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**Regulation of Ethylene Biosynthesis in  
*Festuca novae-zelandiae* (Hack.) Cockayne  
and in *Festuca arundinacea* (Schreb.) in  
Response to a Water Deficit**

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## Abstract

Changes in ethylene evolution and the associated biosynthetic enzyme ACC oxidase to a water deficit, were examined in intact leaves of *Festuca novae-zelandiae* and *F. arundinacea* cultivar 'Roa' (syn. *Schedonorus phoenix*). The aim was to establish a role, or otherwise, for ACC oxidase as a regulator of ethylene biosynthesis in response to a water deficit. While ACC synthase has long been recognised as the major rate-limiting enzyme in ethylene biosynthesis, there is mounting evidence to suggest that ACC oxidase may also regulate the ethylene biosynthetic pathway in higher plants.

Leaf tissues from the two species were harvested at regular intervals during the experimental dry-down, and dissected into two leaf zones; regions enclosed by the ligule, comprising the meristematic and elongating leaf zone (the enclosed tissue), and exposed regions comprising the mature green leaf zones. Leaf proline content and the rate of leaf elongation (LER) were used as internal and external indicators of physiological changes in response to the water-deficit. Ethylene evolution in response to a water-deficit was found to be tissue-specific in *F. arundinacea*. In the rapidly expanding leaf zones, i.e. enclosed tissue, ethylene was maintained at levels similar to control tissue. In the mature green regions of leaves, ethylene followed changes in the leaf elongation rate (LER) with observed peaks in ethylene evolution occurring approximately 48 hours after a rapid decline in the LER. This burst of ethylene was found to precede any accumulation of proline. Increases in the proline content in both leaf zones, only became significant after the ethylene evolution had subsided to below base levels. This stage-specific ethylene evolution in leaves suggests preferential protection of the rapidly expanding leaf cells, an observation that has been documented by other authors.

ACO specific enzyme activity was greatest at soil water contents of ca. 9% in the enclosed and 10% in the exposed leaf tissues of *F. arundinacea*. On further purification of the enzyme, two novel proteins were recognised by polyclonal antibodies in water-stressed leaves of *F. arundinacea*. A 32 kDa protein was identified in the enclosed leaf tissue and a 37 kDa protein was identified in the exposed leaf tissue, by SDS-PAGE. These proteins eluted from a Mono Q column at different points in the separation process, i.e at salt concentrations of 320-340 and 300-320 mM NaCl respectively, indicating that they may represent two distinct isoforms of the ACO enzyme. Both proteins are active at pH 7.5 with saturating substrate (ACC) and co-substrate (Na

ascorbate) concentrations of 1 mM and 30 mM respectively, and co-factor concentrations of 0.02 mM  $\text{Fe}^{2+}$  and 30 mM  $\text{NaHCO}_3$ . When compared with results from western analyses, maximum specific enzyme activity correlated well with the water-deficit induced protein from partially purified enclosed leaf tissue, but only loosely with the protein identified in the exposed leaf tissue. The presence of high molecular weight proteins in both the crude and the purified (Mono Q) leaf extracts of *F.arundinacea* together with the novel proteins, suggests that the ACO enzyme in this species may exist as a dimer. In *F.novae-zelandiae*, the presence of high molecular weight molecules in the crude and partially purified (Sephadex G-25) extracts also suggests dimerisation of the enzyme in this species.

From this study however, it is not possible to establish a clear regulatory role for the ACO enzyme in ethylene biosynthesis in either *F.arundinacea* or *F.novae-zelandiae*. While two novel water-deficit-induced proteins were associated with increased ACO activity in purified leaf extracts of *F. arundinacea*, there was no obvious correlation between ethylene evolution and enzyme activity.

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## List of Abbreviations

|                     |  |
|---------------------|--|
| A (518nm)           | absorbance at a wavelength of 518 nm                     |
| ABA                 | abscisic acid  |
| ACC                 | 1-aminocyclopropane-1-carboxylic acid                    |
| ACO                 | ACC oxidase  |
| ACS                 | ACC synthase   |
| Adomet              | S-adenosyl-L-methionine                                  |
| ANOVA               | analysis of variance                                     |
| APS                 | ammonium persulphate                                     |
| ATP                 | adenosine triphosphate                                   |
| BCIP                | 5-bromo-4-chloro-3-indolyl phosphate                     |
| BSA                 | bovine serum albumin                                     |
| °C                  | degree Celsius   |
| ca.                 | <i>circa</i> (approximately)                             |
| CO <sub>2</sub>     | carbon dioxide   |
| DMSO                | dimethyl sulphoxide                                      |
| DTT                 | dithiothreitol   |
| EDTA                | ethylenediamine tetra-acetic acid                        |
| EFE                 | ethylene forming enzyme                                  |
| FPLC                | fast protein liquid chromatography                       |
| FW                  | fresh weight   |
| GACC                | 1-(gamma-L-glutamylamino) cyclopropane-1-carboxylic acid |
| G                   | gram   |
| GC                  | gas chromatography                                       |
| h                   | hour   |
| HCN                 | hydrogen cyanide   |
| HEPES               | N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid       |
| HSP                 | heat shock protein                                       |
| kDa                 | kilodaltons  |
| K <sub>m</sub>      | substrate concentration at half maximum reaction rate    |
| L                   | litre  |
| LEA                 | late embryogenesis abundant protein                      |
| LER                 | leaf elongation rate                                     |
| Log                 | logarithm  |
| MACC                | 1-(malonylamino) cyclopropane-1-carboxylic acid          |
| M                   | Molar, moles per litre                                   |
| MES                 | 2-(N-Morpholino) ethane-sulfonic acid                    |
| Mg                  | milligram  |
| µg                  | microgram  |
| µL                  | microlitre   |
| MilliQ water        | water purified by a MilliQ ion exchange column           |
| min                 | minutes  |
| mL                  | millilitres  |
| Mr                  | relative molecular mass (g mol <sup>-1</sup> )           |
| MTA                 | 5'-methylthioadenosine                                   |
| n                   | number of replicates                                     |
| N                   | enclosed leaf tissue                                     |
| Na HCO <sub>3</sub> | sodium bicarbonate                                       |
| NBT                 | p-nitro blue tetrazolium chloride                        |
| 2-ODD               | 2-oxoacid dependent dioxygenase                          |
| PA                  | 1, 10-phenanthroline                                     |
| PAGE                | polyacrylamide gel electrophoresis                       |
| PBSalt              | 50mM sodium phosphate, ph 7.4, containing 50 mM NaCl     |

|                  |   |
|------------------|---|
| p.               | page  |
| pH               | - log [H <sup>+</sup> ]                         |
| pI               | isoelectric point                               |
| PIPES            | piperazine-1, 4-Bis (2-ethanesulfonic acid)     |
| ppm              | parts per million                               |
| PVDF             | polyvinylidene difluoride                       |
| resp.            | respectively                                    |
| RO               | reverse osmosis                                 |
| SAM              | S-adenosyl-L-methionine                         |
| SDS              | sodium dodecyl sulphate                         |
| SE               | standard error of mean                          |
| SWC              | soil water content                              |
| TEMED            | <i>N, N, N', N'</i> -tetramethylethylenediamine |
| Tris             | tris (hydroxymethyl) aminomethane               |
| Tween 20         | polyoxyethylenesorbitan monolaurate             |
| V <sub>max</sub> | maximum rate of reaction                        |
| v/v              | volume per volume                               |
| v/w              | volume per weight                               |
| w/w              | weight per weight                               |
| X                | exposed leaf tissue                             |

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# Chapter One: Introduction

## 1.1 Overview

Ethylene is an important signal molecule synthesised in both higher plants and in microorganisms. In addition to its role in plants in sustaining a programme of normal growth and development, ethylene regulates plant responses to biological and environmental stresses. It is one of several signalling compounds in plants that are induced following early signalling events such as calcium influx and protein phosphorylation/dephosphorylation. Changes in global gene expression in response to these primary and secondary signals eventually alters the metabolism/physiology of plants and leads to their adaptation to the new environment (Liu and Zhang, 2004).

In higher plants, the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to the gaseous plant hormone ethylene (C<sub>2</sub>H<sub>4</sub>) by the enzyme ACC oxidase (ACO) is the final step in ethylene biosynthesis. Together with ACC synthase (ACS), ACO is one of two crucial enzymes (each catalyses an irreversible reaction) in the pathway. ACS has long been recognised to play a major role in the regulation of ethylene biosynthesis, i.e. is regarded as the rate-determining step, while ACO has often been regarded as constitutive in plant tissues. However, there is increasing evidence to suggest that ACO is an additional regulator of ethylene biosynthesis in plants. Initially, support was provided by a multigene family for ACO. The temporal and spatial expression of ACO to a wide range of developmental and physiological signals has been widely observed, and adds further support (Barry *et al.*, 1996; Hunter *et al.*, 1999; Nie *et al.*, 2002; Chen and McManus, 2006). In addition, observed increases in ACO activity correlative with increases in ethylene production and ACC content, suggest a regulatory role (Hunter, 1998; Gong, 1999).

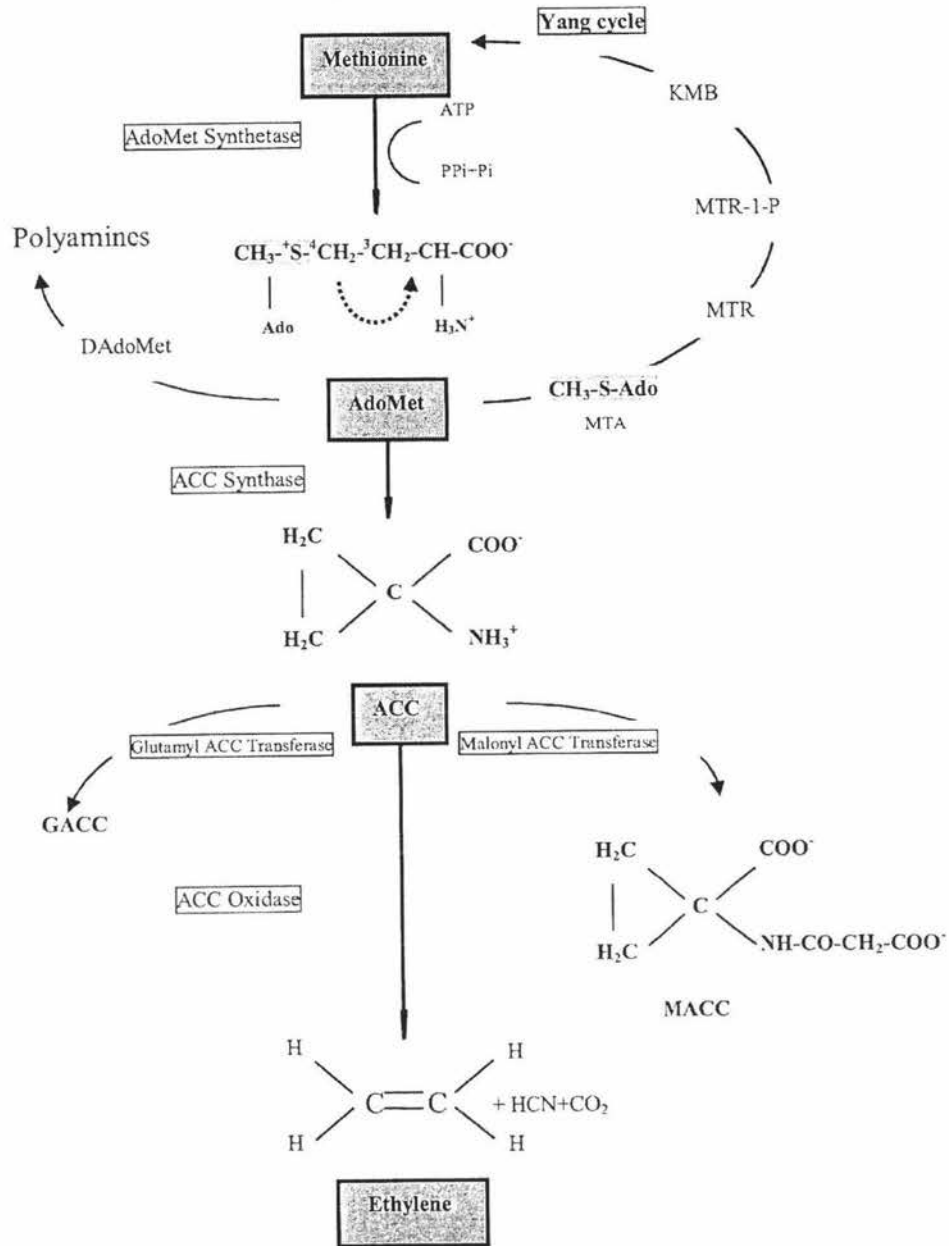
Two distinct ACO isoforms from the same tissue at different developmental stages (mature green and senescent leaves) have been isolated and characterised in white clover (Gong and McManus, 2000). This shows that the widely observed transcriptional regulation of the ACO gene family is also expressed at the protein level by differential regulation of enzyme isoforms (isozymes). While many studies have shown that ethylene evolution is induced by a water deficit, none have examined whether particular ACO isoform/s are induced.

This study sets out to observe changes in ethylene evolution and ACO activity and protein levels in response to a water-deficit in the leaf tissues of two closely related grass species, *Festuca novae-zelandiae* and *F. arundinacea*. Physiological /biochemical measurements such as the leaf elongation rate and proline content of leaves were made, to act as external and internal indicators of physiological responses to a water-deficit. Isoform analysis was attempted in the immature and the mature leaf zones of both species and water-deficit responses in the two tissue types are compared. The aim is to establish a role or otherwise for ACO in regulating the biosynthesis of ethylene.

## 1.2 The Ethylene Biosynthesis Pathway

The biosynthesis of ethylene is a multi-step enzymatic pathway that converts the amino acid methionine to (gaseous) ethylene. In higher plants, the major biosynthetic pathway, commonly called the ACC-dependent pathway (figure 1.1, p. 3) includes  $L$ -methionine, *S*-adenosylmethionine and ACC as the intermediates.

Methionine is converted by the enzyme *S*-adenosyl- $L$ -methionine (Adomet) synthetase in an ATP-dependent reaction to *S*-adenosyl- $L$ -methionine (Adomet). Adomet is an important metabolic intermediate in a wide range of metabolic reactions as well as ethylene biosynthesis, and is therefore not regarded as a committed step in ethylene biosynthesis (Imaseki 1999). The cleavage of Adomet to release ACC and 5'-methylthioadenosine (MTA) in a reaction catalysed by 1-amino-cyclopropane-1-carboxylic acid synthase (ACC synthase) is the first committed step in the ethylene biosynthetic pathway (Kende 1993, Imaseki 1999). MTA is recycled via the Yang cycle to regenerate methionine. This recycling is critical because without regeneration, cellular levels of methionine could become limiting for translation (Yang & Hoffman 1984). At this point in the pathway there are at least two possible fates for ACC: malonylation of ACC by ACC-malonyltransferase to form 1-(malonylamino) cyclopropane-1-carboxylic acid (MACC) in an essentially irreversible reaction (Satoh & Esashi 1984), or the generation of ethylene together with HCN and CO<sub>2</sub> from ACC by the enzyme ACC oxidase (Imaseki 1999). The latter reaction is regarded as the second committed step in the ethylene biosynthetic pathway.



**Figure 1.1** The ethylene biosynthetic pathway in higher plants [adapted from Du (2005)]

**ACC**, 1-aminocyclopropane-1-carboxylic acid; **Adomet**, S-adenosyl-L-methionine; **DAdoMet**, decarboxylated AdoMet; **MTA**, 5'-methylthioadenosine; **MTR**, 5'-methylthioribose; **MTR-1-P**, 5'-methylthioribose-1-phosphate; **KMB**, 2-keto-4-methylthiobutyrate; **GACC**, 1-(gamma-L-glutamylamino) cyclopropane-1-carboxylic acid; **MACC**, 1-(malonylamino) cyclopropane-1-carboxylic acid.

As MACC is unable to act as a precursor of ACC, the malonylation reaction can limit ethylene production by limiting endogenous levels of ACC, as ACC levels are regarded as being the primary determinant of the rate of ethylene production (Satoh & Esashi 1984). A second conjugation reaction occurs in a limited number of species such as tomato (*Lycopersicon esculentum*) and involves the gamma-glutamyltranspeptidase catalysed conversion of ACC into 1-(gamma-L- glutamylamino) cyclopropane-1-carboxylic acid (GACC). However, it appears that GACC constitutes only a minor portion of the conjugated ACC in tomato fruit discs. Therefore, this form of conjugation may be relatively unimportant with regard to the regulation of endogenous ACC levels (Peiser & Yang, 1998).

### 1.3 Regulation of the Ethylene Biosynthetic Pathway

Ethylene acts as a promoter of ageing/senescence in cells and can cause a shift from a programme of growth to one of senescence. Hence, intricate controls are needed in plants to regulate the production of C<sub>2</sub>H<sub>4</sub>. That plants have evolved to tightly regulate the production of ethylene is supported by the fact that multiple genes encode key enzymes in the C<sub>2</sub>H<sub>4</sub> biosynthesis pathway, for example, ACC synthase (ACS) and ACC oxidase (ACO) (Fluhr & Mattoo 1996).

Ethylene production may be regulated developmentally, environmentally and/or by other plant hormones, i.e. by both endogenous and exogenous factors. The basic programme of ethylene biosynthesis during regular development is laid out genetically and C<sub>2</sub>H<sub>4</sub> biosynthesis increases and decreases in specified tissues or organs, depending on developmental stage i.e. the transient production of C<sub>2</sub>H<sub>4</sub> that soon dissipates according to an epigenetic programme (Imaseki, 1999). For example, during seed germination, fruit ripening, leaf abscission and flower senescence, endogenous C<sub>2</sub>H<sub>4</sub> production is induced. Because the physiological effects of ethylene are diverse, this tissue-specific and stage-specific regulation of ethylene biosynthesis is important in sustaining normal plant development.

Ethylene production can also be induced by a variety of external factors such as photoperiod, thermo period, drought, flooding, high and low temperatures, wounding (Kim and Yang, 1994; Barry *et al.*, 1996), wind, insects and animals, touch, or infection by pathogens. In many cases, ethylene is an effector that modulates multiple metabolic processes that lead to the acquisition of tolerance to harsh environmental changes or

the healing of wounds(). Ethylene biosynthesis is induced by the hormone auxin in vegetative tissues (Zimmerman and Wicoxon, 1935). Auxin action is in turn synergistically enhanced by cytokinin, and antagonized by ABA. Therefore, any factors that affect endogenous levels of auxin, cytokinin or ABA will also regulate the synthesis of C<sub>2</sub>H<sub>4</sub>. Ethylene itself has both a positive and a negative effect on its own biosynthesis. It stimulates a tissue's ability to convert ACC to ethylene as well as to sequester ACC as MACC (Fluhr and Mattoo, 1996). Ethylene also stimulates the development of ACC oxidase activity in a number of vegetative (Kim and Yang, 1994) and reproductive tissues (Liu *et al.*, 1985). This stimulation is correlated with the accumulation of transcripts of ACC oxidase (Kim and Yang, 1994; Tang *et al.*, 1994; Barry *et al.*, 1996) suggesting transcriptional regulation (Fluhr & Mattoo, 1996).

Initially, ACO was thought to be constitutive because exogenously applied ACC led to a rapid increase in C<sub>2</sub>H<sub>4</sub> production in tissues that normally produced ethylene at very low rates (Yang & Hoffman 1984), indicating that the ethylene forming enzyme (EFE, later re-named ACO) is induced earlier than ACC synthase (ACS). That is, ACS is the rate-limiting enzyme of the biosynthesis of C<sub>2</sub>H<sub>4</sub> (Imaseki 1999). Regulation by ACO then, was not considered a major control point in the ethylene biosynthetic pathway. It is now recognised that C<sub>2</sub>H<sub>4</sub> evolution increases correlatively with any increase in ACO activity and is not constitutive (Hyodo *et al.* 1993; Tang *et al.* 1994; Kato *et al.* 2000, Upreti *et al.* 2000; Calvo *et al.*, 2004), and a role for ACO in regulating C<sub>2</sub>H<sub>4</sub> biosynthesis has developed in recent years. It is suggested that ACO expression also increases when C<sub>2</sub>H<sub>4</sub> production is maximised, such as during fruit ripening (Kende, 1993; Owino *et al.*, 2002). There is increased evidence to suggest that ACO now constitutes an extra tier of control for C<sub>2</sub>H<sub>4</sub> biosynthesis in higher plants. Further evidence for the support of ACO as an important regulatory step in ethylene biosynthesis is provided by the discovery that ACO is a multi-gene family (Hamilton *et al.* 1990) and that ACC oxidase has been demonstrated to be rate limiting in the ethylene biosynthesis of *Rumex palustris* during submergence (Vriezen *et al.* 1999).

### 1.3.1 ACO Belongs to a Multigene Family

The first indication that multi-gene families for ACO existed in plants came from the discovery that there were three related genomic clones to the pTOM 13 cDNA in tomato (Hamilton *et al.* 1990). Since then, ACO multi-gene families have been characterised for many plant species. For example, there are five gene members in tomato (Barry *et al.*, 1996; Nakatsuka *et al.*, 1998; Sell and Hehl, 2005); four in *petunia*

*hybrida* flowers (Tang *et al.* 1993); three in tobacco (*Nicotiana glutinosa*) (Kim *et al.* 1998); and three in white clover leaves (Hunter *et al.* 1999), melon fruits (*Cucumis melo* L.) (Lasserre *et al.* 1996), broccoli floral tissue (*Brassica oleracea* L.) (Pogson *et al.* 1995; Yang *et al.* 2003), cucumber floral buds (*Cucumis sativus* L.) (Kahana *et al.* 1999), rice seedlings (*Oryza sativa* L.) (Chae *et al.* 2000), sunflower seedlings (*Helianthus annuus*) (Liu *et al.* 1997), potato (Nie *et al.* 2002; Zanetti *et al.* 2002), and papaya (Chen *et al.*, 2003; Lopez-Gomez *et al.*, 2004). There are two gene members in: mung bean (*Vigna radiata*) hypocotyls (Kim & Yang 1994, Jin *et al.* 1999), banana fruits (Huang and Do, 1998), apricot fruits (Mbeguie *et al.*, 1999), peach fruits (*Prunus persica* L.) (Ruperti *et al.* 2001), and carnation (Wang and Woodson, 1991), and one in beech seeds (*fagus sylvatica* L.) (Calvo *et al.* 2004).

### 1.3.2 Differential Expression of the ACO Gene Family

Spatial and temporal differential expression of ACO genes has been demonstrated in a wide range of plant tissues, for example, in tobacco leaves (Kim & Yang 1994), broccoli florets (Pogson *et al.* 1995), tomato (Barry *et al.* 1996), melon fruits (Lasserre *et al.* 1997), sunflower seedlings (Liu *et al.* 1997), passionfruit (Mita *et al.* 1998), white clover leaves and mung bean hypocotyls (Yu *et al.* 1998; Song *et al.*, 2005).

The isogenes in tomato, the most widely studied fruit model, have been found to be developmentally regulated. For example, *LE-ACO1* and *LE-ACO3* transcripts accumulate during fruit ripening; leaf, flower and fruit senescence (Barry *et al.* 1996). During flower development, *LE-ACO1* is expressed predominantly in petals, stigma and style. *LE-ACO3* is expressed in all floral organs except the sepals and *LE-ACO2* expression is confined to the anther cone. Similarly, in white clover, the four distinct isogenes, *TR-ACO1*, *TR-ACO2*, *TR-ACO3* (Hunter *et al.*, 1999) and *TR-ACO4* (Chen and McManus, 2006) are expressed in leaves at different stages of development. For example, *TR-ACO1* is expressed specifically in the apex, *TR-ACO2* is expressed in the apex and in the developing and mature green leaves, and *TR-ACO3* and *TR-ACO4* are expressed in senescent leaf tissue (Hunter *et al.* 1999; Chen and McManus, 2006).

Differential expression of members of the ACO multigene family also occurs in response to physiological stimuli. For example, in tomato, only *LE-ACO1* is inducible in wounded leaves (Barry *et al.*, 1996). In mature green white clover leaves, *TR-ACO3* transcripts increase following wound treatment (Hunter, 1998; Gong, 1999). In potato, *ACO1* is induced markedly in leaves by wounding, soil flooding, heat (40°C) and cold

(0°C) treatments. Induction of ACO<sub>2</sub> by wounding and soil flooding is much lower (Nie *et al.*, 2002). *ST-ACO1* but not *ST-ACO2* or *3* transcripts were induced in leaves, stems and tubers by graft inoculating a Potato Virus A (PVA) resistant cultivar with PVA (Nie *et al.*, 2002).

This differential expression of the ACC oxidase multi-gene family in response to a wide range of developmental and physiological signals provides strong support for the recognition of ACO as a critical regulatory enzyme in the ACC-dependent ethylene biosynthetic pathway in vegetative tissues, ripening fruit and flowers.

#### **1.4 Isolation and Characterisation of the Ethylene-Forming Enzyme, ACC Oxidase (ACO)**

Once ACC was established as an immediate precursor of ethylene, the next step was to develop an assay *in vitro* for the ethylene-forming enzyme (EFE) (Adams & Yang 1979). In converting ACC to ethylene *in vivo*, this enzyme showed stereo specificity towards different isomers of 1-amino-2-ethyl-cyclopropane-1-carboxylic acid (AEC) (Fluhr & Mattoo 1996). None of the initial *in vitro* systems was able to discriminate between isomers, and EFE activity proved difficult to demonstrate. In most plant organs activity of the EFE *in vivo* was high (Yang and Hoffman, 1984) compared with the activity of the enzyme responsible for converting ACC to ethylene (*in vitro*). Originally then, a membrane-associated system, i.e. a cell wall/cell membrane association, was implicated because membrane integrity was found to be important for activity *in vitro*. Before the absolute requirement of the ACO enzyme for the addition of ascorbate and Fe<sup>2+</sup> in extraction media and assay reaction mix was known, (Ververidis and John, 1991), membrane integrity appeared to be needed to retain authentic EFE activity in isolated cell-free preparations (refer to section 1.3), suggesting that ACO was membrane bound in the apoplast (Rombaldi *et al.* 1994; Ramassamy *et al.* 1998). Physiological evidence showed that membrane perturbations affected ethylene production, largely due to an effect on the conversion of ACC to ethylene (Mattoo and White, 1991). Indeed, authentic ethylene-forming activity was retained *in vitro*, in vacuoles from pea (*Pisum sativum* L.) and bean (*Vicia faba* L.) (Guy and Kende, 1984), and in membrane vesicles from kiwifruit (Mitchell *et al.*, 1988). However it was noted that none of the ACO cDNAs predicted a signal sequence encoding a peptide that would facilitate transport to the apoplast. It was also suggested that more efficient regeneration of ascorbate would occur in the cytoplasm compared with the apoplast

and this could explain the apparent membrane requirement for ACO activity *in vitro* (Prescot and John, 1996). Hence there were two possible sub-cellular locations for ACO. It wasn't until recently with much improved techniques, e.g. highly specific antibodies raised against ACO and enzyme recovery methods, a cytosolic location for ACO has been shown. Chung *et al.* (2002) re-examined the sub-cellular location of ACO in apple fruits. It was demonstrated that apple ACO, previously thought to be located extracellularly (Rombaldi *et al.* 1994; Ramassamy *et al.* 1998), is located mainly, if not solely, in the cytosol of apple fruit mesocarp cells.

A major development of historical significance relating to the ethylene-forming enzyme was the sequencing of the pTOM 13 clone from tomato, together with the proposal that this cDNA might represent the gene encoding EFE (Hamilton *et al.*, 1990). The deduced amino acid sequence was similar to that for flavanone 3-hydroxylase. Transgenic tomato plants with a pTOM 13 cDNA insert under the control of the CaMV 35S promoter and terminator, in an antisense orientation, showed much reduced ethylene production, indicating that its gene product is essential for ethylene biosynthesis (Fluhr and Mattoo, 1996). Using conditions similar to those used for isolating and assaying flavanone 3-hydroxylase activity, Ververidis and John (1991) successfully isolated and assayed *in vitro* authentic soluble EFE activity from melon fruits. EFE extraction required anaerobic conditions and the addition of Fe<sup>2+</sup> and Na-ascorbate to the assay reaction mixture. Similar activity was then quickly recovered from avocado fruit, apple, winter squash and pear (Fluhr & Mattoo 1996). The enzyme was shown to require CO<sub>2</sub> for activation (Imaseki 1999). These studies made it possible to obtain homogeneous ACC oxidase from a wide range of fruit tissues (see Section 1.3.1) as well as to characterise the recombinant enzyme from tomato cDNA's expressed in *E. coli* (Fluhr & Mattoo 1996).

From these early characterization studies, it soon became clear that EFE catalysed the oxidation of ACC and its co-substrate ascorbate in the following reaction in the presence of Fe<sup>2+</sup> and CO<sub>2</sub>:



The nature of this reaction called for renaming EFE to ACC oxidase, the most obvious choice of name since the enzyme is a soluble oxidase (Imaseki 1999). Enzyme activity *in vitro* for ACO has been demonstrated in a large number of plant tissues.

### 1.4.1 Enzymatics (Biochemical Characterisation) of ACC Oxidase

The ACO protein has now been isolated and characterised from a number of plant species, mostly in fruit tissues but also in some vegetative tissues. The first successful extraction was from melon fruits (Ververidis and John, 1991). Since then the ACO protein has been isolated and partially purified from fruits of apple (Dong *et al.*, 1992; Kuai and Dilley, 1992; Dupille *et al.*, 1993; Pirrung *et al.*, 1993), avocado (McGarvey and Christoffersen, 1992), kiwifruit (MacDiarmid and Gardner, 1993), pear (Vioque and Castellano, 1994; Kato and Hyodo, 1999), banana (Moya-Leon and John, 1995), tomato (English *et al.*, 1995), citrus peel (Dupille and Zacarias, 1996), cherimoya, (Escribano *et al.*, 1996), papaya (Dunkley and Golden, 1998), and breadfruit (Williams and Golden, 2002). The protein has also been isolated and characterised from senescing carnation floral tissue (Kosugi *et al.*, 1997), white clover leaves (Butcher, 1997; Hunter *et al.*, 1999), barley leaves (Kruzmane and levinsh, 1999), pine needles (*Pinus sylvestris* L.) (Kruzmane and levinsh, 1999) and in seedlings belonging to two orders of the Gymnospermae (the Coniferales and Gnetales): *Pinus nigra*, *Pinus radiata*, *Pseudotsuga menziesii*, *Cupressocyparis leylandii*, *Ephedra major* and *E. navadensis* (Reynolds and John, 2000). The protein has been purified to homogeneity or near homogeneity in apple, banana, papaya, pear and breadfruit (references as above). However, fewer ACOs have been partially purified to homogeneity from vegetative tissues, for example only in white clover (Gong and McManus, 2000).

ACO enzyme preparations in varying degrees of purification, show that the enzyme is a 27.5 kDa (in papaya fruits, Dunkley and Golden, 1998) to 42.3 kDa (in breadfruit, Williams and Golden, 2002) monomer with  $K_m$  values for ACC varying from 20  $\mu\text{M}$  for the enzyme from apple, to 56  $\mu\text{M}$  for banana enzyme and 60  $\mu\text{M}$  for melon enzyme (Fluhr & Mattoo 1996). In pine needles (*Pinus sylvestris* L.) and in barley leaves,  $K_m$  for ACC is 61  $\mu\text{M}$  and 77  $\mu\text{M}$  respectively (Kruzmane and levinsh, 1999). Gong and McManus (2000) reported  $K_m$ s for ACC of 34.7  $\mu\text{M}$  and 110  $\mu\text{M}$  for the white clover isoforms MGI and SEII respectively. Optimum PH for maximum enzyme activity ranges from 7.0-7.2 in barley leaves and pine needles (Kruzmane and levinsh, 1999), and 7.5-8.5 in white clover leaf tissues (Gong and McManus 2000). In all of the plant species studied, ACO is active as a monomer with one exception. In cherimoya fruits, Escribano *et al.* (1996) provided evidence that the ACO enzyme may be active as a

dimer, as indicated by the different molecular masses obtained for native (62, 66 kDa) and denatured (35 kDa) ACO protein.

#### 1.4.2 Evidence for the Occurrence of ACC Oxidase Isoforms in Plants

The presence of a multigene family, together with biochemical characterisation of ACO in many plant tissues, supports the notion that the protein may exist as isoforms in plants. Initial evidence for isoforms came from a study in avocado, using differential ammonium sulphate precipitation at extraction. Two authentic ACO proteins with differences in  $K_m$  for ACC were isolated and designated EFE1 and EFE2. Optimal temperature differences in pear fruits (Vioque and Castello, 1994), chickpea (Derueda *et al.*, 1995) and citrus peel (Dupille and Zacarias, 1996) implied the presence of more than one ACO in these plant tissues.

Further evidence for ACO isoforms has been found in leaf and root tissues of corn (*Zea mays*) and sunflower (*Helianthus annuus*) (Finlayson *et al.*, 1997), in which tissue-specific ACO activity was demonstrated. Observed differences in pH dependence,  $K_m$  for ACC, dioxygen, ascorbate and  $CO_2$  for root and leaf ACO in both species, suggests that ACO isoforms may have evolved to adapt to the environment in which each organ of the plant is exposed (Du, 2004). Three isoforms of tomato ACO when expressed in yeast showed differences in  $K_m$  for ACC, pI and specific activity (Bidonde *et al.*, 1998). Gong and McManus (2000) purified two ACO isoforms (MGI and SEII) to homogeneity from white clover leaf tissue at different developmental stages. MGI was isolated from mature green leaf tissues and SEII from senescent leaf tissues of white clover. Differences in molecular mass, the pH optimum, iso-electric point (pI),  $K_m$  for ACC and optimal requirements for the co-substrate ascorbate shows that these two ACOs are distinct isoforms.

### 1.5 Ethylene as a “Stress” Indicator in Plants

Ethylene influences a diverse range of plant processes including seed germination, diageotropism, flowering, abscission, senescence, fruit ripening and pathogenesis responses (Imaseki 1999). In addition to its role in growth and development, ethylene regulates plant responses to biotic (biological) and abiotic (environmental) stresses. Early studies found that when plants were subjected to chemical defoliant, microbial diseases or mechanical injury, they showed symptoms usually associated with

exposure to ethylene (Hall, 1952; Williamson, 1950). Increased (endogenous) ethylene production in response to stresses represented by drought, mechanical wounding and radiation has been observed in plant tissues that normally produce very little ethylene (Apelbaum & Yang 1981).

In general, stress increases ethylene production. The term "stress ethylene" was coined by Abeles (1973) to refer to the accelerated biosynthesis of ethylene associated with environmental or biological stresses experienced by plants. The reactions and relationships connecting stress with ethylene-mediated responses are complex. A number of stress responses involve interactions with other hormones, for example auxin and abscisic acid (Gomez-Cadenas *et al.* 1996). "Stress ethylene syndrome" has been used by Morgan & Drew (1997) to describe the variable ethylene-mediated stress responses brought about by stress effects on ethylene perception and the signal transduction pathway. Variable responses to stress may include: an increase, a decrease, or no effect on ethylene biosynthesis (Morgan and Drew, 1997). Stress can alter many steps in the ethylene biosynthesis pathway. For example, the enzymes Adomet synthetase, ACC synthase and enzymes that conjugate ACC, including ACC oxidase, are all regulated by stress (Morgan and Drew, 1997).

