Drought resistance mechanisms in “Mediterranean” perennial ryegrass (*Lolium perenne* L.) and potential for introgression of “Mediterranean” germplasm into New Zealand commercial cultivars

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Abstract

The unique topography of New Zealand creates a wide variation in rainfall and temperature between and within the two islands of the country. As a result, successful use of perennial ryegrass (*Lolium perenne* L.), the backbone of New Zealand's agricultural economy, has been restricted to only the higher rainfall and cooler areas of the country. However, there has been only limited analysis of drought resistance in forage grasses at the trait level. This PhD study was conducted on a perennial ryegrass cultivar "Medea" developed in Adelaide in the 1960's from reportedly drought resistant and summer dormant germplasm of North African origin. The main objectives of the study were to compare Medea with a high yielding but drought susceptible current New Zealand cultivar, Grasslands Samson for their drought resistance potential and to evaluate Medea for its suitability for introgression with Grasslands Samson, in a plant improvement programme. Drought resistance strategies of Tolosa, Matrix and Ceres One50 were also evaluated.

In total six glasshouse experiments were conducted. Experiment 1 (April – September 2008) compared winter vegetative growth of potted plants of Grasslands Samson and Medea. Yield of Medea was <50% that of Grasslands Samson, but glasshouse temperature at times exceeded 25°C, so it is possible that this temperature was high enough to partially trigger summer dormancy in Medea.

In Experiment 2 (summer 2008 – 2009) techniques for assessing drought resistance were developed, and in Experiment 2 and Experiment 5 (summer 2009 – 2010) drought resistance strategies exhibited by individual cultivars were evaluated. Experiment 2 included Medea, Grasslands Samson, an unreleased tetraploid breeding line developed from Grasslands Samson and Tolosa. Experiment 5 evaluated Matrix and Ceres One50, in addition to Grasslands Samson and Medea. Drought resistance strategies observed in Medea included deep rootedness and high leaf proline contents, but there was some evidence for lack of transpiration reduction in water deficit stress. Medea had prolific flowering. Grasslands Samson and its tetraploid were more productive than Medea in these experiments. However, Tolosa produced the same shoot DW as Grasslands Samson with greater retention of soil moisture, indicating higher water use efficiency.

Experiment 3 (March 2009 – February 2010) compared five family groups, each comprising a Grasslands Samson and a Medea parent, and three of their F_1 progeny. In this experiment plants were 11 months old when root traits were evaluated and for these older plants, Grasslands Samson had a higher root to shoot ratio and deep rootedness than Medea. Medea plants had similar shoot DW to Grasslands Samson plants during winter, but 46% lower shoot DW in summer. The F_1 progeny showed positive mid-parent heterosis for deep rootedness, but negative mid-parent heterosis for shoot DW, and tended to reflect the prolific flowering of the Medea parent.

Experiment 4 (December 2009 – June 2010) compared six family groups of F_2 progeny for traits related to drought resistance. Although plant numbers were small compared with a commercial breeding programme, it was evident some family groups combined both drought resistance and productivity traits.

Experiment 6 (September 2011 – February 2012) evaluated Grasslands Samson, Medea, and F_1 and F_2 progeny for drought resistance traits. Some useful traits expressed strongly in the F_1 generation reverted to mid-parent values in the F_2 generation. Some genotypes of Grasslands Samson exhibited higher water use efficiency (reduced soil moisture extraction with high shoot DW) and this warrants further research.

It is concluded that some desirable genes for traits contributing to drought resistance, such as deep rootedness and osmotic adjustment might be obtained from Medea. However, the drought resistance strategy of Medea involving reduction in plant size in summer, deep rooting and comparatively high transpiration would have pros and cons for New Zealand farmers as a trait combination. Reduced depletion of soil moisture under water deficit might assist survival of companion plants such as white clover; but high transpiration would decrease water use efficiency. Therefore, improving the water use efficiency of Grasslands Samson or use of material such as Tolosa, which has a comparatively low soil water use per unit of dry matter produced among the cultivars tested, would appear to be a preferred breeding strategy for future breeding programmes in New Zealand.

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Dedication

To my parents, wife, brothers and sisters

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Glossary of Abbreviations

Abbreviation	Full name/meaning	Units
2n	Diploid	-
4n	Tetraploid	-
A_{Lf}	Leaf appearance interval	days $leaf^{-1}$
ANOVA	Analysis of Variance	-
Ci	Internal CO_2 concentration	Ppm
c.w.	Controlled watering	-
d1	Upper soil depth in experimental pots	-
d2	Middle soil depth in experimental pots	-
d3	Lower soil depth in experimental pots	-
DADW	Days after differential watering	
DR:S	Deep root (soil depths 2 and 3) to shoot ratio	-
DW	Herbage dry weight	G
EL	Electrolyte leakage	%
Evp	Evapotranspiration	m mol m^2s^{-1}
Fs	Site Filling	
FW	Fresh weight	g or mg
G. Samson	Grasslands Samson	-
Gener	Generation	
H%	Ratio of seed-head weight to shoot dry weight expressed as a percentage	
Harv	Harvest	-
HN	Seed-head number	Count
HN:TN%	Ratio of seed-head number to tillers number expressed as a percentage	
HW	seed-head weight	g
HW:HN	Ratio of seed-head weight to seed-head number	-
IndexDR	Index of deep rooting, i.e. ratio of root weight in depth2 and depth3 to total root weight	-
IndexWU	Index of water use; ratio of shoot dry weight to soil moisture content at soil depth 2	-
IRT	Infrared thermometer	-
Lcs	Leaf colour score	Score
Ldead%	Ratio of dead leaves to shoot dry weightexpressed as a percentage	%
Lds	Visual score for amount of leaf death	Score
LED	Leaf elongation duration	days
LER	Leaf extension rate	mm d^{-1}
LL	Leaf lamina length	mm
Llam%	Ratio of leaf lamina weight to shoot dry weight expressed as a percentage	%
LN	Whole plant leaf number	count
Lrs	Leaf rolling score	Score

LT	Leaf temperature	°C
LW	Leaf width	mm
LWP	Leaf water potential (often denoted Ψ)	MPa
Lws	Leaf wilting score	Score
MANOVA	Multivariate Analysis of Variance	
NLL	Number of live leaves	count
Ns	Non-significant	-
NZ	New Zealand	-
OA	Osmotic adjustment	-
OP	Osmotic potential (often denoted Ψ_p)	MPa
P	Probability	-
PC	Principal component	
PCA	Principal component analysis	-
PEG	Polyethylene glycol	-
Pn	Photosynthetic rate	µ mol m^2s^{-1}
PP	Pressure potential	MPa
Proline	Proline contents	mg g^{-1}.DW
Ps:Llam	Pseudostem:leaf lamina ratio	
PsL	Leaf pseudostem length	mm
R:S	Root Shoot Ratio	-
Rc d1	Coarse root weight at depth1	g
Rc d2	Coarse root weight at depth2	g
Rc d3	Coarse root weight at depth3	g
Rep	Experimental replication	-
Rf d1	Fine root weight at depth1	g
Rf d2	Fine root weight at depth2	g
Rf d3	Fine root weight at depth3	g
Rt	Total root weight	g
Rt d1	Total root weight at soil depth 1	g
Rt d2	Total root weight at soil depth 2	g
Rt d3	Total root weight at soil depth 3	g
RTAR	Relative tiller appearance rate	Tiller $tiller^{-1}$ d^{-1}
RWC	Relative water content	%
SAS	Statistical Analysis System	-
SC	Stomatal conductance (often denoted g_c)	m mol m^2s^{-1}
SEM	Standard error of mean	-
SMC d1	Soil moisture content at depth1	%
SMC d2	Soil moisture content at depth2	%
SMC d3	Soil moisture content at depth3	%
SMD	Soil moisture deficit	-
SS	Sum of squares (in ANOVA)	
Str	Water deficit treatment	-
Tc-Ta	Canopy-Air temperature difference	°C
TDR	Time domain reflectometer	-
TFW	Turgor fresh weight	mg
T_L	Leaf temperature	°C
TN	Tiller number	count
TW	Tiller weight	g
Var	Cultivar	-
Var × Harv	Cultivar × harvest interaction	-

Var × Wreg	Cultivar × water regime interaction	-
Var × Wreg × Harv	Cultivar × water regime × harvest interaction	-
Wreg	Water regime	-
Wreg × Harv	Water regime × harvest interaction	-
WUE	Water use efficiency	-

1

Introduction

1.1 General background

From a global perspective grasslands of various categories occupy around 5.25 billion ha or 40% of the world's land surface area and often support livestock which provide human populations with food, fibre, and other needs (Suttie et al., 2005). The variety and diversity of world grassland systems is indicated by the fact that in 12 chapters and some 500 pages, Suttie et al. (2005) provided little or no coverage of grassland production systems in more developed areas of the world such as Europe, North America, or of smaller countries like Japan or New Zealand. Furthermore, in contrast to the perspective presented in the FAO review cited above, recent European research has focused on grassland as a multifunctional resource with a role in balancing biogeochemical cycles and meeting recreational needs of urban populations (Lemaire et al., 2005).

New Zealand has a unique status as a small country with a population of just over 4 million people and 27 million ha total land area, regarded as a developed nation, yet obtaining more than 41 % of its foreign exchange earnings in 2011 from the pastoral sector. In view of current high economic returns from dairy farming (around $NZ 3,000 ha^{-1} yr^{-1} gross margin, based on $5.20 kg^{-1} milk solids (Schilling et al., 2010) and 930 kg MS ha^{-1} yr^{-1} on average (Anonymous, 2011), more productive land is increasingly used for dairying. There are currently around 11,000 New Zealand dairy farmers with a total of some 6 m dairy cows (Abell et al., 2011), and they produce over one third of the world's traded dairy produce from a land area of about 2 m ha. These dairy farmers, together with more intensively farmed sheep and beef properties occupying a similar area, use perennial ryegrass (*Lolium perenne* L.) as the primary botanical component of new pasture sowings (Belgrave et al., 1990). However, both the dairy and the sheep and beef industries need to achieve a low cost of production to compete on world markets and this need in turn leads to a reliance on pasture grazed *in situ* as a primary feed source. Therefore, designing pastoral systems with well matched seasonal feed supply and demand, and developing plant

varieties that exhibit resilience to climatic variation and persistence, is a priority for research (Palmer, 2009).

Because New Zealand spans from 34ºS to 47ºS and the two main islands possess central mountain chains that produce orographic climate variation with rainfall (Kenny et al., 1995) reducing from west to east, there is considerable regional variation even within New Zealand in the grassland environment and in farming systems employed. Factors contributing to pasture persistence issues in New Zealand include the occurrence of summer-autumn droughts in some regions, wet winter-early spring conditions, and high summer temperatures. This climatic pattern typical to many parts of New Zealand also drives seasonality of forage production. Late spring and early summer is the peak growth periods with a sharp decline in growth during summer followed by another lesser peak in autumn (Verkerk, 2003). Farmers have traditionally made silage from peak period pasture growth but in recent years there has been a tendency for dairy farmers to increase stocking rates during the milking season and graze cows off farm in winter (Matthew et al., 2010). More intensive systems like these tend to be more vulnerable to summer moisture deficit, and feed deficit which may occur as a consequence (Palmer, 2009). In such feed deficit situations, farmers have a range of options such as: (i) increasing rotation length to reduce herbage intake of animals, (ii) increasing use of supplements, (iii) drying off poor performing cows, (iv) reducing milking frequency from twice to once per day, or (v) reducing stocking rates (Tait et al., 2005). A parallel situation of gradual intensification over time applies on many sheep and beef farms. The pressure on pastures from this gradual intensification over time appears to be adversely affecting the performance of perennial ryegrass pastures, particularly in regions of New Zealand with warmer, drier summers (Macdonald et al., 2011).

Perennial ryegrass was a component of bush burn pastures established by mainly European settlers in the late 18th and early 19th centuries and as will be reported in more detail in Chapter 2. The first New Zealand-bred perennial ryegrass cultivar was released from DSIR Grasslands Division Palmerston North in 1936, and was renamed Grasslands Ruanui in 1964 (Easton, 1983). A brief account of the development of perennial ryegrass in New Zealand appears in Section 2.6. However, suffice to say, within the last 20 years increasing attention has been directed by perennial ryegrass breeders towards achievement of improved tolerance of summer

moisture deficit. Emerging awareness of global climate influences such as the Southern Oscillation Index that determines so-called El Niño and La Niña weather patterns (NIWA 2011), and global warming (Kenny et al., 1995), coupled with farmer concerns about poor persistence of 'new' ryegrass pastures as mentioned above have increased the interest in enhanced moisture deficit and heat tolerance in perennial ryegrass. Breeding strategies employed in perennial ryegrass development in New Zealand in the recent past have included introgression of Spanish germplasm (e.g. cultivars Grasslands Impact, and Trojan marketed by NZ Agriseeds Ltd, cultivar Ceres One50 [Agricom], and introgression of meadow fescue (*Festuca pratensis* L.) germplasm (e.g. cultivars Matrix and Revolution, marketed by Cropmark Ltd.).

Plant material for the present study was provided by Dr H. S. Easton of the New Zealand Crown Research Institute, AgResearch Ltd, and the research involved an evaluation of the agronomic traits contributing to drought resistance in a cultivar 'Medea' first released in 1967 (Barnard, 1972; Oram, 1990) in Australia and derived from germplasm of North African origin (Silsbury, 1961). The cultivar Medea is said to be characterized by high winter growth (Reed et al., 1980) and reduced summer yield, even when fully watered (Norris and Thomas, 1982). This behaviour is presumably associated with summer dormancy, a drought resistance strategy found in a number of perennial grass species of Mediterranean origin (Volaire and Norton, 2006; Volaire et al., 2009). Dr Easton was interested to know whether Medea possessed unique traits associated with drought resistance, that could be transferred into New Zealand breeding populations by introgression as a means to enhance drought resistance of the New Zealand material.

1.2 Objectives

Based on the above considerations the following objectives for the study were identified:

1. To develop an experimental protocol for identifying traits contributing to drought resistance in perennial ryegrass;

2. To assess and compare the drought resistance traits exhibited by Medea, Grasslands Samson, and a small selection of other New

Zealand cultivars or breeding lines representing different approaches to breeding for moisture deficit tolerance;

3. To assess and compare the expression of yield and drought resistance traits in plants of Medea, Grasslands Samson, and their F_1 and F_2 progeny as a preliminary assessment of the potential value of Medea for use in perennial ryegrass breeding programmes in New Zealand.

1.3 Thesis structure

The thesis comprises eight chapters. Following this introduction, Chapter 2 gives a review of literature on plant response to water deficit, with an emphasis on research relating to temperate grassland, and perennial ryegrass in particular. Experimental work is then presented in logical rather than chronological sequence as indicated below.

Chapter 3 provides a winter vegetative growth comparison between the cultivar Medea, derived from Mediterranean germplasm, and Grasslands Samson, a current New Zealand commercial perennial ryegrass cultivar (Experiment 1).

Chapter 4 reports two experiments (Experiments 2 and 5) comparing responses to water deficit of Medea and a range of commercial New Zealand cultivars including Grasslands Samson, but also an unreleased tetraploid line from the same germplasm as Grasslands Samson, and cultivars Tolosa and Ceres One50 (cultivars from NZ Agriseeds Ltd. and Agricom, respectively, and understood to have incorporated Spanish germplasm in the breeding process) and cultivar Matrix (developed by Cropmark using introgression of meadow fescue germplasm into perennial ryegrass breeding populations).

Chapter 5 reports an experiment (Experiment 3) comparing reaction to water deficit of Grasslands Samson, Medea, and their F_1 progeny. Experiment 3 evaluated response to water deficit and the presence of heterosis in 5 ‘family groups’. Each family group comprised one Grasslands Samson and one Medea genotype and 3 F_1 progeny of those parents. Hence, 15 F_1 progeny in total were evaluated.

Chapter 6 reports Experiment 4 which compared 30 F_2 hybrids from six family groups each having five F_2 progeny, but no parents, for their response to water deficit.

Chapter 7 reports an experiment (Experiment 6) in which plant morphological characters and drought adaptations of 3 generations of plant material (Grasslands Samson and Medea and their F_1 and F_2 hybrids) were evaluated under common growing conditions, but without consideration of any family group relationships that might have existed among the plant genotypes included in the experiment.

Chapter 8 presents an integrative discussion together with a summary and conclusions and recommendation for future work.

2

Literature review

2.1 Introduction

Plant water relations in the broader sense, is a topic of immense relevance to securing the future food supply of a rapidly increasing world population, which currently sits around 7 billion and is expected to exceed 8 billion within 15 years. In New Zealand, with its economic dependence on pastoral industries, and with perennial ryegrass typically being the primary botanical component of newly sown pastures (Section 1.1), there is a major pastoral industry research effort directed at perennial ryegrass improvement, with water deficit tolerance being a topic of increasing focus within plant improvement research. This review then, will first seek to define terminology relating to water deficit and water deficit tolerance, and then identify the plant responses that contribute to water deficit tolerance, especially in forage grasses. Finally, key historical developments in the breeding of perennial ryegrass in New Zealand and of the cultivar Medea in Australia will be summarised, and research questions identified relating to determination of potential for use of Medea germplasm in future New Zealand perennial ryegrass breeding to enhance water deficit tolerance. In the course of compiling this literature review, the writer generated an 'Endnote' data base containing almost 900 publications, many more than could be realistically cited here. Therefore, this review has been compiled selectively, identifying key papers defining concepts or describing methodology in water relations research, and providing a contextual basis for the planned research into the drought resistance traits of Medea perennial ryegrass and their potential value in improving drought resistance of New Zealand perennial ryegrass cultivars.

2.2 Definitions of "Drought" and related terms

It is notoriously difficult to define the term "drought". Various scientific disciplines like ecology, hydrology, meteorology and agriculture have their own definitions, though each one of them is open to criticism. Thus, there is no universally accepted definition of drought (Passioura, 1996). However, a widely accepted definition of agricultural' or 'agronomic' drought is "sub-optimal rainfall that limits plant

productivity" (Mishra and Singh, 2010), while a more comprehensive definition is that of Pereira et al. (2009): "a natural but temporary imbalance of water availability, consisting of persistent lower-than average precipitation, of uncertain frequency, duration and severity, whose occurrence is difficult to predict." The significance for these authors of limiting the definition of drought to natural events is indicated in Table 2.1.

Table 2.1: Terminology for distinguishing between different categories of moisture deficit

Water scarcity regime	Natural event	Induced by human activity
Permanent	**Aridity**	**Desertification**
Temporary	**Drought**	**Water shortage**

Reproduced from Pereira et al. (2009).

It is worth noting at this point that to define plant water deficit or drought as a situation where transpiration exceeds water uptake over some period of time is not an unambiguous basis for definition of drought, since there are a number of plant related factors that may lead to an excess of transpiration over water uptake, even in periods of high water availability (Kramer, 1980). Factors which can limit plant water uptake even when water is available include soil salinity, poor soil aeration, and root damage from physical disturbance or pathogens. Kramer (1980) advocates the use of the term "plant water stress" rather than "drought" where water supply is potentially sufficient, but plant water uptake is reduced by some abiotic factor. Moreover, Kramer (1980) also considers that diurnal reduction in plant water status around solar noon (see below) should not be termed drought.

Drought resistance has been defined as a plant's survival ability and production capacity under water deficit conditions (Luo, 2010), although some scientists, including Barker and Caradus (2001), have criticized this terminology based on the logic that plants are actually incapable of resisting drought because of high evaporative demand as compared to the normal level of water reserves. Therefore, Kramer (1980) has suggested replacing the term 'drought resistance' with 'drought

tolerance'. However, a vast majority of plant physiologists are still using the term drought resistance to avoid confusion arising when the term drought resistance is used to describe specific mechanisms exhibited by plants that aid endurance of drought. The writer uses "drought resistance", as discussed further below.

A common misunderstanding is that water use efficiency (WUE), defined as a ratio of dry matter yield produced per unit of water transpired (Araus et al., 2004) or ratio of CO_2 exchange rate to rate of water loss (El-Hafid et al., 1998), is analogous to drought resistance. It will become clear from discussion below that a drought resistant cultivar does not necessarily need to have high WUE and conversely a plant with high WUE will not necessarily be drought resistant (Luo, 2010). One reason why WUE is not necessarily a measure of drought resistance is that a major contribution to drought resistance can come from a greater ability to extract soil moisture through deep rooting as a means to meet transpiration demands (Blum, 2009).

2.3 Fundamentals of plant water relations

Plant water relations involve principles of physics, whereby water will spontaneously move from a 'high potential' state to a state of 'more negative' potential. Under normal plant growth conditions a water potential gradient exists with water vapour in the atmosphere at more negative potential than water in the plant cell which in turn has a potential more negative than soil. When stomata are open to admit CO_2 for photosynthesis, water therefore escapes down the potential gradient to the atmosphere at a rate determined by humidity and temperature and must be replenished by drawing water via the xylem from the soil. This flow of water from soil to atmosphere through the plant is known as transpiration and typically ranges from approximately 1 – 2 mm d^{-1} (rainfall equivalent) in winter in New Zealand up to 5 – 6 mm d^{-1} in summer, and requiring 300 – 1000 litres of water per kg DM produced, depending on plant species and growing conditions.

The energy status of the symplastic water in the plant leaf is termed the leaf water potential (Ψw), and is simplistically defined by the equation (adapted from Turner, 1986):

-

$$\Psi w = \Psi s + \Psi p \quad \text{or} \quad \Psi w = -\pi + P \qquad \text{Eq. 2.1}$$

where Ψs or π (a negative value, often measured in bars) is the osmotic potential drawing water into the plant cell, and Ψp or P is the turgor pressure exerted by the cell wall on the water within. Since water movement from soil to the leaf is not instantaneous and the rate of moisture loss from leaves increases during the day, there is diurnal variation in Ψw, typically with the least negative values around dawn and the most negative values soon after solar noon.

2.3.1 Methods of measuring soil and plant water status

The methods available for measuring soil and plant water status when conducting research on drought have been broadly categorised into those which rely on (i) amount and (ii) energy status of water (Jones, 2007).

2.3.1.1 Amount of water

The amount of water in soil is estimated through measuring soil moisture contents (SMC). Two critical thresholds for SMC are field capacity (the amount of moisture left in soil after gravitational drainage of water), and the permanent wilting point, the level of SMC which is no longer sufficient to support normal plant growth (Veihmeyer and Hendrickson, 1950).

The importance of available soil moisture determination for assessing the extent of plant water uptake, and thus the degree of limitation to plant growth, has led to the development of a number of methods for its measurement (Kramer and Boyer, 1995). Stafford (1988) has given a comprehensive review of methods of SMC determination. The most commonly used method is the gravimetric determination i.e., weighing the soil samples before and after oven drying at 105°C (Stafford, 1988). However, because the gravimetric method is labour intensive and disturbs the soil, it has often been replaced by *in situ* methods like the neutron probe, the time domain reflectometer (TDR), or gypsum blocks (Stafford, 1988), which measure SMC in volumetric terms. A few studies on water relations of forage grasses, including that of Karsten and MacAdam (2001), have used the gravimetric method whereas others like DaCosta and Huang (2006) and DaCosta et al. (2004), have used TDR for determination of soil water status.

The amount of water in plant tissues is estimated by measuring relative water content (RWC). RWC, formerly known as relative turgidity (Barrs, 1968), is a simple way to estimate plant water status. It does not require any sophisticated equipment but is still a powerful technique for quantifying plant water status. For determination of RWC, the technique of Barrs and Weatherley (1962) is mostly employed. This requires measurement of fresh weight of plant tissue soon after excision (FW), its turgid weight (TFW) after floating the leaves for a number of hours on de-ionized water, and dry weight (DW) of the same tissue. These values then give RWC as a percentage using the formula

$$\mathrm{RWC} = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TFW} - \mathrm{DW})} \times 100 \qquad \text{Eq. 2.2}$$

However, disadvantages of this technique are the lapse of considerable time between sampling and obtaining results, and its reliance on the three time consuming and monotonous weighing operations (Smart and Bingham, 1974). Results obtained may also vary somewhat depending on the time for which the plant tissue for a given plant species is soaked, and the temperature difference between the field and during floating (Shepherd, 1977). Despite all these limitations the technique of Barrs and Weatherley (1962) has been employed as a standard technique for estimation of RWC in almost all studies of plant water relations.

2.3.1.2 Energy status of water

Energy status of plant water is mostly expressed as total water potential (Richter, 1997), generally measured on foliar tissues, and therefore termed leaf water potential (LWP). Two instruments in use for measurement of LWP are Scholander pressure chamber and the hygrometer (Kirkham, 1985). The former is more commonly used because of its speed, ease of operation, versatility (Turner, 1988) and lack of need for any temperature control. This piece of equipment was actually developed by (Dixon, 1914) and later modified by Scholander and his group (Scholander et al., 1965; Scholander et al., 1964) into its present form (Kirkham, 1985). The pressure chamber is based on the principle of forcing xylem cell sap out of a cut surface of a leaf or petiole by means of gas pressure applied to the leaf inside the chamber, and measuring the amount of gas pressure required for sap to appear at the cut surface (Scholander et al., 1965; Scholander et al., 1964) .

The hygrometer is basically an instrument for measurement of relative humidity and is also employed for plant water relations measurements. It measures the vapour pressure in a small chamber that is in equilibrium with the vapour pressure of a tissue sample and is used either in psychrometer (wet bulb/dry bulb) or dew-point mode (Kirkham, 1985).

For estimation of total water potential the pressure chamber is the instrument of first choice; a 1997 survey of methodology for studying plant water relations reported that more than 90% of recent studies had used it (Richter, 1997).

Osmotic pressure of leaf tissues can be determined using a psychrometer after freezing the tissue in liquid nitrogen followed by thawing at room temperature (Nilsen and Orcutt, 1996). Turgor pressure or pressure potential, the physical pressure exerted by cell cytoplasm against the cell membrane to maintain structural integrity of the cell, can be calculated by subtracting leaf osmotic potential from LWP or by pressure probe technique (Nilsen and Orcutt, 1996). Among the three components of LWP, turgor potential is most sensitive to overall change in plant water status (Nilsen and Orcutt, 1996).

There are also some indirect measurements of energy status of plant water. These include visible observation of physical changes like wilting (stem, leaf or fruit shrinkage, or altered leaf colour), measurement of rate of cell expansion and growth, and measurement of leaf temperature (often by infrared thermometry) (Begg and Turner, 1976). However, Jones (2007) pointed out that because of their weak linkage with underlying measures of plant water status, these indirect measurements should be used with circumspection. Even so, such methods may be useful in crop improvement research to identify individual plants possessing particular drought resistance traits.

2.3.2 Plant responses to drought

In drought, the soil water potential becomes more negative as soil dries. This more negative soil water potential will, in turn, tend to lead to a lowering of Ψw (and by Eq. 2.1, a reduction in turgor, unless the plant can compensate in some way). Plants

may undergo one or more of a number of modifications in their structures and processes as a consequence of such drought exposure. In the leaf, common responses are reduction of stomatal aperture, or increased osmotic potential, Ψs (Pugnaire et al., 1999). Increased leaf temperature may result (Farooq et al., 2009). Various physiological changes also occur, including activation of specific enzymes, and hormonal changes, often accompanied by growth inhibition (Fitter and Hay, 2002). Morphologically, increased root shoot ratio (Farooq et al., 2009), or changes in root morphology and distribution (Fitter and Hay, 2002), may increase soil water extraction, while leaf rolling and wilting, among others, may reduce leaf water loss. The term 'drought resistance' therefore reflects the combined effects of a number of traits which are usually reported to be complex. Blum (2011), however, is of the opinion that "complexity" of drought related traits is a relative term for various scientific disciplines. For an agronomist, plant breeder or crop physiologist plant responses like deep rooting or osmotic adjustment are easily comprehensible "reactions" of plants under drought. However, from a genomic perspective, where each plant response is controlled by a hundred thousand genes and gene expression is a function of number of up- or down-regulated genes, drought resistance is reasonably labelled a complex trait.

Researchers have generally grouped the range of physical, physiological and biochemical responses according to the mechanisms or strategies plants adopt to endure drought. For example, Levitt (1972) classified plant responses to drought as *escape*, *avoidance* and *tolerance*, while the same author (Levitt, 1980), later recognized just two categories of response: drought avoidance (where plants maintain a comparatively high degree of hydration or less negative water potential) and drought resistance (involving more negative plant water potential). This raises a question of how the difference between drought escape and drought avoidance is defined. Many authors, including Clarke and Durley (1981) used the term drought escape to refer to plants which adjust the timing of their reproductive growth so as to complete their reproductive cycle within seasonal periods of water availability. Since drought escape refers to the situation where plants complete their life cycle before serious seasonal moisture stress, this category is more relevant to annuals than perennials (Johnson and Asay, 1993; Volaire et al., 1998b). By contrast, the "true" drought avoiders (Clarke and Durley, 1981) attain drought resistance either by

conserving water through stomatal control or by increasing water supply to above-ground parts through root proliferation. These ideas on classification of plant drought resistance strategies are well encapsulated by Turner (Turner, 1986) (Table 2.2), who used the term *dehydration postponement* and *dehydration tolerance* rather than drought avoidance and drought tolerance, and whose terminology is adopted here.

Under Turner's (1986) framework, key mechanisms of drought resistance in perennial forage grasses are dehydration postponement and dehydration tolerance, and these will be discussed in greater detail below. It is important to note, however, that the various plant mechanisms that confer drought resistance are not mutually exclusive, since one mechanism can work synergistically with another (Nilsen and Orcutt, 1996) or one or more traits may combine additively (Kramer, 1980). For example, some cultivars of sorghum are early maturing, conferring a measure of drought escape, whereas sorghum as a plant species also has a comparatively extensive root systems (Kramer, 1980), conferring a measure of dehydration postponement by increased water capture.

Table 2.2 Turner's (1986) classification of drought resistance mechanisms.

Mechanisms of adaptation to water deficits and their influence on productive processes

Mechanism	Productive process reduced?
Drought escape	
Rapid phenological development	No
Developmental plasticity	No
Dehydration postponement	
Maintenance of turgor	
Maintenance of water uptake	
Increased root density and depth	No
Increased liquid-phase conductance	No
Reduction of water loss	
Reduction of leaf area	Yes
Increase in stomatal & cuticular resistance	Yes
Reduction in radiation absorbed	Yes
Osmotic adjustment	No
Maintenance of volume	
Increase in elasticity	No
Dehydration tolerance	
Protoplasmic tolerance	Yes

With respect to dehydration tolerance, most crop plants die once dehydration has reached a critical level at which membrane function becomes disordered (Saxena, 2003), although certain plants, sometimes called "resurrection plants" can reconstitute their membranes and can become functional within hours of re-watering (Gaff, 1980). However, Volaire et al. (2009) pointed out that unlike perennial grasses of temperate origin (North America and Europe) perennial grasses of Mediterranean origin can endure periods of long and intense summer drought by becoming dormant during summer and resuming active growth when summer drought has ended. This kind of survival strategy is very similar to that of resurrection plants and has gained attention in the last two decades (Volaire et al., 2009). Therefore, Volaire et al. (2009) have introduced summer dormancy as a special case of dehydration tolerance in perennial forage grasses of Mediterranean origin.

Norton et al. (2009) defined the term summer dormancy as "an endogenously controlled and coupled series of processes comprising the cessation or reduction of leaf growth, the complete or partial senescence of herbage, and in some cases the endogenous dehydration of meristems". When subjected to severe water deficit, temperate perennial grasses also undergo a series of adaptations such as decrease in leaf elongation rate followed by cessation of elongation and senescence, and eventually leading to a stage when only meristems survive (Volaire et al., 1998a). However, these adaptations in temperate grasses are distinguished from summer dormancy (Volaire and Norton, 2006) on the basis that true summer dormancy is expressed only under conditions typical of Mediterranean summers and dormant plants will not respond to summer rainfall but resume growth in a programmed fashion in autumn, whereas the similar responses in temperate plants may occur in any season when water is withheld and the quiescent plants will respond immediately to rainfall (Norton et al., 2009).

So to summarise, drought resistance in grasses may be viewed as a combination of varying degrees of expression of dehydration postponement, dehydration tolerance and summer dormancy, sometimes with a measure of drought escape through early flowering, and is genetically controlled. For example, cocksfoot (*Dactylis glomerata* L.) has been found to cope with drought through efficient dehydration tolerance whereas tall fescue (*Festuca arundinacea* Schreb.) does so through a more developed

dehydration postponement involving traits like deep rooting (Norton et al., 2008). Likewise, Mediterranean and temperate cultivars of tall fescue have been found to cope with drought differently, as the Mediterranean cultivar, Maris Kasba, exhibited primarily morphological adaptations like increased root shoot ratio, and diminished evaporative surface area as a result of reduced leaf elongation rate whereas the temperate cultivar, El Palenque, exhibited primarily physiological adaptations like lower stomatal conductance and higher osmotic adjustment (Assuero et al., 2002).

Breeders of perennial forage grasses have in some cases utilised germplasm of Mediterranean origin in breeding improved cultivars for temperate regions of the world. Interest in the Mediterranean material is based on qualities like (i) high drought resistance and persistence, (ii) rapid autumn re-growth, and (iii) higher growth rates in autumn and winter (Lelièvre and Volaire, 2009). Mediterranean countries (or countries having some degree of Mediterranean climate) like France, Italy, and Portugal are among those to have developed cultivars of perennial forage grasses using germplasm of Mediterranean origin (Lelièvre and Volaire, 2009). Mediterranean germplasm has also been collected and utilised in several temperate areas of Europe and in Australia (Lelièvre and Volaire, 2009). Australian use of Mediterranean perennial ryegrass germplasm is discussed further in Section 2.8 below.

2.4 Progress towards drought resistance in forage grasses

Identification and incorporation of mechanisms of drought resistance are a pre-requisite for a successful drought resistance breeding programme. However, there is a comparatively small amount of literature on variation in drought resistance mechanisms of forage grasses, most of which addresses variation in drought resistance from a species perspective (e.g. comparison of tall fescue and perennial ryegrass) or sometimes within a species (e.g. due to tetraploidy) (Sugiyama and Nikara, 2004).

2.4.1 Dehydration postponement

As discussed above, dehydration postponement is mainly achieved through improved water uptake and control of transpirational loss, so this sub-section will highlight the

importance of these two factors and variations found in each of them, with a particular focus on forage grasses.

2.4.1.1 Improved water uptake

The amount of water available for plant growth depends on soil water uptake by roots (Johnson and Asay, 1993). Root growth is mostly reduced by drought (Wang and Yamauchi, 2006). However, it is usually less inhibited than that of shoot growth and may even be promoted in some cases (Sharp et al., 2004), which in perennial grasses results in a higher root to shoot ratio through deeper-rooting. In this way, a greater proportion of moisture from lower soil layers is extracted (Clarke and Durley, 1981). Deep rooting is a common feature of species found in drought prone areas (Nilsen and Orcutt, 1996). Deep rooted plants not only promote water uptake from lower soil layers to aid in meeting evaporative demand, but have also, at times, been found to release some water into the drier upper soil layers, a phenomenon known as hydraulic lift (Horton and Hart, 1998). However, because of technical difficulties, root characters have been given less attention in selection programmes (Crush et al., 2007).

Bonos et al. (2004) compared a turf-type diploid cultivar of perennial ryegrass APR120 with a forage-type tetraploid cultivar, Bastion, under glasshouse conditions using PEG in root trainers of 63.5 cm length made up of PVC material. They observed that Bastion initially produced a very shallow root system, but after two cycles of selection the progeny showed a 367% gain in total root production, and a gain of 130% in root production in the lower 30 cm of the root trainers.

Wedderburn et al. (2010) conducted a trial on a diverse range of cultivars and ecotypes of perennial ryegrass of New Zealand origin in a 60 cm deep bin and concluded that, though roots grew to a maximum depth of 42.5 cm under drought, the increase in root count in drought conditions was most pronounced in shallower depths (0 – 15 cm).

Crush et al. (2009) conducted a trial on 26 wild accessions (from Portugal, Spain, Morocco, Tunisia, Italy, Afghanistan, Iran, Azerbaijan and Uzbekistan) and a pool of bred material (including cultivars Matrix, and Bronsyn and 40 breeding lines) in 100

cm deep and 9 cm diameter tubes. One of their conclusions was that wild types had higher root shoot ratios than the bred material. Significant variation was found among wild type accessions for shoot dry weight distribution in the upper 0 – 10 cm of the soil profile (Crush et al., 2009). However, none of the above studies measured soil moisture or soil water uptake differences arising from the deeper root growth.

2.4.1.2 Control of transpiration loss

2.4.1.2.1 Reduction in leaf area

Forage grass leaf formation is a continuous process, persisting over a number of days of cell division and enlargement of the newly formed cells (Skinner and Nelson, 1995). Hsiao (1973) has ranked plant processes with respect to sensitivity to drought and notes that cell enlargement is the plant process most vulnerable to water deficit.

In grasses leaf extension rate (LER) is a reflection of rate of cell enlargement (Volaire and Lelievre, 2001). In the Netherlands van Loo (1992) conducted an experiment on two perennial ryegrass cultivars, Wendy (diploid) and Condesa (tetraploid), using a hydroponic system in a glasshouse and using PEG to obtain solutions of low (-1.3 MPa) and normal (0 MPa) water potential. While both cultivars had the same rates of leaf extension, the low water potential of -1.3 MPa reduced LER by 36%, TN by 20% and shoot DW production by 64%. This raises the question of whether reduced growth in moisture deficit is a direct consequence of reduced water availability, or a plant mediated response that has the effect of reducing demand when reduced supply is sensed.

LER in forage grasses of contrasting climatic niches was found by Cooper (1964) to be highly sensitive to low temperature and water stress. Perennial ryegrass populations of Mediterranean origin were found to show more rapid leaf extension in winter than those of temperate origin (Cooper, 1964). Robson (1967) also found the same trend of higher leaf growth in winter in North African than in British cultivars of tall fescue. However, a reverse pattern appears to apply in summer. For potted plants of cocksfoot in a glasshouse at Montpellier, France, Volaire (2002) compared the summer dormant cultivar Kasbah (Australian bred from germplasm of Moroccan origin), the drought resistant summer active cultivar Medley, and the drought sensitive summer active cultivar Lutetia and found that cessation of leaf elongation

occurred at soil moisture contents of 5.6%, 3.3% and 4.0%, respectively, indicating that the Mediterranean cultivar was the least summer active. In plants in the field, summer dormancy and senescence of aerial tissues of Kasbah was observed even in irrigated treatments (Volaire, 2002).

2.4.1.2.2 Control of stomata, photosynthesis and transpiration

Stomata are the gateways of gaseous exchange between plant and atmosphere. They not only allow incoming CO_2 for photosynthesis but also allow removal of moisture produced as a result of photosynthesis, and hence evaporative cooling of leaves in normal conditions. However, stomata close when LWP drops too much to sustain normal rates of photosynthesis and transpiration. With stomatal closure, transpirational cooling halts and thus plant leaves experience a higher temperature than the atmosphere. Jackson et al. (1981) used energy balance equations to derive an index of crop water status based on the difference between canopy temperature and ambient temperature (Tc-Ta), as measured by infrared thermometry. In the raw data of these authors, leaf temperatures observed ranged from approximately 10°C below ambient to 5°C above ambient (their Fig. 2); while the calculated index moved from approximately 0.2 to 0.9, closely reflecting soil water extraction by the crop, A limitation to the use of this index is that its calculation requires an estimate of the ratio r_c/r_a, where r_c and r_a are, respectively, canopy and air resistances to water vapour movement. A less complex index based on the difference between leaf temperature and wet and dry reference surfaces in the same environment has since been proposed by Jones (1999). Following these developments, there has been interest from researchers in using leaf temperature variation between plants in a breeding population as an indicator of water use efficiency. Discussion is ongoing as to the relationship between leaf temperature and water use efficiency, and whether the superior plant would have warmer or colder leaves than the average for the population. Araus et al. (2002) pointed out that evaporative cooling of leaves is an indicator of stomatal conductance which in turn is correlated with rate of photosynthetic metabolism and vascular transport. Blum (2009) in the context of cereal crop yield, argues that breeding for decreased stomatal conductance (i.e. warmer leaves) might result in lower yields through reduced extraction of available soil moisture during crop growth, and argues that a plant with cooler leaves is demonstrating superior extraction of soil moisture. Blum (2009) therefore proposes it

is more important to focus on effective use of water, than on water use efficiency. However, from a theoretical perspective, high canopy temperature depression does not define one particular plant growth strategy: a greater canopy temperature decrease would be expected both where a plant is growing faster with a low water use efficiency (in conditions of sufficient water supply), or because that plant is comparatively better at extracting water from the soil profile under conditions of mild to moderate water deficit. Meanwhile, another growth strategy of potential interest is the plant which can maintain leaf elongation with comparatively lower water use. Such a plant should give farmers more DW production in drought than a plant which depleted soil water faster for the same DW production, but the plant depleting soil water more slowly would be expected to have less transpiration and *warmer* leaves. This latter strategy equates to a shift in the crop coefficient of water use "*m*" in Eq. 4 of Blum (2009).

2.4.1.2.3 Leaf physical responses

Two types of physical response that plant leaves commonly exhibit in drought are leaf rolling (Jordan, 1983; O'Toole and Cruz, 1980) and leaf wilting. Leaf rolling not only reduces leaf area but also leads to marked reduction in canopy temperature (Kadioglu and Terzi, 2007). Hardy et al. (1995) surveyed stomatal distribution and function of 20 C3 and C4 meadow and rangeland grass species and found that most of C3 grasses tend to roll their leaves adaxially and have an adaxial:abaxial stomatal density greater than 1.0. In the case of cocksfoot, Hardy et al. (1995) found this ratio to be 4.25. Hence, when leaves roll in response to water deficit, a majority of the stomata are enclosed and stomatal conductance is greatly reduced. Therefore, Kadioglu and Terzi (2007) maintained that leaf rolling is a means of dehydration postponement. Genetic differences have been observed for leaf rolling (Blum, 1989). However, these kinds of morphological changes are not often recorded for forage species, or where they are recorded, visual ranking the extent of leaf rolling is commonly used, much like that in rice (O'Toole et al., 1979).

Leaf wilting is noted by Jordan (1983) to result in reduction of irradiation load on leaves through change in leaf angle. However, this would apply to dicot plants where turgid leaves tend to be orientated horizontally and wilted leaves droop. By contrast, in grasses turgid leaves tend to be orientated more vertically and wilting could

actually result in increased irradiation load. So wilting can not be considered to be a drought resistance mechanism in grasses.

2.4.2 Dehydration tolerance

Drought tolerance refers to the ability of plants to withstand drought at tissue dehydration level and is a means of sustaining metabolism and thus growth at extremely low water potential (Turner et al., 2001).

2.4.2.1 Osmotic adjustment

Munns (1988) defined osmotic adjustment (OA) as "an increase in osmotic pressure of cell sap resulting from more solute molecules per cell rather than from a lower cell volume". OA is also termed osmoregulation (Morgan, 1984) or osmoprotection because the accumulated solutes function to protect against dehydration and increase cell turgidity and thus protect the cell. Munns (1988), however, criticized the term osmoregulation for the fact that osmoregulation as a phenomenon specifically relates to some freshwater walled algae which maintain their internal osmotic pressure at a constant level against variations in the external osmotic pressure. Regardless of the term used, OA as a physiological process has become one of the most important measurements in almost all abiotic stress studies, including those on drought resistance (Farooq et al., 2009). However, it is believed that there is not strong evidence for a consistent increase in crop yield in response to OA (Blum, 1996; Gosal et al., 2009).

Because of the importance of OA as a physiological process scientists have evolved a number of methodologies for its measurement in plant water relations studies. They include (a) psychrometeric determination of OP followed by subtracting that value from LWP to get an estimate of PP and (b) an array of methods meant to mathematically estimate PP from OP and RWC (Babu et al., 1999) . However, method (a) is more widely used.

Among the various solutes involved in OA sugars, inorganic ions, amino acids (like proline) and minerals have been widely studied. Thomas (1991) made a thorough study on the nature of solutes involved in OA in perennial ryegrass and found that mineral ions (Ca^{+2} and Mg^{+2}) were the major contributors to OP. Proline contents in

leaf laminae were generally very low as compared to those in the base and laminae proline contents rose only at a higher drought stress which supports the assumption that proline accumulation in laminae is a drought injury response (Thomas, 1991). Perennial ryegrass has been shown to accumulate six to nine times (Volaire et al., 1998a) or nine to twelve times (Volaire et al., 1998b) the amount of proline under drought as compared to that when irrigated. It has been proposed that the role of proline is not so much in contributing to OA in grasses, but that it has a role in stabilizing cell membranes and proteins (Ashraf and Foolad, 2007). Therefore, determination of proline contents in perennial ryegrass might be a useful indicator of drought protection.

2.4.2.2 Cell membrane stability

Like most other abiotic stresses such as high temperature, chilling or freezing (McDaniel, 1982) drought stress also disrupts the normal structure and function of cell membranes, and this damage can result in leakage of electrolytes by the cells.

Volaire (2002) found no difference in membrane stability between dormant and non-dormant cultivars of cocksfoot and showed that though membrane stability of a summer dormant cultivar Kasbah remained higher than that of drought resistant and drought sensitive cultivars Medley and Lutetia, drought resistance of plants appeared to be more closely linked to presence of dehydrin proteins.

2.4.3 Drought escape

Unlike annual plants, perennial forage grasses cannot escape drought by early flowering (Volaire et al., 1998b). Among perennial forage grasses early flowering has been found to be associated with greater drought survival in cocksfoot but less so in perennial ryegrass (Volaire et al., 1998b). However, for one Australian ecotype of perennial ryegrass ‘Kangaroo Valley’, an early flowering habit appears to be an adaptation for growth in summer dry areas, and the ecotype is used in the south east of New South Wales (Aitken, 1966).

Adding to the above mentioned physiological determinants of drought resistance of forage grasses is the relatively recent discovery that perennial ryegrass is often naturally infected with a fungal endophyte, *Neotyphodium lolii* which protects the

grass host from attack by Argentine stem weevil [*Listronotus bonariensis* (Kuschel)] and other invertebrates (Easton et al., 2001b).

Because of the fact that endophyte is known to enhance drought resistance of tall fescue (Assuero et al., 2006), it has often been assumed that a similar enhancement of drought tolerance would occur in endophyte infection of perennial ryegrass. However, experimental evidence is scarce and to some extent contradictory. Some scientists report that *N. lolii* affects water relations of perennial ryegrass positively (Amalric et al., 1999; Ravel et al., 1997) whereas others report no effect (Barker et al., 1997) or even detrimental effects (Cheplick, 2004). More recently Kane (2011) reported *N. lolii* to be responsible for an improved resistance to drought stress in perennial ryegrass through an increased tiller count and root mass.

