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The Mechanism of Action of Liquid Seaweed Extracts in the Manipulation of Frost Resistance in Winter Barley (Hordeum vulgare. L)

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The Mechanism of Action of Liquid Seaweed Extracts in the Manipulation of Frost Resistance in Winter Barley (*Hordeum vulgare.* L).

by

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Abstract

The Mechanism of Action of Liquid Seaweed Extracts in the Manipulation of Frost Resistance in Winter Barley (*Hordeum vulgare.* L)
By Stephen Burchett

Frost assays carried out on winter barley (*Hordeum vulgare* cv Igri) showed that a single (10ml l) application of liquid seaweed extract (LSE) marginally increased the frost resistance of non-acclimated (NA) plants by 2.3% compared to NA controls and cold-acclimated (CA) plants by 2.1% compared to CA controls. Three applications of LSE increased the frost resistance of NA plants by 16% compared to NA controls and CA plants by 7.5% compared to CA controls. These observations were durable in a small scale field trial where LSE increased plant dry weights (control 0.55, single LSE, 0.611 and multiple LSE 0.621 log dry weight), but rain following LSE application reduced LSE mediated frost resistance.

Glasshouse growth trials illustrated that LSE enhanced tiller production (control 2.8, one LSE 3.8 and three LSE 4.5 tillers) and dry weight gain, but where precipitation followed LSE application, up to 3 days post application, the LSE mediated effect was not sustained.

Protein analysis demonstrated that cold-acclimation and LSE treatments increased the total soluble protein content of winter barley. A single application of LSE increased the soluble protein content of NA plants by 36.7% and three applications of LSE to NA plants increased protein concentration by 86.5%. There was not a significant increase in the soluble protein concentration of LSE treated CA plants. There was a significant increase in the number of high molecular weight proteins and the up-regulation of a 118kDa and a 57kDa protein when plants were treated with LSE. However precipitation following LSE application adversely affected LSE mediated protein expression. A tentative immunological identification of the up-regulated proteins suggested that the 118kDa protein is a dehydrin.

There was a 2 fold decrease in plant water potential of NA plants treated with three applications of LSE compared to controls and a similar decrease in plant water potential was observed in cold-acclimated plants. The duration of LSE mediated decline in water potential lasted for 6 days, post LSE application. However there was no significant reduction in the percentage water content of cold-acclimated and LSE treated plants.

Differential scanning calorimetry demonstrated that both cold-acclimated and LSE treated plants had significantly less frozen water in their crown tissue compared to non-acclimated controls. Further thermal analysis (infrared thermography and thermocouple data) showed that both cold-acclimation and LSE treatments reduced the speed of water removal from plant cells to the extracellular ice (NA 4.06, NA3LSE 13.4, CA 15.7 and CA3LSE 19.31 minutes). It is hypothesised that both CA and LSE treatments are modifying plant water status, so that water becomes more structured at the physico-chemical level, and thus alters the osmotic behaviour of cellular water. This higher level of water structuring reduces frost damage by conserving the cellular water environment and thus reducing protein denaturation and membrane damage.

Contents Page

Chapter 1: Literature Review On The Mechanism of Plant Freezing Tolerance 1

1.0 Introduction 2

1.1 Ice Formation In Plants 3

1.2 Intracellular Freezing 4

1.3 Extracellular Freezing 5

1.4 Cold Acclimation 6

1.5 Accumulation of Cryoprotectants 8

1.6 Membranes 9

1.7 Properties of water 16

1.8 Protein Metabolism 27

1.9 Low Temperature Responsive Genes (LTR) 35

1.10 Frost Protection Strategies 41

1.11 Biological Effects of Seaweed Extracts 42

1.12 Biologically Active Compounds in Seaweed Extracts 43

1.13 Aims and Objectives of Study 48

Chapter 2: Frost Testing and Evaluation of Growth Effects Mediated by Liquid Seaweed Extract. 51

2.0 Introduction 52

2.0.1 Rationale 52

2.0.2 Measurement of Freezing Tolerance 52

2.1 Experiment 1: Frost assays 56

2.2 Experiment 2: Effect of 3 applications of LSE on the frost resistance of winter barley and scoring scheme assessment 65

2.3 Experiment 3: Field trial on the effects of LSE on frost resistance of winter barley cv Pastoral 67

2.4.0 Growth responses to liquid seaweed extract 70

2.4.1 Experiment 4: Evaluation of the effects of LSE on shoot dry weights in field grown winter barley plants 70

2.4.2 Evaluation of dry weights and tiller production in glasshouse grown plants 70

2.5 Experiment 5: Evaluation of the effects of simulated rain on LSE mediated growth observed in winter barley 72

2.6 Experiment 6: Evaluation of the site of LSE uptake on plant dry weights and tiller production 73

2.7 Experiment 7: The effect of growth stage on the frost resistance of winter barley 73

2.8 Results of experiment 1: Frost assays 75

2.9 Results of experiment 2: Effect of 3 applications of LSE on the frost resistance of winter barley and scoring scheme assessment 79

2.9.1 Results of Assessment of scoring scheme 81

2.10 Results of experiment 3: Field trial on the effects of LSE on frost resistance of winter barley cv Pastoral 87

2.11 Results of experiment 4: Evaluation of the effects of LSE on shoot dry weights in field grown winter barley plant 91

2.12 Results of experiment 5: Evaluation of the effects of simulated rain on LSE mediated growth observed in winter barley 94

2.13 Results of experiment 6: Evaluation of the site of LSE uptake on plant dry weights and tiller production 94

2.14 Results of the effect of growth stage on the frost resistance of winter barley 96

2.15 Discussion 99

2.16 Conclusions 107

Chapter 3: Protein Analysis 109

3.0 Introduction 110

3.1 Experiment 3.1: Development of protein extraction protocol 111

3.2 Experiment 3.2: The effect of cold-acclimation and liquid seaweed extract on the concentration of total soluble proteins 113

3.3 Experiment 3.3: The effect of artificial rain and application site on liquid seaweed enhanced protein expression

3.4 Experiment 3.4: Electrophoretic comparison of protein expression in winter barley plants subjected to normal growth conditions, cold-acclimation and liquid seaweed extract treatments 115

3.5 Experiment 3.5: Electrophoretic comparison of protein expression in winter barley plants subjected to liquid seaweed extract and simulated rain events 116

3.6 Experiment 3.6: Electrophoretic comparison of heat stable proteins 116

3.7 Experiment 3.7: Western Blotting 118

3.8 Results of experiment 3.1: Development of protein extraction protocol 119

3.9 Results of experiment 3.2: The effect of cold-acclimation and liquid seaweed extract applications on the concentration of total soluble proteins 120

3.10 Results of experiment 3.3: The effect of artificial rain and application site on liquid seaweed enhanced protein expression 120

3.11 Results of experiment 3.4: Electroporetic comparison of protein expression in winter barley plants subjected to normal growth conditions, cold-acclimation and liquid seaweed extract treatments 124

3.12 Results of experiment 3.5: Electrophoretic comparison of protein expression in winter barley plants subjected to liquid seaweed extract and simulated rain events 133

3.13 Results of experiment 3.6: Electrophoretic comparison of heat stable proteins 135

3.14 Results of experiment 3.7: Western Blotting 137

3.14 Discussion 138

3.15 Conclusion 144

Chapter 4: Water Relations and Thermal Analysis 145

4.1 Introduction 146

4.2.0 Experiment 1: Changes in leaf water potential of winter barley plants with reference to cold acclimation 148

4.2.1 Experiment 2: Changes in plant water potential of winter barley plants subjected to applications of liquid seaweed extract and/or cold acclimation treatments 148

4.2.2 Experiment 3: Temporal changes in plant water potential following applications of liquid seaweed extract 149

4.2.3 Experiment 4: determination of percentage water content in shoot tissues of winter barley seedlings 149

4.2.4 Experiment 5: Determination of the percentage of unfrozen water in leaf and crown tissue samples by differential scanning calorimetry 150

4.2.5 Experiment 6: Detection and comparision of plant freezing curves with respect to cold-acclimation and liquid seaweed extract treatment using thermography and thermocouple analysis 151

4.3.0 Results 154

4.3.1 Experiment 1: Changes in leaf water potential of winter barley plants with reference to cold acclimation 154

4.3.2 Experiment 2: Changes in plant water potential of winter barley plants subjected to applications of liquid seaweed extract and/or cold acclimation treatments 154

4.3.3 Experiment 3: Temporal changes in plant water potential following applications of liquid seaweed extract 154

4.3.4 Experiment 4: Determination of percentage water content in shoot tissues of winter barley seedlings 158

4.3.5 Experiment 5: Determination of the percentage of unfrozen water in leaf and crown tissue samples by differential scanning calorimetery 159

4.3.6 Experiment 6: Detection and comparision of plant freezing curves with respect to cold-acclimation and liquid seaweed extract treatment using thermography and thermocouple analysis 161

4.4 Discussion 169

4.5 Conclusion 175

Chapter 5: General Discussion

5.0 Introduction 176

5.1 Observed changes in plant water relations 177

5.2 The effects of cold-acclimation 178

4.2.2 Experiment 3: Temporal changes in plant water potential following applications of liquid seaweed extract 149

4.2.3 Experiment 4: determination of percentage water content in shoot tissues of winter barley seedlings 149

4.2.4 Experiment 5: Determination of the percentage of unfrozen water in leaf and crown tissue samples by differential scanning calorimetery 150

4.2.5 Experiment 6: Detection and comparision of plant freezing curves with respect to cold-acclimation and liquid seaweed extract treatment using thermography and thermocouple analysis 151

4.3.0 Results 154

4.3.1 Experiment 1: Changes in leaf water potential of winter barley plants with reference to cold acclimation 154

4.3.2 Experiment 2: Changes in plant water potential of winter barley plants subjected to applications of liquid seaweed extract and/or cold acclimation treatments 154

4.3.3 Experiment 3: Temporal changes in plant water potential following applications of liquid seaweed extract 154

4.3.4 Experiment 4: Determination of percentage water content in shoot tissues of winter barley seedlings 158

4.3.5 Experiment 5: Determination of the percentage of unfrozen water in leaf and crown tissue samples by differential scanning calorimetery 159

4.3.6 Experiment 6: Detection and comparision of plant freezing curves with respect to cold-acclimation and liquid seaweed extract treatment using thermography and thermocouple analysis 161

4.4 Discussion 169

4.5 Conclusion 175

Chapter 5: General Discussion

5.0 Introduction 176

5.1 Observed changes in plant water relations 177

5.2 The effects of cold-acclimation 178

Contents	Page
5.4 The effect of liquid seaweed extract on frost resistance	185
5.5 How and when to use liquid seaweed extract	187
5.6 Critique and further work	188
5.7 Conclusions	189
Appendix	191-204
References	205-219

Table	Page
Table 1.1: Freezing tolerance of *A.thaliana* plants grown in vitro compared with RAB18 protein levels at normal growth temperatures (NGT) and during drought (D), low temperature (LT) and ABA treatments	35
Table 1.2: Low temperature genes for *A. thaliana* and *H. Vulgare* and their associated family members and their response to drought and ABA stress	36
Table 1.3: Response of *A. thaliana* low temperature responsive genes to low temperature (LT), drought (D) and ABA in wild type (WT), ABA-null mutant (aba-1) and the ABA insensitive mutant (abi-1)	37
Table 1.4: Characteristics of *H. Vulgare* lipid transfer genes	38
Table 1.5: Mineral analysis of 2 commercially available seaweed extracts	44
Table 1.6: Concentrations of PGRs characterised by Kingman *et al* (1982).	45
Table 1.7: Concentration and variability in cytokinin levels in 3 commercially available seaweed extracts. (after Blunden and Wildgoose, 1981)	45
Table 1.8: Characterisation and quantitative estimation of betaine content in 4 commercially available seaweed extracts	47
Table 2.0: Spraying and sampling dates for field trial	69
Table 2.1: Mean survival scores of 3 crop species following freezing and Damaging temperature 50 values	75
Table 2.2: Mean survival scores of winter barley and Damagingl temperature 50 values subjected to multiple applications of LSE and a standard frost test	79
Table 2.4: Summary of analysis of variance on the regression coefficients for the assessment of the inter-variation observed in the scoring scheme employed for winter barley	81

Table 2.5: Summary of analysis of variance on the regression coefficients for the assessment of the intra-variation observed in the scoring scheme employed for winter barley 82

Table 2.6: Mean survival scores of field grown winter barley plants subjected to a standard frost test 87

Table 2.7: The effect of sampling date on the survival scores of field grown winter barley subjected to a standard frost test at -3°C 88

Table 2.8: The effect of sampling date on the survival scores of field grown winter barley subjected to a standard frost test at -5°C 88

Table 2.9: Log dry weights of winter barley treated with LSE 91

Table 2.10: Mean tiller production of glasshouse grown winter barley following LSE treatments 91

Table 2.11: Effect of plant growth stage on the frost resistance of winter barley 97

Table 3.1: Comparison of protein yields from winter barley using six different buffer systems 119

Table 3.2: The effect of simulated rain events of the number of high molecular weight proteins, enhanced by LSE treatments 133

Table 3.3: Effect of simulated rain on the expression of the 118kDa protein, enhanced by LSE treatments 134

Table 3.4: The effect of simulated rain on the expression of the 57kDa protein, enhanced by LSE treatments 134

Table 4.1: Percentage water content of winter barley plants subjected to non-acclimating and cold-acclimating conditions and with two levels of liquid seaweed application 158

Table 4.2: Percentage unfrozen water in leaf samples of winter barley subjected to non-acclimating and cold-acclimating conditions and two levels of liquid seaweed applications 159

Table 4.3: Percentage unfrozen water in crown samples of winter barley subjected to non-acclimating and cold-acclimating conditions and two levels of liquid seaweed extract 160

Table 4.4: Mean exotherm temperature ofn non-acclimated and cold-acclimated leaf samples treated with liquid seaweed extract 162

Table 4.5: Mean exotherm temperature of non-acclimated and cold-acclimated crown samples treated with liquid seaweed extract 162

Table 4.6: Time elapsed between apoplastic freezing and full extracellular freezing in leaf samples of non-acclimated and cold-acclimated plants treated with liquid seaweed extract 163

Table 4.7: Time elapsed between apoplastic freezing and full extracellular freezing in crown samples of non-acclimated and cold-acclimated plants treated with liquid seaweed extract 163

Table 1A: Data on band position and percentage area generated by densitometry for molecular weight standard 200

Table 2A: Data on band position and percentage area generated by densitometry for a non-acclimated plant 201

Table 3A: Data on band position and percentage area generated by densitometry for a non-acclimated plant treated with 3 applications of liquid seaweed extract 202

Table 4A: Data on band position and percentage area generated by densitometry for a cold-acclimated plant 203

Figures Page

Figure 1.0: Comparison of atomic orbitals 17

Figure 1.1: The hydrogen bond 18

Figure 1.2: Dissolution of salt 18

Figure 1.3: Structured water surrounds a hydrophobic domain 25

Figure 1.4: Structured water surrounds a hydrophobic domain 25

Figure 1.5: Flow diagram illustrating the components of cold-acclimation 40

Figure 1.6: Flow diagram illustrating possible interactions between liquid seaweed extract and the cold-acclimation process 50

Figure 2.1: Survival curves of young winter cauliflower plants subjected to a frost test 77

Figure 2.2: Survival curves of young winter oil seed rape plants subjected to a frost test 77

Figure 2.3: Survival curves of young winter barley plants subjected to a frost test: 1 application of liquid seaweed extract 78

Figure 2.4: Survival curves of young winter barley plants subjected to a frost test: 3 applications of LSE 80

Figure 2.5: Assessment regression lines of 5 observers scores compared to originators score for non-acclimated winter barley plants 83

Figure 2.6:Assessment regression lines of 5 observers scores compared to originators score for non-acclimated, plus 3 applications of liquid seaweed extract, winter barley plants 83

Figure 2.7: Assessment regression lines of 5 observers scores compared to originators score for cold-acclimated winter barley plants 84

Figure 2.8: Assessment regression lines for 5 observers scores compared to orignators score for, cold-acclimated, plus 3 applications of liquid seaweed extract, winter barley plants 84

Figure 2.9: Assessment regresion lines for original score compared to 3 subsequent scores of non-acclimated winter barley plants 85

Figure 2.12: Assessment regression lines for original score compared to 3 subsequent scores of cold-acclimated plus 3 applications of liquid seaweed extract to winter barley plants 85

Figure 2.13: Survival curves of field grown winter barley at -7°C with reference to sampling date 90

Figure 2.14: Weather station data for the fourth application of LSE. 90

Figure 2.15: The effect of simulated rain following liquid seaweed application on log dry weights of winter barley. (Letters denote statistically significant differences) 95

Figure 2.16: The effect of simulated rain followinf liquid seaweed extract application on tiller production of winter barley. (Letters denote statistically significant differences) 95

Figure 2.17: Effect of site of uptake of liquid seaweed extract on log dry weights of winter barley. (Letters denote statistically significant differences) 96

Figure 2.18: Effect of site of uptake of liquid seaweed extract on tiller production of winter barley. (Letters denote statistically significant differences) 96

Figure 2.19: Survival scores of winter barley plants at three different growth stages following a conductive frost test 98

Figure 3.1: Concentration of total soluble protein in winter barley subjected to cold-acclimation and liquid seaweed extract treatments 121

Figure 3.2: The effect of simulated rain on the concentration of LSE enhanced soluble protein 122

Figure 3.3: The effect of LSE application site on the concentration of total soluble protein 123

Figure 3.4: The effect of cold-acclimation and liquid seaweed extract on the expression of high molecular weight proteins in winter barley 130

Figure 3.5: The effect of cold-acclimation and LSE treatments on the expression of a 118kDa protein from winter barley plants at day 0 131

Figure 3.6: The effect of cold-acclimation and LSE treatments on the expression of a 118kDa protein from winter barley plants at day 4 131

Figure 3.7: The effect of cold-acclimation and LSE treatments on the level of expression of a 57ka protein from winter barley plants 132

Figure 3.8: Schematic representation of Western Blotting on 4 day post LSE application 137

Figure 4.1: Changes in plant water potential with respect to cold-acclimation at 4°C. 155

Figure 4.2: Changes in plant water potential with respect to cold-acclimation and applications of liquid seaweed extract. 156

Figure 4.3: Temporal changes in plant water potential in non-acclimated plants treated with liquid seaweed extract 157

Figure 4.4: Temporal changes in plant water potential in cold –acclimated plants. 157

Figure 4.5: Exotherm curves of a non-acclimated plant treated with LSE and a untreated non-acclimated plant 164

Figure 4.6: Exotherm curves of a non-acclimated plant treated with seaweed extract and a untreated non-acclimated plant. 165

Figure 4.7: Exotherm curves of a non-acclimated plant treated with LSE and an untreated cold-acclimated plant 166

Figure 4.9: Mean survival score of winter barley plants subjected to thermographic analysis 168

Figure 5.0: Schematic model illustrating the freezing of non-acclimated cells 181

Figure 5.1: Schematic model illustrating the freezing of cold-acclimated cells 184

Figure 1A: Example of the procedure used to estimate the DT_{50} values 191

Figure 2A: Weather station data for five days following liquid seaweed extract application 192

Figure 3A: Weather station data for five days following liquid seaweed extract application 192

Figure 4A: Weather station data for five days following liquid seaweed extract application 193

Figure 5A: Weather station data for five days following liquid seaweed extract application 193

Figure 6A: Weather station data for five days following liquid seaweed extract application 194

Figure 7A: Weather station data for five days following liquid seaweed extract application 194

Figure 8A: Densitometry trace of molecular weight standard of the 4 day sampling 200

Figure 9A: Densitometry trace of non-acclimated plant from the 4 day sampling 201

Figure 10A: Densitometry trace of a non-acclimated plant treated with 3 applications of liquid seaweed extract, sample from 4 day 202

Figure 11A: Densitometry trace of a cold-acclimated plant from the 4 day sampling 203

List of Plates Page

Plate 2.1: The glycol freezing tank and PZI programmer 60

Plates 2.2 to 2.11winter barley scoring scheme; 61-65

Plate 2.2: Score = 0 61

Plate 2.3: Score = 1 61

Plate 2.4: Score = 2 62

Plate 2.5: Score = 3 62

Plate 2.6: Score = 4 63

Plate 2.7: Score = 5 63

Plate 2.8: Score = 6 64

Plate 2.9: Score = 7 64

Plate 2.10: Score = 8 65

Plate 2.11: Score = 9 65

Plate 2.12: Effect of LSE on tiller production: control plant 92

Plate 2.13: Effect of LSE on tiller production: 1LSE plant 92

Plate 2.14: Effect of LSE on tiller production: 3LSE plant 93

Plate 3.0: Electrophoretic gel of 0 day smaples 126

Plate 3.1: Electrophoretic gel of 4 day samples 125

Plate 3.2: Electrophoretic gel of 7 day samples 127

Plate 3.3: Electrophoretic gel of 10 day samples 128

List of Plates

	Page
Plate 3.4: Electrophoretic gel showing the effect of precipitation on liquid seaweed mediated protein expression	134
Plate 4.1: View of set-up of winter barley plants used in the radiative frost test and infrared thermograpgy	167

Dedication

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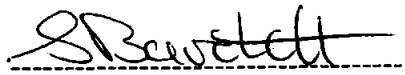
Declaration

At no time during the registration for the Degree of Doctor of Philosophy has the author been registered for any other University award.

I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance is acknowledged



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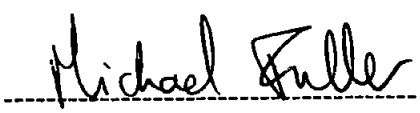

Director of Studies

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Chapter 1: Literature review on the mechanism of plant freezing tolerance

1.0 Introduction

Adaptation to cold climates is a limiting factor in the distribution of many plant species and both latitudinal and altitudinal displacement of wild and cultivated species occurs. Decreasing latitudinal and increasing altitude creates a decline in overall temperature and a reduction in the growing season (that is a decline from optimal growth conditions to sub-optimal). Temperate species grown in or around the boundary of these areas have to, and can, adjust their physiology to survive winter freezing by acclimating at the molecular, physiological and biochemical level in response to cool autumn weather (Pearce, Dunn, Rixon, & Hughes 1996). There is, however, a continuous spectrum of plant adaptation and survival to cold climates, from marginal frost tolerance to extreme sub-zero Celsius survival. This variation is reflected in nature according to the environment in which the plant is grown, and mankind selects plants for cultivation in a given area according to their ability to survive in the prevailing climatic conditions.

There is a growing awareness in the scientific community and by the general public (via media coverage) that the industrial activities of mankind are having detrimental effects on the long term stability of the world's climate. It has been documented that increased carbon dioxide emissions are giving rise to increased atmospheric levels of CO_2 (Baird, 1999). This hinders the escape of reflected light energy (thermal infrared) from the Earth's atmosphere and may account for some of the increased air temperatures experienced since the last century. Another factor which may account for one-third of this increase, since 1970, is the increased rate of solar energy emission (about 0.24%) from the sun (Baird, 1999). There is considerable political and scientific debate about the consequence of global warming but evidence suggests that the polar ice caps could be melting and a potential rise in sea levels from melted polar ice and thermal expansion of water molecules is anticipated.

It is expected that climate change will lead to warmer winters, but frost events are still expected to occur. The cold-acclimation (CA) of crop plants will still occur, but plants may acclimate less or de-acclimate earlier, and thus frost intensities that at the moment do not damage plants could cause damage in the future (Pearce, Dunn, Rixon, Harrison &

Hughes 1996). Furthermore the increase in sea levels will eventually submerge low lying areas, displacing local populations and reducing the growing area for temperate plant species, which may result in increased local frosting risk due to altitudinal displacement.

There has been considerable disruption in world weather patterns in recent years such as extensive flooding in Europe, Bangladesh and Central America but it is not clear whether this is due to cyclic climatic fluctuations such as an *El Nino* event or to global warming. However, untimely frosts in Europe have caused considerable economic loss to food producers. For example in the last week of April and the first week of May 1997 some of the most devastating frost for decades caused an estimated 90% reduction in grapes of the Cotes du-Rhone vintage (Lichfield, 1997). In South East Britain frost caused serious economic loss to soft fruit and top fruit crops (Anon. 1997a,b).

The occurrence of untimely frosts threaten the supply of food products and the financial security of food producers. The frequency of these events may increase due to global warming. These phenomena may increase seasonal fluctuations in temperatures, which could adversely affect the cold-acclimation of hardy crop plants, and therefore this review on plant adaptation and survival at sub-zero temperatures will focus on ice formation in plants, cold acclimation, the role of membranes, protein metabolism and genetic response to low temperature. Similarities between cold acclimation and drought stress will be considered, followed by a short discussion on the role of seaweed products in agriculture.

1.1 Ice Formation In Plants

The basic structure and organisation of most plant tissues and cells dictate to a large extent the site of initiation of ice crystallisation, and determine the location and development of cell water freezing. The freezing process in plant tissue is affected by the following factors: a) The water saturated gaseous environment surrounding cells; b) the relatively small amount of osmotically available extracelluar relative to the amount available inside living cells; c) the lower solute concentration of the apoplastic solution compared to the cell; d) the greater freezing point depression of cellular water because of

its higher solute concentration; e) a functionally intact cell membrane is an effective barrier to the propagation of ice crystals; f) liquid water can freely move across the plasma membrane in either direction; g) the effectiveness of the cell membrane as a barrier to ice may vary with cold acclimation or temperature; h) the presence of heterogeneous nucleators inside the cell is minimised, excluded or masked; and i) in many tissues a portion of the extracellular volume is free air space normally saturated with water vapour. The other major factor in the freezing of plant cells is the rate of cooling (Cited in Guy 1990). Thus ice formation may occur intracellularly or extracellularly (Guy 1990).

1.2 Intracellular Freezing

Intracellular freezing is suspected to occur when cooling rates are rapid or where supercooling has taken place (Pearce, 1999; Levitt, 1980). Supercooling is where a liquid (or plant water) is cooled in a rapid and continuous fashion allowing its temperature to drop below its melting points without ice formation, and the supercooling point is the lowest subfreezing temperature attained before ice formation (Levitt, 1980). Intracellular freezing occurs in sudden flashes cell by cell and is universally accepted as causing lethal non-reversible injury (Levitt, 1980, Guy, 1990). Intracellular freezing disrupts cellular integrity by converting most of the cell sap to ice (Levitt, 1980) concentrating unfrozen sap (potentially increasing cellular solute effects) and causing membrane damage.

Crystallisation of cellular water may occur by internal ice nucleation or by penetration into the cells by an external ice crystal (Pearce, 1999). The rate of cooling required for the formation of intracellular ice, in isolated leaf protoplasts, was between 3-16^{o}C/min^{-1} (Steponkus, Dowgert and Gordon-Kamm, 1983). Cooling rates in excess of 3^{o}C/hr^{-1} may favour intracellular freezing in whole plants (Steffen, Arora and Palta, 1989).

Plants can avoid intracellular ice formation by supercooling, which depresses the freezing point of tissues, and this may afford slight protection in mild frosts for plants during early spring growth and in floral tissue such as blossom. Ashworth (1984) illustrated that xylem water in several hardy fruit trees can deep supercool to -40^{o}C. However when ice nucleation does occur rapid intracellular freezing leads to a large Gibbs free energy event

(at the point of ice formation) which provides the free energy for destructive work, resulting in plant death (Chen, Burke and Gusta, 1995). Intracellular freezing is an example of non-equilibrium freezing. However, there is no evidence of intracellular freezing (Pearce, Personal Communication). Reported scientific evidence supports the theory of extracellular freezing.

1.3 Extracellular Freezing

Extracellular freezing occurs in the spaces between cells, in water transporting elements or on the external surface of plants. Ice will spread from the initial nucleation point through the extracellular spaces and, as long as the plasma membrane is intact and the cooling rate is slow (1-2^{o}C/hr), ice will remain confined to the extracellular regions. The presence of extracellular ice crystals will impose a dehydrative force on the unfrozen cellular water (Guy, 1990) concentrating the cellular sap, which in turn will depress the freezing point of intracellular water (Burke, Gusta, Quamme, Weiser and Li, 1976). Extracellular freezing is an example of equilibrium freezing. Equilibrium is reached when the chemical potential of the cell water equals the chemical potential of the extracellular ice. The result of this process is freeze dehydration, reduction in cell volume, concentration of cell solutes, including salts, and membrane damage (Chen, Burke and Gusta, 1995). Extracellular freezing occurs in frost resistant plants such as winter annuals, biennial vegetable crops like cabbage and cauliflower, overwintering perennials and many tree species.

Mazur (1963, 1969) described a theoretical model to explain why extracellular ice formation leads to cellular dehydration. There are two major components to this theory. First, freezing of the extracellular solution leads to a rapid rise in the solute concentration and an equally rapid decline in vapour pressure of the unfrozen portion as a result of the freeze-concentration of solutes. Secondly, the vapour pressure of ice declines faster than the vapour pressure of liquid water as temperature decreases. Thus for slow cooling rates, a water potential gradient is established, with liquid water moving down the gradient. During extracellular freezing in plants, liquid water will move out of the cell to the extracellular solution or the ice crystal.

Much of Mazur's theory has been widely accepted as explaining the general behaviour of extracellular freezing in plants. However, Mazur's analysis assumed ideal behaviour of both the surrounding medium and the cell during freezing. Other workers have pointed out that while Mazur's theoretical treatment was based on cells immersed in a liquid medium, the freezing of plant tissues may be slightly different. Guy (1990) gives a full account of the debatable points, nevertheless studies have confirmed that the freezing of water in plant tissues could follow the basic pattern of equilibrium freezing (Hansen & Beck 1988) and fit existing theory (Mazur 1969).

1.4 Cold Acclimation

Tender plants have no ability to cold-acclimate and cannot tolerate any freezing of their tissues, for example banana (*Musa. sp*) leaves exposed to a temperature of -1.5°C for 10-15 minutes are killed (Shmueli, 1960, cited in Levitt, 1980). However hardy plants or frost resistant plants can, after a period of cold acclimation, survive freezing temperatures. The ability to survive freezing is dictated by the genetic make up of the plant, stage of development and environmental factors (Levitt, 1980). Hardy plants respond to seasonal fluctuations in temperature and photoperiod.

The importance of temperature was recorded in the literature over 100 years ago. Goppert (1830, cited in Levitt, 1980) illustrated a decrease in the freezing tolerance of overwintering plants when these plants were exposed to 2 weeks of warmer temperatures. Conversely freezing resistance of evergreen trees was increased when these plants were exposed to low, non freezing, temperatures (Irmscher, 1912. Cited in Levitt, 1980). The process of cold acclimation is commonly referred to by growers as "Hardening Off". Hardy plants are exposed for 2 or 3 weeks to temperatures just above freezing, 0-10°C generally increase their frost hardiness. The exact temperature for hardening induction is difficult to determine and can vary with each particular species and variety. A temperature regime of 0 to 5°C will generally induce greater hardening in plants than 5°-10°C (Levitt, 1980). Once the maximum possible degree of hardening has been obtained at these warmer temperatures a second stage of hardening occurs at slightly lower temperatures, including sub-zero temperatures (Levitt, 1980, Abe and Yoshida, 1997).

The influence of temperature on cold-acclimation (CA) is not independent of photoperiod. Low temperatures in the absence of light are incapable of inducing CA in winter annuals (Levitt, 1980). Light induced CA in winter rape seedlings was reversed by far-red light (Kacperska-Palacz, Debska and Jakubowska, 1975) and short term illumination of winter wheat, or part of the leaves, for a longer time period increased the freezing tolerance of plants kept in the dark (Levitt 1980). Steponkus and Lanphear (1968) found that light results in the production of a translocated CA promoter in *Hedera helix* which was transported to a darkened receptor, and C^{14} labelling indicated that the translocatable factor was sucrose. Lawrence, Cooper and Breese (1973) found that the freezing tolerance of *Lolium perenne* was dependent on the total light energy received by the plants. This light dependent induction of CA is due to the need for photosynthesis because chlorotic leaves are unable to CA (Levitt, 1980).

Cold acclimation is improved by short days in both woody plants and herbaceous plants (Levitt, 1980). The annual curve of CA is correlated with change in photoperiod as with temperature. The CA of peach and nectarine trees occurs from November to January even if the temperature remains above 15°C (Buchanan, Briggs and Bartholic, 1974). Conversely normal autumn CA can be prevented if plants have been maintained in long photoperiods. Shoots of trees and shrubs in cities close to street lights can be damaged by frosts compared to similar plants positioned away from street lights (Levitt, 1980). This lack of CA in long photoperiods is due to the continued growth of plants. In general plants that are rapidly growing cannot CA (Levitt, 1980) but when actively growing plants are treated in a manner that retards growth (withholding water will induce some wilting and reduce growth) an increase in frost resistance is observed. If nitrogen fertiliser is applied to plants they frequently lose their ability to CA on exposure to normal CA conditions (Levitt, 1980). The reduction in the ability to CA is thought to be related more to the stage of plant development than growth *per se*. Buds of fruit trees lose their ability to CA when they begin to develop into shoots in the spring. Treatments that affect growth but have little effect on development (wilting and nitrogen) have the least detrimental effect on freezing tolerance (Levitt, 1980).

1.5 Accumulation of Cryoprotectants

In many plants exposure to cold acclimating conditions causes the accumulation of substances that have cryoprotective activity (Levitt, 1956, 1980, Sakai and Larcher, 1987, Guy, 1990, Chen, Burke, and Gusta, 1995). Known cryoprotectants include disaccharide and trisaccharide sugars, proline, polyamines, sorbitol and glycine-betanie (Guy, 1990). The carbohydrates sucrose, raffinose, sorbitol (Guy, 1990) and fructans in winter wheats (Abe and Yoshida, 1997) are the primary cryoprotectants in plants. Sucrose is the most mobile and its concentration can increase 10-fold during exposure to low temperatures (Guy, 1990).

Sakai and Larcher (1987), working with herbaceous and woody plants, illustrated a seasonal increase in soluble carbohydrates during the autumn to a maximum in winter followed by a decline in spring. This increase was correlated with an increase in osmotic potential and an increase in freezing tolerance (Chen, Burke and Gusta, 1995). There is species variation in the strength of the correlation between increased sugar content and increased freezing tolerance. Poplar held at 15^{o}C for 2 months had an increased freezing tolerance, with the killing temperature being -30^{o}C, compared to the previous killing temperature of -2^{o}C, but there was no appreciable increase in the sugar content (Sakai and Yoshida, 1968; cited in Levitt, 1980). Conversely the sugar content of potatoes increases at low temperature but there is no increase in freezing tolerance (Levitt, 1980). Levitt (1980) attributes these discrepancies to the increased osmotic potential of plants associated with changes in sugar levels. Purely osmotic effects should increase the freeze dehydration avoidance of plants. Therefore, a hardened plant with a higher tolerance of

freeze dehydration will show a greater increase in freezing tolerance per unit increase in sugar than an unhardened plant with a lower tolerance to freeze dehydration.

The overall importance of sugars in frost resistance can be illustrated by sugar feeding experiments. The maximum hardiness of winter cereals and callus tissues of woody plants could not be obtained under normal CA conditions unless the plants and tissues were fed sucrose (Sakai, 1962; Ogolevets, 1976. Cited in Chen, Burke and Gusta, 1995). The evidence that sugars play a role in frost resistance is considerable and suggestions on how sugars and cryoprotectants ameliorate freezing injury are;

1. Osmotic effect. Sugars decrease crystallisation of water and thus reduce freeze-induced dehydration.
2. Metabolic effect. Metabolism of sugars during cold acclimation produces unknown protective substances and/or metabolic energy changes.
3. Cryoprotective effect. Sugars may protect cellular constituents including membranes during freeze/thaw cycle.
4. Glass effect. High sugar concentrations may stop all biochemical and most physical activity (dehydration) when they form a solid glass (Chen, Burke and Gusta, 1995).

1.6 Membranes

During extracellular freezing the chemical potential of the intracellular solution must come into equilibrium with the extracellular ice. This can be achieved by intracellular ice formation or cell dehydration (Steponkus, 1984). The plasma membrane is the primary determinant of the manner of equilibrium (Steponkus, 1984). The intact plasma membrane is an effective barrier to extracellular ice (Mazur, 1969) and because of the absence of intracellular ice nucleators the intracellular solution remains unfrozen (Steponkus, 1984). The semipermeable nature of the plasma membrane allows the cell to behave as an osmometer and dehydrate in response to the lower chemical potential of the extracellular solution. Whether the cell achieves equilibrium by continued dehydration or intracellular ice formation is a function of membrane stability (Steponkus, 1984).

Observations of isolated protoplasts from rye (*Secale cereale* L. cv. Puma) have highlighted four fundamental forms of injury: 1) **expansion enduced lysis** during warming and thawing of the suspending medium the decreasing osmolarity of this medium results in osmotic expansion of the protoplasts; 2) **loss of osmotic responsiveness** after slow cooling where protoplasts became osmotically inactive after warming; 3) **altered osmotic behaviour during warming** this suggests a prior transient loss of intracellular solutes or leakiness of the membrane; 4) **intracellular ice formation** during rapid cooling decreased efficiency of the plasma membrane, to the extracellular ice, leads to seeding of supercooled intracellular solution, (Steponkus, 1984).

Expansion-induced lysis accounts for the injury observed in non-acclimated (NA) protoplasts cooled slowly to the lethal temperature of 50% of the population (LT_{50}) of -3 to -5°C. However, cold-acclimated (CA) protoplasts seldom express expansion-induced lysis following slow freezing to any temperature. The predominant form of injury in CA protoplasts cooled to the LT_{50} of -25 to -30°C is the loss of osmotic responsiveness, (Steponkus, 1984). Both NA and CA protoplasts are subjected to intracellular ice formation at rapid cooling rates.

Cryomicroscopic studies (Dowgert and Steponkus, 1983) have shown that cold acclimated protoplasts were more resistant to expansion induced lysis than non-acclimated protoplasts because cold-acclimated protoplasts are able to undergo larger increases in area at lower tensions than NA protoplasts, this is known as the tolerable surface area increment. The surface area increment of NA protoplasts is rate dependent with a maximum proportional change of 1.25 compared to the rate independent increase in CA protoplasts with a maximum increase of 1.6 (Steponkus, 1984). Observations of large amounts of membrane vesicles in close proximity to the plasma membrane in CA cells (before freeze-thaw cycle) could indicate a reservoir of membrane material and may account for the increased expansion of CA protoplasts (Kupila-Ahvenniemi, Pihakaski and Pihakaski, 1978; Niki and Sakai, 1981 and Pomeroy and Siminovitch, 1971).

In contrast to expansion-induced lysis observed in NA protoplasts, CA cells suffer injury via the loss of osmotic responsiveness following slow cooling. Protoplasts exhibit characteristic osmotic behaviour during cooling but are osmotically inactive during warming and remain contracted (Steponkus, 1984). This form of injury corresponds to frost plasmolysis commonly observed in freeze-killed cells. The loss of osmotic responsiveness is a reflection of the semipermeability of the plasma membrane, which is altered in the contracted state. The loss of membrane semipermeability may arises from protein loss (Yoshida, 1984) solute concentration, electrical perturbations, thermotropic phase transitions and or removal of water. Nuclear magnetic resonance techniques show that the loss of semipermeability occurs in the frozen state at the lethal temperature (Steponkus, 1984). Gusta, Rajashekar, Chen and Burke (1982) have demonstrated that this form of injury results in the irreversible loss of intracellular electrolytes. Frequently measured as electroconductivity as an indication of frost damage (Wisniewski and Arora, 1993).

There are similarities between the loss of osmotic responsiveness observed in isolated protoplats and the observed destabilisation of the plasma membrane Rye and Wheat plants samples (Pearce, 1999). In a study of wheat plants membrane phase separation constitutes a removal of protein particles from areas of the membrane (Pearce and Willison, 1985). Break-up of the membrane is mediated by local changes in phase from lamellar ($L\alpha$) to non-lamellar, including hexagonal II (H_{II}) (Steponkus and Webb, 1992; Pearce 1999). The precise sequence of events causing this is unclear, but it is believed that proteins are stabilised in the membrane by association with non-lamellar lipids such as phosphatidylethanolamine (PE), which stabilises the non-lamellar lipids in the bilayer structure (Williams, 1990). Thus loss of protein particles from areas of the membrane could leave the non-lamellar lipids in these areas without stable interactions. Several such molecules would form local non-lamellar structures and cause fragmentation of the membrane (Pearce, 1999).

One of the consequences of cellular dehydration, during freezing or hypertonic simulation, is the concomitant rise in cellular salts and/or acids which have a detrimental chemical

effect on cell processes. Experimental studies support the idea that lipoprotein complexes are disrupted by increased electrolyte concentration, pH and removal of water (Steponkus, 1984). Garber and Steponkus (1976) illustrated that freeze-thaw injury in chloroplast thylakoids was the result of protein release. Coupling factor 1 and plastocyanin were released from the thylakoid membrane and this was confirmed by Volger, Heber and Berzborn (1978, cited in Steponkus, 1984). These researchers also reported that ferredoxin-$NADP^+$ reductase was released. The theory for protein release is the consequence of non-specific suppression of intramembrane ionic interactions by high ionic concentrations resulting from freeze-induced dehydration (Steponkus, 1984).

Further solute effects on membrane stability may arise from direct interactions of solutes and the membrane or by decrease in water structure by solutes. There is considerable evidence that solutes (inorganic electrolytes, organic acids, amino acids, sugars and cryoprotectants) have either a detrimental or positive effect on membrane stability Steponkus, 1984). There is a negative effect of solutes on water structure, altering bonding energies and chemical potential of water. This would increase the interaction of partially polar membrane components with the aqueous phase and facilitate bilayer dissociation (Steponkus, 1984). Other effects of altered water structure would be dissociation of membrane proteins, which if these proteins transport charged ions, may lead to altered charge distribution and lead to the opening of hydrophobic channels and membrane collapse (Jensen, Heber and Oettmeier, 1981).

Another consequence of water removal from the plasma membrane is that many phospholipids undergo a phase transition from a lamellar form to a hexagonal II phase (H_{II}) at water contents below approximately 20% (Steponkus, 1984). During the H_{II} transition phospholipids form long cylinders with the polar head groups orientated in an aqueous core, this is not unexpected because the polar heads would have an affinity for each other but would be repelled from the desiccated cellular environment. Suggestions that H_{II} phase transitions cause loss of semipermeability, implicate the arrangement of the aqueous cores of the hexagonal arrays in the membrane surface (Steponkus, 1984).

Effect of Cold Acclimation on Osmotic Responsiveness

The possible ameliorating effects of cold acclimation on the loss of osmotic responsiveness are, in general terms, a reduction in cellular dehydration, lessening the detrimental effects of dehydration-induced solute concentration, or an increased membrane stability and changes in enzyme and protein isoforms.

The effect of cellular dehydration can be reduced by the accumulation of compatible solutes and Cryoprotective compounds such as sugars and proline. As mentioned previously there are numerous reports of increased concentration of compatible solutes with cold acclimation (Levitt, 1980) and these changes will decrease the extent of cellular dehydration. In isolated protoplasts of *S.cereale* there is a lower incidence of hypertonic-induced loss of osmotic responsiveness in CA protoplasts and this can be accounted for by increased intracellular solute concentration (Steponkus, 1984). Another advantage of compatible solutes is that they will confer protection against specific toxic solutes (Mazur, 1969) by non-specific colligative dilution of toxic substances. There is an observed series of effectiveness of compatible sugars: trisaccharides> disaccharides>monosaccarides. Reports also implicate such solutes with direct membrane stabilisation (Santarius, 1973; Steponkus, 1971). The precise nature of the sugar-membrane interaction is unknown but it is suggested that substances like trehalose prevent dehydration-induced membrane fusion by acting as membrane spacers (Crowe and Crowe, 1982; Crowe, Crowe and Mouradian, 1983). The cryoprotective properties of substances like proline and betaine is because they interact with proteins to stabilise their biological function during water stress (Steponkus, 1984). Work with isolated protoplasts suggests two roles for proline in membrane stabilisation. Hypertonic-induced loss of osmotic responsiveness is significantly less where proline is present (Liu and Steponkus, 1983a) and proline has a specific effect on the behaviour of the plasma membrane during surface contractions (Liu and Steponkus, 1983b).

Temperature and water content affect the physical properties of membranes and hence their function during exposure to cold. Two phase transitions are of particular importance (Pearce 1999). 1) At low temperatures and very low water contents polar membrane lipids

in a lamellar phase undergo a change from a solid-phase (Lα) to a gel-phase (Lβ) and 2) dehydration causes polar lipids in the lamellar phase to enter the non-lamellar phase, the best known of which is the hexagonal II phase (H_{II}). Lipid structural features favouring the non-lamellar phase are a large hydrophobic group and a small head-group (Whilliams, 1990). Because the gel-phase may inhibit membrane functioning, and because non-lamellar lipids may destabilise the bilayer structure, it is suggested that these phase transitions may explain damage to membranes (Whilliams, 1990).

The consequence of these phase transitions is an expected change in membrane lipid Many workers implicate that the degree of unsaturation of fatty acids increases during CA. However, Uemera and Yoshida (1984) could only demonstrate a small change in the unsaturation of fatty acids in the plasma membranes of *S. cereale* L. cv Puma and in the plasma membranes of orchard grass *(Dactylis glomerata* L.) but there was a significant increase in the degree of unsaturation of endomembranes (endoplasmic reticulum, golgi apparatus and mitochondria membranes) (Yoshida and Uemera, 1984). These workers also reported a significant change in the polypeptide complements of *S. cereale* plasma membranes during CA. In both *S.cereale* and *D. glomerata* there was an increase in the phospholipid to protein ratio during CA. Another well observed change is in the composition of leaf plasma membranes. Cold-acclimated leaf material has a higher proportion of di-unsaturated phospholipids (PL) (Uemura, Joseph and Steponkus, 1995; Uemura and Steponkus, 1994). There is direct evidence that these changes contribute to the increase in freezing-tolerance, which occurs during cold-acclimation (Steponkus, Uemura, Balsamo, Arvinte and Lynch 1988.)

These studies on the behaviour of the plasma membrane during CA highlight the central role the membrane plays in ameliorating the effects of freezing damage, but as illustrated, membranes do not function independently from other cellular systems such as cryoprotectant accumulation and protein metabolism and the effects these components have on the cellular water environment.

1.7 Properties of water

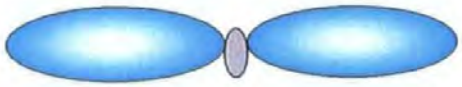
The unique properties of water can be attributed to the bond angle between the 2 hydrogen atoms. X-ray studies show this angle to be 105°, close to the tetrahedral angle of 109° (Ramsden, 1990; Salisbury and Ross, 1992). The explanation for this arrangement lies in the atomic orbitals used for bonding. If the oxygen atom used two P orbitals for bonding the angle would be 90°, but it is believed that hybridisation occurs between the S orbital and the P orbitals of the oxygen atom. Of the four SP3 hybrid orbitals, two are occupied by bonding pairs of electrons and the other two by lone pairs. This gives rise to the dipole moment of water, a pair of separated opposite charges, figure 1.0, (Ramsden, 1990; Anon, 1991g). The difference between the bond angle of 105° for water and that of 109° for the tetrahedron is explained by the greater repulsion between the lone pairs than between the bonding orbitals.

The slight positive charge (+ve) of the hydrogen atoms is cancelled by the negative (-ve) charge of the oxygen giving a net neutral charge to the water molecule. Such a molecule is said to be polar (Salisbury and Ross, 1992). Attraction of the -ve side of one water molecule to the +ve side of another water molecule gives rise to a weak hydrogen bond (Figure 1.1). The angle between the two hydrogen bonds is not stable but represents an average sharing of electrons and distribution of charge (Salisbury and Ross, 1992). In ice, however, this angle is exact. Each water molecule in ice is surrounded by four others, forming a tetrahedral structure, with each oxygen atom attracting two extra hydrogen atoms (Ramsden, 1990; Salisbury and Ross, 1992; Anon, 1991g; Franks, 1975). The tetrahedrons of ice are regularly spaced giving the ice crystal a hexagonal shape, this spaces the water molecules further apart than they are in water and accounts for the 9% increase in size. As the ice crystal melts the volume decreases because the molecules are more tightly packed, each molecule in the liquid is surrounded by five or more other molecules (Salisbury and Ross, 1992).

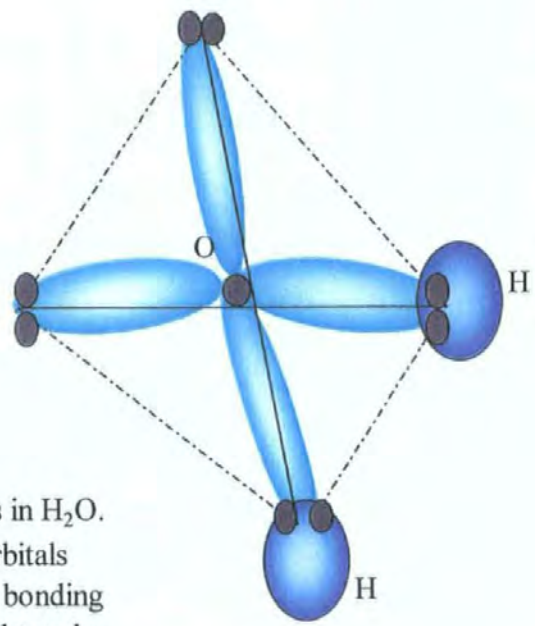
To convert 1g of water at 20°C to 1g of water vapour requires 586 cal of energy, the latent heat of vaporization. In water this is unusually large because of the tenacity of the hydrogen bond. It requires a large amount of energy to separate one water molecule from

Figure 1.0: Comparison of atomic orbitals. After Ramsden (1990)

One p orbital



One sp^3 orbital



The four sp3 orbitals in H_2O. Of the four hybrid orbitals two are occupied by bonding pairs of electrons and two by lone pairs.

Figure 1.1: The hydrogen bond

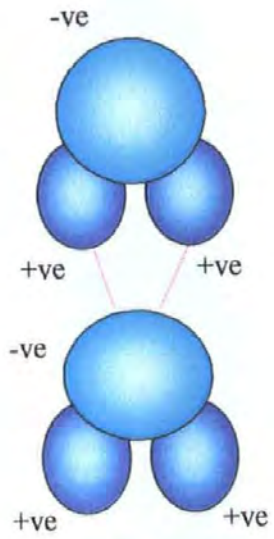
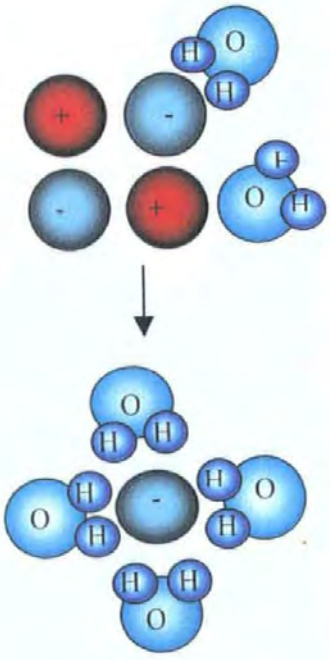


Figure 1.2: Dissolution of salt. After Ramsden (1990)



another. The dipole moment of water is also affected when conversion to vapour occurs. In a molecular dynamics study of water (Dang and Chang, 1997) it was estimated that the average dipole moment of a water molecule far from the water/vapour interface is 2.75D, (*D = self diffusion coefficent*) similar to that of bulk water, but as a molecule of water approaches the water/vapour interface the average dipole moment monotonically decreases and reaches a value close to that of the vapour phase.

Polar properties of water

When a crystal of salt is dropped into a container of water it dissolves. This occurs because water molecules are attracted to the ions in the salt crystal. The -ve oxygen atoms are attracted to the cations and the +ve hydrogens are attracted to the anion. This process is dynamic and the forces of attraction separate the salt ions, (Figure 1.2) energy is released which compensates for the energy required, and eventually each ion of salt is surrounded by water molecules and thus hydrated (Ramsden, 1990).