Ethylene is an early indicator of stress in plants and as a mediator of the stress-response pathway. It functions as an important signal molecule upstream of any receptors, kinases or transcription factors. Stress-induced activation of the mitogen-activated protein kinases (MAPKs), SIPK, a tobacco (*Nicotiana tabacum*) MAPK and its orthologues, MPK6 in *Arabidopsis thaliana*, SIMK (alfalfa, *Medicago sativa*), IeSIPK in tomato (*Lycopersicon esculentum*) and PcMPK6 in parsley (*Petroselinum crispum*) have been shown to occur within several minutes and represent one of the earliest responses in plants to stress. It has been known for some time that protein kinases are involved in stress responses, however, it is only very recently that the MAPK cascade (figure 1.2 on the following page) has been linked to the induction of ethylene biosynthesis in plants under stress (Liu and Zhang, 2004).

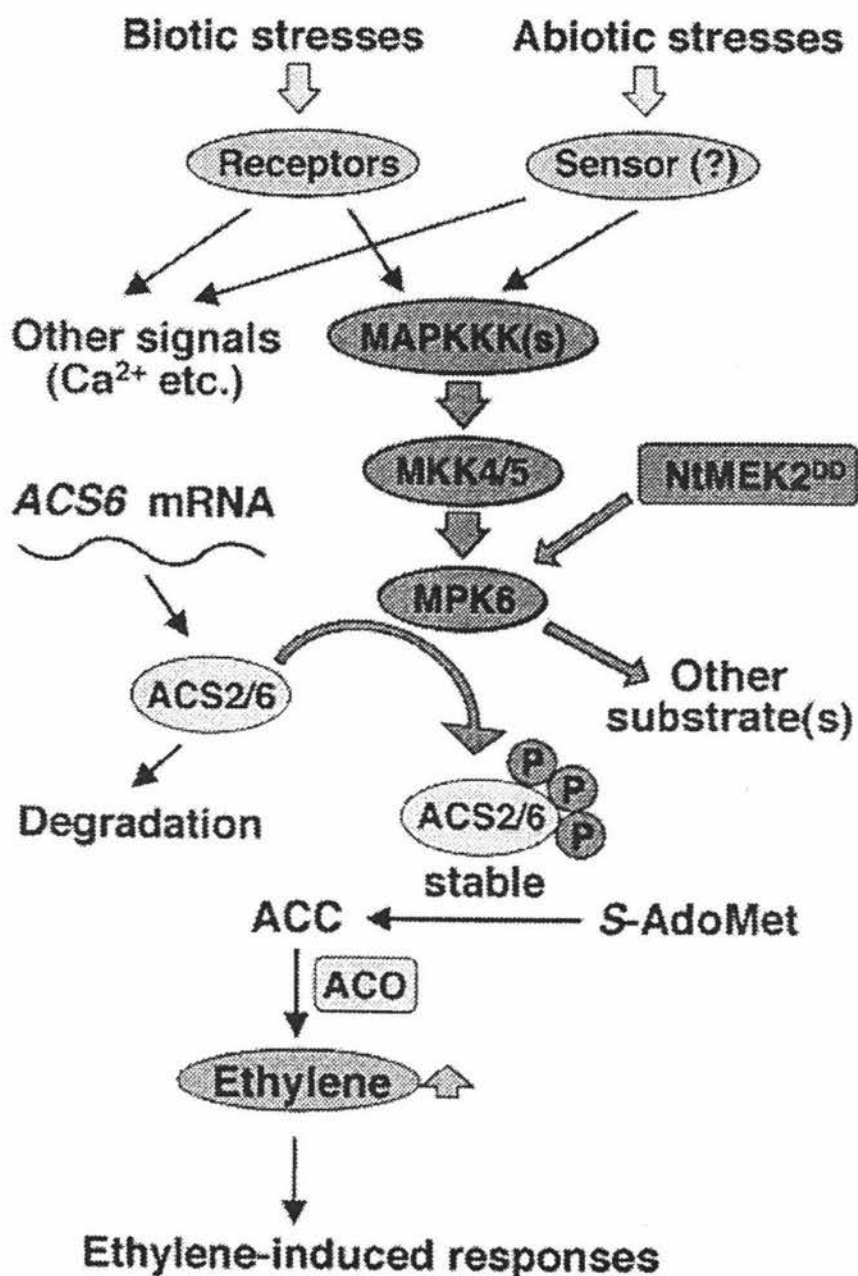


Figure 1.2 MAPK cascade showing responses upstream of ethylene evolution [adapted from Liu and Zhang (2004)]

**MAPKs** are mitogen-activated protein kinases; **NtMEK2<sup>DD</sup>** is the active mutant (gain of function) of a MEK2 gene knockout in tobacco; **ACS** is ACC synthase; **ACC** is 1-aminocyclopropane-1-carboxylic acid; **S-Adomet** is S-adenosyl-L-methionine; **ACO** is ACC oxidase

## 1.6 Water Deficit Stress in Plants

“Stressed” plants are described as those prevented from expressing their full genetic potential (Boyer 1982). Reductions in plant growth due to stress can be considerable, and are measured as losses in fresh weight. Of all the environmental factors that limit plant growth, for example, drought, cold, salinity, alkalinity, flood, wind, anoxia, and pest organisms, drought or water-deficit stress is the most limiting on plant performance (Boyer 1982).

Loss of turgor is the earliest significant biophysical effect of water stress in plants (Taiz and Zeiger, 1998). Therefore, plant turgor-dependent processes are the most sensitive to water deficit. Reductions in turgor cause decreases in growth rates as described by the following relationship:

$$GR = m (\Psi_p - Y)$$

where GR is growth rate,  $m$  is wall extensibility (responsiveness of the wall to pressure),  $\Psi_p$  is turgor and  $Y$  is the yield threshold (the pressure below which the cell resists plastic or non-reversible deformation). As the plant water content decreases, cell shrinkage leads to a relaxation of the cell wall. A subsequent decrease in cell volume results in lowered hydrostatic pressure or turgor. Cell expansion is turgor-dependent and therefore extremely sensitive to water deficit. Since leaf expansion depends mostly on cell expansion, a decrease in leaf area is one of the earliest (adaptive) responses by plants to water deficit. Smaller leaf areas transpire less water and allow conservation of a limited water supply in soil for use over a longer period. Water stress not only affects leaf area but may also affect plant size i.e. a decrease in the number of leaves/shoots on an indeterminate plant.

Drought tolerance has been associated with the presence of considerable quantities of non-reducing di- and oligosaccharides, e.g. mannitol, fructan and trehalose, compatible solutes, e.g. proline, and specific proteins, e.g. the late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs). Proline accumulation to very high levels in plant tissues has been shown to correlate with tolerance to drought and salt stress (Kavi Kishor *et al.*, 1995). Deamination during water-stress-induced proteolysis results in characteristic changes in the levels of free amino acids and amides. Proline increases proportionately faster than other amino acids in plants under stress (Magne and Larher, 1992). Increases can be dramatic. For example, in Bermuda grass (*Cynodon dactylon* L.) shoots under water stress, increases of up to 100-fold have been reported (Barnett and Naylor, 1966). This phenomenon was first observed in

wilted ryegrass by Kemble and McPherson (1954). Proline is believed to be involved primarily in osmotic adjustment, as an osmoprotectant, and, as a hydroxyl radical scavenger but has also been reported to play a role in protecting enzymes from denaturation, stabilising machinery of protein synthesis, regulating cytosolic acidity, increasing water-binding capacity and acting as a reservoir for carbon and nitrogen (Kavi Kishor *et al.*, 1995). Proline also has an effect on ubiquitination of damaged proteins for degradation by the proteasome. In this study, proline is used as a biochemical marker of physiological changes associated with a water-deficit.

Current models in plants for sensing osmotic stress, propose that osmotic stress is firstly sensed by plant cells as perturbations in plasmalemmae caused by a loss of turgor pressure (Skriver and Mundy, 1990). This is followed by changes in cell pH, and then an increase in cytosolic and apoplastic ABA because of *de novo* synthesis and/or release of ABA from organelles (Abernethy, 1996). ABA induces changes in gene expression including expression of the LEA proteins during seed maturation and the RAB (responsive to ABA) group of genes (Abernethy, 1996). LEA proteins are heat soluble and highly hydrophilic, and are located primarily in the cytoplasm and nuclei of cells. Some LEAs are thought to coat intracellular macromolecules with a cohesive layer of water and this is based on high numbers of polar residues within their structures. Small HSPs have also been associated with tolerance to desiccation. These proteins are induced by the same stresses as LEAs and are synthesized with the acquisition of stress tolerance. They have been assigned a role in stabilizing enzymes.

### 1.6.1 Drought Resistance Mechanisms in Plants

Plants use a variety of strategies to resist drought, including desiccation postponement (the ability to maintain tissue in a hydrated state), desiccation tolerance (the ability to function while dehydrated) or escape. Accordingly, plants may be divided into two basic categories: drought avoiders and those tolerant of drought conditions. Escape plants complete their lifecycle during the wet season before the onsets of drought and are the only true "drought avoiders". Drought resistance (avoidance or tolerance) mechanisms may be ecological, developmental, morphological, physiological and/or biochemical.

Desiccation postponement strategies are adaptations that have evolved in plants, by a process of genetic selection in response to prevailing environmental conditions. However, they may be insufficient to prevent water deficit stress from occurring during unusually dry periods. Such traits may be developmental or morphological and occur

typically in mesophytes which are species of temperate (climatic) zones. Desiccation tolerance strategies may be physiological or biochemical, for example, osmolyte accumulation. Such strategies are employed by species of arid or semi-arid places, for example the xerophytes. Traits at the developmental level are more complex organisationally, than morphological or physiological or biochemical traits, and in that order (McCue and Hanson (1990)). The smaller number of gene products involved in biochemical traits than in developmental traits makes them more suited for practical reasons to applications of genetic manipulation.

### 1.6.2 Water Deficit Stress and Ethylene

The imposition of water-deficit stress leads to marked effects on plant growth and development (Abou Hadid *et al.* 1986). McMichael *et al.* (1972) suggested that increased ethylene production under drought conditions causes leaf abscission, and thereby reduces water loss. This in turn aids plant survival.

There is a complexity of documented responses to "drought" involving intact leaves, detached leaves, short and long water stress duration (Upreti *et al.* 2000) and young and mature leaves (Abou Hadid *et al.*, 1986). It is generally accepted that ethylene is induced by water-deficit stress, and many studies in both leaves of intact plants and detached leaves have shown this. For example, in intact cotton petioles (Jordan *et al.*, 1972, McMichael *et al.*, 1972), in detached leaves of Valencia orange, *Citrus sinensis* 'Osbeck', (Ben Yehoshua & Aloni, 1974); and in excised wheat leaves (Apelbaum & Yang, 1981). Thirty-fold increases in ethylene production were observed in intact wheat leaves, within 4 hours of being subjected to a water-deficit (Apelbaum & Yang, 1981). Under normal conditions, wheat leaves produce very little ethylene. Increases in ethylene have been attributed to increased ACC content and ACO activity. In other studies, however, there is no evidence for a water stress induced increase in ethylene production for intact plants, for example, in *Vicia faba* (El-Beltagy & Hall, 1974), in aeroponically grown sunflower, (Hubick *et al.*, 1986; and in wheat seedlings, (Narayana *et al.*, 1991).

When Narayana *et al.* (1991) compared ethylene production rates in dried, excised leaf segments of eight day old and six week old wheat seedlings with intact six week old water stressed seedlings, they concluded that the commonly reported surge in ethylene production by excised leaves subjected to rapid drying represented an artefact, and has little relevance to water stress response of intact wheat plants. Morgan *et al.*,

(1990) also compared the response to desiccation of detached leaves with that of leaves from intact plants. It was found that with regard to ethylene production, detached leaves react differently to rapid drying when compared with leaves attached to intact plants drying out more slowly in soil. In the three species examined (bean, *Phaseolus vulgaris*; cotton, *Gossypium hirsutum* L. cv. 'Stoneville'; and the miniature rose, *Rosa hybrida* L. cv. Bluesette), no evidence for a significant increase in ethylene production with water deficit stress, imposed in a way that resembled a natural drought situation (slowly over several days), was found. In most cases, water deficit stress decreased ethylene production.

Morgan & Drew (1997) have suggested the term "water-deficit stress" be used to describe plants (or plant parts) subjected to rapid drying conditions, such as experienced by excised leaves (or very small volumes of soil) such that a severe deficit develops in one day or less, and "drought" to describe slow drying in pots with volumes of soil large enough so that the water supply declines more slowly over several days. Other workers consider that a drought is where water deficit is influenced by other environmental factors (eg wind, temperature, humidity) and approximates a field situation (Abernethy, 1996). The terms used here are those used by the relevant authors. In glasshouse conditions, it is possible to simply remove water from the plant and keep all other environmental factors identical, and this is usually considered a water deficit. In both situations, when the growth of the plant is affected they are considered to be under drought-stress or water-deficit stress.

In this study, intact plants are used. It is known that the excision of leaves induces ethylene production without the need for drought stress (i.e. wound stress). Therefore, to determine any role for ethylene in plants experiencing a water-deficit, leaves will be excised from whole plants at the appropriate sampling points. The measurement of evolved ethylene as the sole indicator of response to a water-deficit or water-deficit stress is also of limited value. It is possible that responses to a water-deficit may be mediated by changes in the ethylene biosynthetic pathway, and it is these changes that will be examined in this study. Thus far in the literature, there have been no studies on the control of ACC oxidase activity in response to a water deficit or water deficit stress.

## 1.7 The Plants: *Festuca novae-zelandiae* and *Festuca arundinacea*

Many of the traits associated with water-deficit stress have been discovered and researched in crop and pasture species, especially cereals, and in a few unusual plants, for example, *Craterostigma plantagineum*, the desiccation tolerant plant; *Mesembryanthemum crystallinum*, a facultative halophyte; the desert plant *Anastatica hierochuntica*; and the extremely xerophytic liverwort, *Exormotheca holstii* (Abernethy, 1996). In this study, tissue-specific responses to a water-deficit stress will be examined in the two grass species: *Festuca novae-zelandiae* and *Festuca arundinacea* cv. 'Roa' (syn. *Schedonorus phoenix*). Both are members of the Gramineae (grasses) family, however there are important differences. While *F. arundinacea*, or "tall fescue", is an improved forage species of pasture and a mesophyte, that is, it inhabits temperate climatic zones; *F. novae-zelandiae* is a native (unimproved) xerophyte i.e. an inhabitant of harsh, semi-arid environments. *Festuca novae-zelandiae*, commonly known as "hard tussock", is a dominant member of the short tussock grasslands that extend east of the main divide from Marlborough to Southland, in areas of low rainfall to elevations of 1450m, and in isolated regions of the volcanic plateau in the central North Island (Abernethy, 1996). *F. novae-zelandiae* conforms to the accepted definition of a xerophytic plant species in terms of anatomy, morphology, habit and distribution (Connor, 1960). For example, morphological adaptations include rolled leaves that form a tight cylinder enclosing a void which is densely packed with trichomes and a continuous leaf margin gap whose aperture is controlled by the turgidity of bulliform cells located between the vascular ribs (Abernethy *et al.*, 1998).

Studies in several species of the Gramineae show tissue-specific responses to a water deficit. The differential accumulation of metabolites, for example proline, ABA and glycine-betaine (selected species only), suggests preferential protection of growing tissues in response to a water deficit. For example, in wheat (Munns *et al.*, 1979), barley (Matsuda and Riazi, 1981), maize (Michelena and Boyer, 1982) and in *F. arundinacea* (West *et al.*, 1990; Barlow, 1986), the leaf base regions show a greater resistance to water-deficit stress effects than the mature leaf regions. Similarly, in the xerophytic grasses, *Agropyron dasystachyam* and *A. smithii*, maximum osmotic adjustment was observed in the meristematic regions of leaves (Abernethy *et al.*, 1998).

In previous studies in *F. novae-zelandiae*, proline was shown to accumulate preferentially in the leaf bases compared with the mature leaf tissue when subjected to a water deficit (Abernethy *et al.*, 1998). Together with the patterns of protein ubiquitination in leaf bases, immature and mature leaf regions of *F. novae-zelandiae* in response to water deficit (Abernethy and McManus, 1999), this supports the concept of preferential protection of rapidly expanding cells (in the meristematic and elongating leaf regions) in xerophytes during a water deficit (Maxwell and Redmann, 1978). By protecting growing tissue in this way, it is proposed that growth can resume once conditions become favourable (Redmann, 1976).

Biochemical characterisation of the ethylene biosynthetic enzyme, ACC oxidase, will be attempted firstly in the xerophytic native species, *Festuca novae-zelandiae* and secondly in the mesophytic pasture species, *F. arundinacea*. The question central to this project is: Does *Festuca novae-zelandiae*, a xerophytic plant species, produce "ethylene" in response to water-deficit? Previous studies of the plant's response to a water deficit reveal a series of specific cellular responses that result in targeted protection of meristematic tissue (Abernethy *et al.*, 1998) and there may be no induced stress response in the meristem. That is, the plant already exists in a "prepared" state, or signals do not permeate to the protected meristem.

This study will seek to answer this question by examining the existence or otherwise of a pronounced response to a water-deficit in meristematic leaf tissue through the induction of specific ACC oxidase isoforms. Mature, immature and meristematic leaf tissue types will be examined for the presence of such a response. A major aim therefore, will be to establish a role, particularly in a tissue specific context, for ACCO as a regulator of ethylene biosynthesis to a water deficit. The results from this study will add to our knowledge of drought response strategies used by plants that grow in arid environments and, unlike crop species have not undergone plant-breeding improvements. Further, any information gained might be used to aid the improvement of commercial crop species.

## 1.8 Project Objectives

- To compare some responses to a water deficit in the xerophyte *F. nove-zelandiae* with those of the mesophyte *F. arundinacea*.
- To examine ethylene production in meristematic, elongating and mature leaf tissues of *Festuca novae-zelandiae* and *Festuca arundinacea* subjected to a water deficit.
- To examine the activity of the ethylene biosynthetic enzyme ACC oxidase in meristematic, elongating, and mature leaf tissues in response to a water deficit.
- To identify any specific isoforms of ACC oxidase in meristematic, elongating and mature leaf tissues specifically involved in response to a water deficit.

## Chapter Two: Materials and Methods

### 2.1 Plant Material

#### 2.1.1 *Festuca novae-zelandiae* (Hack.) Cockayne

Several potted plants of *Festuca novae-zelandiae* were obtained from Otari Botanical Garden, Wellington. Originally, specimens (ascension number 75000390) were sourced from Molesworth Station in the South Island. Clonal plants were raised in a glasshouse by repeated division of 3 stock plants. In the midwinter of 2003, 12 plants of near equal size were selected for the experimental dry-down. Roots were teased out, washed and, trimmed by 1/3 before transplanting into 4 litre plastic pots containing Kairanga silt loam soil obtained from the Aorangi Research Station, AgResearch Grasslands, Palmerston North. Osmocote Plus 3 month slow release fertiliser (Scotts 3-4 Month Osmocote Plus Controlled Release Fertiliser) was added to soil at a rate of 100g/50L soil. Plants were left to settle for a period of 6 weeks prior to experimental dry-down. Kairanga silt loam soil was chosen because of its ability to dry-down slowly over a period of days, best resembling drought conditions in the field.

#### 2.1.2: *Festuca arundinacea* cultivar 'Roa' (syn. *Schedonorus phoenix*)

Endophyte-free seed of *Festuca arundinacea* 'Roa' was obtained from AgResearch Grasslands, Palmerston North. Seed was sown and raised in seedling trays containing a Massey-sourced bulk generic potting mix. Seedlings were potted-on into plastic planter bags to encourage 'clumping' (~6 months old plants) before being transplanted into 4L plastic experimental pots containing the same soil mix used for *F. novae-zelandiae* (Section 2.1.1). Plants were allowed to settle for 9 weeks before commencement of experimental dry-down.

## 2.2 Physiological Measurements

### 2.2.1 Temperature and Relative Humidity Measurements

Glasshouse temperature and relative humidity measurements were taken at 30-minute intervals throughout each experiment using a datalogger.

### 2.2.2 Soil Water Content (SWC)

SWC was measured as a percentage relative to an empirically determined container capacity (CC) for the soil. CC is the water content of potting medium, initially thoroughly wetted, after free drainage from holes in the base of the container ceases. CC was calculated by: thoroughly saturated soil in a 4L pot and covering the soil surface with polythene to prevent evaporation. The pot was left to drain by gravity for 2 days, after which a representative sample of soil (~200g) was then transferred onto a metal tray of known weight and baked at 105°C for ~48 hours. For drying, soil was spread occasionally to eliminate clumping.

If the gravimetric water content is equal to the mass of water divided by the mass of the dried soil, then:

$$CC = \frac{\text{wet weight (with container)} - \text{dry weight (with container)}}{\text{dry weight (with container)} - \text{container weight}}$$

For the Kairanga silt loam, CC was determined to be 0.5 or 50% saturation i.e. 50% of air spaces are filled with water. Therefore, 70% of CC is calculated as  $0.7 * 50\% = 0.35$  or 35% saturation. Therefore, SWC in control pots was held at or near 70% of the CC that is, around 30% as measured by the TDR apparatus (see below).

SWC was monitored electronically in each pot during the course of the dry-down experiments using a Time Domain Reflectometer (TDR). The TDR measures the speed at which a microwave pulse travels between two stainless steel electrodes. The pulse speed is dependent on the dielectric constant (k) of the material between the electrodes. Soil particles and air, as well as water exhibit a measurable dielectric constant, but since the dielectric constant of water (k=80) is much greater than that of mineral particles (k=2-3) or air (k=1), the speed of the microwave pulse between

electrodes immersed in soil is predominantly dependent on the SWC. The TDR equipment has been calibrated at manufacture by using test cells containing known volumes of water.

The TDR is independent of soil type and any minor differences are due only to differences in bonding of water molecules to different minerals in the soil. The estimated error of the TDR is  $\pm 2\%$  (TRASE operating manual). The TDR is zeroed in air before taking measurements, and only need be corrected once for electrode length just prior to using. A pairs of stainless steel electrodes (or probes) was inserted into each pot before the commencement of each experiment. Probes remained in place throughout the experiment meaning minimal soil disturbance when taking SWC measurements.

### **2.2.3 Leaf Elongation Rate (LER)**

LER is essentially a measure of cell enlargement and is measured in the immature leaf (leaf 1) of 3 randomly chosen tillers of each plant (figure 2.1, the following page). The LER was measured in mm growth over a 24 h period and was recorded in mm/day. From these measurements mean LER for each pot was calculated.

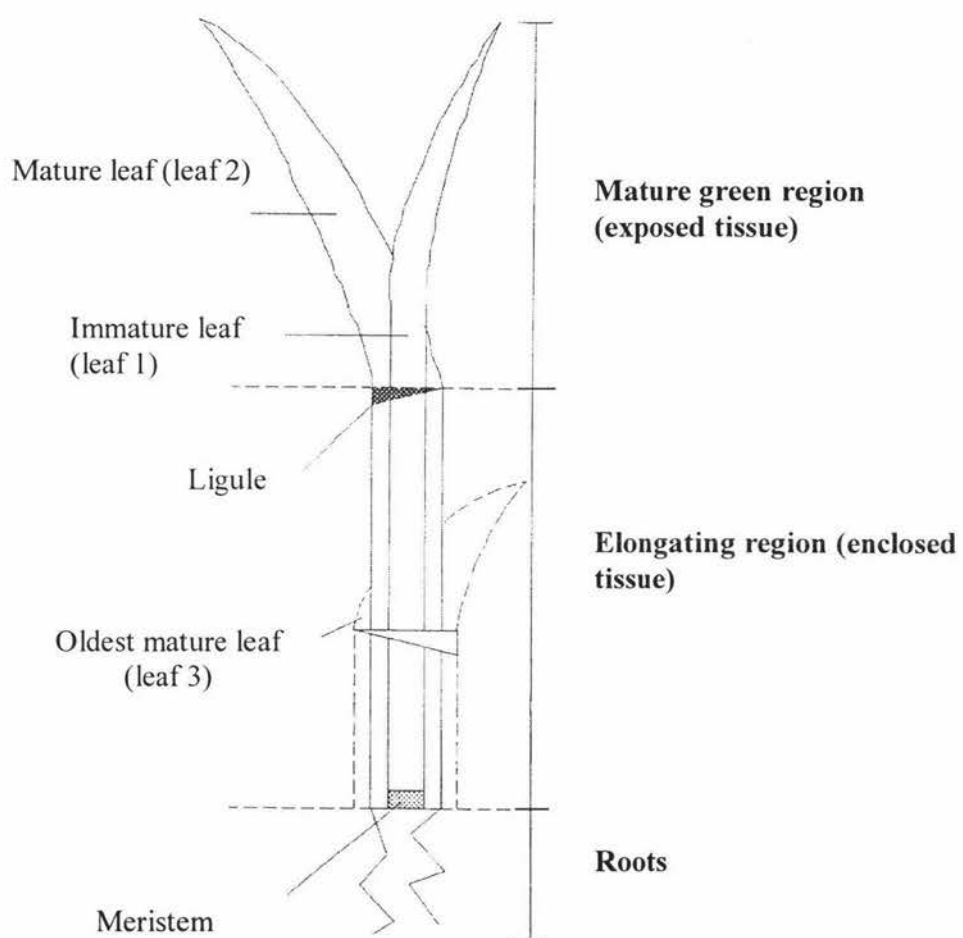
No allowance was made for lengthening day length since this would be the same for all pots. Tillers were tagged with jeweller's tags at hour zero, typically around 8.00am in the morning. A mark with an indelible pen was made on the adjoining mature leaf to show the length of the immature leaf at time zero. The change in length of the tagged leaf was measured 24 h later and recorded for each pot.

## **2.3 Leaf Sampling Methods**

For biochemical analysis, leaf tissue of immature leaf 1 (refer to figure 2.1) was harvested at regular intervals throughout the dry-down experiments. Two tissue types were harvested, and designated enclosed tissue [comprising leaf 1 below the ligule of the next oldest leaf (leaf 2)], and exposed tissue (comprising leaf 1 tissue above the ligule of leaf 2). The enclosed tissue includes the meristematic and elongating leaf zones, while the exposed tissue is the mature green tissue. All sheath material was removed and discarded. As the LER in each of the water-deficit pots reached zero, all leaf material was harvested from the glasshouse and placed on ice, then dissected in

the lab, snap frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until required at a later date for biochemical analysis.

**Figure 2.1 A typical tiller showing the leaf tissue sampled**



## **2.4 Ethylene Measurements**

### **2.4.1 Ethylene Evolution from Leaf Tissues**

Ethylene production was measured using gas chromatography in leaf tissues at different developmental stages i.e. meristematic and elongating (enclosed) and mature green (exposed), at various time points throughout the dry-down, i.e. on days 0, 4, 7, 10 and 14. Ethylene evolution is expressed as ppm/g FW i.e. ppm ethylene gas evolved/ gram (fresh weight) of leaf tissue.

### **2.4.2 Ethylene Measurements by Gas Chromatography**

The concentration of ethylene in gas samples produced by ACC oxidase was measured using a Shimadzu Model GC-8A Gas Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with flame ionisation detector. Nitrogen was used as the carrier gas at a flow rate of 50 mL/min. The detector flame was generated by hydrogen (at 60 kPa) and air (at 50kPa). For ethylene analysis, a 2.5m x 3mm (I.D.) glass column (Shimadzu 8G2 6-2.0) prepacked with Porapak-Q with a mesh size of 80/100 (Alltech Associates Inc., Deerfield, Il., USA) was used. At least 1h before use the column was incubated at 200°C with nitrogen carrier gas at 150kPa. For sample measurement, the oven and injector/detector temperatures were set at 85°C and 150°C respectively. 1 mL of gas from sample reaction containers was injected onto the column. From standard calibration gas of 0.1ppm ethylene in nitrogen (BOC Gas (NZ) Ltd., Palmerston North, New Zealand), the peak retention time for ethylene was observed to be between 1.2 and 1.3 min.

### **2.4.3 Ethylene Calculations**

The concentration of ethylene from the headspace of the reaction containers was measured in ppm by comparing the peak height to that of 1mL of a 0.1 ppm standard (refer to Section 2.4.2).

## 2.5 Biochemical Measurements

### 2.5.1 Proline Measurements

Free proline was measured in leaf tissue (Section 2.3) using the method of Magné and Larher (1992). This method is based on a previous one described by Bates *et al.* (1973) but is proposed to be free from carbohydrate interference. It is a simplified rapid colourimetric method suitable for use in the presence of large amounts of carbohydrates where sugars are known to interfere with the determinations of alpha-amino nitrogen and free proline (Magné and Larher, 1992).

Free proline was determined relative to a set of standards from 0 to 30 µg/mL proline, since both authors reported that the standard curve of absorbance measured at 518 nm, was linear up to a concentration of 30 µg/mL. Standards were prepared from a ready-made stock solution of L-proline (600 µg/mL) stored at 4°C (refer to Appendix 1 for the proline assay calibration curve used; p. 117). To save on time, standards were assayed only once weekly alongside sample batches to check for the consistency of the method. Good consistency was observed, as determined by a readily reproducible standard assay calibration curve.

### 2.5.2 Magné and Larher Method

#### Reagents:

- Ninhydrin reagent: 1% (w/v) ninhydrin in 60% (v/v) glacial acetic acid. Fresh reagent was prepared daily.
- 3% (w/v) sulphosalicylic acid
- toluene

Duplicate aliquots (of 20 to 50 mg) of powdered leaf tissue were weighed into 1.5 mL centrifuge tubes and suspended in 1.2 mL of 3% (w/v) sulphosalicylic acid to precipitate protein. Samples were vortexed to mix and then centrifuged at 12,000g for 10 minutes at room temperature. The resulting supernatant was transferred to fresh 1.5 mL eppendorfs. A 200 µL aliquot was combined with 400 µL of water ( a 1:2 dilution) and reacted with 800 µL of 1% ninhydrin reagent for 1 hour at 98°C in a water bath. The

reaction was stopped by plunging into ice and then returned to room temperature. Products (protein-ninhydrin chromophore) were extracted in 800uL of toluene. After vortexing for 15-20 seconds, vials were left to stand for approximately 5 minutes for phase separation to occur. The upper toluene phase was transferred to a 1 mL quartz cuvette for spectrophotometric analysis at 518 nm against a toluene blank. Sample absorbances were compared to a set of separately prepared proline standards (0 to 30µg/mL).

## 2.6 Extraction of ACC Oxidase

ACC oxidase was extracted from leaf tissues of *F.novae-zelandiae* and *F.arundinacea*, by a modification of the procedure described by McGarvey and Christoffersen (1992) and Fernandez-Maculet and Yang (1992).

### Reagents:

- Extraction buffer [100 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 2 mM DTT, 30 mM sodium ascorbate and 10 uM PA (1,10-phenanthroline)]

Frozen leaf tissue was ground with liquid nitrogen in a pre-cooled mortar and pestle, and then scraped directly into three volumes (v/w ratio of 3:1) of chilled extraction buffer. Typically, to obtain enough enzyme to perform a single activity assay, in duplicate, 1g of powdered sample was added to 3mL of extraction buffer. The sample was incubated on ice, with continuous gentle stirring for 45 minutes and then filtered through two layers of miracloth (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) into pre-chilled microfuge tubes. After centrifugation at 26,000 x *g* for 30 minutes at 4°C, the resulting supernatant was collected and either used directly (i.e. the crude extract) or purified further by ammonium sulphate precipitation (Section 2.6.1) and then Sephadex G-25 chromatography (Section 2.6.2) (the partially purified extract). In some experiments, the partially purified extract was purified further using Mono-Q ion exchange column chromatography (section 2.6.4).

### 2.6.1 Protein precipitation Using Ammonium Sulphate

Precipitation of proteins by ammonium sulphate is dependent on the hydrophobic properties of the surface of proteins (Harris, 1989). It is the most commonly used method to reduce crude extract volume and to aid in protein purification.

#### Reagents:

- Ammonium sulphate
- Resuspension buffer: (50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT and 10  $\mu$ M PA)

The supernatant volume was measured, and then adjusted to 40% (w/v) saturated (226 g/L at 0°C) ammonium sulphate. The mixture was incubated on ice with gentle stirring for 45 min, and then centrifuged at 26,000 x *g* for 15 min at 4°C. The supernatant was transferred to a fresh pre-chilled beaker and adjusted to 80% (w/v) saturated (by adding a further 258 g/L at 0°C) ammonium sulphate. After incubating for 1 h, with stirring, on ice, the protein was pelleted by centrifugation at 26,000 x *g* for 15 min at 4°C. The supernatant was discarded and the protein pellet resuspended in a ½ volume (1.5mL per g tissue) of pre-chilled resuspension buffer.

### 2.6.2 Sephadex G-25 Gel Filtration Chromatography

Gel filtration chromatography separates proteins based on their size (Preneta, 1989). Sephadex G-25 was used to remove ammonium sulphate (desalting) and any other small molecules including proteins with a molecular mass of less than 5 kDa from the protein extract. This is a modification of the method described by Neal and Florini (1973).

#### Reagents:

- Sephadex G-25 resin (Pharmacia Biotech, Uppsala, Sweden)
- Resuspension buffer: [50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 30 mM sodium ascorbate, 2 mM DTT and 10  $\mu$ M PA (extracts for further purification only)]

To prepare the sephadex G-25 column, a 10 mL syringe barrel (Becton-Dickensen, USA) pre-plugged with glass fibre discs (Whatman International Ltd., Maidstone, England) in the bottom, was poured with washed and expanded Sephadex G-25 resin and allowed to settle. The column was then pre-equilibrated with resuspension buffer before carefully loading the protein suspension from Section 2.6.1 onto the column. Eluted protein was collected as 0.5 mL fractions. Bio-Rad assays were carried out on all fractions to determine, visually, those containing the most protein. These fractions were then pooled (typically fractions 4, 5 and 6) and retained either for assaying for enzyme activity, or for further purification.

To retain ACC oxidase activity, the protein extraction, ammonium sulphate precipitation and Sephadex G-25 gel filtration chromatography were carried out within the same day. The prepared extracts were stored overnight at  $-20^{\circ}\text{C}$  for purification the next day.

### **2.6.3 Fast Protein Liquid Chromatography (FPLC)**

For some experiments, the eluate from Sephadex G-25 (Section 2.6.2) was further purified using the fast liquid protein chromatography (FPLC) system with a purpose-made pre-packed anion-exchange column (Mono Q HR 5/5). All Buffer solutions for FPLC were filtered through a  $0.22\mu\text{m}$  hydrophilic polypropylene membrane filter (GelmanSciences, Ann Arbor, Michigan, USA) immediately before use. Protein samples were centrifuged at  $10,000 \times g$  for 20 min at  $4^{\circ}\text{C}$  immediately prior to being loaded onto the column. FPLC was carried out at  $4^{\circ}\text{C}$  and eluted fractions were placed immediately on ice.

### **2.6.4 Anion Exchange Chromatography**

Ion exchange chromatography achieves high resolution separation of proteins based on their overall net surface charge at a given pH. Depending on the pH of the environment, side chains at the surface of proteins may be protonated or deprotonated such that their surface distribution and net overall charge determines the unique behaviour of the protein in an ion exchange environment. Separation then is achieved by differences in equilibrium charge distribution between a buffered mobile phase (protein in elution buffer) and a stationary phase (column matrix). The stationary phase acts as an anion or cation exchanger and consists of a matrix carrying charged functional groups that interact with proteins primarily through ion pairing. A protein will

bind to an anion exchanger when above its isoelectric point (pI), i.e. an overall positive surface charge, and to a cation exchanger when below its pI, i.e. an overall negative surface charge. The proteins that bind to ion exchange chromatography columns are generally eluted by increasing the ionic strength of their elution buffer. For a positively charged protein, such as ACO, NaCl is most commonly used. The sodium ions compete with positively charged groups on the protein for binding to the column.

Care was taken before and after use of the Mono Q column, to ensure that it was thoroughly cleaned using the procedure described by Du (2004).

