

SCREENING MALUS SEEDLINGS

FOR COLD RESISTANCE

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Dedicated to the memory of my Mom
Mary Angela (Larkin) Mathers
June 28, 1920 to June 18, 1988
an excellent mother and friend
who is missed more than words and tears can tell

and to my Dad
a man of strength, character, and determination
who has always encouraged me
to work with diligence and perseverance

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ABSTRACT

Cold stress is the most important factor limiting the northern expansion of *Malus* cultivation, and breeding for improved cold resistance is time-consuming and laborious. Members in the genus *Malus* are characteristically heterozygous and reveal a high variability in the hardiness of their seedlings. This study was conducted to develop an appropriate acclimation routine to identify cold hardy transgressive segregants from *Malus* seedling populations. The effects of frosts (16 hours at -3°C) and lag-times (10 and 11 days at $+3$ to $+5^{\circ}\text{C}$), short (8 hour) day and cool ($+3$ to $+5^{\circ}\text{C}$) temperature exposures, and different screening temperatures (-20 , -30 , and -40°C) were investigated on seedlings grown in a greenhouse from open-pollinated Golden Delicious (*Malus pumila*), Antonovka (*Malus baccata*) X (*Malus pumila*) and Rescue Crab (*Malus baccata*) X (*Malus pumila*).

Differentiation in hardiness response of the seedling populations was not achieved until after exposure to short days at cool temperatures for six weeks. Further population differentiation was achieved by exposure to one or more frosts compared to no frost exposures. Rescue gave a 74% increase in survival, Antonovka a 62% increase, and Golden Delicious a 51% increase, when compared to survival with no frosts.

After the acclimation response had been initiated by exposure to short days at cool temperatures, up to 11 days in the same conditions caused no significant decrease in hardiness. Additionally, no significant decrease in survival was observed in seedlings held at cool temperatures and short days for up to 10 days after a frost exposure.

Hardiness levels of acclimated and non-acclimated seedlings agreed with known inherent hardiness responses for all three cultivars evaluated. Cultivar seedling response to different freeze temperatures was pronounced. For crosses representing a full range of cold hardiness capabilities, a screening temperature close to -30°C was found to be most effective. The response of the different cultivar seedlings to the three different freeze temperatures indicated that the screen could be tailored to fit the minimum survival requirements of a particular region.

A binomial form of regrowth data collection, percent seedling survival, was determined to be the most efficient and precise measure of evaluation. The controlled three-step freeze procedure and thawing rate that was developed, will facilitate rapid, repeatable screening of large numbers of progeny. An examination of replication means revealed that the probability of survival was influenced by when the seedlings grew in the greenhouse. It is recommended that, for subsequent screens, all the material should be grown at the same time.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
TABLE OF CONTENTS.....	vi
LIST OF TABLES.....	x
LIST OF FIGURES.....	xii
1. INTRODUCTION.....	1
2. REVIEW OF THE LITERATURE.....	4
2.0 Fruit production in northern areas: problems of low temperature stress.....	4
2.0.0 Approach to selection methodology.....	4
2.0.1 Seedling research: prospects of mass selection.....	5
2.1 Freeze resistance: avoidance or tolerance.....	8
2.1.0 Improper conceptualization.....	11
2.2 Winter dormancy.....	12
2.2.0 Dormancy terminology.....	13
2.2.1 Stages of dormancy.....	13
2.3 Environmental factors influencing cold acclimation.....	17
2.3.0 Photoperiod.....	17
2.3.0.0 Translocatable cold hardiness promoter.....	17
2.3.0.1 Changes in growth regulator balance.....	18
2.3.0.2 Quantity or quality of light.....	20
2.3.1 Temperature.....	21
2.3.2 Moisture.....	22
2.3.3 Nutrition.....	23

2.4	Internal physiological changes.....	24
2.4.0	Changes to the plasma membrane.....	25
3.	MATERIALS AND METHODS.....	29
3.0	Controlled acclimation experiment.....	29
3.0.0	Experimental design.....	29
3.0.1	Cultivar seedlings studied, seed collection and stratification.....	31
3.0.2	Growing conditions and artificial cold acclimation	32
3.0.3	Controlled freeze procedures.....	33
3.0.4	Data collection methods.....	34
3.0.4.0	Percent seedling survival.....	34
3.0.4.1	Percent bud survival.....	35
3.0.4.2	Viable growth score.....	35
3.0.5	Statistical analysis.....	35
3.1	Freeze without acclimation experiment.....	37
3.1.0	Experimental design.....	37
3.1.1	Growing conditions.....	38
3.1.2	Controlled freeze procedures.....	38
3.1.3	Data collection methods.....	40
3.1.4	Statistical analysis.....	40
3.2	Cool temperature duration experiment.....	41
3.2.0	Experimental design.....	41
3.2.1	Growing conditions and artificial cold acclimation.....	43
3.2.2	Data collection methods.....	43
3.2.3	Statistical analysis.....	44
4.	RESULTS.....	46

4.0	Controlled acclimation experiment.....	46
4.0.0	Data collection methods.....	46
4.0.0.0	Percent seedling survival data.....	46
4.0.0.1	Percent bud survival data.....	46
4.0.0.2	Viable growth score.....	49
4.0.0.3	Transformation of data.....	49
4.0.0.4	Optimum method of data presentation.....	55
4.0.1	Cultivar seedling response.....	55
4.0.2	Frost requirement.....	59
4.0.3	Screening temperature.....	61
4.0.4	Replication significance.....	62
4.1	Freeze without acclimation experiment.....	63
4.1.0	Data collection methods.....	63
4.1.0.0	Percent seedling survival data.....	63
4.1.0.1	Percent bud survival data.....	63
4.1.0.2	Viable growth score.....	65
4.1.0.3	Transformation of data.....	70
4.1.0.4	Optimum method of data presentation.....	70
4.1.1	Cultivar seedling response.....	70
4.1.2	Replication significance.....	74
4.2	Cool temperature duration experiment.....	74
4.2.0	Data collection methods.....	74
4.2.0.0	Percent seedling survival data.....	76
4.2.0.1	Percent bud survival data.....	76
4.2.0.2	Viable growth score.....	81
4.2.0.3	Transformation of data.....	86

4.2.0.4	Optimum method of data presentation.....	86
4.2.1	Cultivar seedling response.....	90
4.2.2	Frost requirement.....	90
4.2.3	Replication significance.....	96
5.	DISCUSSION.....	98
5.0	Cultivar seedling response.....	98
5.0.0	Non-acclimated seedlings.....	98
5.0.1	Acclimated seedlings.....	99
5.1	Frost requirement.....	101
5.1.0	Response to frost.....	101
5.1.1	Relationship of frost response to cultivar seedlings.....	101
5.1.2	Evaluation of increased time in cooler after frost exposure.....	102
5.2	Optimum screening temperature.....	103
5.2.0	Inherent cultivar seedling hardiness.....	103
5.2.1	Dependence on acclimation.....	104
5.3	System of data collection.....	105
5.4	Replication significance.....	105
6.	CONCLUSIONS.....	108
7.	REFERENCES.....	111

LIST OF TABLES

Table 3.1.	Outline of analysis of variance for the controlled acclimation experiment.....	36
Table 3.2.	Outline of analysis of variance for the greenhouse experiment.....	42
Table 3.3.	Outline of analysis of variance for the cooler time experiment.....	45
Table 4.1.	Analysis of variance for the controlled acclimation experiment using percent seedling survival data.....	47
Table 4.2.	Analysis of variance for the controlled acclimation experiment using percent bud survival data.....	48
Table 4.3.	Analysis of variance for the controlled acclimation experiment using viable growth score data.....	52
Table 4.4.	Analysis of variance of the arc sine of square root transformed percent seedling survival data (expressed in radians) for the controlled acclimation experiment.....	54
Table 4.5.	Analysis of variance of the arc sine of square root transformed percent bud survival data (expressed in radians) for the controlled acclimation experiment.....	56
Table 4.6.	Analysis of variance of the arc sine of square root transformed viable growth score data (expressed in radians) for the controlled acclimation experiment.....	57
Table 4.7.	Analysis of variance for the freeze without acclimation experiment using percent seedling survival data.....	64
Table 4.8.	Analysis of variance for the freeze without acclimation experiment using percent bud survival data.....	66
Table 4.9.	Analysis of variance of the arc sine of square root transformed percent seedling survival data (expressed in radians) for the freeze without acclimation experiment.....	69
Table 4.10.	Analysis of variance for the freeze without acclimation experiment using viable growth score data.....	71

Table 4.11.	Analysis of variance of the arc sine of square root transformed percent bud survival data (expressed in radians) for the freeze without acclimation experiment.....	72
Table 4.12.	Analysis of variance of the arc sine of square root transformed viable growth score data (expressed in radians) for the freeze without acclimation experiment.....	73
Table 4.13.	Analysis of variance of the cool temperature duration experiment using percent seedling survival data.....	77
Table 4.14.	Analysis of variance of the cool temperature duration experiment using percent bud survival data.....	78
Table 4.15.	Analysis of variance of the cool temperature duration experiment using viable growth score data.....	82
Table 4.16.	Analysis of variance of the arc sine of square root transformed percent seedling survival data (expressed in radians) for the cool temperature duration experiment.....	87
Table 4.17.	Analysis of variance of the arc sine of square root transformed percent bud survival data (expressed in radians) for the cool temperature duration experiment.....	88
Table 4.18.	Analysis of variance of the arc sine of square root transformed viable growth score data (expressed in radians) for the cool temperature duration experiment.....	89

LIST OF FIGURES

- Figure 2.1. Diagram of degree growth stage ($^{\circ}$ GS) model for identifying ontogenetic stages of development (A), point events (B), and hardiness (C) of vegetative buds of temperate woody plants.....15
- Figure 3.1. Arrangement in the tray of 18 seedlings, per cultivar seedling population, for the controlled acclimation experiment. The three populations (R) Rescue, (A) Antonovka, and (G) Golden Delicious were randomly ordered in the tray.....30
- Figure 3.2. Arrangement in the tray of 10 seedlings, per cultivar seedling population, for the freeze without acclimation experiment. The three populations (R) Rescue, (A) Antonovka, and (G) Golden Delicious were randomly ordered in the tray.....39
- Figure 4.1. Temperature X frost interaction of percent seedling survival data of the controlled acclimation experiment, sampled from three apple cultivar seedling populations.....50
- Figure 4.2. Temperature X frost interaction of percent bud survival data of the controlled acclimation experiment, sampled from three apple cultivar seedling populations.....51
- Figure 4.3. Temperature X frost interaction of viable growth score data of the controlled acclimation experiment, sampled from three apple cultivar seedling populations.....53
- Figure 4.4. Cultivar seedlings X temperature interaction of percent seedling survival data of the controlled acclimation experiment, sampled from six frost treatments.....58
- Figure 4.5. Cultivar seedlings X frost interaction of percent seedling survival data of the controlled acclimation experiment, sampled from three freeze temperatures.....60
- Figure 4.6. Replication means for the controlled acclimation experiment. The first value represents the months of the year grown in the greenhouse. The second value (in brackets) represents the months of the year regrown in the greenhouse.....62

- Figure 4.7. Cultivar seedlings X temperature interaction of percent seedling survival data of the greenhouse experiment.....67
- Figure 4.8. Cultivar seedlings X temperature interaction of percent bud survival data of the greenhouse experiment.....68
- Figure 4.9 Replication means for the freeze without acclimation experiment. The first value listed represents the months of the year grown in the greenhouse. The second value (in brackets) represents the months of the year regrown in the greenhouse.....75
- Figure 4.10. Temperature X frost interaction of percent bud survival data of the cool temperature duration experiment, sampled from three apple cultivar seedling populations.....79
- Figure 4.11. Temperature X frost interaction of percent seedling survival data of the cooler time experiment, sampled from three apple cultivar seedling populations.....80
- Figure 4.12. Temperature X frost interaction of viable growth score data of the cool temperature duration experiment, sampled from three apple cultivar seedling populations.....83
- Figure 4.13. Cultivar seedlings X temperature interaction of percent seedling survival data of the cooler time experiment, sampled from two frost treatments.....84
- Figure 4.14. Cultivar seedlings X temperature interaction of viable growth score data of the cool temperature duration experiment, sampled from two frost treatments.....85
- Figure 4.15. Cultivar seedlings X frost interaction of percent seedling survival data of the cool temperature duration experiment, sampled over three freeze temperatures.....92
- Figure 4.16. A comparison of frost response with Rescue between the controlled acclimation experiment and the cool temperature duration experiment, for the percent seedling survival data.....93
- Figure 4.17. A comparison of frost response with Antonovka between the controlled acclimation experiment and the cool temperature duration experiment, for the percent seedling survival data.....94

- Figure 4.18. A comparison of frost response with Golden Delicious between the controlled acclimation experiment and the cool temperature duration experiment, for the percent seedling survival data.....95
- Figure 4.19. Replication means for the cool temperature duration experiment. The first value represents the months of the year grown in the greenhouse. The second value (in brackets) represents the months of the year regrown in the greenhouse.....97

1. INTRODUCTION

Woody plants of the temperate zone have the ability to acclimate to the cold. Cold acclimation involves a series of phenomena which enable a tree to increase its ability to survive low temperatures. The inability to withstand extreme cold has been one of the major factors limiting the distribution and cultivation of *Malus*.

Two important factors which have inhibited progress in breeding for improved cold resistance are the lack of a simple, reproducible seedling screening method to facilitate the evaluation of large numbers of progeny, and the poor understanding of the genetic control of cold hardiness.

Stushnoff et al. (1985) reported that young apple seedlings could be hardened by manipulating temperature and day length at very early stages of growth and development. The hardening allowed for a screening for cold resistance using a controlled freezing process (Stushnoff et al. 1985). This approach has the potential to enhance the scope of breeding some plants for cold resistance by saving time and space, and by providing an opportunity to study the breeding behaviour with adequate sized populations under controlled and easily repeated conditions.

At the whole plant level, resistance to cold is a complex quantitatively inherited trait or is a character under the control of many genes (Stushnoff and Quamme 1983). It is greatly influenced by the environment, that is, it has low heritability (Cummins and Aldwinckle 1983). Because cold resistance is multigenic, one problem faced by the fruit breeder is the vast number of ways in which the

genes can combine. The possible number of gene recombinations for even a small number of genes is astronomical. This means that the breeder must be able to screen very large numbers of progeny in order to select the individual with the optimum combination of genes (Allard 1960). The seedling screen technique facilitates the identification of such hardy transgressive segregants (Stushnoff et al. 1985).

A seedling screen procedure could allow the breeder to conduct genetic studies under controlled environment conditions on large populations (Stushnoff and Quamme 1983). Appropriate crosses could be made to obtain a better understanding of the mode of inheritance. By studying the effects of frosts and lag times it is possible to determine specific gene combinations for different types of acclimation within different species and genera (Stushnoff et al. 1985).

The primary objective of this study was to determine an appropriate controlled acclimation routine to be used in a seedling screen by investigating the influence of short days at cool temperatures and repeated exposures to temperatures below freezing. The secondary objectives of this study were to determine the optimum temperatures at which to screen for a specific geographic region and to develop an appropriate test and measure of viability to evaluate the screen. To meet these objectives, this experiment followed six of the steps proposed by Stushnoff and Quamme (1983) in breeding for cold hardiness in fruit crops: 1) identify the major cold injury problems for the specific region; 2) determine the nature and extent of some of the environmental controls on acclimation and deacclimation; 3) develop an appropriate preconditioning or handling routine for standardized

testing; 4) incorporate a controlled freezing procedure and standardize freezing and thawing rates for testing purposes; 5) develop an appropriate viability test using standard cultivar seedling populations for comparative purposes; and 6) utilize screening at the juvenile seedling stage to facilitate rapid progress in the breeding program.

2. REVIEW OF THE LITERATURE

2.0 Fruit production in northern areas: problems of low temperature stress

Urban development has gradually encroached on Canada's most productive fruit growing regions. As a result, there is an increasing pressure to shift the location of fruit production to what had been previously considered marginal regions. These marginal regions are often well beyond the range of optimum adaptation (Stushnoff and Quamme 1983). In order to ensure that fruit crops survive and produce in these new environments, breeding and selection for improved adaptation and stress resistance has received attention in most fruit breeding programs.

2.00 Approach to selection methodology

In 1970 Schmadlak introduced the concept that "Selection should begin with those characteristics from which the most rapid and most extensive reduction of the available plant material may be anticipated" (Cummins and Aldwinckle 1983). In breeding fruit crops for northern climates this character is freeze resistance. Two principal types of selection are required in breeding fruit trees for cold resistance: 1) screening of those individuals in a segregating population which exhibit the desired level of resistance; 2) a selection for desirable combinations of pomological traits among that portion of the population

earlier judged to carry the desired level of cold resistance (Dayton et al. 1983). The development of a cold hardiness seedling screening technique eliminates the requirement for several years of evaluation before the second level of selection can begin. With the seedling screening method, the first level of selection can be completed 12 weeks after germination. The development of such a technique can provide very significant advances to the field of tree fruit breeding by eliminating the need for years of maintaining a large acreage of field plantings for seedling evaluations.

2.01 Seedling research: prospects of mass selection

In the seedling screen procedure, the method for detecting superior individuals in the breeding stock is called mass selection. Individuals are selected on the basis of their phenotypic response to cold so that they can be subsequently mated at random *inter se*. Mass selection increases the frequency of favorable alleles in the breeding stock if the effects of the genes influencing the trait of interest are primarily additive (Hansche 1983). Of all breeding methods, mass selection at the seedling level is the most simple, the least expensive and the most time efficient. A hybrid program must generate twice the amount of response per cycle to provide a rate of response equivalent to mass selection (Hansche 1983). At present, little work has been done in the area of seedling cold stress physiology. Some differences in response to controlled acclimation treatments have been discovered and these suggest the possibility of selection procedures specifically

designed for particular environmental conditions (Stushnoff et al. 1985). Selection of the hardiest seedlings can reduce by 90% the number of trees which normally need to be grown under field conditions for at least 10 years. In the past, breeders relied on selecting the best performing individuals from several years of test winter data. The inability to control natural environments restricted the rate of progress and resulted in sporadic unpredictable data which was not repeatable (Stushnoff and Quamme 1983).

The heritability of cold resistance is greatly influenced by the environment: the greater the effects of the environment, the lower the heritability of the trait (Hansche 1983). Thus, considerable motivation is provided for breeders to reduce the effects of environmental variation. The seedling screen technique reduces environmental variation in four distinct ways: 1) by reducing the environmental variation in the rearing of the breeding stock; 2) by improving measurement techniques; 3) by estimating the effects of environment and adjusting measurements accordingly; and, 4) by replicating the measurements (Hansche 1983).

A major deterrent to breeding for cold resistance is the long juvenile period required by many woody plants. The ultimate test for resistance to cold is the response of the adult trees; nevertheless, there is a need to screen seedlings as early as possible to reduce maintenance costs and to increase the rate of progress (Stushnoff et al. 1985). Lapins (1962) noted that the relative ranking of one-year-old apple seedlings was similar to the adult phase, although the seedlings were slightly hardier. Stushnoff et al. (1983) found that

ten-week-old apple seedlings hardened appreciably with an LT_{50} of -17°C for Golden Delicious and -26°C for Dolgo. With the addition of a four week short day (eight hour) treatment, the hardiness of the Dolgo population was not increased. However, there was an appreciable gain in hardiness of the Golden Delicious population (Stushnoff et al. 1985). In Stushnoff et al.'s (1985) study it was determined that two cultivar seedling populations reacted differently to different acclimation regimes. The two populations were divided into two classes. The two classes of seedlings may have resulted from specific gene combinations for adaptation to two different types of acclimation (Stushnoff et al. 1985).

Stushnoff and Sawatzky (unpublished 1986) suggested a methodology for acclimating apple seedlings that could be used in a cold hardiness seedling screen. Holubowicz and Pieniazek (1976) also conducted studies on the acclimation of apple seedlings and found that eight-month-old seedlings acclimated best at temperatures of 0°C . Immediate transfer to temperatures of -5°C and -10°C killed the seedlings.

Fuchigami et al. (1982) derived a model which classified the process of hardening into two stages. The first stage was dependent on short day induction at warm temperatures. The second stage was dependent on exposure to low temperatures near or at 0°C (Fuchigami et al. 1982; Howell and Weiser 1970; Siminovitch 1982). Holubowicz and Pieniazek (1976) supported the acclimation model of Fuchigami. Stushnoff et al. (1985) determined that a period of short days at warm temperatures did not increase the survival of a population of Dolgo seedlings but increased survival in Golden Delicious seedlings. Substantial evidence

supports a three-stage model, of which the third stage is induced by temperatures below freezing (Glerum 1985; Irving and Lanphear 1967; Stushnoff et al. 1985). Different cultivar seedlings fit different acclimation models (Stushnoff et al. 1985).

2.1 Freeze resistance: avoidance or tolerance

According to Levitt's terminology of stress (1972), stress resistance can be subdivided into two major components. Plants are equipped with mechanisms to either avoid or tolerate ice within their tissues. The mechanism by which many temperate fruit crops avoid ice-induced injury in critical tissues is supercooling. Recent research has revealed that supercooling determines the northern growing range of these temperate fruit crops (Burke et al. 1976; Quamme et al. 1982; Burke and Stushnoff 1979). These plants have been shown to have the ability to maintain supercooled cellular water in the xylem ray parenchyma and flower primordia. The lower limit of the liquid state of water is the homogeneous nucleation point, -38°C for pure water and somewhat lower for cellular water because of dissolved solutes (Stushnoff and Quamme 1983). Freezing avoidance occurs in these tissues by supercooling. In deep supercooled tissue, nucleation of the aqueous phase to ice is sudden and lethal when it occurs (Burke and Stushnoff 1979).