Interactions of water and carbohydrates

Examination of the structure of carbohydrates shows that there are many chemical groups which potentially interact with water, for example OH groups, O atoms, C-H or CH2 groups, these groups are involved in the composition of simple sugars. Consideration of other, more complex groups, such as carboxyl, N-acetyl, sulphate, methyl and methoxyl may show further diverse interactions with water (Sugget, 1975). However, for the most important sugar-water interaction (the hydrogen bond) these individual group effects cannot simply be considered in isolation to the detailed stereochemistry of the sugar molecule. It is the spatial orientations of the hydroxyl groups which determine the hydration properties of the sugar molecule (Sugget 1975).

Detailed thermodynamic and spectroscopic studies on a series of sugars has shown that different sugars interact very differently with water and the most favourable hydration interactions are those which involve equatorial -OH substitutions on the pyranose sugars (Franks, 1975).

The preferential hydration of equatorial -OH groups has been related to the compatibility of such groups with the organisation of water molecules in the bulk liquid. The distance between equatorial -OH groups is 4.85 angstroms and this distance matches the O-O spacing in liquid water, (Franks, 1975) and consequently is incompatible to the second neighbour distance in ice of 4.5 angstroms. Equatorial substituted sugars and polyos may prevent ice nucleation because of this incompatibility between ice lattice geometry and the carbohydration structure (Franks, 1975). A good example of the importance of molecular stereochemistry and hydration is the dehydration of DNA from *Escherichia coli*. The organised structure of DNA was lost following dehydration. However, by adding myo-inositol (a sugar like molecule with 5 equatorial -OH groups) prevention of loss of DNA structure was observed following dehydration (Sugget, 1975). The resemblance of the O-O spacings in myo-inositol with those of liquid water replace the hydration requirements of the DNA structure (Sugget, 1975).

The ability of saccharides to participate in hydrogen bonding to neighbouring water molecules and thus effectively alter the physical state of water was considered by Kawai, Sakurai, Inoue, Chujo and Kobayashi, (1992). These workers used a combination of differential scanning calorimetery and nuclear magnetic resonance (NMR) studies to elucidate the hydration ability of 8 saccharides (trehalose, sucrose, maltose, maltotriose, maltotetraose, maltopentose, maltohexose and maltoheptose). Calculations of the number of unfrozen water molecules (estimated from DSC measurements) indicated an increase in unfrozen water with increasing saccharide size, however, when the number of monosaccharides is considered it was illustrated that trehalose had the greater capacity for hydration. These workers indicated that the unfrozen region of these solutions consists mainly of water molecules directly hydrogen bonded with the -OH groups of the saccharides. Furthermore these researchers illustrated that the number of water molecules bound to a given saccharide corresponded well with the number of equatorial -OH groups.

There were two exceptions, maltotriose and maltoheptose. These two saccharides had smaller quantities of unfreezable water than predicted by the number of equatorial -OH groups. This observation may be accounted for by the conformational changes in long saccharides. Computer models of carbohydrates predicts that long saccharides composed of glucose units tend to form a helix like structure and thus reduce the number of equatorial -OH groups. Conformation of computer models must be carried out to confirm this speculation (Kawai *et al*, 1992).

The effect of equatorial -OH groups on hydrogen bonding of water would be an overall reduction in water mobility. Kawai *et al* (1992) measured the mobility of water, using NMR techniques, and found that it was reduced according to the number of equatorial -OH groups. Once again the mobility of water in maltotriose and maltoheptose solutions was greater than predicted by equatorial -OH groups. These workers concluded that the position of equatorial -OH groups closely resembles that of liquid water and thus trehalose can readily fit into the surrounding water structure.

Interactions of water with amino acids and proteins

The most commonly held view of intracellular water is that all but a small fraction of cellular water has the physical properties and behaviour of pure bulk water. Conventional views tell us that the factors involved in cellular response to extracellular osmotic pressure changes is the balance between solute particles and hypotonic/hypertonic extracellular conditions. Thus the large number of small inorganic particles are generally thought to be the main source of intracellular osmolarity while small organic molecules make a significant, but smaller, contribution to the number of osmotically active solutes in cells. Proteins contribute relatively few additional solute particles but, because of their large size, constitute the major dry mass of the cell (Cameron, Kanal, Keener and Fullerton, 1997).

Kelly, Butler and Macklem (1995) studied the osmotic counterbalancing systems involved during the exposure of Xenopus eggs to hypotonic solutions and considered osmolyte efflux, elevation of intracellular pressure, plasma membrane impermeability to water and

water and the osmotic properties of intracellular water and solutes. These workers discovered that neither osmolyte efflux, elevation of intracellular pressure or membrane impermeability to water accounted for the survival of Xenopus eggs in hypotonic solutions. They looked at the osmotic properties of intracellular water to explain the non-ideal behaviour of Xenopus eggs. Under the same hypotonic conditions Xenopus oocytes rupture, whereas eggs do not. Exposure of a fully grown oocyte to progestrone will induce the excised oocyte to mature into a fertilizable egg, in about 4 hours. During maturation there is little change in protein composition but there is significant changes in ion content and structural re-organisation (Kelly *et al*, 1995). The conclusion drawn was that maturation caused almost all of the egg water to become osmotically unresponsive due to changes in interactions with intracellular proteins during the oocyte maturation process.

This work questions the assumptions behind ideal osmotic behaviour because it appears that all of the water in the Xenopus egg is osmotically un-responsive. Can this non-ideal behaviour of water in Xenopus eggs be explained on the basis of solute-induced water perturbation (motion or structure that differs from that of water molecules in bulk water) caused by the surface characteristics of cellular solutes ? The answer was initially sought by the synthesis of available information on the osmotic and motional properties of water in cells. However, further work is required to elucidate the mechanism of osmotically un-responsive water (Cameron *et al* 1997).

Water interactions with amino acids and glycine peptides

Freezing point depression analysis of water soluble hydrophobic and hydrophilic (both polar and ionic) amino acids and on varying sized homo-peptides of the amino acid glycine illustrated the non-ideality of these molecules. Amino acids with large hydrophobic surfaces (alanine, valine, isoleucine, leucine, phenylalanine and methionine), were shown to have positive I values (non-ideality constant) implicating increased water structuring adjacent to hydrophobic surfaces (Cameron *et al*, 1997). Conversely hydrophilic amino acids had negative I values which implies a decrease in water structuring adjacent to the polar surface.

This observation was attributed to dielectric alignment. Dielectrically aligned dipolar water molecules are not in the optimum orientation for hydrogen bonding with one another (Cameron *et al*, 1997). Therefore dielectrically aligned water molecules cannot form as many hydrogen bonds with one another as those structured water molecules that occur over hydrophobic surfaces, or as the multiple-hydrogen-bonded water molecules of bulk water. (Bulk water at 37°C is estimated to have 15% of the water molecules transiently hydrogen bonded to four other water molecules in an assembly refered to as a flicker cluster (Cameron *et al*, 1997). Since the dielectric alignment of water molecules allows fewer hydrogen bonds with adjacent water molecules, these water molecules are expected to have increased motion.

A comparison of the non-ideality of amino acids with progressively larger hydrophobic side chains illustrated increased water structuring with increased hydrophobic surface area at a rate of one water molecule perturbed per nine square angstroms of hydrophobic surface area (Keener, Fullerton, and Cameron, 1995). The water molecules near hydrophobic surfaces develop hydrogen bonds between one and another to form a series of pentagonal structures (Cameron *et al*, 1997). These structures occur adjacent to hydrophobic surfaces because water cannot direct its dipolar charges towards the non-polar (hydrophobic) surface. This results in a five sided water structure because the angle of divergence between the oxygen and two hydrogen atoms is 104.5°, a 3.5° divergence from the 108° angle of a symmetrical pentagon. A square of four water molecules would require a 14.5° divergence (Cameron *et al*, 1997). Multiple pentagons of water can fit together to form a closed 'clathrate' structure which can conform to irregular surfaces and even enclose a purely hydrophobic molecule of polymer (Figure 1.3).

Teeter (1984) illustrated a pentagonal water arrangement around protein crystals of the plant protein crambin. In this example five water pentagons are fused together. Four cover the hydrophobic patch on the protein with the fifth extending into the solvent.

Several factors can destructure the pentagonal water structure (heat, electrical charge and hydrostatic pressure) that occurs over hydrophobic surfaces (Cameron *et al*, 1997; Urry,

1995). The effect of a destabilized pentagonal water structure has on the stability of a molecule was illustrated with a weight stretched, linerized, man made hydrophobic, elastin like protein. Following an input of energy (heat) the hydrogen bonds of the pentagon water structure would break, giving way to bulk water state, and result in the folding and consequently the shortening of the elastin like polymer, (Urry, 1995)

This model would explain the changes in the amounts of structural water. The presence of a charged or hydrophilic side chain in a large hydrophobic polymer will tend to destroy the pentagonal structure by forcing structured water molecules to break their hydrogen bonds and realign their poles around the charged group (Figure 1.4).

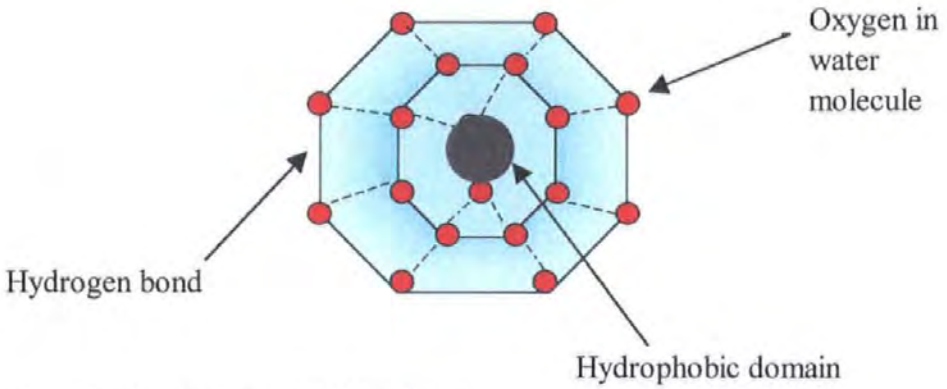
The new radial alignment will reduce translational motion of the bound water molecules closest to the charged group, but, at greater distances from the charged group, electrical realignment of the water dipoles will result in fewer hydrogen bonds and thus will increase translational motion, even to an extent where the activity coefficient will be higher than that observed in bulk water (Cameron *et al*, 1997).

Interactions of water with proteins

Recent studies on the hydration status of Bovine Serum Albumin (BSA) have demonstrated that the I value (non-ideality constant) of BSA (above 50mMolar and at a pH near its isoelectric point) was 4.1g water/g BSA (Cameron *et al*, 1997). This implies that BSA in its native globular state perturbs 4+g water/g of protein. It is noted that this amount of osmotically un-responsive water per unit of dry cellular mass would account for all the intracellular water in most cell types (Cameron *et al*, 1997).

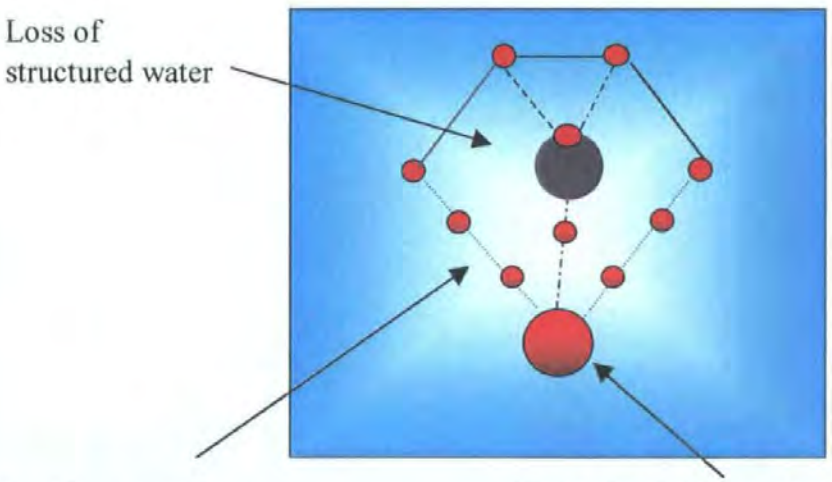
By studying the amount of osmotically un-responsive water associated with native folded protein, Miller, Janin, Lesk and Ghothia, (1987a,b) illustrated that an unfolded protein had 2.78g of water per gram of protein, in the first monolayer, compared to 0.59g of water per gram of protein for the folded protein. Although the unfolded protein had a 4.72 fold increase in surface area this was still not enough to explain the 4.1 g water/g native BSA

Figure 1.3: Structured water surrounds a hydrophobic domain. Adapted from Urry (1995)



Dense shading represents highly ordered water molecules

Figure 1.4: Water destructured by charged hydrophilic group. Adapted from Urry (1995).



Water destructuring elements such as charged hydrophilic groups force water molecules to realign and thus destructure the ordered state of water surrounding hydrophobic domains. Dense shading represents potential energy gradient.

that displays non-ideal behaviour. The conclusions from this study were that the large quantities of perturbed water associated with native BSA may exist in multiple layers of structured water (Cameron *et al*, 1997). This perturbed water is not considered to be bound to BSA but is thought to be approximately the amount of water one would find in the first few layers of water (Cameron *et al*, 1997).

X-ray diffraction studies on crystals of cubic insulin revealed evidence of multiple hydration layers with perturbations extending to a distance of 18 angstroms, equivalent to five or six layers of water molecules (Badger, 1993).

Motional properties of cellular water compared to pure bulk water.

The implications of multiple layers of structured water associated with proteins is that the motional properties of cellular water should be slowed compared to that of pure bulk water. To illustrate this, two methods have been applied to various animal cell types. The first method involves proton pulsed gradient spin echo nuclear magnetic resonance spectroscopy (PGSE-NMR) to measure the water self diffusion coefficient (D). This approach clearly demonstrated that D for Bovine lens (nuclear and cortex homogenates) was slower than that of pure bulk water (Cameron *et al*, 1997). However, this approach could not distinguish between the slower diffusion rate of structured water and that due to obstructions. The second method applied was quasielastic neutron scattering (QENS). The QENS method gives information on water diffusion rates over short distances (a few angstroms) corresponding to a time interval of 10-12 sec. This method does not allow enough time for the average water molecule to encounter cellular obstructions. Once again this method illustrated that pure bulk water had greater D value (2.2 cm2 sec-1 x^{10-5}) than that of Artemia cyts and skeletal frog muscle (0.69 and 0.75 cm2 sec-1 x^{10-5}) respectively (Cameron *et al*, 1997).

Another trend from these studies was, as the percentage of cellular water increased so did the D value. This may indicate that water molecules further away from the protein surfaces are more mobile. This data corresponds well with that of Ross (1978). Here differential scanning calorimetery of the nonfreezable water in solute-macromolecule-

water systems illustrated that at low contents of active water there is little difference between the amounts of bound water and free water. As the active water content rises so does the amount of bound water. This implies that at low amounts of active water, water is well structured and physically altered so as to mimic bound water.

Cameron *et al* (1997) explain the non-ideal osmotic behaviour of protein solutions by the fact that much of the water of the hydration fraction does not appear to participate in the osmotic equation of Vant Hoff. Water amounts that are greater than needed for hydration of the solute have the osmotic behaviour of bulk water.

The observation that a large fraction of cellular water has perturbed osmotic and motional properties can be accounted for by a few layers (2 to 8) of structured water molecules, slowed in motion by two fold, compared to water molecules of pure bulk water. Under these conditions any cell with less than 70 to 80% water by mass might be expected to have all of its water in a state that differs from pure bulk water (Cameron *et al*, 1997). This observation supports Abe and Yoshida (1997) theory that the 2nd phase of cold-acclimation is associated with changes in the physical properties of water.

1.8 Protein Metabolism

Guy (1990) reported that at least two lines of evidence suggest a possible molecular basis for the adjustment of metabolism to low nonfreezing temperatures and perhaps freezing tolerance. Repeated observations that a number of enzymes show shifts in isozymic composition upon exposure to low temperature (LT) and numerous studies have shown both qualitative and quantitative differences in protein content between NA and CA tissues.

Enzyme variation

Early studies (McCown, McLeester Beck & Hall, 1969; cited in Guy, 1990) demonstrated that changes in peroxidase isozymes were induced by low temperatures. They showed that peroxidase activity was increased in hardened stems of four widely unrelated woody species. In three of the species, several peroxidase isozymes present in CA tissue were not

CA tissue were not expressed in NA tissue. Roberts (1974) demonstrated that invertase in wheat leaves at LT undergoes a shift from a low-molecular-weight form to a higher-molecular-weight form. The larger form exhibits different kinetic properties and seems to functionally replace the smaller form in CA plants (Roberts, 1978). A series of studies on enzyme activity in alfalfa by Krasnuk and colleagues (Krasnuk, Jung & Witham, 1975, 1976 and Krasnuk, Whitham & Jung 1976; cited in Guy, 1990) observed increased activity of a number of dehydrogenases associated with respiratory pathways, including glucose-6-phosphate dehydrogenase, lactate and isocitrate dehydrogenase during winter (Krasnuk *et al*, 1976). The increase in enzyme activities paralleled increases in soluble protein content during winter, suggesting that the increase in activity may result from higher amounts of enzyme (Guy, 1990). In this sense, increased soluble protein content and enzyme activity could be part of the adjustment of metabolism to the kinetic constraint imposed by LT. For many of these enzymes, freeze stability was increased during winter. It is not clear, however, where this increased stability arises from. Possible origins may be from the new isozymic variants or from an intrinsic property. Guy & Carter (1984) studied the properties of glutathione reductase from spinach leaves of CA and NA tissues. It was demonstrated that the enzyme had increased activity, freeze stability, altered kinetic behaviour and the expression of new isozyme variants in CA plants. The enzyme from CA plants was better suited, as judged by K_m values for substrates, to function at LT. Furthermore the enzyme from CA plants was more stable to freeze/thaw stress. It can be seen from these studies that there is potential for alterations in enzyme response to LT exposure and these shifts in enzyme characteristics in response to LT can also be demonstrated for other protein families.

Expression of Novel Protein Families

As far back as 1949 Siminovitch and Briggs studied the seasonal variations in protein content in bark tissue of the black locust tree, and demonstrated the accumulation of soluble proteins as temperatures declined and the accumulation of these proteins was correlated with freezing tolerance (Siminovitch & Briggs, 1953). Throughout the winter the protein concentration remained high but declined rapidly during the breaking of dormancy and the resumption of growth in the spring. The decline in soluble protein content closely

matched the loss of freezing tolerance (Guy, 1990). This pioneering work of Siminovitch & Briggs prompted numerous studies on the protein content in plant tissues during cold acclimation (CA) which established that the accumulation of soluble proteins during CA was a general response but was not universal (Guy, 1990).

Despite the constraints of the primitive electrophoretic techniques available, Siminovitch & Briggs (1953) were able to report that not only a quantitative rise in protein content but also a qualitative shift had occurred in response to CA. Since this early work there has been a profusion of electrophoretic studies on protein shifts in plants showing both quantitative and qualitative changes in protein content of NA and CA plants. Most subsequent studies have confirmed the notion that CA and freezing-tolerant plants contain new protein species not present in NA plants (Guy, 1990)

Information on the subcellular location of proteins that vary in response to CA and LT is scant. Existing evidence includes several studies of purified plasma membranes from NA and CA tissue. In CA leaf plasma membranes, more than 20 proteins were found to decline or disappear, and 11 increased in concentration, while 26 proteins were new and unique to membranes from hardened tissue (Uemera & Yoshida, 1984). Yoshida (1984) and Yoshida & Uemera (1984) demonstrated increased levels of high-molecular-weight glycoproteins in plasma membranes of mulberry bark cells (*Morus bombycis* Koidz cv Goroji) and Orchard grass (*Dactylis glomerata L*). In another membrane system, only a single M_r 20,000 protein was accumulated during CA in the endoplasmic reticulum of oil seed rape (*Brassica napus*) (Johnson-Flanagan & Singh, 1987; cited in Guy, 1990). Volger, Heber and Berzborn (1978) located cold induced, cryoprotective proteins that may reside in the chloroplast of spinach. A study on polysomes from CA and NA Rye, showed that CA tissues contained an acidic M_r 140,000 protein. This protein was not found to be expressed in NA tissue (Guy, 1990). In spinach nuclei, higher-molecular-weight proteins predominate at LT, while lower-molecular-weight proteins predominate at warmer temperatures (Guy & Haskell, 1989).

Since the review by Guy (1990) there has been significant advances in the molecular characterisation of expressed novel proteins induced by CA. Dure (1993) and Dure, Crouch, Harada, Ho and Mundy (1989) identified a family of proteins, in cotton, that they classified as "late embryogenesis abundant" (LEA) proteins. Of particular interest is a subgroup of these proteins known as the LEA D-11 family, commonly referred to as dehydrins. Dehydrins are characterised by conserved amino acid motifs, including DEYGNP, and a lysine- rich block (KIKEKLPG) often present in two or more copies (Close, Fenton, Yang, Asghar, De Mason, Crone, Meyer, and Morgan, 1993a; Close, Fenton and Morgan 193b, and Dure, 1993).

Another interesting family of stress-related proteins is the HS70 family of heat-shock proteins (Vierling, 1991). HS70s have been reported by Anderson, Li, Haskell, and Guy, 1994 a; Anderson, Haskell and Guy, 1994b) as being differentially expressed at the gene level, in spinach, during CA, heat-shock and water stress. Heat-shock proteins are an evolutionary conserved family of 70-kDa proteins that belong to a class of proteins known as molecular chaperons (Anderson *et al*, 1994a) which interact transiently with a wide variety of other peptides. The HS70 family has been divided into two groups depending on whether they are heat-shock inducible. Those that are strictly heat-shock inducible are referred to as HSP70s, and those that are constitutive and not strongly heat-shock inducible are known as heat-shock cognates (HSC70) (Wisniewski, Close, Artlip & Arora 1996).

There were three genes reported by Anderson *et al* (1994 a,b) as being expressed by different stress factors: an ER-luminal HSC70 (BiP, endoplasmic reticulum binding protein), a cytosolic HSC70, and a nonconstitutively expressed HS70. It is suggested by the authors, that, since HS70s are considered to be involved in molecular chaperoning and folding of proteins, they may have important roles in the maintenance of cellular homeostasis and proper protein function during CA and possibly in subsequent freeze dehydration stress.

Wisniewski *et al* (1996) studied the seasonal expression of both dehydrins and HS70s in the bark tissue of eight woody species of plants. The species included in the study were peach (*Prunus persica* cv. Loring) apple (*Malus domestica* cv. Golden Delicious), thornless blackberry (*Rubus sp.* cv Chester), hybrid popular (*Popular nigra*), weeping willow (*Salix babylonica*), flowering dogwood (*Cornus florida*) sassafras (*Sassafras albidum*) and black locust (*Robinia pseudo-acacia*). Among these there was a wide array of proteins that were immunologically related to dehydrins. Although some of these proteins appeared to be constitutive in several species (e.g. poplar, flowering dogwood and black locust), all eight species appeared to express dehydrins with a distinct seasonal pattern. In general, certain dehydrins slowly accumulated during the autumn, 10 reached a maximum in December-February and then slowly declined in March-July and relatively few were observed in May-July (Wisniewski *et al,* 1996). This general pattern of dehydrin expression correlated to seasonal hardiness of the woody species involved (Wisniewski *et al,* 1996).

The expression of the HS70 family of heat-shock proteins, induced by a treatment of CA in the eight woody species outlined above was also observed by Wisniewski *et al,* (1996). Using the commercially available monoclonal antibody, produced by using purified HSP70 from bovine brain as an immunogen, Wisniewski *et al* (1996) observed a 70-kDa protein in only four of the eight species examined: willow, peach, hybrid poplar and Sassafras. A seasonal pattern of protein expression was observed, for peach and willow with the highest level of accumulation in December-May, in sassafras significant levels were present only in December. In three of the species (willow, peach and hybrid popular) levels appeared to be expressed constitutively, although in some months levels were quite low (Wisniewski *et al* 1996).

Using a HSC70 monoclonal antibody Wisniewski *et al* (1996) revealed a diversity in the pattern of expression for this protein among the eight species examined. In flowering dogwood and blackberry HSC70 is expressed constitutively but there is a seasonal variation in the levels of HSC70, with the highest levels being detected in the Autumn and Winter months. In the other species the protein was expressed constitutively with the

levels in each species being relatively constant or irregular. It was also noted that for some species (e.g. flowering dogwood, apple and peach) there were distinct isoforms of the protein expressed during certain months.

The BiP monoclonal antibody directed against the 70-kDa endoplasmic reticulum luminal HSC70 protein, revealed a series of expression patterns for this protein that were similar to the HSC70 protein. This protein was constitutively expressed in all eight of the woody species examined, with a seasonal peak in Autumn and Winter for flowering dogwood, willow and blackberry (Wisniewski *et al,* 1996).

Although dehydrins are typically associated with induction by stress it has been noted in another study (Robertson and Chandler, 1994) that a dehydrin in pea (*Pisum sativum*) was also constitutively expressed. Whether or not the dehydrins identified by Wisniewski and colleagues and that identified by Robertson and Chandler are up-regulated in response to reduced photoperiod and/or low temperature remains to be determined. As with the dehydrins it was also noted that several heat-shock proteins were expressed constitutively, and of these some were detected at higher levels in the Autumn and Winter months. Anderson *et al* (1994a) indicated that although the genes coding for HSC70 and BiP were cold inducible, corresponding levels of the actual protein remained relatively constant. They hypothesised that increased levels of mRNA were needed to maintain a constant level of these proteins as greater amounts of mRNA were used to maintain proper protein biogenesis at low temperatures.

Cellular dehydration is a common element between freezing tolerance and water stress, and provides a basis for a universal physiological response to these stress factors. It has been noted by many workers that increased levels of abscisic acid (ABA) are commonly observed in plants that are subjected to both low temperatures and to water stress (Chen & Gusta 1983; Johnson-Flanagan, Huiwen Thiagarajah & Saini, 1991). There are several lines of evidence that support the theory that ABA is involved in plant adaptations to environmental stress: (a) endogenous ABA levels have been shown to increase during both exposure to low temperatures (Daie & Campbell, 1981; Chen & Gusta, 1983; Chen,

Li & Brenner, 1983; Lalk & Dorffling, 1985 and Lang, Mantyla, Welin, Sundberg & Palva, 1994) and to drought (Guerrero & Mullet, 1986; Bray, 1988; Lang *et al,* 1994); (b) plants develop freezing tolerance when treated with ABA under NA conditions (Chen & Gusta, 1983); (c) an ABA-deficient mutant (*aba-1*) (Koorneef, Jorna, Brinkhorst-van der Swan & Karssen, 1982) was shown to be impaired in CA and the defect could be complemented by the addition of exogenous ABA (Heino, Sandman, Lang, Nordin & Palva, 1990); (d) several of the proteins induced by low temperature and drought are induced by exogenous applications of ABA (Cattivelli & Bartels, 1992; Palva, 1994).

Initial evidence for ABA-regulated gene expression frequently comes from corresponding changes in endogenous ABA levels and in the levels of a particular gene product; this generally occurs after an imposition of a stress such as dehydration. Lalk and Dorffling (1985) observed increased levels of endogenous ABA in several plant species during a CA regime and further evidence to support this observation comes from Lang *et al* (1994). They observed a 3-fold increase in endogenous ABA levels during an acclimating regime of ($4^{o}C/2^{o}C$). This increase is seen in both soil grown and axenically grown plants (Hughes & Dunn, 1996).

Work on two wild types of *Arabidopsis thaliana* from two different ecosystems (LE originating from Poland and CO-1 originating from Portugal) and two ABA mutants in the LE background, an ABA-insensitive mutant (*abi1*) and an ABA-deficient mutant (*aba-1*) (Koorneef *et al,* 1982) demonstrated the accumulation of low temperature induced (LTI) proteins and these proteins were correlated to the presence or absence of ABA (Mantyla, Lang & Palva, 1995).

Workers observed the accumulation of a LTI protein (RAB18) for which the genes had been previously characterised and their expression analysed at the mRNA level (Nordin, Heino & Palva 1991; Lang and Palva 1992). The regulation of the *rab18* gene has been shown to be ABA mediated in vegetative tissue, with transcripts abundantly accumulating in wild-type plants during ABA treatment and drought stress (Lang & Palva, 1992; Lang, Mantyla, Welin, Sundberg & Palva, 1994). Exposure of LE and CO-1 plants to low

temperature resulted in a weak but detectable expression of RAB18. Conversely both the ABA mutants were defective in the RAB18 induction (Mantyla *et al* 1995). In the *abi1* mutant accumulation of this protein was delayed and only showed a weak increase in concentration above the background levels after 5 and 7 days of treatment. In the ABA-deficient mutant (*aba-1*) no RAB18 was detected at all during the low temperature treatment (Mantyla *et al* 1995).

Applications of exogenous ABA resulted in a massive accumulation of the RAB 18 protein in both ecotypes LE and CO-1, as well as in the *aba-1* mutant. As expected the abi1 mutant did not respond significantly to exogenous ABA. Some accumulation of the RAB18 protein was seen after the third day indicating the leaky nature of this mutation to prolonged exposure to high levels of ABA (Mantyla *et al,* 1995).

To illustrate the inter-relationships between low temperature stress, water stress (drought) and ABA Mantyla *et al* (1995) subjected the four different types of *A.thaliana* to a progressive drought stress (70% RH). They showed that the RAB18 protein accumulated to high levels in both ecotypes, LE and CO-1. No trace of the protein could be detected in the aba-1 mutant, whereas *abi1* showed a weak signal for the RAB18 protein. These authors concluded that the accumulation of the RAB18 protein is mediated by ABA and correlated the accumulation of the RAB18 protein in *A.thaliana* with increased freezing tolerance (by scoring the extent of irreversible damage to the rosette leaves after sub-zero temperature treatments) after a visual inspection two days after the freezing event. Freezing tolerance was calculated as the LT_{50}, (Table 1.1).

Table 1.1: Freezing tolerance of *A.thaliana* plants grown in vitro compared with RAB18 protein levels at normal growth temperatures (NGT) and during drought (D), low temperature (LT) and ABA treatments. Where - is no protein detected and +++ is maximum protein accumulation. (Adapted from Mantyla, Lang and Palva, 1995).

Treatment	D		LT		ABA		NGT	
Mutant/Ecotype	RAB18	FT	RAB 18	FT	RAB18	FT	RAB18	FT
LE	++	-7.0	+	-7.4	++	-6.5	-	-2.9
abi1	+-	-3.5	+-	-4.1	+-	-3.0	-	-3.0
aba-1	-	-3.0	-	-3.8	+++	-7.5	-	-3.0
CO-1	+	-6.0	+	-6.3	+++	-7.0	-	-2.5

Low Temperature Responsive Genes (LTR)

The biochemical and physiological changes observed in plants during cold-acclimation (CA) have been studied at the gene level and there is a plethora of reports on the genetic analysis of frost resistance. This ponderous body of information is far too detailed to discuss in any depth and therefore this review will consider gene expression of just two plant species, *Arabidopsis thaliana* and *Hordeum vulgare,* for reviews see (Pearce, 1999 and Hughes and Dunn, 1996).

In both *A. thaliana* and *H. vulgare* some of the genes identified and studied are members of small multigene families (Hughes *et al*, 1996) and are responsive to cold, drought, ABA and in the case of *H. vulgare,* pathogens. There are 10 genes isolated from *A. thaliana* and 6 from *H. vulgare* (Table 1.2) of significance.

Table1.2: Low temperature genes for *A. thaliana* and *H. vulgare* and their associated family members and their response to drought and ABA stress. (Adapted from Hughes *et al*, 1996)

Species	Gene	Family Members	Stress Induction
A. thaliana	*cor* 6.6 (kin 1)	*kin* 2	Drought, ABA
	cor 78	*lti* 78, *lti* 65, *rd* 29a, *rd* 29b	Drought, ABA, (Salt)
	cor 15A	*cor* 15B	Drought, (ABA)
	cor 47	*lti* 45	Drought, ABA
	lti 30	-	Drought, ABA
	lti 140	-	Drought, ABA
	rab 18	-	Drought, ABA
	C*cr* 1	C*cr* 2	(Drought)
	RCI 1	RCI 2	-
	Adh	-	Drought, hypoxia
H. vulgare			
	blt 14	*blt* 14.2, *blt* 14.1 *A* 086	-
	blt 4	*blt* 4.2, *blt* 4.6, *blt* 4.9	Drought, ABA -
	blt 101	*blt* 101.2	-
	blt 63	-	NK
	HVA 1	-	ABA
	blt 801	-	ABA

Adapted from Hughes *et al* 1996. Stress response in ( ) indicates response to other family members. NK = not known.

In *A. thaliana cor* 6.6 (*kin* 1) and *kin* 2 are linked in tandem but are expressed at different levels in response to low temperature (LT) and also differ in their response to drought (Kurkela and Borg-Franck, 1992). The *cor* 15A and *cor* 15B from *A. thaliana* are also linked in tandem with *cor* 15A being responsive to both drought and LT and *cor* 15B only responding to LT (Lin and Thomashow, 1992; Wilhelm and Thomashow, 1993). The

interrelationship between gene expression LT, drought and ABA for six *A. thaliana* genes is illustrated in (Table 1.3). All of the genes are responsive to ABA, drought and LT but there is variation in the level of expression of these genes with treatment. With the exception of *rab* 18 (whose response to LT is weak) (Hughes *et al*, 1996), all of these genes are upregulated by LT in all the three genotypes studied; wild type (WT), ABA-null mutant (aba-1) and ABA insensitive mutant (abi-1). However, the ABA response of these 3 genotypes differs; in the WT and aba-1 mutant gene response to ABA is similar but in the abi-1 mutant ABA did not induce a response. This suggest that genes are upregulated independently by LT and ABA (Hughes *et al*, 1996).

Table 1.3: Response of *A. thaliana* low temperature responsive genes to low temperature (LT), drought (D) and ABA in wild type (WT), ABA-null mutant (aba-1) and the ABA insensitive mutant (abi-1).

Gene	LT			D			ABA		
	WT	aba-1	abi-1	WT	aba-1	abi-1	WT	aba-1	Abi-1
lti 140	+++	+++	+++	+++	NK	++	+++	++	0
lti 30	+++	+++	+++	++	+	++	++	+++	0
lti 45	++	++	+++	+	0	0	+	++	0
lti 78	+++	+++	+++	+	0	+	+	+	+
rab 18	+	0	+/-	+++	0	+/-	+++	+++	+/-
adh	+++	++	++	+	0	0	++	+	0
LT50	-7.4°C	-3.8°C	-5°C	-7°C	-3°C	-3.5°C	-6.5°C	-7.5°C	-3°C

After Hughes *et al* (1996).

This level of complexity is also seen in LT induced genes in *H. vulgare*. The *blt* 4 gene has four family members, *blt* 4.1, *blt* 4.2 and *blt* 4.9 which are responsive to LT, drought and ABA but the relative response to these factors varies with each gene. In *blt* 4.9 induction is strongly related to LT but only slightly to drought, whereas *blt* 4.1 is equally responsive to LT and drought, *blt* 4.2 is only expressed in seedlings (White, Dunn, Brown and Hughes, 1994). In the *blt* 101 there are only two family members and the level of expression of *blt* 101 is ten times that of *blt* 101.2.

The *blt* 4 gene in *H. vulgare* has been predicted to encode a group of non-specific lipid transfer proteins (White *et al*, 1994). The lipid transfer nature of *blt* 4 is also observed in several other *H. vulgare* genes known as lipid transfer protein (LTP) genes (Hughes *et al*, 1996). There are two specific LTP genes, *ltp* 1 and *ltp* 3, and two cognates *ltp* 2 and *ltp* 4 of the *blt* 4 LTP genes. The gene structure of *ltp* 1 and *ltp* 3 distinguishes these genes from *blt* 4 genes. In *ltp* 1 and *ltp* 3 there is an intron in the gene and this leads to the insertion of nine bases in the mRNA coding sequence, which leads to the addition of 3 amino acids at residue 85 in the polypeptide (Hughes *et al,* 1996). The response of these genes to LT, drought, ABA and pathogens is complex; the control of *ltp* 1 is unknown (Table 4), *ltp* 3 responds to pathogens and ABA, while the *blt* 4 gene family all respond to LT, drought, pathogens and ABA, but there is variation in gene response to these stimuli across the *blt* 4 family (Table 1.4) (Hughes *et al,* 1996).

Table 1.4: Characteristics of *H. vulgare* lipid transfer genes.

Gene	Introns	Addition of 3 amino acids	Response LT	D	P	ABA
ltp1	++	++	NK	NK	NK	NK
ltp 3	++	++	-	NK	++	++
blt 4.1 (ltp 2)	NK	-	++	++	++	++
blt 4.2 (ltp 4)	-	-	++	NK	++	++
blt 4.6	-	-	++	++	NK	NK
blt 4.9	-	-	++	++	++	++

++ = present and/or response, - = absent and/or no response and NK = not known. Adapted from Hughes *et al.* (1996).

The location of these genes is tissue specific with *ltp* 1 being isolated from the aleurone layer during germination (Mundy and Rogers, 1986), the location of *ltp* 3 is unknown, *blt* 4.1 is located in the leaf, *blt* 4.2 in the coleoptile and *blt* 4.9 in the shoot meristem but the location of *blt* 4.6 is unknown (Hughes *et al,* 1996).

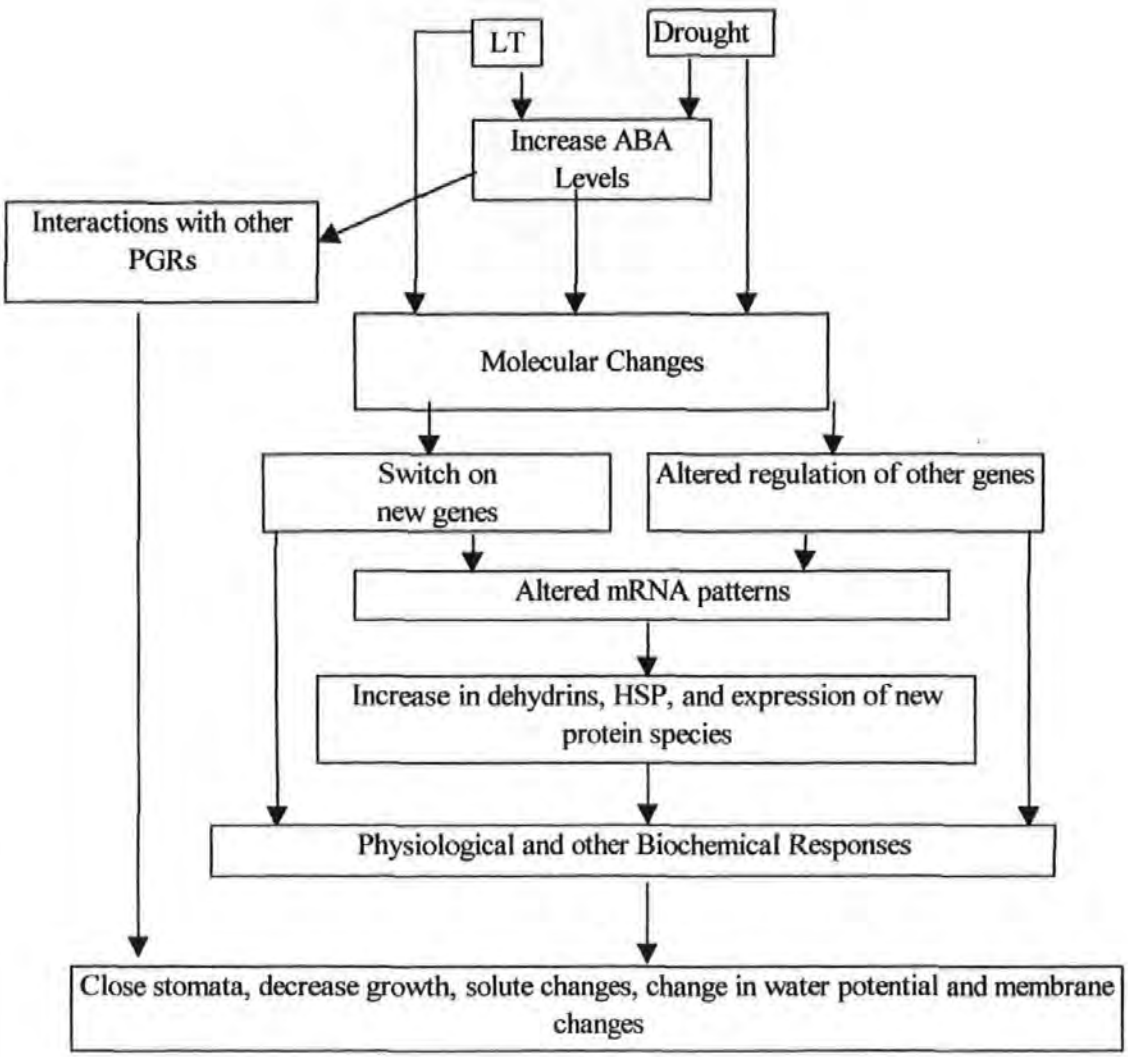
There is debate about whether *H. vulgare blt* 4 genes have LTP function because all the *blt* 4 gene family possess a consensus N-terminal signal sequence for extracellular transport (White *et al*, 1994) and this may not be consistent with lipid transfer function related to

changes in membrane lipids during cold-acclimation. Lipid transfer proteins have been defined by their ability to transfer lipids between membranes *in vitro* (Hughes *et al*, 1996). An extracellular wax associated LTP has been isolated from broccoli (*Brassica oleracea*) leaves and is a member of a LTP gene family expressed in leaf epidermal cells (Pyee, Yu and Kolattukudy, 1994; Pyee and Kolattukudy, 1995 cited in Hughes *et al*, 1996) and it is suggested that these LTP may be involved in wax synthesis and /or secretion. The *blt* 4.9 gene has been isolated in epidermal cells of leaf sheaths and may have a role in leaf surface biochemistry (Hughes *et al*, 1996).

In a study of the low temperature responsive genes (LTR) in *H. vulgare* (Pearce, Dunn, Rixon, Harrison and Hughes, 1996) have illustrated that the level of gene expression for three LTR genes (*blt* 4.9, 14 and 101), isolated from crown tissue, increased during cold - acclimation (CA) and that this leads to increased frost resistance (LT_{50} regrowth test).

It is apparent that the molecular, biochemical and physiological response of hardy plants to CA is complicated and involves the interaction of multiple genes (a polygenic trait) with several external stimuli ranging from reduced photoperoid, seasonal reduction in air temperature, water deficit, salt stress and pathogens. These external stimuli initiate complex changes in the balance of plant growth regulators, which may be involved in genetic signalling, leading to modifications in plant water status, solute accumulation, membrane changes and altered protein metabolism. Figure 1.5 attempts to summarise the effects in a diagrammatic manner. The final outcome of these changes is reduced plant growth but conversely there is a significant increase in frost resistance and thus the ability to survive winter frost and continued survival for future reproduction.

Figure 1.5: Flow diagram illustrating components of the cold-acclimation process.



1.10 Frost Protection Strategies

There are few strategies growers can adopt in the fight against frost damage, these include 1) escape, 2) reduction of heat loss, 3) heat input, 4) the manipulation of ice+ bacteria by the addition of competitive bacteria, 5) selection of frost resistance and 6) genetic engineering.

Escaping spring frost is possible for annual spring sown crop species but in locations with a short growing season this method may lead to early autumn frost damage. The flowering of perennial crops can be delayed by overhead misting where evaporative cooling delays the onset of bud break by as much as two weeks (Janick, 1979) but this may lead to reduced fruit set. In the fruit industry escaping spring frost by choosing the right location and site is still a viable strategy. Reduction of heat loss can be achieved by the use of plastic tunnels, an option adopted by soft fruit growers. Wind machines are used to protect citrus crops by mixing the air layers (Janick, 1979) and heat can be directly added to an orchard by solid petroleum wax heaters placed in strategic locations. The manipulation of ice+ bacteria is a method which is in its infancy (Lindow 1995). In 1992 the United States licensed three strains (*Pseudomonas fluorescens* strain A506, one naturally occuring strain *P.syringae* and one of *P.fluorescens*) (Lindow, 1995). As these bacteria are considered to have pesticide action the cost of registration ($150,000) is prohibiting future development. The breeding and selection of frost resistant crops is ongoing but the release of new varieties and cultivars is the result of lengthy breeding programmes. This may be accelerated in future by genetic engineering but recent public resistance to genetically modified crops suggest that this route will meet considerable obstacles before full commercial acceptance.

All of the above frost protection strategies have commercial limitations and therefore necessitate the development of a commercially viable product which can be used on field grown crops to improve the survival of crop plants following freezing. Liquid seaweed extract has been commercially used as a plant bio-stimulant for several decades and requires no pesticide registration and therefore requires evaluation for its potential use as a frost protectant.

1.11 Biological Effects of Seaweed Extracts

Many claims have been made about the beneficial effects of seaweed extracts (SE) on plant productivity including the amelioration of biotic and abiotic stresses. At times these claims appear mystical and flavour the observed phenomena with a 'muck and magic' image. Since the mid to late 1960's there has been substantial investigation into the SE effect. Much of this has been industry led but scientific papers have reported several biological effects of SE's.

Increased Nutrient Uptake

Commercial field trials on potatoes (Booth, 1966) reported that potato plants treated with SE had a 20% yield increase compared to untreated controls. Mineral analysis of the foliage showed that SE treated plants had 7% more nitrogen, 6% more phosphorus, 11% more potassium, 16% more calcium, 28% more magnesium and 12% more iron compared to control plants.

Increased Resistance to Fungal Disease

Senn, Martin, Crawford and Darting (1961; cited in Abetz, 1980) observed that mildew infestations on melon was considerably less on SE treated plants compared to controls. Similar results were reported by Booth (1966) with turnips and Stephenson (1966; cited in Abetz, 1980) reported that the leaf area of turnips infected with mildew was 15% in SE treated plants compared to 85% in the controls. The treated plants also had a statistically significant increase in root yield. Also the infection of strawberries with *Botrytis* has been reduced on SE treated plants compared to controls. Weekly overhead irrigation of plants with a 1:120 dilution of SE reduced the incidence of fruit infection to 4.6% in SE treated plants compared to 22.5% in untreated plants.

Reduced Pest Attack

Field observations by hop growers in the United Kingdom (Abetz, 1980) report that tank mixes of SE and insecticides were more efficient in controlling aphid infestations compared to insecticides alone and this has been backed up by scientific research.

(1966) reported that aphids on SE treated sugar beet leaves were unsettled and would migrate to untreated controls. The mean percentage distribution was: treated 20%, untreated 83%. Stephenson also reported that SE treated apple trees resulted in reduced red spider mite fecundity.

Increased Yield

Blunden and Wildgoose (1977) found that foliar applications of SE to King Edward and Pentland Dell potatoes 6 weeks after emergence gave a significant yield increase in the King Edward but not in the Pentland Dell. These workers suggested that the increased yield observed in SE treated plants may be due to the cytokinin content of seaweed extracts. Applications of an equivalent amount of the plant growth hormone kinetin to these varieties gave a significant yield increase in the King Edwards but not in the Pentland Dell. The increase in yield may well be an expression of the other effects observed such as reduced disease and pest pressure and/or the effect of soil nutrient status and additional mineral supplement. Goh (1971) reported that applications of SE to white clover did not significantly increase the dry weight of plants (Abetz, 1980). A criticism of Goh is that he used the SE as a complete fertiliser which is in contradiction to manufacture recommendations. The soils Goh used were low in nitrogen, phosphorus, potassium and one was significantly contaminated by a pesticide (Abetz, 1980).

1.12 Biologically Active Compounds in Seaweed Extracts

Mineral Composition of Seaweed Extracts

The mineral analysis published by two manufacturers is presented in Table 1.5 and highlights that if SE's are used at recommended rates the total amount of plant nutrients applied per hectare in a season is minimal (Abetz, 1980). This observation also holds for the micro-nutrients and Blunden (1977; cited in Abetz, 1980) concluded that seaweed extracts could not supply any significant proportion of the annual requirements of micro-nutrients to crops. Therefore the observed effects of SE on plant growth and pest resistance must be due to other compounds (probably organic) present in the seaweed extract.

Table 1.5: Mineral analysis of two commercially available seaweed extracts (after Abetz, 1980.)

Mineral Element	Maxicrop Concentrate g l^{-1}	Seaol Liquid
Nitrogen	3.5	1.80%
Phosphorus	1.2	0.18%
Potassium	5.5	2.55%
Calcium	0.5	0.20%
Magnesium	1.4	0.16%
Sulphur	1.4	0.14%
Iron	1.1	24ppm
Iodine	2.5	-
Boron	0.011	0.5ppm
Copper	0.05	54ppm
Cobalt	0.014	-
Manganese	0.044	3ppm
Molybdenum	0.014	3ppm
Zinc	0.180	15ppm
Sodium	-	480ppm
Chloride	-	0.67ppm

1.10.2 Plant Growth Regulating Substances

Kingman and Moore (1982) used gas liquid chromatography to identify PGRs in *Ascophyllum nodosum* a common constituent seaweed species in SE's. Comparing sample peaks with known reference material they were able to detect 3 different PGRs; adenine (a cytokinin) abcisic acid (ABA) and indole-3- acetic acid (IAA) in dried samples of *A. nodosum*. The concentration of these PGRs was also estimated and are reproduced in (Table 1.6). IAA activity in SE was later confirmed by Stirk and Van Staden (1997).

Cytokinin levels in commercially available SE were determined by Blunden and Wildgoose (1981) using a leaf bioassay and kinetin as the reference cytokinin. Highly significant cytokinin like activity was detected in 3 commercial SE with activity equivalent to 10mg -

250mg/kinetin l^{-1}. However there was considerable variation between the samples and within each product (Table 1.7).

Table 1.6: Concentrations of PGRs characterised by Kingman *et al* (1982).

Compound	Concentration (g/15 ml H_2O)
Adenine	0.03
ABA	0.02
IAA	0.05

Table 1.7: Concentration and variability in cytokinin levels in 3 commercially available seaweed extracts (after Blunden and Wildgoose, 1981)

Product	Variation*
Marinure	10-50mg l^{-1}
Maxicrop	25-200mg l^{-1}
S.M.3.	15-250mg l^{-1}

*Based on two batches of each product

Since this early work on cytokinin activity in SE further studies have identified some of the cytokinin species involved as *trans*-zeatin (tZ), *trans*-zeatin riboside (tZR), dihydrozeatin (DHZ), dihydrozeatin riboside, zeatin-O-glucoside (ZOG) and dehrozeatin-O-glucoside (DHZROG) (Stirk and Van Staden, 1997). As in Blunden and Wildgoose (1981) there was considerable variation in the reported concentrations of these cytokinins (Stirk *et al*, 1997) and Stirk *et al* (1997) note that caution must be exercised in interpreting the results of bioassays. Structurally different compounds respond differently in various bioassays with some being less sensitive to different compounds than others. Also various compounds show different levels of activity e.g. zeatin is the most active naturally occurring cytokinin

in higher plants (Letham, 1978). Thus bioassay activity will vary according to the nature of the compounds present in the SE and the type of bioassay used. Another consideration when comparing biological activity associated with SE is the time of harvest. It is documented that cytokinin levels vary according to season (Featonby-Smith and Van Staden, 1984) and ideally comparisons should be made on SE made from seaweed harvested during the same growing phase and season.

Betaines

The levels of PGRs reported in SE are not sufficiently high to produce all the claimed beneficial effects of the extracts (Nelson and Van Staden, 1985 and Tay, MacLeod, Palni and Letham, 1985). Blunden, Rogers and Barwell (1984; cited in Blunden *et al*, 1986) found major discrepancies in cytokinin activity of SE and concluded that other compounds must be present that have cytokinin like activity. Wheeler (1973; cited in Blunden *et al*, 1986) demonstrated that glycinebetaines have cytokinin like activity. Blunden *et al* (1986) has characterised 3 betaines from 4 commercially available SE and quantified the concentration of these species with ^{1}H NMR spectroscopy (Table 1.8).

