ DM yield (g)	- DM yield (g)	D1 vol. %
Block 1	172.75	197.50	6.50
Block 2	11.18	244.14	6.50
Block 3	178.24	340.41	8.05

The low block 2 + yield is compounded by the high block 3 - drought high yield. There is a case for excluding data from the low block 2 + yield. However, the drought treatment plants would still have produced a greater yield than the control plants. The block 3 – irrigation plant not receiving a severe drought (8.05 vol. %) further compounded the result regarding genotype E2.

Drought tolerance indices

Drought tolerance indices harmonic mean (HARM), Fig. 2.14 and drought tolerance efficiency (DTE), Fig. 2.13 are more powerful at dissecting potential drought tolerance than Fig. 2.12 or the Fig. 2.11 the yield results.

Tolerance Against Stress (TOL) and drought tolerance efficiency (DTE) indicators for screening drought tolerant genotypes. The following indices supported stable and high yield in both non-stress and stress treatments: mean productivity (MP), stress tolerance index (STI), geometric mean productivity (GMP), harmonic mean (HARM). For biomass plantation exposed to occasional drought stress, this latter group of indices form the more appropriate selection criteria as they will select for high yield in wetter and drier years.

Drought infliction methodology

The methodology of exposing plants within the drought whole plots to the same duration of water stress will not be repeated in future work. Whilst it is desirable to have the same drought duration, a more precise drought can be applied on a per plant basis. Fig. 2.26 shows the difficulties of attempting to induce drought on low yielding genotypes. The low yielding, small plants shown to the bottom right of Fig. 2.26 represent plants with a small leaf area which were not able to dry down. They also illustrate the challenge of attempting to provide homogeneous conditions for a broad range of genotypes with different yield potentials, from biomass varieties (Tordis) to a slow growing unimproved native (nwc453 *S. aurita*).

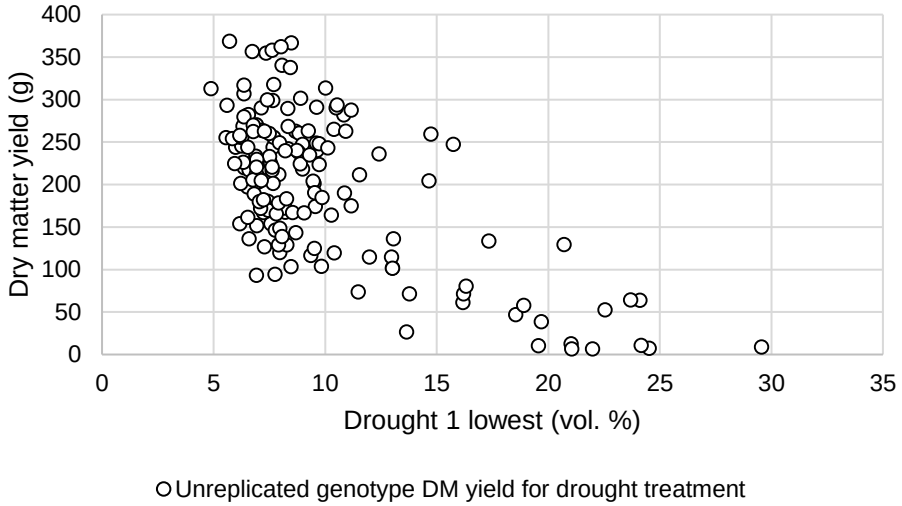

Figure 2.26 Final yield of droughted plants and lowest growth media moisture content during D1.

A more uniform soil moisture was obtained at D2, Fig. 2.27, as plants were larger and had a higher leaf count and therefore potentially higher transpiration rates (Fig.2.20). Despite this, some plants required 21 days to reach low soil moisture conditions, with replicates four genotypes' pots, nwc453, nwc844, nwc432 and F2, not receiving a drought. nwc453 *S. aurita*, nwc844 *S. purpurea*, nwc432 *S. daphnoides* are unimproved pure species with low yield potential compared with other test genotypes. Natural variation between test genotypes explain the performance of these small varieties.

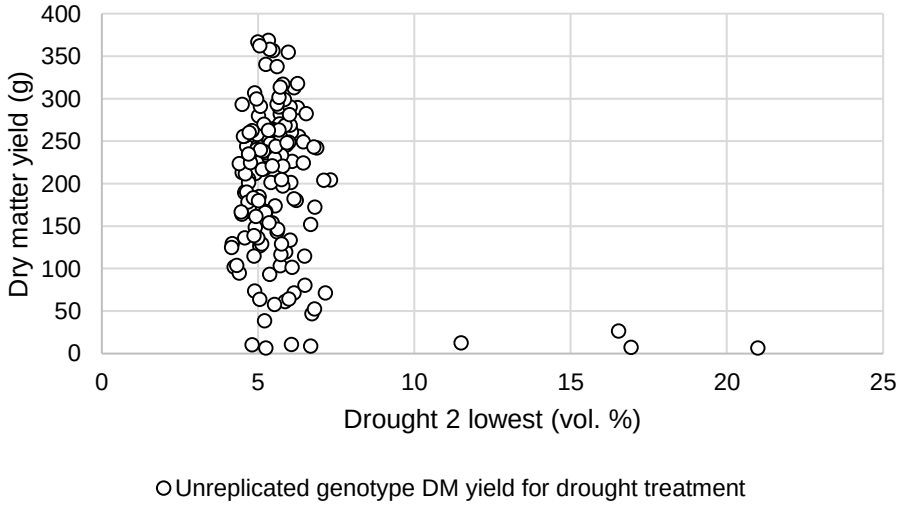

Figure 2.27 Final yield of droughted plants and lowest growth media moisture content during D2.

The genotype diversity included in the pot trial made optimising conditions, pot size, watering regime difficult. Figs. 2.28 – 2.30 show the duration of D1, D2 and D1 + D2 and the drought treatments whole plots dry matter yields. These plots show the low yielding plants take longer to reach a water deficit after irrigation is withheld.

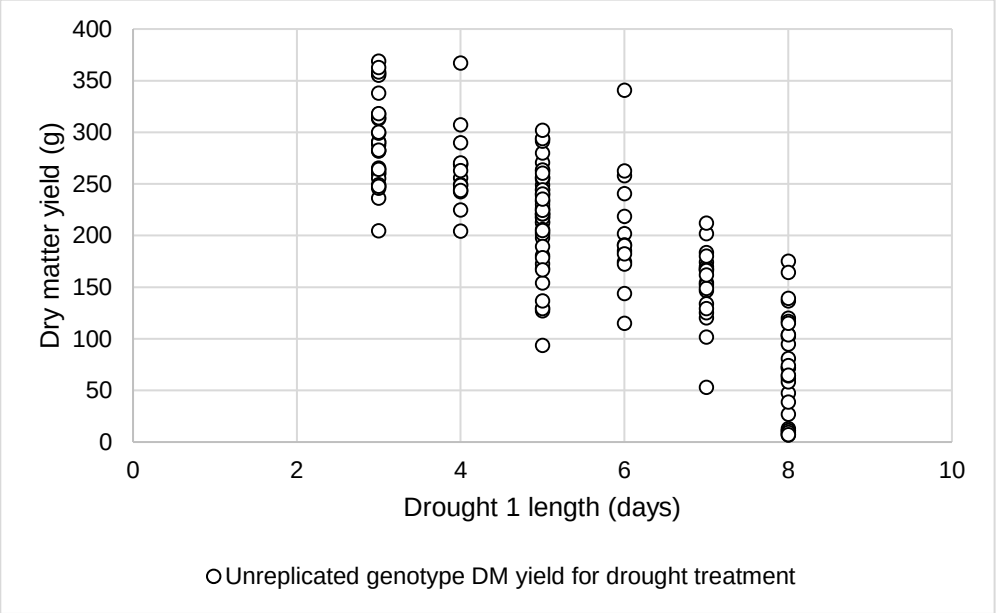

Figure 2.28 Final yield of droughted plants and the number of days drought days D1 lasted.

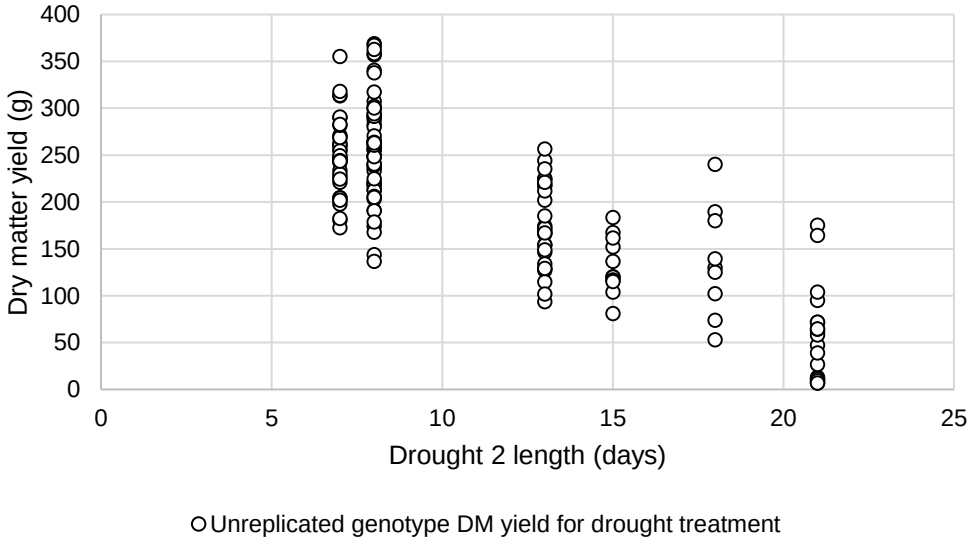

Figure 2.29 Final yield of droughted plants and the number of days D2 lasted.

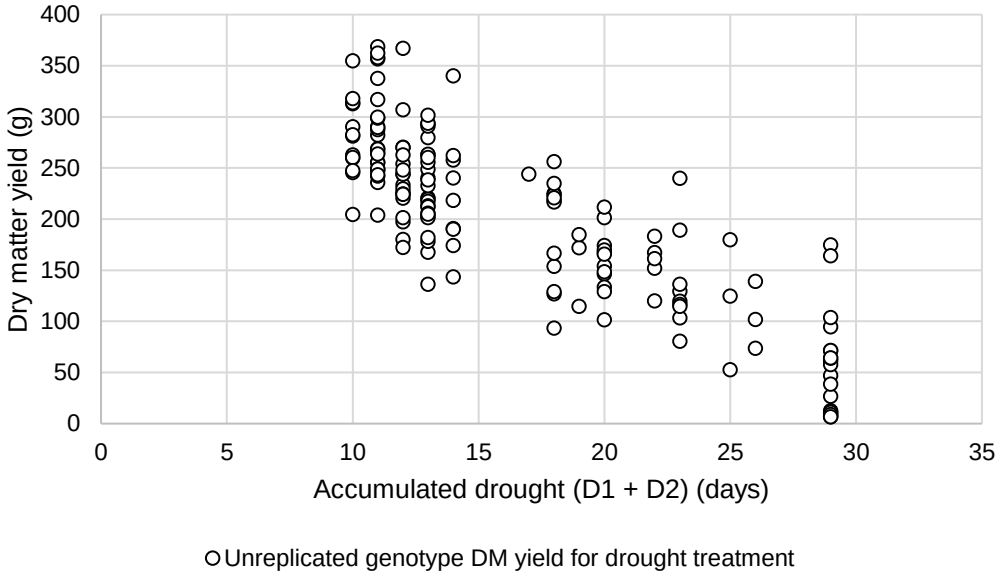

Figure 2.30 Final yield of droughted plants and accumulated drought days.

A water retention curve of the growth media should be conducted to demonstrate the relationship between the water content, θ, and the soil water potential, ψ. This soil moisture characteristic of the growth media used would be more informative than the Delta-T SM200 Soil Moisture data alone and would allow the wilting point (WP) and field capacity for the growth media to be determined.

Syllepsis

A sylleptic shoot is the newly developed lateral axis without the apical meristem passing through a dormant period (Remphrey and Pearn, 2006). The observation of wide spread syllepsis concurs with (Verwijst and Wen, 1996) who suggested that sylleptic shoots in *S. viminalis* mainly occur on establishment-year growth but are less prevalent on subsequent-years growth. This trait is therefore only likely to be relevant in field conditions in the establishment year. Syllepsis is a growth response that is part of a suite of responses which allows a rapid increase in carbon fixation capacity by increasing leaf area. Although no sylleptic leaf measurements were formally taken (only counts performed), sylleptic leaves were generally smaller and more ovate than proleptic shoot derived leaves. This also agrees with Verwijst and Wen's (1996) finding that sylleptic leaves (more ovate) differ from more proleptic shoot leaves in size, shape and specific leaf area. Our finding that there was no interaction with water treatment at all time points, including before a drought treatment was initiated (BD1, measurements taken 41 days after planting) could be explained by a finding in *Populus* where syllepsis was

linked to nitrogen availability. Pot experiments using *Populus balsamifera* ssp. *trichocarpa* × *deltoides* 'UCC-1' found syllepsis was induced by nitrogen and that the response could be seen as early as 14 days after N was applied (Cooke et al., 2005). Functions of sylleptic shoots can differ between the genera; with sylleptic shoots being larger and more permanent in *Populus*, and relatively small and prone to abscise at the end of the growing season in *Salix* (Ronnberg-Wastljung and Gullberg, 1999). Despite this potential difference in scale and longevity between the genera, (Ronnberg-Wastljung and Gullberg, 1999) found there was a genotype × nutrient interaction in *Salix viminalis* where more sylleptic shoots were produced in a higher nutrient environment. Wikberg and Ogren (2004) hypothesised that sylleptic shoots could be prone to abscission under drought stress due to a branch-sacrificing strategy. However, sylleptic branch abscission was not observed in the 2014 pot trial. As the experiment was harvested before autumn, no comments could be made to qualify the observation that leaf abscission from sylleptic shoots was more prevalent than on leaves from the main stem (Wikberg and Ogren, 2004).

The 2014 fertilization regime aimed to avoid nitrogen being growth limiting, so that the study could focus on drought effects. As nitrogen has been cited to affect syllepsis more than drought and in *Salix* it's likely to only be important in the establishment year, syllepsis will not be investigated in 2015. Counting 60,220 sylleptic leaves at BD1, 110,445 sylleptic leaves at AD1 and 182,261 sylleptic leaves at BD2 took a significant effort, which in future studies could be used elsewhere.

Lead stem leaf count

Lead stem leaf counts after AD1 are more informative than whole plant leaf counts. The main effect for irrigation on the lead stem AD1 was (p=0.13, F-test), compared to (p=0.13, F-test) for whole plant leaf counts. The AD1 lead stem leaf count also shows a significant interaction between genotype and irrigation (p=<0.001, F-test).

Stem numbers varied from 1 to 5 (mean 3.0) stems per plant in 2014. The variation in stem numbers is likely to mask the effect of drought on a whole stem basis. The lead stem leaf count is useful at capturing abscised leaves, but observations in the field suggest that leaf counts remain similar, but internode

spaces are reduced by drought, Fig. 2.31. Internode measurement or stem height maybe a more appropriate additional metric to capture this.

Figure 2.31 Internode space on winter dormant nwc99 one-year old stem in January 2019 after the drought of 2018.

Arrow to indicate short internode gaps caused by 2018 summer drought

Selection of material for further work

Previous studies in willow and poplar have found that productive genotypes displayed low levels of drought tolerance (reduction of biomass yield under drought stress), while the less productive genotypes had a greater potential to exhibit drought tolerance (Monclus et al., 2006; Weih, 2001). This study contradicts this previous work, finding that when exposed to two drought periods, productive willows are likely to perform best under drought and well-watered conditions. This should be tested under a more severe drought stress.

