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TISSUE-SPECIFIC RESPONSES TO WATER DEFICIT IN THE NEW ZEALAND XEROPHYTIC TUSSOCK SPECIES *Festuca novae-zelandiae*

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ABSTRACT

Festuca novae-zelandiae (Hack.) Cockayne is an endemic New Zealand perennial tussock forming grass of the family Poaceae. Morphologically F. novae-zelandiae exhibits a number of leaf adaptations associated with dehydration postponement as reflected in the climatic distribution of this species and its occurrence as a physiognomic dominant grass in semi arid short-tussock grasslands. Biochemical studies into the drought tolerance of this species have indicated the occurrence of tissue specific responses with respect to abscisic acid (ABA) and proline accumulation and protein turnover suggestive of a preferential protection of the tiller base and associated meristematic zones at the expense of lamina tissues. Further tissue specific biochemical responses to water-deficit stress in F. novae-zelandiae have been investigated. Changes in water-soluble carbohydrates (WSC) were monitoured over a 49-day dry-down period (decline in soil water content from 30% to 4%) in consecutive leaf segments comprising the leaf base (meristem region), elongation zone, the enclosed and exposed lamina, as well as basal sheath segments from the two next oldest leaves. In fully hydrated leaf tissues polymers of fructose (fructans) were the main WSC present and were mainly low molecular weight fructans of the inulin and neokestose series with the average degree of polymerization (DP) of fructan pools from 6 to 9. The highest fructan concentrations were present towards the leaf base. Fructan concentrations decreased over the course of the dry-down, although remained significantly higher in the meristem region of the tiller base with respect to any other tissue, until tissue water content fell below 45%. By day 49 of the dry-down period, the average DP of the fructan pool in tissues was from 3 to 5. Sucrose content increased in each tissue during the course of the dry-down, and was highest at the leaf base, where a concentration of 200 μ mol g⁻¹ dry weight was measured after 49 days of dry-down.

The negative correlation between fructan and sucrose content, which indicates an inter – conversion dependent on tissue water content, suggests that, in this specises, fructans serve as a carbohydrate storage pool, while sucrose stabilises the meristem during extreme water deficit.

I

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Thanks to Balance and the other characters from the Lab

To Mum and Dad

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CHAPTER 1

Introduction and Literature Review

1.1 Overview

Plant water deficits occur when the rate of water loss through transpiration exceeds the rate of water uptake. The resulting reduction in cellular turgor pressure, i.e. below that of the maximal potential pressure, is defined as water deficit stress (Osmond *et al.*, 1987). Even under moist soil conditions and at maximum water absorption rates, plants may experience transient water deficits on a daily basis, progressing from a state of full turgor predawn after hours with closed stomata, to a water deficit state midday when photosynthetic rates, and correspondingly transpirational rates, are at their highest (Schulze *et al.*, 1985).

In drying soils or soils of increasing salinity, as the soil water potential becomes increasingly negative the water potential gradient favouring water movement from the soil into a plant's root system diminishes. If the rate of water uptake becomes too slow to replace the daily water loss from a plant, permanent water deficits develop impairing metabolic functions and potentially leading to tissue or plant death (Pugnaire *et al.*, 1996).

Globally, water deficit stress is the major abiotic factor limiting crop productivity (Boyer 1982) and is set to become an increasing problem as the human population increases. Forecasts based on recent population trends have placed the global population at around 9 billion by the year 2050 (World Population Prospects, 1999). This growth will inevitably place greater demands on fresh water reserves for domestic requirements alone (Postel *et al.*, 1996). Additionally, projected climate change scenarios suggest that increased aridity is a likely predicament in many regions of the globe (Petit *et al.*, 1999). In view of these projections it is important to understand the mechanisms that underline plant survival in arid or drought prone environments. An understanding of such mechanisms can potentially through classical breeding programmes or gene transfer methods, equip crop and forage species for drier environmental conditions (Volaire and Thomas 1995, McManus *et al.*, 2000, Rathinasabapathi 2000).

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1.2 Ecological division of plants based on water requirements

Based on the relative amounts of water for the completion of the lifecycle, plant species can be grouped, ecologically, as hydrophytes, mesophytes or xerophytes (Levitt 1980, Blum 1988).

Hydrophytes are plants that require either full or partial submersion in free water and, in this environment, complete their lifecycle free from water-deficit stress. Terrestrial plants are divided either into mesophytes, which are plants that require a regular supply of water to maintain metabolic activity, growth and lifecycle progression, or xerophytic plant species that are indigenous to arid or semi-arid environments, and exhibit physiological, morphological, metabolic and lifecycle adaptations to persist during prolonged periods of drought.

Further division within the xerophytes exists based on the overall strategy or mode of drought survival (Kramer 1980, Turner and Jones 1980). These are (i) drought escape, (ii) drought tolerance at low plant water potential (tissue dehydration tolerance), and (iii) drought tolerance at high plant water potential (dehydration postponement).

1.2.1 Drought escape

For plants that employ a drought escape strategy, seed germination, plant growth, tissue development, and seed (or spore) production has evolved to coincide with seasonally favourable environmental conditions. At these times water is available for these phases in the lifecycle to be completed before severe physiological water deficits occur. For these species, drought periods are essentially "escaped" in a desiccant tolerant phase as seeds or spores.

Also common amongst drought escape species is that the onset of water deficit stress may accelerate the transition from a vegetative growth phase to the sexually mature and reproductive phase. In cereal species for example, senescence of vegetative tissues is required for seed set and maturation to occur (Yang *et al.*, 2001), and studies have shown that water deficit stress increases processes associated with senescence including elevated leaf absciscic acid (ABA) concentrations, increased lipid peroxidation, and leaf nitrogen and chlorophyll loss. Further processes associated with reproduction and seed development such as reserve carbohydrate mobilisation and translocation from stem

tissues to developing grains are stimulated (Gebbing and Schnyder 1999, Yang et al., 2001, Yang et al., 2003).

Often environmental cues in addition to the presence of water are required to break seed dormancy in these species so that the growth phase of the lifecycle coincides with additional favourable environmental growth conditions. For example some annual species of the Asteraceae, endemic to south-western Australia, germinate during cool winter periods insuring that their vegetative growth phase is completed before summer and associated high temperatures, high evaporative demand and periods of less frequent rainfall (Schutz *et al.*, 2002).

Plants that employ a water deficit escape strategy may also posses a high degree of developmental plasticity so that the period of seed production in sexually mature plants is determined by the duration of accessible water in the environment. For example, in *Centaurea ergophore*, a plant endemic to the Mediterranean region, a second flowering phase follows the primary or normal flowering period of spring and early summer if rains persist into the usually dry summer months (Ruiz de Clavijo 2002). This second flowering allows a second seed crop to be produced if favourable conditions for seed development endure thereby increasing the reproductive efficiency of the species.

1.2.2 Dehydration postponement

Dehydration postponement strategies in contrast to water deficit escape strategies, involve persistence of the vegetative plant through a drought period by means of adaptations that minimize tissue water loss. As approximately 90% of the total water loss from vascular plants occurs as transpiration from stomata (Monneveux and Belhassen 1996), tissue dehydration postponement involves mechanisms associated with minimising water loss during carbon assimilation and maximising water gain through root development. The most effective method to reduce water loss from leaf surfaces is via stomatal closure, but stomatal closure concurrently reduces gas exchange and therefore limits photosynthetic CO₂ assimilation (Flexas & Medrano 2002). For many xerophytes, evaporation from the leaf surface is reduced by limiting the period of maximal stomatal conductance to the night period when temperatures are generally lower and atmospheric humidity higher. This is observed commonly in plants that utilise the crassulacean acid metabolic (CAM) photosynthetic pathway (Ting 1985). In these plants, CO₂ is fixed in the dark initially into

C-4 organic acids (e.g. malic acid) by phosphoenolpyruvate carboxylase (PEPC). These C-4 organic acids are stored in the vacuoles of leaf cells and subsequently decarboxylated during the light period generating an internal CO₂ concentration which is re-assimilated in the chloroplast by rubisco, followed by the conventional (C-3) photosynthetic carbon reduction cycle. This fixation of CO_2 at night, followed by release inside the leaf during the daytime, negates the requirement for stomatal opening during the daytime when evapotranspiration rates are highest. Thus under comparable conditions plants exhibiting CAM photosynthesis may exhibit several fold higher rates of water use efficiency (ratio of grams of water lost to grams of CO₂ fixed) when compared with mesophytic C-3 species (ratios of 50 and 600, respectively) (Weier et al., 1982, Drennan and Nobel 2000). In plants exhibiting CAM photosynthesis, a number of additional morphological traits that minimize water loss under dehydrating conditions are also observed. These include thick cuticles with associated waxy layers, low surface to volume ratios of leaves, specialised cells for water storage (succulence) (as is observed in members of the Cactaceae), and a reduction in stomatal frequency and pore size (Zimmermann and Milburn 1982, Christensen-Dean and Moore 1993, Cushman 2003).

In perennial C-3 xerophytes, a range of morphological and developmental traits are evident that minimise transpiration rates under high evaporation conditions. For example, seasonal leaf dimorphism is observed in *Cistus incanus* L. subsp. *Incanus*, a plant endemic to the Mediterranean (Aronne and De Micco 2001). In summer this species develops brachyblasts, which are small leaves covered in trichomes with stomata localised within crypts on the lower leaf surface. Both stomatal crypts and trichomes reduce airflow across the surface of the stomata and in doing so reduce evapotranspiration rates and tissue water loss when the stomata are open. In contrast in winter when evapotranspiration rates are lower and rainfall is more frequent, dolichoblasts with large leaves develop, with a total leaf area five times that of the summer leaf. Winter leaves are also found to possess fewer trichomes, and the stomata are not localised in crypts but instead are uniformly distributed across the lower epidermal surface. This leaf dimorphism helps the plant to be water efficient in dry-hot seasons when evaporation rates are high, and competitive in the cooler wetter months when abiotic conditions are favourable for growth through increased photosynthetic area (Aronne and De Micco 2001).

As an alternative to leaf dimorphism, a reduction in the leaf transpiration area is observed. In members of the genus *Euphorbia*, leaves are shed at the beginning of the dry season (Monneveux and Belhassen 1996) or leaves senesce as water deficit stress develops (Tardieu 1996) as is observed in some Mediterranean adapted perennial grass species under drought conditions (Volaire *et al.*, 1998a). Loss of leaf area effectively reduces the surface area of the plant from which water can be lost, and helps to prevent water loss from perenniating vegetative tissues, such as tiller bases.

1.2.3 Dehydration tolerance

Although mechanisms associated with water deficit avoidance allow plants to inhabit arid environments, prolonged exposure to water deficit stress ultimately leads to severe tissue dehydration and plant death. There are some plants, however, that can tolerate severe tissue dehydration. These xerophytes are collectively referred to as poikilohydric or resurrection plants and exhibit a survival strategy of dehydration tolerance in which tissue water deficit is almost complete such that the plant has no bulk cytoplasmic water in its tissues [~23% water on a fresh weight basis, or ~0.3 $(gH_2O)(g dry weight)^{-1}$], and may reach a level of hydration in equilibrium with the surrounding air (Hoekstra et al., 2001, Chaves et al., 2003). In the desiccated state, resurrection plants remain viable in a quiescent mode until water becomes available and metabolism and normal plant functions resume. In some species, e.g. Chamaegigas intrepidus an aquatic resurrection plant, complete tissue recovery can still occur following 11 months in the desiccated state. In *Craterostigma* species inhabiting granite outcrops in southern Africa, over two years may be endured in the dry-down state without loss of viability, while for some ferns up to five years in the desiccated state does not inhibit resurrection on the re-supply of water (Hartung et al., 1998, Iturriaga et al., 2000, Scott 2000).

Attainment of a quiescent state is not simply achieved through tissue dehydration. It is a regulated process initiated in response to dehydration and elevated levels of the plant hormone ABA, and initially involves the accumulation of specific osmotically active compounds in a process termed osmotic adjustment (Scott 2000). Osmotic adjustment is the active accumulation within cells of low-molecular-weight compatible solutes (metabolically non-toxic) that via their accumulation decrease the cellular osmotic potential promoting turgor maintenance and the continuity of turgor-dependent processes such as stomatal opening, photosynthesis and leaf elongation at low water potentials

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(Sambo 1981, Kramer 1993). Although the maintenance of growth under declining soil water availability appears inversely correlated to plant survival (Knight 1973), osmotic adjustment may aid in extending the lifetime of metabolically active tissues between ephemeral showers, and allow a period of metabolic and physiological adjustment to changing environmental water availability in preparation for drought (drought hardening) (Mooney *et al.*, 1983). Solutes actively accumulated as components of osmotic adjustment include amino acids (e.g. proline), sugar alcohols (e.g. pinitol), water-soluble carbohydrates (e.g. sucrose and fructans), and quaternary ammonium compounds (e.g. glycine betaine) (Bray 1997, Kerepesi and Galiba 2000). In addition to being compatible to cellular metabolism, osmolytes appear to be readily available as intermediates of normal metabolic pathways, such as protein and carbohydrate biosynthesis so that accumulation is derived from the disturbance of metabolic activity rather than the synthesis *de novo* of specific compounds (Turner and Jones 1980).

Osmolytes may also have direct roles in the conformational protection of cellular components against stresses associated with dehydration. In this role, osmolytes are referred to as osmoprotectants. In particular, water-soluble carbohydrates appear to be essential in the dessication tolerance of resurrection plant species with sucrose the predominant carbohydrate present in the preserved desiccated state (Ingram and Bartels 1996, Scott 2000). Sugars appear to function in desiccation protection in two ways. Firstly through the conformational stabilisation of macromolecular components of membranes and proteins by substituting the hydrogen bonding derived from water in the hydrated state, and secondly by stabilising the internal spatial cellular arrangement through vitrification (Crowe *et al.*, 1992, Crowe *et al.*, 1998, Alison *et al.*, 1999). In terms of sugars that accumulate in response to water stress, fructans comprise a major group in many plant species, including the agriculturally important grasses (Pollock *et al.*, 1996).

1.3 Plant fructans

1.3.1 Fructan occurrence in plants

Starch represents the main form of storage carbohydrate for the majority of vascular plants. However, for ca. 12 to 15% of the Angiosperms the main storage carbohydrate is water-soluble linear and/or branched polymers of fructose know as fructans (Pollock and Cairns 1991, Hendry 1993, Cairns 2003). Included amongst the ca. 45,000 fructan accumulating species are cultivated plants such as the forage and cereal grasses, including

wheat, barley, oats, fescues and perennial rye grass; vegetables, e.g. onions, asparagus, artichoke and lettuce; and ornamentals e.g. dahlia, tulip and daylilies (Bieleski 1993, Hendry 1993, Vijn and Smeekens 1999).

Unlike bacterial fructans, which occur predominantly as unbranched levans (linear $\beta(2-6)$ – linked fructosyl units) synthesized by a single fructosyl transferase, plant fructans in general exhibit a higher degree of structural diversity and are synthesised via a multiple fructosyl transferase enzyme pathway (Vijn & Smeekens 1999). Five structural classes of plant fructans can be differentiated: inulin, levan, mixed levan, inulin neoseries, and levan neoseries, and to date four plant fructosyl transferases have been characterized [(1-SST, (E.C. 2.4.1.99); 1-FFT, (E.C. 2.4.1.100); 6G-FFT; 6-SFT, (E.C. 2.4.1.10)] that account for this structural diversty (Pollock *et al* 1996, Vijn & Smeekens 1999, Cairns 2003).

Inulins (linear β (2-1-linked fructans) are considered the simplest of the plant fructans and are synthesised by the concerted activity of two enzymes: sucrose:sucrose 1fructosyltransferase (1-SST; E.C. 2.4.199) and fructan:fructan 1-fructosyltransferase (1-FFT, E.C. 2.4.100). 1-SST catalyzes the initial fructosyl transfer from a donor substrate sucrose molecule to a recipient sucrose molecule to form the trisaccharide 1-kestotriose (1-kestose). 1-FFT is also a fructosyl transferase but uses 1-kestotriose or higher degree of polymerisation (DP) fructans as the fructosyl donor, and either fructans (1-kestotriose or >DP inulins) or sucrose as the fructosyl recipient (Fig 1). Through the activity of 1-FFT, inulin fructans of DP>3 are synthesized and it appears that the enzymatic characteristics of 1-FFT determines the specific inulin pattern (mean DP) present within a species.

<u>Chapter</u> 1	Introduction and Literature Review						
	1-SST:	G1-2F	+ G1-2F	→	G1-2F1-2F	+ G	
		sucrose	+ SUCIOSE	→	1-kestose	+ glucose	
	1-FFT:	G1-2F(1-2F)m	+ G1-2F(1-2F)n	i →	G1-2F(1-2F)m-1	+ G1-2F(1-2F)n+1	
		Elongation of fructan chain, $m > 0$ and $n \ge 0$					
	6-SFT:	G1-2F	+ G1-2F1-2F	→	G1-2F1(6-2F)-2F	+ G	
		sucrose	+ 1-kestose	-)	bilurcose	+ glucose	
		G1-2F	+ G1-2F	→	G1-2F6-2F	+ G	
		sucrose	+ sucrose	→	6-kestose	+ glucose	
	6G-FFT:	G1-2F	+ G1-2F1-2F	→	F2-6G1-2F	+ G1-2F	
		SUCTOSE	+ 1-kestose	→	neokestose	+ sucrose	
	FEH:	G1-2F(1-2F)n		→	G1-2F(1-2F)n-1	+ F	
		fructan, $n > 0$			fructan	+ fructose	

Figure 1. Enzymatic activities of different plant fructosyltransferases involved in fructan synthesis and of fructan exohydrolase (FEH), an enzyme involved in fructan degradation.

Inulins appear to be the sole fructan isomeric form accumulated in dicotyledonous plants, and the inulin biosynthetic pathway has been characterized at the gene and protein level for the economically important Asteraceae species chicory (Cichorium intybus L.), artichoke (Cynara scolymus) and Jerusalem artichoke (Helianthus tuberosus) (Van den Ende and van Laere 1996, Koops & Jonker 1996, Lüscher et al., 1996, Van der Meer 1998, Hellwege et al., 1998). The mean DP of fructans accumulated in vivo in the storage organs of Jerusalem artichoke is DP 8-10, and for artichoke, DP 65 (Hellwege et al., 1998). Hellwege and colleagues (Hellwege et al., 1998) showed that tobacco (a non fructan accumulator) protoplasts engineered with 1-FFT genes from either artichoke or Jerusalem artichoke and incubated in the presence of DP3, DP4 and DP5 inulin fructans, resulted in the accumulation of protoplast fructan pools of mean DP equivalent to that found in vivo for each species - i.e. DP 8-10 for Jerusalem artichoke and DP 65 for artichoke. This result indicates that the 1-FFT enzymes of C. scolymus and H. tuberosus have different affinities for the same inulin substrate. The homology in amino acid sequence between the 1-FFT enzymes for both species was high at 78%, although was sufficiently different to impart different catalytic properties of each enzyme (Hellwege et al., 1998).

Fructans of the Liliales and Poales (monocotyledonous orders) exhibit a greater structural complexity than the inulin fructan series of dicotyledonous species. In addition to the inulin series, members of the Liliales, e.g. onion and asparagus, synthesize fructan molecules with an internal glucose unit. This requires the activity of a third enzyme, 6Gfructose: fructose transferase (6G-FFT). The enzyme 6G-FFT uses 1-kestoriose as substrate and catalyses the transfer of the terminal fructosyl residue to the glucose end of a recipient sucrose molecule forming a β -2,6 linkage between the glucose and transferred fructose unit. The trisaccharide product formed is neokestose (6-G-fructosyl sucrose) on which higher DP neo-inulin molecules are built by the β -1,2 linkage of fructosyl residues to either or both fructose ends of the trisaccharide catalysised by 1-FFT. In essence, neoseries inulins are composed of two linear β -1,2 linked fructosyl chains, one attached to the fructosyl residue of the base sucrose molecule, and the other attached to the glucose residue of the sucrose base. The 6G-fructosyltransferase activity of 6G-FFT was characterized by Vijn and colleagues (Vijn et al., 1997) who cloned the 6G-FFT gene from onion using a fructosyl transferase gene from barley as a probe, and then introduced the 6G-FFT cDNA into chicory, a plant that produces inulin fructans only. The chicory transformants were able to produce both inulin and inulin neoseries fructans while in contrast, tobacco plants transformed with the onion 6G-FFT cDNA were able to produce an active protein but only neokestose, if 1-kestotriose was supplied as a substrate. Greatest fructan structural complexity is to be found in members of the Poales, particularly temperate grasses of the Poaceae. In addition to inulins and inulin neoseries fructans characterised from members of the Liliales and Asterales, species of the Poaceae may synthesise levans (referred to as phleins in plants), mixed levans (also called graminans) containing both 2,1- and 2,6-linked β -fructosyl units, and the levan neoseries: polymers predominantly of β -2,6-linked fructosyl residues with an internal glucose moiety.