Glycinebetaines and other betaines have been shown to be components in cytoplasmic osmotica in some higher plants adapted to salt and water stress. Glycinebetaine has been reported to be involved in frost resistance (Blunden *et al*, 1986). Glycinebetaine when incorporated into agar media enables the growth of a bacterium *Klebsiella pneumoniae* O1 in the presence of growth inhibitory levels of sodium chloride (Le Rudulier, Bernard, Goas and Hamelin, 1984). The suppression of nitrogen fixation in *K. pneumoniae* caused by salt stress was elleviated by the incorporation of glycinebetaine and γ-aminobutyric acid betaine (Le Rudulier *et al*, 1984).

The ability of SE to improve plant health and alleviate stress must be linked to the biologically active compounds described. In the case of frost resistance, however, there is a dichotomy. Tender plants that cannot withstand freezing do not benefit from applications

Table 1.8: Characterisation and quantitative estimation of betaine content in 4 commercially available seaweed extracts.

Seaweed Extract	Betaine Content mg l^{-1}		
	Glycinebetaine	γ-aminobutyric acid betaine	δ-aminovaleric acid betaine
S.M.3.			
Batch 1	35.9	21.3	9.6
2	18.2	19.9	11.6
3	21.0	19.2	10.3
4	19.0	21.3	10.2
5	25.3	21.0	8.7
6	31.6	24.9	9.9
Seamac			
Batch 1	6.8	14.3	7.3
2	2.3	7.9	5.9
Maxicrop			
Batch 1	trace	5.4	7.3
2	7.8	11.0	3.7
Alginex			
Batch 1	15.7	19.9	9.8
2	14.1	25.2	10.1
3	20.9	27.2	10.3

of SE compared to hardy plants. Saunders (1997) illustrated no cryoprotective effect of SE on frost susceptible potato (*Solanum tuberosum*) but could demonstrate increased frost tolerance in two hardy species winter barley (*Hordeum vulgare*) and winter cauliflower (*Brassica oleracea var botrytis*). This observation suggests that one possible mechanism of action of SE in the alleviation of abiotic stress is the mediation of inherent hardiness. A schematic representation of the possible role of action of SE in the manipulation of frost resistance is outlined in figure 1.6

Aims and Objectives of Study

The overall aim of this study was to elucidate the mechanism of action of liquid seaweed extract (LSE) in the manipulation of frost resistance in winter barley. In order to achieve this a null hypothesis was developed:

HO: Exogenous applications of liquid seaweed extract have no beneficial effect on the frost resistance of winter barley.

HI: Exogenous applications of liquid seaweed extract have a beneficial effect on the frost resistance of winter barley and this effect may be mediating natural cold-acclimating mechanisms (Figure 1.6).

A series of aims and objectives were designed to investigate the above null hypothesis:

Aim 1) (Chapter 2) Does liquid seaweed extract affect the frost resistance, growth and development of plants ?.

Objectives:

a) To establish the effect of LSE on the frost resistance of three crop species.

b) To establish if LSE increases the frost resistance of both non-acclimated and cold-acclimated plants.

c) To evaluate the effect of single and multiple LSE treatments in the manipulation of frost resistance.

d) To investigate if LSE mediated frost resistance is stable in a field situation.

e) To establish if single or multiple LSE treatments increase tiller production and dry weight gain.

Aim 2) (Chapter 3) What is the influence of cold-acclimation and LSE treatments on soluble protein expression in winter barley ?.

Objectives:

f) To establish whether cold-acclimation affects the soluble protein content in winter barley.

g) To investigate the effect of single and multiple applications of LSE on the soluble protein expression in winter barley.

h) To investigate the similarity of cold-acclimation and LSE treatments in bringing about identical changes in soluble protein expression in winter barley.

Aim 3) (Chapter 4) Does cold-acclimation and LSE affect the water relations of winter barley ?.

Objectives:

i) To establish if cold-acclimation has any effect on plant water potential.

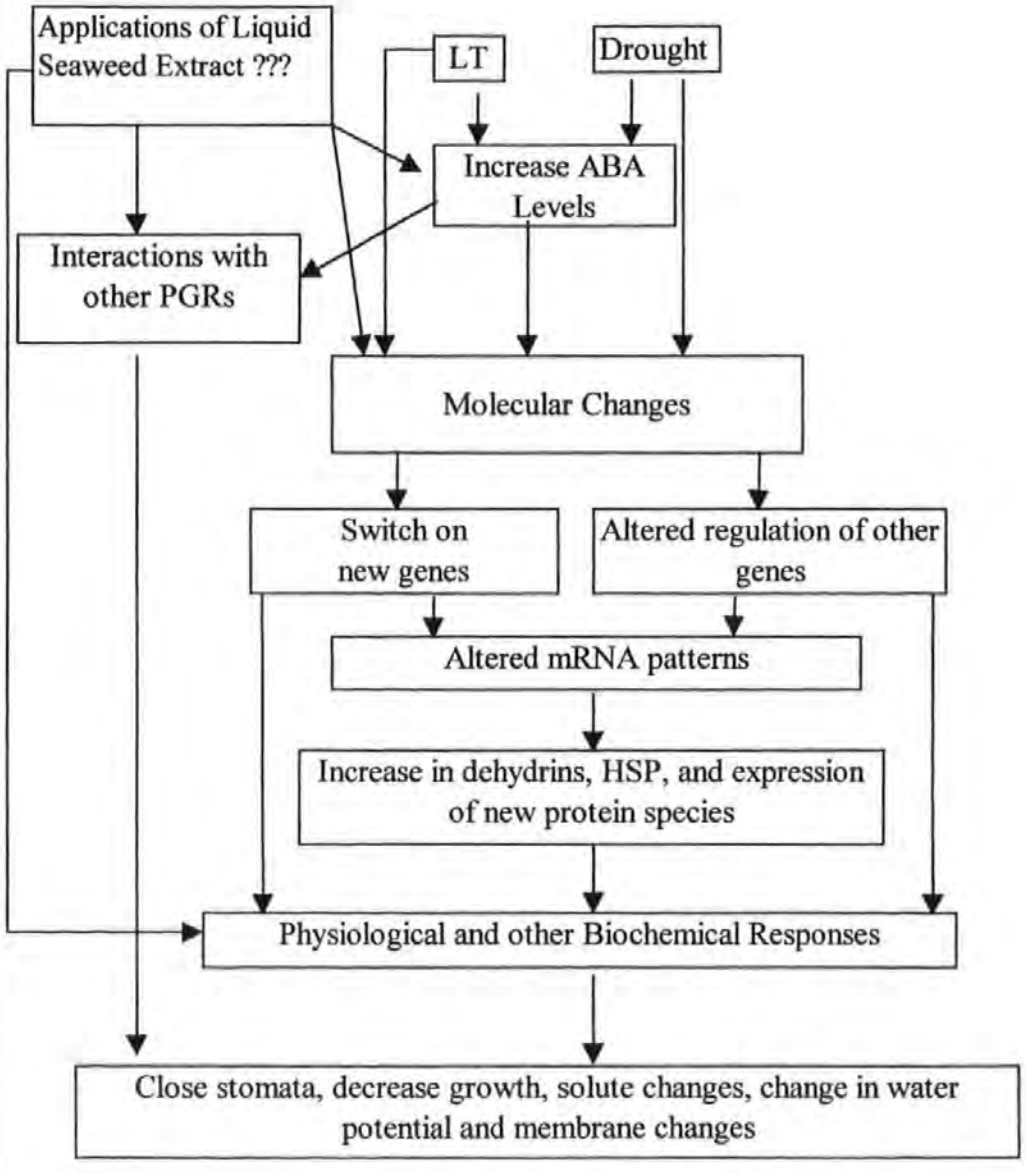
j) To establish if single or multiple applications of LSE affect plant water potential.

k) To establish whether there are any effects on plant water content following cold-acclimation and LSE treatments.

l) To investigate if old-acclimation and LSE treatments decrease the amount of freezable water in leaf and crown tissue of winter barley.

m) To investigate whether the rate of water release from cells to the extracellular ice crystal mediated by cold-acclimation and LSE treatments.

Figure 1.6: Flow diagram illustrating possible interactions between liquid seaweed extract and the cold-acclimation process.



Chapter 2: Frost, growth and development assays

Chapter 2: Frost, growth and development assays

2.0 Introduction

2.0.1 Rationale

The ability of liquid seaweed extract (LSE) to enhance frost resistance of plants is documented for tomatoes (Abetz, 1980), and anecdotal field observation by growers suggest that applications of LSE to Top Fruit crops (apples and pears) has a beneficial effect on field survival and final yields, Dan Neuteboom (personal communication). A recent study on the ability of LSE to enhance frost resistance of crop plants found that only plants that can cold acclimate respond to foliar applications of LSE, e.g. winter barley (*Hordeum vulgare*) and winter cauliflower (*Brassica oleracea var botrytis*) Saunders (1997). Before any biochemical and/or physiological studies can take place it is necessary to confirm the reports of Saunders (1997).

The aim of this series of experiments is to establish whether exogenous applications of liquid seaweed extract can improve the frost resistance of winter crop species.

2.0.2 Measurement of Freezing Tolerance

There are several techniques used to evaluate freezing injury in plants such as regrowth (Palta, Levitt and Stabelmann, 1977a; Levitt, 1980), visual estimates of discoloration/browning (Kraut, Walsh and Ashworth, 1986), vital staining (Stadelmann and Kinzel, 1972), protoplasmic streaming and the ability of the cell to plasmolyse (Arora and Palta, 1988; Palta, Levitt and Stadelmann, 1977), triphenyl tetrazolium chloride reduction (TTC) (Levitt, 1980) leakage of amino acids (Pomeroy, Simimovitch and Wightman, 1970), differential thermal analysis (Quamme, Stushnoff and Weiser, 1972) and electrolyte leakage (Levitt, 1980).

Electrolyte leakage is the basis of the electrical conductivity test developed by Dexter, Tottingham and Garber (1937) and is commonly used in conjunction with observations of the degree of browning/water-soaking of tissues. The test is based on the assumption that where plants sustain more injury there will be greater efflux of solutes from plant tissues (ions, sugars and proteins). Electrical conductivity of effusate is recorded after freeze-injured tissue is incubated and shaken in deionized water. Conductivity is then recorded

once more after the samples have been heat killed, in the same solution, this provides a measure of total ions present in the tissue. Freezing injury is calculated as a percentage of total ions. The advantage of this method is that it removes the element of subjectivity from visual scores and data can be collected immediately or a few hours after freezing, compared to visual scores which require several days of recovery.

There are, however, a few problems with the conductivity method; 1) a substantial amount of solutes leak out of unfrozen tissue when incubated in water, 2) sometimes not all the solutes leak out from heat-killed tissue, even when tissue is irreversibly injured due to freeze-thaw stress (Wisniewski and Arora, 1993). Zhang and Willison (1987) modified the conductivity method to overcome these problems, by using vacuum infiltration to exclude air which facilitates the filling of extracellular spaces with distilled deionised water and improves ion leakage. Fuller, Grout and Tapsell (1989) highlighted difficulties with interpretation of conductivity data. In field experiments with cauliflower a transient increase in conductivity was recorded, following some frosts, and was interpreted as damage but following thawing and recovery leaves did not show any damage. This observation implicates an important component of the physiology of freezing, that is the ability of injured tissues to recover following moderate stress (Arora and Palta, 1991; Palta, Levitt and Stadelmann, 1977a) and thus a true measure of frost hardiness is ultimately the ability of a plant to survive a freezing event and maintain or recover its metabolic activity (Wisniewski and Arora, 1993).

The visual scoring of plant damage (browning/water-soaking, percentage kill) is a standard assay procedure for assessing frost damage and can be used to estimate the 50% killing point (LT_{50}) of a species or variety, but like conductivity measures this system does not take into account the potential for regrowth. In winter annuals if estimates of injury are based on percentage killing of plant foliage this may show little relationship to subsequent yield, since a plant with all or nearly all its foliage killed may still yield well (Levitt, 1980). A good example is wheat, where crown survival, instead of foliage survival, is better correlated with field survival observations (Landi, 1974; cited in Levitt, 1980).

Another criticism of scoring assays is that there is no indication of the freezing point, but this can readily be obtained by determining the exotherm temperature using copper constantan thermocouples. This approach is not an indication of freezing tolerance *per se* but in the case of tissues or organs that owe their resistance to supercooling this technique gives a measure of freezing avoidance (Levitt, 1980).

Therefore experimental tests assessing low temperature should be designed to take recovery into account, but, as with any visual scoring scheme there is always an element of error, subjectivity and a question of repeatability. Scoring schemes have been employed by plant pathologists for many years and there has always been an element of subjectivity to disease assessment. The need for reliable assessment of disease severity in a crop is becoming increasingly more important, particularly where growers are using decision based systems before applying a chemical treatment (Parker, Whelan and Royle, 1995). This has given rise to several methods by which the reliability and the "Truth" of a scoring scheme can be reviewed. O'Brien and Van Bruggen (1992) (Cited in Nutter and Schultz, 1995) defined the "truth" to be disease estimates provided by the originator of the disease score and disease assessments provided by other raters compared with the "expert". In the context of a regrowth score a visual score for one cohort of plants may be provided by the originator and then compared to scores of other observers, this will give a measure of inter-observer variation. To evaluate intra-observer variation several data sets derived by a single observer (the originator) may be compared with the original estimate by the originator. The accuracy of the assessment can then be analysed using linear regression and analysis of variance on the coefficients of the regression lines (Parker, Whelan and Royle, 1995).

In order to establish true differences between treatments freezing assays must be conducted under standardised conditions and Levitt (1956) proposed five steps as basic requirements;

1) Plants must be inoculated (ice seeded or INA bacteria inoculated) to ensure freezing
2) Cooling must be at a standard rate
3) Sample freezing must be of a standard length of time
4) Thawing must be at a standard rate

5) Post-thawing conditions must be standardised

The scoring scheme's employed below, for the evaluation of frost damage in both the winter brassica species and the winter barley plants, were developed from earlier scoring schemes used by Saunders (1997). These earlier schemes were based on a five point scale of plant damage, however, after initial frost trials on both winter cauliflower and winter barley (data not shown) it was considered that these earlier schemes were not detailed enough to account for all the symptoms observed.

2.1.0 Experiment 1 Frost assays.

Materials and Methods

Plant Material and Culture Conditions:

Initial frost assays were carried out on 3 crop species, winter cauliflower (*Brassica oleracea* var botrytis cv Arcade an F1 hybrid), winter oil seed rape (*Brassica napus* cv Cobra) and winter barley (*Hordeum vulgare* cv Igri an old variety which is not longer NIAB listed but one in which previous frost resistance work and genome characterisation has been carried out (Pearce, Dunn, Rixon, Harrison and Hughes, 1996).

Seed was sown in 286 x $10cm^3$ module trays filled with a mix of moist multi-purpose peat based compost:John Innes No2 (60:40 by volume) and germinated on a heated mist bench. Non-acclimated (NA) plants were sown 10 days after plants destined for cold-acclimation in order to harmonise plants to the same physiological growth stage. After germination, plants were raised in a heated glasshouse with a 16 hour photoperiod, extended by supplementary sodium vapour lighting, with a PAR output of 145.25 μ mol/sec/m^2 with a standard deviation of 25 μmol/sec/m^2. Module trays were placed on sand filled trays onto capillary matting and base watered.

Treatments: Cold-acclimation was achieved by placing a proportion of young plants in a constant 4°C phytotron with a 9 hour photoperiod for 14 days. With a PAR level of 10 μ mol/sec/m^2. Liquid seaweed extract (LSE) treatments were applied to both NA and CA plants via a hand held mist sprayer at a concentration of $10ml/l^{-1}$ until run-off was observed (after Saunders, 1997). Plants were then frost tested 4 days post LSE treatment, full treatment structure for the 3 crop species was as follows;

a) Non-acclimated plants, control plants (C),

b) Non-acclimated plus 1 application of LSE (NA1LSE)

c) Cold-acclimated plants (CA)

d) Cold-acclimated plus 1 application of LSE (CA1LSE)

Frost Testing: All 3 crop species were subjected to a frost test in a glycol tank (Fuller, Grout and Tapsell, 1989) (Plate 2.1) in a completely randomised factorial design with 4

replicates of winter cauliflower, 3 replicates of winter oil seed rape and 5 replicates of winter barley. Plants were placed in 75ml boiling tubes complete with their plug of soil and roots. The freezing temperature regime was as follows; hold at 0°C for 30 minutes then nucleate with 1 spray of tap water and sequentially drop to test temperatures of -1.5, -3.0, -6.0, -7.5, -9.0 and -12.0°C at a rate of approximately 1-2°C/h^{-1}. Plants were held at each test temperature for 30 minutes then removed just before the next temperature drop. The temperature regime was controlled by a microprocessor, PZ1 programmer (Plate 2.1, Grant Instruments).

Each species was tested independently and replicated over time, with each frost run containing 4 treatments x 6 test temperatures x 10 plants for each test temperature. A total of 240 plants were used for each run (replicate).

Scoring: Plants were allowed to thaw overnight at 4°C and were then potted on into seed trays filled with the compost mix previously described and then replaced in the heated glasshouse to recover. Scoring for damage and re-growth was carried out 14 days post frost testing using a 10 point scoring scheme which attempted to evaluate both plant damage and re-growth. Two schemes were designed one for the brassica species and one for winter barley.

Scoring Scheme for winter barley (Plates 2.2 to 2.10).

Score	Description
0	Dead no green tissue
1	All leaves necrotic but apical meristem still green.
2	Apical meristem still green and 1 leaf still green.
3	Apical meristem still green and 2 leaves still green.
4	Whole plant still green but leaf tips showing chlorotic and necrotic damage.
5	Whole plant still green and no damage
6	Whole plant still green + 1 poorly developed tiller
7	Whole plant still green + 1 Strong tiller
8	Whole plant still green + 2 tillers
9	Whole plant still green + 3 tillers

Scoring scheme for brassica species

Score	Description
0	Dead no green tissue.
1	All leaves necrotic, external apex necrotic but internal meristems still green.
2	All leaves necrotic but apex still green.
3	1st, 2nd leaves full necrosis, 3rd leaf showing cell damage, apex still green.
4	1st leaf full necrosis, 2nd & 3rd leaves showing cell damage, apex still green.
5	Cell damage on 1st and 2nd leaves, 3rd leaf undamaged, no regrowth.
6	Cell damage on 1st leaf, 2nd & 3rd leaves undamaged, no regrowth.
7	No damage, no regrowth.
8	No damage, some regrowth (new leaf initials).
9	Active regrowth.

Statistical Analysis: Statistical analysis was conducted using multiple analysis of variance in Statgraphics version 5.1 for DOS.

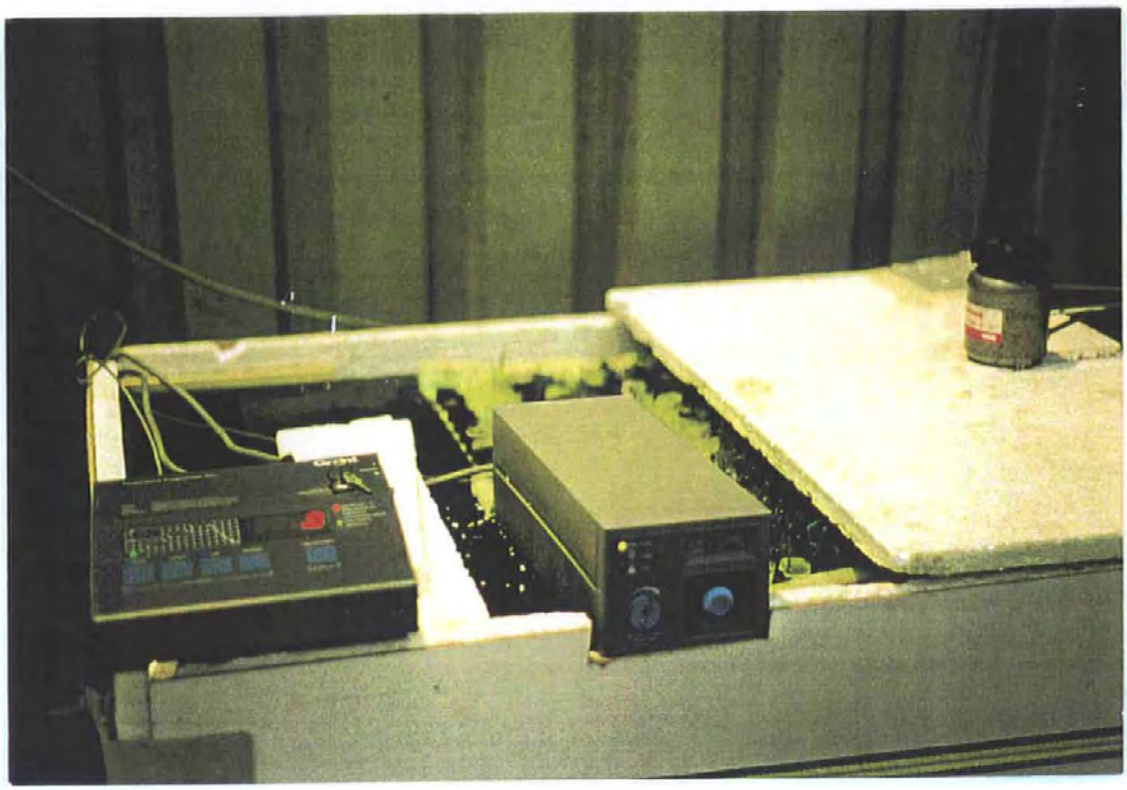
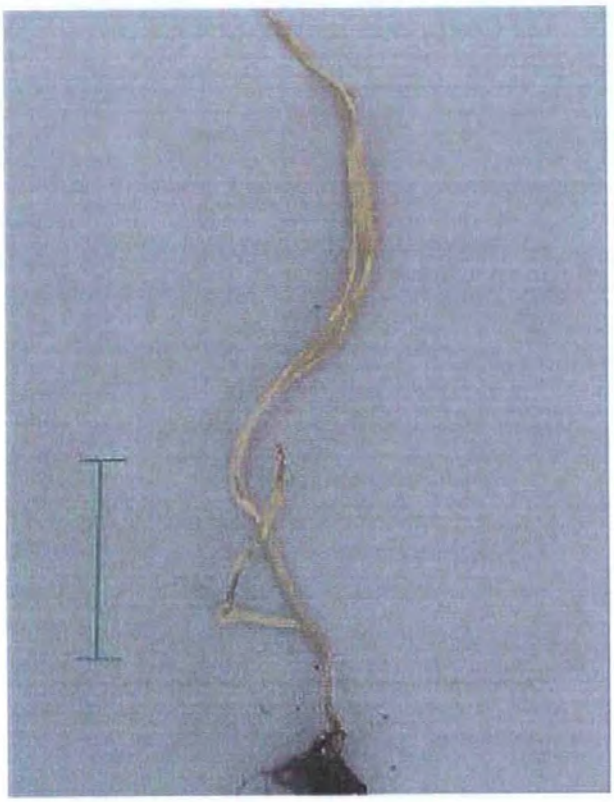


Plate 2.1: The glycol freezing tank illustrating the suspension of boiling tubes and the PZI programmer (Grant Instruments)

Plate 2.2: Score = 0



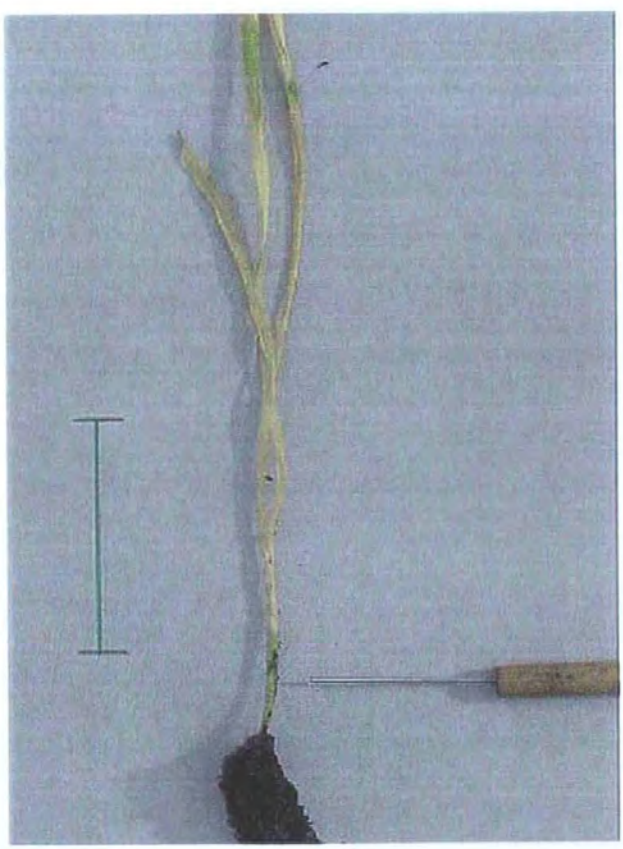


Plate 2.3: Score = 1

Plate 2.4: Score = 2

Plate 2.5: Score = 3

Plate 2.6: Score = 4

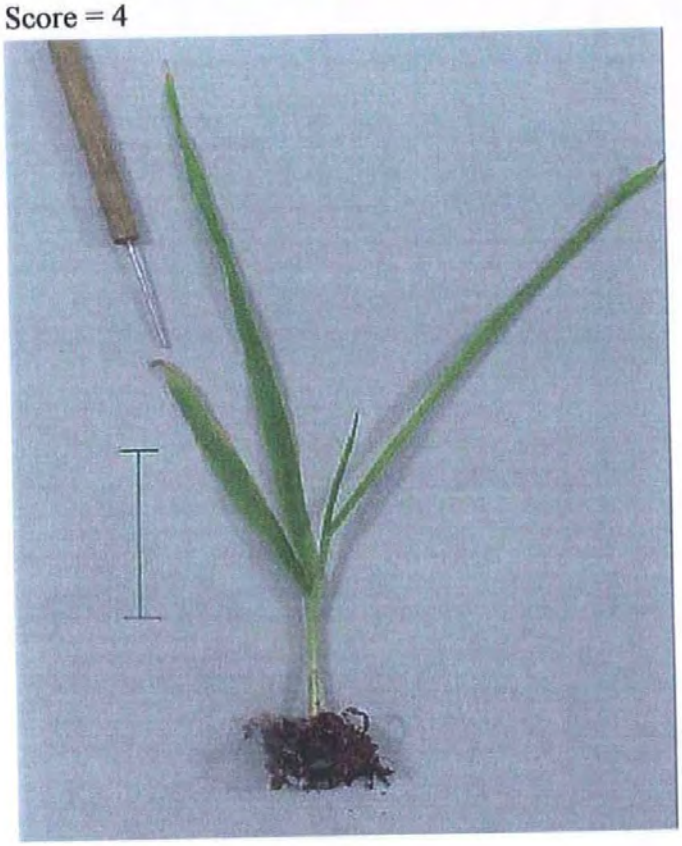


Plate 2.7: Score = 5

Plate 2.8: Score = 6

Plate 2.9: Score = 7

Plate 2.10: Scoe = 8

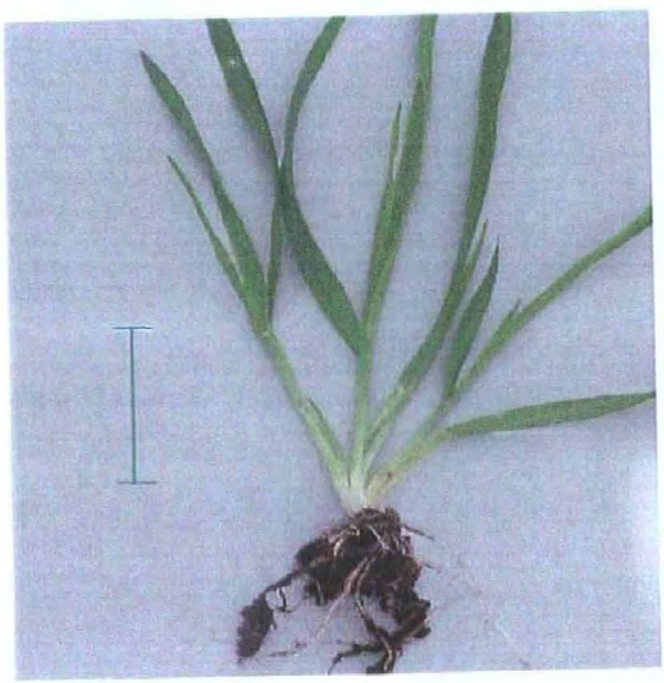


Plate 2.11: Score = 9

2.2 Experiment 2 Effect of 3 applications of LSE on the frost resistance of winter barley and scoring scheme assessment.

Materials and methods

Plant culture, cold-acclimation, frost testing, LSE application, recovery, scoring and statistical analysis was as described in experiment 1 (section 2.3.1) but in this experiment LSE was applied 3 times; the first application just after the emergence of the first leaf, the second after the emergence of the second leaf and the third just after the emergence of the third leaf. Full treatment structure was as follows;

a) non-acclimated, control plants (C)

b) non-acclimated plus 3 applications of LSE (NA3LSE)

c) cold-acclimated (CA)

d) cold -acclimated plus 3 applications of LSE (CA3LSE).

Frost testing was conducted 4 days after the final application of LSE.

Experimental Design: A completely randomised factorial design was employed with 5 replicates (replicated over time). Each replicate had 4 treatments x 6 test temperatures x 10 plants per test temperature.

Assessment of scoring scheme

The first replicate of the above experiment was used to evaluate the robustness of the winter barley scoring scheme for both inter-variation and intra-variation.

Inter-variation: five assessors were given instruction on the scoring scheme and asked to assess the sample plants for damage and re-growth, independently of each other. The originator of the scheme (the author) also assessed the same batch of plants. A regression of the originators score against the originators score was carried out to find the optimum linear correlation to determine the actual score axis, subsequent observations of the five raters were regressed against the actual score. Analysis of variance was carried out on the coefficients of these regressions and on the coefficient of determination or R^2. Any significant deviation from the originators score was expressed in the ANOVA result and

the precision of the assessment is given by the coefficient of determination where 100% = a precise score. The accuracy of the assessment is determined by the closeness of the slope to unity and the intercept to zero (Parker, Whelan and Royle, 1995).

Intra-variation assessment: the approach was similar to the inter-variation approach but in this case only the originator assessed the same batch of plants 3 times over an 8 hour period, to give 3 replicates for statistical analysis. Statistical analysis for both the inter and intra-variation was carried out using a statistical computer package Statgraphics version 5.1 for DOS.

2.3 Experiment 3: Field trial on the effects of LSE on the frost resistance of winter barley cv Pastoral

Materials and Methods

Seed Selection: Winter barley cv Pastoral was selected for the field trial. Pastoral was chosen because this variety has Igri in its parentage (obtained by crossing Igri with Matador) (Anon, 1998h). A change of variety was necessary as germination problems with the Igri seed stock would have created unacceptable field plant populations for this trial and it was not possible to obtain Igri commercially.

Sowing and Treatments: Seed was sown in a 12 x 8m plot, to establish a target final plant population of approximately 250 plants/m^2. The plot was netted to prevent bird damage and fenced with wire mesh to prevent rabbit damage. The plot was divided into 3 blocks with 3 treatments per block: control, (C) where no LSE was applied; a single application of LSE (1LSE); and multiple applications of LSE (MLSE). Liquid seaweed extract was applied via an industry standard knapsack sprayer (Cooper Peglar CP3) at a concentration of 10ml/l^{-1}, (the same concentration as used in the glasshouse trials) and was applied to treatments on a 1 pass basis at a slow walking pace (1ms^{-1}). Two flat fan nozzles were used; a) giving a 1.8m fan and b) giving a 0.5m fan. Nozzle (a) was used on the MLSE plots and nozzle (b) on the 1LSE plots, where 2 rows of plants were sprayed at each spraying interval. This assured that plants selected for frost test were either a single application or a multiple application.

Initial LSE application was at 14 day intervals from October 10th 1997 until the mid November 1997. Two further applications were made in January and February 1998 at a 20 day interval (Table 2.1). Plants were sampled for frost testing 5 days after each LSE application.

Frost Assays and Scoring: Plants were removed from the field complete with roots, washed and subjected to a frost test in the glycol freezing tank previously described. The freezing regime was as follows: hold at 0°C for 30 minutes then nucleate with tap water,

temperatures were then sequentially dropped to test temperatures of -3, -5 and -7°C at approximately 1-2°C /h^{-1}. Plants were held for 15 minutes at each test temperature before sampling. The temperature regime was controlled using a PZ1 programmable microprocessor (Grant Instruments Ltd). After removal from the freezing environment plants were treated and scored for damage/re-growth as described in experiment 1 until tiller production was evident then the scoring scheme outlined below was applied.

Scoring scheme for field trial winter barley

Score	Description
0	Dead, no green tissue
1	All leaves and tillers dead but apical meristem still green.
2	Youngest leaf and apical meristem still green on main shoot. No live tillers.
3	Youngest leaf and apical meristem still green on main stem, one or more tillers showing green apical tissue.
4	Main shoot healthy, all tillers showing chlorotic and necrotic damage.
5	Main shoot healthy, 75% of tillers showing chlorotic and necrotic damage.
6	Main shoot healthy, >50% of tillers showing chlorotic and necrotic damage.
7	Main shoot healthy, >25% of tillers showing chlorotic and necrotic damage.
8	Main shoot healthy, <25% of tillers chlorotic
9	Main shoot healthy, with all older tillers undamaged and new tillers and stem extension

Table 2.1: Spraying and sampling dates for field trial

Run	LSE Applied	Frost Test Date
1	10/10/97	15/10/97
2	23/10/97	28/10/97
3	6/11/97	10/11/97
4	20/11/97	25/11/97
5	15/1/98	5/1/98
6	5/2/98	9/2/98

2.4.0 Growth Response to Liquid Seaweed Extracts

2.4.1 Experiment 4: Evaluation of the effects of LSE on shoot dry weights and tiller production in winter barley

Materials and Methods

Evaluation of dry weights from field trial plot: Ten plants were harvested for each treatment and each block 6 days after LSE application, for each frost test, in total 6 sampling dates. Roots were removed and plants were carefully washed. Samples were then placed in a forced air oven for 48 hours at 90°C. Dry weights were obtained directly after plants were removed from the oven. Treatments were the same as those for the field frost assay (section 2.3.1).

2.4.2 Evaluation of dry weights and tiller production in glasshouse grown plants

Materials and Methods

Culture conditions: Plants were raised in an identical manner to that described earlier except they were grown in 1 litre pots.

Treatments: plants were divided into 3 treatments;

a) control (C) no LSE application, (plants were sprayed with water)

b) 1 LSE application (1LSE)

c) 3 applications of LSE (3LSE)

The application of LSE to the 1LSE plants was as previously described in section 2.1.1 and applications of LSE to the 3LSE plants was as described in section 2.2.1.

Experimental Design and Sampling: A completely randomised design was employed where 10 replicates were used in the shoot dry weight evaluation and 40 replicates were employed in the evaluation of tiller production. All plants in the growth analysis experiments were sampled 18 days after the final LSE application. The rationale for this approach is to maintain similar physiological and developmental stage between growth analysis plants and frost assay plants. In the frost assay experiments plants were tested at 4 days post LSE application but were allowed a further 14 days to recover before scoring of plants for frost damage/re-growth, totalling 18 days.

Tiller numbers were recorded and dry weights were obtained following the procedure for field trial dry weights (section 2.4.1). Statistical analysis was carried out using Statgraphics version 5.1 for DOS.

2.5 Experiment 5: Evaluation of the effects of precipitation on the LSE mediated growth observed in winter barley.

Material and Methods

Culture conditions: as previously described in experiment 4.

Treatments: All LSE treated plants received 3 applications of LSE within the same time period and application method as previously described for the glasshouse plants in section 2.4.2. However, 1 hour after each LSE treatment precipitation treatments were applied with a hand held mist sprayer. Artificial rain was applied as very fine drops to leaf surfaces and allowed to run-off and soak the soil surface, simulating a heavy rain fall. Each precipitation rain treatment was a one off event of 200cm^3 of tap water applied per pot.. Rain treatments were as follows:

Control no LSE no precipitation

Day 1 precipitation after 1 hour following LSE application

Day 2 precipitation after 24 hours following LSE application

Day 3 precipitation after 48 hours following LSE application

NR No precipitation following LSE application

Experimental design and sampling: a completely randomised design was employed with 9 replicates for each treatment in both the dry weight evaluation and tiller counts. Sampling was as described in experiment 4.

2.6 Experiment 6: Evaluation of the site of LSE uptake on plant dry weights and tiller production.

Materials and Methods

Culture conditions: as previously described in experiment 4 except that fibre matting was fitted over the soil around the stems of plants in the leaf treatment so that LSE could not contact the soil.

Treatments: All LSE plants received 3 applications of LSE in the same time period as described for the glasshouse plants in section 2.6.2 but in this experiment, treatments were applied by three methods; a) leaves, b) leaves and allowed to run-off and c) just to the soil. Full treatment structure is was:

Treatment	Abbreviation
No liquid seaweed extract	Control (C)
Liquid seaweed extract applied to leaves	Leaves (L)
Liquid seaweed extract applied to leaves and allowed to run off	Leaves and Roots (LR)
Liquid seaweed extract applied to soil only	Roots (R)

Experimental Design and Sampling: A completely randomised design was employed were 9 replicates were used to evaluate both dry weights and tiller production. Sampling and was as described in section 2.4.2.

2.7: Experiment 7: The effect of growth stage on the frost resistance of winter barley plants.

Culture Conditions: Plants were grown and cold-acclimated as previously described for winter barley (section 2.1.0) with the following modifications; sowing was at 24 day interval to establish a population of test plant at different growth stages, no LSE was applied and all plant were cold-acclimated.

Freezing Regime: Plants were subjected to a standard frost test in a glycol freezing bath (Fuller, Gout and Tapsell, 1989). The freezing temperature regime was as follows; hold at

0°C for 30 minutes then nucleate with 1 spray of tap water and sequentially drop to test temperatures of –1.5, -3.0, -6.0, -7.5 and –9°C at a rate of approximately 1-2°C h^{-1}. Plants were held at the test temperature for 30 minutes and then removed and stored at 4oC overnight. Recovery and scoring were as described in section 2.1.0.

Section 2.8: Results of experiment 1: Frost assays

There was a significant interaction between treatment and temperature for all 3 crop species tested, (Figures 2.1 to 2.3). There was a significant increase in the frost resistance of plants acclimated for 14 days at 4°C but there was no significant increase in the frost resistance of either NALSE and CALSE treated plants, (Table 2.1)

Table 2.1 Mean survival scores and estimated lethal temperature 50 values of 3 crop species following freezing. (0 = Dead, 9 = undamaged and active regrowth)

Species	Treatment	Mean Score	DT_{50}°C
Cauliflower	NA	2.74 a	-4.4
	NALSE	2.93 a	-4.51
	CA	4.55 b	-6.45
	CALSE	4.87 b	-6.65
SED		0.559	
Oil Seed Rape	NA	2.89 a	-4.45
	NALSE	3.10 a	-4.65
	CA	4.89 b	-7
	CALSE	4.96 b	-6.7
SED		0.573	
Winter Barley	NA	2.85 a	-4.4
	NALSE	2.93 a	-4.5
	CA	3.19 b	-4.8
	CALSE	3.4 b	-4.9
SED		0.609	

Letters denote statistically significant differences .

Damaging temperature 50 (DT_{50}) figures were obtained directly from survival plots (appendix 1a) and illustrate a slight increase in the DT_{50} temperatures of plants treated with LSE, except for CALSE treated oil seed rape plants, (Table 2.1).

From the DT_{50}'s it can be calculated that NALSE treated cauliflower and barley there was a 2.5% and 2.27% increase in frost resistance respectively. In the CALSE cauliflower and barley plants there was a 3% increase in the frost resistance of plants compared to the CA plants. The NALSE treated oil seed rape plants had a 4.5% increase in frost resistance compared to the NA plants, but there was a 4.29% decrease in the frost resistance of CALSE treated rape plants compared to CA plants. But these are all marginal improvements.

Figure 2.1: Survival curves of young winter cauliflower plants subjected to a freezing test

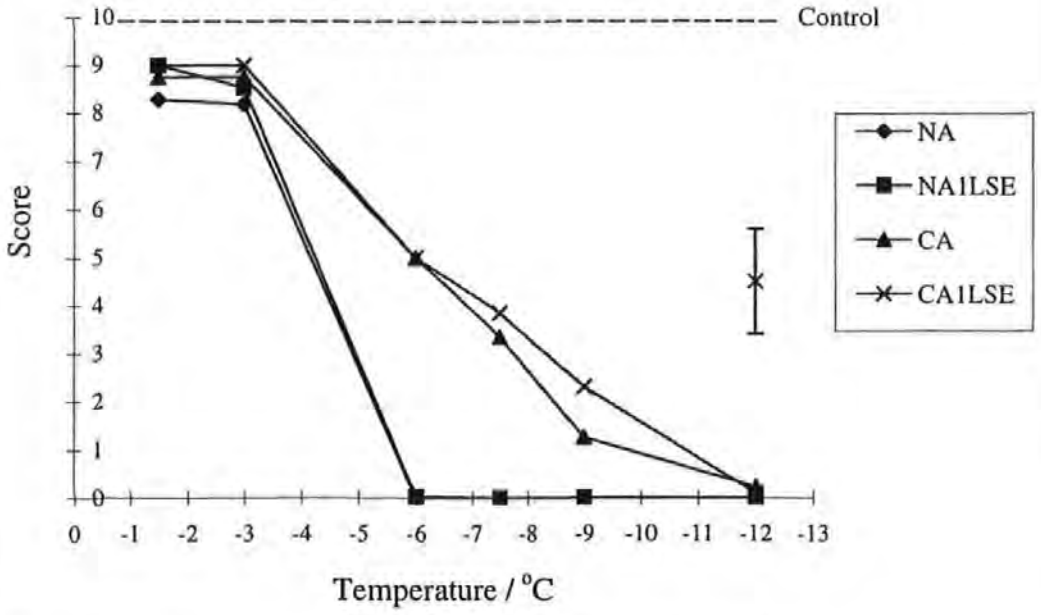


Figure 2.2: Survival curves of young winter oil seed rape plants subjected to a freezing test

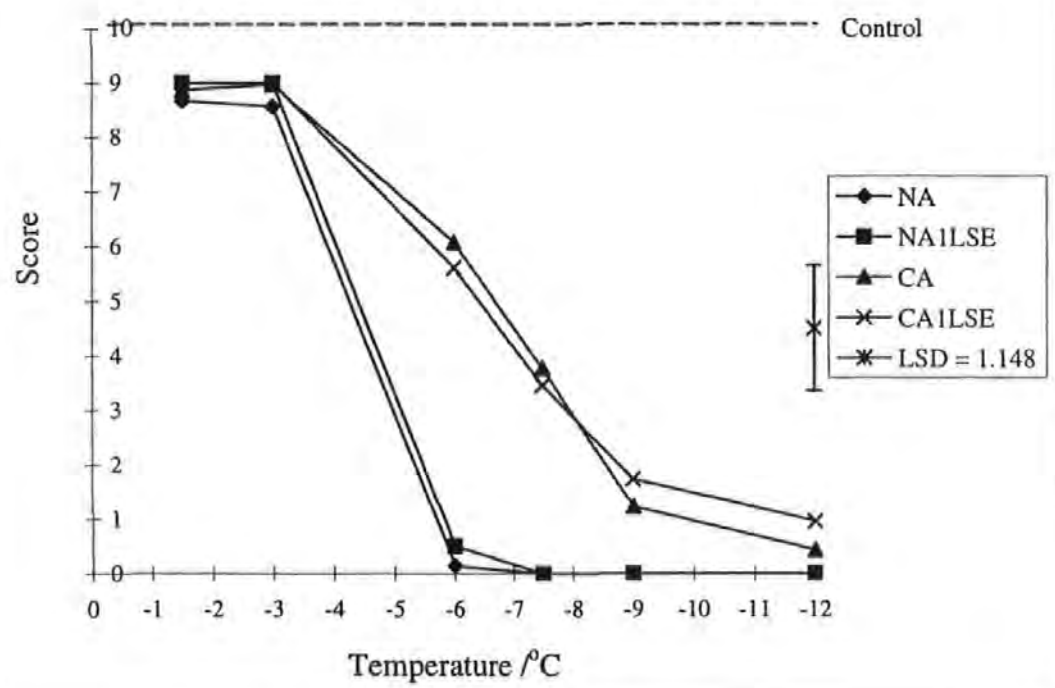
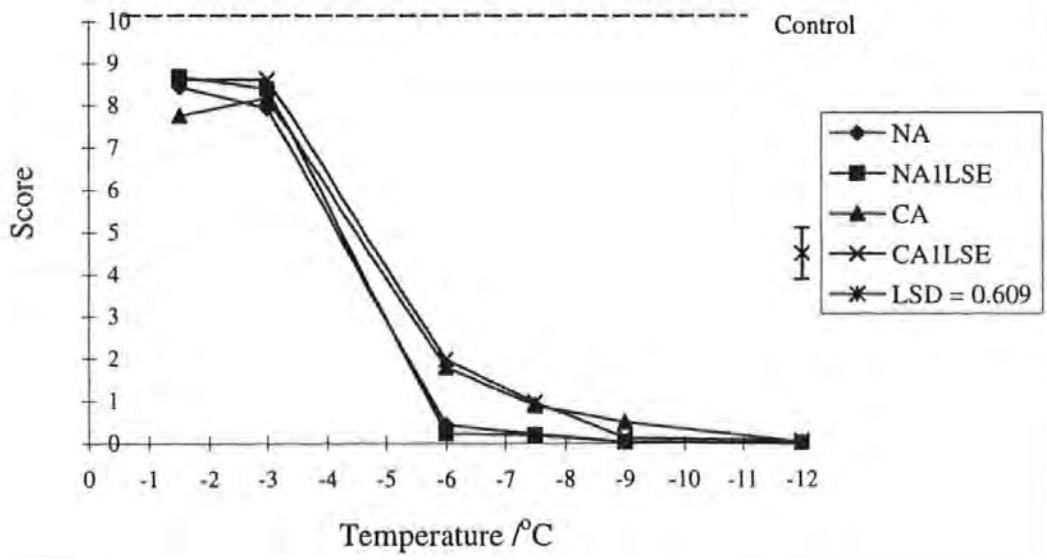


Figure 2.3: Survival curves of young winter barley plants subjected to a freezing test



Section 2.9: Results of experiment 2: effect of 3 applications of LSE on the frost resistance of winter barley and scoring scheme assessment.

There was a significant interaction ($p < 0.001$) between treatment and temperature, (Figure 2.4). At -1.5°C NA3LSE and the CA3LSE plants maintained a high survival score compared to NA and CA plants.

There is a significant increase ($p<0.001$) in the frost resistance of plants subjected to the 14 day CA treatment, but unlike the single application approach, (experiment 1) there is also a significant ($p<0.001$) increase in the frost resistance of the LSE treated plants, (Table 2.2).

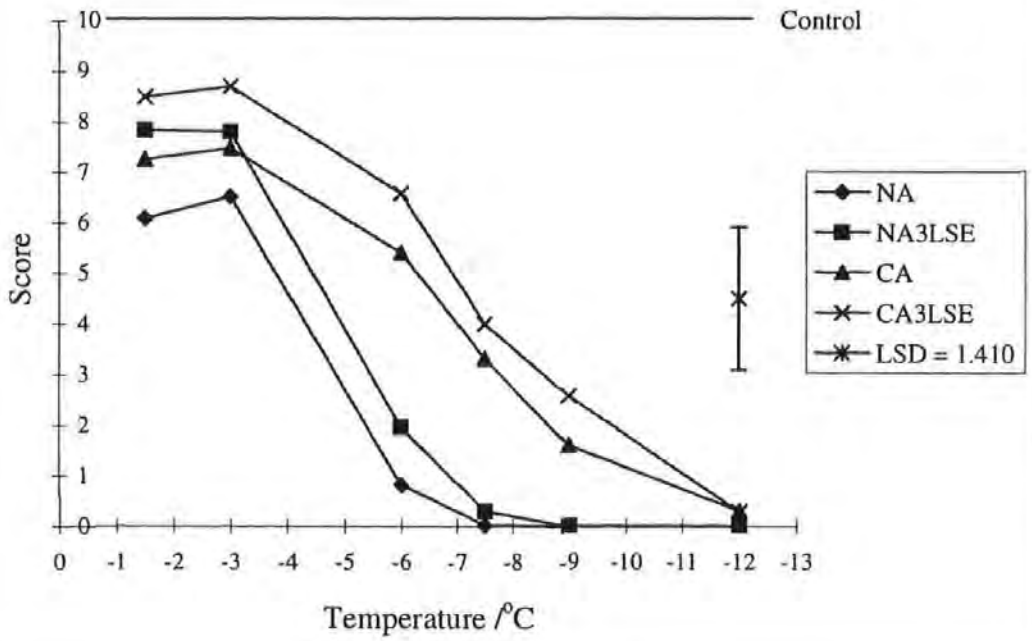
Table 2.2: Mean survival scores and damaging temperature 50 values for winter barley subjected to multiple applications of LSE and a standard frost test.

Species	Treatment	Mean Score	DT_{50}°C
Winter Barley	NA	2.24a	-4.1
	NA3LSE	2.98b	-4.75
	CA	4.23c	-6.7
	CA3LSE	5.11d	-7.2
	SED	0.7195	

Letters denote statistically significant differences.

There was a 15.9% increase in the DT_{50} of NA3LSE plants compared to the NA plants and a 7.46% increase in the frost resistance of CA3LSE treated plants compared to CA plants, (Table 2.3)

Figure 2.4: Survival curves of young winter barley plants subjected to freezing test: 3 applications of LSE



Section 2.9.1 Results of the assessment of scoring scheme

Inter-variation: Plotting the regression lines of the 5 independent observers against the originators score indicated that there was close agreement in observers score and the originators score (Figure 2.5 to 2.8) However, in the NA treatment observer 1 constantly underscored, there was also a scatter of scores around the higher re-growth score values indicating that observers disagreed in the re-growth of plants at -1.5^{o} and -3^{o}C. Plants at the lower end of the temperature range were scored more reliably because the score for a dead plant is not ambiguous. The regression lines of NA 3LSE treated plants are in close agreement across the temperature range. In the CA plants, regression lines are similar to NA plants. The regression lines for CA3LSE treated plants are in close agreement across the temperature range, except observer 3 constantly over scored. The regression coefficients and the coefficient of determination, R^2, were subjected to analysis of variance to highlight any significant difference between the originators score and the observers score for the intercept, slope and line (Table 2.4)

Table 2.4: Summary of analysis of variance on the regression coefficients for the assessment of the inter-variation observed in the scoring scheme employed for winter barley.

Treatment	Intercept	Slope	Line
NA	*	ns	ns
NA3LSE	ns	ns	*
CA	ns	ns	ns
CA3LSE	ns	ns	ns

Key: * = significant at $p< 0.05$, ns = non significant.

The intercept for the regression lines in the NA plants is significantly different from the originators score, indicating a negative bias in scoring, observers tended to under score. The regression lines for NA3LSE plants are significantly different from the originators score suggesting variation in repeatability. All other observations in table 2.5 indicate no significant difference between observers and the originators score.

