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**Response to Water Deficit Stress
in the Native New Zealand Tussock,
*Festuca novae-zealandiae***

Submitted in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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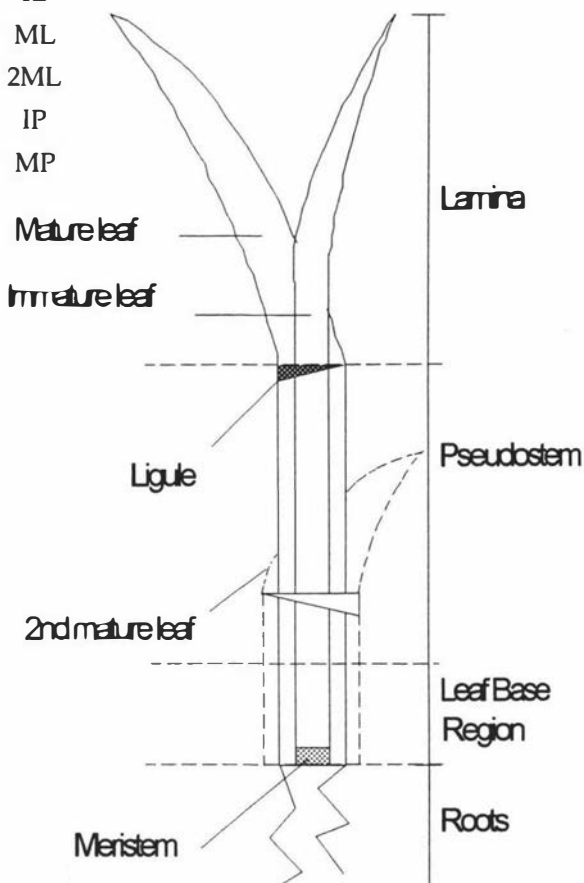
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Quick Reference Card

Tiller region	Abbreviation
Leaf Base Region	LB
Immature Lamina	IL
Mature Lamina	ML
Second Mature Lamina	2ML
Immature Pseudostem	IP
Mature Pseudostem	MP



Common Abbreviations

D_s	Mean degree of leaf tip scorching
H_L	Leaf hydration
LER	Leaf elongation rate
LWP	Leaf xylem water potential
M_T	Mean tiller mass
N_T	Mean number of green leaves per tiller
NSD	Not significantly different
RWC	Relative water content
SWC	Soil water content
T_{Num}	Mean number of green tillers per plant

Dedication

To my parents,
Maxwell and Stephanie Abernethy,
whose encouragement, support and advice
has made this dissertation possible.

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Abstract

Responses to water-deficit stress by a xerophytic tussock native to sub-alpine grasslands of New Zealand have been investigated. *Festuca novae-zealandiae* possesses some structural adaptations such as curled laminae, limited stomatal distribution, and dense trichomes characteristic of dry-land adapted species. These features were compared with those of a mesophyte, *F. arundinacea*. In addition to structural adaptations, evidence that metabolic changes contribute to the drought tolerance of *F. novae-zealandiae* was sought.

Plants of *F. arundinacea* and *F. novae-zealandiae* were grown in glasshouse pots and were subjected to water-deficit. Concentrations of the phytohormone ABA and the osmoprotectants proline and glycine-betaine increased in mature laminae of *F. arundinacea* as SWC declined to 8%. In *F. novae-zealandiae*, ABA concentration increased from 25-50 ng/gDW in two steps. At the first step (at 9-10% SWC), the increase was greatest in leaf bases (to 150 ng/gDW), and the second increase (at 6% SWC) was greatest in laminae (to 320 ng/gDW) and non-existent in leaf bases. In water-sufficient tillers, proline concentration was elevated in leaf bases (5-8 mg/g DW) compared to laminae (2 mg/g DW) and increased to 28 mg/g DW in leaf bases, and to 14 mg/g DW in laminae as SWC declined to 8-9%. Glycine-betaine concentration (40-100 μ mol/gDW) was unchanged by water-deficit stress.

Leaf and tiller counts, and estimates of leaf tip scorching indicated that leaf death progressed with increasing water-deficit. Although leaf elongation declined, it did not cease until the last tillers had died. SDS-PAGE and Western analysis of soluble proteins using antisera to ubiquitin indicated that protein turnover increased in lamina, but not in leaf bases in response to water-deficit. It was concluded that in response to water-deficit stress, leaf bases of *F. novae-zealandiae* retained turgor and remained viable at the expense of existing leaf laminae, which died back.

Southern analysis suggested that the *F. novae-zealandiae* genome contains sequence(s) with homology to group 2 LEA proteins. Western analysis using antisera raised against the dehydrin consensus sequence KIKEKLPG revealed several related proteins in seeds of *F. novae-zealandiae*, but these were not induced by water-deficit stress in leaves.

Table of Abbreviations

Abbreviation	Meaning
2ML	Second mature lamina
A _x	Absorbance at x nm
ABA	Abscisic acid ((±) 5-[1-Hydroxy-2,6,6,-trimethyl-4-oxocyclohex-2-en-1-yl]-3-methyl-[2Z,4E]-pentadienoic acid)
ANOVA	Analysis of variance
APS	Ammonium persulphate
AU	Absorbance unit
BCIP	5-bromo 4-chloro 3-indolyl phosphate
Bisacrylamide	N N'-Methylene bis-acrylamide
BSA	Bovine serum albumin
C _x	Watered control plant number x
c.f.	Compare with
D _s	Mean degree of leaf tip scorching
D _x	Drought treatment plant number x
DMSO	Dimethylsulphoxide
DW	Dry weight
EDC	1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme-linked immuno-sorbent assay
FDW	Freeze-dried weight
FW	Fresh weight
G-B	Glycine-betaine
GC	Gas chromatography
H _L	Leaf hydration
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
IL	Immature lamina
IP	Immature pseudostem
kD	Kilodaltons
LER	Leaf elongation rate
LWP	Leaf water potential
MAb	Monoclonal antibody
ML	Mature lamina
MP	Mature pseudostem
N _T	Mean number of green leaves per tiller
n	Number of replicates
NBT	Nitro-blue tetrazolium, <i>p</i> -toluidine salt
NMR	Nuclear magnetic resonance
NPK	Nitrogen, phosphate, potassium ratio
NSD	Not significantly different
P	Probability

Abbreviation	Meaning
PBSalt	50mM sodium phosphate buffer, pH 7.4, containing 250mM NaCl
Pers. comm.	Personal communication
PMSF	Phenylmethanesulphonyl fluoride
Pro	Proline
PVC	Polyvinyl chloride
PVDF	Polyvinylidene difluoride
QAC	Quaternary ammonium compound
R _f	Relative distance, compared to solvent front
RIA	Radioimmunoassay
RSD	Relative standard deviation
RuBisCO	Ribulose-1,5-bisphosphate carboxylase-oxygenase
RWC	Relative water content
s.e.	standard error of the mean
SDS	Sodium dodecyl sulphate
SDS- PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
Std. Dev.	Standard deviation
SWC	Soil water content
TDR	Time-domain reflectometer
TEMED	N N N' N'-tetramethylethylenediamine
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)methylamine
T _{Num}	Mean number of green tillers per plant
Tween-20	Polyoxyethylenesorbitan monolaurate
UV	Ultra-violet radiation

S.I. Unit Abbreviations

The System Internationale (S.I.) symbol for the unit litre is by convention a lower case "L". However, in typed manuscript it is possible to misread this lower case letter as either the numeral "1", or as an upper or lower case "l". Therefore, to avoid such difficulties, the uppercase character has been used in this dissertation. Note that this convention applies to S.I. units sharing the base unit litre, such as millilitre (mL) or microlitre (μ L). All other quantities used in this volume are expressed in conventional S.I. units.

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Chapter One

Introduction

1.1 Drought in Agriculture

Plants growing in natural or agricultural environments are often prevented from expressing their full genetic potential and are considered to be "stressed". The reduction of plant growth due to stress can be considerable. For example, by comparing stressed plants with those growing in non-limiting conditions, environmental stresses were found to limit the productivity of United States agriculture to 25% of its potential (Boyer, 1982). Of all the environmental factors evaluated (drought, cold, salinity, alkalinity, flood, wind, anoxia, and pest organisms), drought was by far the most limiting to plant performance (Boyer, 1982). Although the occurrence of drought is unpredictable, drought does occur on a regular basis. For example, in Australia drought occurs in 80 out of every 100 years. This 80 years is made up of 60 years of drought covering less than 20% of the continent, 15 years of drought affecting 20-50% of the continent, and in 2 out of every 100 years drought will encompass more than 50% of the continent (McWilliam, 1986). In New Zealand, the economic cost of drought can be substantial, as exemplified by a drought that affected the east coast of the North Island during the summer and autumn of 1988-89. This drought was estimated to reduce regional farm income by \$240 M, and total regional income by \$1000 M (Nield 1990).

Avoidance strategies, such as growing seasonal crops, and technological improvements in agricultural practices, such as irrigation methods that have been developed over thousands of years, can be used to help reduce the deleterious effects of drought. However, the use of irrigation may not always be the best solution. Not all areas can be irrigated, due to terrain restrictions and the requirement for a local, sustainable water supply. Modern irrigation schemes are expensive, and the cost has to be offset against the relatively low profit margins of primary industry, since domestic and industrial water users offer economic returns fifty times larger than can be obtained by irrigating crops (Boyer, 1982). Irrigation, accompanied by poor drainage can also bring about a gradual salinisation of the land. This can be a significant problem; for

example, in California almost 30% of the irrigated land is affected by saline soils (McCue and Hanson, 1990). Although selective breeding may make more salt-tolerant cultivars available (McWilliam, 1986), the problem of salinity is persistent, and likely to become more widespread in the future. The economic and environmental costs of remedying water deficit stress in crops through irrigation suggests that this agricultural practice, although useful for short-term relief, is not the best solution.

The use of cultivars with improved water-deficit tolerance properties is a positive alternative to irrigation and the successful targeting of species used in agriculture toward drought has been demonstrated. A great deal of the cost of the 1988-89 drought of the east coast of the North Island was attributed to farmers reliance on *Lolium perenne* (ryegrass) and *Trifolium repens* (white clover) based pasture. From 1990 to 1992, a number of *T. repens* based demonstration pastures were established using *Festuca arundinacea* (tall fescue), *Dactylis glomerata* (cocksfoot), *Phalaris aquatica* (phalaris) and *Cichorium intybus* (chicory) cultivars developed by DSIR Grasslands in the 1970's, which had been shown to grow longer into droughts, and recover faster than *L. perenne* (Milne *et al.*, 1993; Smith *et al.*, 1993). On these demonstration pastures farmers reported an average 112% increase in grazing over the existing pastures, with the largest increases occurring over the summer (Milne *et al.*, 1993).

1.2 Progress toward Drought Tolerant Cultivars through Conventional Breeding

New drought tolerant cultivars can be developed in dryland areas by selecting against growth performance. For example, improvements in the persistence and forage yield of *T. repens* breeding lines was achieved by evaluating groups of cultivars, including populations that originated from dryland areas, for two years in a drought prone east coast North Island site (van den Bosch *et al.*, 1993).

Such breeding programs can develop cultivars suited to a particular geographical region. However, progress has been slow, and the cultivars produced have been developed to suit the prevailing conditions, and may still be susceptible to the unusually dry conditions of drought. Increased knowledge in the fundamental principles behind plant adaptations toward drought may allow the elucidation of traits important in

drought tolerance. By selecting against these traits (rather than by selection against yield alone), plant breeders could then direct their programs toward the development of cultivars more tolerant of water deficit stress. For example, osmotic adjustment (that is, maintenance of turgor by the accumulation of osmolytes) was shown to be involved in the adaption of plants in response to drought stress (Sambo, 1981). Morgan (1977) found that *Triticum aestivum* (wheat) genotypes differed markedly in their ability to undergo osmotic adjustment, and suggested that this difference could be exploited for improving drought hardiness. Later it was shown that measuring osmotic adaption in plants grown under non-stress conditions in a glasshouse could be used to develop breeding lines with a high, or low capacity for osmotic adjustment. Experiments have shown that plants with a high capacity grew better, and produced more grain under conditions of water deficit, and that these differences did not exist under optimal water conditions (Morgan, 1984). Hence the feasibility of using trait-directed plant breeding to advance drought tolerance has been demonstrated.

1.3 Drought Tolerance Traits found in Plants

Drought can be described as an climactic character resulting from unseasonal absence of rainfall, and usually results in "water deficit" stress in plants. In addition to osmoregulation, plants have evolved many adaptations to counteract water-deficit stress. McCue and Hanson (1990) classified these adaptations into four categories (Table 1.1) which can be considered to be mechanisms of drought avoidance (developmental and morphological traits) or drought tolerance (physiological and biochemical) (Skirver and Mundy, 1990). To this list, we may also add ecological traits, such as the symbiotic relationships between grass species and *Acremonium* endophyte. The effects of such endophyte relationships on the drought tolerance ability of pasture grass species has been reviewed by West and Gwinn (1993).

The most complex mechanisms within a plant, involving many gene products, are at the developmental level (Table 1.1). The simplest traits, involving a small number of genes, are at the biochemical level. Because of the practical requirement for biochemically defined traits for use in genetic engineering, only a relatively few genes involved with biochemical adaptations to water-deficit stress could be genetically exploited at this stage (McCue and Hanson, 1990).

Table 1.1: Classification of water-deficit stress adapted traits found in plants, with some examples relevant to this dissertation. This table was adapted from McCue and Hanson (1990).

<p>Whole Plant</p> <p>↑</p> <p>Level of Organisation</p> <p>↓</p> <p>Gene Product</p>	<p>Low</p> <p>↓</p> <p>Biotechnology Potential</p> <p>↓</p> <p>High</p>	<p>▶ Ecological traits</p> <ul style="list-style-type: none"> ◆ Endophyte relationships, such as <i>Acremonium</i>-type grass infections.
	<p>▶ Developmental traits</p> <ul style="list-style-type: none"> ◆ Annual habit. ◆ Tiller production during the spring season. 	
	<p>▶ Morphological traits</p> <ul style="list-style-type: none"> ◆ Reduced leaf surface area. ◆ Curled leaves, enclosing stomata and trichomes. ◆ Waxy cuticle. ◆ Kranz anatomy. ◆ Root structure, including deep or fibrous roots. 	
	<p>▶ Physiological traits</p> <ul style="list-style-type: none"> ◆ Closing of stomata and wilting. ◆ Leaf curling. ◆ Accelerated leaf turnover. ◆ Slow growth and resource acquisition. ◆ Compartmentalisation of ions into vacuole. ◆ Changes in growth and photosynthetic rates. 	
	<p>▶ Biochemical traits</p> <ul style="list-style-type: none"> ◆ Hatch-Slack (C4) pathway. ◆ Accumulation of osmolytes, e.g. sugar alcohols, mono- and disaccharides, polyols, fructans, inorganic ions. ◆ Accumulation of osmoprotectants, e.g. proline or glycine-betaine. ◆ Accumulation of phytohormones, e.g. ABA. ◆ Accumulation of certain proteins, e.g. dehydrins. ◆ Induction of proteases or protease inhibitors. ◆ Changes in the spectrum of metabolic enzymes, e.g. osmolyte/ osmoprotectant synthesis enzymes. 	

1.3.1 Morphological and Physiological Adaptions

Plants may contain many structural features which reduce water loss to the environment, and as such can be considered to be mechanisms of drought avoidance. Examples of structural adaptations to water-deficit common to many plants include a small ratio of leaf surface area to volume, small intercellular space volume, abundant sclerenchyma, thick cell walls, thick cuticles with associated waxy layers, abundant stomata which are often located in stomatal crypts or grooves, abundant trichomes (Esau, 1977; Schonherr, 1982; Raven *et al.*, 1986) and organs adapted for water storage such as the leaves and stems of succulents (Zimmermann and Milburn, 1982).

These adaptations are most likely to have evolved in response to prevailing environmental conditions, and may be insufficient to prevent water-deficit stress from occurring during unusually dry conditions. Upon the occurrence of water-deficit stress, the growth pattern and physiology of plants may alter to minimise further water loss. Examples include changes in leaf structure by rolling, wilting or leaf movements, closure of stomata, reduction in leaf growth rate with continuation of root growth, reduced photosynthetic rate and nutrient uptake, and increased leaf turnover which are physiological adaptations common to many plants (Hsiao, 1973; Bradford and Hsiao, 1982; Raven *et al.*, 1986; Ludlow and Muchow, 1990). Many of these traits are mediated by reduced turgor in specialised cells such as stomatal guard cells or leaf motor (bulliform) cells, while reduction in leaf elongation may be due to reduced turgor which is required for cell elongation. In some cases, such as ABA/Ca²⁺ mediated stomatal closure, biochemical processes that regulate changes in physiology have been identified (Allan *et al.*, 1994).

1.3.2 Biochemical Adaptions to Water-Deficit Stress

In response to water-deficit stress, plants accumulate a range of low molecular weight solutes (osmolytes) which reduce solute potential as leaf water potential (LWP) decreases. This preserves cell turgor pressure and allows turgor dependent processes such as photosynthesis¹ and leaf elongation to continue at low LWP (Sambo, 1981). Osmolytes that accumulate in the cytoplasm must be "compatible" (non-toxic) with respect to metabolic function. That is, the solutes must not interfere with enzyme structure or activity even when present at high concentrations (McCue and Hanson, 1990). Potassium ions are the predominant compatible solute in halophilic methanogens (Lai and Gunsalus, 1992). However, K⁺, and other inorganic ions such as Na⁺, Cl⁻ and SO₄²⁻ may be toxic to plant metabolism (McCue and Hanson, 1990), and simple electrolytes do not accumulate in higher plants as osmolytes (Yancey *et al.*, 1992). Halophytes do sequester these ions in the vacuole, often up to high concentrations, but this is probably as a mechanism for removal of their toxicity toward the cytoplasm; compatible compounds are still required in the cytosol to balance the osmotic potential of the vacuole (Manetas *et al.*, 1986).

¹ Stomatal guard cells require turgor to remain open which allows CO₂ to enter the plant for photosynthesis.

Neutral polyhydric compounds such as glycerol and glucosyl-glycerol, sugars such as fructans, trehalose, fructose and sucrose, and a range of sugar alcohols, such as mannitol and pinitol, are common cell osmolytes, and occur in a wide range of species, including bacteria, cyanobacteria, unicellular algae, fungi and higher plants (Warr *et al.*, 1988; Yancey *et al.*, 1992; Tarczynski *et al.*, 1992; Pilon-Smits *et al.*, 1995). Amino acids and their derivatives, such as proline, alanine, taurine, β -alanine, proline-betaine and glycine-betaine (Delauney and Verma, 1993; McCue and Hanson, 1990, Yancey *et al.*, 1992), polyamines (Turner and Stewart, 1988), β -carotene (Cowan *et al.*, 1992) and abscisic acid (ABA) (Hartung and Davies, 1991) also accumulate in plants in response to water-deficit stress.

1.3.2.1 Osmoprotectants

The accumulation of amino acids, polyols and related compounds often contributes little to osmotic changes that occur in response to water-deficit stress in comparison with other osmolytes (Barker *et al.*, 1993; Thomas, 1991). These compounds have been postulated to afford a defensive mechanism against water-deficit stress by interacting with proteins and stabilising the native protein structure. For example, glycine-betaine was shown to inhibit the heat denaturation of bovine serum albumin (Winzor *et al.*, 1992). Glycine betaine, myoinositol and proline were effective in preventing loss of *Cicer arietinum* (chickpea) glutamine synthetase activity by high temperature, although enzyme activity was reduced above 30 °C in the presence of proline (Laurie and Stewart, 1990). Both proline and glycine-betaine have been shown to preserve the growth of bacteria in normally lethal hypersaline solutions (Le Rudulier *et al.*, 1984; Csonka, 1989) and prevent NaCl inhibition of phosphoenolpyruvate carboxylase activity from two members of the Poaceae [*Cynodon dactylon* (Bermuda grass) and *Sporobolus pungens*], although only glycine-betaine was effective at stabilising the same enzyme from one species of Chenopodiaceae, *Salsoda soda* (tumbleweed, Manetas *et al.*, 1986).

The ability of osmoprotectants to function in this way is thought to be attributable to an increase in the water-binding capacity of proteins due to flooding of the protein surface by highly soluble osmoprotectant molecules. This allows the proteins to remain soluble (and active) in the cytosol which may become more concentrated with the loss of water (Winzor *et al.*, 1992).

1.3.2.2 ABA and the Sensing of Osmotic Change

Abscisic acid (ABA) is a plant growth regulatory compound which is likely to be a ubiquitous compound in the plant kingdom (Creelman, 1989). ABA inhibits germination and regulates seed maturation, stomatal closure (Munns and Sharp, 1993), increased root growth and reduced shoot growth (Creelman *et al.*, 1990), and the adaptation of plant metabolism in response to osmotic stress (Chandler and Robertson, 1994).

Current models suggest that osmotic stress is first sensed by plant cells as plasma-membrane perturbations caused by a loss of turgor pressure (Skirver and Mundy, 1990). Although the exact nature of the sensory apparatus is not known, osmosensory proteins, such as those coded for by the *osm* family of genes in bacteria (Yim and Villarejo, 1992) may be involved. Sensing of the osmotic change may be followed by a change in cellular pH (Cowan *et al.*, 1992), followed by an increase in cytosolic and apoplastic ABA due to *de novo* synthesis and/or release of ABA from organelles (Zeevaert and Creelman, 1988). This induction of ABA synthesis is rapid (~30 min) and requires gene expression (Guerrero and Mullet, 1988).

ABA induces changes in gene expression (Skirver and Mundy, 1990), and genes induced by this mechanism are often referred to by the acronym RAB (responsive to ABA). It is not known how the cell perceives ABA (Allan and Trewavas, 1994), although the location of ABA perception in guard cells of *Commelina communis* and *Hordeum vulgare* (barley) has been localised to the outer surface of the plasma membrane (Anderson *et al.*, 1994; Gilroy and Jones, 1994). A two tiered perception has also been proposed, where plasma membrane reception of transient signals induces rapid cellular responses, while cytoplasmic receptors may be slower, requiring longer occupancy times for ABA, and are concerned with more prolonged facets of response (Allan and Trewavas, 1994). An ABA responsive element (CCACGTGG) has been identified in the promoter regions of several genes which are regulated by ABA, such as the Em gene from *T. aestivum* (Marcotte *et al.*, 1989), RAB16a from *Oryza sativa* (rice; Mundy *et al.*, 1990) and RAB28 from *Zea mays* (maize; Pla *et al.*, 1993).

Calcium ions are the putative secondary messenger in ABA mediated gene expression (Cowan *et al.*, 1992). An ABA-insensitive mutant of *Arabidopsis thaliana* has been

characterised, and is mutated in the *ABI1* locus which is predicted to encode an early step in the ABA signalling process. The *ABI1* gene product was identified as a Mg^{2+} dependent protein phosphatase, which also has a Ca^{2+} binding site. This suggests that the ABA and Ca^{2+} signals are integrated with protein phosphorylation-dependent response pathways (Meyer *et al.*, 1994; Leung *et al.*, 1994). An ABA induced protein phosphatase has been implicated in the regulation of stomatal aperture by the mediation of Ca^{2+} -induced inactivation of K^+ channels (Luan *et al.*, 1993).

1.3.2.3 Induction of Proteins

A large number of proteins are induced in plants in response to water deficit stress. Many of these are also regulated by ABA, as well as hyper-osmotic, cold, heat, and injury stresses, which suggests a complex and possibly general stress response transduction pathway in plants (Bray, 1993).

Many of the water-deficit stress induced genes share regions of homology. At least five groups of genes have been identified from a diverse range of species, and have been termed LEA groups, following the homology of the members of each group to a particular late embryogenesis abundant (LEA) gene (Dure *et al.*, 1989). During seed maturation ABA induces the expression of LEA proteins, which are highly hydrophilic, and remain soluble even after boiling which suggests that these proteins have a high affinity for water (Close *et al.*, 1989). These proteins are thought to maintain a minimal water content and to stabilise cytoplasmic structures in the embryo (Lane 1991); hence LEA transcripts expressed in water-deficit stressed leaves may serve a similar role.

Although the exact function of most water-deficit induced genes are unknown, functions have been proposed for transcripts from the five LEA groups (Bray, 1993). LEA group 1 proteins such as LEA D19 from *Gossypium hirsutum* (cotton; Baker *et al.*, 1988), p8B6 from *Raphanus sativus* (radish; Raynal *et al.*, 1989) and Em from *T. aestivum* and *O. sativa* (Morris *et al.*, 1990; Litts *et al.*, 1992) are predicted to have enhanced water-binding capacity due to a high percentage of charged amino acids and glycine.

LEA group 2 proteins (often referred to as "dehydrins") have a consensus sequence EEKKGIMDKIKEKLPG at the C terminus and at least once internally, and are thought to be involved with preserving protein structure. At least 30 different genes have been identified with this group (Bray, 1993), including LEA D11 from *G. hirsutum*, dehydrin from *H. vulgare* and *Z. mays*, cyanodehydrin from cyanobacteria, RAB16 and RAB25 from *O. sativa*, RAB17 and RAB28 from *Z. mays*, RAB15 and WSP23 from *T. aestivum*, TAS14 from *Lycopersicon esculentum* (tomato) and PsB12 from *Pisum sativum* (pea) (Baker *et al.*, 1988; Close *et al.*, 1989; Close and Lammers, 1993; Mundy *et al.*, 1990; Kusano *et al.*, 1992; Vilardell *et al.*, 1990; Pla *et al.*, 1993; King *et al.*, 1992; Joshi *et al.*, 1992; Godoy *et al.*, 1994; Robertson and Chandler, 1992; respectively).

LEA Groups 3 and 5 proteins have an 11-mer sequence TAQAAKEKAGE repeated as many as 13 times (although the degree of specificity in each amino acid position is more highly conserved in the Group 3 LEA proteins) and are proposed to function by sequestering ions which are concentrated by cellular dehydration (Bray, 1993). This group includes LEA D7 from *G. hirsutum* (Baker *et al.*, 1988), LEA 76 from *Brassica napus* (rape; Harada *et al.*, 1989) and HVA1 from *H. vulgare* (Straub *et al.*, 1994). The Group 4 LEA proteins such as lea D29 from *G. hirsutum* (Baker *et al.*, 1988) and pcC3-06 from *Craterostigma plantagineum* (the resurrection plant; Piatkowski *et al.*, 1990) have a conserved N terminus, and may replace water to preserve membrane structure (Bray, 1993). In addition to the LEA groups, many other genes which are regulated by water-deficit stress and ABA have been identified (reviewed in Skirver and Mundy, 1990; Chandler and Robertson, 1994).

The regulation of water-deficit associated genes is complex and poorly understood. Exemplifying this is the regulation of osmotin from NaCl stressed *Nicotiana tabacum* (tobacco) cell cultures (Singh *et al.*, 1989). The promoter for this gene has been partially characterised (Nelson *et al.*, 1992) and was induced by ABA, ethylene, NaCl, wounding and tobacco mosaic virus infection. Regulation was also tissue dependent, with a higher expression of osmotin in roots than shoots, and no expression in seeds; expression also declined with tissue age. In many cases, osmotin mRNA was expressed, but the associated protein was not found, indicating that post-transcriptional regulation was involved (Nelson *et al.*, 1992; La Rosa *et al.*, 1992).

Although many water-deficit associated genes have been isolated from embryo or seed genomic libraries (for example Em, LEA, and RAB genes), and have therefore been implicated in the developmental process of seed maturation, many of these genes are also expressed under stress conditions elsewhere in the plant. For example, RAB21, an ABA responsive gene activated during embryogenesis in seeds of *O. sativa*, is also expressed in leaves during water-deficit stress (Mundy and Chua, 1988). However, whether the function of the RAB21 gene product is similar in both embryo and stressed tissues is not known. Illustrating this is a related protein from LEA group 2, RAB17, which is expressed in embryos and leaves of *Z. mays* in a similar fashion to RAB21; but in this instance, embryonic RAB17 is susceptible to phosphorylation while leaf RAB17 is not (Vilardell *et al.*, 1990).

Other members of the same LEA group 2, such as CAP85 from *Spinacea oleracea* (spinach; Neven *et al.*, 1993), cas17 and cas18 from *Medicago sativa* (alfalfa; Wolfrain and Dhindsa, 1993; Wolfrain *et al.*, 1993), c17 from *Solanum tuberosum* (potato; van Berkel *et al.*, 1994), and PCA 60 from *Prunus persica* (peach; Arora and Wisinewski, 1994), appear to be induced mainly during cold-acclimation. This raises the possibility that despite the extensive sequence homology, two sub-groups exist within the group 2 Lea proteins; those which are induced by ABA and in response to drought, and those which are expressed independently of ABA and in response to cold (Lang *et al.*, 1994). A further dehydrin-related gene, PsB61 from *Pisum sativum*, has extensive homology to other LEA group 2 proteins, but is not responsive to ABA, nor to low (4 °C) or high (37 °C) temperature stress (Robertson and Chandler, 1994).

1.4 Potential for Biotechnology

While conventional breeding has the capacity to produce cultivars with improved drought-tolerance properties, it must do so within the confines of the available gene pool. Proline accumulation is a trait common to many plants (Delaunay and Verma, 1993). Hence the wide species distribution of this trait should provide no constraint to the plant breeder. However, not all traits are so widespread. For example, the occurrence of glycine-betaine is limited to members of the Chenopodiaceae, Poaceae, Asteraceae (Weretilnyk and Hanson, 1990), and halophilic cyanobacteria (Arakawa *et al.*,

1992) while proline-betaine is limited to the Fabaceae and Lamiaceae, and β -alanine-betaine to the Plumbaginaceae (McCue and Hanson, 1990). Therefore, genes associated with traits of limited species distribution may have to be introduced into the gene pools of other plant species if advantage of such traits are to be taken. Genetic engineering need not be restricted to between-plant transformations, since the accumulation of polyols by fungi and bacteria (Richardson, 1992; Tarczynski *et al.*, 1992), and the use of sarcosine and trimethylamine-N-oxide as osmolytes in animals and bacteria (Yancey *et al.*, 1982) are possible sources of genetic material. Increased knowledge of the osmosensory mechanisms working in plants and bacteria and of the regulation of pathways leading to the accumulation of metabolites may also provide stock for further refinement of the genetic make-up of elite cultivars.

Investigations into the relevance of solute concentration as selective criteria for plant breeding are continuing. One question which needs to be addressed is whether solute accumulation in plant tissues provides an adaptive advantage to the plant under water deficit stress, or is merely indicative of other stress-induced changes in metabolism (Delauney and Verma, 1993; Hanson and Hitz, 1982; Vartanian *et al.*, 1992). For example, the accumulation of ABA in response to two levels of hydration by 21 hybrids of *Z. mays* has been examined, with the conclusion that ABA concentration was mainly an indicator of drought severity, rather than an agent limiting the negative effects of water shortage (Landi *et al.*, 1995). However, positive progress has been made with several other water-deficit related compounds. The expression of a bacterial gene, *mtlD* from *Escherichia coli*, encoding for the enzyme mannitol-1-dehydrogenase in transgenic *N. tabacum*, with CaMV 35S promoter control led to the accumulation of the osmolyte mannitol, which does not occur naturally in this plant species (Tarczynski *et al.*, 1992). Plants containing mannitol were later shown to have enhanced ability to tolerate high salinity (Tarczynski *et al.*, 1993). This group of authors has also isolated a second gene from *Mesembryanthemum crystallinum* (the ice plant), *imt1*, which encodes a myo-inositol O-methyl transferase, that catalyses the first of two steps required for synthesis of the putative osmoprotectant pinitol, and have also expressed this gene in transgenic *N. tabacum*. The transformants accumulated the intermediate ononitol (Vernon *et al.*, 1993). These two examples demonstrate the feasibility of diverting carbohydrate metabolism by the introduction of new genetic material. However, in the latter

case, the levels of ononitol continued to accumulate in the transgenic plants as they matured which suggested that this osmolyte was a metabolic "dead end", and could not be catabolised by the existing enzyme complement of the plant (Vernon *et al.*, 1993).

Nicotiana tabacum has been transformed with the rate limiting enzyme in glycine-betaine synthesis, betaine aldehyde dehydrogenase (BADH) from *E. coli* (Holmstrom *et al.*, 1994) and *H. vulgare* (Ishitani *et al.*, 1995). In the former report, BADH activity was successfully directed to the chloroplast using an N-terminal transit peptide, and transformants converted exogenously supplied betaine aldehyde to glycine-betaine. In the second report, BADH enzyme and activity was expressed at high levels in leaves and roots, and transcription was increased in response to NaCl, drought, water-deficit and by the application of ABA. Hence the natural compartmentalisation and regulation properties of BADH have been successfully transferred to a novel species.

A fructan synthesis gene, levan-sucrase, from *Bacillus subtilis* has also been introduced in an active form into *N. tabacum* (Pilon-smits *et al.*, 1995), and *O. sativa* has been transformed with the LEA group 3 gene HVA-1 from *H. vulgare* (Xu *et al.*, 1995). Both genes were transformed with 35S promoters. In both cases, the introduction of the foreign trait into novel species mediated enhanced water-deficit or salt tolerance. These experiments with transgenic plants are useful for the study of the role of various solutes in response to water-deficit stress, and demonstrate that these traits do indeed confer adaptive advantage to plants in response to water-deficit stress.

1.5 Thesis Introduction

Many of the traits associated with water-deficit stress, including those discussed above, have been discovered and researched in crop and pasture species, especially cereals. Notable exceptions to this is the work conducted with the desiccation tolerant plant *C. plantagineum* (e.g. Piatkowski *et al.*, 1990; Michel *et al.*, 1994) and the facultative halophyte *M. crystallinum* (e.g. Vernon *et al.*, 1993; Yen *et al.*, 1995), although other "unusual" plants have also been examined, such as a desert plant *Anastatica hierocluntica* (Abou-Mandor and Hartung, 1995) or the extremely xerophilic liverwort

Exormotheca holstii (Hellwege *et al.*, 1994). The investigation of species that are tolerant of water-deficit conditions may lead to the elucidation of associated (and possibly novel) biochemical traits that may then become available for incorporation into the gene pools of important agricultural mesophytes. In New Zealand, the subalpine tussock *Festuca novae-zealandiae* presents such an opportunity to examine a relatively unknown xerophyte for water-deficit stress tolerance mechanisms.

This thesis describes an initial investigation of the water-deficit adaptations of *F. novae-zealandiae*. The natural dry-land distribution of this species, along with some morphological features of the leaves, such as curled laminae with dense trichomes and abundant sclerenchyma (Connor, 1960) suggested that this species has adaptations that have evolved in response to dry environments. In addition, changes in the growth dynamics of this species in response to environmental fluctuations has been proposed (Lord, 1992), which suggested that *F. novae-zealandiae* may adapt physiologically to water-deficit stress. However, it was not known if the drought-tolerance of this species resulted from the morphological and growth characteristics of the plant alone, or whether the drought-tolerance was also in part attributable in changes in the metabolism of *F. novae-zealandiae* in responses to water-deficit stress. Hence, the aim of the project work described in this thesis was to determine if metabolic responses to increasing water-deficit stress occur in *F. novae-zealandiae*. Confirming this would then form the basis of future, more in depth investigations into the biochemical and molecular response of *F. novae-zealandiae* to water deficit stress.

1.5.1 *Festuca novae-zealandiae* (Hack) Cockayne

Festuca novae-zealandiae (Hard tussock) is indigenous to New Zealand, where it is a dominant member of the short tussock grasslands which extend from Marlborough to Southland in areas of low rainfall east of the Main Divide, to elevations of 1450 m, and in isolated regions of the volcanic plateau in the central North Island (Newsome, 1987). The ability of this plant to grow in semi-arid, sub-alpine regions make it an interesting plant for studying the processes of water-deficit stress tolerance.

Details of this species in the literature are scarce, and are generally deal with the ecology of short tussock grasslands (e.g. Scott *et al.*, 1988; Williams, 1989). Although *F.*

novae-zealandiae is not sown for pasture, it is a dominant species in high country sheep stations, where the tussock may provide a favourable micro-climate for associated fodder species (Espie, 1990). *Festuca novae-zealandiae* is a perennial grass with rigid tightly rolled leaves to 600 mm and culms to 800 mm in height forming a tightly packed erect tussock (Plate 4.2, page 99). It is relatively unpalatable to stock, and is slow growing. The life span of a single tussock could exceed fifty years, and it spreads mainly by tillering, since recruitment from seedlings is low (Lord, 1992). This species is tolerant of nutrient-limiting conditions but will grow rapidly in response to fertilizer, especially to added phosphorus and nitrogen (Espie, 1990). A chromosome count indicated that *F. novae-zealandiae* was hexaploid ($2n = 6x = 42$) (Beuzenberg and Hair, 1983).

Understanding the growth dynamics of this species may be important in understanding the drought avoidance mechanisms it employs. The modular structure of tussock grasses means that tussocks (genets) are potentially immortal, but are comprised of modules (tillers) each only a few years old. The continual replacement of modules allows the size of the plant to increase or decrease in response to environmental fluctuations (Lord, 1992). Hence it is possible that *F. novae-zealandiae* may respond to a progressive water-deficit stress, not only by adapting the cellular environment to cope with the increased evaporative demand, but also by increasing the turn over of damaged tillers, and by the replacement of tiller modules.

1.5.2 *Festuca arundinacea* (Schreb)

Festuca arundinacea (Tall fescue var. "Grasslands Roa") is an exotic, perennial pasture grass (Plate 4.1, page 98). This cultivar was developed in New Zealand (Brock, 1983). As a relatively drought-tolerant agronomic grass species (Brock, 1983), it was chosen as a suitable species to compare with *F. novae-zealandiae* under water-deficit stress conditions. It has dense, flat, broad leaves, which are rolled in the young shoot, short ligules, tiny trichomes, and distinct lines of well developed bulliform cells on the upper surface of the leaf. This plant is found naturally in high rainfall areas (Clouston, 1960). *F. arundinacea* is a host plant for *A. coenophialum* endophyte, and this symbiosis increases the drought tolerance of the grass, although the mechanisms are largely unknown (West and Gwinn, 1993; Richardson *et al.*, 1992; Elbersen, 1994). The *F. arundinacea* cultivar used in this study was endophyte free (Margot Forde Forage

Germplasm Center). Like many graminaceae, *F. arundinacea* has been reported to undergo changes in metabolism and physiology in response to water-deficit stress. This species has been shown to accumulate protein (Belesky *et al.*, 1984), nitrate and total nitrogen (Belesky *et al.*, 1982), proline (Belesky *et al.*, 1982, Bokhari and Trent, 1985), total water-soluble carbohydrate (Spollen and Nelson, 1994), sucrose (Richardson *et al.*, 1992; Spollen and Nelson, 1994), hexose (Spollen and Nelson, 1994), glucose and fructose (Richardson *et al.*, 1992) in response to water deficit. Leaf rolling, decreases in biomass production (Belesky *et al.*, 1982), leaf elongation rate (LER), and the concentrations of fructans with both high and low degrees of polymerisation (Spollen and Nelson, 1994) have also been observed in response to water-deficit stress in *F. arundinacea*.

1.6 Experimental Methods for Imposing Water Deficit Stress on Plants

1.6.1 Dry-Down Techniques

Although the development of drought is usually associated with water-deficit stress in plants, there are several other environmental factors which are commonly associated with drought, such as increased light intensity, temperature or wind, and changes in the soil structure and patterns of insect predation. Therefore any technique to study the water deficit stress tolerance mechanism of a plant should isolate the plant from stress arising from these other sources. Since drought progresses slowly in nature, water-deficit stress should also be applied gradually to allow plants to adapt to the changing conditions. To fulfil these requirements, Pennypacker *et al.* (1990) developed a glasshouse based approach for investigating water-deficit adaption in *Medicago sativa*. This method used 900 mm tall by 200 mm wide pots which allowed ample room for root growth, and allowed for a slow dry-down of the soil. Lateral soil tensiometers were used to monitor the progress of the dry-down. The method of Pennypacker *et al.*, (1990) was adapted for use in the current study since it avoids a range of problems (briefly mentioned below) encountered with many other commonly used experimental procedures for imposing water-deficit stress. Krizek (1985) categorised these methods into those that:

- i: rely on the addition of exact quantities of water to each plant.
- ii: incorporate solutes such as PEG, mannitol or salt into the growth medium.
- iii: use semipermeable membranes to separate plant roots from the osmoticum.
- iv: apply water pressure from water columns of various heights.

Other methods involve the desiccation of whole seedlings (Close *et al.*, 1989), or of excised leaves (Riov and Hausmann, 1988) by dry air.

The use of external osmoticum to impose water deficit may have adverse effects on plants. PEG may be phytotoxic (Emmert, 1974) and salt may be both toxic or may induce confounding salt stress responses. Semipermeable membranes used to prevent these adverse effects may be degraded by soil micro-organisms and thus require sterile conditions or chemical treatments to prolong membrane life (Sikurajapathy *et al.*, 1983). Methods which rely on controlling the amount and/or timing of watering may not wet pots uniformly, with the possibility of leaving the bottom of the pots dry if the pots are watered at the surface. Many experimental methods, such as the use of callus tissue cultures (Singh *et al.*, 1987) or excised leaves, subject the plant to unnatural conditions, and the results may not be applicable to plants in the field. For example, the phytohormone ethylene has been reported to accumulate in response to drought (Yang and Hoffman, 1984; Riov and Hausmann, 1988). However other reports suggest that this observation was an artefact, manifested by the experimental use of excised (wounded) leaf samples (Morgan *et al.*, 1990; Narayana *et al.*, 1991; Eklund *et al.*, 1992). Finally, the use of small pots or dehydrating lamps and desiccators may result in plants drying too quickly, causing injury rather than adaption to water deficit stress.

1.6.2 Water Status Measurements

Monitoring the progress of dry-down experiments can be carried out in various ways, and usually relies on the quantitation of water in either the soil or plant. Soil moisture or the osmotic potential of the soil (or of osmolyte solutions if used) can be measured by direct soil mass changes, tensiometers or by time domain reflectometry (TDR). The loss of leaf turgor may be determined by wilting or leaf rolling, but may be subjective and cannot be applied to all species evenly (Hsiao, 1973). Several direct and indirect measurements of water status are commonly used, such as leaf water potential (LWP),

leaf osmotic potential (ψ_{π}) and the turgor (pressure) potential (ψ_p) which are related by the equation $LWP = \psi_{\pi} + \psi_p$ (Sambo, 1981). The chemical potential of water (A_w) or the absolute relative water content (RWC) are also in common usage. However, since the mechanism(s) with which plants sense changes in water status are unknown, the hydraulic parameter(s) that are the most relevant to physiological or metabolic processes are under debate (Bradford and Hsiao, 1982; Sinclair and Ludlow, 1985; Kramer, 1988; Passioura, 1988; Schulze *et al.*, 1988; Boyer, 1989). LWP can be conveniently measured by the pressure apparatus of Scholander *et al.* (1965), and is based on thermodynamics. LWP gradients are important for water transport, however, it is not known if LWP *per se*, is critical for biological functions (Bradford and Hsiao, 1982). The absolute water content and A_w are related to LWP, but may change little before metabolic activities occur and may be confounded by tissue size and dry matter content (Bradford and Hsiao, 1982). Measuring RWC accounts for tissue size and dry matter contents, but involves uncertainties because the measured saturation point is not clear cut (Bradford and Hsiao, 1982). Hence, no single parameter is totally suitable as an indicator of plant water-deficit stress (Bradford and Hsiao, 1982) although the current literature generally reports LWP or RWC. Hence these parameters were chosen for the work described in this thesis.

An experimental inconvenience with using these hydraulic measurements to indicate the stress condition within a plant is that these values may fluctuate widely due to sunlight levels, temperature, stomatal movements and humidity, and due to diurnal cycles. Therefore care needs to be taken to minimise the effect of relatively short-term environmental changes on measurements made during long-term experiments. In some cases (e.g. LWP or RWC) the method used requires the excision of leaves from the plant which may lead to changes in the leaf water content or pressure (Savage *et al.*, 1984).

1.6.3 The "Trigger Point", and use of Water-Deficit Response Traits as Metabolic Markers

Regardless of the parameter used, it is helpful to have a reference point of water-deficit stress. Since several compounds such as proline, glycine-betaine and ABA are known to accumulate after the water-deficit stress has reached a certain threshold

value (Bradford and Hsaio, 1982) and the chemical analyses of these compounds are now straightforward (Bates *et al.*, 1973; Lever *et al.*, 1992; Walker-Simmons, 1987), the onset of the accumulation of these compounds in a plant can be used as metabolic markers which indicate that adaptation to water-deficit stress has occurred (see Machackova *et al.* (1989) for an example of this technique). Hence monitoring plant response to water-deficit treatment need not be limited to the measurement of leaf or soil water status using the methods mentioned in section 1.6.2. For the purposes of this dissertation, the onset of accumulation of the metabolic markers with respect to SWC or time, is referred to as the "trigger point" [many other trigger points may also occur, since other events such as stomatal closure, reduction in leaf growth, leaf senescence and death may occur after the water-deficit stress has reached other threshold values (Bradford and Hsaio, 1982)]. Future work on novel adaptations to water-stress (e.g. dealing with changes in gene expression) could then be related back to the water-deficit stress condition of the plant by comparison with the trigger point.

The concentration of a large number of metabolites, such as osmolytes and osmoprotectants, are known to change in response to water-deficit. However, practical limitations mean that the concentrations of only a small number of representative compounds could be measured during this study. Proline, glycine-betaine and ABA were chosen as metabolic markers to indicate that metabolic adaptation to water-deficit stress has occurred, because the response of these metabolites to water-deficit is relatively well defined, and the species distribution of these compounds meant that these compounds were likely to occur in *F. novae-zealandiae*. Changes in osmotic potential, and changes in the concentrations of compounds which predominantly contribute to osmotic adaptation in other species, such as fructans, mono- and disaccharides, sugar alcohols and inorganic ions, were not measured in this study.

1.7 Experimental Approach

No doubt *F. novae-zealandiae* has many morphological adaptations to survive in the semi-arid environment which it inhabits, and it could be expected that many of these adaptations would reduce the possibility of water-deficit stress occurring during drought. However, of principal interest was whether *F. novae-zealandiae* possessed the ability to

adapt metabolically to the onset of water-deficit stress. Evidence that this occurs could then lead to the examination of this species for novel water-deficit tolerance biochemical traits. The practical work for this dissertation was divided into three parts:

1.7.1 Part I: Dry-down of *F. arundinacea*

The aim of the initial part of this dissertation, which was described in Chapter Three, "Experimental Dry-Down of *Festuca arundinacea*" (page 66), was to conduct a trial of the experimental protocols (which are described in Chapter Two, "Materials and Methods", page 21), which were used to impose water-deficit stress upon *F. novae-zealandiae*. For this purpose, the native species was replaced with *F. arundinacea*, because the relatively well defined drought response of the exotic species afforded a useful model with which to evaluate the method protocols. In particular, the cessation of LER and the accumulation of the osmoprotectants proline and glycine-betaine, and the accumulation of the phytohormone ABA were examined. The achievement of a measurable response from these physiological and metabolic traits would then form the basis for the use of these stress-adaption markers to indicate that adaption to the imposed water-deficit stress had occurred in later experiments with *F. novae-zealandiae*.

1.7.2 Part II: Dry-down of *F. novae-zealandiae*

Plants of *F. novae-zealandiae* were subjected to water-deficit stress, and the induction of physiological and metabolic adaption markers (cessation of LER and certain other tiller measurements, and the accumulation of ABA, proline and glycine-betaine) was determined to seek evidence for the occurrence of metabolic adaption in response to water-deficit in *F. novae-zealandiae* (described in Chapter Four, "Experimental Dry-Down of *F. novae-zealandiae*", page 93). Tillers of *F. novae-zealandiae* were also dissected into various tissues to determine if a tissue specific element to metabolic adaption existed (described in Chapter Five, "Experimental Dry-Down of *F. novae-zealandiae* II", page 156). Hypotheses were proposed on how *F. novae-zealandiae* responds to water-deficit stress.

1.7.3 Part III: Initial Investigation of the Molecular Basis of Water-Deficit Stress Adaption in *F. novae-zealandiae*

A more detailed examination of the response of relevant protein moieties was initiated (described in Chapter Six, "Induction of Proteins in Response to Water-deficit Stress", page 197). The protein concentration, and the profile of the most abundant proteins in extracts from plants subjected to a dry-down were determined using polyacrylamide gel electrophoresis (SDS-PAGE). The proteins separated by SDS-PAGE were also examined using Western blotting techniques, using specific antibodies to ubiquitin and the LEA group 2 protein, dehydrin. The former was conducted to seek evidence of controlled protein turnover in the laminae, while the latter was to seek evidence for the induction of a known class of water-deficit stress response proteins in various tiller tissues. Confirmation of the presence of a group 2 LEA gene in the genome of *F. novae-zealandiae* was sought by Southern analysis using an insert from pRAB16a as a probe.

The conclusion to these experiments, along with suggestions for further research, is presented in Chapter Seven, "Conclusions" (page 243).

Chapter Two

Materials and Methods

2.1 Plant Material

2.1.1 *Festuca arundinacea*

Seed of *F. arundinacea* cv. "Grasslands Roa" (Tall Fescue, AgResearch accession number T1347) was obtained from the Margot Forde Forage Germplasm Centre, Grasslands Research Centre, AgResearch, Palmerston North, New Zealand. Seeds were germinated on moist tissue paper in a growth room at 26 °C. When seedlings reached *ca.* 40 mm in height, they were transferred to sandy potting mix supplemented with Osmocote Plus™ (Grace-Sierra International BV, Nijverheidsweg 5, 6422PD, Heerlen, The Netherlands) slow release fertiliser pellets (NPK = 15, 4.8, 10.8 + trace elements²). After the first experiment with this species where seed derived plants were used (Chapter Three, page 66), all of the plants used were of a single genotype, which was designated TF-1. These plants were clonally propagated from tillers of one plant that was derived from seed. Plants were maintained in a temperature controlled glasshouse (22 °C), under natural light, and with regular watering.

2.1.2 *Festuca novae-zealandiae*

Seeds of *F. novae-zealandiae* (Hack.) Cockayne (also known as Hard tussock or Fescue tussock) were donated by Dr. Janice Lord, Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand, and were collected by Dr. Lord from the Cass River region, North Canterbury in 1990. Seeds were germinated on moist tissue paper in a growth room at 26 °C, and transferred to potting mix, and maintained in the glasshouse using the same conditions described for *F. arundinacea* above. Potted plants of *F. novae-zealandiae* were obtained from a commercial nursery, Omahanui Native Plants, Oropic Rd, RD 3, Tauranga. These plants were maintained in the glasshouse using the same conditions described above. New plants were clonally propagated from tillers, so that (unless otherwise stated) all the plants of *F. novae-zealandiae* used were a single genotype, which was designated T2.

² Micronutrient composition of Osmocote Plus™: S (3.6%), Mg (1.2%), Ca (3%), Fe (0.15%), Mn (0.06%), Cu (0.05%), Mo (0.02%), B (0.02%), Zn (0.015%).

2.1.3 Dissection of Leaf Regions

Plant material was sampled from the leaf base (which included the tiller meristem, as well as the elongation zones of the immature leaves), pseudostem or lamina, and the age of the leaf was described as the immature, mature or second mature leaf. A summary of the tissues referred to in this dissertation is presented in Table 2.1, and in Figure 2.1.

The immature leaf was the topmost leaf on a tiller, and was the youngest exposed leaf. Almost without exception, this leaf did not have an exposed ligule, since a new (immature) leaf began to extend out of the sheath of this leaf before the ligule appeared. A mature leaf was defined as a leaf that had a fully emerged lamina and ligule. For the purposes of this dissertation, “mature leaf” denotes the first mature leaf down from the immature leaf; “second mature leaf” denotes the next leaf beyond that. Note that the second mature leaf was the third, and usually the oldest green leaf on a given tiller, since older leaves on the same tiller were usually senescent. The leaf lamina consisted of the leaf blade from the tip down to the ligule of the same leaf. In the case of the immature leaf, the lamina extended from the leaf tip to the ligule of the first mature leaf.

To dissect the various tiller tissues, all the leaves present on the tiller except for the first mature leaf (and the leaves contained within the sheath of this leaf) were discarded. The IL and ML (Table 2.1) were excised at the ligule of the mature leaf (Figure 2.1). The basal 25 mm of the tiller was excised, and retained as the LB. The remaining portion consisted of the pseudostem, from which the mature leaf sheath (MP) was peeled back to leave the sheath of the immature leaf (IP).

Table 2.1: Abbreviations used for tiller tissues.

Tiller region	Abbreviation
Leaf Base Region	LB
Immature Lamina	IL
Mature Lamina	ML
Second Mature Lamina	2ML
Immature Pseudostem	IP
Mature Pseudostem	MP

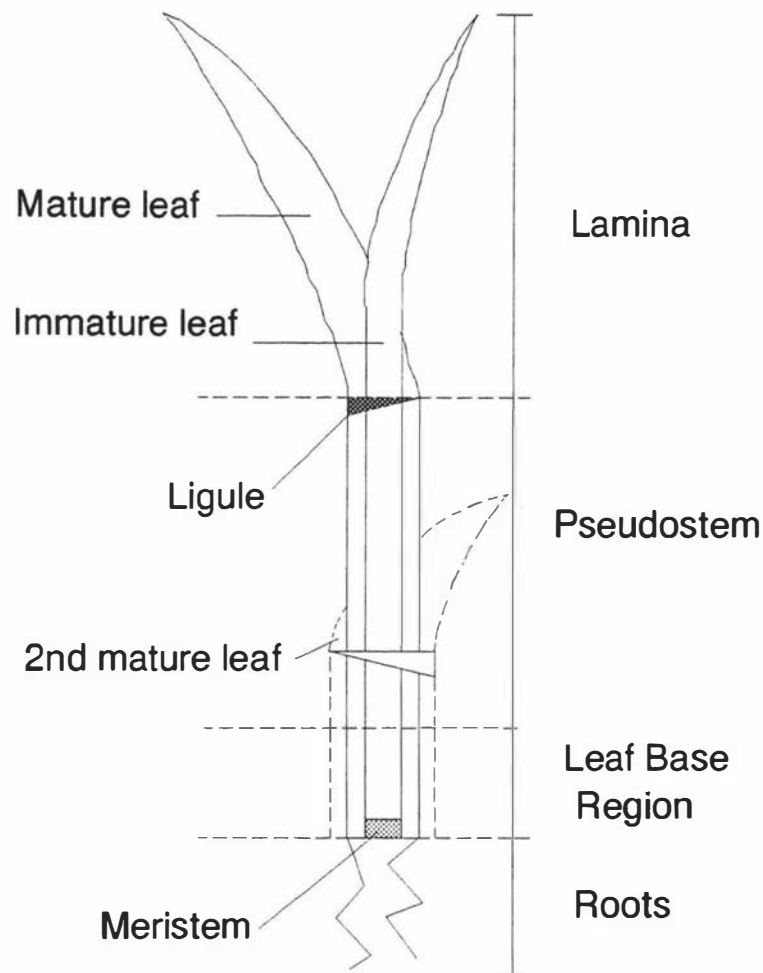


Figure 2.1: Nomenclature of tillers of *Festuca* spp..

2.2 Imposition of Water-Deficit Stress

Although the development of drought is usually associated with water-deficit stress in plants, there are several other environmental factors which are commonly associated with drought, such as changes in light intensity, temperature, humidity, wind, soil structure and patterns of predation. Therefore, the experimental conditions used to impose water deficit stress were intended to isolate plants from stresses arising from these other sources and also to apply the water-deficit gradually to allow plants to adapt to the increasing water deficit.

2.2.1 The 4 L Pot System

Plants were established in the glasshouse in 4.2 L plastic greenhouse pots filled with sandy potting mix containing Osmocote Plus™ slow release fertiliser pellets. The glasshouse temperature was partially controlled, and ranged from 10 to 23 °C,

depending on sunlight and weather. The water-deficit treatment pots were sited in a row on a dry glasshouse bench, and associated control pots were sited on an adjacent wet bench lined with polythene sheet. Pots were watered from the bottom, i.e., the wet bench was filled with water which was then absorbed by the pots. Few weeds germinated from the potting mix, and any that did were removed.

2.2.2 The 24 L Pot System

Plants were grown to maturity using the 4 L pot system described in section 2.2.1. The plants were then transferred, and allowed to establish in 23.6 L pots constructed from PVC piping (Figure 2.2). These pots were based on the design of Pennypacker *et al.* (1990), although the soil matrix used was field soil (Kairanga Silt Loam) of medium fertility³ (Dr. D. Barker, Pers. Comm.), and TDR electrodes were used instead of the soil tensiometers used by Pennypacker *et al.*, (1990). Soil was packed evenly into each pot to a density⁴ of 0.96 g/cm³. Before planting, the pots were top-dressed with 1 kg/m³ of Osmocote Plus™ fertiliser pellets. Water-deficit treatment and watered control pots for each experiment were sited randomly in a row on a wire grid-glasshouse bench. Pots were watered from the top when required, and any weeds which germinated from the field soil were removed.

2.3 Soil Water Content (SWC)

During the progress of the dry-down experiments, the SWC was monitored electronically using a Time-Domain Reflectometer (TDR, TRASE™, Soil Moisture Equipment Corp., PO Box 30025, Santa Barbara, California 93105, USA). The TDR measures the speed at which a microwave pulse travels between two stainless steel electrodes. The pulse speed is dependent on the dielectric constant (k) of the material between the electrodes. Soil particles and air, as well as water exhibit a measurable dielectric constant, but since the dielectric constant of water ($k=80$) is much greater than that of mineral particles ($k=2-3$) or air ($k=1$), the speed of the microwave pulse between electrodes immersed in soil is predominantly dependent on the SWC. The TDR equipment was calibrated by the manufacturer, using test cells containing known volumes of water.

³ Duplicate soil samples were analysed for macronutrient composition and acidity (Soil Fertility Service, AgResearch, Private Bag, Ruakura). The soil used contained 0.22% total nitrogen, 37ppm P, 21ppm K, 10.5ppm S, 7ppm Na, 27.5ppm Mg, 8.5ppm Ca, and had a pH of 5.2.

⁴ Soil density was calculated by dividing the soil volume by dry soil mass; weighed samples of each batch of soil used to fill the pots were oven dried overnight and reweighed to determine the dry soil mass.

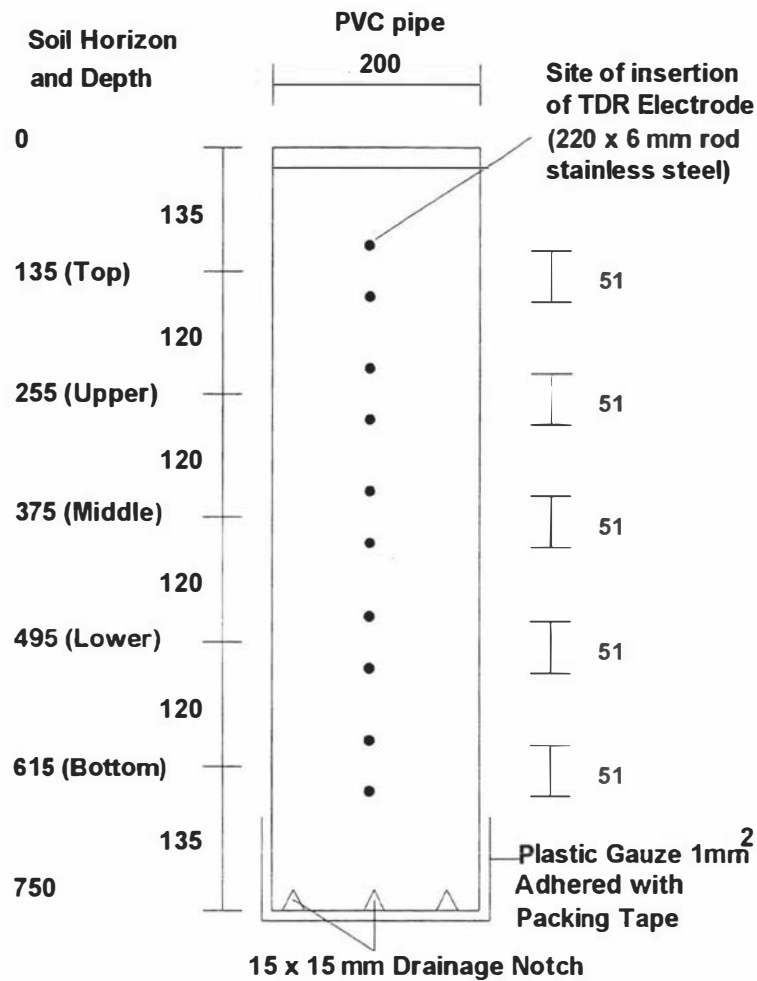


Figure 2.2: Side elevation of the 24L PVC pot. Measurements are in mm.

The TDR needs only to be corrected for the length of the electrodes exposed to the soil sample, which was carried out prior to measurement. The TDR equipment has an error estimated at $\pm 2\%$ SWC (TRASE operating manual).

Pairs of 220 mm electrodes were inserted either vertically into the centre of the pot (4 L pots, and the final experiment with the 24 L pots) or horizontally through the sides of each pot (24 L pots), and remained in place for the duration of each experiment. The TDR equipment was connected to each electrode station in turn for SWC measurements. In control pots, the SWC measurements were taken before watering.

2.4 Leaf Water Status Measurements

2.4.1 Leaf Water Potential (LWP)

On each tiller to be measured, the ML was excised with a razor blade adjacent to the ligule and the LWP was immediately determined with a pressure-bomb apparatus using the method described by Scholander *et al.* (1965). The rubber seal which held the leaves had a narrow groove cut especially for laminae of *F. novae-zealandiae*, and was coated with silicone grease. A magnifying glass was usually used to observe the return of sap to the cut surface. The equipment allowed for measurements in the range of 0 to -3.5 MPa and determinations were carried out in duplicate. For each experiment, the LWP was determined at the same time of day (as described in the methods section for each experiment) on each sampling date to minimise fluctuations in the values caused by diurnal light intensity and temperature changes.

2.4.2 Relative Water Content (RWC)

Tillers were excised with a razor blade at the root-shoot junction and were immediately taken on ice back to the laboratory. Each tiller was dissected into five portions (ML, MP, IL, IP, and LB; section 2.1.3) and each portion was cut into 30-40 mm lengths, which were then pooled and weighed (to give the fresh mass). Samples were then completely immersed in water in individual vials, and incubated overnight at 4 °C in the dark. Samples were blotted dry, re-weighed (to give hydrated mass), and placed into a drying oven for seven hours at 80 °C, and again re-weighed to give the dry mass. The RWC and leaf hydration (H_L) were calculated as below (from Thomas 1991).

$$RWC = \frac{(\text{mass of water in fresh tissue})}{(\text{mass of water in rehydrated tissue})} = \frac{(\text{fresh mass} - \text{dry mass})}{(\text{hydrated mass} - \text{dry mass})}$$

$$H_L = \frac{(\text{mass of water in fresh tissue})}{(\text{dry mass})} = \frac{(\text{fresh mass} - \text{dry mass})}{(\text{dry mass})}$$

2.5 Leaf Elongation Rate (LER)

A number of tillers per pot were tagged with jeweller's labels⁵, and the length of the IL was measured on consecutive days to determine the LER. In the event that the measured leaf matured during the experiment (and hence stopped elongating),

⁵ 3, 4 or 10 tillers per pot were tagged, as described in the methods section for each experiment.

measurements were continued on the next emerging immature leaf. In the event that the measured leaf stopped elongating for some other reason, measurements on that tiller were discontinued; a new tiller was tagged, and measurements were continued on the IL of that tiller. While the latter event did not occur during the dry-down experiments with *F. arundinacea*, it was frequently the case with *F. novae-zealandiae*.

2.6 Root Distribution

At the conclusion of each drydown experiment conducted with the 24 L pot system, the distribution of the roots of each plant was determined. Soil columns were removed intact from the PVC pots, and cut into 70 or 100 mm long sections. Roots in each section were hand-washed free of soil, dried overnight at 80 °C, and weighed. A limitation of this technique was that a portion of the root material, especially the fine roots, was lost, and the roots may have been contaminated with soil particles. However, this was assumed to be equal for all samples.

2.7 Chemical Reagents

Unless otherwise stated, the chemical reagents used were Analar grade; further purification or validation of the purity of chemical reagents was not carried out. The laboratory supply of purified water was produced by reverse-osmosis, followed by microfiltration (Milli-Q, Millipore Corp., 80 Ashley Rd., Bedford, MA 01730).

2.8 Measurement of Abscisic Acid

A variety of approaches are currently employed for the measurement of ABA in plant tissues. High pressure liquid chromatography (HPLC) methods are widely used for the purification and analysis of ABA (Parry and Horgan, 1991; Sweetser and Vatvars, 1976; Walker-Simmons, 1987). However, GC based protocols are now the most commonly used methods for the purification of ABA, prior to quantitation on a variety of detectors such as UV, e^- capture, flame ionisation or mass spectrometry (Parry and Horgan, 1991; Lenton *et al.*, 1971; Quarrie *et al.*, 1988; Vernieri *et al.*, 1989; Ross *et al.*, 1987; Hubick and Reid 1980; Leroux *et al.*, 1985), the most sensitive of which can detect 1 pg of ABA (Seeley and Powell, 1970). While physicochemical methods are extensively used, these techniques have disadvantages. Samples in most cases need to be highly purified, requiring solvent phase partitioning and concentration steps, and

often one or more chromatographic steps before a reliable determination is possible (Weiler, 1979). Numerous steps in a method can affect the recovery of ABA, requiring the use of radio-labelled internal standards (Parry and Horgan, 1991), and may also be time consuming.

The specificity of monoclonal antibodies (MAb) raised against ABA has allowed the development of radioimmunoassay (RIA) (Mertens *et al.*, 1983; Quarrie *et al.*, 1988; Vernieri *et al.*, 1989), and fluoroimmunoassay (Banowetz, 1989) which utilise MAb to measure ABA in either purified samples or crude plant extracts. Enzyme linked immunosorbent assay (ELISA) protocols based on a direct binding of ABA to excess immobilised antibody have been widely used (Daie and Wyse, 1982; Weiler, 1982; Philosoph-Hadas *et al.*, 1983; Leroux *et al.* 1985; Harris *et al.*, 1988), and a commercial assay kit utilising this method is available (Phytodetek-ABA from Idetek, 1057 Sneath Lane, San Bruno, CA90466). Indirect ELISA methods that are more conservative with antibody than the direct ELISA or RIA have also been developed (Walker-Simmons and Sesing, 1987; Ross *et al.*, 1987), and can quantitate 5 pg of ABA in as little as 10 mg of plant tissue, with minimal extract preparation (Walker-Simmons, 1987).

MAb to isomerically pure (+)-*cis-trans*-ABA coupled to bovine serum albumin (BSA) through the C4' position have been obtained by several groups (Mertens *et al.*, 1983; Quarrie *et al.*, 1988; Vernieri *et al.*, 1989), and are now commercially available (Idetek). MAb raised to (+)-*cis-trans*-ABA-4'-BSA conjugate have less than 0.1% cross-reactivity with (-)-ABA, *trans-trans*-ABA, methyl- and glucosyl- ABA esters, the ABA catabolites phaseic acid and dihydrophaseic acid (Walker-Simmons *et al.*, 1991) or the metabolic precursor xanthoxin (Phytodetek-ABA technical brochure).

The procedure used in this dissertation was a modification of the indirect ELISA described by Walker-Simmons (1987), and used MAb from Idetek. The ELISA component ABA-4'-BSA conjugate was not commercially available, and was synthesised as described in section 2.8.1. Several validation experiments, including an independent physico-chemical validation, were carried out as described in sections 2.8.4 to 2.8.7.

2.8.1 Synthesis of ABA-4'-BSA Conjugate

The ABA-4'-BSA conjugate was synthesised using a method based on the three step procedure of Weiler (1979). The most technically demanding of the three steps, the preparation of tyrosylhydrazone substituted ABA, was carried out with the help of Mr Simon Fielder, HortResearch, Fitzherbert West, Palmerston North.

2.8.1.1 Preparation of *p*-Aminohippuric Acid Substituted BSA

A solution of 1 g of *p*-aminohippuric acid (Sigma Chemical Co., St. Louis, USA) and 1 g of BSA (Fraction V, Sigma) in 200 mL of water was prepared, and the pH adjusted to 8 with 1 M NaOH. To this, 1 g of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC, Sigma) was added and the pH adjusted to 6.4 with 0.25 M HCl. After stirring for 6 h at room temperature in the dark, another 0.5 g of EDC was added and the reaction was continued overnight. The product was separated from unreacted reagents by dialysis against three changes of water, and then lyophilised.

2.8.1.2 Preparation of Tyrosylhydrazone Substituted ABA

(±)ABA (45 mg; Sigma) and 150 mg of tyrosine hydrazide (Sigma) were dissolved in 10 mL of methanol and 100 µL of glacial acetic acid. The flask was sealed under nitrogen and the reaction allowed to proceed for eight days. Reaction progress was monitored by thin layer chromatography (TLC) on silica plates, with a solvent system of dichloromethane, methanol and acetic acid (80:20:1). Visualisation of ABA was made under UV light or with acid-vanillin spray reagent [0.5% (v/v) H₂SO₄ and 3% (w/v) vanillin in absolute ethanol] followed by heating to 120 °C. Tyrosine hydrazide and reaction products were detected with ninhydrin spray reagent [3% (w/v) ninhydrin and 3% (v/v) glacial acetic acid in *n*-butanol] followed by heating to 110 °C. The reaction yielded two products, distinguishable on the basis of *R_f* (Table 2.2). The major product [(±)ABA-4'-tyrosyl hydrazone] was separated from unreacted tyrosine hydrazide and a minor by-product using preparative TLC.

Table 2.2: *R_f* values of reactants and products.

Compound	<i>R_f</i>
(±)ABA	0.7
Tyrosine hydrazide	0.1
Major product: (±)ABA-4'-tyrosyl hydrazone	0.3
Minor product: identity unknown	0.2

2.8.1.3 Coupling of ABA-4'-tyrosylhydrazone to *p*-Aminohippuric Acid Substituted BSA.

Substituted BSA (64 mg) was suspended in 5 mL of water, and the pH was adjusted to 1.2 with HCl. An ice-salt bath cooled this solution to -9 °C, and 60 mg sodium nitrite dissolved in 0.5 mL of water was added. This solution was then added dropwise to a chilled solution of 13 mg of (\pm)ABA-4'-tyrosyl hydrazone in 0.5 mL methanol and 10 mL borate buffer (1M, pH 9.0). After 30 min the product was separated from unreacted reagents by dialysis against water (4 days, with daily changes). The product (>13 mL) was divided into 1.0 mL aliquots for storage at -20 °C.

2.8.2 Extraction of ABA from Plant Material

Aliquots (100 mg) of frozen, powdered leaf tissue were weighed into 1.5 mL centrifuge tubes, and extracted in 10% (v/v) PBSalt (50 mM phosphate buffer, pH 7.4, containing 250 mM NaCl) at the ratio of 0.5 mL of buffer per 100 mg tissue. Extracts were mixed overnight at 4 °C and then centrifuged for 10 min at 12000 xg. The supernatant was transferred to fresh 1.5 mL centrifuge tubes. For ELISA, two or three dilutions (usually 1:2, 1:5, and 1:10, occasionally 1:20 or 1:40) of each sample were made up to 120 μ L in PBSalt. Samples were extracted and assayed in duplicate.

2.8.3 ELISA Protocol

2.8.3.1 Monoclonal Antibody

Mouse MAb to *cis, trans* (+)ABA (2 mg; Idetek) were dissolved in 2.0 mL of PBSalt and aliquots were stored at -20°C. Before ELISA an aliquot was thawed, and diluted 1:300 in PBSalt containing 0.05% (w/v) BSA.

2.8.3.2 Method

Aliquots of plant extracts (120 μ L) and MAb dilution (120 μ L; the optimal working dilution of MAb was determined in section 2.8.5) were added to 1.5 mL centrifuge tubes, spun briefly to mix and then incubated overnight at 4 °C. A 100 μ L aliquot of ABA-4'-BSA conjugate [diluted 1:50 in 50 mM sodium carbonate buffer, pH 9.7. (The optimal working dilution of conjugate was determined in section 2.8.4)] was added to

each well of a microtitre plate (Maxisorp F16, 96 well module plates, Nunc, Denmark). Plates were covered with plastic film (which was done for each incubation step that follows), and then incubated at 4 °C overnight. After incubation, the contents of the wells were discarded, and the wells rinsed three times with washing buffer [PBSalt with 0.5 g/L Polyoxyethylenesorbitan monolaurate (Tween-20; Sigma)]. The third rinse was allowed to stand for 10 min before being discarded. A 240 µL aliquot of blocking solution [0.5% (w/v) low fat milk powder (Anchor; Hawkes Bay Milk Corporation, Hastings, New Zealand) in PBSalt] was added to each well, and the plates were incubated at 37 °C for 1 h.

After blocking the wells were again rinsed as above. Aliquots (100 µL) of the sample incubated with MAb were added to duplicate wells. The plates were then incubated at 26 °C for 2.5 h, and were rinsed. A 100 µL aliquot of secondary antibody (alkaline phosphatase conjugated to goat anti-mouse IgG (Sigma), diluted 1:2000 in PBSalt) was then added to each well, and the plates were incubated at 26 °C for 2 h. The plates were then rinsed, and enzyme substrate (100 µL of *p*-nitrophenyl-phosphate (1 mg/mL) in coating buffer containing 1 mM MgCl₂) was added to each well. Plates were covered with aluminium foil, and incubated at 26 °C until the absorbance at 405 nm (A_{405}) of the control sample (containing no ABA) was *ca.* 0.7. The absorbance of each well was measured at 405 nm on a Bio-Rad model 3550 Microplate Reader (Bio-Rad, 2000 Alfred Nobel Drive, Hercules, CA 94547).

A standard curve consisting of serial dilutions from 100 to 10,000 pg/mL (equivalent to 5-500 pg per well) of (\pm)*cis, trans* ABA (Sigma) in PBSalt was included on each plate. A polynomial regression was fitted to each set of standards, using commercial computer software (SlideWrite™, Advanced Graphics Software, Carlsbad, CA 92008-4404). The ABA concentration in each sample was calculated from the regression coefficients using computer software written especially for the purpose (Appendix 2.1). The results for the three dilutions of each sample were averaged.

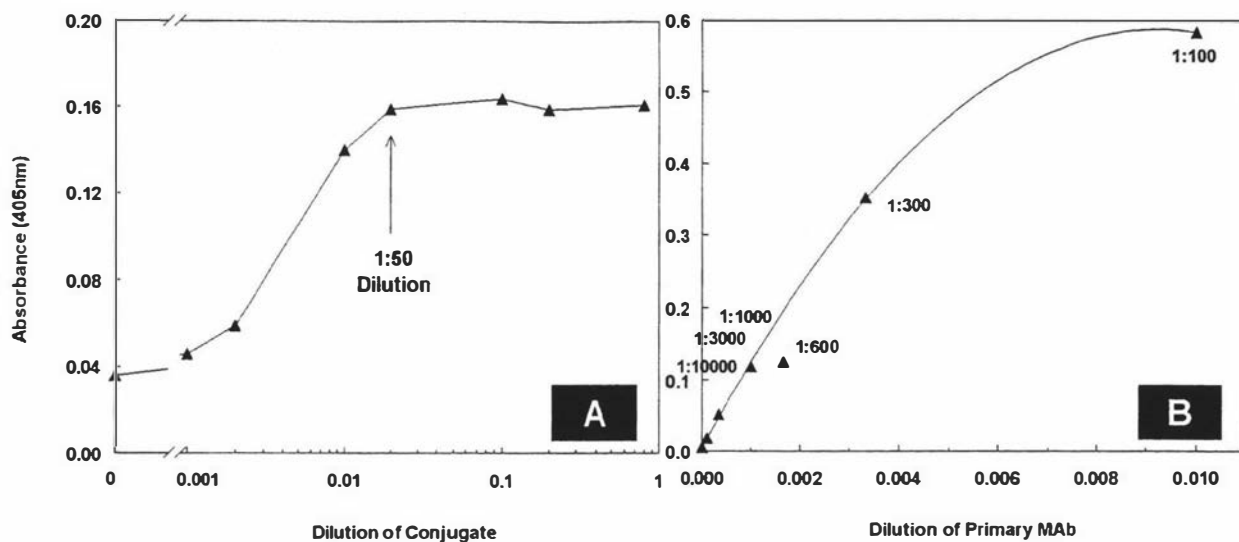


Figure 2.3: A: Determination of optimal concentration of ABA-4'-BSA conjugate. B: The dilution effect of the primary antibody on ELISA absorbance (405 nm). The outlying point at 1:600 was not included in the fitted curve. Data were single determinations.

2.8.4 Determination of the Optimal Working Dilution of ABA-4'-BSA Conjugate

Because both the efficiency of the coupling procedure and the final concentration of the ABA-4'-BSA conjugate were not known, the dilution of ABA-4'-BSA conjugate to be used for the ELISA procedure had to be determined. The ABA-4'-BSA conjugate was prepared in 50 mM sodium carbonate buffer, pH 9.7, in dilutions ranging from 4:5 to 1:1000, and was coated onto duplicate wells overnight. The wells were blocked with reconstituted milk powder, rinsed with washing buffer as described in section 2.8.3.2, and 100 μ L antibody (1:600 dilution) was added to each well and incubated at 26 $^{\circ}$ C for 2.5 h. The plate was then developed using the ABA ELISA protocol above. The wells were saturated at a dilution of 1:50 (Figure 2.3A). Hence this dilution of conjugate was chosen for use in the ABA ELISA protocol.

2.8.5 Verification that the MAb Dilution was Non-Saturating

Aliquots (100 μ L) of a range of dilutions of the primary antibody (from 1:100 to 1:10,000) were allowed to bind (2 h at 26 $^{\circ}$ C) to microtitre plate wells which had previously been coated with ABA-4'-BSA conjugate and blocked with re-constituted low fat milk powder as described in section 2.8.3.2. The microtitre plate was then developed following the protocol for the ABA ELISA above.

The absorbance was dependent on the concentration of primary antibody used. As can

be seen from Figure 2.3B (excluding the outlier at 1:600 dilution), the increase in absorbance was approximately linear from 1:10,000 to 1:300 dilution of primary antibody. At higher MAb concentrations, the slope of the graph declined. Hence a working dilution of the primary antibody of 1:600, obtained by mixing 1:300 dilutions of MAb stock solution with an equal volume of sample extract was considered satisfactory for use with the ELISA procedure.

2.8.6 Reproducibility of the ELISA Protocol

The ELISA gave reproducible results. When the same extract was assayed several times, and when the same sample was extracted and assayed several times, a good degree of reproducibility was obtained (Table 2.3).

The method was free from interference arising from the plant material. When ABA standard was added to plant extract, and then the ABA measured in the extracts alone and with ABA added, the method was sensitive enough to measure the exogenously added ABA (Table 2.3). Further, the addition of a constant amount of plant extract (at a dilution of 1:20 in PBSalt) to increasing concentrations of ABA standards shifted the standard curve, but did not change the linearity of the immunoassay (Figure 2.4).

Table 2.3: Reproducibility of the ABA immunoassay. All values were ng/g FW.

	Multiple assay of one extract	Multiple extractions of one sample.	Multiple assay of one extract, with ABA standard added ¹ .
Plant material:	<i>F. arundinacea</i> , water-deficit stressed	<i>F. arundinacea</i> ² water-sufficient	<i>F. arundinacea</i> water-sufficient
Replicates:	146 150 134 139 <u>138</u>	15.6 10.1 17.0 17.7 <u>21.0</u>	13.0 17.5 13.6 17.5 —
Mean	141	16.3	15.4
s.e.	2.8	1.8	1.2
¹ The extract had 587 pg of (\pm)ABA added to an endogenous level of ca 420 pg ABA, before analysis of ABA concentration by ELISA. This quantity of ABA added was numerically subtracted from the result, and the remainder was expressed on a FW basis.			
² The same tissue sample was used for the assays in the middle and right columns.			

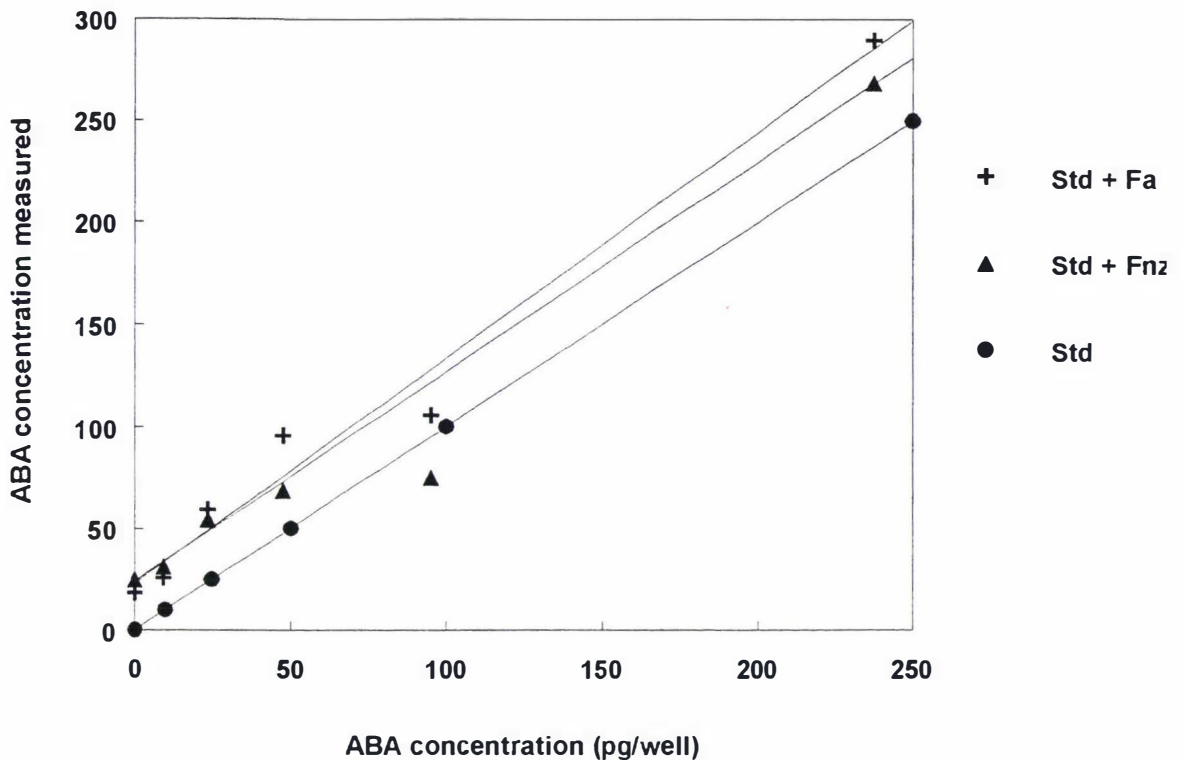


Figure 2.4: Concentration curve for ABA standards, and for ABA standards with *Festuca spp.* extract (1:20 dilution) added to each sample. Key: Std : Standard curve only; Std+Fa: Standard curve with *F. arundinacea* extract added; Std+Fnz: Standard curve with *F. novae-zealandiae* extract added. Data were single determinations.

2.8.7 Independent Validation of the ELISA Protocol

The ELISA method used with *Festuca spp.* was based that described by Walker-Simmons (1987), for the analysis of ABA in embryos of *T. aestivum*. To address the possibility that the procedure may suffer from interference when used with novel tissues or species, ELISA results were compared with a physico-chemical analytical procedure on identical grass samples. The independent estimation of ABA concentration of *Festuca spp.*, was carried out by Dr. John Lenton and Dr. Steve Croker, AFRC Institute of Arable Crops Research, Long Ashton Research Station, Long Ashton UK, in June 1994.

Two samples of ML from *F. arundinacea*, and one from *F. novae-zealandiae*, were frozen in liquid nitrogen and powdered using a mortar and pestle. The powder was then lyophilised and stored at room temperature. Triplicate aliquots of the three samples were assayed by ELISA, and the results were presented in Table 2.4. The same samples were then sent to Dr Lenton's laboratory for ABA quantitation using the following method based on Whitford and Croker (1991). The ELISA results were used as a guide to take aliquots of the samples with approximately the same amount of ABA and a

single determination of each sample was carried out. Each aliquot was extracted in water (Table 2.5) by stirring overnight at 4 °C, and internal standards of 25 ng $^2\text{H}_3$ -ABA and 50 μL ^3H -ABA (40 kdpm) were then added to each sample. Extracts were filtered, followed by a water wash, and the filtrate adjusted to pH 3 with 2 M HCl. The filtrates were then loaded onto a C_{18} Sep-Pak cartridge (Millipore, Watford, UK). The cartridge was washed twice with 2 mM acetic acid in 1% (v/v) methanol and eluted with 5 mL of 50% methanol. The eluate was reduced in volume under vacuum and then applied to reverse phase HPLC (C_{18}). The fractions collected containing the radioactive standard (tritiated ABA) were combined, methylated using diazomethane, and the ABA concentration was quantitated using GC-MS (Whitford and Croker, 1991). The results were compared to the original estimation obtained using ELISA (Table 2.6).

The ELISA estimations of ABA concentration in the grass samples were in good agreement with the physico-chemical method (Dr. Lenton, pers. comm.). Of the three tissue samples sent to England for validation, one (sample FAC) had an ABA concentration within the standard deviation of the ELISA result. The GC-MS results of the remaining two samples were slightly lower than the ELISA measurement. However, the ELISA method was shown to be able to discriminate well between the ABA levels in the three samples.

Table 2.4: Estimation of ABA in the three *Festuca spp.* samples by ELISA.

sample	Moisture Loss upon drying	Individual estimates			Mean Estimate ng/gFDW ¹ , \pm std. dev.	ABA Content ng/g FW
		1	2	3		
FNZ	70%	1,370	1,560	1,460	1460 \pm 77	439 \pm 23
FAD	75.4%	485	507	433	475 \pm 31	117 \pm 8
FAC	91%	245	140	261	215 \pm 54	17 \pm 4

¹ Key: FDW: Freeze Dry Weight; FNZ: *F. novae-zealandiae*; FAD: droughted *F. arundinacea*; FAC: control (non-droughted) *F. arundinacea*.

Table 2.5: Summary of extraction procedure used by Dr. John Lenton.

Sample	ABA Content by ELISA ¹ ng/mg	Tissue Aliquot taken mg	Estimated ABA content ng total	Extracted in (mL) of water	GC-MS Result ng total
FNZ ²	1.5	25	36.6	1	28
FAD	0.5	75	35.6	3	30
FAC	0.2	150	33.5	6	31

¹ Data from Table 2.4
² See Table 2.4 for Key.

Table 2.6: Comparison of ELISA and GC-MS estimates of the ABA concentration of three *Festuca spp* samples.

sample	ELISA estimate ng/g FDW \pm std. dev.	GC-MS estimate ng/g FDW
FNZ ¹	1460 \pm 77	1,120
FAD	475 \pm 31	396
FAC	215 \pm 54	206

¹ See Table 2.4 for Key.

2.8.8 Discussion on the ELISA Method

The protocol used for the synthesis of a covalent link between ABA and BSA through position C4' was somewhat inefficient, with large quantities of protein precipitating after coupling with *p*-aminohippuric acid, and again during coupling to tyrosine hydrazone derivatised ABA. Hence, the product yield of soluble protein was probably low. This observation was consistent with Quarrie and Galfre (1985). These authors, as well as Ross *et al.*, (1987) have described other methods for linking 4'ABA to proteins. Although the reaction yield was not determined, the working concentration of the product was evaluated directly, and at a one to fifty dilution there was sufficient yield of soluble ABA-4'-BSA to carry out a large number (*ca.* 70 plates) of ABA assays.

The addition of BSA to the Idetek MAb solution (Walker-Simmons (1987) stored the MAb solution in the presence of a similar quantity of BSA) was necessary for the estimation of ABA in samples containing plant extracts. Without added BSA, absorbances larger than those generated by the standard curve were routinely observed, making the estimation of ABA impossible (data not presented). The exact nature of this interference was not known.

There is good evidence that the selectivity of this assay was high. The commercial suppliers of the MAb used (MAb 15-I-C5), claim that this MAb has no cross reactivity with ABA isomers or metabolites. This claim was supported by Walker-Simmons *et al.* (1991). The selectivity of the assay was also supported by the results of the GC-MS validation (Table 2.5).

The standard curve was sigmoidal (Figure 2.5), and no attempt to linearise this curve was made. Instead, a polynomial equation was fitted to the data using SlideWrite™, which gave an R value that was usually greater than 0.99. The maximum slope obtained was between 5 and 1000 pg ABA, which compares favourably with the sensitivity of 5 to 250 pg reported by Walker-Simmons and Sesing (1986).