If the selection criteria were to select varieties for commercial production in drought prone areas, genotypes Tordis, IA162 and IA159 (Fig. 2.11) would be selected as they produce good DM yields under drought conditions, but also have the ability to produce higher yields if a drought does not occur. This is important as

willow plantations have a long lifetime of (over 20 years) and may not be exposed to drought annually. It should be noted that many of the genotypes included in the 2014 trial originate from mapping populations and do not have the yield potential of commercial varieties or the high productivity associated with *S. viminalis*, the species of members of the association mapping population. Moreover, this comparison should not be made as the aim of the 2014 pot trial was to select interesting families, populations and genotypes for further study. Although population numbers in this experiment are limited, the progeny of K8 and F produce segregating yield differences to the water treatments making them good candidates for further investigation. Populations K8 and F were therefore selected for further study in the 2015 pot trial.

2.5 Conclusions

This broad-range genotype pot experiment found that;

1. Potentially useful genetic variation exists for drought tolerance traits in the genus *Salix* and it is possible to identify it in a pot experiment.
2. Effective methodologies for screening drought tolerant willows have the potential to be developed.
3. Some measurements have the potential to identify and select for drought tolerant genotypes in SRC willow (with further development).
4. Segregating progeny from defined mapping populations can be identified and selected for further study.

Conducting the 2014 pot experiment highlighted methodology improvements that are needed to allow pot experiments to be performed more precisely in the future. The expected main effects of the irrigation treatment for fresh or dry weight above ground biomass yield were not widely observed. Changes in the methodology of inducing the drought will be implemented in future work to address this. Changes will be achieved by returning replicates of a genotype to watering at separate time points, based on water deficit conditions in individual pots. In 2014 taking a mean soil moisture reading meant that some replicates were not exposed to a water deficit. The timing of the water deficits will also be changed so drought periods will coincide with the plants exponential growth phase. These two methodologies will be changed in future work.

Final harvest dry matter % gave the greatest main effect of irrigation ($p = 0.002$, F-test). The top leaf length measurement could be a useful relatively high throughput measurement for breeders assessing the impact of drought on germplasm. The timing of the measurement to capture the physiological changes in the leaf due to reduced cell division and cell expansion caused by a drought needs to be refined.

The significant genotype and drought treatment interaction ($p = <.001$, F-test) BD2 for bottom leaf width suggest drought-induced leaf senescence is an important mechanism. Assessing drought-induced leaf senescence also has the potential to be a relatively high throughput measurement for breeders assessing the impact of drought on germplasm.

The choice of drought tolerance indices is important when considering them as selection criteria. Geometric Mean Productivity (GMP), Mean Productivity (MP) and Harmonic Mean (HARM) support the selection of stable and high yield in both non-stress and stress treatments. These indices are appropriate for selecting material for biomass plantations exposed to occasional drought stress. These should be used if selecting for performance under both non-stress and stress conditions.

Drought tolerance indices against stress (TOL) and drought tolerance efficiency (DTE) are useful tools for screening drought tolerant genotypes. All drought tolerance indices tested are useful for helping select segregating mapping populations for further study. mpF and mpK8 have been selected for further study based on output showing segregation from DTE and HARM indices.

Chapter 3. mpF and mpK8 pot experiment

3.1 Introduction

This second pot experiment aimed to screen greater numbers of progeny from mpK8 and mpF to further evaluate their potential to segregate for drought tolerance traits.

The central hypothesis of this MPhil is: ‘It is possible to identify and select drought tolerant genotypes in short rotation coppice (SRC) willow’

Hypotheses to be investigated in this chapter are;

1. Useful genetic variation exists for drought tolerance traits in the mpF and mpK8.
2. Effective methodologies for screening drought tolerant willows can be developed.
3. It is possible to identify and select for drought tolerant genotypes in the mpF and mpK8.
4. The inclusion of progeny from mapping populations will allow segregating mapping populations to be identified for further study at the field scale.
5. Changes to the pot trial methodology will lead to a greater main effect of irrigation.

3.2 Materials and Methods

The methodology used in the 2015 pot experiment was broadly similar to that used in 2014 and detailed in the 2.2 materials and methods section. Differences to the 2014 experiment are described below.

3.2.1 Planting and growth conditions

Pots and growth media were prepared using the same protocol as in 2014. In 2015 additional pots were prepared so a ‘guard’ could be added, Fig. 3.2. Guard plants received the same water treatment as adjacent plants. Guard plants on the north and south edge could potentially receive water from precipitation as they were within 110 cm of the open edge of the rain out shelter, this gap had previously (2014) been left unoccupied to prevent rain blowing onto test pots.

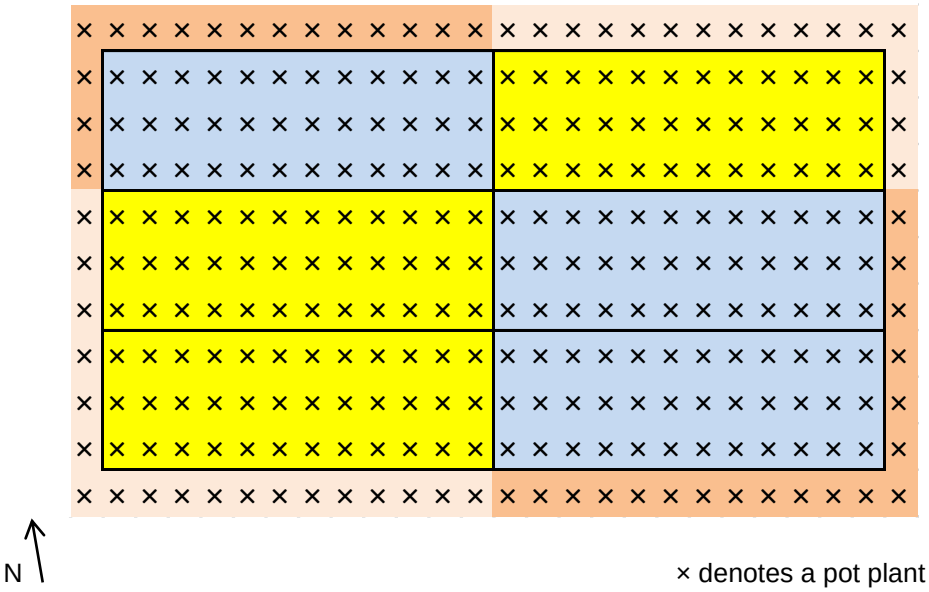

Figure 3.1 *S. viminalis* 'Bowles Hybrid' guard rows surrounding the experiment.

The same rain out shelter, GH44, and irrigation equipment and timers were used to conduct the experiment as in 2014. Pots were placed in GH44 using a twin row design, with a spacing of 26 cm between pots between twin rows and 65 cm between pairs of rows. Pots were spaced at 26 cm within each row. Once arranged, pots were watered by filling to the brim individually with a hosepipe and lance fitted with a rose nozzle prior to planting.

The pot experiment was planted on 1st May 2015. Cuttings were inserted into the centre of each pot. *Circa* 1 cm of the cutting was left above the growth medium surface. Pots were watered to field capacity after planting.

Pots were weeded as necessary. Insecticide Hallmark (100 g/l lambda-cyhalothrin) was applied on 17th June 2015 at the recommended rate to control Terminalis midge (*Dasineura* spp.), aphids, willow beetles (C*hrysomelidae)* and sawfly larvae. Pots were each fertilised after the first drought stress period with 5g of Osmocote Exact Standard 3-4M per m^3 (Scotts UK Professional, Ipswich, Suffolk) on 22nd July 2015 to insure plants had sufficient nutrients.

Slug damage was observed in 2015 and Slugoides containing 3% w/w metaldehyde (Doff, Hucknall, UK) were applied on 28 May 2015 at the recommended rate.

3.2.2 Irrigation regime

Two periods of water stress were applied to the drought whole plots by stopping the irrigation. Drought periods occurred on; 19th June and 10th August in 2015. During the drought periods, pot moisture was monitored daily using a Delta-T SM200 Soil Moisture Sensor and HH2 logger (Delta-T Devices Ltd, Cambridge, UK). The sensor was calibrated as per the manufacturer's instructions. Three measurements were taken per pot and the mean of these measurements was used. In 2015 pots were returned to watering when individual pots soil water content of 7 vol. % was reached.

All pots were watered by hand with a hosepipe through a rose nozzle until the 5th June when a drip irrigation system was installed. Galcon 9001 irrigation controllers (Kfar Blum, Israel) were used to schedule watering. Water was delivered to each pot using a single Octa-Mitter adjustable stake dripper (Access Irrigation, Northampton, UK). From 5th June pots were watered for three minutes per watering when needed. Watering occurred daily from 23rd July and twice a day from 1st August. The drippers delivered approximately 0.4 l of water per minute to each pot. Irrigation was scheduled when the saucers were dry in an attempt to keep pots at pot capacity and avoid waterlogging.

Two periods of water stress were applied to the drought whole plots and adjacent guard row plants by stopping the irrigation. The first drought period (D1) began on 19th June 2015 and lasted 14-31 days for individual genotypes, depending on their soil moisture level. The second drought period began on 10th August 2015 and lasted for 10-28 days, again with the length dependent on soil moisture level.

During the drought periods, pot soil moisture was monitored daily using a Delta-T SM200 Soil Moisture Sensor and HH2 logger (Delta-T Devices Ltd, Cambridge, UK). The sensor was calibrated as per the manufactures instructions. Three measurements were per pot and the mean of these measurements was used. Soil moisture measurement were taken daily taken daily on drought whole plots thought drought periods. In 2015 pots were returned to watering when individual pots soil water content of 7 vol. % was reached, unlike in 2014 when each replicate of each genotype was exposed to the same duration of water stress.

3.2.3 Plant material

The 2015 pot experiment contained 36 willow genotypes from two different diploid families, 15 from mpF and 21 from mpK8. K8 290, from mpK8 is the male parent of mpF. Pedigrees of the 2015 study genotypes can be seen in Table 3.1 and in the pedigree plot, chapter 2 Fig. 2.1.

Table 3.1 *Salix* germplasm 2015 pot experiment

ID	Pedigree	Comment
nwc901 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42'	F female parent
K8 290 *	S3 × R13	F & I male parent
F1 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F2 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F3 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F4 *	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F6	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F7	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F8	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F9	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F10	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F11	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F12	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F13	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F14	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
F15	901 *S. x alberti* (*S. integra* × *suchownesis*) 'Pan 42' × K8 290	
S3 *	Astrid × SW930984	K8 female parent
R13 *	Astrid × SW930984	K8 male parent
K8 011	S3 × R13	
K8 013	S3 × R13	
K8 014	S3 × R13	
K8 018	S3 × R13	
K8 023	S3 × R13	
K8 040	S3 × R13	
K8 043	S3 × R13	
K8 044	S3 × R13	
K8 050	S3 × R13	
K8 057	S3 × R13	
K8 059	S3 × R13	
K8 060	S3 × R13	
K8 300	S3 × R13	
K8 307	S3 × R13	
K8 337	S3 × R13	
K8 341	S3 × R13	
K8 350	S3 × R13	
K8 353	S3 × R13	

* Genotype also present in 2014 pot experiment

In addition to the test genotypes cuttings of nwc672 *S. viminalis* ‘Bowles Hybrid’ were collected to act as a guard to prevent any edge effect.

Dormant woody cuttings were collected from field trials in January, cut to 15 cm lengths, labelled and wrapped in plastic and stored in a -4°C freezer until removal in late April.

mpF material was collected from CS/690 and Bowles Hybrid guards were collected from CS/564 Rothamsted Research (RRes), Harpenden, UK (51°48′30″N, 0°21′22″W) and mpK8 material was collected from CS/697 at Woburn Experimental Farm, Husborne Crawley, UK (52°51.0"N, 0°35'33"W)

3.2.4 Experiment design

A split plot design with three blocks of two plots was used to conduct an experiment to assess the differences between the performance of 36 genotypes in 2015 under well-watered and droughted conditions. The water treatment was applied on whole plots and genotype was applied on sub-plots. The ‘Design’ function in GenStat for Windows, 16th edition was used to plan the experiment. Appendix 3 details the experiment layout.

Figure 3.2 2015 pot experiment on 22nd June, 52 days after planting.

3.2.5 Experiment monitoring

LogTag (Dorset, DT11 9EX, UK) temperature and humidity loggers were place in each of the 6 whole plots (See Appendix 4 for LogTag locations). The sensors were suspended at 1 m above the ground from beams in GH44. The sensors were shielded from direct solar radiation by an 18 cm wide 8 cm deep cone covered in aluminium foil (See Fig 2.5). Sensors logged hourly temperature and humidity values from 18th May 2015. LogTags were changed on 15th July for a model that recorded temperature only and 27th July for a model that recorded temperature and humidity. These changes were made because of concerns about battery life and because of procurement issues. Meteorological data was provided by the Environmental Change Network (ECN) automatic weather station at Rothamsted Research. This is located 400 m south of GH44 and data was available for the duration of the experiment.

3.2.6 Phenotypic measurements

Leaf emergence scores

Pots were assessed at three-day intervals post planting and scored using the 1-7 key presented in Fig. 2.6 until score 7 was reached (plants fully emerged, and stem extension begun).

Time points

The experiment was planted on 1st May 2015. Measurements were taken, before drought 1 (BD1), during drought 1 (D1), starting 19th June and lasting 14-31 days, before drought 2 (BD2), during drought 2 (D2), starting 10th August 2015, lasting 10-28 days and after drought 2 (AD2).

Leaf counts

Leaves derived from the main proleptic stems were counted and recorded at two time points, BD1, 15th June and BD2, 6th August. The protocol described in chapter 2 was used.

Leaf measurements

Leaf length and width measurements were taken on leaves on the dominant stem at two time points, BD1, 17th June and BD2, 6th August. The leaf selection and measurement protocol described in chapter 2 were used.

Leaf area calculations

The leaf area calculations described in chapter 2 based on Verwijst and Wen (1996) non-linear regression model.

3.2.7 Yield measurements

After D2, droughted plants were returned to watering. The experiment was harvested a block at a time with block 1 being harvested on 14th September, block 2 on 15th September and block 3 on 17th September. Stems were cut at the soil surface using secateurs. Leaves (both proleptic and sylleptic) were separated from the stems and fresh and dry weight analysis was performed on these two components; stem and leaf. Dry weights were taken after the stems were cut into *circa* 2 cm sections and the biomass was dried in aluminium trays at 80°C for 48 hours.

3.2.8 Statistical analyses

The following split-plot analysis of variance (ANOVA) was used:

$$y \sim (Family/Genotype)*Irri + Block/MainPlot/SplitPlot$$

where *y* represents any particular response, *Family* is the fixed model term denoting the family, *Genotype* is the fixed model term denoting the genotype, *Irri* is the fixed model term denoting the treatment effect (-Irrigation or +Irrigation), *Block* is the random model term denoting the *Block* The slash (/) indicates the nesting of model terms, in this case of *Genotype* in *Family* and the star (*) indicates that main effects and interactions should be fitted. The statistical significance of fixed effects was tested using F-tests.

Following the modelling, plots of the residuals for the best model were made. The predicted means for the relevant statistically significant model terms were output with standard error of the difference (SED), degrees of freedom and least significant difference (LSD) values at the 5% (p = 0.05) level of significance for their comparison.

The Genstat (2015, 18th edition, © VSN International Ltd, Hemel Hempstead, UK) statistics package was used for all analyses.

3.2.9 Drought tolerance indices

The drought tolerance indices defined in Table 2.2 will be calculated from the dry matter yield results. Ys are stress (drought treatment) and Yp the optimal (potential

or well-watered) yield of any given genotype. $\bar{Y}s$ and $\bar{Y}p$ are average yields of all genotypes under stress and optimal conditions.

Table 3.2 Drought tolerance indices

Index Name	Equation
Drought tolerance efficiency (DTE)	$DTE = \left(\frac{Ys}{Yp}\right)$
Yield Index (YI)	$YI = \frac{Ys}{\bar{Y}p}$
Mean Productivity (MP)	$MP = \frac{Ys + Yp}{2}$
Stress Tolerance Index (STI)	$STI = \frac{(Ys)(Yp)}{(\bar{Y}p)^2}$
Tolerance Against Stress (TOL)	$TOL = (Ypi - Ysi)$
Geometric Mean Productivity (GMP)	$GMP = \sqrt{(Ys)(Yp)}$
Harmonic Mean (HARM)	$HARM = \frac{2(Yp \times Ys)}{Yp + Ys}$

3.3 Results

3.3.1 Meteorological data

Fig. 3.3 shows the maximum and minimum daily temperatures inside the rainout shelter and at the Rothamsted Meteorological site during the experiment period in 2015.

