

THE ROLE OF ICE NUCLEATION ACTIVE BACTERIA IN FROST DAMAGE TO  
EARLY SOWN *Solanum tuberosum* var. JERSEY ROYAL.

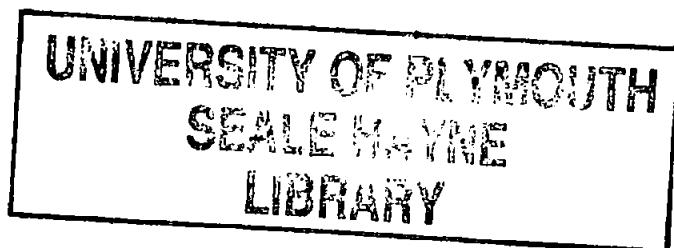
by

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fulfilment for the degree of Doctor of Philosophy.

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*Philip L. L.*

**Abstract.**

The role of Ice Nucleation Active (INA) bacteria in frost damage  
to early sown *Solanum tuberosum* var. Jersey Royal.

by

Philip Le Grice.

The present study indicated that *S. tuberosum* var. Jersey Royal could supercool to temperatures as low as  $-6^{\circ}\text{C}$  during *in-vitro* frost tests, whereas in the field freezing occurred at immediate sub-zero temperatures (between  $0$  and  $-1^{\circ}\text{C}$ ). The microbial flora upon the early sown *S. tuberosum* var. Jersey Royal did not contain INA bacterial species active at warm sub-zero temperatures. Plants inoculated with a strain of *Ps. syringae* (84:27) containing type 1 active nuclei readily froze at  $-2$  to  $-3^{\circ}\text{C}$ . Applications of leaf surface water applied to plants during *in-vitro* frost tests resulted in nucleation temperatures comparable to those measured in the field. Applications of leaf surface water initiated freezing in whole plants during *in-vitro* frost tests at temperatures warmer than those caused by *Ps. syringae* (84:27). Thus field frost kill and ice nucleation in the presence of leaf surface water occurred at temperatures warmer than type 1 INA bacterial nucleation temperatures.

Exogenous foliar applications of ethylene glycol applied in the presence of leaf surface water led to enhanced supercooling and frost avoidance during *in-vitro* frost tests and during field frost events. It was concluded that modifying the formation and freezing of leaf surface water would be more likely to provide a frost control strategy applicable to the field crop than frost control through manipulation of phylloplane microorganisms.

CONTENTS.		page
Copyright Statement.		i
Abstract.		ii
List of Contents.		iii
List of Figures.		vi
List of Tables.		viii
List of Plates.		ix
Acknowledgments.		x
Declaration.		xi
<b>Chapter 1. INTRODUCTION.</b>		<b>1</b>
1.1. Economic importance of frost damage.		1
1.2. Ice formation.		4
1.3. Ice formation within plants.		6
1.4. Plant freezing and injury.		8
1.5. Plant Freezing Resistance.		13
1.6. Frost protection strategies.		22
1.7. Premise of this study.		23
1.8. Experimental Hypotheses.		27
<b>Chapter 2. EQUIPMENT AND TECHNIQUE DEVELOPMENT.</b>		<b>29</b>
2.1. Temperature measurement and recording.		29
2.2. Microbiological techniques.		33
2.3. Agronomy of Jersey Royal Plant Populations and Plant Freezing.		40
2.4. Characterisation of Environmental test chamber.		43
<b>Chapter 3. MATERIALS AND METHODS.</b>		<b>46</b>
<b>Section 1. Investigation of the supercooling ability         of <i>S. tuberosum</i> var. Jersey Royal.</b>		<b>46</b>
Experiment 1. A comparison of in-vitro and in-vivo frost events and the effect of applied ice nucleating bacteria and antibiotic on nucleation temperatures of the early sown <i>S. tuberosum</i> var. Jersey Royal.		46
Experiment 2. Investigations of in-vitro supercooling ability.		51
Experiment 3. A comparison of the effects of dew and a known ice nucleation active strain of <i>Pseudomonas syringae</i> on supercooling ability.		56

<b>Section 2. An investigation of Ice Nucleating Bacteria upon Early Sown <i>S. tuberosum</i> (var. Jersey Royal).</b>	<b>58</b>
Experiment 4. An investigation of INA bacteria on field grown plants at Seale-Hayne.	58
Experiment 5. Pilot survey of microflora associated with the indigenous crop.	59
Experiment 6. Detailed survey for Ice Nucleating Bacteria upon early sown <i>S. tuberosum</i> var. Jersey Royal on the Island of Jersey.	60
<b>Section 3. Phylloplane Microflora of the Early Sown <i>Solanum tuberosum</i> var. Jersey Royal in relation to microclimatology.</b>	<b>62</b>
Experiment 7. Microclimatology and its effect on bacterial populations on crop plants.	62
Experiment 8. Long term temperature survey on the Island of Jersey within a commercial crop.	63
<b>Section 4. The promotion of supercooling of the early sown <i>S. tuberosum</i> var. Jersey Royal as frost avoidance mechanism using exogenous foliar applications.</b>	<b>65</b>
Experiment 9. A preliminary screening of agents to promote supercooling of excised leaves.	65
Experiment 10. The effect of foliar applied ethylene glycol upon the supercooling ability of plants during in-vitro frost tests.	67
Experiment 11. The effect of foliar applied ethylene glycol upon the supercooling of crop plants <i>in-vivo</i> .	68
<b>Chapter 4. RESULTS.</b>	<b>70</b>
Section 1. Investigation of the supercooling ability of <i>S. tuberosum</i> var. Jersey Royal.	70
Section 2. Investigation of Ice Nucleating Bacteria upon Early Sown <i>S. tuberosum</i> var. Jersey Royal.	90
Section 3. Phylloplane Microflora of the Early Sown <i>S. tuberosum</i> var. Jersey Royal in relation to microclimatology.	99

Section 4. The promotion of supercooling of the early sown <i>S. tuberosum</i> var. Jersey Royal as frost avoidance mechanism using exogenous foliar applications.	107
Chapter 5. DISCUSSION OF RESULTS.	119
Chapter 6. GENERAL DISCUSSION.	134
6.1. Critique of the present study.	143
6.2. Directions for future study.	146
APPENDIX 1. The potential of thermal imaging technology in the study of frost damage to sensitive plants:- Visualisation of ice nucleation and plant freezing.	149
A.1. Introduction.	149
A.2. Materials and Methods.	150
A.3. Results.	153
A.4. Discussion.	164
APPENDIX 2. Examples of primary data files, their manipulation and statistical analysis.	167
REFERENCES.	173

## List of Figures.

### Chapter 1.

- 1.1. Early potato cropping patterns in the UK. 3
- 1.2. Geographical location of the island of Jersey. 24
- 1.3. Market prices of *S. tuberosum* var. Jersey Royal. (1991). 25

### Chapter 2.

- 2.1. Typical plant tissue exotherm. 30
- 2.2. Temperature characteristics of cold stage. 37
- 2.3. Temperature characteristics of cold stage following anti condensation measures. 38
- 2.4. Temperature characteristics of cold stage after final modifications. 39
- 2.5. Typical cooling curve measured within the environmental test chamber (Mountford Instruments Ltd.) 45

### Chapter 3.

- 3.1. Experimental design of field trial. Section 1, Experiment 1. 48
- 3.2. Effect of interruption of temperature stability systems of the environmental test chamber. 52

### Chapter 4.

- 4.1. Typical supercooling and exotherm response of in-vitro frozen *S. tuberosum* var. Jersey Royal. 71
- 4.2. The effect of foliar applied treatment on the probability of an exotherm occurring. 72
- 4.3. Average air temperature compared to temperature measured at the plant during a damaging frost event. 73
- 4.4. Effect of simulated dew on exotherm temperature. 84
- 4.5.a,b. Droplet freezing spectra of soil suspension pre and post autoclaving. 85, 86
- 4.6. Effect of foliar treatments on exotherm temperature. 89
- 4.7. Bacterial populations recovered from the field crop through sampling period. 91
- 4.8. Bacterial populations retrieved on nutrient agar and following replica plating onto *Pseudomonas* selective agar. 94

4.9.	Normal probability plot of Log10 transformed total population and of Log10 transformed populations retrieved on <i>Pseudomonas</i> selective (cfc) agar.	95
4.10.	Comparison of nucleation spectra of bacteria retrieved from <i>Pseudomonas</i> selective agar with that of <i>Ps. syringae</i> 84:27.	96
4.11.	Proportions of bacterial species isolated from the <i>Solanum tuberosum</i> var. Jersey Royal crop.	98
4.12.a, b and c.	Patterns of plant temperature, air temperature and relative humidity over a commercial crop of <i>S. tuberosum</i> var. Jersey Royal potatoes.	100
4.13.	Variations of bacterial populations recovered from the field crop in relation to plant temperature, air temperature and relative humidity.	101
4.14.	Cyclical pattern of heating and cooling including three consecutive frost events measured at Jersey trial site.	102
4.15.a and b.	Tautochrones showing differences in development of a frost event at two points within the Jersey trial site.	105, 106
4.16.	Mean exotherm temperatures of excised leaves treated with kaolin, ethylene glycol and seaweed extract in the presence of artificial dew applied to runoff.	109
4.17.	Tautochrones illustrating development of frost event at Seale-Hayne. 20/03/93.	113
4.18.	Typical <i>in-vivo</i> exotherm measured 20/03/93 at the Seale-Hayne trial site.	114
4.19.	Temperature trace of damaged plant not displaying an exotherm (20/03/93 Seale-Hayne trial site.).	115
6.1.	Comparison of nucleation spectra of dew treated and dry <i>S. tuberosum</i> var. Jersey Royal plants frozen <i>in-vitro</i> .	138
Appendix 1.		
1.	Layout of a typical thermal imaging experiment.	152
2.	Comparison of exotherm temperatures of the freezing events monitored using the Agema Thermovision 487.	163



Appendix 2.

1. Graph of exotherm taken from retrieved data file.

170

List of tables.

Chapter 4.

1.	Effect of treatment on mean damage levels.	77
2.	Effect of replicate on mean damage levels.	77
3.	Comparison of minimum temperatures across replicates.	78
4.	Comparison of thaw temperatures across replicates.	78
5.	Effect of bacterial treatment on glasshouse and field bacterial populations.	79
6.	Effect of bacterial treatments upon proportions of <i>Ps. syringae</i> upon the foliage of the outdoor crop.	80
7.	Comparison of mean tissue nucleation temperature.	83
8.	Effect of dew and bacterial treatment on nucleation temperatures of plant tissue.	88
9.	Effect of developmental stage on nucleation temperature of plant tissue.	88
10.	Effect of level of dew application on mean nucleation temperatures of excised leaves of the glasshouse grown <i>S. tuberosum</i> var. Jersey Royal.	108
11.	The effect of seaweed extract on mean nucleation temperature of excised leaves of the glasshouse grown <i>S. tuberosum</i> var. Jersey Royal in the presence of artificial dew applied to runoff.	108
12.	The effect of foliar applied ethylene glycol on the mean nucleation temperature of glasshouse grown <i>S. tuberosum</i> var. Jersey Royals.	111
13.	Effect of treatment on mean frost damage levels on the crop established at Howard Davis Farm.	117
14.	Effect of treatment on mean exotherm temperatures of the crop established at Seale-Hayne.	117
15.	Effect of treatment on mean frost damage levels on the crop established at Seale-Hayne.	115

## Appendix 2.

1. Example of comma separated variable (.csv) file  
retrieved from data logger. 169
2. Example analysis of variance report subsequent  
to collection of nucleation temperatures. 171
3. Example of Fishers LSD report subsequent to  
ANOVA. 172

## List of Plates

### Chapter 2.

- |      |                               |    |
|------|-------------------------------|----|
| i.   | Modified cold stage.          | 36 |
| ii.  | Plants pre and post freezing. | 42 |
| iii. | Environmental test chamber.   | 44 |

### Appendix.

- |      |             |   |     |
|------|-------------|---|-----|
| i.   | a, b and c. | Progression of freezing event through dry foliage using Cyclops TI35.         | 155 |
| ii.  | a, b and c. | Progression of freezing event through dew treated foliage using Cyclops TI35. | 157 |
| iii. | a, b, c, d. | Close up thermal images of dry foliage using Agema Thermovision 487.          | 159 |
| iv.  | a, b, c, d. | Close up thermal images of dew treated foliage using Agema Thermovision 487.  | 160 |
| v.   | a, b and c. | Thermal image of a cauliflower curd using Agema Thermovision 487.             | 162 |

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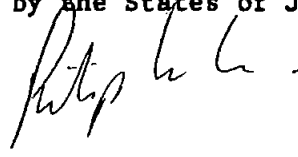
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## Chapter 1. INTRODUCTION.

### 1.1. Economic Importance of Frost Damage

Of the earth's surface only 7.6% is cultivated, the remainder is too hot, too cold, too dry, too steep or too salty. Of these factors drought and cold are the most widespread limitations to cultivated boundaries (Franks 1982). Frost injury is a serious abiotic disease of plants (Lindow *et al* 1978a) and the risk of frost damage is perhaps one of the greatest determinants of the boundaries of agricultural production worldwide. For example, the risk of frost damage is a primary factor limiting deciduous fruit tree production in the Pacific Northwest U.S.A. Cody *et al* (1987) and Yelenosky (1988) stated that in the USA losses in agriculture as a result of frost damage amount to millions of dollars, and 'billion dollar' crops such as citrus are especially vulnerable. Gusta and O'Connor (1987) reflect this statement by claiming that untimely spring and autumn frosts cause agricultural losses of millions of dollars as a result of quality reduction with frost induced lesions allowing bacterial and fungal infection. In crops such as canola (oil-seed rape) and mustard direct loss of yield can also occur due to the sowing of lower yielding later varieties in an effort to avoid frost damage.

Losses to particular crops grown in regions specialising in early production can lead to devastating economic loss as a result of frosting. Cornish growers of winter cauliflower (*Brassica oleracea* var. *botrytis*) bore a loss estimated at £1.25M in the 1985/86 growing season and £3.5M in the 1986/87 season (MAFF 1987, Sheppard 1986). Similarly the most serious limitation to blackcurrant (*Ribes*

*nigrum L.*) production in northern temperate areas of Europe is the risk of frost damage causing death and damage to early flower buds (Brennan, 1991).

Taylor (1990) reported that untimely frosting in 1989 caused an estimated shortfall in revenue to the Texas sugar cane industry of £18.6 million dollars. He stated that 'the subsequent reduction in buying potential of those involved in the sugar industry would have financial repercussion through the whole Rio Grande Valley'.

The potato (*S. tuberosum*), a frost sensitive crop, has become a staple food crop throughout the world and is cultivated in 126 countries (Food and Agriculture Organisation of the United Nations 1992). Although grown on a smaller area than the seven grain staples (wheat, rice, maize, millet, barley, oats and rye), its fresh weight yield is greater than any one of these (Burton 1966). Richardson and Weiser (1972) stated that the threat of economic losses due to frost injury is present in almost every region of the world where potatoes are cultivated and therefore the consequences of frost damage to the potato are of global importance. Within the UK, potato growing can be divided into main crop and early production. Main crop, or ware production, represents the greater land area sown (103 302 ha). (Potato Marketing Board 1991) and fresh weight harvested and provides a UK staple food crop. This later sown crop can suffer localised yield and financial penalties due to late frosts. Early production although occupying only 15,192 ha (Potato Marketing Board 1991), can account for some of the highest prices per tonne realised in the market and are grown in traditionally warmer agricultural areas (figure 1.1.). Despite these planting patterns early potatoes



1.1. Map of UK showing areas of first early potato production. (Source Potato Marketing Board)

are at risk from Spring frosts. Early potato production was first established in Jersey (Thomas and Eyre 1950). The island exported around 850 imperial tons to London in 1809. By World War Two the potato export was worth in the region of £0.5M. Today the export value of the Jersey potato crop is £21.5M (Anon 1991). Production of early potatoes in Cornwall followed a similar history to that of Jersey with its origins in the last century. The early production from Pembrokeshire (now part of Dyfed) however, was of a more recent origin, first grown on a farm scale in the 1920s (Thomas and Eyre 1950).

More recently, due to improved and cheaper transport, imports of early potatoes from Cyprus, Spain, Portugal and Egypt, where frost risk is not present, have affected marketing and prices from traditional UK early areas. This pressure has forced even earlier UK and Channel Island sowings and increased potential frost risk to the crop.

#### 1.2. Ice formation.

The freezing of water is a first order phase transition, that is, at the normal freezing point the potentials of the co-existing solid and liquid phases are equal. However the fact that water has the ability to supercool suggests that a nucleation event must occur. A nucleation event can be regarded as the occurrence of a solid/liquid interface and freezing involves the transfer of molecules from the liquid phase to the solid phase at this interface (Franks 1982). This interface or ice nucleus can be a foreign particle, however, pure water samples do freeze. Laboratory studies have shown small quantities of pure water cooling to as low as  $-40^{\circ}\text{C}$  before ice

nucleation occurred (Bigg, 1953). This suggests that the water molecules themselves must arrange in a way that acts as a nucleus. When foreign particles act as ice nuclei the event is described as heterogenous nucleation. The spontaneous nucleation triggered by water molecule arrangement without a foreign nucleus is termed homogeneous nucleation.

The study of the supercooling and nucleation of water has a long history, with a review as early as 1775 ( in Ahn 1976). It is now accepted that the crystallisation of ice is not a property of water itself but a function of the nuclei present (Franks 1982) whether these nuclei are homogeneous clusters of water molecules, or foreign particles. This fact makes the repeatable study of homogeneous nucleation difficult, due to the problems involved in screening for ice nuclei. Similarly, nucleation events require fluctuations in time and space therefore experimental design can have great effects on the results to be interpreted.

The theory of heterogeneous ice nucleation is not well developed (Franks 1982), but is related to the molecular geometry of the particle. As a result the effectiveness of the particle as a nucleator is dependent on the free energy at the solid/water interface. The range of freezing temperatures will be wide due to the large variety of nucleating particles. Similarly, the freezing point of a given sample will depend on the effectiveness of the nuclei present. The form that a distribution of heterogeneous nucleation temperatures takes has been described by Mason (1957) who suggested that the fraction of foreign particles active as nuclei at a given temperature over a given time is an exponential function of

the degree of supercooling.

### 1.3. Ice Formation within Plants.

The study of ice formation in plants is a study of a heterogeneous nucleation system. Nucleation sites of some form must be active in or on plants in the field since freeze damage frequently occurs at temperatures close to 0°C. The picture however, is complicated due to the differing effectiveness of nucleators and their variable behaviour in relation to species, sample size and tissue maturity (Kaku 1975). Such evidence from the field is further complicated by a wide range of freezing and supercooling temperatures observed in the laboratory by numerous teams recording freezing temperatures from -10.5 °C to -2 °C (Lindow, 1983, Burke et al. 1976, Rajashekar et al 1983, Ashworth and Anderson 1985). The formation of hoar frost is thought to be the most effective cause of plant freezing (Marcellos and Single, 1979). An ice nucleus active at temperatures close to 0°C must however, be present to initiate hoar frost formation. Atmospheric ice nucleators active at warm temperatures in the range -3°C to 0°C are virtually unknown (Lindow, 1983), and studies have shown that initiation of frost on vegetal surfaces by airborne particles is very unlikely (Marcellos and Single, 1976). It would appear likely that nucleation of dew may be caused by some characteristic of the epicuticular surface but as yet such a characteristic remains unquantified. Recent studies have demonstrated the presence of intrinsic plant ice nuclei active at temperatures close to 0°C. Cody *et al.* (1987) working with pear (*Pyrus communis* L.) presented evidence of intrinsic ice nuclei active in the range -1.5 °C to -5 °C.

Colonization of the leaf surface by certain bacterial species, which have been shown to be ice nucleation active (INA) have been demonstrated as having an observable role in increasing or inciting frost damage in many plants, (Maki *et al.* 1974, Yankofsky *et al.* 1981). Marshall (1988) demonstrated that certain cultivars of oat (*Avena sativa*) facilitated greater proliferation of such bacteria with resultingly greater frost damage. This supported the hypothesis that specific leaf epicuticular morphology may enhance nucleation, which in this case may have been due to the favourable conditions for bacterial proliferation.

Levitt (1980) suggested that, if ice nucleated externally, internal nucleation was due to contact of such external ice with internal water through wounds or stomata. In contrast, ice forming internally appeared to be associated with cells of the xylem vessels i.e. those areas of bulk water. Once ice forms in the plant a vapour pressure and temperature gradient is established between the ice and protoplasm. Water is drawn from the cells to freeze in the intercellular spaces until an equilibrium is reached. At equilibrium the cell solute is at its freezing point. If the temperature continues to decline, the vapour pressure of the ice continues to drop and more water is drawn from the cells until a new equilibrium is reached (Levitt, 1980).

Experimental evidence for ice formation travelling rapidly through the vascular system exists for many species including mulberry (*myrtilis* sp)(Kituara 1967) and lemon (*Citrus sinensis*) (Yelenosky 1975). The advance of ice into cells is thought to be arrested by characteristics of the lipid plasma membrane (Chambers and Hale,

1932, in Levitt, 1980).

Outside the vascular system ice formation is harder to follow. This is due to the small spaces and volumes involved and the consequent difficulty of exothermal assay (Marcellos and Single 1979). Only after long periods at sub-zero temperatures (when large volumes of water have frozen) is it possible to start to determine patterns of ice formation outside the vascular tissue. Nath and Fisher (1971) showed epidermal lifting in alfalfa (*Medicago sativa*) leaves (after prolonged exposure to  $-8^{\circ}\text{C}$ ) as a result of freezing between the mesophyll and epidermis.

Freezing rate influences the path of ice growth and crystal size. The faster the freeze then generally, the smaller is the ice crystal formed. Rapid freezing does not permit migration of water to favoured extracellular freezing sites and can be more damaging as a consequence.

#### 1.4. Plant freezing and injury.

Freeze injury is the result of ice formation in the tissues of plants which are damaged if they freeze. Ice can form either intracellularly or extracellularly. Intracellular freezing is generally fatal and tends to be the result of fast freezing (e.g. subsequent to supercooling) with cell by cell flash freezing resulting in thousands of small ice crystals forming throughout the vacuole and protoplast and injury probably due to cataclysmic stress (Asahina, 1956). Despite studies noting that intracellular ice formation is fatal, few hypotheses addressing why it is injurious



have emerged (Steponkus 1984). It has been postulated that ice ruptures the cells or that ice in contact with the plasma membrane is damaging (Levitt 1980). Steponkus *et al.* (1982) postulated that intracellular freezing was a result of damage to the plasma membrane, not a cause.

Extracellular freezing results in a spectra of injury from no damage to fatal depending on the severity and duration of the sub-zero temperatures experienced and on the species of plant and its ability to tolerate extracellular ice formation. Such factors are further discussed in section 1.5. (Freezing Resistance).

Subjective methods of measuring or recognising injury post-freezing include noting changes in texture, odour, discolouration and water soaked appearance of plant tissue (White *et al.* 1989). Water soaked appearance has been correlated with ion leakage (largely K<sup>+</sup>) (Palta *et al.* 1977). Indeed ion leakage has been used as a basis for quantitative assay for frost damage by virtue of its relationship with electrical conductivity (Dexter *et al.*, 1932, Fuller *et al.* 1989). It has been assumed that such leakage is a result of membrane rupture or loss of semi-permeability (Steponkus and Weist, 1978) however Palta and Li (1980) indicated by microscopic examination that onion membranes remained intact after freezing and the cell could later be plasmolysed. On freezing, *S.tuberosum* exhibits loss of turgor and ion leakage, however electron microscopy indicated that although 75% of ions had leaked from the cell at -3°C, cell membranes, mitochondria, chloroplasts and nuclei all appeared normal. At freezing temperatures lower than those required to initiate damage *S.acuale* and *S.tuberosum* both displayed

protoplasm swelling, mitochondria and chloroplast damage followed finally by loss of cell compartmentalisation. Such findings suggest that freeze injury is not initiated by rupture of the plasma membrane (Li and Fennel 1985).

Palta and Li (1980) compared freeze damaged (exhibiting cytoplasmic streaming) live onion cells and undamaged onion cells in relation to their permeability to water and non-electrolytes to ascertain if alterations in the plasma membrane were responsible for observed increases in ion efflux. They found no differences in permeability to methyl-urea, urea or water yet once again a sharp increase in leakage of  $K^+$  ions was recorded. They suggested that intrinsic membrane proteins involved in active ion transport were altered or inactivated on freezing resulting in channels for the passive export of  $K^+$ .

Ice formation, subsequent to nucleation, draws water from the cell to freeze in the intercellular spaces. Thus a form of dehydration stress is operating during freezing which may be responsible for freezing injury. Sukumaron and Weiser (1972) working with potatoes demonstrated that damage was greater in samples exposed to freezing than in samples subjected to identical levels of dehydration through desiccation. This suggests that freezing must have some injurious effect other than dehydration.

Iljin (1933) hypothesised that mechanical forces exerted upon the plasma membrane in a non-uniform manner during freezing caused damage to the membrane. Tao *et al.* (1983) produced work to support this theory by examining the freezing tolerance of protoplasts and

untreated cells of *S.tuberosum*. They discovered that freezing tolerance of protoplasts was the greatest, followed by cells in which cytorrhysis or plasmolysis had been induced with no difference between the two. The worst tolerance was exhibited by the intact cell. They concluded that the level of dehydration stress was equal for all groups and thus the difference in tolerances was due to the mechanical strains associated with the relative ease of expansion and contraction of the plasma membrane related to the strength of its attachments to the rigid cell wall. In the intact cell the plasma membrane is less able to shrink thus inducing cell wall collapse. This was perhaps the mechanical stress Iljin discussed in 1933.

Steponkus *et al.* (1982) suggested that during freezing contraction deletion of membrane material occurred and upon thawing this did not reincorporate into the membrane resulting in membrane lysis. The work of Singh (1982) supported this theory. He noted invaginations and folds in the plasma membrane during non-lethal freeze dehydration and contraction (-5°C) of Rye. At lethal freezing temperatures the bilayer structure was lost, and multi-layered vesicles were formed. Kinnucan (1980) found that in cold acclimated *S.tuberosum* callus membrane lipids accumulated into large crystallised areas inside the membrane bilayer when cells were subjected to lethal freezing temperatures (-9°C). Singh (1982) suggested that fusion of the membrane at these points would occur and not return to the original state upon thawing. Freeze fracture of *S.tuberosum* did not show similar lipid patches but there was evidence of membrane rupture.

As well as freezing, rapid thawing has also been shown to cause plant injury (Levitt, 1980, Gusta and Fowler, 1977). As ice in intercellular spaces melts osmotic pressure and vapour pressure between the cell and extracellular water rises which can lead to rupture of the plasmalemma and death. Slow thawing enables the vapour pressure to rise with temperature, maintaining the equilibrium between ice and cells, allowing rehydration with little or no osmotic shock. Using a model for membrane compression and collapse which demonstrated evidence that tangential membrane pressures do occur in an osmotically stressed cell, Williams (1981), re-enforced the experimental evidence that cell death equates with some form of membrane damage in response to pressure changes. The model also presented evidence that the osmotic potential which can be developed in cells by mechanical resistance has the same potential energy limits as the surface pressure which the lipids can support without collapse.

Steponkus (1984) concluded that a lack of understanding still surrounds the subject of freeze injury and cited two reasons for the deficiency:

a) attempting to attribute all forms of injury to a single stress, with the apparent goal of excluding other stresses.

b) insufficient understanding of what constitutes cellular injury.

He further stated that a comprehensive theory of freeze injury requires that data be accommodated in the hypothesis, rather than simply judged as secondary or inconsequential.

### 1.5. Plant Freezing Resistance.

Not all plants are killed by frost and ice formation as a result of sub zero temperatures. Mechanisms exist which render plants either resistant or susceptible to freeze damage. Much literature has been written on the subject of freeze tolerance in plants (reviewed in Steponkus 1984). No attempt will be made to review all literature on this subject, however some regard must be paid to this work because the results are valuable to the agriculturalist interested in freezing injury to sensitive crops and in particular freeze avoidance and its environmental modifications. Furthermore, a review of freeze tolerance is necessary since it has produced banks of data invaluable to the worker interested in frost avoidance.

Levitt (1980) states that a plant has two routes to freeze resistance:-

- a) Freeze tolerance
- b) Freeze avoidance

#### 1.5.1. Freeze Tolerance

Tolerance of freezing can take one of two possible forms:- tolerance of intracellular freezing or tolerance of extracellular freezing. As intracellular freezing is invariably fatal in natural systems (cryopreservation however, can involve some non-fatal intracellular freezing characteristics) only tolerance of extracellular ice formation is of use to the plant. Mechanisms exist within certain plants to enable survival of extracellular ice formation including its ability to harden (acclimation). A plant must have the ability to harden if it is to avoid damage and this

hardening trait is more useful if hardening can occur rapidly, especially if there is a risk of the plant being exposed to de-hardening conditions (Habeshaw and Swift, 1978, Fuller and Eagles 1978).

#### 1.5.2. Freeze avoidance.

Several mechanisms of freeze avoidance are recognised:-

##### Avoidance of freezing conditions.

It has long been established that the most freeze resistant stage in the life cycle of a plant occurs during its most dehydrated form such as seed or spore (Lipman, 1936). Hence a plant which reverts to seed during a season when the vegetative portion would otherwise be exposed to freezing conditions is demonstrating an avoidance strategy. Similarly, tender plants cannot in nature colonise areas which would predispose them to damaging frosts and are by default avoiding freezing conditions

##### Freezing point depression or avoidance of freeze dehydration.

Freezing point depression or avoidance of freeze dehydration can be regarded as avoidance mechanisms. If physiological processes render a plant tolerant to freezing (by avoidance of freeze dehydration), it is avoiding damage that a non-acclimated plant would have sustained. Burke *et al.* (1976) state however, that freezing point depression itself is not an important means of freeze avoidance in all plants but may be significant in plants of limited hardiness.

## Avoidance of freezing (supercooling).

To avoid intracellular freezing a plant must supercool or form ice extracellularly and be tolerant of the resultant dehydration stress (freeze tolerant). Through supercooling a plant can avoid all of the stresses associated with ice formation within tissues.

In plants normally tolerant of extracellular ice formation the issue of supercooling presents a paradox, if a nucleation event occurs on/in a plant close to 0°C then ice may form extracellularly and can result in a slow dehydration stress which the plant must tolerate to escape injury. Yet if a plant supercools and a nucleation event subsequently occurs, rapid fatal intracellular freezing may be more likely to take place. Lindow (1982a) stated that 'whilst supercooling of frost sensitive tissues is required for their survival, it may be detrimental to certain tolerant plant species by allowing rapid freezing of nucleated supercooled water in these plant tissues'. Rajashekar *et al.* (1983) reinforced this statement working with tuber bearing *Solanum* species. They demonstrated that in the absence of supercooling non-hardened and cold hardened leaves tolerated extracellular freezing between -3.5°C and -8.5°C. If ice initiation occurred however, at any temperature below -2.6°C the leaves were lethally injured.

Water can remain supercooled in plant tissues as long as the temperature remains above the homogeneous nucleation temperature, or above the temperature of the most effective heterogeneous ice nuclei (Burke *et al.* 1976). Li and Fennel (1985) stated that the extent of nucleation in nature was dependent on the nucleation ability of the

plant and its surroundings. Kaku (1973) postulated that ice nucleating ability was a characteristic of a given species, yet Marcellos and Single (1976) presented evidence which suggested that variation in nucleation ability occurred within species as a result of tissue maturity. Rajashekar *et al.* (1983) demonstrated that nucleation ability in some varieties of *Solanum* species was decreased markedly following cold acclimation. They also found (in contrast to Burke *et al.* (1976)) that increasing exposure time to a sub-zero temperature increased the chances of a nucleation event occurring, and that the extent of supercooling decreased in response to a decreasing cooling rate.

The paradox of supercooling can perhaps be resolved by stating that tender plants require supercooling to survive, as any ice in their tissues is detrimental, whereas those plants that are tolerant of ice that forms within their tissues may be less prone to intracellular freezing if ice nucleation occurs as close to 0°C as possible (Anderson *et al.* 1982, Lindow, 1982a).

The discovery that epiphytic bacterial populations were able to incite ice formation at temperatures close to 0°C (Maki *et al.* 1974) has enabled supercooling to be classified as an avoidance mechanism, since without the presence of INA bacteria plant tissues are notably poor nucleators and are able to supercool thus avoiding frost damage (Lindow, 1982b, Marcellos and Single, 1976).

Ashworth (1986) stated that three criteria must be met before INA bacteria can be implicated in inciting freeze damage:-



a) Bacteria must be active ice nucleators at or above the same temperature range that frost injury occurs.

This first point has been well documented and many studies have been published (reviewed by Lindow 1982a, 1983) since the discovery of bacterial ice nucleation by Maki *et al.* (1974). More recent studies are still highlighting bacteria acting as ice nuclei at warm sub zero temperatures (Obata *et al.* 1990).

b) Bacteria must be present in sufficient numbers to initiate ice formation.

The determination of surface bacterial populations has also stimulated much research but the results are not straightforward. Early reports stated the ubiquity of INA bacteria in nature (Lindow *et al.* 1978b) and furthermore that injury was directly proportional to the logarithm of the population (Lindow, 1978a). More recently however, Gross *et al.* (1983) in a study of two climatically different areas of the Pacific North West, found that INA bacteria were not ubiquitous. In fact INA bacteria were undetectable in many fruit orchards during the critical bloom period when frost damage was likely to occur. Ashworth *et al.* (1985) supported such findings with a study that demonstrated that the mean ice nucleation temperature (MNT) was relatively steady through the year. On only two occasions were INA bacteria present in measurable quantities and these did not coincide with measurements exhibiting a higher ice nucleation temperature. The picture is further complicated by the fact that not every cell in an INA population is active at a given time (Maki *et al.* 1974, Yankofsky *et al.* 1981). It appears that the temperature of nucleation is a function of the size and nucleation

frequency of the population (Lindow *et al.* 1982a, 1982b). In addition to these factors, studies indicate that:- bacterial species (Maki *et al.* 1974, Lindow, 1982a), strain (Hirano *et al.* 1978, 1985), population phase (Yankofsky *et al.* 1981) growth temperature (Anderson *et al.* 1982), and time at a sub zero temperature (Rogers *et al.* 1987) may affect nucleation activity. Yankofsky *et al.* (1981) stated that if given enough time 'a cell capable of initiating ice formation at -10°C will also do so at -4°C' thus highlighting that a time dependence factor may affect results. Furthermore Maki *et al.* (1974) demonstrated that dead bacterial cells can retain ice nucleating ability and studies have shown that warm temperature nucleation characteristics do require cellular integrity (Yankofsky *et al.* 1981). The issue of whether sufficient bacterial numbers exist on leaf surfaces to incite frost damage was further explored by Hirano and Upper (1989). They demonstrated the importance of the influence that the microclimate of the crop must exert on leaf residing populations. They estimated a mean doubling time of 4.9 hrs and measured a 28 fold increase in *P.syringae* populations on snap bean (*Phaseolus vulgaris*) leaflets during 12 hrs of a 24 hr sampling period.

Such a wealth of interacting complications to the question of bacterial populations and their levels on plants led Ashworth (1986) to state that the derivation of a threshold value appropriate for the numerous situations confronted by the horticulturalist is not likely to be established.

c) Plants must be able to supercool in the absence of INA bacteria.

As it is postulated that INA bacteria interrupt the supercooling

response it must be shown that plants are able to supercool. Several reports state the inefficiencies of plants as ice nuclei at temperatures above  $-8$  to  $-10^{\circ}\text{C}$  (Arny *et al.* 1976, Lindow *et al.* 1982b and Marcellos and Single, 1979). In these assessments leaf homogenates or leaf discs were frozen according to variations on droplet freezing techniques. Yet it has been noted that the levels of supercooling demonstrated by such techniques have not been mirrored by whole plant or field studies (Ashworth, 1986). Rajashekar *et al.* (1983) working with *Solanum* species demonstrated a lack of intrinsic nuclei active about  $-6.9^{\circ}\text{C}$ , however, when whole plants were cooled, levels of supercooling ranged from  $-5.2^{\circ}\text{C}$  to  $-6.9^{\circ}\text{C}$ . These workers also noted that cooling rate had an effect on supercooling, in that the slower the plants were cooled the less supercooling occurred.

Recent work has demonstrated that intrinsic ice nuclei are associated with certain species and that ice nucleation activity of plant tissue is mass dependent. Anderson and Ashworth (1986) found that sample weight markedly increased the freezing temperature of tomato plants (*Lycopersicon esculentum*). Plants that were harbouring INA bacteria showed freezing at  $-2$  to  $-3^{\circ}\text{C}$  and that mass had no effect. They concluded that this was because the bacteria and not plant associated nuclei were initiating the ice formation. The results indicated that reducing INA bacterial populations on tomato plants with a mass exceeding 50g would not affect freezing temperature, yet frost protection of small seedlings may be afforded by bacterial control. Anderson (1988) in a study using six tomato cultivars showed that differences in frost avoidance capability between cultivars was not apparent, yet plant mass and INA bacteria

significantly affected the freezing temperatures. He concluded that future studies must use uniform plant mass or include plant mass as a covariate.