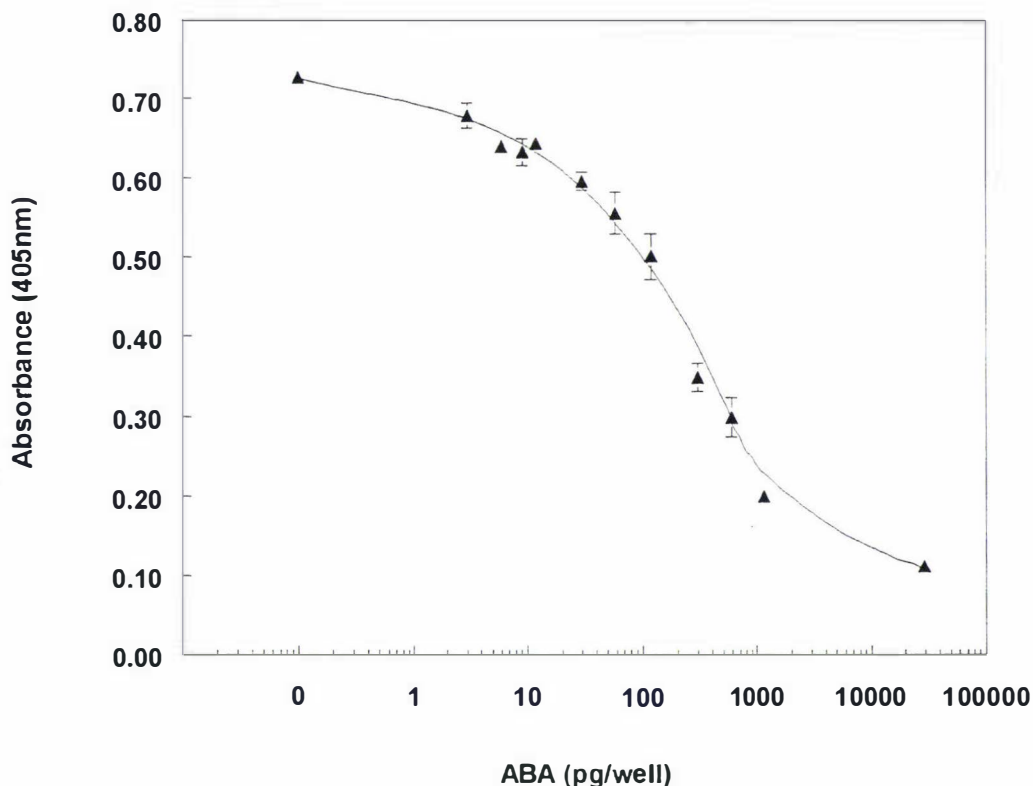


Figure 2.5: Calibration Curve for ABA ELISA. Data were means \pm s.e., n=3.

2.9 Measurement of Glycine-betaine

Several methods for the estimation of glycine-betaine levels in plant material have been reported. A common procedure was acid precipitation of quaternary ammonium compounds (QAC's) as periodide complexes, followed by spectrophotometric quantitation (Ladyman *et al.*, 1983). The separation of individual QAC periodides for spectroscopy is difficult, and not suited to the analysis of large numbers of samples (Grieve and Grattan 1983). This separation was overcome by the use of QAC-periodate extraction followed by nuclear magnetic resonance (NMR) quantitation (Arakawa *et al.*, 1990). Mass Spectroscopy has been used to quantitate glycine-betaine both as the underiv-

atised compound and also as the n-butyl ester (Wood *et al.*, 1991; Lerma *et al.*, 1990). Glycine-betaine has been measured by NMR in *H. vulgare* (Jones *et al.*, 1986) and *T. aestivum* (Naidu *et al.* 1991), and by GC in 22 graminaceous species (Hitz and Hanson 1980). Glycine-betaine has also been identified and measured by thin layer chromatography- densiometry (Koheil *et al.*, 1992) and as a reineckate complex (Gorham 1984).

All of these methods utilise ion-exchange columns to purify aqueous or methanol-chloroform-water extracted samples. Selectivity of the assays require lengthy purification of samples which render these methods inappropriate for routine analysis of glycine-betaine.

An improved method for the quantitation of betaines has recently been reported (Lever *et al.* 1992), and has been adapted for use in this dissertation. Developed initially for the measurement of glycine-betaine, proline-betaine and carnitine in medical samples (blood sera, urine and organ tissue extracts), this method is highly selective, and requires no lengthy extraction nor purification of betaines prior to measurement by analytical HPLC (Lever *et al.* 1992).

2.9.1 Method

2.9.1.1 Reagents

Glycine-betaine (Sigma), magnesium oxide and triethanolamine (Sigma) were a gift from Dr. Michael Lever, Department of Clinical Biochemistry and Infectious Diseases, Christchurch Hospital, Christchurch, New Zealand. HPLC mobile phase solvents (HPLC grade isopropanol (BDH) and water) were filtered⁶ before use. Two batches of the derivatising reagent (4-bromophenacyl triflate) were used, with no apparent differences in response; these batches were donated by Dr. Michael Lever, and by Dr. Alan Happer (Department of Chemistry, University of Canterbury, Christchurch, N.Z.), who synthesised both batches by the method of Ingalls *et al.* (1984). Fresh solutions of derivatising reagent (34.5 mg/mL in acetonitrile) were prepared daily.

⁶ A 0.45 μm filter was used for the aqueous buffer; 0.22 μm filter for isopropanol. The different filters used were to ensure solvent/filter compatibility.

2.9.1.1.1 Drying Reagent

A mixture of 5% (w/w) silver (I) orthophosphate in anhydrous disodium hydrogen phosphate was prepared and was stored in the dark. Silver (I) phosphate is a satisfactory alternative to silver (I) oxide used in the method of Lever *et al.* (1992; Dr. Lever, pers. comm.). Silver (I) phosphate was prepared by mixing 1M solutions of potassium orthophosphate and silver nitrate. The yellow precipitate was collected with #1 filter paper (Whatman Ltd., Springfield mill, Kent ME142LE, England), and washed three times with water on a Buchner funnel, and dried overnight at 60 °C. All steps were carried out in darkness to prevent photo-oxidation of the silver (I) ions.

2.9.1.2 Extraction and Derivatisation of Betaines

Aliquots (10-20 mg) of powdered plant tissue were weighed into pre-weighed 1.5 mL centrifuge tubes; samples were assayed in duplicate. About 50 mg of drying reagent was added to each tube followed by 1.0 mL of extraction solvent [10% (v/v) methanol and 90% (v/v) anhydrous acetonitrile]. Samples were mixed for 10 min and centrifuged at 12,000 $\times g$ for 3 min. An aliquot of supernatant (200 μL) was transferred to a 1.5 mL centrifuge tube containing 20 μL of a 10% suspension of magnesium oxide in water. After mixing, 50 μL of derivatising reagent was added and the mixing continued. After 5 min the tubes were centrifuged for 7 min at 12,000 $\times g$, and *ca.* 150 μL of the supernatant was transferred to a fresh vial ready for HPLC analysis.

Putative glycine-betaine peaks in each extract were compared quantitatively with glycine-betaine standards which were processed with each batch of samples. Standards were prepared by diluting 10 μL to 50 μL of standard (5 mM or 20 mM glycine-betaine in water) into 1.0 mL acetonitrile, and then derivatised in preparation for HPLC as described above.

2.9.1.3 High Performance Liquid Chromatography

The HPLC equipment consisted of an LKB 2249 gradient pump (Pharmacia LKB Biotechnology Ltd., Björksgatan 30, Uppsala, Sweden) and a LC-UV detector (Pye-Unicam Ltd., Cambridge, England), set at 260 nm, with output to either a HP-3393A integrator (Hewlett-Packard, Route 41, Avondale, Pennsylvania 19311) or to a C-R1B chromatopac integrator (Shimadzu Corp., Analytical Instruments Plant, Kyoto 604,

Japan). The column used was a 100 mm x 4.6 mm (5 μ m silica) Brownlee cartridge (Applied Biosystems Ltd., Foster City, CA 94404) with a 15 mm guard column and a 2 μ m prefilter column. The columns were stored in hexane, and were rinsed before use with successive 0 to 100% (v/v) gradients of miscible solvents, from hexane to dichloromethane to acetonitrile to isopropanol to aqueous buffer.

The eluting solvent was isocratic, consisting of triethanolamine and citric acid buffer in an isopropanol : water mixture. The HPLC apparatus was used to measure glycine-betaine in plant extracts on three occasions during the course of this work, and the exact composition of the eluting solvent (Table 2.7) differed between these occasions (discussed below). The eluting solvent was degassed with helium with an LKB 2156 (Pharmacia) solvent conditioner. Samples were centrifuged at 12,000 \times g to clarify before quantitation. Aliquots of sample (5 to 10 μ L) were manually injected (no injection loop was used) and eluted with the solvent phase. Separations were performed at room temperature.

Table 2.7: Composition of HPLC mobile phase used during the three HPLC runs.

Set of assays	Samples Assayed	Citric Acid (mM)	Triethanol-amine (mM)	Isopropanol : water	Flow Rate (mL/min)
first	FA ¹ , Chapter three. FNZ, experiment I, Chapter four.	1.6	4	85 : 15	1.5
second	FNZ, experiment II, Chapter four.	3.2	8	85 : 15	1.5
third	FNZ, Chapter Five.	4.8	12	80 : 20	1

¹ FA: *F. arundinacea*; FNZ: *F. novae-zealandiae*

2.9.2 Discussion

2.9.2.1 Chromatography Conditions

When using the solvent system described by Lever *et al.* (1992), comprised of isopropanol/ water (3:1), derivatised glycine-betaine co-eluted with an interfering compound that was assumed to have arisen from a solvent impurity (data not shown). By reducing the water content of the mobile phase to 15%, while keeping the ethanolamine and citric acid concentrations the same, separation was achieved between the contaminant and glycine-betaine (Figure 2.7A). Lever *et al.* (1992) suggest that the

interfering compound could be due to triethylamine contamination of acetonitrile. This was confirmed by running a chromatogram on derivatised acetonitrile, which displayed a single peak which co-eluted with the contaminant (Figure 2.7B). No attempt to further identify the contaminant was made.

As mentioned above, a total of three sets of assays were conducted using the method described above. During the first set of assays, glycine-betaine was quantified in both *F. arundinacea* and *F. novae-zealandiae* leaf tissues. Representative chromatograms from these samples are presented (Figure 2.7C, D).

A new batch of anhydrous acetonitrile was used for the extraction and derivatisation of betaines during the second set of assays, and contained about 75% less contaminant. Also, following a suggestion from Lever *et al.* (1992), the concentration of citrate/triethanolamine buffer was increased, which shortened the retention times for the glycine-betaine derivative and solvent contaminant peaks, while still maintaining separation. Retention times for glycine-betaine and the solvent contaminant under the different eluant and buffer conditions are presented in Table 2.8.

The solvent flow rate was reduced from 1.5 to 1.0 mL/min for the third set of assays, due to increased pump pressure originating from an unknown source. This flow rate change had the effect of increasing the retention time of derivatised glycine-betaine. Also, HPLC grade acetonitrile was used for the extraction and derivatisation of betaines, and no detectable contaminant was found after derivatisation with triflate (Figure 2.7F). For these two reasons, both the buffer concentration and the solvent composition were changed slightly to induce a shorter retention time for glycine-betaine derivative (following a suggestion from Lever *et al.*, 1992), while still retaining separation of analyte and the non-cationic material following the solvent front.

Derivatised glycine-betaine was the only compound found in samples of *F. novae-zealandiae* assayed during the third set of assays (Figure 2.7I). The identification of this peak was confirmed by comparison of retention time with standard glycine-betaine (Figure 2.7G); and by spiking samples of *F. novae-zealandiae* extract with authentic glycine-betaine, which yielded a single peak (Figure 2.7H).

Table 2.8: Retention times of chromatography peaks.

Set of assays ¹	Retention Time (decimal min.)		
	Glycine-Betaine	Acetonitrile Contaminant	Minor betaines ²
first	9.3	7.5	5 to 6
second	6.3	5.2	ND
third	4.5	ND ³	ND

¹ See Table 2.7 for chromatography conditions for each set of assays.
² Found in significant quantities in *F. arundinacea* extracts only.
³ ND: Not Detected.

2.9.2.2 Assay Performance

The assay calibration curve was linear, up to 1 mM glycine-betaine (Figure 2.6). All glycine-betaine concentrations detected in the plant samples were within this range. Lever *et al.* (1992) report linearity from 5 μM to 10 mM under similar conditions. When the same extract was assayed several times, the assay was reproducible (Table 2.9). The relative standard deviation of 4% to 9% is comparable to that reported by Lever *et al.* (1992). Sample requirements were small, with 10-20 mg FW of leaf tissue routinely used; glycine-betaine was measured in as little as 2.5 mg of fresh weight leaf tissue.

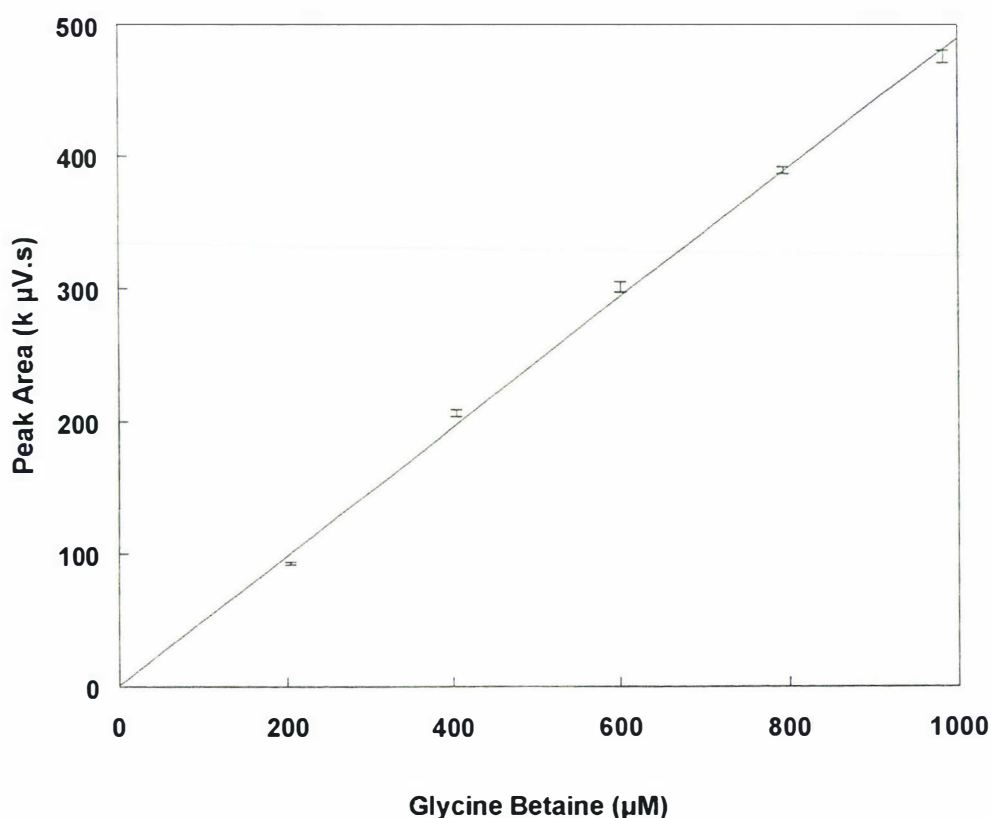
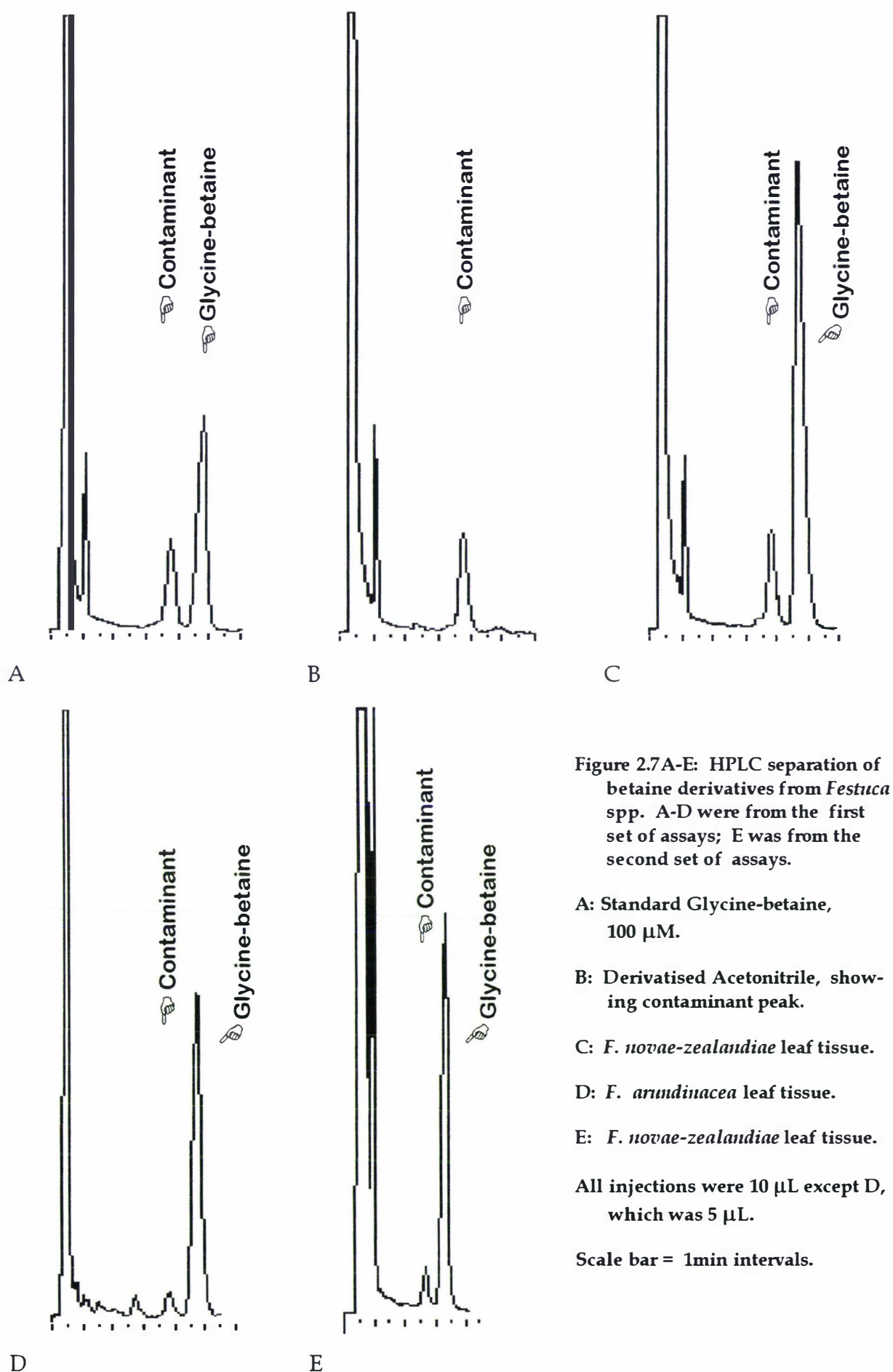


Figure 2.6: Calibration curve for the glycine-betaine assay. Data were means \pm std. dev., $n=3$.



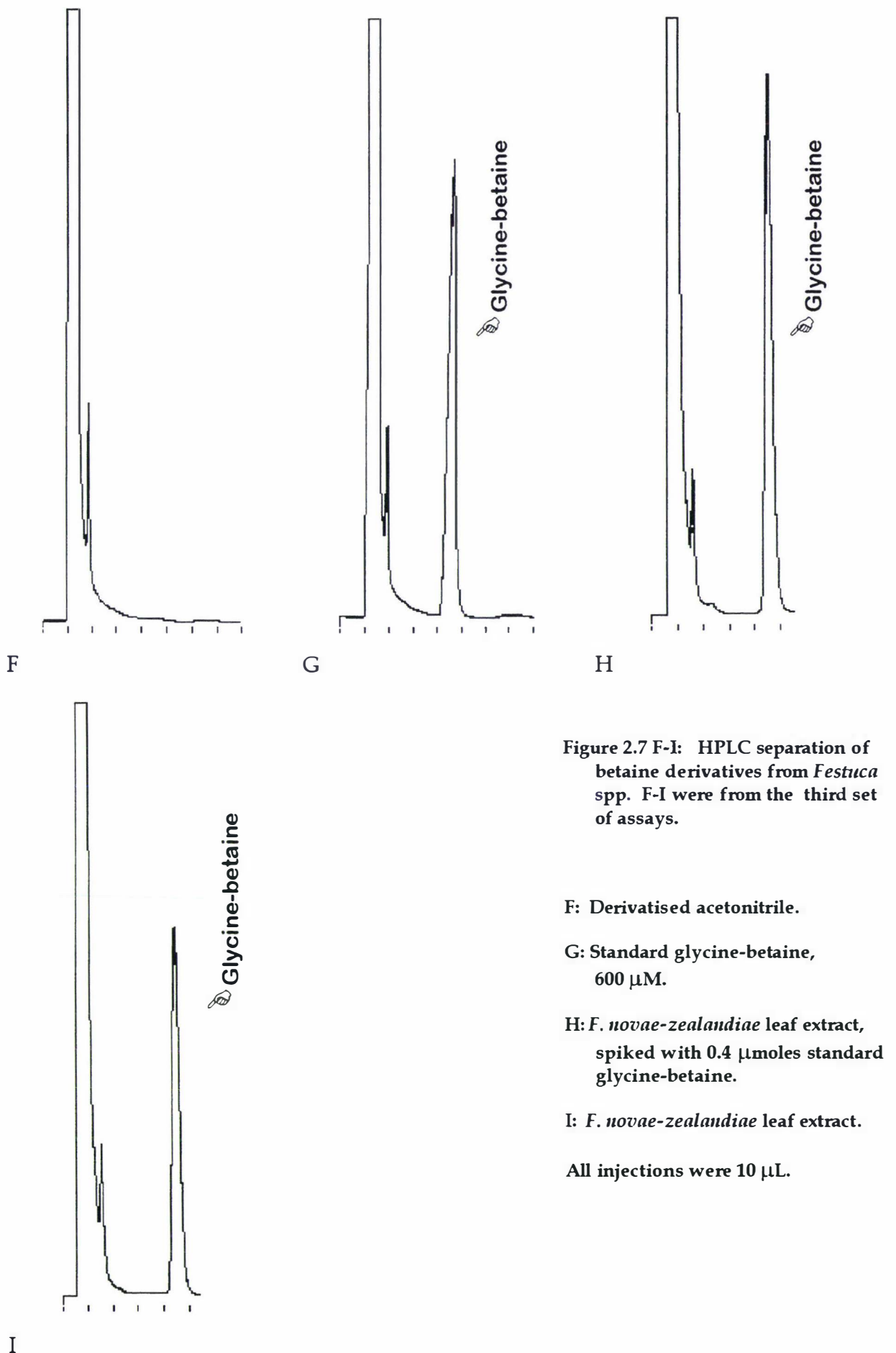


Figure 2.7 F-I: HPLC separation of betaine derivatives from *Festuca* spp. F-I were from the third set of assays.

F: Derivatised acetonitrile.

G: Standard glycine-betaine, 600 μM .

H: *F. novae-zealandiae* leaf extract, spiked with 0.4 μmoles standard glycine-betaine.

I: *F. novae-zealandiae* leaf extract.

All injections were 10 μL .

Table 2.9: Reproducibility of the Glycine-betaine Assay. All values were $\mu\text{mol/g}$ FDW.

Assay treatments ¹ :	Multiple assay of one extract	Multiple extractions of one sample.	Multiple assay of one extract, with standard added ² .
Replicates:	13.8	14.0	19.3
	13.5	13.0	22.9
	13.8	12.0	21.0
	14.2	11.0	15.3
	<u>15.1</u>	<u>11.9</u>	<u>19.1</u>
Average:	14.1	12.4	19.5
Std. dev.:	0.6	1.0	2.5
RSD (%):	3.9	8.3	13.0

¹ The same batch of freeze-dried *F. novae-zealandiae* lamina was used for all three assay treatments.

² Each assay had 0.43 μmoles of standard glycine-betaine added to the extract which contained an endogenous level of *ca* 0.2 μmoles glycine-betaine, before analysis of glycine-betaine concentration. The quantity of glycine-betaine added was numerically subtracted from the result, and the remainder was expressed on a FDW basis.

2.10 Measurement of Proline

The method of Bates *et al.* (1973) has been used extensively by researchers when measuring the proline content of plants as part of environmental stress studies. The ninhydrin reaction yields a coloured product with all amino acids. However, the interference caused by other amino acids on the proline assay is minimal, due to the disproportionately large increase in proline levels over the levels of other amino acids in water-stressed plants (Singh *et al.*, 1973; Bates *et al.*, 1973). Also, the imino acid proline yields a product with a different absorbance maximum from the α -amino acids product with ninhydrin (Magne and Larher 1992). However, Magne and Larher (1992) revealed that the method used by Bates *et al.* (1973) suffered from interference by many non-nitrogenous compounds including sucrose, monosaccharides and sugar alcohols. These authors thus introduced several modifications to the assay to avoid carbohydrate interference. This modified method was used in this dissertation from August 1994. Proline assays completed before that date (including the experimental dry-down of *F. arundinacea* and *F. novae-zealandiae* reported in Chapters Three and Four) followed the method of Bates *et al.* (1973).

2.10.1 Method

2.10.1.1 Ninhydrin Reagent, as Described by Bates *et al.* (1973):

2.5% (w/v) ninhydrin (Sigma) in 60% (v/v) glacial acetic acid and 40% (v/v) 6 M phosphoric acid. Fresh reagent was prepared daily.

2.10.1.2 Ninhydrin Reagent, as Described by Magne and Larher (1992):

1% (w/v) ninhydrin in 60% (v/v) glacial acetic acid. Fresh reagent was prepared daily.

2.10.1.3 Protocol

Duplicate aliquots (20-50 mg) of powdered tissue were weighed into 1.5 mL centrifuge tubes and suspended in 1.2 mL of 3% (w/v) sulphosalicylic acid to precipitate protein. Samples were vortex mixed and centrifuged at 12,000 $\times g$ for 7 min. The supernatant was then transferred to a fresh 1.5 mL centrifuge tube. An aliquot (usually 500 μL) of supernatant was made up to 1.0 mL with water. This dilution was reacted with either 1.0 mL of glacial acetic acid and 1 mL of ninhydrin reagent (as described by Bates *et al.*, 1973) or with 2.0 mL of ninhydrin reagent (as described by Magne and Larher, 1992) for 1 h at 98 $^{\circ}\text{C}$, and the reaction was stopped by cooling the tubes in an ice-water bath. The products were extracted in 2.0 mL of toluene by mixing on a vortex mixer for 15-20 sec. The toluene (upper) phase was separated by standing for 5 min, transferred to a glass cuvette, and read at 518 nm against a toluene blank. Sample absorbances were compared to those from a set of separately prepared proline standards (0 to 30 $\mu\text{g}/\text{mL}$) which were assayed alongside each batch of samples.

2.10.2 Discussion

Bates *et al.* (1973) reported this method for use with 0.5 g FW of plant tissue (*Glycine max* and *Sorghum bicolor*). By scaling down the volumes of reagents from the 15 mL test-tube method used by Bates *et al.* (1973) to 1.5 mL centrifuge tubes, the method has been adapted to work with *ca.* 20 mg FW grass tissue, which is more amenable to the smaller size of *F. novae-zealandiae* leaves.

For both methods the standard curve was linear up to a concentration of 30 $\mu\text{g}/\text{mL}$ proline (Figure 2.8), which was the same as that reported by both authors [Bates *et al.*, 1973; Magne and Larher, 1992). Good precision was obtained for both assay methods,

which was exemplified by the small s.e. bars on the standard curves (Figure 2.8). In addition, when the same sample was assayed several times, a good degree of reproducibility was obtained for the method described by Bates *et al.*, (1973; mean proline concentration was $15.6 \pm 0.06 \mu\text{g}/\text{mL} \pm \text{s.e.}$, $n = 7$).

Although the possibility of interference arising from the use of these methods on a novel species (*F. novae-zealandiae*) was not examined experimentally, the possibility of serious interference (from sources other than amino acids or sugars) from tissues of this species was likely to be low as the ninhydrin methods described above have been used on crude extracts from a wide variety of plant material, such as *Malus domestica* (apple) fruit (Magne and Larher, 1992), leaf discs of *Brassica napus* (Larher *et al.*, 1993), root nodules of *G. max* (Kohl *et al.*, 1991), roots of *Z. mays* (Ober and Sharp, 1994) and the leaves of many graminaceous species such as *H. vulgare* (Zuniga *et al.*, 1989), *Lolium perenne* (Perennial ryegrass; Thomas 1990), *O. sativa* (Dingkuhn *et al.*, 1991), *S. bicolor* (sorghum; Sivaramakrishnan *et al.*, 1988), *F. arundinacea* and *Agropyron smithii* (wheat-grass; Bokhari and Trent, 1985).

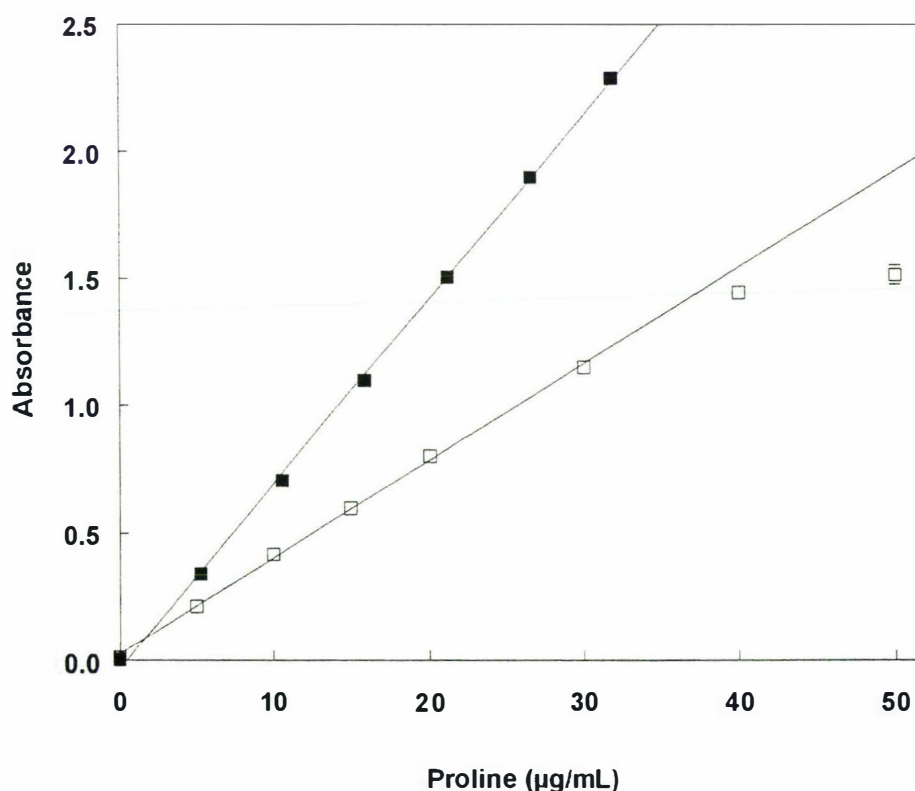


Figure 2.8: Proline assay calibration curves. The curves were obtained by using the methods described by Bates *et al.*, 1973 (closed symbols) and Magne and Larher, 1992 (open symbols). Data were means \pm s.e., $n=3$. Error bars not visible were smaller than symbols.

2.11 Protein Extraction

Aliquots (200 mg to 300 mg) of tissue that had been frozen and ground in liquid nitrogen were weighed into 1.5 mL centrifuge tubes. The tissue was extracted in a ratio of 1:3 fresh mass: extraction buffer (100 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl, 1 mM EDTA, 20 mM 2-mercaptoethanol) for 15 min on ice, with regular mixing. The tubes were then centrifuged at 12,000 $\times g$ for 10 min at 4 °C, the supernatant was transferred to a fresh tube and then centrifuged again for 8 min. Before storage at -20 °C, the extract was mixed with an equal volume of gel loading buffer [GLB: 50 mM Tris-HCl, pH 6.8, containing 10% (w/v) glycerol, 2.5% (w/v) sodium dodecyl sulphate (SDS) and 0.001% (w/v) Bromophenol Blue dye and 5% (v/v) 2-mercaptoethanol].

2.12 Protein Estimation

Protein concentrations were estimated by a microassay version of Bradford's method (Bradford 1976), using a commercially available Coomassie brilliant blue G-250 dye preparation (Bio-Rad). An aliquot of extract from section 2.11 (usually 10 μL) was diluted into 1.0 mL with water. To 200 μL of this dilution was added 600 μL of water and 200 μL of dye reagent. After mixing, the reaction was allowed to stand for 5 min, and the absorbance was measured at 595 nm. Absorbances were compared to a standard curve prepared in the same manner as above using up to 25 $\mu\text{g}/\text{mL}$ bovine serum albumin [BSA Fraction V, (Sigma), Figure 2.9].

BSA was selected as the reference standard protein, since the use of this protein is widely referenced in the literature. However, since albumin proteins yield a greater colour development than other proteins with the dye reagent (Bio-Rad products catalogue, 1995), the protein concentrations determined are liable to be underestimates of the actual protein contents of the samples. In any assay, the best protein to use as a standard is a purified sample of the protein being assayed. However, the protein extracts from section 2.11 contained many different plant proteins. Therefore the use of a purified preparation as an absolute standard was not possible and BSA standard was used for estimating the relative protein concentrations between samples.

The dye reagent is useful for measuring proteins with a relative molecular mass greater than 3000 to 5000, depending on the charge groups (Bio-Rad products catalogue, 1995). Some reagents are not compatible with the Bio-Rad dye preparation. However, the components of the protein extraction buffer (section 2.11) do not provide interference with this assay, at the concentrations employed (Bio-Rad products catalogue, 1995).

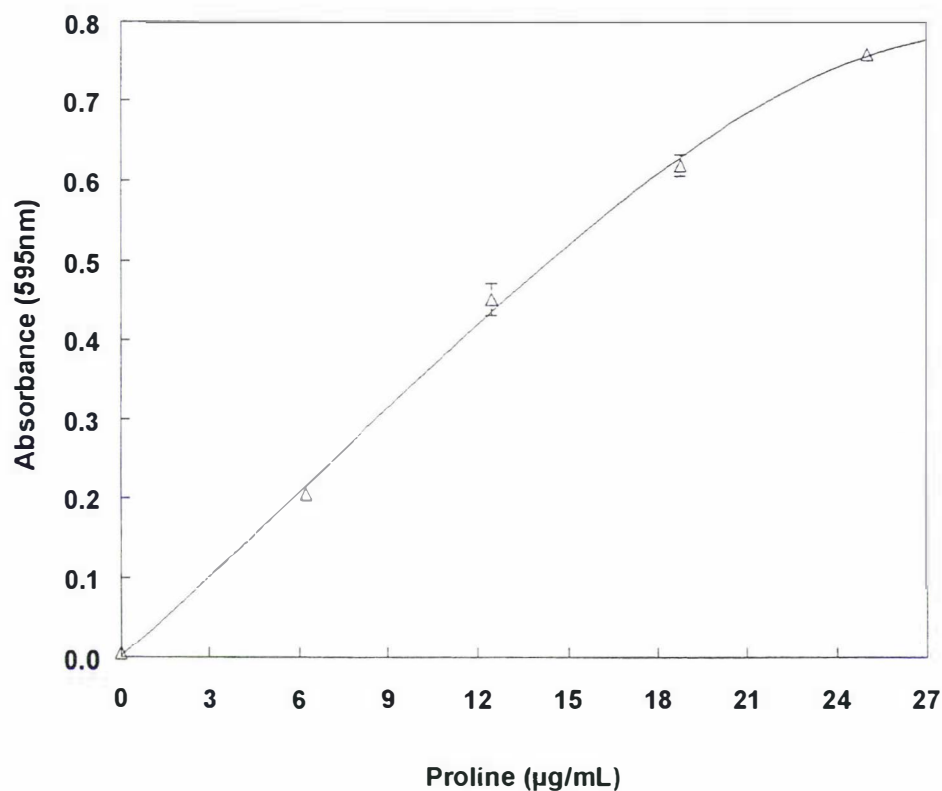


Figure 2.9: Standard curve for the Bio-Rad protein assay microassay procedure. Data were means \pm s.e., n=2.

Table 2.10: Composition of the heavy and light solutions used in the SDS-PAGE gradient gels.

	5% to 15% Gradient		10% to 20% Gradient	
	Heavy	Light	Heavy	Light
Sucrose:	4.5 g	none	4.5 g	none
Separating gel buffer¹:	7.5 mL	7.5 mL	7.5 mL	7.5 mL
Stock acrylamide²:	11.25 mL	3.25 mL	15.0 mL	7.5 mL
Water:	8.75 mL	18.75 mL	5.0 mL	15.0 mL
APS³:	200 μ L	200 μ L	200 μ L	200 μ L
TEMED⁴:	3 μ L	3 μ L	3 μ L	3 μ L

¹ 1.5 M Tris-HCl, pH 8.8, containing 0.4% (w/v) SDS.

² 40% (w/v) acrylamide and 1.04% (w/v) bis-acrylamide.

³ A 10% (w/v) solution of APS was made up fresh for each gel.

⁴ TEMED was added immediately prior to pouring the gel

2.13 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This method is essentially the same as that described in Sambrook *et al.*, 1989.

2.13.1 Separating Gel

A gel comprised of a linear gradient of polyacrylamide was created by mixing two solutions (called 'heavy' and 'light', referring to the density of the solutions created by the addition of sucrose) as they were pumped into a glass plate assembly. Two types of gradient gels were used; either a 10-20% (w/v) gradient, or a 5-15% (w/v) gradient. The composition of the heavy and light solutions required to pour these gradients are listed in Table 2.10. After the gel was poured, a layer of water was pipetted onto the gel surface to protect from atmospheric oxidation, and the gel was allowed to set (*ca.* 20 min). The layer of water was then discarded, the gel surface was rinsed with separating gel buffer (Table 2.10), and the gel was allowed to stand overnight under a layer of separating gel buffer. Prior to sample loading, the gel was pre-run in separating gel buffer for *ca.* 1 h at 25 mV, or overnight at less than 10 mV.

2.13.2 Stacking Gel

The pre-run buffer solution was discarded, and the gel surface was rinsed with stacking gel buffer mix [1.8 mL of stock acrylamide (Table 2.10), 10 mL of 2x stacking gel buffer (250 mM Tris-HCl, pH 6.8, containing 0.2% (w/v) SDS), 25 μ L of TEMED and

8 mL of water]. An aliquot (180 μ L) of 10% (w/v) ammonium persulphate (APS) was added to the stacking gel buffer mix, and the mixture was pipetted onto the separating gel. A fourteen-well comb was inserted, the gel was allowed to set. The comb was then removed, and the apparatus was filled with gel running buffer (25 mM Tris-HCl, containing 200 mM glycine, and 0.1% (w/v) SDS).

Table 2.11: Molecular masses of prestained protein standards (from Bio-Rad).

Protein	Calibrated MW (Daltons)
Phosphorylase B	112,000
Bovine serum albumin	84,000
Ovalbumin	53,200
Carbonic Anhydrase	34,900
Soybean trypsin inhibitor	28,700
Lysozyme	20,500

2.13.3 Loading of Samples

Samples to be loaded (up to 150 μ L, inclusive of GLB) were thawed, incubated for two minutes in a boiling water bath, and then pulsed in a microfuge before loading onto the gel. The two lanes closest to the outside were not used for samples if possible, and instead were loaded with a similar volume of GLB. One lane of each gel was reserved for an aliquot (10 μ L) of prestained molecular weight standards (Bio-Rad). The masses of these standards (documented by the supplier) are listed on Table 2.11. Electrophoresis was conducted at 25 mA (100 to 200 V), and was terminated when the dye front approached the base of the gel (usually 4 to 6 h). Visualisation of separated proteins was either by staining with Coomassie Blue reagent or by Western analysis.

2.13.4 Staining of SDS-PAGE Gels with Coomassie Blue

SDS-PAGE gels were immersed in Coomassie Blue stain [0.1% (w/v) Coomassie Brilliant Blue R250 (Sigma) in a solution of 50% (v/v) methanol and 10% (v/v) acetic acid (Sambrook *et al.*, 1989)] for *ca.* 1h, with gentle agitation. The gels were then rinsed in several changes of 30% (v/v) ethanol over the next 48 h to destain the non-proteinaceous background of the gels, and were then photographed.

2.13.5 Estimation of Protein Molecular Mass

After the proteins had been visualised on the gels, the size of the proteins in individual bands were estimated by reference to the electrophoretic mobility of the size-standard marker proteins. In detail, the distance between the origin and the centre of each of the six size marker protein bands was used to plot a calibration curve. Regression equations were fitted to the calibration curve using commercial software (SlideWrite™) and the sizes of unknown protein bands were determined from the regression curve. Minor difficulties arose with the extrapolation of protein sizes beyond the range of the molecular mass size markers (20 kD to 112 kD), because the regression equations used tended toward asymptotes or were discontinuous beyond the range of the size markers. To overcome this, the software was used to calculate two regression lines for each calibration curve, using both linear and exponential models (Figure 2.10). The linear model was used for protein sizes above 112 kD, and the exponential model was used for protein sizes below 20 kD (Figure 2.10). Both of the models gave similar regression curves within the range of the size standard markers, and the protein sizes determined from both regressions within this range were averaged to obtain the result.

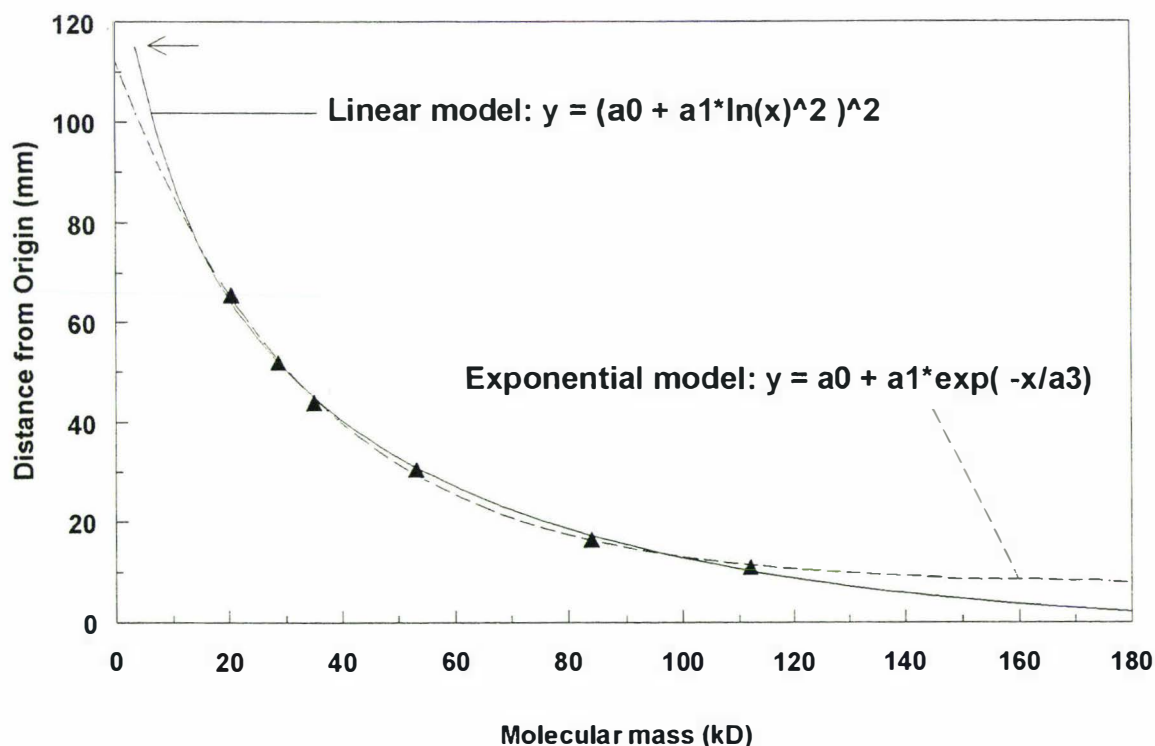


Figure 2.10: Example of a calibration curve drawn from the migration distance of a set of molecular mass size standard marker proteins. The linear model (solid line) was used to determine the size of unknown proteins from 20 to 180 kD; the exponential model (dashed line) was used between 0 and 112 kD. The discontinuity in the linear model is arrowed. Data were single measurements.

2.14 Western Analysis of SDS-PAGE Gels

2.14.1 Primary Antibodies used

2.14.1.1 Anti-Ubiquitin Monoclonal Antibodies

Supernatant from a mouse hybridoma cell culture secreting monoclonal antibodies to bovine ubiquitin was gifted from Dr. Philip Beesley, Biochemistry Department, Royal Holloway College, University of London, UK. Before use, the supernatant was diluted 1:5 with PBSalt. The supernatant was reused several times, and was stored at 4 °C with the addition of 0.02% (w/v) sodium azide (as a preservative).

2.14.1.2 Anti-Dehydrin Polyclonal Antibodies

Polyclonal antibodies specific to the consensus KIKEKLPG amino acid sequence common to the dehydrin class of proteins were kindly donated by Dr. Timothy J. Close, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA, in the form of freeze-dried sera. The antiserum was prepared in rabbits by immunisation with a synthetic polypeptide CTGEKKGIMDKIKEKLPQGH, which was covalently linked to BSA (Close *et al.*, 1993). Prior to the immunisation procedure, serum was obtained from the same rabbit, and a sample of this pre-immune sera was also gifted by Dr. Close. Proteins that were detected by the immune sera, but not by the pre-immune sera were likely to be dehydrins, although the immune sera also contained antibodies to BSA, and to various components of Freund's complete adjuvant (Dr. T. Close, pers. comm.). The sera were reconstituted in 150 µL or 300 µL of water (according to Dr. Close's instructions), and were stored in aliquots at -20 °C [sodium azide 0.02% (w/v) was added prior to freeze-drying]. The sera was diluted 1:500 in PBSalt prior to use.

2.14.2 Protocol

Proteins separated on a SDS-PAGE gel were transferred to a PVDF membrane (Immobilon-P, Millipore, Bedford MA 01730) using a Trans-Blot cell (Bio-Rad), which was filled with transfer buffer (25 mM Tris, containing 200 mM glycine and 10% (v/v) methanol, pH 8.3). Transfer took place at 25V overnight at 4 °C. Protein transfer from polyacrylamide gel to PVDF membrane was confirmed by staining the gel after transfer with Coomassie Blue as described above. The transfer efficiency was not checked on every gel, but the transfer of proteins was adequate on all of the gels examined.

After transfer, the PVDF membrane was immersed in blocking solution [12.5% (w/v) solution of low-fat milk powder (Anchor) in PBSalt)] for *ca.* 1 h at room temperature, with gentle mixing. The blocking solution was then discarded, and the PVDF membrane was rinsed four times with washing buffer (0.05% (w/v) Tween-20 in PBSalt). The PVDF membrane was immersed in a solution of primary antibody (from sections 2.13.1.1 and 2.13.1.2), and then incubated for one hour at 37 °C, with occasional mixing.

The primary antibody solution was removed from the PVDF membrane and retained for re-use. After four to five rinses with washing buffer, the PVDF membrane was immersed in a solution of 0.03% (v/v) of alkaline phosphatase conjugated to either goat-anti-mouse IgG or goat anti-rabbit IgG antibody (Sigma) in PBSalt. Incubation was for 45 min at room temperature, with gentle mixing. The secondary antibody solution was discarded, and the PVDF membrane was rinsed five times in washing buffer. The membrane was then rinsed in assay buffer (150 mM Tris-HCl, pH 9.7), and immersed in 200 mL of freshly prepared assay solution [assay buffer containing 5 mM MgCl₂ and 10 mg of nitro-blue tetrazolium (Sigma); immediately before use, 1 mL of 10 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in dimethylsulphoxide was added]. The reaction was incubated at room temperature in darkness for up to 15 min for colour development to occur. To terminate the reaction, the assay solution was discarded, and the PVDF membrane was rinsed several times in deionised water. The membrane was stored under water, with the addition of 0.02% (w/v) sodium azide if storage was to be for a prolonged period. The developed membranes were photographed before air drying.

2.15 Extraction of Genomic DNA

2.15.1 Aseptic Technique

The work concerning DNA extraction or manipulation described below was carried out under sterile conditions. Aqueous reagents (including purified water) were autoclaved for 15 min. Those reagents which cannot be autoclaved (for example SDS or 80% ethanol) were prepared in sterile water. All of the disposable plasticware used (for example, 10 mL and 1.5 mL centrifuge tubes and pipette tips) was either autoclaved for 15 min, or came from commercially sterilised packets.

2.15.2 DNA Extraction

The extraction protocol used was based on that described by Dellaporta *et al.* (1983). Approximately 1 g of leaf tissue was frozen in liquid nitrogen, and powdered with pestle and mortar. The powder was then transferred to a 10 mL centrifuge tube, containing 7 mL of extraction buffer (50 mM Tris-HCl, pH 8, containing 10 mM EDTA, 100 mM NaCl, 1% (w/v) SDS and 10 mM 2-mercaptoethanol) and was quickly mixed. The tube was capped, vortex mixed and incubated at 65°C while the next tissue samples were ground and extracted. After all the tubes had incubated for at least 5 min, 2.5 mL of 5 M potassium acetate was added to each tube. The tubes were capped, shaken vigorously, and incubated on ice for at least 15 min. After incubation, the tubes were centrifuged at 8000 xg for 10 min at 4 °C. The supernatant was carefully removed, and transferred to a fresh 10 mL centrifuge tube containing 5 mL of isopropanol. The tubes were capped, and inverted several times to precipitate the DNA. The DNA was then collected by spinning at 8000 xg for 5 min at 4 °C.

The DNA was rinsed once with 80% (v/v) ethanol, and air dried by inverting the tube over a paper towel for approximately 5 min. The DNA was then dissolved in 500 µL of TE buffer (10 mM Tris, pH 8, containing 1 mM EDTA), and transferred to 1.5 mL centrifuge tube. An aliquot (50µL) of 3 M sodium acetate, pH 5, was added, and the DNA was precipitated with the addition of 500 µL of isopropanol. The DNA was collected by pulsing (20 s) in a microfuge, washed once with 80% ethanol, and then re-dissolved in 500 µL of TE. The DNA was stored at -20 °C, and before use was centrifuged for 5 min at 12,000 xg to pellet any insoluble material.

2.15.3 Qualitation and Quantitation of DNA

An aliquot (10 to 20 µL) of DNA solution was made up to 1.0 mL with water. The absorbance ratio A_{258nm} / A_{280nm} against a water blank was used as an indication of the quality of the DNA since the A_{258nm} / A_{280nm} of a pure nucleic acid solution equals approximately = 1.8 (Sambrook *et al.*, 1989).

The DNA concentration was calculated as below, where $k = 50 \mu\text{g}/\text{mL}$ DNA since this concentration has an A_{258nm} of 1.0 AU (Sambrook *et al.*, 1989):

$$A_{258nm} \cdot k \cdot \frac{1000}{\text{aliquot}(\mu\text{L})} = [\text{DNA}] (\mu\text{g}/\text{mL}),$$

2.15.4 Agarose Gel Electrophoresis of DNA

2.15.4.1 Electrophoresis Protocol

Electrophoresis was used to separate DNA fragments on the basis of molecular mass. Gels were composed of TAE (40 mM Tris-HCl, 20 mM acetic acid and 1 mM EDTA, pH 8) containing 0.5 $\mu\text{g}/\text{mL}$ ethidium bromide and 0.8% (w/v) or 2% (w/v) of electrophoresis grade agarose (Life Technologies, 8400 Helgerman Court, Gaithersburg, MD 20884-9989) for minigels (90 x 70 mm) and standard gels (150 x 150 mm), respectively. This solution was weighed, heated by microwave oven to dissolve the agarose, and then re-weighed. Extra water was added to account for any loss during heating. The gel was poured, and a fifteen-well comb was inserted into the gel, which was allowed to set before immersion in running buffer (TAE containing 1 $\mu\text{g}/\text{mL}$ ethidium bromide). The comb was removed immediately before loading the gel.

Samples (1 μl to 10 μL) of DNA to be loaded onto the mini-gel were first made up to ca. 20 μL with Gel Loading Buffer [GLB: 10 mM Tris-HCl, containing 1 mM EDTA, pH 8, 20% (v/v) glycerol and 0.025% (w/v) Bromophenol Blue]. One or two lanes of each gel were usually loaded with 10 μL of DNA size standard markers (section 2.15.4.2). Electrophoresis was conducted at 50 V at room temperature, for 30 to 60 min. Visualisation of the DNA was by short-wavelength UV and photographs were taken of each gel with UV illumination.

2.15.4.2 DNA Size Standard Markers (λ H+HE Markers)

The standards used for DNA electrophoresis were from a laboratory stock prepared by the restriction of λ phage DNA with two endonuclease enzymes, HindIII and EcoRI (New England Biolabs, Inc., Beverly, MA, USA). Equal quantities of λ phage DNA (80 μg) were digested with either HindIII, or a combination of HindIII and EcoRI (100 units of each enzyme in 800 μL of buffer B), at 37 $^{\circ}\text{C}$ for 2.5 h. Aliquots (200 μL) of each of these two digests were mixed with 540 μL of water, and 60 μL of dye buffer (100 mM EDTA, pH 8.0, containing 50% (v/v) glycerol, 1% (w/v) SDS, 0.025% (w/v) Bromophenol Blue), to make 1.0 mL of λ H+HE DNA size standard markers. This mixture contains fourteen DNA fragments of known size (Sambrook *et al.*, 1989, Table 2.12).

Table 2.12: Molecular masses of λ H+HE DNA size standard markers.

DNA fragment	Size (bp)	DNA fragment	Size (bp)
1	21,000	8	2,027
2	9,416	9	1,904
3	6,557	10	1,548
4	4,973	11	1,330
5	4,268	12	947
6	3,530	13	831
7	2,322	14	564

2.16 Southern Analysis of Genomic DNA

The Southern analysis method was essentially as described by Sambrook *et al.* (1989).

2.16.1 Restriction of High Molecular Weight DNA

Samples of genomic DNA were digested using three restriction endonuclease enzymes, HindIII, BamHI, and EcoRI (New England Biolabs). Reaction mixtures consisted of 30 μ L (30 μ g) of DNA from section 2.15.2, 20 μ L of endonuclease (20 units/ μ L), 20 μ L of buffer B (for HindIII and BamHI) or buffer H (for EcoRI) [New England Biolabs], 5 μ L DNAase-free RNAase (10 mg/mL, Sigma) and 125 μ L of water. The reactions were incubated at 37 °C overnight, and then supplemented with an additional 10 μ L of endonuclease, 10 μ L of buffer B or H, (as appropriate) and 80 μ L of water, and were again incubated at 37 °C for 3 h.

An aliquot (0.1 volumes) of 3 M sodium acetate (pH 5.1) was added to each reaction, and the digested DNA was precipitated by the addition of one volume of isopropanol, and incubated at -80°C for 10 min. The DNA was collected by centrifugation at 12,000 xg for 5 min, and was washed with 80% (v/v) ethanol. The DNA was then re-dissolved in 20 μ L of 10% (v/v) dye buffer (described in section 2.15.4.2).

2.16.2 Electrophoresis of DNA Digests

Samples (20 μ L) of EcoRI, BamHI and HindIII digested DNA from section 2.16.1 were loaded onto adjacent lanes on a 0.8% (w/v) standard size agarose gel (section 2.15.4.1). Molecular mass markers (10 μ L) were loaded into two lanes on the gel. As a control,

an aliquot (20 μ L) of pRAB16 plasmid which had been digested with Sall and SacI restriction endonucleases (section 2.16.4) and diluted 1:50 in 10% (v/v) dye buffer (section 2.15.4.2) was also loaded onto one lane of the gel. Electrophoresis was conducted at 25 V overnight, until the dye front had migrated *ca.* 150 mm. Gels were then photographed under UV illumination.

2.16.3 Transfer of DNA to Nylon Membrane

Electrophoresis gels from section 2.16.2 were immersed in a depurinating solution (250 mM HCl) with gentle shaking until 10 min after the blue dye-front had changed to yellow (about 30 min). The depurinating solution was then discarded, and gels were rinsed twice with water, before immersion in denaturing solution (1.5 M NaCl and 0.5 M NaOH). Fifteen minutes after the dye-front had changed back to blue, the denaturing solution was discarded. The gels were then rinsed twice with water, and then washed twice for 15 min each in neutralising solution (0.5 M Tris-HCl, pH 7.2, containing 1.5 M NaCl and 10 mM EDTA), and again rinsed twice with water.

The DNA was blotted onto a nylon membrane (Hybond N⁺, Amersham International, Little Chalfont, HP79NA, UK) overnight at room temperature. The apparatus used consisted of a stack of paper towels sited above the nylon membrane, which was placed on the gel. The gel was sited on a blotting paper wick through which a solution of 20 xSSPE (3.6 M NaCl, 200 mM NaH₂PO₄·2H₂O and 20 mM EDTA, pH 7.7) was drawn up, and into the paper towels. Once the blotting was completed, the nylon membrane was placed on a pad of blotting paper soaked with 0.4 M NaOH for 30 min. The nylon membrane was then rinsed in 5 xSSPE and sealed in a plastic bag containing 5 xSSPE, before storage at 4 °C.

DNA transfer from agarose gel to nylon membrane was confirmed by staining the gel after transfer with a solution of 0.5 μ g/mL ethidium bromide, for 30 min, and then examining under UV illumination. The transfer efficiency was adequate on all the gels examined (data not presented).

2.16.4 Preparation of Dehydrin (RAB16a) DNA probe

A gift of pRAB16a cDNA clone (Mundy and Chua, 1988) was received from Dr. Nam-Hai Chua, The Rockefeller University, 1230 York Avenue, New York. The pRAB16a DNA was digested with Sall and SacI to yield a 450 bp sequence within the reading frame [from +135 to +668 (this region of the gene also includes an 83 bp intron)], which was sub-cloned into pBluescript (Stratagene, La Jolla, CA 92037) by Dr. Michael McManus (AgResearch, Grasslands Research Centre, Private Bag, Palmerston North, New Zealand).

An aliquot (*ca.* 2 μ g) of the pBluescript containing the RAB16a insert was digested with SacI and Sall (New England Biolabs) in a reaction mixture comprised of 3 μ L of SacI (20 units/ μ L), 3 μ L of buffer L, 3 μ L of DNAase-free RNAase and 11 μ L of water. The reaction mixture was incubated at 37 °C overnight, before it was supplemented with 3 μ L of Sall (20 units/ μ L), 3 μ L of buffer H, and 24 μ L of water, and incubated for a further 3 h at 37 °C. The digested DNA (60 μ L) was mixed with 6 μ L of dye buffer (section 2.15.4.2) and separated through a 2% agarose minigel (Plate 2.1A). Also loaded onto the gel was an aliquot of undigested pBluescript plasmid (0.5 μ L with 10 μ L of sterile water and 2 μ L dye buffer (section 2.15.4.2) and 10 μ L of DNA size standard markers.

The portion of the agarose gel containing the DNA bands at *ca.* 450 bp was excised, and dissolved in three volumes of 6 M NaI at 50 °C. To this solution, 10 μ L of Glassmilk (Biol101 Inc., LA Jolla CA 92038-2284) was added, the mixture was shaken for 10 min, and then pulsed in a centrifuge. The collected material was washed three times with 0.5 mL of GeneClean (Biol 101 Inc.) wash solution, with intervening centrifuge pulses. After the final centrifugation, the GeneClean supernatant was discarded, the pellet dried and re-suspended in 20 μ L of sterile water at 50 °C. The suspension was centrifuged at 12,000 \times g for 1 min, and the supernatant was transferred to a fresh 1.5 mL centrifuge tube. Confirmation that the 450 bp DNA sequence (designated the RAB16a probe) was present in the supernatant was made by running an aliquot (6 μ L) on a 2% agarose minigel (section 2.15.4.1), alongside one lane of DNA size standard markers (data not presented).

2.16.5 Incorporation of ^{32}P Labelled dCTP into the Dehydrin (RAB16a) Probe

The RAB16a *sacI/salI* 450 bp DNA fragment was made up to 45 μL with water, incubated in a boiling water bath for 3 min, chilled on ice for 2 min, and then pulsed in a microfuge. The DNA was added to a 1.5 mL centrifuge tube containing Ready To Go™ reaction mixture (Pharmacia), 5 μL of ^{32}P -dCTP (3000 Ci/mmol, ICN Pharmaceuticals, Irvine, CA 92715) was then added and the mixture was incubated for 15 min at 37 °C. Confirmation that the radioactivity was incorporated into the probe was made by TLC using cellulose plates (Merck, Darmstadt, Frankfurter Strasse 250, Germany) and 0.75 M phosphate buffer, pH 3.5, as the solvent phase. Radioactivity on the TLC plate was visualised by autoradiography (2 min exposure, Plate 2.1B).

2.16.6 Southern Hybridisation Procedure

The nylon membrane from section 2.16.3 was added to a Hybaid bottle (Hybaid Ltd., Teddington, Middlesex TW118LL) containing 25 mL of pre-hybridisation solution (5 xSSPE containing 5% (v/v) Denhardt's solution [2% (w/v) BSA, 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrrolidone] and 0.5% (w/v) SDS), and incubated at 65 °C for 1 h. An aliquot (1 mL) of herring sperm DNA (5 mg/mL, Sigma) was incubated in a boiling water bath for 5 min, chilled on ice for 2 min, and then added to the prehybridisation solution. The ^{32}P -labelled RAB16a probe (section 2.16.5) was incubated in a boiling water bath for 5 min, and then added to the mixture in the Hybaid bottle, which was then incubated overnight at 65 °C. The next day, the hybridising solution was discarded, and the nylon membrane was washed twice (15 min each) in 2 xSSPE, containing 0.1% (w/v) SDS. The nylon membrane was then washed in either 1 xSSPE, 0.1% (w/v) SDS, or in 0.2 xSSPE, 0.1% (w/v) SDS at 65 °C for 20 min. Radioactivity on the nylon membrane was detected over 72 h using a phosphorimage detector (BioRad).

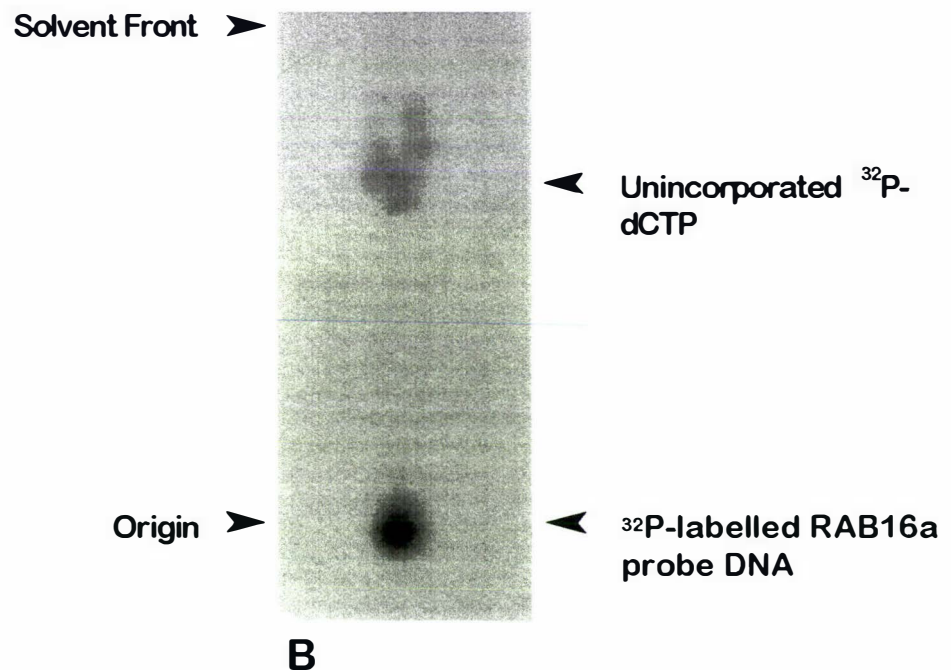
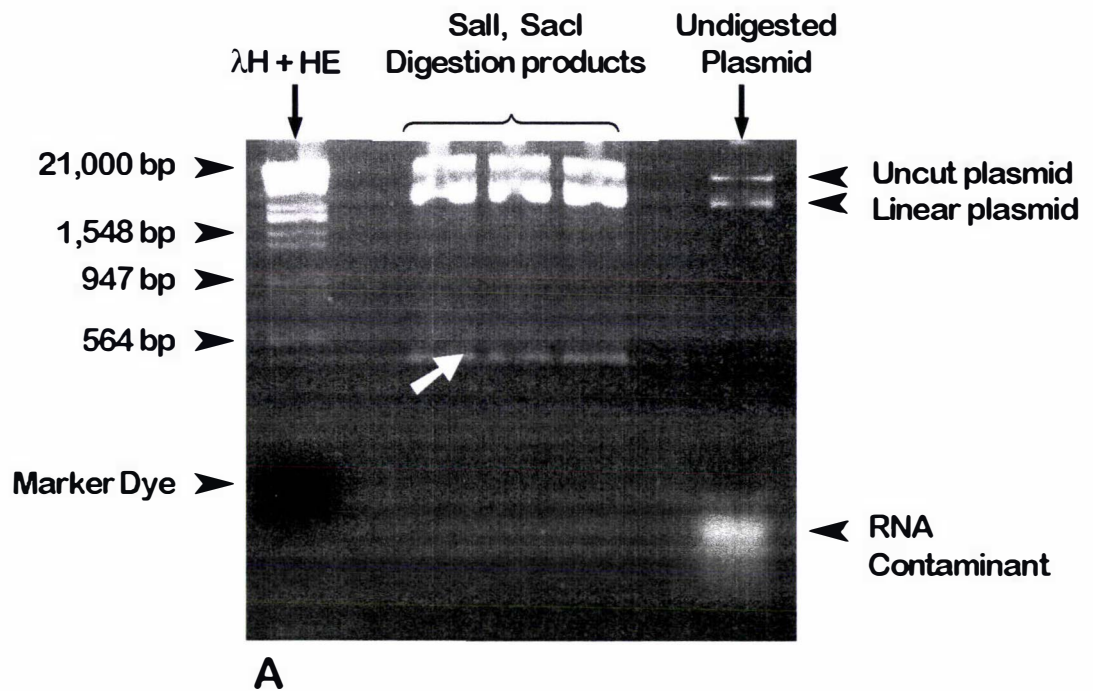


Plate 2.1: **A:** Separation of the 450 bp RAB16a insert (arrowed) from pBluescript plasmid after digestion with SalI and SacI restriction endonucleases. The linear plasmid was produced by a single enzymatic restriction of the circular pBluescript plasmid. λ H+HE: size standard markers. **B:** Thin layer chromatogram showing the incorporation of ^{32}P -dCTP into the RAB16a DNA probe, visualised by autoradiography.

2.17 Scanning Electron Microscopy

Segments of laminae were sliced into *ca.* 1 mm sections in fixative (100 mM phosphate buffer, pH 7.2, containing 3% (v/v) glutaraldehyde and 2% (v/v) formaldehyde). The samples were then prepared, examined, and photographed under a Scanning Electron Microscope (SEM) by Mr Doug Hopcroft, Electron Microscope Unit, HortResearch, Palmerston North. The preparation protocol was as follows: Once sliced, the samples were vacuum infiltrated, fixed at room temperature overnight, and then dehydrated in a graded acetone/water series to two changes of 100% acetone. The samples were dried in a Polaron Critical Point Drier, using liquid CO₂ as the critical point fluid, mounted with double sided tape onto aluminium stubs, coated with gold using a Polaron E5100 cold stage sputter coater, and examined in a Cambridge 250 MkII scanning electron microscope (SEM).

2.18 Statistical Analysis

Sample means have been used throughout this dissertation, and are usually presented with an estimate of the standard error (s.e.) of the sample. The mean and s.e. were calculated using the formulae below (Sokal and Rohlf, 1981). Where means have been presented on figures, the data is represented as a symbol wrapped by error bars of a magnitude of \pm s.e.. In many instances, error bars are not visible on graph points where $n > 1$. In these cases, the error bars are smaller than the graphical symbols.

Mean (\bar{Y}) = $\frac{\sum Y}{n}$, where Y is an observation, and n is the total number of Y .

Standard Error (s.e.) = $\frac{\sigma}{\sqrt{n}}$, where σ (standard deviation of sample) = $\sqrt{\frac{\sum(Y - \bar{Y})^2}{n - 1}}$

Where the statistical difference between two or more means have been compared, an Analysis of Variance (ANOVA) model has been used. ANOVA analysis was carried out on a PC-compatible computer using SAS/STAT™ (SAS Institute, Inc., Cary, NC, USA) software. Since the majority of the ANOVA models used in this dissertation were unbalanced (that is, an unequal n was used to calculate each mean), the SAS/STAT™ procedure for general linear models (PROC GLM) was used. The results of each ANOVA performed are presented in an ANOVA table, which lists the sources of variation in the model, degrees of freedom, the sum of squares and mean square

statistics, and the calculated F-value. The probability P , that the means compared by the ANOVA were sampled from the same population is also provided. Following convention, a difference between means at the 5% level ($P \leq 0.05$) was considered significant, with a difference at the 1% level ($P \leq 0.01$) highly significant. Where a two-way ANOVA was performed, a second ANOVA table is presented, which lists the F- and P values of each component of the model, and of the interaction of those components (denoted by $A \times B$, where A and B are the two types of treatment used in the model). As already mentioned, the probability P , listed on each ANOVA Table indicates the significance of any difference between the means examined with the ANOVA model. In order to determine which means were significantly different from which other means, a Duncan's Multiple Range Test was performed, and the results presented as significance groupings on a table of means. Those means which are connected by the same bar on such a table were not significantly different. ANOVA analyses were not carried out between experimental pots on a basis of comparing water-deficit *versus* control pots as treatment effects. This is because in most experiments reported in this dissertation, the rate of drying in individual water-deficit treatment pots was not controlled. Therefore individual pots dried out at different rates, so that each pot could be considered to have received a unique level of water-deficit treatment. Hence the ANOVA models included each experimental pot as an individual treatment.

Appendix 2.1

Algorithm used for determining ABA sample concentrations from the standard curve

The code below was written in PASCAL, for an IBM PC compatible machine. Although this program was extended to incorporate procedures for the run-time input of filenames, polynomial coefficients, required precision and x value limits; this code was not essential for the running of the algorithm, and so was omitted for clarity. Examples of these data are included in the CONSTANT statement, and in PROCEDURE SetLimits. The algorithm reads ABA ELISA absorbances (y-values) from a text file; and uses an iterative bisection method to interpolate the corresponding ABA concentration (x-value) from polynomial coefficients which were fitted to ABA standards absorbances using SlideWrite™. Note that x-values are reported in LOG(x) form, the anti-log gives the ABA concentration.

PROGRAM Iterate;
{Written by Grant Abernethy, September 1993}

```
VAR                                     {Defines the Type of each variable}
  InputFile, OutputFile :Text;
  ANumberLine           :String;      {A Line read from file InputFile}
  AnError               :Integer;     {Returns error code for procedure Val}
  Number, BottomxLimit,
  TopXLimit, TopYLimit,
  BottomYLimit, x , y   :Real;
  TheEnd               :Boolean;     {Yes/No operand}

CONSTANT                               {for entering the equation parameters }
  c = -0.004955;                       {Constant for polynomial equation }
  m1 = 0.687728;                       {m1 to m6 are the polynomial coefficients}
  m2 = -0.226718;
  m3 = 0.017296;
  m4 = 0;
  m5 = 0;
  m6 = 0;
  DefinedLimit = 0.001;                {level of decimal place accuracy}
  DataSetFile = 'ABADATA';             {File containing y values to be read off curve}

FUNCTION Calc (Inp: real):real;
{Calculates Y from polynomial equation}
VAR
  Answer :Real;

BEGIN
```

```

    Answer := c + m1*Inp + m2*Inp^2 + m3*Inp^3 + m4*Inp^4 + m5*Inp^5 + m6*Inp^6;
    Calc := Answer;
END;                                     {END of FUNCTION Calc}

PROCEDURE SetLimits;                     {defines limits to polynomials graph}
BEGIN
    BottomXLimit := 2.0;                 {units in LOG(ABA concentration) }
    TopXLimit := 4.0;
    BottomYLimit := Calc( BottomXLimit ); {calculates y limits from supplied x limits}
    TopYLimit := Calc( TopXLimit );      {calculates y limits from supplied x limits}
END;                                     {END of PROCEDURE SetLimits}

PROCEDURE Loop;                          {returns the x value for the y read from file}
BEGIN
    TheEnd := False;
    SetLimits;
    IF (Number > BottomYLimit ) OR (Number < TopYLimit ) {check supplied value is within limits}
        THEN                                           { if not within limits, then say so and EXIT}
        BEGIN
            Writeln('Y= ', Number:5:4, ' This Y ordinate out of range!');
            Writeln( OutputFile , 'ERROR' );
            Exit;
        END;
    x := (BottomXLimit + TopXLimit )/2;                {define x between limits before starting Loop}
    REPEAT                                             {REPEAT UNTIL TheEnd returns True }
        y := Calc ( x );                               {calculate y from x }
        IF (Sqrt (Sqr (Number - y)) < DefinedLimit)   {check precision of y}
            THEN
                TheEnd := True                         {Y is within decimal precision required}
            ELSE                                       {Y is not within decimal precision required}
                BEGIN
                    IF ((Number - y) > 0)              {determine which direction to adjust x}
                        THEN                          {Number - y > 0 }
                            TopXLimit := x           {set TopXLimit to x value}
                        ELSE                            {Number - y < 0}
                            BottomXLimit := x;       {set BottomXLimit to x value}
                    END;
                    x := (BottomXLimit + TopXLimit )/2; {select new x midway between limits}
                    Writeln( 'New x := ', x:5:4 );     {report new x }
                UNTIL TheEnd;                          {.. UNTIL required precision is reached}
                Writeln(' y= ', Number:5:4, ' x= ', x:5:4 ); {Report the y from file, and the corres. x}
                Writeln( OutputFile , x:5:4 );        {write x to a new line in OutputFile}
    END;                                               {END of PROCEDURE Loop }

BEGIN                                             {BEGIN PROGRAM Iterat}
    SetLimits;                                       {run PROCEDURE Setlimits, and report data}
    Writeln(' BottomXLimit = ', BottomXLimit:5:4, ' BottomYLimit = ', BottomYLimit:5:4 );
    Writeln(' TopXLimit = ', TopXLimit:5:4, ' TopYLimit = ', TopYLimit:5:4 );
    Assign( InputFile, DataSetFile + '.TXT' );      {assign InputFile to a filename}
    Assign( OutputFile, DataSetFile + '.OUT' );     {assign OutputFile to a filename }
    Reset( InputFile );                             {Open Inputfile }
    ReWrite( OutputFile );                          {create and open new OutputFile}
    WHILE NOT EOF( InputFile ) DO                  {Repeat read lines until End Of File}
        BEGIN
            Readln( InputFile, ANumberLine );      {read line of file, store in ANumberLine }
            Writeln(' Next Y Value from File ' + DataSetFile + '.TXT := ', ANumberLine );
            Val( ANumberLine, Number, AnError );    {convert Line to numerals, store in Number}
            Loop;                                   {calculate x for the y value from file }
        END;                                       {we're at the end of file }
    Close( OutputFile );
    Close( InputFile );
    Writeln( ' FINISHED, Press Enter to Continue...'); {Report completion of program }
    Readln;                                       {wait for enter to be pushed }
END.                                             {of PROGRAM Iterat}

```

Chapter Three

Experimental Dry-Down of *F. arundinacea*

3.1 Introduction

The principal aim of the work described in this chapter was to conduct trial runs of the experimental protocols which were intended to be used for imposing water-deficit stress and for measuring the concentrations of marker compounds in *F. novae-zealandiae*. The native species was replaced with *F. arundinacea* in these experiments, because the relatively well defined drought response of the exotic species afforded a useful model with which to evaluate the method protocols.

The specific aims of these experiments were to determine if under water-deficit conditions, *F. arundinacea* responds by the cessation of leaf elongation, by the accumulation of the osmoprotectants proline and glycine-betaine, and by the accumulation of the phytohormone ABA. The achievement of a measurable response from these experiments would form the basis for the use of these stress-adaption markers in later experiments with *F. novae-zealandiae*.

3.2 Methods

Two experiments were carried out. For experiment I, 10 to 14 plants of *F. arundinacea* derived from seed (cv. "Grasslands Roa") were established over 8 weeks in each of six 4 L pots (described in section 2.2.1, page 23). Single plants (TF-1 genotype) were established over 9 weeks in each of six 24 L pots (section 2.2.2, page 24) for experiment II. A comparison of the methods used for each experiment is presented in Table 3.1.

In experiment I, water-deficit was applied to three pots (D1 to D3) while three were used as watered controls (C1 to C3). Water-deficit was applied to four of six pots (D6 to D8) used for experiment II, while the other two were used as controls (C5 and C6). In addition, two of the water-deficit treatment pots (D5 and D6) were rewatered toward the end of experiment II.

Table 3.1: Comparison of the methods used for experiments I and II.

	Experiment I	Experiment II
Pot system used:	4L (potting mix)	24L (silt loam)
Number of pots : Water deficit treatment:	3	4
Control:	3	2
Pot numbers:	D1 to D3, C1 to C3	D5 to D8, C5 and C6
Number of plants per pot:	10-14	1
Plant genotype:	seed (Grasslands Roa)	TF-1
Number of TDR electrode pairs:	1	5
Number of tillers measured for LER:	4	3
LWP measured:	✓	✗
ABA, proline, glycine-betaine measured:	✗	✓
Root distribution determined:	✗	✓

Water-deficit stress was initiated by withholding water; the soil water content (SWC) of each pot was monitored using the method described in section 2.3 (page 24), and control pots were watered to maintain the SWC at or near soil saturation (30 to 35% SWC). For SWC measurements, either one pair of vertical electrodes (experiment I), or five pairs of horizontal electrodes (experiment II) were used.

The leaf elongation rate (LER) was calculated from measurements of the IL of four tillers per pot during experiment I, and from three tillers per plant in experiment II, using the method described in section 2.5 (page 26). The LWP was measured between 2:00 and 2:30 PM on each sampling day during experiment I using the method described in section 2.4.1 (page 26), but was not measured during experiment II as the apparatus became inoperable.

Samples of mature laminae (ML) were taken at regular intervals during experiment II. A number of ML were excised and pooled to supply at least 300 mg of fresh weight tissue for biochemical analyses. In most instances, a single *F. arundinacea* leaf supplied sufficient material. Samples were weighed, frozen in liquid nitrogen and stored at -80 °C until processing, which consisted of cooling in liquid nitrogen and powdering with pestle and mortar. The powder was then stored at -80 °C. Samples of tissue powder were analysed for ABA, glycine-betaine and proline concentration, using the methods described in sections 2.8, 2.9 and 2.10 (pages 27, 37, 45, respectively).

At the end of experiment II, the root distribution of two of the experimental plants (C5 and D8) were determined as described in section 2.6 (Page 27). In addition, the roots of plant D5 were washed free of soil and photographed.

On one occasion that was separate from the two dry-down experiments, three tillers were excised from a plant of *F. arundinacea* grown in water-sufficient conditions. These tillers were dissected for RWC analysis, as outlined in section 2.4.2 (page 26).

3.3 Results from Experiment I

3.3.1 Experimental Time Course

The experimental measurements were started on 31 August 1992, and the final date of watering of the dry-down treatment pots (D1, D2 and D3) was on September 1. Pots C1, C2, and C3 were maintained as watered controls. Measurements from this experiment were continued for up to 18 days.

3.3.2 Soil Water Content (SWC)

The water-deficit treatment plants dried down at a similar rate, to reach a lower level of *ca.* 5% (v/v) SWC after 13 days (Figure 3.1). With regular watering, the SWC of the control pots remained above 23% SWC throughout the experiment (Figure 3.1).

3.3.3 Leaf Elongation Rate (LER)

The lengths of the IL of four tillers per pot were measured during the dry-down to determine the LER (Figure 3.2). The LER was between 18 and 48 mm.d⁻¹ under water-sufficient conditions (plants C1 to C3; Figure 3.2), although individual LER measurements of more than 50 mm.d⁻¹ were recorded in some instances.

The LER declined in response to the water-deficit treatment. The LER plot of D1 to D3 was essentially bimodal, where the LER was comparable to that of the controls during the initial stages of the experiment, until the SWC had declined to 6-7%. The LER then declined, before cessation of growth at 4-5% SWC (Figure 3.2).

3.3.4 Leaf Water Potential (LWP)

The LWP of the water-deficit treatment plants remained similar to that of the controls (-0.4 to -1.3 MPa) for the initial period of the experiment, until the SWC had decreased to 7-8% (Figure 3.3). After this time the LWP declined below -1.3 MPa, and continued to decline to become less than -3.0 MPa by the end of the dry-down (Figure 3.3).

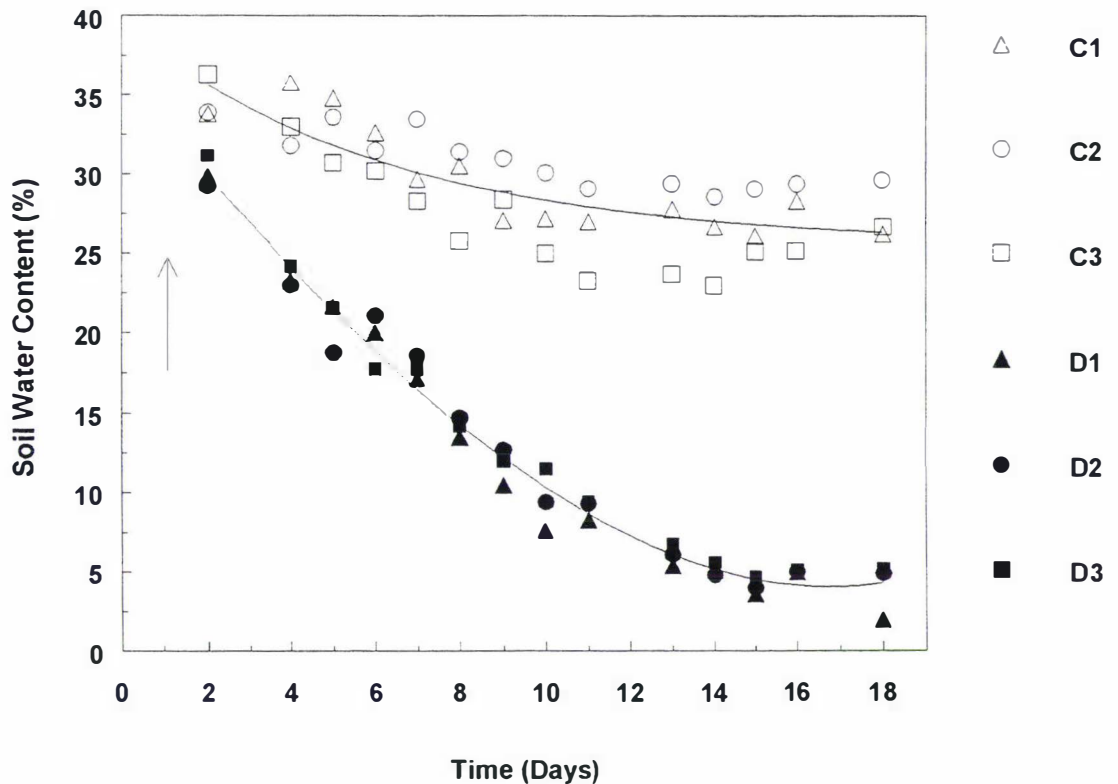


Figure 3.1: The SWC measured during experiment I. The legend indicates pot numbers. The arrow indicates the last date of watering for the water-deficit treatment pots. Data were single TDR measurements.

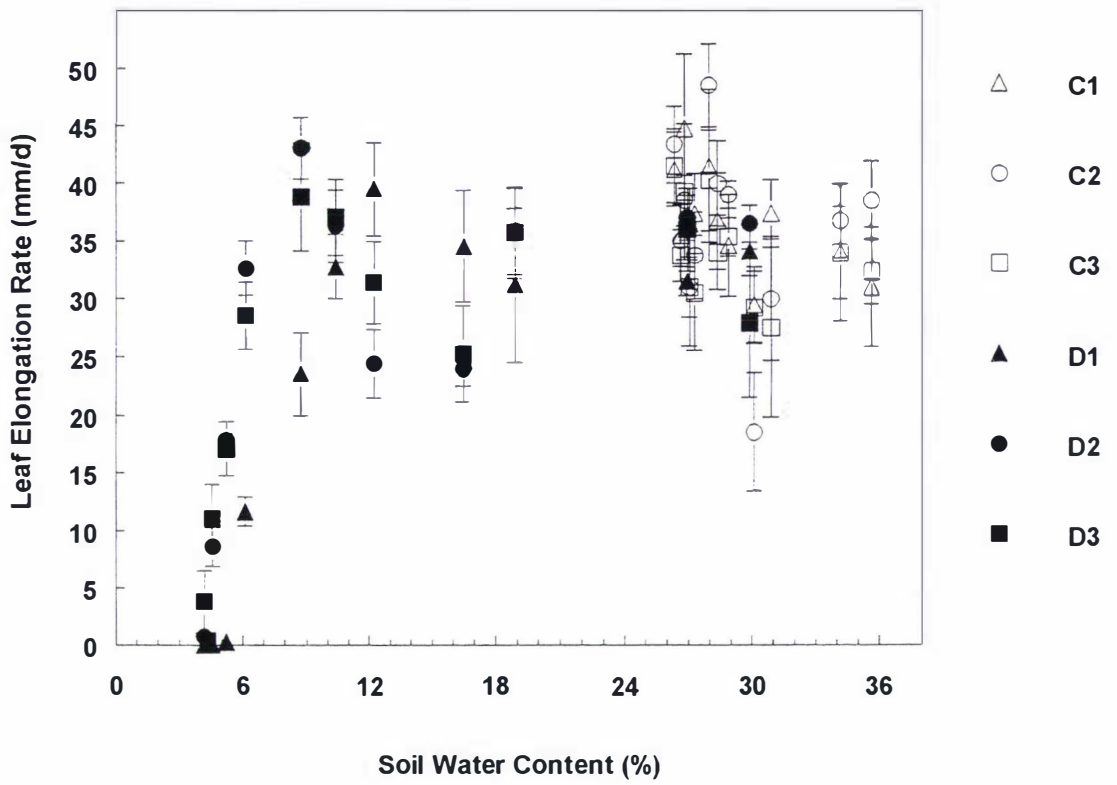


Figure 3.2: The effect of declining SWC on LER during experiment I. The legend indicates pot numbers. Data were means \pm s.e., n=4.

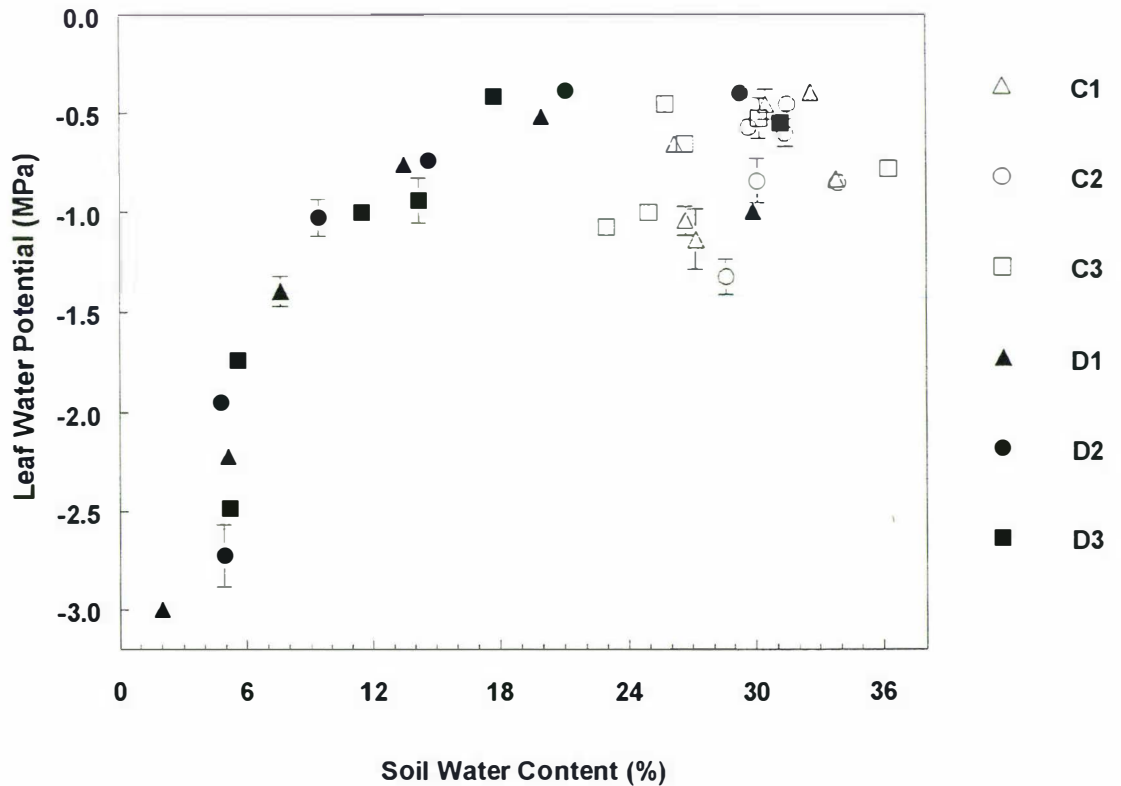


Figure 3.3: The effect of declining SWC on LWP during experiment I. The legend indicates pot number. Data were means \pm s.e., n=2.

