Seed Factors Involved in Early Seedling Establishment of *Festuca arundinacea* (Tall Fescue)

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Plant Biology and Biotechnology at Massey University, New Zealand.

Patricia Alison Murray

1995

Abstract.

New Zealand pastures are commonly based on vigorously growing *Lolium* (ryegrass) species. In many situations, however, it is often advantageous to combine the vigorous qualities of *Lolium* species with the versatility of other species such as *Festuca arundinacea* (tall fescue) to result in a pasture which is high in quality all year. *F. arundinacea*, however, has poor seedling vigour which places it at a competitive disadvantage when sown with *Lolium*.

During seedling establishment, the seedling is dependent upon the food reserves present in the seed. The nature and amount of these reserves and the ability of the seed to mobilise them are therefore likely to have an affect on seedling performance. While much knowledge exists about the processes involved in seed reserve mobilisation in some cereals, little information is available for pasture grasses.

An in depth investigation of the behaviour of one seed lot of *F. arundinacea* was undertaken in order to gain a more detailed understanding of germination, reserve mobilisation and establishment processes in pasture grasses and how they relate to the processes of seedling growth. Comparisons between this species and *Lolium multiflorum* (Italian ryegrass) were undertaken throughout the study.

Germination and seedling growth of the *F. arundinacea* seed lot was found to be heterogenous and slower than in *L. multiflorum* due to later radicle emergence. Mobilisation of reserves and the onset of α-amylase activity correlated well with the utilisation of reserves in *L. multiflorum*. In both prechilled and non prechilled *F. arundinacea* seeds, however, anomalies were identified in the process which indicated that reserve mobilisation was less tightly coupled to seedling growth in *F. arundinacea*. Reciprocal plot analyses indicated that the beneficial effects of prechilling in most seeds of the *F. arundinacea* seed lot were not related to residual dormancy but were a thermal time benefit. It appears prechilling was allowing rate limiting steps in embryo growth to be advanced before visible germination. Apart

from this, no real differences were detected in the way the two species mobilised reserves.

Differences in the appearance of α-amylase isoenzymes of *F. arundinacea* at different times during germination indicated that gene expression may be under some complex differential control mechanisms during germination and reserve mobilisation. Prechilling was not found to change the spectrum of isoenzymes, but merely to advance the time-course in which different isoenzymes appeared. There were also distinct differences in α-amylase isoenzyme patterns between *F. arundinacea* and *L. multiflorum*, and also wheat. Preliminary studies indicated that exogenous gibberellic acid was more effective in promoting α-amylase production in *L. multiflorum* than in *F. arundinacea*. However, α-amylase production in *F. arundinacea* was more susceptible to promotion by prechilling.

This study has identified a wide range of variables impacting on germination and seedling establishment in *F. arundinacea*. This, together with the lack of previous detailed studies on grass seed germination and seedling growth and the lack of literature on *F. arundinacea* germination in particular, highlights the enormity of the task ahead of extending key areas of this study to different seed lots and species.

Acknowledgements

I wish to thank the many people who have helped in the production of this thesis. In particular, I would like to thank my supervisors, Dr. Peter Coolbear and Dr. Clive Cornford, for their patience, advice and encouragement. Thank you also to Dr. Paula Jameson for helpful advice, and to Dr. Michael McManus for encouragement and patience during the last year.

I gratefully acknowledge the cheerful help of:

- The technical staff of the Department of Plant Biology and Biotechnology and the Seed Technology Centre, and also Cathy for solving my computing hassles.

- Megan for her patient help with statistics and production of graphs, and Grant for help with statistics.

- Guy, Sandra, Margaret and Merie for the proof-reading.

I would like to thank Don for his 'extended' computer loan and ongoing encouragement and friendship. Thank you also to the following people for their friendship, advice and support: Sheree and Pete, Merie, Megan, Sandra, Sal and Mike, Sally and Paul, Kirsty, Katherine, Malcolme, Richard, Lise, Howard, Ross, Bruce and my AgResearch workmates.

A huge thank you goes to my family for their tireless support and encouragement.

Table of Contents

PAGE

Acknowledgements iv

List of Figures viii

List of Plates x

List of Tables xi

Abbreviations xii

1. Introduction 1

1.1 Context of Study 1

1.2 Objectives and Experimental Approach 2

1.3 Organisation of Thesis 3

2. Literature Review 5

2.1 Pasture Grasses 5

2.2 Effects of Seedling Vigour on Seedling Establishment 8

2.3 Seed Structure and Development 11

2.3.1 Seed Coat 13

2.3.2 Endosperm 13

2.3.3 Aleurone Layer 15

2.3.4 Embryo 15

2.4 The Transition from Seed Development to Germination 16

2.5 Seed Ageing 18

2.6 Dormancy 18

2.7 Germination Requirements 20

2.8 The Germination Process 21

2.9 Reserve Mobilisation 23

2.10 α-Amylase 27

2.11 α-Amylase Isoenzymes 28

2.12 The Effects of Calcium 30

2.13 Abscisic Acid Interactions 31

2.14 Conclusion 31

3. Materials and Methods **33**
3.1 Plant Material 33
3.2 Seed Size Variation 33
3.3 Calculation of the Percentage of Empty Seed 33
3.4 1000 Seed Weight 34
3.5 Tetrazolium Testing 34
3.6 Seed Germination 35
3.7 Starch Assay 38
3.8 α-Amylase 39
3.9 Hormone Response Experiments 40
3.10 Isoelectric Focusing 40
3.10.1 β-Limit Dextrin Overlay Gel 41
3.10.2 Estimation of pI 42

4. Optimisation of *F. arundinacea* germination Conditions **43**
4.1 Results 43
4.1.1 Determination of Loss of Residual Dormancy
Versus Thermal Time Effects of Prechilling 52
4.2 Discussion 56
4.2.1 Characterisation of the *F. arundinacea* Seed Lot 56
4.2.2 Determination of Optimum Germination Conditions 56
4.2.3 Determination of Dormancy of Thermal Time Effects . . . 59

5. Comparative Time-Course Studies Between *F. arundinacea*
and *L. multiflorum* **63**
5.1 Results 63
5.1.1 Final Percentage Germination 63
5.1.2 Germination Rates 68
5.1.3 Seedling Dry Weight Changes 70
5.1.4 Seed Reserve Changes 70
5.1.5 α-Amylase Activity 76
5.1.6 α-Amylase Isoenzymes 79

5.2 Discussion 84
5.2.1 Germination and Seedling Growth Rate Comparisons . . . 84
5.2.2 α-Amylase Activity 87

6. Hormone Responsiveness 90
6.1 Results 90
6.2 Discussion 94
6.2.1 Hormone Responsiveness of Half-Seeds to GA_3 94
6.2.2 Storage Effects 94
6.2.3 Prechilling Effects 95
6.2.4 Other Variables 96

7. General Discussion 98
7.1 Heterogeneity of the *F. arundinacea* seed lot 98
7.2 Evidence that Reserve Mobilisation may be Limiting 100
7.3 Responsiveness to GA_3 102
7.4 Limitations of Study and Scope for Further Work 103
7.5 Conclusions 104

Appendix 1 106
Appendix 2 107
Appendix 3 108
Appendix 4 109
Appendix 5a 110
Appendix 5b 111

Bibliography 112

List of Figures

Figure Page

1 Period of Grass Seedling Establishment Showing Relative Importance of the Various Stages of Seedling Growth 9
2 Transverse Section of a Wheat Grain 14
3 Germination Described by the Imbibition Curve 22
4 Mobilisation of Food Reserves in Barley Grains 25
5 The Seed Weight Distribution of Dehusked *F. arundinacea* Seeds 45
6 Time-course of *F. arundinacea* Seed Germination at Different Temperature Treatments 47
7 Reciprocal Plot of Median Germination Time Against Various Germination Temperatures Showing the Base Temperature for *F. arundinacea* and *L. Multiflorum* 54
8 The Effect of Prechilling on the Germination at 20°C of *F. arundinacea* and *L. multiflorum* 64
9 The Effect of Prechilling on Seedling Dry Weight Changes of *F. arundinacea* and *L. multiflorum* 71
10 The Effect of Prechilling on the Shootlength of *F. arundinacea* and *L. multiflorum* 72
11 The Effect of Prechilling on the Rootlength of *F. arundinacea* and *L. multiflorum* 73
12 The Effect of prechilling on the Seed Dry Weights of *F. arundinacea* and *L. multiflorum* 75
13 Starch Levels of Prechilled *F. arundinacea* and *L. multiflorum* over a 14 Day Time-Course 77
14 The Effect of Prechilling on the α-Amylase Activity of *F. arundinacea* and *L. multiflorum* 78
15 α-Amylase Activity of *F. arundinacea* and *L. multiflorum* Embryoless Half-Seeds in Response to Exogenous GA_3 91

16 α-Amylase Activity of *F. arundinacea* and *L. multiflorum* Embryoless Half-Seeds in Response to Exogenous GA_3 and Prechilling 92
17 Variables Effecting Germination Rate and Seedling Growth Rate 99

List of Plates

Plate Page

1 Variation in *F. arundinacea* Half-Seeds Following Tetrazolium Staining .. 46

2 A Comparison of Prechilled *F. arundinacea* and *L. multiflorum* Germination at Day 0 65

3 A Comparison of Prechilled *F. arundinacea* and *L. multiflorum* Germination and Early Seedling Growth at Day 2 66

4 A Comparison of Prechilled *F. arundinacea* and *L. multiflorum* Germination and Early Seedling Growth at Day 4 67

5 α-Amylase Isoenzymes of *F. arundinacea* Seeds Over a 14 Day Time-Course 80

6 α-Amylase Isoenzymes of *F. arundinacea* Seeds Over Days 4-8 of a Germination Time-Course 81

7 α-Amylase isoenzymes of *F. arundinacea*, *L. multiflorum* and Wheat Seeds at Times of High Levels of α-Amylase Activity 83

List of Tables

Table Page

1 Physical Characteristics of *F. arundinacea* Seed Used in this Study 44

2 The Effects of Prechilling and Various Temperature Treatments on the Final % Germination, the Median Germination Time and the Uniformity of Germination of *F. arundinacea* 48

3 The effects of Prechilling and Various Temperature Treatments on the Shoot and Root Length of *F. arundinacea* Following 16 Days Germination 51

4 Correlation Analyses Between T_{50}'s and Subsequent Seedling Growth of Seeds Germinated at Different Temperatures, With and Without Prechilling 53

5 Changes in T_{50} and Final % Germination of *F. arundinacea* During Storage 60

6 The Effects of Prechilling on the Final % Germination, the Median Germination Time and the Uniformity of Germination of *F. arundinacea* and *L. multiflorum* 69

7 A Comparison of Linear Growth (Dry Weight) in *L. multiflorum* and *F. arundinacea* 74

8 A Summary of Germination and Early Seedling Growth Data of Prechilled and Non Prechilled *F. arundinacea* and *L. multiflorum* over a 16 Day Time-Course 86

Abbreviations

ABA	- Abscisic Acid
cv.	- Cultivar
d	- Day
ER	- Endoplasmic Reticulum
GA_3	- Gibberellic Acid
h	- Hour
IEF	- Isoelectric Focusing
PGR	- Plant Growth Regulator
SE	- Standard Error
T_{50}	- Median Germination Time
T_{90}-T_{10}	- Uniformity of Germination

1. Introduction

1.1 Context of study

New Zealand pastures are dominated by species of vigorously growing *Lolium* which establish rapidly and are able to tolerate heavy grazing under a variety of environmental conditions. However, *Lolium* cannot tolerate extreme climatic conditions such as hot, dry summers or cold winters (Langer 1990). Therefore it is desirable to introduce some other grass species into *Lolium* pasture to complement its growth characteristics. One such option is *Festuca arundinacea* which is slow to establish but is an attractive pasture grass as it is hardy. It can grow on shallow, droughty soil and can also withstand waterlogging, flooding and considerable grazing (Barnes 1990).

In difficult climates it is often advantageous to combine the vigorous qualities of *Lolium multiflorum* (Italian ryegrass) with the versatility of *F. arundinacea* (tall fescue) to result in a pasture which is high in quality all year. However, the vigour of *L. multiflorum* which is advantageous in many situations can jeopardise the establishment of other species in mixed pastures (Langer 1990). *F. arundinacea* has poor seedling vigour which places it at a competitive disadvantage in mixed sowings (Charles 1965; Rhodes 1968). Therefore it is strongly recommended not to sow *F. arundinacea* with *L. multiflorum* (AgResearch 1993).

A study by Brock *et al.* (1982) has shown that after 10 days germination, *Lolium perenne* (perennial ryegrass) seedlings utilise 47% of their endosperm reserves compared with only 14% for *F. arundinacea* seedlings. The ideas that emerged from this study were that these differences in vigour were either due to relative growth rate differences, or that there were differences in the mobilisation process of the endosperm. However, as will be discussed in Chapter 2, this study had severe limitations and needs re-examination.

At these early stages of germination and seedling growth the seedling is dependent upon the food reserves present in the seed. The nature and amount of these reserves and the ability of the seed to mobilise them are therefore likely to have an affect on seedling performance. While much knowledge exists about the processes involved in seed reserve mobilisation in cereals, little information is available for pasture grasses. In addition, these reserve mobilisation processes are based on only a few species such as barley and wheat.

Reserve mobilisation of barley involves the synthesis and secretion of gibberellins by the scutellum. This stimulates a response by the aleurone layer to synthesise α-amylase and other hydrolytic enzymes *de novo* and to secrete them into the endosperm where they are involved in the mobilisation of starch reserves (Fincher 1989). Many differences have been observed between the model system of seed reserve mobilisation processes in barley and other cereal species. These include species variations in aleurone tissue responsiveness to added gibberellin, the presence of α-amylase inhibitor activity and the spectrum of α-amylase isoenzymes (Cornford and Coolbear 1992). It would therefore be dangerous to assume that these processes in pasture grasses would be identical to that described for barley. There is an obvious need for the range of activities and species studied to be broadened.

1.2 Objectives and experimental approach

This project aimed to determine whether reserve mobilisation in general and α-amylase activity in particular are limiting components of seedling growth and establishment in *F. arundinacea*. The objectives of the study were:

- to identify the optimum germination conditions of *F. arundinacea*, enabling differences between *L. multiflorum* (which has been the focus of previous research in this laboratory) and *F. arundinacea* to be identified.

- to compare changes in physical characteristics of *F. arundinacea* and *L. multiflorum*

as germination and seedling growth proceed.

- to measure the activity of α-amylase in *F. arundinacea* and *L. multiflorum* during these stages.

- to characterise α-amylase isoenzymes present at different stages of germination in the two species.

-to try to identify mechanisms for the differences during germination and seedling growth in *F. arundinacea* and *L. multiflorum.*

Different cultivars and even different seed lots have been found to vary enormously in their germination characteristics and behaviour (Anslow 1964; Elgersma and Sniezko 1988; Flintham and Gale 1988). This is of concern in relation to selection of seed for testing procedures (Wiesner and Grabe 1972). This project could have been carried out in two ways - 1) a relatively superficial comparative study involving many seed lots and cultivars of each species, or 2) a more in depth approach with just one seed lot of each type. Due to interest in fundamental processes the latter approach was chosen. An obvious limitation is that 1) still needs to be done, however, the data from this project is intended to highlight key aspects that need to be addressed in a multi-seed lot survey.

1.3 Organisation of thesis

To meet the above objectives, this thesis has been organised into five chapters. Following and expanding on this introduction, Chapter 2, includes the description of relevant literature. The methods and materials of experiments performed to meet the

above objectives are described in Chapter 3. Results and discussion of these experiments have been written up in three chapters, 4) Optimisation of *F. arundinacea* germination conditions, 5) Comparative time-course studies between *F. arundinacea* and *L. multiflorum*, and 6) Hormone responsiveness. The separate discussions of these results are tied together in a general discussion in Chapter 7.

2. Literature Review

All grasses belong to the family *Gramineae*, which comprises an estimated 620 different genera and 10 000 species. The family is distributed throughout the world with grasses adapting to a diverse range of environments. Their adaptability is attributed largely to the fact that the growing points of most of the species are close to the soil where they are fairly sheltered from climatic conditions, grazing and fire (Lambrechtsen 1986).

Grasses are the world's most important agricultural plants. They include cereals, forage grasses for farm animals, sugarcane, bamboos and grasses that stabilise various environments. Ornamental grasses are also of importance, with a large group used in horticulture, to provide lawns, public parks and various kinds of sports turfs (Chapman and Peat 1992). Turf grasses have recently become one of the largest seed markets in the United States of America (Kidd 1993).

Many valuable industrial products such as starch, fibres, adhesives and aromatic oils are made from grasses (Lambrechtsen 1986). In addition, the major source of carbohydrate and protein in human nutrition is from grains of the common cereals. Grains are also used extensively in the production of alcoholic beverages (Fincher 1989).

2.1 Pasture grasses

In New Zealand, where the national economy depends mainly on production from grazed pastures, grasses are of extreme importance (Lambrechtsen 1986). The long term aim of grassland farming has been high quality pastures based on ryegrass and white clover (AgResearch 1993). Most New Zealand farmers renewing pastures in fertile lowland regions prefer to sow perennial ryegrass and white clover for long term pastures and Italian ryegrass for annual pastures (Charlton 1991). Both ryegrass and clover monoculture pastures are vigorous in establishment and easy to manage.

However, pasture stresses such as drought, pests and disease, soil fertility, grazing management (stocking rate, winter treading damage and summer overgrazing) and animal disorders (ryegrass staggers and facial eczema) have combined to reduce the production, persistence and acceptability of ryegrass monoculture (Moloney 1991). This has lead to an increased awareness of the need for more sustainable and ecologically sound pastoral systems (Woodman and Fraser 1991) and a demand for alternative pastures based on, or including, species more tolerant of these problems (AgResearch 1993). Although there are a large number and variety of grass species world wide, only approximately 40 species have come into use as pasture grasses, with many of these having been improved by plant breeding to produce a range of cultivars (Langer 1990).

In a study using careful selections of compatible species to address key pasture problems and requirements, Moloney (1991) has demonstrated both annual and seasonal yield advantages of grass mixtures over grass monocultures. In general, pastures are renewed with a mixture of herbage species to suit the following objectives: to increase total and seasonal herbage yields, to provide a nutritive balanced diet for livestock without animal health problems such as ryegrass staggers, to allow for soil drainage and fertility variation within a paddock and grazing management flexibility for a range of livestock types (Charlton 1991).

Lolium (ryegrass) species are common pasture grasses in New Zealand, favoured because of their rapid establishment, vigorous growth and their ability to tolerate heavy grazing under a variety of environmental conditions. *Lolium multiflorum* has the ability to grow well in the autumn and winter and produces high quality green feed in winter. However, *L. multiflorum* cannot tolerate extreme climatic conditions such as hot dry summers or cold winters (Langer 1990). In many situations such as in high hill country and drought prone areas, there are a variety of other species such as *Festuca arundinacea* that are better adapted (Cornford and Coolbear 1992).

F. arundinacea is a versatile temperate pasture grass (Hill *et al.* 1985[a]). It has many agronomic and forage attributes such as hardiness and desirable chemical

composition. Once established it exhibits competitive and aggressive growth (Barnes 1990) giving a high annual yield (AgResearch 1993). The high summer-autumn growth of *F. arundinacea* and the ability to persist under a wide range of environmental conditions are important attributes for New Zealand agriculture (Brock *et al.* 1982). *F. arundinacea* is also useful in more difficult environments, including dryland, peat and saline soils and where pests such as grass grub and Argentine stem weevil are a problem (AgResearch 1993), and can tolerate acid and alkaline soils (Langer 1990). The coarse, deep root system forms a dense sod which withstands water logging and flooding. The dense green turf is resistant to animal traffic and can withstand considerable grazing (Barnes 1990). New Zealand varieties do not contain endophyte fungus, which is present in wild plants and can cause circulatory disorders in livestock (AgResearch 1993).

In addition, recent work has shown *F. arundinacea* to be superior in animal and pasture performance to *L. multiflorum* under certain conditions. In a 3 year AgResearch trial it had a higher growth rate over spring, summer and autumn. Grazing *F. arundinacea* pastures instead of *L. multiflorum* dramatically improved beef production by 74% and also mutton production over a particularly dry summer. Two other trials at different locations showed similar performance advantages. Recent discoveries showed that *F. arundinacea* also has much lower levels of zearlalenone and facial eczema toxins (Anon. 1993).

In difficult climates it is often advantageous to combine the vigorous qualities of *L. multiflorum* with the versatility of *F. arundinacea* to result in a pasture which is high in quality all year. However, the vigour of *L. multiflorum* which is advantageous in many situations can jeopardise the establishment of other species (Langer 1990). *F. arundinacea* has poor seedling vigour (as described in detail on the following pages) which places it at a competitive disadvantage in mixed sowings (Charles 1965; Rhodes 1968). Therefore at present it is strongly recommended not to sow *F. arundinacea* with *L. multiflorum* (AgResearch 1993).

2.2 Effects of seedling vigour on seedling establishment

The rate at which seeds grow into successful, fully self-supporting plants, seedling vigour, is an important characteristic of seedling establishment. Following germination the seedling is dependent upon the food reserves present in the seed until it is able to rely on its own photosynthetic products for growth (Figure 1) (Langer 1990).

During the establishment stage, intense competition from within the species and from other species may be high and result in the loss of many seedlings. The ability of a species to compete in mixed sowings depends largely on its rate of germination and the vigour of the emerging seedling (Brock *et al.* 1982). Similarly under unfavourable environmental conditions only those plants with a high degree of seedling vigour will be able to survive (Youngner and McKell 1972).

There are many factors that affect germination and post germination processes such as primary and secondary dormancy mechanisms (Cornford and Coolbear 1992), water absorption (McWilliam *et al.* 1970), the ambient temperature and the germination, emergence and early growth characteristics of the genotype (Hill *et al.* 1985[b]). These include the amount of seed reserves (Bryant 1985), time to emergence (Naylor 1980), root extension (Rhodes 1968), tillering and seed size (Harkess 1956; Black 1957; Brown 1977).

In contrast to cereals, pasture grasses do not have large seeds with extensive food reserves, however, various seedling characteristics have evolved which may be influential on seedling establishment (Youngner and McKell 1972). Early seedling root growth has been shown to be correlated with seed size (Kittock and Patterson 1962; Tadmor and Cohen 1968) and is largely dependent on endosperm reserves as photosynthetic activity of leaves is initially insufficient to support root growth and metabolism (Youngner and McKell 1972). Although the size of endosperm (seed) is positively correlated with seedling vigour within a cultivar, there is less relationship between cultivars of a species and little or none between species (Wright

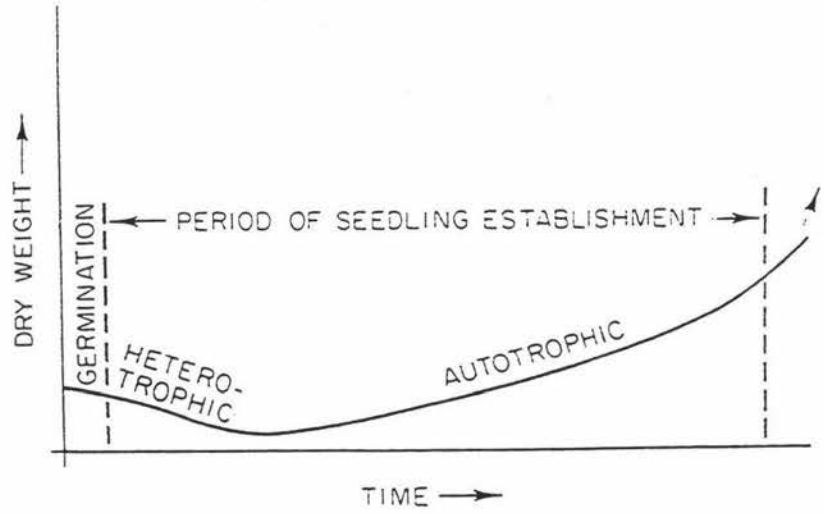


Figure 1. Period of grass seedling establishment showing relative importance of the various stages of seedling growth (Youngner and McKell 1972).

1971 cited in Brock *et al.* 1982).

Within a given species the extension of the mesocotyl, which is the first internode formed above the endosperm and scutellum, tends to be similar for seeds of different weight with the lighter seeds producing a thinner structure. Comparisons between species, however, have shown mesocotyl extension to vary with the size of the seed, with potential mesocotyl extension being least in small seeded species (Jones 1972 cited in Jones and Lazenby 1988).

Seed age can be a negative factor in seedling vigour unless storage of seeds is under conditions that retard embryo and endosperm deterioration. Older seeds have been shown to germinate more slowly, they may produce malformed seedlings and the resulting seedlings are often slower in growth (Kittock and Law 1968). Similarly, loss of resistance to unfavourable environmental conditions such as plant pathogens, disease, mechanical damage, temperature and humidity may also be a direct expression of reduced seedling vigour (Youngner and McKell 1972).

The above factors all may contribute to the varying abilities and rates of seedling establishment (Hill *et al.* 1985[b]). One key factor, related to these is the ability of the seed to mobilise carbohydrate reserves rapidly in the endosperm and to transport these to the growing points. There are very large differences among pasture species in their early seedling growth, and part of this appears to be due to differences in mobilisation of reserves (Langer 1990).

The poor seedling vigour of *F. arundinacea* has been attributed to slow mobilisation of seed reserves, slow root growth (Brock *et al.* 1982) and slow tillering (Hill *et al.* 1985[b]). The inherent root relative growth rates (Brock *et al.* 1982) (which may or may not be dependent on the efficiency of reserve mobilisation) have been suggested as the critical limiting component in the establishment of this species (Cornford and Coolbear 1992). Studies by Brock *et al.* (1982) and Hayes (1976) found that root appearance was earlier, root elongation rates faster, and endosperm utilisation rates higher in *L. perenne* than *F. arundinacea.* Brock *et al.* (1982), in an attempt to

identify the characters responsible for the slow establishment of *F. arundinacea*, showed that after 10 days germination, *L. perenne* seedlings utilise 47% of their endosperm reserves compared with only 14% for *F. arundinacea* seedlings. The ideas that emerged from this study were that these differences were either due to relative growth rate differences, or that there were differences in the mobilisation processes of the endosperm. These results were based on somewhat limited data, in that not enough replicates were used and changes in seed, root and shoot dry weights were not measured. In addition, not enough germination counts were done to determine an accurate T_{50} and thus clearly differentiate between differences in rates of radicle emergence and subsequent post-germinative events such as reserve mobilisation. These limitations indicate a need for further detailed study.

If as in cereals, nutrient uptake from the soil often commences well before the exhaustion of endogenous reserves (McWilliam *et al.* 1970), plants with greater root surfaces would be able to take up nutrients rapidly, enhancing their growth and consequently their competitive ability compared with plants with smaller root systems. *L. Perenne*, with its longer and quicker developing roots would have a clear advantage over *F. arundinacea* in this way (Brock *et al.* 1982). In addition, root competition for nutrients has been shown to be of more importance than above ground competition (Wheeler 1987).

2.3 Seed structure and development

Seeds are very complex structures which are made up of genetically and physiologically distinct components whose functions change during their development (Bewley and Black 1985). It is important to have an awareness of the changes a seed undergoes during development as these changes have an influence on the seeds' behaviour during germination and early seedling growth. They may also be responsible for the variation in germination both within and between *Gramineae* species.

The conversion of an ovule into a seed is triggered by fertilisation with the developing grain remaining within the spikelet until germination (Chapman and Peat 1992). Before fertilisation, the ovule consists of one or two outer folds of tissue (integuments), inner tissue (nucellus) which surrounds the megagametophyte (embryo sac), and a funiculus which connects the ovule to the placenta of the ovary (Crouch 1987). Within grasses there is a great variety of breeding systems ranging from apomixis to complete sexuality. Many of the annual species tend to be self fertilised, while many perennials such as *F. arundinacea* and *L. multiflorum* are cross fertilised and may remain sterile if selfed (Langer 1974). Two fertilisation events occur in the *Gramineae*. One male gamete from the pollen tube fuses with the haploid egg nucleus to form the zygote and to initiate the process of embryogenesis. The second male gamete fuses with the two female polar nuclei to form triploid endosperm (Goldberg *et al.* 1989).

Although the development of the seed coat, endosperm and embryo are usually triggered by the same events, their development proceeds at different rates (Crouch 1987). For example, in a study of perennial ryegrass seed development, Elgersma and Sniezko (1988) demonstrated a big lag in early embryonic versus endospermic division, and this has been found to vary considerably between species (Hill 1971).

Literature describing seed development in the *Gramineae* tends to be focused on cereals with little known about the development of seeds in forage grasses. However, the pattern of reproductive growth in the pasture grasses has been found to be a much less uniform process than in cereals such as wheat or barley (Anslow 1964; Elgersma and Sniezko 1988). Reeder (1957), in an investigation of about 700 species from 150 genera of *Gramineae* showed that there were six different categories of mature embryo types. This emphasises the diversity of developmental patterns within the *Gramineae*. Harvested seed may be of variable quality due not only to variation in production conditions between lots, but also to the inherent heterogeneity of the grass seed crop (Coolbear and Hill, unpublished). This could present a problem in studies on grass seed behaviour.

2.3.1 Seed coat

The integuments of the ovule develop into the seed coat (testa). It is often the only protective barrier between the embryo and the external environment (Bewley and Black 1985). In the *Gramineae*, the ovary wall (pericarp) and the testa are fused (Figure 2). Hence, a cereal or grass seed is technically known as a one-seeded, non-dehiscent fruit termed a caryopsis (Jones and Lazenby 1988). The caryopsis alone forms the grain of the 'naked-grain' cereals such as wheat and maize and can vary enormously in size. However, in most grasses, including *F. arundinacea* and *L. multiflorum* the caryopsis is retained within protective accessory structures, the palea and lemma (Chapman and Peat 1992).

The testa bears a scar, the hilum, marking the point at which the seed was attached to the ovary wall (Bewley and Black 1985). Following the completion of nutrient transfer from the maternal plant to the embryo, the testa can become specialised for its function in the mature seed and in subsequent germination. The testa may be involved in the inhibition of germination due to permeability restriction, physical constraint of the embryo, the presence of chemical inhibitors or modification of the light reaching the embryo. Special substances may accumulate in the seed coat to help regulate water entry during germination, or enzymes for storage reserve degradation may be localised in the inner seed coat wall. Thus, the mature seed coat is an assemblage of specialised cell types that finally become desiccated and compressed when the seed is ready to be shed (Crouch 1987).

