Winter Hardiness in Barley
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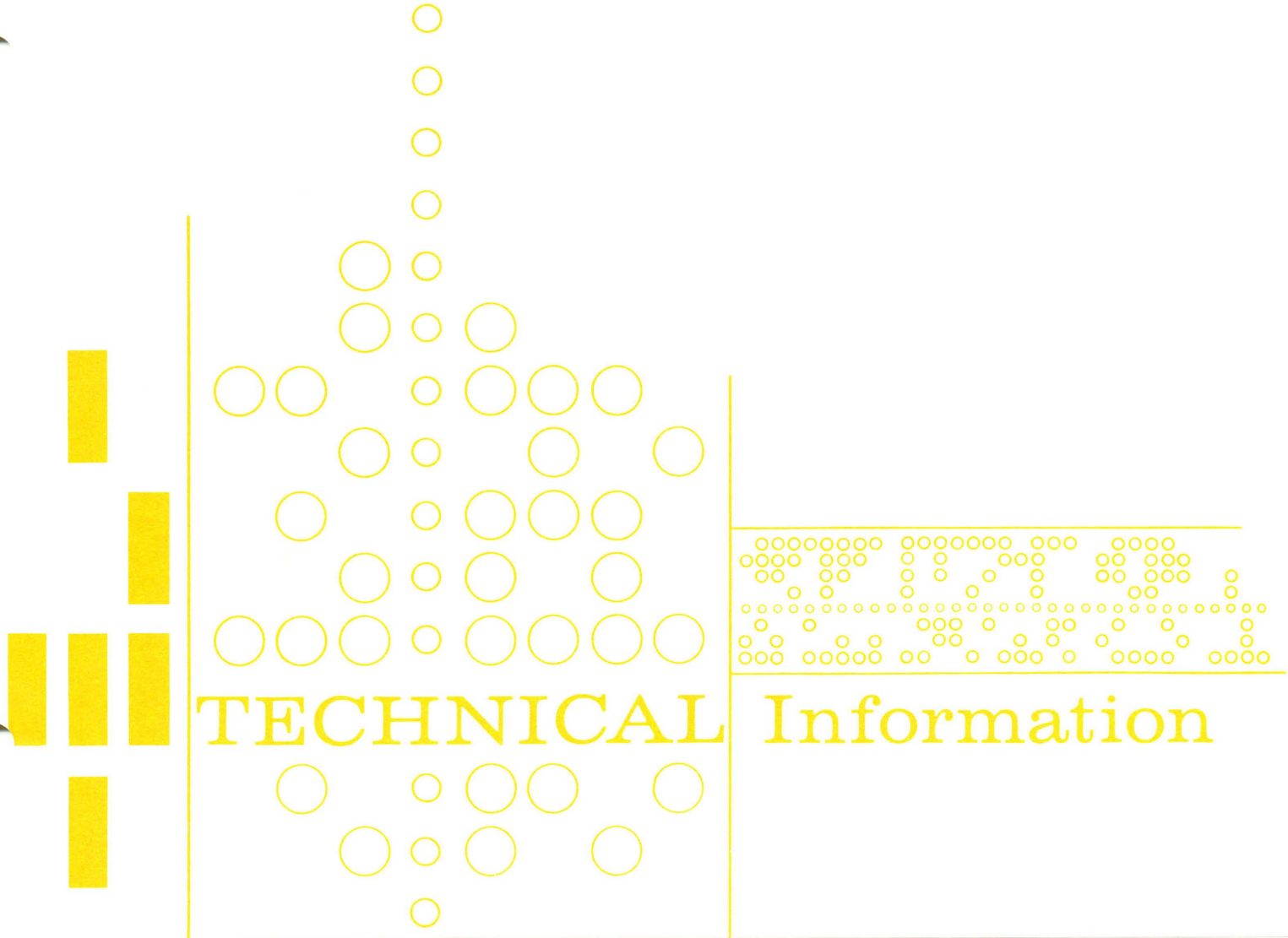

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STRESS ANALYSIS

by C. R. Olien[2]

INTRODUCTION

In winter hardiness, as in most biological problems, we are concerned with a large body of information. More than 5,000 papers have been published on this subject. Such a large amount of information can be organized and viewed from many different perspectives.

The following chapter is a review of research leading toward a quantitative concept of freezing stress. Transitional energy of water distribution is considered as a unifying principle for stress analysis. An expanded expression of transitional energy that consistently inter-relates descriptive parameters was developed to characterize interacting components. This analysis of stress energies requires coordinating data obtained by several techniques.

Because a genetic approach to winter hardiness improvement would be aided by techniques for specific identification of effective genetic units in terms of their physiological action, analyses of freezing stress and injury were undertaken. Components of hardiness have been defined in terms of protective physiological systems that modify stress, injury, or recovery. Our next objectives are as follows:

Biological Variability—

Find range and distribution of specific hardiness components within *Hordeum* and *Triticum* species.

Heritability—

Evaluate complexity of genetic systems that determine specific hardiness components and determine heritability of these systems.

Physiological Interactions—

Develop isogenic lines to study physiological effects of specific hardiness components in several uniform genetic backgrounds.

Statistical Development of a Unified Concept—

Quantitatively interrelate all essential features and energy relationships that affect freezing stresses and survival.

Reality determined by predictability of effects caused by modifying genetic and environmental variables.

Define optimum genotypes for specific environments and for balance with other desirable plant characteristics.

WINTER HARDINESS

Winter barley survival depends on hardening of established vegetative plants to withstand different types of stress. Degree of hardiness, however, involves environmental conditions other than exposure to low temperature (16, 17). Healthy barley plants start to harden in early fall. After 2 to 4 weeks of growth at temperatures just above freezing in an environment supplying adequate light and nutrients, physiological changes permit the plant tissues to withstand freezing. New leaves developing in this period are usually darker and more appressed.

In growth chambers kept at 0.5°C, new tissues develop slowly and pigments frequently accumulate in older leaves. The plants are more readily attacked by root-rotting organisms, and after 2-3 months, evidence of physiological degeneration is observed. If the temperature drops slightly below freezing after the hardening period, the plants retain their hardiness and do not degenerate in other respects. Partial frost dehydration apparently is important in retarding metabolic processes harmful to winter cereals under prolonged periods of mild, low-temperature stress.

The type of freezing stress that first causes a lethal effect as the temperature decreases depends on the test environment and the plant's physiological condition. Transition from the tender to hardened state involves metabolic activity. During this period, the protoplasmic membrane changes so ice no longer forms easily in the protoplasts.

The minimum temperature a plant can withstand in a freezing test is not determined exclusively by this transition, however, and temperature *per se* is not a measure of stress. Variations in the nature and intensity of stress are induced by the amount of water and by the redistribution energy and pattern.

The nature and intensity of stress are also affected by many plant characteristics. Differences between species and cultivars of hardy plants may depend more on

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[1]This biophysical analysis of freezing stress was conducted as a U. S. Department of Agriculture, Agricultural Research Service project at Michigan State University in cooperation with the Crop & Soil Science Dept.

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nonadaptive than adaptive features. Protective mechanisms that modify stress involve control of freezing energy and its effect on critical meristematic tissues.

PROPERTIES OF WATER

Quantitative interrelation of heritable plant characteristics and environmental variables is needed for evaluating the nature and energy of freezing stresses. The most important plant characteristic is water and its interaction with plant substances.

Water molecules are associated by two types of bonds. The H-bond involves the mutual attraction of two strongly electronegative atoms (oxygen) for a proton. The hybridization of electronic energy states induced by two protons coupled with an oxygen results in a strongly vectorized tetrahedral (105°) bonding pattern between water molecules (coordination number = 4). This type of bonding predominates in normal ice (ice I_h).

The second type of bonding involves weaker dipole and multipole attractions or van der Waals-London bonding that occurs when the rigidly vectorized structure is distorted. These bonds are weaker but the coordination number is higher. The H-bond structure is less dense than the van der Waals-London structure.

Liquid water is bonded by both types and the energy required to break an H-bond is the difference between the bond energies. Water is held within tissues by bonding with hydrophilic plant components. In very wet tissues, some additional surface tension may hold unassociated water between structural features of plants. Generally, the properties of water in plants differ from those of pure water since association with plant components greatly affects bonding interactions. Some plant substances, such as inorganic ions, are structure breakers; some nonpolar substances are structure formers that induce clathrate or hydrotactoid formation. Much water in biological systems is in gradient association with hydrophilic interfaces (7).

The properties of liquid water are greatly affected by association with plant components, especially the insoluble hydrophilic polymers (15). Early studies of this association were reviewed by Gortner (5). New techniques have been used to study various water interactions and less distinction found between bound and free water (4, 7).

Water can be part of various stable molecular compositions or part of special configurations such as clathrates or hydrotactoids around solutes (11). Most water in plants, however, is associated by bonds having a short half-life (10^{-8} to 10^{-11} seconds). These dynamic patterns of association are affected by the insoluble hydrophilic polymers. Association of water with hydrophilic polymers has an extended effect on water structure. The physical properties of water in plants, such as viscosity and freezing point, are functions of distance in a gradient zone extending from a tissue structure in water. Though little water is tightly bound, there is no free water or homogeneous liquid. The liquid becomes more heterogenous as freezing selectivity isolates water in crystals and draws plant components together with less liquid between them. All tissue components and solutes compete in structuring the intervening liquid. Ice is a new phase that causes redistribution of water associations. Ice is the novelty, but liquid is the common denominator, the medium in common for all water interactions in barley plants. Analyses of the shifting freezing point in barley are discussed later under the section "Stresses in barley."

FREEZING STRESS

Patterns of water redistribution found in various tissues of barley are discussed in several publications (see Outline p. 6). Although all patterns have some hysteresis, most are nearly reversible when supercooling is prevented, when heat is withdrawn or added slowly (less than 1 cal/gm/hr), and when tissue has been hardened so ice forms only along the cell walls and not in the protoplasts. Water redistribution is irreversible after injury.