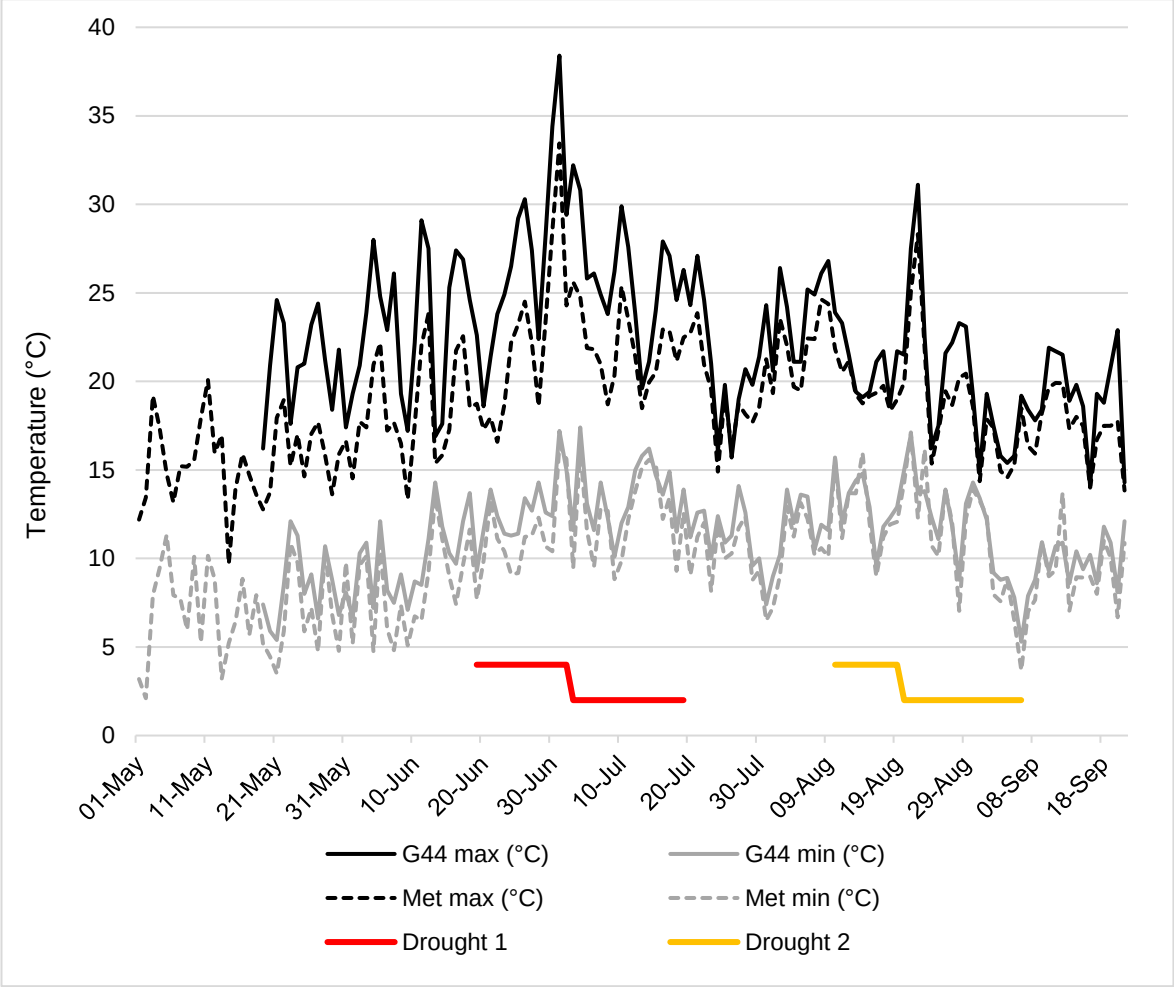

Figure 3.3 2015 maximum and minimum daily temperatures from Rothamsted. Meteorological site and LogTag sensors in GH44.

Drought periods marked in a stepped line to represent drought time range, D1, 14 – 31 days, D2, 10 – 28 days.

Whilst minimum temperatures were broadly similar to readings from the meteorological site (+1.10°C on average), maximum daily temperatures were elevated in the rain out shelter (+3.13°C on average). Compared to the 2014 experiment in the same location, the difference in minimum temperatures was elevated by 0.52°C whereas the difference between maximum daily temperatures were broadly similar. A maximum temperature in the rain out shelter of 38.4°C (33.4°C at the Met site) was recorded on 1st July, 12 days into D1. Days recorded with a maximum temperature exceeding 30°C were 4 and 32 days exceeded 25°C. The temperatures >30°C that occurred during drought 1 mean that heat stress could have been a factor during this period in addition to drought stress.

3.3.2 Results for key varieties in 2015 pot experiment

Summary statistics

The effect of the induced droughts on dry matter yield was greater in 2015 than in 2014 (Table 3.3). Control plants yielded 48.58% higher than the drought treatment in 2015. Missing values were higher in 2015. This was due to slug damage during the establishment period in and resulted in the higher numbers of missing values. Members of mpF were more vulnerable to slug damage than mpK8, with 27 and 7 missing values respectively. Whist slug damage is an occasional issue during the establishment phase of field plantings, damage had not been observed before in pot experiments. Slugs caused damage to emerging shoots below the growth media surface and was not observed until too late (27 days after planting). This application was timed too late to protect test material.

Table 3.3 2014 and 2015 summary statistics for above ground biomass dry matter yield (g).

	2014 +	**2014 -**	**2015 +**	**2015 -**
Number of observations	160	160	90	91
Number of missing values	2	2	18	17
Mean	223.8	200.3	204.5	103.1
Median	228.2	217.3	205.3	98.4
Minimum	3.776	6.78	17.5	20.6
Maximum	572.6	368.6	363.5	220.3
Lower quartile	158.6	145.1	150.9	60.88
Upper quartile	283.5	260	258.9	144.2

The square root of the residual was similar for experiments; 46.16 in 2014 and, 43.71 in 2015. This allows for comparison to be made between the two experiments.

Table 3.4 presents ANOVA results for varieties from three time points during the 2015 pot experiment. The residual plots indicated a random scatter with broadly homogeneous variability across the genotype by treatment combinations, so there was no need to transform the data.

Table 3.4 ANOVA results for key varieties in 2015 pot experiment.

Timing	Variate	*Irri*	*Family*	*Family. Geno*	*Family. Irri*	*Family. Geno. Irri*
		F pr.	F pr.	F pr.	F pr.	F pr.
Leaf counts (leaf number)						
BD1	15/6/15 Lead stem	0.806	<.001	<.001	0.665	0.255
BD1	15/6/15 Whole plant	0.482	<.001	<.001	0.746	0.938
BD2	06/8/15 Lead stem	0.126	<.001	<.001	0.359	0.46
BD2	06/8/15 Whole plant	0.082	<.001	<.001	0.666	0.939
Leaf measurements (cm)						
BD1	17/6/15 Top leaf L	0.11	<.001	<.001	0.219	0.668
BD1	17/6/15 Top leaf W	0.166	<.001	<.001	0.328	0.609
BD1	17/6/15 Middle leaf L	0.038	<.001	<.001	0.781	0.458
BD1	17/6/15 Middle leaf W	0.174	<.001	<.001	0.011	0.562
BD1	17/6/15 Bottom leaf L	0.363	<.001	<.001	0.171	0.284
BD1	17/6/15 Bottom leaf W	0.486	<.001	<.001	0.866	0.651
BD2	06/8/15 Top leaf L	0.019	0.004	<.001	0.018	0.468
BD2	06/8/15 Top leaf W	0.153	0.078	<.001	0.827	0.405
BD2	06/8/15 Middle leaf L	0.003	0.497	<.001	0.002	0.048
BD2	06/8/15 Middle leaf W	0.007	0.005	<.001	0.042	0.448
BD2	06/8/15 Bottom leaf L	0.026	<.001	<.001	0.657	0.613
BD2	06/8/15 Bottom leaf W	0.168	0.007	<.001	0.7	0.504
Leaf area calculations						
BD2	6/8/15 Top leaf LxW	0.068	0.004	<.001	0.451	0.606
BD2	6/8/15 Top leaf LxW adj[a]	0.067	0.005	<.001	0.445	0.602
BD2	6/8/15 Middle leaf LxW	0.002	0.026	<.001	0.002	0.212
BD2	6/8/15 Middle leaf LxW adj[a]	0.002	0.026	<.001	0.002	0.224
BD2	6/8/15 Whole plant adj[b]leaf area	0.043	<.001	<.001	0.129	0.742
Final harvest yield responses (g)						
AD2	Above ground biomass DW yield	<.001	0.018	<.001	0.359	0.029
AD2	Above ground biomass FW yield	<.001	<.001	<.001	0.119	0.681
AD2	Stem DW yield	0.001	<.001	<.001	0.053	0.55
AD2	Stem FW yield	0.001	<.001	<.001	0.215	0.662
AD2	Leaf DW yield	<.001	<.001	<.001	0.155	0.515
AD2	Leaf FW yield	<.001	<.001	<.001	0.036	0.666

[a] Calculated using a non-linear regression (Verwijst and Wen, 1996)
[b] Adjusted leaf area (Verwijst and Wen, 1996) to whole plant level

For all varieties investigated there was no interaction between genotype and drought treatment at the BD1 timepoint apart from middle leaf width. This middle leaf width result was unexpected as the drought regime had not been initiated at this time point.

Final harvest yield responses

There was a main effect of irrigation for all final harvest yield responses. ANOVA revealed an interaction ($p = 0.029$, F-test) between family, genotype and irrigation for above ground biomass dry matter yield. There was no *Family.Irrigation* interaction, but there was a significant ($p < 0.001$, F-test) *Family.Genotype* integration and there were differences between the mpF and mpK8 families ($p < 0.018$, F-test).

Fig. 3.4 – 3.6, 3.9 and 3.10 show final harvest yield responses sorted by family and performance for the well-watered control yield. Fig. 3.7 and 3.8 and Table 3.4 present drought indices results.

mpK8 and mpF have similar average above ground dry matter yield losses due to drought, 49.33% and 48.60% respectively, but Fig 3.5 shows the variation in response to the drought treatment. Mean mpK8 above ground dry yield are ~10% above mpF for both water treatments.

Similar to 2014, mpF and mpK8 show useful segregation for yield under drought conditions. nwc901 and K8 290 (male parent K8 290, grouped with other mpK8 family members) segregate at opposing ends of the yield range for mpF. Genotypes of mpK8 segregate for yield; however their parents (S3 and R13) rank is similar. This may be expected for mpK8 as S3 and R13 are full siblings.

mpF and mpK8 drought responses vary from 19% – 95% and 30% – 71% respectively, Fig. 3.5 is useful to illustrate this variation. Fig. 3.4 shows the potential of F10, F11 and K8 043 have the desirable smallest decrease in yield under drought conditions, relative to the yield under well-watered conditions. F11, K8 043 and F10 have a yield loss of 19%, 40 % and 42 % respectively.

For remaining final harvest yield responses; above ground biomass fresh weight yield, and fresh and dry weight stem and leaf components of yield, ANOVA revealed significant differences between the mpF and mpK8 families ($p < 0.001$, F-

test) and a *Family.Genotype* ($p < 0.001$, F-test) interaction. There was a *Family.Irrigation* interaction for leaf fresh weight yield only.

Drought tolerance indices

Table 3.4 shows that drought tolerance indices Geometric Mean Productivity (GMP) and Harmonic Mean (HARM) produce the same results, similar to in 2014. These GMP and HARM indices highly correlate with Mean Productivity (MP) ($r = 0.99$), Yield Index (YI) ($r = 0.99$) and the Stress Tolerance Index (STI) ($r = 0.97$). The Drought tolerance efficiency (DTE) and Tolerance Against Stress (TOL) have an inverse relationship, but these indices are not as closely associated as 2014. Figs 3.7 (DTE) and 3.8 (HARM) show results from these two broad classes of drought tolerance indices that can be used for selecting genotypes response to drought stress.

Leaf counts

Similar to 2014, lead stem leaf counts showed potential to be an informative measurement, although there was no significant main effect of irrigation. However, whole plant leaf counts, (p 0.082, F-test), had the potential to be more informative than lead stem leaf counts (p 0.0126, F-test), (Fig. 3.13) at BD2.The lead stem was more informative in 2014.

There were significant differences between the mpF and mpK8 families ($p < 0.001$, F-test) and a significant (*Family.Genotype*: $p < 0.001$, F-test) interaction for leaf counts performed on the lead stem and whole plant basis BD1 and BD2. There were no interactions at the *Family.Irrigation* or *Family.Genotype.Irrigation* levels.

Performing leaf counts on a whole plant basis will be slower through-put. Maximum mpK8 family whole plant leaf counts at BD2 were 396 leaves (S3), 207 leaves/plant average and maximum leaves/plant for mpF were 367 for F14, with 174 leaves/plant average. Lead stem leaf counts offer higher throughput with a maximum leaf count/stem of 124 for K8 059, average 88.2 leaves/stem and a maximum leaf count/stem of 121 for F7, 79.3 leaves/stem average.

Stem numbers varied from 1 to 6 (mean 2.7) stems per plant in 2015 and this variation has not impacted on the whole plant leaf counts unlike in 2014.

Leaf measurements

There was no interaction between genotype and drought treatment at the BD1 timepoint apart from Middle leaf width. This result was unexpected as the water treatment had not been initiated. Significant differences between the mpF and mpK8 families ($p < 0.001$, F-test) and a significant (*Family.Genotype*: $p < 0.001$, F-test) interaction was observed BD1. The mean middle leaf length was 15.8cm for droughted plants in mpK8 which was less than the control 16.84%, in mpF middle leaves were 8.89% shorter than the control at 16.4cm.

BD2 there was a significant main effect of irrigation for top leaf length, (p 0.019, F-test), Fig. 3.12, middle leaf length, (p 0.003, F-test) Fig. 3.13, middle leaf length, (p 0.007, F-test) and bottom leaf length, (p 0.026, F-test).

There were significant differences between the mpF and mpK8 families for top leaf length, (p 0.004, F-test), middle leaf width, (p 0.005, F-test), bottom leaf length, ($p <0.001$, F-test) and bottom leaf length, (p 0.007, F-test). There was a significant *Family.Genotype* interaction ($p < 0.001$, F-test) for all leaf length and width measurements at BD1. A significant *Family.Irrigation* interaction is seen for top leaf length (p 0.018, F-test), the strongest interactions are for the middle leaf at BD2. The middle leaf length has the only *Family.Genotype.Irrigation* interaction (p 0.048 F-test).

Leaf area calculations

Leaf area calculations are more significant, main effect of irrigation (p 0.002 F-test), but adjustments to leaf area using Verwijst and Wen's (1996), non-linear regression model did not improve results apart from the significance of the between family difference. The calculated whole plant leaf area is reduced by 30.71% over the control in mpK8 and 25.59% in mpF when means of the families are compared.