**Reagents:**

- Buffer A: (50 mM Tris-HCl, pH 7.5, 30 mM sodium ascorbate, 2 mM DTT and 10  $\mu$ M PA)
- Buffer B: Buffer A, pH 7.5, with 1.0 M NaCl (FPLC grade)

The clean Mono Q prepacked HR 5/5 anion exchange column (Pharmacia Biotech) was pre-equilibrated with pre-chilled (on ice) Buffer A. Protein samples were loaded onto the column manually and allowed to interact with the column matrix. Bound proteins were then eluted using a linear increasing gradient concentration of sodium chloride from 0 to 1.0 M at a flow rate of 0.5 mL/min. Fractions (1.0 mL) were collected automatically in 1.5 mL eppendorfs placed on ice and assayed for protein content using the method of Bradford (1976), (see section 2.5.8.1). The activity of all fractions was determined by ACO activity assays *in vitro* (Section 2.7).

**2.7 ACC Oxidase Activity Assay (*in vitro*)**

The activity of ACC oxidase was measured *in vitro* using the method of Du (2004). This method is a modification of that described by Hunter (1998).

**Reagents:**

- Buffer A (2x stock); 100 mM Tris-HCl, pH 7.5, 20% (v/v) glycerol
- 1 mM FeSO<sub>4</sub>·7H<sub>2</sub>O (freshly prepared)
- 40 mM ACC (stored frozen)
- 1 M DTT (stored frozen)
- 1 M NaHCO<sub>3</sub> (freshly prepared)

- 1 M sodium ascorbate (freshly prepared)

A standard reaction mixture for 10 samples was prepared (see Table 1 below). After all components were mixed, ACC was added to the mixture just prior to assaying and the pH of the mix was readjusted to 7.5 with concentrated HCL. The final concentrations of all components are also shown in Table 2.1. To perform each assay, 0.8 mL of the reaction mixture was pipetted into 4.5 mL Vacutainer tubes and placed into a heating block held at 30°C with shaking at 180 rpm for 7 min. Enzyme preparations (0.2 mL) were aliquotted into Eppendorfs tubes and equilibrated in another heating block for 1 min at 30°C. To start the reaction, 0.2 mL of enzyme preparations was mixed with 0.8 mL of the reaction mixture, the Vacutainers sealed immediately and incubated with shaking at 200 rpm for 20 min at 30°C. At completion of the incubation period, 1 mL of gas was withdrawn from the headspace of each Vacutainer and the ethylene content determined (Section 2.4.2).

**Table 1 Formulation of standard reaction mixture for in vitro ACC oxidase activity assay**

| Component                                 | Final Concentration (mM) | Amount for 10 Samples (mL) |
|---|--------------------------|----------------------------|
| Buffer A (x 2)                            | -                        | 5                          |
| Tris-HCl                                  | 50                       | -                          |
| Glycerol                                  | 10% (v/v)                | -                          |
| 1 M DTT                                   | 2                        | .02                        |
| 40 mM ACC                                 | 1                        | .25                        |
| 1 mM FeSO <sub>4</sub> .7H <sub>2</sub> O | 0.02                     | 0.2                        |
| 1 M sodium ascorbate                      | 30                       | 0.3                        |
| 1 M NaHCO <sub>3</sub>                    | 30                       | 0.3                        |
| H <sub>2</sub> O                          | -                        | 1.93                       |

## 2.8 Protein Quantification

The protein concentration of samples was determined using the method of Bradford, as described by Bradford (1976).

### 2.8.1 Bradford Method

#### Reagents:

- Protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA)
- BSA (1 mg/10 mL stock solution, stored at  $-20\text{ }^{\circ}\text{C}$ )

Samples were compared to standards (of known protein concentration) prepared from bovine serum albumin (BSA) (see Appendix 2 for protein standard curve). Typically, 10  $\mu\text{L}$  aliquots of sample, in triplicate, were pipetted into wells of a microtitre plate. In each well, the volume was made up to 160  $\mu\text{L}$  with water and then 40  $\mu\text{L}$  of protein assay added. Reaction mixtures were mixed by gentle up and down pipetting then allowed to stand for 5 min, before absorbance readings were taken, at 595 nm, using an AnthosHT II plate reader (Anthos Labtech Instruments, Salzburg, Austria). Protein concentration was estimated by comparison to a protein standard curve (Appendix 2; p. ). Only absorbances within the linear range of the curve were used.

## 2.9 Protein Analysis by SDS-PAGE (SDS polyacrylamide-gel electrophoresis) Gels

SDS-PAGE was used to separate analytically different proteins based on molecular mass. The method used is for linear slab gels as described by Laemmli (1970)

#### Reagents:

- 40% (w/v) acrylamide stock solution (Bio Rad)
- 4X resolving gel buffer (1.5M Tris-HCL, pH 8.8, 0.4% (w/v) SDS)
- 2X stacking gel buffer (0.25M Tris-HCL, pH 6.8, 0.4% (w/v) SDS)
- 10% (w/v) APS (ammonium persulphate; Univar, Auburn, NSW, Australia) prepared fresh each time

- TEMED (*N,N,N',N'*-tetramethylethylenediamine) (Riedel-de haen ag seelze, Hannover, Germany) (stored at 4°C)
- 10X SDS running buffer (30g Tris, 144g glycine, 10g SDS, water to 1L)pH should be approximately 8.3, stable indefinitely at room temperature
- 2X SDS gel loading buffer (60mM Tris-HCL pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4mM 2-mercaptoethanol, 0.1% (w/V) bromophenol blue) Stable for months at -20C.

**Table 2 Composition for a 12% acrylamide gel of resolving and stacking gels used for SDS-PAGE with the Mini-Protean apparatus**

| Order of adding to mix | Components                | Resolving gel Solution (mL) | Stacking gel Solution (mL) |
|------------------------|---------------------------|-----------------------------|----------------------------|
| 1                      | water                     | 4.5                         | 4.0                        |
| 2                      | 4X resolving gel buffer   | 2.5                         |                            |
| 3                      | 2x stacking gel buffer    |                             | 5.0                        |
| 4                      | acrylamide stock solution | 3.0                         | 1.0                        |
| 5                      | APS                       | 0.1                         | 0.1                        |
| 6                      | TEMED                     | 0.01                        | .01                        |

A resolving gel solution was prepared by mixing the above components in Table 2 in the order shown. This solution was then transferred between clean glass plates of a gel sandwich until the level was around 20mm below the top edge of the glass plates (or 10mm below the base of wells as indicated by the placement of the well-forming comb). To prevent oxidation by air, milliQ water was then overlaid and the gel left to polymerise for around 50 minutes. Once polymerised this layer of water was discarded and the gel washed twice with a 1x resolving buffer. After insertion of the comb, a 2X stacking gel solution (4%) was added and after polymerisation of around 30 minutes, the comb was removed for loading and the gel placed in the electrophoresis chamber. Samples were prepared for loading by adding an equal volume of 2X loading buffer and then boiling in a water-bath for 3 minutes followed by centrifugation at 10,000 x *g* for 1 min. All samples were made to the same volume with sample buffer. Routinely, one lane was reserved for a 10uL aliquot of pre-stained molecular weight standards (Bio-Rad Laboratories, Richmond, CA, USA). Running buffer was added to both the inner and the outer electrophoresis chambers, and electrophoresis was conducted at 150V for a minimum of 50 min.

## 2.10 Western Analysis

Western blotting and antibody detection was carried out using Extraction Procedure 2 of Towbin *et al* (1979).

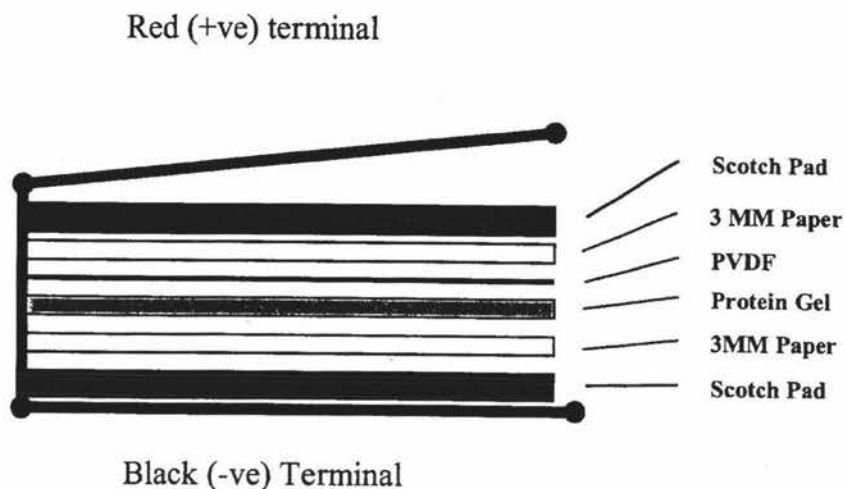
### 2.10.1 Electrophoresis transfer of protein to PVDF Membrane

After electrophoresis was complete (section 2.9), the gel was removed from the electrophoresis chamber and soaked for approximately 22 minutes in transfer buffer immediately prior to transfer.

#### Reagents:

- Transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/v) methanol)
- Polyscreen PVDF transfer membrane (NEN LifeScience Products Inc. Boston, MA, USA) cut to size of gel.
- 100% methanol
- water

The transfer cell was assembled as shown on the following page in figure 2.2, taking care to remove all air bubbles. The membrane was soaked for a few seconds first in 100% methanol, then in milliQ water and then in transfer buffer immediately before placing in the transfer cell. Blotting occurred for 30 minutes at 12V.



**Figure 2.2** Assembly of the semi-dry blot for the transfer of proteins from gel to membrane.

### 2.10.2 Antibody Source and preparation

In this thesis, two antibodies to TR-ACO2 were available. The first, designated TR-ACO2 #5, was raised and described by Hunter *et al.*, (1998). The second was raised to a recombinant TR-ACO2 protein expressed in *E.coli* (designated G 52). A second antibody, anti-TR-ACO1, (designated B34), which is raised to the TR-ACO1 isoform of white clover (Hunter *et al.* 1998) was also used. Serum containing B34 was from the terminal bleed of a rabbit injected with a recombinant white clover TR-ACO1 protein.

### 2.10.3 Immunodevelopment of the Membrane

#### Reagents

- 1X PBSalt ( 50 mM sodium phosphate pH 7.4 in 250 mM NaCl)
- PBSalt-Tween 20 (0.05% (v/v) Tween 20 in 1XC PBSalt)
- Primary antibody [anti-TR-ACO1/TR-ACO2 rabbit IgG antibody (Section 2.10.2)]
- Secondary antibody [anti-rabbit alkaline phosphatase conjugate raised in goat (Sigma)]
- Substrate (150 mM Tris-HCL, pH9.7 containing 0.01% (w/w) 5-bromo-4-chloro-3-indoylphosphate (BCIP), 0.02% (w/v)  $p$ -nitro blue tetrazolium chloride (NBT), 1% (v/v) dimethyl sulphoxide (DMSO), 1M MgCL<sub>2</sub>)
- 12.5% (w/v) milk powder solution

After the conclusion of transfer (Section 2.10.1), the membrane was peeled from the gel, placed protein-side up into an appropriate container and blocked in a 12.5% (w/v) (skim) milk powder solution (Anchor Milk Products, New Zealand) at room temperature for 1 hour or overnight at 4°C. The blocking solution was discarded, and the PVDF membrane rinsed briefly with PBSalt-Tween 20. The membrane was then bathed with a solution of the rabbit-raised primary antibody at a concentration of 1:1000 (diluted in 1X PBSalt) at 37°C for 1 h with gentle shaking. After which the primary antibody was discarded and the membrane washed three times for ~5 min with PBSalt-Tween 20 and the secondary antibody (anti-rabbit alkaline phosphatase conjugate raised in goat) at a concentration of 1: 1000 was added. After incubation at room temperature for 1 h with gentle shaking, the membrane was washed three times, each for ~5 min, with PBSalt-Tween 20, twice with 150mM Tris pH 9.7 for ~ 5 min each, the substrate added, and the reaction allowed to continue in the dark. The reaction was stopped when sufficient colour had developed, by discarding the substrate solution and rinsing the membrane several times with RO water.

## **2.11 Estimation of Protein Molecular Mass**

Protein size was estimated by comparing the electrophoretic mobility of proteins to the known sizes of standard marker proteins. Marker proteins were run concurrently in an adjacent well. All estimates of molecular mass are from mobility plots of mobility in mm relative to the point of origin (at wells) against Log MW. This is an accepted method for sizing electrophoresis protein bands as described in Hames and Rickwood (1981). Refer to Appendix 3 (p.118) for an example.

## **2.12 Statistical Analysis**

All statistical analyses were performed with an Excel spreadsheet programme (Version 2003; Microsoft, USA). In this thesis, unless otherwise stated, all errors associated with the means are standard errors (SE). Within the treatments, average standard deviation is sometimes quoted, and this is a good indication of the accuracy of results since it is the variation within a data set that is important. For statistical reasons  $n < 30$  (in this case  $n=6$ ) is considered to be a very small sample size and it not surprising that the variation in some of the data is considerable. Significant differences between data were tested using an Analysis of Variance (ANOVA) where criteria were met. Where the

variances between data sets were unequal, a Student's *t*-test was instead performed to test for significant differences. In this work, differences at the 5% level ( $p \leq 0.05$ ) are considered significant with differences at the 1% level ( $p \leq 0.01$ ) being highly significant. Refer to Appendix 4 (p. 119) for statistical analyses.

## Chapter Three: Experiment 1: Experimental Dry-Down of *Festuca novae-zelandiae*

### 3.1 Experimental Design

The experimental dry-down of *Festuca novae-zelandiae* took place in the spring of 2003 [from 01/09/03 (day 0) to 06/10/03 (day 35)], to coincide with the main season of growth. A balanced design of n=6 dry-down pots and n=6 control pots was chosen to detect, with the greatest degree of accuracy, any differences in physiological measurements. The experiment took place in the Massey (Ecology and IMBS shared) glasshouse under partially controlled conditions, i.e. temperature and water. Pot placement was randomised at regular intervals throughout the experiment to account for possible variances in light intensity, effects of direct sunlight or shading, and air movement or humidity pockets, etc., in the glasshouse.

Water-deficit stress was imposed on six plants, randomly chosen from a total of 12, by withholding water for a period of 35 days. Pots were of large enough soil volume so that the water supply declined slowly over several days. This is important since, with regard to ethylene production, detached leaves have been shown to react differently to rapid drying when compared with leaves attached to intact plants drying more slowly in soil (Morgan *et al.* 1990). A control set of 6 plants were maintained in a hydrated state, i.e. at or near 70% container capacity, by regular watering (refer to Section 2.3.1 for soil saturation theory).

### 3.2 Soil-Water Measurements

During the course of the experiment, the soil water status of each individual pot was measured at regular intervals: i.e. on days 0, 4, 7, 10, 12, 15, 17, 21, 23, 27, 30 and 35. Measurements were recorded as percentage soil water content (% SWC) relative to an empirically determined container capacity (refer to Section 2.3.1 for method).

The SWCs in the control and the dry-down pots are compared in figure 3.1 (on the following page). At the start of the experiment, i.e. day 0, the average % SWC, for the dry-down and the control pots was the same at  $30.5 \pm 0.5\%$ . During the course of the dry-down, an average soil water content of  $27.0 \pm 0.5\%$  was maintained in control pots (data not shown). In all six dry-down pots, % SWC declined exponentially over time

(days). A lower than expected  $R^2$  value of 0.66 can be explained by slower dry-down in one pot compared with the others.

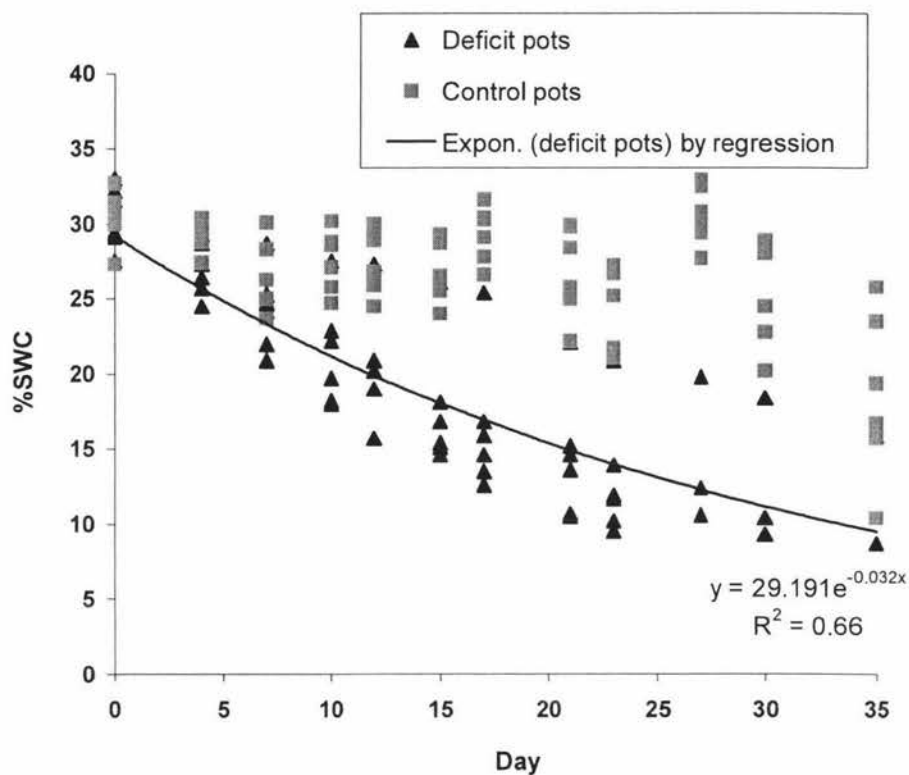


Figure 3.1 Soil water content over the experimental period in the dry-down and the control pots with the regression outcome.

Figure 3.2; p. 40, shows the individual dry-down curves for each of the six dry-down pots with regression outcomes. The  $R^2$  values range from 0.97 to 0.99 and are higher than the  $R^2$  value of 0.66 which describes the data set as a whole. The rate of dry-down was greatest in pot ND6, slowest in pot ND5 and similar in pots ND1, ND2, ND3 and ND4. For example, pot ND6 reached a minimum SWC of 9.5% by day 23. In comparison, pots ND1, ND2, ND3, ND4 and ND5 recorded SWCs of 10.2%, 11.7%, 11.9%, 13.9% and 20.9% respectively by day 23. Pot ND5 reached a minimum SWC of 15.9% on day 35 compared to a similar SWC for pots ND1, ND2, ND4 and ND6 on days 12, 17, 20 and 12 respectively.

The lowest value for %SWC value was recorded in pot ND3, i.e. 8.7% on day 35. In comparison, minimum SWCs for pots ND1, ND2, ND4, ND5 and ND6 were 10.2%, 11.7%, 10.4%, 15.9% and 9.5% respectively. Pot ND5 dried down the least to a SWC of 15.9% at day 35.

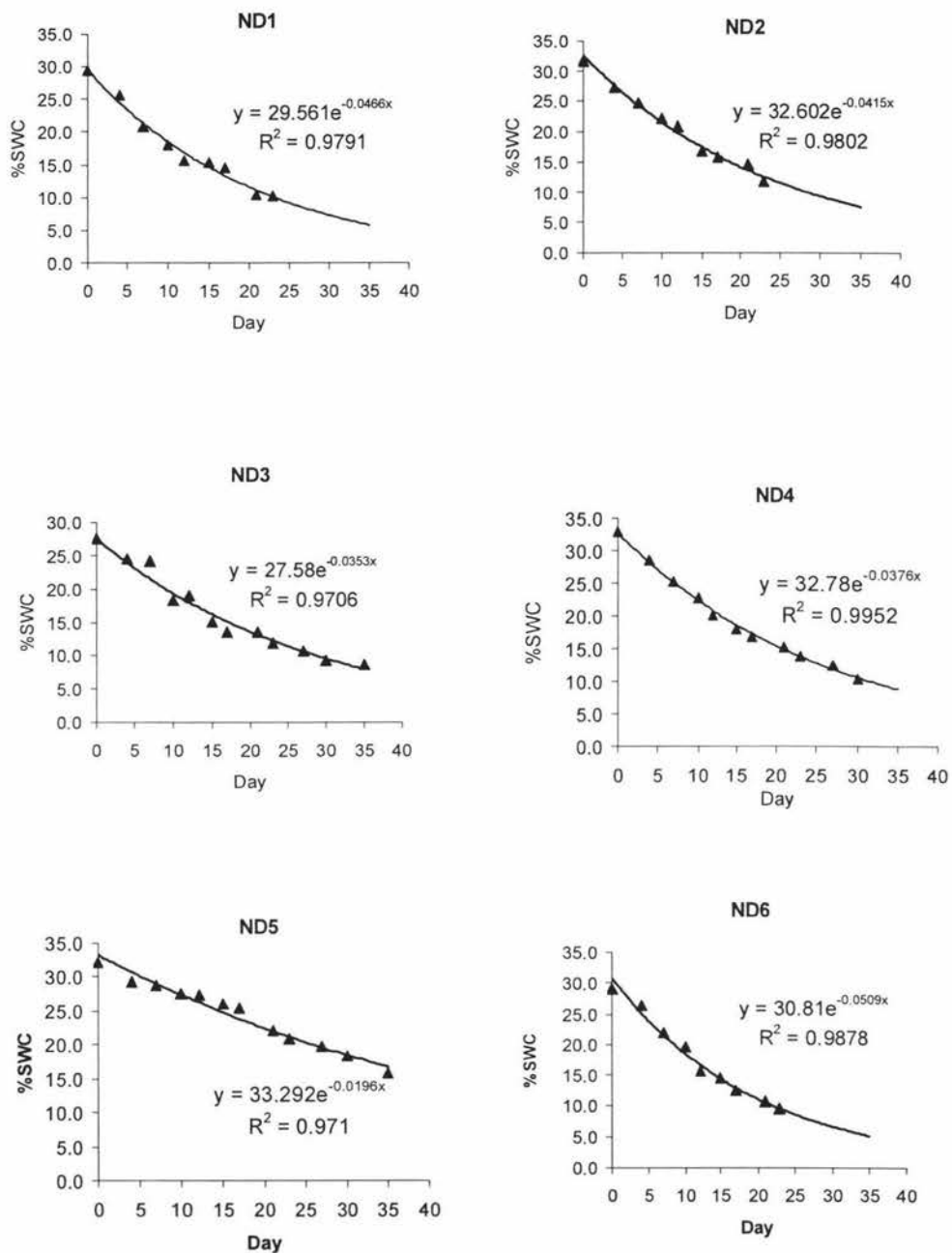


Figure 3.2 Dry-down curves for each of the six water-deficit pots over the experimental period with the regression outcomes.

### 3.3 Leaf Elongation Rate

To relate any observed physiological and biochemical changes within individual plants to cell hydration, a measure of plant-water status in pots was necessary. LER was the preferred method (see sections 2.3.2 and 2.3.3 for method), because it is non-destructive, is a simple and straightforward technique, and is extremely sensitive to changes in leaf water potential. Non-destructive sampling of leaf material was important in this species because of limited leaf material.

Mean leaf elongation rate (LER), in mm/day, was measured for each plant (deficits and controls) at regular intervals: i.e. on days 1, 4, 8, 12, 17, 20, 24, 28 and 32 (figure 3.3 on the following page) (see section 2. for method). Over the dry-down period, the LER declined linearly in all six water-deficit pots to reach zero in all but one pot (pot ND5). The LER in the water-deficit plants became significantly different from the controls, at the 5% level, on day 12 (by a student *t*-test assuming unequal variance). The LER reached zero first in pot ND6 at day 17 (figure 3.4; p. 43), followed by pots ND1 and ND2 at day 20. The LER reached zero in pot ND4 at day 28 and in pot ND3 on day 32. In contrast, the LERs in control pots never reached zero (data not shown). The mean LER over the course of the experiment for controls was  $9.4 \pm 0.4$  mm/day compared with  $5.2 \pm 0.6$  mm/day for the dry-down pots.

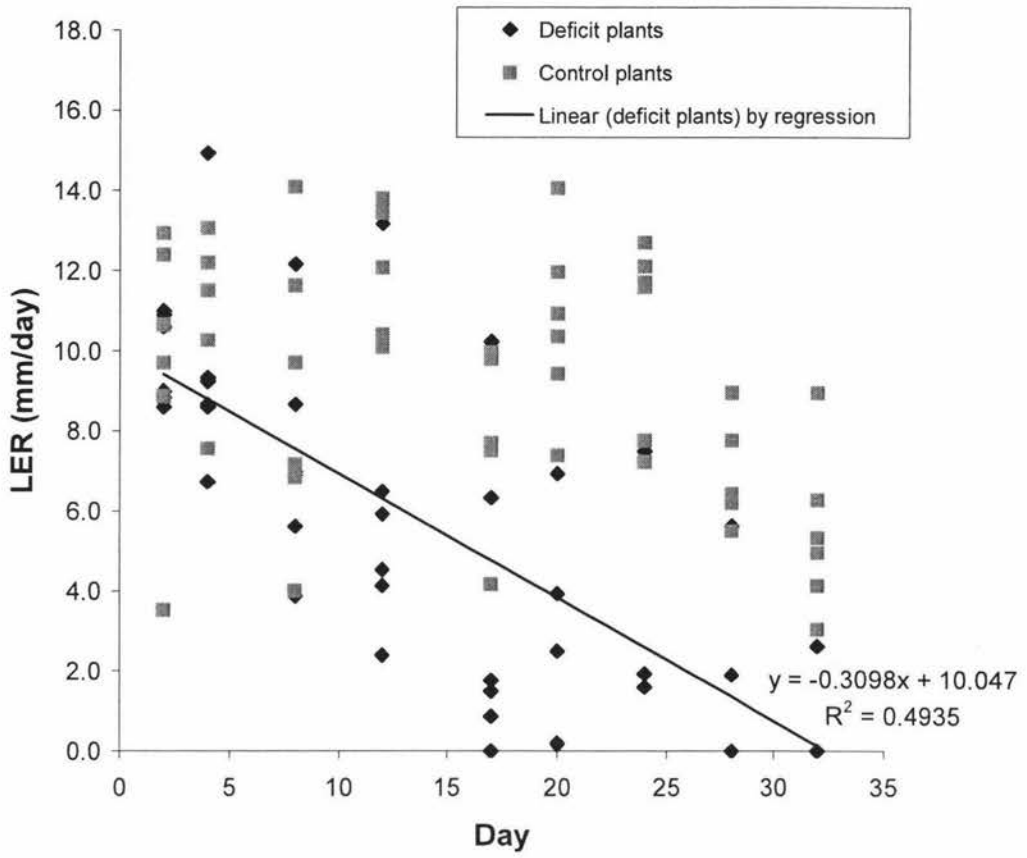
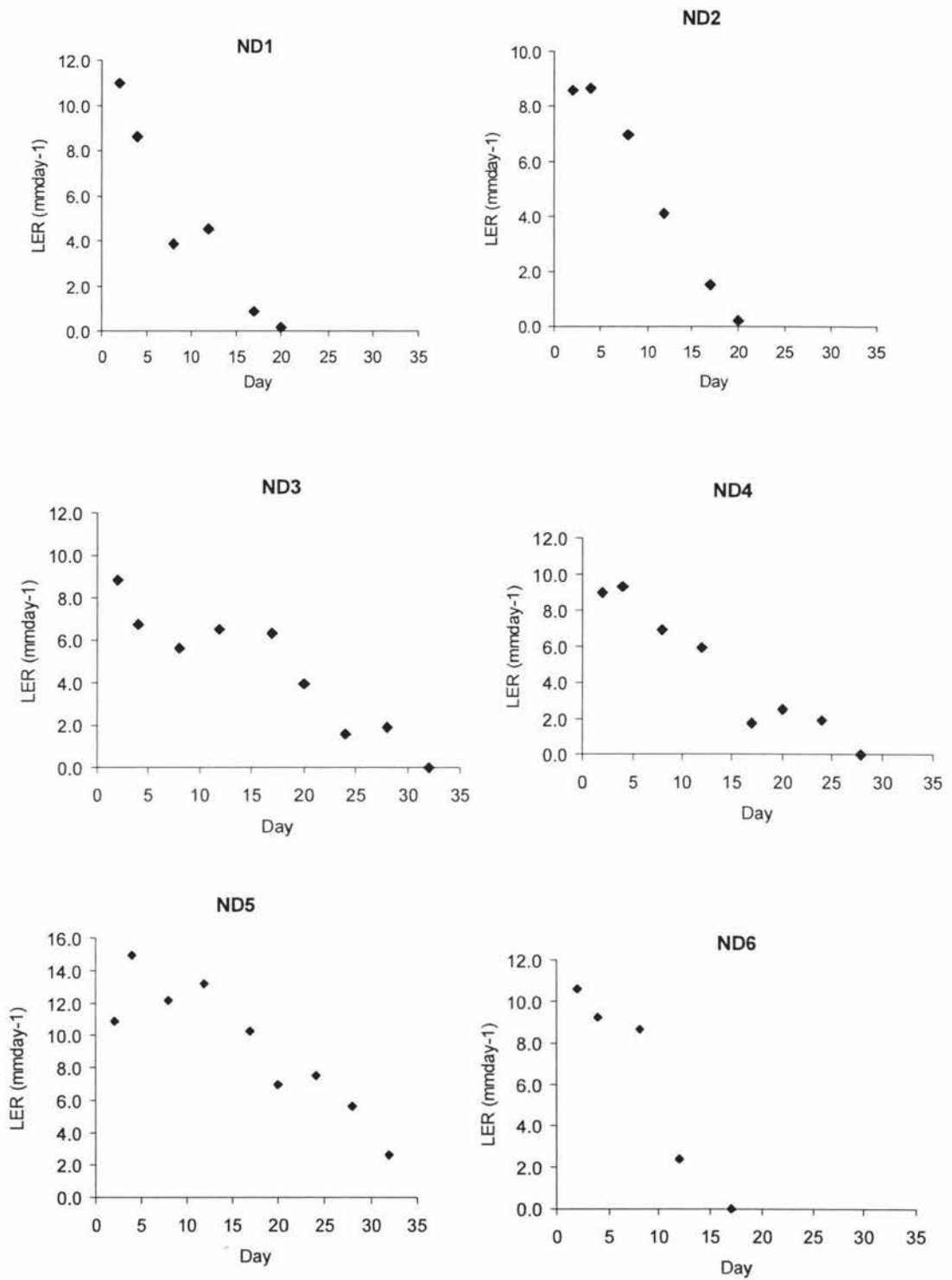


Figure 3.3 Leaf elongation rates in the water-deficit and the control plants of *F. novae-zelandiae* over the experimental period.



**Figure 3.4** Leaf elongation rates in each of the six dry-down plants (the mean of three replicates) over the experimental period.

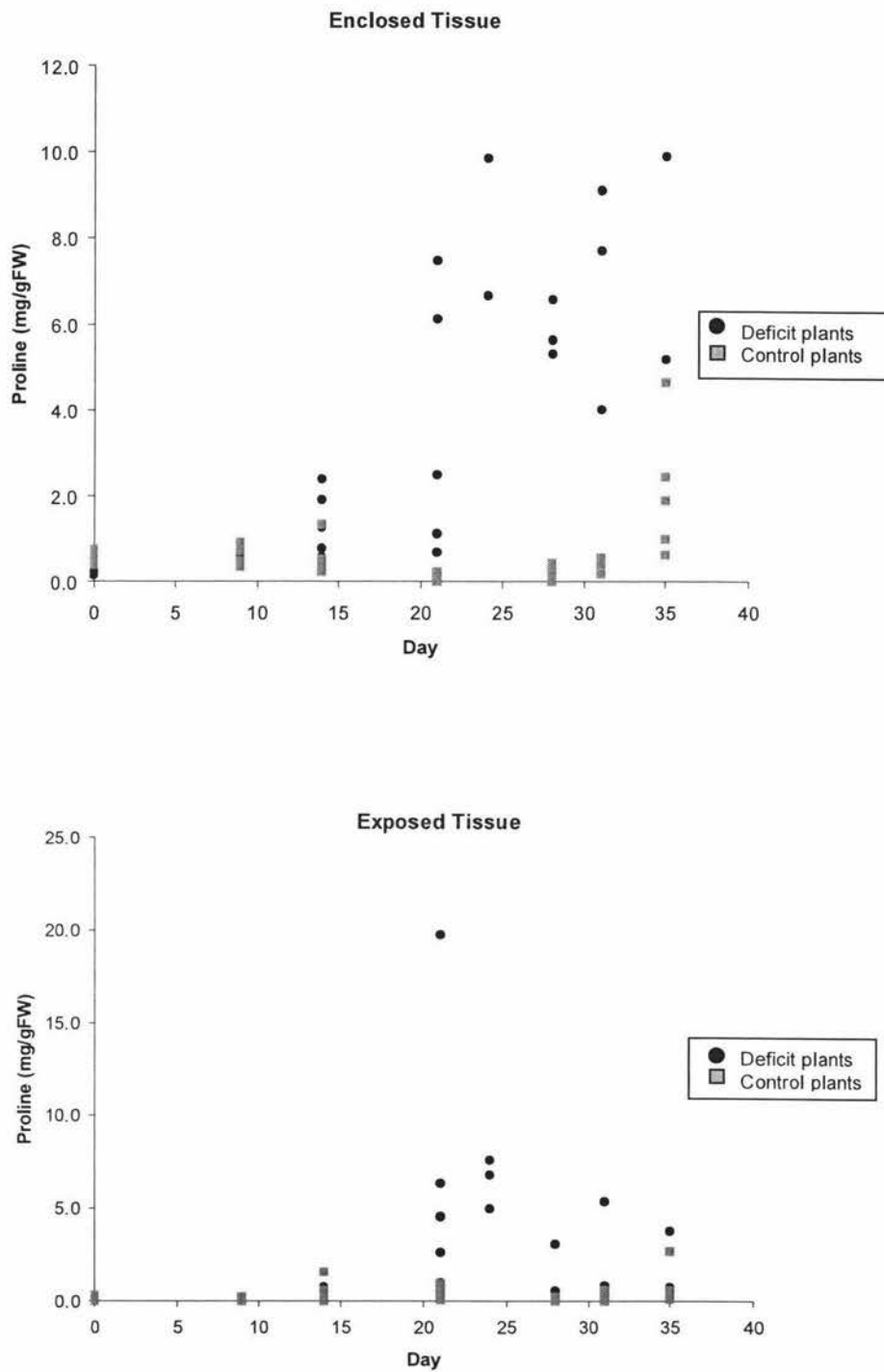


### 3.4 Proline Accumulation

A significant increase in the accumulation of proline in leaves is one of the most dramatic characteristics of severe water stress. Proline is therefore a very useful indicator of water-deficit stress.

Over the course of the experiment, proline levels were measured in both the enclosed and the exposed tissue of each plant. Two tillers were chosen at random from leaf material harvested at regular intervals throughout the experimental dry-down. Leaf 1 from each tiller was dissected into enclosed and exposed leaf tissue (refer to section 2.3), and then analysed for proline content using the method of Magne and Larher (1992) (described in section 2.5.2).

At day 0, proline levels in the dry-down and the control plants were similar, but then proline levels increased significantly in the water-deficit plants compared with the controls (figures 3.6 and 3.7 on the following pages, pp. 46 and 47 resp.). These increases were between 50 to 100-fold in the water-deficit plants with results becoming significant at the 5% level on day 14 in enclosed tissue (by a Student's *t*-test, refer to Appendix 4 for statistical analysis) . In the exposed tissue, the proline content in the water-deficit leaves was significantly different from control tissue on day 21 only, at the 5% level (by a Student's *t*-test). For example, in the dry-down pots, the mean proline in enclosed tissue was  $1.47 \pm 0.30$  mg/gFW on day 14 from mean base levels of  $0.30 \pm 0.05$  mg/gFW with levels climbing thereafter to reach a mean proline content on day 35 of  $7.54 \pm 2.36$  mg/gFW (equivalent to a 25-fold increase); and in exposed tissue mean proline of  $6.5 \pm 2.76$  mg/gFW on day 21, from a base level of  $0.09 \pm 0.05$  mg/gFW on day 0, or a 72 -fold increase. In comparison, the mean free proline level in control leaves was  $0.62 \pm 0.13$  mg/gFW in enclosed and  $0.33 \pm 0.08$  mg/gFW in exposed tissue throughout the experiment (data not shown).