Since drought resistance of perennial ryegrass is at least potentially affected by infection with *N. lolii* fungal endophyte, this needs to be taken into account when interpreting data from experiments on drought resistance of perennial ryegrass.

2.4.4 Measurements to quantify drought resistance

It follows from the discussion above on potential plant adaptations to drought (Section 2.3.2; Table 2.2) and on findings from studies on drought response (Section 2.4), that in order for a researcher to fully define the drought resistance strategy of plants under study, measurements need to be made across a number of 'domains' of plant growth and development: root growth and function, shoot growth, plant water status, and stomatal and cellular control of gas exchange. A primary aim in a plant breeding programme is improved forage yield. Recognising the domains of plant growth and development that may contribute to drought resistance, and that yield is also an important outcome in plant breeding, a list of desirable measurements for the present study can be drawn up as in Table 2.3.

Table 2.3: A list of four domains of plant growth and development, which contribute to differing mechanisms of drought resistance recognised by Turner (1986) and proposed trait measurements to define the drought resistance strategy of test plants.

Domain	Traits to be measured
Shoot growth	Shoot DW per plant; dead leaf, leaf lamina, and leaf pseudostem components of shoot DW; Tiller number per plant; Leaf number per plant.
Root development and water uptake	Root mass at three soil depths; Root:shoot ratio; Soil moisture content at three soil depths.
Plant water status	Leaf water potential, osmotic potential and pressure potential; Level of osmolytes such as proline; Relative water content.
Stomatal and cellular control	Net photosynthesis; Stomatal conductance; Leaf temperature difference from ambient; Leaf rolling; Electrolyte leakage

2.5 New Zealand's climate in relation to the adaptive range of perennial ryegrass

Successful growth of any forage plant, including perennial ryegrass depends on three major edaphic factors: rainfall, temperature and soil fertility (Chapman and Macfarlane, 1985). In the case of hill country farming, edaphic variability due to slope, aspect and microtopography are additional factors affecting the performance of particular plant species (Chapman and Macfarlane, 1985). Hence, consideration of the extent to which warmer or drier areas of New Zealand may fall outside the adaptive range of perennial ryegrass will assist in the development of objectives for plant improvement research.

Regional climate variation in New Zealand is controlled by two key features. First the central range of mountains in both main islands produces an orographic effect with a higher rainfall to the West, particularly in the South Island (2,875 mm mean annual rainfall at Greymouth), and lower rainfall in the East, particularly from Napier to Christchurch (666 mm mean annual rainfall at Lincoln). Second, there is a gradient of decreasing temperature from North (18.9°C/10.7°C January/July mean at Kaikohe) to South (13.7°C/5.1°C January/July mean at Invercargill) (Figure 2.1 below).

In Fig. 2.1a drier areas in the east and in Fig. 2.1b warmer areas in the north are highlighted by shades of orange or red. However, agricultural drought is not predicted by any single climatic factor but arises from an extended deficit between rainfall and plant water use, to the point that soil moisture is depleted. Despite some detailed historic investigation of regional variation in water balance (e.g. New Zealand Meteorological Service miscellaneous publications 150, 163, 177 and 185), literature which quantifies summer moisture deficit stress for pasture species like perennial ryegrass is scarce. To overcome this gap in the literature, rainfall, temperature and evaporation data were assembled from climate records (Anonymous, 1980) for selected sites, modelled potential evapotranspiration values were obtained from a published pasture growth model, and a simple cumulative water deficit over the summer months of November to March was calculated to

indicate the extent to which seasonal moisture deficit could potentially occur across the above climatic gradients in an 'average' year (Table 2.4).

Table 2.4: Long-term annual rainfall and January/July temperature data for selected New Zealand sites ranging from high to low rainfall and warm to cool temperature and modelled soil moisture deficit or surplus for months November to March inclusive. Sites are ranked in order of severity of summer moisture deficit. Data were extracted from Anonymous (1980).

Region	Years	An. Rain (mm)	Average Temp. (°C)		SMD (Rain-ET) Nov-March
			January	July	
Greymouth	1947-1980	2451	16.1	8.0	401(S)
Stratford	1960-1980	2053	15.4	7.1	131(S)
Gore	1971-1980	918	14.0	4.4	115
Invercargill	1948-1980	1037	13.7	5.4	132
Kaikohe	1956-1978	1573	18.9	11.7	160
Palmerston North	1928-1980	1000	17.5	8.9	231
Lincoln	1881-1980	666	16.5	6.4	287
Havelock North	1950-1980	798	18.0	8.1	332

Rain = Rainfall, ET = Evapotranspiration. ET values were obtained by entering monthly mean rainfall and temperature data into the 'Grow' pasture growth rate model developed by B. Butler and briefly described by Butler et al. (1990).

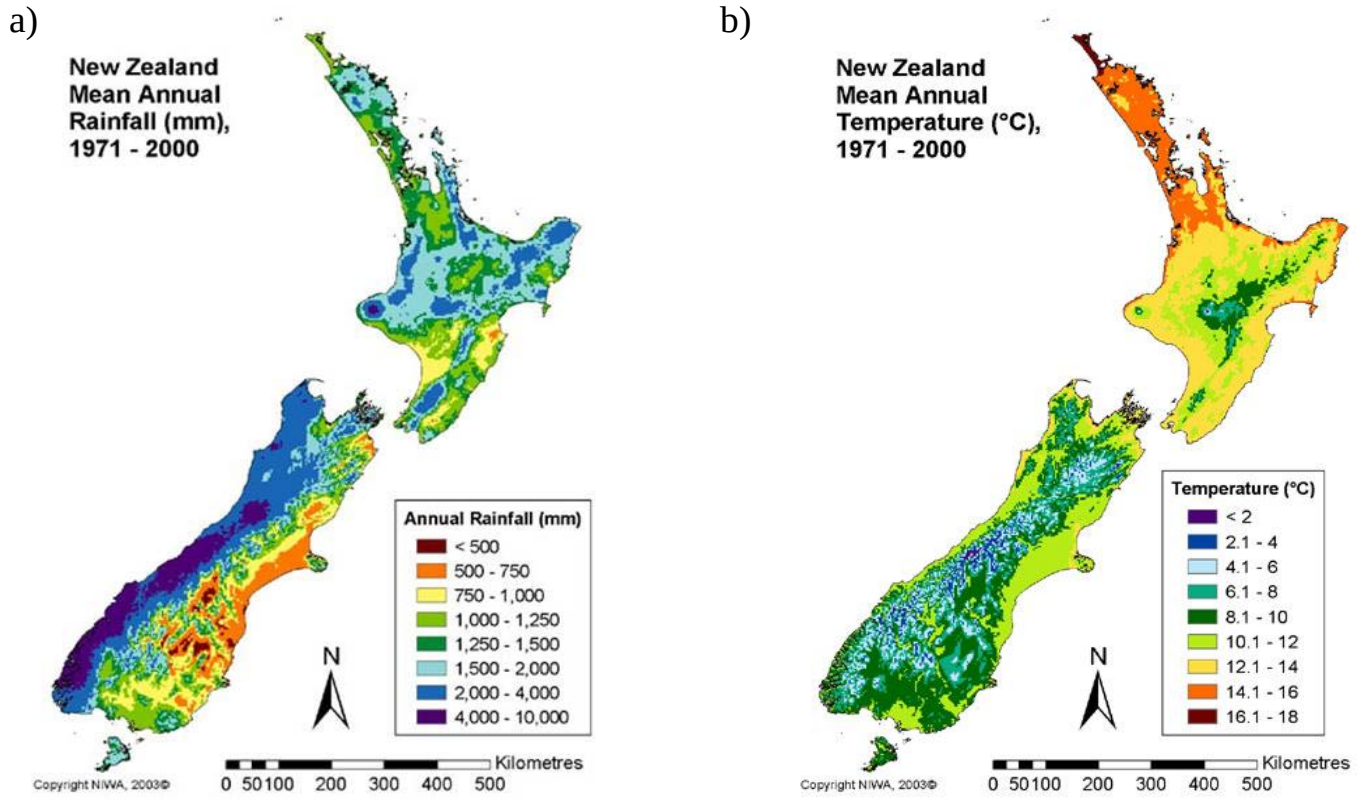

Figure 2.1: Regional variation in (a) mean annual rainfall (mm) and (b) temperature (^{o}C) of the North and South islands of New Zealand. Source: http://www.niwa.co.nz/education-and-training/schools/resources/climate/overview

Perennial ryegrass is regarded as being sensitive to warmer temperature and to drought (Moore, 2003), but there is a lack of clarity in the literature as to the climatic limits for perennial ryegrass persistence. In a sports turf context, Thorogood (2003) stated that perennial ryegrass requires at least 475 to 635 mm rainfall per annum and temperatures cooler than 20-25°C in summer, but that a day time temperature of 31°C and night time temperature of 25°C, irrespective of moisture availability, reduces its growth. Against that, (Mitchell and Lucanus, 1962) reported in their Fig. 1 that in growth chamber experiments the optimum temperature for ryegrass herbage accumulation (15% increase in plant size per day) was around 70°F (21°C) and that at 85°F (29°C) ryegrass herbage accumulation was reduced to around 10% increase in plant size per day. The optimum temperature for tillering tended to be lower than the optimum temperature for herbage accumulation, especially when photoperiod was reduced. Taking these data collectively, it is not hard to imagine that sites like Kaikohe or Havelock North with average January temperatures near the physiological optimum for ryegrass growth (Table 2.4) might have extended periods in summer when ryegrass growth is suppressed by supra-optimal temperature.

Evidence from field studies also suggests perennial ryegrass is intolerant of warmth and moisture deficit. For example in a field study in Victoria, Australia, Hill (1985) found that under 675 mm annual rainfall and a January temperature of 30.5°C persistence of perennial ryegrass was markedly less than phalaris and cocksfoot and perennial ryegrass was no longer present 3 - 5 years after sowing. In addition, there is a growing body of evidence of poor persistence of perennial ryegrass especially in Waikato region (Lane, 2011) or of invasion of perennial ryegrass based pastures by C4 grasses like kikuyu (*Pennisetum clandestinum* Hochst. ex Chiov.), and paspalum (*Paspalum dilatatum* Poir.) generally in northern areas of New Zealand (Campbell et al., 1996).

While soil moisture deficits in different districts of New Zealand are largely determined by climate, soil differences which influence soil moisture holding capacity are also important (Gradwell, 1968). Woodward et al. (2001) with reference to Gradwell (1968), Gradwell (1971) and Gradwell (1974) reported that water holding capacities of a vast majority of soils of New Zealand are in the range of 70 to 176 mm to 76 cm depth. In general agreement with these published available soil

water values, Salinger (2003) considered SMD values of 100 mm to be “significant” while McAneney et al. (1982) held SMD values of 150 mm to be “severe”. It follows from the analysis in Table 2.4 above, based on cumulative water deficit for an ‘average’ summer that significant or severe water deficit stress is likely to be experienced by perennial ryegrass pastures at Kaikohe, Palmerston North, Lincoln, and Havelock North. This point was explored and confirmed by Matthew et al. (2012), by modelling actual weather data from several sites for a ten year period.

In addition to this regional variation in rainfall and potential evapotranspiration there is also strong inter-annual variation in rainfall in both islands of the country (Ummenhofer and England, 2007). These authors indicate values for inter-annual rainfall variation of +/- 400 mm when mountainous areas are included. A compilation of 4 years rainfall data for Palmerston North (July 2000 to June 2004; Fig. 2.2) was made as an indication of inter-annual variation at a lowland site. November-to-March cumulative soil moisture deficit values based on modelled potential evapotranspiration were, respectively, 332, 69, 395, and 24 mm, for the 4 years 2000/01 to 2003/04, indicating inter-annual variation of at least +/- 150 mm. It is clear, even from this short run of 4 years data at one site, that inter-annual rainfall fluctuations (Fig. 2.2) are generally at least as large as regional variation, so that even in regions where long term average data suggest the climate should be suitable for perennial ryegrass, there are likely to be seasons when perennial ryegrass is seriously challenged by moisture deficit. Hence, frequency and severity of drought events will also be an issue for persistence of grass cultivars.

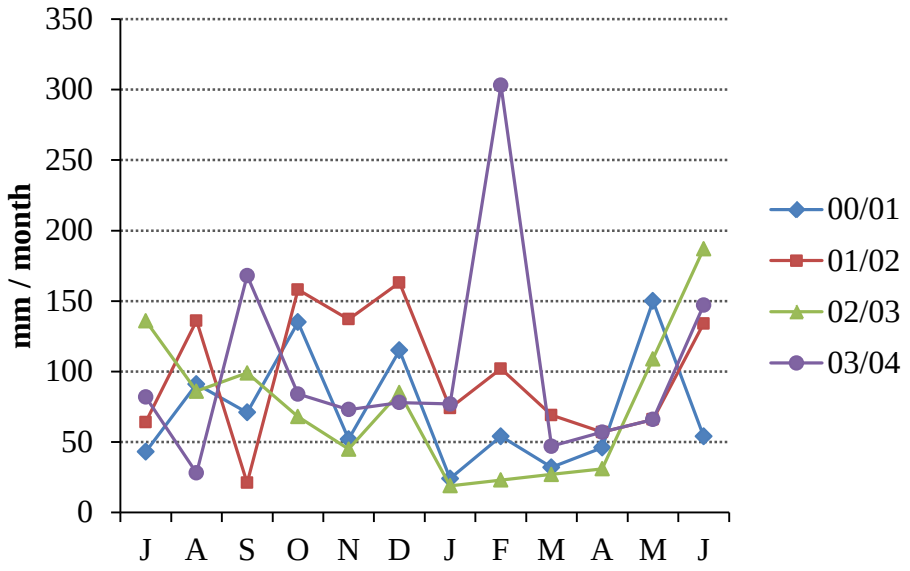

Figure 2.2: Inter-annual variation in monthly rainfall recorded at AgResearch Palmerston North from July 2000 to June 2004.

This issue has recently gained industry attention after many parts of New Zealand experienced a prolonged period of below average rainfall during the years 2007 - 2009. In affected areas, a prolonged severe moisture deficit occurred in the first three months of 2008. Affected areas received less than 10 mm rainfall (i.e. about 10% of normal) in January, dry conditions (<50% normal rainfall) continued until March, and the Waikato region experienced its driest January for 100 years (Renwick et al., 2010).

In 2009, seasonal and regional rainfall variability was not as strong as that in 2008; still it was the driest year for Taupo in its rainfall recording history (Renwick et al., 2010). A number of agriculturally important areas of New Zealand experienced an unprecedented high number of days of water deficit in 2008 - 2009 (Renwick et al., 2010); Table 2.5). This drought cycle of 2007/08 and 2009/2010 has caused a huge loss to New Zealand's agricultural economy. The dairy sector, being the hub of country's agricultural economy, has suffered the most and 44% of this loss was concentrated in the Waikato region (Anonymous, 2009).

Table 2.5: Extreme soil moisture deficit statistics for 2008 and 2009 in agriculturally important areas of New Zealand. In each case the reported deficit was ranked first or first equal for severity in the region in the period 1972 – 2009.

Location of sample site	Dry period	Total days of deficit over 3 months	Modelled deficit level (mm)
Waikato	Jan to Mar 2008	69	130
South Canterbury	Apr to Jun 2008	91	75
Central Hawke's Bay	Nov 2008 to Jan 2009	84	110
Dunedin	Nov 2008 to Jan 2009	82	75
Central Otago	Oct to Dec 2009	59	110

Reproduced from Renwick et al. (2010).

2.6 History of perennial ryegrass plant breeding work in New Zealand

Introduction of perennial ryegrass germplasm to New Zealand from the UK and elsewhere, followed by plant breeding work and the eventual evolution of modern cultivars from the introduced germplasm is a "story" spanning almost 200 years. Three historical perspectives will be highlighted here: (a) a chronology of the more important developments and milestones achieved and the scientists, organisations and farmers involved, (b) the source of germplasm from which important New Zealand cultivars have originated, and (c) the breeding methods and objectives employed. Some of the more important sources of information about perennial ryegrass breeding in New Zealand are Corkill et al. (1980), Easton (1983), Rumball (1983), Burgess (1987), Charlton and Stewart (1999) and Stewart (2006) and these have been used in drafting the following information.

Perennial ryegrass was first introduced to New Zealand by British immigrants around 1820 and during the following 60 years progressively more material was imported (Stewart, 2006). By 1912, most of the seed sold in New Zealand was locally produced in old pastures of North and South islands and thus imports were greatly reduced (Stewart, 2006). Naturalised ryegrass plants from various New

Zealand regions, especially the drier regions of Hawke's Bay / Poverty Bay and Canterbury were collected and observed in 1920's by E.B. Levy and W. M. Davies, of the Plant Research Station (later Grasslands Division of the Department of Scientific and Industrial Research (DSIR Grasslands), now AgResearch) at Palmerston North (Easton, 1983). Levy & Davies classified their plant material into 5 (Burgess, 1987) or 6 (Easton, 1983) plant types. Plants collected from the Hawkes Bay were considered by Levy to be superior. This germplasm evaluation was a major milestone and laid the foundation for subsequent plant breeding work on perennial ryegrass in New Zealand. Realizing the potential contribution of the superior strains identified from old pastures, a Government Seed Certification Scheme was initiated in 1929 with the aim of providing farmers a source of perennial ryegrass seed of reliable provenance, and expected to be true to type and persistent (Stewart, 2006). Under this scheme elite plants of the Hawke's Bay/Poverty Bay ryegrass ecotype identified by Levy were bred to produce a "New Zealand pedigree" strain of perennial ryegrass (Stewart, 2006), which was available by 1936. The main selection criteria were increased leaf production, persistence and resistance to crown rust (*Puccinia coronata* Corda). This breeding line after re-selection and modification provided the genetic base for New Zealand's first released cultivar "Grasslands Ruanui" in 1955 (Stewart, 2006).

Release of a New Zealand strain of Italian ryegrass (*Lolium multiflorum* Lam.) followed soon after the development of the NZ pedigree perennial ryegrass. Later, the first specialist plant geneticist at DSIR Grasslands, Dr L. Corkill, developed a cultivar "H1" by hybridization of elite plants of the previously selected perennial ryegrass and Italian ryegrass breeding lines. This hybrid ryegrass was released in 1943 and became known as "Short Rotation", or "H1" ryegrass, and was later renamed "Grasslands Manawa". Corkill further backcrossed H1 ryegrass to perennial ryegrass in order to improve persistence of H1, while maintaining greater winter growth potential, and this work resulted in the release of "Grasslands Ariki" in 1965 (Stewart, 2006). In this context, Lamp et al. (1990) noted that for plant material introduced to New Zealand from the UK and adapted to comparatively cold winters and mild summers, breeding for reduced winter dormancy and improved summer drought tolerance would be a logical requirement.

Perennial ryegrass breeding in New Zealand took an unexpected turn in the 1960s when a farmer from Mangere in South Auckland, named T.R. Ellett, reported occurrence on his farm of a perennial ryegrass ecotype with summer growth superior to Grasslands Ariki. This "Mangere ecotype" was further investigated and compared with "Grasslands Ruanui", "Grasslands Ariki" and "Grasslands Manawa" at Palmerston North and this work resulted in release of the cultivar "Grasslands Nui" in 1977 (Armstrong, 1977). Concurrently with the development of Grasslands Nui at DSIR Grasslands, the same Mangere ecotype was used by the Yates Corporation (a New Zealand family company well known at that time for providing planting materials for home gardeners) to develop the cultivar "Ellett" released in the early 1980s. There is little published data comparing yield of Grasslands Ariki and Grasslands Ruanui with Grasslands Nui and Ellett, but the consensus is that both cultivars Grasslands Nui and Ellett developed from the Mangere ecotype outyield Grasslands Ruanui and Ariki. Bahmani et al. (2001) in a comparison between Ellett and Grasslands Ruanui noted a 13% higher total herbage accumulation for Ellett than Grasslands Ruanui.

The technology to create artificial tetraploids of perennial ryegrass (naturally diploid with 2n=14) using colchicine was another new development in perennial ryegrass breeding and commercial tetraploid cultivars started to emerge in the 1960s, following some pioneer work by Myers (1939) in the USA and Shalygin (1941) in the USSR. Scientists in the Netherlands were early adopters of this technology. By that time it had been clarified that characteristics of tetraploid plants included increased cell size and sucrose content, and increased leaf lamina length and tiller size, but also a reduction in tiller number and dry matter percentage (Ahloowalia, 1967). Charlton and Stewart (1999) also note that tetraploids have a larger seed size than the diploid cultivars.

"Grasslands Tama", a tetraploid of an extreme-annual form of ryegrass, was the first New Zealand tetraploid ryegrass cultivar, and was released in 1968. Following the release of "Grasslands Tama" a number of other tetraploid cultivars (both of perennial and "hybrid" ryegrass) namely Grasslands Greenstone, Nevis, Quartet, Ceres Horizon, Grasslands Sterling, Bealey and Banquet have been released in the last two decades (Stewart, 2006). Bealey and Banquet have reportedly Spanish

germplasm in ancestry (Stewart, 2006). Minneé et al. (2010) contrary to their hypothesis of higher annual DM from tall fescue cultivars tested in irrigated pastures of Canterbury and Waikato regions found a higher annual yield of perennial ryegrass tetraploid Banquet II in establishment year. Tetraploid cultivars, in general, fill a niche market, catering for situations where growing conditions for ryegrass are good (adequate summer moisture and high soil fertility) and the farmer wishes to focus on improved livestock performance. The tetraploid cultivars tend to have a lower population density of larger tillers as well as being more palatable to animals compared to diploids, hence the tetraploids require lax grazing management as well as high soil fertility and adequate moisture for best results (Stewart and Charlton, 2003), so they are not suited to all farming situations, but anecdotal information suggests significant numbers of farmers are using them in an informed way where conditions are favourable to ryegrass performance.

As discussed above, improved winter growth had been achieved by the 1970s in Grasslands Ariki and in Grasslands Nui and Ellett developed from the Mangere ecotype. At that point breeders began to more actively consider the second objective of improved performance in summer conditions, particularly the warmer and drier regions of New Zealand where ryegrass was sown. It was quickly realised that there are strong climatic similarities between north west Spain (province Galicia) and New Zealand's North Island and also that introductions from Mediterranean region (notably Spain and Portugal) were already being utilised in successful plant breeding work at DSIR, New Zealand (Forde and Easton, 1986). Therefore, a more systematic and planned germplasm collection tour was made by M. B. Forde and H.S. Easton of DSIR in collaboration with INRA (France) and IBPGR (Italy) to Portugal, Spain, France and Italy in 1986 (Forde and Easton, 1986). The total collection (1244 samples) of various grasses and legumes contained 209 samples of *Lolium* (Forde and Easton, 1986). Hence, this collection contained material that exhibited a number of traits highly relevant to New Zealand plant breeding programmes, including winter activity, late flowering, a low vernalisation requirement and excellent crown rust resistance in addition to drought tolerance traits.

Another development at this time was the promulgation of the Plant Variety Rights Act in 1987 that provided a commercial environment conducive to cultivar

development by private seed companies. In hindsight it is clear that the combination of availability of new germplasm and a commercial environment that would allow breeders to protect their intellectual property, has led to a large increase in the number of ryegrass cultivars available to New Zealand farmers.

Two examples of cultivars which emerged in New Zealand in the 1990s in this changed operational environment for plant breeders are Grasslands Impact and Grasslands Samson. The difference between the two in the germplasm used highlights the range of breeding options now available. The breeding programme for Grasslands Impact primarily utilised germplasm from a sub-population of Grasslands Nui (i.e. from the Mangere ecotype) and from north west Spain (Stewart, 2006), whereas Grasslands Samson combined germplasm of the Mangere ecotype (Grasslands Nui and Ellett) with persistent plants collected from drier eastern regions of New Zealand (Gisborne to North Canterbury) (pers. comm. H.S. Easton; (Stewart, 2006)). It is seen then, that there are various distinct candidates available when selecting germplasm for experimental crossing with Medea.

The use of germplasm of Spanish origin in New Zealand plant breeding has been ongoing. Grasslands Impact was subsequently licensed by AgResearch to NZ Agriseeds Ltd, who marketed it for some years, and Grasslands Impact germplasm was used by PGG Wrightson to breed a tetraploid cultivar, Banquet. Later cultivars to incorporate Spanish germplasm include Tolosa (Stewart, 2006) and Trojan (NZ Agriseeds Ltd.) and Ceres One50 (PGG Wrightson / Agricom).

Assessment of performance of the various cultivars has not produced clear conclusions. Company brochures tend to be positive about the product. For example, a promotional brochure about Grasslands Samson by the seller states “Grasslands Samson has proven its yield advantages in trial situations as well as in farmer evaluations. This, combined with high rust tolerance, leads to optimum animal performance.” (http://www.agricom.co.nz/userfiles/files/Samson%20AR37.pdf). On the other hand, more conventional yield trial data (Easton et al., 2001a) shows yield of Grasslands Samson as being not statistically different from other recently released cultivars and the best conclusion that could be drawn was that the average annual

yield for seven cultivars released after 1993 was 6% higher than the average yield of seven cultivars available before 1993.

Another New Zealand ryegrass cultivar of interest when attempting to categorise sources of germplasm used to develop existing cultivars, is Matrix developed by Cropmark Ltd. In general, members of the genus *Festuca* show more drought (and cold) tolerance than the genus *Lolium* (Ghesquière et al., 2010; Humphreys et al., 2003), and tall fescue is closely related to perennial ryegrass, but being a natural hexaploid, does not readily hybridise with *Lolium.* However, because meadow fescue is a diploid like perennial ryegrass, and also closely related, introgression (for discussion of this term see Section 2.7) with perennial ryegrass has been possible. The first Cropmark cultivar produced in this way was Matrix, released in 2000's incorporating Aries HD and Grasslands Impact with 8% introgression of meadow fescue (Stewart, 2006). However, despite the presumption that the breeding of Matrix may have conferred some drought tolerance there has been little or no experimental data published that explores and confirms this point.

2.7 Use of hybrids in plant breeding

There are two reasons for hybridization of divergent genotypes within a species (a) to exploit heterosis in hybrids; and (b) to incorporate useful genes from an exotic source (introgression). So this section will cover these two aspects.

2.7.1 Concept of heterosis/hybrid vigour

The term "heterosis" was originally coined by G. H. Shull of Princeton University, New Jersey in 1914 (Shull, 1948) and as a phenomenon of plant breeding has revolutionized maize crop production over the 20th century. The term refers to the "superiority of progeny arising from crossing genetically divergent parents" (Barrett et al., 2010) and is usually quantified as the % superiority of progeny over mean of the two parents for a particular trait (mid parent heterosis; MPH).

The terms heterosis and hybrid vigour are taken as synonymous in today's plant breeding world. Whaley (1944), however, discriminated between the two terms by pointing out that hybrid vigour is a name for an "end product" of developmental

stimulation from union of different gametes while heterosis is a mechanism leading to hybrid vigour. Sometimes the terms hybrid and heterosis are erroneously taken as synonyms (Posselt, 2010b). Heterosis is not characteristic of every hybrid but cannot occur without hybridisation (Lamkey and Edwards, 1999).

Most of the success stories in the use of hybrids that capture heterosis come from naturally out-crossing species (maize being a classic example) that are forced to self-pollinate. Though such crossing results in inbreeding depression (loss of vigour), selection of genotypes with good combining ability from amongst them makes the further step easy. The hybrids produced by such selected genotypes are much more vigorous than the parents. However, it is very difficult to produce commercial hybrids from naturally self-fertile species like wheat. Though it is easy to produce inbred lines, but absence of transgressive heterosis inhibits production of vigorous hybrids.

A few plant breeders have tried to exploit hybrid vigour in ryegrass. But the process needs (a) a good male sterility system which is a very expensive technique and raises the price of the resulting seed lot; and (b) commitment for 25 – 30 years. As a result, production of synthetic cultivars is the system commonly used in perennial ryegrass breeding in New Zealand.

Synthetic cultivars are a population derived from a limited number of elite founder plants (usually 7 – 20) that are carefully evaluated, multiplied for a number of years and finally sold to farmers. However, Barrett et al. (2010) note that the resulting cultivar delivers to the farmer only a portion of the potentially available hybrid vigour as there is comparatively little capture of heterosis in a synthetic cultivar.

A system designed to increase the capture of hybrid vigour in perennial ryegrass breeding is the production of semi-hybrids (Brummer, 1999). If seed of two cultivars with good combining ability is sown together at equal density, then the seed harvested will consist of 50% from interpopulational crosses and 50% from intrapopulational pollination (Brummer, 1999), hence the term semi-hybrid (Posselt, 2010b).

Another important point is that expression of heterosis is not an automatic consequence of crossing genetically divergent parents, but the divergent populations must also prove to be 'good combiners' (Scotti and Brummer, 2009). Therefore, selection of parents should involve assessment of combining ability (Posselt, 2010a).

2.7.2 Introgression

The introduction of useful genes from an exotic source is called introgression. Plant breeders persist in crossing closely related, high yielding cultivars, which results in a high yielding cultivar (Tanksley and McCouch, 1997). However, traits like yield are actually polygenic, so under this breeding approach not all yield-related loci end up with the best alleles for yield (Tanksley and McCouch, 1997). Cultivars Tolosa and Matrix have been introgressed in New Zealand (section 2.6). This thesis deals with exploring a summer dormant cultivar, Medea, as an exotic source, since it may potentially carry superior genes at some loci, compared to the ryegrass breeding material in use in New Zealand.

Introgression relies to some extent on chance to bring together in one plant desirable alleles from the divergent parents; hence it is common for large numbers of plants, often as many as 10,000 or more to be screened in the early generations. Introgression also relies on observation and recording, to detect and utilise a plant that has inherited desirable trait combinations.

2.8 Medea as a summer dormant drought resistant cultivar

The issue of drought resistance of perennial ryegrass is even more relevant to a number of areas of Australia (especially in areas of 500 – 1000 mm annual rainfall in the states of New South Wales and Victoria), than to New Zealand and one of the tactical approaches employed there has been the use of Mediterranean germplasm. Reed et al. (1987) note that introduction of Mediterranean germplasm to facilitate forage grass improvement in Australia was proposed as early as 1920. Whyte (1957) has given a historic account of germplasm collection missions of a Commonwealth Scientific and Industrial Research Organization (CSIRO)-FAO joint project for exploration and collection of grass and legume germplasm from Mediterranean region. According to Whyte (1957), C. A. Neil-Smith from CSIRO first visited the

Mediterranean countries of interest and later returned for collection in 1954 . The collection consisted of six hundred lines of various species of legumes and grasses notably *Phalaris*, *Lolium* and *Trifolium* that was equally divided by CSIRO and FAO (Whyte, 1957).

Silsbury (1961) tested the Mediterranean collection (2 lines from Cyrenaica, 28 lines from Algeria and 8 lines from Greece) of perennial ryegrass for flowering, dormancy, survival and growth characteristics against one line each from Australia "Victorian"[1], New Zealand (New Zealand Mother[2]) and UK (S-24). He concluded that the Mediterranean collection has varying degrees of summer dormancy while the Australian, New Zealand or UK (S-24) germplasm does not have this characteristic. More specifically, both the lines from Cyrenaica flowered 12 days earlier than Victorian and showed 100% dormancy. Out of the 28 lines of Algerian origin 12 were early flowering (-8 to 0 days against Victorian) while 16 were mid-season flowering (+2 to 12 days after "Victorian"). In these two sub-groups dormancy ranged from 70 to 100 and 30-100%. Likewise germplasm of Grecian origin flowered very late (+28 to +38 days after Victorian) and also showed relatively low degree (20 – 60%) of dormancy. Silsbury (1961) attributed variation in flowering dates to summer dormancy and thus drought escape.

A set of lines (CPI 19003, CPI 19004, and CPI 19006) collected from Medea near Algeria was used as the basis for development of a cultivar "Medea" by J.H. Silsbury of the Waite Agricultural Research Institute, University of Adelaide that was registered in 1967 (Barnard, 1972; Oram, 1990; Silsbury, 1961). This cultivar though morphologically similar to the Victorian ecotype is characterized by its high degree of summer dormancy. Medea was not properly maintained and promoted (Reed, 1996). Some of the reasons for lack of adoption of Medea at the industry level were poor seed production, susceptibility to crown rust, lack of marketing and possible contamination with seeds of *L. rigidum* (Cunningham et al., 1994). Still, it is clear some interest in the traits possessed by Medea remained, because Valley Seeds Pty Ltd used a paddock previously sown to Medea as source material for crossing with

[1] A strain of perennial ryegrass referred to by some authors as a cultivar but more correctly regarded as a local ecotype originating from older-established pastures in Victoria, Australia.
[2] Later renamed Grasslands Ruanui.

the Victorian ecotype and released a cultivar named Brumby in 1987. This cultivar has proved its worth for regions of Australia with marked Mediterranean climate (Reed, 1996).

There are very few research studies of the performance of Medea. Vartha (1975) in a multi-year (1971 to 1974) yield trial in Canterbury of Grasslands Ruanui and Medea confirmed Silsbury's (1961) finding that Medea has summer dormancy. In that trial summer yield of Medea was about 60% of Grasslands Ruanui, while winter yield was slightly greater. Vartha (1975) also linked summer dormancy of Medea to higher drought resistance. Likewise, Hill (1985) also found Medea to be more persistent than other recognised cultivars in a multi-year (1971 through to 1976) yield trial where other cultivars (Kangaroo Valley and the Victorian ecotype) had died after first two years of the trial, whereas some plants of Medea persisted one or two years longer. Superiority of Medea over cultivars of European origin (Ellett, Kangaroo Valley and Brumby) in Victoria (600 mm rainfall) for persistence through drought was reported by Anderson et al. (1999). Stewart and Aberdeen (1997) too noted improved persistence and winter activity in some turf grasses of Mediterranean origin with Medea in ancestry, compared to the Victorian ecotype. Hence, the combination of qualities like summer dormancy, winter growth and persistence through summer make Medea a material of interest to perennial ryegrass breeding in New Zealand, especially if producing a cultivar suited to areas of Canterbury and other areas in New Zealand of similar climate where annual rainfall is similar to that of Victoria.

Despite all the positive points about Medea, its summer dormancy still raises questions that need to be answered before Medea can be used in perennial ryegrass breeding in New Zealand. To answer these questions it is important to explore the mechanisms at the trait level which contribute to the reduction in plant size in summer, and establish how Medea differs from present commercial cultivars (for example, Grasslands Samson) currently used in New Zealand.

2.9 Conclusions

It is apparent from this brief review that while there is an extensive literature on mechanisms of plant drought resistance and that perennial ryegrass breeding in New Zealand has been the subject of extensive scientific and commercial input over decades, there is a comparative lack of work that explores drought resistance mechanisms of perennial ryegrass, especially in New Zealand. This is not to say that there has been no interest in drought resistance, as it is clear that introgression of germplasm from Northwest Spain by commercial breeders was partly aimed at improving summer performance of the cultivars released. These two points then provide a logical basis to define the research directions for this Ph D study. In accepting a proposal from Dr H. S. Easton of AgResearch that this study assess potential for improving perennial ryegrass drought tolerance by introgression with germplasm of cultivar Medea, derived from summer dormant North African material, the following lines of research are indicated:

- In general, previous studies on drought tolerance of forage grasses have focussed on particular traits and there is not a developed methodology for performing a general evaluation of how different morphological and physiological traits expressed above and below ground combine to determine the overall water deficit response of a plant. Development of methodology for determining the contribution of a broad range of above- and below ground morphological and physiological traits to drought resistance would be useful.
- Most of the historical studies involving Medea have been field studies to determine forage yield and persistence. In research in Victoria, Australia, Medea was found to be a little more persistent than perennial ryegrass cultivars of European origin, and the increased survival was attributed to ‘summer dormancy’. There has been little or no quantitative measurement of traits contributing to drought resistance. It would be desirable to have more detailed information, both on how summer dormancy is triggered and expressed, and on any other drought resistance traits Medea may possess, in addition to the summer dormancy habit.

- It would be of interest to compare drought resistant strategies of Medea, and perennial ryegrass cultivars currently in commercial use in New Zealand.
- Once traits of interest are identified, investigation of patterns of inheritance of those traits and the extent to which heterosis occurs when plants of Medea are crossed with New Zealand perennial ryegrass germplasm would be a first step to assessing the prospects for cultivar development based on Medea introgression.

3

Comparison of morphogenetic traits in perennial ryegrass (*Lolium perenne* L.) cultivars Grasslands Samson and Medea in winter

3.1 Introduction

As mentioned in Section 1.3, the first experiment conducted was a winter comparison of the plant material to be studied (perennial ryegrass cultivar Medea) and a current New Zealand commercial perennial ryegrass cultivar (Grasslands Samson). Details of the breeding of Medea from germplasm of Mediterranean origin by J. H. Silsbury in Adelaide in the 1960s were covered in Section 2.8. The rationale for this experiment was that since Medea is believed to exhibit the trait of summer dormancy (Silsbury, 1961) commonly seen in forage grass germplasm of Mediterranean origin (Volaire et al., 2009), and has been observed at Palmerston North to have flaccid leaves in summer (H. S. Easton, personal communication), it would be useful to first gain an appreciation of how the productive capacity of Medea compares to that of a current commercial New Zealand cultivar in growth conditions optimal for Medea. The suite of measurements was planned so as to determine not only the dry matter yield of Medea relative to Grasslands Samson, but also to detect any differences in tillering strategy or pattern of leaf morphogensis. To assess tillering capacity, the approach taken was to derive average relative tiller appearance rate (Bahmani et al., 2000) for early and late phases of the experiment. For study of leaf morphogenesis, the approach taken was to monitor date of appearance of new leaves, and leaf elongation rate for marked tillers on each plant (Gastal et al., 1992).

3.1.1 Aims

Given the above background, the aims for Experiment 1 could therefore be defined as follows:

(i) to provide familiarization with morphology and growth of grass, a new area of research for the writer;

(ii) to quantify winter dry matter production per plant, tiller appearance, and leaf formation in ryegrass cultivar Medea bred from germplasm of Mediterranean origin and compare it with that of a current commercial New Zealand cultivar Grasslands Samson.

3.2 Materials and Methods

On 14 April, 2008, 4-tiller plantlets of perennial ryegrass ('ramets' with 2 adult and 2 daughter tillers) were established in 100 mm diameter pots of 280 mm rooting depth in a glasshouse at the Institute of Natural Resources, Massey University, Palmerston North. There were two cultivars (Grasslands Samson and Medea) and ten seedlings of each cultivar included in the experiment, with two replicates of each genotype of the two cultivars. Soil composition was: builder's sand 50% and B horizon of a Manawatu alluvial soil 50%; with addition of 'Osmocote' slow release fertiliser (15% N, 4.8% P, 10.8% K and 1.2% Mg + trace elements, release time 3 – 4 months). This produced a visually uniform population of plants which produced 3 – 4 leaves per tiller between transplanting and the start of measurement. The pots were covered with opaque, heavy, black polythene plastic sleeves to 120 mm above soil surface level to simulate shading from neighbouring plants in a sward and plants were initially allowed to grow undefoliated, then trimmed to the top of the sleeve on 14 May 2008 (Day 0). A suite of measurements was then conducted over approximately 120 days, as described below. The glasshouse was fitted with heaters, thermostatically controlled, to switch on when air temperature fell below 5°C.

3.2.1 Morphogenetic data

The following measurements were recorded or derived to identify any major morphogenetic similarities and differences between the cultivars:

Leaf lamina length (LL): Two randomly selected adult tillers of each plant were marked using coloured plastic rings and these marked tillers were inspected every 2 – 3 days from Day 0, for approximately 50 days, and for LL of those leaf laminae appeared since clipping on Day 0 was measured (mm from the tip of the target leaf to the ligule of the subtending leaf). For recording purposes, the next leaf lamina tip to appear from the pseudostem whorl after clipping was designated Leaf 1 (L1). Measurements continued until L4 of all marked tillers was nearly fully expanded. In this way, 3 complete leaf appearance cycles were monitored between mid May and early July.

Leaf psuedostem length (PsL): PsL (mm) was measured as the distance from the L1 ligule to the soil surface.

Leaf elongation duration (LED): From the LL data described above, LED (days leaf^{-1}) for the main shoot of marked tillers was derived as the number of days from the date a leaf was first seen until the date the maximum value of LL for that leaf was recorded. LED values were also converted to °C.day values using temperature data collected from probes placed in the glasshouse near the plants (see below).

Leaf appearance interval (A_{Lf}): Related to LED, but not identical to it, A_{Lf} (days leaf^{-1}) was determined as the number of days interval between appearance of successive leaf tips.

Leaf elongation rate (LER): LER (mm d^{-1}) was calculated by dividing final leaf length by LED.

Tiller number (TN): Tiller number per plant was counted on 10 and 17 July, and values for **relative tiller appearance rate (RTAR**, tillers tiller^{-1} d $^{-1}$) and **site filling** (**Fs**, tillers tiller^{-1} (leaf appearance interval)$^{-1}$ derived from the TN data. This choice of tiller counting dates allowed mean values of A_{Lf} or Fs to be determined for the plant establishment and measurement period from 14 April to 10 July, and for newly defoliated plants from 10 July to 17 July. RTAR was estimated by the formula: ln(TN2)-ln(TN1)/days interval (T2)-(T1), where TN2 and TN1 are the number of tillers per plant at time T2 and T1, respectively. Fs was estimated by multiplying RTAR by A_{Lf} to obtain Fs tillers tiller^{-1} (leaf appearance interval)$^{-1}$. It should be

noted that this methodology generates whole plant average values for a defined period of time.

Whole plant leaf number (LN): The total number of live leaves on each plant, including all tillers, was determined from a simple count and recorded.

Leaf width (LW): LW was measured to the nearest 0.1 mm by setting a graduated eyepiece of a 15× zoom microscope to read 100 divisions per cm, and recording width (at mid point) of two leaves on each of the target tillers.

Number of live leaves per tiller (NLL): Number of live leaves per tiller was recorded on day 57 from defoliation when L1 had started to senescence. For the senescent leaf of marked tillers, the proportion of the lamina remaining green was visually estimated to the nearest 10% (expressed as 0.1 to 0.9), the emergence status of the youngest leaf was also estimated by dividing the current LL divided by the final LL of the leaf below, and the number of fully emerged live leaves between the senescent leaf and emerging leaf was counted, and these three values added together.

Plant Dry Weight (DW): DW (g $plant^{-1}$) was measured by lowering plastic sleeves and cutting plants to ground level on 8 July. The cut foliage was oven-dried for 48 hours at 80^{o}C and dry weight measured to the nearest 0.01g.

Tiller weight (TW): An estimate of average tiller weight per plant (TW, mg) at the end of the experiment was obtained by dividing plant DW by TN.

3.2.2 Leaf gas exchange data

Although not the main focus of the experiment, and because relevant equipment was available it was decided to collect a set of leaf gas exchange data. The instrument used was a CIRAS-2 Portable Photosynthesis System manufactured by PP Systems, Ltd. provided with PLC6 (U) Automatic Universal Leaf Cuvette with 18 mm diameter window able to measure CO_2 and moisture concentrations of an air stream before and after passing through the cuvette containing an enclosed leaf. After calculations by the onboard computer from the raw data the CIRAS-2 can report estimated values for leaf photosynthesis (Pn, µMol CO_2 m^{-2} leaf sec^{-1}), evapotranspiration (Evp, µMol H_2O m^{-2} leaf sec^{-1}), stomatal conductance (SC, m mol water vapour m^{-2} leaf sec^{-1}), leaf internal CO_2 concentration (Ci, ppm) and leaf

temperature (T_L, °C), among others. A practise run to learn how to use the equipment was carried out on 18 July and on 26 August, after plants had regrown satisfactorily from defoliation to determine plant DW, gas exchange measurements for 20 plants were carried out (one plant from each of the 10 genotypes of Grasslands Samson and Medea). The CIRAS-2 was set to perform the measurements at a photosynthetically active radiation (PAR) level of 1000 µMol photons m^{-2} sec^{-1}.

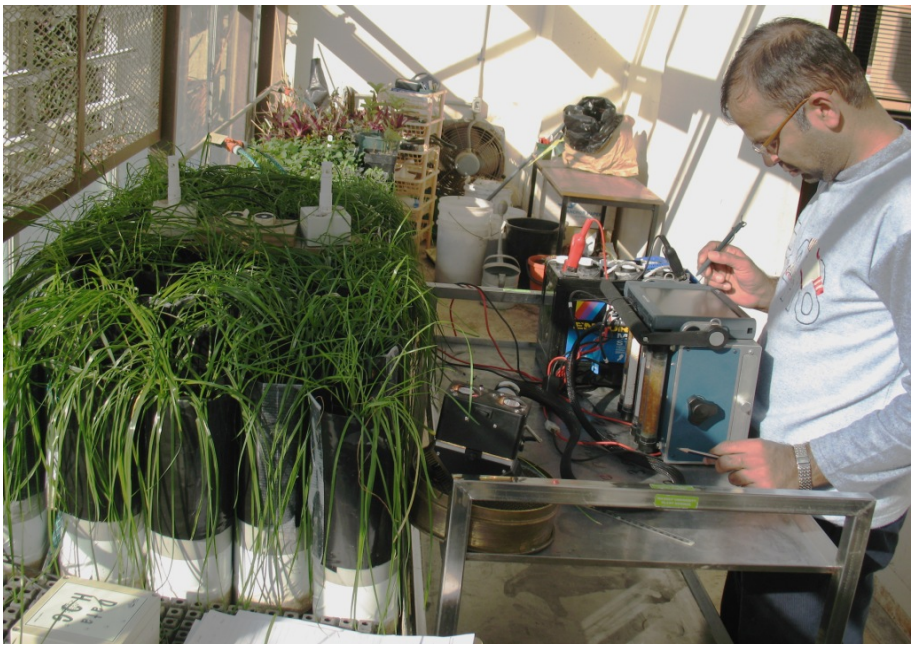

Figure 3.1: The author measuring leaf gas exchange parameters i.e., photosynthesis, evapotranspiration and stomatal conductance on leaves, Medea and Grasslands Samson using the CIRAS-2 Portable Photosynthesis System. Vertical white rods among plants in the middle-left background are Skye temperature sensors (See Section 3.2.3 below).

3.2.3 Glasshouse temperature recordings

To assist the interpretation of morphogenetic data, a 5 channel data logger DataHog "SDL 2830", a product of Skye Instruments Ltd, U.K., with a pair of pyranometer sensors (SKP 1110/l) and a pair of air temperature probes (SKH2021/1) (both manufactured by the company named above) attached to it was used. Hourly data on glasshouse temperature and solar radiation were recorded over 22 days (19 June to 10 July) by placing the assembly of temperature and solar radiation sensors between the two replicates of the experiment on a raised surface so the sensors were on the same level as the plant canopy, as shown in Fig. 3.1.