2.3.2 Endosperm

The endosperm occupies a large part of the seed and represents the nutrient store that is mobilised during germination to nourish the growing seedling. Cells of the endosperm are packed with starch granules in a matrix of storage proteins and constitute the target tissue for enzymes secreted by the aleurone and scutellum (Fincher 1989).

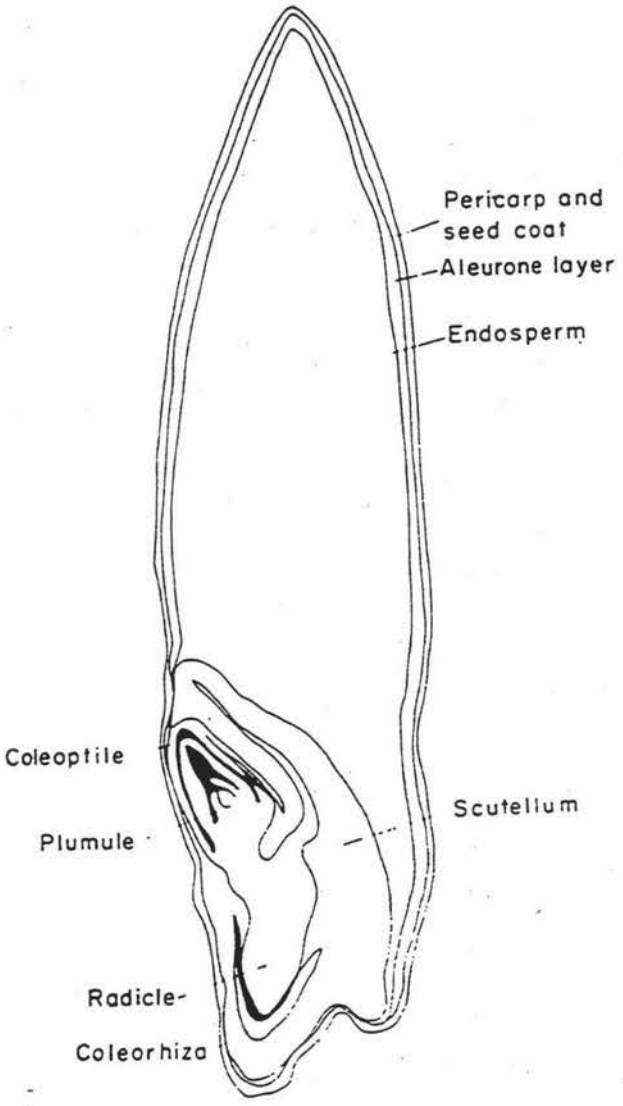


Figure 2. Transverse section of a wheat grain (Mayer and Poljakoff-Mayber, 1966)

When endosperm development is complete, the inner cells of the endosperm are generally dead tissue. The accumulation of insoluble food reserves is such that any surviving cytoplasm is very distorted and does not survive desiccation (Bewley and Black 1985).

2.3.3 Aleurone layer

During grain development, the outer endosperm differentiates into the aleurone which is a morphologically and functionally distinct layer. It may range from one to several layers in thickness depending on the species. Little information, however, seems to be available on details of endosperm structure in pasture grasses. Although the origin of the aleurone and the starchy endosperm cells is the same, only the cells of the aleurone layer remain alive after the storage reserves of the endosperm are deposited and the grain matures and dies (Fincher 1989).

Some enzymes capable of starting the process of food reserve breakdown are contained in the cells of the aleurone layer. Upon germination, their release in an active form is triggered by gibberellic acid and/or calcium ions. The most efficient enzymes for reserve mobilisation, however, are synthesised *de novo* in the aleurone layer as germination begins. This is discussed in detail in Chapter 2.9.

2.3.4 Embryo

During seed development, the embryo of the *Gramineae* differentiates into two organ systems, the embryonic axis and a much reduced and modified cotyledon - the scutellum (Goldberg *et al.* 1989). The scutellum is a shield of tissue which separates the embryo from the endosperm where it can participate in enzyme secretion (Fincher 1989) and absorption for the embryo. It takes up the mobilised reserves from the endosperm during germination and early seedling growth (Mayer and Poljakoff-Mayber 1966).

The embryonic root (radicle), the hypocotyl to which the cotyledons are attached, and the shoot apex with the first true leaves (plumule) are incorporated into the axis. A coleoptile covering the first leaves is formed from the elongated basal sheath of the cotyledons and in several species such as maize, the hypocotyl is modified to form a mesocotyl. The base of the hypocotyl which covers the radicle is the coleorhiza (Bewley and Black 1985). At seed maturity the embryonic axis can be clearly distinguished from the scutellum. The aleurone layer, embryonic axis and scutellum are the only living tissue in the quiescent seed (Fincher 1989).

The relative proportions of embryo and endosperm vary among the grass subfamilies. In the festucoid grasses, which include both *F. arundinacea* and *L. multiflorum*, the embryo is usually very small relative to the caryopsis, but this is not always the case with panicoid species where the embryo may constitute 50% of the seed (Reeder 1957).

2.4 The transition from seed development to germination

Morphologically and biochemically, the most dramatic events of seed development occur during maturation, preparing the seed for germination and subsequent development of the mature plant. During maturation, the developing seed increases dramatically in volume and mass due to cell expansion and the accumulation and storage of protein and lipid to be used as nitrogen and carbohydrate sources during germination (Thomas 1993).

The plant embryo is capable of germination during its late development but is constrained from doing so while attached to the mother plant. A variety of evidence suggests that something actively maintains embryogenesis until a termination switch is thrown that allows subsequent germination, however, there is little agreement about the identity of the maintenance factors and switches or when they act (Galau *et al.* 1991). During late embryogenesis, abscisic acid (ABA) levels have been found to reach a maximum, suppressing precocious germination prior to desiccation and

modulating gene expression (Dure 1985; Morris *et al.* 1988). Desiccation ensures seeds do not germinate even in the absence of ABA (Thomas 1993).

In most *Gramineae* species, desiccation is the terminal phase of seed development leading to a state of metabolic quiescence which acts as the boundary between development and germination (Kermode and Bewley 1985). The moisture content of the whole seed can fall from 80% to as low as 10% in a few days. Despite this extreme dehydration, all the cells of the aleurone and embryo remain alive (Osborne 1983).

Desiccation dramatically changes the subsequent biochemical activity of the living cells of the embryo. Some of the proteins produced during seed development are no longer synthesised when water again becomes available, and other novel proteins essential to germination are translated.

The performance of the aleurone is also changed. For example, the production of one enzyme (α-amylase) in the cells of the aleurone of wheat and barley can be induced by gibberellins only after the tissue has been subjected to a period of dehydration (Armstrong *et al.* 1982; Osborne 1983). It has been suggested that desiccation is necessary for the development of complete sensitivity to the gibberellins involved in germination of cereals (Evans, Black and Chapman 1975). This transformation to the gibberellin responsive condition marks the normal termination of seed development on the mother plant (Osborne 1983).

Desiccation may operate by decreasing the ABA concentration in the seed, increasing the gibberellic acid concentration, changing the sensitivity of the tissues to these hormones or by having a direct effect on the genome. These options may all operate in different species and/or on different aspects of metabolism of the same seed (Kermode *et al.* 1989).

There is much variation in the onset of germinability even within species, with environmental conditions thought to have major effects. For example, the onset of

germinability of ryegrass seeds formed on early heads compared to those emerging more than a week later on the same plant have been found to vary (Anslow 1964). Variation also occurs in the rate of seed development depending on the position of the floret within the ear (Elgersma and Sniezko 1988).

2.5 Seed ageing

Depending on species and storage conditions, the desiccated embryos and aleurone of the *Gramineae* may remain alive for weeks, years or decades. However, the only requirement for renewed synthetic activity and the reinitiation of embryo growth and development is the addition of water. Nevertheless, the condition of the air dry seed does not remain static. Throughout the period it remains dry, continued slow changes occur within the living cells (Osborne 1983). As a result of this, the performance capability of seeds declines during storage with the ageing or loss of vigour characterised by delayed germination and emergence, slower growth, increased susceptibility to environmental stresses and, ultimately, a decline in germinability (Parrish and Leopold 1978; Petruzzelli and Taranto 1990; Livesley and Bray 1991).

2.6 Dormancy

Upon hydration, most mature seeds germinate readily over a range of environmental conditions (Morris *et al.* 1991). However, at this stage of their life many seeds are dormant: they will not germinate when placed in conditions normally considered to be adequate for germination such as suitable temperature, adequate water and oxygen (Roberts 1972). Dormancy helps to ensure that the seed does not germinate if it becomes rehydrated while still on the parent plant (Bryant 1985) and also has adaptive significance as the basis of a temporal dispersal mechanism for many seeds (Villiers 1975).

Dormant seeds can remain viable in a hydrated state for a prolonged period without

cell elongation until a signal is recognised by the embryo which initiates germination. For the first few hours following imbibition, dormant and nondormant seeds exhibit similar physiological responses, including the same rates of water uptake, respiration and total protein synthesis (Bewley and Black 1985). In dormant seeds, however, there appear to be molecular and biochemical restrictions which prevent cell expansion and germination (Morris *et al.* 1991). There is a large variety of dormancy mechanisms including those attributed to the seed coat such as chemical inhibitors and oxygen restriction, impermeability of the seed coat to water and gases and mechanical restriction to embryo growth and development. Embryo immaturity, special temperature or light requirements or the presence of inhibitors are also contributing factors (Murray 1984; Morris *et al.* 1991).

Environmental changes during seed production may have variable effects on induced dormancy levels within seeds. For example, variation in temperature during seed development has been found to effect dormancy of seed in several species with other species showing no effect in dormancy to the maturation temperature (Wiesner and Grabe 1972). Jensen and Pierpoint (1961) cited in Wiesner and Grabe (1972), have shown ryegrass seed dormancy to vary with season and area of production, as well as with species. Dormancy was more pronounced and persistent in *L. multiflorum* than *L. perenne*. Other studies have shown similar variation in ryegrass seed development (Harrison 1954).

A large number of species can be released from dormancy when, in the hydrated condition, they experience relatively low temperatures, generally in the range of 1-10° C. Prechilling is effective in seeds with embryo, coat imposed, primary, relative and secondary dormancy. Dormancy in some herbaceous species is broken by just a few days, or even a few hours, of low temperatures. Temperature alternations have also been found to be effective in breaking dormancy (Bewley and Black 1985).

The molecular basis for dormancy breaking in seeds is not yet known. Ultimately the low temperature response could involve activation of specific genes - a switching of the morphogenetic program in response to low temperatures (Salisbury and Ross

1985). Effects of low temperatures might include differential changes in enzyme concentration or in enzyme production (Bewley and Black 1985). Both inhibitor disappearance and hormone accumulation in whole seeds have been observed during prechilling, but there are numerous contradictions and it is very likely that different mechanisms may operate in different species (Salisbury and Ross 1985).

2.7 Germination requirements

The germination of a viable seed is largely dependent on a favourable temperature and an adequate supply of water and oxygen. At temperatures below about 5°C, germination rate and percentage are both impaired to an extent that varies with species (Chippendale 1949 cited in Jones and Lazenby 1988). Above the species' base temperature (the temperature below which germination cannot occur), only the rate of germination varies, increasing with temperature up to about 20° C (Jones and Lazenby 1988). Alternating temperatures have been found to promote germination in some species (ISTA 1985; Garcia-Huidobro *et al.* 1982[b]).

The germination rate, which is defined as the reciprocal of the time taken for half the population to germinate, usually increases linearly with temperature when this exceeds the base temperature, at least within a well defined range. When the relation between germination rate and temperature is linear, the thermal time or accumulated temperature for germination is a constant and this can be used to compare germination in different species, climates and locations (Garcia and Huidobro *et al.* 1982[a])

$$(T - T_b) \times t = K$$

t = time to radicle emergence

T = ambient temperature

T_b = base temperature below which no germinative metabolism can occur

K = thermal time constant

2.8 The germination process

Imbibition initiates a rapid resumption of metabolic activity in the quiescent seed (Figure 3). The ability to reinitiate metabolism so quickly indicates that the components necessary to sustain metabolism, such as enzymes and the membrane system, survive the dehydration process. This renewal of metabolic activity is directed at growth, although some of the metabolic activity during the early stages of germination is related to repair and replacement processes (Bryant 1985).

Heterotrophic growth of *Gramineae* seedlings depends upon utilisation of proteins, carbohydrates and other nutrients stored in the endosperm (Grierson and Covey 1985; Brown and Ho 1986). The reserve proteins, carbohydrates, lipids, phosphates, calcium and cofactors, together with hydrolytic enzymes and mitochondria allow the rapid proliferation of the intracellular membrane system and the protein synthesising machinery and provide amino acids necessary for the synthesis of hydrolytic enzymes after the initiation of germination (Bewley and Black 1985).

Germination plays a central role in the propagation of cereal crops, therefore a large research effort has been accorded the regulation of enzymes responsible for endosperm mobilisation in germinating grains (Fincher 1989). The nature and location of food reserves and their fate have been the focus of extensive studies (Cornford and Coolbear 1992). Many studies have been based on barley, where the breakdown of storage products during the malting process has economic significance (Akazawa and Miyata 1982; Akazawa *et al.* 1988), and also on wheat and triticale, in which premature grain sprouting is a problem (Fincher 1989). However, knowledge of reserve mobilisation processes in other grasses is extremely limited and assumptions are often made that the processes determined for cereals will be directly applicable to pasture grass species (Cornford and Coolbear 1992).

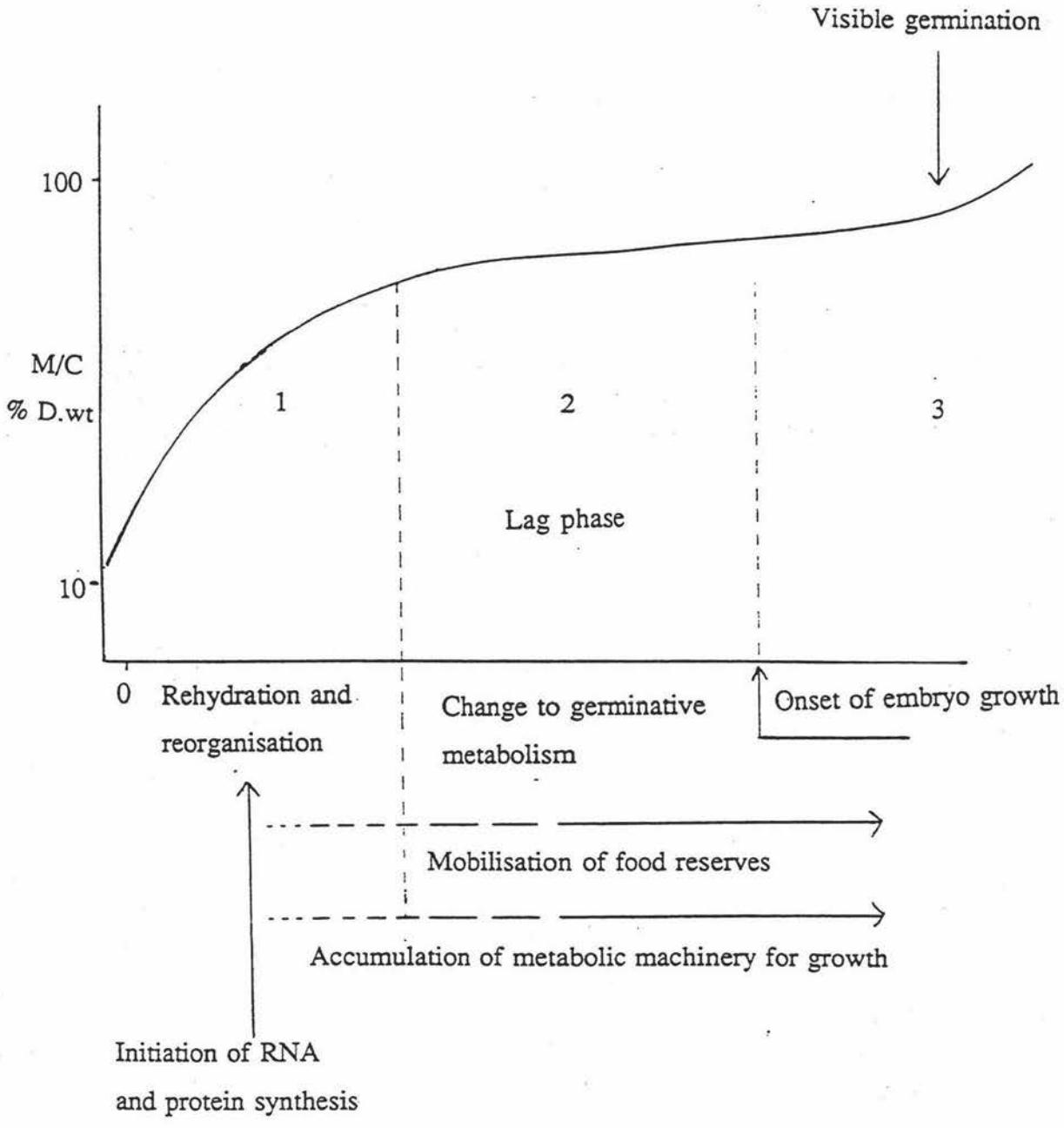


Figure 3. Germination described by the imbibition curve (Coolbear)

Germination consists of those processes which begin with water uptake and which successfully terminate with the emergence of the radicle or hypocotyl through the seed coverings. All events subsequent to this have been taken to be part of or associated with seedling growth. Thus mobilisation of food reserves according to this definition is not strictly a component of germination, however, since it is uniquely associated with the germinated seed it is nevertheless best considered in this context (Bewley and Black 1985).

2.9 Reserve mobilisation

Mobilisation of food reserves commences after radicle elongation. The high molecular-weight reserves contained within the storage areas of the seed are all polymers and as such cannot be transported to the growing embryonic axis and must therefore be hydrolysed to yield smaller, readily transportable molecules (Bewley and Black 1985; Bryant 1985).

Although the endosperm is the major source of carbohydrate reserves in cereals, some low molecular-weight sugars are stored in much smaller quantities within the embryo. These provide an early source of respirable substrate during germination and early seedling growth until the hydrolytic products of starch are made available from the endosperm. Although these reserves are only present in small amounts, the products of their hydrolysis might be important for early seedling establishment. For example, sucrose and raffinose have been found to be present in the dry grains of barley, cv. Proctor with both declining to imperceptible levels within the first 1-2 days from the start of imbibition; replenishment of carbohydrate from the endosperm as sucrose began on the third day (Bewley and Black 1985). Reliance on the stored reserves diminishes as the seedling emerges above the soil and becomes photosynthetically active, i.e. autotrophic.

A few days following imbibition, mobilisation of the stored reserves is quite noticeable as the endosperm becomes partly liquefied. Transverse sectioning of the

endosperm at different times after imbibition shows that the solubilisation process begins at the outside, in association with the cells of the aleurone layer, and progresses inwards. Further, if the embryo is removed from the endosperm at an early stage following imbibition no solubilisation is found (Grierson and Covey 1985).

There have been observations that isolated barley embryos have been found to synthesise gibberellins, whereas embryoless half-grains or isolated aleurone layers generally fail to synthesise hydrolytic enzymes, and that with the addition of gibberellins or gibberellin-synthesising embryos to the half grains or the aleurone layers, enzyme synthesis is induced in a dose dependent way (Schuurink *et al.* 1992). It is generally accepted, therefore, that gibberellins released from the embryo during the initial stages of germination, diffuse to the aleurone layer and induce reserve mobilisation and the synthesis and secretion into the starchy endosperm of hydrolytic enzymes (Figure 4) (Fincher 1989). These hydrolases then break down storage materials into small components, which are used by the seedling to maintain growth until photosynthesis commences (Nolan and Ho 1988).

The process of gibberellin-induced synthesis and secretion of hydrolases has a temporal component. In cereals, hydrolase activity secreted from aleurone layers soon after hormone treatment (<9 hours) include phosphoesterases, β-glucanase and carboxypeptidases. For these enzymes, gibberellic acid (GA_3) is apparently only required for secretion of the activities as they are already present within the aleurone layers when incubated without hormone. Enzyme activities secreted 10-24 hours after GA_3 induction include α-amylase and β-1,3:1,4-glucanase. The hydrolases, including nuclease, are secreted from aleurone layers following prolonged exposure (>24 hours) to GA_3 (Brown and Brodl 1988). All of these later induced hydrolytic enzymes are synthesised *de novo* during germination but only α-amylase and proteases require the presence of GA_3 (Grierson and Covey 1985).

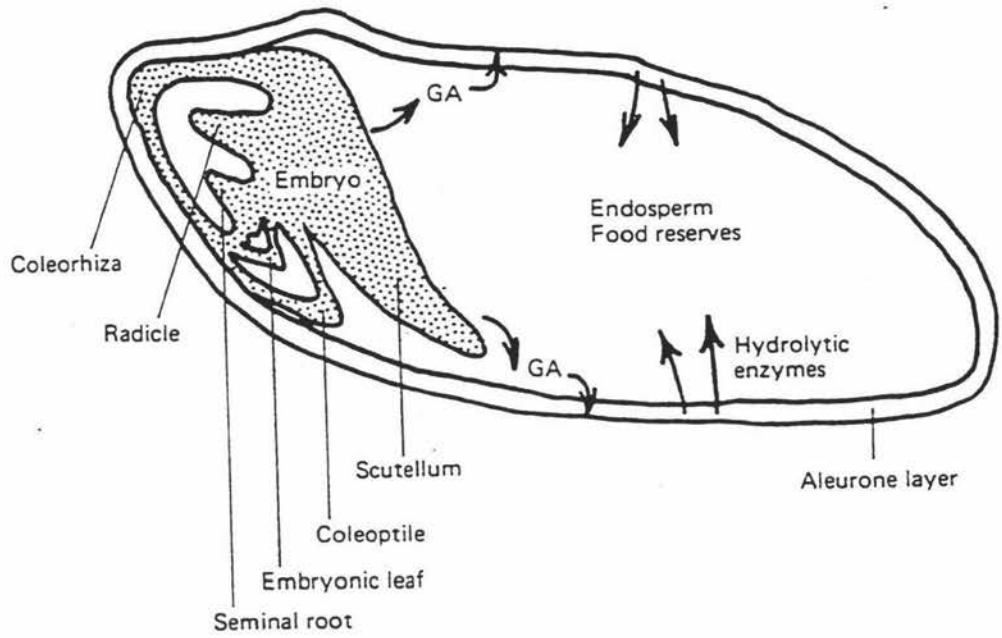


Figure 4. Mobilisation of food reserves in barley grains. The synthesis and secretion of gibberellic acid by the scutellum of the barley embryo stimulates the aleurone layer to synthesise and secrete hydrolytic enzymes which digest the stored food reserves in the endosperm (Grierson and Covey 1985).

Although it is widely accepted that both the aleurone layer and scutellum of cereal grains produce α-amylase, the relative contributions of each to total starch breakdown are disputed (Jones 1985; McFadden *et al.* 1988; Pogson *et al.* 1989; MacGregor *et al.* 1984; Palmer 1988 and Miyata *et al.* 1981). Anatomical differences between species may partly explain the varying conclusions which have been drawn. In barley for example, the aleurone layer which is as many as three to four cells thick is the principal source of hydrolases, while in corn, oat, rice and wheat where the aleurone layer is generally only one cell thick it may be a less important source of these enzymes (Jones 1985).

The processes of seed reserve mobilisation in pasture grasses can clearly not be assumed to be the same as that just described for cereals. Studies have already shown that in grasses such as *L. multiflorum* and *Phalaris aquatica* the spectrum of α-amylase isoenzymes is distinct from those of wheat and from each other. In addition, species differences in aleurone tissue responsiveness to added gibberellin and the presence of α-amylase inhibitor activity have been identified (Cornford and Coolbear 1992).

In barley, the first change that the embryo appears to undergo is the digestion of the intermediate layer adjacent to, and between the cells of the absorptive epithelium of the scutellum. The enzymes suggested to be responsible for digestion of the cell walls of the intermediate layer are endo-β-glucanases, a class of enzymes capable of degrading hemicellulose (glucans containing β-1,3 and β-1,4 links). In some cereal grains (eg. those of the oat family, but not barley or wheat) the scutellum and its' epithelial cells then elongate, thus presenting a larger surface area for absorption of the hydrolytic products of the endosperm reserves (Bewley and Black 1985).

Cell wall degradation allows access of α-amylase and proteases to their substrates within the endospermal cells (McFadden *et al.* 1988). α-Amylase degrades intact granules of cereal starches to form a complex mixture of saccharides (Crouch 1987). This is accomplished primarily through the random 1,4-α-endoglycolytic cleavage of

amylose and amylopectin, the principal components of starch granules in plant cells (Huang *et al.* 1992).

Although details of regulation of enzyme synthesis by GA_3 are beginning to be understood, far less is known about the mechanisms of intracellular transport and secretion of the inducible proteins. An aspect of GA_3 action related to enzyme secretion in both barley and wheat is the substantial synthesis and proliferation of endoplasmic reticulum (ER) elicited by the hormone. This is associated with increased membrane synthesis, formation of golgi and the stimulation of polyribosomal formation (Brown and Brodl 1988; Grierson and Covey 1985; Sakai-Wada and Nakada 1987).

Barley α-amylases are synthesised on the ER and exocytosis probably occurs through the golgi apparatus (Gubler *et al.* 1986). During transport through the endomembrane system α-amylases are post-translationally modified in the lumen of the ER by addition of Ca^{2+} which activates and stabilises the enzyme (Jones *et al.* 1993).

Following the release of gibberellins from the embryo, there is also an increase in β-amylase activity which appears to be due to the transformation of an inactive or latent form of the enzyme to an active form (Duffus and Duffus 1984). β-Amylase is also involved in the digestion of barley starch by attacking the ends of the macromolecules, but it is not *de novo* synthesised, nor is it released from either the scutellum or aleurone layer. It is present in the starchy endosperm of the mature ungerminated grain where, presumably it becomes active (or activated) after initial digestion of the starch grains, by α-amylase (Bewley and Black 1985).

2.10 α-Amylase

α-Amylase is by far the most abundant protein synthesised and secreted by GA_3-induced aleurone cells (up to 70% of the total protein synthesised in germinating

barley in the first 24 hours: Deikman and Jones, 1985) and has been the focus of extensive studies (Brown and Brodl 1988; San Segundo *et al.* 1990). It is the enzyme primarily responsible for the hydrolysis of the starchy endosperm to produce the sugars needed to sustain the growth of the emerging seedling. For this reason, studies on rice have shown expression of α-amylase activity to be positively correlated to important agronomic traits such as germination rate, seedling vigour, yield and cold tolerance (Williams and Peterson 1973).

The synthesis of poly A-containing mRNA, including that coding for α-amylase, is also promoted by GA_3. When placed in an *in vitro* protein synthesising system, this mRNA catalysed the synthesis of α-amylase and several other proteins, evidence that GA_3 controls α-amylase synthesis at the transcriptional level. Further evidence in support of transcriptional regulation of α-amylase by GA_3 was obtained from nuclear run off transcription experiments within isolated aleurone protoplast nuclei. The amount of initiated α-amylase gene transcripts increased by up to 14 fold when cells were treated with GA_3 (Brown and Brodl 1988).

More recently α-amylase mRNA has been measured by hybridising nick-translated cDNA probes to aleurone layer RNA after Northern blotting. This more sensitive technique shows that α-amylase mRNA appears within 1 h of applying GA_3 to aleurone layers and continues to increase with time. The appearance of mRNA is prevented if cycloheximide is added at the same time as GA_3 suggesting that there is a requirement for protein synthesis in order for α-amylase mRNA to be made. Such a protein might function as a receptor for GA_3, in promoting transcription of the α-amylase genes or in stabilising the mRNA (Grierson and Covey 1985).

2.11 α-Amylase isoenzymes

Multiple isoenzymes of α-amylase have been found to exist in several *Gramineae* species. Two groups of α-amylase isoenzymes (low and high pI) have been identified in barley on the basis of their migration on isoelectric focusing gels

(Jacobsen and Higgins 1982). High and low pI α-amylase can also be distinguished by different amino acid sequences, in their affinity for the Ca^{2+} ion and in their antigenic properties (Jones and Jacobsen 1983; Bush *et al.* 1989). Each isoenzyme group is encoded by a set of genes with high and low pI gene sets on different chromosomes (Brown and Jacobsen 1983). Wheat α-amylase genes have been divided into three subfamilies and in rice, several α-amylase genes have been isolated and characterised (Ranjhan *et al.* 1991; Huang *et al.* 1992).

In barley, incubation of aleurone layers without hormone results in a low level of low pI α-amylase expression, whereas high pI α-amylase is not expressed (Jacobson and Chandler 1987; Nolan, Lin and Ho 1987). The addition of GA_3 results in a dramatic increase in the expression of both α-amylase isoenzyme groups within 4-8 h. The expression of high pI α-amylase is greatly reduced after 24 h of hormone treatment, whereas the expression of low pI α-amylase isoenzymes remains at a high level. Several *Gramineae* species appear to produce only low pI α-amylase isoenzymes (Marchylo *et al.* 1987 cited in Cornford and Coolbear 1992).

The differential expression of α-amylase isoenzymes is observed at both the protein and RNA levels. This, along with evidence discussed in the previous section, indicates that GA_3 controls the induction of α-amylase isoenzymes at the transcriptional level.

Many abundant proteins in the endosperm of barley and other cereals are inhibitors of α-amylases and proteases (Mundy and Rogers 1986). Inhibition of α-amylase activity and immunochemical studies have lead to the detection of an endogenous α-amylase inhibitor in barley, wheat, rye and triticale, but not in sorghum, oats, millet, rice or maize (Weslake *et al.* 1985). Little information is available for pasture grasses. The precise role of these inhibitors is not known, but the general assumption is that they may protect the grain from exogenous hydrolases (Mundy and Rogers 1986), or possibly premature activity.

2.12 The effects of calcium

The importance of calcium for the production of α-amylase by the barley aleurone layer was established by Chrispeels and Varner (1967). They showed that while GA_3 alone was sufficient to promote α-amylase production in embryoless half seeds, Ca^{2+} in addition to GA_3 was necessary to obtain a high rate of α-amylase production in isolated aleurone layers.