Most *Malus* species have the ability to maintain supercooled cellular water in their xylem ray parenchyma. The xylem is the only tissue that acclimates by deep supercooling. The cambium, phloem and

bark all acclimate through dehydration (Hong and Sucoff 1982). In several other woody species, the flower primordia as well as the xylem parenchyma acclimate by supercooling (Andrew and Proebsting 1983; Burke and Stushnoff 1979). Supercooling in tissue can only happen when effective barriers are created to allow for the internal cell solution to remain in its supercooled state (i.e. barriers that prevent external nucleation of the supercooled tissue). Gusta et al. (1983) stated that deep supercooling in the xylem of plants was the result of two conditions: 1) the cells were dispersed into independent freezing units made possible by the cell walls (i.e. ice does not penetrate through the cell wall to nucleate intracellular water) (Hong and Sucoff 1982); and, 2) the plasma membrane remained intact, preventing the flow of extracellular water into the cell, so that the solutes within the cell were concentrated by dehydration, thus lowering the homogeneous nucleation temperature (Gusta et al. 1983). For the first condition to be met, secondary thickening must have been present in the cell walls (Gusta, personal communication 1986). When plant tissue freezes, ice forms either inside the cell (intracellular) or outside the cell walls (extracellular). Intracellular freezing disrupts the integrity of the cell and is invariably lethal. Intracellular freezing is common in tender plants which are unable to acclimate, and occurs in plants which are capable of acclimating but have not yet done so (Stushnoff and Quamme 1983).

The oldest hypothesis of how intracellular ice formation causes injury proposes that cell rupture from the expansion of ice causes cell death. A more recent view is that intracellular ice formation is a

consequence of injury to the plasma membrane (Steponkus 1984).

Observations at the microscopic level of isolated protoplasts have revealed that the freeze-thaw cycle induces many disruptions of the plasma membrane. These disruptions all cause alterations in the semipermeable characteristics of the membrane and cause cell injury (Dowgert and Steponkus 1983; Ketchie et al. 1972). The disruptions produce three distinct forms of injury. The first form is intracellular ice formation at large fractional cell volumes associated with the immediate physical disruption of the plasma membrane. The other two forms come from cellular dehydration and can be seen as expansion-induced lysis during warming or the loss of osmotic responsiveness following cooling. Any injury is dependent on the freeze-thaw circumstances (i.e. cooling rate and minimum temperature imposed), composition of the suspending medium, and the hardness of the tissue studied (i.e. acclimated versus non-acclimated).

Freeze avoidance is the prevention or delay of ice formation in the tissue and is accomplished through supercooling. The series of changes that make supercooling successful are stimulated by the external environment. Freeze tolerance, on the other hand, is unlike supercooling in its ability to tolerate freezing and not avoid it. Freeze tolerance is dependent on the hardening capacity of the tissues and is produced by the adaptive processes within the protoplasm in response to environmental conditions. Both freeze avoidance and freeze tolerance are components of the terms "freeze resistance" or "cold resistance".

2.10 Improper conceptualization

For years some scientists have approached the study of cold resistance looking for a single compound or factor responsible for cold hardiness. They also believed that the greater the quantitative increase (or decrease) in a given compound, the more likely it was to be responsible for cold hardiness. Another misconception in the study of cold hardiness has been the assumption that freezing injury was the result of the same stress in all plants, or more important, the same stress at different stages of acclimation. Too often it is inferred that different types of freezing stress are distinct, mutually exclusive pressures on a plant. According to Steponkus (1978), it is more appropriate to conclude that the stresses which arise during freezing are a sequential series of events.

The sequential series of events during freezing may be understood as successive barriers, and survival depends on the successful sequential avoidance or tolerance of each individual stress barrier (Steponkus 1978). Hence, cold acclimation can be thought to involve a successive series of changes which allow each stress barrier to be overcome, and not as a particular change which is responsible for the total increase (or decrease) in hardiness. With this view it is easier to understand why numerous biochemical and physiological changes may be associated with the cold acclimation process and not just one single biochemical event (Ormrod and Layne 1974).

2.2 Winter dormancy

The degree of cold hardiness attained by a plant is under genetic control but is readily altered by environmental and cultural conditions. For example, to harden conifers, a short photoperiod combined with warm temperatures induces dormancy. Once the plants are dormant, they are able to cold harden under cool temperatures. This process is time dependent; the length of time depends on the genotype and area of collection (Glerum 1985).

Dormancy is induced in response to a shortening photoperiod under warm temperatures (Fuchigami et al. 1982), whereas cold hardiness is induced by exposing dormant trees to cool temperatures. Tumanov et al. (1972) stated that dormancy was essential to maintain a proper hormonal balance for hardening and accumulation of reserves for overwintering. Genetic evidence has indicated that a large number of genes are involved in winter dormancy, and hence a large number of enzymes and regulating substances may also be involved (Perry 1971). Leopold et al. (1972), working with white pine, and Siebel and Fuchigami (1978), working with dogwood, showed that ethylene production declined prior to the development of winter dormancy. Siebel and Fuchigami (1978) suggested that ethylene could be used as a predictor for vegetative maturity.

2.2.0 Dormancy terminology

The terms in the literature describing the period of winter dormancy in temperate woody perennials are numerous and confusing (Lang et al. 1985; Doorenbos 1953). In this paper, dormancy will be referred to as quiescence, correlated inhibition and rest, the three categories usually thought to comprise dormancy. These terms are accepted by most horticulturists and physiologists (Fuchigami and Cheng-Chu Nee 1987). Quiescence is regulated by environmental factors, while correlated inhibition is regulated by physiological factors outside the affected structures. These affected structures will remain dormant in a favorable environment, but rapidly resume growth if neighbouring organs (leaves, buds) are removed, thus removing the source of inhibitors. Rest is regulated by physiological factors inside the affected structures. These affected structures remain dormant for prolonged periods even if the environment is favorable for growth and competitive/inhibitory organs are removed. The plant will not resume growth without exposure to winter cold, chilling, or other special treatments (Childers 1976; Fuchigami and Cheng-Chu Nee 1987; Levitt 1980; Perry 1971).

2.2.1 Stages of dormancy

In an attempt to further delineate the various annual physiological growth stages of perennial temperate woody plants Fuchigami et al. (1982) developed a conceptual numerical procedure

called the Degree Growth Stage ($^{\circ}$ GS) Model. This Model quantifies the annual development of temperate woody species (see Fig.2.1). Cold acclimation in most woody species appears to be controlled by certain internal changes and several environmental factors: light, temperature, moisture, and nutrients. The process of hardening normally occurs in two stages (Bervaes et al. 1978; Glerum 1985; Hong and Sucoff 1982; Howell and Weiser 1970; Irving and Lanphear 1967; Nitsch 1957; Siminovitch 1982; Weiser 1970). The first stage of acclimation in dogwood is normally induced by short days and warm temperatures after the photoreceptive point (90° GS) (see Fig. 2.1) (Fuchigami and Cheng-Chu Nee 1987; Fuchigami et al. 1982; Kobayashi et al. 1982). Dogwood, however, does not experience a rapid increase in low temperature survival until after vegetative maturity (180° GS) is obtained. Ketchie (1985) reported that until vegetative maturity (VM) is reached, the tree will not acclimate to its full potential regardless of the temperature of exposure.

Short day induction of hardiness involves a translocatable factor which promotes hardiness by causing the cessation of vegetative growth (Tumanov et al. 1972; Perry 1971; Fuchigami et al. 1982). Acclimation can be imposed in some woody plants without exposure to short days. The rate, however, is much faster under the influence of a short day photoperiod (Howell and Weiser 1970; Siminovitch 1982). Growth cessation is important to the accumulation of reserves that will be utilized in the hardiness period: an actively growing woody plant does not acclimate (Levitt 1980). Analysis has shown that actively growing trees have an inadequate amount of reserve carbohydrates and they

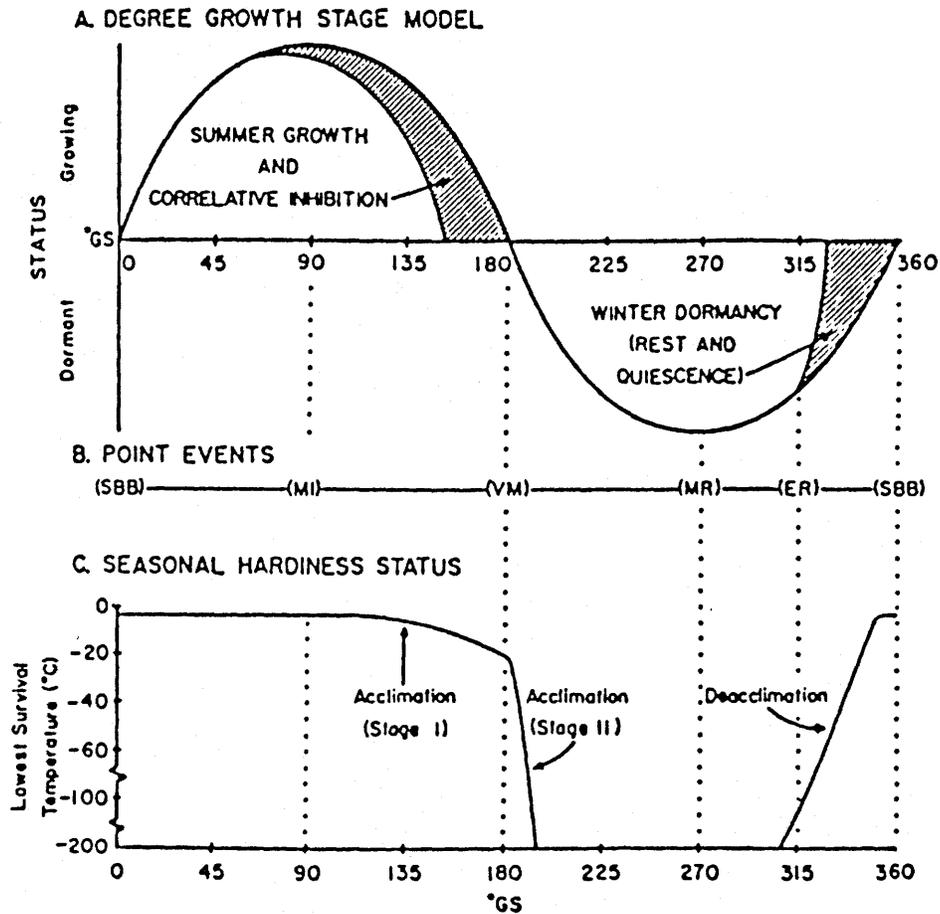


Figure 2.1. Diagram of degree growth stage (GS) model for identifying stages of development (A), point events (B), and hardiness (C) of vegetative buds of temperate woody plants. Point events are indicated: spring bud break (SBB); maturity induction point (MI); vegetative maturity (VM); maximum rest (MR); end of rest (ER); and end of dormancy/spring bud break (SBB) (Fuchigami et al. 1982).

cannot be hardened (Tumanov et al. 1973). Polysaccharides and oligosaccharides are predominantly accumulated in trees that have ceased to grow. Because the effects of this first stage are translocatable, it is also reversible until the second stage is initiated (Fuchigami and Cheng-Chu Nee 1987; Fuchigami et al. 1982; Kobayashi et al. 1982).

The second stage begins at vegetative maturity and is induced by exposure to low temperatures (Fuchigami et al. 1982). As the first hardening stage advances, plants become increasingly responsive to temperatures near or just below 0°C which causes the initiation of the second stage (see Fig. 2.1 (C)). The effects of this second stage in the acclimation process are non-translocatable and non-reversible until after the chilling requirements of the tree are fulfilled (Howell and Weiser 1970; Kobayashi et al. 1982). Although the "stage" concept of cold acclimation has been assumed by several investigators to be applicable to woody plants native to temperate climates (Glerum 1985; Kobayashi et al. 1982; Weiser 1970), there is still no consensus on the validity of this concept among cold hardiness scientists.

Some researchers have suggested a modification to the two stage model. Stushnoff et al. (1985) presented evidence that in apple seedlings of Dolgo and Golden Delicious a period of short days and warm temperatures for the first stage of acclimation was not essential. Instead, they proposed that a period of exposure to short days at temperatures near but above 0°C, was effective for cold acclimation. They also showed that with subsequent exposures to temperatures below but near 0°C, hardiness resistance levels of certain cultivar seedling

populations could be significantly increased. Stushnoff et al. (1985) called these subsequent exposures to temperatures below 0°C, frost cycles.

2.3 Environmental factors influencing cold acclimation

2.3.0 Photoperiod

The role of light in the acclimation process relates not only to its photosynthetic character, but also its photoreceptive character (Tumanov et al. 1973). In experiments reported by Holubowicz (1978) there was a beneficial influence by foliated shoots on the development of cold resistance in defoliated shoots. This was also supported by the work of Howell and Weiser (1970) and Tumanov et al. (1972). It was proposed by Fuchigami et al. (1971) that a winter hardiness stimulator is produced in leaves exposed to short days and translocated to other parts of the plant.

2.3.0.0 Translocatable cold hardiness promoter

Holubowicz's (1978) data supported the hypothesis that a "hardiness stimulator" was produced in the leaves and translocated from shoots to the rest of the tree. Research by Mityga and Lanphear (1971) indicated that some promoter from the top of the plant, exposed to short days, was translocated to the roots, thus influencing mature root hardiness. If the phloem was girdled, the promoter movement was

blocked and the mature roots did not acclimate (Mityga and Lanphear 1971).

Mityga and Lanphear (1971) proposed that only mature roots could harden because the high levels of gibberellic acid produced in the root tips of the young roots nullified the effect of the growth regulator. Suggestions have been made that AbA (Abscisic acid) is the hardiness promoter (Bray 1982; Irving and Lanphear 1968; McKenzie et al. 1974). The primary role of AbA would be to change the plant's water status (Bray 1982; McKenzie et al. 1974). The ability of the plant to decrease its water content during the period of acclimation is very important (Levitt 1980). The decrease in plant water content, under AbA control, results from open stomata and high root resistances (Parsons 1978). Less AbA was found in short day leaves, and this finding supports the assumption that stomata would be open more in short day exposed plants, thus increasing transpiration (Bray 1982).

Previous research on woody plants indicated that the long day leaf was a source of hardiness inhibitors just as the short day leaf was a source of hardiness promoters (Bray 1982; Fuchigami et al. 1971; Howell and Weiser 1970; Irving and Lanphear 1967). The studies on partially defoliated plants were consistent with the inhibitor/promoter concept (Bray 1982; Fuchigami et al. 1971; Holubowicz 1978).

2.3.0.1 Changes in growth regulator balance

In terms of photoperiodic control, there seem to be four stimulated endogenous events necessary for efficient acclimation in

hardy woody plants (Fuchigami et al. 1971): growth cessation, a substrate for synthesis, a high proportion of hardiness promoter to hardiness inhibitor, and an efficient metabolic machinery for translating the three preceding events into the biophysical or physiological changes responsible for resistance.

Photoreceptive plants, such as dogwood, not exposed to short days acclimate more slowly and less efficiently than short day exposed plants because a low hardiness promoter/inhibitor ratio is present in the tree (Fuchigami et al. 1971). The difference between a genotype which displays a very hardy character and a genotype which does not display much hardiness may be attributed to a lower promoter/inhibitor threshold and thus acclimation begins earlier.

The sequence of vegetative growth followed by dormancy, proposed by Kuzina et al. (1984), is a multicomponent system of hormonal regulation. It is fitting to present this concept in a discussion of photoperiod, because much of the findings which corroborate the theory of hormonal regulation of dormancy has been obtained from research on woody plant extracts of natural inhibitors obtained from leaves exposed to short days. These extracts increased cold resistance by inducing or deepening dormancy (Kuzina et al. 1984).

Several different growth regulators have been employed in agricultural production and research in efforts to regulate dormancy by means of foliar applications (Holubowicz 1983; Khamis et al. 1979; Kuzina et al. 1984; Lockhart and Bonner 1957; Tumanov et al. 1974). The general consensus of these studies is that the natural influence of short photoperiods is a more powerful factor for cold hardening than

the treatment of plants with extracts of natural inhibitors. The primary reason is that the plant's physiological state is of major importance in its ability to harden (Holubowicz 1983; Ketchie 1985; Tumanov et al. 1974). Short days are very effective in regulating the physiological state, and thus the hardening capacity.

2.3.0.2 Quantity or quality of light

McKenzie (1974) pointed out that woody plants growing in northern latitudes are exposed to longer periods of twilight. Twilight has an increased ratio of far red to red light. McKenzie (1973) and Williams et al. (1972) found that the first stage of acclimation, and growth cessation could be promoted by exposing dogwood to end-of-day (twilight) far-red light. Bjornseth (1983) revealed that different ecotypes have different abilities to react to the spectral changes in the twilight. Northern ecotypes were adapted to perceive the signal of the beginning of darkness at a higher irradiance than ecotypes from lower latitudes. Therefore, higher irradiances and relatively more far red light are needed to prevent growth cessation in a northern ecotype (Junttila and Kaurin 1983). Seedlings of the northern ecotype did not respond to a night break treatment indicating a light-dominant response (Vince-Prue 1976). Compared to red irradiation, far red light at the end of the photoperiod, increased the frost tolerance of dogwood (Kaurin 1983). McKenzie et al. (1974) concluded that the initiation of cold acclimation in dogwood was a photomorphogenetic phenomenon which is mediated by phytochrome. They suggested that the level of P_r must

be below a critical threshold value for acclimation to proceed.

2.3.1 Temperature

Temperature is a key environmental parameter for synchronizing a plant's capacity to withstand freezing temperatures with prevailing ambient temperatures (Steponkus 1978). Temperatures just above 0°C are essential to induce maximum rates of cold acclimation of roots (Bigras 1987; Mityga and Lanphear 1971; Weist and Steponkus 1977). Johnson and Havis (1977) found that both short days and low temperatures were necessary for root acclimation. Most authors, however, cite temperature as the key and often the only factor regulating hardening in roots (Bigras 1987; Lindstrom 1986). Temperature influences the deepening rest phase (Fig. 2.1 B between 180° to 270°GS). Low temperature is essential for the attaining of maximal acclimation potential. Before short days initiate the first stage in the acclimation process, however, the process is independent of temperature (Bervaes et al. 1978; Glerum 1976; Ketchie 1985). Regardless of ambient temperature, the xylem and bark are cold resistant to -8° and -10°C respectively. After vegetative maturity, further cold hardiness is temperature dependent. Ketchie (1985) found that -4°C was the optimum temperature for acclimation of the xylem and bark after vegetative maturity. Ketchie also found that 7 days of exposure to temperatures of 0 to -5°C were sufficient to maximize acclimation. More than 7 days of exposure gave no further acclimation. During the period of 180 to 270° (270°= maximum rest (MR)) (see Fig. 2.1) high

temperatures resulted in no deacclimation occurring. Between maximum rest and the completion of rest, apple trees deacclimated when temperatures were above 4°C, but reacclimated when temperatures were below 4°C. Maximum cold resistance was reached during the period between 180 and 315° GS (see Fig. 2.1) (Fuchigami et al. 1982; Ketchie 1985).

In apple trees (Ketchie 1985) and other woody species (Friesen and Stushnoff 1985; Fuchigami et al. 1982; Glerum 1976; Tumanov et al. 1973), the physiological stage of development is the single most significant factor, and thus physiological development is not always predominantly related to temperature. Most observations show, however, that there is an interrelationship between temperature and physiological stage which governs cold resistance in trees.

2.3.2 Moisture

The effects of light and temperature on acclimation are mediated through the development of resistance, whereas moisture content directly affects the stresses the plant must tolerate. The reduction of available water within the tree is the most important quantitative change associated with hardiness (Gusta et al. 1983). Moisture can also have an indirect effect on hardiness. If moisture is withheld during the summer, hardiness is increased as a result of growth cessation (Glerum 1976). Prolonged droughts in summer should be avoided by irrigation, as should any kind of stress, since they will interfere with the physiological processes, which in turn interfere

with the cold acclimation process (Levitt 1982).

Compared with the shoots, roots have relatively high moisture percentages (Pellet and White 1969). It is known that hardy plants lose water in the fall (Levitt 1980). Water binding substances have been noted to inhibit cellular dehydration during water movement to regions of intracellular ice formation (Levitt 1980). Cells having a higher percentage of bound water due to less actual moisture would have greater tolerance to freeze damage. On the basis of shoots having lower moisture percentages than roots, greater hardiness would be developed in the shoots. The decrease in shoot moisture is caused by an increase in bound water which, in turn, is caused by an actual increase in water binding substances (Pellet and White 1969; Levitt 1980).

2.3.3 Nutrition

Nitrogen tends to reduce hardiness or prevent the cold acclimation process (Fuchigami and Weiser 1981); potassium and phosphorus tend to increase hardiness (Pellet 1973). Nutrients stimulate or reduce the rate of growth in woody plants and thus affect cold hardiness indirectly. Nitrogen stimulates growth, and when applied late in the growing season can prevent growth cessation and thus reduce the potential for cold hardiness in a woody plant. Nitrogen can also improve hardening when applied after bud set (Glerum 1976). Conversely, potassium and phosphorus can assist in growth cessation and bud development (Pellet 1973). The amounts of nutrients, their

relative proportion to each other, timing of application, and availability of moisture for uptake are all significant (Glerum 1976; Pellet 1973).

The widely-held belief that fertility may predispose plants to severe winter injury is not universally supported by research data. Most plants fertilized at levels which promote optimum growth will cold acclimate at a similar rate and to the same degree as plants grown under a lower fertility regime, and may even exceed cold hardiness development of plants grown under severe nutrient deficiencies. Extremely high levels of fertility, however, can retard cold acclimation (Pellet and Carter 1981; Pellet 1973; Pellet and White 1969). Brierly and Landon (1946) reported that a well grown plant in good health, not weakened by insects, disease or excessive production will survive cold stress better than a plant in poor condition.

2.4 Internal Physiological Changes

The internal changes during acclimation are of equal significance to external factors and are usually initiated by environmental cues. Hardening is normally accompanied by an accumulation of one or more substances synthesized by the plant. During hardening, the concentration of sugars, proteins and unsaturated fatty acids increase (Levitt 1980). The increase in the accumulation of these substances is supported by the visible increase in the amount of protoplasm per cell (Siminovitch 1981). The purpose which this accumulation of substances may play in plant freeze resistance, however, is yet to be fully

understood.

Felker et al. (1983) provided evidence that starch accumulates in dormant tissues during the second stage in the cold acclimation process. In the winter dormancy period the starch is hydrolyzed to low molecular weight carbohydrates. These carbohydrates function as cryoprotectants in the cells and also facilitate the energy requirements needed to express cold resistance (Felker et al. 1983). Cold hardiness is an inducible character, and because the cold acclimation process is a dynamic one, the expression of cold hardiness is dependent upon a source of energy and specific environmental factors. Because cold hardiness is inducible, it can be called a latent trait, which exhibits an annual periodicity (Steponkus 1978).

Many of the annual internal changes which occur during the hardening process directly affect the stability of the plasma membrane. Weist and Steponkus (1977) showed that changes in the membrane fluidity of roots was of great importance to their surviving freezing temperatures. They proposed that the inability of young roots to acclimate was associated with a lack of structural alterations in the plasmalemma, limiting membrane fluidity (Weist and Steponkus 1977).