1.3.2 Fructans as plant osmoprotectants

In early transgenic studies, fructans have been shown to be particularly promising as a means of improving plant performance under drought stress without negatively impacting on plant growth or yield under non-stressed conditions. Pilon-smits *et al.*, (1995) transformed tobacco, a non-fructan accumulating plant with the *Sac B* gene from *Bacillus subtilis*. This gene encodes for levan sucrase, the enzyme that catalyses the transfer of fructose from a sucrose molecule to a recipient sucrose molecule to form the simplest fructan of the levan series, the main fructan type of bacteria. Fructans of larger degree of

polymerization (DP) are synthesized by the fructosyl transferase activity of the levan sucrase adding fructose units from substrate sucrose molecules to the fructosyl end of fructan molecules. These fructans are characterized by $\beta(2-6)$ -linked fructosyl residues, and in bacteria may consist of over 100,000 fructose units (DP>100,000) (Vijn & Smeekens 1999). Under polyethylene-glycol mediated water stress conditions tobacco plants expressing the SacB gene and consequently accumulating levan fructans of DP>25,000 were found to have a growth rate of 55% greater than wild-type tobacco plants and increased fresh and dry weight yields by 33 and 59%, respectively, were observed when compared to wild-type plants under the same conditions (Pilon-smits et al., 1995). Under non-stressed conditions, there was no difference in these growth parameters between wild type or SacB transformants indicating that the addition of fructan biosynthesis had not affected the allocation of photosynthate to plant growth. What was not understood in this study was how the addition of fructan metabolism in the non-fructan accumulating species tobacco had improved its tolerance to water stress, as the level of fructan accumulation was not thought to be high enough to have resulted in an osmotic effect (Pilon-smits et al., 1995).

More recent studies also support an osmoprotectanct role for fructans in drought tolerance. Several studies have shown that fructans are able to stabilise biological membranes in a dehydrated state (Demel et al., 1998, Hincha et al., 2000, Vereyken et al., 2001, Hincha et al., 2002). As cellular organisation is derived from a hydrophobic effect that drives the assembly of phospholipids into selectively permeable barriers, membrane dehydration results in the disruption of this assembly and complete desiccation causes the irreversible loss of cellular organization and cell function (Tanford 1978). Loss of membrane integrity is considered a major component of desiccation and freezing injury to plant cells (Oliver et al., 2001). Fructans have been shown to be able to stabilise the liquid-crystalline bilayer of membrane systems under dehydrated conditions (Hincha et al., 2000). In the hydrated state, 10-12 water molecules are hydrogen bonded to each phosphate head group of the membrane phospholipids. In this state, the lateral spacing between the head groups is sufficient to minimize van der Waals interactions of the hydrocarbon chains thus keeping the lipid bilayer in a liquid crystalline state. As water is removed, the head groups become more densely packed and the spacing between the hydrocarbon chains diminishes such that the van der Waals interactions between the hydrocarbon chains increase. In effect, this increases the phase transition temperature required to maintain the membrane in a liquid crystalline state (e.g. a shift from -10° C to 60° C). Thus at biological temperatures, the dehydrated membrane will exist in a gel phase (Crowe *et al.*, 1992). Phase changes from the liquid to gel state and *vice versa* are associated with increased membrane permeability or leakage and loss of cellular function (Demel *et al.*, 1998). Demel *et al.*, (1998) suggested that fructans were able to substitute for the water molecules between lipid polar head groups and in doing so maintain the lateral spacing between the hydrocarbon chains minimizing van der Waals interactions and preventing phase transition. Vereyken and colleagues (Vereyken *et al.*, (2001) recently showed in a follow up study to that of Pilon-Smits *et al.*, (1995), that high DP fructans produced by *Sac B* levan sucrase are able to interact with the headgroups of phospholipids in hydrated membrane systems stabilising the liquid-crystalline-phase, a role that is consistent with cellular water deficit protection and drought tolerance.

Using unilamellar liposomes composed of egg phospatidylcholine as a model system, Hincha *et al.*, (2000) showed that fructan extracts from chicory roots and dahlia tubers were able to stabilize liposomes during freeze drying, and that the fructans were hydrogen bonded to the phosphate in the phospholipid headgroups. This result strongly supports the role of fructans as osmoprotectants during cellular water deficits.

1.3.3 Evolution of the fructan flora

Fossil records indicate that the evolution of the majority of the fructan rich angiosperm families (comprising approximately 15% of the group) coincided with a climatic shift towards contrasting seasons of wet and dry periods during the Oligocene to mid-Miocene epochs (32-15 Ma ago) (Hendry 1993). The mid-Miocene is also the period in which large dry open grassland habitats established, and the divergence of the Pooidea, the only subfamily of the Poaceae to accumulate fructans as their major reserve carbohydrate, occurred (Bonnett *et al.*, 1997, Jacobs *et al.*, 1999, Kellogg 2001). Within this subfamily are the agronomically important forage grasses, e.g. the *Lolium* species, and the cereals *Aveneae* (oats) and *Triticea* (wheat, barley and rye) (Kellogg 1998). Geographically, fructan-accumulating plants are concentrated in temperate climatic zones and are almost absent from tropical regions. This specific distribution combined with the timing of their appearance in the fossil record suggests that fructan metabolism in angiosperms may have evolved under the selective pressure of seasonal water shortages. Hendry (1993) using this line of thought proposed that in addition to fulfilling the roll of reserve carbohydrate, an

additional natural function of fructan metabolism was osmoregulation during cell expansion and tissue growth under conditions of limited water availability.

If fructan metabolism evolved in the Poaceae under the selective pressure of water-deficit stress and plays a functional role in the alleviation of water deficit stress, this is likely to be most evident in wild Poaceae species endemic to arid or semi arid grasslands.

1.4 Festuca novae-zelandiae (Hack.) Cockayne

Festuca novae-zelandiae (Hack.) Cockayne is an endemic New Zealand perennial tussock forming grass of the family Poaceae. It is widespread throughout New Zealand from swamp and high rainfall (>6000 mm per annum) areas to semi arid regions east of the main divide (southern alps) where the annual rainfall may be less than 350 mm (Edgar and Connor 2000). In these semi arid regions dominated by short-tussock grasslands *F. novae-zelandiae* is often the physiognomic dominant species (Lord 1993, Connor 1998).

Within this habitat *F. novae-zelandiae* is exposed to annual precipitation that in some years may not exceed 200 mm. In addition high evapotranspiration conditions are frequent as a consequence of hot dry föhn-type winds generated over the Southern Alps, a mountain range with peaks in access of 3,000 m (Wardle 1991). Despite these desiccating conditions the life span of individual tussocks may exceed 50 years (Moore 1976), and through vegetative clonal fragmentation single clones may persist for hundreds of years (Lord 1993).

Many xeromorphic features have been identified in F. novae-zelandiae that represent adaptations to either a high evapotranspiration environment, a semi arid environment, or a combination of both (Baker 1953, Lord 1992, Abernethy *et al.*, 1998). These include a longitudinally curled leaf lamina which creates an almost entirely enclosed air space over the adaxial leaf surface, the only part of the lamina in which stomata are present and the presence of numerous trichomes on the leaf surface, which may disrupt and slow air movement over the leaf increasing the boundary layer effect. The leaves grow erect, and are structurally supported by subepidermal sclerenchyma (Connor 1960). They do not abscise following senescence and so remain intact and erect within the tussock creating a microclimate effect by reducing air movement through the tussock and providing a level of shade. In addition to adaptations that reduce water loss, the root system of F. novaezelandiae seems adapted to utilising both short-term water supplies in the topsoil that may occur in sporadic summer rainfall, or longer-term water sources that may occur deeper in the soil profile. This is indicated by a deep-root system (down to two metres) with a welldeveloped system of laterals in the topsoil characteristic of plants adapted to regions where transpiration and desiccation rates are high (Baker 1953).

Investigations into biochemical responses to water deficit stress in F. novae-zelandiae have also revealed that this species adjusts biochemically on exposure to water deficit stress. In addition, this adjustment appears to occur in a tissue specific manner suggestive of preferential protection of meristematic tissues in the tiller base at the expense of mature lamina tissues. These responses include the accumulation of ABA in mature leaf tissues and elevated levels of ubiquitinated protein preceding tissue senescence (leaf burn). In contrast, in the tiller base containing the apical, and lamina intercalary, meristematic tissues, the osmolyte proline accumulates and this tissue remains at a relatively higher hydration level with respect to corresponding mature lamina tissues.

1.5 Thesis aims

To further investigate the mechanisms of drought survival of this xerophytic grass, the aim of this current work was to detail water-deficit induced cellular responses with particular focus on solute potential and the water-soluble sugar composition of aerial tiller tissues. Specifically changes in tissue specific water-soluble carbohydrates were to be documented during an imposed water-deficit period to identify preferentially accumulated or depleted sugars that may underlie a specific drought tolerant function important to the vegetative survival of *F. novae-zelandiae* during prolonged dry periods.

This thesis sets out to test the hypothesis that vegetative meristematic tissues of the tiller base of the xerophytic grass *F. novae-zelandiae* undergo preferential osmotic adjustment and accumulate specific water soluble carbohydrates at the expense of differentiated lamina tissues as a component of water deficit stress acclimatisation and persistence in drought prone environments.

For this work *F. novae-zelandiae* tussocks were cultured in pots containing 4 L of soil and water-deficit conditions were imposed through withholding water from the soil. Periods over which watering was ceased were termed dry-downs. During these periods specific leaf tissue zones were harvested at specific time points in the dry-down to isolate tissues at different stages of water-deficit stress and allow a comparative analysis of specific

parameters i.e. solute potential and/or specific water soluble carbohydrate composition relative to soil water status. Three such dry-down periods were imposed over three consecutive spring/summer periods to coincide with periods of maximal potential leaf growth, metabolic activity and correspondingly vulnerability to water-deficit stress. The results from dry-down I and II are reported in chapter 3, while the final dry-down is reported in chapter 4 and discussed in chapter 5.

CHAPTER 2

Materials and Methods

2.1 Plant material

A *Festuca novae-zelandiae* (Hack.) tussock consisting of approximately 200 tillers was gifted from the Otari Botanical Gardens, Wellington, New Zealand. The ancestral plant material, from which this tussock was vegetatively descended, had been collected from the grasslands of Molesworth Station, South Island, New Zealand and was catalogued under the Otari accession number 750039.

The tussock was divided into 6 smaller individual tussocks comprising 20-40 tillers and each was potted into separate PB-12 black polythene bags containing a horticultural grade bark/peat/pumice mix at a ratio of 50:30:20, respectively (Dalton Nursery Mix, Tauranga New Zealand). The mix was supplemented with Osmocote PlusTM fertiliser pellets at a rate of 1 kg/m³ of potting mix (Abernathy 1996). A second group of eight *F. novae-zelandiae* tussocks was purchased from Naturally Native NZ Plants Ltd, 30 Gammon Mill road, RD3, Tauranga, New Zealand. These plants had been propagated from seed produced at the Naturally Native NZ Plants Ltd nursery, and were descended from an original seed stock obtained from Southern Seeds Ltd in 1988. No information was available on the origin of the original seed stock. Each of these eight tussocks were divided into 3 smaller tussocks of approximately 40 tillers and potted into 4-L capacity pots containing Kiranga silt loam soil with one tussock per pot.

Both sets of plants were maintained in a Glasshouse at the Plant Growth Unit (PGU), Massey University, Palmerston North, New Zealand. Within the glasshouse, partial temperature control was provided, maintaining temperatures above 10°C at all times with automatic venting at temperatures exceeding 25°C. Watering occurred every other day in summer and was reduced to twice weekly by winter. Water was supplied at the soil surface until seepage from the drainage holes in the base of each pot was observed to insure the soil was well wetted and plants were maintained in a hydrated state. Pest control, when necessary was managed by the PGU staff with the insecticide spray AttackTM (Crop Care Holdings Ltd., Richmond, Nelson, New Zealand).

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The *Lolium perenne* cultivar used in this study was *L. perenne* (L.) ABY-BA 1689.81U. This grass was originally obtained from the Institute of Grassland and Environmental Research, Aberystwyth, UK and was maintained in 25-L capacity pots containing a free draining potting mix in a vented greenhouse without climate control at the Max Planck Institute of Molecular Plant Physiology, Golm, Germany.

2.2 Soil conditions

2.2.1 Description of soil used for dry-downs

The soil medium used during the dry-down experiments was a local Palmerston North soil, Kairanga silt loam obtained as topsoil from a pasture paddock at Aorangi Farm AgResearch, Grasslands Palmerston North, New Zealand. Following removal from the paddock, the soil was air dried on a glasshouse concrete floor for 7 days and then crushed under foot, followed by sifting through a 6 mm gauge sieve. The sieved soil was used to fill cubic 4-L capacity free draining plastic pots, and the soil was top-dressed with Osmocote plus pellets, a general slow release fertiliser, at a rate of 25 g/pot. This soil type was recommended by Dr David Baker a pastoral scientist at AgReasearch Grasslands, Palmerston North, New Zealand, for its slow drying rate relative to commercially available bark and pumice potting mixes and sand based soils. In general silt loam soils have a greater available water capacity (AWC) than either sand or clay-type soils. (McLaren & Cameron 1993). Available water capacity is defined as the amount of water that a soil can store for plant growth, and is equal to the amount of water held at field capacity less the amount held at the permanent wilting point. Field capacity is described as the stable water content at complete soil saturation at which rapid drainage has effectively ceased (i.e. water held in the soil against gravity), and the permanent wilting point is the soil water content at which the amount of accessible water in the soil is too low for wilted plants to recover to full turgor within a 24 h cycle (McLaren & Cameron 1993). At the permanent wilting point most of the soil water content is tightly bound in the soil matrix with little if any free water accessible to the plant root system. Field capacity for a number of silt loam soils in New Zealand has been reported at between 33.8% and 47% (v/v), and the permanent wilting point at between 14.5% and 23.9% (v/v) (Gradwell 1976).

2.2.2 Calculation of soil water content

For non-water stress conditions (ie for hydrated plants) it was recommended that the soil water content be maintained at between 80 and 85% of the field capacity of the soil (A. Carran, AgReasearch Grasslands personal communications). To determine the field capacity of the Kairanga silt loam, four pots of soil were watered to saturation by standing the pots half submerged in a container of water for 2 h and additionally adding water to the surface of the soil. At the completion of the 2 h period the pots were removed from the water and stood to free drain for a further 2 h period. After this time, water had ceased draining from the base of each pot and the soil was deemed to be fully saturated or at container capacity (Cassel & Nielsen 1986). The volumetric water content of the soil was then determined electronically using a Time-Domain Reflectometer (TDR, TRASETM, Soil Moisture Equipment Corp., PO Box 30025, Santa Barbara, California 93105, USA). For the TDR measurements the stainless steel electrodes were 15 cm long and were inserted vertically into the soil profile in the centre of the pot. The measured water content of the four pots ranged between 36.9% and 38.6% with a mean of 37.8%. Thus an 80% to 85% container capacity was equivalent to a volumetric water content (based on the mean container capacity) of 30.24% to 32.13% (v/v).

2.3 Imposition of a water-deficit

To expose plants to a water deficit, a dry-down period was imposed in which the soil water concentration was decreased by withholding water from the soil. The progression of the dry-down at the soil level was quantified using a TDR. The effect of the declining soil water content on plant growth was quantified by monitoring changes in leaf extension rate (LER mm day⁻¹) at weekly intervals throughout each dry-down experiment. Using randomly selected tillers within tussocks, a continuous indelible black line was drawn across the surface of the youngest sheath emerged leaf and the adjacent non-elongating mature tiller leaves. After a 72 h interval, a second line was drawn on the surface of the youngest leaf reconnecting on either side the lines still present on the adjacent mature leaves. The longitudinal distance between the two lines on the youngest leaf represented the leaf extension that had occurred over the past 72 h, and was used to calculate the LER per day by dividing the distance by 3. Throughout the dry-down period randomly selected pots were maintained at pre dry-down water levels to create hydrated control plants.

2.4 Dissection of tillers

At determined time points in each dry-down experiment, destructive harvests were carried out to collect tissue samples for analyses. In dry-downs I and II (nursery grown plants), individual tillers from randomly selected treatment pots were harvested and pooled (3 tillers per sample, 3x replication), while in dry-down III (clonal Molesworth plants) whole tussocks were harvested at each harvest date and each sample for analysis was derived from 20-40 tillers from a single tussock.

Each harvest was carried out one hour pre-dawn and involved excising the aerial component of the plant (harvested material) from the root system, at the root/crown junction (soil surface). The leaf material was immediately frozen in liquid nitrogen prior to storage at -80°C. For dissection, frozen plant material was dissected on a glass plate cooled on ice to minimize tissue warming and reduce evaporative water loss.

Tissue zones were determined by differential staining for mitotically active regions, nonmitotic regions and lignified regions within the leaf. These zones were viewed using a dissection microscope, and the tissue zones are represented schematically in Fig. 2.1. For this identification, whole tillers were vacuum infiltrated for 15 min with fixative solution: 10% (v/v) formalin (37% (v/v) formaldehyde), 5% (v/v) propionic acid, 70% (v/v) ethanol, 15% (v/v) distilled water, and fixed over night at room temperature. The tissue was submerged in fresh fixative the following day, and stored in a tightly sealed glass vial at room temperature in the dark for a period of 6 weeks prior to staining. Immediately before staining, the tissue was washed twice in 70% (v/v) ethanol (2 min each). For staining, the tissue segments were placed in a 15 ml volume of 70% ethanol to which 100 μ l of safranin stain (1% (w/v) safranin in 70% (v/v) ethanol) had been added, and left at room temperature for a period of 16 h. The following day the stain was poured off and the tissue was brought through a graded alcohol series (75, 85, 95, 100% ethanol) in 10 min steps. The tissue was then transferred to a counter stain solution of 15 ml 100% ethanol to which 50 μ l of fast green solution (0.02% (w/v) fast green in 10% (v/v) clove oil, 10% (v/v) methyl cellosolve, 60% (v/v) ethanol, 20% (v/v) glacial acid). After a 5 min period, the counter stain was poured off and the tissue was brought through an ethanol:histoclear series comprising ratios of 75:25, 50:50, and then 100:0) with 10 min for each step. The tissue was then removed from the histoclear and mounted on microsope slides in DPX, and sealed with cover slips. Slides were left to cure for ca. 7 days.

The meristem region, staining red due to the occurrence of large cell nuclei, consisted of the basal 5 mm of the youngest exposed leaf and included the intercalary meristem, the tiller apical meristem and associated leaf primordia. The adjacent 10 mm of lamina cells stained predominantly green indicating a condensation of nuclei within cells, and a cessation of mitotic activity. Over the next proximal 10 mm section of the leaf (zone 2), the stained tissue progressed from a green colour to a dark red indicating a progression to lignification. The next proximal 10 mm section (zone 3) stained red as did the remainder of the leaf.

A 10 mm sample of sheath enclosed, mature lamina was dissected from just proximal to the ligule of the next oldest leaf (designated the enclosed lamina), as was the adjacent 10 mm of air-exposed lamina (designated the exposed lamina). Sheath samples were collected from the basal 10 mm of the next oldest leaf, and were designated sheath 1. For dry-down III a second 10 mm basal sheath sample was collected from the second oldest sheathed leaf within the tiller and was designated sheath 2 (Fig. 2.1).



Figure 2.1 Schematic diagram of a *Festuca novae-zelandiae* tiller indicating the location of tissue samples.

I. Elongating leaf: Meri, meristem; Zn1, Zn2, Zn3, zones 1, 2, and 3, respectively; EncL, enclosed lamina; ExpL, exposed lamina.

II. Youngest expanded leaf: Sh1, sheath 1.

III. Mature expanded leaf: Sh2, sheath 2.

2.5 Measurement of plant cell water relations

2.5.1 Determination of osmotic adjustment

Osmotic adjustment determination of leaf tissue regions was based on the re-hydration method of Blum (1989), with osmotic adjustment calculated as the difference in osmotic potential at full turgor between well-watered (control plants) and dry-down plants.

For the measurement of osmotic adjustment, randomly selected whole tillers (intact root system) were removed from tussocks at 4 pm on the day of harvest and rehydrated to full turgor by submersion of the root system in distilled water (MilliQ) for a period of 16 h. Tiller re-hydration was carried out at ambient glasshouse temperatures in the dark. At the end of the re-hydration period, tillers were removed from the water, blotted dry with a paper towel and the roots were excised using a scalpel blade and discarded. Immediately, the remainder aerial region of the tillers was wrapped in a plastic film and frozen in liquid nitrogen prior to storage at -80° C.

