EFFECTS OF CROP LOAD ON SEASONAL VARIATION IN PROTEIN, AMINO ACID, AND CARBOHYDRATE COMPOSITION, AND SPRING FROST HARDINESS OF APPLE FLOWER BUDS (*Malus pumila* Mill. cv. McIntosh/M7).

By

Shahrokh Khanizadeh©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

Department of Plant Science
McGill University,
Montreal, Quebec
Canada.

March 1989

ABSTRACT

Shahrokh Khanizadeh

Ph.D.
Plant Science **Horticulture**

EFFECTS OF CROP LOAD ON SEASONAL VARIATION IN PROTEIN, AMINO ACID, AND CARBOHYDRATE COMPOSITION, AND SPRING FROST HARDINESS OF APPLE FLOWER BUDS (*Malus pumila* Mill. cv. McIntosh/M7).

Deblossoming caused initiation of more flowers and heavier buds. During the following season buds contained more carbohydrates on non-cropped than on cropped trees and sorbitol was the predominant carbohydrate. Starch content increased in the autumn and decreased in the late winter and early spring in both non-cropped (deblossomed) and cropped trees. Nitrogen, P, and K content increased throughout the bud development period and were lower on cropped trees. Three different methods (Kjeldahl; sulfuric acid-hydrogen peroxide; and summation of amino acid content) for determining and calculating the protein content of flower buds, were compared A new nitrogen:protein conversion factor (5.51) was calculated based on total amino acid analysis. Ninhydrin reaction of amino acids appeared to be the most sensitive method for the quantification of total protein. A new approach is reported for determining total 4-hydroxyproline-rich glycoproteins found in the extracellular matrices of apple flower buds. Total protein and amino acids increased in concentration during bud development and were higher on non-cropped trees. From "dormant" stage until "first pink" flower buds on non-cropped trees had significantly higher levels of hydrophilic, and acidic amino acids. The absence of fruit in the previous year increased the ability of buds to withstand low temperatures probably because of increased availability of assimilates. Two procedures, differential thermal analysis (DTA) and a conductivity test, were used to assess the spring frost tolerance of flower buds. Both procedures give similar results. DTA showed that buds on trees carrying no crop were more cold tolerant at all stages of development. Conductivity tests showed non-cropped trees had a lower median lethal temperature (MLT). The flower buds retained the capacity to deep supercool until the "first pink" stage, when the low temperature exotherm (LTE) disappeared.

RESUME

Shahrokh Khanizadeh

Ph.D.

Phytologie **Horticulture**

EFFETS DE LA FRUCTIFICATION SUR LA VARIATION SAISONNIERE DE LA COMPOSITION EN PROTEINES, ACIDES AMINES ET HYDRATES DE CARBONE, ET SUR LA RESISTANCE AU GEL PRINTANIER DES BOUTONS FLORAUX DU POMMIER (*Malus pumila* Mill. cv. McIntosh/M7).

L'enlèvement des fleurs a augmenté la quantité de primordia floraux pour saison suivante de même que la grosseur et le poids des boutons floraux. Les lambourdes de pommiers non-fructifères contenaient plus d'hydrates de carbone, où prédominait le sorbitol, que les fructifères Le contenu en amidon a augmenté pendant l'automne et diminué à la fin de l'hiver et au début du printemps dans les deux types de pommiers (fructifères ou non). La teneur en azote, phosphore et potassium des bourses à fruits a augmenté pendant la période de développement de ces dernières et ont été inférieures chez les arbres fructifères. Nous avons comparé trois différentes méthodes de détermination et de calcul du contenu protéique (Kjeldahl; acide sulfurique-peroxyde d'hydrogène, et sommation des teneurs en acides aminés) des boutons floraux du pommier. Un nouveau facteur de conversion azote·protéine (5.51) a été calculé d'après l'analyse des acides aminés totaux. La méthode la plus sensible pour la quantification du contenu total en protéines a semblé être la réaction des acides aminés à la ninhydrine Ce travail a aussi fait état d'une nouvelle approche pour déterminer les glycoprotéines totales riches en 4-hydroxy-proline trouvées dans les matrices extra-cellulaires des bourgeons floraux. La concentration en protéines et acides aminés totaux a augmenté pendant le développement des bourses à fruits et a été supérieure dans les arbres non-fructifères Du stade "dormant" au stade du "bouton rose primaire", les boutons floraux de pommiers non-fructifères ont eu des niveaux d'acides aminés hydrophiles et d'acides aminés acides significativement supérieurs L'absence de fruits pendant l'année précédente a augmenté la rusticité des bourgeons floraux, probablement à cause de la disponibilité accrue des composés d'assimilation. Deux procédés, Analyse Thermale Différentielle [DTA] et un test de conductivité, ont servi à établir la résistance au gel printanier des lambourdes Les deux méthodes ont donné des résultats similaires. La DTA a démontré que les bourgeons d'arbres non-fructifères étaient plus tolérants au froid à tous les stades de leur développement. Les tests de conductivité ont révélé que la température médiane léthale (MLT) était inférieure chez les arbres non-fructifères. Les lambourdes ont conservé la capacité d'entrer en surfusion jusqu'au stade du "bouton rose primaire" lorsque l'exotherme de basse température (LTE) a disparu.

SHORT TITLE:

Effect of crop load on apple flower bud development.

Shahrokh Khanizadeh[©]

Key words: ***conductivity, differential thermal analysis (DTA), freezing tolerance, frost tolerance, freezing injury, spring frost, glycoprotein.***

ACKNOWLEDGMENTS

I wish to extend my appreciation and sincere gratitude to **Dr. D. Buszard** for her continuous guidance, encouragement, support and constructive suggestions in the preparation of this thesis.

I thank **Dr. C. G. Zarkadas** in the Agriculture Canada's Muscle Biochemistry Laboratory for his supervision and the use of facilities in his laboratory, where analyses of the amino acids and sugars were performed. I also thank **Dr. M. A. Fanous** for his support and advice in statistical analysis. I am also grateful to my colleagues and friends for their help and guidance especially **Adolfo Minero** and **Constantinos N. Karatzas.**

I extend my appreciation to my **family** especially my **brother,** whose moral support is always with me.

Last, but not least, I thank **Elise Beausoleil** for her encouragement, help, moral support and love throughout the course of this study.

TABLE OF CONTENTS

PAGE

TABLE OF CONTENTS

PAGE

TABLE OF CONTENTS

PAGE

LIST OF TABLES

Table **page**

LIST OF FIGURES

Figure **Page**

I. GENERAL INTRODUCTION

Crop load and flower bud induction and differentiation

Flower bud formation and flower initiation have been extensively reviewed (Buban and Faust 1982, Evans 1971, Luckwill 1974, Fulford 1965, 1966a, 1966b, 1966c). Flower bud formation normally takes place 3 to 6 weeks after full bloom in apple, while developing fruits are present (Buban and Faust 1982); regular flower initiation is essential for successful fruit production. Buban and Faust (1982) advised that some buds be examined during the winter to determine the severity of pruning needed for an optimum crop load. Trees carrying a heavy crop produce fewer flower buds for the next year. This effect of crop load is considered to be hormonally mediated (Buban and Faust 1982, Luckwill and Silva 1979, Looney et al. 1978, Fulford 1966c).

Buban and Faust (1982) examined the development of apple flower buds and divided the process of flower development into three phases: induction, differentiation of meristems, and differentiation of flower primordia. They reported that "Flower bud development is attained through transformation of the vegetative apex to a reproductive structure. This transformation takes place only when the structure of the vegetative bud is complete. This requires that a certain number of vegetative organ promidia must already be developed on the axis of the bud. Flower initiation occurs only after 9 bud scales and 3 primordia of transition leaves have appeared and 6 true leaflets and 3 bracts have differentiated. Consequently, flower induction occurs only when a critical number of internodes already have developed on the axis of the bud."

Although Marro and Ricci (1962) found no difference between the structures of buds on spurs of non-cropped and cropped trees in the first part of the growing season, Buban and Simon (1978) and Buban and Hesemann (1979) found buds on non-cropped apple trees cv. Jonathan had a higher DNA and RNA content compared to those on cropped trees at the time of flower differentiation.

For many years inhibition of flower bud formation was correlated with depletion of nutrients in trees carrying a heavy crop (Childers 1961, Sachs 1977). Fulford (1970a, 1970b, 1973), however reported that the inhibition of flower formation is hormonal and nutrient content has no effect on flower initiation. Luckwill (1974) and Chan and Cain (1967) concluded that both "auxin" and "gibberellin" are involved in flower induction, and failure of flower bud formation in trees carrying a heavy crop is not because of lack of nutrients

(carbohydrates and nitrogenous compounds).

Buban and Faust (1982), having reviewed 142 papers on the subject of the effect of crop load on flower bud formation, commented that " The development of (a) reproductive apex is the process most sensitive to hormonal, nutritional, and physiological effects.". They concluded that the primary factor which inhibits flower bud induction is hormones (gibberellins) produced by the developing seeds in fruit.

Low temperature injury

One of the major problems for apple growers in Quebec is damage to trees caused by low temperatures during the winter and spring. Low winter temperatures and rapid changes in late spring temperatures cause different types of injuries. All of these injuries are associated with freezing of water in plant tissue (Krueger 1965, Burke et al. 1976, Kubler 1983). Winter injury of apple trees is a severe problem in most regions of Canada (Fisher 1978, Estabrooks et al. 1980, Blackburn 1984). Recently Brown and Blackburn (1987) studied the impact of freezing temperatures on crop production in Eastern Canada. They reported that severe winter injury in 1980/81 killed 56,128 trees in Eastern Ontario, 362,702 trees in Quebec, 22,274 trees in New Brunswick and 25,419 trees in Nova Scotia. Granger (1982) reported that 37% of the apple trees in Quebec died as a result of that winter. Spring frosts come about during the period of apple bloom and frequently damage the buds or flowers and consequently reduce yield (Krueger 1965, Granger 1982, Gross 1983, Brown and Blackburn 1987).

Many different strategies have been investigated to avoid the problems of low temperature injury. Several researchers have evaluated cold resistance of a range of apple cultivars and rootstocks (Coleman 1985, Warner 1987, Granger and Vincent 1988). Granger and Vincent (1988) detected no differences in cold hardiness between "Lobo", "Melba", "Cortland", and "McIntosh" during bloom and early stages of fruit development. Coleman (1985) however reported apple cultivars varied considerably in their relative hardiness from year to year. He ranked "Imperial Red Mac/Antonovka" as the hardiest and "Roger's Red Mac/MM111" as the most susceptible of the cultivars and rootstocks observed during two years testing.

Many tests have been conducted on the effect of rootstocks on scion hardiness (Rollins et al. 1962 , Chaplin and Scheider 1974, Sako 1985, Warmund and Slater 1988). Sako (1985) tested the effect of clonal rootstocks on hardiness of apple trees. He ranked YP (Yltoinen Piikio) rootstock as very hardy, M7 less hardy and M9 as susceptible to frost injury. Layne and Ward (1978) reported that flower buds of "Redhaven" peach (*Prunus persica* L.) had higher levels of total carbohydrates, reducing sugars, and other carbohydrates and were more cold hardy when propagated on "Siberian C" rootstocks than on "Harrow Blood".

Various cryoprotectants or/and plant growth regulators have been used to increase the winter hardiness of apple trees or to prolong spring frost hardiness. It has been shown that Alar (N-dimethylamino succinamic acid / Daminozide) and surfactant WK (dodecyl ether of polyethylene glycol / DEPEG) increased the cold hardiness of apple trees. However, their effects were not consistent from year to year (Coleman and Estabrooks 1985). Holubowicz (1985) reported that application of 1000 and 2000 ppm of DEPEG at full bloom increased the cold resistance of flowers and fruitlets, but application of paclobutrazol [(2RS. 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol / PP333] (1000 ppm) reduced the hardiness of flowers and fruitlets of apple, peach and cherry. Durner and Gianfagna (1988) reported that fall application of ethephon (2-chlororethyl phosphonic acid / Ethrel) increased peach (*Prunus persica* L. Batsch.) flower bud resistance to low temperature injury and delayed the bloom period.

Biochemical changes during cold acclimation

Protein

Accumulation of sugar, inorganic nutrients and protein are processes involved in the cold hardening of many species (Brown 1978, Levitt 1978, Sakai and Larcher 1987). Protein metabolism has been investigated extensively in connection with development of hardiness and cold resistance (Parker 1963, Ghazaleh and Handershott 1967, Pomeroy et al 1970, Gusta and Weiser 1972, Levitt 1980, Trunova 1982, Chen and Li 1982, Li 1987). Heber (1959) hypothesized that some low molecular weight protein fractions are important in maintaining the vital structure of the protoplasm under adverse conditions of low temperatures. He postulated that water of hydration is held by hydrogen bonds of

protein with low molecular weight and CO- and NH- groups of the proteins with high molecular weight. He concluded that proteins of low molecular weight can protect the frost-sensitive proteins of high molecular weight by hydrogen bonding. Kaplya (1961) suggested protein accumulation in the root system of fruit trees during the growing season was a prerequisite for hardening.

Many early workers attempted to relate nitrogen in various forms to cold hardiness. Early works on determination of nitrogenous compounds were based on crude methods, and results were often inconsistent and most investigations were of little value. Levitt (1956) suggested that more refined techniques were necessary, such as analyses of individual nitrogenous substances, rather than mixed groups.

Determination of particular nitrogenous substances in relation to cold hardiness had already begun in 1949 (Siminovitch and Briggs 1949, Briggs and Siminovitch, 1949). They reported that the concentration of water-soluble protein increased in the bark of black locust (*Robinia pseudo-acacia* L.) in the fall along with development of hardiness and declined in the spring with disappearance of frost hardiness. Alden and Hermann (1971) reported that in the flower buds of cold-resistant species, all forms of nitrogen increased during cold acclimation. They observed that nitrogen, including protein nitrogen, in the bark of cold resistant trees increased upon hardening more than in non-cold-resistant species. Many workers have reported a positive relationship between water-soluble protein and cold hardiness (Pomeroy et al. 1970, Brown and Bixby 1973, 1975, Trunova and Zvereva 1977, Chen and Li 1980, Sakai and Larcher 1987). On the other hand some reports indicate that there is no relationship between soluble protein and cold hardening (Ghazaleh and Handershott 1967, Young 1969, Pieninzek and Holubowicz 1973).

Henze (1959) found a greater increase in non water-soluble protein than in water-soluble protein in the bark of fruit trees as cold hardiness developed. Paulson (1968) found that in wheat plants under a constant long photoperiod and decreasing temperatures, more water-soluble protein was synthesized than under a decreasing photoperiod and a constant temperature. He concluded that water-soluble proteins alone are not responsible for hardiness. Water soluble proteis may indicate an indirect response to hardening by being synthesized at an increased rate.

Drozdov and Sycheva (1965) reported that the increase in water-soluble protein that parallels development of frost hardiness results from breakdown of

more complex proteins and not from synthesis of new amino acids per se. Alden and Hermann (1971) reported that high content of water-soluble protein associated with increased frost hardiness resulted from the action of enzymes which hydrolyze the proteins at the peptide bond. Water-soluble protein apparently increases for two reasons: first, because water content decreases as hardening occurs leading to concentration of the cytoplasm and secondly, because of an increase in synthesis and/or decrease in degradation of protein (Li 1987).

Amino acids

Studies on free amino acid content during development of frost resistance were started by Simonovitch and Briggs (1953). They found amino acid content, at least in bark cells of black locust (*Robinia pseudo-acacia* L.), difficult to monitor because the amino acids were rapidly converted into proteins.

Amino acids have been examined in relation to cold hardiness in different plant organs of many species (Sakai and Larcher 1987). Sagisaka and Araki (1983) examined 31 species and grouped them into three categories: i) a group which accumulated arginine alone, ii) a group which accumulated proline alone and iii) a group which accumulated arginine and proline and required chilling temperatures for regrowth. The seasonal cycle of soluble amino acids in relation to cold tolerance in deciduous trees and other plant species has been well documented (Pomeroy et al. 1970, Steponkus 1984, Guy et al. 1985, Tseng and Li 1987, Yelenoski et al. 1987), and in general indicates an autumnal increase in amino acids (Li 1987, Sakai and Larcher 1978).

Li et al. (1965) classified amino acids in Red-Osier Dogwood (*Cornus stolonifera* Michx.) into three groups. Group I decreased during cold acclimation and included aspartate, glutamate, alanine, valine and glycine. Group II amino acids increased in concentration during cold acclimation and included glutamine, phenylalanine, leucine, and isoleucine. Group III fluctuated in concentration during cold acclimation, and included serine, threonine, asparagine and cystine. Kandarova (1964) detected large quantities of proline which appeared before development of deep dormancy in resistant trees. He observed that free amino acid content of the buds and bark of cold-hardy deciduous trees increased sharply in March but, in non cold-hardy deciduous trees, it remained the same or declined.

Free amino acids of cherry flower buds were examined in an attempt to study spring frost hardiness. The content of phenylalanine, proline, threonine, arginine, histidine and glutathione in pollen of cherry was positively correlated

with flower cold resistance (Kan'shina 1964). Isoleucine, valine, alanine, and asparagine content of pollen showed no relation with the cold resistance of the flower buds (Kan'shina 1964). Pauli and Zech (1964) reported that alanine, arginine, aspartic acid, glutamic acid, and histidine content of water-soluble proteins extracted from crowns of winter wheat reached a maximum during the period of greatest hardening and decreased during dehardening. They concluded that with the exception of alanine, these amino acids provided soluble proteins with reactive side chains, which could increase the rate of contact between the protein and other substances within the cytoplasm.

Amino acids with polar side chains have been associated with hardiness by many authors (Parker 1962, Van Huystee 1965, Smith 1968, Sagisaka 1974, Sagisaka and Araki 1983). Proteins with a high proportion of polar amino acids were increased in hardy twig sections of Red Osier Dogwood (*Cornus stolonifera* Michx.) compared with non hardy material. Van Huystee (1965) suggested that proteins rich in polar amino acids increase the hydrophilic capacity of protein colloids, preserve water, and prevent denaturation of protein when the protoplasm is dehydrated by extracellular ice formation.

While many studies have reported changes of amino acids and soluble protein in shoots, bark or leaves, little is known of variation in total protein, insoluble protein and amino acids in the flower buds themselves. Insoluble proteins may be important in cold hardiness. Clarke et al. (1979) reported that arabinogalactan-proteins (AGP) have a water-holding capacity, and may be involved in frost and drought resistance. AGP have been found in many plant species and consist of a small proportion of protein rich in 4-hydroxyproline and a large proportion of carbohydrates. The extreme insolubility of AGP makes them difficult to isolate without a degradative procedure (Hag and Adams 1961, Lamport and Miller 1971).

Carbohydrates

Some investigators have studied the seasonal and daily distribution of carbohydrates and reported sucrose, fructose, glucose and sorbitol are the major water soluble carbohydrates in apple trees (Sakai 1966, Chong 1971, Chong and Taper 1971). Many studies have related seasonal changes of carbohydrate content to cold hardiness in plants (Levitt 1956, Sakai 1966, Smith 1968, Heber et al. 1979, Raese et al. 1977, 1978, Siminovitch 1981, Ichiki and Yamaha 1982, Krause et al. 1982, Santarius 1982, Reich 1985).

Donoho and Walker (1960a, 1960b) reported electrical conductance was well correlated both with survival of peach trees subjected to cold temperatures and with total reducing sugars. Layne and Ward (1978) found that increases in total sugars, sucrose, and reducing sugars in "Redhaven" peach flower buds was associated with increases in their ability to withstand low temperatures. However, they reported that hardiness of flower buds was not correlated with total carbohydrates or starch. Cold hardiness was found to be related to high levels of total sugars (fructose, glucose, sucrose and sorbitol) in combined apple bark and wood samples (Raese et al. 1978). Raese et al. (1977) reported that temperature had a marked influence on the carbohydrate content of excised 2-year-old apple shoots; increases in carbohydrates were greatest at the early stage of hardening in each of the three years studied. They observed starch levels were inversely related to fructose, glucose, and sucrose and levels of soluble sugars in the wood were higher at below -0.6^{o}C than at warmer temperatures.

Taylor and Ferree (1986) observed no differences between cropped and non-cropped apple trees in terms of water-soluble reducing sugars in March or June. However Reich (1985) reported that deblossoming apple trees increased fructose levels, but had no effect on sorbitol and glucose. Pruning had an inverse effect on starch content but had no effect on sorbitol or glucose.

In 1987 Brown and Blackburn reported that "there was a suggestion that a heavy fruit load in the 1980 crop year in Eastern Ontario reduced carbohydrate reserves, thus reducing the hardiness of some trees particularly Northern Spy".

Minerals

Studies of seasonal mineral and carbohydrate content began many years ago (Murneek and Logan 1932). Nitrogen content in deciduous fruit trees changes substantially during autumnal leaf senescence prior to cold acclimation (Oland 1963, Spencer and Titus 1972, O'Kennedy et al. 1975, Kang and Titus 1980). The availability of minerals essential for plant growth is an important factor for development of maximum cold resistance. It has been shown that application of minerals improves the cold resistance of fruit trees and encourages recovery of winter injured trees (Jones 1971).

Cultural practices such as pruning and fruit thinning affect the nitrogen and mineral composition of apple trees. Taylor and Ferree (1986) reported that while cropping did not affect P, Fe, Cu, Zn, or Na content in spur leaves. Leaves

on non-cropped trees had lower N, Ca, Mg, Mn, and Al compared to those on cropped trees.

In 1971 Alden and Hermann reported that while frost resistance could be correlated with mineral content the mechanisms were not understood. Since that time other workers have identified specific roles of individual elements in protecting plants from freezing injury. Content of ions such as potassium and phosphate are higher within plant cells than they are in the extracellular environment. High internal concentrations of these ions maintain turgor pressure, which apparently protect the cell against freezing (Graham and Patterson 1982). It has been reported that frost resistance of tulip poplar foliage and alfalfa roots increased with application of potassium (White and Finn 1964, Calder and Macload 1966). Li et al. (1966) speculated that an increase of inorganic phosphorus causes an increase of organic phosphorus (lipid, protein, nucleic acid, and carbohydrate) which consequently increases in frost hardiness. They observed that dehardening was accompanied by an increase in inorganic phosphorus and a decrease in total organic phosphorus.

Determination of frost resistance

Transformation of liquid-water to ice-crystals and dehydration of cells are the major changes occuring during freezing which lead to injury (Li 1987, Sakai and Larcher 1987). Various methods have been introduced for measuring cold hardiness and studying plant-tissue-water in relation to freezing injury. The most commonly used procedures are as follows:

1) Nuclear Magnetic Resonance (NMR)

NMR signal intensity is proportional to the amount of water in the sample. However, in partially frozen samples, both liquid-water and ice-crystals contribute to the NMR signal. NMR is a type of spectroscopy which employs radio frequency radiation. The area under the NMR curve is proportional to amount of liquid water. Narrow lines appear above the freezing point and become broader as freezing progresses. The spectrum for pure water is a single absorption line (Burke et al. 1976, Gadian 1982).

2) Calorimetry

Exothermic and endothermic events are measured by relative temperature changes in plant tissue with or without a comparable size

dry-sample to monitor the reference temperature. There are three different types of "calorimetry" methods which measure the exothermic and/or endothermic events (Quamme et al. 1972a, George et al. 1974a, Burke et al. 1976).

a) **Thermal analysis.** This technique measures temperature of plant tissue using a thermocouple while the sample is frozen (Quamme 1974, Quamme et al. 1975, Quamme 1978, Ashworth et al. 1981). In this method, sample temperature is plotted against time to detect the exothermic peaks.

b) **Differential thermal analysis (DTA).** This procedure is a modified "thermal analysis". One or more dried samples are used along with the tissue samples. The temperature differential between the dry-reference and sample are plotted against a reference temperature to detect the exothermic peaks (Stushnoff and Junttila 1978, Biermann et al. 1979, Nus et al. 1981, Andrews and Proebsting 1983, Andrews et al. 1983, Ketchie and Kammereck 1987).

c) **Differential scanning calorimetry (DSC).** This method measures the amount of water that freezes or thaws between two experimental temperatures. Liberated heat during cooling or absorbed heat during warming are used to calculate the amount of water (Olien 1974).

3) Viability tests.

There are several techniques for determination of cold injury using viability tests.

a) **Electrolyte leakage (Electrolyte conductivity).** In this method the electrolytic conductivity of tissue diffusates is measured and percent electrolyte leakage is used for determination of hardiness (Ketchie et al 1972, Raese 1983, Ketchie and Kammereck 1987). Different techniques are used to calculated the median lethal temperature based on percent leakage. By far the most reliable method appears to be fitting a logistic function as

demonstrated by Su et al (1987) and Zhu and Liu (1987)

b) **2,3,5-triphenyltetrazolium chloride (TTC)** This method is based on staining with TTC Colourless TTC is reduced to a red colour by accepting electrons from the electron transport system This procedure tests whether or not the reducing enzymes are still intact (Steponkus and Lanphear 1967, Chen et al 1979, Ketchie and Kammereck 1987)

c) **Visual observation** This provides the simplest and fastest way of determination of cold injury (Hudson 1961) Data collected after visual observations are normally ordinal with a subjective scale of measurement e g rating

d) **Regrowth** This is the best method of testing viability, however, the requirement for a large population and subjective scale of measurement (ordinal) make this procedure less popular than others (Larsen 1978)

4) **Field tests.**

In this method exposure of plants to frost under natural environmental conditions is studied This type of study is very difficult to interpret because of the non reproducibility of natural frosts (Estrada 1982)

Among the above procedures, thermal analysis, DTA and conductivity tests are the most frequently used Thermal analysis and DTA have been applied often for detection of "deep supercooling" in many deciduous species especially fruit trees (Quamme et al 1972b George et al 1974b Quamme 1974, Quamme et al 1975 Quamme 1978 Biermann et al 1979, Nus et al 1981 Ashworth et al 1981, Andrews et al 1983 Andrews and Proebsting 1983 Li 1984, Ketchie and Kammereck 1987) Deep supercooling is a phenomenon which is defined as "avoidance of freezing in some but usually not all of plant tissues at temperatures as low as -40°C in midwinter" (Burke et al 1976)

Apple trees are typical of plants able to deep supercool (Burke et al 1976) During summer, injury may happen as a result of intracellular ice

formation at -2°C to -3°C. However after the completion of cold acclimation flower buds and bark tissues become capable of supercooling to some degree and water freezes only extracellularly until the limit of cold tolerance is reached (Burke et al. 1976). Further ice formation between the bud scales and the outer cortex at supercool temperatures apparently causes no damage (Wiegand, 1906, Sakai, 1965).

Whilst there have been some studies of frost injury and possible frost survival mechanisms in apple, no work has been reported up to now on the effect of crop load on the spring frost hardiness of apple flower buds.

The objectives of this research were: i) to obtain precise information on quantitative fluctuations of total protein and amino acids, carbohydrates and N, P, and K in flower buds of cropped and non-cropped trees, and to elucidate the annual cycling of amino acids, carbohydrates and inorganic nutrients in flower buds; ii) to clarify the relative importance of total protein, individual amino acids, sugars, starch and N, P, K during flower induction and development; iii) to determine whether crop load influenced any of the above mentioned constituents of buds; and iv) to determine the effect of crop load on spring frost tolerance of apple flower buds using the techniques of electrolytic conductivity and differential thermal analysis (DTA).

This thesis is submitted in the form of original papers suitable for journal publications. The thesis format has been approved by the **Faculty of Graduate Studies and Research, McGill University**, and follows the conditions outlined in the **"Guidelines concerning thesis preparation,** section 7 **Manuscripts and Authorship"** which are as follows:

"The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in **Guidelines Concerning Thesis Preparation.** Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e g in appendices) to allow a clear and precise judgment to be made of the importance and originality of the research reported The thesis should be more than a mere collection of manuscripts published or to be published. **It must include a general abstract, a full introduction and literature review and a final overall conclusion.** Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion.

It is acceptable for the thesis to include as chapters authentic copies of papers already published, provided these are duplicated clearly on regulation thesis stationery and bound as an integral part of the thesis. Photographs or other materials which do not duplicate well must be included in their original publishable form. **In such instances, connecting texts are mandatory** and supplementary explanatory material is almost always necessary.

The inclusion of manuscripts co-authored by the candidate and others is acceptable but, the candidate is required to make an explicit statement on who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims before the oral committee Since the task of the Examiners is made more difficult in these cases, it is in the Candidate's interest to make the responsibilities of authors perfectly clear. Candidates following this option must inform

The text of the above should be cited in full in the introductory section of any thesis to which it applies."