The interactions of plant mass, intrinsic nuclei and INA bacteria have been further studied in woody deciduous plants. Lindow (1983) reported that pear and citrus blooms can have frost injury reduced by INA bacterial control. Similarly considerable interest in the ability of peach (*Prunus persica*) flowers to supercool and avoid freeze damage exists. Proebstring *et al.* (1982) supercooled detached peach flowers to  $-8^{\circ}\text{C}$ . In a study presented by Cody *et al.* (1987) the existence of an intrinsic ice nucleus active in pear trees at temperatures from  $-1.5^{\circ}\text{C}$  to  $-5^{\circ}\text{C}$  was discovered. Anderson and Smith (1989) found that the presence of the stem and resulting increase in mass (in the absence of INA bacteria), precluded the peach flower from supercooling. Proebstring and Gross (1988) stated that although control of INA bacteria resulted in considerably reduced frost injury in herbaceous species conclusive evidence does not exist for woody plants and they supported this statement experimentally by showing that the MNT of detached flowers contaminated with INA bacteria was consistently  $1^{\circ}\text{C}$  to  $2^{\circ}\text{C}$  lower than those attached to their stems, demonstrating the overriding influence of the wood associated nuclei.

It can be seen therefore that supercooling and its modification is a complex area of study and has led to the formulation of two theories. A stochastic mechanism of ice nucleation has been favoured by recent work (Ashworth *et al.* 1985, Ashworth and Anderson 1985), as opposed to the singular theory (which predicts that freezing

temperature is predominantly a function of ice nucleus character), used by Vali and Stansbury (1966) to describe the freezing characteristics of water samples. Stochastic theory assumes that all samples have the same chance of freezing at any moment and predicts continued freezing over time (Vali and Stansbury, 1966). Ashworth *et al.* (1985) and Yelenosky (1988) working with peach and citrus respectively demonstrated a time dependence in freezing which supports stochastic theory. Ashworth and Anderson (1985) carried out a direct test of singular theory using tomato plant stem sections. Samples were cooled to  $-5^{\circ}\text{C}$  and subsequently warmed to  $-4^{\circ}\text{C}$ . Singular theory would predict that all nuclei active at  $-5^{\circ}\text{C}$  and below would have been expressed at  $-5^{\circ}\text{C}$ , whatever the duration, and upon rewarming no further nucleation would occur. The results showed that a further 33% of samples froze in the 7 hrs at  $-4^{\circ}\text{C}$ . The fact that decreased cooling rate leads to reduced supercooling (Rajashekar *et al.* 1983) is perhaps evidence to support stochastic theory, in that a plant cooled at a slow rate is, in effect, being exposed to a single temperature for a longer duration. Thus investigations of supercooling and its modifications must take into account time/mass interactions. Leaf sample assays are only valid if they are harbouring INA populations great enough to ensure that any intrinsic nuclei (or mass effect) activity is overridden, as leaf sample assays overestimate supercooling ability.

### 1.6. Frost protection strategies.

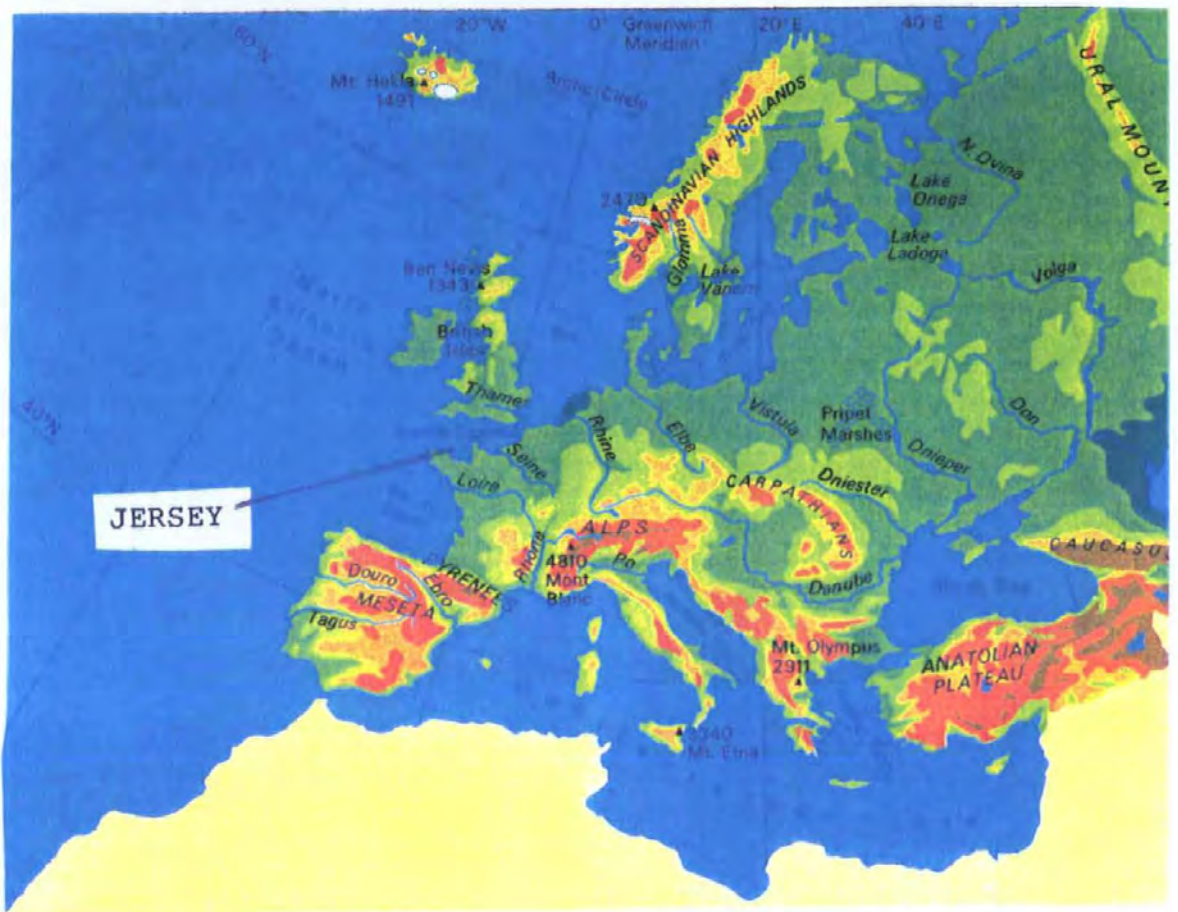
Methods of controlling frost in the field were highlighted by Blanc (1969). These included:-

- 1) Planting in traditionally warm areas.
- 2) The use of wind machines and helicopters to mix warm air layers above the canopy with cold layers surrounding the crop.
- 3) The use of heaters.
- 4) Water irrigation.
- 5) Artificial smokes and fogs.
- 6) Foam like insulation.

However, as a result of the energy intensive nature of some of these methods they are not economically viable in most agricultural situations, although in some high value production systems such as fruit orchards their use can be justified.

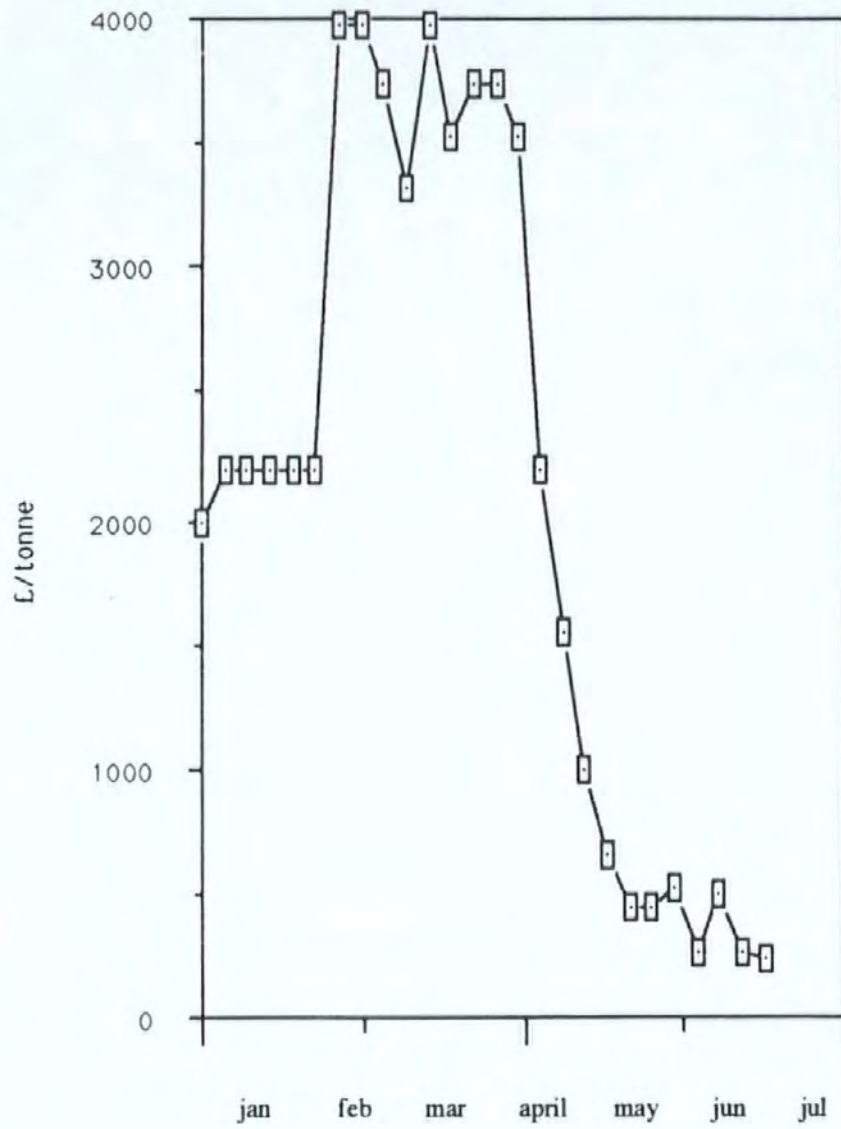
### 1.7. The premise of this study.

The present study is concerned with the investigation of the supercooling ability of the *S. tuberosum* var. Jersey Royal, and the factors that interrupt this supercooling response. As shown in the literature review, the supercooling response is not fully understood or characterised for any particular species. Similarly the effect of INA bacteria as modifiers of supercooling has as yet not been implicated in all agricultural crops. It has been postulated that discrete areas of production are more likely to lead to frost protection regimes applicable in the industry, rather than a general predictive or threshold bacterial population and control regime being found applicable to all species and situations (Ashworth, 1986). The Island of Jersey, with its specialist production of early potatoes, is such a discrete unit in terms of geography and production (fig 1.2). Furthermore the crop is of high economic importance to the agricultural industry of the island accounting for more than £21.5 M annually or in excess of 60% of the islands vegetable export revenue (Anon 1991). Frost can be damaging to the early potato crop, and consequently to the islands economy. Losses occur in the following ways; firstly, reduction in leaf area index following a frost can reduce net assimilation rate and thus reduce yield; more importantly, however, is the fact that such damage can check the crop, causing delay and subsequent missing of peak market prices for the early crop (Fig 1.3). Recent trends in potato supply have seen other imports enter the United Kingdom from countries such as Cyprus, Spain and Egypt reducing prices as the season progresses. The risk of Spring frosts can also retard planting dates causing delayed harvest.



1.2. Geographical location of the island of Jersey.





1.3. Market prices of *Solanum tuberosum* var. Jersey Royal. (1991).

The growers of Jersey could be in an ideal position to exploit a frost protection system. The study is therefore of potential economic value in an applicable area of the agriculture industry and is of scientific value for the chance it presents to clarify the nature of supercooling and its modification in one of the worlds most important food crops.

### 1.8. Experimental Hypotheses.

This study is founded upon the following hypotheses:-

1. *S. tuberosum* var. Jersey Royal is frost sensitive and can not tolerate ice formation within its tissues.
2. As a result of 1. the foliar portion of the crop must supercool to avoid ice formation and subsequent damage.
3. Ice nucleation active bacteria have been found as leaf epiphytes on other crops and have been shown to interrupt supercooling leading to frost damage and may be implicated in frost damage to *S. tuberosum* var. Jersey Royal.

Ashworth (1986) summarised the conditions necessary for implication of INA bacteria in frost damage to sensitive crops as:-

- a) The bacteria must be active ice nucleators at or above the same temperature range that frost injury occurs.
- b) Bacteria must be present in sufficient numbers to initiate ice formation.
- c) The plants must be able to supercool in the absence of INA bacteria.

The experimental strategy of this study was developed to determine whether the frost damage complex of the early sown *S. tuberosum* var. Jersey Royal could be defined by the above conditions and hypotheses, with the aim of utilising this knowledge to develop a

frost control strategy. The experimentation addressed the hypotheses under the following broad headings:-

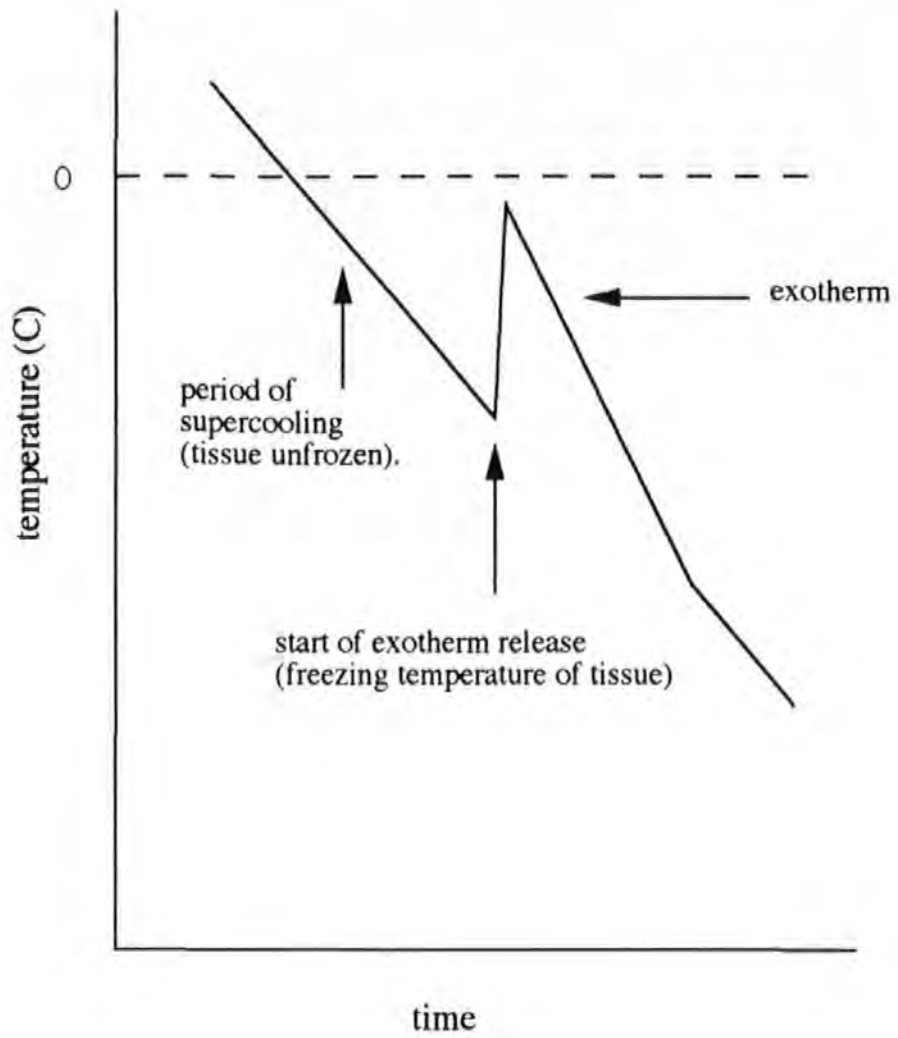
1. The supercooling ability of *S. tuberosum* var. Jersey Royal.
2. The microflora associated with *S. tuberosum* var. Jersey Royal.
3. The microclimatology of *S. tuberosum* var. Jersey Royal.
4. Frost protection strategies applicable to *S. tuberosum* var. Jersey Royal.

## Chapter 2. EQUIPMENT AND TECHNIQUE DEVELOPMENT.

### 2.1 Temperature Measurement and Recording.

Temperature measurement and recording are an integral part of frost damage studies. The essential nature of accurate temperature records for the study of plant freezing has already been highlighted. When a liquid freezes the latent heat of fusion is released and this heat release can be detected as a rise in temperature. This temperature rise is detectable in plant tissue when freezing occurs (fig 2.1.) and is called an exotherm. The temperature attained by the tissue immediately prior to exotherm release is the tissue nucleation temperature, the temperature at which ice starts to form in the tissue. Exotherm detection was employed to determine nucleation temperature of tissue samples in the present study.

A data logger was used to enable exotherm response to be recorded (Delta Logger DL2, Delta T devices, Burwell, Cambs.). The logger was capable of storing up to 65000 recordings over 64 channels using a wide variety of sensor types and time intervals. It was possible to customise data acquisition intervals and sensor types separately for each individual channel. This was important since an exotherm is a transient phenomenon, and as such, the logging device must record at short enough intervals to ensure that a temperature rise was detected. Similarly the actual temperature of nucleation occurs at the very beginning of the exotherm, hence if recording intervals are too protracted the temperature of the nucleation event will be masked. Recorded data was downloaded using a standard RS232 interface to a PC. Opus PCSX386 (Opus Technology Ltd.) equipped with



- 2.1. Illustration of exotherm release in plant tissue following a period of supercooling. (hypothetical).

a numeric co-processor supported with an Epson FX1050 dot matrix printer. (Appendix 2)

Data retrieval was carried out using Supercalc 5 (Computer Associates Ltd ). The design, however, of this spreadsheet limited RAM availability and thus to utilise the data storage capacity of the data logger large data files had to be segmented for study.

Thermocouple thermometry was used for all temperature measurement for the following reasons:-

1. The robust nature of thermocouple thermometry facilitated outdoor use.
2. Although not as accurate as other sensor types, precision was possible through calibration.
3. The small heat sink of thermocouples ensured a rapid response time.
4. Small sensor size allowed intimate contact with the material to be measured.
5. The traditional problem of a reference junction was overcome by the data logger which maintained an electronic reference based upon an inboard precision thermistor circuit.
6. Relatively small capital outlay.

The sensor types used were type T (copper/constantan) 1/0.315mm PTFE coated welded junction thermocouples. Thermocouples were calibrated using a standard calibration technique (British Calibration Service, National Physics Laboratory Queens Rd. Middx.) All thermocouple measuring junctions were placed for 3 hours in a

circulating glycol bath maintained at 0.0C and compared to an accurate nitrogen filled mercury in glass thermometer (BS592). The thermocouples were in turn connected to the logger and temperature recordings taken every 5 minutes and integrated to a single figure every hour. This process was repeated 4 times resulting in 12 readings for each thermocouple, which were then averaged to give a final offset temperature attributable to each individual thermocouple. These offsets were used as a correction value for each particular thermocouple thereafter. An offset value was preferable to a calibration over a range of temperatures because the Delta Logger contained standard calibration curves for mV to °C for type T thermocouples. Logger output was therefore in °C

The welded junction of the thermocouples was inserted into stem and leaf tissue of the plants subjected to frost testing. No other means of attachment was required during in-vitro frost tests. In the field thermocouple wires were tied to the plants using wire ties to further ensure that they were not dislodged. Unless otherwise stated all plants subjected to in-vitro frosting had one thermocouple placed in the stem 1-2cm above soil level and one thermocouple placed in the terminal leaflet of the uppermost leaf.

All plants monitored in the field had one thermocouple placed in the rib of the uppermost terminal leaflet.



## 2.2. Microbiological techniques.

### Bacterial Sampling.

Tissue samples were harvested aseptically from both field and glasshouse grown plants using a scalpel and forceps and immediately placed in sterile bags. Depending on the objective of the experiment either whole plants or individual leaves were harvested. Samples were macerated using a laboratory blender (Colworth Ltd.) in sterile quarter strength Ringers (Oxoid Ltd.) solution to effect a  $10^{-1}$  dilution. Unless stated otherwise the macerates were serially diluted in quarter strength Ringers solution and spread plated onto nutrient agar (Oxoid Ltd).

### Bacterial Identification.

Isolated bacteria recovered were screened for ice nucleation activity at  $-5^{\circ}\text{C}$  using replica plating after the method of Lindow et al (1978a). Bacterial isolates for identification were purified and stored in a laboratory refrigerator on nutrient agar plates and sub-cultured fortnightly to maintain viability.

Bacteria recovered on all plates including those showing confluent growth as well as lower dilutions were assessed for ice nucleation activity. Confluent plates were included in the assessment to account for any INA bacteria which may have been present in low numbers and would have been lost at lower dilutions.

Where appropriate bacterial isolates were identified with the

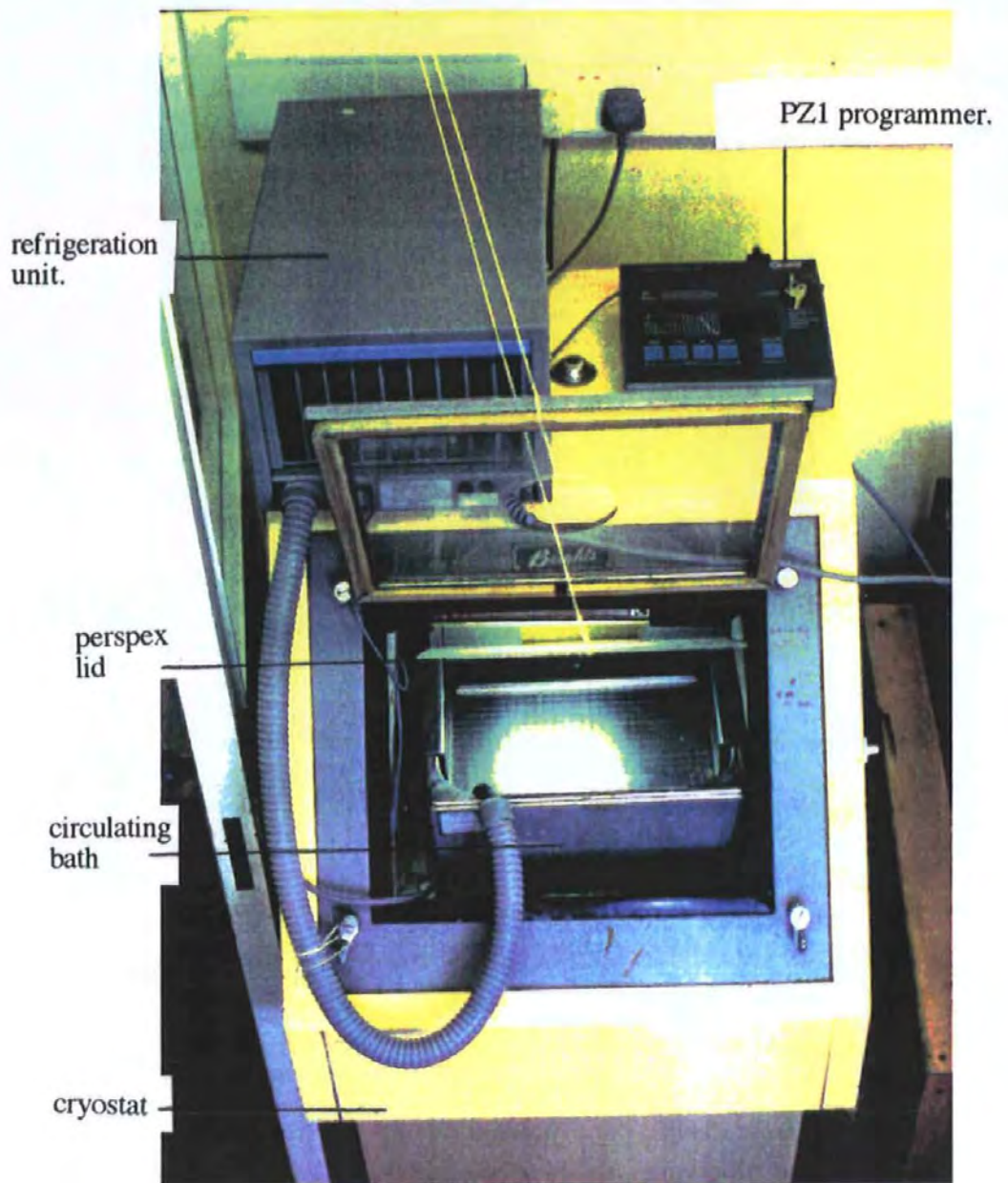
purpose of characterising *Ps. syringae* as part of the total population and illustrating the diversity of the species colonizing *S. tuberosum* var. Jersey Royal. Conventional morphological and biochemical tests were applied for these identifications.

#### Ice Nucleation Testing of Bacterial Isolates.

Droplet freezing spectra analysis (Vali 1971, modified by Lindow *et al* 1982a) and replica plate freezing (Lindow *et al* 1978a) were used to assay bacterial isolates for ice nucleation activity (see literature review). However the following modifications to the spectra analysis technique were necessary to achieve repeatable results. A cold stage was constructed from 2mm thick, food grade stainless steel and suspended from the edges of a circulating ethylene glycol filled refrigerated bath so that the base was in intimate contact with the glycol surface. All droplet spectra tests were carried out by placing one hundred 40ul droplets of the test suspension upon the cold stage and noting when the droplets froze. Droplet temperature was a function of the air temperature surrounding the droplet and the temperature of the cold stage. For each spectrum investigation four thermocouples were mounted on the stage under 40ul droplets of ethylene glycol. Droplet temperature over the whole stage was regarded as the average of these temperatures. Bath ethylene glycol temperature was the major determinant of droplet temperature but the differential between droplet temperature and bath temperature was wider at cooler temperatures and droplet temperature could not be accurately inferred from bath temperature (fig 2.2.).

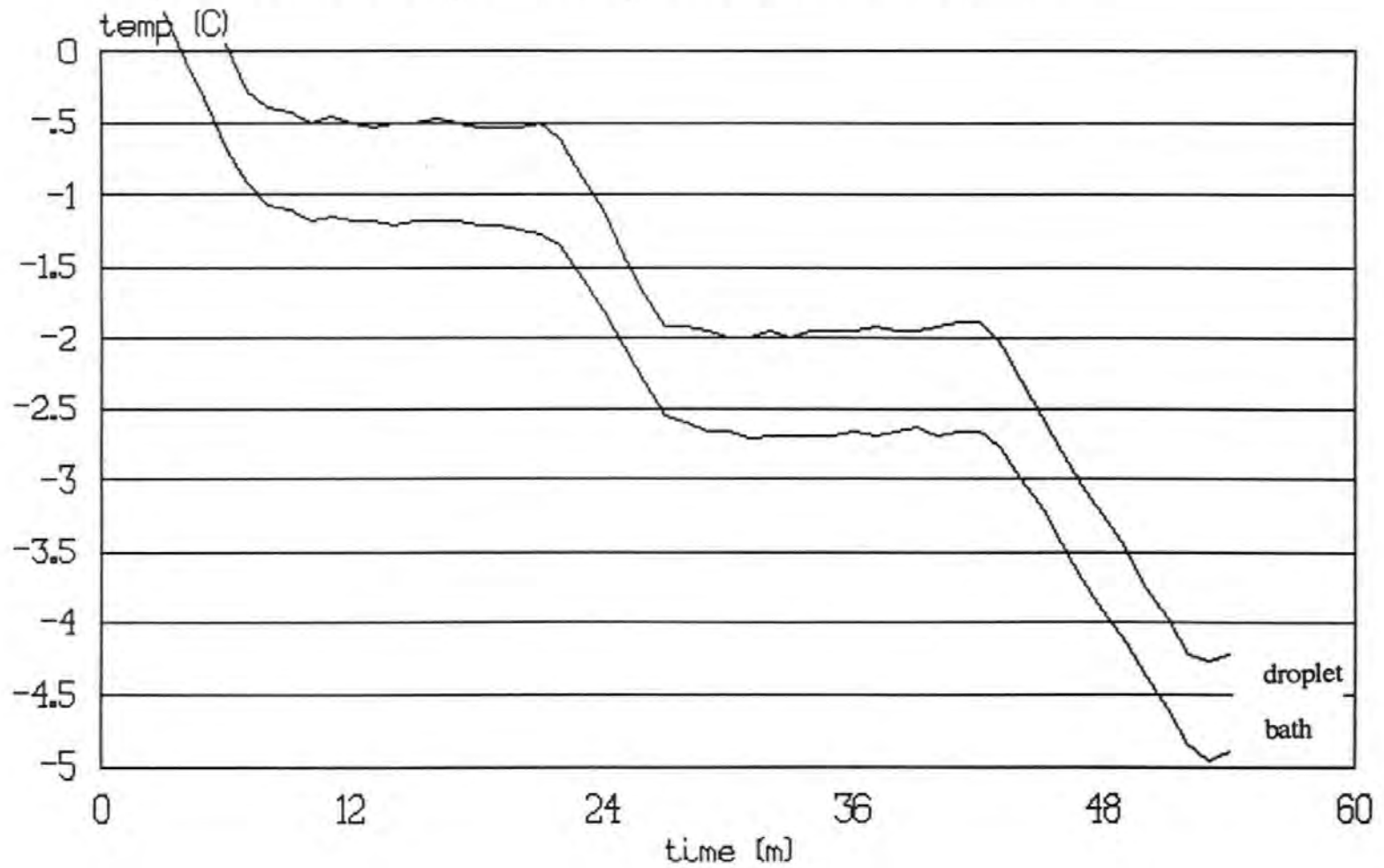
A serious problem of condensation forming upon the cold stage

during cooling was highlighted during early experimentation which is not referred to in the literature. This meant that when droplets of test suspensions were under investigation ice formation within condensation or droplets often seeded freezing in other droplets via networks of nucleating condensation. These problems led to spurious estimations of the nucleating ability of test suspensions. This problem was overcome by placing the circulating bath inside a cryostat (plate i.). With the cryostat running, the air surrounding the circulating bath was effectively de-humidified by frost formation upon the cryostat walls. The fabrication of a transparent perspex lid ensured that bath temperature remained the major determinant of droplet temperature and stopped seeding of the test droplets by ice crystals falling from the walls of the cryostat. These measures however, disrupted the temperature stability of the bath at preset temperatures (fig.2.3.) such that the proportion of droplets frozen at a given test temperature could not be recorded accurately. This problem was overcome by using a continuous cooling programme (fig.2.4.) and recording the time at which droplets froze. This was related to ethylene glycol droplet temperature and time recorded by the Delta logger. Thus each test droplet freezing time was noted during the experiments and compared with corresponding time and temperature recorded by the logger enabling deduction of the freezing temperature of test droplets.

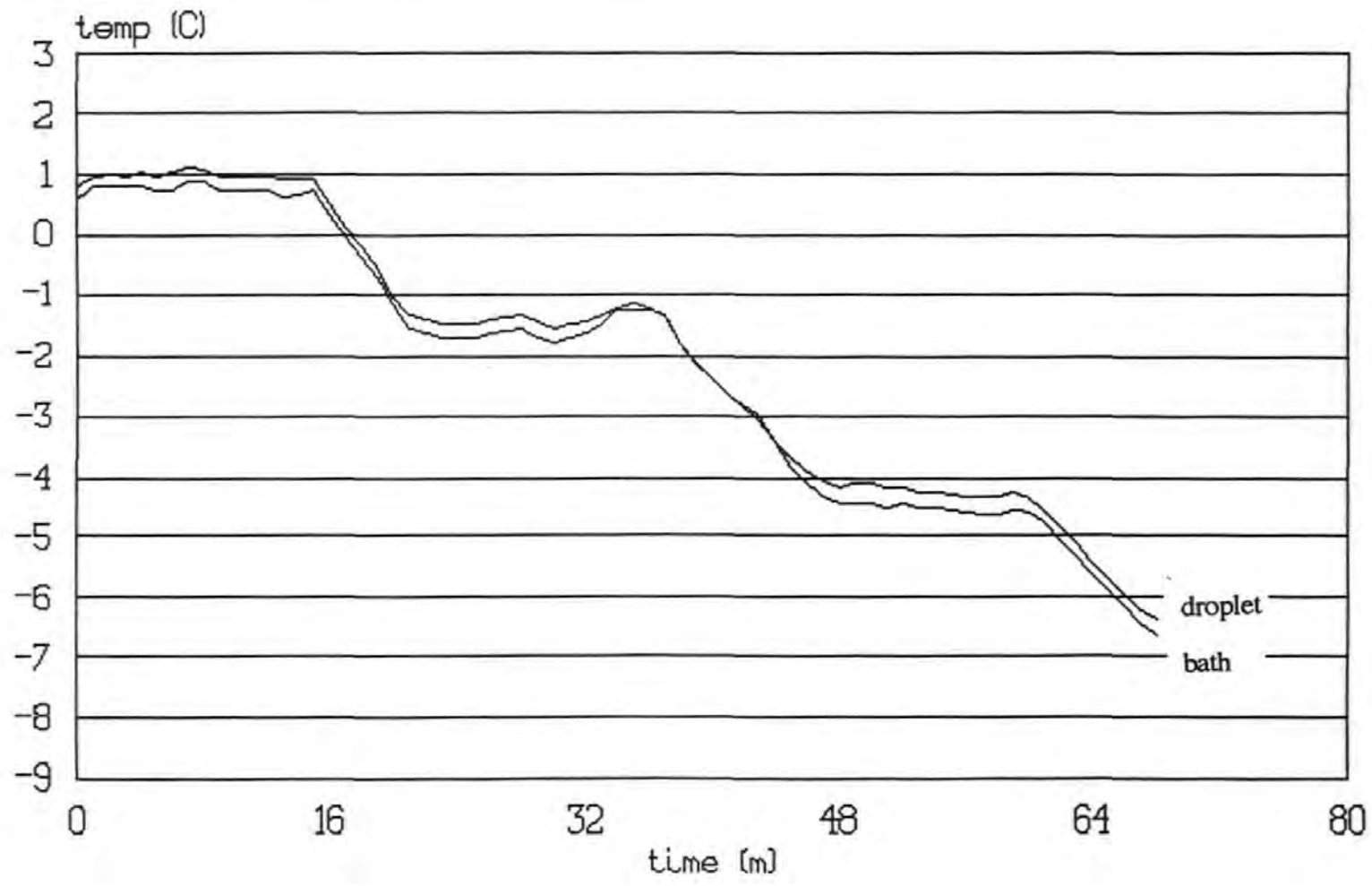


i. Modified cold stage.

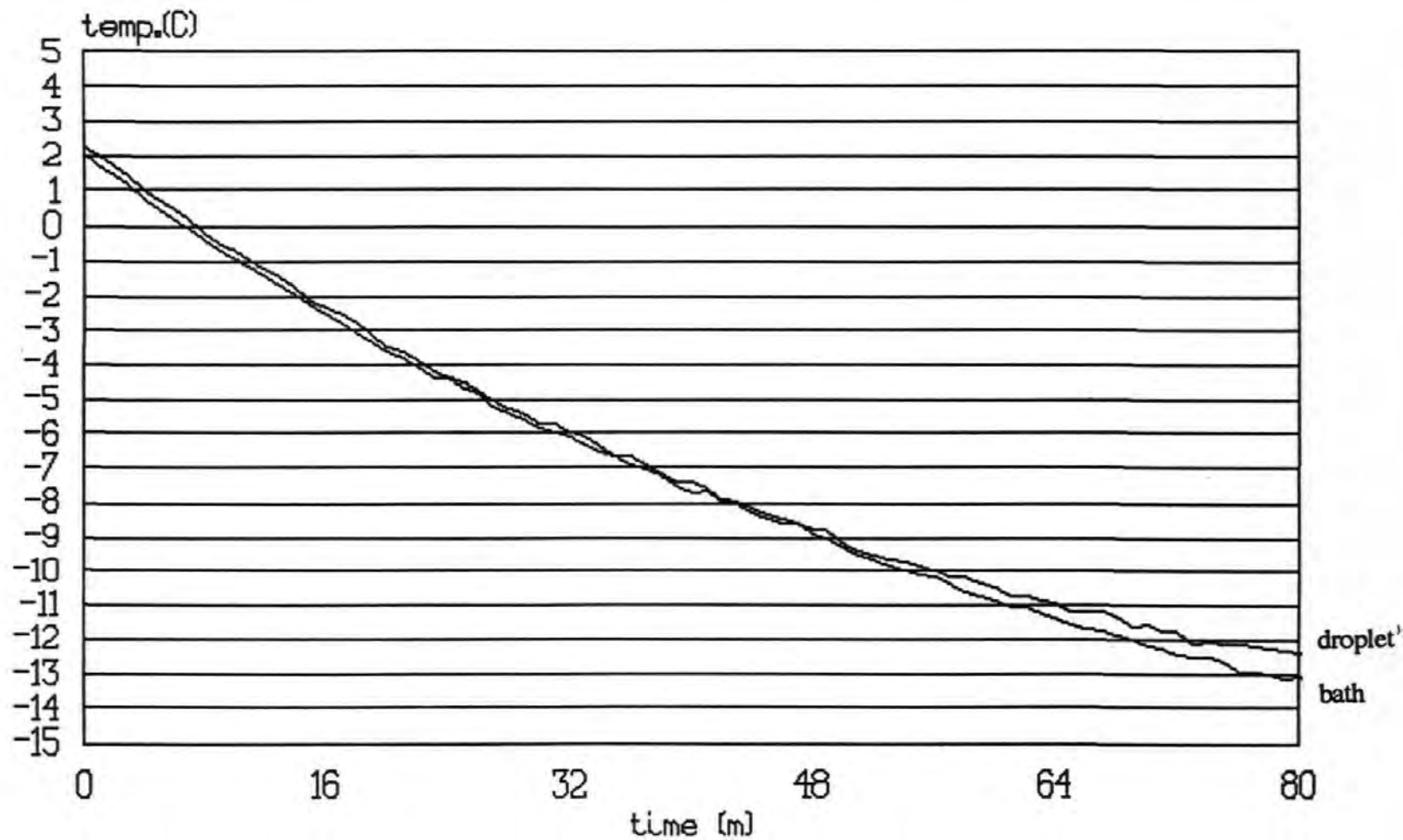
2.2. Droplet vs. bath temperature (pre- anti condensation)



2.3. Droplet temperature vs. bath temperature (post anti-condensation measures).



2.4. Droplet temperature vs. bath temperature  
(continuous cooling programme)



### 2.3. Raising of *S. tuberosum* var. Jersey Royal plant populations, and freezing of plant materials.

#### Field grown populations.

All plants were raised from sprouted seed tubers supplied by the States of Jersey Department of Agriculture and Fisheries. Field crops were raised by planting according to normal agronomic practice at trial sites at Seale-Hayne Faculty Devon, (grid ref. SX97 826732, soil type. clay loam, pH 6.8) and the Howard Davis Farm, Trinity, Jersey (grid ref. NW 5530 6560). Seed tubers were spaced at 15 cm within the rows and 76 cm between rows. Weeds were controlled with a soil acting pre-emergence herbicide (Linuron, PBI Ltd) and plots were covered with polythene sheeting (100 micron, 100 hole per m<sup>2</sup>). Polythene was removed from the plots no later than four weeks after emergence or prior to this in anticipation of frost events. Frost testing of the field crop was as a result of natural *in-vivo* frost events.