Intra-variation: The regression lines for the NA scores illustrated a close relationship between the original score and the 3 subsequent observations. There was no significant difference in the intercept and slope of observations 1, 2 and 3 and the original score, but there was a significant ($p< 0.05$) difference in the regression lines, R^2, of observers 1, 2 and 3 compared to the original observation, suggesting variation in the level of precision of each assessment. In the case of the NA3LSE plants both the intercept and the slope were significantly different indicating a positive and systematic bias. However, there was no significant difference in the line which implies no variation in the level of precision of each score. Indeed the plot of NA3LSE regression lines is tightly clustered compared to that of the NA regression lines (Figures 2.9 and 2.10). The scores for the CA plants, (Figure 2.11) illustrate the same observations as those seen in the NA plants, in that there is some variation in the level of precision of each scoring event. In the case of CA3LSE, (Figure 2.12) the intercept of observations 1, 2 and 3 is only just significantly ($p = 0.0457$) different from the original score, but there is a significant difference in the regression lines between observations 1, 2 and 3 and the original observation, once again indicating variation in the level of precision, (Table 2.5).

Table 2.5: Summary of analysis of variance on the regression coefficients for the assessment of the intra-variation observed in the scoring scheme employed for winter barley.

Treatment	Intercept	Slope	Line
NA	na	ns	*
NA3LSE	*	*	ns
CA	ns	ns	*
CA3LSE	*/ns	ns	*

Key: * = significant at $p< 0.05$, ns = non significant.

The regressions of the original score vs subsequent scores were all highly significant ($p<0.001$) and in each case 99% of the data was explained by the regression models.

Figure 2.5: Assessment regression lines of 5 observers scores compared to originators score for non-acclimated winter barley plants

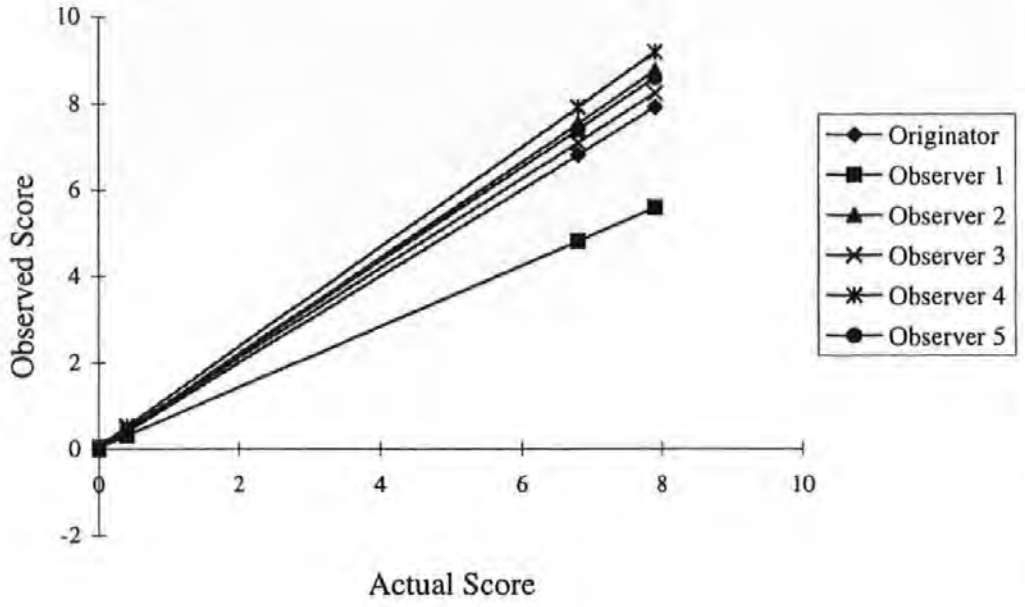


Figure 2.6:Assessment regression lines of 5 observers scores compared to originators score for non-acclimated, plus 3 applications of liquid seaweed extract, winter barley plants

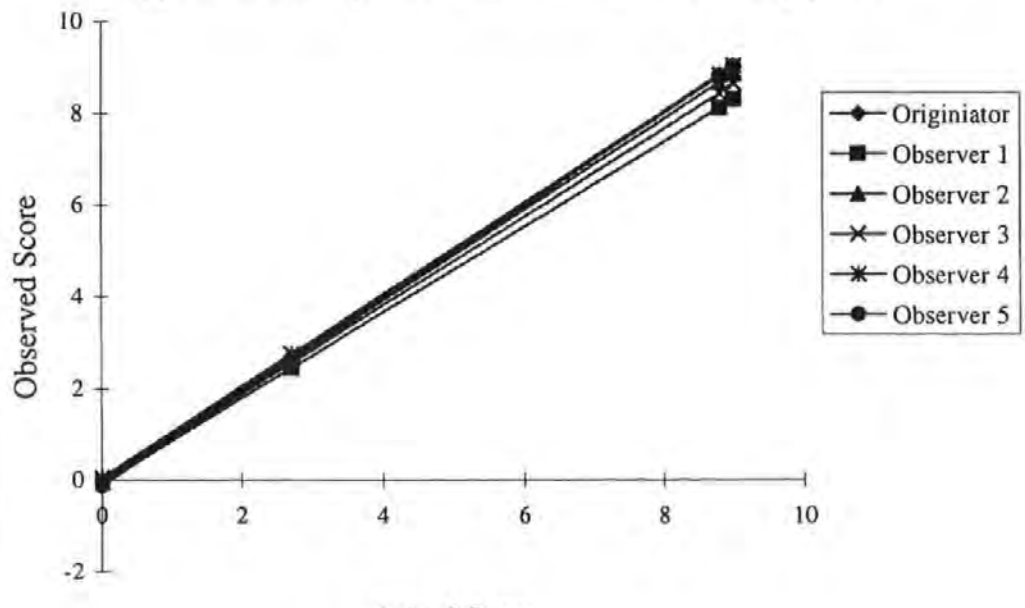


Figure 2.7: Assessment regression lines of 5 observers scores compared to originators score for cold-acclimated winter barley plants

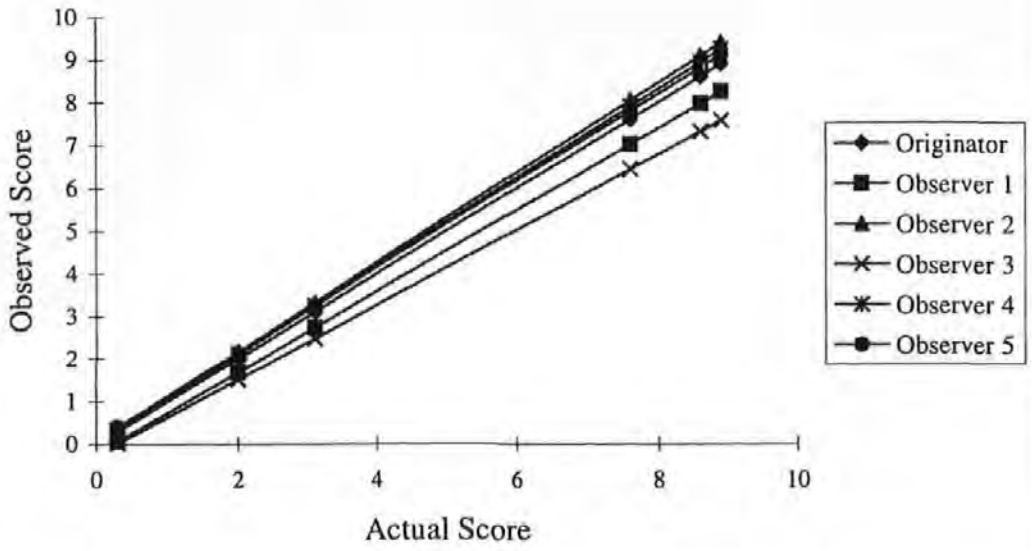


Figure 2.8: Assessment regression lines for 5 observers scores compared to orignators score for, cold-acclimated, plus 3 applications of liquid seaweed extract, winter barley plants

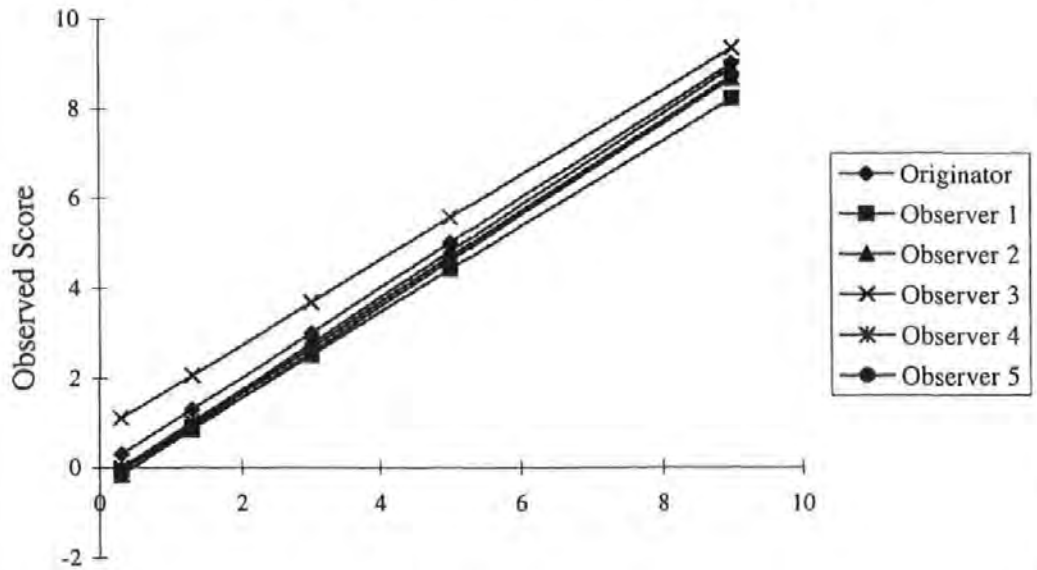


Figure 2.9: Assessment regresion lines for original score compared to 3 subsequent scores of non-acclimated winter barley plants

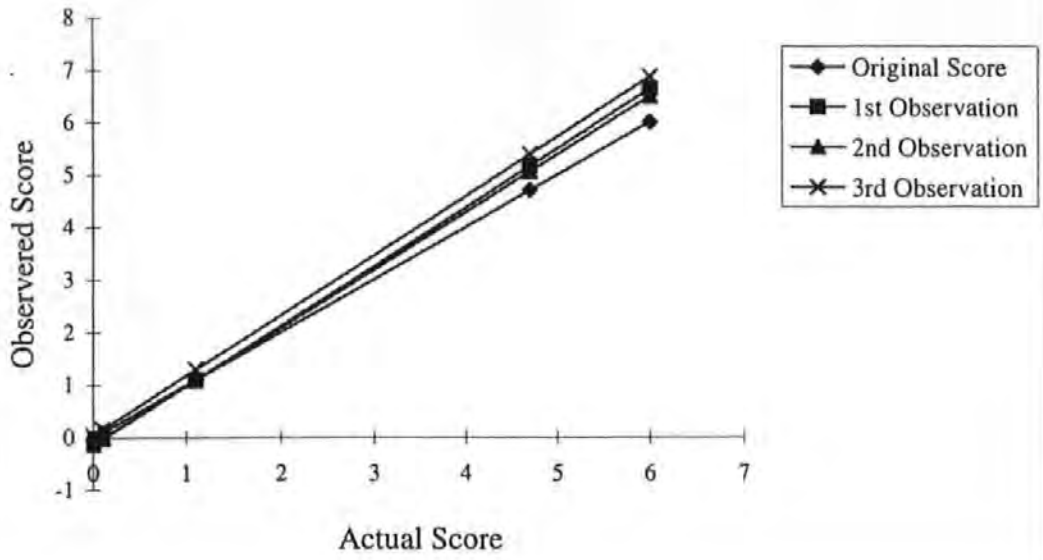


Figure 2.10: Assessment regression lines for original score compared to 3 subsequent observations on non-acclimated, plus 3 applications of liquid seaweed extract, winter barley plants

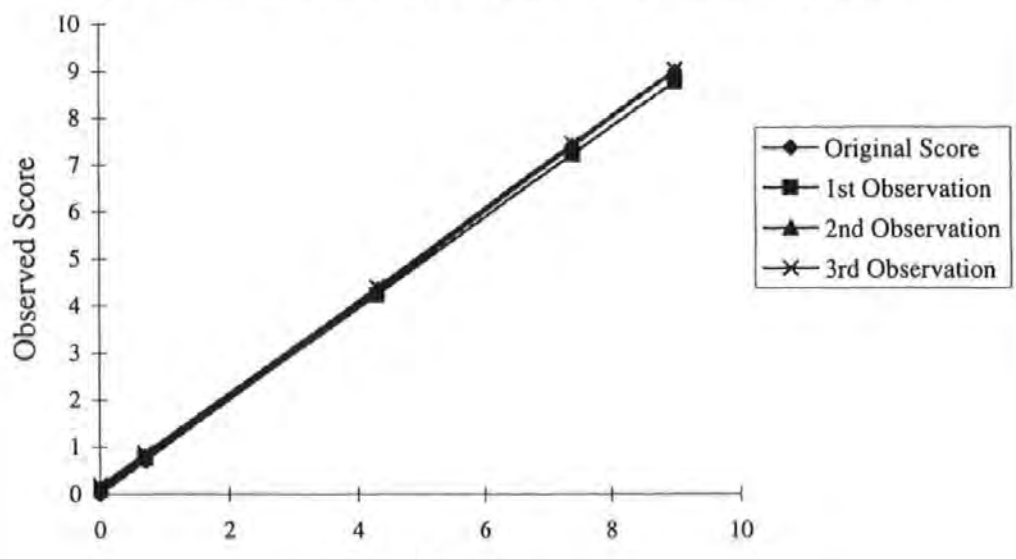


Figure 2.11: Assessment regression lines for original score compared to 3 subsequent observations of cold-acclimated winter barley plants

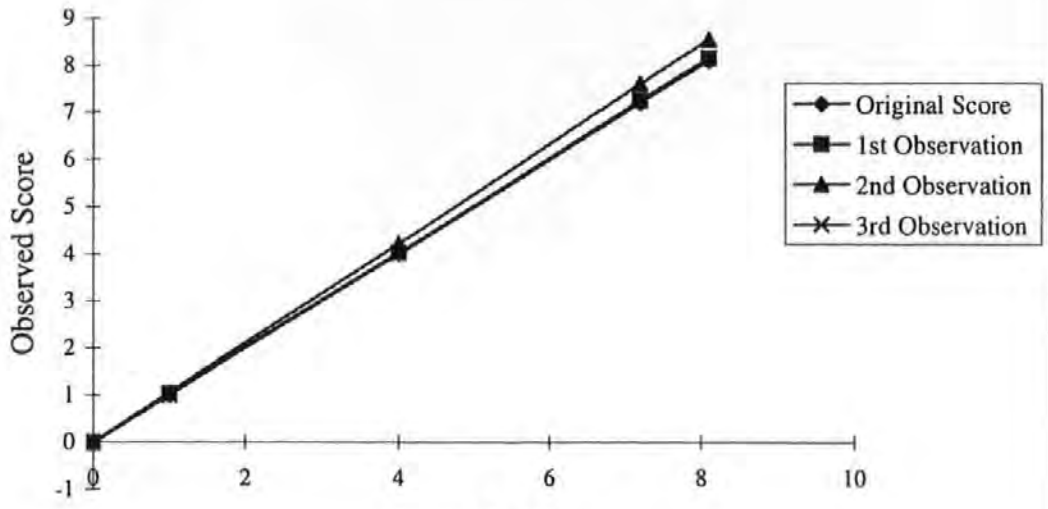
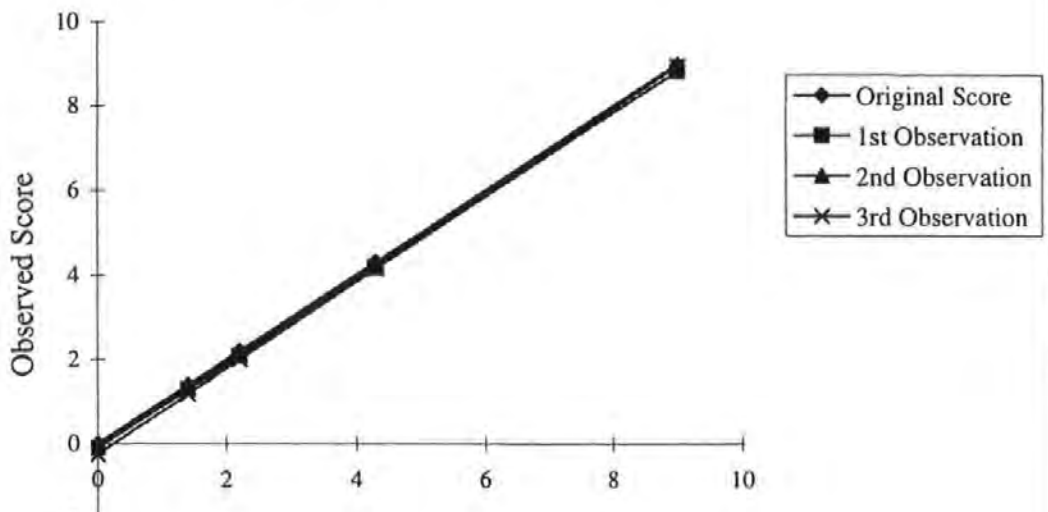


Figure 2.12: Assessment regression lines for original score comparede to 3 subsequent scores of cold-acclimated, plus 3 applications of liquid seaweed extract, winter barley plants



Section 2.10 Results of experiment 3: Field trial on the effects of LSE on the frost resistance of winter barley cv Pastoral

There was no significant interaction for both the -3°C and the -5°C analysis, but the main effects, treatment and sampling data were significant at ($p < 0.001$). Applications of LSE to field grown plants significantly improved the frost resistance of treated plants compared to untreated controls (Table 2.6).

Table 2.6: Mean survival scores of field grown winter barley plants subjected to a standard frost test.

Temperature	Treatment	Mean Score	SED
-3°C	C	8.56	0.157
	SLSE	8.78 a	
	MLSE	8.94 a	
-5°C	C	4.98	0.447
	SLSE	5.85 a	
	MLSE	6.35 a	

Within a temperature means followed by the same letter are not significantly different from each other.

There was no significant improvement in the frost resistance of multiple LSE plants compared to single LSE plants.

At -3°C there was not a significant relationship between plant survival scores and time (Table 2.7). There was a significant rise in the frost resistance of plants from sampling date 1 to sampling date 2, and then there was a steady level of frost resistance in plants until sampling date 4. After this there was a significant decline in the frost resistance of plants at sampling date 5 followed by a significant increase in the frost resistance of plants at sampling date 6 (Table 2.7)

Table 2.7: The effect of sampling date on the survival scores of field grown winter barley subjected to a standard frost test at -3°C.

Sampling Date	Mean Survival Score
1	8.24
2	8.95 a
3	8.83 a
4	9.00 a
5	8.58
6	8.97 a

Means followed by the same letter are not significantly different from each other.

The effect of sampling date on the overall mean survival scores at -5°C was significant at $p<0.001$ and the data show an increase in plant survival with time (Table 2.8). This relationship is significant at ($p<0.05$) and explains 89.43% of the data.

Table 2.8: The effect of sampling date on the survival scores of field grown winter barley subjected to a standard frost test at -5°C.

Sampling Date	Mean Survival Score
1	0.50 a
2	4.67 b
3	5.13 b
4	7.24 c
5	8.16 d
6	8.67 d

Letters denote statistically significant differences.

There was a significant interaction between treatment and sampling date (Figure 2.13) for the -7°C analysis, the response of plants to -7°C temperatures is complex. The first 4 sampling dates demonstrate a general increase in the frost resistance of control plants over time, with a further significant increase in the frost resistance of LSE treated plants, with

multiple LSE treated plants being significantly more hardy than either single LSE and control plants. However, at sampling date 5 there is no significant difference between treatments but there is a continued, background, increase in the frost resistance of plants, presumably due to decreasing air temperatures and thus increasing natural CA. At sampling date 6 there is a decline in the frost resistance of control and single LSE plants, but there is a significant increase in the frost resistance of multiple LSE plants.

Another observation from figure 2.13 is that both the LSE treated plants show a parallel decline in their frost resistance between the 10th and the 25th of November, but the control plants continue to increase their frost resistance during this time period. This may be a reflection of the prevailing weather conditions post LSE application (Figure 2.14 and Appendix 2a).

Figure 2.13: Survival scores of grown winter barley subjected to a frost test at -7oC.
SED = 0.441

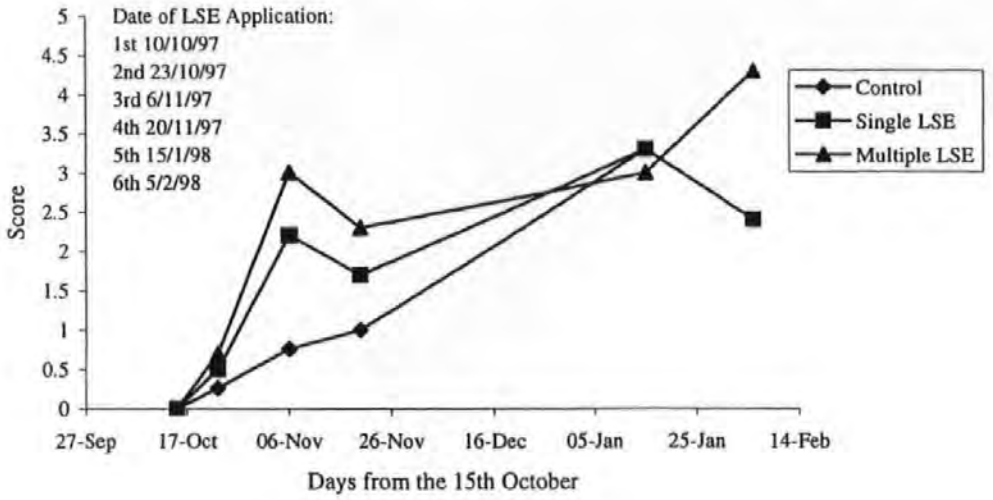
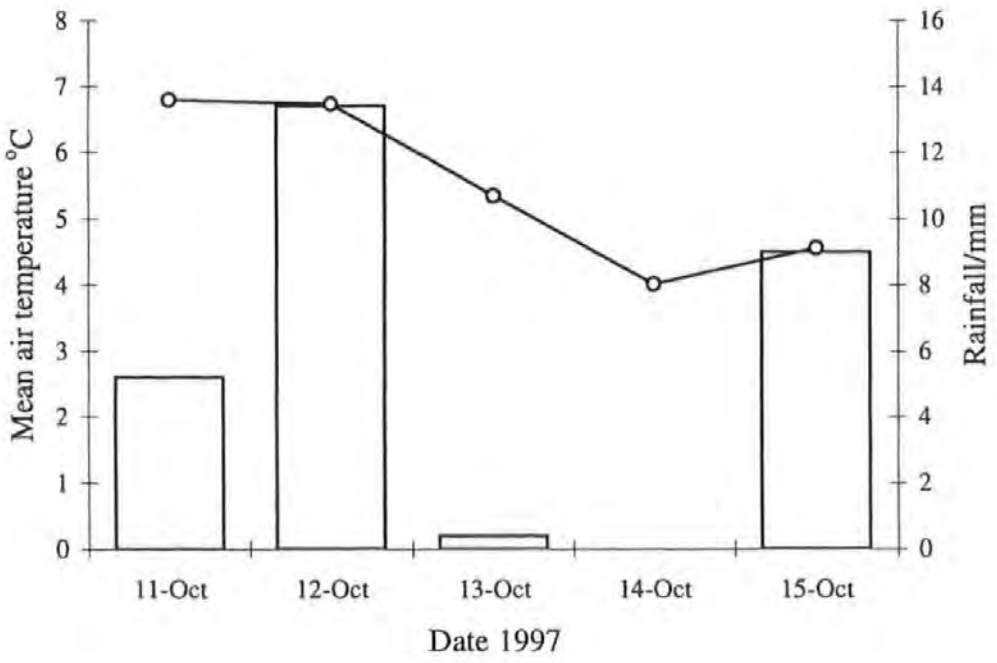


Figure 2.14: Weather station data for the five days following the fourth application of liquid seaweed extract application



2.11 Results of Experiment 4: evaluation of the effects of LSE on shoot dry weights and tiller production in winter barley

Both field grown and glasshouse grown barley plants had a significant increase in shoot dry weights when treated with LSE, and there was a small but non-significant increase between single LSE and multiple LSE treated plants, (Table 2.9).

Table 2.9: Log dry weights of winter barley treated with LSE.

Culture Conditions	Treatment	Log Dry Weight	S.E.D
Field	C	0.550a	0.0513 (d.f. = 20)
	SLSE	0.611b	
	MLSE	0.621b	
Glasshouse	C	0.318a	0.0240 (d.f.= 27).
	SLSE	0.435b	
	MLSE	0.480b	

Letters denote statistically significant differences.

The application of LSE to glasshouse grown barley plants significantly improved tiller production with 3 applications having the most significant increase, (Table 2.10) (Plates 2.11, 2.12 and 2.13).

Table 2.10: Mean tiller production of glasshouse grown winter barley following LSE treatments.

Treatment	Tiller Count	SED (d.f. = 117)
C	2.78a	0.2378
1LSE	3.80b	
3LSE	4.45c	

Letters denote statistically significant differences.

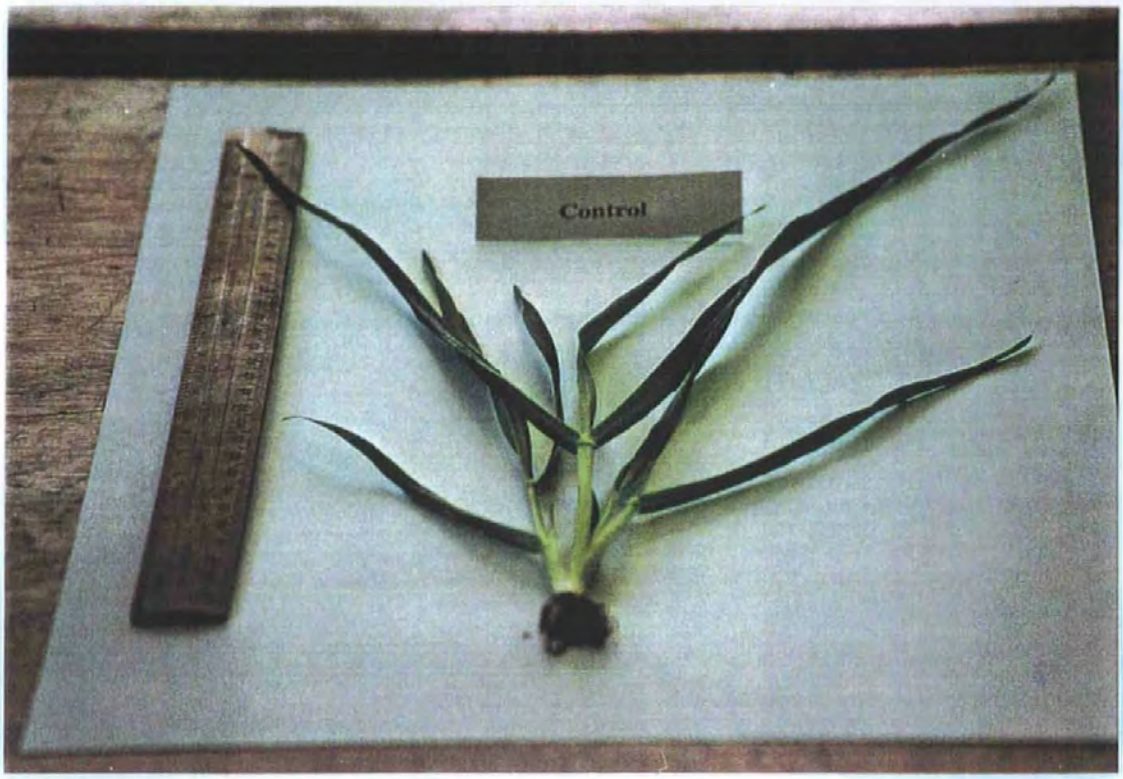


Plate 2.12: The effect of liquid seaweed extract on tiller production; control plant.

Plate 2.13: The effect of liquid seaweed extract on tiller production; 1 application of liquid seaweed extract.

Plate 2.14: The effect of liquid seaweed extract on tiller production; 3 applications of liquid seaweed extract.

2.12 Experiment 5: evaluation of the effects of simulated rain on the LSE mediated growth observed in winter barley.

Simulated rain 1 or 2 days after LSE spraying significantly reduced shoot dry weights compared to rain after 3 days and those which did not receive any artificial rain, (Figure 2.15). Although simulated rain events at day 1 and day 2 did reduce the effect of LSE on shoot dry weights there was still a significant increase in dry weights compared to the control plants.

The effect of simulated rain at day 1 and 2 on tiller production was to reduce tiller numbers to that observed in control plants. At day 3 there was a reduced effect of artificial rain on LSE enhanced tiller production and tiller counts recovered to the same level as those observed in experiment 4. Where no simulated rain events were applied tiller production was significantly greater than either days 1, 2 and 3 rain treatments, (Figure 2.16).

2.13 Results of Experiment 6: evaluation of the site of LSE uptake on plant dry weights and tiller production.

The site of LSE application significantly ($p<0.001$) affected shoot dry weights. Where LSE was applied to leaves and excluded from the soil there was a significant increase in shoot dry weights compared to control plants, but where LSE was allowed to run-off the leaf surface and soak the soil there was no further significant increase in shoot dry weights compared to the leaf treatment. However, if LSE was applied directly to the soil there was a significant improvement in shoot dry weights, (Figure 2.17).

Tiller production was improved when LSE was applied directly to the soil and even when applications of LSE to leaves was allowed to run-off and soak the soil. Applications of LSE directly to leaves did not significantly improve tiller production compared to control plants, (Figure 2.18).

Figure 2.15: The effect of simulated rain following liquid seaweed application on log dry weights of winter barley. (Letters denote significant differences)

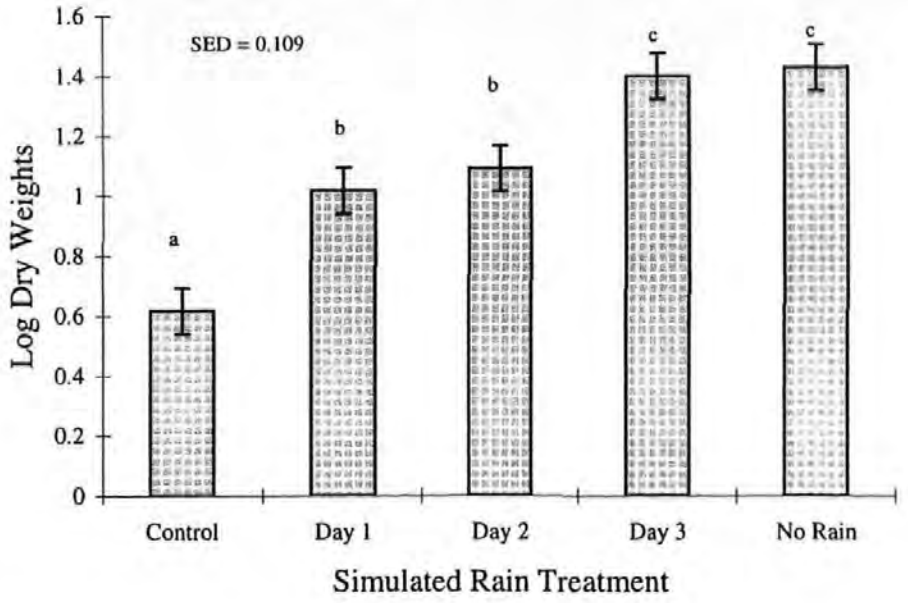


Figure 2.16: The effect of simulated rain following liquid seaweed extract application on tiller production of winter barley. (Letters denote significant difference)

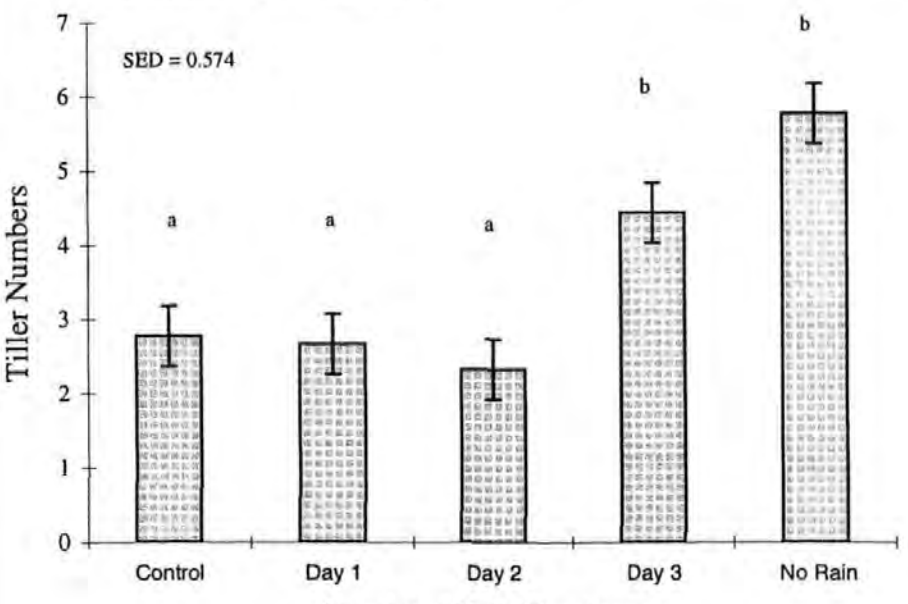


Figure 2.17: Effect of site of uptake of liquid seaweed extract on log dry weights of winter barley. (Letters denote significant difference)

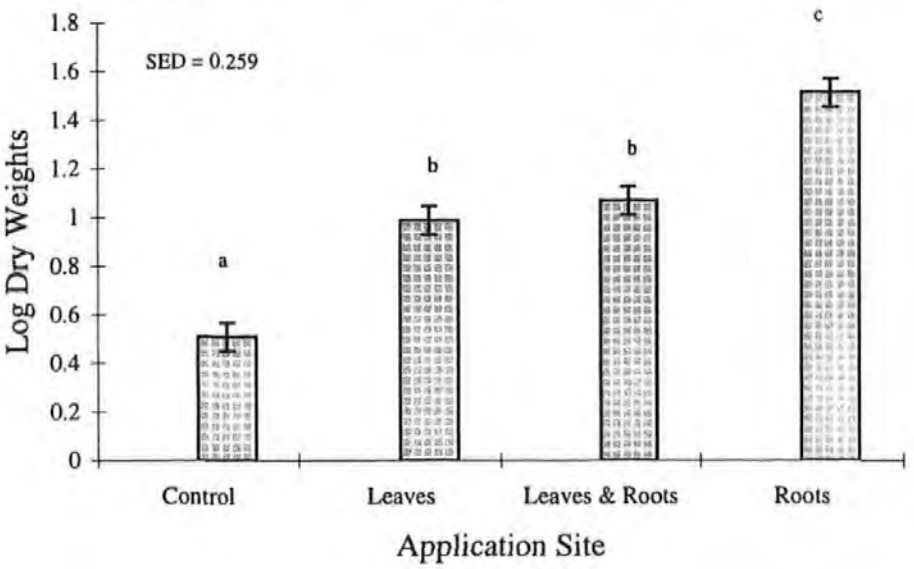
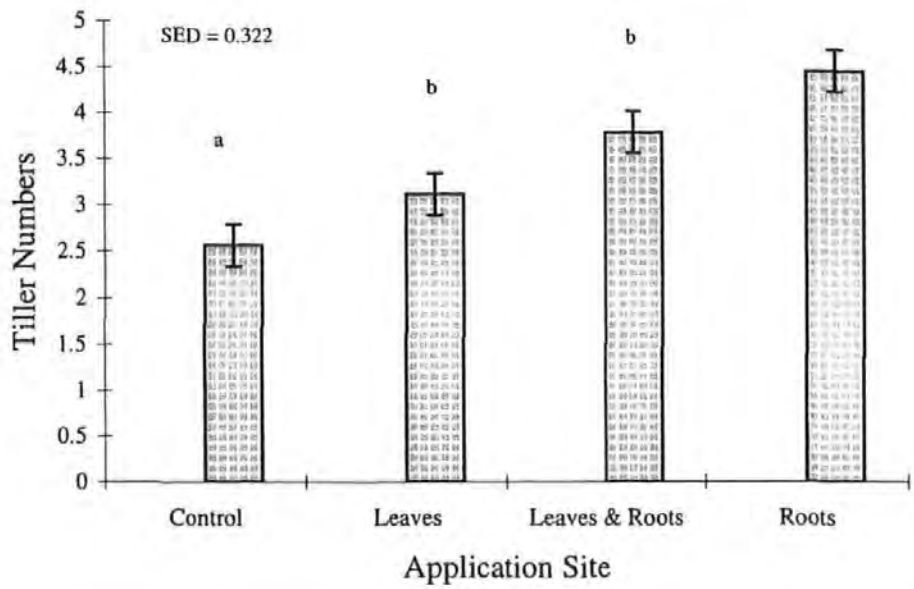


Figure 2.18: Effect of site of uptake of liquid seaweed extract on tiller production of winter barley. (Letters denote significant difference)



Section 2.14 Results of the effect of growth stage on the frost resistance of winter barley plants.

There was a significant interaction between growth stage (GS) and temperature, with GS22 and GS25 plants being significantly more frost resistant than GS13 plants, from -3 to -7.5°C. At -7.5°C there was no significant difference in the frost resistance of the plants at any of the GS measured. However, at -9°C GS25 plants were significantly more frost resistant than either GS13 and GS22 (Figure 2.19).

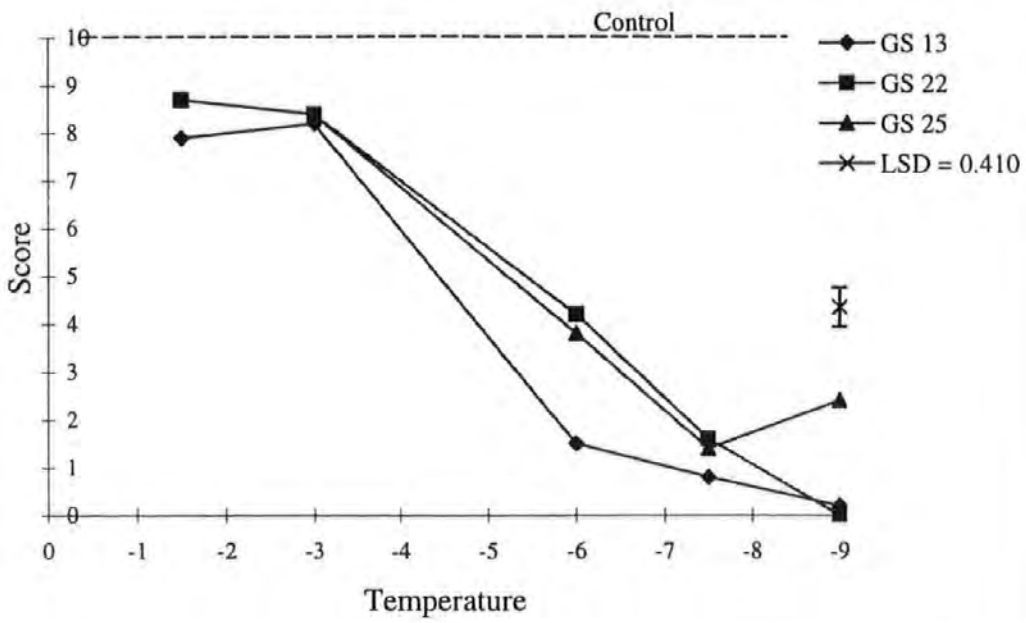
The overall effect of GS is that GS22 and GS25 are significantly more frost resistant than GS13 but they do not differ from each other, (Table 2.11)

Table 2.11: Effect of plant growth stage on the frost resistance of winter barley.

Growth Stage	Mean Survival Score	SED (d.f. = 135)
13	3.72a	0.8036
22	4.58b	
25	4.94b	

Letters denote statistically significant differences.

Figure 2.19: Survival scores of winter barley plants at three different growth stages following a conductive frost test



2.15 Discussion

Frost Assays

A single application of a 1% solution of liquid seaweed extract (LSE), 4 days prior to freezing, did not significantly increase the frost resistance of the 3 crop species tested. However, the percentage increase in the DT_{50} values indicated a trend of increasing frost resistance in all but the cold-acclimated plus LSE *B.napus* plants.

The difference in the survival scores of the CA brassica species and that of the CA barley may be a reflection of their differing physiological response to freezing. In winter brassica species there is a potential for leaves to freeze and then subsequently recover and show no symptoms of frost damage (Fuller, Grout and Tapsell, 1989). This physiological response would be detected by the scoring scheme employed for the brassica crops. In winter barley there was poor survival of leaf tissue but considerable survival of crown tissue below -3°C. Further observations of the growth response of winter barley to freezing injury illustrated the propensity of winter barley to produce new tillers, even from crowns which survived freezing down to -7°C, when there was little or no leaf tissue survival (data not shown). This phenomenon has been reported for field observations on cereal crops (Levitt, 1980), where leaf tissue damage had little impact on the field yields. Many workers have demonstrated that crown survival is crucial in cereals. The expression of cold-inducible genes in the crown meristem of young winter barley plants (cv Igri) correlated well with frost hardiness (Pearce, Dunn, Rixon, Harrison and Hughes, 1996). Gusta, Burke and Kapoor (1975) reported that crown tissue of hardy wheat cultivars could tolerate diminishing quantities of liquid water compared to non-hardy cultivars. Another study has shown that freezing tolerance and fructan metabolism in crowns of rye, wheat, barley and oats respond to a 2nd stage of CA, with a decrease in crown fructans but an associated increase in simple sugars (glucose, fructose and sucrose) which was correlated with increased frost resistance ($P<0.05$) (Livingstone III, 1996). This investigation illustrated that re-growth of shoots and roots from crowns, after 3 weeks recovery, was improved when plants were subjected to a 2nd stage of CA at -3°C for 7 days (Livingstone III, 1996).

A visual comparison of the survival curves of CA winter cauliflower and winter barley illustrates a noticeable shift to the right for winter cauliflower, whereas in winter barley there is a rapid decline in survival below -3°C. This is possibly an indication of significant leaf damage to the young barley plants, whereas the curve for winter cauliflower suggest reduced leaf damage.

The degree of frost resistance observed in the CA plants test was marginal and may be partially arributed to the low PAR levels in the 4°C phytotron. Low light levels would give rise to slow CA as a result of limiting photosynthesis and thus a reduction in metabolic process associated with CA. Another factor that would contribute to the low level of frost resistance observed in CA plants is that the CA regime (4°C for 14 days) was not sufficiently cold enough to induce full CA.

There is potentially little difference indicated between the 3 crop species in response to LSE treatments. Therefore the choice of crop species as a plant model should take into account other factors particularly that in the published literature on the molecular, physiological and biochemical responses to CA and freezing stress. There is considerable literature on the response of cereal plants to CA and freezing stress. Another factor to consider must be the ease of recording the degree of plant damage/re-growth. In the case of winter barley tiller production is an easy and reproducible component to measure. Also there may be some biochemical response associated with tillering that is being enhanced by LSE application. In conclusion winter barley was chosen as the model species and therefore all subsequent work was based on observations made on *Hordeum vulgare.*

Multiple LSE Applications

In contrast to the 1 application approach multiple applications of LSE (3 doses) resulted in a significant increase in the frost resistance of both CA and NA plants. The recorded survival scores for CA plants was significantly greater than that of NA plants and superimposing 3LSE treatments onto CA and NA plants significantly improved the frost resistance of these plants. Multiple applications of LSE could have a stimulating effect on

the natural CA process in young barley plants, possibly accelerating the 1st phase of CA or bringing about an early initiation of the 2nd phase of CA.

Livingstone III (1996) suggests that the 2nd stage of CA is possibly a continuation or acceleration of adaptations initiated by low, non-freezing, cold acclimating temperatures of the 1st phase. Therefore the more hardy the plant is after the 1st phase of CA, the greater the freezing tolerance increase during the 2nd phase.

Alternatively as LSE applications stimulate enhanced growth it is possible that 3 applications simply increase plant size and therefore result in an increase in frost resistance. However, this is unlikely to explain completely the significant increase in frost resistance observed, in the 3LSE treated plants, because care was taken to ensure that test plants were of similar size and development (i.e. 3 fully expanded leaves and no tillers). Furthermore, in experiment 8, where the comparative differences between growth stage and frost resistance was evaluated, results suggest that there would have to have been significant growth stage differences between plants before increased frost resistance could be attributed to growth stage differences alone.

Pearce *et al* (1996) have shown, however, that expression of cold-inducible genes increases more rapidly in older than in younger plants and the down regulation of these genes is slower in older plants, typically mRNA levels of younger plants fell to their long term levels during the first day. They also suggest that there is a critical age or developmental stage (approximately when the 4th leaf is beginning to emerge) when the expression of cold-inducible genes can change substantially within 1 day, in response to a marked change in temperature, whereas in younger plants (the 3rd leaf emerging) the response is slower.

In the current work with multiple LSE applications all plants had 3 fully expanded leaves, but, some of these plants had a 4th leaf emerging. This could explain the increased frost resistance observed in the multiple LSE plants compared to the single application or

control plants, i.e. that, 3LSE treatments enhance growth which may have a beneficial effect on the speed of response of cold-inducible genes.

Field Trial

The development of a small scale field trial clearly illustrated that applications of LSE to field grown winter barley cv Pastoral did enhance their frost resistance. At -3 and -5^{o}C there was a significant improvement in the survival scores of single and multiple applications compared to the control plants and there was no significant difference between single and multiple applications. At -7^{o}C, there was a significant increase in the frost resistance of multiple application plants compared to a single application. This may indicate a threshold temperature where increased ice crystal growth leads to ever increasing freeze dehydration and plant damage and therefore plants that are more resistant to freeze dehydration damage will have an increased survival score, compared to less resistant plants. At warmer sub-zero temperatures critical damage may not have occurred in the time period of the test and therefore no significant differences will be observed.

If multiple LSE treatments are having a genuine effect on plant CA processes, e.g. the continuation or acceleration of 1st and 2nd stages of hardening, it would be reasonable to postulate that multiple applications of LSE firstly enhance CA and subsequent applications maintain this effect and further enhance CA mechanisms, leading to increased frost resistance.

Further evidence to support this hypothesis arose from the interaction plot of the -7^{o}C plants. Both curves for single and multiple applications illustrated parallel increases in frost resistance and then a parallel decline after sampling date 4, whereas the control plants illustrated a slow but continuous increase in CA over the entire sampling period. Studying the weather station data for this sampling period highlights certain weather patterns that may explain these observations. In the 1st three sampling dates there was no significant rainfall post LSE application and the air temperature was slowly declining. At the 4th sampling date there was a measurable increase in rainfall immediately after LSE

application, which coincided with a decline in the frost resistance of LSE treated plants despite declining air temperature. It is possible, then, that rainfall immediately after LSE application had an adverse effect on the interaction of LSE and cold-acclimation. This may have arisen from dilution effects or leaching. In the control plants there was no decline in frost resistance at sampling date 4, indeed there was a small but noticeable increase in the frost resistance of control plants at sampling date 4 and this may indicate an upper threshold temperature (10°C) where CA does not take place or is extremely slow. Identification of threshold temperatures is notoriously difficult (Fuller *et al,* 1989; Pearce *et al,* 1996). Variation in the air temperature of the field environment would affect the expression of cold-inducible genes, especially for younger plants, where maximum possible expression is unlikely in such an environment.

The significant linear relationship between sampling date and frost resistance of plants at -5°C can be explained by plant development and age. The low frost resistance scores of young plants must be the result of low levels of cold-induced gene expression as a result of a) variation in air temperature and b) the young and immature development of the test plants, at the early sampling dates. But as plants developed over time and the air temperature continued to decline there was a consistent rise in the frost resistance of these test plants and this must be a reflection of developmental stage and steady state cold-induced gene expression (Pearce *et al,* 1996). This rise in frost resistance with age could have been accelerated by applications of LSE which a) speed up plant development and b) induce and enhance CA processes.

The significant overall effect of sampling date on the frost resistance of plants, at the -3°C test temperature did not conform to a linear relationship, but describes a pattern of increased frost resistance from sampling date 1 to sampling date 2, followed by a steady state of frost resistance to sampling date 4. At sampling date 5 there was a significant decline in the frost resistance of plants followed by a significant rise in the frost resistance of plants at sampling date 6. It is unlikely that variations in air temperature can fully explain this pattern because -3°C temperatures are non-damaging to winter barley plants. The most plausible explanation is that during the first 4 sampling dates applications of

LSE were at consistent fortnightly intervals and the results could reflect the general growth response of plants to LSE. However, there was a long break between the 4th and 5th sampling period (56 days) and the significant decline in frost resistance at this date could be a reflection of this break. If this is the case then the increase in the frost resistance at

Growth Trials

The beneficial effects of LSE treatments applied to plants during the early stages of growth is reported by Featonby-Smith (1983) for tomatoes, and by Steveni, Norrington-Davies and Hankins, (1992) for hydroponically grown spring barley. The results from experiment 5 confirm those of Steveni *et al,* (1992) in that a single application of LSE increases shoot dry weights, but there is no further significant increase in shoot dry weights with multiple applications, in either the field or glasshouse grown plants. However, in both cases there is always a non significant increase in shoot dry weights of multiple LSE plants over single LSE plants.

Many workers have attributed this growth increase enhanced mineral supplementation from the LSE. However, if growth was a direct response to mineral supplementation then it would be expected that multiple applications would bring about a significant weight gain over single applications, but this was not observed in experiment 5. Indeed, Steveni *et al,* (1992) illustrated that a mixture of mineral nutrients that represent the mineral composition of LSE did not produce significant increases in plant growth. These observations suggest that enhanced growth may be mediated by the presence of a plant growth regulator or a plant growth regulator like substance and there is speculation in the literature that cytokinins are the most likely group. Cytokinins have been shown to promote root growth at low concentrations, (Wrightman, Schneider and Thimann, 1980), and to increase lateral-bud break in pea stem sections (Wickson and Thimann, 1958) and partially overcome apical dominance. Medford *et al* (1989) inserted a bacterial gene encoding isopentenyl AMP synthase (an enzyme responsible for cytokinin production) together with a heat shock promoter into the genome of tobacco and arabidopsis plants. This new gene was turned on by subjecting the transgenic plants to short periods of 40°C

to 45°C, resulting in an increase in zeatin riboside monophosphate, zeatin riboside and zeatin levels, (23, 46 and 80 times respectively). The transgenic plants which were overexposing cytokinins exhibited multiple lateral bud break and an overall lack of apical dominance.

There have been several cytokinins identified in LSE (Blunden and Wildgoose, 1981; Stirk and Van Standen, 1997) and therefore applications of LSE to barley plants may change the ratio of cytokinin to auxin and favour development of tillering, which is equivalent to lateral bud break in dicots, and the suppression of apical dominance. Increased tiller production was observed in glasshouse grown plants that were treated with single and multiple applications of LSE.

Abscisic acid (ABA) is regarded as a growth inhibitor and the application of ABA to intact plants may inhibit shoot elongation and initiate rosette formation. In experiments with maize plants the response of the terminal and axillary inflorescence to water stress and applied ABA are a special example of a general effect on apical dominance (Aspinall, 1980). In the maize plant, water stress at the time of terminal inflorescence initiation causes a marked increase in the endogenous ABA content of the developing tassel, an inhibition of its growth and following stress relief, a promotion of the growth of the axillary inflorescence (Aspinall, 1980). Growth of the axillary inflorescence is controlled by the developing tassel and it is speculated that increasing ABA concentration in the developing tassel during water stress diminishes its capacity to inhibit the growth of the axillary inflorescence (Aspinall, 1980). A similar response has been found in vegetative pea seedlings, and these examples are reminiscent of the effect of a period of water stress on vegetative cereal plants where re-watering is followed by prolific tillering (Aspinall, 1980).