3.2.4 Statistical analyses

For those morphogenetic measurements performed on all 40 plants in the experiment (2 cultivars, 10 genotypes within cultivars and 2 replicates, a nested ANOVA model for genotypes within cultivars was carried out using PROC GLM of SAS (SAS command code is reproduced in Appendix 3.1). To assist evaluation of trait associations, a table of correlation coefficients was compiled using PROC CORR of SAS and also a principal component analysis (PCA) was carried out in SAS using PROC PRINCOMP. Variables included in the PCA were: LER, LED, LL, LW, TN, PsL, DW, LTW, LN, Fs and RTAR. Structure coefficients for PCs 1-4 of the 11 PCs generated are reported. To aid interpretation of PCs, PC scores were statistically analysed for cultivar and genotype effects as above. To perform ANOVA of PC scores, the PCA was re-run in Minitab 15 Statistical Software.

For gas exchange measurements on one replicate of each genotype, genotypes were considered as replicates but to remove a possible diurnal time trend, successively sampled pairs of Grasslands Samson and Medea plants were treated as blocks and statistical analysis was done by GLM in Minitab, to test for cultivar difference of the mean for all genotypes after removal of the diurnal effect. PCA and ANOVA of PC scores was also carried out for gas exchange measurements, with the variables Pn, SC, Evp, Ci, $T_L$and LW after adjusting the other data for leaf width.

3.3 Results

3.3.1 Glasshouse temperature data

Mean daily maximum and minimum temperatures for a 22 day period (19 June to 10 July) were respectively 9.0°C at 7.00 am and 17.6°C at 2 pm, with a mean for hourly measurements of 12.1°C, though on some days afternoon temperatures in the glasshouse exceeded 20.0°C, and occasionally 25°C (Fig. 3.2). Solar radiation as measured with a Skye instruments SKP1110/1 ‘pyranometer’ sensor at leaf canopy level in the glasshouse averaged 1.2 MJ dy^{-1} over the same period with peaks of up to 200 W m^{-2} on sunny days (Fig. 3.3).

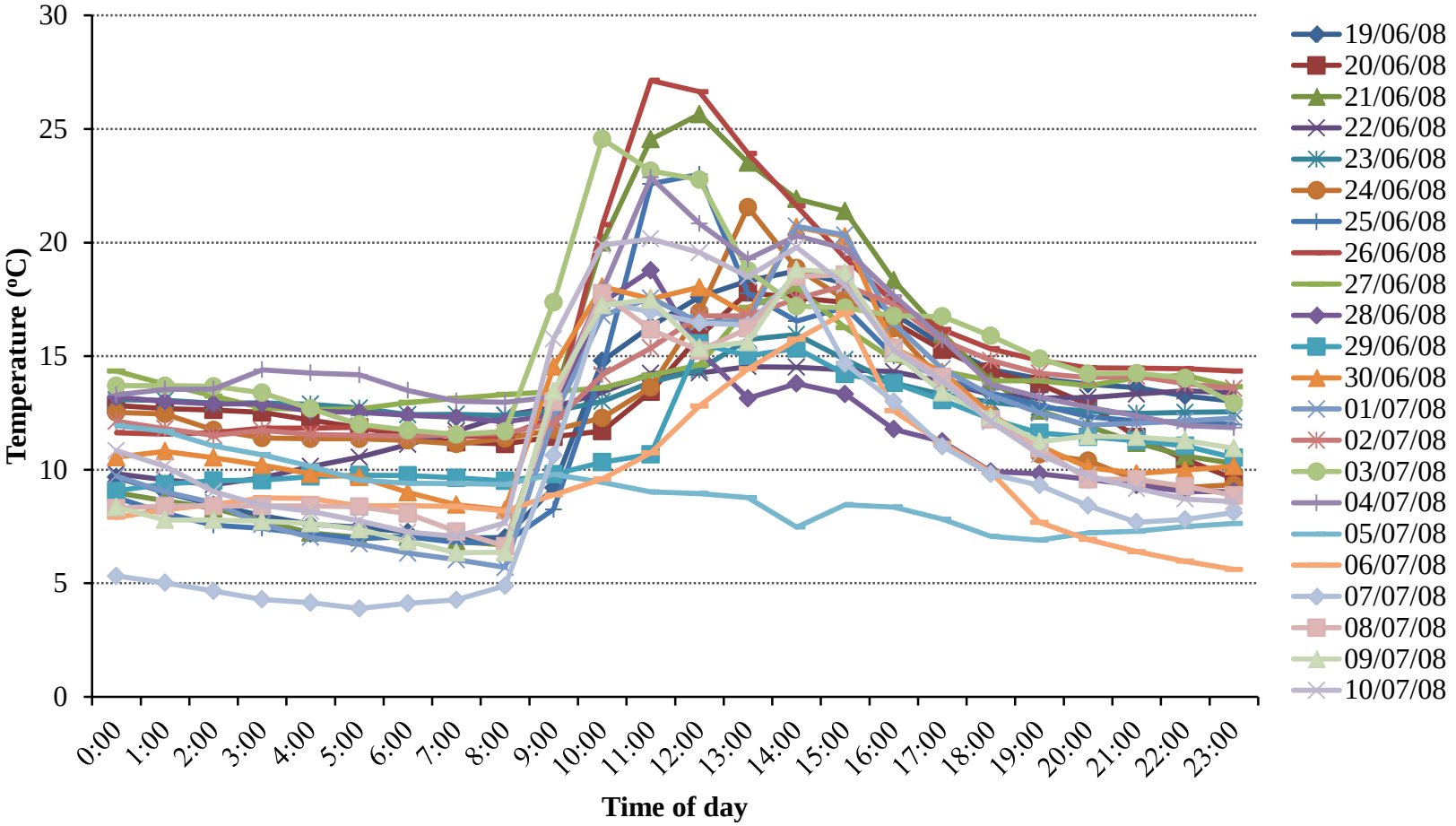

Figure 3.2: Hourly temperature data in the glasshouse measured with a Skye Instruments data logger from 19 June to 10 July 2008.

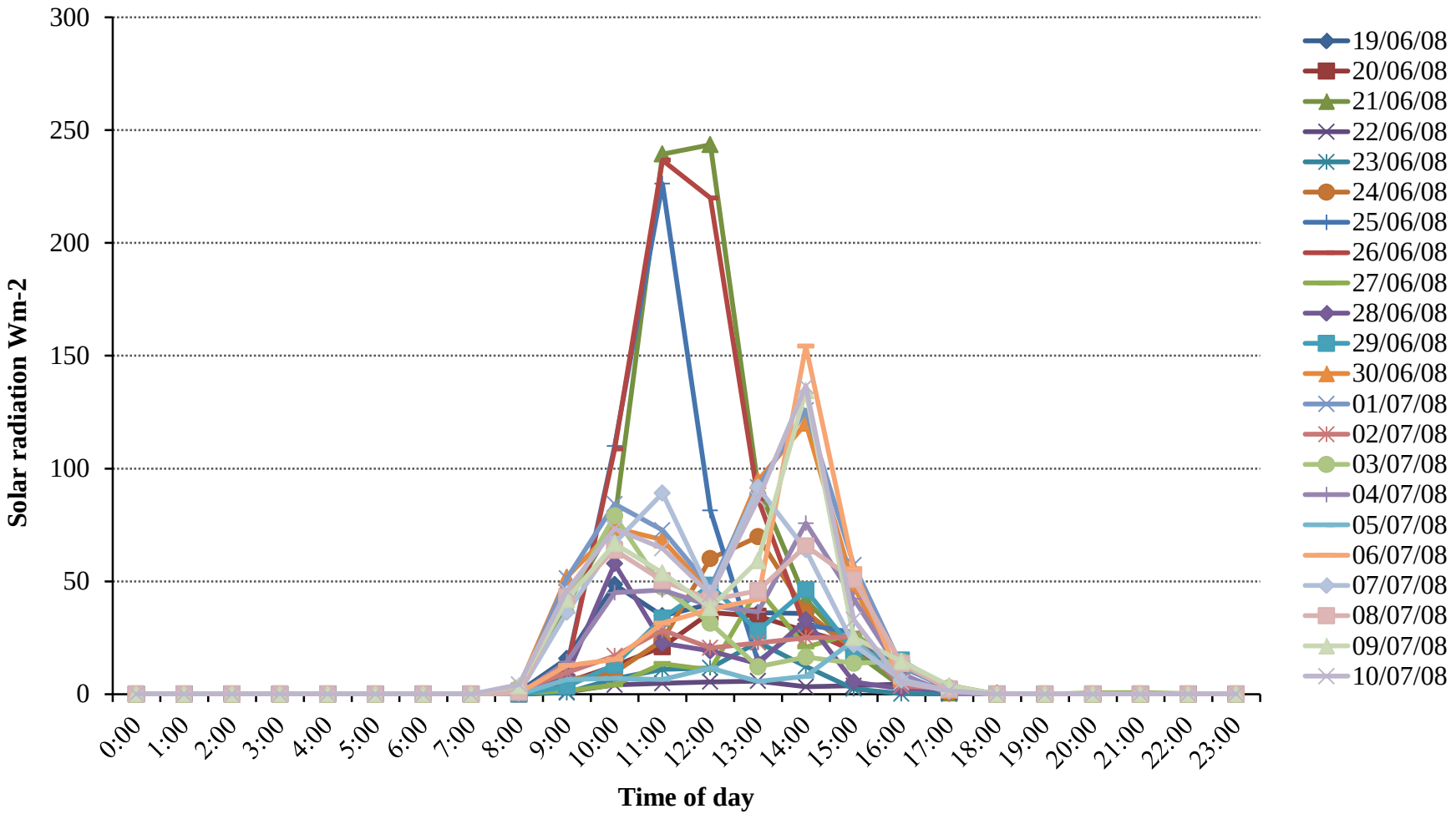

Figure 3.3: Hourly solar radiation data for the glasshouse measured with a Skye Instruments data logger from 19 June to 10 July 2008.

3.3.2 Leaf morphogenesis

For the first and second leaf after defoliation, Medea plants had lower LL and LER than Grasslands Samson plants (Table 3.1). However, in the later stages of the experiment, growth of Medea was similar to that of Grasslands Samson.

Table 3.1: Mean values of leaf length (LL), and leaf elongation rate (LER) for the first three leaves (leaves 1-3) appearing after defoliation on 15 May 2008. Probability values for effect of cultivar [P(Cv)] and genotype-within-cultivar [Pgen(Cv)] are shown, with the least significant difference at 5% for cultivar effects.

Trait	Leaf	G.Samson	Medea	P(Cv)	Pgen(Cv)	LSD5%(Cv)
LL (mm)	1	337	280	<0.001	0.105	22.13
	2	361	332	0.038	0.205	27.47
	3	383	361	0.073	0.063	23.00
	Mean	360	324	0.004	0.083	22.99
LER (mm day^{-1})	1	22.2	18.6	<0.001	0.053	1.377
	2	25.2	23.3	0.029	0.048	1.7322
	3	24.1	22.5	0.124	0.069	2.0996
	Mean	23.8	21.5b	0.002	0.038	1.354

In contrast to LL and LER, data for LED of the same three leaves was nearly identical for the two cultivars (Fig. 3.4). Although calculated values for LED of around 12 – 16 days for different plants over the three leaf appearance intervals monitored showed no significant cultivar effect, the average date at which successive leaves were first seen did progressively separate, so that L3 was first seen and ceased elongating three days earlier in Grasslands Samson than in Medea (P = 0.041; Fig. 3.4). It is also notable that in both the cultivars the overlap of leaf elongation periods for L4 and L3 was greater than for L3 and L2, and for L2 and L1 (Fig. 3.4).

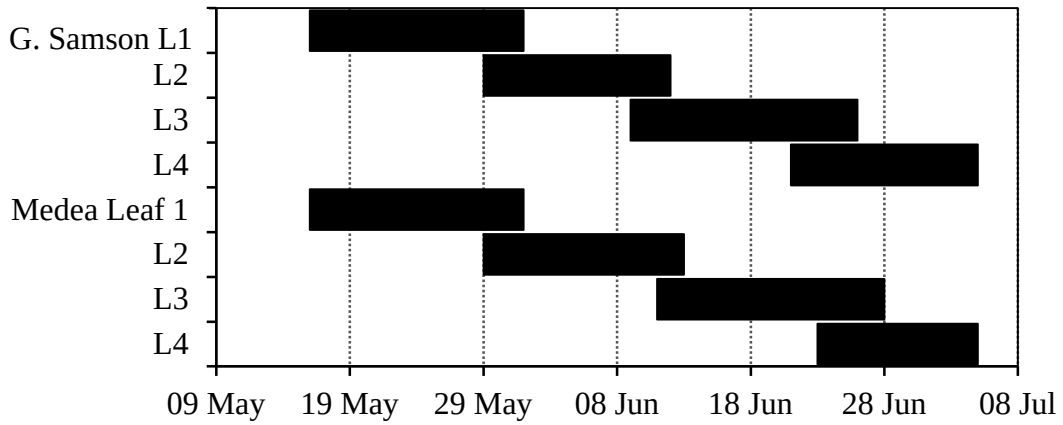

Figure 3.4: Comparison of leaf extension duration (LED) for the first 4 leaves (L1 to L4) appearing after defoliation on 14 May for the two cultivars, Grasslands Samson and Medea.

3.3.3 Other morphogenetic traits

The remaining morphogentic measurements were largely focussed on TN attained by plants towards the end of the experiment and its rate of increase during the experiment, and on plant DW and derived as measured by a ground level cut made on 8 July. These data are presented here as cultivar means for the relevant time periods or dates (Table 3.2). Notable features of the data are: (i) in early July after regrowth following defoliation to a common height on 15 May, Grasslands Samson plants had higher DW and TN than Medea plants; (ii) while Medea had a lower relative tiller appearance rate (RTAR) than Grasslands Samson from planting until early July, both cultivars showed increased RTAR after defoliation on 7 July with no significant cultivar difference at this time; (iii) Medea had narrower LW and longer PsL than Grasslands Samson (Table 3.2).

Table 3.2: Mean values of other morphogenetic traits measured for the perennial ryegrass cultivars Grasslands Samson and Medea in Experiment 1 from 15 May to 17 July 2008.

Trait	G. Samson	Medea	P(Cv)	Pgen(Cv)	SEM
LW (mm)	3.90	3.40	<0.001	<0.001	0.093
PsL (mm)	158.0	197.0	<0.001	<0.001	5.49
A_{Lf} (days)	12.3	13.0	0.055	0.044	0.23
LED (days leaf^{-1})	15.5	15.6	ns	ns	0.33
LN (leaves plant^{-1})	70.0	30.2	<0.001	ns	5.06
NLL (leaves tiller^{-1})	3.89	3.69	0.054	0.015	0.066
TN at harvest (tillers plant^{-1})	20.4	8.7	<0.001	ns	1.41
TW (mg)	165.0	143.8	0.030	<0.001	6.37
DW (g)	1.930	0.907	<0.001	0.038	0.0939
RTAR 1-planting to harvest (tillers tiller^{-1} day^{-1})	0.018	0.008	<0.001	0.027	0.0008
RTAR 2-post defoliation	0.037	0.034	ns	ns	0.0043
Fs	0.216	0.104	<0.001	0.019	0.0088

A_{Lf} = Leaf appearance interval, LL = Leaf lamina length, LER = Leaf elongation rate, LED = Leaf elongation duration, LW = Leaf width, TN = Tiller number per plant, LN = Leaf number per plant, NLL = Number of live leaves per tiller, PsL = Pseudostem height, DW= Plant Dry Weight, TW = Tiller weight, Fs = Site Filling, RTAR-planting to harvest = Relative tiller appearance rate averaged from planting on 14 April to defoliation in early July. RTAR-post defoliation = Relative tiller appearance rate between counts of tillers plant^{-1} carried out on 10 and 17 July, following defoliation of plants on 8 July.

3.3.4 Plant dry weight variation for genotypes within cultivars

DW was plotted against one of its main yield components, TN per plant, for all 40 plants in Experiment 1, to assess variation between genotypes within cultivars and

overlap between cultivars. It is seen that in this experiment the largest 10 Medea plants have similar DW to the smallest 10 plants of Grasslands Samson, but that plants of Grasslands Samson have more variation in TN for a given DW, than plants of Medea (Fig. 3.5).

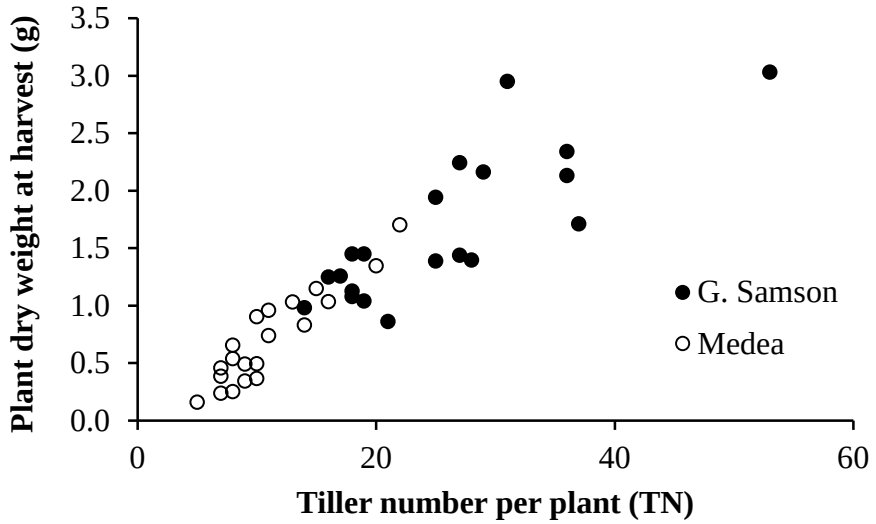

Figure 3.5: Shoot dry weight harvested (DW, g plant^{-1}) for 20 individual plants of Grasslands Samson and 20 plants of Medea cut to ground level on 8 July 2008, plotted against tiller number (TN) per plant.

To check the extent to which the two yield components TN and TW contributed to DW per plant in the growing conditions of this experiment, DW was regressed on standardised values for TN and LL. LL data were used in place of TW as an independently measured variable correlated with TW ($R = 0.57$, $P < 0.001$, Table 3.3) whereas TW had been derived as DW/TN. The equation obtained was:

$$DW\ (g\ plant^{-1}) = 1.42 + 0.603\ TN + 0.242\ LL.$$

3.3.5 Trait associations as assessed by correlation analysis and PCA

To examine the patterns of association between the various morphogenetic traits and plant size measurements, coefficients of correlation were calculated and PCA was also employed.

The correlation matrix of statistically significant coefficients was assembled (Table 3.3) for a group of 13 variables selected from those reported in Tables 3.1 & 3.2. In general there were high positive correlations between variables reflecting plant size, DW, LN, TN and RTAR. In this data set TW was markedly less strongly correlated with DW than TN, but positive associations between TW and LL and NLL were detected. As expected, LED was positively correlated with A_{Lf}, but negatively correlated with LER (Table 3.3).

From PCA of the same data, 4 of the 13 available PCs are reported (Table 3.4), and these explain 84.9% of the variation in the data set. A conceptual interpretation of the PCA is that PC1 reflects variation between cultivars for plant size, PC2 reflects variation between genotypes within cultivars in tiller size (especially in Grasslands Samson), PC3 reflects variation between genotypes for leaf appearance interval (especially in Medea) and PC4 reflects variation between genotypes for NLL.

Table 3.3: Matrix of coefficients of correlation between thirteen selected variables in forty plants of perennial ryegrass cultivars Grasslands Samson and Medea during winter 2008. Correlations with P > 0.10 have been omitted. Approximate thresholds for correlation coefficients to achieve 0.05, 0.01, and 0.001 levels of statistical probability are, respectively, R = 0.31, R = 0.40, and R = 0.51.

	LL	LW	PsL	A_{Lf}	LER	LED	LN	NLL	TN	TW	DW	RTAR
LW	0.619											
PsL												
A_{Lf}												
LER	0.809	0.437	0.326	-0.360								
LED		0.340		0.348	-0.289							
LN			-0.335	-0.446								
NLL		0.349										
TN1			-0.380	-0.414			0.980					
TW	0.571	0.680	0.374		0.503			0.298				
DW	0.522	0.450		-0.441	0.521		0.831		0.842	0.264		
RTAR	0.297		-0.419	-0.368	0.303		0.919		0.938		0.887	
Fs	0.346		-0.472		0.271		0.834		0.862		0.832	0.967

LL = Leaf lamina length (mm), LW = Leaf width (mm), PsL = Pseudostem length (mm), A_{Lf} = Leaf appearance interval (days), LER = Leaf elongation rate (mm dy^{-1}), LED = Leaf elongation duration (days), LN = Leaf number, NLL = Number of live leaves, TN = Tiller number, TW = Tiller weight, DW = Plant Dry Weight, RTAR = Relative tiller appearance rate planting to harvest, Fs = Site Filling.

Table 3.4: Principal component coefficients for the first four principal components (PCs) generated by principal component analysis (PCA) of morphological data for Grasslands Samson and Medea perennial ryegrass cultivars. Coefficients < 0.3 have been suppressed.

Traits	PC1	PC2	PC3	PC4
% variation explained	40.6	23.7	13.7	6.9
Cumulative % variation	-	60.3	78.0	84.9
LL	-	-0.409	-	0.3
LW	-	-0.393	-0.314	-
PsL	-	-0.343	0.384	-
LER	-	-0.358	-	0.372
A_{Lf}	-	-	-0.506	-
LED	-	-	-0.599	-
LN	-0.389	-	-	-
NLL	-	-	-	-0.826
TN1	-0.392	-	-	-
TW	-	-0.48	-	-
DW	-0.42	-	-	-
RTAR	-0.415	-	-	-
Fs	-0.391	-	-	-
P Cv	<0.0001	0.907	0.191	0.624
P gen(Cv)	0.087	<0.001	0.003	0.009
Ratio SEM G.Sam/Medea	1.21	1.67	0.70	0.90

Trait abbreviations are as for Table 3.3. P cv = statistical probability of cultivar effect. Pgen(Cv) = statistical probability of genotype within cultivar effect. Ratio SEM G. Sam./Medea indicates the extent to which the genotype effect is more prominent in Grasslands Samson.

3.3.6 Leaf gas exchange data

Leaf gas exchange measurements with the CIRAS-2 portable photosynthesis system conducted on the late morning of 26 August 2008 indicated that mean stomatal conductance of Medea leaf segments in the CIRAS chamber was around 40% higher than that of Grasslands Samson (P = 0.038) and this difference remained statistically significant (P = 0.043) after adjustment of the data to correct for difference in mean leaf width between Medea and Grasslands Samson leaves sampled (Table 3.5).

Table 3.5: Cultivar means for gas exchange parameters measured or calculated by the CIRAS-2 Portable Photosynthesis System for the second youngest leaf of a randomly selected tiller on 10 plants of Grasslands Samson and 10 plants of Medea on 26 August 2008.

Trait	G. Samson	Medea	P Cv	SEM
Pn (µ mol m^2s^{-1})	3.67	3.80	> 0.100	0.458
SC (m mol m^2s^{-1})	51.90	71.80	0.005	3.780
Evp (m mol m^2s^{-1})	0.82	1.01	0.008	0.038
LW (mm)	3.60	3.95	> 0.100	0.175
Ci (ppm)	279.50	300.30	> 0.100	10.300
T_L (C)$^-$	21.40	21.00	> 0.100	0.190
Pn-adj (µ mol m^2s^{-1})	4.03	3.80	> 0.100	0.486
SC-adj (m mol m^2s^{-1})	56.90	71.80	0.020	3.720
Evp-adj (m mol m^2s^{-1})	0.91	1.01	0.100	0.039

Pn = Photosynthetic rate, SC = Stomatal conductance, Evp = Evapotranspiration, Ci = Leaf internal CO_2 concentration, T_L = Leaf temperature, LW = Leaf width. Suffix 'adj' indicates data adjusted for cultivar difference in mean leaf width. PC2 from PCA = scores for Principal Component 2, from Principal Component Analysis.

When adjusted data for Pn, SC and Evp were entered into a PCA, together with variables Ci and T_L, PC1 appeared to reflect differences between individual plants while PC2 appeared to combine the stomatal conductance difference between Grasslands Samson and Medea with some statistically non significant trends in other variables to express the overall pattern of difference between the cultivars in gas exchange patterns (Table 3.6). Plants of cultivar Medea had a lower mean score PC2 than plants of Grasslands Samson.

Table 3.6: Principal components (PC) structure for PC1 and PC2 from Principal Components analysis (PCA) of gas exchange data for 20 perennial ryegrass plants (10 plants of Grasslands Samson and 10 plants of Medea).

Traits	PC1	PC2
% variation explained	56.2	27.0
Cumulative % variation	-	83.3
Pn-adj	0.558	-0.195
SC-adj	0.495	0.457
Evp-adj	0.538	0.261
Ci	-0.384	0.546
T_L	0.077	-0.621
P cv	0.704	0.042

3.4 Discussion

3.4.1 Plant response to the growth environment

It should be noted that although termed a 'winter' experiment, temperatures in the heated glasshouse lacked the overnight minima experienced by plants in the field at this time of year, with the thermostat set to keep the glasshouse temperature above 5°C. Meanwhile early afternoon temperatures briefly exceeded 25°C on some days (Fig. 3.2). To place these temperature data in context they were compared (Table 3.7) with data collected by Cooper (1964) in a series of experiments to compare leaf agronomic performance of *Lolium* and *Dactylis* seed lines of Mediterranean and north European origin.

Table 3.7: Comparison of temperature regimes for research of Cooper (1964) and for the present experiment.

Country	Months	Heating in glasshouse	Max. (°C)	Min. (°C)	Mean (°C)
UK	Oct – Feb	No	8	2	5.5
UK	Jan – Mar	No	19	6	12.8
UK	Mar – May	No	25	11	17.8
UK	Oct – Dec	Yes	21	13	16.7
NZ (present expt.)	Apr – Jul	Yes	25	4	12.1

It is seen that there is not an exact fit to any of the regimes reported in Cooper's (1964) work but that the temperature conditions in the present experiment approximated those of Cooper (1964) October – December experiment in a heated glasshouse, with maximum temperature similar to that of summer in the UK.

Another issue in interpreting these data is the light level. Intercepted radiation at canopy level in the present experiment was comparatively low at 1.2 MJ d^{-1}. This combination of high temperature and reduced light level would be expected to increase respiration and decrease photosynthesis relative to that of field plants though the extent to which plants experienced carbohydrate deficit stress as a result is uncertain. Since Cooper (1964) reports light in his experiments as hours of sunlight it is difficult to compare those data with the present ones, but it can be assumed that with shorter day lengths in Wales than in Palmerston North at the corresponding time of year, light levels might have been even lower in Cooper's (1964) experiments than in the present work. However, such conditions are not unusual in glasshouse experiments. For example, it can be calculated that thermal time (base temperature 4°C) was 9.3°C.d d^{-1}, and solar energy 0.13 MJ $(°C.d)^{-1}$ in this experiment. By comparison, corresponding values for an 'autumn' experiment of Sartie et al. (2011) to detect quantitative trait loci for herbage yield traits were 17°C.d d^{-1}, and 0.15 MJ $(°C.d)^{-1}$. Plant growth potential in the present experiment should therefore have been adequate for the purposes of cultivar comparison to assess salient differences in growth pattern.

The two cultivars had very similar A_{Lf} but Grasslands Samson plants attained larger size than Medea plants, meaning that the two cultivars differed significantly for most leaf and tiller characteristics, including LL, LW, LER, TN and TW, these component traits contributing to a more than 2-fold mean size increase in DW of Grasslands Samson plants compared to Medea plants. The only other paper known to the author to compare morphogenetic behavior of perennial ryegrass of temperate and Mediterranean origin is that of Cooper (1964), mentioned above. Direct comparison of the results is difficult because Cooper's (1964) results are mainly reported as comparative rates of leaf expansion for the first 6 leaves of seedlings. However, Cooper (1964) reported relative leaf expansion at 5°C relative to 25/12°C of 1.40 and 1.34 for two Algerian lines compared to 1.4 and 1.09, respectively, for 'local varieties' from New Zealand and Oregon. Cooper (1964) also indicated (in his Fig. 3) a higher leaf expansion rate for Algerian material than for the new Zealand local cultivar at 5 and 10°C than at 25/12°C, and his overall conclusion was that in winter growth conditions, Mediterranean populations of perennial ryegrass exhibit higher leaf area expansion than European lines.

Therefore, two explanations are available for the significantly lower leaf and tiller growth of Medea than Grasslands Samson in the present experiment: either that the breeding of Medea did not capture the potential for rapid winter growth for which Mediterranean forage grass material has become well known (Cooper, 1964; Lelièvre and Volaire, 2009) or that temperatures in the present experiment were too warm for this winter growth potential of Mediterranean germplasm to have been expressed. Considering only the temperature regime comparison in Table 3.7 the possibility of comparatively high mid-day temperatures on some days causing commencement of summer dormancy and a suppression of leaf growth in Medea cannot be ruled out, but the results do seem to indicate that Medea does not possess the potential for high cool-season growth often seen in forage grasses of Mediterranean origin.

Further evidence on this point is available from work of Silsbury (1969), who reported that at 10°C in a growth chamber with approximately 8 MJ d^{-1} light exposure (140 W m^{-2} with 16 h photoperiod), potted plants of Mediterranean ryegrass selection 'Ga40' accumulated seedling weight after germination more slowly than Grasslands Ruanui (2.2 v. 2.7 mg $plant^{-1}$) and Medea also grew more

slowly at 10°C, but not to the extent reported here (0.8 v. 0.9g pot^{-1}, respectively, from 32 days growth). Also, in a field experiment near Christchurch, Vartha (1975) recorded similar yields for Medea and Grasslands Ruanui during winter, although lower total yield for Medea than Grasslands Ruanui on an annual basis. Thus, it is difficult to interpret the present results with certainty. On the one hand, comparison between the present results and those of Cooper (1964)would seem to indicate that in the breeding of Medea, the selection focus on summer dormancy and drought survival might have incurred a loss of winter growth potential of the Mediterranean material. However, based on results of Silsbury (1969) and Vartha (1975), Medea growth in winter would have been expected to be close to that of a temperate cultivar, so the twofold superiority in yield of Grasslands Samson compared to Medea points to a partial expression of summer dormancy of Medea as a possible factor in the present results.

3.4.2 Comparison of morphogenetic traits in Grasslands Samson and Medea

Besides the larger tiller and plant size of Grasslands Samson than Medea discussed above, two other notable points relating to morphogenesis were the similarity in A_{Lf}, and a progressive loss of difference in LER between Medea and Grasslands Samson (Tables 3.1 and 3.2). Evidently, response of A_{Lf} in ryegrass to temperature lacks variation across a diverse range of plant material because Cooper (1964) also reported little or no difference in A_{Lf}, for germplasm of diverse origins. It is interesting to note that LER of Medea increased from 84% of Grasslands Samson for the first leaf observed after transplanting (L1) to 93% of Grasslands Samson for L3 (Table 3.1). A parallel trend was seen in LL, and similarly RTAR did not differ significantly between the cultivars in the regrowth after defoliation in early July (Table 3.2). It is unclear if this comparatively larger difference in growth rate between Medea and Grasslands Samson in the earlier part of the experiment represents slower seedling establishment of Medea as reported by Silsbury (1969) for Ga40 ryegrass of Mediterranean origin, comparatively better growth of Medea in response to falling temperatures in the glasshouse with decreasing day length from May to July, or some other factor. The increasing overlap in time for elongation of L3 and L4 compared to L3 and L2, and L2 and L1 (Fig. 3.4) would have contributed

to increasing LL of successive leaves (Table 3.1), and this is a well known feature of recovery from defoliation in forage grasses.

Since TN and TW are yield components of DW, it has been a point of discussion among forage agronomists whether TN or TW is the more important contributing factor to DW. More recently a consensus has emerged that the answer to this question depends on sward conditions. Where a sward has low leaf area either, in the plant establishment phase or following defoliation, TN is important. In closed canopy conditions larger TW is more commonly the principal contributing factor to increased DW or yield. The stronger correlation between DW and TN than between DW and TW (Table 3.3) is consistent with the plants in this experiment being developing seedlings in the establishment phase, and this point is confirmed by the larger coefficient for TN (0.603) than TW (0.242) when DW was regressed on standardised data for TN and TW, removing the effect of comparative scale of the variables TN and TW.

Increased tiller production is necessarily associated with increased relative tiller appearance rate and site filling, and this explains the significantly higher RTAR and Fs. for Grasslands Samson in the current results.

Average values for site filling (Fs), a measure of readiness of leaf axillary buds to develop new tillers (Neuteboom and Lantinga, 1989), were 0.216 and 0.104 for Grasslands Samson and Medea, respectively. By comparison, the maximum value of site filling in perennial ryegrass is 0.693 when prophyll buds of each tiller develop (Neuteboom and Lantinga, 1989), or 0.481 where they do not (Davies and Thomas, 1983). Values near the maximum were reported by Neuteboom and Lantinga (1989). The lower values of site filling in the present study may well indicate sub-optimal growth conditions arising from the comparatively low light level per unit of thermal time as discussed above.

Correlations between measured variables can arise for a number of reasons. Some variables (e.g. TN, Fs and RTAR) are correlated because they are mathematically interdependent. Other variables correlate because they at least partly measure the same thing, for example variables like LL, LER, TN and TW, which are all expected

to vary with plant size, DW. A third category of correlation is when variables have a common influence. An example of this in the present data set occurs because Medea plants normally have a lower DW than Grasslands Samson plants (Fig. 3.5), but a longer PsL, leading to a negative correlation in the data between PsL and traits like LN, TN, RTAR, and Fs, which contribute to DW.

Of greatest interest are those correlations that reveal something about the functional inter-relationship of morphogenetic variables. Results in Table 3.3 that possibly fall into this category include: (i) the positive correlations of PsL with LER and TW; (ii) the negative correlations between A_{Lf} and LED, and LER; (iii) the lack of a significant negative correlation between TN and TW; and (iv) the negative correlation between A_{Lf} and TN.

In relation to point (i), a long PsL in Grasslands Impact ryegrass, compared to Grasslands Samson, was found by Sartie et al. (2009) to affect tiller morphogenesis, leading to a longer than expected leaf length in Grasslands Impact, relative to tiller size. This same mechanism operating in Medea compared to Grasslands Samson could explain why PsL was positively correlated with LER and TW, when other measures linked to DW were negatively correlated to DW, reflecting the generally lower DW in Medea than in Grasslands Samson, but it was outside the scope of this experiment to follow up on this point.

In relation to points (ii) to (iv), a similar analysis of a plant population (Sartie et al., 2011) also found negative correlation between A_{Lf} and LER, yet there is no *a priori* reason why a longer A_{Lf} and LED should not be associated with a longer LL, rather than a reduced LER. However, in contrast to the present study where there was no significant correlation between TN and TW or between TN and LER, and a negative correlation between TN and A_{Lf}, Sartie et al. (2011) found strong negative correlations between TN and TW, a weaker negative correlation between TN and LER, and positive correlations between TN and A_{Lf}. Theoretically, longer A_{Lf} means fewer shoot buds produced over a period of time, and therefore a lower TN development over time, unless there are compensatory changes in Fs. Complex interactions like this are hard to unravel, and it is unclear in this case if they relate to

differences between cultivars between plant growth strategy, or differences in plant growing conditions.

PCA can assist in unraveling complex trait associations, partly because each PC represents a set of trait associations uncorrelated with all other PCs, partly because the proportion of the total data variation explained by each PC is represented by the eigenvalue, and partly because coefficient structures of PCs indicate the contribution of particular variables after correction for the effects of other variables. In this way, 'hidden' associations can emerge, analogous to a situation where the residuals from a regression analysis of two primary variables are found to be correlated with a third variable, but this correlation is evident from calculating the correlation between either of the primary variables and the third variable. As noted above in Section 3.3.5, PC1 can be understood as separating individual plants based on size, so there is a strong cultivar effect in ANOVA of scores for this PC (Table 3.4), while PC2 and PC3 involve, respectively, TW and related variables, and a contrast between PsL and A_{Lf} or LED. PCs 2 and 3 are shown by ANOVA of their scores to reflect variation between genotypes within cultivars (Table 3.4) and the ratio of SEM for the two cultivars was calculated as a measure of whether or not a particular cultivar contributed more to variation between genotypes in scores for this PC. In this way PC2 is revealed to reflect the wider horizontal spread between individual plants of Grasslands Samson than plants of Medea for TN at a given DW (Fig. 3.5) and PC3 is revealed to relate more strongly to Medea and its tendency for greater PsL. Hence, it can be said that in this data set, no mechanistic trait associations are uncovered by PCA.

3.4.3 Comparison of gas exchange traits in Grasslands Samson and Medea

The single measurement of leaf gas exchange parameters in Experiment 1 indicated similar net assimilation rate for the two cultivars, but a significantly higher stomatal conductance (SC) for Medea than for Grasslands Samson. Since the primary data collected by the CIRAS is amount of CO_2 removed from, and the amount of water added to the air stream passing around the leaf in the chamber of the instrument, it is expected that derived variables like Ci will also show effects consistent with

increased SC. It is reassuring, however, that the independently measured variable T_L was also lower in Medea, consistent with greater evaporative cooling expected in a plant with high SC. Coefficients for PC1 from PCA of the data also linked the measured variables in a manner consistent with increased SC. Increased SC is consistent with the observation of plants of Medea in a glasshouse at AgResearch during the planning of the experiment having more flaccid leaves than other ryegrass plants. This observation raises interesting questions for further study, as published reports on Medea appear to make no mention of this trait.

3.5 Conclusions

- Medea ryegrass was significantly less productive than Grasslands Samson, in this Experiment, possibly because temperature within the glasshouse was high enough to trigger partial onset of summer dormancy, and possibly because it is inherently less productive as a result of emphasis on summer survival rather than winter growth in the selection of Medea.
- Smaller plant size in Medea arose from reduced LER and Fs leading to fewer and smaller tillers per plant in Medea than Grasslands Samson.
- A_{Lf} of the two cultivars was similar and there was no evidence that Medea had a different pattern of morphogenetic development from Grasslands Samson.
- These differences in LER and Fs were less pronounced at the end of the experiment, possibly reflecting a tendency to slower seedling development in Medea, or possibly a response to cooler temperatures in July. However, it maintained a steady state of growth at later leaf stages after seedling establishment. It is presumed that glasshouse conditions provided environmental conditions such as to suppress growth of Medea plants; otherwise they could produce as high as Grasslands Samson.
- In a single measurement of gas exchange Medea exhibited higher stomatal conductance than Grasslands Samson. This trend corroborates similar studies on Mediterranean cultivars of tall fescue and also requires further study.

4

A survey of traits contributing to drought resistance in Medea and some current New Zealand commercial cultivars of perennial ryegrass (*Lolium perenne* L.)

4.1 Introduction

The potential for summer dormant germplasm of Mediterranean origin to be used in forage grass breeding programmes aimed at enhanced drought resistance has been long known and was discussed in Section 2.8. However, studies to explore specific drought resistance mechanisms of this Mediterranean germplasm were only started in the late 1990s and most of those studies that have been carried out have focussed on tall fescue and cocksfoot with almost no information available on perennial ryegrass. Therefore, before using a Mediterranean cultivar of perennial ryegrass such as Medea (reportedly possessing summer dormancy) for introgression with a temperate cultivar (in this case Grasslands Samson) it was imperative to know what particular mechanisms the Mediterranean cultivar possesses to endure drought and how these mechanisms differ from those of Grasslands Samson. A secondary question also explored in this experiment for two arbitrarily selected cultivars was whether or not there are differences in drought resistance mechanisms between existing New Zealand commercial cultivars in drought resistance mechanism. Finally, it was of interest to develop a set of measurements that would define the drought resistance mechanism of individual plants in terms of the logical framework expressed in Section 2.4.4. Technique development was required because historically a majority of studies on drought resistance have monitored specific mechanisms. For example, Thomas (1990) and Thomas (1991) studied osmotic adjustment in perennial ryegrass and identified the nature of osmolytes contributing to osmotic adjustment. Root development and soil water extraction in isolation of other mechanisms was studied by Crush et al. (2007) and Wedderburn et al. (2010). Likewise plant water relations and shoot growth were studied by Thomas and Evans (1989). Few studies, including

those of Volaire et al. (1998b) and Wang and Bughrara (2008) have presented a holistic picture of multiple traits, ranging from root growth and water uptake, shoot growth, plant water relations to osmotic adjustment contributing to drought resistance of perennial ryegrass.

These considerations were the basis for two experiments, with aims as defined below, Experiment 2 (September – December 2008), and Experiment 5 (September 2010 – January 2011).

4.1.1 Aims for Experiment 2 and Experiment 5

(i) To gain experience with equipment and techniques for investigation of drought resistance mechanisms in perennial ryegrass (Experiment 2);

(ii) To develop a methodology for defining drought resistance as a suite of traits operating in an integrated way for a plant as a whole and use this methodology to explore drought resistance mechanisms of Medea (Experiment 2);

(iii) To compare drought resistance mechanisms of Medea identified in (ii) above with those of Grasslands Samson and with other current commercial New Zealand perennial ryegrass cultivars of differing breeding background (Experiment 5).

The current New Zealand cultivars chosen for inclusion in Experiment 2 besides Medea were: Grasslands Samson, an unreleased tetraploid breeding line GAT 101 derived from Grasslands Samson, designated here Samson (4n), and Tolosa. Cultivars chosen for Experiment 5 besides Medea and Grasslands Samson were Matrix and Ceres One50. According to Stewart (2006) Tolosa has a breeding background of introgression of Spanish germplasm, and Matrix has a mix of genetic backgrounds including the so-called Mangere ecotype and meadow fescue introgression. Ceres One50, marketed by Agricom, according to company promotional literature, was developed by crossing elite New Zealand and north west Spanish germplasm. Some further details of cultivar origins were discussed in sections 2.6 and 2.8.

4.2 Materials and Methods

4.2.1 Experiment 2 (September – December 2008)

4.2.1.1 Location, design and setting up

Experiment 2 was conducted from 2 September to late December 2008, in a glasshouse at the Plant Growth Unit, Massey University, Palmerston North, New Zealand. The experiment comprised two water treatments (a well watered control and a water deficit regime) and two destructive harvests intended to indicate change in plant response with increasing severity of water deficit. Seeds of the three cultivars Grasslands Samson, Medea, and Tolosa and of Samson (4n) were pre-germinated at 20°C and transferred on 2 - 3 September 2008 to PVC pots in the glasshouse, with four seeds retained in every pot. There were three replications, and hence 48 pots in total, arranged as shown in Fig. 4.1 below. For convenience of reporting, the tetraploid derived from Grasslands Samson is referred to here as a 'cultivar' even though it is technically a breeding line and not a named cultivar

R3	Harvest 1	C4 D	C4 W	C1 W	C1 D	C3 D	C3 W	C2 D	C2 W
	Harvest 2	C1 W	C1 D	C4 D	C4 W	C2 W	C2 D	C3 W	C3 D
R2	Harvest 2	C2 D	C2 W	C4 D	C4 W	C3 W	C3 D	C1 D	C1 W
	Harvest 1	C3 W	C3 D	C1 W	C1 D	C4 W	C4 D	C2 W	C2 D
R1	Harvest 1	C3 D	C3 W	C2 D	C2 W	C4 W	C4 D	C1 D	C1 W
	Harvest 2	C4 W	C4 D	C2 W	C2 D	C1 D	C1 W	C3 W	C3 D

Figure 4.1: Randomised complete block layout used for plants in Experiment 2. R1, R2 and R3, denote replicates with two harvests in each case. C indicates cultivar or breeding line. 1 = Grasslands Samson, 2 = Medea, 3 = Samson (4n), 4 = Tolosa. W = well watered; D = dry.

Pots were constructed from pipes of 100 cm length and 15 cm diameter split longitudinally and held together with nylon cable-ties. The pots were filled with a mixture of B horizon of a Manawatu Silt Loam recent alluvial soil and builder's sand in the ratio 3:1 and fertilised with a slow release proprietary fertiliser, 'Osmocote', at approximately 6 g per 150 litres of soil and judged sufficient to facilitate unrestricted plant growth. The pots were lined internally with a transparent heavy duty plastic tubular sleeve of the same diameter as the pot and in each pot two vertical cuts (each

of 5 cm length) through the sleeve were made near the base to ensure drainage of excess water. Holes were also drilled in the pots to allow horizontal insertion of time domain reflectometer (TDR) probes for soil moisture measurement. Before transplanting, the endophyte status of the Medea, Grasslands Samson, and Samson (4n) plants was evaluated using an ELISA procedure (Appendix 4.1). For testing, a single tiller from each plant was severed about 2 cm above ground level with a scalpel and the cut end dabbed onto paper impregnated with rabbit serum antibodies to *Neotyphodium* endophyte. The specially prepared paper was provided by AgResearch, Palmerston North. The seed of Tolosa was supplied by NZ Agriseeds Ltd. as 'endophyte free' and was not tested for the presence of endophyte.

Plants were watered through a PVC tube of 27 cm length and 2 cm diameter in the centre of each pot and penetrating 20 cm below the soil surface to deliver water directly to the root zone rather than the soil surface (Fig. 4.2). This tube was kept in place between waterings though it was removed, cleaned and replaced occasionally to remove any soil blocking the flow of water. Water was fed into the tubes via another PVC tube of the same diameter and 70 cm long, and connected to the lower tube by a tap (Fig. 4.3). When applying water, a plastic funnel was inserted into the top of the watering tube to avoid spillage.

Figure 4.2: Experiment 2 in October 2008 before the introduction of differential watering. A watering tube can be seen in the pot nearest the camera.

Figure 4.3: Plants of Experiment 2 in late November 2008 with 70 cm watering tubes and taps in place. The pipes provided static head pressure to promote water infiltration into the soil.

Water was applied at a rate of 200 ml per pot per week until 30 October 2008 after which a system of differential watering was introduced. For control plants 900 ml of water per pot was applied at each watering whereas the stressed plants were given 300 ml of water at each watering. Watering was initially weekly, but became more frequent as day length and temperature increased with the onset of summer, and plants grew larger. To determine when water was needed, randomly chosen pots were weighed every 3 – 4 days (data not shown) to estimate the plant water use. The water ration was increased on 24 November from 900 ml to 1350 ml of water per pot per week for control plants and 450 ml for stressed plants and was kept unchanged until 8 December when the water allocation was again increased to 1500 and 500 ml per pot per week for control and stressed plants, respectively.