Although all the work reported here is the responsibility of the candidate, the project was supervised by **Dr. Deborah Buszard**, Department of Plant Science, Macdonald College of McGill University. Analysis of tissue samples for amino acids and sugars was done by the candidate in the Muscle Biochemistry Laboratory, Food Research Centre, Saint Hyacinthe, Agriculture Canada Research Branch, under the supervision of **Dr. Constantinos G. Zarkadas.**

For the first, second, and third manuscripts, **Dr. D. Buszard** and **Dr. C. G. Zarkadas** are the co-authors. For the fourth manuscript **Dr. D. Buszard, Dr. C. G. Zarkadas,** and **Dr. M. A. Fanous,** a statistician, are the co-authors. The First manuscript has been submitted to the HortScience. The second manuscript is in press in the Journal of Agricultural and Food Chemistry. The third manuscript has been submitted to Hortscience. The fourth manuscript is in press in the Canadian Journal of Plant Science.

References

Alden, J. and Hermann, R. K. 1971. Aspects of cold-hardiness mechanism in plants. Bot. Rev. 37:37-142.

Andrews, P. K. and Proebsting, E. L. 1983. Differential thermal analysis and freezing injury of deacclimating peach and sweet cherry reproductive organs. J. Am. Soc. Hort. Sci. 108:755-759.

Andrews, P. K. and Proebsting, E. L. and Campbell, G. S. 1983. An exotherm sensor for measuring the cold hardiness of deep-supercooled flower buds by differential thermal analysis. HortScience. 18:77-78.

Ashworth, E. N., Lightner, G. W. and Rowse, D. J. 1981. Evaluation of apricot flower bud hardiness using a computer-assisted method of thermal analysis. HortScience. 16:754-756.

Biermann, J., Stushnoff, C. and Burke, M. J. 1979. Differential thermal analysis and freezing injury in cold hardy blueberry flower buds. J. Am. Soc. Hort. Sci. 104:444-449.

Blackburn, W. J. 1984. Apple tree losses in Canada due to winter injury. A climatic perspective. Resources and Environment report No. 84-3. Agriculture Canada, Ottawa.

Briggs, D. and Siminovitch D. 1949. The chemistry of the living bark of the black locust tree in relation to frost hardiness. II. Seasonal variations in the electrophoresis patterns of the water soluble proteins of the bark. Arch. Biochem. 23:18-28.

Brown, D. M. and Blackburn, W. J. 1987. Impacts of freezing temperatures on crop production in Canada. Can. J. Plant Sci. 67:1167-1180.

Brown, G. N. 1978. Protein synthesis mechanisms relative to cold hardiness. In Plant Cold Hardiness and Freezing. pp. 153-163, (Li, P. H., and Sakai, A. eds.), Academic Press, N. Y.

Brown, G. N. and Bixby, J. A. 1973. Quantitative and qlalitative changes in total protein and soluble protein during induction of cold hardiness in black locust stem tissues. Cryobiology 10:529-530.

Brown, G. N. and Bixby, J. A. 1975. Soluble and insoluble protein pattern during induction of freezing tolerance in black locust seedlings. Physiol. Plant. 34:187-191.

Buban, T. and Faust, M. 1982. Flower bud induction in apple trees: Internal control and differentiation. Hort. Rev. 4:174-203.

Buban, T. and Hesemann, C. U. 1979. Cytochemical investigations in spices of the shoot apex of apple trees. I.DNA, DNA- and RNA-, and histone content of meristematic cell nuclei in terminal buds of spurs with and without fruits. Acta Bot. Acad. Sci. Hung. 25:53-62.

Buban, T. and Simon, I. 1978. Cytochemical investigations in apices of applwe buds with special references to flower initiation. Acta Hort. 80:193-198.

Burke, M. J.; Gusta, L. V.; Quamme, H. A.; Weiser, C. J. and Li, P. H. 1976. Freezing and injury in plants. Ann. Rev. Plant Physiol. 27:507-528.

Calder, F. W. and Macload, L. B. 1966. Effect of cold treatment on alfalfa as influenced by harvesting system and rate of potasium application. Can. J. Plant Sci. 46:17-26.

Chaplin, C. E. and Scheider, G. W. 1974. Peach rootstock/scion hardiness effects. J. Am. Soc. Hort. Sci. 99:231-234.

Chen, H. H. and Li, P. H. 1982. Potato cold acclimation In Plant Cold Hardiness and Freezing Stress, Vol. II pp. 5-22, (Li, P. H. and Sakai, A. eds.), Academic Press, New York.

Chen, H. H. and Li, P. H. 1980. Characteristics of cold acclimation and deacclimation in tuber-bearing *Solanum* species. Plant Physiol. 65:1146-1148.

Chen, H. H., Gavinlertvatana, P. and Li, P. H. 1979. Cold acclimation of stem cultured plants and leaf callus of *Solanum* species. Bot. Gaz. 140:142-147.

Chen, P. M. and Li. P. H. 1977. Induction of frost hardiness in stem cortical tissues of *Cornus stolonifera* Michx. by water stress. II. Biochemical changes. Plant Physiol. 59:240-243.

Childers, N. F. 1961. Modern fruit science. Horticulture Publishers, New Jersey.

Chong, C. 1971. Study of the seasonal and daily distribution of sorbitol and related carbohydrates within apple seedlings by analysis of selected tissues and organs. Can. J. Plant Sci. 51:519-525.

Chong, C. and Taper, C. D. 1972. *Malus* tissue culture. I. Sorbitol (D-glucitol) as a carbon source for callus initiation and growth. Can. J. Bot. 50:1399-1404.

Clarke, A. E., Anderson, R. L. and Stone, B. A. 1979. Form and function of arabinogalactans and arabinogalactan-proteins. Phytochemistry 18:521-540.

Coleman, W. K. 1985. Variations in cold resistance among apple cultivars during deacclimation. J. Exp. Bot. 36:1159-1171.

Coleman, W. K. and Estabrooks, E. N. 1985. Chemical modification of cold hardiness in apple trees in Eastern Canada. Can. J. Plant Sci. 65:969-975.

Donoho, C. W., Jr., and Walker, D. R. 1960a. The effect of controlled temperature on hardiness of Elberta peach trees. Proc. Amer. Soc. Hort. Sci. 75:148-154.

Donoho, C. W., Jr., and Walker, D. R. 1960b. The effect of temperature on certain carbohydrate and nitrogen fractions in the bark and roots of peach trees during the dormant season. Proc. Amer. Soc. Hort. Sci. 75:155-162.

Drozdov, S. N. and Sycheva, Z. F. 1965. Relatioship of frost resistance of potato haulms with the level of nitrogen metabolism. Sov. Plant Physiol. 12:274-279.

Durner, M. R. and Gianfagna, T. J. 1988. Fall application increases peach flower bud resistance to low-temperature stress. J. Amer. Soc. Hort. Sci. 113:404-406.

Estabrooks, E. N., MacMillan, J. K., Reed, G. W. and Smith, P. J. 1980. A study of New Brunswick apple orchards-winter injury 1979. Plant Industry Br., Dep. of Agriculture and Rural Development, Fredericton, New Brunswick.

Estrada, N. 1982. Breeding wild and primitive potato species to obtain frost resistant cultivated varieties. In Plant Cold Hardiness and Freezing Stress. pp. 615-633, (Li, P. H. and Sakai, A. eds.), Academic Press, New York.

Evans, T. 1971. Flower induction and florigen concept. Ann. Rev. Plant Physiol. 22:365-394.

Fisher, K. H. 1978. Winter injury in fruit trees. Ontarion Ministry of Agriculture and Food Fact sheet 78-082. Agdex 210.

Fulford, R. M. 1965. The morphogenesis of apple buds. I. The activity of the apical meristem. Ann. Bot. 29:167-180.

Fulford, R. M. 1966a. The morphogenesis of apple buds. II. The development of the bud. Ann. Bot. 30:25-38.

Fulford, R. M. 1966b. The morphogenesis of apple buds. III. The inception of flowers. Ann. Bot. 30:597-606.

Fulford, R. M. 1966c. The morphogenesis of apple buds. IV. The effect of fruit. Ann. Bot. 30:207-219.

Fulford, R. 1970a. The effect of chemical defoliation on the development of apple spurs. Ann. Bot. 34:1079-1080. Rpt. East Malling Res. Sta. 1972. p. 93.

Fulford, R. 1970b. Leaves, fruit and flower initiation. Proc. 18th Intern. Hort. Congr. 4:143-150.

Fulford, R. 1973. Flower initiation-effect of gibberellin sprays. Rept. East Malling Res. Sta. 1972. p. 93.

Gadian, D. G. 1982. Nuclear Magnetic Resonance and Its Application to Living Systems. Clarendon, Oxford.

George, M. F., Burke, M. J., Pellett, H. M., and Johnson, A. G. 1974a. Low temperature exotherms and woody plant distributions. HortScience. 9:519-522.

George, M. F., Burke, M. J., and Weiser, C. J. 1974b. Supercooling in overwintering azalea flower buds. Plant Physiol. 54:29-35.

Ghazaleh, M. Z. S. and Hendershott, C. H. 1967. Effect of drought and low temperature on leaf freezing points and water soluble proteins and nucleic acid content of sweet orange plants. Proc. Amer. Soc. Hort. Sci. 90:93-102.

Graham, D. and Patterson, B. D. 1982. Responses of plants to low, nonfreezing temperatures; proteins, metabolism, and acclimation. Ann. Rev. Plant Physiol. 33:347-372.

Granger, R. L. 1982. Cultural practices which facilitated survival of apple trees in the severe winter of 1980-1981. Western New York Apple Growers Newsletter. Feb. and Mar. issue of 1982. Rochester, New York.

Granger, R. L. and Vincent, C. 1988. Effects of spring frosts on four apple cultivars. Fruit Var. J. 42:5-8.

Gross, D. C. 1983. Differential thermal analysis and freezing injury of deacclimating peach and sweet cherry reproductive organs. J. Amer. Soc. Hort. Sci. 108:755-759.

Gusta, L. V. and Weiser, C. J. 1972. Nucleic acid and protein changes in relation to cold acclimation and freezing injury of Korean boxwood leaves. Plant Physiol. 49:91-96.

Guy, C. L.; Niemi, K. J.; Bramble, R. 1985. Altered gene expression during cold acclimation of spinach. Proc. Natl. Acad. Sci. U.S.A. 83:3673-3677.

Hag, S. and Adams, G. A. 1961. Structure of an arabinogalactan from Tamarack (*Larix laricina*). Can. J. Chem. 39:1563-1573.

Heber, U. 1959. Ursachen der Frostresistenz bei Winterweizen. III. Die Bedeutung von Protein fuer die Frostresistenz. Planta 54:34-67.

Heber, U., Volger, H, Overback, V. and Santarious, K. A. 1979. Membrane damage and protection during freezing. Adv. Chem. 180:159-189.

Henze, J. 1959. Untersuchungen ueber den Kohlenhydrat-Eiweisshaushalt der Rinde in seiner Beziehung zur Frostresistenz von Obstgehoelzen. Zeitschr. Bot. 47:42-87.

Holubowicz, T. 1985. Influence of growth regulator application on frost resitance of fruit tree shoots, spus, flowers and fruitlets. Acta Hort. 168:139-143.

Hudson, M. A. 1961. The limitation of a cut leaf test for assessing the frost resistance of the tuber-bearing *Solanum*. Euphytica. 10:169-179.

Ichiki, S. and Yamaha, H. 1982. Sorbitol in tracheal sap of dormant apple (*Malus domestica* Borkh.) shoots as related to cold hardiness. In Plant Cold Hardiness and Freezing Stress pp. 181-187, (Li, P. H., and Sakai, A. eds.) Academic Press, New York.

Jones, R. E. 1971. Aid to winter injured trees. Canada Horticulture and Agriculture, April 1971, p. 8.

Kandarova, I. V. 1964. Features of the nitrogen metabolism of frost-resistant and non-frost-resistant woody plants. In: The physiology of frost resistance of woody plants. Nauka, Moscow, pp. 61-63. (Biol. Abstr. 46:63289. 1965)

Kang, S. M. and Titus, J. S. 1980. Qualitative and quantitative changes in nitrogenous compounds in senescing leaf and bark tissues of the apple. Plant Physiol. 50:285-290.

Kan'shina, N. V. 1964. Correlation between the free amino acid content in the pollen and pistils of cherry plants and the winter resistance of the flower buds. Sov. Plant Physiol. 11:783-785.

Kaplya, A. V. 1961. Carbohydrate metabolism and the frost resistance of the root system of stocks. Visnyk Kyyivs;koho Univ. Seria Biol. 4:49-66. (Biol. Abstr. 45:83849. 1964)

Kawana, A.; Nakahara, M.; Sugimoto, B. and Huruhata, H. 1964. The effect of fertilization on the growth and frost damage of sugi (*Cryptomeria japonica* D. Don) and hinoki (*Chamaecyparis obtusa* E.) J. Jap. Forestry Soc. 46:355-363.

Ketchie, D. O. and Kammereck, R. 1987. Seasonal variation of cold resistance in *Malus* woody tissue as determined by differential thermal analysis and viability tests. Can. J. Bot. 65:2640-2645.

Ketchie, D. O. Breeman, C. H. and Ballard, A. L. 1972. Relation of electolytic conductance to cold injury and acclimation in fruit trees. J. Amer. Soc. Hort. Sci. 97:403-406.

Krause, G. H. Klosson, R. J. and Troster, V. 1982. On the mechanism of freezing injury and cold acclimation of spinach leaves. In Plant Cold Hardiness and Freezing Stress, Vol. II, pp. 55-76, (Li, P. H., and Sakai, A. eds.) Academic Press, New York.

Krueger, K. R. 1965. The geography of the orchard industry of Canada. Geog. Bull. 7:27-71.

Kubler, H. 1983. Mechanism of frost crack formation in trees-A review and synthesis. Frost. Sci. 29:559-568.

Lamport, D. T. A. and Miller, D. H. 1971. Hydroxyproline arabinosides in the plant kingdom. Plant Physiol. 48:454-456.

Larsen, A. 1978. Freezing tolerance in grasses. Meldinger fra norges landbrukshogskole. 57:1-56.

Layne, R. E. C. and Ward, G. M. 1978. Rootstock and seasonal influences on carbohydrate levels and cold hardiness of 'Redhaven' peach. J. Amer. Soc. Hort. Sci. 103:408-413.

Levitt, J. 1978. An overview of freezing injury and survival and its interrelationships to other stress. In Plant Cold Hardiness and Freezing pp. 3-15, (Li, P. H., and Sakai, A. eds.) Academic Press, New York.

Levitt, J. 1980. Responses of plants to environmental stresses. Vol. I. Chilling, freezing, and high temperature stresses, 2nd edn. Academic Press, New York.

Levitt, J. 1956. The hardiness of plants. Academic Press, New York. 278 pp.

Li, P. H. 1987. Plant Cold Hardiness. Alan R. Liss, Inc., New York, pp. 381.

Li, P. H. 1984. Subzero temperature stress physiology of herbaceous plants. Hort. Rev. 6:373-416.

Li, P. H.; Weiser, C. J., and Van Huystee, R. B. 1965. Changes in metabolites of red-osier dogwood during cold acclimation. Proc. Amer. Soc. Hort. Sci. 86:723-730.

Li, P. H.; Weiser, C. J., and Van Huystee, R. B. 1966. The relationship of cold resistance to the status of phophorous and certain metabolites in red-osier dogwood (*Cronus stolonifera* Michx.). Plant and Cell Physiol. 7:475-484.

Looney, N. E., Kamienska, A., Regge, R. L. and Pharis, R. P. 1978. Metabolism of ^{3}H-gibberellin A_4 in relation to flower initiation in apple. Acta Hort. 80:105-114.

Luckwill, L. C. 1974. The control of growth and fruitfulness of apple trees. In Physiolog of tree crops. pp. 237-254, (Luckwill, L. C. and Cutting, C. V. eds.), Academic Press, New York.

Luckwill, L. C. and Silva, J. M. 1979. The effects of daminozide and gibberellic acid on flower initiation, growth and fruiting of apple cv Golden Delicious. J. Hort. Sci. 54:217-223.

Marro, M. and Ricci, A. 1962. Research on differentiation of flower buds in *Malus communis*. Riv. Ortoflorofruttic. Ital. 46:450-457.

Murneek A. E. and Logan J. C. 1932. Autumnal migration of nitrogen and carbohydrates in the apple trees. Uni. Missouri, Agr. Exp. Stn. Res. Bull. 171:1-30.

Nus, J. L., Weigle, J. L. and Schradle, J. J. 1981. Superimposed amplified exotherm differential thermal analysis system. HortScience. 16:753-754.

O'Kennedy, B. T., Hennerty, M. J. and Titus, J. S. 1975. Changes in the nitrogen reserves of apple shoots during the dormant season. J. Hort. Sci. 50:321-329.

Oland, K. 1963. Changes in the content of dry matter and major nutrient elements of apple foliage during senescence and abscission. Physiol. Plant. 16:682-694.

Olien, C. R. 1974. Energies of freezing and frost dessication. Plant Physiol. 53:764-767.

Parker, J. 1963. Cold resistance in woody plants. Bot. Rev. 29:123-201.

Parker, J. 1962. Relatioships among cold hardiness, water-soluble protein, anthocyanins, and free sugars in *Hedera helix*. Plant Physiol. 37:809-813.

Pauli, A. and Zech, A. C. 1964. Cold hardiness and amino acid content of winter wheat. Trans. Kan. Sci. 66:255-259.

Paulson, G. M. 1968. Effect of photoperiod and temperature on cold hardening in winter wheat. Crop Sci. 8:29-32.

Pieniazek, J. and Holubowicz, T. 1973. The effect of ABA and GA on the cold hardiness of apple seedlings and explants. Acta Hort. 34:247-251.

Pomeroy, M. K.; Siminovitch, D., and Wightman, F. 1970. Seasonal biochemical changes in the living bark and needles of red pine (*Pinus resinosa*) in relation to adaptation to freezing. Can. J. Bot. 48:953-967.

Quamme, H. A. 1974. An exothermic process involved in the freezing injury to flower buds of several *Prunus* species. J. Amer. Soc. Hort. Sci. 99:315-318.

Quamme, H. A. 1978. Mechanism of supercooling in overwintering peach flower buds. J. Amer. Soc. Hort. Sci. 103:57-61.

Quamme, H. A., Evert, D. R., Stushnoff, C. and Weiser, C. J. 1972a. A versatile temperature control system for cooling and freezing biological materials. HortScience. 7:24-25.

Quamme, H. A., Layne, R. E. C., Jackson, H. O. and Spearman, G. A. 1975. An improved exotherm method for measuring cold hardiness of peach flower buds. HortScience. 10:521-523.

Quamme, H. A., Stushnoff, C. and Weiser, C. J. 1972b. The relationship of exotherms to cold injury in apple stem tissues. J. Amer. Soc. Hort. Sci. 97:608-613.

Raese, J. T. 1983. Conductivity tests to screen fall-applied growth regulators to induce cold hardiness in young 'Delicious' apple trees. J. Amer. Soc. Hort. Sci. 108:172-176.

Raese, J. T.; Williams, M. W. and Billingsley, H. D. 1977. Sorbitol and other carbohydrates in dormant apple shoots as influenced by controlled temperatures. Cryobiology 14:373-378.

Raese, J. T.; Williams, M. W. and Billingsley H. D. 1978. Cold hardiness, sorbitol, and sugar levels of apple shoots as influenced by controlled temperature and season. J. Amer. Soc. Hort. Sci. 103:796-801.

Reich, L. 1985. Carbohydrates in the apple tree:Effects associated with pruning and deblossoming. Hortscience 20:238-240.

Rollins, H. A. Jr., Howlett, F. S. and Emmert, F. H. 1962. Factors effecting apple hardiness and methods of measuring resistance of tissue to low temperature injury. Ohio Agr. Exp. Sta. Bul. 901.

Sachs, R. M. 1977. Nutrient diversion: An hypothesis to explain the chemical control of flowering. HortScience. 12:220-222.

Sakai, A. 1965. Survival of plant tissue at super-low temperatures III. Relation between effective prefreezing temperatures and the degree of frost hardiness. Plant Physiol. 40:882-887.

Sakai, A. 1966. Seasonal variations in the amounts of polyhydric alcohol and sugar in fruit trees. J. Hort. Sci. 41:207-213.

Sakai, A. and Larcher, W. 1987. Frost Survival of Plants, Responses and Adaptation to Freezing Stress. Springer-Verlag, New York, pp. 303.

Sako, J. 1985. Winter hardiness problems on fruit growing in Finland. Acta Hort. 168:31-36.

Sagisaka, S. 1974. Transition of metabolisms in living poplar bark from growing to wintering stage and *vice versa.* Plant Physiol. 54:544-549.

Sagisaka S. and Araki, T. 1983. Amino acid pools in perennial plants at the wintering stage and at the beginning of growth. Plant Cell Physiol. 24:479-494.

Siminovitch. D. 1981. Common and disparate elements in the processes of adaptation of herbaceous and woody plants to freezing - a perspective. Cryobiology 8:166-185.

Siminovitch, D. and Briggs, D. R. 1949. The chemistry of the living bark of the black locust tree in relation to frost hardiness. I. Seasonal variations in protein content. Arch. Biochem. 23:8-17.

Siminovitch, D. and Briggs, D. R. 1953. The chemistry of the living bark of the black locust tree in relation to frost hardiness. IV. Effects of ringing on translocation, ,protein synthesis, and the development of hardiness. Plant Physiol. 28:177-200.

Smith, D. 1968. Varietal chemical differences associated with freezing resistance in forage plants. Cryobiology 5:148-159.

Santarius, K. A. 1982. The mechanism of cryoprotection of biomembrance systems by carbohydrates. In Plant Cold Hardiness and Freezing Stress, Vol. II, pp. 475-486, (Li, P. H., and Sakai, A. eds.) Academic Press, New York.

Spencer, P. W. and Titus, J. S. 1972. Biochemical and enzymatic changes in apple leaf tissue during autumnal senescence. Plant Physiol. 49:746-750.

Steponkus, P. L. 1984. Role of the plasma membrane in freezing injury and cold acclimation. Ann. Rev. Plant Physiol. 35:543-584.

Steponkus, P. L. and Lanphear, F. O. 1967. Refinement of the triphenyl tetrazolium chloride method of determination cold injury. Plant Physiol. 42:1423-1426.

Stushnoff, C. and Junttila, O. 1978. Resistance of low temperature injury in hydrated lettuce seed by supercooling. In Plant Cold Hardiness and Freezing Stress, Mechanisms and Crop Implications. pp. 241-247, (Li, P. H. and Sakai, A. eds.), Academic Press, New York.

Su, W. A., Mi, R. Q. and Wang, W. Y. 1987. A relationship among stress intensity, duration, and strain. In Plant Cold Hardiness. pp. 141-153, (Li, P. H. ed.), Alan R. Liss, Inc., New York.

Taylor, B. H. and Ferree, D. C. 1986. The influence of summer pruning and fruit cropping on the carbohydrate, nitrogen, and nutrient composition of apple trees. J. Amer. Soc. Hort. Sci. 111:342-346.

Trunova, T. I. 1982. Mechanism of winter wheat hardening at low temperature. In Plant Cold Hardiness and Freezing Stress, Vol. II pp. 41-54, (Li, P. H. and Sakai, A. eds.), Academic Press, New York.

Trunova, T. I. and Zvereva, G. H. 1977. Effect of protein synthesis inhibitors on frost hardiness of winter wheat. Soviet Plant Physiol. 24:311-316.

Tseng, M-J. and Li, P. H 1987. Changes in nucleic acid and protein synthesis during induction of cold hardiness. In Plant Cold Hardiness. pp. 1-27, (Li, P. H. ed.), Alan R. Liss, Inc., New York.

Van Huystee, R. B. 1965. Cold acclimation and accompanying metabolic changes in red-osier dogwood with emphasis on proteins. Ph.D. thesis, Univ. Minn. (Diss. Abstr. 25(6):4989-4899.

Warmund, M. R. and Slater, J. V. 1988. Hardiness of apple and peach trees in the NC-140 rootstock trials. Fruit Var. J. 42:20-24.

Warner, J. 1987. Freeze susceptibility to fruit fruit buds of selected apple cultivars in east-central Ontario, 1986. Fruit Var. J. 41:105-110.

White, D. P. and Finn, R. F. 1964. Frost damage in a tulip poplar plantation as related to foliar potassium content. Papers of the Mich. Acad. Sci. Art. and Letters. 49:75-80.

Wiegand, K. M. 1906. Some studies regarding the biology of buds and twigs in winter. Bot. Gaz. 41:373-424.

Yelenosky, G.; Mauk, C. S.; Bausher, M. G., and Kushad, M. M. 1987. Chemical bioregulation of growth and cold hardiness in citrus. In Plant Cold Hardiness. pp. 299-321, (Li, P. H. ed.), Alan R. Liss, Inc., New York.

Young, R. 1969. Cold hardening in "redblush" grapefruit as related to sugars and water soluble proteins. J. Amer. Soc. Hort. Sci. 94:252-254.

Zhu, G. H. and Liu, Z. Q. 1987. Determination of median lethal temperature using logistic function. In Plant Cold Hardiness. pp. 291-298, (Li, P. H. ed.), Alan R. Liss, Inc., New York.

II. QUANTITATIVE DETERMINATION OF TOTAL PROTEIN AND GLYCOPROTEIN CONTENT IN DEVELOPING APPLE FLOWER BUDS (*Malus pumila* Mill. cv. McIntosh/M7).

Introduction

Quantitative measurements of the protein content of apple flower bud tissues present many difficulties, partly because of the presence of large quantities of other complex plant constituents (McNeil et al. 1984), but primarily due to the relatively small amounts of protein present in buds. In addition, the extracellular matrices of the primary cell walls of angiosperms are known to be composed of approximately 90% polysaccharide and 10% protein (Stuart and Varner 1980). These proteins are apparently all glycoproteins in which a number of proline residues are post-translationally modified to 4-trans-hydroxyproline (Lamport 1980). These 4-hydroxyproline-rich glycoproteins, viz. extensins, arabinogalactan proteins, etc., which are major structural components of the primary cell walls of all dicotyledonous plants (Lamport and Epstein 1983, McNeil et al. 1984, Cooper et al. 1987), are essentially insoluble and difficult to extract with any of the conventional protein solvents.

The most commonly used procedures for total protein determination include the Folin phenol method (Lowry et al., 1951), the biuret method (Gornall and Bardawill 1949), the 280/260 nm ultraviolet (UV) absorption method (Warburg and Christian 1941, 1942) or the improved 224-236 nm isoabsorbance method (Groves et al., 1968), the 280/205 nm absorption procedure of Scopes (1974). the relatively new Coomassie Blue dye binding method of Bradford (1976), and the classical Kjeldahl nitrogen procedure (AOAC 1984). The Folin phenol, the Bradford (1976) and the UV absorption methods are not well suited for measuring total proteins in intact plant tissue since complete protein extraction and solubilization is required and the sensitivity of these methods is affected by the presence of numerous interfering compounds, especially phenolics and tannins (Stock et al. 1968, Robinson 1979, Peterson 1983, Marks et al. 1985). The Kjeldahl nitrogen method (AOAC 1984) is the procedure most frequently used by investigators to measure protein, owing to its simplicity and precision. However, because a substantial quantity of the Kjeldahl nitrogen in plant tissues is derived from non-protein nitrogenous compounds, i.e., amides, nitrogenous glucosides, nucleic acids, porphyrins, fats, alkaloids, ammonium salts, and hormones (Maynard and Loosli 1969, Westwood 1978), a considerable analytical error can be introduced if the

conventional Kjeldahl nitrogen conversion factor of 6.25 is used to calculate the actual protein content (Jones 1931). While the Kjeldahl method for determination of total protein has been valuable in meeting the most general as well as specialized applications, Heidelbaugh et al. (1975) found that the best estimate of the protein content of a biological sample is the summation of the amino acid nitrogen content and recommended that whenever accurate data on the protein content of individual tissues is required, conversion factors based on the actual amino acid nitrogen content should be used.

Absolute protein concentration in biological materials can be determined if the total number of amino acids and the average molecular weight of the amino acid residues in the protein composition of the mixture are known. The amino acid method of protein quantitation, particularly as presented by Horstmann (1979) and Peterson (1983), is based upon knowledge of the amino acid composition of a protein or protein mixture. Horstmann (1979) used an amino acid analyzer, bypassing normal column separation, and assumed that the ninhydrin reaction with all amino acids was essentially equivalent. This method has been subjected to extensive evaluation recently (Nguyen et al. 1986, Zarkadas et al. 1988a, 1988b) and found to be the least variable with respect to a variety of proteins and plant and animal tissues, and yields accurate estimates of the amount of protein present.

The present study extends the work reported earlier (Zarkadas et al. 1988a) on oil seeds, cereal grains and other plant tissues and includes the quantitative determination of total protein content in developing apple flower buds. The purpose of the present study was to compare three different methods for determining the protein content of typical apple flower bud tissues: 1) multiplication of Kjeldahl nitrogen by 6.25 and the new conversion factor; 2) multiplication of the nitrogen content of apple flower buds measured by the sulfuric acid-hydrogen peroxide ashing procedure of Thomas et al. (1967) by 6 25 and the new conversion factor; and 3) determination of total protein by summation of the amino acid content of apple flower buds as described by Horstmann (1979). In this study, an attempt was also made to determine the total amount of 4-hydroxyproline-rich glycoproteins present in the extracellular matrices of apple flower buds from the quantity of 4-hydroxyproline present in their acid hydrolysates.