It is possible then that the levels of cytokinins in the LSE combined with the presence of ABA in the LSE act together and remove apical dominance and promote lateral growth and tillering.

Assessment of scoring scheme

Inter-variation: The minimal variation recorded for inter-rater variation suggests a degree of robustness in terms of scores being repeatable by different observers. There were only two significant deviations from the originators score. In the non-acclimated (NA) plants the intercept of the regression lines was significantly different from the actual score and this identified a significant negative bias (Nutter and Schultz, 1995) by raters, (a tendency to underscore) compared to the originators score, but the slope was not significantly different suggesting no systematic bias between raters. In addition the R^2 was not significantly different from the originators score indicating consistency and reproducibility. In the non-acclimated plus LSE treated plants there was a significant difference in the raters R^2 compared to the originators R^2 indicating variation in the reproducibility of each observer, but there was no systematic or constant bias. In all the other plants there was no significant difference between raters coefficients, thus data collected by the 5 observers and the originator are in close agreement (high precision) Nutter and Schultz, 1995).

Intra-variation: Compared to the assessment of inter-variation the analysis of the intra-variation illustrated a complex pattern of accuracy and precision where:

1) accuracy describes how close the mean of an estimate is to the true value of the quantity of damage and re-growth assessed (adapted from Martin, 1971).

2) Precision describes the repeatability or variation associated with an estimate regardless of the average value (Martin, 1971).

In the case of NA and CA plants the data suggest that where accuracy in damage/re-growth is achieved there was a loss of repeatability, significant deviation in the R^2. This may also hold true for CALSE plants because the significant difference in the intercept of these plants was a border line case ($p = 0.0457$) and may be improved by replication. This observation does give rise to some concern on the repeatability of assessments made using the designed scheme.

Repeated assessments may give rise to variation in;

a) recorded damage

b) errors in re-growth assessments

c) from rapid and imprecise assessments

The scoring scheme was designed to be objective in that counts of leaves and tillers are used to measure survival instead of percentage damage. Therefore it is unlikely that true variation in damage and re-growth would account for all the variation observed. More likely errors arising from rapid and relatively imprecise assessments could account for the majority of the variation observed. By comparison there was only 1 significant deviation from precision (line) by the slower and inexperienced observers in the inter-variation assessment. This is probably due to care and attention to detail of the various, novice, observers in this group, compared to the rapid assessments made by the experienced originator. Experiments on the reliability measurements of disease severity have shown that on 1 occasion a rapid observer might provide a very precise assessment but on another, a relatively imprecise assessment (Parker, Whelen and Royle, 1995).

Data from these experiments suggest that the designed scoring scheme is relatively robust and that there is a reliable measure of truth in the assessments of frost resistance in winter barley. However, caution must be exercised by observers not to make to rapid and hence imprecise assessments.

2.15 Conclusions

It was clearly demonstrated that foliar applications of LSE marginally increase the frost resistance of winter cauliflower, winter oil seed rape and winter barley and where multiple LSE applications were applied to both non-acclimated and cold-acclimated winter barley there was a further significant improvement in the frost resistance of this species. This was subsequently illustrated to be stable in the field environment following a small scale field trial. Further experiments illustrated that LSE does increase growth and development of winter barley, which maybe involved in the observed increase in frost resistance.

Observations from the field trial implied that heavy precipitation following LSE application adversely effects LSE mediated frost resistance. Further glasshouse growth trials demonstrated that precipitation up to three days post LSE application significantly reduces LSE mediated growth effects which may reduce LSE enhanced frost resistance.

Chapter 3: Protein Analysis

3.0 Introduction

The frost assay experiments clearly demonstrated exogenous applications of liquid seaweed extract (LSE) increased the frost resistance of winter barley. These results give rise to questions concerning the mechanism of action of liquid seaweed extracts on the manipulation of frost resistance in crop plants.

The frost assays support previous work of Saunders (1997) indicating that LSE applications do not prevent the plant from freezing but appear to reduce frost damage and enhance plant re-growth post freezing. This observation, together with those of Suanders (1997), which suggested that LSE only works on hardy plant species, suggest that LSE may induce normal cold-acclimation (CA) processes, such as membrane changes, solute changes and altered protein metabolism.

In order to pursue the question of how LSE enhances the frost resistance of barley, a series of protein studies were conducted to elucidate whether LSE has any effect on protein metabolism. The strategy employed was firstly to establish a suitable extraction protocol, then evaluate the concentration of total soluble protein. This would indicate if CA and LSE are having any effect on protein metabolism. This was then followed by electrophoretic separation of protein extracts, after Sambrook, Fritsch, and Maniatis, (1989) for visualisation of protein species being affected by CA and LSE, followed by Western Blotting dehydrin expression, Towbin, Staehelin and Gordon (1979) and Sambrook, Fritsch, and Maniatis, (1989) .

3.1 Experiment 3.1: Development of protein extraction protocol

Materials and Methods

Culture conditions: Winter barley seed (cv Igri) was sown and plants raised as previously described in experiment 2.1.

Protein Extraction Protocol: six buffer systems were investigated in order to establish the most appropriate system for protein analysis.

1)20ml Borate buffer pH 9 to 4g of fresh plant material

2)12ml Borate buffer pH 9 to 4g of fresh plant material

3)12ml Tris-HCL pH 7 to 4g fresh plant material

4)12ml Tris-HCL pH 7 + 2% SDS to 4g fresh plant material

5)12ml Tris-HCL pH 8 to 4g fresh plant material

6)12ml Tris-HCL pH 8 +2% SDS to 4g fresh plant material

The composition of the buffer systems were as follows;

a) Borate buffer

50mM Sodium borate

50mM Ascorbic acid

1mM PMSF adjust to pH 9 with concentrated HCL

b) Tris-HCL Buffer

50mM Tris

1mM PMSF adjust to pH 7 or 8 with concentrated HCL

c) Tris-HCl +2% SDS Buffer

As above, with the addition of 2% SDS

Non-acclimated shoot material was harvested when plants had 3 fully expanded leaves. Any senescing leaves were removed and samples were washed and sterilised in a 1% bleach solution, for 1 minute, then rinsed in double distilled water (ddH_20) and cut into

small sections and crushed, in a pre-chilled (4°C), pestle and mortar, with liquid nitrogen. The slurry was then centrifuged at 15,000g for 5 minutes at 4°C. The supernatant was filtered through a No 1 Whatman filter paper then sub-divided into 1ml aliquots and stored at -80°C.

Determination of Total Soluble and SDS soluble Proteins: Protein concentration was determined by the Bradford Assay procedure, (Bio-Rad). A standard calibration curve was established by diluting a known concentration of Bovine Serum Albumin (BSA) in 1ml of ddH_2O; 0.2, 0.4, 0.5, 0.6, 0.8 and 1ml of BSA was added to the appropriate volumes of ddH_2O. Then 100µl of each dilution was pipetted into a test tube to which 5ml of Bradford reagent was added. Preparations were allowed to react for 30 minutes. Absorbency was then measured with a spectrophotometer (Pye Instruments) at 595 nm with a yellow filter.

Experimental Sample Preparation: 100µl of samples were reacted with the Bradford reagent for 30 minutes and measured by the same procedure as the calibration curve. Tris-HCL samples were diluted 2:1 (vol:vol) to ensure absorbency readings were within the scale available (visual estimate of colour development suggested that pure samples would be too concentrated for the available scale).

3.2. Experiment 3.2: The effect of cold-acclimation and liquid seaweed extract applications on the concentration of total soluble proteins

Materials and Methods

Culture conditions: standard culture conditions were applied (section 2.1.1) to winter barley (cv Igri). Sequential sowing was applied to ensure similar development of CA and NA samples. Cold acclimation was achieved by placing young plants in a constant 4°C phytotron with a 9 hour photoperiod for 14 days.

Treatments: Plants were divided into 6 treatments as follows;

1) Non-acclimated (NA)
2) Non-acclimated + 1 liquid seaweed extract (NA1LSE)
3) Non-acclimated + 3 liquid seaweed extract (NA3LSE)
4) Cold-acclimated (CA)
5) Cold-acclimated + 1 liquid seaweed extract (CA1LSE)
6) Cold-acclimated + 3 liquid seaweed extract (CA3LSE)

The first application of LSE (to the 3LSE plants) was just after emergence of the first leaf, the second a week later and the third just after the emergence of the third leaf. The first application of LSE (to the 1LSE plants) was just after the emergence of the third leaf. Application method was as previously described in section 2.1.1.

Sampling: Plant material was sampled at 0, 4, 6 and 10 days, where; 0 day = no application of LSE and 4, 6 & 10 days = days after final LSE application, this sampling regime is designed to be within the time scale of LSE enhanced frost resistance observed by Saunders (1997).

Extraction protocol and colourimetric determination: Samples were prepared as described in section 3.2. Colourimetric procedure was as described in section 3.1.0.

Experimental design and statistical analysis: Plants were randomly positioned in both the glasshouse and the 4°C phytotron, and 2 replicates of each treatment were analysed for protein. Calculated protein concentrations were subjected to analysis of variance using Statgraphics v 5.1 for DOS.

3.3. Experiment 3.3: The effect of artificial rain and application site on liquid seaweed enhanced protein expression

Culture conditions, treatments and sampling: As described in section 2.5.0 for artificial rain experiment and as section 2.6.0 for the application site experiment.

Experimental design: A completely randomised design was employed where 3 replicates were evaluated for both the artificial rain plants and the site of application plants.

Extraction protocol and colourimetric determination were achieved as described in section 3.1.

3.4. Experiment 3.4: electrophoretic comparison of protein expression in winter barley plants subjected to normal growth conditions, cold-acclimation and liquid seaweed extract treatments

Culture conditions: As described in experiment 1.1

Treatments and sampling: As described in section 3.3.

Extraction protocol: As described in experiment 3.1.

Experimental Design: Plants were randomly distributed in both the glasshouse and 4°C phytotron, with 3 independent replicates for each treatment.

Electrophoresis protocol

Gel casting: Stock solutions were prepared as outlined in appendix 3A.

Procedure for casting resolving gel and stacking gel: mix all the components, of the resolving gel, except APS and TEMED. Degas for 15 minutes then add APS and TEMED and pour resolving gel into casting plates, overlay with water saturated butyl and allow 2 hours drying time. Rinse with ddH_20 then with 0.5M Tris-HCL pH 6.8. Mix and degas stacking gel, as above, add catalysts and pour over resolving gel. Insert comb, leaving a gap of 1 to 1.5cm between resolving gel and the comb, allow 1 hour drying time. Resolving gels were cast between an aluminium oxide plate and a glass plate.

Sample and standard preparation: Samples were mixed with the sample buffer at a ratio of 40µl:80µl (sample:buffer) and heated to 100°C for 4 minutes. A Bio-Rad broad spectrum molecular weight standard was used for molecular weight calibration and 10µl was mixed with 190µl of sample buffer and then treated as above.

Running conditions: 12µl of the sample cocktail was loaded into the wells of the stacking gel, the power pack was set at 115mV and gels were run for 1 hour and 45 minutes. A

Hoffer Mighty II electrophoretic system was used and ice cold water was circulated around the electrophoresis unit.

Stain and Destain Conditions: following electrophoresis, gels were stained for 1 hour in coomassie blue stain and then destained overnight. A second destain step in fresh solution for 1 hour was applied before photography and densitometery.

Photography: Gels were photographed using a Polaroid camera with white back lighting supplied by a transilluminator. Aperture F32 shutter speed 1/125 seconds with a 45 second development time at room temperature.

Densitometry: Gels were scanned wet, with an LKB 2222-020 ultrascan laser densitometer, between 2 glass plates with the following settings;

X width 3

Y step 2

Smoothing 2

Peak width 1

(empirically determined, data not shown)

3.5. Experiment 3.5: electrophoretic comparison of protein expression in winter barley plants subjected to liquid seaweed extract and simulated rain events

Culture conditions, treatments and sampling were as described in section 2.5.0. Protein extraction was as described in section 3.1. and electrophoresis was as described in section 3.3.

3.6. Experiment 3.6: electrophoretic comparison of heat stable proteins

Samples of the 4 day plants, from experiment 4, were subjected to a 15 minute heat denaturation step just after the initial centrifugation step, in the protein extraction

protocol. Samples were centrifuged once more, with the same settings as the initial centrifugation step, and decanted into 1ml aliquots.

Analytical Strategy: A standardised strategy was devised for gel interpretation and statistical analysis.

1) Construct molecular weight calibration curve

2) Determine apparent molecular weight of unknown protein species

3) Measure the number of bands detectable by densitometer and cross reference gel by eye

4) Observe any reproducible patterns detected by densitometer

5) Construct analysis of variance test with data generated by densitometer

3.7. Experiment 3.7: Western Blotting

Immuno-blotting was carried out using a Bio-Rad immuno-blot assay kit, with a goat anti-rabbit secondary antibody and horseradish peroxidase conjugate substrate protocol. The primary antibody was a rabbit monoclonal antibody raised against the consensus lysine rich peptide of plant dehydrins (T. Close, personal communication).

Immuno-blotting was carried out on the electrophoretically separated proteins (from the 4 day gel) of the protein comparison study carried out on NA, CA and LSE treated barley plants. Transfer of separated proteins to a nitrocellulose membrane was carried out electrophoretically, in a Towbin buffer, (appendix 4A) overnight, at 29.5mV and with circulating cold water.

Strategy: After electrophoretic transfer, the nitrocellulose membrane was immersed into the blocking solution and gently agitated on an orbital shaker for 1 hour, at room temperature. The membrane was then washed with TTBS washing solution and inoculated with the primary antibody and incubated overnight. Unbound antibody was washed off with TTBS twice for 5 minutes for each washing step. The second antibody was incubated for 2 hours, using gentle agitation, at room temperature. Two final, TTBS, washes were applied and just prior to colour development a final wash with TBS was carried out, to remove residual Tween 20. Following this final wash the membrane was immersed for 45 minutes in colour development solution then washed, twice, in ddH_20 at room temperature.

3.8. Results of experiment 3.1: development of protein extraction protocol

The preliminary investigation into the 6 buffer systems indicated that Tris-HCL pH 7 buffer, at a ratio of 3 parts buffer to 1 part fresh plant material, yielded the highest protein concentration (Table 3.1).

Table 3.1: Comparison of protein yields from winter barley using six different buffer systems; (n = 3).

Buffer System	Protein Yield mg/ml	s.e.
Borate pH 9		
20ml	0.997	0.020
12ml	1.912	0.025
Tris-HCL pH 7		
12ml	2.235	0.025
Tris-HCL + 2%SDS pH 7		
12ml	1.404	0.014
Tris-HCL pH 9		
12ml	1.991	0.038
Tris-HCL + 2% SDS pH7		
12ml	1.332	0.025

3.9. Results of experiment 3.2 The effect of cold-acclimation and liquid seaweed extract applications on the concentration of total soluble proteins

There was no significant interaction between treatment and day (sampling period) but both main effects were significant. The treatment level was significant at ($p< 0.001$) and illustrates an increase in total soluble protein in plants subjected to CA treatments compared to the NA plants. There was also a significant increase in total soluble protein extracted from NA plants treated with LSE but there was no significant increase in total soluble protein in CA plants treated with LSE (Figure 3.1).

Single applications of LSE to NA plants increases protein concentration by 36.7% and 3 applications increase protein concentration by 86.54%.

3.10. Results of experiment 3.3: the effect of artificial rain and application site on liquid seaweed enhanced protein expression

There was a significant increase, in LSE enhanced protein concentration, following simulated rain events at day 3 and where no rain event occurred (Figure 3.2) however, there was a significant reduction in the concentration of total soluble protein from LSE treated plants which received simulated rain events at day 1 and day 2. This treatment effectively reduced protein concentration to that of control plants, where the soluble protein concentration is similar to that observed in experiment 2.

The site of LSE application has a significant effect on the concentration of plant protein, with soil application expressing the highest yield of proteins followed by leaf and soil and then leaf application. The control plants had the least protein (Figure 3.3).

Figure 3.1: Concentration of total soluble protein in winter barley subjected to cold-acclimation and liquid seaweed extract treatments (Letters denote significant differences)

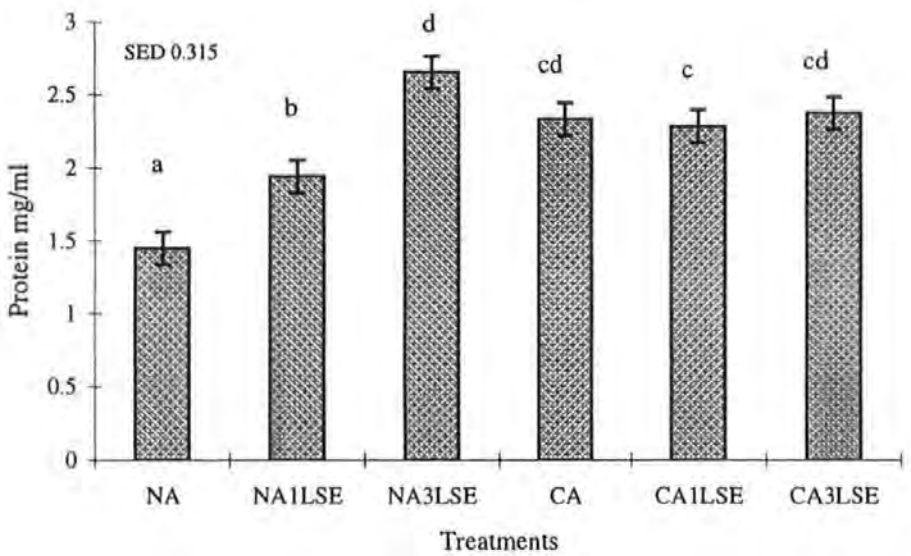


Figure 3.2: The effect of simulated rain on the concentration of LSE enhanced soluble protein. Letters denote significant difference

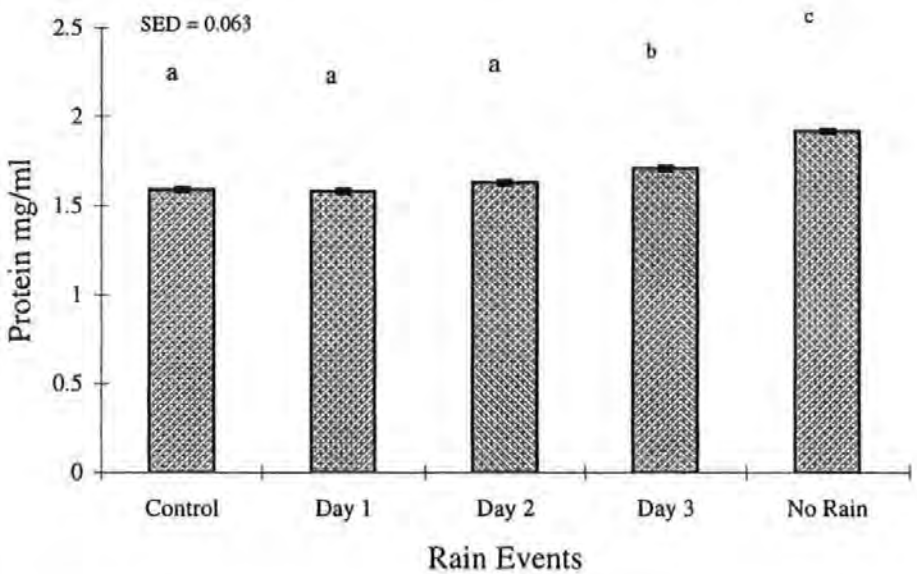
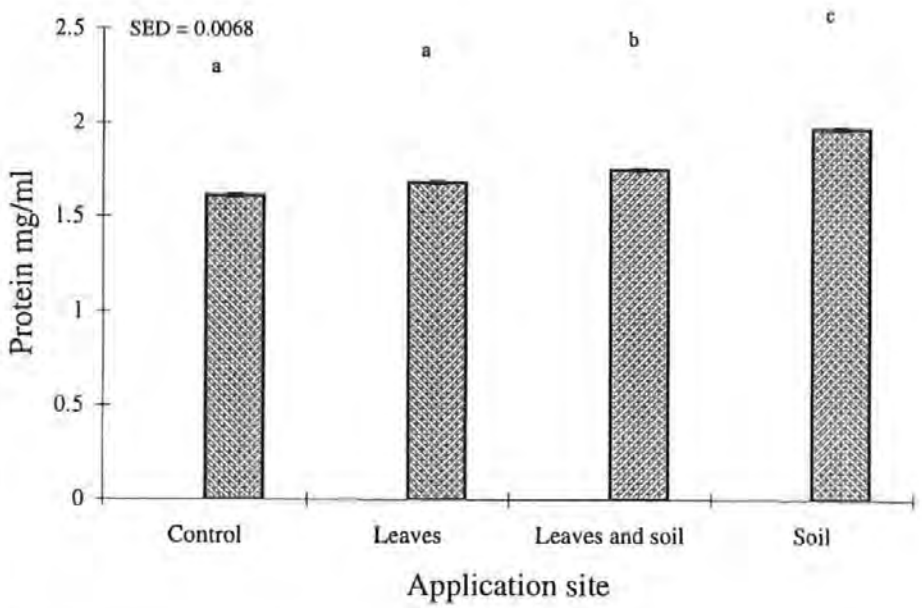


Figure 3.3: The effect of LSE application site on the concentration of total soluble protein (Letters denote significant differences)



3.11. Results of experiment 3.4: electrophoretic comparison of protein expression in winter barley plants subjected to normal growth conditions, cold-acclimation and liquid seaweed extract treatments

There was no significant difference between the total number of proteins, detected by densitometry, across the treatment structure and this is consistent with visual observations (Plates 3.1, 3.2, 3.3 & 3.4). However, there was a significant ($p< 0.05$) increase in the number of high molecular weight proteins (HMW) (proteins between 200kDa and 57kDa) being expressed across the treatment structure (Figure 3.4)

Non-acclimated plants had the lowest number of HMW proteins and were not statistically different from the NA1LSE plants. Multiple applications of LSE to NA plants resulted in a significant increase in the number of HMW proteins and the numbers of HMW proteins, in NA3LSE plants were similar to CA plants. Applications of LSE to CA plants resulted in no further increase in the number of HMW proteins.

Two protein species; 1) a 118kDa protein and 2) a 57kDa protein were observed to be up-regulated by both CA and LSE applications. The reported position of the 118kDa protein is the average position of this protein, from three samples analysed by densitometry, for example figure 10A (appendix 7A) illustrates a novel protein at peak 7 with a relative MW of 125kDa (Table 3A).

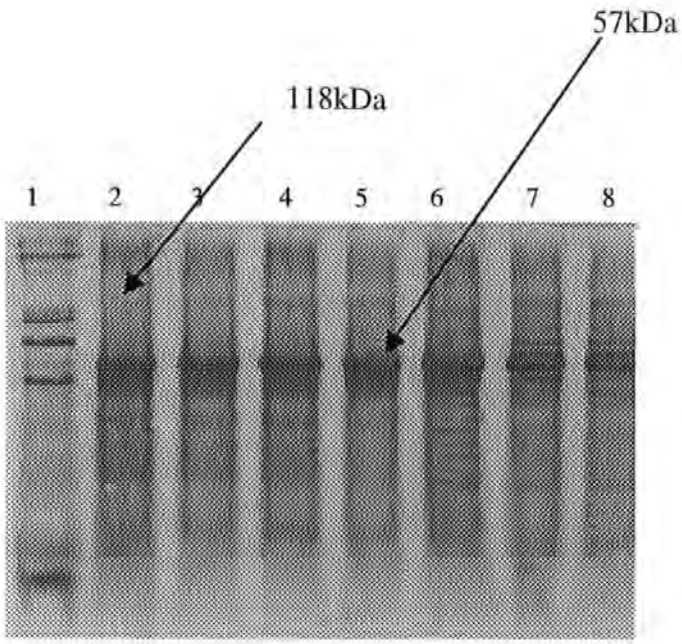
The level of expression, measured as relative percentage expression, of the 118kDa protein was subjected to analysis of variance for the 0 day and 4 day sampling periods. The 7 and 10 day sampling periods were too variable for analytical procedures.

At day 0 there was a significant difference in the level of expression of the 118kDa protein across the treatment structure. This protein species was virtually absent from NA plants then increased either with CA proportionally with increasing LSE to NA plants (Figure 3.5).

At day 4 there was a distinct division in 118kDa expression. Non-acclimated and NA1LSE plants are not significantly different from each other and had the lowest level of expression. Conversely NA3LSE, CA, CA1LSE and CA3LSE plants had the highest level of 118kDa expression and were significantly different from NA and NA1LSE plants (Figure 3.6).

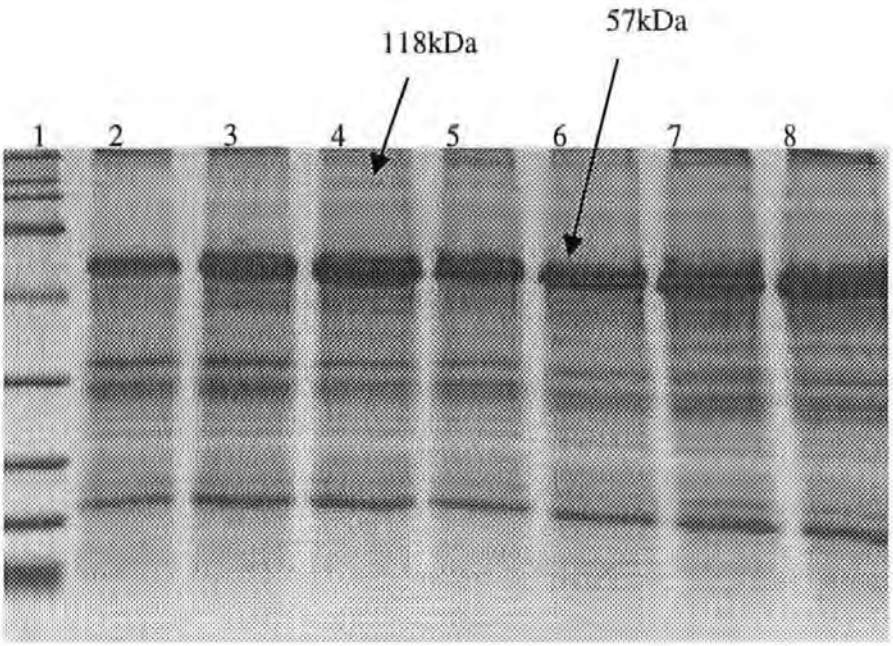
Since data was available for each sampling period the level of expression of the 57kDa protein was subjected to a factorial analysis. There was no significant interaction between sampling period and treatment and no significant sampling effect. However, there was a significant treatment effect ($p<0.05$) with NA plants expressing the lowest levels of the 57kDa protein, with the levels of this protein in NA1LSE plants being statistically similar to NA plants and CA plants. Multiple applications of LSE to NA plants increased the level of 57kDa expression. Indeed, CA, CA1LSE, and NA3LSE plants had very similar levels of expression with CA3LSE plants having the highest levels of expression (Figure 3.7)

Plate 3.0: Electrophoretic gel of 0 day samples. Note low background expression of the 118kDa and 57kDa proteins in non-acclimated lane.



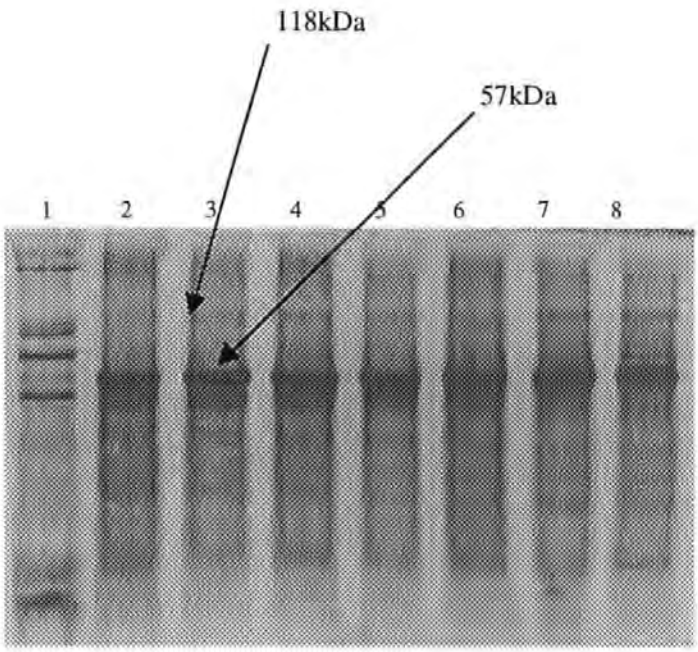
Lane 1 = Standard, lane 2 = Non-acclimated, 3 = Non-acclimated plus 1application of liquid seaweed, 4 = Non-acclimated plus 3 applications of liquid seaweed, 5 = Cold-acclimated, 6 = Cold-acclimated plus 1 application of liquid seaweed and lanes 7 & 8 = Cold-acclimated plus 3 applications of liquid seaweed. Molecular weight standard; from top to bottom, 200kDa, 116.25kDa, 97.4kDa, 66.2kDa, 45.0kDa, 31.0kDa, 21.5kDa, 14,4kDa and 6.6kDa.

Plate 3.1: Electrophoretic gel of 4 day samples. Note up-regulation of high molecular weight proteins in the cold-acclimated and liquid seaweed treated plants and the up-regulation of the 118kDa protein and the heavy 57kDa band in these plants.



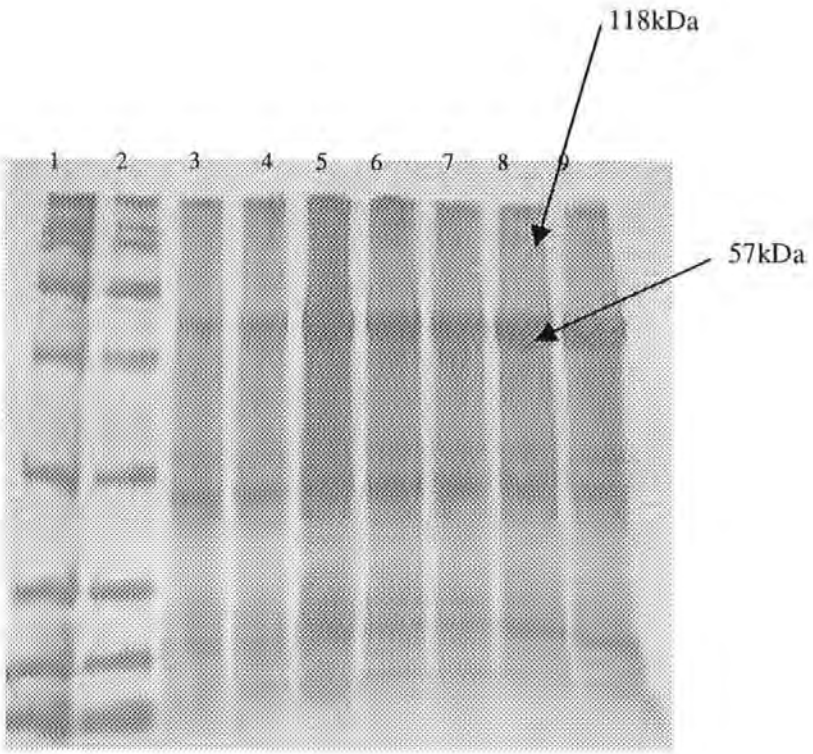
Lanes: 1 = Standard, 2 = Non-acclimated, 3 = Non-acclimated plus 1 application of liquid seaweed, 4 = Non-acclimated plus 3 applications of liquid seaweed, 5 = Cold-acclimated, 6 = Cold-acclimated plus 1 application of liquid seaweed and 7 & 8 = Cold-acclimated plus 3 applications of liquid seaweed. Molecular weight standard; from top to bottom, 200kDa, 116.25kDa, 97.4kDa, 66.2kDa 45.0kDa, 31.0kDa, 21.50kDa, 14.4kDa & 6,500kDa

Plate 3.2: Electrophoretic gel of 7 day samples. Note that the 118kDa and 57kDa bands are less dense in the Non-acclimated plus liquid seaweed treated lanes, especially where only one application was applied.



Lanes: 1 = Standard, 2 = Non-acclimated, 3 = Non-acclimated plus 1 application of liquid seaweed, 4 = Non-acclimated plus 3 applications of liquid seaweed, 5 = Cold-acclimated, 6 = Co acclimated plus 1 application of liquid seaweed and 7 & 8 = Cold-acclimated plus 3 applications of liquid seaweed. Molecular weight standard; from top to bottom, 200kDa, 116.25kDa, 97.4kDa 66.2kDa, 45,0kDa, 31.0kDa, 21.5kDa, 14.4kDa & 6.5kDa

Plate 3.3: Electrophoretic gel of 10 day sample. Although the resolution of this gel is poor it can be clearly seen that there is a decline in the expression of the 118kDa protein in both the treated non-acclimated lanes.



Lanes: 1 & 2 Standard, 3 =Non-acclimated, 4 = Non-acclimated plus 1 application of liquid seaweed, 5 = Non-acclimated plus 3 applications of liquid seaweed, 6 = Cold-acclimated, 7 = Cold-acclimated plus 1 application of liquid seaweed and 8 & 9 = Cold-acclimated plus 3 applications of liquid seaweed. Molecular weight standard; from top to bottom, 200kDa, 116.25kDa, 97.4kDa, 66.2kDa, 45.0kDa, 31.0kDa, 21.5kDa,14.4kDa & 6.5kDa.

Figure 3.4: The effect of cold-acclimation and liquid seaweed extract on the expression of high molecular weight proteins in winter barley (Letters denote significant differences)

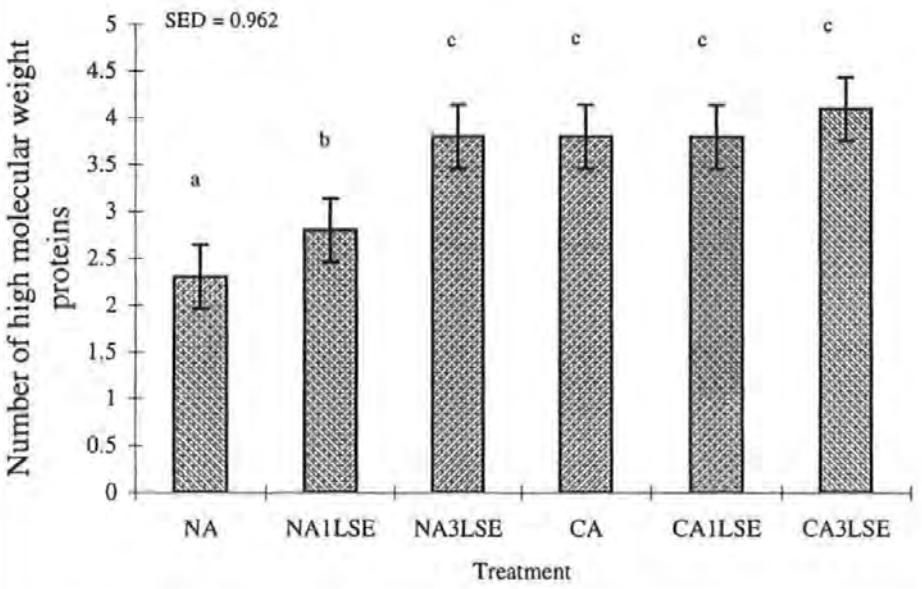


Figure 3.5: The effect of cold-acclimation and LSE treatments on the expression of a 118kDa protein from winter barley plants at day 0. Letters denote significant difference.

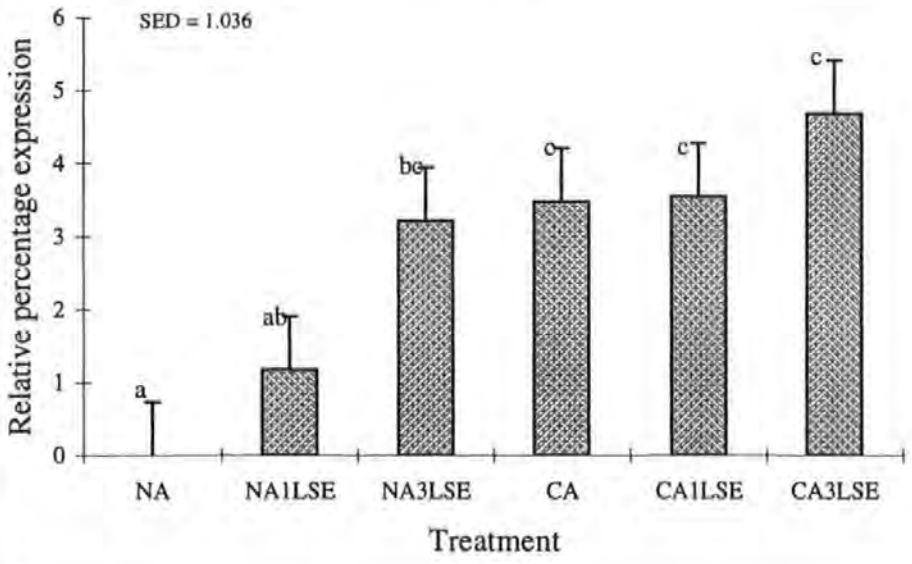


Figure 3.6: The effect of cold-acclimation and LSE treatments on the expression of a 118kDa protein from winter barley plants at day 4. Letters denote significant difference.

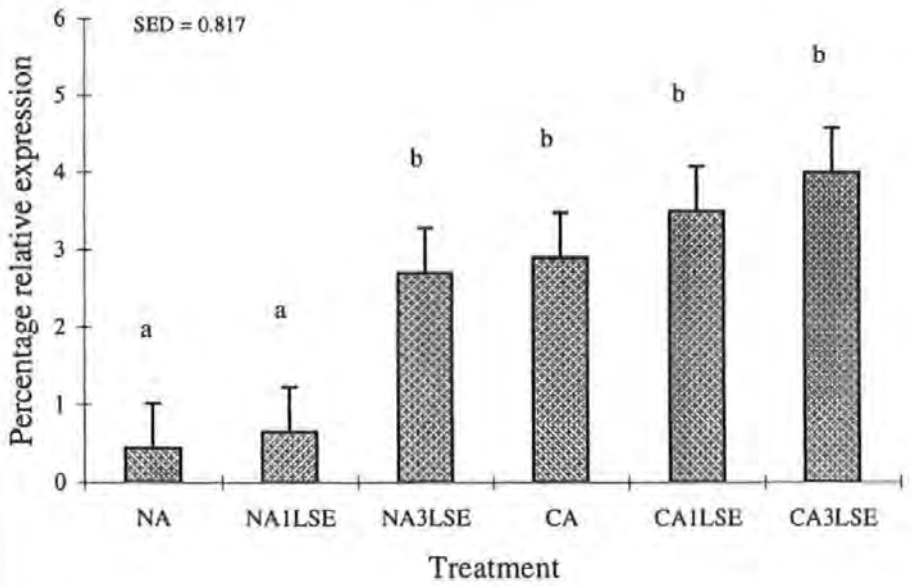
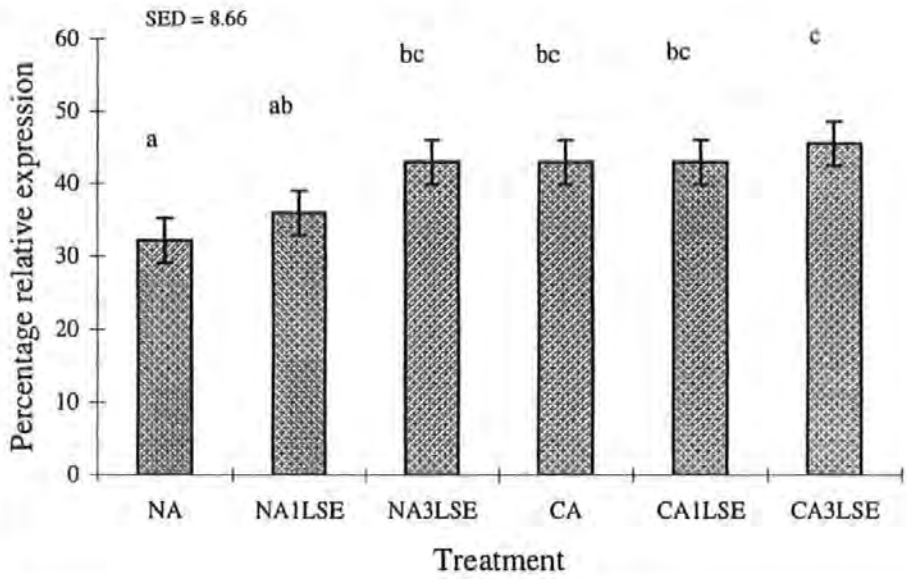


Figure 3.7: The effect of cold-acclimation and LSE treatments on the level of expression of a 57kDa protein from winter barley plants. Letters denote signifcant difference.



3.12. Results of experiment 3.5: electrophoretic comparison of protein expression in winter barley plants subjected to liquid seaweed extract and simulated rain events

Simulated rain events had a significant effect on the expression of HMW proteins, with artificial rain at day 1, 2 and 3 reducing the number of HMW proteins (square root transformed data) compared to the no rain plants. Day 3 was not significantly different from the no rain plants. In all cases there were significantly more HMW proteins in the LSE treated plants compared to the control plants (Table 3.2) (Plate 3.4).

Table 3.2: The effect of simulated rain events of the number of high molecular weight proteins, enhanced by LSE treatments.

Rain event	Number of high molecular weight proteins	s.e.
Control	1.410	0.0001
Day 1	1.730	0.0001
Day 2	1.730	0.0001
Day 3	1.900	0.1700
No rain	2.08	0.0800

Effect of simulated rain on the expression of the 118kDa protein, enhanced by liquid seaweed extract

Simulated rain events at day 1, 2 and 3 significantly reduced the concentration of the 118kDa protein compared to the "no rain" plants. Once again all LSE treated plants had a significant increase in the concentration of the 118kDa protein compared to the control (Table 3.3) (Plate 3.4).

Table 3.3: Effect of simulated rain on the expression of the 118kDa protein, enhanced by LSE treatments.

Rain event	Percentage expression of 118kDa protein	s.e.
Control	0.000a	0.000
Day 1	3.043b	0.344
Day 2	3.460b	0.248
Day 3	3.370b	0.242
No rain	5.140d	0.468

Letters denote statistically significant differences.

Effect of simulated rain on the expression of the 57kDa protein, enhanced by liquid seaweed extract

Simulated rain events significantly reduced the concentration of the 57kDa protein at day 1 compared to either day 3 and the "no rain" plants. At day 2 the concentration of the 57kDa protein was statistically similar to day 1 and the "no rain" plants. There was no significant difference in the concentration of the 57kDa protein in the day 3 plants and the no rain plants. All LSE treated plants had a significantly higher concentration of the 57kDa protein compared to the control plants (Table 3.4)

Table 3.4: The effect of simulated rain on the expression of the 57kDa protein, enhanced by LSE treatments.

Rain event	Expression of the 57kDa protein	s.e.
Control	23.580 a	3.343
Day 1	34.890 b	0.883
Day 2	35.290 bc	2.390
Day 3	45.433 cd	1.816
No rain	41.983 d	1.330

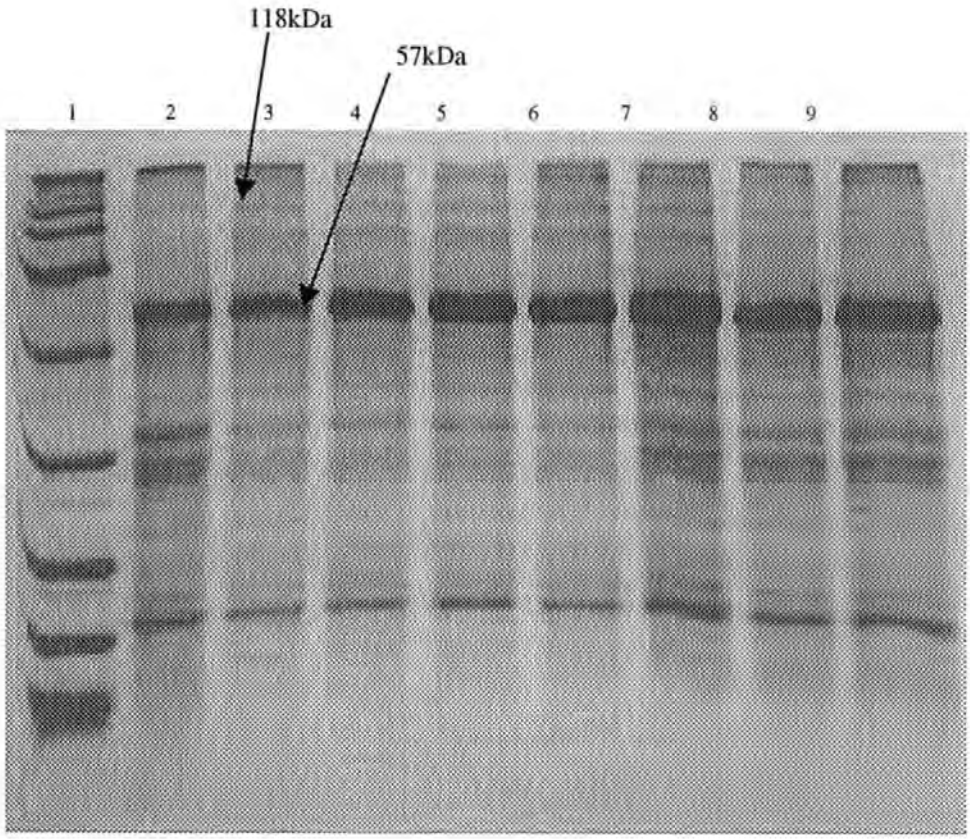
Suffix denote statistically significant differences.

Poor resolution and separation of proteins in the application site gels made valid comparisons between treatments impossible.

3.13. Results of experiment 3.6: electrophoretic comparison of heat stable proteins

No statistical analysis was carried out on these gels due to poor electrophoretic resolution. However, visual observations suggest that some of the HMW proteins are heat stable and that there is a particular heat stable protein around the 57kDa position, which appears darker in the CA plants.

Plate 3.4: Electrophoretic gel showing the effect of precipitation on liquid seaweed mediated protein expression. Note the density of the 57kDa band is reduced in lanes 3, 4 and 5 compared to lane 6. Also the expression of the 118kDa protein is also reduced in lanes 3, 4 and 5 compared to lane 6. However, the expression of both these proteins is still being up-regulated compared to those ob served in the control lane, lane 2.



Lanes: 1) = Standard, 2) = Control (no liquid seaweed), 3) = Rain at day 1, 4) = Rain at day 2, 5) = Rain at day 3, 6) = No Rain. 7) = Non-acclimated plus 3 applications of liquid seaweed from 4 day sample, 8) = Cold-acclimated from 4 day sample and 9) = Cold-acclimated plus 3 applications of liquid seaweed from 4 day sample, for comparision. All rain treated plants received 3 applications of liquid seaweed extract. Molecular weight standard; from top to bottom, 200kDa, 116.25kDa, 97.4kDa, 66.2kDa, 45.0kDa, 31.0kDa, 21.5kDa, 14.4kDa & 6.5kDa.

3.14 Western Blotting

The tentative identification of the 118kDa was partially achieved using Western Blotting techniques with a monoclonal antibody raised against the conserved dehydrin motif. There were consistent reports of a 118kDa protein in the three Western Blotting procedures carried out on the 4 day gels. However, the level of expression was very low making image capture impossible, furthermore, there were no other dehydrin proteins identified on the Western Blots. This observation in inconsistent with the literature on dehydrin expression in winter barley. The possible reasons for this observation are that transfer of proteins from polyacrylamide gel to nitro-cellulose membrane were incomplete, the second antibody was inoperative, the colour development was faulty or the primary antibody (the dehydrin probe) was inoperative. Results suggest that the epitope of the dehydrin probe was non-functional. Successful transfer of proteins from gel to nitro-cellulose membrane was complete (checked by staining the gel after transfer which showed visible depletion of proteins indicating successful transfer). The secondary antibody appeared to be functioning correctly because both the 200kDa and the 97.4kDa proteins in the standard (which are sourced from rabbit muscle) were detected. This also suggests that the colour development system was in working order. A schematic representation of the above results illustrates the trends observed in the 3 Western Blots conducted on the 4 day post LSE application gels (Figure 3.8).

Figure3.8: Schematic representation of Western Blotting procedures carried out on 4 day post LSE application.

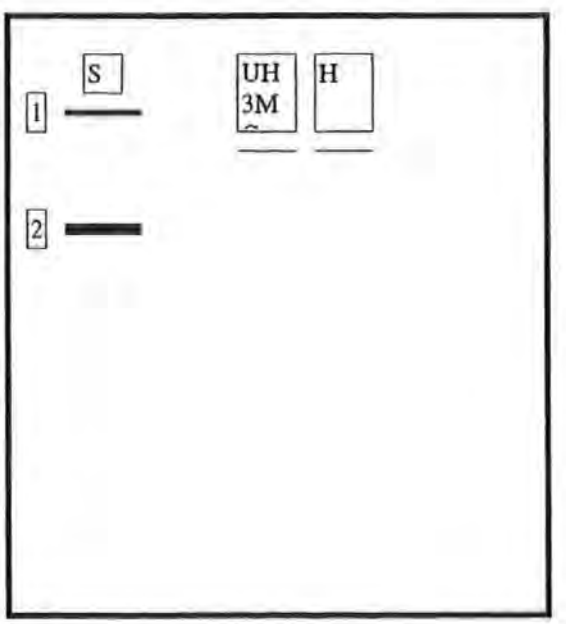
Key:
S = MW Standard

UH3MC = 3 applications of LSE lane

H = Hardened Lane

1 = 200kda Protein Myosin, rabbit skeletal muscle

2 = 97.4kda Protein Phosphorylase B, rabbit muscle



3.14.Discussion

Extraction buffer protocol

It is clear from this series of experiments that the ratio of buffer to plant material is crucial, with 3 parts buffer to 1 part plant material being the optimum ratio. This was the case for both the Tris-HCL and Borate buffers. The addition of 2% sodium dodecylsluphate (SDS) to the buffer system did not improve protein yield, but this may be due to interference of the SDS with the Bradford reagent. Treatments with SDS in the presence of a reducing agent causes the polypeptide chains to unfold and assume a rod like structure, in which the polypeptide core is coated with SDS molecules (Svasti and Panjipan, 1977). The bound SDS molecules may well block the binding of the Bradford reagent and give a spurious result.

The importance of the buffer:material ratio is quite relevant to plant tissues. Only a small fraction of the volume of plant tissue is truly intracellular, large vacuoles (which can be regarded as extracellular) and intracellular spaces means that on disruption liquid is released, making additional extractant liquid almost unnecessary (Scopes, 1987). The residue after centrifugation may occupy only 20 - 40% of the volume of the original plant tissue. However, because plant tissue contains phenolic compounds, which oxidise (mainly under the influence of endogenous phenol oxidases) to form dark pigments, which attach to proteins and react covalently to inactivate them (Scopes, 1987), addition of some extractant liquid will control this undesirable process. The volume of extractant per gram of material will tend to be a compromise between maximum extractant and minimum volume.

Taking into account the variability of protein extraction methods data from the standardised procedure implicated Tris-HCL pH7 as the most suitable extractant buffer for the extraction of total soluble protein from winter barley.

The effect of cold-acclimation and liquid seaweed extract application on the concentration of total soluble protein

Data from experiment 2 confirmed reports that CA increases the concentration of total soluble protein (Graham and Patterson, 1982). The nature of this increase and the identity of the protein species involved cannot be determined from this series of experiments. It is possible to speculate that a) protein turn-over has been slowed and/or b) that new, cold inducible, stress proteins may be involved in the process of CA (Guy 1990; Cattivelli and Bartels, 1992). These data also illustrate that multiple applications of LSE to NA plants increases the concentration of soluble protein, to levels that approximate those seen in CA plants.