Barley α-amylase is a Ca^{2+}-containing metalloprotein (Jones and Jacobsen 1983). The activity and stability of the barley α-amylase molecule is dependent on the binding of at least 1 atom of Ca^{2+} per mole α-amylase, and Ca^{2+} binding requires a Ca^{2+} concentration of at least 10 μM (Bush *et al.* 1989).

Experiments with isolated aleurone layers and aleurone protoplasts show that Ca^{2+} regulates secretion of barley α-amylase at the intracellular transport level rather than through an ion exchange process in the cell wall. Individual isoenzymes of α-amylase are affected differentially by Ca^{2+} and GA_3. Ca^{2+} does not effect the accumulation of α-amylase mRNA, which suggests that it has no effect on the transcription of these genes (Deikman and Jones 1985).

Calcium ions also influence the release of other enzymes in the aleurone layer of barley (Jones and Jacobsen 1983). These enzymes, unlike α-amylase, are not metalloenzymes, therefore any effects of Ca^{2+} on their activity are unrelated to effects of this ion on α-amylase (Carbonell and Jones 1984).

Although Ca^{2+} may have an affect at several levels, the molecular mechanism for Ca^{2+} participation in GA_3-induced responses is not yet clear (Fincher 1989). Once again, there is no information on the role Ca^{2+} might play in reserve mobilisation in pasture grasses.

2.13 Abscisic acid interactions

Abscisic acid (ABA) is another hormone which effects gene expression, protein synthesis and secretion in barley aleurone layers (Brown and Brodl 1988). ABA and gibberellin play major and opposing roles in the development and germination of cereal grains, with levels of ABA reaching a peak during seed development and declining during seed maturation (Bewley and Black 1985; Lanahan *et al.* 1992). ABA is involved in the regulation of embryo maturation and suppression of precocious germination (Finkelstein and Crouch 1986).

ABA has been found to antagonise GA_3 action in a number of responses in barley aleurone layers, such as preventing the expression of GA-induced synthesis and secretion of hydrolases (Brown and Brodl 1988; Lanahan *et al.* 1992; Nolan and Ho 1988). For α-amylase isoenzymes, these effects are mediated at the mRNA level (Nolan, Lin and Ho 1987). Little is known of the molecular events that connect ABA or GA with activation or suppression of genes, and less is known about the mechanisms that direct their tissue specific expression (Riviv and Grudt 1991; Lanahan *et al.* 1992).

2.14 Conclusion

Although reserve mobilisation in cereals such as barley and wheat has been studied extensively, knowledge in this area for grasses is very limited. Given the variation that occurs in cereal species it is dangerous to assume that model systems can be generalised to *L. multiflorum* and *F. arundinacea.* In addition, the timing of events between seed lots of the same species may vary depending on pre- and post- harvest conditions.

A more detailed understanding is required not only of the reserve mobilisation

processes in grasses but also how they relate to the processes of germination and especially seedling growth. Only in this way will we begin to understand whether successful reserve mobilisation is a rate limiting step in seedling establishment.

3. Materials and Methods

3.1 Plant material

Seeds of *Festuca arundinacea.* Schreb [Tall fescue] cv. Roa (harvested December 1990) and *Lolium multiflorum* Lam. [Italian ryegrass] cv. Paroa (harvested September 1991) were supplied by AgResearch Grasslands, Palmerston North, New Zealand. Until January 1992, seeds were stored at 0° C with 30% relative humidity. Seed batches were then stored in plastic bags in opaque 20 l moisture-proof containers at 5°C.

3.2 Seed size variation

To determine the level of variation in seed size in the *F. arundinacea* seed lot, 200 seeds were completely dehusked and weighed individually using a five decimal place balance. The mean seed weight was also determined.

3.3 Calculation of the percentage of empty seeds

A number of *F. arundinacea* seeds were found to be empty following the removal of the husk. In order for this to be taken into account when comparisons were made with *L. multiflorum* (which didn't have this noticeable number of empty seeds), the percentage of empty seeds in the *F. arundinacea* seed lot was estimated.

Groups of 100 seeds, which had been subsampled using a soil divider, were dehusked and the average empty seed % was calculated. Dehusking individual seeds determined accurately the empty seed % but it was destructive and time consuming. Further groups of 100 subsampled seeds were then spread over a light box. The light

shining through the seeds showed up seeds which were empty with these seeds being picked out. Several of these groups of 100 seeds observed over the light box were then dehusked to validate the light box method. The use of the light box method to determine the average % empty seed of the seed lot was found to not be as accurate as dehusking the seeds. When seeds were observed over the light box 1.11 ± 0.18% appeared to be empty, however, dehusking the seeds found the average % empty seed to be higher at 4.45 ± 0.45%.

3.4 1000 Seed weight

F. arundinacea seed was spread over a light box for the detection and removal of empty seeds and impurities. Eight replicates of 50 full seeds were weighed, with each replicate being returned to the original sample prior to the selection of the next 50 seeds.

3.5 Tetrazolium testing

When staining with tetrazolium, a red colour develops when hydrogen from respiration processes of living cells combines with absorbed tetrazolium solution. Distinct colour changes between normally stained, firm tissues and white, flaccid tissues are evidence that the unstained tissues are dead. Grabe (1970) describes in detail the features of staining which determine the viability of the seed.

Two hundred *F. arundinacea* seeds were tested in replicates of 50 seeds. Seeds were selected from subsampled pure seed and were prepared for sectioning by placing them on moist blotters for 18 h in darkness at room temperature. Following imbibition, the seeds were bisected longitudinally, exposing the main structures of the embryo. One half of each seed was transferred immediately to a watch glass containing 20 ml tetrazolium solution (0.5% w/v 2,3,5-triphenyltetrazolium chloride solution in distilled water). The pH of the solution was required to be between 6 and

8 for optimum staining. Seeds were incubated in the tetrazolium solution for 2.5 h at 35° C. The seeds were then examined using a dissecting microscope. Photographs showing the variation in seed viability were taken using a stereo photo-microscope. The seeds photographed were selected as being representative of the whole sample.

3.6 Seed germination

The optimum germination conditions for *F. arundinacea* were found by carrying out germination tests under a variety of temperature treatments within International Seed Testing Association (ISTA) recommendations as described below. These germination conditions were then used in subsequent time-course experiments comparing the germination and early seedling growth characteristics of *F. arundinacea* and *L. multiflorum*. In all experiments, seeds were sown on blotting paper of double thickness on which grids of 25, (2.5 cm x 2.5 cm,) squares had been drawn. The blotting papers were saturated equally with 20 ml 0.2% (w/v) KNO_3 and placed in sealed containers with opaque lids in darkness.

a) Optimisation

To determine the optimum germination conditions for *F. arundinacea*, eight replicate samples of 50 seeds were used for each of four different temperature treatments, with two seeds sown per square. Four replicates from each temperature treatment were prechilled for 7 d at 5° C. These replicates were set up 7 d earlier than the non prechilled samples. Following prechilling all samples were randomised in growth cabinets at their different temperature treatments of a) alternating temperatures of 15° C for 16 h and 25° C for 8 h, b) alternating temperatures of 20° C for 16 h and 30° C for 8 h, c) constant 20° C, d) constant 30° C. The germinated seeds from each treatment were counted daily for the first 7 d and then every 2 d until they had been exposed to their different temperatures for 17 d. The median germination time (T_{50}) and the uniformity of germination (T_{90} - T_{10}) were calculated as described below. The final root and shoot lengths were measured using a graduated ruler.

b) Time course experiments

For germination time-course experiments, between three and six replicate samples of 25 *F. arundinacea* and *L. multiflorum* seeds were sown one seed per square. The seeds were prechilled (5° C for 7 days) and then incubated at 20° C, conditions which had been determined as optimum for germination of *F. arundinacea*. Replicates were removed every two days from day 0 to day 16 of the time-course. Seeds to be used for α-amylase and starch assays were frozen following the removal of their roots and shoots. The germination %, root and shoot lengths and root, shoot and seed fresh and dry weights were measured for the remaining seeds. The root and shoot lengths were measured using a graduated ruler. The dry weights were obtained by incubation at 80° C for 48 hours.

c) Median germination time (T_{50}) and uniformity of germination (T_{90} - T_{10}) calculations

The method described by Coolbear *et al.* (1984) was used to determine the time to 50% radicle emergence (T_{50}) and the time interval between 10% and 90% germination (T_{90}-T_{10}), which is a measure of the uniformity of germination.

$$T_{50} = t_i + \frac{(N + 1)/2 - n_i}{(n_j - n_i)} \times (t_j - t_i)$$

n_i and n_j are the number of seeds germinated at times t_i and t_j,
where $n_i < (N + 1)/2 < n_j$
N = final number of seeds germinated.

$$T_{10} = t_j + \frac{(N + 1)/10 - n_i}{(n_j - n_i)} \times (t_j - t_i)$$

Where $n_i < (N + 1)/10 < n_j$

$$T_{90} = t_i + \frac{9(N + 1)/10 - n_i}{(n_j - n_i)} \times (t_j - t_i)$$

Where $n_i < 9(N + 1)/10 < n_j$

d) Calculation of base germination temperature

As both seed lots showed a prechilling response it was necessary to determine whether prechilling (5° C for 7 d) breaks residual dormancy or if accumulated thermal time could explain all the chilling advantage. The Garcia-Huidobro *et al.* (1982[b]) method was used to determine the effective base temperature of the two seed lots as most germinating seeds follow the thermal-time relationship:

$$(T - T_b) \times t = K$$

t = time to radicle emergence (T_{50})
T = ambient temperature
T_b = base temperature below which no germinative metabolism occurs
K = thermal time constant

The reciprocal of the T_{50} values were plotted against temperature of germination, giving a linear relationship:

$$1/t = (T - T_b)K$$

When $T = T_b$, $t = \infty$, and the base temperature can thus be determined as the intercept of the linear relationship with the x-axis. The calculated base temperatures of the two species can then be used to determine whether germination responses of prechilled seed can be explained by accumulated thermal time alone or whether a change in physiology (i.e. dormancy breaking) has occurred.

T_{50} values were obtained over a range of temperatures for *F. arundinacea* and *L. multiflorum*. Four replicates of 25 seeds were sown, as in the germination conditions described previously, for each temperature treatment used. The germination temperatures used were 7°C, 12°C, 15°C, 20°C and 25°C without prechilling and 20°C with prechilling. The germinated seeds were counted daily until approximately 50% of the seeds had germinated, then seeds were counted every two days until they had been exposed to their temperature treatments for 25 days.

3.7 Starch assay

Starch assays followed the procedure described by Megazyme (1992), Australia. Samples (25 seeds) were ground using a mortar and pestle and transferred to test-tubes. Aqueous ethanol (0.2 ml, 50% v/v) and 1.0 ml, 40 U/ml thermostable α-amylase solution in 50 mM MOPS buffer, pH 7.0, containing 5 mM $CaCl_2$ and 0.02% sodium azide were added to each sample. Samples were then placed in a vigorously boiling water bath. Following 2 min of incubation, the samples were stirred for 10 s on a vortex mixer and returned to the water bath. After exactly 4 min from the time of initial incubation in the water bath, samples were removed and immediately treated with 4.0 ml of diluted pullanase (2.5 U/ml)/β-amylase (125 U/ml) solution in 100 mM sodium acetate buffer (pH 4.5) containing bovine serum albumin (0.5 mg/ml) and 0.2% sodium azide. The test-tubes were then transferred to a 50°C water bath for 1 h.

Each sample was transferred to 100 ml flasks where the volumes were adjusted to the mark with distilled water and mixed. Solutions were then filtered through

Whatman No. 1 filter circles and aliquots of the filtered solution (0.1 ml) were transferred to the bottom of three test-tubes. Amyloglucosidase (0.1 ml, 2 U) in 100 mM sodium acetate buffer (pH 5.0) containing bovine serum albumin (0.5 mg/ml) and sodium azide (0.02%) was added to two of the test-tubes and the tubes were incubated for at 50°C for 10 min. To the third tube (the sample blank) was added 0.1 ml sodium acetate buffer (pH 5.0). This tube was concurrently incubated at 50°C for 10 min.

Glucose oxidase/peroxidase reagent (3.0 ml) was added to each tube, including the glucose standards and reagent blanks and the tubes were incubated for 20 min at 50°C. The absorbance at 510 nm was read for each sample, the sample blanks and the glucose standards against the reagent blank. Glucose standards consisted of 0.1 ml sodium acetate buffer (pH 5.0), 0.1 ml glucose standard (50 μg/0.1 ml and 100 μg/0.1 ml) and 3.0 ml of glucose oxidase/peroxidase reagent. Reagent blanks consisted of 0.1 ml distilled water, 0.1 ml sodium acetate buffer and 3 ml glucose oxidase/peroxidase reagent. Glucose standards and reagent blanks were in duplicate, and the weight of starch present was calculated on the basis of the determined absorption of 100 μg of free glucose (see Appendix 1).

3.8 α-Amylase assay

Seeds were extracted in 3 ml 0.05 M Tris-maleate buffer, pH 6.2, containing 10 mM $CaCl_2$, using a pestle and mortar. The homogenate was centrifuged at 3000 rpm for 5 min and the supernatant was assayed for α-amylase activity. Enzyme activity was determined according to the method of Cornford *et al.* (1987), using Phadebas blue starch powder (Pharmacia Diagnostics Ltd.) as substrate. An appropriate dilution of the extract was made up to 4 ml with fresh buffer. This was pre-incubated at 37°C for 3 min followed by the addition of 1 ml substrate (10 mg/ml) in the same buffer. After 20 min incubation, the reaction was stopped by the addition of 1 ml 0.5 M NaOH and the mixture was centrifuged at 3000 rpm for 5 min. The absorbance of

the supernatant was read at 620 nm and the readings converted to enzyme units using a standard curve constructed with barley malt α-amylase (Sigma). One enzyme unit was defined as that amount of enzyme which would hydrolyse 1.0 g of maltose from starch at pH 6.2 at 37° C. A specimen standard curve is shown in Appendix 2.

3.9 Hormone response experiments

Seeds of *F. arundinacea* and *L. multiflorum* were prechilled for seven days at 5° C in 0.2% KNO_3 as in previously described germination conditions. Non prechilled and prechilled seeds (when required) were then dehusked and the embryo containing portion of the seed removed. The embryoless half seeds were surface sterilised in 30% v/v janola for 20 min and washed with copious amounts of sterile distilled water using sterile funnel and filter paper (Whatman No. 1) in a flow hood. Ten half seeds per replicate were incubated via filter sterilisation in 3 ml of sterile hormone solution at 25° C with shaking for 72 h in the dark. Hormone solutions used were a) a control of 10 mM $CaCl_2$, b) 10^{-6} M GA_3 in 10 mM $CaCl_2$, c) 10^{-5} M ABA in 10 mM $CaCl_2$ and d) 10^{-6} M GA_3 + 10^{-5} M ABA in 10 mM $CaCl_2$.

Following incubation, seeds were washed with 1.0 ml 0.05 M Tris-maleate buffer, pH 6.2, containing 10 mM $CaCl_2$ which was then added to the incubation solution. α-Amylase activity was determined separately from the incubation solution (secreted) and the half seeds (extracted) using the assay described above.

3.10 Isoelectric focusing

Isoelectric focusing (IEF) of α-amylase was based on a method used by Garcia-Maya *et al.* (1990). The isoelectric focusing gel was run using a LKB-Multiphor unit according to the manufacturers instructions. Three ml of kerosine was dispersed over the cooling plate on to which the pre-cast Ampholine polyacrylamide gel (PAG) plate

(LKB Pharmacia, Sweden) pH range 3.5 - 9.5 was placed, ensuring no air bubbles were trapped. Electrode strips were placed on opposite edges of the gel, with the cathode soaked in 1 M NaOH and the anode in 1 M H_3PO_4.

Twenty five seeds (four when using wheat) were ground with a pestle and mortar in 1.5 ml of 0.04 mM Tris - HCl buffer, pH 8.6, containing 10 mM $CaCl_2$ and centrifuged at 3000 rpm for 5 min. Volumes of 20 µl (or differing volumes adjusted to the same α-amylase activity) were placed on segments of filter-paper applied to the surface of the gel on the cathode side. Markers for determining the pI were also applied to the gel (as described later).

The running conditions used were 1 500 V, 50 mA and 20 W for a whole gel and the duration of the run was for 1.5 h. When half a gel was used the current was reduced to 25 mA and the power to 15 W. In each case, the filter application pieces were removed after 45 minutes. The whole operation was performed at 5°C.

On completion of the run, α-amylase activity was determined after by applying the IEF gel to one containing β-limit dextrin (see below) and incubated at 25°C for 1 h. The β-limit dextrin gel was stained with iodine solution (0.2% KI, 0.02% I_2 in 0.1 M HCl). Staining of marker proteins is described on the following page.

3.10.1 β-Limit dextrin overlay gel

This was prepared according to a method adapted from Garcia-Maya *et al.* (1990). Three grams of soluble starch (Merckt, Darmstadt, FRG) were dissolved by boiling in about 50 ml of 20 mM Tris-maleate buffer, pH 6.2, containing 10 mM $CaCl_2$. This was cooled, made up to 100 ml with buffer, followed by the addition of 100 µl β-amylase type 1-B from sweet potato (Sigma) and then incubated at 35°C for 15 h. The solution was then boiled and filtered through Whatman no.1 filter paper. The β-limit dextrin gel was prepared by mixing the β-limit dextrin solution with 16 ml of a solution containing 30% acrylamide and 0.8 bisacrylamide, 25 µl N,N,N'N'-

tetramethylethylenediamine (TEMED), and 200 µl of 10% ammonium persulphate. The solution was poured between glass plates (1.5 mm) and left to set at room temperature. All gel reagents were supplied by Bio-rad, Richmond, UK.

3.10.2 Estimation of pI

Two lanes of pI marker proteins (LKB Pharmacia, Sweden) were run with every gel to determine the isoelectric point of the α-amylase isoenzymes in the pH range 3.5 - 9.5. This was done according to Pharmacia (1990) instructions.

Following the exposure of the Ampholine PAG plate to the β-limit dextrin gel for activity staining, the marker proteins were stained as follows. The Ampholine PAG plate was fixed for 30 min in a 250 ml solution containing 29 g TCA and 8.5 g sulphosalicylic acid dissolved in distilled water. The gel was then washed for 5 min in destaining solution containing 25% v/v ethanol and 5% v/v acetic acid and then stained for 18 h in a 250 ml solution containing 0.29 g Coomassie Blue R250 dissolved in destaining solution. Prior to use the staining solution was heated with stirring to 60°C and filtered. The gel was then destained by soaking several times in fresh staining solution until the background of the gel became clear. Following focusing and staining, the distances migrated from the cathodic edge of the gel to the different marker protein bands were plotted against the corresponding pI's of the marker proteins. The isoelectric points of the unknown proteins were interpolated from the calibration curve by measuring their migration distance. A specimen calibration curve is shown in Appendix 3.

4. Optimisation of *F. arundinacea* germination conditions

4.1 Results

On commencing this project the optimal germination conditions for *L. multiflorum* had already been established by others in the laboratory (C. Cornford, Pers. comm.). It was, however, necessary to determine the optimum germination conditions of this seed lot of *F. arundinacea*. We wanted to identify and eliminate any residual effects of dormancy in order to gain a high percentage germination and uniformity of germination for the biochemical time-course studies.

Table 1 describes features of the *F. arundinacea* seed lot used in this study which may effect germination performance. The % empty seed within the seed lot was low (4.5 ± 0.5%). It was therefore felt unnecessary to process the seed lot to remove these prior to further experimentation. The mean seed weight of the dehusked seed was 2.04 ± 0.05 mg, but with considerable variation (0.49-4.4 mg). Seed weight distribution is shown in Figure 5. Using the tetrazolium stain to test for seed viability, there was found to be 34.5% incompletely stained seed. Plate 1 shows the variations in staining patterns of seeds stained with tetrazolium. These variations included seeds with embryos which were poorly developed (10.5%), partially stained (4%) or completely unstained (20%). The empty seeds were not taken into account in this result as they were removed prior to the selection of seeds for staining. The 1000 seed weight (complete caryopsis) was determined as 2.43 g, thus, on average, 16.04% of the seed is husk.

Experiments were carried out to determine optimal germination conditions for *F. arundinacea* within the range prescribed by the International Seed Testing Association (ISTA) (1985). Figure 6 shows the effects of various temperature treatments, with and without prechilling, on the germination of *F. arundinacea*. A summary of results expressed in terms of T_{50} and T_{90}-T_{10} is presented in Table 2.

Table 1. Physical characteristics of *F. arundinacea* seed used in this study.

	$\bar{x}$	SE
Empty seed %[a]	4.5	± 0.5
Weight of dehusked seed[b] (mg)	2.04	± 0.05
1000 seed weight (g)	2.43	± 0.06
Estimate of non-viable embryos(%)[c]	34.5	± 4.3

[a]Result obtained as the mean of 11 replicates of 100 seeds.

[b]Mean and SE of 200 individually weighed dehusked seeds. A frequency distribution graph of this data is shown in Figure 5.

[c]Mean result of 4 replicates of 50 seeds. The estimate includes partially stained, completely unstained and poorly developed embryos.

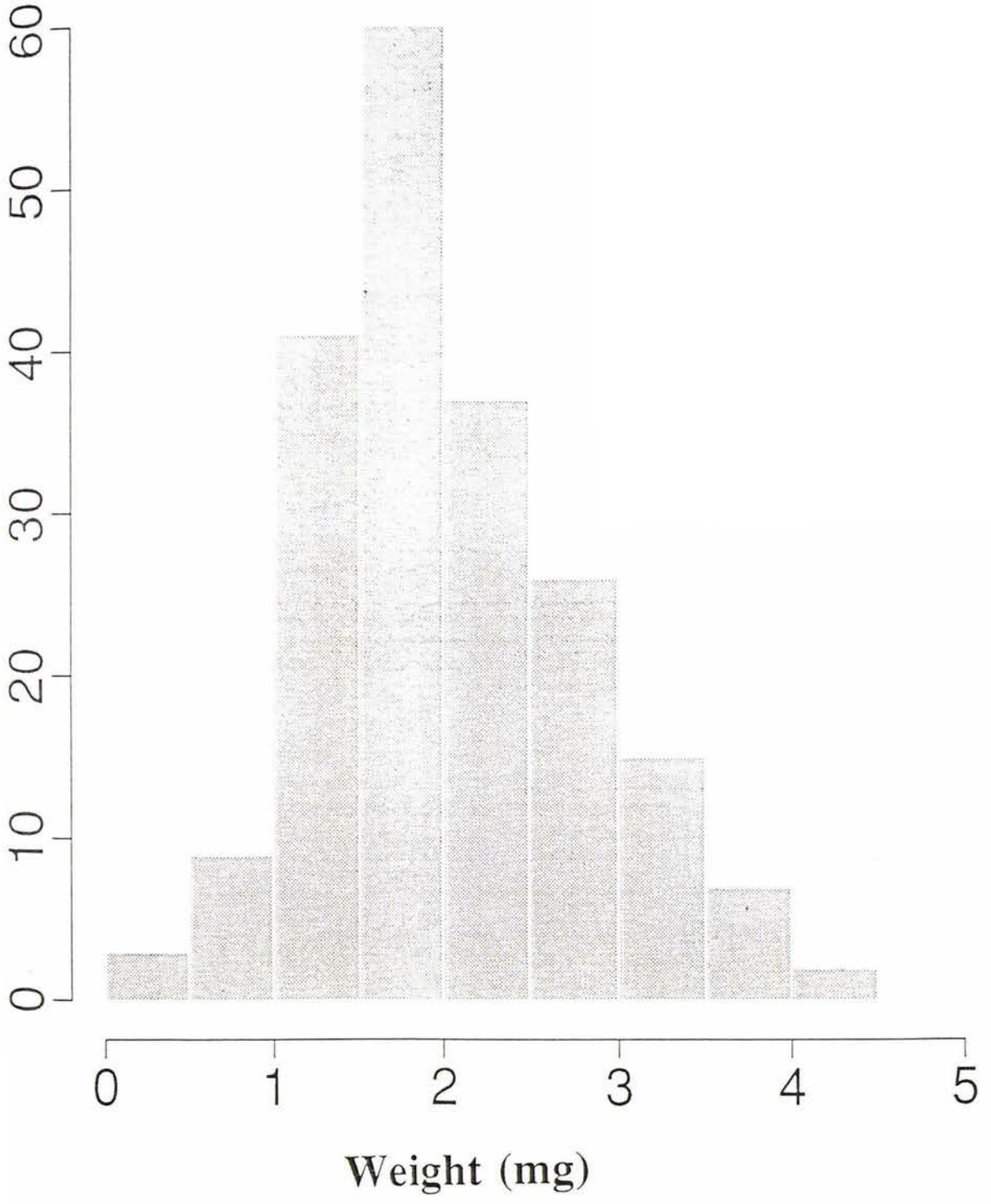


Figure 5. The seed weight distribution of dehusked *F. arundinacea* seeds.

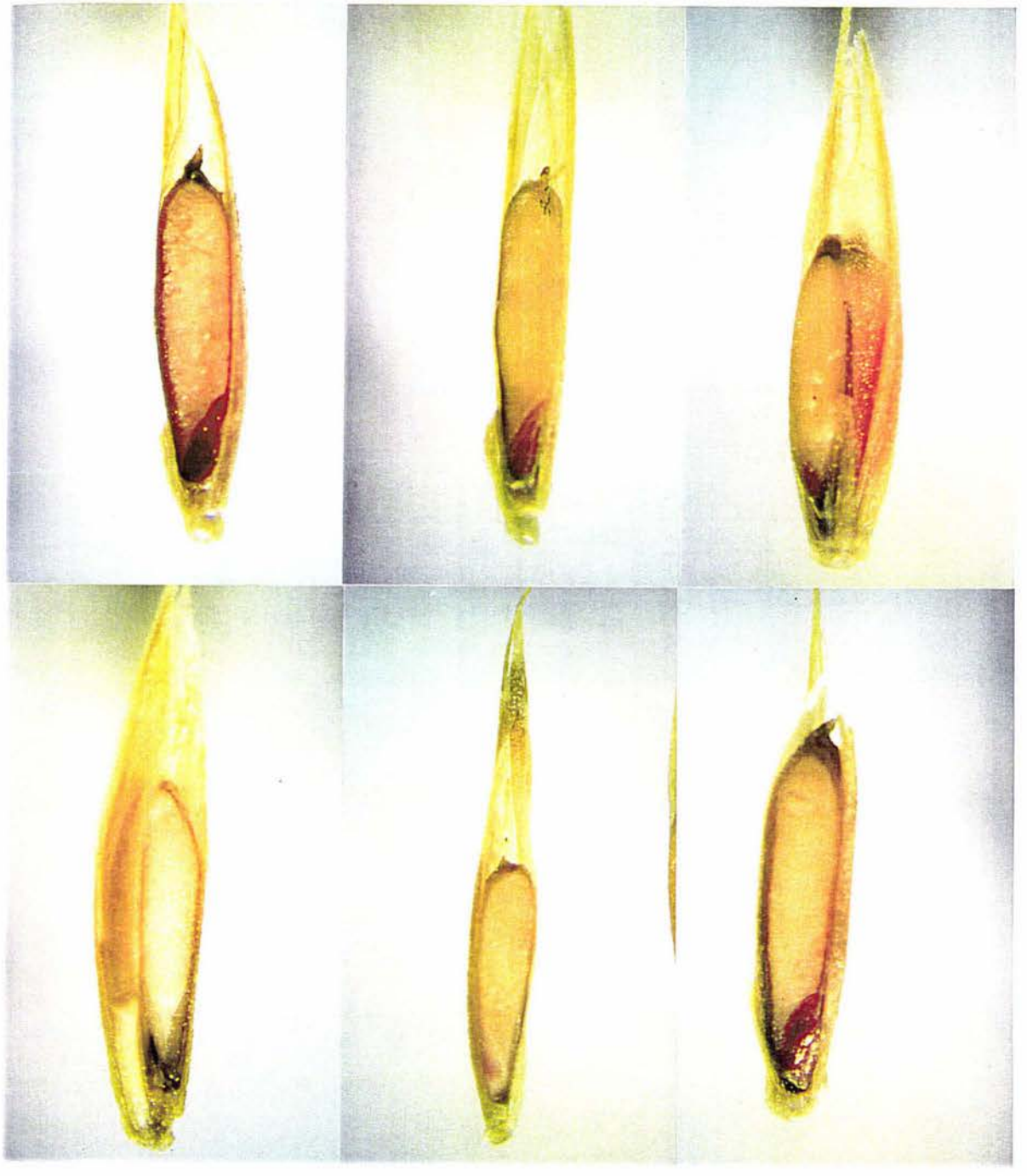


Plate 1. Variation in *F. arundinacea* half seeds following tetrazolium staining. Seeds shown were selected as being representative of the whole sample.