#### Glasshouse raised populations

For *in-vitro* frost testing glasshouse raised plants were used. Sprouted seed tubers were planted in 15cm diameter, 2 litre plastic pots containing a multi purpose peat based compost, placed in a glasshouse (minimum temperature 10°C) and irrigated manually ensuring that no water was applied to the foliar portion of the crop. Plants recieved no further agronomic treatments.



Frost testing of the glasshouse raised plants took place *in-vitro* using an environmental test chamber (Mountford Instruments Ltd. Model K800) and were repeated at each of three developmental stages, early post emergence, two weeks subsequent to emergence (plant 10 to 15 cm tall with the first two leaf branches exhibiting fully unfurled terminal, sub-terminal and secondary leaflets), and 4 weeks subsequent to emergence (15 to 25 cm tall, four leaf branches and leaflets fully developed).

#### Frost Damage Assessment.

A visual assessment of damage symptoms was employed following both *in-vitro* and *in-vivo* frost events. Tissue turgor and a water soaked, darkened appearance was scored as an all or nothing response (0= no damage and 1= evidence of damage symptoms). Plate ii. shows examples of Jersey Royal plants pre and post freezing. Nucleation temperatures and damage recordings were statistically analysed using analysis of variance and Fishers least significant difference (LSD) test. Results are quoted using the protocol that different letters following the measured parameter indicate significant difference ( $p < 0.05$ ).



pre-freezing.



post -freezing.

- ii) Frost damage symptoms of *S. tuberosum* var Jersey Royal.

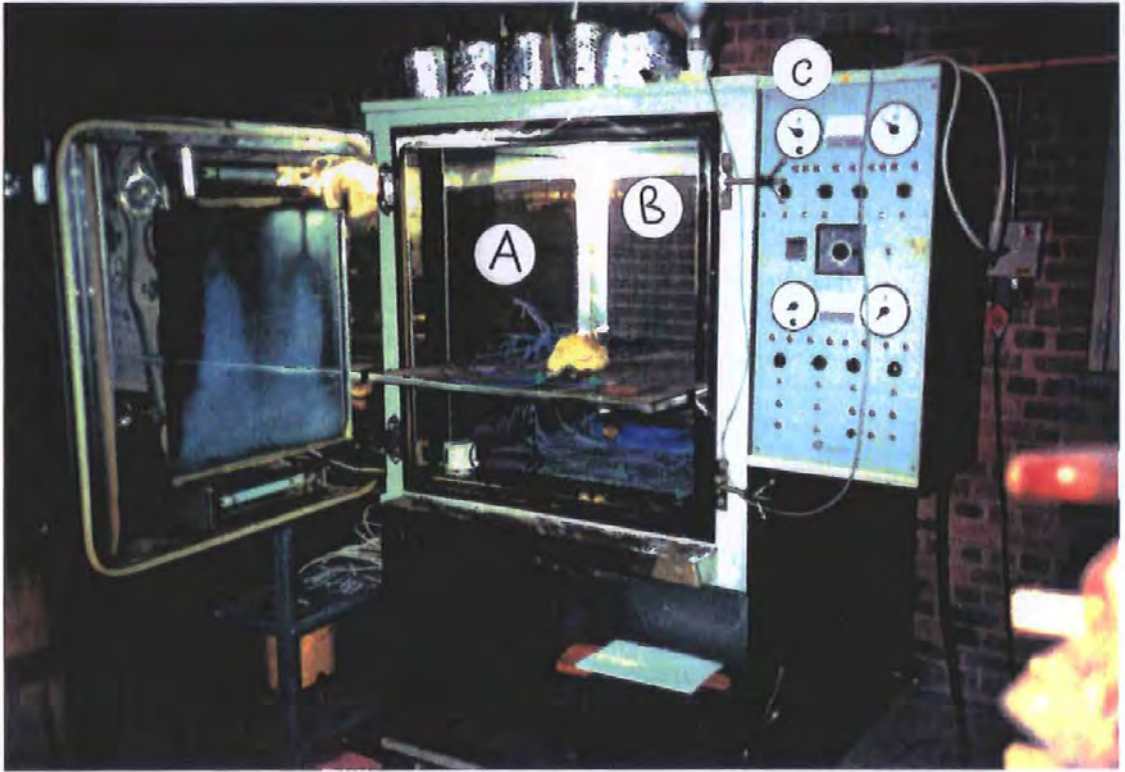
## 2.4. Characterisation of environmental test chamber.

### Description of environmental test chamber.

A large environmental test chamber (1m<sup>3</sup>) (Mountford Instruments Ltd. Model No. K800) (plate iii.) was employed for all *in-vitro* whole plant frost tests. The chamber was cooled by forced air circulation over a rear mounted refrigerated battery. The chamber had no reliable programming capability and as a result test temperatures, duration at test temperature and cooling rates were actuated manually. Repeatability was obtained through meticulous attention to the manual settings of the heating and cooling controls. The inherent nature of the system is one of temperature correction thus a fluctuation about the desired temperature could be observed, with the amplitude of the overshoot oscillation reducing through time as a steady state was achieved (fig 2.5.).

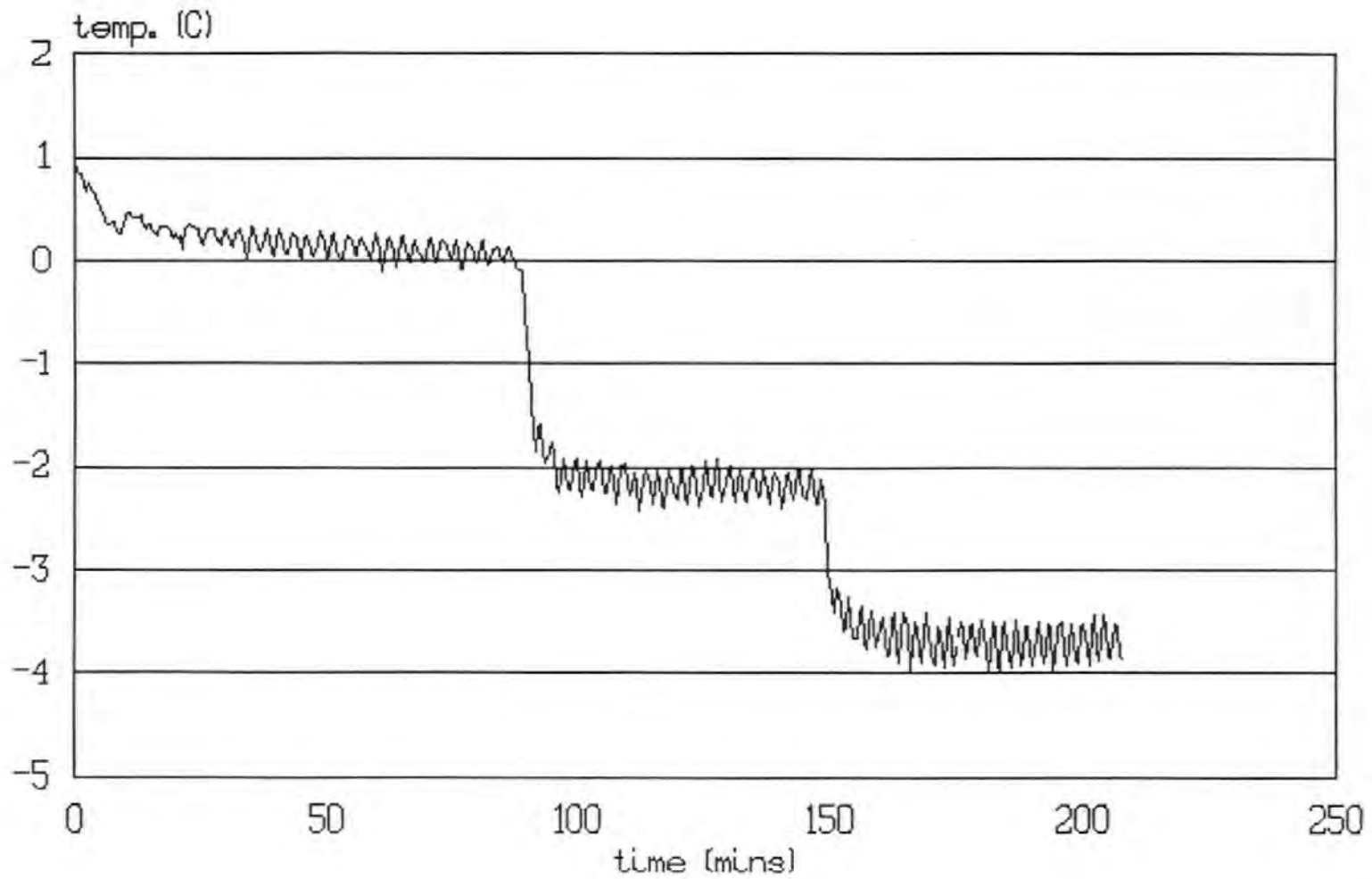
### 2.4.2. Calibration of environmental test chamber.

The test chamber was calibrated using 9 type T thermocouples attached in an equidistant 3 x 3 square on a removable shelf, placed in the three positions within the chamber. The thermocouples were connected to the data logger set to record at 5 minute intervals, integrating the readings every hour. Target temperatures were maintained for two hours. Analysis of variance revealed no significant differences across a shelf or between shelf position at any test temperatures ( $p < 0.05$ ).



iii) Environmental test chamber.  
(Mountford Instruments Ltd.)

- A) Access port
- B) Cooling bank.
- C) Temperature control system



2.5. Typical cooling curve measured within the environmental test chamber (Mountford Instruments Ltd.)

### Chapter 3. MATERIALS AND METHODS.

#### Section 1. Investigation of the supercooling ability of *Solanum tuberosum* var. Jersey Royal.

**Experiment 1.** A comparison of *in-vitro* and *in-vivo* frost events and the effect of applied ice nucleating bacteria and antibiotic on nucleation temperatures of the early sown *S. tuberosum* var. Jersey Royal.

This preliminary investigation was designed not only as a study of bacterial manipulation upon the early sown *S. tuberosum* var. Jersey Royal but also had important developmental objectives. These objectives were:-

- Familiarisation with the husbandry of *S. tuberosum* var. Jersey Royal.
- isolation of INA bacteria associated with the crop.
- measurement of frost damage to *S. tuberosum* var. Jersey Royal.

Two populations of early sown *S. tuberosum* var. Jersey Royal plants were established, one in the field to observe frost events *in-vivo*, the second in the glasshouse for frosting *in-vitro*.

The field raised population comprised of a 36 x 42 m plot planted on 3 sowing dates (19th Feb, 7th March, 14th March 1991) at the Seale-Hayne site (fig 3.1).

Glasshouse raised seed tubers were planted on 3 sowing dates (5th

March, 19th March and 26th March. 36 plants per sowing, n=108). The three foliar treatments (see below) were also applied to the glasshouse raised plants. In addition a hardening regime was incorporated as a covariate by placing 50% of the plants from each treatment and sowing date (6 plants) four weeks after sowing into a phytotron at 4°C, 8 hr days and 2°C, 16 hr nights for 14 days prior to frost testing. The unhardened plants were frost tested after four weeks growth.

Three treatments were applied to the plants:-

Treatment 1. a 48 hr culture of *Pseudomonas syringae* 1205 (Harpenden plant pathogenic bacteria collection) suspended in nutrient broth (applied once to run off using a hand held plant sprayer). The nucleation frequency of the bacteria was quantified by S.E. Lindow (Pers. comm.) as  $2.8 \times 10^{-5}$  nuclei per cell at -9°C. This treatment was designed to encourage maximum levels of INA bacteria upon the treated plants.

Treatment 2. 100 mg/l of streptomycin + 100 mg/l of oxytetracycline (applied weekly to run off using a hand held plant sprayer). This treatment was designed to suppress both total bacterial populations and the contingent of the population which might have displayed ice nucleation activity.

Treatment 3. No foliar treatment. (control)

The inhibitory effects of the antibiotics were confirmed in the laboratory using nutrient agar plates amended with 100mg/l streptomycin and 100mg/l oxytetracycline. Serial dilutions of potato plant macerate were spread plated onto both antibiotic amended and unmodified nutrient agar plates. All plates were incubated for 48

R1	R2	UPHILL		R3	R4
S2	S3	S1			S2
S1	S1	S3			S3
S3	S2	S2			S1
TREATMENT 2	TREATMENT 3	TREATMENT 3			TREATMENT 1
S2	S1	S1			S1
S1	S3	S2			S2
S3	S2	S3			S3
TREATMENT 3	TREATMENT 2	TREATMENT 1			TREATMENT 3
S1	S2	S3			S2
S2	S3	S1			S3
S3	S1	S2			S1
TREATMENT 1	TREATMENT 1	TREATMENT 2			TREATMENT 2

TREATMENT 1 applied bacteria

TREATMENT 2 applied antibiotic

TREATMENT 3 control

DOWNHILL

sowing date

S1 19/2/91

S2 7/3/91

S3 14/3/91

3.1. Experimental design of field trial. Section 1, Experiment 1.



hrs at 24°C prior to enumeration.

Bacterial populations on plants were monitored by harvesting 5 plants from each replicate and treatment at weekly intervals. Each plant was cut at the soil surface with a scalpel and placed in a sterile bag. The whole plant was macerated and bacteria enumerated as described earlier (Equipment and Technique Development, section 2.2). A subsample of two bacteria per plate from the counted plates was taken and identified. These bacteria were regarded as representative of the bacterial species on the crop. The characterisation of isolated bacteria was not designed to provide precise identification to species level but instead broad categories were used. Gram positive bacteria were categorised into spore formers and non spore formers. Attempts at precise identification of Gram positive bacteria was unnecessary since Gram positive organisms have not been demonstrated as ice nucleation active. Most importance was placed on the species *Ps. syringae* and tests towards the characterisation of *Ps. syringae* allowed identification of other groups of gram negative bacteria. Tests used for the characterisations were:-

- a) Oxidase reaction. *Pseudomonas sp.* oxidase positive, *Ps. syringae* and *Enterobacteriaceae* oxidase negative.
- b) Aerobic and anaerobic breakdown of glucose (using glucose broth and bromocresol purple indicator). *Ps. syringae* no fermentation. *Enterobacteriaceae* acid and gas production in aerobic and anaerobic conditions.

All colonies identified as *Ps. syringae* were further tested for arginine di-hydrolase activity using semi solid arginine medium (Thornley 1960, in Harrigan and McCance 1976), and fluorescence on Kings medium B.

In the latter weeks of the experiment plants were becoming too large for macerating as a whole. Therefore a sub-sample of 10 to 15g of tissue was taken for processing.

#### Frost Testing.

The glasshouse raised plants were frost tested using the following freezing regime:- cooling the chamber from ambient to the first of three sub-zero holding temperatures ( $-1^{\circ}\text{C}$  for 75 min, followed by  $-2.5^{\circ}\text{C}$  for 75 min and  $-4.5^{\circ}\text{C}$  for 75 min) (all holding temperatures  $\pm 1^{\circ}\text{C}$ ) at a rate of approximately  $0.5^{\circ}\text{C min}^{-1}$ , Equilibration of tissue temperature and test chamber occurred within the first holding period. The temperature was ramped between holding temperatures at approx  $0.5^{\circ}\text{C min}^{-1}$ . The plants were allowed to warm to ambient temperature at approx  $0.5^{\circ}\text{C min}^{-1}$ .

Frosting in the field occurred during a natural radiative frost event. Temperature measurement and ice nucleation detection were carried out as previously described.

**Experiment 2.      Investigations of in-vitro supercooling ability of  
*S. tuberosum* var. Jersey Royal**

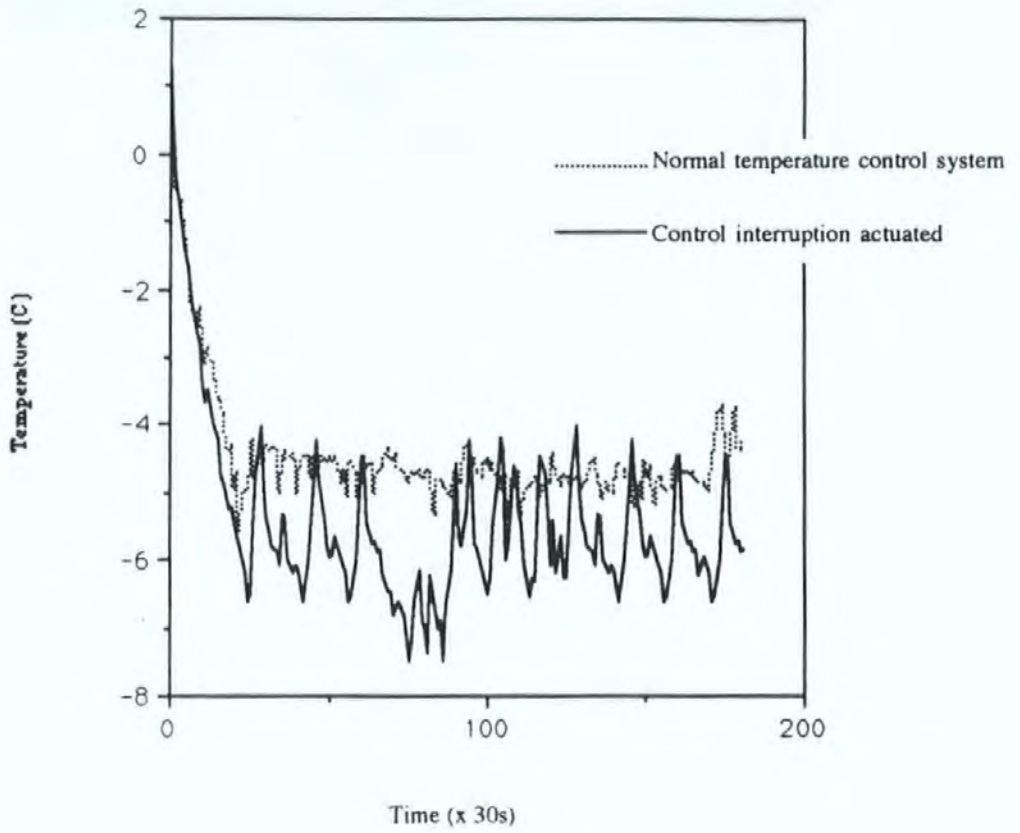
Four hundred and eight glasshouse raised plants sown on the 20th of February 1992 were apportioned to this experiment which investigated the following five aspects of in-vitro freezing.

2.1. The effect of cooling rate on the supercooling ability.

Twelve plants of each developmental stage for each cooling rate were used. The rates of cooling employed were  $0.7\text{ }^{\circ}\text{C min}^{-1}$ ,  $0.25^{\circ}\text{C min}^{-1}$  and  $0.15\text{ }^{\circ}\text{C min}^{-1}$  to a minimum of  $-5^{\circ}\text{C}$ . Once this temperature was attained the trial was stopped. Temperature of freezing was determined by exotherm detection as described previously.

2.2. The effect of fluctuating temperature on supercooling ability.

This trial required a fluctuating temperature environment within the environmental test chamber. Manual raising and lowering of the temperature was impractical and the required conditions were therefore achieved by influencing the normal temperature control of the cooling chamber. Firstly the pulse controls were set to ensure maximum fluctuation about the set temperature. Secondly the action of the correction mechanism inherent in the test chamber was interrupted by placing a bank of three 200 watt lamps below the temperature sensor of the chamber. These lamps were switched on and off at regular repeatable intervals using an electronic time switch



3.2. Effect of interruption of temperature stability systems of the environmental test chamber.

operating a relay via a 5v\240v transformer. This resulted in a regular pulse of heat influencing the built in sensor which the chamber then attempted to correct by a pulse of cooling. These measures were effective in creating a fluctuating temperature environment (Fig.3.2.).

Twelve plants of each developmental stage were subjected to either fluctuating temperature about -5 °C or a steady temperature of -5 °C for 1 hour.

2.3. The effect of leaf soiling and leaf damage on the supercooling ability.

The following treatments were applied to the foliar portion of twelve plants of each developmental stage:-

1) Soiling. A soil suspension containing 200g of soil vigorously shaken in 1 litre of distilled water was applied to plants using a hand held plant sprayer until runoff.

2) Damage. The vegetative portion of the plants was lacerated by supporting the plant in the palm of the left hand and applying four firm strokes of a 30 cm<sup>2</sup> piece of 600 grit wet and dry paper across the foliage.

3) Soiling + Damage. Both the soil suspension and abrasive process of the above treatments were applied to the plants. (The abrasion process was executed first to ensure that soiling was not removed with abraded plant tissue.)

4) Untreated control.

The nucleation characteristics of the soil suspension was

investigated using the droplet freezing spectra assay. The suspension was then autoclaved and the freezing spectrum investigated once more. One hundred 40ul droplets of each of three tenfold dilutions (the first of which was the concentration applied as the foliar treatment) was used to determine the nucleation spectra.

The plants were then frozen in the environmental test chamber and subjected to 4 test temperatures (-1.25 °C, -2.5 °C, -5.0 °C and -7.5 °C) maintained for a duration of 20 minutes.

#### 2.4. The existence of intrinsic ice nuclei.

Twelve plants from each developmental stage were harvested from the glasshouse raised population and subjected to destructive tube nucleation tests. Each plant was dissected into three 1g portions (+/-0.1g) representative of the basal stem, mature leaf and apical meristem. Each of these portions was suspended individually in test tubes containing 9ml sterile distilled water. In this way no mass component would affect the monitored freezing temperatures. A type T thermocouple was inserted in each tube. The tubes were then stoppered and placed in the circulating temperature controlled refrigerant bath programmed to cool at 1 °C per hour to a minimum of -10 °C (+/- 0.1 °C). Twelve tubes of sterile distilled water also containing type T thermocouples were cooled simultaneously as a control. On thawing the tubes were shaken for 30 min and 0.1 ml of the diluent was sampled, serially diluted and plated on nutrient agar for enumeration of bacteria and ice nucleation testing at -5.0 °C. Following freezing and sampling all tubes and their associated

tissue samples were autoclaved and the process was repeated.

#### 2.5. The effect of simulated dew on the supercooling ability.

Twelve plants of the two oldest developmental stages were apportioned to two treatments. Plants were either frozen dry or treated with artificial dew. Artificial dew was created by spraying sterile distilled water onto all surfaces of the plants with a hand held plant sprayer. All plants were subjected to 4 test temperatures (-1.25 °C, -2.5 °C, -5.0 °C and -7.5 °C) each maintained for a duration of 20 minutes.

**Experiment 3.** A comparison of the effects of dew and a known ice nucleation active strain of *Pseudomonas syringae* on supercooling ability.

Seed tubers (n=108) were raised in a controlled environment growth chamber maintaining a 12 hr photoperiod with a day temperature of 15 °C and a night temperature of 10 °C. Plants were watered automatically using a drip irrigation system, ensuring that the foliage remained dry, and received no further agronomic treatment. The following experimental treatments were applied to the foliar portion of the plants at three developmental stages:-

1. aqueous suspension of *Ps. syringae* 84:27 ( $10^6$  cfu/ml, nucleation frequency (NF) of  $7.2 \times 10^{-5}$  at  $-2.5$  °C) applied 7 days prior to freeze testing.
2. aqueous suspension of *Ps. syringae* 84:27 ( $10^6$  cfu/ml, nucleation frequency (NF) of  $7.2 \times 10^{-5}$  at  $-2.5$  °C) applied 1 day prior to frost testing.
3. untreated control.

The Nucleation Frequency of *Ps. Syringae* 84.27 was calculated from nucleation spectra analysis (described earlier) according to the formula  $N(T) = -\ln(f)/V$  (Lindow et al 1982a)

where,

$N(T)$  is the nucleation frequency at a given temperature.

$f$  is the proportion of droplets unfrozen at temperature  $T$ .

$V$  is the volume of the individual droplets.



The bacterial suspension was sprayed onto the foliage of the plants to run off with a hand held plant sprayer. Immediately prior to freeze testing artificial dew was applied as a mist of sterile distilled water using a hand held plant sprayer to 50% of the plants in each treatment. The remaining 50% were frost tested with dry foliage.

#### Frost testing.

Plants were cooled simultaneously from ambient to three test temperatures (-2.5 °C, -5.0 °C and -7.5 °C +/- 0.5 °C) at approximately 1 °C min<sup>-1</sup>, and held at each for 15 min. Plants were allowed to warm to ambient temperature at approximately 1 °C min<sup>-1</sup>.

#### Bacterial ice nucleation.

Following freezing the existence of the applied nucleating bacteria upon the foliage was confirmed by plating foliar macerate onto *Pseudomonas* selective CFC agar (Oxoid). After incubation at 24 °C for 48 hrs colonies were harvested with a sterile loop and placed on the aluminium cold stage. Each colony was covered with 40ul of sterile distilled water. The freezing point of these droplets was compared with that of similarly prepared droplets of *Pseudomonas syringae* 84:27 as a positive control. Those that froze at the same temperature as the control were regarded as ice nucleation active and likely to be *Ps. syringae* 84:27.

**Section 2. An investigation of Ice Nucleating Bacteria upon Early  
Sown *S. tuberosum* (var. Jersey Royal).**

**Experiment 4. An investigation of INA bacteria on field grown  
plants at Seale-Hayne.**

Plant material for this experiment was gathered from the trial site described in experiment 1. Five plants were harvested at weekly intervals from the control plots of each replicate and sowing date until the frost event of 21 April 1991. Twenty plants were thus harvested weekly for 5 weeks. Each plant was macerated in sterile Ringers solution for enumeration and ice nucleation testing as described earlier. In the latter weeks of the experiment the plants were becoming too large to macerate as whole plants. As a result a sub-sample of 10 to 15g of tissue from each harvested plant was processed only.

From the same site four leaves of similar age and position in the canopy were harvested from all plots of the first two sowing dates (96 leaves harvested i.e. 32 leaves per treatment). These were directly assessed for their nucleation ability by being suspended in sterile Ringers solution and held in an environmental chamber at -2.5 °C for 30 mins followed by another 30 mins at -5 °C. After each holding period the number of tubes frozen was recorded.

**Experiment 5. Pilot survey of microflora associated with the indigenous crop.**

**Swab Sampling of Polythene Sheeting.**

The early sown potato crop is protected through early post emergence by transparent perforated polythene sheet. This pilot study addressed the possibility that the polythene may have been harbouring ice nucleating bacterial populations.

During the immediate post-planting and emergence period bacterial swabs of the polythene sheeting covering a commercial crop of early sown *S. tuberosum* var. Jersey Royals were taken from field grid ref NW 5530 6560, Howard Davis Farm, Trinity, Jersey. Four swabs per week for 4 weeks were processed at Seale-Hayne. Swabs were vortex stirred in 10ml of sterile Ringers solution for 30 s. The diluent was then serially diluted, enumerated and screened for ice nucleation activity using the replica plating method.

**Tube Ice Nucleation Assay of Leaf Samples.**

One hundred and twenty leaves harvested randomly from throughout the canopy of the crop described above were screened for ice nucleation activity using a tube nucleation test. Each leaf was placed in 9 ml of sterile distilled water and held at -5 °C for 30 minutes within the environmental test chamber. After 30 minutes any tubes that were frozen were thawed and the contents plated on nutrient agar and screened for INA bacteria using the replica plating method.

**Experiment 6.** Detailed survey for Ice Nucleating Bacteria upon early sown *S. tuberosum* var Jersey Royal on the Island of Jersey.

The following experiment was designed to screen thoroughly a commercial Jersey Royal potato crop for the existence of INA bacteria. It was further designed to test the hypothesis that INA bacterial populations on individual leaves can be described by the lognormal distribution. Such a distribution in other crop species has enabled frost risk to be predicted from measured INA bacterial levels on individual leaves (Hirano et al 1982). A final objective was to characterise the bacterial populations upon the Jersey crop.

A plot measuring 6 m x 6 m was established in the centre of a commercial field crop three weeks after emergence (grid ref. NW 5540 6562, Howard Davis Farm, Trinity, Jersey). Forty individual leaves were harvested aseptically and placed individually in sterile bags from plants growing within the plot at 09:00 hrs on five consecutive days (6th-10th April 1992)

Each leaf was then suspended in 9 times v/w sterile quarter strength Ringers solution ( $10^{-1}$  dilution) and macerated for 1 minute in a Colworth stomacher. Two dilutions ( $10^{-1}$  and  $10^{-2}$ ) were plated onto nutrient agar using a spiral plater (Don Whitley Scientific Ltd.) and incubated at room temperature for 7 days. Two hundred bacterial isolates (40 per day) representative of the most abundant members of the leaf borne bacterial flora were collected by randomly selecting two colonies from the perimeter of each plate of the  $10^{-2}$  dilution. These were purified and identified according to the scheme detailed earlier (experiment 1).

Three methods were employed to ensure a thorough screening of ice nucleation activity of the isolates from the Jersey Crop.

1. Each  $10^{-1}$  and  $10^{-2}$  spiral plate was tested for ice nucleation activity at  $-5\text{ }^{\circ}\text{C}$  by replica plating.
2. Each  $10^{-1}$  plate was replicated onto CFC *Pseudomonas* selective agar (Oxoid Unipath) using a replica plating block. Following incubation of the replica plates at  $22\text{ }^{\circ}\text{C}$  for 3 days all colonies were individually harvested from the CFC plates with a sterile loop and placed on an aluminium cold stage, covered with a 40ul droplet of sterile distilled water and cooled at  $5\text{ }^{\circ}\text{C/hr}$  to  $-10\text{ }^{\circ}\text{C}$ . A record of the nucleation spectra for these bacteria was recorded by noting the proportion of droplets frozen through time.
3. Each of the 200 isolates collected as representative of the most abundant members of the leaf borne flora were grown on nutrient agar for 3 days at  $24\text{ }^{\circ}\text{C}$ , harvested with a loop and subjected to the freezing process outlined above.

In all the nucleation tests *Ps. syringae* 84:27 was employed as a positive control. This was done by growing it under similar conditions to the test isolates and testing its nucleation activity alongside the test isolates using the same methodology.

**Section 3. Phylloplane Microflora of the Early Sown *Solanum tuberosum* var. Jersey Royal in relation to microclimatology.**

The aim of this series of experiments was to monitor the microclimate within a commercial crop and record the effects of microclimate upon the bacterial species identified upon the crop. In particular to see if field frost damage temperatures could be related to the activity of any recovered INA bacteria. This was made possible because the detailed bacterial survey described in experiment 6 took place within the same trial area. A further aim was to carry out a longer term temperature survey within the trial area with the objective of measuring frost events on the Island of Jersey and recording the development of such events within a commercial crop.

**Experiment 7. Microclimatology of the early sown *S. tuberosum* var. Jersey Royal and its effect on bacterial populations.**

Microclimate was monitored within a commercial crop 3 weeks after crop emergence (grid ref. NW 5540 6562). Leaf temperature was recorded at fourteen locations within the plot. Data were recorded by inserting the measuring junction of type T thermocouples into the rib of the uppermost terminal leaflet. The rib was used as the site of attachment as the leaf tissue was too fragile to be able to attach the thermocouples and windy and wet weather conditions were less likely to dislodge the thermocouples from rib tissue. A final thermocouple attached to a mast (using cable grips so that the

measuring junction was not in contact with the mast) was used to monitor air temperature at the top of the canopy at a similar height to the thermocouples attached to the plant. In addition temperature profiles were recorded at two stations within the trial area with data being recorded on a second data logger (similar to that previously described, except equipped with memory and input boards to enable the use of only 15 thermocouples). Temperature was recorded every 30 s.

A channel of the data logger was configured to record relative humidity within the canopy and logged every 30 mins using a Delta T Devices R1 relative humidity sensor. Relative humidity was recorded less frequently than temperature due to the power consumption of the instrument.

**Experiment 8. Long term temperature survey within a commercial crop on the Island of Jersey .**

The field site on the Howard Davis experimental husbandry farm (field grid ref NW 5540 6562) used in experiment 7 was also the site for this trial. Temperature profiles were monitored using Type T thermocouples positioned at two stations within the trial plot. Seven type T thermocouples were attached to each of two 1 metre tall masts at 0 cm, 10 cm, 20 cm, 30 cm, 40 cm, 50 cm and 100 cm above soil surface in such a way that measuring junctions were not touching the masts. Recording started on February 1st 1992, whilst the field was still under grass, prior to cultivations for the potato crop monitored in experiment 7. Data were recorded upon a data logger No. 2.

The thermocouples were not shielded from radiation and thus errors due to radiative heating and cooling of the measuring junction could be present. In practice, however, these errors have been proved to be negligible even in the worst examples of radiation incidence and windspeed (Biscoe et al 1975)



**Section 4. The promotion of supercooling of the early sown *Solanum tuberosum* var. Jersey Royal as a frost avoidance mechanism using exogenous foliar applications.**

**Experiment 9. A preliminary screening of agents to promote supercooling of excised leaves.**

The objective of the following experiment was to test substances for their ability to promote supercooling of plant tissue in the presence of artificial dew. The aim was to test any substances successful in promoting supercooling in further experiments using whole plants subjected to *in-vitro* frost testing, and in a field grown crop under conditions of a natural frost.

**Plant treatment.**

Glasshouse raised plants were treated 5 weeks after sowing with three substances aimed at promoting supercooling (potential cryoprotectants), and compared with a fourth untreated control. The treatments were:-

1. Ethylene glycol applied to run off with a hand held plant sprayer 1 day prior to freezing. Ethylene glycol was applied to assess if it could 'antifreeze' the dew thus promoting supercooling.
2. Kaolin powder dusted over the plants with a sieve 1 day prior to freezing. Kaolin was applied to assess if its absorbent properties could disrupt leaf/surface water complex which could be responsible for supercooling interruption on the foliage of *S. tuberosum* var. Jersey Royal.
3. Proprietary seaweed extract applied according to manufacturers

instructions 1 and 10 days prior to freezing. Seaweed extract was used as a currently available agricultural product which may have the ability to confer freeze resistance on sensitive crops.

Simulated dew was applied to runoff to all foliar treatments after the leaves were mounted in the environmental test chamber. Leaves treated with seaweed extract were subjected to a further level of dew treatment. This was applied after mounting the leaves in the environmental test chamber using one depression of the plunger of a perfume atomiser held 30 cm away from the leaf. This resulted in a just visible covering of very fine droplets across the leaf surface. Twenty leaves of each treatment and dew level were mounted for frost testing. Leaves for frost testing were selected randomly and excised from the plants by cutting at the junction of leaf petiole and stem using a scalpel.

#### Freezing Procedure.

Two wires were stretched horizontally across the inside of the environmental test chamber. Ten excised leaves were attached to each line by the stalk using a miniature crocodile clip (Farnell Electronic Components Ltd). This clip also held a type T thermocouple such that the measuring junction was in intimate contact with the leaf surface. Temperature of freezing was noted using exotherm detection.

**Experiment 10.** The effect of foliar applied ethylene glycol upon the supercooling ability of *S. tuberosum* var. Jersey Royal during *in-vitro* frost tests.

One hundred and eight sprouted tubers were raised in a glasshouse and treated with ethylene glycol either 1 or 7 days prior to freezing. Immediately prior to freezing plants were divided into two groups (n=54), one group was sprayed with sterile distilled water (dew) the remainder were kept dry. Frosting took place in the environmental test chamber at three sequential test temperatures, -2.5 °C, -5.0 °C and -7.5 °C. Each test temperature was maintained for 15 minutes.

**Experiment 11. The effect of foliar applied ethylene glycol upon plant supercooling in the field.**

Two field trial sites were established, one at the Howard Davis Experimental Farm, Trinity, Jersey, within a commercial field crop. The other at Seale Hayne.

**Site Description and Experimental Design:- Howard Davis Farm.**

Single sprouted seed tubers were sown on 12 February 1993 into twenty four 40 m long ridged rows. (grid ref. NW 5650 7554, sandy textured soil, pH 6.8). Within this crop the following treatments were imposed on 4.5 m segments of randomly selected rows, each replicated 4 times:- untreated control, ethylene glycol applied to runoff at weekly intervals from 19th March 1993 and sterile distilled water applied between 1600 hrs and 1700 hrs on the evening of an anticipated frost event. Ethylene glycol and sterile distilled water were applied with a hand held plant sprayer.