When osmotic potential measurements were to be made, individual frozen tillers were dissected on a glass plate (cooled on ice) and each dissected tissue segment was sealed into a separate microfuge tube maintained on ice. Tissues from three tillers were pooled per sample, and this was replicated three times to provide three replicate samples per tissue for analysis. Osmotic potential was measured on each sample using a wescor C-52 sample chamber connected to a wescor HR-33T microvoltmeter. Measurements were made in the hygrometric mode (dew-point depression) with a consistent cooling period of 15 s applied for each sample, as per the instructions supplied with the C-52 sample chamber instruction manual. For the measurement of the osmotic potential of leaf tissues, the pooled frozen segments making up each sample were transferred to the Wescor sample holder and sealed in the C-52 sample chamber. Before cooling the thermocouple and measuring the dew point microvolt output, a 45 m equilibration time was imposed. This period allowed temperature and vapour pressure equilibrium to be achieved within the chamber (as indicated from repeated stable measurements at this time point). Stable microvolt out put readings for each sample were converted to osmotic potential according to the equation of the calibration curve (Fig. 2.2) generated from NaCl standards over the range 0.05 to 1.4 molality (-2.3 to -64.87 bars) as specified in the C-52 sample chamber instruction manual. The calibration curve was drawn from triplicate measurements of the NaCl solutions and was checked against a single NaCl standard each day of tissue

measurements to determine if recalibration was necessary. No recalibration was required over the period of sample measuring.



Figure 2.2 Calibration curve derived from the psychrometric output of the C-52 sample chamber in the dew-point mode as a function of the osmotic potential of NaCl standard solutions over the molality range of 0.05 to 1.4.

2.5.2 Determination of tissue water content

Samples of tissue were sealed in pre-weighed microfuge tubes and weighed to determine fresh weight (FW). Dry weight (DW) was recorded after drying the samples at 80°C for 24 h, and water content (as % FW) was calculated as (FW – DW) x (100/FW).

2.5.3 Solute potential determination

The solute potential of water-soluble tissue extracts was measured in leaf regions in the tiller over the course of the dry-down. For this, 40 mg of oven dried tissue samples were ground to a powder in liquid nitrogen using a mortar and pestle and transferred to a microfuge tube maintained on ice. Five hundred μ l of ice-cold water was used to rinse the mortar and pestle and the recovered volume was added to the microfuge tube. The tube was sealed, vortexed for 30 s and then mixed on a rotary mixer for 15 min. The samples were then centrifuged at 8600 g for 10 min. Following centrifugation, the supernatant was transferred to a clean microfuge tube and placed on ice. The pellet was re-suspended in a second 500 μ l volume of ice-cold water and re-extracted as above. Following centrifugation the second supernatant was pooled with the first and the combined sample was dehydrated overnight in a Speed Vac (SC200, Savant Instruments Inc. NY, USA). The dried samples were then stored at -20°C.

To determine the contribution of sucrose and fructans in the different leaf regions, to changes in the solute potential of the tissue over the course of dry-down III, each of the solute extracts were re-suspended in a 160 μ l volume of water, and the total solute potential of the suspension measured. Solute potential was measured by thermocouple psychrometry utilising a SC-10 Thermocouple Psychrometer Sample Changer and NT-3 Nanovoltmeter (Decagon Devices, Pullman, Washington, USA). This system allowed for 9 samples to equilibrate at the same time and replaced the single sample system of the Wescor C-52 sample chamber. Before the osmotic potential of tissue extract samples was measured, a calibration curve was generated from sucrose solutions over the molar concentration range of 0.05 to 1.0. For this, 80 μ l of each representative molar concentration was pipetted on to a single filter paper disc (cut with a standard paper hole punch) and placed at the base of each of the SC-10 sample cups. After all sample cups had been loaded with different standard solutions, the sample chamber was sealed and a 30 m temperature and vapour equilibration period was imposed before solute potential

measurements were made. Calibration was carried out before and after each batch of tissue extract samples was measured, and the combined sucrose calibration values were used to generate calibration curves relating μ V, molality, and solute potential (Fig. 2.2 and 2.3). The solute potential of each sucrose solution was obtained from the table of Gusev (1960).

For solute potential measurements, 80 μ l of sample extract was pipetted onto a single filter paper disc (cut with a standard paper hole punch) placed at the base of each of the SC-10 sample cups and measured as per the calibration solution. The μ V output of the NT-3 Nanovoltmeter for each sample was converted to units of solute potential (bar) calculated from the equation of the calibration line. Because the sucrose and total fructan concentration of each tissue sample harvested had been determined by high performance anion exchange chromatography (HPAEC), the molar concentration of these sugars in the 160 μ l solute extracts was known. Using the equation of the line for Fig. 2.2, the μ V output derived from the molar concentration of either sucrose or fructan present within each extract suspension was calculated. The difference between the total measured solute potential of each extract and that calculated for each individual sugar present in the extract represented the contribution of each sugar to the total solute potential. In addition, differences in the solute potential between the 160 μ l extract suspensions of the same leaf region harvested at different time periods in the dry-down indicated solute loss or gain within the tissue in response to the water deficit conditions.



Figure 2.3 Calibration curve of sucrose solutions vs μV output of the NT-nanovoltmeter.



Figure 2.4 Calibration curve relating NT-nanovoltmeter μV output derived from the sucrose standard solutions to solute potential.

2.6 Carbohydrate analysis

Water-soluble carbohydrates were extracted from *F. novae-zelandiae* leaf tissue samples based on the method of Naidu (1998). Water-soluble carbohydrate analysis was based on the high performance anion exchange chromatography (HPAEC) method described by Hellwege *et al.*, (1997).

2.6.1 Extraction of water-soluble carbohydrate (WSC)

Frozen (-80°C) leaf samples (either 6 pooled leaves per sample or 40 mg FW samples pooled from 20-40 tillers were ground to a powder in liquid nitrogen using a mortar and pestle and then transferred to a microfuge tube maintained on ice. Five hundred μ l of ice-cold methanol:chloroform:water (MCW; 60:25:15) was used to rinse the mortar and pestle and the recovered volume added to the microfuge tube. The tube was sealed and the sample vortexed for 30 s and then placed on ice. Ice-cold distilled water (300 μ l) was then added to the sample. The sample was then vortexed for a further 10 s before being centrifuged at 5000 g for 5 min. This separated the extractant/sample mixture into a clear (upper) methanol:water phase containing polar and water soluble compounds, and a chloroform phase into which the lipid was partitioned, and a solid phase in the base of the tube.

The upper methanol-water phase was transferred to a new microfuge tube and stored on ice. The chloroform phase was discarded. To the solid phase 300 μ l of ice-cold distilled water was added and the sample was vortexed for a further 40 s. The suspension was then centrifuged at 5000 g for 5 min, and the water phase was pooled with the methanol-water phase collected earlier. Samples were deionised by adding approximately 1/3 weight per volume of ion-exchanger resin (AG 501-X8, 20-50 mesh, Bio-Rad Laboratories, Hercules, CA, USA) and then mixed on a rotary mixer for 15 min. The deionised solutions were transferred to pre-washed (with 2 x 500 μ l aliquots of distilled water) centrifuge filters (Microcon YM-10, Millipore Corporation, Bedford, Mass, USA) and centrifuged at 8600 g until the solution had passed through the filter membrane (approximately 30 m). The filtrates were then dehydrated overnight in a Speed Vac (SC200, Savant Instruments Inc. NY, USA) and stored dry at -20°C. For carbohydrate analysis, samples were re-hydrated in 200 μ l of distilled water from which aliquots were removed when required.
To extract WSCs from *Lolium perenne*, whole leaves (sheath and lamina) were excised from the glasshouse-grown plants and induced to accumulate fructans by placing the cut end into a solution of 150 mM sucrose. These leaves were then maintained in continuous light, at ambient temperature. After 72 h, the leaves were dissected to excise the basal tissues (leaf base, zones 1, 2 and 3 see Fig. 2.1). These tissues were pooled, homogenised in liquid nitrogen and extracted twice with 1 ml of 80% (v/v) ethanol at 80°C, and then once with 1 ml of distilled water at 60°C, each for 30 min. The ethanol extracts were dried down under vacuum and the dried powder dissolved in the water extracts, after which the samples were deionised, and filtered as described for *F. nova- zelandiae* leaf extracts.

2.6.2 Qualitative and quantitative analysis of WSC

This work was undertaken at the Max Planck Institute of Molecular Plant Physiology, Golm, Germany. Water-soluble carbohydrates (WSCs) were analyzed by high performance liquid anion exchange chromatography (HPAEC) using a CarboPac PA-100 column on the Dionex DX-500 gradient chromatography system (Dionex Corporation, Sunnyvale, CA, USA) coupled with pulsed amperometric detection (PAD) by a gold electrode. The detector settings were: $T_1 = 0.4$ s, $T_2 = 0.2$ s, $T_3 = 0.4$ s, $E_1 = 0.05$ V, $E_2 =$ 0.75 V, $E_3 = -0.15$ V, sensitivity range = 0.1 μ C, and integration range = 0.2 - 0.4 s. The solvents used were A = 0.15 M NaOH, B = 1M NaAc in 0.15 M NaOH, C = 1 M NaOH, and D = H₂O. Where appropriate, different chromatographic methods were used for the qualitative analysis of WSCs extracted from Dry–down plants.

HPAEC method 1: 10 min linear gradient from 33% A and 67% D to 100% A, 20 min linear gradient to 85% A and 15% B, 10 min linear gradient to 75% A and 25% B, 2 min linear gradient to 100% B, 5 min 100% B, 2 min linear gradient to 100% A, 10 min 100% A.

HPAEC method 2: This method was developed to provide maximum separation and quantification of fructose, glucose and sucrose. Standard curves generated for glucose, fructose and sucrose using method 2 are shown in Fig. 2.5a, 2.5b, and 2.5c respectively. The chromatographic conditions of HPAEC method 2 were: 13 min linear gradient from 5% C and 95% D to 12.5% C and 87.5% D, 2 min linear gradient to 100% B, 5 min 100% B, 2 min linear gradient to 12.5% C and 87.5% D, 8 min gradient to 5% C and 95% D.





Figure 2.5 Calibration curves for glucose, a; fructose, b; and sucrose, c; obtained with HPAEC method 2.

HPAEC method 3: This method was a modification of HPAEC method 1, with the difference being the elimination of the initial 10 min 33% A and 67% D step used in HPAEC method 1 for the chromatographic separation of fructose and glucose. The conditions for HPAEC method 3 were: 20 min linear gradient from 100% A to 85% A and 15% B, 10 min linear gradient to 75% A and 25% B, 2 min linear gradient to 100% B, 5 min 100% B, 2 min linear gradient to 100% A, 6 min 100% A.

2.6.3 Quantification of fructans

For fructan quantification (dry-down III extracts only), resuspended samples were hydrolyzed with 0.5% (w/v) oxalic acid at 65° C for 3 h and neutralized with 0.2 N NaOH. Released glucose and fructose molecules were quantified by HPAEC using HPAEC method 2. Endogenous glucose, fructose and sucrose concentrations predetermined for each sample were subtracted from the oxalic acid digested samples for calculation of the total fructan concentration on a hexose equivalent basis. Mean degree of fructan polymerisation was calculated by dividing the fructan derived fructose concentration by the fructan derived glucose concentration. In addition this represents the total fructan molar concentration within a sample assuming a single glucose residue per fructan molecule.

Dry-down II fructan isomer identification was achieved by chromatographic comparison with peak retention times for commercial standards of the inulin series DP3-DP5, and water soluble carbohydrate extracts from onion bulb tissue and barley leaf tissue, the chromatographic profiles of which had been previously determined for the current chromatographic method by E. M. Hellwege (Max Planck Institute for Molecular Plant Physiology, Golm, Germany).

Under the chromatographic conditions of HPAEC method 1 sucrose eluted from the column at 6.5 minutes (Fig. 2.6.A,B,C,D). For the inulin series (Fig. 2.6.A) the trisaccharide 1-kestotriose eluted first at 9.5 minutes follow by 1,1-kestotetraose a tetrasaccharide at 13 minutes and the pentosaccharide 1,1,1-kestopentaose at 17 minutes. Each of these retention times corresponded to peaks in the chromatogram generated form a pooled lamina and sheath tissue extract of *F. novae-zelandiae* indicating the presence of these fructan isomers in *F. novae-zelandiae* (Fig. 2.6.B). Comparison with the onion chromatogram also indicated the presence of low DP fructans of the inulin neoseries (6G-

kestotriose; 1&6G-kestotetraose; and 1&6G,1-kestopentaose) peaks 3, 5 and 8 respectively, as well as 6G,6-kestotetraose (peak 6) an isomer of the levan neoseries. Bifurcose (1&6-kestotetraose) and 6-kestotriose both fructans common in barley (Fig. 2.6.D) were not present in the pooled *F. novae-zelandiae* extract. Also occurring in the chromatogram of *F. novae-zelandiae* were many unidentified peaks, including the large peak present at the 34 min mark. With acid hydrolysis of a second pooled sample, and subsequent HPAEC analysis, all fructan-associated peaks and the unidentified peaks present in the initial chromatogram were undetected in the hydrolyzed sample while conversely glucose and fructose levels had increased. This indicated that the unidentified peaks were also fructan isomers.



Figure 2.6 HPAEC-PAD chromatographic comparison of inulin standards (A), with fructan isomers extracted from leaf tissue of *F. novae-zelandiae* (B), onion (C), and barley (D). S, sucrose; 1, 1-kestotriose; 2, 6-kestotriose; 3, 6G-kestotriose; 4, 1,1-kestotetraose; 5, 1&6G-kestotetraose; 6, 6G, 6-kestotetraose; 7, 1,1,1-kestopentaose; 8, 1&6G, 1-kestopentaose; a, 1&6-kestotetraose

Dry-down III fructan isomer identification was achieved by chromatographic comparison with peak retention times for leaf extracts of *Lolium perenne*. The structure of fructan isomers synthesised by this grass species had been previously determined by Pavis *et al.*, (2001) using both mass spectrometry and a similar HPAEC-PAD analytical method to HPAEC method 3. Spiking the *L. perenne* extract with purified commercial inulin standards identified inulin peaks in the *L. perenne* chromatogram. This allowed the identification of unspiked peaks within the *Lolium perenne* chromatogram through the direct comparison with the chromatographic WSC profile presented in Pavis *et al.*, (2001). The identified peaks within the WSC chromatographic profile of *L. perenne* and the corresponding peaks within the WSC profile of a pooled sample of meristem, zone 1, 2 and 3 extracts from *F. novae-zelandiae* are shown in Fig. 2.7.



Figure 2.7 HPAEC-PAD chromatographic comparison of fructan isomers present in leaf extracts of *Lolium perenne* (A), and *Festuca novae-zelandiae* (B). Numbers indicate the peaks with elution times corresponding to known standards. 1, 1kestotriose; 2, 6-kestotriose; 3, 6G-kestotriose; 4, 1,1-kestotetraose; 5, 1&6Gkestotetraose; 6,6G, 6-kestotetraose; 7, 1,1,1-kestopentaose; 8, 1&6G,1kestopentaose; L, loliose; R, raffinose.

2.7 Confocal microscopy

Differentially stained cells (see section 2.4) of the lamina base (first subepidermal layer), and tiller apical meristem were imaged using a Leica TCS4D confocal laser scanning microscope. An excitation wavelength of 568 nm was used and emissions beyond 590 nm were collected. Images were taken as an optical slice optimized with 32-fold line averaging. The objective lens was 63x, NA 1.4 oil-immersion and a 1.5 zoom factor was applied. Bright field transmission on the confocal microscope was used to distinguish between the stained cell nuclei (bright red) and non-stained cytoplasm of cells, both of which auto-fluoresced at the 568 nm wavelength.

2.7.1 Cell length measurements

To determine cell length, a 100 μ m scale bar was superimposed into each intercalary meristem confocal micrograph (using the confocal software) parallel to the longitudinal leaf axis. Confocal images were captured 2.5 mm above the point of the leaf attachment to the tiller crown. For plants subjected to the water deficit treatment, the average cell length within the 100 μ m scale bar was calculated by dividing the number of complete cells (within a cell column) over the length of the scale bar. Three such measurements were made within the first sub-epidermal layer of each leaf, and the mean of these measurements was recorded as the average cell length within the intercalary meristem of the measured leaf. Ten randomly selected leaves were measured in this way at each harvest in dry-down III. For control plants, the average cell length within the intercalary meristem was calculated in the same way from a total of nine leaves harvested at days 0, 21 and 49 of the dry-down (three leaves at each harvest).

2.8 Statistical analysis

Determination of mean values, analysis of variance (ANOVA) and the calculation of degrees of freedom were determined from the appropriate equations in Sprinthall (1990).

CHAPTER 3 Dry-down I and II

An investigation into tissue specific osmotic adjustment and watersoluble carbohydrate composition in response to water deficit by *Festuca novae-zelandiae*

3.1 Introduction

In a previous study investigating the drought tolerance of F. novae-zelandiae a number of biochemical adjustments were found to occur within specific leaf tissue regions in response to declining soil water content (Abernethy *et al.*, 1998). These adjustments included elevated levels of protein:ubiquitin conjugation in mature photosynthetic lamina tissue relative to the tiller base, and preferential accumulation of proline within the leaf base as soil water content decreased. It is believed that these processes are indicative of a regulated preferential protection of the tiller base (meristematic tissues) at the expense of mature photosynthetic lamina tissues during periods of drought.

Osmotic adjustment is thought to be a major component of tissue turgor maintenance and turgor dependent processes in plants under water-deficit stress (Turner 1986, Zhang *et al.*, 1999). Osmotic adjustment is known to occur within guard cells aiding the maintainance of the stomatal aperture and photosynthetic activity, in plants under mild water deficits (Turner *et al.*, 1978, Ackerson *et al.*, 1980, Ludlow *et al.*, 1985), while in perennial grass species water-soluble carbohydrate accumulation and osmotic adjustment within tiller base tissues appears to be strongly associated with meristematic tissue survival and tiller recovery from drought (Thomas 1991, Spollen and Nelson 1994).

To investigate the role of osmotic adjustment and water-soluble carbohydrate accumulation as potential mechanisms of tissue survival in *F. novae-zelandiae* during periods of water-deficit stress dry-downs I and II were under taken. The focus of dry-down I was to measure tissue osmotic potential within designated developmental regions of *F. novae-zelandiae* tillers and determine if tissue osmotic adjustment occurs in response to declining soil water content. Dry-down II was carried out to determine if declining soil water content, 1, induced tissue specific water-soluble carbohydrate accumulation and 2, affected the chemical composition of the water-soluble carbohydrate content within these tissues.

3.2 Experimental approach to dry-down I

Dry-down I was carried out under glasshouse conditions (see section 2.1) over a 28-day period commenced on the 7th of November 1999. The plant material consisted of 18 individual tussocks comprising 80 to 100 tillers each, of the nursery *F. novae-zelandiae* cultivar. Individual tussocks had been established eight weeks prior to commencing the dry-down in 4-L capacity pots containing Kairanga silt loam (section 2.2). Nine of the tussocks were randomly selected and kept as hydrated (control) plants by maintaining the soil water content above the 30% volumetric water content (>80% of the maximum water holding capacity). This was achieved by the addition of ca. 300 ml of tap water to the soil surface every other day.

The water-deficit treatment was applied to the remaining nine tussocks by withholding water from the soil for the duration of the dry-down period. To monitor the progression of the soil water loss during the dry-down, the volumetric soil water content was monitored by time domain reflectometry (TDR) every second or third day for the duration of the dry-down. Both control and water-deficit tussocks were placed on a free draining (wire mesh) table in the glasshouse. Pot positions on the table were randomised every other day. Growth responses to the dry-down conditions were measured as changes in lamina elongation rate (averaged from three consecutive days growth) at weekly intervals. Measurements were made on the youngest emerging leaf (most rapidly elongating) within three randomly selected tillers per pot.

