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CHILLING INJURY AND MEMBRANE FATTY ACID SATURATION IN IMBIBING AND GERMINATING SEEDS OF PHASEOLUS VULGARIS L.

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CHILLING INJURY AND MEMBRANE FATTY ACID SATURATION IN IMBIBING AND GERMINATING SEEDS OF *PHASEOLUS VULGARIS* L.

By

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ABSTRACT

CHILLING INJURY AND MEMBRANE FATTY ACID SATURATION IN IMBIBING AND GERMINATING SEEDS OF PHASEOLUS VULGARIS L.

By

William David Wolk

Section I

High and low moisture seeds of two Phaseolus vulgaris L. cultivars, 'Tendercrop' and 'Kinghorn wax,' were chilled at 5°C during imbibtion and then transferred to 25°C. Percent germination was scored at the end of 15 days and compared to that of non-chilled controls. Low seed moisture contributed to increased chilling injury in both cultivars. Chilled 'Kinghorn Wax' seeds germinated significantly better than did chilled 'Tendercrop' seeds of the same moisture level indicating that 'Kinghorn Wax' is a more chilling-resistant cultivar than 'Tendercrop.'

Section II

The major phospholipids of the seeds of two Phaseolus vulgaris L. cultivars, 'Tendercrop' and 'Kinghorn Wax,' were determined. The fatty acids of the individual phospholipids from high and low moisture seeds were analyzed and the relative degree of saturation for each phospholipid was determined. 'Kinghorn Wax' had greater relative amounts of linolenic acid and lesser amounts of oleic and linoleic acids than 'Tendercrop.' These differences are indicative of more highly unsaturated phospholipids in 'Kinghorn Wax' and more highly saturated phospholipids in 'Tendercrop.'

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INTRODUCTION

Seeds of many plant species are injured or fail to germinate at temperatures in the range of 0° to 12°C while others germinate and grow satisfactorily at the same temperatures. The earliest attempt to delineate this observation by a modern, scientific approach was in 1778 when Bierkander reported that plants of eight species were killed at a non-freezing temperature of 2°C (Levitt, 1972). A century later, Molisch suggested that low temperature injury sustained by plants above 0°C should be called chilling injury (Erkältung) in order to avoid confusion with that injury incurred by plants at temperatures below 0°C (Erfrieren or freezing injury) (Levitt, 1972). Over the ensuing years, a wide range of plants have been categorized as being either chilling-sensitive or chilling-resistant. As a general rule, chilling-sensitive species tend to be tropical or sub-tropical in origin while chilling-resistant species usually evolved in temperate and sub-arctic regions (Lyons, 1973).

Chilling injury is a function of both temperature and time (Lyons, 1973). The longer a chilling-sensitive plant is exposed to a chilling environment and the lower the temperature, the greater the injury it sustains.

Plants and animals growing in warmer climates tend to synthesize more saturated fats than do identical species in colder

climates (Pearson, Raper, 1927). In 1929, McNair hypothesized that the degree of lipid saturation is a key factor in determining a plant's ability to withstand chilling temperatures. Lewis (1956) narrowed the scope of this hypothesis by suggesting that the degree to which the fatty acids within the phospholipid fraction are saturated is the most crucial aspect in a plant's chilling sensitivity or tolerance. Since that time most of the work attempting to explain the nature of chilling injury has concerned the role played by the plant's phospholipids. From this the Lyons-Raison or membrane phase change hypothesis emerged suggesting that the phospholipids in cellular membranes of chilling-sensitive plants undergo a phase change from a liquid-crystalline state to a solid gel at some specific temperature. The temperature at which the transition occurs is primarily determined by the degree to which the phospholipids' fatty acyl constitutents are saturated or unsaturated. Phospholipids made up of more highly saturated fatty acids undergo the phase change at higher temperatures than do those comprised of more highly unsaturated fatty acids. The metabolic consequences of such an alteration in the physical state of the membrane lead to chilling injury. Thus, highly saturated membrane phospholipids give a plant a greater chilling sensitivity while highly unsaturated membrane phospholipids give a plant greater chilling tolerance.

LITERATURE REVIEW

Phospholipids and Cell Membranes

With the exception of seed oils and cuticular leaf waxes, most cellular lipids are membrane-bound (Kates, 1970). All cellular membranes contain significant amounts of phospholipids. It has even been speculated that phospholipids are unique to biological membranes (Williams and Chapman, 1970).

Today, the most widely accepted conceptualization of membrane structure is Singer's fluid mosaic membrane model (Singer and Nicolson, 1972). In this model, phospholipids are arranged in a bilayer to form a fluid, liquid-crystalline core. Interspersed throughout the membrane are globular proteins. The structure is not static or fixed. Both the lipids and the proteins are free to migrate laterally in two dimensions.

In the past, the phospholipid component of membranes has been viewed as being nothing more than structural. However, recent research has relegated a greater importance to the role played by phospholipids. Evidence now points to the necessity of phospholipids for the proper functioning of several membrane-bound enzyme systems (Cronan and Vagelos, 1972; Yamaki and Uritani, 1974).

Several different types of phospholipids are found in plant cell membranes. The most common are: phosphatidylinositol (PI),

phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG) (Kates, 1970). The requirements and roles for the various phospholipid classes are not fully understood. It has been observed, however, that the phospholipid composition of a given membrane under given conditions is constant (VanDeenen, 1965). Williams and Chapman (1970) suggest that the various phospholipids found in a given membrane may be responsible for the proper physical state of the membrane.

Other possible roles, heretofore unsuspected, have recently been attributed to certain classes of phospholipids. Most notably, PC has been implicated in the process of fatty acid desaturation leading to linoleic and linolenic acids (Roughan, 1975). The 18-carbon fatty acid is desaturated while bonded to PC. Following desaturation, the chain can apparently be transesterified to other phospholipids thus allowing the cell to alter the degree of saturation of its membranes.

Physical Properties of Phospholipids in Membranes

The capillary melting points of anhydrous plant phospholipids are well beyond the range of physiological temperatures (0° to 40°C). For examples, the capillary melting point of PE is 200°C and that of PC is 230°C (Chapman and Wallach, 1968). Strong ionic bonds between the phospholipid's polar head groups account for these relatively high values. Fatty acyl chain length and degree of saturation have no appreciable influence on a phospholipid's melting point (Chapman and Wallach, 1968).

Phospholipids, however, exhibit the phenomenon of mesomorphism. That is, they do not pass directly from a crystalline state to a liquid upon heating. Infared spectroscopy (Byrne and Chapman, 1964), thermal analysis (Chapman and Collin, 1965), and spin label studies (Chapman, et al., 1965) all confirm that phospholipids exist in various thermotropic mesomorphic states within the range of physiological temperatures. These transitions are a result of the fatty acyl chains melting while the polar head groups of the phospholipid remain ionically bound in a crystalline state (Chapman and Wallach, 1968). The term "liquid-crystalline" accurately describes such a structure: a non-polar liquid region enclosed by two layers of crystalline lattice. The properties inherent in this arrangement of lipid molecules allow for membrane flexibility and fluidity and yet maintenance of structural integrity at the same time.

While it is true that fatty acid chain length and degree of saturation do not influence a phospholipid's capillary melting point, these two factors are crucial in determining the temperature at which a mesomorphic phase transition occurs (Tc). The shorter the chain length and/or the greater the degree of unsaturation, the lower the Tc will be (Chapman and Wallach, 1968; Williams and Chapman, 1970).

Using model membrane systems, Lyons and Asmundson (1965) have demonstrated the relationship between the degree of fatty acyl saturation and the Tc. As the mole percent of polyunsaturated fatty acids increases, the Tc decreases. Using phospholipid mixtures containing fatty acid compositions approximating those found in

plants, they found that a change of less than 5% in the total unsaturated fatty acid content could profoundly affect the Tc.

The foregoing discussion only considers the behavior of anhydrous phospholipids. In cell membranes, however, these lipids are in a hydrated state. Hydration of phospholipids leads to a further depression of the Tc due to lyotropic mesomorphism (Williams and Chapman, 1970). Polar water molecules became bound to the phospholipids' polar head groups which "loosen" the crystal lattice and leads to a lowering of the Tc. When the temperature falls below the Tc of a hydrated phospholipid bilayer, the liquid-crystalline core solidifies. The resulting structure is termed a solid-gel. Thus, a biological membrane can exist in two basic states in the range of physiological temperatures. Above the Tc, the membrane is liquid-crystalline and below the Tc it exists as a solid-gel.

The Influence of Temperature on the Phospholipids of Living Organisms

In 1901, Henriques and Hanson reported that the composition of adipose tissue from swine could be altered by environmental temperature. Since then similar observations have been made for bacteria (Cronan and Vagelos, 1972), fish (Knipprath and Mead, 1968), and plants (Canvin, 1965). In every instance, lower environmental temperature has resulted in a greater proportion of unsaturated fats in the phospholipid fraction of the organism.

E. coli shifts its lipid metabolism toward more monounsaturated fatty acids at lower temperatures (Cronan and Vagelos, 1972).

Unsaturated fatty acid auxotrophs supplied only with saturated fatty acids, lyse when moved to lower temperatures (Haest, et al., 1969).

Plants grown in colder climates tend to produce seeds higher in unsaturated fats and oils than do plants of the same species grown in warmer climates (Stumpf and Bradbeer, 1959). Oat grains that develop at 12°C have more unsaturated fats than those developing at 28°C (Beringer, 1971). Flax, rape and sunflower growing at 10°, 16°, 21° and 26.5°C during seed development, produce seeds with more unsaturated fatty acids at the lower temperatures (Canvin, 1965). The same holds true for the chilling-sensitive castor bean (Harris and James, 1969b).

After frost hardening treatments at 13°C, the roots of alfalfa have increased levels of linolenic acid. The increase is greatest for those cultivars that display the greatest resistance to chilling injury (Grenier and Willemot, 1974).

Daffodil bulbs have an increased rate of unsaturated fatty acid metabolism with a decrease in temperature (Harris and James, 1969a).

The phospholipids of winter wheat are more unsaturated when imbibed seeds are incubated for 72 hours at 2°C as compared to 24°C (DeLaRoche, Andrews and Kates, 1973).

Leaves of wheat (DeLaRoche, Andrews, Pomeroy and Weinberger, 1972), rye (Farkas, et al., 1975), and snap bean (Wilson, 1976) increase the degree of unsaturated fatty acids associated with phospholipids when the whole plant is grown at lower temperatures.

Moreover, it is important to note that the more chilling-resistant cultivars have a tendency to produce greater amounts of linolenic acid than the less chilling-resistant cultivars (DeLaRoche, et al., 1972; Farkas, et al., 1975).

On a subcellular level, the fatty acid composition of membranes of various organelles show a greater degree of unsaturation in response to lower environmental temperatures than from plants at higher temperatures. The plasmalemma of soybean roots becomes more highly unsaturated in 48 hours when the temperature is lowered from 30°C to 15°C. Soybean root mitochondria display a similar shift in membrane composition in periods as short as 24 hours (Rivera and Penner, 1978). Mitochondria isolated from wheat grown at 2°C show a marked increase in levels of linolenic acid as compared to plants grown at 24°C (Miller, et al., 1974). Chloroplast membranes from chilling-resistant alfalfa cultivars show a greater increase in polyunsaturates when grown at low temperatures than do the chloroplast membranes from chilling-sensitive cultivars (Peoples, et al., 1978).