The reversible processes are divided into two categories. In one category, the freezing point gradually shifts as freezing progresses (a function of depth of liquid water associated with plant components). In the other category, a large part of tissue moisture is relatively unassociated and has nearly a single freezing point. With normal rates of temperature decrease, water redistribution in tissues with a gradually shifting freezing point stays near equilibrium. The balance of water distribution gradually shifts as the temperature is lowered and energies of association increase and eventually cause injury. Large crystals grow in spaces inside or outside the plant because there is time for diffusion and little energy is available for disrupting tissues to make space. In tissues with an abruptly shifting freezing point, freezing occurs more quickly when the plant is in a heat sink with a steadily decreasing temperature, such as in soil.

The displacement of water distribution from equilibrium determines the intensity of freezing. High intensity freezing can result either from supercooling the water in a plant tissue before freezing begins or from having the tissue at a high moisture content in an efficient heat sink without supercooling. Either condition causes a nearly instantaneous high energy of crystallization when ice inoculation occurs. Supercooling causes a high energy per water molecule. It typi-

cally occurs in spring frosts and increases in severity with decrease in moisture content.

Rapid transfer of latent heat from wetter tissues can occur after a mid-winter thaw. This involves lower energy per molecule but many more molecules contribute to the freezing energy released in the tissues. Abrupt freezing is a nonequilibrium process when displacement from equilibrium provides enough excess energy to significantly alter the nature of the process from that which would have occurred if the heat had been withdrawn slowly as freezing progressed.

The excess potential energy driving the crystallization process is available to disrupt tissue structure that resists crystal growth. The results differ from the equilibrium process with respect to the distribution and type of ice crystals in the tissues, and these differences become especially significant when the killing temperature is affected. Substances that either lower the energy of crystallization by interacting with the liquid water making it less easily available or help control crystal growth by interacting with the ice directly can modify the freezing stress.

NATURE OF FREEZING PROCESSES IN CRITICAL TISSUES OF BARLEY

The crown is the most critical region of winter cereals. Destruction of this region always kills the plant. If any other region is destroyed, the plant is weakened, but will survive in an environment suitable for growth. The crown is the transitional zone between the roots and leaves and contains the meristematic tissues capable of regenerating either. The crown consists of three regions distinguished by major differences in the tissues and type of freezing processes that occur in specific plants. (See Fig. 1.) Unless the moisture content is exceptionally high, equilibrium freezing occurs in the upper region containing the apical meristem. Nonequilibrium freezing usually occurs in the region where the large vascular elements of the mesocotyl and roots enter the base of the crown and continue toward the central transitional region. This type of freezing does not occur when the moisture content of the tissues is low. Either freezing process occurs in the lateral region where much finer vascular elements branch out from the central region to the leaf sheaths. The freezing process in the lateral region depends on the moisture content and the cultiver. The extent of injury in this region is important because the meristematic tissues from which new roots arise are located in this region.

The nature of the freezing stress, the killing temperature and symptoms of injury are greatly affected by the moisture content of the crown tissues. Cyclic thawing and freezing greatly increase the severity of injury from ice crystal growth in the lower part of the crown so repetition results in death at higher temperatures. The killing temperature of hardened vegetative winter barley is the lowest (between —10° and —20° C in a single freeze test) at 65% tissue moisture and increases to about 0° C when the tissue moisture approaches 80%. The killing temperature at low tissue moisture and the change in killing temperature with increasing moisture are heritable characteristics. Both systems are important.

Under conditions causing high freezing intensity, varietal differences in hardiness greatly depend on factors modifying stress. Stress can be modified by heritable systems that affect: 1) supercooling (14), especially in relation to summer frost (6); 2) amount of water in the tissue (9); 3) redistribution of water as freezing progresses (see Dynamics of Freezing in Outline, p. 6); 4) freezing velocity as a function of free energy (see Energy Relationships in Outline, p. 6); 5) temperature and heat transfer rate (18); and 6) histological organization involving essential tissue location in relation to plant regions having high freezing intensity (16); also see Energy Relationships in Outline, p. 6). In this research, protective systems and substances have been identified by physiological function rather than correlation of chemical composition with survival of a freezing test.

The systems that modify stress from growth of intercellular ice crystals act in one of two ways. They either enhance diffusion to crystallization sites, permitting large crystals to grow in noncritical regions with low-freezing intensity or they restrict displacement of water, preventing the growth of any crystal to a destructive size.

In the former situation freezing proceeds as a reversible equilibrium process. When freezing is nearly an equilibrium process, water is redistributed with such little energy that no excess is available to increase stress and ice only grows where the potential energy at the ice surface is minimum. Diffusion over long distances is possible since time is not limiting.

Any more abrupt freezing causes a nonequilibrium process where displacement from distribution equilibrium provides extra energy for tissue disruption and decreases time for diffusion. Kinetics inhibitors modify this type of freezing process, by preventing ice crystals from growing into critical systems. When ice does form, energy is dissipated while forming additional ice interface and imperfect crystals rather than by distorting plant structure.

With high crystallization energy from supercooling or rapid heat transfer, ice crystal growth can be destructive if the energy is opposed by tissue structure. Ice growth in a leaf does little damage if it only lifts

the epidermis, but if crystals grow in a crown imbedded in frozen soil, significant injury can occur.

Kinetics inhibitors are substances in cell walls which interact directly with ice to give a plant control over the location and macro structure of ice formation. More energy is required to maintain crystal growth when these substances are present (see Fig. 2). They coat the crystal and tend to block its growth. These substances are extractable arabo-xylans and the activity of these extracts can be rated.

With low crystallization energy, other interactions become important and effects of crystals separated by a gas phase from wet tissue components are very different from ice in the interface. When the temperature of a system at equilibrium with ice is lowered, a crystallization energy develops. If the ice is separated by a gas phase, the ice crystal grows as long as the wet polymer system can provide water vapor. The free energy is dissipated as the vapor pressure drops.

This does not happen in an ice-polymer interface. Here the free energy is dissipated by shifts in the activation energies of transition of water from the polymer to ice. The shift in activation energies reduces the latent heat of freezing; then, a latent heat of transition of water from the interface to free liquid develops. This in effect provides an energy of adhesion which causes the ice to stick to the polymer. If it sticks at points on a membrane, stress could arise if the crystal was displaced or the membrane pulled by frost desiccation. There are probably other more important potential energy interactions, but this is easy to explain and an obvious effect.

The difference in the nature of the interaction between ice and a polymer system when the two are separated by a gas phase rather than by liquid water may account for the difference between freezing and desiccation as stress vectors. The difference in the way the driving energy of water crystallization is satisfied for comparable isothermal processes and the possibility of different interactions in a freezing matrix with ice as compared to a matrix without ice are significant. Potential energy interactions modify stress energies acting on polymer organization and are determined by specific composition of plant systems.

Activation energies of transition characterize interactions of plant systems with liquid water. These can be determined by coordinating distribution and calorimetric data. Potential energies of water associations are evaluated from the shift in freezing point as changes in liquid amounts in associated phases are determined from mobility of probes (diffusion or electrophoresis of indicator molecules), in sublimation cells where the liquid and ice are separated by a vapor phase so they can be evaluated independently or by NMR or other methods. Latent heats and net enthalpies are determined calorimetrically.

Chemical potential is the partial derivative of free energy that describes the mean effect of water transition. A distribution partition of chemical potential was developed to describe the dynamic states of water and the interactions in plants. Chemical potential is partitioned on a basis of phase or potential energy of association and on a basis of kinetic energy with respect to activation limits (see Figs. 3 and 4). Potential energies of interactions and activation energies of transitions involving liquid water, entropy changes in matrices of plant substances, and inhibition of freezing kinetics by plant substances that block ice lattices are measurable heritable plant characteristics. The energy of various freezing stresses and the killing temperature can be predicted by interrelating these plant characteristics with environmental variables (moisture, external ice nucleation, rate of temperature change, thermal conductivity, and minimum temperature).

Researchers are looking for parental lines with diversity in specific heritable characteristics. These characteristics alone might not cause a plant to be hardy but would contribute to hardiness in the right combination with other plant characteristics. Also, attempts are underway to develop new combinations of hardiness characteristics starting with the male-sterile populations created by G. A. Wiebe and D. A. Reid (21). J. E. Grafius has developed a system of inbreeding in balance with outcrossing to get maximum recombination with sufficient stability to recognize and retain improvements in hardiness.

ANALYTICAL TECHNIQUES

Critical data are needed to develop a realistic concept of freezing stress. Ice formation can be estimated from change in volume (dilatometry) and release of dissolved gas (eudiometry). Nuclear magnetic resonance is being developed to evaluate water structure stability. Water potential in frozen tissue has been evaluated by pressure methods. The transition from liquid to ice can be estimated by calorimetry or differential thermal analysis if the latent heat is assumed to be the same as in bulk water. The error in this assumption increases as freezing progresses and interactions between plant components increase. These methods do not permit study of water redistribution as ice crystals grow in a small specified region. Ice crystals interfere with direct optical techniques, though freeze substitution or freeze etching can be used to study ice in some situations.

One technique for plant tissues with well-developed cell walls involves measuring fluidity of the unfrozen

liquid as freezing progresses (see Methods of Analysis in Outline, p. 6). This is determined by mobility of indicators—molecular probes of the liquid along the cell walls. Both random mobility of diffusion and directional mobility of electrophoresis are studied under a microscope. The indicators are nontoxic molecules such as amaranth (which is not taken up by protoplasts) or small charged particles. Radioactive isotopes improve mobility measurement (1). Mobility can also be estimated from conductivity data under special conditions—the contact resistance must be low, the electrical resistance of the tissues must be known, the voltage, current, and frequency must be below a level affecting protoplasts, the ionic strength of the liquid must be low, and the amount of electrolyte must be constant.

Although precise conductivity data are easily obtained, accuracy of interpretation with respect to fluidity depends on mobility evaluations. Conductivity measurements can be made quickly but require rather large, uniform tissues and several control experiments for each test to interpret data. Electrophoretic mobility measurements are more directly interpretable but require more time for each measurement.