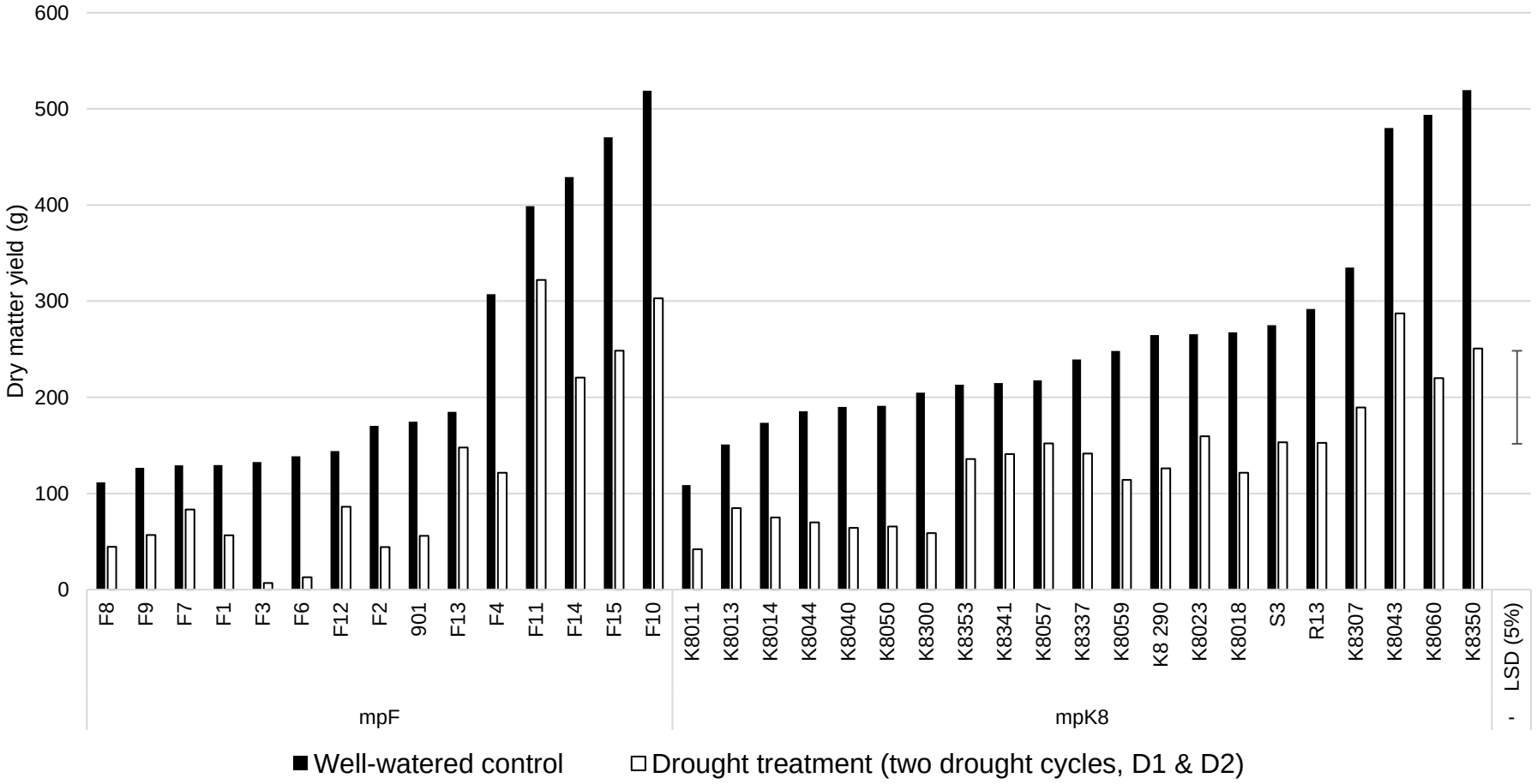

Figure 3.4 2015 Final harvest above ground biomass dry matter yield.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

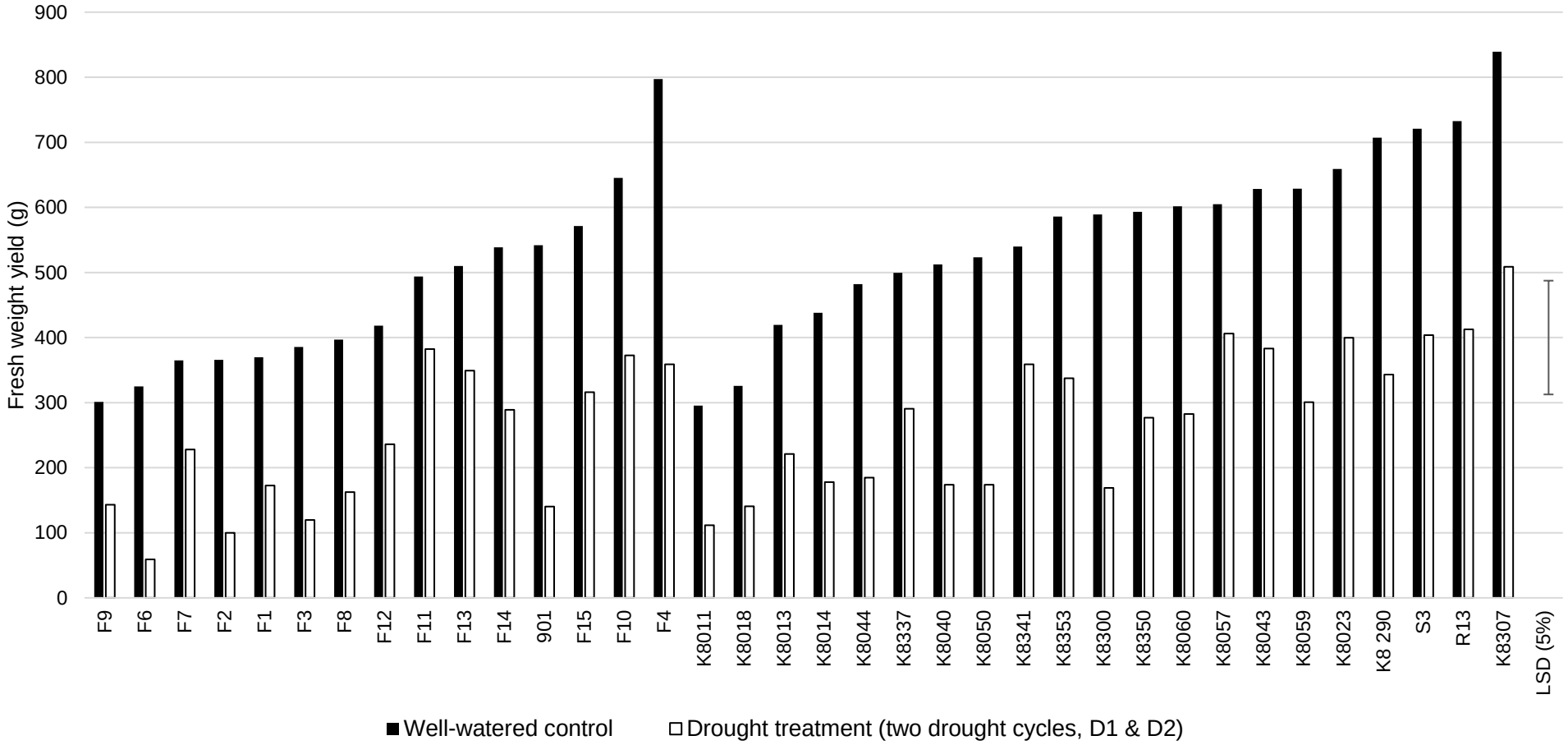

Figure 3.5 2015 Final harvest above stem biomass fresh weight yield (stem and leaf).

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

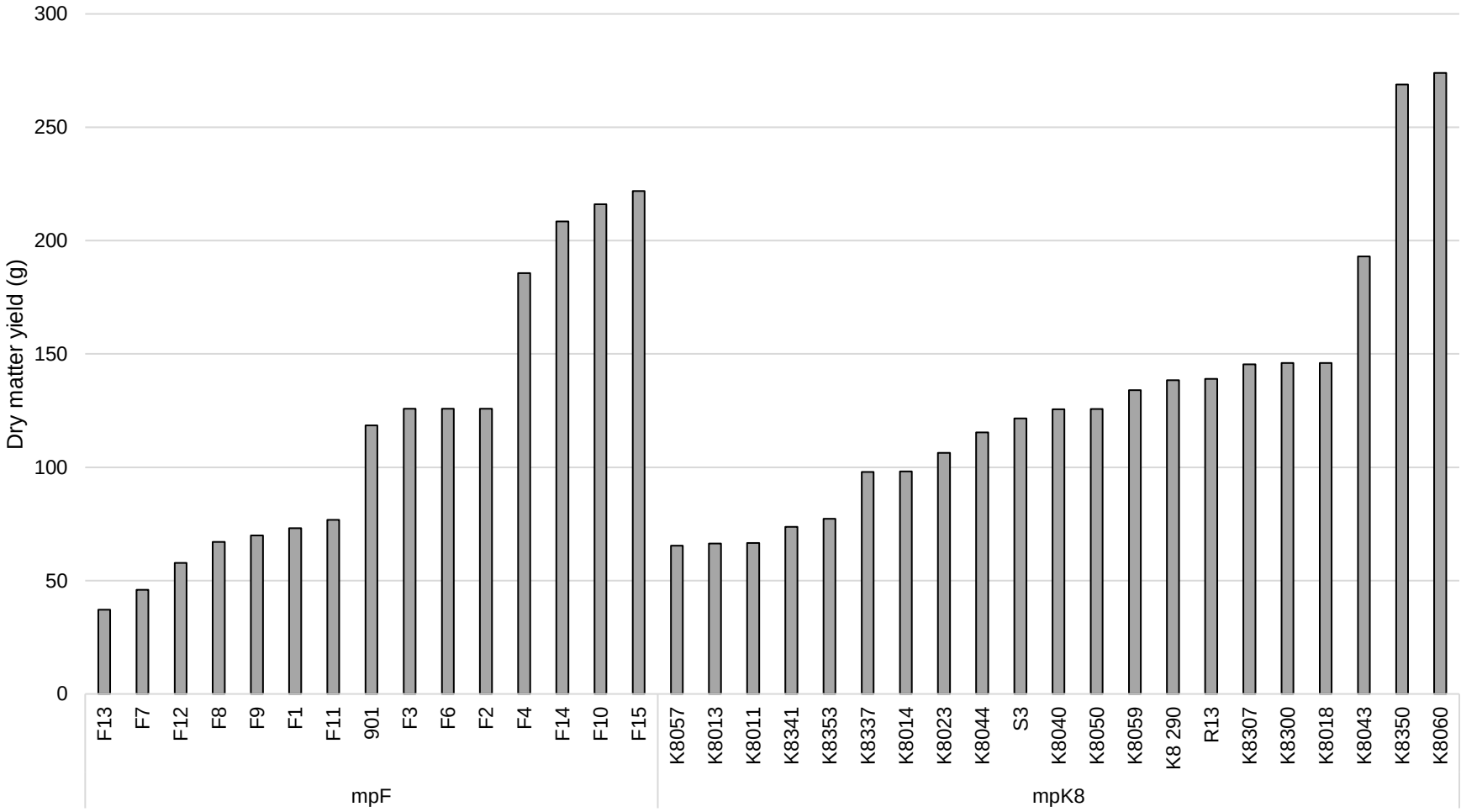

Figure 3.6 2015 Difference between (control – drought) in final harvest above ground biomass dry matter yield.

Table 3.5 Correlation coefficients between drought tolerance indices and 2015 final harvest dry matter yield

	DTE	YI	MP	STI	TOL	GMP	HARM	Well-watered control DM Yield (Yp)	Drought treatment DM Yield (Ys)
DTE	-								
YI	0.64	-							
MP	0.46	0.97	-						
STI	0.42	0.96	0.98	-					
TOL	-0.22	0.53	0.72	0.69	-				
GMP	0.53	0.98	1.00	0.98	0.67	-			
HARM	0.58	0.99	0.99	0.97	0.62	1.00	-		
Well-watered Control DM yield (Yp)	0.32	0.91	0.99	0.96	0.83	0.97	0.95	-	
Drought treatment DM yield (Ys)	0.64	1.00	0.97	0.96	0.53	0.98	0.99	0.91	-

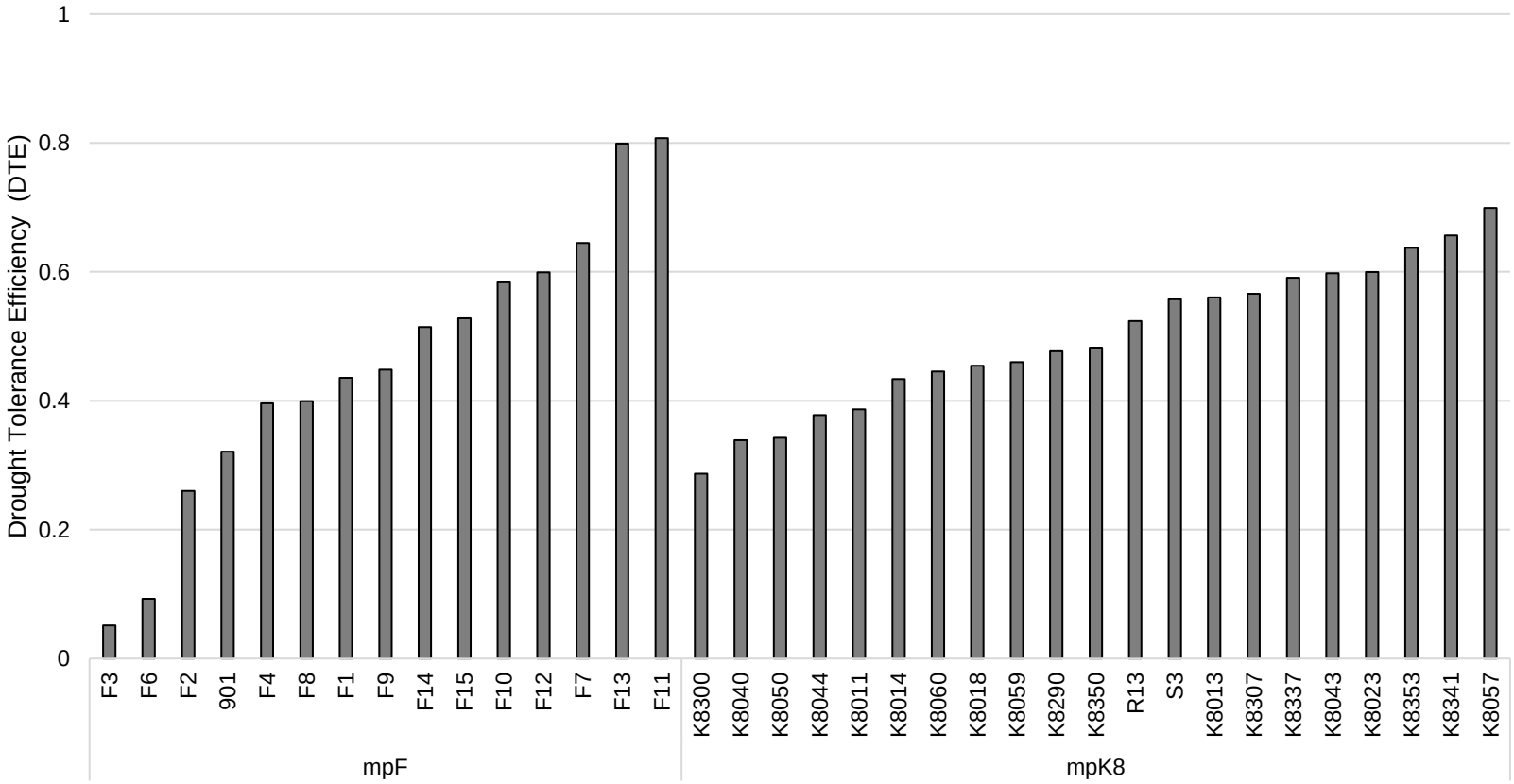

Figure 3.7 2015 Drought tolerance efficiency (DTE) index for final harvest above ground biomass dry matter yield