2.4.0 Changes to the plasma membrane

In acclimated protoplasts, the plasma membrane poses an effective barrier to external ice. The increase in the plasma membrane efficiency, however, is not due to an increase in its water permeability following cold acclimation as proposed by Levitt (1982),

because there is no significant difference in the hydraulic conductance of acclimated and non-acclimated protoplasts (Dowgert and Steponkus 1984).

Immediately before intracellular ice formation, the plasma membrane flutters, or eddy-like flow patterns occur in the cytoplasm, and the intracellular solution flows outward. Upon ice nucleation and attendant gas bubble formation, gas bubbles emerge from the protoplast in the region of the plasma membrane. Upon thawing, gas bubbles and cellular contents emerge from the same area. These observations suggest that mechanical breakdown of the plasma membrane precedes intracellular ice formation and exposes the supercooled intracellular solution to extracellular ice (Steponkus et al. 1983). Thus, the decreased incidence of intracellular ice formation in acclimated protoplasts is attributable to an increase in the stability of the plasma membrane which precludes nucleation of the supercooled intracellular solution. One source of freeze-thaw injury to isolated protoplasts is the contraction and expansion of the surface area of the plasma membrane during freezing and thawing. The surface area of the plasma membrane contracts during cellular dehydration. This can be attributed to the removal of intracellular water to extracellular areas. The contraction is not reversible and lysis occurs during the subsequent expansion of the thaw cycle. Expansion-induced lysis is the predominant form of injury in non-acclimated protoplasts (Dowgert and Steponkus 1983). The implication is that the expansion potential of isolated protoplasts is characterized by an absolute Tolerable Surface Area Increment (TSAI) (Dowgert and Steponkus 1978).

The TSAI is a constant and is independent of the extent of contraction. The $TSAI_{50}$ value denotes the absolute magnitude of change in the mean surface area of a population of protoplasts, in which lysis occurs within 50% of the population. Larger cells have larger TSAI values. Large surface area contractions beyond the $TSAI_{50}$ value are reversible in acclimated protoplasts. After acclimation the tolerance of the plasma membrane to contraction is increased, and a lethal stress-strain situation is overcome (Dowgert and Steponkus 1978).

During contraction in acclimated protoplasts, plasma membrane material deposits into a reservoir from which material is retrieved during expansion. These reservoirs are called exocytotic vesiculations. This demonstrates that the elastic surface area contraction and expansion ability of the plasma membrane is limited to 2%. Surface area deformations which occur during osmotic manipulation or during a freeze-thaw cycle greatly exceed this 2% value. In contrast, non-acclimated protoplasts showed contractions of endocytotic vesiculations (Gordon-Kamm and Steponkus 1984).

The osmiophilic nature of these endocytotic regions indicated a high lipid content. These areas appeared readily exchangeable with the plasma membrane during contraction and subsequent expansion. During contraction and subsequent loss of membrane surface area, intramembrane particle (IMP) density (representing the ratio of protein particles per membrane surface area) increased. Because the IMP density increased during contraction, it appears that the surface area loss is due to the preferential loss of lipid material from the membrane into the lipid extrusion regions. In this way, the protein particles remain in place

in the plane of the plasma membrane (Gordon-Kamm and Steponkus 1984).

3. MATERIALS AND METHODS

3.0 Controlled acclimation experiment

3.00 Experimental design

A three-way treatment structure in a randomized complete block design was used from February to September 1987. The experiment was replicated four times with 18 sub-samples per experimental unit. The three factors that were analyzed in the experiment were number of frosts, freeze temperatures, and cultivar seedlings. Six levels of frost, three levels of freeze temperatures, and three open-pollinated *Malus* cultivar seedling populations were studied. The three freeze temperatures were -20, -30, and -40°C. The six frost treatments were zero, one, two, three, four and five frosts. Each of the 216 experimental units (three cultivar seedling populations X three freeze temperatures X six frost treatments X four replications) consisted of 18 seedlings.

The seedlings were grown in styrofoam trays. Each tray consisted of 130 centers. Two rows around the outside of each tray, totalling 76 centers, were filled with medium to act as insulation during the freeze. The inside 54 centers were seeded. Groups of 18 seedlings of each of the three cultivar seedling populations were planted in each tray, and the three cultivar seedling populations were ordered randomly in each tray (Figure 3.1).

3.0.1 Cultivar seedling populations studied, seed collection and stratification

The three open-pollinated *Malus* seedling populations investigated were Golden Delicious (*Malus pumila*), Antonovka (*Malus baccata*) X (*Malus pumila*) and Rescue Crab (*Malus baccata*) X (*Malus pumila*). These cultivar seedling populations were selected to represent a full range of inherent cold resistance levels. The cultivar Rescue crab was originally developed at Scott, Saskatchewan (Strang 1974) and was the most cold resistant cultivar seedling investigated. The cultivar Antonovka was introduced from Russia, although the seed used in this experiment was from Summerland, BC and therefore represented cultivar seedling parentage of intermediate cold resistance, because in all likelihood the pollen parent was from a tender cultivar. The Golden Delicious cultivar was originally selected in Pennsylvania and was the least cold resistant cultivar seedling studied.

The Rescue Crab fruit was collected from trees grown in the research plots of the Department of Horticulture Science at the University of Saskatchewan in Saskatoon in September 1986. The Antonovka fruit was collected from trees grown in the orchards of the British Columbia Fruit Growers Association (B.C.F.G.A.), Summerland, BC. The Golden Delicious fruit was grown in orchards in Washington, U.S.A. and obtained from grocery stores in Saskatoon.

The Antonovka seed was sent dried from the B.C.F.G.A.. The fruit of the Rescue and Golden Delicious were cut open and the seeds were

removed and immersed in water in order to loosen particles of fruit clinging to the seeds. Next, the seeds were laid on paper towels to dry for 24 hours. The dried seed was then stored in a +6°C cooler for one month before stratification.

To begin the stratification process, the seeds were soaked in water for 24 hours. They were then placed in plastic bags containing silica sand and a small quantity of captan (0.2 grams per one kilogram of sand) for 90 days and planted when the radicals emerged uniformly within the bags.

3.0.2 Growing conditions and artificial cold acclimation

The seedlings were grown for six weeks in a greenhouse under long (12 to 16 hours) days and 20 to 30°C temperatures. After this period the seedlings were transferred to a cooler to grow for six more weeks under short (eight hour) days and cool temperatures (+3 to +5°C). The planting schedule was staggered. Only two trays could be frozen in the computerized freezer at one time. In order that all seedlings were 12 weeks old when their frost cycles began, the planting schedule was delayed for each frost treatment.

The growing medium was one part medium grade vermiculite, one part peat and one part perlite. The seedlings were fertilized twice a week with 20-20-20, 19 grams per 8 litres of water (this solution would cover approximately seven trays). Fertility and moisture were kept at levels during the entire six week growing period required to minimize stress factors other than those imposed by the cold treatments. In the

cooler, plants were watered once a week and the temperature was recorded hourly on an Omega digital data logger.

After the six week period in the cooler, the trays were treated with an earlier determined frost exposure. One frost exposure consisted of approximately 16 hours (4:30 p.m. to 8:30 a.m.) at -3°C . A tray which was assigned zero frosts did not receive a -3°C exposure before its evaluation freeze at -20 , -30 , or -40°C . A tray which was scheduled to receive two frosts was put through the following regime: 1) after six weeks at $+3$ to $+5^{\circ}\text{C}$ the tray was moved to a deep freeze for 16 hours at -3°C ; 2) after 16 hours at -3°C , the tray was returned to the $+3$ to $+5^{\circ}\text{C}$ cooler for 32 hours; 3) the tray was again moved to the -3°C deep freeze for 16 hours to complete the second frost exposure; 4) the tray was moved back to $+3$ to $+5^{\circ}\text{C}$ for 32 hours; 5) the tray was frozen to its respective freeze evaluation temperature. After each frost exposure the trays were allowed a 32 hour period back in the $+3$ to $+5^{\circ}\text{C}$ cooler so that the root mass could thaw.

3.0.3 Controlled freeze procedures

Before the trays were frozen, a 0.5 cm thick layer of moist peat was applied to the top of each tray to act as insulation. The tray was then placed in a box made of insulating styrofoam 2.54 cm thick. This box, the layer of insulating peat, and the two outside rows of medium around each tray protected the roots during the freeze. The trays were frozen two at a time to -20 , -30 , or -40°C . Each tray was removed after its assigned freeze temperature was reached. The freeze was

conducted in three stages: 1) a holding period at -3°C for 10 hours; 2) a temperature drop period of 2°C per hour to -16°C ; and, 3) a temperature drop period of 4°C per hour to the desired temperature, i.e. -20 , -30 or -40°C .

When the freezing cycle was over, the seedlings were moved as quickly as possible back to the $+3$ to $+5^{\circ}\text{C}$ cooler for overnight to prevent sudden thawing. The next morning, the top 0.5 cm layer of peat was removed from the trays. The trays were then moved to the greenhouse, defoliated with scissors, and left to regrow for four weeks. At the end of the four weeks regrowth data was collected.

3.0.4 Data collection methods

Seedling regrowth was measured in three ways; percent seedling survival, percent bud survival, and viable growth score. All measurements were taken on all 216 experimental units of 18 seedlings each.

3.0.4.0 Percent seedling survival

Percent seedling survival was collected as binomial data. Each seedling was given a rating of either one or zero. Only if the seedling were completely dead would it be recorded as a zero. Percentages of plant population survival were then calculated by dividing the number of plants given a rating of one over the total number of seedlings per tray per cultivar seedling population.

3.0.4.1 Percent bud survival

Percent bud survival was also collected as binomial data. A bud was rated as either dead or alive. Percentages of bud survival were calculated by dividing the number of buds alive by the total number of buds per seedling. Percent bud survival for each experimental unit was calculated as the average of percent bud survivals of 18 seedlings.

3.0.4.2 Viable growth score

Viable growth was scored on a range of one to 100. Each seedling was scored by visual comparison of the amount of green stem to the total height of original growth before the freeze. Mean scores for up to 18 seedlings were used as the viable growth scores of individual experimental units.

3.0.5 Statistical analysis

Analyses were conducted on the calculated means of the eighteen plants in each tray for all three measures (percent seedling survival, percent bud survival, and viable growth score). Sometimes seedlings were missing, and so means were not always based on 18 seedlings per tray. The SAS procedure ANOVA was used to perform the analysis outlined in Table 3.1 for each of the three regrowth measures (SAS Institute Inc. 1985 and Milliken 1984). All factors were considered fixed effects, therefore all terms were tested for significance against the error mean

Table 3.1. Outline of analysis of variance for the controlled acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	M1	M1/M9
Cultivar seedlings(C)	2	M2	M2/M9
Freeze temperature (T)	2	M3	M3/M9
Frost treatment (F)	5	M4	M4/M9
C X T	4	M5	M5/M9
C X F	10	M6	M6/M9
T X F	10	M7	M7/M9
C X T X F	20	M8	M8/M9
Experimental error	159	M9	

square. In the case of significant frost effects, orthogonal comparisons were used to compare means. Since all the data was expressed in percentages, an arc sine of square root transformation was performed (Steel and Torrie 1980), the data sets were reanalyzed, and the two analyses for each regrowth measure were compared.

3.1 Freeze without acclimation experiment

3.1.0 Experimental design

A two-way treatment structure in a randomized complete block design was used from May to July 1987. The experiment was replicated four times with 10 sub-samples per experimental unit. The two factors that were analyzed in the experiment were freeze temperatures and cultivar seedlings. Three levels of freeze temperature, and three *Malus* cultivar seedling populations were studied. The three freeze temperatures were -5, -10, and -15°C. The three cultivar seedling populations were the same as those studied in the controlled acclimation experiment. The seed was collected and stratified in the same manner as well (Section 3.0.1). The seedlings were grown in styrofoam trays of 10 seedlings per treatment per cultivar seedling population per replication. Each tray consisted of 90 centers. Two rows around the outside of each tray, totalling 60 centers, were filled with medium to act as insulation during the freeze. The inside 30 centers were seeded. Groups of 10 seedlings of each of the three cultivar seedling populations were planted in each tray, and the three

cultivar seedling populations were ordered randomly in each tray (Figure 3.2).

3.1.1 Growing conditions

The seedlings were grown for six weeks in a greenhouse under long (12 to 16 hours) days and 20 to 30°C temperatures just as they were in the controlled acclimation experiment. In the freeze without acclimation experiment, however, the seedlings were given no preconditioning time in a +3 to +5°C cooler.

The planting schedule was staggered. Three trays could be frozen in the computerized freezer at one time. In order that all seedlings were six weeks old when their controlled freeze began, the planting schedule was delayed for each replication.

The growing medium was one part medium grade vermiculite, one part peat and one part perlite. The seedlings were fertilized twice a week with 20-20-20, 19 grams per 8 litres of water (this solution would cover approximately 10 trays). Fertility and moisture were kept at levels during the entire six week growing period required to minimize stress factors other than those imposed by the cold treatments.

3.1.2 Controlled freeze procedures

Before the trays were frozen, a 0.5 cm thick layer of moist peat was applied to the top of each tray to act as insulation. The tray was then placed in a box made of insulating styrofoam 2.5 cm thick. This

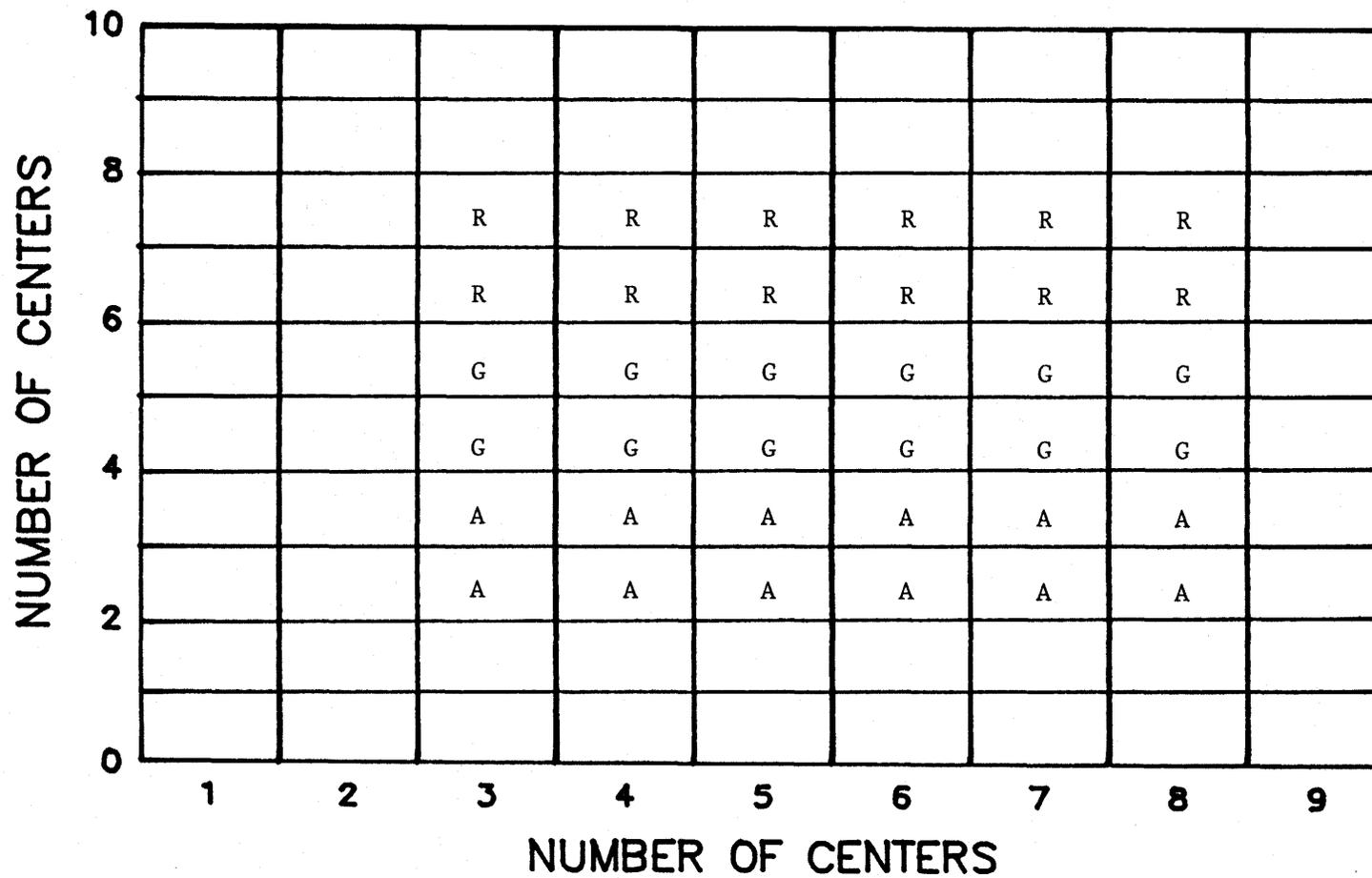


Figure 3.2. Arrangement in the tray of 10 seedlings, per cultivar seedling population, for the freeze without acclimation experiment. The three populations (R) Rescue, (A) Antonovka, and (G) Golden Delicious were randomly ordered in the tray.

box, the layer of insulating peat, and the two outside rows of medium around each tray protected the roots during the freeze. The trays were frozen three at a time to -5, -10, or -15°C. Each tray was removed after its assigned freeze temperature was reached. The freeze was conducted in two stages: 1) a holding period at -3°C for 10 hours; and, 2) a temperature drop period of 2°C per hour to -15°C.

When the controlled freeze was over, the seedlings were moved as quickly as possible to a +3 to +5°C cooler for overnight to prevent sudden thawing. The next morning the top 0.5 cm layer of peat was removed from the trays. The trays were then moved to the greenhouse, defoliated with scissors, and left to regrow for four weeks. At the end of the four weeks, regrowth data was collected.

3.1.3 Data collection methods

Seedling regrowth was measured in the same ways as outlined for the controlled acclimation experiment (Section 3.0.4). All measurements were taken on all 36 experimental units, of 10 seedlings each.

3.1.4 Statistical analysis

Analyses were conducted on the calculated means of the 10 plants in each tray for all three measures (percent seedling survival, percent bud survival, and viable growth score). Sometimes seedlings were missing, and so the means were not always based on the average of 10 seedlings per tray. The analysis of means was on 36 observations. The

SAS procedure ANOVA was used to perform the analysis outlined in Table 3.2 for each of the three regrowth measures (SAS Institute Inc. 1985 and Milliken 1984). All factors were considered fixed effects, therefore all terms were tested for significance against the error mean square. Since all the data was expressed in percentages, an arc sine square root transformation was performed (Steel and Torrie 1980), the data sets were reanalyzed, and the two analyses for each regrowth measure were compared.

3.2 Cool temperature duration experiment

3.2.0 Experimental design

A three-way treatment structure in a randomized complete block design was used from August to October 1987. The experiment was replicated four times with 18 sub-samples per experimental unit. The three factors that were analyzed in the experiment were frosts, freeze temperatures, and cultivar seedlings. Two levels of frost, three levels of freeze temperatures, and three *Malus* cultivar seedling populations were studied. The three freeze temperatures were -20, -30, and -40°C. The two frost treatments were zero plus 11 days in the cooler, and one frost plus 10 days in the cooler.

The seedlings were grown in styrofoam trays. Each tray consisted of 130 centers. Two rows around the outside of each tray, totalling 76 centers, were filled with medium to act as insulation during the freeze. The inside 54 centers were seeded. Groups of 18 seedlings of

Table 3.2. Outline of analysis of variance for the freeze without acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	M1	M1/M5
Cultivar seedlings (C)	2	M2	M2/M5
Freeze temperature (T)	2	M3	M3/M5
C X T	4	M4	M4/M5
Experimental error	24	M5	

each of the three cultivars were planted in each tray, and the three cultivar seedling populations were ordered randomly in each tray (see Figure 3.1 of the controlled acclimation experiment). The cultivar seedlings studied, seed collection methods and stratification procedures were all the same as in the controlled acclimation experiment (Section 3.0.1).

3.2.1 Growing conditions and artificial cold acclimation

The seedlings were grown for six weeks in a greenhouse and six weeks in the cooler just as they were in the controlled acclimation experiment (Section 3.0.2). After the six week period in the cooler the trays were treated with their earlier determined frost exposure. The one frost plus 10 days in the cooler treatment consisted of 16 hours (4:30 p.m. to 8:30 a.m.) at -3°C , and 10 days back in the $+3$ to $+5^{\circ}\text{C}$ cooler. A tray which was assigned a zero frost plus 11 day treatment did not receive a -3°C exposure, but stayed in the cooler six weeks plus 11 days before its evaluation freeze at -20 , -30 , or -40°C . The freeze procedures were identical to those outlined for the controlled acclimation experiment (Section 3.0.3).

3.2.2 Data collection methods

Seedling regrowth was measured in the same ways as outlined for the controlled acclimation experiment (Section 3.0.4). All measurements were taken on all seedlings of three cultivar seedling

populations evaluated in three controlled freeze temperatures, two frost treatments, and four replications.

3.2.3 Statistical analysis

Analyses were conducted on the calculated means of the eighteen plants in each tray for all three measures (percent seedling survival, percent bud survival, and viable growth score). Sometimes seedlings were missing, and so means were not always based on 18 seedlings per tray. The SAS procedure ANOVA was used to perform the analysis outlined in Table 3.3 for each of the three regrowth measures (SAS Institute Inc. 1985 and Milliken 1984). All factors were considered fixed effects, so all terms were tested for significance against the error mean square. Since all the data was expressed in percentages, an arc sine of square root transformation was performed (Steel and Torrie 1980), the data sets were reanalyzed, and the two analyses for each regrowth measure were compared.

Table 3.3. Outline of analysis of variance for the cool temperature duration experiment.

Source of variation	df	Mean square	F
Replication	3	M1	M1/M9
Cultivar seedlings (C)	2	M2	M2/M9
Freeze temperature (T)	2	M3	M3/M9
Frost treatment (F)	1	M4	M4/M9
C X T	4	M5	M5/M9
C X F	2	M6	M6/M9
T X F	2	M7	M7/M9
C X T X F	4	M8	M8/M9
Experimental error	51	M9	

4. Results

4.0 Controlled acclimation experiment

4.0.0 Data collection methods

Analyses of variance were conducted on all three methods of data collection.