3.4 Results from Experiment II⁷

After the experience of experiment I (section 3.3), some amendments were made to the method used for imposing water-deficit on experimental plants. These amendments focused on increasing the length of the dry-down period in order to increase the time in which the plants had to adapt to the increasing water-deficit stress (before the stress became injurious to the plant). The amendments included reducing the number of plants per pot from about 12 to only one, and increasing the depth of the soil from *ca.* 200 to 750 mm to increase its volume from 4 to 24 L, and to prevent the plants becoming root-bound (the roots of *F. arundinacea* were observed to fill the 4 L pots). Field soil was substituted for the potting mix used in experiment I, and was also intended to slow the rate of drying by increasing the clay content (and hence the density) of the soil.

3.4.1 Experimental Time Course

Six plants of *F. arundinacea* were established in six 24 L pots under water-sufficient conditions, and the dry-down was initiated (except in the control plants C5 and C6) on 26 April, 1993. Due to a technical problem with the TDR, experimental measurements were started on 29 April. SWC measurements were carried out for all plants for 47 days. The soil in the pots containing the water-deficit treatment plants dried at different rates, with the dry-downs taking 20, 22, 35, and 37 days (plants D6, D5, D7, and D8 respectively, Figure 3.5).

3.4.2 Soil Water Content (SWC)

The progress of the dry-down was followed by monitoring the SWC of each of five soil horizons within each pot (Figure 3.4). The SWC data from the five soil horizons was used to calculate the mean SWC for the entire pot. To take into account moisture gradients down each soil column, the total water content of each pot was calculated as the sum of the average SWC between electrode stations, multiplied by the distance between stations. This value was then divided by the height of the soil column to obtain the mean SWC (Figure 3.5).

⁷

As only one plant per pot was used, the plant references are the same as the pot references.

3.4.2.1 Changes in Mean SWC

At the start of the experiment, all pots were watered to saturation (*ca.* 30% SWC) and by the time measurements were started, the mean SWC of the soil in the water-deficit treatment pots had declined to 16-21%. The mean SWC levels initially declined at a fast rate until the SWC had been reduced to 6-7%, when the rate of drying slowed appreciably (Figure 3.5). With regular watering, the mean SWC of pot C6 remained relatively constant with only a marginal decline after 36 days (Figure 3.5). In C5, the mean SWC declined in a linear fashion to reach *ca.* 12% by the end of the experiment (Figure 3.5). The reduction in SWC of this pot was attributed to shrinkage of the soil, which left a small gap (2-3 mm) between the soil column and the inside of the pot wall (this also occurred in the water-deficit treatment pots), and was probably due to the clay content in the soil since it not observed when using potting-mix based soils. This gap facilitated the drainage of water applied to the top of the pot, making the maintenance of a constant water content difficult. Attempts to alleviate the problem by scoring the soil surface to improve the absorption of water, or to push extra soil into the gap were ineffective. Evidence to indicate that this gap was allowing excessive drainage was found when two of the water-deficit treatment pots (D5 and D6) were re-watered after the experiments had been completed for these two pots. Although the pots were watered from the top, the lowest soil horizons absorbed water fastest (Figure 3.4); evidence that the applied water did indeed drain down the inside of the pot to wet the base of the soil column.

3.4.2.2 Changes in SWC Gradients

The SWC was found to exist in a gradient within each soil column. The top soil horizon was usually found to be the driest of the five horizons measured, and the SWC increased with depth, to reach a maximum in the lowest soil horizon. C6 exhibited a close to a model control response, where the gradient of SWC (which ranged from 8-15% SWC in the top soil horizon and to 33-40% SWC in the bottom horizon) remained constant throughout the experiment (Figure 3.4). The SWC of C5 declined gradually in all soil horizons, with water being removed from the deepest soil horizon last.

In all the water-deficit treatment pots, the SWC declined in all the soil horizons. The gradient of SWC was preserved as the water was lost from the soil, with the top

horizons losing water the most rapidly. The SWC of all the soil horizons eventually fell to a lower level of 7-8% SWC (which required 7, 12, 12 and 25 days for pots D5, D6, D7 and D8, respectively, Figure 3.4).

3.4.3 Leaf Elongation Rate (LER)

The LER of the control plants (C5 and C6) remained above 16 and 10 mm.d⁻¹ (respectively) during the experiment (Figure 3.6). However, the LER of these plants was not constant, with day to day fluctuations of up to 17 mm.d⁻¹. A general increase (*ca.* two-fold) in the LER occurred between 6 and 15 days, and was likely to be due to environmental conditions such as an increase in sunlight or glasshouse temperature (data was not collected).

The LER declined in response to the water-deficit treatment (Figure 3.6). Although the LER of the two fast-drying plants (D5 and D6) declined from the outset of the experiment, it was not until after 6 or 7 days (D5 and D6, respectively) that the LER of these plants declined below that of the controls (10-12 mm.d⁻¹). The LER continued to decline to become close to zero (indicating the cessation of growth) after 8-9 days.

The LER of plants D7 and D8 remained between 10 and 32 mm.d⁻¹ for the first 20 days, before declining to low levels (Figure 3.6). In D7, the LER remained low for the rest of the experiment. The dry-down of D8 (and to a lesser extent, D7) was irregular in that there appeared to be two cycles of growth. The LER of D8 had almost ceased by day 27, resumed by day 32, and had ceased again by day 40. This second cycle of growth may possibly have been caused by a small amount of rain water leaking onto the plant, although every effort was made to locate plants in a leak-free area of the glasshouse. The rain leak at day 30 also appeared to effect a small growth increment (up to 5 mm.d⁻¹) in D7. This event was not accompanied by a measurable change in the SWC (Figure 3.4), indicating that the quantity of water involved was not sufficient to wet the soil down to the depth of the topmost TDR electrode station.

The LER was dependent on the SWC. Most of the LER measurements were between 10 and 30 mm.d⁻¹, but when the SWC declined to less than 10%, the LER in the water-deficit treatment plants also declined; cessation of leaf elongation occurred at a SWC of *ca.* 7-8% (Figure 3.10).

Soil Water Gradients: Experiment II

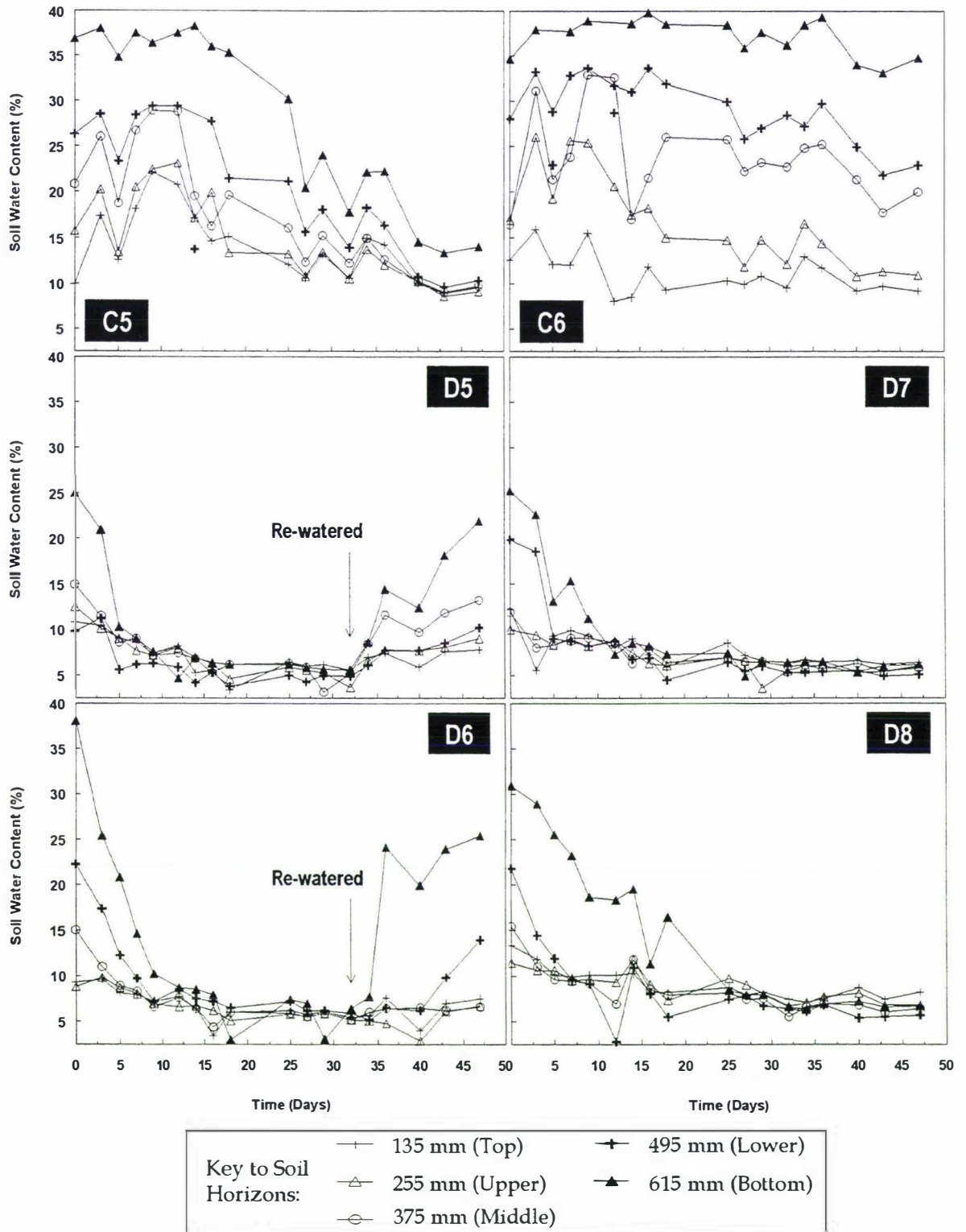


Figure 3.4: The SWC measured in the five soil horizons during experiment II. The labels indicate pot numbers. Pots D5 and D6 were re-watered on day 32 (arrowed).

Mean Soil Water Content: Experiment II

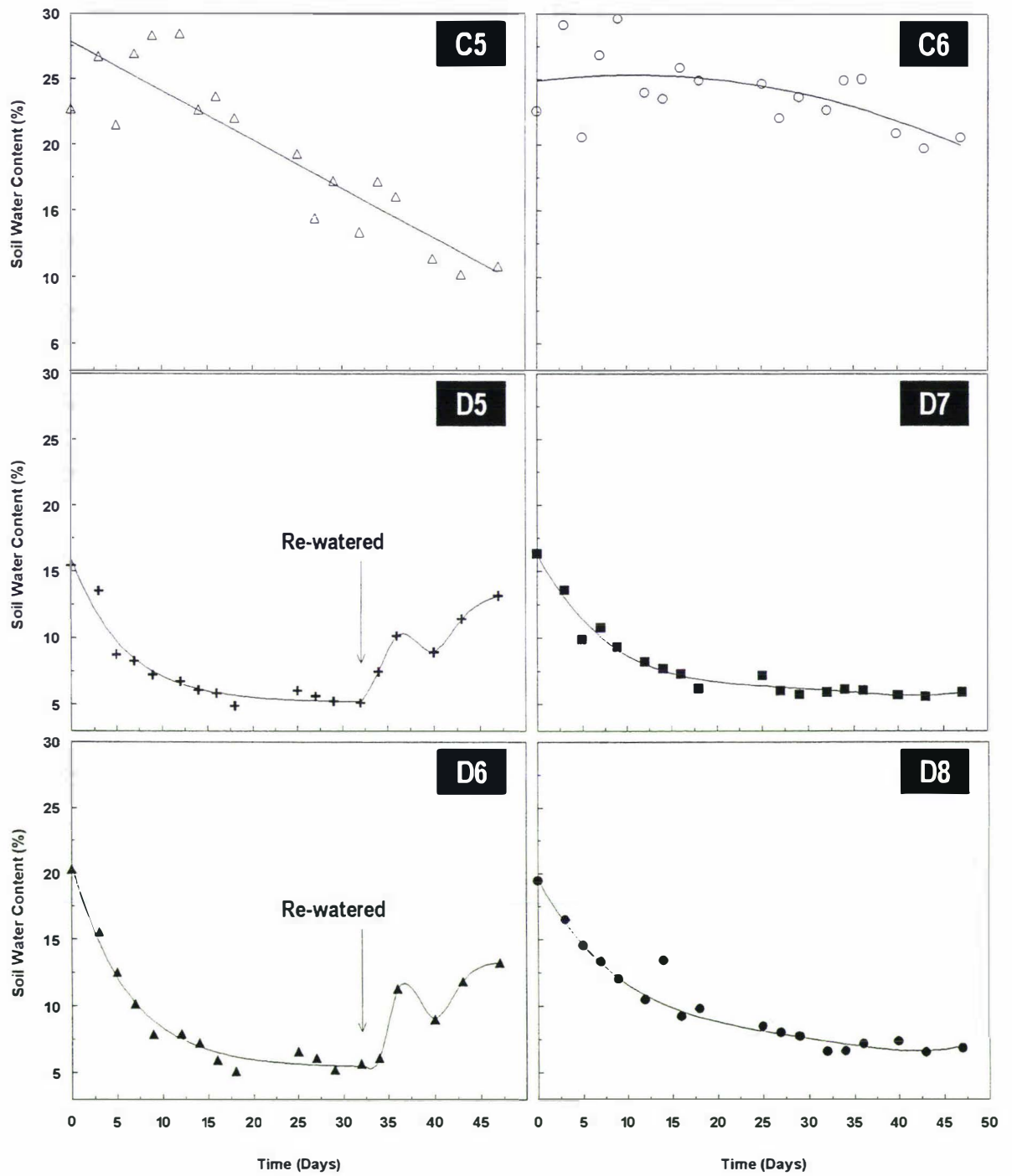


Figure 3.5: Mean SWC measured during experiment II. The labels indicate pot numbers. Pots D5 and D6 were rewatered on day 32 (arrowed).

Leaf Elongation Rate: Experiment II

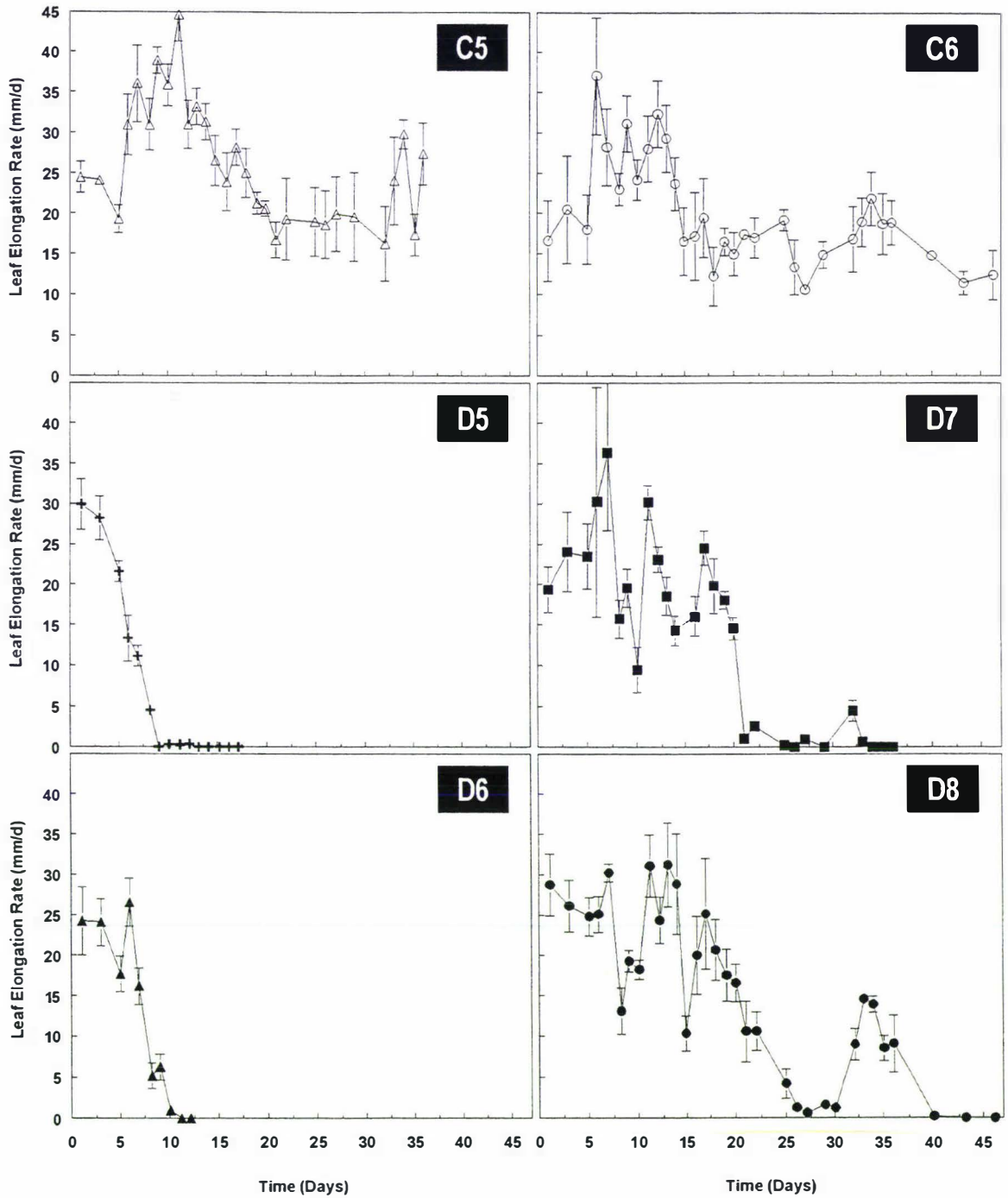


Figure 3.6: The LER measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., $n=3$.

3.4.4 Water-Deficit Stress Induced Metabolites

3.4.4.1 Leaf ABA Concentration

The ABA concentration of ML excised from control plants (C5 and C6) was in the range of 5 to 30 ng/g FW (Figure 3.7). The ML from water-deficit treatment plants accumulated ABA up to *ca.* 200 ng/g FW (Figure 3.7), which represented an order of magnitude increase in concentration in response to the treatment. The onset of accumulation occurred after 3, 6, 20 and 22 days (D5, D6, D7 and D8, respectively), although transient increases in ABA concentration above the levels measured in the ML from control plants occurred in plant D7 before this date (Figure 3.7).

The water-deficit induced accumulation of ABA appeared to be to some extent reversible, since the ABA concentration in D7 and D8 decreased quickly in response to the rain-water leak on day 30 (Figure 3.7). Further dry-down of D8 resulted in the return of ABA concentration to high levels. The onset of this second accumulation occurred after 36 days (Figure 3.7).

A temporary decline in the ABA concentration of three of the four water-deficit treatment plants occurred on day 11 (Figure 3.7). Since this occurred in several plants, the cause of the latter event was attributed to environmental conditions. However, the nature of the cause was not known.

The ABA concentration in laminae of *F. arundinacea* was found to be related to the SWC, where the onset of ABA accumulation occurred after the SWC had declined below *ca.* 8% in each of the water-deficit treatment pots (Figure 3.11).

3.4.4.2 Leaf Proline Concentration

The lower levels of proline in the ML of *F. arundinacea* were in the range 20 to 70 $\mu\text{g/g}$ FW, and remained constant throughout the experiment so long as the plants remained water-sufficient (C5 and C6, Figure 3.8). Proline concentrations increased in response to the dry-down treatment (Figure 3.8). The actual timing of the onset of the accumulation of proline varied between plants, and occurred after 8 (plants D5 and D6), 21 (D7) and 25 days (D8). From this time, proline levels continued to rise for the rest of the experiment in D5 and D6, and peaked on day 30 in D7. The proline concentration of the

ML responded to re-watering. Coincident with the rain-water leak on day 30, the proline concentration declined toward the end of the dry-down in D7 (Figure 3.8). In D8, two phases of proline accumulation were observed. In this plant, proline began to accumulate after day 25, peaked at day 30, and then declined to relatively low levels. The second cycle of proline accumulation had commenced by day 40, and proline continued to accumulate in this plant for the rest of the experiment.

The maximum increase in leaf proline concentration (up to 2.3mg/gFW) was observed in D5. This reflects an *ca.* 50-fold increase over the mean value of the control plants. The proline concentration in ML of *F. arundinacea* was related to the SWC, where the onset of proline accumulation in the water-deficit plants occurred after the mean SWC had declined to *ca.* 7-8% (Figure 3.12).

3.4.4.3 Leaf Glycine-Betaine Concentration

The glycine-betaine concentration in the ML from water-sufficient plants (C5 and C6) remained between 6-9, and 9-11 mmol/g FW, respectively (Figure 3.9) during the experiment. A single datum (C6, day 30) was sufficiently separated from the data trend to be considered as an outlier.

The glycine-betaine concentration increased approximately linearly from the outset of the experiment to around two-fold in response to the water-deficit treatment (Figure 3.9). The glycine-betaine concentration in D5 increased from 12 mmol/g FW to over 24 mmol/g FW by day 20. In the ML from D6 and D7, the glycine-betaine concentration increased above the levels of the controls by 8 and 17 days, respectively, and continued to increase for the rest of the experiment. Although fluctuations in glycine-betaine concentration were observed in D8, an accumulation of this metabolite above that of the controls was not observed until after day 30. In contrast to the proline and ABA concentration of D7 and D8, the glycine-betaine concentration did not decline after the rain-water leak on day 30.

The glycine-betaine concentration of the ML of *F. arundinacea* was related to SWC, where the onset of glycine-betaine accumulation occurred after the mean SWC had declined below 8% (Figure 3.13).

ABA concentration: Experiment II

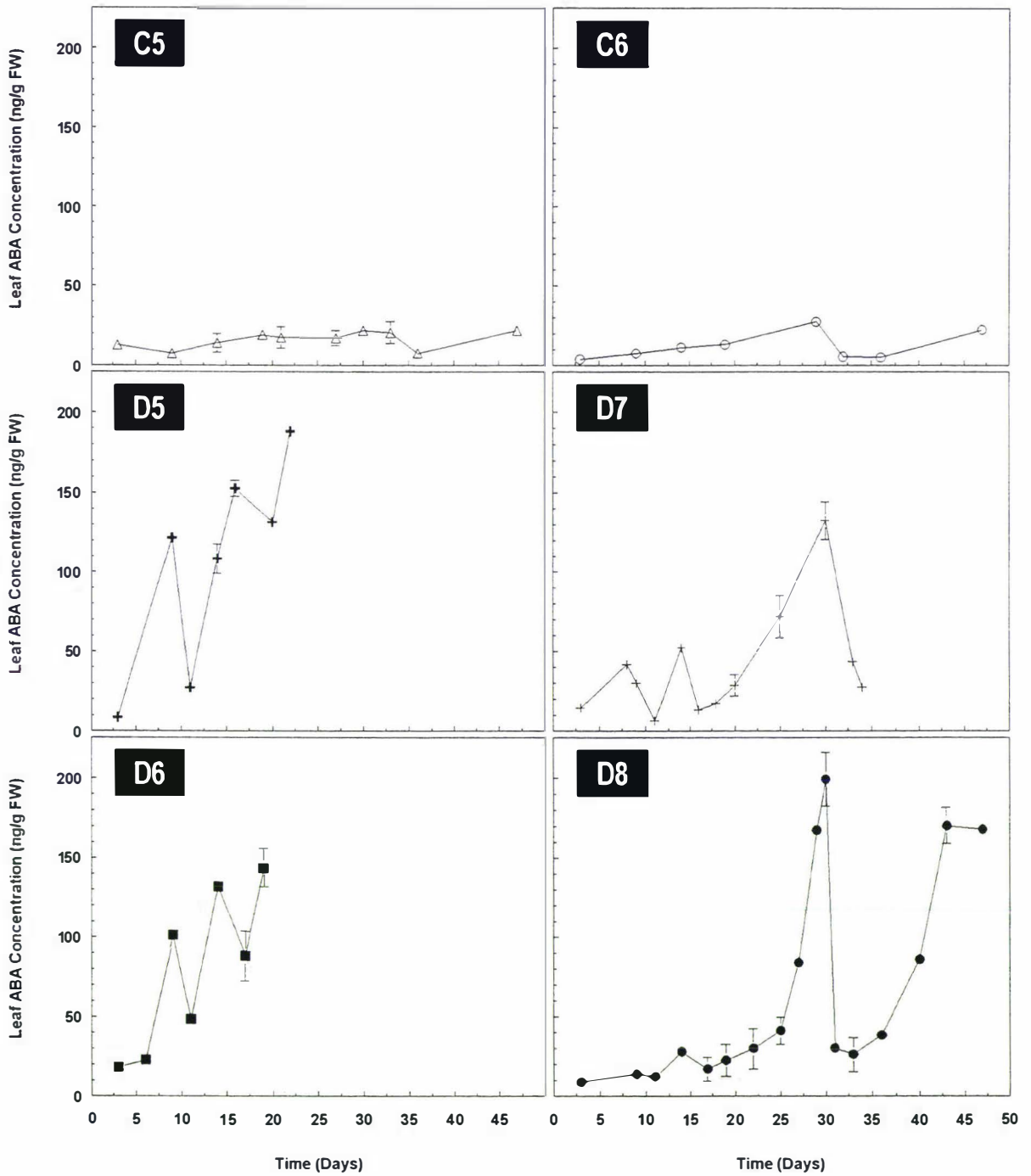


Figure 3.7: ABA concentration measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., n=2.

Proline concentration: Experiment II

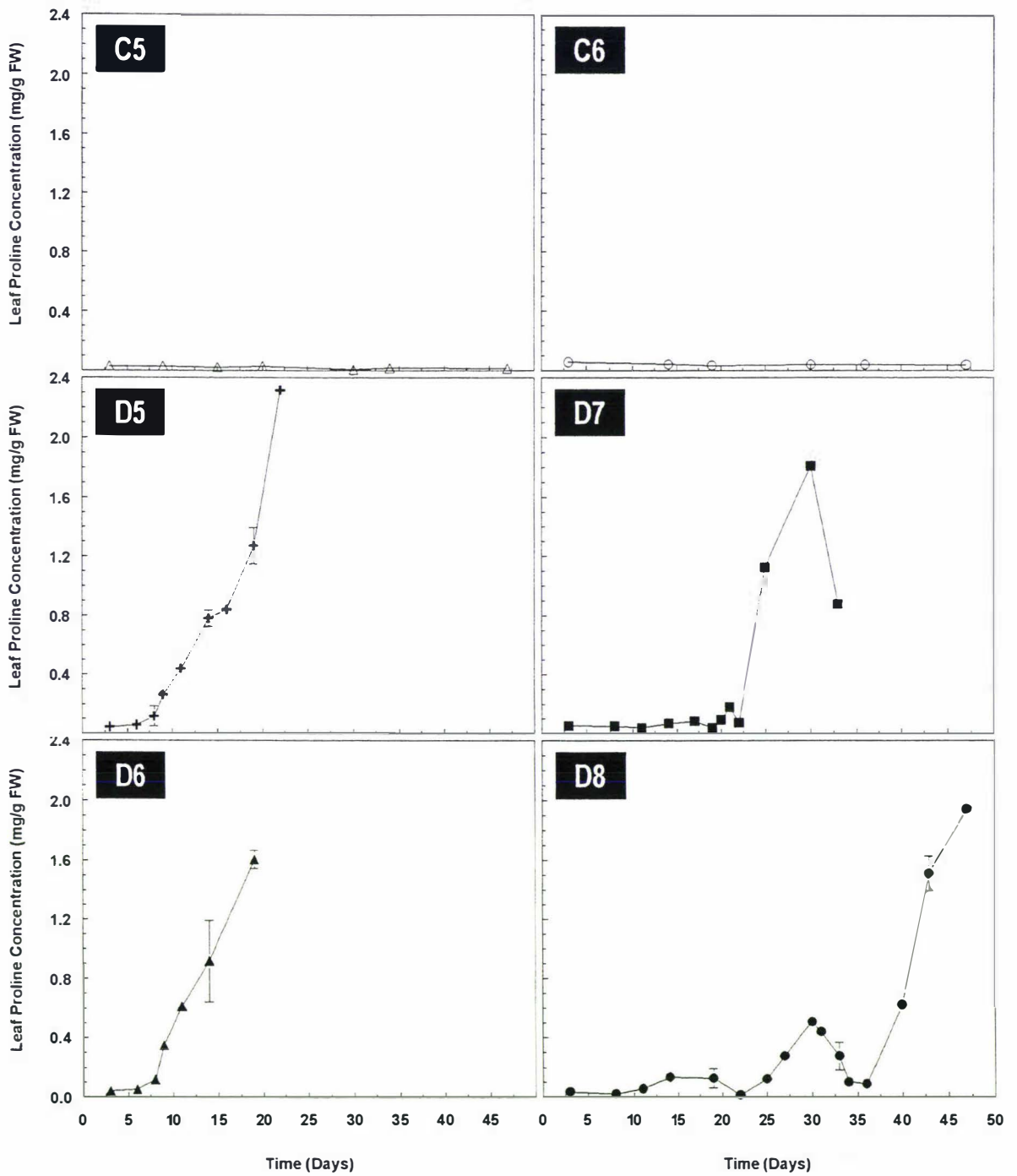


Figure 3.8: Proline concentration measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., $n=2$.

Glycine-betaine concentration: Experiment II

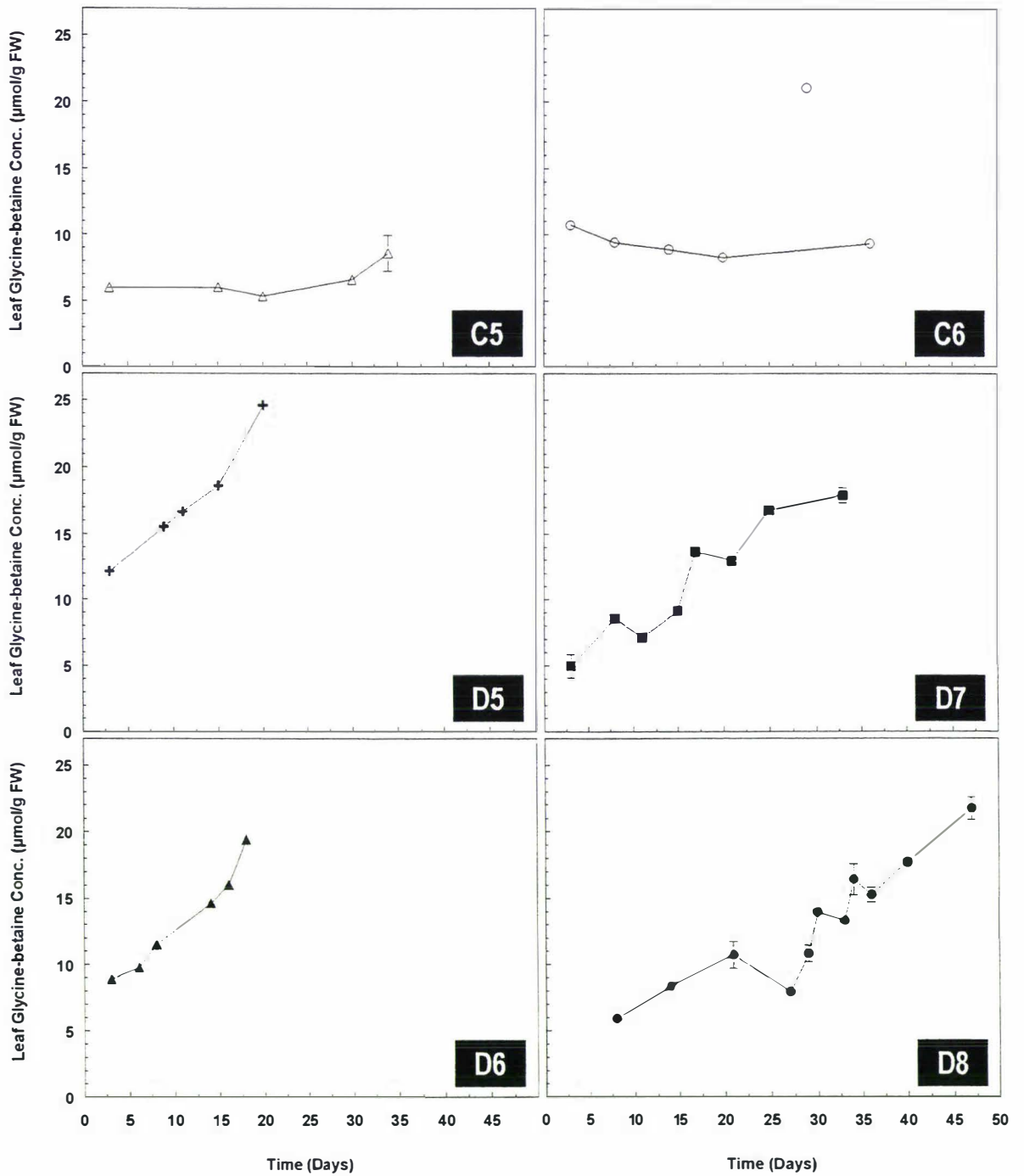


Figure 3.9: Glycine-betaine contents measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., $n=2$, except for D5 and C6 ($n=1$).

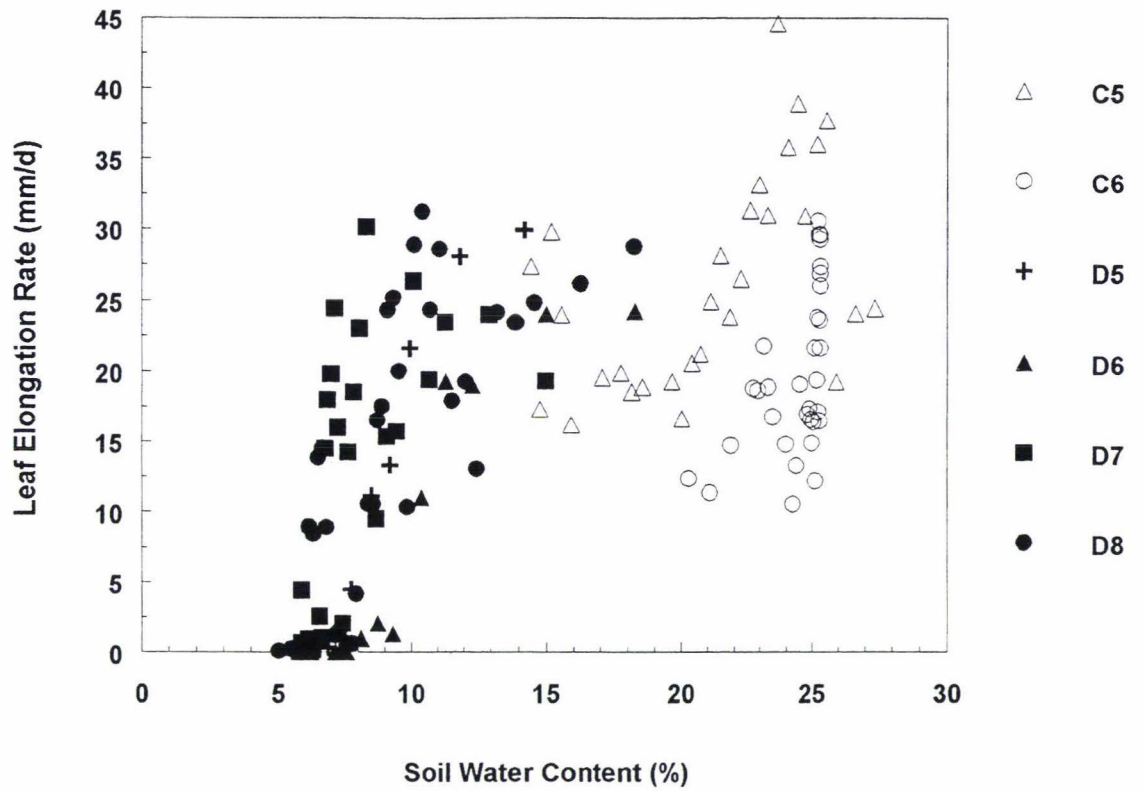


Figure 3.10: The effect of declining SWC on LER during experiment II. Data from Figure 3.6 has been replotted as a function of SWC. The legend indicates plant numbers. Data were means, $n=3$ (Error bars omitted for clarity, see Figure 3.6).

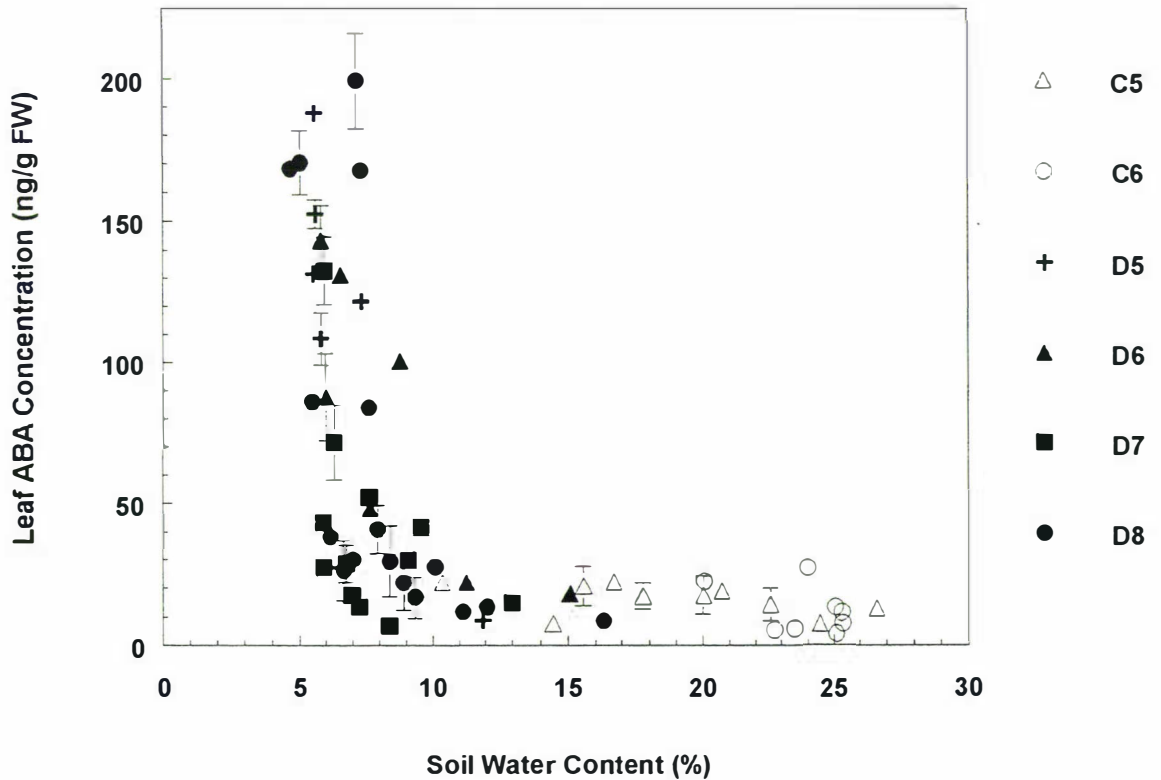


Figure 3.11: The effect of declining SWC on leaf ABA concentration during experiment II. Data from Figure 3.7 has been replotted as a function of SWC. The legend indicates plant numbers. Data were means \pm s.e., $n=2$.

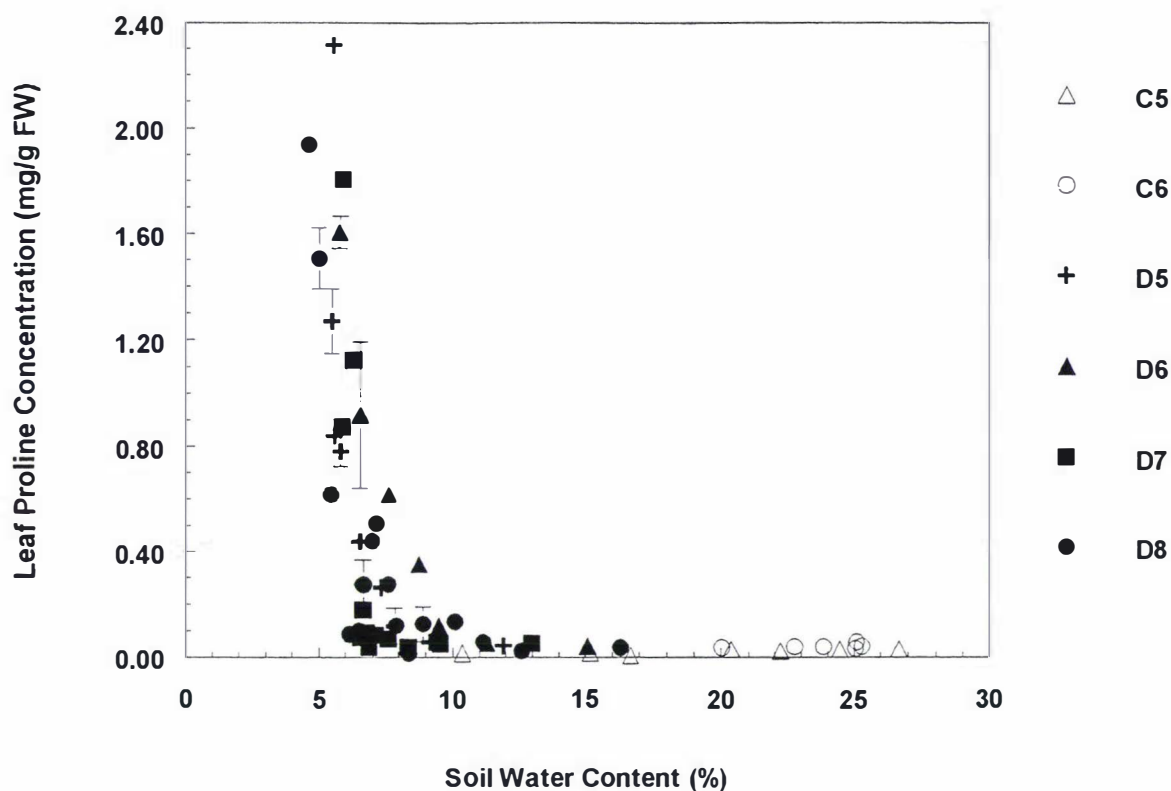
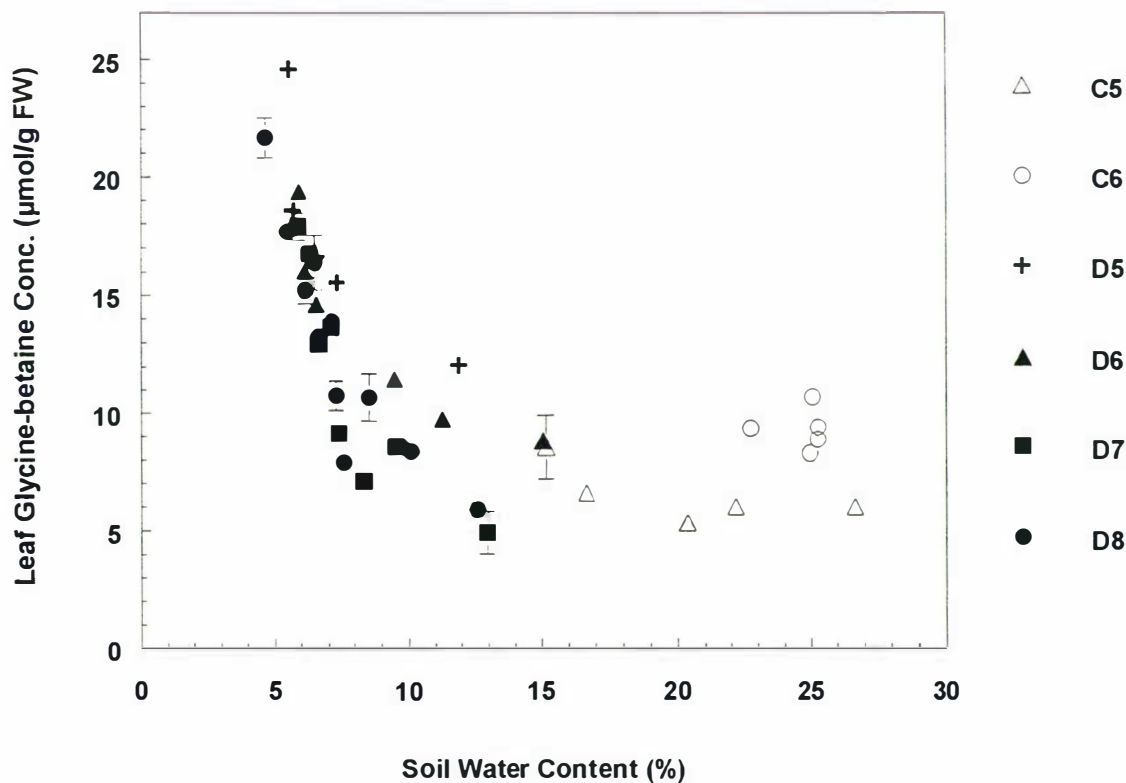


Figure 3.12: The effect of declining SWC on leaf proline concentration during experiment II. Data from Figure 3.8 has been replotted as a function of SWC. The legend indicates plant numbers. Data were means \pm s.e., n=2.



3.4.5 Distribution of Root mass

At the conclusion of experiment II, the distribution of roots from one water-deficit treatment (pot D8), and one control plant (pot C5) were determined (Figure 3.14). In both cases, the root mass was greatest near the top of the soil column, and root density decreased with soil depth. Toward the bottom of the soil column root density increased, presumably as a result of deep roots striking the base of the pot and continuing to grow in that region. The roots at the top of the soil column appeared to contain more fine root material (although some loss of fine roots occurred during preparation), and the deeper roots were more coarse in structure (Plate 3.1).

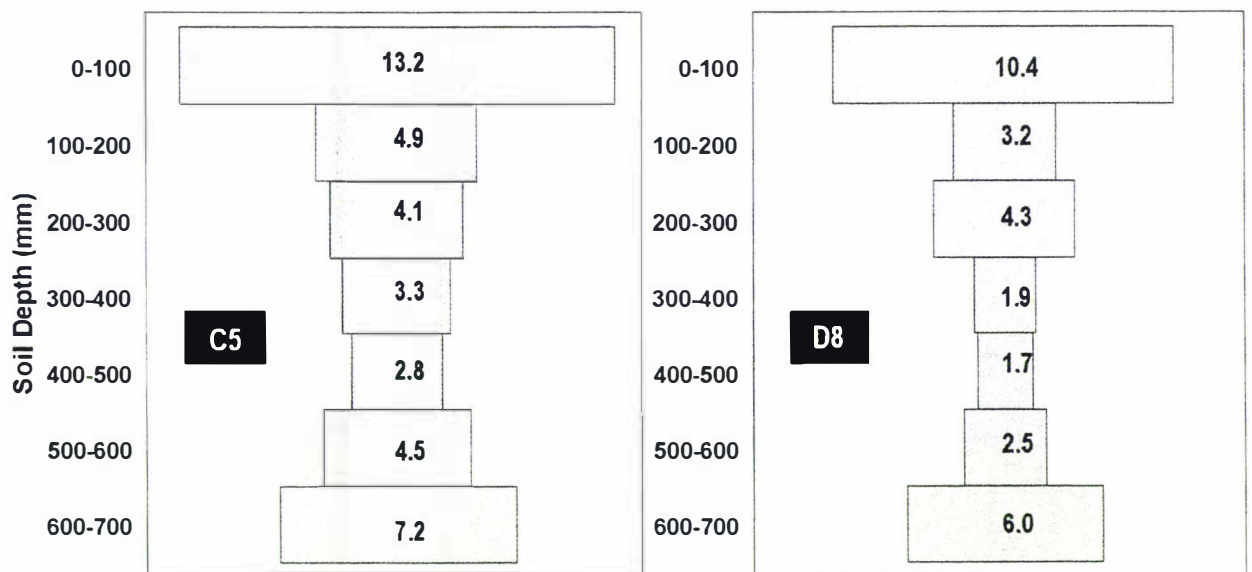


Figure 3.14: Root distribution of representative plants of *F. arundinacea* grown in the 24 L experimental pots. Labels indicate pot number. Bar numbers represent root mass (gDW).



Plate 3.1: Distribution of the roots of *F. arundinacea*, within the soil column from one of the 24 L pots (plant D5).

3.4.6 The Relative Water Content (RWC) of Water-Sufficient *F. arundinacea*

The RWC of several tissues derived from tillers of *F. arundinacea* was determined to trial the method of RWC estimation with this species (Table 3.2). The RWC data was also used to gain an estimate of the fresh mass: dry mass ratio, which was useful as an aid for interpreting published data for this species.

Table 3.2: The RWC of tiller tissues from *F. arundinacea*. Data were means \pm s.e., n=3.

Tissue		RWC	H _L	FW: DW ratio
Leaf base	(LB)	0.864 \pm 0.01	7.04 \pm 0.35	0.13 \pm 0.01
Immature pseudostem	(IP)	0.693 \pm 0.02	7.75 \pm 1.30	0.12 \pm 0.02
Mature pseudostem	(MP)	0.781 \pm 0.02	4.52 \pm 0.43	0.18 \pm 0.02
Immature lamina	(IL)	0.831 \pm 0.00	5.23 \pm 0.47	0.16 \pm 0.01
Mature lamina	(ML)	0.782 \pm 0.06	4.05 \pm 0.54	0.21 \pm 0.03

3.5 Discussion

Before the start of experiments I and II, each pot was watered to saturation, and the progression of the dry-downs was followed by monitoring the decline in SWC. The SWC of each dry-down pot declined in an expected exponential decline, where the rate of water loss was greatest at the start of the drydown, and the rate slowed as SWC declined. The loss of this water was assumed to be predominantly *via* plant transpiration, and to a small extent by evaporation from the soil surface (Dr. D. Barker, pers. comm.).

The decline in SWC during experiment I was almost linear for the first 15 days (Figure 3.1). However, additional attempts in the summer to dry-down *F. arundinacea* using the 4 L pot system were abandoned, because the SWC declined up to three times faster, from saturation to *ca.* 5% in 3-5 days (data not shown). This period was considered to short to allow plants to adapt to the increasing water-deficit stress. The difference in the rate of transpiration between the 4 L pot experiments may have been due to warmer summer weather, and may also have reflected differences in plant size. Although leaf area was not measured, larger leaf surface areas would allow more transpiration to take place.

Therefore to increase the period of the dry-down, larger pots of greater soil volume and with single plants were chosen for further experiments with this species. In addition, the larger pots would be better able to accommodate plants of *F. arundinacea* as the roots quickly become bound when grown in the 200 mm deep, 4 L pots.

The 24 L pot system used in experiment II successfully slowed the rate of dry-down. The SWC decline measured in these pots varied with soil depth. Water was removed from all five soil horizons during the experiment, with the shallow soil horizons losing water at the greatest rate (Figure 3.4). This was consistent with the pattern of root distribution within the 24 L pot, where the greatest density of roots was located in the top soil horizon (Figure 3.14 and Plate 3.1). In all the water-deficit treatment pots from experiment II, the initial rate of SWC decline in each soil horizon was rapid, and approached zero when the SWC reached *ca.* 7%, as compared to 5% during experiment I.

In one of the control pots (C6) used during experiment II, the SWC was maintained at a relatively high level for most of the experiment. However, despite regular watering, the SWC in C5 declined in all soil horizons during the experiment (Figure 3.4), although the SWC of C5 did not fall to a level low enough to cause water-deficit stress on the plant, as evidenced by the LER, and metabolite analyses from this plant (figures 3.6 to 3.9) where responses observed in the water-deficit treatment plants (discussed below) were not observed in C5.

Since the roots were found to occupy the entire depth of the 24 L pots, the SWC of all the five soil horizons was averaged to follow the progress of the mean SWC within each pot during the dry-down (Figure 3.5). The water-deficit treatment pots dried at different rates, which may have resulted from variation in plant size, although the leaf area was not measured. The mean SWC declined from soil water capacity to 7-8% in *ca.* 8, 14, 20, and 28 days (D5, D6, D7 and D8, respectively). Therefore in the latter two cases, the use of the 24 L pot system was able to significantly increase the period of the dry-down over the 4 L pot system. It is also interesting to note that some of the roots (especially near the surface) may have become water-deficit stressed before others, due to the gradients of water formed in the 24 L pots. Therefore if plants are able to sense dry soil, and produce a signal that is transmitted to the shoots (Tardieu *et al.*, 1992), the plants may have sensed the water-deficit stress by some roots before the lack of water absorption by all of the roots became limiting to growth, thus allowing a longer period of water-deficit adaption to take place.

The cessation of LER was consistent between the two dry-downs, occurring at 6-7% and 7-8% SWC for experiments I and II, respectively. The mean LER of water sufficient IL was $36.4 \pm 1.2 \text{ mm.d}^{-1}$ during experiment I (Figure 3.2), and $22.7 \pm 1.0 \text{ mm.d}^{-1}$ during experiment II (Figure 3.6). Seasonal differences in day length and temperature between the two experiments (early spring versus autumn, respectively) may account for the difference in LER, since Gastal *et al.* (1992) reported that the LER of nitrogen sufficient *F. arundinacea* was closely related to temperature. The continuous measurement of light and temperature during dry-down experiments using a datalogger type device may provide a means of experimentally testing this possibility.

ABA accumulated *ca.* ten-fold in response to water-deficit treatment in *F. arundinacea* (Figure 3.7), which was comparable to a recent report where a 16-fold increase in ABA concentration was observed during drought acclimation in this species (Joost and Holder, 1994). Other than the abstract published by Joost and Holder (1994), no further reports of ABA accumulation in response to water-deficit stress by *F. arundinacea* have been published. When adjusted for FW to DW differences (the dry mass of the ML was calculated from the RWC data (Table 3.2) to be an average of 20% that of the fresh mass), the levels of ABA were estimated to be 50 to 100 ng/gDW in control tissue and up to 1,000ng/gDW in stressed tissue (Figure 3.7). These values were similar in magnitude to data reported (200 to 300 ng/gDW in control tissue, and up to 1,200 ng/gDW in drought-stressed tissue) for *O. sativa* leaf material (Dingkuhn *et al.*, 1991).

The rain-water leak that occurred in D7 and D8 on day 30 of experiment II caused a reduction in ABA concentration to non-stressed levels. Similar reductions in ABA concentration to re-watering have been described (e.g. Dingkuhn *et al.*, 1991). The responsiveness of ABA to water-deficit stress and re-watering supports the view that ABA concentration was likely to serve as a useful metabolic marker for use in further dry-down studies.

The accumulation of proline by *F. arundinacea* was consistent with a report by Bokhari and Trent (1985) where the proline concentration of the vegetative tissue of *F. arundinacea* increased from 3.94 mg/gDW to 7.48 mg/gDW, in response to a glasshouse water-deficit experiment. This was comparable to the current study after adjustment for the difference between FW and DW as above. The proline accumulation in response to water-deficit treatment in experiment II was up to 11.5 mg/gDW, although Bokhari and Trent's estimate of proline concentration from water-sufficient tissue was high, compared to <350 µg/gDW from the control plants of experiment II (which meant that the *ca.* two-fold accumulation reported by Bokhari and Trent was relatively low). The non-specificity of the tissue sampled ("vegetative tissue") by Bokhari and Trent (1985), may account for the difference in proline concentration, since the ML contained less proline than the LB of water-sufficient *F. arundinacea* (*c.f.* section 5.3.1.2, page 160). However, the proline concentration of *F. arundinacea* may also be a genetically

determined factor, as indicated by variation in proline accumulation by various cultivars of *F. arundinacea* (Belesky *et al.*, 1982). The concentration of proline accumulated in response to water-deficit stress reported by Bokhari and Trent (1985) compares favourably to that observed in experiment II, where it increased to 8-11.5 mg/gDW. The degree of proline accumulation observed (40-fold increase), was not considered to be unusually great, since it has been reported to accumulate to high levels in many other species in response to drought, including monocots (for example: 195-fold in *T. aestivum* (Munns *et al.*, 1979), see Delauney and Verma (1993) for a review).

The existence of glycine-betaine in tissues of *F. arundinacea* has not been previously reported. However, this compound has been reported to exist in a number of graminaceous species, including *T. aestivum* (Naidu *et al.*, 1991), *H. vulgare* (Jones *et al.*, 1986) and *Z. mays* (Lerma *et al.*, 1991; Yang *et al.*, 1995), and the accumulation of glycine-betaine in response to drought in *H. vulgare* has also been reported (Ladyman *et al.*, 1983; Zuniga *et al.*, 1989). The concentration of glycine-betaine in *F. arundinacea* was comparable to that measured by Ladyman *et al.*, 1983. In this report, the glycine-betaine concentration in whole shoots of *H. vulgare* increased from 20 to 31 $\mu\text{g/gDW}$ to 48 to 67 $\mu\text{g/gDW}$ during a controlled-environment study, and in laminae was measured to a maximum of 84 $\mu\text{g/gDW}$, in response to a field drought experiment. For the purposes of comparison, the data from experiment II can be converted from molarity to mass units, and adjusted to take into account the FW/DW difference. After these conversions, control levels of glycine-betaine were observed to be in the range of 30 to 40 $\mu\text{g/gDW}$, which increased to 90 to 125 $\mu\text{g/gDW}$ in response to the water-deficit treatment. Hence the two-fold accumulation of glycine-betaine observed in experiment II was considered to be sufficient in magnitude to serve as a potential metabolic indicator of water-deficit stress in *Festuca* spp.

3.5.1 The Occurrence of a Trigger Point

Although differences occurred in the time taken for individual plants both between experiments, and within each experiment to dry-down, a common element of timing occurred as to the onset of changes in each marker used within each plant.

During experiment I, both the LWP and LER of the water-deficit treatment plants declined below those of the control plants, after the SWC had been reduced to *ca.* 7-8%. The water-deficit stress responses during experiment II are summarised in Table 3.3. As can be seen from this table, there was a consistent timing in the onset of changes in each marker used (LER, ABA, proline), which indicated that the plants began to respond to the water-deficit treatment after *ca.* 6, 6, 20, and 22 days (D5, D6, D7, and D8, respectively). However, the onset of glycine-betaine accumulation did not coincide with the other markers followed. In fact, a gradual increase in glycine-betaine concentration occurred in all water-deficit treatment plants, throughout the experiment, and a clear response to the rain-water leak on day 30 in D7 and D8 was not observed (Figure 3.9). It is possible, then, that the response of glycine-betaine concentrations are not under the same metabolic control as are LER, ABA or proline accumulation. Glycine-betaine is known to accumulate in many species in response to saline (osmotic) water-stress (McCue and Hanson, 1990). Since the removal of water from the soil will doubtless increase the ionic concentration in the soil, the changes in glycine-betaine concentration observed during experiment II may have been a response to an increase in salinity, and not a direct response to a reduction in SWC. However, this possibility does not negate the usefulness of glycine-betaine as a metabolic marker to indicate water-deficit stress within a plant, since changes in the concentration of solutes in the soil are an inevitable consequence of any drought. The accumulation of glycine-betaine may also have been a passive response to the water-deficit treatment, resulting from the concentration of cellular osmolytes due to a reduction in water content.

Table 3.3: Summary of the timing of water-deficit responses during experiment II.

Plant	LER ¹ (SWC)	Proline (SWC)	ABA (SWC)	Glycine-betaine (SWC)
D5	6 (9%)	7-8 (8%)	>3 (12%)	<1 (15%)
D6	6-7 (10%)	7-8 (9-10%)	6-8 (9-10%)	6-8 (9-10%)
D7	20-21 (7%)	20-21 (7%)	20 (7%)	15-17 (7.5%)
D8 ²	22-23 (8%)	22-25 (8%)	22-25 (8%)	27-30 (7.5%)
D8	36-37 (7%)	36-40 (7%)	36 (7%)	-

¹ Data presented is the day of the first apparent increase above (for the three metabolites) or decrease below (LER) control-plant levels.

² Due to the rain-water leak, D8 had two cycles of drought, separated by re-wetting.

In this experiment also, changes in the markers were linked to the SWC, where the onset of metabolic adaption occurred when the SWC had declined below 10% (Table 3.3). Together, the data is indicative of a trigger-point type phenomenon occurring in water-deficit stressed *F. arundinacea*. That is, that changes in the metabolism of *F. arundinacea* were induced once the water-deficit had reached a certain level of severity. In this case, the trigger-point appeared to occur when the SWC had declined to 8%. However, care needs to be taken when using SWC in interpreting the response of plants to water-deficit stress, since the SWC does not necessarily measure the availability of water to the plant (the TDR averaged the pot SWC, which does not take into account the distribution of roots within the soil), SWC should be used as a guide, and not as an absolute indicator, as to the water-status of a plant. The results reported here show that internal markers, such as the accumulation of ABA and osmoprotectants by the plant, may be more useful in the latter role.

3.6 Conclusion

The methodology used for these experiments, particularly the use of the 24 L pot system, and the use of biochemical traits as markers have been shown to be suitable for the study of water-deficit adaption. Using the 24 L pot system, dry-down periods running up to 47 days were achieved, which was considered to be an adequate time for the process of water-deficit adaption to occur. Adaption of the metabolism of *F. arundinacea* to increasing water-deficit stress was verified. Cessation of LER, and increases in ABA, proline and glycine-betaine concentrations in the ML of *F. arundinacea* were observed, and a common timing element to the induction of these changes indicated a "trigger point" during water-deficit stress adaption. Hence these results confer some confidence to expand the study of adaption to water-deficit stress to the native xerophyte, *F. novae-zealandiae*.

Chapter Four

Experimental Dry-Down of *F. novae-zealandiae*

4.1 Introduction

As an initial examination of the drought tolerance mechanisms of *F. novae-zealandiae*, a comparison of leaf anatomy from *F. novae-zealandiae* and *F. arundinacea* was carried out. This was conducted to gather evidence that the native tussock possesses morphological adaptations that could reduce the possibility of water-deficit stress affecting the plant.

This chapter also reports the results from two experiments conducted to determine if *F. novae-zealandiae* responds physiologically and/or metabolically to water-deficit stress. These experiments used the same methodology as described for the those with *F. arundinacea*, using the 24 L PVC pot system. Data was gathered by monitoring the LER, the number of tillers per plant, the degree of scorching on each leaf of several tillers per plant, and by measuring the leaf contents of the metabolic marker compounds proline, glycine-betaine, and ABA. The principal aim of this chapter was to use the data collected to propose a hypothesis on how *F. novae-zealandiae* responded to water-deficit stress, and to propose means for the experimental examination of aspects of this hypothesis.

4.2 Methods

4.2.1 Plant Material

Six *F. novae-zealandiae* plants of genotype T2 were used in experiment I; two of these plants were maintained as watered controls (C1 and C2), and water-deficit was applied to the remaining four (D1 to D4).

Six *F. novae-zealandiae* plants were used for experiment II. Three of these plants were T2 genotype, of which two were exposed to water-deficit (D8 and D9) and one was used as a watered control (C8). The other three plants used in this experiment were derived from seed that was collected from North Canterbury (section 2.1.2, 21). Of these, two

were exposed to water-deficit (D6 and D7) and one was used as a watered control (C6). For the SEM study, plants of *F. novae-zealandiae* (T2 genotype), or *F. arundinacea* (TF-1 genotype) were used.

4.2.2 SEM Examination of *Festuca spp.*

Portions (10 mm) of the ML, occurring about one third of the length of the laminae down from the leaf tip, were excised from water-sufficient plants of both *Festuca* species. Once sampled, the leaf segments were sliced in fixative and examined under a Scanning Electron Microscope (SEM) as described in section 2.17 (page 62). The density of stomata present on each surface of the leaves was estimated from the electron-micrographs, using scale bars to calculate the surface area in view.

4.2.3 Experimental Dry-Down

Plants were established over nine or seven weeks (experiments I and II, respectively) in the 24 L pot system described in section 2.2.2, page 24. Water-deficit was initiated by withholding water; watering was continued in the control plants for the duration of each experiment. The SWC during both experiments was monitored by TDR, using five electrode stations down the side of each pot. An additional pair of 220 mm electrodes was inserted vertically into the soil surface, for use during experiment II. A comparison of the methods used for each experiment is presented in Table 4.1.

Samples of the ML were taken at regular intervals during each experiment. A number of laminae were excised at the ligule, and pooled to supply at least 300 mg FW of tissue for metabolite analyses. Samples were frozen in liquid nitrogen and stored at -80 °C until processing, which consisted of cooling in liquid nitrogen and powdering with pestle and mortar. The powder was then stored at -80 °C until required for analytical assay. Samples of frozen tissue were analysed for ABA, glycine-betaine or proline concentration as required, using the methods described in sections 2.8, 2.9 and 2.10 (pages 27, 37 and 45, respectively).

On each sampling date during experiment II only, duplicate ML were excised at the ligule for LWP measurements, which were carried out using the method described in section 2.4.1, page 26.

Table 4.1: Comparison of the methods used for experiments I and II.

	Experiment I	Experiment II
Pot system used:	24L (silt loam)	24L (silt loam)
Number of pots : Water deficit treatment:	4	4
Control:	2	2
Pot numbers:	D1 to D4, C1 and C2	D6 to D9, C6 and C8
Number of plants per pot:	1	1
Plant genotype:	T2	T2 or seed derived ¹
Number of TDR electrode pairs:	5 (side)	5 (side) + 1 (vertical)
Number of tillers measured for LER:	3	3
Tiller count (T_{Num}):	End of experiment	During experiment
Tiller mass (M_T), number of green leaves per tiller (N_T), leaf-tip scorching (D_s) measured:	✓	✓
LWP measured:	✗	✓
ABA, proline, glycine-betaine measured:	✓	✓
Root distribution determined:	✓	✓
¹ Plants C8, D8 and D9 were T2 genotype; plants C6, D6 and D7 were derived from seed.		

Throughout both experiments, the LER was measured on three tillers per plant, using the method described in section 2.5, page 26.

It became clear during experiment I that plants that had been subjected to water-deficit became visibly distinct from the watered plants (the former became almost entirely fawn-brown in colour; watered plants were predominantly green with some brown leaf-tip scorching; Plate 4.2). Therefore, several tiller measurements were taken, and assessed for use as physiological indicators of water-deficit stress severity. On the last day of experiment I, the total number of tillers in each plant were counted and scored by colour as either green or brown. Tillers were scored on outward appearance only; dissection of tillers to locate green tissues within the tiller was not carried out. A number of tillers (up to 12) per plant were stripped of the older, brown leaves, weighed, and the number of green leaves on each tiller (N_T) were counted. The length of each lamina on the tiller was measured, as was the length of any scorching present at the tip. This procedure was also followed at the end of experiment II. However, the number of tillers in each plant was also counted on several occasions during this

experiment. At the conclusion of each experiment, the distribution of the roots of all but one plant (D3) were determined using the method described in section 2.6 (page 27). Plant D3 from experiment I was washed free from the soil column intact, and photographed.

4.3 Results

4.3.1 Morphological Comparison of *Festuca* spp.

Tillers of *F. arundinacea* hold three to four dark-green leaves. The ML were up to ca. 15 mm wide, and 450 mm long, and appeared to be supported predominantly by cell turgor. Consequently the leaves wilted severely when subjected to water-deficit stress (Plate 4.1). Leaf rolling was also apparent in water-stressed *F. arundinacea* leaves (Plate 4.1), and upon senescence, the leaves collapse to ground level.

Tillers of *F. novae-zealandiae* were tightly packed, and tussocks had a range of colours, ranging from dark-green to lime-green and cyan. Each tiller held three to four green leaves, although the older leaves were generally scorched severely at the tips. Older leaves did not yellow upon senescence, but leaf turnover appeared to progress *via* increasing leaf-tip scorching, until the entire leaf had browned off. Dead leaves did not wilt appreciably.

Portions of the ML from both *Festuca* spp. were examined using a SEM. Leaves of *F. arundinacea* were characterised by a smooth abaxial surface, and a convoluted adaxial surface, composed of vascular ribs separated by fans of bulliform cells (Plate 4.3). Vascular bundles were supported by abaxial and adaxial girders and sclerenchyma (fibre) bundles at each leaf surface. A low density of trichomes occurred on the adaxial surface only. Stomata were found on both surfaces of the leaf, with a greater density occurring on the adaxial surface (Table 4.2).

Leaves of *F. novae-zealandiae* are similar in structure to those of *F. arundinacea*, but with the leaf curled such that the adaxial surface is almost entirely enclosed (Plate 4.5A). The convoluted adaxial surface is composed of ribs of vascular tissue separated by fans of bulliform cells. Vascular bundles do not appear to have associated girders to the

surface, and the fibre bundles have extended to form an almost continuous ring of cells inside the epidermis. Fibres were especially concentrated at the leaf margin (Plate 4.5A). The trichomes on the adaxial surface were larger and more dense than on *F. arundinacea*, filling a large proportion of the internal region of the leaf (Plate 4.5A). A lower density of smaller trichomes existed on the abaxial surface, along with numerous circular scars which may have been caused by the loss of trichomes (Plate 4.5B). Stomata were not found on the abaxial surface; but a density of stomata comparable to that of *F. arundinacea* was found on the adaxial surface (Table 4.2).

The adaxial surface of both species was coated with a powdery substance, which was visible under the higher-power SEM scans (Plates 4.4B, and 4.6B). This layer was considered likely to be a waxy cuticle, but no analysis was undertaken to confirm this. The powdery deposits were not observed on the abaxial surface of either *Festuca* species.

Table 4.2: Estimation of stomatal density.

		mean stomata / cm ²	No. of counts
<i>F. arundinacea</i>	abaxial (bottom surface)	9,897	2
	adaxial (top surface)	20,511	3
<i>F. novae-zealandiae</i>	abaxial (outside surface)	0	3 ¹
	adaxial (enclosed surface)	18,693	3

¹ Stomata were not observed on this leaf surface during examination under the SEM.



A

B

Plate 4.1: Foliage of *F. arundinacea*. A: Control plant (C6) grown in water sufficient conditions. B: Water-deficit stressed plant D5, from experiment II, described in Chapter Three.



A



B

Plate 4.2: Foliage of *F. novae-zealandiae*. A: Plant grown in water-sufficient conditions; B: Water-deficit stressed plant. Both photographs are of plant D2 from experiment I.

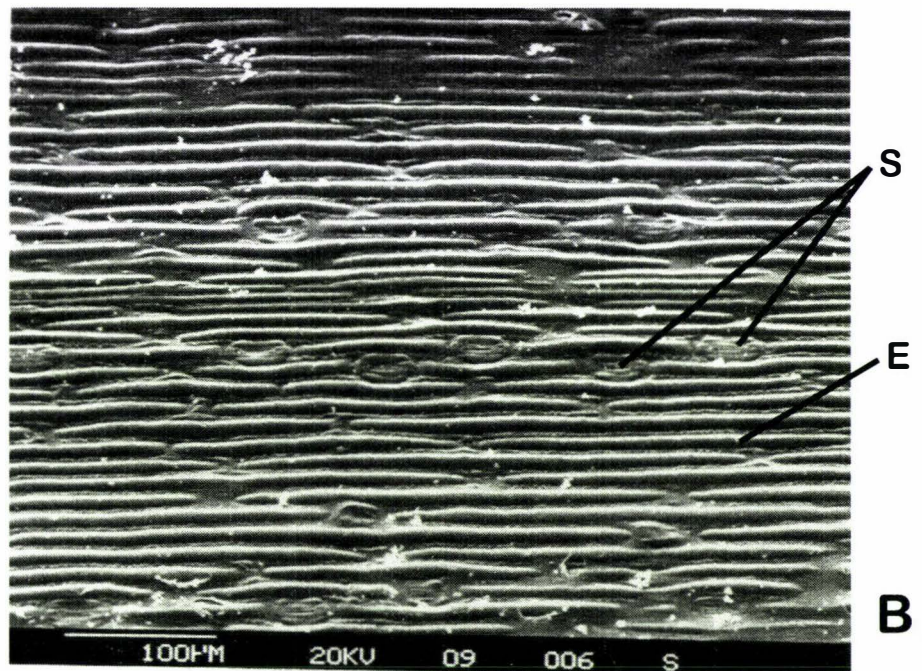
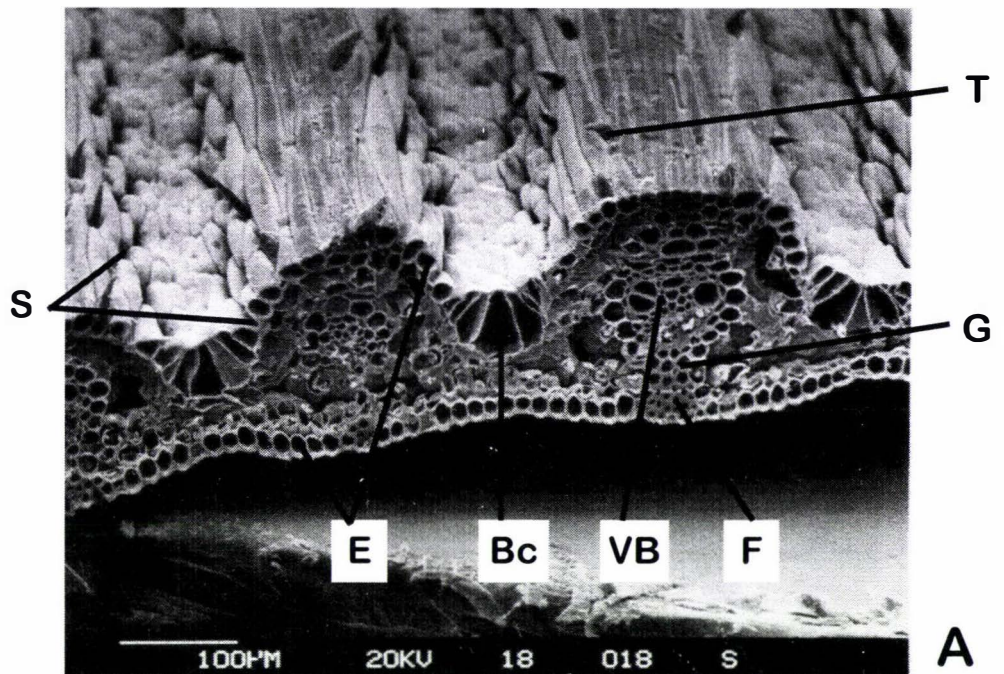


Plate 4.3: Scanning electron micrographs of ML of *F. arundinacea*. A: Transverse section (100x); B: Abaxial (lower) surface of the lamina (200x). Key: Bc: bulliform cell; E: epidermal cell; F: fibre cell; G: girder cell; S: stomata; T: trichome; VB: vascular bundle.

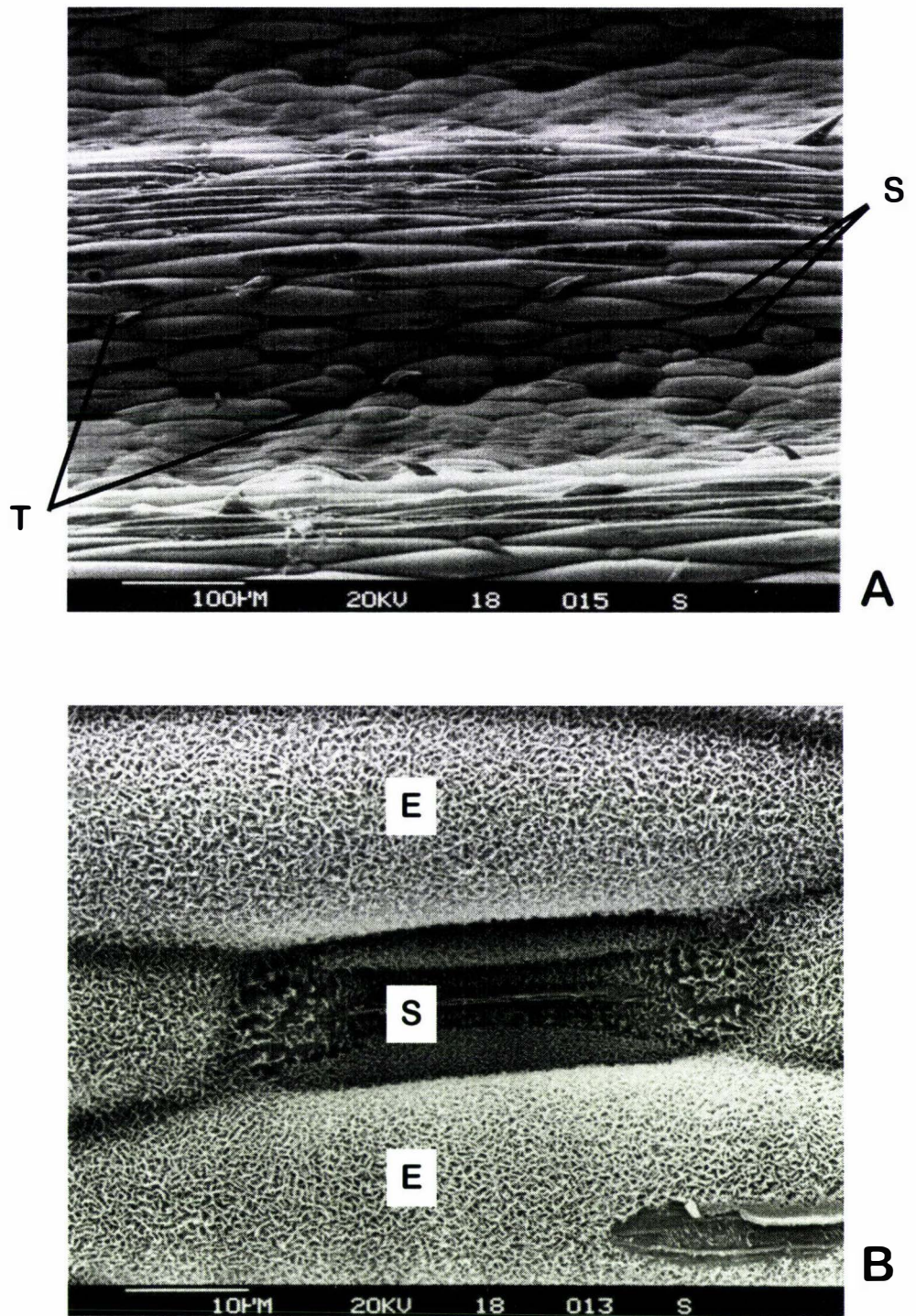


Plate 4.4: Scanning electron micrographs of ML from *F. arundinacea*. A: Adaxial (top) surface of the leaf, location of stomata on side of the vascular trace (170x); B: Stomata present on the adaxial surface (1700x). Key: E: epidermal cell; S: stomata; T: trichome.

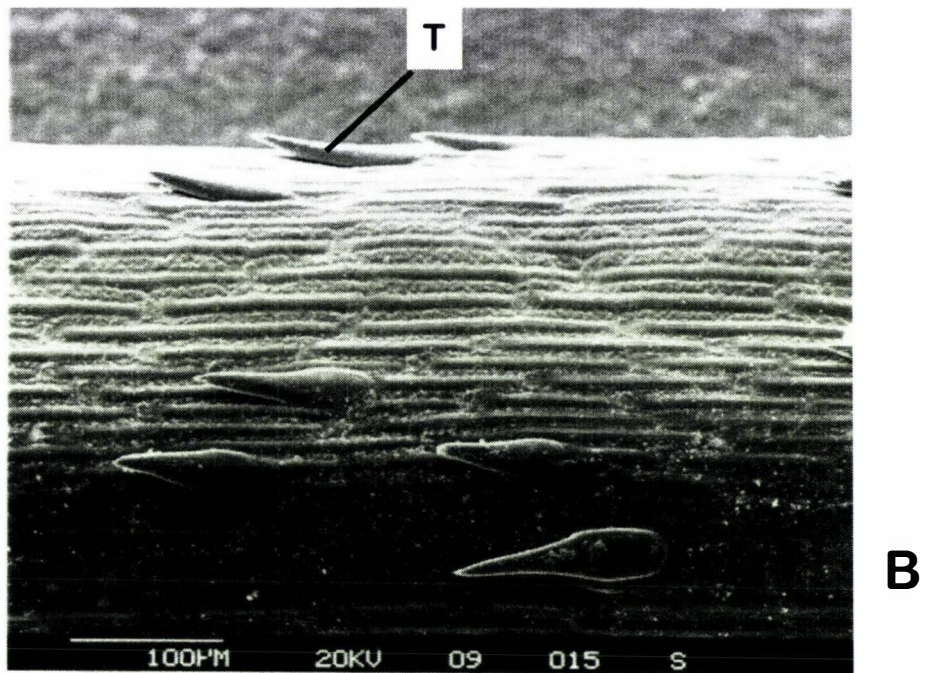
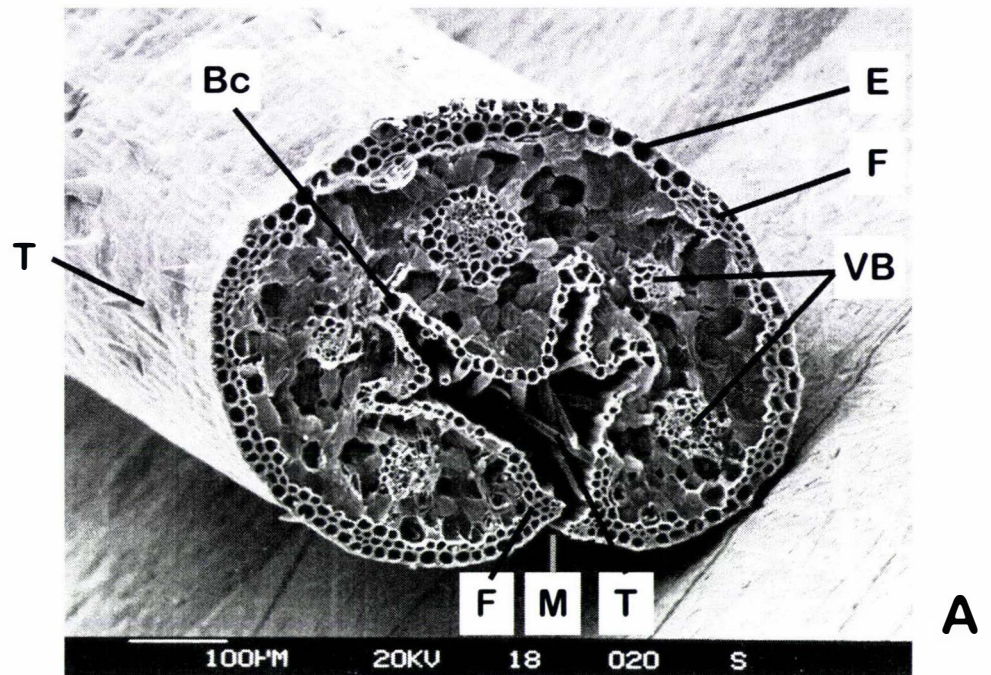


Plate 4.5: Scanning electron micrographs of ML from *F. novae-zealandiae*. A: Transverse section (120x); B: Abaxial (outside) surface of the lamina (200x). Key: Bc: bulliform cell; E: epidermal cell; F: fibre cell; M: leaf margin gap; T: trichome; VB: vascular bundle.

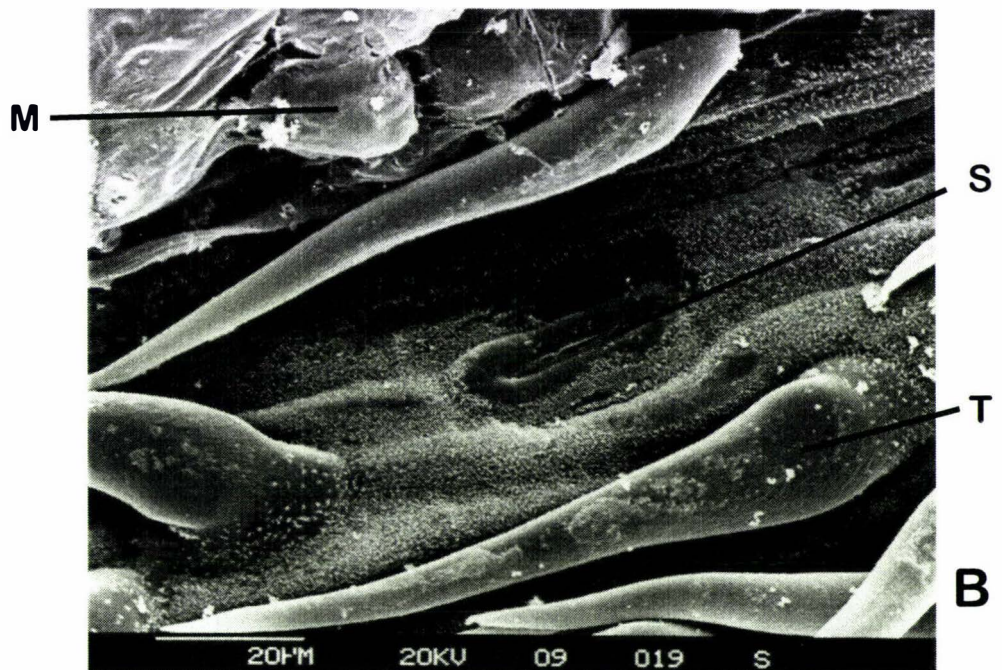
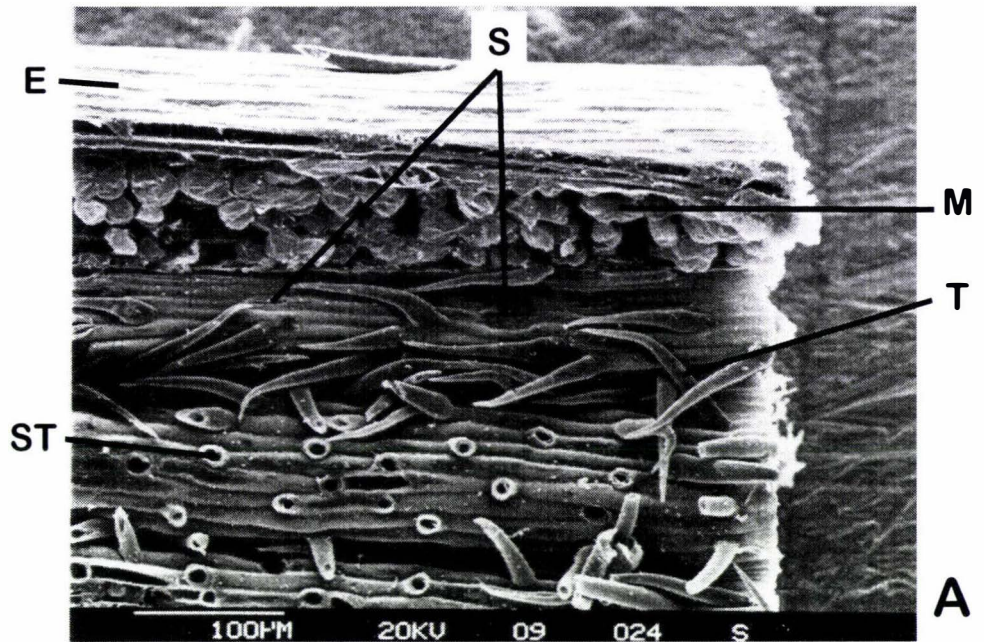


Plate 4.6: Scanning electron micrographs of ML from *F. novae-zealandiae*. A: Adaxial (inside) surface (200x); B: Adaxial surface (1000x). Key: E: epidermis; M: mesophyll cell; S: stomata; ST: severed trichome; T: trichome.

4.3.2 Dry-Down Experiments with *F. novae-zealandiae*

4.3.2.1 Time Course

Plants of *F. novae-zealandiae* were established in the 24 L pots under well-watered conditions, and then water was withheld (except in the control pots) for the period between 26 April to 15 June 1993, and from 24 November 1993 to 21 February 1994 (experiments I and II, respectively). Measurements for experiment I were started on 29 April 1993, and were continued for up to 102 days. For experiment II, measurements were started on 24 November 1993, and were carried out for 83 days.

4.3.2.2 Soil Water Content (SWC)

The progress of each dry-down was monitored by measuring the SWC using the five TDR electrode stations down the side of each pot (Figures 4.1 and 4.2, for experiments I and II, respectively) and also by using a vertical electrode station during experiment II (Figure 4.4). The mean SWC from the experiment I is presented in Figure 4.3.

4.3.2.2.1 Changes in SWC Gradients

During both experiments, the SWC of the soil column of each pot formed a gradient. In each case, the top horizon was usually the driest, and the bottom horizon was usually the wettest of the five soil horizons measured (Figures 4.1 and 4.2). With regular watering, the gradient of SWC of the control pots was preserved with little change in the SWC of each soil horizon during each experiment (Figures 4.1 and 4.2).

In experiment I, the gradients of SWC ranged from 16-27% (top) to 32-40% (bottom) in C1, and from 22-28% (top) to 28-39% (bottom) in C2 (Figure 4.1). Some noise (std. dev. = ca. 4% SWC) in the SWC data was apparent, possibly arising from the TDR equipment (for example from poor electrode contacts). The noise was not attributable to possible large SWC changes as a direct result of watering, since the SWC was always measured before water was added to the control pots. Some points from SWC data were sufficiently far from the trends to be considered as outliers (Figure 4.1). These points have been included in the graphs, but were not connected to the series lines for the purposes of clarity. During experiment II, the SWC gradients were similar between the two control pots, ranging from 22-27% (top) 32-35% (bottom) in C6, and from 23-27% (top) to 32-36% (bottom) in C8 (Figure 4.2). With regular watering, little

change in the SWC gradients occurred in the soil column of these two pots during the experiment. In comparison with experiment I, the background noise in the SWC data sets of experiment II was significantly reduced, probably due to maintenance of the electrical contacts.