In summary, then, whether one looks at the whole plant or at various plant organs such as leaves, roots, seeds or bulbs, or at subcellular organelles, there is a definite metabolic shift toward a greater degree of unsaturated fatty acids associated with membrane phospholipids. Furthermore, those species and cultivars that display greater chilling-resistance also seem to have an enhanced ability in such a metabolic shift. The biosynthesis of linolenic

acid is especially high in chilling-resistant plants under low temperature regimes.

The Effects of Chilling Temperatures and Membrane Phase Changes on Enzyme Systems Associated with Membranes

The rate of enzyme-catalyzed reactions decreases as the temperature decreases. This phenomenon is often described by a temperature coefficient Q_{10}, that is, the factor by which the reaction decreases for every 10°C decrease in temperature. The rate of change can also be predicted by using an integrated form of the Arrhenius equation

$$\ln\left(\frac{K_2}{K_1}\right) = \frac{Ea}{R}\ \frac{1}{T_1} - \frac{1}{T_2}$$

where K_1 and K_2 are rate constants at temperatures T_1 and T_2 (°K), R the universal gas constant and Ea the Arrhenius activation energy. An Arrhenius plot consists of a graph of $\log_{10}K$ vs. the reciprocal of T. For most enzyme-catalyzed reactions, the Arrhenius plot yields a straight line with a slope of Ea/R x 2.3 from which the Ea can be calculated. Thus, for most biological reactions, the Ea value remains constant in the range of physiological temperatures (0° - 40°C). However, membrane-bound enzyme systems in chilling-sensitive plants exhibit a break in the linearity of their Arrhenius plots at a specific temperature in the chilling range. The break is indicative of an abrupt increase in the Ea for that particular reaction (Raison, 1973). Significantly, the temperature at which the sudden rise of the Ea is observed is the same temperature at

which the membrane undergoes a phase change from the liquid-crystalline to the solid-gel state. Moreover, no break in the Arrhenius plot is observed for soluble enzyme systems taken from the same chilling species in the 0° -40°C range (Raison, 1973). Thus, there is a strong implication that a low temperature-induced phase change in the membrane produces a subsequent change in the kinetics of membrane-bound enzyme systems. If so, the membrane itself can be viewed as playing a regulatory role in certain rate controlling processes.

Respiration of intact castor bean seedlings, a chilling-sensitive plant, and oxidation of succinate by isolated castor bean mitochondria show discontinuities in plots of reaction velocities. The soluble gluconeogenic enzymes of the glyoxysomes from the same plants do not display concomitant changes in Ea (Breidenbach, et al., 1974).

Isolated mitochondria from homeothermic rat liver and chilling-sensitive sweet potato each display an increase in Ea for the membrane-bound succinate dehydrogenase, succinate cytochrome c reductase, and cytochrome c oxidase systems (Raison, et al., 1971). Yet malate dehydrogenase, a soluble enzyme from the same tissues, has a constant Ea throughout the same temperature range (Waksman and Rendon, 1971). Returning the chilled mitochondria to a non-chilling temperature within a few hours of chilling reverses the membrane phase back to a liquid-crystalline state (Raison, et al., 1971) and the Ea returns to its non-chill values (Raison, Lyons and Thomson, 1971).

When the growth rate of mung bean is measured as a function of temperature, the temperature coefficient of growth increases abruptly as the temperature falls below 28°C and again at 15°C. Ea values from Arrhenius plots for mitochondrial succinate oxidase activity increase sharply at precisely the same temperatures. Spin label ESR spectroscopy indicates that the mitochondrial and chloroplast membrane lipids of mung bean undergo phase change at 28°C and 15°C (Raison and Chapman, 1976).

Chloroplasts from maize, tomato and bean, all chilling-sensitive species, show a marked increase in Ea for the photoreduction of NADP at 10°C while chloroplasts from two chilling-resistant species, pea and lettuce, have a constant Ea at temperatures down to 0°C. Spin label studies indicate that the chloroplast membranes of the former group undergo a phase change at 10°C while no phase change is observed in the membranes of the latter above 0°C (Shneyour, et al., 1973).

Photosynthesis of chilling-sensitive alfalfa is greatly reduced at 10°C, the same temperature at which its chloroplast membranes undergo a phase change. In chilling-resistant alfalfa cultivars, photosynthesis is not reduced at 10°C and chloroplast membranes, which are much richer in polyunsaturates than the chilling-sensitive cultivar, remain in the liquid-crystalline state (Raison, et al., 1971).

The tricarboxylic acid cycle and oxidative phosphorylation, both mediated to a large extent by membrane-bound proteins, are

disrupted in chilled banana slices. Glycolytic activity, a cytoplasmic process, from the same tissue proceeds unperturbed (Murata, 1969).

When certain metabolic processes such as the TCA cycle are greatly reduced by chilling temperatures and subsequent membrane phase change while other processes like glycolysis continue at optimum rates, imbalances in intermediary metabolites occur. Watada and Morris (1966) have shown that if chilled snap bean fruits are returned to non-chilling temperatures prior to the onset of irreversible injury, respiration immediately increases to levels considerably higher than those of non-chilled fruits. The increased respiration is a result of excessive pools of glycolytic products being burned off by the reactivated TCA cycle enzymes.

Information concerning the effect of temperature on the protein constituents within the membrane system is sorely lacking. The only detailed work at present is that done by Yamaki and Uritani using sweet potato mitochondria. They have removed phospholipids from mitochondrial membranes and have used these lipid-depleted mitochondria (LDM) in their research. LDM chilled for two days retain their capacity to rebind exogenous phospholipids. However, LDM chilled for 14 days show little ability to do so (Yamaki and Uritani, 1973).

The membrane-bound succinate oxidase system in sweet potatoes is irreversibly altered by the membrane phase change encountered at low temperatures. Prolonged chilling irreversibly

changes the protein and phospholipids are subsequently released from the system (Yamaki and Uritani, 1973).

Membrane proteins interact with the hydrophobic region of the membrane. Chilling treatments disturb this relationship and eventually lead to cold denaturation of the protein (Yamaki and Uritani, 1974). The dissociation of phospholipid and protein at the Tc may be dependent upon the lipophilic nature of the protein as well as that of the phospholipid.

Membrane Permeability and Chilling Injury

One of the most striking and immediate symptoms of chilling injury in herbaceous plants is the wilting of leaves. Chilling-sensitive species show signs of wilting within a few hours of being placed in a chilling environment (Simon, 1974). Chilled cucumber leaves lose 75% of their original fresh weight in 48 hours due to water loss (Wright and Simon, 1973). This dehydration is not a result of an increase in resistance of the root to the passage of water (Kramer, 1969).

When chilling-sensitive plant tissue is held at temperatures below the Tc of its membranes, permeability, measured as electorlytic leakage with a conductivity bridge, increases greatly. This allows intracellular substances to pass out of the cell. The increase in permeability is due to a rapid, temperature-dependent phase change in the phospholipid component of the membrane rather than an actual loss of phospholipid (Simon, 1974). The quantity of electrolytic

loss is proportional to the time the tissue is chilled (Wright and Simon, 1973).

Chilling tissue at 100% relative humidity deters the onset of wilting, slows electrolyte loss and forestalls the point of irreversible damage (Simon, 1974). A saturated atmosphere does not, however, alter the temperature at which a break in the Arrhenius plot is observed for previously mentioned metabolic events, nor does it prolong the time before the break can be measured. This evidence suggests that a saturated atmosphere simply acts to slow up the mass flow of water from the chilled tissue rather than preventing membrane permeability.

Seed Germination and Chilling Injury

Seeds of chilling-sensitive plants are themselves subject to chilling injury. In fact, germination of such seeds often represents the most chilling-sensitive stage of growth in the entire life cycle of the plant. When dry seeds are immersed in water or planted in moist soil, a variety of substances begin to leak out of them almost immediately (Simon, 1974). Several researchers have shown a positive correlation between leakage and pre-emergence mortality (Flentje and Saksena, 1964; Matthews and Bradnock, 1968; Hayman, 1969).

The leakage of electrolytes from seeds declines rapidly during the first few hours of immersion at non-chilling temperatures. Larson (1968) has shown that the decline can be extremely rapid. Leakage from pea embryos decreased five-fold during the first ten

minutes of immersion. Seeds that are re-dried once leakage has diminished and then subsequently reimmersed, leak large quantities of electrolytes initially which, in turn, decrease in the same manner as they had during the first immersion (Simon and RajaHaron, 1972).

The substances leaking out of seeds during water immersion include amino acids, glucose, fructose, sucrose, maltose, organic acids, gibberellic acid, phenolics and phosphates (Simon, 1974). These compounds represent such a wide range of various metabolic activities as to suggest a general leakage of cell contents.

The discussion above concerns dry seeds, that is, seeds containing moisture levels that are generally considered acceptable for storage--less than 14% moisture in most instances. Seeds with high moisture levels do not leak nearly as much material as seeds with low moisture levels. Pollock (1969) has shown that only a negligible amount of material leaks from lima bean seeds with 20% initial moisture at 25°C. Other workers have shown similar results with different seeds (RajaHaron and Simon, 1972). It appears, then, that a certain level of seed moisture is required to prevent the leakage of solutes during imbibition. The decrease of leakage in low moisture seeds only minutes after immersion in water may be due to the hydration of the seed's outer layers of cells which, in turn, prevent the inner cells from losing their soluble contents. High moisture seed has sufficient cell hydration prior to imbibition and, as a result, shows no significant leakage.

Pollock (1969) has demonstrated that the temperature of imbibition is an important factor in determining the total quantity of solutes lost during imbibition over a given period of time. Dry seeds of chilling-sensitive species do not show the same rapid dissipation of solute leakage when immersed at chilling temperatures. The seeds, when imbibed at low temperatures (< 12°C) have a much higher initial level of solute leakage than those seeds of chilling-resistant species. The subsequent decline of solutes lost is much slower and never reaches levels as low as chilling-resistant species. High moisture chilling-sensitive seeds, however, follow a pattern similar to that of chilling-resistant seeds with the exception that they consistantly leak slightly more solutes than the chilling-resistant species.