Osmotic adjustment determination was based on the re-hydration method of Blum 1989. This involved the removal of a single whole tiller (intact root system) from each tussock plant at 4 pm on the day of harvest and complete submersion of the root system and tiller base in distilled water (MilliQ) for a period of 16 h. The tillers were maintained in the dark at ambient glasshouse temperatures for the duration of the rehydration period. At the end of the 16 h, each tiller was removed from the water and the central emerging leaf including the tiller base excised from the tiller and immediately rapped in parafilm and then tinfoil prior to freezing in liquid nitrogen and storage at -80°C. From each of the frozen tillers the developing lamina was excised from the tiller and dissected into six designated tissue regions (see section 2.4). These were the meristem, zones 1, 2, 3, the enclosed lamina, and the exposed lamina. Tissues from three tillers were combined per sample for osmotic potential. Osmotic potential was measured directly on these tissue samples by the dew

point thermocouple psychrometry method (see section 2.5.1). Harvests for osmotic adjustment measurements were carried out on day 0, 7, 14, and 28 of the dry-down.

3.3 Results

3.3.1 Soil water depletion

With regular watering, the soil water content (SWC) of control pots remained at a volumetric water content between 28 and 35% during the experiment (Fig. 3.3.1). For dry-down pots, withholding water from the soil had a significant effect on the soil water status such that over the duration of the dry-down the soil water content decreased from 30% at day 0 to 14% by day 30 (Fig. 3.3.1). Of the total water loss ca. 50% occurred within the first 7 days of the dry-down at a volumetric rate of 0.89% per day. In contrast over the final 7 days of the dry-down the rate of water loss had slowed to 0.32% per day.



Figure 3.3.1 Change in soil water content (SWC) over the course of Dry-down I. Soil water contents were measured at the days indicated using TDR. Data points represent the mean SWC of all pots per treatment (control or dry-down).

Bars represent \pm standard errors.

3.3.2 Changes in leaf extension rate (LER) during dry-down I

Prior to commencing the dry-down the average elongation rate of newly emerging leaves for all tussocks was ca. 5.5 mm per day (Fig. 3.3.2). For plants that were watered over the dry-down period (controls), the average LER then increased to 7.8 mm per day. In contrast, for plants exposed to the drying soil, LER was impaired (Fig. 3.3.2). By day 7 of the dry-down, the LER had declined to 2.4 mm per day. This decline coincided with the steepest drop in soil water content (Fig. 3.3.1) and was the fastest weekly decline in LER observed over the dry-down. By day 14, the average LER was 0.75 mm per day indicating that leaf elongation had ceased in some of the tillers as daily elongation rates below 1 mm per day were below the level of detection. By day 21 of the dry-down, measurable leaf extension had ceased for all tillers.



Key — control — dry-down



3.3.3 Tissue specific osmotic adjustment

To determine if tissue regions within the *F. novae-zelandiae* tiller under go osmotic adjustment in response to a drying soil, tissue solute potentials were measured for each of the tissues harvested during the dry-down (see section 2.6 for method details). In fully hydrated plants (control), a significant difference (P<0.01) in osmotic potential was evident between the basal and distal regions of the leaf resulting in a -1.5 MPa gradient along the leaf axis (Fig. 3.3.3A). The lowest potential of this gradient (-0.5 MPa) was recorded for the meristem and the adjacent zone 1 tissue, while the highest osmotic potential (ca. -2.0 MPa) was recorded for both the enclosed lamina and adjacent exposed lamina region. Intermediate osmotic potentials between that of the basal and distil regions of the leaf were observed for zones 2 and 3 with osmotic potentials of -1.0 MPa and -1.6 MPa respectively (Fig. 3.3.3A). Over the duration of the dry-down, these values remained stable for the hydrated control tissues. (Fig. 3.3.3 A-D).

In contrast, changes in tissue osmotic potential were observed in plants for which water had been withheld for the duration of the dry-down (Fig. 3.3.3 A-D). For these plants, by day 7, the osmotic potential gradient between the meristem tissue (leaf base) and the exposed lamina tissue had increased to -3.75 MPa. This was due to a significant 2-fold decrease (P<0.01) in the osmotic potential of the meristem to -0.25 MPa and a significant 2-fold increase (P<0.05) in the osmotic potential of the exposed lamina to -4.0 MPa, due to an osmotic adjustment of ca. -2.2 MPa (Fig. 3.3.3 E). Significant increases (P<0.05) in osmotic potential with respect to corresponding tissues of watered control plants were also measured for zone 2, zone 3, and the enclosed lamina (Fig. 3.3.3 E).

At day 14 of the dry-down, the differences in osmotic potential evident at day 7 between the leaf tissues of the control plants and corresponding tissues of the dry-down plants were, with the exception of the meristem, still evident. For the meristem, a recovery in osmotic potential to the level of the control tissue was recorded, indicating an accumulation of osmotically active compounds between day 7 and 14 of the dry-down. (Fig. 3.3.3E).

At the conclusion of the dry-down (day 28), tissues with significantly different levels of solute potential with respect to the equivalent tissue regions of the hydrated control plants

(see Fig. 3.3.3E) were the exposed lamina with a net solute loss equivalent to 0.5 MPa, the enclosed lamina with a net solute loss equivalent to 0.4 MPa, and the meristem with an increase in solute potential (osmotic adjustment) equivalent to 0.25 MPa. However, when comparison is made between the solute potential of the same tissues of dry-down plants at day 14 and 28 of the dry-down (Fig. 3.3.3C-D), a decline in solute potential (i.e. solute depletion) had occurred for zone 3 of ca. 1.25 MPa, the enclosed lamina of ca. 1.9 MPa, and the exposed lamina tissue of ca. 2.0 MPa. Similar comparisons between the osmotic adjustment of tissues at day 7 and day 28 of the dry-down (Fig. 3.3.3B vs 3.3.3D), indicate that a net accumulation of osmotically active compounds (increase in osmotic potential)

down after an initial solute depletion within the first 7 days of the dry-down.

equivalent to 0.5 MPa had occurred for the meristem over the final 21 days of the dry-





Figure 3.3.3 Osmotic potential of leaf tissues at designated days in Dry-down I after a 16 h re-hydration period.

A = day 0, B = day 7, C = day 14, D = day 28. n = 3, bars represent \pm standard errors. Tissues are as designated in 2.6, Figure 2.1.



Figure 3.3.3E Osmotic adjustment in tissues of the youngest emerging leaf of F. *novae-zelandiae* at the indicated time points. Significant osmotic adjustment with respect to hydrated control tissues is indicated by * (P>0.05).

3.4 Experimental approach Dry-down II

Dry-down 11 was carried out under glasshouse conditions (see section 2.1) over a 42-day period during late spring and early summer (November and December).

The plant material of Dry-down II consisted of individual tussocks of 80 to 100 tillers each, of the nursery F. novae-zelandiae cultivar (see section 2.1). Individual tussocks had been established eight weeks prior to commencing the dry-down in 4-L capacity pots containing Kairanga silt loam (see section 2.1). To monitor the progress of the dry-down, soil water content was measured by time domain reflectometry (TDR) every second or third day over the duration of the dry-down period. Growth response to the changing soil water content was measured as changes in leaf extension rate (averaged from three consecutive days growth) at weekly intervals (see section 2.3). Water-soluble carbohydrate analysis of tissues harvested throughout the dry-down was carried out by high performance liquid anion exchange chromatography (HPAEC) as described for HPAEC method 1 (see section 2.6.2). The tissues dissected for analysis were six segments from the youngest sheath emerged lamina (the meristem, zones 1, 2, 3, the enclosed lamina, and the exposed lamina) as well as an additional 10 mm section from the base of the youngest sheathed leaf (see section 2.4). Sampling took place at weekly intervals over the course of the dry-down and each sample consisted of pooled tissue excised from six tillers harvested from the same tussock. This pooled sampling was replicated over 3 randomly selected tussocks at each harvest date.

3.5 Results

3.5.1 Changes in leaf extension rate during dry-down II

In this experiment tillers were harvested from control and dry-down tussocks at weekly intervals over a 42-day dry-down period. During this time the soil water content decreased from 30% maintained for control plants to 6% in the dry-down pots (Fig. 3.4.A) In common with Dry-down I, water loss was most rapid over the early stages of the dry-down with ca. 30% of the total soil moisture loss occurring within the first 7 days. Concomitant with this decline, was a reduction in the rate of leaf extension from ca. 9 mm/day at day 0 to ca. 5.5 mm/day at day 7 (Fig. 3.4.B). By day 21 of the dry-down the soil water content had declined to ca. 11% in dry-down pots and leaf extension had ceased (Fig. 3.4.A-B).



Figure 3.4Soil water content (A), and leaf extention rate (B) during Dry-down II.For A n = 9, for B n = 12.Bars represent \pm standard errors

3.5.2 Identification of water-soluble carbohydrates in the leaf tissue extracts of *F*. *novae-zelandiae*

The water-soluble carbohydrate composition of individual *F. novae-zelandiae* leaf tissues was determined by HPAEC method 1 (see section 2.6.2). Sucrose and individual fructan isomer identification was achieved by chromatographic comparison with peak retention times for commercial standards of the inulin series DP3-DP5, and fructan isomers isolated from onion bulbs and barley leaves (see section 2.6.3)

Under the chromatographic conditions used in the separation, sucrose eluted from the column at 6.5 min (Fig. 3.5.A,B,C,D). Of the inulin series (Fig. 3.5.A), the trisaccharide 1-kestotriose eluted first at 9.5 min follow by 1,1-kestotetraose a tetrasaccharide at 13 min and the pentosaccharide 1,1,1-kestopentaose at 17 min. Each of these retention times corresponded to peaks in the *Festuca novae-zelandiae* chromatogram indicating the presence of these fructan isomers in *Festuca novae-zelandiae* (Fig. 3.5.B). Comparison with a chromatogram from onion bulb tissue also indicated the presence of low DP fructans of the inulin neoseries 6G-kestotriose (peak 3); 1&6G-kestotetraose (peak 4); and 1&6G,1-kestopentaose (peak 8), as well as 6G,6-kestotetraose (peak 6) an isomer of the levan neoseries. Bifurcose (1&6-kestotetraose) and 6-kestotriose that are fructans common in barley (Fig. 3.5.D) were not present in the *F. novae-zelandiae* extract. A series of unidentified peaks were also observed in the chromatogram of *F. novae-zelandiae*, including a large peak that eluted at 34 min.



Figure 3.5 HPAEC-PAD chromatographic comparison of inulin standards (A), with fructan isomers extracted from leaf tissue of *Festuca novae-zelandiae* (B), onion (C), and barley (D).

S, sucrose; 1, 1-kestotriose; 2, 6-kestotriose; 3, 6G-kestotriose; 4, 1,1-kestotetraose; 5, 1&6G-kestotetraose; 6, 6G,6-kestotetraose; 7, 1,1,1-kestopentaose; 8, 1&6G,1-kestopentaose; a, 1&6-kestotetraose

3.5.3 Changes in the water-soluble carbohydrate composition within the elongating leaf of *F. novae-zelandiae* in response to a water deficit

In fully hydrated plants, glucose, fructose and sucrose were present throughout the elongating leaf (Fig. 3.5.A). In contrast, fructans were localised preportionately in the meristem and tissues of the elongation zone, and appeared to consist of the entire range of fructans synthesised by the leaf tissue of *F. novae-zelandiae* (Fig. 3.5.B). In tissues distil to the meristem and adjacent zone 1, 2, and 3 tissues, i.e. the enclosed and exposed lamina tissues. Only trace amounts of fructans were found. In the enclosed lamina, there appears to be no low DP fructan isomers (at least at the sensitivity of detection used), but a small peak at the end of the chromatogram indicates the presence of higher DP fructans. In the sheath chromatogram, 1-kestotriose (peak1) and higher DP fructans (at 33.5 min) are identified but no other low or intermediate DP isomers appear to be present in this tissue. For the exposed lamina, fructans appear to be completely absent.

At seven days into the dry-down (Fig. 3.5.B), there is no significant change apparent in the WSC composition of most tissues. The exception was the enclosed lamina in which trace amounts of 1-kestotriose, 6G-kestotriose, 1,1-kestotetraose, and 6G,6-kestotetraose are now detectable.

By 14 days (Fig. 3.5.C) there is a decrease in the level of Low DP fructans relative to sucrose in zone 2. In contrast the peak at 34 min in the chromatogram of zone 2 is still a dominant peak indicating the continued presence of higher DP fructans in this tissue. The chromatogram for the exposed lamina indicates the accumulation of trace amounts of 1-kestotetraose, 6G-kestotriose, and 1&6G,1-kestopentaose in this tissue and is the first time in the dry-down that fructans appear in this tissue. Also, trace amounts of 6-kestose can be found in the meristem, zone 1, and zone 2. This fructan is not present in any of the leaf tissues examined previously (Fig. 3.5.A, Fig. 3.5.B).

At day 21 (Fig. 3.5.D), sucrose has become the dominant sugar in all tissues. The reduction in the higher DP fructans (at 34 min) for tissue zones 1, 2 and 3 (relative to the previous week) indicate that these fructans are no longer accumulating, but are declining as a proportion of the total water-soluble carbohydrate pool. This decline is also evident over the range of low DP fructans in these tissues.

At days 28 and 35 (Fig. 3.5.E, and 3.5.F, respectively) the trends evident in the previous two weeks have continued. Sucrose is still the dominant sugar and all fructan isomers are declining from the tissues of the elongation zone. However, one difference occurs in the meristem where higher DP fructans (peak at 34 min) appear to be accumulating.

By day 42 (Fig. 3.5.H), it is clear that the dry-down has had a significant affect on the water-soluble carbohydrate composition of the leaf. The major trend is an accumulation of sucrose in nearly all tissues, seemingly at the expense of all types of fructan isomers. This trend, however, does not appear to apply to the meristem. In this tissue, levels of 1-kestotriose also appear to be maintained throughout the dry-down, while higher DP fructans appear to accumulate concomitantly with the loss of low and presumably intermediate DP fructans (Fig. 3.5.G, H).





Figure 3.5A/B HPAEC-PAD chromatograms of water-soluble carbohydrates in leaf tissue extracts of *F. novae-zelandiae* at days 0 (hydrated control) and 7 of the dry-down.



Retention Time (mins)

Figure 3.5C/D HPAEC-PAD chromatograms of water-soluble carbohydrates in leaf tissue extracts of *F. novae-zelandiae* at days 14 and 21 of the dry-down.



Retention Time (mins)

Figure 3.5 E/F HPAEC-PAD chromatograms of water-soluble carbohydrates in leaf tissue extracts of *F. novae-zelandiae* at days 28 and 35 of the dry-down.



Retention Time (mins)

Figure 3.5G/H HPAEC-PAD chromatograms of water-soluble carbohydrates in leaf tissue extracts of *F. novae-zelandiae* at days 0 (hydrated control) and 42 of the dry-down.

3.5.4 Quantitative changes in sucrose, 1-kestotriose, and 1,1-kestotetraose in the leaf tissues of *F. novae-zelandiae* during dry-down II

Using standards of known concentration for sucrose, 1-kestotriose, and 1,1-kestoteraose, calibration curves were generated for the HPAEC system and using the Dionex software the quantity of these compounds per tissue sample calculated automatically. As tissue samples were measured on the bases of length and not weight, sugar concentrations are presented as per mm of tissue.

The imposition of a water-deficit had an effect on the concentration and distribution of water-soluble carbohydrates in the elongating leaf of *F. novae-zelandiae* (Fig. 3.6). Under non-stressed hydrated conditions (day 0) sucrose was widespread throughout all tissues while 1,-kestotriose and 1,1-kestotetraose were present in the meristem, zone 1, zone 2, zone 3 and the sheath only. In terms of concentration none of these carbohydrates were found to exceed levels of 1 nmol per mm of tissue in any of the tissues of the hydrated leaf.

By day 7 of the dry-down, water-soluble carbohydrates accumulated in a tissue-specific manner. The meristem had the highest concentration at 9 nmol per mm of tissue followed by zone 1, zone 2, and zone 3 with 5, 4 and 3 nmol/mm, respectively. Each of these values were significantly (P<0.05) higher than the value for the same tissue in fully hydrated plants. Small increases were also evident for the enclosed lamina and sheath 1 tissue. However, these were not significant when compared with controls. No change in the level of sucrose in the exposed lamina was recorded at this stage of the dry-down. In contrast to sucrose, in which the meristem was the site of greatest accumulation, highest accumulation of 1-kestotriose, and 1,1-kestotetraose occurred in zone 1 with values of 6, and 3 nmol/mm, respectively. At this stage, there was no significant difference between the level of sucrose or 1-kestotriose in this tissue. For 1,1-kestoteraose at 3 nmol/mm, this was the highest concentration of this fructan to occur in any tissue at any stage of the dry-down.

At day 14 the average concentration of sucrose in the meristem tissue had increased to 16 nmol/mm, but due to the large standard deviation for samples extracted from replicate plants this was not determined to be significant, from concentrations observed in the

meristem at day 7. No change in sucrose, 1-kestotriose or 1,1-kestotetraose concentrations were recorded for any of the other tissues examined at this stage.

By day 21, significant (P<0.05) increases in sucrose concentrations to 4, 3 and 9 nmol/mm had occurred for zone 3, the enclosed lamina, and sheath 1, respectively. Increases in sucrose were also recorded for the meristem and zone 1, as well as decreases in the concentration of 1-kestotriose in these tissues. However, these gains/losses were not significant.

For the remaining three weeks of the dry-down, there was predominantly little change in the concentration of sugars in the leaf. However, there was a significant increase (P<0.05) in the level of sucrose in the enclosed lamina from 3 nmol/mm (the concentration maintained through out the dry-down since week two) to 7 nmol/mm by day 42 (P<0.05), and an increase in sucrose in the meristem at day 42 with respect to day 28 (P<0.01). By the conclusion of the dry-down, the meristem had accumulated more sucrose (P<0.001) than any other tissue assayed, and had increased from control levels (below 1

nmol/mm of tissue) to 34 nmol/mm of tissue by day 42 of the dry-down.



Figure 3.6Quantitative changes in sucrose, 1-kestotriose, and 1,1-kestoteraose inleaf tissues of *F. novae-zelandiae* during Dry-down II.

n = 3. Bars represent \pm error bars.

3.6 Discussion of dry-down I and II

When looking through the results of dry-down's I and II, a pattern emerges suggestive of how tissue specific osmotic adjustment and changes in water-soluble carbohydrate composition of tissues may contribute to the survival of *F. novae-zelandiae* tillers during periods of drought.

With regards to osmotic adjustment, F. novae-zelandiae appears to under go a biphasic response dependent on tissue type and the severity of the water deficit stress. The primary response takes place during the developing stages of a soil water deficit, e.g. in dry-down I a decrease from 30% SWC to 20% SWC over 14 days, as osmotically active compounds appear to accumulate primarily in the mature (differentiated) photosynthetic lamina (a region of stomatal conductance and therefore an area of transpiration and potential dehydration). This initial osmotic adjustment may be of benefit to the plant in at least two ways. Firstly, osmotic adjustment is the main mechanism of turgor maintenance under water deficit conditions (Turner 1986). In this case, solute accumulation in response to cellular water deficits decreases the cellular osmotic potential resulting in the maintenance of a higher turgor potential at a given leaf water potential (Zhang et al., 1999). As turgor potential is required for stomatal opening, osmotic adjustment within the guard cells may allow the plant to maintain stomatal aperture and photosynthetic activity under mild water deficits (Turner et al., 1978; Ackerson et al., 1980; Ludlow et al., 1985). An osmotic adjustment of 0.8 MPa was observed to maintain turgor pressure and keep stomates open in leaves of sugarbeet under mild water deficit stress (McCree and Richardson 1987). The osmotic adjustment measured for the lamina tissue of F. novae-zelandiae in the early stages of dry-down I was 1.5 MPa. Secondly, osmotic adjustment within cells lining the stomatal chambers of the lamina reduces the water potential gradient between the inside of the leaf and the immediate atmosphere. This results in increased water retention within lamina cells and a reduction in water loss through transpiration (Hare *et al.*, 1998; Zhang et al., 1999).

The second phase of osmotic adjustment is confined to the tiller base and occurs at a drier soil water content, i.e. later into a soil drying episode. With respect to the osmotic adjustment of the mature lamina, in dry-down I this had occurred at a soil water content of 15% and corresponding to day 28 into the dry-down period. This apparent solute accumulation in the meristem (tiller base region) is accompanied by a depletion of

osmotically active compounds from the osmotically adjusted mature lamina region. This shift in osmotic adjustment between tissues within drought treated tillers suggests the possibility of a translocation of osmotically active compounds. Re-mobilisation of carbohydrate reserves to support reproductive growth including grain filling is considered an important metabolic trait for adaptation to terminal drought stress (Ludlow and Muchow 1990; Gebbing and Schnyder 1999; Yang *et al.*, 2001; Yang *et al.*, 2003). For perennial grass species, osmotic adjustment of the leaf base and their associated meristematic tissues appears to be an important mechanism of drought tolerance (West *et al.*, 1990). Often water-soluble carbohydrates form a large part of this osmotic adjustment particularly sucrose (Schnyder and Nelson 1987; Thomas 1991; Spollen and Nelson 1994) and may be derived from the hydrolysis of storage carbohydrates such as fructans in the absence of photosynthate (Volaire and Lelievre 1997).