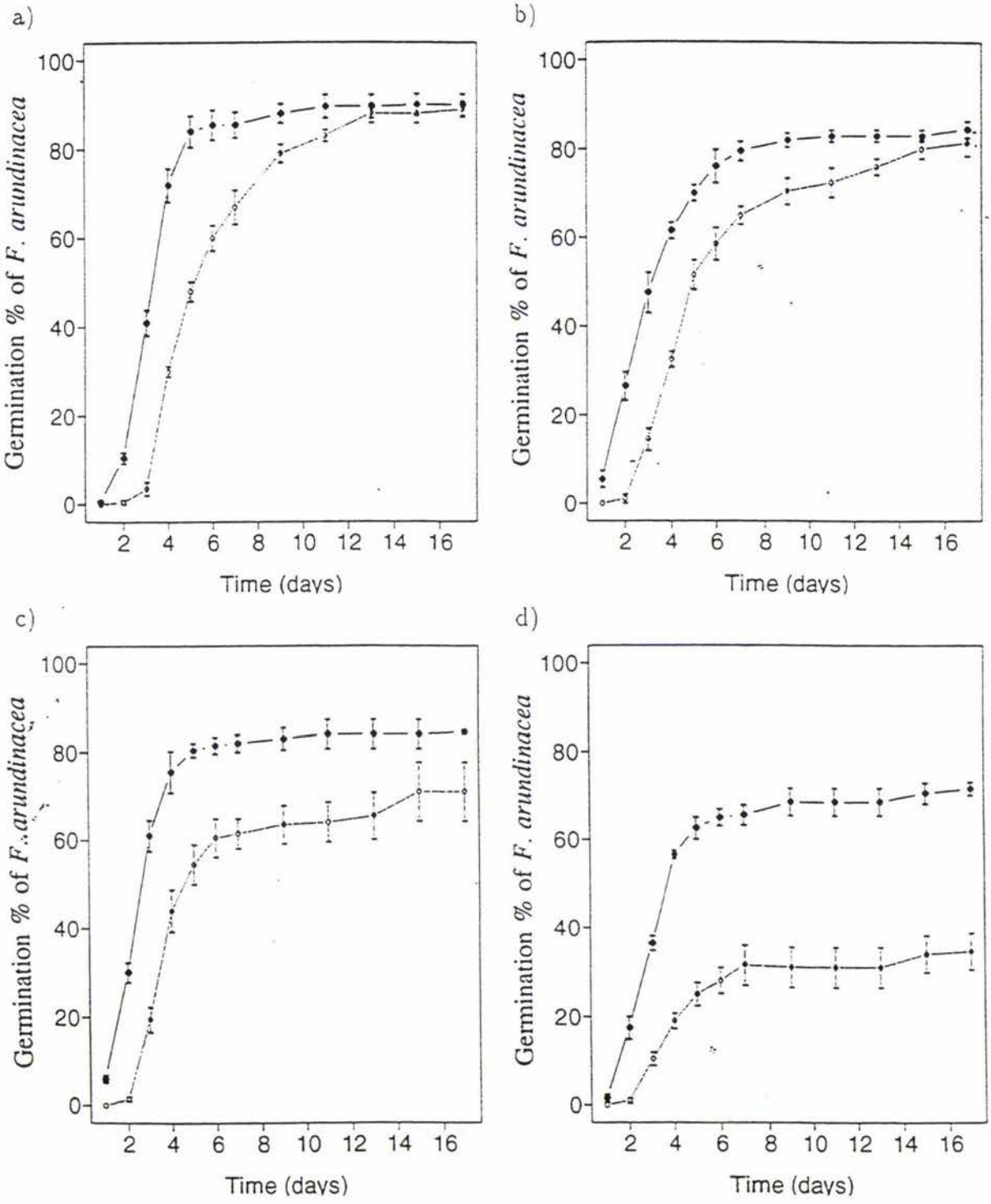


Figure 6. Time-course of *F. arundinacea* seed germination at temperature treatments of a) alternating temperatures of 15°C for 16 h and 25°C for 8 h, b) alternating temperatures of 20°C for 16 h and 30°C for 8 h, c) constant 20°C, and d) constant 30°C. The temperature treatments are each with (●) and without (○) prechilling (5°C for 7 d). Data are means of 4 replicates. Vertical bars represent SE's of individual means.

Table 2. The effects of prechilling and various temperature treatments on the final germination %, the median germination time (T_{50}) and the uniformity of germination (T_{90}-T_{10}) of *F. arundinacea*.

Treatments	T_{50}(hrs)	T_{90}-T_{10}(hrs)	Germination
15-25°C + prechilling	76 ± 1	87 ± 15	90 ± 2
15-25°C - prechilling	118 ± 4	170 ± 14	89 ± 2
20-30°C + prechilling	68 ± 4	131 ± 7	85 ± 2
20-30°C - prechilling	108 ± 2	213 ± 33	82 ± 3
20°C + prechilling	**59 ± 2**	**90 ± 23**	**85 ± 1**
20°C - prechilling	**89 ± 3**	**224 ± 35**	**71 ± 7**
30°C + prechilling	72 ± 2	174 ± 43	72 ± 2
30°C - prechilling	90 ± 6	191 ± 62	35 ± 4

$LSD_{[0.01]}$=12.9

Prechilling consisted of imbibing seed for 7 days at 5°C in 0.2% (w/v) KNO_3. The seeds were then transferred for germination to various temperature treatments along with the non-prechilled seeds. There was constant darkness throughout except when seeds were monitored for germination. Values represent the means of 4 replicates ± SE. The treatments adopted for all subsequent germination experiments are highlighted in bold type.

Significant differences were found in the final % germination of seeds from the four temperature treatments (P = 0.001). A comparison of prechilled seeds germinated at temperatures of alternating 15-25°C, alternating 20-30°C and constant 20°C (Figure 6a-c) showed similar final % germination ranging from 85-90%. Prechilled seeds germinated at constant 30°C had a significantly lower final % germination of 72% (P = 0.001) (Figure 6d). There was considerably more variation in the final % germination of non prechilled seeds with values ranging from 89% for seeds germinated at alternating 15-25°C to 35% for seeds at constant 30°C (Figure 6a-d).

Alternating temperatures substituted for prechilling in permitting germination, but prechilling still determined rate and uniformity. At 30°C, prechilling had little effect on the T_{50} or T_{90}-T_{10} of those seeds that do germinate but doubled the germination % (P = 0.001). The final % germination of prechilled seeds germinated at constant 20°C was also significantly higher than in non prechilled seeds (Table 2).

Seeds from all prechilled treatments reached near to their maximum germination % after 7 d germination, with the increase in germination % of non prechilled seeds being more gradual (Figure 6a-d). The effects of the different temperatures were also reflected by differences in T_{50} and T_{90}-T_{10} values. For example, the germination rate varied between treatments with the T_{50} ranging from 59 h for constant 20°C to 76 h for alternating 15-25°C in prechilled treatments. In treatments without prechilling the range was from 89 h for constant 20°C to 118 h for alternating 15-25°C. The range in T_{90}-T_{10} of prechilled seeds was 87 h for alternating 15-25°C to 174 h for constant 30°C and in non prechilled seeds it was from 170 h for alternating 15-25°C to 224 h for constant 20°C (Table 2). There was no significant effect of temperature on T_{90}-T_{10} (P = 0.4170). However, high variation in the T_{90}-T_{10} results between replicates within the treatments may have contributed towards this.

Prechilling of *F. arundinacea* appeared to affect the rate of germination by eliminating a lag period. This was shown by a delay in the onset of visible germination of non prechilled seeds of up to 2 d compared to prechilled seeds for each treatment (Figure 6a-d). The T_{50} values for prechilled seeds ranged from being

less than the non prechilled values by 18 h for constant 30°C to 42 h for alternating 15-25°C. Prechilled seeds were also more uniform in their germination than non prechilled seeds, as demonstrated by their significantly lower T_{90}-T_{10} values (P = 0.0027) (Table 2). No significant interaction was found between the four temperature treatments and the presence or absence of prechilling on T_{90}-T_{10} values (P = 0.3962).

Prechilled and non prechilled seeds had significantly different final % germination (P = 0.001). There was also a significant difference on the final % germination due to the interaction of the various temperature treatments with prechilling effects (P = 0.001).

Although certain conditions may promote germination, they may also adversely effect the growing seedling. Table 3 shows the mean final root and shoot lengths of seeds from each treatment. Although prechilling of seeds resulted in a lower T_{50} at all temperatures, it did not appear to have a marked effect on the overall seedling growth. No significant differences were found between prechilled and non prechilled treatments in shootlength (P = 0.0648) or rootlength (P = 0.6643). Greater differences were observed between the different temperature regimes. Variation in mean shoot length ranged from 5.5 to 10.9 cm between treatments, while the mean root length ranged from 1.0 to 3.1 cm. The mean shoot and root lengths were shortest for seeds germinated at 30°C both with and without prechilling. Comparisons between seeds germinated at constant 20°C, alternating 15-25°C and alternating 20-30°C found no significant difference in final rootlength (P = 0.0637), however, there was a significant difference between each temperature treatment in final shootlength (P = 0.0001). There was no significant interaction effect between prechilling and these three temperature treatments on both rootlength (P = 0.1184) and shootlength (P = 0.3064). Variation in the root/shoot ratios was also observed. Germinating seeds at alternating 15-25°C resulted in the highest ratio for both prechilled (0.30) and non prechilled seeds (0.35). The lowest root/shoot ratio was

Table 3. The effects of prechilling and various temperature treatments on the shoot and root lengths of *F. arundinacea* following 16 days germination.

Treatments	Mean shoot length (cm)	Mean root length (cm)	Root/ shoot ratio
15-25°C + prechilling	9.1 ± 0.6	2.7 ± 0.3	0.30
15-25°C - prechilling	8.0 ± 0.1	3.1 ± 0.3	0.39
20-30°C + prechilling	7.4 ± 0.3	2.2 ± 0.3	0.30
20-30°C - prechilling	7.5 ± 0.1	2.5 ± 0.1	0.33
20°C + prechilling	**10.9 ± 0.4**	**2.9 ± 0.1**	**0.27**
20°C - prechilling	**9.9 ± 0.5**	**2.5 ± 0.2**	**0.25**
30°C + prechilling	5.5 ± 0.1	1.0 ± 0.6	0.18
30°C - prechilling	5.9 ± 0.1	1.0 ± 0.2	0.17

Prechilling consisted of imbibing seed for 7 days at 5°C in 0.2% (w/v) KNO_3. The seeds were then transferred for germination to various temperature treatments along with the non-prechilled seeds. There was constant darkness throughout except when seeds were monitored for germination. Values for shoot and root lengths represent the means of 4 replicates ± SE.

in seeds germinated at constant 30°C both with prechilling (0.18) and without (0.17), reflecting a much lower shoot/root growth at this temperature. Differences between treatments in SE's reflect the variation in uniformity of the different treatments.

Correlation analyses were carried out in order to determine whether final lengths of roots and shoots were influenced by the T_{50}'s of seeds after different treatments (Table 4). For example, it might be assumed that treatments with shorter T_{50}'s would have longer root and shoot lengths. However, since no R^2 value was higher than 18.2% it was concluded that treatment effects on T_{50}'s were not correlated with treatment effects on subsequent seedling growth. There is, however, clear evidence from this that 30°C slows down seedling growth, especially root growth.

Based on the results presented above, conditions of constant 20°C with prechilling were used in all subsequent experiments. This temperature resulted in the lowest T_{50} and T_{90}-T_{10} values, a high % germination and significantly longer mean shoot lengths ($P = 0.0001$). In much of the subsequent experimental work, constant 20°C without prechilling was used in addition to the prechilled treatment. This was to compare the relative effects of prechilling on *F. arundinacea* and *L. multiflorum* and thus enable a link between this study and previous studies on *L. multiflorum* using constant 20°C without prechilling to be created.

4.1.1 Determination of loss of residual dormancy versus thermal time effects of prechilling

As shown in Table 2, apart from an increased percentage final germination, *F.arundinacea* also responded to prechilling by having a lower T_{50} and T_{90}-T_{10}. To determine whether these effects were due to the loss of residual dormancy or thermal time effects, T_{50} values were determined for *F. arundinacea* over a range of temperatures. *L. multiflorum* seeds were included at this stage for comparative purposes. The reciprocal plot of T_{50} against temperature was plotted for each species (Figure 7). A line of best fit was drawn between the first four points on the graph

Table 4. Correlation analyses between T_{50}'s and subsequent seedling growth of seeds germinated at different temperatures with and without prechilling.

Treatment comparisons	R^2 values
T_{50} + prechilling / shootlength	18.20%
T_{50} - prechilling / shootlength	0.02%
T_{50} + prechilling / rootlength	17.15%
T_{50} - prechilling / rootlength	1.12%

Prechilling consisted of imbibing seed for 7 days at 5°C in 0.2% (w/v) KNO_3. The seeds were then transferred for germination to various temperature treatments along with the non prechilled seeds. There was constant darkness throughout except when seeds were monitored for germination. T_{50} and mean final root and shoot length calculations were determined from treatments consisting of 4 replicates of 50 seeds.

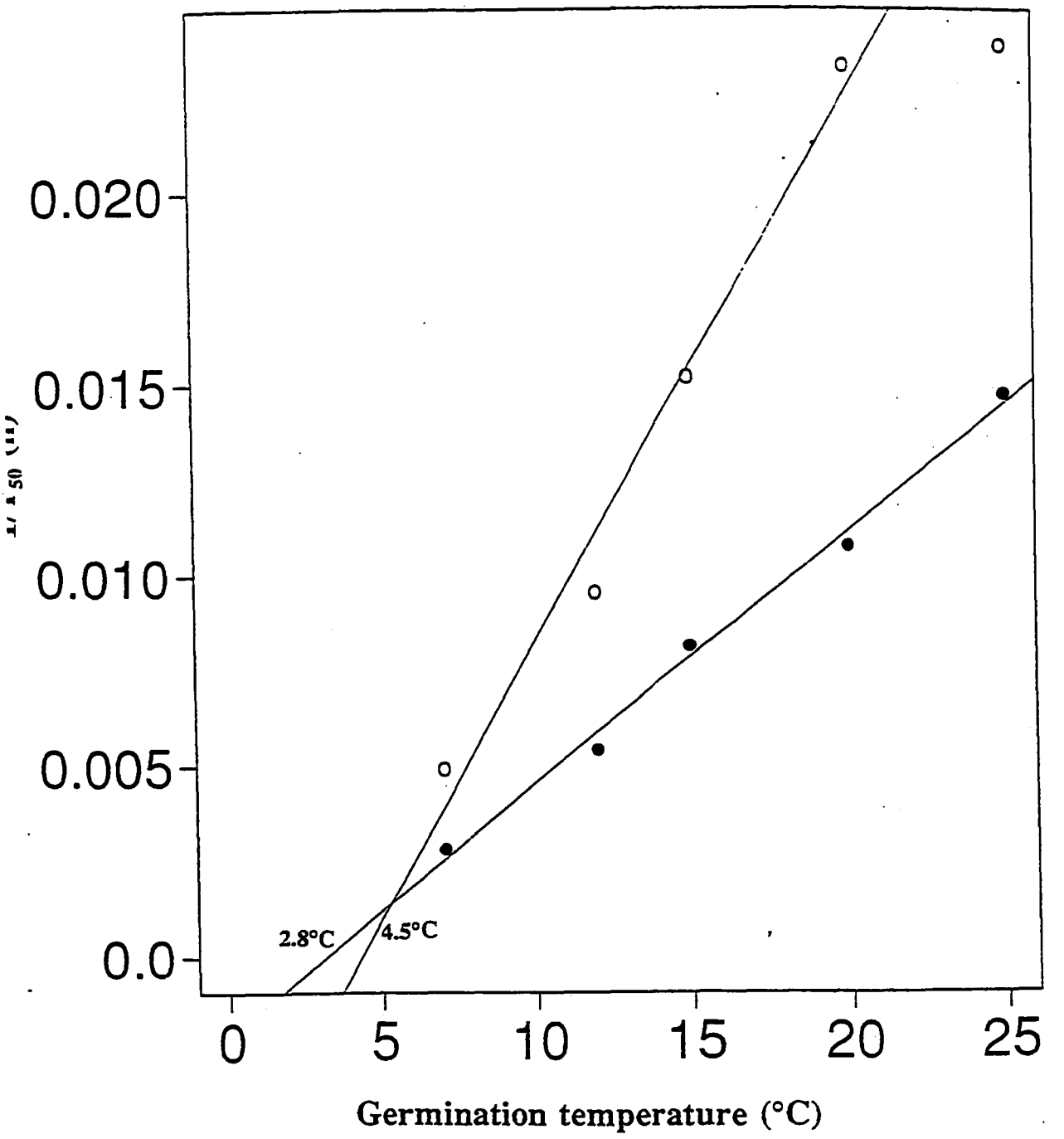


Figure 7. Reciprocal plot of median germination time (T_{50}) against various germination temperatures showing the base temperature for *F. arundinacea* (●) and *L. multiflorum* (○). Data are means of 4 replicates. Vertical bars represent SE's of individual means.

for each species. The fifth points were excluded as the graphs appeared to turn over at 25°C and R^2 values were higher when using four data points compared to five. The R^2 value for *L. multiflorum* using the first four data points was 97.5% compared to 89.9% when using all five data points. There was less difference in R^2 values in *F. arundinacea* when comparing four data points (99.2%) with five data points (98.5%).

The base temperature (T_b), determined from the intercept of the linear relationship with the x-axis, was 2.8°C for *F. arundinacea* and 4.5°C for *L. multiflorum* (Figure 7). From these base temperatures, a prechilling advantage of 15.4 d°C can be expected due to thermal time effects during the prechilling period for *F. arundinacea.* The observed advantage was 18.9 d°C, very close to the expected value given the variation in the raw data. On this basis there is no clear evidence for a general stratification response in this species (or at least in this seed lot). In *L. multiflorum,* however, there was a large difference between the expected prechilling advantage of 3.5 d°C and the observed prechilling advantage (12.4 d°C) indicating a stratification response had occurred. Calculations are presented in Appendix 4.

4.2 Discussion

Although this study focuses on germination in *F. arundinacea* with other information being obtained from studies of *L. multiflorum*, the literature on details of *Gramineae* germination is very limited in general. For example, most literature (such as studies described by Brock *et al.* 1982, and Hill *et al.* 1985[a]) does not distinguish between rates of germination *per se* and rates of subsequent seedling growth.

4.2.1 Characterisation of the F. arundinacea seed lot

Tetrazolium staining is a routinely used method of determining seed viability. The variation in staining patterns of *F. arundinacea* was large (Plate 1). Tetrazolium staining also confirmed the presence of live aleurone tissue in *F. arundinacea* seeds, however, this was not obvious in all stained seeds.

The germination time-course data (Figure 6) do not support the assumption that all incompletely stained seeds (as described in Table 1) are non-viable, as implied by Grabe (1970). This therefore questions the way in which staining of embryos in this species should be interpreted. In a study of tetrazolium activity, Perl *et al.* (1978) found that misinterpretations occurred as seed staining can vary due to several responses including the enzyme activity, the level of reducing substrate and the competition between hydrogen acceptors in the cells. Furthermore, the extent of non-lethal damage which can occur in seeds of different species has been poorly characterised.

4.2.2 Determination of optimum germination conditions

a) Germination behaviour

In determining the optimum germination conditions for *F. arundinacea* (constant 20° C with prechilling), it was clear that the use of different temperature treatments resulted in variations in the final % germination, the median germination time and the uniformity of germination (Table 2). This variation was larger in non prechilled seeds than prechilled seeds, although in both cases the temperature treatment of constant 30° C resulted in a significantly lower final % germination ($P = 0.001$) than in other temperature treatments.

The observed variation in uniformity of germination may have some relation to the large variation in seed size observed in the *F. arundinacea* seed lot (Figure 5). The effects of seed size on germination could be investigated by separating the seed lot into size grades prior to experimental work. Unfortunately this was beyond the scope of the present study. While seed size may affect subsequent growth in many species, there are often no clear relationships between size and germination rates *per se* (Coolbear, pers. comm.).

b) Prechilling effects

The effect of prechilling appeared to be in the elimination of a lag period in the germination of *F. arundinacea* seeds in each treatment. This was reflected by lower T_{50} values of prechilled seeds compared to non prechilled seeds. However, at 30° C, although prechilling resulted in double the % germination, it had no effect on T_{50} or T_{90}-T_{10}. Prechilling also resulted in seeds reaching their maximum germination % more rapidly than for non prechilled seeds. However, the use of alternating temperature treatments resulted in similar final % germination in both prechilled and non prechilled seeds. Alternating temperatures appeared to substitute for prechilling in permitting germination in some seeds within the population, although, prechilling still determined the overall rate and uniformity of germination. Garcia-Huidobro *et al.* (1982[b]) found that some seed populations of *Pennisetum typhoides* (pearl millet) which remain dormant when held at a constant temperature have an increase in the maximum fraction that germinate in a population when exposed to alternating temperatures. In addition, they found a small increase in the rate of germination in

response to alternations in temperature. Their study did not, however, compare the effects of prechilling with those of alternating temperatures.

c) Seedling growth measurements

Final root and shoot lengths of *F. arundinacea* seeds used in the time-course for determination of optimum germination conditions were measured to evaluate normal seedling growth. This was done as a treatment may promote germination but adversely affect the germinated seedling. Although variations in final mean shoot and root lengths and shoot/root ratio were observed between different temperature treatments, it was found that the treatment effects on T_{50}'s were not correlated with treatment effects on subsequent seedling growth. Similarly, prechilling had no significant effect on the final shoot ($P = 0.0648$) and rootlengths ($P = 0.6643$) in the various treatments. This was surprising as it might be expected that treatments with shorter T_{50}'s would have longer root and shoot lengths. It is clear, however, that 30°C was suboptimal for seedling growth, especially root growth.

Two possible explanations for these results are either that the root and shoot lengths may have been determined by the temperature optimum for seedling growth to occur, not by the rate at which germination occurred, or, alternatively, after sixteen days germination, seed reserves may have been exhausted which may explain the similarities in final shoot and root lengths between prechilled and non prechilled seeds of each treatment. Starch levels and seedling growth measurements determined in later experimental work (described in Chapter 5) tend to support the second possibility.

Future work looking at effects of different temperatures on seedling growth may be benefitted by including dry weight data in addition to shoot and root length measurements. It has been found that in a given species, the darkness response of mesocotyl extension tended to be similar for seeds of different weight, with lighter seeds producing thinner structures (Jones, 1972, cited in Jones and Lazenby 1988).

Knowledge of optimum temperatures for maximum shoot and root elongation is probably of equal importance or greater to germination rate as often nutrient uptake and photosynthesis commence long before seed reserves are exhausted (McWilliam *et al.* 1970). In these instances, plants with greater root surfaces would be able to take up nutrients rapidly, enhancing their growth and competitive ability compared with plants with smaller root systems. *F. arundinacea*, with its slow seedling growth, which may or may not be due primarily to slow root growth (Brock *et al.* 1982), is at a clear disadvantage to *L. multiflorum* with its longer and quicker developing roots. Seedling growth was explored in more detail in later experiments (described in Chapter 5).

4.2.3 Determination of dormancy breaking or thermal time effects

In *L. multiflorum*, prechilling advantages were demonstrated to be largely due to a stratification response. *F. arundinacea*, with a much lower base temperature, appeared to have no overall stratification requirement for a reduced T_{50} or T_{90}-T_{10}. However, the differences due to prechilling effects (Figure 6a-d) in final % germination (Table 2) indicate that the *F. arundinacea* seed lot may not be entirely homogenous as a small proportion of seed were only able to germinate after stratification.

A confounding factor in this study is that the seeds were in storage for 11 months between the germination time-course study of Figure 6 and the determination of dormancy breaking or thermal time effects. During this time the seed lot apparently lost its stratification requirement for maximal final germination (Table 5). Thus direct comparisons between the time-course and thermal time studies cannot be made.

In addition to possible stratification requirement differences within the seed lot, the chance that these results may differ to other seed lots of the same cultivar are emphasised by other studies. Variations in temperature during seed development has been found to affect seed dormancy in several grass species (Wiesner and Grabe

Table 5. Changes in T_{50} and final % germination of *F. arundinacea* during storage.

Storage time following harvest (months)	T_{50} (hrs) for prechilled treatments	Final % germination + prechilling	Final % germination - prechilling
14[a]	59 ± 2	85 ± 1	71 ± 7
16[b]	65 ± 5		
25[c]	76 ± 3	77 ± 5	85 ± 5

Seeds were stored following harvest at 0°C with 30% relative humidity for 13 months. Seeds were then stored at 5°C. Prechilling consisted of imbibing seed for 7 days at 5°C in 0.2% (w/v) KNO_3. The seeds were then transferred for germination to 20°C. There was constant darkness throughout except when seeds were monitored for germination. Values represent means of: [a]4 replicates of 50 seeds, [b,c]3 replicates of 25 seeds.

1972). Jensen and Pierpont (1961) cited in Wiesner and Grabe (1972) have found the seed dormancy of *Lolium* species to vary with season and area of production, as well as with species. The International Seed Testing Association normally recommends up to seven days at between 5° C and 10° C as a stratification treatment. For many species a complete dormancy break may not occur as this may be too high a temperature or too short a time period. Seeds used in all germination time-course studies were imbibed in 0.2% (w/v) KNO_3 which has been found to be effective in many species in promoting dormancy breaking and is another variable which might be usefully explored in future work.

When determining the base temperatures of *F. arundinacea* and *L. multiflorum*, the fifth points on the graph representing the $1/T_{50}$ values for the 25° C treatment were excluded for each species. This was due to lower R^2 values being determined when using the five point correlation compared with the four point correlation analysis. This implies that the linear increases in rate of germination ($1/T_{50}$) started turning over at 25° C. Garcia-Huidobro *et al.* (1982[a]) have found the rate of germination to increase linearly with temperature from a base temperature to a sharply defined optimum beyond which the rate decreased linearly with temperature. The temperature treatment of 25° C appears to exceed this optimum range. In the 25° C treatment there was also a lower % germination when compared to other treatments (results not included) which is supported by Thompson (1970) who found that the fraction of seeds which germinate usually remains constant over a wide range of temperatures and sharply declines on either side of this range. Danielson and Toole (1976) found that final germination of *F. arundinacea* declined at higher and lower temperatures compared to its optimum germination temperature.

Time-course data (Figure 6 and Table 2) show that although not many seeds germinate at 30° C, those that do germinate do so rapidly. The use of the 30° C treatment also resulted in a huge loss of uniformity, the shortest shoot and root length and the lowest shoot/root ratio. As shown in Figure 6 the final % germination in non prechilled seeds germinated at 30° C was significantly lower than in prechilled seeds. These results, indicate that a temperature sensitive process may be completed during

the prechilling period (although there is no indication of this in root and shoot growth). This may be emphasised by the final % germination not being as high in the 25°C treatment used in the above experiment and in non prechilled seeds germinated at alternating 20-30°C (Figure 6) when compared to prechilled seeds in this treatment. A study on *F. arundinacea* and *Bromus catharticus* (prairie grass) (Hill *et al.* 1985[a]) has shown temperatures above 20°C to exceed the optimum germination temperature of the seed lot. In addition, Blacklow (1972) found the extension rate of shoots and roots of young maize plants to also be affected by high temperatures. There was a linear increase in extension rate with increased temperatures until reaching temperatures above 30°C where the extension rate turned over.

5. Comparative time-course studies between *F. arundinacea* and *L. multiflorum*

5.1 Results

A series of time-course experiments were performed to compare changes in the physical germination characteristics of *F. arundinacea* with *L. multiflorum* as germination and seedling growth proceeded. Comparative time-course experiments were also performed to determine changes in activity of α-amylase during germination and early seedling growth and to characterise the α-amylase isoenzymes present during these stages. Typical germination and seedling growth data is shown in Figures 8-12 from the main time-course study undertaken in this project. Two previous time-courses did not include non prechilled seeds, with another two time-courses only having one replicate for seedling growth data (other replicates were used for enzyme and starch assays). Apart from variations in levels of α-amylase activity, there were no major differences observed between the different time-course studies.

5.1.1 Final percentage germination

Figure 8 shows the comparative % germination for prechilled and non prechilled *F. arundinacea* and *L. multiflorum* seeds over a 16 day time-course at 20°C constant. Due to the destructive nature of the sampling, which involved new replicates of seeds being harvested for each time point, the final germination % was not necessarily the same as the maximum germination %, although these differences were not significant ($P = 0.2807$). When the two species were compared over days 12-16 there was found to be no significant difference in their % germination ($P = 0.0529$). The effect of prechilling on the % germination in each species over these days was also not significant ($P = 0.0529$). Differences in growth and development of the two species are illustrated for prechilled seeds in Plates 2-4.

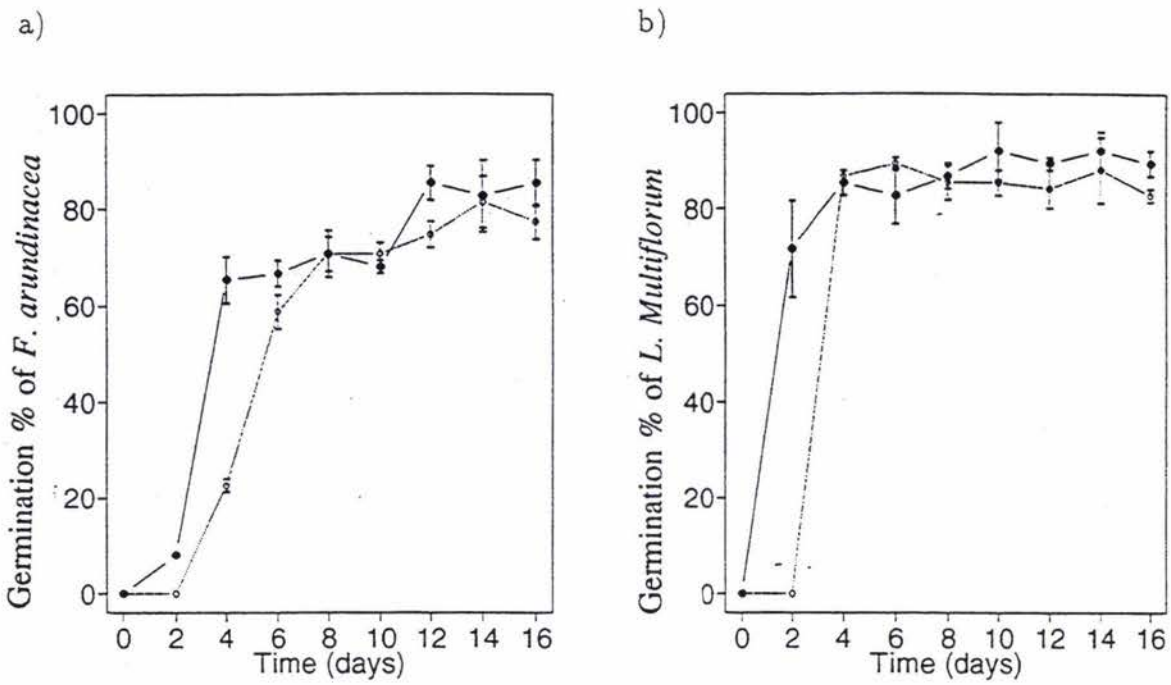


Figure 8. The effect of prechilling (5°C for 7 d) on the germination at 20°C of a) *F. arundinacea* and b) *L. multiflorum* seed. Data are means of 4 replicates. Vertical bars represent SE's of individual means. Closed symbols represent + prechilling, open symbols represent - prechilling.