Temperature in the glasshouse was monitored with a datalogger and thermocouple probes (Skye Instruments, Llandrindod Wells, Wales). When the maximum temperature on a sunny day in early December was observed to reach 47°C action was taken and two days later on 9 December the glasshouse was covered with an exterior nylon mesh shade cloth stated by the manufacturer to provide 50% reduction

in solar radiation. The shade cloth was effective in preventing a recurrence of excessive temperatures.

Twenty four pots designated for Harvest 1 (Harv1) were destructively harvested between 24 and 30 November, 24 days after differential watering (DADW) was commenced, and Harvest 2 (Harv2) was carried out between 22 and 26 December (52 DADW). Pots of Harv2 were defoliated on 28 November, to 5 cm above soil surface level.

4.2.1.2 Measurements (Experiment 2)

A suite of measurements was constructed to gain information about behaviour differences between the ryegrass cultivars for each of the four water deficit plant response domains identified in section 2.4.4.

4.2.1.2.1 Measures of shoot growth

Tiller number per plant (TN) for Harv1 plants was counted before harvesting on 24 November (24 DADW) whereas tiller number in Harv2 plants was counted on 30 November (30 DADW), with numbers of both flowering and non-flowering tillers recorded.

Leaf elongation rate (LER) was measured on 3 December, five days after defoliating the plants to 5 cm above soil surface by measuring the length of the emerging leaf for one tiller in each of the four plants per pot. Data were collected using a ruler and rounded to the nearest mm.

Four components of plant dry weight leaf laminae (Llam), pseudostem (Ps), dead leaves (Ldead) and sead-head (H) were determined for each of the harvests by sorting a subsample of about 25% of the herbage in each pot. All the four herbage components together with the unsorted herbage were oven dried at 80°C for 48 hours and their respective dry weights recorded. Finally the dry weights for the herbage components were used to compute percentage of each of the components in the bulk sample (reported below as Llam%, Ps:Llam, Ldead% and H%).

4.2.1.2.2 Measures of root development and water uptake

Root biomass was determined at the Harv1 and Harv2 destructive harvests in late November and late December, respectively, for 'coarse' and 'fine' root fractions. Pots were cut open and the internal plastic sleeve with enclosed soil separated into three segments, 0 – 30, 30 – 55 cm and below 55 cm from the soil surface (d1, d2, and d3, respectively). To extract 'coarse' roots (Rc) from each segment, soil was tipped into a plastic box of 30 litres volume and visible roots handpicked until no more could be found on mixing the soil. To extract 'fine' roots (Rf) left behind by the hand picking process, approximately 500 g of soil was sub-sampled and stored in a plastic bag at 4°C for later washing, with subsample and total soil weights recorded to the nearest g. Rf were extracted from the soil a few weeks later using the root washer described by Matthew (1992). Retrieved roots, both Rc and Rf, were oven dried at 80°C for 48 hours and weighed. It is the author's understanding that the separation of fine and coarse root fractions in this way has been practiced in Palmerston North for 20 years or more and is a methodology anecdotally attributed to Professor S. Barber of Purdue University.

Volumetric soil moisture content (SMC) was determined at three soil depths (20 cm, 45 cm and 70 cm below the soil surface) using TDR (Trace System-Soil Moisture Equipment Copr., Santa Barbara, California, USA) on 24 November (24 DADW) for Harv1 pots and on 6th November (6 DADW) for Harv2 pots.

4.2.1.2.3 Measures of plant water status

RWC was determined according to the method of Barrs and Weatherley (1962) using Eq. 2.2 (Section 2.3.1.1). Four leaf laminae segments, each of 2 cm length, chopped from a leaf lamina of leaf position No. 2 were made to float on 10 ml of de-ionized water after measuring their fresh weight. Turgid fresh weight was determined after an imbibition period of four hours. Dry weight was determined after incubation of the leaf segments in oven at 80°C for 48 hours. RWC was measured on 5 November (7 DADW) and on 14 November (24 DADW) in Harv1 plants only. These two measurements of RWC are designated RWC1 and RWC2 in the subsequent sections of this chapter.

4.2.1.2.4 Measures of stomatal and cellular control and dehydration tolerance

Instantaneous rates of Pn, Evp and SC were measured on all plants of Harv1 and Harv2 on 3 November 2008 (3 DADW) using a CIRAS-2 Portable Photosynthesis System manufactured by PP Systems Ltd. Further details of this instrument are given in section 3.2.2. Measurements were performed between 1100 h and 1300 h.

4.2.1.2.5 Statistical analysis

Data for the various measurements of water deficit response in the 48 pots were analysed in a factorial model, extracting sums of squares for replicates, and for cultivars, harvests, water regime, and their two- and three-way interactions. For some measurements where only pots of one harvest were measured (24 plants), the ANOVA was reduced accordingly. Both SAS 9.2 (SAS Institute Inc., NC, USA) and Minitab 16 (Minitab Inc., 2009) were used to perform the ANOVA at different times, but the choice of software was not considered important as an early trial analysis confirmed both gave identical results.

4.2.2 Experiment 5 (September 2010 – January 2011)

4.2.2.1 Location, design and setting up

Experiment 5 was conducted from 20 September 2010 to late January 2011, in a glasshouse at the Plant Growth Unit Massey University, near to the one used for Experiment 2. This experiment was housed in centre of the glasshouse where two replications of another experiment (Experiment 6; reported in Chapter 7) were placed. In this experiment seeds of cultivars Ceres One50 and Matrix (obtained from Agricom and Cropmark Seeds, respectively, and supplied as endophyte free) and those of Grasslands Samson and Medea (obtained from AgResearch, and known from prior testing to be endophyte free) were sown on soil on 20 September 2010 in pots constructed from PVC plastic water pipes of 10 cm diameter and 100 cm height filled with a mixture of B horizon of a Manawatu Silt Loam alluvial soil and sand in the ratio 3:1 and fertilised with the slow release fertiliser ‘Osmocote’ as in Experiment 2. The pots had gauze taped to their bases to facilitate watering by immersing their lower ends into a drum of water to a predetermined depth. Topsoil was kept moist by sprinkling about 100 ml of water daily on the soil during the

germination phase. Most of seeds germinated within 7 – 10 days of planting. One robust seedling in each pot was retained and the remaining seedlings were uprooted. Pots arranged in a Randomised Complete Block design (Fig. 4.4), were placed in the glasshouse in 200 litre drums which had a tap at the base (Fig. 4.5). During the plant establishment phase of the experiment the plants were watered by filling the drums to a level of 45 cm below the soil surface. Soil was allowed to aerate by draining the water from the drums through the tap for 8 hours once every 7 – 10 days, and then refilling the drums. This water level was maintained until imposition n of water deficit as described below.

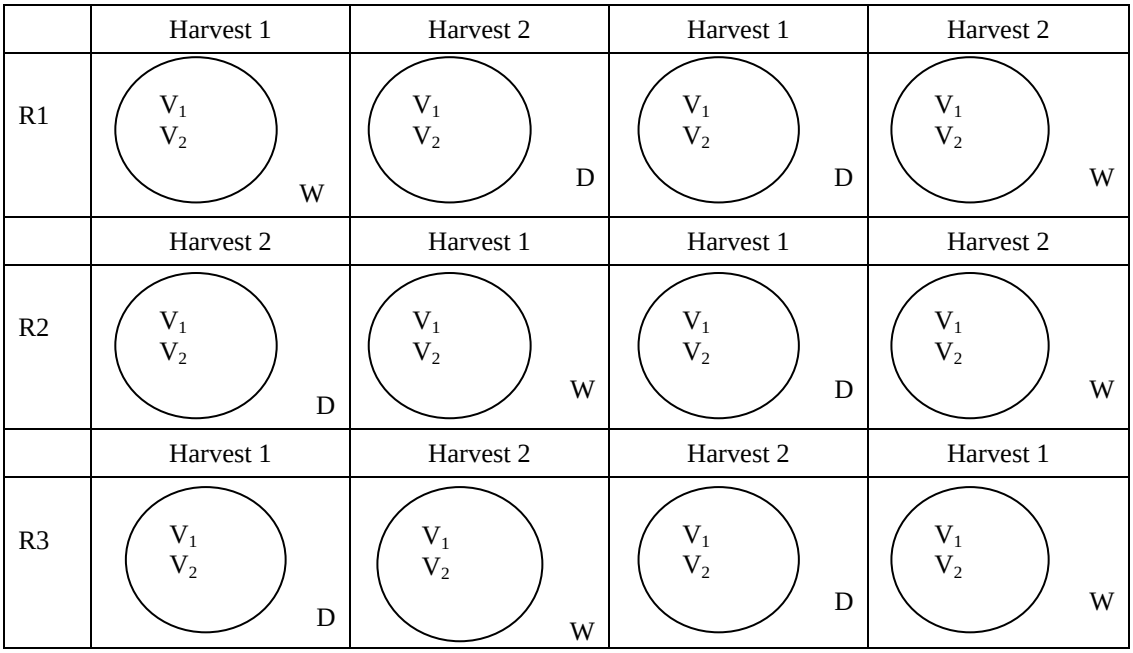

Figure 4.4: Randomized Complete Block layout used for plants in Experiment 5. R1, R2 and R3, denote replicates with two harvests in each case [W = well watered; D = dry]. Circle represents the drum of water in which the four cultivars (V_1 to V_4) were randomized. V_1 = Grasslands Samson, V_2 = Medea, V_3 = Ceres One50, V_4 = Matrix.

Figure 4.5: Arrangement of pots in 200 litre drums fitted with a plastic tap at the bottom in Experiment 5. Pots were constructed from 100 cm sections of PVC plastic waterpipe. Watering of plants was controlled by manipulating the water levels in the drums.

Plants of Harv1 were subjected to different water treatments from 23 November 2010. On that date water was drained from Harv1 drums designated as "dry", keeping 5 cm of water at the base of each drum - just enough to provide some moisture to the lower soil horizon. Plants were allowed to grow for another week. On 29 November the remaining water was removed from these drums and from that point no further water was added. Meanwhile, the water level in "wet" drums was maintained at 45 cm below the soil surface, with periodic drainage for aeration.

Water levels in "dry" drums of Harv2 plants were lowered to 5 cm from the base of the drums on 7 January 2011, while the water level of "wet" drums of Harv2 were maintained at 45 cm below the soil surface.

Temperature in the glasshouse was monitored for the period 21 December to 26 January with the datalogger and thermocouple probes (Skye Instruments, Llandrindod Wells, Wales) used in Experiment 2.

Twenty four pots designated for Harv1 were destructively harvested between 7 and 9 December (14 DADW), and those of Harv2 between 20 and 22 January (13 DADW).

4.2.2.2 Measurements (Experiment 5)

4.2.2.2.1 Measures of shoot growth

TN per plant was counted on 17 December (88 days after sowing) by subsampling the larger plants into groups of 2 or 4.

The plants started producing seed-heads by the end of October. Emerged seed-heads were removed approximately twice weekly by cutting at node number 2 of the stem (counting from top to bottom). Seed-head number (HN) was recorded, as well as seed-head weight (HW) after oven drying the seed-heads at 65°C.

LER was recorded on 20 January, 2011 on plants of Harv2 after one day of defoliation to 7 cm from soil surface.

Herbage to 7 cm height from soil surface was taken on 19 January, dried and weighed. Stubble from ground level to 7 cm recovered when doing root sampling and added to the previous sample weight.

4.2.2.2.2 Measures of root development and water uptake

Plants of Harv1 were brought to "AgHort C" field sample laboratory at Massey University on 7 December 2010 for recovering stubbles and root retrieval. Plastic sleeves containing the soil and plants were slid out of the pots and the soil column was cut with a heavy knife into three depth segments: 0 – 40 cm, 40 – 70 cm and below 70 cm. (Note that boundaries between d1, d2 and d3 were not identical to those used in Experiment 2 (refer Section 4.2.1.2.2).

A slice of approximately 5 cm length was removed from the middle of each of the three soil depth segments to obtain a subsample of soil for determination of

gravimetric SMC. After hand removal of Rc, the soil subsamples were stored briefly (typically less than 24h) in sealed plastic bags with care taken to prevent condensation forming inside the bags. Gravimetric SMC was calculated (after weighing the moist samples, oven drying at 105°C for 48 hours, and reweighing the dry samples) as weight of moisture lost on drying: weight of dry soil.

Rc and Rf fractions of roots were extracted and dried as described in Section 4.2.1.2.2, with Rc collected from subsamples for determining gravimetric SMC added back to the main sample in each case. Rf were extracted on 13 and 14 December.

Plants of Harv2 were brought to the field sample laboratory on 20 January 2011 and processed as described above for plants of Harv1, to obtain data for gravimetric SMC and the Rc and Rf root fractions. Rf were extracted on 25 and 26 January**.**

4.2.2.2.3 Measures of plant water status

Experiment 5 used a procedure that had been evolved during earlier experiments whereby a predawn measurement of LWP was made on plants of one replicate each day and immediately following measurement of LWP, further leaf lamina samples for determination of OP, RWC and lamina proline levels were collected from the same plants. This sampling strategy aimed to ensure that detection of any inter-relationship between the various measures of plant water status was not compromised by their being measured at different times.

Harv1 plants were measured in this way from 30 November to 2 December (7 – 10 DADW). The pre-dawn LWP measurement was taken using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara. CA; (Scholander et al., 1965) between 0500 h and 0700 h. A lamina tip of about 5 cm length from leaf 2 of a healthy tiller was sampled with a scalpel blade, wrapped in a plastic sleeve and loaded immediately into the pressure chamber with the cut end protruding out. Pressure in the chamber was then gradually increased using nitrogen gas and LWP (bars) was read from an analogue meter on the pressure chamber when the exudation of xylem cell sap was first detected and later converted to MPa for data presentation.

For OP the lower 2 cm part of the sample leaf for LWP was clipped and immediately immersed in liquid nitrogen for 15 – 20 seconds, then stored in a clip-seal plastic sample bag. Samples snap-frozen in this way were then placed in a box of dry ice until sampling for the day was completed, and then transferred to longer-term storage at -80°C for subsequent determination of OP with a thermocouple psychrometer Wescor HR 33 T. The samples were later thawed at room temperature and loaded into sample chambers of the psychrometer. Readings of thermocouple output (πv) were determined after 2 hours of equilibration. Final values of OP (in bars) were calculated by conversion of πv values through a standard curve prepared from NaCl solutions of different strengths. PP was estimated by subtracting OP from LWP values (see Section 2.3).

Samples for RWC determination were collected immediately following determination of LWP and collection of OP samples. RWC was determined using the procedure of Barrs and Weatherley (1962) but with slight modification. Three leaf lamina tips, each about 5 cm in length, from three leaves at leaf position 2 of three healthy tillers were clipped and immediately weighed to give the fresh weight. The cut segments of leaves were then placed with their ends resting in 1 ml of de-ionized water in a test tube while keeping the remainder of the leaf laminae dry. After 24 hours of imbibition the leaves were taken out of the water, the wet ends were blotted dry using tissue paper and the samples were weighed immediately to give turgid fresh weight. Finally, dry weight was recorded after oven-drying the samples at 65°C for 48 hours.

For Harv2 plants, measurement of LWP with concurrent sampling for OP was carried out pre-dawn between 17 January and 19 January 2011 (10 – 12 DADW); one replication each day. Samples for measurement of OP were tested subsequently (after temporary storage at -80°C) and PP calculated by subtraction as mentioned above.

Sampling for proline determination was carried out each morning between 0700 h and 0730 h after measuring LWP and sampling for OP and RWC determination. Proline contents were analysed using the method of Bates et al. (1973). Leaf samples (approximately 0.1 g) were snap frozen in liquid nitrogen and then homogenized in 5 ml of 3% sulphosalicylic acid using a mortar and pestle. The homogenate was

filtered through a Whatman No. 2 filter paper; 2 ml of the filtrate was placed in a test tube with 2 ml of glacial acetic acid and 2 ml of acid-ninhydrin (1.25 g ninhydrin dissolved in a blend of 30 ml glacial acetic acid, and 20 ml of 6 M phosphoric acid). The reaction mixture was boiled in a water bath at 100°C for 1 hour. After cooling, 4 ml of toluene was added to the reaction mixture. After vortexing for 15 – 20 seconds the chromophore containing toluene was separated and absorbance was read at 520 nm using a Bausch & Lomb Spectronic 20 Spectrophotometer. Final values for proline contents (mg/g DW) were calculated from a standard curve obtained by plotting absorbance values of 0, 4, 8, 12, 16 and 20 µg proline standards.

4.2.2.2.4 Measures of stomatal and cellular control and dehydration tolerance

Visual scoring for leaf rolling (Lrs), leaf wilting (Lws) and leaf colour (Lcs) was carried out on the plants. Scores from 1 to 3 were given to plants depending on the extent to which leaves were rolled, wilted or exhibited a "blue" colour change indicative of water deficit. Details of the ranking criteria appear in Table 4.1.

Table 4.1: Criteria for scores (1 to 3) for leaf rolling (Lrs), leaf wilting (Lws) and degree of blue colour change (Lcs).

Score	Leaf Rolling	Leaf Wilting	Degree of blue colour change
1	No rolling to slightly rolled	Not wilted or slightly wilted: Leaf 2 linear and near vertical	Leaves showing no change from normal hydrated colour
2	Moderately rolled: opposite edges forming an angle of more than 30° from horizontal	Wilted: Leaf 2 with strong curvature but leaf tip less than 45° below the horizontal	Visually discernible increase in blue-green hue of leaf laminae compared to fully hydrated colour
3	Tubular: opposite edges almost touching or touching	Severely wilted: Leaf two drooping and leaf tip more than 45° below the horizontal	Blue green hue of leaf lamina subjectively very obvious
Reference	modified from Bittman and Simpson (1989)	Modified from Engelbrecht et al. (2007)	Personally developed.

4.2.2.2.5 Statistical analysis

Statistical analysis for Experiment 5 was identical to that used in Experiment 2.

4.3 Results

The experimental design used for Experiments 2 and 5 generated a large volume of data with a range of different statistical effects extracted in the ANOVA, therefore it was felt helpful to adopt a standardised presentation where practicable. On this basis, the format adopted is that the statistical significance of all terms in the ANOVA model is presented first, then means with their SEMs for the main effects of cultivar and water regime (whether statistically significant or not), followed by graphical or textual description of any statistically significant main effects and interactions. Data are grouped into the 'plant response domains' (Section 2.4.4), with data related to plant yield presented first, followed by data on root development and water uptake, plant water status, and stomatal and cellular control, in that order. To extract greater biological insight from the data collected during conduct of the experiments some data were transformed into ratios or percentages, where applicable, and presented in separate tables..

4.3.1 Experiment 2

4.3.1.1 Glasshouse temperatures

Daily minimum temperature inside the glasshouse generally ranged between 10 and 18°C, and daily maximum temperature between 20 and 35°C but with a peak of 46°C on 9 December, after which a shade cloth was installed over the exterior of the glasshouse, to prevent any similar events (Fig. 4.6).

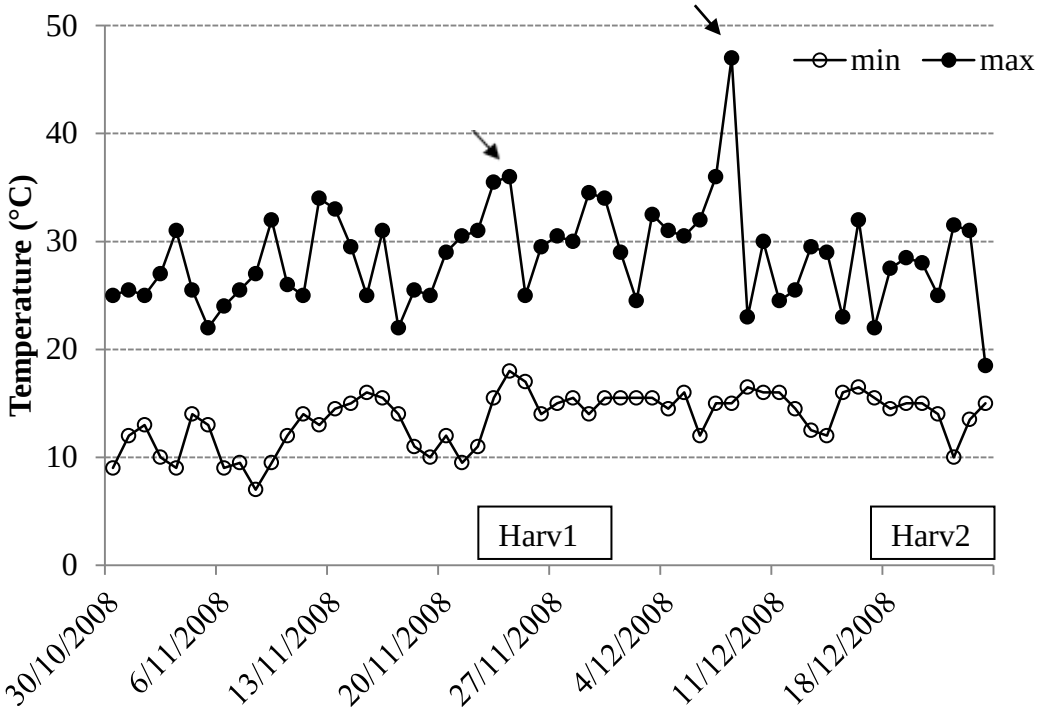

Figure 4.6: Daily maximum and minimum glasshouse temperature during the application of drought treatments in Experiment 2. The upper arrows show two extreme maxima that occurred. Following the 46°C peak on 9 December shade cloth was installed and the temperature was subsequently controlled. The lower arrows show the dates (24 November and 22 December 2008) of Harv1 and Harv2.

4.3.1.2 Shoot growth and growth components

Plants of Medea were smaller than those of the other cultivars while, in line with expectations, water deficit reduced DW, TN, and LER and increased Ldead% and Ps:Llam ratio (Tables 4.2 & 4.3). Most of the interaction terms were non-significant, or at least small by comparison with the three main-effects (Table 4.2).

For TN, cultivars Tolosa (55.1) and Grasslands Samson (51.9) had the highest average number per plant while Medea and Samson (4n) were much lower with values of 31.3 and 36.1, respectively. For shoot DW, Medea, with a value of 18.34 g, produced almost 35% less herbage than the other cultivars, which were statistically equal. The lower TN for Samson (4n), but similar shoot DW, when compared to Grasslands Samson and Tolosa indicates a larger average tiller size in the tetraploid (Table 4.3). A notable feature of Medea was that this cultivar had much higher values for seed-head related traits such as H% (34.4%) and also for Ps:Llam (3.59).

Tolosa did not produce any seed-head throughout the experimental period. Linked to the increased H% and Ps:Llam, Medea had the lowest Llam% (19.4% compared with 38 to 51% for the other cultivars), though it also had the lowest percentage of dead leaf (Ldead% 15.2).

Table 4.2: ANOVA f-ratios and their P values for measures of shoot growth in Experiment 2. TN, tiller number per plant; DW, herbage dry matter harvested (g) at defoliation to soil surface in Harvest 1 or to 7 cm height in Harvest 2; Ldead%, Llam%, H% are the percentage of dead leaves, leaf laminae and seed-head, respectively, to DW; Ps:Llam, pseudostem:leaf lamina ratio; LER, leaf extension rate (mm d^{-1} measured for Harvest 2 only).

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
TN	1.79	**13.89**	**14.76**	0.95	2.34	1.71	0.19	1.05
	ns	**<0.001**	**<0.001**	Ns	ns	ns	ns	
DW	3.4	**23.93**	**64.24**	0.27	**58.71**	2.01	**5.61**	ns
	0.05	**<0.001**	**<0.001**	Ns	**<0.001**	ns	**0.025**	
Ldead%	2.33	**14.77**	**9.31**	0.53	0.72	**3.37**	**3.42**	1.67
	ns	**<0.001**	**0.005**	Ns	ns	**0.031**	**0.074**	
Llam%	4.66	**44.24**	**31.17**	1.30	3.38	**3.12**	**3.83**	ns
	0.017	**<0.001**	**<0.001**	Ns	0.076	**0.041**	**0.060**	
H%	1.61	**52.86**	**15.06**	**6.97**	**24.97**	**21.8**	**8.31**	**3.09**
	ns	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.007**	
Ps:Llam	1.50	**8.01**	**3.43**	1.02	**7.63**	**2.67**	0.57	**0.042**
	ns	**<0.001**	**0.074**	Ns	**0.010**	**0.065**	ns	
LER	4.40	**28.20**	**106.34**	**2.62**	-	-	-	1.37
	0.033	**<0.001**	**<0.001**	**0.092**				

Traits most strongly reduced by water deficit were percentage of seed-head weight to DW i.e., H% and LER (approximately 50% and 45%, respectively; Table 4.3). DW and Llam% were reduced under water deficit by 27% and 26%, respectively. Ps:Llam, however, increased by 55% in water stressed plants compared to control plants.

For Harv2 plant management factors such as timing of defoliation in December led to significant harvest and harvest × water regime effects in the ANOVA for DW (Table 4.2) but these differences were not felt to reflect differences in cultivar growth pattern so are not reported further here. However, H% and Ps:Llam showed indications of a seasonal change from spring to summer indicated by a significant harvest effect in ANOVA (Table 4.2). Mean values of H% for Harv2 were 7.31, compared with 18.24 in Harv1 (mean values extracted from ANOVA output but data not shown here).

Table 4.3: Cultivar and water regime main effect means for measures of shoot growth in Experiment 2. Statistical significance of effects and details of abbreviations used are presented in Table 4.2.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Samson (4n)	Tolosa	SEM	c.w.	Str	SEM
TN	31.3	51.9	36.1	55.1	3.13	49.6	38.0	2.21
DW	18.34	29.68	30.91	31.09	1.25	32.53	22.47	0.88
Ldead%	15.2	31.0	22.7	22.7	1.68	20.3	25.4	1.19
Llam%	19.4	42.3	37.9	51.3	2.02	43.3	32.1	1.43
H%	34.4	1.7	15.0	0.0	2.19	17.0	8.5	1.55
Ps:Llam	3.59	1.18	1.14	1.26	0.42	1.40	2.18	0.30
LER	3.25	6.36	5.99	7.09	0.32	7.30	4.04	0.22

A trait that was notable for the occurrence of statistically significant interactions in the ANOVA was H%, with cultivar × water regime, cultivar × harvest, water regime × harvest and cultivar × water regime × harvest interactions all significant (Table 4.2). The cultivar × water regime and cultivar × harvest interactions are accounted for by differences between Medea and the other cultivars in flowering behaviour. Medea showed high flowering activity for control plants at Harv1, with a marked reduction in stressed plants (Fig. 4.7a) and in Harv2 (Fig. 4.7b). The cultivar × water regime × harvest interaction is shown graphically in Fig. 4.8 and appears to arise partly from the same tendency for Medea to flower prolifically early in the season

(i.e. at Harv1) when unstressed, as discussed above, and partly from Samson (4n) expressing its flowering peak at Harv2, particularly in unstressed plants.

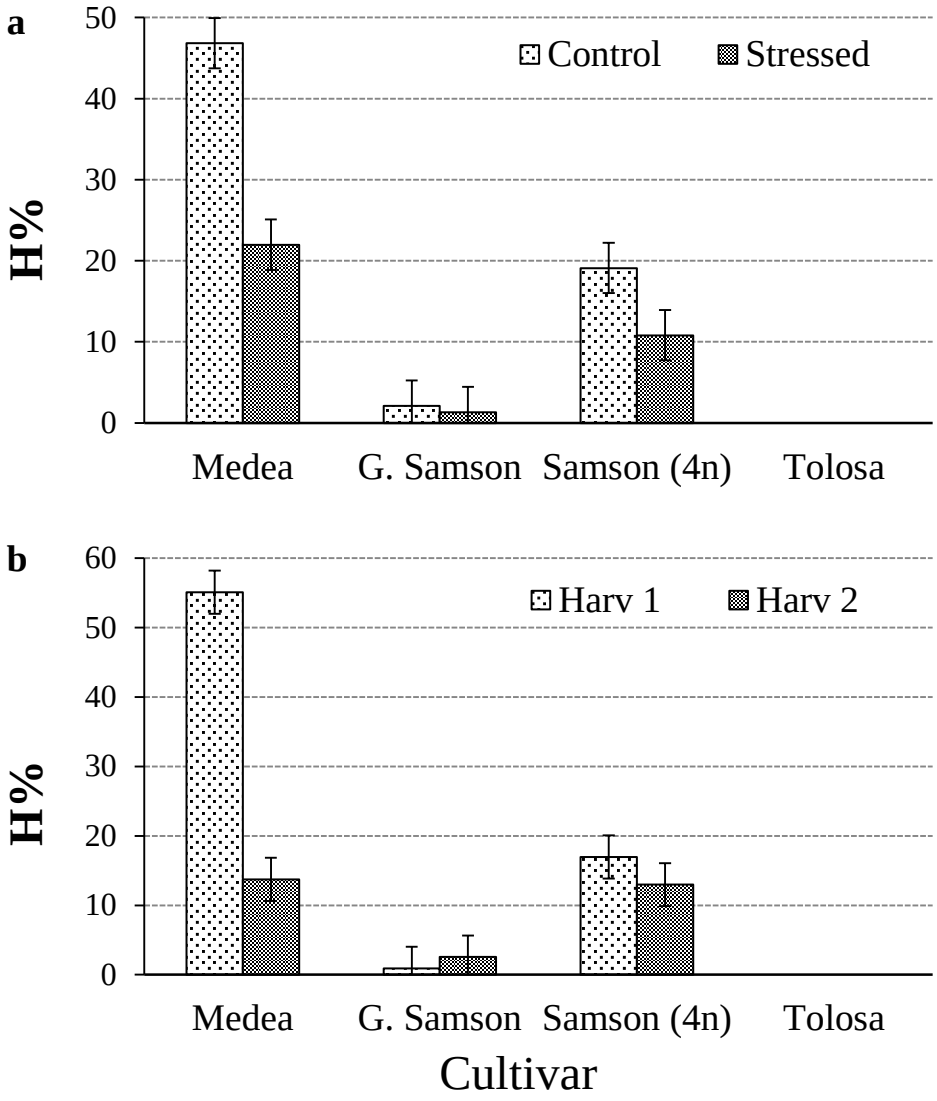

Figure 4.7: Percentage of seed-head to shoot dry weight, H (%), in four cultivars: Medea, Grasslands Samson, Samson (4n) and Tolosa, for (a) two water regimes (i.e., control and stressed), and (b) Harv1 and Harv2 in Experiment 2. These figures illustrate, respectively, the statistically significant cultivar × water regime and cultivar × harvest date interactions reported in Table 4.2.

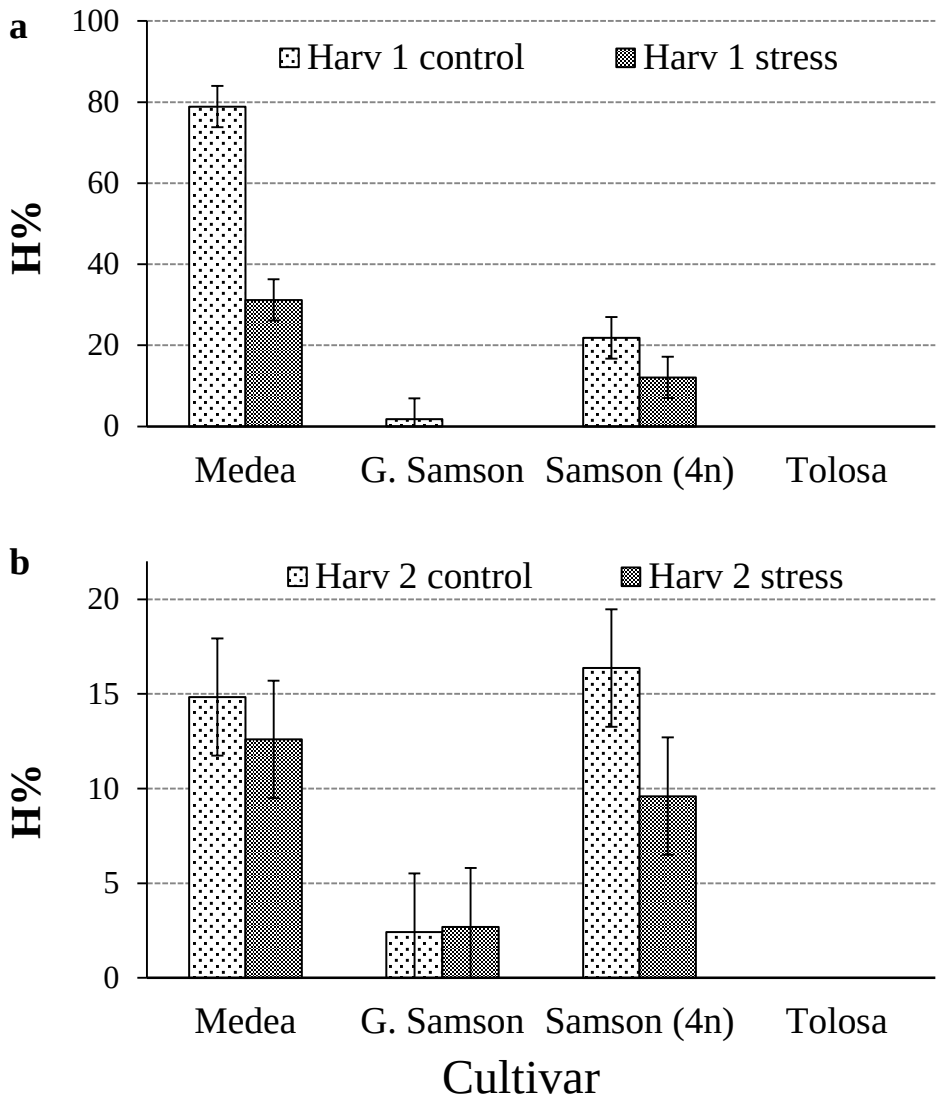

Figure 4.8: Percentage of seed-head weight to shoot dry weight (H%), under control and water stressed conditions of the four cultivars, Medea, Grasslands Samson, Samson (4n) and Tolosa for (a) Harv1, and (b) Harv2 in Experiment 2.

4.3.1.3 Root development and water uptake

ANOVA of root development and water uptake data of Experiment 2 revealed cultivar, water regime and harvest effects for most measured traits (especially SMC), with several interactions between these main effects also detected (Table 4.4), especially for SMC and traits associated with deep rootedness (Table 4.4).

Table 4.4: ANOVA f-ratios and their P values for measures of root development and water uptake in Experiment 2. Rc d1, Rc d2 and Rc d3 are weights (g) of coarse root at depth 1, 2 and 3, respectively; Rf d1, Rf d2 and Rf d3 are those of fine root at those soil depths, respectively; SMC d1, SMC d2 and SMC d3 are soil moisture percentage at the three soil depths, respectively; Rt is the total root weight (g); R:S is the root shoot ratio; DR:S is sum of root masses at depths 2 and 3 to that of shoot DW; Index DR is the ratio of sum of total roots at depths 2 and 3 to total root mass (Rt).

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
Rc d1	2.24	**9.01**	**28.83**	1.88	**9.21**	0.72	0.03	0.46
	ns	**<0.001**	**<0.001**	ns	**0.005**	ns	ns	ns
Rc d2	3.18	**2.79**	0.15	0.61	**6.27**	0.46	**3.89**	**3.89**
	0.05	**0.06**	ns	ns	**0.018**	ns	**0.06**	**0.018**
Rc d3	0.95	**9.72**	0.05	0.37	**3.67**	0.86	1.9	2.05
	ns	**<0.001**	ns	ns	**0.06**	ns	ns	ns
Rf d1	0.28	0.95	**21.83**	1.35	0.55	0.61	0.00	0.34
	ns	ns	**<0.001**	ns	ns	ns	ns	ns
Rf d2	0.83	**2.76**	**11.07**	0.44	**5.61**	0.70	0.11	1.34
	ns	**0.059**	**0.002**	ns	**0.024**	ns	ns	ns
Rf d3	1.09	1.33	1.94	**5.66**	2.19	0.31	1.44	0.17
	ns	ns	ns	**0.003**	ns	ns	ns	ns
SMC d1	3.62	**4.27**	**77.58**	**5.39**	**44.08**	1.45	**14.87**	0.95
	0.039	**0.013**	**<0.001**	**0.004**	**<0.001**	ns	**<0.001**	ns
SMC d2	1.57	**9.87**	**37.04**	**12.28**	**24.45**	2.19	**5.50**	1.46
	ns	**<0.001**	**<0.001**	**<0.001**	**<0.001**	ns	**0.026**	ns
SMC d3	0.86	**2.39**	**4.30**	**4.77**	**37.67**	1.96	0.65	**3.64**
	ns	**0.089**	**0.047**	**0.008**	**<0.001**	ns	ns	**0.024**
Rt	3.01	**4.19**	**34.73**	1.04	**14.39**	0.17	0.29	0.82
	0.06	**0.01**	**<0.001**	ns	**<0.001**	ns	ns	ns
R:S	3.48	**26.21**	2.06	**6.34**	**11.53**	**4.34**	**7.24**	1.94
	0.04	**<0.001**	ns	**0.002**	**0.002**	**0.012**	**0.012**	ns
DR:S	0.42	**48.40**	**17.42**	**3.91**	**7.31**	**5.68**	2.44	**2.8**
	ns	**<0.001**	**<0.001**	**0.018**	**0.011**	**0.003**	ns	**0.06**
Index DR	0.74	**17.17**	**10.27**	0.88	0.33	0.89	0.32	1.00
	ns	**<0.001**	**0.003**	ns	ns	ns	ns	ns

Cultivar and water regime effects are presented in Table 4.5 and the significant interactions in Figs. 4.9 & 4.10. Compared to the other cultivars, Medea developed more Rc at d2 and d3, and higher R:S, DR:S, and Index DR. However, SMC d1 and d2 was less depleted by Medea than by the other cultivars in the control treatment but not in the stressed treatment, and Tolosa depleted SMC d2 and d3 less than G. Samson. The water deficit treatment reduced GSMC by 3.5% in d1, 1.6% in d2, and 0.8% in d3, compared to control plants, with a lowest value of 5.2% for Stress treatment plants at d2. The Cv × Wreg interaction effects largely arise from a greater variation between Control and Stress treatment plants for Medea than for the New Zealand cultivars (Figs 4.9 & 4.10).

Table 4.5: Cultivar and water regime main effect means for measurements of root growth and plant water uptake of Experiment 2. Statistical significance of effects and details of abbreviations used are presented in Table 4.4.

	Main Effect							
	Cultivar					Water Regime		
	Medea	G. Samson	Samson (4n)	Tolosa	SEM	c.w.	Str	SEM
Rc d1	3.34	5.93	5.66	4.01	0.42	5.86	3.61	0.29
Rc d2	1.27	1.19	1.06	0.84	0.11	1.07	1.11	0.08
Rc d3	0.58	0.21	0.26	0.25	0.05	0.33	0.32	0.038
Rf d1	1.09	1.11	1.00	1.27	0.115	1.38	0.85	0.08
Rf d2	0.57	0.42	0.39	0.34	0.06	0.53	0.33	0.042
Rf d3	0.34	0.24	0.27	0.29	0.037	0.31	0.26	0.026
SMC d1	9.44	7.71	7.67	7.96	0.407	9.9	6.4	0.288
SMC d2	7.00	5.28	5.35	6.33	0.263	6.8	5.2	0.186
SMC d3	7.63	6.74	7.16	8.18	0.402	7.8	7.0	0.284
Rt	7.19	9.11	8.64	7.01	0.51	9.49	6.48	0.36
R:S	0.42	0.31	0.29	0.23	0.01	0.30	0.33	0.01
DR:S	0.17	0.07	0.07	0.05	0.007	0.07	0.11	0.005
Index DR	0.39	0.23	0.24	0.25	0.018	0.25	0.31	0.13

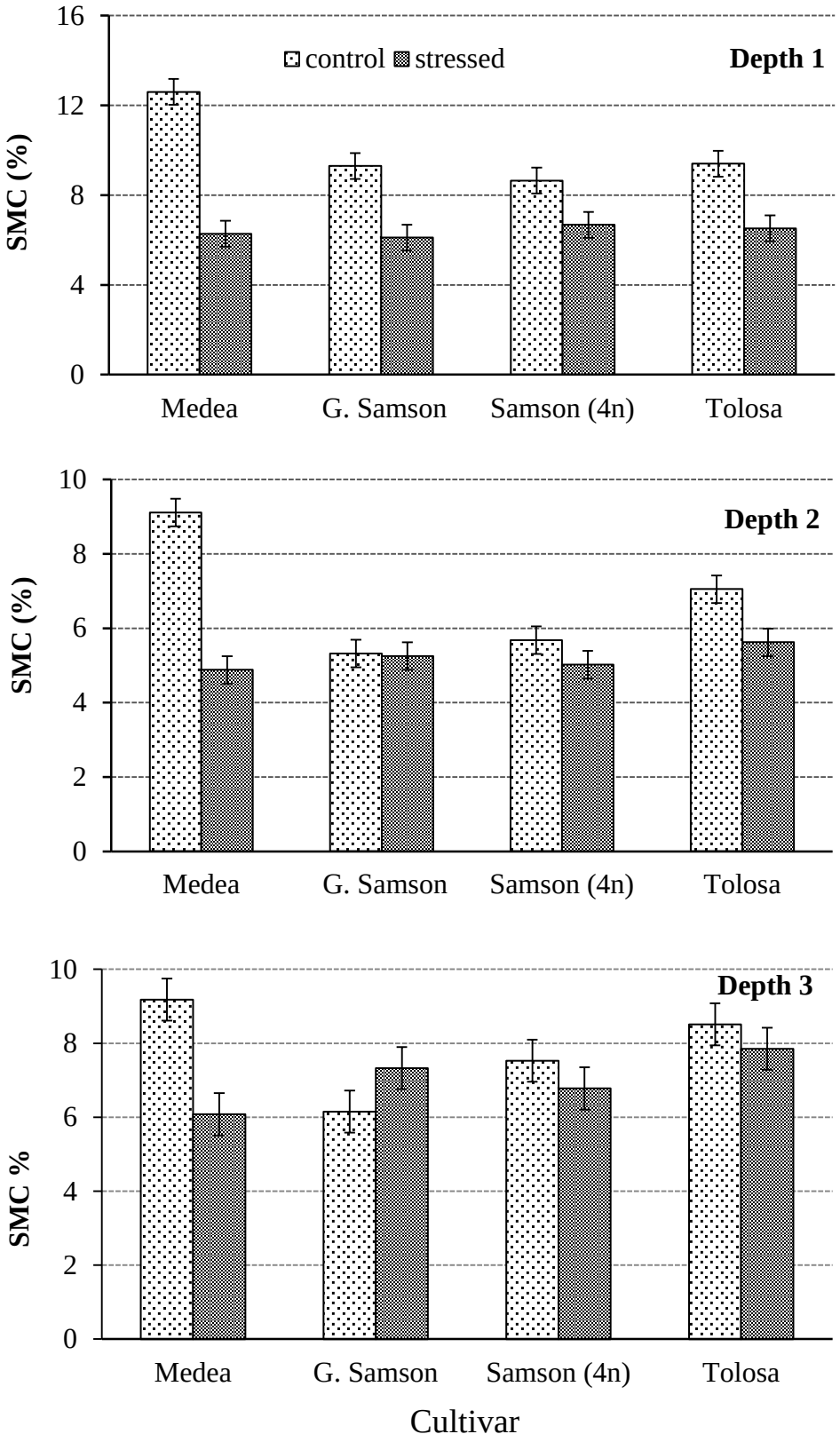

Figure 4.9: Interaction of cultivar × water regime in the four cultivars, Medea, Grasslands Samson, Samson (4n) and Tolosa, for soil moisture contents (SMC %) at

soil depth 1 (a), depth 2 (b) and depth 3 (c) in Experiment 2. The bar in columns presents the standard error.

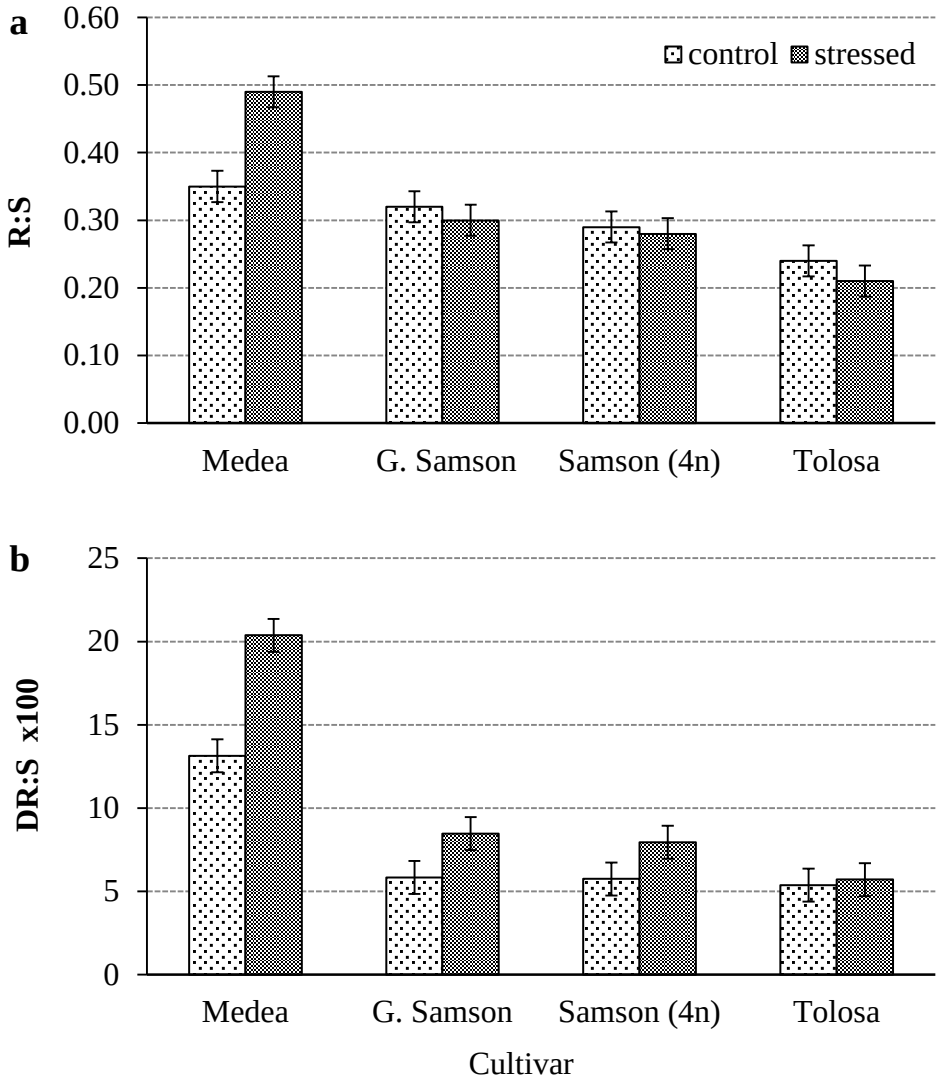

Figure 4.10: R:S, root shoot ratio (a) and DR:S x100, deep root (soil depths 2 and 3) to shoot ratio (b) of the four cultivars: Medea, Grasslands Samson, Samson tetraploid and Tolosa for controlled and stressed watering. The bars in columns indicate the standard error.