To investigate the role of water-soluble carbohydrates in the drought survival of *F. novae-zelandiae*, the water-soluble carbohydrate composition of fully hydrated tiller leaf tissues was determined and changes in the water-soluble carbohydrate profile that occurred under the dry-down conditions recorded. As determined by HPAEC-PAD chromatographic elution times, *F. novae-zelandiae* is a fructan producing grass synthesising a fructan series similar to that reported for *Festuca arundinacea* (Lüscher and Nelson 1995). In *F. novae-zelandiae* the profile is composed mainly of the inulin neoseries with an internal glucose residue and $\beta(2-1)$ linked fructose residues, and the inulin series with a terminal glucose characterised by an internal glucose residue and $\beta(2-6)$ linked fructose residues, and low levels of 6-kestotriose, a trisaccharide of the linear $\beta(2-6)$ series with a terminal glucose residue.

In common with other members of the super tribe Pooidae, with the exception of *Dactylis* (Chatterton *et al.*, 1993), *F. novae-zelandiae* appears not to synthesis 1& 6-kestotetraose (bifurcose), a branched DP4 fructan isomer common to *Bromus* (Chatterton *et al.*, 1993), *Hordeum* (Simmen et al., 1993), and *Triticum* (Carpita et al., 1989), members of the super tribe Triticodae.

The qualitative fructan analysis under taken during Dry-down II indicates that fructans are predominantly associated with the lamina intercalary meristem and adjacent elongation

zone at the base of the expanding leaf. This location of highest fructan concentration is also reported for perennial rye grass, wheat, tall fescue, timothy and barley (Spollen & Nelson 1988; Guerrand *et al.*, 1996, Roth *et al.*, 1997). Also in common with these species, fructan accumulation within the lamina zone of expansion of *F. novae zelandiae* is likely to serve a similar role. That is the unloading of sucrose from the phloem, maintaining cellular osmotic potential necessary for cell expansion, buffering metabolism against temporary shortages of carbon and energy supply from photosynthate, and providing carbohydrates for cell differentiation and secondary cell wall synthesis on cessation of cell expansion.

Lowest fructan concentrations occur in the photosynthetic region of the lamina, and the leaf sheath. As the photosynthetic tissue is a site of photosynthate production and translocation and not a carbohydrate storage tissue in grass species, a low concentration of fructans in this part of the leaf is to be expected. On the other hand the leaf sheath of fructan accumulating grass species may act as a water-soluble carbohydrate storage organ. In the case of *F. novae-zelandiae* however, the sheath does not appear to have this role as has been suggested for *F. arundinacea* (Housley and Volenec 1988) but rather may serve as a thoroughfare for carbohydrates between leaf blades and the tiller base as has been suggested for *L. perenne* (Guerrand *et al.*, 1996).

Drought, as simulated in dry-down II, has a significant effect on the WSC composition of the aerial leaf tiller tissues of *F. novae-zelandiae*. Here too, there appears to be a biphasic response, at least in terms of the accumulation of fructans. At the onset of soil water depletion, fructans accumulate in the leaf base and elongation zone. This accumulation corresponds to a decrease in leaf elongation rate and may be directly related to the slowed growth rate and reduced utilisation of translocated photosynthate in growth related processes. However as soil water deficits increase and growth is further reduced, the initial fructan accumulation becomes depleted and sucrose accumulates to be the dominant water-soluble carbohydrate throughout the leaf. In later stages of water-deficit stress, sucrose accumulation becomes concentrated in the tiller base at approximately twice the concentration of any other part of the tiller leaf. This result provides an insight into the mechanisms operating within the *F. novae-zelandiae* tiller to adapt and presumably protect vital tissue regions from water-deficit stress. The accumulation of high concentrations of sucrose is characteristic of desiccation tolerant seeds (Williams and Leopold 1998) and as
well, the predominant from of water soluble carbohydrate in dessication tolerant tissues of resurrection plants (Wolkers *et al.*, 1998). In these plants, tissue dehydration and attainment of quiescence is concurrent with the accumulation of sucrose. Two hypotheses have been proposed to explain the role of sugars, especially sucrose, in the dehydration tolerance of plant tissues (Crowe *et al.*, 1992, Crowe *et al.*, 1998, Allison *et al.*, 1999). Firstly, sugars may interact with membranes and proteins facilitating stability by substituting the hydrogen bonding within and between macromolecules, usually derived from a molecular interaction with water. Secondly sucrose is thought to participate in the vitrification and subsequent stabilization of internal cellular structures, slowing metabolic interactions and maintaining the relative spatial distribution of macromolecules within the cell. High sucrose concentrations have also been observed in the leaf base of a number of grass species during prolonged periods of drought (Volaire *et al.*, 1998b; Thomas & James 1999) and might reflect a similar role of sucrose in these species as described for resurrection plants.

Sucrose accumulation during water deficit stress requires a carbohydrate source. For *C. Plantagineum*, this role is fulfilled by the eight-carbon carbohydrate, 2-octulose which is accumulated during hydrated conditions and is rapidly metabolized during water-deficit stress concurrently as sucrose concentrations increase (Bianchi *et al.*, 1991). The depletion of fructans from leaf tissues in this current study and the concurrent accumulation of sucrose in the tiller base, suggests that fructans form a large part of the carbon source for this accumulation in drought exposed *F. novae-zelandiae*. This also indicates that fructan synthesis is under some form of metabolic control, and not simply proportional to tissue sucrose concentration as suggested by Schnyder and Nelson (1987), and shown for *F. arundinacea* (Spollen and Nelson 1994).

In support of the hypothesis of this thesis, osmotic adjustment has been shown to occur in the tiller base of *F. novae-zelandiae* when exposed to drought like conditions. In addition preferential accumulation of the water-soluble carbohydrate sucrose occurs in the tiller base seemingly at the expense of fructans, and sucrose in adjacent lamina tissues. What has not however been tested of the proposed hypothesis is whether or not these biochemical changes are simply artefacts of water-deficit stress as the plant slowly dies or alternatively actual adaptations associated with the survival of the tiller base. This was to be tested in dry-down III.

CHAPTER 4 Dry-down III

An investigation into the effect of water-deficit stress on meristematic activity and leaf extension at a cellular level and the contribution of water-soluble carbohydrates to osmotic adjustment and water-deficit stress recovery in *Festuca novae-zelandiae*

4.1 Introduction and experimental approach

To test the ability of the tiller base to survive drought, a third dry-down was undertaken with the specific aim of determining the effect of water-deficit stress on mertistematic activity and recoverability of this region post water-deficit stress. Additionally, due to the observed accumulation of sucrose in the tiller base during dry-down II and the osmotic adjustment of this tissue during dry-down I, dry-down III was also to be used to assess the importance, i.e. contribution, of sucrose to osmotic adjustment of this tissue.

Interestingly the results from dry-down II also suggested that fructan synthesis is inhibited during the later stages of a developing drought period consequentially allowing sucrose to accumulate. It is hypothesised that fructans may also play an important role in drought recovery such that they become repositories for the accumulated sucrose allowing high concentrations of cytoplastic sucrose to be unloaded rapidly from adjusted tissues allowing pre-drought metabolic activity and function to return upon tissue re-hydration. This hypothesis was also to be tested in dry-down III.

The plant material used for dry-down III consisted entirely of clonal tussocks of the semi arid *F. novae zelandiae* plants (Otari accession number 7500039). The aim of dry-down III was to assess the role of water-soluble carbohydrates in the preferential protection of the tissues comprising the tiller base of *F. novae-zelandiae* during water deficit stress. Specific aims were to determine if tissue hydration levels, lamina elongation rates, meristematic activity or conversely plant dormancy were correlated with the accumulation of specific water-soluble carbohydrates during water deficit stress.

To investigate how these processes interrelate, leaf elongation, lamina intercalary meristematic activity and tissue hydration status in conjunction with changes in the WSC composition of specific tiller leaf tissue regions were monitored over a 49-day dry-down period (Dry-down III). Dry-down III was conducted under climate control conditions at the New Zealand Controlled Climate Laboratory, Horticulture & Food Research Institute

of New Zealand Ltd. Palmerston North, New Zealand. The conditions were set to represent seasonal conditions of temperature and solar radiation that *F. novae-zelandiae* (7500039) would encounter during the most drought prone period of the year (summer) in the region from which it had originally been collected (i.e. Molesworth station in the eastern region of the south island of New Zealand (Coulter 1978; Moore 1976)). The climate conditions were set at 25/14°C day/night temperatures (\pm 0.5°C), 75% continuous humidity, a photosynthetic photon flux density of 1067 µmol m⁻²s⁻¹ in the photosynthetically active range (400-700 nm) with a 14 h photoperiod.

To quantify the contribution of WSC to tissue osmotic adjustment, the osmotic potential of the total solute content of harvested leaf tissues was compared with the osmotic potential derived solely from the WSC content within the tissue (determined by HPAEC-PAD). Total solute potential measurements were made on water-soluble extracts extracted as described in section 2.5.3. WSC qualitative and quantitative analyses were carried out by HPAEC (see section 2.6.2). In addition to the qualitative analysis of fructans, changes in total fructan concentration (expressed as hexose equivalents) of tissues during dry-down III was determined by measuring the concentration of glucose and fructose molecules released from the fructan pool by acid hydrolysis (see section 2.6.3). From the ratio of released glucose to fructose units the average degree of polymerization of the fructan pool of each tissue was calculated allowing the molar concentration to osmotic potential to be calculated (see section 2.5.3).

During dry-down III, visual observations at the cellular level were undertaken using confocal microscopy (see section 2.7). This part of the investigation was aimed at gaining an understanding of how the lamina growth zone of developing leaves responds to water deficit conditions in terms of cell expansion and mitotic activity, and how the two processes interrelate as leaf elongation is suspended (or ended) during severe water deficits as indicated from dry-down I and II.

Following the imposed 49-day dry-down period, tussock plants were re-watered and selected tissues were analysed for changes in WSC composition over a 24 h period. The aim was to determine if a change in the qualitative WSC profile of selected tissues occurred after rewatering and if so was the speed of this response equal between mature

and meristematic regions of the tiller. In addition, did these responses precede a return to mitotic activity in meristematic tissues.

In dry-down II, pooled replicate tissues from six individual tillers of *F. novae-zelandiae* within a single tussock were extracted to create each sample for HPAEC-PAD analysis. Although a complex mixture of WSC, and the loss of fructans from the meristem, zone 1, zone 2 and zone 3 tissues were observed, the concentration of water soluble carbohydrates in the sheath enclosed lamina, exposed lamina, and sheath tissue extracts was too low to assess how the dry-down conditions had effected the WSC composition of these tissues. To improve the analysis of WSC extracts of leaf tissues harvested during dry-down III (with respect to dry-down II), each extract was prepared from a 40 mg FW sample of pooled tissue which, depending on tissue type and hydration level, contained material harvested from between 20 and 80 individual tillers.

4.2 Results

4.2.1 Soil water depletion

Changes in soil water content were measured by TDR and the results are shown in Fig. 4.1. For control plants watering was continued throughout the dry-down and the soil water content to which these plants were exposed remained between 28% and 35%. In contrast, two distinct phases of water loss were observed in the soil of pots from which water was withheld. The initial phase occurred over the first 16 days of the dry-down with the soil water content declining in an approximately linear fashion from ca. 31% at day 0 to ca. 8% by day 16, an average daily water loss of 1.44%. The second phase of water loss occurred over the remainder of the dry-down (day 16-49) and was considerably slower at 0.12% day⁻¹. By the end of the dry-down the soil had dried to a water content of around 4.13% in all pots from which water had been withheld.



Figure 4.1 The soil water content of pots during dry-down III as measured by TDR. Data points represent the mean of 3 control pots, and 13 dry-down pots. Bars represent \pm standard error.

4.2.2 Changes in leaf extension rate

Under control conditions (soil water content of ca. 30%) a mean lamina elongation rate (LER) of 7 mm per day was observed for emerging leaves over the 49-day dry-down period. This LER was set as the 100% reference for the water deficit treatment (Fig. 4.2a). In response to the decrease in soil water content to 20% over the first 7 days of the dry-down, the LER of the youngest exposed tiller leaf decreased by a significant amount (P<0.05) to 70% of the rate observed for the watered controls. As the dry-down progressed, the LER continued to decline, falling to 40% by day 14 and 20% by day 21 with respect to hydrated control LERs. By day 28 of the dry-down the soil water content in dry-down pots had declined to ca. 7% and lamina elongation had ceased for plants in these pots and did not resume over the remainder of the dry-down period.

4.2.3 Changes in Leaf tissue water content

In fully hydrated plants, leaf tissues could be divided into 3 groups with respect to water content. The tissues with the highest water content were the basal three adjacent tissues of the elongating lamina; i.e. the meristem, zone 1, and zone 2. For these tissues at full turgor, water made up approximately 70% of the tissue fresh weight (day 0, Fig. 4.2b). The second group consisted of zone 3, the enclosed lamina, the exposed lamina, and sheath 1. For these tissues the water content at full turgor was significantly less (P<0.01) than that of the basal 3 tissues and ranged from ca.50 to 60% of the tissue fresh weight (day 0 Fig. 4.2b). The driest of the measured tissues (significantly so at P<0.01) was sheath 2 with an average tissue water content comprising ca. 42% of this tissues fresh weight (day 0 Fig 4.2b).

Over the course of the dry-down all tissues became significantly dehydrated (P<0.001) with respect to tissues of control plants (Fig. 4.2b). However, unlike leaf elongation rate (Fig 4.2a), for most tissues a significant decrease (P<0.05) with respect to corresponding hydrated control tissues was not observed until the soil water content had declined to ca. 7.5% at day 21 of the dry-down. The exception to this was the exposed lamina tissue, which did not show a significant water loss (P<0.05) until day 35 of the dry-down. By the time the soil had declined to ca. 6% at day 35 of the dry-down, all leaf tissues had lost a significant amount of water at the P<0.001 level of significance with respect to controls. By day 49 of the dry-down (SWC 5%), each tissue had lost in excess of 50% of its fully hydrated water content. As observed under hydrated conditions, significant differences in

terms of tissue water content existed between tissues at day 49 of the dry-down. Significantly, the meristem tissue was more hydrated (P<0.01) than any other lamina or sheath tissue with water contributing 30% of the tissue fresh weight. The driest tissues were sheath 1, sheath 2 and the exposed lamina with less than 50% of the water content of the meristem at this stage of the dry-down. For the sheath-enclosed tissues, excluding the meristem, i.e zones 1,2,3 and the enclosed lamina, the water content comprised between 15 and 25% of the fresh weight of these tissues, and each value was not significantly different between each tissue. These sheath-enclosed tissues did however contain significantly higher water content at this stage of the dry-down than either sheath 1, sheath 2 or the exposed lamina (P<0.05).



Figure 4.2 Changes in the leaf extension rate (LER) of *F. novae zelandiae* leaf tissue as a function of the soil water content (SWC) during dry-down III. Values are means \pm s.e. for n=13

4.2.4 Water deficit effects on cell density within the meristematic zone at the base of the elongating lamina

To determine if the intercalary meristem of *F. novae-zelandiae* enters a quiescent state during a water deficit, cell density, cell expansion and mitotic activity within a mitotically active zone 2.5 mm above the point of attachment of the lamina to the tiller base was examined by confocal microscopy at different time points during the dry down. Over the course of the dry-down the cell density (number of cells) within the zone of cell division of the lamina increased by ca. two-fold (Fig. 4.3). The initial significant increase (P<0.001) occurred between days 14 and 21 coinciding with a significant decrease in tissue water content from 70% to 60% (Fig. 4.2b), a reduction in leaf elongation rate from 40% to 20% of the control rate (Fig. 4.2a), and a reduction in cell length along the longitudinal axis of the lamina from ca. 13 mm to 8 mm (Fig. 4.3). The confocal images of cells at day 21 of the dry-down (Fig. 4.4B), indicate that this decrease in cell length occurred at the expense of cell cytoplasmic volume, with the nuclei, although no larger in these cells when compared with those of the hydrated control tissue (Fig. 4.4C), occupying a greater percentage of the internal cellular area i.e. ca. 50% compared to ca. 25%.

At day 28 of the dry-down (Fig. 4.4C), mitotic activity is still evident within the zone of division despite these cells, at least in terms of cell length, being ca. 50% reduced in size with respect to the corresponding cells of hydrated control plants (Fig. 4.4A).

By day 35 of the dry-down, mitotic activity was no longer apparent in the lamina intercalary meristematic zone (Fig. 4.4D). At this stage, cell density appears to have achieved a regular value of ca.17 cells per 0.1 mm^{-1} (through the longitudinal axis of the lamina meristematic zone). Cell length has stabilised at ca. $6 \mu m$ (Fig. 4.3) as indicated from the confocal micrographs of the corresponding cells at day 35 (Fig. 4.4D) and day 49 (Fig. 4.4E) of the dry-down. The final 14-day period of the dry-down (days 35 to 49) was accompanied by a significant dehydration of the meristem tissue, with the water content of 45% at day 35 declining to ca. 30% by day 49 (Fig. 4.2b). However, this is not accompanied by a reduction in cell length and presumably cell volume (although this was not measured directly) suggesting that a minimal cell size has been obtained which may be fixed by the elasticity of the primary cell wall and is independent of reduced turgor pressure. This effect can be seen in the representative micrograph of the meristem tissue at day 49 (Fig. 4.4E) in which cells appear to be no longer under turgor pressure as indicated

by the presence of void areas where cell protoplasts appear to have shrunken away from the encasing primary cell wall. In addition, the cytoplasm of these cells appears condensed around the nuclei (Fig, 4.4E; cy), and several small areas within the cytoplasm have become visible (Fig. 4.4E; v) which may represent the concentrated contents of small vacuoles as these to have become condensed.



Figure 4.3 Changes in length and density of the meristematic cells in the lamina intercalary meristem during dry-down III. Values are means \pm s.e. for n = 9.



4.2.5 Changes in the water-soluble carbohydrate (WSC) composition of *F. novaezelandiae* leaf tissues during dry-down III

During dry-down III, changes in the leaf tissue WSC composition of *F. novae-zelandiae* was quantified using HPAEC method 2. The Fructan analysis included determining the total concentration of the fructan pool within tissues based on the size of the fructan pool in terms of hexose equivalents, and measuring changes in the average degree of polymerization of tissue fructan pools determined from the ratio of glucose to fructose after complete fructan hydrolysis. For the protocol for fructan analysis see section 2.6.3.

4.2.5.1 Comparison of the WSC composition of leaf tissues at several time points during dry-down III

In the fully hydrated lamina and sheath tissues of control plants, the concentration of fructose and glucose for all tissues was less than 30 μ mol g⁻¹DW (Fig. 4.5, day 0). In each fully hydrated leaf tissue with the exception of the exposed lamina tissue the combined concentration of fructose and glucose accounted for less than 15% of the total water-soluble carbohydrate concentration of these tissues. For the exposed lamina, fructose and glucose at approximately equivalent concentrations made up ca. 50% of the total WSC concentration of this tissue. Over the 49-day dry-down period, the fructose and glucose concentration in leaf tissues did not change significantly in response to the dry-down conditions with both sugars remaining at levels below 30 μ mol g⁻¹DW in all tissues.



Figure 4.5 Tissue comparisons of the glucose, fructose, sucrose and total fructan concentration at different time points during dry-down III. Meri, meristem; Znl, Zn2, Zn3, zones 1, 2, and 3, respectively; Encl, enclosed lamina; Expl, exposed lamina; Sh1, and Sh2, sheath 1, and 2, respectively. Values are means \pm s.e. for n=3.

In contrast, changes in tissue fructan and sucrose content were evident in response to the imposed dry-down conditions in all tissues (Fig. 4.5, day 0 vs day 49). At full hydration, fructans collectively were the main WSC present within each tissue (Fig. 4.5 day 0). Highest concentrations occurred in the meristem and zone 1 tissues with ca. 300 μ mol g⁻¹ DW and 350 μ mol g⁻¹ DW, respectively. For zones 2 and 3, and the enclosed lamina, the total fructan content was ca. 200 μ mol g⁻¹ DW. However, due to large variation between replicated tissue samples the difference in fructan content between these tissues was not considered statistically significant within a 95% level of confidence. The meristem and zone 1 tissues did however contain a significantly higher fructan content (*P*<0.05) than the exposed lamina, sheath 1 and sheath 2 tissues with fructan concentrations of 50 μ mol g⁻¹ DW, 150 μ mol g⁻¹ DW and 12 μ mol g⁻¹ DW, respectively. In fully hydrated tissues, sucrose was the least concentrated of the detected WSC at < 5 μ mol g⁻¹ DW in all leaf tissues.