4.0.0.0 Percent seedling survival data

The analysis of variance of the percent seedling survival data (Table 4.1) showed significant differences due to replication, cultivar seedling, temperature, and frost. The interactions of temperature X frost and cultivar seedlings X temperature were also significant. The cultivar seedlings X frost and cultivar seedlings X temperature X frost interactions were nonsignificant. The residual of the orthogonal contrast indicates that the significance of the frost effect was due to the difference between no frost and one or more frosts. Subsequent frost exposure after one frost gave no significant increase in cold resistance.

4.0.0.1 Percent bud survival data

The analysis of variance of percent bud survival data (Table 4.2)

Table 4.1. Analysis of variance for controlled acclimation experiment using percent seedling survival data.

Source of variation	df	Mean square	F
Replication	3	5276	16.97**
Cultivar seedlings (C)	2	13904	44.71**
Freeze temperature (T)	2	91815	295.24**
Frost treatment (F)	5	1233	3.96**
0 Frost vs Others	(1)	5552	17.85**
Residual	(4)	153	0.49
T X F	10	626	2.01*
C X T	4	1327	4.27**
C X F	10	215	0.69
C X T X F	20	400	1.09
Error	159	311	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.2. Analysis of variance for controlled acclimation experiment using percent bud survival data.

Source of variation	df	Mean square	F
Replication	3	2983	13.82**
Cultivar seedlings (C)	2	9225	43.04**
Freeze temperature (T)	2	83318	379.36**
Frost treatment (F)	5	838	3.91**
0 Frost vs Others	(1)	4064	18.96**
Residual	(4)	32	0.15
T X F	10	237	1.10
C X T	4	1584	7.39**
C X F	10	171	0.80
C X T X F	20	222	1.04
Error	159	214	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

showed similar significant differences to those indicated by the percent seedling survival data analysis (Table 4.1), except that the temperature X frost interaction was not significant. To better demonstrate this difference, graphs of the interactions from the two data sets are presented in Figures 4.1 and 4.2. Each point on each of the two graphs represents 216 seedlings, the means of three cultivar seedling populations evaluated in four replications. The two graphs are very similar. However, since the percent seedling survival graph (Figure 4.1) represents a significant interaction between temperature and frost and the percent bud survival graph (Figure 4.2) does not, we conclude that the percent seedling survival analysis is more precise. Percent bud survival is not consistent enough to indicate the significance.

4.0.0.2 Viable growth score

The analysis of variance of the viable growth score data (Table 4.3) exhibits the same significant differences as displayed in the percent bud survival data (Table 4.2). The graph of the viable growth score data (Figure 4.3) shows the same trends as percent bud survival (Figure 4.2) and percent seedling survival (Figure 4.1).

4.0.0.3 Transformation of data

An arc sine square root transformation was conducted on all three sets: percent seedling survival data (Table 4.4), percent bud survival

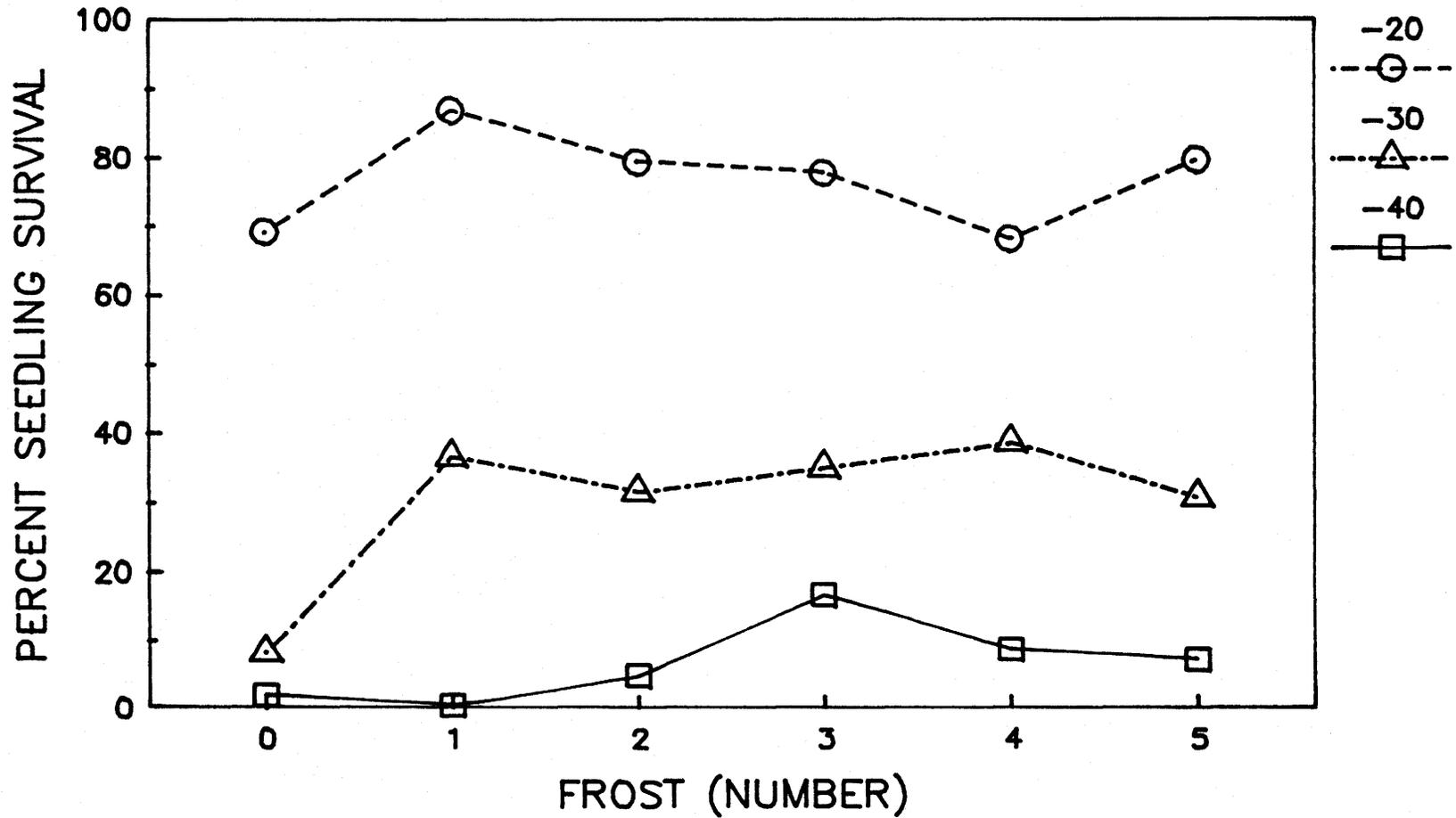


Figure 4.1. Temperature X frost interaction of percent seedling survival data of the controlled acclimation experiment, sampled from three apple cultivar seedling populations.

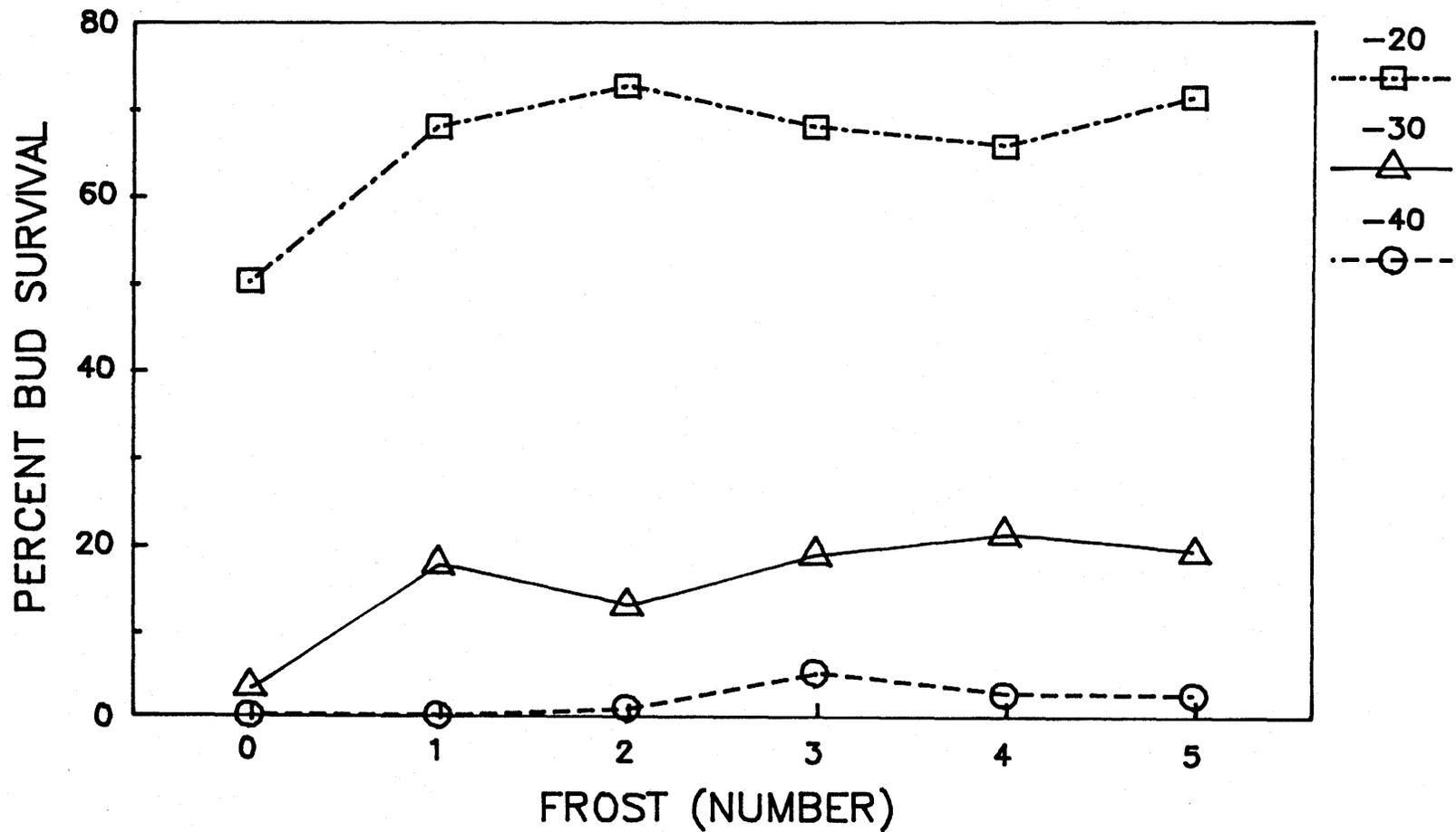


Figure 4.2. Temperature X frost interaction of percent bud survival data of the controlled acclimation experiment, sampled from three apple cultivar seedling populations.

Table 4.3. Analysis of variance for controlled acclimation experiment using viable growth score data.

Source of variation	df	Mean square	F
Replication	3	3295	15.13**
Cultivar seedlings (C)	2	9676	44.42**
Freeze temperature (T)	2	82947	380.81**
Frost treatment (F)	5	831	3.81**
0 Frost vs Others	(1)	4071	18.69**
Residual	(4)	21	0.09
T X F	10	240	1.10

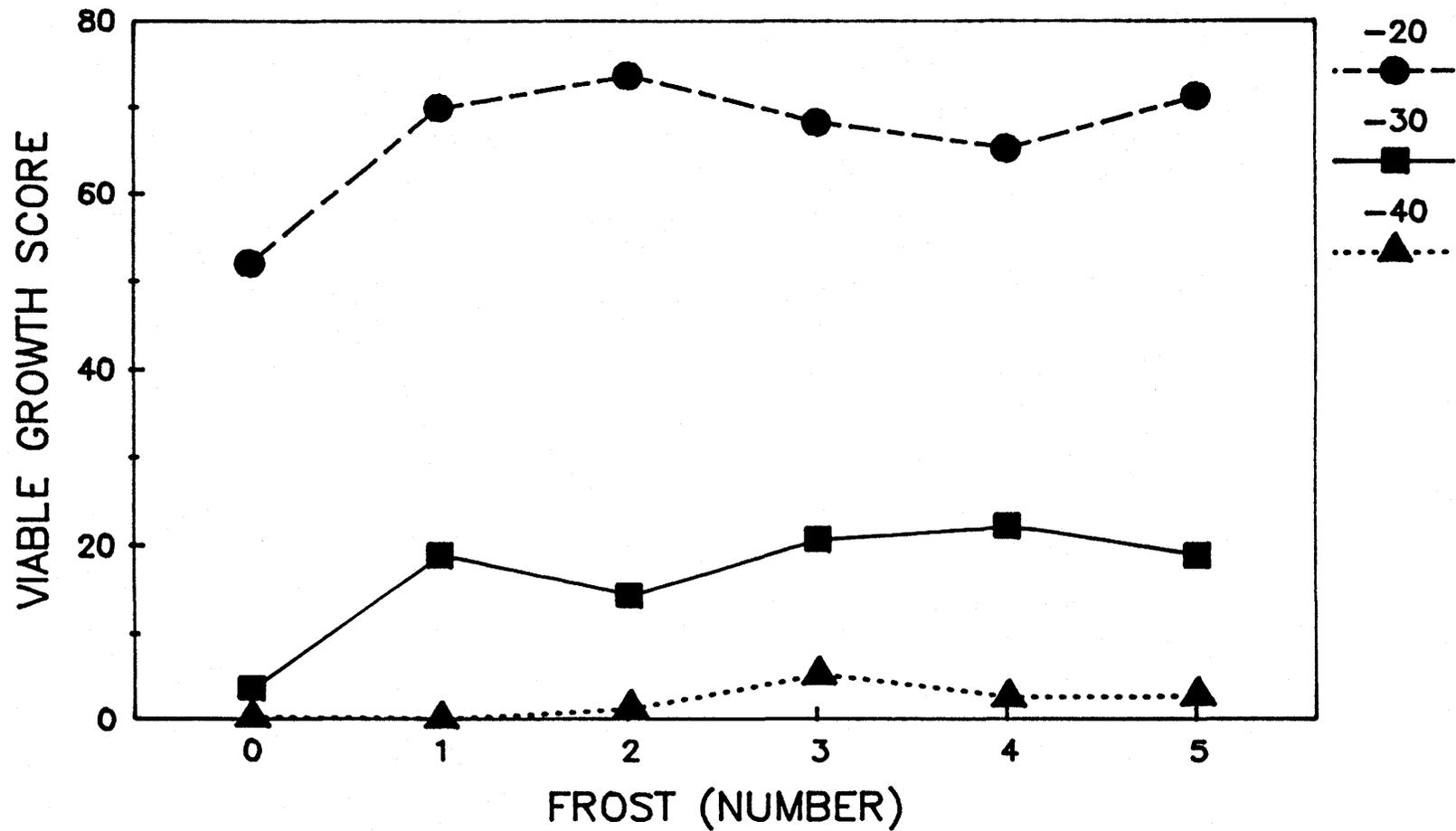


Figure 4.3. Temperature X frost interaction of viable growth score data of the controlled acclimation experiment, sampled from three apple cultivar seedling populations.

Table 4.4. Analysis of variance of the arc sine of square root transformed percent seedling survival data (expressed in radians) for the controlled acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	1.155	19.44**
Cultivar seedlings (C)	2	3.124	52.58**
Freeze temperature (T)	2	18.934	318.73**
Frost treatment (F)	5	0.246	4.13**
0 Frost vs Others	(1)	1.082	18.21**
Residual	(4)	0.037	0.62
T X F	10	0.142	2.39**
C X T	4	0.191	3.22**
C X F	10	0.041	0.68
C X T X F	20	0.071	1.19
Error	159	0.059	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

data (Table 4.5), and viable growth score data (Table 4.6). The analyses of variance of the transformed data indicated the same significant differences as displayed in the non-transformed data analysis.

4.0.0.4 Optimum method of data presentation

Since the transformation proved nonessential and the percent seedling survival data analysis gave the most precise results over the percent bud survival and viable growth score analyses, only the non-transformed percent seedling survival data tables and figures will be presented for the remainder of the controlled acclimation experiment results and discussion.

4.0.1 Cultivar seedling response

As indicated in the percent seedling survival data analysis of variance (Table 4.1), cultivar seedling main effects were significant. The cultivar seedlings X temperature interaction graph (Figure 4.4) indicates cultivar seedling differences are quite pronounced following acclimation. Each point on the graph represents 432 seedlings, means of six frost treatments in each of four replications. The level of seedling survival decreased as the screening temperatures decreased. Rescue was known to be inherently the hardiest cultivar seedling. Golden Delicious was known to be the most tender of the three and Antonovka was recognized as of inherent intermediate hardiness. The

Table 4.5. Analysis of variance of the arc sine of square root transformed percent bud survival data (expressed in radians) for controlled acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	0.689	19.80**
Cultivar seedlings (C)	2	2.113	60.71**
Freeze temperature (T)	2	16.153	464.08**
Frost treatment (F)	5	0.194	5.57**
0 Frost vs Others	(1)	0.905	25.99**
Residual	(4)	0.016	0.46
T X F	10	0.042	1.20
C X T	4	0.194	5.56**
C X F	10	0.029	0.83
C X T X F	20	0.033	0.93
Error	159	0.035	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.6. Analysis of variance of the arc sine of square root transformed viable growth score data (expressed in radians) for the controlled acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	0.763	21.21**
Cultivar seedlings (C)	2	2.180	60.61**
Freeze temperature (T)	2	16.516	459.11**
Frost treatment (F)	5	0.194	5.39**
0 Frost vs Others	(1)	0.906	25.18**
Residual	(4)	0.016	0.44
T X F	10	0.045	1.26
C X T	4	0.201	5.59**
C X F	10	0.029	0.82
C X T X F	20	0.033	0.92
Error	159	0.036	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

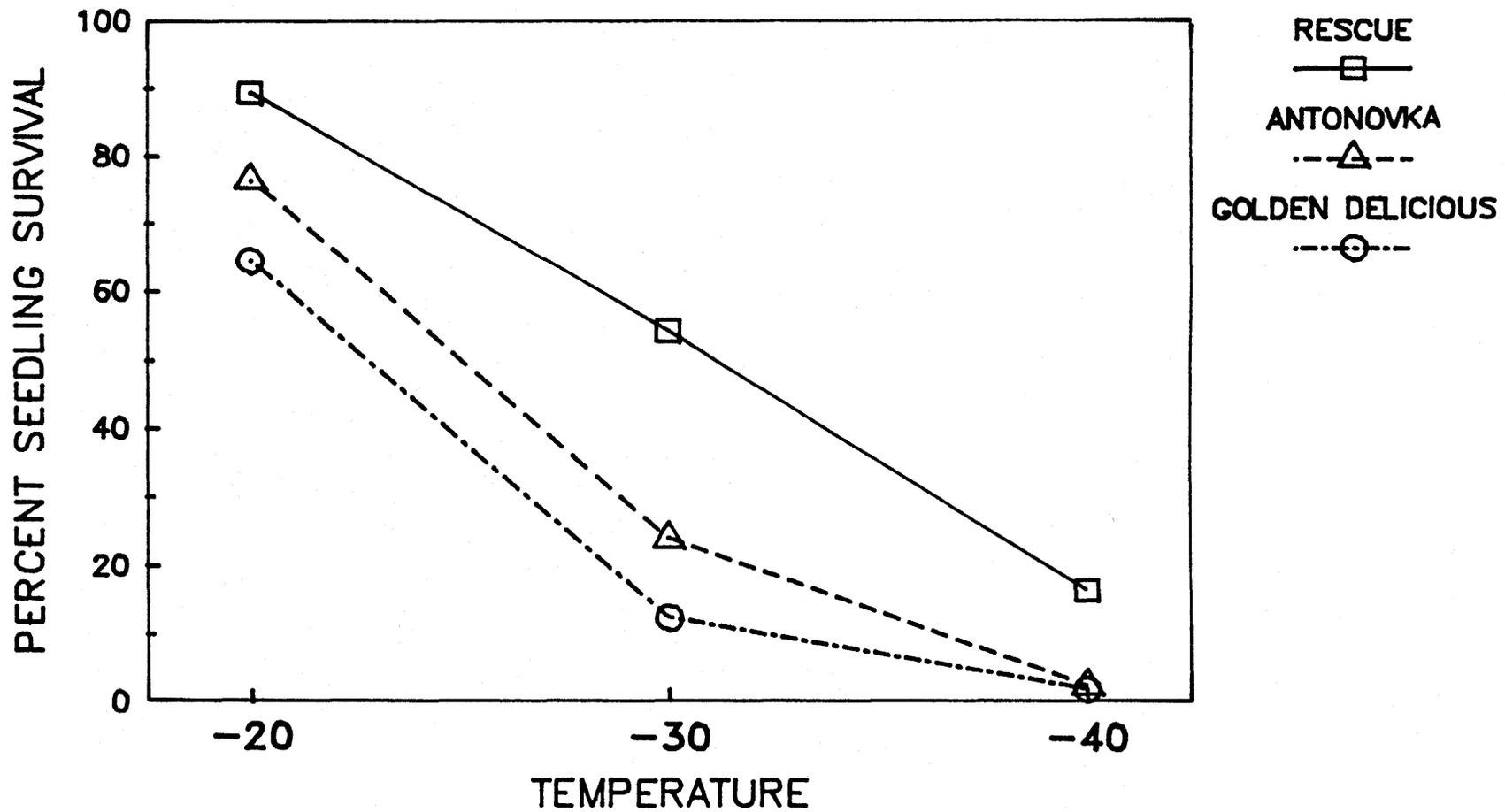


Figure 4.4. Cultivar seedlings X temperature interaction of percent seedling survival data of the controlled acclimation experiment, sampled from six frost treatments.

graph supports the expectations of inherent hardiness response.

The interaction of cultivar seedlings X temperature is significant. The decrease in survival was much more rapid for less hardy cultivars seedlings, with both Antonovka and Golden Delicious going to zero at -40°C . The decrease in survival for Rescue was linear over the three screening temperatures with approximately 20 percent of the seedling population surviving to -40°C .

4.0.2 Frost requirements

The main effects of the frost treatments were highly significant for percent seedling survival (Table 4.1). Each point on the cultivar seedlings X frost interaction graph (Figure 4.5) represents 216 seedlings or means of three temperatures in each of four replications. The interaction was nonsignificant, indicating that all three cultivar seedling populations responded similarly to the frost treatments. The nonsignificant residual of the orthogonal contrast (Table 4.1) indicates that the main frost effect was due to the difference between zero and one or more frosts. The graph supports this conclusion. The relative increase in seedling survival was different for each cultivar seedling population. One or more frosts with the Rescue gave a 74% increase in survival over no frost exposures. The Antonovka responded with a 62% increase in survival and Golden Delicious with an increase of 51% over zero frosts.