The mechanism of seek leakage is a function of the physical state of the seed's cellular membranes. The structure of the membrane is greatly influenced by its immediate aqueous environment (Simon, 1974). As discussed earlier, the structure of biological membranes is believed to consist of a bilayer of phospholipid molecules arranged so that the polar alcohol moieties face outward into the polar aqueous cell regions with the non-polar fatty acid tails sandwiched in between (Singer, 1972). Luzzati and Husson (1962) have shown that this type of arrangement is completely dependent upon the amount of water in the vicinity of the membrane. When the ratio of water to phospholipid is greater than 20% (approximately ten molecules of water/molecule phospholipid), hydrophilic forces are predominant and the phospholipid molecules are arranged in the manner

described by Singer. When the water content drops below 20%, hydrophobic forces result in a completely different arrangement of phospholipid and water molecules. The sheet-like molecular bilayer of phospholipids is disrupted and the phospholipid molecules cluster around water molecules in hexagonal arrays. Chapman et al. (1967) have confirmed that 20% water content marks the limiting value below which the structure of biological membranes is severely altered. Such a membrane would by definition be extremely porous. Hexagonal channels of water surrounded by phospholipids would exist in place of a membrane. During imbibition there would be a period of time in which intracellular substances would be free to move through the area normally bounded by the membrane. Shortly after the influx of water, a hydrophilic environment would be re-established and there would be a subsequent rearrangement of phospholipid molecules back into the molecular bilayer. At this time there would be a dramatic decrease of solute leakage. Recently Webster and Leopold (1977) have made electronmicrographs of the membranes in dry and imbibed soybean seeds. They clearly show the disrupted and discontinuous state of the plasma membrane and various organelle membranes when the seed is dry. After 20 minutes of imbibition, the plasma membrane is seen as a continuous unit and phospholipid droplets which are visible in regions near the disrupted membrane in dry seeds are no longer present.

Dry seeds of chilling-sensitive plants are most susceptible to injury during the same period that the membrane is undergoing

reorganization. Cotton (Christiansen, 1963), snapbean (Pollack, et al., 1969) and lima bean (Pollack, 1969) are injured after 30 minutes of imbibition at chilling temperature. High moisture (> 20%) seeds in which the water content is great enough so that the membrane has maintained its biological structure, show a marked increase in chilling resistance during the same imbibitional period. In the chilling-sensitive species of soybean (Bramlage, et al., 1978), corn (Cohn and Obendorf, 1978), cotton (Christiansen, 1963), lima bean (Pollock, 1969) and snapbean (Pollock, et al., 1969), imbibitional chilling injury can almost be entirely overcome with sufficiently high initial seed moisture levels. It seems, then, that one difference between the seeds of chilling-sensitive and chilling-resistant plants is in their ability to reorganize the membrane molecules at chilling temperatures.

Although there is no literature that addresses this specific point, one can draw upon the general body of information on chilling injury and speculate as to the mechanism of chilling injury during seed imbibition.

Chilling-sensitive plants are characterized as having a greater proportion of saturated fatty acids in their membranes causing the membrane to undergo a phase change somewhere in the range of chilling temperatures. When dry seeds from such species are imbibed at non-chilling temperatures, one would expect the disorganized membrane to rearrange and become a continuous, functional membrane as described above. However, if the same seeds were to be

imbibed at chilling temperatures, as soon as the phospholipids became hydrated and began to reorganize, they would instantly solidify into a solid-gel. Fluidity would be lost and reorganization would be prevented. The membrane would remain porous and there would be a continuation of cell leakage. Various metabolic functions attributed to membrane structure would be impaired if not completely absent and death of the seed would follow. If, on the other hand, the seed's moisture content was elevated prior to imbibition allowing the membrane to reorganize before chilling, one would expect to see an increase in chilling resistance.

This particular scheme does not preclude the other aspects of chilling injury discussed earlier in this review. One would expect the various metabolic consequences of membrane phase changes to affect the seed in the same way as they affect the other plant parts. It may be that the seed must deal with an additional factor in the problem of chilling injury that other plant parts do not; that of membrane reorganization. If so, it may explain why seeds are often more chilling-sensitive than the rest of the plant from which they originate.

THESIS OBJECTIVES

Evidence strongly suggests that a relationship exists between the physical state of cellular membranes and a cell's ability to withstand the adverse effects of chilling temperatures. The physical state of a membrane at chilling temperatures is greatly dependent upon the degree to which its constituent phospholipids are comprised of unsaturated fatty acids in that a greater degree of unsaturation imparts a greater degree of chilling resistance. This is supported by a wealth of evidence concerning lipid comparisons between chilling-resistant and chilling-sensitive species and between chilling-sensitive species displaying different sensitivities to chilling temperatures. The physical behavior of model membrane systems gives further support to this idea. The purpose of this study was to find two cultivars of a chilling-sensitive species that differ in their ability to withstand chilling temperatures during imbibition and germination and after identifying two such cultivars, to determine if any differences existed in the fatty acid composition of their membrane phospholipids. The results were compared to existing information to see if the findings coincided with the general hypothesis of chilling injury concerning membrane lipid saturation.

SECTION I

CHILLING SENSITIVITY OF PHASEOLUS VULGARIS L., DURING IMBIBITION: CULTIVAR DIFFERENCES AND THE EFFECTS OF INITIAL MOISTURE CONTENT

Introduction

The seeds of chilling-sensitive plants are themselves subject to chilling injury during imbibition and germination (Levitt, 1972). Several researchers have shown that the initial seed moisture content has a great influence on the degree of chilling injury sustained by the seeds of chilling-sensitive crops (Pollock, 1969; Bramlage, et al., 1978; Cohn and Obendorf, 1978; Christiansen, 1963). In general, higher initial seed moisture results in greater chilling-resistance while low seed moisture renders the seed more chilling-sensitive.

Cultivars of a given chilling-sensitive species often display striking differences in their sensitivity to chilling temperatures. These cultivar differences are observed in seedling, mature plants, plant organs, harvested plant parts and at the subcellular level concerning various metabolic activies (Grenier and Willemot, 1974; DeLa-Roche, et al., 1972; Farkas, et al., 1975; Peoples, et al., 1978; Pollock, et al., 1969).

For the purposes proposed in the following experiments, it was necessary to identify two cultivars of a given crop whose seeds displayed differences in chilling-sensitivity during the imbibition and

germination stages of growth. It was also pertinent to determine the degree to which initial moisture content and the temperature of seed moisture adjustment might influence the cultivar's chilling-sensitivity/resistance. Previous work (Pollock, et al., 1969) indicated that the cultivars of garden snapbeans (Phaseolus vulgaris L.), 'Tendercrop' and 'Kinghorn Wax,' might be suitable for such work.

Two separate experiments were used to investigate the chilling sensitivity of these cultivars. In the initial experiment, seed moisture content was adjusted at a single temperature (25°C). In the follow-up experiment, seed moisture content was adjusted at one of three temperatures (5°, 15° or 25°C).

Materials and Methods

Phaseolus vulgaris L., cvs. 'Tendercrop' and 'Kinghorn Wax,' were purchased from Joseph Harris Seed, Inc., Rochester, New York and Ferry Morse Seed Co., Mountain View, California. Care was taken to assure that seeds of each cultivar were obtained from more than one seed lot. Seeds used were from one to two years old and were of high germinability. Laboratory sorting consisted of removing cracked, broken, discolored and extremely small seeds.

To determine the effects seed moisture had on germination at a chilling temperature, seed moisture was altered by equilibration in desiccators at 25°C for 10 to 12 days. High moisture seeds were equilibrated over distilled water. Low moisture seeds were equilibrated over oven-dried $CaSO_4$ (Drierite).

After 12 days moisture content was determined on three replicates consisting of ten seeds each. Seeds of each replicate were

weighed, oven dried at 100°C for 48 hours and reweighed. Moisture content was expressed as percent of fresh weight.

Seeds were planted in ten inch plastic post containing pre-moistened, coarse vermiculite. Units used for chilling treatments were filled with vermiculite, moistened and placed at 5°C for 24 hours prior to planting.

Chilling treatments consisted of subjecting planted seeds to 5°C for 0, 24, 48, or 72 hours. After chilling, the pots were removed to the greenhouse (30° day, 20-25° night) for observation. The number of seeds germinated per pot was recorded for a 15 day period. Each experiment consisted of 2 cultivars x 2 moisture levels x 4 chilling durations = 16 treatments. Each treatment was replicated three times with ten seeds per replicate. The percent germination was determined at the end of the 15 day greenhouse observation period. Data presented here are the means of three separate experiments.

For experiments to determine the effect of temperature during seed moisture equilibration on subsequent germination at a chilling temperature, seeds were allowed to reach moisture equilibrium in desiccators at 5°, 15° or 25°C. High moisture seeds were equilibrated over distilled water. Phosphorus pentoxide (P_2O_5) was used as the desiccant for low moisture seeds. Medium moisture seeds were placed in a desiccator over air. Seeds were equilibrated for 21 days at 5° and 15°C and for 14 days at 25°C. Thus, high, medium and low moisture seed levels were attained at three different temperatures. Moisture content was determined as described above. The seeds were planted in pre-moistened (and pre-chilled when applicable)

vermiculite and either chilled for 72 hours at 5°C and then moved to the greenhouse or planted and moved directly to the greenhouse. As before, germination was observed for a 15 day period and is expressed as percent germination.

Results

For seeds that had been equilibrated at 25°, overall germination decreased as the duration of stress increased for the first 48 hours. After 48 hours, no further injury occurred (Figure 1.1). The initial 24 hours of stress gave the greatest amount of injury to 'Tendercrop' while the second 24 hour period contributed only slightly to the total chilling injury. Germinability of 'Kinghorn Wax' was only slightly reduced by temperature stress (Figure 1.2).

The initial moisture content also contributed significantly to observed differences in germination (Figure 1.3). The decreased germination of low moisture seed was apparent for non-chilled treatments as well as for those placed under chilling stress. After 24 hours of chilling, the low moisture seed had sustained heavy injury. During the same period, high moisture seed was only minimally damaged. Even after 72 hours' chilling, the high moisture seed was still capable of germinating nearly as well as non-chilled low moisture seeds and much better than those of low moisture seeds after 24 hours' chilling.

Germination was lower for low moisture seeds with each cultivar (Figure 1.4). While each cultivar displayed decreased