**Site Description and Experimental Design:- Seale-Hayne.**

A 10 x 30 m area of single sprouted seed tubers was established by hand sowing into ridges following a split plot design (grid ref SX97 820730, clay loam, pH 6.9). Tubers were sown on three sowing dates (12 February 1993, 8th March 1993 and 15th March 1993). Three foliar treatments were applied prior to an expected frost event:- ethylene glycol, sterile distilled water and an untreated control. Ethylene glycol and sterile distilled water were applied to runoff with a hand held plant sprayer.

Temperature of plants representative of all treatments on both the Seale-Hayne trial site and the Howard Davis trial site were monitored for exotherm response as previously described. Relative humidity within the canopy was also recorded at the Seale-Hayne trial site.

Symptoms of frost damage were noted 24 hours after a frost event and scored on a scale where 0 = no damage, and 1 = damage symptoms present.

## Chapter 4. RESULTS.

### Section 1. Investigation of the supercooling ability of *S. tuberosum* var. Jersey Royal.

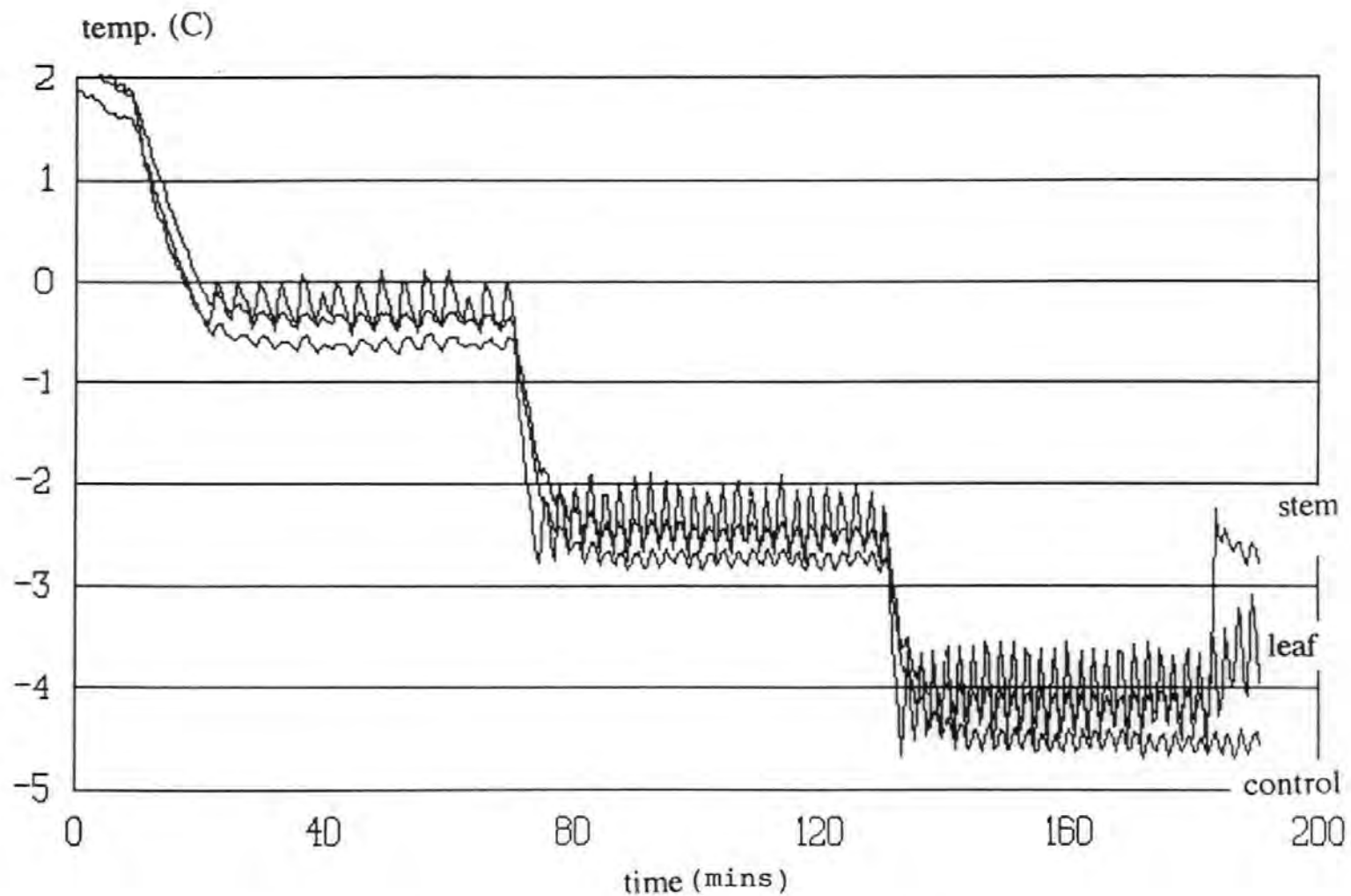
Experiment 1. A comparison of *in-vitro* and *in-vivo* frost events and the effect of applied ice nucleating bacteria and antibiotic on nucleation temperatures.

Frost damage and exotherm detection.

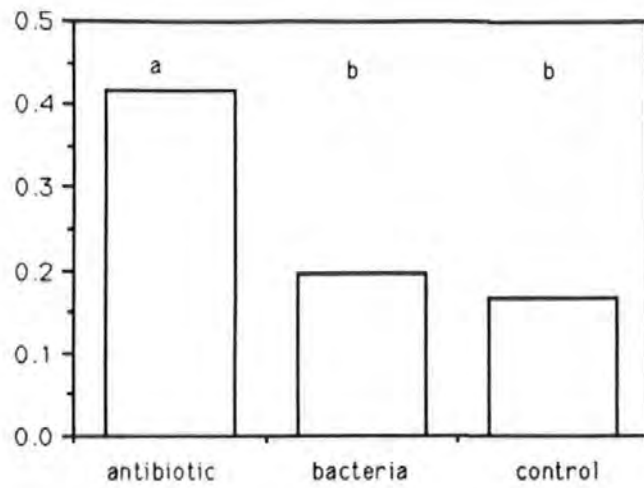
Glasshouse Population.

Exotherms were detected during *in-vitro* freezing following supercooling and a perfect positive correlation between the occurrence of exotherms and total plant damage was recorded ( $r=1.0$ ), i.e. when an exotherm was recorded complete plant death occurred. A typical trace of a freezing test for a plant exhibiting supercooling is shown in Fig.4.1. and in this example the foliage experienced a period of supercooling of 180 mins prior to freezing.

Nucleation temperatures (the temperature attained by the tissue immediately prior to an exotherm) were not significantly affected by the applied treatments with mean nucleation temperatures for antibiotic treated, bacteria treated and control plants  $-3.8^{\circ}\text{C}$ ,  $-3.9^{\circ}\text{C}$  and  $-4.1^{\circ}\text{C}$  respectively. Treatments did, however, significantly affected the probability of an exotherm occurring with antibiotic treated plants more likely to display an exotherm than the other treatments, (figure 4.2.). Importantly this suggests that some plants were not freezing. Indeed of the 108 plants frost tested only



4.1. Typical supercooling and exotherm response of in-vitro frozen *Solanum tuberosum* var. Jersey Royal.

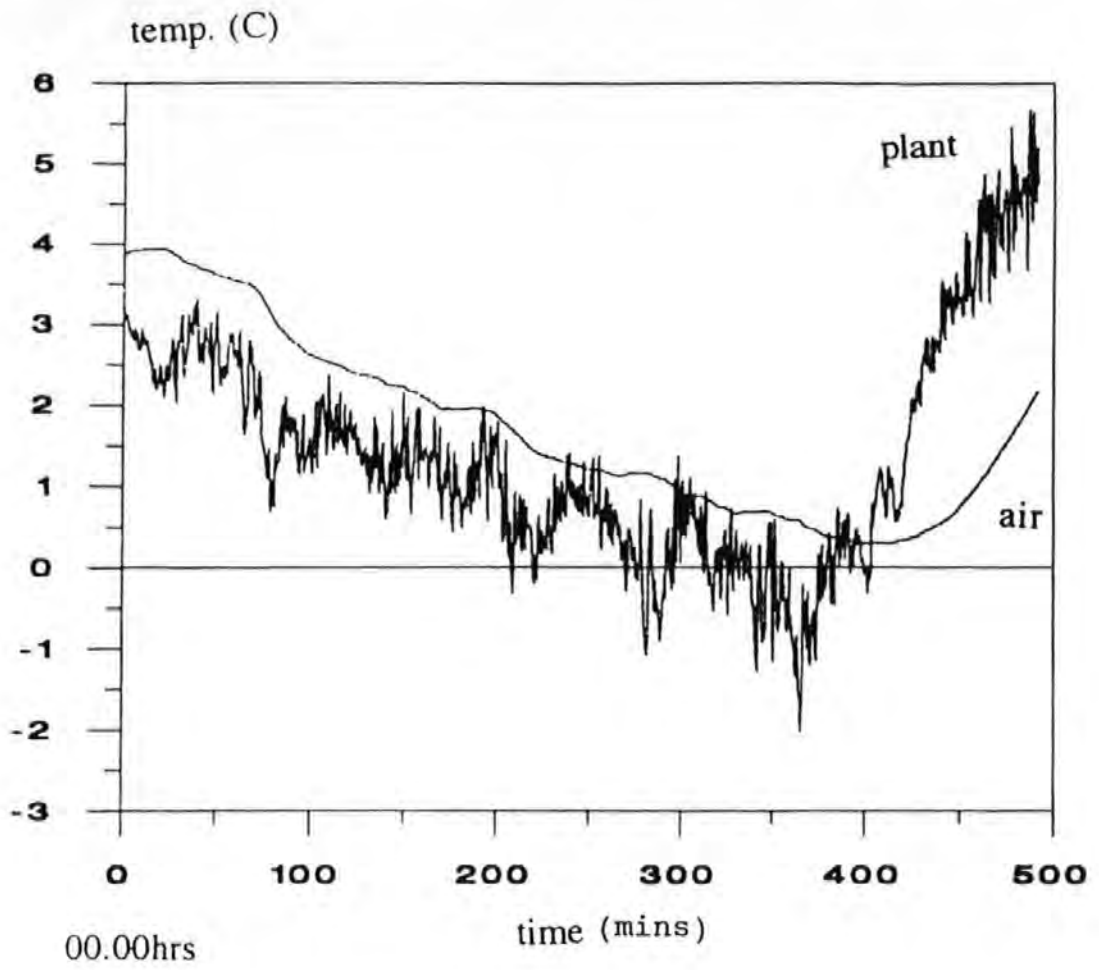


4.2. The effect of foliar treatment on the probability of an exotherm occurring.

(s.e. 0.042)

1 = exotherm, 0 = no exotherm.





4.3. Average air temperature compared to temperature measured at the plant during a damaging frost event.

40 displayed an exotherm, despite being subjected to sub-zero temperatures for 225 minutes including a minimum temperature of  $-4.5^{\circ}\text{C}$  for 75 minutes. The hardening regime did not affect either temperature of nucleation nor the probability of an exotherm occurring ( $p=0.17$  treatment s.e.  $0.057$ ).

#### Field Population.

A general correlation was apparent between air temperature and leaf temperature (fig.4.3.) As the air temperature fell towards zero leaf temperature was consistently  $1-2^{\circ}\text{C}$  below air temperature. This situation was reversed at sunrise when the plant warmed more quickly than the air, resulting in a leaf temperature up to  $3^{\circ}\text{C}$  warmer than the air. Sub-zero temperatures were monitored at the leaf between 260 mins to 400 mins into the frost event, however the average air temperature above the crop never fell below  $0^{\circ}\text{C}$ . It can be seen from figure 4.3. that instantaneous leaf temperature fluctuation was so great that it was impossible to be certain which of the rises in temperature was an exotherm. Air temperature fluctuation was so minimal that differential thermal analysis does not aid exotherm detection.

Visual assessment of the outdoor crop revealed that plants were damaged by the frost. Both applied treatments and replicate position significantly affected damage levels ( $p<0.05$ ) whilst sowing date had no significant effect. In plots treated with *Ps. syringae* 1205 significantly lower levels of damage occurred compared to antibiotic treated and control plots (table 1.). Replicates 3 and 4 (easterly)

had significantly lower damage scores than replicates 1 and 2 (westerly) (table 2.).

Temperature differences across the replicates were investigated further in order to try and explain the significant effects that replicate position was having on recorded damage levels. Minimum temperatures recorded across replicates were significantly different ( $p < 0.05$ ) with replicates 2 and 3 giving significantly colder minimums than the other replicates, (table 3.), but this result did not correlate with recorded damage levels. Analysis of thawing temperature (the average reading of 3 sets of 8 readings for each thermocouple from 6.00 am onwards) also showed significant differences across replicates, with replicates 1 significantly warmer than replicate 3 suggesting more rapid thawing, with replicates 2 and 4 intermediate (table 4.), but this did not correlate with crop damage records either. It was therefore difficult to account for the replicate effect and possibly some factor other than temperature influenced damage patterns recorded in the field.

#### Bacterial investigation.

Results of the bacterial investigation indicated that treatments significantly affected bacterial populations upon both glasshouse and outdoor plants with the antibiotic treatment significantly lowering bacterial populations (table 5.). It can be seen from table 5. that application of *Pseudomonas syringae* 1205 did not significantly affect population sizes measured on the plants relative to the untreated control. Identification of the bacteria

recovered from the outdoor crop indicated that the treatments also altered the proportions of bacterial species on the crop (table 6.). Plots treated with antibiotic exhibited a greater proportion of colonies identified as *Ps. syringae*, whereas plots treated with *Ps. syringae* 1205 showed a reduced proportion of bacteria identified as *Ps. syringae*. This change in proportions was reflected by a reduction of Gram negative and an increase of Gram positive bacteria isolated from plots treated with bacteria.

Foliar treatment	Mean damage score
applied bacteria	0.64 a
applied antibiotic	0.71 b
control	0.70 ab

Table 1. Effect of applied treatment on mean damage levels measured on the field grown *S. tuberosum* var. Jersey Royal.

(treatment s.e. 0.035)

Replicate position	Mean damage score
1	0.91 a
2	0.98 a
3	0.43 b
4	0.41 b

Table 2. Effect of replicate on mean damage levels recorded on the field grown *S. tuberosum* var. Jersey Royal.

(treatment s.e. 0.041)

Replicate position	Mean minimum temperature (C)
1	-1.8 b
2	-2.3 a
3	-2.2 a
4	-2.1 ab

Table 3. Comparison of minimum temperatures recorded across replicates.

(treatment s.e. 0.124)

Replicate position	Mean thaw temperature (C)
1	-0.8 a
2	-1.2 c
3	-1.3 b
4	-1.1 c

Table 4. Comparison of thaw temperatures across replicates.

(treatment s.e. 0.051)

Foliar treatment	Field grown plants (treatment s.e. 0.102)	Glasshouse grown plants (treatment s.e. 0.202)
Applied antibiotic	5.84a	8.83 a
Applied Ps. syringae 1205	6.34b	10.01 b
Control	6.53b	10.60 b

Table 5. Effect of treatments on mean total bacterial counts (log<sub>10</sub> cfu/g) recovered from field crop and glasshouse raised plants.

Foliar treatment	% Gram +ve	% Gram -ve	% of Gram -ve identified as <i>Ps. syringae</i> .
Applied <i>Ps. syringae</i> 1205	37.2	62.8	60
Applied antibiotic	29.4	71.6	90
Control	18.8	81.2	85

Table 6. Effect of bacterial treatments on proportions of *Ps. syringae* isolated from the foliage of the outdoor crop.



**Experiment 2. Investigation of in-vitro supercooling ability.**

The mean nucleation temperature of plant tissue across the series of experiments was  $-4.9^{\circ}\text{C}$  (s.d.=1.4). The nucleation temperature of plants treated with artificial dew was significantly higher with a mean nucleation temperature of  $-0.8^{\circ}\text{C}$  (figure 4.4.). The investigation of intrinsic nuclei indicated that tissue samples froze at temperatures significantly warmer than the distilled water controls. There was no significant difference between leaf and stem nucleation temperatures but the meristematic tissue froze at significantly colder temperatures (table 7.). Autoclaving significantly reduced mean nucleation temperature of tissue samples from  $-5.6^{\circ}\text{C}$  to  $-7.6^{\circ}\text{C}$  (treatment s.e. 0.076). The nucleation temperature of leaf tissue in the fluctuating temperature experiment was significantly lower than that of the stem tissue with mean nucleation temperatures of  $-5.1^{\circ}\text{C}$  and  $-3.6^{\circ}\text{C}$  respectively (treatment s.e. 0.330).

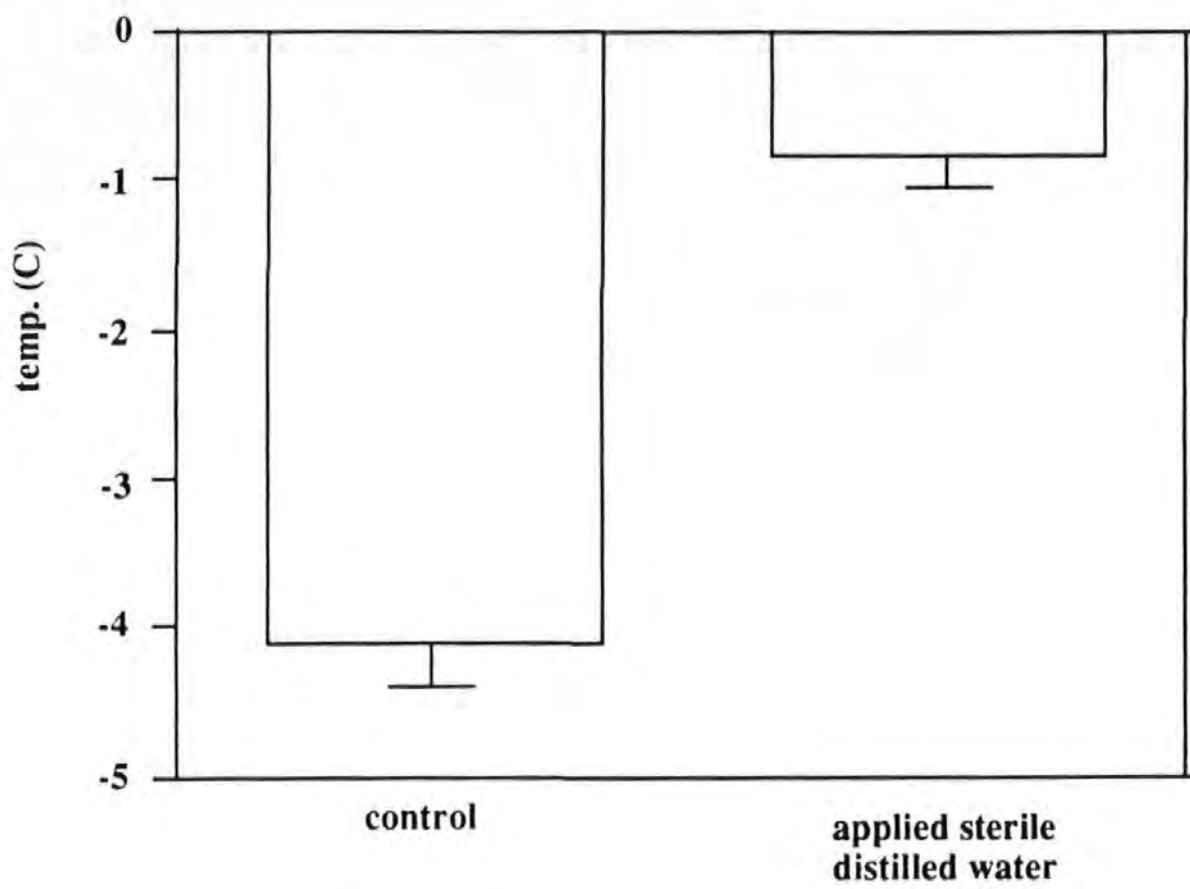
The freezing spectra of the applied soil suspension are presented in figure 4.5a (pre autoclaving) and figure 4.5b (post autoclaving). It can be seen that there were no nuclei active above  $-5^{\circ}\text{C}$  pre autoclaving. After autoclaving there were no nuclei active above  $-7.5^{\circ}\text{C}$ . The plants treated with soil suspension, and soil suspension + damage showed mean nucleation temperatures of  $-4.8^{\circ}\text{C}$  and  $-4.9^{\circ}\text{C}$  respectively (treatment s.e. 0.260). These temperatures were significantly warmer than the untreated controls and the damaged plants, nucleation temperatures of all treatments were within one standard deviation of the mean nucleation temperature recorded for the series of experiment. Ice nucleation testing of bacteria sampled

during the intrinsic ice nuclei investigation revealed no isolates acting as ice nuclei at -5.0 °C.

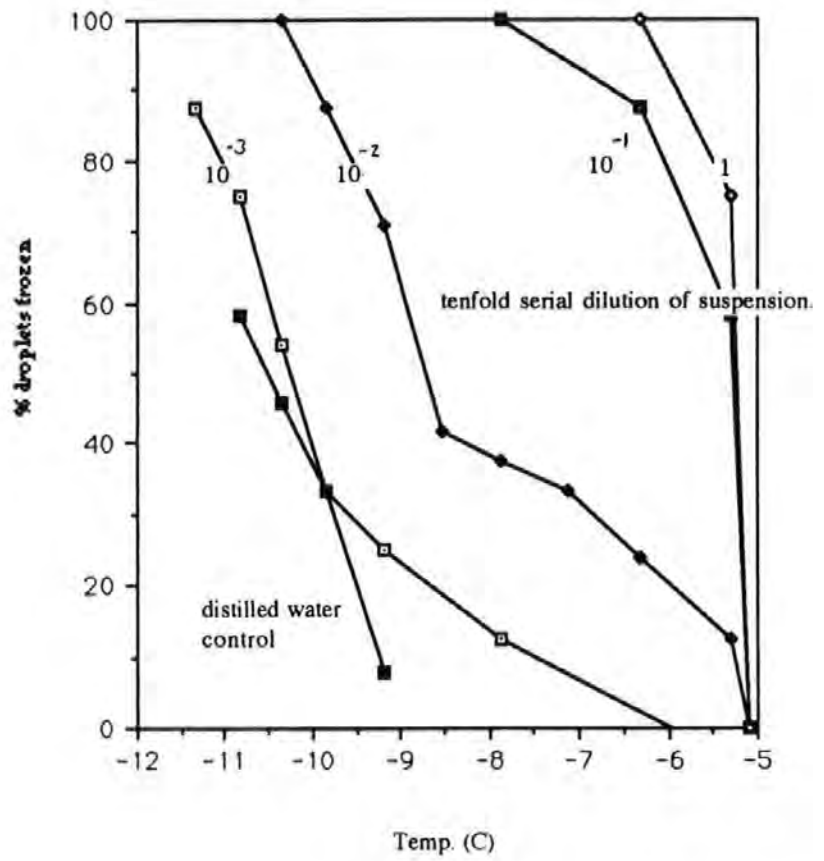
tissue sample	mean nucleation temperature (C)
control	-7.4 a
meristem	-6.8 b
leaf	-6.0 c
stem	-5.9 c

Table 7. Comparison of mean nucleation temperature of different tissue samples.

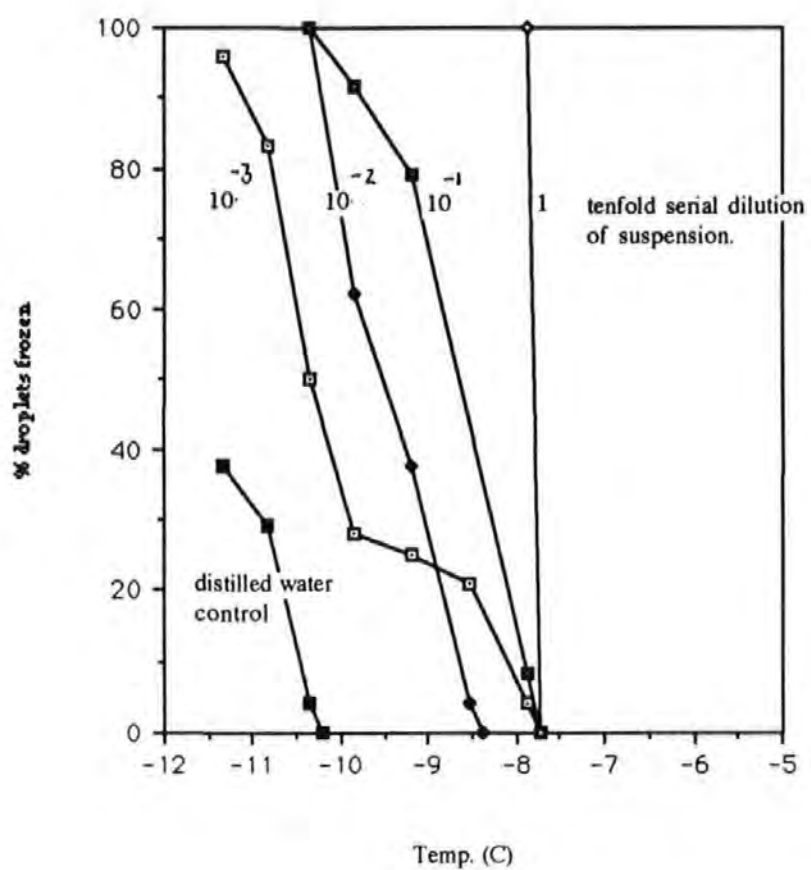
(treatment s.e. 0.108)



4.4. Effect of simulated dew on exotherm temperature.  
(vertical bars are standard errors)



4.5a. Droplet freezing spectra of applied soil suspension pre-autoclaving.



4.5b. Droplet freezing spectra of applied soil suspension post-autoclaving.

**Experiment 3.** A comparison of the effect of dew and a known ice nucleating strain of *Pseudomonas syringae* on supercooling ability.

All plants frost tested in the experiment displayed an exotherm and exhibited symptoms of frost damage which proved fatal.

Ice nucleation active (INA) bacteria were recovered from those plants inoculated with *Pseudomonas syringae* 84:27. No INA bacteria were recovered from untreated plants.

Plants treated with artificial dew froze at significantly warmer temperatures than those plants that were dry during the frost test with mean nucleation temperatures of  $-1.3^{\circ}\text{C}$  and  $-2.7^{\circ}\text{C}$  respectively (treatment s.e. 0.109). Similarly, applied INA bacteria reduced nucleation temperature (table 8.). The mean nucleation temperature however of plants treated with dew was approximately  $0.5^{\circ}\text{C}$  warmer than that of plants treated with INA *Ps. syringae*. This can be seen in figure 4.6. where the effect of dew on nucleation temperature is compared across the bacterial treatments.

The developmental stage of the plants at freezing was also significantly affecting nucleation temperature, with the first developmental stage, equating to early post emergence, freezing approximately  $1^{\circ}\text{C}$  cooler than stages two and three (table 9.).

Foliar treatment	Mean nucleation temperature. (C)
control	-2.5 b
applied <i>Ps. syringae</i> 84:27 1 day prior to freezing	-1.8 a
applied <i>Ps. syringae</i> 84:27 7 days prior to freezing	-1.7 a

Table 8. The effect of foliar treatment on mean nucleation temperatures of *S. tuberosum* var. Jersey Royal.

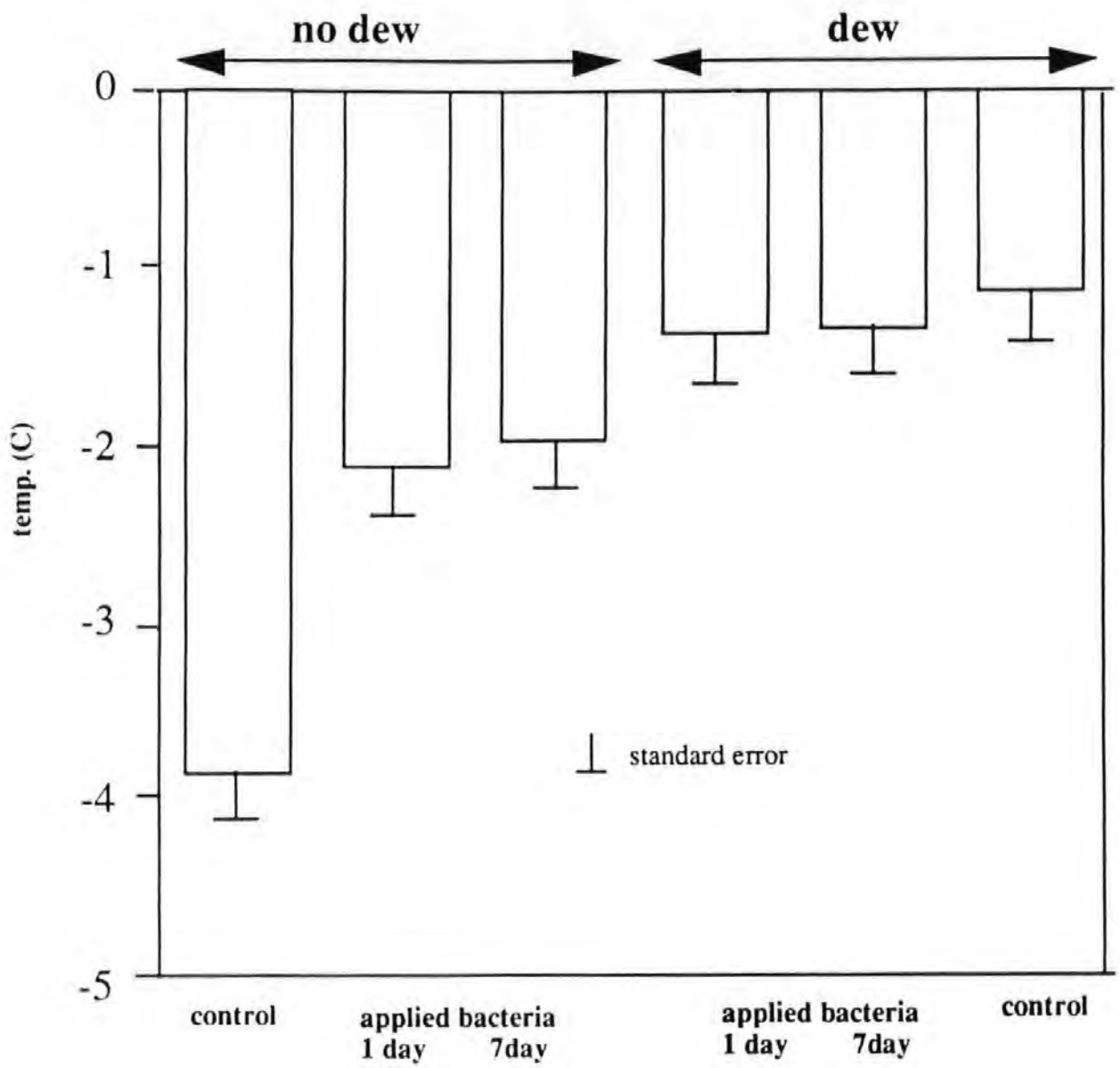
(treatment s.e. 0.134)

Plant developmental stage	Mean nucleation temperature (C)
stage 1	-2.7 a
stage 2	-1.5 b
stage 3	-1.7 b

Table 9. The effect of developmental stage on nucleation temperature of *S. tuberosum* var. Jersey Royal.

(treatment s.e. 0.134)





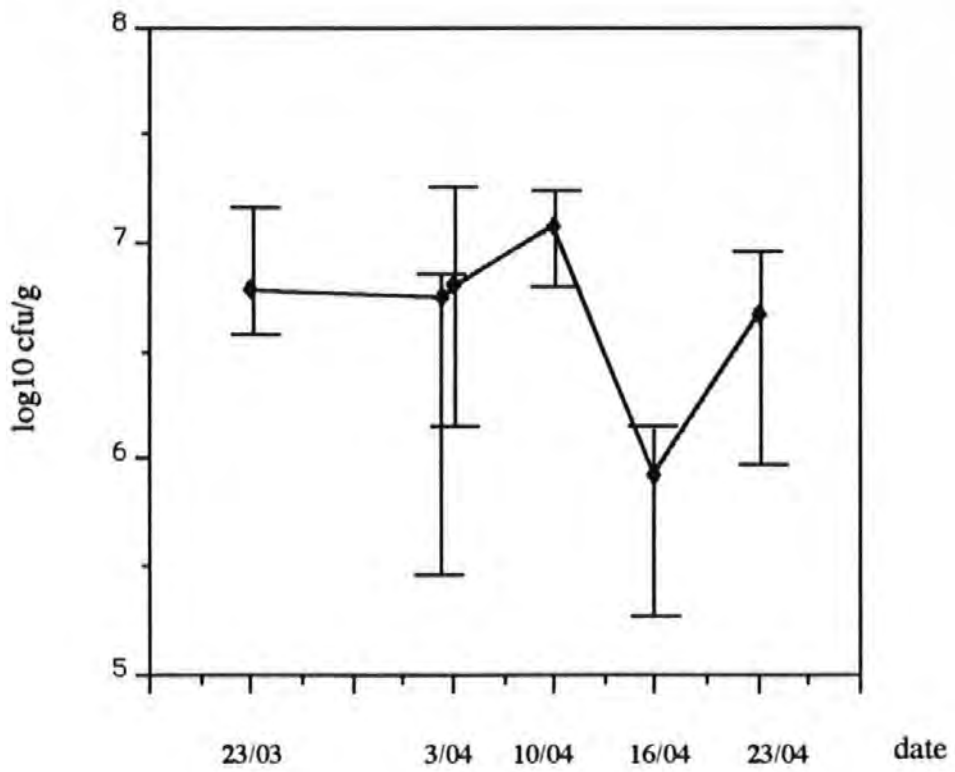
4.6. Effect of foliar treatments on exotherm temperature.

Section 2. An investigation of Ice Nucleating Bacteria upon Early Sown *S. tuberosum* (var. Jersey Royal).

Experiment 4. An investigation of INA bacteria on field grown plants at Seale-Hayne.

Bacterial populations were relatively constant throughout the sampling period (fig.4.7.) with a Log<sub>10</sub> mean of 6.6 cfu/g recovered from the crop. Bacterial identification indicated that 85% of the isolates were *Ps. syringae* species. Ice nucleation testing revealed no bacteria inciting ice formation at -5 °C.

The tube nucleation test revealed that no tubes were frozen at the end of the temperature holding periods.



Error bars indicate maximum and minimum populations isolated.

Bacterial populations isolated from *Solanum tuberosum* var. Jersey Royal foliage at the Seale-Hayne trial site. March-April 1990.

**Experiment 5. Pilot survey of microflora associated with the indigenous crop.**

Swab analysis revealed an average population size of  $1.5 \times 10^5$  cfu/m<sup>2</sup> existing on the polythene throughout its time on the crop. Counts never exceeded the order of magnitude  $10^5$  or went below  $10^4$  cfu/m<sup>2</sup>. Replica freezing of these isolates elicited no ice nucleation activity.

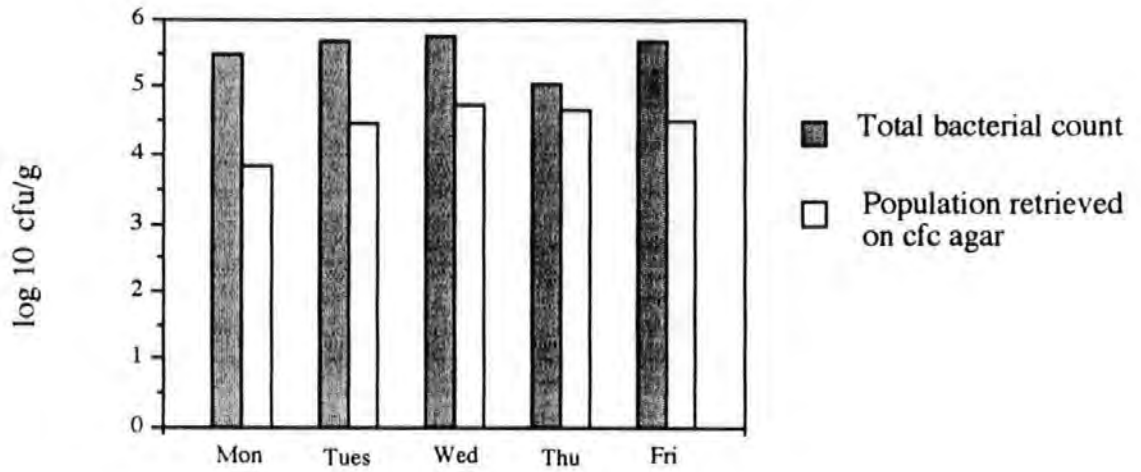
Tube nucleation of leaf material resulted in only 1 tube freezing at -5.0 °C. Four colonies were isolated as possibly INA positive. These colonies proved INA negative at temperatures warmer than -5 °C when investigated using the droplet spectra freezing method.