Materials and methods

Plant material and sample preparation

Flower buds were collected from six 30-year-old apple trees (*Malus pumila* Mill. cv. McIntosh/M7) grown at Macdonald College of McGill University, Ste. Anne de Bellevue, Quebec. Forty buds were harvested from each tree at bud break (March 29, 1985), and each bud weighed immediately after harvest. All the buds from each tree replicate (about 2.68 g) were combined, frozen in liquid nitrogen (-170^{o}C), freeze dried, pulverized in a standard electrically-driven Thomas-Wiley mill equipped with a 64 mm stainless steel hopper and stationary blades (Arthur H. Thomas Company, Philadelphia, PA.) and then stored at -20^{o}C in air-tight containers until needed.

Materials

Type W-3 cation-exchange spherical resin, sized to 9.0±0.5 mm (Beckman Instruments Inc., Palo Alto, CA.), type DC-6A 11.0±1.0 mm spherical resin (Dionex Corp., Sunnyvale, CA.), L-Tryptophan, D-glucosamine.HCl, D-galactosamine.HCl, and 4-hydroxyproline (Calbiochem-Behring Corp., La Jolla, CA.) and 3-Nitro-L-tyrosine (Aldrich Chemical Co., Milwaukee, WI.), purified as described previously (Zarkadas et al. 1987) were used. The standard amino acid calibration mixture (type H) and norleucine were purchased from Pierce Chemical Co., Rockford, IL. In the case of single column methodology for determining tryptophan, 4-hydroxyproline and standard amino acids, the chemicals and reagents used were as follows: sodium citrate.2H_2O (crystals) (Allied Fisher Scientific, Fairlawn, NJ.) sodium acetate buffer, pH 5.51 (4M), Piersolve (ethylene glycol monomethyl ether), ninhydrin, and stannous chloride.2H_2O (Pierce Chemical Co., Rockford, IL.). Kjeltabs (Tecator, Hoganas, Sweden) containing 3.5 g potassium sulfate and 3.5 mg selenium used as catalysts during Kjeldahl digestion were obtained from Allied Fisher Scientific, Fairlawn, NJ. All other chemicals and reagents were used without further purification.

Total nitrogen determination

Kjeldahl nitrogen

The total nitrogen content of the freeze dried samples of apple flower buds was determined by the Kjeldahl method (Section 2.058, AOAC 1984). The digestion of 0.5 g of sample was carried out using the Kjeltec digestion system (Model 20, Tecator AB, Hoganas, Sweden) with Selenium as the catalyst. Total N was determined by distilling the ammonia from the Kjeldahl digests into 4% boric acid and titrating with standard acid using the Kjeltec distilling and titration unit (Model 1002).

Automated analysis

The sulfuric acid-hydrogen peroxide wet ashing procedure of Thomas et al. (1967) was used and the total nitrogen in these digests was determined as ammonia by the indophenol blue method of Ferrari (1960) using the automated Technicon AAII analyzer (Technicon Instruments Co., Tarrytown, NY).

Procedure for amino acid analyses

Amino acid analyses were carried out using a conventional semi-automated amino acid analyzer (Beckman Spinco Model 120C) modified for accelerated chromatography to accommodate both the 0.6 cm and 0.9 cm diameter columns with adjustable column fittings as described previously (Zarkadas et al. 1986). These minor modifications increased the sensitivity of the semi-automated amino acid analyzer so that amino acid analyses could be carried out at the nanomole level using single column methodology as follows:

Hydrolysis of apple flower bud tissue. Powdered apple flower bud samples (50 mg) were hydrolyzed in Pyrex test tubes (18 x 150 mm) under vacuum (below 20 mm of mercury) with 5.0 mL of triple-glass distilled constant boiling HCl (6.0M) at 110^{0}C in duplicate for each of four times (24, 48, 72 and 96 h) with the usual precautions described by Hunt (1983). Standard amino acid analyses were carried out on a 40 x 0.6 cm column of Beckman type W-3 cation exchange resin using the elution buffers recommended by Fauconnet and Rochmont (1978) for ninhydrin analysis. The data reported for serine, threonine and tyrosine represent the average of values extrapolated to zero time of hydrolysis. Addition of phenol (10-15 uL) to the hydrolysates

prevented chlorination of tyrosine. The values for valine, isoleucine, leucine and phenylalanine are averages of data from 48, 72 and 96 h of hydrolysis. All others are reported as the average values from 24, 48, 72 and 96 h of hydrolysis.

4-Hydroxyproline was determined separately from a concentrated hydrolysate (equivalent to 0.1 mg of protein/analysis) by the procedure of Zarkadas et al. (1986). In this system, 4-hydroxyproline and aspartic acid were completely separated and emerged from the the column at 28.5 and 32.7 min, respectively. Recoveries of 4-hydroxyproline were calculated relative to alanine, which elutes at 68.5 min.

Methionine and cyst(e)ine were determined separately by the performic acid procedure of Moore (1963). Norleucine was added to the hydrolysate as an internal standard, and the recovery of cyst(e)ine as cysteic acid and methionine as methionine-S,S-dioxide were calculated in proportion to the yields obtained by the performic acid treatment of standard solutions of these amino acids and relative to the amount of alanine and leucine present in the samples.

Tryptophan in 100-mg apple flower bud samples was also determined separately after alkaline hydrolysis (Hugli and Moore, 1972) by a rapid method (Zarkadas et al. 1986) using 3-nitrotyrosine as the internal standard. Elution times for 3-nitrotyrosine and tryptophan were 28.5 and 56.2 min, respectively.

Determination of total protein

Data processing and quantification of protein in the hydrolysates were carried out by the method described by Horstmann (1979). According to this method, a mean residue weight (WE in µg/nmol) is calculated for the amino acids constituting the proteins in the apple flower bud tissue by the following equation:

$$(1) \qquad WE = \sum_{i=1}^{19} (a_i \cdot b_i)$$

where a_i is the mole fraction of a specific amino acid i found in the analyzed aliquot and b_i is the molecular weight of amino acid residue i. A conversion factor, F, which is the apparent average residue molecular weight (in mg/nmol) of the apple flower bud protein mixture, increased in proportion to the missing

tryptophan and cyst(e)ine residues from acid hydrolysates, was used for determining the protein mass in each hydrolysate sample analyzed as follows:

$$F = \frac{WE}{1 - (a_{\underline{Trp}} + a_{\underline{Cys}})} \qquad (2)$$

where $a_{\underline{Trp}}$ and $a_{\underline{Cys}}$ are the mole fractions of the specific amino acids tryptophan and cyst(e)ine per mole of total amino acid composition. Similarly, the F' factor is also calculated from eqn (2) but in the absence of tryptophan, cyst(e)ine, proline and 4-hydroxyproline. Both F and F' calculated values are constants characteristic of apple flower buds and can be used in all subsequent quantitations of this tissue following standard procedures as described by Horstmann (1979) and Peterson (1983).

The protein concentration (P) of each hydrolysate was then calculated from the following expression:

$$P = F \sum_{i=1}^{17} X_i \qquad (3)$$

where X_i are the namomoles of amino acid i found in the analyzed aliquot.

Determination of the extracellular matrix cell wall 4-hydroxyproline-rich glycoproteins

In this study, an attempt was made to relate the amounts of the protein-bound 4-hydroxyproline, a unique amino acid which occurs exclusively in the 4-hydroxyproline glycoproteins of the primary cell walls of all dicotyledonous plants, i.e., extensins, arabinogalactan proteins, and salt-extractable glycoproteins (McNeil et al, 1984, Cooper et al. 1987), to the major components of these extracellular matrix proteins in apple flower buds. This quantitation is based on three major findings: first, that the 86 KDa carrot extensin monomer consists of 35% protein and 65% carbohydrate; secondly, that the 30 KDa protein moiety contains 306 amino acids in its primary sequence (Chen and Varner 1985a, 1985b, Smith et al. 1986) and has a mean residue weight: WE = 0.1095 ng/nmol; and third, that 4-hydroxyproline makes up 45.5% of the polypeptide backbone, corresponding to 455 4-hydroxyproline residues per

thousand amino acid residues. The anhydrous of 4-hydroxyproline M_{ri} is 113.12.

A general method to calculate the amount of a specific protein i present in apple flower buds from the quantitative determination of a given unique amino acid i which is known to occur exclusively in that specific protein (j) is as follows:

$$(4) \qquad P_i = C_i \cdot \frac{[1000]}{n'_i} \cdot \frac{WE_{pi}}{M_{ri}}$$

where P_i is the concentration of a specific primary cell wall glycoprotein i expressed in g per kg total protein, C_i is the mean concentration of a unique protein-bound amino acid i, i.e., 4-hydroxyproline (in g/kg of total apple flower bud protein), WE_{pi} is the weight equivalent of a specific protein i determined from its amino acid composition using eqn (1) according to Horstmann (1979), and n'_i is the number of residues of a unique amino acid residue i per 1000 amino acid residues.

The amino acid composition of the 86 KDa carrot extensin monomer, which has been the most characterized of the 4-hydroxyproline-rich glycoproteins (Stuart and Varner 1980, Van Holst and Varner 1984, Stafstrom and Staehelin 1986, Cooper et al. 1987) has been used as a standard for comparison in this study. Substituting the computed parameters for extensin from the data reported by Van Holst and Varner (1984) [i.e., extensin: n'_i = 455; WE_{pi} = 0.1095; M_{ri} = 0.1131] in eqn (4), the total 4-hydroxyproline-rich glycoproteins in g/kg total protein in apple flower buds can be calculated as shown below:

(4a) amount of extensin [P_{ext-1}] = amt of Pro[4-OH] x 2.128

Results and discussion

One complication encountered in determination of protein by the analysis of nitrogen in apple flower buds by the Kjeldahl procedure was that a substantial quantity of Kjeldahl nitrogen is derived from non-protein nitrogenous constituents of this tissue (Benedict 1987). The commonly used protein conversion factor of 6.25 calculates a higher than actual protein value in apple flower buds (Table 1).

It was found that the total nitrogen of replicate samples determined either by the Kjeldahl or the Thomas et al. (1967) method was significantly higher than the total protein nitrogen determined by the summation of amino acids nitrogen (Table 1). The three methods used gave nitrogen levels which differed by as much as 42.87%. Similar differences between methods have been reported previously by Heidelbaugh et al. (1975) and Benedict (1987).

To correct for this variance, a new factor (5.51) for the conversion of Kjeldahl nitrogen to protein content was devised, which was based on the summation of the amino acid nitrogen content of this plant tissue (Table 2). While these results are in accord with the factors reported for almonds (5.18), peanuts and Brazil nuts (5.46), tree nuts and coconuts (5.30), etc. (Benedict 1987), the data of Table 1 show that even with the use of the new factor, a higher protein content was calculated in apple flower buds analyzed by the Kjeldahl procedure than that calculated from amino acid analyses as described by Heidelbaugh et al. (1975) and Horstmann (1979).

These authors found that the best estimate of the true protein content of a biological sample is the summation of the amino acids released after total acid hydrolysis in 6M HCl, and because it takes into consideration their different contributions to the total protein weight. Analysis of acid hydrolysates of apple flower buds is presented in Table 2. The values in Table 2 show deviations of less than $\pm$3.0% from the average values obtained between three replicates within the same treatment. The least variability in amino acid content was found when the results were expressed as g of amino acids per Kg of anhydrous, fat and ash-free tissue protein, because the influence of all non-protein constituents of this tissue was eliminated.

Table 1. Comparison of three methods for determining total nitrogen and protein content of apple flower buds harvested at bud break (g per 100 g dry matter)

Component	Methods for determining total nitrogen and protein nitrogen						% differences between methods		
	A		B		C		A and B	A and C	B and C
	Sulfuric acid.hydrogen peroxide ashing (Thomas et al. 1967)		Kjeldahl, (AOAC 1984)		Sum of amino acid nitrogen (Heidelbaugh et al 1975)		(A-B)/A x 100	(A-C)/A x 100	(B-C)/B x 100
	mean±SEM†	CV	mean±SEM†	CV	mean±SEM¥	CV			
Nitrogen	1.917±0 107[a]	1 51	1 654±0 045[b]	4 75	1.095±0 015[c]	2 37	13.71	42 87	33.79
Methods for calculating total protein									
Sum of amino acids					6 029±0 058	2 37			
N x 6 25	11 979±0 104[a]	1 51	10 337±0 284[b]	4 75	6 800±0 032[c]	2 37			
N x 5 51§	10 555±0 129[a]	1 51	09 108±0 250[b]	4 75	6 031±0 084[c]	2 37			

† Mean values and standard error of the means (SEM) for 6 replicates and 30 determinations, CV, coefficient of variation

¥ Mean values and standard error of the means (SEM) for 3 replicates and 24 determinations

[a,b,c] Means, within a row with different supercripts are significantly diffferent at the 1% level by Duncan's new multiple range test

§ The new factor for converting Kjeldahl nitrogen into protein content was calculated by the following analytical convention

$$\text{Protein conversion factor} = \frac{100 \text{ g total nitrogen}}{\text{percent amino acid nitrogen (\%N)}}$$

where N%=18 16 calculated from the amino acid data of Table 2

Table 2 The amino acid composition and protein content of apple flower bud tissues harvested at bud break , (March 29, 1985)

Amino acid (AA)	g of AA per Kg of total protein		Mean mole fraction, %	Mean residue weight , μg
	mean±SEM†	CV	$(a_i)^{¥}$	$(a_i \ b_i)^{¥}$
Aspartic acid	108.76±1.07	2 42	10 36	0 011919
Threonine	43 13±0 77	4 36	4 68	0 004727
Serine	41 31±1.51	8 96	5 20	0 004526
Glutamic acid	124 62±1 40	2 75	10 59	0 013659
Proline	46 35±0 64	3 40	5 58	0 005413
Glycine	52 35±0 50	2 35	10 06	0 005731
Alanine	50 09±0 46	2 25	7 73	0 005487
Cyst(e)ine	11 04±0 25	5 56	1 17	0 001210
Valine	57 41±0 84	3 58	6 35	0 006290
Methionine	27 32±0 67	6 02	2 28	0 002992
Isoleucine	51 83±0 59	2 77	5 02	0 005674
Leucine	80 89±0 90	2 74	7 84	0 008857
Tyrosine	50 84±1 24	5 98	3 42	0 005570
Phenylalanine	51 69±0 43	2 02	3 85	0 005662
Histidine	75 91±1 38	4 45	6 49	0 008314
Arginine	65 92±0 98	3 65	4 63	0 007129
Tryptophan	5 35±0 13	5 82	0 32	0 000585
4-Hydroxyproline	23 95±0 37	3 82	2 32	0 003043
Total AA recovered	994 84±0 41	0 11	100 00	
Ammonia	27 42±0 60	5 15		
Total AA nitrogen (g/kg total protein)§	181 60±0 80	1 15		
Mean residue wt (WE), μg (defined by eqn 1)¶				0 109685±0 00007
Conversion factors, μg (defined by eqn 2)¶, F				0 111343±0 00006
F'				0 121056±0 00016
Total protein (g/kg dry weight calculated from eqn 3)¶	60 29±0 59	2 37		
Extracellular matrix glycoprotein (defined by eqn 4)‖				
g kg total protein	50 95±4 43	20 43		
g/kg dry matter	3 09± 0 25	20 43		

Table 2 continued

Table 2 (continued)

† Mean values and standard error of the means (SEM) for 3 replicates and 24 determinations, Cv, coefficient of variation.

¥ a_i is the mole fraction of a specific amino acid i found in the analyzed sample, b_i is the molecular weight of amino acid residue i; and $\sum_{i=1}^{19}(a_i \; b_i)$ is the mean weight equivalent (WE) of AA in that protein mixture

§ Calculated according to Heidelbaugh et al (1975)

¶ The total protein , WE, F and F' constant were calculated according to Horstman (1979)

|| Data for 4-hydroxyproline-rich glycoproteins were calculated from the amounts of 4-hydroxyproline found in the acid hydrolysates of apple flower buds according to eqns (4) and (4a), and represent the mean values of 12 determinations from three replicates

The protein concentration of individual hydrolysate samples was determined by the procedure described by Horstmann (1979). The average weight equivalent (WE, µg/nmol) and conversion factors, F and F' (µg/nmol) obtained are listed in Table 2 and can be used in all subsequent quantitations of protein in this tissue following standard procedures (Horstmann, 1979). Because tryptophan is destroyed during acid hydrolysis of proteins in 6M HCl, cystine is converted to cysteine, and asparagine and glutamine are converted respectively to aspartic and glutamic acids, these considerations were taken into account in calculating the conversion factor F, which is the apparent average residue molecular weight of this protein mixture increased in proportion to the missing tryptophan and cyst(e)ine residues [eqn (2)]. Similarly, the F' factor is also calculated from eqn (2) but in the absence of tryptophan, cyst(e)ine, proline and 4-hydroxyproline. Apple flower buds harvested at bud break contained 6.03% total protein on a dry weight basis (Table 2). The moisture content of this plant tissue averaged 47.3 %.

To determine whether 4-hydroxyproline could be used as an index for determining the 4-hydroxyproline-rich glycoproteins of the primary cell wall of plant tissues, 4-hydroxyproline content of apple flower buds (cv. McIntosh/M7) was determined at the nanomole range by the single-column chromatographic method described previously (Zarkadas et al. 1986). Typical chromatographic separations obtained for 4-hydroxyproline and other amino acids by this system are illustrated in Fig. 1A. As may be seen in Fig. 1B, 4-hydroxyproline from a 24 h apple flower bud acid hydrolysate sample separated completely from aspartic acid and they eluted from the column at 32.2 and 36.7 min, respectively. Recoveries of 4-hydroxyproline can be calculated relative to alanine, which elutes at 74.5 min. Fig. 1B also shows the complete separation of three major and two minor, as yet unidentified ninhydrin-positive peaks with a characteristic higher absorbance at 440 nm compared to 570 nm. Each of the five unknown peaks was assigned an Arabic number to indicate its relative order of elution from the column, followed by its retention time in minutes as follows: peak 1, 12.7; 2, 17.5; 3, 23.0; 4, 27.2 and 5, 32.2 min. The identity of these compounds has not yet been established and further detailed studies are required to ascertain their nature.

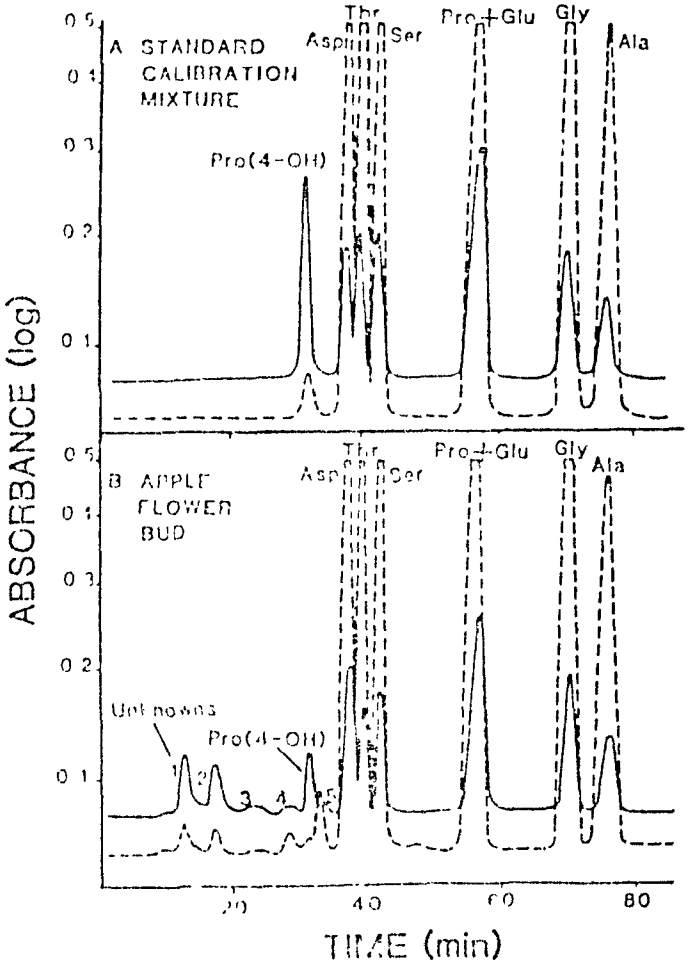

Fig. 1. Chromatographic separations of 4-hydroxyproline in apple flower bud tissue hydrolysates. A, separation of a synthetic amino acid calibration mixture; B, typical chromatographic separation of a 24-h hydrolysate of apple flower bud tissue. The upper curve shows absorbance at 440 nm and the lower curve (- - - -) the absorbance at 570 nm. Each of the five unknown ninhydrin positive peaks that has been separated was assigned an Arabic number (1 to 5) to indicate its relative order of elution from the column (Fig. 1B).

The presence of considerable amounts of 4-hydroxyproline in the acid hydrolysates of apple flower buds is highly significant. The values obtained for protein-bound 4-hydroxyproline show low coefficients of variation, and within the precision of the present methodology (±3.0%), recoveries were found to be quantitative (Table 2). Based on the known distribution of 4-hydroxyproline in the primary sequence of the glycoprotein moiety of carrot extensin (Cooper et al. 1987), which has been used as a standard for comparison in this study, the content of 4-hydroxyproline-rich glycoproteins in the primary cell walls of apple flower buds was calculated by multiplying the amounts of 4-hydroxyproline found in their acid hydrolysates (Table 2) by the conversion factor 2.128 [eqn (4a)]. The results summarized in Table 2 show that the amounts of 4-hydroxyproline-rich glycoproteins in the primary cell walls of apple flower buds average 5.09% of total protein corresponding to 0.31% of the dry weight or 0.16% of the fresh weight of this plant tissue. Using this sensitive method, further studies are now being carried out to quantitatively establish the levels and variation of these important primary cell wall glycoproteins in flower buds during the seasonal cycle of this deciduous tree.

References

AOAC, 1984. "Official Methods of Analysis", 14th ed., Association of Official Analytical Chemists, Washington, DC.

Benedict, R.C. 1987. Determination of nitrogen and protein content of meat and meat products. J. Assoc. Off. Anal. Chem. 70:69-74.

Bradford, N.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Chen, J. and Varner, J.E. 1985a. Isolation and characterization of cDNA clones for carrot extensin and a protein-rich 33 kDa protein. Proc. Natl. Acad. Sci. USA. 82:4399-4403.

Chen, J. and Varner, J.E. 1985b. An extracellular matrix protein in plants: Characterization of a genomic clone for carrot extensin. EMBO J. 4:2145-2152.

Cooper, J.B., Chen, J.A., Van Holst, G.J. and Varner, J.E. 1987. Hydroxyproline rich glycoproteins of plant cell walls. TIBS 12:24-27.

Duncan, D.B. 1955. Multiple range and multiple F tests. Biometrics 11:1-42.

Fauconnet, M., and Rochemont, J.A. 1978. A single column amino acid analysis method which resolves hexosamines and several cysteine derivatives. Anal. Biochem. 91:403-409.

Ferrari, A. 1960. Nitrogen determination by a continuous digestion and analysis system. Ann. N.Y. Acad. Sci. 87:792-800.

Gornall, A.G. and Bardawill, C.J. 1949. Determination of serum protein by the means of the Biuret reaction. J. Biol. Chem. 177:751-766.

Groves, W.E , Davis, F.L. Jr., and Shells, B.H. 1968. Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Anal. Biochem. 22:195-210.

Heidelbaugh, N.D., Huber, C.S., Bednarczyk, J.F., Smith, M.G., Rambart, P.C. and Wheeler, H.O. 1975. Comparison of three methods for calculating protein content in foods. J. Agric. Food Chem. 23:611-613.

Horstmann, H.J. 1979. A precise method for the quantitation of protein taking into account their amino acid composition. Anal. Biochem. 6:230-138.

Hugli, T.E. and Moore, S. 1972. Determination of the tryptophan content of proteins by ion exchange chromatography of alkaline hydrolysates. J. Biol. Chem. 247:2828-2834.

Hunt, S. 1983. Degradation of amino acids accompaning *in vitro*. In Chemistry of amino acids. Edited by G.C. Barrett. Chapman and Hall, London. pp. 376-398.

Jones, D.G. 1931 (slightly revised, Feb. 1941). Factors for converting percentages of nitrogen in foods and feeds into percentages of protein. U.S. Dept. Agr. Circ. 183.

Lamport, D.T.A. 1980. Structure and function of plant glycoproteins. Pages 501-541 in The Biochemistry of plants. Vol. 3. J. Preiss, ed. Academic Press, New York. pp. 501-541.

Lamport, D.T.A. and Epstein, L. 1983. A new model for the primary cell wall: A concatenated extensin-cellulose network. Pages 73-83 in Current Topics in Plant Biochemistry and Physiology. Vol. 2. D.D. Randall, D.J. Blevins, R.L. Larson, B.J. Rupp, S.G. Palardy, C.J. Nelson, J.C. Polacco, and M.F. George, eds. University of Missouri, Columbia, MO.

Lowry, O.L., Rosebrough, N.J., Farr, A.L. and Randall, R.S. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Marks, D.L, Buchsbaum, R. and Swain, T. 1985. Measurement of total protein in a plant sample in the presence of tannins. Anal. Biochem. 147:136-143.

Maynard, L.A. and Loosli, J.K. 1969. Animal nutrition (6th ed.), McGraw-Hill Book Company, New York. P. 613.

McNeil, M., Darvill, A.G., Fry, S.C. and Albersheim, P. 1984. Structure and function of the primary cell walls of plants. Pages 625-663 in Annual Review of Biochemistry. Vol. 53. C.G. Richardson, P.D. Boyer, and A. Meister, eds. Annual Reviews Inc., Palo Alto, CA.

Moore, S. 1963. On the determination of cysteine as cysteic acid. J. Biol. Chem. 238:235-237.

Nguyen, Q., Fanous, M.A., Kamm, L.H., Khalili, A.D., Schuepp, P.H. and Zarkadas, C.G. 1986. Comparison of the amino acid composition of two commercial porcine skins (rind). J. Agric. Food Chem. 34:565-572.

Peterson, G.L. 1983. Determination of total protein. Pages 95-119 in Methods in Enzymology. Vol. 91. C.H.W. Hirs and S.N. Timasheff, eds. Academic Press, New York, NY.

Robinson, T. 1979. The determination of protein in plant extracts that contain polyphenols. Plant Sci. Lett. 15:211-216.

Scopes, R.K. 1974. Measurement of protein by spectrophotometry at 205 nm. Anal. Biochem. 59:277-282.

Smith, J.J., Muldoon, E.P., Willard, J.J. and Lamport, D.T.A. 1986. Tomato extensin precursors P1 and P2 are highly periodic structures. Phytochemistry. 25:1021-1030.

Stafstrom, J.P. and Staehelin, L.A. 1986. Cross-linking patterns in salt-extractable extensin from carrot cell walls. Plant Physiol. 81:234-241.

Stock, D.M., Anderson, J.W. and Rowan, K.S. 1968. The isolation of mitochondria from potato-tuber tissue using sodium metabisulphite for preventing damage by phenolic compounds during extraction. Phytochemistry. 7:1509-1512.

Stuart, D.A. and Varner, J.E. 1980. Purification and characterization of a salt-extractable hydroxyproline-rich glycoprotein from aerated carrot discs. Plant Physiol. 66:787-792.

Thomas, R.L., Sheard, R.W. and Moyer, J.R. 1967. Comparison of conventional and automated procedures for nitrogen, phosphorus and potassium analysis of plant material using a single digestion. Agron. J. 59:240-243.

Van Holst, G.J. and Varner, J.E. 1984. Reinforced polyproline II conformation in a hydroxyproline-rich cell wall glycoprotein from carrot root. Plant Physiol. 74:247-251.

Warburg, O. and Christian, W. 1941/42. Isolierung und kristallisation des garungsferments enolase. Biochem. Z. 310:384-421.

Westwood, M.N. 1978. Dormancy and plant hardiness. Pages 299-332 in Temperate Zone Pomology. W.H. Freeman and Company.

Zarkadas, C.G., Drouliscos, N.J. and Karatzas, C.N. 1988a. Comparison of the total protein, nitrogen and amino acid composition of selected additives and ingredients used in composite meat products. J. Agric. Food Chem. (in press).

Zarkadas, C.G., Meighen, E.A., Zarkadas, G.C., Karatzas, C.N., Khalili, A.D., Rochemont, J.A. and Berthelet, M. 1988b. The determination of the myofibrillar and connective tissue proteins in the bovine diaphragm. J. Agric Food Chem. (in press).