Plate 2. A comparison of prechilled *F. arundinacea* and *L. multiflorum* seeds at Day 0 of a germination time-course. Top row shows *F. arundinacea* seeds, bottom row shows *L. multiflorum* seeds. The seeds shown were selected as being representative of the whole sample.

Plate 3. A comparison of prechilled *F. arundinacea* and *L. multiflorum* germination and early seedling growth at Day 2 of a germination time-course. Top row shows *F. arundinacea* seeds, bottom row shows *L. multiflorum* seeds. The seeds shown were selected as being representative of the whole sample.

Plate 4. A comparison of prechilled *F. arundinacea* and *L. multiflorum* germination and early seedling growth at Day 4 of a germination time-course. Top row shows *F. arundinacea* seeds, bottom row shows *L. multiflorum* seeds. The seeds shown were selected as being representative of the whole sample.

5.1.2 Germination rates

Germination and seedling growth was faster for *L. multiflorum* than *F. arundinacea* both with and without prechilling. In the absence of a prechilling treatment *L. multiflorum* seeds germinated rapidly reaching a maximum germination of 87% by day 4 (Figure 8). This was reflected in a T_{50} of 72 h (Table 6). In contrast only 23% of *F. arundinacea* seeds (also without prechilling) had germinated by day 4, eventually reaching a peak in germination of 71% by day 8 (Figure 8), with a T_{50} of 121 h (Table 6). This pattern was repeated when prechilled seeds of the two species were compared.

The effect of prechilling on the germination of both species was an acceleration in rate of germination and thus a reduction in T_{50}. In the case of *L. multiflorum*, 72% of prechilled seeds had germinated after 2 days incubation compared to 0% germination in the non prechilled seeds (Figure 8). The T_{50} was 39 h longer for non prechilled seeds (72 h) than for prechilled seeds (33 h) (Table 6). Prechilled seeds of *F. arundinacea* had also begun to germinate after 2 days although with a much lower % germination (8% compared to prechilled *L. multiflorum* 72%). The T_{50} of *F. arundinacea* in the absence of prechilling (121 h) was 45 h longer than when prechilled (76 h) (Table 6).

F. arundinacea was much more uniform in its germination when prechilled, having a T_{90}-T_{10} of 94 h compared to 222 h when without prechilling. In *L. multiflorum*, however, prechilling had no impact on T_{90}-T_{10} values, these being comparable with that of *F. arundinacea* without prechilling. As noted previously, the effects of prechilling resulted in no significant difference in the final germination percentage (Table 6).

Table 6. The effects of prechilling on the final germination %, the median germination time (T_{50}) and the uniformity of germination (T_{90} -T_{10}) of *F. arundinacea* and *L. multiflorum*.

Treatments	T_{50} (hrs)	T_{90}-T_{10} (hrs)	Germination %
F. arundinacea + prechilling	76 ± 3	94 ± 42	77 ± 5
F. arundinacea - prechilling	121 ± 2	222 ± 58	85 ± 5
L. multiflorum + prechilling	33 ± 6	247 ± 32	89 ± 3
L. multiflorum - prechilling	72 ± 1	229 ± 31	83 ± 1

Prechilling consisted of imbibing seed for 7 days at 5°C in 0.2% (w/v) KNO_3. The seeds were then transferred to constant 20°C. There was constant darkness throughout except when seeds were monitored for germination. Values represent the means of 3 replicates ± SE.

5.1.3 Seedling dry weight changes

Data shows seedlings moved into a linear growth phase after radicle emergence - as described by Blacklow (1973) for maize. The time of onset of linear growth (as indicated by increasing seedling dry weight) was earlier in *L. multiflorum* than in *F. arundinacea* by 2 d in prechilled seeds and 4 d in non prechilled seeds (Figure 9). Regression analysis comparing linear growth rates of non prechilled *F. arundinacea* (days 8-12) with non prechilled *L. multiflorum* (days 4-8) found these rates to not be significantly different (P = 0.461). This was also the case when comparing linear growth rates of prechilled *F. arundinacea* (days 4-8) with prechilled *L. multiflorum* (days 2-6) (P = 0.053).

In both species, linear increases in dry weight coincided with plumule elongation (Figure 10) rather than radicle elongation which was at least 2 d earlier (Figure 11).

Regression analysis found that the linear growth rate of prechilled *L. multiflorum*, although starting 2 d earlier, was not significantly different to that of non prechilled *L. multiflorum* (rate = 0.494 mg/d in both species) (P = 0.226). Prechilling in *F. arundinacea* resulted in the onset of linear growth being 4 d earlier, however, it also resulted in a significantly slower linear growth rate (0.1919 mg/d for non prechilled *F. arundinacea*, 0.1027 mg/d for prechilled *F. arundinacea*) (P = 0.003). These results are summarised in Table 7 for easy comparison.

5.1.4 Seed reserve changes

The decrease in seed reserves of non prechilled *L. multiflorum* started after 2 days incubation and was observed to reach a maximum rate after 4 days (Figure 12). These events coincided with the increase in seedling dry weight (Figure 9). However, in non prechilled *F. arundinacea* there was a dislocation of these events. Dry weight losses occurred (Figure 12), but seedling dry weight increases were 2 d behind (Figure 9). The onset of root length increases (Figure 11) correlated more

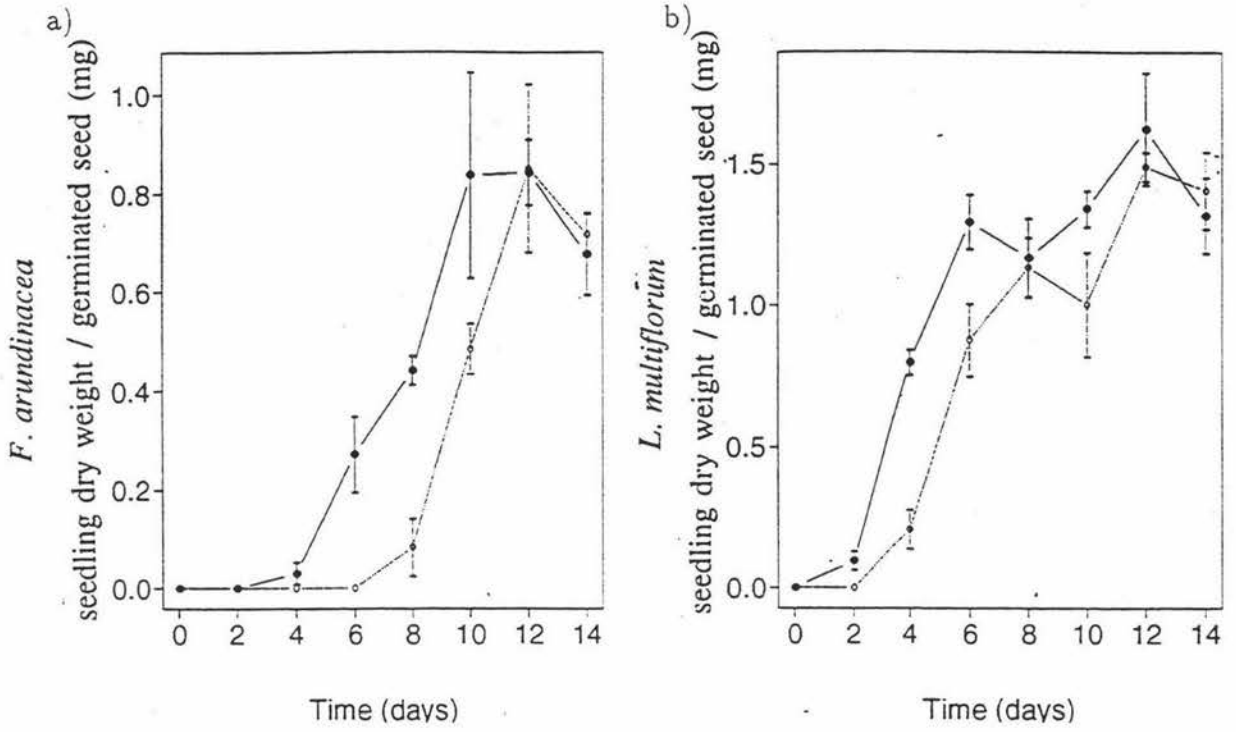


Figure 9. The effect of prechilling (5° C for 7 d) on the seedling dry weight changes of a) *F. arundinacea* and b) *L. multiflorum* seed. Data are means of 4 replicates. Vertical bars represent SE's of individual means. Closed symbols represent + prechilling, open symbols represent - prechilling.

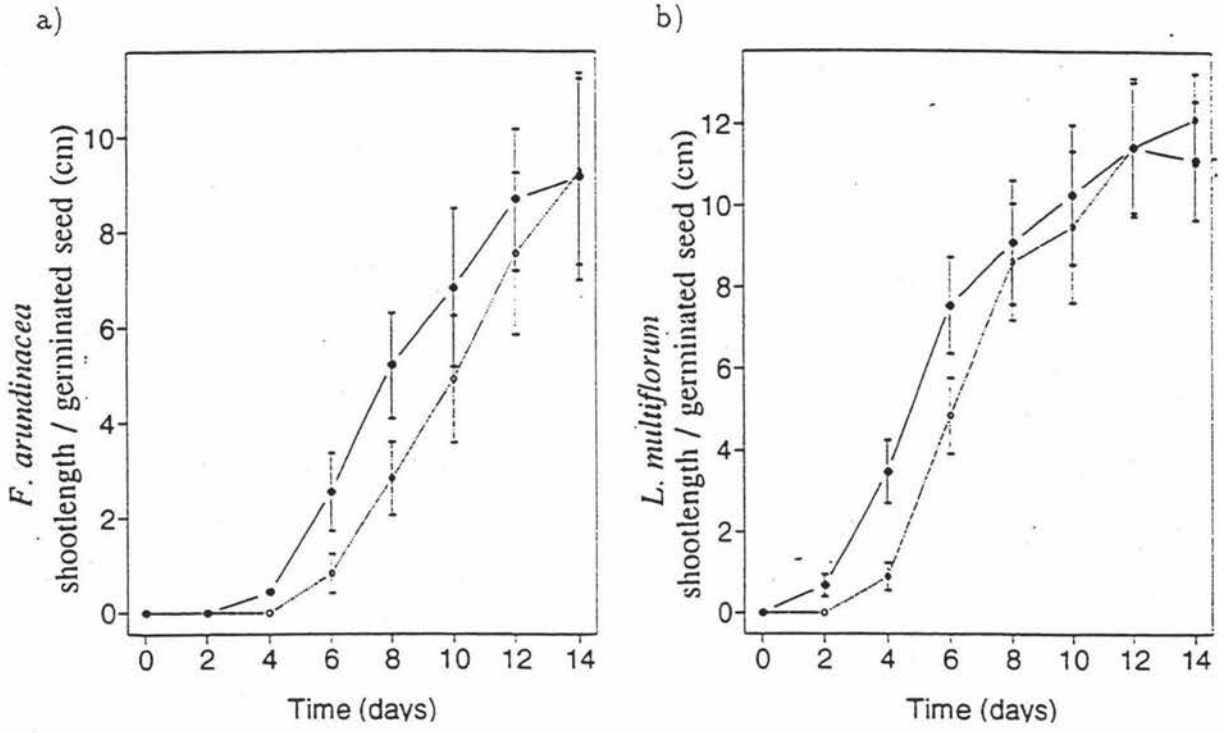


Figure 10. The effect of prechilling (5°C for 7 d) on the shootlength of a) *F. arundinacea* and b) *L. multiflorum* seeds. Data are means of 4 replicates. Vertical bars represent SE's of individual means. Closed symbols represent + prechilling, open symbols represent - prechilling.

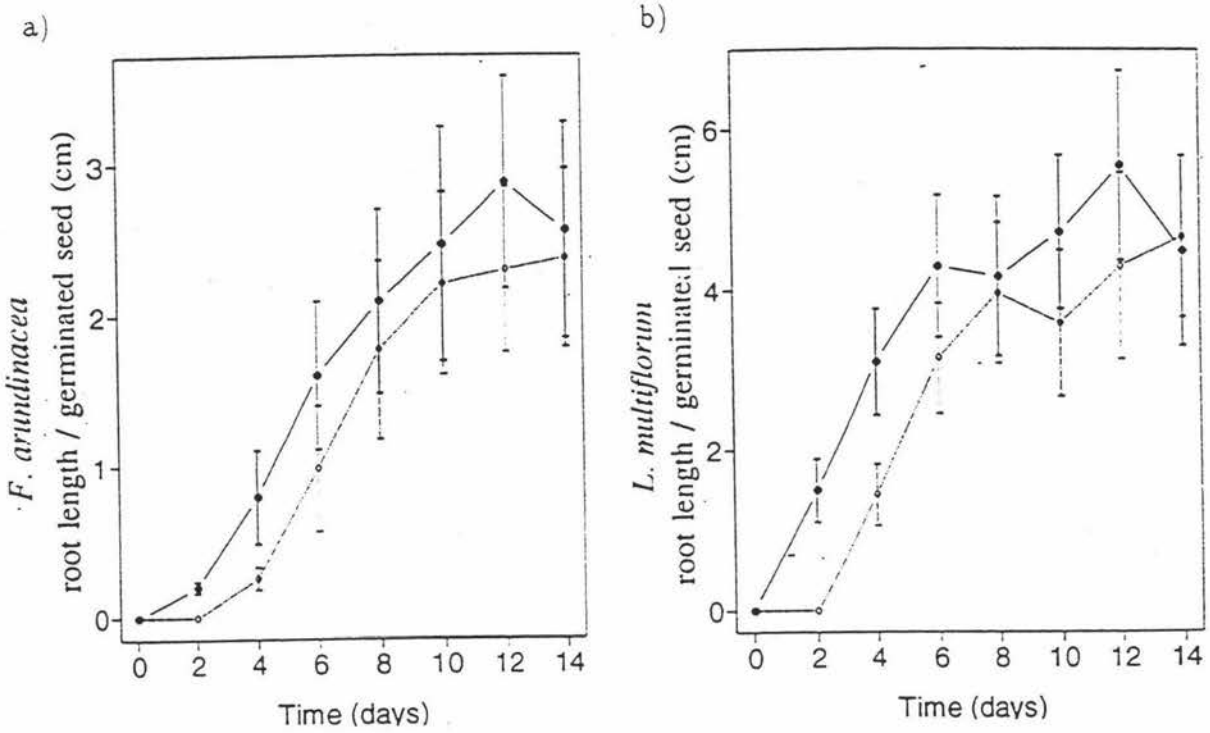


Figure 11. The effect of prechilling (5°C for 7 d) on the root length of a) *F. arundinacea* and b) *L. multiflorum* seed. Data are means of 4 replicates. Vertical bars represent SE's of individual means. Closed symbols represent + prechilling, open symbols represent - prechilling.

Table 7. A comparison of linear growth (dry weight) in *L. multiflorum* and *F. arundinacea.*

Species	Prechilling	Duration of linear growth phase	Rate of linear growth (mg/d)	P value for rate comparisons with	
				L.m. + P	*F.a.* - P
L. multiflorum	-	4-8 d	0.494	0.226	0.461
L. multiflorum	+	2-6 d	0.494	-	n.a.
F. arundinacea	-	8-12 d	0.1919	n.a.	-
F. arundinacea	+	4-12 d*	0.1027	0.053	0.003

* Regression analysis calculated 4-8 d because of high variation of data at 10 d. Prechilling consisted of imbibing seed for 7 days at 5°C in 2% (w/v) KNO_3. The seeds were then transferred to constant 20°C. There was constant darkness throughout except when seeds were monitored for germination. Values represent the means of 3 replicates ± SE.

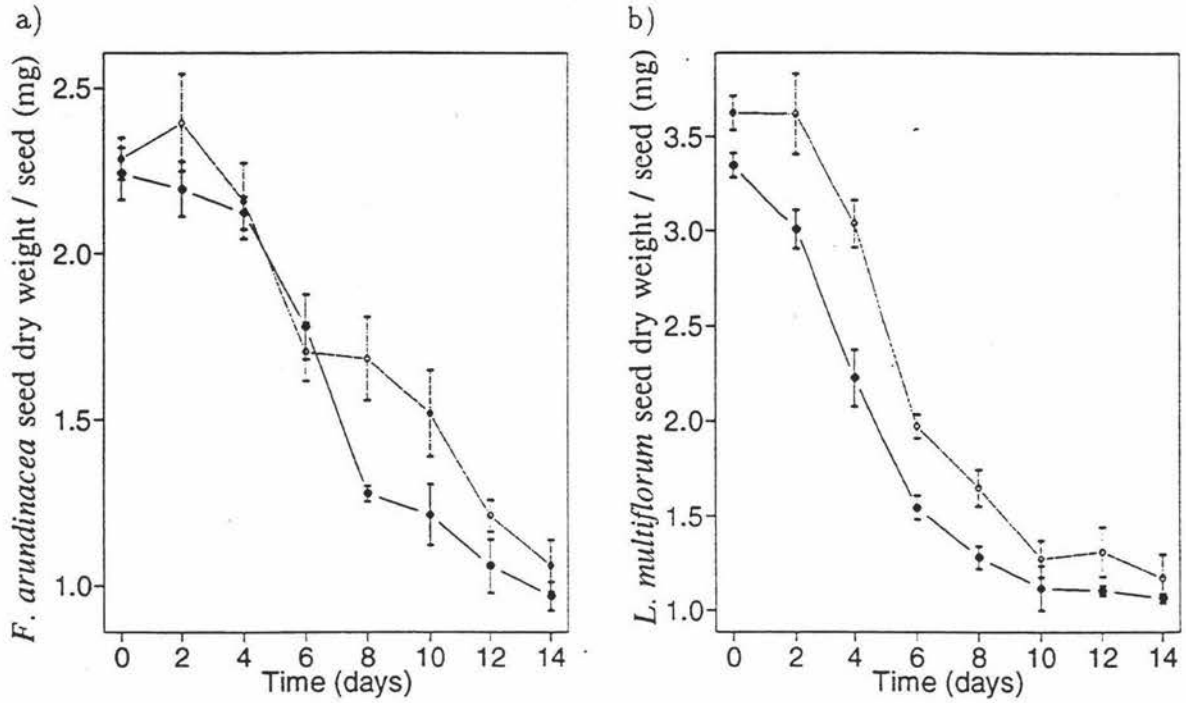


Figure 12. The effect of prechilling (5° C for 7 d) on the seed dry weight of a) *F. arundinacea* and b) *L. multiflorum* seed. Data are means of 4 replicates. Vertical bars represent SE's of individual means. Closed symbols represent + prechilling, open symbols represent - prechilling.

closely with the decrease in seed dry weight here (Figure 12).

The effect of prechilling for both species was again a reduction in the time to the onset of reserve mobilisation events with decreases in seed dry weight of prechilled and non prechilled seeds being detected by day 2 of the time-course (Figure 12). The effects of prechilling thus mirrors the effects of this treatment on the onset of germination. There was a strong quantitative correlation between patterns of seedling growth and reserve mobilisation for prechilled and non prechilled *L. multiflorum* and for prechilled *F. arundinacea.*

Figure 13 shows that decreases in starch levels of prechilled *L. multiflorum* and *F. arundinacea* reflect the trends found for seed dry weight changes (Figure 12).

The proportion of seed dry weight as starch was found to be 29.6% in *L. multiflorum* and 26.4% in *F. arundinacea.* After 14 d germination these starch levels were reduced to 4.8% in *L. multiflorum* and 7.3% in *F. arundinacea.* Decreases in starch levels accounted for 41% of total losses in seed dry weight in *L. multiflorum* and 46% in *F. arundinacea.*

5.1.5 α-Amylase activity

In the absence of prechilling there was a 2 d delay before a peak in α-amylase activity was reached in seeds of *F. arundinacea* (day 8) compared to *L. multiflorum* (day 6) (Figure 14). These peaks in activity were at the times of large changes in the dry weights of the seeds and seedlings and followed rapid increases in % germination (Figures 8, 9 and 10). The level of α-amylase activity reached per mg of dry weight was similar in non prechilled seeds of *F. arundinacea* (0.28 enzyme units/mg) (day 8) and *L. multiflorum* (0.29 enzyme units/mg) (day 6).

When prechilled, however, *F. arundinacea* (0.36 enzyme units/mg, day 6) had a lower peak level of α-amylase activity than *L. multiflorum* (0.56 enzyme units/mg,

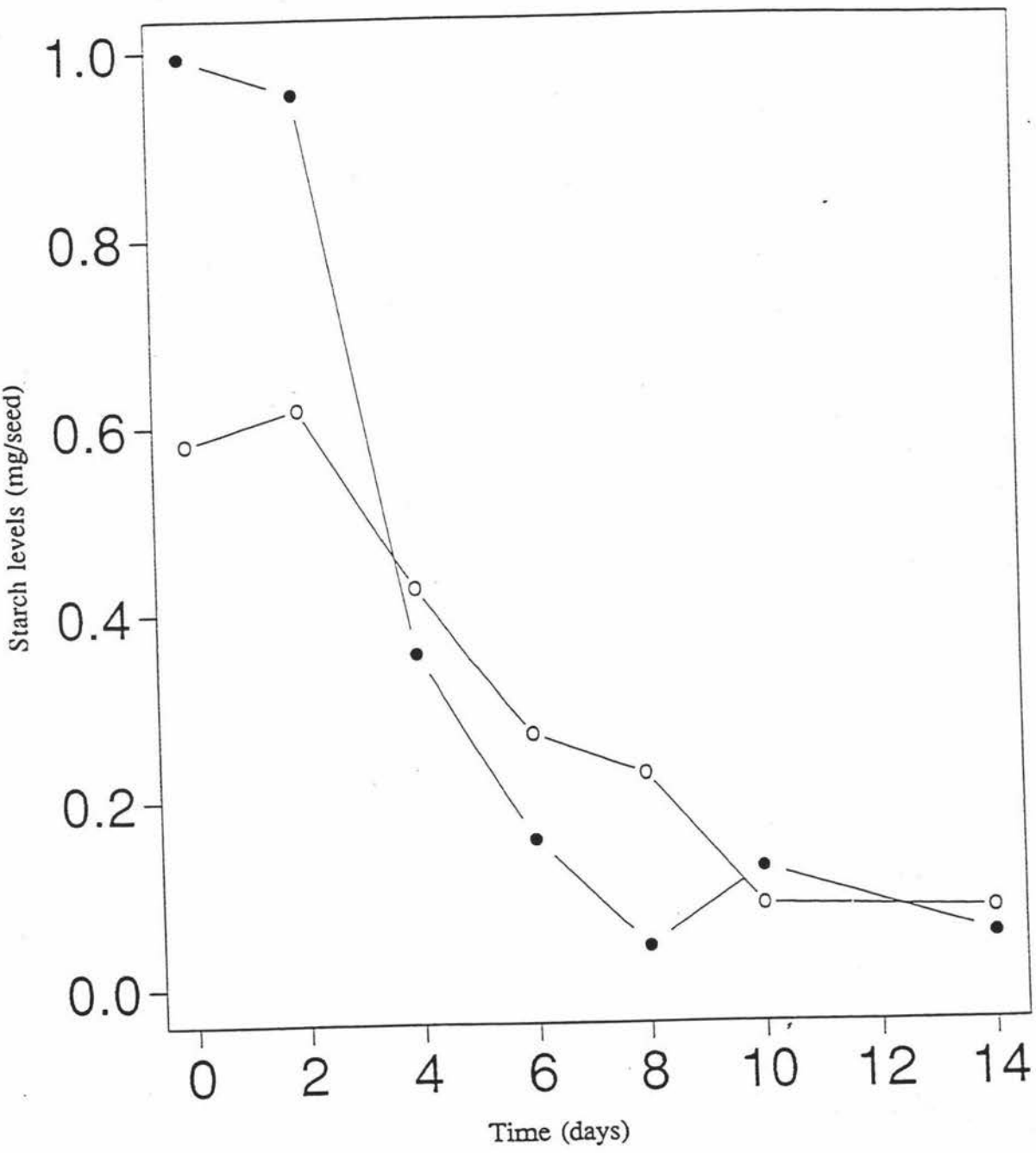


Figure 13. Starch levels of prechilled *F. arundinacea* (o) and *L. multiflorum* (•) over a 14 day time-course. Prechilling consisted of imbibing seed for 7 days at 5° C in 0.2% (w/v) KNO_3. The seeds were then transferred to constant 20° C. There was constant darkness throughout except when seeds were monitored for germination. Data are means of 2 replicates.

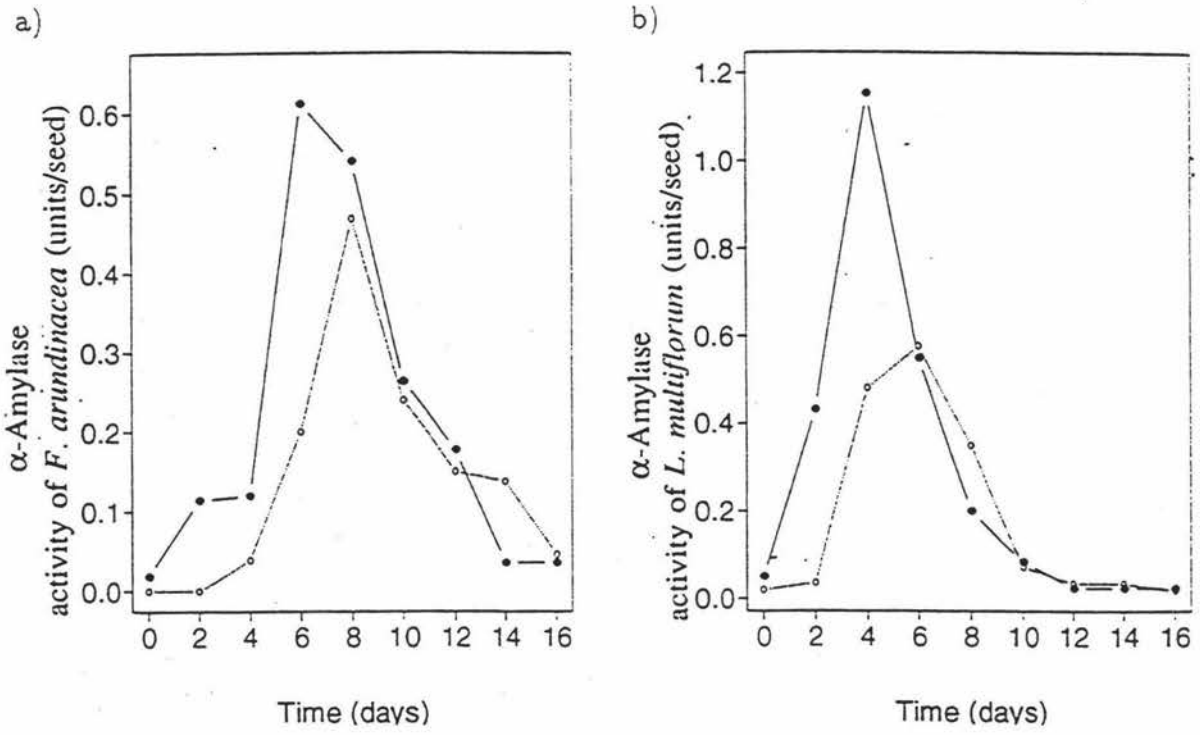


Figure 14. The effect of prechilling (5°C for 7 d) on the α-amylase activity of a) *F. arundinacea* and b) *L. multiflorum* seed. Closed symbols represent + prechilling, open symbols represent - prechilling.

day 4). Prechilling resulted in double the peak level of α-amylase activity in *L. multiflorum* but only approximately 25% higher levels in *F. arundinacea*. As in non prechilled seeds there was a 2 day delay of *F. arundinacea* reaching a peak in activity (day 6) compared with *L. multiflorum* (day 4). These peaks in activity also followed the rapid increase in germination % (Figure 8).

The data presented in Figure 14 was from the same time-course as that used to determine the seed and seedling dry weight changes and % germination. The α-amylase activity profile was representative of the many other time-course experiments. Although considerable differences were found in the absolute levels of total α-amylase assayed, trends from growth data were consistent. Other time-courses of α-amylase activity are in Appendix 5a and b.

5.1.6 α-Amylase isoenzymes

α-Amylase exists as multiple isoenzymes which can be separated by isoelectric focusing. Plate 5 shows the isoenzyme profiles of *F. arundinacea* from days 0-14 following imbibition. Two distinct bands (pI 4.75 and 5.0) were found to be present from day 2 to day 14. From day 6 further bands were apparent (pI 4.35, 4.55, 4.65 and 5.25) which correlated with the peak in α-amylase activity in Figure 14. Due to the possibility of undetected, low levels of α-amylase isoenzymes being present, differing volumes of *F. arundinacea* seed extracts were used to result in equal α-amylase levels over days 4-8. There were no quantitative differences observed over these days in isoenzyme profiles, however, distinct qualitative changes in the darkness of the bands (pI 4.35, 4.55 and 4.65) were observed (Plate 6). A densitometer scan was not done as the background was too varied.

A similar comparative time-course analyses of α-amylase isoenzymes for prechilled seeds showed no clear evidence of any differential gene expression induced by prechilling (data not shown).