This LSE enhanced increase in protein may be a potential response to an 'elicitor like' component in the LSE, which pre-conditions the plant for resistance to stress, in a similar way to salicylic acid as a known elicitor of the systemic acquired resistance mechanism in plants against plant pathogens (Arteca, 1996 and Raskin, 1992). Alternatively the increase in soluble protein may be the result of increased chlorophyll content and associated enzymes and carotenoids, (Anon, 1997e; Hankins, personal communication) and presumably an increase in ribulose 1,5-bisphosphate carboxylase (RUBISCO). Interestingly there is no significant increase in soluble protein in LSE treated CA plants, an observation which cannot easily be explained. Perhaps the CA state has a physiological maximum with respect to protein metabolism.

The effect of artificial rain and application site on liquid seaweed enhanced protein expression

If LSE has a component that initiates changes in protein metabolism and this change is associated with the increase in frost resistance, it would not seem unreasonable to suggest that periods of high rain fall, immediately after LSE application, would reduce this effect. That being the case this phenomena would reduce the concentration of soluble protein, an hypothesis supported by the data from experiment 3.3

These data clearly show a fall in protein concentration following rain events at day 1 and day 2. However, after 2 days simulated rain events had little effect on the interaction of LSE and protein metabolism. Indeed high rainfall would certainly dilute and leach out the LSE solution from the soil and data from the site of application experiments illustrated that, applications of LSE to the soil had the most significant effect on plant growth and protein metabolism.

Electrophoretic Separation

Electrophoresis gives evidence to suggest that CA is having a profound effect on protein expression. There are several protein species in the high molecular weight (HMW) region (between 200KDa and 57KDa) that are being up-regulated by CA, but it is not clear from these gels whether these are new protein species or up-regulation of constitutively expressed proteins. This increase in HMW proteins has been reported for Spinach, (Guy and Haskell, 1987 and Guy, Haskell, Neven, Klein and Smelser, 1992) where CA of Spinach was at 5^{o}C.

Immunoblot analysis for two Spinach proteins, (160KDa and 85KDa) from the leaves of seedlings subjected to CA, revealed a low temperature-induced rise in the steady-state concentration of these proteins (Guy et al, 1992). These proteins were present at low levels in non-stressed plants grown at 25^{o}C and increased in amount when transferred to 5^{o}C (Guy et al, 1992).

Further evidence to suggest that some cold-stress related proteins may be constitutively expressed, at NA temperatures, was illustrated in a study on the seasonal patterns of dehydrin and heat shock protein expression in the bark tissue of eight species of woody plants (Wisniewski, Close, Artlip and Arora, 1996). In this study Wisniewski and colleagues showed that both a 60KDa dehydrin and HSP 70, HSC70 and BiP were constitutively expressed.

The expression of these proteins appeared to be highest from Autumn to Spring (Wisniewski *et al,* 1996).

Multiple applications of LSE to NA plants also results in the up-regulation of HMW proteins and this level of expression approximates that seen in CA plants. This observation is repeated in LSE treated CA plants, once again there is an increase in the number and density of HMW proteins.

The reproducible expression of a protein band at the apparent MW of 118KDa in CA, CALSE, CA3LSE and the NA3LSE plants implies that this protein is involved in the CA processes and may be an adaptable response to low temperature stress. Furthermore, there is evidence to suggest that multiple applications of LSE increase the expression of this protein to similar levels as that seen in CA plants, 4 days after final LSE application.

The non significant difference in the expression of the 118KDa protein between NA1LSE and the NA plants suggest that the up-regulation of this protein requires at least 3 applications of LSE to bring about a significant increase in protein expression. It may be that the expression of this protein is under the influence of developmental age (Pearce *et al,* 1996) and clearly 3 applications of LSE has the most significant effect on plant growth and frost resistance. Conversely the expression of this protein may be under the influence of mild osmotic shock around the rhizosphere, due to the K^+ salt content of LSE, or responding to ABA or an ABA like compound.

Guy *et al* (1992) reported that when water was withheld from young Spinach seedlings, water stressed leaves accumulated both the 160 and 85KDa proteins in amounts at least equal to or even greater than could be obtained by low temperature. When the water stress was removed the concentration of these proteins declined to that in unstressed plants after about one week.

If the 118ka protein is responding in a similar fashion as that just described, then this observation could partly explain the variability in 118kDa expression at days 7 and 10, where results were too variable for analytical procedures.

Another protein, of apparent MW 57kDa, was repeatedly observed to respond to CA and LSE treatments. There are, however, several problems with this observation. Firstly RUBISCO is a 560kDa protein made up of 8 56kDa subunits and 8 14kDa subunits (Salisbury and Ross, 1992). It can be seen from plates 3.1 to 3.4 that there is a regular occurrence of these 2 subunits across the treatments structure. It has been shown that in both monocots and dicots that CA suppresses the synthesis of both RUBISCO subunits (Hahn and Walbot, 1989 and Meza-Basso, Alberdi, Raynal, Ferrero-Cadinanos and Delseny, 1986). In rice (Hahn and Walbot, 1989) the smaller subunit was suppressed more than the larger subunit. The suppression of RUBISCO synthesis by cold was shown to result from reduced levels of mRNAs encoding both subunits (Hahn and Walbot, 1989).

The results from experiment 4 appear to contradict these observations. Here the protein band in the region of 57kDa is arguably the large subunit of RUBISCO and does appear to be up-regulated by CA and LSE application. In the case of LSE application increase in the concentration of the 57kDa protein (or presumably the large subunit of RUBISCO) would be expected because LSE enhances chlorophyll production and photosynthesis (Anon 1997e).

There are, however, several reports of a dehydrin and/or a dehydrin like protein around 55kDa to 60kDa (Wisniewski et al 1996; Muthalif and Rowland, 1994 and Danyluk, Perron, Houde, Limin, Fowler, Benhamou and Sarhan, 1998) and these reports demonstrate that proteins in this region are up-regulated by CA. Data from experiment 6 report the presence of a protein around the 57kDa MW which is heat stable. Unfortunately there was no reaction of this protein with the dehydrin antibody. It may be possible that the large RUBISCO subunit and dehydrin, or a dehydrin like protein, occupy similar MW sites in the resolving gels used in these experiments and thus give rise to conflicting

results, for example CA could be up-regulating a 57kDa dehydrin and simultaneously down-regulating the large RUBISCO subunit.

If LSE is genuinely up-regulating cold inducible proteins then it is expected that simulated rain events, following LSE application, will have an adverse effect on this process. This is certainly the case, because the number of HMW proteins, the 118kDa and the 57kDa proteins were down-regulated where simulated rain was applied in the first 48 hours following LSE application. After this time period the deleterious effect of artificial rain was greatly reduced. Furthermore, in all cases multiple applications of LSE increase the number of HMW proteins and the concentration of the 118 and the 57kDa proteins. These independent observations add further support to the hypothesis that multiple LSE applications induce protein changes that are potentially involved in cold-acclimation.

Western Blotting

A tentative identification of the 118kDa protein was partially achieved using Western Blotting techniques. There was a weak response of this protein to the monoclonal antibody raised against the conserved motif of plant dehydrins. Problems of weak reaction were investigated, with the variables of inactive colour development solution, inactive conjugate and/or poor transfer of separated proteins studied. Staining gels following transfer confirmed acceptable transfer of proteins to nitrocellulose membrane. The conjugate and colour developer were functioning correctly because both the 200kDa and the 97.4kDa proteins in the standard (which are sourced from rabbit muscle) were clearly detected. This suggest that the primary antibody was inactive.

Interestingly Guy et al (1992) reported that a 117kDa protein from Spinach, subjected to CA, responded to a 2H8 antibody raised against a 160kDa protein also isolated from Spinach. The expression of this 160kDa protein was also regulated by water stress, where periods of water stress resulted in up-regulation of this protein. These results may suggest that both the 117kDa and the 160kDa proteins from Spinach are dehydrins, (because the

2H8 antibody may be responding to the conserved motif of the general plant dehydrin) this being the case 118kDa may well be a dehydrin.

3.15. Conclusion

This series of protein experiments clearly demonstrates that cold-acclimation and multiple applications of LSE increase protein expression and up-regulate HMW proteins. This phenomena is reduced if precipitation occurs in the first three days following LSE application and is consistent with field observations of reduced frost resistance in LSE treated plants following heavy rainfall. There is evidence that two proteins (118kDa and a 57kDa protein) respond to both CA and LSE applications in a similar fashion and thus maybe an adaptable response to environmental stress, in particular dehydration.

Chapter 4: Water Relations and Thermal Analysis

4.0 Water Relations and Thermal Analysis

4.1 Introduction

The analysis of protein metabolism in chapter 3 indicated that both cold-acclimation (CA) and liquid seaweed extract treatments (LSE) had a profound effect on plant protein expression. In both cases there was an increase in total soluble proteins and also an increase in the expression of two specific proteins (118kDa & 57kDa), which may have a role in increased frost tolerance. These latter 2 proteins may be involved in stress relief through chaperon activity or they may be isomer variants of house keeping proteins (Guy, 1990). Furthermore, it may be possible that these two protein species are involved in modifications to the hydration status of the cellular environment (Abe and Yoshida, 1997; Guy *et al*, 1992). Indeed the doubling of water soluble proteins in the CA and LSE treated plants must have an effect on the matric potential of these plants, which in turn will affect the final water potential.

In a complex system such as a cell, water interacts with the hydrophilic surfaces of solutes and colloidal substances (proteins, starch and simple ions) and thus cellular water is in a dynamic state of colloidal associations (Olien, 1977); such associations are influenced by the surface properties of the colloid and the distance between the colloid and the absorbed water molecule. Water molecules located nearer the colloidal surface are more tightly bound than those furthest away (Salisbury and Ross, 1992).

The interaction between water molecules and colloids in plants gives rise to the matric potential (T) of the water potential equation;

$$\Psi = P + \Psi s + T$$

where; Ψ = water potential, P = pressure or turgor potential, Ψs = osmotic potential and T = matric potential.

It is considered that P and Ψs are the major components of Ψ and often T is considered so small that it is ignored in measurements of Ψ. However, in viable cells the final water

potential will be determined not only by the pressure and solute potentials but also by the proteins and other colloidal substances of the cell (Salisbury and Ross, 1992).
The biological reactions of life occur on surfaces and it is easy to see how relatively large surfaces can exist in a single cell. It is also easy to see how hydration (matric forces) can influence the water milieu of cells (Salisbury and Ross, 1992).

It is logical then to hypothesise that both CA and LSE treated plants should have a decreased water potential compared to non-acclimated plants (NA). If this decreased water potential is the consequence of changes in solute and protein concentrations this should be detectable by thermal analysis (differential scanning calorimetery and thermocouple measurements). The rationale for this is that when water freezes it releases the latent heat of fusion and results in a detectable exotherm. The size of the exotherm is directly proportional to the amount of water frozen (assuming that only water is freezing and not water like substances e.g. dehydrins) (Close, 1996). Therefore if CA and LSE plants have less free water then the area under the exotherm curve should be significantly less then that observed in NA plants.

4.2.0 Experiment 1: changes in leaf water potential of winter barley plants with reference to cold acclimation

Materials and Method

Culture conditions: Winter barley cv Igri was raised as described in section 3.1.0 with the following modifications: seed was sown in standard seed trays and the seedlings were transferred to a large phytotron at growth stage 10. Trays were placed on capillary matting with a 20°C/16°C day/night temperature with a 16 hour photoperiod; plant hardening was carried out in a phytotron at a constant 4°C with a 9 hour photoperiod.

Sampling: Water potential was measured with a J14 press (Bristow, Van Zyl and De Jager, 1981) on the second youngest leaf of winter barley plants starting at growth stage 15. Two measurements were carried out, on 2 consecutive days, at 20°C/16°C before plants were transferred to the 4°C phytotron. Further water potential measurements were carried out after 48h, 72h, 96h, 120h, 144h and 168h, at 4°C treatment. All water potential measurements were carried out in the last 2 hours of the light period.

Experimental design and statistical analysis: A completely randomised design was employed and data was analysed using one-way analysis of variance based on 16 replicates. This approach was also used in experiment 2.

4.2.1 Experiment 2: changes in plant water potential of winter barley plants subjected to applications of liquid seaweed extract and/or cold acclimation treatments

Culture conditions: Plants were raised as described in section 4.1.0 but CA was carried out for 14 days before water potential measurements. Sequential sowing was applied to ensure a similar developmental stage for CA and NA plants. Liquid seaweed extract (LSE) was applied as described in section 2.1.

Sampling: As per section 4.2.0.

4.2.2 Experiment 3: temporal changes in plant water potential following applications of liquid seaweed extract

Culture conditions and treatments: Plants were raised and treated as described in section 4.2.0.

Sampling: Water potential measurements were carried out on the second youngest leaf at day 0, 2, 4, 6, 8 and 14 post final LSE treatment. (Day 0 is where NA1LSE and CA1LSE plants have had no LSE applications but NA3LSE and CA3LSE plants had received 2 LSE applications, at 10 day intervals). Non-acclimated plants were sampled in the 20°C/16°C phytotron and CA plants were sampled in the 4°C phytotron.

Experimental design and statistical analysis: A completely randomised design was employed with 5 replicates for each treatment and data were analyzed using analysis of variance.

4.2.3 Experiment 4: determination of percentage water content in shoot tissues of winter barley seedlings

Culture conditions and treatments: Plants were raised, CA and treated with LSE as described in section 4.2.0.

Experimental design and sampling: A completely randomised design was employed with 6 replicates for each treatment. Sampling was at 4 days post final LSE application. Roots were removed, samples weighed and then placed in a fan assisted oven for 48 hours at 80°C. Dry weights were obtained directly after samples were removed from the oven. Percentage water content was calculated as follows:

$$(FW - DW)/FW * 100$$

where FW = fresh weight, DW = dry weight.

Data analysis: Data was subjected to arcsin squareroot transformation and analyzed using one-way analysis of variance.

4.2.4 Experiment 5: determination of the percentage of unfrozen water in leaf and crown tissue samples by differential scanning calorimetery

Culture conditions and plant treatments: Plants were raised, CA and treated with LSE as described in section 4.2.0.

Sample preparation and calorimetery: Two tissue types (leaf and crown) were subjected to differential scanning calorimetery (DSC). Sections of leaf, 0.3cm long, were taken from the fully expanded section (3.5cm from the leaf tip) of the second youngest leaf. Samples were weighed and sealed in aluminium crucibles. Crown tissue samples were prepared from the main stem of plants, 0.3cm samples were dissected, 2cm from the base of the stem, weighed and sealed in aluminium crucibles.

Samples were loaded into an automatic dispenser of the Mettler^{TM} Toledo DSC 820 (courtesy of Professor W.Block of the British Antartic Survey, Cambridge) and run to a pre-programmed cooling and heating cycle. Cooling rate was $1^{o}C\ min^{-1}$ from $5^{o}C$ to $-40^{o}C$. Samples were held at $-40^{o}C$ for 5 minutes then heated to $35^{o}C$ at $1^{o}C\ min^{-1}$. The DSC unit was linked to a Pentium PC and data were collected directly from the DSC unit as the experiment proceeded. Curves obtained from the heating/cooling cycle were analysed using integration of the area under the endotherm peak (the ice melt peak) using custom Mettler^{TM} software. The procedure used for determining the percentage of unfrozen water was that described by Roos, (1986).

Following the DSC scan, samples were placed in a fan assisted ovens for 48 hours at $80^{o}C$ and then re-weighed to obtain an estimate of the total water content of the DSC samples.

Statistical analysis: Two replicates of each treatment were analyzed using one-way analysis of variance. Arcsin squareroot transformation was applied to the data set.

4.2.5 Experiment 6: Detection and comparison of plant freezing curves with respect to cold-acclimation and liquid seaweed extract treatment using infrared thermography and thermocouple analysis.

Plant culture, cold-acclimation and liquid seaweed extract treatments: Plants were raised and CA applied as previously described, except that plants were raised in individual $10cm^3$ module trays. Liquid seaweed extract was applied to both CA and NA plants using the previously described method, section 2.2.0. However, the single application approach was dropped in favour of multiple (3) applications. The rationale for this is that single applications have little effect in the improvement of the frost resistance of plants and freezing chamber space was limited. The full treatment structure was as follows;

Non-acclimated (NA)

Non-acclimated plus 3 applications of LSE (NAMLSE)

Cold-acclimated (CA)

Cold-acclimated plus 3 applications of LSE (CAMLSE)

Freezing Chamber and Protocol: The frost chamber used was that described by Fuller and Le Grice (1998) which mimics overnight radiative freezing. A radiative cooling plate at the top of the chamber, cooled to -45°C using Hydro-Flouro Carbon refrigerant (HFC) acts as a black body absorbing long wave radiation from materials placed in the chamber. To prevent the walls of the chamber radiating energy to test materials they are lined with cooled side plates which can be adjusted from 5 to -5°C. The overall size of the chamber is (1m x 1m x 2m high).

To maintain a slow freezing rate (1 to 2°C h^{-1}) the side plates were set at -5°C and allowed to run for 2 hours prior to testing to remove residual heat. After plants were positioned in the chamber the side plate setting was reduced to -1°C and the top plate was switched on. Empirical evidence (data not shown) indicated that this protocol was sufficiently stable and gave an initial cooling rate between 4 and 7°C h^{-1} until a temperature just below 0°C was reached. After this rate of temperature drop reduced steadily. This data is in agreement with that of Fuller and Le Grice (1998).

Infrared Thermography: infrared thermography was carried out using an imaging radiometer (model 760, Inframetrics, North Billerica, MA) with an HgCdTe long wave (8 -12μm) detector. Infra-red images were captured in real time and recorded on video tape for future analysis. The temperature span was set at 2°C, since temperature spans higher than 5°C would not detect the small exothermic event associated with plant freezing (Wisniewski, Lindow and Ashworth, 1997). Temperatures below the selected span are displayed as black and those above as white and within the temperature span as a series of colour shades or in grey scale.

Freezing protocol: plants were removed from modules, complete with compost and roots, and supported on cork tiles, using large plastic headed pins, which helped minimise artificial ice nucleation. The plug of compost was insulated with foam rubber. The cork tile, complete with plants, was positioned in a box at the centre back of the freezing chamber. Copper/constantan thermocouples were positioned under the test leaf (second youngest leaf on the main shoot) and in the middle of the of the main shoot. Thermocouples were connected to a multi channel Delta-T Data logger (Delta-TDevices Ltd, UK) with logging events at every 30 seconds.

Ice nucleation was controlled by using a suspension of *Pseudomonas syringae* strain 29E, supplied by Dr M Wisniewski of the Appalachian Fruit Research Station, West Virginia, USA, with a nucleating temperature of 2.95°C +/- 0.058 s.e. The radiometer was mounted on a tri-pod and positioned in the chamber so that test plants were clearly visible and both the 2nd youngest leaf and the crowns of the main stem of test plants were clearly imaged. Ice nucleating bacteria (35μl drops) were positioned on the 2nd youngest leaf, approximately 14 cm from the top of the leaf sheath. Plants were frozen for 1 hour after the initial freezing of the last nucleated plant. This ensured that at least 1 hour of extracellular freezing was recorded for each test plant.

Data collection: thermocouple data was downloaded to a PC computer and analysed using dedicated software Delta-TView (Delta-TDevices Ltd, UK). The integral of the exotherm curve during a 60 minute period from the appearance of the exotherm was recorded and

analysed. This was achieved by marking the thermocouple trace with a vertical cursor line at the beginning of the exotherm and a second after 60 minutes and integrating the area under the trace over this period. Data was then exported to Excel 5 for (Microsoft Ltd) for graph drawing. Freezing of both leaf and crown tissue was analysed.

Infrared data was collected from the colour video tapes. Tapes were viewed in real time and the freezing event timed, with two Lorus R23 stopwatches, one for each plant. Timing was started at the rapid flash of blue colour, an hypothesised apoplastic flush, and stopped when the cellular water was readily freezing out, to the extracellular ice crystal (green colour). The choice of cut off colour (green) was selected because not all samples reached the final yellow to red colours, but all samples did obtain the green colour.

Plant Recovery and assessment of survival: following freezing plants were allowed to thaw for 24h at 4°C before being potted up into standard seed trays, using the previously described soil medium (section 2.1.0). Plants were then transferred to a glasshouse and the standardised culture conditions were applied (section 2.1.0). Survival scores were assessed after 14 days recovery using the 10 point scoring scheme previously described for the glasshouse frost assays in chapter 2.

Experimental design and data analysis:

Two plants were frozen in each frost test and there were 10 frost tests in total. Plants were randomly paired with 5 replicates per treatment. Data was pooled ($\log_{10}$ transformation was applied, because residual plots of the raw data illustrated increasing variance with increasing means) and analysed using one-way analysis of variance.

4.3.0 Results

4.3.1 Experiment 1: changes in leaf water potential of winter barley plants with reference to cold acclimation

After 24h in the 4°C phytotron water potential of winter barley plants significantly ($P<0.001$) increased (less negative) followed by a significant ($P<0.001$) decrease (more negative) after 48h, to a relatively constant water potential from 72h (Figure 4.1). (note: observations are based on 16 replicates).

4.3.2 Experiment 2: changes in plant water potential of winter barley plants subjected to applications of liquid seaweed extract and/or cold acclimation treatments

Cold-acclimation and exogenous applications of LSE significantly ($P<0.001$) decreased the water potential of young winter barley plants (Figure 4.2). The water potential of NA and CA plants was comparable to those in experiment 1. Single applications of LSE to NA plants significantly decreased plant water potential compared to NA controls (Figure 4.2). Three applications of LSE to NA plants significantly decreased plant water potentials to similar levels as those observed in CA and CA1LSE plants (Figure 4.2). Finally, 3 applications of LSE to CA plants resulted in the most significant decrease in plant water potentials (note: observations based on 16 replicates).

4.3.3 Experiment 3: temporal changes in plant water potential following applications of liquid seaweed extract

There was a significant interaction between plant treatments and time. In both NA and CA plants water potentials were similar to those reported in sections 4.3.1 and 4.3.2 and showed no significant deviation from their respective base values. However, applications of LSE to both NA and CA plants resulted in a significant decrease in water potentials from day 2 to day 6 post LSE application (Figures 4.3 and 4.4). In both the NA and CA plants 3 applications of LSE resulted in the most significant decrease in plant water

Figure 4.1: Changes in plant water potential with respect to cold-acclimation at 4°C. SED =0.0048

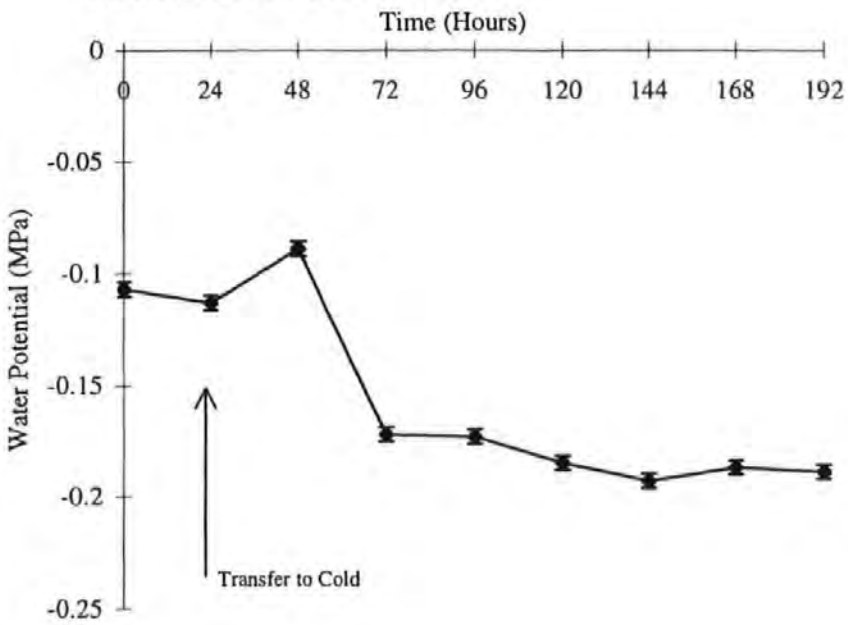


Figure 4.2: Changes in plant water potential with respect to cold acclimation and applications of liquid seaweed extract. Data are for samples at 4 days post final liquid seaweed extract application. SED = 0.00993

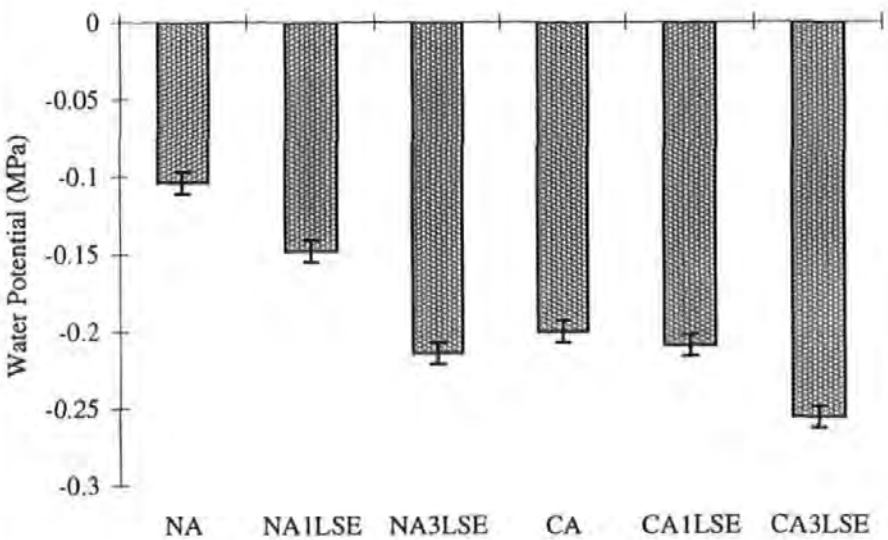


Figure 4.3: Temporal changes in plant water potential in non-acclimated plants treated with liquid seaweed extract.(Note: at day 0, NA1LSE plants had no LSE applied and the NA3LSE plants had 2 previous LSE applications at 10 day intervals).

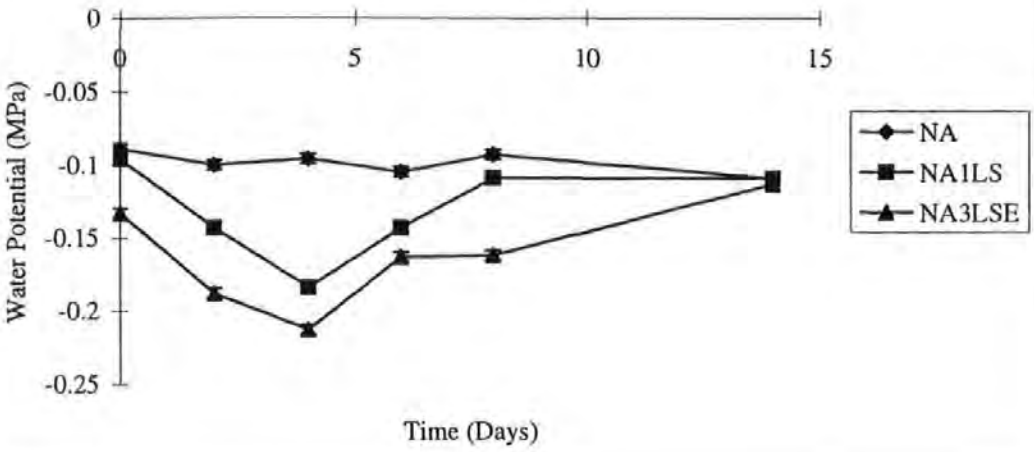
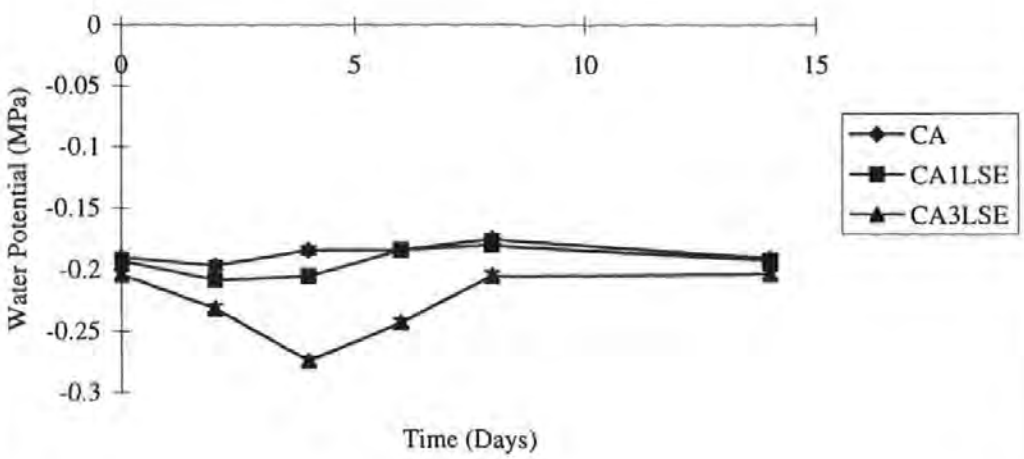


Figure 4.4: Temporal changes in plant water potential in cold-acclimated plants. (Note: at day 0, CA1LSE had no LSE applied and CA3LSE had 2 previous LSE applications at 10 day intervals).



potentials. In the CA plants 1 LSE application only illustrated a significant difference from the CA controls at day 4 post LSE application. Where NA plants were treated with 3 applications of LSE a more negative water potential at day 0 was observed, compared to the NA1LSE plants and the NA controls. This was probably due to the 2 applications of LSE applied to these plants over the previous 20 days.

4.3.4 Experiment 4: determination of percentage water content in shoot tissues of winter barley seedlings

There was no statistically significant difference in the water content of winter barley plants subjected to LSE and CA treatments (Table 4.1), although the data does show steady decline (not significant) in water content with increasing frost resistance (based on DT_{50} values from section 2.8.0).

Table 4.1: Percentage water content of winter barley plants subjected to non-acclimating and cold-acclimating conditions and with two levels of liquid seaweed application.

Treatment	Angular Transformed Means	Back Transformed (Percent Water Content)
NA	0.01640	88.3
NA1LSE	0.01638	88.1
NA3LSE	0.01636	87.9
CA	0.01633	87.5
CA1LSE	0.01631	87.3
CA3LSE	0.01630	87.2
SED	6.17×10^{-5}	

4.3.5 Experiment 5: determination of the percentage of unfrozen water in leaf and crown tissue samples by differential scanning calorimetery

The percentage water content of leaf and crown samples showed no significant difference between treatments and consequently data was pooled and average values was estimated for both leaf and crown samples, which was then used in the calculations of the percentage of unfrozen water content.

Water content of leaf samples = 87.3% +/- 2.4 s.d.

Water content of crown samples = 91.5% +/- 1.5 s.d.

Crown tissues contained 4.5% more water than leaf tissues.

Leaf Samples: Although there was a significant ($p<0.05$) treatment effect on the percentage of unfrozen water in leaf tissue samples only CA samples were significantly different from all other treatments (Table 4.2). The large reduction in the percentage of unfrozen water in the CA samples is probably due to supercooling of these samples. The mean ice nucleation point of NA samples was -14.8^{o}C +/- 0.98 s.d. compared to -16.74^{o}C +/- 0.74 s.d. for CA samples.

Table 4.2: Percentage unfrozen water in leaf samples of winter barley subjected to non-acclimating and cold-acclimating conditions and two levels of liquid seaweed applications.

Treatment	Angular Transformed Means	Back Transformed (Percentage Unfrozen Water)
NA	0.00500 a	8.2
NA1LSE	0.00545 a	9.75
NA3LSE	0.00570 a	10.67
CA	0.00345 b	3.9
CA1LSE	0.00495 a	8.04
CA3LSE	0.00475 a	7.41
SED	3.958×10^{-4}	

letters denote statistically significant differences.

Crown Tissue: There was a significant increase in the percentage of unfrozen water in the NA3LSE samples compared to NA samples and the percentage of unfrozen water in the NA3LSE samples was not statistically different to the percentage of unfrozen water observed in CA plants (Table 4.3). The trend of unfrozen water observed in NA, NA1LSE and NA3LSE samples is consistent with observations from earlier experiments such as the frost resistance studies, protein metabolism and in the water potential studies. However, there were no significant differences in any of the CA treatments (Table 4.3).

Table 4.3: Percentage unfrozen water in crown samples of winter barley subjected to non-acclimating and cold-acclimating conditions and two levels of liquid seaweed extract.

Treatment	Angular Transformed Means	Back Transformed (Percentage Unfrozen Water)
NA	0.00275 a	2.48
NA1LSE	0.00340 ab	3.79
NA3LSE	0.00555 c	10.11
CA	0.00440 b	6.36
CA1LSE	0.00530 c	9.22
CA3LSE	0.00510 bc	8.54
SED	7.269×10^{-4}	

Letters denote statistically significant differences.

4.3.6 Experiment 6: detection and comparison of plant freezing curves with respect to cold-acclimation and liquid seaweed extract treatment using thermography and thermocouple analysis.

Thermocouple data: Calculated values for the mean exotherm temperatrure (for the first hour of the exotherm) for the leaf samples illustrates a significant difference between treatments. Non-acclimated had a significantly larger mean exotherm temperature than either non-acclimated plants treated to 3 applications of LSE, cold-acclimated plants and cold-acclimated plants treated with 3 applications of LSE (Table 4.4). The mean exotherm temperature line for the crown samples also illustrated a significant difference, with the NA plants having the greatest value compared to the CA and LSE treated plants, which had lower mean exotherm temperature values (Table 4.5).

Thermography data: There was a significant difference in the time period between apoplastic freezing and full cellular freezing, in both leaf and crown samples. In the leaf samples there was no significant difference between NA plants and NA3LSE treated plants, however, in the NA3LSE plants the "time elapsed" between freezing events was statistically similar to that observed in CA and CA3LSE plants (Table 4.6).

In the crown tissue the time elapsed between freezing events mimics that seen in the exotherm curves of crown tissue (thermocouple data). Only the NA plants were statistically different (Table 4.7) in that these samples were readily freezing cellular water in 4.06 minutes compared to the average value of 16.4 minutes observed for the other treatments. It is noteworthy that there is a trend of increasing "time elapsed" between freezing events and increasing frost resistance. Comparisons of "time elapsed" for the crown tissues in NA, NALSE and CA plants (Figures 4.5, 4.6 and 4.7 illustrate the data presented in Table 4.7. The associated thermal images in figures 4.5, 4.6 and 4.7 demonstrate that water is still being released to the extracellular ice crystal in LSE treated plants whereas the untreated crowns have a reduced heat release signature. Figure 4.8 illustrates how the plants where set-up and can be used to visualise the scale of the thermal images in figures 4.5, 4.6 and 4.7.

The recovery scores of test plants clearly demonstrates that both multiple LSE treatments and CA increase the frost resistance of these plants (Figure 4.9) and thus implicate the importance of cellular water status in plant survival following freezing events.

Table 4.4: Mean temperature above base line for the first hour of thermocouple recorded exotherm for non-acclimated and cold-acclimated leaf samples treated with liquid seaweed extract.

Treatment	Mean Temperature ^{o}C	s.e.m
NA	-20.36a	0.402
NA3LSE	-15.54b	0.330
CA	-15.14b	0.503
CA3LSE	-14.34b	0.627
SED	0.677	

Letters denote statistically significant differences.

Table 4.5:Mean temperature above base line for the first hour of thermocouple recorded exotherm for non-acclimated and cold-acclimated crown samples treated with liquid seaweed extract.

Treatment	Mean Temperature ^{o}C	s.e.m
NA	-20.09a	0.706
NA3LSE	-14.46b	0.104
CA	-13.57b	0.592
CA3LSE	-13.40b	0.480
SED	0.738	

Letters denote statistically significant differences.

Table 4.6: Time elapsed between apoplastic freezing and full extracellular freezing in leaf samples of non-acclimated and cold-acclimated plants treated with liquid seaweed extract

Treatment	Log time elapsed	s.e.m	Time elapsed/minutes (Back transformed)
NA	1.289 a	0.129	3.63
NA3LSE	1.855 ab	0.293	6.39
CA	2.372 b	0.362	10.72
CA3LSE	2.667 b	0.277	14.4
SED	0.419		

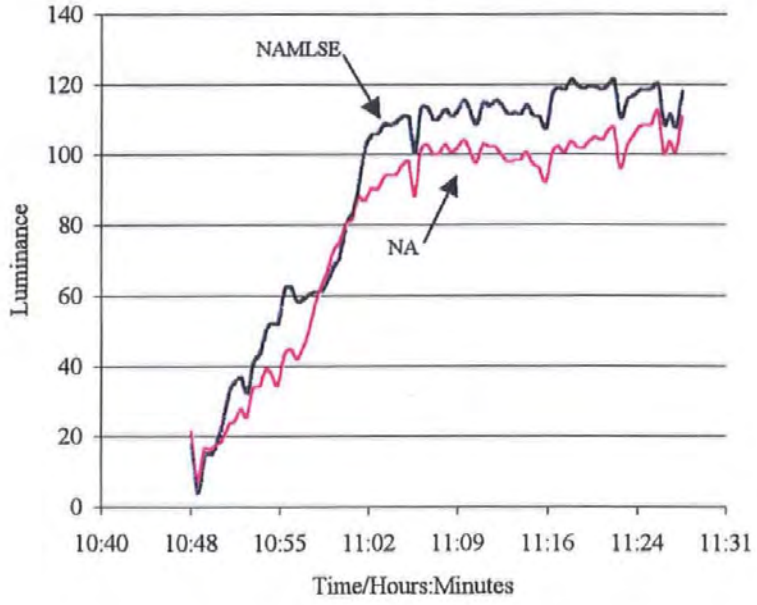
Letters denote statistically significant differences.

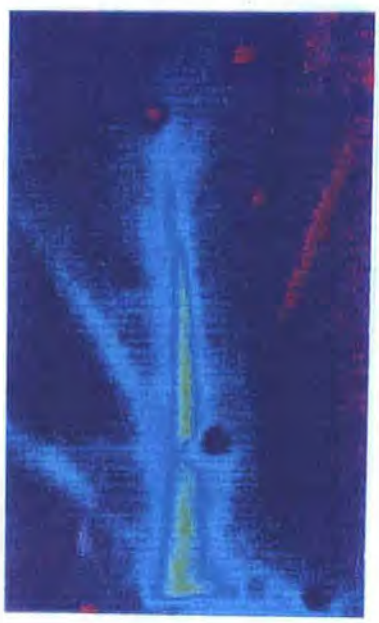
Table 4.7: Time elapsed between apoplastic freezing and full extracellular freezing in crown samples of non-acclimated and cold-acclimated plants treated with liquid seaweed extract

Treatment	Log time elapsed	s.e.m	Time elapsed/minutes (Back transformed)
NA	1.401 a	0.303	4.06
NA3LSE	2.595 b	0.130	13.39
CA	2.754 b	0.332	15.7
CA3LSE	2.961 b	0.391	19.31
SED	0.377		

Letters denote statistically significant differences.

Figure 4.5: Exotherm curves of a non-acclimated plant treated with liquid seaweed extract and a untreated non-acclimated plant

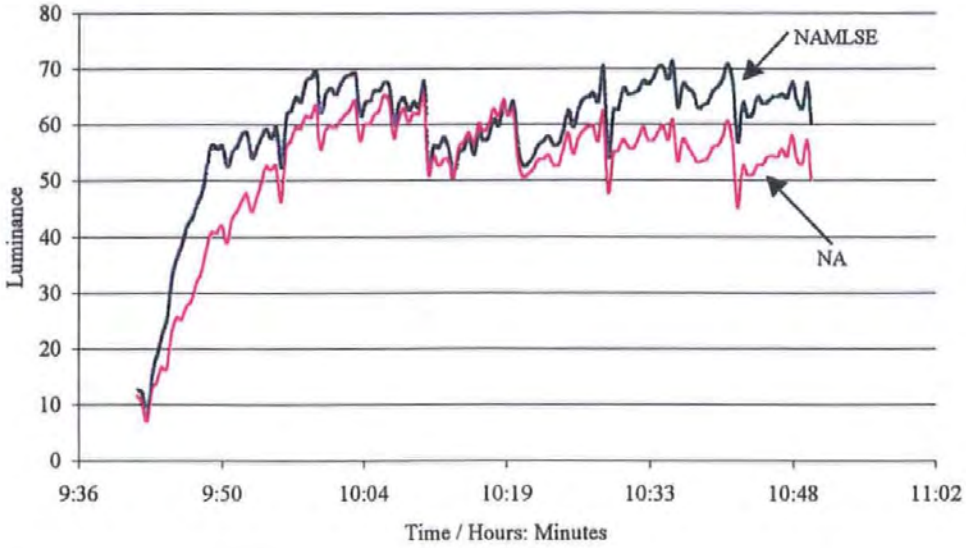




a) Non-acclimated plant treated with liquid seaweed extract. Note that stem tissue is still releasing cellular water to the extracellular ice crystal. Plant froze at -4.7°C. Picture captured at the end of frost test.

b)An untreated non-acclimated plant. Note that the stem tissue has completely frozen compared to that of the NAMLSE plant. Plant froze at -4.7°C. Picture captured at the end of frost test.

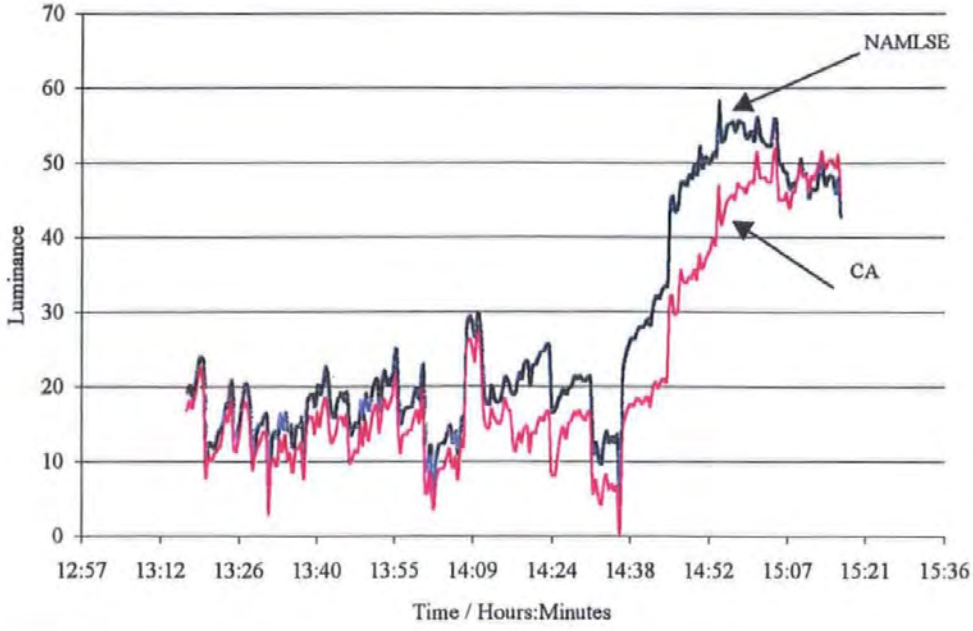
Figure 4.6: Exotherm curves of a non-acclimated plant treated with liquid seaweed extract and a untreated non-acclimated plant



a) A non-acclimated plant treated with liquid seaweed extract. Note light blue colour of crown tissue, which shows that cellular water is still being released to the extracellular ice crystal. This observation correlates with the continued rise in the luminance axes in the above graph for this plant. Plant froze at -4.4°C. Image captured at end of test.

b) An untreated non-acclimated plant. Note the much reduced light blue area of the crown tissue, which suggest that little or no cellular water is being released to the extracellular ice crystal. This observation correlates with the decline in the luminance axes in the above graph for this plant. Plant froze at -4.4°C. Image captured at end of test.

Figure 4.7 Exotherm curves of a non-acclimated plant treated with liquid seaweed extract and an untreated cold-acclimated plant

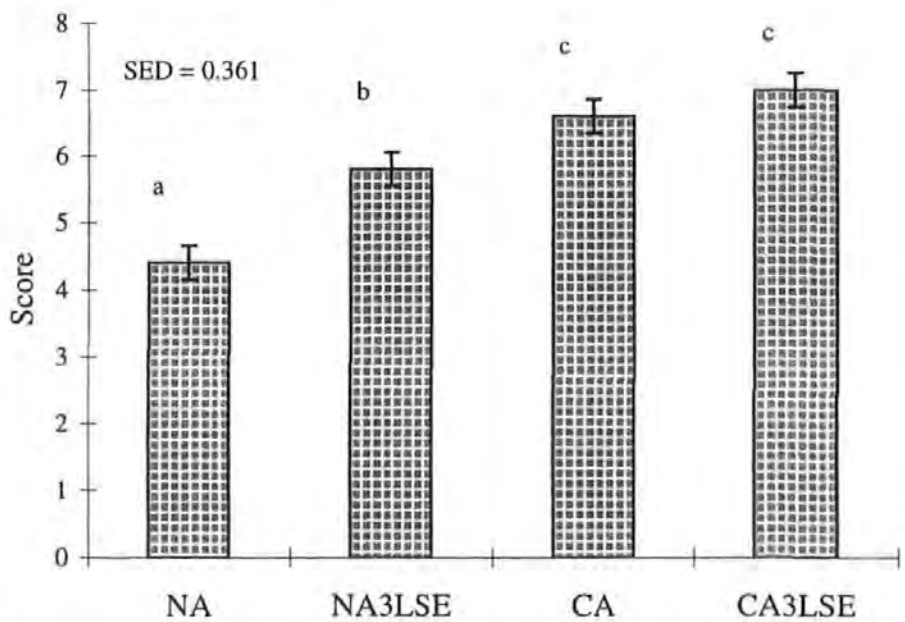


a) A non-acclimated plant treated with liquid seaweed extract. Note that the rate of cellular water release to the extracellular ice crystal, observed as luminance in the above graph, closely mimcs that of the cold-acclimated plant until after, approximately, 14:45 when the rate of cellular water release increased. Plant froze at -4.6°C. Image captured at end of test.

b) An untreated cold-acclimated plant. The rate of cellular water release from the crown tissue of this sample was reduced compared to the NAMLSE plant after, approximately 14:45. Observed as luminance in the above graph.This sample did not reach full luminance within the peroid of the frost test. Plant froze at -4.6°C. Image captured at end of test.

Plate 4.1: View of set-up of winter barley plants used in the radiative frost test and infrared thermography experiments.

Figure 4.9: Mean survival score of winter barley plants subjected to thermographic analysis



4.4. Discussion

Water Potential

The observed decreases in water potential of winter barley plants, when subjected to a 4°C treatment, is consistent with observations in CA dogwood (Parsons, 1977). A decrease in water potentials was also reported for wild type *Arabidopsis thaliana* plants when treated to 4°/2°C day/night temperatures (Lang, Mantyla, Welin, Sundbery and Palva, 1994) and water potential also decreased in these plants when treated with exogenous applications of abscisic acid (ABA) (Lang *et al*,1994). Decreases in shoot water potential was also observed in maize when the roots were subjected to chilling (8°C) temperatures. In this study shoot water potential remained considerably lower than that observed before chilling.

The transient rise in water potential in winter barley after plants were transferred to the 4°C phytotron may be a reflection of stomatal aperture. When plants were transferred to the 4°C phytotron stomates may have been closed or partially closed (because transfer occurred during the latter part of the light period) and thus the 4°C treatment could have physically slowed down the rate of stomatal response. In Dogwood fully closed stomates correlated to a rise in leaf water potential (Parsons, 1977). Decreases in the water potential of barley plants was also observed when NA and CA plants were subjected to applications of LSE. In particular NA plants subjected to 3 applications of LSE had a similar water potential to CA plants. This observation, (combined with that of parallel changes in protein metabolism) implies that multiple LSE treatment is producing a CA response in NA plants. In the CA plant only multiple applications of LSE result in a significant decrease in water potential. This may reflect the onset of the 2nd stage of CA (Trunova, 1965; Abe and Yoshida, 1997) or the acceleration of the 1st phase of CA (Livingstone III, 1996).

These observations are consistent with the current understanding of water relations and CA. It is known that solute changes occur in plants subjected to CA (Levitt, 1980) and also when leaf water potential declines (Morgan, 1980). Furthermore, Guy *et al*, (1992) report the accumulation of an 85kDa and a 160kDa proteins when water potentials fall

below -0.5MPa in Spinach. In chapter 3 it was shown that protein metabolism was affected by CA and LSE treatments.

Saunders (1997) reported that LSE enhanced frost resistance was optimal if applied between 4 and 10 days prior to freezing. Temporal water potential measurements of LSE treated winter barley plants showed a decrease in water potential from day 2 to a maximum at day 4 and a return to base values at day 10. It can be speculated that if LSE is having a temporal effect on plant water potentials and frost resistance this may be explained by the accumulation of solutes in the cell. This would bring about a fall in water potential due to osmotic adjustment (Morgan, 1980). As more water is absorbed by the plant then water potentials would begin to rise back to their respective base value. Indeed the estimated base value was very consistent in all three water potential experiments and does suggest that the water potential data are reliable. Bristow *et al* (1981) demonstrated that the water potential of wheat can be reliably measured with a J14 press and the data from J14 press measurements correlated well with measurements made with a Scholander pressure chamber.

Water content

Water content is generally inversely related to cold hardiness (Levitt, 1980). In Dogwood the water content of short day plants decreased as hardiness increased (Parsons, 1977) and Abe and Yoshida, (1997) illustrated a reduction in water content of winter wheat with the induction of the 1st stage of CA. Conversely an abrupt decrease in hardiness is correlated with an increase in tissue water content (Sakai and Larcher, 1987). Results from the water relations work on CA and LSE treated winter barley showed no significant decrease in water content (although there was a trend of decreasing water content with increasing cold hardiness) these results appear to be in conflict with the literature. Perhaps the method used to estimate the percentage water content was open to error, especially in the DSC estimates. Here sample size was very small and evaporation losses could have been significant. However, Guy *et al* (1992) found that there was only a 2% decline in water content of Spinach plants as water potential fell below -0.5MPa. Similar results have been demonstrated in water relations work on wheat plants.

Morgan (1980) reported a marked departure from ideal behaviour in some wheat genotypes. In one example there was a bimodal response to turgor maintenance where in the initial phase there was a change in osmotic potential from -1.5 to -3.0 Mpa with only a slight change in tissue water content (Morgan, 1980). This response was attributed to osmotic adjustment. In the second phase, water content fell in line with osmotic potential. In the case of cold-acclimation water content falls in the 1st phase of CA but remains stable in the 2nd phase (Abe and Yoshida, 1997). In the case of the CA and LSE treated plant, from this series of experiments, perhaps the 1st phase of CA has only just begun and a threshold limit in CA needs to be reached before any significant decline in tissue water content is observed. Certainly LT_{50} values for winter barley suggest that the CA regime (constant $4^{o}C$ for 2 weeks) was insufficient to induce significant declines in tissue water content. The water relations data generated for CA and LSE treated winter barley suggest that the physical state of water is being altered. Indeed Abe and Yoshida (1997) consider the physical state of water important in the process of CA in crown tissue of wheat plants.

Thermal analysis

The importance of the physical state of water is demonstrated in this series of thermal analysis experiments. Differential scanning calorimetery measurements illustrated a complex response to freezing; in the leaf samples there were no significant differences in the amount of unfrozen water but in the crown samples there was a significant difference in the unfreezable water content with increasing plant hardiness. This observation is in disagreement to that reported by Gusta, Burke and Kapooar, (1975). These workers could not correlate the amount of unfrozen water, in cereal crowns, with cold hardiness. However, there was a trend of increasing unfreezable water with plant hardiness. This study showed that as crown tissues harden they increase their tolerance to freezing. For example, hardy Frontier rye with a 2g water/g dry weight tolerated 88% of its freezable water frozen before injury occurred, whereas tender Manitou spring wheat with 5.6g water/dry weight was killed with only 18% of its water frozen.

The most apparent difference between crown tissues of cultivars differing in hardiness is the ability of hardier crowns to tolerate diminishing quantities of liquid water (Gusta *et al*, 1975).