In all the water-deficit treatment pots, water was removed from all the soil horizons. The rate of water loss was negatively correlated with soil depth, where water was removed from the deepest soil horizon last. Hence a gradient of soil moisture was preserved as the SWC declined.

Pot D1 did not dry-down to the same extent as the other water-deficit treatment pots during experiment I (Figure 4.1). After 100 days, the lower four soil horizons still contained over 22% SWC. In particular, the SWC of the upper and middle horizons was similar to one of the controls (C2). The top soil horizon also dried at a relatively slow rate, losing only 4% SWC during the first 65 days before the rate of loss of SWC increased, to become *ca.* 15% by the end of the experiment.

In the remaining three water-deficit treatment pots (D2, D3 and D4) from experiment I, the SWC of the top soil horizons declined to a lower limit of *ca.* 7-8% SWC (Figure 4.1). This required *ca.* 32, 50, and 78 days (D2, D3 and D4, respectively). In D2, the SWC of the upper soil horizon also declined to the lower level of 7-8% after 66 days. In all three pots, the remaining soil horizons dried at a relatively slower rate than top horizons, to reach between 9 and 23% SWC by the end of the experiment (Figure 4.1).

During experiment II, the SWC declined in all the soil horizons of each water deficit treatment pot in a close to linear fashion (Figure 4.2). By the end of the experiment, the SWC gradient had declined from initial control levels to a range of 8-15% in D6, and 8-17% in D7. The gradient of SWC of these two pots was preserved during the experiment, being driest at the top horizon and wettest at the bottom.

In D8 and D9, the gradient of SWC declined to between 9-13%, and 10-16% (respectively) by the end of the experiment (Figure 4.2). However, the gradient of SWC was not preserved in these two pots. After about 40 days, the rate of SWC decline of the bottom horizon of D9, and the lowest three horizons of D8 increased relative to the

other horizons. By the end of the experiment, the lower and bottom horizons of D8, and the bottom horizon of D9 were the second driest horizons in each respective pot, and within 2% SWC of the driest top soil horizon. The reason why the lower portion of the soil columns in these two pots began to dry at such an increased rate was not clear.

4.3.2.2.2 Changes in the SWC of the Top Soil Horizon

After experiment I, it was found that the roots of *F. novae-zealandiae* were not evenly distributed within the soil column (*c.f.* section 4.3.2.7). Because most of the roots were located close to the soil surface, an additional pair of TDR electrodes were introduced during experiment II to measure the SWC in the vicinity of the majority of the roots. This modification to the method was made to gain a better estimate of the SWC available to the plant than could be gained by averaging the data from the side electrode stations in the same manner as with the experiments with *F. arundinacea* (section 3.4.2.2, page 72). Because the vertical electrodes were not used in experiment I, the SWC measured by the top side electrode station was used as a measure of SWC available to the plant. Polynomial curves were fitted to the top (experiment I, Figure 4.3), or vertical (experiment II, Figure 4.4) electrode station data, to determine the SWC for each date on which samples were taken for metabolite analyses.

At the start of each experiment, all pots were watered to saturation (*ca.* 30% SWC), and by the time SWC measurements for experiment I were started, the SWC of the water-deficit treatment pots had been reduced to as low as 20% (Figure 4.3). In D2, D3 and D4, the top horizon SWC declined steadily for the first 30 to 40 days. After this time, the rate at which the soil dried declined, to reach a close to zero value (horizontal slope on the graph) after 50 to 60 days, when the SWC had declined to 7-8%. Data from the upper soil horizon (Figure 4.1) in these pots indicated that water was absorbed by the plants from this horizon, after the top horizon had reached the lower level of 7-8% SWC; hence the use of the SWC data from the top soil horizon can only be used as a guide as to the water availability to the plant.

As already mentioned above, the SWC in D1 did not appear to decline to a great extent for the first 65 days, and after 100 days, the SWC of the top horizon had declined to only 15% (Figure 4.3). The discrepancy in the rate of drying between pots receiving the

same treatment is not obvious. Although leaf area was not measured, D1 was not considered unusually small. However, root mass data from the soil sections (section 4.3.2.7) suggested that the roots from this plant may have been smaller than from at least two of the water-deficit treatment plants (D2 and D4).

During experiment II, the decline in SWC measured in the four water-deficit treatment pots was more uniform than that found during experiment I. The SWC measured in D6, D7, D8 and D9 declined steadily to reach a lower limit of 8-10% SWC. The decline in SWC measured in D8 lagged behind that of the other water-deficit treatment pots for most of the experiment (Figure 4.4). With regular watering, the SWC of the top-most portion of the controls (C1 and C2 from experiment I, C6 and C8 from experiment II) remained above 15% and 25% (respectively) for the duration of the experiment (Figures 4.3 and 4.4).

4.3.2.3 Leaf Elongation Rate (LER)

During both experiments, the length of the IL of three tillers per plant was regularly measured to determine the mean LER.

In experiment I, the LER of the control plants (C1 and C2) ranged between 4 and 21 mm.d⁻¹ (Figure 4.5), with a mean LER of 10.6 ± 0.5 and 11.9 ± 0.6 mm.d⁻¹ (respectively). Although meteorological data was not recorded during the experiment, it was considered likely that fluctuations in the LER reflected changes in the weather. The LER of the control plants (C6 and C8) were slightly lower during the experiment II, occurring between 1 and 19 mm.d⁻¹ (Figure 4.6), with a mean LER of 10.4 ± 0.8 and 8.5 ± 0.7 mm.d⁻¹ (respectively).

In both experiments, the LER was observed to decrease in response to the water-deficit treatment. However, cessation of growth was not observed.

Differences in the response of LER to water-deficit treatment were apparent between the plants used in experiment I (Figure 4.5). The LER of D1 remained above 4 mm.d⁻¹ during the experiment, and hence was not different from that of the controls. This

Soil Water Gradients: Experiment I

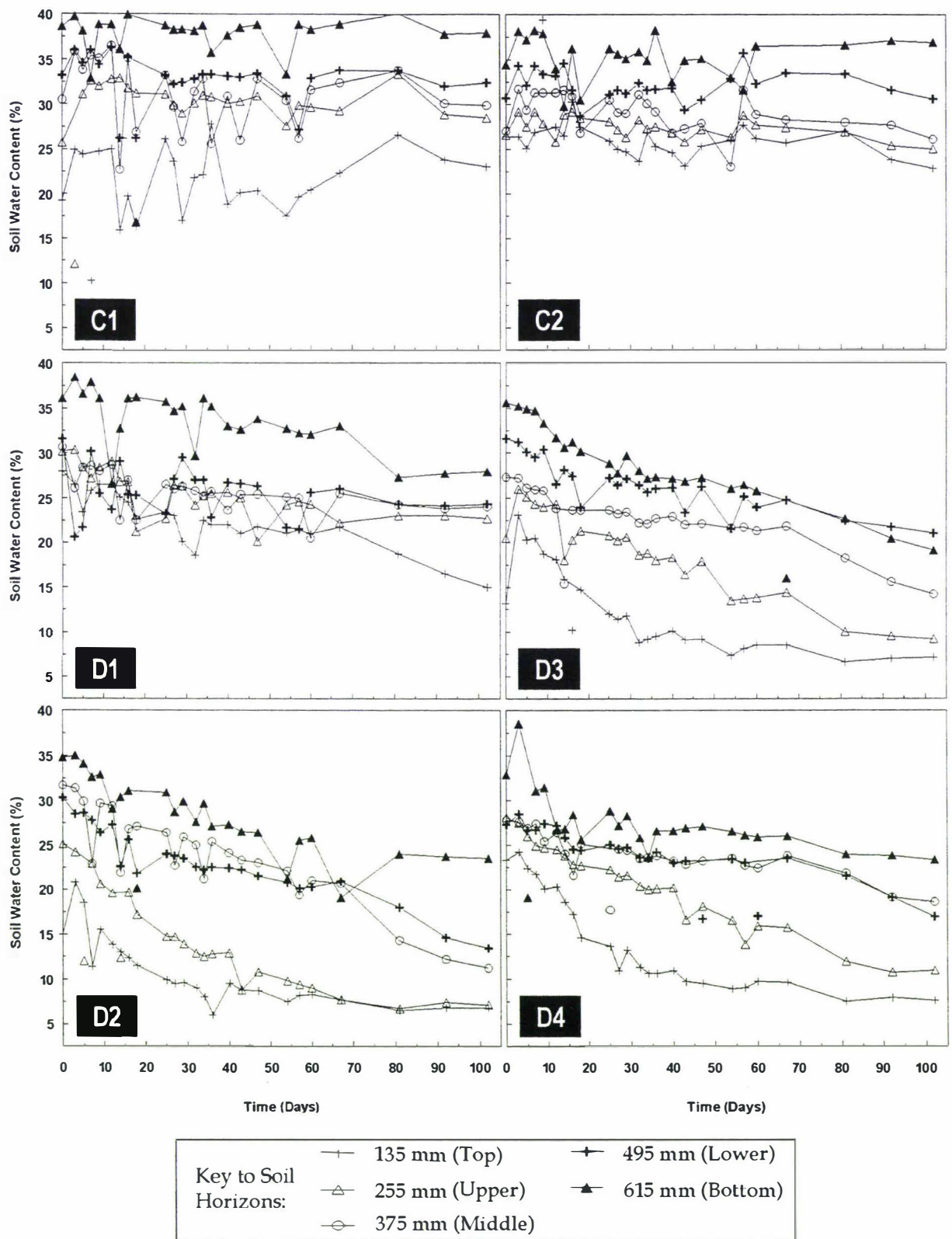


Figure 4.1: The SWC of the five soil horizons in each pot during experiment I. The labels indicate pot numbers.

Soil Water Gradients: Experiment II

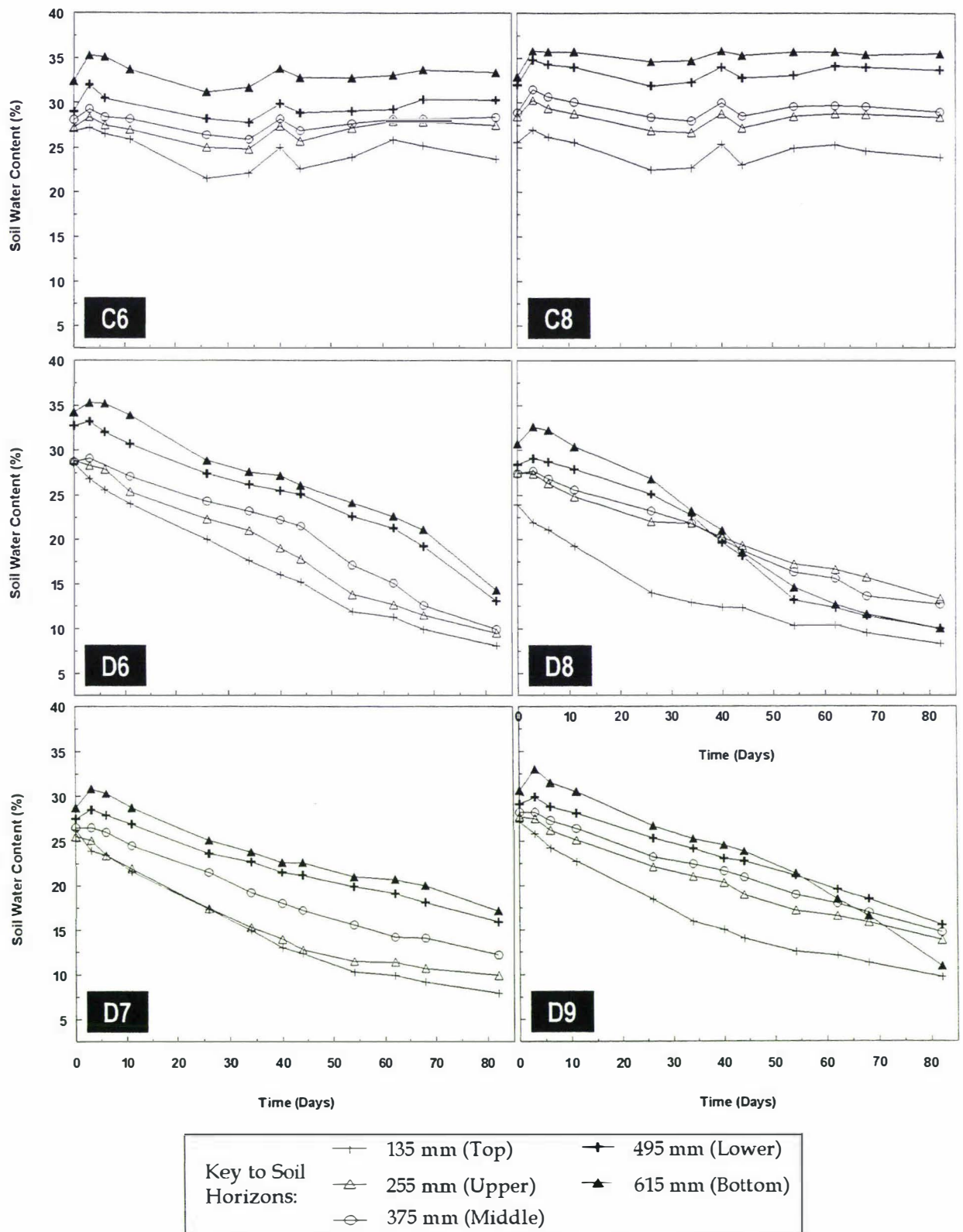


Figure 4.2: The SWC of the five soil horizons in each pot during experiment II. The labels indicate pot numbers.

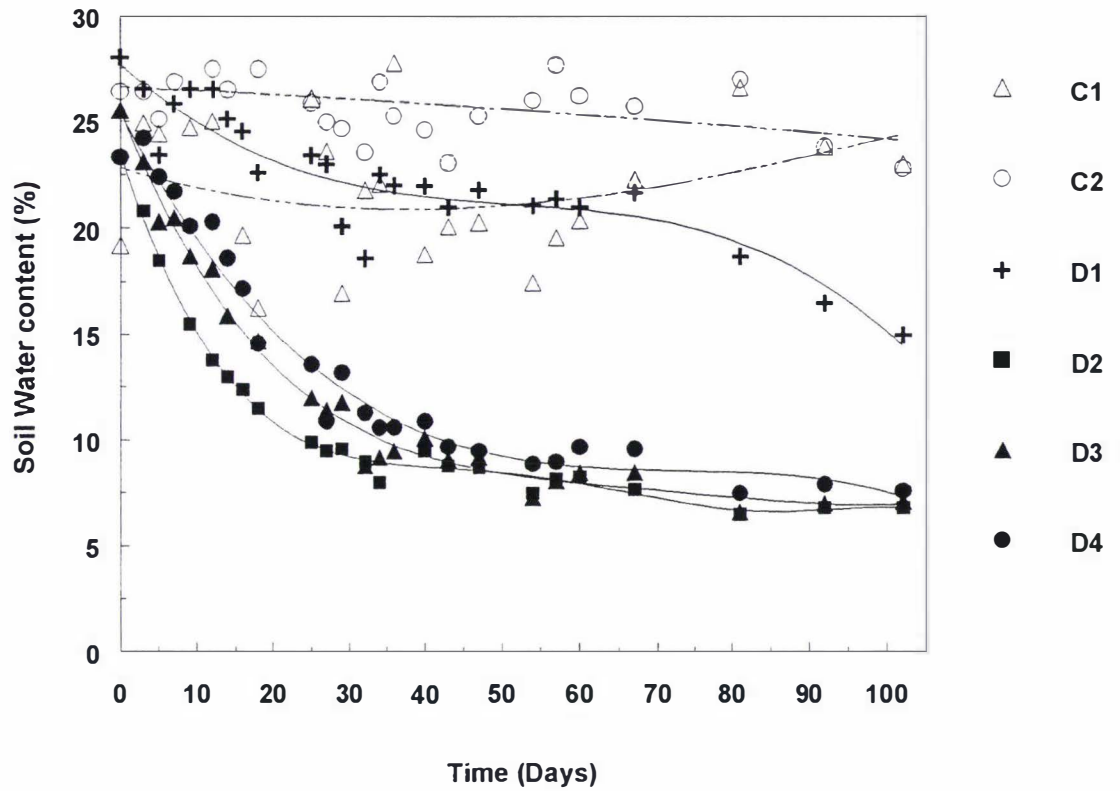


Figure 4.3: The SWC of the top soil horizon for each pot used during experiment I. The legend indicates pot numbers. Data were single TDR measurements and were fitted with polynomial curves (C1 and C2 have dashed curves).

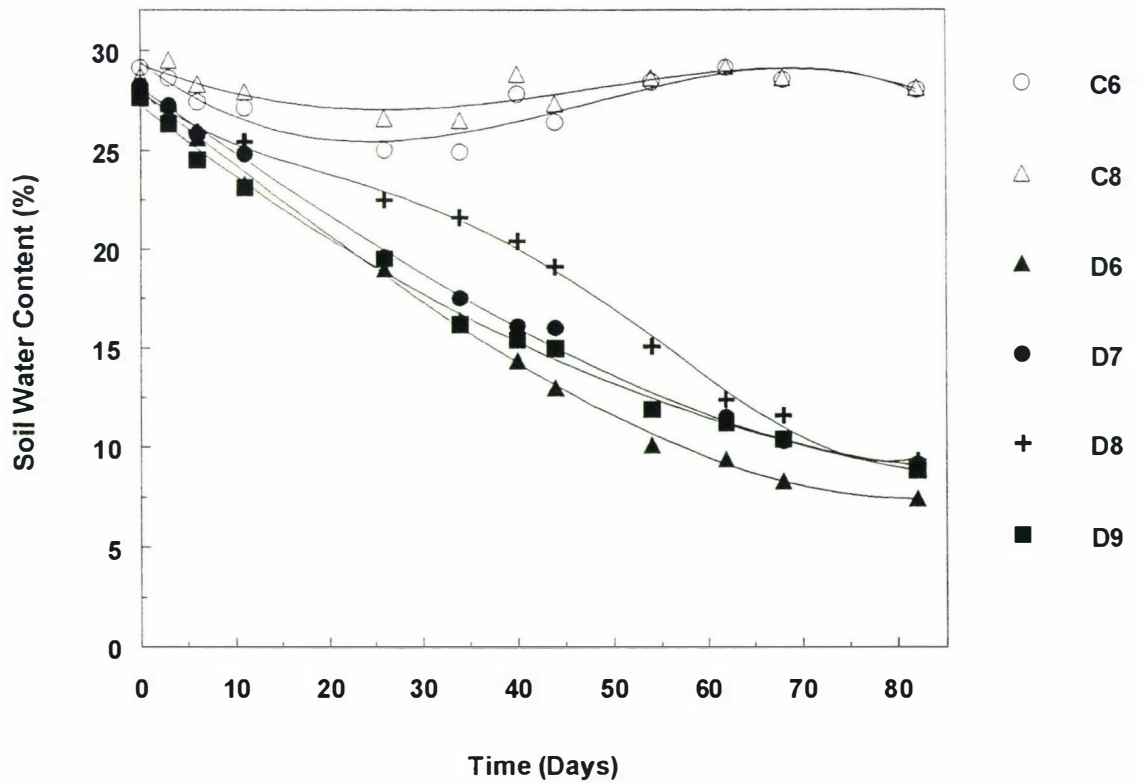


Figure 4.4: The SWC recorded by the vertical electrode station during experiment II. The legend indicates pot numbers. Data were single TDR measurements and were fitted with polynomial curves.

was consistent with the SWC data from D1, which suggested that this particular plant may not have experienced water deficit stress (section 4.3.2.2). The LER of D2 underwent a gradual decline for about the first 70 days, and except for the final point, remained low (2-6 mm.d⁻¹) for the rest of the experiment. In D3, the LER declined from 12-14 mm.d⁻¹ to below 4 mm.d⁻¹ after 21 days. The LER then remained below this level for the next 40 days, before increasing to 5-7 mm.d⁻¹ for the rest of the experiment. In D4, the LER remained between 6 to 10 mm.d⁻¹ for most of the experiment, ending in a decline in LER during the last 12 days. A period of relatively lower LER in this plant was also observed between days 20 and 29 (Figure 4.5).

In contrast to experiment I, the LER changes measured in experiment II were similar amongst the water-deficit treatment plants. In each of these plants, the LER at the start of the experiment was *ca.* 11-12 mm.d⁻¹. Although some fluctuations in the data were observed, the LER declined gradually in all four of the plants, to reach *ca.* 2 mm.d⁻¹ in D8, and close to zero in D6, D7, and D9 by the end of the dry-down (Figure 4.6).

The data collected from both experiments indicated that cessation of LER did not occur in the plants subjected to the water-deficit treatment. However, because *F. novae-zealandiae* turns over tillers as a normal cycle of growth, cessation of LER did occur in individual tillers which were being measured. When this occurred, a new tiller was selected, and measurements on the new IL were continued. Therefore the data presented above does not reflect the mean LER of all the tillers in a particular plant. Rather, the LER data may reflect the growth rate of a random selection of (the remaining) elongating tillers.

Leaf Elongation Rate: Experiment I

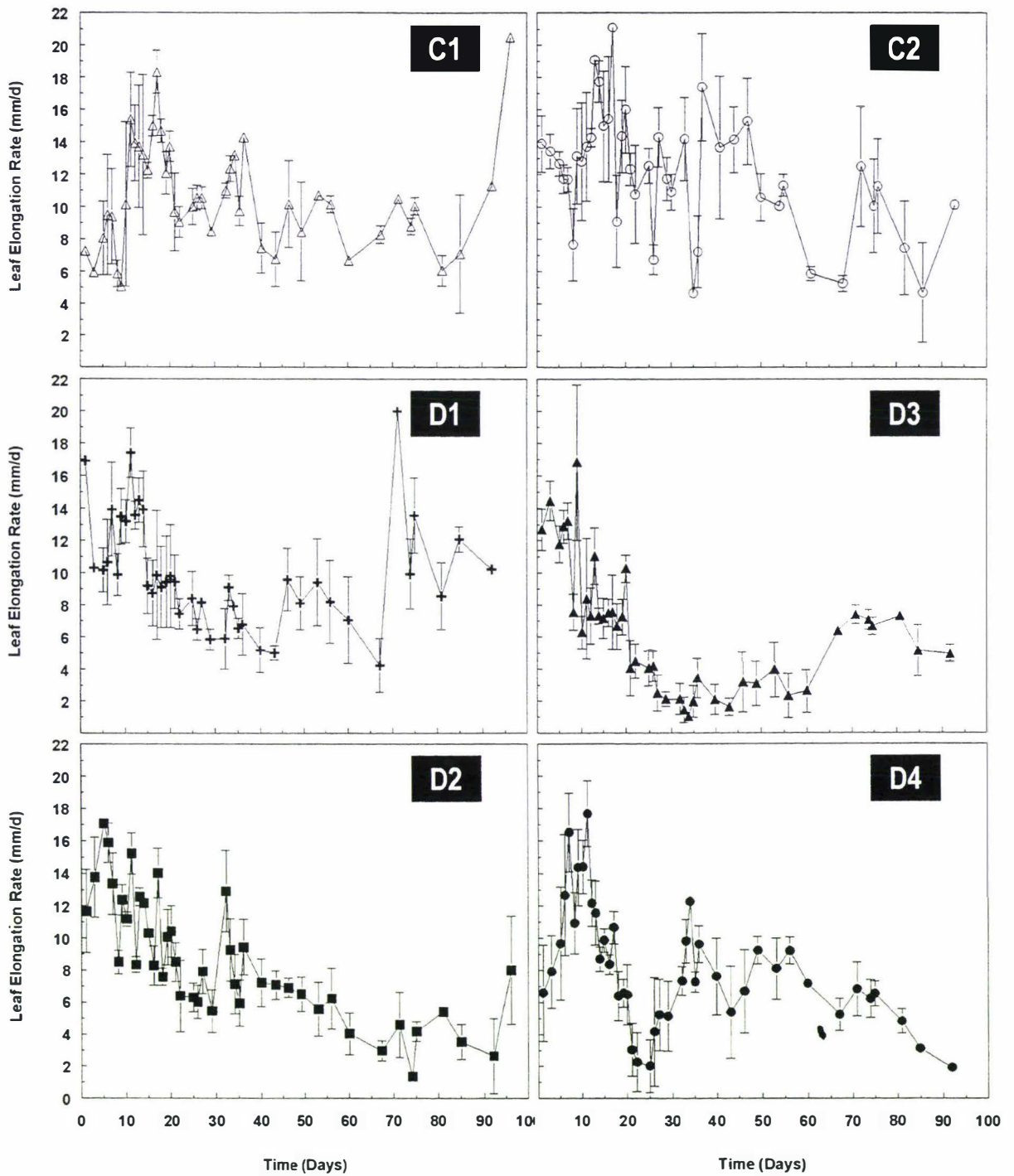


Figure 4.5: The LER measured during experiment I. The labels indicate plant numbers. Data were means \pm s.e., (n=3).

Leaf Elongation Rate: Experiment II

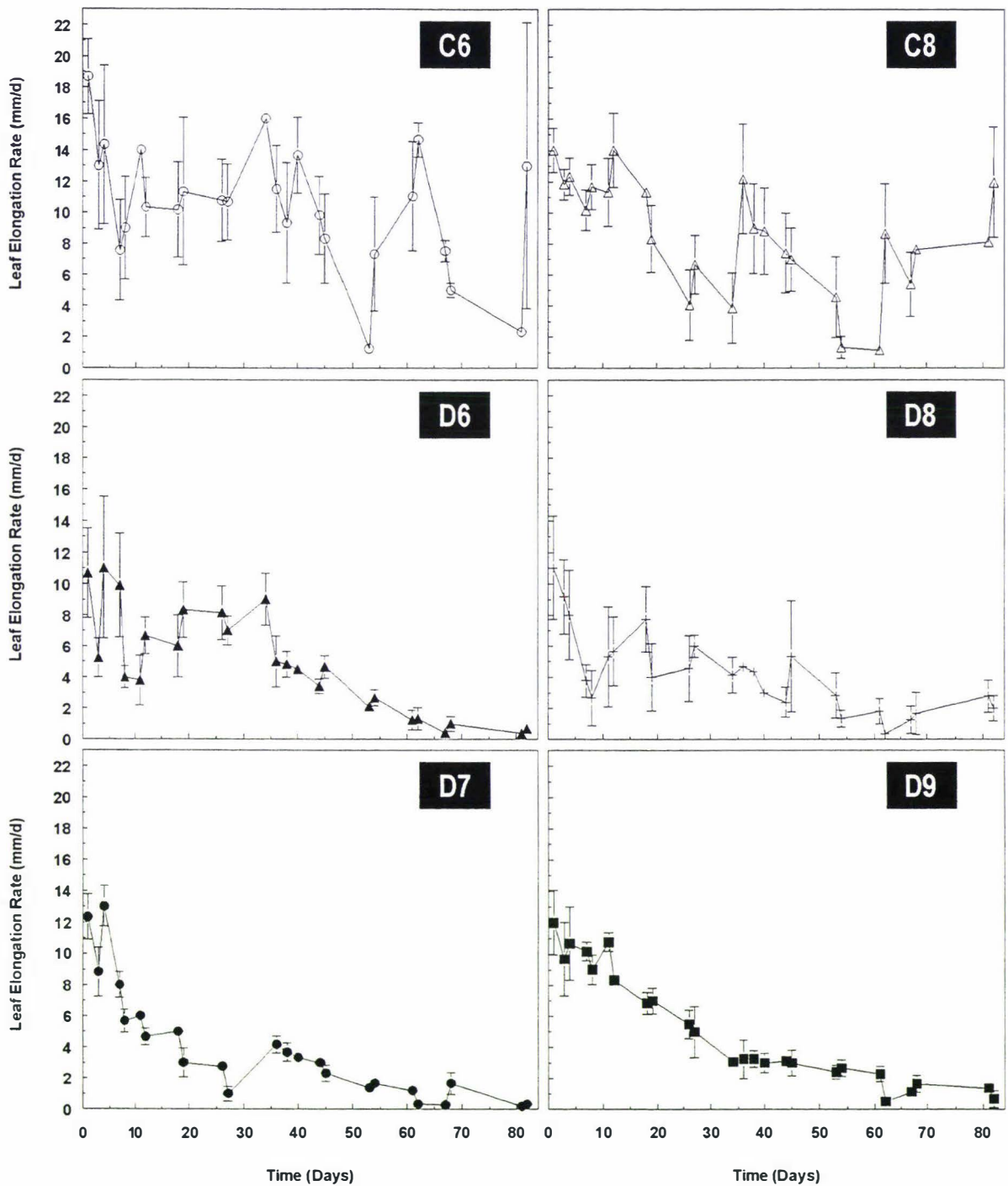


Figure 4.6: The LER measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., (n=3).

4.3.2.4 Additional Physiological Measurements

During the dry-down experiments with *F. arundinacea*, the LER was followed as a physiological indicator of the growth status of the plant. The decline of the LER to zero in response to water deficit stress in this species correlated with the onset of metabolic adaptation. However, for *F. novae-zealandiae*, the LER declined gradually, and did not cease in response to the water-deficit treatment. Therefore, other physiological measurements were recorded in the expectation that a replacement for LER could be used to indicate the onset of water-deficit stress. Several measurements of the whole tussock, and of individual tillers within each tussock were made. These included the number of green tillers in each tussock clump (T_{Num}), the mean tiller mass (M_T), the mean number of green leaves on each tiller (N_T), and the mean extent of leaf-tip scorching (D_S) on the IL, ML and 2ML.

4.3.2.4.1 Tiller Number (T_{Num})

At the conclusion of the experiment I, the number of tillers in each plant were counted *in situ*, and were scored by colour as either green or brown. It was intended that by using this scoring method, the living tillers could be distinguished quickly and easily from those which were dead. Only the outward appearance of the tillers was taken into account; no dissection of the tillers, nor confirmation of cell vitality using chemical reagents, was carried out for this purpose.

The T_{Num} at the end of experiment I appeared to correlate with the treatment (Figure 4.7). The control plants (C1 and C2) were composed of *ca.* 50% green tillers, and the tussock as a whole appeared green to the eye. Plants D2, D3 and D4 had 10% or less green tillers, and these plants were distinctly brown to the eye. Plant D1 contained an intermediate T_{Num} (19%, Figure 4.7).

From this experiment, it became clear that future T_{Num} counts would have to take into account the ratio of tillers before the experiment, since some plants may have started with a greater proportion of dead tillers. It was also expected that the proportion of dead tillers would always increase, regardless of the treatment. This was because of the continuous turnover of the tiller population coupled with the absence of a mechanism for the removal of dead tillers. Therefore, before the start of experiment II, most of the dead tillers were excised from each plant.

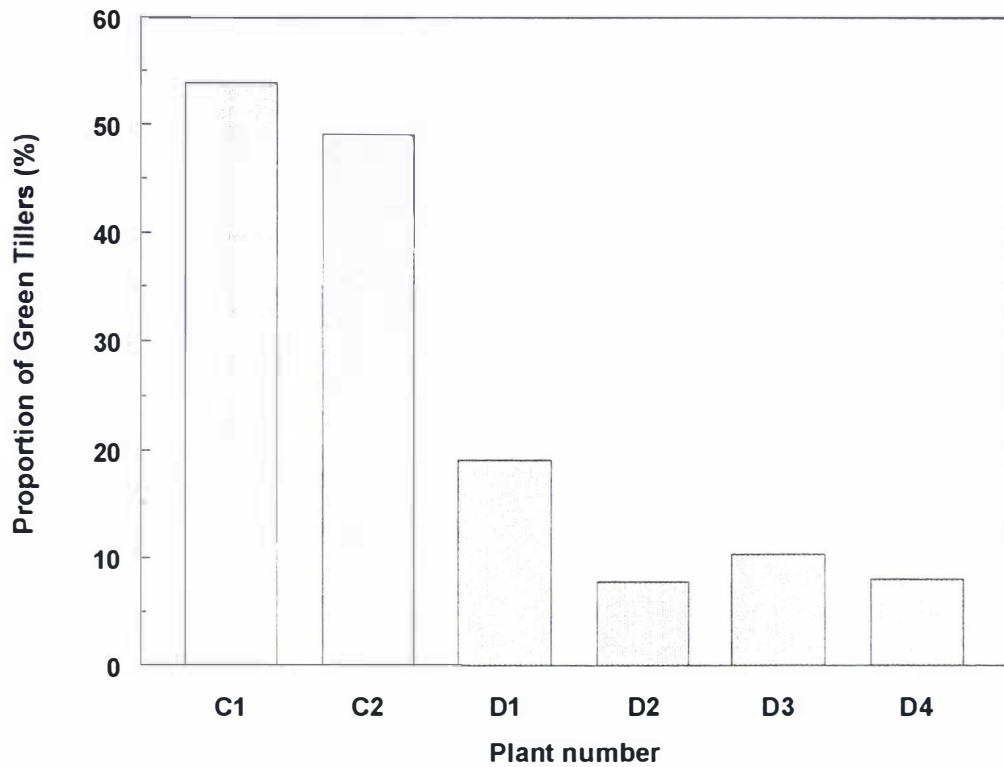


Figure 4.7: The proportion of green tillers per plant (T_{Num}) at the conclusion of experiment I. Data were single counts.

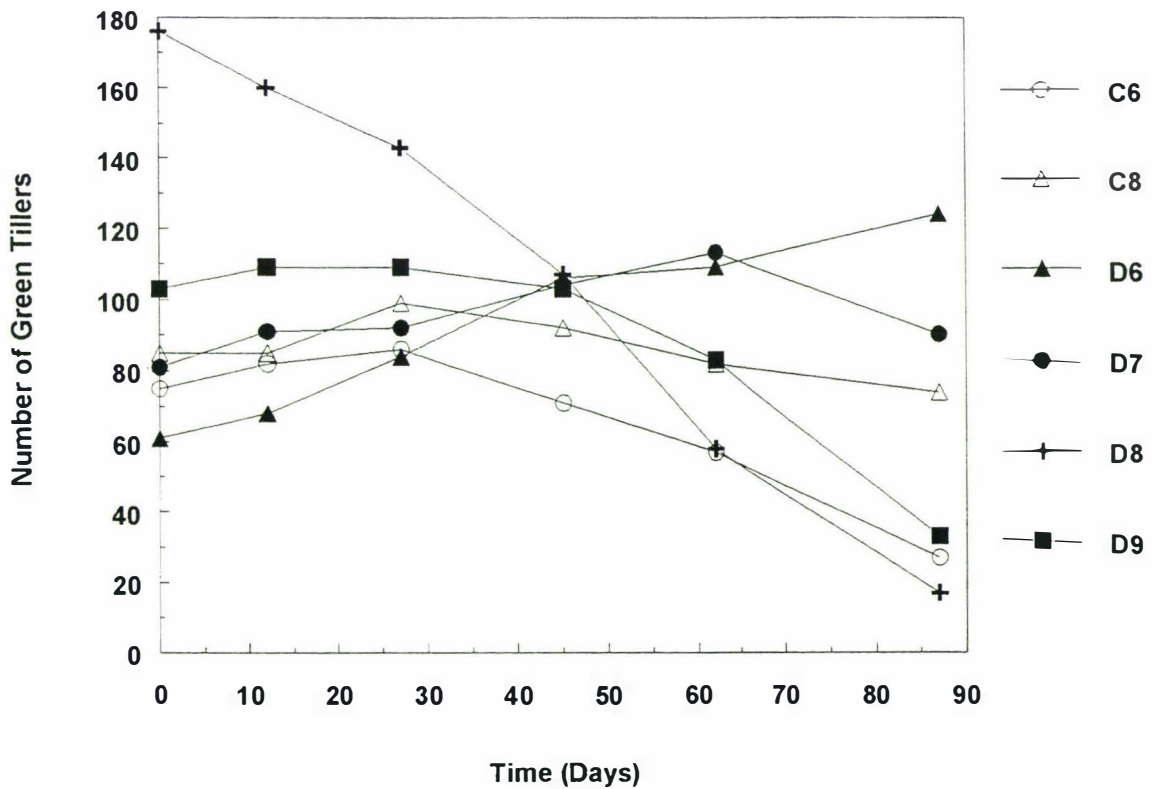


Figure 4.8: Changes in the number of green tillers in each plant during experiment II. The legend indicates plant numbers. Data were single counts.

Throughout experiment II, the T_{Num} was counted at regular intervals. Both control plants (C6 and C8) started with a T_{Num} of about 80, which increased marginally after 28 days, and then declined to 27 and 75 (respectively) by the end of the experiment (Figure 4.8). Differences were found amongst the water-deficit treatment plants. The T_{Num} continually increased in D6 (from 60 to 120), and continually decreased in D8 (from 175 to 23) during the experiment. The T_{Num} of D9 declined from *ca.* 105 after 45 days for the rest of the experiment. A similar reduction in T_{Num} occurred in D7, after T_{Num} had increased from 80 to 114 during the first 62 days.

4.3.2.4.2 Tiller Mass (M_T)

At the end of each experiment, up to 12 tillers per plant were excised at the base and were stripped of all the older, brown leaves prior to weighing. The M_T ranged from 0.13 to 0.37g in experiment I, and from 0.07 to 0.2g in experiment II (Figure 4.9). The mean tiller mass was compared between plants using one way ANOVA models, which in both experiments were found to be highly significant (Appendix 4.1 Table 4.3 and Table 4.5 for experiments I and II, respectively). The tillers from two plants (D3 and D2) from experiment I were significantly smaller than the tillers sampled from the control plants (C1 and C2); the M_T from D1 and D4 were not significantly different (NSD) from the controls (Table 4.3).

The M_T from experiment II were divided into three groups based on significant difference (Table 4.6). However, there was no correlation between the significance groupings and the treatment (water-deficit or control) applied to each plant, since tillers sampled from the control plants were some of the heaviest (C8) and the lightest (C6) of the tillers sampled (Figure 4.9).

It was concluded that the measurement of M_T was not adequate as a physiological indicator of water-deficit stress in *F. novae-zealandiae*. Although a trend was apparent where control tillers were heavier than those which were stressed, the difference was not always significant. In addition, the mass of each tiller was likely to have been influenced from sources other than water-deficit stress alone. The differences observed in M_T between plants may have arisen from three sources. Firstly, due to the water

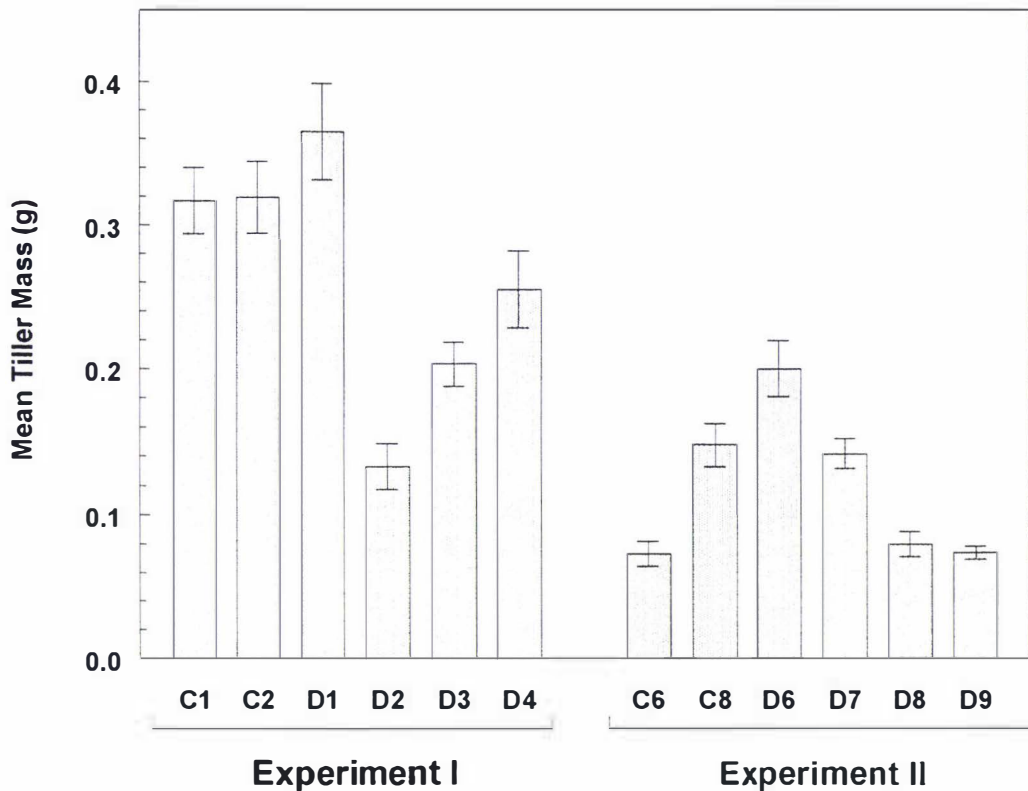


Figure 4.9: Mean tiller masses from both experiments. The x-axis indicates plant numbers. Data were means \pm s.e., $n=8$ to 12 (see Tables 4.4 and 4.6).

deficit treatment, some tillers may have been less hydrated than the controls, and hence lighter in mass. Secondly, plant size would have a substantial effect on tiller mass. This was particularly evident between the T2 genotype plants used in the two experiments, where C8 (experiment II) had an M_T of about half of the control plants (C1 and C2) from experiment I (Figure 4.9). Finally, it was observed during the processing of these tillers, that many of the tillers subjected to water-deficit stress carried only the IL and one mature leaf (the ML). However, the control tillers usually carried at least two mature leaves (ML and 2ML), in addition to the IL. Hence the number of leaves per tiller (N_T) would have an important contribution to tiller mass. Because of this, a more detailed examination of N_T was carried out.

4.3.2.4.3 Mean Number of Green Leaves per Tiller (N_T)

The number of green leaves per tiller was counted for all the tillers used for mass measurements above. All tillers sampled carried between 1 and 4 green leaves, although the N_T was between 1.9 and 3.0 (Figure 4.10). The N_T was dependent on the treatment, where the control plants had either a similar, or greater N_T than the water-deficit treatment plants (Figure 4.10). The N_T was compared between plants using a one-way

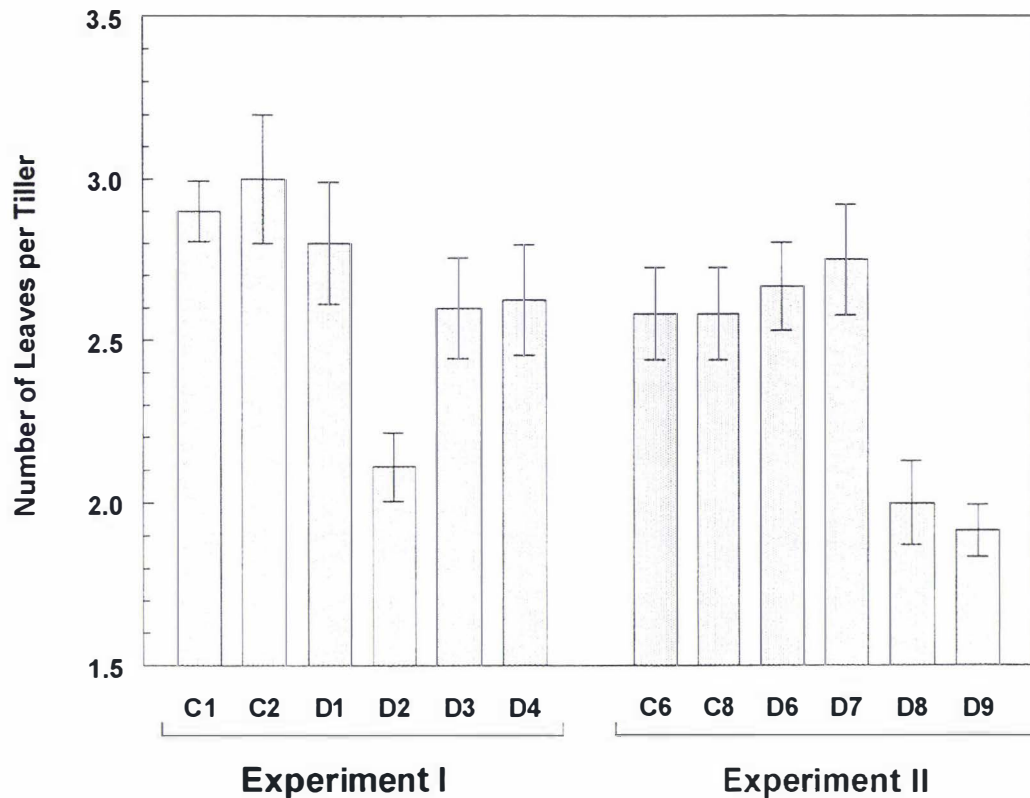


Figure 4.10: The N_T determined at the end of both experiments. The x-axis indicates plant numbers. Data were means \pm s.e., $n = 8$ to 12 (see Tables 4.8 and 4.10).

ANOVA model, which was significant at levels of 0.9% (Table 4.7) and 0.01% (Table 4.9) for experiments I and II, respectively. However, the N_T amongst all the plants from experiment I were NSD, with the exception of plant D2 (Table 4.8).

Plants D8 and D9 from experiment II had a significantly smaller N_T than the controls (Figure 4.10), while D6 and D7 had an N_T of ca. 2.7, which was NSD from the controls (Table 4.10).

Because the N_T was dependent on the treatment, it was concluded that counting N_T may be useful as an indicator of water-deficit stress in *F. novae-zealandiae*. Of the three leaves usually present on a tiller of *F. novae-zealandiae*, the 2ML appeared to be the first leaf lost in response to water-deficit stress. However, increased leaf-tip scorching was observed on all the leaves, including the IL. In some instances, the entire exposed portion of the IL was scorched. Since an increase leaf-tip scorching may precede leaf loss, it was proposed that scorching may provide a better indicator of water-deficit than N_T alone.

4.3.2.4.4 Degree of Leaf Tip Scorching (D_s)

The D_s was determined for each of the leaves that were counted for N_T above to determine which leaf was more susceptible to water-deficit stress. The D_s was calculated as the percentage of the length of each leaf lamina from the tip down which was scorched. The D_s was calculated from three leaf types during experiment I (IL, ML, and 2ML), but only from IL and ML from experiment II. This was because many of the tillers sampled during experiment II, including all but one of the tillers from D7, D8 and D9, had only two green leaves per tiller. In many cases, it was not clear if this was because the tiller had recently divided from an existing tiller (therefore 2ML was non-existent), or whether the third leaf had senesced, and fallen away from the tiller (therefore $D_s = 100\%$). Hence an incomplete data set was obtained for the 2ML from experiment II.

For the comparison of means, a two-way ANOVA model was used. This model simultaneously compared the mean D_s between the six experimental plants, and also between three (or two) leaf types. Both ANOVA comparisons were highly significant at levels of 0.01% (Table 4.11 and Table 4.15 for experiments I and II, respectively).

In experiment I, the comparison of means between leaf types, and the comparison of means between plants were highly significant at levels of 0.01% (Table 4.12). This indicates that D_s was dependent on leaf maturity, and was also affected by water-deficit. However, the interaction effect (plant \times leaf type) was NSD ($P > 0.05$, Table 4.12).

In experiment II, the ANOVA comparisons of means between plants, and the interaction effect were both highly significant, at levels of 0.01% and 0.04% respectively (Table 4.16). This indicates that the D_s was influenced by the water-deficit treatment, and that this treatment affected each leaf type differently. However, the D_s was not dependent on leaf maturity ($P = 0.9740$), which was consistent with the fact that the D_s of both leaf types increased in response to the water-deficit treatment (Table 4.16).

In both experiments, the D_s measured in the control plants was dependent on leaf maturity. Of the three leaf types, the IL was affected by scorching the least (with D_s less than 1%). The D_s increased with leaf maturity, rising up to 6% in the ML, and up to 19% in the 2ML (Figures 4.11 and 4.12). However, this difference between leaf types was significant in only one of the four control plants (C8) (Tables 4.13 and 4.17).

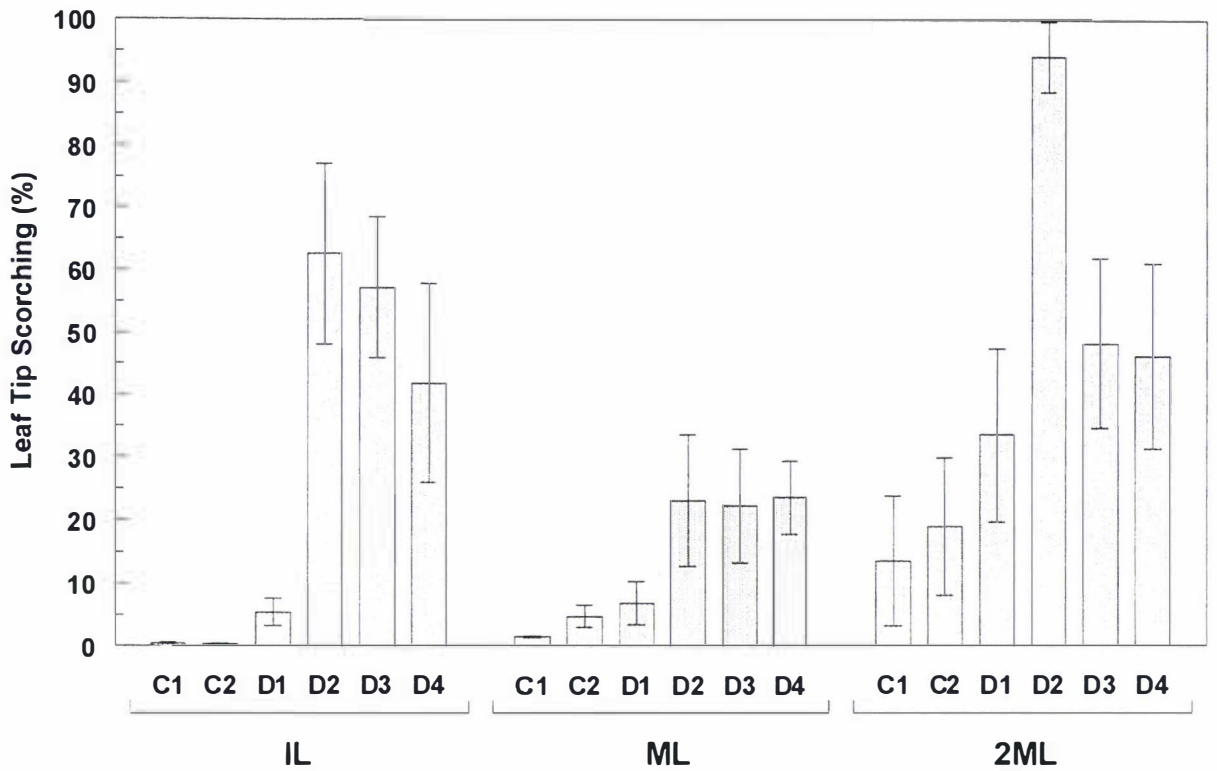


Figure 4.11: The D_s of each leaf type measured at the end of experiment I. The x-axis indicates plant numbers and leaf types. Data were means \pm s.e., $n = 8$ to 10 (see Table 4.13).

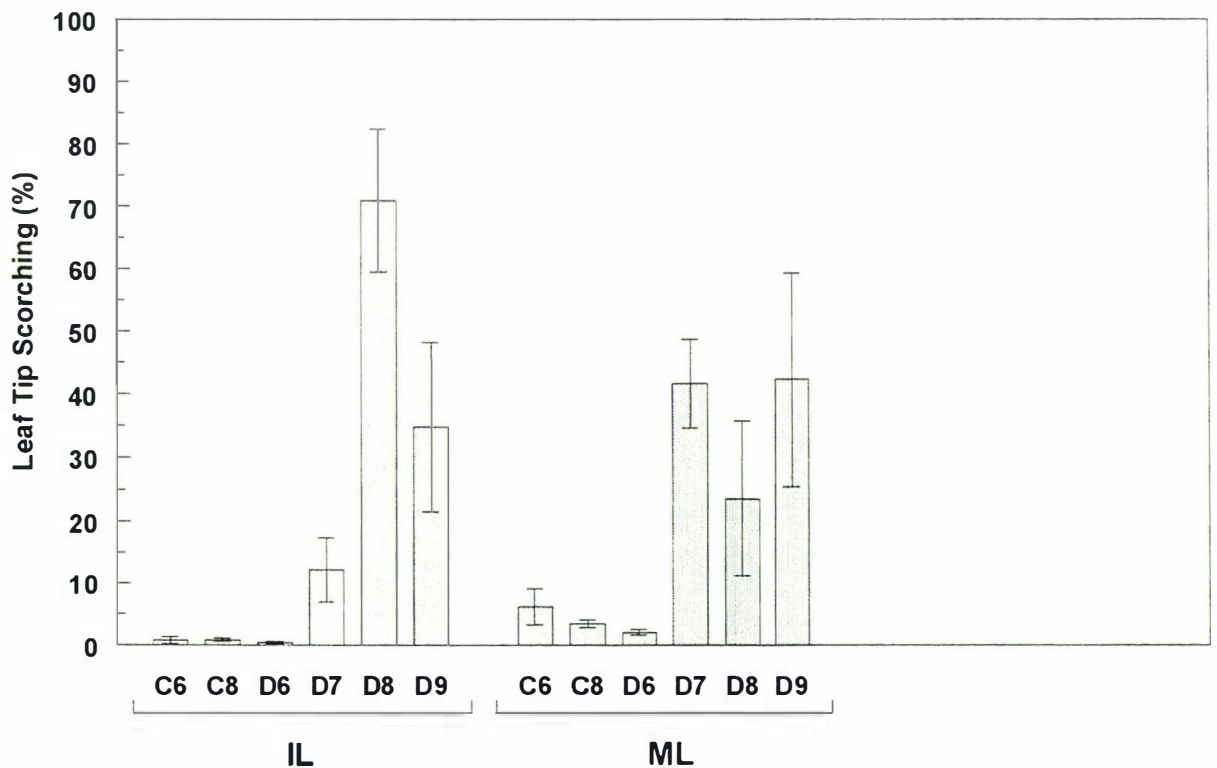


Figure 4.12: The D_s of each leaf type measured at the end of experiment II. The x-axis indicates plant numbers and leaf types. Data were means \pm s.e., $n = 6$ to 12 (see Table 4.17).

In response to the water-deficit treatment, the D_s increased in all the leaf types. At the end of experiment I, the D_s was greater than the controls in all of the water-deficit plants (Figure 4.11). In addition, the leaf types were differently affected by the treatment. In plants D1 to D4, the ML exhibited a similar or lower D_s than the IL or 2ML tissues (Figure 4.11). However, the difference in D_s between leaf types was significant only in plant D2 (Table 4.13). The comparison between the control and water-deficit plants for each leaf type are presented in Table 4.14. The controls had the least D_s for each leaf type, and D1 was NSD from either of the controls. Whether the D_s from the remaining water-deficit treatment plants was significantly different from the controls depended on the leaf type. When examining the data from the IL and 2ML tissues, only D2 was significantly different from the controls. However, with respect to the ML tissue, all the water-deficit treatment plants were NSD from at least one of the control plants (C2).

At the end of experiment II, plants D7, D8 and D9 had an elevated D_s (Figure 4.12), while D6 had a smaller D_s with respect to the controls (4 and 7). In most of the plants, the D_s of the IL was less than that of the ML. However, in D8, the D_s of the IL was significantly greater than that of the ML (Table 4.17).

The comparison between the control and water-deficit stressed plants for each leaf type from experiment II are presented in Table 4.18. As can be seen from this table, three significance groupings were allocated to the means from the IL. In this case, D7 and D6 were NSD from the control plants, while D8 and D9 had a significantly different (and greater) D_s on the IL than the controls. With respect to the ML, D9 and D7 had a significantly greater D_s , while D6 and D8 were NSD from the controls (Table 4.18).

In summary, in the water-sufficient plants, the D_s was dependent on the maturity of the leaf. When subjected to water-deficit stress, the D_s increased in all of the leaf types. However, the IL often had the greatest D_s of the leaf types examined. Because the greatest difference between the D_s of the control and the water-deficit treatment leaf types occurred on the IL, this leaf was considered to be the most suitable to indicate water-deficit stress. However, due to the high variation in the data, the D_s of the IL had to be greater than *ca.* 50%, before a significant difference from the controls could

be found. Hence measuring the D_s was not considered to be an accurate indicator of water-deficit stress in whole *F. novae-zealandiae* plants, although the low variation of D_s in the control IL tissue suggested that the D_s may be useful in determining whether an individual tiller had become water-deficit stressed.

4.3.2.5 Leaf Water Potential (LWP)

The LWP measurement in *F. novae-zealandiae* proved difficult. The small diameter of the lamina meant that obtaining a good seal around the leaf while inside the pressure chamber was difficult, especially as the pressure approached 3 MPa. Observation of the return of sap to the cut surface of the leaf was problematic, since the small volume of sap expressed was often barely visible. This difficulty became worse as the dry-down experiments progressed. Since the expressed sap had to exceed a minimum size to be seen clearly, and because the thin leaves of this species must by volume constraint contain a relatively small amount of sap, the end-point pressure could have been overestimated.

However, some trends in the data were apparent. The LWP of one of the controls (C6) was measured in the range -0.9 to -1.8 MPa, for the duration of experiment II. However, the LWP measured in the second control (C8) declined as low as -2.8 MPa, and remained below -2.0 MPa for the last 35 days (Figure 4.13). If the data from C8 was disregarded, a decline in LWP below the controls (<-2.0 MPa) occurred in the water-deficit treatment plants (D6, D7, D8 and D9) after *ca.* 45, 68, 40 and 40 days, respectively. A polynomial regression was fitted to the combined data from all the plants, with the exception of D8 (Figure 4.14). At higher SWC, the regression curve was essentially horizontal, at *ca.* -2.0 MPa, and the slope of the curve began to increase after the SWC had declined below *ca.* 16%.

Leaf Water Potential: Experiment II

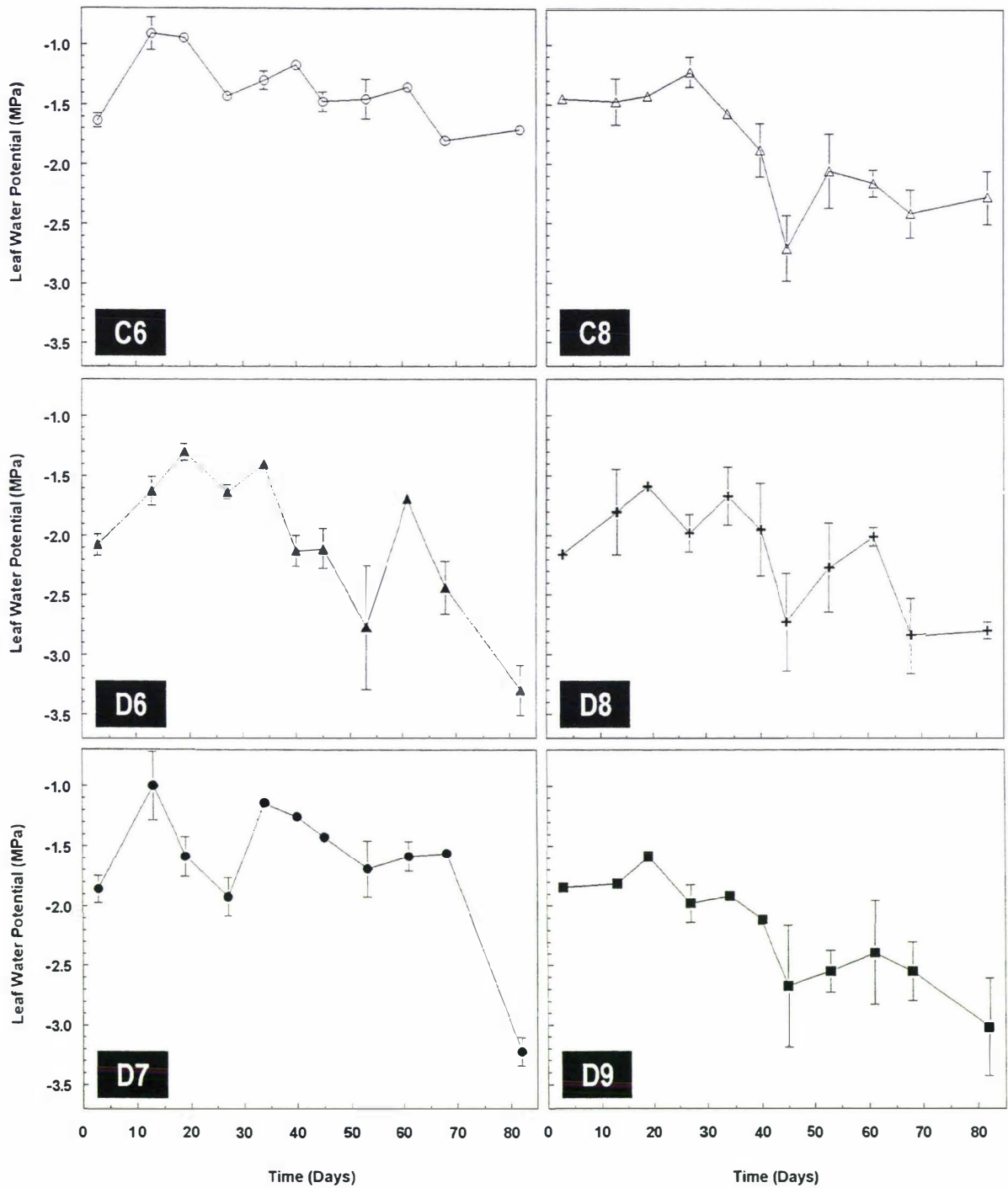


Figure 4.13: The LWP during experiment II. The labels indicate plant numbers. Data were means \pm s.e., $n=2$.

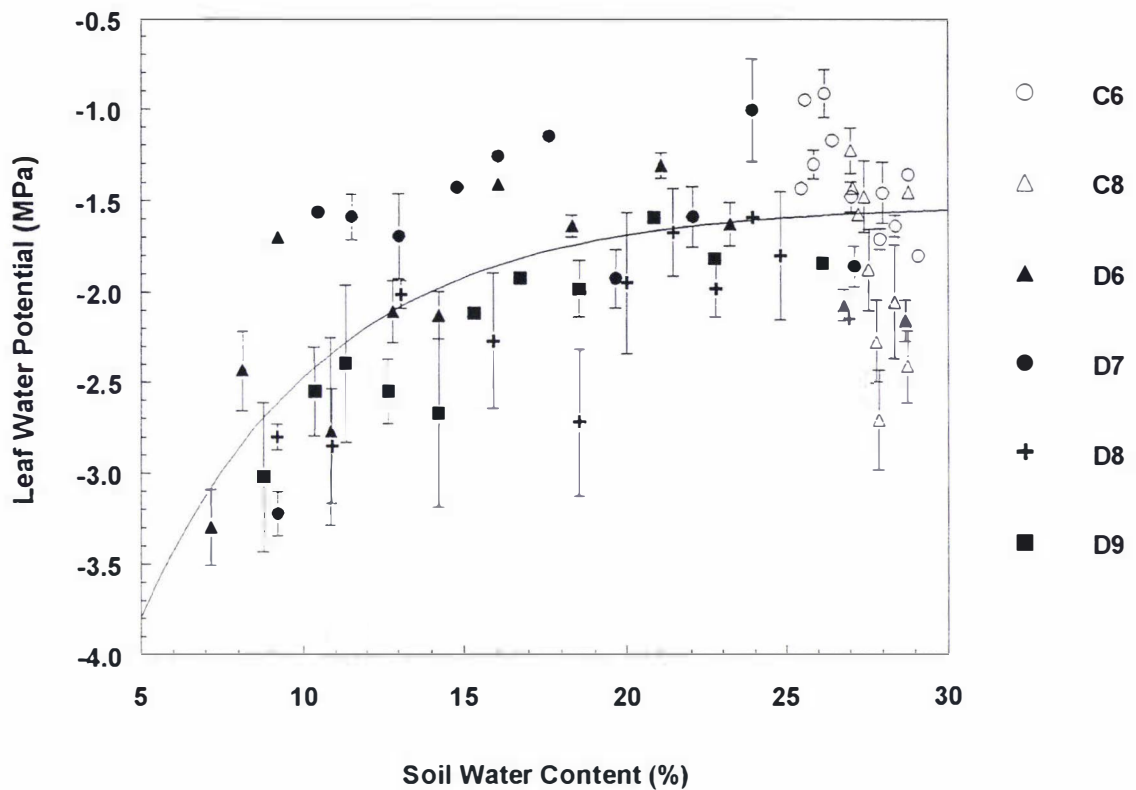


Figure 4.14: The effect of declining SWC on LWP during experiment II. Data from Figure 4.13 has been replotted as a function of SWC. The legend indicates plant numbers. The regression curve has been fitted to all data, except data from plant D8. Data were means \pm s.e., $n=2$.

4.3.2.6 Water-Deficit Stress Induced Metabolites

4.3.2.6.1 Leaf Abscisic Acid Concentration

The ABA concentration measured in the control plants remained relatively constant throughout both experiments, with the exceptions of C6 and C8, which had relatively high data recorded on days 2 and 52, respectively (Figure 4.16). Discounting these two unusually high measurements, the concentration of ABA in the ML of control plants (C1 and C2 from experiment I, C6 and C8 from experiment II) was 4 to 30 ng/gFW (with an average of 13.1 ng/gFW). This range was similar to that in the water-sufficient tissue of *F. arundinacea* (5 to 30 ng/gFW; section 3.4.4.1, page 77).

The ABA concentration in the ML of water-deficit treatment plants was elevated with respect to the controls. The maximum ABA concentration measured was *ca.* 80 ng/gFW in D2 and D8 (from experiments I and II, respectively), which represented a *ca.* six-fold increase over the average ABA concentration of water-sufficient plants. This

concentration was lower than the highest value of *ca.* 200 ng/gFW measured in *F. arundinacea* (section 3.4.4.1, page 77).

The water-deficit treatment plants accumulated ABA at different times during experiment I. The ML of D1 had a peak ABA concentration after 20 days which then declined to a similar level to the controls for the rest of the dry-down (Figure 4.15). Plant D2 had an elevated level of ABA compared to the controls during the first 30 days, which then declined to the level of the controls for the next 30 days, and accumulated toward a peak of ABA concentration on day 72 before it declined again to less than 40 ng/gFW by the end of the dry-down. The ABA concentration in D3 remained at control levels for at least 55 days before increasing to reach a peak of 47 ng/gFW on day 72. Plant D4 underwent relatively minor accumulations of ABA (about 40 ng/gFW) which peaked on days 8, 20 and 67 (Figure 4.15).

Although there were differences in the pattern of changes in ABA concentration between the water-deficit treatment plants, there may be common elements, such as accumulation events on days 8, 20 and 72. The former two events can be discounted as water-deficit induced ABA accumulation because the mean SWC was still relatively high (>14%, *c.f.* Figure 4.3). The cause of these ABA accumulations was not obvious. The accumulation of ABA which occurred at day 72 in D2, D3 and D4 may have been induced by water-deficit stress, since by this stage of the experiment, the SWC of the top soil horizon of these three plants had declined to *ca.* 8% (Figure 4.1). However, any correlation between ABA concentration and SWC was difficult to determine, although a general increase in the ABA concentration above 30ng/g FW occurred when the SWC had declined below 9% (Figure 4.17).

At the start of experiment II, the ABA concentration of all plants (including the controls) was relatively high, and declined during the first 20 days (Figure 4.16). After remaining low for the next two weeks, the ABA concentration in all plants increased. The fact that ABA levels also increased in the watered control plants at this time suggests that the accumulation of ABA that commenced around day 40 was not a water-deficit induced event. However, the magnitude of the accumulation of ABA in the water-deficit treatment plants was greater than that observed in the controls. The

accumulation of ABA peaked in the water-sufficient plants (C6 and C8) on days 61 and 53 (respectively), and peaked in D6, D8 and D9 on days 68, 61 and 53 (respectively, Figure 4.16). The ABA concentration in D7 remained below 30ng/gFW for the rest of the experiment, a range similar to that of the controls. Hence only three of the water-deficit treatment plants (D6, D8 and D9) displayed a net accumulation of ABA above that of the control plants (Figure 4.16).

No clear correlation between SWC and the ABA concentration of the ML was found (Figure 4.18). The accumulation of ABA observed in the water-deficit treatment plants occurred at *ca.* 13% in plants D8 and D9, while the concentration of ABA in D6 appeared to peak after the SWC had declined to less than 8%.

4.3.2.6.2 Leaf Proline Concentration

The proline concentration measured in ML from the control plants were in the range 10 to 155 $\mu\text{g/gFW}$ during experiment I (Figure 4.19), and 2 to 500 $\mu\text{g/gFW}$ during experiment II (Figure 4.20). The leaf proline concentration increased in response to the water-deficit treatment.

In experiment I, the proline concentration measured in D1 remained low during the dry-down, with the exception of a single datum on day 20 (Figure 4.19). Plant D2 accumulated proline to *ca.* 400 $\mu\text{g/gFW}$ which peaked on day 80, and was followed by a decrease during the last 20 days. In D3, the proline concentration remained low for the first 70 days, followed by an increase to *ca.* 300 $\mu\text{g/gFW}$. The proline concentration of D4 remained within the range of the control plants throughout the experiment (Figure 4.19).

During experiment II, no accumulation of proline occurred in D6, with the concentration remaining below 200 $\mu\text{g/gFW}$ (Figure 4.20). Plant D7 was unusual in that it had an elevated proline concentration of between 400 and 500 $\mu\text{g/gFW}$ throughout the experiment. Increases above this level occurred around days 35 to 40, and again after 67 days (Figure 4.20). Plant D8 had the clearest proline accumulation, with levels rising above a baseline of *ca.* 100 $\mu\text{g/gFW}$ after 45 days.

Leaf ABA Concentration: Experiment I

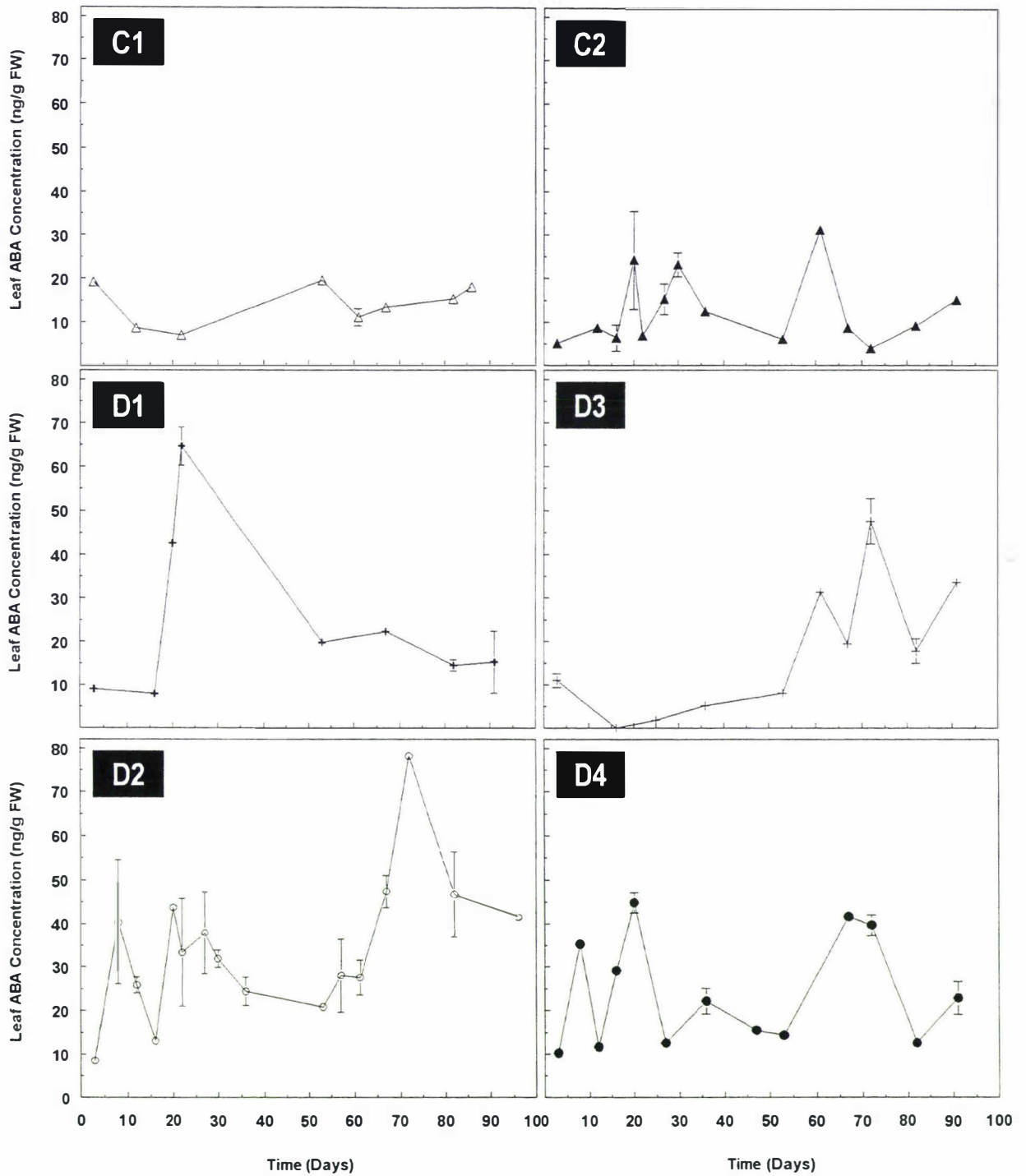


Figure 4.15: Leaf ABA concentration measured during experiment I. The labels indicate plant numbers. Data were means \pm s.e., $n=2$.

Leaf ABA Concentration: Experiment II

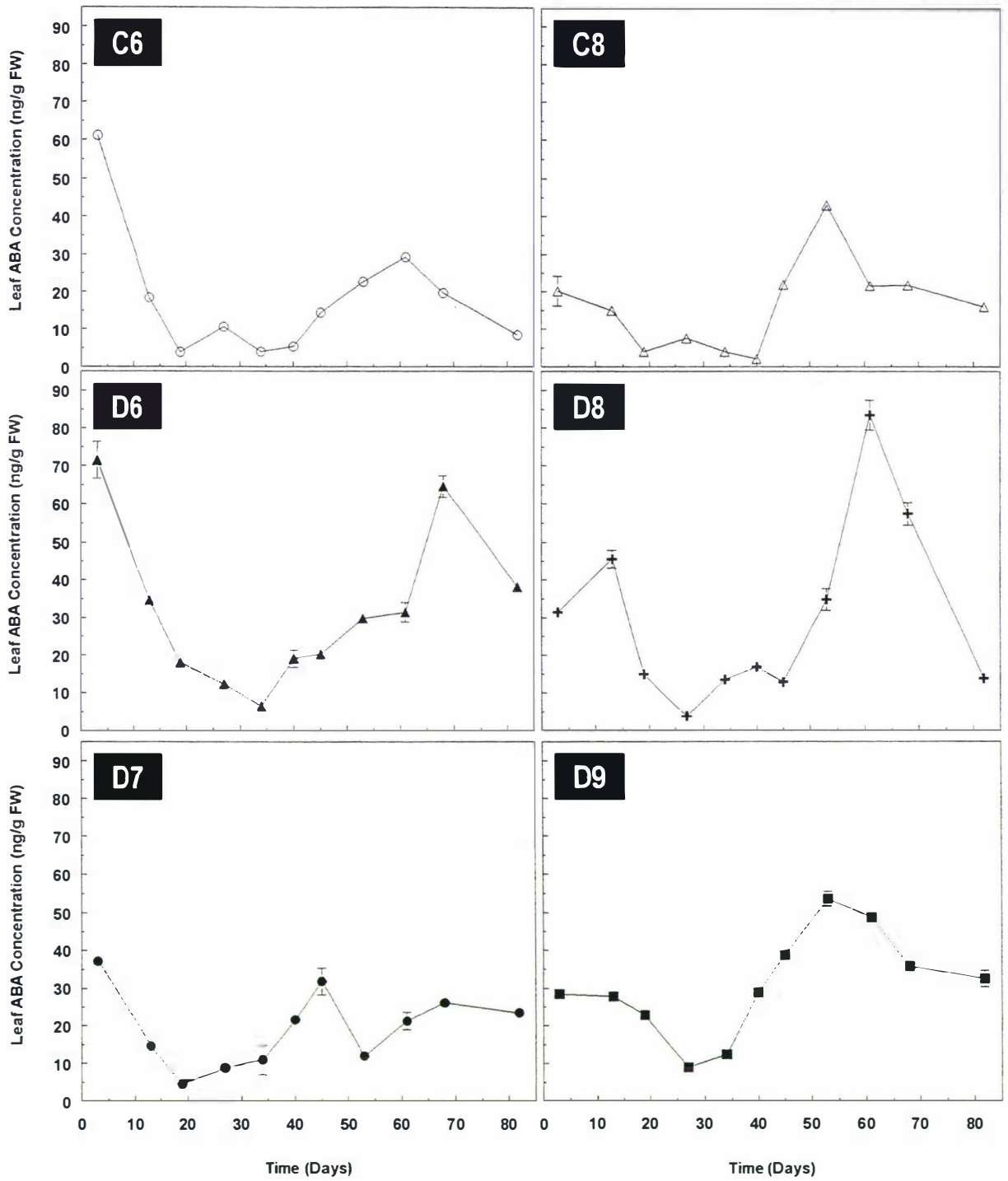


Figure 4.16: Leaf ABA concentration measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., $n = 2$.

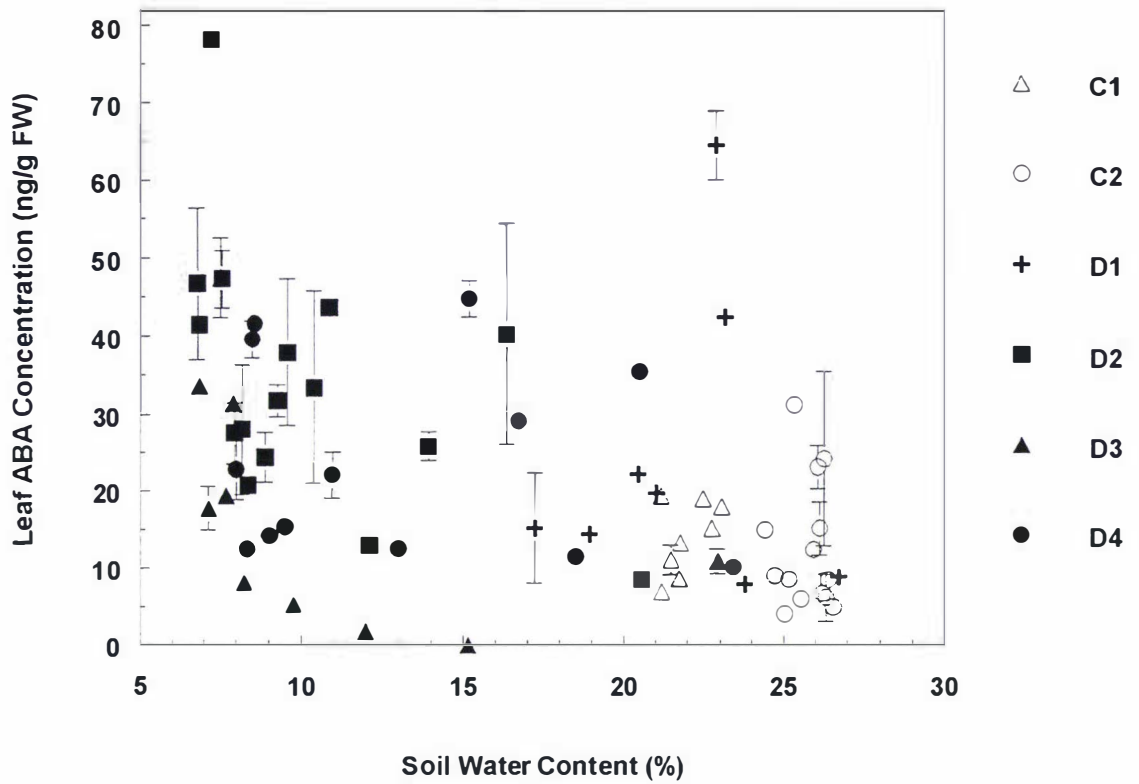


Figure 4.17: The effect of declining SWC on leaf ABA concentration during experiment I. Data from Figure 4.15 has been replotted as a function of the SWC of the top soil horizon. The legend indicates plant numbers. Data were means \pm s.e., $n=2$.

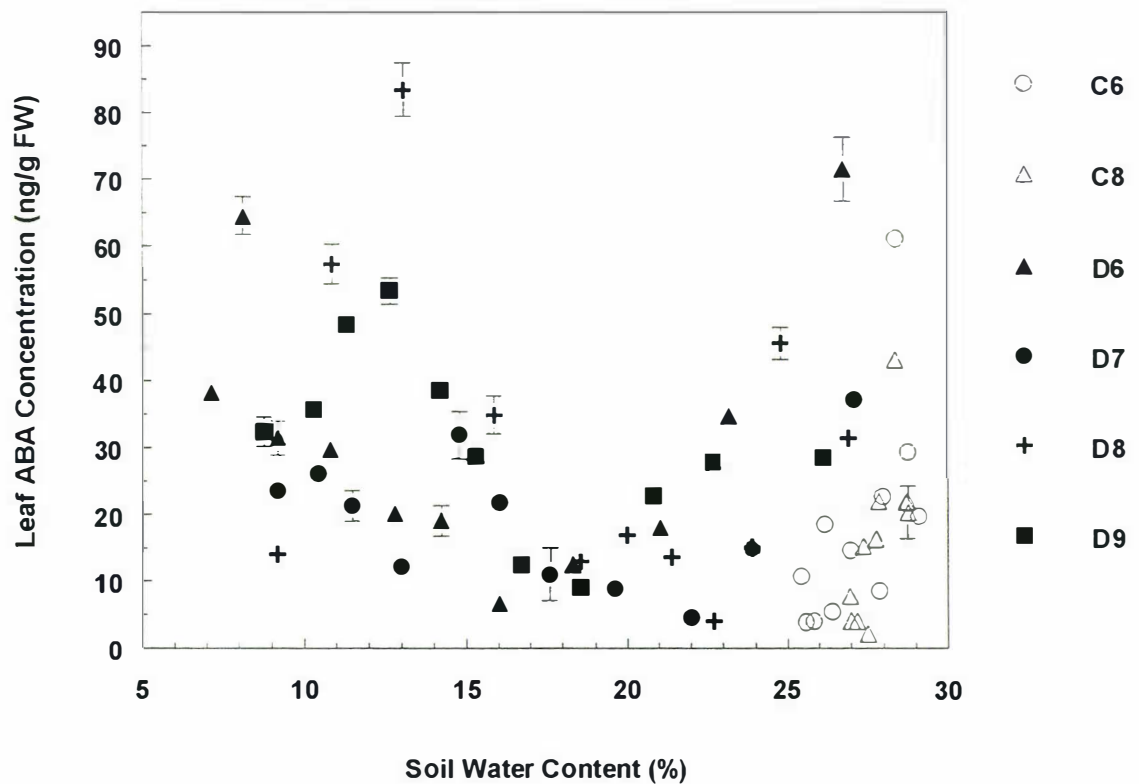


Figure 4.18: The effect of declining SWC on leaf ABA concentration during experiment II. Data from Figure 4.16 has been replotted as a function of the SWC from the vertical electrode station. The legend indicates plant numbers. Data were means \pm s.e., $n=2$.

The peak proline concentration of about 1000 $\mu\text{g/gFW}$ occurred in this plant after 60 days, before the proline concentration declined to about 200 $\mu\text{g/gFW}$ by the end of the experiment. No clear accumulation of proline was observed in D9, although a temporary rise in proline concentration to about 600 $\mu\text{g/gFW}$ occurred during the first 20 days.

In summary, the accumulation of proline by *F. novae-zealandiae* in response to water-deficit was an inconclusive result. Four of the total of eight plants subjected to the water-deficit treatment (D1 and D4 from experiment I, D6 and D9 from experiment II) did not accumulate proline. In addition, the levels that proline attained in D2 and D3 from experiment I (300 to 400 $\mu\text{g/gFW}$) were similar in magnitude to some individual data from the water-sufficient plants during experiment II. The maximum increase in proline concentration was less than eight-fold and ten-fold during experiments I and II, respectively. These data were low compared to the *ca.* fifty-fold increase in proline found with *F. arundinacea* during a similar experiment (section 3.4.4.2, page 77).

The onset of the accumulation in plants that did accumulate proline in experiment I occurred after the mean SWC had declined below 8% (plants D2 and D3, Figure 4.21). However, in experiment II, proline began to accumulate at *ca.* 10% SWC in D7, and *ca.* 13% in D8. This variability in the SWC suggests that factors other than the SWC may have been involved. However, problems in accurately determining the SWC available to the plants were also encountered (discussed in section 4.4.2).

Leaf Proline Concentration: Experiment I

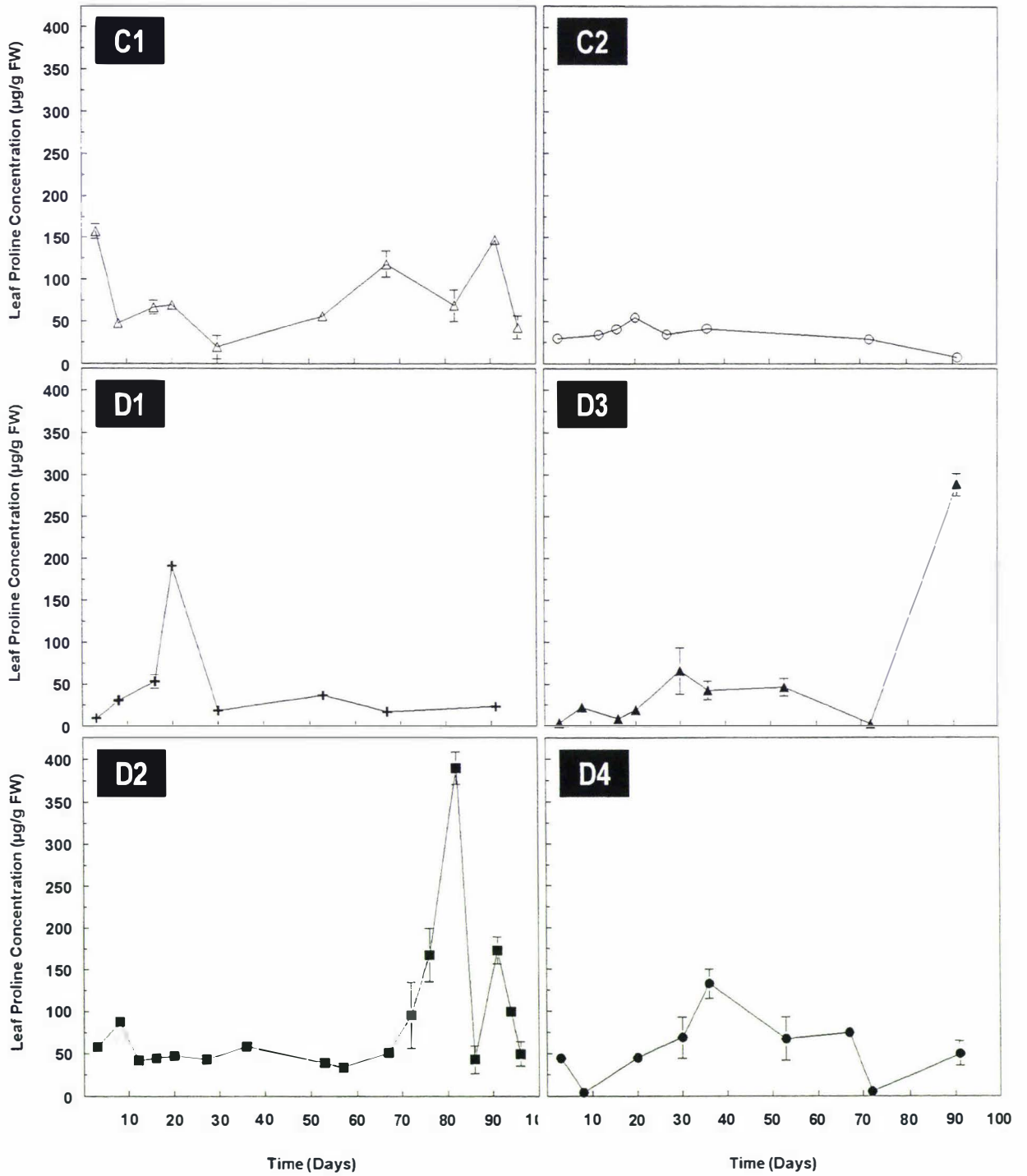


Figure 4.19: Leaf proline concentration measured during experiment I. The labels indicate plant numbers. Data were means \pm s.e., n=2.