**Figure 3.6 Proline accumulation in the control and the dry-down plants, in the two tissue types over the experimental period**

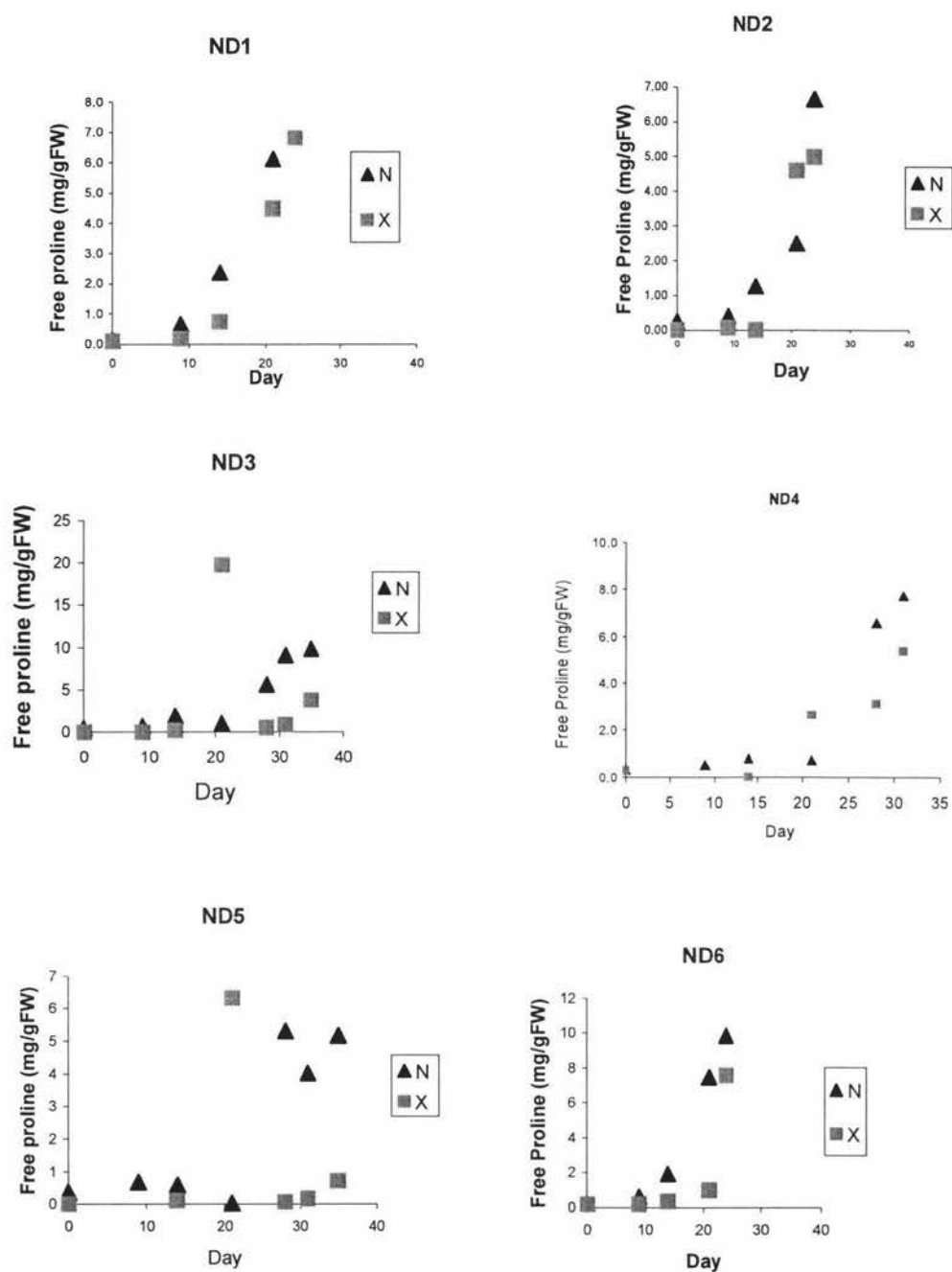
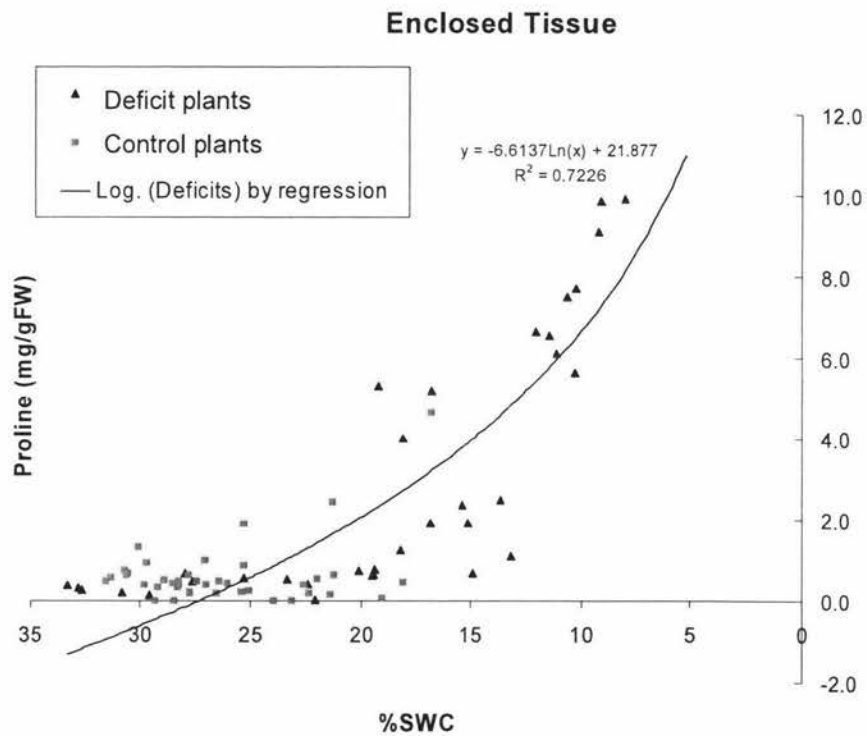


Figure 3.7 Individual proline content in each of the dry-down pots over the experimental period in the two tissue types. N is enclosed tissue, X is exposed tissue.

As the SWC decreased, proline levels increased in both tissue types of the dry-down pots (figure 3.8 below and figure 3.9 on the following page). In enclosed tissue (figure 3.8, below), accumulation is very nearly exponential at soil water contents below ca. 20% ( $R^2 = 0.67$ ), however the regression equation shown is for a logarithmic curve with a slightly better  $R^2$  value of 0.72. In the exposed tissue (figure 3.9; p. 49), only ca. 20% of the variation in proline accumulation in the exposed tissue could be accounted for by the variation in SWC, as indicated by a very poor  $R^2$  value of 0.20 (regression analysis not shown).



**Figure 3.8** Free proline content with decreasing SWC in the enclosed leaf tissue for the dry-down and the control plants of *F. novae-zelandiae*.

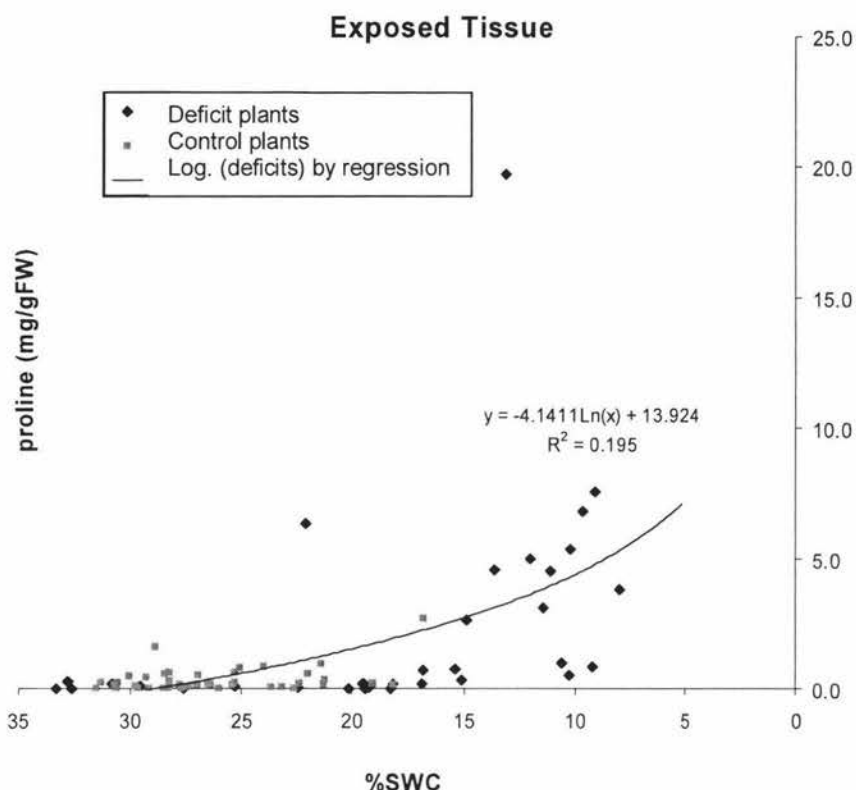


Figure 3.9 Free proline content with decreasing SWC in the exposed tissue for the dry-down and the control plants of *F. novae-zelandiae*.

### 3.5 Ethylene Measurements

The preliminary measurements of ethylene evolution in response to a water deficit for *F. novae-zelandiae* determined that very low levels were being evolved (data not shown). It became clear that any ethylene production was at the limits of detection of the flame-ionisation detector. Clearly, 1.0 gram of fresh weight leaf tissue was not sufficient for measuring ethylene evolution. Because of the finite amount of leaf material, large number of harvest dates and destructive sampling methods, increasing the amount of tissue by weight was not an option. Therefore, no further ethylene evolution measurements were attempted for this species.

### 3.6 ACC Oxidase (ACO) Activity Assays

ACO enzyme activity assays for *F. novae-zelandiae* were carried out *in vitro* after pooling the ground, frozen (-80°C) leaf tissue according to soil-water status, and after the removal of non-typical dry-down material. Non-typical dry-down material was deemed as any tissue showing a wide deviation in proline content from the regression equations given in figures 3.8 (p. 48) and 3.9 (p. 49), i.e. leaf tissue corresponding to  $R^2$  values of less than 0.50. For example, all of the exposed tissue harvested from pots ND3 and ND5, enclosed tissue harvested on day 21 from pot ND5 and exposed tissue harvested on day 21 from pot ND6.

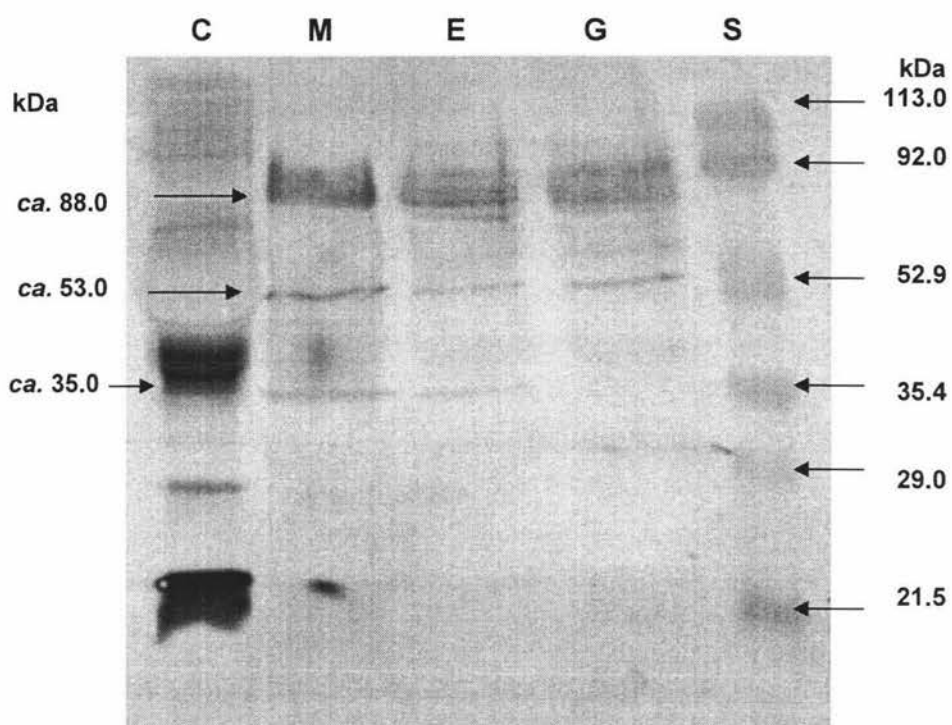
Extract preparation involved a partial purification step using gel filtration (Sephadex G-25) (refer to Section 2.6.2), and ACO enzyme activity was assayed using the method of Du (2005) (Section 2.7), with ethylene evolution measured using a gas chromatograph (Section 2.4.2). Initial assays were carried out at a pH of 7.5 and an ACC concentration of 1 mM. These conditions were based on biochemical characterisation of ACO isoforms in white clover, as determined by Gong and McManus (2000). No ethylene peaks of any significance when compared to a blank (no plant extract) were observed, for either enclosed or exposed tissue. Assay conditions were then altered for enclosed tissue extracts only, to a pH of 8.5 and a 10-fold increase in substrate concentration of 10mM ACC. Tissue weights were also increased from 0.5 to 1.0 g. Again no peaks of significance were observed. Therefore, no further characterisation of ACO enzyme activity for this particular grass species was attempted as part of this thesis.

### 3.7 Western Analysis

Initial immunodevelopment of *F. novae-zelandiae* crude leaf extracts using two different primary antibodies to ACO gave good recognition responses. The antibodies used were anti-MD-ACO1 (from apple) and anti-TR-ACO2 (from white clover and designated rabbit #5). Both of these antibodies are specific to ACC oxidases, but it was decided to use the white clover ACO-specific antibody because of a stronger signal in the expected size range for ACO of molecular mass of 35 to 50 kDa (data not shown). In later experiments, a second antibody was used (anti-TR-ACO1, designated B34), which is raised to the TR-ACO1 isoform of white clover (Hunter *et al.* 1998) (refer to Section 2.10.2).

### 3.7.1 Western Analysis of Crude Leaf Extracts in Water Sufficient Plants

Before dry-down, crude extracts (i.e. no purification steps including Sephadex G-25) prepared from water-sufficient fresh-frozen leaf material, (harvested 4 weeks earlier and stored at -20 °C) and dissected into three leaf regions [meristematic, M; elongating, E; and mature green, G (refer to Section 2.3)], were separated by SDS-PAGE and challenged with the white clover anti-TR-ACO2 antibody. The enclosed tissue comprised the meristematic and elongating regions, while the exposed tissue comprised the mature-green regions. The antibody recognised bands of high molecular mass between 52.9 and 92 kDa (figure 3.10 on the following page). A higher molecular diffuse band of ca. 88.0 is present in all three tissues, as is a discrete band of ca.53.0 kDa. An additional minor recognition band of ca. 35kDa is visible in the elongating and mature green tissue but not in the meristematic tissue. This band is around the expected size for an ACO protein, as confirmed by a similar mobility with a positive control (C) comprising partially purified white clover ACO extracted from white clover leaves.



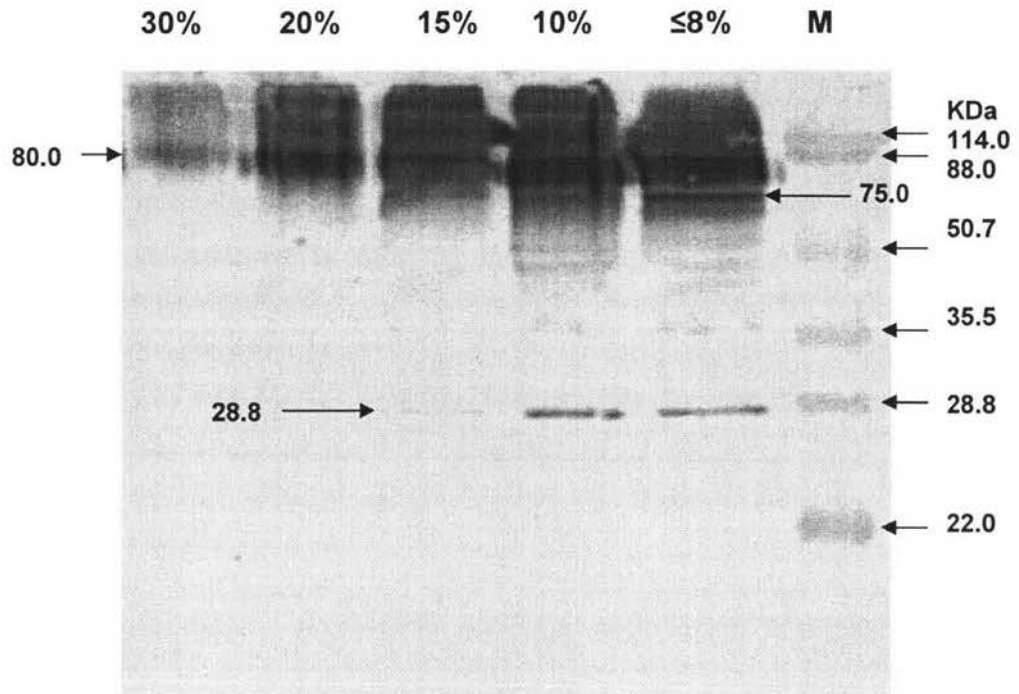
**Figure 3.10** Immunoblot of crude extracts for the three leaf tissue zones, meristematic (Lane M), elongating (Lane E) and mature green (Lane G), using the anti-TR-ACO2 antibody.

Lane S: pre-stained molecular mass markers (molecular masses are indicated); Lane C is a positive control (partially purified ACO from white clover leaves)

### 3.7.2 Western Analysis of Dehydrated and Water Sufficient Partially Purified Leaf Extracts

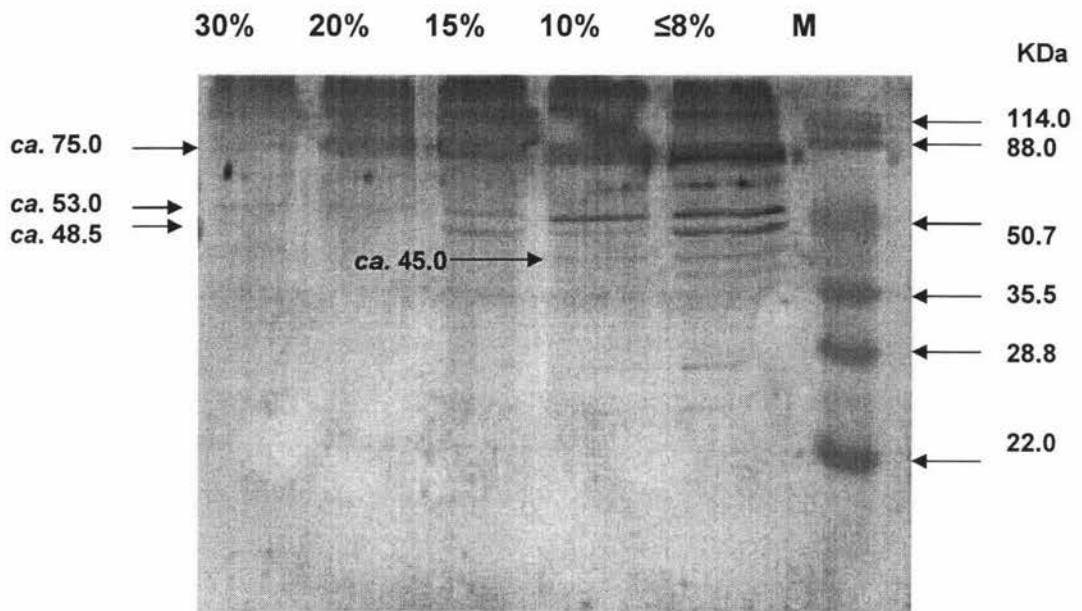
Crude extracts of pooled leaf tissue at SWCs of 30%, 20%, 15%, 10%, and  $\leq 8\%$  were subjected to a partial purification step using gel filtration (Sephadex G-25). Western analysis was carried out on the resulting pooled fractions. Only those fractions containing the higher levels of protein, (data not shown) as determined by BioRad Assay (section 2.8.1), were pooled. Subsequent immunodevelopment of membranes was carried out using one of two primary antibodies, anti-TR-ACO2 or anti-TR-ACO1.

For enclosed tissue (N), comprising the meristematic and elongating leaf zones, the anti-TR-ACO2 antibody recognised a band with a molecular mass of ca. 28.8 kDa at SWCs of 15%, 10% and  $\leq 8\%$  (refer to figure 3.11 on the following page). These bands are not present in tissue at soil water contents of 20% or 30%, i.e. in leaf tissue in a fully hydrated state. A major band of ca. 80.0 kDa is recognised in all of the extracts. An additional band of ca. 75 kDa is also recognised by the antibody in tissue at SWCs of 15% and less. The antibody has also recognised a pair of diffuse high molecular weight bands of  $\geq 114.0$  kDa. The anti-TR-ACO1 antibody (figure 3.12; p. 55) recognised bands of high molecular mass between ca. 50.7 and  $>114.0$  kDa in all extracts and especially at the lower SWCs. Of these, there is a major recognition band with a molecular mass of ca. 75 kDa, with higher recognition at the lower SWC values. Multiple diffuse bands are recognised in the 10% and  $\leq 8\%$  SWC extracts. A well-resolved band of ca. 53.0 kDa is present in all extracts and increases in intensity with decreasing SWC. For example, this band is most intense at SWCs of 10% and  $<8\%$ . This band is associated with another with a molecular mass of ca. 48.5 kDa at SWCs of 15% and less. The TR-ACO1 antibody has also recognised minor band of ca. 45.0 in the tissue at 10% and  $\leq 8\%$  SWC.



**Figure 3.11** Immunoblot of *F. novae-zelandiae* partially purified enclosed leaf tissue extracts at varying SWCs using the anti-TR-ACO2 antibody.

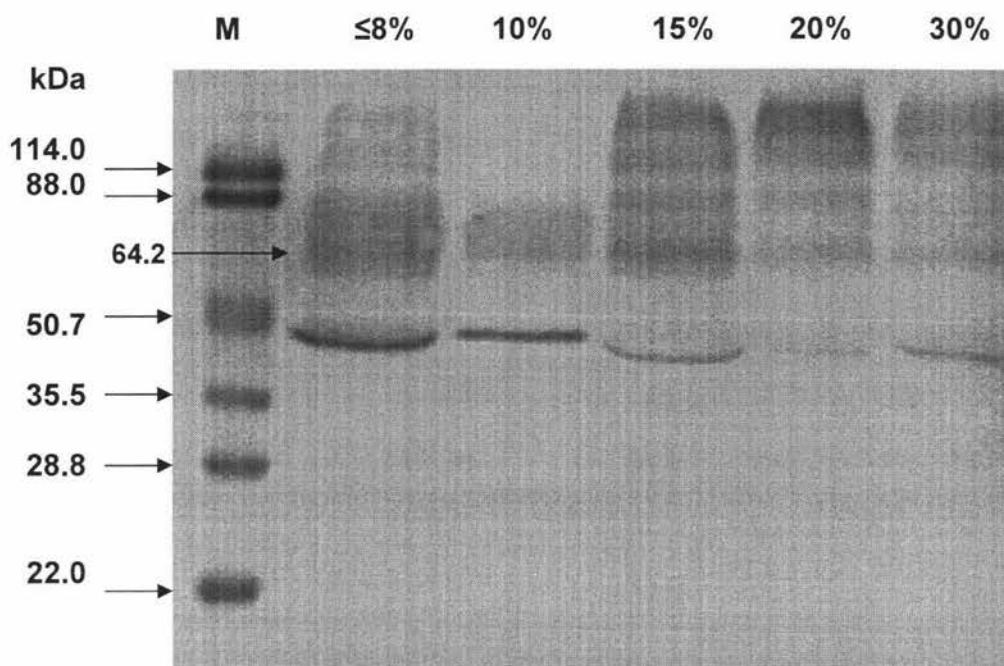
Leaf tissue is at various soil water contents as indicated from water-sufficient (30% SWC) to dehydrated ( $\leq 8\%$ ). Lane M is pre-stained molecular mass markers (molecular masses are indicated).



**Figure 3.12** Immunoblot of *F. novae-zelandiae* partially purified enclosed leaf tissue extracts at varying SWCs using the anti-TR-ACO1 antibody.

Leaf tissue is at various soil water contents as indicated, from water-sufficient (30 %SWC) to dehydrated ( $\leq 8$  %SWC). Lane M is pre-stained molecular mass markers (molecular masses are indicated).

For exposed tissue, the anti-TR-ACO2 antibody clearly recognised a band of ca. 50.0 kDa at all SWCs (figure 3.13, below). This band appears more intense at the lower SWCs of 10% and <8%. The antibody also recognised a diffuse band of ca. 64.2 kDa in all extracts. Bands of high molecular weight (>~60kDa) are visible in all extracts especially for SWCs of 15%, 20% and 30% but these are more diffuse in nature.



**Figure 3.13 Immunoblot of *F. novae-zelandiae* partially purified extracts of exposed leaf tissue at various SWCs using the anti-TR-ACO2 antibody.**

Leaf tissue is at various SWCs as indicated, from dehydrated ( $\leq 8\%$  SWC) to water-sufficient (20 and 30 %SWC). Lane M is pre-stained molecular mass markers (molecular masses are indicated).

At the conclusion of this western analysis, no further purification of *F. novae-zelandiae* leaf tissue extracts was attempted.

**Summary of results:****Table 3 Bands of major recognition by the polyclonal antibodies TR-ACO1 and TR-ACO2 in partially purified leaf extracts of *F. novae-zelandiae*, as indicated:**

| <b>Antibody</b> | <b>Enclosed Tissue</b>           | <b>Exposed Tissue</b> |
|-----------------|----------------------------------|-----------------------|
| <b>TR-ACO1</b>  | ca. 75.0<br>ca. 53.0<br>ca. 48.5 |                       |
| <b>TR-ACO2</b>  | ca. 80.0<br>ca. 75.0<br>ca. 28.8 | ca. 50.0<br>ca. 64.2  |

## Chapter Four: Experiment 2: Experimental Dry-down of *Festuca arundinacea* cv. 'Roa' (syn. *Schedonorus phoenix*)

### Part I: Physiological Measurements

#### 4.1 Experimental Design

The experimental design and set-up for Experiment 2 was similar to Experiment 1 (Section 3.1). That is, 6 water-deficit pots and 6 control pots were used. Experiment 2 commenced on 23/09/03 (day 0) and ended on 08/10/03 (day 15).

Water-deficit stress was imposed on six plants randomly chosen from a total of 12, by withholding water for a period of 15 days. As before, a control set of 6 plants was maintained in a hydrated state, i.e. at or near 70% of container capacity, by regular watering (refer to Section 2.2.2 for soil saturation theory).

#### 4.2 Soil Water Measurements

Over the 15-day experimental period, %SWC was measured in each pot, relative to an empirically determined container capacity of 50%, using the same method as in Experiment 1 (Section 2.3.1). Measurements were made on days 1, 4, 7, 10 and 14.

At the start of the experiment, all pots were at or near a SWC of ca. 30%. During the course of the experiment, an average SWC of  $23.8 \pm 0.7\%$  was maintained in control pots (data not shown). The mean SWC in the water-deficit pots decreased over time (figure 4.1; p. 60) to become significantly different from controls at the 5% level by day 4 (by a student *t*-test assuming unequal variance, refer to Appendix 4 for statistical analysis). At day 14 (the final SWC measurement made), the mean SWC in the dry-down pots was  $8.1 \pm 0.4\%$ . In each pot, SWC was recorded until dry-down was complete (as determined by zero LER, see Section 4.3) at which point no further SWC measurements were made, and this explains reductions in 'n' in figure 4.1 as the experiment progressed.

The individual dry-down curves for the six water-deficit pots are shown in figure 4.2 (p. 61) with regression outcomes. While the  $R^2$  values for the individual pots were much higher than the  $R^2$  value obtained from the whole data set, analysing the data collectively as in figure 4.1(p. 60) is a much better assessment of the degree of accuracy of the prediction model described by the regression equation. The rates of dry-down were similar in all six pots as indicated by similar standard deviations i.e. similar amounts of variation among the data. The dry-down followed a (natural) logarithmic decrease. A minimum SWC for the experiment was recorded in pot AD2 on day 10, i.e. at 7.2%. In comparison, SWCs of 9.4%, 7.7%, 9.6%, 9.8% and 8.5% were recorded in dry-down pots AD1, AD3, AD4, AD5 and AD6 respectively. These were recorded on day 10, except for pot AD3, which was recorded on day 14.

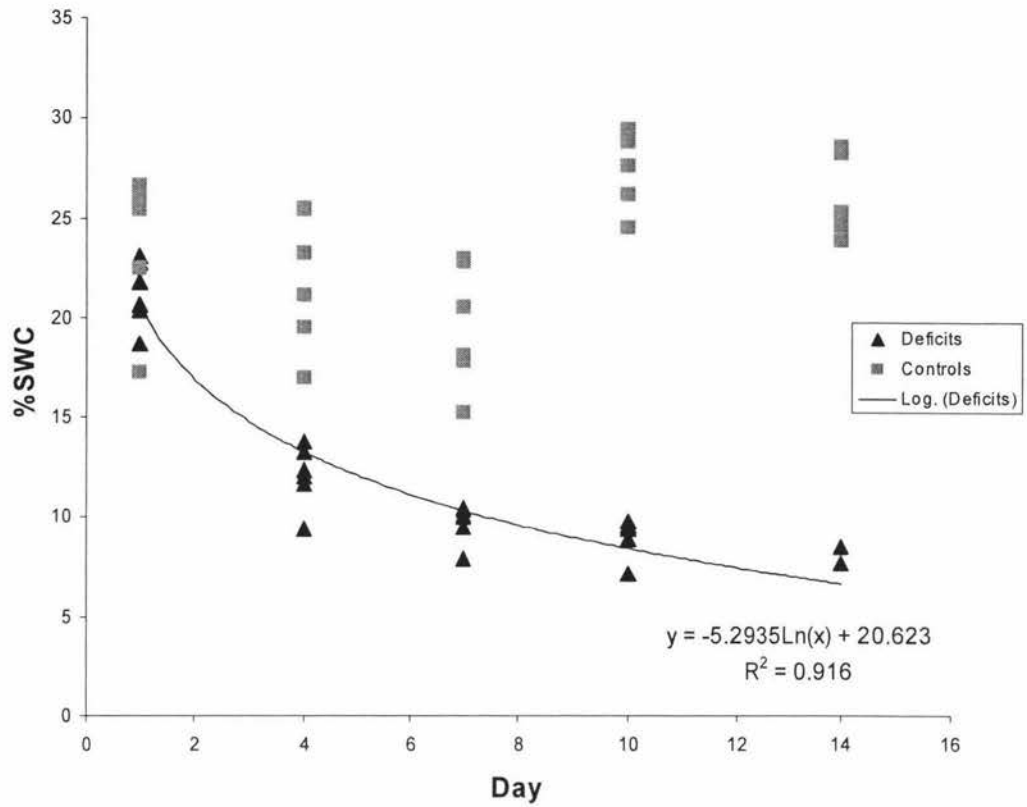


Figure 4.1 Soil water content over the course of the experiment in the water-deficit and the control pots (n=6).

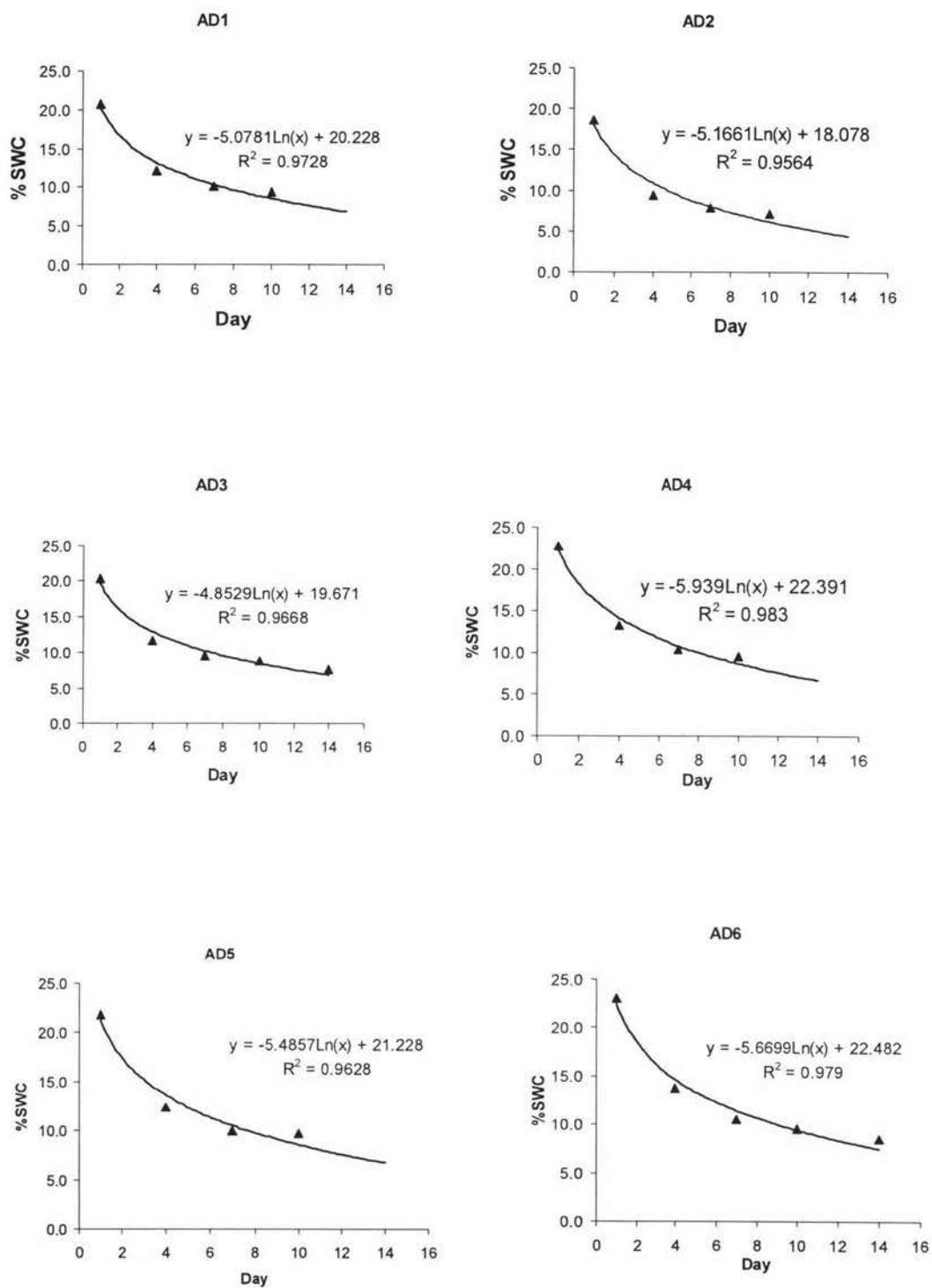


Figure 4.2 Individual dry-down curves for the six water-deficit pots over the experimental period

### 4.3 Leaf Elongation Rate (LER)

The mean leaf elongation rate for all plants was measured using the method described in sections 2.3.2 and 2.3.3, in mm/day. Measurements were made at regular intervals i.e. on days 1, 4, 7 and 14, for each pot. As the LER for each plant reached zero, no further measurements were recorded. For example  $n=6$  at day 0 and diminished with dry-down.