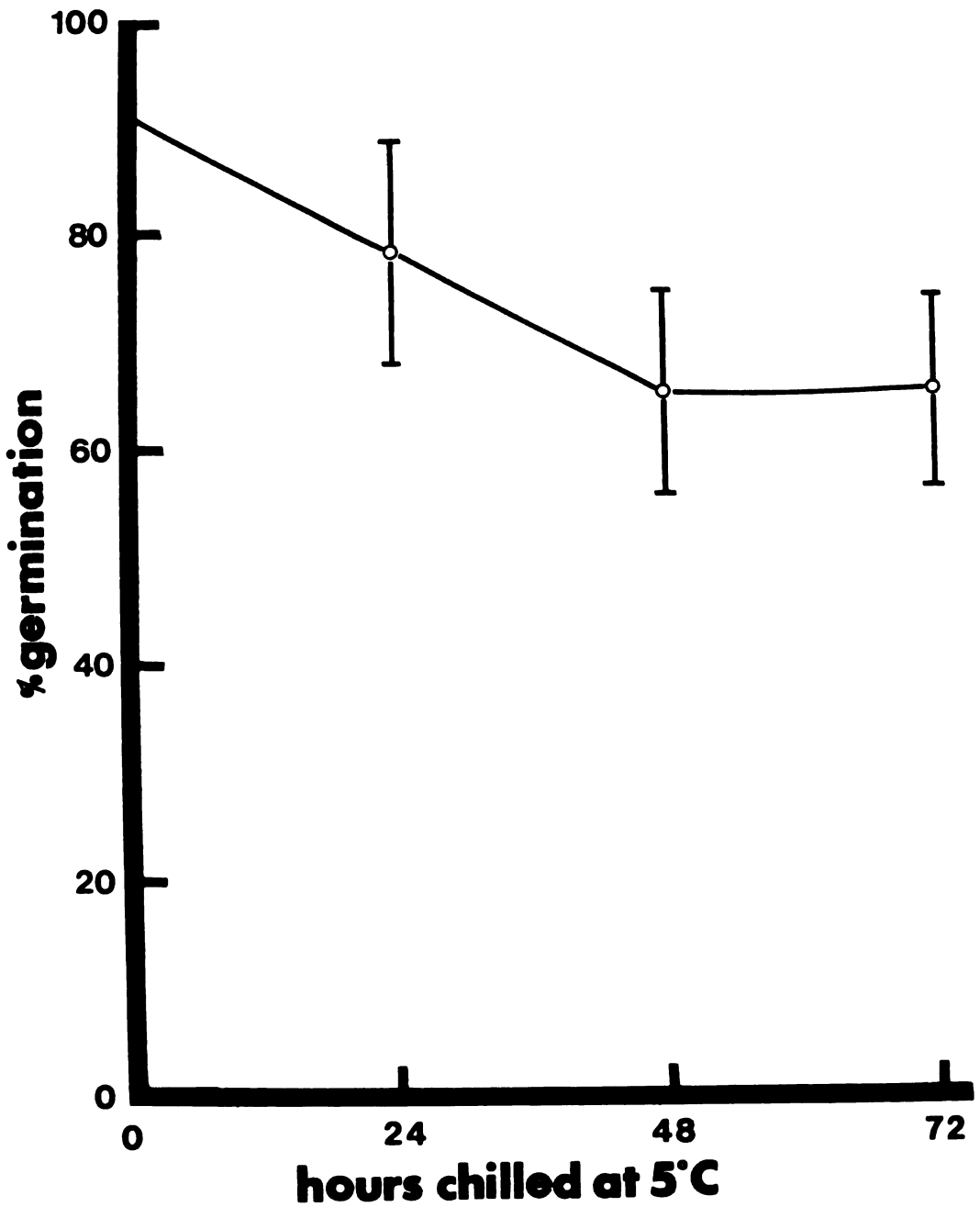

Figure 1.1--Overall germination response of two Phaseolus vulgaris L., cultivars to imbibitional chilling (5°C) for 0, 24, 48, or 72 hours.

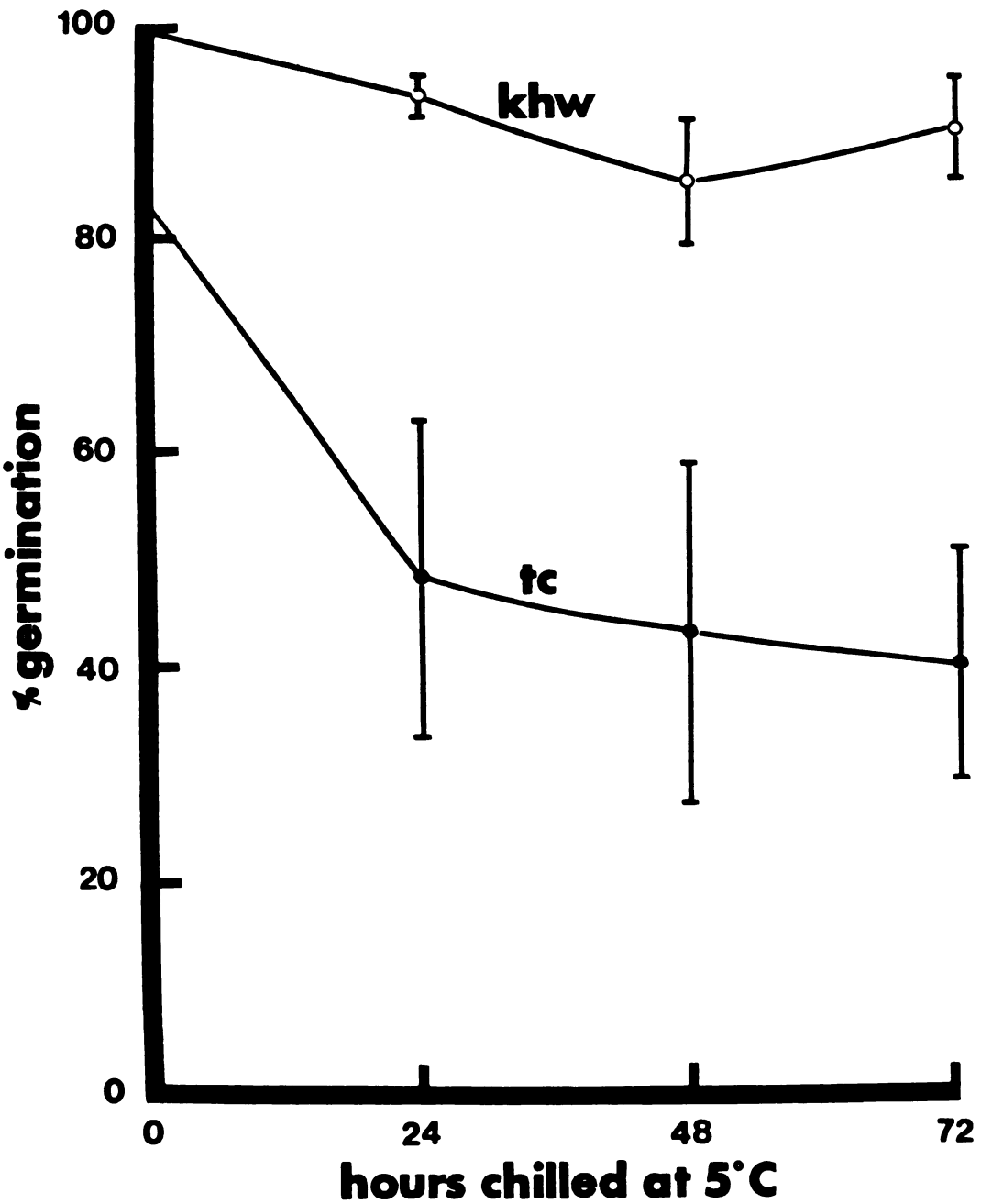

Figure 1.2--Germination of 'Tendercrop' (tc) and 'Kinghorn Wax' (khw) (Phaseolus vulgaris, L.) after imbibitional chilling at 5°C for 0, 24, 48, or 72 hours.

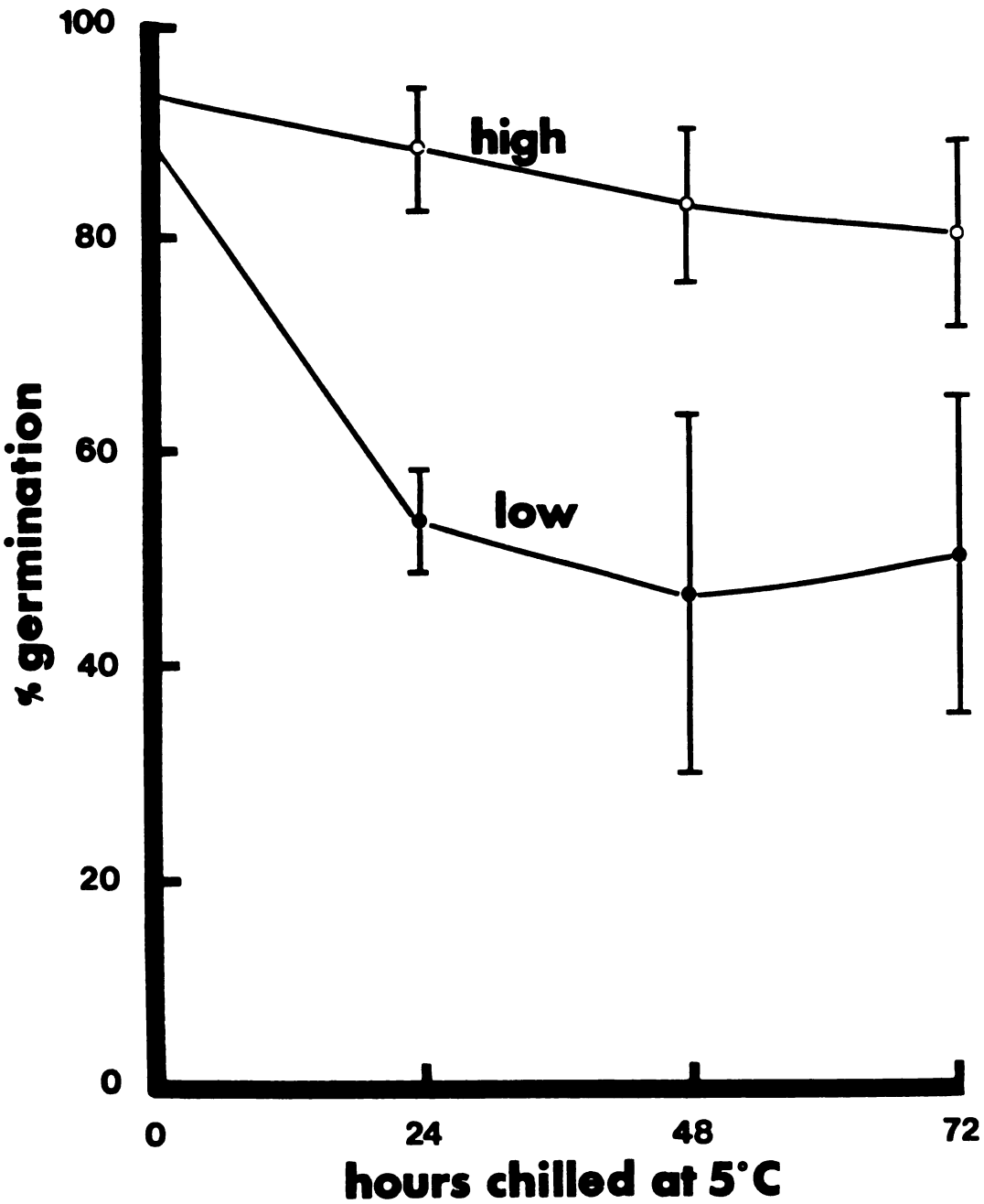

Figure 1.3--Effect of initial seed moisture content on germination of two Phaseolus vulgaris, L. cultivars after imbibitional chilling at 5°C for 0, 24, 48, or 72 hours. high = high moisture (23%), low = low moisture (8%).