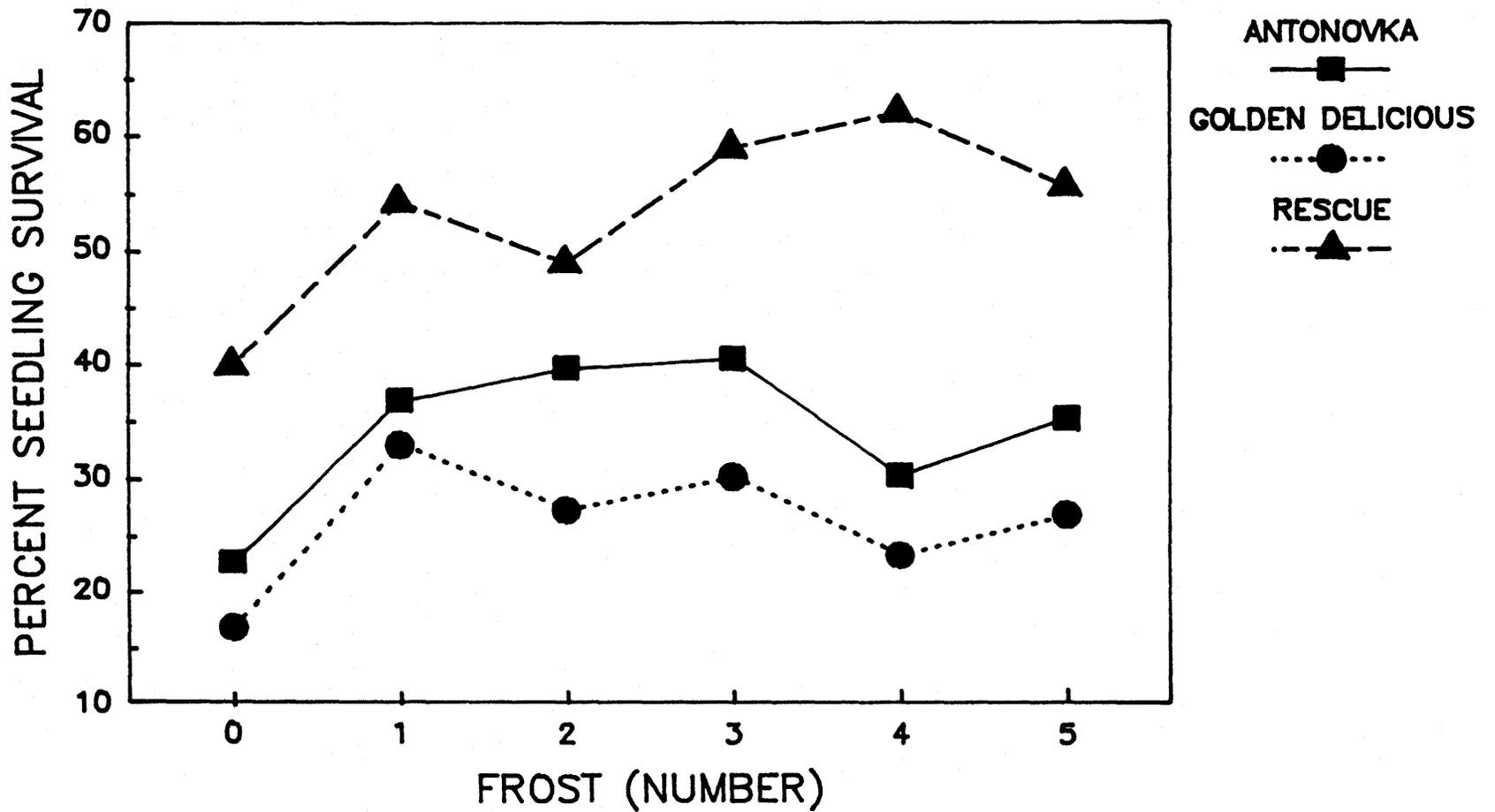


Figure 4.5. Cultivar seedlings X frost interaction of percent seedling survival data of the controlled acclimation experiment, sampled from three freeze temperatures.

4.0.3 Screening temperature

The main effects of temperature were highly significant (Table 4.1). The temperature X frost interaction graph (Figure 4.1) illustrates that the optimum screening temperature is -30°C . Very little of the population survived a freezing temperature of -40°C regardless of the frost preconditioning treatment applied. Well over 70% of the seedling population survived a freezing temperature of -20°C regardless of the number of frosts they were exposed to. A screening temperature approaching an LT_{50} (the temperature at which 50% of the plant population is killed) would be ideal. As indicated on the graph, the temperature that best differentiates that 50% of the population which is most hardy is somewhere between a freeze temperature of -20°C and -30°C .

The cultivar seedlings X temperature graph (Figure 4.4) indicates that for the hardiest cultivar seedling population, Rescue, a screening temperature of -30°C is actually too high. A freeze temperature below -30°C would better approach the LT_{50} . The less hardy cultivar seedling populations, Antonovka and Golden, could best be screened at approximately -25°C . The interaction is significant, suggesting that each cultivar seedling has an optimum LT_{50} screening temperature.

4.0.4 Replication significance

The examination of the replication means (Figure 4.6) reveals that there was a trend to decreasing survival as the summer progressed. The

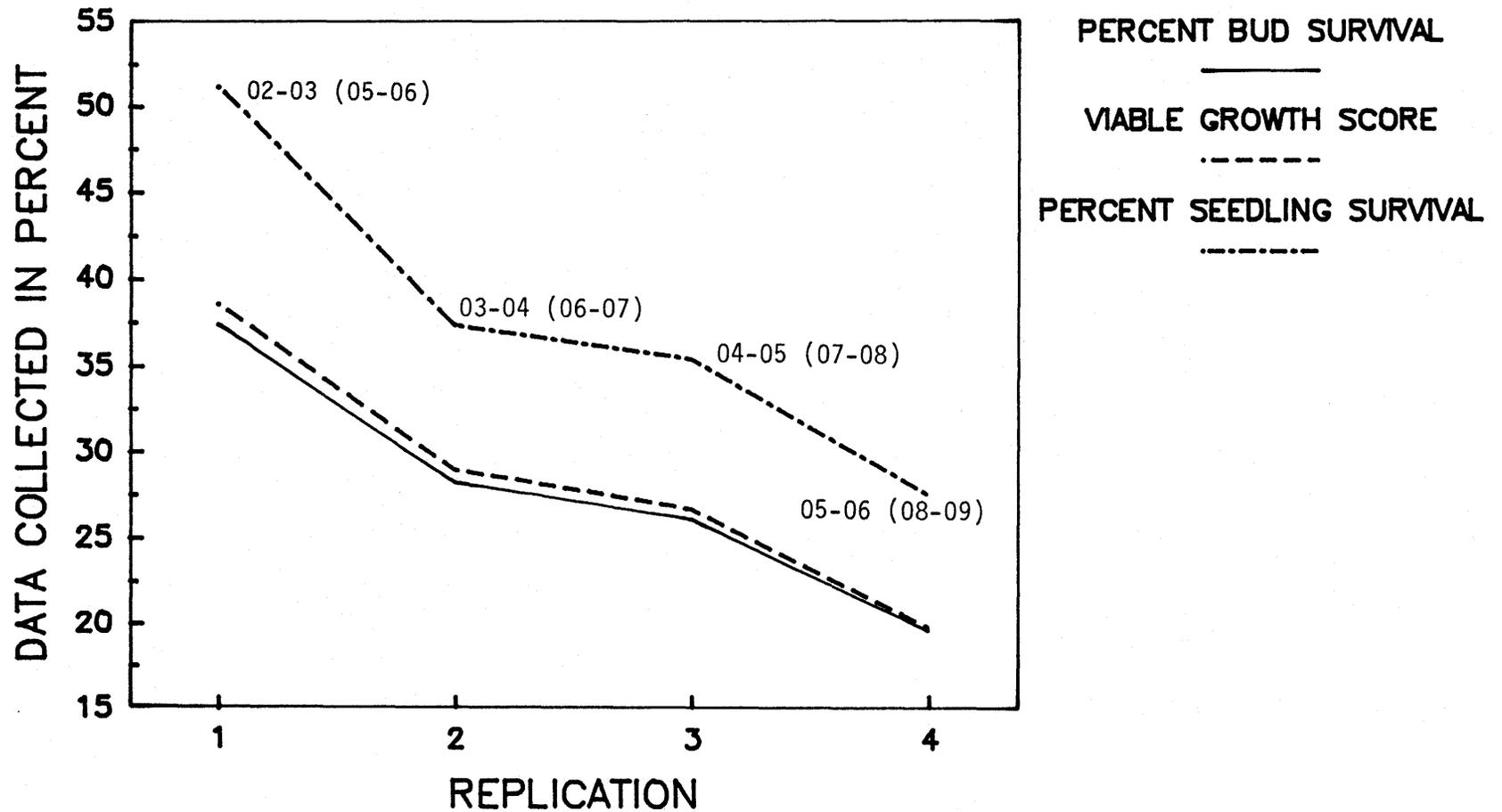


Figure 4.6. Replication means for the controlled acclimation experiment. The first value listed represents the months of the year grown in the greenhouse. The second value (in brackets) represents the months of the year regrown in the greenhouse.

fourth replication was damaged by Diazinon which resulted in a major decline in survival. The analysis of variance of the percent seedling survival data (Table 4.1) showed that replications were highly significant.

4.1 Freeze without acclimation experiment

4.1.0 Data collection methods

Analyses of variance were conducted as in the controlled acclimation experiment on all three data collection methods.

4.1.0.0 Percent seedling survival data

The analysis of variance of the percent seedling survival data (Table 4.7) showed significant differences due to temperature and replication. Cultivar seedling was nonsignificant, as was the cultivar seedlings X temperature interaction.

The first orthogonal contrast was highly significant indicating that -5°C was different than the average of -10 and -15°C (Table 4.7). The second contrast was also highly significant indicating -10°C was different from -15°C (Table 4.7).

4.1.0.1. Percent bud survival data

The analysis of variance of the percent bud survival data (Table

Table 4.7. Analysis of variance for the freeze without acclimation experiment using percent seedling survival data.

Source of variation	df	Mean square	F
Replication	3	1883	4.94**
Freeze temperature (T)	2	7975	20.91**
(C1) -5 vs -10 & -15°C	(1)	12238	32.09**
(C2) -10 vs -15°C	(1)	3713	9.73**
Cultivar seedlings (C)	2	42	0.11
C X T	4	970	2.54
Error	24	381	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

4.8) resulted in the same significant differences as exhibited by the percent seedling survival data analysis (Table 4.7), except that the contrast between -10 and -15°C was not significant for bud survival. To better demonstrate this difference, graphs of the cultivar seedlings X temperature interactions from the two data sets are presented (Figure 4.7 and 4.8). Each point on each of the two graphs represents 40 seedlings, the means of four replications. The percent seedling survival graph suggests that Antonovka may not be hardy at -5°C. This possibility can be entertained, but it is not conclusive. The percent seedling survival C X T interaction is only significant at the 7% level and the variability in the data could account for such a small measure of probability. Antonovka was probably hardy at -5°C as suggested in the percent bud survival graph (Figure 4.8). This conclusion is supported by the nonsignificance of the second contrast in the percent bud survival analysis (Table 4.8), and by the high significance of the C X T interaction for the transformed percent seedling survival data (Table 4.9). Because the C X T interaction becomes highly significant with the transformation, it is reasonable to suggest that the response of Antonovka at -5°C is irregular in Figure 4.7. The conclusion that all the cultivar seedlings were hardy to between -5 and -10°C also agrees with known hardiness levels of these cultivar seedlings in their non-preconditioned state.

4.1.0.2 Viable growth score

The analysis of variance of the viable growth score data (Table

Table 4.8. Analysis of variance for the freeze without acclimation experiment using percent bud survival data.

Source of variation	df	Mean square	F
Replication	3	2034	6.88**
Freeze temperature (T)	2	10992	37.18**
(C1) -5 vs -10 & -15°C	(1)	21451	72.57**
(C2) -10 vs -15°C	(1)	532	1.80
Cultivar seedlings (C)	2	67	0.23
C X T	4	221	0.75
Error	24	296	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

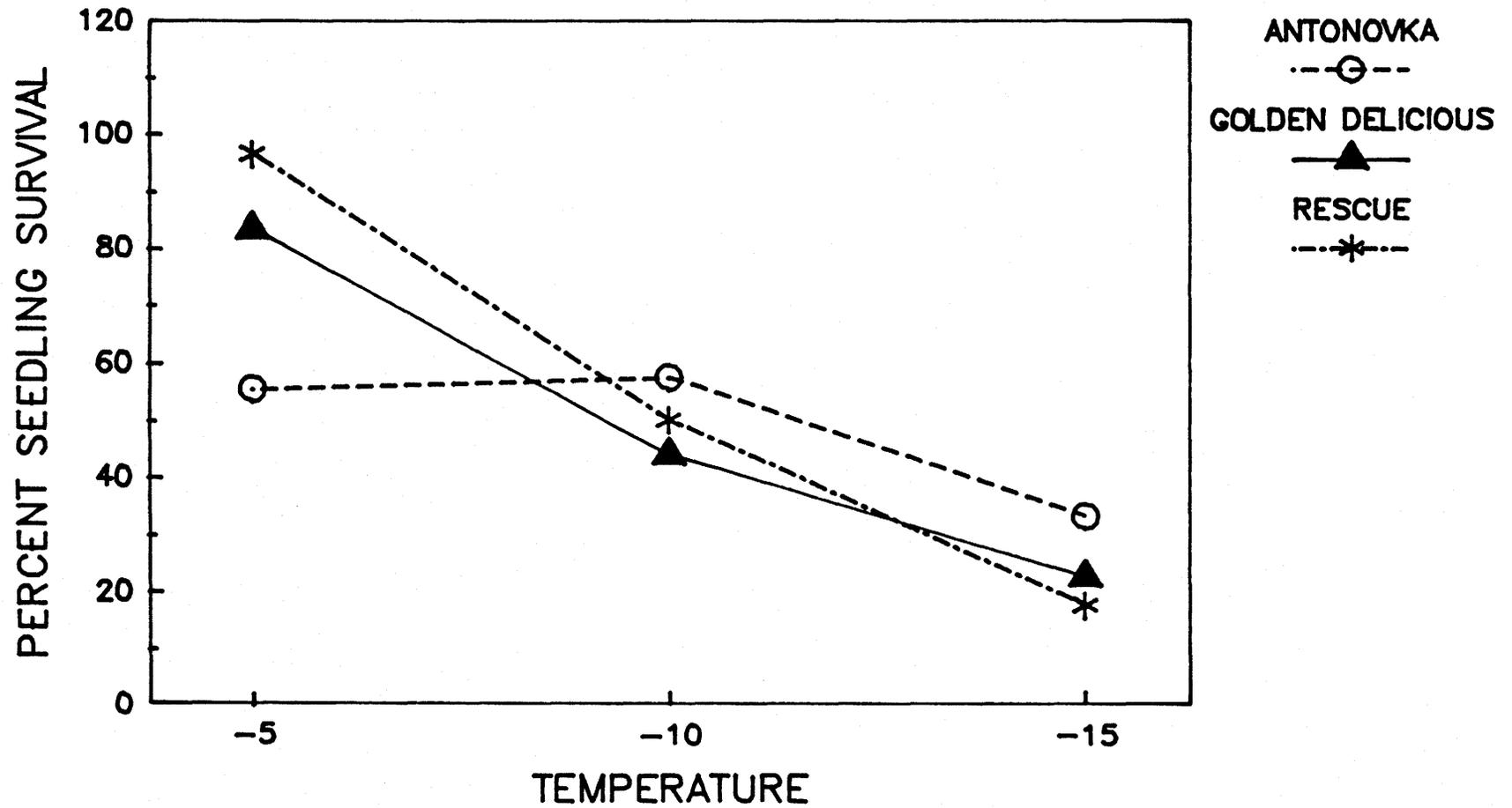


Figure 4.7. Cultivar seedlings X temperature interaction of percent seedling survival data of the freeze without acclimation experiment.

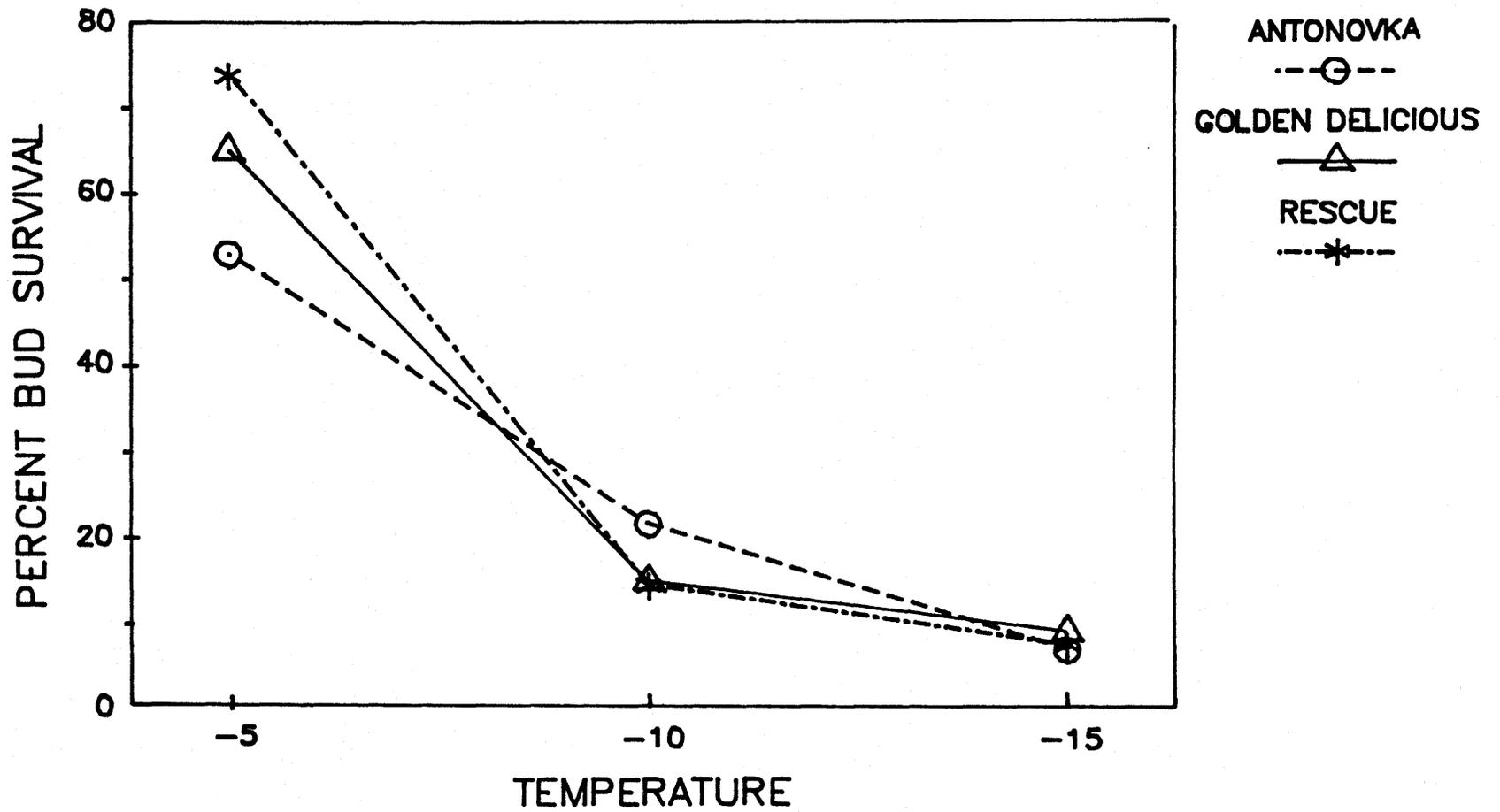


Figure 4.8. Cultivar seedlings X temperature interaction of percent bud survival data of the freeze without acclimation experiment.

Table 4.9. Analysis of variance of arc sine square root transformed percent seedling survival data (expressed in radians) for the freeze without acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	0.336	5.28**
Freeze temperature (T)	2	1.618	25.42**
(C1) -5 vs -10 & -15°C	(1)	2.498	39.24**
(C2) -10 vs -15°C	(1)	0.738	11.59**
Cultivar seedlings (C)	2	0.016	0.25
C X T	4	0.231	3.63**
Error	24	0.064	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

4.10) exhibited the same significant differences as the percent bud survival data (Table 4.8).

4.1.0.3 Transformation of data

Analyses of variance of arc sine square root transformations were conducted on all three data sets: percent seedling survival data (Table 4.9), percent bud survival data (Table 4.11), and viable growth score data (Table 4.12). The analyses of variance of the transformed data indicated the same general trends as displayed in the non-transformed data analyses (Tables 4.7, 4.8 and 4.10), with the C X T interaction for seedling survival being highly significant in the transformed scale.

4.1.0.4 Optimum method of data presentation

The transformation proved nonessential and the percent seedling survival data analysis gave the most precise results over the percent bud survival and viable growth score analyses. Only the non-transformed percent seedling survival data tables and figures are presented for the remainder of the freeze without acclimation experiment results and discussion.

4.1.1 Cultivar seedling response

The analysis of variance of the seedling survival data (Table 4.7)

Table 4.10. Analysis of variance for the freeze without acclimation experiment using viable growth score data.

Source of variation	df	Mean square	F
Replication	3	1929	5.81**
Freeze temperature (T)	2	11053	33.26**
(C1) -5 vs -10 & -15°C	(1)	21731	65.40**
(C2) -10 vs -15°C	(1)	375	1.13
Cultivar seedlings (C)	2	24	0.07
C X T	4	228	0.69
Error	24	332	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.11. Analysis of variance of arc sine square root transformed percent bud survival data (expressed in radians) for the freeze without acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	0.464	8.57**
Freeze temperature (T)	2	2.026	37.43**
(C1) -5 vs -10 & -15°C	(1)	3.848	71.10**
(C2) -10 vs -15°C	(1)	0.203	3.75
Cultivar seedlings (C)	2	0.015	0.27
C X T	4	0.045	0.83
Error	24	0.054	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.12. Analysis of variance of arc sine square root transformed viable growth score data (expressed in radians) for the freeze without acclimation experiment.