Simple diffusion of microdots, with no electric field, can be used to study extremely small regions in plant tissues, but this technique requires maintenance of a stable test condition for several days. Fluidity depends on the amount of unfrozen liquid and is a function of distance from the polymer in the gradient zone for a specific system. It can be used to characterize the gradient zones of different systems (freezing patterns). These systems are calibrated by comparing mobility with the liquid content determined by weight in a diffusion chamber with controlled vapor pressure. Deviations in mobility of molecular probes perhaps caused by the presence of ice are checked by freezing the same system, but at different water contents, to a specified temperature and plotting fluidity against the total water content.

Mobility of indicators as an index of water structure, weight as an assay of water content, and temperature as an index of exchangeable kinetic energy that determines the vapor pressure of ice are three interrelated variables. One mobility test relates to liquid water content, another relates to temperature. The third combination, weight versus temperature, is evaluated in a sublimation cell.

Sublimation cells are used to obtain adsorption isotherms at temperatures below freezing (see Methods of Analyses in Outline, p. 6). In these cells, ice is separated from the rest of the system by a vapor phase and change in liquid content is determined by change in weight. This technique provides accurate equilibrium data for homogeneous systems, such as polysaccharide or protein models or rather uniform pieces of plant tissue, when ice forms as a completely independent phase. If the total water is extracellular, if the polymers are not injured or altered by the presence of ice, and if the effect on potential energies of associations with water are negligible, then the sublimation data obtained with ice separated from the rest of the system will agree with the mobility data. Deviations characterize outer versus inner free space, stability of the test system, and bonding interactions that change water association energies.

When the temperature is changed slowly (less than 1°C/hr) in a system inoculated with ice, the amount of liquid remaining can be evaluated as a function of temperature. This equilibrium shift in freezing point is a quantitative characteristic distinguishing tissues and regions within a plant. After the equilibrium relationship has been established, faster temperature changes permit a quantitative study of freezing kinetics. In this case, freezing velocity is evaluated as a function of the displacement from equilibrium and the free energy of freezing. Relative freezing velocity in a specific system equals the product of an equilibrium constant, a kinetics inhibition constant, and an energy variable. The equilibrium constant is determined by the interaction of plant components with liquid water, and the kinetics constant quantitatively discribes interaction with the ice lattice.

The thermodynamics of water interactions can be evaluated partly from water redistribution patterns, but it also requires microcalorimetry to evaluate changes in latent heat and entropy. As freezing progresses and plant components are drawn into close association, interactions become more complex and physical constants of bulk water become invalid. The latent heat can be measured if the equilibrium relationship between freezing point and the amount of water remaining liquid is known. The latent heat measured with a calorimeter as freezing progresses is the net effect. It equals the latent heat of ice formation minus the latent heat of water association with other plant components.

The latent heat of ice formation is a function of temperature and the Person-Kirchoff equation expresses the rate of change, $\frac{d\Delta H}{dT} = -\Delta Cp$ where ΔCp is the difference between the heat capacities of ice and liquid. The latent heat of association increases as freezing progresses and water more closely associated with plant components becomes involved. The latent heat of association is important for calculating activation energies of water association to characterize plant systems, as components of winter hardiness.

COMPONENTS OF WINTER HARDINESS

Data and detailed discussion of a biophysical approach to freezing stress analysis can be found in the following publications.[3]

A. Definition of Problems

Physiology of winter hardiness in barley.
Olien, C. R. (1961). Agriculture Handbook 338, USDA. 121-127.

Winter hardiness of barley.
_____ (1963). Barley Genetics Symposium I: 356-363.

Freezing stresses and survival.
_____ (1967). Ann. Rev. Plant Physiol. 18:387-408.

Effect of Michigan environments on barley and wheat winter hardiness.
_____ (1970). Mich. State Univ. Agr. Expt. Sta. Res. Rep. 123:4-8.

B. Methods of Analysis

1. Water distribution

   a. Mobility of indicators

      A method of studying stresses.
      _____ (1961). Crop Sci. 1:26-28.

      Liquid water content of frozen systems.
      Olien, C. R. and Su-En Chao (1973). Crop Sci. 13:674-676.

   b. Sublimation cells and adsorption isotherms

      Freezing and desiccation as stress vectors.
      _____ (1971). Cryobiology 8:244-248.

2. Calorimetry

   Energy of freezing and frost desiccation.
   _____ (1974). Plant Physiol. 53:764-767.

C. Patterns of Interaction

Freezing processes in the crown of 'Hudson' barley.
_____ (1964). Crop Sci. 4:91-95.

Freezing processes in wheat stems.
Single, W. V. and C. R. Olien (1967). Australian J. of Bio. Sci. 20:1025-1028.

Ice structure in hardened winter barley.
Olien, C. R., B. L. Marchetti and E. V. Chomyn (1968). Mich. State Univ. Agr. Expt. Sta. Quart. Bul. 50: 440-448.

Crown moisture content and killing temperature.
Metcalf, E. L., C. E. Cress, C. R. Olien and E. H. Everson (1970). Crop Sci. 10:362-365.

Freezing patterns in stems of cherry and azalea.
Dennis, F. G., G. P. Lumis and C. R. Olien (1972). Plant Physiol. 50:527-530.

D. Energy Relationships

1. Kinetics of freezing

   Interference of cereal polymers with freezing.
   _____ (1965). Cryobiology 2:47-54.

   Preliminary classification of freezing inhibitors.
   _____ (1967). Crop Sci. 7:156-157.

   Characterization of inhibitors from wheat.
   Shearman, L. L., C. R. Olien, B. L. Marchetti and E. H. Everson (1973). Crop Sci. 13:514-519.

2. Dynamics of freezing

   Freezing stresses in barley.
   _____ (1969). Barley Genetics Symposium II:356-363.

   Thermodynamic components of freezing stress.
   _____ (1973). J. Theoret. Biol. 39:201-210.

3. Stress energies

   Desiccation and freezing as stress vectors.
   _____ (1971). Cryobiology 8:244-248.

   Energy of freezing and frost desiccation.
   _____ (1974). Plant Physiol. 53:764-767.

This research provides the basis for identifying heritable plant characteristics that: 1) modify stress in critical tissues; 2) increase the ability of tissues to withstand stress; or 3) improve their ability to recover from an injury.

SPECIFIC COMPONENTS OF HARDINESS BEING EVALUATED

Properties of critical plant systems

1. Water balance with environment
2. Plant histology and water distribution
3. Water structure in plant systems

Kinetics of freezing

4. Inhibitor activity of cell wall araboxylans

Dynamics of freezing

5. Water distribution patterns
6. Activation energies of water transitions

Patterns of injury from freezing

7. Low intensity tests
8. High intensity tests

General resistance to injury from physical stress

9. Stability tests, especially of protaplasmic membrane structure

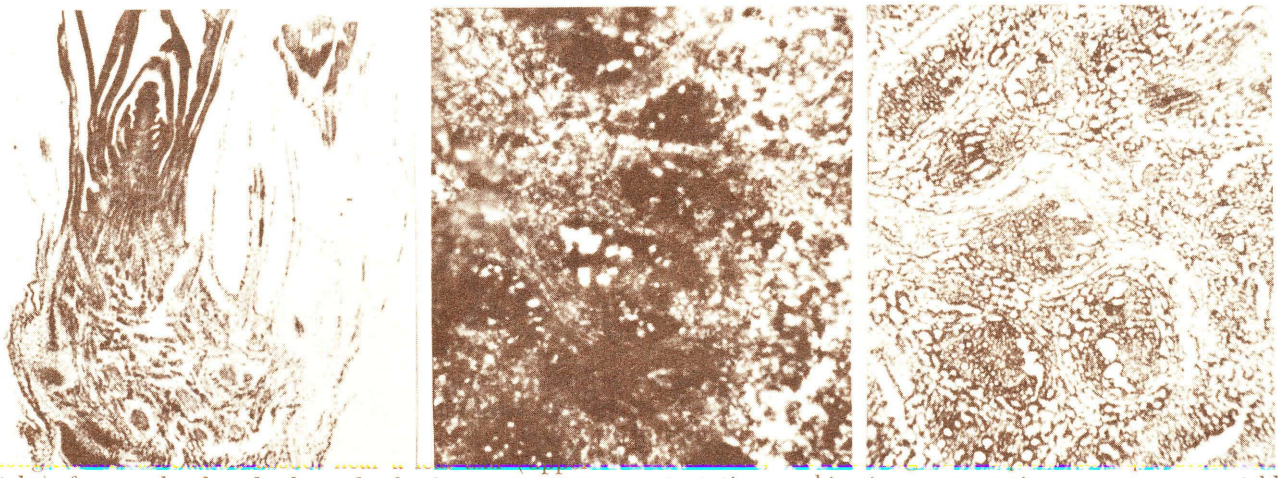
Recovery ability

10. Time required to recover from a specified injury

The essential feature of evaluations 1-8 are described in the preceding outline. For screening cultivars, number 3 involves evaluating indicator mobilities and freezing point shifts as functions of the amount of liquid water associated with the plant system. Evaluation 5 can be made by the methods published. However, for screening cultivars only a few points on the curve that relate liquid water content to temperature need be obtained.

[3]Publications in the above outline were written by C. R. Olien unless indicated otherwise.

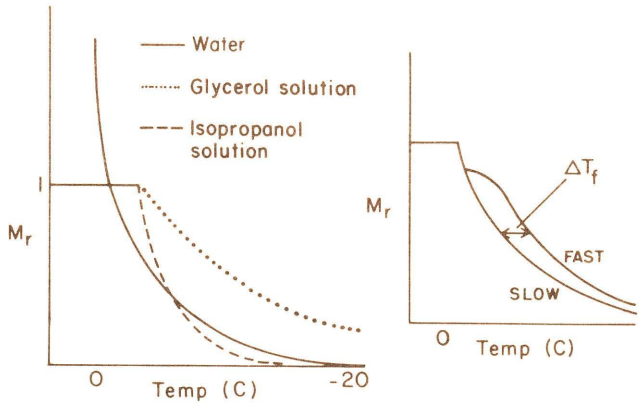
APPENDIX



right) from a hardened plant slowly frozen to 1°C below killing temperature.