**Experiment 6. Detailed survey of the existence of ice nucleating bacteria upon plants on the Island of Jersey**

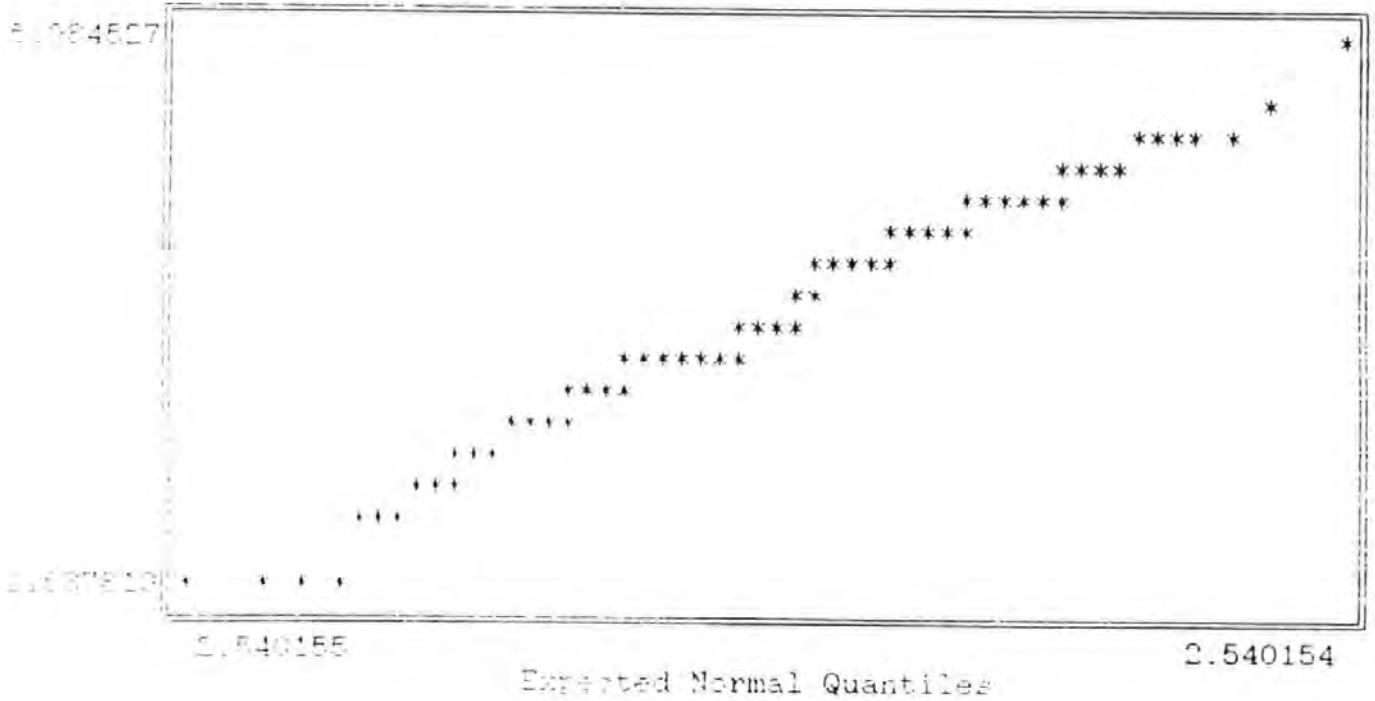
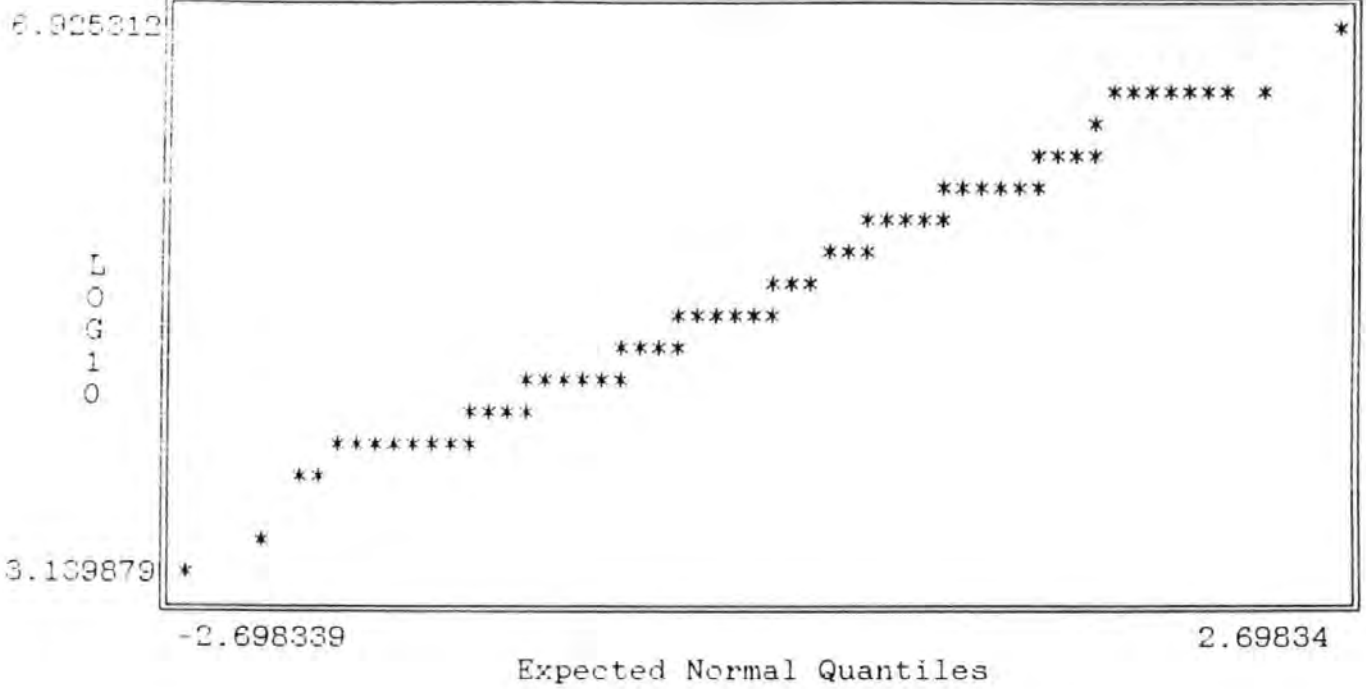
Mean total bacterial counts, and mean populations retrieved on *Pseudomonas* selective (CFC) agar can be seen in figure 4.8. Total populations on individual leaves ranged from a maximum of  $8.42 \times 10^6$  cfu/g to a minimum of  $1.38 \times 10^3$  cfu/g. Populations retrieved on *Pseudomonas* selective (cfc) agar ranged from a maximum of  $9.65 \times 10^5$  cfu/g to a minimum of less than 10 cfu/g.

Bacterial populations recovered from the foliage could not be described by the normal distribution. Transformation by Log<sub>10</sub> resulted in both total counts and bacterial populations isolated on the selective media fitting the normal distribution (fig 4.9.)

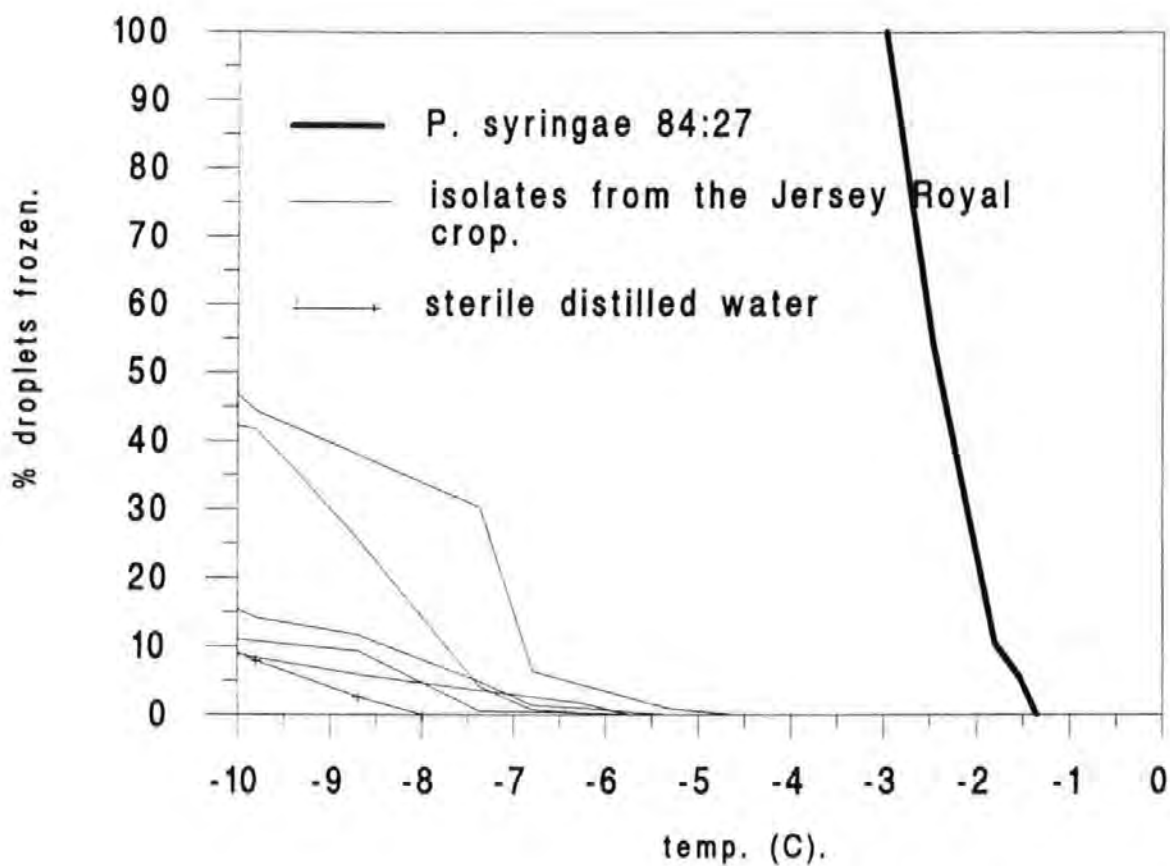
Replica plate ice nucleation testing of all the spiral plates revealed no ice nucleation activity at -5 °C. Furthermore the screening of each isolate identified as *Pseudomonas* sp. by replica plating onto *Pseudomonas* selective (CFC) medium for ice nucleation revealed no activity at -5 °C. Nucleation activity was measured in these isolates but only at temperatures below -5 °C. Nucleation spectra illustrating this colder temperature nucleation activity can be seen in Fig. 4.10. and the nucleation spectrum of *Ps. syringae* 84:27 can be seen in the same figure. It is apparent that none of the isolates recovered from the crop were active at warm temperatures. No ice nucleation activity at -5 °C was recorded in any of the 200 bacterial isolates selected as representative of the most abundant members of the foliar population.



- 4.8. Bacterial populations isolated from *Solanum tuberosum* var. Jersey Royal on nutrient agar followed by replica plating onto *Pseudomonad* selective (cfc) agar.



4.9. Normal probability plot of Log<sub>10</sub> transformed total population and of Log<sub>10</sub> transformed populations retrieved on *Pseudomonas* selective (cfc) agar.



4.10. Nucleation spectra of bacterial colonies isolated on *Pseudomonas* selective (cfc) agar in comparison with sterile distilled water and *Ps. syringae* 84:27.

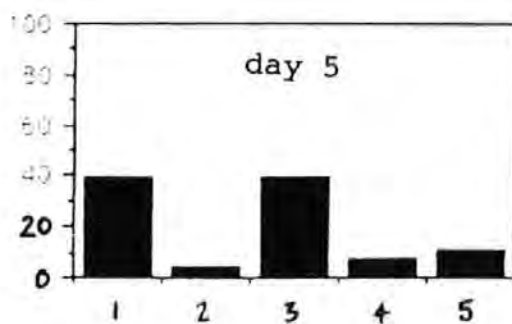
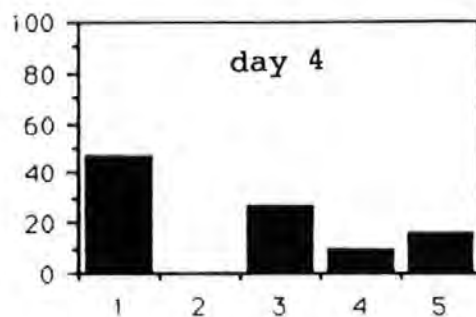
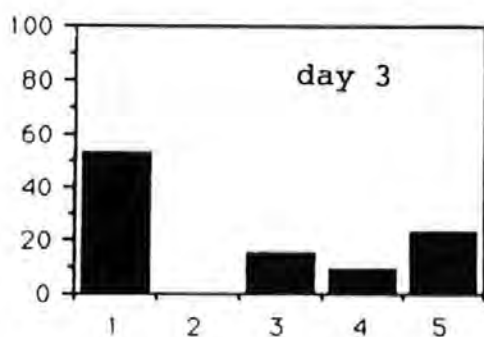
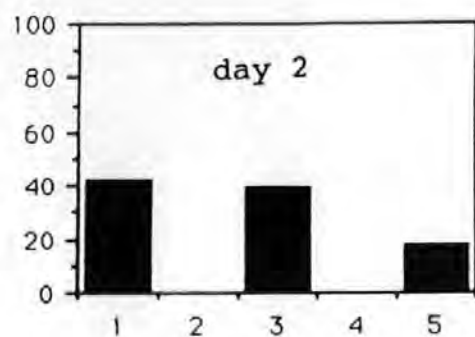
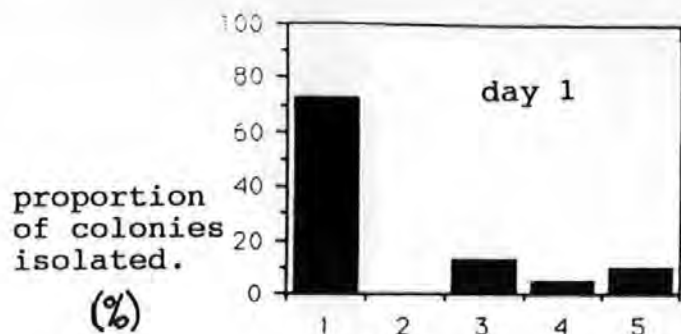


#### Bacterial Identification.

Of the 200 colonies selected, 16 were lost in culture, 103 isolates were Gram positive and 81 colonies were Gram negative. *Bacillus cereus* var *mycoides* was apparent on the original spiral plates of leaf samples taken on Monday and Tuesday. The spreading growth of these colonies meant that isolating bacterial colonies as representative of the most abundant species on the leaf without *B. cereus* contamination was difficult. It is possible therefore that the proportion of Gram positive spore forming bacteria recovered from the leaves harvested on Monday and Tuesday overestimate the true proportions residing on the crop. The process of identification allowed the isolates to be grouped as follows:-

- 1) Gram positive spore formers. (*Bacillus* spp)
- 2) Gram positive non-spore formers. (*Coryneforms*)
- 3) Gram negative, oxidase positive. (*Pseudomonas* spp.)
- 4) Gram negative, oxidase negative fermentative, (*Enterobacteriaceae*)
- 5) Gram negative, oxidase negative non-fermentative. (*Ps. syringae*)

The relative proportions of these groups isolated from the leaves on each day of the sampling period is shown in figure 4.11.



KEY.

1. Gram +ve spore forming.
2. Gram +ve non-spore forming.
3. Gram -ve. Oxidase +ve.
4. Gram -ve. Oxidase +ve. Fermentative.
5. Gram -ve. Oxidase -ve. Non-Fermentative.

4.11. Bacterial populations recovered from the field crop through sampling period.

Section 3. Phylloplane Microflora of the Early Sown *S. tuberosum* var. Jersey Royal in relation to microclimatology.

Experiment 7. The effect of microclimate on bacterial populations.

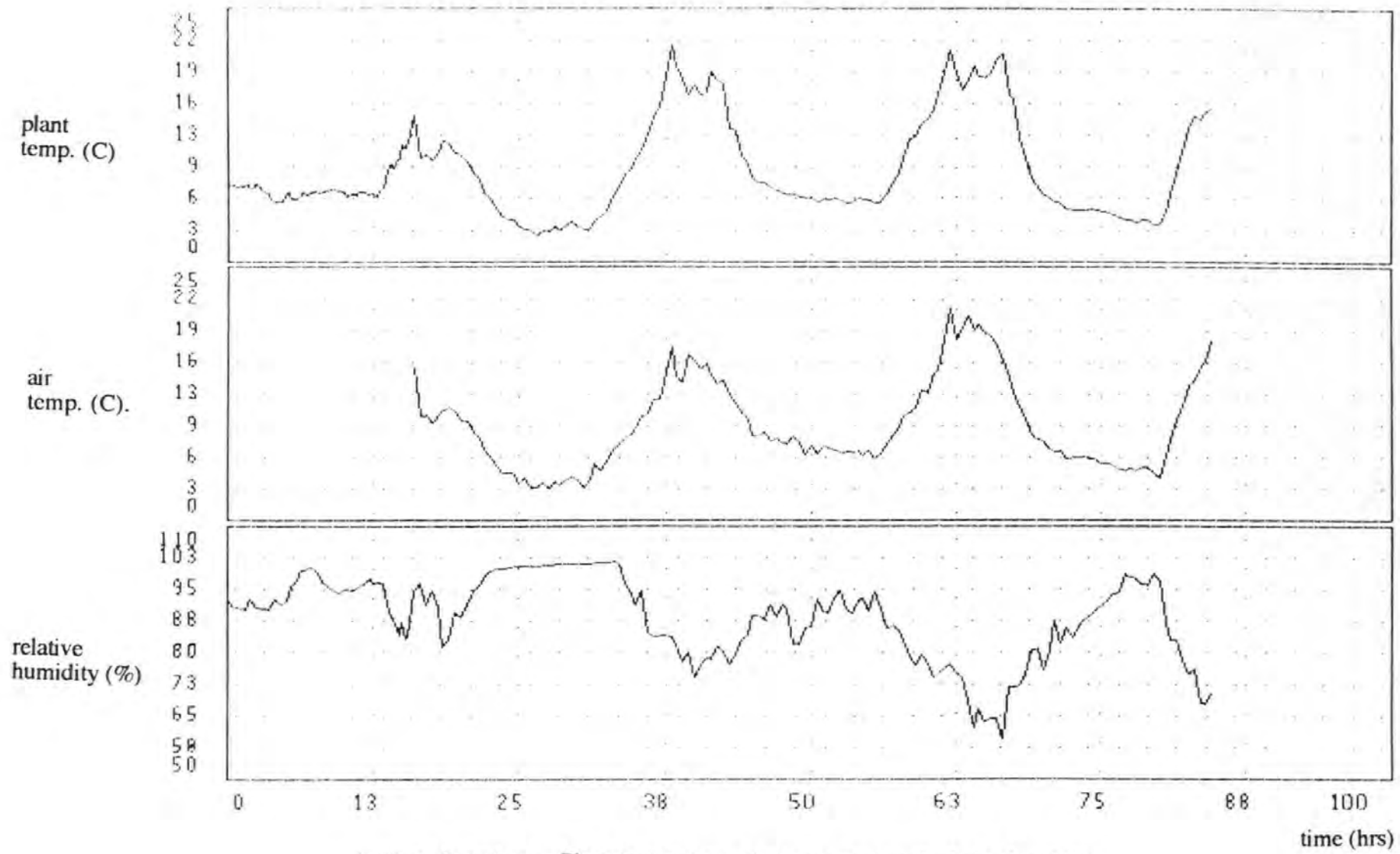
Microclimatic Data.

The period studied did not include any frost events. It is possible to see the cyclical pattern of heating and cooling recorded during the week (fig 4.12a). This pattern was reflected in the measured plant temperatures (fig 4.12b). Temperatures measured at the plant showed less instantaneous fluctuation than air temperatures but maxima and minima were very similar.

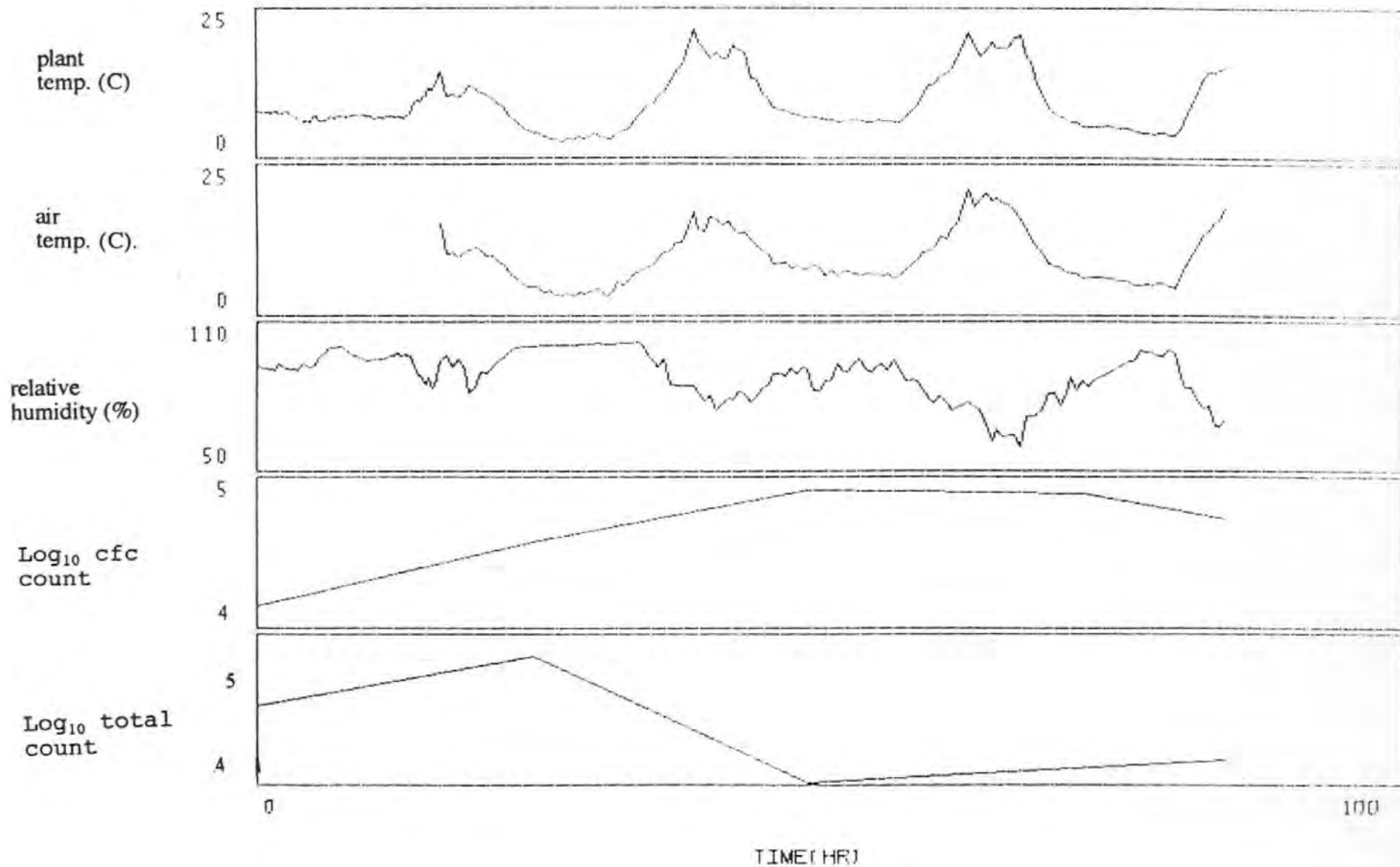
The curve representing relative humidity traces the approximate inverse of the temperature curves (figure 4.12c.). A period of apparently erroneous readings was observed in the period 20 hrs to 35 hrs into the trial, where relative humidity was consistently over 100%. This related to a continuous mist and fine drizzle which was prevalent on that day, causing wetting of the sensor (despite screening) giving elevated readings.

It is important to note the differences in plant temperature across the trial site. Variations of 3-4 °C were recorded for the measured maxima. These variations did not appear so large for the minima with differences across the site of up to 1 °C.

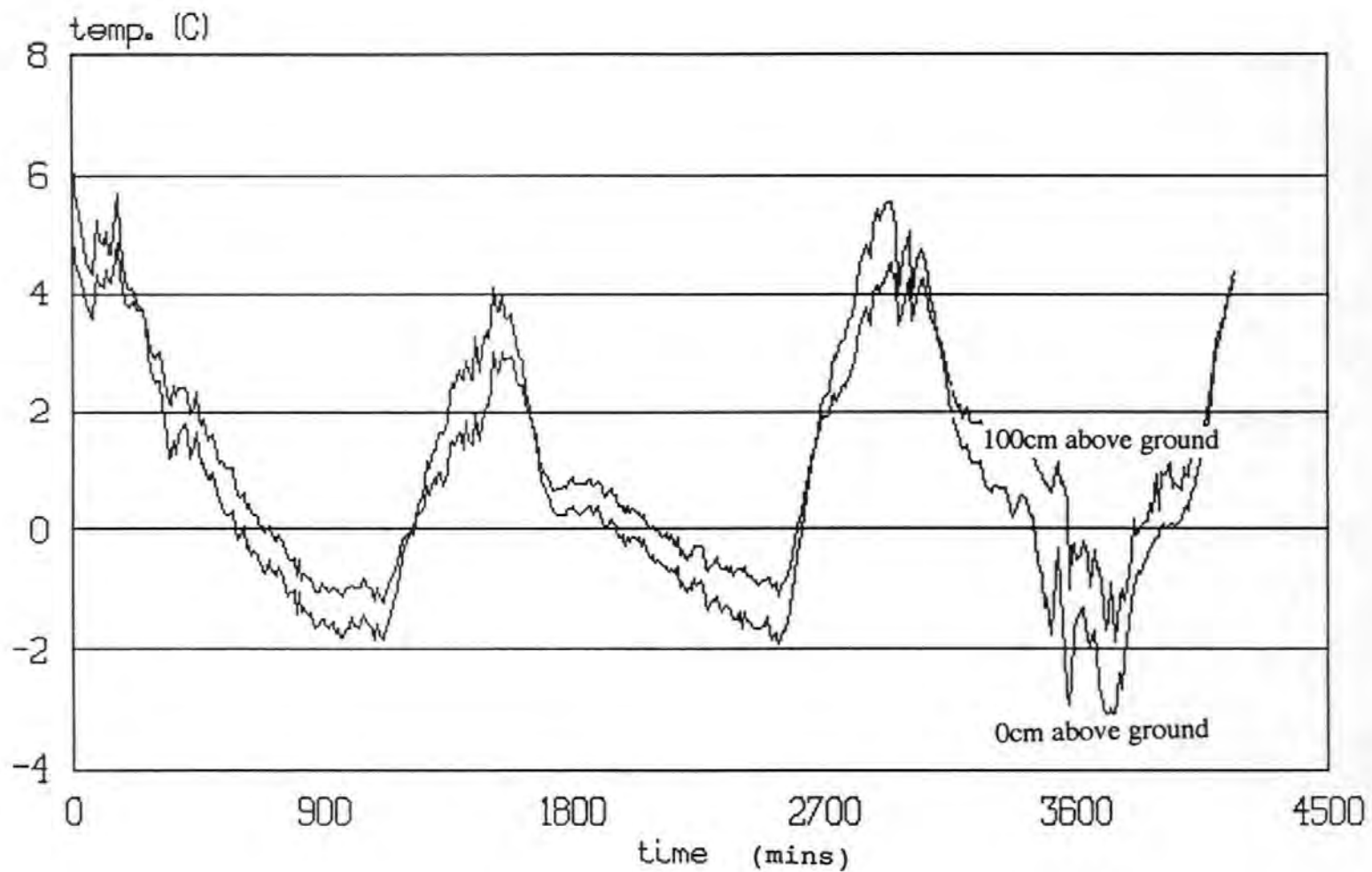
Effect of microclimate on bacterial populations.



4.12.a, b and c. Plant temperature, air temperature and relative humidity measured at the Jersey trial site.



4.13. Bacterial populations (log<sub>10</sub> cfu/g) recovered from *Solanum tuberosum* var. Jersey Royal from the Jersey trial site compared with prevailing temperature and relative humidity.



4.14. Cyclical pattern of temperature including three consecutive frost events measured at the Jersey trial site.

The measured trend in the total populations recovered from the individually harvested leaves (as described in experiment 6) was one of an increase from Monday until Wednesday, followed by a decrease toward the end of the week. This trend was mirrored in the counts of those bacteria tentatively characterised, by virtue of their selection media, as *Pseudomonads*, except the decline in population occurred a day earlier.

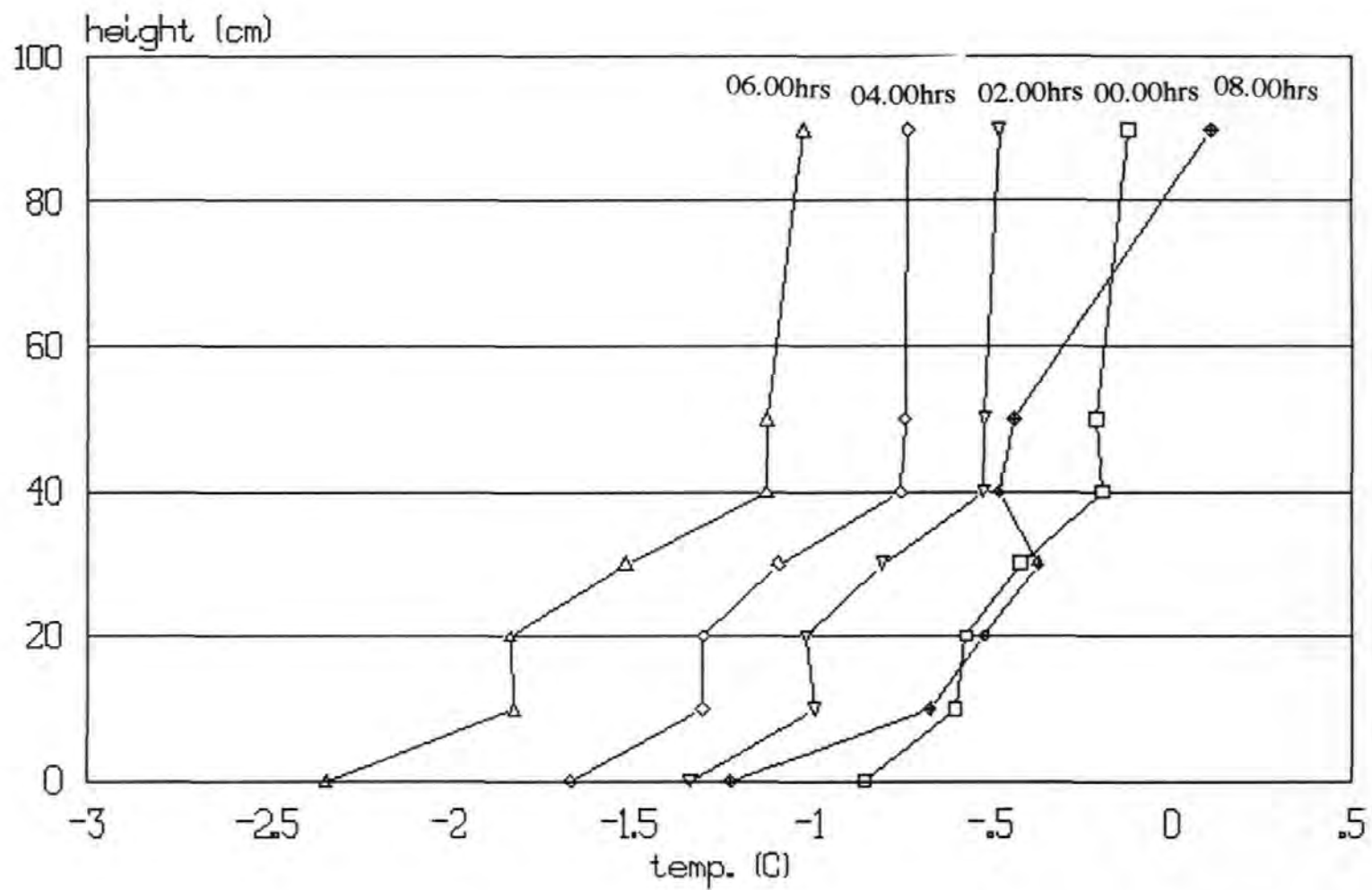
The mean daily populations can be seen in comparison with the prevailing temperature and humidity conditions in figure 4.13. There appeared to be no obvious pattern of temperature or humidity affecting the population trends. Precipitation occurred on the Sunday (3 mm) before the start of sampling, and on Monday (2 mm) and Tuesday (2 mm). This was accompanied by an increase in measured bacterial populations, which started to decline on the days when precipitation was absent. The mean populations recovered from leaves were significantly larger on days during which precipitation occurred with a mean of 4.93 log<sub>10</sub> cfu/g when precipitation was absent, compared to 5.34 log<sub>10</sub> cfu/g when precipitation was present ( $p < 0.05$ ). Numbers of bacteria isolated on *Pseudomonas* selective media were not significantly affected by precipitation.

**Experiment 8. Long term temperature survey within a commercial crop on the Island of Jersey.**

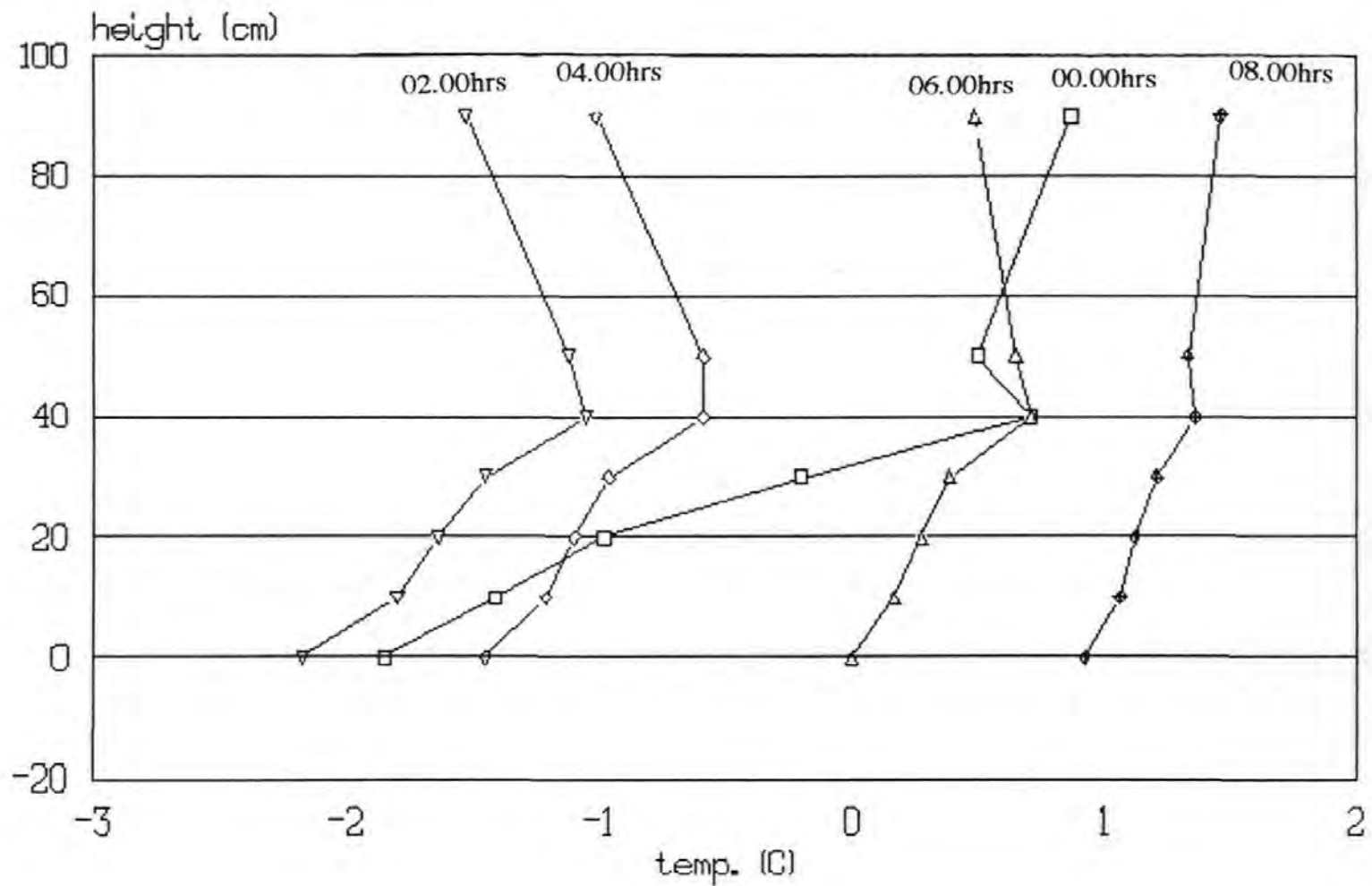
Data collection in this experiment was hampered due to a logger malfunction. This meant that no data were collected through the growth period of the crop. Thus data for modification of temperature profiles through crop establishment remain unavailable. However, data was collected prior to this breakdown which included the recording of three frost events during the pre-emergence period of the crop.

The diurnal pattern of air temperature is illustrated in fig 4.14. and approximates to the sine curve during the period 18th to the 21st February 1992. It was evident that the maximum and minimum temperatures occurred at ground level as expected. Three frost events, where the recorded temperature fell below 0 °C, are also shown in figure 4.14. The frost events of the morning of the 19th of February are shown as temperature profiles in figure 4.15a (station 1) and figure 4.15b. (station 2). These graphs indicate that instantaneous temperature profiles varied across the 2 m distance between the instrument stations. The frost event of the 19/02/92 indicated a minimum temperature at the ground of approaching -2.5 °C at 06:00 hrs at station 1, whereas at station 2 the corresponding temperature recording was approaching -2.0 °C.