Source of variation	df	Mean square	F
Replication	3	0.440	7.16**
Freeze temperature (T)	2	2.054	33.43**
(C1) -5 vs -10 & -15°C	(1)	3.951	64.29**
(C2) -10 vs -15°C	(1)	0.158	2.57
Cultivar seedlings (C)	2	0.009	0.15
C X T	4	0.046	0.75
Error	24	0.061	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

showed that cultivar seedling differences were nonsignificant. The cultivar seedlings X temperature interaction (Figure 4.7) was also nonsignificant. For Rescue and Golden Delicious, survival decreased linearly with decreasing temperatures (Figure 4.7). However, the Antonovka had similar survival at -5 and -10°C. Without preconditioning or cold acclimation, *Malus* should be hardy to approximately -7°C. There is no differentiation of cultivar seedlings based upon the screening temperature because all cultivar seedlings are hardy to the same level without preconditioning. Each point on the graph represents 40 seedlings, that is, means of four replications.

4.1.2 Replication significance

The graph of the replication means (Figure 4.9) reveals a trend to increasing survival, without preconditioning, as the summer progressed. The analysis of variance of the percent seedling survival data indicated that replications were highly significant (Table 4.7).

4.2 Cool temperature duration experiment

4.2.0 Data collection methods

Analyses of variance were conducted on all three methods of data collection.

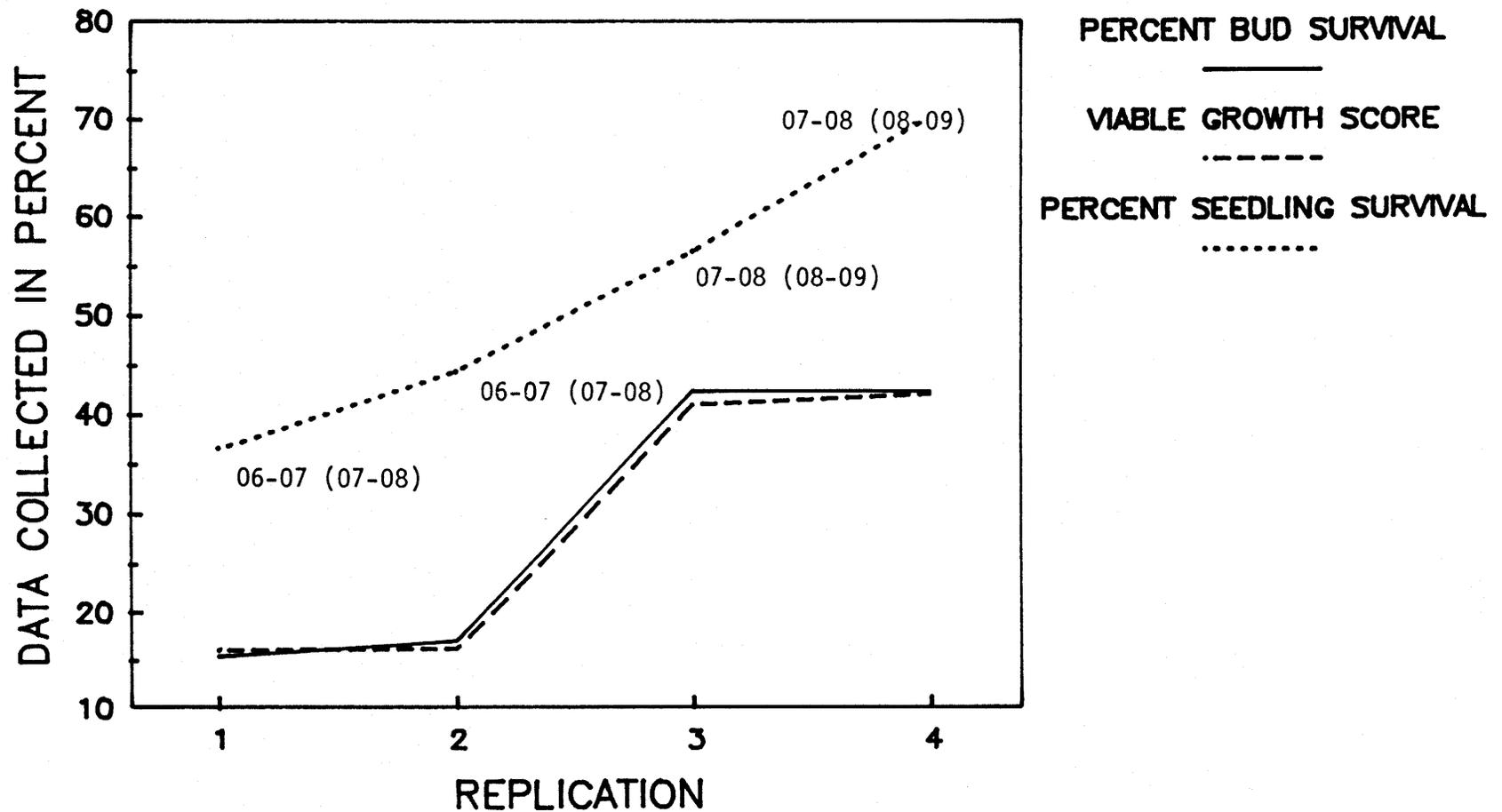


Figure 4.9. Replication means for the freeze without acclimation experiment. The first value listed represents the months of the year grown in the greenhouse. The second value (in brackets) represents the months of the year regrown in the greenhouse.

4.2.0.0 Percent seedling survival data

The analysis of variance of the percent seedling survival data (Table 4.13) indicated significant differences due to replication, cultivar seedling, temperature and frost. The interaction of cultivar seedlings X temperature was also significant. The temperature X frost, cultivar seedlings X frost and cultivar seedlings X temperature X frost interactions were nonsignificant for percent seedling survival.

4.2.0.1 Percent bud survival data

The analysis of variance of the percent bud survival (Table 4.14) indicated the same significant differences as the percent seedling survival data analysis (Table 4.13), except that the temperature X frost interaction was significant for bud survival. To better illustrate this difference, the two graphs of the interactions are presented (Figures 4.10 and 4.11). Each point on each of the two graphs represents 216 seedlings, that is, the means of three cultivar seedling populations evaluated in four replications. The -20°C line is slightly steeper in the percent bud survival graph (Figure 4.10) and the -40°C line makes only a small departure from the zero axis. In the percent seedling survival graph (Figure 4.11) all three temperature lines make relatively the same rise off the x-axis. In the percent seedling survival graph, the points on the graph represent higher values than on the percent bud survival graph. The expansion of the y-axis resulting from the higher values explains why the percent seedling

Table 4.13. Analysis of variance of the cool temperature duration experiment using percent seedling survival data.

Source of variation	df	Mean square	F
Replication	3	1264	5.00**
Cultivar seedlings (C)	2	4583	18.14**
Freeze temperature (T)	2	34087	134.89**
Frost treatment (F)	1	2547	10.08**
T X F	2	251	0.99
C X T	4	837	3.31**
C X F	2	100	0.40
C X T X F	4	562	2.22
Error	51	253	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.14. Analysis of variance of the cool temperature duration experiment using percent bud survival data.

Source of variation	df	Mean square	F
Replication	3	756	4.00**
Cultivar seedlings (C)	2	2237	11.85**
Freeze temperature (T)	2	26387	139.69**
Frost treatment (F)	1	2605	13.79**
T X F	2	1261	6.67**
C X T	4	626	3.46**
C X F	2	199	1.05
C X T X F	4	426	2.26
Error	51	189	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

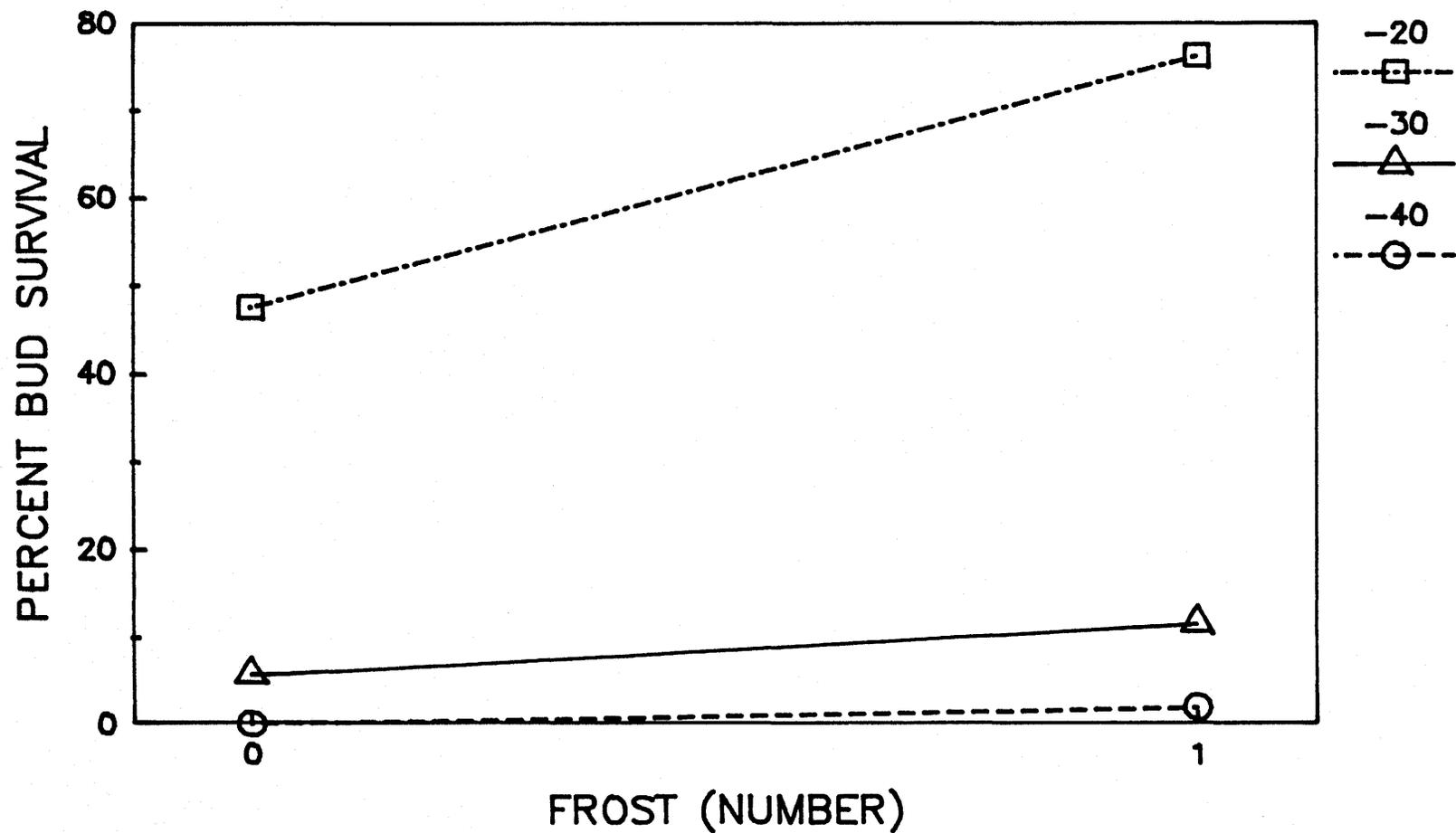


Figure 4.10. Temperature X frost interaction of percent bud survival data of the cool temperature duration experiment, sampled from three apple cultivar seedling populations.

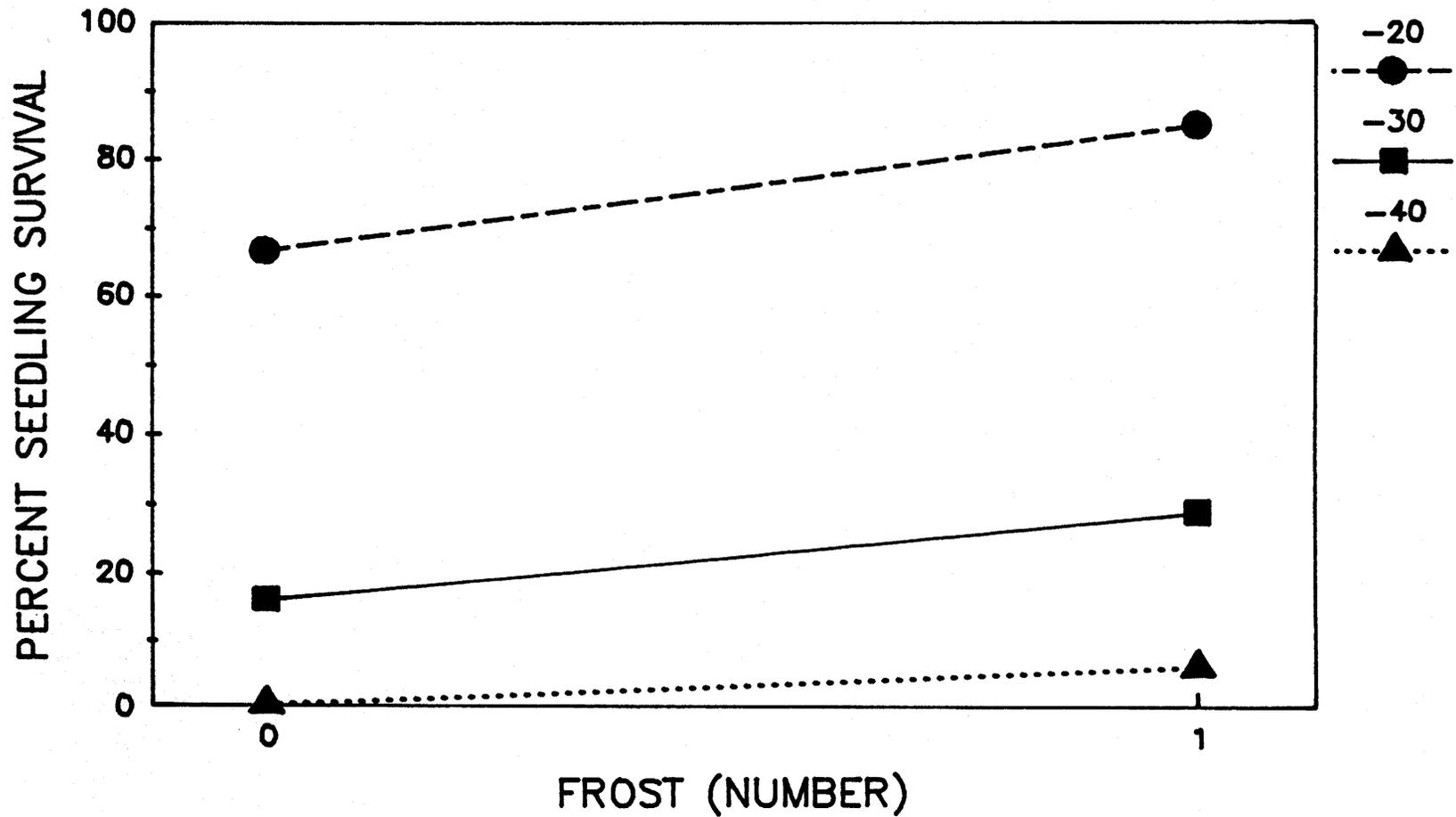


Figure 4.11. Temperature X frost interaction of percent seedling survival data of the cooler time experiment, sampled from three apple cultivar seedling populations.

survival data presents a nonsignificant interaction.

4.2.0.2 Viable growth score

The analysis of variance of the viable growth score data (Table 4.15) showed the same significant differences as in the percent bud survival analysis (Table 4.14), except that the cultivar seedlings X temperature interaction was not significant for the viable growth score. An examination of the temperature X frost interaction graph (Figure 4.12) for the percent viable growth score data shows exactly the same situation as explained in Section 4.2.0.1 for the percent seedling survival and percent bud survival data. A comparison of Figure 4.10 (percent bud survival data) and Figure 4.12 (viable growth score data) indicates identical trends. Therefore, the explanation given for the percent bud survival data versus percent seedling survival data holds as well for viable growth score data.

In the percent seedling and percent bud analyses the cultivar X temperature interaction was significant. In the viable growth score the interaction was not significant. To better illustrate this difference between seedling survival and viable growth, graphs of the interactions are presented in Figures 4.13 and 4.14. Each point on each of the two graphs represents 144 seedlings, that is, means of two frost treatments evaluated in four replications.

The two graphs, Figure 4.13 and Figure 4.14, show similar trends. In the percent seedling survival graph (Figure 4.13), however, Rescue shows a more steady decline in mortality than it does in the viable

Table 4.15. Analysis of variance of the cool temperature duration experiment using viable growth score data.

Source of variation	df	Mean square	F
Replication	3	1038	4.36**
Cultivar seedlings (C)	2	1908	8.02**
Freeze temperature (T)	2	24827	104.32**
Frost treatment (F)	1	3191	13.41**
T X F	2	1598	6.71**
C X T	4	532	2.23
C X F	2	205	0.86
C X T X F	4	345	1.45
Error	51	238	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

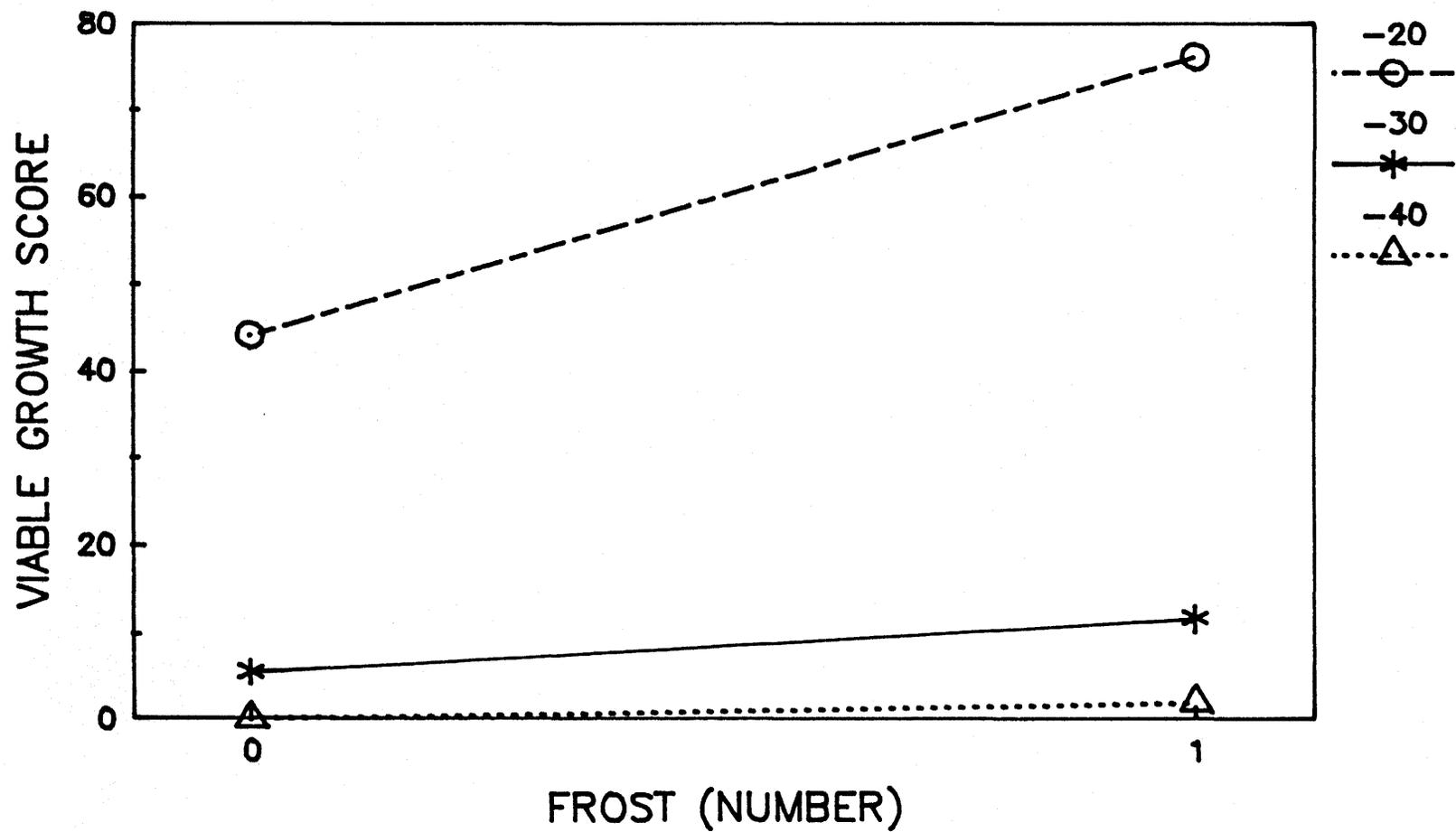


Figure 4.12. Temperature X frost interaction of viable growth score data of the cool temperature duration experiment, sampled from three apple cultivar seedling populations.