4.3.1.4 Plant water status

For RWC1 most of main and interaction effects were statistically non-significant (Table 4.6) whereas RWC2 measured on plants of Harvest 2 differed for cultivar, water regime and their interaction (at $P < 0.1$).

Cultivars ranged from 83.32 to 91.39% for RWC2 (Table 4.6) with Tolosa having the highest value (91.39%) and Samson (4n) the lowest (83.32%). Drought treatment reduced RWC2 from 91.94 to 85.43% (Table 4.7).

Table 4.6: ANOVA f-ratios and their P values for plant water status measurements in Experiment 2. RWC1 and RWC2 are Relative Water Contents (%) measured on 5.11.2008 and 14.11.2008, respectively. RWC2 was measured on plants of harvest 2 only.

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
RWC1	2.16 ns	1.84 ns	0.00 ns	1.07 ns	2.05 Ns	**2.68 0.065**	0.92 ns	1.05 ns
RWC2	3.16 0.073	**6.28 0.006**	**20.09 0.001**	**2.70 0.086**	-	-	-	-

Table 4.7: Cultivar and water regime main effect means for plant water status measurements in Experiment 2. Statistical significance of effects and details of abbreviations used are presented in Table 4.6.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Samson (4n)	Tolosa	SEM	c.w.	Stress	SEM
RWC1	76.74	80.56	81.57	74.70	2.37	78.47	78.31	1.68
RWC2	89.77	90.26	83.32	91.39	1.45	91.94	85.43	1.03

4.3.1.5 Stomatal and cellular control

The four cultivars did not differ significantly for Pn, SC and Evp. However, water regime affected these parameters significantly (Table 4.8).

Table 4.8: ANOVA f-ratios and their P values for measurements of stomatal and cellular control in Experiment 2. Pn, photosynthetic rate (μ mol m^2s^{-1}); Evp, rate of evapotranspiration (m mol m^2s^{-1}); SC, stomatal conductance (m mol m^2s^{-1}) measured 3 days after differential watering (DADW).

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
Pn	1.18 ns	0.65 ns	**117.13 <0.001**	1.27 ns	0.35 ns	**2.35 0.092**	0.00 ns	**2.29 0.098**
Evp	2.56 0.094	0.31 ns	**87.36 <0.001**	0.43 ns	0.40 ns	2.03 Ns	0.00 ns	**2.33 0.094**
SC	1.26 ns	0.64 ns	**60.24 <0.001**	1.24 ns	1.45 ns	**2.47 0.081**	0.68 ns	2.2 ns

Interaction terms were non-significant in most, though some interaction effects were marginally significant. All three parameters fell drastically under the Stress treatment (Table 4.9).

Table 4.9: Cultivar and water regime main effect means for measurements of stomatal and cellular control in Experiment 2. Statistical significance of effects and details of abbreviations used are presented in Table 4.8.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Samson (4n)	Tolosa	SEM	c.w.	Stress	SEM
Pn	3.09	3.75	3.3	2.94	0.44	5.66	0.88	0.31
Evp	0.93	1.09	1.05	1.08	0.13	1.65	0.42	0.09
SC	39.08	44.75	32.99	42.58	6.41	54.75	14.96	4.53

4.3.2 Experiment 5

4.3.2.1 Glasshouse temperatures

The daily minimum temperature inside the glasshouse for Experiment 5 generally ranged between 12°C and 22°C, and daily maximum temperature between 18°C and 43°C (Fig 4.11). The mean temperature over the entire period was 21.8°C. The temperature record in Fig 4.11 is broken down into Harv1 (7 January 2011) and Harv2 (20 January 2011) of Experiment 5 and is also extended to cover Experiment 6 to avoid later repetition (refer Section 7.3.1).

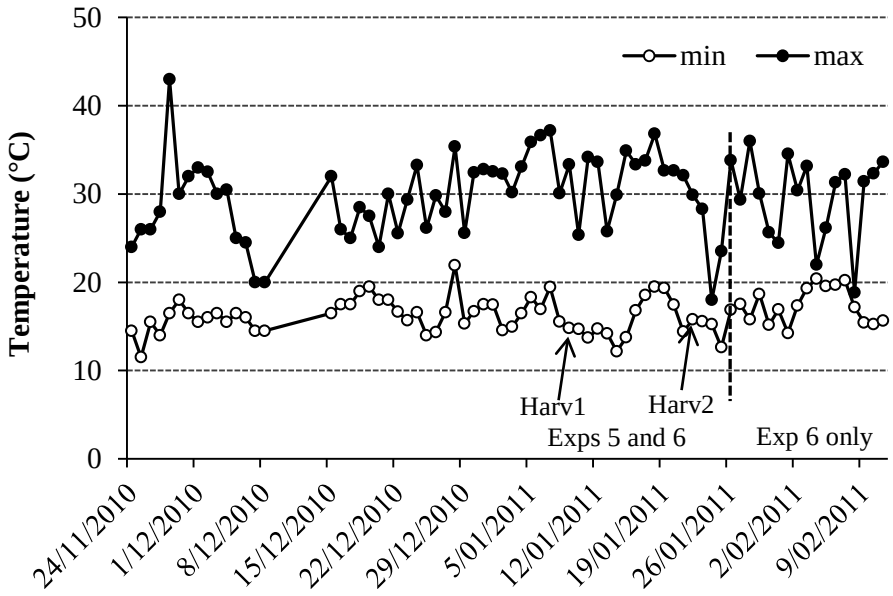

Figure 4.11: Daily maximum and minimum glasshouse temperatures for the period 24 November 2010 to 26 January 2011 of Experiment 5. The temperature record is continued until the end of Experiment 6 (refer Section 7.3.1). For Harv1 and Harv2 arrows indicate dates (7 and 20 January 2011) on which the two harvests were commenced.

4.3.2.2 Shoot growth and growth components

ANOVAs for measures of shoot growth and flowering behaviour revealed significant effects of cultivar for all traits and of water regime for some traits related to seed-head count (Table 4.10). Harvest varied for shoot DW as well as some traits of seed-head count. Several interaction effects of the three main treatments were also detected (Table 4.10).

Table 4.10: ANOVA f-ratios and their P values for measures of shoot growth in Experiment 5. TN, tiller number per plant; DW, shoot dry weight (g); LER, leaf extension rate (mm d^{-1}); HN, number of seed-heads; HN:TN%, ratio of seed-head number to tiller number expressed as a percentage; HNspring, total number of seed-heads emerged in spring (i.e., early October to end of November, 2010); HNsummer, total number of seed-heads emerged in summer (i.e., early December to the end of experiment in late summer 2010 – 11); HWspring and HWsummer, their corresponding weights of seed-heads in spring and summer; HW:HN, ratio of weight (g) to the number of total seed-heads.

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
TN	0.04	**26.96**	0.58	0.17	0.90	**3.04**	0.95	0.06
	ns	**<0.001**	ns	ns	ns	**0.046**	Ns	ns
DW	1.69	**37.33**	2.05	0.20	**9.78**	**5.88**	0.17	1.44
	ns	**<0.001**	ns	ns	**0.004**	**0.003**	Ns	ns
LER	0.33	**11.00**	0.01	0.70	-	-	-	-
	ns	**<0.001**	ns	ns				
HN	2.55	**26.29**	**3.34**	0.58	**15.40**	**13.03**	**3.34**	2.55
	0.095	**<0.001**	**0.077**	ns	**<0.001**	**<0.001**	**0.077**	0.095
HN:TN%	2.18	**42.3**	**5.69**	**2.31**	**37.78**	**16.83**	0.01	2.18
	ns	**<0.001**	**0.024**	**0.099**	**<0.001**	**<0.001**	Ns	Ns
HNspring	1.5	**8.06**	**2.54**	0.71	0.91	1.93	0.91	0.38
	ns	**<0.001**	**<0.001**	ns	ns	ns	Ns	Ns
HNsummer	3.68	**19.03**	1.44	0.22	**13.31**	**9.96**	2.18	1.89
	0.037	**<0.001**	ns	ns	**<0.001**	**<0.001**	Ns	Ns
HWspring	1.01	**5.89**	**5.56**	1.19	0.22	0.75	**3.64**	0.53
	ns	**0.003**	**0.025**	ns	ns	ns	**0.066**	Ns
HWsummer	1.77	**8.34**	2.02	1.33	0.42	0.28	**6.22**	**5.48**
	ns	**<0.001**	ns	ns	ns	ns	**0.018**	**0.004**
HW:HN	1.8	**6.42**	**2.97**	1.00	2.75	1.82	**3.94**	2.03
	ns	**0.002**	**0.095**	ns	ns	ns	**0.056**	Ns

The ranking among cultivars for values DW, TN and LER was generally Matrix > Ceres One50 > Grasslands Samson > Medea (Table 4.11). Shoot DW for Medea was 83 – 85% less than that of Matrix and Ceres One50 and 67% less than that of

Grasslands Samson. Reduction of TN of Medea from that of Matrix and Ceres One50 was in the order of 80% and from that of Grasslands Samson was in the order 62.7% while LER of Medea was, on an average, 38% lower than that of other cultivars.

Matrix did not flower throughout the experimental period (Table 4.11), while Ceres One50 flowered significantly less than Grasslands Samson and Medea (HN 21.25 and 9.83), respectively. However, for ratio of seed-head to tiller number (HN:TN%) Medea was almost double (36.94) that of Grasslands Samson with a value of 19.70% (Table 4.11).

Under the Stress water regime HN and HN:HN% were reduced to around 35% than their values in the Control treatment (Table 4.11).

Table 4.11: Cultivar and water regime main effect means for herbage-yield-related measurements in Experiment 5. Statistical significance of effects and details of abbreviations used are presented in Table 4.10.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Ceres One50	Matrix	SEM	c.w.	Str	SEM
TN	38.15	102.33	183.82	215.48	15.32	140.78	129.12	10.89
DW	3.80	11.58	21.93	25.35	1.61	16.82	14.51	1.14
LER	14.80	23.12	23.91	25.27	1.43	21.71	21.84	0.99
HN	9.83	21.25	2.25	0.00	1.87	10.04	6.62	1.32
HN:TN%	36.94	19.70	1.03	0.27	2.67	17.54	11.15	1.89
HNspring	4.25	3.67	0.25	0.00	0.78	2.67	1.41	0.55
HNsummer	5.58	17.58	2.00	0.00	1.80	7.37	5.20	1.27
HWspring	0.50	0.64	0.09	-	0.13	0.46	0.16	0.09
HWsummer	0.76	7.9	0.48	-	1.30	3.21	1.36	0.92
HW:HN	0.16	0.11	0.07	-	0.02	0.11	0.06	0.02

The higher HN of Grasslands Samson than Medea was more obvious around the first two weeks of December (Fig. 4.12). However, by the time of Harv2, Grasslands Samson had produced almost 3 times the HN of Medea (Figs 4.13a & 4.13b).

However, the high HN value in Grasslands Samson was related to plant size as the seed-head count to tiller count ratio (HN:TN%) was higher for Medea, especially at Harv2 (Fig. 4.13c).

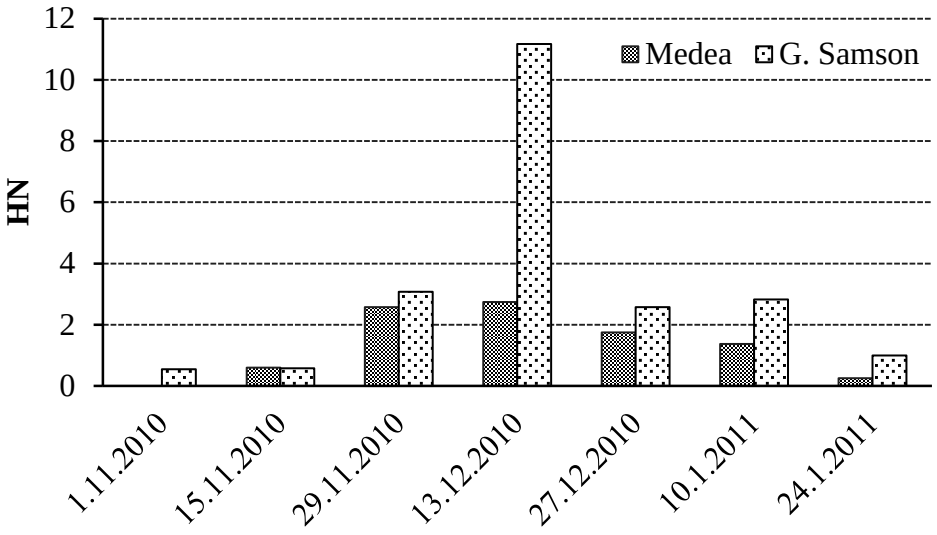

Figure 4.12: Comparison between Medea and Grasslands Samson for phenological development of HN (seed-head number) during spring (November, 2010) and summer (December 2010 to January 2011).

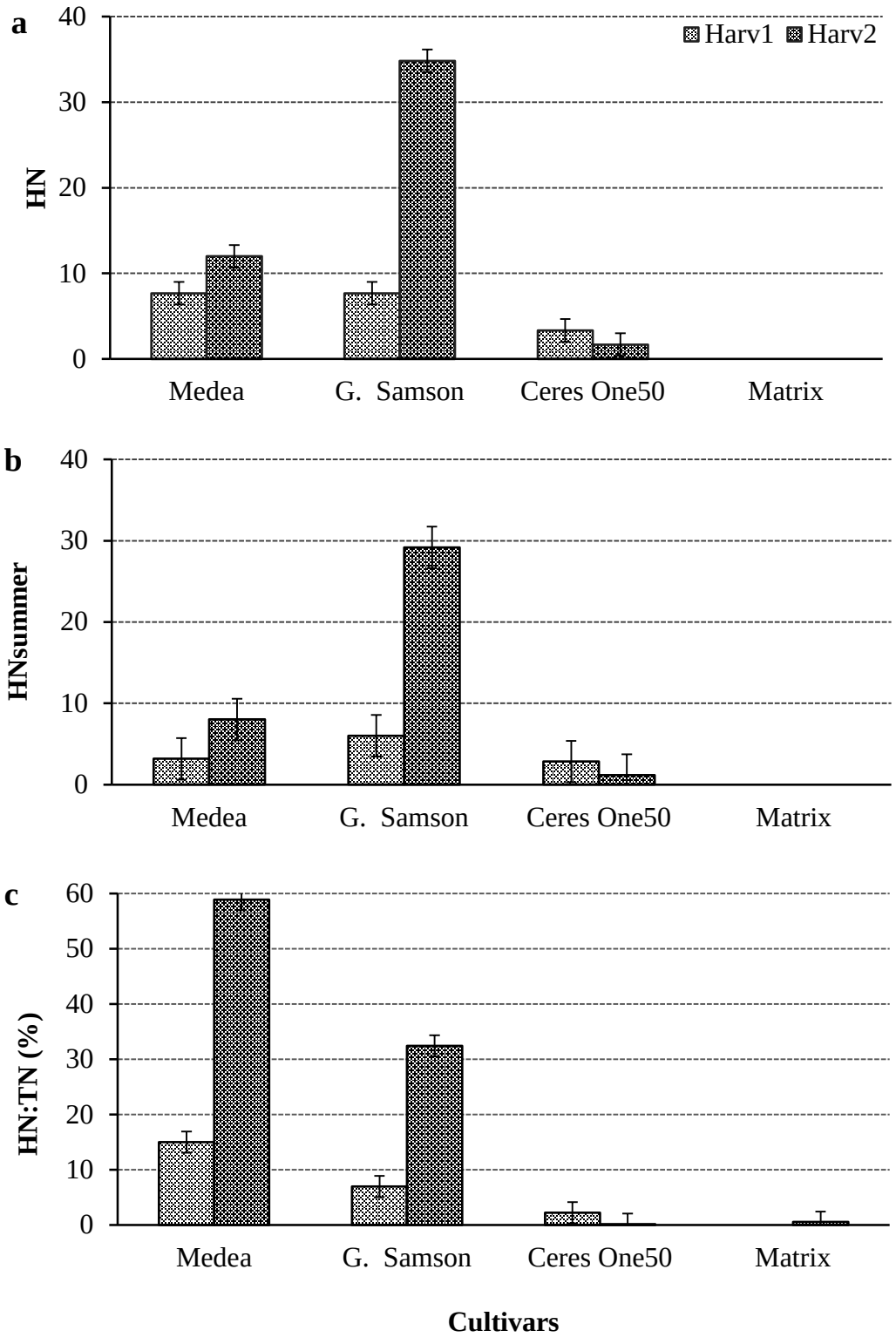

Figure 4.13: HN, total seed-head number (a), HNsummer, number of seed-heads emerged in summer (b) and HN:TN (%), percentage of total seed-head number to tiller number (c) of the four cultivars: Medea, Grasslands Samson, Ceres One50 and Matrix between two harvests of Experiment 5. The bar in columns presents the standard error of the mean.

4.3.2.3 Root development and water uptake

Effects of water regime were highly significant (Table 4.12) for SMC in all depths with associated effects on root development. In particular, fine root mass at Rf d1 was almost halved in the Stress treatment (0.38 cf. 0.67 g pot^{-1}), while Rc d2 was increased almost nine-fold in the Stress treatment relative to Control plants (0.04 cf. 0.34 g pot^{-1}; Table 4.13). Likewise, Index DR and DR:S were almost doubled between the Control and Stress treatments (Table 4.13).

Cultivar effects were seen in data for Rc d1 Rf d1, as well as for various root:shoot ratios (Table 4.12), and for data for SMC (especially at d3). The differences in root mass for both Rc d1 and Rf d1 (Table 4.12) appear to be linked to plant herbage mass differences (Table 4.11), with cultivars ranking in the order Matrix = Ceres One50 > Grasslands Samson = Medea (The correlation between plant herbage mass and d1 total root mass was 0.691 ($P < 0.001$)). The key effect identified by the R:S and DR:S is that (as in Experiment 2) Medea invests proportionately more of its total DW in deeper roots than the other cultivars (Table 4.13). The cultivar differences for SMC d3 reflect reduced water extraction by Medea and Ceres One50 at this soil depth, compared to Grasslands Samson and Matrix (Table 4.13) and this trend was maintained even under Stress condition (Fig. 4.14).

Table 4.12: ANOVA f-ratios and their P values for measures of root development and water uptake in Experiment 5. Abbreviations are as defined for Table 4.4.

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
Rc d1	2.98	**3.70**	0.34	0.8	0.02	**3.38**	1.84	0.72
	0.067	**0.023**	ns	ns	ns	**0.032**	Ns	Ns
Rc d2	3.56	**2.67**	**47.2**	2.35	0.33	0.32	0.35	0.93
	0.046	**0.072**	**<0.001**	ns	ns	ns	Ns	Ns
Rc d3	0.62	1.09	0.79	0.65	1.02	0.39	**3.24**	0.88
	ns	ns	ns	ns	ns	ns	**0.083**	Ns
Rf d1	0.73	**6.52**	**17.44**	1.05	0.07	**3.1**	0.12	1.48
	ns	**0.002**	**<0.001**	ns	ns	**0.043**	Ns	Ns
Rf d2	0.37	1.56	**3.24**	0.42	0.05	**3.91**	0.01	0.24
	ns	ns	**0.083**	ns	ns	**0.019**	Ns	Ns
Rf d3	1.61	0.14	0.06	2.23	1.64	0.64	3.55	1.42
	ns	ns	ns	ns	ns	ns	0.07	Ns
SMC d1	0.61 ns	**2.62 0.070**	**18.96 <0.001**	0.86 ns	0.43 ns	1.62 ns	**4.15 0.051**	1.25 Ns
SMC d2	0.71 ns	**2.35 0.095**	**62.52 <0.001**	1.46 ns	**15.22 <0.001**	0.36 ns	**28.86 <0.001**	0.54 ns
SMC d3	0.13 ns	**11.12 <0.001**	**53.64 <0.001**	**10.8 <0.001**	**31.67 <0.001**	**9.99 <0.001**	**117.7 <0.001**	**5.15 0.007**
Rt	1.9	**3.45**	0.14	0.08	0.00	2.72	0.7	0.55
	ns	**0.031**	ns	ns	ns	0.065	Ns	Ns
R:S	1.49	**5.88**	1.66	0.44	0.23	0.78	0.58	0.17
	ns	**0.003**	ns	ns	ns	ns	Ns	Ns
IndexDR	2.80	**3.22**	**16.49**	1.25	0.01	1.92	2.73	1.81
	0.018	**0.005**	**<0.001**	ns	ns	ns	Ns	Ns
DR:S	012	**10.05**	**6.59**	**2.53**	0.09	0.11	**3.74**	1.02
	ns	**<0.001**	**0.016**	**0.078**	ns	ns	**0.063**	Ns

Table 4.13: Cultivar and water regime main effect measurements of root development and water uptake in Experiment 5. Statistical significance of effects is presented in Table 4.12. Abbreviations are as in Table 4.4.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Ceres One50	Matrix	SEM	c.w.	Str	SEM
Rc d1	1.01	1.58	3.80	4.59	0.88	2.49	3.00	0.62
Rc d2	0.08	0.23	0.19	0.25	0.04	0.04	0.34	0.03
Rc d3	0.026	0.005	0.005	0.03	0.012	0.023	0.011	0.009
Rf d1	0.33	0.43	0.60	0.74	0.07	0.67	0.38	0.048
Rf d2	0.16	0.18	0.21	0.15	0.024	0.01	0.02	0.017
Rf d3	0.105	0.102	0.096	0.106	0.013	0.100	0.103	0.009
SMC d1	9.47	8.85	6.70	7.25	0.82	9.85	6.28	0.58
SMC d2	15.48	15.05	13.76	13.08	0.75	17.31	11.38	0.53
SMC d3	16.87	15.88	17.79	15.94	0.28	17.65	15.60	0.20
Rt	1.83	2.48	4.24	4.95	0.77	3.23	3.53	0.54
R:S	0.56	0.22	0.21	0.23	0.07	0.26	0.35	0.05
Index DR	0.28	0.24	0.16	0.15	0.02	0.15	0.26	0.018
DR:S	0.15	0.05	0.04	0.03	0.018	0.04	0.09	0.0133

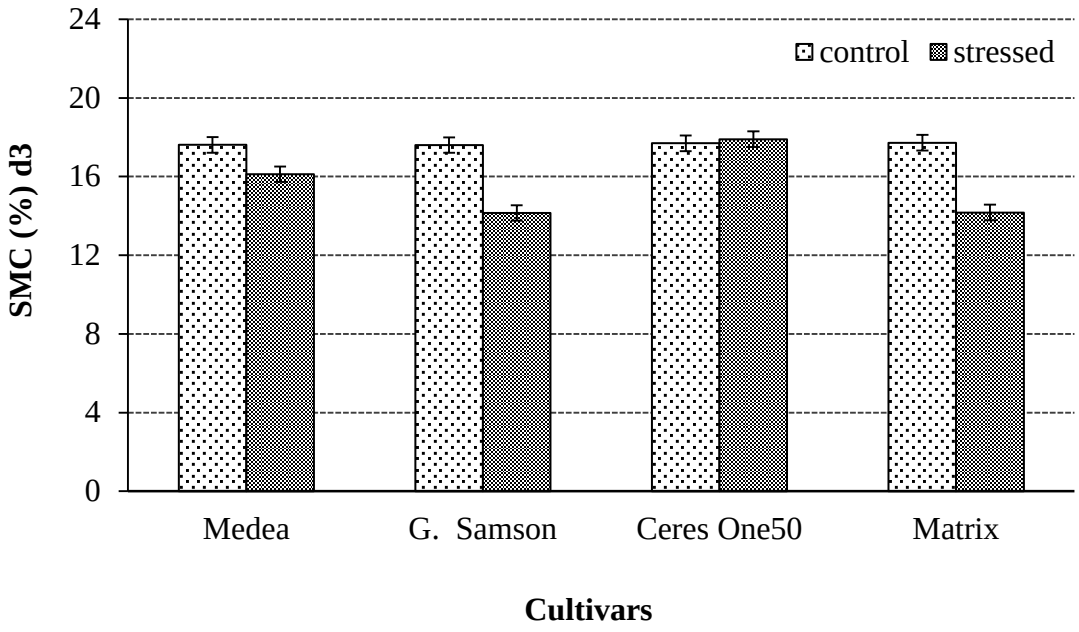

Figure 4.14: Comparison of SMC d3 (soil moisture contents at depth 3) under control and stress conditions in the four cultivars: Medea, Grasslands Samson, Ceres One50 and Matrix in Experiment 5. The bar in columns presents the standard error of the mean.

4.3.2.4 Plant water status

Among the main effects, cultivars differed significantly for LWP, OP and proline but not PP and RWC, while water regime significantly affected LWP and proline (Table 4.14). Values for OP were more negative while that of proline were significantly less at Harv2 than Harv1. Most of interaction terms were non-significant except the cultivar × harvest interaction for LWP and water regime × harvest interaction for proline (Table 4.14).

Table 4.14: ANOVA f-ratios and their P values for plant water status measurements in Experiment 5. LWP, leaf water potential (MPa); OP, osmotic potential (MPa); PP, pressure potential (MPa); RWC, percentage of relative water content; Proline, the amount of free proline (mg g^{-1} DW) amino acid in leaf lamina tissue.

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
LWP	0.49 ns	**4.74 0.008**	**28.97 <0.001**	1.91 ns	2.36 Ns	**2.93 0.051**	0.18 ns	0.76 ns
OP	0.11 ns	**3.28 0.036**	0.61 ns	1.09 ns	**3.04 0.093**	0.35 ns	0.30 ns	0.65 ns
PP	0.03 ns	2.14 ns	0.39 ns	0.72 ns	1.84 Ns	0.95 ns	0.35 ns	0.61 ns
RWC	5.49 0.01	0.23 ns	0.07 ns	0.22 ns	2.69 Ns	1.92 ns	0.85 ns	0.55 ns
Proline	0.59 ns	**2.46 0.087**	**28.87 <0.001**	1.72 ns	**32.16 <0.001**	1.97 ns	**7.18 0.013**	0.93 ns

Medea and Ceres One50 had a more negative LWP averaged over harvests than the other cultivars (Table 4.15) but in the case of Medea the LWP was markedly more negative at Harv2 than Harv1, whereas this was not the case with the other cultivars (Fig. 4.15). Medea also exhibited a high proline concentration compared to the other cultivars (Table 4.15).

LWP of plants under water stress was -1.16 MPa, compared to -0.80 MPa for control plants and under water stress the concentration of proline more than doubled from 1.30 to 2.72 m g g^{-1} DW (Table 4.14).

Table 4.15: Cultivar and water regime main effect means for measurements of plant water status in Experiment 5. Statistical significance of effects is presented in Table 4.14. LWP, leaf water potential (MPa); OP, osmotic potential (MPa); PP, pressure potential (MPa); RWC, percentage of relative water contents; Proline, the amount of free proline (mg g^{-1} DW) amino acid in leaf lamina tissue.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Ceres One50	Matrix	SEM	c.w.	Str	SEM
LWP	-1.12	-0.82	-1.09	-0.90	0.07	-0.80	-1.16	0.04
OP	-3.08	-2.05	-2.16	-2.16	0.26	-2.26	-2.46	0.19
PP	1.95	1.20	1.06	1.25	0.27	1.45	1.28	0.19
RWC	85.43	80.88	83.82	84.34	3.97	84.02	82.95	2.8
Proline	2.55	2.06	1.51	1.91	0.262	1.30	2.72	0.18

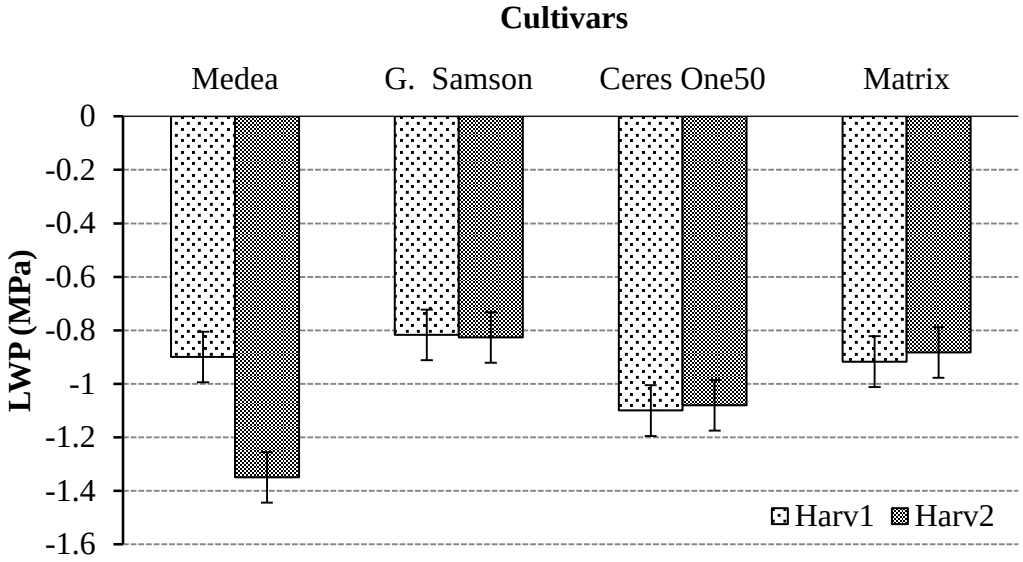

Figure 4.15: Comparison of leaf water potential (MPa) for the four cultivars: Medea, Grasslands Samson, Ceres One50 and Matrix at Harvest 1 (Harv1) and Harvest 2 (Harv2) in Experiment 5. The bar in columns presents the standard error of the mean.

4.3.2.5 Stomatal and cellular control

Of the three main effects measurements of stomatal and cellular control differed statistically for cultivar and water regime but differed little for harvest date. Most of the interactions between the three main effects were non-significant (Table 4.16).

The four cultivars varied significantly for Lrs (P = 0.043), Lws (P< 0.001) and for Lcs (P = 0.076) but Tc-Ta were non-significant (Table 4.16). All cultivars rolled their leaves. However, Grasslands Samson and Medea rolled their leaves more than Ceres One50 and Matrix. The same trend was observed for Lws. For Lcs, Medea developed comparatively less and Grasslands Samson comparatively more dark blue colour than the other cultivars (Table 4.17).

Table 4.16: ANOVA f-ratios and their P values for measurements of stomatal and cellular control in Experiment 5. Lrs, leaf rolling score; Lws, leaf wilting score; Lcs, leaf colour score. These are visual scores on a scale from 1 to 3 with 1 the lowest and 3 the highest. Tc-Ta, difference between canopy and air temperatures.

Variable	Rep	Cv	Wreg	Cv × Wreg	Harv	Cv × Harv	Wreg × Harv	Cv × Wreg × Harv
Lrs	0.39	**3.1**	**31.07**	**3.94**	1.10	**2.55**	2.61	**6.28**
	ns	**0.043**	**<0.001**	**0.018**	Ns	**0.076**	Ns	**0.002**
Lws	2.95	**8.65**	**47.35**	**3.99**	0.02	**2.39**	0.31	0.88
	0.069	**<0.001**	**<0.001**	**0.017**	Ns	**0.090**	Ns	Ns
Lcs	3.29	**2.55**	**12.01**	2.09	0.57	1.38	0.09	0.79
	0.052	**0.076**	**0.002**	ns	Ns	ns	Ns	Ns
Tc-Ta	3.15	0.13	0.00	0.37	-	-	-	-
	0.083	ns	ns	ns				

Table 4.17: Cultivar and water regime main effect means for measurements of stomatal and cellular control in Experiment 5. Statistical significance of effects is presented in Table 4.16. Lrs, leaf rolling score; Lws, leaf wilting score; Lcs, leaf colour score are visual score from 1 to 3 with 1 the lowest and 3 the highest; Tc-Ta, difference of canopy and air temperatures.

	Main effect							
	Cultivar					Water regime		
	Medea	G. Samson	Ceres One50	Matrix	SEM	c.w.	Str	SEM
Lrs	2.47	2.55	2.00	2.17	0.14	1.88	2.71	0.10
Lws	2.43	2.35	1.67	2.08	0.11	1.72	2.54	0.08
Lcs	1.91	2.49	2.17	2.00	0.16	1.87	2.42	0.11
Tc-Ta	1.18	1.08	1.68	1.18	0.76	1.26	1.31	0.55

4.4 Discussion

4.4.1 Methodology development

As indicated in the objectives for this chapter, one purpose of Experiment 2 was to gain experience in simultaneous measurement of multiple traits across different plant growth and development domains. The key point about making multiple measurements is that each one is time consuming making it difficult to complete all in an acceptable time period. This means plant status might have changed during measurement of the traits under consideration. For example, in Experiment 2, Harv1, LWP was scheduled for measurement on 29 October 2008 this was never completed because of problems with the rubber seal breaking the vascular tissues of the sample leaves (later solved by placing leaves in plastic sleeves), and RWC was measured on 5 November, a time gap which would have raised questions of interpretation, even if the LWP measurement had been available. However, in Experiment 5 LWP measurements were conducted successfully with leaf samples for determination of RWC and OP collected on the same day and within 2 hours of LWP determination. The solution devised to bring leaf sampling for different measurements closer together in time was to freeze leaf samples intended for determination of OP in liquid N for later analysis rather than trying to conduct OP measurements on the same day leaves were sampled. Subsequently, it has been noted that this methodology used in

Experiment 5 is the same as that suggested by Turner (1986) in order to reduce PP to zero. As a result of better time use during the intensive measurement phase of Experiment 5 additional measurements such as canopy temperature by IRT were possible, though some other measurements which had not been productive in Experiment 2 (e.g. EL) were dropped.

4.4.2 Statistical interactions involving harvest date

Tables 4.2, 4.4, 4.6, 4.8, 4.10, 4.12, 4.14 and 4.16 report a total of 49 interactions for Cv × Harv, WReg × Harv, and Cv × WReg × Harv with statistical significance less than $P < 0.10$, only seven of which (Figs 4.7, 4.8a, 4.8b, 4.13a, 4.13b, 4.13c & 4.15c) are reported in the results. However, all were reviewed and a summary follows.

"Harvest" effects for seed-head number and related traits (Tables 4.2, 4.3, 4.10 & 4.11) are easily understood as reflecting timing of the measurement cycles in relation to flowering, while the Cv × Harv interaction reflects differing flowering dates of the various cultivars. From a biological perspective, the key point to emerge from the data is that Medea tends to reach peak flowering about 2 weeks earlier than Grasslands Samson (Fig. 4.12) and early flowering is a trait commonly seen in perennial ryegrass germplasm from lower rainfall regions (Volaire and Lelievre, 2002). Since the current New Zealand industry preference appears to be for later flowering cultivars, earlier flowering of Medea would be a point for consideration when designing a crossing programme for introgression of Medea germplasm to New Zealand breeding programmes.

Other statistically significant harvest effects appear to arise more from the impact of experimental procedures on plants than from biological differences in the plants themselves. For example, in Experiment 2 the statistically significant Harvest effect for shoot DW (Table 4.2) reflected mean yields of 27.53 and 17.79 g pot^{-1}, for Harv1 and Harv2, respectively (data not presented), and the lower yield for Harv 2 arose because the weight reported is for herbage recovered on cutting at 7 cm in that harvest to allow for post-defoliation LER assessment, whereas shoot DW for Harv1 was measured to soil level.

4.4.3 Differences in methodology of watering and their implications for plant growth

It is evident from inspection of the SMC data for Experiments 2 and 5 (Tables 4.5 & 4.13, respectively) that the differing water application techniques in the two experiments resulted in differing plant growth environments. In Experiment 2, the plants were top-watered in the effective root zone but water did not penetrate and lower soil layers became very dry (SMC d2 5.2%, SMC d3 7.0% in the Stress treatment; Table 4.5). However, in Experiment 5, bottom-watering was carried out by placing the potted plants in drums of water and then gradually lowering the water table to simulate the natural soil conditions. As a result, lower soil layers remained much wetter in Experiment 5 (SMCd2 11.38%, SMC d3 15.6% in the stress treatment; Table 4.13) than in Experiment 2. Associated with these differences in SMC, the plants in Experiment 2 had a well-developed deep root system (~ 8.8 g total root weight) whereas plants had a shallow and less developed root system in Experiment 5 (~ 3.4 g total root weight) in response to capillary rise of water in the pots. Interestingly, R:S ratio was similar in the two experiments (0.33 and 0.35 for Experiments 2 and 5, respectively). As a result of well-developed water stress in Experiment 2 the differences in water regime significantly affected traits of shoot growth like TN, Ldead%, Llam% and LER (Table 4.2) while in Experiment 5 (Table 4.10) these differences were not statistically significant. Likewise, difference in SMC at various soil depths in Experiment 2 were more “stronger” statistically (Tables 4.4 & 4.5) than those in Experiment 5 (Tables 4.12 & 4.13).

Comparison of wettest and driest values of SMC (Tables 4.5 & 4.13) suggest (volumetric) SMC was around 17% at field capacity with plants able to draw SMC down to around 5%, which allowing for adjustment for bulk density (1.33 g/cc) would place the soil mix used to fill the pots somewhere between a sandy loam and a Loam on the diagram of McLaren and Cameron (1996; Fig.4.16). Both experiments used a 3:1 ratio of alluvial B horizon soil and builder’s sand which seems consistent with the above. One question raised for future experiments then, is whether the onset of water deficit could have been slowed and plant responses more clearly measured if a finer textured soil had been used. For Experiment 2, the mean SMC values of under 7% except for soil depth 1 of Control plants (Table 4.5) indicate SMC had been

reduced to near permanent wilting point in the lower portions of the pots and plants were surviving on the water added on a regular basis via pipes releasing the water at 15 cm soil depth. By contrast, in Experiment 5 SMC values of nearly 8% in depth 1 and nearly 16% in depth 3 suggest plants should have been less stressed in that experiment and this may at least partly explain the comparative absence of drought resistance response in the domain of leaf water relations.

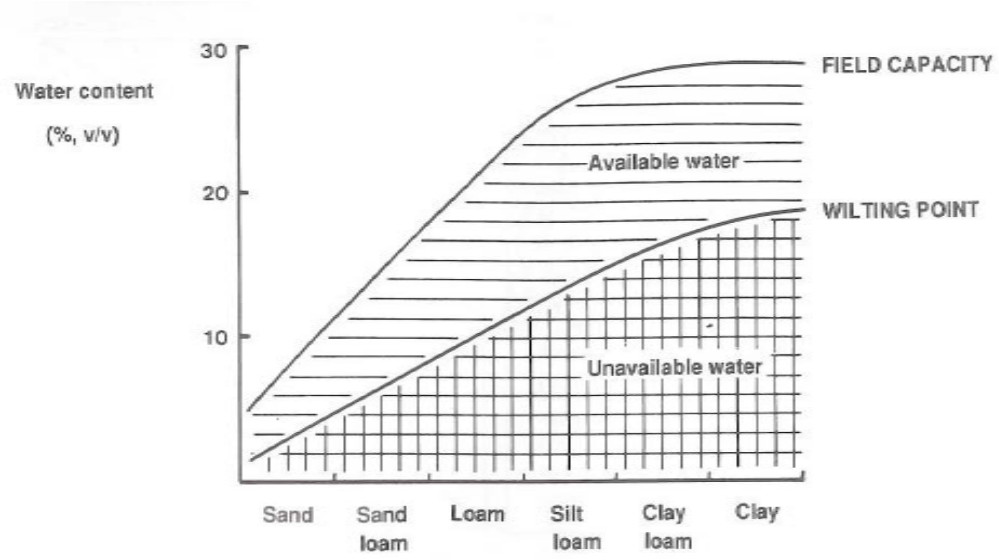

Figure 4.16: A generalized relationship between soil texture and moisture contents at field capacity and permanent wilting point (McLaren and Cameron, 1996) adapted from Cassel and Klute (1986).

It is not entirely clear if the less pronouced water deficit seen in Experiment 5 should be attributed to the changed watering regime or if other factors contribute, such as slow establishment of plants from seed in Experiment 5, resulting in plants remaining small in that experiment compared to plants in Experiment 2, and as a result having a lower water demand.

4.4.4 Effects of water deficit on plant processes

Forage plants, in general, have been reported to show different trends when subjected to flooding or drought ((Volence and Nelson, 2003); Fig. 4.17). According to the diagram below when forage plants are subject to drought, shoot growth declines more rapidly and becomes static even at a much lower level of water deficit than root growth.

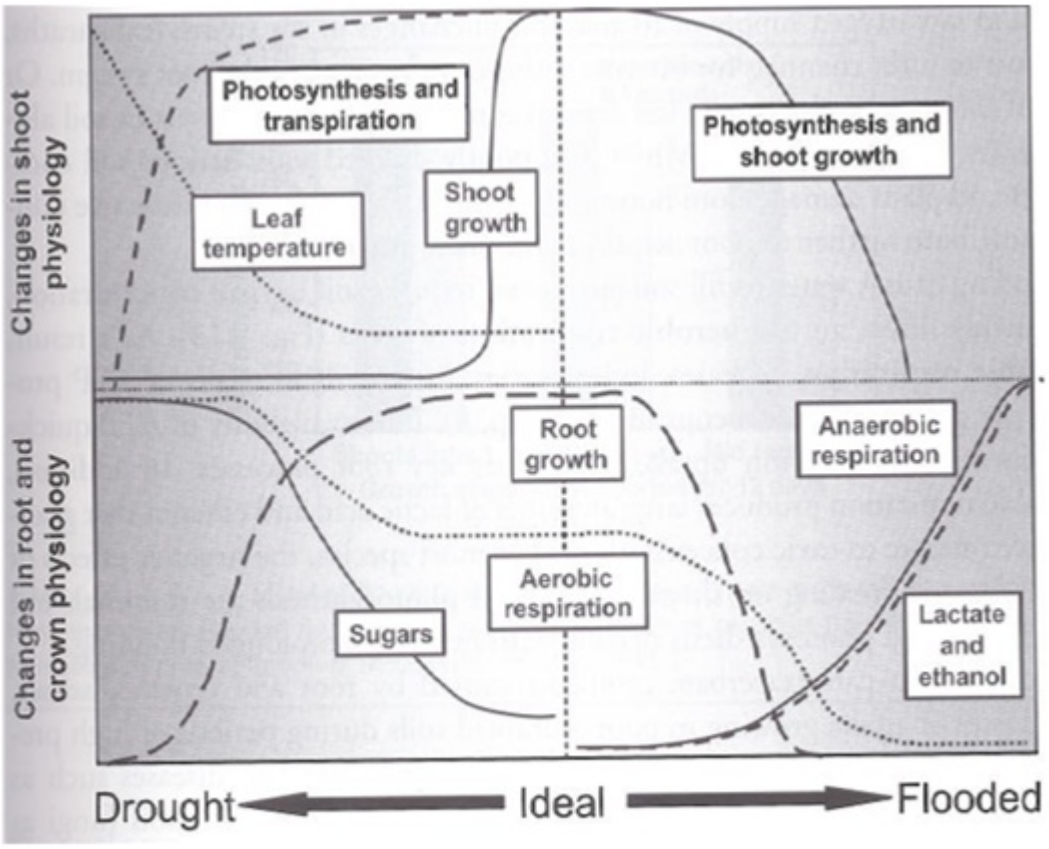

Figure 4.17: A comparative response of shoot growth and root development traits in forage plants for drought and flooding.

Available data for the two experiments mainly covered the traits of shoot and root growth. The results suggest that due to a higher moisture deficit experienced by plants in Experiment 2 than those in Experiment 5, TN, shoot DW and LER significantly differed for water regime (wet and dry) in Experiment 2 (Table 4.2) but not significantly affected in Experiment 5 (Table 4.10), so as root growth (represented by Rt) for Experiment 2 (Table 4.4) and Experiment 5 (Table 4.10). Mean values of LER (7.30 and 4.04 mm d^{-1}) between Control and Stress treatments of Experiment 2 (Table 4.3) and almost 3 - 5 times higher values of LER in Experiment 5 (Table 4.11) also give a clue to the fact that plants in Experiment 5 did not vary a lot for X axis of the diagram above.

4.4.5 Cultivar differences in water deficit response

With respect to choice of cultivars, it was felt important to include Grasslands Samson and Medea in both experiments, and time budget calculations in planning the

experiments indicated that more than two additional cultivars might result in logistical difficulties to complete the various measurements. Therefore the decision was whether or not to include 2 different comparison cultivars with Grasslands Samson and Medea in Experiment 5, or the same two cultivars as in Experiment 2. In Experiment 2 the rationale for the choice of Samson (4n) as one of the two additional cultivars was to test a diploid and tetraploid cultivar derived from related germplasm, while the reason for the inclusion of Tolosa was to test Grasslands Samson and Medea against a cultivar that used introgression with Spanish germplasm in the breeding process (Stewart, 2006). In Experiment 5 the two cultivars chosen were Ceres One50 (a second cultivar incorporating Spanish germplasm during the breeding process), and Matrix. The latter cultivar utilised introgression with meadow fescue in the breeding process. This germplasm was of interest because it is widely recognised that the genus *Festuca* could be a source of genes for drought resistance in ryegrass improvement [see e.g. Humphreys and Pasakinskiene (1996)] and because of the commercialisation of the material by Cropmark Ltd. Cropmark Ltd claim on their website that meadow fescue introgression confers a strong root system. Since the work reported here was completed, DairyNZ has published a Forage Value Index in which Matrix scores first for yield among cultivars tested in the Northern North Island. Against that, Cooper (1996) reported after testing naturalised meadow fescue in Northland "Unless a specific role for the species can be determined further work is largely unjustified, as productivity did not surpass that of control species."