4.15a. Tautochrones of frost event at Station 1. (19th February 1992)



4.15b. Tautochrones of frost event at Station 2. (19th February 1992)

**Section 4. The promotion of supercooling of the early sown *Solanum tuberosum* var. Jersey Royal as frost avoidance mechanism using exogenous foliar applications.**

**Experiment 9. A preliminary screening of agents to promote supercooling of excised leaves.**

**Freezing of leaves treated with seaweed extract.**

The mean nucleation temperature of leaves treated with light or no dew were not significantly different to each other, regardless of the application of seaweed extract. Nucleation temperatures of leaves treated with dew to runoff levels were significantly warmer (table 10). At run off levels of dew, leaves treated with seaweed extract froze at significantly warmer temperatures than untreated controls (table 11). Kaolin significantly increased nucleation temperatures relative to the untreated dew covered control (fig. 4.16.). It can be seen from figure 4.16. that the application of ethylene glycol significantly reduced nucleation temperatures to a mean of -8.2 °C compared to a mean of -5 °C in the control leaves.

Applied dew level	Mean nucleation temperature (C)
no dew	-9.9 a
light dew	-9.8 a
run off	-2.9 b

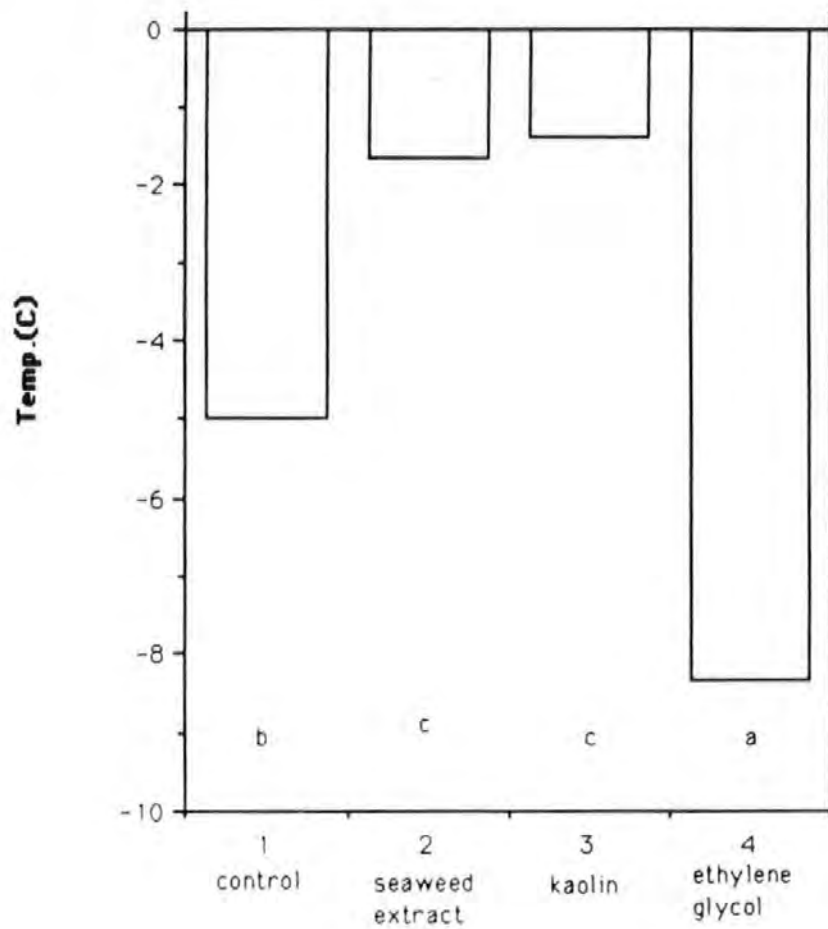
Table 10. The effect of level of dew application on mean nucleation temperatures of excised leaves of the glasshouse grown *S. tuberosum* var. Jersey Royal.

(treatment s.e. 0.347)

Applied seaweed extract	Mean nucleation temperature (C)
1 day prior to freezing	-1.6 a
10 days prior to freezing	-2.3 a
control	-5.0 b

Table 11. The effect of the application of seaweed extract on mean nucleation temperatures of excised leaves of the glasshouse grown *S. tuberosum* var. Jersey Royal in the presence of artificial dew applied to runoff.

(treatment s.e. 0.347)



4.16. Mean exotherm temperatures of exised leaves treated with kaolin, ethylene glycol and seaweed extract in the presence of artificial dew applied to runoff.

(treatment s.e. 0.485)

**Experiment 10. The effect of foliar applied ethylene glycol upon supercooling ability of whole plants during *in-vitro* frost tests.**

Applied dew, and the applied foliar treatments significantly affected nucleation temperature of the plant tissue. Plants treated with dew froze approximately 2.5 °C warmer than dry tissue (mean nucleation temperatures of -2.1°C and -4.6°C respectively, treatment s.e. 0.196). Plants treated with applied ethylene glycol froze at significantly cooler temperatures than untreated plants (table 12.).

Examination of plants treated with ethylene glycol showed signs of phytotoxic response, only when applied at the final developmental stage seven days prior to frosting. In these plants necrotic patches at leaf edges giving a brown scorched appearance were noted. The patches were dry and extended to approximately 10% of leaf area on the worst affected leaves.

The range of temperatures tested ensured that all plants displayed an exotherm, and all plants upon removal of the test chamber exhibited signs of frost damage including stem collapse and water soaked appearance.

Applied ethylene glycol	Mean nucleation temperature (C)
1 day prior to freezing	-3.8 a
7 days prior to freezing	-3.5 a
untreated control	-2.6 b

Table 12. The effect of foliar applied ethylene glycol on the mean nucleation temperatures of glasshouse raised *S. tuberosum* var. Jersey Royal.

(treatment s.e. 0.240)

**Experiment 11. The effect of foliar applied ethylene glycol upon the supercooling plants in the field.**

Frost event at Howard Davis Farm trial site.

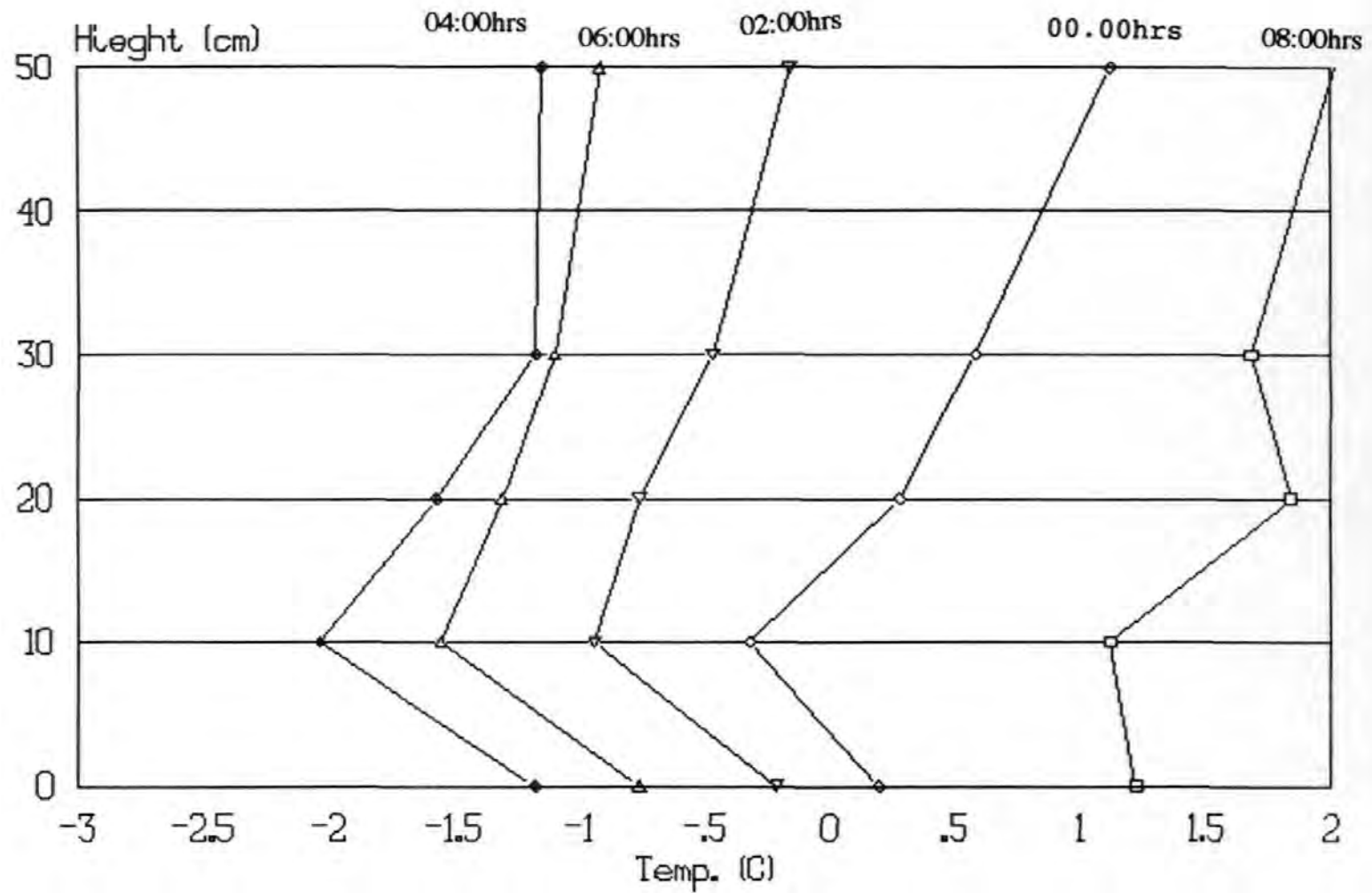
A damaging frost event was recorded on the morning of 15th March 1993 but exotherm detection was unsuccessful. Plants did suffer damage however and the applied treatments significantly affected the measured damage levels. Plants treated with sterile distilled water prior to the frost were damaged to a greater extent than untreated controls and plants treated with ethylene glycol. The difference in damage levels recorded in control plants and those treated with ethylene glycol was not significant (table 13.).

Frost event at Seale-Hayne trial site.

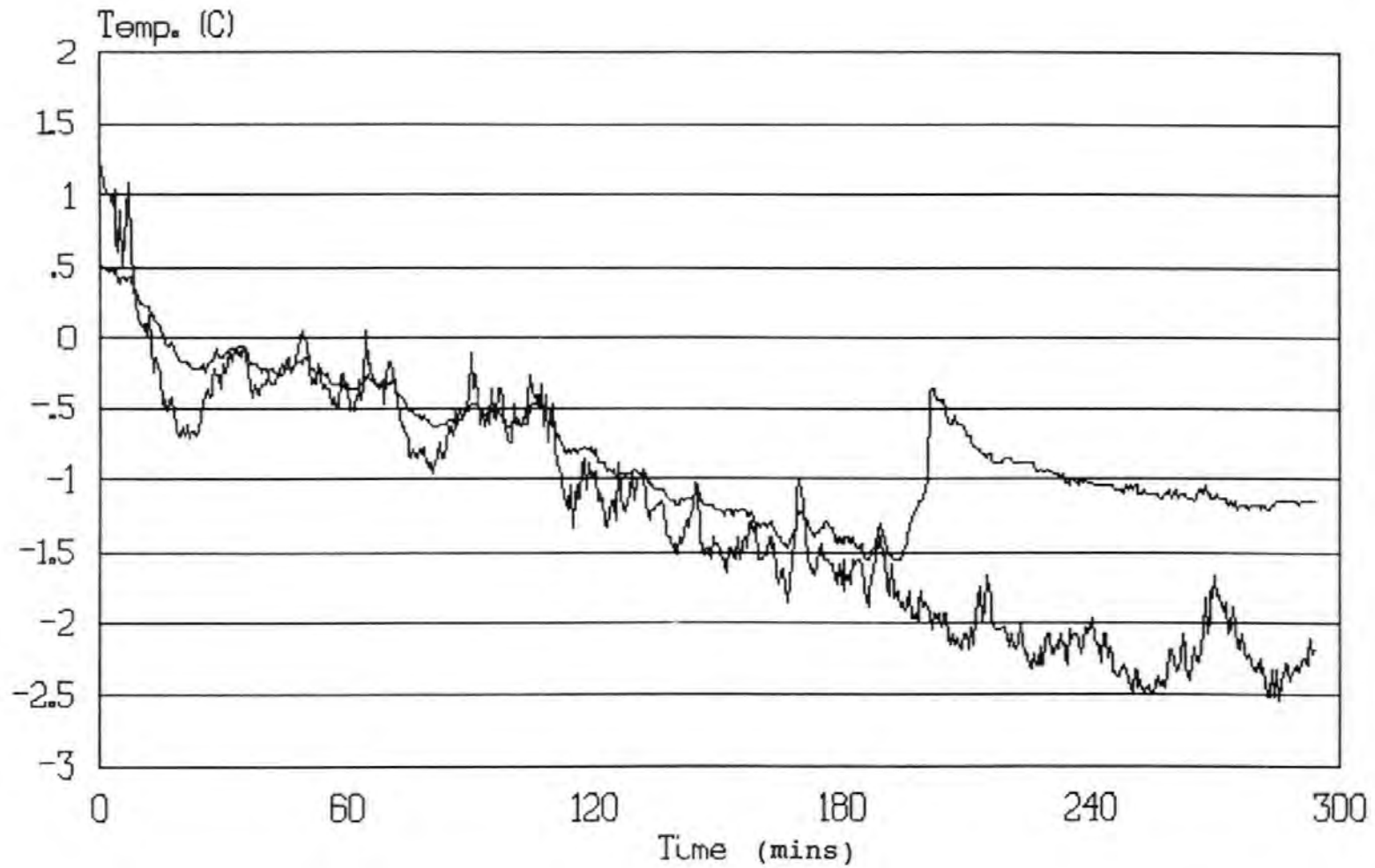
A frost severe enough to cause damage to the plants of the first sowing date occurred during the morning of the 20th of March 1993. The plants of the second and third sowing had not emerged. Tautochrones showing the temperature profile through the development of the frost are shown in figure 4.17.

Exotherm detection was successful in 16 of the 36 attached thermocouples. An example of a typical exotherm is shown in figure 4.18. Of the 36 monitored plants 22 were damaged and 14 were undamaged with exotherms not detected in 6 of the damaged plants. A typical cooling curve of a damaged plant not exhibiting an exotherm is shown in figure 4.19. It can be seen that sub-zero temperatures were attained, and damage consistent with ice formation was

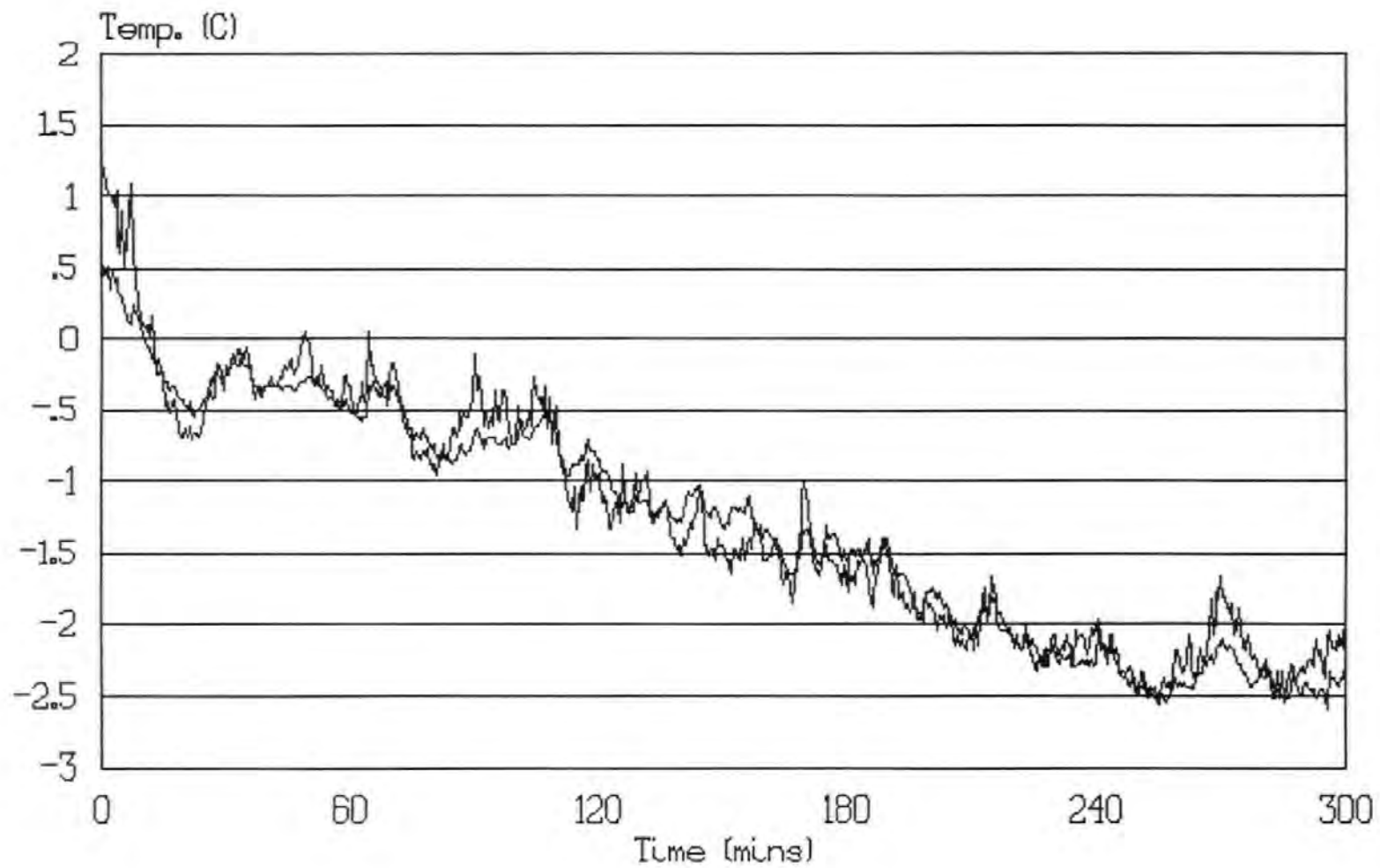




4.17. Tautochrones illustrating development of frost event at Seale-Hayne. 20/03/93.



4.18. Typical in-vivo exotherm measured 20/03/93 at the Seale-Hayne trial site.



4.19. Typical temperature trace of damaged plant not displaying an exotherm. (Scale-Hayne trial site 20/03/93.).

observed. It is likely that no exotherm was recorded because no ice formed in close proximity to the thermocouple. No exotherms were recorded in the undamaged plants. The lowest exotherm temperatures were recorded in plots treated with ethylene glycol, and the warmest exotherm temperatures were recorded in plots treated with sterile distilled water (table 14.).

The foliar applied treatments significantly affected recorded frost damage levels, with the untreated control showing the least damage. There was no significant difference in damage levels between plants treated with ethylene glycol and those treated with sterile distilled water (table 15.). It is difficult to comment on the relationship between the temperature of exotherm and the levels of frost damage recorded in the trial because not enough exotherm temperatures were recorded. Furthermore the exotherm temperatures indicated that ethylene glycol treated plants froze at colder temperatures than the other treatments but they still froze. This suggests that the frost was severe enough to mask treatment effects on damage levels subsequent to thawing.

The maximum and minimum relative humidities during the frost event were 99.8% and 94.6% respectively.

Foliar treatment	Damage level
control	0.42 a
ethylene glycol	0.51 a
sterile distilled water	0.64 b

Table 13. Effect of treatment on mean frost damage levels on the crop established at Howard Davis Farm.

(treatment s.e. 0.052)

Foliar treatment	Exotherm temperature (C)	Standard deviation
control	-1.8	0.44
ethylene glycol	-2.1	0.33
sterile distilled water	-1.4	0.65

Table 14. Effect of treatment on mean exotherm temperatures of the crop established at Seale-Hayne.

Foliar treatment	Damage level
control	0.35 a
ethylene glycol	0.53 b
sterile distilled water	0.63 b

Table 15. Effect of treatment on mean frost damage levels on the crop established at Seale-Hayne.

(treatment s.e. 0.051)

## Chapter 5. DISCUSSION OF THE RESULTS.

The field grown populations in the present study highlighted the susceptibility of *S. tuberosum* var. Jersey Royal potatoes to a spring frost with plants severely damaged despite relatively mild frosts (circa  $-1^{\circ}\text{C}$  to  $-2^{\circ}\text{C}$ ) which suggested a lack of exploitable supercooling ability. *In-vitro* freezing of the glasshouse raised populations however effectively demonstrated that plants could supercool to temperatures lower than those monitored in the field and thus avoid frost damage. Nucleation temperatures of  $-4^{\circ}\text{C}$  to  $-6^{\circ}\text{C}$  were frequently recorded. All of the experiments confirmed that freezing subsequent to supercooling was fatal and there were no exceptions to the finding that complete kill followed exotherm detection (i.e. freezing). Other authors including Richardson and Weiser (1972), Warren et al (1987) and Li and Fennel (1985) have also noted that freezing *in-vitro* favours freezing of plant tissue at much cooler temperatures than those measured in the field.

The results presented in experiment 1 showed that *S. tuberosum* var. Jersey Royal had no exploitable hardening mechanism, which corroborates the study of Lindstrom et al (1981) and Collet et al (1991) who also found that cold acclimation had no effect on the supercooling ability of *S. tuberosum*. The only route, therefore, to promote field frost survival of *S. tuberosum* must be to enable the significant supercooling ability recorded *in-vitro* to be expressed *in-vivo*.

It has been postulated that ice nucleation active bacteria are agents capable of interrupting supercooling in the field (Maki et al

1974, Lindow 1982ab). The bacterial and antibiotic treatments applied to test this hypothesis (experiment 1) did affect levels of frost damage recorded in the field and indicated that it was possible to modify the supercooling response by modifying bacterial populations. These treatments however, affected the damage levels in an unexpected manner. The ice nucleating bacteria apparently reduced damage, whilst the antibiotic treatment predisposed plants to greater damage. In the experiment antibiotic treated plots exhibited higher numbers of isolates identified as *Pseudomonas syringae* species than those plots inoculated with the bacterial suspension and it is possible that the antibiotic may have been selecting for ice nucleation active strains and predisposing the plants subjected to this treatment to damage. This does not concur with the work of Proebstring et al (1982), who showed that streptomycin and oxytetracycline at 100mg/litre were able to suppress bacterial populations to a negligible level. A further unexpected effect was that the applied *Ps. syringae* 1205 appeared to suppress *Ps. syringae* populations. This may have resulted in reduced levels of natural INA bacteria, resulting in a corresponding reduction in damage levels. Investigations however, of the untreated control plants did not reveal any natural ice nucleators on the crop. The applied *Ps. syringae* 1205 did not significantly increase total bacterial levels which suggests that it either competitively excluded other populations of the phyllosphere or it may not have affected total populations due to its failure to establish upon the foliage. These findings suggest that the bacterial inoculation of potatoes with *Pseudomonas* species may be problematic.

No INA bacteria species were isolated during surveys of the



commercial *S. tuberosum* var. Jersey Royal crop on the Island of Jersey. Swab samples (experiment 4) suggested a stable population of bacteria residing on the polythene surface but again no INA bacteria were isolated. Results of the tube nucleation assays of leaf material revealed no INA bacteria associated with the harvested plant material. Tube nucleation assays during the present study also highlighted the disparity between *in-vitro* and *in-vivo* nucleation temperatures. This does not reflect the findings of Hirano *et al.* (1985) who employed a tube nucleation test as a descriptor and predictor of frost damage in the field however the leaves harvested for the experiment of the present study were in transit at ambient temperatures for up to 2 days before being processed and such conditions would suggest that the monitored microflora may not be strictly representative of that found in the field. The survey of microflora associated with the Seale-Hayne trial site and the results of the week long survey of a commercial crop on Jersey also failed to identify INA bacteria active at -5 °C. In these instances plant material was processed immediately after harvesting and the criticism put forward above did not apply.

This absence of INA bacteria does not support evidence of other workers who describe the ubiquitous nature of ice nucleating bacteria (Lindow *et al* 1978a, Olive and McCarter 1988), but is supported by Gross *et al* (1983) who surveyed deciduous fruit trees and found that INA bacterial populations were negligible. Elsherif and Grossman (1990), demonstrated that crop rotation had an effect on levels of fluorescent *Pseudomonads* and it is possible that low levels of ice nucleators recorded in the present study resulted from the varied nature of cropping at the trial sites.

The study of Wood (1989) illustrated that *Ps. syringae* was the dominant INA species residing upon the Northern Irish potato crop. Wood, however, was also unable to identify INA strains active at temperatures warmer than -5 °C. Tube nucleation assay of leaf material in that study demonstrated ice nuclei active at -5 °C only occasionally and in very small numbers, and such warm freezing was not noted until 11th June, well after the peak in frost risk. The present study reflected the scarcity of INA bacteria and 'warm' temperature nuclei noted by Wood. Furthermore the present study suggested that *P. syringae* strains were just one member of a diverse population present upon the leaf surface of *S. tuberosum* var. Jersey Royal. Although no nuclei active at 'warm' (0 °C - 5 °C) temperatures were isolated, bacterial ice nucleation activity was noted between -5 °C and -10 °C. The work of Lindow (1982b) elicited abundant -9 °C nuclei on potatoes in California and suggested that their source was the soil.

This study suggested that both total populations of bacteria and those isolated on *Pseudomonas* selective media could be described by the lognormal distribution. This corroborates the work of Hirano *et al* (1982) who determined that bacterial populations on individual leaves followed a lognormal distribution and were able to use this relationship to help predict risk of frost damage. It appears that this fact can not be used to determine risk of frost damage to *S. tuberosum* var. Jersey Royal crop as no INA bacteria were detected upon the crop and consequently did not increase the frost risk to early sown *S. tuberosum* on the island of Jersey.

The ice nucleation spectra for the bacteria isolated on individual days of the week were not constant. This could be due to species fluctuation from day to day, or modification of the ice nucleation activity of a particular species due to altering growth phases and conditions. There appears to be no relationship between the categories of bacteria identified and the monitored ice nucleation activity. This suggests that only a small proportion of the population is displaying ice nucleation activity and that their activity is modified by the environment, or that there is a continually fluctuating population of INA bacterial species, each with different levels of INA activity.

It is apparent from the present study that precipitation appears to favour larger populations of leaf epiphytes on the foliar portion of *S. tuberosum* var. Jersey Royal than dry conditions which supports the findings of other teams (Henis and Bashan 1986, Fokkema and Schippers 1986, Dickenson 1986, Hirano and Upper 1986). The portion of the population isolated on *Pseudomonas* selective media was not affected by precipitation, suggesting that *Pseudomonas* species are more adaptable leaf epiphytes. No bacteria expressing ice nucleation activity at -5 °C were recovered on any day and thus no facet of the microclimate appeared to be favouring their colonization. Furthermore no frost events occurred within the sampling period so it was not possible to relate frost kill temperatures to bacterial ice nuclei.

The lack of INA bacteria recovered could possibly be because sampling took place before ice nucleation active species had colonised the crop, thus none were detected. However if this was the

case INA species would be proliferating after the period of highest frost risk. Wood (1989) in a study of INA bacteria on early potatoes in Northern Ireland noted that few ice nucleating bacteria were present at emergence, but ice nucleating strains of *P. syringae* were established very rapidly post emergence. The present study was of a crop two weeks post emergence, suggesting that nucleating populations would have had time to establish on the foliage, furthermore, bacterial sampling occurred in the morning and Hirano and Upper (1985) postulated that epiphytic populations were likely to be greatest at such times.

Bacteria representative of *Pseudomonas* species were isolated from the plants. It was possible that these populations were competitively excluding ice nucleation active strains. Similarly it was possible that some facet of the leaf ecology was not favouring the establishment of INA populations. Marshall (1988) working with oat cultivars and Hirano and Upper (1985) working with snap beans demonstrated that certain plant varieties were less prone to harbouring proliferations of INA bacteria. It is tempting to speculate that the closed cropping that has continued for many years on Jersey has perhaps selected against INA bacterial proliferation. This is however unlikely because this study has suggested that frost damage in the *S. tuberosum* var. Jersey Royal occurred at temperatures warmer than those at which bacterial ice nuclei are expressed.

The literature notes modification of nucleation activity of the order of 1.0 °C to 2.0 °C as a result of modifying culture conditions (Yankofsky *et al* 1981, Anderson *et al* 1982, Lindow 1982a). Even if

this degree of 'improvement' of ice nucleation activity occurred within the isolates recovered in the present study, the temperature of activity of the tested isolates would still be cooler than the temperatures noted in the field as causal to frost damage. O'Brien and Lindow (1988) stated however, that the activity of a given isolate is liable to be greater on its host plant tissue than *in-vitro*. It is therefore possible that bacterial isolates collected in this study would exhibit ice nucleation activity upon the crop. The increase in temperature of nucleation activity of the isolates required in order to be causal to frost damage would however, be in the order of 5 °C - 10 °C. Such 'improvements' are unreported in the literature.

A point of vital importance has been demonstrated in this study, in that, ice nucleation occurs in the field between 0 °C and -2 °C, and often before -1 °C. The microbiological surveys highlight the lack of ice nucleation activity of bacterial isolates in the temperature range that other studies suggest may have a role in inciting frost damage in the field (Lindow 1982a). Ice nucleation activity was noted, but in the range -5.0 °C to -10.0 °C, well below that at which field frost damage occurred. Thus it appears that an agent other than INA bacteria is causal to the interruption of supercooling of the early sown *S. tuberosum* var. Jersey Royal.

Experiment one which monitored damage in a field crop subsequent to a natural frost event highlighted the fact that environmental factors over small areas could have significant effects on recorded crop damage levels. Furthermore it appeared that these factors exerted a greater influence on damage levels than the manipulation

of bacterial populations. It proved difficult however, from the measurements of temperature taken in the field, to attribute recorded frost damage levels in each replicate to any facet of the temperature data recorded. It therefore seemed likely that factors additional to temperature were influencing the frost damage recorded in the field. These findings corroborate those of Richardson and Weiser (1972) who found that seemingly minor microclimatic differences, supercooling and spontaneous nucleation are just as important to the survival of sensitive species as minimum temperature and frost duration. The series of trials described in experiment two were designed to address such factors and determine what agents could be causal to an interruption of supercooling. The nucleation temperature results continued to demonstrate the supercooling ability of the foliar portion of the *S. tuberosum* var. Jersey Royal across a variety of treatments representative of field conditions. *S. tuberosum* var. Jersey Royal did contain intrinsic ice nuclei, as highlighted in experiment 2.4. These were destroyed under conditions of high temperature and pressure and were thus likely to be of biological origin. No INA bacteria were isolated in this experiment and these nuclei are therefore likely to be proteins inherent in plant tissue. These nuclei were not however active at temperatures warm enough to be responsible for the field frost kill temperatures recorded. These results supported other studies which have illustrated the supercooling ability of plant tissues (Lindow *et al* 1982, Lindow and Connell 1984 and Marshall 1988).

Plant developmental stage, cooling regime and leaf soiling and leaf damage moderately affected the degree of supercooling attained by the tissue, but did not interrupt supercooling to the extent

observed in the field. It is unlikely therefore that they are responsible for field freezing at temperatures of 0 °C to -2 °C. The only treatment in the study which incited nucleation temperatures comparable to field frost kill temperatures was the application of sterile distilled water analogous to dew formation. In a total of 516 frost tests this was the first example of the *S. tuberosum* var. Jersey Royal plant freezing *in-vitro* at immediately sub-zero temperatures. Simulated dew appeared causal to a dramatic interruption of supercooling. This supports the work of other studies including Cary and Mayland (1970), Yelenosky (1983) and Kackperska and Kulesza (1987) who also demonstrated that leaf surface water interrupted supercooling. Ashworth *et al* (1985) working with corn, soyabean, bean and cotton seedlings noted that surface water limited supercooling to a greater extent than inoculation with INA bacteria (nucleation temperatures of up to 3 °C warmer, between -1 °C and -2 °C, depending on plant species) whilst Cary and Lindow (1986) suggested that free water on the plant surface (common in radiation frost events) may limit supercooling to the same extent as INA bacteria. The dew treated plants of the present study froze after very little supercooling (mean nucleation temperature -0.8°C), yet when removed from the test chamber exhibited damage symptoms routinely observed as consistent with a freezing event after up to 6.0 °C of supercooling. Rajashekar *et al* (1983), working with *Solanum* species, indicated that frosting following little or no supercooling was less damaging than a freezing event occurring after protracted supercooling but this finding was not supported by the present study.

Since no INA bacteria were recovered from the plants it is unlikely

that INA bacteria were responsible for the modification of supercooling characteristics recorded in the present study. Similarly the temperature of freezing was so warm (mean  $-0.8^{\circ}\text{C}$ ) as to require an extremely active ice nucleus and evidence of bacterial ice nuclei active at such temperatures is unreported in the literature. Most bacteria implicated as inciting frost damage are active in the range of  $-2$  to  $-5^{\circ}\text{C}$  (Lindow *et al* 1982b, Lindow 1982a).

The hypothesis that dew was causal to supercooling interruption was further tested in experiment 3 where the nucleation temperatures of *S. tuberosum* var. Jersey Royal plants treated with known ice nucleating bacteria and artificial dew were compared. Applied bacteria did indeed interrupt supercooling, however plants treated with dew froze at significantly warmer temperatures than those treated with INA bacteria. This finding suggested that bacteria noted as highly INA at temperatures of  $-2^{\circ}\text{C}$  range could not be causal in limiting supercooling of the early sown *S. tuberosum* var. Jersey Royal in a situation where dew has formed since dew formation would have incited a nucleation event before the critical temperature range of INA bacterial nuclei was reached. Thus a field crop on which dew had formed would already be frozen before the INA bacteria 'triggered' a nucleation event. The study of Cary and Lindow (1986) indicated that water droplets upon leaves limited supercooling to a similar extent as applied bacteria. During the study however, the nucleation temperature of the plant tissue was not recorded, only the temperature to which the test chamber was lowered and therefore it was not possible to state which treatment was inciting ice formation and damage at the warmest temperature. The effect of bacteria acting as 'dew inciting' as well as ice



nucleating has been noted by Cary and Lindow (1986) and Sands *et al* (1985) and it is possible to speculate that, in the case of frost damage to the early sown *S. tuberosum* var. Jersey Royal, such bacteria would be more important as a causal agent than INA bacteria. In the study of Cary and Lindow (1986) INA bacterial strains were noted as being 'dew inciting' bacteria. As no INA bacteria were isolated during this series of experiments other bacterial species would have to act as 'dew inciting' bacteria to have been responsible for the measured interruption of supercooling.

The results of the present study suggest an explanation for the events surrounding frost damage of the early sown *S. tuberosum* var. Jersey Royal in the field. Plants in the field are subjected to varying cooling rates disrupted by instantaneous temperature fluctuation even on calm clear nights (Ashworth *et al* 1985). As the environment cools, sub-zero temperatures are attained earlier in the leaf than either stem tissue or surrounding air due to the radiative nature and smaller mass and therefore lower heat capacity. This is the beginning of supercooling. Evidence from the *in-vitro* tests suggested that the supercooling process should continue for up to 4 to 6 degrees, with no damage to the plant. However, in the field during the radiation event dew condenses upon the cooler leaf tissue, and as leaf temperature drops below zero a nucleation event is likely to occur and subsequent damage is likely.

Freezing *in-vitro* always resulted in total plant death, whereas in the field plant death did not always occur, but damage symptoms such as leaf scorch, loss of turgor or dark and wet patches on the leaves were noted. *In-vitro* the termination temperature and duration of

sub-zero temperature provided an environment suitable for the continued progression of ice through the plant subsequent to nucleation, whereas in the field sub-zero duration or severity may not always allow progression of the ice frontier through regions of the plant which are warmer than the initial site of nucleation. It is also possible that dew formation may be uneven across the plant or crop due to leaf borne factors such as chemical application, leaf soiling and damage or 'dew condensing' bacteria, which may affect the number and position of nucleation sites and the extent of ice progression. Furthermore, microclimatological factors such as temperature variations through the crop canopy, or air turbulence may affect dew condensation levels, distribution and plant temperatures, thus influencing supercooling and resultant frost damage patterns. The results of the microclimatological recordings in the field supported the high degree of leaf temperature variation and site to site temperature variation which could be responsible for field frost damage patterns.

The indication that the application of leaf surface water to the foliar portion of *S. tuberosum* var. Jersey Royal reduced supercooling to levels comparable to field frost kill temperatures mitigates against a bacterial control strategy for protection of the crop from frost damage. The possibility of promoting supercooling by interrupting the dew/leaf association which appears causal to warm temperature nucleation is an alternative strategy. It was demonstrated that it was possible to increase the supercooling ability of dew covered tissue by the application of ethylene glycol. It is likely that this was acting as an antifreeze within the dew droplets thus stopping ice nucleation. It is difficult to speculate

on attainable freezing point depression because the concentration of the ethylene glycol present within dew droplets was not known. Furthermore, the droplet size distribution would have resulted in many different concentrations of ethylene glycol in solution across one leaf. It is unlikely that it affected the tissue physiologically as it was applied immediately prior to freezing. Both seaweed extract and kaolin applied prior to an *in-vitro* frost event did not enhance tissue supercooling ability, indeed they appeared to predispose leaves to ice formation at temperatures warmer than those measured in the untreated controls. This suggested that these treatments neither acted as 'antifreezes' to the dew nor interrupted the water droplet/leaf association responsible for causing ice nucleation. This could be due to reduction of dew surface tension, allowing a more even coverage of the leaf surface with water which could result in more of the potential nucleation sites associated with the leaf being in contact with water molecules and being given the opportunity to be 'nucleation active'. An earlier droplet freezing spectra trial (not presented in this study) indicated that neither seaweed extract nor kaolin contained nuclei active at the temperatures highlighted above.