Drought tolerance efficiency (DTE) formula $DTE = \left(\frac{Ys}{Yp}\right)$

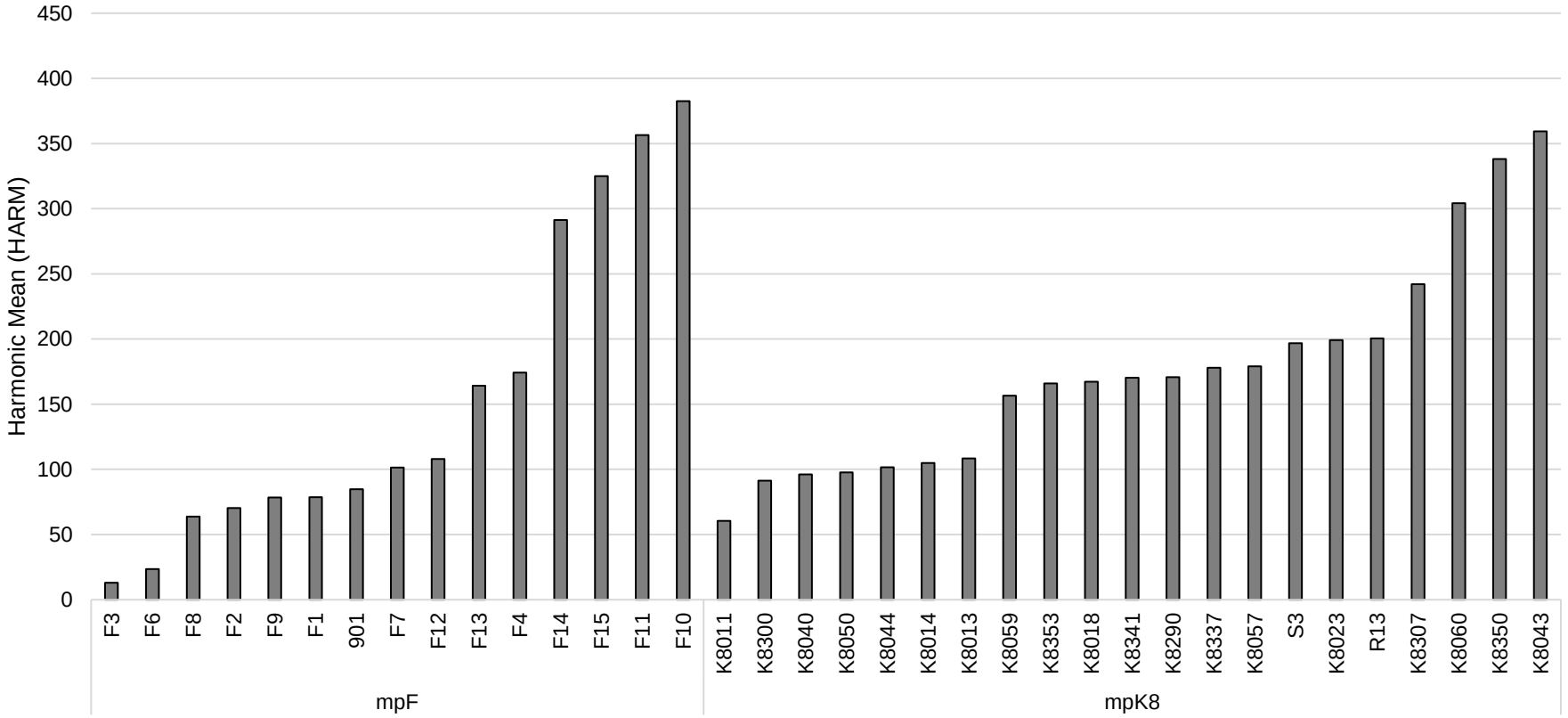

Figure 3.8 2015 Harmonic Mean (HARM) index for final harvest above ground biomass dry matter yield

Harmonic Mean (HARM) formula $HARM = \frac{2(Yp \times Ys)}{Yp+Ys}$

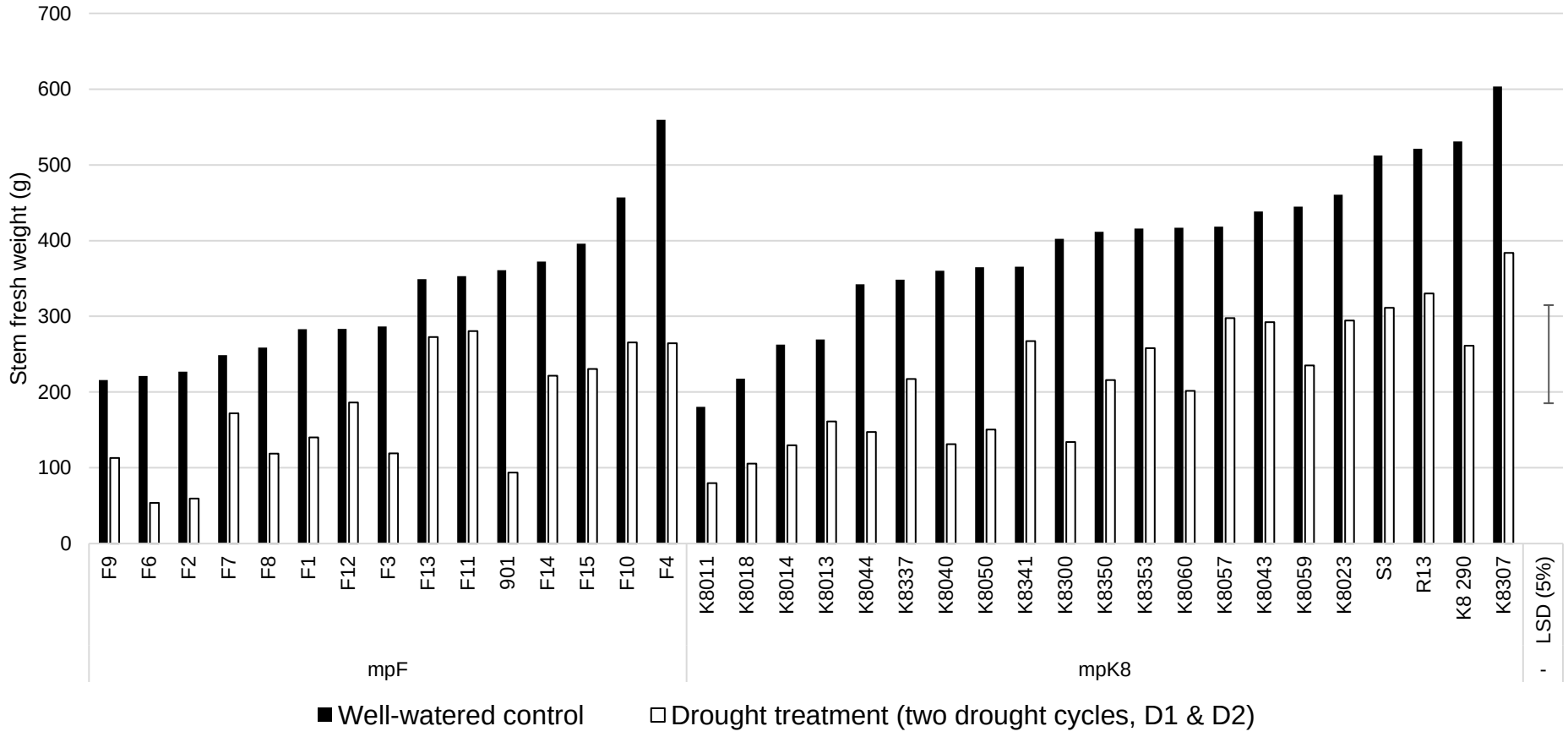

Figure 3.9 2015 Final harvest above stem biomass fresh weight yield.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

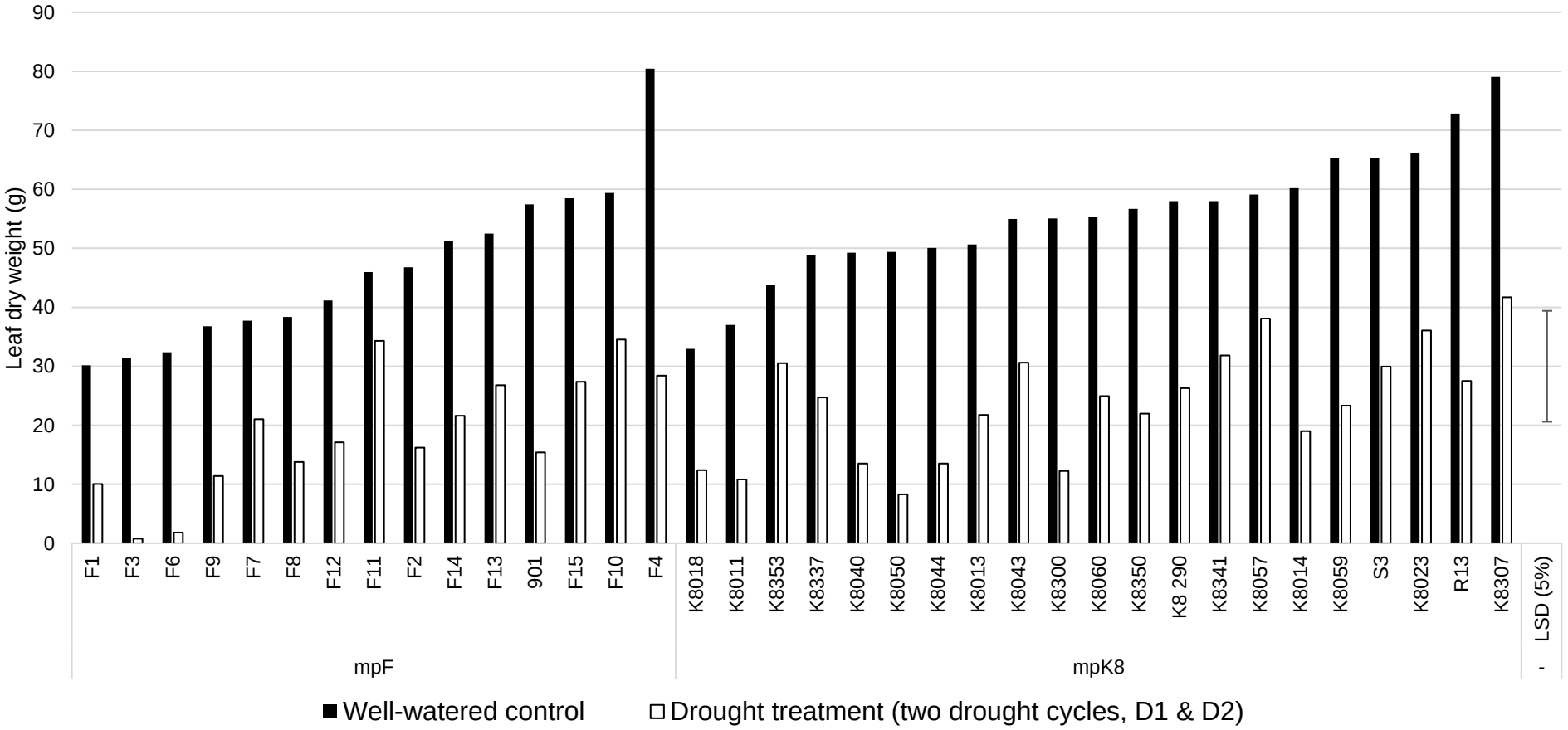

Figure 3.10 2015 Final harvest leaf dry matter yield.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

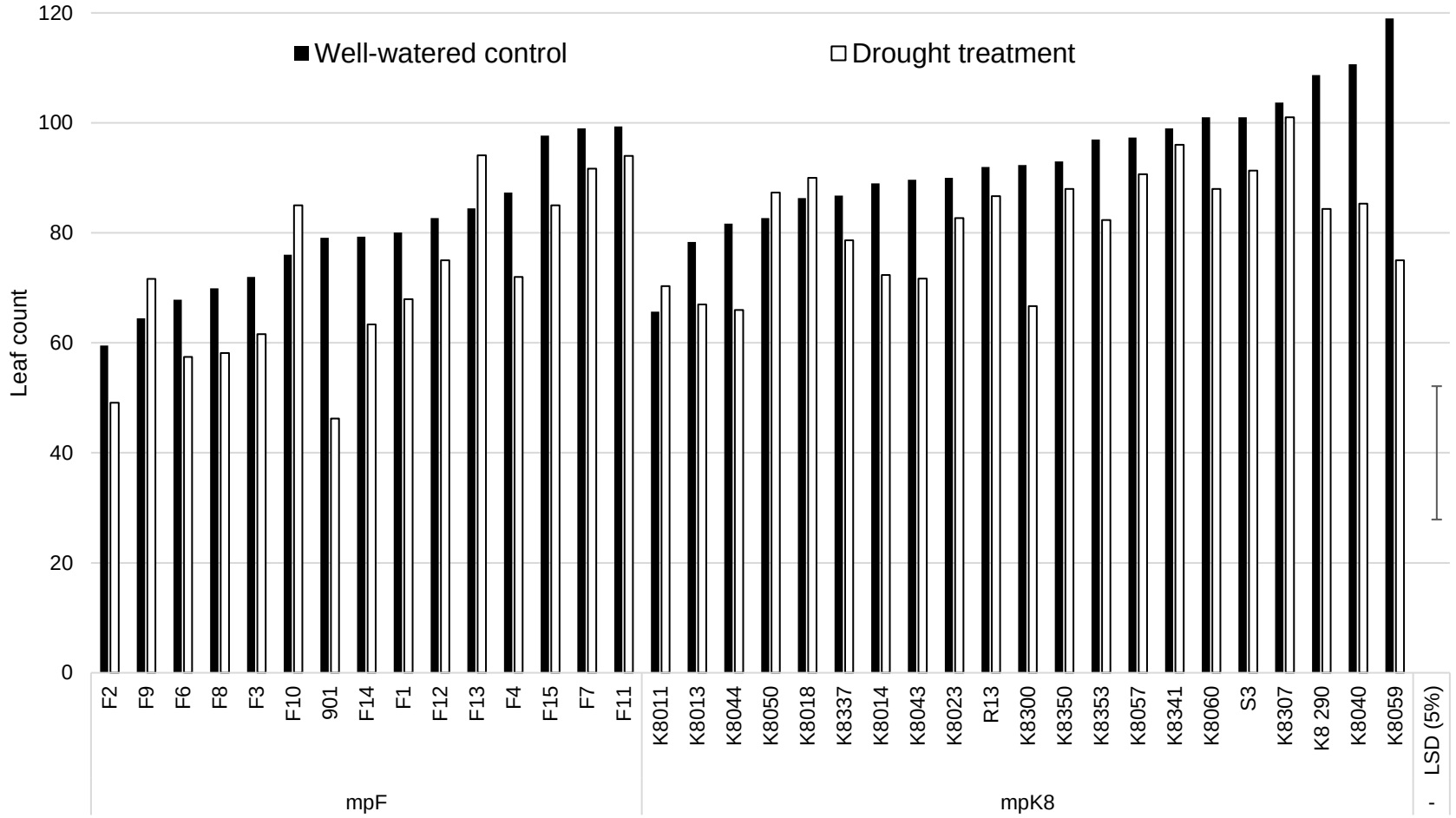

Figure 3.11 2015 Before drought 2 (BD2) lead stem leaf count.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