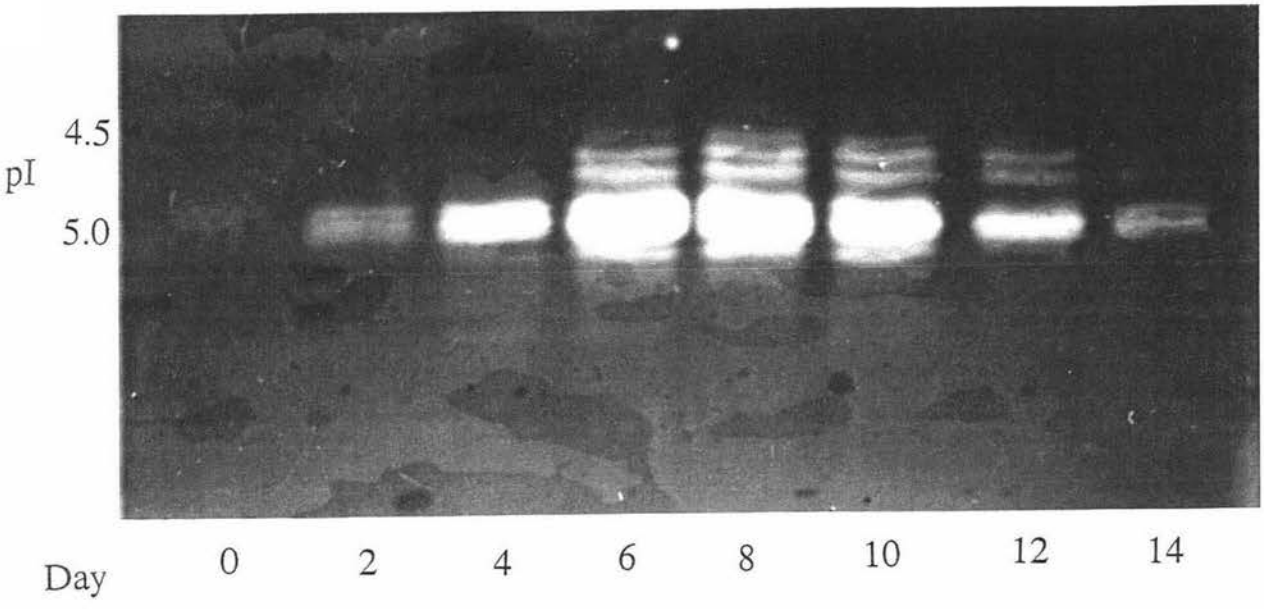


Plate 5. α-Amylase isoenzymes of *F. arundinacea* seeds over a 14 day germination time-course. Equal numbers of seeds were extracted and the extracts were separated on an IEF gel (pH range 3.5-9.5). α-Amylase was visualised using β-limit dextrin. Equal volumes were applied to the gel for each time point.

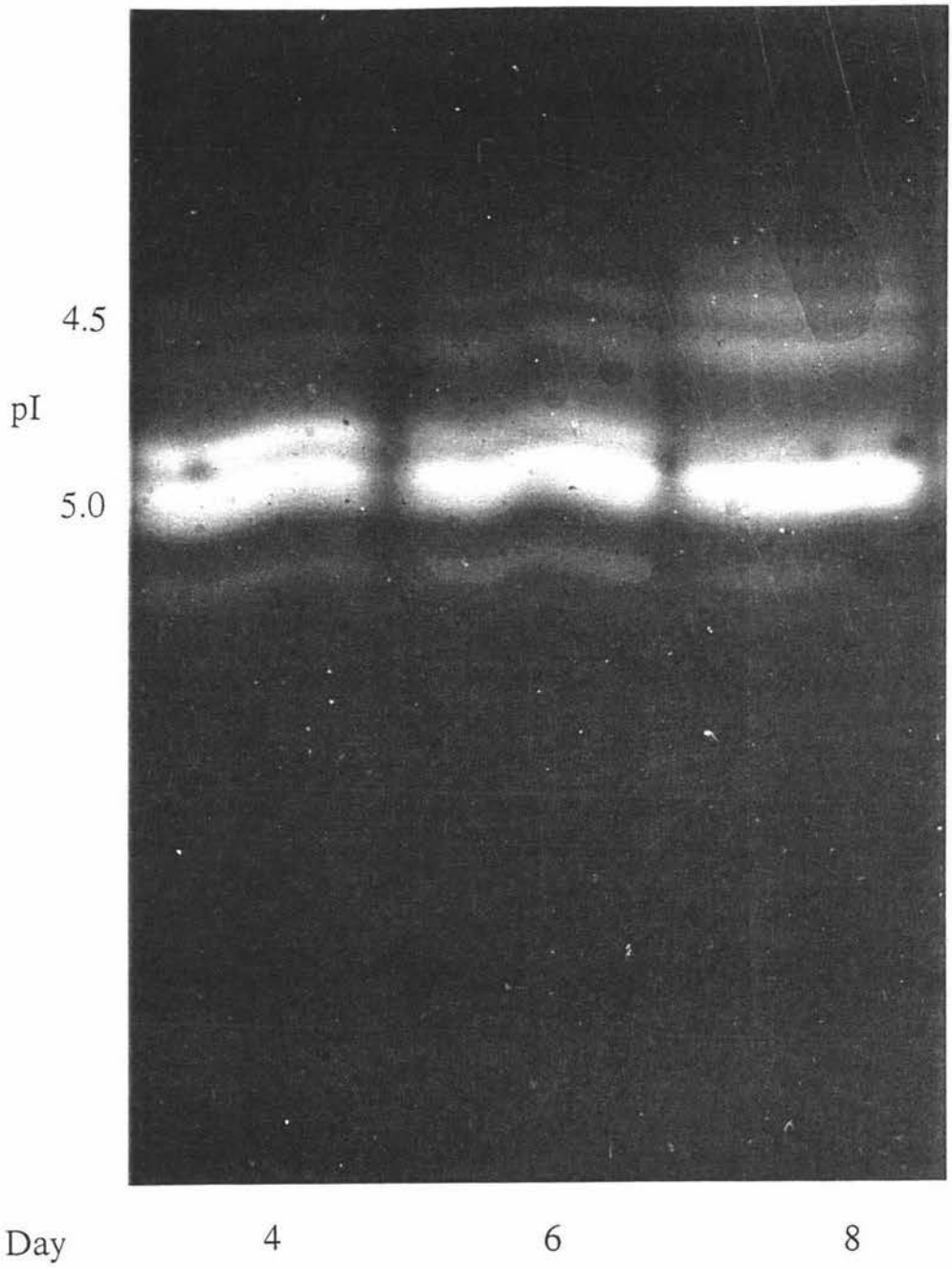


Plate 6. α-Amylase isoenzymes of *F. arundinacea* seeds over Days 4-8 of a germination time-course. Differing loading volumes were used to result in approximately equal levels of α-amylase activity (as previously determined from α-amylase assays). Equal numbers of seeds were extracted and the extracts were separated on an IEF gel (pH range 3.5-9.5). α-Amylase activity was visualised using β-limit dextrin.

Plate 7 shows a comparison between wheat, *L. multiflorum* and *F. arundinacea* at times when they were known to have high levels of α-amylase activity. Although *F. arundinacea* and *L. multiflorum* had similarities in their α-amylase isoenzyme patterns (both had distinct bands at pI 4.65, 4.75 and 5), differences were also obvious between these species such as *L. multiflorum* having additional bands (pI 5.35 and 5.55). There were marked differences between these species and wheat in the nature of these isoenzymes. Although in wheat there were bands present at pI 4.35 and 4.65, and very faint bands present at 5.0 and 5.25 in common with *F. arundinacea* and *L. multiflorum*, these bands showed no similarity in their groupings to the other two species. In addition, wheat had two obvious pI groups compared to one in both *F. arundinacea* and *L. multiflorum*, with wheat bands in the additional higher pI group being considerably more distinct than those previously described in the lower pI group.

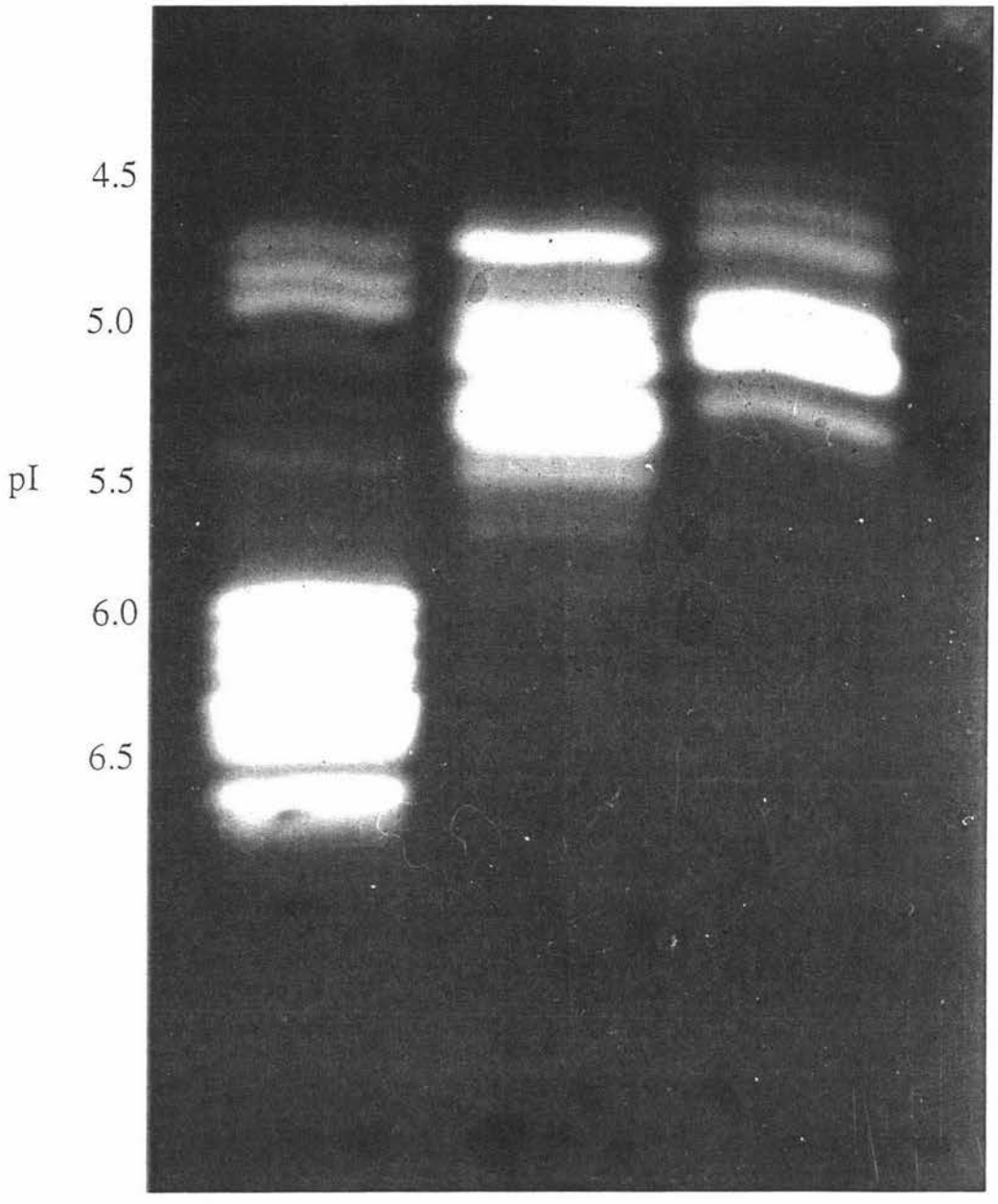


Plate 7. α-Amylase isoenzymes of *F. arundinacea*, *L. multiflorum* and wheat seeds at times of high levels of α-amylase activity (as previously determined from α-amylase assays) during a germination time-course. Equal numbers of *F. arundinacea* and *L. multiflorum* seeds (25) and 4 wheat seeds were extracted and the extracts were separated on an IEF gel (pH range 3.5-9.5). α-Amylase activity was visualised using β-limit dextrin. Equal volumes were applied to the gel for each species.

5.2 Discussion

5.2.1 Comparative studies between F. arundinacea and L. multiflorum: germination and seedling growth rate comparisons

There was no significant difference between *L. multiflorum* and *F. arundinacea* in final % germination. The effects of prechilling on the final % germination were also not significant (P = 0.0529). This differs to results described previously (Chapter 4.1), where prechilled and non prechilled *F. arundinacea* had significantly different final % germination (P = 0.001). This may be a reflection of the lack of homogeneity or the loss of a stratification requirement during storage of the *F. arundinacea* seed lot (as described in Chapter 4.2.3).

Germination and seedling growth were faster for *L. multiflorum* than *F. arundinacea* in both prechilled and non prechilled seeds. The onset of linear growth (as described by Blacklow 1973 and Coolbear *et al.* 1987) was earlier in *L. multiflorum* than in *F. arundinacea*, however, there was no significant difference in linear growth rates in non prechilled seeds (P = 0.461) of both species.

F. arundinacea growth rates were linear until day 10 in prechilled seeds and day 12 in non prechilled seeds, when the phase of rapid seedling growth stopped (Figure 9). The subsequent levelling off in linear growth rate indicates that seed reserves had been depleted. This is supported by a correlation with low starch levels in prechilled seeds at the same times (Figure 13). In addition, apparent decreases in seedling dry weight (Figure 9) after day 12 in both species (with and without prechilling), although not significant, may be due to an exhaustion of food reserves in some seeds and losses of seedling dry weight via respiration. Decreases in starch levels during the time-course accounted for total losses in seed dry weight of 46% in *F. arundinacea* and 41% in *L. multiflorum.*

Although the conversion of seed reserves to seedling growth would not be expected to be on a weight to weight basis, the efficiency of reserve utilisation was found to

be similar for both species with and without prechilling. Over days 0-12 of the time-course, seedling dry weight increases accounted for approximately 75% of the decreases in seed dry weight (*F. arundinacea* with prechilling = 76%, without prechilling = 72%, *L. multiflorum* with prechilling = 74%, without prechilling = 65%).

A linear growth pattern in *L. multiflorum* was less clearly defined than in *F. arundinacea*, however, when tying the decrease in starch levels (Figure 13) with seedling dry weight increases (Figure 9), seedling growth rates did appear to be linear until being limited by low levels of starch (day 6 in prechilled seeds). A second increase in seedling dry weight (day 8 in prechilled seeds and day 10 in non prechilled seeds) is unexplained at this stage (Figure 9).

In *L. multiflorum*, seedling growth for both prechilled and non prechilled seeds coincided with seed dry weight decreases. In non prechilled *F. arundinacea,* however, there was a 3 d delay in the onset of rapid seedling growth following the onset of rapid loss of reserves (Table 8, Figures 9 and 12). Increases in root length (Figure 10) correlated to these decreases in seed dry weight.

This dislocation of events is only observed in non prechilled *F. arundinacea.* Prechilling appeared to help in the utilisation of reserves (see Table 8) as there was a more rapid onset of linear seedling growth, although, interestingly, this linear growth was at a significantly slower rate than in non prechilled *F. arundinacea* ($P = 0.003$). Prechilling did not appear to affect the rate of growth in *L. multiflorum* ($P = 0.226$). Caution is needed in the interpretation of this data, however, because as already stated, *L. multiflorum* fits the linear growth model less well than *F. arundinacea* (Figure 9). The anomaly in Table 7, where the linear growth rate of prechilled and non prechilled *L. multiflorum* (0.494 mg/d) is not significantly different to that of non prechilled *F. arundinacea* (0.1919 mg/d), but is significantly different to prechilled *F. arundinacea* (0.1027 mg/d), further emphasises the variation around the linear regression in *L. multiflorum.* In addition the apparent decrease in

Table 8. A summary of germination and early seedling growth data of prechilled and non prechilled *F. arundinacea* and *L. multiflorum* over a 16 day time-course.

	F. arundinacea		*L. multiflorum*	
	- prechilling	+ prechilling	- prechilling	+ prechilling
Onset of germination	2-3 d	2 d	2 d	~1 d
T_{50}	5 d	3 d	3 d	1.5 d
Onset of rapid seedling growth	7 d	4 d	2-3 d	2 d
Onset of rapid loss of reserves	4 d*	2-4 d	2 d	1-2 d
Onset of increased α-amylase activity	4 d	2 d	~3 d	~1d

* but lag phase of 6-8 d

Prechilling consisted of imbibing seed for 7 days at 5°C in 0.2% (w/v) KNO_3. The seeds were then transferred to constant 20°C. There was constant darkness throughout except when seeds were monitored for germination.

seedling growth in prechilled *F. arundinacea* may be an artifact of the timing of measurements. Nevertheless, the present results are interesting in that they indicate that radicle emergence and mobilisation of reserves may be less tightly linked than in *L. multiflorum.*

Under optimal germination conditions there was no real difference in the way the two species mobilised reserves, with decreases in starch levels accounting for similar total losses in seed dry weight in both species. Bigger delays were apparent in the utilisation of *F. arundinacea* reserves, supporting the idea that the processes are not as tightly coupled in *F. arundinacea* as in *L. multiflorum.*

Largely, prechilling allowed seedling growth to start earlier via affects on germination. In prechilled *F. arundinacea* seeds, the reserve mobilisation capacity may be ahead of the embryo growth capacity. It may be that reserves are being mobilised but all they can do initially is drive cell expansion without any real accumulation of anabolites. The embryo size may be limiting or the efficiency of growth metabolism may be limiting. Conversely, in non prechilled *F. arundinacea* seeds, the embryo growth capacity may be ahead of the reserve mobilisation capacity.

5.2.2 α-Amylase activity

There was a close relationship between α-amylase activity and reserve mobilisation in both species. The onset of increased α-amylase activity (Figure 14) was seen either just prior to or at approximately the same time as the rapid loss of seed reserves in both prechilled and non prechilled seeds of both species. α-Amylase activity tied in with the onset of rapid mobilisation of reserves.

A lower level of increase of α-amylase activity in *F. arundinacea* following prechilling compared with *L. multiflorum*, supports the earlier suggestion (Chapter 5.2.1) that *F. arundinacea* may be reserve mobilisation limited following prechilling.

It is important to understand the nature of the variation found in the α-amylase assays from different time-courses (Appendix 5a and b). It may be due in part to the small seed sample sizes used for enzyme assays. The varying lengths of time the seeds had been in storage may also have had an effect.

Differences were apparent in the appearance of α-amylase isoenzymes at different times during the germination of prechilled *F. arundinacea* which were not reflected in any changes in grass reserves. Two distinct bands were present from day 2 (Plate 5) which correlated with α-amylase activity shown in Figure 14, and the onset of rapid reserve losses (Figure 12). There were further bands apparent from day 6 which did not tie in with seedling growth data. By day 6, 67% of prechilled *F. arundinacea* seeds had already germinated (Figure 8) and the onset of rapid loss of reserves and rapid seedling growth had occurred at least two days previously (Figures 9 and 12). The appearance of these later forming isoenzymes tied in with the peak in α-amylase activity (Figure 14) suggesting that the different isoenzymes are active at different stages of germination. It is the earlier forming isoenzymes that appear to be important for the onset of reserve mobilisation, especially as non prechilled seeds of both species have lower overall α-amylase levels and yet their germination rates are not significantly different to those of prechilled seeds. The delay in the onset of linear increases in seedling growth rate of non prechilled *F. arundinacea* correlated to the 2 d delay observed in the appearance of α-amylase isoenzymes for this treatment (data not shown). Prechilling did not change the spectrum of isoenzymes, it just advanced it.

There may be some complex controls of differential α-amylase gene expression during germination and reserve mobilisation. Different α-amylase isoenzymes may be active in different locations in the seed, or be under different hormonal or temporal control, as in barley (Jacobsen and Chandler 1987, Nolan *et al.* 1987, Brown and Brodl 1988) and wheat (Garcia-Maya 1990) or possibly have different specificity for the substrate they are breaking down.

Differences in the darkness of bands (Plate 6) could not be determined quantitatively

using a densitometer as the background was too varied. The relative amounts of α-amylase components present in the extracts might be determined in future experiments using chromatofocusing techniques (MacGregor *et al.* 1988).

Different isoenzyme patterns in wheat, *L. multiflorum* and *F. arundinacea* (Plate 7) suggest that different biochemical mechanisms may be involved in the germination processes of these seeds. Both *L. multiflorum* and *F. arundinacea* have only one pI group compared to two in wheat. There is differential control of the two wheat pI groups (Fincher 1989), however, in *L. multiflorum* there is only one major group and the efficiency at which reserve mobilisation occurs does not seem to be impaired. An α-amylase inhibitor found to be present in *F. arundinacea* (Cornford and Hill 1994) may either have no effect on endogenous enzymes or it may be masking a high pI group.

6. Hormone responsiveness

6.1 Results

Preliminary experimental work was carried out in an attempt to identify whether a hormonal mechanism might contribute to the differences in germination and early seedling growth between *F. arundinacea* and *L. multiflorum*. This work studied the response of aleurone tissue to exogenous plant growth regulators (PGR's) and prechilling by measuring the levels of α-amylase produced.

F. arundinacea and *L. multiflorum* seeds used for the experiments summarised in Figure 16 had been in storage for 18 months longer than those used in Figure 15. In addition, the seeds used in Figure 16 had been dehusked up to three months prior to the experiment. Although there was the risk of contamination, it was decided not to use antibiotics due to the possibility that they may affect the biochemical processes occurring within the seed at this time.

Accepting that this work is limited as these were only single experiments (although replicates within each treatment were used) some trends within and between the two species in response to prechilling and exogenous PGR's have emerged.

F. arundinacea and *L. multiflorum* embryoless half-seeds were shown to vary in levels of α-amylase produced in response to their incubation in solutions of PGR's (Figures 15 and 16). Incubation in gibberellic acid (GA_3) alone resulted in the maximum production of α-amylase in *L. multiflorum* and in *F. arundinacea* (Figure 16), although in Figure 15 α-amylase levels of GA_3- incubated *F. arundinacea* were not significantly different to that of the control. More aleurone tissue may have been present with the larger *L. multiflorum* species, allowing potentially higher levels of α-amylase production, and Figure 15 shows significantly higher levels of α-amylase were present in this species in comparison to *F. arundinacea* when incubated in GA_3 over 72 h.

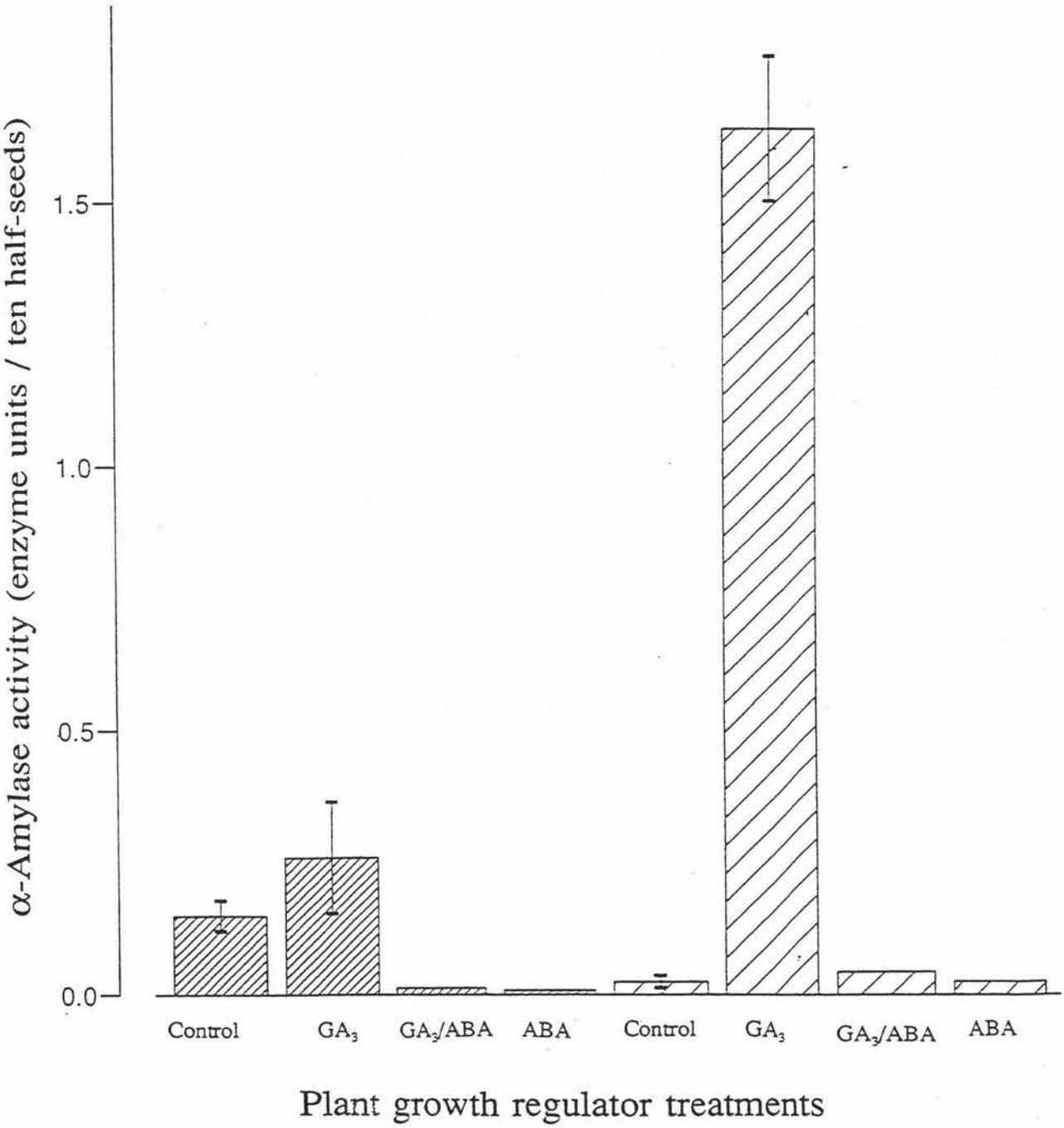


Figure 15. α-Amylase activity of *F. arundinacea* and *L. multiflorum* embryoless half-seeds in response to exogenous plant growth regulators. These are 10^{-6} mM gibberellic acid (GA_3), 10^{-5} mM abscisic acid (ABA), 10^{-5} abscisic acid + 10^{-6} gibberellic acid (GA_3/ABA) and a control. All solutions contain 10 mM $CaCl_2$. Half-seeds were incubated in the PGR solutions at 25°C for 72 h with constant darkness and stirring throughout. Seeds were not prechilled. Data are means of 3 replicates. Vertical bars represent SE's of individual means.

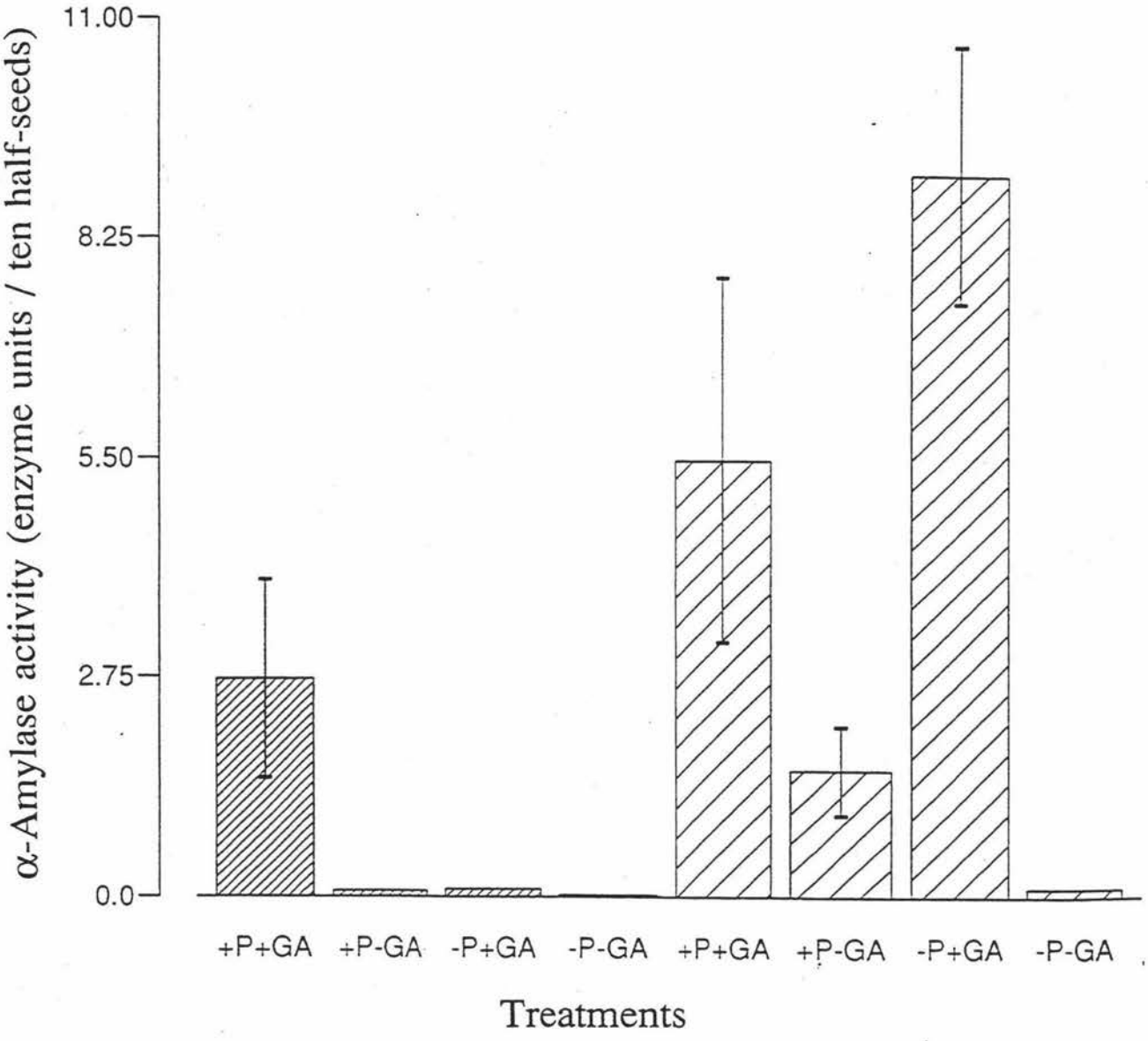


Figure 16. α-Amylase activity of *F. arundinacea* and *L. multiflorum* embryoless half-seeds in response to gibberellic acid (GA_3) and prechilling (7 d at 5°C in 0.2% w/v KNO_3). Prechilled (+P) and non prechilled (-P) half-seeds were incubated in 10^{-6} mM GA_3 (labelled + GA) and a control (labelled - GA). Both solutions contained 10 mM $CaCl_2$. Incubations were at 25°C for 72 h with constant darkness and stirring throughout. Data are means of three replicates. Vertical bars represent SE's of individual means.

The responsiveness of *F. arundinacea* to GA_3 was greatly enhanced by prechilling, however, prechilling did not enhance the responsiveness of *L. multiflorum*. These results should be treated with caution however: while the mean α-amylase levels in GA_3 treated non prechilled *F. arundinacea* were similar in both experiments, in *L. multiflorum*, Figure 16 shows mean αamylase levels to be over five times higher than in Figure 15.