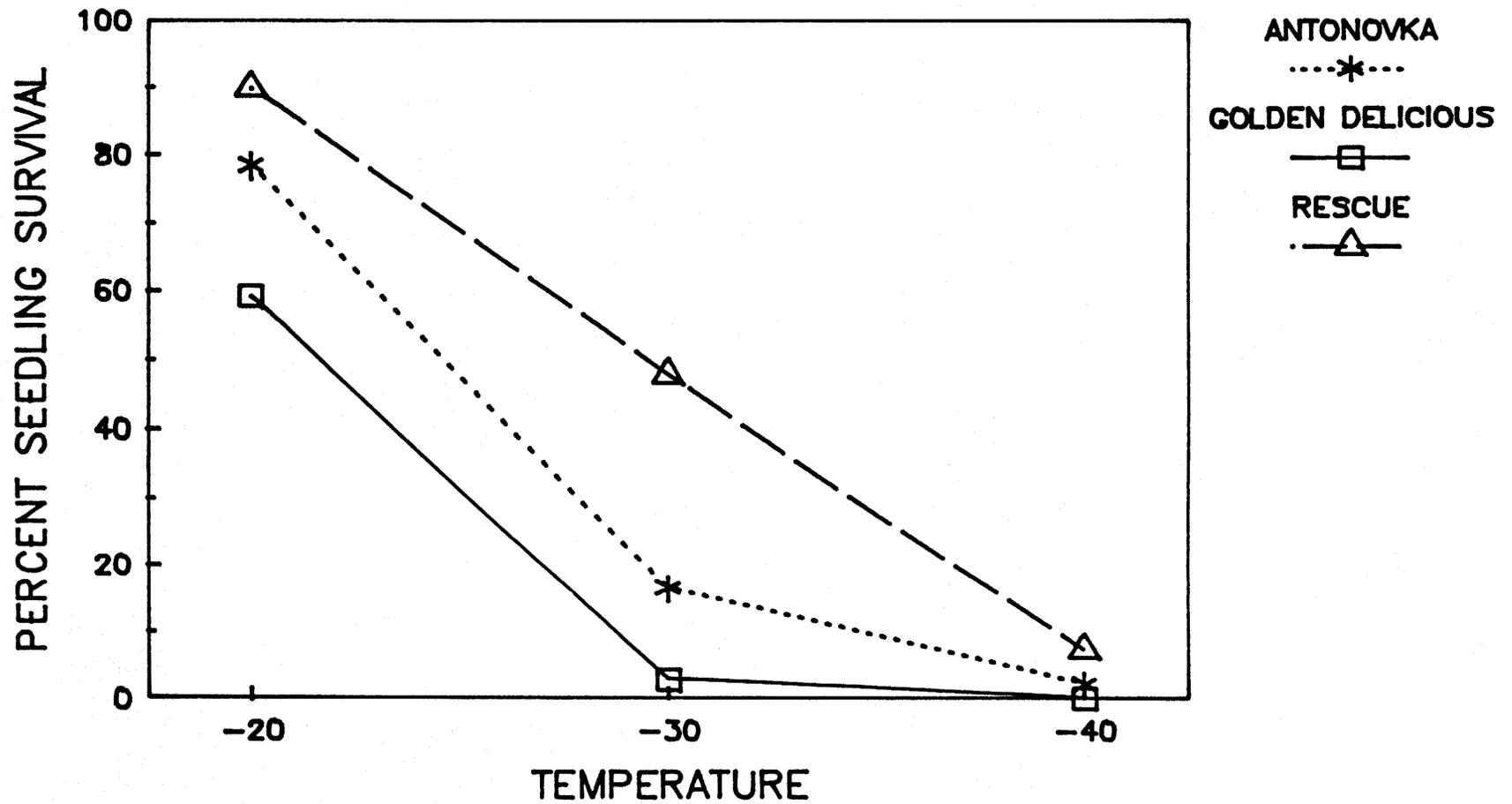


Figure 4.13. Cultivar seedlings X temperature interaction of percent seedling survival data of the cooler time experiment, sampled from two frost treatments.

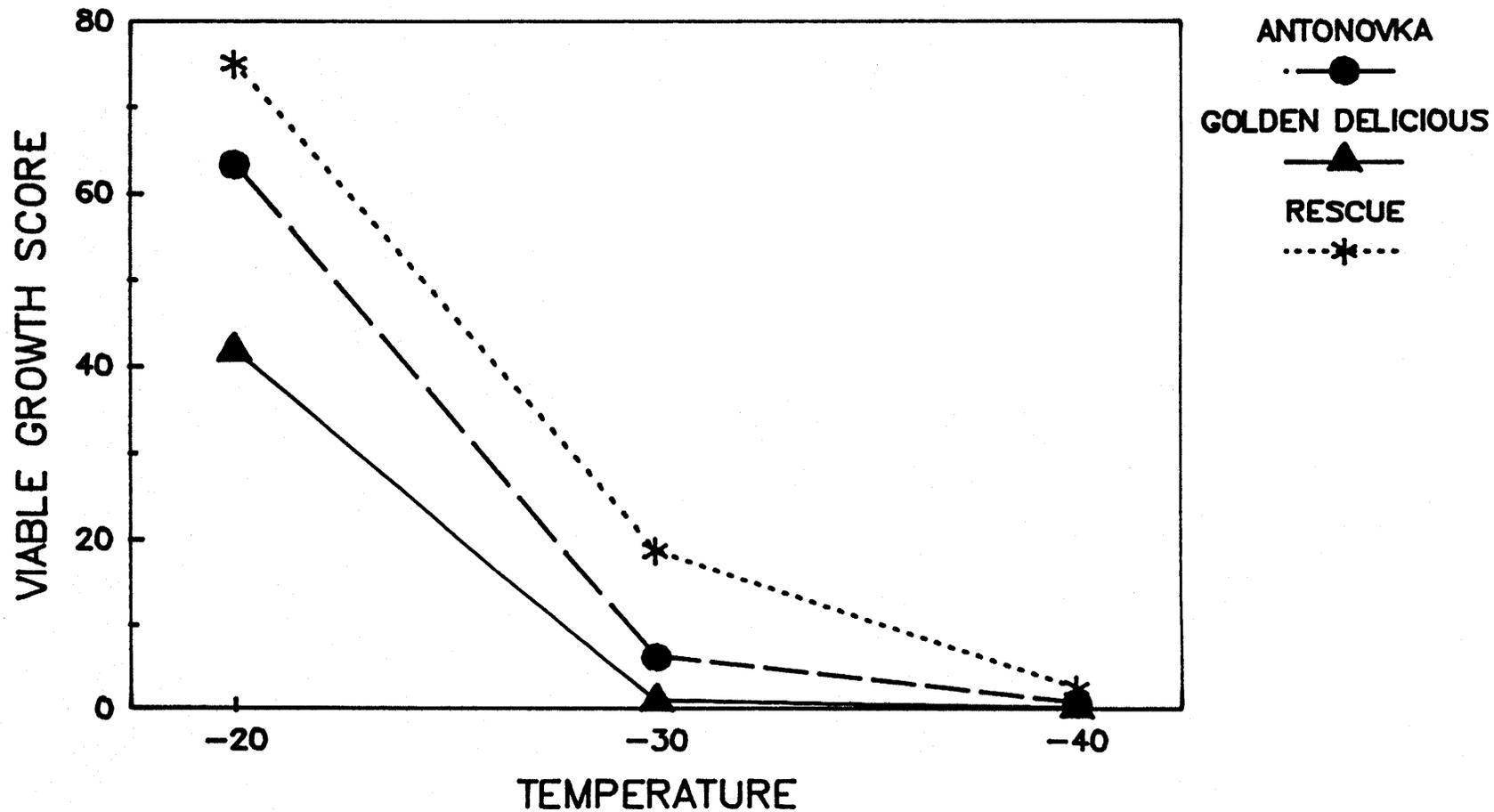


Figure 4.14. Cultivar seedlings X temperature interaction of viable growth score data of the cool temperature duration experiment, sampled from two frost treatments.

growth score graph (Figure 4.14). Since the percent seedling survival analysis presents a significant interaction and the viable growth score analysis does not, percent seedling survival appears to be the more precise test.

4.2.0.3 Transformation of data

An arc sine square root transformation was conducted on all three data sets: percent seedling survival data (Table 4.16), percent bud survival data (Table 4.17), and viable growth score data (Table 4.18). The analyses of variance of the transformed data indicated the same significant differences as in the non-transformed data analyses, the two exceptions being the cultivar seedlings X temperature interaction in both the seedling and bud survival analyses. In the non-transformed data the cultivar seedlings X temperature interaction was significant. In the transformed data analysis the interaction was nonsignificant. Since the non-transformed data represents a significant interaction and the viable growth score data does not, we conclude the non-transformed data has a higher level of precision.

4.2.0.4 Optimum method of data presentation

Since the non-transformed data gave more accurate results over the transformed data and the percent seedling survival analysis presented the same relative trends as the other two data sets, only the non-transformed percent seedling survival data tables and figures will be

Table 4.16. Analysis of variance of the arc sine square root transformed percent seedling survival data (expressed in radians) for the cool temperature duration experiment.

Source of variation	df	Mean square	F
Replication	3	0.275	4.80**
Cultivar seedlings (C)	2	1.006	17.56**
Freeze temperature (T)	2	6.698	116.94**
Frost treatment (F)	1	0.621	10.84**
T X F	2	0.036	0.63
C X T	4	0.146	2.55
C X F	2	0.025	0.43
C X T X F	4	0.104	1.82
Error	51	0.057	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.17. Analysis of variance of the arc sine square root transformed percent bud survival data (expressed in radians) for the cool temperature duration experiment.

Source of variation	df	Mean square	F
Replication	3	0.017	4.07**
Cultivar seedlings (C)	2	0.512	12.37**
Freeze temperature (T)	2	5.540	133.83**
Frost treatment (F)	1	0.664	16.04**
T X F	2	0.195	4.71**
C X T	4	0.075	1.80
C X F	2	0.292	0.71
C X T X F	4	0.076	1.83
Error	51	0.041	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

Table 4.18. Analysis of variance of the arc sine square root transformed viable growth score data (expressed in radians) for the cool temperature duration experiment.

Source of variation	df	Mean square	F
Replication	3	0.216	4.51**
Cultivar seedlings (C)	2	0.446	9.33**
Freeze temperature (T)	2	5.295	110.73**
Frost treatment (F)	1	0.784	16.39**
T X F	2	0.240	5.02**
C X T	4	0.064	1.34
C X F	2	0.031	0.65
C X T X F	4	0.062	1.31
Error	51	0.048	

*, ** Significant at the 0.05 and 0.01 levels of probability, respectively.

presented for the remainder of the cool temperature duration experiment results and discussion.

4.2.1 Cultivar seedling response

As indicated in the percent seedling survival data analysis of variance (Table 4.13), cultivar seedling main effects were highly significant. The cultivar seedlings X temperature interaction graph (Figure 4.13) indicates cultivar seedling differences are quite pronounced following acclimation. Each point on the graph represents 144 seedlings, that is, means of two frost treatments in each of four replications. The level of seedling survival decreased as the screening temperatures decreased. Rescue was known to be inherently the hardiest cultivar seedling. Golden Delicious was known to be the most tender of the three and Antonovka was recognized as of inherent intermediate hardiness. The graph supports the expectations of inherent hardiness response. The interaction of cultivar seedlings X temperature was significant. The fatality rate was higher for the less hardy cultivars seedlings, with both Antonovka and Golden Delicious going to zero at -40°C . The fatality rate for Rescue was most steady over the three screening temperatures and maintained approximately a 10 percent seedling survival at -40°C .

4.2.2 Frost requirement

The main effects of the frost treatments were highly significant

(Table 4.13). The cultivar seedlings X frost interaction graph (Figure 4.15) is presented to better demonstrate the cultivar seedling response to frost. Each point on the graph represents 216 seedlings, that is, means of three freeze temperatures evaluated in four replications. The interaction was nonsignificant, showing that all three cultivar seedlings responded the same to the frost treatments. It was previously described in Section 4.0.2 of the controlled acclimation experiment that the main frost effect was due to the difference between zero and one or more frosts. Values for the relative increases in seedling survival associated with the frost effect were similar for each of the three cultivar seedlings.

A series of three figures are presented, one for each cultivar seedling: Figure 4.16 (Rescue), Figure 4.17 (Antonovka), and Figure 4.18 (Golden Delicious). The figures provide a comparison for each cultivar seedling of the frost response in the controlled acclimation experiment, with the frost response in the cool temperature duration experiment. The figures show that, with the exception of Golden Delicious, there is a general increase in survival with a lag time after frost exposure. These figures indicate that there is no relative detrimental effect to leaving seedlings in the cooler for up to 10 days after a frost exposure, and that up to 11 days can elapse before frosts are applied to seedlings without significantly varying the freeze test results.

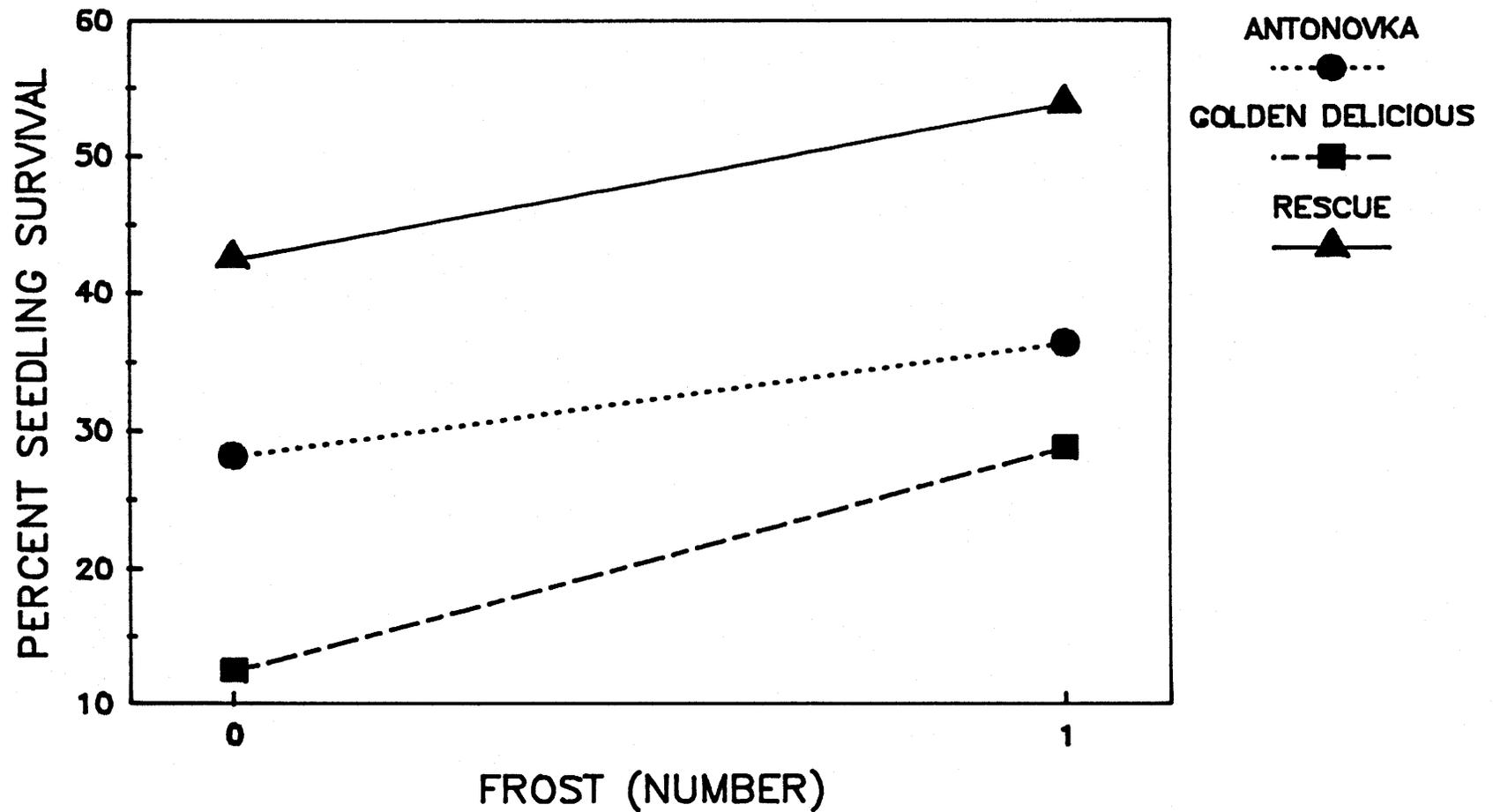
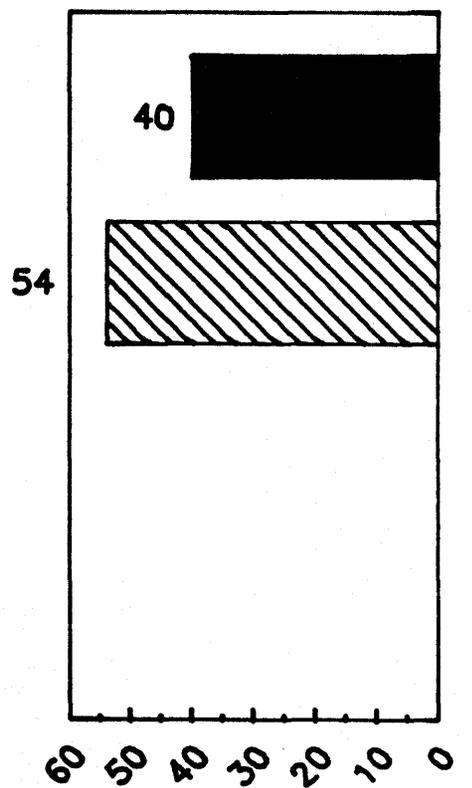


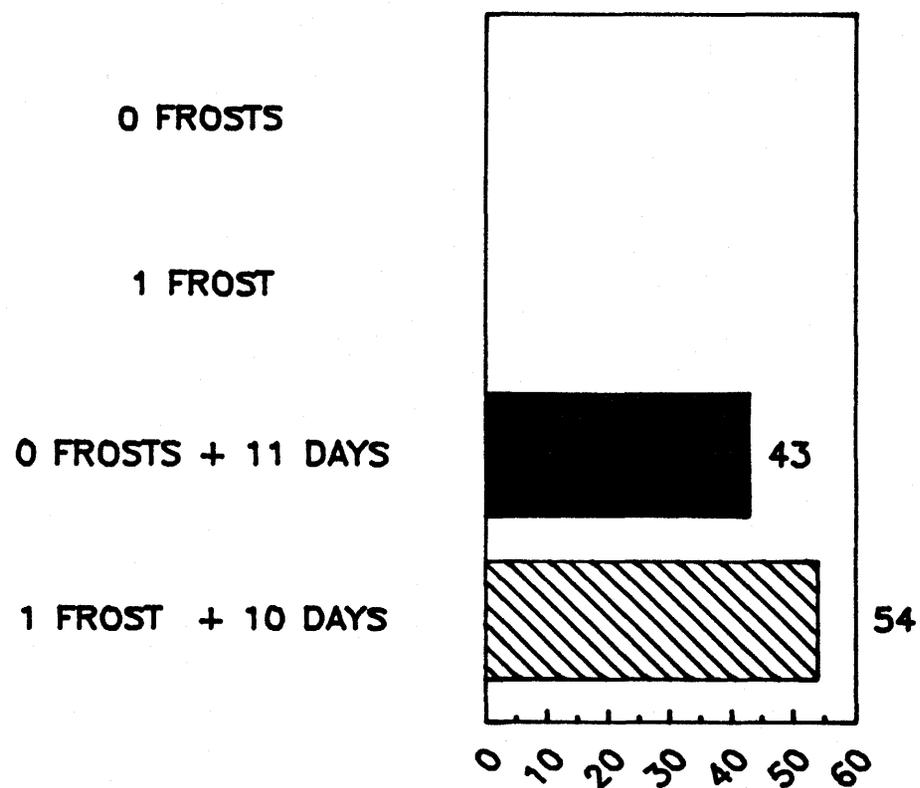
Figure 4.15. Cultivar seedlings X frost interaction of percent seedling survival data of the cool temperature duration experiment, sampled over three freeze temperatures.

CONTROLLED ACCLIMATION EXPERIMENT



PERCENT SEEDLING SURVIVAL

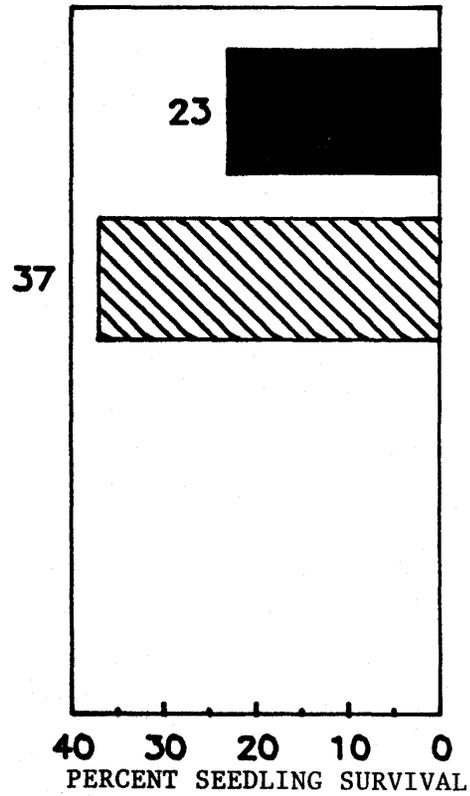
COOL TEMPERATURE DURATION EXPERIMENT



PERCENT SEEDLING SURVIVAL

Figure 4.16. A comparison of frost response with Rescue between the controlled acclimation experiment and the cool temperature duration experiment, for the percent seedling survival data.

CONTROLLED ACCLIMATION EXPERIMENT



COOL TEMPERATURE DURATION EXPERIMENT

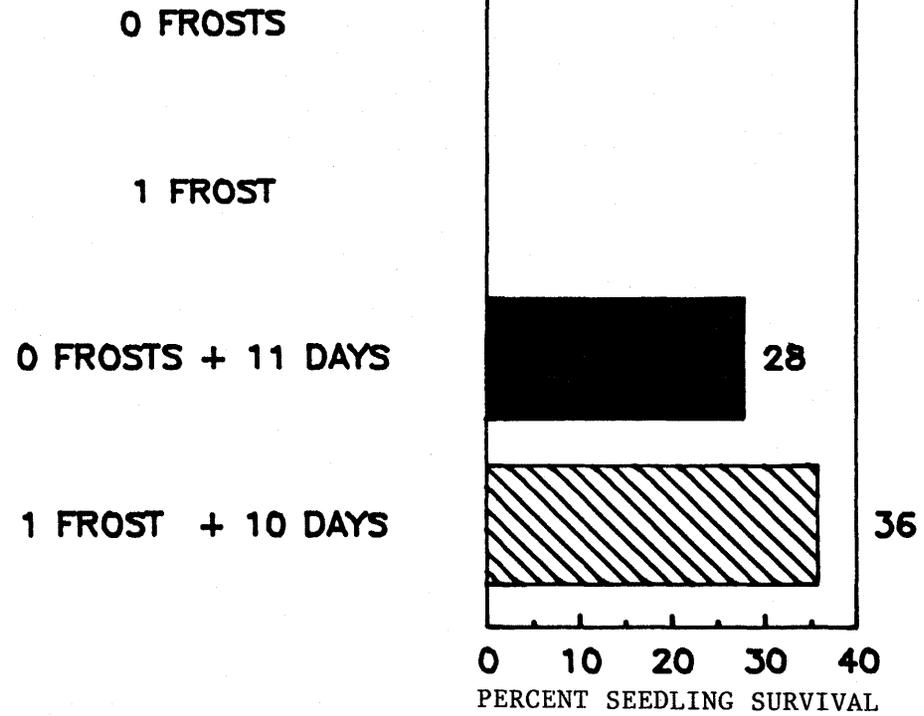
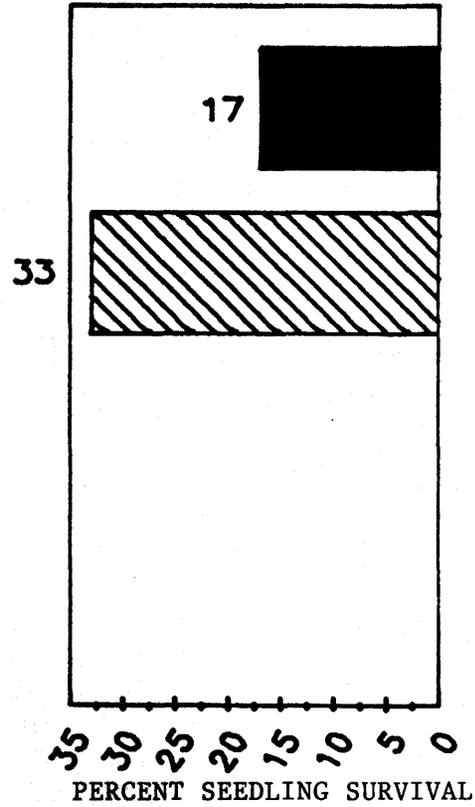


Figure 4.17. A comparison of frost response with Antonovka between the controlled acclimation experiment and the cool temperature duration experiment, for the percent seedling survival data.