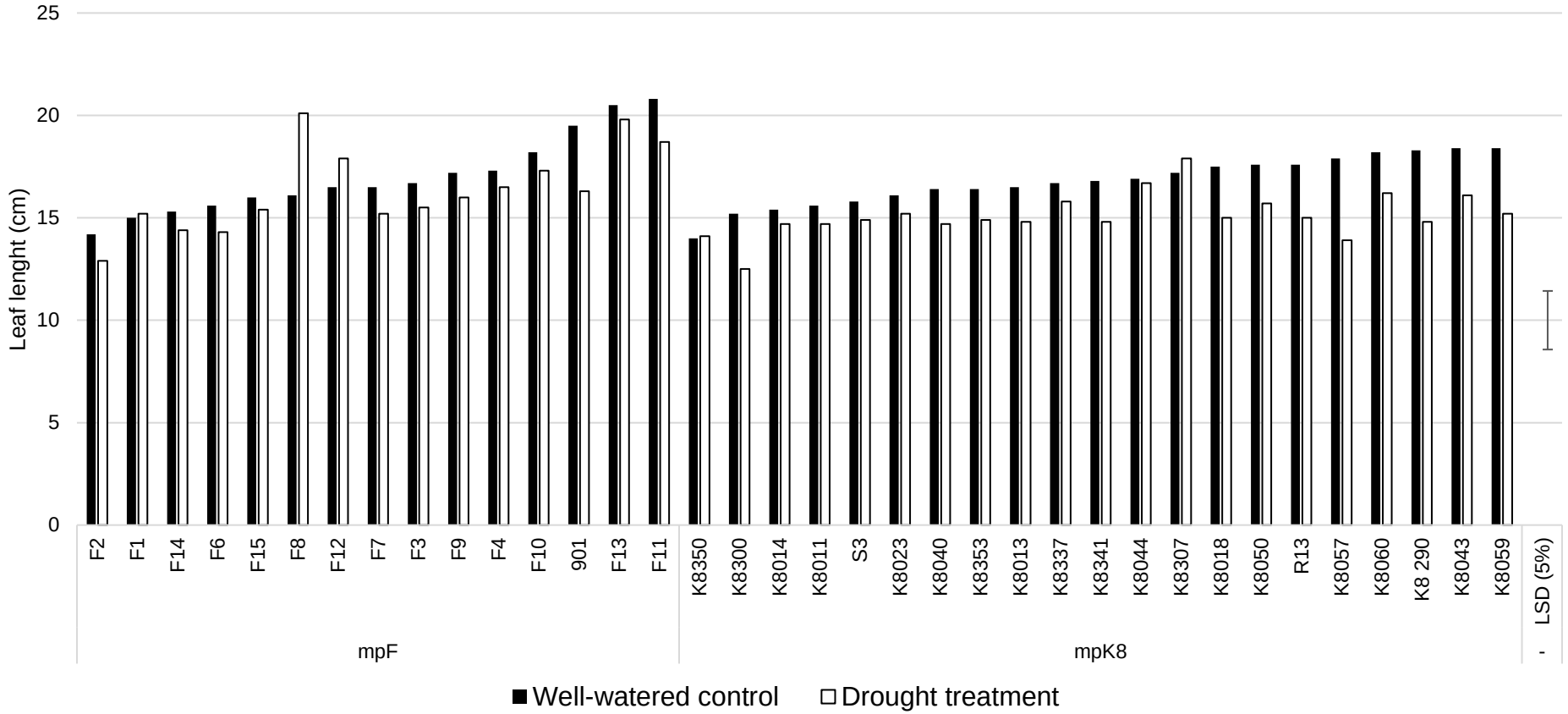

Figure 3.12 2015 Before drought 2 (BD2) top leaf length.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

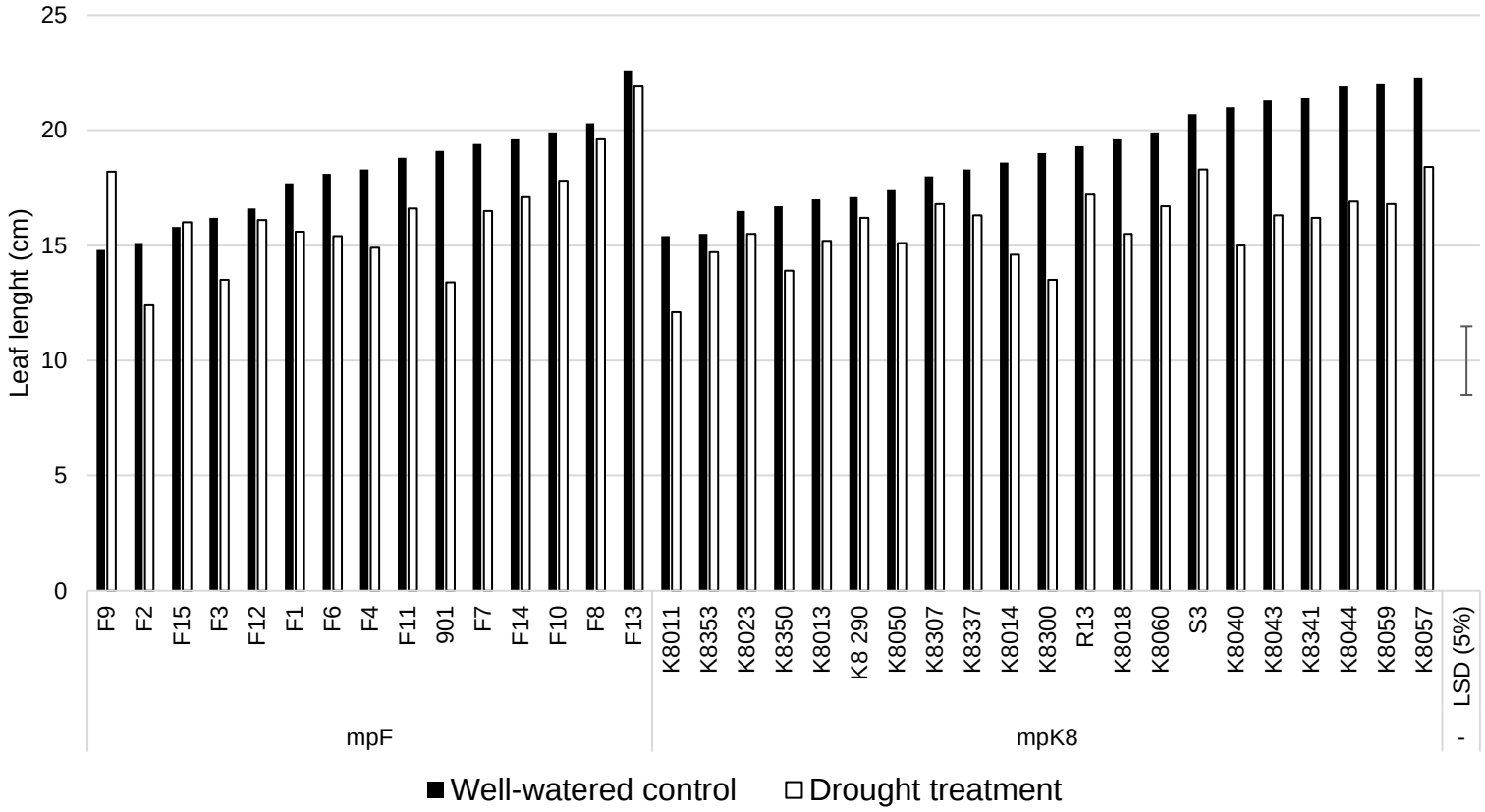

Figure 3.13 2015 Before drought 2 (BD2) middle leaf length.

With LSD required for significance at p =0.05, and for comparing genotypes between different irrigation levels.

3.4 Discussion

The greater drought effect in 2015 can be explained by the different methodology used between the two experiments. The timing of the drought periods, the length of the drought periods and the method of ending the drought period were changed. In 2015 the first drought took place 32 days earlier than in 2014 and coincided with the exponential phase of the logistic growth curve. Fig. 3.14 shows mean growth curves of lead stem height measurements for mpK8 male parent R13 for the well-watered control and droughted plants. It demonstrates that bringing the drought periods forward will coincide with a higher growth rate.

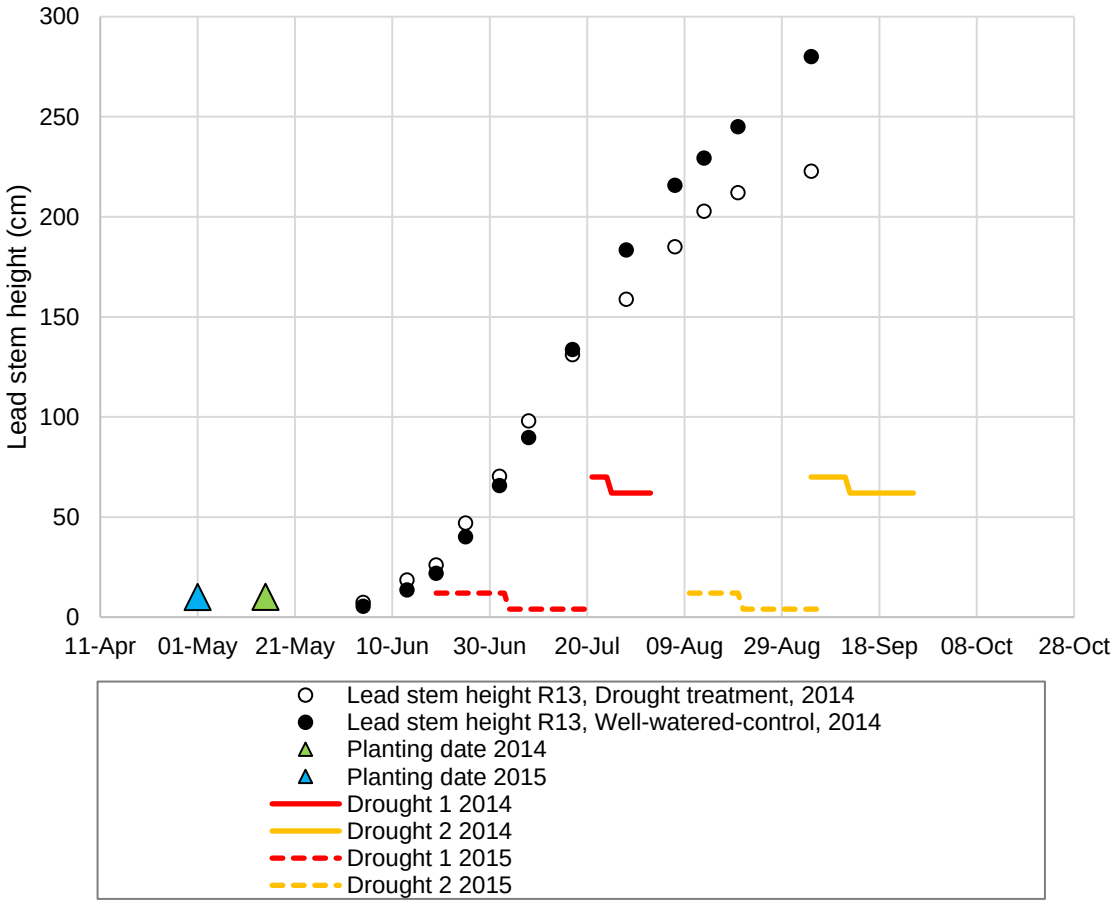

Figure 3.14 Mean weekly lead stem height measurements of mpK8 male parent R13 in 2014 pot experiment.

2015 planting date and drought periods included for comparison. Drought periods shown in red (D1) and yellow (D2) (2015 drought periods indicated with a dashed line). Higher level of drought line indicates all plants being droughted, lower level of drought line indicates some plants have been re-watered.

Inducing drought stress early in the growth phase has a greater impact on dry matter yield than a later drought. This agrees with Richard et al., (2019) who found

that an early drought has a negative effect on canopy development, resulting in lower SRC willow yields.

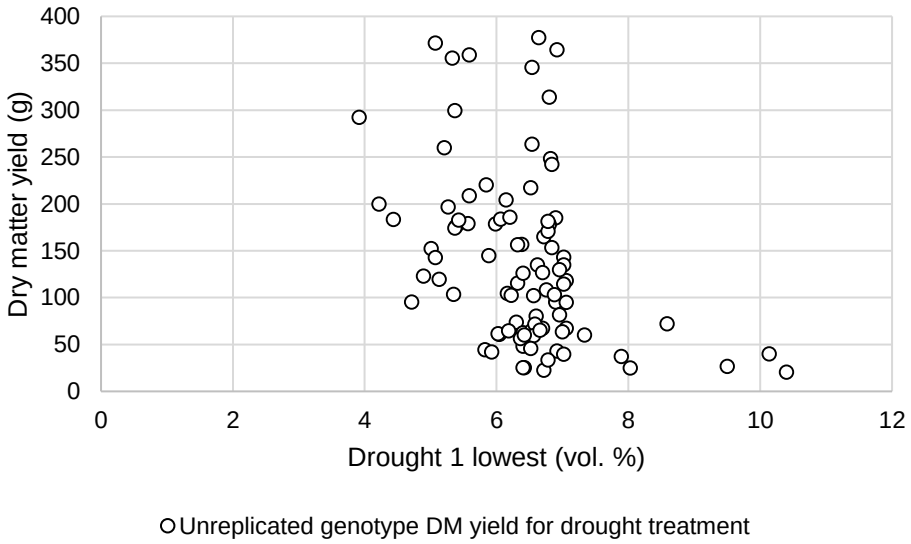

Figure 3.15 Final yield of droughted plants and lowest growth media moisture content during D1.

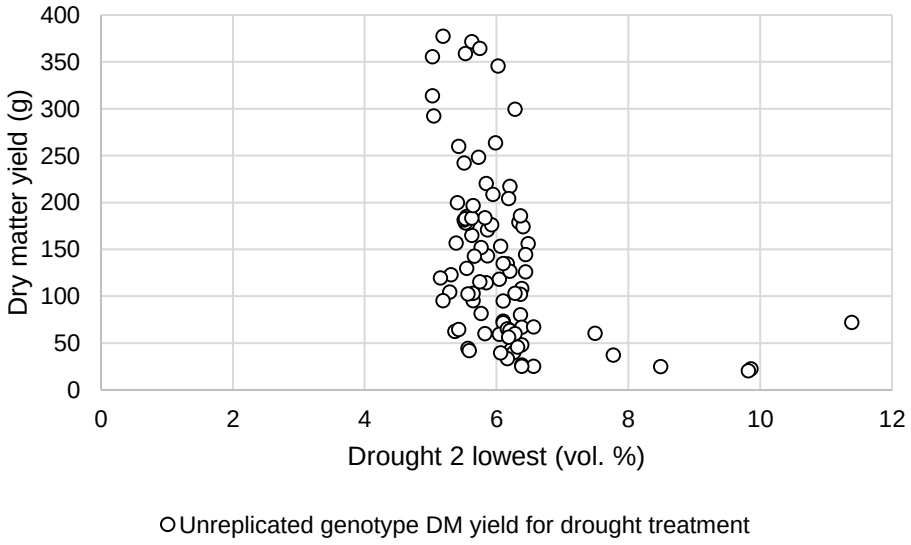

Figure 3.16 Final yield of droughted plants and lowest growth media moisture content during D2.

The method of ending the drought period also contributed to an increased difference between the droughted and well-watered yields. When plants were re-watered on a pot by pot basis based on their individual soil moisture content a more constant drought stress was imposed. This more consistent approach can be

seen in the soil moisture content plots, Fig. 3.15 & 3.16. These graphs both highlight small plants that were not exposed to a drought period, despite irrigation being withheld. F1, F7, nwc901, K9040 and K8044. All were slow to emerge and had low leaf areas.

Drought tolerance indices harmonic mean (HARM), Fig. 3.8 and drought tolerance efficiency (DTE), Fig. 3.7 are more powerful at dissecting potential drought tolerance than Fig. 3.6 or the Fig. 3.4 yield results.

Drought tolerance efficiency (DTE) is a useful indicator for screening drought tolerant genotypes. DTE values below 1 indicate that the genotype performs better in well-watered conditions than in the drought treatment. DTE values were generally lower in 2015 in a comparison across the two experiments. Of the common F population genotypes, the ranks of F1 (1), F4 (2) and F2 (4) were consistent across the experiments, the other comparable genotypes, nwc901, F3, S3, R13 and K8290 switched ranks between the experiments.

Table 3.5 shows that drought tolerance indices Geometric Mean Productivity (GMP) and Harmonic Mean (HARM) produce the same results, similar to in 2014. These GMP and HARM indices highly correlate with Mean Productivity (MP) 0.99, Yield Index (YI) 0.99 and the Stress Tolerance Index (STI) 0.97. The Drought tolerance efficiency (DTE) and Tolerance Against Stress (TOL) have an inverse relationship, but these indices are not as closely associated as 2014. Figs 3.7 (DTE) and 3.8 (HARM) show results from these two broad classes of drought tolerance indices that can be used for selecting genotypes response to drought stress.

The indices supported stable and high yield in both non-stress and stress treatments: mean productivity (MP), stress tolerance index (STI), geometric mean productivity (GMP), harmonic mean (HARM). These make more appropriate selection criteria when selecting for biomass plantations exposed to occasional drought stress, this latter group of indices form the more appropriate selection criteria as they will select for high yield in wetter and drier years.

Yield reductions under drought were more similar to the findings of other drought studies in the Salicaceae conducted in pots with *Salix* (Bonosi et al., 2010; McIvor, 2005), in field trials with *Salix* (Bonosi et al., 2013; Linderson et al., 2007) and in pots with *Populus* (Monclus et al., 2006).