Caution must be exercised in the interpretation of the DSC results as the rate of cooling was far in excess of cooling rates observed in the field and thus the manner and pattern of sample freezing may not reflect that observed in slower cooling rates, i.e. supercooling may have occurred in the rapidly cooled DSC samples. Furthermore, the final test temperature (-40°C) was only held for 5 minutes and therefore not all water may have been frozen. However, -40°C is believed to be a realistic end point for the freezing of the freezable water fraction in DSC samples (Prof. W.Block personal communication). Finally, the small size of sample (typically 3mm) could have been subjected to evaporative losses and thus leading to modifications in the water status of test samples which could lead to spurious results.

The values for the percentage unfrozen water content observed in the winter barley samples (in both crown and leaf) are within the range reported for carrots (Roos, 1986).

There are similarities between frost tolerance of crowns and drought response of crown tissues. In a study of water relations of wheat plants (Barlow, Munns and Brady, 1980) where 3 tissue types were examined (the apex, an expanding leaf enclosed in older leaf sheaths (leaf 7) and an exposed fully expanded leaf (leaf 4)) over a 13 day drought period, illustrated differences in the way these 3 tissue types responded to drought. The water potential of leaf 4 declined sharply after 6 days of drought and the leaf eventually turned yellow and died, whereas in the apex and leaf 7, water potentials illustrated a parallel decline until day 6 and thereafter maintained similar steady state water potentials throughout the drought period. In the case of tissue water content this study illustrated that as drought proceeded leaf 4 lost all its water after 8 days, at an exponential rate, but the apex and leaf 7 responded to drought in a similar manner. These tissues reduced their water content in the first 4 days of drought, leaf 7 at a quicker rate then the apex, thereafter the water content of both tissue types remained constant for the remaining drought period.

The conservation of water in the apex and young growing tissue appears to play a significant role in plant survival. Evidence from the DSC measurements illustrates again the importance of water conservation in crown tissue and is in agreement with the literature.

How is water conserved in the apex and young growing tissue ?

It has been illustrated that in CA Poaceae crowns there is an increase in solutes (Livingstone III, 1997; Castonguay and Nadeau, 1998; Abe and Yoshida, 1997) and furthermore soluble sugar concentration in leaf 7 and the apex of wheat doubles after 2 days of drought, however, there is a much lower accumulation of solutes in mature leaves (leaf 4) (Barlow *et al*, 1980). Solutes are not the only compounds to accumulate in crown tissue, proline in response to drought (Barlow *et al*, 1980), and low molecular weight proteins in response to CA and drought, have been shown to accumulate (Pearce *et al*, 1996). Evidence suggests that these accumulated compounds alter the physical state of water. In a study of vertical relaxation times of water molecules by pulsed ^{1}H-NMR spectrometer Abe and Yoshida (1997) correlated crown tissue hardiness with a decrease in relaxation time of water molecules. Their explanation for this observation suggested that free water was completely used and cell water was bound. These workers suggest that the physical state of water together with a decrease in water content caused the important increase in cold hardiness at the transition period from the 1st phase of CA to the 2nd phase of CA. These researchers indicate that the tolerance to freeze-induced dehydration is of great importance rather then the ability to retain water, i.e. the avoidance of the dehydration strain in sub-zero environments.

This theory is supported by the thermocouple data and the infra-red video footage. In these experiments it was clearly demonstrated that crown tissues show increasing resistance to freeze-induced dehydration strain compared to leaf samples. In the thermography data crown tissue illustrated significant tolerance to freeze-induced dehydration whereas the leaf tissues were less resistant to freeze dehydration. Thermocouple data support thermography observations. However, care must be exercised in the results of the

thermocouple work because there was no base line data for comparisons of treatment differences in exotherm size (i.e. is the observed differences in the mean exotherm temperature a true difference or a trend). In defence of the exotherm data the thermography data do illustrate a true difference between treatments, in that there is a difference in the freezing equilibrium of these samples (seen in the depression of the exotherm curves of NA plants compared NA3LSE plants in figures 4.9 and 4.10). It is noteworthy that freezing point depression is directly related to solute content and this observation is consistent with the theory that water is being conserved.

Wisniewski and Fuller (1999) have demonstrated, for potato, using thermography, that it was not the freezing process that was directly responsible for injury but rather the extent of dehydration brought about by the loss of cellular water to the extracellular ice crystal. The survival scores of winter barley plants, after 1 hour of freezing, demonstrate the importance of freeze-induced dehydration. In non-acclimated plants survival was greatly reduced and corresponds to a statistically larger exotherm than that of the healthier cold-acclimated plants. Non-acclimated plants released their cellular water, to the extracellular ice crystal, quicker than cold-acclimated plants and applications of liquid seaweed extract to non-acclimated plants improved both survival and tolerance to freeze-induced dehydration strain. However, it is believed that at equilibrium all cell water is frozen and therefore would the observed differences in the water release times observed in the thermography experiments result in an increase in frost resistance in the field.

In criticism of the thermography data collection: data was collected by visual observations of colour change in infra-red videos, which must have an element of subjectivity (when is green green ?). This situation could be improved by collecting data using PC. software now available for recovering data from stored black and white video tapes, (Thermoteknix 95-Plot, ThermaGram). Data is collected in binary format, luminance or temperature over time. However, because of thermal variation in the radiative chamber fluctuations in image colour, and thus temperature, was extremely variable. Recorded data illustrated large peaks and troughs in the exotherm event, flushing of colour in video tapes, making treatment comparisons meaningless. This was inconsistent with real time observations. In

support of the method applied here collected data is supported by simultaneous thermocouple data and the trend of water resistance to freeze-induced dehydration is consistent with the literature.

4.4.4 Conclusions

It was clearly demonstrated that cold-acclimation does decrease plant water potential and that this is consistent with increased frost resistance. Further, replicated experimentation illustrated that multiple applications of LSE to both non-acclimated results in a decrease in plant water potential and that this decrease is similar to that observed in cold-acclimated plants. Also multiple applications of LSE to cold-acclimated plants results in a further decline in water potential of these plants compared to standard cold-acclimated plants. The duration of decreased water potential observed in LSE treated plants corresponds to the observed duration of LSE mediated frost resistance (i.e. 4 -10 days). However, there was no observed decrease in plant water content with increasing frost hardiness and although differential scanning calorimetery illustrated a significant decrease in the amount of freezable water in winter barley crowns this was not sufficient to explain fully the observed increase in frost resistance in cold-acclimated and LSE treated plants. The combination of these data suggest that both cold-acclimation and LSE are altering the physical properties of cellular water and thus increasing the time it takes for water to be removed from the cell by the new extracellular osmotic environment generated by the extracellular ice crystal. This theory was supported by freezing experiments which measured heat release over time (thermocouple data and infrared thermoghraphy). In both the cold-acclimated and LSE treated plants it took longer for the cellular water to be removed by the extracellular ice crystal, with crown tissue water being highly conserved.

5.0 Chapter 5: General discussion

5.1 Introduction

There is an ongoing demand from the agricultural industry for an affordable and safe compound that could be employed as a frost protection agent, in a sustainable frost protection strategy. Such a compound would need to be readily available and compatible with field crop production systems. Observations by fruit growers (Dan Neautoboom, personal communication) suggested LSE as a candidate as repeated applications of LSE to apple orchards improves the frost resistance of apple crops. The use of LSE as a frost protectant would meet growers needs in terms of production system compatibility (LSE can be tank mixed and applied during normal spraying regimes) and also LSE is an organic and safe product. However, before LSE can be fully recommended as a frost protectant it is necessary to understand how the product manipulates plant frost resistance. Earlier work with LSE on frost hardy and frost susceptible plants demonstrated that LSE can only increase the frost resistance of frost hardy plants (Saunders, 1997); this implied that LSE is manipulating intrinsic cold-acclimation mechanisms in frost hardy plants. Therefore an understanding of these mechanisms was necessary before progress could be made on the question of how LSE manipulates frost resistance. It was this underlying philosophy that resulted in the formulation of three questions to elucidate the mechanism of action of LSE in the manipulation of frost resistance in winter barley and determined the course of experimentation: 1) Does LSE effect the frost resistance, growth and development of plants ?, 2) What is the influence of cold-acclimation and LSE treatments on soluble protein expression in winter barley ? and 3) Does cold-acclimation and LSE affect the water relations of winter barley ?. The findings of the experiments designed to answer these questions will be discussed in context with the observations that both cold-acclimation and LSE treatments alter the physical properties of cell water and thus reduce the extent of frost damaged observed.

5.2 Observed changes in plant water relations

The decrease in water potential observed in winter barley plants subjected to CA and LSE must reflect the altered metabolism of the cell, i.e. the accumulation of compatible solutes (such as sugars, proline and proteins) which enable the plant to survive freezing stress. The beneficial effect of this increased solute content to cells is the modification of the physical status of cellular water. Indeed data from this series of experiments did not show any significant reduction in percentage water content across the treatment structure. This observation in conjunction with that of decreased water potential, increased protein content and the increased residence time of cellular water, in CA and LSE treated plants, during radiative freezing, strongly supports the hypothesis that cellular water status is being modified at the physio-chemical level. This is presumably because of interactions between water compatible solutes and the bulk water fraction of the cell.

Differential scanning calorimetry suggested that both cold-acclimated crowns and non-acclimated crowns, treated with 3 applications of LSE, have more unfrozen water than non-acclimated crowns. However, this difference is insufficient to account for the difference in frost resistance, a view supported by Gusta *et al* (1975). It appears that in frost hardy tissue it is the ability of cold-acclimated cells to retain water in their cells, for longer periods, that results in increased frost resistance rather than the greater percentage of unfrozen water.

How then does this phenomenon account for increased frost resistance ?

5.3 The effect of cold acclimation

Life is a process of biochemical reactions catalysed by enzymes and contained by the plasma membrane. It follows then that survival following a desiccation stress requires the maintenance and recovery of normal biochemical reactions. Therefore, the physical and biochemical changes observed in cold-acclimated, hardy, plants is the consequence of evolution and it is therefore the interaction of these changes with the cellular water environment that is fundamental to plant survival following a desiccation stress. Freezing is a desiccation stress.

In non-acclimated plants there is little or no resistance to extracellular ice and consequently water rapidly leaves the cell following extracellular freezing; this can be explained by several factors. Firstly the rate of water loss from the cell is partially dependant on the amount of interstitial water and the least resistant pathway for either water migration to the site of ice nucleation or the pathway of ice growth (Reaney and Gusta, 1999). In experiments with bromegrass (*Bromus inermis* Leyss), cells frozen to -2°C in a large volume of interstitial water, (Reaney *et al*, 1999) illustrated that upon nucleation the ice crystal grows rapidly through the interstitial water and eventually comes into contact with the cell wall. The resistance to water flow is thus reduced because the ice crystal is adjacent to the cell wall. In addition, the pathway of water efflux is directly through the liquid phase and thus eliminates the resistance of the vapour phase. In frozen (-2°C) bromegrass cells, with a reduced volume of interstitial water, the pathway of water efflux was via both the liquid and the vapour phase, thus additional resistance to water mobility would be expected.

The second factor that may account for the rapid release of cellular water to the extracellular ice crystal is that the plasma membrane is in a tender state, and may allow ice penetration of the cellular environment and thus ice nucleation. Furthermore, the composition of the plasma membrane in non-acclimated plants is different to that of cold-acclimated plants. Presumably the interaction of the non-acclimated membrane with the cellular water environment does not protect the membrane from freeze-dehydrative destabilization. This would account for the water soaked appearance of frozen non-acclimated cells. The third factor is that the protein composition of non-acclimated cells is different to that of cold-acclimated cells, in that it is deficient in stress proteins such as antifreeze proteins, (which depress the freezing point of solutions and modify the shape of the ice crystal) (Anikainen, Griffith, Zhang, Hon, Yang and Pihakaski-Maunsbach, 1996), dehydrins and/or heatshock proteins. The fourth factor is that the solute concentration of non-acclimated cells is lower than that of cold-acclimated cells. The different protein and solute composition of non-acclimated cells will have profound consequences on the physical properties of cellular water, in that most of the water in non-acclimated cells is in a state that mimics pure bulk water and thus is more osmotically responsive to the

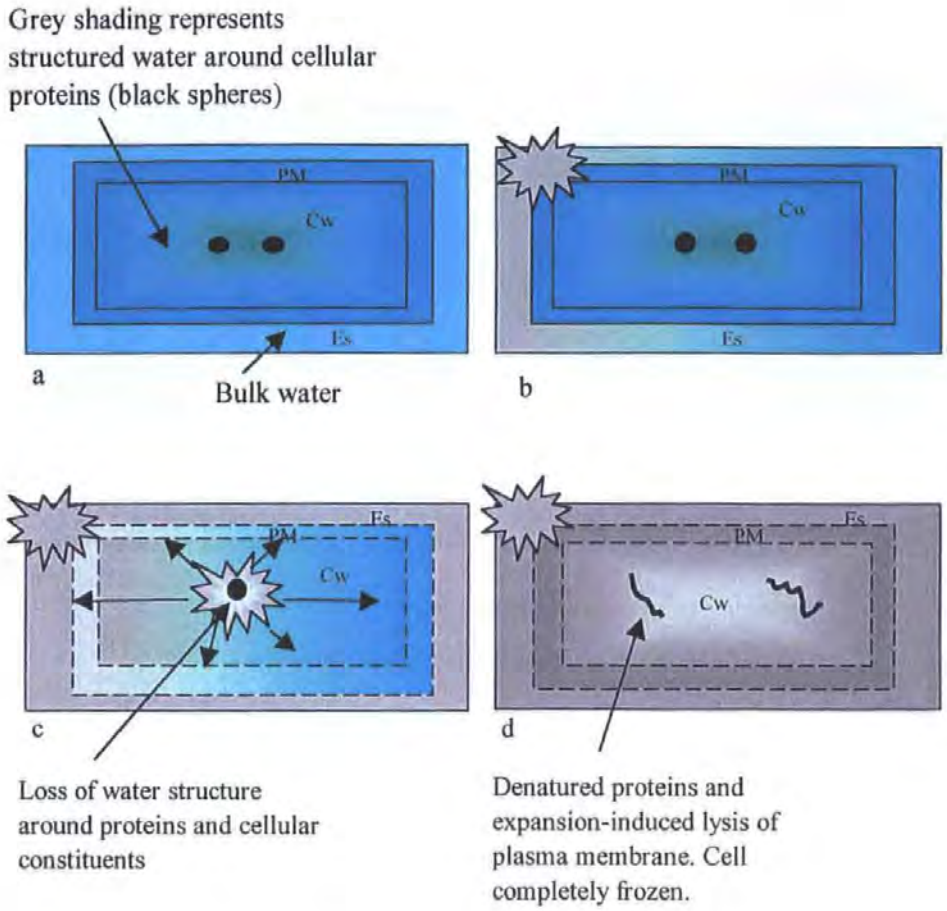
extracellular ice crystal. In this situation, the readily osmotically responsive water of non-acclimated cells would rapidly respond to the new osmotic environment of the frozen extracellular space; an observation consistent with thermal imaging data. Cellular water would move down the new osmotic gradient to the extracellular ice crystal. The consequence of this would be rapid cellular desiccation, protein and plasma membrane destabilization and ultimately cell death (Figure 5.0).

During cold-acclimation, hardy plants go through a series of physiological and biochemical changes that include changes in the composition of the plasma membrane, increased protein production, variation in isoenzymes, up-regulation of cold-inducible protein, and the accumulation of solutes such as sugars and amino acids. Physiological changes include a fall in water potential, a reduction in relative water content, stomatal closure, reduced photosynthesis and a reduction in growth rate. The consequence of these changes is that the new plasma membrane configuration can withstand freeze-dehydrative destabilization and thus protect the cell from the extracellular ice crystal.

Recent studies using *A.thaliana* protoplasts have shown that a COR15a gene encodes a COR15am polypeptide that interacts with the inner membrane of the chloroplast envelope and alters the intrinsic curvature of the inner membrane. This leads to a shift in the lamellar-to-hexagonal II phase transition temperature and thus increased resistance to freeze dehydrative destabilization (Steponkus and Uemera, 1999).

Secondly the increased production of proteins and the appearance of novel stress proteins and the accumulation of cellular solutes exert a stabilising effect on the physical properties of water. This results in a new cellular water environment that is more resistant to the freeze-induced extracellular osmotic gradient, (i.e. a cellular water environment exists in cold-acclimated cells that is essentially less osmotically responsive than that of non-acclimated cells). Consequently cellular water has increased residence time during a freezing event, the overall effect being protein stabilisation. How is this achieved ? It appears, from the literature, that solutes protect proteins from freeze-induced destabilization, by being preferentially excluded from the protein surface (Carpenter and

Figure 5.0: Schematic model illustrating the freezing of non-acclimated cells.



Hypothesised events during the freezing of non-acclimated cells: a) Unfrozen cell, water is structured around proteins and membranes but most of the cell water and that in the extracellular space is in a form that mimics pure bulk water, b) Ice nucleation has occurred in the extracellular space and rapidly spreads (shown as grey scaling from left to right) throughout the extracellular compartments. This sets up a new osmotic gradient and water begins to leave the cell, c) Membrane and cellular water rapidly freezes out to extracellular ice crystal and consequently there is a loss of water structure around the plasma membrane and cellular proteins, d) This results in excess pressure on the membrane giving rise to expansion-induced lysis (shown by dashed lines) and the loss of structured water around protein molecules results in protein denaturation and thus cell death.

Key to symbols: Cw = cellular water, Es = Extracellular space, PM = Plasma membrane,

= Site of ice nucleation ● = Protein

Crowe, 1988; Close, 1996). Furthermore, this preferential exclusion theory extends to proline (Carpenter and Crowe, 1988) and to dehydrins (Close, 1996). It is possible that the ordered hydration status of proteins would be disrupted if hydrophilic groups were bound to the surface of these proteins, these groups would re-orientate the dipoles of water and destabilize the ordered pentagonal structure of the hydrophobic protein surface. This would lead to protein destabilization. Conversely the ordered water shells of, say, trehalose would increase the surface tension of the solute-water structure. This would leave the less energetic bulk water to preferentially hydrate the protein. The net effect would be a cellular water environment that is far less osmotically responsive than that of non-acclimated cells. This situation would continue when cellular water content declines during the 2nd phase of hardening and would result in a stabilized protoplasm which could survive freeze desiccation. This model predicts a condition which is analogous to the increased hydraulic resistance observed in drying soils (Williams and Burt 1974; cited in Reaney and Gusta, 1999) and goes some way to confirming the observations of Reaney and Gusta (1999) that dehydration of any matrix increases its resistance to water and solute flow. This phenomena may be reflected in the results obtained by the thermal imaging experiments, in that CA plants and LSE treated plants released their cellular water to the extracellular ice crystal slower than NA plants. But it must be highlighted that crown tissues are more complex than leaf tissue containing several layers of leaves, leaf sheaths and connecting tissue that may well slow the rate of water loss in the crown compared to leaves. However, as there were observed differences between the rate of water loss from crowns of NA and NALSE and CA plants it is unlikely that the above could account for all the observed resistance of cellular water to the extracellular ice crystal. The observed increase in the resistance of cellular water, to the extracellular ice crystal, in NALSE, CA and CALSE treated plants, must be a reflection of increased matric content of these cells. This would lead to an increase in the water structuring of CA and LSE treated plants and consequently slower rates of water removal from their cells.

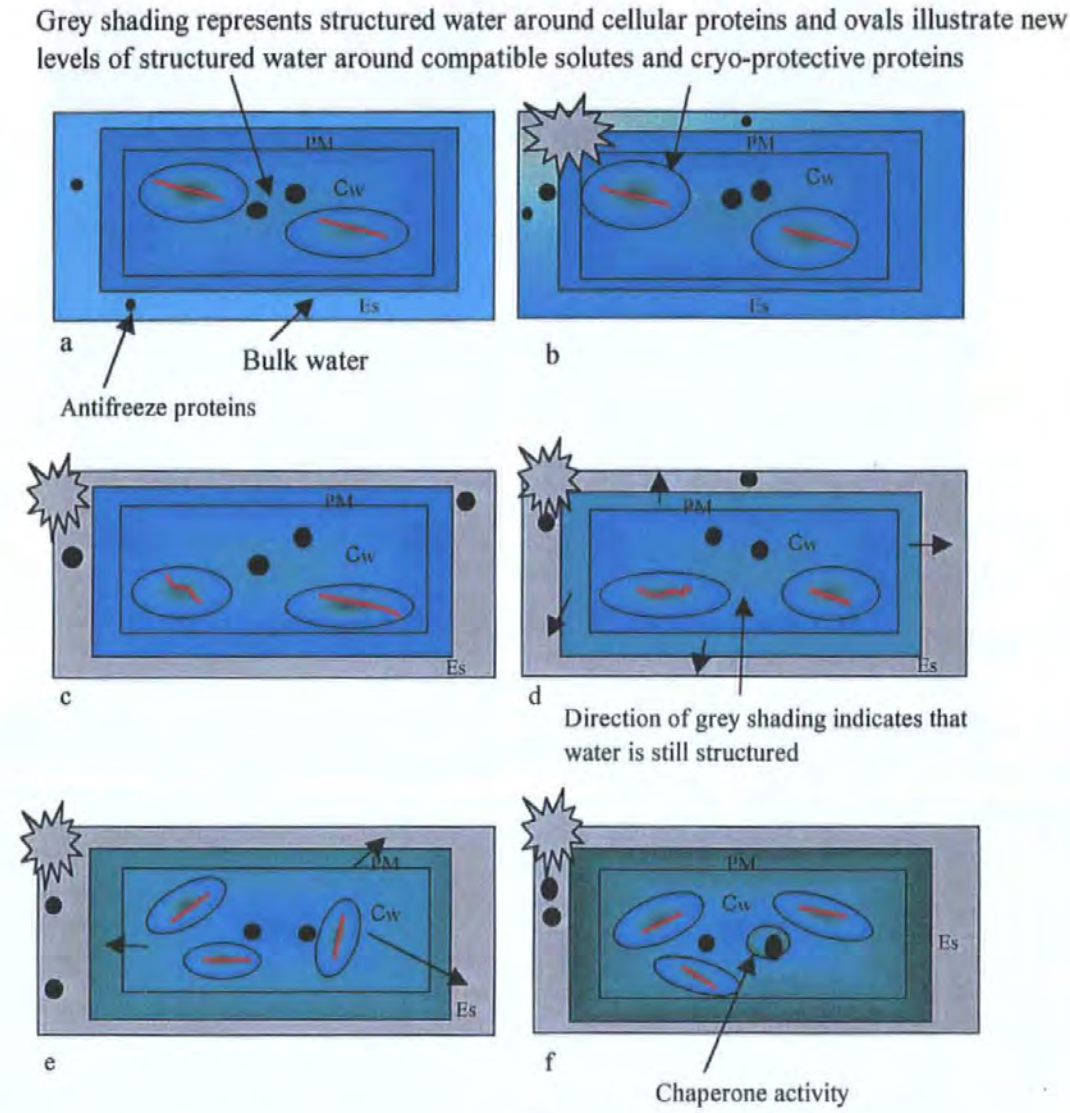
The resistance of cellular water to the extracellular ice crystal would be negated if there were large quantites of interstitial water, because this water would dilute the effect that proteins and solutes have on water structure. This observation may account for the

increase in the water potential of plants treated with liquid seaweed extract after day 6 (section 4.2.3) and thus also the decrease in the frost resistance of these plants from day 10 (Saunders, 1997). In these plants water potential returned to the base line value, observed in the untreated plants, and suggests that these treated plants have taken up more water and thus diluted the effect of solutes on the water structure of these plants.

The advantage of increased water struture on the behaviour of cold-acclimated cells is that these cells would be expected to contain more water after freezing, an observation that is consistant with the differential scanning calorimetery results. Furthermore, this observation is supported by measurements of the cellular water content of bromegrass cells following controlled freezing and thawing. In non-acclimated cells frozen to -3^{o}C (plants still viable at -7^{o}C) there was a decline in relative water content from 1 to 0.59 but cold-acclimated cells were unaffected at this temperature and only showed a decline in water content (0.50) at -9^{o}C or lower (Reaney and Gusta, 1999).

The overall effect of cold-acclimation is a profound change in plant biochemistry and physiology that results in membrane changes (Steponkus and Uemera, 1999), up-regulation and expression of novel proteins and the accumulation of solutes. These changes lead to membrane stability, thus the exclusion of the extracellular ice from the cytoplasm and prevention of freeze-dehydrative destabilization, and a cellular water environment that is less osmotically responsive to the extracellular ice crystal and thus the maintenance of cellular viability (Figure 5.1).

Figure 5.1: Schematic model illustrating the freezing of cold-acclimated cells.



Hypothesised events during the freezing of cold-acclimated cells: a) Unfrozen cell expressing compatible solutes which aid in water structuring events and reduces the bulk water fraction in the cell, b) Ice nucleation has occurred in the extracellular space but growth and spread (shown as grey scaling from left to right) is modified by antifreeze proteins, c) Extracellular space is frozen, however, cellular water unfrozen, d) Cellular water begins to move out of membrane and cell to the extracellular ice crystal, e) Cell water freezing out, but ordered hydration shells around compatible solutes slows this process, f) Cell water readily freezing out but hydration shells around compatible solutes maintains cellular integrity and complementary action of chaperone proteins force unfolding cellular proteins back into conformational state.

Key to symbols: Cw = cellular water, Es = Extracellular space, PM = Plasma membrane.

= Site of ice nucleation = Protein

5.4 The effect of liquid seaweed extract on frost resistance

Data presented in this thesis suggest that liquid seaweed extract promoted cold-acclimation in non-acclimated plants and increased water structuring in liquid seaweed treated cold-acclimated plants. The influence of liquid seaweed extract on the process of cold-acclimation could be through several different mechanisms; 1) increased growth via a cytokinin mediated pathway, 2) an ABA mediated response, 3) plant growth regulator like activity of an unknown oligosaccharides and 4) increased K+ content of the rhizosphere (i.e. osmotic shock). Any of these mechanisms could be responsible for the promotion of the cold-acclimated state in non-acclimated plants treated with 3 applications of liquid seaweed extract.

An increase in plant growth has been correlated with cytokinin levels and there is also correlation between growth responses of plants to liquid seaweed extracts (Blunden *et al* 1981). It is possible that the increased growth of plants treated with liquid seaweed extract has given these plants an advantage over untreated plants, by increasing the number of lateral apices and increased probability of frost survival and/or via the rapid genetic response of older plants to environmental stimuli (Pearce *et al* 1996). However, there are two problems with these observations: firstly the physiological and developmental stages of both untreated and LSE treated plants were very similar at the time of frost testing. Secondly, the biochemical and physiological responses of non-acclimated plants treated with 3 applications of liquid seaweed extract were very similar to those observed in cold-acclimated plants. This observation would not be expected if enhanced frost resistance was due exclusively to growth and development, because the non-acclimated plants treated with liquid seaweed extract received no normal cold-acclimating stimuli.

If the biochemical, physiological and water mobility responses of non-acclimated plants subjected to 3 applications of LSE were similar to those of cold-acclimated plants, then it would be expected that the frost resistance of these plants should be similar. This, however, was not observed. The frost resistance of non-acclimated plants subjected to 3 applications of LSE was notably reduced compared to the frost resistance of cold-acclimated plants. This implies that certain genes or gene products that are required for

full cold-acclimation are not being affected by the LSE. The blt4 barley gene family contains a gene, blt4.9, that responds to low temperature, abscisic acid and other environmental cues such as drought (Hughes, Brown, Vural and Dunn, 1999; White, Dunn, Brown and Hughes, 1994). Conversely there is another gene blt101 that only responds to low temperatures, (Hughes, Brown, Vural and Dunn, 1999). A similar situation occurs in *Arabidopsis thaliana,* the COR gene family is responsive to low temperature, drought and high salinity and studies on COR78 show that gene regulation is via a 9bp promoter, referred to as a drought responsive element (DRE) (Thomashow, 1999). This element has a 5bp sequence of CCGAC designated as the low temperature responsive element (LTRE) or also named C-repeat (CRT) (Medina and Salinas, 1999). In contrast to this Medina and Salinas (1999) describe two genes from *Arabidopsis*, CBF2 and CBF3, which are expressed very early during the process of cold-acclimation but do not respond to abscisic acid or drought.

This suggests that the LSE seaweed extract mediation of frost resistance is via a PGR like compound or through a transient K+ salt induced osmotic shock in the rhizosphere. Certainly these compounds would be adversely affected by rain, and experiments on plant growth and protein metabolism demonstrate a loss of the LSE effect when rain follows application, up to three days post LSE application.

It is proposed that LSE enhances frost resistance of winter barley via a PGR or a K+ induced osmotic shock, or possibly both. Non-acclimated plants treated with liquid seaweed extract then respond to these stimuli (potentially via blt4.9 or similar genes) by up-regulating protein expression, especially high molecular weight proteins like the 118kDa protein, reduce their water potential and thus result in a less mobile cellular water environment. This falls short of full cold-acclimation in non-acclimated plants treated with three or more applications of liquid seaweed (DT_{50} values of NA3LSE plants are lower than CA plants) and may be due to the lack of response of other low temperature responsive genes such as blt101.

Experimental data also illustrate that multiple applications of LSE to cold-acclimated plants increases water structuring (experiment 6, section 4.2.6) and may explain the increase in the frost resistance of these plants over the normally cold-acclimated plants (section 2.8.0). This observation supports the above hypothesis because the enhanced frost resistance must be mediated by a separate gene to the low temperature responsive genes like blt101, because these plants received the same cold-acclimating regime as the normal cold-acclimated plants. The observed increase in the water structuring of cold-acclimated plants treated with multiple applications of LSE must be due to factors other than protein metabolism alone. There was no significant difference in the protein metabolism of these plants compared to normal cold-acclimated plants, but there was a further decrease in the water potential of treated cold-acclimated plants, which suggest that other solutes such as sugars and proline may be involved. The implication that multiple applications of LSE may be mediating different genes in the cold-acclimation response is not unreasonable. Uemura and Steponkus, (1998) observed an increase in the freezing tolerance (~1^{o}C) of excised leaves from transgenic *A.thaliana* lines that expressed both the COR6.6 gene and COR15a gene compared to leaves from *A.thaliana* lines that expressed either one of these genes alone.

5.5 How and when to use liquid seaweed extract

Data suggest that multiple low dose applications of LSE should be applied to the soil in the autumn until cold-acclimating temperatures are achieved. It would be beneficial to apply LSE during warmer spells (above 7 to 10^{o}C) in the winter and early spring. However, LSE should not be applied if 2 to 3 days of fine weather cannot be guaranteed following application although it is unlikely that low volumes of fine misty rain would be detrimental to LSE applications. One consequence of global warming may be the early onset of spring growth in perennial crops and loss of acclimation leading to early spring frost damage on the new growth. Applications of LSE to these plants, around this time, could improve plant survival and consequently final yield by manipulating intrinsic frost resistance mechanisms.

5.6 Critique and further work

The major shortfall of this study is that no single compound or groups of compounds in the LSE were identified to be involved in the manipulation of frost resistance in winter barley. This necessitates further study on the LSE product which could take the form of isolating promising compounds and testing the response of treated plants. Further studies on the ability of compounds in the LSE to induce blt genes could aid the isolation of the underlying mechanism.

Implications of results obtained from thermal imaging techniques suggest that the infra-red camera is capable of detecting changes in the physical properties of water. This hypothesis needs testing by simultaneous study. This would involve careful freezing of a series of plants in different states of hardiness, imaging the plants during freezing and corroborating the results using nuclear magnetic resonance techniques.

Furthermore, if results are promising and technology is available for the resolution of single cells, an artificial environment could be constructed, e.g. cell and extracellular space. Then components of cold-acclimation could be manipulated to test the hypothesis that cells physically alter their water environment.

Another consequence of the data generated by this study is that the infra-red camera could be developed as a non-invasive tool for water relations studies, in particular water potential work. A series of water potential measurements could be made on a sequentially desiccated population of plants and then following freezing, water release curves, obtained by thermal imaging, could be constructed and correlated to water potential measurements. This could be done for species favoured by water relations workers.

5.7 Conclusions

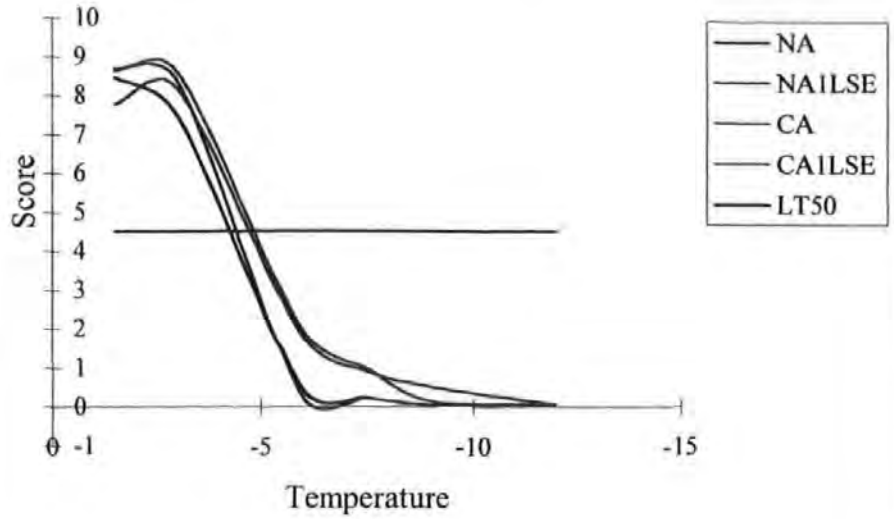
It was clearly demonstrated, by replicated experimentation, that multiple applications of LSE improved both the frost resistance and growth of winter barley and that this observation was stable in a small scale field trial. Protein analysis showed that cold-acclimation and LSE application increased the soluble protein content of winter barley and up-regulated the expression of high molecular weight proteins. However, where precipitation followed LSE application there was a reduction in LSE mediated frost resistance, growth and protein expression. This observation clearly implicated the up-regulation of high molecular weight proteins with improved frost resistance and suggest that when precipitation diluted and/or leached LSE from the soil there was a loss of LSE mediated protein expression and frost resistance.

There was a decrease in plant water potential following cold-acclimation and this decrease was consistent with observed protein changes and frost resistance. It was subsequently shown that multiple applications of LSE to both non-acclimated and cold-acclimated plants also decreased plant water potential, and, the water potential of non-acclimated plants, treated to multiple applications of LSE, was similar to that observed in cold-acclimated plants. However, there was no significant reduction in the relative water content of cold-acclimated and LSE treated plants. This implied that both cold-acclimation and LSE treatments were modifying the physical properties of plant cellular water. Replicated experiments with differential scanning calorimetry, thermocouple data and infrared thermography suggest that both cold-acclimated and LSE treated plants release their cellular water to the extracellular ice crystal slower than non-acclimated controls. These results clearly demonstrate that exogenous applications of LSE do enhance the frost resistance of winter barley and this appears to be by the manipulation of intrinsic cold-acclimation pathways. Therefore the null hypothesis is rejected in favour of the alternative hypothesis.

The data presented in this thesis suggest that there is a role for LSE in agriculture, as a frost protectant. The product could be applied by foliar spray or fertigation programme from the beginning of autumn, on a regular basis, until stable winter temperatures induce

full cold-acclimation. A further role for LSE in the manipulation of frost resistance could be during a late spring frost, where considerable economic loss can occur in a de-hardening crop. A frost protection programme that used a strategy of multiple, low dose, applications of LSE could reduce the amount of frost damage caused by these late spring frosts. Indeed, one of the expected consequences of global warming is an increase in the incidence of untimely spring frosts and therefore potentially a greater demand for a frost protection agent by growers.

Figure 1a: Example of the procedure used to estimate the DT_{50} values



Appendix A2

Sample calculation of percentage increase in LT_{50} values

Increase in LT_{50} for winter cauliflower: Increase in the frost resistance of NA1LSE over NA

NA = -4.4
NA1LSE = -4.51
Ignoring the sign
So: 4.51 - 4.4 = 0.11
0.11/4.4 = 0.025
0.025 x 100 = 2.5% Increase in frost resistance

Figure 2A: Weather station data for the five days following liquid seaweed extract application

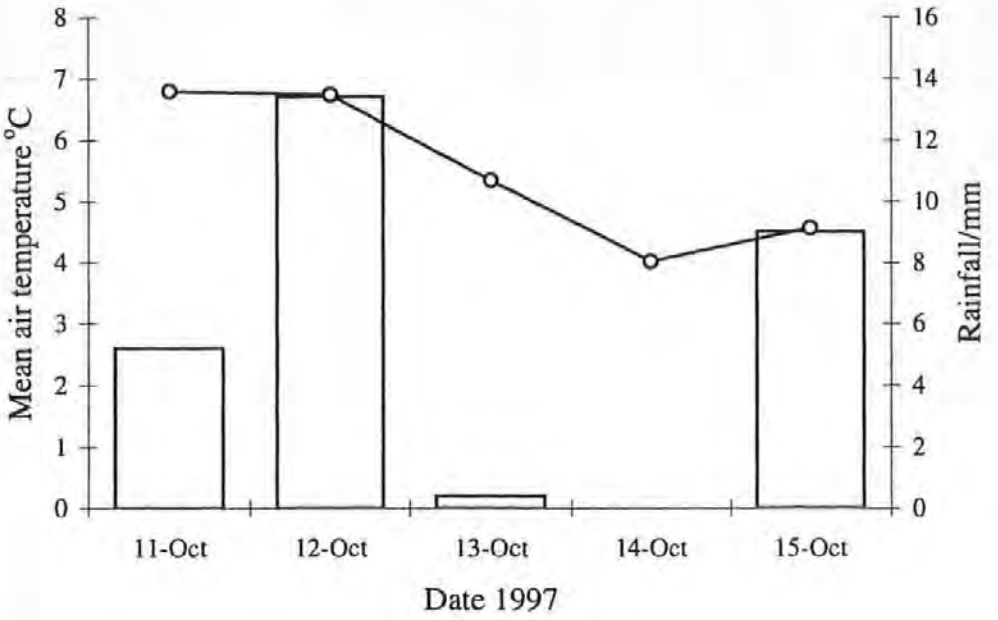


Figure 3A: Weather station data for the five days following liquid seaweed extract application

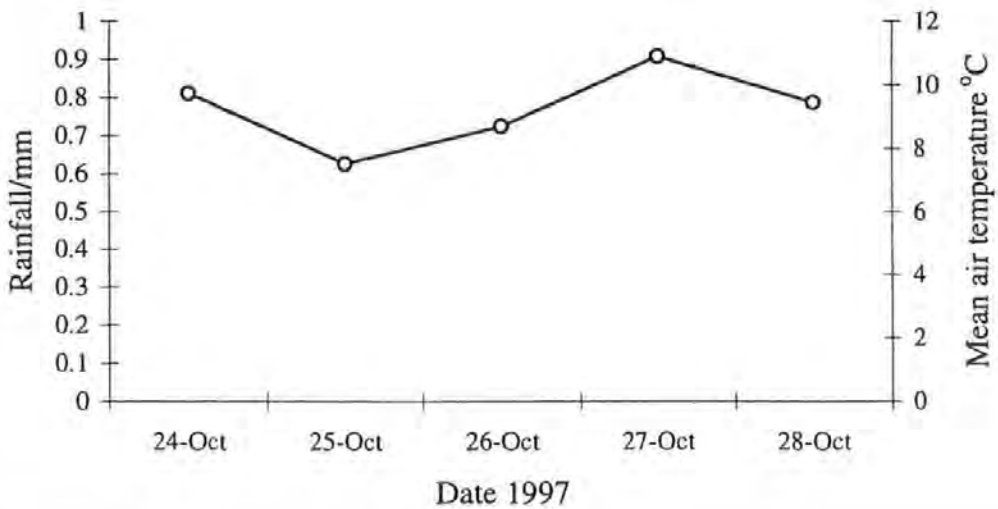


Figure 4A: Weather station data for the four days following liquid seaweed extract application

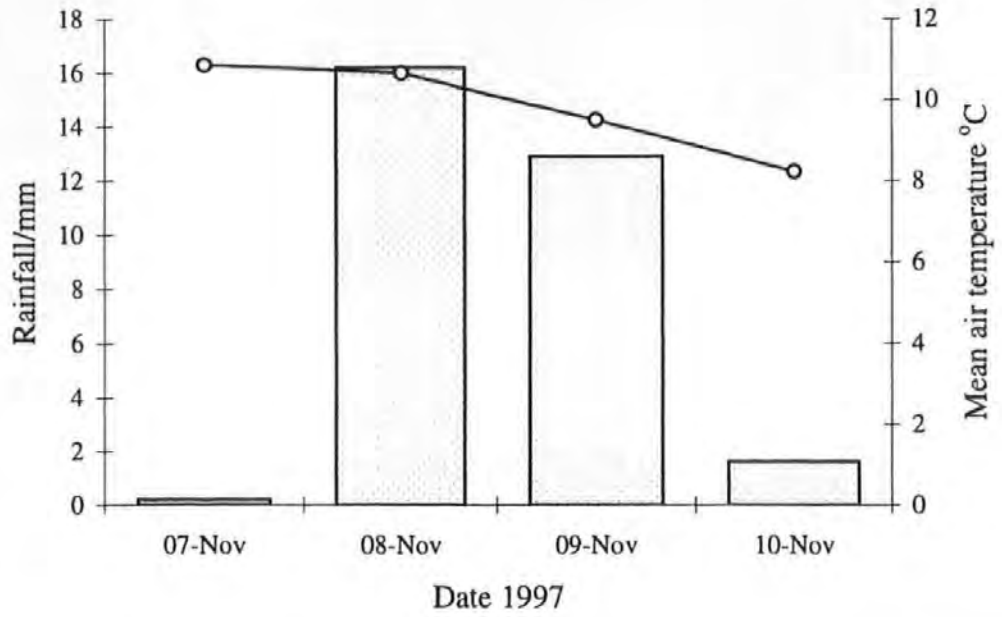


Figure 5A: Weather station data for the five days following liquid seaweed extract application

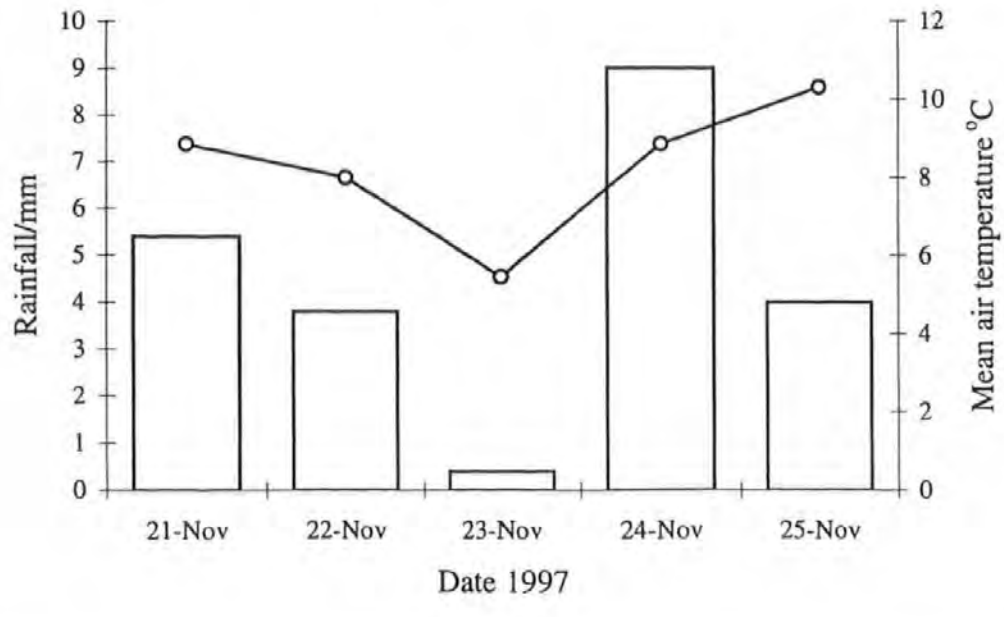


Figure 6A: Weather station data for the five days following liquid seaweed extract application

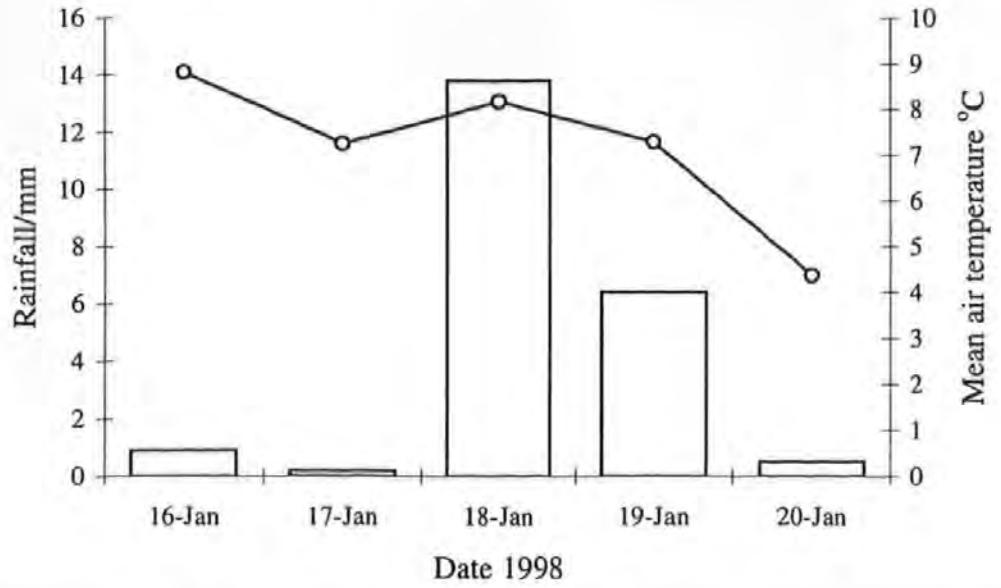
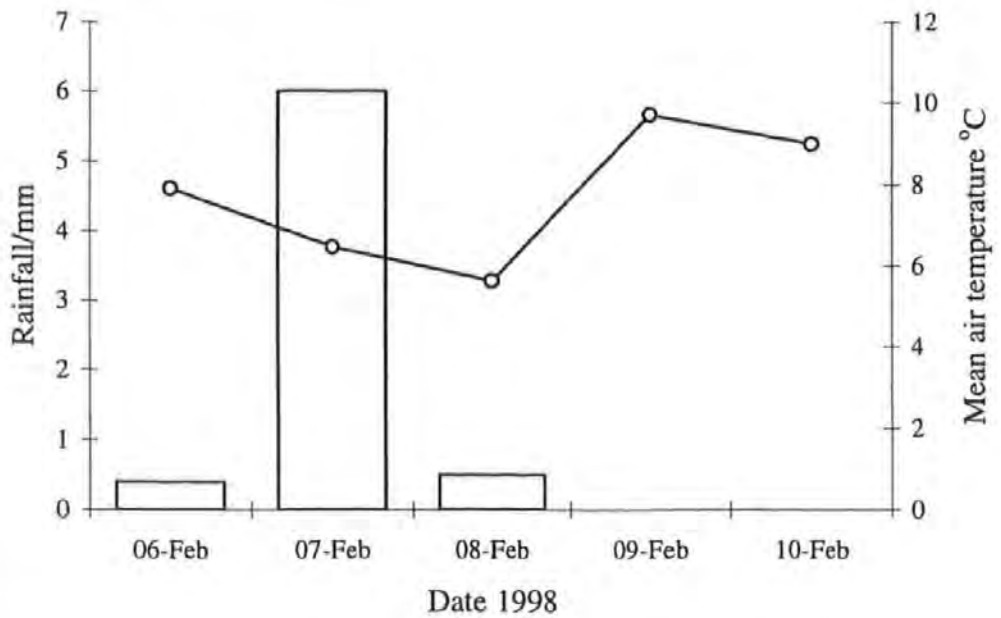


Figure 7A: Weather station data for the five days following liquid seaweed extract application



Appendix 3A Electrophoresis Recipe

a) Acrylamide solution:

29.2g acrylamide

0.8g N'N'-Bis methylene acrylamide

make to 100ml with double distilled water (ddH_20)

b) Tris buffers:

1.5M Tris-HCL pH 8.8;

18.15g Tris base

50ml ddH_20

adjust to pH 8.8 with concentrated HCL

make to 100ml with ddH_20

0.5M Tris-HCL pH 6.8;

6g Tris base

50ml ddH_20

adjust to pH 6.8 with concentrated HCL

make to 100ml with ddH_20

c) 5X Stock running buffer pH 8.3:

113.03g Tris base

14.42g Glycine

10ml 10% SDS

d) 10% SDS

10g SDS

100ml ddH_20

e) Sample Buffer

7.6ml ddH_20

2ml 0.5M Tris-HCL pH 6.8

1.6ml Glycerol

3.2ml SDS

1% Bromophenol blue

f) 10% Ammonium persulfate (APS)

100mg APS

1ml ddH_20

Make fresh every time

(Stock solutions are after Bio-Rad Industries Ltd).

Gel Recipes

a) 12.5% Resolving Gel:

3.2ml ddH_20

2.5ml 1.5M Tris-HCL pH 8.8

0.1ml 10% SDS

4.17ml Acrylamide-bis

0.05ml 10% APS

0.005ml TEMED

(enough monomer solution for 2 X 0.75mm gels)

b) 4% Stacking Gel:

6.1ml ddH_20

2.5ml 0.5M Tris-HCL pH 6.8

0.1ml 10% SDS

1.33ml Acrylamide-bis

0.05ml 10% APS

0.005ml TEMED

(After Bio-Rad)

Staining recipes:

a) Coomassie blue

1g Coomassie blue

450ml methanol

100ml acetic acid

filter through whatman No 1

b) Destain

350ml acetic acid

1l methanol

(After Bio-Rad)

Appendix 4A: Working solutions for Western Blotting

Solutions:

a) Towbin buffer

3.03g Tris

14.4g Glycine

200ml methanol

make to 1l with ddH_2o, degas with nitrogen gas.

b) Working solution

Tris-buffered saline, 20mM Tris, 500mM NaCl, pH7.5 (TBS).

100ml of stock TBS

900ml ddH_20 = 1:10 TBS working solution.

c) Wash solution

Tween Tris-buffered saline, 20mM Tris, 500mM NaCl. 0.05% Tween 20, pH 7.5 (TTBS).