Leaf Proline Concentration: Experiment II

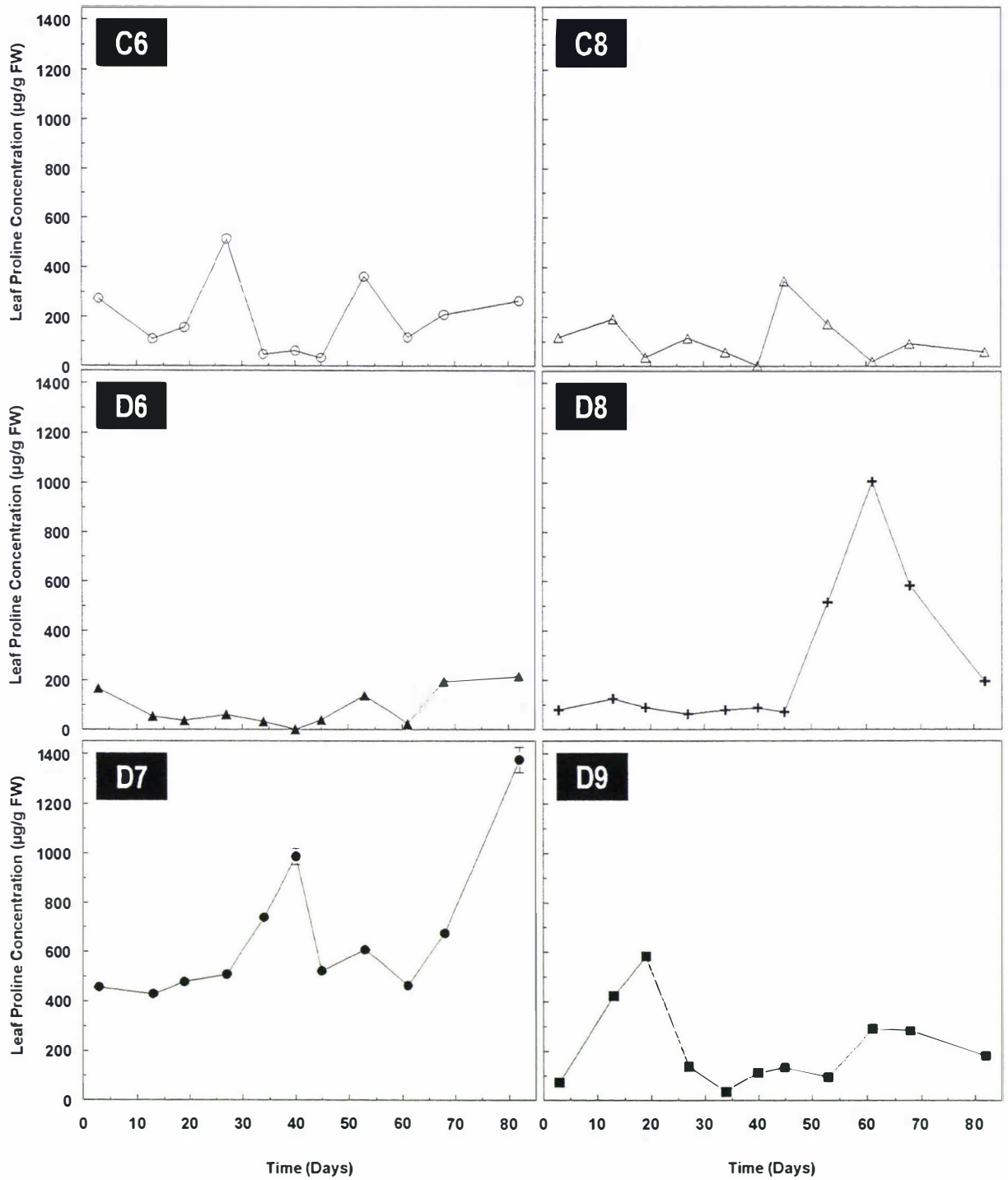


Figure 4.20: Leaf proline concentration measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., n=2.

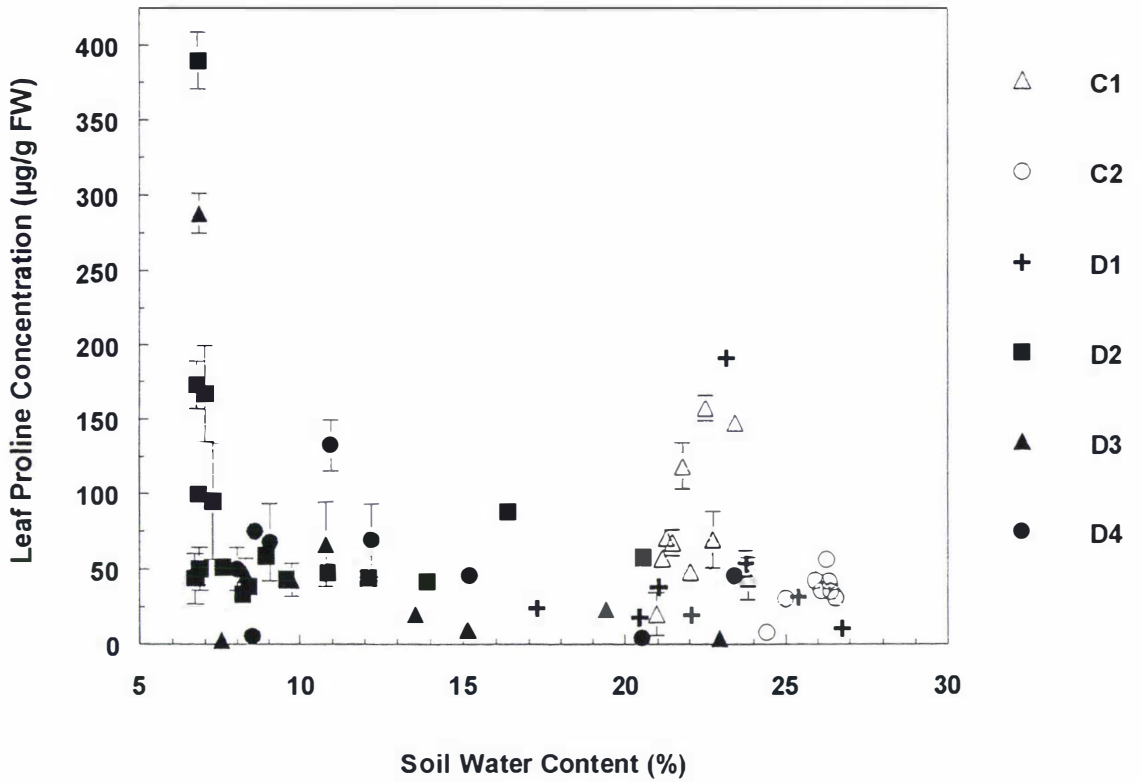


Figure 4.21: The effect of declining SWC on leaf proline concentration measured during experiment I. Data from Figure 4.19 has been replotted as a function of the SWC of the top soil horizon. The legend indicates plant numbers. Data were means \pm s.e., n=2.

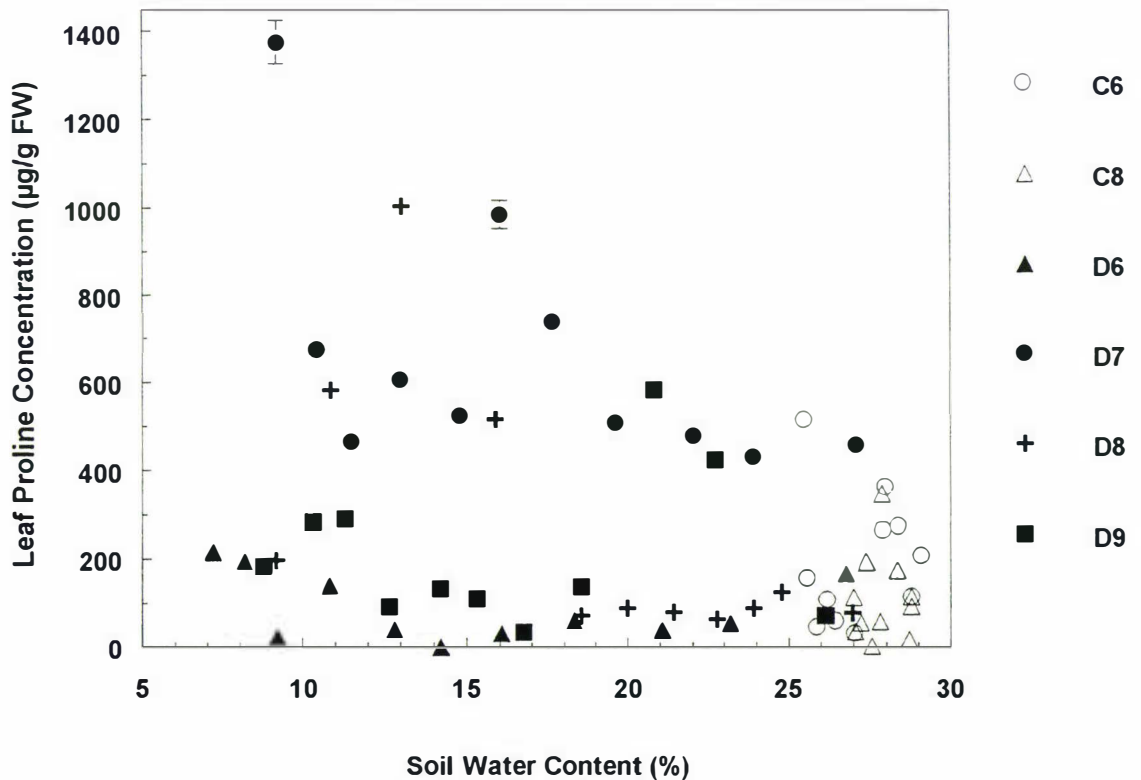


Figure 4.22: The effect of declining SWC on leaf proline concentration measured during experiment II. Data from Figure 4.20 has been replotted as a function of the SWC from the vertical TDR electrode station. The legend indicates plant numbers. Data were means \pm s.e., n=2.

4.3.2.6.3 Leaf Glycine-Betaine Concentration

Because insufficient tissue was available following the previous analyses, the glycine-betaine contents were determined in the ML from only three plants (D2, D4 and C2) from experiment I (Figure 4.23). All six plants were assayed during experiment II (Figure 4.24).

During experiment I, the glycine-betaine concentration in the control (C2) remained in the range of 7 to 18 $\mu\text{mol/g}$ FW. However, a single datum on day 91 was relatively high, and was considered to be an outlier (Figure 4.23). In the water-deficit treatment plants (D2 and D4), glycine-betaine increased gradually throughout experiment I to approximately double by the end of the experiment (Figure 4.23), although the data did not exceed values observed in the control until at least 72 days. It was not clear if the increase in glycine-betaine concentration was in response to the water-deficit treatment. The outlying datum observed in the control plant was the highest measured during the experiment, and if it was not considered to be an outlier, would mean that the glycine-betaine concentration of the water-deficit treatment plants did not differ from the control.

During experiment II, glycine-betaine concentrations occurred in the range 18 to 33 $\mu\text{mol/g}$ FW in the control plants (C6 and C8), which was about double that observed in experiment I. In experiment II, the water-deficit treatment plants did not accumulate glycine-betaine. A similar concentration to the controls were observed in D6 and D8, and lower ranges (up to 16 and 24 $\mu\text{mol/g}$ FW, respectively) were measured in D7 and D9; the glycine-betaine concentration remained constant in these four plants throughout the experiment (Figure 4.24).

Discounting the outlier from experiment I, glycine-betaine concentration was dependent on SWC. The glycine-betaine concentration of the water-deficit treatment plants (D2 and D4) exceeded that of the control plant (C2) after the SWC had declined below 10% (Figure 4.25). No correlation between SWC and glycine-betaine concentration was observed during experiment II (Figure 4.26).

Leaf Glycine-betaine Concentration: Experiment I

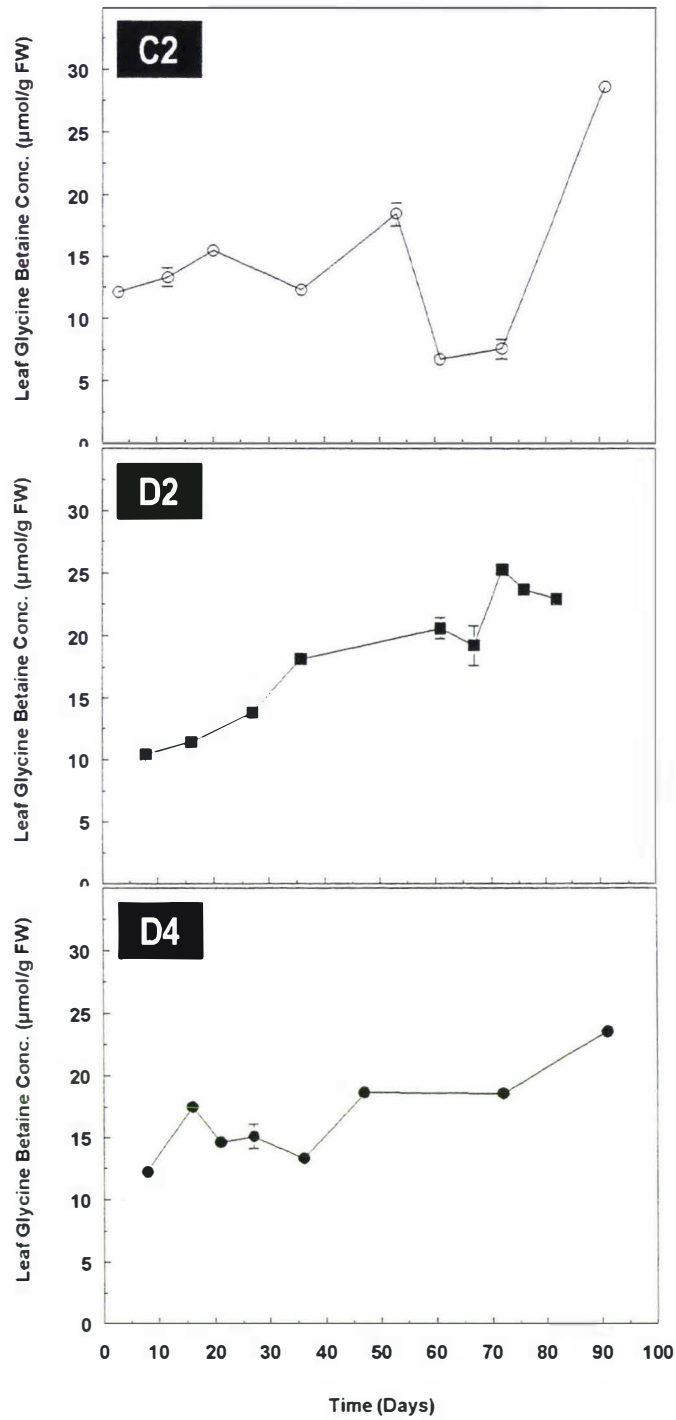


Figure 4.23: Leaf glycine-betaine concentration measured during experiment I. The labels indicate plant numbers. Data were means \pm s.e., $n=2$, except for data from day 91 ($n=1$).

Leaf Glycine-betaine Concentration: Experiment II

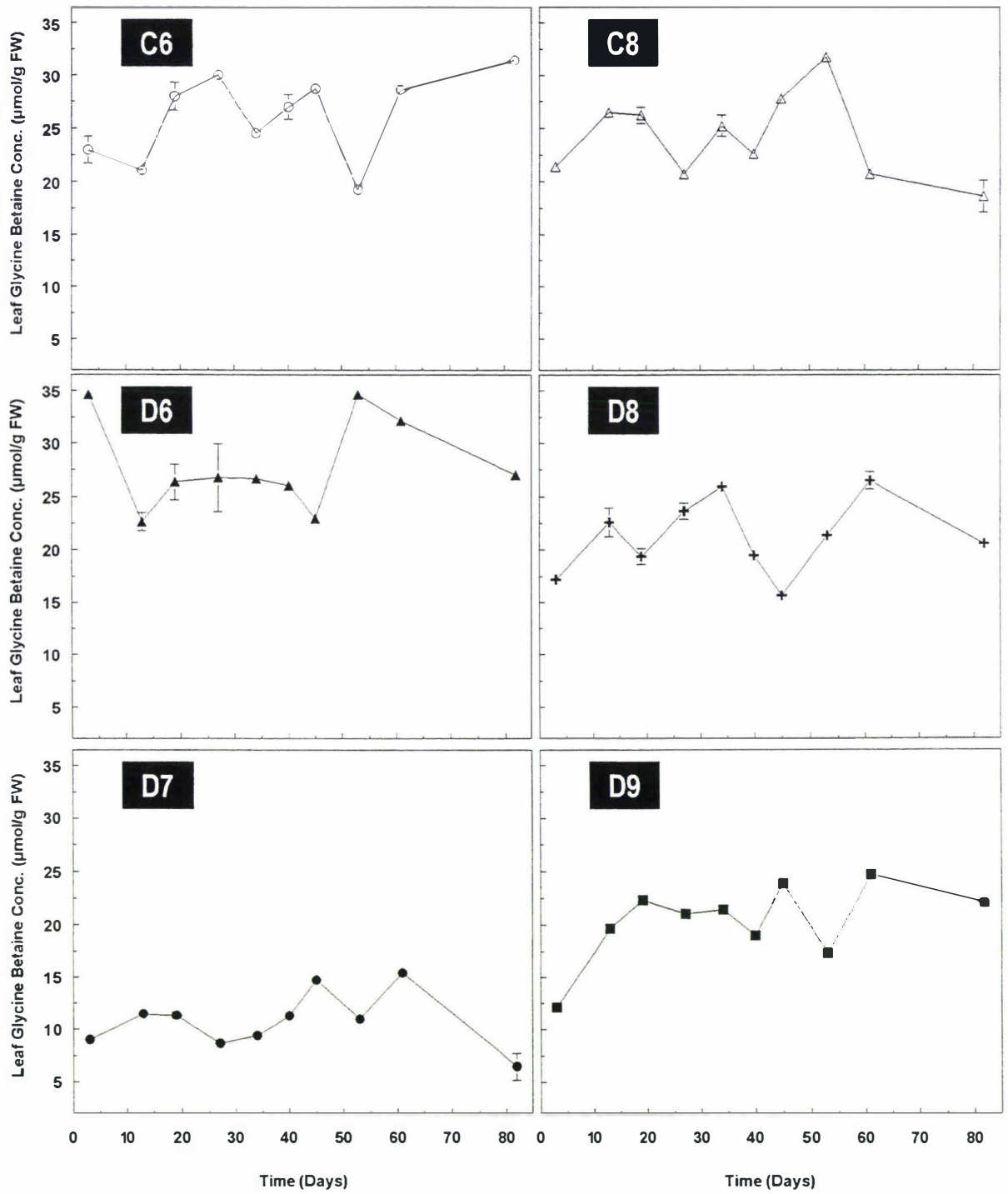
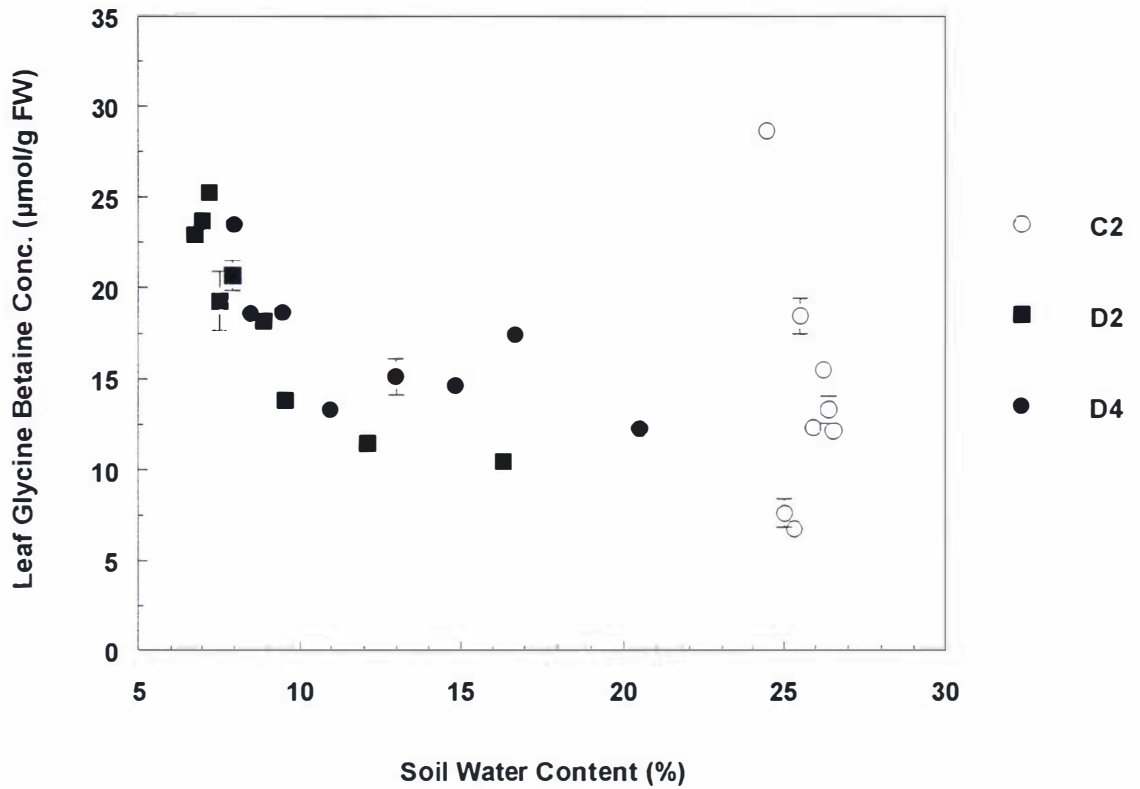


Figure 4.24: Leaf glycine-betaine concentration measured during experiment II. The labels indicate plant numbers. Data were means \pm s.e., $n=2$.



Figures 4.25: The effect of declining SWC on leaf glycine-betaine concentration during experiment I. Data from Figure 4.23 has been replotted as a function of the SWC of the top soil horizon. The legend indicates plant numbers. Data were means \pm s.e., $n=2$, except for data from day 91 ($n=1$, see Figure 4.23).

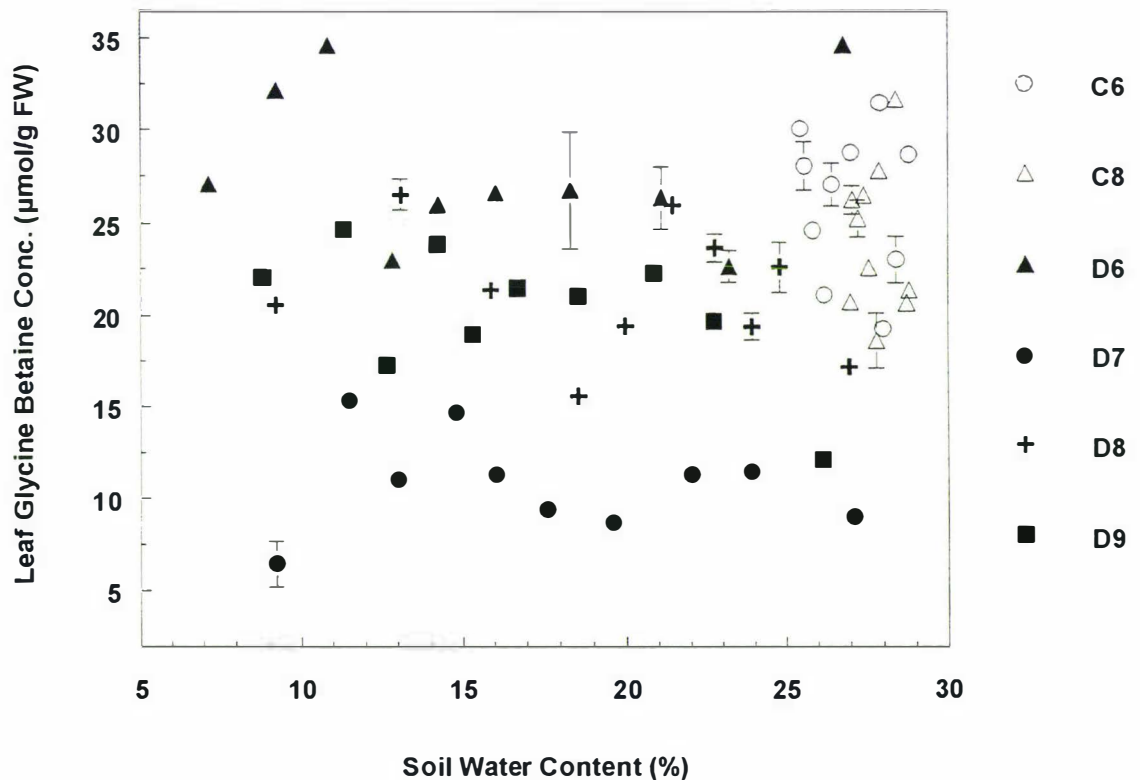


Figure 4.26: The effect of declining SWC on leaf glycine-betaine concentration during experiment II. Data from Figure 4.24 has been replotted as a function of the SWC from the vertical TDR electrode station. The legend indicates plant numbers. Data were means \pm s.e., $n=2$.

4.3.2.7 Root Distribution

At the conclusion of both experiments, the soil column was removed intact from each 24 L pot and cut into sections. The roots in each section were washed free of soil, dried, weighed and the distribution of the bulk of the roots was determined (Figure 4.27 and Figure 4.28, for experiments I and II, respectively).

For each of the plants examined, the roots were predominantly in the top 200 mm of soil (Figure 4.27 and Plate 4.7). This region also contained one of the five side-mounted TDR electrodes (the top soil horizon station) and also the 200 mm deep vertical electrodes. Two of the three water-deficit treatment plants (D2 and D4) from experiment I had a few deep sparsely branched roots along with the mass of fibrous roots located near the surface.

The root distribution obtained from experiment II were similar to those from experiment I, with the roots of each plant predominantly in the top two soil sections (up to 140 mm in depth, Figure 4.28). A few sparsely branched roots from each plant penetrated up to the fifth soil section (a depth of 450 mm). Only plant D6 had significant root mass deeper than this section. In this case, the roots extended to the base of the pot (*ca.* 745 mm).

Root Distribution: Experiment I

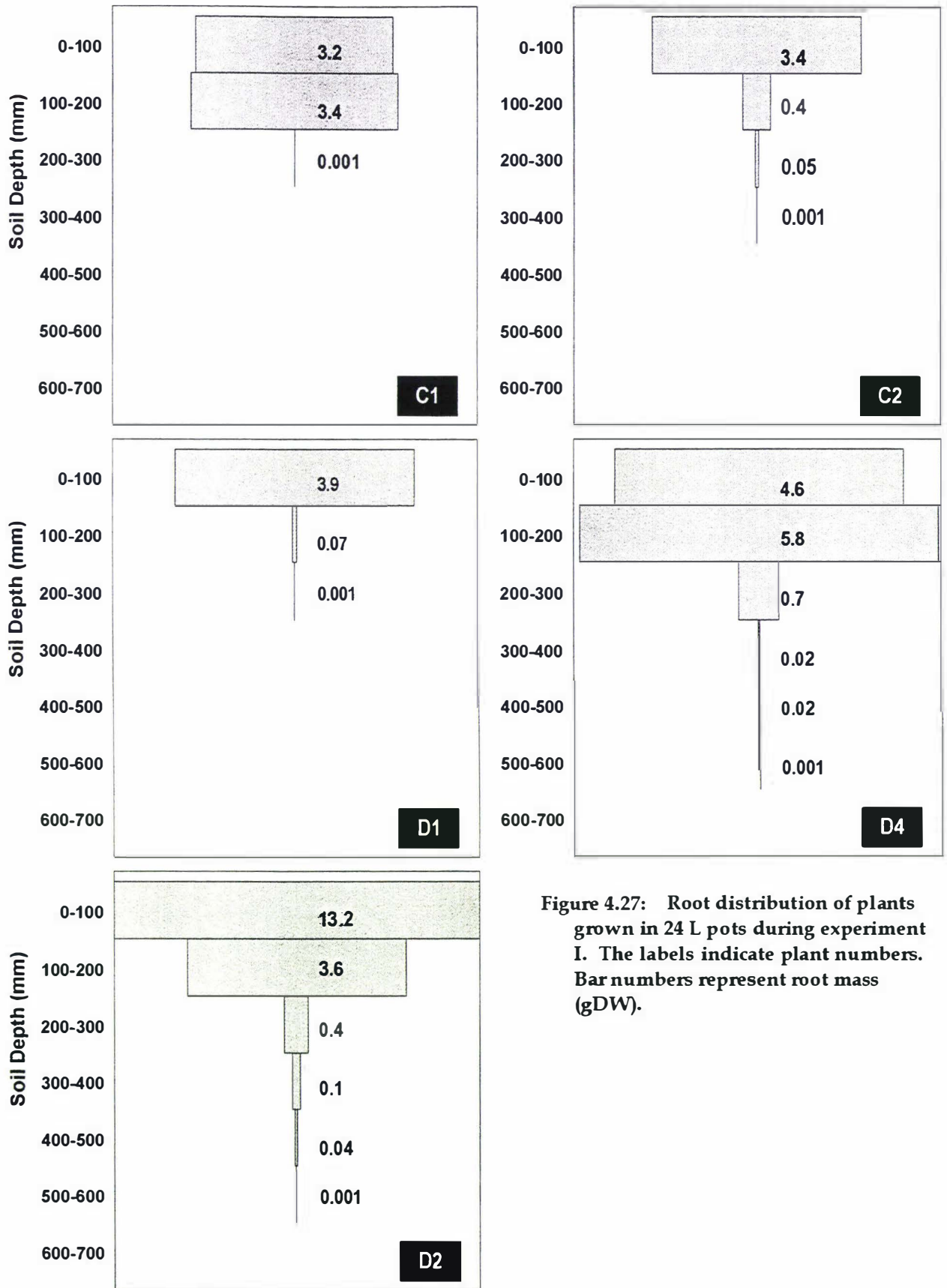


Figure 4.27: Root distribution of plants grown in 24 L pots during experiment I. The labels indicate plant numbers. Bar numbers represent root mass (gDW).

Root Distribution: Experiment II

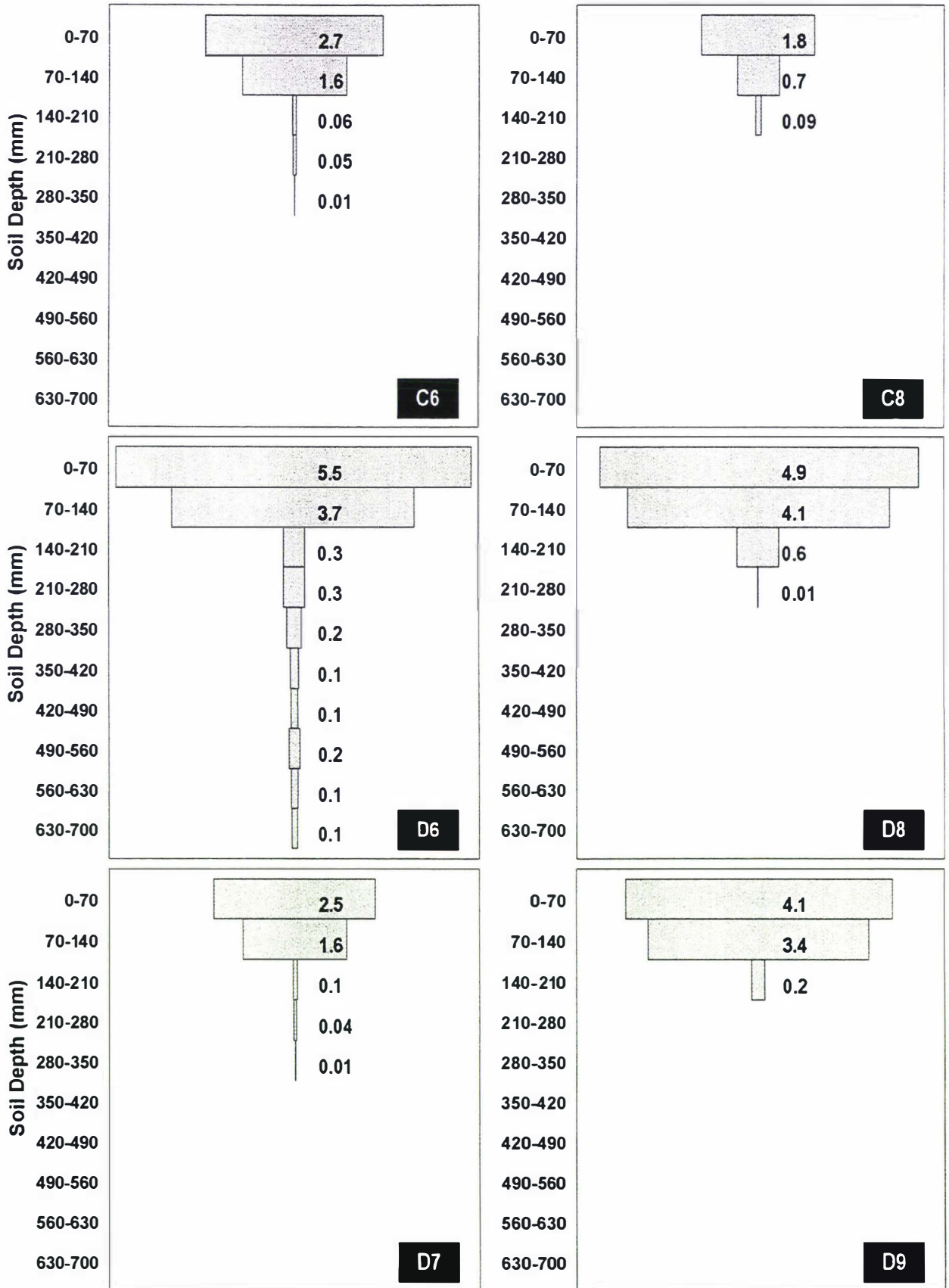


Figure 4.28: Root distribution of plants grown in 24 L pots during experiment II. The labels indicate plant numbers. Bar numbers represent root mass (gDW).

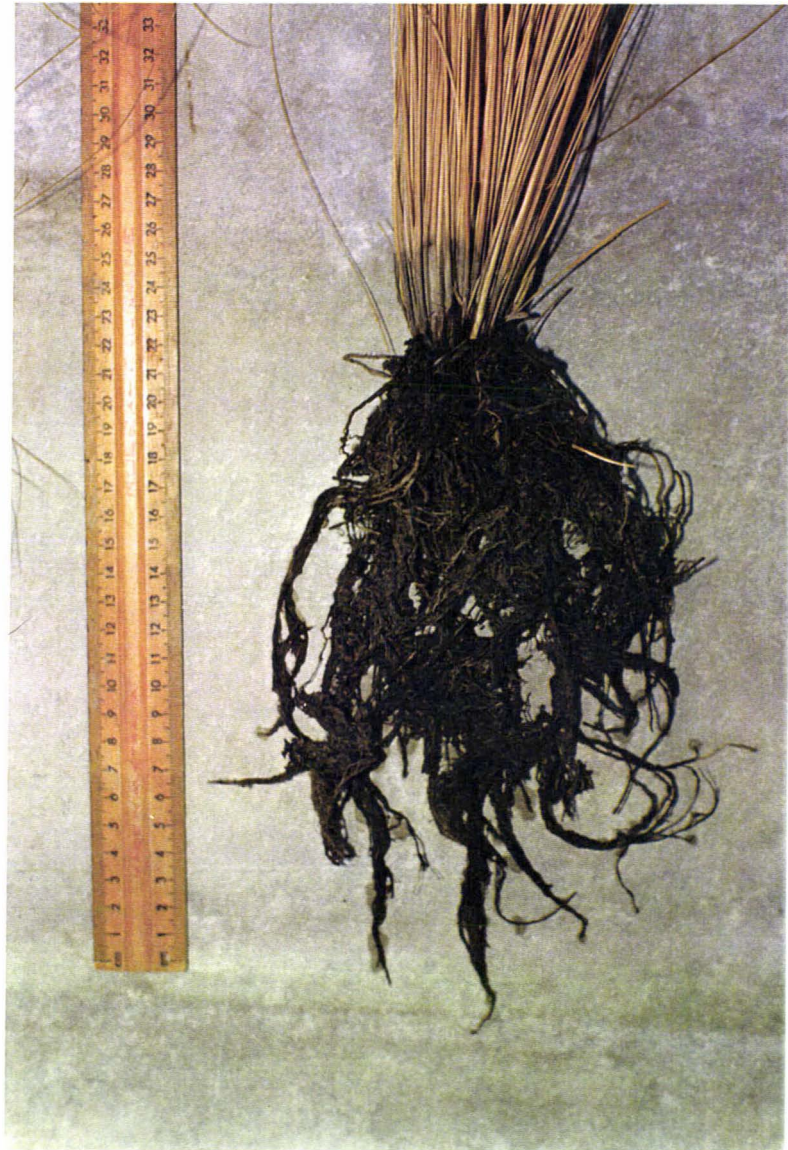


Plate 4.7: Distribution of the roots of *F. novae-zealandiae* (plant D3 from experiment I) within the soil column of a 24 L PVC pot.

4.4 Discussion

4.4.1 Morphological Characteristics

Festuca novae-zealandiae had morphological adaptations to arid environments that were not observed in *F. arundinacea*. The most striking feature of the tussock was the dry, fibrous appearance, which is consistent with one of the plants common names "hard tussock". When leaves senesce they do not collapse to the ground, as was the case with *F. arundinacea*, but are kept upright by their fibrous structure. This habit may affect the drought tolerance of this plant in two ways. Firstly, large numbers of upright tillers (alive or dead) can effectively shade living tillers from wind and sunlight, reducing transpiration within the clump. Secondly, the reduced palatability of dead tillers may act as a feeding deterrent. This may be an important adaptation, since plants that are adapted to harsh environments are generally characterised by a reduced ability to assimilate resources and to replace tissues lost to grazing (Chapin, 1991). The fibrous nature of *F. novae-zealandiae* was attributed to bundles of sclerenchyma (fibre) cells occurring around the perimeter of the leaf. The distribution of these fibre bundles within the lamina of *F. novae-zealandiae*, and the fusion of bundles in the older (lower) portions of the lamina to form continuous rings of fibres has been studied in detail (Connor, 1960).

Festuca novae-zealandiae does not possess an extensive root structure. This agrees with Wardle (1991), who described the roots as "sparse and relatively coarse", and as "characteristic of slow growing tussocks on undisturbed soils". The shallow roots were in contrast to the deep, fibrous root system of *F. arundinacea*, a species which may employ a drought avoidance strategy of accessing water from deeply below the soil surface.

Several adaptations to prevent water loss from individual leaves of *F. novae-zealandiae* were identified. The long, narrow leaves may be an efficient structure for heat exchange, thus cooling the leaves during hot conditions and reducing the need for transpirational cooling. The leaf lamina is rolled, which entirely encloses one surface of the leaf and forms an internal region that is lined with a waxy cuticle, and is densely packed with trichomes. All the stomata found on the leaves of this species were located within this enclosed region; no stomata were located on the exposed surface of the leaf.

This region has only a small gap at the leaf margin opening to the atmosphere. Fans of bulliform cells were present between the vascular traces, and may allow *F. novae-zealandiae* to control the aperture of the margin gap. Gas exchange between the central region of the leaf and the atmosphere would occur at a low rate, resulting in an increase in local humidity. The enclosed surface also prevents exposing stomata to wind currents or direct sunlight. The above factors are all involved in reducing water evaporation from the leaf surface. This kind of anatomy, where stomata are located in sunken depressions in leaves, is common to xerophytes (Esau, 1977; Raven *et al.*, 1986).

Many grasses exhibit a modified photosynthetic metabolism, termed the C₄ (or Hatch-Slack) pathway. This pathway has been cited to be an adaptive advantage to plants living in arid environments (Raven *et al.*, 1986). Pronounced bundle-sheath cells, characteristic of the Kranz anatomy associated with C₄ metabolism, were not evident in the leaves of either *Festuca* species. This suggests that both species use a C₃ carbon fixation pathway (agreeing with Clifford and Watson (1977), for *F. arundinacea*; the C₃/C₄ nature of *F. novae-zealandiae* has not been reported).

4.4.2 Experimental Dry-Down

The experiments described in this chapter were carried out using the 24 L pot system that was used for the final experiment with *F. arundinacea* (Chapter Three, page 66). However, these pots were not as suitable for use with *F. novae-zealandiae* as with the former species. In most cases, the SWC of only the top soil horizon of each water-deficit treatment pot declined appreciably during the two experiments. This was because the root system of *F. novae-zealandiae* was found to be relatively shallow, with most of the roots penetrating no more than 200 mm into the soil. Therefore, averaging the SWC of all the soil horizons in a similar manner to that carried out for *F. arundinacea* was misleading, since this average would have included data from soil horizons that were devoid of roots. SWC data from either the top soil horizon, or the vertical electrode station (for experiments I and II, respectively) was used to monitor the progress of the experiments. Because both of these electrode stations measured the SWC within the top 200 mm of the soil column, the data from these electrodes more closely reflected the SWC near the majority of the roots than the mean SWC. However, care is still needed to be taken when using the SWC as a basis for comparison between

experimental plants, since root density differed between plants (Figures 4.27 and 4.28). In addition, a few roots penetrated deeper than the top soil horizon, and hence would tap into a water supply which was not being measured; it was also not known which of the plants tillers (alive or dead) were connected to these deep roots.

The physiological data indicated that the experimental plants were affected by the water-deficit. After a period of continued growth, a general decline in the health of water-deficit stressed plants was observed. The LER declined in response to the water-deficit treatment, but did not cease. This result may be interpreted as an indication that the plants were either subjected to a non-limiting stress, or they were able, in part, to overcome the deleterious effects of the stress. The latter appears to be the case, since the continuation of elongation was accompanied by a decline in the number of surviving tillers. However, the LER data and tiller counts were not completely satisfactory as means of indicating water-deficit stress. Because of this, several other measurements of the leaves were made.

The M_T and N_T measurements were related, such that the tillers with the fewest green leaves (plant D2 from experiment I, D8 and D9 from experiment II) also had the smallest tiller mass. However, because of the variation of tiller size within an individual tussock, and variation in tiller size between experiments as well as between treatments (control *versus* water-deficit), the M_T was considered to be unsuitable for use as an indicator of water-deficit stress. However, it became apparent during the experiment that *F. novae-zealandiae* tillers lost leaves in response to the water-deficit treatment. The tillers of this species normally carry three (occasionally four) green leaves at one time. These leaves were turned over in a developmental cycle with the immature leaf expanding as the most mature leaf senesces. Upon water deficit stress, the number of leaves per tiller decreased, as the rate of turn over of the most mature leaf was increased. This decline in N_T was usually accompanied by a increase in the extent of leaf-tip scorching. The IL and 2ML were affected to a greater extent than the ML, hence the D_S did not appear to correlate with leaf maturity. This result suggested an element of control in the leaf turnover. Of all the leaf types, scorching of the IL was the most severe. The IL of the control plants usually had a negligible amount of scorching (<1%). However, this was observed to increase in all the water-deficit stressed plants, with

the exception of D6 from experiment II. Hence, the D_s of the IL was considered to be a useful indicator of water-deficit stress, although the statistical significance of this was reduced by the relatively high variation in the data. This variation may have been a consequence of the modular structure of tussock. Since each tiller within a tussock plant can act as an individual (White 1979), it is possible that individual tillers could become exposed to a limiting water supply at different times. Indeed, it was observed in some cases that some tillers were badly scorched, whilst neighbouring tillers were not. Hence the D_s of the IL may be more useful as an indicator of water-deficit stress of individual tillers.

The physiological and soil data indicated that plants responded differently to the water-deficit treatment. From experiment I, D1 responded less to the water-deficit treatment than other plants from this experiment. The SWC of D1 declined to only about 15%, and the LER, N_T , and M_T , did not differ from the controls. Although the T_{Num} and D_s both showed differences from the controls, the latter was not found to be significant. The SWC of the remaining three water-deficit treatment plants from experiment I was reduced to about 8% by the end of the experiment (Figure 4.3). This, and the physiological data, indicated that these plants were exposed to a limiting water-deficit stress. Changes that were observed included a general decline in LER, reductions in T_{Num} , M_T and N_T , and increases in D_s , with respect to the controls. Where tested, these data from plant D2 were always significantly different from the controls. However, this was not always the case with D3 and D4. From the physiological and soil data, the plants from experiment I could be ranked in order of increasing response to the treatment as follows:

plant D1 < plant D4 < plant D3 < plant D2.

Because the plants responded differently to the water-deficit treatment, statistical comparisons between treatment and control plants were not considered. Instead, each plant was regarded as an individual treatment.

During experiment II, the SWC data indicated that the four water-deficit treatment pots dried at similar rates (Figure 4.4), and the LER of all four plants declined below that of the controls (Figure 4.6). However, dissimilar responses were observed in the

remaining physiological measurements. The T_{Num} counted in plant D6 increased throughout experiment II, and this plant showed no difference in N_T and D_S from the controls. This plant had an unusually deep root system, which may account for the fact that it did not appear to become stressed. D7 may have become water-deficit stressed in the latter stages, as evidenced by the decline in T_{Num} during the last 25 days of the dry-down (Figure 4.8). The N_T and M_T of this plant were similar to those of the controls. However, an increase in the D_S of D7 was observed, although it was significantly different from the controls in the ML only. The remaining two plants (D8 and D9) from experiment II appeared to be affected by the water-deficit treatment. Both of these plants showed reductions in T_{Num} and N_T , and significantly increased D_S during the drydown. Hence, the plants from experiment II could be ranked in order of increasing response to the treatment as follows:

plant D6 < plant D7 < plant D9 \leq plant D8.

Plants D8 and D9 from experiment II were of T2 genotype, while D6 and D7 were derived from seed. This may account, in part, for some of the observations above, such as the deep root system of D6, or the increased level of proline observed in D7. However, larger sample sizes would be required to confirm that these differences were caused by genotype differences.

The LWP measured during experiment II was not able to satisfactorily distinguish between water-deficit treatment and control plants, although a general decrease in the LWP occurred during the second half of this experiment (Figure 4.13). The LWP in water-sufficient leaves of *F. novae-zealandiae* was generally low (-1.0 to -2.0 MPa). The time of sampling, and difficulties that arose in the measurement of LWP (section 4.3.2.5) may have contributed to the relatively low measurements. Changing the sampling time closer to dawn (when the LWP is at its daily maximum value), and the measurement of relative water concentration (RWC) to gauge the leaf water status may overcome some of the practical difficulties which were encountered with the measurement of LWP.

Quantitation of metabolites from *F. novae-zealandiae* has not been reported previously. The concentration of ABA in the control plants (up to 30 ng/gFW) was similar to that measured in water-sufficient *F. arundinacea*. However, glycine-betaine and proline

concentration in the controls were relatively high, with up to 35 $\mu\text{mol/gFW}$ (c.f. 11 in *F. arundinacea*) and up to 150 $\mu\text{g/gFW}$ (c.f. 70 in *F. arundinacea*), respectively.

The fresh-weight concentration of ABA and proline increased in response to the water-deficit treatment. However, this response was plant-dependent, and followed a similar pattern to the ranking of the plants based on the physiological results above. Hence the greatest accumulation of ABA and proline was observed in those plants that were considered to be the most severely stressed (D2 and to a lesser extent, D3 from experiment I, and D8 from experiment II). However, as mentioned in the respective results sections, the accumulation of these two compounds was not conclusive. The actual peak concentrations of ABA observed in the water-deficit treatment plants (up to 80 ng/g FW) were less than half the concentration found in water-deficit treated *F. arundinacea* (Figure 3.8, page 79), and fluctuations in the data were comparatively large. The fresh-weight proline concentrations (up to 0.4 and 1 mg/gFW⁸ in experiments I and II, respectively) were also comparatively low (c.f. 2.4 mg/gFW in water-deficit stressed *F. arundinacea*).

Unlike the case with *F. arundinacea*, glycine-betaine was not observed to accumulate in response to water-deficit stress in *F. novae-zealandiae*.

There existed the possibility that *F. novae-zealandiae* may not have the capacity to accumulate ABA, glycine betaine nor proline to the levels characteristic of water-deficit stressed *F. arundinacea*. This may be due to some endogenous limitation, such as constraints acting on the respective biosynthetic pathways (for example, regulatory activity or insufficient precursor pools) or genotypic differences between the two species. ABA, glycine betaine and proline were measured in physiologically relevant concentrations in *F. novae-zealandiae*, indicating that the biosynthetic pathways probably do exist. However, the induction of these pathways may not be linked to water-deficit stress in *F. novae-zealandiae* as they are proposed to occur in other species. The sensitivity of the plant toward ABA also needs to be considered. ABA does induce metabolic adaptation in other species (Chandler and Robertson, 1994), and the threshold concentration of ABA required for this may differ between species. It cannot be determined from these

⁸ This does not take into account the unusually high proline concentration of the seed-derived plant D7.

experiments whether the relatively low accumulation of ABA was sufficient to induce metabolic adaptation in *F. novae-zealandiae*.

4.4.2.1 Growth versus Decline

The physiological data (T_{Num} , N_T , D_S) indicated that some of the *F. novae-zealandiae* plants were subjected to a water-deficit of sufficient severity to induce a decline in the viable foliage. In addition, leaf loss was to some extent dependent on leaf maturity, indicating an element of control in the tissue loss. Although the LER declined, it did not cease, indicating that the meristematic and elongation zones of the leaves remained viable. Hence, there was an apparent paradox, whereby the plants were dying back in response to the water-deficit treatment, but they were still actively elongating at the same time. In some extreme instances, the IL of water-deficit stressed tillers was observed to elongate, even though the exposed portion of the IL was emerging completely scorched. These observations were particularly intriguing, since the positive turgor required for cell elongation (Hsiao, 1973) was not considered likely to occur during a severe water-deficit. It appeared that the growing leaf meristems of *F. novae-zealandiae* were protected whilst the existing leaves died back.

Therefore, a hypothesis was proposed to encompass all the observations made from the experiments with *F. novae-zealandiae* thus far. This hypothesis was used to guide further experimental investigation into the response of this species to water-deficit stress; hence it was referred to as the "Working Hypothesis".

4.4.2.2 Working Hypothesis

Tillers of *F. novae-zealandiae* undergo tissue-dependent responses to survive water-deficit stress. The viability of the meristematic regions, and hence the capacity for continued growth, is maintained by the accumulation of osmolytes and/or osmoprotectant compounds, while the existing leaves die-back during a process of controlled leaf turnover.

This strategy could be regarded as mechanism by which the plant survives drought, rather than a strategy involved with overcoming the adverse effects of the water-deficit stress. The latter could be considered to occur in *F. arundinacea*, which is a species in

which osmoprotectants and osmolytes accumulate in the existing tissues. These compounds modify the cellular environment, retaining turgor and protecting cellular constituents from the deleterious effects of water deficit. Once the water-deficit stress has been relieved, the osmoprotectant and osmolyte concentration return toward normal. However, if the drought continues, the water-deficit stress becomes too great and cellular injury results. Therefore this strategy can be considered to be short-term, since the adaptations are not sufficient to overcome long drought periods. Unlike *F. arundinacea*, *F. novae-zealandiae* is a plant that has adapted to environments where prolonged droughts are likely to occur, and this plant has morphological adaptations which reduce the effects of water-deficit stress when it occurs. In addition to these pre-existing adaptations, the metabolism of *F. novae-zealandiae* also adapts to reduce the water-deficit stress. According to the working hypothesis, *F. novae-zealandiae* does not accumulate osmoprotectant compounds in the existing leaves. Maintaining leaves in this way in an arid environment would be at considerable metabolic cost to the plant, since they are likely to be damaged during a prolonged drought, regardless of the biochemical adaptations. Instead, those tissues that are the most important for re-growth, the meristems, are kept viable. In addition, some biomass may be salvaged from the existing leaves as they are turned over. Hence this species has adapted to virtually shut-down during periods of drought, and can quickly re-grow from viable meristems once the water-deficit has been relieved. This aspect may be particularly important for the ecology of *F. novae-zealandiae*, since recruitment of seedlings to mature plants is extremely low (Lord 1992). Therefore surviving prolonged drought by means of seed dispersal may not be efficient in this species.

Several aspects of the working hypothesis could be examined experimentally. The hypothesis stated that the viability of the meristems is maintained by the accumulation of osmoprotective compounds. Since the accumulation of ABA and proline in the ML was inconclusive, further evidence that *F. novae-zealandiae* has the metabolic capacity to accumulate these compounds was required. This aspect was examined experimentally in two parts in Chapter Five (page 156). Firstly, evidence that the various tissues of *F. novae-zealandiae* have the capacity to accumulate these compounds was sought in a bench-top dry-down experiment. For the second part, another dry-down experiment

was conducted with *F. novae-zealandiae*, and tillers were dissected into several tissues before metabolite analysis.

The working hypothesis also stated that the mature tissues are turned over in a controlled manner. Evidence to support this aspect was collected by carrying out another dry-down experiment with *F. novae-zealandiae*. In this experiment, large quantities of leaf material were harvested, and evidence for the turnover of proteins within the ML was sought. The results of this experiment are presented in Chapter Six (page 197).

Appendix 4.1

ANOVA Tables Referred from Text

A: *Tiller Mass (M_T)*

Table 4.3: ANOVA comparison of M_T from each plant used in experiment I.

<i>ANOVA Table; Dependent variable = tiller mass</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Plants	5	0.352	0.070	11.25	0.0001
Within Plants	51	0.320	0.006		
Total	56	0.671			

Table 4.4: Grouping of plants based on the means tested by ANOVA in Table 4.3.

NSD Grouping	Mean Mass	n	Plant
	0.366	10	D1
	0.320	10	C2
	0.317	10	C1
	0.256	8	D4
	0.204	10	D3
	0.133	9	D2

Means connected by the same bar were NSD.

Table 4.5: ANOVA comparison of M_T from each plant used in experiment II.

<i>ANOVA Table; Dependent variable = tiller mass</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Plants	5	0.163	0.033	17.13	0.0001
Within Plants	65	0.124	0.002		
Total	70	0.287			

Table 4.6: Grouping of plants based on the means tested by ANOVA in Table 4.5.

NSD Grouping	Mean Mass	n	Plant
	0.201	12	D6
	0.148	12	C8
	0.142	12	D7
	0.080	11	D8
	0.074	12	D9
	0.073	12	C6

Means connected by the same bar were NSD.

B: Number of Green Leaves per Tiller (N_T)

Table 4.7: ANOVA comparison of N_T from each plant used in experiment I.

<i>ANOVA Table; Dependent variable = number of green leaves</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Plants	5	4.652	0.930	3.47	0.0089
Within Plants	51	13.664	0.268		
Total	56	18.316			

Table 4.8: Grouping of plants based on the means tested by ANOVA in Table 4.7.

NSD Grouping	Mean count	n	Plant
	3.0	10	C2
	2.9	10	C1
	2.8	10	D1
	2.63	8	D4
	2.6	10	D3
	2.1	9	D2

Means connected by the same bar were NSD.

Table 4.9: ANOVA comparison of N_T from each plant used in experiment I.

<i>ANOVA Table; Dependent variable = number of green leaves</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Plants	5	7.657	1.532	6.35	0.0001
Within Plants	65	15.667	0.241		
Total	70	23.324			

Table 4.10: Grouping of plants based on the means tested by ANOVA in Table 4.9.

NSD Grouping	Mean count	n	Plant
	2.75	12	D7
	2.67	12	D6
	2.58	12	C6
	2.58	12	C8
	2.0	11	D8
	1.92	12	D9

Means connected by the same bar were NSD.

C: *The Degree of Leaf Tip Scorching (D_s): Experiment I*

Table 4.11: ANOVA of D_s from experiment I.

<i>ANOVA Table; Dependent variable = Degree of leaf tip scorching</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Plants	17	107,030	6,295.9	6.72	0.0001
Within Plants	151	141,386	936.3		
Total	168	248,417			

Table 4.12: ANOVA of D_s from experiment I, breakdown of model components.

<i>ANOVA Table; Dependent variable = Degree of leaf tip scorching</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Leaf Types	2	22,824	11,412	12.19	0.0001
Among Plants	5	67,211	13,442	14.36	0.0001
Plant x Leaf Type	10	16,881	1,688	1.8	0.0645

Table 4.13: Grouping of the means within each plant from experiment I.

Plant Number	Leaf Type	NSD Grouping	n	Mean	s.e.
D1	2ML	■	10	33.4	14.55
D1	ML	■	10	6.8	3.61
D1	IL	■	10	5.5	2.31
D2	2ML	■	9	94.1	5.90
D2	IL	■	9	62.6	15.40
D2	ML	■	9	23.1	11.00
D3	IL	■	10	57.2	11.92
D3	2ML	■	10	48.1	14.36
D3	ML	■	10	22.1	9.42
D4	2ML	■	8	46.1	15.95
D4	IL	■	8	41.8	17.01
D4	ML	■	8	23.5	6.12
C1	2ML	■	9	13.5	10.83
C1	ML	■	10	1.6	0.19
C1	IL	■	10	0.4	0.16
C2	2ML	■	10	18.9	11.38
C2	ML	■	9	5.1	2.07
C2	IL	■	10	0.3	0.13

Means connected by the same bar were NSD.

Table 4.14: Grouping of the means within each leaf type from experiment I. Individual means are presented on Table 4.13.

IL		ML		2ML	
NSD Grouping	Plant	NSD Grouping	Plant	NSD Grouping	Plant
	D2		D4		D2
	D3		D2		D3
	D4		D3		D4
	D1		D1		D1
	C2		C2		C2
	C1		C1		C1
	Means connected by the same bar were NSD.				

D: The Degree of Leaf Tip Scorching (D_s): Experiment II

Table 4.15: ANOVA of D_s from experiment II.

<i>ANOVA Table; Dependent variable = Degree of leaf tip scorching</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Among Plants	11	62,566	5,687	9.34	0.0001
Within Plants	116	70,658	609		
Total	127	133,224			

Table 4.16: ANOVA of D_s from experiment II; breakdown of model components.

<i>ANOVA Table; Dependent variable = Degree of leaf tip scorching</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Between Leaf Types	1	0.64	0.65	0.00	0.9740
Among Plants	5	40,138	8,028	13.18	0.0001
Plant x Leaf Type	5	15,168	3,033	4.98	0.0004

Table 4.17: Grouping of the means within each plant from experiment II.

Plant Number	Leaf Type	NSD Grouping	n	Mean	s.e.
D6	ML	[Grouping bar]	12	2.1	0.40
D6	IL		12	0.4	0.17
D7	ML	[Grouping bar]	12	41.7	7.30
D7	IL		12	12.2	5.48
D8	IL	[Grouping bar]	11	70.9	11.97
D8	ML		7	23.5	13.27
D9	ML	[Grouping bar]	6	42.4	18.53
D9	IL		12	34.8	5.23
C6	ML	[Grouping bar]	8	6.1	3.15
C6	IL		12	0.8	0.61
C8	ML	[Grouping bar]	12	3.4	0.66
C8	IL		12	0.8	0.23

Means connected by the same bar were NSD.

Table 4.18: Grouping of the means within each leaf type from experiment II. Individual means are presented on Table 4.17.

IL		ML	
NSD Grouping	Plant	NSD Grouping	Plant
[Grouping bar]	D8	[Grouping bar]	D9
	D9		D7
	D7		D8
	C8		C6
	C6		C8
	D6		D6

Means connected by the same bar were NSD.

Chapter Five

Experimental Dry-Down of *F. novae-zealandiae* II: Dissection of Tillers

5.1 Introduction

The previous experiments conducted with *F. novae-zealandiae* (Chapter Four, page 93) showed that this species responded physiologically to water-deficit stress. However, evidence of metabolic adaption using the compounds ABA, proline or glycine-betaine as markers, was inconclusive. A working hypothesis concerning the response of *F. novae-zealandiae* to water-deficit stress was presented in the discussion of Chapter Four. The aim of the two experiments described in this chapter was to examine two aspects of this hypothesis.

The first was to determine if *F. novae-zealandiae* has the capacity to accumulate ABA and/or proline under artificially severe water-deficit, and if so, to determine whether the accumulation is localised to any particular regions within the tiller. This experiment was referred to as the "bench-top dry-down", since stress was applied by allowing tillers to dry on a bench surface overnight.

In the second experiment, evidence was sought for the accumulation of the metabolic adaption markers, ABA, proline and glycine-betaine, by different tiller tissues during a longer (pot-based) dry-down period. Further physiological evidence was also sought that supports the hypothesis that a process of controlled tiller turnover takes place, and that growth is maintained in response to water-deficit stress.

5.2 Methods

5.2.1 Plant Material

The plant material used was either T2 or TF-1 genotype (*F. novae-zealandiae* and *F. arundinacea*, respectively).

5.2.2 Bench-Top Dry-Down of *Festuca spp.*

Plant material was grown under well-watered conditions, using the 4 L pot system described in section 2.2.1. (page 23). Two repeats of this experiment were carried out. For the first experiment, tillers were sampled at 3:00 PM. The tillers of *F. arundinacea* were slightly wilted and leaf rolling was apparent, but *F. novae-zealandiae* tillers had no indications of wilting. For the second experiment, the tillers were harvested at about 11 am, to avoid wilting induced by the "heat of the day". Tillers were removed intact from the plant, and most of the roots were removed.

Several tillers from each species were brought on ice to the laboratory. Half of these were frozen in liquid nitrogen, and kept frozen during processing. The rest of the tillers were laid out on a bench, and allowed to dry overnight. Three or six (*F. arundinacea* or *F. novae-zealandiae*, respectively) tillers of each treatment were dissected in the following manner: Roots were severed at the crown, and all leaves were removed until only the first mature leaf, and the leaves contained within the pseudostem of this leaf, remained. The LB was excised 25 mm from the tiller base. The IL, and ML were excised at the ligule of the mature leaf, and the MP was separated from the remaining portion of the tiller (IP). Each of the five leaf tissues were frozen and ground in liquid nitrogen, transferred to a pre-weighed 1.5 mL centrifuge tube, which was re-weighed, and stored at -80 °C. In the case of *F. arundinacea*, there was sufficient material in each plant sample to allow aliquots for both ABA and proline analysis to come from the same tiller. Separate *F. novae-zealandiae* tillers had to be dissected to gain sufficient material for each assay. For analysis, the appropriate extraction buffer was added directly to the sample tubes, and the samples were assayed for ABA and proline concentration by the methods described in sections 2.8 and 2.10 (pages 27 and 45, respectively).

5.2.3 Experimental Dry-down of *F. novae-zealandiae*

Plants were established over six weeks in the 4 L pot system described in section 2.2.1 (page 23). Seven dry-down plants (numbered D1 to D7) and two control plants (C1 and C2) were used. The dry-down was initiated by withholding water, and after 49 days, plants D1 to D4 were re-watered, and the experiment was continued for an additional eight days. Watering was continued in the control plants for the duration of the experiment. A summary of the experimental conditions is presented in Table 5.1.

Table 5.1: Summary of the experimental dry-down method.

	Conditions	Tissues selected
Pot system used:	4L (Potting mix)	
Number of pots :	7	
Water-deficit treatment:	7	
Control:	2	
Pot numbers:	D1 to D7, C1 and C2	
Number of plants per pot:	1	
Plant genotype:	T2	
Number of TDR electrode pairs:	1	
Number of tillers measured for LER:	3	
Tiller counts (T_{Num}):	✓	
Number of green leaves per tiller (N_T), leaf-tip scorching (D_S) measured:	During experiment (n=3) and again close to end of experiment (n=20)	IL, ML, 2ML
LWP measured:	✗	
RWC and H_L measured:	✓	LB, IP, MP, IL, ML
ABA, proline, glycine-betaine measured:	✓	LB, IP, IL, ML
Glasshouse temperature measured:	✓	
Root distribution determined:	✗	

During the experiment, the SWC in each pot was monitored by TDR (section 2.3, page 24) using one pair of electrodes inserted vertically into the soil surface. Three tillers per plant were tagged with jewellers labels, and the length of the IL was measured on consecutive days to determine the LER, in accordance with section 2.5 (page 26). Several times during the experiment, the number of tillers in each plant were counted and scored by colour as either green or brown, using the method from the previous experiment (section 4.3.2.4.1, page 114). Most of the dead tillers were removed before the start of this experiment (but not during the experiment) to facilitate counting. Close to the end of the experiment (on day 49), 20 tillers from each plant were tagged and measured. The number of green leaves per tiller and the lengths of each green leaf, including the length of any leaf tip scorching present, were recorded.

Tiller samples were taken at regular intervals during the experiment. For each sample, a total of 11 tillers were excised from one plant only. At 8:00 AM on the day of sampling, three tillers were excised for RWC and H_L measurements using the method

described in section 2.4.2 (page 26). Eight more tillers were excised between 8:00 and 9:00 AM and transferred back to the laboratory on ice for metabolite analyses. The number of green leaves per tiller (N_T), the length of each leaf, and the length of any leaf tip scorching present, were recorded for each tiller. Each tiller was then dissected into the same portions as used for the determination of RWC above, except that the MP portion was discarded. Each leaf portion was cut to 20-40 mm lengths and placed into a plastic vial, frozen with liquid nitrogen and stored at -80°C . For analysis, samples were ground in liquid nitrogen using a pestle and mortar, and added to a pre-weighed 1.5 mL centrifuge tube. The tubes were re-weighed, an aliquot of the appropriate extraction buffer was added, and the samples were assayed for either ABA (three tillers), proline (three tillers) or glycine-betaine (two tillers) using the methods described in sections 2.8, 2.9 and 2.10 (pages 27, 37 and 45, respectively).

The experiment was commenced in early Spring, when mean daily temperature was expected to increase over the coming months. The glasshouse temperature was recorded at 8:00 AM on each day of sampling.

5.3 Results

5.3.1 Bench-Top Dry-Down of *Festuca* spp.

5.3.1.1 ABA Concentration

ABA was evenly distributed within the tissues excised from water-sufficient *Festuca* spp. tillers, at levels of *ca.* 15 to 20 ng/g FW (Figure 5.1). However, the levels of ABA in most *F. arundinacea* tissues from the first experiment were approximately doubled with respect to the second. This may be attributable to the time of harvest, since tillers for the first experiment were wilted, and hence may have had some degree of mild water-deficit stress, whereas tillers for the second repeat were not.

In response to the drying treatment, the mean ABA concentrations in all tissues were significantly increased, to levels of 200-850 ng/g FW (Figure 5.1). In *F. arundinacea*, the ABA concentration was tissue dependent, with higher levels located in the ML and IL with respect to the rest of the tiller (Figure 5.1). ABA accumulation was related to the age of the tissue, with the greatest fold-increase occurring in the ML, and the smallest increase in the LB and IP (Table 5.2). Tillers of *F. novae-zealandiae* accumulated ABA to high levels in response to the applied stress, and the concentration of ABA was evenly distributed throughout the tiller (Figure 5.1). In this species, the fold-increase in ABA followed the same trend to that of *F. arundinacea*, where the increase in ABA concentration was lowest in the LB (Table 5.2).

5.3.1.2 Proline Concentration

The proline concentration in most of the control tissues were less than 100 $\mu\text{g/g}$ FW in *F. arundinacea*, and less than 200 $\mu\text{g/g}$ FW in *F. novae-zealandiae* (Figure 5.2). These levels were consistent with those from previous measurements with water-sufficient *Festuca* spp. (sections 3.4.4.2 and 4.3.2.6.2, pages 77 and 126). However, proline was not distributed evenly throughout the tiller. The LB regions of both species, and the IP of *F. novae-zealandiae* had elevated proline contents when compared to the rest of the tiller (Figure 5.2).

Proline levels increased in all tissues in response to the drying treatment (Figure 5.2). In *F. arundinacea*, proline concentration increased to 470 to 2100 $\mu\text{g/g}$ FW, with the highest contents in the ML. The greatest fold increase in proline (ca. 150-fold) was in the laminae (Table 5.3). Proline also accumulated to high levels (ca. 1300 $\mu\text{g/g}$ FW) in the LB. However the fold-increase in this tissue was comparably low (4-fold) due to the high proline concentration observed in the LB of the control.

In contrast to *F. arundinacea*, proline did not accumulate to a relatively high degree in the laminae of *F. novae-zealandiae* when compared to the LB (Figure 5.2), although the greatest increase (20-fold) occurred in these tissues (Table 5.3). Instead, proline concentrations were highest in the LB and IP, although the increase in proline concentration in these tissues was low (up to 3-fold, Table 5.3), due to the relatively high proline concentration found in these tissues in the water-sufficient tillers.

Table 5.2: Fold-increase of ABA concentration of bench-dried *Festuca* spp. tillers⁹.

		<i>F. arundinacea</i> ¹	<i>F. novae-zealandiae</i>
Leaf Base	(LB)	17	20
Immature Pseudostem	(IP)	15	46
Mature Pseudostem	(MP)	27	51
Immature Lamina	(IL)	27	43
Mature Lamina	(ML)	37	31

¹ Data for the water-sufficient tillers from the first experimental run in this species were not used for this calculation.

Table 5.3: Fold-increase of proline concentration of bench-dried *Festuca* spp. tillers.

		<i>F. arundinacea</i>	<i>F. novae-zealandiae</i>
Leaf Base	(LB)	4	3
Immature Pseudostem	(IP)	14	2
Mature Pseudostem	(MP)	19	6
Immature Lamina	(IL)	164	22
Mature Lamina	(ML)	146	18

⁹ Data are the fold-difference between bench-dried and water-sufficient tiller tissues. Data from both experimental runs were averaged for the purposes of this calculation, unless indicated otherwise.

ABA Concentration: Bench-top Drydown

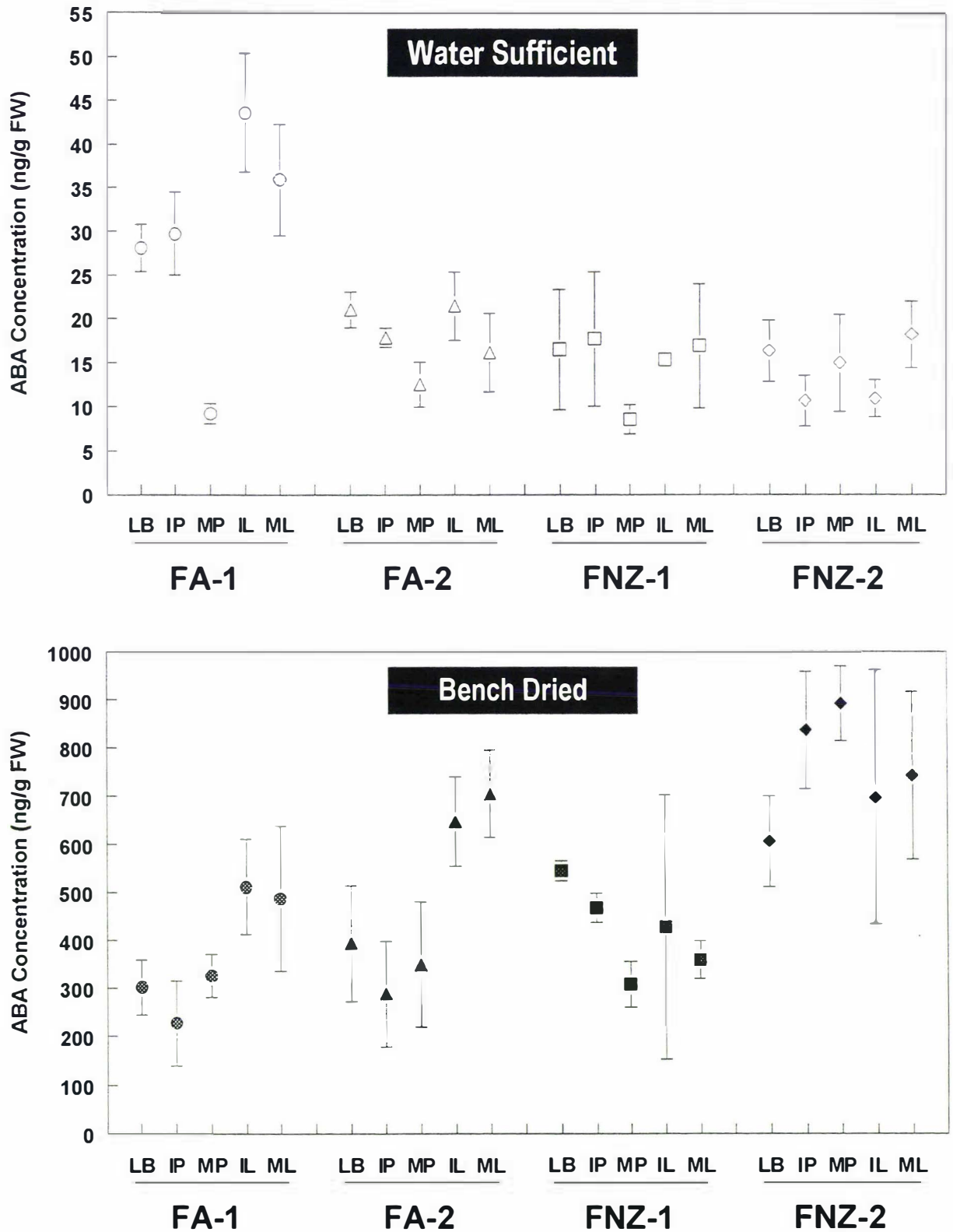


Figure 5.1: The distribution of ABA in water-sufficient and bench-dried tillers. Key: FA-1, FA-2: *F. arundinacea*, first and second experiment, respectively. FNZ-1, FNZ-2: *F. novae-zealandiae*, first and second experiment, respectively. The x-axis indicates leaf types; data were means \pm s.e., n=3.

Proline Concentration: Bench-top Drydown

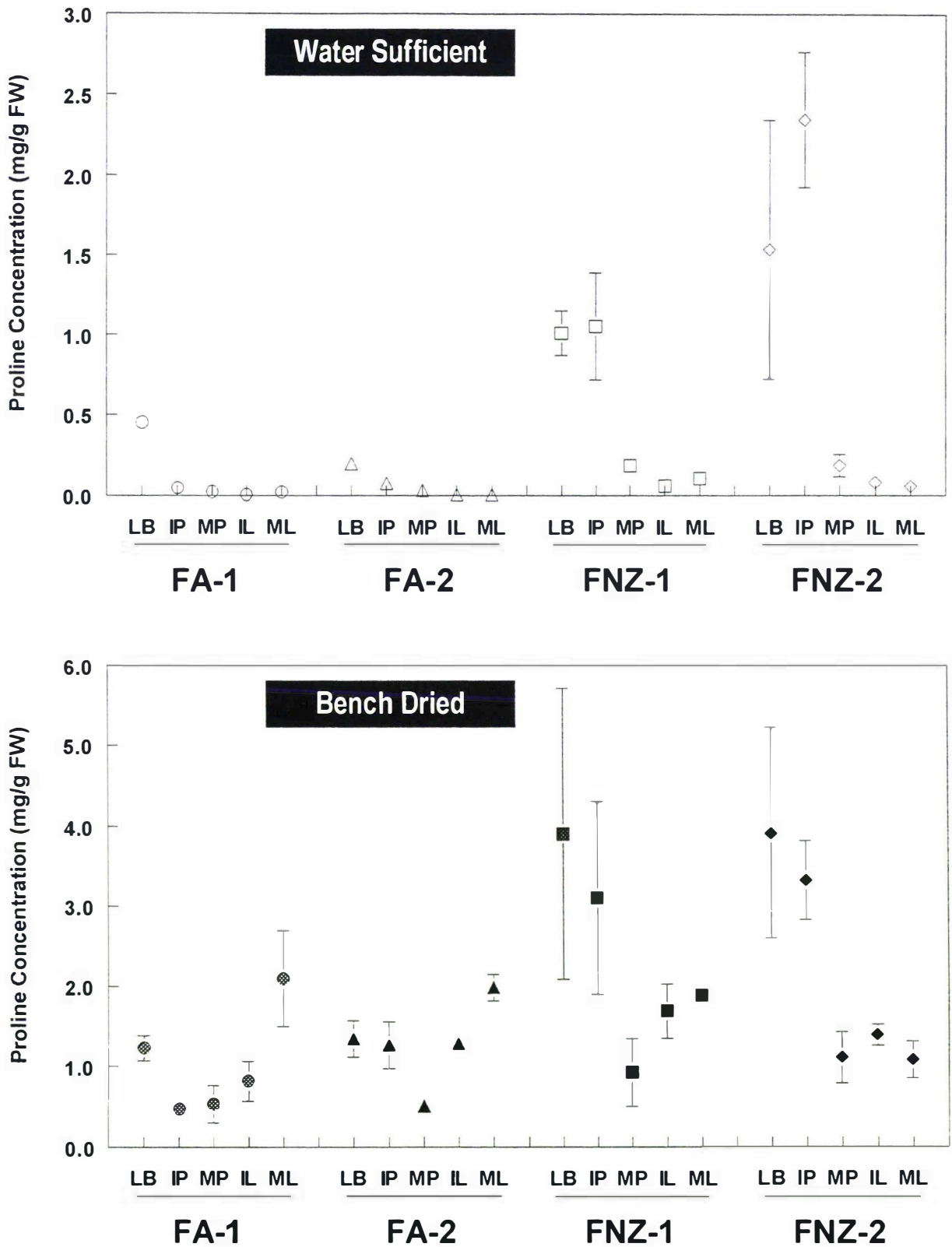


Figure 5.2: The distribution of proline in water-sufficient and bench-dried tillers. Key: FA-1, FA-2: *F. arundinacea*, first and second experiment, respectively. FNZ-1, FNZ-2: *F. novae-zealandiae*, first and second experiment, respectively. The x-axis indicates leaf types; data were means \pm s.e., n=3.

5.3.2 Experimental Dry-Down of *F. novae-zealandiae*

5.3.2.1 Time Course

The experiment was started on 21 September 1994, and measurements were continued for 57 days. After 49 days, four of the seven dry-down plants (D1 to D4) were re-watered, and samples from these plants were taken at 1, 2, 5 and 8 days after re-watering. Control samples were taken on three dates. The timetable of sampling is summarised in Table 5.4.

Table 5.4: Summary of Experimental Sampling Regime.

Date	Days	Plant Sampled	SWC (%)	Temperature (°C) ¹	Comments	
September	21	0	C1	34	12	Control
	26	5	D1	30	11	water-deficit
October	3	12	D3	22	8	water-deficit
	10	19	D6	17	14.5	water-deficit
	14	23	D7	10	10.5	water-deficit
	25	34	D4	8	16	water-deficit
	26	35	C1	32	15	Control
November	3	43	D5	6	15	water-deficit
	8	48	D2	5.5	15	water-deficit
	10	50	D3	34	15	1 day after re-watering
	11	51	D2	34	18	2 days after re-watering
	14	54	D3	35	17	5 days after re-watering
	16	56	C2	32	14	Control
	17	57	D2	28	20	8 days after re-watering
17	57	D5	5.6	20	water-deficit	

¹ Glasshouse temperature at time of sampling.

5.3.2.2 Soil Water Content (SWC)

With regular watering the SWC of the control pots (C1 and C2) was maintained at 27-35% (Figure 5.4). The decline in SWC was uniform between the seven dry-down pots (Figure 5.4); this was emphasised by the low standard error of the meaned data (Figure 5.3). Because of this, the dry-down pots were considered to be replicates of the same treatment (and the data could be pooled), unlike the previous experiments in Chapter Four where each plant was considered to have experienced a unique level of treatment. After 34 days, the SWC of these pots had fallen from 33-35% to less than 8%. By this time the rate of water loss from the soil had declined to a low level, and the SWC did not decrease below 5.5-6% for the rest of the experiment (Figure 5.3).

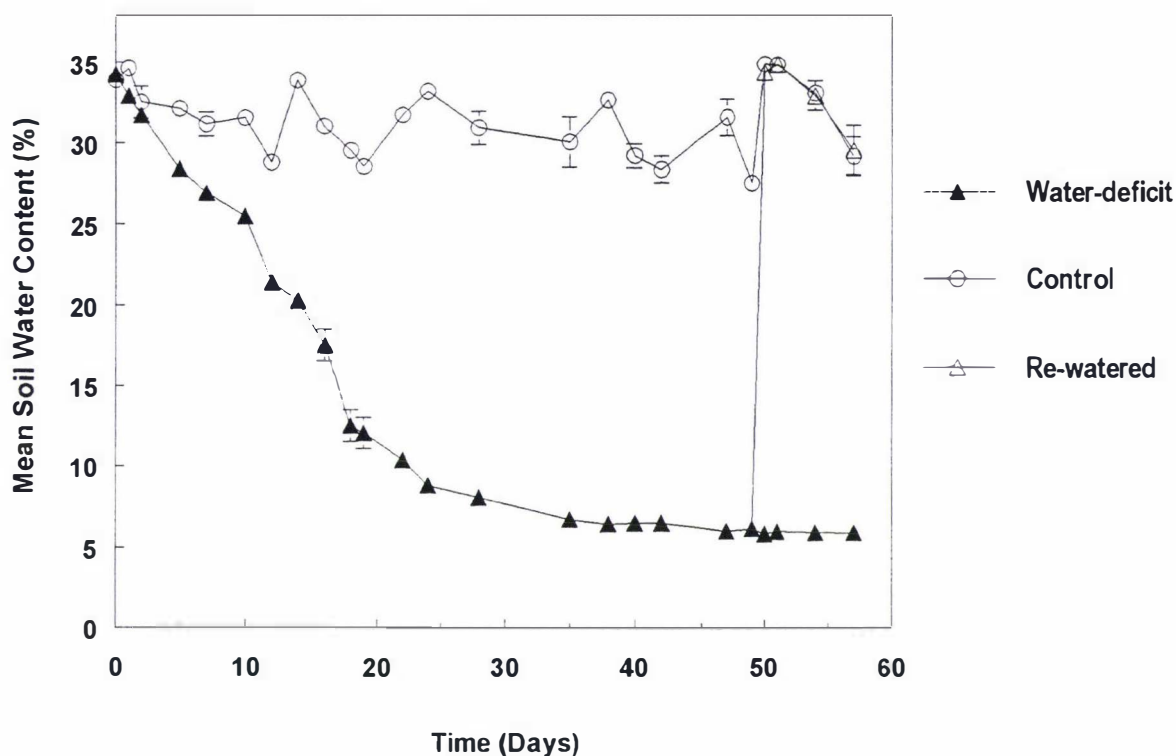


Figure 5.3: The mean SWC during the dry-down. The data was the mean \pm s.e., $n=7$ or 2 (water-deficit and control, respectively) of the data from Figure 5.4. Plants D1 to D4 were re-watered on day 49.

The SWC of D1 to D4 responded quickly to re-watering, and closely followed the SWC of the control pots for the remainder of the experiment (Figure 5.4).

5.3.2.3 Leaf Elongation Rate (LER)

The LER of the IL was constant throughout the experiment under water-sufficient conditions, with the exception of two low measurements in C2 on days 24 and 34 (Figure 5.5). The LER of the water-deficit treatment plants was constant for about the first 20 days, after which the LER declined to a low value (<2 mm.d⁻¹) in six of the plants and to zero in plant D1 (Figure 5.5), although the LER of D5 and D6 increased during the final 10 or 20 days (respectively).

A re-commencement of elongation occurred in D2 and D3 after re-watering (Figure 5.5). The growth rate of D2 quickly assumed the normal LER of *ca.* 8 mm.d⁻¹, however there was a lag of 3-4 days before renewed elongation was observed in D3. The other two re-watered plants (D1 and D4), did not resume growth after re-watering. In fact, neither of these two plants recovered, and both were completely dead shortly after the end of the experiment.

Soil Water Content

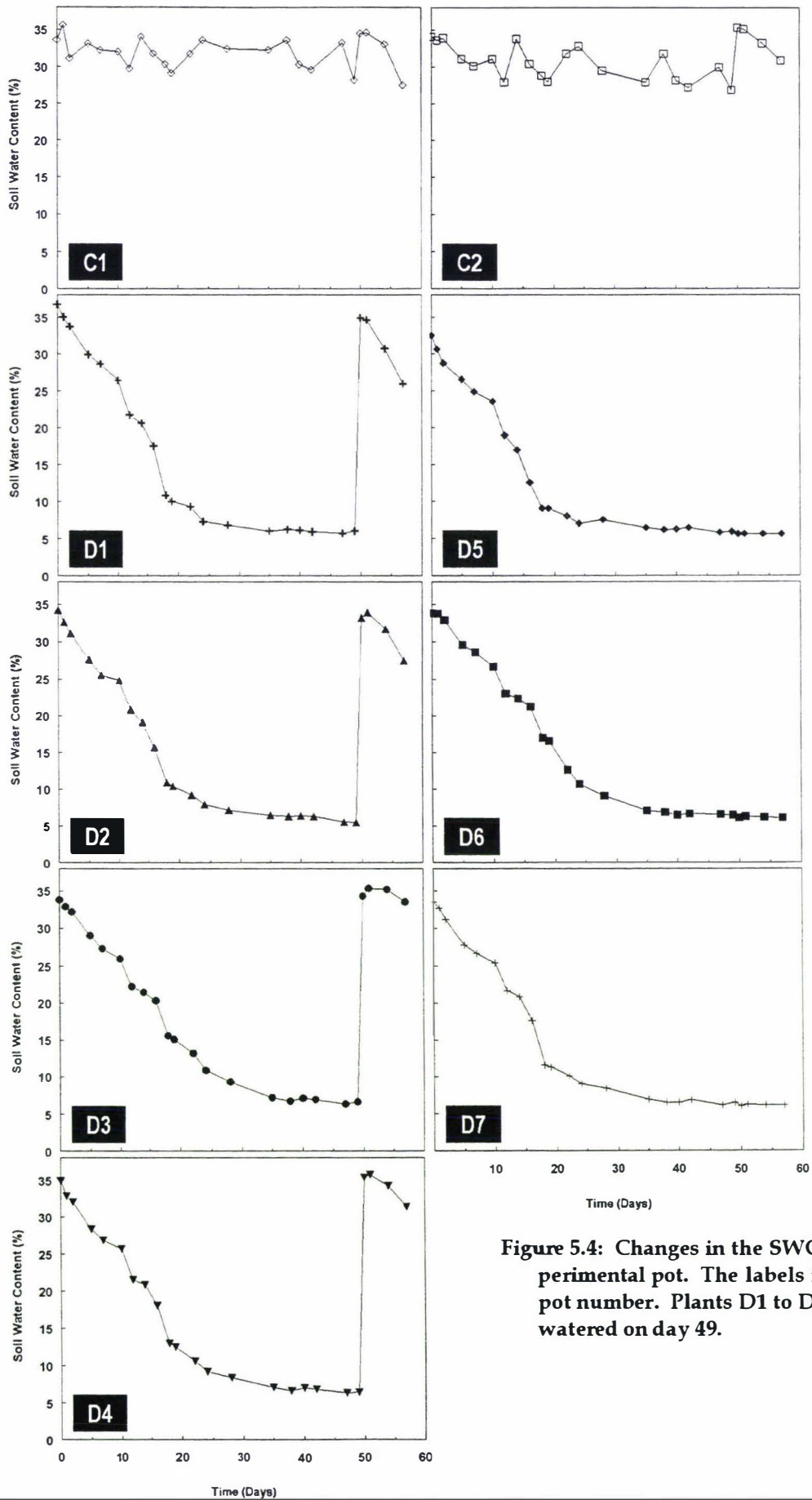


Figure 5.4: Changes in the SWC of each experimental pot. The labels indicate the pot number. Plants D1 to D4 were re-watered on day 49.

Leaf Elongation Rate

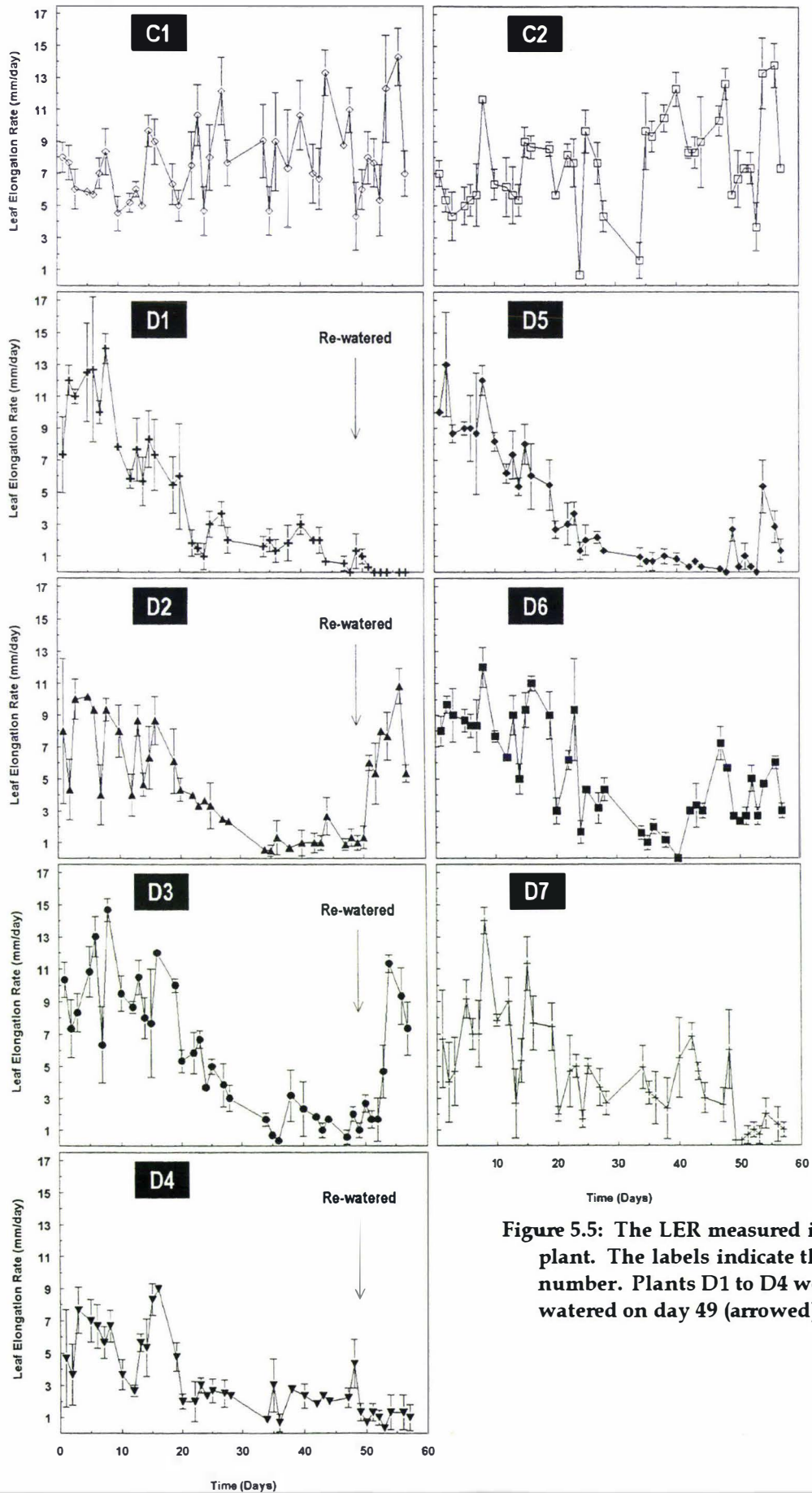


Figure 5.5: The LER measured in each plant. The labels indicate the plant number. Plants D1 to D4 were re-watered on day 49 (arrowed).