6.2 Discussion

6.2.1 Hormone responsiveness of L. multiflorum and F. arundinacea half-seeds to GA_3

L. multiflorum embryoless half-seeds were found to be more responsive to exogenous GA_3 than *F. arundinacea* during a 72 h incubation period, as determined by α-amylase production. To determine whether the lower production of α-amylase in *F. arundinacea* was due to less responsive aleurone tissue, future work could include a GA_3 dose response curve comparing both species. In previous results (Chapter 5.1.6), peak α-amylase activity in *F. arundinacea* was found to occur after 6 d germination in prechilled seed and 8 d in non prechilled seed. This was a delay of 2 d in peak α-amylase activity compared with both prechilled and non prechilled *L. multiflorum*. Thus, if the incubation period of the half-seeds was longer (eg 120 h for *F. arundinacea*), α-amylase production in response to GA_3 may have been nearer to that of *L. multiflorum*. A half-seed germination time-course over an eight day period using GA_3 and control treatments may also be useful as a species comparison to determine whether *F. arundinacea* is slower or less responsive to GA_3 than *L. multiflorum*.

As *L. multiflorum* half-seeds are larger than *F. arundinacea*, there was the potential for higher levels of *L. multiflorum* α-amylase production due to more tissue being present. Therefore the relative size of the seed of each species needs to be taken into account. However, as SE's were large, it is difficult to accurately determine seed size effects. In addition, the large amount of variation occurring between replicates may have masked other species or treatment differences.

6.2.2 Storage effects

The effects of storage on the seed lot, as noted previously (Chapters 4.2.3 and 5.2.2),

may explain the variation observed in non prechilled *L. multiflorum* mean α-amylase levels, which are over five times higher in Figure 16 than in Figure 15. Half-seeds used in Figure 16 had been in storage for 31 months compared to 13 months for those used in Figure 15. It may be that the observed stratification response in *L. multiflorum*, although still present, was considerably reduced during the 18 months storage time between experimental work of Figure 15 and 16. This may have resulted in a difference in responsiveness of the aleurone tissue to GA_3. An investigation by Schuurink *et al.* (1992) with dormant and after-ripened *Avena fatua* grains have shown that the aleurone cells differ substantially in their capacity to respond to exogenous GA_3. However, as the loss in stratification requirement of *F. arundinacea* (Table 5) did not result in any change in response of half-grains to GA_3 (Figures 15 and 16), the increase in α-amylase levels over time in *L. multiflorum* may be due to a deterioration in the quality of the seed during storage time. Wheat varieties exhibiting preharvest deterioration have been found to have higher α-amylase levels (Flintham and Gale 1988). In addition, the older seeds used in Figure 16 had been dehusked up to three months prior to experimental work, which may have contributed to seed deterioration during storage, or resulted in them being more susceptible to temperature effects on residual dormancy.

6.2.3 Prechilling effects

In *F. arundinacea*, where non prechilled-GA_3 treated half-seeds showed no significant difference to the control in the production of α-amylase, prechilling greatly enhanced the half-seed in α-amylase production in response to GA_3 (Figures 15 and 16). Non prechilled *L. multiflorum* seeds were responsive to GA_3 and when prechilled showed no significant difference in their response. Prechilling may have changed the sensitivity of the *F. arundinacea* aleurone cells to GA_3, which together with thermal time effects described in Chapter 4.2.3 may have resulted in the onset of earlier germination and also may be involved in linking reserve mobilisation to reserve utilisation. Prechilling of whole grains (Chapter 5.1.6) resulted in increased levels of α-amylase in both species. Trewavas (1982) proposed that a change in sensitivity

of the aleurone cells to gibberellins is as important for the production of α-amylase during germination as the levels of gibberellins. The effects of low temperatures have been suggested to include differential changes in enzyme concentration or in enzyme production (Bewley and Black 1985). In addition, inhibitors may disappear with prechilling (Salisbury and Ross 1985). Future work including a half-seed germination time-course, both with and without prechilling, may be useful in determining whether prechilling induces a change in sensitivity of the half-seeds to exogenous GA_3 in the two species.

6.2.4 Other variables

It is essential that studies such as these are coupled with measurements of endogenous hormone levels, particularly ABA and GA_3. Nicholls *et al.* (1986) have suggested that sufficient physiologically active gibberellins may be present in the aleurone at the start of incubation, or that the GA_3 synthesis responses are triggered during kernel maturation. Atzorn and Weiler (1983) claimed that one batch of barley endosperm halves appeared to have active gibberellins synthesised during the incubation period.

The nature of the endogenous gibberellins involved is not clear. Detailed information is required on the nature, activity, tissue distribution and physiological significance of several gibberellins in germinating seeds (Atzorn and Weiler 1983). Furthermore, it is not clear whether experiments with isolated tissue fragments truly reflect the behaviour of the tissue *in vivo*. Marchylo *et al.* (1981) have found the surgical removal of seed coats and embryo-scutella from mature wheat caryopses to have a marked effect on the production of α-amylase and requirement for exogenous GA_3. Observing the IEF pattern of half-seeds with respect to α-amylase production in comparison to that of whole grains may be beneficial in future work.

There is also a need to evaluate the potential effects of routine disinfection procedures used in the half-seed experiments, especially as whole seeds used in

experimental work in the previous chapters of this study were not surface sterilised. Tittle *et al.* (1988) found that disinfection with 1% compared to 0.1% hypochlorite significantly reduced the synthesis of α-amylase by isolated barley aleurone layers, although it was not found to differentially affect the synthesis of each group of isoenzymes in barley. The potential benefits of including antibiotics in the incubation media in future half-grain work also need to be evaluated.

Nicholls (1982) has found environmental factors during development to influence the hormone response of mature aleurone tissue such that the behaviour of different batches of the same barley cultivar grown in different locations can vary considerably. Therefore, it is important that future work in this area be extended to different seed lots of *F. arundinacea* and *L. multiflorum* and also to other species, as different mechanisms may be operating in different species. The relative contribution of the scutellum and the aleurone layer in the production of α-amylase may also vary with different species, making it difficult to compare species in their α-amylase production in response to GA_3.

7. General discussion

During the establishment of mixed pastures with complementary species such as *F. arundinacea* and *L. multiflorum*, the comparatively poor seedling vigour of *F. arundinacea* places it at a competitive disadvantage. Results from a previous study (Brock *et al.* 1982) suggested that differences in seedling vigour between the two species were either due to relative growth rate differences, or that there were differences in the mobilisation process of the endosperm. The limitations of this study (described in Chapter 2.2) strongly showed a need for re-examination.

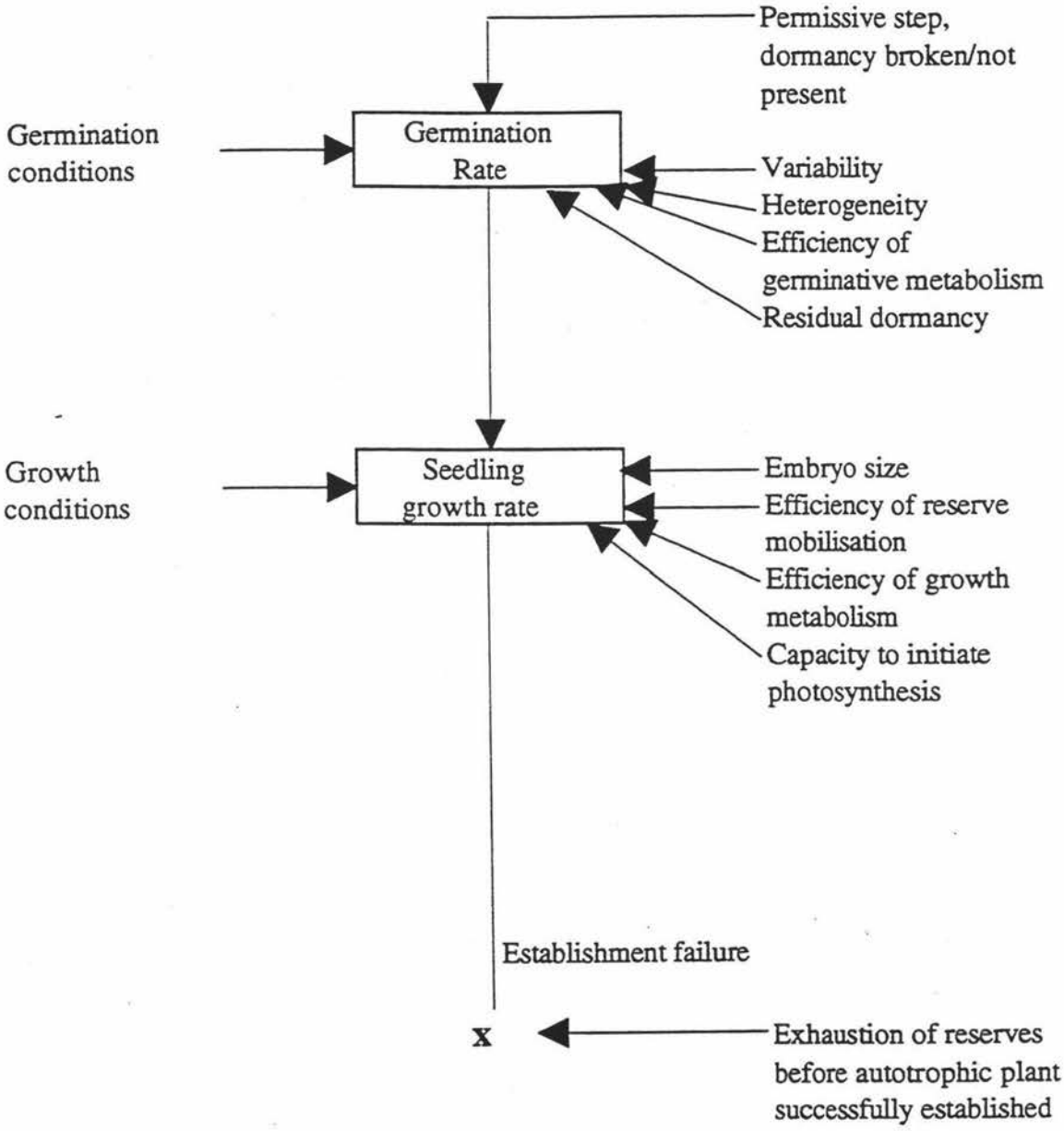
In addition, while much knowledge exists about the seed reserve mobilisation process in cereals, particularly barley and wheat, little information is available for pasture grasses. With many differences having been observed between the model system of seed reserve mobilisation in barley and other species (Chapter 2.2) these processes in pasture grasses would not be expected to be identical to that described for barley. Most literature available on such previous studies does not distinguish between rates of germination and rates of seedling growth, making it difficult to compare previous studies with data obtained in this study.

There was therefore an obvious need for a detailed study on single seed lots of *F. arundinacea* in comparison with *L. multiflorum*, highlighting key areas requiring broadening with a multi-seed lot survey. The aim of this project was to determine whether reserve mobilisation in general and α-amylase activity in particular are limiting components of seedling growth and establishment in *F. arundinacea*. Figure 17 summarises the variables affecting germination and seedling growth rates and indicates where in the germination and seedling growth process they have an effect.

7.1 Heterogeneity of the F. arundinacea seed lot

When looking at the *F. arundinacea* seed lot in detail over time it was found to be

Figure 17. Variables affecting germination rate and seedling growth rate



Changes in germination and seedling growth conditions would change the influence of the other factors.

very heterogenous in its germination and seedling growth. Tetrazolium staining showed a large variation in the staining pattern of *F. arundinacea* seeds, and also showed that the presence of live aleurone tissue was not obvious in all stained seeds (Plate 1). Further work is needed in the accurate interpretation of tetrazolium results and also in the determination of the extent of non lethal damage which can occur in seeds of different species. The seed lot also varied largely in seed size (Figure 5) which may have played some part in the observed variation in the uniformity of germination (Table 2). In addition, there was a small proportion of empty seeds within the seed lot (Table 1) which may have contributed to the overall heterogeneity observed. Separating the seed lot into size grades could be useful in looking at the effect of seed size on germination.

A prechilling advantage, which in *L. mutltiflorum* was demonstrated to be largely due to a stratification response, appeared to result in a day degree effect in *F. arundinacea* (there was no overall stratification requirement for a reduced T_{50} or T_{90}-T_{10}). However, as shown by prechilling effects on final % germination, a lack of homogeneity of the *F. arundinacea* seed lot was indicated by a small proportion of the seed lot only being able to germinate following stratification (Table 2, Figure 6a-d). As seeds were in storage for 11 months between these studies, during which time the seed lot apparently lost its stratification requirement (as indicated by final % germination data shown in Table 5), a direct comparison cannot be made between experiments.

The above factors indicative of heterogeneity would be expected to have an overall influence on the germination and seedling growth behaviour of the *F. arundinacea* seed lot, with a large effect being on the germination rate (Figure 17).

7.2 Evidence that reserve mobilisation may be limiting

Germination and seedling growth of *F. arundinacea* was slower than in *L. multiflorum* due to later radicle emergence. Mobilisation of reserves and the onset

of α-amylase activity correlate well with the utilisation of reserves in *L. multiflorum*, but anomalies have been identified in the process for both prechilled and non prechilled *F. arundinacea*. This delay indicates that reserve mobilisation is not limiting in non prechilled *F. arundinacea*, it is the rate at which reserves are being used that is limiting. The reverse may be the case after prechilling.

Scott and Hampton (1985) have found seedling growth of ryegrass and cocksfoot cultivars to be directly proportional to seed weight. Thus larger seeds give seedlings a better start in the intense competition that occurs during pasture establishment. Bremner *et al.* (1963) found that in contrast with little or no effects from the embryo, it was the volume of the endosperm which had a considerable effect on seedling growth. Chapter 2.2 describes seed size correlations in more detail. It may be that the larger seeds have larger meristems which would give rise to faster multiplying cells and faster germination. Future work could involve comparisons of meristem size of *F. arundinacea* and *L. multiflorum*, possibly by using flow cytometry techniques (Lanteri *et al.* 1994). Although *F. arundinacea* is a smaller seed than *L. multiflorum*, the difference between the two species in the reserve mobilisation process appears to be due to more than size.

Prechilling in *F. arundinacea* does not break dormancy in most of the seed lot (Chapter 4.1.1), therefore prechilling is allowing something to happen in the embryo that allows the rate limiting step in embryo growth to be advanced before visible germination. Future work could look for a cell division cycle in the embryos of *F. arundinacea* during the 7 d prechilling period. This could explain the differences resulting from prechilling as there would suddenly be more meristem activity to drive growth. Techniques of Lanteri *et al.* (1994) could be used for this work. In addition, changes in sensitivity of the aleurone tissue may have occurred during prechilling; a start was made in addressing this issue in Chapter 6.

Apart from the results discussed above, no real differences were detected in the way the two species mobilised reserves, with decreases in starch levels accounting for similar total losses in seed dry weight in both species. There was also a close

relationship between α-amylase activity and reserve mobilisation in both species.

Although differences were apparent in the appearance of α-amylase isoenzymes at different times during germination in prechilled *F. arundinacea*, it was demonstrated that prechilling did not change the spectrum of isoenzymes but just advanced it (data not included). These α-amylase isoenzymes appeared to be active at different stages of germination in prechilled *F. arundinacea* (Plate 6). Their gene expression may be under some complex differential control mechanism during germination and reserve mobilisation.

Differences in isoenzyme patterns of α-amylase between *F. arundinacea* and *L. multiflorum*, and also between wheat, indicate that there may be different biochemical mechanisms involved during the germination process of these species and clearly identifies a need for further work in this area.

7.3 Responsiveness to GA_3

The preliminary half-seed studies on *L. multiflorum* and *F. arundinacea* found *L. multiflorum* embryoless half-seeds to be more responsive to exogenous GA_3 (as determined by α-amylase production) than *F. arundinacea* during a 72 h incubation period, but that α-amylase production in *F. arundinacea* was more susceptible to prechilling. In future experimental work, this incubation period needs to be extended as peak α-amylase levels in *F. arundinacea* were 2 d behind that of *L. multiflorum*. A GA_3 dose response curve comparing both species also needs to be done to determine whether the lower α-amylase production in *F. arundinacea* was due to less responsive aleurone tissue than in *L. multiflorum*. In addition, a half seed germination time-course with GA_3 and control treatments may also be a useful indication of whether *F. arundinacea* is less responsive to GA_3 or just slower in its response than *L. multiflorum*.

Future half-seed experimental work needs to be set up on a larger scale as the

variation occurring between replicates within treatments may have masked other species or treatment differences in this study due to large SE's. The storage status of seeds, the relative seed size of each species and endogenous hormone levels also need to be taken into account.

7.4 Limitations of the study and scope for further work

With different species and even different cultivars of the same species having been found to vary enormously in their germination characteristics and behaviour, it cannot be assumed that the germination behaviour and characteristics of the seeds used in this study reflect that of seeds from different seed lots and cultivars of the same species. Therefore, the main limitation of this study is that it focuses on only one seed lot of each species. This, however, was to enable the major differences between the two species, which may be involved in limiting the establishment of *F. arundinacea* compared to *L. multiflorum*, to be identified. Detailed comparisons were required of the two cultivars used before further species comparisons and cultivars within the same species comparisons can be made.

The time-courses analysed when looking at different stages of germination reflect the average behaviour of a variable population at different stages of germination. As this study is aiming to identify a detailed process occurring in the seed, the heterogeneity observed in the *F. arundinacea* seed lot made it difficult to work with. This observed heterogeneity reinforces the use of single seed lots as it is important to have a clear understanding of the different components of overall seedling establishment processes. Effects of seed size variation within the seed lot population on germination and seedling growth need to be characterised further. Once this is completed a useful study might be a detailed comparison of meristem size between different seed lots of *F. arundinacea* and *L. multiflorum*.

The effects of storage on the seeds was also a major limitation as there appeared to be changes in residual dormancy of the *F. arundinacea* seed lot, which together with

possible deterioration in seed quality during storage time, made it difficult to link results from experimental work done over time. Variations in α-amylase production over time may also be attributable to storage effects, possibly due to a change in responsiveness of the aleurone tissue to GA_3 occurring.

Other variables not fully characterised include the effects of dehusking, disinfection, KNO_3 supplements on dormancy, and any possible contamination due to the avoidance of antibiotics in the half-seed experiments.

Residual dormancy and thermal time effects and effects on rapid seedling growth are the most important areas requiring further study. Not only do these areas of study need to be extended to further seed lots, but a storage time-course also needs to be done using 5-6 different seed lots and several different storage types.

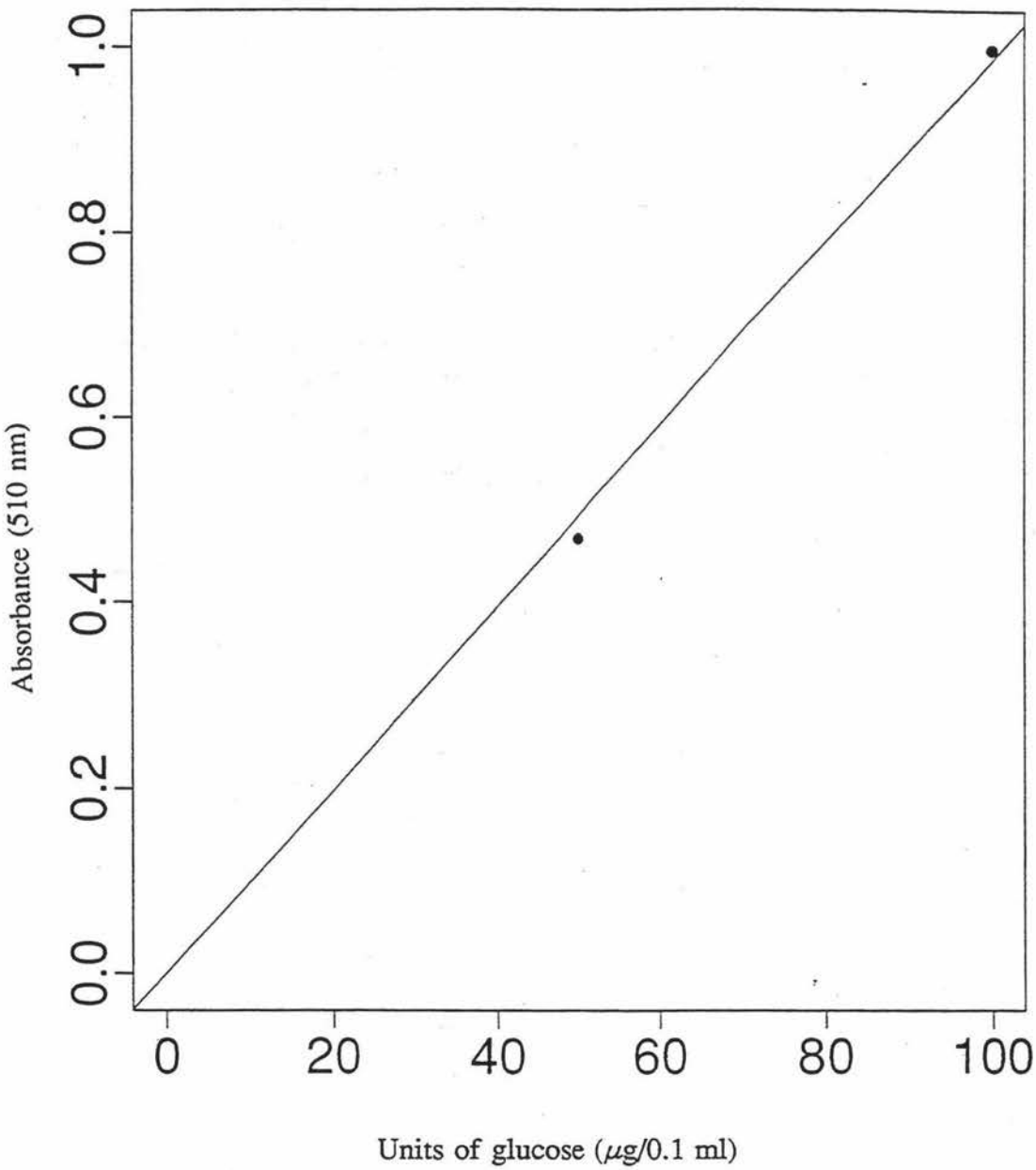
7.5 Conclusions

The suggestion has been made in this thesis that one of the reasons why *F. arundinacea* establishes less well than *L. multiflorum* is that the processes of seedling growth and reserve mobilisation are less tightly coupled in *F. arundinacea.* However, one of the main outcomes of this study has been the detailed characterisation of the wide range of variables limiting germination and seedling establishment. The identification of these factors, together with the lack of previous detailed studies on the physiology of grass seed germination and seedling growth and the lack of literature available on *F. arundinacea* germination in particular, justifies the use of single seed lots for comparative studies. It also highlights the enormity of the task ahead in extending key areas of study to different seed lots and species. These areas most requiring further attention include the relationship between thermal time and final % germination and the relationship between mobilisation of reserves and their translation into seedling growth.

It is obviously dangerous to assume, given the variation in cereal species, that model

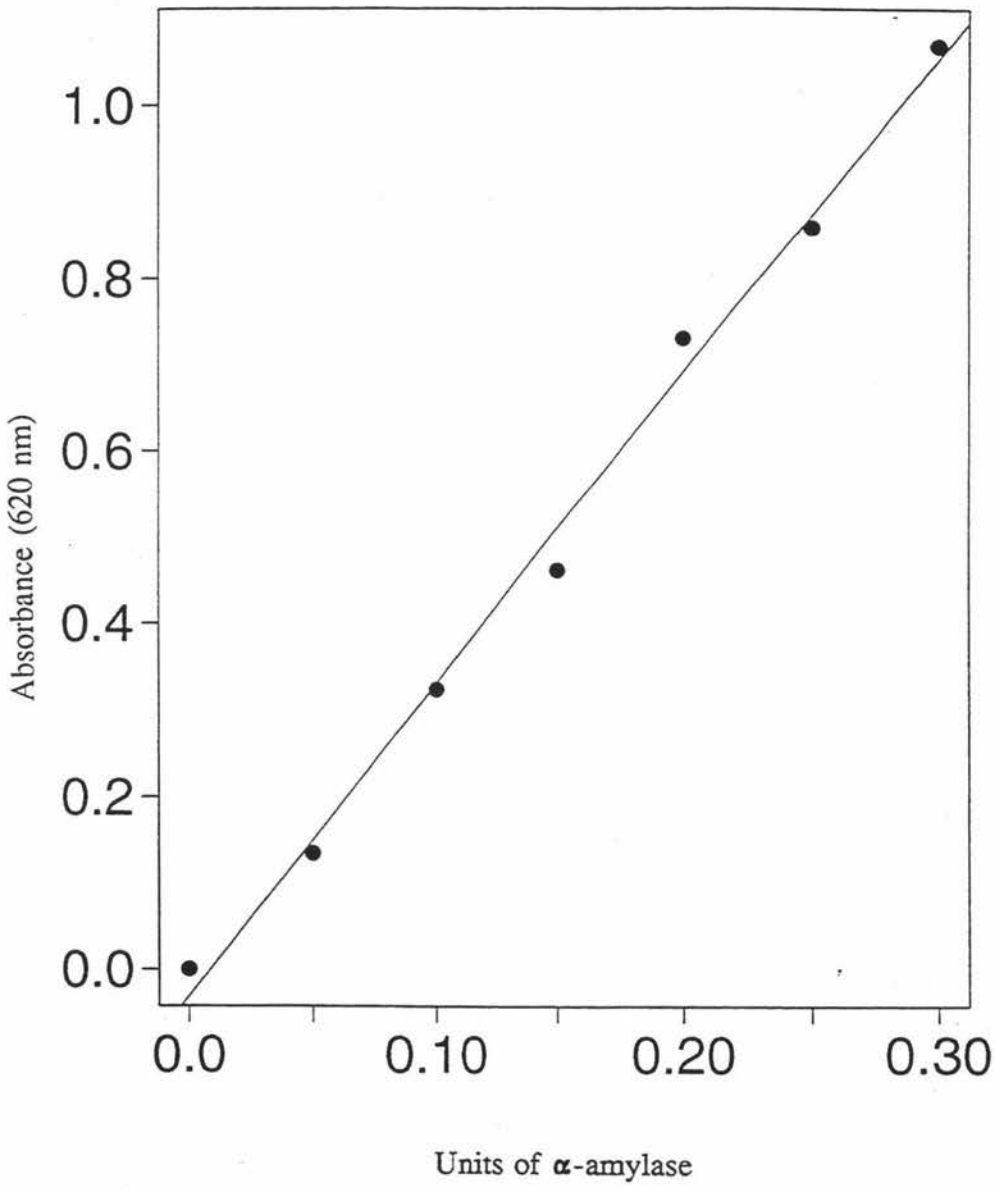
systems of reserve mobilisation can be generalised to *F. arundinacea* and *L. multiflorum*. In addition, the timing of events between seed lots of the same species may vary depending on pre- and post- harvest conditions. A more detailed understanding is required not only of the reserve mobilisation processes in grasses, but also how they relate to the processes of germination and especially seedling growth. Only in this way will we begin to understand where the rate limiting steps in seedling establishment occur. Without this underlying knowledge, the prospect of radicle advances in the development of more versatile grass seed mixtures for pastures seems a long way off.

Appendix 1.



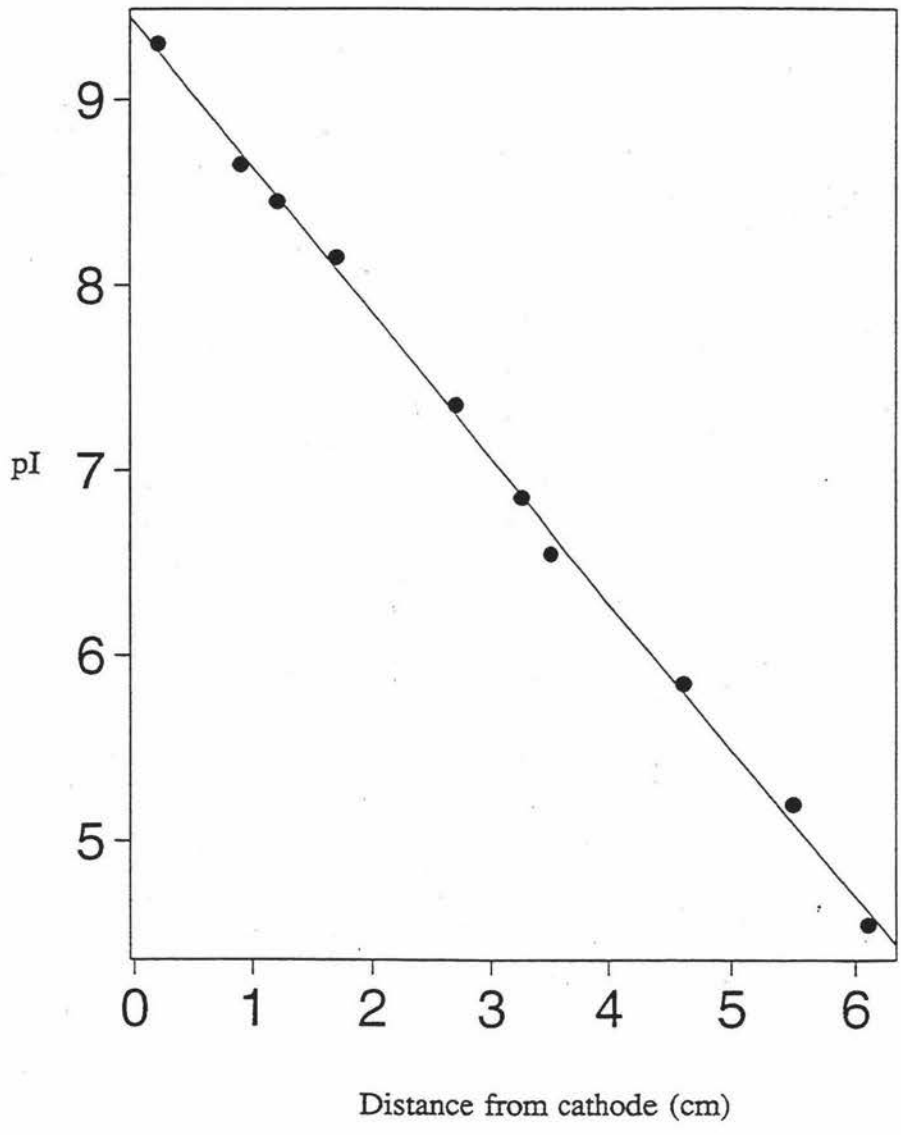
Standard curve for determination of free glucose levels

Appendix 2.