The *in-vitro* frost test of whole plants further demonstrated that ethylene glycol could significantly enhance the supercooling ability of *S. tuberosum* var. Jersey Royal, providing protection of up to 1 °C extra supercooling over untreated controls. There was no significant difference in this enhancement of supercooling ability between plants treated either 1 or 7 days prior to frosting. In this study the application of ethylene glycol did however, appear to be phytotoxic with leaf necrosis occurring. This could have reduced

tissue water content and perhaps have reduced the chance of tissue nucleation. These preliminary *in-vitro* studies support the work of Coulter (1962) who noted ethylene glycol conferring freeze resistance to a number of crop plants although the study did not postulate a mode of operation. Similar work presented by Wilson and Jones (1980), (1983a) and (1983b) demonstrate long chain polymers active in conferring frost resistance to Blackcurrant.

The action of ethylene glycol as a promoter of supercooling was tested in a field trial plot at Seale-Hayne and within a commercial crop on Jersey. The greatest damage levels recorded in the field were on plants treated with sterile distilled water at both sites. This corroborates *in-vitro* studies showing that sterile distilled water interrupted supercooling and predisposed plants to damage. At Seale-Hayne similar damage was, however, associated with applied ethylene glycol, and on Jersey ethylene glycol treated plants displayed damage levels similar to untreated control plants. Superficially, it would appear that the effect of ethylene glycol in promoting supercooling *in-vivo* did not work. Exotherm measurement indicated however, that plants treated with ethylene glycol froze at colder temperatures than the other treatments, suggesting that ethylene glycol did enhance supercooling in the field. Damage of equal severity in ethylene glycol treated plots and control plots will of course have appeared if the freezing temperature dropped below the temperature of freezing of the ethylene glycol, and it is postulated that this happened in this crop. Fewer exotherms were recorded in plants treated with ethylene glycol and it can be speculated that this was due to enhanced supercooling leading to frost avoidance and consequently fewer measured exotherm responses,

but this is based upon a small data set. Importantly the mean exotherm temperatures of plants treated with ethylene glycol recorded *in-vivo* (-2.1 °C) were comparable to those recorded *in-vitro* (-2.0 °C) when in the presence of artificial dew. These findings also suggest that "real" dew formed upon the leaf surfaces during the frost *in-vivo*. The fact that relative humidity measurements of around 99% were recorded in the crop further suggests that it was likely that dew had formed on these plants and predisposed them to damage.

## Chapter 6. GENERAL DISCUSSION.

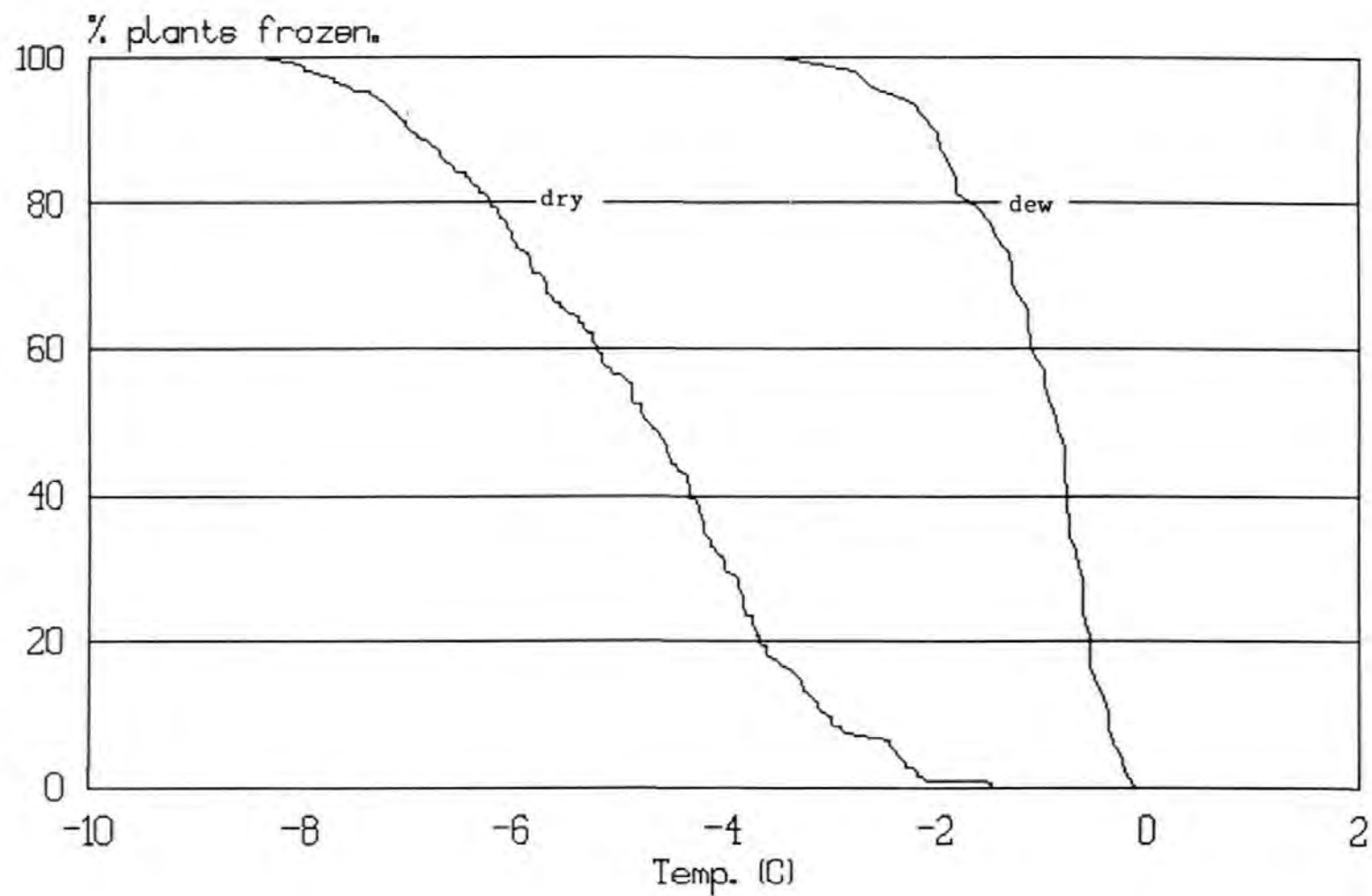
In the present study supercooling of the field grown *S. tuberosum* var. Jersey Royal was limited and damage occurred at immediate sub-zero temperatures (0 °C to -2 °C). The study of Marshall (1988) also noted field frost damage occurring in the range 0 °C to -2 °C in oat cultivars. During *in-vitro* frost testing ice nucleation events occurred at around -4 °C to -7 °C and thus for an ice nucleus to be construed as causal to *in-vivo* frost damage it would have to be active at a temperature in the range 0 °C to -2 °C. The extensive literature on ice nucleators cites no evidence of bacteria active at such warm temperatures (Yankofsky *et al* 1981, Lindow 1982a, Lindow 1983) and no bacterial ice nuclei active in this temperature range were detected in the present study. Published evidence of intrinsic non bacterial ice nuclei active at such warm temperatures does however, exist (Proebstring and Gross 1988, Anderson and Smith 1989). A nucleus active within this temperature range appeared to predispose *S. tuberosum* var. Jersey Royal to frost damage however, unlike the studies of Proebstring and Gross (1988) and Anderson and Smith (1989) leaf surface water must be present before it initiates freezing. This suggests that the nucleus must be on the leaf surface, yet not in contact with plant tissue water. A paradox appears to exist since these nuclei cease to be active in proximity to large volumes of water (eg in a tube nucleation test), yet are highly active in the presence of leaf surface water. This phenomenon has also been demonstrated by Ashworth *et al* (1985), who stated that the reason for this was not 'readily apparent'. It is generally considered that the probability of a nucleation event occurring should increase with sample volume for both homogeneous and

heterogeneous systems (Franks 1982). For both homogeneous and heterogeneous nucleation the groups of water molecules that lead to further ice crystallisation are the same (Franks 1985). The difference is that in a heterogeneous system (such as dew upon the surface of the *S. tuberosum* var. Jersey Royal) ice nuclei are present and act as catalysts that enhance the probability that such a cluster can form. One hypothesis explaining this paradox is that the surface tension operating in small dew droplets, perhaps by interrupting diffusional motion, enhances molecular clustering in such a way that a nucleation event and ice crystallisation is favoured. Although the aggregation of water molecules into a cluster required for ice formation results from random density fluctuations within the liquid such a phenomenon being modified by molecular energetics within smaller droplets is not accommodated in nucleation theory. Furthermore, studies using such small volumes of water have been aimed at describing homogeneous nucleation theory (Bigg 1953, Franks 1985). This area of speculation reflects problems in homogeneous nucleation theory, particularly the assumption that the number of clusters capable of initiating ice crystal growth within a unit volume is described by a Boltzmann distribution. Such a distribution is a statistical representation of molecular association (Franks 1982) and does not take into account the possible effects of hydrogen bonding in the clustering process which may be operating within the liquid. Franks (1982) stated that refinements to the theory taking into account such factors and their effect on molecular clustering have been presented but in view of other 'even more uncertain assumptions' the Boltzmann expression is retained.

Govindarajan and Lindow (1988), using radiation inactivation techniques, measured the molecular mass of the ice nucleation protein associated with an INA bacteria active at  $-3^{\circ}\text{C}$  as 8,700,000 Da. As leaf surface water on the *S. tuberosum* var. Jersey Royal can incite nucleation up to  $2.5^{\circ}\text{C}$  warmer than this any nucleus associated with the leaf surface must be even larger. Burke and Lindow (1990) calculating theoretical nuclei sizes and shapes suggested that a disk shaped nucleator with the height equal to the diameter up to 75 A, then of constant height with further increases in diameter provided the most likely nucleator shape. Furthermore, these dimensions were consistent with the growth of a high molecular weight protein. They calculated that a  $-3^{\circ}\text{C}$  nucleus of this shape, with a height of 75 A would be around 300 A in diameter. They speculated that a nucleus active at  $-1^{\circ}\text{C}$  (as are apparent in the *S. tuberosum* var. Jersey Royal associated with leaf surface water) would be 9.3 times larger (83,700,000 Da, and 900A diameter). These workers stated that this was 'very large indeed' and ' suggests a reason why  $-2$  and  $-1^{\circ}\text{C}$  ice nuclei are very rare'. The work of Fletcher (1970) demonstrated that to achieve heterogeneous nucleation at temperatures approaching  $0^{\circ}\text{C}$  the ice nucleus would be of a radius between  $10^3$  and  $10^4$  A with a wetting parameter ( $m$ ) of 1.0. The present study has demonstrated that nuclei active at immediate sub-zero temperatures are routinely expressed in the *S. tuberosum* var. Jersey Royal associated with leaf surface water. This suggests that either the theoretical considerations are overestimating nuclei size, or that the nucleator associated with the *S. tuberosum* var. Jersey Royal is not a protein.



The present study appears to corroborate the work of Fletcher (1962), who demonstrated that nucleation temperatures were a specific property of the ice nuclei present. Figure 6.1. presents the nucleation spectra of the pooled data from the investigations of the present study where nucleation temperatures of dry and dew treated plants were measured. It is apparent that two distinct freezing spectra are described depending on the presence of leaf surface water, suggestive of two characteristic sets of nuclei. The fact, however, that exotherm detection both in the field, and *in-vitro* has shown that plants often experience colder temperatures without freezing (due to fluctuating temperatures) than the temperature at which the nucleus is finally expressed indicates that despite the 'singular' characteristics of the nuclei, a stochastic mechanism was operating. This observation is important as it explains why minimum temperature is not likely to be a predictor of frost risk. Furthermore it helps to explain why damage patterns measured in the field did not correlate with measured temperature minima. This finding corroborates the work of Ashworth and Anderson (1985) who carried out a direct test of singular theory using tomato plant stem sections. Samples were cooled to  $-5^{\circ}\text{C}$  and subsequently warmed to  $-4^{\circ}\text{C}$ . Singular theory would predict that all nuclei active at  $-5^{\circ}\text{C}$  would have been expressed at  $-5^{\circ}\text{C}$ , whatever the duration, and upon rewarming no further nucleation would occur. The results showed that a further 33% of the samples froze after 7 hours at  $-4^{\circ}\text{C}$ . In the case of *S. tuberosum* var. Jersey Royal plants associated with leaf surface water the freezing behaviour can be described by the work of Vali and Stansbury (1966). They stated that the probability function that characterises the propensity of a nucleus to become active at a particular time at a temperature other than



6.1. Comparison of nucleation spectra of dew treated and dry *Solanum tuberosum* var. Jersey Royal plants frozen in-vitro.

its characteristic nucleation temperature varies so rapidly with temperature that nucleation is almost bound to take place within  $\pm 0.25$  °C of its characteristic temperature and it can be seen from figure 6.1. that 40% of nucleation events occur within  $\pm 0.25$  °C of the median nucleation temperature for wet plants. This theory, however, does not explain the freezing spectra of dry *S. tuberosum* var. Jersey Royal foliage so adequately. In this case it appears that either there are many nuclei each with very specific active temperatures, or that there are perhaps fewer nuclei each with a wider active temperature. Such results corroborate those of Ashworth et al (1985) who suggested that plant freezing conforms to a 'modified singular hypothesis'. The fact that freezing spectra of *S. tuberosum* var. Jersey Royal associated with dew follows a singular theory of nucleation closer than the spectra of dry *S. tuberosum* var. Jersey Royal foliage begins to suggest that perhaps some nuclei are described by singular theory, whereas others are not, and therefore one hypothesis is not applicable to all heterogeneous nucleation systems. From the experimental evidence collected it is not possible to prove this because many different types of nuclei (complexes of size, surface free energy) may be present, each rigorously conforming to singular theory. This seems unlikely, (unless the nuclei are transient or constantly changing particles, and are not necessarily active when their nucleation temperature is first reached), due to the fact that ultimate nucleation temperature is not necessarily the lowest experienced by the plant (or foliar associated nucleus).

Whatever the description of the nucleus this study has demonstrated that, in the presence of dew, supercooling of *S. tuberosum* var.

Jersey Royal is interrupted. Using the collected data of figure 6.1 it is possible to speculate on facets of field frost control. These data indicate that, in the presence of dew, supercooling can not be relied upon as a frost avoidance mechanism. From the spectra of dew treated plants it can be estimated that for a frost event of  $-1\text{ }^{\circ}\text{C}$  60% of plants are likely to experience a nucleation event. Plant death will not necessarily occur, because as this study has shown frost events are transient, and conditions favourable to continued ice propagation and progression may not occur. Whereas promoting supercooling to the same extent as keeping the plant dry, likely nucleation within 60% of the plants does not occur until  $-5.5\text{ }^{\circ}\text{C}$ . However, ice propagation and progression through the plant at such low temperatures, should the effect of the protection strategy be exceeded, is likely to be rapid and will result in ice spreading through the plant and causing death. This is not, however, a drawback of promoting supercooling because without such protective action the untreated crop could have been fatally damaged at previously attained warmer temperatures.

The results of the present study and the work of others (Ashworth (1986), Anderson and Ashworth 1985) suggests that the 'supercooling ability' must be addressed as a complex of duration at sub zero temperature, the severity of the subzero temperature and the influence of plant borne or environmental influences modifying the supercooling response. The present study demonstrates that the latter is especially important when trying to utilise *in-vitro* supercooling ability as an indicator of likely survival in the field. *In-vitro* frosting of *S. tuberosum* var. Jersey Royal favours nucleation at much cooler temperatures than those causing damage in the field. Similar discrepancies were noted by authors including Richardson and Weiser (1972), Warren *et al* 1987, Li and Fennel (1985).

The finding, in this and other studies, that considerable supercooling can occur during *in-vitro* frost testing highlights the need to monitor plants during frost tests for temperature of nucleation. There are many reports in the literature where freezing events were assumed to have occurred and no measurement of freezing temperature was made e.g. Anderson *et al* (1982), Lindow *et al* (1978b), Cary and Lindow (1986), van Swaijj *et al* (1987), Kim *et al* (1987) and Wilson and Jones (1980). Since little or no damage is incurred by the process of supercooling such reports are prone to an over estimation of frost resistance. Such over estimations may explain the frequently reported disappointments of laboratory evaluation of frost resistance being overridden by some unmeasured factor during field experimentation.

It is of paramount importance to define the temperature at which nucleation is occurring in the field for any crop. Without this information it can only be possible to speculate on whether control agents or causal nucleants tested *in-vitro* can be active in modifying frost damage in the field. Considerable effort can be expended on scientific investigations of 'potential economic importance in frost control' without ever actually addressing the crux of the problem. This fact has important connotations for shorter term studies and studies investigating the devastating occasional frost that occur in favoured climates. It is quite possible that a natural frost providing the vital first information on frost damage temperatures *in-vivo* can be so infrequent as to be very difficult to detect. The present study has shown that anecdotal evidence on damaging frost temperatures, and also inference from Stevenson screen temperature measurements are not adequate in defining the actual temperature at which nucleation and frost damage occurs within a crop.

### 6.1. Critique of the present study.

Data collection in the present study hinged on the correct installation and operation of the electronic data logger. Failure to do this in one instance resulted in a period of no data collection. Careful operation, however, at Seale-Hayne ensured that *in-vitro* frost events, providing information fundamental to the study, were measured.

A problem of the exotherm detection procedure employed in the present study was that thermocouples were placed in intimate contact with the plant tissue. Thus choices about the mode, site and number of attachment points, and type of thermometer had to be made. Furthermore any attachment to the tissue under investigation was altering the thermodynamics of the system being measured. However the choice of very small type T thermocouples attempted to overcome this problem. A more important problem of this technique was that to monitor exotherm temperatures the freezing appliance had to be lowered to temperatures that ensure freezing occurred in the tissue samples thus allowing an exotherm to be measured, and freezing temperatures to be compared. This raises the possibility of confusing freezing temperature and frost kill temperature, because some plants under test may freeze at temperatures warmer than the final tissue temperature upon removal from the chamber. This means that the length of time that a plant is frozen and therefore prone to continuing freeze dehydration stress may also affect survival, or that small degrees of frost tolerance were possibly masked. It is unlikely that frost tolerance was masked in the early sown *S. tuberosum* var. Jersey Royal as it is highly frost sensitive. Indeed,

damage has been recorded after very mild sub-zero temperatures, not even approaching the extreme temperature minima employed *in-vitro*. The thermal imaging study of appendix 1 demonstrated, however, that although ice had formed in a cauliflower curd, frost kill had not occurred. This effectively highlighted the difference between nucleation and kill temperature. Baker and Upper (1991) developed a tube nucleation system linked to a continuous readout electronic temperature recorder which allowed removal of sample on freezing and negated the need to attach a thermometer directly to the tissue sample which addressed the possible confusion between nucleation and kill temperature. However the tubes limited the tissue size that could be frost tested which can lead to overestimation of supercooling ability. In addition nucleation sites could not be visualised.

Another problem associated with the use of thermocouple thermometry is that when exotherms were viewed after data retrieval it was not possible to differentiate between nucleation sites and the passing of an ice frontier because the exotherm response appeared the same. This means that even if many thermocouples are placed on one plant or piece of test tissue it is still not possible to count nucleation sites. The ability to view freezing in real time, and spot sites of nucleation, demonstrated the potential usefulness of thermal imaging.

A further criticism of the present study is that all *in-vitro* frost testing was carried out in an advective frost chamber. This environment was not representative of the damaging radiation frosts which occur in the field on the Island of Jersey. It was therefore



impossible to say what component of the supercooling distributions were artifacts of the test chamber. Despite this limitation however, field frost damage temperatures were re-created *in-vitro* on whole plants, so that control measures were targeted at the correct temperature range. As discussed earlier a major criticism of plant freezing studies is that the field frost kill temperature is not known before investigating causal agents, and that these temperatures are thus not re-created during *in-vitro* trials.

Tube nucleation assays were employed in the present study, and have been used by other teams as an assay for assessing potential frost risk (Hirano *et al* 1985). The work of the present study suggests that this test is not perhaps applicable to assessing potential frost hazard to the early sown *S. tuberosum* var Jersey Royal. This is because leaves of whole plants in association with small amounts of surface water analogous to dew froze at immediate sub-zero temperatures. Whereas when individual leaves of *S. tuberosum* var. Jersey Royal are suspended in test tubes of sterile distilled water supercooling appeared to be favoured. Similar results were presented by Ashworth *et al* (1985). This observation reiterates the importance of knowing field frost kill temperatures to ensure that laboratory assays are adequately representing field frost conditions.

## 6.2. Directions for future study.

The present study suggests four main avenues for the progression of study of frost damage of early sown *S. tuberosum* var. Jersey Royal:-

1. The ice nucleus associated with the foliar portion of *S. tuberosum* var. Jersey Royal must be characterised. It is possible that alteration of this nucleus may be possible through plant breeding. Description of the nucleus may provide clues to the identity of intrinsic ice nuclei implicated by other workers in other crops. Furthermore characterisation of the nucleus will yield information of interest to other branches of science working with ice nuclei.

2. Study of the microclimate of the field crop must take place to quantify and describe the conditions that influence dew formation. It appears from the present study that dew played an instrumental role in the interruption of the supercooling ability. Understanding the parameters which lead to dew formation will start to define more explicitly the damaging frost event, and the nature of the conditions that control measures must address.

3. The interaction of foliar nuclei and surface water must be further investigated. The nature of the relationship between nucleus activity, droplet size and resultant nucleation temperature must be explored. A possible frost control mechanism might involve the interruption of the optimum droplet size/nucleus configuration that appears to predispose the plant to damage. Furthermore, the results of such work will be valuable in further refining heterogeneous

nucleation theory.

4. The route through which ice formed within dew upon the leaf surface seeds plant tissue water should be further investigated. Frost control may be possible by attempting to 'break the bridge' of external ice nucleating supercooled plant tissue water.

In addition to the above directions aimed directly at the *S. tuberosum* var. Jersey Royal the present study suggests further directions of study in the area of plant frost studies:-

1. The continued use of thermal imaging techniques to enable both visualisation and quantification of nucleation sites. The techniques present the opportunity and should be used to characterise differences between frost kill temperature and nucleation temperature. In addition the potential of thermal imaging should be used to explore the above areas of study in the field, and to study field frost damage patterns.

2. The development, use and characterisation of radiation frost chambers, allowing better simulation of *in-vivo* microclimatic factors *in-vitro*. The use of such chambers would provide the opportunity to describe what components of earlier plant freezing studies are artifacts of advective freezing techniques, and facilitate experiments on the mechanisms and measurement of dew formation.

3. The development of cryogenic electron microscopy techniques to

show the location and progression of ice within the tissues of plants, and to relate this to temperature of freezing, as modified by techniques of *in-vitro* and *in-vivo* freezing, and plant injury.

4. The measurement of *in-vivo* frost damage temperatures of those plant species for which nuclei and experimental modifications to physiology may have been implicated as causal to damage *in-vitro*. This would ensure that experimental effort, particularly on correlative physiological studies aimed at promoting frost control, was correctly targeted.

**APPENDIX 1. The potential of thermal imaging technology in the study of frost damage to sensitive plants:- Visualisation of ice nucleation and plant freezing.**

**A.1 Introduction.**

Previous plant freezing studies in the literature and in the present study utilise two main strategies of temperature measurement:-

1) Inference. In these experiments temperature of the freezing apparatus is monitored and frost kill or freezing temperature is inferred from the temperature of the freezing apparatus.

2) Direct. In this case plant temperature is measured directly, employing some form of thermometer usually connected to an electronic data storage and retrieval system. Freezing temperature is commonly noted using exotherm detection subsequent to the retrieval of the stored data.

The nature of both of the above methodologies mean that a frost event or the results of a frost event can only be observed historically. Even field observations of frost damage are historical. This is due to the fact that frosts occur at sporadic times, freezing can take place without damage and it is difficult to be sure that ice has formed and furthermore, damage symptoms take time to express themselves in the tissue. The development of thermal imaging in recent years has reduced the price and improved the quality and portability of the technology. With quoted temperature resolutions of 0.1 °C available these systems are perhaps becoming

useful to the scientist interested in plant temperature studies. The present study furthermore, has postulated that supercooling interruption of the *S. tuberosum* var. Jersey Royal is a function of foliar associated ice nuclei which are able to express themselves at warm sub-zero temperatures in the presence of dew. The visualisation of nucleation events may allow the testing of this hypothesis.

The objectives of the study were twofold:-

- 1) To assess the potential of thermal image devices to record exotherm response.
- 2) To assess if it was further possible to visualise and quantify nucleation sites.

#### A.2. Materials and Methods.

Two commercially available thermal imaging systems were investigated:-

1. Land, Cyclops TI 35 TIS and LIPS Image Processing System, Land Infrared, Division of Land Instruments International Ltd., Dronfield, Sheffield. S18 6DJ
2. Agema Thermovision 487, Agema Infrared Systems Ltd. Arden House, West Street, Leighton Buzzard, Bedfordshire. LU7 7DD.

#### Plant Material

Glasshouse grown *S. tuberosum* var. Jersey Royal (raised as described earlier) and a cauliflower curd (cv. Arcade) were used.

## Imaging Protocol.

The ability of the systems to detect exotherm response was tested by cooling test plants within a chest deep freeze monitored by a thermal imaging camera (figure 1.). Each system was used to image one dry potato plant, and one treated with sterile distilled water analogous to dew formation.

The ability to visualise nucleation sites was tested using the Agema system by taking close up thermal images of plants (such that individual leaves were shown clearly). The Agema Thermovision 487 was also used to take thermal images of ice progression through a cauliflower curd.

Each system stored digitised images at preset intervals which were later retrieved via dedicated computer software. This capability, however, was predicted not to be adequate to ensure that exotherms were detected because the point of freezing is an instantaneous event and the chances of capturing this manually over a protracted freezing experiment was likely to be impossible, especially as it was not possible to anticipate what a freezing event would look like when viewed as a thermal image. Therefore each thermal imaging camera was connected to a video recorder and the freezing experiments were recorded on video tape (VHS and Video 8 formats). In this way digitised images of exotherms could be retrieved from video, and the course of an experiment could be reviewed, and re-imaged when necessary.

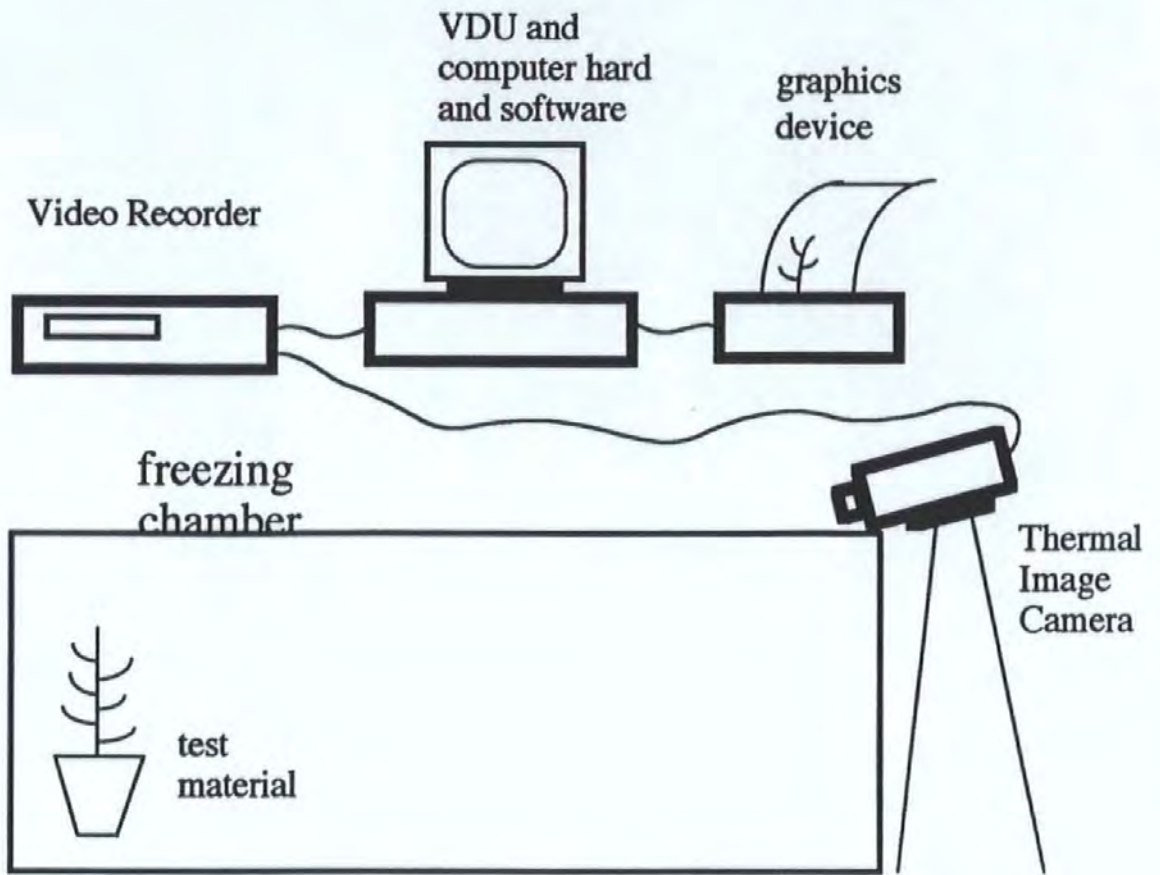


figure 1. Layout of a thermal imaging experiment.



### A.3. Results.

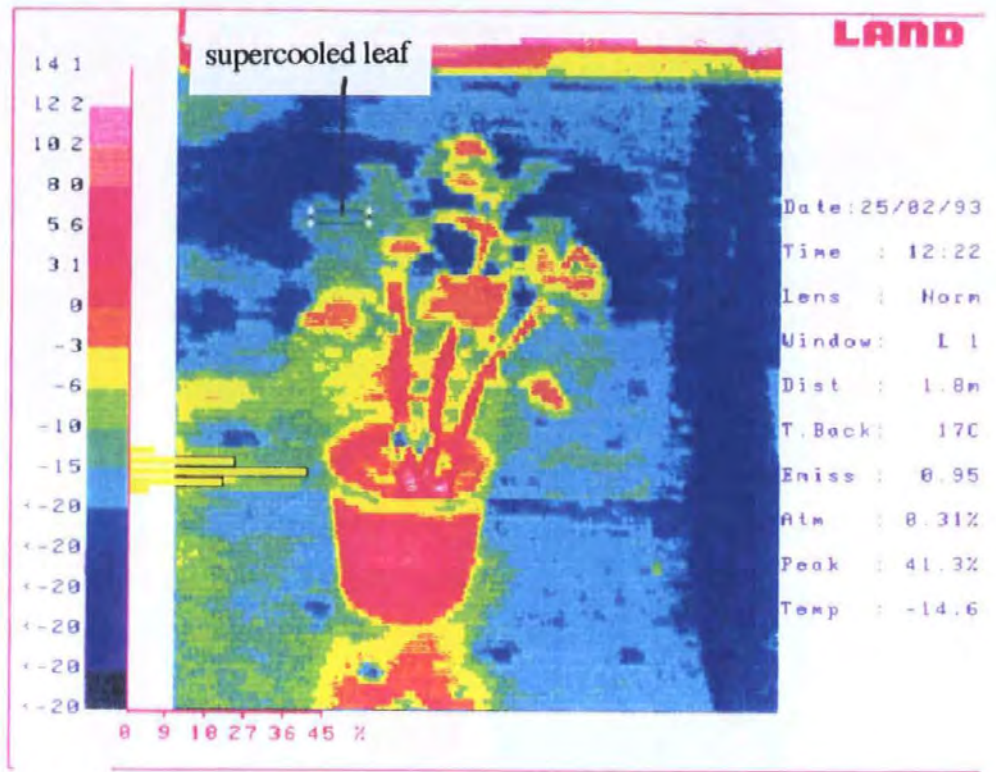
#### Thermal Imaging of *S. tuberosum* var. Jersey Royal plants

Plates i a,b and c show the progression of a freeze event through a portion of dry foliage using the Cyclops TI35. As the ice boundary progressed the colour changed on the image. Plates ii a,b and c show the same equipment monitoring a freeze event in a dew treated plant. The ice boundary was less distinct, and in this case its advance was only visible by studying the video recording. However, the image processing executed on the stills and the histogram of the temperature distribution (left hand side of images) indicated that the distribution had become warmer, consistent with the release of the latent heat of fusion of ice. Both plants upon rewarming showed complete frost damage.

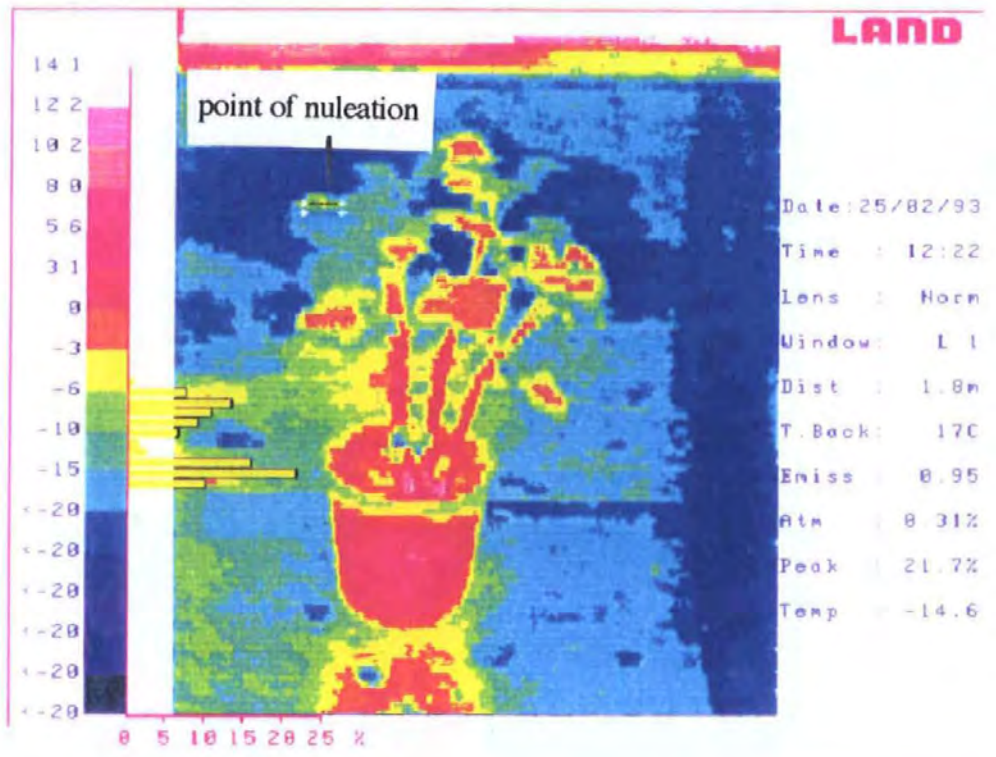
Plates iii a,b,c and d show images taken of individual leaves of a dry *S. tuberosum* var. Jersey Royal plant using the Agema Thermovision 487. Figures iv a,b,c and d are images of the dew treated plant. Freezing was being initiated at warmer temperatures in the dew treated plant. Furthermore, nucleation was incited at three points upon the leaf and initiated three frontiers of ice travelling through the leaf, whereas ice nucleation occurred at only one point on the dry leaf before spreading through the remaining tissue. The temperature trace of these freezing events can be seen in figure 2, which were recorded simultaneously. All plants frozen died on thawing after removal from the frost chamber.

## Thermal Imaging of cauliflower curd.

Plates v a, b and c show the progression of the ice frontier through the cauliflower curd. On removal from the chamber the curd was frozen and was hard to the touch and had the characteristic 'glassy' appearance. Upon rewarming and maintenance at room temperature for 48 hrs the curd showed only slight signs of frost damage.