H. Normal degeneration of original roots and growth of new roots in a surviving plant.

I. Cross section of a root from a plant slowly frozen, thawed, and then incubated for 1 week in a normal environment for growth (20°C). Only tissues within the endodermis were killed by freezing.



M_r-Relative content of liquid water

Fig. 2. Kinetics of freezing. (See Outline, p. 6.)

Water in plant tissue has shifting freezing points that are functions of the amount of liquid in each specified system. The equilibrium relationship between temperature and the relative amount of liquid water (Mr) is obtained by slowly adjusting the temperature of a paritially frozen system.

Freezing rate $\frac{dMr}{dt} = \frac{dMr}{dT}\frac{dT}{dt}$ where T represents temperature and t represents time. $\frac{dMr}{dT}$ for a water solution = $\frac{dMr}{dT}$ for pure water times $\left(1 - \frac{d\Delta Te}{dT}\right)$ or $e^{\frac{-d\Delta Te}{dT}}$, factors that describe the change in shape of the equilibrium curve induced by a solute.

Heat can be withdrawn faster than water can freeze, stant times a kinetics constant times an energy variable.

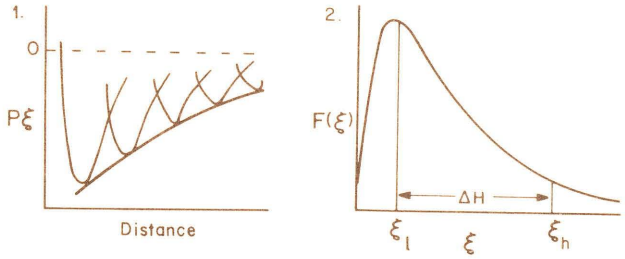


Fig. 3. Dynamics of freezing. (See Outline, p. 6).

3. $\left(\frac{dG}{dn}\right)_{T,P} = \frac{dG>}{dn_t}\frac{dn_t}{dn} + \frac{dG<}{dn_s}\frac{dn_s}{dn} + \frac{dG_o}{dn_o}\frac{dn_o}{dn}$

$G>$ = free energy of ice molecules escaping to liquid.
$G<$ = free energy of liquid molecules being trapped in ice.
G_o = free energy of other water in the interface.
n = water in the interface, n_t = associated, n_s = dissociated.

4. $\frac{\Delta G>}{n_t} = \int_{\xi_h}^{\infty}(\xi - \xi_h)\,F(\xi)\,d\xi$, $\frac{\Delta G<}{n_s} = \int_{o}^{\xi_l}(\xi - \xi_l)F(\xi)\,d\xi$

5. $\Delta G_{trs} = \frac{\Delta G>}{n_t}\,n_t + \frac{\Delta G<}{n_s}\,n_s$

Potential energy-kinetic energy interaction and free energies of water transitions are evaluated from a distribution partition of chemical potential (see J. Theoretical Biol. 39:201-210, 1973).

1. Gradient association of water with a plant polymer, represented as successive potential energy patterns of water molecules diminishing with distance from the polymer. This pattern represents an average effect in a highly dynamic system.

2. Frequency of molecules with a specified energy (ξ) = $\frac{2\pi}{(\pi RT)^{3/2}}\,\xi^{\frac{1}{2}}\,e^{\frac{-\xi}{RT}}$. The derivation is based on the simplest system (or thermometer) where only translational kinetic energy is exchanged (as with a perfect gas molecule). At any given temperature all systems have the same average exchangeable kinetic energy and this is assumed to be distributed normally.

A. Longitudinal section of a tiller. (A new root is forming.)

B. Cross section of a tiller from a plant frozen in a normal test before sectioning.

C. Cross section similar to B, but photographed after ice in the section melted. (Numerous ruptures previously contained ice crystals.)

D. Longitudinal section near base of crown prepared by freeze substitution after initial freezing of the whole plant. (Many of the cells in the lower crown are elongate. Some xylem vessels have large ruptures.)

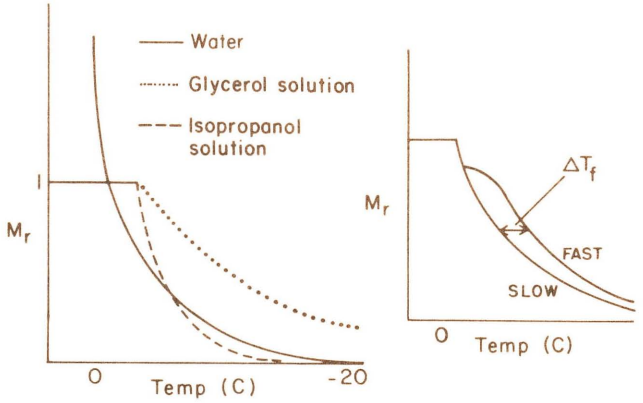
E. Cross section of vascular tissues in a frozen crown.

F. Cross section similar to that in E, but photographed as the ice melted. There is obvious rupture of xylem vessels.

G. Longitudinal section of tissues near a leaf axil (upper right) from a hardened plant slowly frozen to 1°C below killing temperature.

H. Normal degeneration of original roots and growth of new roots in a surviving plant.

I. Cross section of a root from a plant slowly frozen, thawed, and then incubated for 1 week in a normal environment for growth (20°C). Only tissues within the endodermis were killed by freezing.



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Heat can be withdrawn faster than water can freeze, so temperature displacement (ΔT_f) occurs when the temperature of a partially frozen system is decreased rapidly. The free energy of freezing and the activity of water with respect to freezing (Aw_f) are functions of ΔT_f and calculated from the temperature displacement. The data is obtained with an oscilloscope.

$$\text{The free energy of freezing} = RT\ln\frac{L}{I} = RT\ln(1 + Aw_f)$$

where RT is the gas constant times the absolute temperature and where L and I represent the activities of liquid water and ice. The activity of water with respect to freezing is the difference in activities of liquid water and ice divided by the activity at equilibrium which equals the activity of ice.

$Aw_f = e^{-i}\frac{dT}{dt}$ where e^{-i} is a kinetics inhibition constant.

Therefore, the freezing rate equals an equilibrium constant times a kinetics constant times an energy variable.

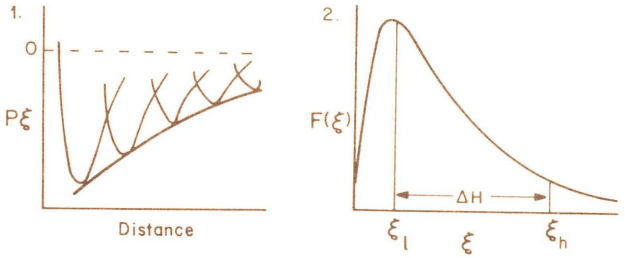

Fig. 3. Dynamics of freezing. (See Outline, p. 6).

3. $\left(\frac{dG}{dn}\right)_{T,P} = \frac{dG>}{dn_t}\frac{dn_t}{dn} + \frac{dG<}{dn_s}\frac{dn_s}{dn} + \frac{dG_o}{dn_o}\frac{dn_o}{dn}$

$G>$ = free energy of ice molecules escaping to liquid.
$G<$ = free energy of liquid molecules being trapped in ice.
G_o = free energy of other water in the interface.
n = water in the interface, n_t = associated, n_s = dissociated.

4. $\frac{\Delta G>}{n_t} = \int_{\xi_h}^{\infty}(\xi - \xi_h)\,F(\xi)\,d\xi$, $\frac{\Delta G<}{n_s} = \int_{o}^{\xi_l}(\xi - \xi_l)F(\xi)\,d\xi$

5. $\Delta G_{trs} = \frac{\Delta G>}{n_t}\,n_t + \frac{\Delta G<}{n_s}\,n_s$

Potential energy-kinetic energy interaction and free energies of water transitions are evaluated from a distribution partition of chemical potential (see J. Theoretical Biol. 39:201-210, 1973).

1. Gradient association of water with a plant polymer, represented as successive potential energy patterns of water molecules diminishing with distance from the polymer. This pattern represents an average effect in a highly dynamic system.

2. Frequency of molecules with a specified energy (ξ) = $\frac{2\pi}{(\pi RT)^{3/2}}\,\xi^{\frac{1}{2}}\,e^{\frac{-\xi}{RT}}$. The derivation is based on the simplest system (or thermometer) where only translational kinetic energy is exchanged (as with a perfect gas molecule). At any given temperature all systems have the same average exchangeable kinetic energy and this is assumed to be distributed normally.

The energy exchangeable with a thermometer is assumed to be nearly the energy exchanged between molecules at phase transitions, especially considering the dynamically disrupted state of water interfaces (half life = 10^{-5} seconds for ice-liquid water). R is the constant that equates temperature with the product of mass times the mean squared velocity (the mean squared velocity with respect to 1 axis is the second moment about the mean, the variance).

3. Partition of chemical potential expressed as a differential equation, based on phase of association and on distribution of exchangeable kinetic energy with respect to activation limits for association (E_l) and dissociation (E_h). The effective potential energy determines the activation energy for dissociation. The activation energy for association equals that for dissociation minus the latent heat of transition (ΔH).

4. Integrate the partial derivatives involved in dynamics of transitions to determine the free energies of these partial distributions.

5. Multiply by the phase densities of the interface to determine the free energies of transitions.

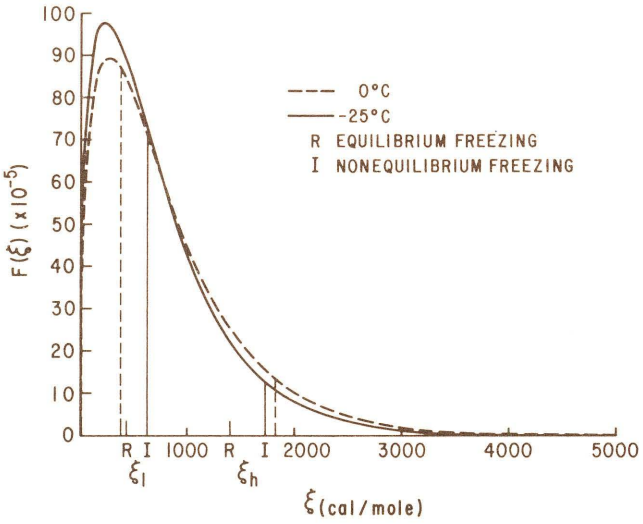

Fig. 4. Energy distribution over shifting activation energies of water transitions.