With respect to the comparison between Medea and Grasslands Samson, which was the main objective of this research, there was clear evidence in both Experiment 2 and Experiment 5 of a trait combination in Medea of an increased proportion of the root mass at depth and reduced herbage accumulation resulting in lower shoot weight compared to Grasslands Samson. Results which reflect this difference between the two cultivars in growth strategy in Experiment 2 include higher coarse and fine root mass in depth 2 for Medea than Grasslands Samson (despite smaller plant size of Medea) (Tables 4.4 & 4.5) and similar root mass in Experiment 5 for both cultivars, again despite smaller plant size of Medea (Tables 4.11 & 4.12). The index DR was devised as an indicator of this growth strategy difference and is seen to be very much higher for Medea than for any of the other cultivars in either of the experiments

(Tables 4.5 & 4.13). Reduced shoot size of Medea compared to other cultivars would decrease water demand while a similar or increased root mass would ensure or enhance water supply. In these two experiments the net effect of the decreased shoot size and increased root mass at depth in Medea was generally an increase in SMC at depth for those pots containing Medea plants and this trend achieved statistical significance in depth 2 of Experiment 2 and depth 3 of Experiment 5. On this basis it can be assumed that Medea is better adapted to survive moderate drought than the European germplasm and this difference can be classified as morphological (i.e. the shoot growth and growth components domain) rather than physiological. This is not to say that there are not other effects operating simultaneously in other plant functional domains, however. A similar effect of increased root mass at depth with decreased shoot size was previously noted in tall fescue germplasm of Mediterranean origin (Assuero et al., 2002). Perhaps the overall effect of ongoing exposure to moderate summer moisture deficit is to generate selection pressure on populations that favours survival of plants that do not excessively deplete soil moisture.

Also in the 'shoot growth' domain, another feature of Medea's behaviour is a strong expression of flowering activity. In Experiment 2, 34% of Medea tillers had flowered by late November whereas very few Grasslands Samson tillers had flowered at that time (Table 4.3). In Experiment 5, Medea plants had much lower TN and DW harvested than Grasslands Samson, but almost double the % of tillers flowering (Medea 37%, Grasslands Samson 20%) and with flowering activity beginning earlier than Grasslands Samson in spring and continuing through summer (Table 4.11). It follows that in a breeding programme, where Medea parents were used for introgression of deeper rooting characteristics, the issue of flowering behaviour of the progeny would be a major one. The current trend for recently released New Zealand perennial ryegrass cultivars is towards late flowering cultivars with a shorter flowering period (Easton et al., 2002).

As noted above (Section 4.3.2.2), other characteristics of Medea in the 'shoot growth' domain were small plant size and an increased ratio of pseudostem:lamina compared to Grasslands Samson. Since the DW size reduction of Medea compared to Grasslands Samson was much greater in the late spring experiment (Experiment 2) and the summer experi,emt (Experiment 5) (Section 4.3.1.2) than in winter (Section

3.2) this seasonal reduction in Medea growth should probably be regarded as a form of summer dormancy though less absolute that the summer dormancy described for *Dactylis* and *Festuca* spp. by Clark and Harris (2009). If Medea were to be used in the breeding of a New Zealand forage ryegrass a decision would need to be made as to whether or not to select for reduced summer growth as a water saving strategy as indicated by SMC values for Medea (Tables 4.5 & 4.13) or for increased summer growth. In general, current industry demand for new perennial ryegrass cultivars in New Zealand is for better summer growth, but this could compromise survival potential.

With respect to variation in pseudostem:lamina ratio (for which Medea had higher values than the other cultivars in Experiment 2; Table 4.3), it can be predicted from work of J.L. Durand in France and Matthew et al. (2001) that a longer pseudostem would increase leaf elongation duration by delaying the signal for initiation of elongation in successive emerging leaves, with the expected result being a shift to a smaller number of larger tillers. Hence it might be that the long pseudostem in Medea acts as a mechanism contributing to reduced DW in summer. Another ryegrass cultivar with comparatively long pseudostem length is Grasslands Impact (Sartie et al., 2009), and these authors also noted the role of pseudostem length as a determinant of tiller morphology.

With respect to plant water status, RWC data are typically found to be responsive to increase in water deficit stress (Barrs, 1968). However, in Experiment 2, RWC effects were statistically significant only at Harv2 while in Experiment 5, only the replicate effect in RWC data was statistically significant (Table 4.14). This may indicate an unresolved procedural issue with the measurement such as inconsistent drying of leaf segments when weighing after hydration and this would need to be resolved in any future work. In Experiment 2, RWC was assessed using four leaf lamina segments each of 2 cm length floated on water. In Experiment 5 on advice of N.C. Turner (Pers. Comm.) RWC was assessed using 5 cm leaf lamina segments in a closed test tube and with their ends dipped in 1 ml deionized water. Another issue that makes measurement of RWC in forage grasses difficult is a wide variation in technique for establishing the equilibration point when tissue is rehydrated. Some examples include: 4 h at 20°C in Kentucky bluegrass (Liu et al., 2008), overnight at

0°C in perennial ryegrass (Thomas, 1991), overnight at 4°C in white clover (Marshall et al., 2001), 18 h at 4°C in Kentucky bluegrass (DaCosta et al., 2004), and 24 h (with no temperature mentioned) in Kentucky bluegrass and tall fescue (Fu and Huang, 2001). Various authors have noted that the hydrated tissue weight may be affected by water vapour loss from cut edges, or photosynthesis or respiration occurring during incubation. The author investigated the effect of varying incubation temperature and time on RWC, but no substantive effects were found.

However, LWP, OP, and PP measurements in particular did detect changes in plant water status associated with cultivar, water regime, and harvest date in Experiment 5 (Tables 4.14 & 4.15). In particular there were some indications in Medea of an atypical response to water deficit, consistent with the high stomatal conductance measurement obtained in Experiment 1 (Table 3.5). Among these were: in Experiment 2 the wider difference in SMC between Control and Stress water regimes for Medea than for the other cultivars (Fig. 4.9), in Experiment 5 a similar effect for LWP (Fig. 4.15), and a high Lws (Table 4.17). Medea also had more negative LWP and OP and higher leaf proline concentrations averaged across the Experiment than other cultivars (Table 4.15). This reliance on small plant size to reduce water use without physiological conservation of water is somewhat counterintuitive, and suggests what has elsewhere been termed an ‘anisohydric’ response to. This means that when breeding forage grasses for water deficit tolerance, a comprehensive awareness of multiple plant response domains and their inter-relations would be needed for best results.

With respect to the other cultivars tested, in Experiment 2, Samson (4n) differed little in behaviour from its diploid relative, Grasslands Samson, apart from a higher flowering percentage (Table 4.3). So in this study there was no indication of any drought resistance advantage conferred by tetraploidy. However, the cultivar Tolosa, derived from Spanish germplasm, despite the lack of seed-head development, produced similar shoot DW (Table 4.3) to the other New Zealand material but with significantly higher SMC at the destructive harvest (Table 4.5), indicating a probable increase in water use efficiency. In Experiment 5 the fact that cultivars Ceres One50 and Matrix both had a higher TN, shoot DW (Table 4.11) and Rt (Table 4.13) than

Grasslands Samson might be best interpreted as indicating that in warm conditions (temperature range 12 °C and 22 °C, Fig. 4.11) but without serious moisture deficit (LWP ~ -0.9 MPa) these cultivars perform well in terms of herbage production.

4.5 Conclusions

- Experiment 2 and Experiment 5, with their differing watering methodologies and management of planting stressed the plants in different ways and to different degrees. The overall effect was that plants in Experiment 5 had markedly higher SMC at depth 2 and depth 3 and were therefore likely to be less stressed than those in Experiment 2.
- Medea showed dramatic differences from the New Zealand cultivars it was compared with, having a reduced summer growth rate, decreased TN and plant DW and a high % of tillers flowering, with an increased R:S ratio and index of deep rootedness.
- Several results for Medea indicate a lack of control of water loss, compared to current New Zealand germplasm, in the leaf water relations and stomatal and cellular control domains.
- In Experiment 2 Grasslands Samson and Samson (4n) attained similar shoot DW although with a lower TN in Samson (4n), while Tolosa indicated possible evidence of higher water use efficiency than the two Samson lines. In Experiment 5 Ceres One50 and Matrix both attained much higher shoot DW than Grasslands Samson but this may relate to tolerance of warm temperature rather than tolerance of water deficit.
- Further work is needed to determine if desirable traits in Medea like the deeper-rooted growth habit and undesirable traits like the high H% will segregate in a breeding population to provide progeny with lower seed-head production but retaining the deep-rooted trait of Medea.
- Medea could be used as a source of genes for deep-rootedness, compared to Grasslands Samson and other current New Zealand cultivars tested, but traits such as a high % of shoots flowering and reduced shoot growth in summer would need to be bred out.

5

Patterns of trait inheritance in Medea × Grasslands Samson F_1 progeny

5.1 Introduction and aims

Having established growth characteristics of Grasslands Samson and Medea (Chapter 3) and finding a range of drought resistance mechanisms in a wider set of cultivars of perennial ryegrass and a standardised methodology (Chapter 4), the logical next step in the study was to explore the potential for introgression of the drought resistance characteristics of Medea by studying a structured population of hybrids and parents of Medea and Grasslands Samson cultivars of perennial ryegrass.

To realise this objective, a series of three experiments were conducted. The first of these (Experiment 3) is reported here and involved five family groups. Each group contained a Medea and a Grasslands Samson parent with 3 of their F_1 progeny. There were 2 clonal replicates making 50 plants in total in the experiment.

5.2 Materials and Methods

5.2.1 Location, design and setting up

This experiment was conducted from March 2009 to February 2010 in the same glasshouse at Plant Growth Unit of Massey University in which Experiment 2 was conducted. Each of the twenty five plants was divided into two ramets to get two clonal copies i.e., replications for a 25 × 2 = 50 plants in the experiment. The ramets were transplanted into 15 cm diameter PVC pipes of 100 cm length, lined internally with transparent plastic sleeves and filled with a mixture of B horizon of a Manawatu alluvial soil and builder's sand in the ratio 3:1 and fertilised with 'Osmocote' on 16 March, 2009. The plastic sleeves lining the pots were given two vertical cuts at the base (each cut 5 cm length) to ensure drainage. The experiment comprised a plant establishment period from March to October 2009, a treatment standardisation and measurement period from November 2009 to February 2010. During the

establishment period the plants were watered to 80% of "pot field capacity" by weighing the pots on a 30 kg capacity balance. "Pot Field Capacity" was measured in March 2009 by the methodology given in Appendix 5.1. The heavy labour requirement for weighing the pots led to a change of water application methodology, and from late October 2009, the pots were placed in 200 litre plastic drums with taps at the base (Section 4.2.2.1).

During the treatment standardisation and measurement period, the level of water in the drums was kept to 50 cm (unstressed) from the soil surface until 23 November 2009 when the level was lowered to 70 cm below the surface to start the onset of water deficit. Drums were emptied of water every 7 - 10 days to ensure aeration of roots. Later, on 22 January 2010, the drums were emptied of water and dried meaning plants had to continue growth using water already in the PVC pipes.

5.2.2 Measurements

Measurements were made in three phases of the experiment: unstressed plants (2 to 20 November 2009); mildly stressed plants (7 to 21 December 2009) and severely stressed plants (1 to 26 February 2010). DWunstress, DWstressed, HN, HNspring and HNsummer were taken at various times in the unstressed, stressed phases and spring and summer seasons during the experiment. DW is a cumulative of the respective individual measurements i.e., DWunstress and DWstress taken on 28 August, 22 October, 21 December and during final harvest. HN is a cumulative of the respective individual measurements - HNspring (taken on 21 November) and HNsummer (taken on 17 December 2009 and 18 January 2010). TN was measured at harvesting by dividing the plant into 4 - 5 groups and counting the number of tillers in each of the sub-groups.

Plant water relations measurements (LWP, RWC, proline and SMC) and a measurement from the stomatal and cellular control domain (Tc-Ta) were conducted in each of the three phases as indicated in Tables 5.1, 5.2 and 5.3.

Pre-dawn LWP (MPa) was measured using a Scholander pressure chamber and a 5 cm tip of the leaf blade of a mature, healthy tiller as detailed in section 4.2.2.2.3. Measurements for plants of one replicate were completed each day. RWC was

measured by the method of Barrs and Weatherley (1962) on a 5 cm long leaf tip as mentioned in section 4.2.2.2.3 Proline contents were measured according to the method of Bates et al. (1973) as mentioned in section 4.2.2.2.3. During the severely stressed phase, SMC was measured gravimetrically as SMC d1, SMC d2 and SMC d3 at harvesting by weighing immediately on extraction, then oven-drying and re-weighing a 5 cm length of the soil column sliced with a knife from the midpoint of each of the 3 soil depths 1, 2 and 3. Tc-Ta (°C) was measured using a non-contact Infrared thermometer (IRT) (model 8828H, Shenzhen Everbest Machinery Industry Co., Ltd P. R. China.

Table 5.1: List of measurements and their dates for the unstressed phase of Experiment 3. LWP, pre-dawn leaf water potential (MPa), RWC, relative water content (%), OP, osmotic potential (MPa), Tc-Ta, canopy- air temperature difference, SMC (TDR), volumetric soil moisture content by time domain reflectometry (%).

Activities	Days (November 2009)														
	Week 1					Week 2					Week3				
	2	3	4	5	6	9	10	11	12	13	16	17	18	19	20
LWP													x	x	
RWC													x	x	
OP													x	x	
Proline						x	x								
Tc-Ta				x											

Table 5.2: List of measurements and their dates for the mild stress phase of watering for Experiment 3 plants. Abbreviations are as for Table 5.1.

Activities	Days (December 2009)												
	Week 6					Week 7					Week 8		
	7	8	9	10	11	14	15	16	17	18	21	22	23
LWP	x	x											
RWC	x	x											
OP	Not measured												
Proline			x	x									
Tc-Ta						x							
Defoliation for DM											x		

Table 5.3: List of measurements and their dates for the severely stressed phase of watering for Experiment 3 plants. Abbreviations are as for Table 5.1. Additional abbreviations are as defined in the footnote.

Activities	Days (February 2010)																			
	Week 14					Week 15					Week 16					Week 17				
	1	2	3	4	5	8	9	10	11	12	15	16	17	18	20	22	23	24	25	26
LWP	x	x																		
RWC	x	x																		
OP	x	x																		
Proline			x	x																
Tc-Ta					x															
Coarse and fine root extraction									Commenced 11 February, concluded 26 Februrary.											
SMC d1, d2 d3 (grav.)									Commenced 11 February, concluded 26 Februrary.											

SMC d1, d2 and d3 are gravimetric soil moisture contents at soil depths 1, 2 and 3, respectively.

About 2.5 cm of each leaf blade was sampled for OP after LWP measurement each day and was frozen immediately in liquid nitrogen and later stored at -80°C for subsequent measurement of OP with a Wescor HR 33T psychrometer (Section 4.2.2.2.3). PP (MPa) was calculated by subtracting OP from LWP.

Destructive harvest of roots to determine Rc d1, Rc d2, Rc d3, Rf d1, Rf d2, Rf d3 was carried out at the end of the severe stress phase according to the methodology described in Section 4.2.1.2.2.

Mid parent heterosis (%) was calculated according to the formula $(F_1\text{-MP})/MP \times 100$ where F_1 is the value for a particular trait for the mean of the replicates of an F_1 genotype and MP (mid-parent value) is average of the two parents in that family group.

5.2.3 Statistical analysis

Effects of interest for statistical testing were (i) significance of difference between family groups, (ii) significance of any difference in means between trait means for the two parents or between the mean for each parent and the mean of their three progeny, and (iii) significance of the parent × progeny interaction among families for the various traits. A factorial ANOVA model in Proc GLM of Minitab v. 16 was used. Sums of squares were extracted for replicate, family group, “Class” (Class 1 =

one of the progeny, Class 2 = either a Grasslands Samson or Medea parent plant), and the family group × class interaction with 4 degrees of freedom. To separate the question of variation across families between the progeny mean and the combined mean for both parents, and variation across families between the progeny mean and the individual parent means, a second ANOVA was made redefining "Class" (1 = one of the progeny, 2 = Grasslands Samson parent plant, 3 = Medea parent plant). In this second ANOVA the family group × class interaction has 8 degrees of freedom. A composite ANOVA was then compiled incorporating the Class effect calculated from ANOVA 2 considering the two parents and their progeny as three discrete groups and the family group × class interaction from ANOVA 1 based on the variation among families between the progeny mean and the combined mean of both parents. In the composite ANOVA an "interaction remainder" with 4 degrees of freedom representing the variation of individual parental means from the combined mean of both parents was calculated as the difference between the interactions of ANOVA 1 and ANOVA 2. For an example of the construction of the composite ANOVA for partitioning of the family group × class interaction in this way, see Appendix 5.2.

5.3 Results

5.3.1 Parent / progeny and Family group water deficit responses

In this experiment the interest was to identify specific traits in parents that were transferred to the progeny and might be useful selection targets in a plant breeding programme. Therefore, in this chapter the data for shoot growth, root development and water uptake, and plant water status and stomatal and cellular control domains are presented with minimal comments in Tables 5.4, 5.5 and 5.6, respectively, and summary tables (Tables 5.7 and 5.8) are compiled to overview those data.

The key points about shoot growth are that Grasslands Samson had high TN and low HN compared to Medea while the progeny were intermediate between Grasslands Samson and Medea for TN and similar to Medea for HN (Table 5.4).

Table 5.4: Parent and F_1 means, P values and mid-parent heterosis (MPH), for variables of the "shoot growth" domain of Experiment 3: TN, tiller count, DW, shoot dry weight (g), HN, seed-head number. DWstress and LERstress are shoot dry weight and leaf elongation rate (mm d^{-1}) during stress. DWunstress, shoot dry weight (g) during unstressed phase. HNspring and HNsummer are seed-head numbers during spring and summer.

Variable	Mean values for parents and progeny						P values		
	Medea	G. Samson	Progeny	SE (parents)	SE (progeny)	MPH	Family Group	G. Samson-Medea-Progeny	Family group × class[1] interaction
TN	331.3	437.5	380.2	32.88	18.98	-1.08	0.017	0.029	ns
DW	43.73	50.85	46.97	3.72	2.15	-0.67	Ns	ns	0.008
DWunst	35.6	36.42	36.68	3.09	1.78	1.86	0.024	ns	0.005
DWstress	8.14	14.43	10.29	1.04	0.60	-8.82	Ns	ns	0.096
HN	50.5	28.3	47.43	5.42	3.12	20.38	0.02	0.008	0.006
HNspring	8.8	2.5	12.6	2.31	1.33	123.00	Ns	0.002	ns
HNsummer	41.7	25.9	34.83	4.63	2.67	3.05	0.009	0.066	0.015
LER stress	4.06	6.02	4.42	1.35	0.78	-12.30	Ns	ns	ns

[1]Test as described in Section 5.2.3 to statistically evaluate the Family group variation of the progeny trait mean around the combined parental mean.

For traits of root development and water uptake, Grasslands Samson had higher Rt (and its components at d1 and d2) and higher R:S, DR:S and Index DR than Medea (Table 5.5). Progeny had Rt, Rf d3, R:S, DR:S and Index DR intermediate between Grasslands Samson and Medea, though for Rf d3 and Index DR the progeny had values similar to Grasslands Samson while for Rt and R:S the progeny values were similar to Medea.

Table 5.5: Parent and F_1 means, P values and mid-parent heterosis (MPH) for variables of the plant response domain "root development and water uptake". All measurements are from the destructive harvest in early February 2010 under severe water stress.

Variable	Mean values for parents and progeny						P values		
	Medea	G. Samson	Progeny	SE (parent)	SE (progeny)	MPH	Family Group	G. Samson-Medea-Progeny	Family group × class[1] interaction
SMC d1	5.32	3.18	4.56	1.12	0.64	7.29	Ns	Ns	ns
SMC d2	7.72	4.70	5.72	1.32	0.77	-7.89	Ns	Ns	ns
SMC d3	8.78	7.57	8.20	1.49	0.86	0.30	Ns	Ns	ns
Rt	28.57	45.30	31.80	4.75	2.74	-13.90	Ns	0.032	0.054
Rt d1	17.46	23.98	17.02	2.18	1.26	-17.85	0.074	0.028	0.011
Rt d2	9.96	19.31	12.55	2.53	1.46	-14.26	Ns	0.032	0.081
Rt d3	1.14	2.01	2.22	0.52	0.30	40.95	Ns	Ns	ns
Rc d1	15.12	20.78	14.37	2.11	1.22	-19.94	0.081	0.067	0.021
Rc d2	8.54	17.85	11.34	2.55	1.47	-14.05	Ns	0.014	0.090
Rc d3	0.81	1.25	1.55	0.55	0.32	50.48	Ns	Ns	ns
Rf d1	2.34	3.20	2.65	0.443	0.25	-4.33	Ns	Ns	0.091
Rf d2	1.41	1.46	1.21	0.21	0.12	-15.68	Ns	Ns	ns
Rf d3	0.33	0.75	0.67	0.096	0.05	24.07	Ns	0.004	0.090
R:S	0.63	0.88	0.66	0.061	0.035	-12.58	0.012	0.007	0.004
DR:S	0.22	0.41	0.30	0.041	0.02	-4.67	0.061	0.009	0.015
Index DR	0.33	0.45	0.44	0.032	0.018	12.82	Ns	0.012	ns

[1]See footnote to Table 5.4.

SMC d1, SMC d2 and SMC d3 are gravimetric soil moisture contents (%) at depths 1, 2 and 3, respectively. Rt, total root mass (g), Rt d1, Rt d2 and Rt d3, total root mass (g) at depths 1, 2 and 3. Rc d1, Rc d2 and Rc d3 are coarse root weights (g) at depths 1, 2 and 3. Rf d1, Rf d2 and Rf d3 are fine roots weights (g) at depths 1, 2 and 3. R:S, root shoot ratio, DR:S, deep root (soil depths 2 and 3) to shoot ratio, Index DR, ratio of total roots mass at depths 2 and 3 to total root mass (Rt).

For traits of "plant water status" and "stomatal and cellular control" (combined together in Table 5.6 below), it emerges that under unstressed conditions progeny had a significantly higher LWP while they also had a higher (though similar to Medea) RWC. Under severely stressed conditions, again progeny had a higher LWP and a higher concentration of proline contents. Medea, however, had a higher cooling effect under severe water stress. Looking at differences across the four

Table 5.6: Mean, P values and mid-parent heterosis (MPH), for variables of domains "plant water status" and "stomatal and cellular control" during unstressed, mild stressed and highly (Sev.) stressed phases of Experiment 3. Osmotic potential (OP) and pressure potential (PP) were not measured for mildly stressed plants. Dates indicated are for measurement of replicate 1. Replicate 2 was normally measured the following day.

Trait		Mean values for parents and progeny					MPH	P values		
		Medea	G. Samson	Progeny	SE (parents)	SE (progeny)		Family Group	G. Samson-Medea-Progeny	Family group × class interaction[1]
LWP	Unstressed (18 Nov)	-0.56	-0.59	-0.46	0.051	0.029	-20.0	ns	0.046	0.038
	Mild stress (7 Dec)	-0.85	-0.80	-0.76	0.065	0.038	-7.87	ns	ns	ns
	Sev. stressed (2 Feb)	-1.04	-1.32	-0.95	0.084	0.049	-17.39	ns	0.003	ns
OP	Unstressed (18 Nov)	-1.11	-1.18	-1.13	0.068	0.039	-1.31	<0.001	ns	ns
	Mild stress	-	-	-	-	-	-	-	-	-
	Sev. stressed (2 Feb)	-1.73	-1.75	-1.71	0.113	0.065	-6.30	0.094	ns	0.051
PP	Unstressed (18 Nov)	0.55	0.59	0.67	0.077	0.044	17.54	<0.001	ns	0.005
	Mild stress	-	-	-	-	-	-	-	-	-
	Sev. stressed (2 Feb)	0.69	0.43	0.75	0.156	0.09	12.59	0.082	ns	0.045
RWC	Unstressed (18 Nov)	94.43	92.43	94.67	0.81	0.46	1.37	ns	0.066	ns
	Mild stress (7 Dec)	96.57	96.19	95.45	1.85	1.06	-0.95	ns	ns	ns
	Sev. stressed (1 Feb)	89.05	89.25	89.32	0.96	0.55	0.19	ns	ns	ns
Proline	Unstressed (9 Nov)	0.027	0.023	0.025	0.0042	0.002	<0.001	ns	ns	ns
	Mild stress (9 Dec)	0.014	0.015	0.017	<17	0.03	17.24	ns	ns	ns
	Sev. stressed (3 Feb)	0.16	0.22	0.39	0.164	0.094	105.2	<0.001	0.001	<0.001
Tc-Ta	Unstressed (4 Nov)	-3.38	-5.45	-4.05	0.767	0.44	-8.26	ns	0.065	ns
	Mild stress (14 Dec)	-3.50	-3.36	-3.27	0.458	0.26	-4.66	0.019	ns	0.078
	Sev. stressed (5 Feb)	-6.74	-4.18	-3.03	0.728	0.42	-44.50	ns	0.018	ns

[1]See footnote to Table 5.4.

domains, notable separates between Medea and Grasslands Samson are high HN and leaf cooling in Medea and high TN, R:S, DR:S, IndexDR and highly negative LWP in Grasslands Samson (Table 5.7). The traits where progeny significantly exceeded the parents were HN and proline contents.

Table 5.7: Standard deviations of Medea, Grasslands Samson and progeny means from the population means for traits where statistically significant differences were detected. Values of less than 0.8 standard deviation are suppressed.

Trait	Medea	Grasslands Samson	Progeny
TN	-1.57	1.65	
HN	1.55	-2.54	0.99
HN spring		-2.36	2.01
HN summer	1.63	-1.78	
Rc d1		1.91	-1.13
Rc d2	-1.58	2.07	
Rf d3	-2.63	1.73	
R:S	-1.53	2.56	-1.04
DR:S	-2.19	2.44	
Index DR	-2.39	1.35	1.04
LWP (unstressed)		-2.57	1.82
Proline (stressed)			0.81
Tc-Ta (stressed)	-2.74		2.11

The MPH values in Tables 5.4 to 5.6 present a complex picture in terms of the 'combining ability' of Medea and Grasslands Samson. F_1 progeny tended to inherit the lower DW of Medea, leading to negative MPH for DW, LER, and Rt d1 and d2; but the deep rootedness and prolific heading behaviour of Medea, resulting in positive MPH for Rt d3 and spring and summer HN. F_1 progeny also tended to display higher proline accumulation under stress than either parent and less negative LWP, ranging from strongly positive to strongly negative MPH for those two traits.

Family groups were compared by the same method used to compare the two parents and their F_1 progeny in Table 5.1, and each group was found to exhibit different

combinations of the parental traits (Table 5.8). Family group 1 had strong shoot production traits (TN and DW) when plants were unstressed, with the increase in plant size also associated with high values for Tc-Ta and Rc d1 (and Rt d1), but not R:S. Family group 2 was notable for reduction in several root traits and for proline accumulation and more negative OP under high stress. Family group 3 had below average DW and Rc d1 and reduced heading. Family groups 4 and 5 both expressed high R:S and DR:S. In the case of family group 5 these root traits were associated with high DW and low TN, indicating large tiller size, but also with strong heading behaviour.

Table 5.8: Standard deviations of family group means from the population means for traits where statistically significant differences were detected. Values of less than 1 standard deviation are suppressed.

	Family groups				
Trait	1	2	3	4	5
Unstressed phase					
TN	1.07				-3.33
DW	1.69		-1.98	-1.87	1.37
HN	-1.53		-1.87		2.61
HNsummer	-1.79		-1.84	1.13	2.82
OP	-1.59	2.56	-2.41	-1.12	2.56
PP	2.31	-3.24	2.59		-2.17
Medium stress					
Tc-Ta	2.25	1.71	-1.43	-1.66	
High stress					
Rt d1	1.29	-1.24	-1.76		1.74
Rc d1	1.42	-1.38	-1.50		1.69
R:S		-2.39	-1.59	1.91	1.78
DR:S		-1.66	-1.36	1.79	1.46
OP		-1.54	-1.17		2.04
PP			1.62		-2.45
Proline		1.25			

5.3.2 Parent / progeny × family group interaction

For a number of traits, across the plant response domains, there were statistically significant interactions between family group and parent / progeny difference (Figs. 5.1 – 5.3).

Although not analysed statistically here, there was an indication of a cultivar × time interaction in that in family groups 2 and 5 the cumulative shoot DW over unstressed period (from March to November 2009) was greater for the Medea parent than for the Grasslands Samson parent. By contrast, for the stressed plants in warm growing conditions in January / February 2010 (DWstressed) Medea never outproduced Grasslands Samson. This appears as an interesting shift of equally productive Medea and Grasslands Samson plants in Fig. 5.1a to highly productive Grasslands Samson only in Fig. 5.1c.

However, despite the negative MPH for DWunstr and the strong positive MPH for HNspring (Table 5.4) (i.e. a tendency for F_1 progeny to have low DW under stress and prolific seed-head production) family groups varied in their characteristics and F_1 progeny of some family groups had DW (Fig. 5.1a) and HN (Fig. 5.1d) similar to Grasslands Samson, family group 3 being a good example.

Another important feature to emerge from these interactions is that the higher R:S and DR:S observed in Grasslands Samson plants (Table 5.5) was particularly seen in family groups 4 and 5 (Figs. 5.2c and 5.2d). However, the positive MPH for proline at severe stress phase of the experiment (Table 5.6) was seen to be scattered over all family groups (Fig. 5.3b).

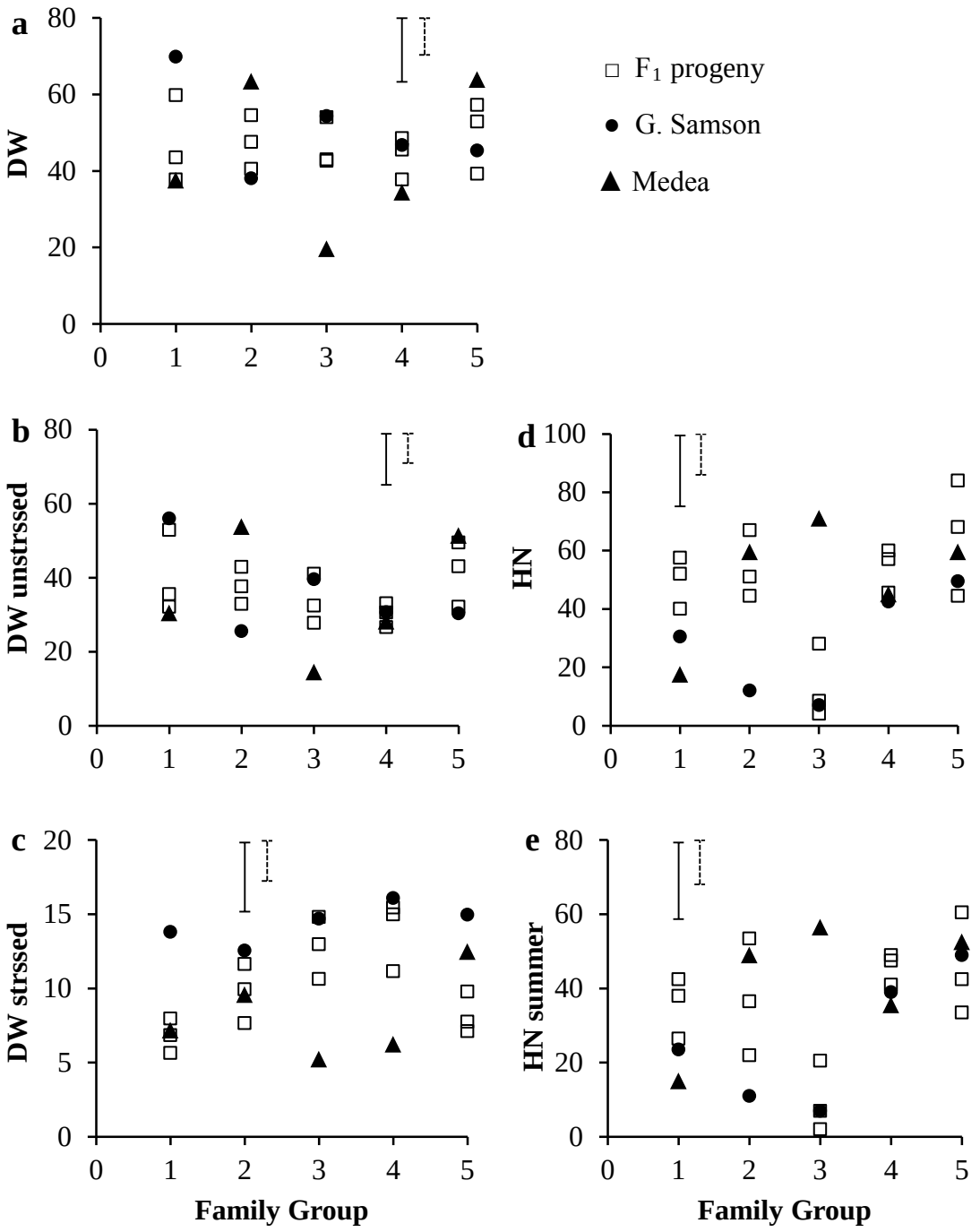

Figure 5.1: Graphical representation of statistically significant family group × parent/progeny interaction for plant responses of the shoot growth domain: (a) DW, shoot dry weight; (b) DWunstress, shoot dry weight during unstressed phase; (c) DWstressed, shoot dry weight during stressed phases; (d) HN, seed-head number; and (e) HNsummer, seed-head number during summer. Solid and dash bars are the standard error (± SE) of parents and progeny, respectively.

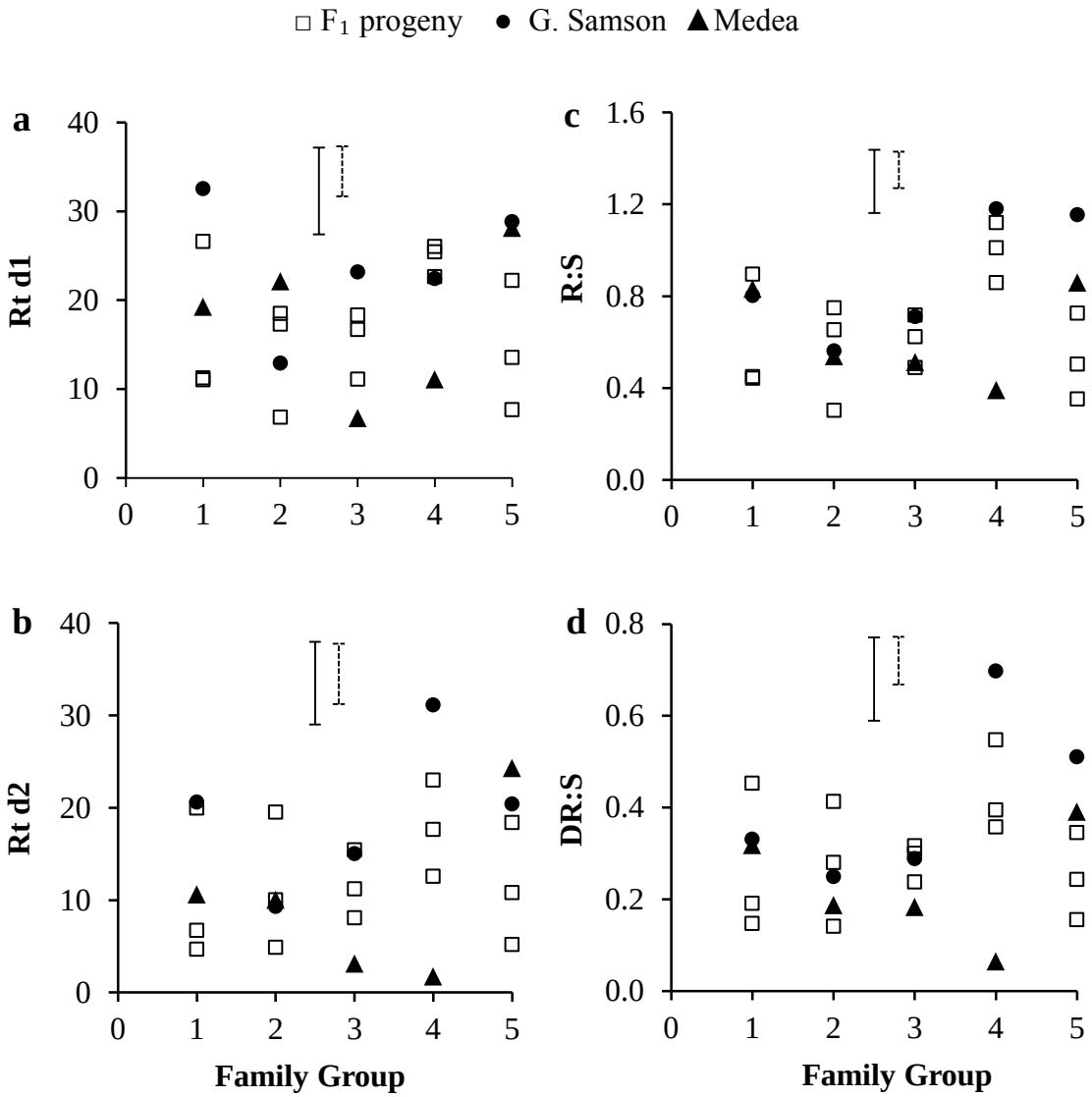

Figure 5.2: Graphical representation of statistically significant family group × parent/progeny interaction for plant responses of the root development and water uptake domain: (a) Rt d1, total root weight (g) at soil depth 1; (b) Rt d2, total root weight (g) at soil depth 2; (c) R:S, root to shoot ratio; and (d) DR:S, deep root (soil depths 2 and 3) to shoot ratio. Solid and dash bars are the standard error (± SE) of parents and progeny, respectively.

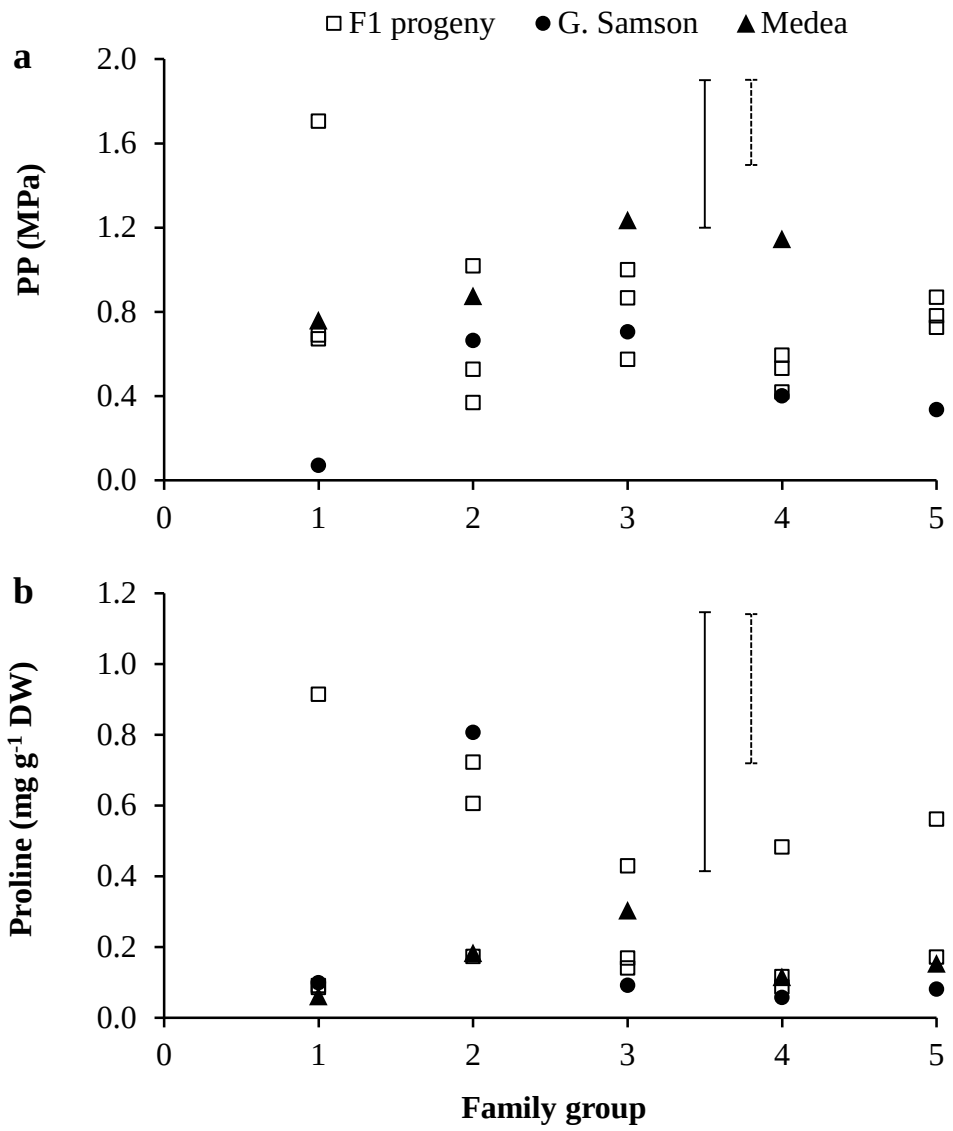

Figure 5.3: Graphical representation of statistically significant family group × parent/progeny interaction for plant responses of the plant water status domain (a) PP, pressure potential (MPa) and (b) proline contents (mg g^{-1} DW). Solid and dash bars are the standard error (± SE) of parents and progeny, respectively.

5.4 Discussion

5.4.1 Choice of statistical analysis

Variation between family groups for expression of parental traits in the progeny is of interest in understanding the results of this experiment. Such variation in trait inheritance is tested statistically in a data set of this structure by the significance of the parent × progeny interaction. However, an interesting question arises in setting up the statistical analysis, of how to partition within the ANOVA, the contribution to

the interaction SS of (a) variation among the genotypes of the two parents and (b) variation of the separation between the parent mean and the progeny mean. The solution adopted, of first calculating the interaction SS with 4 degrees of freedom for separation of the progeny mean from the combined parental mean, next calculating the interaction SS with 8 degrees of freedom for separation between the progeny and each parental mean separately, then subtracting the smaller interaction from the larger one to partition the interaction in the second ANOVA allows significance testing of both Medea / Grasslands Samson differences, and the progeny variation around the mean of both parents, in a single ANOVA. The greater the statistical significance of this family group × "class" interaction (Tables 5.4 – 5.6), the greater the opportunity to screen a number of family groups in an expanded plant breeding programme, for variation in the progeny mean for the trait in question. For example, in Fig. 5.1d family group 3 is seen to have a low value for HN and so could potentially be a candidate for breeding a Medea-like line without the prolific flowering normally seen in most of the F_1 progeny. Trait distribution in the progeny is discussed further in Section 5.4.3 below.

It would also be possible to statistically test variation of the progeny mean of a family group trait across family groups for statistical association with variation of one or other of the parents from the mean of genotypes of each parent. However, partly because 5 family groups is a rather small number for such analysis to work well, and partly because of time pressure, this point was not explored further here.

5.4.2 Comparison of Medea and Grasslands Samson

In this experiment in an unheated glasshouse, shoot DW (i.e. herbage production) did not vary between Medea and Grasslands Samson during winter, but Medea DW was 44% lower than Grasslands Samson (8.14 g plant^{-1} cf. 14.43 g plant^{-1}, Table 5.4) in conditions of summer drought. The similar growth of Medea and Grasslands Samson in this experiment in winter adds weight to the suggestion that growth of Medea plants in Experiment 1 could have been reduced by warm glasshouse temperatures and triggering of a summer dormancy response, while the reduced growth of Medea in summer coupled with the high HN compared with Grasslands Samson, further confirm results of Experiments 2 & 5 previously reported in Chapter 4. The question

then arises whether New Zealand farmers prefer summer dormancy (presumably with better survival) in a ryegrass cultivar, or whether they prefer a ryegrass with a strategy of maintaining growth during moisture deficit (implying that ryegrass would not be sown in sites subject to more severe moisture deficit), or whether plant breeders should consider producing 'niche' cultivars for each of those situations. The answer to this question would determine the approach to be taken if Medea was to be used in a breeding programme.

An unexpected but important point to emerge when comparing results for Medea and Grasslands Samson in Experiment 3 is that in these plants which were 11 months old when destructively harvested, Grasslands Samson rather than Medea had greater Rt d2, R:S, DR:S, and Index DR than Medea (Table 5.5). This shows that plant breeders need to consider the age of their material when selecting for root related traits, and that traits expressed in seedling plants or newly transplanted tillers may not reflect the behaviour of older, more mature plants.

With respect to leaf water relations Medea plants again showed evidence of cooling under severe water deficit (Table 5.6). Plants of Grasslands Samson had more negative values for LWP than Medea, indicating that plants with a growth strategy like that of Grasslands Samson are likely to encounter significant physiological stress in more severe water deficit situations, and this highlights the breeder's choice mentioned above of selecting for summer survival or selecting for summer growth.

5.4.3 Trait expression in F_1 progeny

Though parent / progeny × family group interactions were observed for a number of traits and are graphically presented in Figs. 5.1 – 5.3, it was only seed-head production (i.e. HN and HN summer,Table 5.4); PP and proline under severe stress (Table 5.7) for which an appreciable amount of positive MPH was recorded. The trend for higher HN and HN in summer in the F_1 progeny that seems to have been inherited from Medea and is seen particularly in family groups 1, 4 and 5 (Fig. 5.1 d) is something not favoured by New Zealand farmers, since higher flowering leads to lower herbage quality. However, the exceptionally high value of MPH for proline contents and that this was observed in most of the five family groups (Table 5.6)

under study is a promising result. Higher proline contents are often physiologically inter-linked to higher PP (Section 2.4.2.1). Higher OA has been found to assist plants with soil moisture extraction (Morgan, 1984), and it is also clear that proline accumulation has a substantial role in OA (Ashraf and Foolad, 2007), it is far from clear from the existing literature on proline whether any such effects are typically associated with observation of higher proline levels. From the physiological link between proline accumulation and OA, it can be assumed that the higher proline contents in the progeny provide a possibility of selection of individual plants from the progeny for OA. Accumulation of osmolytes in cell cytoplasm to maintain its turgidity and thus water retention, has always been valued to impart an improved plant yield under drought (Zhang et al., 1999) and has also been reported to be a heritable trait in perennial ryegrass (Thomas, 1990).

5.5 Conclusions

- In this experiment DW of Medea was no less than Grasslands Samson in winter but 44% reduced compared to Grasslands Samson in summer, suggesting a summer dormancy response;
- In mature plants R:S, DR:S and Index DR of Medea were less than those of Grasslands Samson. For younger plants in Experiments 2 and 5 (Chapter 4) the reverse was true. This indicates that plant breeders should not assume that root traits of seedlings reflect those of mature plants when making selections for root behaviour;
- In general, mature F_1 progeny of Medea × Grasslands Samson parents showed prolific heading, and lower R:S than Grasslands Samson; traits similar to Medea. However, existence of family group × parent / progeny interactions for many traits means that a range of trait combinations could be obtained.
- F_1 Medea × Grasslands Samson progeny exhibit leaf proline levels (associated with PP) in water deficit much higher than either parent. Further study of the implications of proline accumulation might be worthwhile.