Day 14 of dry-down III (Fig. 4.5, day 7) was the initial time point in the dry-down when a significant difference between the fructan content of the sheath-enclosed lamina tissues (meristem, zones 1, 2, 3, and enclosed lamina) was evident. At this stage, the meristem tissue with a total fructan content of ca. 320 μ mol g⁻¹ DW contained the highest fructan content of the leaf tissues sampled. This was significantly higher (*P*<0.05) than Zones 1 and 2 the tissues of next highest fructan content at 183 μ mol g⁻¹ DW and 172 μ mol g⁻¹ DW respectively. Zone 3 and the enclosed lamina contained significantly less fructan than the meristem tissue with total fructan contents of ca. 102 μ mol g⁻¹ DW and ca.108 μ mol g⁻¹ DW, respectively. These tissues, however, were not significantly different in fructan content (at the 95% confidence level) with respect to zone 1, zone 2 or sheath 1 each with a fructan contents of less than 30 μ mol g⁻¹ DW, contained significantly (*P*<0.01) the lowest concentration of fructan at this stage of the dry-down, with respect to the sheath enclosed tissues (meristem, zones 1, 2, 3 and the enclosed lamina).

By day 21 of the dry-down (Fig. 4.5 day 21), the fructan concentration of all tissues with the exception of the meristem was less than 100 μ mol g⁻¹ DW. For the meristem, the total concentration of the fructan pool on a hexose equivalent basis was 320 μ mol g⁻¹ DW and was a significantly higher (*P*<0.001) fructan content with respect to all other leaf tissues.

At this stage of the dry-down, elevated levels in sucrose content were evident with respect to day 14 of the dry-down such that sucrose and fructan content on a hexose equivalent basis were no longer significantly different for zones 1, 2, 3, the enclosed lamina, the exposed lamina and sheath 2. However, in both sheath 1 and the meristem tissue, the concentration of fructan remained significantly higher than sucrose (P<0.05).

By day 35 of the dry-down (Fig. 4.5 day 35), the sucrose concentration (on a hexose equivalent bases) within each tissue was equivalent to the total fructan concentration of the tissue. For the meristem, both total fructan and sucrose concentration of ca. 200 μ mol g⁻¹ DW (hexose equivalents) was significantly higher than the total fructan or sucrose concentration in any of the other leaf tissues sampled. With a combined WSC concentration of ca. 400 μ mol g⁻¹ DW (hexose equivalents), the meristem contained nearly three-fold more WSC than any other tissue. At this stage in the dry-down, the combined total WSC concentration within each non-meristem tissue did not differ significantly (at a confidence level of 95%) between tissues, and ranged between ca. 80 and 160 μ mol g⁻¹ DW (hexose equivalents).

At the final harvest date of dry-down III (day 49), the total fructan concentration of the meristem had declined to ca. 150 μ mol g⁻¹ DW and when compared with all other leaf tissues was no longer a significantly higher concentration (*P*<0.05) (Fig. 4.5 day 49). In contrast, sucrose had accumulated in the meristem to a concentration of ca. 400 μ mol g⁻¹ DW, a significantly higher concentration (*P*<0.05) than the adjacent zone 1 tissue with the second highest sucrose concentration of ca. 158 μ mol g⁻¹ DW. For all other leaf tissues, the sucrose concentration was less than 100 μ mol g⁻¹ DW and did not differ significantly between each other within the 95% confidence level.

4.2.5.2 Changes in tissue specific WSC compositions during dry-down III

In the meristem tissue of fully hydrated plants, the total fructan concentration was ca. 300 μ mol g⁻¹ DW (hexose equivalents). In comparison to the sucrose, fructose and glucose concentrations of the tissue this equated to at least a 200-fold higher concentration when compared on a hexose equivalent bases (Fig. 4.6).

Over the first 21 days of the dry-down, in which the soil water content declined from ca 32% to 7.5%, the total fructan concentration of the meristem did not change significantly. By day 35 however, in which the soil water content declined to ca. 6%, a significant decrease (P<0.05) of ca.100 µmol g⁻¹ DW was observed.

Over the final 14 days of the dry-down, as the soil water content decreased to ca. 4.8%, the total fructan content of the meristem declined to $150 \ \mu mol \ g^{-1}$ DW indicating a ca. 50% loss of the total fructan concentration of the tissue from hydrated plants. In contrast to the decline in fructan concentration, sucrose accumulated in the meristem tissue over the same period. This accumulation was apparent first at day 21 but was not statistically significant (P < 0.05) with respect to the hydrated control plants (day 0) until day 35 of the dry-down. At this stage, the sucrose concentration had increased to 200 µmol g⁻¹ DW (hexose equivalents) and on a hexose equivalent basis was equivalent in concentration to the total fructan content within the tissue. Over the final 14 days of the dry-down, as the soil dried from a water content of ca. 7.5% to ca 4.8% a significant two-fold increase (P<0.05) in sucrose concentration within the meristem occurred so that by the final day of the drydown (day 49) sucrose made up ca. 70 % of the total WSC content of the tissue at a concentration of 400 μ mol g⁻¹ DW (hexose equivalents). For the meristem tissue during dry-down III, a net accumulation of 150 μ mol g⁻¹ DW in WSC on a hexose equivalent basis had occurred, although no significant change in the concentration of fructose or glucose within the tissue was evident over the dry-down period.



Figure 4.6 Changes in glucose, fructose, sucrose and total fructan concentration (in μ mol hexose g⁻¹ DW) in selected *F. novae-zelandiae* leaf tissues as a function of soil water content during dry-down III. Values are means \pm s.e. for n = 3.

For the zonel designated lamina tissue (Fig. 4.6), the total fructan concentration was approximately 350 μ mol g⁻¹ DW in hydrated plants in contrast to the < 5 μ mol g⁻¹ DW sucrose concentration in the tissue. During the decline in soil water content from control levels of ca. 32% to ca. 11% at day 14 of the dry-down, the fructan concentration of zone 1 decreased to ca. 200 μ mol g⁻¹ DW. With respect to control plants however, a statistically significant decrease (*P*<0.05) in fructan concentration was not evident until day 21 of the dry-down when the tissue fructan content had dropped to ca. 100 μ mol g⁻¹ DW. Over the remainder of the dry-down, there was no further change in the fructan concentration of this tissue. In contrast, sucrose levels began to increase in the zone 1 lamina tissue at soil water contents below ca. 11% (day 21 of the dry-down), so that by day 49, a significant increase (*P*<0.05) from ca. 2 μ mol g⁻¹ DW in hydrated control tissues to ca.160 μ mol g⁻¹ DW (hexose equivalents) in Zone 1 of the dry-down plants had occurred. In contrast to the meristem tissue, for zone 1 a net loss in total WSC concentration of 100 μ mol g⁻¹ DW on a hexose equivalent basis had occurred during dry-down III. Over the dry-down period there was no significant change in the fructose or glucose concentration of this tissue.

In hydrated plants, the average fructan concentration of zones 2, 3, and the enclosed lamina was ca. 200 μ mol g⁻¹ DW. For these tissues, significant decreases (*P*<0.05) in fructan concentration did not occur until the soil water content had to declined below 11%. At this stage in the dry-down (day 14) the fructan concentration had declined to ca. 75 μ mol g⁻¹ DW for zone 2, and ca. 50 μ mol g⁻¹ DW for both zone 3 and the enclosed lamina tissue. Over the remainder of the dry-down, slight fructan increases of ca. 25 μ mol g⁻¹ DW occurred in zones 2 and 3 but these were not statistically significant. In the enclosed lamina, the fructan concentration remained stable over the remainder of the dry-down.

For zones 2, 3, and the enclosed lamina tissues, significant increases (P<0.05) in sucrose concentration with respect to the corresponding hydrated control tissues, were not observed in plants at soil water contents below 7.5% (day 21 of the dry-down). For both zone 2 and the enclosed lamina the sucrose levels increased from concentrations below 2 μ mol g⁻¹ DW in control plants to ca. 80 μ mol g⁻¹ DW in dry-down plants at day 49. In zone 3, the increase in sucrose concentration was from less than 2 μ mol g⁻¹ DW in hydrated control plants to ca 60 μ mol g⁻¹ DW in dry-down plants at day 49. At the completion of the dry-down period, concentrations of total fructan and sucrose were approximately equal on

a hexose equvalent bases in these three tissues (zones 2, 3, and the enclosed lamina tissues). Fructose and glucose concentrations within these tissues did not significantly change over the course of the dry-down.

For the exposed lamina the total fructan concentration in hydrated plants was ca. 50 μ mol g⁻¹ DW and was not observed to differ significantly in response to the declining soil water content over the dry-down (Fig. 4.6). However, a significant increase (*P*<0.05) in sucrose levels within the exposed lamina from control concentrations of < 2 μ mol g⁻¹ DW to ca. 75 μ mol g⁻¹ DW (hexose equivalents) was observed in dry-down plants as the soil dried from an 11% soil water content to ca. 4.8%. No significant change in fructose or glucose concentration was observed for this tissue during the dry-down.

In sheath 1 of fully hydrated plants, the total fructan concentration was ca. 150 μ M g⁻¹ DW (Fig. 4.6). For this tissue, a decline in soil water content from ca. 30 to ca. 11% over the first 14 days of the dry-down resulted in a significant decline (*P*<0.05) in fructan content to ca. 50 μ mol g⁻¹ DW. By the end of the dry-down period however, the fructan concentration had increased to ca. 100 μ mol g⁻¹ DW and was not significantly different from the fructan concentration in sheath 1 of the hydrated control plants. For sucrose in this tissue, the pattern of accumulation followed that of sucrose accumulation in the tissues of the developing lamina. At soil water contents above 11 % there was no significant change in the sucrose content (< 2 μ mol g⁻¹ DW) of the tissue with respect to control plants. However, below a soil water content of 11% sucrose began to accumulate and by day 49 of the dry-down at ca. 75 μ mol g⁻¹ DW (hexose equvivalents) a significant increase (P<0.001) with respect to the sucrose content in hydrated control plants had occurred. No significant alteration in fructose or glucose concentration occurred in this tissue over the dry-down.

In sheath 2 of hydrated plants (Fig. 4.6), both sucrose and fructan concentrations were below 5 μ mol g⁻¹ DW. During the dry-down, a significant increase (*P*<0.01) in both total fructan and sucrose concentrations to ca. 70 μ mol g⁻¹ DW and 60 μ mol g⁻¹ DW, respectively was observed by day 35. These levels did not significantly change over the final 14 days of the dry-down period. This tissue also had no significant change in concentration of glucose and fructose over the duration of the dry-down.

4.2.6 Changes in the mean degree of polymerisation (DP) of the fructan pool of

F. novae-zelandiae leaf tissues during dry-down III

In the fully hydrated control *F. nova- zelandiae* plants, the average DP of the fructan pool for the sheath enclosed lamina tissues (meristem, zones 1, 2, 3, and the enclosed lamina) and the youngest sheath tissue (sheath 1) ranged between 7.8 and 9.4 (Fig 4.7a) and was not significantly different between these tissues at the 95% confidence level. For the exposed lamina and the oldest sheath tissue (sheath 2), the average DP of the fructan pool of these tissues was ca. 6 and was significantly lower than the DP of the fructan pool of sheath 1 and the sheath enclosed lamina tissues (P<0.05). This indicates that the fructan pool of low DP fructans when compared with the other leaf tissues sampled.

Degree of polymerisation

11 а 10 Т 9 8 7 6 5 4 3 Meri Zn1 Zn2 Zn3 EncL ExpL Sh 1 Sh 2 11 10 b 9 8 7 6 5 4 3 Meri Zn1 EncL Zn2 Zn3 ExpL Sh 1 Sh 2

Tissues

Figure 4.7 Average degree of polymerisation of the total fructan pool of leaf tissues of a, control plants; or b, plants harvested at day 7 of the dry-down. Meri, meristem; Zn1, Zn2, Zn3, zones 1, 2, and 3 respectively; Encl, enclosed lamina; Expl, exposed lamina; Sh1, and Sh2, sheath 1, and 2 respectively. Values are means \pm s.e. for n = 3.

After 7 days of the dry-down, significant reductions in the average DP of the fructan pool of several of the lamina tissues was evident (Fig. 4.7b). In zones 1, 2, 3 and the enclosed mature lamina tissue the average DP of the fructan pool had declined from ca. DP 9 to ca. DP 8 (P<0.01 for zones 1 and 2, P<0.05 for zone 3 and the enclosed lamina).

By day 14 of the dry-down further significant decreases in the average DP of the fructan pool of the meristem (P<0.05), zone 1 (P<0.01), zone 2 (P<0.05), zone 3 (P<0.05) and sheath 1 (P<0.01) (Fig. 4.8a) had occurred with respect to corresponding tissues in plants harvested at day 7 of dry-down III (Fig. 4.7b). In the meristem, zones 1, 2, and 3 the average DP of the fructan pool had decreased to between DP 6 and DP 7 while for sheath 1, the average DP had declined to ca. 5.5.

At day 21 of the dry-down, the greatest change in the fructan composition of leaf tissues occurred in the fructan pool of the enclosed lamina tissue with a significant decrease (P < 0.001) in the average DP of the fructan pool from ca. DP 7.4 at day 14 (Fig. 4.8a) to ca. DP 4.0 by day 21 (Fig. 4.8b).



Tissues

Figure 4.8 Average degree of polymerisation of the total fructan pool of leaf tissues of a, plants harvested at day 14; or b, plants harvested at day 21 of the drydown. Meri, meristem; Zn1, Zn2, Zn3, zones 1, 2, and 3 respectively; Encl, enclosed lamina; Expl, exposed lamina; Sh1, and Sh2, sheath 1, and 2 respectively. Values are means \pm s.e. for n = 3.

From day 7 to 14 of the dry-down, significant decreases in the average DP of the fructan pools of zone 2 (P<0.01) and zone 3 (P<0.01) of ca. DP2 were also measured (Fig. 4.8 a,b). This lowered the mean DP of the fructan pools of these two tissues to ca. 4 and ca. 3.8 respectively so that for the first time in the dry-down they were significantly lower (had a relatively higher concentration of low DP fructans) (P<0.05) than either the meristem or zone 1 tissues with fructan pools averaging ca. DP 6.

A significant decrease (P < 0.01) in the average DP of the fructan pool of sheath 2 also occurred from day 14 to day 21 of the dry-down, with a decline in mean DP from ca. DP 6 at day 14 (Fig 4.8a) to ca. DP4 by day 21 (Fig. 4.b).

By day 35 of the dry-down, the average DP of the fructan pool of most tissues was <5 with the exception of zone 2 of the enclosed lamina for which the average fructan DP was ca. DP5.8, and was significantly larger than for any other leaf tissue (P<0.05) (Fig. 4.9a).

In comparison to the WSC make up of tissues at day 21 of the dry-down, only the meristem and sheath 1 had significant decreases in the mean DP of their respective fructan pools over this period. For the meristem, the DP decreased from ca. 6 to ca. 4.7 (significant at P < 0.05) at day 21, while for sheath 1, the average DP of the fructan pool decreased from ca. 5 to ca. 4 (significant at P < 0.01) over the same period (Fig. 4.8b vs Fig. 4.9a). By day 49 of the dry-down, further significant decreases in the average DP of the fructan pool of the meristem, zone 2, zone 3, and the enclosed lamina tissue had occurred. For the meristem, zone 3 and the enclosed lamina the average DP of the fructan pool of these tissues dropped to ca. 3.5. While for zone 1, the average DP of the fructan pool of this tissue decreased to ca. 5 and remained significantly higher than in any other tissue (P < 0.05) (Fig. 4.9b).





Figure 4.9 Average degree of polymerisation of the total fructan pool of leaf tissues of a, plants harvested at day 35; or b, plants harvested at day 0 (hydrated controls), and day 49 of the dry-down. Meri, meristem; Zn1, Zn2, Zn3, zones 1, 2, and 3 respectively; Encl, enclosed lamina; Expl, exposed lamina; Sh1, and Sh2, sheath 1, and 2 respectively. Values are means \pm s.e. for n = 3.

4.2.7 Tissue-specific fructan identification

To identify compounds detectable by HPAEC-PAD within each leaf tissue of F. novaezelandiae, elution times of individual peaks were compared with the WSC chromatographic profile of leaves of *Lolium perenne* (Fig. 4.10) induced to synthesise fructans (see section 2.6.1). The structure of fructan isomers synthesised by this grass species had been previously determined by Pavis et al., (2001) using both mass spectrometry and HPAEC-PAD analytical methods. Using a similar HPAEC-PAD system (HPAEC method 3), specific peaks within the *Lolium perenne* fructan chromatographic profile were identified by spiking extracts with specific inulin standards and locating the corresponding transformed peaks and elution times within the chromatographic profile. Using this method, the identification of unknown peaks within the Lolium perenne chromatogram was achieved through the direct comparison with the chromatographic WSC profile presented in Pavis et al., (2001). The identified peaks within the WSC chromatographic profile of Lolium perenne and the corresponding peaks within the WSC profile of a pooled sample of meristem, zone 1, 2 and 3 extracts from *Festuca novae*zelandiae are shown in Fig. 4.10. The elution times corresponding to the fructan representative peaks of Fig. 4.10 were used to identify the fructan isomers (detected by HPAEC-PAD) in tissue extracts prepared from harvested tissues collected during drydown III. This allowed relative increases or decreases between individual fructan isomers within a tissue over the course of dry-down III to be monitored.



Figure 4.10 HPAEC-PAD chromatographic comparison of fructan isomers present in leaf extracts of *Lolium perenne* (A), and *Festuca novae-zelandiae* (B). Numbers indicate the peaks with elution times corresponding to known standards.

1, 1-kestotriose; 2, 6-kestotriose; 3, 6G-kestotriose; 4, 1,1-kestotetraose;

5, 1&6G-kestotetraose; 6, 6G,6-kestotetraose; 7, 1,1,1-kestopentaose;

8, 1&6G,1-kestopentaose; L, loliose; R, raffinose.

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4.2.8 Comparison of the WSC composition of fully hydrated *F. novae-zelandiae* leaf tissues and equivalent water deficit tissues harvested at day 49 of dry-down III

Comparison of the fructan chromatographic profiles of the hydrated (control) leaf tissues (Fig. 4.11A) indicates that the fructan composition throughout the elongating leaf (meristem, zones 1, 2, 3, the enclosed and exposed lamina tissues) and the sheath tissues of the older tiller leaves (sheath 1 and sheath 2) are similar in that each tissue consists of a heterogeneous fructan pool containing isomers of the inulin, inulin neoseries and levan neoseries. The representative chromatograms of the meristem, zones 1, 2, 3, the enclosed lamina and sheath 1 extracts have relatively large peaks representing 1-kestotriose (peak 1), 6G-kestotriose (peak 3), 1,1-kestotetraose (peak 4), and 1&6G-kestoteraose (peak 5) and a relatively small peak representing 6-kestotriose (peak 2). This indicates that the fructans in F. novae-zelandiae are mainly composed of the inulin and inulin neoseries. Unidentified peaks appearing in the chromatograms of these tissues at retention times above 15 min including the large unresolved end peak eluting at 33 min represent fructans of DP 5 and higher. In conjunction with the determined average DP of the fructan pool of these tissues (DP ca. 8-9), these results indicate that within each tissue a wide range of fructan isomers of different molecular weight occur ranging from trisaccharides through to fructan polymers in excess of DP 10. In addition, because each chromatographic profile was generated from extracts of 40 mg FW tissue samples of equal dilution, the difference in relative peak size between chromatograms indicates that under hydrated conditions fructan concentrations are highest in the lamina base (meristem and zone 1) and then decline along a gradient towards the exposed lamina. A second declining soluble sugar gradient also occurs between the basal tissues of adjacent leaves such that highest soluble sugar concentrations occur in the meristematic lamina tissue of the youngest tiller leaf, with sequentially lower sugar concentrations occurring in sheaths 1 and 2 of the sequentially older leaves (Fig. 4.11A).

The smaller fructan peaks in each of the representative chromatograms of tissues harvested at day 49 of the dry-down (Fig. 4.11B) with respect to the corresponding chromatograms of hydrated tissues (Fig. 4.11A), indicate that in all tissues the individual fructan isomers have decreased in concentration during the dry-down. A massive increase in sucrose concentration within leaf tissues over the dry-down period is also suggested when the corresponding leaf tissue chromatographic profiles of control and day 49 dry-down plants are compared.