The pattern of water redistribution in response to a temperature change below freezing is a variable plant tissue characteristic. Dynamics of interaction between the effective potential energy of water in a plant system and its exchangeable kinetic energy determine the redistribution pattern and can be studied most simply in a low intensity process where supercooling is prevented by ice inoculation and where heat transfer is very slow. Under these conditions kinetic effects do not limit or alter the interactions. The problem can be further simplified by considering model systems which simulate plant systems.

Sample Calculations for Systems at —25°C

Ice-Liquid water system—The latent heat of freezing (−1435 cal/mole at 0°C) changes with decrease in temperature, and according to the Person-Kirchhoff relationship ($\Delta H = -\int \Delta C_p dT$), it equals −1097 cal/mole at −25°C. This corrects only for internal heat capacity of the phases and assumes no change in the amount of work done in the system when freezing occurs at different temperatures. The latent heat is −970 cal/mole at −25°C for reversible isothermal (equilibrium) freezing.

In such a system, the free energy could be dissipated in transport of liquid water to ice. Since ΔG is nearly zero for this as an equilibrium process the change in entropy for freezing at −25°C equals 3.90 cal/mole. The free energy for nonequilibrium freezing at −25°C equals −127 cal/mole ($\Delta H - T\Delta S$). The activation limits at −25°C can be found by solving the following equation since ΔG and ΔH are known and $\Delta H = \xi_h - \xi_l$.

The free energy of ice formation (ΔG) equals $n_t \int_{\xi_h}^{\infty} (\xi - \xi_h) F(\xi) d\xi + n_s \int_{o}^{\xi_l} (\xi - \xi_l) F(\xi) d\xi$ which is the mean energy greater than the activation energy for escape minus the mean energy less than the activation energy for trapping. n_t represents the number of water molecules in the ice, and n_s the number in the liquid at the ice-liquid interface. The activation energy for melting (ξ_h) is the kinetic energy an ice molecule at the ice-liquid interface must exceed to escape into the liquid phase. This was found to be 1400 cal/mole for equilibrium freezing at −25°C (1725 cal/mole for nonequilibrium freezing) by approximate integration of the preceding equation.

The activation energy for freezing (ξ_l) is the kinetic energy a liquid water molecule at the interface can not exceed in the liquid in order to be drawn into an ice lattice site and arrive with less kinetic energy than the limit for escape. ξ_l was found to be 430 cal/mole for equilibrium freezing at −25°C (628 cal/mole for nonequilibrium freezing).

Ice-liquid water-cellulose system—when ice and a polymer compete for the same intervening liquid water, a more complex interface develops where the potential energies of water associations with ice and with the polymer overlap. The simplest interaction lowers the activation energies for freezing and for melting and reduces the ΔH of freezing to the equilibrium freezing value. Also, a new ΔH develops, the ΔH of transition from this intervening liquid to free liquid water. The sum of these ΔH's equals the ΔH of freezing a corresponding ice-liquid water system.

The new latent heat is an index of adhesion between ice and the polymer as they compete for the intervening liquid water. When ξ_h decreases as a result of simple interaction between potential energy curves, ξ_l decreases proportionally. When the temperature is reduced, the interaction continues to develop, ice grows closer to the polymer in an isothermal process until the free energy for growth of ice is zero.

If ice growth involves secondary work terms, such as tissue disruption or water transport, the free energy is applied as stress acting against the interference. The potential energy curves cannot remain independent much below freezing. Interactions (inductive, resonance, etc.) must change bond energies and the shape of the induced gradient curve extending from a polymer must be especially susceptible. The change in activation energies changes the latent heat of water transition (See Outline, p. 6, Stress Energies).

ANALYSIS OF INJURY

by Su-En Chao[1]

LITERATURE REVIEW

Freezing injury in overwintering plants is usually assessed by shoot and root growth from the crown tissues after spring-thaw. Simulation of natural freezing stress can be performed by rigidly controlling the tissue moisture, photoperiod, and temperature (19). The injury estimated reflects a conglomerate of strains imposed on various cell types during freezing and thawing. More specific evaluations of injury would require manipulation of plant parts under conditions such that a uniform freezing stress could be operated and weighted.

For example, even with slow freezing (temperature lowered at 1°C/hr to —20°C) desiccation alone does not account for the freezing injury in leaf segments of several barley varieties (11). Interactions between ice crystals and matrices of plant substances, especially cell membrane components, were inferred. Efforts to delineate physical stresses of freezing and physiological strains in winter barley and wheat have been attempted in several laboratories, particularly with the awareness that interbreeding among existing winter-hardy varieties in the past was not adequate for breeding desirable winter barley and wheat.

A complete analysis of inheritable components of winter hardiness in barley and wheat of diverse genetic origins may add to the wealth of breeding materials from which geneticists can synthesize varieties for different regions. Hence, the intrinsic physiochemical factors governing the plant's response to freezing and thawing is our major concern.

The cell membrane is a traffic regulating system. It promotes uptake of metabolites and electrolytes, and waste secretion; and restricts the loss of substances essential to the cell and the entry of unwanted substances. It also helps the cell wall control cell volume, and protects the cell from osmotic shocks. Freezing stress could affect this multiple function of the cell membrane and result in cell injury or death.

Drastic changes of membrane permeability after freezing and thawing are detected quantitatively by leakage of electrolytes from the tissues or qualitatively by the efflux of neutral red from the stained tissues. Non-specific physiological tests like plasmolysis of living cells in hypertonic salt solutions or oxidation of triphenyl tetrazolium chloride (7), though effective in distinguishing living and dead tissues, are not specific enough to elicit the primary physico-chemical responses of plant tissues to freezing and thawing.

While intact plant protoplasmic membrane is difficult to obtain, mitochondria can be isolated from other cellular components. Mitochondrial membrane structure is evidently similar to that of protoplasmic membrane. It also possesses built-in enzymatic complexes for substrate oxidation and oxidative phosphorylation (13). Hence, mitochondrial membrane can be tested for catalytic functions, and the effects of freezing and thawing on membrane permeability can be determined. These findings generally apply to cell membrane.

Functional plant mitochondria are passive osmometers, as are chloroplasts, since their membranes are flexible. They swell in hypotonic solutions and contract in hypertonic solutions. The swelling and contraction of mitochondria in suspensions are accompanied by a quantitative change in light absorption at a specific wavelength which can be monitored on a double-beamed spectrophotometer. This property was studied in isolated functional mitochondria from two groups of vegetables differing in their sensitivity to chilling injury (0°-10°C) (9). The chilling-resistant mitochondria showed rapid osmotic adjustment in hypotonic solutions in contrast to chilling-sensitive mitochondria which adjusted more slowly.

The flexibility of mitochondrial membrane is a function of the fluidity of lipid-protein in the membrane structure. The fluidity of lipid-protein in the membrane is determined by the composition of the phospholipid, the amount of unsaturated fatty acids, the length of the carbon chains of fatty acids, and the amount of interstitial water. Lyons et al. (9) reported an apparent correlation of high unsaturated fatty acid content to high chilling-resistance in the group of horticultural plants studied. A similar study on fatty acid composi-

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tion in hardy and nonhardy alfalfa varieties failed to show any quantitative difference in their unsaturated fatty acid constituents (4). Nevertheless, a substantial increase of linoleic and linolenic acids in alfalfa root lipids during cold hardening was detected.

These findings may seem inconsistent with the concept that the genetic make-up of a plant predetermines the fatty acid composition in its mitochondrial membrane. One study (22) showed that isolated sweet potato mitochondria contain more unsaturated fatty acids than the mitochondria of potato, which is relatively cold-resistant. During the cold-storage (0° to 1°C for 14 days), four major phospholipids decreased as much as 40% in isolated sweet potato mitochondria. Hence, the lipid composition of mitochondria is not metabolically stable and even at low temperatures is subjected to enzymatic modification such as oxidation.

These membrane-bound oxidative enzymes are regulated by lipid-protein interactions in the presence of interstitial water. A shift of lipid metabolism in plants does not necessarily reflect an adaptive change in the cell membrane to low temperatures. Similar seasonal shifts of lipid metabolism have been reported in the bark tissue of the black locust tree (18). An increase of polar phospholipids in the overwintering bark took place apparently at the expense of non-polar lipids.

PROPOSED RESEARCH

Rigid lipid-protein interrelation may not be necessary to insure flexibility of the mitochondrial membrane and the optimal activities of membrane-bound enzyme complexes at low temperatures. To maintain the constancy of the membrane fluidity, mechanisms for varying the phospholipid composition and their chain length may be essential to environmental adaptation. Studies correlating conformational changes and catalytic properties of membrane-bound mitochondrial enzymes would help explain the effects of freezing and thawing. Three experimental approaches toward this goal are reviewed in the following and incorporated in my proposal for testing freezing injury.

1. Titration of sulfhydryl groups in cell extracts:

Levitt (8) proposed that the formation of disulfide bonds in proteins is greatly accelerated at sub-freezing temperatures and results in irreversible protein aggregation. He showed that the sulfhydryl group (SH) contents in crude cell extracts of two hardy wheat varieties actually increased during cold hardening at 3°C while that of tender plants (leaves of sunflower and soybean) showed no appreciable changes unless a substantial amount of water was withdrawn before cold hardening.

The increase of SH content was interpreted as an indication of protein conformational changes. When this occurs in tender plants disulfide bonds will form and cause further protein aggregation. In hardy plants, the disulfide bonds will not form even when more SH groups are exposed on the protein surface during cold hardening. While the existence of a cryoprotective mechanism is questionable, the experimental facts are interesting since they have been confirmed by many in vitro experiments with pure proteins. Likewise, changes of SH content in some mitochondrial membrane proteins may be related to conformational changes in the organelle.