6

Evaluation of F_2 Medea × Grasslands Samson hybrids for drought resistance traits

6.1 Introduction and aims

In the testing of parents and their F_1 hybrids in family groups reported in the previous chapter it was found that particular family groups in the F_1 generation varied in the combination of productive and drought tolerance traits (water capture or leaf water relations) displayed. For example: family group 1 displayed higher than average TN, DW and PP but no improvement in root traits like R:S, DR:S or OP and proline level, for which family significant differences were detected. Family groups 4 and 5 though exhibited higher than average root traits but also higher HN or HNsummer (Table 5.8). In addition, F_1 progeny in general showed higher levels of proline than the parents (Table 5.7). However, it is well recognised in plant breeding that the allelic diversity of two parents recombines to yield hybrids that express dominant allele phenotypes with the recessive alleles being suppressed. Then, in the F_2 generation, recessive alleles present in only one parent may be expressed and masked traits are visible in this filial generation in ratios that permit genetic analysis.

To explore this further, in this experiment (Experiment 4) a population of F_2 hybrids previously created by Dr H.S. Easton at AgResearch was screened for production and drought resistance traits using the same methodology as for parents and their F_1 progeny in Experiment 3. Hence the aim of Experiment 4 was to evaluate randomly chosen family groups of F_2 hybrids to:

i. Describe how differences in a suite of measurements of plant water relations (especially those mentioned above) are distributed between and within family groups in a structured F_2 population of hybrids of Grasslands Samson and Medea; and to
ii. For those traits that could be measured non-destructively, to evaluate the relationships among traits in the F_2 hybrid population, under contrasting mild

and more severe water deficit stress levels, as water deficit increased with time after cessation of watering.

6.2 Materials and Methods

6.2.1 Location, design and setting up

This experiment was conducted between December 2009 and June 2010 in the same glasshouse of Plant Growth Unit of Massey University as reported for previous experiments, and used 30 Grasslands Samson × Medea hybrids of the F_2 generation. The plants obtained from AgResearch consisted of 8 family groups numbered at AgResearch 1, 3, 5, 7, 10, 12, 13 and 15 (not related to the family groups in Experiment 3) and numbered here 1 – 8, respectively. Five genotypes within each family group were randomly chosen from each of the eight family groups. Two clonal copies were obtained from each of the 40 genotypes, making a total of 80 plants in the experiment. PVC pipes 100 cm long and 10 cm in diameter were pre-filled with a soil mixture (as described in Section 5.2.1) on 18 December 2009 and ramets were transplanted on 25 December 2009. To allow water ingress, pipes were closed at the bottom end with two layers of nylon cloth fastened with adhesive tape at the base. To keep the numbers of plants in the experiment manageable it was decided to include family groups 1 to 6 only in the experiment (6 family groups, 60 plants), while discarding the plants of family group 7 and 8. However, when differences between genotypes in plant survival were noted among the discarded plants which had been left unwatered in the glasshouse, some measurements were undertaken to investigate the basis for the differential survival of these unwatered plants.

After transplanting of ramets, the 60 pots were randomly placed into two wooden frames to hold them upright. The plants were then top-watered for 97 days with an amount of water sufficient to facilitate good root and shoot growth while the discarded plants remained unwatered. During that period the watered plants were defoliated twice (10 February and 7 March 2010) 7 cm above the soil surface, and the DW of clippings recorded.

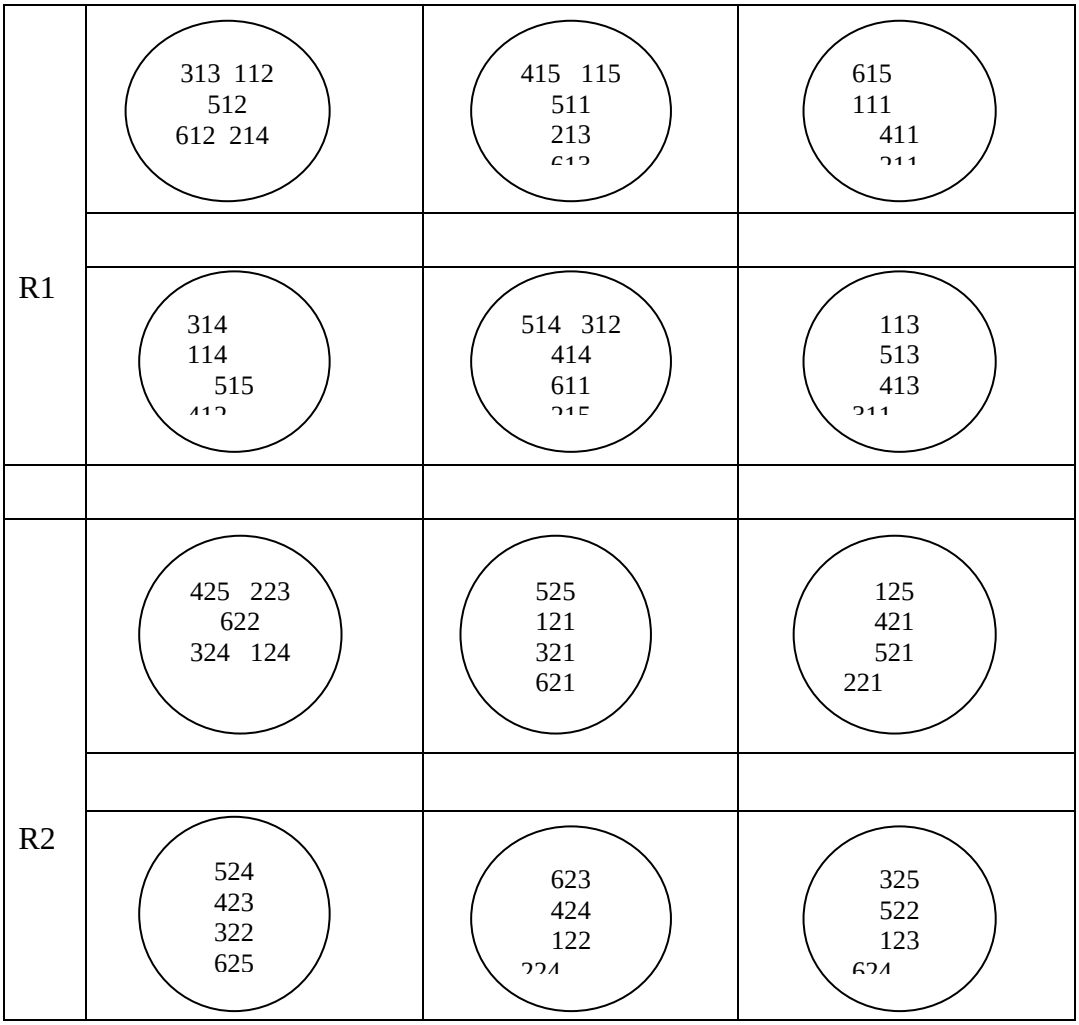

Figure 6.1 Layout used for plants in Experiment 4. R1 and R2 denote replicates. Circles represent the drums of water, each of which held 5 randomly allocated plant genotypes. Three digit numbers identify plant genotypes with the first digit indicating the family group, the second digit the replication number, and the third digit the genotype within family group.

6.2.2 Treatment application and measurements

It was planned to carry out non-destructive measurements for the watered plants in both mild (-0.5 MPa) and severely stressed (-1.0 MPa) phases of water deficit, based on their pre-dawn LWP (Lucero et al., 1999). On 31 March the pots of each replicate were randomly allocated to six 200 L drums, 5 plants per drum (Fig. 6.1), as described in the previous chapter, for control of water level. At first the water level in the drums was maintained 45 cm below the soil surface in the pots with the aim of maintaining a mild water deficit. From time to time LWP was measured on randomly

selected plants to monitor plant water status. On 7 April, average LWP had reached -0.7 Mpa, and therefore, a measurement cycle was started. However, measurement was discontinued soon afterward because the plants developed powdery mildew. To control the disease, plants were sprayed with Systhane on 13 April while the water level was maintained at 45 cm below the soil surface. On re-measuring LWP on randomly selected plants on 19 April, it was found to be still -0.7 MPa and so measurements of plant water relations as outlined in Table 6.1 were conducted between 19 April and 3 May.

On 4 May the drums were drained and residual water in bottom of the drums was wiped out with a dry towel to commence the severely stressed phase of the experiment. On 17 May, LWP was measured on randomly selected plants and found to be -0.82 MPa. Anticipating a further drop in LWP, measurements for the severely stressed phase of the experiment were started a week later (24 May) and continued until 6 June (Table 6.2). LWP, OP, PP, RWC and proline contents during both phases of the experiment were measured following the techniques described in Section 4.2.2.2.3. Tc-Ta (°C) was measured using a non-contact Infrared thermometer (IRT) (model 8828H, Shenzhen Everbest Machinery Industry Co., Ltd P. R. China on 19 April and 31 May for mildly and severely stressed phases of the experiment, respectively.

In addition to two defoliation events as mentioned in Section 6.2.1, two other defoliations 7 cm above the soil surface were carried out during mild and severely stressed phases on 28 April (Table 6.1) and 2 June (Table 6.2), respectively for measurement of LER. The herbage DW thus obtained from the cut foliage was added to previously obtained DW data (refer Section 6.2.1). LER of mild and severely stressed plants was measured, respectively, five and four days after defoliation following the methodology described in Section 4.2.2.2.1. TN was determined for mildly stressed plants, only, on 3 May by dividing each plant into groups of 2 or 4 (depending on plant size), counting the number of tillers in each of the sections and finally multiplying the count by 2 or 4 as appropriate.

At each defoliation as described above, emerged seed-heads were clipped, the HN counted, and their DW determined by drying at 65°C.

On 6 June the plants were shifted to a refrigerator in a field laboratory at Massey University and stored at 4°C until destructive measurements of root development and soil moisture content could be carried out. This occurred between 8 and 18 June. Pots were sawn apart transversely at 30 cm and 60 cm from soil surface to divide the soil column into upper, middle and lower segments. A 5 cm section of the soil column from each soil depth was cut out with a large knife, placed in a plastic bag, and within 24 hours weighed, then oven dried over 48 hours, and reweighed to determine gravimetric SMC (%). Coarse roots were retrieved from the SMC samples, and coarse and fine roots from the remaining soil of each soil depth segment, as described in Section 4.2.1.2.2.

Table 6.1: Schedule of measurements carried out in the mild stress phase of Experiment 4. Abbreviations: LWP, leaf water potential; OP, osmotic potential; PP, pressure potential; RWC, relative water content; Proline, leaf proline concentration, Tc-Ta, canopy and ambient temperature difference determined using an infra red thermometer; LER, leaf extension rate; TN, tiller count per plant.

Activities	Days (April 2010-May 2010)														
	Week 1					Week 2					Week 3				
	19	20	21	22	23	26	27	28	29	30	3	4	5	6	7
LWP	x	x													
OP	x	x													
PP	x	x													
RWC	x	x													
Proline			x	x											
Tc-Ta	x					x									
Defoliation								x							
LER											x				
TN											x				
Lowering water table												X			

Table 6.2: Schedule of measurements carried out in the severe stress phase of Experiment 4. Abbreviations are as for Table 6.1.

Activities	Days (May 2010-June 2010)																		
	Week 6					Week 7					Week 8				Week 9				
	24	25	26	27	28	31	1	2	3	4	6[a]	8	9	10	11	14	15	16	17	18
LWP	x	x																		
OP	x	x																		
PP	x	x																		
RWC	x	x																		
Proline							x	x												
Tc-Ta						x														
Defoliation								x												
LER											x									
Coarse root and SMC												Commenced 8 June, completed 18 June								

[a]June 7 was used preparing for the destructive harvest carried out from June 8 – 18.

6.2.2.1 Unwatered plants

When survival of some genotypes among the unwatered plants after 90 days without water was noted (Fig. 6.2 below), a small data set was collected on an impromptu basis. Foliage was cut at ground level and sorted into green and dead, dried in a hot air draft oven, and weighed. Holes (5 cm diameter) were drilled in the sides of each of 10 pots (5 plant genotypes × 2 replicates) at 15 cm, 45 cm, and 75 cm below the soil surface (i.e. the midpoint of soil depths d1, d2, and d3) and approximately 200 g soil extracted and placed in a sealed plastic bag for SMC determination.

6.2.3 Data analysis

Data were analysed separately for mild and highly stressed phases of the experiment using the GLM command of Minitab version 16 to perform an ANOVA to test for differences between family groups and between genotypes within each family group for each trait. PCA, performed using Minitab, was used to assess the pattern of association between traits. Results of PCA were interpreted from the size and sign of coefficients for trait contributions to individual scores, and by ANOVA of principal component scores using the same model as used for the original data. Though some statisticians criticize the use of ANOVA on PC scores because PCA as a statistical tool only considers the total variability of data but ignores structure of the experiment

(Jackson, 1991), many other statistical commentators believe that PCA of PC scores (especially higher order PCs explaining a greater % of data variation) iss a valid approach (Jolliffe, 2002). Differences between genotypes of the unwatered plants were tested using an ANOVA as for a completely randomised design, again performed using Minitab.

6.3 Results

6.3.1 Family group differences

For the non-destructive measurements made in the mild stress phase of Experiment 4, statistically significant family differences were found only for DW, TN and HN (Table 6.3). Mean DW per plant (summed across the two defoliation events) of family groups ranged from 14.6 to 25.0 g, and TN per plant from 185 to 298. On this basis it can be calculated that mean yield per tiller ranged from 79 mg in family group 2 to 101 mg in family group 3. Family groups differed greatly in head number formed per plant during the experiment.

Table 6.3: Family group means of traits non-destructively measured during the mild water deficit phase of Experiment 4. Data are grouped by plant functional domains: shoot growth, plant water status, and stomatal and cellular control. Abbreviations are as defined for Table 6.1. Additional abbreviations are defined in footnotes.

	Family group							
Functional domain Trait	1	2	3	4	5	6	SEM	P value
Shoot growth								
DW[a] g plant^{-1}	22.4	14.6	20.8	25.0	18.3	21.9	1.33	<0.001
TN plant^{-1}	224	185	206	298	206	238	16.7	<0.001
LER mm tiller^{-1} d^{-1}	12.9	15.6	14.4	12.4	12.8	13.6	1.16	ns
HN[b] plant^{-1}	9.1	13.7	0.8	17.3	0.3	0.0	0.60	<0.001
Plant water status and stomatal and cellular control								
LWP MPa	-0.78	-0.81	-0.86	-0.80	-0.75	-0.75	0.030	ns
OP MPa	-1.07	-1.13	-1.11	-1.07	-1.03	-1.04	0.042	ns
PP MPa	0.29	0.32	0.24	0.27	0.32	0.28	0.049	ns
RWC %	95.6	94.5	96.7	94.6	94.7	94.6	1.07	ns
Proline mg g^{-1}DW	0.53	0.43	0.36	0.28	0.33	0.38	0.111	ns
Tc-Ta °C	-0.38	-0.21	-1.40	-0.77	-0.58	-1.48	0.426	ns

[a]Dry weight of leaves harvested on 10 February, 7 March and 28 April.
[b]HN was measured by clipping and counting the number of seed-heads at each defoliation event. DW, shoot dry weight (g), TN, number of tillers per plant, LER, leaf extension rate (mm d^{-1}).

For the measurements of severely stressed plants, including those data obtained from destructive harvesting, significant differences between family group means were found for 7 of 24 measurements carried out: DW, Index DR, R d2, Rc d2 and d3, SMC d2 and proline (Table 6.4).

Table 6.4: Family group means of traits measured during the severe water deficit phase of Experiment 4. Data are grouped by plant functional domains: shoot growth, root development and water uptake and plant water status and stomatal and cellular control. Abbreviations are as defined for Tables 6.1 and 6.3. Additional abbreviations are defined in footnotes.

	Family group							
Functional domain Trait	1	2	3	4	5	6	SEM	P value
Shoot growth								
DW g plant^{-1}	20.4	13.9	20.3	21.7	17.4	24.8	1.64	0.002
LER mm tiller^{-1} d^{-1}	12.4	12.1	11.5	12.6	10.4	11.9	0.55	0.085
Root development and water uptake								
R:S	0.66	0.63	0.69	0.53	0.76	0.52	0.065	Ns
DR:S	0.22	0.18	0.31	0.21	0.24	0.24	0.037	Ns
IndexDR	0.23	0.24	0.35	0.32	0.25	0.36	0.036	0.05
Rt g	12.80	8.00	14.05	11.61	13.15	13.08	1.640	Ns
Rt d1 g	9.93	6.04	8.50	7.94	9.85	7.89	1.155	Ns
Rt d2 g	2.36	1.59	4.88	3.14	2.71	4.44	0.742	0.03
Rt d3 g	0.51	0.36	0.66	0.53	0.59	0.74	0.088	0.08
Rc d1 g	9.38	5.56	7.89	7.31	8.99	7.29	1.191	Ns
Rc d2 g	1.82	1.24	4.46	2.58	2.19	4.03	0.745	0.03
Rc d3 g	0.21	0.10	0.34	0.25	0.32	0.49	0.082	0.04
Rf d1 g	0.55	0.48	0.62	0.63	0.85	0.60	0.102	Ns
Rf d2 g	0.54	0.35	0.42	0.57	0.52	0.41	0.048	Ns
Rf d3 g	0.30	0.26	0.32	0.31	0.28	0.25	0.032	Ns
SMC d1 %	7.75	7.19	5.79	6.45	7.29	5.85	0.619	Ns
SMC d2 %	11.64	12.12	9.38	10.57	11.01	9.63	0.701	0.06
SMC d3 %	19.53	16.62	17.77	15.85	18.16	13.98	1.957	Ns
Plant water status and stomatal and cellular control								
LWP MPa	-1.20	-1.17	-1.14	-1.18	-1.20	-1.18	0.032	Ns
OP MPa	-1.63	-1.56	-1.68	-1.61	-1.63	-1.99	0.142	Ns
PP MPa	0.43	0.40	0.56	0.43	0.43	0.80	0.133	Ns
RWC %	94.35	94.93	93.24	93.96	94.81	91.68	0.920	Ns
Proline mg g^{-1}DW	0.37	0.84	0.75	0.96	0.48	1.19	0.223	0.016*
Tc-Ta °C	1.79	1.60	1.60	1.48	1.88	1.61	0.198	Ns

DW, dry weight of leaves harvested on 8 June and during final harvest (between 8 to 18 June, 2010). R:S, root to shoot ratio; DR:S is sum of root masses at depths 2 and 3 to that of shoot DW, IndexDR is the ratio of sum of total roots at depths 2 and 3 to total root mass (Rt). Rt d1, Rt d2 and Rt d3 are weights (g) of total root at depth 1, 2 and 3. Rc d1, Rc d2 and Rc d3 are weights (g) of coarse root at depth 1, 2 and 3 while Rf d1, Rf d2 and Rf d3 are those of fine root at the three soil depths. SMC d1, SMC d2 and SMC d3 are soil moisture contents the three soil depths.
*Denotes P value for ANOVA of log-transformed data.

Family group mean DW at destructive harvest (Table 6.4) was strongly correlated (R = 0.847; P = 0.035) with mean DW of clippings from mildly stressed plants (Table 6.3). The 276 correlation coefficients from pair-wise comparison of the 24 traits reported in Table 6.4 were all calculated and 34 were found to be statistically significant (P < 0.05), which greatly exceeds the number of "false significance effects" expected if the significant correlations arose from "Type I" statistical error (see further discussion below), so some biological relationship can be assumed. In some cases this could be attributed to the measurements essentially capturing the same information (e.g. R = 0.998, P < 0.001) for correlation of R d2 and Rc d2) but in other cases correlations involving DW, Index DR, SMC d2, RWC, and proline appeared to have plant functional significance (Table 6.5).

Table 6.5: Selected statistically significant correlations among the 6 family group means for measurements performed on severely stressed plants. The threshold to attain P < 0.05 is R > 0.810.

Trait	Correlated with (R)
DW	RWC (-0.850); IndexDR (R = 0.729, P = 0.10)
IndexDR	SMC d1 (-0.981); SMC d2 (-0.939); RWC (-0.863)
SMC d2	Rt d2 (-0.991); Rt d3 (-0.994)
RWC	SMC d1 (0.807); SMC d2 (0.819); R d2 (-0.829); R d3 (-0.820)
Proline	R:S (-0.819); SMC d3 (-0.973)

Notably: high IndexDR was associated with larger plant size, reduced SMC for d1 and d2, and reduced plant RWC; higher SMC for d2 at destructive harvest was associated with lower root mass (R d2, R d3); higher plant RWC was associated with higher SMC for d1 and d2; and elevated leaf proline levels were associated with a low R:S and with greater moisture extraction at d3.

To assess the salient characteristics of family groups that might be retained if further breeding work aimed at cultivar development were carried out, for those traits where statistically significant differences were detected either in the mild stress or the severe stress phase of the experiment, the population mean was subtracted from the family group mean and the deviation divided by the standard error of the mean

(Table 6.6). It is seen that family group 2 has small plant size and family group 4 was notable for seed-head production. The notable family group from a plant breeding perspective was family group 6, which had above average plant DW in mild stress conditions and the highest mean DW of any family group in severe stress conditions with several drought resistance traits including deep-rootedness, strong soil moisture extraction in d2, and elevated proline (Table 6.6).

Table 6.6: Standard deviations of family group means from the population means for traits where statistically significant differences were detected. Values of less than 1 standard deviation are suppressed.

	Family group					
Trait	1	2	3	4	5	6
Mild stress phase						
DW (g)	1.45	-4.44		3.39	-1.67	1.05
TN		-2.46	-1.22	4.31	-1.22	
LER (mm tiller^{-1} d^{-1})		1.72		-1.05		
HN	3.72	11.39	-10.11	17.39	-10.94	-11.44
Severe stress phase						
DW		-3.55		1.20	-1.42	3.07
LER (mm tiller^{-1} d^{-1})	1.10			1.47	-2.64	
Index DR	-1.54	-1.29	1.46		-1.04	1.71
Rt d2 (g)	-1.12	-2.16	2.29			1.69
Rt d3 (g)		-2.22	1.11			1.89
SMC d2 (%)	1.31	1.99	-1.92			-1.56
Proline (mg g^{-1} DW)	-1.80				-1.30	1.93

6.3.2 Analysis of trait expression at the genotype level

To screen the population of plants from Experiment 4 for variation in drought resistance strategy at the genotype level, a principal component analysis (PCA) was performed to examine the pattern of association between some selected drought resistance traits representative of the four plant functional domains. The selected variables were mostly from the severe water stress phase of the experiment but TN from the mild stress phase was included as TN was not recorded in the concluding destructive harvest. Rf was entered as the sum for soil depths d2 and d3 to capture information from those measurements while minimising the number of traits

included in the PCA. The first five PCs explaining 69.6% of the variation in data are presented in Table 6.7. PC1 explained 31.7% of the variation in the data, and (based on the size and sign of coefficients) differentiated between plants primarily on plant size, with larger plants also having increased soil moisture extraction. PC1 coefficients for DW, Rc d2, Rc d3, and SMC d2 were, respectively, 0.359, 0.279, 0.319, and -0.378 (Table 6.7). Proline accumulation was not a trait of plants with high scores for PC1 (coefficient -0.081); nor did other leaf water relations traits (LWP, PP, and RWC) contribute strongly to scores of PC1 (Table 6.7).

PC2 and PC3 (respectively, 12.5 and 10.7 of data variation explained) both establish a link between plant water relations at the time of the destructive harvest and LER, but not DW. PC2 indicates a category of plant where high LWP and low OP is associated with high LER (or the reverse), while coefficients for PC3 indicate a picture of a plant in water deficit exhibiting low LER associated with elevated proline, more negative LWP and OP and warmer leaves (or the reverse; coefficients: LER 0.391; Proline 0.459; LWP 0.311; OP 0.462; Tc-Ta 0.371). PC4 links higher LWP, OP and RWC with reduced DR:S under stress. None of these 3 PCs exhibited family group differences in PC score, but PC 2 and PC4 did show statistically significant effects for plant genotype.

PC5 (6.5% of data variation explained, with highly significant family group and plant genotype effects) identified a plant type with high TN in mild stress but elevated proline and low Rc d1 and Rc d2 and RWC at destructive harvest (or the reverse; coefficients: TN 0.354; proline 0.404; Rc d1 -0.404; Rc d2 -0.377; RWC -0.541; Table 6.7).

Table 6.7: Coefficients indicating trait contributions to principle coefficient (PC) scores from Principal Coefficient Analysis (PCA) of seventeen selected traits across the four plant functional domains. Abbreviations are as defined in Tables 6.3 and 6.4. Statistical significance of family group and plant genotype differences are also indicated as well as the % data variation explained by each PC. Coefficients with an absolute value greater than 0.2 which have more influence on scores are in bold type.

	PC1	PC2	PC3	PC4	PC5
% variation explained	31.7	12.5	10.7	8.2	6.5
Cumulative % variation		44.2	54.9	63.1	69.6
Trait					
LWP (MPa)	-0.001	**0.466**	**-0.311**	**0.365**	0.024
RWC (%)	-0.142	0.017	0.172	**0.398**	**-0.541**
OP (MPa)	0.091	**-0.424**	**-0.462**	**0.253**	0.09
PP (MPa)	-0.08	**0.625**	**0.229**	-0.018	-0.065
Tc-Ta (°C)	-0.129	**-0.347**	**0.371**	0.115	-0.043
SMC d1 (%)	**-0.284**	0.048	-0.04	-0.105	-0.108
SMC d2 (%)	**-0.378**	0.047	-0.14	-0.09	0.071
SMC d3 (%)	**-0.32**	-0.026	**-0.222**	0.032	-0.06
Rc d1 (g)	**0.301**	0.073	0.092	0.002	**-0.404**
Rc d2 (g)	**0.279**	-0.105	0.046	-0.13	**-0.377**
Rc d3 (g)	**0.319**	0.01	-0.08	**-0.296**	-0.123
Rf (d2+d3) (g)	0.028	-0.003	-0.089	**-0.631**	-0.101
Proline (mg g^{-1} DW)	-0.081	0.042	**0.459**	-0.105	**0.404**
LER (mm $tiller^{-1}$ d^{-1})	0.084	**0.241**	**-0.391**	-0.167	0.061
DWhs g	**0.353**	0.056	0.035	**0.212**	**0.222**
DWtotal g	**0.359**	0.081	0.043	0.1	-0.011
TN	**0.291**	0.027	0.089	0.123	**0.354**
P family group	0.01	ns	ns	ns	0.005
P genotype within f. group	0.02	0.005	ns	0.07	0.029

6.3.3 Data from unwatered plants

After 90 days without water application, both replicates of some plant genotypes remained visually green and leafy while both replicates of other genotypes were dead (Fig. 6.3). One replicate of plant genotype 8-5 had died early and the replicates of plant genotype 8-1 responded inconsistently to water deficit. So data for these plant genotypes are not presented. However, for the other three plant genotypes there were

statistically highly significant differences in green and dead herbage DW and in soil moisture extraction (Table 6.8). SMC in the pot where the plant died early was 14.6, 15.6, and 14.3 for d1, d2, and d3, respectively. It is interesting to note that in this series of three plants, the visual progression from green to dead (Fig. 6.2) is associated with reduced extraction of soil moisture (Table 6.8).

Figure 6.2: Condition of plants from 3 plant genotypes of family groups 7 and 8 in late March 2010 after remaining unwatered for over 90 days. The two replicates of each genotype are included in the photograph. Shoot dry weight (DW; g) and soil moisture content (SMC; %) data for these plants are presented in Table 6.8. Plant genotypes from left to right are (Family group-plant) 7-5, 8-1, 7-4, 8-4 and 8-5.

Table 6.8: Comparison of three unwatered genotypes of Experiment 4 for shoot dry weight (DW; g), green and dead (g plant^{-1}), and SMC, soil moisture contents (%) at soil depths 1, 2 and 3. The plants were harvested in June 2010 after more than 90 days of being unwatered.

	Plant genotype				
	7-5	7-4	8-4	SEM	P
Green herbage DW (g plant^{-1})	2.85	1.60	0.18	0.258	0.012
Dead herbage DW (g plant^{-1})	0.45	1.48	0.84	0.212	0.090
SMC d1 (%)	4.6	8.1	9.7	0.53	0.014
SMC d2 (%)	5.0	10.4	11.3	0.16	<0.001
SMC d3 (%)	9.1	12.2	13.4	1.68	ns

6.4 Discussion

6.4.1 Findings about proline concentrations and its relationship to OA and plant yield

Comparing traits of plant water status between mild and severe water stress phases, it emerges that as expected with lowering LWP under severe water stress OP becomes more negative (Tables 6.3 and 6.4). Numerically the shift in average OP values across the six family groups between the mild and severely stressed phases in the experiment was 0.61 MPa, and this shift in OP can be termed OA (refer to Section 2.4.2.1). This increase in OP was associated with an approximate doubling of proline concentration between mild and severe water stress phases (Tables 6.3 and 6.4). However, this increase in proline concentration is small compared to other studies like Volaire et al. (1998b) (a 12-fold increase) and Volaire et al. (1998a) who reported a 6 – 9 fold increase in proline concentration between control and stressed plants. From these observations several questions emerge:

(i) Is this shift in proline concentration and in OP values (0.61 MPa) typical of other studies in perennial ryegrass?

(ii) Is proline one of the major osmolytes contributing to OA?

(iii) Does OA (whichever osmolyte is involved) really help in increasing in plant yield?

In answer to the first question above, Thomas and James (1993) in their study on perennial ryegrass genotypes subject to drought found similar values i.e., a 1.1 MPa increase in OP associated with a 2.9 times increase in proline concentration while Thomas (1990) found an increase in 0.59 MPa in OP associated with a 6.35 times increase in proline concentration. So, the present results are in normal range for perennial ryegrass when compared to other literature. Proline concentration in plants varies with plant age, developmental stage of leaves (Claussen, 2005) and leaf position (Morgan, 1984). So no such generalisation for comparison between different studies can be made with respect to increase in proline concentration for drought stressed plants.

In answer to the second question, Thomas (1991) reported that Ca^{+2} and Mg^{+2} ions were the major contributors to OA while Barker et al. (1993) reported that even 20

times increase in proline concentration was insufficient to affect OP in some C3 and C4 forage grasses. Our results support those of Barker et al. (1993) since our calculation (Appendix 6.1) of the contribution of proline concentration to OP in this experiment indicates that proline is a minor osmolyte contributing to OA. This conclusion agrees with that of Jiang and Huang (2001) in a study of Kentucky blue grass. However, in some cases proline does make a major contribution.

In answer to the third question, there is controversy in the literature on the impact of OA on plant yield (Serraj and Sinclair, 2002). Even in one species (for example, wheat) there was difference of opinion among scientists for the relationship between OA and plant yield (Serraj and Sinclair, 2002). Though, Munns (1988) argued that OA occurs at the expense of accumulation of solutes from other plant processes like protein synthesis and thus negatively affects plant yield, Thomas and Evans (1990) reported a positive correlation between shoot DW and OA at $P<0.05$. These divergent observations indicate that OA has both a cost and a benefit to plants. So, our results of positive (though indirect) correlation between proline and DW (Table 6.5) are in agreement with those of Thomas and Evans (1990) and indicate that in the conditions of this experiment positive effects of OA outweighed the negative.

6.4.2 Trait combinations in F_2

One of the objectives of evaluating a number of family groups in a plant breeding programme could be to search for families possessing desirable trait combinations for use in further breeding work. Our results show that Family Group 6 had the greatest number of desirable traits combined together (Table 6.6), namely shoot DW, Index DR, Rt d2 and Rt d3 and increased proline without the undesirable traits of Medea like prolific heading. This indicates both that it is possible in a breeding programme to produce plants that express the desirable traits of both parents, and that plants from that family group might be used for future breeding programmes. Equally, it would be possible to implement a larger breeding programme with more family groups with the aim of finding a combination superior to that in Family Group 6.

6.4.3 PCA highlights

PCA was performed on selected traits from severe water stress across the four plant domains to screen individual genotypes (Table 6.7). PC1 identifies plants of larger size with an associated increase in soil moisture depletion. However, it is expected that larger plants would use more moisture. The near-zero coefficients for DW in PCs 2 and 3 that describe associations between traits of plant water relations and stomatal and cellular control (Table 6.7) suggests that selection for traits of plant water relations and cellular control alone might not result in hybrids with higher shoot DW. Moreover, the negative association between proline contents and SMC d3 (as shown in PC3) could be interpreted as indicating that proline is a response to developing water deficit rather than acting to protect plants from dehydration by facilitating soil moisture extraction. Waldren and Teare (1974) propose that proline accumulation occurs only after a threshold of soil moisture depletion is reached which in our case was indicated by excessive soil moisture depletion from soil depth 3. This model would explain why there was not a large increase in proline levels under severe water stress compared to mild stress (Tables 6.3 and 6.4) but is difficult to reconcile with studies that reported as much as a 12 fold rise, for example Volaire et al. (1998b).

PC4 detects genotypes that though apparently shallow rooted (as indicated by coefficients of -0.296 and -0.631 for Rc d3 and Rf (d2 + d3), respectively) are none the less better hydrated as indicated by higher coefficients for LWP, RWC and OP and also tend to have a higher shoot DW during the water stressed phase. This could indicate a commercially useful drought resistance trait that would be worth further investigation and study.

PC5 is of high interest because of its strong statistical difference between family groups and because genotypes identified in PC5 are drier (negative coefficient of RWC) and have smaller root mass at d1 and d2, yet have ability to maintain higher shoot DW and TN. The trait combination of plants identified by this PC also includes high leaf proline. In colloquial language PC5 describes a plant able to maintain growth as it dries out. Such a strategy might lead to plant death in severe drought but would be commercially valuable where a breeder sought to produce a cultivar with improved capacity to maintain growth in drought, particularly if the trait described in

PC5 could be combined with deep-rootedness from Medea and/or reduced soil moisture depletion as seen in Tolosa (Section 4.3.1.3). It is interesting to note that plants of Family Group 6 seem to possess such a trait association because they have a high score for PC5 (Fig. 6.2), as well as a high value for Index DR (Table 6.6), compared to other family groups.

6.4.4 Findings from unwatered plants

The unwatered plants that died failed to extract available water from their pots (Table 6.8) and this indicates variability between genotypes within a population that may also be a factor in loss of ryegrass plants from new sowings on farms in New Zealand. Further research aimed at producing populations with fewer individuals of this type could be useful. For those genotypes that survived withholding of watering for more than 90 days it seems intuitively likely that increased soil moisture extraction alone would be insufficient to produce this response, so the question arises what other mechanisms could be involved. One possibility is that soil moisture was partially recharged on a daily basis from morning dew as reported by Kosmas et al. (1998) in the semi-arid Mediterranean climate of Greece. If so further questions arise as to whether this type of soil moisture recharge is plant mediated and whether plants need specific adaptations for this soil moisture recharge to occur. It is possible that the high stomatal conductance seen in this study in Medea and also in tall fescue of Mediterranean origin is related to moisture capture from morning dew. Further research into these observations might be worthwhile

6.5 Conclusions

- The F_2 family groups compared demonstrated recombination of traits from Medea and Grasslands Samson and that it is possible to obtain summer active plants with desirable Medea traits like deep rootedness, but without prolific heading.

- PC 4 and PC 5 possibly indicate the existence of novel drought resistance traits that with further investigation might be commercially useful.

- Family Group 6 stood out as combining desired traits from the two parents, Medea and Grasslands Samson. Family Group 6 also had a high score for PC

5. So plants of this family group can readily be utilised in future breeding programmes.

- Some family groups that do not possess desired traits across the four plant domains still have useful traits from one or more domains and therefore might be considered for use in further breeding work. For example, Family Group 4 showed higher shoot DW while Family Group 3 showed higher root weights at depth and IndexDR.

- In this study proline concentration was strongly linked to other measured traits, yet made a small contribution to the measured OA. Other osmolytes such as water-soluble carbohydrates might be principally responsible for OA and the exact roles of proline and other osmolytes needs further research.

7

Inheritance of drought resistance traits from parents to F_1 and F_2 progeny

7.1 Introduction and aims

The previous chapters have reported differences between Grasslands Samson and Medea in key traits related to drought resistance (Chapter 4), inheritance of drought resistance traits from those parents to the F_1 generation (Chapter 5) and an evaluation of differences between F_2 family groups in the expression of particular traits (Chapters 6). While these studies used a limited number of F_1 and F_2 genotypes, some clear findings emerged. The next logical step therefore was to test the parent, F_1 and F_2 generations under the same growth environment. The present experiment was thus planned to test inheritance of root and shoot traits in particular, but also those of plant water status and stomatal and cellular control to the extent that time allowed, across three generations from parent to F_2.

7.2 Materials and Methods

7.2.1 Location and experimental set up

This glasshouse experiment was also conducted at the Plant Growth Unit, Massey University, Palmerston North. Experiment 5 (Chapter 4) ran concurrently in the same glasshouse. On 22 September 2010, a total of 36 genotypes (10 genotypes of Grasslands Samson, 6 of Medea, 8 of F_1 progeny, and 12 genotypes of the F_2 generation) were selected from a nursery stock of plants held at AgResearch Grasslands, Palmerston North for transplanting to pots. Each of the 36 plants was divided into four ramets to obtain 144 plants so that each of the 36 genotypes could be subjected to two water regimes (wet and dry) with two replications. A randomised complete block design was used.

Plants were transplanted on 24 September into PVC pots of 10 cm diameter and 100 cm height filled with a soil mix as detailed in section 5.2.1 and placed in sixteen 20 litre drums (eight drums in each replication). Each of the drums contained 9 plants

(normally two plants each of Grasslands Samson, Medea, and the F_1 progeny, and three plants of the F_2 generation). After first top-watering the pot, water was supplied to plants by filling drums to 40 cm below the soil surface. For a few plants, roots did not reach the artificial water table at 40 cm before the surface soil dried, and so the plants died. Dead plants were replaced by taking a small ramet from the live plants of another replicate. Drums were emptied of water every 7-10 days for root aeration and also rotated within the glasshouse by moving the last two drums of each replicate to the first positions, and moving the remaining drums down the row. During a period of hot weather conditions in December 2010 the plants showed signs of wilting, so the water level in the drums was temporarily raised to 30 cm from the soil surface of the pots. In late December, symptoms of aphid attack appeared on plants and they were sprayed with the insecticide Nuprid on 29 December and with neem oil on 5 January 2011.

7.2.2 Treatment application

The introduction of water deficit for the designated "dry" plants was commenced on 16 January 2011, when plants were 114 days old. In four of the eight drums in each replication, the water level was dropped from 30 cm to 80 cm below the soil surface in the pots. However, on 18 January, the plants were given additional water by raising the water in "dry" drums to 60 cm from the soil surface for approximately 8 hours, then returning to 80 cm, while keeping the water level of "well watered" drums at 30 cm from the soil surface. This was done because of high glasshouse temperature.

7.2.3 Measurements

Temperature in the glasshouse was monitored with a datalogger and thermocouple probes (Skye Instruments, Llandrindod Wells, Wales; for details see Section 3.2.3).

Plants started flowering by the end of October. Seed-heads were removed every 10-15 days and data on number and dry weights of seed-heads were recorded from that time, until the end of the experiment.

Measurements to determine plant response to water deficit commenced on 25 January 2011. Measurements of plant water status included LWP, OP and RWC while those on stomatal and cellular control were Tc-Ta and Lrs, Lws, and Lcs were visually assessed. Additionally, an estimate of leaf senescence was taken by visually ranking the foliage for dead leaf score (Lds). Pre-dawn LWP was recorded using a Scholander pressure chamber commencing on 25 January. Work was carried out between 4.30 a.m. and 7.30 a.m. daily with half of the plants of each replicate measured each day and plants from the same replicate measured on consecutive days. Measurement of RWC and sampling and storage of leaves for measurement of OP was co-ordinated with measurement of LWP, with the harvesting of leaf tissue for those measurements occurring just after the measurement of LWP, following the methods given in section 4.2.2.2.3. Lrs, Lws, and Lcs were scored on a scale from 1 to 3 according to the criteria set in Table 4.1. Lds was recorded on a 1 – 6 scale as set out in Table 7.1.

Table 7.1: Criteria for visually scoring foliage dead leaf score (Lds).

% dead leaves	Score
0%	1
Up to 20%	2
20-40%	3
40-60%	4
60-80%	5
80-100%	6

On 7 February 2011 all plants were brought to the field sample laboratory at Massey University for destructive harvesting to determine shoot and root dry weight. Plants were stored in a refrigerator at 4°C. Following defoliation at the soil surface, the whole soil column enclosed in the plastic sleeve was pulled out of the pot and was sliced transversely at 40 and 70 cm from the soil surface, thus giving the three soil depths. Coarse and fine roots were extracted from each of the three soil depths following the procedure detailed in section 4.2.1.2.2. A 5 cm section of soil from each of the soil depths was conserved (after first picking out coarse roots and adding those to the relevant sample) for subsequent determination of gravimetric soil moisture contents. Dry weights of root (both coarse and fine from all three depths)

and shoot were taken after drying the samples at 80°C for 48 hours. By summing these fractions of root weights a value for total root weight at each soil depth (Rt d1, Rt d2 or Rt d3) was obtained. Besides root shoot ratio (R:S), deep root to shoot ratio (DR:S) and index of deep rooting (Index DR) calculated as in previous Chapters a ratio introduced in this experiment was Index of water use (Index WU) which was calculated as follows:

Index WU= shoot DW/(0.2 - SMC d2)

where the constant 0.2 was chosen to just exceed the greatest value of SMC d3 data (the average of SMC d3 was 0.16).

7.2.4 Data analysis

Tables of data means were compiled and the effect of parental cultivar and generation effects (Grasslands Samson, Medea, F_1 and F_2), the effect of water regime, and the generation × water regime interaction tested for statistical significance, using Proc GLM of SAS 9.2 (SAS Institute Inc., NC, USA). Trait associations in Experiment 6 were assessed through a multivariate analysis of variance using the MANOVA subcommand in Proc GLM of SAS 9.2 for a selected subset of data, rather than by PCA as in previous experiments.

7.3. Results

7.3.1 Glasshouse temperature

Daily glasshouse temperature is presented in Section 4.3.2.1 (Fig. 4.6). The daily minimum temperature inside the glasshouse for Experiment 6 generally ranged between 12°C and 22°C, and daily maximum temperature between 18°C and 47°C, though typically between 25 and 30°C for the last month of the experiment. The mean temperature over the entire period was 21.8°C.

7.3.2 Trait characteristics of parents and F_1 and F_2 generations

In the warm conditions of this experiment a DW and TN reduction in Medea compared to Grasslands Samson was particularly pronounced (83% and 87% reduction for TN and DW, respectively). However, R:S, DR:S, and Index DR of Medea were higher (0.54, 0.16 and 0.29, respectively) than Grasslands Samson

(0.16, 0.02 and 0.13, respectively). Medea also had more SMC at all soil depths than any other generation. Meanwhile, Index WU for Medea (38.55) was almost 6 times less than that of Grasslands Samson (247.37).

For most of the measured traits, values for the F_1 and F_2, generations were intermediate between the two parents. However, for some root traits, the F_1 generation surpassed both parents. For example, Rt of the F_1 plants (3.29 ± 0.353 g) was similar to the Grasslands Samson plants (2.92 g) and almost 3 times higher than the Medea plants (0.83 g). For traits DR:S and Index DR, the F_1 generation had values 19% and 62% higher than those of Medea (the parent with a tendency to express this trait) but values for the F_2 generation were similar to those of Grasslands Samson (Table 7.2).

As expected, the water deficit treatment decreased SMC of all three soil depths (Table 7.2). These decreases were in the order of 40%, 30% and 12% for d1, d2 and d3, respectively. Decrease in Index WU values for stressed as compared to control plants was 52%.

As regards the traits of plant water status and stomatal and cellular control, Medea exhibited more negative OP than Grasslands Samson and the F_1 and F_2 generations with this difference also reflected in PP (Table 7.3). Most of the measured traits showed statistically significant changes in the water deficit treatment, including a more negative LWP from (-1.36 cf. -1.00 MPa) and increased Lrs, Lws, Lcs and Lds (Table 7.3).

All four generations dropped their SMC d2 and d3 when subjected to drought (data not shown). This reduction was least in case of Medea. Numerically drop in SMC d2 for Grasslands Samson, Medea, F_1 and F_2 was 40%, 17%, 29% and 34% while that for SMC d3 was 18%, 0%, 10% and 12%.

Table 7.2: P and f values (in parenthesis) for the main effects i.e., generations (gener.) and water regime (Wreg) and their interaction (gener. × Wreg) and means values of the four plant populations (G. Samson, Medea, F_1 and F_2) and water regime (control and stressed) for TN, tiller number, DW, shoot dry weight (DW), Rt, total root mass (g), Rt d1, total root mass (g) at depth 1, Rt d2, total root mass(g) at depth 2 (g), Rt d3, total root mass (g) at depth 3, Rc d1, weight of coarse roots(g) at depth 1, Rc d2, weights of coarse root (g) at depth 2, and Rc d3, weight of coarse root mass (g) at depth 3, Rf d1, fine root mass(g) at depth 1, Rf d2 fine root mass (g) at depth 2, Rf d3, fine root mass (g) at depth 3, R:S, root to shoot ratio, DR:S, deep root (soil depths 2 and 3) to shoot ratio, Index DR, index of deep rooting, SMC d1, soil moisture contents (%) at depth 1, SMC d2, soil moisture contents (%) at depth 2 and SMC d3, soil moisture contents (%) at depth 3 and Index WU, index of water use.