Zarkadas, C.G., Rochemont, J.A., Zarkadas, G.C., Karatzas, C.N. and Khalili, A.D. 1987. Determination of methylated basic, 5-hydroxylysine, elastin crosslinks, other amino acids, and the amino sugars in proteins and tissues. Anal. Biochem. 160:251-266.

Zarkadas, C.G., Zarkadas, G.C., Karatzas, C.N., Khalili, A.D. and Nguyen, Q. 1986. A rapid method for determining desmosine, isodesmosine, 5-hydroxy-proline, tryptophan, lysinoalanine and the amino sugars in proteins and tissues. J. Chromatogr. 378:67-76.

Connecting text

Comparison of three different methods for determination and calculation of protein, and amino acid content of apple flower buds at bud break (*Malus pumila.* Mill. cv. McIntosh/M7) showed great variabilty in the amounts calculated depending on the method of calculation. This difference is because of incorporation of nitrogen from other sources (nucleic acids, amide, etc.) when using the Kjeldahl or hydrogen peroxide sulfuric acid procedures (Benedict 1987). By far the most precise method for the quantitation of protein in plant tissues appears to be the ninhydrin reaction of amino acids. This method is based on the summation of amino acid content, as determined by chemical analysis. Using this method, the next experiment was carried out to determine if the levels of protein and amino acids changed during the development of apple flower buds from inception to flowering, including the period of cold acclimation and dormancy.

Benedict, R. C. 1987. Determination of nitrogen and protein content of meat and meat products. J. Assoc. Off. Anal. Chem. 70:69-73.

III. SEASONAL VARIATION OF PROTEINS AND AMINO ACIDS IN APPLE FLOWER BUDS (*Malus pumila* Mill., cv. McIntosh/M7)

Introduction

The level of nitrogenous compounds in deciduous fruit trees changes substantially during autumnal leaf senescence when much of the leaf nitrogen is translocated into spurs and smaller twigs and thence into older shoot bark (Murneek and Logan, 1932; Oland, 1963; Spencer and Titus, 1972; O'Kennedy et al., 1975; Kang and Titus, 1980). Amino acids stored in the woody tissue of fruit trees are of particular importance in the early stages of spring growth since at this time environmental conditions for absorption and translocation of nutrients are not always optimal (Batjer and Rogers, 1952; Boynton, 1954). This reserve nitrogen is utilized early in the spring for the development of flowers and leaves (Tromp and Ovaa, 1971, 1979; O'Kennedy and Titus, 1979). Most studies on nitrogen metabolism in apple trees report changes of amino acids and soluble protein in shoots, bark or leaves, but little is known of the variation in the levels of these cellular constituents in the buds themselves.

The molecular mechanisms that control higher plant growth and development, dormancy, and remarkable adaptation to low temperatures are not well understood (Goldberg, 1987; Tseng and Li, 1987). It is not known, for example, how meristems originate and give rise to the morphological pattern specific for vegetative organ systems (leaf, stem, root) and floral organ systems (petal, stamen, pistil), nor is it known how the underlying molecular processes can regulate differential gene expressions that determine flower patterns in higher plants (Kamalay and Goldberg, 1980; Singer and McDaniel, 1986). There is some evidence that oligosaccharide fragments from plants' cell walls may play a role in flowering (Tran Thanh Van et al., 1985); however, this result awaits confirmation.

The seasonal cycle of soluble cellular constituents in relation to cold tolerance in deciduous trees and other plant species, has been well documented (Levitt and Siminovitch, 1940; Siminovitch and Briggs, 1949; Siminovitch, 1963; Pomeroy et al., 1970; Steponkus, 1984; Guy et al., 1985; Tseng and Li, 1987; Yelenoski et al., 1987), and in general indicates an autumn increase in soluble proteins, sugars, and nucleic acids. While these studies have emphasized

that the development of cold tolerance in shoots, bark or leaves is closely associated with a general augmentation of total soluble proteins in the protoplasm, including augmentation of cell organelles and other membranous components of the cell (Pomeroy et al., 1970; Singh et al., 1977a, 1977b; Johnson-Flanagan et al., 1986; Johnson-Flanagan and Singh, 1987), there is little information on the insoluble cell wall or membranous proteins of higher plants, and very little has been done toward studying the problem from the biochemical point of view. The work of Lamport (1980) demonstrated that the extracellular matrices of the primary cell walls of angiosperms are comprised of glycoproteins in which a number of proline residues are post-translationally modified to 4-trans-hydroxyproline. The 4-hydroxyproline-rich glycoproteins, i.e., extensins, arabinogalactan proteins, and salt-extractable glycoproteins and agglutinins, which are major structural components of the primary cell walls of all dicotyledonous plants (Lamport and Epstein, 1983; Fincher et al., 1983; McNeil et al., 1984; Cooper et al., 1987; Averyart-Fullard et al., 1988), are difficult to extract with conventional protein solvents. Jian et al. (1987) have recently found increased levels of glycoproteins at the cell surface of different hardy wheat varieties suggesting that one of their functions is to increase freezing tolerance in plants.

The present study attempts to establish the levels and variation of total protein as well as the individual amino acids in apple spur buds (*Malus pumila* Mill. cv. McIntosh/M7) from bud inception (July) to the first pink stage (April). The aims were 1) to obtain information on quantitative fluctuations of these cellular constituents in this species; 2) to clarify the importance of each amino acid during flower induction and accumulation of storage proteins in overwintering apple buds; and, 3) to ascertain whether or not similar seasonal changes occur in the total amount of 4-hydroxyproline-rich glycoproteins found in the extracellular matrices of the primary cell wall of apple flower buds.

Materials and methods

Materials

Type W-3 cation-exchange spherical resin, sized to 9.0 ± 0.5 um was obtained from Beckman Instruments Inc., Palo Alto, CA, while type DC-6A 11.0 ± 1.0 um spherical resin was purchased from Dionex Corporation, Sunnyvale, CA. L-Tryptophan, D-glycosamine.HCl, D-galactosamine.HCl, and 4-hydroxyproline were obtained from Calbiochem-Behring Corp., LaJolla, CA. 3-Nitro-L-tyrosine was purchased from Aldrich Chemical Co., Milwaukee, WI, and was purified as described previously (Zarkadas et al., 1987). The standard amino acid calibration mixture, Piercesolve (ethylene glycol monomethyl ether), ninhydrin, and stannous chloride.H_2O were purchased from Pierce Chemical Co., Rockford, IL. Sodium citrate, dihydrate (crystals) was obtained from Allied Fisher Scientific, Fairlawn, NJ. All other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Experimental Procedure.

Collection of Plant Material and Sample Preparation.

Buds were collected from six 30-year-old apple trees, *Malus pumila* Mill. cv. McIntosh/M7, at the Macdonald College of McGill University orchard, Ste. Anne de Bellevue, Quebec. Forty buds from spurs on two- to three-year-old wood, assumed to be flower buds (90%), from each tree were combined, and quickly frozen at -170°C. All tissues were lyophilized for 24 h, ground in a standard electrically driven Thomas-Wiley Intermediate Model mill equipped with a 64 mm stainless steel hopper and stationary blades (A.H. Thomas Company, Philadelphia, PA), and stored in small plastic bottles at -20°C for subsequent analysis.

Extraction Procedure for Apple Flower Bud Tissues.

To remove all traces of soluble amino acids and other compounds from apple flower bud tissues, samples (2-3 g) of the pulverized tissues were extracted with a mixture of 0.1 M HCl in 75% ethyl alcohol (Rangeley and Lowrie, 1976). The samples were suspended in 200 mL of extraction solvent, homogenized for 3 min in a VirTis Model 45 (VirTis, Gardiner, NY) homogenizer (speed set at 30/100), and the homogenates were centrifuged at 50,000 x g

(SS-34 Sorvall rotor) for 30 min at 2°C. The supernatants were removed and dried under vacuum (Buchi, Rotavapor, Switzerland) at 45°C. The pellet was suspended in the same extraction solvent and the extraction procedure was repeated twice. The final pellets were suspended in 20 volumes acetone, and the suspension was again centrifuged as before. The pellets from the final centrifugation were dried at 50°C overnight, and then placed under vacuum to remove the last remnants of solvent. The dried pellets were finally ground to pass through a 40 mm screen and stored at -20°C until needed.

Procedures for Amino Acid Analyses.

Amino acid analyses were carried out on a semiautomated amino acid analyzer (Beckman Spinco Model 120C) using single-column methodology as described previously (Zarkadas et al. 1986).

Seasonal changes in amino acids and proteins of apple flower buds were determined on 50-mg samples of lyophilized powders. Samples were hydrolyzed in Pyrex test tubes (18 x 150 mm) under vacuum (below 10 μm of mercury) with 15.0 mL of triple-glass distilled constant boiling HCl (6.0 M) at 110°C in duplicate for each of four times, 24, 48, 72 and 96 h, respectively, with the usual precautions described by Hunt (1985) and Zarkadas et al. (1988).

The data reported for serine, threonine, and tyrosine represent the average of values extrapolated to zero time of hydrolysis. Addition of phenol (10-15 μL) to the hydrolysates usually prevented chlorination of tyrosine. The values for valine, isoleucine, leucine and phenylalanine are averages of data from 48, 72 and 96 h of hydrolysis. All others are reported as the average values from 24, 48, 72, and 96 h of hydrolysis as described previously (Zarkadas et al., 1988).

The 4-hydroxyproline was determined separately from concentrated hydrolysate (equivalent to 0.1 mg of protein/analysis) by the procedure of Zarkadas et al. (1986). Recoveries of 4-hydroxyproline were calculated relative to alanine. Methionine and cyst(e)ine were determined separately (50-mg) by the performic acid procedure of Moore (1963). The recovery of cystine plus cysteine as cysteic acid and of methionine as the methionine S,S-dioxide was calculated relative to the yields obtained by the performic acid treatment of standard solutions of these amino acids and relative to alanine and leucine present in the samples.

Tryptophan in apple flower bud samples (50 mg) was also determined separately after alkaline hydrolysis (Hugli and Moore, 1972) by a rapid method (Zarkadas et al., 1986), using 3-nitrotyrosine as the internal standard.

Data processing and regression analysis of the amino acid data were carried by the General Linear Model (GLM) procedure of the Statistical Analysis System (SAS, 1985).

Predicting Properties of Proteins From Amino Acid Compositions.

Since amino acid compositions represent a large body of easily accessible data that is not clearly related in any simple way to useful structural properties of proteins, it would be useful if there were unequivocal ways of grouping the amino acids into classes with distinct properties in the hope that such classes correlate to some extent with the rather general properties of the proteins in mixtures. One feature of protein structures that is fairly reliable is the tendency of the side chain of charged or very polar amino acid residues to be external, interact strongly with water, and have high solubility in water. At the opposite end of the polarity scale are the apolar or hydrophobic side chains which tend to have low solubility in water and therefore will be internal (Bigelow, 1967; Nozaki and Tanford, 1971).

One feature of protein structures that should be attainable from amino acid composition is predicting whether a protein or protein mixture is associated with membranes, and thus should have a larger number of nonpolar residues and fewer charged residues, or whether a protein mixture is located in the cytosol in which case it should resemble a typical soluble nonmembrane protein(s). Barrantes (1973, 1975) has grouped the amino acids into four classes: total charged; hydrophylic; hydrophobic; and apolar, and simply compared the ratio (R) of the frequencies of occurrence (X) of whatever particular side chains of proteins one wishes to stress, e.g.,

$$R = \frac{\sum_k x_k}{\sum_j x_j} \qquad (1)$$

where k can be hydrophilic and j hydrophobic side chains, or k polar and j nonpolar as defined by Barrantes (1973) below:

Basic: histidine + lysine + arginine;

Acidic: aspartic + glutamic + asparagine + glutamine;

Total charged: basic + acidic;

Hydrophilic: total charged + threonine + serine;

Hydrophobic: valine + methionine + isoleucine + leucine + tyrosine + phenylalanine + tryptophan;

Apolar: hydrophobic - tyrosine;

Ratio 1 (R1): hydrophilic/hydrophobic;

Ratio 2 (R2): hydrophilic/apolar;

Ratio 3 (R3): total charged/hydrophobic; and

Ratio 4 (R4): total charged/apolar.

Although the particular choice of residues used to construct these ratios is somewhat arbitrary (Barrantes, 1973, 1975) one particular ratio scale that reliably weights the tendency of charged or very polar residues to be external is R3. This ratio (R3) is convenient because it spreads out different proteins over a wide scale range, from 0.36 to 2.03, and gives a measure with more information about the system.

Determination of 4-hydroxyproline Glycoproteins.

Total protein in each apple flower bud acid hydrolysate was determined by the procedure of Horstmann (1979) as described previously (Nguyen et al., 1986, Zarkadas et al., 1987). In this study, an attempt was also made to relate the amounts of protein-bound 4-hydroxyproline, which occurs exclusively in the 4-hydroxyproline-rich glycoproteins of the primary cell walls of the Angiosperms, i.e., extensin, arabinogalactan protein, and salt-extractable glycoproteins (Lamport, 1980; McNeil et al., 1984; Cooper et al., 1987), to the contents of these extracellular matrix proteins in apple flower buds.

Previous studies (Zarkadas et al., 1988) have shown that a general method to calculate the amount of a specific protein j present in apple flower buds from the quantitative determination of a given unique amino acid j which is known to occur exclusively in that specific protein (j) is as follows:

$$P_j = C_j \cdot \frac{[1000]}{n'_j} \cdot \frac{WE_{pj}}{M_{rj}} \qquad (2)$$

where P_j is the concentration of a specific primary cell wall glycoprotein j, i.e., extensin, expressed in g per kg total protein, C_j is the mean concentration of a unique protein-bound amino acid j, i.e., 4-hydroxyproline, in g per kg of total protein, WE_{pj} is the weight equivalent of a specific protein j determined from its known amino acid composition according to Horstmann (1979), and n'_j is the number of residues of a unique amino acid residue j per 1000 amino acid

Since the 86KDa carrot extensin monomer has been the best characterized (Cooper et al., 1987), its amino acid composition reported by Stuart and Varner (1980) and Van Holst and Varner (1984) has been used as a standard for quantitating the 4-hydroxyproline-rich glycoprotein content in apple flower buds in this study. This quantitation is based on three major findings: first, that the 86KDa carrot extensin monomer (ext-1) consists of 35% protein and 65% carbohydrate; second, that the 30KDa protein moiety contains 306 amino acids in its primary sequence (Chen and Varner, 1985a, 1985b; Smith et al., 1986) and has a calculated mean residue weight: WE = 0.1095 ng/nmol (Horstmann, 1979); and third, that 4-hydroxyproline makes up 45.5% of the polypeptide backbone, corresponding to 455 4-hydroxyproline residues per thousand amino acid residues. The anhydrous M_{rj} of 4-hydroxyproline is 113.12. Substituting the computed parameters for extensin in eqn (2), the total 4-hydroxyproline-rich glycoproteins in g/kg total protein in apple flower buds can be calculated by the following analytical convention:

$$\text{amount of extensin } (P_{ext\text{-}1}) = \text{amt of Pro(4-OH)} \times 2.128 \qquad (2a)$$

Results and discussion

Flower buds of 30-year-old apple trees (cv. McIntosh/M7) were chosen as an example of a potentially hardy tree tissue because it would be expected that the amplitude of protein change in the cells of the vegetative shoot meristem will be greatest and easiest to detect in this fast-growing tissue, even in autumn or winter, when tree buds are exposed to the greatest extremes of low temperatures, 10°C to -50°C or lower. Accurate determination of total protein and amino acids in apple spur buds, at various stages of growth and development, was carried out at the nanomole range by use of analytical chromatographic methods described previously (Zarkadas et al., 1986), and the results obtained

are summarized in Table 3. The analytical capability of the present methodology and its application to complex tissue hydrolysates, i.e., apple flower buds, have been presented previously (Khanizadeh et al., 1987; 1988).

Seasonal cycle of amino acids.

The seasonal changes of amino acids in the buds and levels of statistical significance obtained from analysis of variance as presented in Table 3 represent the average values of six replicates and duplicate determinations obtained from duplicate 24, 48, 72 and 96 h acid hydrolysates. These values show deviations of less than ±3.0% from the average values obtained between replicates within the same sampling period. The least variability occurred when the amino acid data were expressed as g of amino acids per 100 g of anhydrous fat- and ash-free protein. The main advantage of this unit of expressing the composition of a complex protein mixture is that comparison can be made on a protein basis, and that the percentage recovery by weight can be found by simple summation (Tristram and Smith, 1963; Zarkadas et al., 1987). The mean protein concentration of each sample was determined by the procedure described by Horstmann (1979). The mean residue weight (WE, mg/nmol) and conversion factors F and F' (µg/nmol) obtained are listed in Table 3, and can be used in all subsequent quantitations of the same tissue following standard procedures as described by Horstmann (1979), Peterson (1983) and Zarkadas et al. (1987).

The results show that although the amino acid profiles of apple flower buds at various stages of development appeared to be similar and highly characteristic of this plant tissue, there was a preferential accumulation of individual amino acids in buds throughout the season (Table 3). The seasonal variation of amino acids in apple bud tissues was found to be highly significant ($P<0.01$) for each amino acid analyzed, except for aspartic acid.

It would therefore be useful if the constituent amino acids of apple flower buds could be grouped into classes with distinct properties so that such classes correlate to some extent with the general properties of the proteins in this plant tissue. As mentioned earlier, Barrantes (1973, 1975) has grouped the amino acids into four classes: totally charged; hydrophylic; hydrophobic; and apolar, and simply compared the ratio (R) of the frequencies of occurrence of whatever particular side chains of proteins one wishes to stress, especially in terms of the proportion of total charged and hydrophobic residues (ratio R3).

Table 3. Seasonal changes of protein-bound amino acids and proteins in the flower buds of apple trees, Malus pumila Mill. cv. McIntosh/M7).

(g of amino acid per 100 g of total protein)

Amino acid	date of sampling												significant levels betw. test periods	
	July 2?, 1984		August 24, 1984		October 24, 1984		March 29, 1985		April 11, 1985		April 28, 1985			
	mean±SEM[3]	CV	mean±SEM[3]	CV	mean±SEM[3]	CV	mean±SEM[3]	CV	mean±SEM[3]	CV	mean±SEM[3]	CV	CV[3]	F[3]
[illegible] acid	12.4?±0.??	6.17	12.60±0.26	5.54	1?.05±0.?4	5.65	12.04±0.35	10.30	11.90±0.21	6.00	12.37±0.17	3.81	6.89	0.99^{ns}
threonine	4.68±0.10^{c,d,e}	5.55	5.13±0.04^{b}	4.20	4.35±0.07^{e}	4.63	4.59±0.13^{d,e}	9.60	4.88±0.09^{b,c,d}	6.60	5.03±0.27^{b,c}	15.00	8.59	4.20**
serine	5.01±0.06^{b}	4.2?	4.91±0.12^{b}	7.11	4.47±0.0?	5.1?	4.42±0.12^{c}	9.00	4.36±0.07^{c}	5.81	4.00±0.16^{d}	11.11	7.26	10.72**
glutamic acid	11.12±0.14^{d}	3.?4	10.24±0.1?	2.?6	12.19±0.04^{c}	0.98	12.12±0.13^{c}	3.72	12.24±0.21^{c}	6.01	13.20±0.19^{b}	4.01	4.19	36.12**
[illegible]	4.13±0.03?	5.54	4.57±0.0?	2.0?	4.70±0.1?^{b}	[illegible]	4.65±0.04^{b,c}	[illegible]	4.74±0.05^{c}	3.81	3.97±0.06^{c}	4.15	4.22	19.69**
[illegible]	5.?6±0.?^{b}	4.30	[illegible]	9.4?	5.23±0.12^{b}	[illegible]	4.94±0.05^{b,c}	5.44	4.72±0.12^{c}	8.6?	4.74±0.09^{c}	5.10	6.86	3.94**
[illegible]	[illegible]	3.3?	5.39±0.1?^{b}	3.47	4.67±0.0?	[illegible]	4.9?±0.03^{c}	2.03	4.62±0.05^{d}	4.08	5.44±0.20^{b}	10.33	5.17	21.77**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.02±0.02^{b}	5.80	1.19±0.01^{b}	2.41	10.33	2.51*
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	3.77	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	2.7?±0.?^{b}	5.45	2.54±0.0?	9.30	2.94±0.03	2.95	7.29	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	4.6?±0.0?^{d}	4.0?	4.63±0.07^{e}	?.35	3.66	23.04**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	7.75±0.1?	[illegible]	7.80±0.?	3.5?	[illegible]	12.4?**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	2.43±0.33	[illegible]	3.35	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	5.6?	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]**

Table 3 continued...

[a]Mean values and standard error of the means (SEM) for 3 replicates and 24 determinations. Significance: F, values from analysis of variance; **, P<0.01; *, P<0.05, key ns, not significant. CV, coefficient of variation.

[b,c,d,e]Means along a horizontal column with different superscripts are significantly different at the P<0.01 level by Duncan's new multiple range test (Duncan, 1955).

[f]Calculated [illegible] (1975).

[illegible]

[illegible] Barnantes [illegible]

[illegible]

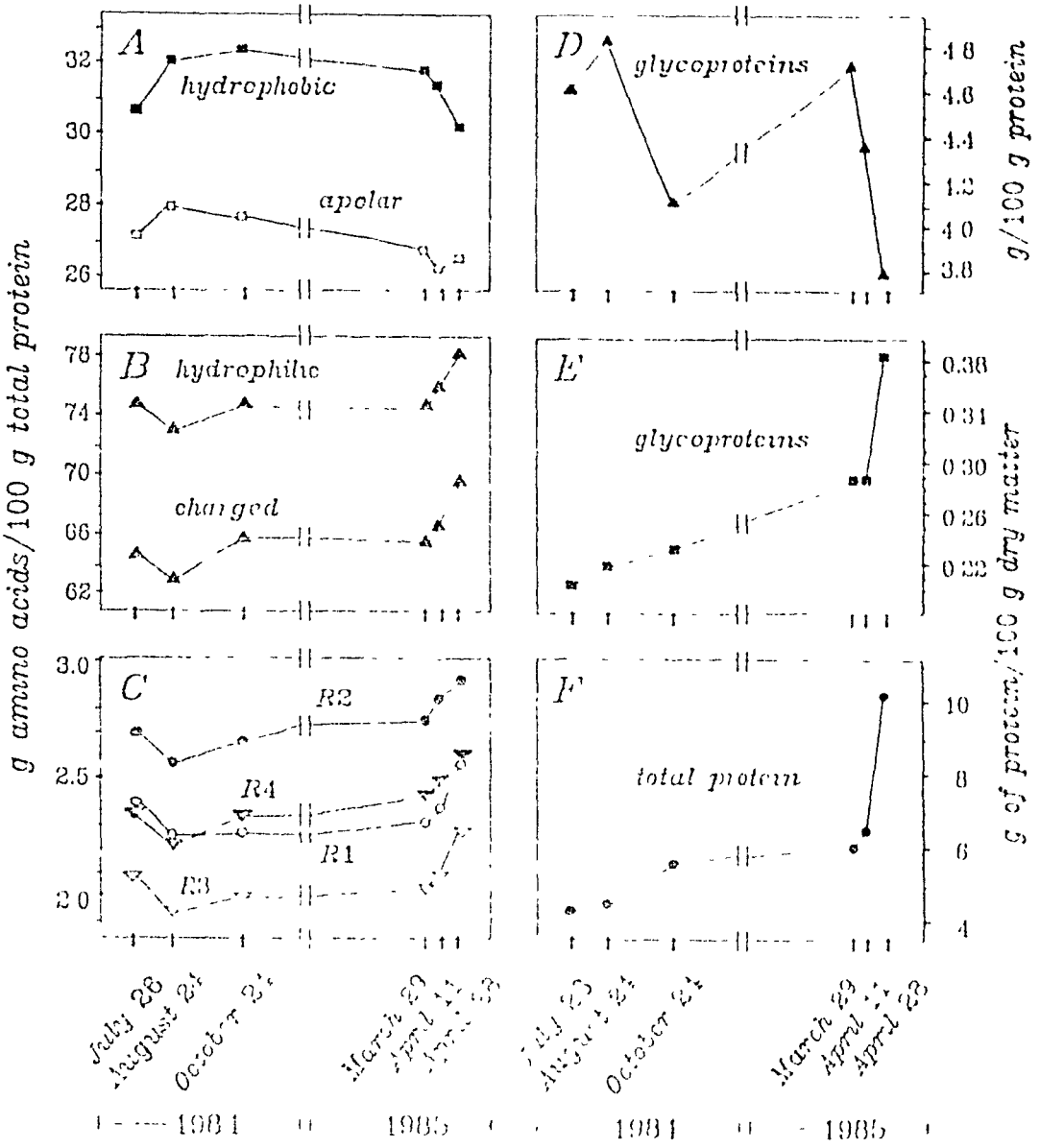

Fig. 2. Variation of proteins and amino acids in apple flower buds (Malus pumila Mill c.v. McIntosh N17) during the sampling period.

A. hydrophobic (▽) and apolar (○) amino acids; B hydrophilic (△) and total charged (□) amino acids, C. ratios defined by Barrantes (1973) as: R1, hydrophilic/hydrophobic (◇), R2, hydrophilic/apolar (●), R3, total charged/hydrophobic (▽), R4, total charged/apolar (▼), D 4-hydroxyproline-rich glycoproteins expressed on a total protein basis (▲), E. 4-hydroxyproline-rich glycoproteins expressed on a dry weight basis (■); F. total protein (●)

The seasonal cycle of proteins

The results presented in Table 3 show that the protein content of buds started to increase just before cold acclimation and after the fruit had been harvested. Unlike bark storage nitrogen, which increases before tree dormancy and decreases in early spring (Siminovitch, 1963; Titus and Kang, 1982), bud storage proteins increased constantly throughout the season. As may be seen in the composite Figure 2F, the increases in protein content (g/100 g dry weight basis) occurred principally from August 24 to October 24, and continued during the winter into early spring (March 29), while the major change in net protein synthesis occurs in the period from March 29 to April 28. It is not known what triggers these synthetic processes but the net effect is a gradual increase in protein content within the existing cells which may change their physical properties so that it enables them to withstand or adapt to adverse low temperatures of -20°C to -50°C or lower, prevalent in the north temperate zone. The results are in accord with those reported by other investigators (Kacperska-Palasz et al., 1977; Brown and Bixby, 1975; Trunova and Zvevera, 1977; Chen and Li, 1980).

To ascertain whether or not other than the soluble proteins and protoplasmic proteins of the apple flower buds are increased during dormancy and adaptation to low temperatures, as suggested by Siminovitch (1963) and Pomeroy et al. (1970), the ratio R3 was calculated (Figure 2C). The results of Table 3 show that from July 26 to August 24 there is a change in the R3 ratio and hydrophobicity of the proteins in the apple flower buds. This suggests that some of the newly synthesized proteins in August must have higher levels of hydrophobic amino acids in their primary sequence but lower solubility and fewer charged amino acids. It should also be noted that the R3 ratio of the same protein mixtures in October and March are markedly lower in comparison with the high R3 values obtained in late April (Figure 2C), which coincides with the period of increased protein synthesis in the apple flower buds (Figure 2F) at that time. The identity and biological significance of these newly synthesized proteins during dormancy has not yet been established, nor is it known how the underlying molecular processes can regulate differential gene expressions that determine protein transformation patterns in the buds (Singer and McDaniel, 1986).

Further detailed studies to ascertain their nature, function and location in the plant cells, including cell organelles and other membranous protein components of the cell, may prove a very fruitful area for future research.

The presence of considerable amounts of the predominant amino acid 4-hydroxyproline in the acid hydrolysates of apple flower buds, ranging from 1.79 g to 2.27 g/100 g of total protein throughout the season (Table 3), is highly significant. The values obtained for protein-bound 4-hydroxyproline per unit of protein show low coefficients of variation between replicates, and within the precision of the present methodology (±3.0%), recoveries were found to be quantitative (Table 3), indicating that the analytical errors were also small relative to the biological variability ($P<0.01$) observed between bud tissue harvested at different intervals during the season. From the known distribution of 4-hydroxyproline in the primary sequence of the glycoprotein moiety of carrot extensin (Stuart and Varner, 1980; Van Holst and Varner, 1984; Chen and Varner, 1985a; 1985b), which has been used as a standard for comparison in the present study, the content of 4-hydroxyproline-rich glycoproteins of apple flower buds was calculated by multiplying the amounts of 4-hydroxyproline found in their acid hydrolysates by 2.128 [equation (2a)], as described previously (Khanizadeh et al., 1988).