5.3.2.4 Tiller Measurements during the Dry-Down

5.3.2.4.1 Number of Tillers

The total number of tillers in all plants increased during the experiment. Under water-sufficient conditions, this increase in tillers was near linear (Figure 5.6). In the water-deficit treatment plants, the rate of increase in tiller number declined after 23 days, so that these plants had put on *ca.* 30% less tillers than the controls by the end of the experiment (Figure 5.6). However, these tiller counts were not considered to be an effective indicator of the living plant size. This is because the number of tillers in a living tussock will always increase, since new tillers are formed as older tillers senesce, and in the absence of a removal system for dead tillers, their number will steadily increase. Because of this, the tillers counted during the experiment were scored by colour as either green or brown, as an indication of the viability of the tillers. The change in the number of living tillers in each plant is presented in Figure 5.7.

At the start of the experiment, all plants had a positive tillering rate (that is, the number of new tillers forming was exceeding the number of tillers dying). In plants D1, D4 and D7, the number of living tillers reached a peak after 23 days and then started to decline. By about 27 days, these three plants comprised their original number of living tillers and by the end of the experiment, these plants had lost 80% to 90% of their tillers (Figure 5.7). The tiller number in D5 and D6 also peaked at day 23, and lost tillers at a slower rate, to become 40% to 50% smaller than their starting size by the end of the experiment. The tiller number in D3 reached equilibrium between days 23 to 34, at which time the number of live tillers was at its peak. Plant D3 then lost tillers at about the same rate as D5 and D6. In contrast to the other water-deficit treatment plants, the tiller number in D2 declined at a relatively slow rate after peaking at day 23, and had reached equilibrium by the end of the experiment (Figure 5.7).

In the control plants (C1 and C2), the number of live tillers increased steadily throughout most of the experiment, with the exception of the final measurement interval when numbers declined (Figure 5.7).

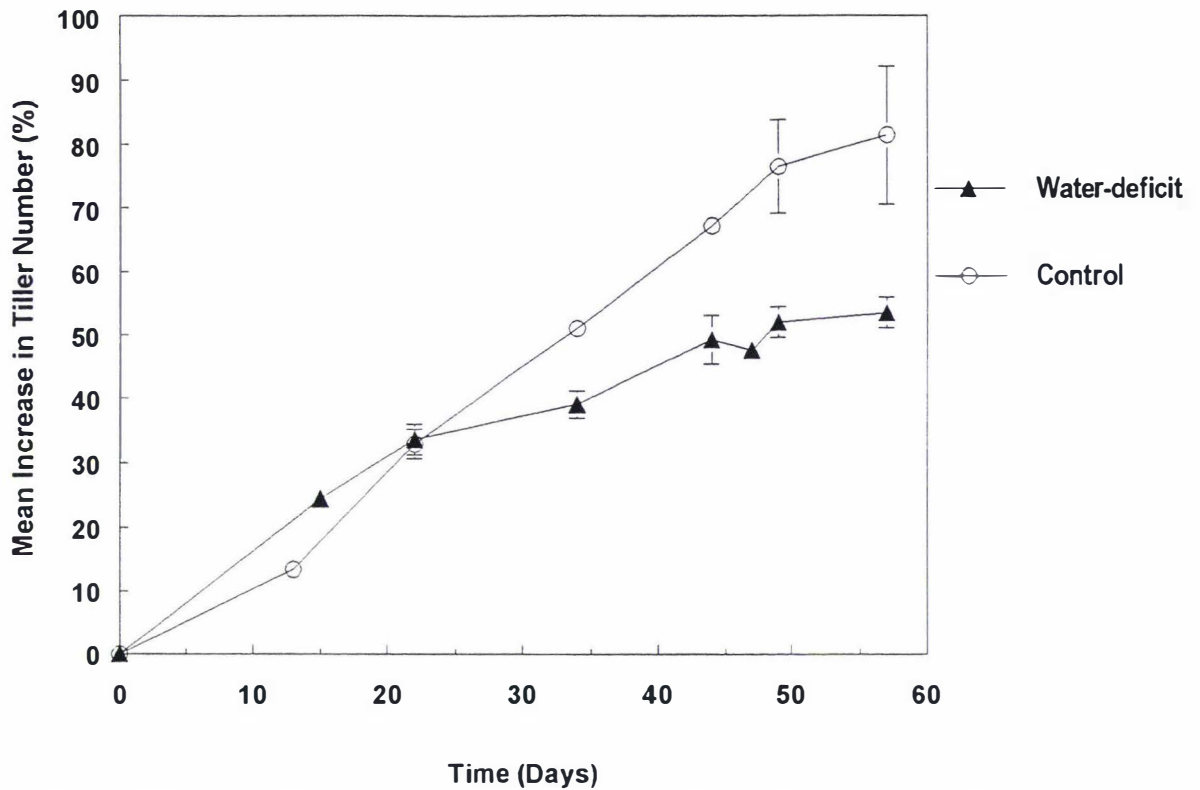


Figure 5.6: The differences in the increase in total number of tillers per plant between water-deficit treatment, and watered control plants. Data were means \pm s.e., $n=7$ or 2 (water-deficit and control respectively).

5.3.2.4.2 Number of Green Leaves per Tiller (N_T)

Tillers in the control plants comprised approximately three green leaves during the experiment (Figure 5.8). For the first 23 days, water-deficit treatment plants also had three (or more) green leaves per tiller. After this time, the N_T began to decline, and by 43 days, the N_T had declined to the lower level of about two. These tillers were excised for the purposes of sampling, and therefore required a minimum of two green leaves (an immature leaf, and at least one mature leaf). Hence the expected range for this measured parameter was from 2.0 to 3.0 (or more). The N_T did not respond to re-watering within the time frame of the experiment, which was expected, since the immature leaves would have to mature, and new immature leaves emerge before an increase in this parameter would be detected.

Number of Green Tillers per Plant

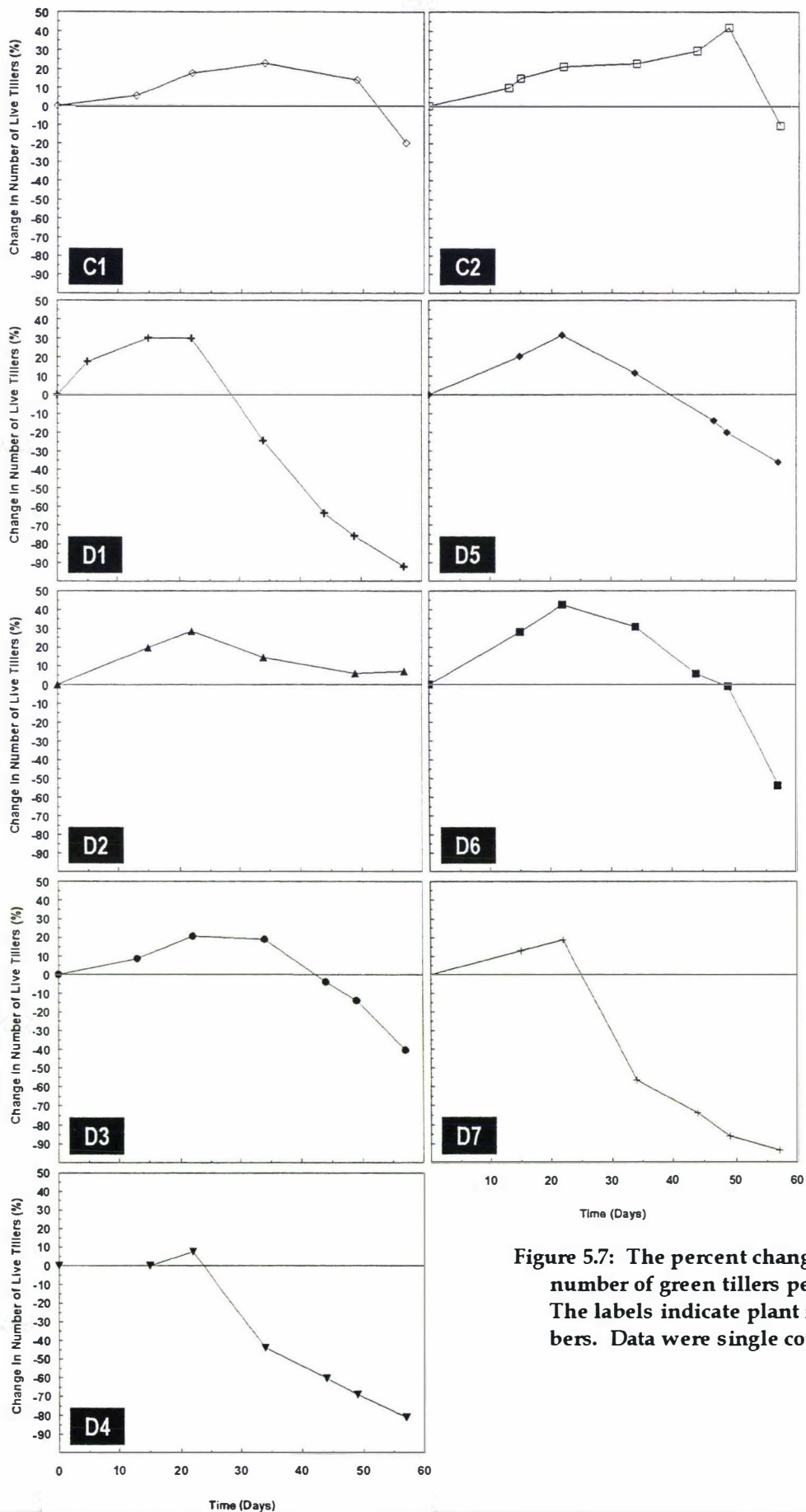


Figure 5.7: The percent change in the number of green tillers per plant. The labels indicate plant numbers. Data were single counts.

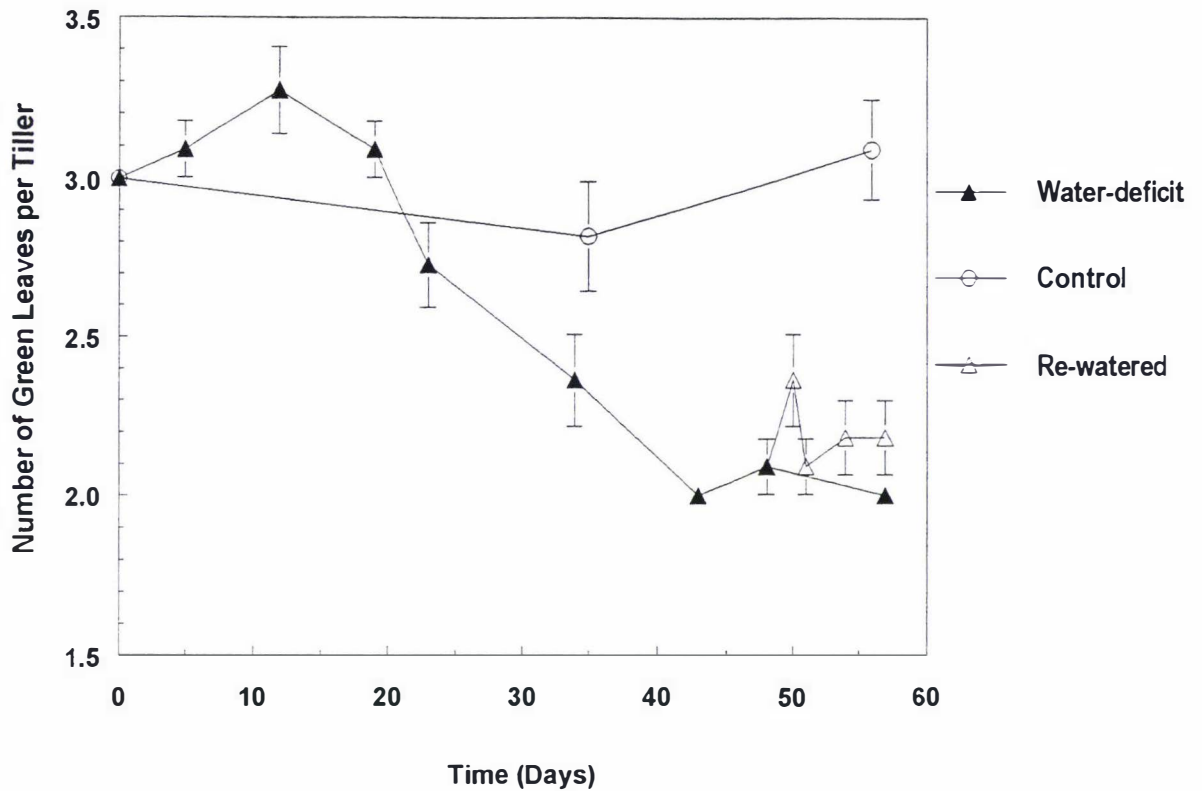


Figure 5.8: The differences in the number of green leaves per tiller between the experimental treatments. Data were means \pm s.e., $n=11$.

5.3.2.4.3 Degree of Leaf Tip Scorching (D_s)

The individual leaves on each tiller excised during the experiment were measured, and the length of leaf tip scorching was calculated as a percentage of the length of the exposed lamina. In the case that a tiller had only one or two green leaves, the D_s of the remaining (dead) leaves was scored as 100%.

The D_s measured on the IL was negligible in the control plants throughout the experiment (Figure 5.9). An increase in D_s occurred in the IL in the water-deficit treatment plants after 23 days and continued to rise to reach *ca.* 30% by the end of the experiment. On the ML, the D_s in control plants was higher than in the IL, but still less than 10% (Figure 5.9). The water-deficit treatment plants did not have a D_s greater than the controls until after *ca.* 50 days; the D_s did not exceed 20% of the ML. The D_s of the 2ML remained at *ca.* 15% in the control plants (Figure 5.9). By 23 days, the D_s on the 2ML of the water-deficit treatment plants had exceeded that of the control plants. By 40 days, the D_s of this leaf had reached 100%, indicating that only two green leaves per tiller remained. The D_s remained at this level on the 2ML for the rest of the experiment. The D_s did not respond to re-watering, although fluctuations were apparent in the

2ML and the D_s declined during the last measurement interval follow both the IL and ML. A decrease in D_s was expected for the IL only, since it continues to elongate following re-watering without further increase in length, thereby decreasing the ratio of scorched tip length to leaf length. Since the IL in plants 2 and 3 increased growth after re-watering (Figure 5.5) a contribution of this effect to the decrease in D_s cannot be discounted. However, these apparent responses to re-watering were also likely to be due to random sampling effects.

The three leaf types were ranked in order of the change in D_s in response to the water-deficit treatment. The 2ML had the greatest response both in magnitude (up to 100%) and earliest onset of increase of scorching (ca. 23 days). A lesser response was recorded (up to 30%) at 23 days for the IL, while there was virtually no response for the ML, with a small increase in D_s (ca. 10%) late in the experiment. This ranking is in contrast to the order of D_s of water-sufficient *F. novae-zealandiae* plants, where there was practically no scorching on the IL, and 10% and 15% on the ML and 2ML (respectively).

5.3.2.5 Tiller Measurements Towards the end of the Dry-Down

Although N_T and D_s were measured for all tillers sampled during the experiment, there was an inherent bias in the sampling method, reflecting the need to sample tillers which had a sufficiently emerged IL to supply enough material for biochemical analyses. Hence although measuring the D_s of the sampled tillers was able to give an indication of changes during the experiment, this data may not have accurately reflected the tiller population. Therefore, before the re-watering of plants D1 to D4, the N_T and D_s of 20 tillers per plant were measured as a means of determining if changes in the tillers had occurred in response to the water-deficit treatment.

5.3.2.5.1 Number of Green Leaves per Tiller (N_T)

The N_T was dependent upon the treatment; all of the water-deficit treatment plants had a reduced N_T compared to the controls (Figure 5.10). The N_T was compared between plants using an ANOVA model, which was highly significant at a level of 0.01% [appendix 5.1 (page 194), Table 5.5. With average of three or more, the control plants

Leaf-Tip Scorching

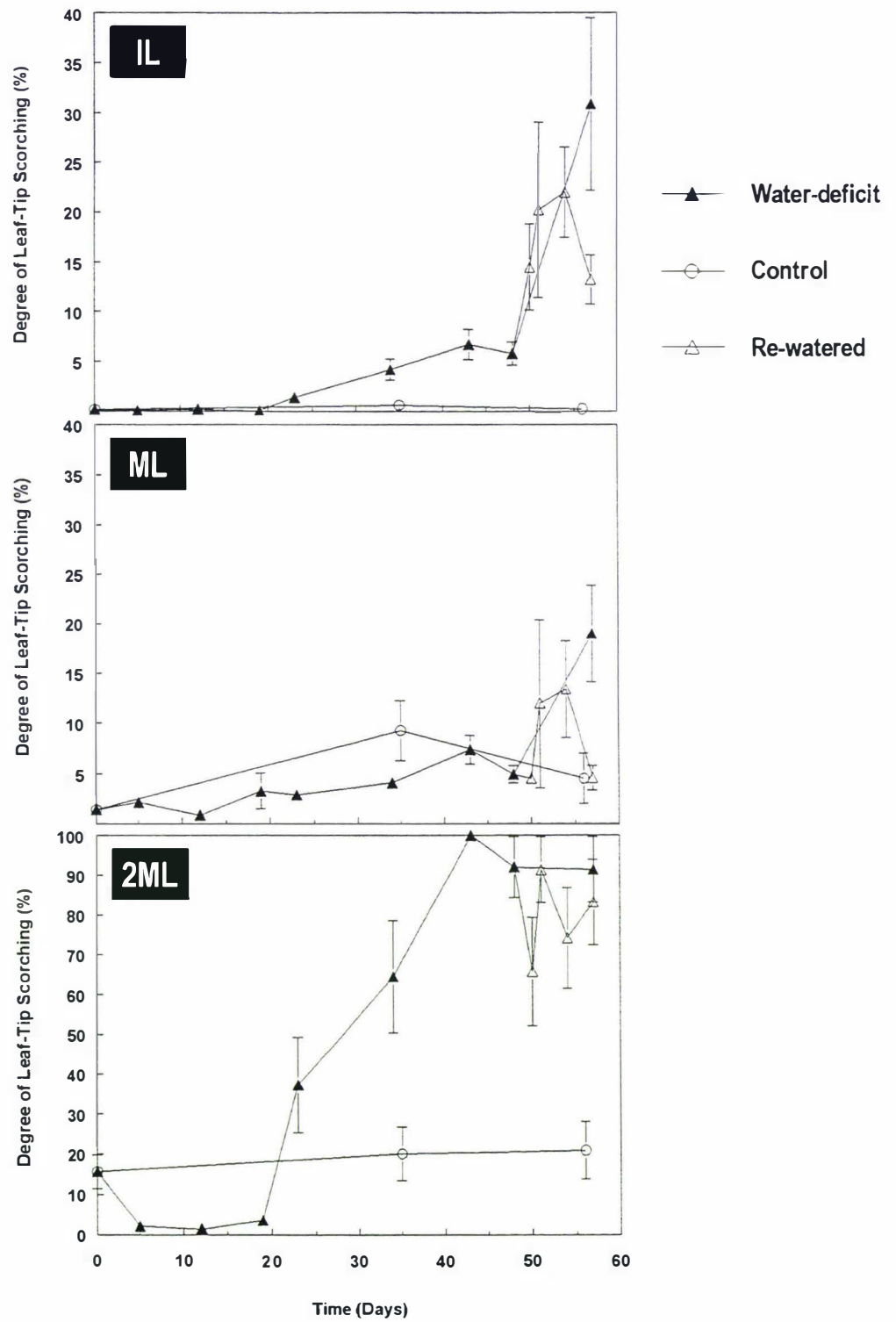


Figure 5.9: Differences in the degree of leaf-tip scorching between treatments. The labels indicate the leaf types. Data were means \pm s.e., n=11.

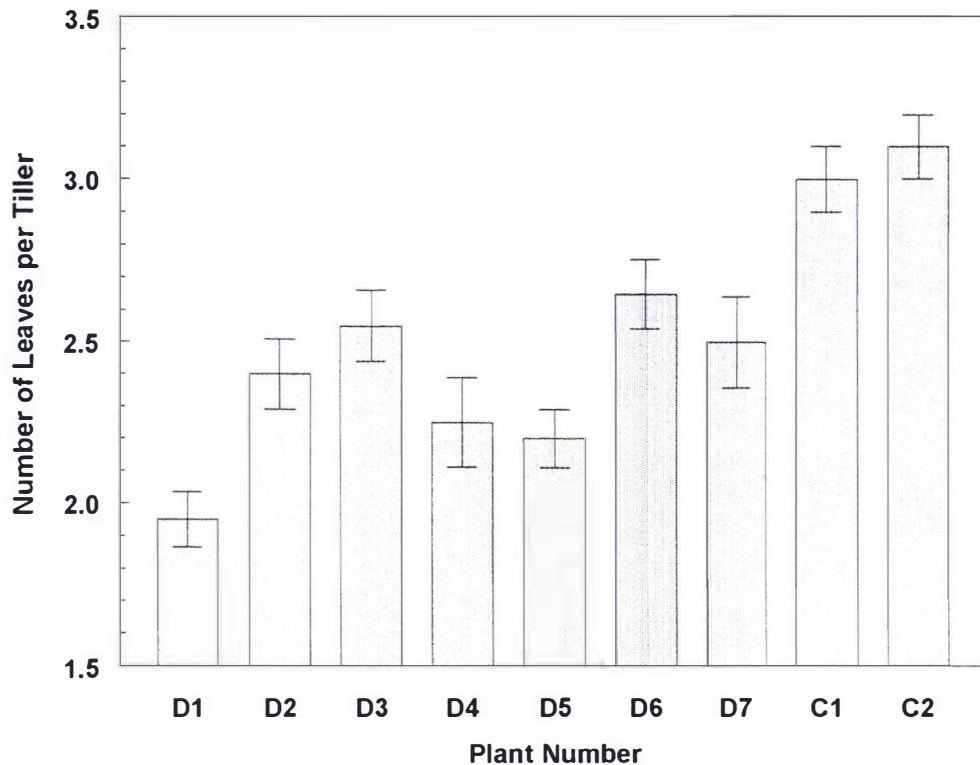


Figure 5.10: The number of green leaves per tiller for each plant. Data was collected on day 49 of the experiment. Data were means \pm s.e., $n=20$.

(C1 and C2) had significantly more green leaves per tiller than any of the water-deficit treatment plants, which all had a N_T of less than 2.6 (Table 5.6).

5.3.2.5.2 Degree of Leaf Tip Scorching (D_s)

The mean D_s from 20 tillers per plant were compared using a two-way ANOVA model. The model was highly significant, at a level of 0.01% (Table 5.7), as were the component comparisons between plants, between leaf types, and the interaction effect between plants and leaf type (Table 5.8). Hence the model indicated that the D_s was dependent on the maturity of the leaf, on the type of treatment, and that an interaction effect existed whereby the effect of leaf type on D_s was dependent on which treatment (water-deficit versus control) was examined.

In the control plants (C1 and C2), D_s was dependent on the age of the leaf. The IL and ML had a D_s from <1% to 10%, respectively, although the means between these leaf types were NSD (Table 5.10). The D_s of the 2ML was significantly greater (up to 41%) when compared with the IL and ML (Figure 5.11).

The D_s of all leaf types increased in response to the water-deficit treatment. In the IL, D_s ranged from 5 to 44% (Figure 5.11), and in all the plants except for D4 and D7, D_s was significantly greater than the controls (Table 5.9). In the ML, D_s ranged from 8.5 to 23.5% (Figure 5.11). In this leaf type, the mean D_s from all the water-deficit treatment plants were NSD from the controls (Table 5.9). The 2ML was affected to a greater extent than the IL and ML, where most of the water-deficit treatment plants had a D_s of more than 50%, and in D1 the D_s approached 100%. However, the D_s in only three of the seven water-deficit treatment plants (D1, D5 and D4) was significantly greater than in the controls (Table 5.9). As already mentioned, the D_s of the IL was low in the water-sufficient plants (<0.5%). In response to the water-deficit treatment, the D_s of the IL increased. Although the high variation in the water-deficit treatment data meant that differences between the treatments (water-deficit versus control) were not always statistically significant (Table 5.9), the relatively large difference in the D_s of the IL between the treatments suggested that this leaf was the most suitable leaf for use as an indicator of water-deficit stress. The high variation in the data did not allow the D_s of the ML and 2ML to be such a sensitive indicator as the IL.

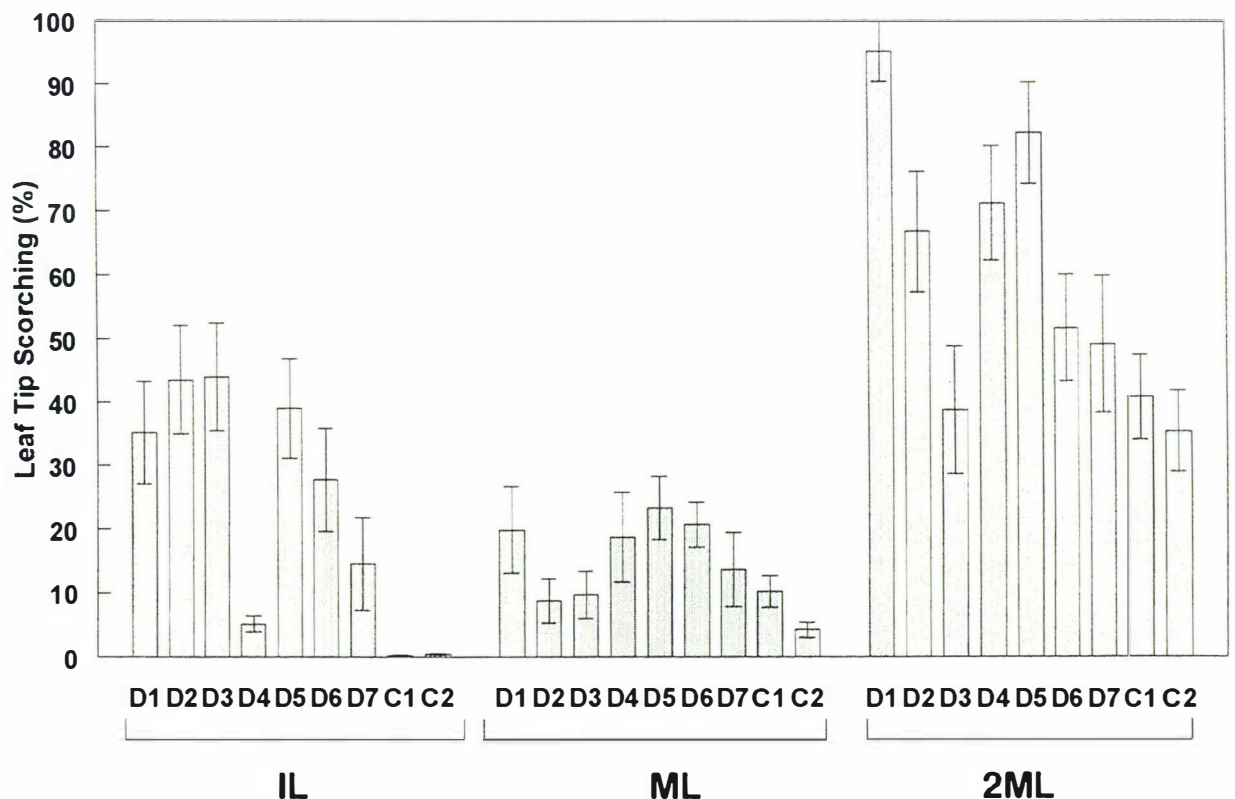


Figure 5.11: The D_s of tillers sampled on day 49 of the experiment. The x-axis indicates plant numbers. Data were means \pm s.e., $n=20$.

5.3.2.6 Leaf Water Status

5.3.2.6.1 Relative Water Content (RWC)

In water-sufficient conditions, the RWC remained between 0.7 and 0.8 in all tissues except the MP, where the RWC was 0.48-0.64 (Figure 5.12). In response to the water-deficit treatment, most of the tiller tissues (except the MP) had RWC levels comparable to the controls for at least the first 34 days, after which the RWC declined in these tissues. The magnitude of the RWC reduction was tissue dependent, with a relatively small decline in the LB (down to 0.58, Figure 5.12), and larger reductions in the IP, ML and IL (down to 0.5, 0.43 and 0.4, respectively (Figure 5.12). The RWC of the MP declined to 0.4 by the end of the experiment. However, in this case, the decrease in RWC may not have been significant until after 44 days.

The RWC responded promptly to re-watering on day 49. Within one day, RWC values returned to the non-stress levels in all tissues (Figure 5.12).

5.3.2.6.2 Leaf Hydration (H_L)

In watered plants, H_L was tissue dependent. The H_L was greatest in the LB and IP, where levels between 3 and 4.5 g water/gDW were measured (Figure 5.13). The IL and ML had *ca.* 2 to 3g water/gDW, and H_L levels in the MP were less than 2g water/gDW (Figure 5.13).

After 35 days, a reduction in H_L occurred in the water-deficit treatment plants, and the rate of H_L decline was tissue dependent. This rate of decline was greatest in the LB and IP. The rates found in the remaining three tissues (IL, ML and MP) were lower, indicating that the rate of dehydration was negatively correlated with the maturity of the tissue. The H_L values from the different tissues all declined to a similar level of *ca.* 1.2 g water/gDW by the end of the experiment, hence the differences in the rates of H_L decline may reflect the relative hydration of each tissue before water was withheld.

The H_L responded to rehydration. However, this response was not as fast as that displayed by the RWC, since it took at least eight days to return to the level of the controls in all tissues (Figure 5.13).

Relative Water Content

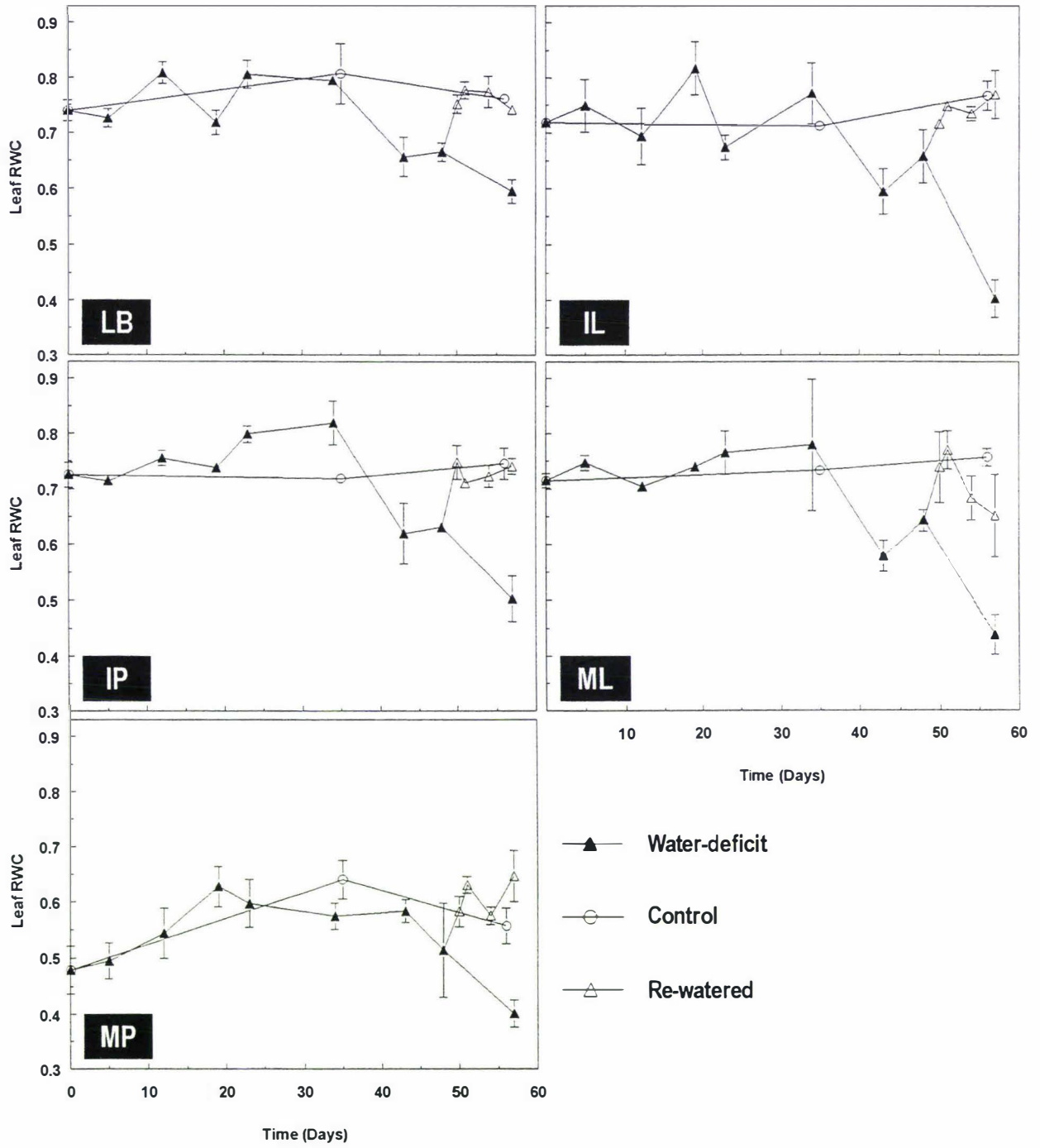


Figure 5.12: Differences in the RWC between experimental treatments. The labels indicate tissue types; Data were means \pm s.e., $n=3$.

Leaf Hydration

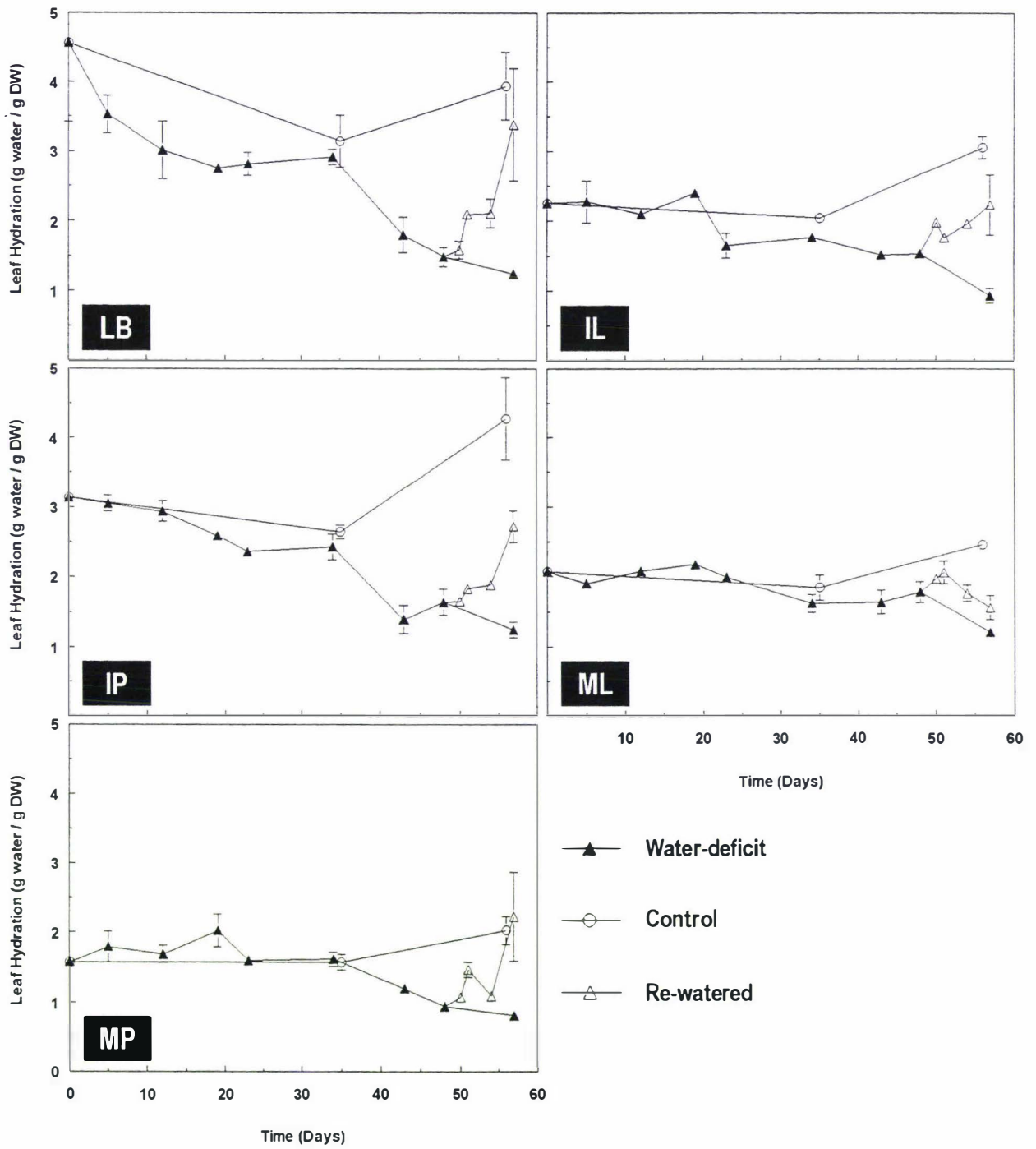


Figure 5.13: Differences in the H_L between experimental treatments. The labels indicate tissue types; Data were means \pm s.e., $n=3$.

5.3.2.7 Water-Deficit Stress Induced Metabolites

5.3.2.7.1 Leaf Abscisic Acid Concentration

The ABA concentration in all tissues from the control plants was *ca.* 25-50 ng/gDW throughout the experiment (Figure 5.14). An increase in ABA concentration in response to the water-deficit treatment was apparent in all tissues by 23 days, and the magnitude of ABA accumulation differed between tissues (Figure 5.14).

ABA accumulation was greatest in the IL, where the measured ABA concentration peaked at 315 ng/gDW, followed by the ML and IP (230 and 215 ng/gFW, respectively) (Figure 5.14). The peak accumulation of ABA in the IP preceded that in the IL and ML by about four days (43 and 47 days, respectively). The LB accumulated ABA during this experiment, but not to the same extent as the other tissues. In this tissue, ABA concentration had increased to 155 ng/gDW by 23 days, and then remained at a slightly lower level for the rest of the experiment (Figure 5.14).

The ABA concentration decreased promptly in response to re-watering (Figure 5.14). Two days after re-watering, the ABA concentration in all tissues had returned to the level of the controls, and remained low for the rest of the experiment, except in the ML. In this tissue, a single elevated measurement was recorded 8 days after re-watering.

In all tissues, ABA accumulation was dependent on SWC where the increase in ABA concentration occurred after the SWC had declined to below 10% (Figure 5.15).

5.3.2.7.2 Leaf Proline Concentration

The proline concentration in water-sufficient plants was inversely related to tissue age, with 8, 4, 1.5 and 1.3 mg/gDW in the LB, IP, IL and ML, respectively, at the start of the experiment (Figure 5.16). Proline contents declined in all tissues of the controls during the experiment (Figure 5.16).

An increase in proline concentration was apparent in all tissues after 23 days, and the magnitude of proline accumulation differed between tiller tissues (Figure 5.16). The greatest accumulation of proline was in the LB, where proline concentration increased

Leaf ABA Concentration

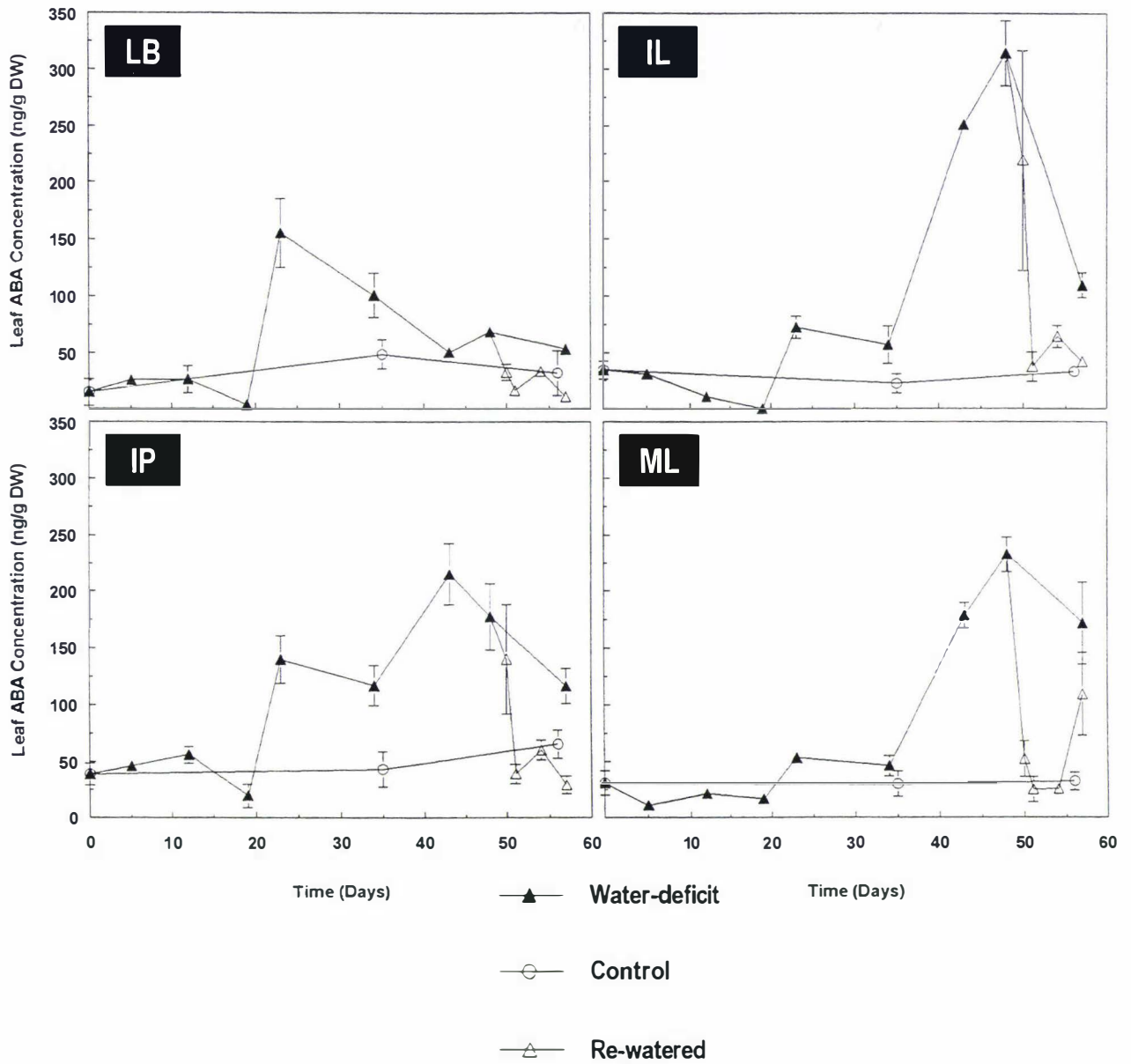


Figure 5.14: Differences in the ABA contents of each tiller tissue between treatments. The labels indicate tissue types. Data were means \pm s.e., $n=3$.

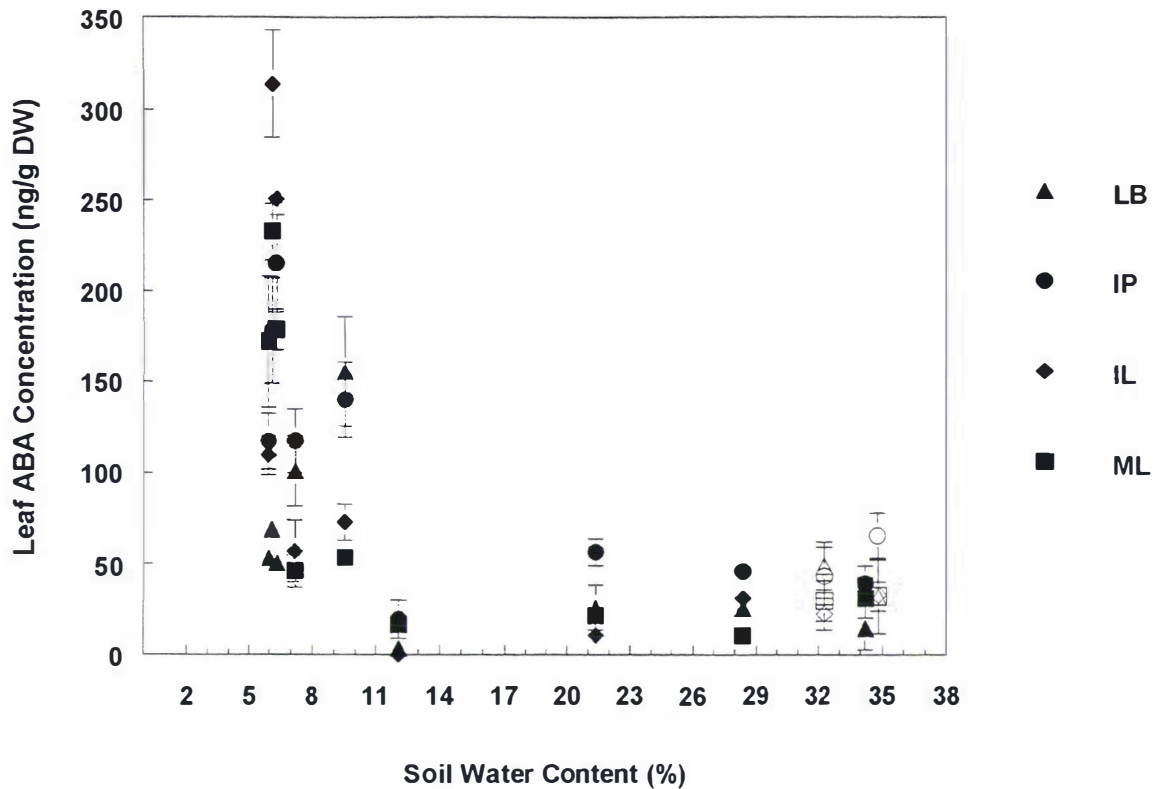


Figure 5.15: The effect of declining SWC on ABA concentration. Data from Figure 5.14 (except for the re-watered data) has been replotted as a function of SWC. The legend indicates tissue types; closed symbols are water-deficit treatment data, open symbols are watered control data. Data were means \pm s.e., $n=3$.

to more than 28 mg/gDW (Figure 5.16). Peak proline contents found in the IP, IL and ML were less than half that of the LB, and in the latter tissues occurred *ca.* 10 days after the peak accumulation in the LB, and either remained at a similar level (LB and IP) or decreased to low levels (IL and ML) during the last stages of the dry-down.

The proline concentration in the LB decreased in response to re-watering with a time-lag of at least two days, and by the eighth day after re-watering, was still significantly greater than in the control tissue (Figure 5.16). Proline contents in the other three tissues either did not decrease in response to rewetting (IP), or declined at a rate similar to that of the water-deficit treatment tissue (IL and ML, Figure 5.16).

In all tissues, proline accumulation was dependent on the SWC, where the onset of accumulation occurred after the SWC had declined below 10% (Figure 5.17).

Leaf Proline Concentration

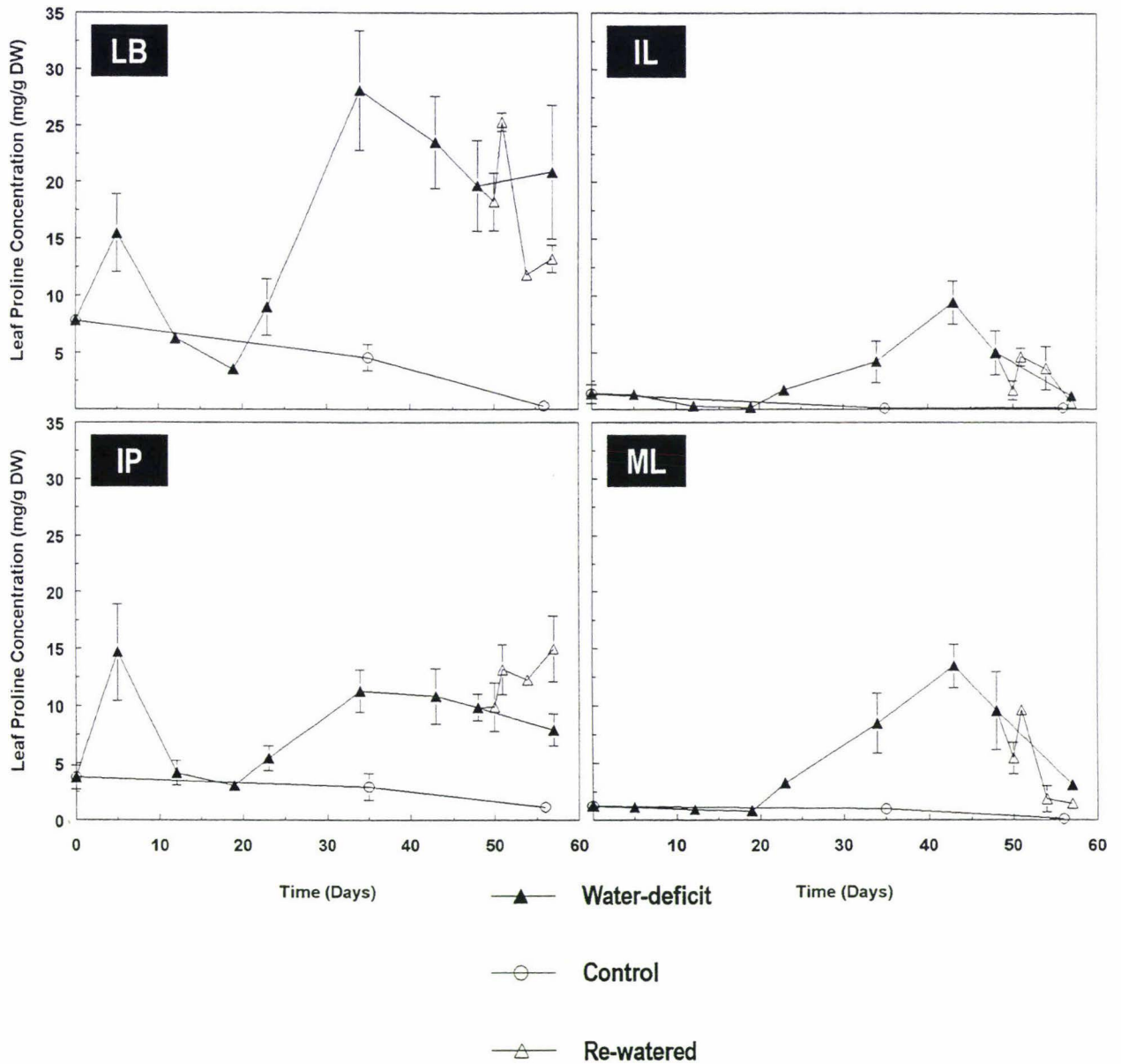


Figure 5.16: Differences in the proline concentration of each tiller tissue between treatments. Data were means \pm s. e., n=3.

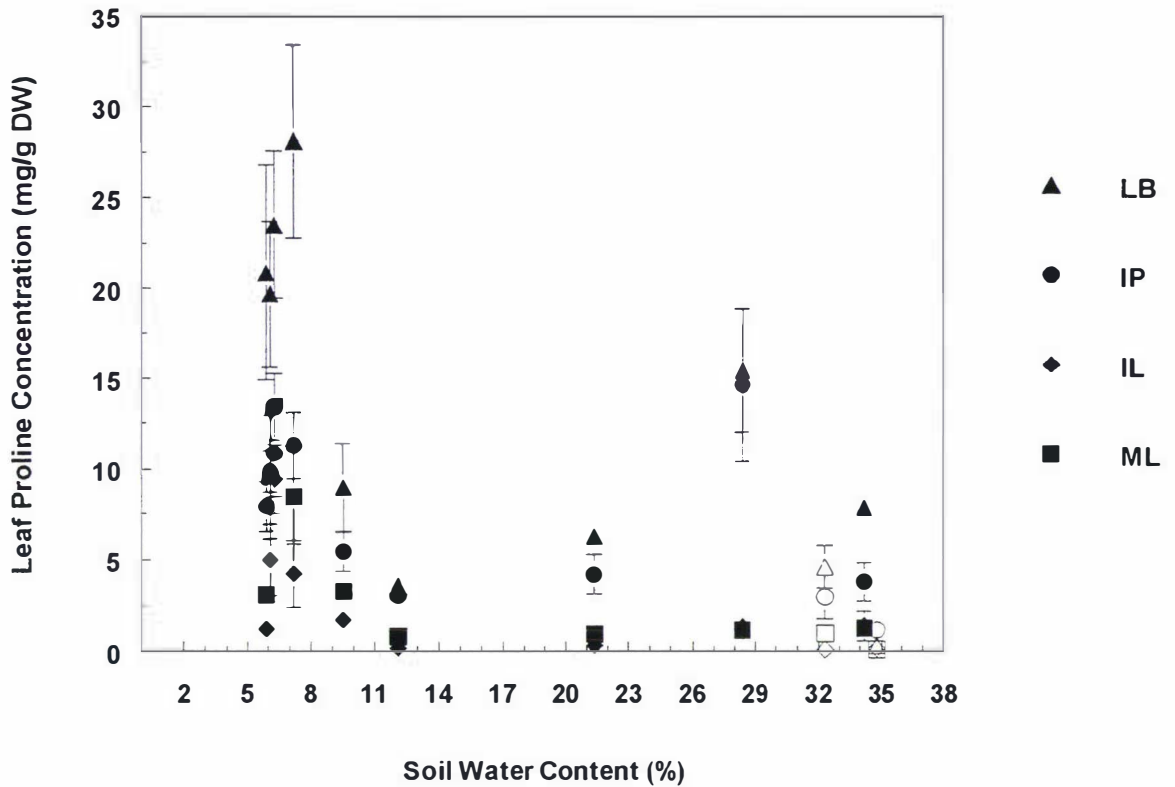


Figure 5.17: The effect of declining SWC on proline concentration. Data from Figure 5.16 has been replotted as a function of SWC. The legend indicates tissue types; closed symbols are water-deficit treatment data, open symbols are watered control data. Data were means \pm s.e., $n=3$.

5.3.2.7.3 leaf Glycine-Betaine Concentration

Glycine-betaine concentrations in tiller tissues from *F. novae-zealandiae* were between 20 to 160 $\mu\text{mol/gDW}$ (Figure 5.18), and did not accumulate in any tissue in response to the water-deficit treatment (Figures 5.18 and 5.19), although a slight increase in glycine-betaine concentration over that of the controls occurred in the ML close to the end of the experiment (day 48). Re-watering the water-deficit treatment plants had no effect on the glycine-betaine concentration of these tillers (Figure 5.18).

Leaf Glycine-betaine Concentration

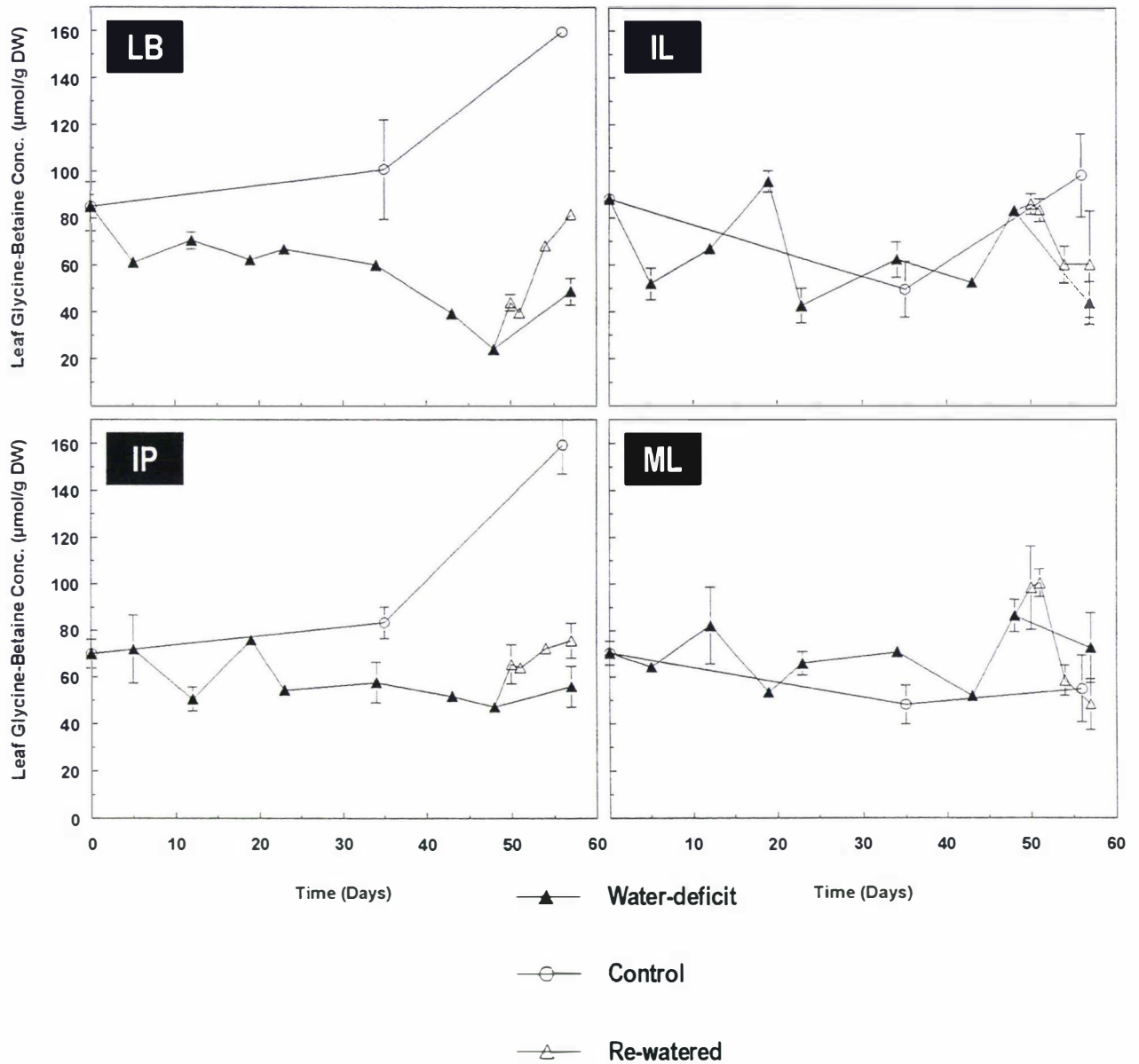


Figure 5.18: Differences in the glycine-betaine concentration of each tiller tissue between experimental treatments. The labels indicate the tissue type. Data were means \pm s. e., $n=2$.

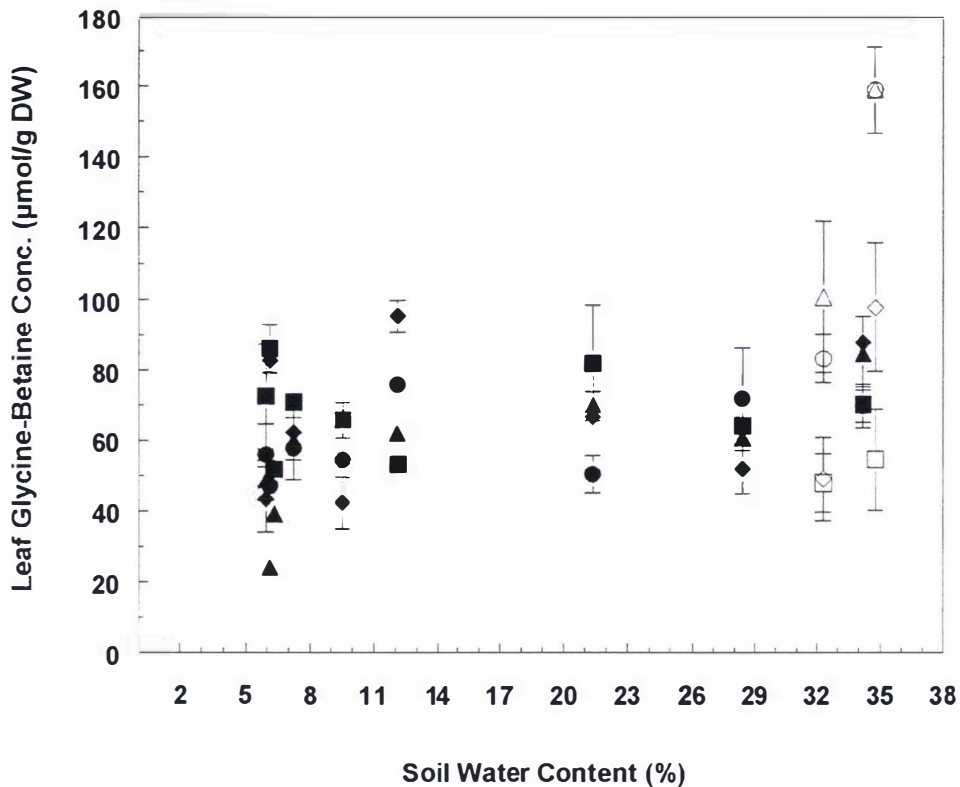


Figure 5.19: The effect of declining SWC on glycine-betaine concentration. Data from Figure 5.18 has been replotted as a function of SWC. The legend indicates tissue types; closed symbols are water-deficit treatment data, open symbols are watered control data. Data were means \pm s.e., $n=2$.

5.3.2.8 Glasshouse Temperature

This experiment was started in early Spring, when mean daily temperature was expected to increase. The temperature of the glasshouse at the time of sampling (8:00 AM) increased from 12 °C to 20 °C during the experiment (Table 5.4). This data was plotted against the experimental measurements below, to examine the possibility that these measurements were affected by a response of the water-deficit treatment plants to temperature change. However, this was not the case. No correlation was observed between the temperature at the time of sampling, and the RWC (Figure 5.20A), H_L (Figure 5.20B), leaf ABA concentration (Figure 5.20C), leaf proline concentration (Figure 5.20D) or leaf glycine-betaine concentration (Figure 5.20E).

The Effect of Glasshouse Temperature on Experimental Measurements

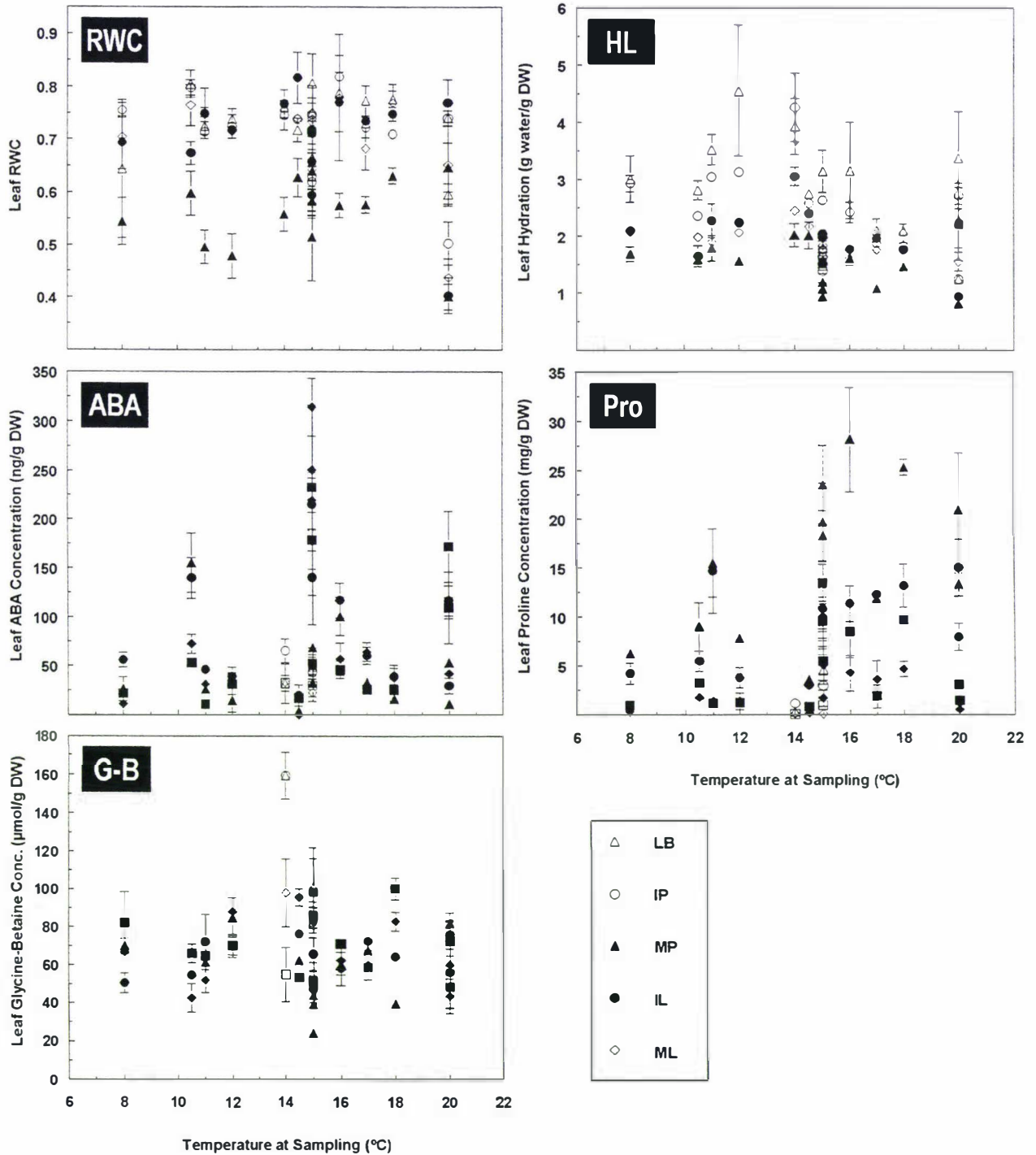


Figure 5.20: The effect of glasshouse temperature on experimental measurements. RWC data from Figure 5.12; H_L data from Figure 5.13; ABA data from Figure 5.14; proline data from Figure 5.16; and glycine-betaine data from Figure 5.18 were re-plotted as a function of the glasshouse temperature at the time of sampling. The legend indicates tissue types. Data were means \pm s.e., $n=3$, except for glycine-betaine data ($n=2$).

5.4 Discussion

5.4.1 Bench-Top Dry-Down Experiment

Results from this experiment supported the aspect of the working hypothesis from Chapter Four (section 4.4.2.2, page 152), that *F. novae-zealandiae* has the capacity to accumulate the metabolites ABA and proline in response to water-deficit stress. However, caution is required if these results are to be compared with those from experiments where plants had been exposed to a dry-down over a longer time. This is because the bench-top dry-down experiment imposed a rapid and severe stress upon tillers of *Festuca spp.* which may have been composed of several factors arising from different sources, such as wounding, gravitropic stress and water-deficiency, and because the increase in ABA and proline content would have been due in part to concentration of the cytoplasm via dehydration, as well as by changes in metabolic activity. Due to the short duration of this experiment, adaption of the metabolism of the experimental plants may have been dominated by injury responses rather than responses to the change in environmental conditions.

The tissue-dependence of proline accumulation suggested that various tissues of *F. novae-zealandiae* may respond differently to the water-deficit treatment. Hence, in the subsequent longer term experiments with *F. novae-zealandiae*, sampling different tissues was conducted to gain a fuller understanding of the process of adaption to water-deficit stress.

5.4.2 Experimental Dry-down of *F. novae-zealandiae*

This experiment examined a further aspect of the working hypothesis, that there exists a differential accumulation of ABA and osmoprotectants by various tiller tissues in response to water-deficit stress.

This experiment used the 4 L instead of the 24 L pot system used in the previous experiments with *F. novae-zealandiae* (Chapter Four, page 93). Since the majority of roots of *F. novae-zealandiae* were located within 200 mm of the soil surface, pots deeper than this were not required. The smaller soil volume of the 4 L pots encouraged a faster dry-down, which was more homogenous within the soil column of each pot. The SWC data indicated that the plants dried down at similar rates. Therefore, an

experimental design where plants were sampled sequentially was suitable; this was in contrast to the dry-down experiments reported in Chapters Three and Four, where each plant was regarded as a separate water-deficit treatment.

5.4.2.1 Timing of the Trigger Point

The physiological data collected during this experiment largely confirms the results from the previous experiments with *F. novae-zealandiae* (Chapter Four, page 93). The gradual decline (but not cessation) of LER, and also a decline in the number of tillers is characteristic of *F. novae-zealandiae* in response to the water-deficit treatment. The event chosen as indicative of the trigger point was a relatively pronounced down-turn in the trend of accumulating tillers in all water-deficit treatment plants which occurred after *ca.* 23 days (Figure 5.6), and corresponded to a SWC of 8-9% (*c.f.* Figure 5.3). This was caused by a decline in the number of green tillers per plant (Figure 5.7), although the total number of tillers increased throughout the experiment which suggested that water-deficit treatment does not prevent the formation of new tillers. This supports the report of Lord (1992) that the continual replacement of tillers allows the size of *F. novae-zealandiae* to increase or decrease in response to environmental fluctuation. However, it was not clear from the present study whether the reduction in living plant size during the dry-down was due to an increased rate of loss of existing tillers or to a reduction in the rate of new tiller formation (or both). Since the leaf-tips of tillers became increasingly scorched as the dry-down experiment progressed (Figure 5.9), the former would seem to be the case. However, a more detailed study of the tiller dynamics in response to water-deficit stress would be required to confirm this.

Changes in D_s supported the occurrence of the trigger point at 23 days. The leaf most affected by the water deficit treatment in terms of D_s was the 2ML, which had become completely scorched by 42 days (Figure 5.9). This was supported by the N_T data (Figure 5.8), which indicated that at least one leaf per tiller was lost by the same time. The onset of the increase in D_s of the 2ML and IL and also in the N_T was at about 23 days (Figures 5.9 and 5.8, respectively). Because of the large differential in the D_s between the control and water-deficit treatment IL (which was also noted in the previous experiment in Chapter Four), this measurement may be a particularly useful indicator of the timing of the trigger point in *F. novae-zealandiae*.

The D_5 measurements were collected during, and also on one occasion toward the end of the dry-down. This was done because of concern that a bias might arise in the data collected during the experiment, which could have originated from the need for sample tillers with an adequately emerged and living IL to supply sufficient material for biochemical analysis. The D_5 measured toward the end of the dry-down was generally greater than those measured during the experiment, especially in the IL [up to 45% in some plants towards the end of the dry-down (Figure 5.11), compared to about 6% (Figure 5.9) from the D_5 data collected at a similar time (48 days) during the experiment]. This indicates that some bias may have occurred in the data collected during the experiment. A possible consequence of the bias was that the ABA and osmoprotectant measurements may have been similarly affected; hence these data may not reflect the respective metabolite concentration of the average tiller within the plant sampled. Because ABA and proline accumulate in response to water-deficit stress, measurements of their concentration may have been underestimated, although the timing of the onset of their accumulation were not likely to have been affected.

5.4.2.2 Accumulation of Metabolites

In the previous dry-down experiments with *F. novae-zealandiae* (Chapter Four), only the ML was examined for metabolite concentrations, and the evidence for metabolite accumulation was inconclusive. In the current dry-down experiment, ABA and proline accumulated in response to the water-deficit treatment. The measurement of RWC during this experiment meant that metabolite concentrations could be expressed on a dry-weight basis, thus accounting for the concentration of metabolites by dehydration.

The onset of the accumulation of ABA and proline in all tiller tissues coincided with the trigger point estimated from the physiological measurements (N_T , D_5) at about 23 days, and began after the SWC had declined below 10%. The accumulation of ABA and proline was tissue-dependent which supported one aspect of the working hypothesis which was under examination in this experiment, that a differential accumulation of osmoprotectants occurs in different tissues in response to water-deficit stress.

ABA concentration was similar in all tissues of the controls, but the accumulation of ABA in response to the water-deficit treatment was greatest in the laminae, and least in

the LB. This pattern appeared to be the reverse of the proline accumulation, where the LB both contained higher concentrations of proline in the water-sufficient state, and also accumulated proline to higher levels than the other leaf tissues. In this regard, the accumulation of proline during this dry-down was consistent with the bench-top dry-down experiment.

Glycine-betaine was the only metabolic marker that did not accumulate in response to the water-deficit treatment. This was consistent with the previous experiment (Chapter Four), and with the suggestion from Chapter Three that glycine-betaine concentration may not be under the same metabolic control as ABA or proline. The high proline concentration in the LB was not observed with glycine-betaine, which was present in all the tissues in about equal quantities. This suggests that this compound may not be involved directly in metabolic adaptation to water-deficit stress. The marginal increase in glycine-betaine observed in the previous experiments (Chapter Four) may have been due to a concentration effect caused by tissue dehydration.

5.4.2.3 Proposed Injury Response

The leaf water status was monitored using the RWC and H_L . These measurements were used because of the practical difficulties encountered when measuring LWP in the previous dry-down experiments with *F. novae-zealandiae* (Chapter Four). In all leaf tissues, the RWC and H_L declined in response to the water-deficit treatment. However, the timing of the decreases were not co-incident with the trigger point; the RWC and H_L of the water-deficit treatment plants first diverged from those of the controls after 43 days, some 20 days past the trigger-point.

This may be explained by an injury response to the water-deficit treatment. It is possible that *F. novae-zealandiae* has the capacity to retain water in the leaves to a high degree during a period of water-deficit. Certainly, some features of the morphology of this plant, such as the location of stomata on the inside of the curled leaf, where dense trichomes and a waxy cuticle are located (*c.f.* section 4.3.1, page 96), support this. Hence a reduction in water loss from the leaves may be a physiological adaptation which enables the plant to reduce its demand for water once the supply of water becomes limiting (*i.e.* once the trigger point has occurred). However, this situation cannot be

maintained indefinitely, and if the water-deficit is not relieved, the leaves will eventually lose sufficient water that cellular disruption occurs. At this point, the ability to efficiently retain water may be lost. Hence, a decrease measured in the RWC or H_L of leaves of *F. novae-zealandiae* may indicate injury.

Changes in the D_5 and in metabolite concentrations which correlate with the timing of changes in RWC and H_L have also been observed in this experiment. As mentioned above, ABA accumulated in all the tissues in response to the water-deficit treatment. This commenced after 23 days which correlated with the timing of the trigger point. However, ABA concentrations did not reach maximum until after the reduction in RWC and H_L were observed (after 42 days, Figure 5.14). In fact, from Figure 5.14, it appears that two inductions of ABA accumulation may have occurred during the dry-down (Figure 5.21). The first correlated with the trigger point where ABA may have accumulated as part of the plants metabolic adaption to the water-deficit treatment. The second, and much greater increase in ABA concentration after 42 days may have been due to the effects of water-deficit induced injury such as the loss of metabolic control of ABA synthesis, or changes in the extraction efficiency of ABA.

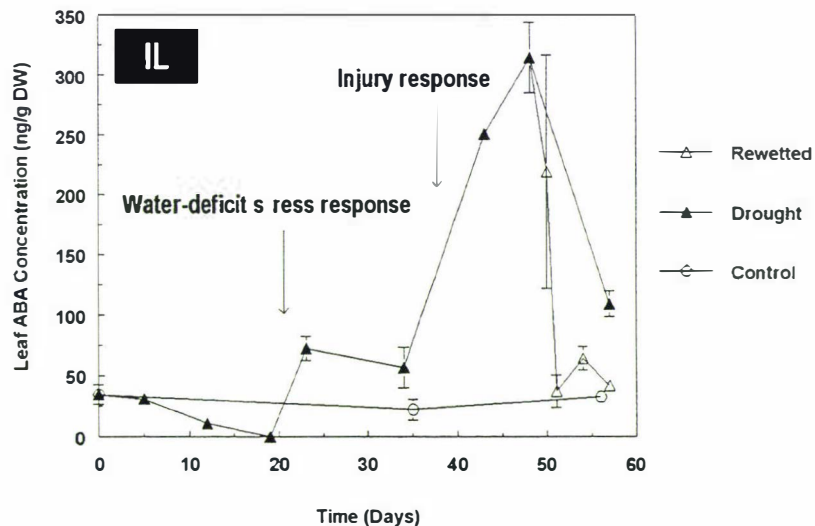


Figure 5.21: The two phase accumulation of ABA in response to water-deficit stress and proposed injury response (as indicated). This figure was redrawn from Figure 5.14.

The second "injury response" increase in ABA concentration did not occur in the LB (Figure 5.14). This is consistent with the hypothesis that this tissue is protected from water-deficit stress by metabolic adaption. ABA accumulated in response to the water-deficit treatment in all tiller tissues. The greatest accumulation of ABA after the trigger

point was in the LB and IP (*ca.* 150ng/gDW, Figure 5.14), followed by the IL (*ca.* 75ng/gFW) and ML (*ca.* 50ng/gFW). However, there was no increase in the ABA concentration of the LB after 40 days as there was in the other tissues, despite the fact that the RWC also declined in this tissue. Hence, the LB appeared to have been protected from water-deficit induced injury. Unlike the other tissues, in the LB the RWC declined slower than the H_L , which suggested that osmoprotection may have occurred in this tissue. The measurement of osmolytes implicated in osmotic adaption in other species, such as mono- and disaccharides, sugar alcohols, and polyols would be required to confirm this. The occurrence of metabolic protection of the LB was also supported by the proline data, which showed that proline accumulated to a high degree in the LB (*ca.* 28mg/gDW, Figure 5.16), and to only a relatively small extent in the IL and ML (less than *ca.* 15mg/gDW). The time lag of several days between the peak accumulation of proline in the LB and the laminae (IL and ML) suggested that some upward transport of proline within the leaf may have occurred.

The accumulation of proline did not appear to follow the 'two phase' induction which was seen with ABA, although the maximal accumulation of proline also occurred after 42 days. This may reflect the difference in roles between the two metabolites: proline is an osmoprotectant, and it may be continually accumulated as the dry-down progresses, whereas ABA is a phytohormone, and may require only one pulse of accumulation (at the trigger point) to signal changes in the plants metabolism.

The D_5 of the IL and especially the ML increased at a relatively faster rate after the RWC and H_L had declined (after 48 days, Figure 5.9). Although there was a time-lag of about six days between the two events, this supports the possibility of an injury response, since some delay would be expected between cellular disruption, and the visible effects of injury.

This data supported the previous experiment, described in Chapter Four (page 93). Although ABA accumulation by *F. novae-zealandiae* was found to be inconclusive during the previous dry-down, the level of ABA measured after the trigger point (up to 40ng/gFW) in this experiment also occurred in peaks in many of the water-deficit treatment plants from Chapter Four [plants D2, D3 and D4, D6, D8 and D9 (Figures 4.15

and 4.16, pages 127 and 128, respectively)]. Hence in the previous experiment, the plants may have responded to the dry-down metabolically by an increase in ABA concentration, although a large increase in ABA due to injury response was not recorded. The proline accumulation measured in the previous experiment was also inconclusive. However, this may be because proline was measured only in the ML, which in the present experiment has been shown to accumulate little proline in response to water-deficit stress.

5.4.2.4 Modified Hypothesis on the Response of *F. novae-zealandiae* to Water-Deficit Stress

Together, the physiological and biochemical data from this experiment supported the hypothesis from Chapter Four. The hypothesis on the response of *F. novae-zealandiae* to water-deficit stress was amended to include the results from the current chapter:

Festuca novae-zealandiae has morphological and biochemical adaptations (exemplified by the limited distribution of stomata, and a relatively high concentration of proline in the LB of water-sufficient tillers, respectively), that reduce water loss to the environment and reduce the probability of water-deficit stress occurring. Tillers undergo tissue-dependent responses when water-deficit stress occurs, and many of these responses (such as changes in leaf ABA and proline concentration, LER, T_{num} and D_5) occur at a common point in the dry-down (the "trigger point") when the SWC has declined to ca. 8%. In response to water-deficit, the viability of the meristematic regions, and hence the capacity for continued growth, is maintained (possibly by the accumulation of osmolytes and/or osmoprotectant compounds), as evidenced by the continuation of leaf elongation which requires turgor for cell extension, and by the disproportionately large increase in proline concentration in the LB compared to the other tissues. In contrast to the LB, the existing leaf tissues die-back during a process of controlled leaf turnover, as evidenced by an increase in D_5 and reductions in the number of tillers and the number of leaves per tiller. In addition to the trigger point, a secondary response occurs, when reductions in RWC and H_L , and a relatively large increase in ABA in the older leaf tissue are observed. This secondary response may indicate the point when water-deficit induced injury has been sustained, and loss of metabolic control has occurred.

Appendix 5.1

ANOVA Tables Referred from Text

A: *Number of Green Leaves per Tiller*

Table 5.5: ANOVA of the N_T between plants.

<i>ANOVA Table; Dependent variable = number leaves per tiller</i>					
Source	DF	Sum Squares	Mean Square	F Value	P
Model	8	21.9	2.75	10.92	0.0001
Error	169	42.5	0.25		
Corrected Total	177	64.5			

Table 5.6: Grouping of means for the N_T between plants.

NSD Grouping	Mean	n	Plant
	3.10	20	C2
	3.00	20	C1
	2.60	20	D6
	2.55	20	D3
	2.50	18	D7
	2.40	20	D2
	2.25	20	D4
	2.20	20	D5
	1.95	20	D1

Means connected by the same bar were NSD.

B: Degree of Leaf Tip Scorching

Table 5.7: Two-way ANOVA of the D_s between leaf type and plants.

ANOVA Table; Dependent variable = Degree of leaf tip scorching					
Source	DF	Sum Squares	Mean Square	F Value	P
Model	26	329,973	12,691	13.59	0.0001
Error	507	473,614	934		
Corrected Total	533	803,587			

Table 5.8: Two-way ANOVA of the D_s : component comparisons.

ANOVA Table; Dependent variable = Degree of leaf tip scorching					
Source	DF	Sum Squares	Mean Square	F Value	P
Between Plants	8	7,235	9,404	10.1	0.0001
Between Leaf Types	2	198,289	99,144	106	0.0001
Plant x Leaf Type	16	555,278	3,471	3.7	0.0001

Table 5.9: Grouping of the means within each leaf type. Means are presented on Table 5.10.

IL		ML		2ML	
NSD Grouping	Plant	NSD Grouping	Plant	NSD Grouping	Plant
[Bar 1]	D3	[Bar 1]	D5	[Bar 1]	D1
	D2		D6		D5
	D5	D1	D4		
	D1	D4	D2		
	D6	D7	D6		
	D7	C1	D7		
	D4	D3	C1		
	C2	D2	D3		
C1	C2	C2			

Means connected by the same bar were NSD.

Table 5.10: Means of all possible combinations of Leaf Type and Plant number.

Plant Number	Leaf Type	NSD Grouping	n	Mean	s.e.
D1	2ML		20	95.12	4.88
D1	IL		20	35.16	8.33
D1	ML		20	19.86	6.96
D2	2ML		20	66.75	9.64
D2	IL		20	43.51	8.80
D2	ML		20	8.77	3.58
D3	IL		20	44.00	8.75
D3	2ML		20	38.70	10.33
D3	ML		20	9.70	3.79
D4	2ML		20	71.24	9.17
D4	ML		20	18.74	7.21
D4	IL		20	5.12	1.30
D5	2ML		20	82.25	8.23
D5	IL		20	39.00	8.03
D5	ML		20	23.32	5.10
D6	2ML		20	51.68	8.66
D6	IL		20	27.71	8.32
D6	ML		20	20.72	3.61
D7	2ML		18	49.11	11.09
D7	IL		18	14.58	7.45
D7	ML		18	13.71	5.97
C1	2ML		20	40.77	6.87
C1	ML		20	10.28	2.55
C1	IL		20	0.21	0.10
C2	2ML		20	35.38	6.56
C2	ML		20	4.24	1.23
C2	IL		20	0.46	0.14

Means connected by the same bar were NSD.

Chapter Six

Molecular Response to Water-Deficit Stress

6.1 Introduction

The principal aim of the experiments in this chapter was to test an aspect of the hypothesis which was presented in the conclusion of Chapter Four (page 93). That is, leaves of *F. novae-zealandiae* were examined for evidence that would be indicative of a process of controlled leaf turnover in response to water deficit stress. The evidence sought included changes in the range of soluble proteins, and the range of ubiquitinated proteins in the LB, IL and ML tissues in response to water-deficit stress. A second aim of these experiments was to gather evidence for the existence of dehydrin-class proteins in *F. novae-zealandiae*, and for their induction in leaf tissue in response to water-deficit stress.

6.2 Methods

6.2.1 Plant Material

All the plants of *F. novae-zealandiae* were a single genotype (T2).

6.2.2 Experimental Dry-Down

Experimental plants were established over six weeks using the 4 L pot system (section 2.2.1, page 23). Six water-deficit treatment plants, and six control plants were used. The dry-down was initiated by withholding water; watering was continued in the control pots throughout the experiment. The SWC of each pot was monitored during the dry-down as described in section 2.3 (page 24). A summary of the experimental conditions is presented in Table 6.1.

With the exception of the first sampling date, when only one plant was sampled, plants were sampled in pairs comprised of one control, and one water-deficit treatment plant. Sampling dates were determined using SWC as a guide, with the intention that plants would be sampled when the SWC had declined to levels of *ca.* 20%, 15%, 10%, and 8% SWC. Two further samples were then sampled over the next 12 days.

Table 6.1: Summary of the experimental dry-down conditions.

	Conditions	Tissues selected
Pot system used:	4L (Potting mix)	
Number of pots : water-deficit treatment:	6	
Control:	6	
Number of plants per pot:	1	
Plant genotype:	T2	
Number of TDR electrode pairs:	1	
Number of tillers measured for LER, N_T, D_S	10	for D_S : IL, ML, 2ML
Sampling procedure:	Each plant sampled once only	
LWP measured:	✗	
RWC and H_L measured:	✓	LB, IP, MP, IL, ML
Proline measured:	✓	LB, IL, ML
ABA and glycine-betaine measured:	✗	
Glasshouse temperature measured:	✓	
Protein content measured:	✓	LB, IL, ML
Proteins separated by SDS PAGE:	✓	LB, IL, ML

One day before each sampling date, ten tillers per plant were tagged with jewellers labels, and the length of the IL, the N_T , and D_S of each lamina (IL, ML, and 2ML) on the tagged tillers was measured. On the sampling date, the length of the IL of each tagged tiller was re-measured to determine the LER. At 8:00 AM the glasshouse temperature was recorded, and tillers were excised for RWC measurements. Within one hour tillers were excised for proline and protein analysis (see below).

Initially, three tillers per plant were sampled, dissected, and the RWC and H_L were determined using the method described in section 2.4.2 (page 26). All the remaining tillers in the plant were then excised at the base, and were kept on ice while the LB, IL and ML from each tiller were dissected and pooled using the method described in section 2.1.3 (page 22). The tissues were frozen and ground in liquid nitrogen, and stored at -80 °C. About 20-30 mg of each sample were analysed for proline concentration using the method described in section 2.10 (page 45). Aliquots (*ca.* 200 mg) were transferred to 1.5 mL centrifuge tubes, for the extraction of soluble proteins by the method described in section 2.11 (page 48). The concentration of protein in each extract was determined using the method described in section 2.12 (page 48). Protein extracts were separated by SDS-PAGE using the method described in section 2.13 (page 50).

6.2.3 SDS-PAGE Separation of Soluble Proteins

6.2.3.1 Protein Profiles, Visualised by Coomassie Blue

A series of both 10% to 20% gradient gels and 5% to 15% gradient gels were prepared as described in section 2.13.1 (page 50), and were loaded with protein extracts from either the LB, IL or ML from tillers harvested during the experiment (for a total of six gels). The loading of extracts was on the basis of equal protein content in each lane; usually 150 μg total protein per lane was used. However, in some instances, where the protein concentration of extracts were too dilute the maximum volume of sample (100 μL) was loaded (the protein contents loaded are given on Plates 6.1 to 6.3). After electrophoresis had been completed, these gels were developed with Coomassie Blue solution, as described in section 2.13.4 (page 51).