Figure 4.11 Representative HPAEC-PAD chromatographic profiles of WSC extracts of leaf tissues of hydrated (control) plants (A), and plants harvested at the end of dry-down III (day 49) (B). Numbers indicate the peaks with elution times corresponding to known standards. 1, 1-kestotriose; 2, 6-kestotriose; 3, 6G-kestotriose; 4, 1,1-kestotetraose; 5, 1&6G-kestotetraose; 6, 6G,6-kestotetraose; 7, 1,1,1-kestopentaose; 8, 1&6G,1-kestopentaose; S, sucrose

4.2.9 Contribution of WSC to leaf tissue solute potential in *F. novae-zelandiae* during dry-down III

The WSC analysis of leaf tissue extracts indicated that sucrose accumulated throughout the lamina and sheath tissues of *F. novae-zelandiae* when intact plants were exposed to soil water contents below 7.5%. This occurred at approximately day 21 in dry-down III, and was also accompanied by a significant decrease in the water content of leaf tissues. As part of the dry-down III experiment, the contribution of sucrose to changes in the total solute potential of leaf tissues during dry-down III was assessed.

For this the total water-soluble fraction of 40 mg DW tissue samples harvested at various time points in dry-down III were dissolved in 160 µl of pure distilled water and the solute potential determined by thermocouple psychrometry (for method details see section 2.5.3). Because the sucrose concentration on replicate tissues harvested during dry-down III had been determined by HPAEC, the osmotic potential of the sucrose concentration within each 160 µl extract could be calculated, using the equation of the osmotic potential standard curve generated for sucrose solutions of known concentration (see section 2.5.3) allowed the contribution of sucrose to the osmotic potential of each tissue extract to be calculated and the contribution of sucrose to osmotic adjustment to be estimated. The solute potential of the extracts from the control (hydrated) leaf tissues are presented in Fig. 4.12. These results do not represent actual measured tissue osmotic potentials but rather indicate which tissues contain higher concentrations of osmotically active compounds. As indicated in Fig. 4.12, the osmotically active compounds of the elongating lamina are not uniformly distributed along the lamina but occur along a declining gradient with significantly higher (P < 0.01) solute concentrations in the lamina base (meristematic tissue) and lowest concentrations in the exposed mature lamina. The lowest solute concentrations occurred in the two sheath tissues (sheath 1 and 2).



Tissue

Figure 4.12 Solute potential of standardised tissue extracts of control (fully hydrated) tissues. Meri, meristem; Zn1, Zn2, Zn3, zones 1, 2, and 3 respectively; Encl, enclosed lamina; Expl, exposed lamina; Sh1, and Sh2, sheath 1, and 2 respectively. Values are means \bullet s.e. for n = 3.

The solute potential of the control tissue extracts was set as the zero solute potential point for each tissue so that a direct comparison could be made between the changes in the solute composition of different tissues over the dry-down. The changing solute potential of tissues as determined from the tissue solute extracts is presented in Fig. 4.13, as are the changes in solute potential respective to sucrose accumulation. Also included in this Figure is the calculated solute potential that would be associated with the fructan concentration in each tissue extract (as estimated from the HPAED-PAD analysis of replica tissue samples) assuming that the osmotic potential of one mole of sucrose was equal to the osmotic potential of one mole of fructan regardless of degree of polymerisation. Fructan concentration was derived from the total fructan concentration expressed on a hexose equivalent basis divided by the average degree of polymerisation calculated for the total fructan pool of the extract (see section 2. 5.3).



Figure 4.13 Differences in fructan, sucrose and total solute potential of resuspended tissue extracts harvested at different soil water contents during drydown III. Control tissue water extracts (30 % soil water content) were used as the zero point. Values are means \pm s.e. for n = 3 and do not include the error derived from controls (zero point).

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As indicated in Fig. 4.13, over the first 7 days of the dry-down the meristem, zone 1 and the youngest sheath tissue (sheath 1) accumulated significant levels of osmotically active compounds (P < 0.05). At this stage of the dry-down, WSC were not a part of this osmotic adjustment. Over the next 7 days of the dry-down corresponding to a decrease in soil water content to ca. 11%, both the meristem and sheath 1 tissues continued to accumulate water soluble compounds. These tissues, along with the exposed lamina and sheath 2 displayed a significantly increased solute potential (with respect to control tissues) at this stage in the dry-down. In contrast, a decrease in solute potential was indicated for zone 1 of the elongating lamina between day 7 and 14 of the dry-down. At this stage in the drydown, sucrose and fructan concentrations had not directly effected a change in the osmotic potential of leaf tissues. By day 35 of the dry-down however, significant sucrose accumulation had occurred in the meristem and correspondingly resulted in an increase in the osmotic potential of the extract. For this tissue between day 14 and day 35, the increased sucrose concentration accounted for ca. 80% of the total increase in solute concentration indicated from the difference in the osmotic potential of the meristem extracts over this period.

Over the final 14 days of the dry-down, increased sucrose levels contributed ca. 70 % of the measured increase in solute potential of the meristem extracts between days 35 and 49 (-0.36 MPa verses -0.48 MPa, respectively). Increased sucrose levels in zone 1 over the final 14 days of the dry-down (accounting for an increase in the solute potential of the extracts of day 35 and 49 of ca. -0.06 MPa) accounted for ca. 75% of the total solute accumulation over this period. In contrast, although significant increases were recorded in the solute potential of all other leaf tissue extracts (zone 2, zone 3, the enclosed and exposed lamina tissues, and sheath 2) when compared over the final 14 days of the dry-down, low levels of sucrose accumulation in each of these tissues over this period indicate that this sugar was only a minor component of the solute accumulation (< 10%).

Interestingly although large changes (decrease) in the fructan concentration measured on a hexose equivalent basis had occurred within tissues over the dry-down (Fig. 4.6), the concurrent reduction in the average DP of the fructan pool within tissues (Fig. 4.9b) meant that the molar concentration of fructans within tissues did not significantly change over the

course of the dry-down. Because of this, fructans did not directly contribute to changes in the solute potential of tissues during dry-down III.

By the end of the dry-down (day 49), sucrose accounted for ca. 27% of the accumulated osmotically active compounds in the meristem tissue over the entire dry-down period (Fig. 4.14). For zones 1, 2 and 3, in which similar concentrations of osmolyte compounds had been accumulated the proportion of sucrose as a percentage of the total accumulated osmolyte content was ca. 35% for zone 1, ca. 12.5% for zone 3, with zone 2 intermediate at ca. 17%. For the enclosed lamina and exposed lamina, sucrose accounted for ca. 13.5% and 10%, respectively of the total solute accumulated over the dry-down period. For sheath 1 and 2, sucrose was not a significant component of the solute accumulated over the dry-down comprising less than 5% (Fig. 4.14).
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Figure 4.14 Contribution of sucrose to osmotic adjustment of *F. novae-zelandiae* leaf tissues at day 49 of dry-down III. Values are means \pm s.e. for n = 3 and do not include the error derived from total solute potential.

When different tissues are compared, the meristem and sheath 1 were the leaf regions in the tiller with greatest solute accumulation over the dry-down period, comprising ca. 24% and ca. 22%, respectively of the total solute accumulated by the designated leaf tissues (Fig. 4.15). Between the other leaf tissues sampled, there was no significant difference in the solute levels accumulated on a dry-weight basis.



Figure 4.15 Distribution of the osmotically active compounds accumulated by selected leaf tissues during dry-down III as a percentage of the total net solute accumulation of these tissues combined. Values are means \pm s.e. for n = 3 and do not include the error derived from total accumulated solute potential.

4.3. The fructan composition of selected *F. novae-zelandiae* leaf tissues following rewatering

At the end of the 49-day dry-down period, the tussocks were re-watered and HPAEC profiles prepared for WSC extracts of the lamina meristem, zone 2, the sheath enclosed lamina, and sheath 1 tissues extracted at 1.5 h, 8 h, and 24 h post re-watering. The representative chromatograms for each tissue are presented as Fig. 4.16. Comparison of the chromatograms for the meristem tissue show that by 1.5 h post rewatering, the sugar composition of the tissue has changed such that the level of 1-kestotriose (peak 1) has increased in the tissue relative to sucrose (peak S). By 8 h post re-watering, this trend has continued and 1-kestotriose appears to be accumulating at the expense of sucrose as indicated from the increase in the representative 1-kestotriose peak (peak 1) and the contrasting decrease in the sucrose peak (peak S) when comparing the chromatogram at 1.5 h with the chromatogram at 8 h. By 24 h post re-watering the contrast in the representative chromatograms for the meristem prior to watering, and at 24 hrs post rewatering indicate a further decrease in the level of sucrose in the tissue of at least 37% based on the relative difference in the size of peak S between chromatograms. In contrast the proportion of low DP fructan levels have increased. Comparison between the different time points indicate that 1-kestotriose is initially accumulated as early as 1.5 h post rewatering, but has plateaued by 8 h. Between 8 and 24 h post re-watering 1,1-kestotetraose (peak 4) has accumulated and has now become the second highest accumulated fructan at this stage after re-watering.

For zone 2, the enclosed lamina, and sheath 1, resumption of WSC metabolism appears to be delayed in each tissue until at least 8 hrs post re-watering. By 24 h however, and in common with the meristem, a decrease in the level of sucrose (peak S) and an increase in the level of low DP fructans of the inulin series, i.e. 1-kestotriose (peak 1) in zone 2 and the mature enclosed lamina tissue and, additionally 1,1-kestotetraose (peak 4) in sheath 1, is apparent. In sheath 1, between 8 and 24 h post re-watering, the decline in sucrose concentration of the tissue was ca. 50% as indicated from the change in the size of peak S in the representative chromatograms. For zone 2 and the enclosed lamina tissue the decline in sucrose concentration is less at ca. 40% for both tissues 24 h post re-watering.





Figure 4.16 Representative HPAEC-PAD chromatograms of sucrose and fructans extracted from selected leaf tissues at day 49 of dry-down III, and 1.5, 8, and 24 hours post rewatering at day 49.

4.3.1 Confocal microscopy visualisation of cells of the apical meristem in waterdeficit and rehydrating cells of *F. novae-zelandiae*

Confocal microscopy was used to visualize the cells of the apical meristem of individual *F. novae-zelandiae* tillers at days 35 and 49 of the dry-down, and day 49 plus 24 h post rewatering, to determine if the morphology of cells had been preserved as the meristem tissue had dehydrated, and if there was any indication of a post-water-deficit recovery.

At day 35 of the dry-down (Fig. 4.17A) the cells of the meristem are still under turgor pressure as indicated from the apparent continuum of cytoplasm between neighbouring cells. None of the cells appear to be mitotically active, suggesting that the apical meristem has become quiescent. In contrast to observations at day 35, cells of the apical meristem after 49 days exposure to the dry-down (Fig. 4.17B) show distinct signs of cellular dehydration. No longer does the cytoplasm appear to fill the volume of the cell, but rather the cells appear plasmolysed such that the protoplast has retracted away from the primary cell wall, and the cytoplasm appears to have condensed around the nuclei in the centre of the cell. This has also occurred in the adjacent basal lamina tissue (lb).

The micrograph of the apical meristem post 24 h re-watering (Fig. 4.17C), indicates how rapidly the tiller base tissue recovers from severe dehydration. Already at this relatively early stage post re-watering, the quiescent state of the lamina tissue has ended as indicated by the resumption of mitotic activity (Fig. 4.17C*) in the lamina base adjacent to the apical meristem (lb). The cells of the apical meristem appear to have re-hydrated although they do not appear to be at full turgor at this stage as suggested by the defined outline of each cell, which contrasts with the fuller volume and pressed appearance between adjacent cells of the apical meristem cells at day 35 of the dry-down (Fig. 4.17A). In common with the continued lamina elongation of control plants under the artificial summer type conditions produced in the climate laboratory, the lamina mitotic activity (Fig. 4.17C*) indicates that *F. novae-zelandiae* is not a summer dormant species, but rather becomes mitotically inactive (quiescent) under water deficit conditions. The rapidity of the reversal of the quiescent state is indicated from the WSC metabolic activity in the meristem as early as 1.5 h post rewatering (Fig. 4.16A) and the mitotic activity in the lamina base 24 hours post re-watering.



Figure 4.17 Confocal micrographs of the apical meristem of individual *F. novaezelandiae* tillers after 35 and 49 days of dry-down (A and B, respectively), and 49 days of dry-down plus 24 h post rewatering (C). n, nuclei; lp, leaf primordia; lb, leaf base; cy, cytoplasm; *, mitotically active cell showing condensed chromosomes. Bar represents 10 μ m scale, magnification = 63x10.

CHAPTER 5

Discussion

This study has investigated cellular responses to water deficit in the indigenous New Zealand perennial grass Festuca novae-zelandiae. The natural habitat, in which F. novae zelandiae forms the sole physiognomic dominant vegetation, is characterised by low annual rainfall, extensive dry-periods and a high evaporotranspiration demand as a result of frequent dry northerly winds (Baker 1953). Already documented for this species are a number of anatomical features associated with water conservation that underlines the fitness of this plant species to this habitat (Baker 1953, Connor 1960, Lord 1993, Abernethy et al., 1998). These include a deep root system with a well developed system of laterals in the topsoil, tightly rolled narrow leaves in which stomate distribution is localised to the inner curled (adaxial) lamina surface, dense adaxial trichome coverage associated with stomate distribution, and an abundance of leaf sclerenchyma providing the structural support of permanently erect leaves. These features are common amongst other plant species endemic to rainfed Mediterranean-type climates where prolonged drought periods are also regular climatic events (Distel and Fernandez 1988, Pugnaire et al., 1996, Valladares and Pugnaire 1999). Together, these morphological traits are associated with limiting the rate of water loss from leaf surfaces, lowering the water demand of the plant and aiding in the conservation of soil water supplies, features associated with delaying tissue dehydration under water-limiting conditions and characteristic of a drought avoidance strategy (Levitt 1980, Ludlow 1989).

In the current study, the aim was to determine whether *F. novae-zelandiae*, in addition to its xeromorphic traits, employed specific water deficit induced biochemical responses that would provide a degree of protection against tissue dehydration that may occur under prolonged drought situations when soil water has become severely depleted. In other words, in addition to a drought avoidance strategy determined largely by the anatomy of the leaf and deep root system, does *F. novae-zelandiae* possess water deficit adapted metabolic traits that underline a strategy of drought tolerance? In particular, the aim was more specifically to assess the role of water-soluble carbohydrates in the water deficit survival and recovery of the perenniating meristematic tissues of the tiller base.

In work by Abernethy et al., (1998) on F. novae-zelandiae, tissue specific biochemical responses were induced in the leaves of plants exposed to drying soil conditions, suggestive of a preferential protection of the tiller base region including meristematic tissues, at the expense of both immature and mature leaf tissue (Abernethy *et al.*, 1998). These responses included a sustained elevated concentration of the osmoprotectant proline in the leaf base in contrast to an initial increase and then decline in proline within mature and immature lamina tissues. Proline decline was also accompanied by changes in the pattern of protein ubiquitination specifically through enhanced conjugation of three polypeptides of Ku18, Ku26 and Ku29, which may indicate a scavenging of material from the lamina tissue for translocation to the tiller base (Abernethy and McManus 1999). In the leaf base where proline levels remained high, and in water sufficient plants, no such changes in the pattern of protein ubiquitination was observed suggesting no accelerated ubiquitin-mediated protein degradation. Abernethy et al., (1998) reported a similar accumulation pattern for ABA as to that which had been observed for proline. ABA is thought to play a role in coordinating whole plant adjustment and adaptation of metabolism to osmotic stress (Chandler & Robertson 1994).

In terms of tissue responses to water-deficit stress in F. novae-zelandiae, fructans were of interest for a number of reasons. Firstly, fossil records indicate that the evolution of the majority of the fructan rich angiosperm families (comprising approximately 15% of the group) coincided with a climatic shift towards contrasting seasons of wet and dry periods during the Oligocene to mid-Miocene epochs (32-15 Ma ago) (Hendry 1993). The mid-Miocene is the period during which large dry open grassland habitats established with the divergence of the Pooidea, the only subfamily of the Poaceae to accumulate fructans as their major reserve carbohydrate (Kellog 2001; Jacobs et al., 1999, Bonnett et al., 1997). Within this subfamily of graminae are the agronomically important forage grasses eg Lolium species, and the cereals Aveneae (oats) and Triticea (wheat, barley and rye), and the Festuca including F. novae-zelandiae (Kellog 1998). Secondly studies have shown that specific fructan molecules may interact and stabilize membrane systems in a dehydrated state (Hincha et al., 2000; Vereyken et al., 2001) potentially this may be one of the roles of fructans in the water deficit survival of F. novae-zelandiae. Alternatively or in addition to, fructans could play an indirect role in cellular survival during water deficit periods as a source of WSC for synthesis of sucrose, one of the main osmoprotectant WSCs accumulated in desiccation tolerant tissues of resurrection plant species (Scott 2000) as well as in meristem associated tissues of many grass apecies during drought (Thomas 1991; Spollen and Nelson 1994).

The fructan profile of *F. novae-zelandiae* is mainly composed of the inulin neoseries, the inulin series, and the levan neoseries. In common with other members of the super tribe Poodae, with the exception of *Dactylis* (Chatterton *et al.*, 1993), *F. novae-zelandiae* does not synthesis the branched DP4 fructan isomer 1& 6-kestotetraose (bifurcose) which is common to *Bromus* (Chatterton *et al.*, 1993), *Hordeum* (Simmen *et al.*, 1993), and *Triticum* (Carpita *et al.*, 1989), members of the super tribe Triticodae. This finding supports the suggestion that the presence of specific fructan isomers within grasses may underlie a phylogenetic relationship that could potentially be used as an aid for taxonomic classification (Smouter and Simpson 1989; Bonnett *et al.*, 1997). As far as the author is aware this is the first study to isolate and identify fructan isomers from tissues of *F. novae-zelandiae*.

Apart from the structure of the fructan isomers produced, different grass species, and different tissues within grasses, accumulate fructans of varying degrees of polymerisation (Smouter and Simpson 1989) the formation of which may also be dependent on environmental conditions (Suzuki 1989; Thomas and James 1999). Spollen and Nelson (1988) showed that in common within the Gramminae, at least two fructan pools are present within fructan accumulating tissues: a high DP fructan pool (mean DP > 10) and a low DP fructan pool (DP ≤ 10). For F. arundinacea a high DP pool predominates in the leaf blade of ca. DP 72 and ranging up to DP 90, while in contrast a low DP pool (\leq DP 10) is predominant in the elongation zone. In contrast for *Phleum pratense* L. a high DP fructan pool predominates through the elongation zone and a low DP pool in the leaf blade (Spollen and Nelson 1988). Of the tissues analysed for F. novae- zelandiae, the difference in mean DP between tissues was not as marked, and ranged from DP 6 (for the exposed lamina of the youngest leaf, and the oldest sheath tissue (sheath 2)) to DP 9.5 (for zone 1, 2, and 3 (the elongation and differentiation tissue of the youngest emerging leaf)). Although a distinction between fructan pools based on mean DP was not made in this study, from the representative chromatographic profiles of each tiller region it appears that both low DP (≤ 10) and high DP fructan pools are present in the tissues although low DP fructans appear to dominate the profile in each of the fructan accumulating tissues (leaf base, zone 1, 2, 3, the enclosed lamina and sheath 1). The tissue-specific fructan analysis of F. novae-zelandiae in this study also suggests (at least under the imposed experimental

conditions) that the mature sheath tissue does not act as a storage tissue of non-structural carbohydrates as has been suggested for F. arundinacea (Housley and Volenec 1988) but rather may serve as a thoroughfare for carbohydrates between leaf blades and the tiller base as suggested for L. perenne (Guerrand et al., 1996). In common with other members of the grass subfamily Pooideae (Meier & Reid 1982), Fructans constitute the major portion of WSC in fully hydrated leaf tissues of F. novae-zelandiae. The results from this current study also show that the highest fructan concentrations occur in the lamina intercalary meristem and adjacent elongation zone in the base of the expanding leaves. This location of highest fructan concentration is also reported for perennial rye grass, wheat, tall fescue, timothy and barley (Spollen & Nelson 1988, Guerrand et al., 1996, Roth et al., 1997). Also in common with these species, fructan accumulation within the lamina zone of expansion of F. novae-zelandiae is likely to serve a similar role. That is, unloading of sucrose from phloem, maintaining cellular osmotic potential necessary for cell expansion, buffering metabolism against temporary shortages of carbon and energy supply from photosynthate, and providing the carbohydrate for cell differentiation and secondary cell wall synthesis on cessation of cell expansion. Lowest fructan concentrations occurred in the photosynthetic lamina from which photosynthate is translocated and so is not readily available for fructan accumulation, and in the oldest sheath of the two older leaves sampled. Low fructan levels in this sheath suggests that this tissue is either operating as a thoroughfare for carbohydrates between the leaf blade of the same leaf and sink tissues within the tiller via the point of attachment in the tiller base as reported for Lolium perenne L (Guerrand et al., 1996), or conversely, the WSC reserves of this leaf have been re-assimilated into younger tissues of the tiller prior to leaf senescence.