The LER declined (in the water-deficit plants) as the dry-down progressed (figure 4.3 on the following page), to become significantly different from the controls at the 5% level, by day 4 (by a student  $t$ -test assuming unequal variance). The LER reached zero i.e. cessation of cell enlargement, in dry-down plants AD1, AD2, AD4 and AD5 on day 7 and on day 14 in plants AD3 and AD6 (figure 4.4, p.64). In comparison, minimums LERs for the six control plants were 13.8, 5.1, 8.0, 10.3, 14.2 and 20.7 mm/day. The mean LER over the 16-day dry-down period was  $5.7 \pm 2.0$  mm/day for the water-deficit plants and  $17.7 \pm 1.2$  mm/day for the control plants.

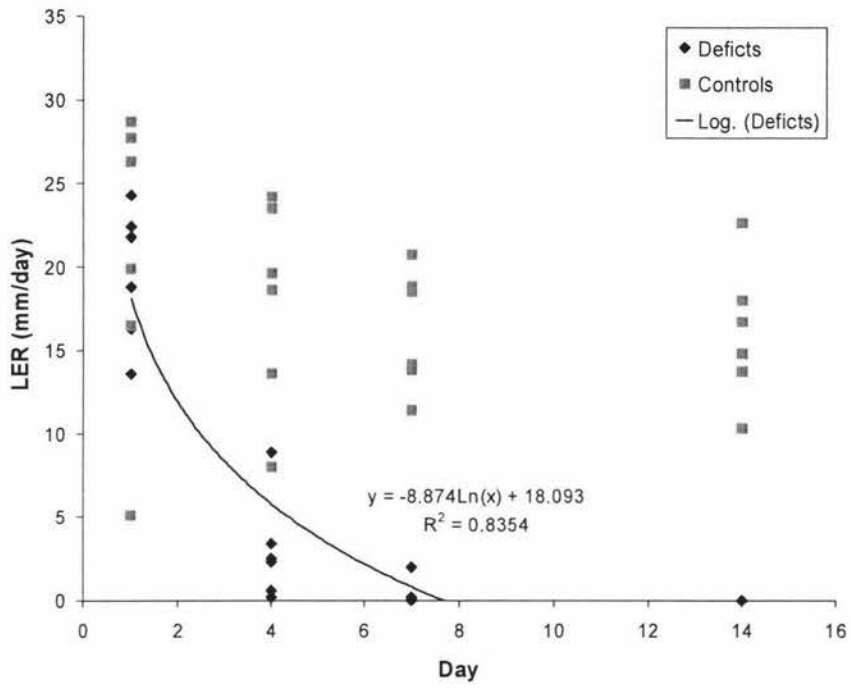
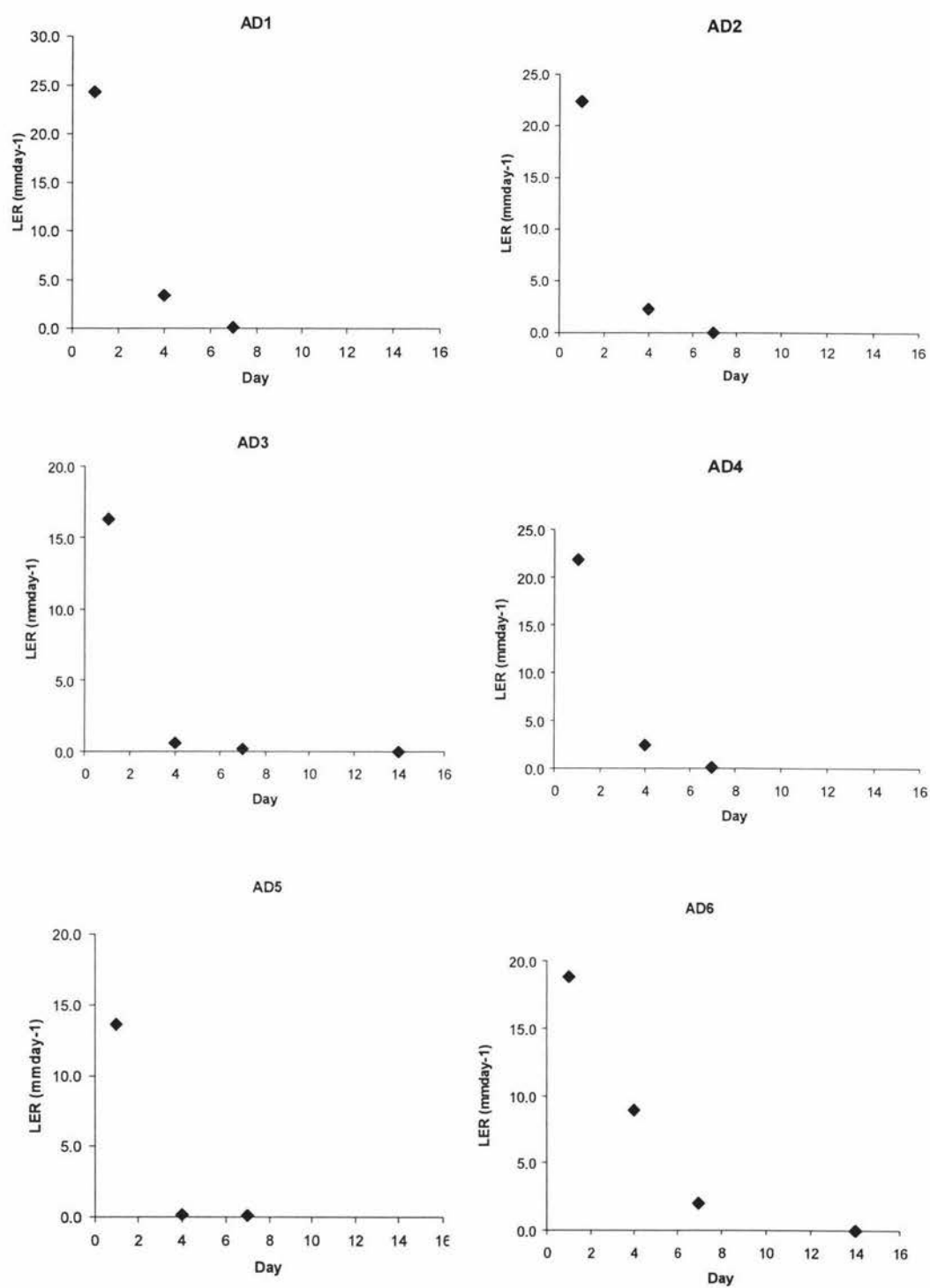


Figure 4.3 Leaf elongation rates in the water-deficit and the control plants over the experimental period (n=6 at day 0, and diminishing thereafter as zero LER was reached).



**Figure 4.4** Leaf elongation rates (the mean of three replicates) in each of the six dry-down plants over the experimental period

The LER declined as SWC decreased (figure 4.5 below) to become significantly different from controls at a mean SWC of  $12.1 \pm 0.6\%$ . The LERs, with decreasing SWC, for each of the six dry-down pots are shown separately in figure 4.6 (p. 66) with regression analysis outcomes. Note that the  $R^2$  values shown ( $R^2 = 0.86$  to  $0.99$ ) are better than for the pooled data. Analysing the data collectively however (figure 4.5), gives a more accurate confidence level (an  $R^2$  value of  $0.83$ ). That is,  $83\%$  of the variation observed in the LER is accounted for by variation in the SWC. The LER declined in a logarithmic manner in all six pots, with respect to a decline in SWC. Zero LER corresponded to SWCs of  $10.3\%$ ,  $8.0\%$ ,  $10.8\%$  and  $10.6\%$  (from interpolated data; data not shown) in pots AD1, 2, 4 and 5 respectively, and  $6.9\%$  and  $7.5\%$  in pots AD3 and AD6 respectively. This is an average SWC of  $9.0 \pm 0.7\%$ .

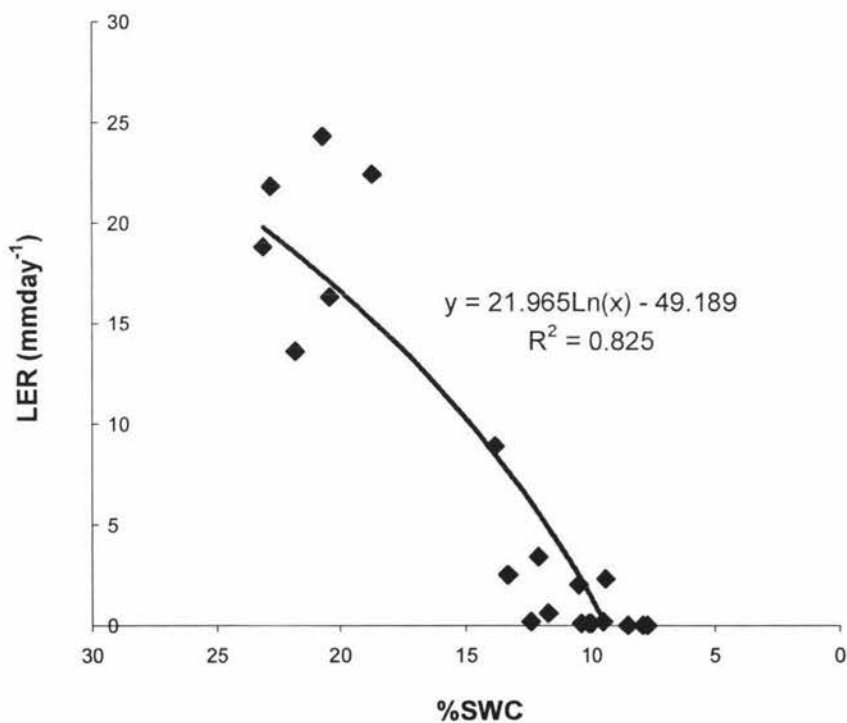


Figure 4.5 Leaf elongation rates in dry-down plants with decreasing SWC.

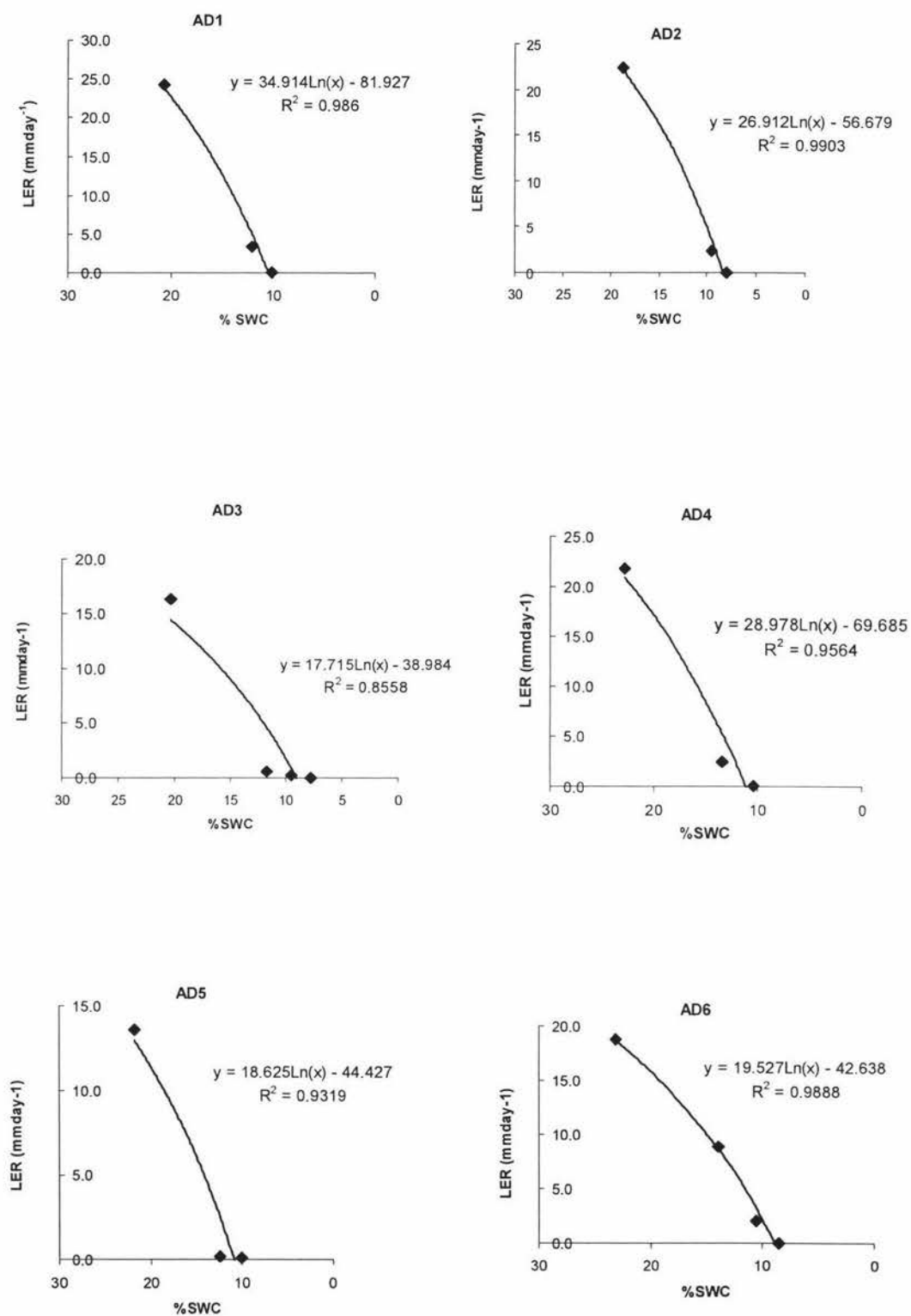


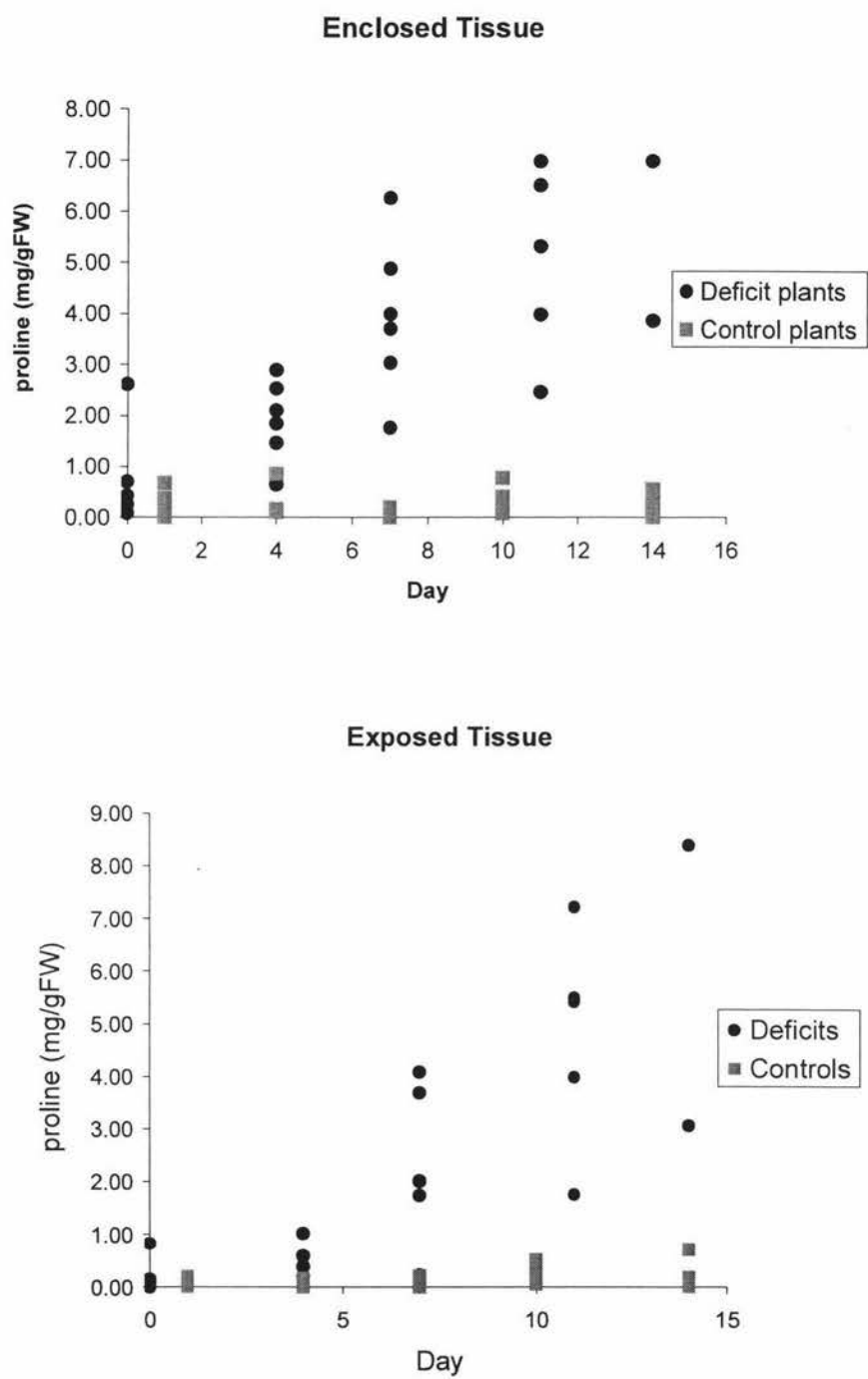
Figure 4.6 Leaf elongation rates (the mean of three replicates) in each of the six dry-down plants with decreasing SWC.

## Part II: Biochemical Analysis

### 4.4 Proline Accumulation

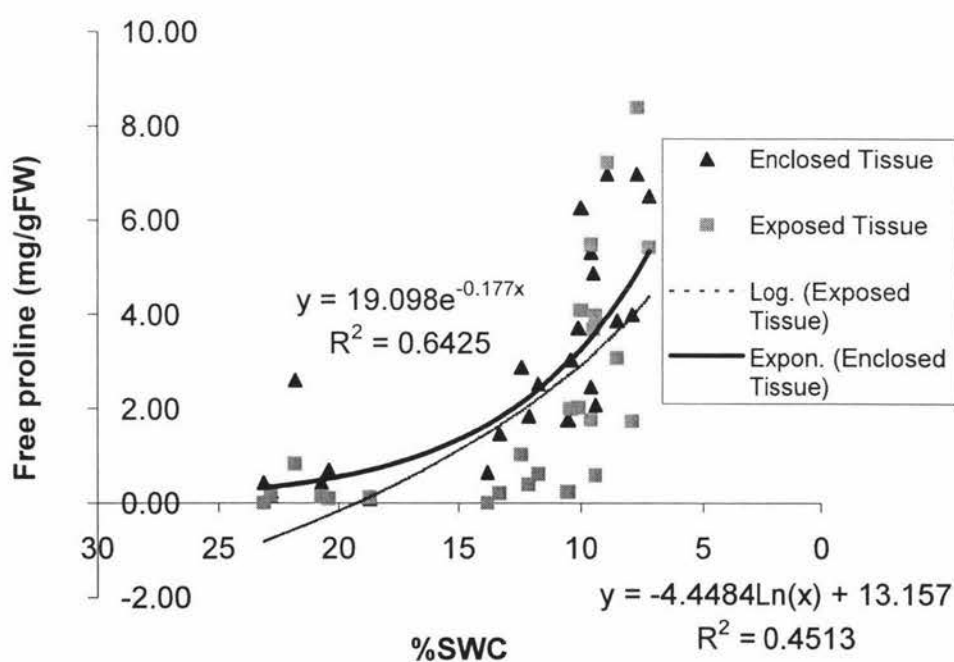
Free proline was measured in leaf tissues on days 0, 4, 7, 11 and 14 for all plants, and expressed in units of milligrams of proline per gram of fresh weight tissue (mg/gFW). Proline levels were also determined in the two tissue types: enclosed and exposed (refer to section 2.5.1 for method). Levels increased significantly in both tissue types as the dry-down progressed (figure 4.7 on the following page). In enclosed tissue, proline accumulation in the dry-down pots became significantly different from that in control leaves (at the 5% level) from day 4 onwards (by a Students *t*-test). In exposed tissue, accumulation in the dry-down pots became significant from day 7 onwards (at the 5% level by a Students *t*-test).

In enclosed tissue, levels increased from a base level of *ca.*  $0.53 \pm 0.20$  mg/gFW at day 0, to a maximum of  $5.42 \pm 1.56$  mg/gFW on day 14, a *ca.* 10-fold increase. For exposed tissue, mean daily free proline increased from a base level of *ca.*  $0.17 \pm 0.06$  mg/gFW to a maximum of  $5.7 \pm 2.66$  mg proline/ gFW tissue, a *ca.* 28-fold increase. In control pots, the mean daily free proline levels remained at less than 0.5 mg/gFW throughout the experiment. On average, across the 16-day period, the mean daily proline level in deficit pots was approximately 15 times that of controls.

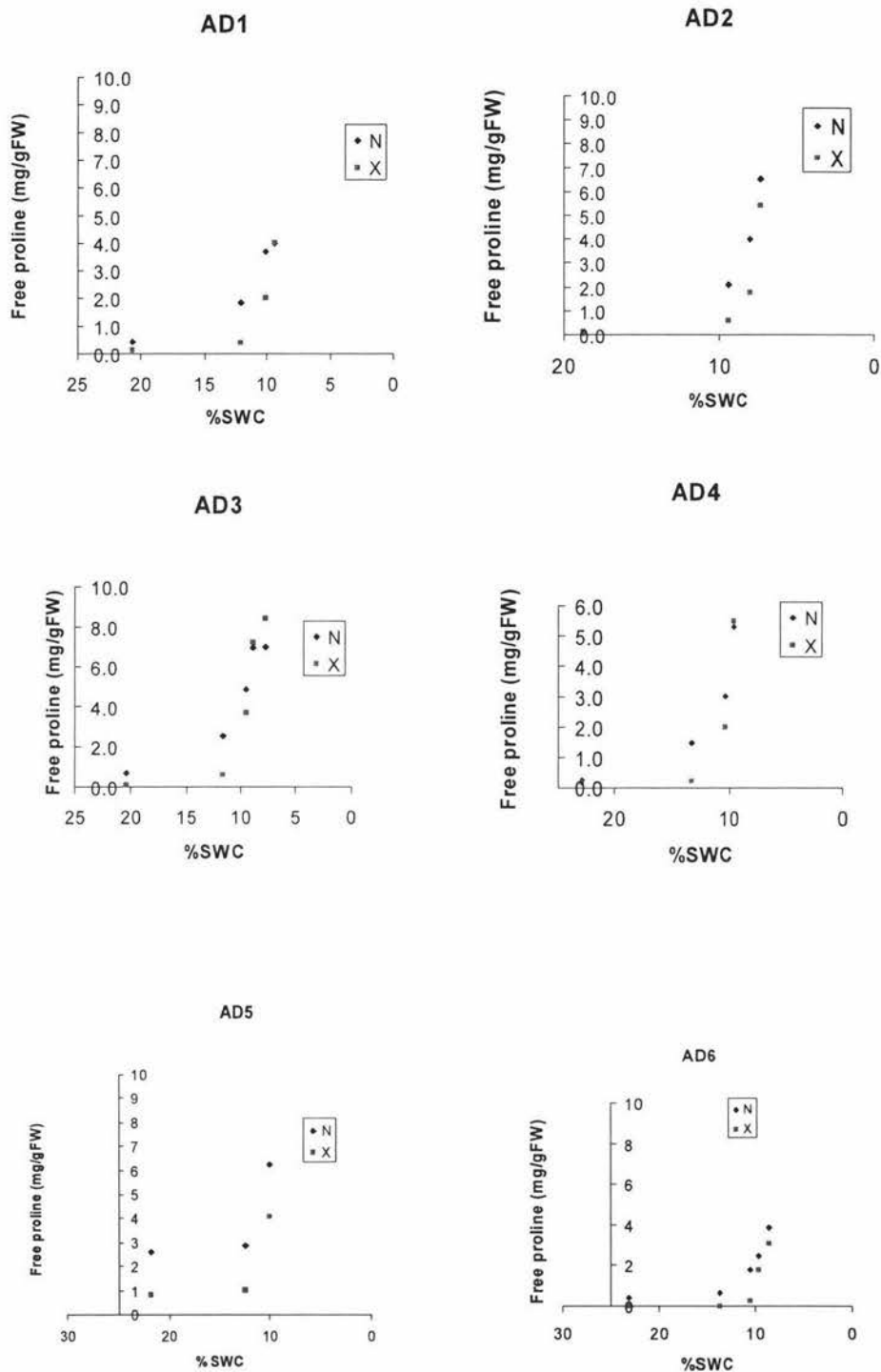


**Figure 4.7 Free proline in the dry-down and the control plants over the experimental period in the two tissue types**

As the percentage soil water content decreased, the levels of free proline increased in both tissue types (figure 4.8 below). Exponential functions describing proline accumulation in the enclosed tissue (with  $R^2 > 0.89$ ), can be fitted to five of the six dry-down pots (AD1, 2, 3, 4 and 6) (figure 4.9 on the following page) (regression analysis not shown). Using the data set as a whole, 64% of the variation observed in proline content is accounted for by variation in the SWC. For exposed tissue, increases were generally slower (as indicated by flatter polynomial or linear curves, with  $R^2$  values of from 0.55 to 0.91), but reach final levels similar to those for the enclosed tissue. Proline levels became significantly different in the enclosed tissue as the mean SWC approached 12%, and in the exposed tissue at a mean SWC of ca. 10%. Proline continued to accumulate in both tissue types throughout the experimental dry-down, reaching maximum recorded accumulation at SWC's of around 8% or LERs of ca. 4.0 mm/day in enclosed and ca. 2.0 mm/day in exposed leaf tissue (figure 4.5; p. 65).



**Figure 4.8** Free proline accumulation in the two tissue types of the dry-down plants using all of the data points (n=6).



**Figure 4. 9** Free proline accumulation in the two tissue types for each of the six water-deficit plants. N is enclosed and X is exposed leaf tissue.

Once the measurements for the LER and proline accumulation were complete, this meant that critical periods in cellular adjustment to the water deficit could be identified, and these stages were then targeted for enzyme measurement.

## 4.5 Ethylene Analysis

### 4.5.1 Ethylene Measurements

During the experimental dry-down of *F. arundinacea*, the ethylene evolved from leaves was measured for both the enclosed and the exposed leaf tissue (refer to Method 2.4).

Ethylene production increased in the leaves of both the water-deficit and the control plants during the dry-down. Scatter plots (figures 4.10 and 4.11; pp. 73 and 74 resp.) for the water-deficit plants, reveal different patterns of ethylene evolution for the two tissue types: enclosed and exposed leaf tissue.

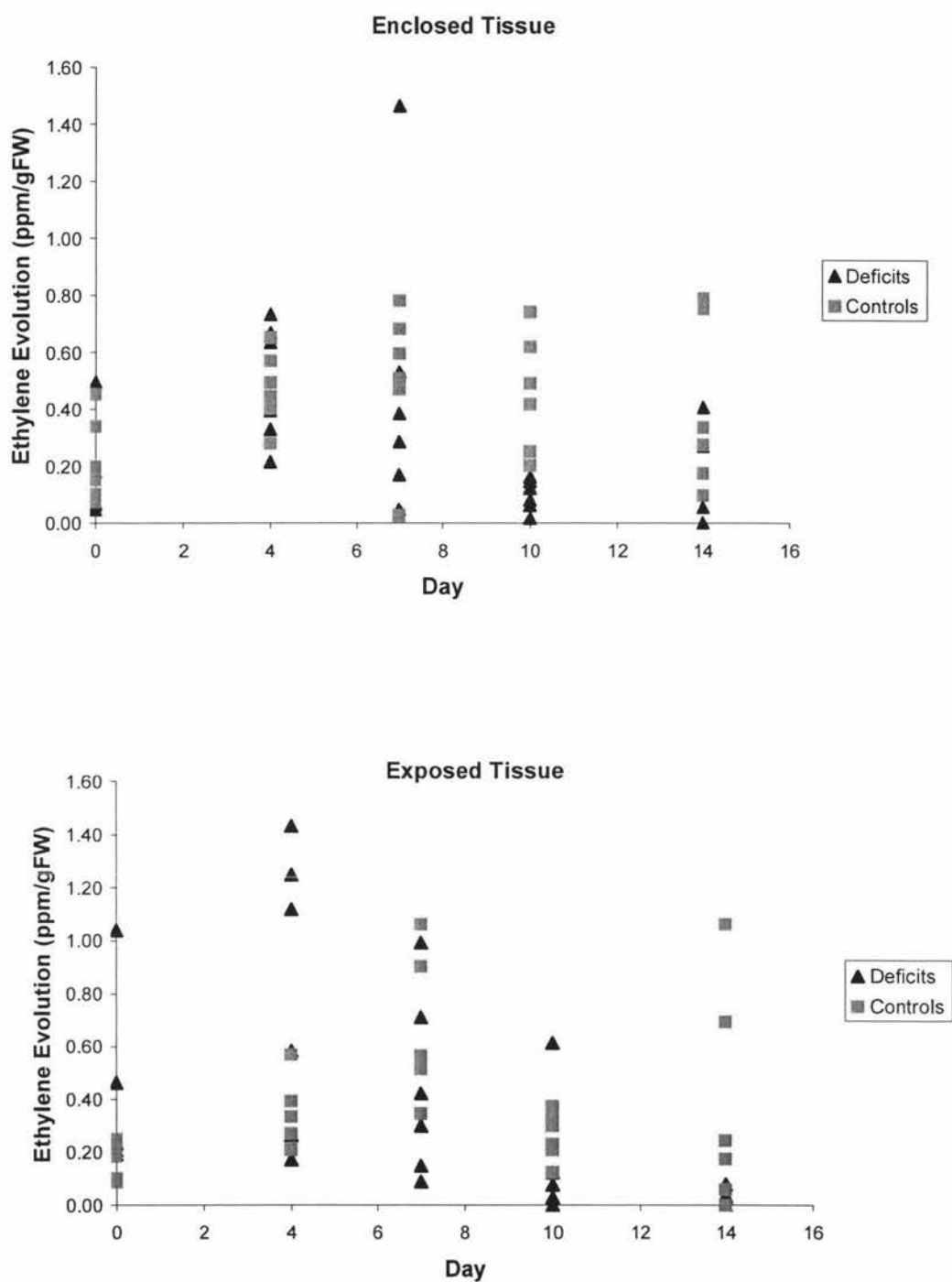
At the start of the experiment, i.e. day 0, ethylene evolution was similar in the enclosed tissue of the dry-down and the control leaves. For example, the mean ethylene evolution was  $0.16 \pm 0.07$  ppm/gFW in the dry-down tissue (figure 4.11 upper panel (p. 74), and  $0.22 \pm 0.06$  ppm/gFW in the control tissue (means for control data not shown). Over the experimental period, ethylene evolution in the enclosed tissue of the water-deficit leaves was not significantly different from the control leaves except on day 10, as determined by a Student's *t*-test (figure 4.11, upper panel). At this point in the dry-down, the mean ethylene evolution in the enclosed tissue of the water-deficit leaves was almost zero ( $0.10 \pm 0.02$  ppm/gFW). Differences are significant at the 5% level. In contrast, in the enclosed tissue of the control leaves, the mean ethylene evolution remained constant throughout the experiment at ca.  $0.41 \pm 0.04$  ppm/gFW (mean data not shown).

In the exposed tissue, the mean ethylene evolution was ca.  $0.40 \pm 0.13$  ppm/gFW on day 0 in the dry-down leaves (figure 4.11, lower panel), and  $0.17 \pm 0.03$  ppm/gFW in the control leaves (mean control data not shown). This difference between the dry-down and the control leaves at the start of the experiment is explained by an unusually high measurement for ethylene evolution of 1.04 ppm ethylene/gFW in plant AD6. If this value is removed from the data set, mean ethylene evolution in the dry-down plants

at day 0 ( $0.17 \pm 0.05$  ppm ethylene/gFW) is the same as the controls. This level is comparable to that recorded in the enclosed tissue on day 0.

The mean ethylene production in the exposed tissue of the water-deficit leaves was significantly different from control leaves, at the 5% level (by a student *t*-test assuming unequal variance), on days 4 and 14 (figure 4.10 lower panel, p. 73). Ethylene peaked around day 4 in the majority of the water-deficit plants, i.e. in AD2, AD3, AD4 and AD6. For example, on this day, the mean ethylene in leaves of the water-deficits was  $0.80 \pm 0.22$  ppm/gFW compared with  $0.33 \pm 0.06$  ppm/gFW for the control leaves (figure 4.11, lower panel). Ethylene maximums, on day 4 in pots AD2, AD4 and AD6 were  $1.12 \pm 0.54$ ,  $1.25 \pm 0.54$  and  $1.43 \pm 0.54$  ppm/gFW respectively. In AD1, ethylene levels peaked later on day 7 and in AD5, no significant ethylene peak was observed. Ethylene production then steadily declined to reach a mean ethylene evolution of zero on day 14, in all of the water-deficit plants.

Ethylene measurements for the exposed tissue of the control leaves fluctuated over the experimental period and averaged  $0.36 \pm 0.05$  ppm/gFW. The variation among the controls was least on day 0 (and this is a good sign) and day 4, and greatest on day 14. In contrast, the variation in ethylene evolution among the water-deficit plants was greatest on day 4 and least on day 14 (as ethylene approached values of zero and this is expected since values negative values are not valid). This is an indication then that any observed differences between the treatments on these days is likely to be real. Indeed results are significant at the 5% level.



**Figure 4.10** Ethylene evolution over the experimental period for the two tissue types in both the control (n=6) and the water-deficit leaves (n=6). Note that some points are hidden behind others.