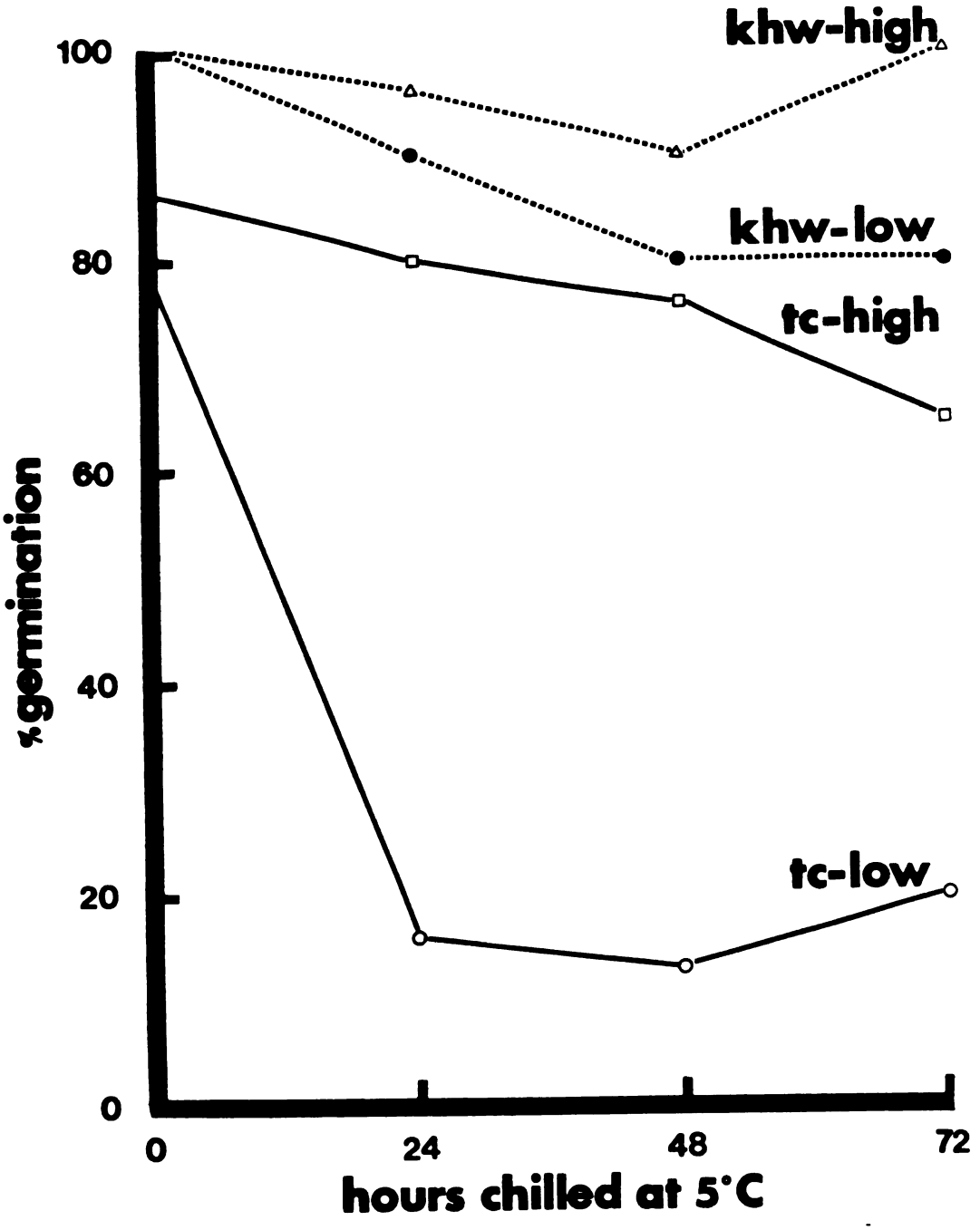

Figure 1.4--Germination of high (23%) and low (8%) moisture 'Tendercrop' (tc) and 'Kinghorn Wax' (khw) seeds after imbibitional chilling at 5°C for 0, 24, 48, or 72 hours.

germination at low seed moisture levels under chilling conditions, the drop is dramatic with 'Tendercrop' and only slight with 'Kinghorn Wax.'

The seed moisture levels reached during moisture equilibrium over water (high moisture), air (medium moisture) or P_2O_5 (low moisture) at 5°, 15° and 25°C are expressed in Table 1.1.

Table 1.1--Seed moisture levels attained over water (high), air (med), and P_2O_5 (low) at 5°, 15° or 25°C.

	'Tendercrop'				'Kinghorn Wax'			
	5°	15°	25°	$\bar{X}$	5°	15°	25°	$\bar{X}$
High	22.8	22.9	18.5	21.4	24.1	24.1	20.7	22.9
Med	12.3	12.7	12.0	12.2	13.3	12.8	14.2	13.4
Low	5.3	4.2	5.3	4.9	6.1	4.5	6.0	5.6
$\bar{X}$	13.5	13.2	12.0		14.5	13.8	13.6	

The results of the subsequent germination tests under chilling or non-chilling conditions are shown in figures 1.5 through 1.8. Statistical analysis of the germination data indicated that in most instances there were no significant differences (LSD, 0.05) in germination caused by the temperature at which the seed's moisture level was altered. In two cases, however, significant differences were detected. These were both found to occur in non-chilled 'Tendercrop' seed brought to moisture equilibrium at 25°C (Figure 1.5). The low moisture 25°C 'Tendercrop' seed had significantly

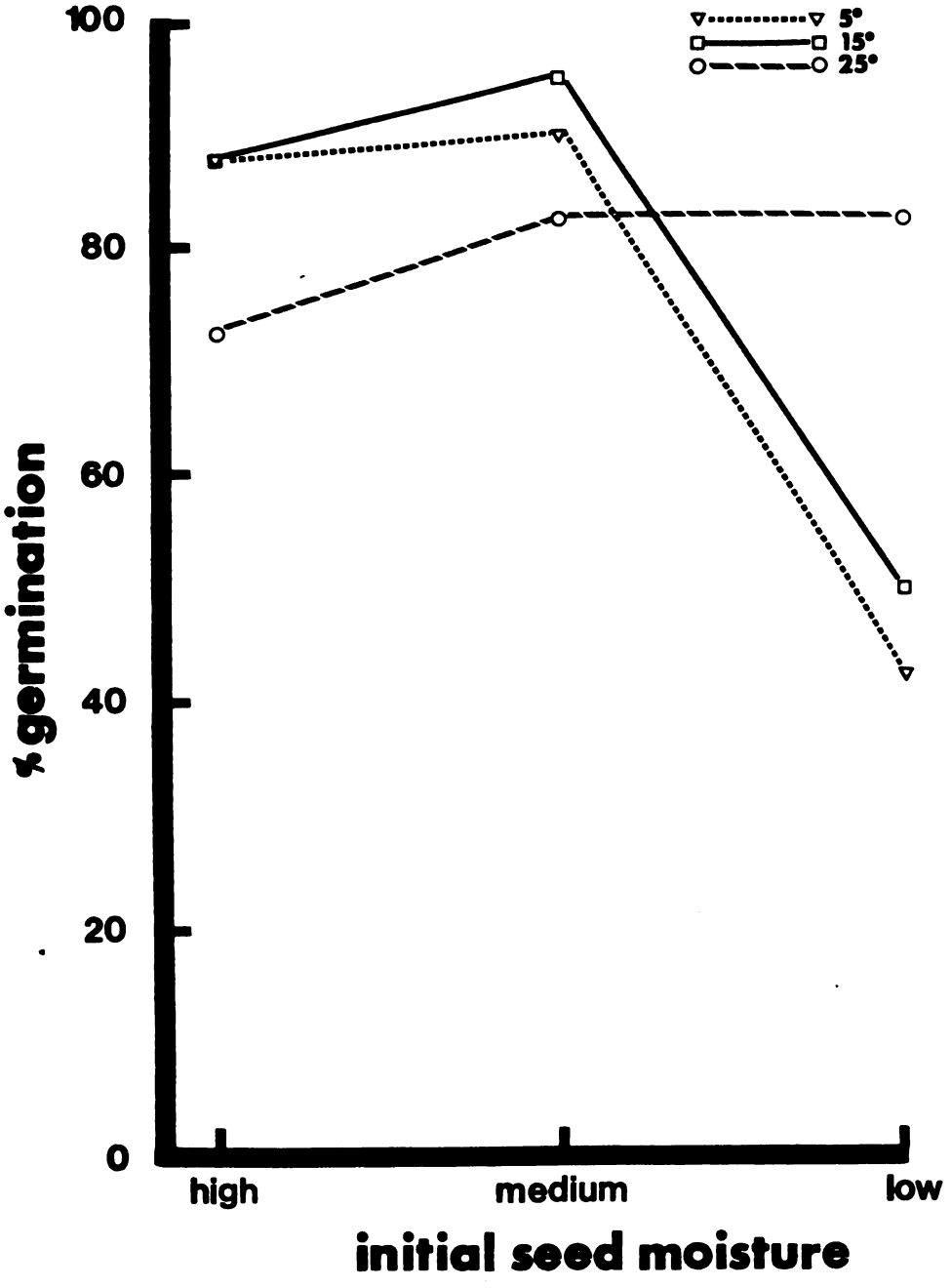

Figure 1.5--Effect of temperature at which seed moisture equilibrium is reached and initial seed moisture content on the germination of Phaseolus vulgaris L., cv. 'Tendercrop' seed. high = 21%, medium = 12%, low = 5% seed moisture.