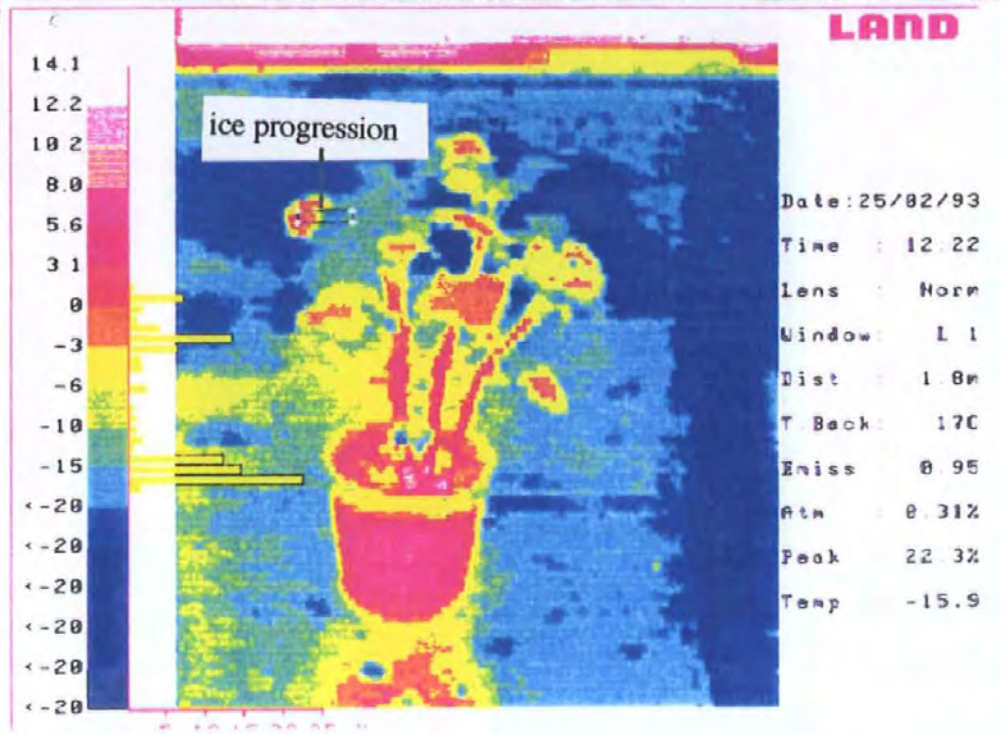


a

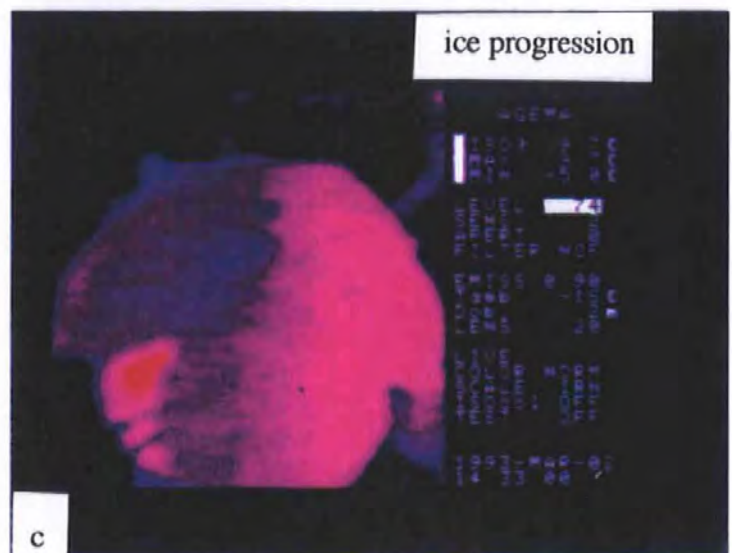


b

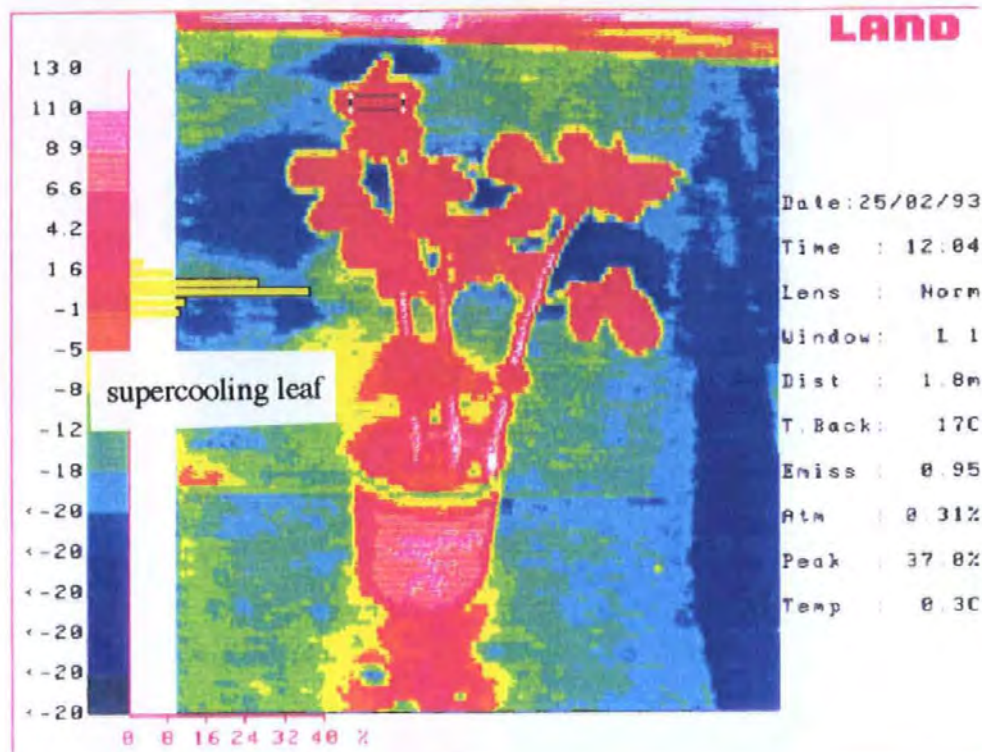
i a, b and c. Progression of freezing event through dry foliage using Cyclops TI35.



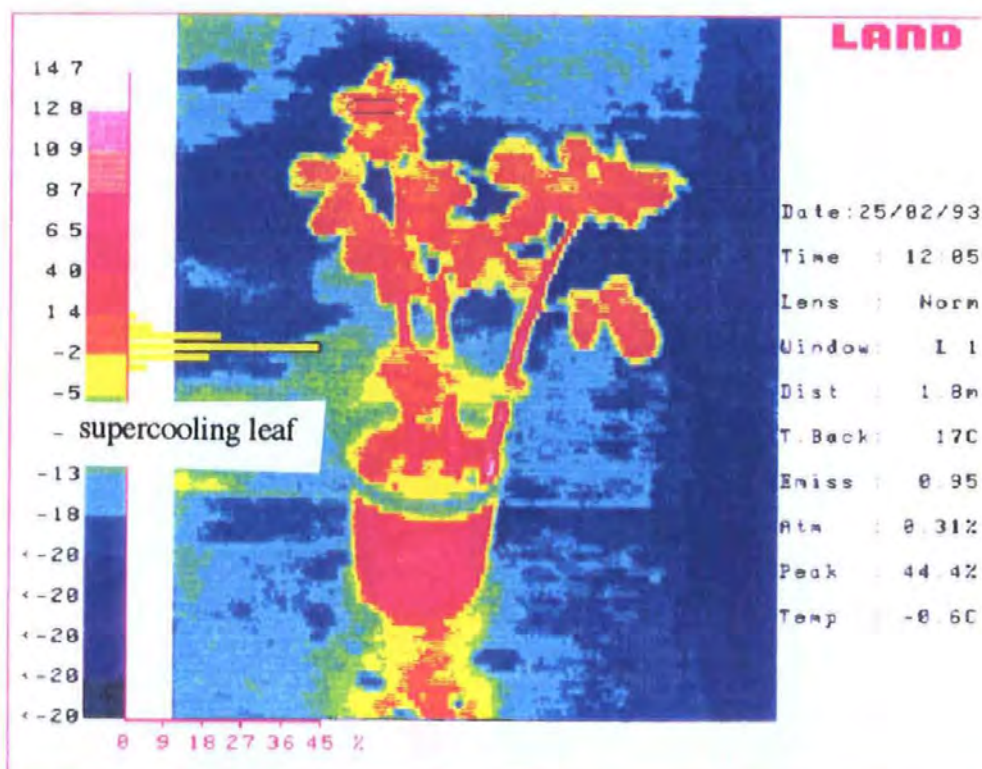
C



iii a, b and c) Close-up thermal images of freezing in dry foliage using Agema Thermovision 487.



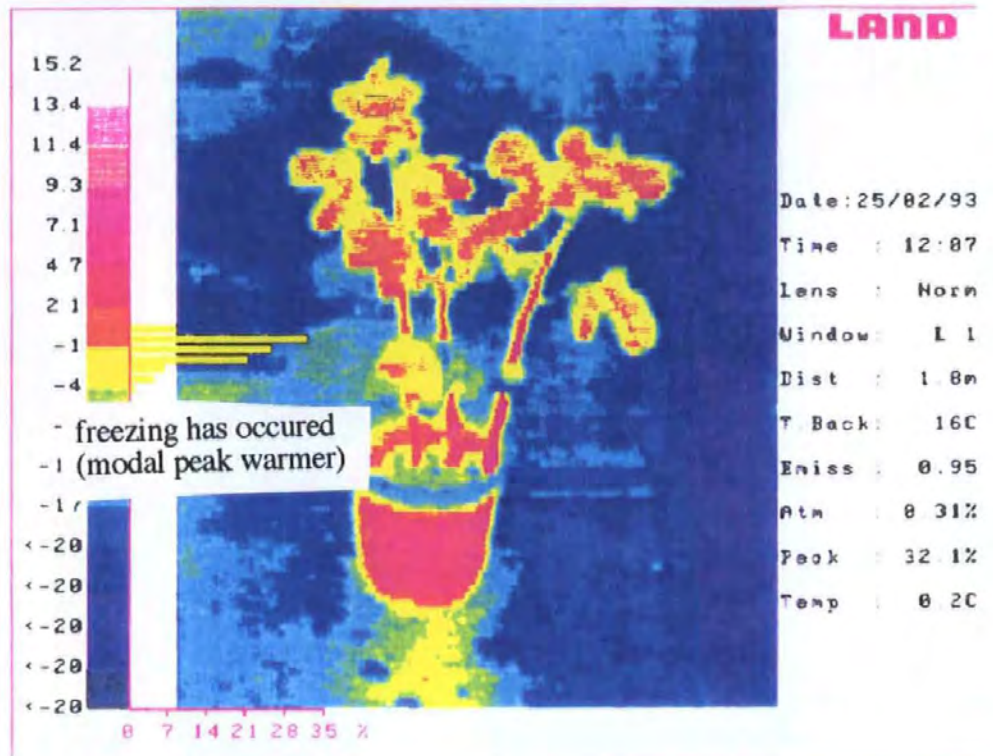
a



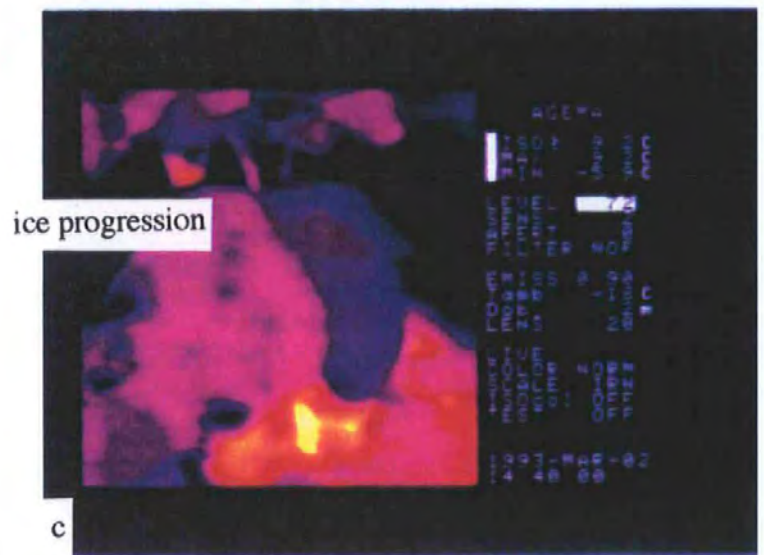
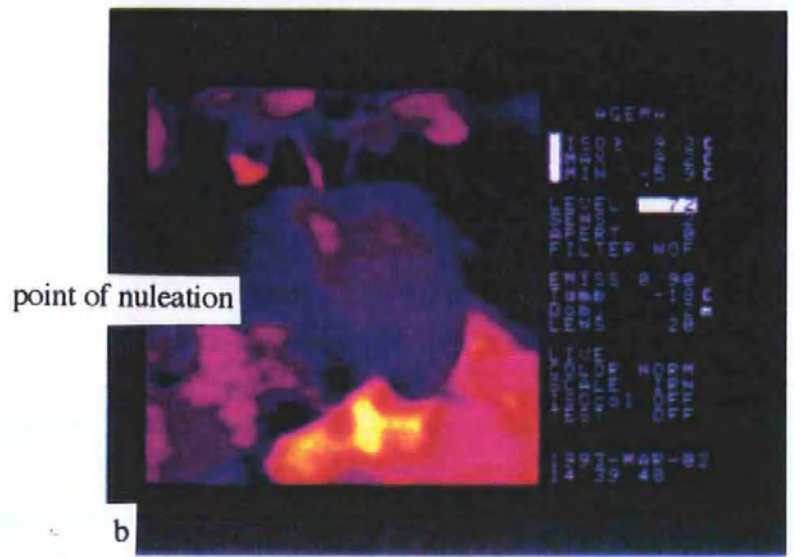
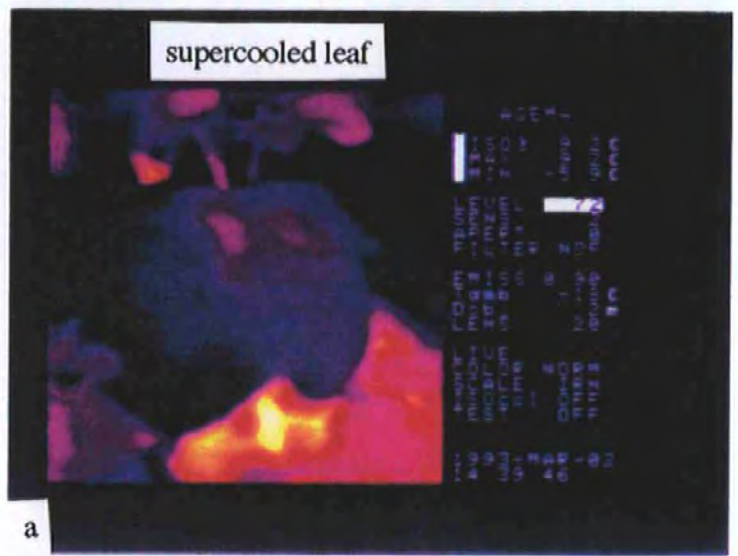
b

ii a, b and c. Progression of freezing event through dew treated foliage using Cyclops TI35.

Point of nucleation not visible.  
 Freezing can be inferred from the temperature distribution histograms.



c

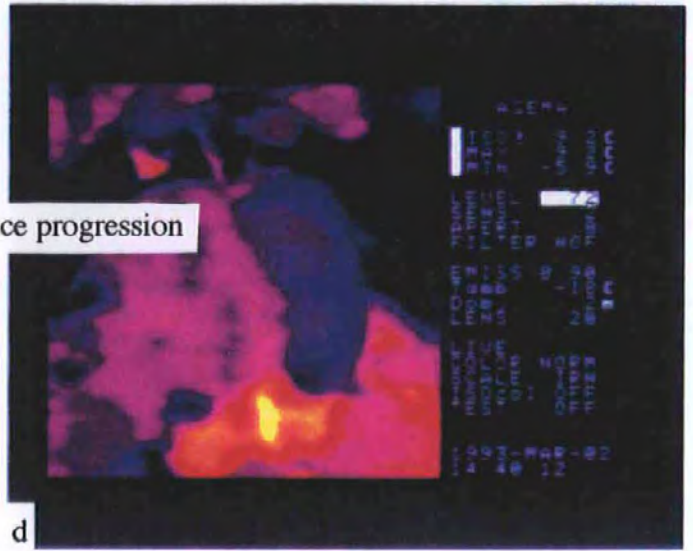


iv a, b, c, d and e)

Close up thermal images of freezing in dew treated folia using Agema Thermovision 487.



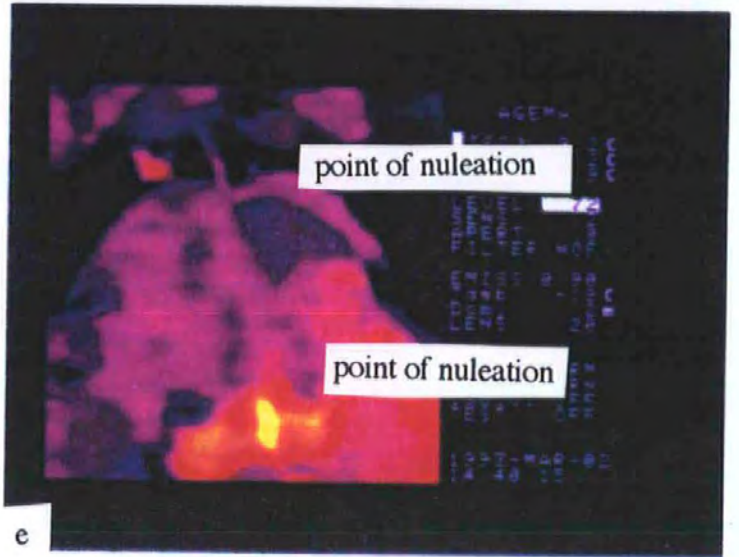
ice progression



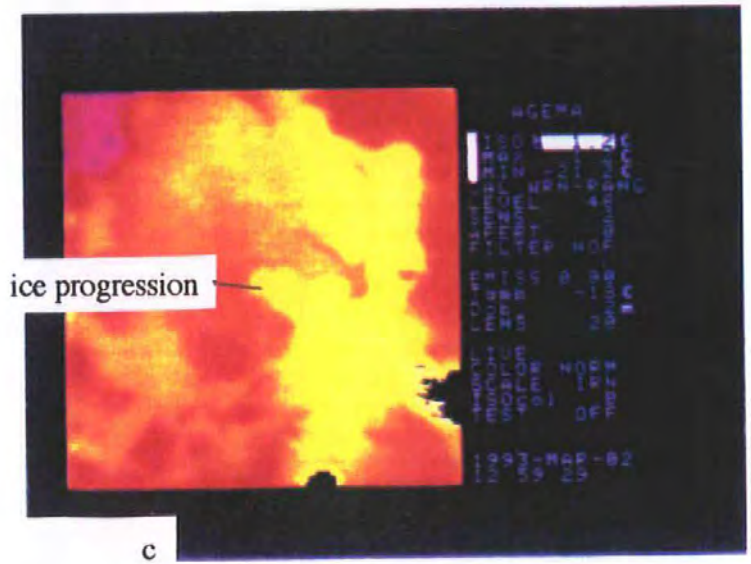
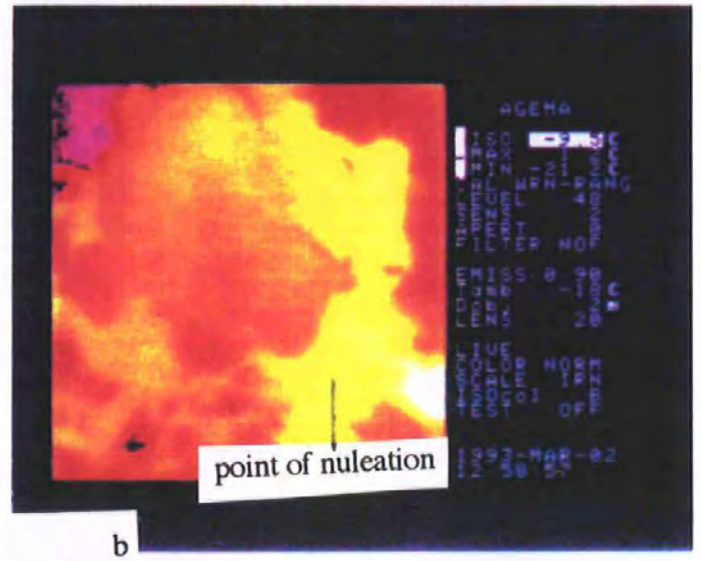
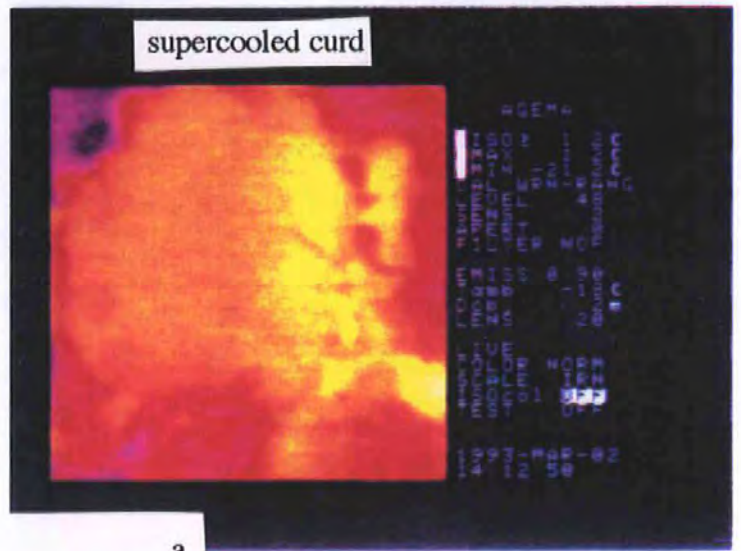
d

point of nucleation

point of nucleation



e



v a, b and c. Thermal image of a cauliflower curd using Agei Thermovision 487.

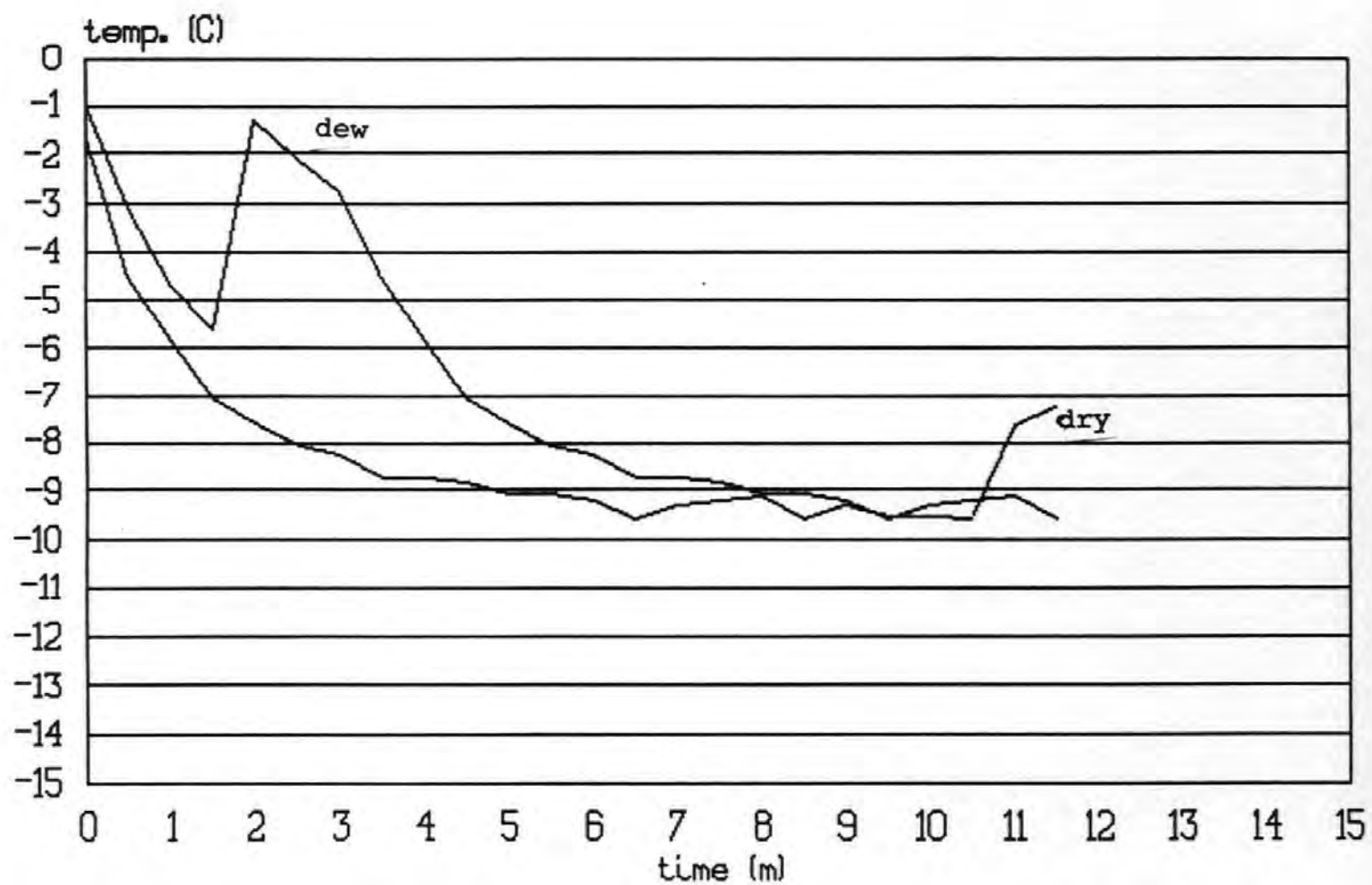


figure .2 Comparison of exotherm temperatures of dew treated and dry plants.

#### A.4. Discussion.

##### Plant Thermal imaging.

This series of experiments corroborate the earlier trials of the present study which indicate that leaf surface water variables are reducing the supercooling ability of the *S. tuberosum* var. Jersey Royal. It is possible to see from both the Agema images, the Land images and the temperature trace recorded using thermocouples attached to the data logger that dew treated plants froze at warmer temperatures than dry plants (dew treated plant  $-5^{\circ}\text{C}$ , dry plant  $-9^{\circ}\text{C}$ ). These nucleation temperatures were, however, noticeably cooler than nucleation temperatures of plants treated similarly but frozen in the environmental test chamber. This was due to the rapid cooling rates of the chest deep freeze used as the test chamber (circa  $4^{\circ}\text{C min}^{-1}$ ) faster rates of cooling typically enhanced supercooling (Rajashekar *et al* 1983).

It was postulated earlier that this warmer freezing must be allied to more numerous warm temperature ice nuclei. The results of this investigation support this in that it is possible to see more nucleation sites initiating freezing at warmer temperatures in the dew treated plants. The historical and single point nature of thermocouple thermometry does not allow this 'counting' of nucleation sites, as demonstrated in the exotherm traces recorded in this trial. This is because the only exotherm a thermocouple can record is the first ice frontier which travels over it. Even if the thermocouple happens to be placed on an initial nucleation site it is not possible to differentiate this from the passing of an ice

frontier as the exotherm response, studied historically, is identical. If many thermocouples are placed on one plant or piece of test tissue it is still not possible to precisely differentiate between site of nucleation, or the passing of an ice frontier, or to count nucleation sites. The ability to view freezing in real time, and spot sites of nucleation demonstrates the potential usefulness of thermal imaging to the plant scientist.

The freezing of the cauliflower curd demonstrated an exciting area of development in which thermal imaging could be important. The fact that the freezing was viewed in real time allowed the curd to be removed from the chamber immediately subsequent to freezing. However, the curd remained undamaged after thawing. This experiment effectively demonstrated the difference between nucleation temperature and frost kill temperature, and the potential ease with which this difference could be studied through employing thermal imaging. It is possible that plant species that have been difficult to categorise in terms of their frost sensitivity could be re-examined effectively using thermal imaging as an investigative tool.

System summary:-

Agema:- Good resolution, exotherms visible. Appear much better in close up work, however further experimentation may improve whole plant imaging. Useful 'on image' notation, especially time flags measured to the nearest second.

Colour scaling, modification, intensities are altered upon the camera during experimentation. Therefore initial camera set up is more difficult, as the video storage is the final image, and

reflects camera colour settings. This is a disadvantage as interesting events cannot be modified or enhanced using the software subsequent to filming.

Land:- Poorer resolution, yet exotherms visible. Clearer on screen notation, yet very poor time flags, resolved only to the nearest minute. A major advantage is the capability to alter the colour ranges of the recorded image, thus allowing clarification of freeze events. The ability to print images from the retrieval software onto standard bubble jet printers is also an advantage, as the images are larger than the photographic retrieval available from the Agema system.

These systems are comparable in terms of price and results. The capital outlay is considerable, (circa £20,000 to £30,000), but they are both tailored to industrial usage and as such facilities required by the plant scientist to allow the formulation of a rigorous methodology are perhaps lacking. A full demonstration of each lens type and software configuration would be necessary not just to decide which system is most suitable to the individual scientist and experimental objectives, but perhaps more importantly, which system matches the chosen freezing facility. Interestingly Agema produce a system dedicated to the requirements of the scientist, but the cost is appreciably more (Agema Thermovision 900 Series, plus data storage circa £62,000), and was not investigated in this trial.

## APPENDIX 2. Example of exotherm data collection and analysis.

Data was collected as described in Chapter 2. This appendix shows the format of the data produced by the data logger, and the way in which it was possible to note the temperature of exotherm release.

Figure 1 shows a portion of a typical data file subsequent to conversion of primary binary data (as collected by the data logger) into a comma separated variable (.CSV or .DAT) file readable by PC spreadsheet programmes.

Column TM1 represents the temperature of the on board reference thermistor of the data logger against which the temperature output of the thermocouples (columns headed TCT) is measured.

It is possible to see in the rows time flagged as 17/02/94 10:51 and 17/02/94 10:52 of column TCT 3 a temperature rise of  $-1.15\text{ }^{\circ}\text{C}$  to  $-.29\text{ }^{\circ}\text{C}$  is recorded in that 30s interval. Data files were so large as to preclude storage as hard copies. The .CSV files were studied on computer only. All files were stored on hard and floppy disc as both binary and .CSV files.

The temperature rise noted in figure 1 can be seen as a graph in figure 2 which confirms the temperature rise as an exotherm. The temperature rise is a significant deviation from the fluctuation of the cabinet temperature. The exotherm temperature recorded in this case was  $-1.15\text{ }^{\circ}\text{C}$ . This figure was then corrected using the measured offset for the particular thermocouple giving a temperature which could then be entered into a statistical analysis with all the other temperatures recorded in this manner for the particular experiment.

All nucleation temperatures were analysed using Solo, a statistical analysis package.

An example of an analysis of variance report is shown in figure 3. If analysis of variance suggested that the applied treatments were significantly affecting recorded nucleation temperatures Fishers LSD test was employed to differentiate between treatment nucleation temperatures. An example of a Fishers LSD report is shown in figure 4. Following analysis all temperatures were quoted to one decimal place.



Table 1 Example of Comma Separated Variable (.CSV) data file retrieved from data logger.

DELTA LOGGER				
17/02/94 10:44				
17/02/94 11:27				
TIMED				
Channel number	15	1	2	3
Sensor type	15	TM1	TCT	TCT
Label	15			
Unit	15	deg C	deg C	deg C
Minimum Value	15	14.62	-8.31	-2.15
Maximum Value	15	15.43	0.28	4.75
17/02/94 10:44	0	15.43	0.05	4.75
17/02/94 10:44	1	15.4	0.28	4.14
17/02/94 10:45	2	15.38	0	3.65
17/02/94 10:45	3	15.33	-0.47	3.01
17/02/94 10:46	4	15.31	-0.94	2.48
17/02/94 10:46	5	15.29	-1.34	1.94
17/02/94 10:47	6	15.26	-1.75	1.43
17/02/94 10:47	7	15.24	-2.02	0.94
17/02/94 10:48	8	15.22	-2.54	0.48
17/02/94 10:48	9	15.19	-2.64	0.02
17/02/94 10:49	10	15.19	-2.02	-0.21
17/02/94 10:49	11	15.17	-1.7	-0.4
17/02/94 10:50	12	15.14	-2.25	-0.68
17/02/94 10:50	13	15.12	-2.54	-1.08
17/02/94 10:51	14	15.1	-1.99	-1.15
17/02/94 10:51	15	15.1	-1.75	-1.15
17/02/94 10:52	16	15.07	-2.28	-0.29
17/02/94 10:52	17	15.05	-2.56	-0.47
17/02/94 10:53	18	15.03	-1.99	-0.53
17/02/94 10:53	19	15	-1.68	-0.61
17/02/94 10:54	20	15	-2.25	-0.68
17/02/94 10:54	21	14.98	-3.69	-0.89
17/02/94 10:55	22	14.96	-4.73	-1.13
17/02/94 10:55	23	14.96	-5.57	-1.26
17/02/94 10:56	24	14.94	-4.78	-1.28
17/02/94 10:56	25	14.92	-3.95	-1.23
17/02/94 10:57	26	14.92	-4.73	-1.28
17/02/94 10:57	27	14.92	-5.36	-1.36
17/02/94 10:58	28	14.9	-4.6	-1.31
17/02/94 10:58	29	14.9	-4.08	-1.23
17/02/94 10:59	30	14.88	-4.91	-1.34
17/02/94 10:59	31	14.88	-5.17	-1.36
17/02/94 11:00	32	14.86	-4.52	-1.34
17/02/94 11:00	33	14.86	-4.08	-1.28
17/02/94 11:01	34	14.84	-5.12	-1.36
17/02/94 11:01	35	14.84	-4.91	-1.41
17/02/94 11:02	36	14.82	-4.42	-1.39
17/02/94 11:02	37	14.82	-4.23	-1.31
17/02/94 11:03	38	14.8	-5.33	-1.41
17/02/94 11:03	39	14.8	-4.89	-1.41
17/02/94 11:04	40	14.78	-4.31	-1.39
17/02/94 11:04	41	14.78	-4.34	-1.34
17/02/94 11:05	42	14.78	-5.41	-1.47
17/02/94 11:05	43	14.76	-6.17	-1.55

Fig. 1 Graph of exotherm taken from data file (fig. 1.)

Axis Label

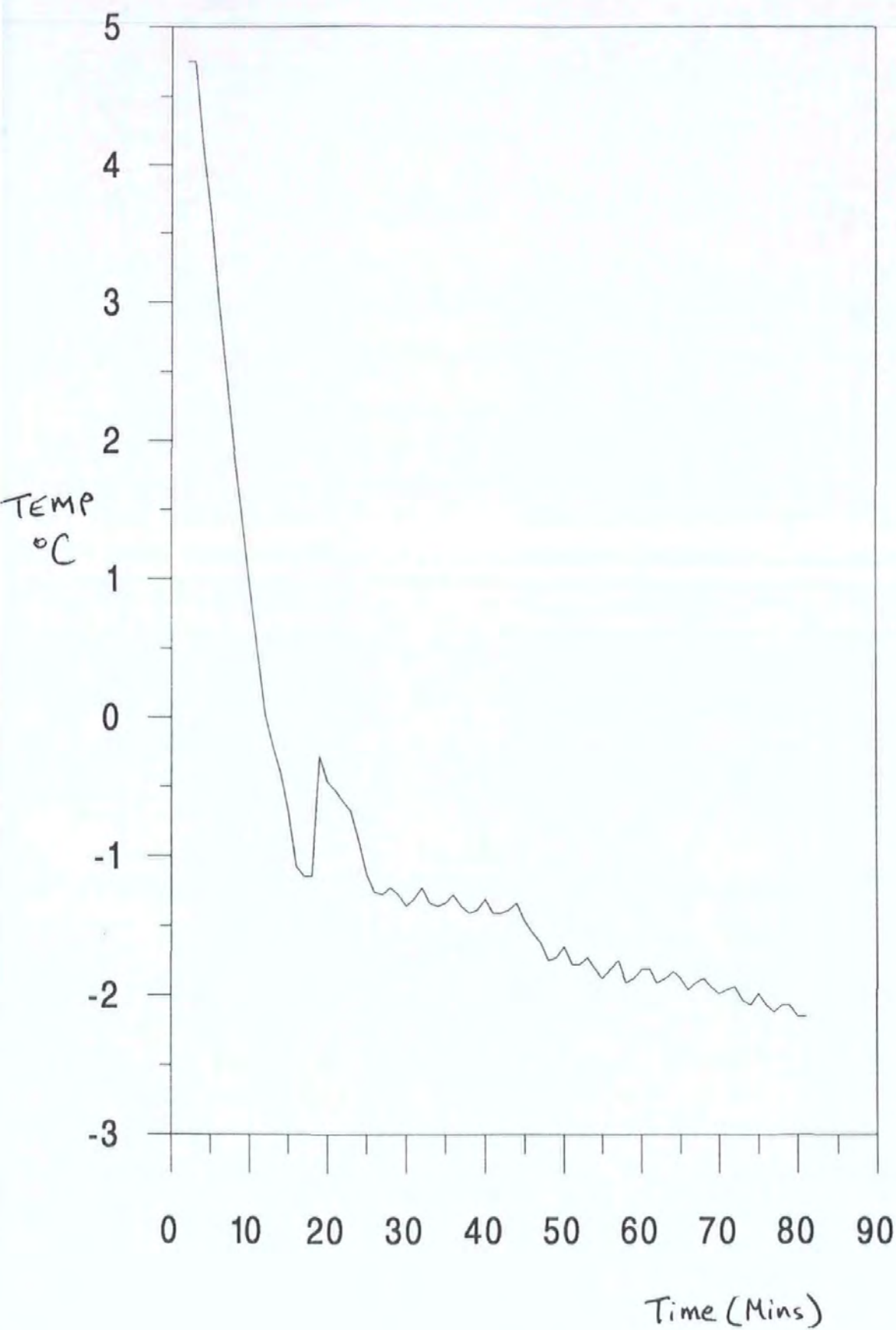


Table 3 Example analysis of variance report subsequent to collection of nucleation temperatures.

Unweighted Means ANOVA (Analysis of Variance Report)

---C:\solo\exp92\LEAF

---

ANOVA Table for Response Variable: EXOTEMP.

Source	DF	Sum-Squares	Mean Square	F-Ratio	Prob>F	Error Term
A (DEWLEVL )	2	1916.8	958.4001	132.56	0.0000	ERROR
B (	2	52.76381	26.3819	3.65	0.0281	ERROR
AB	4	75.21495	18.80374	2.60	0.0379	ERROR
ERROR	170	1229.044	7.229671			
TOTAL(Adj)	178	3235.366				

Table 2 Example of Fisher's LSD report subsequent to ANOVA.

—  
Fisher's LSD Comparison Report

Response Variable: EXOTEMP. Factor(A,DEWLEVL) Error Term: ERROR

Summary Results  $\alpha = .1$  Level Codes

Code(Level)	Mean	ABC
A(1)	-9.985	..S
B(2)	-9.844426	..S
C(3)	-2.993621	SS.

—  
Fisher's LSD Comparison Report

Response Variable: EXOTEMP. Factor(B, .) Error Term: ERROR

Summary Results  $\alpha = .1$  Level Codes

Code(Level)	Mean	ABC
A(1)	-8.369	.SS
B(3)	-7.444746	S..
C(2)	-7.197667	S..

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