The role of the sheath as a longterm WSC storage organ in *F. novae-zelandiae* is also questionable from the results of this current study. In the youngest sheath tissue, WSC were low with respect to the WSC concentration of the elongating lamina and, in addition, the average DP of the fructan pool was similar (DP ca. 8) between both tissues. Hously and Volenec (1988) suggested that in grasses, leaf sheaths function metabolically as a reserve of non-structural carbohydrates on the basis that the sheath tissues of *Festuca arundinacea* had higher concentrations of fructan and of proportionally higher molecular weight than expanded blades or expanding leaves. In contrast, Borland and Farrar (1989) reported that in *Poa pratensis* L, most of the sugars imported into the sheath from the leaf blade were subsequently re-exported, resulting in similar concentrations of fructans in

blade and lamina tissues. In this current study, the similar mean DP of the fructan pools of sheath 1 and the lamina base (meristem and zone 1) suggest that these fructan pools are of a similar age, i.e. one to two days (the age of the cells in zone 1). This time period is insufficient presumably to allow the synthesis or accumulation of significant amounts of high DP fructans (Pavis *et al.*, 2001). Thus the fructan pool of the relatively older cells of sheath 1 is presumably turned over within the same period of time such that the average DP stays at ca. 8 and total fructan concentrations stay low.

A possible reason for the establishment and success of the fructan accumulating grasses is the utilization of fructan metabolism as a source of osmoregulation during periods of drought to maintain tissue hydration or cell expansion and growth (Hendry 1993). A functional role of fructans in osmoregulation has been reported in a number of studies. In daylilies for example, fructan hydrolysis into glucose and fructose monosaccharides within petal cells of developing flower buds, increases cell osmolality and turgor driven petal expansion leading to flowering (Bieleski 1993). In onions, fructan hydrolysis and concurrent fructose accumulation is thought to be a mechanism for cell osmoregulation driving water uptake and turgor-dependent cell expansion during bulb formation. In bulbs of Lachenalia minima, a plant species endemic to dry-desert regions of South Africa, in response to rewatering following rainfall, fructans are mobilized from the outer bulb leaf scales and accumulated as low DP fructans within the inner bulb leaf scales. The associated increase in osmolality within this tissue and resulting preferential hydration is a prerequisite for bulb sprouting (Orthen 2001). A role of low DP fructans as osmoticum supporting cell expansion and leaf growth in F. arundinacea has also been proposed (Housley and Volenec 1988).

More recent studies support an osmoprotectanct role for fructans in drought tolcrance. Several studies have shown that fructans are able to stabilise biological membranes in a dehydrated state (Demel *et al.*, 1998, Hincha *et al.*, 2000, Vereyken *et al.*, 2001, Hincha *et al.*, 2002). As cellular organization is derived from a hydrophobic effect that drives the assembly of phospholipids into selectively permeable barriers, membrane dehydration results in the disruption of this assembly and complete desiccation causes the irreversible loss of cellular organization and cell function (Tanford 1978). Loss of membrane integrity is considered a major component of desiccation and freezing injury to plant cells (Oliver *et al.*, 2001). Vereyken and colleagues (Vereyken *et al.*, 2001) showed recently that high DP

fructans produced by *Sac B* levan sucrase are able to interact with the headgroups of phospholipids in hydrated membrane systems stabilising the liquid-crystalline-phase, consistent with a role in cellular water deficit protection and drought tolerance. Using unilamellar liposomes composed of egg phospatidylcholine, as a model system, Hincha and colleagues (Hincha *et al.*, 2000) showed that fructan extracts from chicory roots and dahlia tubers were able to stabilise liposomes during freeze drying, and that the fructans were hydrogen bonded to the phosphate in the phospholipid headgroups. The role of fructans in the drought tolerance of grasses may, however, be indirect. For example, if drought conditions have been severe enough to promote the senescence of photosynthetic leaf area, fructans replace the lost photosynthetic capacity to become the major source of carbohydrates for the growth of new leaf tissue and drought recovery of the plant (Volaire *et al.*, 1998*b*; Thomas and James 1999). A similar role for fructans in the growth of new leaf tissue has been reported for *Lolium perenne* plants recovering from lamina defoliation (Morvan-Bertrand *et al.*, 2001).

To investigate the role of fructans in drought tolerance of *F. novae-zelandiae*, tussocks of *F. novae-zelandiae* grown in 4 L soil volumes were exposed to a diminishing water supply by withholding water from the soil, and changes in the water-soluble carbohydrate composition of distinct developmental regions monitored during the drought period. A similar method was employed in Abernethy *et al.*, (1998) water deficit study of *F. novae-zelandia* and was considered to allow a soil water depletion rate slow enough to allow controlled water deficit acclimation responses within plants such as the synthesis of osmoprotectant compounds, while limiting direct cellular injury such as membrane disruption that may occur when water stress is imposed at a faster rate (Turner and Jones 1980; Hanson and Hitz 1982, Navari-Izzo *et al.*, 1989).

During the dry-down the soil water content reduced from ca. 30% to less than 5% and resulted in a significant dehydration of leaf tissues to between 14 and 30% (fresh weight water volume) with the meristematic tissues of the tiller base retaining the highest water content. In terms of leaf growth, leaf expansion completely ceased when the soil water content decreased below 7%, i.e. at day 28 of the dry-down. This, however, was not concurrent with a cessation of mitotic activity in the lamina zone of division at this time point of the dry-down. This continued cell division in the absence of lamina extension through cell elongation is the likely mechanism responsible for the accumulation of

smaller sized cells in the lamina base following week 4 of the dry-down. Accumulation of cells in water deficit stressed plants has been reported for wheat seedlings in which the mitotic activity of mesophyll cells was reduced in response to mild water stress (Schuppler *et al.*, 1998), but at the same time the period of mitotic activity was extended such that the final number of cells produced in stressed leaves reached 86% of the controls. In common with observations for other species, cell division in *F. novae-zelandiae* appears less sensitive to water stress than does cell enlargement (Hsiao 1973). Doley and Leyton hypothesised that cell divisions are limited by a minimal size that a meristematic cell must attain before division can take place (Doley and Leyton 1968). This would explain why no cell division was observed after 35 d, when cells were universally small in the leaf base. However, the difference could also depend on the steep reduction of tissue water content that occurred between day 28 and 35 of the dry-down, when water content dropped from around 65% to less than 50%.

In some forage grasses (perennial rye grass, and tall fescue) leaf growth post water deficit occurs at an elevated rate when compared to non-water stressed plants (Horst and Nelson 1979, Thomas 1991). It may be that this compensatory growth is in part due to the concurrent expansion of undifferentiated cells in the base of leaves as is observed in *F. novae-zelandiae*, after leaf expansion had ceased but prior to the cessation of cell division. This may function as a mechanism for the rapid recovery of photosynthetic leaf area post-drought, and may be why in forage species tillers growing before drought are responsible for rapid recovery of leaf tissue in the first week post drought before growth is evident from axillary buds (Volaire *et al.*, 1998b).

By day seven of the dry-down, an accumulation of osmotically active compounds in the lamina zone of elongation had occurred without a measurable change in the water content of this tissue. This implies that the turgor pressure within cells of this region had increased. Despite this, a significant decrease of ca. 30% in the elongation rate of the lamina had occurred over this period of the dry-down. Cell expansion and the resulting leaf elongation is dependent not only on hydrostatic pressure but also two distinct metabolically controlled phases, the acceleration phase in which the cell wall loosens, and the decelerating phase in which the cell wall becomes rigid and resistant to turgor pressure (Tomos and Pritchard 1994, Kutschera 1994). It is likely that either or both of these phases have been modified to affect the growth reduction in spite of an indicated increase in

turgor pressure. A similar effect was observed in a study on the elongation of maize leaves during mild water stress (Van Volkenburgh and Boyer 1985). This study showed a correlation between a reduction in proton extrusion into the apoplast of cells in the leaf elongation zone and a concurrent decrease in the rate of leaf elongation. Decreased growth was attributable to an inhibition of the acidification of the cell wall, and a concurrent suppression of low pH activated expansins. These enzymes are polysaccharide hydrolases and transferases that catalyse the slippage between the cellulose microfibrils and the polysaccharide matrix of the wall enabling the cell wall to stretch under turgor pressure (McQueen-Mason and Cosgrove 1995). Changes in tissue auxin levels are also thought to effect mechanisms that effect cell expansion in a process independent of cell-wall acidification (Kutschera 1994). How the decrease in leaf elongation was affected specifically in F. novae-zelandiae in this current study was not determined, although metabolically controlled suppression of growth during water-deficit conditions is considered to be an important mechanism for maintaining tissue viability at the expense of tissue production (Barlow et al., 1980). In rainfed Mediterranean areas survival of cultivated perennial forage grasses during the dry summer months is inversely correlated to growth (Knight 1973). For Poa bulbosa L, an extremely drought tolerant grass, summer drought is endured in a state of dormancy with the onset of which associated with a sharp decline in leaf growth at the beginning of summer, which is independent of soil water status (Volaire et al., 2001).

For *F. novae-zelandiae*, leaf extension continued to decline over the dry-down until a soil water content of ca. 7% had been reached at which stage leaf elongation ceased. In fully hydrated plants, watered throughout the experiment, the rate of leaf extension of rapidly expanding leaves remained at a fairly constant rate of ca. 7 mm per day. Because the dry-down experiments were conducted under long day conditions and as growth was not inhibited in the watered control plants, it can be concluded that *F. novae- zelandiae* is not a summer dormant species but rather the cessation of leaf growth is in response to a depletion of soil water. This was also supported from the confocal study of the meristematic tissue of tiller bases post 24 h re-watering which indicated the resumption of mitotic activity, which had ceased over 14 d earlier in the dry-down. This partial dormancy or quiescence is common amongst plant species endemic to semi-arid climates such as the Mediterranean. It allows plants to make use of sporadic rainfall to exploit resources during a period when competition from dormant species is absent and before the

germination and establishment of new plants (Distal and Fernandez 1988, Pugnaire et al., 1996, Scott 2000). Complete suspension of growth and attainment of quiescence (the ability to recover full metabolic activity and resume growth rapidly on rehydration from a dehydrated state) (Volaire et al., 1998b, Scott 2000) is also characteristic of some of the most drought tolerant perennial forage grasses utilised in Mediterranean areas (Volaire et al., 1998b, Volaire 2001). Considered one of the most drought tolerant is the Dactylis glomerata cultivar KM2. Vegetative persistence of this cultivar during severe water deficit is associated with a number of drought survival strategies as described by Ludlow (1989). These include a slow rate of desiccation of perennating organs (dehydration postponement) afforded by a deep root system and consequent access to water held deep in the soil profile; the ability to tolerate extensive tissue dehydration, i.e. below 35% water content on a fresh weight basis; and ultimately dehydration tolerance through the development of a quiescent state (Volaire et al., 1998b). In comparison, water deficit tolerance by F. novae zelandiae can be considered a composite trait. A dehydration postponement strategy is evident from the xeromorphic characteristics of the lamina tissue and the deep root system; tolerance of dehydration was shown as the apical meristem recovered from dehydration to a water content of 30% (fresh weight basis) after entering a quiescent state in which cell activity had decreased as evident from the suspension of leaf

The confocal imaging of the dehydrated meristem tissues suggests how the organization of the meristematic cells are affected by dehydration and how the arrangement of the cell contents in the dehydrated state may protect the viability of the tiller base. By day 49 of the dry-down, cells of the tiller base no longer appeared to be under hydrostatic pressure. As dehydration occurred, the protoplasts within cells have contracted from the cell walls but appear to have remained as complete structures. As the protoplasts have contracted the nucleus appear to have remained centralised within the cytoplasm such that the cytoplasm is evenly distributed around the nuclei of cells. At a water content of 30% (fresh weight basis) the meristematic cells of the tiller base although severely dehydrated will still contain a bulk cytoplasmic water content (Hoeksra *et al.*, 2001). Contraction of the protoplast although most likely a passive dehydration response has effectively confined the contents of the cytoplasm including the cell water content to a smaller volume and will have aided in the retention of a viscous state and the hydration and conformation of macromolecules suspended in the cytoplasm.

elongation and mitotic activity in the surviving meristematic tissues of the tiller base.

For *F. novae-zelandiae* during the water deficit period, and in parallel to cessation of growth, mobilisation of reserve carbohydrates occurred in tissues with high fructan contents, causing a decrease in the total amount of WSC and decrease in mean fructan DP such that 1-kestotriose became the dominant fructan isomer within each tissue. Simultaneously, total WSC increased in the meristematic region where it remained higher than in any other tissue during the entire dry-down experiment. However, by day 49 of the dry-down fructan had declined in the leaf base and sucrose became the dominating soluble sugar, accumulating to ca. 200 μ mol g⁻¹ DW from control levels of less than 5 μ mol g⁻¹ DW. Over the final 14 days of the dry-down sucrose made up ca. 70% (on a molality basis) of the osmotically active compounds accumulated over this period in the meristem.

Sucrose is the predominant WSC accumulated within dessication-tolerant tissues of resurrection plants (Wolkers et al., 1998). In these plants the attainment of a quiesent dehydrated state is concurrent with the accumulation of sucrose. The sugar may accumulate to concentratations as low as 72 µmol g⁻¹ DW, as observed for the plant species Tripogon jacquemontii (Ghasempour et al., 1998), or as high as 2000 µmol g⁻¹ DW as reported for Craterostigma plantagineum (Bianchi et al., 1991). Sucrose is proposed to aid in the dehydration tolerance of plant tissues in at least two ways (Crowe et al., 1992, Crowe et al., 1998, Allison et al., 1999). Firstly, sugars may facilitate membrane and protein stability by substituting the hydrogen bonding within and between macromolecules, usually derived from a molecular interaction with water. Secondly sucrose is thought to stabilise internal cellular structures through vitrification which aids in slowing metabolic interactions and maintaining the relative spatial distribution of macromolecules within the cell. Sucrose accumulation during water deficit stress requires a carbohydrate source. For C. plantagineum, this role is fulfilled by the eight-carbon carbohydrate, 2-octulose which is accumulated during hydrated conditions and is rapidly metabolised during water-deficit stress concurrently as sucrose concentrations increase (Bianchi et al., 1991).

The depletion of fructans from leaf tissues in this current study and the concurrent accumulation of sucrose in the tiller base, suggests that fructans form a large part of the carbon source for this accumulation in drought exposed *F. novae-zelandiae*. This also indicates that fructan synthesis is under some form of metabolic control, and not simply

proportional to tissue sucrose concentration as already suggested by Schnyder and Nelson (1987), and shown for *F. arundinacea* (Spollen and Nelson 1994). How this is achieved can only be speculated at this stage and requires in depth molecular study of the fructan biosynthetic pathway of *F. novae-zelandiae*. However, for fructan depletion to occur at least invertase activity would have to exceed the rate of both SST and FFT activity. In the fructan metabolism of *Lolium perenne*, invertase activity and fructan hydrolysis appears to remain at a constant rate, with fructan accumulation or depletion within tissues regulated at the level of SST activity (Guerrand *et al.*, 1996).

In addition to fructans having a role as a carbohydrate source for sucrose accumulation during water-deficit stress, a second role is the unloading of accumulated sucrose, presumably from the cytosol, and restorage in the vacuole in the form of fructan. This is suggested from the rapid re-synthesis of fructans post re-watering at the completion of dry-down III. This would in effect be a reversal of the water-deficit state promoting the recovery of cytoplasmic viscosity akin to predrought through the reduction in WSC concentration. This should aid in the rehydration and return of biological function of any macromolecules that were osmoprotected through a molecular intereaction with the accumulated sucrose. Additionally, for resurrection plants, sucrose is often accumulated together with oligosaccharides, commonly those of the raffinose family.

It is not clear whether oligosaccharides play a functional role in protection of cellular components, either by preventing crystallization of sucrose during drying (Smythe 1969) or by increasing the glass transition temperature (Wolkers *et al.*, 1998). For several species it has been demonstrated that the presence of oligosaccharides correlates with seed longevity (Brenac *et al.*, 1997, Sun and Leopold 1997, Buitink *et al.*, 2000). Since it has been shown that vitrification is not sufficient to prevent damage during dehydration (Crowe *et al.*, 1998), the fructan in the meristematic region in *F. novae-zelandiae* might play a role in protection of membranes even at the reduced concentrations that have been shown to occur. As fructans appear to be localised within the vacuoles of cells it is likely that this membrane reaction would be localised at the tonoplast only.

CHAPTER 6

Conclusions

F. novae-zelandiae has a wide geographic distribution in New Zealand encompassing areas of high rainfall (>6000 mm per annum) and conversely semi arid regions where the annual rainfall may be less than 350 mm (Edgar and Conner 2000). In addition *F. novae-zelandiae* occurs in regions of the country where high evapotranspiration conditions are frequent as a consequence of drying fohn type winds (Wardle 1991).

Morphologically, with respect to leaf structure (stomata localized to the adaxial leaf surface, an area enclosed within the permanently rolled leaf), and root system (extensive lateral development within topsoil zones and deep soil root penetration up to 2 m), *F. novae-zelandiae* appears well suited to semi arid and drought prone regions. Biochemical responses to imposed water-deficit conditions outlined by Abernethy and McManus (1999), and Abernethy *et al.*, (1998), suggest that in addition to morphology, *F. novae-zelandiae* undergoes preferential protection of the tiller base during the onset of water-deficit conditions with an increase in turnover rate of proteins in mature lamina tissues and the accumulation of the osmotically active compound proline in the tiller base. Proline may have a dual role under such conditions. Firstly that of a component of osmotic adjustment aiding in tissue hydration and water retention, and secondly as a nitrogen rich storage compound utilised for protein synthesis during post drought recovery and lamina tissue re-synthesis.

This thesis set out to test the hypothesis that vegetative meristematic tissues within the tiller base of the xerophytic grass F. novae-zelandiae undergo preferential osmotic adjustment and accumulate specific water soluble carbohydrates at the expense of differentiated lamina tissues as a component of water deficit stress acclimatisation and persistence in drought prone environments. The results from the three dry-down experiments carried out in this study strongly support this hypothesis. At the conclusion of the two dry-down experiments in which osmotic adjustment was measured, the tiller base had the greatest level of osmotic adjustment. In terms of the water-soluble carbohydrate composition, changes within the plant occurred such that the mobilisation of fructans from higher DP molecules to lower DP molecules throughout all leaf tissues was coupled to a net decline in fructan concentration and concurrent sucrose accumulation in the

meristematic region of the tiller base. Additionally, the accumulation of sucrose over the imposed drought period within the tiller base appears not simply to represent the accumulation of photosynthate as a consequence in the decline of sink strength for translocated photosynthate for growth. This accumulation appears to be a regulated adjustment in water-soluble carbohydrate metabolism, with fructan hydrolysis and sucrose accumulation favoured over fructan synthesis. Also, the water-deficit stress was severe enough to inhibit mitotic activity in the tiller base of F. novae-zelandiae plants. On rewatering, however, this activity was regained rapidly (within 24 h) indicating that under severe water deficits the meristem tissues had achieved a quiescent state. Associated with this state was an accumulated sucrose concentration of 200 µmol g DW⁻¹ This concentration is well above the minimum of 72 μ mol g DW⁻¹ necessary to protect cellular structures in a desiccated state (Scott 2000). This suggests that the increase in sucrose concentration in the meristem of F. novae-zelandiae plants under extreme water-deficit may be associated with desiccation tolerance and indicative of a drought escape strategy. During post water deficit recovery a rapid reduction in sucrose concentration and resumption in fructan accumulation occurred. This indicates that fructans may not only be a direct source of carbohydrate for sucrose synthesis during water deficit stress, but also through fructan synthesis provides a means of rapidly unloading the accumulated sucrose from the cell cytoplasm and associated membranes into vacuoles allowing the hydrating tissue to recover to a normal hydrated physiological state.

Further research in this area particularly an enzymology study with a focus on WSC metabolism and the enzymes associated with sucrose and fructan synthesis and hydrolysis, coupled with measurements of photosynthetic rates and tissue carbohydrate translocation during periods of water deficit adjustment, would help complete the physiological understanding of the xerophytic nature of *F. novae-zelandiae*.

CHAPTER 7

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