The data on protein composition, as presented in Table 3 on a dry weight basis (DWB), show that the 4-hydroxyproline-rich glycoprotein content of apple flower buds vary significantly ($P<0.01$) throughout the season, ranging from a low of 0.21% (DWB) during the summer to a high of 0.39% (DWB) in the following spring. As may be seen in composite Figure 2E, the increase in 4-hydroxyproline-rich glycoproteins occurs principally in the period from July 26 to August 24, after which it gradually increased which was maintained throughout the winter, while the major change in glycoprotein content appears to occur in the period from April 11 to April 28. From a comparison of the seasonal cycles of glycoproteins and the total protein in apple flower buds (Figures 2E and 2F), it appeared that a relatively close relationship existed between the seasonal variation in 4-hydroxyproline-rich glycoprotein content and the seasonal cycle of total proteins, when the results were expressed on the basis of unit dry weight of tissue. However, when the 4-hydroxyproline-rich glycoprotein content of buds was expressed on a total protein basis, it was evident that the seasonal increase in primary cell wall glycoproteins is not a gradual one, but consists of two peaks (Figure 2D), one which coincides with the time of increase in

hydrophobic amino acids in the buds (Figure 2A) and the other a smaller peak which slightly precedes the period of most intense synthesis of hydrophilic amino acids and protein from April 11 to April 28 (Figures 2B and 2F). The results obtained on the seasonal cycle of proteins in apple buds are compatible with those reported by Siminovitch (1963) on black locust bark and Li et al. (1965) on dogwood. These authors have consistently demonstrated, for example, that a close correlation exists between seasonal variation in protein content and winter hardiness in these two deciduous tree species. Similar studies on apple trees have not been reported previously. Nevertheless, the striking and consistent biochemical changes observed in the flower buds of apple trees during the early autumn and spring test periods, pointing to an increase in many macromolecular components, suggest that augmentation of total protein, including the 4-hydroxyproline-rich glycoproteins of the primary cell wall, in some way increase the adaptation of the bud cells to withstand the stress of the winter temperatures of the north temperate zone. The biochemical mechanism by which this increase in freezing tolerance is achieved remains to be elucidated.

References

Averyhart-Fullard,V.; K. Data; Marcus, A. "A hydroxyproline-rich Protein in the Soybean Cell Wall." Proc. Natl. Acad. Sci. U.S.A. 1988, 85, 1082-1085.

Barrantes, F.J. "A Comparative Study of Several Membrane Proteins from the Nervous System." Biochem. Biophys. Res. Commun. 1973, 54, 395-402.

Barrantes, F.J. "The Nicotinic Cholinergic Receptor. Different Compositions Evidenced by Statistical Analysis." Biochem. Biophys. Res. Commun. 1975, 62, 407-414.

Batjer, L.P.; Rogers, A.L. "Fertilizer Applications as Related to Nitrogen, Phosphorus, Potassium, Calcium, and Magnesium Utilization by Apple Trees." Proc. Amer. Soc. Hort. Sci. 1952, 60, 1-6.

Bigelow, C.C. "On the Average Hydrophobicity of Proteins and the Relation Between it and Protein Structure." J. Theoret. Biol. 1967, 16,187-211.

Boynton, D. Nutrition by Foliar Application. Arnon, D.I.; Machlis, L. Eds.; Annual Review Plant Physiology 5; Annual Review:Stanford, CA, 1954, pp 31-54.

Brown, G.N.; Bixby, J.A. "Soluble and Insoluble Protein Pattern During Induction of Freezing Tolerance in Black Locust Seedling." Physiol. Plant. 1976, 34, 187-191.

Chen, H.H.; Li, P.H. "Characteristics of Cold Acclimation and Deacclimation in Tuber-Bearing Solanum Species." Plant Physiol. 1980, 65, 1146-1148.

Chen, J.; Varner, J.E. "An Extracellular Matrix Protein in Plants:Characterization of a Genomic Clone for Carrot Extensin." EMBO J. 1985a, 3, 2145-2152.

Chen, J.; Varner, J.E. "Isolation and Characterization of cDNA Clones for Carrot Extensin and a Protein-Rich 33 kDa Protein." Proc. Natl. Acad.Sci. U.S.A. 1985b, 82, 4399-4403.

Cooper, J.B.; Chen, J.A.; Van Holst, G.T.; Varner, J.E. "4-hydroxyproline-Rich Glycoproteins of Plant Cell Walls." TIBS 1987, 12, 24-27.

Duncan, D.B. "Multiple Range and Multiple F Tests." Biometrics 1955, 11, 24-27.

Fincher, G.B.; Stone, B.A.; Clarke, A.E. Arabinogalactan-Proteins: Structure, Biosynthesis, and Function. Briggs, W.R.; Jones, R.L.; Walbot, V. Eds; Annual Review of Plant Physiology 34; Annual Reviews:Palo Alto, CA, 1983, pp 47-70.

Goldberg, R.B. "Emerging Patterns of Plant Development." Cell 1987, 49, 298-300.

Guy, C.L.; Niemi, K.J.; Bramble, R. "Altered Gene Expression During Cold Acclimation of Spinach." Proc. Natl. Acad. Sci. U.S.A. 1985, 83, 3673-3677.

Horstmann, J.H. "A Precise Method for the Quantitation of Protein Taking into Account their Amino Acid Composition." Anal. Biochem. 1979, 247, 130-138.

Hugli, T.E.; Moore, S. "Determination of the Tryptophan Content of Proteins by Ion Exchange Chromatography of Alkaline Hydrolysates." J. Biol. Chem. 1972, 247, 2828-2834.

Hunt, S. Degradation of Amino Acids Accompanying In Vitro. Chemistry and Biochemistry of Amino Acids. Barrett, G.C., Ed.; Chapman and Hall; London. 1985, pp 376-398.

Jian, L.; Sun, L H.; Sun, D.L. Glycoproteins of the Cell Surface in Cold Hardy and Cold Tender Wheat (Triticum asetivum L.). Plant Cold Hardiness; Li, P.H, Ed.; Alan R. Liss.New York, NY, 1987, pp. 50-66.

Johnson-Flanagan, A.M.; Barron, L.R.; ıngh, J. "L-Methionine Transport During the Induction of Freezing Hardiness by ABA in Brassica napus Cell Suspension Cultures." J. Plant Physiol. 1986, 124, 309-319.

Johnson-Flanagan, A.M.; Singh, J. Induction of Freezing Tolerance in Suspension Cultures of Brassica napus by ABA at 25˚C: Studies on Protein and RNA Changes; Plant Cold Hardiness; Li, P.H., Ed.; Alan R. Liss:New York 1987, pp 29-41.

Kacperska-Palacz, A , Dlugokecka, K.; Breitenwald, J.; Wcislinska, B. "Physiological Mechanism of Frost Tolerance: Possible Role of Protein in Plant Adaptation to Cold." Biol. Plantarium. 1977, 19, 10-17.

Kamalay, J.C.; Goldberg, R.B. "Regulation of Structural Gene Expression in Tobacco." Cell 1980, 19, 935-964.

Kang, S.M., Titus, J.S. "Qualitative and Quantitative Changes in Nitrogenous Compounds in Senescing Leaf and Bark Tissues of the Apple." Plant Physiol. 1980, 50, 285-290.

Khanizadeh, S.; Buszard, D.; Zarkadas, C.G. "Quantitative Determination of Total Protein Content of Apple (Malus pumila Mill. cv. McIntosh/M7) Flower Buds: a Comparative Study." Hort Sci. 1987, 22, 1061. Abst. 185.

Khanizadeh, S.; Buszard, D.; Zarkadas, C. G. "Seasonal variation of protein and amino acids in apple flower buds (Malus pumila Mill. cv. McIntosh/M7). Hort Sci. 1988, 23 744. Abst. 174.

Lamport, D.T.A. Structure and Function of Plant Glycoproteins. The Biochemistry of Plants 3; Preiss, J. Ed.; Academic Press:New York, 1980, pp 502-541.

Lamport, D.T.A.; Epstein, L. A New Model for the Primary Cell Wall: a Concatenated Extensin-Cellulose Network. Current Topics in Plant Biochemistry and Physiology 2; Randall, B.D.; Blevins, D.J.; Lavson, R.L.; Rupp, B.J.; Palardy, S.G.; Nelson, C.J.; Palasco, J.C ; George, M.F. Eds.; University of Missouri, Columbia, MO. 1983, pp 73-83.

Levitt, J.; Siminovitch, D. "The Relation Between Frost Resistance and the Physical State of Protoplasm." Can. J. Res. C. 1940, 18, 550-561.

Li, P.H.; Weiser, C.J.; Van Huystee, R B. "Changes in Metabolites of Red-Osier Dogwood During Cold Acclimation." Proc. Amer. Soc. Hort. Sci.1965, 86, 723-730.

McNeil, M.; Darville, A.G.; Fry, S.G.; Albersheim, P. Structure and Function of the Primary Cell Walls of Plants. Richardson, D.D., Boyer, P.D., Meister, S. Eds.; Annual Reviews of Biochemistry, 53; Annual Reviews:Palo Alto, CA, 1984, pp 625-663.

Moore, S. "On the Determination of Cysteine and Cysteic Acid." J. Biol. Chem. 1963, 238, 235-237.

Murneek, A.E.; Logan, J.C. "Autumnal Migration of Nitrogen and Carbohydrate in the Apple Tree with Special Reference to Leaves " Univ. Missouri Agr. Expt. Sta. Res. Bull. 1932, 171, 3-30.

Nguyen, Q.; Fanous, M.A.; Kamm, L.H.; Khalili, A.D., Schuepp, P.H.; Zarkadas, C.G. "Comparison of the Amino Acid Composition of Two Commercial Porcine Skins (rind)." J. Agri. Food Chem. 1986, 34, 565-572.

Nozaki, R.; Tanfor, C. "The Solubility of Amino Acids and Two Glycine Peptides in Aqueous Ethanol and Dioxane Solutions." J. Biol. Chem. 1971, 246, 2211-2217.

O'Kennedy, B.T.; Titus, J.S. "Isolation and Mobilization of Storage Proteins from Apple Shoot Bark." Physiol. Plant. 1979, 35:419-424.

O'Kennedy, B.T.; Hennerty, M.J.; Titus, J.S. "Changes in the Nitrogen Reserves of Apple Shoots During the Dormant Season." J. Hort. Sci.1975, 50, 321-329.

Oland, K. "Changes in the Content of Dry Matter and Major Nutrient Elements of Apple Foliage During Senescence and Abscission." Physiol. Plant.1963, 16, 682-684.

Peterson, G.L. Methods in Enzymol. Determination of total protein. Methods in Enzymology. Hirs C. H. W. and Timasheff, S. N. Eds.; Academic Press:New York, NY, 1983, 95-119.

Pomeroy, M.K.; Siminovitch, D.; Wightman, F. "Seasonal Biochemical Changes in the Living Bark and Needles of Red Pine (Pinus resinosa) in Relation to Adaptation to Freezing." Can. J. Bot. 1970, 48, 953-967.

Rangeley, W.R.D.; Lawrie, R.A. "Methylated Amino Acids as Indices in Meat Products. I. Development and Validity of an Analytical Procedure." J. Food Technol. 1976, 11, 143-159.

Siminovitch, D. "Evidence from Increase in Ribonucleic Acid and Protein Synthesis in Autumn for Increase in Protoplasm During the Frost-Hardening of Black Locust Bark Cells." Can. J. Bot. 1963, 41, 1301-1308.

Siminovitch, D.; Briggs, D.R. "The Chemistry of the Living Bark of the Black Locust Tree in Relation to Frost Hardiness. I. Seasonal Variations in Protein Content." Arch. Biochem. 1949, 23, 8-17.

Singer, S.R.; McDaniel, C.N "Floral Determination in the Terminal and Axillary Buds of Nicotiana tabacum L." Dev. Biol. 1986, 118, 587-592.

Singh, J ; De La Roche, I A.; Siminovitch, D. "Differential Scanning Calorimeter Analyses of Membrane Lipids Isolated from Hardened and Unhardened Black Locust Bark and from Winter Rye Seedlings." Cryobiology 1977a, 14, 620-624.

Singh, J.; De La Roche, I.A.; Siminovitch, D. "Relative Insensitivity of Mitochondria in Hardened and Nonhardened Rye Coleoptile to Freezing in situ." Plant Physiol. 1977b, 60, 713-715.

Smith, J.J.; Muldoon, E.P.; Willard, J.J.; Lamport, D.T.A. "Tomato Extensin Precursors P1 and P2 and Highly Periodic Structures." Phytochemistry.1986, 25:1021-1030.

Spencer, P.W.; Titus, J.S. "Biochemical and Enzymatic Changes in Apple Leaf Tissue During Autumnal Senescence." Plant Physiol. 1972, 49, 746-750.

Statistical Analysis System (SAS). User's Guide:Basics; Version 6.0; SAS Institute:Cary, NY, 1985.

Steponkus, P.L. Role of the Plasma Membrane in Freezing Injury and Cold Acclimation. Briggs, W.R.; Jones, R.L.; Walbot, V. Eds.; Annual Reviews of Plant Physiology 35; Annual Reviews:Palo Alto, CA, 1984, pp 543-584.

Stuart, D.A.; Varner, J.E. "Purification and Characterization of a Salt-Extractable Hydroxyproline-Rich Glycoprotein from Aerated Carrot Discs." Plant Physiol. 1980, 66, 787-792.

Tanford, C. "Contribution of Hydrophobic Interactions to the Stability of the Globular Conformations of Proteins." J. Amer. Chem. Soc. 1962, 84:4240-4247.

Titus, J.S.; Kang, S.M. Nitrogen Metabolism, Translocation, and Recycling in Apple Trees. Janick, J. Ed.; Horticultural Reviews 4; AVI Publishing Company:Westport, CO. 1982, pp 204-246.

Tran Thanh Van, K.; Toubart, P.; Couson, A.; Darvill, A G ; Gollin, D.J.; Chelf, P.; Albersheim, P. "Manipulation of the Morphogenetic Pathways of Tobacco Explants by Oligosaccharins." Nature (London) 1985, 314, 615-617.

Tristram, G.R.; Smith, R.H. The Amino Acid Composition of Some Purified Proteins. Anfinsen, C.G.; Anson, M.L.; Edsall, J.T., Eds.; Advances in Protein Chemistry 18; Academic Press:New York, NY, 1963, pp 227-318.

Tromp, J.; Ovaa, J.C. "Spring Mobilization of Storage Nitrogen in Isolated Shoot Sections of Apple." Physiol. Plant. 1971, 25, 16-22.

Tromp, J.; Ovaa, J.C. "Uptake and Distribution of Nitrogen in Young Apple Trees After Application of Nitrate or Ammonium, With Special Reference to Asparagine and Arginine." Physiol. Plant. 1979, 45, 23-28.

Trunova, T.I.; Zvereva, G.H. "Effect of Protein Synthesis Inhibitors on Frost Resistance of Winter Wheat." Soviet Plant Physiol. 1977, 24, 311-316.

Tseng, M-J.; Li, P.H. Changes in Nucleic Acid and Protein Synthesis During Induction of Cold Hardiness. Plant Cold Hardiness; Li, P.H. Ed.; Alan R. Liss:New York, NY, 1987, pp 1-27.

Van Holst, G.J.; Varner, J.E. "Reinforced Polyproline II Conformation in Hydroxyproline-Rich Cell Wall Glycoproteins from Carrot Root." Plant Physiol. 1984, 73, 247-251.

Yelenosky, G.; Mauk, C.S.; Bausher, M.G.; Kushad, M.M. Chemical Bioregulation of Growth and Cold Hardiness in Citrus. Plant Cold Hardiness. Li, P.H. Ed.; Alan R. Liss:New York, NY, 1987, pp 299-321.

Zarkadas, C.G.; Zarkadas, G.C.; Karatzas, C.N.; Khalili, A.D.; Nguyen, Q. "Rapid Method for Determining Desmosine, Isodesmosine, 5-Hydroxyproline, Tryptophan, Lysinoalanine and the AminoSugars in Proteins and Tissues." J. Chromatogr. 1986, 378, 67-76.

Zarkadas, C.G.; Rochemont, J.A.; Zarkadas, G.C.; Zaratzas, C.N.; Khalili, A.D. "Determination of Methylated Basic, 5-Hydroxylysine, Elastin Crosslinks, Other Amino Acids, and the Amino Sugars in Proteins and Tissues." Anal. Biochem. 1987, 160, 251-266.

Connecting text

Total protein and amino acid-nitrogen of spur buds of apple trees (*Malus pumila* Mill. cv. McIntosh/M7) increased in concentration (mg/100g protein) from bud inception to bud break. Grouping amino acids based on their distinct chemical properties and comparing their ratios indicated that, at least in apple flower buds there is a high proportion of polar amino acids and their levels increased during bud development.

Other workers have suggested a relationship between protein content, particular amino acids (hydrophobic and charged) and cold hardiness (Sagisaka and Araki 1983, Sagisaka 1974) and high content of hydrophilic amino acids has been reported to increase the ability of trees, including apples, to withstand low temperatures (Li 1987, Sakai and Larcher 1987).

The next experiment was conducted to determine the effect of crop load on seasonal variation of protein, amino acids and spring frost tolerance of apple flower buds. It has been speculated that an excessively heavy crop load increases the susceptibility of apple trees to low temperature injury perhaps because of nutrient depletion (Granger 1982, Brown and Blackburn 1987).

Brown, D. M. and Blackburn, W. J. 1987. Impacts of freezing temperatures on crop production in Canada. Can. J. Plant Sci. 67:1167-1180.

Granger, R. L. 1982. Cultural practices which facilitated survival of apple trees in the severe winter of 1980-1981. Western New York Apple Growers Newsleter, Feb. and Mar issue of 1982.

Sagisaka, S. 1974. Transition of metabolisms in living polar bark from growing to wintering stage and *vice versa* Plant Physiol. 54.544-549

Sagisaka, S. and Araki, T. 1983 Amino acid pools in perennial plants at the wintering stage and at the beginning of growth Plant Cell Physiol. 24:479-494.

Sakai, A. and Larcher, W. 1987. Frost survival of plants Responses and adaptation of freezing stress. Springer-Verlag, New York, 303 pp.

Li, P. H. 1987. Plant cold hardiness Alan R. Liss , New York 381 pp.

IV. EFFECT OF CROP LOAD ON PROTEIN, AMINO ACIDS AND SPRING FROST RESISTANCE OF APPLE FLOWER BUDS.

Introduction

One of the major problems affecting apple trees in Quebec is low temperature injury occurring during the winter and spring. Deacclimation of temperate zone deciduous fruit trees makes the developing flowers increasingly vulnerable to spring frost damage.

Some researchers have suggested protein synthesis is involved in cold hardiness in a wide range of species (Brown 1978, Levitt 1978, Sakai and Larcher 1987). The seasonal cycle of metabolism of soluble proteins and amino acids, in relation to frost resistance in deciduous trees and other plant species, has been well reviewed (Sakai and Larcher 1987, Li 1987). In general, these indicate an autumnal increase in soluble proteins, sugars, and nucleic acids. These studies have emphasized that the development of frost resistance in shoots, bark or leaves is closely associated with a general augmentation of total soluble proteins in the protoplasm, including augmentation of cell organelles and other membranous components of the cell (Singh et al. 1977). However, other workers have reported that soluble protein content is not changed during cold hardening or that increases in soluble protein are not associated with increased cold hardiness (Pieninzek and Holubowicz 1973). Increased soluble protein content during cold acclimation could be due to increases in synthesis and/or decreased degradation (Li 1987).

Many investigators have studied the amino acid content and composition of plants in an attempt to find correlations with frost hardiness. In some cases, the amino acid content may increase with hardiness, in other it may not show any relationship. Where a relationship exists, it seems to reflect a general accumulation of organic nitrogen during fall (Sakai and Larcher 1987). Free amino acids present during dormancy and the beginning of growth were analyzed in 31 species by Sagisaka and Araki (1983). They separated the 31 species into three distinct groups: a group which only accumulated arginine; a group which only accumulated proline and a group which accumulated arginine and proline and required chilling temperatures for regrowth. Further investigation showed that arginine and proline play a role not only in the storage of nitrogen, but also, in combination with sugars, in protection from freezing injury (Sagisaka and Araki

1983, Sagisaka 1987, Sakai and Larcher 1987). While there is much information on free and soluble amino acids, there is little information available on the amino acid composition of soluble and insoluble proteins.

Supercooling is a phenomenon which is characteristic of many dormant deciduous fruit species (Burke and Stushnoff 1979). It has been known for a long time that a typical DTA profile of supercooling buds consists first of a large exotherm (high temperature exotherm HTE), corresponding to freezing of water in the bud scales and stem axis, followed by a smaller exotherm (low temperature exotherm LTE) representing freezing of shoot primodia or deep supercooled cells (Sakai and Larcher 1987, Graham 1971, Graham and Mullin 1976).

Many researchers have studied cold resistance in *Malus* woody tissue (Brown 1978, Li 1987, Sakai and Larcher 1987) but no work on cold resistance of apple flower buds has so far been reported in relation to crop load; although it has been shown that trees with no crop accumulate more nutrients and initiate more flowers for the next year (Luckwill 1974, Buban and Faust 1982). Trees in Eastern Ontario which had carried a heavy crop of apples in 1980 were more severely injured during the winter of 1980/1981, apparently due to nutrient depletion (Brown and Blackburn 1987). If higher protein and amino acid content increases the potential degree of cold hardening, and if the absence of a crop increases the accumulation of nutrients (e.g. protein, amino acids, etc.), then the question that arises is: "Are the flower buds of cropped-trees more susceptible to spring frost injury than those on non-cropped trees?"

Materials and methods

Type W-3 cation-exchange spherical resin, sized to 9.0 ± 0.5 um (Beckman Instruments Inc., Palto Alto, Ca), type DC-6A 11.0 ± 1.0 *um* spherical resin (Dionex Corporation, Sunnyvale, CA.), L-tryptophan. HCl, and 4-hydroxyproline (Calbiochem-Behring Corp., LaJolla, CA.), 3-Nitro-L-tyrosine (Aldrich Chemical Co., Milwaukee, WI), were used in the procedure. The standard amino acid calibration mixture, Piercesolve (ethylene glycol *monomethyl ether*), *ninhydrin*, *stannous chloride*.H_2O (Pierce Chemical Co., Rockford, IL.), sodium citrate, dihydrate (crystals), (Allied Fisher Scientific, Fairlawn, NJ.) were used and all other chemicals and reagents were of the highest purity commercially available and were used without further purification.

Sample preparation for amino acids analysis

Six thirty-year-old apple trees (*Malus pumila* Mill. cv. McIntosh/M7), uniform in size and development, were selected. at Macdonald College of McGill University, Quebec, Canada. The selected trees were divided into two groups. One group of 3 trees was kept as a control while the second group was deflowered in 1984. Deflowering involved pinching off the blossom at the late pink stage of development. Forty flower buds on each tree were collected from non-fruiting spurs on 3-4 year-old wood from July in the year of deblossoming until April 28 in the next year. A parallel sample of buds from each tree type was checked visually from August until the end of the sampling period to confirm the presence of flowers. Visual recognition of flower buds as such proved to be effective 95-100% in each sample. The buds were separated from the spur at the base of the bud scales with a minimum amount of woody tissue. Forty buds per tree were combined, and quickly frozen to -170^{o}C. All tissues were lyophilized for 24 h, and ground in a standard electrically driven Thomas-Wiley Mill (A. H. Thomas Company, Philadelphia, PA). Composite samples were stored in small plastic bottles at -20^{o}C for subsequent analysis. Extraction procedure, amino acid analyses, and prediction of properties of proteins from amino acid composition were according to previously described methods (Zarkadas et al. 1987, Khanizadeh, et al. 1989). The protein content of individual samples was determined from the amino acid data according to Horstmann (1979). A new conversion factor (CF) for converting Kjeldahl nitrogen to protein content was devised, based on the summation of total amino acid nitrogen (Table 4).

Evaluation of spring frost tolerance of apple flower buds by differential thermal analysis (DTA)

Fourteen buds were selected randomly for each test (7 from deblossomed and 7 from cropped trees) Freezing tests were repeated six times for each stage of growth. dormant bud, silver tip, green tip, half-inch green, tight cluster, first pink and full pink. A FTS Multi-Cool mechanically refrigerated bath with a controller (FTS System Model TC-44) and freezing chamber (FTS System, Inc., Stone Ridge, NY.) was used to determine freezing tolerance of buds collected from non-cropped and cropped trees. A program for an AT-PC (Micro AL, Nepean, Ontario) IBM compatible equipped with a MetraByte MBC-488 interface card for the IEEE-488 (FTS System, Inc., 158, Stone Ridge, NY), was developed to decrease and control the temperature of the freezing chamber (Appendix I). Exothermic differential temperatures were observed as tissue samples were

cooled from 0°C to -35°C. Buds were collected and prepared for DTA directly in the field to prevent the buds from warming and tested immediately. Flower buds were excised from the tree along with a small portion of spur tissue approximately 1-2 mm in length. Sixteen thermocouples (36 gauge copper constantan) were used. The thermocouple was placed next to the bud or among several buds (Fig. 3) to facilitate optimal thermal contact between sensor and specimen and wrapped with a moisture barrier (Parafilm). Two dried samples of comparable size along with a T-type thermocouple were used to monitor the reference temperature. Prepared Samples (7 non-cropped, 7 cropped and two dried references) were placed in cold test tubes, sealed and secured in a test tube rack inside the freezing chamber (Fig. 4). Specimens and sensors were cooled at a constant rate of 0.5°C/h. The temperatures of the specimens were monitored until both exotherms were observed for each sample. The signals derived from sensors were passed through an ADC-1 Data Acquisition System (Remote Measurement Systems, Inc. Seattle, WA) and recorded with a Tandy 100 portable computer. Data were then transformed from millivolts to degrees Celsius according to the manufacturers procedure. Differences in temperature between the sample and the reference were recorded as a function of temperature (°C).

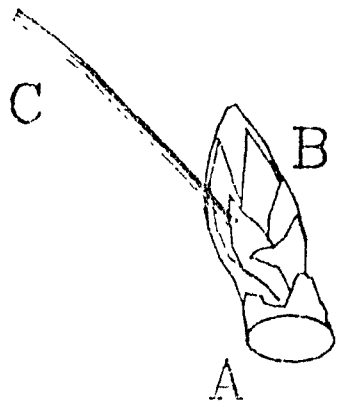

Fig 3 Attachment of a thermocouple on a flower bud between the scales. (A) twig (B) flower bud (C) thermocouple The whole (A), (B), and (C) wrapped with a moisture barrier.

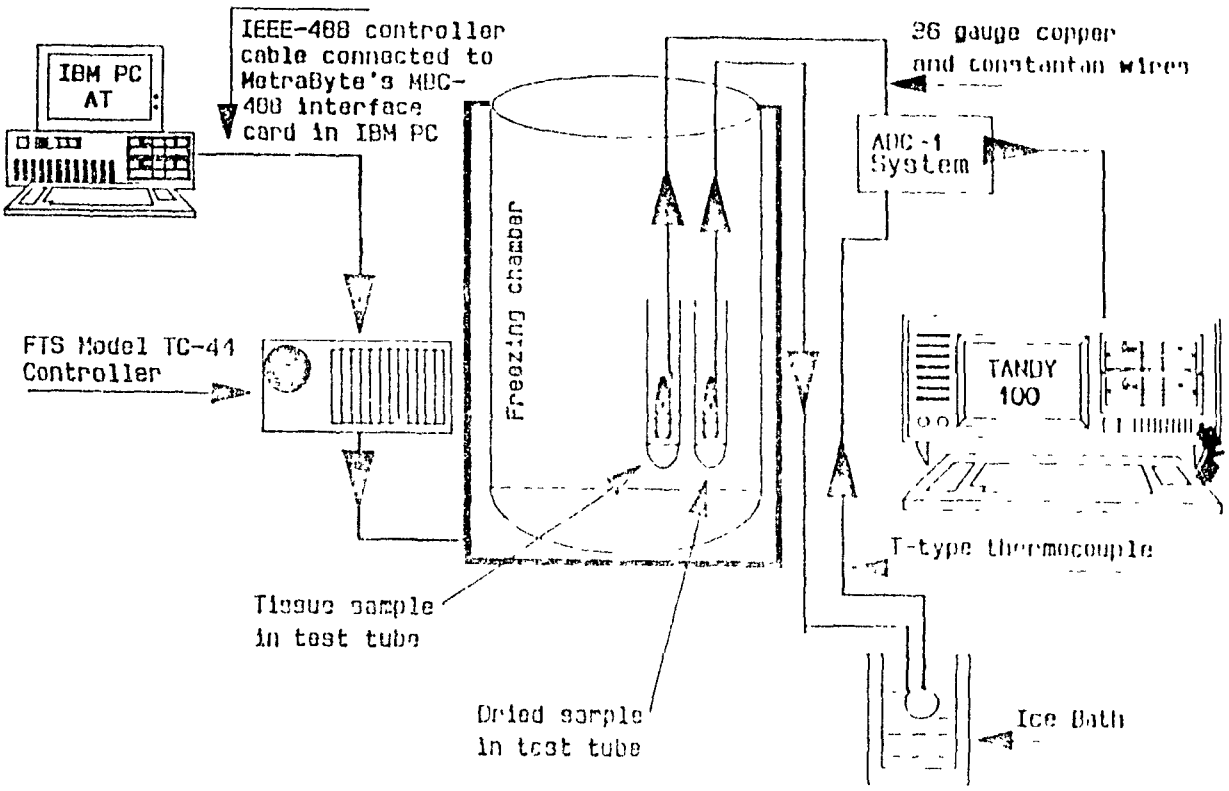

Fig. 4. Construction of the freezing chamber and other components to monitor the temperature of a buds.