2. Measurement of phase-transition temperature in isolated mitochondrial membrane:

The phase transition of phospholipids from liquid-crystal to crystalline is generally accompanied by a marked heat absorption. The temperature at which phase transition takes place characterizes the class of phospholipids present and their relations to the hydrophilic surroundings—proteins and water. The phase-transition temperatures of mitochondrial membrane monitored by a calorimeter along a temperature scan are comparable to those of saponified and deproteinized membrane preparations. The phase-transition temperature range of the membrane of *Mycoplasma laidlawii* remained unchanged after it had been deproteinized and was similar to that of an aqueous dispersion of the phospholipids extracted from the same membrane preparation (15).

3. Testing the functions of mitochondria and chloroplasts:

Abnormal activities in the electron transport chain either in whole or in segments can indicate freezing injury. The rate of O_2 uptake was measured in isolated barley mitochondria from the crown tissues of two hardy varieties, Wong and Dicktoo, acclimated (2°C for 4 weeks) and non-acclimated (held at 16°C for 4 weeks) (6). Higher rates of O_2 uptake were observed for mitochondria isolated from plants acclimated at 11°C and 31°C than for those from non-acclimated plants. The oxidative activity of mitochondria isolated from two groups of plant tissues were compared; both were chilling-sensitive and resistant between 0° to 10°C (10). The logarithm of substrate respiration rates was plotted against the temperatures of the reactions (Arrhenius plots). The mitochondrial oxidative activity of chilling-resistant plants; namely beet roots, potato tubers, and cauliflower buds had more or less constant Q_{10} values ranging from 1.3 to 1.6 (assayed at 1° to 36°C). Those from chilling-sensitive plants (sweet potato roots, tomato fruits, and cucumber fruits) had a marked increase in their Q_{10} values from 2.2 to 6.3 as

the assay temperature was lowered to 10°C. This relationship remained unchanged even when the Mitochondria were disrupted by sonication, hypotonic swelling or freezing and thawing (14). The change of Q_{10} at the critical temperature or the discontinuity in the Arrhenius plot could be eliminated by solublizing the lipids in the mitochondrial membrane (14).

Results suggest a phase change in the membrane is coupled with an inductive conformational change in the membrane-bound enzymes. Thus, loss of some membrane proteins and the lability of others could happen subsequently. Cytochrome oxidase, for example, is located at the outer membrane of mitochondria. Its activity in isolated beef heart mitochondria can be enhanced by adding phospholipids to the incubation media (20).

Likewise, 80% activity of wheat mitochondrial cytochrome oxidase is lost from plants frozen at —20°C for 15 hours. Most cytoplasmic enzymes are stable at low temperature but mitochondrial adenosine triphosphatase is labile at 0°C. Studies showed that the loss of this enzymatic activity was due to a partial dissociation of the molecule which could be further accelerated in the presence of various salts at concentrations even below 0.1 M (12).

Like mitochondria, the isolated chloroplast membrane also provides an excellent system for evaluating freezing injury. The inactivation of its cyclic photophosphorylation can be related to cell freezing injury (16). Apparently, as the water freezes out the electrolyte concentration is elevated and a negative hydrostatic pressure is exerted on the thylakoid membrane. This causes the later to fail to perform photophosphorylation.

Taking up protons by isolated spinach grana also shows the integrity of the membrane. Isolated spinach grana lost proton uptake capacity following freezing (5). The same effect can be brought about by suspending the isolated membrane in a high concentration of electrolyte solutions (21).

A PROPOSAL FOR EVALUATING FREEZING INJURY IN BARLEY AND WHEAT

Dependent variables

Conformation of mitochondrial membrane components.

Oxidative activity of mitochondria.

Independent variables

Fresh vs. frozen mitochondria.

Tender vs. hardy varieties.

Nonhardened vs. hardened mitochondria.

Mitochondria hardened in vivo vs. in vitro.

PRELIMINARY EXPERIMENTS

Plant Materials

Mitochondria were isolated from etiolated seedlings of Hudson barley and Genesee wheat. Since recovery of functional mitochondria depends largely on tissue tenderness, 7-day old wheat and 4-day old barley seedlings were most suitable. Before isolating mitochondria, coleoptiles and plumules were collected and chilled at 6°C for about 1 hour. Usually, 10 g of fresh material yielded mitochondria equivalent to 1 mg protein.

Mitochondria were isolated according to a modified procedure of Sarkissian and Srivastava in a cold chamber where temperature ranged from 6° to 14°C. The process took about 45 minutes. Thirty grams of the chilled tissue were ground for one minute in a porcelain mortar with 80 ml of the grinding medium consisting of 0.4 M sucrose, 1.0 mM (EDTA), 0.1% Bovine serum albumin (BSA), and 0.05% cysteine in a HEPES-HCl pH 7.5 buffer. The tissue homogenate was then filtered through a double-layered Miracloth. The filtrate was centrifuged in a clinical centrifuge at 500 xg for 5 minutes.

The pellet was discarded and the supernatant was again centrifuged in a Beckman RC-2B refrigerated centrifuge (set at 0°C) at 40,000 xg for 2 minutes. The yellow pellet was gently resuspended in the wash medium of 0.4 M sucrose, 1.0 mM EDTA in a 0.05 M HEPES-HCl pH 7.5 buffer and centrifuged at 20,000 xg for 2 minutes. The resulting yellow pellet carried very little white starch and was mainly mitochondria. The isolated mitochondria were suspended in 4 ml of the wash medium and stored in crushed ice for further assays.

Titration of Reactive SH Groups on Mitochondrial Proteins

The three methods usually used for quantitative determination of SH groups in protein are amperometric titration (2), p-hydroxy mercuribenzoic acid titration (1), and dithionitrobenzoic acid titration (3). These methods differ in their specificities and reactivities with native proteins. Hence, the reproducibility of the data obtained by employing different methods for SH group titration depends on the assay conditions and the results must be carefully interpreted.

I have tried titration of SH group with the latter two methods (PHBA and DTNB) on isolated barley and wheat mitochondria with purified BSA and chicken egg albumin (EA) for reference proteins with glutathione and cysteine as standards. Mitochondrial proteins and BSA could be titratable only with DTNB but not PHBA, whereas the reverse relation is true for EA. The reason for differential reactivity is not

clear but apparently is pH dependent (the reaction pH for PHBA is 4.5 and for DTNB is 8.0).

With PHBA, 4 SH equivalents per mole of EA were detected from the titration curve. With DTNB there was 0.5 SH equivalent per mole of BSA. The quantitations of SH groups on both reference proteins were highly reproducible. A procedure for titrating SH groups in mitochondria with DTNB follows:

> To one ml of the sample protein containing 0.5-2.0 mg mitochondrial protein, add 5 ml of 0.1 M potassium phosphate buffer pH 8.0 and 1 ml of 0.01 M DTNB (in 0.1M potassium phosphate buffer, pH 7.0). Mix and read the increased optical density at 410 nm with either a spectrophotometer or a double beam scanning spectrophotometer. The increase of optical density due to the release of nitrophenyl sulfite is proportional to the number of SH groups on the proteins complexed with DTNB (each mole contains 2 moles of nitrophenyl sulfite linked with a disulfide bond) and is expressed in the number of SH equivalents per mg of mitochondrial proteins with reference to either gutathione or cysteine.

Results of comparing titratable SH group on proteins before and after freezing are given in Table 1.

Table 1. Sulfhydryl group titration in frozen and unfrozen protein samples

Protein samples	SH eq/mole protein	
	Unfrozen	Frozen
Bovine serum albumin	0.50	0.53
Chicken egg albumin	4.0	1.0
Barley mitochondria	2.5	1.7
Wheat mitochondria		
Exp. 1	3.2	—
Exp. 2	3.8	—
Exp. 3	3.0	—
Exp. 4	1.7	—
Exp. 5	4.0	0
Exp. 6	2.0	0
Exp. 7	2.9	1.8

Note: The average molecular weight of mitochondrial proteins is arbitrarily assumed to be 10,000 daltons. The SH titrations were done with DTNB for all protein samples except chicken egg albumin which was titrated with PHMB.

Whenever frozen protein solution is thawed a considerable amount of protein precipitate is formed, presumably due to protein aggregation. The SH assays are then based on those protein fractions remaining in the solution after freezing and thawing, and hence represent a high estimate of the amount of SH groups. The preliminary data in Table 1 showed a tendency toward diminishing titratable SH groups in proteins subjected to freezing and thawing. The DTNB titration method here is sensitive enough to detect any quantitative changes in SH groups on mitochondria before and after freezing.

Oxidative Activity of Isolated Mitochondria

Oxygen uptake and other enzymatic oxidations were measured polarographically using Clark-type oxygen electrodes (Yellow Spring Instrument Model 53SA) equipped with a constant temperature circulator. The O_2 electrodes were calibrated with air-saturated water at the set temperatures. The concentration of oxygen in air-saturated water was calculated from Handbook of Chemistry and Physics 42nd edition p. 1706 (Chemical Rubber Publishing Co., Cleveland).

The 3 ml reaction mixture contained 60 mmoles HEPES-HCl pH 7.5 buffer, 1.2 mmoles of sucrose, 30 μmoles of inorganic phosphate, 0.2 mg of mitochondria, in the presence/absence of 12.5 μmoles of sodium succinate, 200 mμ moles of NADH, and 3 mμ moles of reduced cytochrome c (horse heart cytochrome c from Sigma Chemical Co., St. Louis).

Numerous attempts were made to characterize the mitochondrial activity by measuring the P/O ratio indicating the efficiency of O_2 uptake coupled with oxidative phosphorylation. The results were erratic particularly when the mitochondria preparations were subjected to more than 6 hours of standing as the freezing experiments required. Subsequently, only oxidative activities with respect to added succinate, NADH, and cytochrome c were concerned.

The "endogenous respiration" served as the basal level and also showed the extent of mitochondria breakage during the preparation and the treatments. The results of two typical experiments with wheat mitochondria are presented in Table 2.