6.2.3.2 Investigation of Ubiquitinated Polypeptides

An identical series of polyacrylamide gradient gels were prepared and loaded as described for the Coomassie Blue stained gels (section 6.2.3.1). Upon completion of the electrophoretic separation, the proteins were transferred to a PVDF membrane using the protocol described in section 2.14.2 (page 53). Ubiquitinated proteins were visualised using the Western analysis procedure detailed in section 2.14.2 (page 53), using monoclonal antibodies to ubiquitin (section 2.14.1.1, page 53).

6.2.3.3 Investigation of Polypeptides with Homology to Dehydrin

A series of 10% to 20% polyacrylamide gradient gels were prepared, and were loaded with the same protein extracts used for the 5% to 15% polyacrylamide gels described in section 6.2.3.1. After the completion of electrophoretic separation, the proteins were transferred to PVDF membranes, and dehydrin-like proteins were detected using the Western analysis method described in section 2.14.2 (page 53), using anti-dehydrin antisera (section 2.14.1.2, page 53).

6.2.3.3.1 Examination of Tissues Sampled from a Bench-Top Dry-Down Experiment

A bench-top dry-down experiment was conducted using the method described in section 5.2.2 (page 161). Tillers were dried out on a laboratory bench overnight (*ca.* 18 h). The LB, IL and ML were dissected from the tillers, pooled then frozen with liquid nitrogen and ground using a pestle and mortar. The presence of dehydrin-like proteins in each extract was determined using SDS-PAGE and Western analysis.

6.2.3.3.2 Examination of Dehydrin-Like Seed Proteins

Approximately 20 embryos from *H. vulgare*, or 0.9 g of seeds from *F. arundinacea*, or 0.8 g of seeds from *F. novae-zealandiae* were ground with a pestle and mortar in a ratio of 1:2.5 tissue mass: volume of extraction buffer (section 2.11, page 48). The extracts were centrifuged at 12,000×g for 10 min at 4 °C, and the supernatants transferred to a fresh tube. The supernatant was mixed with an equal volume of gel loading buffer (section 2.11, page 48) and stored at -20 °C. The presence of dehydrin-like proteins in each seed extract was determined using SDS-PAGE and Western analysis.

6.2.3.3.3 Identification of Dehydrins

As an aid to the identification of putative dehydrins in *Festuca spp.* tissue samples, a heat denaturation step was carried out whereby aliquots of protein extracts were immersed in a boiling water bath for 10 min. After cooling to room temperature, the denatured proteins were pelleted by centrifugation for 10 min at 12,000×g. The supernatant was prepared for SDS-PAGE without concentration, and was designated as the heat-stable protein fraction.

6.2.4 Southern Analysis of *F. novae-zealandiae* Genome

Genomic DNA was extracted from the ML of *F. novae-zealandiae* using the method described in section 2.15.2 (page 55), and quantified using the method described in section 2.15.3 (page 55). The DNA was digested using three restriction endonucleases (HindIII, BamHI and EcoRI), separated on a 0.8% agarose gel and transferred to a nylon membrane using the methods described in section 2.16.3 (page 58). The nylon membrane was probed with RAB16a DNA from section 2.16.4 (page 59), using the Southern hybridisation method described in section 2.16.6 (page 60). The DNA loading onto the agarose gel was carried out in duplicate, so that identical Southern blots could be washed with two levels of stringency (with 1 × SSPE and 0.2 × SSPE).

6.3 Results

6.3.1 Results from the Experimental Dry-Down

6.3.1.1 Experimental Time Course

Water was withheld from experimental plants from 25 January to 28 February 1995. Tillers were harvested from one water-deficit treatment and one control plant on six dates, except the first sampling date when only one plant was harvested. At the end of the experiment, tillers from two water-deficit treatment plants were sampled, and pooled. This was because the number of green tillers on the water-deficit treatment plants had declined to the level where insufficient material for the protein analyses could be harvested from a single plant. A summary of the sampling regime is presented in Table 6.2.

Table 6.2: Summary of the harvesting regime used during the dry-down experiment.

Date (1995)	Days	Sample Designation ¹	SWC (%)	Temperature (°C) ²
January 26	1	D1	31.2	21
February 3	9	C2	20.4	21
3	9	D2	20.4	21
10	16	C3	13.8	23
10	16	D3	13.8	23
17	23	C4	8.9	21
17	23	D4	8.9	21
23	30	C5	5.5	19
23	30	D5	5.5	19
28	34	C6	3.9	20
28	34	D6	3.9	20

¹ D: Water-deficit treatment sample; C: Control sample.
² Glasshouse temperature at the time of sampling.

6.3.1.2 Soil Water Content (SWC)

The SWC of the soil column of each pot was averaged within each treatment (water-deficit or control). The number of measurements used to calculate the mean was reduced as plants were destructively sampled during the experiment. Hence the initial data point (at time = 0 days) was the average of seven measurements, the data points up to the first sampling on day eight were the mean of six measurements, and so on. The SWC of the watered control pots was maintained above 24% (Figure 6.1),

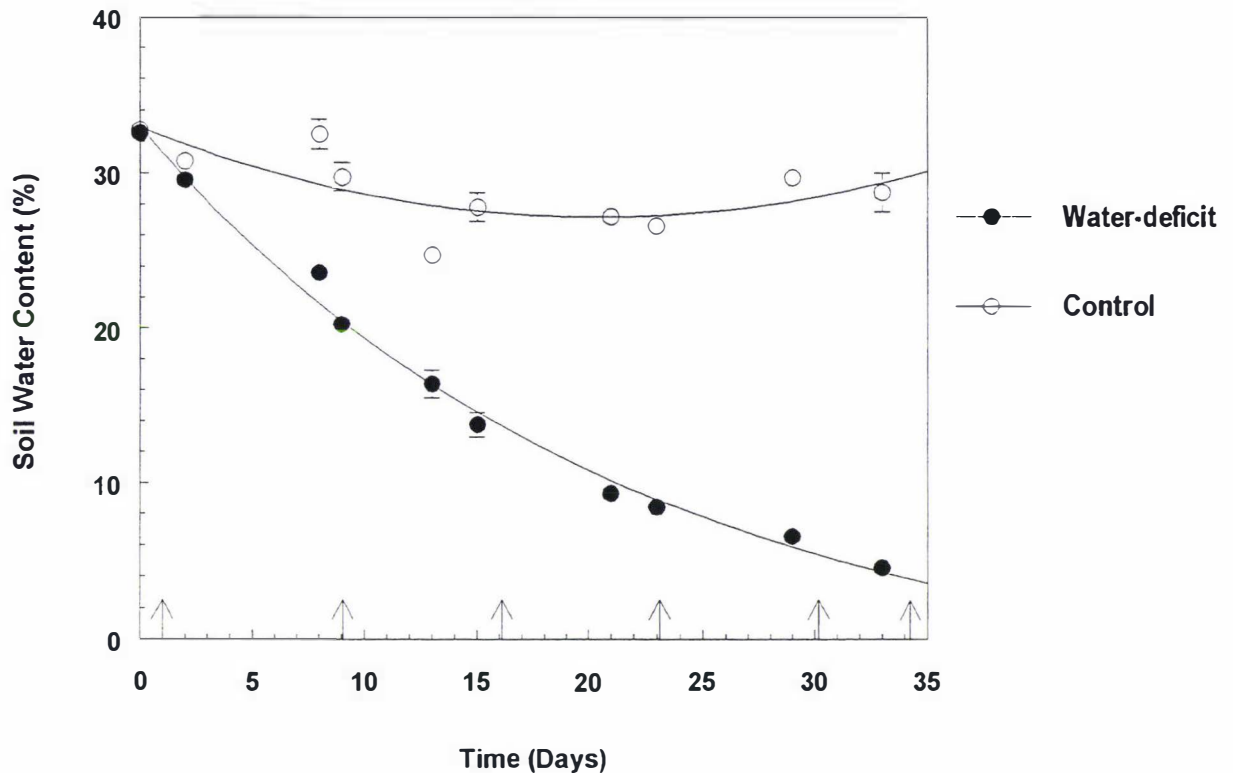


Figure 6.1: The mean SWC of the water-deficit treatment and watered control pots during the dry-down. The x-axis arrows indicate the dates that tillers were harvested. Data were means \pm s.e., $7 \geq n \geq 2$.

while the SWC of the water-deficit treatment pots declined steadily from saturation (ca. 33%), to less than 5% by the end of the dry-down (Figure 6.1).

6.3.1.3 Leaf Elongation Rate (LER)

The lengths of ten IL from each plant to be harvested were measured on consecutive days to determine the LER. In the control plants, the IL were found to extend at rates of ca. 7 to 10 mm.d⁻¹ (Figure 6.2) throughout the dry-down. In the water-deficit treatment plants, the LER declined almost linearly to ca. 1 mm.d⁻¹ by the end of the experiment (Figure 6.2).

6.3.1.4 Mean Number of Green Leaves per Tiller (N_T)

The N_T was counted on the same sample of ten tillers used to determine the LER. In the control plants, the N_T remained between 3.3 and 3.7 (Figure 6.3). The plot of N_T in the water-deficit treatment plants was similar to that of the control plants for the first 23 days, and then declined to become less than 2.3 by the end of the experiment (Figure 6.3). By this stage, the water-deficit treatment plants were distinctly tan-brown in colour, whereas the control plants were still predominantly green.

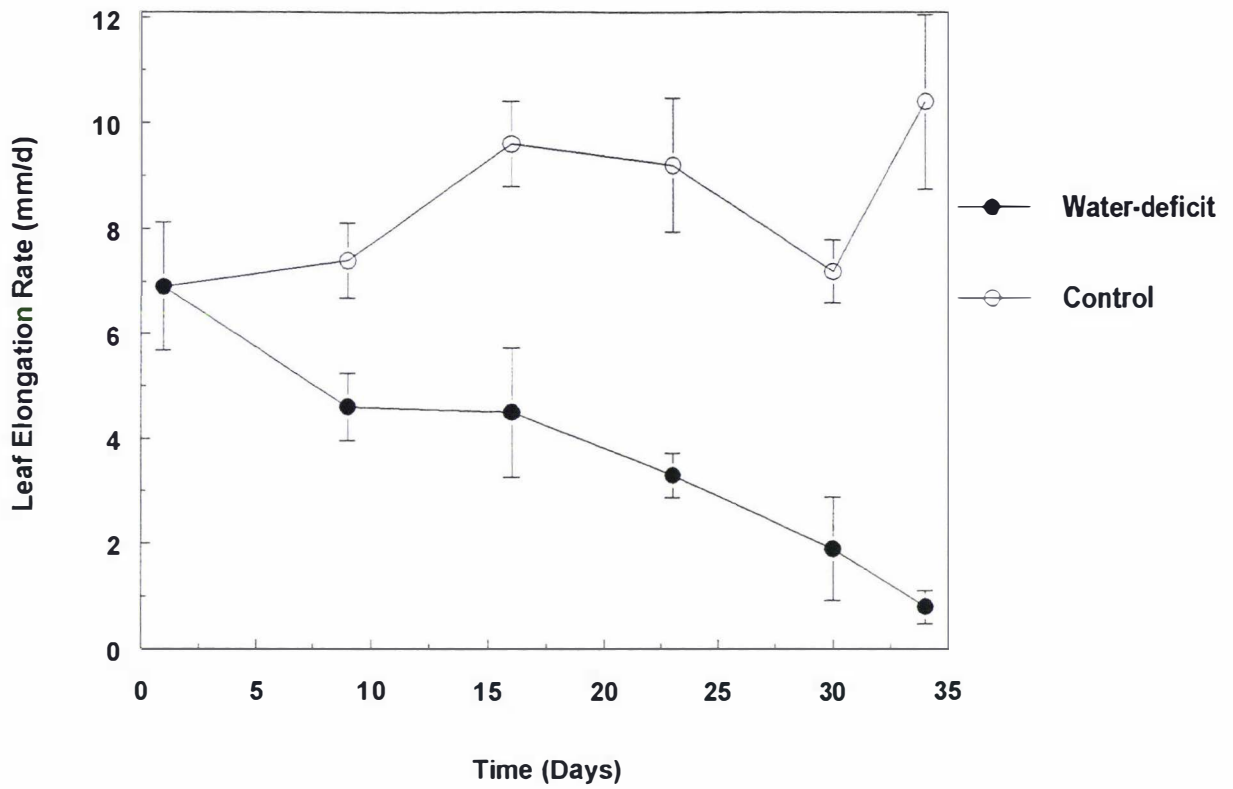


Figure 6.2: Differences in the mean LER of the IL between water-deficit treatment plants and watered control plants during the dry-down. Data were means, \pm s.e., $n=10$.

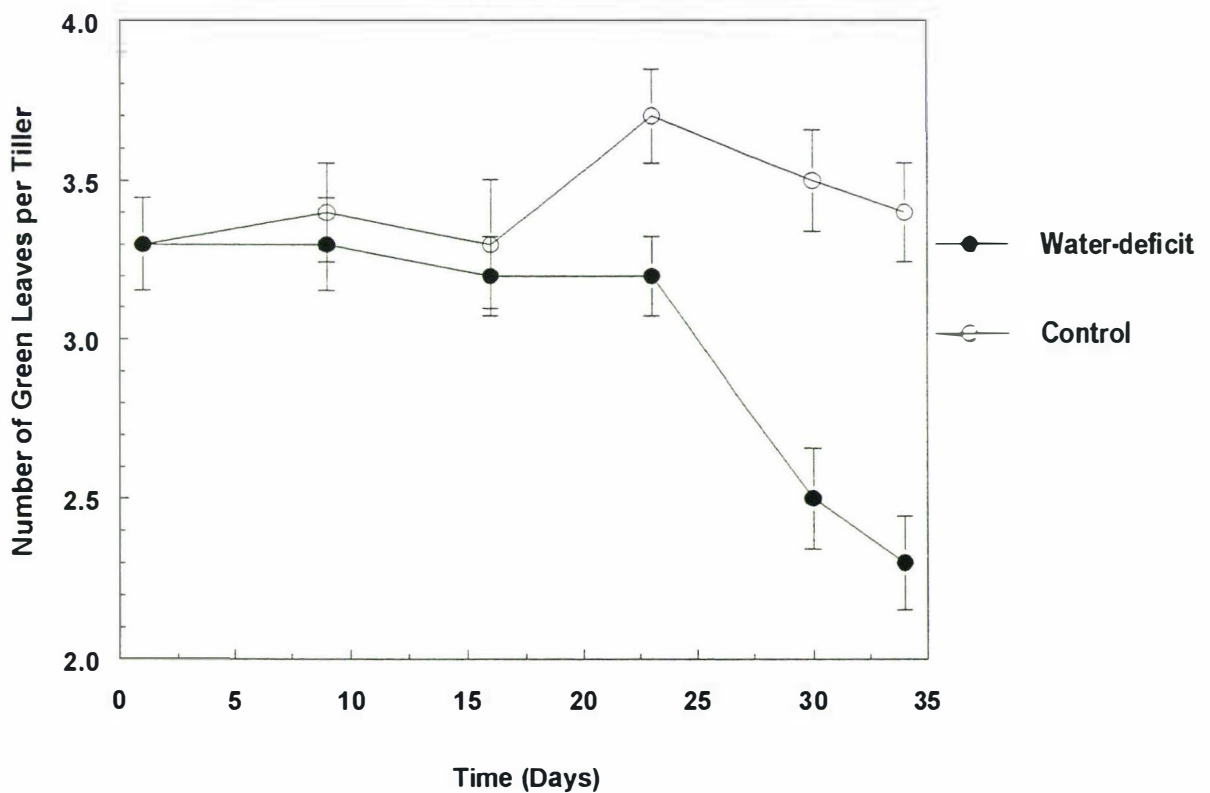


Figure 6.3: Differences in N_T between tillers from water-deficit treatment and watered control plants during the dry-down. Data were means \pm s.e., $n=10$.

6.3.1.5 Degree of Leaf Tip Scorching (D_s)

The D_s of the IL, ML and 2ML, was measured on ten tillers per plant on each sampling date. In water-sufficient plants, D_s increased with increasing leaf age, with up to 4%, 20% and 26% scorching measured in the IL, ML and ML, respectively (Figure 6.4). No increase in the D_s within any leaf type was observed in the water-sufficient plants during the experiment.

An increase in the D_s was observed within each leaf type in response to the water-deficit treatment. In the IL, the D_s was less than 4% for at least 23 days, after which time the D_s of the IL increased to *ca.* 20% (Figure 6.4). The D_s of the ML of water-deficit plants remained at a similar level to that of the control plants for most of the experiment, increasing above the controls (up to 28%) on the final sampling date. An increase in D_s of the 2ML was observed after 23 days, when the D_s progressed to become over 80% by the end of the experiment (Figure 6.4).

6.3.1.6 Relative Water Content (RWC)

On each harvest date, three tillers from each plant to be sampled were excised and dissected before the RWC of five tiller tissues (LB, IP, MP, IL, ML) was determined. The RWC determined in most of the tissues was within the range 0.68 to 0.83, although in the MP it was lower, at 0.55 to 0.70 (Figure 6.5). In each of the five tissues, the trend of RWC measurements was similar, with little difference between the water-deficit and control treatments, and with little change in the magnitude of RWC measurements within each tissue type during the experiment (Figure 6.5).

6.3.1.7 Leaf Hydration (H_L)

The data collected for the determination of the RWC values was also used to calculate the H_L in each of the five leaf tissue samples. The H_L was negatively correlated with leaf age. The LB consistently had the greatest H_L , with a ratio of about 4.5 g water/g DW. The IP and IL contained 3 to 4 g water/gDW and 2 to 3 g water/gDW, respectively, and the MP and ML contained *ca.* 2 g water/gDW (Figure 6.6). In each of the five tissues, the trend of H_L measurements was similar, with little difference between the water-deficit and control treatments, and with little change in H_L within each tissue type during the experiment (Figure 6.5).

Degree of Leaf-Tip Scorching

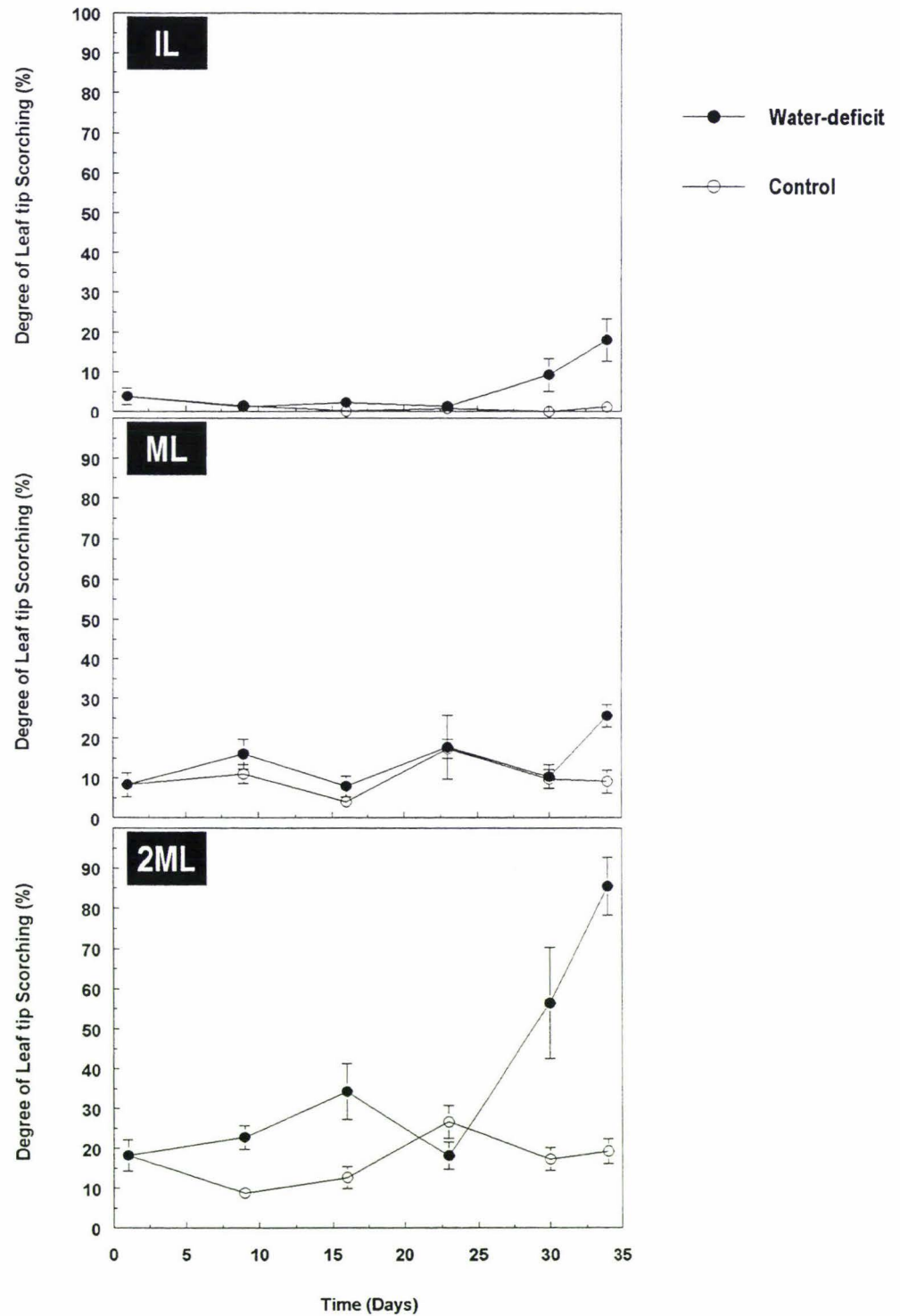


Figure 6.4: The difference in the D_s of each leaf type between water-deficit treatment and watered control plants during the experiment. The labels indicate leaf type. Data were means \pm s.e., $n=10$.

Relative Water Content

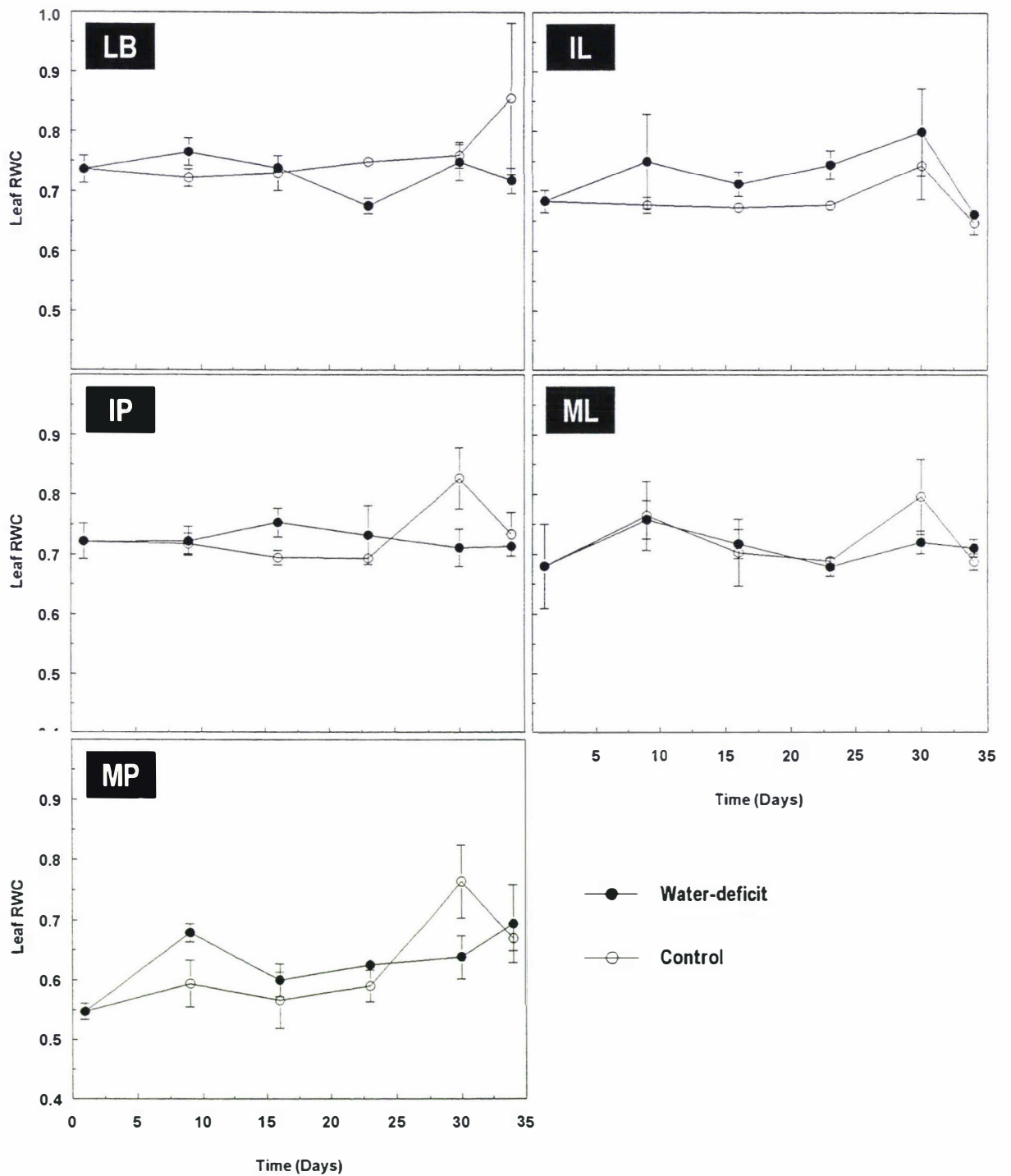


Figure 6.5: Differences in the RWC in each of the five tiller tissues sampled from water-deficit treatment plants and watered control plants during the dry-down. The labels indicate tissue type. Data were means \pm s.e., $n=3$.

Leaf Hydration

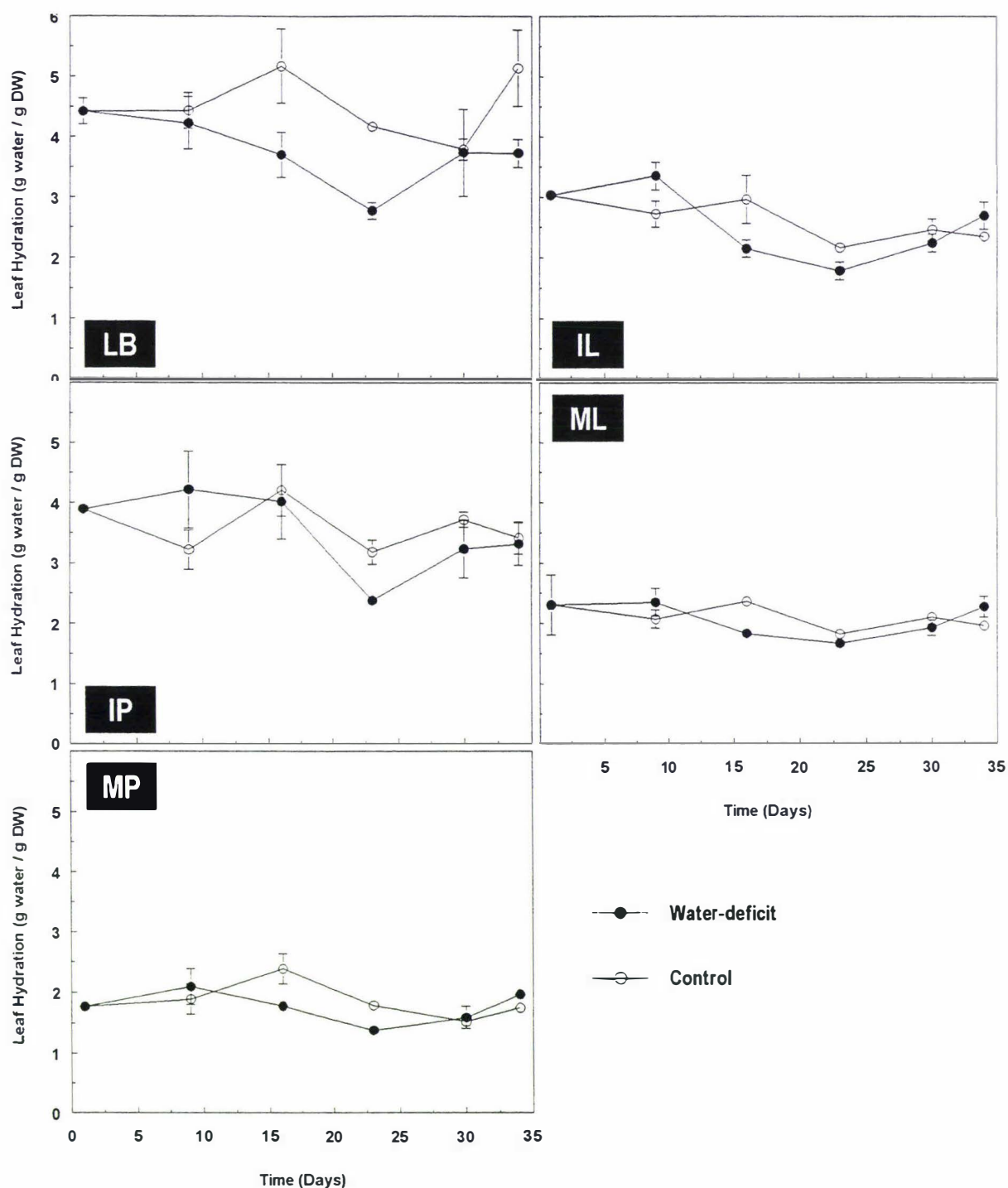


Figure 6.6: Differences in the H_L in each of the five tiller tissues sampled from water-deficit treatment plants and watered control plants during the dry-down. The labels indicate tissue type. Data were means \pm s.e., $n=3$.

6.3.1.8 Glasshouse Temperature

The glasshouse temperature at the time of sampling was relatively constant, at 20.8 ± 0.5 °C during the dry-down (Table 6.2). Hence an examination of the effect of temperature on RWC, H_L or leaf proline concentration was not required.

6.3.1.9 Leaf Proline Concentration

Samples from three tissues (LB, IL and ML) from each harvest date were analysed for proline concentration, as an aid for determining the timing of the trigger point. Since the experiments in this chapter focus on the differences between the laminae (IL and ML) and the LB, in response to the water-deficit treatment, the proline concentration of the pseudostem was not measured.

In the control plants, the proline concentration in the LB was relatively high (4.8 to 7.9 mg/g DW) compared to the proline contents of the IL and ML (where five out of six determinations were in the range 0.4 to 2 mg/gDW, Figure 6.7). The single measurement from the LB on day nine of 12 mg/gDW was considered to be an outlier (Figure 6.7). This point was the greatest of all the determinations from this experiment, and was uncharacteristically high when compared to the proline concentrations from the other control tissues from the same sample date. Discounting this outlier, the proline concentration from the LB of water-deficit treatment plants were similar in magnitude to those of the controls throughout the dry-down (Figure 6.7).

The proline concentration in the IL was essentially constant in the control plants (Figure 6.7). However, the final control plant datum on this graph (at day 34) may also be an outlier, since large increases in proline concentration from other control tissues from this sample date were not observed. Discounting this point, the proline concentration measured in the IL of water-deficit treatment plants were similar to those of the controls for the first 16 days, at *ca.* 1.8 mg/gDW after which time the proline concentration increased to *ca.* 4 mg/gDW for the rest of the experiment. This pattern of proline accumulation was also observed in the ML, where proline levels of the water-deficit treatment plants did not exceed that of the control plants for the first 16 days. In this case, proline concentration also increased to about 4 mg/gDW.

Leaf Proline Concentration

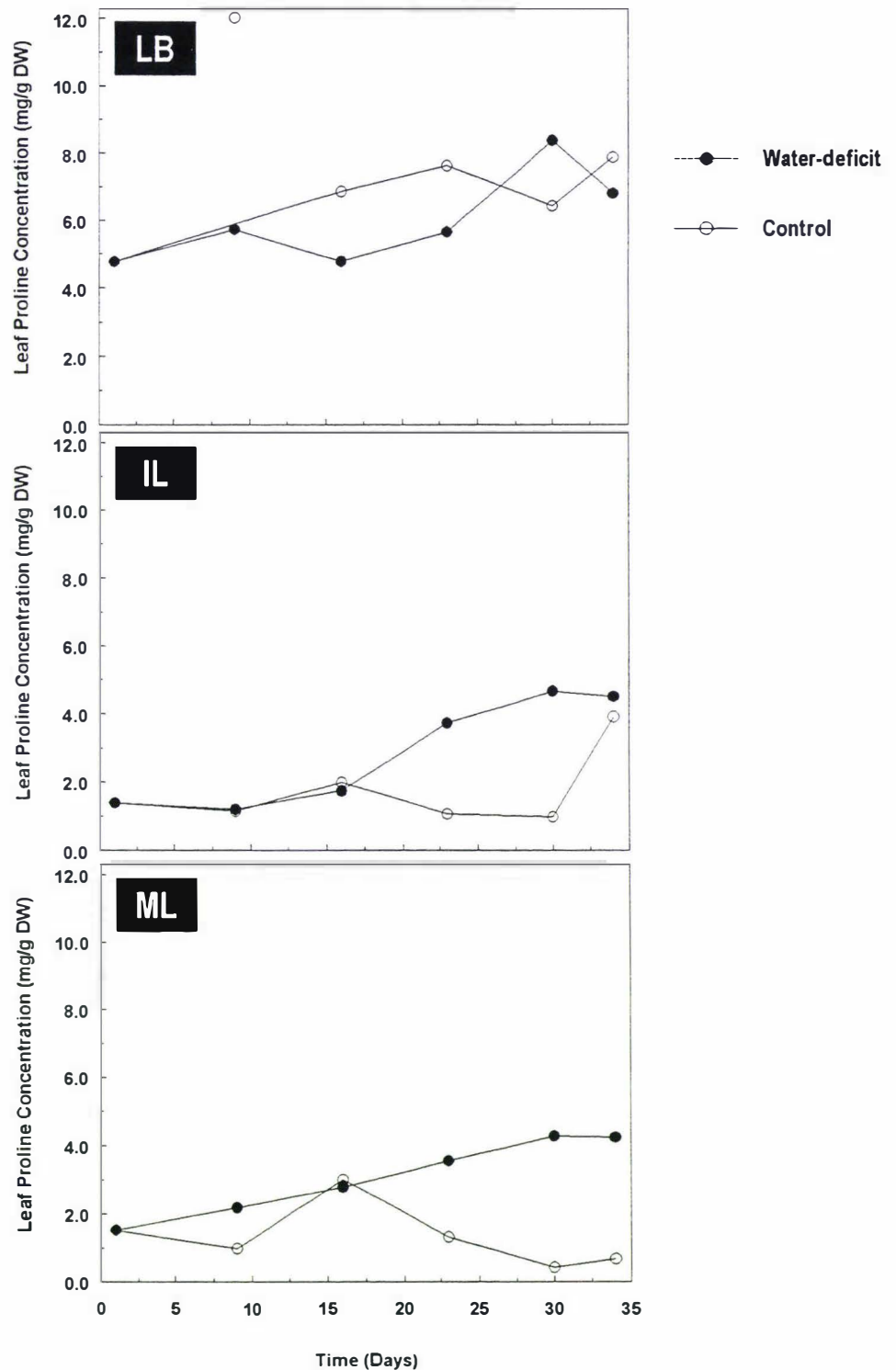


Figure 6.7: Differences in the proline concentration of the three tiller tissues sampled from water-deficit treatment plants and watered control plants during the experiment. The labels indicate tissue types. Data were single determinations.

6.3.1.10 Soluble Protein Concentration

On each sampling date, leaf tissue was harvested for the extraction of soluble proteins to be analysed using SDS-PAGE. The protein concentration of each extract was determined to facilitate the loading of equal quantities of protein in each lane of the polyacrylamide gels.

The soluble protein content of the leaf tissue was dependent on the age of the tissue (Figure 6.8). On each harvest date, the greatest protein content was in the ML, with successively lower contents in the IL and LB, respectively. Under water-sufficient conditions, the levels of protein approximated 20mg/gFW, 15 mg/gFW and 5mg/gFW (equivalent to 2%, 1.5% and 0.5% of the fresh mass of the tissue) in the ML, IL and LB, respectively (Figure 6.8).

Although some variation in the data was observed between samples from both the dry-down and control plants, a reduction occurred in the soluble protein concentration in all tissues in response to the water-deficit treatment, with the lowest concentration occurring in the water-deficit treatment tissues sampled from days 29 and 33 (Figure 6.8).

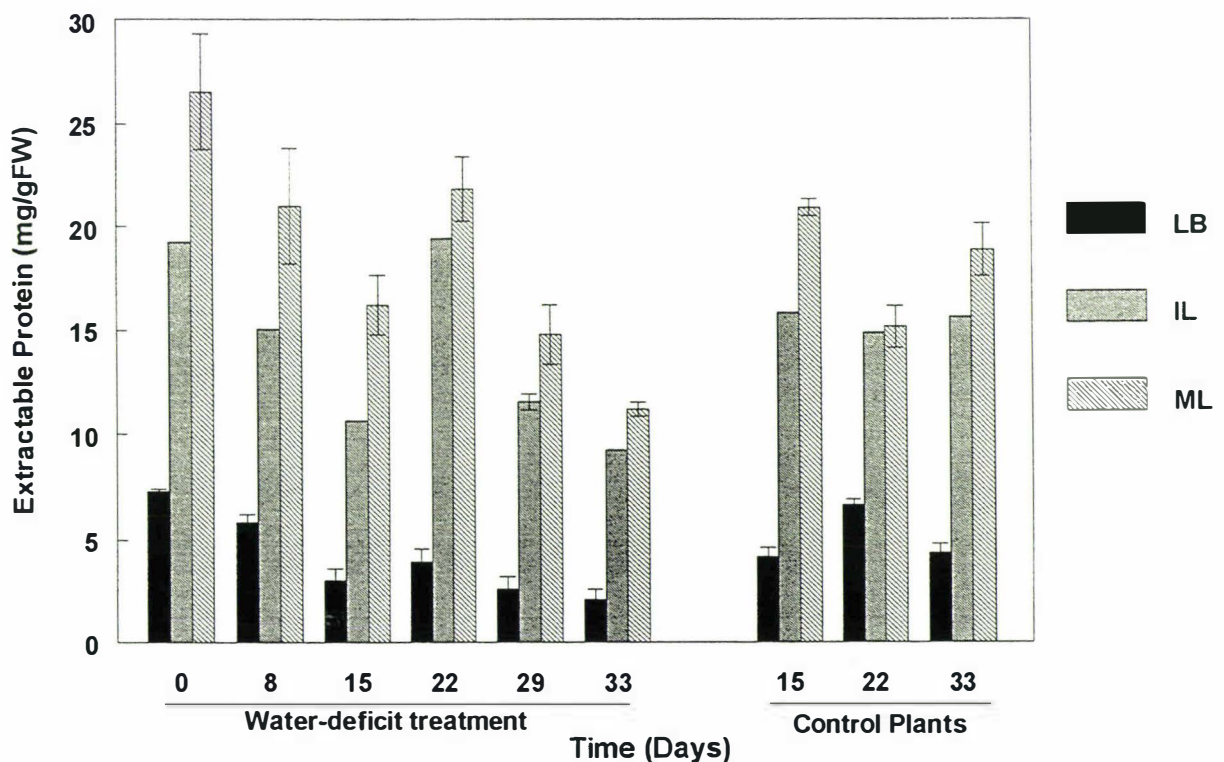


Figure 6.8: The soluble protein concentration in each of the protein extracts of tissue harvested during the dry-down. Data were means \pm s.e., $n=3$ for LB, $n=2$ for ML and for water-deficit treatment IL data from days 29 and 33. The remaining IL data were single determinations.

6.3.2 SDS-PAGE Separation of Soluble Proteins

6.3.2.1 Visualisation of Protein Profiles by Coomassie Blue Stain

6.3.2.1.1 Protein Profiles from the ML

Protein extracts from samples of the ML separated on a 10% to 20% and 5% to 15% gradient polyacrylamide gels (Plates 6.1A and 6.1B, respectively) showed *ca.* 50 of the most abundant proteins present in the extracts when visualised using Coomassie Blue stain. Of these, three proteins of *ca.* 54, 50, and 17 kD were present as major protein constituents.

The profile of separated proteins (i.e., the banding pattern observed in each lane of the gel) were generally similar amongst all the samples loaded onto the two polyacrylamide gels. However, both the electrophoretic mobility of all the proteins, and the relative separation of the higher molecular mass proteins was as expected greater in the 5% to 15% gradient polyacrylamide gel.

There was particular similarity amongst the extracts from the first four water-deficit treatment samples (D1 to D4), and also with the extracts from the three control samples (C3 to C6). Together, this group of seven samples are referred to as the "control group". This feature was evident on both of the gradient gels (Plates 6.1A and 6.1B), although some differences in the intensity of certain proteins within this group of similar protein profiles were observed. For example, the proteins of *ca.* 170, 95 and 71 kD on the 10% to 20% gradient gel, were less intense in sample lanes D1, D3, less intense in C4 and C6, and more intense in lane C6, respectively (Plate 6.1A). The two proteins of *ca.* 15 kD on the 5% to 15% gradient gel were not represented with even intensity on all the lanes of this group (Plate 6.1B).

The banding pattern of samples D5 and D6 (referred to as the "response group") were similar to each other, but had many differences in the intensity, and the pattern of separated proteins when compared to the control group (Plates 6.1A and 6.1B). A list of the individual protein changes between the response group and the control group is presented in Table 6.3. The intensity of several proteins was reduced in the response group. Most notably, the intensity of the major protein of *ca.* 50-55 kD, and also many

of the proteins of greater than 50 kD were reduced. Also, the intensity of a number of proteins was greater than that of the control group, and these proteins were generally less than *ca.* 50 kD (Table 6.3). Numerous small proteins (<15 kD) were observed in the response group on the 5% to 15% gradient gel, which were not seen on the other lanes of this gel, nor on the 10% to 20% polyacrylamide gradient gel (Plate 6.1).

Table 6.3: Differences between the pattern of proteins observed in the response group of samples (D5 and D6) with respect to the control group of samples (D1 to D4, and C3 to C6), from the ML (Plate 6.1). Data are the estimated protein size in kD.

Proteins induced ¹		Proteins reduced	
10% to 20%	5% to 15%	10% to 20%	5% to 15%
-	94.6	168.4	138.6
28.4	-	137.1	132.9
27.7	27.8	125.7	121.0
23.5	-	-	113.3
-	21.0	103.0	110.1
16.1	-	-	99.3
15.5	-	-	92.4
13.6	12.1	-	84.5
-	10.5	-	80.8
		75.1	75.8
		62.8	66.9
		-	62.9
		60.8	60.3
		52.8 m	54.6 m
		48.9 m	50.4 m
		-	41.3
		-	39.7
		42.9	39.0
		27.0 nc	27.2
		-	24.1
		-	23.1
		19.0	19.8
		16.8	17.5
		15.1	-

¹ Proteins listed on the same row of the half table were considered likely to be the same protein on both gradient gels (based on the relative intensity of each protein); Key: dash: a comparable protein was not observed on this gel; m: major protein; nc: No change in the intensity of this protein between the lanes on this particular gel were observed.

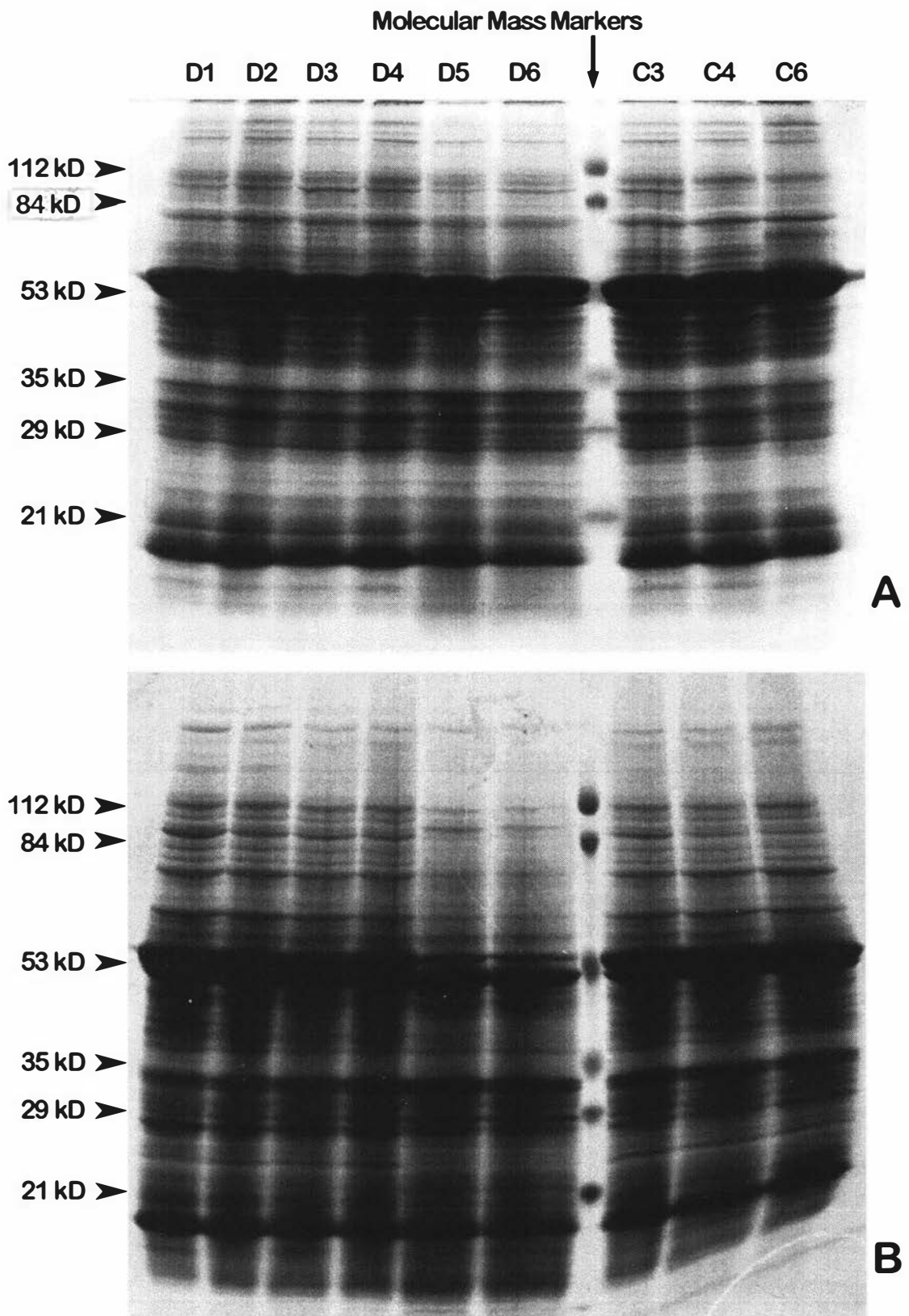


Plate 6.1: SDS-PAGE analysis of protein extracts from the ML of tillers sampled during the dry-down. The proteins were visualised by Coomassie Blue stain. A: 10% to 20% gradient gel, B: 5% to 15% gradient gel. Lanes D1 to C6 (see table 6.1) were loaded with A: 200µg/lane, and B: 150µg/lane of protein.

6.3.2.1.2 Protein Profiles from the IL

Protein extracts from the IL harvested during the experiment were separated on 10% to 20% gradient and 5% to 15% gradient polyacrylamide gels, and were visualised using the Coomassie Blue stain (Plates 6.2A and 6.2B, respectively). Three proteins, of *ca.* 55, 50, and 17.5 kD were present as major protein constituents of the samples.

A high degree of similarity was observed amongst the control group on both gels (Plates 6.2A and 6.2B). However, some differences in the intensity of a few proteins within this group were apparent, such as the protein of *ca.* 17.5 kD in sample D2 on both of the gradient gels, which was not present to the same extent in the other samples (Plates 6.2A and 6.2B).

The pattern of separated proteins of the response group were similar to each other, but displayed many differences in the intensity, and in the pattern of proteins when compared with the control group (Plates 6.2A and 6.2B). A list of the individual protein differences between the response group, and the control group is presented in Table 6.4. The intensity of several proteins was reduced in the response group. Most notably, the intensity of the major protein of *ca.* 50 kD, and also of many of the proteins of *ca.* >50 kD were reduced. Also, the intensity of a number of proteins was greater than that of the control group, and these were generally less than 50 kD (Table 6.3). Numerous small proteins (<15 kD) were observed in the response group on the 5% to 15% gradient gel, which were not seen on the other lanes of this gel, nor on the 10% to 20% polyacrylamide gradient gel (Plates 6.2A and 6.2B).

6.3.2.1.3 Protein Profiles from the LB

The exhaustion of samples 5D and 6D limited the separation of proteins from the LB to the 10% to 20% gradient gel (Plate 6.3). Because of the dilute nature of the protein extracts, loading targets of 150 µg/lane protein were not possible. Instead, an aliquot (50 µL) of extract (with 50 µL of loading buffer) was loaded onto each lane. This loading contained between 53 and 120 µg/lane protein (Plate 6.3). This gel was stained with Coomassie Blue stain, and about 35 proteins were visualised.

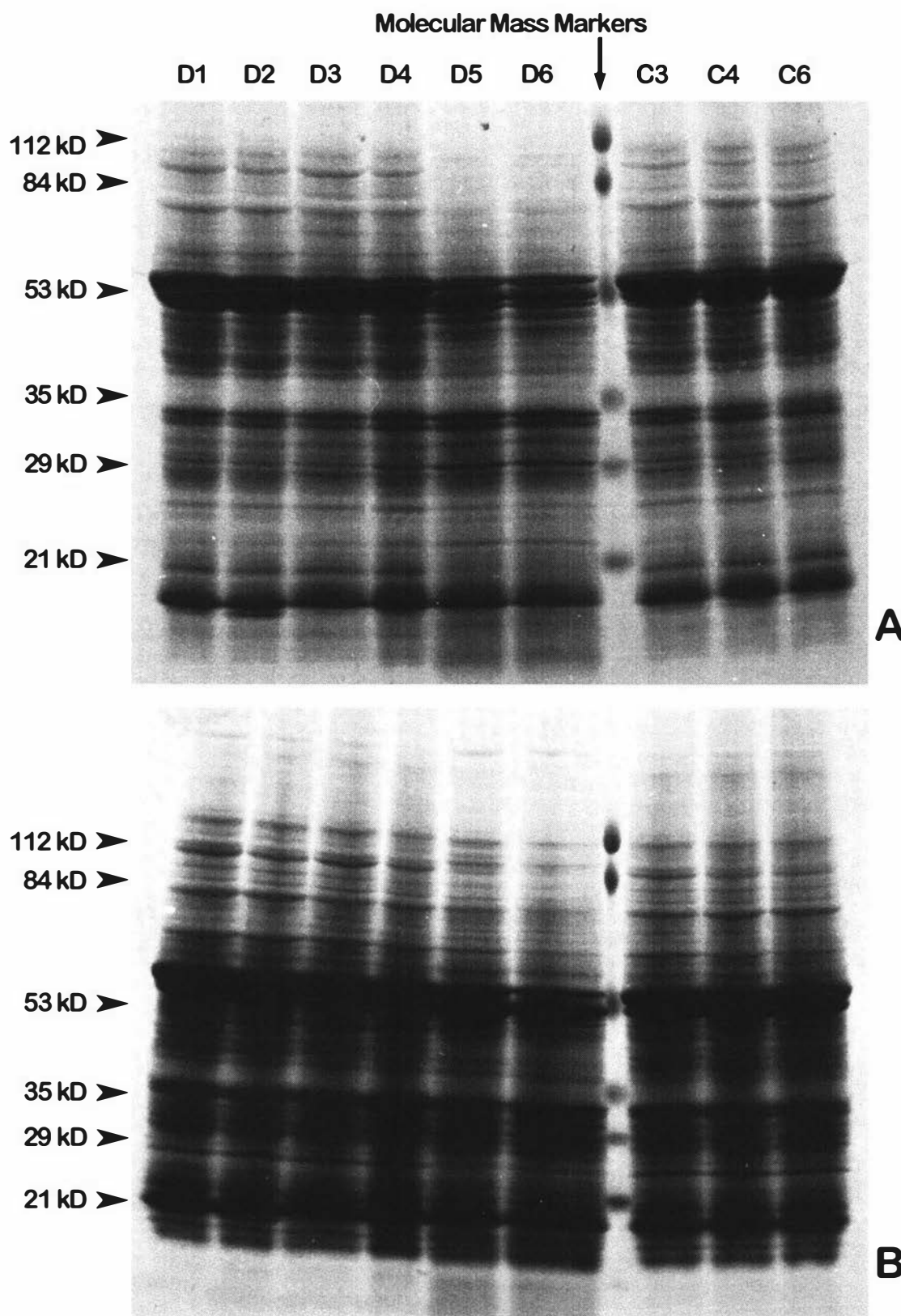


Plate 6.2: SDS-PAGE analysis of protein extracts from the IL of tillers sampled during the dry-down. The proteins were visualised by Coomassie Blue stain. A: 10% to 20% gradient gel, B: 5% to 15% gradient gel. Lanes D1 to C6 (see table 6.1) were loaded with 150 μ g/lane of protein.

Table 6.4: Differences between the pattern of proteins observed in the response group of samples (D5 and D6) with respect to the control group of samples (D1 to D4, and C3 to C6), from the IL (Plate 6.2). Data are the estimated protein size in kD.

Proteins induced ¹		Proteins reduced	
10% to 20%	5% to 15%	10% to 20%	5% to 15%
23.0	25.9	213	-
16.5	16.7	154.6	155.0 nc
14.4	14.6	142.0	142.8
-	11.6	103.4	110.7
-	8.0	-	100.0
		91.2	92.9
		88.6	86.5
		81.6	82.6
		73.9	76.1
		61.8	63.0
		-	60.2
		58.1	57.7
		55.8 m	53.8 m
		51.6 m	49.4 m
		43.3	42.3
		41.3	40.5
		38.5	38.8
		25.0	25.9
		20.2	24.1
		15.9	16.2

¹ Proteins listed on the same row of the half table were considered likely to be the same protein on both gradient gels (based on the relative intensity of each protein); Key: dash: a comparable protein was not observed on this gel; m: major protein; nc: No change in the intensity of this protein between the lanes on this particular gel were observed.

The pattern of proteins in each lane on the gel shared a high degree of similarity, although differences in the general intensity of all of the bands in different lanes were apparent, which reflected the inequality in the protein content loaded. Only two proteins of *ca.* 27.3 and 23.8 kD appeared to be present at lower levels in the response group, when compared to the control group. The differences in the protein content loaded between lanes may have contributed to the low visibility of these bands in lanes D5 and D6. However, the sample D3 was also loaded with a comparable quantity of protein, and both proteins were visible in this lane (Plate 6.3).

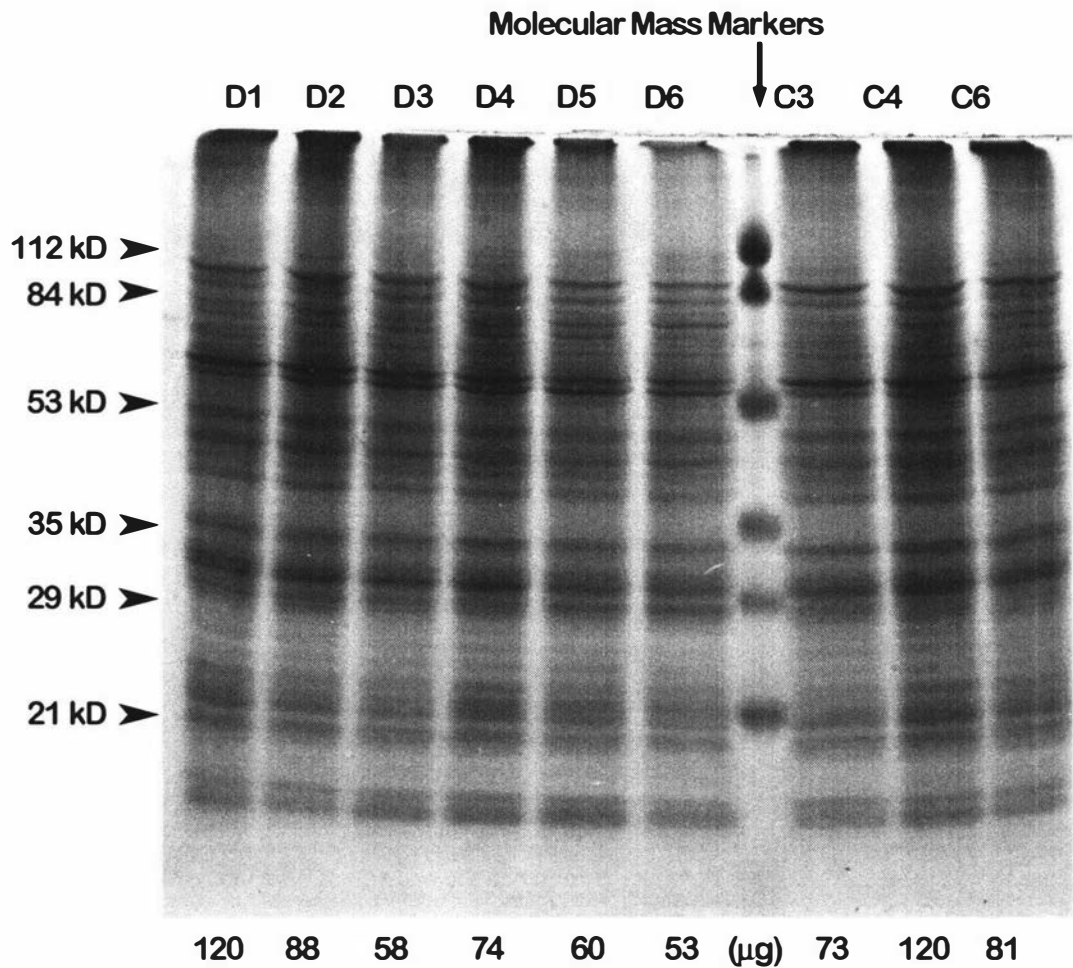


Plate 6.3: SDS-PAGE analysis of protein extracts from the LB of tillers sampled during the dry-down. The proteins on this 10% to 20% gradient gel were visualised by Coomassie Blue stain. Lanes D1 to C6 (see table 6.1) were loaded with 50μL of extract; the amount of protein (μg) loaded onto each lane is presented under each lane.

6.3.2.2 Detection of Ubiquitinated Polypeptides

Several features were common to all of the gels analysed with ubiquitin antisera. A single protein of low molecular mass (*ca.* 14 kD) occurred in the lane allocated to the prestained molecular mass standards, and was assumed to be a contaminant of the commercial product. Also, a major protein migrated with the Bromophenol Blue dye front in all samples, on each of the gels (Plates 6.4 to 6.6), was thought to be free ubiquitin, which has a molecular mass of 8.5 kD (Schulz *et al.*, 1993). A significant amount of streaking in each of the samples (caused by a large number of indistinguishable proteins) occurred at high molecular mass, possibly due to polyubiquitinated proteins. In IL and ML extracts (Plates 6.4 and 6.5), a clear patch within the protein streaking occurred at *ca.* 50 to 55 kD, which indicates that these proteins were not conjugated with ubiquitin. In each of the samples, a number of discrete proteins (<20) were visible after Western analysis using the anti-ubiquitin antisera. These proteins were generally in the region of 15 to 50 kD in size. Changes in the pattern of these proteins for each tissue are detailed in the following subsections.

6.3.2.2.1 Ubiquitinated Proteins from the ML

Eight or nine ubiquitinated proteins (respectively) were observed in the ML extracts on both the 5% to 15%, and the 10% to 20% gradient polyacrylamide gels (Plate 6.4). These proteins occurred at a similar intensity in all the extracts on each gel, with the exception of the response group. In the latter two extracts, the intensity of the proteins was either increased with respect to the control group, or remained the same. A list of the estimated molecular masses of these ubiquitinated proteins in the ML extracts is presented (Table 6.5).

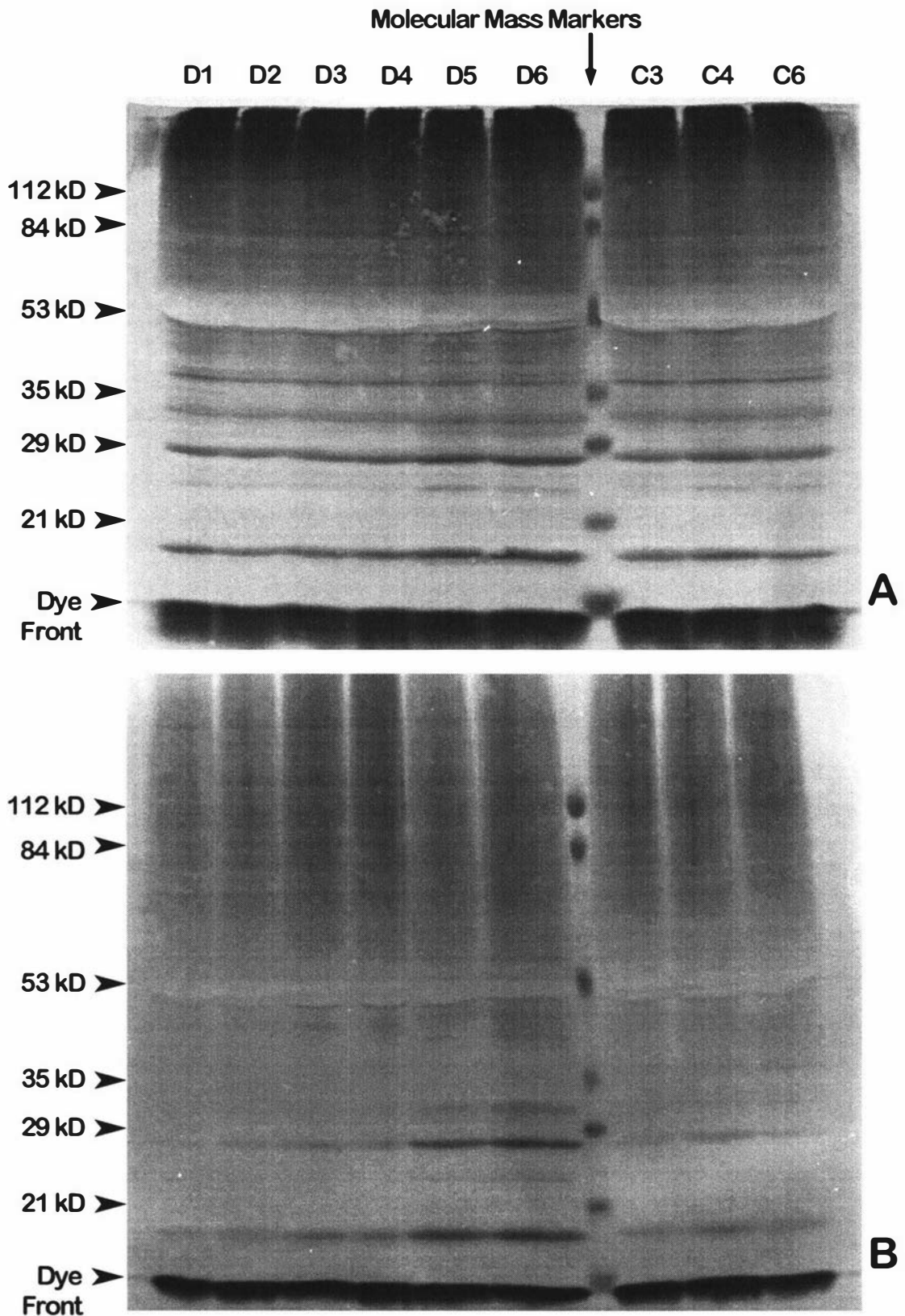


Plate 6.4: Western analysis of protein extracts from the ML of tillers sampled during the dry-down. The proteins were separated by SDS-PAGE through either A: 10% to 20% gradient gel, B: 5% to 15% gradient gel, and were visualised using antisera to ubiquitin. Lanes D1 to C6 (see table 6.1) were loaded with A: 200 μ g/lane, and B: 150 μ g/lane of protein.

Table 6.5: Differences between the pattern of proteins observed in the response group of samples (D5 and D6) with respect to the control group of samples (D1 to D4, and C3 to C6), from the ML (Plate 6.4.). Data are the estimated protein size in kD.

Protein Bands Reduced ¹		Protein Bands Induced	
10% to 20%	5% to 15%	10% to 20%	5% to 15%
46.3	48.9	-	42.0
37	36	30.8	32.5
25.4	-	29.8	30.8
		27.1 m	27.9 m
		23.8	24.2
		17.5 m	18.4 m
		16.9	-

¹ Proteins listed on the same row of the half table were considered likely to be the same protein on both gradient gels (based on the relative intensity of each protein); Key: dash: a comparable protein was not observed on this gel; m: major protein.

6.3.2.2 Ubiquitinated Proteins from the IL

The pattern of ubiquitinated proteins from the extracts of the IL from the control group shared a high degree of similarity, with only one protein (*ca.* 40.5 kD) present at a lighter intensity in samples D1 and D2 (Plate 6.5). Six ubiquitinated proteins were present at a relatively higher intensity in the response group (Plate 6.5), and the intensity of two bands was decreased in the same extracts (Table 6.6). Although the electrophoretic mobility of all the proteins was not the same between the two gradient polyacrylamide gels, no difference in the pattern of ubiquitinated proteins was apparent.

Table 6.6: Differences between the pattern of proteins observed in the response group of samples (D5 and D6) with respect to the control group of samples (D1 to D4, and C3 to C6), from the IL (Plate 6.5). Data are the estimated protein size in kD.

Protein Bands Reduced ¹		Protein Bands Induced	
10% to 20%	5% to 15%	10% to 20%	5% to 15%
48.9	49.4	42.6	42.0
40.7	36.6	32.4	31.8
		27.2 m	27.5 m
		24.5	23.8
		18.4 m	17.7 m
		17.8	17.2

¹ Proteins listed on the same row of the half table were considered likely to be the same protein on both gradient gels (based on the relative intensity of each protein). Key: m: major protein.

6.3.2.2.3 Ubiquitinated Proteins from the LB

The exhaustion of samples 5D and 6D limited the separation of proteins from the LB to the 10% to 20% gradient gel (Plate 6.6).

The pattern of ubiquitinated proteins in each extract shared a high degree of similarity, although slight differences in the intensity of all the bands in different lanes were apparent, which reflected the inequality in the protein content loaded. Only two ubiquitinated proteins (ca. 32.2 and 38.2 kD) were present at lower levels in the response group of samples, when compared with the control group (Table 6.7). The differences in the protein content loaded between lanes may have contributed to the low visibility of these bands in the extracts from samples D5 and D6. However, five other ubiquitinated proteins which were visible in all the samples (Table 6.7) did not appear to be as greatly reduced in lanes D5 and D6, indicating that the 32.2 and 38.2 kD proteins may have been reduced in intensity to a greater extent than can be explained by unequal protein loading alone.

Table 6.7: Differences between the pattern of proteins observed in the response group of samples (D5 and D6) with respect to the control group of samples (D1 to D4, and C3 to C6), from the LB (Plate 6.6). Data are the estimated protein size in kD.

Protein Bands Reduced	Protein Bands Unchanged
10% to 20% gel	10% to 20% gel
32.2	45.6
38.2	42.3
	27.7
	18.0
	14.9

6.3.2.3 Detection of Polypeptides with Sequence Homology to Dehydrin

6.3.2.3.1 Extracts from Tissues Harvested during the Dry-Down Experiment

The protein extracts from the ML of tillers harvested during the dry-down were separated through replicate 10% to 20% polyacrylamide gradient gels. Both gels were subjected to Western analysis, one using anti-dehydrin antiserum, and the other using pre-immune serum. In neither of the Western analyses were any proteins in the sample lanes recognised by the antisera (Data not shown).

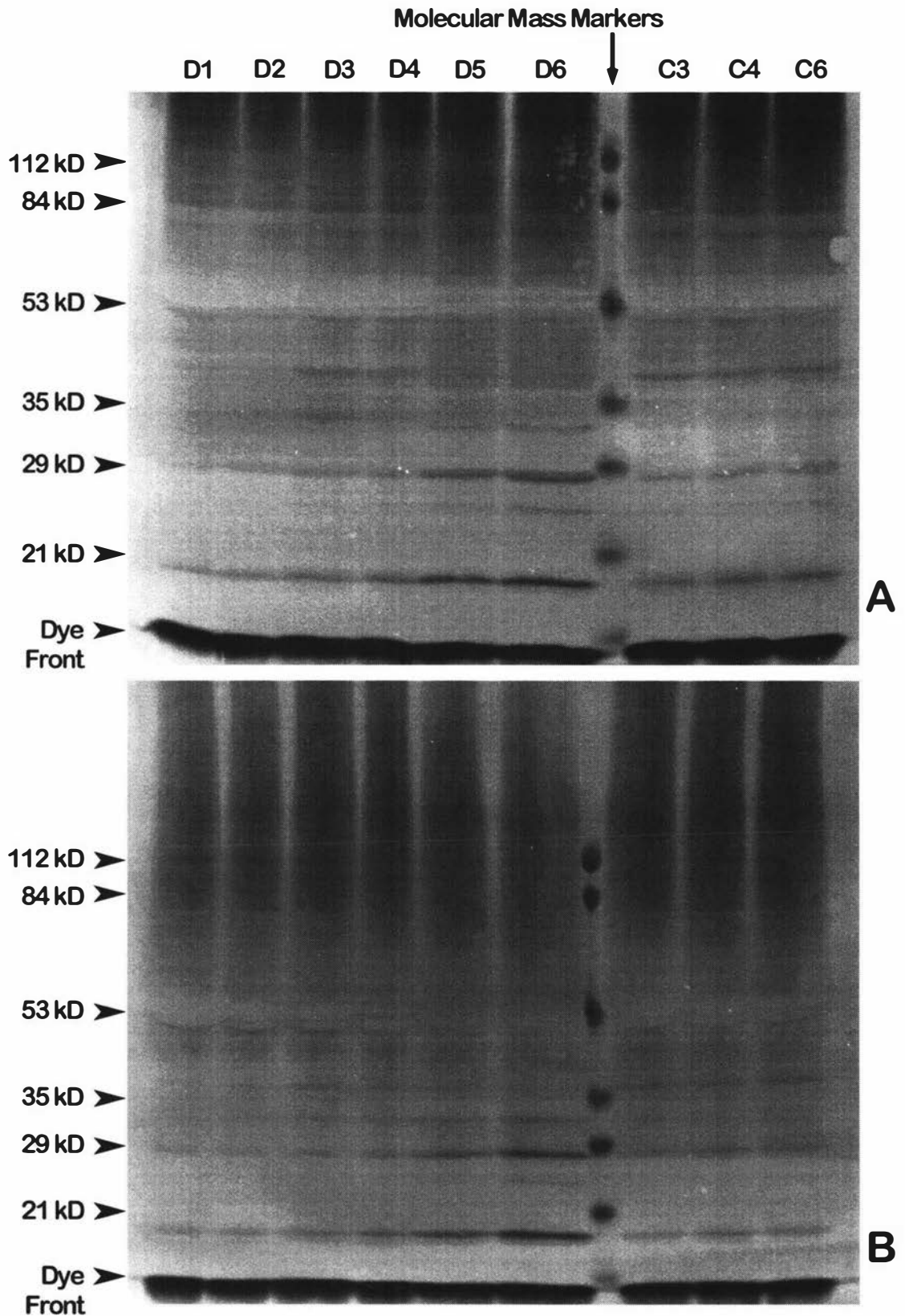


Plate 6.5: Western analysis of protein extracts from the IL of tillers sampled during the dry-down. The proteins were separated by SDS-PAGE through either A: 10% to 20% gradient gel, or B: 5% to 15% gradient gel, and were visualised using antisera to ubiquitin. Lanes D1 to C6 (see table 6.1) were loaded with 150 μ g of protein.

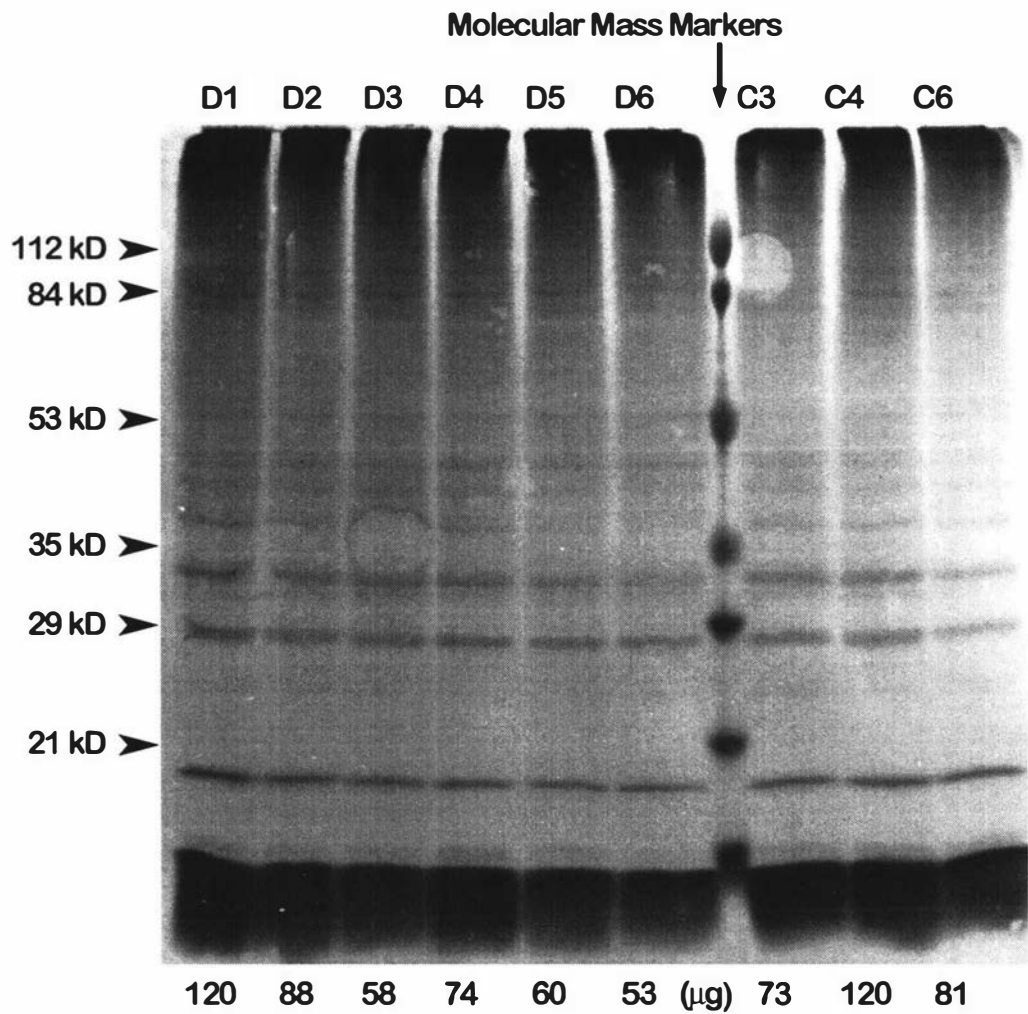


Plate 6.6: Western analysis of protein extracts from the LB of tillers sampled during the dry-down. The proteins on this 10% to 20% gradient gel were separated by SDS-PAGE, and were visualised using antisera to ubiquitin. Lanes D1 to C6 (see table 6.1) were loaded with 50 μ L of extract; the amount of protein (μ g) loaded onto each lane is presented under each column.

Protein extracts from the IL, and LB from tillers harvested during the dry-down were separated through 10% to 20% polyacrylamide gels, both of which were subjected to Western analysis, using antisera to dehydrin. No proteins were detected in the extracts of the IL (Plate 6.7A) or from the LB of water-deficit treatment tillers (Plate 6.7B). However, one protein (*ca.* 34.5 kD), was detected in the LB extracts C3 and C4 (Plate 6.7B). This protein was designated LB-1, for future reference.

A single lane of BSA (6.25 μ g) was introduced onto these gels to act as a positive control (the antisera used was polyclonal, and detected BSA as well as dehydrin). On both of these gels, multiple bands in the BSA sample were detected, including a major protein of *ca.* 70 kD.

6.3.2.3.2 Tissues Sampled from the Bench-Top Dry-Down Experiment

An attempt to induce or increase the content of dehydrin-like proteins within the tissues of *F. novae-zealandiae* was made by subjecting tillers to an overnight dry-down treatment. The bench-top dried tillers were dissected into LB, IL, and ML tissues. Protein extracts from these tissues, and from the LB, IL and ML tissues from the water-sufficient tillers from sample C4, were separated through a 10% to 20% gradient gel, and were subjected to Western analysis using either antisera to dehydrin (Plate 6.8A). Two proteins, *ca.* 36.7 kD (which corresponds to LB-1), and *ca.* 20.5 kD (designated LB-2) were detected in the LB tissue from both the control, and the bench-top dried treatments (Plate 6.8A). However, there was no difference in the intensity of these bands between the two treatments. No further proteins were detected in either the IL or ML tissue from either control or bench-top dried treatments. The BSA standard was also detected on this gel.

In order to determine if the two proteins LB-1 and LB-2 were detected with the pre-immune sera, aliquots of the extracts of the bench-top dried tissues were separated on a further 10% to 20% gradient gel. This gel was subjected to Western analysis using pre-immune sera (Plate 6.8B). No proteins were detected on this gel, in either the sample extracts or in the BSA standard.

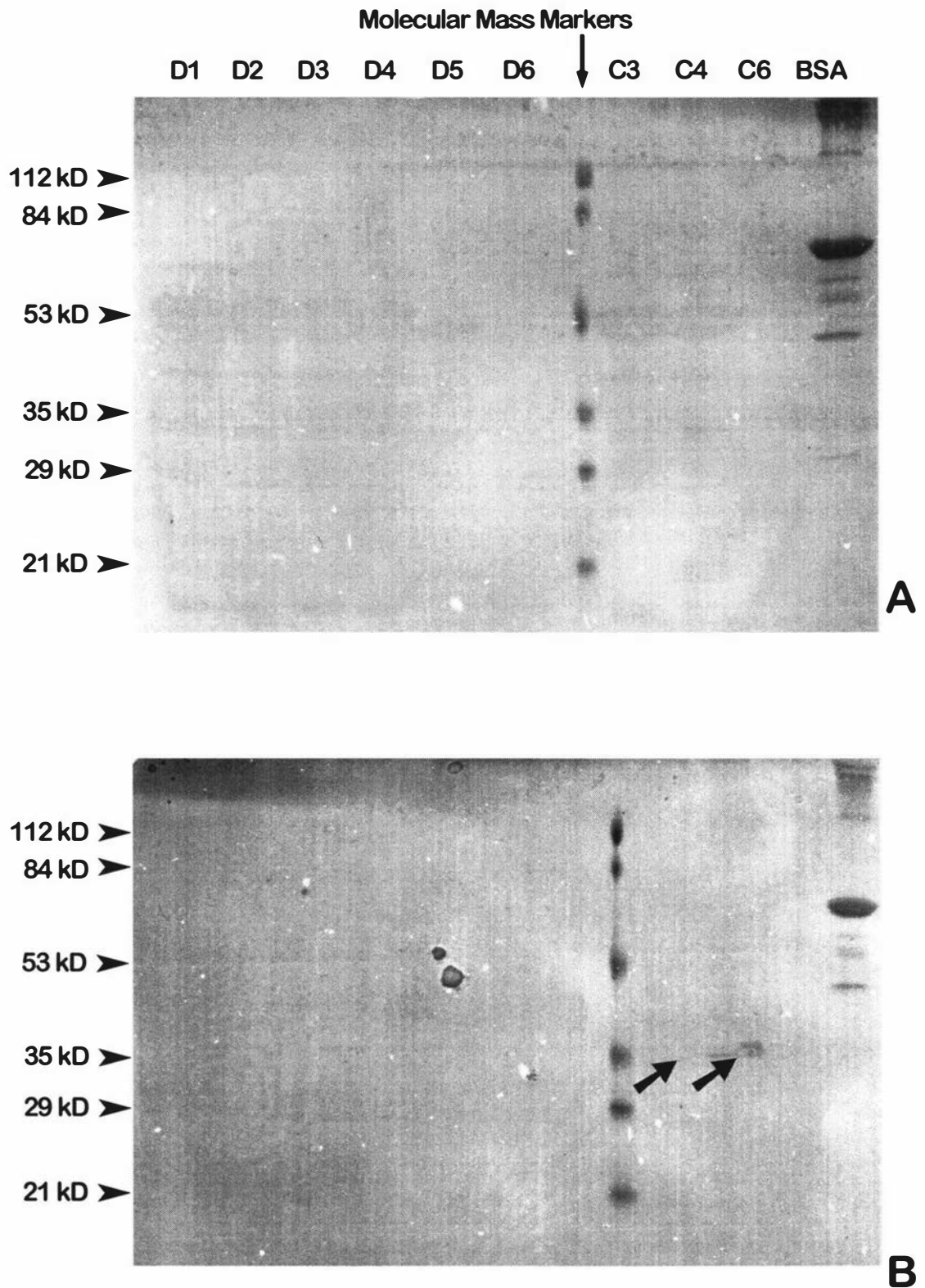


Plate 6.7: Western analysis of protein extracts from A: IL, and B: LB of tillers sampled during the dry-down. The proteins on these 10% to 20% gradient gels were separated by SDS-PAGE, and were visualised using anti-dehydrin immune serum. Lanes D1 to C6 (see table 6.1) were loaded with either A: 150 μ g/lane (except lane D6= 142 μ g/lane) or B: 100 μ g/lane of protein; 6.25 μ g of BSA was loaded onto the lane indicated. The arrows indicate the protein designated LB-1.

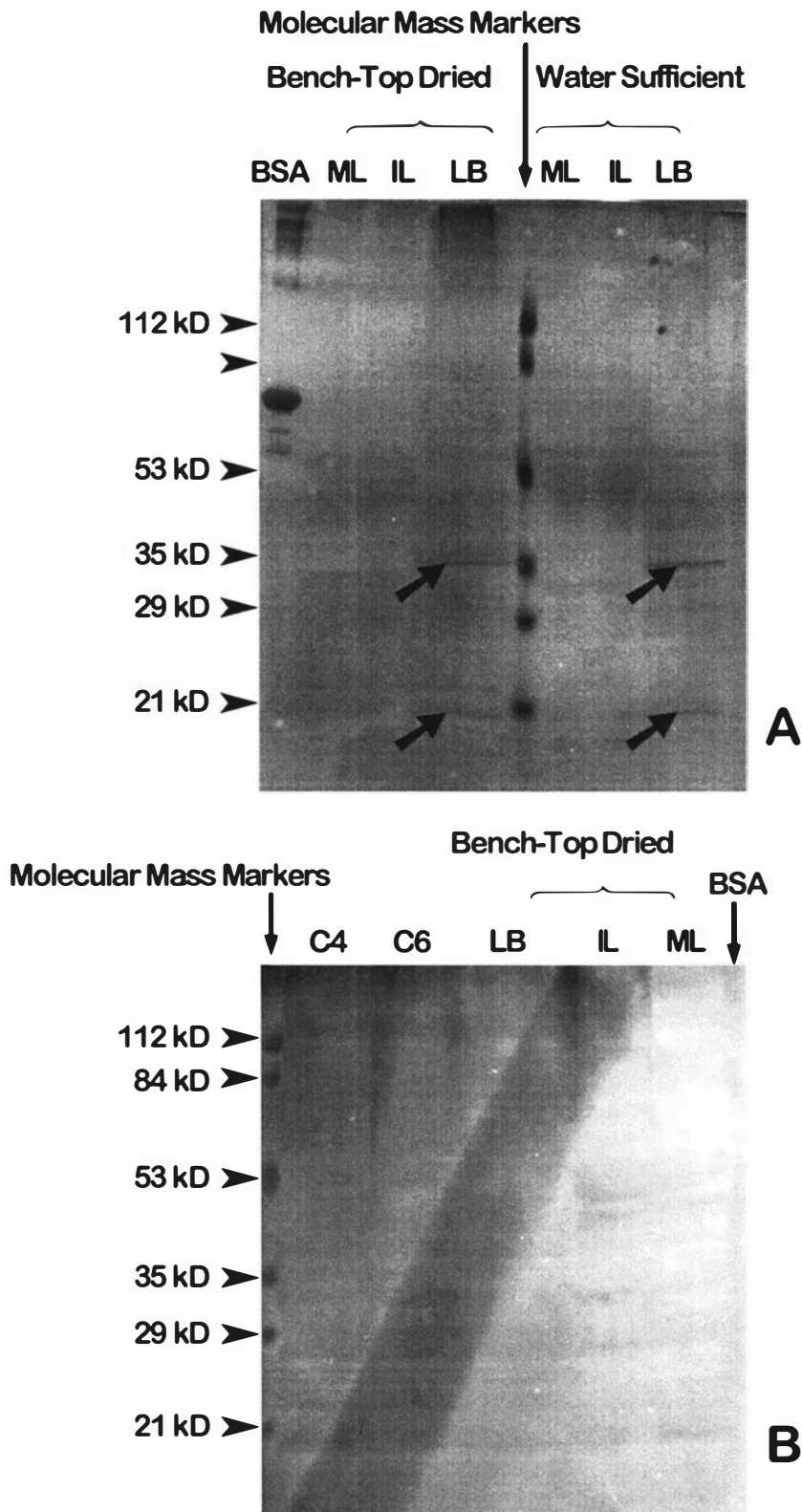


Plate 6.8: Western analysis of protein extracts from *F. novae-zealandiae* tillers that were subjected to a bench-top dry-down treatment. The proteins on these 10% to 20% gradient gels were separated by SDS-PAGE, and were visualised using: A: Anti-dehydrin immune serum or B: Pre-immune serum. The leaf tissue (IL, ML and LB) extracts were loaded with 150 μ g/lane of protein, except for the LB extracts C4 and C6 (on gel B) which were 100 μ g/lane. Arrows indicate proteins LB-1 (top) and LB-2 (bottom arrow). Some distortion of the background intensity of gel B was caused by the tray used for the Western analysis, and should be disregarded.

6.3.2.3.3 Examination of LB-1 and LB-2 as Dehydrin-Like Proteins

No bands were detected in the heat-treated LB extract from harvest C4 (Figure 6.9A), indicating that LB-1 and LB-2 were heat-labile, and hence unlikely to be dehydrins.

6.3.2.3.4 Examination of Seed Proteins

Protein extracts from the seeds of *F. arundinacea*, *F. novae-zealandiae*, and from embryos of *H. vulgare* were separated through a 10% to 20% gradient gel and were subjected to Western analysis using either anti-dehydrin immune or pre-immune sera (Plate 6.9). Samples of seed extract from *F. arundinacea* and *F. novae-zealandiae* which had been heat-treated by boiling for 10 min, and clarified by centrifugation and extracts of the LB from harvest 4C (which contained LB-1 and LB-2) which were also heat treated were included (Plate 6.9).

Many proteins were detected by the immune sera in the seed extracts of all three species, these included four major proteins (ca. 55.3, 28, 24, and 22 kD) in the extract from *H. vulgare*, two (ca. 35.5, 12 kD) in the seed extract from *F. novae-zealandiae*, and four (ca. 36, 32, 25, and 22 kD) in the seed extract from *F. arundinacea*, as well as many proteins detected in relatively minor abundance in all three extracts (Plate 6.9). Many of the proteins from *Festuca spp.* were also detected in the fraction of the extract which was heat-stable, including all the major proteins listed above (Plate 6.9). The major band of the BSA standard was also detected by the immune sera on this gel (Plate 6.9).

Proteins were also detected using the pre-immune serum. One protein (ca. 32 kD) was detected in the seed extract from *F. arundinacea*, two proteins (ca. 13.5 and 12.3 kD) in the seed extract from *F. novae-zealandiae*, and four proteins (ca. 50, 47, 33.5, and 33 kD) were visible in the embryo extract from *H. vulgare* (Plate 6.9). All but the 13.5 kD protein from *F. novae-zealandiae* were present in minor quantities.

Since many of the proteins observed were both heat-stable, and were not visualised by Western analysis using the pre-immune sera (Plate 6.9, Table 6.8), they were likely to share homology with the dehydrin class of proteins (Dr. Close, pers. comm.). A summary of all of the bands visible in the seed extracts is presented in Table 6.8.