	P and F values for the main and interaction effects			Mean values for the four generations				Mean values for water regime			
Variable	Gener	Wreg	Gener × Wreg	G. Samson	Medea	F_1	F_2	SEM	C.W.	Str	SEM
TN	**<0.001 (22.56)**	**0.002 (10.17)**	ns (0.8)	**193.0**	**37.26**	**144.58**	**140.44**	11.301[b]	**148.81**	**108.83**	8.83[d]
DW	**<0.001 (21.67)**	ns (0.1)	ns (0.78)	**18.04**	**2.27**	**9.57**	**10.32**	1.204[a]	10.26	9.84	0.9514[c]
Rt	**<0.001 (6.27)**	ns (0.02)	ns (0.93)	**2.92**	**0.83**	**3.29**	**2.93**	0.353[a]	2.51	2.48	0.2793[c]
Rt d1	**<0.001 (7.69)**	ns (0.19)	ns (0.97)	**2.57**	**0.65**	**1.76**	**2.57**	0.277[a]	1.82	1.95	0.2185[c]
Rt d2	**<0.001 (19.54)**	ns (1.6)	ns (0.52)	**0.29**	**0.10**	**1.44**	**0.26**	0.125[a]	0.61	0.43	0.0993[c]
Rt d3	ns (1.25)	ns (0.29)	ns (0.63)	0.06	0.07	0.10	0.10	0.0161[b]	0.08	0.09	0.012[d]
Rc d1	**0.002 (5.31)**	ns (0.34)	ns (1.08)	2.05	0.53	1.34	2.04	0.263[b]	1.40	1.57	0.21[d]
Rc d2	**<0.001 (20.43)**	ns (1.44)	ns (0.53)	**0.19**	**0.02**	**1.34**	**0.17**	0.121[a]	0.51	0.35	0.0962[c]
Rc d3	ns (0.92)	ns (0.00)	ns (0.96)	0.004	0.005	0.01	0.005	0.003[b]	0.007	0.006	0.002[d]
Rf d1	**<0.001 (7.18)**	ns (0.47)	ns (1.76)	**0.56**	**0.15**	**0.42**	**0.53**	0.057[a]	0.44	0.39	0.0458[c]

Table 7.2 Continued

Rf d2	ns (0.26)	ns (0.83)	ns (0.17)	0.09	0.08	0.1	0.09	0.095[a]	0.1	0.08	0.0143[c]
Rf d3	ns (0.96)	ns (0.23)	ns (0.47)	0.05	0.07	0.08	0.09	0.015[b]	0.07	0.08	0.044[d]
R:S	**<0.001 (19.85)**	ns (0.89)	ns (0.25)	**0.16**	**0.54**	**0.39**	**0.29**	0.128[a]	0.33	0.36	0.0241[c]
DR:S	**<0.001 (38.13)**	ns (0.69)	ns (0.22)	**0.02**	**0.16**	**0.19**	**0.03**	0.013[a]	0.086	0.098	0.01021[c]
Index DR	**<0.001 (62.19)**	ns (0.00)	ns (0.49)	**0.13**	**0.29**	**0.47**	**0.13**	0.02[a]	0.254	0.255	0.01571[c]
Index WU	**<0.001 (20.49)**	**<0.001 (34.07)**	ns (2.06)	**247.37**	**38.55**	**150.88**	**155.19**	16.11[a]	**199.69**	**96.3**	12.72[c]
SMC d1	**<0.001 (7.44)**	**<0.001 (81.5)**	0.07 (2.39)	**0.07**	**0.1**	**0.08**	**0.07**	0.004[a]	**0.1**	**0.06**	0.0032[c]
SMC d2	**<0.001 (6.08)**	**<0.001 (204.02)**	**0.005(4.49)**	**0.117**	**0.135**	**0.129**	**0.124**	0.0028[a]	**0.148**	**0.104**	0.0022[c]
SMC d3	**<0.001 (10.12)**	**<0.001 (34.85)**	**0.006(4.39)**	**0.152**	**0.174**	**0.164**	**0.157**	0.0025[a]	**0.17**	**0.15**	0.1536[c]

[a]SEM of G. Samson while that of Medea, and F_1 and F_2 generations can be obtained by multiplying that value by 0.747, 0.906 and 1.468, respectively.

[b]SEM of G. Samson while that of Medea, and F_1 and F_2 generations can be obtained by dividing that SEM value with 1.347, 0.887 and 1.0893, respectively.

[c]SEM of stressed watering while that of controlled watering can be obtained by dividing that SEM with 1.0372.

[d]SEM of stressed watering while that of controlled watering can be obtained by dividing that SEM with 1.15.

Table 7.3: P and F values (in parenthesis) for the main effects i.e., generations (gener.) and water regime (WReg) and their interaction (gener. × WReg) and means values of the four plant populations (G. Samson, Medea, F_1 and F_2) and water regime (control and stressed) for LWP, leaf water potential (MPa), OP, osmotic potential (MPa), PP, pressure potential (MPa), RWC, relative water contents (%),Tc-Ta, canopy and air temperature difference (°C), Lrs, leaf rolling score, Lws, leaf wilting score, Lcs, leaf colour score and Lds, dead leaves score.

	P and F values for the main and interaction effects			Mean values for the four generations					Mean values for water regime		
Variable	Gener	WReg	Gener × WReg	G. Samson	Medea	F_1	F_2	SEM[a]	c.w.	Str	SEM[b]
LWP	ns (1.65)	**< 0.001(34.94)**	ns (0.42)	-1.19	-1.29	-1.08	-1.19	0.046	**-1.00**	**-1.37**	0.047
OP	**0.013(3.75)**	**0.024(5.21)**	ns (1.06)	**-2.45**	**-3.53**	**-2.86**	**-2.64**	0.150	**-2.64**	**-3.10**	0.153
PP	**0.01(4.00)**	ns(0.21)	ns (0.97)	**1.26**	**2.23**	**1.78**	**1.45**	0.144	1.63	1.73	0.144
RWC	ns(0.23)	ns(0.6)	ns (1.56)	80.75	84.46	83.46	81.68	2.694	83.94	81.23	2.572
Tc-Ta	ns(2.11)	**0.004(8.51)**	ns(0.69)	1.05	1.74	-0.09	0.07	0.448	**-0.12**	**1.51**	0.420
Lrs	ns(0.46)	**0.003(9.47)**	ns(0.48)	1.55	1.57	1.45	1.63	0.105	**1.35**	**1.75**	0.094
Lws	ns(1.29)	**0.020(5.54)**	0.002(5.27)	1.83	1.54	1.66	1.58	0.102	**1.50**	**1.81**	0.094
Lcs	ns(0.93)	**<0.001(24.67)**	ns(1.19)	1.93	1.66	1.71	1.79	0.101	**1.45**	**2.10**	0.093
Lds	ns(0.14)	**<0.001(10.75)**	ns(1.23)	2.50	2.56	2.35	2.34	0.242	**1.96**	**2.91**	0.216

[a]SEM of G. Samson while that of Medea, F_1 and F_2 generations can be obtained by dividing that SEM value with 0.56, 0.86 and 1.08, respectively

[b]SEM of stressed watering while that of controlled watering can be obtained by dividing that SEM with 1.12.

7.3.3 Trait association as indicated by MANOVA analysis

For discrimination between Grasslands Samson, Medea and F_1 and F_2 generations, MANOVA identified two statistically significant canonical factors ($P < 0.001$) from the three available when 4 groups are analysed. The first of these captured 72.5% of the data variation and most reflected differences in root properties especially root mass in d2 and deep rootedness. The second canonical factor (21.9 % data variation explained) most reflected differences in DW and Index WU. For multivariate definition of the interaction between water regime and parent cultivars and F_1 and F_2 generations, one of the three available canonical factors was significant ($P = 0.0014$). This factor explained 69.7% of data variation and was most influenced by SMC (Tables 7.4 & 7.5).

Table 7.4: Standardised canonical coefficients for statistically significant canonical factors from MANOVA of traits measuring plant response to water deficit. Coefficients represent the magnitude of contribution of named traits to canonical scores when assessing differences between plants of the two parent cultivars and their F_1 and F_2 hybrids (Generation) and the interaction between Generation and water regime.

	Generation		Generation × water
	Canonical 1	Canonical 2	Canonical 1
TN	-0.39904	**0.862641**	**1.20484**
DW	-0.09493	-0.182027	-1.34755
Rt d1	0.23471	0.122322	-0.36702
Rt d2	0.52450	-0.327501	-0.30423
Rt d3	**0.69967**	-0.919914	-1.27669
Rf d1	0.16093	0.111004	0.05258
Rf d2	-0.72481	-0.310311	-0.40197
Rf d3	-0.66531	**0.688941**	**1.25593**
R:S	0.16951	-0.800134	0.71120
DR:S	-0.13968	**0.730836**	-0.58848
Index DR	**1.65888**	**0.642252**	0.91114
SMC d1	0.20975	0.263975	0.69960
SMC d2	-0.00256	-0.223850	-0.20779
SMC d3	0.26326	-0.145206	0.36309
Index WU	-0.09594	**0.723551**	**1.64809**
Eigenvalue	2.8182	0.8518	0.5628
% var. Expl.	72.5	21.9	69.7
P	<0.001	<0.001	0.0014
r^2	0.859	0.678	0.600

Table 7.5: Standardised canonical structures for statistically significant canonical factors from MANOVA. Coefficients approximately represent the correlation between the score and the raw data of the named variable when assessing differences between plants of the two parent cultivars and their F_1 and F_2 hybrids (Generation) and the interaction between Generation and Water regime.

	Generation		Generation × water	
	Canonical 1	Canonical 2	Canonical 1	
TN	-0.4744	**0.8468**	**0.8485**	
DW	-0.5688	**0.8220**	-0.4775	
Rt d1	-0.6823	0.5421	-0.6350	
Rt d2	**0.7840**	0.5543	0.1610	
Rt d3	0.3618	-0.3721	-0.4499	
Rf d1	-0.5800	**0.7359**	0.4417	
Rf d2	0.1809	**0.9129**	-0.4550	
Rf d3	0.2449	-0.4312	-0.4354	
R:S	**0.8018**	-0.5975	**0.9008**	
DR:S	**0.9864**	-0.1081	0.5487	
Index DR	**0.9847**	0.1718	**0.9271**	
SMC d1	**0.6910**	-0.6198	**0.9663**	
SMC d2	**0.8163**	-0.5766	**0.8805**	
SMC d3	**0.7564**	-0.5793	**0.9722**	
Index WU	-0.4865	**0.8728**	**0.8687**	
Generation mean canonical scores			Wet	Dry
Samson	2.302	1.333	466.0	279.8
Medea	4.827	-1.004	79.8	54.6
F_1	6.353	1.186	342.8	170.9
F_2	2.813	-0.103	303.2	185.2
P[1]	<0.001	<0.001	0.0014	

[1]Statistical significance of Generation and Water regime × Generation effects in MANOVA.

To explore the extent to which deep rootedness (as reflected by Canonical 1 for discriminating between Grasslands Samson, Medea and their F_1 and F_2 generations) and high DW occur together in individual plants, a biplot was constructed (Fig. 7.1). As indicated in Tables 7.3 and 7.5, Grasslands Samson has higher DW and lower scores for Canonical 1 (i.e., deep rooting) than Medea, with the F_1 progeny sometimes having high DW and always segregating transgressively compared to

Medea for deep rootedness, but the F_2 generation are generally intermediate between the two parents for both DW and deeper rooting. However, there is a group of 4 F_2 progeny which to some extent combine the deep rooting of the Medea parent and the DW of the Grasslands Samson parent (Fig. 7.1).

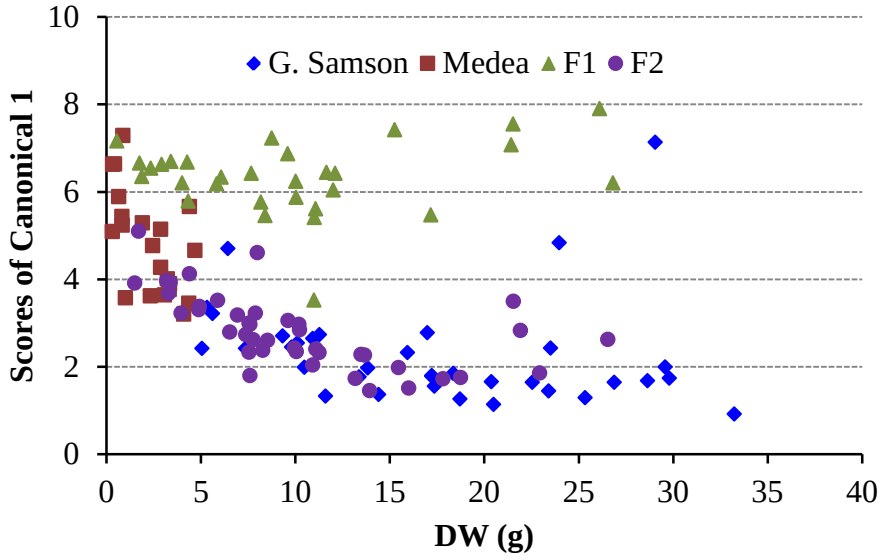

Figure 7.1: A biplot of scores of Generation Canonical 1 with raw data for shoot dry weight (DW; g).

7.3.4 Indices of effectiveness of water use

Since in Section 4.4.5 (Table 4.5) a point of difference between cultivars was the soil moisture remaining, despite similar plant DW, a biplot of SMC d2 with DW (Fig. 7.2) was constructed and showed that Medea sits at one side of the data cloud with average-to-high SMC d2 and plants of low DW while the Grasslands Samson parent showed marked variation between individual plants on both axes, especially among the stressed plants. The F_1 and F_2 generations lie between the two parents. Notably, five plants of Grasslands Samson and one plant of the F_2 generation have the ability when grown under moisture deficit to produce high plant DW with reduced depletion of soil moisture.

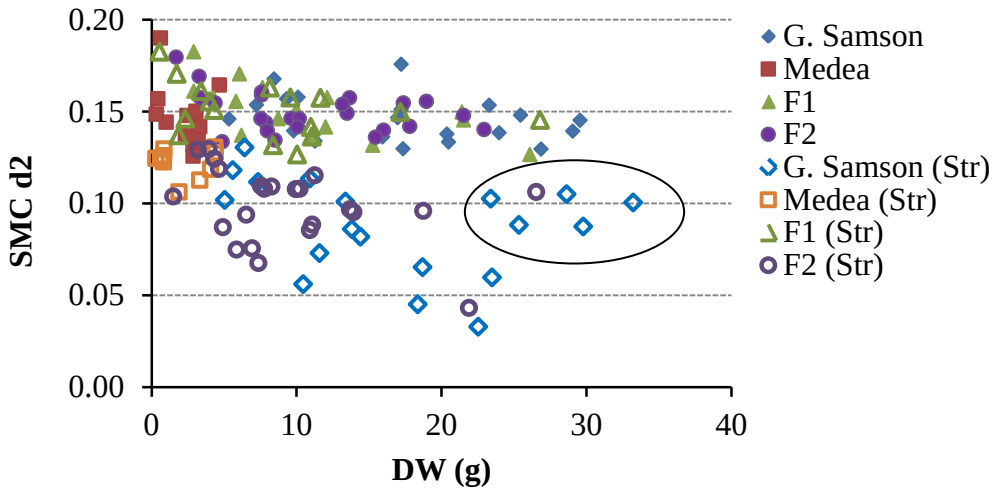

Figure 7.2: Variation between individual plants of Grasslands Samson, Medea and their F_1 and F_2 hybrids in soil moisture depletion and herbage production, as shown by a biplot of soil moisture contents at soil depth 2 (SMC d2; %) and shoot dry weight (DW; g). Control plants are indicated by solid symbols and stressed plants by open symbols. Plants of potential interest to a plant breeder are circled.

7.4 Discussion

7.4.1 Experiment management

In this experiment, plant behaviuor was somewhat different from the other experiments in that the soil profile at d3 was largely unexplored by the plants with comparatively low root mass and minimal soil moisture extraction in d3 (Table 7.2), even though plants were of a similar age at the end of the experiment, to plants in earlier experiments. The reasons for this are unclear, but one possibility is in the watering methodology. Since daily maximum temperatures were frequently above 30°C in the early stages of seedling establishment (Fig. 4.11), and pots were not top-watered, it seems likely in hindsight that seedling development was slowed by the dry soil conditions in d1, although this was less obvious at the time. Despite this limitation it will be shown below that the experiment has produced a number of useful results.

7.4.2 Methodology for results presentation

With respect to choice of MANOVA instead of PCA, both techniques combine information from measurement of multiple (correlated) traits into a smaller number of uncorrelated scores. However, in MANOVA the matrix algebra equations are

written to maximise the separation of groups of scores (treatments) rather than the values of single observations. This makes MANOVA logically relevant to compare parents, F_1 and F_2 generations in this experiment.

"MANOVA tests whether mean differences among group scores on a combination of dependent variables are likely to have occurred by chance. In MANOVA, a new dependent variable that maximises group differences is created from the set of dependent variables. The new dependent variable is a linear combination of measured dependent variables, combined so as to separate the groups as much as possible" (Tabachnick and Fidell, 2007). The number of these "canonical factors" (SAS), also called canonical variables or discriminant functions (Rencher, 1992), is T-1 where T is the number of treatments (Matthew et al., 1994) and in this experiment T = 4: Grasslands Samson, Medea and F_1 and F_2 generations. The MANOVA in this experiment therefore yielded three canonical factors describing differences between Grasslands Samson, Medea and the F_1 and F_2 generations, which is a sufficient number to allow different facets of plant behavior to be mathematically described in different scores. The same number of canonical factors is also available for the Generation × Water regime interaction. By contrast, for the two water regimes, there is just one canonical factor available from MANOVA of the data, meaning that all facets of plant behavior are condensed into a single score, and therefore confounded. For that reason MANOVA of the water regime effect is not presented here. Had PCA been used instead, 15 sets of PC scores would have been available from the analysis of 15 variables used in MANOVA, but further analysis would have been required to determine which if any of those scores separated the plants on the basis of plant generation, water regime or the interaction effects.

A further point is that as MANOVA operates in SAS, if any trait has a missing value, all data for that experimental unit are omitted from the analysis. Here, the inclusion of variables in the MANOVA was largely decided by the number of missing values. Those variables with more than two or three missing values were not included.

7.4.3 Key findings

7.4.3.1 Comparison of Grasslands Samson and Medea

The thinking in calculating Index WU was that at least for the stressed plants where growth was water-limited, this statistic might reveal whether either of the parents or progeny generations exhibited reduced soil moisture depletion for a given dry weight accumulation. Index WU differs from the physiological trait of WUE (Blum, 2009) in that the latter refers to total water use, not calculated in this experiment. However, the growth reduction of Medea in this experiment, presumably reflecting summer dormancy, was so large that this cultivar had a very low Index WU, despite what was effectively a water-saving growth strategy based on dormancy and growth reduction. This point of understanding is relevant to situations where the aim is to optimise herbage production from limited water resources; providing that there is enough water supply to ensure survival of a faster growing plant, that faster growing plant can be expected to produce more herbage per unit of water used. However, a biplot of SMC d2 at destructive harvest and DW (Fig. 7.2) was used instead as another means to identify genotypes with desirable economy of water use and this graphically shows the drought resistance strategy of conserving SMC through smaller plant size in Medea. The contrasting strategies of moisture conservation expressed by Medea and effective use of water in Grasslands Samson (which likely imply a lowering of transpiration loss per unit of DW through faster growth), respectively, have their own agronomic pros and cons. Up to the present time plant breeders and farmers in New Zealand appear to have presumed that the solution to industry problems around summer forage supply is to breed for improved productivity during summer water deficit stress. The alternative drought resistance strategy of Medea raises the question of whether seeking to also develop a cultivar with lower production but enhanced survival might be a useful option. This then raises a follow up question of whether water saving from using a ryegrass with reduced summer growth would facilitate enhanced summer performance of companion species such as white clover, or whether water extraction by companion species sown with a summer dormant ryegrass would result in stress and plant deaths for the summer dormant plant. Shallower rooting species, however, might coexist with a deeper rooted ryegrass like Medea, without seriously affecting the ryegrass survival. Moisture conserved in soil by Medea can be utilised by neighbouring plants

when forage is grown as mixed swards while effective use of water can increase chances of survival under drought of a more productive cultivar like Grasslands Samson.

7.4.3.2 Insights from MANOVA

It is interesting that when the available data are taken together in a MANOVA analysis that the major point of difference between Grasslands Samson, Medea, and F_1 and F_2 generations lies in data related to deeper rooting (Canonical 1; 72.5% data variation explained). Plant size traits such as TN and DW feature as a much smaller influence in discriminating between the generations along with TN and some other variables like Index WU in Canonical 2 (21.9% of data variation explained). The prominence of root-related traits in the MANOVA results would be partly because statistics like % variation explained in MANOVA are influenced by the number of variables in each category and there are several related root measures included in the MANOVA, but just a single measure of DW. Even so the differences in root behavior are clearly biologically important in understanding behavioural differences between the parents and progeny assessed here.

Superficially, there appear to be some conflicts between values of coefficients (Table 7.4 and scores (Table 7.5). For example, DW had a negative coefficient (-0.1820) for Canonical 2 in Table 7.4 but a positive coefficient (0.8220) for the canonical structure of the same canonical variable in Table 7.5. This is reconciled by considering that the coefficient shows how the canonical score is calculated (i.e., DW was allocated a slight negative weighting in derivation of canonical scores) whereas the structure coefficient shows how the set of canonical scores correlates with the raw data for that variable (i.e., DW is expected to be positively correlated with a score that includes TN as a major component of calculation). The combination of a negative DW coefficient and a positive TN coefficient is suggestive of both plant size and tiller size differences being included in the multivariate discrimination between the two parental types and their F_1 and F_2 progeny.

7.4.3.3 Key findings for plant improvement

From the statistical perspective, a result that stands out in this experiment is the transgressive segregation relative to Medea, for deep rootedness in the F_1 generation (Fig. 7.1). While J.R. Crush and co-workers at AgResearch, Ruakura have carried

out a number of insightful investigations of ryegrass root system properties, including one with a mapping population of 200 F_1 progeny of Grasslands Samson and Grasslands Impact parent plants (Crush et al., 2007), none of those presents a comparison of parent and F_1 means similar to Fig. 7.1, although these authors concluded that their data indicate selection for root traits would be worthwhile. With rice, two studies tracking trait means across generations from parents to F_2 progeny are known to the author, (Chang et al., 1982) and (Ekanayake et al., 1985). For most of the data illustrated in these two studies the F_1 and F_2 trait means are intermediate between the two parents but for some root traits reported the F_1 or the F_2 progeny mean does exceed the better parent or fall below the lower scoring parent. (Chang et al., 1982) notes that root traits differ in their inheritance, sometimes involving dominant and sometimes recessive alleles, while (Ekanayake et al., 1985) concluded that the these types of generational shifts in population means as seen in Fig. 7.1 involve both additive and dominance effects and indicate a polygenic basis for those traits. However, it is worth noting that statistical analysis to determine additive and dominance components of trait inheritance from parents to progeny does not precisely define how many genes are operating or the way they interact to determine the phenotypic effect.

In the present data set, Fig. 7.1 shows two higher DW plants of Grasslands Samson with a Canonical 1 score for deep rootedness similar to that of the F_1 plants and two higher DW plants of the F_2 generation intermediate between the F_1 and Grasslands Samson scores, so it is unclear if it would be possible to stabilize this deep rootedness trait from Medea into subsequent generations of a breeding population developed through introgression of Medea with a current New Zealand cultivar such as Grasslands Samson. Selection for F_2 plants exhibiting deep rooting and/or deeper rooting genotypes within a New Zealand-cultivar parent could be a first step to future research on this point.

Also of interest is the possibility of selecting for a plant able to achieve high DW under moisture deficit with comparatively less reduction of SMC d2, and five Grasslands Samson plants and one plant among the F_2 progeny are of interest (Fig. 7.2). Selection for plants with this drought resistance behaviour may be another option for breeders seeking to improve summer performance of existing ryegrass germplasm. An interesting feature of Fig. 7.2 is that even though there was no plant

generation × water deficit interaction for DW (Table 7.2), the SMC d2 / DW biplot shows that stressed plants of the F_1 progeny exhibited mainly size reduction, whereas stressed plants of Grasslands Samson exhibited either size reduction or increased soil moisture depletion, with a few plants as mentioned above maintaining larger plant size without soil moisture depletion. It does appear from the limited set of data for plants unwatered for 90 days (Section 6.3.3) that the first strategy of reducing plant size without accessing soil moisture tends to lead to plant death.

7.5 Conclusions

- The traits providing the greatest statistical discrimination between Grasslands Samson, Medea, and their F_1 and F_2 progeny in this experiment were related to deep rootedness, as reflected by Canonical 1.

- Medea was confirmed to have increased deep rootedness and less soil moisture extraction than Grasslands Samson during summer. These characteristics are likely to provide Medea resilience against drought and might also lead to beneficial interactions with other cultivars or species (especially shallower rooted ones) when Medea is grown in a mixed sward.

- Stressed plants of Grasslands Samson and of the F_2 progeny showed considerable variation in residual soil moisture at a given DW when plants were destructively harvested. Selection within Grasslands Samson or among the F_2 hybrids for reduced soil moisture depletion in conjunction with high DW accumulation could be a beneficial strategy for commercial breeders.

- The F_1 generation segregated transgressively to exhibit greater deep rootedness than Medea, but this more extreme deep rooting behaviour was not exhibited in the F_2 generation, and in general the F_1 plants exhibited reduced size rather than soil moisture depletion when subject to water deficit.

8

Overview and conclusions

8.1 Rationale for the work

One of the major current thrusts in ryegrass breeding in New Zealand is a search for improved tolerance of summer moisture deficit. The prime objective of this PhD study was to ascertain the potential for further improvement of drought resistance of current commercially released New Zealand perennial ryegrass cultivars by introgression with “Medea”, a cultivar developed from germplasm of Mediterranean origin (Silsbury, 1961) and therefore presumed to be winter active and drought resistant.

Germplasm of Mediterranean origin reportedly reduces or ceases its leaf growth and expansion during summer in response to long and dry summers of that climate (Volaire and Norton, 2006). Such quiescence has been classified as summer dormancy by Volaire and Norton (2006) and confers superior drought survival to the quiescent germplasm (Volaire et al., 2009). A number of commercial cultivars of forage grasses (for example cultivar Maris Kasba and Flecha of tall fescue and Kasbah of cocksfoot) have summer dormant Mediterranean germplasm in their ancestry that confers such traits. For example, tall fescue cultivar Maris Kasba (a decaploid cultivar derived from North African germplasm) was found to have a DW reduction of 40 – 60% in two experiments and a higher R:S ratio than an Argentinean cultivar El Palenque, derived from European germplasm (Assuero et al., 2002). Likewise, the summer dormant Grasslands Flecha tall fescue (selected from a French cultivar of Tunisian parentage) was found to have reduced yield and water use compared to a summer active tall fescue cultivar Demeter. The reduced summer yield of Flecha, was followed by a higher autumn yield, however (Norton et al., 2006b).

As mentioned in Section 2.8, the study of Vartha (1975) reported that Medea had slightly higher winter yield and better persistence through summer than Grasslands Ruanui, while Hill (1985) found in Victoria that 15% of Medea plants were still alive

after 2 years, compared to nil survival for plants of the Victorian ryegrass ecotype. However, these studies give no information at the trait level on mechanisms of drought resistance, and subsequent work on summer dormancy by researchers such as F. Volaire and M. Norton [see e.g. Norton et al.(2006a), Norton et al. (2006b), Volaire and Thomas (1995) Volaire and Gandoin (1996)] has largely focused on tall fescue and cocksfoot, with very little research into summer dormant ryegrass cultivars. Hence, the present study was set out to investigate the behaviour of Medea, as an example of a summer dormant ryegrass cultivar.

Grasslands Samson was identified at the outset by the AgResearch co-supervisor as the current New Zealand cultivar on which the introgression work with Medea would be performed. Among criteria considered in selecting Grasslands Samson, two of the more important were (i) significant recent commercial sales in the New Zealand market, and (ii) the prior use of plants of Grasslands Samson as a parent in other cultivar development work at AgResearch, meaning that experimental populations suitable for the present study were already in existence. As noted in Section 2.6 Grasslands Samson combined germplasm of the Mangere ecotype (Grasslands Nui and Ellett) with persistent plants collected from drier eastern regions of New Zealand (Gisborne to North Canterbury) (pers. comm. H.S. Easton; Stewart, 2006), so could be expected to be among the more drought tolerant new Zealand germplasm. The tetraploid form of Grasslands Samson was also chosen to guage the potential for drought resistance of a tetraploid variety compared to a genetically related diploid form. If an apparent increase in drought resistance in the tetraploid had been found, then inducing tetraploidy would be an option in breeding for drought tolerance. However, no such response was noted.

A question which logically follows is the extent to which other current commercially released New Zealand cultivars share common drought resistance traits with Grasslands Samson or have differing drought tolerance strategies. Therefore investigations were extended to a selected set of New Zealand cultivars (Grasslands Samson, Tolosa, Ceres One50 and Matrix) and a breeding line (Samson tetraploid).

8.2 Protocol development

It was also evident from the review in Chapter 2 that few earlier studies have simultaneously collected a set of plant response data across plant functional domains. Hence, a secondary need of the study was to develop a methodology for doing this.

The philosophical approach in evolving the methodology was to generate data that captured the morphology of the plant (shoot DW, TN, root DW (for different soil depths), and then link those morphological measurements with physiological measurements. In view of the focus on drought resistance, leaf water relations data was considered a priority (LWP, OA, RWC), and then other measurements such as proline, gas exchange, and Tc - Ta were added as time allowed.

In Experiment 2, besides the RWC measurements presented (Section 4.3.1.4) LWP and OP measurements were scheduled and attempted but took 9 days to complete, meaning that plant water status might have changed during the measurement period and interpretation of the data would be problematic. This prompted reflection on ways to streamline the measurements and bring them closer together in time. For example, instead of measuring OP on fresh leaves after completing LWP data collection in the unreported data from Experiment 2 (usually one day per replicate for each procedure), it was realised that a piece of the same leaf used for LWP determination could be snap frozen in liquid nitrogen and OA determined at a later date, and RWC samples could be collected on the same day. In this way, measurement of RWC, LWP, and OP were brought close together in time and successively more complex data sets were built up in later experiments.

Another point that emerged in the process of technique evolution was that measurement of gas exchange in Experiment 2 with the CIRAS-2 equipment consumed very large amounts of time yet yielded comparatively little insight into drought tolerance mechanisms, whereas increased stomatal conductance of Medea compared to Grasslands Samson could be inferred just as usefully, more quickly, and with greater statistical significance by comparing Tc-Ta (see e.g. Table 5.6). Similarly some of the more basic measurements such as HN, TN, and SMC were often more informative than those obtained with sophisticated equipment in this

series of experiments where screening for traits conferring drought resistance was the primary aim. Obviously equipment like the CIRAS-2 is still required where more detailed physiological hypotheses are to be tested.

8.3 Review of experiments and results highlights

In total, six glasshouse experiments were conducted during this PhD study. Experiment 1 (April – August, 2008) was a comparison of leaf extension and tillering characteristics of Medea perennial ryegrass and Grasslands Samson during winter. This experiment established that during winter, despite similar LED, cultivar Medea was less than half as productive as Grasslands Samson (Table 3.2), although the Medea plants appeared to be catching up at the end of their winter growth (Table 3.4). The smaller shoot DW of cultivar Medea than that of Grasslands Samson could be attributed to high glasshouse temperature that presumably was high enough to trigger partial onset of summer dormancy in Medea. A second possibility is that breeding of Medea did not capture the potential for rapid winter growth, but this latter scenario can be ruled out since it is contrary to Vartha's (1975) report of winter growth of Medea being equal to or greater than Grasslands Ruanui.

Experiment 2 (September – December 2008) and Experiment 5 (September 2010 – January 2011) were a wider comparison of moisture deficit tolerance strategies in Medea and Grasslands Samson with a tetraploid breeding line derived from Grasslands Samson, and the commercial cultivars Tolosa (Experiment 2) and Ceres One50 and Matrix (Experiment 5). Key traits of drought resistance possessed by Medea were higher R:S, DR:S and Index DR (Tables 4.5, 4.13 and 7.2) and low soil moisture extraction (Tables 4.5, 4.13 and 7.2). Under drought stress Medea had a higher R:S and DR:S (Fig. 4.10). However, it conserved soil moisture at all three soil depths even when fully watered (Fig. 4.9) and had higher proline contents than Ceres One50 and Matrix (Table 4.15). Medea also exhibited prolific flowering (Tables 4.3 and 5.4). However, shortly before completion of this thesis the author received a personal communication from Dr Alan Stewart who has worked in Australia earlier in his career, stating that "true Medea" does not have a prolific flowering habit, and a seed line that exhibits this trait would be contaminated with *Lolium rigidum* hybrid plants. It was not possible to resolve this information in the short time available as

the author is unaware of any publications detailing the heading behaviour of either Medea or *L. rigidum*, against which data from the author's experiments could be compared. If Medea is to be researched further, then possibly with modern DNA technology. a genetic study could be carried out to confirm or rule out the presence of *L. rigidum* contamination in Medea seed lines to be used in those studies.

As a logical link to Vartha's (1975) observations about summer and winter DW productions of Medea we note that during summer Medea did not produce as much as Grasslands Samson across all experiments in which Medea – Grasslands Samson comparison was done. Reason for this is that either those experiments were conducted in summer season or the temperature inside the glasshouse was high enough to cause an artificial onset of summer dormancy. For example, across all experiments in which Medea – Grasslands Samson comparison for shoot DW was done, ratios of Medea – Grasslands Samson shoot DW of 0.47 (Experiment 1; Table 3.2) at an average temperature of 12.1°C, 0.62 (Experiment 2; Table 4.3) at an average temperature of 27.0°C, 0.56 (Experiment 3; Table 5.4), 0.33 (Experiment 5; Table 4.11) at an average temperature of 21.8°C and 0.12 (Experiment 6; Table 7.2) at an average temperature of 21.8°C were obtained. However, for winter part of Experiment 3 this ratio was 0.98 (35.60 g Medea cf 36.42 g Grasslands Samson; Table 5.4).

Tolosa exhibited some indication of higher WUE, indicated through lower soil moisture extraction (Table 4.5) while maintaining approximately equal shoot DW production, compared to Grasslands Samson (Table 4.3). Matrix produced higher shoot DW than Grasslands Samson (Table 4.11) but the mechanism for this response was not revealed in the data collected (Table 4.13).

Experiment 3 (March 2009 – February 2010) evaluated five family groups of the parents (Grasslands Samson and Medea) and 3 F_1 progeny in each of the family groups. One important finding of this experiment was that some traits including proline content, HN and Index DR had a positive MPH (i.e. progeny behaved more like the Medea parent). In considering the implications for introgression of traits from Medea to improve summer performance of New Zealand ryegrass, this information needs to be taken together with data from Experiments 4 and 6, but may

suggest that some Medea loci exert dominance over the corresponding Grasslands Samson loci. A second point of significance was that Grasslands Samson plants at 11 months of age had higher R:S, DR:S and Index DR than those of Medea. This observation implies that expression of root traits in a plant can change with plant age, meaning that in commercial breeding, selection might need to be carried out on mature plants and not recently established seedlings for sound results.

Experiment 4 (December 2009 – June 2010) compared six family groups of F_2 hybrids. The differing traits expressed by these family groups could be regarded as illustrative of possible trait combinations that might by further breeding be "fixed" into a synthetic cultivar. Family Group 6 had a combination of several potentially desirable traits including high shoot DW, Index DR and proline contents. When some plants prepared for this experiment were discarded and left unwatered for 90 days, some survived. One of the plants that died failed to develop a deep root system and extract available soil moisture, while the surviving plants exhibited better soil moisture extraction capability. If poor root development is a factor in determining which plants will die in a pasture establishment situation, this may be a trait that can be manipulated by selection. Another possibility is that survival of some plant genotypes may have been aided by an enhanced ability to effect water uptake from morning dew in the Palmerston North autumn, a factor shown to be important in Mediterranean climatic conditions (Kosmas et al., 1998). These points might be worth further investigation in the search for traits of value in drought resistance.

Experiment 6 (September 2010 – February 2011) was an evaluation of the two parents, and F_1 and F_2 generations in the same growing conditions. Results confirmed superiority of Medea over Grasslands Samson for root traits like R:S, DR:S and Index DR. These root traits also exhibited poor expression in F_2 seen as negative MPH values (-0.17, -0.67 and -0.38 for R:S, DR:S and Index DR, respectively). One key point from this experiment is that the pattern of shift in trait means across generations indicates complex polygenic inheritance the details of which have never been elucidated, although root traits in rice are known to display similar behaviour (Chang et al., 1982; Ekanayake et al., 1985). The other key point is there is a possibility of selection within Grasslands Samson plants for increased DW per unit of water extraction as shown by five Grasslands Samson plants (Fig. 7.2).

8.4 Commercialisation potential from the results

8.4.1. Drought resistance traits observed and their implications for New Zealand farm practice

8.4.1.1 Production versus survival

Farmers would prefer year round vegetative growth of grass to maximise their annual forage yield. A summer dormancy trait in a sown cultivar, while possibly increasing survival, would increase any drought-related feed deficit and increase farm costs. Therefore, a preferred option for a new cultivar would be improvement of summer survival through enhanced water use efficiency , rather than through summer dormancy.. However, it is a matter of choice whether or not farmers are willing to forego yield potential in exchange for better survival of Medea through improved root growth under water stress and reduced water uptake. When attempting to extend the boundary of ryegrass cultivation in low rainfall areas of New Zealand, farmers also have an option to grow the otherwise drought sensitive forage species in these areas by additional irrigation or by choosing endophyte infected cultivars since endophyte infection reportedly improves drought resistance.

8.4.1.2 Prolific flowering

While in this study Medea expressed a prolific flowering habit that would be undesirable in a commercial cultivar, it is unresolved at this time (Section 8.3) whether or not this relates to contamination of the seed lines used with *L. rigidum.* However, even if the flowering behaviour observed does come from *L. rigidum*, it was clear in Experiment 4 (Table 6.6) that some family groups will emerge in the F_2 generation in a structured crossing programme that exhibit some of the desirable attributes of Medea, without the prolific flowering trait. Hence the prolific flowering observed in Medea seed lines need not be a barrier to its use in introgression work.

8.4.1.3 Physiological traits of Medea (proline contents, flaccid leaves, stomatal conductance, canopy temperature)

Where leaf proline content was measured, it was generally found to be higher in Medea than in Grasslands Samson. However, scientists have not reached a consensus on the role of proline in promoting shoot growth, and its contribution to OA. Some

do not even agree on the significance of OA to plant growth. One of the anecdotal observations on Medea plants before commencing this research was flaccid leaves, though that was not consistently observed during these six experiments. Higher stomatal conductance of Medea (Experiment 1) and lower canopy temperature than ambient temperature were observations for Medea consistent with loss of leaf turgor, however. These are some of the Medea traits observed in this research that are points of difference between Medea and other ryegrass cultivars but have no obvious value for improving on-farm performance of a bred cultivar.

8.4.1.4 High production per unit of water

The measurements made on the selected current New Zealand ryegrass cultivars indicated a basis at the trait level for comparatively strong drought resistance in Tolosa and One50, two cultivars bred by introgression with Spanish germplasm: namely reduced soil moisture depletion per unit of DW grown.

An interesting observation noted about Grasslands Samson was that five plants of Grasslands Samson that produced higher shoot DW under drought stress also exhibited lower soil moisture extraction (Fig. 7.2). This suggests that selection within Grasslands Samson for plants with this trait could be profitable.

The series of experiments described identified three plant types in terms of their productive capacity and drought resistance. These are

1. Medea type: summer dormant (Table 4.11) but water saving (Table 4.5) and, prolific flowering (Table 4.3);
2. Tolosa type: equally productive as Grasslands Samson but with evidence for higher water use efficiency (Table 4.5);
3. Grasslands Samson type: gradual accumulation of root mass over the first year of growth to give high R:S, DR:S and Index DR in one year old seedlings (Table 5.5) and an indication of low soil moisture extraction (relative to plant size) at d2 in some plants (Fig. 7.2).

In addition, the basis of the comparatively good performance at the trait level of Matrix in Experiment 5 was not detected from the measurements made.

8.5 Conclusions

- The main points of difference detected between Medea and Grasslands Samson were in pattern of root development with Medea being deeper rooting. Medea also expressed growth reduction at higher temperatures (even when the high temperature was experienced in winter), with the amount of growth reduction apparently proportional to temperature rather than triggered by a threshold, or some seasonal factor like day length. There was some indication of a high stomatal conductance in Medea also known to occur in other germplasm of North African origin.
- On the question of suitability of Medea for introgression to high yielding New Zealand bred ryegrass cultivars, this cultivar is possibly useful as a source of genes for improved root traits, specifically Index DR and R:S; and for OA, but not for an increased shoot DW. However, due to the polygenic nature of the root traits of interest, expression of some of those traits is less pronounced in the F_2 than in the F1 generation, suggesting that gene harvesting from Medea will pose problems for breeders.
- Cultivars Tolosa and Ceres One50 were shown to maintain growth in water deficit with reduced extraction of available soil water compared to Grasslands Samson; while Grasslands Samson showed variation between plants in soil water depletion per g plant DW. Hence selection for ability to maintain growth in moderate water deficit with lower soil moisture extraction per unit of DW grown is an immediate option for plant breeders seeking to improve drought tolerance of ryegrass.
- The basis at the trait level of good performance of Matrix under summer moisture deficit was not detected from the measurements made in Experiment 5. Further investigation of this point could be worthwhile.
- Since our results indicate a different Grasslands Samson/Medea relativity for deep rootedness of older plants compared to newly established plants, future selection for root traits in ryegrass breeding programmes will need to consider that these traits may be age specific in their expression, and confirm that traits selected for are retained as plants age.

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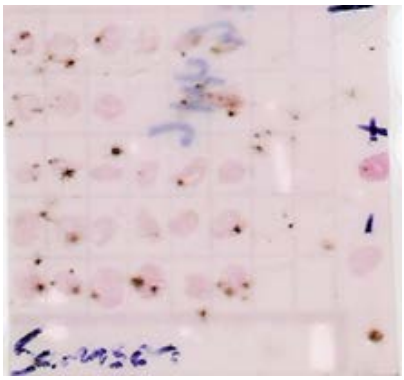
Appendices

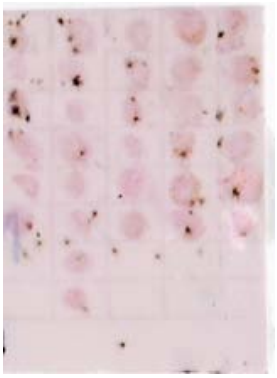
Appendix 3.1: SAS code for analysis of morphogenetic data of Experiment 1

```
proc glm;
class replication cultivar genotype;
model LER1 LER2 LER3 LER LED1 LED2 LED3 LED LL1 LL2 LL3 LL
      LW TN PsL DW TW LN Fs TN1TN2 lnTN2 lnT1 RTAR
= replication cultivar genotype (cultivar) cultivar | genotype (cultivar);
means cultivar | genotype (cultivar)/lsd;
run;
```

Appendix 4.1: ELISA scans for endophyte status of Grasslands Samson, Samson 4n and Medea. All plants were considered by an AgResearch staff member with experience at reading ELISA assays, to be nil-endophyte. A reference result for a tiller confirmed as positive for endophyte is indicated by "+" to the right of the G. Samson card. The positive result shows a darker pink stain.



G. Samson



Samson 4n

Medea

Appendix 5.1: Calculation of "Pot field capacity" and for the amount of water to be topped up

Weight of empty container (A)=79.6 g

Weight of container and air dried soil (B)=180.48 g

Weight of container and oven-dried soil (C)=179.47 g

Soil moisture contents of air dried soil= (B-C)/(C-A)×100=1.01%

Weight of air dried soil=22 kg

Moisture contents of air dried soil=1.01% i.e., 22 kg air dried soil has

22×1.01/100=0.22 kg water

Therefore 22 kg air dried soil contains oven-dried soil=22-0.22=21.78 kg

Field capacity=21.78% w/w

Soil weight at Field Capacity=21.78+(21.78/100×21.78)=Z

Where Z is the final weight of pot after adding required water.

Appendix 5.2: Portioning of two ANOVAs in Experiment 3 (Chapter 5). The trait seed-head count has been presented as an example.

ANOVA 1

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
rep	1	60.5	60.5	60.5	0.21	0.653	
group	4	7683.5	3926.7	981.7	3.34	0.021	
ClassB	2	3238.6	3238.6	1619.3	5.52	0.008	
group×class	4	3752.7	3752.7	938.2	3.20		Imported from ANOVA 2
Interaction Remainder	4	4163.3	4163.3	1040.825	3.55		Obtained by difference from Original interaction of ANOVA 1
Error	34	9982.0	9982.0	293.6			
Total	49	28880.6					
Class versus ClassB	1	2464.2	2464.2	2464.2	8.39	0.0066	
group×ClassB	8	7916.0	7916.0	989.5	3.37	0.006	Original interaction of ANOVA1

ANOVA 2

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
rep	1	60.5	60.5	60.5	0.14	0.708	
group	4	7683.5	6192.4	1548.1	3.64	0.013	
class	1	774.4	774.4	774.4	1.82	0.185	
group×class	4	3752.7	3752.7	938.2	2.20	0.087	Interaction term of interest
Error	39	16609.5	16609.5	425.9			Class described as parent or progeny
Total	49	28880.6					

Appendix 6.1: Calculation of contribution of proline to osmotic potential. Proline concentration averaged across family groups at severe water stress (Table 6.4) was used to calculate proline contribution to OP.

The OP exerted by a solute in solution is approximately given by the formula

$OP\ (Pa) = -i\ C\ R\ T$ Eq. 1

Where i = van't Hoff factor = 1 since proline does not dissociate

C = Molarity = Number of moles of proline/L or Kg of symplastic water

R = gas constant = 8.3145 J K^{-1} mol^{-1}

T = absolute temperature (for 23°C = 23+273 = 296°K)

All factors for Eq. 1 other than C are known. C can be calculated if it is assumed that measured proline resides in the symplastic water. Symplast and apoplast make a ratio of 0.8 and 0.2 in a cell on an average while DM of plants is usually 15% of fresh weight. Weight of symplastic water g^{-1} plant DW (X) is calculated as follows:
X = 1g DM/15%× 85% × 80% = 4.54 g

To obtain C from leaf proline concentration:
Mean leaf proline concentration averaged across family groups (mg g^{-1} DW) from Table 6.4 = (0.37 + 0.84 + 0.75 + 0.96 + 0.48 + 1.19)/6 = 0.765 mg g^{-1} DW
Molecular weight of proline = 115.13 g mol^{-1}
Moles of proline g^{-1} DW = weight in gram/Molecular weight Eq. 2
=0.765 mg g^{-1} DW/115.13 g mol^{-1}

Moles of proline g^{-1} in symplastic water = 6.64×10^{-6} mol proline per 4.54 g symplastic water
= 1.475×10^{-3} mol L^{-1} symplastic water

Putting the values of i, C, R and T in Eq. 1:
$OP = -1 \times 1.475 \times 10^{-3}$ mol $L^{-1} \times 8.3145$ J K^{-1} $mol^{-1} \times 296$ K = -3.63 J L^{-1} = -3.63×10^{-3} MPa compared to an observed OP (average of 6 family groups) of -1.08 MPa.

Appendix 8.1: Published paper Matthew et al. (2012)

Matthew C., Linden A.v.d., Hussain S., Easton H.S., Hatier J.-H.B., Horne D.J. (2012) Which way forward in the quest for drought tolerance in perennial ryegrass?, Proceedings of the New Zealand Grassland Association 74. pp. 195-200.

URL for the paper:
http://www.grassland.org.nz/viewpublication.php?pubID=357**2**