Typical standard curve for determination of α-amylase activity

Appendix 3.



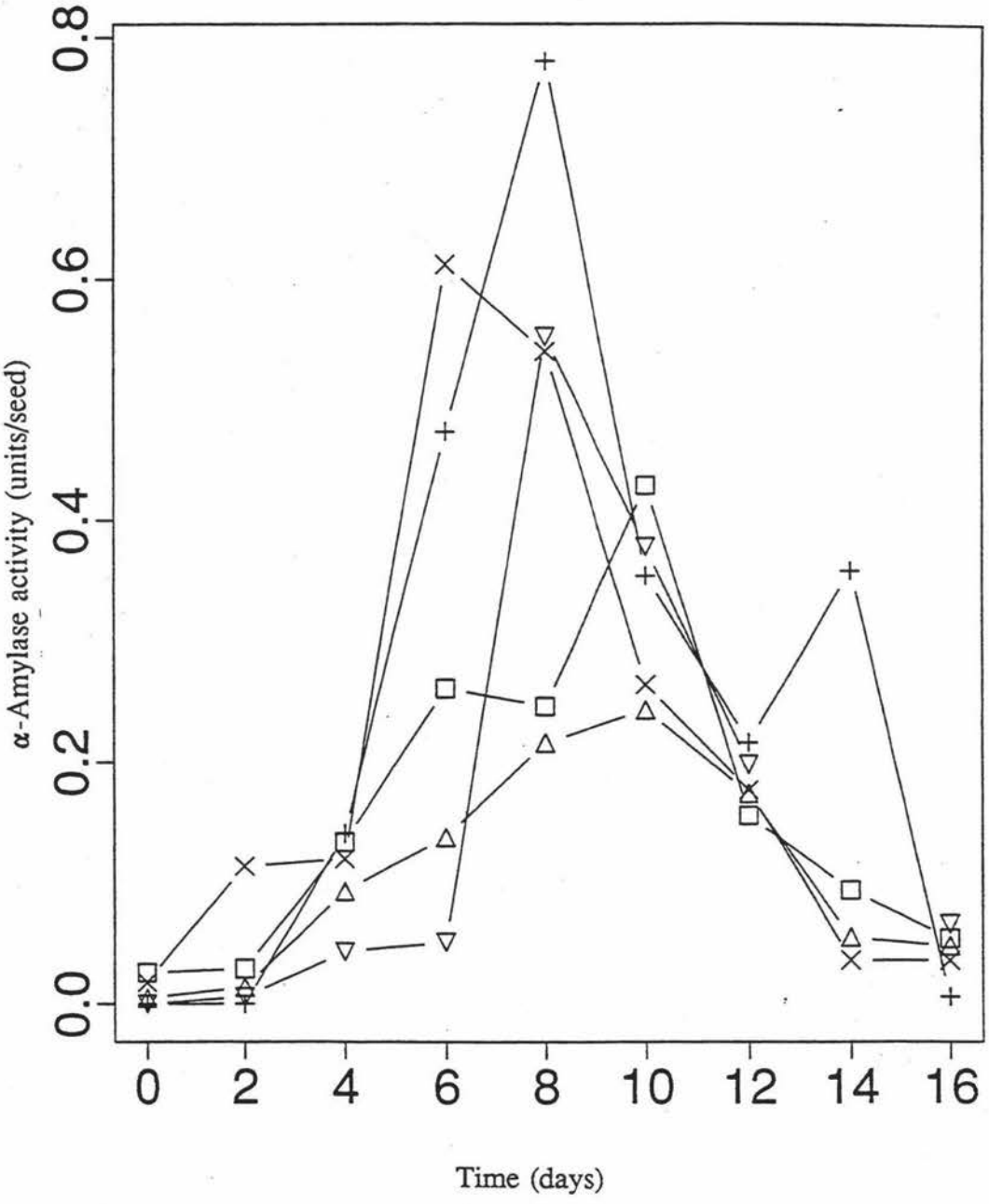
A typical calibration curve for determination of pI

Appendix 4.

Calculations for determination of dormancy breaking or thermal time effects

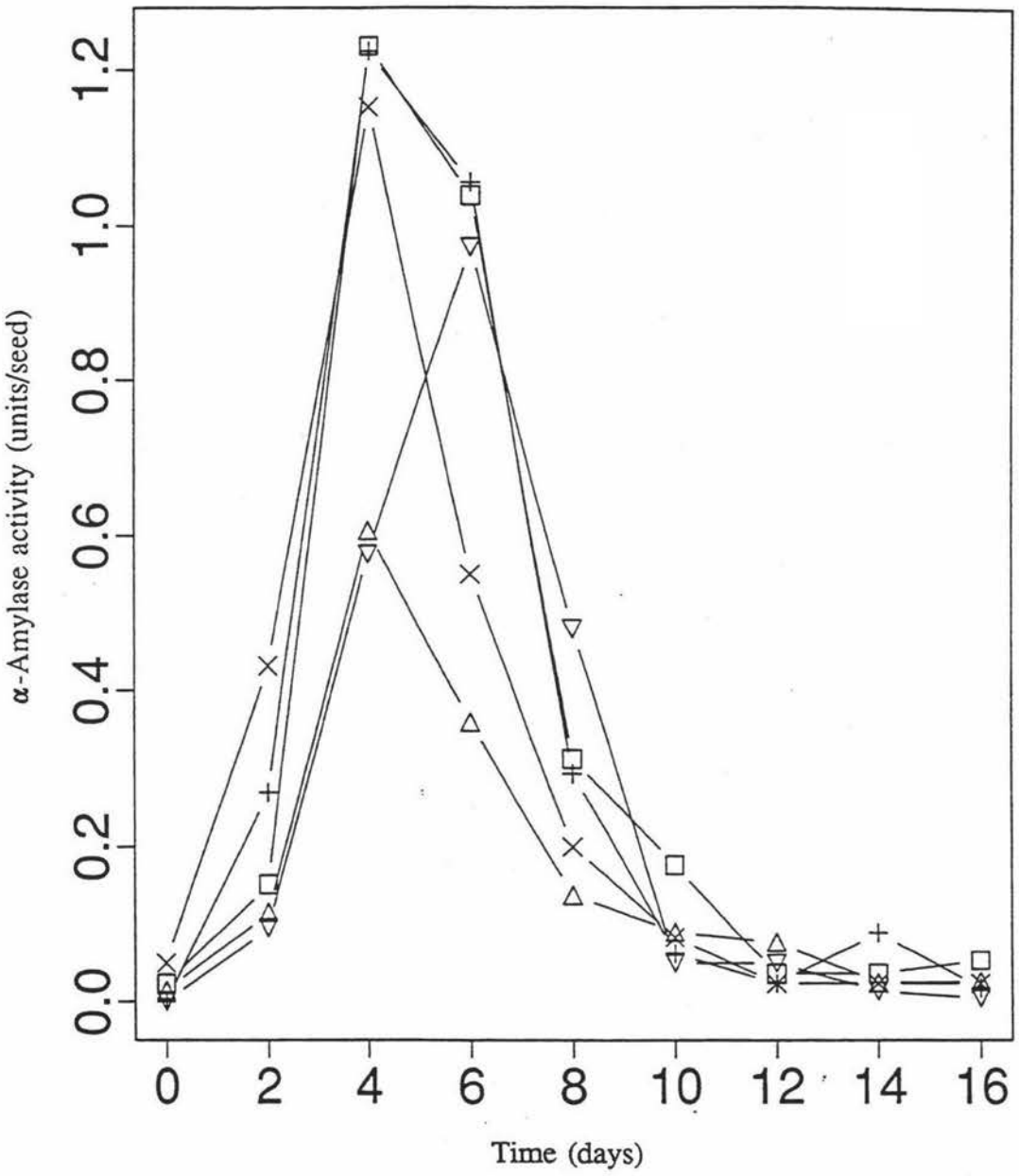
	F. arundinacea	*L. multiflorum*
Estimated T_b	2.8°C	4.5°C
Day degrees accumulated during prechilling (expected advantage)	(5 - 2.8) x 7 = 15.4 d.°C	(5 - 4.5) x 7 = 3.5 d.°C
Observed advantage from prechilling (change in T_{50})	1.1 d	0.8 d
In day degrees (observed advantage)	(20 - 2.8) x 1.1 = 18.9	(20 - 4.5) x 0.8 = 12.4
Observed ≈ expected?	Yes	No
Stratification response?	Not shown	Demonstrated

Appendix 5a.



α-Amylase levels determined from prechilled (5° C for 7 d in 0.2% KNO_3) *F. arundinaca* seeds over a 16 d time-course. Symbols represent individual time-courses with varying storage times of □ = 17 months, △ = 17 months, + = 18 months, × = 24 months and ▽ = 28 months.

Appendix 5b.



α-Amylase levels determined from prechilled (5°C for 7 d in 0.2% KNO_3) *L. multiflorum* seeds over a 16 d time-course. Symbols represent individual time-courses with varying storage times of □ = 17 months, △ = 17 months, + = 18 months, × = 24 months and ▽ = 28 months.

Bibliography

AgResearch (1993) AgResearch Grasslands Publication

Akazawa, T., Mitsui, T. and Hayashi, M. (1988) Recent progress in α-amylase biosynthesis. *The Biochemistry of Plants* **14**, 465-493.

Akazawa, T. and Miyata, S. (1982) Biosynthesis and secretion of α-amylase and other hydrolases in germinating cereal seeds. *Essays in Biochemistry* **18**, 41-73.

Anonymous (1993) Grass study shows fescue a bonus. *Evening Standard*, Palmerston North (15/2/93)

Anslow, R.C. (1964) Seed formation in perennial ryegrass. Maturation of seed. *Journal of the British Grassland Society* **19**, 349-357.

Armstrong, C., Black, M., Chapman, J.M., Norman, H.A. and Angold, R. (1982) The induction of sensitivity to gibberellin in aleurone tissue of developing wheat grains. 1. The effect of dehydration. *Planta* **154**, 573-577.

Atzorn, R. and Weiler, E.W. (1983) The role of endogenous gibberellins in the formation of α-amylase by aleurone layers of germinating barley caryopses. *Planta* **154**, 289-299.

Barnes, R.F. (1990) Importance and problems of tall fescue. pp 1-12 *in* Kasperbauer, M.J. (Ed.) *Biotechnology in tall fescue improvement.* CRC Press, Boston.

Bewley, J.D. and Black, M. (1985) *Seeds. Physiology of development and germination.* Plenum Press, New York.

Black, J.N. (1957) Seed size as a factor in the growth of subterranean clover (*Trifolium subterraneum* L.) under spaced and sward conditions. *Australian Journal of Agricultural Research* **8**, 335-351.

Blacklow, W.M. (1972) Influence of temperature on germination and elongation of the radicle and shoot of corn (*Zea mays* L.). *Crop Science* **12**, 647-650.

Blacklow, W.M. (1973) A mathematical model to predict germination and field emergence of maize (*Zea mays* L.) in an environment of changing temperatures. pp 533-540 *in* Heydecker, W. (Ed.) *Seed ecology*. Butterworths, London.

Bremner, P.M., Eckersall, R.M. and Scott, R.K. (1963) The relative importance of embryo size and endosperm size in causing effects associated with seed size in wheat. *Journal of Agricultural Science* **16**, 139-145.

Brock, J.L., Anderson, L.B. and Lancashire, J.A. (1982) 'Grasslands Roa' tall fescue: seedling growth and establishment. *New Zealand Journal of Experimental Agriculture* **10**, 285-289.

Brown, A.H.P. and Jacobsen, V. (1983) Genetic basis and natural variation of α-amylase isoenzymes in barley. *Genetic Research* **40**, 315-324.

Brown, K.R. (1977) Parent seed weight, plant growth, and seeding in 'Grasslands Tama' Westerworlds ryegrass. *New Zealand Journal of Experimental Agriculture* **5**, 143-146.

Brown, P.H. and Brodl, M.R. (1988) Hormonal and heat-stress regulation of protein synthesis in the aleurone layer of barley seeds. *BioEssays* **8**, 199-202.

Brown, P.H. and Ho, T.H.D. (1986) Barley aleurone layers secrete a nuclease in response to gibberellic acid. *Plant Physiology* **82**, 801-806.

Bryant, J.A. (1985) *Seed physiology.* London: Edward Arnolds.

Bush, D.S., Sticher, L., Van Huystee, R., Wagner, D. and Jones, R.L. (1989) The calcium requirements and enzymatic activity of two isoforms of barley aleurone α-amylase. *Journal of Biology and Chemistry* **264**, 19392-19398.

Carbonell, J. and Jones, R.L. (1984) A comparison of the effects of Ca^{2+} and gibberellic acid on enzyme synthesis and secretion in barley aleurone. *Physiology Plantarum* **63**, 345-350.

Chapman, G.P. and Peat, W.E. (1992) *An introduction to the grasses (including bamboos and cereals).* C.A.B. International.

Charles, A.H. (1965) Interactions of grass, clover and nurse crop in the seeding year. *Journal of the British Grassland Society* **20**, 241-247.

Charlton, J.F.L. (1991) Some basic concepts of pasture grass seed mixtures for New Zealand farms. *Proceedings of the New Zealand Grassland Association* **53**, 37-40.

Chrispeels, M.J. and Varner, J.E. (1967) Gibberellic acid enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. *Plant Physiology* **42**, 398-406.

Coolbear, P. *Seed physiology course lecture notes*, Massey University, Palmerston North.

Coolbear, P., Francis, A. and Grierson, D. (1984) The effect of low temperature pre-sowing treatment on the germination performance and membrane integrity of artificially aged tomato seeds. *Journal of Experimental Botany* **35**, 1609-1617.

Coolbear, P., Newell, A.J. and Bryant, J. (1987) An evaluation of the potential of low temperature pre-sowing treatments of tomato seeds as a means of improving germination performance. *Annals of Applied Biology* **110**, 185-194.

Cornford, C.A., Black, M., Daussant, J. and Murdoch, K.M. (1987) α-Amylase production by pre-mature wheat (*Triticum aestivum* L.) embryos. *Journal of Experimental Botany* **38**, 277-285.

Cornford, C.A. and Coolbear, P. (1992) Reserve mobilisation and seedling growth in grasses. *International Herbage Seed Production Research Group Newsletter* **17**, 8-9.

Cornford, C.A. and Hill, R.D. (1994) α-Amylase isoenzymes and BASI-like proteins in seeds of different grass species. *Seed Science Research* **4**, 285-291.

Crouch, M.L. (1987) Regulation of gene expression during seed development in flowering plants. pp 367-404 *in* Browder, L.W. (Ed.) *Developmental biology, a comprehensive synthesis 5*. New York, Plenum Press.

Danielson, H.R. and Toole, V.K. (1976) Action of temperature and light on the control of seed germination in alta tall fescue (*Festuca arundinacea* Schreb). *Crop Science* **16**(3), 317-320.

Deikman, J. and Jones, R.L. (1985) Controls of α-amylase mRNA accumulation by gibberellic acid and calcium in barley aleurone layers. *Plant Physiology* **78**, 192-198.

Duffus, C. M. and Duffus, J. H. (1984) *Carbohydrate metabolism in plants.* Longman, London and New York.

Dure, L. (1985) Embryogenesis and gene expression during seed formation. *Oxford Survey of Plant Molecular Biology and Cell Biology* **2**, 179-197.

Elgersma, A. and Sniezko, R. (1988) Cytology of seed development in perennial ryegrass, *Lolium perenne* L. pp 377-382 *in* Cresti, M., Gori, P. and Pacini, E. (Eds.) *Sexual reproduction in higher plants*. Springer-Verlag, Berlin.

Evans, M., Black, M. and Chapman, J. (1975) Induction of hormone sensitivity by dehydration is one positive role for drying in cereal seeds. *Nature* **258**, 144-145.

Fincher, G.B. (1989) Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**, 305-346.

Finklestein, R.R. and Crouch, M.L. (1986) Rapeseed embryo development in culture on high osmotica is similar to that in seeds. *Plant Physiology* **81**, 907-912

Flintham, J.E. and Gale, M.O. (1988) Genetics of pre-harvest sprouting and associated traits in wheat: review. *Plant Varieties and Seeds* **1**, 87-97.

Galau, G.A., Jakobsen, K.S. and Hughes, D.W. (1991) The controls of late dicot embryogenesis and early germination. *Physiologia Plantarum* **81**, 280-288.

Garcia-Huidobro, J., Monteith, J.L. and Squire, G.R. (1982[a]) Time, temperature and germination of pearl millet (*Pennisetum typhoides* S. & H.): 1. Constant temperature. *Journal of Experimental Botany* **33**, 288-296.

Garcia-Huidobro, J., Monteith, J.L and Squire, G.R. (1982[b]) Time, temperature and germination of pearl millet (*Pennisetum typhoides* S. & H.): 2. Alternating temperatures. *Journal of Experimental Botany* **33**, 297-302.

Garcia-Maya, M., Chapman, J.M. and Black, M. (1990) Regulation of α-amylase formation and gene expression in the developing wheat embryo. *Planta* **181**, 296-303.

Goldberg, R.B., Barker, S.J. and Perez-Grau, L. (1989) Regulation of gene expression during plant embryogenesis. *Cell* **56**, 149-160.

Grabe, D.F. (1970) Tetrazolium testing handbook for agricultural seeds. Contribution 29 to *Handbook in seed testing*. Association of Official Analysts.

Grierson, D. and Covey, S.N. (1985) *Plant Molecular Biology*. Blackie and Son Ltd. Chapman and Hall, New York.

Gubler, F., Jacobsen, J.V. and Ashford, A.E. (1986) Involvement of the golgi-apparatus in the secretion of α-amylase from gibberellin treated barley aleurone cells. *Planta* **168**, 447-452.

Harkess, R.D. (1956) The effect of seed size on early growth of diploid and tetraploid Italian ryegrass. *Journal of British Grassland Society* **20**, 190-193.

Harrison, C.S. (1954) The technique of ultra-violet testing in New Zealand. *Proceedings of the International Seed Testing Association* **19**, 178-183.

Hayes, P. (1976) Seedling growth of four grasses. *Journal of the British Grassland Society* **31**, 59-64.

Hill, M.J. (1971) *A study of seed production in perennial ryegrass, timothy and prairie grass*. PhD Thesis, Massey University, New Zealand.

Hill, M.J., Kay, G. and Yeates, S.J. (1985[a]) A comparison of the growth of seedlings of mediterranean and temperate tall fescues, phalaris and ryegrass. *Australian Journal of Experimental Agriculture* **25**, 818-823.

Hill, M.J., Pearson, C.J. and Kirby, A.C. (1985[b]) Germination and seedling growth of prairie grass, tall fescue and Italian ryegrass at different temperatures. *Australian Journal of Agricultural Research* **36**, 13-24.

Huang, N., Reinl, S.J. and Rodriguez, R.L. (1992) RAmy2; a novel α-amylase-encoding gene in rice. *Gene* **111**, 223-228.

International Seed Testing Association (1985) International Rules for Seed Testing. *Seed Science and Technology* **13**, 299-355.

Jacobson, J.V. and Chandler, P.M. (1987) Gibberellin and abscisic acid in germinating cereals. pp 164-193 *in* Davies, P.J. (Ed.) *Plant hormones and their role in plant growth and development.* Dordrecht: Martinus Nijhoff.

Jacobsen, J.V. and Higgins, T.J.V. (1982) Characterisation of the α-amylase synthesis by aleurone layers of Himalaya barley in response to gibberellic acid. *Plant Physiology* **70**, 1647-1653.

Jones, L.J., Gilroy, S. and Hillmer, S. (1993) The role of calcium in the hormonal regulation of enzyme synthesis and secretion in barley aleurone. *Journal of Experimental Botany* **44**, 207-212.

Jones, M.B. and Lazenby, A. (1988) *The grass crop. The physiological basis of production.* London, New York. Chapman and Hall.

Jones, R.L. (1985) Protein synthesis and secretion by the barley aleurone: A perspective. *Israel Journal of Botany* **34**, 377-395.

Jones, R.L. and Jacobsen, J.V. (1983) Calcium regulation of the secretion of α-amylase isoenzymes and other proteins from barley aleurone layers. *Planta* **158**, 1-9.

Kermode, A.R. and Bewley, D.J. (1985) The role of maturation drying in the transition from seed development to germination. *Journal of Experimental Botany* **36**, 1906-1915.

Kermode, A.R., Oishi, M.Y. and Bewley, J.D. (1989) Regulatory roles for desiccation and abscisic acid in seed development: A comparison of the evidence from whole seeds and isolated embryos. pp 23-50 *in* Stanwood, P.C. and McDonald, M.B. (Eds.) *Seed Moisture*. CSSA Special Publication 14.

Kidd, G. (1993) Why do agbiotech firms neglect turfgrass? *Biotechnology* **11**, 206.

Kittock, D.L. and Law, A.G. (1968) Relationship of seedling vigour to respiration and tetrazolium chloride reduction by germinating wheat seeds. *Agronomy Journal* **60**, 286-288.

Kittock, D.L. and Patterson, J.K. (1962) Seed size effects on performance of dryland grasses. *Agronomy Journal* **54**, 277-278.

Lambrechtsen, N.C. (1986) *What grass is that?* New Zealand DSIR Information Series 87. Government Printing Office.

Lanahan, M.B., Ho, T.H.D., Rogers, S.W. and Rogers, J.C. (1992) A gibberellin response complex in cereal α-amylase gene promoters. *The Plant Cell* **4**, 203-211.

Langer, R.H.M. (1974) *How grasses grow*. Studies in biology 3. Edward Arnold.

Langer, R.H.M. (1990) *Pastures their ecology and management*. Auckland, Oxford University Press.

Lanteri, S., Saracco, F., Kraak, H.L. and Bino, R.J. (1994) The effects of priming on nuclear replication activity and germination of pepper (*Capsicum annum*) and tomato (*Lycopersicum esculentum*) seeds. *Seed Science Research* **4**, 81-87.

Livesley, M.A. and Bray, C.M. (1991) The effect of ageing upon α-amylase production and protein synthesis by wheat aleurone layers. *Annals of Botany* **68**, 69-73.

MacGregor, A.W., MacDougall, F.H., Mayer, C. and Daussant, J. (1984) Changes in levels of α-amylase components in barley tissues during germination and early seedling growth. *Plant Physiology* **75**, 203-206.

MacGregor, A.W., Marchylo, B.A. and Kruger, J.E. (1988) Multiple α-amylase components in germinated cereal grains determined by isoelectric focusing and chromatofocusing. *Cereal Chemistry* **65**, 326-333.

Marchylo, B.A., La Croix, L.J. and Kruger, J.E. (1981) α-Amylase synthesis in wheat kernels as influenced by the seed coat. *Plant Physiology* **67**, 89-91.

Mayer and Poljakoff-Mayber (1966) *The germination of seeds*. Pergamon Press, Oxford, London.

McFadden, G.I., Ahluwalia, B., Clarke, A.E. and Fincher, G.B. (1988) Expression sites and developmental regulation of genes encoding (1→3, 1→4)-β-glucanases in germinated barley. *Planta* **173**, 500-508.

McWilliam, J.R., Clements, R.J. and Dowling, P.M. (1970) Some factors influencing the germination and early seedling development of pasture plants. *Australian Journal of Agricultural Research* **21**, 19-32.

Miyata, S., Okamoto, K., Watanabe, A. and Akazawa, T. (1981) Enzymic mechanism of starch breakdown in germinating rice seeds. *Plant Physiology* **68**, 1314-1318.

Moloney, S.C. (1991) Performance of tall fescue, cocksfoot and phalaris based pastures compared with perennial ryegrass, in on-farm trials. *Proceedings of the New Zealand Grassland Association* **53**, 41-46.

Morris, C.F., Andergerg, R.J., Goldmark, P.J. and Walker-Simmons, M.K. (1991) Molecular-cloning and expression of abscisic acid-responsive genes in embryos of dormant wheat seeds. *Plant Physiology* **95**, 814-821.

Morris, P.C., Weiler, E.W., Maddock, S.E., Jones, M.G.K., Lenton, J.R. and Bowles, D.J. (1988) Determination of endogenous abscisic acid levels in immature cereal embryos during *in vitro* culture. *Planta* **173**, 110-116.

Mundy, J. and Rogers, J.C. (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells. Comparison to the barley amylase/subtilisin inhibitor. *Planta* **169**, 51-63.

Murray, D.R. (1984) *Seed Physiology 2. Germination and reserve mobilisation.* Academic Press, Sydney.

Naylor, R.E.L. (1980) Effect of seed size and emergence time on subsequent growth of perennial ryegrass. *New Phytologist* **84**, 313-318.

Nicholls, P.B. (1982) Influence of temperature during grain growth and ripening of barley on the subsequent response to exogenous gibberellic acid. *Australian Journal of Plant Physiology* **9**, 373-383.

Nicholls, P.B., MacGregor, A.W. and Marchylo, B.A. (1986) Production of α-amylase isoenzymes in barley caryopses in the absence of embryos and exogenous gibberellic acid. *Australian Journal of Plant Physiology* **13**, 239-247.

Nolan, R.C. and Ho, T.H.D. (1988) Hormonal regulation of gene expression in barley aleurone layers. *Planta* **174**, 551-560.

Nolan, R.C., Lin, L.S. and Ho, T.H.D. (1987) The effect of abscisic acid on the differential expression of α-amylase isoenzymes in barley aleurone layers. *Plant Molecular Biology* **8**, 13-22.

Osborne, D.J. (1983) Biochemical control systems operating in the early hours of germination. *Canadian Journal of Botany* **61**, 3568-3577.

Palmer, G.H. (1988) Enzyme development in the aleurone and embryos of Galant and triumph barleys. *Journal of the Institute of Brewing* **94**, 61-63.

Parrish, D.J. and Leopold, C.A. (1978) On the mechanisms of ageing in soybean seeds. *Plant Physiology* **61**, 345-368.

Perl, M., Luria, I. and Gelmond, H. (1978) Biochemical changes in sorghum seeds affected by accelerated aging. *Journal of Experimental Botany* **29**, 497-509.

Petruzzelli, L. and Taranto, G. (1990) Amylase and loss of viability in wheat. *Annals of Botany* **66**, 375-378.

Pogson, B.J., Ashford, A.E. and Gubler, F. (1989) Immunofluorescence localisation of α-amylase in the scutellum, germ aleurone and "normal" aleurone of germinated barley grains. *Protoplasma* **151**, 128-136.

Ranjhan, S., Litts, J.C., Foolad, M.R. and Rodriguez, R.L. (1991) Chromosomal localisation and genomic organisation of α-amylase genes in rice (*Oryza satura* L.). *Theoretical and Applied Genetics* 1-9.

Reeder, J.R. (1957) The embryo in grass systematics. *American Journal of Botany* **44**, 756-768.

Rhodes, I. (1968) The growth and development of some grass species under competitive stress. *Journal of the British Grassland Society* **23**, 330-335.

Riviv, C.J. and Grudt, T. (1991) Abscisic acid and the developmental regulation of embryo storage proteins in maize. *Plant Physiology* **95**, 358-365.

Roberts, E.H. (1972) *Viability of seeds*. Chapman and Hall, London.

Sakai-Wada, A. and Nakada, M. (1987) Effect of gibberellic acid on the ultra structure and α-amylase activity of aleurone cells of *Avena sativa* L. *Plant Cell Physiology* **28** (8), 1465-1476.

Salisbury, F.B. and Ross, C.W. (1985) *Plant physiology*. Wadsworth Inc. Belmont, California.

San Segundo, B., Ma Casacuberta, J. and Puigdomenech, P. (1990) Hormonal and light regulation of the multiple amylase isoenzyme during seed germination and in vegetative tissues of *Zea mays*. *Plant Science* **69**, 167-177.

Schuurink, R.C., Norbert, J.A.S. and Wang, M. (1992) Dormancy of the barley grain is correlated with gibberellic acid responsiveness of the isolated aleurone layer. *Plant Physiology* **100**, 1834-1839.

Scott, D.J. and Hampton, J.G. (1985) Aspects of seed quality in producing herbage seeds. pp 43-52 *in* Hare, M.D. and Brock, J.L. (Eds.) *Grassland research and practise series 2.* New Zealand Grassland Association, Palmerston North.

Tadmor, N.H. and Cohen, Y. (1968) Pre-emergence root development of mediterranean grasses and legumes. *Crop Science* **8**, 416-419.

Thomas, T.L. (1993) Gene expression during plant embryogenesis and germination: An overview. *The Plant Cell* **5**, 1401-1410.

Thompson, P.A. (1970) Characterisation of the germination response to temperature of species and ecotype. *Nature* **225**, 827-831.

Tittle, T.T., Goudey, J.S. and Spencer, M.S. (1988) Hypochlorite disinfection influences the GA_3-induced synthesis of α-amylase isozymes by isolated barley aleurone layers. *Plant Physiology* **86**, 510-511.

Trewavas, A.J. (1982) Growth substance sensitivity: the limiting factor in plant development. *Physiologia Plantarum* **55**, 60-72.

Villiers, T.A. (1975) *Dormancy and the survival of plants.* London, Edward Arnold.

Weslake, R.J., MacGregor, M.C. and Hill, R.D. (1985) Endogenous α-amylase inhibitors in various cereals. *Cereal Chemistry* **62** (2), 120-123.

Wheeler, J.L. (1987) Pasture and pasture research in Southern Australia. pp 3-31 *in* Wheeler, J.L., Pearson, C.J. and Robards, G.E. (Eds.) *Temperate pastures, their production, use and management.* CSIRO Editorial and Publishing Unit, Australia.

Wiesner, L.E. and Grabe, D.F. (1972) Effect of temperature preconditioning and cultivar on ryegrass (*Lolium* sp.) seed dormancy. *Crop Science* **12**, 760-764.

Williams, J.F. and Peterson, M.L. (1973) Reactions between α-amylase activity and growth of rice seedlings. *Crop Science* **13**, 612-615.

Woodman, R.F. and Fraser, W.J. (1991) Alternative grass options for dry east coast and downland pastoral systems. *Proceedings of the Agronomy Society of New Zealand* **21**, 45-53.

Youngner, V.B. and McKell, C.M. (1972) *The biology and utilisation of grasses*. Academic Press, New york and London.