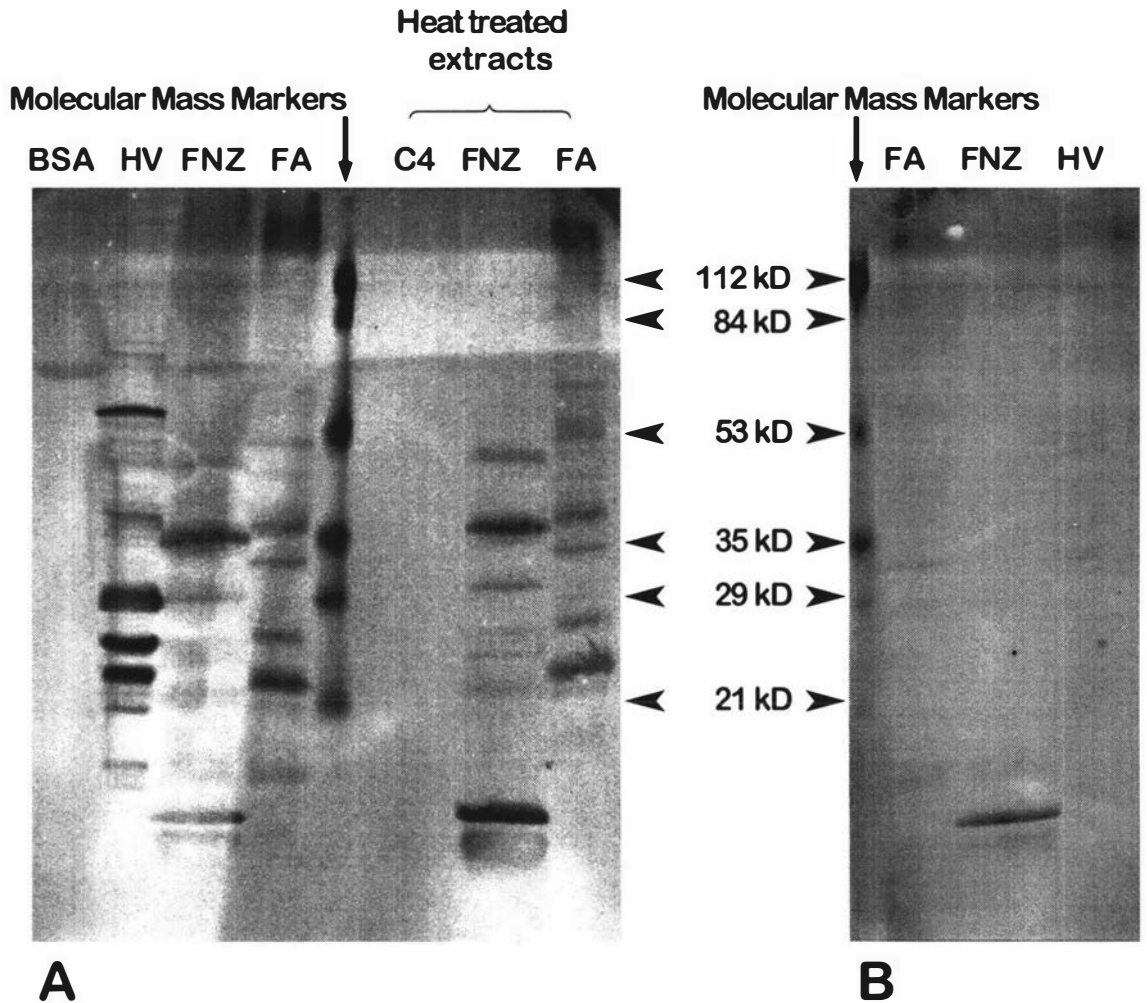


Plate 6.9: Western analysis of protein extracts from seeds of *F. arundinacea* (FA), *F. novae-zealandiae* (FNZ), from embryos of *H. vulgare* (HV) and LB of harvest C4, with or without heat-treatment as indicated. The proteins were separated by SDS-PAGE through a 10% to 20% gradient gel, and were visualised using A: Antisera to dehydrin or B: Pre-immune serum. Each lane of extract contained 150 μ g of protein, except for the heat treated extracts, which were loaded with the same volume as the respective untreated extracts. Some distortion in the background intensity of plate A was caused by the plastic tray used for the Western analysis and should be disregarded.

Table 6.8: Proteins detected by Western analysis using anti-dehydrin immune and pre-immune sera in seed extracts of *Festuca spp.* and in an embryo extract of *H. vulgare*. Those proteins which were heat-stable and were not detected by the pre-immune serum were likely to be dehydrins (Dr. Close, pers. comm.). Data are the estimated protein size in kD.

<i>F. novae-zealandiae</i>	<i>F. arundinacea</i>	<i>H. vulgare</i> ¹
66.9	167.2 h	74.2
46.0 h	78.5 h	70.4
35.5 h m	74.2	55.3 m
28.5 h	60.6 h	52.9
24.5 h	48.6 h	48.6 p
23.1 h	43.0	44.8 p
21.1 h	35.9 h m	38.3
15.6	31.9 h m p	36.8
14.4	24.8 h m	34.2 p
12.0 h m p	21.7 h m	33.4 p
11.1 h p	19.4 h	27.9 m
	17.1 h	24.2 m
	15.1	21.8 m
		20.6
		18.9
		15.1
		13.7

¹ Heat stability of this extract was not tested; Key: h: heat stable; m: major protein; p: detected with pre-immune serum

6.3.3 Southern Analysis of *F. novae-zealandiae* Genome

Nucleic acid was extracted from two samples of approximately 1.0 g of ML tissue from *F. novae-zealandiae*. The concentration of nucleic acid in the DNA extracts was determined by UV-spectroscopy to be *ca.* 2.0 mg/mL in both extracts, which were pooled to give a total yield of about 2.0 mg of nucleic acid. The absorbance ratio A_{260}/A_{280} of the extract was determined to be *ca.* 1.8. The extract contained both high molecular mass DNA and RNA, as evidenced by separation of an aliquot of the extract on a 0.8% agarose minigel (data not shown). Aliquots of the nucleic acid extract were digested with the restriction endonucleases HindIII, BamHI and EcoRI, in the presence of DNAase-free RNAase. The DNA product of each reaction was separated by electrophoresis on a 0.8% agarose gel (Plate 6.10), and then hybridised with a heterologous dehydrin DNA probe (RAB16a), followed by either high (0.2 xSSPE) or low (1 xSSPE) stringency washes before visualisation by phosphorimage detector (Plate 6.11). The nylon membrane subjected to the high stringency wash was found to have a high radioactivity background that masked about half of the membrane (Plate 6.11A). This was most likely due to incomplete mixing of the washing solution, that did not appear to affect all of the nylon membrane. The RAB16a probe hybridised to multiple bands in all three of the restriction endonuclease digests of *F. novae-zealandiae* genetic material. However, this hybridisation was observed only on the nylon membrane that had been subjected to a low stringency wash (Plate 6.11B), indicating a low degree of homology between the probe and the genome of *F. novae-zealandiae*.

Three DNA sequences were detected in the lane containing digested pRAB16 plasmid. These corresponded to circular plasmid, linearised plasmid, and the 450 bp RAB16a insert.

The probe used also hybridised to the λ H+HE size standard markers, even after the high stringency wash (Plate 6.11A), indicating a degree of sequence homology between the probe DNA and DNA from the size standard markers.

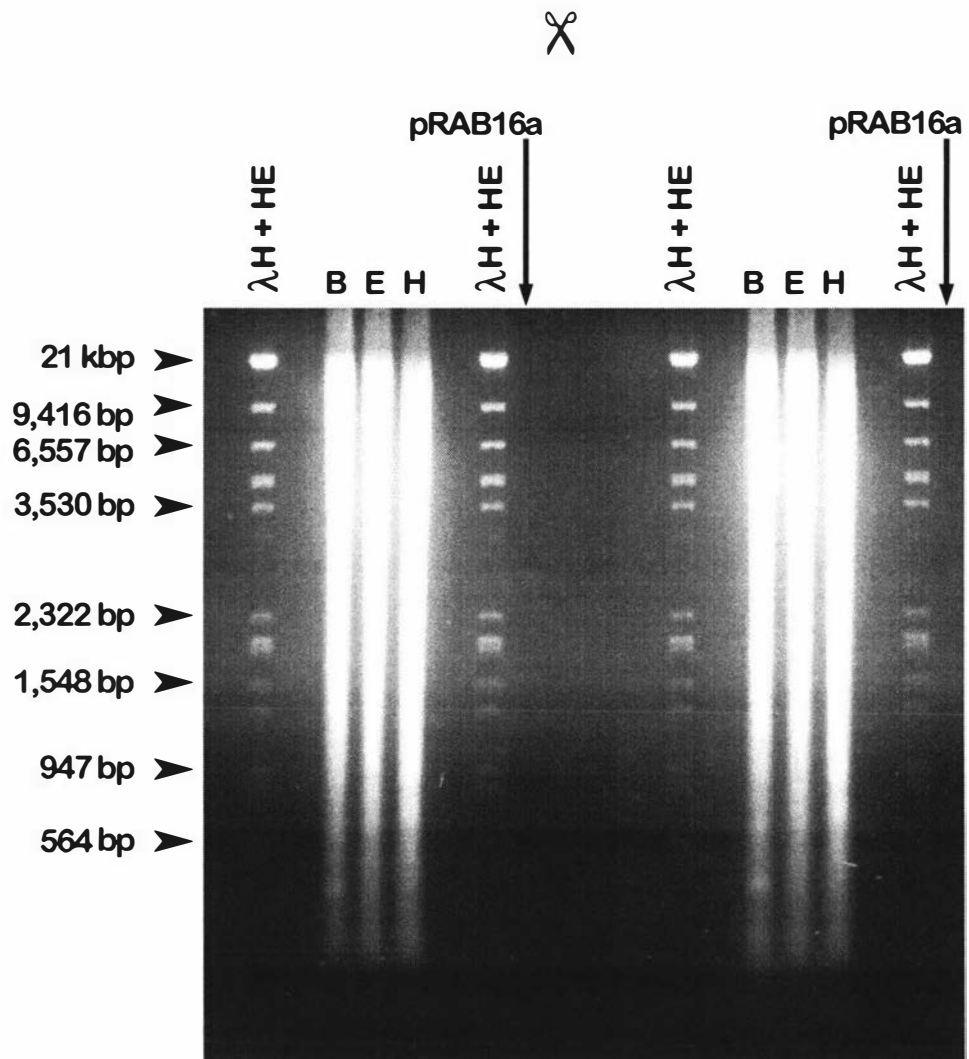


Plate 6.10: Separation of restriction endonuclease digests of genetic material extracted from *F. novae-zealandiae*. The two halves of this 0.8% agarose gel were loaded with aliquots of the same samples. After blotting, the two halves were divided along the dotted line, in preparation for Southern analysis with RAB16a. An equal quantity of genomic DNA (30 μ g) was digested with either BamHI (B), EcoRI (E) or HindIII (H). ca. 0.7 μ g pBluescript plasmid containing the RAB16a insert which was digested with Sall and SacI was loaded into the lanes indicated. λ H+HE: Size standard markers.

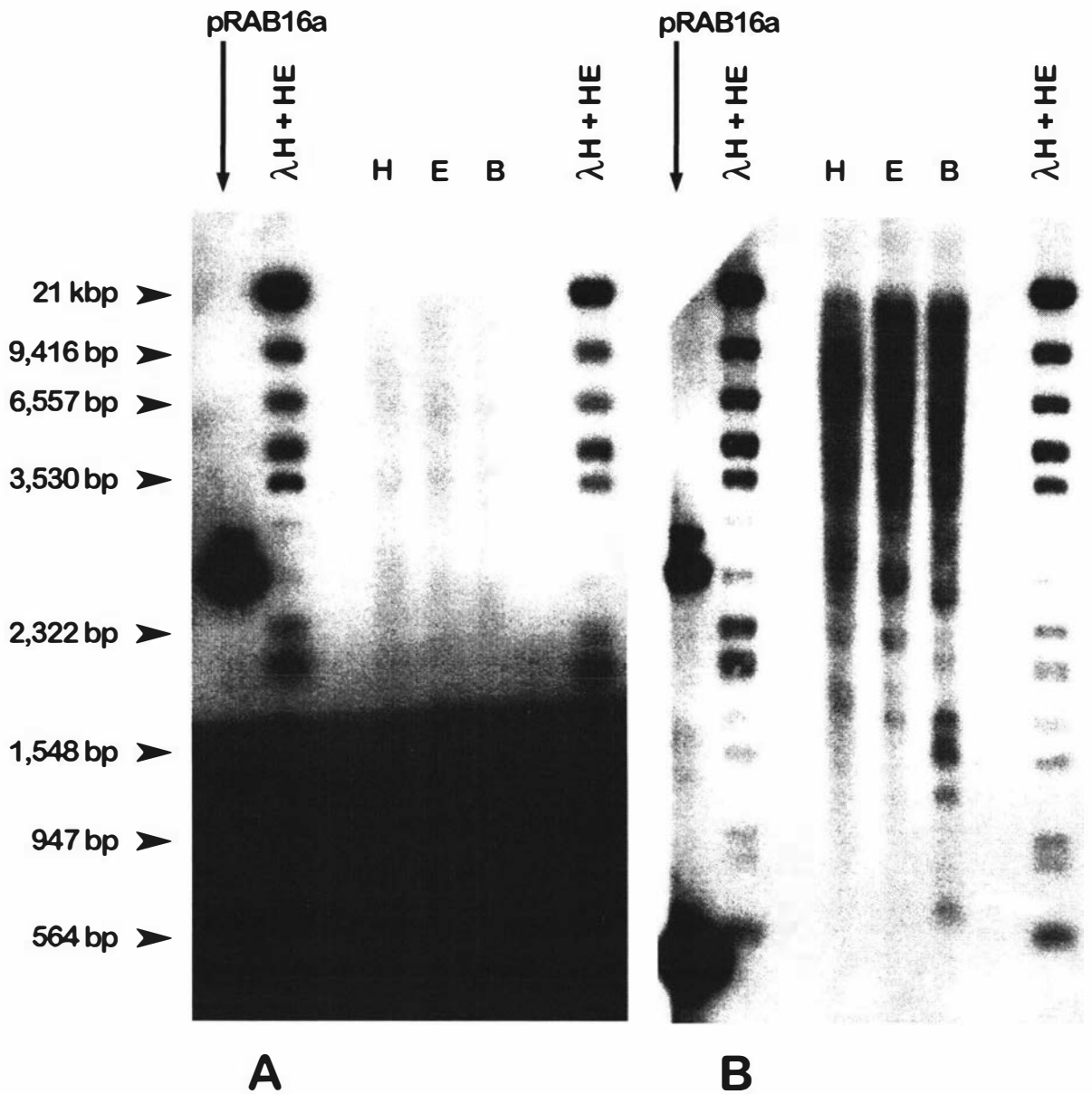


Plate 6.11: Southern hybridisation of RAB16a probe with genetic material from *F. novae-zealandiae*. A: High stringency wash (0.2 xSSPE); B: Low stringency wash (1 xSSPE).

6.4 Discussion

6.4.1 Timing of the Trigger Point

This experiment was conducted to examine an aspect of the hypothesis which was presented in the conclusion of Chapter Four (section 4.4.2.2, page 148). This was to examine the LB and laminae (IL and ML) of *F. novae-zealandiae* for evidence of the onset of a process of controlled leaf turnover in response to water-deficit stress. The experiment was designed so that a large number of tillers were sampled on several dates during a dry-down experiment to supply a sufficient quantity of fresh mass material for protein extractions. The monitoring of several soil and plant growth parameters, including SWC, LER, D_s , N_T as well as leaf proline content was conducted to determine the timing of the trigger point.

All of the pots used for the water-deficit treatment dried down at a similar rate, as indicated by the relatively small standard errors recorded for the SWC measurements (Figure 6.1). The sampling regime was intended to supply tiller harvests at regular intervals, which included samples taken before, during, and after the trigger point had been reached [which was known from previous experiments (Chapters Four and Five) to occur at *ca.* 8% SWC]. This was achieved, with samples taken at *ca.* 30, 21, 14, 9, 5 and 4% SWC (Figure 6.1). With regular watering, the SWC of the control plants was maintained at *ca.* 30% SWC.

In agreement with the previous experiments with *F. novae-zealandiae* (Chapter Five), the measurements of LER during this experiment were not indicative of the timing of the trigger point, since a gradual decline in the LER from about 8 down to 1 $\text{mm}\cdot\text{d}^{-1}$ occurred in the water-deficit treatment plants, but cessation of growth was not observed (Figure 6.2). However, the tiller scorching measurements appeared to indicate the trigger point, which occurred after 23 days for both the N_T (Figure 6.3), and for the D_s of the IL and 2ML (Figure 6.4). Consistent with the previous experiment, the D_s of the ML did not increase above that of the controls until later in the experiment (Figure 6.4).

Changes in the RWC and H_L in response to the water-deficit treatment were not observed during this experiment. In one respect, this was consistent with the previous experiment (Chapter Five), where changes in these two parameters was not coincident with the trigger point. However, changes in the RWC and the H_L were observed some

time after the trigger point in the results presented in Chapter Five. It was possible that the present experiment was not conducted for long enough for changes in the RWC and H_L to be observed. However, this was unlikely to have been the case, since the last sample was taken 11 days after the proposed trigger point, when the SWC had declined to less than 4% (Table 6.2). In addition, the D_5 of all leaf types had by the end of this experiment responded to a similar or greater degree to that observed in Chapter Five (*ca.* 7, 10 and 100% for the IL, ML and 2ML, respectively) at the time when changes in the RWC and H_L were first observed in the previous experiment (43 days). Hence, although changes in the appearance and growth of the leaves were observed by the end of the experiment, hydraulic indications of these changes were not observed.

The higher level of proline in the LB when compared to the IL and ML, and the marginal increase in proline concentration in the LB of water-deficit treatment tillers was consistent with Chapter Five, although the accumulation of proline to the relatively high levels (28 mg/gDW) observed in the previous experiment were not repeated in the current experiment. The greatest fold increase in proline content (*ca.* 5-fold) was in the laminae (IL and ML) tissues, but the concentrations of proline in these tissues did not exceed that of the LB. As discussed in Chapter Five, the highest proline concentrations in the previous experiment were not observed until the RWC had begun to change (after 43 days), and may be indicative of the disruption of cellular compartmentalisation due to water-deficit induced injury (Stewart and Bogges, 1978). Hence the relatively lower maximum proline contents observed in the current experiment, in addition to the absence of a decline in the leaf hydraulic data, suggested that this experiment may not have been conducted for a sufficient period for tillers to be injured by the water-deficit treatment. The proline accumulation was first recorded after 23 days in the IL and ML, which also confirms the timing of the trigger point in this experiment.

The data gathered indicated that the timing of the trigger point (about 23 days) occurred after the SWC had declined to *ca.* 9%. Hence, the tillers harvested for samples D1 to D3 (Table 6.2) were taken before the trigger point, sample D4 was close to the trigger point, and the last two samples (D5 and D6) were taken after adaption to the water-deficit treatment had begun.

6.4.2 Changes in the Protein Complement

Examination of the protein content of tissues from these samples was carried out to gather evidence regarding the controlled turnover of these tissues. Investigations into the nature or content of proteins in *F. novae-zealandiae* have not been previously reported, with the exception of one ecological study (Lord 1993). In Lord's study, an isozyme electrophoresis survey of five enzymes in crude extracts was used as a tool for studying clonal fragmentation as a means of reproduction in this species.

6.4.2.1 Crude Protein Content

The crude protein content of these tissues was found to be dependent on the maturity of the tissue, where the LB contained a lower protein content per unit fresh weight than the laminae (IL and ML). The protein content of the leaves decreased in all tissues after the trigger point (23 days; Figure 6.8), indicating that protein turnover was increased by the water-deficit treatment. Reductions in protein synthesis and protein content are generally thought to occur in response to drought (Hsaio, 1973; Rhodes, 1987), and severe reductions in protein synthesis associated with polysome disassembly may occur in response to rapidly induced drought stress (Hanson and Hitz, 1982). However, reductions in protein synthetic capacity in response to longer-term stress are often relatively slight (Hanson and Hitz, 1982), and may be associated with alterations in the protein complement (Hanson and Hitz, 1982; Bray, 1988). Certainly, many proteins are known to be induced by water-deficit stress, such as those in *O. sativa* (Mundy and Chua, 1988), *L. esculentum* (Bray, 1988), *H. vulgare* and *Z. mays* (Close *et al.*, 1989) and *P. sativum* (Robertson and Chandler, 1992), which suggests that protein synthesis continues during water-deficit stress. Hence the reduction in crude protein content which coincided with the trigger point (Figure 6.8) may reflect alterations in the protein complement of *F. novae-zealandiae* in response to the water-deficit treatment. In order to examine this more closely, SDS-PAGE was used to separate proteins in each of the extracts.

6.4.2.2 Visualisation of Proteins with Coomassie Blue

Separation of proteins followed by visualisation by Coomassie Blue stain was able to visualise about 50 of the most abundant proteins in the ML and IL, and 35 in the LB. This difference may be attributable to the relatively lower protein concentration of the

LB extracts, which meant that smaller quantities of protein from the LB were separated. None of the proteins can be identified from these gels. However, two of the major proteins, with comparative molecular masses of 55 and 17 kD, could be tentatively identified as subunits of the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO, or fraction I protein), since this protein has been reported to occur as the most abundant protein in leaf extracts, and is comprised of large and small subunits of 54-60 kD and 12-16 kD, respectively (Conn *et al.*, 1987). These proteins were reduced in abundance in the LB, which correlates with the lack of green pigmentation (red pigments, probably anthocyanin(s), were present in some cases; Plate 6.3).

The Coomassie-Blue stained gels indicated that the relative content of several proteins changed in response to the water-deficit treatment, since the pattern of protein banding in the post-trigger point samples (D5 and D6) was different to those of the pre-trigger point samples (D1 to D4), or to the controls. Although the abundance of some proteins on the SDS-PAGE gels appeared to increase, many more proteins decreased in abundance after the trigger point (Tables 6.2 and 6.4). Moreover, this appeared to be tissue dependent, since it was observed in the laminae (IL and ML), but not in the LB (Table 6.4). In the IL and ML, the relative abundance of larger proteins (>55 kD), including the larger subunit of RuBisCO, appeared to decrease while the abundance of small polypeptides increased, which supports the hypothesis that protein degradation had occurred in response to the water-deficit treatment. Note that the relative abundance of some of the less abundant proteins may have increased because the mass of some proteins, especially the fraction I protein decreased in the post-trigger point samples, but an equal quantity (150 µg) of protein was still separated in each lane.

If proteins were degraded in the leaves of *F. novae-zealandiae* in response to the water-deficit treatment, then proteases must be involved. Since inhibitors of proteolysis (such as PMSF) were not included in the extraction buffer, it is possible that some of the degradation had occurred during the extraction procedure. However, the fact that certain proteins appeared to increase in relative abundance after the trigger point argues that *in vitro* degradation of proteins was minimal, or at least did not extend to all proteins. To conclusively show the possibility that the smaller polypeptides observed were degradation products of the larger proteins, the sequences of degraded

polypeptide fragments must be matched with sequences of the original, intact proteins. This was not possible in the present study where unknown proteins were separated on the basis of size alone. Evidence that proteases were induced in response to the water-deficit treatment could be sought by protease activity assays, such as colourimetric assays which utilise the degradation of azo-labelled protein substrate (for endopeptidases), or specific aminopeptidase or carboxypeptidase substrates (described in Sarath, 1990).

The use of SDS-PAGE, followed by Coomassie blue stain, allowed only a few dozen of the innumerable proteins in a plant tissue to be observed. The effectiveness of this technique could be enhanced by the use of two-dimensional SDS-PAGE, where proteins are separated both by size, and by isoelectric point. However, this technique would still display proteins on the basis of abundance. Western analysis provides a means of observing certain proteins present in relatively low abundance, without purification of extracts. The same extracts used for the Coomassie Blue stained SDS-PAGE gels were visualised after SDS-PAGE by Western analysis, using antisera to two classes of proteins: ubiquitinated proteins, and dehydrins.

6.4.2.3 Ubiquitin-Labelled Proteins

Several proteins were visualised on Western blots using monoclonal antibodies that were raised against bovine ubiquitin. Although these proteins were immunologically related to the mammalian protein, little idiotypic difference between the animal and plant ubiquitin is likely to exist, since only three amino acid substitutions of the 76 amino acid polypeptide exist between animal and plant forms (Vierstra *et al.*, 1986). Indeed, free ubiquitin from *F. novae-zealandiae* leaf extracts was visualised on all of the Western blots as a relatively large quantity of low molecular mass protein (<14 kD). Because the free ubiquitin appeared to migrate with the dye-front on the SDS-PAGE gels, an accurate determination of its relative molecular mass (which is *ca.* 8.5 kD; Ferguson *et al.*, 1990) could not be made. Separation of free ubiquitin might be achieved if SDS-PAGE gels of a higher percentage polyacrylamide were used.

As well as free ubiquitin, many larger proteins (from 14 to >>112 kD) were observed

on all the Western blots, which were likely to be mono-, or poly-ubiquitinated proteins. Distinct protein bands were apparent below *ca.* 40 kD, while an almost continuous 'smear' of bands occurred above this size. A similar banding pattern has been reported, where ubiquitinated proteins present in crude protein extracts from wheat roots occurred in distinct bands below *ca.* 40 kD, and with a smear of bands from *ca.* 40 to *ca.* 180 kD (Ferguson *et al.*, 1990). None of these ubiquitinated proteins could be identified from the information gained from these experiments. However, the proteins comprising the continuous smear of bands above *ca.* 40 kD were likely to be poly-ubiquitinated, since the conjugation of variable numbers of ubiquitin units to many different sized proteins may account for the large number of relatively minor bands. Conversely the distinct bands were likely to be mono-ubiquitinated proteins. The use of Western analysis using antibodies specific to poly-ubiquitinated proteins, could be used to elucidate the different ubiquitination states.

The large subunit of RuBisCO was not detected by the anti-ubiquitin antibodies, but nevertheless was conspicuous in the IL and ML samples (*ca.* 55 kD; Plates 6.4 and 6.6). Since the content of RuBisCO in the laminae was observed to decrease after the trigger point (Plates 6.1 and 6.2), this data suggests that RuBisCO is not degraded via ubiquitin-mediated proteolysis in *F. novae-zealandiae*. This is consistent with current opinion that the ubiquitin-proteasome system is involved in the degradation of the bulk of short-lived or abnormal proteins (reviewed in Hershko and Ciechanover, 1992), but that long-lived proteins, such as RuBisCO, may be degraded by the proteasome complex without the requirement for ubiquitin (Ciechanover, 1994). However, this contrasts with reports of ubiquitin-stimulated breakdown of RuBisCO in isolated chloroplasts of senescing *Avena sativa* (oat) leaves (Veierskov and Ferguson, 1991), and in chloroplast membranes of mature *Vicia faba* (bean) leaves (Schulz *et al.*, 1993). However, in the present study, verification that RuBisCO is degraded *via* ubiquitin mediated proteolysis *in vivo*, has not been shown.

Ubiquitin-mediated protein turnover is an on-going process in any eucaryotic cell (Hershko and Ciechanover, 1992). Hence many ubiquitinated proteins, such as cyclins, that control the cell division cycle, or the regulatory photoreceptor phytochrome, which are both known to be turned over by the ubiquitin system (Ciechanover, 1994),

should be visible in protein extracts from non-stressed plants. However, changes in the size-species of proteins being ubiquitinated may indicate metabolic adaptation to an applied stress, as exemplified by the response of wheat roots to high temperature stress, where the relative abundance of ubiquitinated proteins was dependent on the level of stress applied (Ferguson *et al.*, 1990). This is particularly relevant for the mono-ubiquitinated proteins, which are more stable than polyubiquitinated proteins, and may possess regulatory activity (Hershko and Ciechanover, 1992; Schulz *et al.*, 1993). In this experiment, many of the distinct protein bands appeared to increase in intensity after the trigger point (samples D5 and D6), in the IL and ML (Plates 6.4 and 6.5). Hence, the process of ubiquitin-mediated turnover of proteins was affected by the water-deficit treatment. However, similar changes in the complement or content of ubiquitin-conjugated proteins were not observed in the LB (Plate 6.6).

Changes in the complement of ubiquitinated proteins, especially the mono-ubiquitinated moieties, and observations of the degradation of proteins such as Ru-BisCO in post-trigger point laminae of *F. novae-zealandiae*, suggests that this species turns over protein at an enhanced rate in response to the water-deficit treatment. Moreover, the observed responses were tissue-specific, with modification of the protein complement and changes in the mono-ubiquitinated proteins observed mainly in the IL and ML, and not in the LB. This tissue-specificity of protein turnover supports one aspect of the hypothesis presented in Chapter Four, that in response to water-deficit, controlled turnover of existing leaves occurs.

6.4.2.4 Dehydrins

Dehydrin-class polypeptides have been reported to occur in embryos of a diverse range of species, such as *P. sativum*; (Robertson and Chandler, 1992), *Ginkgo bilboa* (Maiden-hair Tree), *Pinus edulis* (pinon pine), *Gossypium hirsutum*, *Allium cepa* (onion), *Cucumis sativus* (cucumber), *L. esculentum* [Close *et al.*, 1993], and including several members of the graminaceae, such as *Z. mays*, *H. vulgare* (Close *et al.*, 1989), and *O. sativa*; (Bradford and Chandler, 1992). The occurrence of dehydrins in embryos of *H. vulgare* (Close *et al.*, 1993) was confirmed in the present study by western analysis of a protein extract of this tissue using monoclonal antibodies specific to the consensus amino acid sequence (KIKEKLPG) of dehydrins. Four major proteins were detected (*ca.* 55, 28, 24

and 22 kD), as well as many proteins in lower quantities which could be intact proteins or degradation products of other proteins. This was consistent with Close *et al.* (1993), who reported that about ten size species, including two predominant proteins (*ca.* 25 and 60 kD) were detected in various cultivars of *H. vulgare*. Several proteins were detected in seed extracts from *Festuca spp.*, using the same immune-serum. Many of these were heat stable (a trait that appears to be common to the dehydrin-class polypeptides; Close *et al.*, 1993), and were not detected by pre-immune serum, which suggests that these proteins were likely to be dehydrins (Dr. Close, pers. comm.). Note that only one population of seed (from either cultivar "Grasslands Roa", and from the Cass region of North Canterbury, for *F. arundinacea* and *F. novae-zealandiae*, respectively) was used for the detection of dehydrins in *Festuca spp.*. With regard to the diverse range of dehydrins found in *H. vulgare* cultivars (Close *et al.*, 1993), many more dehydrins may exist in seeds from other populations of *Festuca spp.*. The detection of dehydrins in the seeds of *F. arundinacea* and *F. novae-zealandiae* has not been previously reported. Hence this finding increases the known species distribution of these proteins.

In addition to the localisation of dehydrins in embryos, the presence of dehydrins in leaves can also be induced by the application of water-deficit stress. Such accumulation of dehydrin has been shown for seedlings of *H. vulgare* and *Z. mays* (Close *et al.*, 1989), *O. sativa* (Mundy and Chua, 1988), *T. aestivum* (Close and Chandler, 1990) and *Pisum sativum* (Robertson and Chandler, 1992). In *H. vulgare*, many of the dehydrins accumulating in leaves in response to water-deficit stress were similar in size to those located in the embryo. This suggests that the same proteins may occur in both tissues, although no constant pattern of gene regulation exists, since regulation may depend on the cultivar and tissue, and is under developmental and environmental control (Close *et al.*, 1993). When protein extracts from tissues of *F. novae-zealandiae* were subjected to Western analysis using anti-dehydrin antibodies, no proteins were observed in the extracts from the IL or ML, regardless of the treatment, or from water-deficit treatment LB tissue (Figures 6.6 and 6.7). In addition, subjecting tillers of *F. novae-zealandiae* to an increased level of stress via a bench-top dry-down did not induce the accumulation of dehydrin-like proteins (Plate 6.8). This suggests that the dehydrins located in the seed of *F. novae-zealandiae* are seed-specific, and that the developmental regulation may not be under the same control mechanisms as the dehydrins from the other species listed

above. However, tissue age may also be an important factor in the expression of dehydrins, and this may explain, in part, the difference between the published results above (which involved the use of seedlings of each respective species), and the results from the current experiments (where mature plants were used). It is possible that the ability to synthesise dehydrins is a property of seeds which is maintained in young seedlings as an early defence against water-deficit, but is lost as the seedlings mature. Hence, the examination of seedlings of *F. novae-zealandiae* for dehydrins may be worthwhile. Since whole seeds of *F. novae-zealandiae* were used as the source of dehydrins, an investigation into the compartmentalisation of dehydrins into the embryo, aleurone layer and endosperm may also yield information on the expression pattern of dehydrins in this species.

Two proteins were visualised in extracts from the LB of water-sufficient tillers, termed LB-1 (Plate 6.7) and LB-2 (Plate 6.8). However, these two protein bands were found to be heat-labile (Plate 6.9), indicating that they were probably not dehydrins (Close *et al.*, 1993). These proteins were not detected by Western analysis using pre-immune sera as the primary antibody source, and hence probably shared some homology to the components of either BSA fraction V (sigma), or to Freund's complete adjuvant. In any case, these two proteins were not likely to have been related to the water-deficit treatment.

6.4.2.5 Genetic Analysis of Dehydrins

The proteins visualised in the seed extracts (Plate 6.9) were immunologically related to dehydrin. Confirmation that these proteins were indeed dehydrins was sought by analysis of the genetic material of *F. novae-zealandiae* by Southern hybridisation with a gene known to encode a dehydrin. Extraction or analysis of the genetic material of *F. novae-zealandiae* has not been previously reported, with the exception of one chromosome count, which was hexaploid (Beuzenberg and Hair, 1983).

The DNA sequence used to probe the restricted fragments of *F. novae-zealandiae* DNA was a 450 bp insert from RAB16a, which encodes a dehydrin from *O. sativa* (Mundy *et al.*, 1990). This insert contained code for the amino acid sequence DEYGNP, and for two repeats of the amino acid sequence KIKEKLPG, which are common to dehydrin

genes from *O. sativa* (Mundy and Chua, 1988), *H. vulgare* and *Z. mays* (Close *et al.*, 1989), *L. esculentum* (Godoy *et al.*, 1990), *Craterostigma plantagineum* (Piatkowski *et al.*, 1990), *G. hirsutum* (Galau and Close, 1992) and *P. sativum* (Robertson and Chandler, 1992), and was contained within the polypeptide CTGEKKGIMDKIKEKLPGQH against which the antisera used for the Western analysis was raised.

Some homology between the dehydrin DNA sequence and the genetic material from *F. novae-zealandiae* was found, as evidenced by the binding of RAB16a probe to genomic DNA, after a "low-stringency" wash (1 xSSPE, Plate 6.11). Several bands were observed in each restriction endonuclease digest of genomic DNA from *F. novae-zealandiae*, which suggests that several copies of the dehydrin gene may occur in this species. The complexity of the pattern may in part be due to the hexaploid nature of *F. novae-zealandiae*. However, the binding of probe DNA to genome DNA was reversed by a 0.2% SSPE wash, which indicated a relatively low degree of homology. This may be due to the use of a heterologous probe, which apart from the conserved base-pair regions (e.g. DEYGNP and KIKEKLPG), may have other regions of low-homology to the dehydrin genes from *F. novae-zealandiae*. Cloning the dehydrin gene(s) from this species, and then comparing the sequence(s) with those published from *O. sativa* would be required to confirm this. An insert from a dehydrin gene cloned from *F. novae-zealandiae* might also be used as a homologous probe for use with Southern hybridisations, which should provide a better degree of hybridisation. From the use of such a probe, a better indication of the number of dehydrin loci in the genome of *F. novae-zealandiae* might also be gained. The use of a homologous probe to dehydrin also raises the possibility of conducting Northern analyses to investigate the transcription of dehydrin genes, and the fate of the respective mRNA message from seeds, seedlings or mature plants of *F. novae-zealandiae* in response to water-deficit stress.

Chapter Seven

Conclusions

7.1 Experimental Conditions

The experiments with *F. arundinacea* (Chapter Three, page 66) demonstrated that the methods used for applying water-deficit and for measuring the metabolic and physiological markers were suitable to show that the plants had responded to the water-deficit treatment. In these experiments, a common element to the timing of the induction of the markers was observed. This supported the concept of a trigger point, that is, that there is a point during a progressive dry-down when the supply of water to the plant becomes insufficient to meet the plants requirements. At this time, the water-deficit causes a host of responses. For *F. arundinacea*, these events included the cessation of LER and the accumulation of the phytohormone ABA, and the osmoprotectants glycine-betaine and proline. These events occurred when the SWC had declined to about 8%. It was not clear if these events conferred an advantage to the plant or if they were a result of water-deficit induced injury. However, the usefulness of LER, ABA, proline and glycine-betaine as suitable internal marker compounds to demonstrate the response of plant metabolism to the water-deficit treatment was confirmed.

7.2 Original Aspects of the Study of Water-Deficit Stress Response by *F. novae-zealandiae*

To the knowledge of the current author, no studies of this species with respect to metabolite composition or drought tolerance adaptations have been reported, although several studies of the ecology, and leaf and root structure of *F. novae-zealandiae* have been conducted [Connor, 1960; Lord 1992 (and references within); Wardle, 1991]. Hence the main scope of this study, which included the measurement of ABA, proline and glycine-betaine concentrations in response to water-deficit treatment, extraction and Southern analysis of the genetic material of *F. novae-zealandiae*, and Western analysis of soluble proteins using anti-ubiquitin and anti-dehydrin MAb, was original research.

7.3 Morphological Adaptions of *F. novae-zealandiae*

An examination of the leaf structure of *F. novae-zealandiae* revealed several morphological adaptations which could assist the drought stress-tolerance of this species (section 4.3.1, page 96). Such morphological adaptations were expected to be found, since *F. novae-zealandiae* has evolved to survive in the lowest precipitation regions in New Zealand (Newsome 1987). It was found that upon the water-deficit treatment, this species could survive at least 3-4 times longer than did the closely related *F. arundinacea*. In addition, cessation of leaf elongation was not observed until the last tillers on the plant had succumbed to the water-deficit stress. This was unexpected since the rate of growth is considered to be a sensitive indicator of stress in many plants (Hsiao, 1973). These abilities may be a consequence of the morphology of the leaves, which may make *F. novae-zealandiae* very efficient at preventing water loss to the environment. Indeed, water loss from the leaves was not observed until the advanced stages of the dry-down (section 5.3.2.6, page 176). It was interesting to find that the native tussock does not possess an extensive root system, which could limit water harvest from the soil. Indeed, the continuation of root growth (coupled with a reduction in LER) been reported to be general responses of plants to drought stress (Hsiao, 1973). This implies that a larger root system would be beneficial to the plant in such circumstances. However, since *F. novae-zealandiae* continually turns over tillers as part of normal growth, the shallow root system may be an adaptation to reduce the loss of large quantities of root tissue during this process. These morphological features, coupled to the modular structure of the tussock which allows the size of the plant to change in response to environmental limitations, means that *F. novae-zealandiae* has effective defences employed long before any water-deficit occurs.

Mechanisms of drought avoidance may also be important adaptive traits in *F. novae-zealandiae*. In particular, this species may undergo most of its annual growth during the Spring (when water-deficit stress is less likely to occur), and remain relatively dormant for the remainder of the year. This was exemplified by differences in proline and ABA accumulation of the ML observed between the various drydown experiments. In Chapter Four, two experiments were described; one was conducted during Summer, and the other during Autumn. The results from these two experiments were inconclusive with respect to whether *F. novae-zealandiae* adapted metabolically to the applied

stress. Although protein changes were observed during the experiment described in Chapter Six, the proline data collected from this (Summer) drydown was relatively unresponsive. The remaining drydown experiment (described in Chapter Five) was conducted during Spring, and relatively clear responses to water-deficit stress were observed. Hence, active responses to water-deficit by *F. novae-zealandiae* may occur mainly during Spring, which is the period of the most active tillering and leaf elongation in this species (Lord, 1992). Because of the apparent seasonal differences in the water-deficit response, a further dry-down experiment should be conducted during the Spring to verify the changes observed in metabolite concentrations and protein profiles in response to water-deficit stress.

From a practical viewpoint, this enhanced ability to tolerate water-deficit also meant that the pot system used for imposing water-deficit on *F. arundinacea* was not convenient for use with *F. novae-zealandiae* (see Chapter Four). Hence several changes to the pot system were made to encourage faster dry-downs (see Chapters Five and Six).

7.4 Occurrence of a Trigger Point

In response to the water-deficit treatment, the number of tillers and leaves of *F. novae-zealandiae* declined, and leaf-tip scorching increased. This species also accumulated the ABA and proline. Changes in the protein content of the leaves were also observed. These included a reduction in soluble protein content, which was mainly attributed to a reduction in high molecular mass (>50 kD) proteins, including the major subunit of RuBisCO. Changes in ubiquitin mediated protein turnover were also observed, which suggests that an element of metabolic control existed in the protein degradation response.

The point of induction of the above changes was usually found to occur around a common time, which supports the suggestion that the trigger-point type of water-deficit response regulation found in *F. arundinacea* also occurs in the native species.

7.5 Tissue Specificity of Water-Deficit Stress Response

Many of the response to the water-deficit treatment were tissue dependent. The loss of leaves, and the increase in leaf-tip scorching affected the 2ML, and both the 2ML and IL, respectively, to a greater extent than the ML. Probably, this is because the ML is in the middle stages of the developmental cycle of leaf turnover, and hence was not as susceptible as the younger IL, nor as senescent as the 2ML.

Proline was present in the LB region at higher contents than the laminae (IL and ML). In fact, in some cases the levels of proline in the LB of water-sufficient *F. novae-zealandiae* were more than those found in the bench top-dried *F. arundinacea* (Figure 5.2, page 163). Proline also increased to higher levels in this tissue than the laminae in response to water-deficit.

ABA occurred in much the same quantity in all of the tiller tissues in the water-sufficient state, and accumulated in response to the trigger point. However, disproportionately large increases in ABA occurred in the laminae when compared to the LB during the advanced stages of water-deficit (Figure 5.14, page 180).

The reduction in soluble proteins occurred in all the tissues in response to the water-deficit treatment (Figure 6.8, page 210). However, the greatest decline in soluble protein was observed in the laminae. Probably, this was because the protein content of the laminae, on a fresh weight basis, was greater than the LB, hence the laminae had more protein to lose. However, investigations into the spectrum of proteins in each tissue showed that many more individual protein species declined in the laminae. These proteins included the major subunit of RuBisCO, which was severely reduced in quantity in response to the water-deficit treatment. Since carbon fixation is reduced in response to water-deficit (Hsiao, 1973), the reduction in fraction I protein may follow a loss in photosynthetic capacity, although this was not studied. The fate of the hydrolysis products of RuBisCO are unknown, but may supply nitrogen or carbon skeletons to enable the accumulation of osmolytes. The pattern of ubiquitinated protein species was also changed predominantly in the laminae, which suggests that some controlled

salvaging of leaf material, possibly similar to the process of senescence, occurred in these tissues.

In summary, it appears that the traits linked to tissue turnover, such as necrosis (scorching) and protein turnover were located in the laminae, while the traits linked to tissue survival (proline accumulation and maintenance of turgor for LER) were limited to the LB. It seems then, that *F. novae-zealandiae* does not adapt metabolically to overcome the deleterious effects of water deficit stress, but rather employs a mechanism of water-deficit avoidance, where the viability of the meristematic regions, and hence the capacity for re-growth are maintained for as long as possible, at the expense of the existing leaf tissue.

7.6 Adaption or Injury?

It was not possible to tell from these experiments what role the accumulation of ABA or proline play in the water-deficit response mechanism of *F. novae-zealandiae*. In particular, it was not possible to ascertain whether ABA and proline accumulation was a specifically induced response to water-deficit treatment which conferred some advantage to the plant, or whether they accumulated as a result of water-deficit induced cellular injury.

Some evidence was found to support both of these possibilities. ABA accumulated in all tiller tissues after the trigger point, and may have been a specifically induced response. This accumulation may have been transient, since peaks in ABA content were observed in some plants, especially those in the experiments described in Chapter Four. However, a second, much greater and longer accumulation occurred in the laminae at the same time as the leaf water content first declined (see Chapter Five). This second event may have been injury related, since the LB region was not affected (other evidence, such as the continuation of LER suggest that the LB does not become injured), and increases in the rate of leaf tip scorching were observed at the same time. The cause of the second accumulation was likely to be due increased synthesis (possibly due to disruption of the ABA synthetic pathway) rather than to increased transport of ABA from the roots, since the accumulation did not occur in the intermediary LB region. The peak ABA contents in two of four water-deficit treatment plants from

Chapter Five returned to normal after re-watering, which suggests that the "injury" sustained was not severe enough to cause cell death. However, the other two plants did not recover, and subsequently died, which suggests that for these two plants, the converse was true.

From the data collected during these experiments, it was difficult to determine if proline accumulation in the laminae was injury related, as suggested was the case with *Lolium perenne*, where proline accumulation in the laminae was correlated with symptoms of severe drought such as dehydration, scorching, cessation of LER, and very low LWP (below -2 MPa; Thomas 1991). Certainly the presence of relatively high levels of proline in the LB cannot be injury-related, due to the constitutive nature of its occurrence in water-sufficient tillers. Proline started to accumulate in all tissues after the trigger point, and before the decline in the RWC and hydration of all the tissues was observed which suggests a protective role, although the highest levels were found after dehydration of the leaves commenced.

The reasons for the relatively lower quantity of proline accumulated in the laminae when compared to the LB are not known. The laminae of *F. novae-zealandiae* are quite fibrous, due to numerous sclerenchyma cells which contain no cytosol at maturity. This, and the relatively larger vacuoles in (living) mature cells compared to cells in meristematic tissue mean that the laminae probably have less cytoplasm than the LB, although this was not confirmed. Hence, if proline is located predominantly in the cytoplasm then a difference in proline content between the two tissues could be expected. Some upward transport of proline that was synthesised in the LB may also be involved.

More accurate determination of the leaf water status, or the use of some other measure of tissue integrity, such as sap purity¹⁰, is required to elucidate more clearly the differences between water-deficit response, and injury response accumulation of ABA and proline in this species.

¹⁰ The analysis of the osmotic strength of xylem sap obtained from water-deficit stressed leaves may enable this to be determined, since the near pure water quality of the transpiration stream should become contaminated with cytosolic components in the event of injury.

7.7 Future Prospects in the Study of Water-Deficit Stress Response in *F. novae-zealandiae*

7.7.1 Morphological and Physiological Traits

It appears from this study that *F. novae-zealandiae* has the ability to contain water loss from the leaves. This may be in part because the leaves of *F. novae-zealandiae* contain relatively little water compared to other species such as *F. arundinacea* (H_L as low as 2g water/gDW). It is not clear if leaf morphology coupled to physiological traits, such as the closing of stomata and leaf curling were sufficient to account for the efficient water retention, or if osmotic adaption (that is, the accumulation of osmolytes to increase the osmotic strength of the cytoplasm) also occurs. To examine this aspect, it would be interesting to measure changes in transpiration during a dry-down. A more detailed examination of the stomata and margin aperture by SEM during the progress of a dry-down experiment may also help to answer these questions.

7.7.2 Accumulation of Metabolites

Changes in the proline content of the leaves was indicative that osmoprotectants may have a role in the water-deficit tolerance of this species. However, in drought-stressed laminae of *L. perenne* (Thomas 1991) and five other forage grass species including *F. arundinacea* (Barker *et al.*, 1993), the accumulated proline was responsible for a only small fraction of the total osmoticum within the leaf. Many other compounds, such as inorganic ions, sugars, sugar alcohols and polyols also accumulated in response to the water-deficit, and accounted for the majority of the osmoticum (Thomas 1991). Therefore, an examination of the nature and quantity of the osmolytes present in the various tiller tissues of *F. novae-zealandiae* may be worthwhile. Only one amino acid (proline) was measured in the present study; it may be worthwhile measuring other amino acids, particularly glutamate, aspartate and γ -aminobutyric acid since these have been reported to accumulate in other species, such as *Phaseolus vulgaris* (Bean; Raggi, 1994) in response to drought. There also exists the possibility that *F. novae-zealandiae* may accumulate novel osmolyte (or osmoprotectant) species.

As mentioned above, proline accumulated to relatively low levels in the lamina when compared to the LB. It was not clear if this was a result of transport from the LB region, from *de novo* synthesis *in situ*, or if it resulted from increases in protein degradation that began after the trigger point. Alternatively, the high proline levels in the LB may have partially resulted from inward transport of proline as the plant scavenges material from the laminae. Radiotracer feeding 'pulse/chase' experiments may help to elucidate the mode of proline accumulation in the leaves of water-deficit stressed *F. novae-zealandiae*. Examination of the expression patterns enzymes of proline synthesis, especially the rate limiting step, pyrroline-5-carboxylate synthetase (P5CS; Delauney and Verma, 1993) may help to explain the differences in proline content between the LB and the laminae. It seems likely from this study that P5CS is constitutively expressed in the LB, and may be induced in the laminae. The further accumulation of proline to high levels in the LB after the trigger point may also be due to lessening of the product feed-back inhibition of this enzyme (Delauney and Verma, 1993).

Glycine-betaine was not observed to accumulate in the leaves of *F. novae-zealandiae* in response to the water deficit treatment. This was perhaps surprising, given the fact that the accumulation of glycine-betaine in response to drought has been reported for other grass species, such as *Z. mays* and *H. vulgare* (Ladyman *et al.*, 1983; McCue and Hanson, 1990), and in response to water-deficit stress in *F. arundinacea* (Chapter Three). However, glycine-betaine may function in *Festuca spp.* to protect proteins from high ionic content resulting from saline stress, rather than from water-deficit. Circumstantial evidence for this includes the accumulation of glycine-betaine at a different date from the trigger point in *F. arundinacea*, and also the fact that glycine-betaine was not constitutively expressed in higher levels in the LB when compared to the laminae of *F. novae-zealandiae* tillers as was the case for proline. Certainly, the accumulation of glycine-betaine was not induced by the same mechanism as ABA or proline accumulation in *F. novae-zealandiae*. An examination of the response of this species to saline (NaCl) stress may provide a better understanding of the role of glycine-betaine in this species.

7.7.3 ABA Responsive Genes

In the current study, changes in the concentration of ABA were used as a marker to indicate changes in metabolic processes. However, ABA regulated gene expression in response to water-deficit stress has been widely reported (reviewed in Bray, 1993). Therefore, an examination of the expression of known ABA regulated genes in *F. novae-zealandiae* may increase understanding of drought tolerance in this species. This work was started in the present study with the examination of dehydrins, which are coded for by ABA regulated genes. However, these proteins were not induced by the water-deficit treatment. Several avenues for further work with dehydrin were suggested in Chapter Six. Hence the question may be asked, which, if any, water-deficit induced proteins are regulated by ABA in *F. novae-zealandiae*?. As a starting point, proteins (or genes) representing LEA groups of stress induced proteins and osmotin (Section 1.3.2.3, page 8) could be examined, since these are relatively well described, although in most cases the protein function(s) are unassigned.

7.7.4 Proteases and Protein Turnover

Proteases, and elucidation of the regulation of protein turnover would be good candidates for research, since some evidence for the turnover of proteins was found, such as the reduction in soluble protein, the degradation of RuBisCO and changes in the patterns of ubiquitinated proteins. Therefore proteases or proteosome complexes should be involved. It is possible that the mono-ubiquitinated proteins regulate some aspects of cellular metabolism (Hershko and Ciechanover, 1992), and hence the isolation and amino acid sequencing of these proteins may be worthwhile.

References

- Abou-mandor AA, Hartung W (1995):** Tissue culture of the desert plant *Anastatica hierochuntica*. *Plant Cell Reports* **14**(10), 657-661.
- Allan AC, Fricker MD, Ward JL, Beale MH, Trewavas AJ (1994):** Two transduction pathways mediate rapid effects of abscisic acid in *Commulina guard cells*. *The Plant Cell* **6**, 1319-1328.
- Allan AC, Trewavas AJ (1994):** Abscisic acid and gibberelin perception: Inside or out? *Plant Physiology* **104**, 1107-1108.
- Anderson BE, Ward JM, Schroeder JI (1994):** Evidence for an extracellular reception site for abscisic acid in *Commelina guard cells*. *Plant Physiology* **104**, 1177-1183.
- Arakawa K, Katayama M, Takabe T (1990):** Levels of betaine and betaine aldehyde dehydrogenase activity in the green leaves, and etiolated leaves and roots of barley. *Plant and Cell Physiology* **31**(6), 797-803.
- Arakawa K, Mizuno K, Kishitani S, Takabe T (1992):** Immunological studies of betaine aldehyde dehydrogenase in barley. *Plant and Cell Physiology* **33**(7), 833-840.
- Arora R, Wisniewski ME (1994):** Cold acclimation in genetically related (sibling) deciduous and evergreen peach (*Prunus persica* [L.] Batsch). *Plant Physiology* **105**, 95-101.
- Baker J, Steele C, Dure L III (1988):** Sequence and characterisation of 6 Lea proteins and their genes from cotton. *Plant Molecular Biology* **11**, 277-291.
- Banowitz GM (1989):** Hybrid hybridoma cell lines which express anti-zeatin riboside, anti-abscisic acid and hybrid antibodies. *Plant Physiology* **91**, 144-147.
- Barker DJ, Sullivan CY, Moser LE (1993):** Water deficit affects on osmotic potential, cell wall elasticity, and proline in five forage grasses. *Agronomy Journal* **85**, 270-275.
- Bates LS, Waldren RP, Teare ID (1973):** Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205-207.
- Belesky DP, Fishman ML, Wilkinson SR (1984):** Effect of nitrogen fertilization and mild water stress on the distribution of nitrogen in tall fescue. *Plant and Soil* **77**, 295-303.
- Belesky DP, Wilkinson SR, Pallas JE Jr (1982):** Response of four tall fescue cultivars grown at two nitrogen levels to low soil water availability. *Crop Science* **22**, 93-97.
- Beuzenberg EJ, Hair JB (1983):** Contributions to a chromosome atlas of the New Zealand flora 25. Miscellaneous species. *New Zealand Journal of Botany* **21**, 13-20.
- Bokhari UG, Trent JD (1985):** Proline concentrations in water stressed grasses. *Journal of Range Management* **38**(1), 37-38.
- Boyer JS (1982):** Plant productivity and environment. *Science* **218**, 443-448.
- Boyer JS (1989):** Water potential and plant metabolism: comments on Dr P.J. Kramer's article 'Changing concepts regarding plant water relations', volume 11, number 7, pp. 565-568, and Dr J.B Passioura's response, pp. 569-571. *Plant, Cell and Environment* **12**, 213-216.
- Bradford KJ, Chandler PM (1992):** Expression of "dehydrin-like" proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. *Plant Physiology* **99**, 488-494.
- Bradford KJ, Hsiao TC (1982):** Physiological responses to moderate water stress. In: Encyclopaedia of plant physiology. New series. Physiological plant ecology II. Vol. 12B. (Eds: Lange OL, Nobel PS, Osmond CB, Ziegler H) Springer-Verlag, Heidelberg, 263-324.
- Bradford MM (1976):** A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Chemistry* **72**, 248-254.
- Bray EA (1988):** Drought- and ABA-induced changes in polypeptide and mRNA accumulation in tomato leaves. *Plant Physiology* **88**, 1210-1214.

- Bray EA (1993):** Molecular responses to water deficit. *Plant Physiology* **103**, 1035-1040.
- Brock JL (1983):** "Grasslands Roa" tall fescue: A review. *Proceedings of the New Zealand Grassland Association* **44**, 74-80.
- Chandler PM, Robertson M (1994):** Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**, 113-141.
- Chapin FS III (1991):** Integrated responses of plants to stress. A centralised system of physiological responses. *Bioscience* **41**(1), 29-36.
- Ciechanover A (1994):** The ubiquitin-proteasome proteolytic pathway. *Cell* **79**, 13-21.
- Clifford HT, Watson L (1977):** Identifying Grasses: Data methods and illustrations. University of Queensland Press, St. Lucia, Queensland. 146 pages.
- Close TJ, Chandler PM (1990):** Cereal dehydrins: Serology, Gene mapping and potential functional roles. *Australian Journal of Plant Physiology* **17**, 333-344.
- Close TJ, Fenton RD, Moonan F (1993):** A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Molecular Biology* **23**, 279-286.
- Close TJ, Kortt AA, Chandler PM (1989):** A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Molecular Biology* **13**, 95-108.
- Close TJ, Lammers PJ (1993):** An osmotic stress protein of cyanobacteria is immunologically related to plant dehydrins. *Plant Physiology* **101**, 773-779.
- Clouston D (1960):** Identification of grasses in non-flowering condition. The sports turf research institute, Bingley, Yorkshire. 31 pages.
- Conn EE, Stumpf PK, Bruening G, Doi RH (1987):** Outlines of biochemistry. 5th ed. John Wiley and Sons, New York. 691 pages.
- Connor HE (1960):** Variation in leaf anatomy in *Festuca novae-zealandiae* (Hack.) Cozyane, and *F. matthewsii* (Hack.) Cheeseman. *New Zealand Journal of Science* **3**, 468-509.
- Cowan AK, Rose PD, Home LG (1992):** *Dunaliella salina*: a model system for studying the response of plant cells to stress. *Journal of Experimental Botany* **43**(257), 1535-1547.
- Creelman RA (1989):** Abscisic acid physiology and biosynthesis in higher plants. *Physiologia Plantarum* **75**, 131-136.
- Creelman RA, Mason HS, Bensen RJ, Boyer JS, Mullet JE (1990):** Water deficit and abscisic acid cause differential inhibition of shoot versus root growth in soybean seedlings. *Plant Physiology* **92**, 205-214.
- Csonka LN (1989):** Physiological and genetic responses of bacteria to genetic stress. *Microbiological Reviews* **53**, 121-147.
- Daie J, Wyse R (1982):** Adaption of the enzyme-linked immunosorbent assay (ELISA) to the quantitative analysis of abscisic acid. *Analytical Biochemistry* **119**, 365-371.
- Delauney AJ, Verma DPS (1993):** Proline biosynthesis and osmoregulation in plants. *The Plant Journal* **4**(2), 215-223.
- Dellaporta SL, Wood J, Hicks JB (1983):** A plant DNA mini-preparation: Version II. *Plant Molecular Biology Reporter* **1**(4), 19-21.
- Dingkuhn M, Cruz RT, O 'toole JC, Turner NC, Doerffling K (1991):** Responses of seven diverse rice cultivars to water deficits. III. Accumulation of abscisic acid and proline in relation to leaf water-potential and osmotic adjustment. *Field Crops Research* **27**, 103-117.
- Dure L III, Crouch M, Harada J, Ho Tuan-HD, Mundy J, Quatrano R, Thomas T, Sung ZR (1989):** Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Molecular Biology* **12**, 475-486.
- Eklund L, Cienciala E, Hallgren Jan-E (1992):** No relation between drought stress and ethylene production in Norway spruce. *Physiologia Plantarum* **86**, 297-300.
- Elbersen HW (1994):** Endophyte effects on growth and drought survival of tall fescue. Ph.D. Dissertation, Agricultural University, Wageningen, The Netherlands.

- Emmert FH (1974):** Inhibition of phosphorus and water passage across intact roots by polyethylene glycol and phenylmercuric acetate. *Plant Physiology* **53**, 663-665.
- Esau K (1977):** The leaf: Variations in structure. In: Anatomy of seed plants. 2nd ed. John Wiley and Sons, Inc, New York, 351-353.
- Espie PR (1990):** The impact of nutrients on fescue tussock grasslands. *Whats New in Forest Research* ed. Vol. 189. Forest Research Institute, Rotorua. 4 pages.
- Ferguson DL, Guikema JA, Paulsen GM (1990):** Ubiquitin pool modulation and protein degradation in wheat roots during high temperature stress. *Plant Physiology* **92**, 740-746.
- Galau GA, Close TJ (1992):** Sequences of the cotton group 2 LEA/RAB/Dehydrin proteins encoded by *Lea3* cDNAs. *Plant Physiology* **98**, 1523-1525.
- Gastal F, Belanger G, Lemaire G (1992):** A model of the leaf extension rate of tall fescue in response to nitrogen and temperature. *Annals of Botany* **70**, 437-442.
- Gilroy S, Jones RL (1994):** Perception of gibberelin and abscisic acid at the external face of the plasma membrane of barley (*Hordeum vulgare* L.) aleurone protoplasts. *Plant Physiology* **104**, 1185-1192.
- Godoy JA, Lunar R, Torres-Schumann S, Moreno J, Rodrigo R, Pintor-Toro JA (1994):** Expression, tissue distribution and subcellular localisation of dehydrin TAS14 in salt-stressed tomato plants. *Plant Molecular Biology* **26**, 1921-1934.
- Godoy JA, Pardo JM, Pintor-Toro JA (1990):** A tomato cDNA inducible by salt stress and abscisic acid: nucleotide sequence and expression pattern. *Plant Molecular Biology* **15**, 695-705.
- Gorham J (1984):** Separation of plant betaines and their sulphur analogues by cation-exchange high performance liquid chromatography. *Journal of Chromatography* **287**, 345-351.
- Grieve CM, Grattan SR (1983):** Rapid assay for determination of water soluble quaternary ammonium compounds. *Plant and Soil* **70**, 303-307.
- Guerreiro FD, Mullet JE (1988):** Reduction of turgor induces rapid changes in leaf translatable RNA. *Plant Physiology* **88**, 401-408.
- Hanson AD, Hitz WD (1982):** Metabolic responses of mesophytes to plant water deficits. *Annual Review of Plant Physiology* **33**, 163-203.
- Harada JJ, DeLisle AJ, Baden CS, Crouch ML (1989):** Unusual sequence of an abscisic acid-inducible mRNA which accumulates late in *Brassica napus* seed development. *Plant Molecular Biology* **12**, 395-401.
- Harris MJ, Outlaw WH Jr, Mertens R, Weiler EW (1988):** Water stress induced changes in the abscisic acid content of guard cells and other cells of *Vicia faber* L. leaves as determined by enzyme-amplified immunoassay. *Proceedings of the National Academy of Science USA* **85**, 2584-2588.
- Hartung W, Davies WJ (1991):** Drought-induced changes in physiology and ABA. In: ABA physiology and biochemistry. (Eds: Davies WJ, Jones HG) BIOS Scientific Publishers, Oxford, 63-77.
- Hellwege EM, Dietz Karl-J, Volk OH, Hartung W (1994):** Abscisic acid and the induction of desiccation tolerance in the extremely xerophilic liverwort *Exormothea holstii*. *Planta* **194**, 525-531.
- Hershko A, Ciechanover A (1992):** The ubiquitin system for protein degradation. *Annual Review of Biochemistry* **61**, 761-807.
- Hitz WD, Hanson AD (1980):** Determination of glycine betaine by pyrolysis-gas chromatography in cereals and grasses. *Phytochemistry* **19**, 2371-2374.
- Holmstrom Kjell-O, Welin B, Mandal A, Kristiansdottir I, Teeri TH, Lamark T, Strom AR, Palva ET (1994):** Production of the *Escherichia coli* betaine-aldehyde dehydrogenase, an enzyme required for the synthesis of the osmoprotectant glycine betaine, in transgenic plants. *The Plant Journal* **6**(5), 749-758.
- Hsiao TC (1973):** Plant responses to water stress. *Annual Review of Plant Physiology* **24**, 519-570.
- Hubick KT, Reid DM (1980):** A rapid method for the extraction and analysis of abscisic acid from plant tissue. *Plant Physiology* **65**, 523-525.

- Ingalls ST, Minkler PE, Hoppel CL (1984):** Derivatisation of carboxylic acids by reaction with 4'-bromophenacyl trifluoromethanesulfonate prior to determination by high-performance liquid chromatography. *Journal of Chromatography* **299**, 365-376.
- Ishitani M, Nakamura T, Han SY, Takabe T (1995):** Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Molecular Biology* **27**, 307-315.
- Jones GP, Naidu BP, Starr RK, Paleg LG (1986):** Estimates of solutes accumulating in plants by ¹H nuclear magnetic resonance spectroscopy. *Australian Journal of Plant Physiology* **13**, 649-658.
- Joost RE, Holder TL (1994):** Effect of endophyte infection on ABA content and drought response of tall fescue. *Agronomy Abstracts* **1994**, 140.
- Joshi CP, King SW, Nguyen HT (1992):** Molecular cloning and characterisation of a cDNA encoding a water stress protein (WSP23) from wheat roots. *Plant Science* **86**(1), 71-82.
- King SW, Joshi CP, Nguyen HT (1992):** DNA sequence of an ABA-responsive gene (rab 15) from water-stressed wheat roots. *Plant Molecular Biology* **18**, 119-121.
- Koheil MAH, Hilal SH, El-Alfy TS, Leistener E (1992):** Quaternary ammonium compounds in intact plants and cell suspension cultures of *Atriplex semibaccata* and *A. halimus* during osmotic stress. *Phytochemistry* **31**(6), 2003-2008.
- Kohl DH, Kennelly EJ, Zhu Y, Schubert KR, Shearer G (1991):** Proline accumulation, nitrogenase (C₂H₂ reducing) activity and activities of enzymes related to proline metabolism in drought-stressed soybean nodules. *Journal of Experimental Botany* **42**, 831-837.
- Kramer PJ (1988):** Changing concepts regarding plant water relations. *Plant, Cell and Environment* **11**, 565-568.
- Krizek DT (1985):** Methods of inducing water stress in plants. *Horticultural Science* **20**, 1029-1038.
- Kusano T, Aguan K, Abe M, Sugawara K (1992):** Nucleotide sequence of a rice rab 16 homologue gene. *Plant Molecular Biology* **18**, 127-129.
- Ladyman JAR, Ditz KM, Grumet R, Hanson AD (1983):** Genotypic variation for glycine-betaine accumulation by cultivated and wild barley in relation to water stress. *Crop Science* **23**, 465-468.
- Lai M, Gunsalus RP (1992):** Glycine betaine and potassium ion are the major compatible solutes in the extremely halophilic methanogen *Methanohalophilus* strain Z7302. *Journal of bacteriology* **174**(22), 7474-7477.
- Landi P, Conti S, Gherardi F, Sanguineti MC, Tuberosa R (1995):** Genetic analysis of leaf ABA concentration and of agronomic traits in maize hybrids grown under different water regimes. *Maydica* **40**, 179-186.
- Lane BG (1991):** Cellular desiccation and hydration: developmentally regulated proteins, and the maturation and germination of seed embryos. *FASEB Journal* **5**, 2893-2901.
- Lang V, Mantyla E, Welin B, Sundberg B, Palva ET (1994):** Alterations in water status, endogenous abscisic acid content and expression of freezing tolerance in *Arabidopsis thaliana*. *Plant Physiology* **104**, 1341-1349.
- Lather F, Rotival-Garnier N, Lemesle P, Plasman M, Bouchereau A (1996):** The glycine-betaine inhibitory effect on the osmoinduced proline response of rape leaf discs. *Plant Science* **113**: 21-31.
- LaRosa PC, Chen Z, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992):** Osmotin gene expression is post-transcriptionally regulated. *Plant Physiology* **100**, 409-415.
- Laurie S, Stewart GR (1990):** The effects of compatible solutes on the heat stability of glutamine synthetase from chickpeas grown under different nitrogen and temperature regimes. *Journal of Experimental Botany* **41**(232), 1415-1422.
- Lenton JR, Perry VM, Sanders PF (1971):** The identification and quantitative analysis of abscisic acid in plant extracts by gas-liquid chromatography. *Planta* **96**, 271-280.
- Lerma C, Rich PJ, Ju GC, Yang Wen-JJ, Hanson AD, Rhodes D (1991):** Betaine deficiency in maize. Complementation tests and metabolic basis. *Plant Physiology* **95**(4), 1113-1119.
- Leroux B, Maldiney R, Migiriac E, Sossountzov L, Sotta B (1985):** Comparative quantitation of abscisic acid in plant extracts by gas-liquid chromatography and an enzyme-linked immunosorbent assay using the avidin-biotin system. *Planta* **166**, 524-529.

- Le Rudulier D, Strom AR, Dandekar AM, Smith LT, Valentine RC (1984): Molecular biology of osmoregulation. *Science* **224**, 1064-1068.
- Leung J, Bouvier-Durand M, Morris Peter-C, Guerrier D, Chefdor F, Giraudat J (1994): *Arabidopsis* ABA response gene ABI1: Features of a calcium-modulated protein phosphatase. *Science* **264**, 1448-1451.
- Lever M, Bason L, Leaver C, Hayman CM, Chambers ST (1992): Same-day batch measurement of glycine betaine, carnitine, and other betaines in biological material. *Analytical Biochemistry* **205**, 14-21.
- Litts C, Erdman MB, Huang N, Karrer EE, Noueir A, Quatrano RS, Rodriguez RL (1992): Nucleotide sequence of the rice (*Oryza sativa*) Em protein gene. *Plant Molecular Biology* **19**, 335-337.
- Lord JM (1992): The evolutionary ecology of *Festuca novae-zealandiae* in mid-Canterbury, New Zealand. Ph.D. Thesis, University of Canterbury, Christchurch, New Zealand.
- Lord JM (1993): Does clonal fragmentation contribute to recruitment in *Festuca novae-zealandiae*? *New Zealand Journal of Botany* **31**, 133-138.
- Luan S, Li W, Rusnak F, Assman SM, Schreiber SL (1993): Immunosuppressants implicate protein phosphatase regulation of K⁺ channels in guard cells. *Proceedings of the National Academy of Science USA* **90**, 2202-2206.
- Ludlow MM, Muchow RC (1990): A critical evaluation of traits for improving crop yields in water-limited environments. *Advances in Agronomy* **43**, 107-153.
- Macháková I, Hanisová A, Krekule J (1989): Levels of ethylene, ACC, MACC, ABA and proline as indicators of cold hardening and frost resistance in winter wheat. *Physiologia Plantarum* **76**, 603-607.
- Magné C, Larher F (1992): High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry* **200**, 115-118.
- Manetas Y, Petropoulou Y, Karaboumiotis G (1986): Compatible solutes and their effects on phosphoenolpyruvate carboxylase of C4-halophytes. *Plant, Cell and Environment* **9**(2), 145-151.
- Marcotte WR Jr, Russell SH, Quatrano RS (1989): Abscisic acid-responsive sequences from the Em gene of wheat. *The Plant Cell* **1**, 969-976.
- McCue KF, Hanson AD (1990): Drought and salt tolerance: towards understanding and application. *Trends in Biotechnology* **8**, 358-362.
- McCue KF, Hanson AD (1992): Effects of soil salinity on the expression of betaine aldehyde dehydrogenase in leaves: Investigation of hydraulic, ionic and biochemical signals. *Australian Journal of Plant Physiology* **19**, 555-564.
- McWilliam JR (1986): The national and international importance of drought and salinity effects on agricultural production. *Australian Journal of Plant Physiology* **13**, 1-13.
- Mertens R, Deus-Neumann B, Weiler EW (1983): Monoclonal antibodies for the detection and quantification of the endogenous plant growth regulator, abscisic acid. *Febs Letters* **160**(1,2), 269-272.
- Meyer K, Leube MP, Grill E (1994): A protein 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science* **264**, 1452-1455.
- Michel D, Furini A, Salamini F, Bartels D (1994): Structure and regulation of an ABA- and desiccation-responsive gene from the resurrection plant *Craterostigma plantagineum*. *Plant Molecular Biology* **24**, 549-560.
- Milne GD, Molony SC, Smith DR (1993): Demonstration of dryland species on 90 east coast North Island farms. *Proceedings of the New Zealand Grassland Association* **55**, 39-44.
- Morgan JM (1977): Differences in osmoregulation between wheat genotypes. *Nature* **270**, 234-235.
- Morgan JM (1984): Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology* **35**, 299-319.
- Morgan FW, He Chuan-J, De Greef JA, De Proft MP (1990): Does water deficit stress promote ethylene synthesis by intact plants? *Plant Physiology* **94**, 1616-1624.
- Morris PC, Kumar A, Bowles DJ, Cumming AC (1990): Osmotic stress and abscisic acid induce expression of the wheat Em genes. *European Journal of Biochemistry* **190**, 625-630.

- Mundy J, Chua Nam-H (1988):** Abscisic acid and water-stress induce the expression of a novel rice gene. *The EMBO Journal* 7(8), 2279-2286.
- Mundy J, Yamaguchi-Shinozaki K, Chua Nam-H (1990):** Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice rab gene. *Proceedings of the National Academy of Science USA* 87, 1406-1410.
- Munns R, Sharp RE (1993):** Involvement of abscisic acid in controlling plant growth in soils of low water potential. *Australian Journal of Plant Physiology* 20, 425-437.
- Naidu BP, Paleg LG, Aspinall D, Jennings AC, Jones GP (1991):** Amino acid and glycine betaine accumulation in cold-stressed wheat seedlings. *Phytochemistry* 30(2), 407-409.
- Narayana I, Lalonde S, Saini HS (1991):** Water-stress-induced ethylene production in wheat. A fact or artifact? *Plant Physiology* 96, 406-410.
- Nelson DE, Raghothama KG, Singh NK, Hasegawa PM, Bressan RA (1992):** Analysis of structure and transcriptional activation of an osmotin gene. *Plant Molecular Biology* 19, 577-588.
- Neven LG, Haskell DW, Hofig A, Li Qin-B, Guy CL (1993):** Characterisation of a spinach gene responsive to low temperature and water stress. *Plant Molecular Biology* 21, 291-305.
- Newsome PFJ (1987):** The vegetative cover of New Zealand. National Water and Soil Conservation Authority, Wellington. 139 pages.
- Nield JD (1990):** Regional economic impacts of the 1988/89 drought on the east coast of the North Island. Report prepared for the Regional Policy Manager, MAF Technology, North Central Region, Batchalar Agricultural centre, Palmerston North, January 1990.
- Ober ES, Sharp RE (1994):** Proline accumulation in Maize (*Zea Mays* L.) primary roots at low water potentials. *Plant Physiology* 105, 981-987.
- Parry, Horgan (1991):** Physico-chemical methods in ABA research. In: ABA physiology and biochemistry. (Eds: Davies WJ, Jones HG) BIOS Scientific Publishers, Oxford. pp 5-22.
- Passioura JB (1988):** Response to Dr P.J. Kramers's article, 'Changing concepts regarding plant water relations', Volume 11, number 7, pp 565-568. *Plant, Cell and Environment* 11, 569-571.
- Pennypacker BW, Leath KT, Stout WL, Hill RR (1990):** Technique for simulating field drought stress in the glass-house. *Agronomy Journal* 82, 951-957.
- Philosoph-Hadas S, Hadas E, Aharoni M (1993):** Characterisation and use in ELISA of a new monoclonal antibody for quantitation of abscisic acid in senescing rice leaves. *Plant Growth Regulation* 12, 71-78.
- Piatkowski D, Schneider K, Salamini F, Bartels D (1990):** Characterisation of five abscisic acid-responsive cDNA clones isolated from the desiccation-tolerant plant *Craterostigma plantagineum* and their relationship to other water-stress genes. *Plant Physiology* 94, 1682-1688.
- Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeuken MJW, Weisbeek PJ, Smeekens SCM (1995):** Improved performance of transgenic fructan-accumulating tobacco under drought-stress. *Plant Physiology* 107, 125-130.
- Pla M, Vilardell J, Gultinan MJ, Marcotte WR, Niogret MF, Quatrano RS, Pagès M (1993):** The cis-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene rab28. *Plant Molecular Biology* 21, 259-266.
- Quarrie SA, Galfre G (1985):** Use of different hapten-protein conjugates immobilized on nitrocellulose to screen monoclonal antibodies to abscisic acid. *Analytical Biochemistry* 151, 389-399.
- Quarrie SA, Whitford PN, Appleford NEJ, Wang TL, Cook SK, Henson IE, Loveys BR (1988):** A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* 173, 330-339.
- Raven PH, Evert RF, Eichhorn SE (1986):** Biology of plants. 4th ed. Worth publishers, New York. 425 pages.
- Raynal M, Depigny D, Cooke R, Delsney M (1989):** Characterisation of a radish nuclear gene expressed during late seed maturation. *Plant Physiology* 91, 829-836.

- Rhodes D (1987): Metabolic responses to stress. *The Biochemistry of Plants* 12, 201-241.
- Richardson MD, Chapman GW, Hoveland CS, Bacon CW (1992): Sugar alcohols in endophyte-infected tall fescue under drought. *Crop Science* 32, 1060-1061.
- Riov J, Hausman R (1988): Regulation of water-stress-induced ethylene in citrus leaves. *Israel Journal of Botany* 37, 83-91.
- Robertson M, Chandler PM (1992): Pea dehydrins, characterisation and expression. *Plant Molecular Biology* 19, 1031-1044.
- Robertson M, Chandler PM (1994): A dehydrin cognate protein from pea (*Pisum sativum* L.) with an atypical pattern of expression. *Plant Molecular Biology* 26, 805-816.
- Ross GS, Elder PA, McWha JA, Pearce D, Pharis RP (1987): The development of an indirect enzyme linked immunoassay for abscisic acid. *Plant Physiology* 85, 46-50.
- Sambo EY (1981): Osmotic adjustment as a mechanism of drought resistance in crop and forage species. *Journal of Science and Technology* 2(2), 21-37.
- Sambrook J, Fritsch EF, Maniatis T (1989): Molecular cloning: A laboratory manual. 2nd ed. Vol. 1-3. Cold Spring Harbour Laboratory Press, New York.
- Sarath G, De La Motte RS, Wagner FW (1987): Protease assay methods. In: *Proteolytic enzymes; A practical approach*. (Eds: Benyon RJ, Bond JS) IRL Press, Oxford, 25-55. (N)
- Savage MJ, Cass A, Wiebe HH (1984): Effect of excision on leaf water potential. *Journal of Experimental Botany* 35(151), 204-208.
- Scholander PF, Hammel HT, Bradstreet ED, Hemmingsen EA (1965): Sap pressure in vascular plants. *Science* 148, 339-346.
- Schönherr J (1982): Physiological responses to moderate water stress. In: *Encyclopaedia of plant physiology. New series. Physiological plant ecology II*. Vol. 12B. (Eds: Lange OL, Nobel PS, Osmond CB, Ziegler H) Springer-Verlag, Heidelberg, 154-179.
- Schulz M, Wolf D, Schnabl H (1993): Age dependant appearance of polypeptides with immunoreactivity to ubiquitin antibodies in chloroplast membranes of *Vicia faba* L. *Journal of Plant Physiology* 141, 298-303.
- Schulze ED, Steudle E, Gollan T, Schurr U (1988): Response to Dr P.J. Kramer's article, 'Changing concepts regarding plant water relations', Volume 11, number 7, pp 565-568. *Plant, Cell and Environment* 11, 573-576.
- Scott D, Dick RD, Hunter GG (1988): Changes in the tussock grasslands in the central Waimakariri River basin, Canterbury, New Zealand, 1947-1981. *New Zealand Journal of Botany* 26, 197-222.
- Seeley SD, Powell LE (1970): Electron capture-gas chromatography for sensitive assay of abscisic acid. *Analytical Biochemistry* 35, 530-533.
- Sikurajapathy M, Cappy JJ, Gross HD (1983): A method for inducing controlled moisture stress on seedlings. *Agronomy Journal* 75, 840-843.
- Sinclair TR, Ludlow MM (1985): Who taught plants thermodynamics? The unfulfilled potential of plant water potential. *Australian Journal of Plant Physiology* 12, 213-217.
- Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hemmerson MA, Pfankoch E, Regnier FE, Bressan RA (1987): Characterisation of osmotin. A thaumatin-like protein associated with osmotic adaptation in plant cells. *Plant Physiology* 85, 529-536.
- Singh NK, Nelson DE, Kuhn D, Hasegawa PM, Bressan RA (1989): Molecular cloning of osmotin and regulation of its expression by ABA and adaptation to low water potential. *Plant Physiology* 90, 1096-1101.
- Singh TN, Aspinall D, Paleg LG, Boggess SF (1973): Stress Metabolism. II. Changes in proline concentration in excised plant tissues. *Australian Journal of Biological Sciences* 26, 57-63.
- Sivaramakrishnan S, Patell VZ, Flower DJ, Peacock JM (1988): Proline accumulation and nitrate reductase activity in contrasting sorghum lines during mid-season drought stress. *Physiologia Plantarum* 74, 418-426.
- Skirver K, Mundy J (1990): Gene expression in response to abscisic acid and osmotic stress. *The Plant Cell* 2, 503-512.

- Smith DR, Slay MWA, Bray MH, Milne GD (1993): On-farm establishment of drought-tolerant pastures on the east coast of the North Island. *Proceedings of the New Zealand Grassland Association* 55, 33-38.
- Sokal RR, Rohlf FJ (1987): Biometry. 2nd ed. W.H. Freeman and Company, New York. 860 pages.
- Spollen WG, Nelson CJ (1994): Response of fructan to water deficit in growing leaves of tall fescue. *Plant Physiology* 106, 329-336.
- Stewart CR, Bogges SF (1978): Metabolism of [5-3H] proline by barley leaves and its use in measuring the effects of water stress and proline oxidation. *Plant Physiology* 61, 654-657.
- Staub PF, Shen Q, Ho Tuan-HD (1994): Structure and promoter analysis of an ABA- and stress-regulated barley gene, HVA1. *Plant Molecular Biology* 26, 617-630.
- Sweetser PB, Vatvars A (1976): High performance liquid chromatography analysis of abscisic acid in plant extracts. *Analytical Biochemistry* 71, 68-78.
- Tarczynski MC, Jensen RG, Bohnert HJ (1992): Expression of a bacterial mtd gene in transgenic tobacco leads to production and accumulation of mannitol. *Proceedings of the National Academy of Science USA* 89, 2600-2604.
- Tarczynski MC, Jensen RG, Bohnert HJ (1993): Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* 259, 508-510.
- Tardieu F, Zhang J, Davies WJ (1992): What information is conveyed by an ABA signal from maize roots in drying field soil? *Plant, Cell and Environment* 15, 185-191.
- Thomas H (1990): Osmotic adjustment in *Lolium perenne*; its heritability and the nature of solute accumulation. *Annals of Botany* 66, 521-530.
- Thomas H (1991): Accumulation and consumption of solutes in swards of *Lolium perenne* during drought and after rewatering. *New Phytologist* 118, 35-48.
- Turner LB, Stewart GR (1988): Factors affecting polyamine accumulation in barley (*Hordeum vulgare* L.) leaf sections during osmotic stress. *Journal of Experimental Botany* 39(200), 311-316.
- van Berkel J, Salamini F, Gebhardt C (1994): Transcripts accumulating during cold storage of potato (*Solanum tuberosum* L.) tubers are sequence related to stress-responsive genes. *Plant Physiology* 104, 445-452.
- van den Bosch J, Black IK, Cousins GR, Woodfield DR (1993): Enhanced drought tolerance in white clover. *Proceedings of the New Zealand Grassland Association* 55, 97-101.
- Vartanian N, Hervochon P, Marcotte L, Larher F (1992): Proline accumulation during drought rhizogenesis in *Brassica napus* var *oleifera*. *Journal of Plant Physiology* 140, 623-628.
- Veierskov B, Ferguson IB (1991): Ubiquitin conjugating activity in leaves and isolated chloroplasts from *Avena sativa* L. during senescence. *Journal of Plant Physiology* 138, 608-613.
- Vemieri P, Perata P, Armellini D, Bugnoli M, Presentini R, Lorenzi R, Ceccarelli N, Alpi A, Tognoni F (1989): Solid phase radioimmunoassay for the quantitation of abscisic acid in plant crude extracts using a new monoclonal antibody. *Journal of Plant Physiology* 134, 441-446.
- Vernon DM, Tarczynski MC, Jensen RG, Bohnert HJ (1993): Cyclitol production in transgenic tobacco. *The Plant Journal* 4(1), 199-205.
- Vierstra RD, Langan SM, Schaller GE (1986): Complete amino acid sequence of ubiquitin from the higher plant *Avena sativa*. *Biochemistry* 25, 3105-3108.
- Vilardell J, Goday A, Freire MA, Torrent M, Martinez MC, Tome JM, Pagès M (1990): Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. *Plant Molecular Biology* 14, 423-432.
- Walker-Simmons M (1987): ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. *Plant Physiology* 84, 61-66.
- Walker-Simmons MK, Reaney MJT, Quarrie SA, Perata P, Vemieri P, Abrams SR (1991): Monoclonal antibody recognition of abscisic acid analogs. *Plant Physiology* 95, 46-51.
- Walker-Simmons M, Sesing J (1986): Development of a sensitive immunoassay for abscisic acid in wheat grain utilizing a monoclonal antibody. In: Fourth International Symposium on pre-harvest sprouting in cereals. (Ed: Mares D) Westview Press, CO, 591-597.

- Wardle P (1991): Vegetation of New Zealand. Vol. 1. New Zealand plants. Cambridge University Press, Cambridge.
- Warr SRC, Reed RH, Stewart WDP (1988): The compatibility of osmotica in cyanobacteria. *Plant, Cell and Environment* 11, 137-142.
- Weiler EW (1979): Radioimmunoassay for the determination of free and conjugated abscisic acid. *Planta* 144, 255-263.
- Weiler EW (1982): An enzyme-immunoassay for cis-(+)-abscisic acid. *Physiologia Plantarum* 54, 510-514.
- Weretilnyk EA, Hanson AD (1990): Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. *Proceedings of the National Academy of Science USA* 87, 2745-2749.
- West CP, Gwinn KD (1993): Role of *Acremonium* in drought, pest, and disease tolerances of grasses. Proc. Second Internat. Symp. on *Acremonium*/Grass Interactions: Eds: Hume D.E., Latch G.C.M., Easton H.S.
- White J (1979): The plant as a metapopulation. *Annual Review of Ecology and Systematics* 10, 109-145.
- Whitford PN, Croker SJ (1991): An homogeneous radioimmunoassay for abscisic acid using a scintillation proximity assay technique. *Phytochemical Analysis* 2, 134-136.
- Williams PA (1989): Vegetation of the Inland Kaikoura range, Marlborough. *New Zealand Journal of Botany* 27, 201-220.
- Winzor CL, Winzor DJ, Paleg LG, Jones GP, Naidu BP (1992): Rationalization of the effects of compatible solutes on protein stability in terms of thermodynamic nonideality. *Archives of Biochemistry and Biophysics* 296(1), 102-107.
- Wolfrain LA, Dhindsa RS (1993): Cloning and sequencing of the cDNA for cas17, a cold-acclimation-specific gene of alfalfa. *Plant Physiology* 103, 667-668.
- Wolfrain LA, Langis R, Tyson H, Dhindsa RS (1993): cDNA sequence, expression and transcript stability of a cold-acclimation-specific gene, cas18, of alfalfa (*medicago falcata*) cells. *Plant Physiology* 101, 1275-1282.
- Wood KV, Stringham KJ, Smith DL, Volenec JJ, Hendershot KL, Jackson KA, Rich PJ, Yang WJ, Rhodes D (1991): Betaines of alfalfa. *Plant Physiology* 96, 892-897.
- Xu D, Duan X, Wang B, Hong B, Ho Tuan-HD, Wu R (1996): Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water-deficit and salt stress in transgenic rice. *Plant Physiology* 110, 249-257.
- Yancey PH, Clark ME, Hand SC, Bowles RD, Somero GN (1982): Living with water stress: evolution of osmolyte systems. *Science* 217, 1214-1222.
- Yang SF, Hoffman NE (1984): Ethylene biosynthesis and its regulation in higher plants. *Annual Review of Plant Physiology* 35, 155-189.
- Yang W, Nadolska-Orczyk A, Wood KV, Hahn DT, Rich PJ, Wood AJ, Saneoka H, Premachandra GS, Bonham CC, Rhodes JC, Joly RJ, Samaras Y, Goldsbrough PB, Rhodes D (1995): Near-isogenic lines of maize differing for glycine-betaine. *Plant Physiology* 107, 621-630.
- Yen HE, Grimes HD, Edwards GE (1995): The effects of high salinity, water-deficit, and abscisic acid on phosphoenol pyruvate carboxylase activity and proline accumulation in *mesembryanthemum crystallinum* cell cultures. *Journal of Plant Physiology* 145, 557-564.
- Yim HH, Villarejo M (1992): *OsmY*, a new hyperosmotically inducible gene, encodes a periplasmic protein in *Escherichia coli*. *Journal of bacteriology* 174(11), 3637-3644.
- Zeevaert JAD, Creelman RA (1988): Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* 39, 439-473.
- Zimmermann MH, Milburn JA (1982): Physiological responses to moderate water stress. In: Encyclopaedia of plant physiology. New series. Physiological plant ecology II. Vol. 12B. (Eds: Lange OL, Nobel PS, Osmond CB, Ziegler H) Springer-Verlag, Heidelberg, 135-153.
- Zuniga GE, Argandona VH, Corcuera LJ (1989): Distribution of glycine-betaine and proline in water stressed and unstressed barley leaves. *Phytochemistry* 28(2), 419-420.

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Erratum

Page Position

	general	<i>Festuca novae-zealandiae</i> should read <i>Festuca novae-zealandiae</i>
iii	title	Acknowledgements should read Acknowledgements
iv	Line 2	<i>F. novae-zealandiae</i> should read <i>F. novae-zealandiae</i> (Hack) Cockayne
2	Line 14	The acronym DSIR not given in full, as Department of Scientific and Industrial Research.
4	Line 3-4	exam-pes should read examples
13	Line 17	responses should read response
13	Line 29	and are generally should read and generally
18	Line 15	concentration should read concentrations
19	Line 5	was should read is
19	Line 24	tissues should read parts of the tiller
49	Fig. 2.9	X-axis title should read Protein ($\mu\text{g}/\text{mL}$)
68	Line 2	were should read was
87	Line 12	to short should read too short
117	Fig. 4.9	Y-axis title should read Mean Tiller Mass (g FW)
148	Line 27	survives should read avoids
160	Line 9	second repeat should read second experiment
160	Line 21	concentration should read concentrations
179	Line 8	ng/g FW should read ng/g DW
191	Line 13	plants should read plant's
224	Line 8	antisera should read antiserum
233	Line 18	plants should read pots
239	Line 30	monoclonal should read polyclonal
243	Line 10	plants should read plant's
245	Line 17	omit the
246	Line 3	response should read responses
246	Line 8	than the laminae should read than in the laminae
249	Line 4	contain should read constrain
xxviii	Line 23	Parry, Horgan (1991) should read Parry A, Horgan R (1991)