Table 2. The rates of oxygen uptake in the absence and the presence of cytochrome c by isolated mitochondria from the Genesee wheat

Reaction conditions	Freezing rate	0_2 uptake mμ moles/min/mg protein	
		Unfrozen sample	Frozen sample
Endogeneous respiration	slow	46.05	62.40
	fast	37.29	84.50
Cytochrome c added	slow	90.45	192.80
	fast	85.92	153.07

Note: The general reaction condition is given in the text. Only the net rates of O_2 uptake in the presence of a given substrate are presented here.

In the slow freezing experiment, the mitochondrial preparation was cooled to —2°C and then spread on a sheet of ice kept at the same temperature. The unfrozen mitochondria were inoculated with an ice crystal while the temperature was lowered to —5°C. The mitochondrial suspension was thus frozen rapidly as the heat was quickly removed by the heat sink. The sample was kept frozen for one hour and then rapidly thawed, to recover the mitochondrial suspension, and then assayed.

In the fast freezing experiment, the mitochondria were prepared and assayed the same way, except they were frozen in a deep freezer (—10°C) for 24 hours. The above two experiments are not directly comparable because the modes of freezing and the age of the mitochondria are different. In fact, the oxidative

activity of mitochondria is perhaps so sensitive to the mode of freezing that the control of freezing becomes critical for minimizing experimental variations. Nevertheless, the general freezing injury is reflected by the elevated activity of endogeneous respiration and cytochrome c oxidase in the frozen samples indicating ruptured mitochondria resulted from freezing and thawing.

SUMMARY

The freezing injury of isolated barley and wheat mitochondria can be detected by the decrease in titratable SH groups and the abnormal oxidative activity. However the isolated mitochondria deteriorate too rapidly to be used for analysis of freezing stress. Extending the longevity of isolated mitochondria and minimizing the effects of chemicals added to protect mitochondria during isolation will be the focal points and require more extensive experimentation. Eventually, Dr. Olien and I hope to analyze stress energies, as described in the preceding chapter, and coordinate this with analyses of the injury induced in plant membranes.

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BREEDING FOR RESISTANCE

by John E. Grafius[1]

INTRODUCTION

The crown is the most vulnerable area of a small grain plant to winterkill. The loss of upper leaf tissue and root tissue is of little consequence in survival if the meristems of the crown remain intact and undamaged by freezing injury. The meristem region can regenerate both leaves and roots, providing injury has not walled off the viable cells from the surrounding environment and the meristems have not been destroyed.

Most symptoms describing the effect of winterkill on the plant have been published previously. The observation of the central position of the crown in the syndrome winter injury of a small grain plant, however, is a relatively recent discovery by Olien (16) and of great interest to the plant breeder.

LITERATURE REVIEW

The literature abounds with references on winter hardiness. The genetics of winter hardiness in barley has been reviewed recently by Dantuma (3), Rhode and Pulham (21), and more recently by Nilan (15). One of the early reviews and one of the best from the standpoint of integrating personal observations and ideas with the early literature is by Salmon (22). He lists the primary causes of winter injury as 1) heaving, 2) smothering, 3) physiological drought, and 4) freezing of the plant tissue. In his review of the literature, Salmon develops a formal hypothesis of winter injury.

We will be concerned here with the most of the types of winter injury with the exception of heaving. [The readers are referred to a paper by Bouyoucous and McCool (2). These authors found that heaving is caused not so much by the expansion of the soil and the water contained in it as by the formation of ice, either as a solid or as capillary needles or columns, which push the plants upward, breaking and exposing the roots. A recent review on the subject is given by Lamb et al. (11).]

Salmon (22) then develops the necessary background for artificial refrigeration tests of winter hardiness. As he writes, the puzzling effects of genotype by environment interactions begin to emerge. With the post-knowledge of the developments by Dr. Olien, some of the inconsistencies can be explained. Salmon puzzles over the effects of freezing injury in dry and wet soils, and over the relatively rapid rate of loss of winter hardiness when snow cover is removed. These puzzles are similar to those confronting us 10 years ago. As Salmon continues, he is able to show that artificial freezing tests are comparable to field conditions — sometimes! (We are now relatively sure that the "sometimes" is due to differences in amount of moisture in the crown of the small grain at the time of freezing injury.)

This observation carries over into Salmon's discussion of varietal resistance for several localities in the United States.

According to Salmon, "Red Rock and Berkeley Rock are among the most important red wheats in Michigan and are referred to in the literature of the Michigan Agricultural Experiment Station as being very winterhardy. But at the Minnesota Station, with more severe and quite different conditions, Red Rock is nonhardy." Salmon further states, "As would be expected, a relation between cold resistance and adaptation cannot always be clearly established. The hard wheats, for example, are less winterhardy as a class than the soft winter wheats in the Eastern United States. Trumbull, which is one of the leading varieties in Ohio, is relatively nonhardy as judged by artificial freezing tests."

In general, the well integrated and thoughtful discussion of Salmon leads directly into our present position—that of studying water relationships in the plant relative to freezing injury and the genetics of resistances under various conditions of wetness.

Other factors, of course, enter into winter hardiness. Disease reaction is very important. Even small amounts of injury due to diseases such as mildew, can cause violent disruptions in the rate of achieving winter hardiness. Snow molds are also important in areas where continuous snow cover exists. Poor drainage, ice sheets and soil heaving are also important. The very complexity of the problem demands some limitation to the scope of this discussion.

Before leaving Salmon's interesting paper, one more quote should be added, "Because of a secondary effect,

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probably physiological, which appeared in many of the experiments soon after freezing, it was not possible to use the survival of plants as a criterion of injury. Consequently, the relative injury was estimated and recorded in percentages, based roughly on the portion of visible plant tissue that appeared to be killed." From the evidence given by Olien (16), I feel that Salmon was mistaken. The true criteria of winter hardiness must be survival and death is often the result of crown damage.

Based on the crudest analysis, the sequence of events necessary for winter hardiness are as follows: 1) winter habit, 2) disease and insect resistance, 3) cold resistance to the different kinds of winter injury. The first of these is not genetically complex and rather easily dealt with as only a few genes are responsible for winter habit (19, 24).

In the Southern part of the United States crown damage is usually not the most important factor. More importance is attached to actual resistance to cold injury of the leaves rather than crown injury, because these plants are pastured. Abo-Elenein et al. (1) have published a summary paper on this aspect of winter hardiness. Breeding for this kind of resistance would require a different technique than the one discussed here.

The problem of selecting for resistance to equilibrium and nonequilibrium (with respect to water content of the crown) injury is unique and discussed in this section. We will not consider heaving injury here—I suppose mainly because we know so little about it.

One of the most exhaustive papers in modern genetics regarding winter hardiness is by Rhodes et al. (20, 21) and the re-analysis by Eunus et al. (4). These papers deal with an 18 parent diallel. This experiment, conducted at six locations, was plagued with a high degree of genotype by environment interaction. A variety might exhibit dominance with respect to winter hardiness at certain locations, while at others the reverse was true. Evidently, both dominant and recessive genes were in operation, depending on the particular parents and environment involved.

We, too, have experienced such reversals in our experiments. From one year to the next, the same set of varieties has exhibited a correlation coefficient of —.6 with regard to winter survival. This is striking evidence of two different kinds of winterkilling in operation. Thus, results from the diallel experiment indicate that many genes are involved and that winter hardiness is a complex character. Susceptibility to disease and insect predation confuse the issue, presenting a very perplexing problem.

No discussion of winter hardiness and barley variety would be complete without mentioning a bulletin by Wiebe et al. (27). This bulletin summarizes many years of testing different barley varieties in the United States. From it one may conclude that the most winterhardy variety present in the United States must be Kearney or Dicktoo. Even so, the winter hardiness of these varieties is far short of that attained by wheat and especially rye. Wiebe et al. (27) conclude, "In general, the hardiest of the barleys from the world's collection have not been any more hardy than commercial varieties grown in the United States. Only one variety was a foreign introduction among the 30 varieties with the highest relative survival, while 17 were selections from hybrids and 12 were local strains or varietal selections. It appears that increased hardiness can be obtained by breeding and selection methods under environments conducive to differential killing (27)."

We question the last sentence in the foregoing paragraph, not because we know so much about winter hardiness and its inheritance but because we fail to see any evidence of real progress.

EXPECTED PROGRESS IN WINTER HARDINESS

If past history can be our guide then we can expect little or no improvement in winter hardiness using present methods. Winter hardiness data over the last two decades have reported the supremacy of Dicktoo and Kearney and they still top the list. True, we have consolidated our gains with better lodging resistance, much higher yields, better disease and insect resistance, and better quality grain. Thus, the improvement of barley varieties has not remained stationary, but improvement in winter hardiness has. We do not have varieties better than Kearney, or if we do, they are only slightly better. The chance of obtaining winter hardiness in barley equal to that of wheat in this century, using present methods, appears almost nil. This statement is based on evidence published by Rhode et al. (20, 21), Eunus et al. (4), Reid (17, 18, 19), and on our own data.

Having alienated most barley breeders with these statements, some positive statements are in order. Why have breeding methods so successful for other traits failed here? First, we were selecting against a moving background. Part of the problem may be sorted out by improved testing procedures. Careful attention to moisture content of the crowns during test freezing will give more reliable results. Many of the early freezing tests gave good measures of resistance to equilibrium (dry) freezing injury, but generally the only measure of nonequilibrium freezing has been from field data on a hit or miss basis.

Olien (16) developed equipment to measure nonequilibrium injuries, and Marshall (13) and Warnes et al. (26) developed methods which correlate well with winter injury in the northeast. Presumably, these tests measure nonequilibrium freezing injury as this is the most common type of injury in the area.

Secondly, and of equal importance, we should take a long hard look at expected genetic gain for each kind of injury. Evidence for the existence of separate gene systems governing each type of apparent injury can be found in the diallel study by Eunus et al. (4). It is also apparent that polygenic systems are involved. Reported heritability estimates can be misinterpreted to mean that rapid advance is possible. In fact, crosses of resistant x resistant parents have resulted in low heritabilities (20).