350µl Tween 20

700ml working TBS solution

d) Blocking solution (3% gelatin-TBS)

3g Gelatin

100ml TBS

Heat to 50°C and stir until dissolved

e) Antibody buffer (1% gelatin-TTBS)

2g Gelatin

200ml TTBS

Heat to 50°C and stir until dissolved

f) Primary antibody solution

a 1/1000 dilution of primary antibody with antibody buffer

g) Secondary antibody solution

Appendix 5A Example of analytical strategy applied to densitometry data

Figure 8A: densitometery trace of molecular weight standard of the 4 day sampling

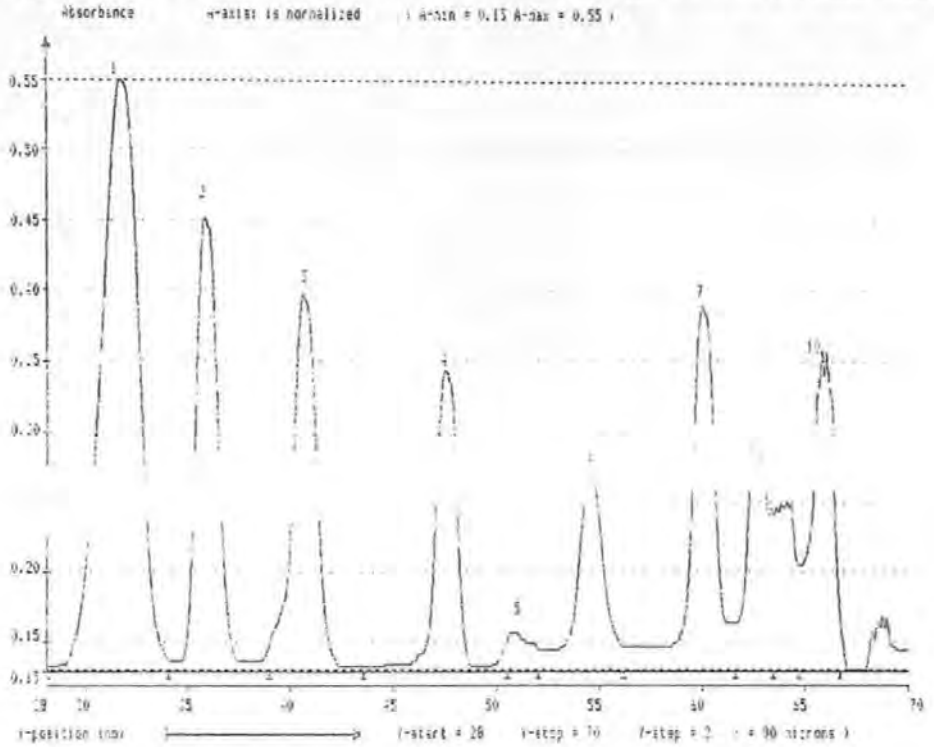


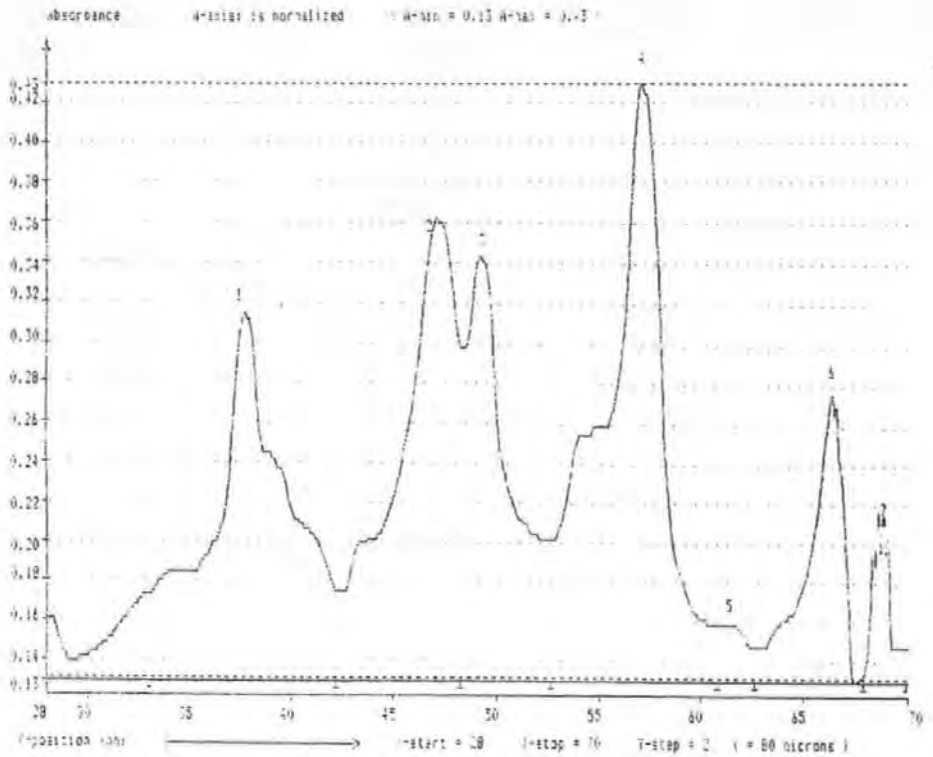
Table 1A: Data on band postion and percentage area generated by densitometery for molecular weight standard.

Peak	Position	Log MW	MW/KDa	% Area
1	NA	NA	NA	NA
2	35.76	1.16	14.4	31.6
3	40.56	1.33	21.5	35.76
4	47.36	1.49	31.0	40.56
5	NA	NA	NA	NA
6	51.00	1.65	45.0	47.36
7	58.00	1.82	66.2	54.4
8	62.4	1.99	97.4	62.0
9	64.0	2.07	116.25	64.0
10	65.68	2.3	200	65.68

Note: peak 1 was ignored because this band was positioned in the lower molecular weight region which was subjected to heat distortion. Peak 5 was also ignored as this was considered to be an artifact. Regression coefficents; a= 4.17, b=28.34, R^2 = 0.984.

Appendix 6A: Example of analytical strategy applied to densitometry data

Figure 9A: densitometery trace of a non-acclimated plant from the 4 day sampling



Peak	Position	Log MW	MW	% Area
1	37.6	1.18	15.1	22.33
2	46.72	1.50	31.7	21.36
3	49.63	1.59	39.3	15.88
4	54.0	1.76	57.3	29.12
5	61.44	2.02	104.9	1.22
6	66.32	2.19	155.9	7.7
7	68.64	NA	NA	

Note: Peak 7 was ignored due to interference from gel edge. Number of bands detected by densitometer was 6 out of 15 visible to the eye.

Appendix 7A Example of analytical strategy applied to densitometry data

Figure 10A: densitometery trace of a non-acclimated plant treated with 3 applications of liquid seaweed extract, sample from the 4 day sampling peroid.

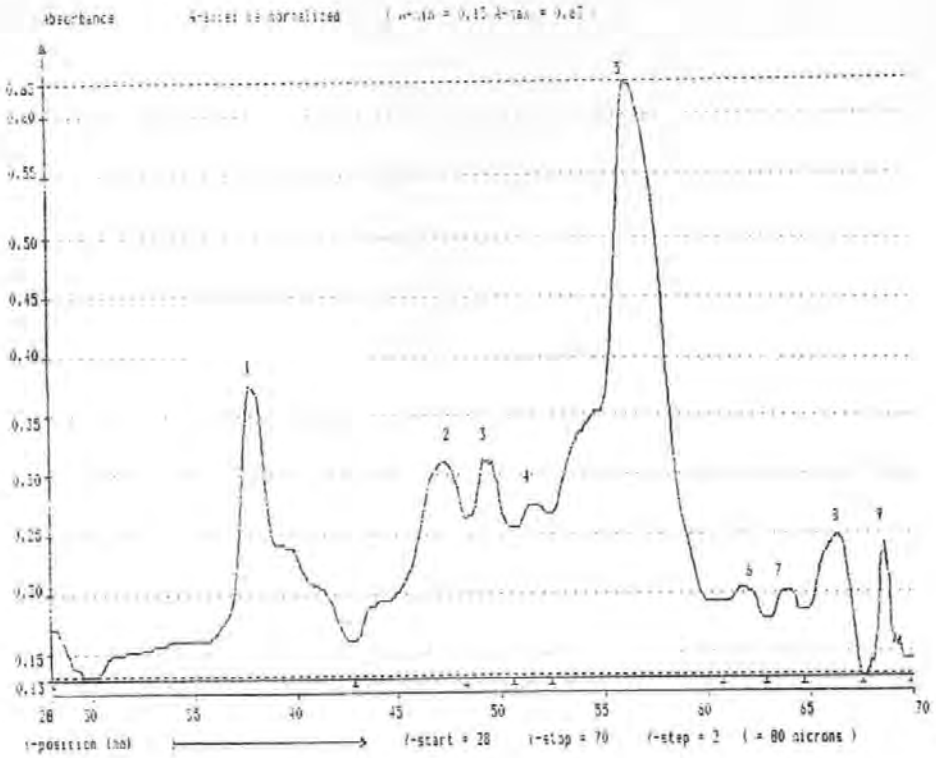


Table 3A: Data on band postion and percentage area generated by densitometry for a non-acclimated plant treated with 3 applications of liquid seaweed extract.

Peak	Position	Log MW	MW	% Area
1	37.84	1.18	15.4	18.56
2	47.36	1.52	33.4	12.55
3	49.28	1.59	38.8	7.9
4	51.44	1.67	47.0	5.45
5	56.08	1.76	57.3	43.33
6	61.92	2.04	109.1	2.99
7	63.6	2.09	125.00	2.50
8	66.32	2.19	156.9	4.87
9	NA	NA	NA	NA

Note: peak number 9 was ignored due to interference from gel edge. 8 bands were detected by densitometry compared to 16 visible.

Appendix 8A Example of analytical strategy applied to densitometry data

Figure 11A: densitometery trace of a cold-acclimated plant from the 4 day sampling

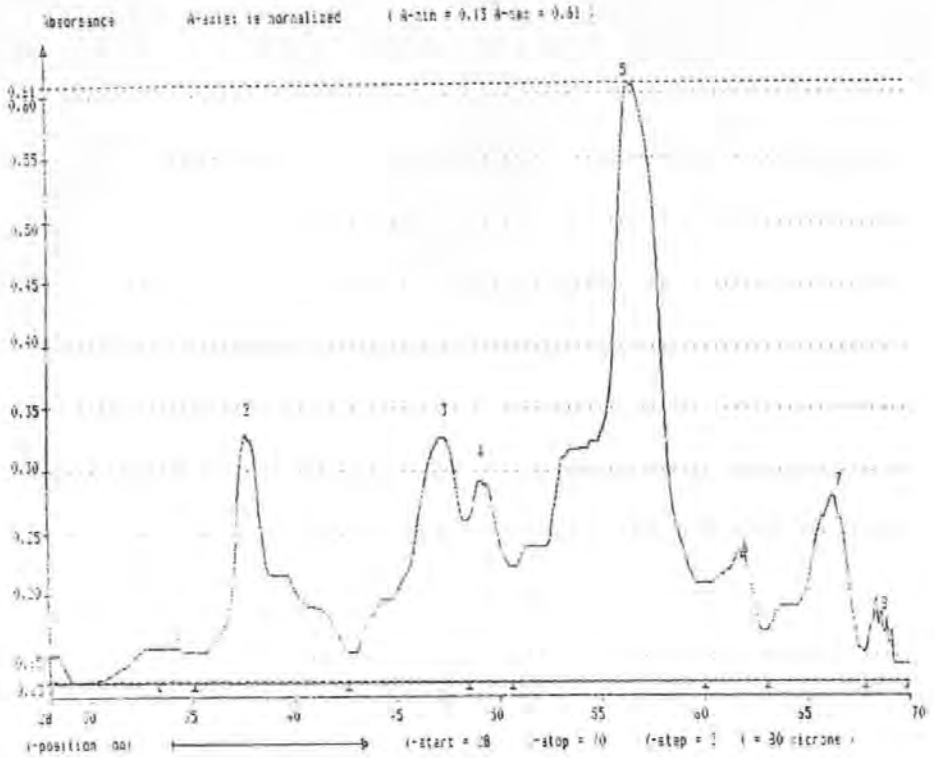


Table 4A: Data on band postion and percentage area generated by densitometry for a cold-acclimated.

Peak	Position	Log MW	MW	% Area
1	34.24	1.06	11.5	1.13
2	37.68	1.18	15.2	13.4
3	47.44	1.53	33.6	14.87
4	49.20	1.59	38.8	7.0
5	56.32	1.76	57.3	46.79
6	62.08	2.04	109.7	5.8
7	66.84	2.19	157.9	9.27
8	NA	NA	NA	NA

Note: peak 8 was ignored due to interference from gel gel edge. 7 band detected by densitometer compared to 12 visible.

Appendix 9A: example calculation of percentage unfrozen water from differential scanning calorimetery experiments.

Estimated energy change for pure water = 342.48 Jg

Estimated percentage of water in the crown tissue of winter barley = 91.53%

Sample is from a non-acclimated plant:

Weight of water: 91.53/100 x 23.6290[a] g = 21.628 g

Energy should be: 21.628 x 342.48 = 7407.157 Jg

Estimated energy of melt: 21.628 x 301.76[b] J = 6526.465 Jg

Percentage of water frozen: 6526.465 / 7407.157 = 88.11%

Unfrozen water: 91.53 - 88.11 = 3.42%

[a] weight of sample

[b] energy change observed in sample

REFERENCES

Abe, J. and Yoshida, M. 1997. Influence of Water and Air Temperature on Cold Hardiness of Wheat. In Proceedings of the International Symposium on Cereal Adaptation to Low Temperature Stress in Controlled Environments. Editor-in-Chief Bedo, Z. Lenia DTP Studio Hungary.

Abetz, P., 1980. Seaweed extracts: Have they a place in Australian agriculture or hotriculture? J. Aust. Inst. Ag. Sci. 46, 23-29.

Anderson, J.V., Li, Q-B., Haskell, D.W. and Guy, C.L. 1994a. Structural organization of the spinach endoplasmic reticulum-70-kilodalton heat-shock cognate gene and expression of the 70-kilodalton heat-shock genes during cold acclimation. Plant Physiol. 104, 1359-1570.

Anderson, J.V., Haskell, D.W. and Guy, C.L. 1994b. Differential influence of ATP on native spinach 70-kilodalton heat-shock cognates. Plant Physiol. 104, 1371-1380.

Anikainen, M., Griffith, M. Zhang, J., Hon W-C Yang, D.S.C. and Pihakaski-Maunsbach, K. 1996. Immunolocalization of antifreeze proteins in winter rye leaves, crowns and roots by tissue printing. Plant Physiol. 110, 845-857.

Anon 1998. NIAB. UK. Recomended lists of cereals. Cereal Variety Handbook. National Institute of Agricultural Botany, Cambridge

Anon, 1991a. Concise Science Dictionary. Oxford University Press.

Anon 1994b. Bio-Rad Protein Assay Instruction Manual. Bio-Rad Laboratories. Printed in Germany.

Anon 1997c. Grower 18. 4.

Anon 1997d. Grower 22. 15-18

Anon 1997e. Mini-Protean^{R} Multi-Casting Chamber Instruction Manual. Bio-Rad Laboratories. Printed in USA.

Anon 1997f. Immun-Blot^{R} Assay Kik Instruction Manual. Bio-Rad Laboratories. Printed in USA.

Anon 1997g Grower 4. 19-20.

Arora, R. and Palta, J.P. 1988. In vivo perturbation of membrane-associated calcium by frezze-thaw stress in onion bulb cells. Simulation of this perturbation in extracelluar KCL and alleviation by calcium. Plant Physiol. 87, 622.

Arora, R. and Palta, J.P. 1991. A loss in the plasma membrane ATPase-activity and its recovery coincides with incipient freeze-thaw injury and postthaw recovery in onion bulb scale tissue. Plant Physiol. 95, 846.

Arteca, R.N. 1996. Plant Growth Substances Principles and Applications. Chapman and Hall

Ashworth. E.N. 1984. Ice nucleation within peach trees. J. Am Soc. Hortic. Sci. 109, 198-201.

Ashworth, E.N. 1990. Formation and spread of ice in plant tissues. In: Hortic. Rev. 12, pp 215-254.

Aspinall, D. 1980. Role of abscisic acid and other hormones in adaptation to water stress. In: Adaptation of plants to water and high temperature stress. (edS. Turner, N.C. and Kramer, P.J.). Wiley and Sons New York. 156-172.

Badger, J. 1993. Multiple hydration layers in cubic insulin crystals. Biophys. J, 65. 1656-1659.

Baird, C. 1999. Environmental chemistry 2nd edition. W.H. Freeman and Company New

York. pp 173-220.

Barlow, E.W.R, Munns, R.E. and Brady, C.J. 1980. Drought Responses of Apical Meristems. In Turner, N.C. and Kramer, P.J. Adaptation of Plants to Water and High Temperature Stress. A Wiley-Interscinece Publication. John Wiley and Sons.

Blunden, G., 1977. Cytokinin activity of seaweed extracts. In : Marine natural products chemistry, eds : Faulkner, D.J., Fenical, W.J. Plenum press, New York.

Blunden, G. and Wildgoose, P.B., 1977. The effects of aqueous seaweed extract and kinetin on potato yields. J. Sci. Fd. Agric. 28, 539-541.

Blunden, G., Wildgoose, P.B., Nicholson, F.E., 1979. The effects of aqueous seaweed extracts on sugar beet. Botanica mar. 22, 539-541.

Blunden, G. and Wildgoose, P.B. 1981. Plant growth regulatory substances in commercial seaweed extracts. Proc. 8th. Int. Seaweed. Symp. 760-763.

Blunden, G., Gordon, S.M. and Keysall, G.R. 192. Lysine betaine and other quaternary ammonium compounds from British species of Laminariales. J. Nat. Prod. 45, 449-452.

Blunden, G., Rogers, D.J., Barwell, C.J., 1984. Biologically active compounds from British marine algae. In : Natural products and drug development, (eds, Krogsgaard-Larsen, P., Brogger-Christensen, S., Kofod, H). Alfred Benzon symposium. 20, Munksgaard, Copenhagen, 179-190.

Blunden, G., Cripps, A.L., Gordon, S.M., Mason, T.G., Turner, C.H., 1986. The characterisation and quantitative estimation of betaines in commercial seaweed extracts. Botanica. mar. 29, 155-160.

Blunden, G., Gordon, S.M., 1988/89. Agrochemicals from marine algae. J. Agric. Sci. University college Wales, 184-204.

Booth, E. 1966. Some properties of seaweed manures. Proc. 5th. Int. Seaweed. Symp. Pergamon Press. 349-57.

Bray, E. 1988. Drought- and -ABA- induced changes in polypeptide and mRNA accumulation in tomato leaves. Plant Physiol. 88, 1210-1214.

Bristow, K.L., Van Zyl, W.H. and De Jagger, J.M. 1981. Measurement of Leaf Water Potential Using the J14 Press. J. Exp. Bot. 32, 851-854.

Buchanan, D.W., Briggs, R.H. and Bartholic, J.F. 1974. Cold hardiness of peach and nectarine trees growing at 29-30oN Latitude. J.Am. Soc. Hortic. Sci. 99, 256-259.

Burke, M.J., Gusta, L.V., Quamme, H.A., Weiser, C.J. and Li, P.H. 1976. Freezing and injury in plants. Ann. Rev. Plant. Physiol. 27, 507-28.

Cameron, I.L., Kanal, K.M., Keener, C.R. and Fullerton, G.D. 1997. A Mechanistic View of the Non-Ideal Osmotic and Motional Behaviour of Intracellular Water. Cell. Biol. Int. 21, 99-113.

Carpenter, J.F. and Crowe, J.H. 1988. The Mechanism of Cryoprotection of Protein Solutes. Cryobiology. 25, 244-255.

Castonguay, Y. and Nadeau, P. 1998. Enzymatic Control of Soluble Carbohydrate Accumulation in Cold-Acclimated Crowns of Alfalfa. Crop Sci. 38, 1183-1189.

Cattivelli, L. and Bartels, D. 1992. Biochemistry and molecular biology of cold-inducible enzymes and proteins in higher plants. In- Inducible plant proteins, Society of experimental biology seminar series 49. (Wray, J.L. eds). University press, Cambridge, UK. 267-288.

Chen, H.H., Li, P.H. 1980. Biochemical changes in tuber-bearing Solanum species in relation to frost hardiness during cold acclimation. Plant. Physiol. 66, 414-421.

Chen, T.H. and Gusta, L.V. 1983. Abscisic acid induced freezing resistance in cultured plant cells. Plant. Physiol. 73, 71-75.

Chen, T.H. Li, P.H. and Brenner, M.L. 1983. Involvement of abscisic acid in potato cold acclimation. Plant Physiol. 73, 71-75.

Chen, T.H.H., Burke. M.J. and Gusta, L.V. 1995. Freezing tolerance in plants an overview. In- Biological ice nucleation and its applications (Lee Jr, R.E., Warren, G.and Gusta, L.V. eds). American Phytopathological Society. St Paul, Minnesota. 115-136.

Close, T.J. 1996. Dehydrins: Emergence of a Biochemical Role of a Family of Plant Dehydration Proteins. Physiol. Plant. 97, 795-803.

Close, T.J., Fenton, R.D., Yang,A., Asghar,R., DeMason, D.A., Crone, D.E. Meyer,N.C. and Morgan, F. 1993a. Dehydrin: the protein - In plant responses to environmental stress (Clos, T.J. and Bray, E.A. eds), pp 104-118. American society of plant physiologists, Rockville, MD. ISBN 0-943088-26-7.

Close, T.J., Fenton, R.D. and Morgan, F. 1993b. A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. Plant. Mol. Biol. 23, 279-286.

Crowe, J.H. and Crowe, L.M. 1982. Induction of anhydrobiosis: Membrane changes during drying. Cryobiol. 19, 317-28.

Crowe, J.H. Crowe, L.M. and Mouradian, R. 1983. Stabilization of biological membranes at low water activities. Cryobiol. 20, 346-56.

Daie, J. and Campbell, W.F. 1981. Responses of tomato plants to stressful temperatures. Plant Physiol. 67, 26-29.

Dang, L.X. and Chang, T-M. 1997. Molecular Dynamics Study of Water Clusters, Liquid, and Liquid-Vapour Interface of Water with Many-Body Potentials. J. Chem. Phyisics. 106, 8149-8159.

Danyluk, J., Perron, A., Houde, M., Limin, A., Fowler, B., Benhamou, N. and Sarhan, F. 1998. Accumulation of an Acidic Dehydrin in the Vicinity of the Plasma Membrane during Cold Acclimation of Wheat. Plant. Cell. 10, 623-638.

Dexter, S.T., Tottingham, W.E. and Garber, L.F. 1937. Investigations of hardiness of plants by measurements of electrical conductivity. Plant Physiol. 7, 63-78.

Dowgert, M.F. and Steponkus, P.L. 1983. Effect of cold acclimation on the plasma membrane area expansion of isolated protoplasts. Plant Physiol. 72, 45 (Suppl.).

Dure, L, III, Crouch, M., Harada, J., Ho, T.-H. D and Mundy J. 1989. Common amino acid sequence domains among LEA proteins of higher plants. Plant Mol. Biol. 12, 475-86.

Dure, L. III. 1993. Structural motifs in LEA proteins of higher plants. In: responses of plants to cellular dehydration during environmental stress. Eds: Close, T.J., Bray, E.A., pp 91-103. American society of plant physiologists, Rockville, MD. ISBN 094088-26-7.

Featonby-Smith, B.C. and Van Standen, J. 1984. The effect of seaweed concentrate and fertilizer on growth and the endogenous cytokinin content of Phaseolus vulgaris. South African J. Bot. 3, 375-379.

Featonby-Smith, B.C. and Van Standen, J. 1984. Identification and seasonal variation of endogenous cytokinins in Ecklonia maxima. Botanica mar. 27, 527-531.

Featonby-Smith, B.C. and Van Standen, J. 1983. The effect of seaweed concentrate on the growth of tomato plants in nematode infested soil. Scientia Hortic. 20, 137-146.

Franks. F. 1975. Water, Ice and Solutions of Simple Molecules. In Water Relations of Foods, edited by R.B.Duckworth. Academic Press.

Fuller, M.P., Grout, B.W.W. and Tapsell, C.R. 1989. The pattern of frost hardening of leaves of winter cauliflower (Brassica oleracea var. botrytis c.v. clause 30). Ann. Appl. Biol. 115, 161-170.

Fuller, M.P. and Le Grice, P. 1998. A chamber for the simulation of radiation freezing of plants. Ann. appl. Biol. 133, 111-121.

Garber, M.P. and Steponkus, P.L. 1976. Alterations in chloroplast thylakoids during an in vitro freeze-thaw cycle. Plant physiol. 57, 673-680.

Gerloff, E.D., Richardson, T. and Stahmann, M.A. 1966. Changes in fatty acids of alfalfa rots during cold hardening. Plant physiol. 41, 1280-1284.

Goh, K.M. 1971. Kelp extract as fertilizer. I. Effect on the germination and dry matter production of white clover. N.Z. J. Sci. 14, 734-48.

Goppert, H.R. 1830. Uber die Warme-Entwickelung in den Pflanzen, deren Geefrieren und die Schutzmittel gegen dasselbe. Max and Comp. Berlin.

Grahan, P. and Patterson, B. 1982. Responses of Plants to Low, Nonfreezing Temperatures: Proteins, Metabolism, and Acclimation. Ann. Rev. Plant. Physiol. 33, 347-372.

Guerrero, F. and Mullet, J.E. 1986. Increased abscisic acid biosynthesis during plant dehydration requires transcription. Plant Physiol. 80, 588-591.

Gusta, L.V., Burke, M.J. and Kapoor, A.C. 1975. Determination of unfrozen water in winter cereals at sub-freezing temperatures. Plant physiol. 56, 707-709.

Gusta, L.V., Rajahekar, C., Chen, P. and Burke, M.J. 1982. Frezze-induced membrane permeability changes in winter rye. Cryo-letters.3, 27-34.

Guy, C.L., Yelenosky, G. and Sweet, H.C. 1980. Light exposure and soluble sugars in citrus frost hardiness. Fla. Sci. 43, 268-273.

Guy, C.L. and Carter, J.V. 1984. Characterization of partially purified glutathione reductase from coldhardened and nonhardened spinach leaf tissue. Cryobiol. 21,. 454-64.

Guy, C.L. and Haskell, D. 1987. Induction of Freezing Tolerance in Spinach is Associated with the Synthesis of Cold Acclimation Induced Proteins. Plant Physiol. 84, 872-878.

Guy, C.L. and Haskell, D. 1989. Preliminary characterization of high molecular mass proteins associated with cold acclimation in spinach. Plant Physiol. Biochem. 27,

Guy, C.L. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Ann.Rev.Plant. Physiol. Plant Mol. Biol. 41, 187-223.

Guy, C., Haskell, D., Neven, L., Klein, P. and Smelser, C. 1992. Hydration-State-Responsive proteins Link Cold and Drought Stress in Spinach. Planta.188, 265-270.

Guy, C.L., Anderson, J.V., Haskell, D.W., Li, Q.B. 1994. CAPS, cors, dehydrins, and molecular chaperones: their relationship with low temperature responses in spinach. In : Biochemical and cellular mechanisms of stress tolerance in plants. Ed :Cherry, J.H. pp 479-499. Springer-Verlag, Berlin.

Hahn, M. and Walbot, V. 1989. Effects of Cold-Treatment on Protein Synthesis and mRNA Levels in Rice Leaves. Plant Physiol. 91, 930-938.

Hansen. J. and Beck, E. 1988. Evidence for ideal and non-ideal equilibrium freezing of leaf water in frosthardy ivy (Hedera helix) and winter barley (Hordeum vulgare). Bot. Acta.101, 76-82.

Heino, P., Sandman, G., Lang, V., Nordin, K. and Palva, E.T. 1990. Abscisic acid deficiency prevents development of frezzing tolerance in Arabidopsis thaliana (L) Heynh. Theor. Appl. Genet. 79, 801-806.

Hillard, J.H. 1975. Eragrostis curvula : Influence of low temperatures on starch accumulation, amylolytic activity and growth. Crop Sci. 15, 293-294.

Hughes, M.A., Brown, A.P.C., Vural, S. and Dunn, M.A. 1999. Low temperature regulation of gene expression in cereals. In: Plant Responses to Environmental Stress (eds, Smallwood, M.F., Calvert, C.M. and Bowles, D.J.) BIOS Scientific Press. 89- 97.

Hughes, M.A. and Dunn, M.A. 1996. The molecular biology of plant acclimation to low temperature. J. Exp. Bot. 47, 291-305.

Irmscher, E. 1912. Under die Resistenz der Laubmoose gegen Austrocknung und Kalte. Jahrd. wis. Bot. 50, 387-449.

Janick, J. 1979. Horticultural Science third edition. W.H. Freeman and Company. San Francisco. 465-463.

Jensen, M., Heber, U. and Oettmeier, W. 1981. Chloroplast membrane damage during frezzing: the lipid phase. Cryobiol. 18, 322-35.

Johnson-Flanagan, A.M. and Singh, J. 1987. Alteration of gene expression during the induction of frezzing tolerance in Brassica napus suspension cultures. Plant Physiol. 85, 699-705.

Johnson-Flanagan, A.M., Huiwen, Z., Thiagarajah, M.R., Saini, H.S. 1991. Role of abscisic acid in the induction of freezing tolerance in Brassica napus suspension cultured cells. Plant. Physiol. 95, 1044-1048.

Jung, G.A., Shih, S.C. and Shelton, D.C. 1967. Seasonal changes in soluble protein, nucleic acids and tissue pH, related to cold hardiness of Alfalfa. Cryobiol. 4, 11-16.

Kacperska-Palacz, A., Debska, Z. and Jakubowska, A. 1975. The phytochrome involvment in frost hardening process of rape seedlings. Bot. Gaz. 136, 137-140.

Kawai. H, Sakurai, M. Inoue, Y. Chujo, R. and Kobayashi, S. 1992. Hydration of Oligosaccharides: Anomalous Hydration Ability of Trehalose. Cryobiol. 29, 599-606.

Kelly, S.M, Butler, J.P. and Macklem, P.T. 1995. Control of Cell Volume in Oocytes and Eggs from Xenopus laevis. Comp. Biochem. Physiol. 111A, 681-691.

Keener, C.R., Fullerton, G.D. and Cameron, I.L. 1995. Solution Nonideality Related to Solute Molecular Characteristics of Amino Acids. Biophyis. J. 68, 291-302.

Kingman, A.R. and Moore, J. 1982. Isolation, purification and quantification of several growth related substances in Ascophyllum nodosum. Bot.mar. 25, 149-153.

Koorneef, M., Jorna, M.L, Brinkhorst-van der Swan, D.L.C. and Karssen, C.M. 1982. The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of Arabidopsis thaliana (L) Heynh. Theor. Appl. Genet. 61, 385-393.

Krasnuk, M., Jung, G.A. and Witham, F.H. 1975. Electrophoretic studies of the relationship of peroxidases, polyphenol oxidase, indoleacetic acid oxidase to cold tolerance of alfalfa. Cryobiol. 12, 62-80.

Krasnuk, M., Jung, G.A. and Witham, F.H. 1976. Electrophoretic studies of several deydrogenases in relation to cold tolerance of alfalfa. Cryobiol. 13, 375-93.

Krasnuk, M., Witham, F.H and Jung, G.A. 1976. Electrophoretic studies of several hydrolytic enzymes in relation to cold tolerance of alfalfa. Cryobiol. 13, 225-42.

Kraut, J.L., Walsh, C.S. and Ashworth, E.N. 1986. Acclimation and winter hardiness patterns in the eastern thornless blackberry. J. Am. Soc. Hortic. Sci. 3, 347.

Kushad, M.M., Yelenosky, G. 1987. Evaluation of polyamine and proline levels during low temperature acclimation of citrus. Plant. Physiol. 84, 692-695.

Kingman, A.R., Moore, J. 1982. Isolation, purification and quantitation of several growth regulatory substances in Ascophyllum nodosum. Botanica. Mar. 25, 149-153.

Krasovitski, B., Kimmel, E and Amir, I. 1995. A simulation of foam protection of plants against frost. J. Agric. Engng. Res. 61, 155-163.

Kupila-Ahvenniemi, S., Pihakaski, s. and Pihakaski, K. 1978. Wintertime changes in the ultrastructure and metabolism of the microsporangiate strobili of Scotch pine. Planta. 144. 19-29.

Kurkela, S. and Borg-Frank, M. 1992. Structure and expression of kin 2, one of two cold and ABA-induced genes in Arabidopsis thaliana. Plant. Mol. Biol. 19, 689-692.

Lalk, I. and Dorffling, K. 1985. Hardening, abscisic acid, proline, and frezzing resistance in two winter wheat varieties. Physiol Plant. 63, 287-292.

Landi, R. 1974. The crown-freezing technique in wheat breeding for cold area. Genet. Agrar. 28, 381-391.

Lang, V., Mantyla, E., Welin, B., Sundberg, B. and Palva E.T. 1994. Alterations in water status, endogenous abscisic acid content, and expression of rab18 gene during the development of frezzing tolerance in Arabidopsis thaliana. Plant Physiol. 104, 1341-1349.

Lang, V. and Palva E.T. 1992. The expression of a rab-related gene, rab18, is induced by abscisic acid during the cold acclimation process in Arabidopsis thaliana (L) Heyn. Plant. Mol. Biol. 29, 951-62.

La Rudulier, D., Bernard, D., Goas, G. and Hamelin, J. 1984. Osmoregulation in Klebsiella pneumoniae: enhancement of anaerobic growth and nitrogen fixation under stress by proline betaine, γ-butyrobetaine andother releated compounds. Can. J. Microbiol. 30, 299-305.

Lawrence, T., Cooper, J.P. and Breese, E.L. 1973. Cold tolerance and winter hardiness in Lolium perenne II - Influence of light and temperature during hardening. J. Agric. Sci. Camb. 80, 341-348.

Lee, S.P., Chen, T.H.H. 1993. Molecular cloning of abscisic acid responsive mRNA's expressed during the induction of freezing tolerance in bromegrass suspension culture. Plant. Physiol. 101, 1089-1096.

Letham, D.S. 1978. Cytokinins. In Phytohormones and related compounds, (eds) Letham D.S., Goodwin, P.B. and Higgins T.J.V.

Le Rudlier, D. And Valentine R.C. 1982. Genetic engineering in agriculture: osmoregulation. Trends in Biochem. Sci. 7, 431-433.

Levitt, J. 1956. The hardiness of plants. 278pp. Academic press, New York.

Levitt, J. 1980. Responses of plants to environmental stresses. Vol 1. Chilling, freezing and high temperature stresses. 2nd edition, Academic press, New York.

Lichfield, J. 1997. The Independent 23 April.

Lin, C. and Thomashow, M.F. 1992. A cold-regulated Arabidopsis gene encodes a polypeptide having potent cryoprotective activity. Biochem. Biophyis. Res. Comm. 183, 1103-1083.

Lindow, S.E. 1995. Control of epiphytic ice nucleation-active bacteria for management of plant frost injury. In- Biological ice nucleation and its applications (Lee Jr, R.E., Warren, G.and Gusta, L.V. eds). American Phytopathological Society. St Paul, Minnesota. 239-257.

Liu, Y.L., Steponkus, P.L. 1983a. Influence of proline on the tolerance of isolated protoplasts to osmotically induced contraction/expansion. Cryobiol. 20, 707.

Liu, Y.L., Steponkus, P.L. 1983b. Influence of proline on the tolerance of isolated protoplasts to osmotically induced contraction/expansion. Plant Physiol. 72,93 (Suppl.)

Livingston, D.P., III. 1996. The second phase of cold hardening: Freezing tolerance and fructan isomer changed in winter cereal crowns. Crop Sci.. 36, 1568-1573.

Mantyla, E., Lang, V. and Palva, T. 1995. Role of abscisc acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LTI78 and RAB18 proteins in Arabidopsis thaliana. Plant Physiol. 107,. 141-148.

Martin, B.R. 1971. Statistics for Physicists. London Academic Press.

Mazur, P. 1963. Kinetics of water loss from cells at sub-zero temperatures and the likelihood of intracellular freezing. J. Gen. Physiol. 47, 347-469.

Mazur, P. 1969. Freezing injury in plants. Ann. Rev. Plant. Physiol. 20, 419-448.

Mazur, P. 1970. Cryobiology: The frezzing of biological systems. Science. 168, 939-949.

McCown, B. H., McLeester, R.C., Beck, G.E. and Hall, T.C. 1969. Environment-induced changes in peroxidase zymograms in the stems of deciduous and evergreen plants. Cryobiol. 5, 410-12.

Medford, J.L., Horgan, R., El-Sawi, Z. and Klee, H.J. 1989. "Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene". The Plant Cell. 1, 403-413.

Medina, J. and Salinas, J. 1999. Identification and molecular characterisation of CBF2 and CBF3, two genes from Arabidopsis that encode AP2 domain-containing proteins and are induced by low temperatures. In: Plant Responses to Environmental Stress (eds, Smallwood, M.F., Calvert, C.M. and Bowles, D.J.) BIOS Scientific Press. 83-97.

Meza-Basso, L., Alberdi, M., Raynal, M., Ferrero-Cadinanos, M-L. and Delseny, M. 1986. Changes in Protein Synthesis in Rapeseed (Brassica napus) Seedlings during a Low Temperature Treatment. Plant Physiol. 82, 733-738.

Miller, S. Janin, J., Lesk, A.M. and Ghothia, C. 1987a. Interior and Surface of Monomeric Proteins. J. Mol. Biol. 196, 641-656.

Miller, S. Janin, J., Lesk, A.M. and Ghothia, C. 1987b. The Accessible Surface Area and Stability of Oligomeric Proteins. Nature. 328,834-836.

Morgan, J.M. 1980. Osmotic Adjustment in Spikelets and Leaves of Wheat. J.Exp. Bot. 31, 655-665.

Morgan, J.M. 1984. Osmoregulation and Water Stress in Higher Plants. Ann. Rev. Plant Physiol. 35, 299-319.

Morton, W.M. 1968. Effects of freezing and hardening on sulphydryl groups of protein fractions from cabbage leaves. Plant physiol. 44, 168-172.

Mundy, J. and Rogers, J.C. 1986. Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparisons to the barley amylase/subtillism inhibitor. Planta. 169,51-63.

Muthalif, M.M. and Rowland, L. 1994. Identification of Dehydrin-Like Proteins Responsive to Chilling in Floral Buds of Blueberry (Vaccinium, section Cyanococcus). Plant Physiol. 104, 1439-1447.

Nelson, W.R. and Van Staden, J. 1984. The effect of seaweed concentrate on wheat culms. J. Plant. Physiol. 115, 433-437.

Nordin, K., Heino, P. and Palva, E.T. 1991. Separte signal pathways regulate the expression of a low-temperature-induced gene in Arabidopsis thaliana. Plant. Mol. Biol. 16, 1061-71.

Nutter, F.W. and Schultz, P.M. 1995. Improving the accuracy and precision of disease assessments: Selection of methods and use of computer-aided training programs. Can. J. Plant. Path. 17, 174-184.

O'Brien, R.D. and Van Bruggen, A.H.C. 1992. Accuracy, precision and correlation to yield loss of disease severity scales for corky root lettuce. Phyopath. 82, 91-96.

Ogolevets, I.V. 1976. Study of hardening of isolated callus tissue of trees with different frost resistance. Fiziol. Rast. 23, 139-145.

Okanenko, A.A. 1974. Dynamics of fats in annual shoots of apple varieties differeing in frost resistance. Fiziol. Biokhim. Kul't Rast. 6, 90-94.

Olien, C, R. 1977. Barley: Patterns of Response to Freezing Stress. Technical Bulletin No 1558. Agricultural Research Service, United States Department of Agriculture. U.S. Goverment Printing Service.

Palta, J.P., Levitt, J. and Stabelmann, E.J. 1977a. Frezzing injury in onion bulbs. II. Post-thawing injury or recovery. Plant Physiol. 60, 398-401.

Palta, J.P., Levitt, J. and Stabelmann, E.J. 1977b. Frezzing injury in onion bulbs. II. Evaluation of the conductivity method and analysis of ion and sugar efflux from injured cells. Plant Physiol. 60, 393-397.

Palta, J.P., Levitt, J. and Stabelmann, E.J. 1977. Freezing tolerance of onion bulbs and significance of freeze-induced tissue infiltration. Cryobiol. 14, 1372.

Palva, E.T. 1994. Gene expression under low temperature stress. In- Stress induced gene expression in plants (Basra, A.S. ed). Harwood Academic, New York. 103-130.

Parker, S.R., Whelan, M.J. and Royle, D.J. 1995. Reliable measurement of disease severity. Assp. App. Biol. 43, 1-8.

Parsons, L.R. 1977. Water Relations, Stomatal Behaviour, and Root Conductivity of Red Osier Dogwood Durring Acclimation to Freezing Temperatures. Plant Physiol. 62, 64-70.

Patterson, B., Murata, T., Graham, T. 1976. Electrolyte leakage induced by chilling in Passiflora species tolerant to different climates. Aust. J. Plant. Physiol. 3, 435-442.

Pearce, R. 1990. Molecular analysis of acclimation to cold. Plant. Gro. Reg. 29, 47-76.

Pearce, R.S, Dunn, M.A., Rixon, J.E., Harrison, P. and Hughes, M.A. 1996. Expression of cold-inducible genes and frost hardiness in crown meristem of young barley (Hordeum vulgare L. cv. Igri) plants grown in different environments. Plant. Cell. Env. 19, 275-290.

Pearce, R. And Willison, J.H.M. 1985. A freeze-etch study of the effect of extracellular freezing on the cellular membranes of wheat. Planta. 163, 304-316.

Pescod, D. 1965. A method of forming dew on plants under controlled conditions. Jour. Agric. Eng. Res. 10, 328-332.

Pommeroy, M.K., Siminovitch, D. and Wightman, F. 1970. Seasonal biochemical changes in the living bark and needles of red pine in relation to adaptation to frezzing. Can. J. Bot. 48, 953.

Pomeroy, M.K. and Siminovitch, D. 1971. Seasonal cytological changes in secondary pholoem parenchyma cells in Robinia pseudoacacia in relation to cold hardiness. Can. J. Bot. 49, 787-95.

Pyee, J., Yu, H. and Kolattukudy, P.E. 1994. Identification of a lipid trasnfer protein as the mojor protein in the surface wax of broccoli (Brassica oleracea) leaves. Arch. Bichem. Biophyis. 311, 460-8.

Pyee, J. and Kolattukudy, P.E. 1995. The gene for the major cuticular wax-associatied protein and three homologous genes from broccoli (Brassica oleracea) and their expression patterns. Plant. J. 7, 49-59.

Quamme, H.A., Stushnoff, C. and Weiser, C.J. 1972. The relationship of exotherms to cold injury in apple tissue. J. Am. Soc. Hortic. Sci. 97, 608.

Ramsden, E.N. 1990. A-Level Chemistry, second edition. Stanley Thornes (Publishers) Ltd.

Raskin, I. 1992. Role of Salicylic Acid in Plants. Ann. Rev. Plant. Physiol. Mol. Biol. 43, 439-463.

Reaney, M.J.T and Gusta, L.V. 1999. Modeling sequential responses of plant cells to freezing and thawing. In: Cold-Adapted Organisms: ecology, physiology, enzymology and molecular biology (eds Margesin, R. and Shinner. F.) Springer. 119-135.

Robertson, M. and Chandler, P.M. 1994. A dehydrin cognate protein from pea (Pisum sativum L.) with an atypical pattern of expression. Plant. Mol. Biol. 26, 805-816.

Roberts, D.W.A. 1974. The invertase complement of cold-hardy and cold-sensitive wheat leaves. Can. J. Bot. 53, 1333-37.

Roberts, D.W.A. 1978. Changes in the proportions of two forms of invertase associated with cold acclimation of wheat. Can. J. Bot. 57, 413-419.

Roos, Y.H. 1986. Phase Transitions and Unfreezable Water Content of Carrots, Reindeer Meat and White Bread Studied using Differential Scanning Calorimetry. J. Food Sci.. 51, 684-686.

Ross, K.D. 1978. Differential Scanning Calorimetry of Nonfreezable Water in Solute-Macromolecule-Water Systems. J. Food Sci, 43, 1812-1815.

Salisbury, F.B. and Ross, C.W. 1992. Plant Physiology fourth edition. Wadsworth.

Sanderson, K.J. and Jameson, P.E. 1986. The cytokinins in liquid seaweed extract : Could they be the active ingredients ? Acta Hortic. 179, 113-116.

Santarius, K. A. 1973. The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost desiccation and heat resistance. Planta.113, 105-14.

Sakai, A. and Yoshida, S. 1968. The role of sugar and related compounds in variations of freezing resistance. Cryobiol. 5, 160-174.

Sakai, A. 1962. Studies on the frost hardiness of woody plants. I. The causal relation between sugar content and frost hardiness. Contrib. Inst. Low. Temp. Sci. B.11, 1 - 40.

Sakai, A. and Larcher, W. Frost Survival of Plants. Springer-Verlag. 1987.

Sambrook, Fritsch and Maniatis. 1989. Molecular Cloning: A Laboratory Manual Second Edition vol 3. Cold Springer Harbor Laboratory Press.

Saunders, N.D. 1997. An evaluation of the effects of extracts of Ascophyllum nodosum and surface water on the frost resistance of crop plants. M. Phill Thesis University of Plymouth.

Scopes, R.K. 1987. Protein Purification Principles and Practice 2nd Edition. Springer-Verlag.

Senn, T.L., Martin, J.A., Crawford, J.H. and Darting, C.W. 1961. S. Carolina Agric. Exp. Stn. Ser. No23.

Shmueli, E. 1960. Chilling and frost damage in banana leaves. Bull. Res. Counc. Isr. Bot. 8D, 225-238.

Siminovitch, D. and Briggs, D.R. 1949. The chemistry of the living bark of the black locust tree in relation to frost hardiness. I. Seasonal variations in protein content. Arch. Biochem. 23, 8-17.

Siminovitch, D. and Briggs, D.R. 1953. The chemistry of the living bark of the black locust tree in relation to frost hardiness. IV. Effects of ringing on translocation, protein sybthesis, and the development of hardiness. Plant Physiol. 28, 177-200.

Stadelmann, E.J. and Kinzel, H. 1972. Vital staining of plant cells. In: Methods in cell physiology. (ed. Prescott, D.M.) Academic Press. New York. 325.

Steffen, K.L., Arora, R., Palta, J.P. 1989. Relative sensitivity of photosynthesis and resperation to frezze-thaw stress in herbaceous species. Plant. Physiol. 80, 1372-79.

Stephenson, W.M. 1966. The effect of hydrolysed seaweed on certain plant pests and diseases. Proc. 5th. Int. Seaweed. Symp. 405-415.

Steponkus, P.L. 1971. Cold acclimation of Hedra helix evidence for a two phase process. Plant Physiol. 47, 175-80.

Steponkus, P.L. and Uemura, M. 1999. The quest to elucidate the role of the COR genes and polypeptides in the cold acclimation process. In: Plant Responses to Environmental Stress (eds, Smallwood, M.F., Calvert, C.M. and Bowles, D.J.) BIOS Scientific Press. 99-110.

Steponkus, P.L. 1984. Role of the plasma membrane in frezzing injury and cold acclimation. Ann. Rev. Plant. Physiol. 35, 543-84.

Steponkus, P.L. Dowgert, M.F. and Gordon-Kamm, W.J. 1983. Destabilization of the plasma membrane of isolated plant protoplasts during a freeze-thaw cycle: the influence of cold acclimation. Cryobiol. 20, 448-65.

Steponkus, P.L., Lanphear, F.O. 1968. The relationship of carbohydrates to cold acclimation of Hedera helix L. c.v Thorndale. Physiol. Plant. 21, 777-791.

Steveni, C.M., Norrington-Davies, J. and Hankins, S.D. 1992. Effect of seaweed concentrate on hydroponically grown spring barley. J. App. Phycol. 4, 173-180.

Stirk, W.A. and Van Staden, J. 1977. Comparison of cytokinin- and auxin-like activity in some commercially used seaweed extracts. J. App. Phycol. 8, 503-508.

Sugget. A. 1975. Water-Carbohydrate Interactions. In Water Relations of Foods, edited by R.B.Duckworth. Academic Press.

Svasti, J. and Panjipan, B. 1977. SDS-Polyacrylamide Gel Electrophoresis A Simple Explanation of Why it Works. J. Chem. Edu. 54, 560-562.

Tay, S.A.B., MacLeod, J.K., Palni, L.M.S. and Letham, D.S. 1985. Detection of cytokinins in a seaweed extract. Phyto-chemistry. 24, 2611-2614.

Teeter, M.M. 1984. Water Structure of a Hydrophobic Protein at Atomic Resolution: Pentagonal Rings of Water Molecules in Crystals of Crambin. Proc. Nat. Ac. Scie. USA. 81, 6014-6018.

Trunova, T.L. 1965. Light and Temperature Systems in the Hardening of Winter Wheat and the Significance of Oligosaccharides for Frost Resistance. Fiziol. Rast. 12, 70-77 (Cited in Abe and Yoshida, 1997).

Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. Proc. Nat. Aca. Sci. USA. 76, 4350-4354.

Thomashow, M.F. 1999. Regulation of plant cold acclimation. In: Plant Responses to Environmental Stress (eds, Smallwood, M.F., Calvert, C.M. and Bowles, D.J.) BIOS Scientific Press. 75-82.

Uemera, M. and Steponkus, P.L. 1998. Freezing tolerance of Arabidopsis thaliana mutants that constiuitively express COR15a, COR6.6 and CBF1 genes. Cryobiol. 37, 390-391.

Uemera, M.and Yoshida S. 1984. Involvement of plasma membrane alterations in cold acclimation of winter Rye seedlings (Secale cereale L. cv. Puma) Plant Physiol. 75, 818-826.

Uemera, M., Steponkus, P.L. 1994. A contrast of the plasma lipid membrane composition of oat and rye leaves in relation to freezing tolerance. Plant. Physiol. 104 (No2), 479-496.

Urry, D.W. 1995. Elastic Biomelecular Machines. Scientific American. 272, 64-69.

Vierling, E. 1991. The roles of heat-shock proteins in plants. Ann. Rev. Plant Physiol. Plant. Mol. Biol. 42, 579-520.

Volger, H., Heber, U. and Berzborn, R.J. 1978. Loss of function of biomembranes and solubilization of membrane proteins during frezzing. Biochim. Biophys. Acta. 511, 455-69.

Wheeler, A.W. 1973. Endogenous growth substances. Rep. Rothamsted. Exp. Stn. Part 1, 101-102.

White, T.C., Dunn, M.A., Brown, K. and Hughes,M.A. 1994. Comparative analysis of genomic sequence and expression of a lipid transfer gene feamily in winter barley. J. Exp. Bot. 45, 1885-92.

Wickson, M. and Thimann, K.V. 1958. "The antagonism of auxin and kinetin in apical dominance" Physiol. Plant. 11, 62.

Williams, W.P. 1990. Cold induced lipid phase transitions. Phil. Trans. Royal. Soc. Lond. Series B. Biol. Sci. 326, 555-570.

Williams, P.J. and Burt, T.P. 1974. Measurement of hydraulic conductivity of frozen soils. Can. Geotech. J. 11,647-650.

Wilhem, K.S. and Thomashow, M.F. 1993. Arabidopsis thaliana cor15b, an apparent homologue of cor15a, is strongly responsive to cold and ABA, but not drought. Plant. Mol. Biol. 23, 1073-7.

Wisniewski, M. and Fuller, M. 1999. Ice Nucleation and Deep Supercooling in Plants: New Insights Using Infrared Termography. In Cold-Adapted Organisms Ecology, Physiology, Enzymology and Molecular Biology. Springer.

Wisniewski, M., Lindow, S.E. and Ashworth, E.N. 1997. Observations of ice nucleation and propagation in plants using infrared video thermography[1]. Plant Physiol. 113, 327-334.

Wisniewski, M., Close, T.J., Artlip, T., Arora, R. 1996. Seasonal patterns of dehydrins and 70-kDa heat-shock proteins in bark tissues of eight species of woody plants. Physiol. Plant. 96, 496-505.

Wisniewski, M.and Arora, R. 1993. Adaptation and response of fruit trees to freezing temperatures. In: Handbook of cytology, histology and histochemistry of fruit tree diseases. (Ed. Biggs. A.R.) CRC Press. Boca Raton. USA.

Wrightman, F. Schneider, E.A. and Thimann, K.V. 1980. Hormonal factors controlling the initiation and development of lateral roots. II. Effects of exogenous growth factors on lateral root formation in pea roots. Physiol. Plant. 49, 304-314.

Yoshida, S. and Uemura, M. 1984. Protein lipid composition of plasma membranes from Orchard grass (Dactylis glomerata L.) and changes during cold acclimation. Plant Physiol. 75, 31-37.

Yoshida, S. 1984. Studies on freezing injury of plant cells. I. Relation between thermotropic properties of isolated plasma membrane vesicles and frezzing injury. Plant Physio. 75, 38-42.

Zhang, M.L.N. and Willison, J.H.M. 1987. An improved method for the measurement of frost hardiness. Can. J. Bot. 40, 630.