Results

Amino acid and protein composition

Total protein and amino acids increased throughout the sampling period in buds on non-cropped and cropped trees. Protein content was always higher in buds on non-cropped trees (Table 4). Aspartic acid, glutamic acid, valine, leucine, lysine, and arginine were the major amino acids in buds on both sets of trees until bud break. Buds on none cropped trees contained more aspartic acid, glutamic acid, proline, valine, and arginine throughout the sampling period until bud break (April 28) when this relationship was reversed, probably because these trees had more flower buds than previously cropped trees (average flower number of cropped vs non-cropped trees: 17483 vs 25108), resulting in more competition between the buds for protein and amino acids stored in the other parts of the tree.

When the amino acids were grouped into four categories based on their physical chemical characteristics: totally charged, hydrophilic, hydrophobic, and apolar (mg/100g protein); and their ratios (R1-R4) compared using the techniques developed by Barrantes (1973) some major differences between the four types were detected (Table 5).

The groups and ratios were defined as follows:

total charged basic + acidic;

Basic histidine + lysine + arginine;

Acidic: aspartic + glutamic + asparagine + glutamine;

hydrophilic: total charged + threonine + serine;

hydrophobic: valine + methionine + isoleucine + leucine + tyrosine + phenylalanine + tryptophan;

Apolar: hydrophobic - tyrosine.

Ratio 1 (R1): hydrophilic/hydrophobic

Ratio 2 (R2). hydrophilic/apolar

Ratio 3 (R3) total charged/hydrophobic; and

Ratio 4 (R4). total charged/apolar.

Buds on non-cropped trees contained proportionally more acidic and hydrophilic amino acids than those on cropped trees from October to April (Table 5), proportions of hydrophobic and apolar amino acids were correspondingly lower.

Table 4 Protein and amino acids content of apple flower buds cv. McIntosh on six sampling dates. (g per kg dry weight)

	1984						1985					
	July 26		August 24		October 24		March 29		April 11		April 28	
amino acids	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped
Aspartic acid	5 97[a][z]	5 07[b]	5 81	5 77	7 28[a]	6 49[b]	8 31[a]	6 56[b]	8 59[a]	7 19[b]	10 92[b]	14 64[a]
Threonine	2 26	1 90	2 41	2 32	2 58	2 41	3 06[a]	2 60[b]	3 32	3 14	4 78	5 48
Serine	2 25	2 19	2 31	2 21	2 68[a]	2 43[b]	2 96[a]	2 49[b]	2 93	2 83	3 85	4 31
Glutamic acid	4 98	4 86	5 01[a]	4 44[b]	7 17[a]	6 76[b]	7 41	7 51	8 82[a]	7 40[b]	11 95[b]	15 27[a]
Proline	2 24[a]	1 96[b]	2 22[a]	2 07[b]	2 86[a]	2 51[b]	2 97[a]	2 89[b]	3 16[a]	3 10[b]	3 59[b]	4 59[a]
Glycine	2 30	2 27	2 15	2 24	3 01[a]	2 88[b]	2 98[b]	3 15[a]	2 98[b]	3 25[a]	4 14[b]	5 66[a]
Alanine	2 51	2 40	2 53	2 43	2 81[a]	2 53[b]	3 12[a]	3 02[b]	3 06	3 05	6 63[a]	4 66[b]
Cysteine	0 55	0 47	0 67	0 46	0 65[a]	0 56[b]	0 71	0 67	0 85	0 79	1 10[b]	1 35[a]
Valine	2 69[a]	2 55[b]	2 89[a]	2 56[b]	3 28[a]	3 00[b]	3 45	3 46	4 20[a]	3 94[b]	5 59[b]	7 35[a]
Methionine	1 43[a]	1 13[b]	1 60	1 33	1 69	1 58	1 72	1 65	1 79	1 70	2 66[b]	3 40[a]
Isoleucine	2 46	2 25	2 37	2 27	3 10	3 11	3 15	3 12	3 25[a]	3 10[b]	4 08[b]	5 49[a]
Leucine	3 34[a]	2 81[b]	3 90[a]	3 62[b]	4 44	4.77	4 82	4 88	5 54[a]	4 76[b]	6 90[b]	9 22[a]
Tyrosine	1 59	1 51	2 27	1 52	2 66	2 65	3 13	3 06	3 50[a]	3 23[b]	3 70	3 63
Phenylalanine	2 48	2 39	2 35	2 25	3 02	3 07	2 99	3 12	2 89	2 96	4 17[b]	5 47[a]
Histidine	1 24	1 23	1 24	1 24	1 48[b]	1 54[a]	1 49	1 58	1 64	1 67	2 66	3 11
Lysine	2 75	2 88	3.29[a]	2 62[b]	4 41[a]	3 78[b]	4 61	4 58	5 07	4 76	7.30[b]	9 38[a]
Arginine	3 77[a]	3.03[b]	3 62[a]	3 00[b]	3 72	3 88	4 14[a]	3.98[b]	5 08[a]	4 81[b]	6.29[b]	7 65[a]
Tryptophan	0 31	0 28	0 33	0.31	0 31	0 31	0 33	0 32	0 33	0 32	0 30	0 30
Hydroxy-proline	0 98	1 04	1.04	1 05	1 19	1 03	1 29	1 44	1 28	1 43	1 79	1 86
Total protein [y]	46.18[a]	42.42[b]	48.24[a]	43 92[b]	58 46[a]	55 71[b]	62 92[a]	60 29[b]	68.55[a]	63 75[b]	90.71[b]	115.25[a]
% amino acids nitrogen	19.27	18.93	17.98	18.62	18 27	18 09	17.50	18 16	17.98	17 74	18.49	18.90
CF [x]	5 19	5 28	5.56	5 37	5 47	5 53	5.71	5 51	5.56	5 64	5 41	5 29
Ammonia	1.69	1 43	1 12	1.39	1 75	1 45	1 28	1 65	1 70	1 24	2 65	3 97

[a,b] Means in the row at each sampling date are significantly different at 5% level using the F ratio.

[z] Each value is the average of 3 replicates and 24 determinations.

[y] Calculated according to Horstman (1979).

[x] A new factor for converting nitrogen into protein content was calculated by following analytical convention: $CF = \frac{\text{100 g total protein}}{\text{\% amino acids nitrogen}}$

Table 5. Amino acid content of apple flower buds, cv. McIntosh for six sampling dates. (g of amino acid per 100 g of total protein)

	1984						1985					
	July 26		August 24		October 24		March 29		April 11		April 28	
amino acids	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped
Charged	64.25[z]	63 66	61.82	62 04	65 89	64 08	66 24[a]	63 49[b]	68 00[a]	63.39[b]	68 27	69.38
Basic	16.84	16.83	16 94[a]	15.60[b]	16 45	16 52	16 27	16 81	17.20[b]	17.63[a]	17 92	17.45
Acidic	47 42	46.84	44 88	46 47	49 43[a]	47 56[b]	49 97[a]	46 68[b]	50 80[a]	45 76[b]	50 35	51.92
Hydrophobic	30.95	30.45	32 57	31 56	31 62[b]	33 17[a]	31.13[b]	32.53[a]	31 37	31.41	30.16	30 31
Hydrophilic	74.00	73.29	71.59	72.37	74.89[a]	72 76[b]	75.81[a]	71.93[b]	77 12[a]	72 77[b]	77.81	77 89
Apoplar	27 51	26 89	27 90	28 11	27 08[b]	28.42[a]	26 16[b]	27 45[a]	26.27	26.35	26.12[b]	27.90[a]

[a,b] Means in the row at each sampling date are significantly different at 5% level using the F ratio.

[z] Each value is the average of 3 replicates and 24 determinations.

Differential thermal analysis (DTA)

Buds on non-cropped trees had a lower HTE and LTE at all stages of development (Table 6). DTA curves for freezing of excised apple spurs for two selected stages "silver tip" and "first pink" are presented in Fig. 5. A typical DTA profile with two exotherms was observed at "dormant", "silver tip", "green tip", "half-inch green", and "tight cluster" stages of bud development (Fig. 5A). However, the LTE was not observed at the "first pink" and "full pink" stage (Fig. 5B) indicating a loss of hardiness.

Discussion

It is generally accepted that accumulation of water-binding substances inside the cell, particularly water-soluble carbohydrates, depresses the freezing point of the cell sap and thus the freezing temperature of living cells (Sakai and Larcher 1987). It has been reported that accumulation of proteins during the hardening period increased the frost hardiness of some plant species, and there is a correlation between total protein content and development of cold hardiness (Li 1987). In our experiment, non-cropped trees with a higher protein content could withstand lower temperatures than cropped trees, suggesting a relationship between protein content and cold resistance. Polar amino acids may depress the freezing point of apple flower buds in a way similar to carbohydrates Protein in buds collected from non-cropped trees had significantly higher levels of hydrophilic and acidic amino acids from October until mid April when the "first pink" stage was reached (Table 5). Hydrophobic and apolar amino acids comprised a smaller proportion of total protein in non-cropped trees than cropped trees in the same period. It appears that crop load changes the proportions of amino acids in protein. The ratios (R1-R4), which compare hydrophilic and charged amino acids with hydrophobic and apolar amino acids, were higher in buds from non-cropped than cropped trees from October to April (Fig. 6). It is possible that total protein *per se* is not responsible for the greater tolerance of buds to cold injury. Rather, an increase in hydrophilicity of the protein may increase the cold tolerance of apple flower buds

Table 6 High and low temperature exotherms (°C) of apple flower bud on cropped and non-cropped trees at different stages of development

Stage of growth	High temperature exotherm			Low temperature exotherm		
	Non-cropped	Cropped	F ratio	Non-cropped	Cropped	F ratio
dormant bud	-5.9	-4.8[z]	8.3*	-28.8	-26.6	42.0**
silver tip	-5.1	-4.6	4.4	-23.6	-21.1	6.5**
green tip	-5.1	-4.7	6.6**	-22.6	-20.7	10.5**
half-inch green	-5.1	-4.7	6.6*	-21.2	-18.2	22.6**
tight cluster	-5.0	-4.5	7.9*	-21.2	-18.0	25.6**
first pink	-5 1	-4 7	6.6*	-	-	-
full pink	-5 1	-4.7	6.6*	-	-	-

[z] Each value is the average of 3 replicates and 18 determinations.

*, ** F values from analysis of variance using General linear model procedure, significant at 5% and 1% levels respectively.

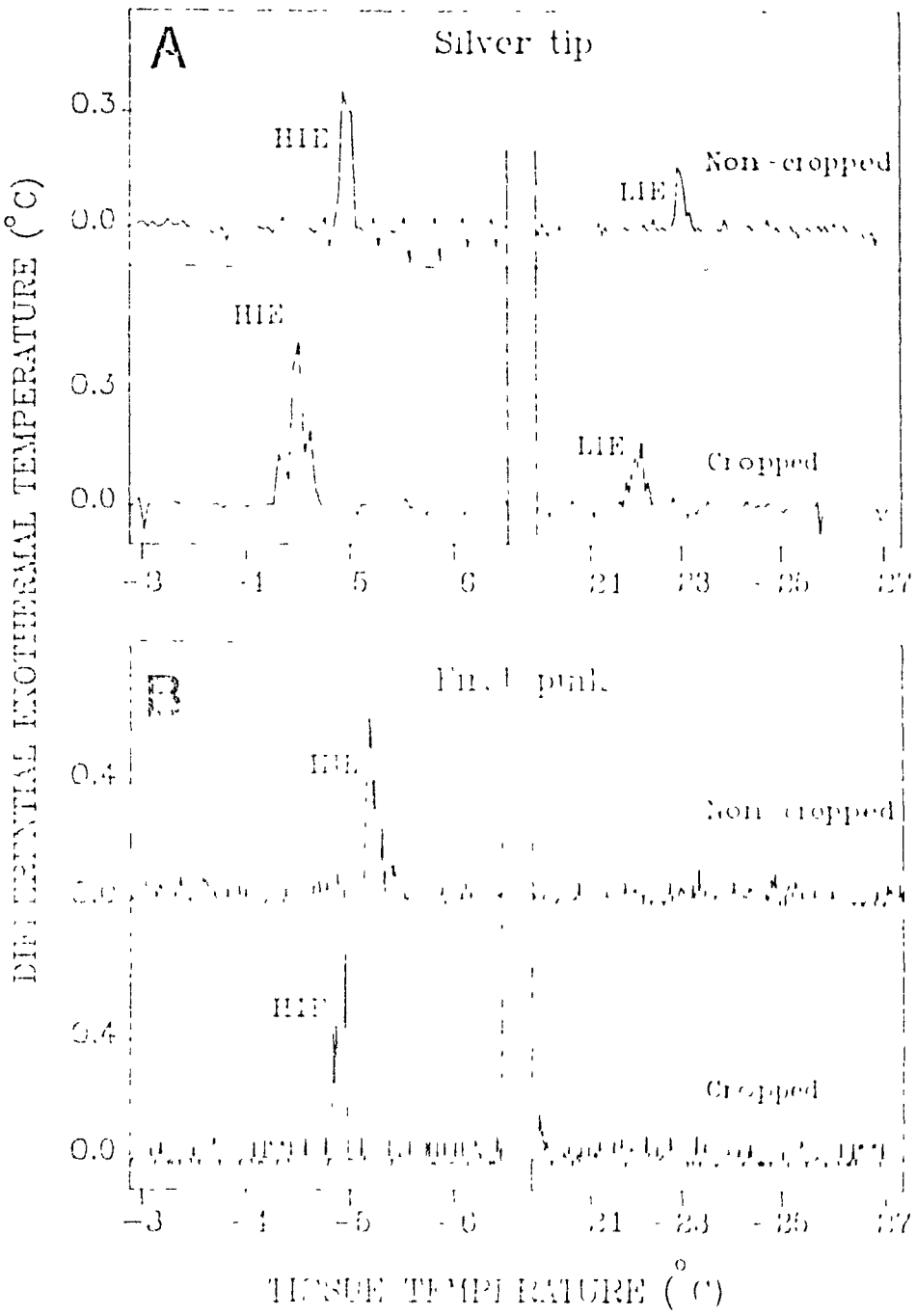

Fig 5 Typical exotherm patterns (HTE, LTE) observed in apple flower bud of M pumila Mill cv. Melrose by MZ at 'Silver tip' and 'First pink' stage for one determination

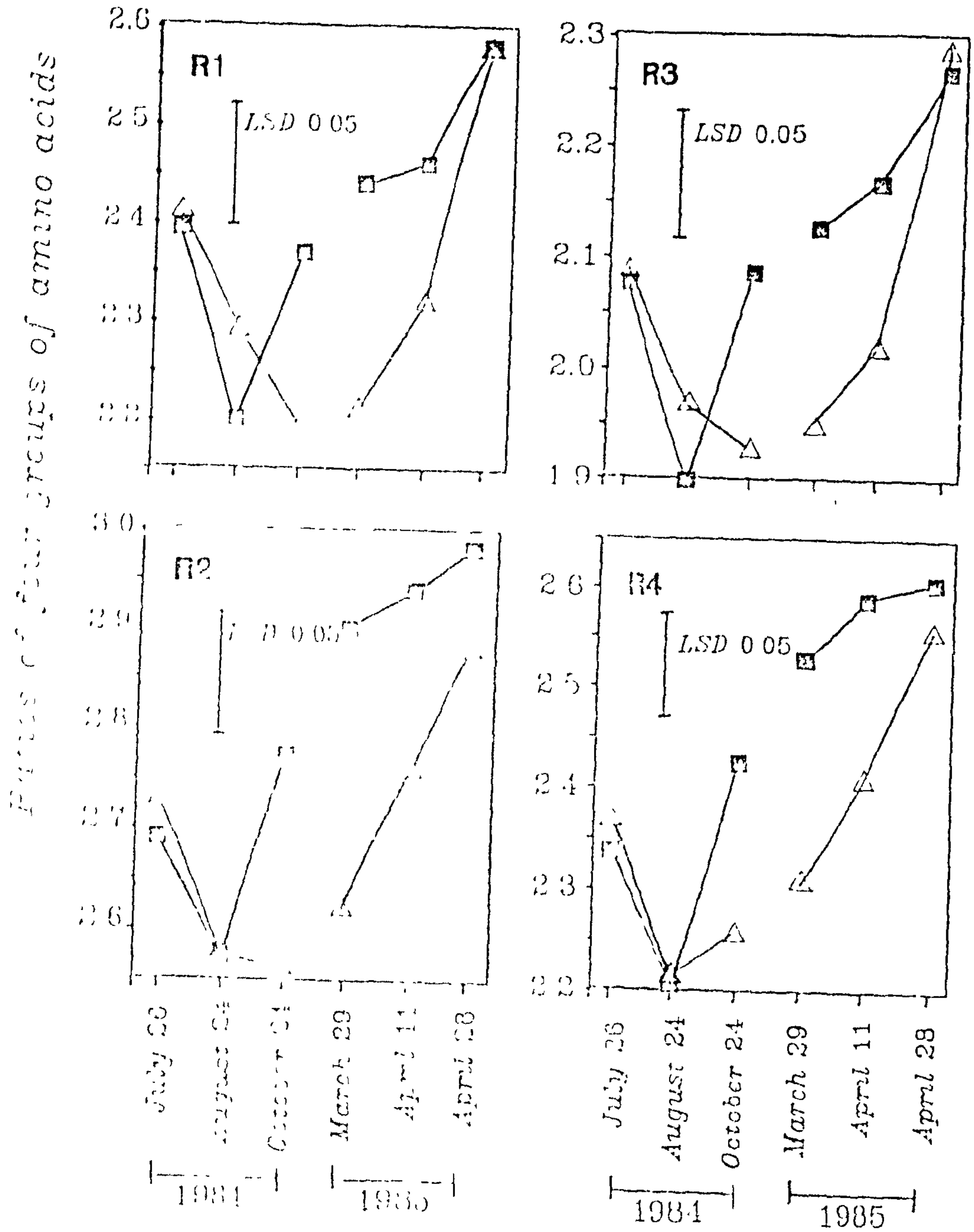

Fig. 6 Effect of crop load on amino acids composition of apple flower buds (Malus pumila Mill. cv. McIntosh M7) during the sampling period. Where (☐ ☐) stand for non-cropped and (△ .... △) for cropped trees. The ratios defined as R1=hydrophilic/hydrophobic, R2 hydrophilic apolar, R3 total charged hydrophobic; and R4=total charged apolar.

Bud hardiness depends on growth stage and previous crop load. Threshold temperatures (LTE) below which tissue damage occured, are presented in Table 6. HTE and LTE of non-cropped trees were significantly lower at all stages of development. Depression of the freezing point (LTE) of the buds on non-cropped trees might result from the greater quantities of hydrophilic and acidic groups of amino acids present in the buds The solubility of these two groups of amino acids means they may act as frost protectants in the same way as water-soluble carbohydrates which depress the freezing point of the cells (Sakai and Larcher 1987). It is possible that these two groups of amino acids may increase the hydrophilic capacity of protein colloids, preserve water, and prevent denaturation of proteins, when the protoplasm is dehydrated by extracellular ice formation. Our observations are similar to the findings of Alden and Hermann (1971) who hypothesied that water-soluble protein fractions and polar amino acids have a stabilizing effect on frost-sensitive proteins of high molecular weight, by increasing their amount of bound water.

Whilst the apparent contribution to protein of hydrophobic and apolar amino acids in non-cropped trees declined during the sampling period, this decline was only a reflection of the greater quantities of hydrophilic and acidic amino acids accumulated during that time (Appendix II)

It has been reported that proline which is in fact an imino rather than amino acid acts as a cryoprotectant (Withers and King 1979) *In vitro* thylakoid membranes may be protected from freezing by addition of proline and arginine (Sakai and Larcher 1987) It seems that, like arginine, proline plays a role not only in storage of nitrogen but also, in combination with sugars, in providing protection from freezing injury (Sakai and Larcher 1987) Non-cropped trees had higher levels of proline thoughout the sampling period until April 28, Arginine was also present in greater quantities except for two sampling dates (October 24 and April 28)

While the Kjeldahl method is recognized as accurate and precise method for nitrogen determination, its application for protein assay in plant materials has been questioned because of the CF used (Havery and Fazio 1983, Benedict 1987). Protein quantitation by the Kejeldahl method makes the assumption that all plant proteins have a mean nitrogen content of 16% We found nitrogen content varied from 17.50 to 19.27% in apple flower buds The nitrogen CF calculated in this study, based on amino acid analysis, ranged from 5 19 to 5 64

for the six sampling dates (Table 4), these data are in accord with the conversion factors reported for almonds (5.18), peanuts and brazil nuts (5.46), and coconuts (5.3), (Benedict 1987). This new CF could be substituted for 6.25 for estimating total protein content of apple flower buds; however using this CF will still give an erroneously high protein content estimate when used with Kjeldhal total nitrogen analysis, since plant tissue contains many non-protein nitrogenous compounds, i.e., amides, nitrogenous glucosides, nucleic acids, porphyrins, fats, alkaloids, ammonium salts and hormones.

Conclusions

From October to April flower buds on non-cropped apple trees had higher levels of protein, hydrophilic and acidic amino acids and were more cold resistant than those on cropped trees from the "dormant" to "first pink" flower stages. It is speculated that high levels of polar amino acids in buds on non-cropped trees increase their cold-hardiness

Calculation of protein using the 6 25 Kjeldhal conversion factor results in an erroneously high estimation of total protein in apple flower buds. The new conversion factor calculated is 5 19 to 5 64 depending on the sampling date.

For accurate protein determination, in this or any other tissue, determination of total amino acids should be made in tandem with Kjeldhal analysis to allow an estimation of non-protein nitrogen in the tissue before using the new CF.

References

Alden, J. and Herman. K. K. 1971. Aspect of the cold hardiness mechanism in plants. Bot. Rev. 37:37-142.

Benedict, R. C. 1987. Determination of nitrogen and protein content of meat and meat products. J. Assoc. Offic. Anal. Chem. 70:69-74.

Barrantes, F. J. 1973. A comparative study of several membrane proteins from the nervous system. Biochem. Biophys. Res. Commun. 54:395-402.

Brown, D. M. and Blackburn, W. J. 1987. Impacts of freezing temperature on crop production in Canada. Can J. Plant Sci. 67:1167-1180.

Brown, G. N. 1978. Protein synthesis mechanisms relative to cold hardiness, p. 153-163. In: P. H. Li and A. Sakai (eds.). plant cold hardiness and freezing. Academic Press, N.Y.

Buban, T. and Faust, M.; 1982. Flower bud induction in apple trees: Internal control and differentiation., Hort Rev 4:174-203.

Burke, M. J. and Stushnoff, C. 1979 Frost hardiness: a discussion of possible molecular causes of injury with particular references to deep supercooling of water, p. 197-225. In· H. Mussell and R C Staples (eds.). Stress physiology in crop plants Wiley, N Y

Graham, R. 1971. Cold injury and its determination in selected Rhododendron species. MS. thesis, University of Minnesota, St. Paul.

Graham, P. R. and Mullin, R. 1976 The determinations of lethal freezing temperatures in buds and stems of deciduous azalea by a freezing curve method. J. Amer. Soc. Hort Sci 101.3-7.

Havery, D. C. and Fazio, T 1983 J Assoc. Offic. Anal Chem. 66 1500-1503.

Horstmann, H. J. 1979. A precise method for the quantitation of protein taking into account their amino acid composition Anal Biochem 96 230-138.

Khanizadeh, S., Buszard, D and Zarkadas, C G 1989 Seasonal variation of proteins and amino acids in apple flower buds (*Malus pumila* Mill McIntosh/M7). (in press. J. Agr. Food Chem.)

Levitt, J.; 1978. An overview of freezing injury and survival and its interrelationships to other stress, p 3-15. In H P. Li and A Sakai (eds.). Plant cold hardiness and freezing Academic Press., N.Y

Li, P. H., 1987. Plant Cold Hardiness, Alan R. Liss, Inc , N Y. 381pp.

Luckwill, L. C.; 1974. A new look at the process of fruit bud formation in apple Proc. 19th Intl. Hort Congr , (Warsaw) 3.237-245

Pieniazek, J. Holubowicz, T. 1973. The effect of ABA and GA on the cold hardiness of apple seedling and explants. Acta Hort. 34:247-251.

Sagisaka S. 1987. Amino acid pools in Herbaceous plants at the wintering stage and at the beginning of growth. Plant and Cell Physiol. 28:171-178.

Sagisaka S. and Araki, T. 1983. Amino acid pools in perennial plants at the wintering stage and at the beginning of growth. Plant and Cell Physiol. 24:479-494.

Sakai, A. and Larcher, W. 1987. Frost Survival of plants. Responses and adaptation to freezing stress. Springer-Verlag, N Y., pp 303.

Singh, J., De La Roche, I. A. and Siminovitch, D. 1977. Differential scanning calorimeter analysis of membrane lipids isolated from hardened and unhardened black locust bark and from winter rye seedlings. Cryobiology 14:620-624.

Withers, L. A. and King, P. J. 1979. A noted cryoprotectant for the freeze preservation of cultured cells of Zea mays L. Plant Physiol. 64:675-678.

Zarkadas, C G , Rochemont, J. A., Zarkadas, G. C., Karatzas, C. N. and Khalili, A. D. 1987. Determination of methylated basic. 5-hydroxylysine, elastin crosslink, other amino acids, and the amino acid sugars in proteins and tissues. Anal. Biochem. 160:251-266.

Connecting text

Cold hardiness of flower buds decreased during spring development and the absence of fruit in the previous year increased the buds ability to withstand low temperatures at all stages. From October to April flower buds on non-cropped trees contained higher levels of acidic and hydrophilic amino acids than those on non-cropped trees. These two groups of amino acids apparently increase the ability of the plant to withstand low temperature injury because of their affinity with water (Sakai and Larcher 1987).

Many other compounds especially carbohydrates (glucose, fructose, sorbitol and starch), and total nitrogen have been reported to increase cold hardiness (Graham and Patterson 1982, Li et al. 1965, Raese et al. 1978). The next experiment was conducted to determine the effect of crop load on carbohydrates, N, P, and K content and cold hardiness of spur buds of apple trees.

Raese, J. J., Williams, M. W. and Billingsley, H. D. 1978. Cold hardiness, sorbitol, and sugar levels of apple shoots as influenced by controlled temperature and season. J. Amer. Soc. Hort. Sci. 103:796-801.

Sakai, A. and Larcher, W. 1987. Frost survival of plants. Responses and adaptation of freezing stress. Springer-Verlag, New York, 303 pp.

Graham D. and Patterson, B. D. 1982. Responses of plants to low, nonfreezing temperatures: Proteins, metabolism, and acclimation. Ann. Rev. Plant Physiol. 33:347-372.

Li, P. H., Weiser, C. J., Van Huystee, R. 1965. Changes in metabolites of Red-Osier Dogwood During cold acclimation. Proc. Amer. Soc. Hort. Sci. 86:723-730.

V. EFFECT OF CROP LOAD ON SEASONAL VARIATION IN CHEMICAL COMPOSITION, AND FROST HARDINESS OF APPLE FLOWER BUDS.

Introduction

The major carbohydrates in apple trees are sorbitol, sucrose, glucose, fructose, and starch and relative levels of these vary seasonally (Sakai 1966b, Chong 1971; Chan et al 1972). Stored carbohydrates are important in the annual growth cycle of the apple tree (Hansen 1967) especially in early spring when bud growth occurs before the onset of photosynthesis.

In herbaceous and woody plants, an increase in soluble carbohydrate occurs with dehardening in the spring (Levitt 1980; Siminovitch 1981). Recent evidence suggests that changes in sugar and polyhydric alcohols may influence osmotic potential changes which accompany the seasonal course of freezing tolerance (Sakai and Larcher 1987, Li, 1987). The determination of total carbohydrates in hardy plants and their relationship to crop load is important in explaining their role at low temperatures and to understand the mechanism of their protective effect (Sakai and Larcher 1987, Li, 1987). Granger (personal communication) noted that apple trees which had carried a heavy crop in 1980 were more severely damaged in the winter of 1980-1981 in Quebec. Numerous studies have examined the carbohydrate content of leaves, shoots and stems of this herbaceous woody species (Raese et al. 1978), but few experiments have focused on the flower buds *per se*.

The objectives of this study were to determine the effect of crop load on seasonal variation in glucose, fructose, sorbitol, starch, and nitrogen (N), phosphorus (P), and potassium (K) in apple flower buds, and to determine whether crop load in the previous season influences the spring frost tolerance of these buds.