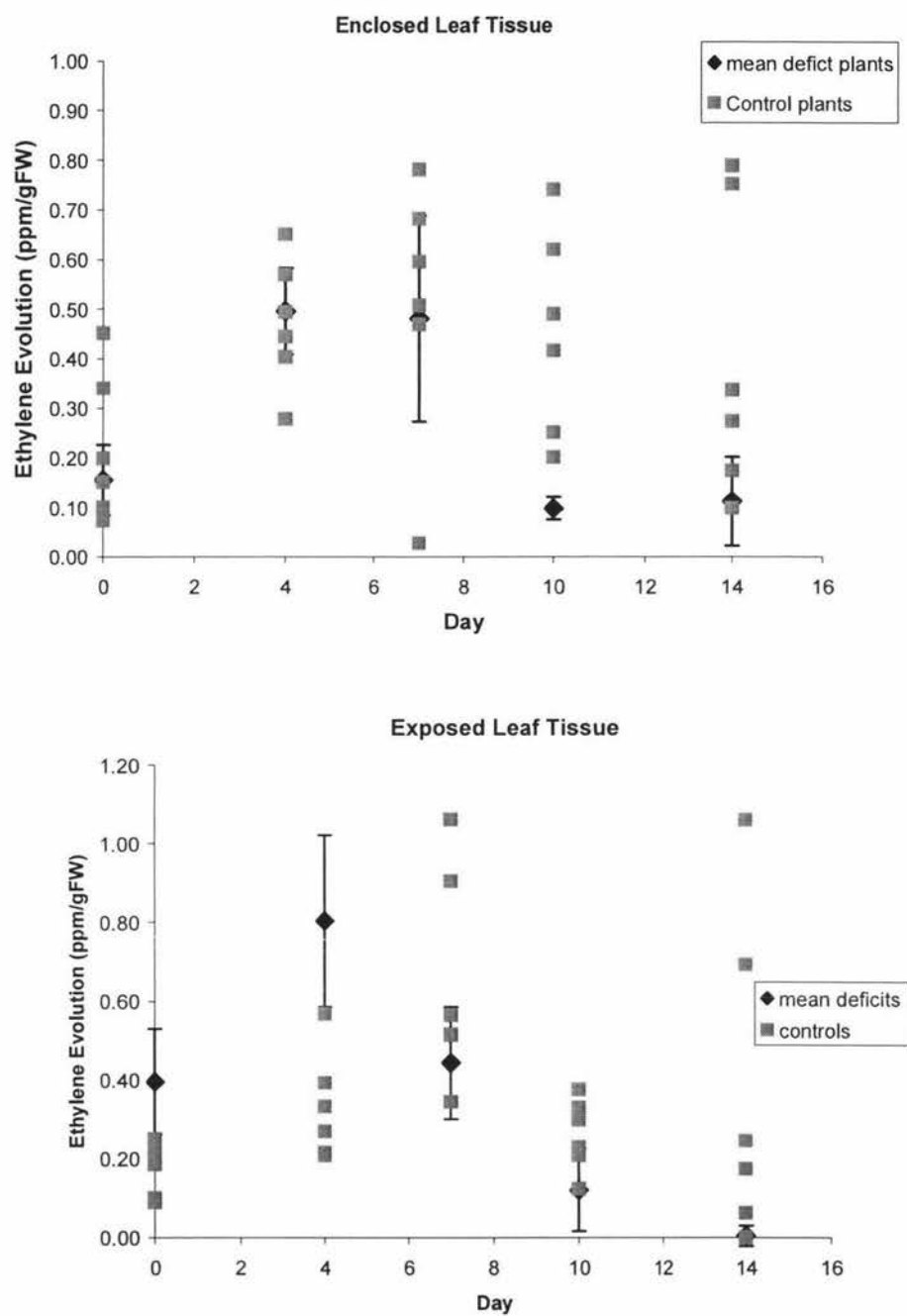
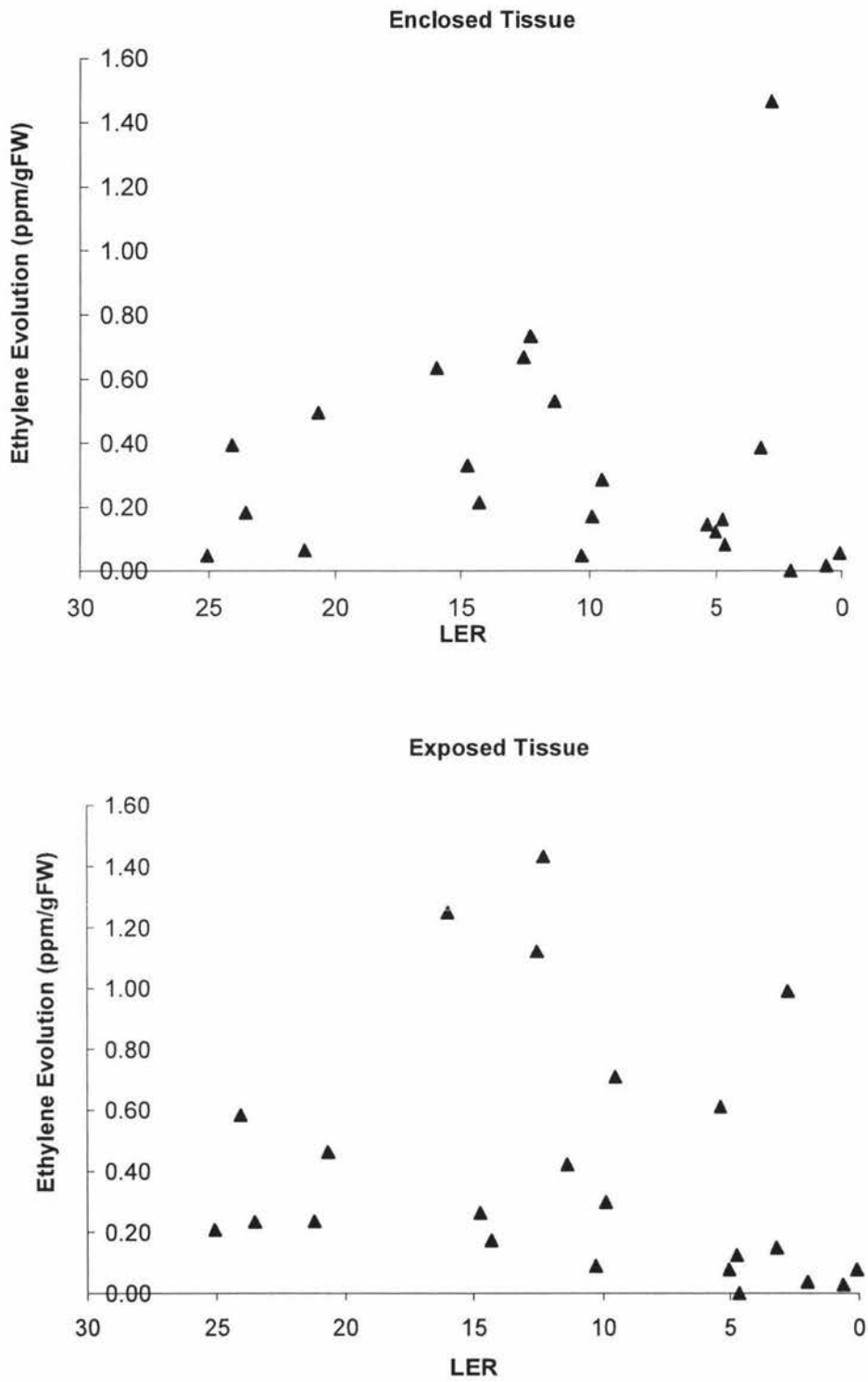


Figure 4.11 Mean ethylene evolution together with average standard deviations in the water-deficit and control leaves for the two tissue types (n=6).

#### 4.5.2 Ethylene Evolution with Decreasing LER

The ethylene evolution in the two tissue types with decreasing LER is shown in figure 4.12 (on the following page). Individual plots for each of the dry-down plants are shown in figure 4.13 (p. 77). In the enclosed tissue, ethylene evolution had declined significantly compared with the control leaves, to below base levels at a mean critical LER of  $5.7 \pm 1.0$  mm/day from interpolated data.

Peaks in ethylene evolution in the exposed tissue of the dry-down plants, occurred at LERs of between *ca.* 15.0 and 10.0 mm/day in the majority of the dry-down plants (from figure 4.12, lower panel), i.e. in AD2, AD3, AD4 and AD6. From the interpolated data, this is a mean LER of  $16.1 \pm 1.7$  mm/day. The corresponding mean SWC is *ca.* 22% SWC from figure 4.5 (plot of LER against SWC) or 21.5% from raw data. AD1 peaked at an LER of 9.5 mm/day from the interpolated data (figure 4.5; p. 65). No peak was observed for AD5. As a general trend, the ethylene evolution approached zero in both tissue types as the LER approached zero.



**Figure 4.12** Ethylene evolution with decreasing LER in the water-deficit plants for the two leaf tissue types.

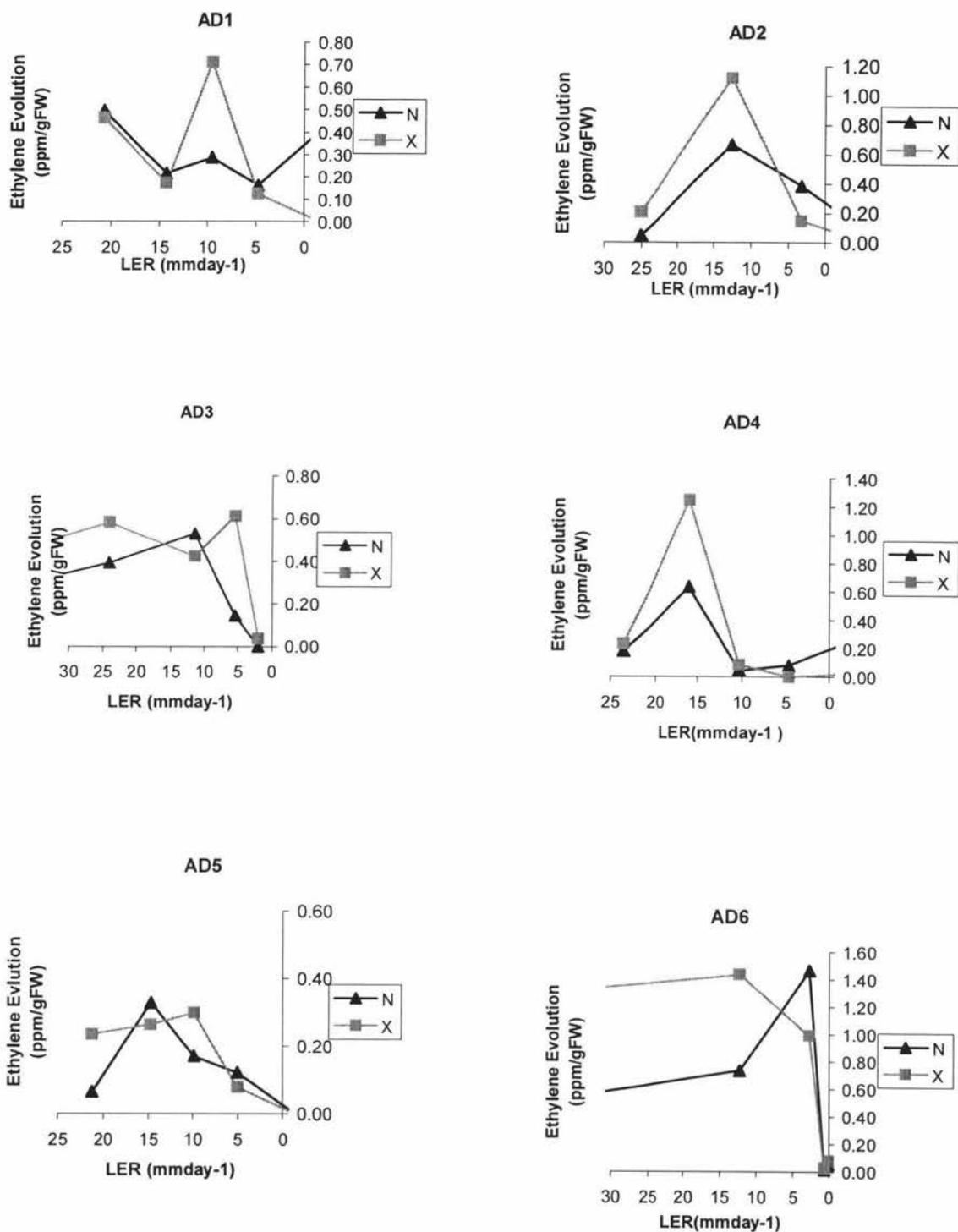
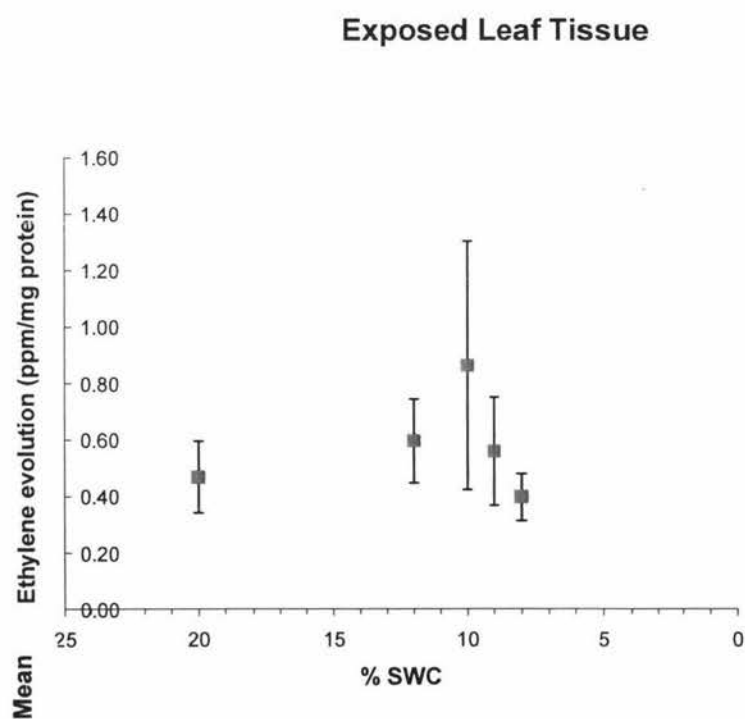
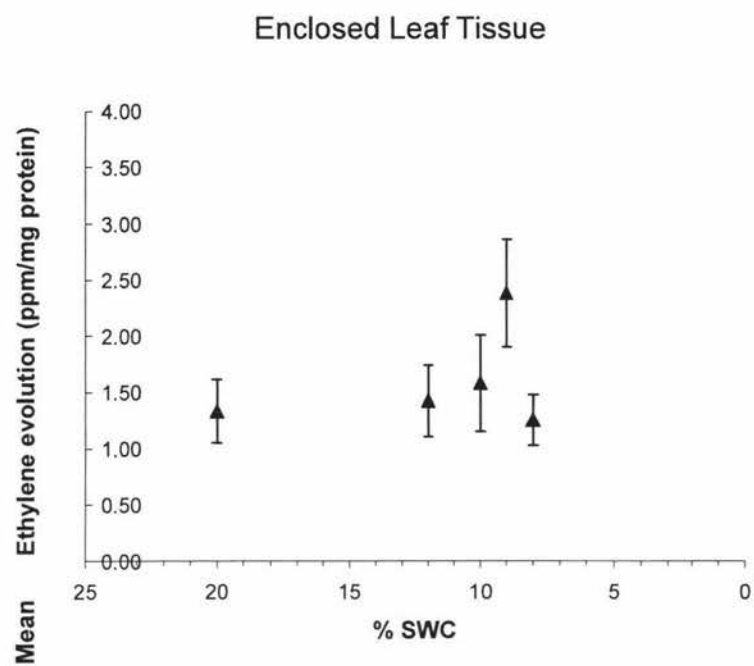


Figure 4.13 Ethylene evolution with declining LER in the individual water-deficit plants. N is enclosed tissue, X is exposed tissue.

## 4.6 ACO Enzyme Activity Assays in Partially Purified Leaf Extracts

Frozen, ground leaf material for the two tissue types (enclosed and exposed) was pooled by soil water content. Extracts were partially purified with a gel filtration step (Sephadex G-25) after extraction, to remove low molecular weight molecules (refer to section 2.6 for extraction method). Fractions containing the greatest amounts of protein, as determined by the BioRad assay (section 2.8), were pooled and *in vitro* activity assays were carried out as described by Du (2005), (section 2.7).

For the enclosed leaf tissue, the enzyme activity was greatest at soil water contents of ca. 9 % (figure 4.14, upper panel; p.79). For the exposed leaf tissue, enzyme activity was greatest at soil water contents of ca.10 % (figure 4.14, lower panel; p. 79). Specific activity maximums ranged from 1.3 to  $3.4 \pm 0.7$  ppm ethylene/mg protein in enclosed tissue at 9% SWC (average standard deviation, data not shown), and from 0.1 to  $1.9 \pm 0.4$  ppm ethylene/mg protein in exposed tissue at 10% SWC (average standard deviation, data not shown). There is a mean peak specific activity of  $2.4 \pm 0.5$  ppm ethylene/mg protein in the enclosed and  $0.9 \pm 0.4$  ppm ethylene/mg protein in the exposed leaf tissue (figure 4.14).



**Figure 4.14** Mean enzyme specific activity (n=4) with decreasing SWC for the partially purified extracts from the two tissue types. Standard errors are shown.

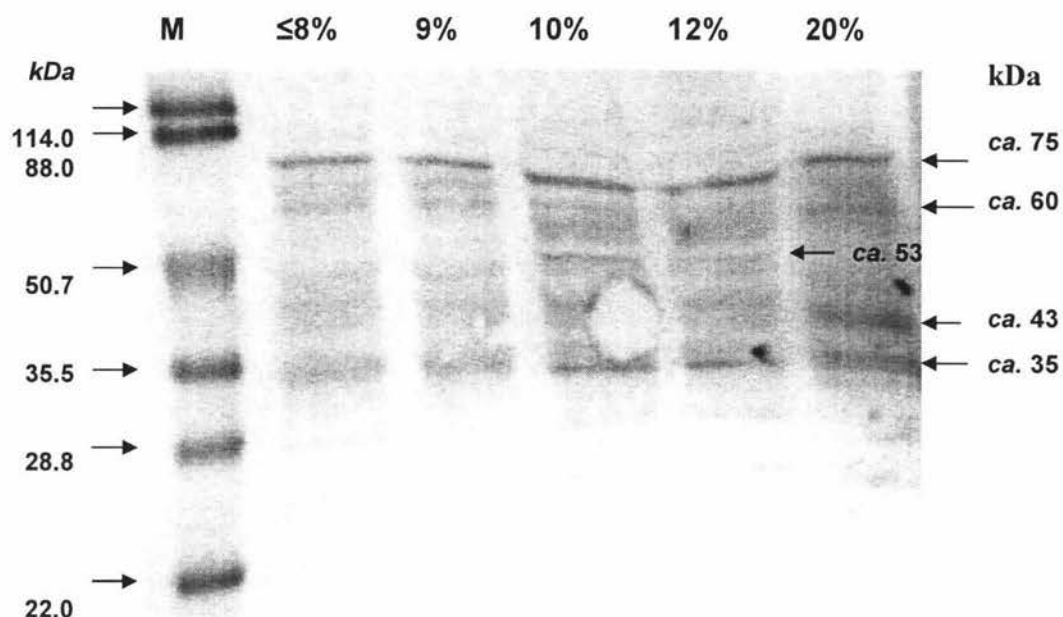
## 4.7 Western Analyses of Partially Purified Leaf Extracts

After a Sephadex G-25 gel filtration step (refer to Section 2.6.2), crude extracts of pooled leaf tissue at SWCs of: 20%, 12%, 10%, 9% and 8% or less, were subjected to Western analysis after separation by SDS-PAGE (Sections 2.9 and 2.10 ).

Immunodevelopment of membranes was carried out using primary antibodies anti-TR-ACO2 (designated rabbit #5) or anti-TR-ACO1 (designated B34) (refer to Section 2.10.2 for a full description of antibody source and preparation). Typically, the amount of total protein loaded into SDS-PAGE wells was less than 5.0  $\mu\text{g}$ . This is very little because protein yields after purification were low in some extracts. Loading was then limited to the maximum volume of extract that the well could physically hold for the extract with the least amount of protein as it was necessary to load equal amounts of protein into wells if the extracts were to be compared.

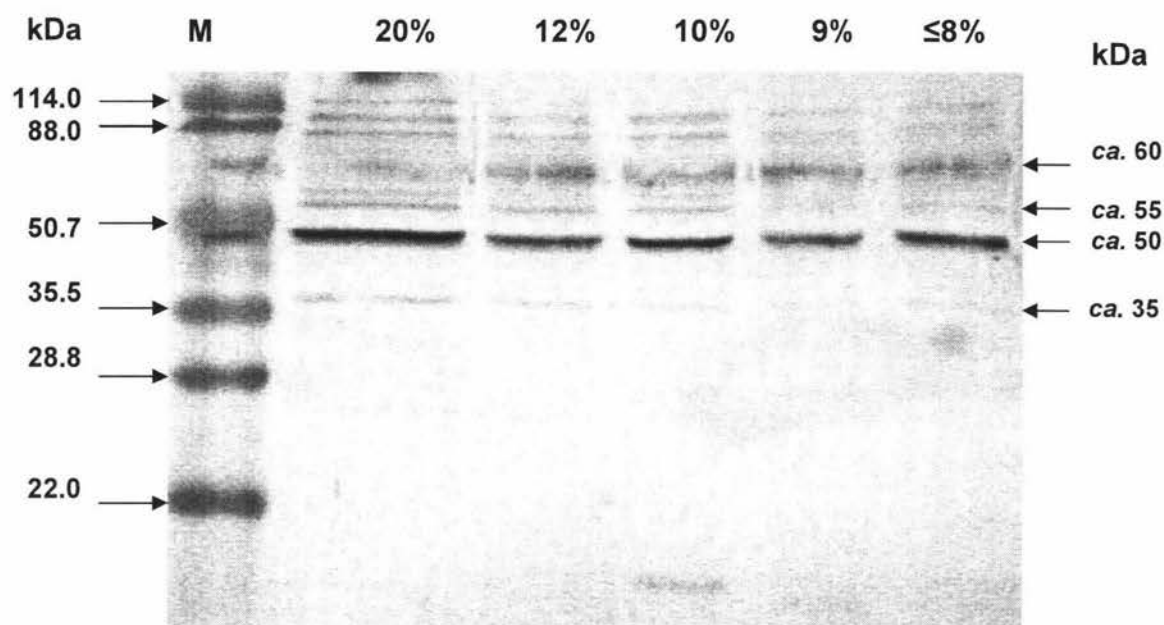
### 4.7.1 Enclosed Tissue

In enclosed leaf tissue extracts of varying soil water content, the anti-TR-ACO1 antibody (B34) (figure 4.15 on the following page), recognised major bands with molecular masses of *ca.* 35 and *ca.* 75 kDa at SWCs of:  $\leq 8\%$ , 9%, 10%, 12% and in water-sufficient tissue at 20% SWC. The 35 kDa band is most intense at SWCs of 12% and 10% and becomes more diffuse with decreasing SWC. There are various smaller bands at *ca.* 28.8 kDa and *ca.* 24.0 kDa in all extracts, as well as the phenomenon of multiple banding between 35 and 75 kDa for example *ca.* 43, 53 and 60 kDa. When a repeat Western was performed on freshly prepared extracts from the same ground tissue (kept frozen at  $-80\text{C}$ ) (refer to figure 4.16 on p. 82), the antibody anti-TR-ACO2 (#5) now recognised major bands of molecular masses *ca.* 50kDa and *ca.* 60 kDa in all extracts. A band of *ca.* 35 kDa is also recognised in all extracts, but is most intense at SWCs of 20%, and decreases in intensity with decreasing SWC. The larger band of *ca.* 75 kDa is now only weakly recognised and is associated with another of molecular mass *ca.* 88 kDa. At the higher SWCs, e.g. 10% SWC, there is a well resolved band at *ca.* 55 kDa. The smaller bands seen in the previous set of extracts are no longer visible, but this could be explained by the reduced protein loading (from 10 to 8  $\mu\text{g}$ ) in this set of extracts.



|  |                |               |               |               |               |
|--|----------------|---------------|---------------|---------------|---------------|
| Mean enzyme specific activity (ppm/mg protein) | 1.26<br>± 0.22 | 2.38<br>±0.48 | 1.58<br>±0.43 | 1.43<br>±0.31 | 1.33<br>±0.28 |
|--|----------------|---------------|---------------|---------------|---------------|

Figure 4.15: Immunoblot of *F. arundinacea* partially purified enclosed leaf tissue extracts at varying SWCs using the anti-TR-ACO1 (B34) antibody. The lower panel represents specific ACO activity as means (n=4) with standard errors (from figure 4.14; upper panel).



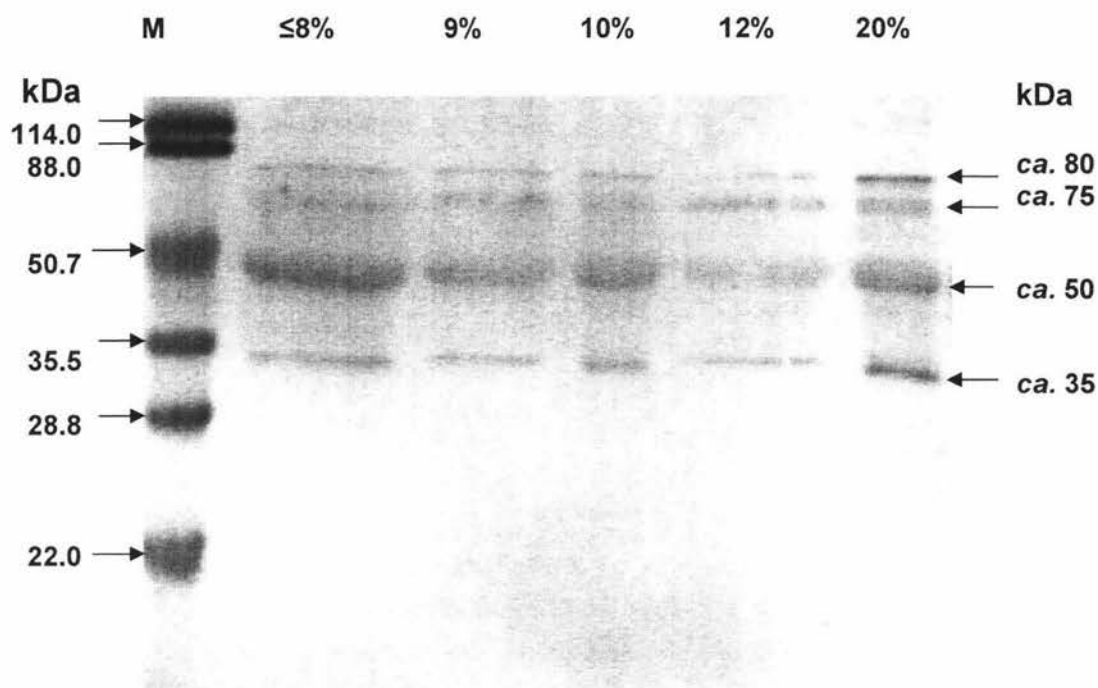
|  |               |               |               |               |               |
|--|---------------|---------------|---------------|---------------|---------------|
| Mean enzyme specific activity (ppm/mg protein) | 1.33<br>±0.28 | 1.43<br>±0.31 | 1.58<br>±0.43 | 2.40<br>±0.48 | 1.26<br>±0.22 |
|--|---------------|---------------|---------------|---------------|---------------|

Figure 4.16: Immunoblot of partially purified *F. arundinacea* enclosed leaf tissue extracts at varying SWCs using the anti-TR-ACO2 antibody (#5). The lower panel represents specific ACO activity as means (n=4) with standard errors (from figure 4.14; upper panel).

#### 4.7.2 Exposed Tissue

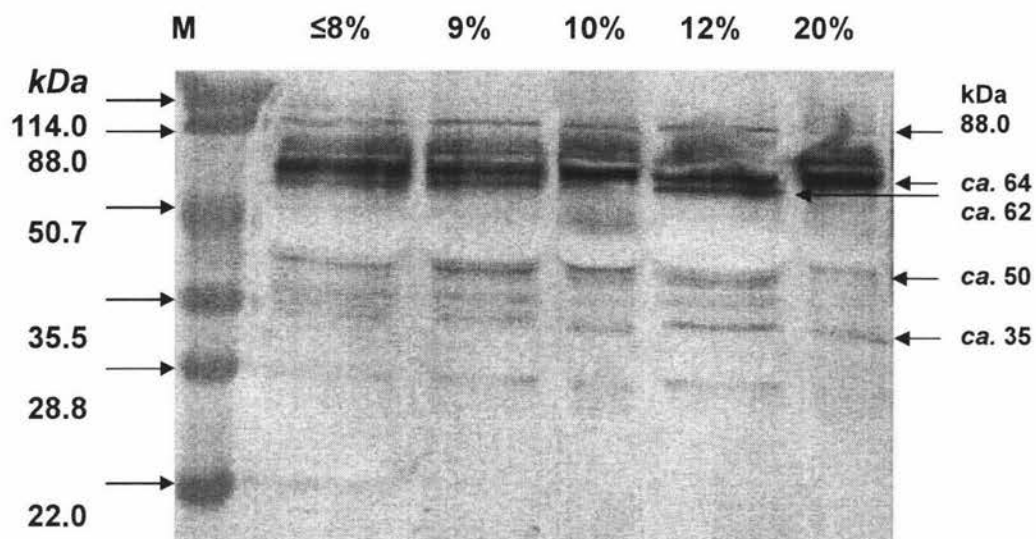
When the exposed leaf tissue partially purified extracts were challenged with the anti-TR-ACO1 antibody (B34) (figure 4.17 on the following page), the antibody recognised a distinct band of molecular mass *ca.* 35.0 kDa in all extracts, i.e. at SWCs of  $\leq 8\%$ , 9%, 10%, 12% and 20%. Recognition is highest in tissue at 20% SWC. A pair of bands of *ca.* 75 and 80 kDa was also recognised in all extracts and is especially clear in fully hydrated tissue (SWC of 20%). In addition, a protein product of *ca.* 50 kDa appears as a diffuse band in all extracts.

When the same extracts were challenged with the anti-TR-ACO2 antibody (#5), the antibody recognised several bands of molecular masses from 28.8 to 50.0 kDa (figure 4.18; p. 85). Of these, there are two distinct protein products at *ca.* 35.0 kDa and *ca.* 50 kDa, in all extracts i.e. at SWCs of  $\leq 8\%$ , 9%, 10%, 12% and 20%. The 35.0 kDa protein is present in greater quantities in tissue at the higher SWCs. A smaller band of *ca.* 28.8 kDa is present at SWCs of  $\leq 8\%$ , 9%, 10% and 12%, but absent from tissue at 20%SWC. In repeat Westerns, after one freeze-thaw cycle (extracts stored at  $-20\text{C}$ ), these smaller bands were no longer visible, but this could be explained by reduced protein loading in this repeated Western analysis. The antibody also recognised major bands of between 60.0 and 100.0 kDa in all extracts, with more clearly defined bands at *ca.* 62, 64 and 88 kDa.



|  |               |               |               |               |               |
|--|---------------|---------------|---------------|---------------|---------------|
| Mean enzyme specific activity (ppm/mg protein) | 0.39<br>±0.08 | 0.56<br>±0.19 | 0.86<br>±0.44 | 0.60<br>±0.15 | 0.47<br>±0.13 |
|--|---------------|---------------|---------------|---------------|---------------|

Figure 4.17 Immunoblot of *F. arundinacea* exposed leaf tissue partially purified extracts at varying SWCs using the anti-TR-ACO1 antibody (B34). The lower panel represents specific ACO activity as means ( $n=4$ ) with standard errors (from figure 4.14; lower panel).



|                   |            |            |            |            |            |
|-------------------|------------|------------|------------|------------|------------|
| Mean enzyme       | 0.40       | 0.56       | 0.86       | 0.60       | 0.47       |
| specific activity | $\pm 0.08$ | $\pm 0.19$ | $\pm 0.44$ | $\pm 0.15$ | $\pm 0.13$ |
| (ppm/mg protein)  |            |            |            |            |            |

Figure 4.18: Immunoblot for *F. arundinacea* exposed leaf tissue partially purified extracts at varying SWCs using anti-TR-ACO2 antibody (#5). The lower panel represents specific ACO activity as means ( $n=4$ ) with standard errors (from figure 4.14; lower panel).

## Summary of Results:

**Table 4 Molecular masses of the major bands recognised by the two antibodies, as indicated ,in partially purified *F. arundinacea* leaf extracts for the two tissue types:**

| <b>Antibody:</b>       | <b>TR-ACO1(B34)</b>                               | <b>TR-ACO2 (#5)</b>                                |
|------------------------|---|--|
| <b>Enclosed tissue</b> | ca. 75.0<br>ca. 60<br>ca.53<br>ca. 43<br>ca. 35.0 | ca. 80-100<br>ca. 60<br>ca. 55<br>ca. 50<br>ca. 35 |
| <b>Exposed tissue</b>  | ca. 80<br>ca. 75<br>ca. 50<br>ca. 35              | ca. 88.0<br>ca. 64, 62 duplex<br>ca. 50<br>ca. 35  |

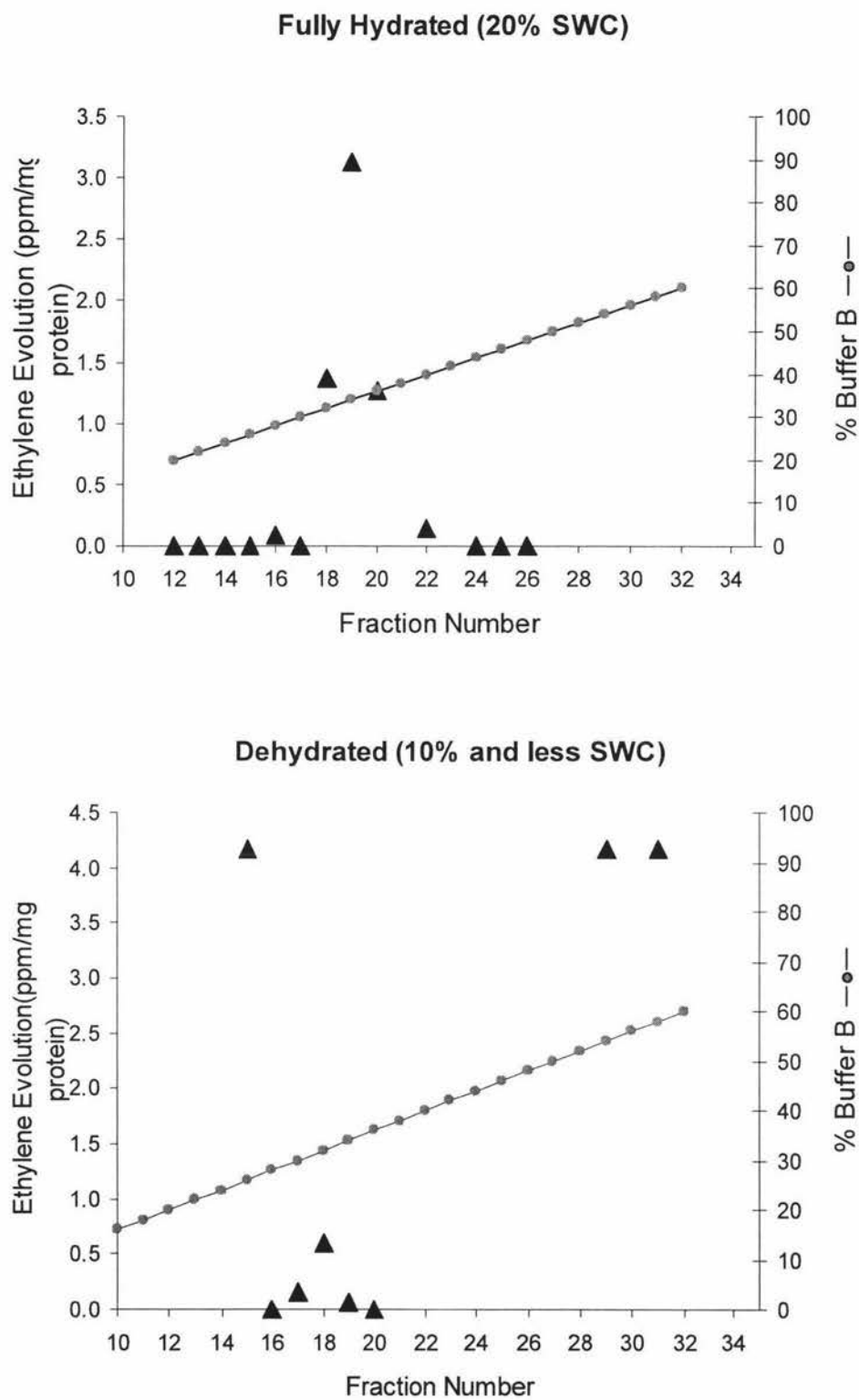
## 4.8 ACO Enzyme Activity Measurements of Leaf Extracts After Further Purification

From the specific activity data for partially purified leaf extracts (figure 4.14; p. 79), i.e. extracts at  $\leq 8\%$  and at 10%SWC for enclosed (but not 9% because of insufficient plant material), and extracts at 10% SWC for exposed tissue, were selected for further purification by ion-exchange chromatography (Mono-Q HR5/5 column) using a fast protein liquid chromatography system (FPLC). These extracts were representative of leaf tissue in states of dehydration. For comparison, leaf tissue in a fully hydrated state, i.e. at SWC of 20%, for enclosed and exposed alike, were also partially purified using the same procedure (Sections 2.6.3 and 2.6.4). After partial purification, assays for enzyme activity were performed on all resulting protein-containing fractions (fractions #8 to #32), as determined by the BioRad assay (Method 2.8.1). Assays were single point measurements i.e.  $n=1$ . The proteins eluted differentially with an increasing salt concentration from 0% buffer B (with no NaCl) to 100% buffer B (with 1M NaCl). In these separations, ACC oxidase eluted between 20 to 50% buffer B, i.e. at salt concentrations of 200-500mM NaCl.