Genotypes in the 2015 experiment originate from mapping populations and do not have the yield potential of commercial varieties. This is reflected in the maximum yield presented in Table 3.2. These maximum yields would equate to 3.3 odt ha^{-1} for droughted plants and 5.5 odt ha^{-1} for well-watered controls if adjusted to a typical SRC plantation density of 15,000 plants/ha^{-1}. This compares to yields to ~4 odt ha^{-1} for droughted plants and ~5 odt ha^{-1} for the well-watered control in 2014 for of SRC biomass variety family means equate. The higher yield in 2015 can be explained by the longer growth season.

Middle leaf length is the most meaningful leaf length measurement 17 – 34 days after a drought. It is a high through put measurement for breeders assessing the impact of drought on germplasm. The timing of measurements to capture the responses to drought is important. In 2014 the top leaf was the most interesting leaf 2 – 7 days after re-watering with effects of the drought being seen 34-39 days later, on both the top and middle leaf.

This also informs which leaf could produce interesting measurements for other physiological measurements.

The experiment aimed to increase the number of individual genotypes per family assessed. Fig. 3.4 shows dry matter differences are both more variable and greater among population mpK8 than population mpF. Population mpK8's establishment was also more reliable, with 30% of mpF plants failing to establish. In the entire 2014 experiment only 1.25% of plants failed to establish. Both populations show useful segregation in their progeny using HARM and DTE drought tolerance indices Fig. 3.8 and 3.7 respectively. mpK8 has delivered a greater contrasting response to imposed drought from its progeny than population mpF. However, a combination on the reliability of mpK8 with regard to slug resilience / confidence in data (less missing values) and the strong multisite legacy of mpK8 field trial data (Hanley et al., 2002; Karp et al., 2011) and pre-existing genetic resources available has led to mpK8 population will being selected for future study.

Future work will focus on linking pot and field trial performance, mapping QTL and potentially analysing candidate genes within them (by transcriptomics and bioinformatic approaches). Ultimately, it is hoped that this will allow the identification of genomic regions linked to drought tolerance and water use traits, and that these can be incorporated into the willow breeding programme, resulting in SRC willow varieties that are more able to cope with drought periods.

3.5 Conclusions

The pot experiment containing mpF and mpK8 members and parents found that;

1. Useful genetic variation exists for drought tolerance traits in the mpF and mpK8.
2. Potentially useful leaf measurements for screening drought tolerant willows can be developed.
3. Drought tolerance indices are suitable to identify and select for drought tolerant genotypes in the mpF and mpK8.
4. The assessment of mpF and mpK8 material has shown that they both segregate for yield under drought conditions and are potentially suitable for further study at the field scale.
5. Changes to the pot trial methodology have led to a greater main effect of irrigation.

The methodology changes in re-watering and the timing of the drought produced a significant main effect of irrigation on all final yield responses. An early drought coinciding with the exponential growth phase has a more negative effect on yield than a drought that occurs later in the growing season. Future work will use routine slug control methods to mitigate the risk of plant damage at establishment. The use of guard plants improved experimental design. Further methodology changes should be considered if wider germplasm is to be studied within the same pot experiment. There are opportunities to monitor the water deficit and plant water status more closely and precisely. Relative growth rates, derived from non-destructive estimates of yield such as stem diameter measurements or stem length should be recorded at regular intervals (weekly) through the experiment.

Assessing the middle leaf length 17 – 34 days after a drought is a potential high throughput measurement for breeders assessing the impact of drought on germplasm. It also informs which leaf could be the subject of other physiological

measurements, for example gas exchange measurements, SPAD etc, making these measurements more impactful.

The choice of drought tolerance indices has proven to be important when considering them as use as selection criteria. Geometric Mean Productivity (GMP) and Harmonic Mean (HARM) support the selection of stable and high yield in both non-stress and stress treatments. These indices are appropriate for selecting material for biomass plantations exposed to occasional drought stress. These should be used if selecting for performance under both non-stress and stress conditions.

4. Final conclusions

Phenotypic measurements

Top and middle leaf lengths may potentially offer the breeder a high throughput method of assessing the impact of drought on germplasm and is sufficiently high throughput to be applied to large genetic mapping populations. The timing of the measurement to capture the physiological impacts of drought is key and likely to vary among species. A further study with more time points would help build on this finding. This knowledge will inform which leaf could be the subject of other physiological measurements, for example gas exchange measurements, SPAD etc, this could make potential low-throughput measurements more impactful.

Assessing drought-induced leaf senescence has the potential to be a relatively high throughput measurement for breeders assessing the impact of drought on germplasm. Results in 2014 revealed a significant genotype and drought treatment interaction ($p = <.001$, F-test) for bottom leaf width one month after a drought event. Drought-induced leaf senescence is an important mechanism and should be monitored during pot trials or during a drought event in a field experiment.

The choice of drought tolerance indices are important when considering them as selection criteria. The specific indices should be matched to the selection goal of the breeder, with the output being suitable for the selected breeding zone. This study suggests that Geometric Mean Productivity (GMP), Mean Productivity (MP) and Harmonic Mean (HARM) support the selection of stable and high yield in both non-stress and stress treatments. These indices are appropriate for selecting material for biomass plantations exposed to occasional water deficit. This criteria of selecting for performance under both non-stress and stress conditions is appropriate for perennial energy crops in UK conditions currently.

Drought tolerance indices against stress (TOL) and drought tolerance efficiency (DTE) are useful tools for screening drought tolerant genotypes. These indices will be more helpful if selecting material for breeding zones that have more regular and severe water deficits.

Relative growth rates, derived from non-destructive estimates of yield such as stem diameter measurements or stem length should be recorded at regular intervals (weekly) through the experiment.

Experiments should be sized so it is possible to perform more regular soil water status monitoring and regular measurements are possible at key time points. Only yield measurements were taken after the second droughts in these studies. There would have been value in continuing regular measurements and scoring to study the consequences/recovery from the water deficit after re-watering the second drought cycle.

If small, slow growing germplasm is to be studied in an experiment with larger material, it should be investigated if the experimental design can incorporate the material separately, so it doesn't get shaded. It should be investigated if small plants can be planted in proportionally smaller pots, so dry down times can be similar to larger plants in bigger pots.

Be mindful not to over-water the well-watered control. Aim not to exceed field capacity, so as not to flood plants and cause yield penalty by creating an anoxic environment.

Integration into a breeding programme

Future work will focus on linking pot and field trial performance, mapping QTL and analysing candidate genes within them (by transcriptomics and bioinformatic approaches). Ultimately, this could allow the identification of genomic regions and linked genetic markers associated with drought tolerance and water use traits, with this knowledge used to increase the efficiency of selection within the willow breeding programme.

Identifying high throughput phenotyping techniques, such as top or middle leaf length measurements, gives the potential to phenotype the large full-sib mpK8, (n. 947) progeny, at Woburn Experimental Farm, Husbourne Crawley, UK. Large populations such as this provide increased power to dissect complex drought traits into more defined components for molecular breeding and gene discovery. Selection via marker-assisted selection (MAS) would be a useful outcome, particularly for traits such as drought tolerance that may not be encountered within the conventional breeding programme selection pipeline.

Drought stress scenario

Drought timings are important, and the two pot experiments have demonstrated that an early drought coinciding with the exponential growth phase has the

potential for a more negative effect on yield than a drought that occurs later in the growth season.

The timing, duration and frequency of the water stress will have implications on the experimental results. The breeder should carefully match drought scenarios in their experiments with meteorology data or future climate forecasts for their target breeding zone to insure maximum impact on their breeding programme.

Flowering is widely recognised as the most drought sensitive plant growth stage in many cultivated crop species. However, in willow cultivation, the breeder is only concerned with stress at the vegetative growth stage. Ideotypes of interest with respect to the vegetative growth are: the plant's capability to continue growing and developing during the drought stress and/or the ability to recover and regrow after a severe stress. For a plant's capacity to maintain high plant water status during a water stress period, dehydration avoidance is highly desirable. This dehydration avoidance strategy could be achieved by osmotic adjustment as reflected in relative water content or by maintaining plant water potential (Blum, 2011).

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Appendix 1 2014 pot experiment design

Block																		
	1 4 - D2	2 47 - 64_CZ	3 2 - K8_165	4 5 - D3	5 42 - 1093	6 17 - F3	7 3 - D1	8 11 - E3	9 27 - K2	10 48 - 13_CZ	11 28 - K3	12 38 - 432	13 53 - IA162	14 37 - nwc453	15 22 - G3	16 20 - G1	17 31 - R13	18 54 - IA143
Block I	19 19 - nwc1059	20 7 - K8_319	21 50 - S_Hallstad1	22 24 - nwc628(Tora)	23 1 - nwc789	24 16 - F2	25 35 - 615	26 26 - K1	27 8 - nwc506	28 46 - 77_CZ	29 30 - S3	30 45 - 33_CZ	31 25 - Baldwin	32 49 - IA159	33 12 - E4	34 14 - K8_290	35 39 - 844	36 40 - Tordis
	37 18 - F4	38 23 - G4	39 44 - 024_CZ	40 51 - IA136	41 52 - IA102	42 10 - E2	43 29 - K4	44 9 - E1	45 13 - nwc901	46 36 - nwc627(Bjorn)	47 33 - K8_2	48 43 - 003_CZ	49 41 - K8_411	50 32 - K8_3	51 34 - 663	52 15 - F1	53 6 - D4	54 21 - G2
	109 15 - F1	110 49 - IA159	111 44 - 024_CZ	112 33 - K8_2	113 50 - S_Hallstad1	114 37 - nwc453	115 19 - nwc1059	116 27 - K2	117 54 - IA143	118 40 - Tordis	119 30 - S3	120 12 - E4	121 31 - R13	122 47 - 64_CZ	123 41 - K8_411	124 6 - D4	125 5 - D3	126 36 - nwc627(Bjorn)
Block II	127 22 - G3	128 7 - K8_319	129 17 - F3	130 52 - IA102	131 14 - K8_290	132 51 - IA136	133 45 - 33_CZ	134 25 - Baldwin	135 43 - 003_CZ	136 23 - G4	137 32 - K8_3	138 8 - nwc506	139 13 - nwc901	140 4 - D2	141 10 - E2	142 24 - nwc628(Tora)	143 34 - 663	144 29 - K4
	145 38 - 432	146 53 - IA162	147 20 - G1	148 35 - 615	149 39 - 844	150 18 - F4	151 9 - E1	152 11 - E3	153 26 - K1	154 2 - K8_165	155 48 - 13_CZ	156 16 - F2	157 3 - D1	158 46 - 77_CZ	159 1 - nwc789	160 42 - 1093	161 28 - K3	162 21 - G2
	217 33 + K8_2	218 30 + S3	219 41 + K8_411	220 45 + 33_CZ	221 18 + F4	222 48 + 13_CZ	223 49 + IA159	224 19 + nwc1059	225 20 + G1	226 54 + IA143	227 7 + K8_319	228 53 + IA162	229 34 + 663	230 25 + Baldwin	231 43 + 003_CZ	232 44 + 024_CZ	233 52 + IA102	234 16 + F2
Block III	235 23 + G4	236 36 + nwc627(Bjorn)	237 46 + 77_CZ	238 1 + nwc789	239 4 + D2	240 32 + K8_3	241 40 + Tordis	242 27 + K2	243 12 + E4	244 13 + nwc901	245 21 + G2	246 47 + 64_CZ	247 22 + G3	248 6 + D4	249 31 + R13	250 38 + 432	251 24 + nwc628(Tora)	252 42 + 1093
	253 29 + K4	254 10 + E2	255 2 + K8_165	256 37 + nwc453	257 26 + K1	258 35 + 615	259 50 + S_Hallstad1	260 51 + IA136	261 28 + K3	262 3 + D1	263 8 + nwc506	264 5 + D3	265 11 + E3	266 9 + E1	267 39 + 844	268 14 + K8_290	269 17 + F3	270 15 + F1

Block																		
	55 3 + D1	56 22 + G3	57 39 + 844	58 35 + 615	59 51 + IA136	60 47 + 64_CZ	61 36 + nwc627(Bjorn)	62 43 + 003_CZ	63 17 + F3	64 21 + G2	65 12 + E4	66 7 + K8_319	67 20 + G1	68 50 + S_Hallstad1	69 54 + IA143	70 6 + D4	71 30 + S3	72 26 + K1
Block I	73 23 + G4	74 32 + K8_3	75 11 + E3	76 45 + 33_CZ	77 25 + Baldwin	78 49 + IA159	79 1 + nwc789	80 33 + K8_2	81 53 + IA162	82 10 + E2	83 48 + 13_CZ	84 27 + K2	85 52 + IA102	86 40 + Tordis	87 46 + 77_CZ	88 5 + D3	89 29 + K4	90 15 + F1
	91 13 + nwc901	92 2 + K8_165	93 38 + 432	94 18 + F4	95 19 + nwc1059	96 24 + nwc628(Tora)	97 34 + 663	98 41 + K8_411	99 31 + R13	100 44 + 024_CZ	101 4 + D2	102 42 + 1093	103 9 + E1	104 37 + nwc453	105 28 + K3	106 8 + nwc506	107 14 + K8_290	108 16 + F2
	163 28 + K3	164 30 + S3	165 2 + K8_165	166 15 + F1	167 5 + D3	168 49 + IA159	169 22 + G3	170 47 + 64_CZ	171 31 + R13	172 24 + nwc628(Tora)	173 1 + nwc789	174 14 + K8_290	175 20 + G1	176 13 + nwc901	177 39 + 844	178 54 + IA143	179 44 + 024_CZ	180 16 + F2
Block II	181 4 + D2	182 53 + IA162	183 38 + 432	184 21 + G2	185 12 + E4	186 48 + 13_CZ	187 19 + nwc1059	188 26 + K1	189 42 + 1093	190 45 + 33_CZ	191 25 + Baldwin	192 18 + F4	193 6 + D4	194 17 + F3	195 29 + K4	196 43 + 003_CZ	197 10 + E2	198 52 + IA102
	199 23 + G4	200 41 + K8_411	201 7 + K8_319	202 46 + 77_CZ	203 51 + IA136	204 3 + D1	205 11 + E3	206 33 + K8_2	207 34 + 663	208 9 + E1	209 8 + nwc506	210 27 + K2	211 35 + 615	212 50 + S_Hallstad1	213 37 + nwc453	214 32 + K8_3	215 40 + Tordis	216 36 + nwc627(Bjorn)
	271 9 - E1	272 43 - 003_CZ	273 1 - nwc789	274 6 - D4	275 23 - G4	276 40 - Tordis	277 52 - IA102	278 16 - F2	279 51 - IA136	280 38 - 432	281 48 - 13_CZ	282 44 - 024_CZ	283 39 - 844	284 49 - IA159	285 14 - K8_290	286 46 - 77_CZ	287 13 - nwc901	288 54 - IA143
Block III	289 10 - E2	290 34 - 663	291 50 - S_Hallstad1	292 53 - IA162	293 19 - nwc1059	294 21 - G2	295 32 - K8_3	296 31 - R13	297 41 - K8_411	298 11 - E3	299 28 - K3	300 22 - G3	301 29 - K4	302 45 - 33_CZ	303 3 - D1	304 8 - nwc506	305 35 - 615	306 24 - nwc628(Tora)
	307 26 - K1	308 36 - nwc627(Bjorn)	309 15 - F1	310 17 - F3	311 30 - S3	312 20 - G1	313 4 - D2	314 2 - K8_165	315 5 - D3	316 33 - K8_2	317 7 - K8_319	318 25 - Baldwin	319 42 - 1093	320 47 - 64_CZ	321 37 - nwc453	322 27 - K2	323 18 - F4	324 12 - E4

Design

Split-plot in 3 blocks, with ± irrigation on main plots and genotypes on Split-plots

Blockstructure

Block/W_Plot/S_Plot (= Block + Block.W_Plot+Block.W_plot.S_plot)

Treatmentstructure

Genotype * Irrigation (= Gentype + Irrigation + Genotype . Irrigation)

Key

1	Plot number
4 -	Genotype code & drought water tratment (-)
D2	Genotype
184	Plot number
21 +	Genotype code & control water tratment (+)
G2	Genotype