Materials and Methods

Plant material

Trees used in this experiment were 30-year-old McIntosh/M7 apple trees (*Malus pumila* Mill.) at Macdonald College of McGill University, Ste-Anne-de-Bellevue, Quebec. A completely randomized design was used with three replicates per treatment. In each of two consecutive years (1984/85 and 1985/86), six trees were selected randomly. Three trees were deblossomed (non-bearing trees) by pinching the flower clusters at first bloom stage (10 May 1984; 15 May 1985); three other trees were allowed to carry a crop to maturity (bearing trees). Samples of forty flower buds (each sample ca 2.7 g fresh weight) per tree were collected from non-fruiting spurs on 3- to 4-year-old wood at intervals of 2-4 weeks between mid-summer (11 July 1984; 12 July 1985) and early winter (24 October 1984; 1 November 1985) and between late winter (8 March 1985; 20 March 1986) and bud break in the spring (28 April 1985; 8 May 1986). Visual inspection of a second set of flower buds collected on each date between August and bud break in each year confirmed a 95-100% presence of flower buds in each sample taken. The buds were separated from the spur at the base of the bud scales. Each sample was frozen in liquid nitrogen, freeze dried, pulverized in an electrically driven mill, and stored at -20°C in an air-tight container until assayed. Data were analysed using the General Linear Model Procedure (GLM) of Statistical Analysis System (SAS 1988).

Percent Fruit Set

In both 1985 and 1986 percent fruit set was determined 1 year after deblossoming by sub-sampling 4 branches randomly selected from each non-cropped and cropped tree (approximately 200 flowers/branch). Total flower number per tree was estimated based on total fruit harvest per tree, and the percent fruit set of the selected branches.

Chemical Analysis

Glucose, fructose and sorbitol analysis. A 10 mg subsample of powdered flower bud was added to 10 ml of double distilled de-ionized water and vortexed for 30 minutes. This suspension was centrifuged for 5 min at 5000 rpm, then 25 ml of supernatant was collected and assayed enzymatically for glucose, fructose and sorbitol (Bergmeyer 1984). In this and subsequent chemical analyses, 6 determinations were made of each subsample.

Starch analysis. A 10 mg subsample of powdered flower bud was added to 2 ml dimethylsulfoxide and 0.5 ml HCl (8 mol/L) in a test tube. The test tube was sealed with parafilm and incubated for 30 min in a 60°C water-bath to hydrolyse starch to D-glucose. The sample was quickly cooled to room temperature, diluted with 6.5 ml of double distilled de-ionized water and vortexed for 30 minutes to obtain a suspension. The pH was adjusted to 4.5 with NaOH (5 mol/L), the mixture centrifuged as above and 25 ml of supernatant enzymatically assayed for starch (Bergmeyer 1984). All chemicals and enzymes were purchased from Boehringer Mannheim GmBH Biochemica Company, Quebec.

Nitrogen, phosphorous and potassium analysis. An Automated Technicon AAII analyzer (Industrial Method, No. 157-71, 1983; Technicon Instruments Co., Tarrytown, N.Y.) was used to determine N, P, K by wet digestion with H_2SO_4-H_2O_2 following the procedures of Thomas et al. (1967).

Freezing Tolerance

Between March and May 1986, the freezing tolerance of buds from non-cropped and cropped trees was determined at the following stages of growth: dormant bud (March 15), silver tip (April 12), green tip (April 20), half-inch green (April 24), tight cluster (April 27), first pink (May 2) and full pink (May 8). On each date 300 buds were selected randomly (150 each from non-cropped and cropped trees). A FTS Multi-Cool mechanically refrigerated bath with a controller (FTS System Model TC-44) and freezing chamber (FTS System, Inc., 158, Stone Ridge, NY.) was used to determine freezing tolerance. Temperature of the freezing chamber was controlled by a computer program written in Basic for an IBM-PC AT compatible (Micro A/L, Nepean, Ontario) equipped with a MetraByte MBC-488 interface card for the IEEE-488 (FTS System, Inc., 158, Stone Ridge, NY.). The source file is available upon request to the authors.

For each test (each stage), the temperature was set at 0°C and decreased to -30°C at a rate of 0.5°C /hour. Following each 1°C drop in temperature, 10 buds (5 each from non-cropped and cropped trees) were removed and allowed to reach ambient temperature. After addition of 10 ml double distilled de-ionized water the electrical conductivity (EC) of the solutions was measured. Following this the samples were boiled and then frozen to -80°C which totally broke down the cells, then a second measurement of conductivity was made to determine maximum leakage (Ketchie et al. 1972).

Percent leakage was calculated according to Eq. 1.

$$[1]\ \%\ \text{Leakage}\ (Y) = \frac{\mu\text{mohs before boiling and freezing} \times 100}{\mu\text{mohs after boiling and freezing } (-80^{0}C)}$$

where μmohs represent the electrolytic conductance.

Since temperature and leakage have a nonlinear relationship (Finney 1978), a nonlinear function to represent the relationship between temperature (T) and the leakage (Y) was developed [Eq. 2].

$$[2] \qquad Y = \frac{e^{(a + b \times T)}}{1 + e^{(a + b \times T)}}$$

This relationship, the logistic response function, was used to determine the median lethal temperature (MLT) (Finney 1978). However, since the percent leakage was used for regression analysis Eq. 2 was changed to:

$$[3]\ \%\ \text{Leakage}\ (Y) = \frac{e^{(a + b \times T)}}{100 + e^{(a + b \times T)}}$$

where e is the base of the natural logarithms, T is the independent variable (temperature), Y is the dependent variable (% leakage), 100 is the maximum leakage and 'a' and 'b' are regression parameters. The regression parameters were estimated by the GLM Procedure of Statistical Analysis System (SAS, 1988). Since the response function (logistic) to be fitted was nonlinear, the following transformation was used (Finney 1978)

$$[4] \qquad Y' = \text{Ln}\left(\frac{Y}{100 - Y}\right)$$

to reduce Eq. 3 to

[5] $$Y' = a + b\,(T)$$

before analysis. The value of T (temperature) at Y = 50% is of considerable practical importance. It is called the MLT. The value of Y = 50% corresponds to a value of Y'=0 as can be seen from Eq. 4. Hence,

[6] $$T = \frac{-a}{b}$$

Where T is inflexion point temperature or MLT. The response Y can then be plotted against T as a symetrical sigmoid curve.

Results and Discussion

Influence of Crop Load (Deblossoming)

In each year, significantly more flowers were produced on non-cropped (deblossomed) trees in the year following deblossoming (Table 7). Buds on these trees were generally larger and heavier (Table 8). In 1986 only, the percent fruit set was also greater on non-cropped trees. Concomitantly, buds on non-cropped trees contained more glucose, sorbitol, starch, N, P, and K throughout most of the sampling period except the last sampling date in each spring where this difference was reversed (Table 9); probably because these trees had more flower buds than cropped trees (Table 7) resulting in more competition between the buds for carbohydrates and nutrients stored in other parts of the tree (Levitt 1980; Siminovitch 1981). Deblossoming did not influence fructose content (Table 9). Relative proportions of glucose:fructose:sorbitol:starch averaged over all dates were 11:5:18:68 in buds on deblossomed trees, and 11:5:18:65 inbuds on cropped trees. It appears deblossoming increased the relative proportion of starch to glucose, fructose and sorbitol.

Seasonal Variation in Carbohydrates and Nutrients

As reported by Rease et al. (1977), sorbitol was the predominant water soluble carbohydrate in flower buds of both non-cropped and cropped trees during the sampling period, accounting for 18-19% of total soluble carbohydrates (Table 9). In both year the sorbitol content rose from the begining of sampling (11 July 1985 and 12 July 1986) until budbreak in the spring. Changes in glucose content paralleled those of sorbitol; levels of fructose were lower than glucose and sorbitol (Table 9).

The pattern of variation in water-soluble reducing sugar is not the same for apple buds as for apple shoots. For example Raese et al. (1978) reported that in two year old apple shoots sorbitol and sucrose concentrations increased before hardening and decreased before growth began in spring. Such a trend was not observed in the apple flower bud where water-soluble reducing sugar content increased throughout the sampling period. This suggests that the flower bud may act as a storage organ for carbohydrates, and also receives nutrients from other storage organs at bud break. The decrease in starch and increase in glucose and sorbitol content in early spring reflects hydrolysis of accumulated starch and mobilization of sugars from woody tissues into the buds (Sakai 1966a). This process is vital to tree growth and development since photosynthesis is not occurring at this time. Nitrogen, P, and K increased in all buds throughout the sampling period, in contrast N levels have been reported to decline in bark and shoots as spring growth occurs (Tromp 1970). This again may indicate mobilization of nutrients from other parts of the tree to buds in the spring, similar to the pattern observed for water-soluble reducing sugars.

Bud Hardiness

Buds from trees which had been deblossomed in the previous year were able to survive lower temperatures at all stages of development during spring (from "dormant" 15 March to "full pink" 8 May 1986) (Table 10). While it is known that hardiness decreases as flower buds develop in the spring it seems that deblossoming can influence bud hardiness and the rate at which it is lost. This result confirms previous reports that hardy trees have high nutrient concentrations (Sakai and Larcher 1987; Li 1987). Increases in the content of glucose, sorbitol and starch in flower buds of deblossomed trees may account for their increased cold hardiness.

Table 7. Flower number, percent fruit set, and yield (fruit number) of three non-cropped and three cropped apple trees (McIntosh/M7) in 1985 and 1986 following deblossoming.

	1985			1986		
Treatments†	Flower number	%Fruit set	Yield	Flower number	%Fruit set	Yield
Non-cropped	25108[a]	6.69[a]	1868[a]	23669[a]	8.66[a]	1869[a]
Cropped	17483[b]	6.23[a]	1089[b]	18590[b]	6.23[b]	1157[b]

†Deblossoming was done in spring of 1984 and 1985 for data taken in 1985 and 1986, respectively.

[a,b]Means within columns followed by the different superscripts are significantly different at 5% level using the F ratio.

Table 8. Diameter and dry weight of apple flower buds from non-cropped and cropped trees.

Sampling date	Diameter (mm)		Dry wt (g)	
	Non-cropped	Cropped	Non-cropped	Cropped
11 Jul 84	3.11†	3.27	0.027	0.019
29 Jul 84	3.70	3.31	0.025	0.020
24 Aug 84	3.70	3.43	0.031	0.026
24 Sep 84	4.33	4.07	0.037	0.031
24 Oct 84	4.74	4 11	0.044	0.034
08 Mar 85	4.85	4.32	0.047	0.039
29 Mar 85	5.16	4.45	0.051	0.045
11 Apr 85	5.70	4.75	0.062	0.047
28 Apr 85	6.44	5.94	0.076	0.063
Overall mean	4.63[a]	4.18[b]	0.044[a]	0.036[b]
12 Jul 85	3.00	2.53	0.013	0.012
26 Jul 85	3.65	3.03	0.016	0.015
01 Aug 85	3.85	3.38	0.022	0.017
16 Sep 85	3.90	3.35	0.032	0.024
01 Nov 85	4.44	3.85	0.036	0.031
20 Mar 86	4.85	4.42	0.043	0.038
12 Apr 86	5.79	5.13	0.050	0.046
27 Apr 86	6.32	5.87	0.058	0.051
08 May 86	6.91	6.10	0.072	0.055
Overall mean	4.74 [a]	4.18[b]	0.038[a]	0.032[b]

†Each value is the average of 18 determinations

[a,b]Mean with different superscript within the row for each variable are significantly different at 5% level using the F ratio.

Table 9 Content of carbohydrate and nutrient constituents (mg/g dry Wt) in apple flower buds from non-cropped and cropped trees

Sampling date	Glucose		Fructose		Sorbitol		Starch		Total[§]		N		P		K	
	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped
11 Jul 84	8 2[†]	8 1	4 2	4 2	16 1	15 4	52 5	54 0	81 1	81 7	13 2	11 2	2 8	1 7	4 6	3 6
29 Jul 84	7 9	7 7	4 4	4 1	16 6	16 0	53 2	54 9	82 1	82 6	14 5	11 8	3 0	2 7	5 9	4 3
24 Aug 84	8 2	8 0	4 0	4 0	15 9	15 5	58 7	60 2	86 8	87 7	15 6	14 7	3 1	2 6	6 2	4 7
24 Sep 84	9 5	8 5	4 2	4 3	16 3	15 4	70 1	68 8	101 0	97 0	17 3	16 7	3 3	3 2	6 3	5 3
24 Oct 84	11 4	10 6	5 2	5 2	19 2	17 4	124 5	90 4	160 2	123 5	18 6	17 3	3 7	3 4	6 4	5 5
08 Mar 85	14 2	12 1	6 0	6 1	19 4	17 5	132 8	100 5	172 4	136 3	19 7	17 5	4 2	3 4	7 1	5 4
29 Mar 85	14 3	12 9	6 2	6 2	22 2	18 4	87 3	66 4	130 0	103 9	22 1	19 2	4 1	3 4	6 5	5 1
11 Apr 85	14 6	13 0	6 3	6 4	25 6	23 3	64 7	57 3	111 2	100 1	23 2	21 8	4 2	4 1	7 5	6 7
28 Apr 85	16 4	21 4	6 7	8 4	26 0	26 4	35 1	34 6	84 1	90 9	27 3	29 3	5 6	6 6	9 8	10 3
Overall mean	11 63[a]	11 36[b]	5 3	5 4	19 7[a]	18 4[b]	75 4[a]	65 2[b]	112 1[a]	100 4[b]	19 5[a]	17 7[b]	3 8[a]	3 5[b]	6 7[a]	5 7[b]
12 Jul 85	8 1	8 0	4 0	4 0	16 1	15 8	52 5	53 4	80 8	81 2	10 6	9 6	2 8	2 0	5 0	3 1
26 Jul 85	8 2	8 0	4 1	4 0	16 2	15 5	58 7	59 3	87 1	86 9	11 3	10 3	3 1	2 4	5 2	3 6
01 Aug 85	9 4	8 8	5 1	4 4	16 2	15 4	69 5	70 1	100 2	98 8	14 4	11 8	3 1	2 9	5 2	3 8
16 Sep 85	11 7	10 1	5 3	5 1	19 2	15 4	84 4	80 3	120 6	111 0	14 0	13 4	3 5	2 9	5 3	4 3
01 Nov 85	11 5	10 3	5 3	5 1	19 5	17 4	124 5	90 4	160 8	123 2	15 2	14 0	3 7	3 0	5 6	4 6
20 Mar 86	14 2	12 1	6 1	6 1	19 2	17 4	132 8	101 6	172 2	137 1	16 4	14 3	3 8	3 0	5 5	4 4
12 Apr 86	14 6	13 0	6 2	6 1	22 2	18 5	88 2	66 7	131 3	104 2	20 5	19 3	3 9	3 6	5 2	4 8
27 Apr 86	14 8	13 0	6 6	6 1	25 2	24 6	64 6	57 4	111 2	101 1	24 0	22 0	4 4	4 0	6 1	4 7
08 May 86	16 4	21 4	7 0	8 1	26 4	26 7	35 0	34 6	84 8	90 8	26 0	23 7	5 3	5 0	9 0	8 0
Overall mean	12 1[a]	11 6[b]	5 5	5 4	20 0[a]	18 5[b]	78 9[a]	68 2[b]	116 5[a]	103 8[b]	16 9[a]	15 3[b]	3 7[a]	3 2[b]	5 6[a]	4 7[b]

[§]Sum of all analyzed carbohydrate constituents

[†]Each values is the average of 18 determinations

[a,b]Means with different superscript within the row for each variable are significantly different at 5% level using the F ratio

Williams and Billingsley (1973) and Williams and Raese (1974) noted that sorbitol content of buds increased when temperature decreased. These observations lend credence to the theory proposed by Sakai (1966b), Raese et al. (1977) and Ichiki and Yamaya (1982) that sorbitol may play an important role in cold hardiness. Substances like sucrose are known to retard growth of ice cristals (Parker 1963), such action may protect the proteins of membranes and enzymes from sudden loss of water during freezing. Sucrose and sorbitol may protect proteins directly by replacing some of the water of hydration, or they may serve to hold the water of hydration more firmly through hydrogen bonding in structures sensitive to dehydration (Doebbler and Rinfred 1962).

Conclusions

Since overcropping causes nutrient depletion and can result in reduced tree hardiness (Brown and Blackburn 1987), the probability of spring frost damage may be reduced by reducing crop load to an optimum level by flower or fruit thinning. Our results confirm this hypothesis. Deblossoming of apple trees resulted in greater accumulation of carbohydrate and nutrient constituents in apple flower buds and increased the cold tolerance of these buds. Similar observations have been made in other plants (Li 1987; Sakai and Larcher 1987). Further studies are required to determine optimum crop load conditions to reduce the risk of spring frost injury to apple flower buds in Quebec.

Table 10. Regression coefficients and median lethal temperatures (MLT) at various stage of bud growth and development between March and May 1986.

		Regression coefficients					
		Intercept †		Slope†		Inflexion point °C (MLT)§	
Date 1986	Stage of bud growth	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped
March 15	dormant bud	2.20±0.09	2 52±0.32	0.095±0 005	0 132±0 017	-23 2	-19 1
April 12	silver tip	2.51±0 28	2 56±0 29	0 152±0 016	0.160±0 017	-16 5	-16 0
April 20	green tip	2.41±0.24	2 45±0 23	0 147±0 013	0 171±0 013	-16 4	-14 3
April 24	half-inch green	2.95±0 31	2 95±0 22	0 195±0 018	0 216±0 014	-15 1	-13 7
April 27	tight cluster	2 91±0 31	2 77±0 23	0 199±0 017	0 209±0 013	-14 6	-13 3
May 2	first pink	0 84±0 23	0 57±0 23	0 217±0 022	0 221±0 022	-3 9	-2 6
May 8	full pink	1.02±0 18	0.29±0 26	0 263±0 017	0 197±0 025	-3.9	-1 5

†Intercept and slope ± standard error were calculated by General Linear Model Procedure (GLM) of SAS (1988), using 30 observations

§Median lethal temperature, calculated according to Eq 5 and 6 using regression coefficients

References

Bergmeyer, H. U. 1984. Methods of Enzymatic Analysis. 3rd ed., vol. VI, Weinheim:Verlag Chemie, Deerfield Beach/Florida. 701 pp.

Chan, W. W., Chong, C. and Taper, C. D. 1972. Sorbitol and other carbohydrate variation during growth and cold storage of McIntosh apple fruits. Can. J. Plant Sci. **52**:743-750.

Chong, C. 1971. Study of the seasonal and daily distribution of sorbitol and related carbohydrates within apple seedlings by analysis of selected tissues and organs. Can. J. Plant Sci. **51**:519-525.

Chong, C. and Taper, C. D. 1972. *Malus* tissue culture. I. Sorbitol (D-glucitol) as a carbon source for callus initiation and growth. Can. J. Bot. **50**:1399-1404.

Doebbler, G. F. and Rinfred, A. P. 1962. The influence of protective compounds and cooling and warming conditions on hemolysis of erythrocytes by freezing and thawing. Biochem. Biophys. Acta. **58**:449-458.

Finney, D. J. 1978. Statistical method in biological assay 3rd ed., Charles Griffen and Co. Ltd., London. 668 pp.

Hansen, P. 1967. ^{14}C-studies on apple trees. 1. The effect of fruit on the translocation and distribution of photosynthates. Physiol. Plant. **20**:383-391.

Ketchie, D. O., Beeman, C. H., and Ballard, A. L. 1972. Relationship of electrolytic conductance to cold injury and acclimation in fruit trees. J. Amer. Soc. Hort. Sci. **87**:403-406.

Ichiki, S, Yamaya, H. 1982. Sorbitol in tracheal sap of dormant apple (*Malus domestica* Borkh.) shoots as related to cold hardiness. In P. H. Li and A. Sakai, Eds. Plant cold hardiness and freezing stress. vol II. Academic Press, New York, pp 181-187.

Levitt, J. 1980. Responses of plants to environmental stresses. Vol I. Chilling, freezing, and hight temperature stresses, 2nd ed., Academic Press, New York. 497 pp.

Li, P. H. 1987. Plant Cold Hardiness. Alan R. Liss, Inc. New York. 381 pp.

Parker, J. 1963. Cold resistance in woody plants. Bot. Rev. **29**:123-201.

Raese, T. J., Williams, M. V., and Billingsley, H. D. 1977. Sorbitol and other carbohydates in dormant apple shoots as influenced by controlled temperature. Cryobiology **14**:373-378.

Raese, T. J., Williams, M. V., and Billingsley, H. D. 1978. Cold hardiness, sorbitol, and sugar levels of apple shoots as influenced by controlled temperature and season. J. Amer. Soc. Hort. Sci. **103**:796-801.

Sakai, A. 1966a. Studies of frost hardiness in woody plants. II: Effect of temperature on hardening. Plant Physiol. **41**:353-359.

Sakai, A. 1966b. Seasonal variation in the amount of polyhydric alcohol and sugar in fruit trees. J. Hort. Sci. **41**:207-213.

Sakai, A. and Larcher, W. 1987. Frost survival of plants. Responses and adaptation to freezing stress. Springer-Verlag, Berlin, New York. 303 pp.

Siminovitch, D. 1981. Common and disparate elements in the process of adaptation of herbaceous and woody plants to freezing - a perspective. Cryobiology. **18**:166-185.

Statistical Analysis System (SAS). 1988. SAS procedures guide for personal computers. Version 6.03. SAS Institute Inc., Carry, N.C.

Thomas, R. L., Sheard, R. W., and Moyer, J. R. 1967. Comparison of conventional and automated procedures for nitrogen, phosphorus and potassium analysis of plant material using a single digestion. Agronomy J. **59**:240-243.

Tromp, J. 1970. Sotage and mobilization of nitrogenous compounds in apple trees with special reference to arginine. In L. C. Luckwill and C. V. Cutting, Eds. Physiology of tree crops. Academic Press, New York, pp 143-159.

Williams, M. W., and Billingsley, H. D. 1973. Watercore development in apple fruits as related to sorbitol levels in the tree sap and minimum temperature. J. Amer. Soc. Hort. Sci. **98**:205-207.

Williams, M. W. and Raese, J. T. 1974. Sorbitol tracheal sap of apple as related to temperature. Physiol. Plant. **30**:49-52.

VI. GENERAL DISCUSSION AND CONCLUSION

Cold tolerance of many plant species has been extensively reviewed and studied (Chen and Li 1980, Gusta et al. 1982, Li 1987, Sakai and Larcher, 1987). Short days and low temperatures are the main factors which induce cold hardiness and subsequently cause biochemical changes in the plant (Chen and Li 1980). Generally it has been accepted that development of cold hardiness is associated with metabolic changes during cold acclimation (Levitt 1980).

One of the obvious metabolic changes before cold acclimation is the dehydration of plant tissues. Water content of flower buds on cropped and non-cropped trees declined at the end of the summer and remained low until the "first pink" stage in the following year (Table 11).

An increase in N, P, soluble sugars, total protein, and polar amino acids especially hydrophilic and acidic amino acids was observed during cold acclimation. It is clear from our data that high levels of salts, amino acids and carbohydrates are associated with increased hardiness of flower buds. These results are in accord with many others reported by Li (1987), Sakai and Larcher (1987) and Levitt (1956, 1980). However it is interesting to note that the level of these components was found to be highest in April when the buds were tender and damaged by temperatures of -2 to -5^{o}C. At this particular time the percent of water in the tissue was 5-15% more than in August, September and October (Table 11). Recently Tyler and Stushnoff (1988a, 1988b) studied the effects of prefreezing and controlled dehydration on cryopreservation of dormant vegetative apple buds. They reported that flower buds which had low survival regardless of prefreezing treatment, could withstand low temperatures (liquid nitrogen) if they were dehydrated prior to prefreezing. Further they concluded that the magnitude of dehydration affected the survival of flower buds in liquid nitrogen. Based on these observations it may be concluded that high levels of nutrients alone are not responsible for cold hardiness and that dehydration is a prerequisite for frost tolerance. Reducing tissue water content increases the water potential of the cytoplasm which decreases the availability of non-bound water for freezing (Levitt 1980).

Table 11. Fresh and dry weights of apple flower buds at different stages of develpments.

sampling dates	Non-cropped Fresh Wt.	Dry Wt.	Diff.	% H2O	Cropped Fresh Wt.	Dry Wt	Diff.	% H2O
26 July 84	0.050	0.020	0.030	60a	0.044	0.020	0 024	55b
24 Aug 84	0.053	0.028	0.025	47c	0.048	0.026	0 022	46d
24 Sep 84	0.062	0.034	0.028	45c	0.054	0 031	0.023	43d
24 Oct 84	0.081	0.044	0.037	46c	0 062	0.034	0.028	45d
11 Apr 85	0.126	0.062	0.064	51b	0 095	0.047	0.048	51c
28 Apr 85	0.206	0.076	0.130	63a	0.182	0.064	0.118	65a

a,b,c,d Mean values for three replicates and 30 determinations, within columns followed by the same letter are not significantly different at 0.05 level using Duncan's new multiple range test.

Changes observed in quantities of total protein, amino acids, carbohydrates and minerals in apple flower buds during the period of cold acclimation are similar to those observed in shoots and bark of many plant species including apple and in general show an accumulation of cell components (Ichiki, Yamaha, 1982, Li 1987, Sakai and Larcher 1987). However during spring growth the levels of nutrients (total protein, carbohydrates, and N, P, K) increase in apple flower buds while in bark and shoots levels have been reported to decline (Siminovitch et al. 1968 , Raese et al. 1978,). This confirms that the apple flower bud acts as a "sink" and receives nutrients from other storage organs at bud break, a time of rapid growth.

In this study two methods of determination of frost resistance were used; differential thermal analysis (DTA), and a viability test (conductivity). Differential thermal analysis of apple flower buds throughout the sampling period gave results similar to those obtained by Quamme et al (1972) working with apple stem tissue. The high temperature exotherm (HTE) remained

relatively constant at all stages of development and ranged from -4.5 to -4.8°C for buds from cropped trees and -5.1 to -5.9°C for buds from non-cropped trees. The low temperature exotherm (LTE) occured at progressively higher temperatures as the buds developed until it disappeared at the "first pink" stage. Flower buds on non-cropped trees had significantly lower HTE and LTE at all stages of development.

In the second experiment on cold tolerance of flower buds conductivity tests were used and gave generally similar results to DTA. The inflection point declined in both types of buds from early to late sampling and buds on non-cropped trees were hardier than buds on cropped trees. However there were differences in results of up to 7°C between the two methods. Results were most similar at the latest stages of development, when buds were least hardy. DTA gave consistantly lower killing temperature than the conductivity test (Table 12).

Table 12. Comparison of differential thermal analysis and conductivity tests at various stages of bud growth (°C).

	Conductivity[z]		LTE[y]	
Stage of growth	Cropped	non-Cropped	Cropped	non-Cropped
dormant bud	-19.1	-23.2	-26.6	-28.8
silver tip	-16.0	-16.5	-21.1	-23.6
green tip	-14.3	-16.4	-20.7	-22.6
1/2" green	-13.7	-15.1	-19.2	-21.2
tigh cluster	-13.3	-14.6	-18.0	-21.2
first pink	-2.6	-3.9	-4.7	-5.1
full pink	-1.5	-3.9	-4.7	-5.1

[z] Each value is the results of 72 determinations.

[y] Each value is the results of 54 determinations.

This difference might be because of the freezing behavior of water in small pores (Homshaw 1980). In 1984 Ashworth and Abeles (1984) observed

that tissues which exibit supercooling have tightly packed cells with common cell walls. Further they reported large intercellular spaces are absent in these tissues and the extracellular water would be in cell wall microcapillaries. They observed that freezing of water within microcapillaries occurs at lower temperatures. They concluded that microcapillaries in cell walls depress the freezing point of the water, impede the spread of ice throughout the tissue and facilitate supercooling.

In our experiment the conductivity test gave a more realistic reading of cold hardiness compared to DTA.

The primary objective of the work reported here was to elucidate the relationship between crop load, and spring frost hardiness of apple flower buds. This information should be usefull in orchard management in Quebec where low temperatures during the winter and spring frosts frequently cause crop losses. Although frost survival capacity depends primarily on the specific hardiness of a plant, various mechanism are involved which help the plant to avoid excessive low temperature stress. Resistance to freezing stress results from the ability of cells to tolerate the various strains exerted by ice formation in the tissues. Hardiness is increased by accumulation of proteins, amino acids, carbohydrates and minerals, and this accumulation is affected by crop load. More studies are needed to find the optimum crop load to increase the flower buds resistance to spring frost damage.

Summary of findings

Three methods for determining and calculating the protein content of apple flower buds were compared: Kjeldahl, sulfuric acid-hydrogen peroxide, and summation of amino acid content . A new nitrogen:protein conversion factor (5.1-5.6) was calculated based on total amino acid analysis. This new conversion factor should be substituted for 6.25 for estimating total protein content of this tissue using the Kjeldahl method. Ninhydrin reaction of amino acids was the most sensitive method for the quantitation of total protein.