CONTROLLED ACCLIMATION EXPERIMENT



COOL TEMPERATURE DURATION EXPERIMENT

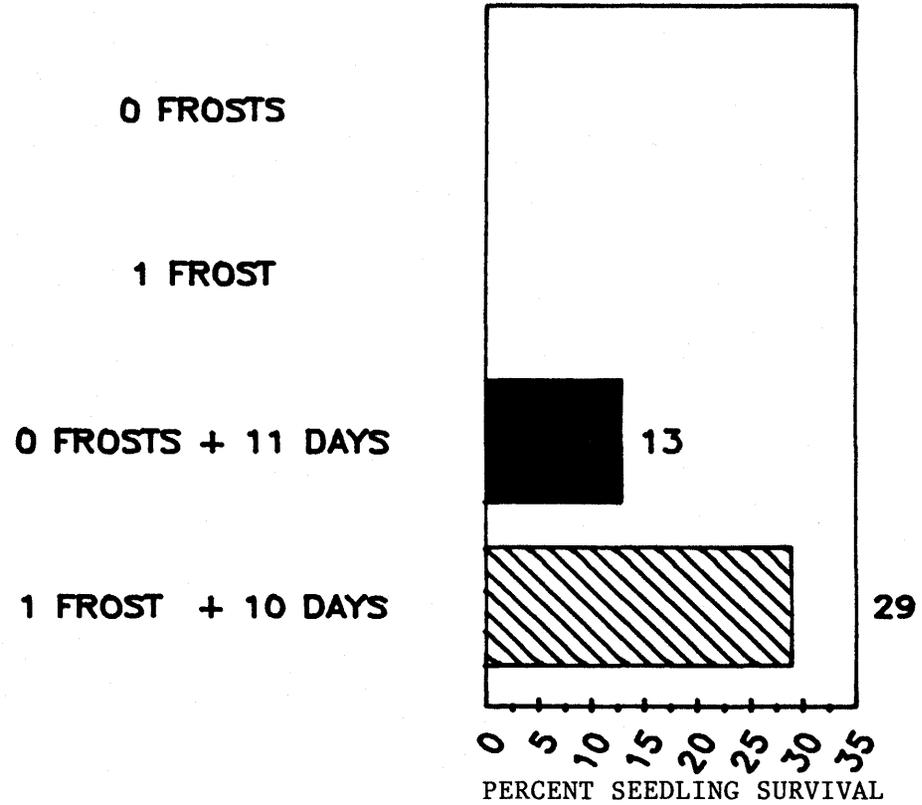


Figure 4.18. A comparison of frost response with Golden Delicious between the controlled acclimation experiment and the cool temperature duration experiment, for the percent seedling survival data.

4.2.3 Replication significance

Examination of the replication means (Figure 4.19) showed that there was a significant increase in survival from replication one to replication two. The analysis of variance of the percent seedling survival data (Table 4.13) indicated that replications were highly significant. Most of the variability of this significance can be accounted for by the first replication.

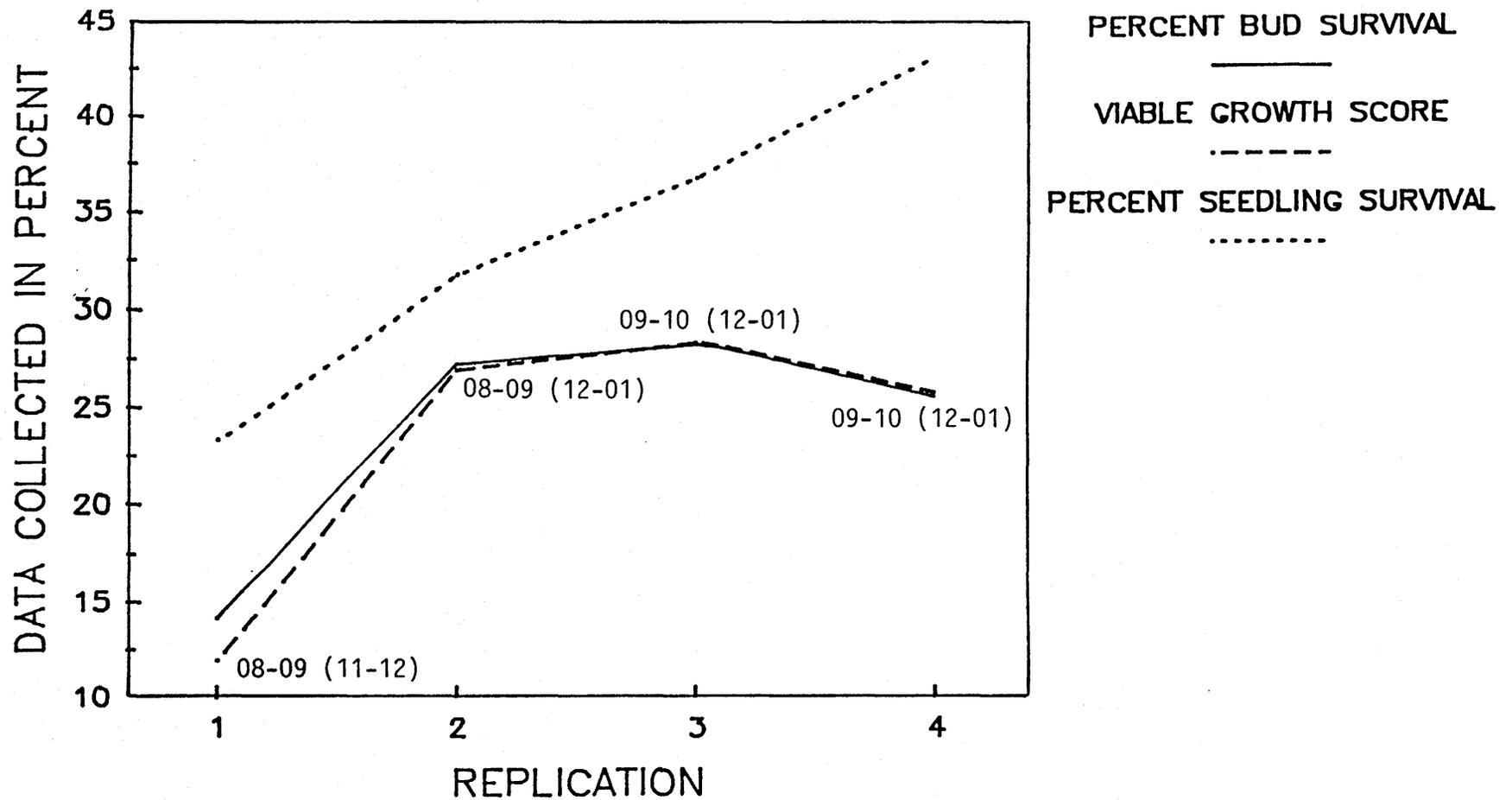


Figure 4.19. Replication means for the cool temperature duration experiment. The first value represents the months of the year grown in the greenhouse. The second value (in brackets) represents the months of the year regrown in the greenhouse.

5. Discussion

5.0 Cultivar response

Cold hardiness is an inducible character dependent on exposure to cold temperatures for expression (Levitt 1980; Fuchigami et al. 1982). Through the interactions of appropriate environmental cues and inherent genetic potential of the species, an increase in cold hardiness can occur (Steponkus 1978). In this experiment, exposure to acclimating conditions resulted in an increase in cold hardiness for seedlings of all three *Malus* cultivars tested. The survival of the seedlings agreed with expectations of inherent hardiness responses before acclimation (Section 5.0.0) and after acclimation (Section 5.0.1). In screening seedling populations of *Malus* for cold resistance, acclimated seedlings were necessary.

5.0.0 Non-acclimated seedlings

In order for woody plants to develop a high degree of cold hardiness, the plants must be fully dormant (Fuchigami et al. 1982). Magness (1929) found that non-acclimated apple trees were killed by temperatures as high as -7°C . Ketchie (1985) found that non-acclimated xylem and bark of *Malus* were hardy to -8 and -10°C respectively. Without proper photoperiods and ambient temperature controls, cold acclimation will not be induced.

Transfer of 6-week-old seedlings from long (12-16 hour) days and

20-30°C temperatures to controlled freezes reaching temperatures of -10 and -15°C resulted in approximately 90% kill of the seedling populations. The seedlings were hardy in a controlled freeze to -5°C and thus supported the findings of the afore-mentioned researchers. The degree of cold hardiness obtained by a plant is under genetic control, but environmental factors must prompt the hardening process. Growth cessation is important to the accumulation of reserves used in the hardiness period: an actively growing woody plant will not be hardy (Levitt 1980). Chandler found (1913) that non-acclimated apple roots were killed in artificial freezes at -3°C. Acclimated roots, however, were hardy to -12°C, even in a rapid freeze. In this experiment, the non-acclimated seedling roots were maintained at temperatures above 0°C.

5.0.1 Acclimated seedlings

Cold acclimation occurred after exposure to short (8-hour) days and cool (+3 to +5°C) temperatures. In this experiment, Rescue and Antonovka represented northern latitude cultivar seedlings and Golden Delicious represented mid-latitude cultivar seedlings. Strang (1974) found that acclimated Rescue was hardy to -40°C. Quamme (1987) found that acclimated Antonovka was hardy to a T_{10} of -37°C (the temperature at which 10% of the shoots were killed) and acclimated Golden Delicious was hardy to a T_{10} of -31°C. The results of this experiment supported the work by Quamme (1987) and Strang (1974) in the relative hardiness ranking of seedlings from the three cultivar seedlings studied: Rescue

would be considered very hardy, Antonovka hardy, and Golden Delicious tender.

Fuchigami et al. (1982) identified two stages in the process of hardening. The first stage was dependent on short day induction at warm temperatures. The second stage was dependent on exposure to low temperatures near or at 0°C (Fuchigami et al. 1982; Howell and Weiser 1970; Siminovitch 1982). Stushnoff et al. (1985) determined that a period of short days at warm temperatures did not increase the survival of seedlings from a northern latitude cultivar Dolgo, but did increase the survival of a seedling population of Golden Delicious, a mid-latitude cultivar.

Vince-Prue (1976) proposed that the acclimation of northern ecotypes was dominated by light. Stushnoff et al.'s (1985) work and this experiment supported Vince-Prue's proposal. The northern ecotype acclimates in response to shortening photoperiods regardless of the ambient temperature. Golden Delicious might have shown an increase in the rate or depth of acclimation, had a short day warm temperature treatment been imposed in this experiment. The Golden Delicious did show an acclimation response, however, following the application of a short day cool temperature treatment. A cultivar seedling response such as described in Section 4.0.1 and indicated in Figure 4.4 suggests an adequate differentiation of the three seedling populations for cold screening purposes. It is accepted, therefore, that the artificial cold acclimation process outlined in Section 3.0.2 is suitable for screening both northern and mid-latitude cultivar seedlings.

5.1 Frost requirement

The results of this experiment supported the work of Stushnoff et al. (1985) where they found that treatment applications of temperatures below but near 0°C, after a period of exposure to short days and cool temperatures, increased the hardiness levels of both Dolgo and Golden Delicious seedling populations. The increase in survival was more pronounced for the northern latitudes cultivar than for the mid-latitudes cultivar.

5.1.0 Response to frost

The exposure to one frost cycle, as outlined in Section 3.0.2., resulted in an increase in survival over no frosts of 74% for Rescue, 62% for Antonovka and 51% for Golden Delicious.

5.1.1 Relationship of frost response to cultivar

As shown in Figure 4.5, the interaction of cultivar seedlings X frost was nonsignificant. All three cultivar seedlings responded similarly to the frost treatments. The nonsignificant residual of the orthogonal contrast (Table 4.1) indicated that the main frost effect was due to the difference between zero and one or more frosts (Section 4.0.2). The conformity of response of all three cultivar seedlings may have resulted from the same gene combinations triggering the acclimation response.

Since secondary thickening must be present in the cell walls for supercooling to occur (Gusta et al. 1983), it is probable that no supercooling occurs in 12-week-old apple seedlings. The system of stress resistance, therefore, may be a tolerance mechanism, perhaps similar to the one described in Section 2.4.0. Williams et al. (1981) performed studies to determine compression/decompression curves for acclimated and non-acclimated cells from the very hardy winter wheat cultivar Kharkov. They proposed that a flattening in the curve of the acclimated cells indicated that hardened cells contained substances which facilitated the reversible removal of lipids during the stress imposed by cell shrinkage. The high degree of freeze resistance exhibited by all three cultivar seedlings resulting from one frost exposure (Figure 4.4) could be attributed to a lipid removal and reincorporation ability (Williams et al. 1981; Gordon-Kamm and Steponkus 1984), but this hypothesis would require testing. The increase in hardness with one frost exposure, followed by a flattening in the curve with subsequent frost exposures, could indicate that the ability to replace stored lipids in the membrane during deplasmolysis had been facilitated by the single frost exposure.

5.1.2 Evaluation of increased time in cooler after frost exposure

The finding that one frost maximized the acclimation response for the differentiation of superior individuals in the seedling screen process is of great importance. Of equal importance, however, is the finding that a lag time of up to 10 days could follow the frost

exposure without a significant reduction in cold survival (Section 4.2.2). In addition, up to 11 days could elapse after the six-week period in the cooler, before the frost treatment was applied, with no significant decrease in the hardiness response (Figures 4.16, 4.17 and 4.18). A comparison of the 0 frost bar and the 0 frosts + 11 days bar in these three figures illustrates this effect. These findings in regard to frost response greatly relieve the time pressure on the researcher.

5.2 Optimum screening temperature

The cultivar seedling response to different freeze temperatures was quite pronounced. Both cultivar seedling and temperature main effects were highly significant, as was the cultivar seedlings X temperature interaction (Table 4.1). Stushnoff et al. (1983) proposed that it might be possible to select cultivar seedlings for specific environmental conditions because definite differences in response to freeze temperatures occurred, and the results of this experiment supported Stushnoff's proposal.

5.2.0 Inherent cultivar seedling hardiness

Apple cultivar seedling hardiness is usually expressed in terms of minimum survival temperature. Many other factors besides minimum survival temperature, however, are important in determining hardiness for a specific region (Strang 1974). It is important to know how

quickly the cultivar seedling acclimates in the fall or deacclimates in the spring, and what response it displays to mid-winter warming trends. Strang (1974) developed a hardiness classification method in which apple cultivar seedlings were placed in hardiness zones. The cultivars' zones were based on the northern-most area in which they had been reported to grow with little or no injury. All three of the cultivar seedlings tested in this experiment were classified by Strang (1974), and their hardiness responses to the three different screening temperatures agreed with Strang's classification. Thus, screening could be tailored to match the requirements of particular hardiness zones (Stushnoff et al. 1983).

5.2.1 Dependence on acclimation

The temperature X frost interaction graph (Figure 4.1), as discussed in Section 4.0.3, illustrates that the optimum screening temperature is -30°C . A screening temperature of -30°C should follow one frost exposure which, in turn, should follow the acclimation procedure as outlined in Section 3.0.2 and 3.2.1, to give the best population differentiation. Again, the screening temperature can be tailored to match the requirements of the specific hardiness zones, but in order to screen a full range of crosses of different hardiness potentials, a temperature close to -30°C is advisable.

5.3 System of data collection

Data was collected on seedling regrowth after the controlled freezes in three ways as outlined in Section 3.0.4, 3.1.3 and 3.2.2. Two of the methods used a binomial system of data collection. The third method used a scoring system, based on a range between one and 100. One of the objectives of this experiment was to develop an appropriate viability test. The most reliable estimate of viability was determined to be regrowth. A precise and efficient measure of regrowth, however, had to be selected.

Of the three data collection methods examined, the measure of percent seedling survival proved to be the most efficient. Percent seedling survival was collected as binomial data. Each seedling was given a rating of either one or zero. Only if the seedling was completely dead would it be recorded as a zero (as outlined in Section 3.0.4.0). Percent seedling survival was the fastest and easiest form of data collection used, and in the statistical analysis it gave the most precise results. The finding that percent seedling survival was the best measure of viability was very useful, because it simplified the data collection process a great deal.

5.4 Replication significance

As discussed in Section 4.0.4, replications were highly significant (Table 4.1). Figure 4.6 reveals that freeze survival decreased as the summer progressed. The experiment was conducted from

February to September 1987. The period of initial greenhouse growth for the controlled acclimation experiment occurred from February to June 1987.

The fourth replication was damaged by the insecticide Diazinon and showed a major decline in survival. This decline might be attributed to ethylene production, induced by wounding (Leopold et al. 1972): the Diazinon caused severe damage to the cortical tissue and premature leaf drop. Siebel and Fuchigami (1978) found that wound-induced ethylene and high normal endogenous ethylene levels reduced the ability of a plant to resist cold by postponing the development of vegetative maturity.

The results of an experiment by Leopold et al. (1972) indicated that a seasonal pattern of ethylene production existed in white pine. Work by Siebel and Fuchigami (1978) suggested that a similar seasonal pattern existed in red-osier dogwood. Endogenous ethylene production increased over the period of March through June, coinciding with spring bud break and bud elongation. After June, a steady decrease in endogenous ethylene levels resulted in a non-detectable level by October. Such a pattern of seasonal ethylene production may exist in *Malus* and may be responsible, at least in part, for the changes observed. It has long been appreciated by workers in cold hardiness that some internal factor of seasonal periodicity or annual endogenous rhythm is implicated in cold acclimation (Levitt 1972; Siminovitch 1982; Steponkus 1978). Such a endogenous rhythm is thought to be inherent in a woody plant (Siminovitch 1982), and thus would be observed in a seed or seedling response to acclimation, as well as the

adult tree. If indeed ethylene production increases in *Malus* from March through June, as it does in white pine and dogwood, the decline in cold survival observed in this experiment over that period (Figure 4.6) could be explained. If the seasonal pattern observed for these species was also similar in *Malus* for the period of June through October, the increase in cold survival that occurred in the freeze without acclimation experiment (Figure 4.9) and the cool temperature duration experiment (Figure 4.19) could also be accounted for. Because the probability of survival was influenced by when the seedlings grew in the greenhouse in this experiment, it is suggested for subsequent screens, all the material should be grown at the same time.

6.0 Conclusions

The seedling screen technique facilitates the identification of hardy transgressive segregants and substantively reduces the time and maintenance costs involved in a fruit breeding program (Stushnoff and Quamme 1983). Examination of three *Malus* cultivar seedling populations revealed that the seedling screen could be tailored to specific environments based on the different responses of the cultivar seedlings to different freeze temperatures. The cold survival of the cultivar seedlings agreed with known inherent hardiness responses as classified by Strang (1974).

An acclimation procedure of short (eight hour) days at cool (+3 to +5°C) temperatures was necessary to identify superior cold resistant individuals in the seedling populations. Without the exposure to short days and cool temperatures, all cultivar seedlings behaved similarly, all being hardy to approximately -7°C. In subsequent experiments, it would be of interest to study the effect of ambient temperatures during the short day exposures to determine the difference in survival potential between southern and northern ecotypes to different acclimation regimes.

Further differentiation in the seedling populations was achieved by the addition of one frost cycle. The one frost significantly increased the cold survival of all three cultivar seedlings. The relative increase, however, was different for each. One or more frosts with Rescue gave a 74% increase in survival over no frost exposures. The Antonovka responded with a 62% increase in survival and Golden

Delicious with an increase of 51% over zero frosts. No significant increase in survival was obtained with more than one frost exposure. The main frost effect was due to the difference between zero and one or more frosts. This finding supported a three stage model of acclimation for *Malus*.

After the acclimation response had been initiated by exposure to short days at cool temperatures, up to 11 days in the same conditions caused no significant decrease in hardiness. As well, no significant decrease in survival was observed in seedlings held at cool temperatures and short days for up to 10 days after a frost exposure.

In matching the screening temperature to the minimum survival temperatures required for a specific region, different freeze temperatures could be applied. For a screen of crosses from a full range of hardiness backgrounds a screen temperature close to -30°C was considered optimum. Regrowth was measured as an appropriate viability test. Data collection in the form of binomial values for percent seedling survival was determined to be the most efficient and precise measure of survival.

This experiment was designed to utilize six of Quamme and Stushnoff's (1983) nine outlined steps useful in breeding for cold hardiness in fruit crops. All six proposed steps were achieved: 1) the ability to survive temperatures as low as -40°C was addressed as the major problem; 2) a determination was made of the environmental controls influencing acclimation; 3) an appropriate pre-conditioning routine was developed for standardized testing; 4) a controlled freezing procedure with a standardized freezing and thawing rate was

developed; 5) an appropriate viability test was outlined; and, 6) 12-week-old seedlings were used to facilitate rapid progress in the breeding program.

The development of a seedling screen for *Malus* could be used in a breeding program for other woody species which have potential for commercial production in the prairie provinces. Further studies on *Malus* and other woody species seedling populations would be beneficial to increase the understanding of plant adaptation to northern environments. As urban development continues to encroach on Canada's most productive fruit growing regions, breeding and selection for improved resistance to cold will become the most important emphasis of any fruit breeding program.

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