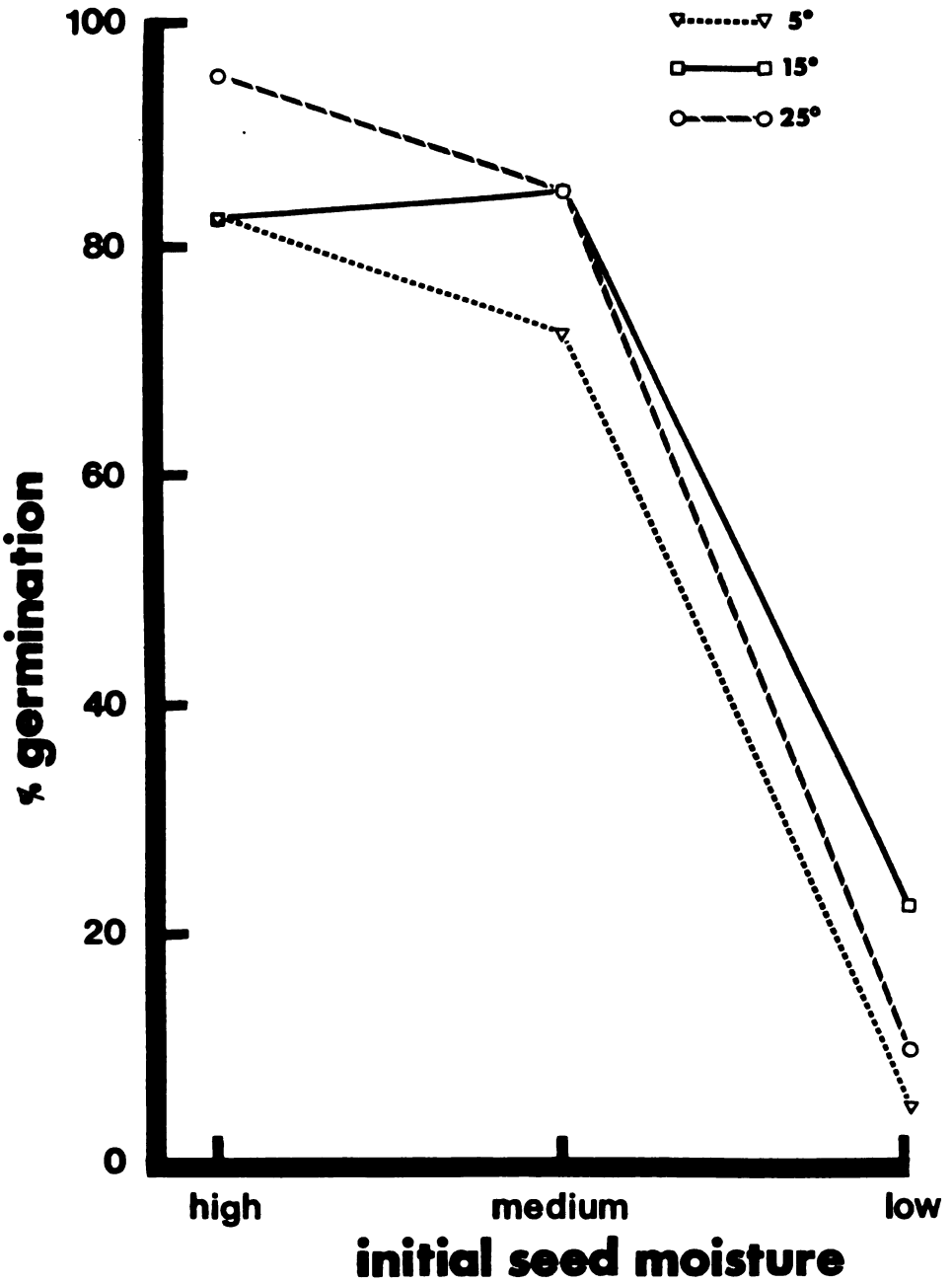

Figure 1.6--Effect of temperature at which seed moisture equilibrium is reached, initial seed moisture content and 72 hours' imbibitional chilling (5°C) on seeds of Phaseolus vulgaris L., cv. 'Tendercrop.' high = 21%, medium = 12%, low = 5% seed moisture.

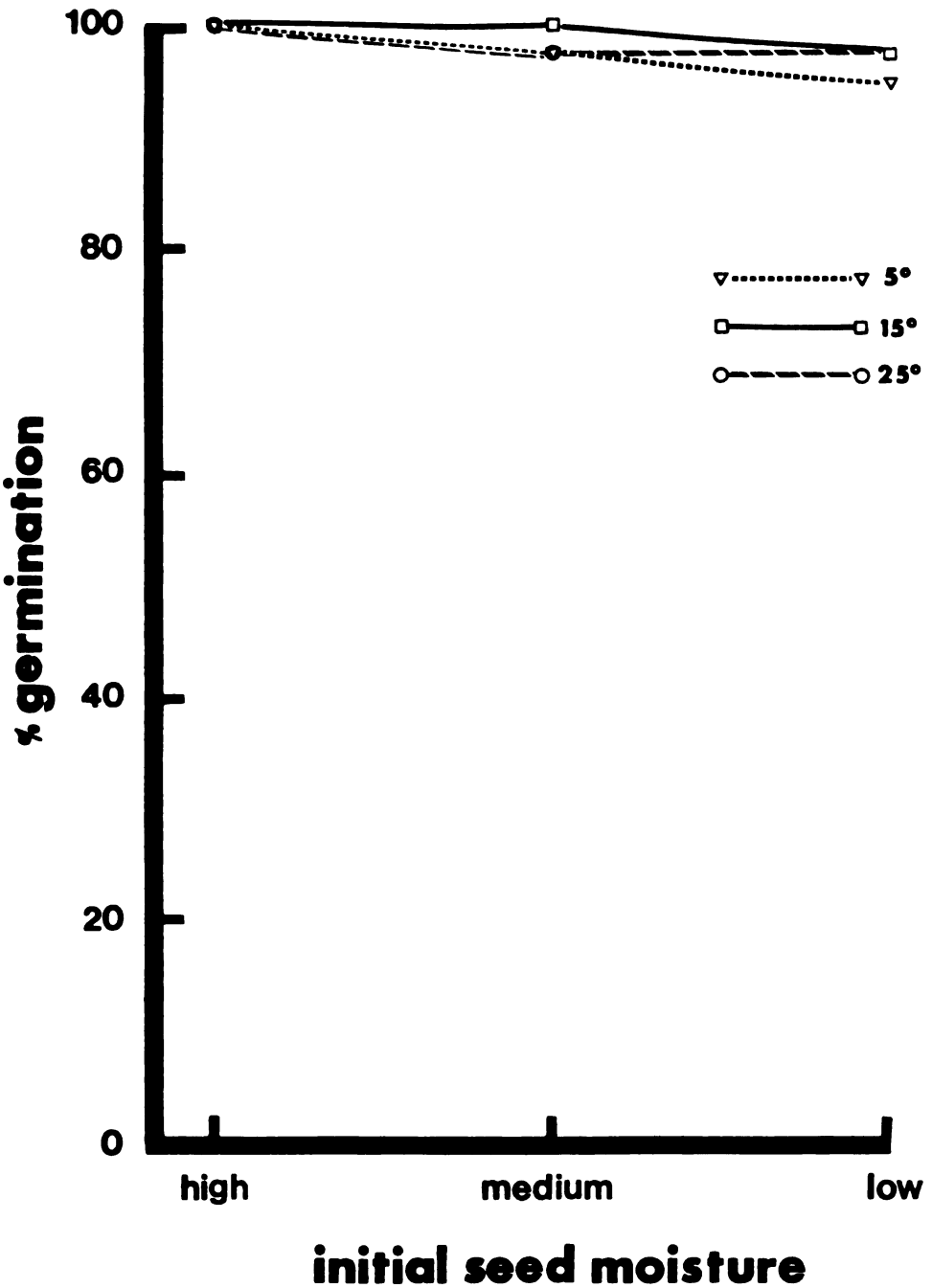

Figure 1.7--Effect of temperature at which seed moisture equilibrium is reached and initial seed moisture content on the germination of Phaseolus vulgaris L., cv. 'Kinghorn Wax.' high = 23%, medium = 13%, low = 6% seed moisture.

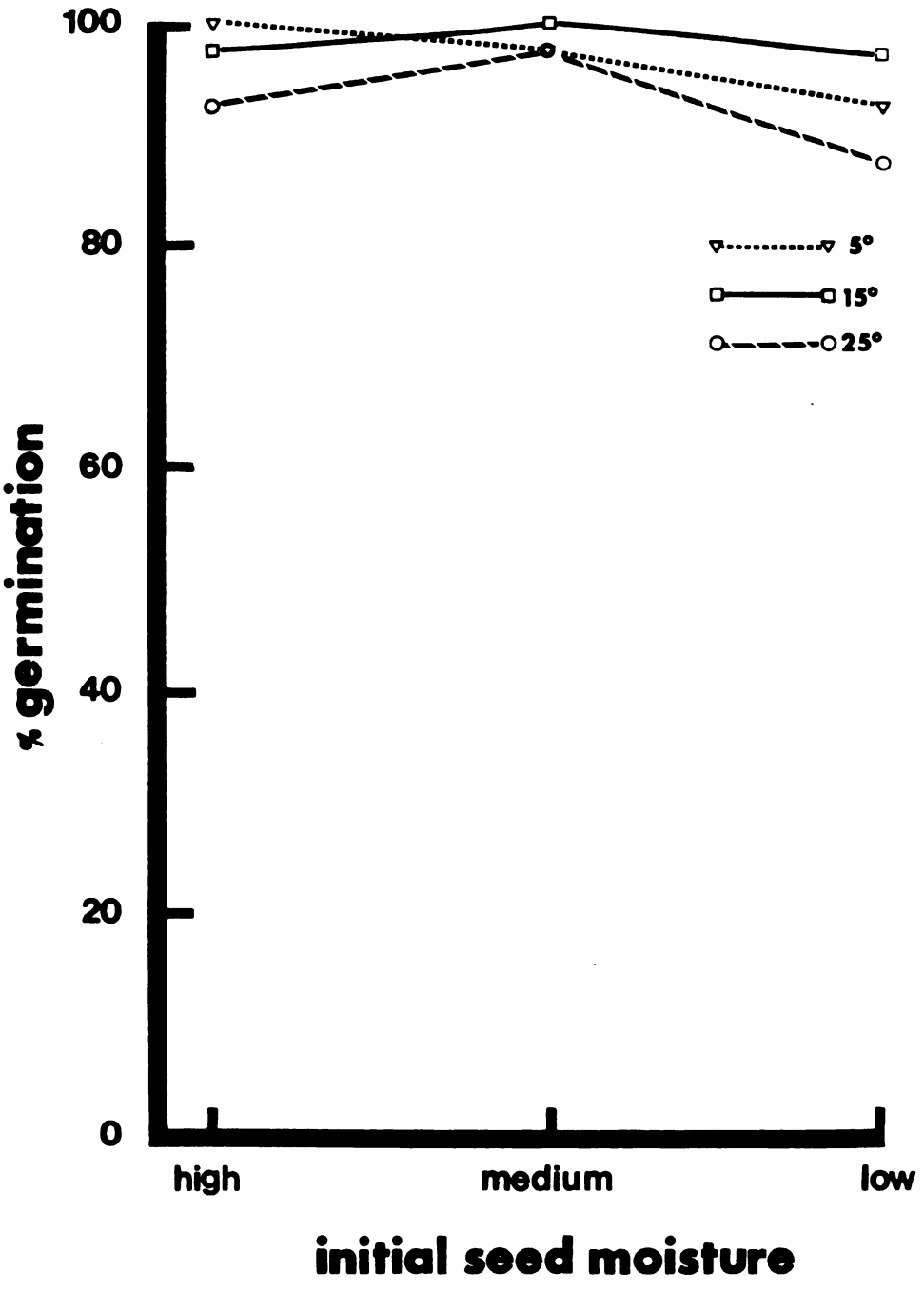

Figure 1.8--Effect of temperature at which seed moisture equilibrium is reached, initial seed moisture content and 72 hours' imbibitional chilling (5°C) on seeds of Phaseolus vulgaris L., cv. 'Kinghorn Wax.' high = 23%, medium = 13%, low = 6% seed moisture.

higher germination than did the seed equilibrated at 5°C and at 15°C. The 'Tendercrop' high moisture at 25°C seed showed significantly lower germination than did the 5° and 15°C treatments. Furthermore, the 'Tendercrop' non-chilled 25°C treatment displayed an overall decrease in germination as the moisture level was increased from low to high.

Discussion

Low temperature stress, initial seed moisture and cultivar all act to determine the seed's ability to germinate.

Phaseolus vulgaris is a warm season crop and sustains chilling injury during germination at low temperatures. The data here coincides with previous reports (Christiansen, 1963; Pollock, 1969; Pollock, et al., 1969) that the bulk of chilling injury occurs during the first hours of imbibition. The results of this study also concur with other work (Christiansen, 1963; Pollock, 1969; Bramlage, et al., 1978; Cohn and Obendorf, 1978) that higher levels of initial seed moisture impart some degree of chilling resistance to the seed during imbibition and germination.