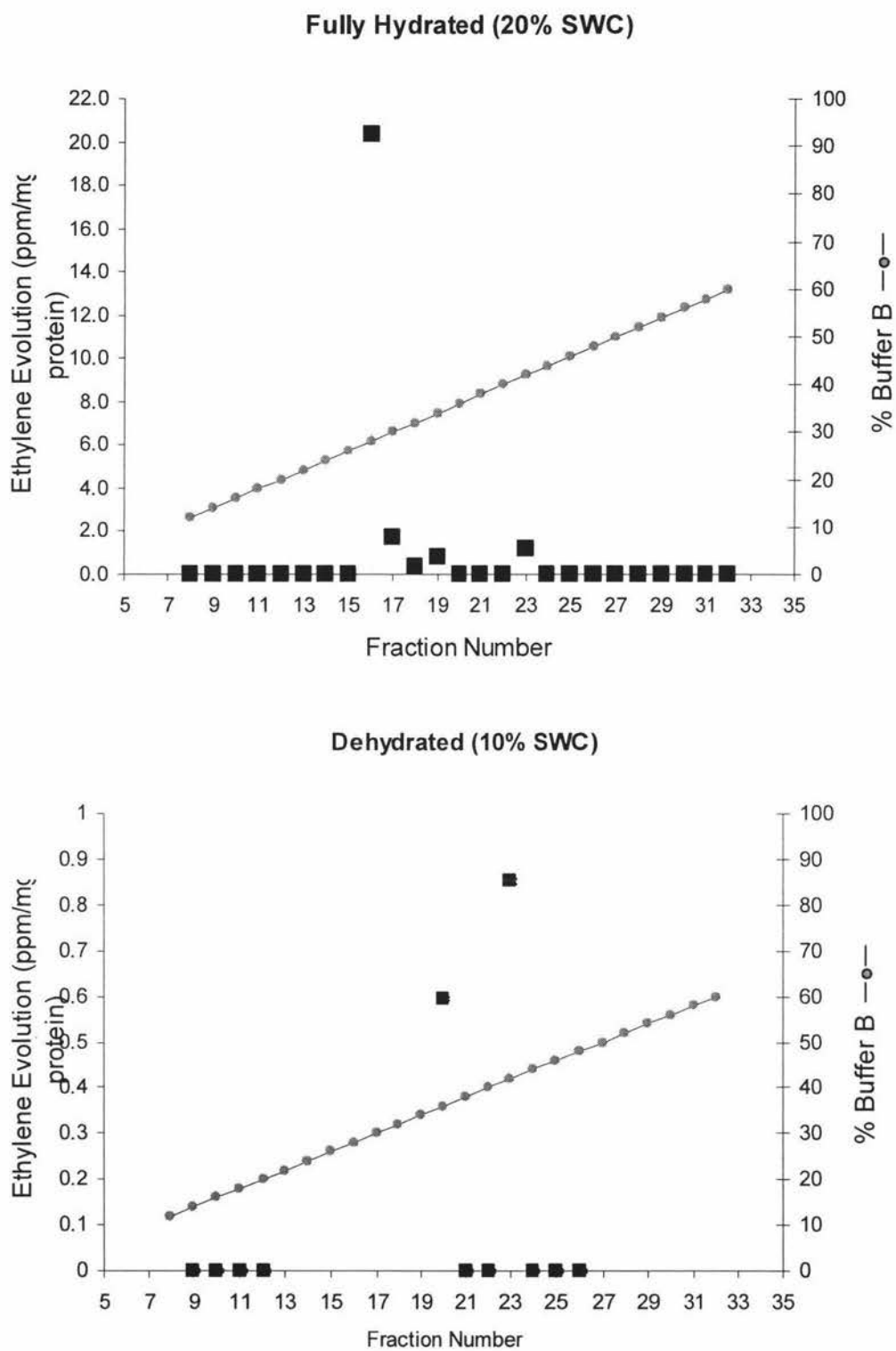
To further identify any ACO isoforms associated with a water-deficit in *F. arundinacea*, ACO-containing leaf extracts from tissue at SWCs of 10% and less for enclosed tissue and at 10% SWC for exposed tissue, were prepared for comparison with water-sufficient tissues (for both tissue types). To confirm whether different isoforms of ACO may be responsible for the induction of ethylene in leaf tissue at different developmental stages, fractionated proteins were then assayed for enzyme activity. Fractions corresponding to activity peaks were subjected to immunoblot techniques using the polyclonal antibodies TR-ACO1 and TR-ACO2.

For enclosed tissue, strong activity peaks (ca. 3.0 ppm/mg protein) coincided with fraction #19 (340 mM NaCl) for fully hydrated tissue, and fractions #15 (260 mM NaCl), #18 (320 mM NaCl), #29 (540 mM NaCl), and #31 (590 mM NaCl) for dehydrated tissue (figure 4.19 upper and lower graphs respectively; p.90). In the dehydrated tissue sample, the peak corresponding to fraction #18 is small (ca. 0.5 ppm/mg protein) in comparison with other peaks (all at ca. 4.0 ppm/mg protein), yet is well resolved.

For exposed tissue (figure 4.20; p. 90), peaks in activity coincided with fraction #16 (280 mM NaCl) for fully hydrated tissue and fractions #20 and #23 (360 and 420 mM NaCl respectively) for dehydrated tissue. At this point in the experiment, further analysis was needed to determine which activity peaks were authentic. Because the measured enzyme activities were single point measurements ( $n=1$ ), it is not possible to include standard errors or even standard deviations for this data set. However, the average standard deviations from the previous data sets in figure 4.14 (p. 79) were considered sufficient for determining the level of accuracy of this data set, as they represent the amount of variation within the activity data. These are given as the errors here.



**Figure 4.19** Specific ACO activity by fraction for Mono Q-purified enclosed leaf tissue at the two states of leaf hydration (n=1).



**Figure 4.20** Specific enzyme activity for Mono Q-purified exposed leaf tissue at the two states of leaf hydration (n=1).

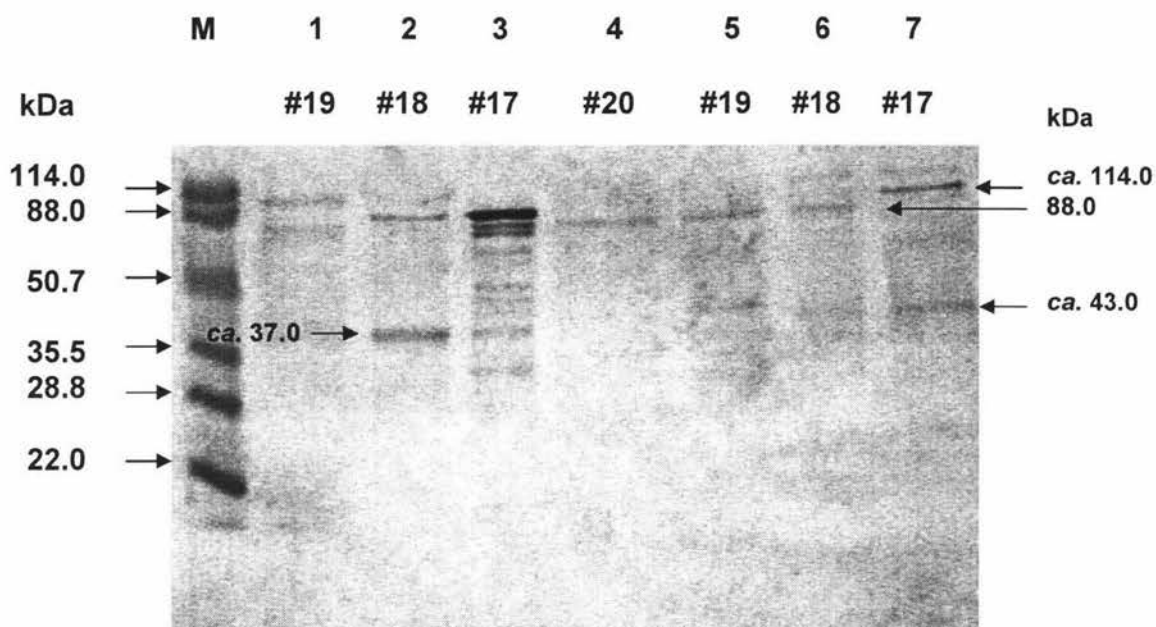
## **4.9 Western Analysis of Leaf Extracts After Purification Using Mono Q Ion-Exchange Column Chromatography**

From enzyme specific activity plots for leaf tissue extracts after purification using Mono Q ion-exchange column chromatography (figures 4.19 and 4.20; pp. 89 and 90 resp.), fractions corresponding to peaks of activity were subjected to Western analysis. Two different ACO-specific primary antibodies were used: a new anti-TR-ACO2 antibody (designated G52) and anti-TR-ACO1 (designated B34). For a full description of antibody source and preparation, refer to Section 2.10.2.

### **4.9.1 Fractions (#17, 18, 19 and 20) Identified in Enclosed Tissue**

Fractions #17, 18 and 19 from dehydrated (10% or less SWC) and for comparison, fractions #17, 18, 19 and 20 from fully hydrated tissue, were subjected to Western analysis (figure 4.21; on the following page). The same amount of protein (3.2 µg) was loaded into each well. The anti-TR-ACO2 antibody, recognised bands of high molecular mass between ca. 80.0 to 100.0 kDa, in all extracts. A band of ca. 88.0 kDa is clearly visible in fractions #17 and #18, but recognition is highest in fraction #17 of dehydrated tissue (lane 3). The equivalent #17 fraction for water-sufficient tissue (lane 7) has a slightly larger band of nearer ca. 114.0 kDa. None of the multiple bands between ca. 30.0 and 90.0 kDa, i.e. a ladder effect, in the dehydrated #17 are present in the fully hydrated fraction.

A novel band of approximately 37.0 kDa is present in lanes 2 and 3 (fractions #17 and #18 of dehydrated tissue) and this band is most clearly seen in lane 2 (#18). This same protein product is absent from all fully hydrated extracts. A slightly larger diffuse band of ca. 43.0 kDa however, is present in fractions #17, #18 and #19 of fully hydrated tissue (lanes 5, 6 and 7 respectively).

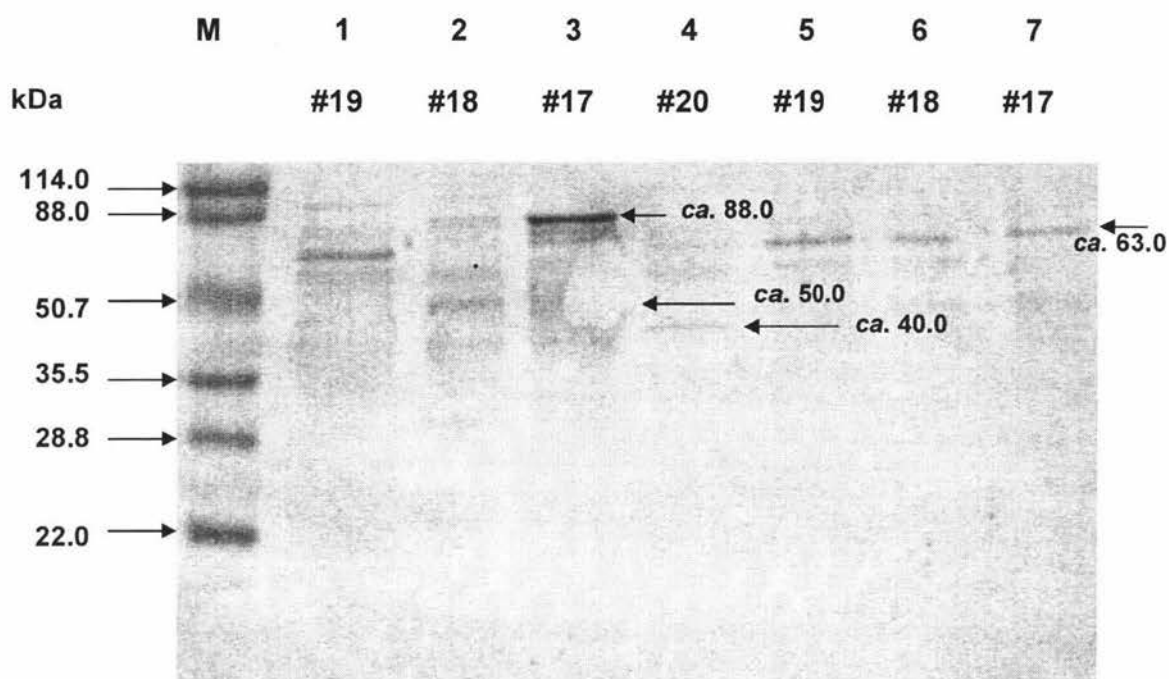


|                  |            |            |            |            |            |            |            |
|------------------|------------|------------|------------|------------|------------|------------|------------|
| Enzyme activity  | 0.06       | 0.60       | 0.16       | 1.27       | 3.12       | 1.36       | 0.0        |
| (ppm/mg protein) | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ |

**Figure 4.21: Immunoblot of *F. arundinacea* Mono Q-purified dehydrated and fully hydrated enclosed leaf tissue fractions, as indicated, using the anti-TR-ACO2 antibody. The lower panel represents specific ACO activity as single points (n=1, from figure 4.19) with the estimated average standard deviation (determined from the data set in figure 4.14, upper panel).**

Lane M: Pre-stained molecular mass markers (molecular masses are indicated); Lanes 1-3: Dehydrated tissue, fractions #19, #18, and #17 respectively from left to right respectively; Lanes 4-7: Fully hydrated tissue, fractions #20, #19, #18, #17 respectively from left to right respectively.

The same prepared extracts were also challenged with anti-TR-ACO1 antibody (figure 4.22, on the following page). The same amount of protein (3.2 $\mu$ g) was again loaded into each well. The antibody recognised an intense band of *ca.* 88.0 kDa in fractions #18 and #17 (lanes 2 and 3 respectively) but not #19 (lane 1) of dehydrated tissue. This band is most intense in lane 3. A band of this size is absent from all fully hydrated extracts, and is a similar result to that using the anti-TR-ACO2 antibody. Again, there is a protein ladder effect in fraction #17 of dehydrated but not fully hydrated tissue. A slightly smaller band of *ca.* 63.0 kDa is present in three of the four fully hydrated extracts, i.e. fractions # 17, # 18 and # 19 in lanes 5, 6 and 7. A band of this approximate size is also clearly visible in fraction #19 of dehydrated tissue (lane 1), but not in other dehydrated extracts. Other minor bands of *ca.* 50.0 kDa are recognised in lanes 2 and 3 and another of *ca.* 40.0 kDa in lane 4 (fraction #20 of fully hydrated tissue).



|                  |            |            |            |            |            |            |            |
|------------------|------------|------------|------------|------------|------------|------------|------------|
| Enzyme activity  | 0.06       | 0.60       | 0.16       | 1.27       | 3.12       | 1.36       | 0.0        |
| (ppm/mg protein) | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ | $\pm 0.54$ |

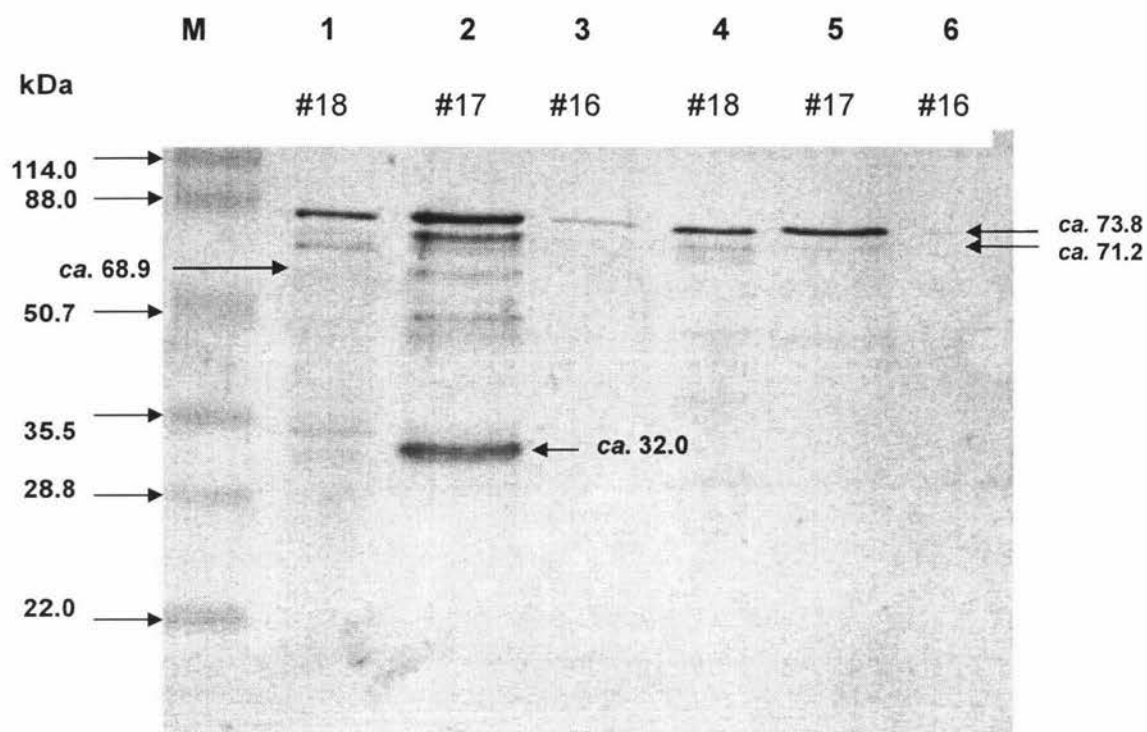
Figure 4.22: Immunoblot of *F. arundinacea* Mono Q-purified dehydrated and fully hydrated enclosed leaf tissue fractions as indicated using the anti-TR-ACO1 antibody. The lower panel represents specific ACO activities as single points ( $n=1$ , from figure 4.19) with the estimated average standard deviation (from the data set in figure 4.14, upper panel).

Lane M: Pre-stained molecular mass markers (molecular masses are indicated); Lanes 1-3: Dehydrated tissue, fractions #19, #18 and #17 from left to right respectively; Lanes 4-7: Fully hydrated tissue; fractions #20, #19, #18 and #17 from left to right respectively.

#### 4.9.2 Fractions #16, #17 and #18 Identified in Exposed Tissue

Fractions # 16, 17 and 18 of dehydrated and fully hydrated partially purified exposed leaf tissue extracts were subjected to immunodevelopment for comparison, using the anti-TR-ACO2 antibody (figure 4.23, the following page). The antibody recognised a band of ca. 73.8 kDa in all extracts, i.e. fractions #16, #17 and #18 for both dehydrated and fully hydrated tissue. A band of ca. 68.9 kDa is also recognised in lane 1 (#18 dehydrated) and another of ca. 71.2 kDa in lane 2 (#17 dehydrated). The amount of protein loaded into wells varied, i.e. four times the amount of protein (2.1µg) for fractions #17 and #18 was loaded than for fraction #16 (0.5µg). Hence, any comparison of band intensity is not valid here.

For fraction #17 of the dehydrated tissue (lane 2), a novel band of good intensity, and with a molecular mass of ca.32.0 kDa, is clearly visible. This band is of the expected size for an ACO protein product. This same band is completely absent from fully hydrated extracts. The phenomenon of multiple bands (ladder) in the medium to high molecular mass range from ca. 50.0 to 88.0 kDa, appears in lane 2 (dehydrated extract #17) but is absent from the equivalent fraction in fully hydrated tissue.



|                                  |    |    |    |               |               |                |
|----------------------------------|----|----|----|---------------|---------------|----------------|
| Enzyme activity (ppm/mg protein) | ND | ND | ND | 0.35<br>±0.20 | 1.72<br>±0.20 | 20.31<br>±0.20 |
|----------------------------------|----|----|----|---------------|---------------|----------------|

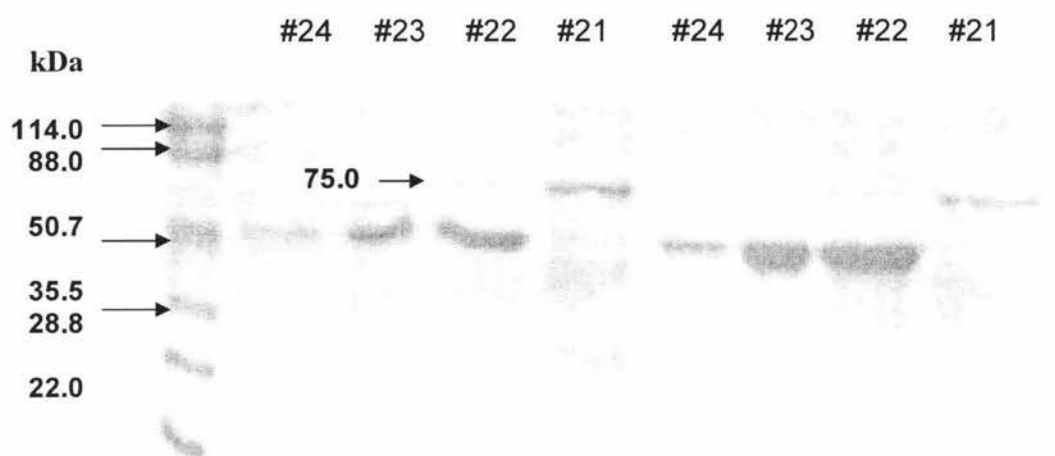
Figure 4.23: Immunoblot of *F. arundinacea* Mono Q-purified dehydrated and fully hydrated exposed leaf tissue fractions, as indicated, using the anti-TR-ACO2 antibody. The lower panel represents specific ACO activities as single points (from figure 4.19, n=1) with the estimated average standard deviation (from the data set figure 4.14, lower panel). ND is not determined.

Lane M: pre-stained molecular mass markers (molecular masses are indicated); Lane 1-3: Dehydrated tissue, fractions #18, #17, #16 from left to right respectively; Lanes 4-6: Fully hydrated tissue, fractions #18, #17, #16 from left to right respectively.

### 4.9.3 Fractions #21 to 24 Identified in Exposed Tissue

When fractions #21, #22, #23 and #24 of dehydrated and fully hydrated partially purified exposed leaf tissue extracts were subjected to immunodevelopment for comparison, using the anti-TR-ACO2 antibody (figure 4.24, the following page), the antibody recognised a (clearly resolved) band of ca. 50.7 kDa in fractions #22, #23 and #24 of both dehydrated and fully hydrated tissue. In fraction #21 the band is more diffuse. This protein product is present in greatest amounts in fully hydrated fractions #22 and #23 (lanes 7 and 6 respectively) and at much reduced amounts in fractions #24 (lane 5) especially in the dehydrated tissue extracts (lane 1). A major recognition band of ca. 75.0 kDa is present in fractions #21 and #22 (lanes 3, 4, 7 and 8) for tissue in both states of hydration, although band intensity is much greater in dehydrated tissue.

Fractions #21, #22, #23 and #24 of dehydrated and fully hydrated separations were also challenged with the anti-TR-ACO1 antibody (Figure 4.25; p. 99). The same amount of protein was loaded into each well (2.3 µg). The anti-TR-ACO1 antibody recognised a band of ca. 64.1 kDa in dehydrated tissue fractions: #24, #23 and #21 (lanes 1, 2 and 4 respectively), but not in fraction #22 (lane 3). Recognition is highest in fraction #24. This same band is absent from fully hydrated tissue extracts. A band of ca. 50.7 kDa is present in all extracts, i.e. in tissue at both states of hydration, but is especially intense in fractions # 22 and #23. This protein is present in larger amounts in the fully hydrated extracts compared with the dehydrated extracts.



|  |     |                    |     |     |     |                    |     |     |
|--|-----|--------------------|-----|-----|-----|--------------------|-----|-----|
| Enzyme activity<br>(ppm/mg<br>protein) | 0.0 | 0.85<br>$\pm 0.20$ | 0.0 | 0.0 | 0.0 | 1.21<br>$\pm 0.20$ | 0.0 | 0.0 |
|--|-----|--------------------|-----|-----|-----|--------------------|-----|-----|

**Figure 4.24:** Immunoblot of *F. arundinacea* Mono Q-purified dehydrated and fully hydrated exposed leaf tissue fractions, as indicated, using the anti-TR-ACO2 antibody. The lower panel represents specific ACO activity as single points ( $n=1$ , from figure 4.20) with the estimated average standard deviation (from the data set in figure 4.14, lower panel).

Lane M: pre-stained molecular mass markers (molecular masses are indicated); Lanes 1-4: dehydrated tissue, fractions #24, #23, #22, #21 from left to right respectively; Lanes 5-8: fully hydrated tissue, fractions #24, #23, #22, #21 from left to right respectively.

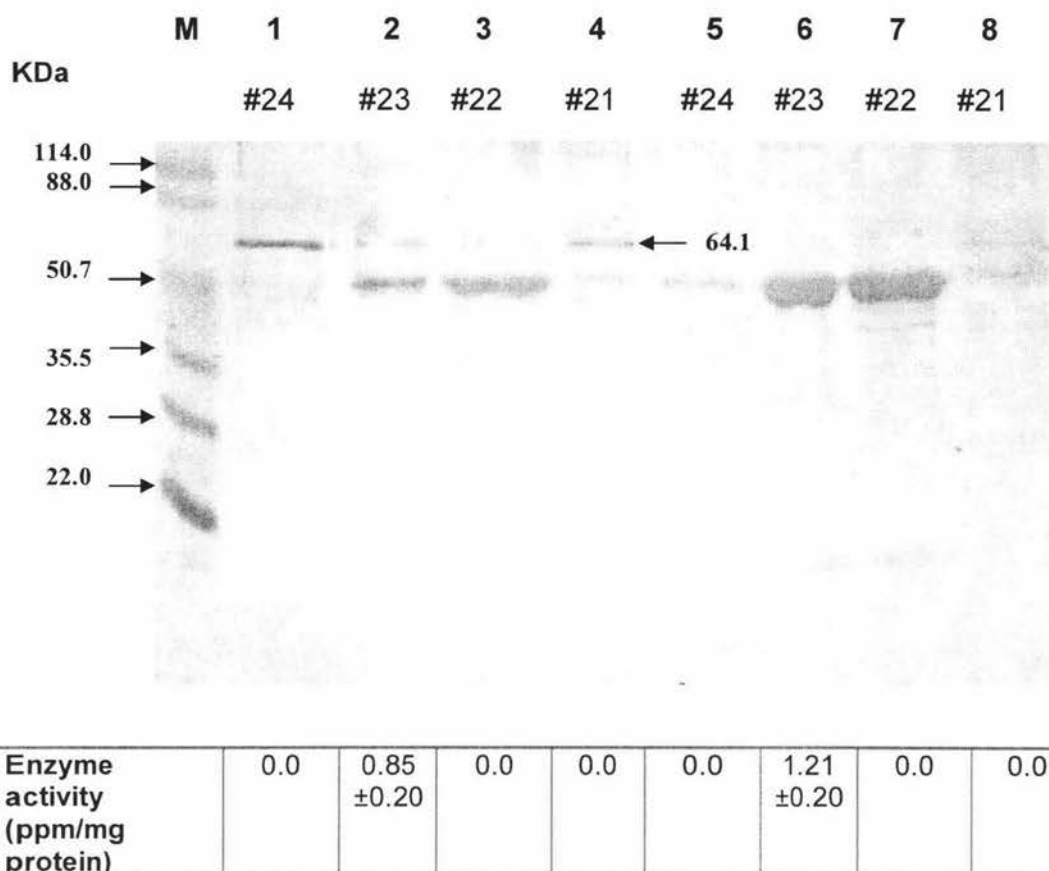


Figure 4.25: Immunoblot of *F. arundinacea* Mono Q-purified dehydrated and fully hydrated exposed leaf tissue fractions, as indicated, using the anti-TR-ACO1 antibody (B34). The lower panel represents specific ACO activity as single points ( $n=1$ , from figure 4.20) with the estimated average standard deviation (from the data set in figure 4.14, lower panel).

Lane (M) are pre-stained molecular mass markers (molecular masses are indicated); Lanes 1-4: dehydrated tissue, fractions #24, #23, #22, and #21 from left to right respectively; Lanes 5-8: fully hydrated tissue, fractions #24, #23, #22, and #21 from left to right respectively.

## Summary of Results:

For the enclosed leaf tissue, activity eluted as single peaks at salt concentrations of 340 and 320 mM NaCl for fully hydrated and dehydrated tissue respectively. In the exposed leaf tissue, ACO activity eluted at salt concentrations of 280 mM and 420 mM NaCl in fully hydrated tissue, and 360 and 420 mM NaCl in dehydrated tissue.

**Table 5 molecular masses of the major bands recognised by the antibodies in the dehydrated Mono Q-purified extracts of the two tissue types:**

| Enclosed       | TR-ACO1  | TR-ACO2  |
|----------------|----------|----------|
| Dehydrated     | ca. 63.0 | ca. 37.0 |
|                | ca. 88.0 | ca. 88.0 |
| Fully hydrated | ca. 40.0 | ca. 43.0 |
|                | ca. 63.0 | ca. 88.0 |

| Exposed        | TR-ACO1  | TR-ACO2  |
|----------------|----------|----------|
| Dehydrated     | ca. 64.1 | ca. 73.8 |
|                | ca. 50.7 | ca. 50.0 |
|                |          | ca. 32.0 |
| Fully hydrated | ca. 75.0 | ca. 73.8 |

## Chapter Six: Discussion

### Primary Research Focus

The primary aim of this thesis is to examine changes in the ethylene biosynthetic pathway in response to a water deficit in *F. novae-zelandiae* and in *F. arundinacea*, and to look at the role of ACO in the context of this process. Previous water-deficit studies in these species (Abernethy, 1996; West *et al.*, 1990; Barlow, 1986) have focused on the physiological (leaf elongation rates, leaf water potentials and relative water content) and biochemical (metabolite accumulation) responses to a water deficit. This study then, serves to confirm some of these measurements and to extend such studies by observing changes in ethylene evolution, ACO activity and ACO protein accumulation in leaves in response to a water deficit. A water deficit is defined as a decrease in SWC such that some changes in physiological status are observed. In this thesis, LER was used in addition to the accumulation of the osmoprotectant proline.

The study compares the response of *F. arundinacea*, an improved pasture species with a mesophytic leaf anatomy/morphology with *F. novae-zelandiae*, a species with a defined xeromorphic anatomy. Important differences in the physiological response to dry-down in the two species were observed.

### Physiological Responses to a Water-Deficit in *Festuca novae-zelandiae* and *F. arundinacea*

A water-deficit was induced in 50% of the pots in Experiments I, and II alike, by withholding water. In control pots, soil-water was maintained at or near 70% of the container capacity (CC). CC is a measure of the mean water content of soil in a container. From the dry-down curves (figures 3.1 and 4.1), soil-water decreased over time in the dry-down pots for both species from around 30% SWC at the start of each experiment to mean soil water contents of around 9%.

Analyses of measurements for soil-water and plant-water status confirm a clear correlation between soil water content (SWC) and leaf elongation rate (LER) in both species (figures 3.5 and 4.5). That there is a significant decline in the rate of leaf cell elongation with increasingly limited water shows the LER to be a useful indicator of a water deficit in plants. Differences are significant at the 5% level in both species. Using

SWCs to describe physiological and biochemical changes can be simplistic and misleading since SWC is a soil-water measurement and not a plant-water determinant. LER is the preferred measure of plant-water because it is non-destructive and is a simple and straightforward technique. Non-destructive sampling was especially important in *F. novae-zelandiae* because of limited leaf material. The plant-water measurements overcome any variation in the soil dry-down characteristics due to differences in the volume or compaction of the soil in the different pots. Essentially the LER is a measure of cell elongation, and as such is extremely sensitive to changes in the leaf water potential since limiting leaf area by reduced cell expansion is often the first response by plants to water-deficit stress/drought stress. Cell elongation ceased at critical soil water contents of around 10% SWC in *F. novae-zelandiae* and 9% SWC in *F. arundinacea*. In the water-sufficient pots, the leaf elongation remained relatively unchanged throughout the experiments. The physiological response of the plants to the dry-down was species-specific and may suggest different drought survival mechanisms after a more prolonged water deficit.

In *F. novae-zelandiae*, there is an early and rapid response to limited water. This is consistent with previous studies by Connor (1960) and Abernethy and Fountain (1998), in which *F. novae-zelandiae* is reported to have morphological adaptations typical of a xerophytic plant species. For example, rolled leaves that form a tight cylinder enclosing a void which is densely packed with trichomes, a continuous leaf margin gap whose aperture is controlled by the turgidity of bulliform cells located between the vascular ribs, and stomata on the adaxial (inside) surface of the leaf only (Abernethy *et al.*, 1998). With obvious higher transpiration rates, as indicated by severe wilting in the glasshouse at the level of water deficit imposition, and with a mesophytic leaf anatomy. *F. arundinacea* has limited ability to conserve water and this constitutes a reduced ability to perceive the threat of drought.

### **Proline Accumulation in Response to a Water-Deficit in *F. novae-zelandiae* and *F. arundinacea***

The metabolite proline accumulated differentially in the two leaf tissue types of both species as dry-down progressed. In the enclosed leaf tissue of *F. novae-zelandiae*, increases were near exponential at SWCs of 20% and less (or mean LER of 4.5 mm/day). In the exposed leaf tissue, proline content was similar to control tissue. In contrast, Abernethy (1996) reported that the onset of a differential accumulation of

proline and ABA in immature and mature leaf tissues of *F.novae-zelandiae* in response to a water-deficit coincided with critical soil water contents of less than 10% SWC.

In *F.arundinacea* leaves, proline became significantly different from the controls at mean SWCs of 12% for enclosed (or a mean LER of  $ca. 4.0 \pm mm/d$ ) and 10% for exposed leaf tissue (a mean LER of  $ca.1.0 \pm mm/d$ ). Accumulation was logarithmic, with the degree of accuracy of prediction (by the regression model) being greatest in the enclosed tissue (an  $R^2$  value of 0.64) when compared with the exposed tissue (an  $R^2$  value of 0.45). Significant increases in proline accumulation occurred earlier in the immature leaf tissue when compared with the mature green leaf zones (exposed tissue). In *F. arundinacea*, Abernethy (1996) reported a "trigger point" of  $ca. 8\%$  SWC that marked the onset of metabolic changes within leaves, e.g. the accumulation of proline, ABA and glycine-betaine, and reductions in the soluble protein content of leaves. Again, in this thesis the onset of accumulation in *F. arundinaecea* occurred earlier in the dry-down (at greater SWCs) than in Abernethy's study (1996). As a cautionary note however, the use of SWC can be simplistic since SWC is a soil-water measurement and not a plant-water determinant. These differences in dry-down characteristics could be due to the smaller pot size used here of 4 L compared to the 24 L pots used by Abernethy (1996).

Nevertheless, increases in proline were 50 to 100-fold in *F.novae-zelandiae* and 10 to 30-fold in *F.arundinacea* (than control plants). In comparison, in a similar dry-down experiment, Abernethy (1996) reported a 40-fold increase in the free proline content for intact *F.arundinacea* leaves. In detached leaves, i.e.bench top dry-down, of *F.novae-zelandiae*, two and three fold increases were recorded in leaf bases and immature pseudostems respectively (together these regions make up the enclosed leaf tissue), and 22-fold increases in the immature leaf laminae (representing exposed leaf tissue) (Abernethy, 1996). Since benchtop dry-down may resemble drying shock treatment and not slow drying (Page et al., 1990), perhaps disagreement is to be expected. However this may also be explained by the smaller pot system (4L) used in this thesis when compared with Abernethy (1996). In Bermuda grass (*Cynodon dactylon* L.) shoots under water stress, Barnett and Naylor (1966) reported increases in proline content of 10 to 100-fold which are similar to those reported in this thesis.