The cold tolerance of flower buds of apple *Malus pumila* Mill. (McIntosh/M7) declined during spring growth. The absence of fruit in the previous year increased the ability of the buds to withstand low temperatures. All buds retained the capacity to deep supercool until first pink stage, but buds on trees carrying no crop were more cold tolerant at all stages of development. The HTE (high temperature exotherm) of the flower buds remained relatively constant throughout the sampling period and was -4.7^{o}C on cropped trees and -5.1^{o}C on non-cropped trees. Non-cropped trees contained higher levels of hydrophilic and acidic amino acids from October until April , probably associated with their greater cold tolerance.

Spring frost hardiness and fluctuations in nitrogen (N), phosphorous (P), potassium (K), glucose, fructose, sorbitol and starch were compared in spur buds of bearing and non-cropped mature McIntosh/M7 trees (*Malus pumila* Mill.). Deblossoming caused more flowers to be initiated for the next season and flower buds were larger and heavier. Sorbitol was the predominant soluble carbohydrate in both years and was higher in the buds of non-cropped trees during most of the year. Buds of deblossomed trees also had higher contents of glucose and starch. In both non-cropped and cropped trees total starch increased during cold acclimation and decreased during late winter and early spring . Fructose content was not effected by crop load. Bud content of N, P, and K was lower on cropped trees throughout the sampling period.

References

Ashworth, E. N. and Abeles, F. B. 1984. Freezing behavior of water in small pores and the possible role in the freezing of plant tissue. Plant Physiol. 76:201-204.

Chen, H. H. and Li, P. H. 1980. Characteristics of cold acclimation and deacclimation in tuber-bearing *Solanum* species. Plant Physiol. 65:1146-1148.

Gusta, A. L., Fowler, D. B. and Tyler, N. J. 1982. Factors influencing hardening and survival in winter wheat. In Plant Cold Hardiness and Freezing Stress pp. 23-40, (Li, P. H., and Sakai, A. eds.) Academic Press, New York.

Homshaw, L. G. 1980. Freezing and melting temperature hysteresis of water in porous materials: Application to study of pore form. J. Soil Sci. 31:399-414.

Ichiki, S. and Yamaha, H. 1982. Sorbitol in tracheal sap of dormant apple (*Malus domestica* Borkh.) shoots as related to cold hardiness. In Plant Cold Hardiness and Freezing Stress pp. 181-187, (Li, P. H., and Sakai, A. eds.) Academic Press, New York.

Levitt, J. 1980. Responses of Plants to Environmental Stresses. Vol. I. Chilling, Freezing, and High Temperature Stresses, 2nd edn. Academic Press, New York.

Levitt, J. 1956. The Hardiness of Plants. Academic Press, New York. 278 pp.

Li, P. H. 1987. Plant Cold Hardiness. Alan R. Liss, Inc., New York. 381 pp.

Quamme, H. A., Stushnoff, C. and Weiser, C. J. 1972. The relationship of exotherms to cold injury in apple stem tissues. J. Amer. Soc. Hort. Sci. 97:608-613.

Raese, J. T.; Williams, M. W. and Billingsley H. D. 1978. Cold hardiness, sorbitol, and sugar levels of apple shoots as influenced by controlled temperature and season. J. Amer. Soc. Hort. Sci. 103(6):796-801.

Sakai, A. and Larcher, W. 1987. Frost Survival of Plants, Responses and Adaptation to Freezing Stress. Springer-Verlag, New York. 303 pp.

Siminovitch, D., Rheaume, B. Pomeroy, K. and Lepage, M. 1968. Phospholipid, protein, and nucleic acid increases in protoplasm and membrane structures associated with development of extreme freezing resistance in black locust tree cells. Cryobiology 5:202-225.

Tyler. N. and Stushnoff, C. 1988a. The effect of prefreezing and controlled dehydration on cryopreservation of dormant vegetative apple buds. Can. J. Plant Sci. 68:1163-1167.

Tyler. N. and Stushnoff, C. 1988b. Dehydration of dormant apple buds at different stages of cold acclimation to induce cryopreservability in different cultivars. Can. J. Plant Sci. 68:1169-1176.

VII. CLAIM OF ORIGINALITY

The following are original contributions to knowledge:

1) **It is proven that a heavy crop load increases the susceptibility of apple flower buds to spring frost damage.**

2) **A new protein nitrogen conversion factor (5.19-5.64 depending on the sampling date) for apple flower buds (*Malus pumila* Mill. cv. McIntosh/M9) is reported. This factor is more accurate and replaces the factor of 6.25 currently used.**

3) **It is reported for the first time that 4-hydroxyproline-rich-glycoprotein content of the primary cell walls of apple flower buds can be estimated from the amount of 4-hydroxyproline in the acid hydrolysates.**

4) **Amino acids of apple flower buds were grouped into four classes according to their physical and chemical properties.**

VIII. SUGGESTIONS FOR FUTURE EXPERIMENTS

1. Study the effect of crop load (i.e. no-crop, 20%, 40%, 60%, 80% and full crop) on nutrient composition and spring cold tolerance of apple flower buds.

2. Study the effect of crop load on protein content and identify the specific protein(s) responsible for cold tolerance of apple flower buds.

3. Study the effects of crop load and summer pruning on spring cold tolerance of apple flower buds.

4. Develop a model based on crop load, nutrient composition, environmental factors, tree age, cultivar and rootstock, etc. to predict the spring frost tolerance of apple flower buds. The model could be started with a few main parameters to avoid complexity, and over the years more parameters added.
   e.g. % injury = tree age + level of sugar + crop + etc.

5. Study the effect of plant growth regulators used for thinning apples on flower buds and spring frost tolerance.

X. APPENDIX I

```
1 '=====================================================
2 ' A program to read/control the temperature of FTS MULTI-COOL
3 ' SYSTEM (R) mechanically refrigerated bath through the FTS SYSTEM
4 ' TC-40 CONTROLLER (R).
5 ' The three given source files are a part of TEMPERATURE
6 ' CONTROLLER, Version 1.0 (c) Copyright 1988, Shahrokh Khanizadeh
7 ' Registration number 367247, File number 280116
8 ' Consumer and Corporate Affairs Canada, Copyright Office.
9 ' All rights reserved.
10'=====================================================
11' This Program may not be used without the above Copyright Notice.
12' Before running this program a MBC-488 board must be installed.
13'=====================================================
60 'FILE NAME:LOGO.BAS
70 '-----------------------------------------------------
80 CLS:KEY OFF:SCREEN 2
90 '----------------------- logo routine ------------------
100 LINE (0,0)-(639,199),,B
110 LINE (4,2)-(635,197),,B
120 LOCATE 10,30:PRINT "TEMPERATURE CONTROLLER
130 LOCATE 12,21:PRINT "A program to receive or transmit data to
140 LOCATE 15,33:PRINT "TC-40 Controller
150 LOCATE 14,50:PRINT "r"
160 CIRCLE (394,108),10
170 LOCATE 20,3:PRINT "Please wait ....":LINE (4,160)-(635,160)
180 '
190 '
200 LOCATE 22,15:PRINT "Version 1.00, Copyright (C) 1988,
210 LOCATE 23,20:PRINT "Registration No. 367247, File No. 280116
220 LOCATE 22,49:PRINT "Shahrokh Khanizadeh
230 '
240 '
250                                   FOR I=1 TO 40000' :NEXT
```

```
260                                     FOR I=1 TO 8000  :NEXT
270 CLS:LOCATE 8,32:PRINT "M A I N    M E N U
280    LOCATE 10,22:PRINT "A) Read data from TC-40 controller
290    LOCATE 12,22:PRINT "B) Send data to TC-40 controller
300    LOCATE 14,22:PRINT "C) Send and read data simultaneously
310    LOCATE 16,22:PRINT "D) new option
320    LOCATE 18,22:PRINT "E) EXIT to system
330 LINE (100,50)-(515,149),,B
340 K$=INKEY$
350 LOCATE 23,3:PRINT "Select your choice A/B/C .... "
360 LOCATE 23,31:PRINT "                    "
370 IF K$="a" OR K$="A" THEN GOTO 440    'read
380 IF K$="b" OR K$="B" THEN GOTO 450    'write
390 IF K$="c" OR K$="C" THEN GOTO 460    'read & write
400 IF K$="d" OR K$="D" THEN GOTO 470    'new
410 IF K$="e" OR K$="E" THEN GOTO 480   'exit
420 IF K$="" THEN GOTO 340
430 IF K$<>"a" OR K$<>"b" OR K$<>"c" OR K$<>"d" OR K$<>"e" THEN GOTO 340
440 CLS:CHAIN "read
450 CLS:CHAIN "write
460 CLS:PRINT "option not available ... ":GOTO 260
470 CLS:PRINT "option not available ... ":GOTO 260
480 LOCATE 1,1:CLS:PRINT "Temperature Controller, Version 1.00, Copyright
       (C) 1988, Shahrokh Khanizadeh"
490 LOCATE 2,1:PRINT "Registration No. 367247, File No. 280116
500 LOCATE 5,1:PRINT "------ END RUN ------"
700 END
```

```
1 'FILE NAME: READ.BAS
2 'READ TEMPERATURE FROM FTS system TC-40 Controller -R-
3 ' ------------------------------------------------
10 CLS:KEY OFF:SCREEN 2
20 COUNT=0 :J=0
30 LINE (2,100)-(630,185),1,B
40 LOCATE 14,3:PRINT "NOTE: filename is consist of 8 char. length with or
       without extension, eg.
50 LOCATE 16,3:PRINT "      C:TEMP.DAT, B:TEMPDATA, B:\SUBDIR\TEMP.DAT,
       A:\TEMPERATU, etc.
60 LOCATE 18,3:PRINT "      A sequential output file will be created on
       the default or specified
61 LOCATE 20,3:PRINT "      device, and Temp. reading from the controller
       will be dump into it.
62 LOCATE 22,3:PRINT "      IF THE FILE ALREADY EXIST THE OLD READING WILL
       BE REPLACED.
70 LOCATE 2,3:INPUT "Please enter the output file name: ",AAA$
71 IF AAA$="" GOTO 70
80 CLS:
90 LINE (2,100)-(630,185),1,B
100 LOCATE 14,3:PRINT "NOTE: Interval time of reading should be entered in
110 LOCATE 16,3:PRINT "      Minimum accepted value for time interval is
120 LOCATE 2,3:PRINT "Please enter number of minutes between each reading:
       ":LOCATE 2,57:LINE INPUT A$:MN=INT(VAL(A$))
130 IF MN<1 THEN CLS:PRINT "  NOTE: your time interval must be 1 or more
       ":FOR  I=1 TO 6000:NEXT I:GOTO 120
140 CLS:
150     LOCATE 4,4:PRINT "     Display of FTS system, model TC-40 will be
       written into "
160     LOCATE 6,4:PRINT "     file: ";AAA$;" for every ";MN;"minutes
170     LOCATE 16,9:INPUT "Is this correct (y/n)  ",ANS$
180 AN$=LEFT$(ANS$,1) :IF AN$="n" OR AN$="N" THEN GOTO 10
190 LINE (2,100)-(630,185),1,B
200 LOCATE 16,9:PRINT "Release the REMOTE button, and set the temperature
210 LOCATE 18,9:PRINT "by pressing DISPLAY and turning the SETPOINT to
       desire Temp.
```

```
220 LOCATE 20,9:PRINT "Hit any key when you are done.
230 A$=INKEY$:IF A$="" THEN 230
240 OPEN "o",#1,AAA$ :CLOSE #1
250 CLS:LINE (1,1)-(630,185),1,B:LINE (430,70)-(530,130),1,B:LINE (1,148)-
      (630,148),1
260 '---------------------- timer to read data ------------------
270 SM=VAL(MID$(TIME$,4,2))
280 NR=SM+MN                        '  next reading will be in nr (next
290 MM=VAL(MID$(TIME$,4,2))
300 IF (NR-MM)>59 THEN NR=NR-60
310 IF INKEY$="E" THEN GOTO 410
320 LOCATE 14,40:PRINT "Current time:    ";TIME$
330 LOCATE 16,40:PRINT "Current Date:    ";DATE$
340 LOCATE 12,14:PRINT "No. of data line(s) is written to file:    ";COUNT
350 LOCATE 21,3:PRINT "       Press ' E ` to interrupt the routine and save
351 LOCATE 18,4:PRINT "Free memory available";FRE(0)
352 DATE=FRE(DATE$) : TIME=FRE(TIME$) : TEMP=FRE(TEMP$)
360 LOCATE 20,3:PRINT "NOTE: DO NOT Press the DISPLAY button since the
      view is being recorded
370 '---------------------- check for time delay -------------
380 IF NR=MM THEN GOTO  390 ELSE 290
390 SM=MM : COUNT=COUNT+1:J=J+1: IF J<2 THEN GOSUB 1000 ELSE GOSUB 1340
400 IF INKEY$="E" THEN GOTO 410 ELSE 270
410 CLS:LOCATE 10,4:PRINT "WAIT ... file ";AAA$;" is being closed":
420 LOCATE 12,4:PRINT "and  data are being saved.":FOR I=1 TO
430 CHAIN "logo
440 '
450 '
460 '
1000 '--------------- routine start reading ------------
1010 LINE (1,1)-(630,185),1,B
1020 LINE (430,70)-(530,130),1,B :LINE (1,148)-(630,148),1
1030 LOCATE 2,3:PRINT "NOTE: Data are being written to file ";AAA$
1040 LOCATE 4,3:PRINT "       Number of minutes between each reading is
1050 '              IF AN$="n" OR AN$="N" THEN GOTO 320
```

```
1060 LOCATE 23,9:PRINT"TEMPERATURE CONTROLLER, COPYRIGHT (C), 1988,
       Shahrokh Khanizadeh"
1070 DEF SEG = &H2800
1080 '
1100 '
1110 '------ Initialize MBC-488 board using "SYSCON" command -- ------
1120 CMD$ = "SYSCON MAD=3, CIC=1, NOB=1, BA0=&H300"
1130 A% = 0 : FLAG% = 0 : BRD% = 0
1140 CALL IE488 (CMD$, A%, FLAG%, BRD%)      'initialize
1150 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in initialization":STOP
1160 '
1170 '------ Set FTS into REMOTE ------------------------------------
1180 CMD$ = "REMOTE 4"
1190 CALL IE488 (CMD$, A%, FLAG%, BRD%)
1200 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in selecting
1210 '
1220 A% =35 : CMD$="TIMEOUT"
1230 CALL IE488 (CMD$, A%, FLAG%, BRD%)      'initialize
1240 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in selecting
1250 '
1260 '--------------- SET MODE TO TRIGGER ON TALK ---------------- --
1270 '
1280 MODE$ = "T1X":CMD$="EOI 4[$]"
1290 CALL IE488 (CMD$, MODE$, FLAG%, BRD%)
1300 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in EOI MODE
1310 '------------------- Read data from FTS ------------------------
1320 CMD$ = "enter 4[$]"
1330 LOCATE 10,70:TEMP$ = SPACE$(8)  'create blank string to receive data
1340 CALL IE488 (CMD$, TEMP$, FLAG%, BRD%)
1350 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in ENTER command":STOP
1360 LOCATE 10,14:PRINT "  Last Multi Cool bath Temperature was:   ";TEMP$
1370 LOCATE 14,40:PRINT "Current time:   ";TIME$
1380 LOCATE 16,40:PRINT "Current Date:   ";DATE$
1390 LOCATE 12,14:PRINT "No. of data line(s) is written to file:   ";COUNT
1400 LOCATE 21,3:PRINT "      Press ' E ` to interrupt the routine and
       save the file ";AAA$
```

```
1410 LOCATE 20,3:PRINT "NOTE: DO NOT Press the DISPLAY button since the
      view is being recorded
1411 OPEN AAA$ FOR APPEND AS 2
1420 BEEP :WRITE #2,DATE$;TIME$;COUNT;TEMP$:CLOSE #2
1430 DATE=FRE(DATE$) : TIME=FRE(TIME$) : TEMP=FRE(TEMP$)
1440 RETURN
```

```
1 'FILE NAME: WRITE.BAS
2 'SEND TEMPERATURE TO FTS system TC-40 Controller -R-
3 ' ------------------------------------------------
10 CLS:SCREEN 2:KEY OFF
20                      DEFINT I,J,K
30                      DIM T$(500)
40 COUNT=0:K=0:I=0
50 '--------------------- DEFINE --------------------------
60 DEF SEG = &H2800
70 LOCATE 23,3:PRINT "Please wait initializing the MBC-488 board.
80 FOR I=1 TO 9000:NEXT:FOR I=1 TO 18:PRINT :NEXT
90 LINE (2,100)-(630,185),1,B
100 LOCATE 14,3:PRINT "NOTE: Before setting up the initial temp. on TC-40
      controllrer be sure
110 LOCATE 16,3:PRINT "      the REFRIGERATION and MAGNETIC STIRRER are
      on, and the REMOTE button
120 LOCATE 18,3:PRINT "      is pushed in.
130 LOCATE 21,3:PRINT "      Press any key when you are ready ...
140 AS$=INKEY$:IF AS$="" THEN 140
150 CLS:LOCATE 4,3:PRINT " NOTE: Temp. of the controller is set to ZERO
160 FOR I=1 TO 10000:NEXT
180 '
200 IE488 = 0
210 '------ Initialize MBC-488 board using "SYSCON" command -- -----
220 CMD$ = "SYSCON MAD=3, CIC=1, NOB=1, BA0=&H300"
230 A% = 0 : FLAG% = 0 : BRD% = 0
240 CALL IE488 (CMD$, A%, FLAG%, BRD%)     'initialize
250 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in initialization":STOP
260 '------ Set FTS into REMOTE ------------------------------------
270 CMD$ = "REMOTE 4"
280 CALL IE488 (CMD$, A%, FLAG%, BRD%)
290 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in selecting
300 '
310 A% =35 : CMD$="TIMEOUT"
320 CALL IE488 (CMD$, A%, FLAG%, BRD%)     'initialize
330 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in selecting
```

```
340 '--------------- SET MODE TO TRIGGER ON TALK --------------------
350 MODE$ = "T1X":CMD$="EOI 4[$]"
360 CALL IE488 (CMD$, MODE$, FLAG%, BRD%)
370 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in EOI MODE
380 '------------------- write data to FTS  -------------------------
390 CMD$ = "output 4[$]"
400 TEMP$ = SPACE$(25):
410 CALL IE488 (CMD$, TEMP$, FLAG%, BRD%)
420 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in output command":STOP
430 IF COUNT=0 THEN GOSUB 560  ELSE GOSUB 920
440 TEMP$=SPACE$(25)
450 TEMP$=T$(K)
460 LOCATE 2,3:PRINT "NOTE: data are being read from file ";BBB$
470 LOCATE 4,3:PRINT "      Total number of data points (temp.) is ";NO
480 LOCATE 10,3:PRINT "Previous Temp. send to controller at ";TIME$;" was:
      ";TEMP$;" C
481 LOCATE 23,9:PRINT "TEMPERATURE CONTROLLER, COPYRIGHT (C), 1988
      Shahrokh Khanizadeh
490 LOCATE 6,3:PRINT "      number of minutes between each sending is
500 COUNT=COUNT+1:BEEP
510 CALL IE488 (CMD$, TEMP$, FLAG%, BRD%)
520 IF FLAG%<>0 THEN PRINT"Error #";HEX$(FLAG%);" in output command":STOP
530 GOSUB 920
540 GOTO 440
550 '--------------------- entering temperature ---------------
560 CLS:
570 LINE (2,100)-(630,185),1,B
580 LOCATE 14,3:PRINT "NOTE: This subroutine will create a file on default
      or specified drive,
590 LOCATE 16,3:PRINT "and record the entered Temp. data points. File name
      can be entered as:
600 LOCATE 18,3:PRINT "C:\SUBDIR\TEMP.DAT, OR B:DATA.LST, etc. Please use
      the below format.
610 LOCATE 20,3:PRINT "Use -0800 for -80.0C, -0201 for -20.1, +0153 for
      +15.3, +0113 for +11.3,
```

```
620 LOCATE 22,3:PRINT "-0001 for -0.1, 0000 for 0 C, etc. DO NOT USE ANY
      OTHER FORMAT.
630 ' ------------ writting and reading file temp.dat ----------------
640 LOCATE 4,3:INPUT "Please enter file name to hold temperature data
650 IF BBB$="" GOTO 640
660 OPEN BBB$ FOR OUTPUT AS #1
670 LOCATE 6,3:INPUT "Enter # of Temp. data points ",NO
680 FOR K=1 TO NO:LOCATE 8,3:PRINT "Enter Temp. ";K;:INPUT T$(K)
690 PRINT #1, T$(K):LOCATE 8,15:PRINT "                    ":NEXT
700 LOCATE 8,3:PRINT "Please Wait ....                          "
710 CLOSE
720 CLS:OPEN BBB$ FOR INPUT AS #1
730 FOR K=1 TO NO
740 INPUT #1,T$(K):PRINT T$(K);" [";K;"],   ";:NEXT
750 LINE (2,130)-(630,185),1,B
760 LOCATE 18,3:PRINT "The above Temp. will be send in the same sequence
      to the controller"
770 LOCATE 19,3:PRINT "Are these values entered with regard to the
      previous example "
780 LOCATE 20,3:PRINT "i.e. Each value start with + or - plus 4 digit
790 LOCATE 22,3:PRINT "ANY OTHER FORMAT WILL SEND UNPREDICTED VALUES AND
      DAMAGE THE CONTROLLER":LOCATE 20,67:INPUT AN$
800 IF AN$="n" OR AN$="N" THEN CLOSE :GOTO 550
810 '----------------- timer    ---------------------------
820 CLS:K=0
830 LOCATE 2,3:PRINT "Please enter number of minutes between each Temp.
      change: ":LOCATE 2,62:LINE INPUT A$:MN=INT(VAL(A$))
840 IF MN<1 OR MN>59 THEN CLS:PRINT "  NOTE: your time interval must be
      between 1 and 60 ": FOR I=1 TO 6000:NEXT I:GOTO 830
841 IF COUNT=0 GOTO 1070
850 CLS
860 '------------------------ Verify input -----------------
870 CLS
880 LOCATE 4,4:PRINT "Temp. of controller will be changed every ";MN"
890 LOCATE 6,4:PRINT "Temp. data will be read from file ";BBB$
900 LOCATE 16,9:INPUT "Is this correct (y/n)  ",ANS$:CLS
```

```
910 AN$=LEFT$(ANS$,1):IF AN$="n" OR AN$="N" THEN GOTO 550
920 SM=VAL(MID$(TIME$,4,2))
930 NR=SM+MN
940 TEMP=FRE(TEMP$):T=FRE(T$):DATE=FRE(DATE$):TIME=FRE(TIME$)
950 LINE (1,1)-(630,185),1,B:LINE (430,70)-(530,130),1,B:LINE (1,148)-
      (630,148),1
960 MM=VAL(MID$(TIME$,4,2))
970 LOCATE 18,4:PRINT "Free memory available "; FRE(0)
980 LOCATE 12,12:PRINT "No. of data points is send to controller:   ";K
990 LOCATE 14,40:PRINT "Current time:   ";TIME$
1000 LOCATE 16,40:PRINT "Current date:   ";DATE$
1010 IF INKEY$="E" OR INKEY$="e" THEN CLS:LOCATE 6,3:PRINT "Routine
      intrupt abnormally .......":GOTO 1090
1020 LOCATE 20,3:PRINT "NOTE: Press ' E ` to intrupt the routine and
      return to main menu.
1040 TEMP=FRE(TEMP$):T=FRE(T$):DATE=FRE(DATE$):TIME=FRE(TIME$)
1050 IF (NR-MM)>59 THEN NR=NR-60
1060 IF NR=MM "HEN GOTO 1070 ELSE 960
1070 SM=MM:CLS :K=K+1:IF K>NO THEN GOTO 1090
1080 RETURN
1090 LOCATE 2,3:PRINT "NOTE: last temperature (";TEMP$;") was read from
      file ";BBB$;
1100 LOCATE 4,3:PRINT "and send to controller at ";TIME$;" on ";DATE$
1110 LOCATE 20,3:PRINT "Press and key to return to main menu
1120 SS$=INKEY$:IF INKEY$="" THEN 1120
1130 CLOSE
1140 CHAIN"logo
```

Seasonal changes of protein and amino acids in the spur buds of apple trees, Malus pumila Mill. cv. McIntosh. (mg of amino acid per 100 g of dry weight)

	1984						1985					
	July 26		August 24		October 24		March 29		April 11		April 28	
Amino acids	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped	Non-cropped	Cropped
Aspartic acid	5.97a[z]	5.07b	5.81	5.77	7.28a	6.49b	8.31a	6.56b	8.59a	7.19b	10.92b	14.64a
Threonine	2.26	1.90	2.41	2.32	2.58	2.41	3.06a	2.60b	3.32	3.14	4.78	5.48
Serine	2.25	2.19	2.31	2.21	2.68a	2.43b	2.96a	2.49b	2.93	2.83	3.85	4.31
Glutamic acid	4.98	4.86	5.01a	4.44b	7.17a	6.76b	7.41	7.51	8.82a	7.40b	11.95b	15.27a
Proline	2.24a	1.96b	2.22a	2.07b	2.80a	2.51b	2.97a	2.89b	3.10a	3.10b	3.59b	4.59a
Glycine	2.30	2.27	2.15	2.24	3.01a	2.68b	2.98b	3.15a	2.98b	3.25a	4.14b	5.66a
Alanine	2.51	2.4	2.53	2.43	2.81a	2.53b	3.12a	3.0 b	3.06	3.05	6.63a	4.66b
Cysteine	0.55	0.47	0.67	0.46	0.65a	0.56b	0.71	0.67	0.85	0.79	1.10b	1.35a
Valine	2.69a	2.55b	2.89a	2.56b	3.28a	3.00b	3.45	3.46	4.20a	3.94b	5.59b	7.35a
Methionine	1.43a	1.13b	1.60	1.33	1.69	1.53	1.72	1.65	1.9	1.7	2.66b	3.40a
Isoleucine	2.46	2.25	2.37	2.27	3.10	3.11	3.15	3.12	3.25a	3.10b	5.05b	6.49a
Leucine	3.34a	2.91b	3.90a	3.62b	4.44	4.77	4.82	4.58	5.54a	4.76b	8.90b	9.22a
Tyrosine	1.59	1.51	2.27	1.52	2.66	2.65	3.13	3.06	3.50a	3.23b	3.70	3.63
Phenylalanine	2.48	2.39	2.35	2.25	3.02	3.01	2.99	3.12	3.58	2.96	5.17b	7.47a
Histidine	1.24	1.23	1.24	1.24	1.48b	1.54a	1.49	1.48	1.64	1.67	2.66	3.11
Lysine	2.75	2.88	3.29a	2.62b	4.41a	3.76b	4.61	4.58	5.07	4.76	7.30b	9.38a
Arginine	3.77a	3.03b	3.62a	3.0b	3.72	3.38	4.14a	3.96b	5.05a	4.81b	6.29b	7.65a
Tryptophan	0.31	0.28	0.33	0.31	0.31	0.31	0.33	0.32	0.33	0.32	0.30	0.36
Hydroxyproline	0.98	1.04	1.04	1.05	1.19	1.05	1.29	11.48	1.28	1.43	1.79	1.66
Total protein[y]	46.18a	42.42b	48.24a	43.92b	58.46a	55.71b	62.92a	60.29b	68.55a	63.75b	90.71b	115.25a
Totally charged	18.71	17.07	18.97	17.07	24.06	22.45	25.96	24.21	29.2	25.83	39.13	50.05
Basic	7.76	7.14	8.15	6.86	9.61	9.2	10.24	10.14	11.79	11.24	16.25	20.14
Acidic	10.95	9.93	10.82	10.21	14.45	13.25	15.72	14.07	17.41	14.59	22.87	29.91
Hydrophilic	23.22	21.16	23.69	21.6	29.32	27.29	31.98	29.3	35.45	31.8	47.75	59.84
Hydrophobic	14.3	12.92	15.71	13.86	18.5	18.49	19.59	19.61	21.5	20.01	27.4	34.66
% amino acids nitrogen	19.27	18.93	17.98	18.62	18.27	18.09	17.50	18.16	17.98	17.74	18.49	18.90
CF[x]	5.19	5.28	5.56	5.37	5.47	5.53	5.71	5.51	5.56	5.64	5.41	5.29
Ammonia	1.69	1.43	1.12	1.39	1.75	1.45	1.28	1.65	1.7	1.24	2.65	3.97

[a,b] Means in the column at each sampling date of sampling are significantly different at the 5% level using the F ratio.
[z] Each value is the average 3 replicates and 24 determinations.
[y] Calculated according to Horstman (1979).
[x] A new factor for converting nitrogen into protein content was calculated by following analytical convention:

$$\text{Protein conversion factor} = \frac{\text{100 g total protein}}{\text{Percent amino acid nitrogen}}$$