It is clear that 'Kinghorn Wax' germinated better than 'Tendercrop' under chilling conditions. At 5°C, 'Kinghorn Wax' had 94 to 85% germination while 'Tendercrop' ranged between 54 to 48%. These differences still stand when one takes into account the slight differences in germination observed between the two cultivars under optimal conditions. Low moisture seed of both cultivars was more sensitive to chilling than the high moisture seed; 'Tendercrop' was

significantly more sensitive than 'Kinghorn Wax.' It would appear that two different factors involved in chilling injury are responsible for the results. First, initial seed moisture alters the seed's chilling sensitivity and second, a genetically based difference between the two cultivars renders 'Kinghorn Wax' more chilling-resistant than 'Tendercrop.'

Low moisture seed must go through a period of membrane reorganization. During this time intracellular solutes can lead out of the seed and, if extensive, can lead to a weakened seedling or death. Low temperatures would increase the visocity of membrane lipids and thus slow the rate of reorganization allowing a greater period for solute leakage. Because all low moisture seed must go through a period of membrane reorganization regardless of the temperature of imbibition, even non-chilled low moisture seed germinates at slightly lower percentages than non-chilled high moisture seed (Figure 1.3).

High moisture seed negates the effects of membrane reorganization since the membranes of seeds contianing more than 20% moisture would already be present in a continuous, organized state. High moisture 'Kinghorn Wax' seed was capable of 100% germination after 72 hours of chilling temperatures (Figure 1.4). Within a given cultivar at a given level of chilling stress, high moisture seed always had higher germination percentages than low moisture seed. The increased chilling sensitivity attributable to low seed moisture diminishes after 24 hours imbibition and is totally absent after 48

hours (Figures 1.3 and 1.4). This may indicate that the membrane requires between 24 and 48 hours to reorganize at 5°C.

Throughout the experiment, the cultivar 'Kinghorn Wax' had higher germination scores under chilling conditions than 'Tendercrop' (Figures 1.2 and 1.4). This held true regardless of seed moisture content. Low moisture 'Kinghorn Wax' germinated better than high moisture 'Tendercrop' for any given chilling period (Figure 1.4). The differences in germination at 5°C between 'Tendercrop' and 'Kinghorn Wax' cannot be ascribed to seed moisture level. These are cultivar differences and, overall, 'Kinghorn Wax' is a more chilling-resistant cultivar than 'Tendercrop.'

Perhaps the most striking aspect of this study was the response of low moisture 'Tendercrop' seed to chilling stress. After the first 24 hours at 5°C, there was a four-fold decrease in germination (Figure 1.4) for this treatment combination. The three-way interaction of cultivar x moisture level x chilling stress was statistically significant for this experiment. Low moisture 'Tendercrop' seed is an example of such interaction; a chilling-sensitive cultivar made even more sensitive by low seed moisture and then chilled at 5°C. The effect of this particular treatment combination is almost complete chilling injury.

In summary then, three basic conclusions can be drawn from this study. (1) The chilling-sensitivity of the seeds used can be altered to some degree by adjusting their seed moisture content. Increased seed moisture imparts an increased resistance to chilling

injury during imbibition and germination while decreased seed moisture lowers the seed's chilling resistance. The basis for this phenomenon might be due to altered membrane structure in low moisture seeds. Although no cytological study of the membranes of low moisture seeds was undertaken here, there has been sufficient evidence reported by other research groups as cited in the Literature Review of this thesis to warrant such speculation. (2) The seeds of 'Kinghorn Wax' are more chilling-resistant than those of 'Tendercrop.' The causes of these differences cannot be determined from these experiments except to say that they are genetically based. (3) Cultivar, seed moisture level and chilling stress can interact and result in an extremely high level of chilling injury. This is evidenced by low moisture 'Tendercrop' seed.

In studies where seed moisture content was adjusted at different temperatures, seed moisture levels attained at each temperature are fairly comparable to one another. However, it can be seen in Table 1.1 that the high moisture treatment at 25°C for both 'Tendercrop' and 'Kinghorn Wax' did not reach quite the same levels as the high moisture treatments at 5° and 15°C. Seed moisture equilibrium is reached more slowly at lower temperatures than at higher temperatures (unpublished data). Therefore, because seeds deteriorate more rapidly at high temperatures and at high moisture levels (Copeland, 1976), seed equilibration was achieved at various durations depending upon the particular moisture level desired and temperature used. High moisture seeds at 25°C were equilibrated for

the shortest period of all the treatments. It is apparent from the data in Table 1.1 that a more optimum moisture level might have been reached had the seeds been allowed to equilibrate for an additional 24 to 36 hours. However, results from a large number of past trials indicate that the moisture levels reached are sufficiently high enough to produce the anticipated results of high moisture seed.

For the most part no differences in germination were statistically attributable to the temperature at which seed moisture was equilibrated. In two instances, however, differences did occur. These were for high and low moisture non-chilled 'Tendercrop' seeds. Those seeds equilibrated at 25°C scored significantly lower germination percentages at the high moisture level than did the 5° and 15°C equilibrated seeds. On the other hand, non-chilled 'Tendercrop' seeds adjusted to low moisture at 25°C germinated significantly better than did the 5° and 15°C equilibrated seeds (Figure 1.5). Two aspects of these data are most interesting: (1) an increase in moisture level resulted in a decrease in germination; and (2) this occurred under non-chilling conditions. These results were entirely unexpected. Figure 1.6 shows that the 25°C seeds behaved in a similar manner as the 5° and 15°C seeds when chilled. It is possible that the high moisture 25°C seed might have suffered some deterioration during equilibration due to the combination of high humidity and high temperature. However, if this was so, it should also be reflected by a similar decrease in germination in the chilled treatments but such was not the case. Villiers (1974) has discussed the

role of repair enzymes and chromosome stability in their relationship with the extended viability of stored seed. He has suggested that the chromatin of high moisture seed stored at elevated temperatures is challenged by a high incidence of mutagenic events resulting in a large number of chromosome abberations. The repair enzymes of air-dried seed apparently function at a very low rate. Velliers suggests that their activity may, in fact, be suspended. The combination of a high level of mutation and low level of repair necessarily reduces seed viability. Can this account for the observed results in this case? It can be used to explain a decrease in germination as the moisture content of the seed is increased as higher moisture levels support an increased number of mutations. Further, those seeds equilibrating at lower temperatures would not suffer from as many chromosomal aberrations and therefore should display a higher rate of germination. This is seen in the non-chilled treatment. However, as before, if the hypothesis of chromosome aberration and repair enzyme activity is used to explain the observations of the non-chilled material, it must also be applicable to the chilled seed treatments. Once again, it is quite obvious that such a hypothesis does not support the data of the 'Tendercrop' chilled seed nor does it apply to any of the data regarding the 'Kinghorn Wax' seed. The data suggest, the possibility of experimental error aside, that the event(s) leading to these unanticipated results occur during imbibition and germination, not during moisture equilibration. In fact, it does not appear that any reasonable explanation based on current knowledge can be used to explain these results.

SECTION II

DEGREE OF MEMBRANE PHOSPHOLIPID FATTY ACID SATURATION IN *PHASEOLUS VULGARIS* L., CV.'S TENDERCROP AND KINGHORN WAX

Introduction

The Lyons-Raison hypothesis of chilling injury holds that chilling injury in plants is a function of membrane phospholipid fatty acids and the degree to which they are either saturated or unsaturated. The degree of saturation dictates the temperature at which the membrane undergoes a phase change from the liquid-crystalline to solid-gel state. Such a change in the physical state of the membrane leads to disruption of the metabolic activities of membrane-bound enzyme systems and to increased membrane permeability. Chilling injury is a result of the subsequent imbalances in intermediary metabolites, breakdown of compartmentalization and solute leakage and loss.

It has been found that the membranes of low moisture seeds exist in a disorganized and discontinuous state (Leopold and Webster, 1977). Upon hydration, the membrane lipids reorganized into a bilayer capable of the various functions attributed to membrane structure.

The previous study indicated that the seeds of two cultivars of *Phaseolus vulgaris*, 'Tendercrop' and 'Kinghorn Wax,' are inherently

different in their ability to withstand chilling temperatures. 'Kinghorn Wax' is only slightly injured during imbibition at 5°C while 'Tendercrop' suffers appreciable injury during imbibition at the same temperature. Although the study presented in Section I did not undertake to prove that observed differences in germination between high and low moisture seeds are a direct result of disorganized membrane lipids in low moisture seeds, it was noted that such an explanation was not contradicted by the data presented and seemed reasonable especially in light of information from other research presented in the Literature Review section of this paper. Regardless of the cause(s) of the differences in germination between high and low moisture seeds, it was further noted that seed moisture content could not account for the differences in germination at 5°C between the two cultivars, 'Kinghorn Wax' and 'Tendercrop.'

If the Lyons-Raison hypothesis is correct, the phospholipids of 'Kinghorn Wax' should be more highly unsaturated than the phospholipids of 'Tendercrop.' Such differences might account for 'Kinghorn Wax's' greater degree of chilling resistance. The following study seeks to establish whether or not this is os.

Materials and Methods

Seeds of Phaseolus vulgaris L., cv. 'Tendercrop' and 'Kinghorn Wax' were purchased from commercial seed sources. Throughout the course of the experiments, seeds were taken from three different seed lots for each cultivar. The seeds were one to two years old

and of high germinability. Cracked, discolored and extremely small seeds were discarded prior to lipid analysis.

Seed moisture content was altered and determined in the manner previously described.

Lipid Extraction

Twenty seeds were used for each lipid sample. Seed coats were peeled off by hand prior to the extraction. Peeled seeds were kept under N_2 gas as the remainder of each sample lot was prepared. The 20 seed samples were reduced to a fine powder by grinding for one minute with a mechanical grinder (J.K., Inc., West Germany). Lipids were extracted immediately from the powdered seed by the method of Folch, et al. (1957). Each sample was extracted three times with a chloroform/methanol solution (2:1, v/v) at 25°C for 40 minutes. After each extraction, the supernatent was decanted and collected. The three extracts were combined and washed with 0.2 volumes of 0.9% NaCl. The lower chloroform lipid-containing phase was drawn off and the aqueous phase discarded. The lipid solution was washed twice with Folch's solution, chloroform/methanol/water (3:48:47, v/v) containing 0.9% NaCl. The lipid solution was then reduced in volume with a rotary evaporator. Samples were evaporated to complete dryness and redissolved in chloroform. Lipids stored were in a chloroform solution under N_2 gas at -17°C until analyzed.

Thin-Layer Chromatography

Thin-layer plates, 20 x 20 cm x 0.25 mm, were prepared in the laboratory using Silica gel H (E. Merck) containing 7.5%

magnesium acetate. Plates were air-dried overnight prior to use. Twenty-five µl of lipid extract was applied to each plate in a spotting chamber under a N_2 atmosphere. Phospholipids were separated from one another and from other lipids using the two dimensional technique described by Rouser, et al (1969). Plates were developed in the first direction with a solution of chloroform/methanol/58% NH_4OH (65/25/5, v/v) allowing the solvent front to reach a level 1.5 cm from the top of the plate. Plates were removed and dried 30 minutes under a N_2 flow in a TCL developing tank fitted with a cardboard top. Plates were subsequently developed in the second direction with a solvent system of chloroform/acetone/acetic acid/methanol/water (60:80:20:20:10, v/v). After development, the plates were removed to the drying tank and dried for 45 minutes under N_2.