In water-sufficient tissue (as represented by the control tissues and dry-down tissue at day 0), the proline content in the enclosed (meristematic and elongating) leaf tissue was elevated approximately three to four times compared to that in the mature leaf

tissue, i.e.  $0.30 \pm 0.05$  mg/gFW and  $0.09 \pm 0.05$  mg/gFW respectively. This phenomenon was also observed by Abernethy, (1996) in leaf bases of *F.novae-zelandiae* when compared with laminae, i.e. 5-8 mg/gDW and 2 mg/gDW, respectively. Note that Abernethy's figures are calculated on a dry weight basis but the ratio of proline in the leaf base to proline in the laminae tissue is similar, at approximately three to four times.

The accumulation of proline to very high levels in plant tissues has been shown to correlate with tolerance to drought (Kavi Kishor *et al.*, 1995). It has been suggested that the differential accumulation of metabolites, for example proline, ABA and glycine-betaine (in selected species only), suggests preferential protection of growing tissues in response to a water deficit (Maxwell and Redmann, 1978) (see later in Discussion).

### **Summary of Dry-down Responses**

In these experiments, the interpolated data for SWC, LER and proline accumulation agree well with the raw data, as indicated by high  $R^2$  values (greater than 0.89) describing the regression equations for predicted values and this is an acceptable level of confidence.

The two species showed different physiological responses to the dry-down as indicated by different dry-down curves (SWC plotted against LER). In *F.novae-zelandiae*, reductions in leaf elongation rates were linear with decreasing % soil water content, while in *F.arundinacea* leaf elongation rates followed a logarithmic decrease.

### **The Induction of Ethylene with an Ensuing Water-Deficit**

In this study, ethylene was found to be evolved differentially in leaf tissue of *F.arundinacea* at different developmental stages, in response to a water-deficit. As water became limiting for growth, ethylene production increased in the mature-green leaf tissue. Increases were rapid, and a peak in ethylene production occurred early in the dry-down at mean leaf elongation rates of  $ca. 16.0 \pm 1.7$  mm/day. In the elongating regions of leaves that included the meristematic tissue, ethylene production remained at levels similar to those in water-sufficient tissue until more than half-way through the dry-down period, with significant decreases occurring late in the dry-down at mean LERs of  $ca. 6.0 \pm 1.0$  mm/day. This point in the dry-down corresponded to zero

ethylene evolution in the immature leaf tissue, and this occurred earlier than for the mature leaf tissue. In the mature green leaf regions, zero ethylene evolution occurred after cell elongation had already ceased and this could represent cell death, since stress ethylene is associated with living tissue and severe stress results in cell death and cessation of ethylene production (Yang and Hoffman, 1984). Chlorophyll measurements however, would be necessary to confirm this. The loss of pigmentation (yellowing) is one of the first symptoms of senescence in leaves.

In *F. arundinacea*, ethylene evolution in the mature green leaf regions appeared to follow changes in the leaf elongation rate. For example, rapid decline in the LER of water-stressed leaves (at ca. day 2 in two of the dry-down plants or day 5 in another two of the dry-down plants) was followed by a burst of ethylene two days later (at ca. day 4 or day 7 respectively). Voisin *et al.* (2006) reported no role for ethylene in controlling the leaf elongation response to soil water deficit in mature leaves (leaves 4 and 5) of maize plants experiencing moderate water deficit, with no change in the LER in plants experiencing either high (>100 pmol/gFW/h) or low (<100 pmol/gFW/h) ethylene levels under these conditions. And in the case of more severe water deficit, Voisin *et al.* (2006) reported that high levels of ethylene were no longer observed. In a study of wheat grains, Yang *et al.* (2006) reported that higher filling rates were associated with lower concentrations of ethylene and ACC. For example, ethylene evolution rates were higher in inferior grains i.e those that fill late and are small, than in superior grains (those that fill earlier and reach a greater size) under all (well watered, moderate and severe) soil drying treatments.

In this thesis, in the immature leaf regions no significant differences in ethylene evolution between the water-deficit and the control leaves were observed until late in the dry-down. This could imply that either the window for observing the ethylene peaks was missed, or, that the enclosed tissue is in some way protected from a water-deficit. It is suggested that preferential protection is the more likely explanation.

### **Preferential Protection of Rapidly Expanding Leaf Cells**

In *F. arundinacea*, the meristematic and elongating leaf tissue appeared less sensitive to a water-deficit. For example, no significant ethylene peaks were observed and ethylene evolution was maintained at levels similar to that of the control tissue. It is highly likely then that rapidly expanding cells in the elongating regions of the leaf are preferentially protected from the potential stress effects of a water-deficit. This is

consistent with previous physiological and biochemical studies in water-stressed grasses. For example, tissue specific osmotic adjustment has been observed in wheat (Munns *et al.*, 1979), maize (Michelena and Boyer, 1982), barley (Matsuda and Riazi, 1981) and in *F. arundinacea* (West *et al.* 1990, Barlow, 1986), and is greatest in the meristematic and elongating leaf regions compared with the mature leaf regions. This confers a greater resistance to any water-deficit-induced stress effect. In *F. novae-zelandiae*, Abernethy (1996) showed that the leaf bases retained turgor and remained viable at the expense of existing leaf laminae, which died back. It is proposed that these preferential changes in the meristematic and elongating leaf regions allow resumed growth once environmental conditions become favourable (Redmann, 1976). In this study, in both the rapidly expanding and the mature leaf zones of *F. arundinacea*, accumulation of the osmoprotectant proline was not significantly different from the control tissue until after the peak of ethylene evolution had subsided to near zero.

It is not possible, however from this study to relate the measured ethylene response directly to the water-deficit. There may have been other factors contributing to the observed induction of ethylene. The variances in the data tend to support this assertion, since fluctuations (i.e. both increases and decreases) in C<sub>2</sub>H<sub>4</sub> evolution over time were observed in control leaves.

This variation among the control leaves on any given day over the experimental period was more or less constant and suggests natural daily fluctuations relating to temperature or sunlight intensity. It is quite possible that there is an associated wound response by leaves to detachment and subsequent dissection. To determine any role for ethylene in plants experiencing a water-deficit, leaves were excised from whole (intact) plants since It is known that the excision of leaves induces ethylene production without the need for drought stress (Narayana *et al.* 1991, Morgan *et al.*, 1990). Leaves were harvested as far as possible around the same time each sampling day i.e. ca. 8.00 am in the morning. The ACO enzyme is believed to be most active between 6.00am and 12 noon and at midnight (Du, 2004). Indeed the ACO enzyme in white clover has been shown by Du (2004) to be under circadian control. Another consideration is that ethylene is both catalytic and an inhibitor of its own biosynthesis (Fluhr and Mattoo, 1996) and this effect cannot be ruled out when using sealed vials to measure ethylene evolution (Page *et al.*, 1990). Page *et al.* (1990) pointed out that the air in these sealed containers is often static. Oxygen is needed to convert ACC to ethylene and CO<sub>2</sub> can either promote or reduce the production of ethylene. However, any autocatalytic or inhibitory effect is expected to be relative to base levels of ethylene in the vials.

## Biochemical Characterisation of ACC Oxidase in *F. novae-zelandiae* and *F. arundinacea*

Initial biochemical assays for enzyme activity in the two species, were performed on crude leaf extracts. The ACO enzyme was isolated from the two leaf zones, enclosed (immature leaf zones comprising the meristematic and elongating regions) and exposed tissue (mature-green leaf zones), using a modified method (Du, 2004) of Hunter (1998). This particular method is Tris-HCl based. Alternative buffers have been used in other studies to extract ACO from plant tissue, for example HEPES, PIPES, MES and Na-phosphate. The extraction process included a size exclusion step using a Sephadex G-25 column (gel filtration column chromatography), to remove any low molecular weight molecules. Kruzmane and Levinsh (1999) reported the presence of low molecular weight inhibitors in pine needle ACO crude extracts that could be efficiently removed by chromatography on a Sephadex G-100 column. By using Sephadex G-25 in this thesis, the total protein was reduced to around 50% for the enclosed and ca. 95% for the exposed leaf regions in *F. novae-zelandiae*. In *F. arundinacea*, the concentration of proteins was higher, at ca. 35-40% (enclosed) and ca. 20-25% (exposed) of crude amounts. Protein yields in excess of 2.5 mg/mL were found to be needed for measuring enzyme activity in *F. novae-zelandiae* and this equates to greater than 1.0g of ground leaf tissue. At concentrations below this, activity was at the limits of detection by the Gas Chromatograph. ACO yields in enclosed tissue of *F. novae-zelandiae*, were found to be almost half that of the exposed tissue. In *F. arundinacea*, 1.0 g of ground frozen leaf tissue was found to be sufficient for measuring enzyme activity.

To aid enzyme extraction, leaf tissue was ground to a very fine powder. To ensure the maintenance of maximum enzyme activity, samples were held on ice at each step in the extraction process, and activity assays were performed as soon after enzyme extraction as possible. Several authors have noted a significant loss of activity of the ACO enzyme with purification. For example, in melon fruits further purification, after anion exchange column chromatography, was prevented by enzyme instability (Smith *et al.*, 1992). Dunkley and Golden (1998) reported that ACO from papaya fruits is somewhat labile with a loss of activity after 4 days at 14°C. Activity was prolonged when the extract was stored at -15°C. The addition of 1,10-phenanthroline (PA), a protease inhibitor, to extraction buffer at (final) concentrations of 10 µM, helps to stabilise the enzyme during purification

## Enzyme Activity Measurements

Enzyme activity assay conditions were those described by Gong and McManus (2000) for white clover ACO. That is, a pH of 7.5 with a saturating substrate concentration of 1mM ACC, co-factor concentrations of 30 mM NaHCO<sub>3</sub> and 0.02 mM Fe<sup>2+</sup>, and a co-substrate (sodium ascorbate) concentration of 30 mM. Good results have been obtained using these concentrations in other species. For barley (*Hordeum vulgare* L.) leaves and pine (*Pinus sylvestris* L.) needles, pH optimums of 7.0 to 7.2 were reported (Kruzmane and levinsh, 1999) using a (final) concentration of 20 mM NaHCO<sub>3</sub> in a HEPES/KOH buffer system. The optimum pH differed only slightly for the different buffer systems, for example, HEPES, PIPES, MES, Na-phosphate and Tris-HCL. From biochemical characterisation studies, Kruzmane and levinsh (1999) provided evidence that extractable ACO from cereals and conifers have similar characteristics *in vitro*.

Biochemical characterisation of the ACO enzyme in *F.novae-zelandiae* failed to detect any significant enzyme activity even after altering the pH to 8.5 and increasing the substrate concentration 10-fold to 10 mM ACC. This modification was based on characterisation studies in white clover, in which the pH optimum and substrate concentration as well as other biochemical properties, differed in the two isoforms characterised. MGI, the isoform from mature green leaf tissue in white clover is most active at pH 7.5, while SEII, the isoform from senescent leaf tissue, has a pH optimum of 8.5 (Gong and McManus, 2000). It is concluded therefore, that ACO activity in *F.novae-zelandiae* is at the limits of detection by the Gas Chromatograph. It is also possible that the presence of phenolic compounds in crude extracts of *F.novae-zelandiae* indicated by a dark brown colouration may have interfered with proteins during the extraction process and caused catalytic inactivation of the enzyme activity (Kruzmane and levinsh, 1999). This interference has been observed in other phenolic rich species such as *Citrus reticulata* and pine needles (*Pinus sylvestris* L.) making gel filtration a necessary step for recovery of ACO activity (Kruzmane and levinsh, 1999).

Although ACO enzyme activity could not be measured, antibodies were used to try to detect ACO protein accumulation. In *F. novae-zelandiae*, western analyses of crude leaf extracts support increased levels of enzyme at critical SWC's of ca.15 % for both elongating and mature green leaf zones (figures 3.11, 3.12 and 3.13). The TR-ACO2 antibody identified a putative ACO protein with a molecular mass of ca. 28.8 kDa in the

enclosed dehydrated tissue but not in similar tissue in a fully hydrated state (at 30% SWC). This is slightly smaller than the expected size for an ACO protein, but is still within the documented ranges of between 27.5 kDa for partially purified enzyme from papaya fruits (Dunkley and Golden, 1998) and 42.3 kDa in breadfruit (Williams and Golden, 2002). A protein of this size is absent from the mature leaf region. The presence of high molecular weight proteins, of good resolution, from ca 64.0 to 80.0 kDa in the crude extract preparations of both the elongating and the mature leaf zones, suggests that the ACO enzyme may exist as a dimer or form protein aggregates. Further characterisation studies would be needed to confirm this however. Because of limited leaf material and because of insufficient activity data, extracts of this species were not further purified. In contrast, in *F.arundinacea*, western analyses of crude leaf extracts generally support decreased levels of ACO with decreasing SWC in leaf tissue at both stages of development. A putative ACO protein with a molecular mass of ca. 35 kDa (by SDS-PAGE) was recognised by both polyclonal antibodies in water-sufficient and in water-deficient tissue, with recognition being highest in the water-sufficient tissue. Other protein products of importance had molecular masses of ca. 50 kDa and from ca. 75 to 100 kDa. The higher molecular mass proteins were often associated with protein duplexes and these warrant further investigation. The 50 kDa protein had reduced recognition at the lower soil water contents in the enclosed tissue. Interestingly, in this study, this protein was associated with a second peak of enzyme activity in the purified extracts. After one freeze-thaw cycle, the smaller proteins (35 kDa and less) detected in the freshly prepared leaf extracts, were no longer recognised by the anti-TR-ACO2 antibody. This observation along with repeated protein quantitation measurements over a period of a few days suggests that the ACO protein is relatively unstable *in vitro* and that it has a relatively short half-life.

In other studies of ACO activity in plant species, typically yields of 1.2% or a two to four fold purification factor (Smith et al., 1992) are obtainable after Mono-Q anion exchange. For homogeneous enzyme preparations, much larger increases can be expected. For example, using a six step chromatography procedure, Williams and Gong (2002) reported an 87-fold increase in yield for ACO from breadfruit, with a recovery of 9.5 %. Dong *et al.* (1992) purified apple ACO 180-fold to near homogeneity with 37% protein recovery using conventional column fractionation (ammonium sulphate precipitation, followed by HIC liquid chromatography and gel filtration. In this thesis, further separation using anion-exchange column chromatography and an increasing salt (NaCl) concentration was used for *F.arundinacea*. The ACO enzyme eluted within the range of 280 to 420 mM NaCl, indicating a net overall negative charge at pH 7.5. This

result is comparable to characterisation studies in white clover, in which ACO activity in mature green and in senescent leaves eluted as single peaks at a salt concentration range of 265-371 mM NaCl (Gong and McManus, 2000). In this study, representative leaf samples of around 1.0 -1.5 g of tissue (~ 5-10 mg of total protein) were found to be sufficient for separation on the mono-Q column. A protein precipitation step using ammonium sulphate was included in the extraction process. Ammonium sulphate fractionation has been used to good effect in previous purification studies. Du (2004) modified an earlier method of Gong (1999) to include a 40% and an 80%  $\text{NH}_2\text{SO}_4$  saturation step. Most of the ACO protein was recovered from crude extracts of white clover leaves using this concentration range.

Two proteins of major importance with approximate molecular masses of 32 and 37 kDa (by SDS-PAGE) were identified by the TR-ACO2 antibody in the eluted fractions of dehydrated mature and immature leaf tissue respectively. Both are absent from similar tissue in a water-sufficient state, indicating that these may be novel proteins induced by the more severe water deficit, i.e. SWCs of 10% and less. The TR-ACO2 antibody has been used by this lab group (in both published and unpublished work) to recognise ACO proteins in other species with good results. For example, in vegetative tissues of apple (*Malus domestica*) and cherry (*Prunus spp.*). The high molecular weight protein complexes from ca. 60 to 100 kDa identified in the crude extract preparations were again recognised by the antibodies in the purified extracts, especially those of dehydrated tissue.

For the enclosed leaf tissue, ACO activity eluted as a single peak at salt concentrations of 320 and 340 mM NaCl in dehydrated and in fully hydrated tissue respectively. Additional peaks of activity in the dehydrated tissue (fractions #15, #29 and #31 in figure 4.19) were assumed to be artefacts of the assay and therefore not considered important. It was found that the rubber bungs used to seal the assay vacu-tainers if not properly dried before use, released compounds that contributed to the evolved ethylene. Because of time constraints, these fractions were not further analysed. In the exposed leaf tissue, ACO activity eluted as double peaks at salt concentrations of 280 mM and 420 mM NaCl for water-sufficient tissue, and 360 and 420 mM NaCl for dehydrated tissue. Enzyme activity for fractions #13 to #19 in figure 4.20) of dehydrated exposed tissue were not determined due to problems with the gas chromatograph.

In the elongating leaf regions, the highest specific enzyme activity in the crude extracts was found to be approximately two to three-fold higher than that in tissue from the mature green leaf regions. This finding has also been reported in characterisation studies of ACO from pine needles. Kruzmane and Levinsh (1999) reported proportionately higher activity in younger needles, in general, than in mature needles. For *F. arundinacea* in this thesis, when the eluted fractions were assayed for enzyme activity, the highest specific activity in the dehydrated immature leaf tissue corresponded to the fraction containing the greatest amounts of the ca. 37.0 kDa protein (fraction # 18; figure 4.21). When compared to the same fractions from similar tissue in a fully hydrated state, activity levels in the dehydrated tissue are low. This agrees with the trend in activity data for the crude extracts in which decreased enzyme activity is observed at the lower SWCs. This result is also consistent with the ethylene measurements for *F. arundinacea* in which ethylene evolution was almost zero and this was significantly lower than in similar tissue in a fully hydrated state. For the mature leaf tissue, the ca. 32.0 kDa protein formed part of a characteristic ladder seen in dehydrated tissue of both types (enclosed and exposed) (fraction # 17; figures 4.21 and 4.23). Enzyme activity was also non-determinable for this fraction. The ca. 50 kDa protein identified in both the crude and purified extracts was initially associated with a second activity peak in dehydrated mature leaf tissue. However, this protein did not correlate with peak activity data.

That the two ACO proteins eluted at different points in the separation suggests different charge properties and this is possibly due to differences in the amino-acids at their surfaces. Together with the differences in molecular mass as determined by SDS-PAGE and subsequent western analyses, these results suggest two distinct isoforms of ACC oxidase in the two tissue types. Indeed in white clover, Gong and McManus (2000) reported the presence of two ACO isoenzymes that were both purified to homogeneity, MGI isolated from mature green leaf tissue and SEII from senescent leaf tissue. These isoforms have different molecular masses (37 and 35 kDa as determined by SDS-PAGE), and display different biochemical preferences. For example, differences in the pH optimum, isoelectric point,  $K_m$  for ACC, and optimal requirements for the co-substrate ascorbate, and  $\text{NaHCO}_3$  and  $\text{Fe}^{2+}$  as co-factors. In this thesis however, further biochemical characterisation of the identified isoforms was not attempted.

The high molecular mass proteins seen repeatedly in this study in the fractions together with the smaller (32 and 37 kDa novel) proteins suggest that the ACO enzyme

may exist as a dimer in *F. arundinacea*. Thus far, dimerisation of the ACO enzyme is uncommon in the literature. From previous studies in fruit tissues and to a much lesser extent in vegetative tissues, in a wide range of plant species, it is generally accepted that the ACO enzyme is active as a monomer. Documented molecular masses for partially purified enzyme range from 27.5 kDa in papaya (Dunkley and Golden, 1998) to 42.3 kDa in breadfruit (Williams and Golden, 2002). For example, molecular masses of: 39 and 35 kDa by gel filtration (native protein) and SDS-PAGE (denatured protein) respectively in apple fruits (Dong *et al.*, 1992); 40 and 36 kDa in banana fruits (Moya-Leon and John, 1995); 41 kDa by gel filtration in melon fruits (Smith *et al.*, 1992) and 42.3 kDa by SDS-PAGE in breadfruit (Williams and Golden, 2002). In vegetative tissues, Kim and Yang (1994) reported a predicted molecular mass for mung bean (hypocotyls) pVR-ACO1 from deduced sequence information of 35.8 kDa and in white clover leaves, Gong and McManus (2000) reported molecular masses of 37.5 kDa by gel filtration, and 37 and 35 kDa by SDS-PAGE for mature green (MGI) and senescent leaf (SEII) isoforms respectively. In cherimoya fruits however, Escribano *et al.* (1996) reported molecular masses of 62 and 66 kDa by native PAGE and gel filtration respectively, and only 35 kDa by SDS-PAGE. This difference in molecular mass for native and denatured protein indicates that the enzyme could be active as a dimer. Escribano *et al.* (1996) related this to its primitive phylogenetic origin. Good recognition of high molecular weight protein complexes (of *ca.* 75 to 88 kDa) together with the smaller protein products (of *ca.* 32 and 35 kDa) in extracts strongly suggests that the ACO enzyme in *F. novae-zelandiae* and in *F. arundinacea* may exist as a dimer (figures 3.10-3.12, 4.15- 4.18 (partially purified) and figures 4.21 and 4.23 (further purified)). Further analysis however, would be necessary to confirm this.

All estimates of molecular mass are from mobility plots of mobility in mm relative to the point of origin (at wells) against Log MW. This is an accepted method for sizing electrophoresis protein bands (Hames and Rickwood, 1981). Because the measured enzyme activity for fractions were single point measurements only ( $n=1$ ), it is therefore not possible to report the accuracy of, or the variation in the data. What is important however are the relative activity values when comparing enzyme activity with western blots. Single point measurements were used due to time constraints because of the large number of fractions to be assayed at the same time for best comparison. Ideally, for accuracy and confidence levels, multiple measurements, in duplicate or triplicate would be preferable.

### **The Relationship between Ethylene Evolution and ACO Activity**

In the mature leaf tissue of *F. arundinacea*, ethylene evolution peaked at mean LERs of ca. 16 mm/d or a SWC of 22%, and this is long before any observable peaks in ACO enzyme activity. In partially purified leaf extracts of *F. arundinacea*, ACO activity was greatest at soil water contents of ca. 9% for the immature tissue and ca. 10% for mature green leaf tissue, or mean LERs of 0.0 mm/day and 1.4 mm/day respectively from enzyme specific activity plots (from figure 4.5). In *F. arundinacea*, no clear correlation between activity of the ACO enzyme and ethylene evolution was observed. In the absence of any significant activity data for *F. novae-zelandiae*, it is also not possible to establish any correlation between evolved ethylene and the ACO enzyme for this species.

In contrast to these findings, Jain *et al.* (2006) reported a positive correlation among ACC content, ACO activity and endogenous ethylene in pigeonpea (*Cajanus cajan* L.) roots experiencing moisture stress. Increases in ACO activity of from 37-66% and increases in ethylene of from 83-270% were recorded as the drought treatment increased from moderate to severe. In wheat grains during filling, concentrations of ACC and ethylene were significantly correlated with concentrations significantly reduced under moderate soil drying but greatly increased under conditions of severe drying treatment (Yang *et al.*, 2006).

### **The Relationship between a Water Deficit and Water Deficit Stress**

As mentioned previously, 'stressed' plants are described as those that do not reach their full genetic potential (Boyer, 1982). In this thesis, a water deficit was applied to two grass species with different apparent adaptations to water loss. Physiological changes were measured that do imply these plants were experiencing stress, and these served as important time points with which to examine changes in the pattern and possible control (through the accumulation of specific ACO isoforms) of ethylene biosynthesis. However, critical measurements of leaf water content and leaf water potential were not undertaken, and so it cannot be concluded that the plants, at the time points examined were experiencing water deficit stress. It can be concluded with more certainty (through measurements of SWC, LER and proline accumulation) that the plants were experiencing a water deficit.

## Conclusions

In the xerophyte *F.novae-zelandiae*, there was an early and rapid physiological response to limited water. Reductions in leaf elongation rates were linear with decreasing % soil water content. In comparison, leaves of *F.arundinacea* appear to have limited ability to conserve water. Leaf elongation rates declined more slowly to begin with in response to the dry-down, and followed a logarithmic decrease. The possibility of severe water stress was evident in this species by much wilting and rolling of the leaves.

Ethylene evolution in the mature green tissues of *F.arundinacea* leaves followed changes in the LER. Reductions in cell enlargement (measured as LER) with decreasing water availability preceded any bursts of ethylene observed in mature-green tissues. That ethylene appeared to follow these changes (both increases and decreases), indicates that there is an ethylene response to water deficit since the LER was correlated with SWC. In *F.arundinacea*, there is an ethylene response to changes in leaf expansion with an ensuing water-deficit, and this trend is for a burst of ethylene approximately 48 hours after a sudden rapid decline in the LER. These findings suggest that ethylene biosynthesis is sensitive to cell elongation rates, or that ethylene may be important in regulating cell elongation. Ethylene is known to be an early indicator of water-deficit stress and in this study observed increases in ethylene in mature green leaf regions, preceded the onset of accumulation of the osmoprotectant proline in leaves.

Under conditions of a water deficit, ethylene evolution in leaf tissue of *F.arundinacea* is tissue-specific, i.e. differentially evolved from immature and mature leaf tissues. This finding adds further support to the notion that there are a number of isoforms involved in regulating ethylene biosynthesis in higher plants, and that accumulation of these may be tissue-specific.

Immature leaf regions exhibited preferential protection from the ensuing water-deficit, as indicated by control tissue. This is supported by the enzyme activity data in the enclosed leaf regions in which specific activity peaked at a lower SWC [of 9% SWC ( $p \leq 0.05$ )] than exposed (mature-green) leaf regions [at 10% SWC ( $p \leq 0.05$ )]. This suggests that rapidly expanding leaf regions are less sensitive, and therefore in some way protected from a water-deficit. Proline analysis supports this theory because proline was found to accumulate differentially in leaf tissue at different stages of

development. This differential accumulation of proline has been documented in previous work.

Biochemical characterisation of the ACO enzyme in *F. arundinacea* indicates that the enzyme is active at a pH of 7.5 and substrate, co-substrate and co-factor concentrations of: 1 mM ACC, 30 mM Na-ascorbate, 30 mM NaHCO<sub>3</sub> and 0.02 mM Fe<sup>2+</sup> (final concentrations). However any relationship between the induction of ethylene evolution and ACO enzyme activity in this species is elusive. It is not possible from the results of this study to assign a regulatory role for ACO in ethylene biosynthesis.

Two distinct ACO isoforms in *F. arundinacea* are induced in response to a water-deficit. Proteins of ca. 37 kDa and 32 kDa were identified by SDS-PAGE and subsequent western analysis in leaves and appear to be tissue specific. The larger 37 kDa isoform was identified in immature leaf tissue and the other (32kDa) in mature-green tissue. Additional high molecular weight protein complexes in the dehydrated tissue, of ca. 88 kDa in enclosed and ca. 75 kDa in exposed, suggests that the enzyme may exist as a dimer and ACO activity associated with these high molecular mass complexes (e.g. figure 4.20) further suggests that ACO may be active as a dimer. The 37 kDa protein corresponded to maximum ACO activity in enclosed tissue.

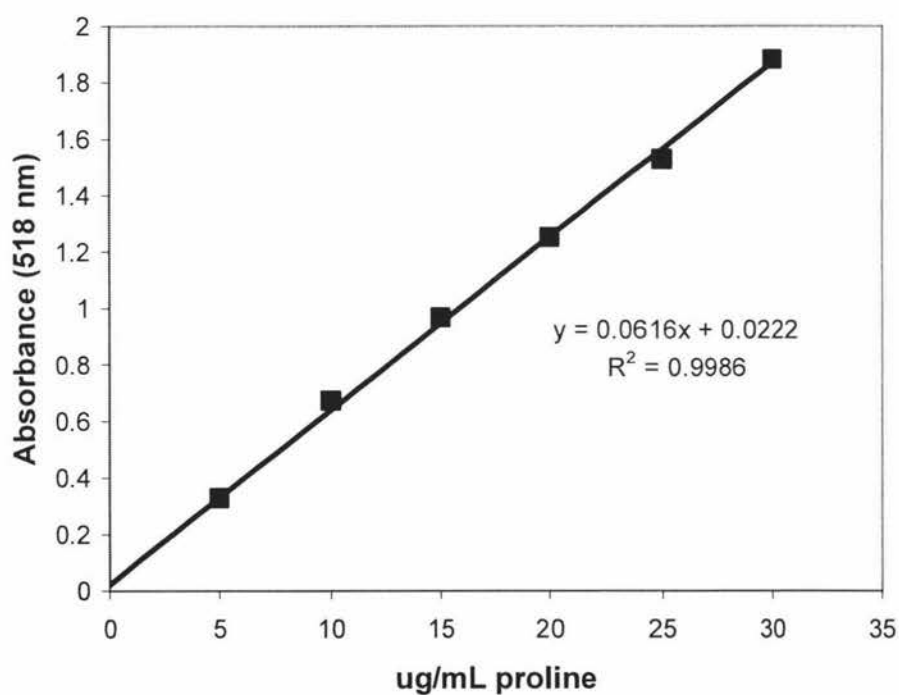
In *Festuca novae-zelandiae*, in the absence of any significant activity or ethylene measurements, the only conclusion that can be made is that ethylene evolution is expected to be in the nanomolar region rather than the micromolar region as for *F. arundinacea*. However, high molecular weight complexes in crude extracts of *F. novae-zelandiae* revealed by western analysis suggest that ACO in this species may exist as a dimer.

## Appendix 1

Free Proline Assay Standard Curve Data:

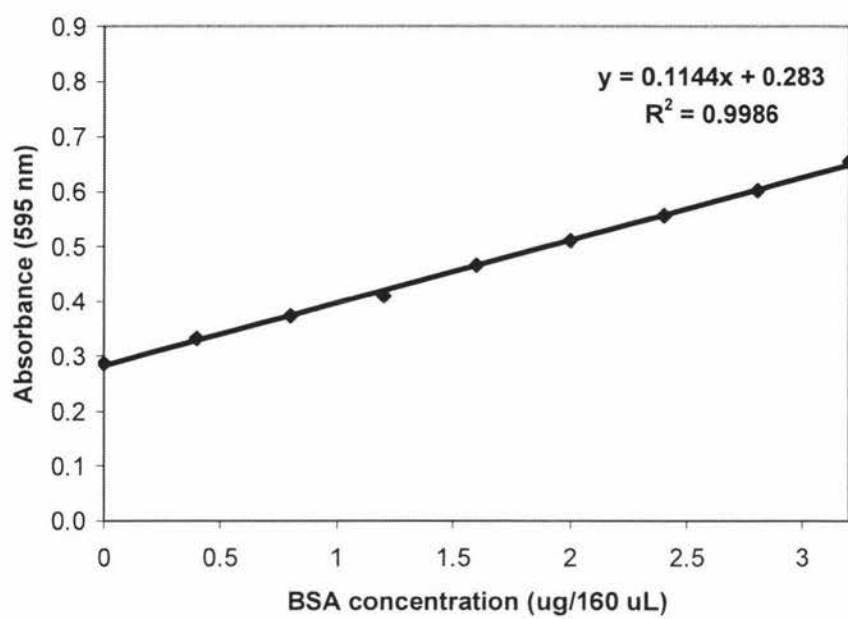
| Sample | Proline concentration (µg/mL proline) | A (518nm) Data set I | A (518nm) Data set II | A(518nm) Data set III | A(518 nm) Mean |
|--------|---------------------------------------|----------------------|-----------------------|-----------------------|----------------|
| 1      | 0                                     | -0.017               | 0.004                 | 0.004                 | -.003          |
| 2      | 5                                     | 0.325                | 0.312                 | 0.350                 | 0.329          |
| 3      | 10                                    | 0.671                | 0.686                 | 0.662                 | 0.673          |
| 4      | 15                                    | 0.966                | 0.927                 | 1.011                 | 0.968          |
| 5      | 20                                    | 1.252                | 1.232                 | 1.270                 | 1.251          |
| 6      | 25                                    | 1.510                | 1.558                 | 1.516                 | 1.528          |
| 7      | 30                                    | 1.908                | 1.884                 | 1.853                 | 1.882          |

Standard Curve For Free Proline Assay (Mean)



## Appendix 2

Typical protein standard curve for the BioRad protein assay

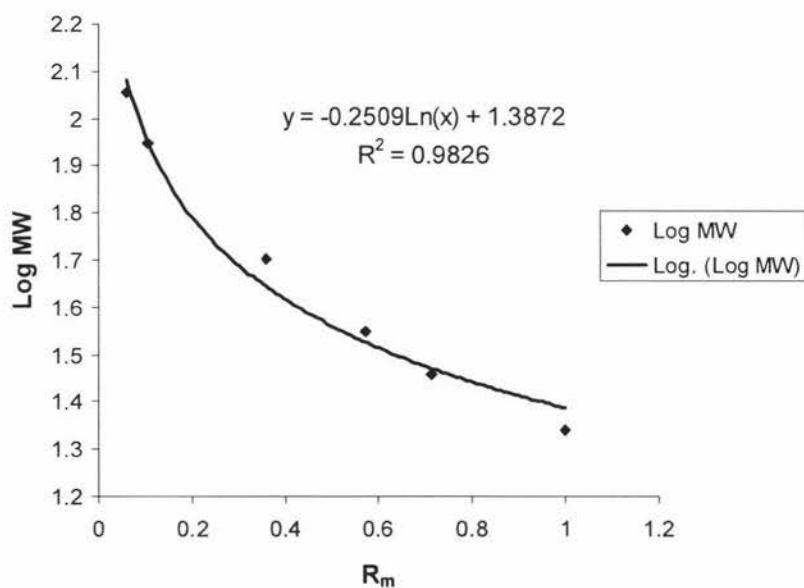


## Appendix 3

An example of a mobility plot using the method of Hames and Rickwood (1981) to determine molecular masses of protein bands

| Marker Proteins | MW (molecular mass) | $R_m$ (relative mobility) | Log MW | Distance (mm) from origin |
|-----------------|---------------------|---------------------------|--------|---------------------------|
| 1               | 114                 | 0.06                      | 2.0569 | 3.5                       |
| 2               | 88.8                | 0.11                      | 1.9484 | 6.0                       |
| 3               | 50.7                | 0.36                      | 1.7050 | 20.0                      |
| 4               | 35.5                | 0.57                      | 1.5502 | 32.0                      |
| 5               | 28.8                | 0.71                      | 1.4593 | 40.0                      |
| 6               | 22                  | 1.00                      | 1.3424 | 56.0                      |

**Mobility Plot**



## Appendix 4

An example of a Student's *t*-test performed on actual proline data:

**Proline Content (mg/gFW) in enclosed leaf tissue of *F. novae-zelandiae***

| Day 14 | Deficit<br>plants | Control<br>plants |
|--------|-------------------|-------------------|
|        | 2.38              | 0.21              |
|        | 1.27              | 1.33              |
|        | 1.91              | 0.40              |
|        | 0.77              | 0.33              |
|        | 0.58              | 0.52              |
|        | 1.91              |                   |

**t-test**

**t-Test: Two-Sample Assuming Unequal  
Variances**

|   | <i>Variable</i><br><b>1</b> | <i>Variable</i><br><b>2</b> |
|---|-----------------------------|-----------------------------|
| <b>Mean</b>                             | 1.47                        | 0.558                       |
| <b>Variance</b>                         | 0.50748                     | 0.19887                     |
| <b>Observations</b>                     | 6                           | 5                           |
| <b>Hypothesized Mean<br/>Difference</b> | 0                           |                             |
| <b>df</b>                               | 8                           |                             |
| <b>t Stat</b>                           | 2.586217                    |                             |
| <b>P(T&lt;=t) one-tail</b>              | 0.016151                    |                             |
| <b>t Critical one-tail</b>              | 1.859548                    |                             |
| <b>P(T&lt;=t) two-tail</b>              | 0.032303                    |                             |
| <b>t Critical two-tail</b>              | 2.306006                    |                             |

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