Lacking any source of high resistance to winterkill in cultivated varieties, the breeder must develop a scheme of recurrent selection against a constant environment based on somatic resemblance and this program must be accompanied by mild inbreeding and selection. It may well be that without mild inbreeding the program will fail to show much progress. For example, schemes using the male sterile gene in composites under selection for winter stress have not shown the expected progress, even after 6 cycles of relatively severe selection pressure (17). The situation may be confounded somewhat by the lack of a constant environment, but selection was undertaken on the basis of test results over a wide region and the surviving populations were recombined each year. The thought remains, however, that the lack of progress is predicted on the basis of Sewall Wright's (28) paper on assortative mating based on somatic resemblance.

Although we are dealing with a form of mass selection rather than assortative mating, the problem has many of the same features. In a polygenic system where, for example, AAbbcc = aaBBcc = aabbCC etc., assortative mating based on somatic resemblance will do little to fix genes in a homozygous state without some inbreeding pressure. The same holds true for recurrent selection. Given recurrent selection plus mild inbreeding and a constant environment, the chances of progress are greatly improved.

Mild inbreeding can be brought about by restricting the number of lines to be consolidated into the next cycle. Wright (29) presents the formula $\triangle P = 1/8\ M + 1/8\ F$, where $\triangle P$ equals the loss in heterozygosity in each generation and M and F equal the number of male and female parents. If these concepts seem radical, see Chapter 14 of Hayes and Immer (9). When continuous ear-to-row selection in an open-pollinated variety of corn was practiced over an 8-year period and where many ears from the better selections were recombined, no progress was made. When only one ear was used to reproduce the next generation, the yield dropped. The only increase in corn yield due to ear-to-row selection occurred when a composite of only four lines was made. Various reasons are given for the apparent failure of this breeding system. The most logical explanation for this can be deduced from Sewall Wright's (28) paper. The analogy between this and the present case should be apparent.

The animal husbandman has long recognized that both the number of progeny and the generation time are important in expected genetic gain. Michael Lerner (12) gives a lucid discussion of this and we would do well to consider what he has to say. The generation time in a standard breeding program with a cycle time of 10-12 years is too long, especially when the expected genetic gain is low and the distance to cover is great. Plant breeders have used greenhouses and southerly climates to decrease generation time, but more can be done.

I am about to suggest a novel approach, intended for use as a starting point. The breeding system is outlined in Fig. 1. Starting with a panmictic population of winterhardy lines which include the male sterile gene, I propose to pollinate 100 male sterile plants using random bulk pollen from the population.

Part of the seed from each plant will be tested in the laboratory for crown injury under freezing stress. The remainder of this seed will be planted that same fall in individual plots. Reduced planting rates to allow approximate space planting will help to obtain as much seed as possible from the various male sterile plants.

On the basis of the previous season laboratory tests, 25 male sterile plants in each of the four best plots will be pollinated by bulk pollen from all four of the plots. The series will be repeated and part of the seed from each plant will be tested in the laboratory; the remainder of the seed will be planted in individual plots. One added feature is that of the bulk winter hardiness test. If desired, the remnant seed from the four plots of the previous season can be used for replicated tests over several environments in the area to check whether other factors are influencing the winter injury process.

This procedure has all of the desirable features of the old ear-to-row breeding system. It includes the test procedures where, in this case, part of the progeny is examined in the laboratory and part of the seed is planted. Only the selections which show superior characteristics in the lab go on to reproduce the next generation. We have added mild inbreeding to the ear-to-row method.

A. B. Schooler. We need money, dedication and time to realize some of the pragmatic advantages apparent in this research.

Our work at Michigan State University got its start through Dr. Arnold B. Schooler at North Dakota State University. We have first and second backcrosses to *H. vulgare* from the original hexaploid *Hordeum jubatum* x *H. Compressum* sent to us, but as yet we do not have the fertility needed to start a recurrent selection program. Cytological studies of doubled F_2, and of BC1 progenies indicate we are not getting random assortment of chromosomes at meiosis and the reduction from a hexaploid to a diploid through backcrossing may be proceeding at a slower rate than expected. This may prove advantageous in the long run by allowing a longer association between the chromosomes of wild and cultivated species.

Other institutions have more vigorous programs of longer standing and it is reassuring that sooner or later the right tack will be discovered. Until that time we must admit that this too, along with the proposed breeding plan for cultivated varieties, lies in the realm of dreams and conjecture. At present there is no historical evidence that any material advance in winter hardiness in cultivated barley is possible in the near future—nevertheless we are optimistic for the reasons given.

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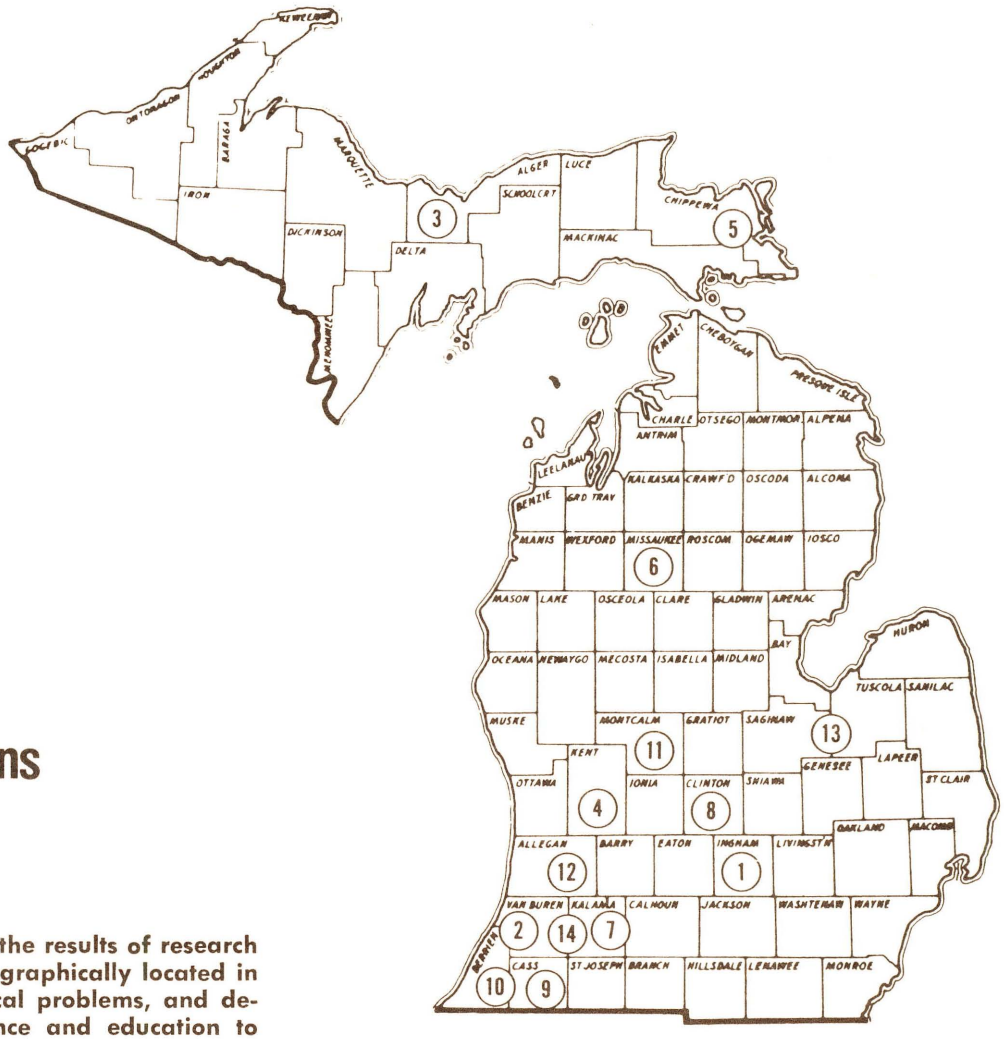

Outlying Field Research Stations

These research units bring the results of research to the users. They are geographically located in Michigan to help solve local problems, and develop a closeness of science and education to the producers. These 14 units are located in important producing areas, and are listed in the order they were established with brief descriptions of their roles.

(1) Michigan Agricultural Experiment Station. Headquarters, 101 Agriculture Hall. Established 1888. Research work in all phases of Michigan agriculture and related fields.

(2) South Haven Experiment Station, South Haven. Established 1890. Breeding peaches, blueberries, apricots. Small fruit management.

(3) Upper Peninsula Experiment Station, Chatham. Established 1907. Beef, dairy, soils and crops. In addition to the station proper, there is the Jim Wells Forest.

(4) Graham Horticultural Experiment Station, Grand Rapids. Established 1919. Varieties, orchard soil management, spray methods.

(5) Dunbar Forest Experiment Station, Sault Ste. Marie. Established 1925. Forest management.

(6) Lake City Experiment Station, Lake City. Established 1928. Breeding, feeding and management of beef cattle and fish pond production studies.

(7) W. K. Kellogg Farm and Bird Sanctuary, Hickory Corners, and W. K. Kellogg Forest, Augusta. Established 1928. Forest management, wildlife studies, mink and dairy nutrition.

(8) Muck Experimental Farm, Laingsburg. Plots established 1941. Crop production practices on organic soils.

(9) Fred Russ Forest, Cassopolis. Established 1942. Hardwood forest management.

(10) Sodus Horticultural Experiment Station, Sodus. Established 1954. Production of small fruit and vegetable crops. (land leased)

(11) Montcalm Experimental Farm, Enrican. Established 1966. Research on crops for processing, with special emphasis on potatoes. (land leased)

(12) Trevor Nichols Experimental Farm, Fennville. Established 1967. Studies related to fruit crop production with emphasis on pesticides research.

(13) Saginaw Valley Beet and Bean Research Farm, Saginaw. Established 1971. Studies related to production of sugar beets and dry edible beans in rotation programs.

(14) Kalamazoo Orchard, Kalamazoo. Established 1974. Research on integrated pest control of fruit crops.

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