N

Appendix 2 LogTag temperature sensor location within 2014 pot experiment

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72
4 -	47 -	2 -	5 -	42 -	17 -	3 -	11 -	27 -	48 -	28 -	38 -	53 -	37 -	22 -	20 -	31 -	54 -	3 +	22 +	39 +	35 +	51 +	47 +	36 +	43 +	17 +	21 +	12 +	7 +	20 +	50 +	54 +	6 +	30 +	26 +
D2	[illegible]	[illegible]	D3	1093	F3	D1	E3	K2	13_CZ	K3	432	IA162	nwc453	G3	G1	R13	IA143	D1	G3	844	615	IA136	64_CZ	[illegible]	[illegible]	F3	G2	E4	K8_319	G1	S_Hallstad1	IA143	D4	S3	K1
19	36783	36	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	73	74	75	76	77	78	36782	81	82	83	84	85	86	87	88	89	90
19 -	[illegible]	50 -	24 -	1 -	16 -	35 -	26 -	8 -	46 -	30 -	45 -	25 -	49 -	12 -	14 -	39 -	40 -	23 +	32 +	11 +	45 +	25 +	49 +	1 +	33 +	53 +	10 +	48 +	27 +	52 +	40 +	46 +	5 +	29 +	15 +
nwc1059	K8_319	S_Hallstad1	nwc628(Tora)	nwc789	F2	615	K1	nwc506	77_CZ	S3	33_CZ	Baldwin	IA159	E4	K8_290	844	Tordis	G4	K8_3	E3	33_CZ	Baldwin	IA159	nwc789	K8_2	IA162	E2	13_CZ	K2	IA102	Tordis	77_CZ	D3	K4	F1
37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108
18 -	23 -	44 -	51 -	52 -	10 -	29 -	9 -	13 -	36 -	33 -	43 -	41 -	32 -	34 -	15 -	6 -	21 -	13 +	2 +	38 +	18 +	19 +	24 +	34 +	41 +	31 +	44 +	4 +	42 +	9 +	37 +	28 +	8 +	14 +	16 +
F4	G4	024_CZ	IA136	IA102	E2	K4	E1	nwc901	nwc627(Bjorn)	K8_2	003_CZ	K8_411	K8_3	663	F1	D4	G2	nwc901	K8_165	432	F4	nwc1059	nwc628(Tora)	663	K8_411	R13	024_CZ	D2	1093	E1	nwc453	K3	nwc506	K8_290	F2
109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
15 -	49 -	44 -	33 -	50 -	37 -	19 -	27 -	54 -	40 -	30 -	12 -	31 -	47 -	41 -	6 -	5 -	36 -	28 +	30 +	2 +	15 +	5 +	49 +	22 +	47 +	31 +	24 +	1 +	14 +	20 +	13 +	39 +	54 +	44 +	16 +
F1	IA159	024_CZ	K8_2	S_Hallstad1	nwc453	nwc1059	K2	IA143	Tordis	S3	E4	R13	64_CZ	K8_411	D4	D3	nwc627(Bjorn)	K3	S3	K8_165	F1	D3	IA159	G3	64_CZ	R13	nwc628(Tora)	nwc789	K8_290	G1	nwc901	844	IA143	024_CZ	F2
127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198
22 -	7 -	17 -	52 -	14 -	51 -	45 -	25 -	43 -	23 -	32 -	8 -	13 -	4 -	10 -	24 -	34 -	29 -	4 +	53 +	38 +	21 +	12 +	48 +	19 +	26 +	42 +	45 +	25 +	18 +	6 +	17 +	29 +	43 +	10 +	52 +
G3	K8_319	F3	IA102	K8_290	IA136	33_CZ	Baldwin	36784	G4	K8_3	nwc506	nwc901	D2	E2	nwc628(Tora)	663	K4	D2	IA162	432	G2	E4	13_CZ	nwc1059	K1	1093	33_CZ	Baldwin	F4	36785	[illegible]	K4	003_CZ	E2	IA102
145	146	147	148	149	150	151	152	[illegible]	154	155	156	157	158	159	160	161	162	199	200	201	202	203	204	205	206	207	208	209	210	[illegible]	[illegible]	213	214	215	216
38 -	53 -	20 -	35 -	39 -	18 -	9 -	11 -	26 -	2 -	48 -	16 -	3 -	46 -	1 -	42 -	28 -	21 -	23 +	41 +	7 +	46 +	51 +	3 +	11 +	33 +	34 +	9 +	8 +	27 +	35 +	50 +	37 +	32 +	40 +	36 +
432	IA162	G1	615	844	F4	E1	E3	K1	K8_165	13_CZ	F2	D1	77_CZ	nwc789	1093	K3	G2	G4	K8_411	K8_319	77_CZ	IA136	D1	E3	K8_2	663	E1	nwc506	K2	615	S_Hallstad1	nwc453	K8_3	Tordis	nwc627(Bjorn)
217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288
33 +	30 +	41 +	45 +	18 +	48 +	49 +	19 +	20 +	54 +	7 +	53 +	34 +	25 +	43 +	44 +	52 +	16 +	9 -	43 -	1 -	6 -	23 -	40 -	52 -	16 -	51 -	38 -	48 -	44 -	39 -	49 -	14 -	46 -	13 -	54 -
K8_2	S3	K8_411	33_CZ	F4	13_CZ	IA159	nwc1059	G1	IA143	K8_319	IA162	663	Baldwin	003_CZ	024_CZ	IA102	F2	E1	003_CZ	nwc789	D4	G4	Tordis	IA102	F2	IA136	432	13_CZ	024_CZ	844	IA159	K8_290	77_CZ	nwc901	IA143
235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306
23 +	36 +	46 +	1 +	4 +	32 +	40 +	27 +	12 +	13 +	21 +	47 +	22 +	6 +	31 +	38 +	24 +	42 +	10 -	34 -	50 -	53 -	19 -	21 -	32 -	31 -	41 -	11 -	28 -	22 -	29 -	45 -	3 -	8 -	35 -	24 -
G4	nwc627(Bjorn)	77_CZ	nwc789	D2	K8_3	Tordis	K2	E4	nwc901	G2	64_CZ	G3	D4	R13	432	nwc628(Tora)	1093	E2	663	S_Hallstad1	IA162	nwc1059	G2	36828	K8_411	E3	K3	G3	K4	33_CZ	D1	nwc506	615	nwc628(Tora)
253	[illegible]	[illegible]	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	307	308	309	310	311	312	[illegible]	[illegible]	315	316	317	318	319	320	321	322	323	324
29 +	36827	[illegible]	37 +	26 +	35 +	50 +	51 +	28 +	3 +	8 +	5 +	11 +	9 +	39 +	14 +	17 +	15 +	26 -	36 -	15 -	17 -	30 -	20 -	4 -	2 -	5 -	33 -	7 -	25 -	42 -	47 -	37 -	27 -	18 -	12 -
K4	E2	K8_165	nwc453	K1	615	S_Hallstad1	IA136	K3	D1	nwc506	D3	E3	E1	844	K8_290	F3	F1	K1	nwc627(Bjorn)	F1	F3	S3	G1	D2	K8_165	D3	K8_2	K8_319	Baldwin	1093	64_CZ	nwc453	K2	F4	E4

Appendix 3 2015 pot experiment design

	1	2	3	4	5	6	7	8	9	10	11	12	37	38	39	40	41	42	43	44	45	46	47	48
	25 +	10 +	23 +	17 +	9 +	14 +	21 +	8 +	35 +	18 +	28 +	19 +	16 -	9 -	28 -	31 -	8 -	15 -	3 -	25 -	36 -	30 -	4 -	26 -
	F3	K8044	F1	K8337	K8043	K8060	901	K8040	F14	K8341	F7	K8350	K8307	K8043	F7	F10	K8040	K8300	K8011	F3	F15	F9	K8013	F4
	13	14	15	16	17	18	19	20	21	22	23	24	49	50	51	52	53	54	55	56	57	58	59	60
Block I	13 +	26 +	1 +	12 +	29 +	33 +	32 +	15 +	6 +	20 +	16 +	22 +	23 -	33 -	24 -	7 -	21 -	27 -	22 -	17 -	18 -	14 -	11 -	32 -
	K8059	F4	S3	K8057	F8	F12	F11	K8300	K8018	K8353	K8307	K8 290	F1	F12	F2	K8023	901	F6	K8 290	K8337	K8341	K8060	K8050	F11
	25	26	27	28	29	30	31	32	33	34	35	36	61	62	63	64	65	66	67	68	69	70	71	72
	36 +	24 +	2 +	4 +	3 +	7 +	11 +	5 +	31 +	27 +	34 +	30 +	29 -	34 -	2 -	13 -	12 -	10 -	6 -	5 -	1 -	35 -	20 -	19 -
	F15	F2	R13	K8013	K8011	K8023	K8050	K8014	F10	F6	F13	F9	F8	F13	R13	K8059	K8057	K8044	K8018	K8014	S3	F14	K8353	K8350
	73	74	75	76	77	78	79	80	81	82	83	84	109	110	111	112	113	114	115	116	117	118	119	120
	25 -	13 -	8 -	31 -	9 -	30 -	2 -	23 -	29 -	12 -	14 -	10 -	11 +	23 +	26 +	29 +	12 +	5 +	32 +	27 +	28 +	10 +	33 +	21 +
	F3	K8059	K8040	F10	K8043	F9	R13	F1	F8	K8057	K8060	K8044	K8050	F1	F4	F8	K8057	K8014	F11	F6	F7	K8044	F12	901
Block II	85	86	87	88	89	90	91	92	93	94	95	96	121	122	123	124	125	126	127	128	129	130	131	132
	24 -	6 -	26 -	17 -	27 -	34 -	21 -	22 -	1 -	16 -	20 -	3 -	15 +	18 +	16 +	8 +	3 +	36 +	7 +	6 +	9 +	35 +	1 +	22 +
	F2	K8018	F4	K8337	F6	F13	901	K8 290	S3	K8307	K8353	K8011	K8300	K8341	K8307	K8040	K8011	F15	K8023	K8018	K8043	F14	S3	K8 290
	97	98	99	100	101	102	103	104	105	106	107	108	133	134	135	136	137	138	139	140	141	142	143	144
	18 -	15 -	36 -	35 -	4 -	28 -	32 -	19 -	33 -	11 -	7 -	5 -	19 +	17 +	24 +	2 +	4 +	13 +	14 +	34 +	30 +	31 +	20 +	25 +
	K8341	K8300	F15	F14	K8013	F7	F11	K8350	F12	K8050	K8023	K8014	K8350	K8337	F2	R13	K8013	K8059	K8060	F13	F9	F10	K8353	F3
	145	146	147	148	149	150	151	152	153	154	155	156	181	182	183	184	185	186	187	188	189	190	191	192
	11 -	1 -	35 -	24 -	29 -	27 -	12 -	20 -	17 -	33 -	5 -	23 -	2 +	8 +	30 +	35 +	21 +	29 +	15 +	5 +	14 +	23 +	20 +	28 +
	K8050	S3	F14	F2	F8	F6	K8057	K8353	K8337	F12	K8014	F1	R13	K8040	F9	F14	901	F8	K8300	K8014	K8060	F1	K8353	F7
	157	158	159	160	161	162	163	164	165	166	167	168	193	194	195	196	197	198	199	200	201	202	203	204
Block III	13 -	18 -	14 -	16 -	21 -	22 -	8 -	6 -	34 -	10 -	32 -	4 -	7 +	11 +	36 +	12 +	17 +	34 +	10 +	19 +	24 +	22 +	13 +	6 +
	K8059	K8341	K8060	K8307	901	K8 290	K8040	K8018	F13	K8044	F11	K8013	K8023	K8050	F15	K8057	K8337	F13	K8044	K8350	F2	K8 290	K8059	K8018
	169	170	171	172	173	174	175	176	177	178	179	180	205	206	207	208	209	210	211	212	213	214	215	216
	7 -	26 -	25 -	3 -	31 -	36 -	28 -	30 -	9 -	19 -	2 -	15 -	4 +	31 +	27 +	26 +	3 +	18 +	16 +	25 +	1 +	32 +	33 +	9 +
	K8023	F4	F3	K8011	F10	F15	F7	F9	K8043	K8350	R13	K8300	K8013	F10	F6	F4	K8011	K8341	K8307	F3	S3	F11	F12	K8043

N

Appendix 4 LogTag temperature sensor location within 2015 pot experiment

1 25 + F3	2 10 + K8044	3 23 +	4 17 +	5 9 + K8043	6 14 + K8060	7 21 + 901	8 8 + K8040	9 35 + F14	10 18 + K8341	11 28 + F7	12 19 + K8350	37 16 - K8307	38 9 - K8043	39 28 - F7	40 31 - F10	41 8 - K8040	42 15 -	43 3 -	44 25 - F3	45 36 - F15	46 30 - F9	47 4 - K8013	48 26 - F4
13 13 + K8059	14 26 + F4			17 29 + F8	18 33 + F12	19 32 + F11	20 15 + K8300	21 6 + K8018	22 20 + K8353	23 16 + K8307	24 22 + K8 290	49 23 - F1	50 33 - F12	51 24 - F2	52 7 - K8023	53 21 - 901			56 17 - K8337	57 18 - K8341	58 14 - K8060	59 11 - K8050	60 32 - F11
25 36 + F15	26 24 + F2			29 3 + K8011	30 7 + K8023	31 11 + K8050	32 5 + K8014	33 31 + F10	34 27 + F6	35 34 + F13	36 30 + F9	61 29 - F8	62 34 - F13	63 2 - R13	64 13 - K8059	65 12 - K8057			68 5 - K8014	69 1 - S3	70 35 - F14	71 20 - K8353	72 19 - K8350
73 25 - F3	74 13 - K8059	75 8 - K8040	76 31 - F10	77 9 - K8043	78 30 - F9	79 2 - R13	80 23 - F1	81 29 - F8	82 12 - K8057	83 14 - K8060	84 10 - K8044	109 11 + K8050	110 23 + F1	111 26 + F4	112 29 + F8	113 12 + K8057	114 5 + K8014	115 32 + F11	116 27 + F6	117 28 + F7	118 10 + K8044	119 33 + F12	120 21 + 901
85 24 - F2	86 6 - K8018	87 26 - F4	88 17 - K8337	89 27 - F6	90 34 - F13	91 21 - 901	92 22 -	93 1 -	94 16 - K8307	95 20 - K8353	96 3 - K8011	121 15 + K8300	122 18 + K8341	123 16 + K8307	124 8 + K8040	125 3 + K8011	126 36 + F15	127 7 + K8023	128 6 + K8018	129 9 + K8043	130 35 + F14	131 1 +	132 22 +
97 18 - K8341	98 15 - K8300	99 36 - F15	100 35 - F14	101 4 - K8013	102 28 - F7	103 32 - F11			106 11 - K8050	107 7 - K8023	108 5 - K8014	133 19 + K8350	134 17 + K8337	135 24 + F2	136 2 + R13	137 4 + K8013	138 13 + K8059	139 14 + K8060	140 34 + F13	141 30 + F9	142 31 + F10		
145 11 - K8050	146 1 - S3	147 35 -	148 24 -	149 29 - F8	150 27 - F6	151 12 - K8057			154 33 - F12	155 5 - K8014	156 23 - F1	181 2 + R13	182 8 + K8040	183 30 + F9	184 35 + F14	185 21 + 901	186 29 + F8	187 15 +	188 5 +	189 14 + K8060	190 23 + F1		
157 13 - K8059	158 18 - K8341			161 21 - 901	162 22 - K8 290	163 8 - K8040	164 6 - K8018	165 34 - F13	166 10 - K8044	167 32 - F11	168 4 - K8013	193 7 + K8023	194 11 + K8050	195 36 + F15	196 12 + K8057	197 17 + K8337	198 34 + F13		201 24 + F2	202 22 + K8 290	203 13 + K8059	204 6 + K8018
169 7 - K8023	170 26 - F4			173 31 - F10	174 36 - F15	175 28 - F7	176 30 - F9	177 9 - K8043	178 19 - K8350	179 2 - R13	180 15 - K8300	205 4 + K8013	206 31 + F10	207 27 + F6	208 26 + F4	209 3 + K8011	210 18 + K8341		213 1 + S3	214 32 + F11	215 33 + F12	216 9 + K8043