Spot Detection

The phospholipid fraction of the lipid extract primarily contained phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Spots were identified by a comparison of their migration on the plate with those of authentic standards (purchased from Supelco, Inc., Bellefonte, Pennsylvania).

Chromogenic visualizing reagents specific for certain phospholipids were used as a second confirmation. Dragendorff's reagent reacts specifically with PC, ninhydrin reagent with PE and periodate-Schiff's reagent with PI. Periodate-Schiff's reagent also reacts with phosphatidylglycerol (PG) and was used to confirm that no detectable amounts of PG were present. Phospholipid spots

used for fatty acid analysis were reacted with a spray of 0.2% 2', 7'-dichlorofloresin in 95% ethanol and visualized briefly under UV light.

Transesterification

Spots of the three phospholipids PI, PC and PE were scraped from the plate into short segments of glass tubing fitted with a small plug of siliconized glass wool. Phospholipids were eluted from the silica gel with three washings of chloroform/methanol/water (50:50:1, v/v). The eluate was collected in 16 x 125 mm test tubes and brought to dryness under N_2. Two ml of 17% BF_3 in methanol (Supelco) was added to each tube and a glass marble was placed on top of the tube to prevent evaporation of the reaction mixture. Test tubes were then placed in a 40°C waterbath. Over a 45 minute period the temperature of the waterbath was raised to 60°C. At the end of the transesterification process the reaction was stopped by adding 1 ml distilled water to each tube. Two ml hexane was added and the sample was mixed with a vortex mixer for two minutes. After phase separation, the upper hexane phase containing the methyl esters was removed with a pipette and the lower aqueous phase discarded. The hexane was evaporated under N_2 and the methyl esters were redissolved in 60 µl chloroform for GLC analysis.

GLC Analysis

The composition of the methyl ester mixture was determined by gas-liquid chromatography (GLC). Analysis was carried out using a Packard model 805 gas chromatograph equipped with a 6', 2 mm i.d.

column paced with 15% diethyleneglycol succinate (DEGS). Methyl ester peaks were identified by comparison with peaks of authentic methyl ester standards (Supelco). Peak areas were determined by triangulation. The relative concentration of each fatty acid within a given phospholipid sample was expressed as percent by weight.

$$\frac{\text{mg } 16:0}{\text{mg}(16:0+18:0+18:1+18:2+18:3)} = \%16:0 \text{ by weight}$$

Further calculations were made using the percentage values. These were:

The Double Bond Index (DBI),

$$\frac{\%18:1 + 2(\%18:2) + 3(\%18:3)}{100}$$

the ratio of unsaturated to saturated fatty acids (UNSAT),

$$\frac{\%18:1 + \%18:2 + \%18:3}{\%16:0 + \%18:0}$$

and the linoleic to linolenic ration (LINO),

$$\frac{\% \ 18:2}{\% \ 18:3.}$$

Each experiment used two cultivars and two seed moisture levels for a total of four treatments. Each treatment was replicated three times. The fatty acid content of three phospholipids was analyzed for each treatment. the data presented are means of three separate experiments.

An abbreviated experiment was performed as described above with the addition of the antioxident BHT (0.2 mg/l) to all organic solvents used in the extraction, transesterification and GLC analysis procedures. TLC plates were sprayed with 0.02% BHT in chloroform/methanol (2:1, v/v) prior to drying during the TLC procedure. Care was taken to determine that the inclusion of BHT did not interfere with any of the analytical procedures. The BHT included in the transesterification reaction was carried over in the sample used for GLC analysis. BHT has a significantly shorter retention time on the DEGS column under the conditions described above than any of the methyl esters and thus did not interfere with the analysis.

Results

Phosphatidylinositol

Analysis of the composition of PI showed palmitic acid (16:0) to be the most abundant fatty acid present regardless of cultivar or moisture level (Table 2.1). Palmitic acid made up 35 to 40% of the total fatty acid fraction. There were no statistical differences between cultivars or moisture levels in the 16:0 fraction.

There were significant differences (LSD 5%) between the two cultivars for each of the four 18-carbon chain fatty acids (Table 2.2). 'Tendercrop' seeds contained greater relative amounts of stearic (18:0), oleic (18:1), and linoleic (18:2) acids than 'Kinghorn Wax.' 'Kinghorn Wax' had higher relative amounts of linolenic acid (18:3).

Table 2.1--Fatty acid analysis of phosphatidylinositol extracted from high and low moisture seeds of *Phaseolus vulgaris* L., cv.'s 'Tendercrop' (TC) and 'Kinghorn Wax' (KHW). (See text for explanation of "calculated ratios.")

Cultivar	Moisture Level	Fatty Acid* (% by wt)					Calculated Ratios		
		16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
TC	High	39.4	8.6	13.7	21.8	16.5	1.1	1.1	1.3
	Low	35.7	8.8	6.8	25.1	23.6	1.3	1.3	1.1
KHW	High	37.1	5.6	4.3	17.8	35.2	1.5	1.5	0.5
	Low	37.0	8.0	5.6	19.8	29.6	1.2	1.4	0.7
	LSD (0.05)	N.S.	N.S.	5.0	N.S.	11.9	N.S.	N.S.	0.4

KEY: *fatty acids
16:0 palmitic acid
18:0 stearic acid
18:1 oleic acid
18:2 linoleic acid
18:3 linolenic acid

Table 2.2--Effect of cultivar on the fatty acid content of phosphatidylinositol extracted from seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' (TC) and 'Kinghorn Wax' (KHW).

Cultivar	Fatty Acid* (% by wt)					Calculated Ratios		
	16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
TC	37.6	8.7	10.2	23.5	20.2	1.2	1.2	1.2
KHW	37.1	6.8	4.9	18.2	32.4	1.3	1.4	0.6
LSD (0.05)	N.S.	1.4	1.3	3.4	2.8	N.S.	0.1	0.2

See Key, page 49

Table 2.3--Effect of initial seed moisture on the fatty acid content of phosphatidylinositol extracted from seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' and 'Kinghorn Wax.' High = 23% moisture; Low = 8% moisture.

Moisture Level	Fatty Acid* (% by wt)					Calculated Ratios		
	16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
High	38.3	7.1	9.0	19.8	25.8	1.3	1.3	0.9
Low	36.4	8.4	6.2	22.4	26.6	1.3	1.3	0.9
LSD (0.05)	N.S.	N.S.	1.9	N.S.	N.S.	N.S.	N.S.	N.S.

See Key, page 49.

The lone significant difference found between high and low moisture levels (Table 2.3) was in the 18.1 fraction. Here, the high moisture seed contained more oleate than the low moisture sample.

The UNSAT ratio revealed no differences between either the two cultivars or the two moisture levels. However, both the DBI and LINO ratio showed significant differences between the cultivars (Table 2.2). In each case, the calculation for 'Kinghorn Wax' is indicative of a greater degree of unsaturation than that of 'Tendercrop.' The UNSAT, DBI and LINO calculations detected no difference between high and low moisture seeds (Table 2.3).

Phosphatidylcholine

The PC fractions of 'Kinghorn Wax' and 'Tendercrop' seeds showed different relative amounts 18:0, 18:1 and 18:3 fatty acids (Table 2.5). The difference in 18:0 is slight. However, the differences in 18:1 and 18:3 between the two cultivars are quite large. PC from 'Tendercrop' contained 14.7% oleic acid while 'Kinghorn Wax' had only 8.7%. This difference is made up by 'Kinghorn Wax' in the linolenic fraction where it had 30.4% to 'Tendercrop's' 22.2%.

There was no statistical difference in the UNSAT ratio between the two cultivars. Because 'Kinghorn Wax's' fatty acid composition was made up of such a large amount of the triene linolenate compared to 'Tendercrop,' both the DBI and LINO ratios showed 'Kinghorn Wax' to be more highly unsaturated than 'Tendercrop' (Table 2.5). No differences were found in either the fatty

Table 2.4--Fatty acid analysis of phosphatidylcholine extracted from high and low moisture seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' (TC) and 'Kinghorn Wax' (KHW). (See text for explanation of "calculated ratios.")

Cultivar	Moisture Level	Fatty Acid* (% by wt)					Calculated Ratios		
		16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
TC	High	24.0	5.7	14.6	34.3	21.4	2.5	1.5	1.7
	Low	24.3	4.8	14.8	33.0	23.1	2.8	1.5	1.5
KHW	High	23.5	3.9	9.4	31.6	31.6	2.8	1.7	1.1
	Low	28.4	4.0	8.1	30.4	29.0	2.1	1.6	1.1
	LSD (0.05)	N.S.	2.1	6.1	N.S.	N.S.	N.S.	N.S.	N.S.

See Key, page 49.

Table 2.5--Effect of cultivar on the fatty acid content of phosphatidylcholine extracted from seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' (TC) and 'Kinghorn Wax' (KHW).

Cultivar	Fatty Acid* (% by wt)					Calculated Ratios		
	16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
TC	24.2	5.2	14.7	33.7	22.2	2.6	1.5	1.6
KHW	25.9	4.0	8.7	31.0	30.4	2.4	1.6	1.1
LSD (0.05)	N.S.	1.1	1.9	N.S.	3.2	N.S.	N.S.	0.2

See Key, page 49.

Table 2.6--Effect of initial seed moisture on the fatty acid content of phosphatidylcholine extracted from seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' and 'Kinghorn Wax.' High = 23% moisture; Low = 8% moisture.

Moisture Level	Fatty Acid* (% by wt)					Calculated Ratios		
	16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
High	23.8	4.8	12.0	33.0	26.4	2.7	1.6	1.4
Low	26.3	4.4	11.5	31.7	26.1	2.4	1.5	1.3
LSD (0.05)	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

See Key, page 49.

acids or calculated ratios that could be attributed to initial seed moisture (Table 2.6).

Phosphatidylethanolamine

The PE fraction showed differences in fatty acid composition between the two cultivars (Table 2.7). These differences were realized in the relative amounts of oleic (18:1), linoleic (18:2) and linolenic (18:3) acids. 'Tendercrop' contained significantly higher levels of oleic and linoleic acids while 'Kinghorn Wax' had a greater percentage of linolenic acid. These differences in percent values of fatty acids led to differences in the DBI and LINO ratios. In each case, these calculations were indicative of a greater degree of unsaturation in the 'Kinghorn Wax' cultivar. There was no cultivar difference in the UNSAT ratio. No differences in either fatty acid percentage or calculated ratios could be attributed to initial seed moisture level.

Discussion

In each of the three phospholipids analyzed, significant differences between 'Kinghorn Wax' and 'Tendercrop' (LSD, 0.05) were detected in the relative quantities of the 18-carbon chain fatty acids. As a rule, 'Tendercrop's' phospholipids contained more stearic (18:0), oleic (18:1), and linoleic (18:2) acids while 'Kinghorn Wax' always had greater relative amounts of linolenic acid (18:3). The only exceptions to this overall trend were that no differences were detected between the cultivars' levels of oleic acid in the PC fraction and stearic acid in the PE fraction.

Table 2.7--Fatty acid analysis of phosphatidylethanolamine extracted from high and low moisture seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' (TC) and 'Kinghorn Wax' (KHW). (See text for explanation of "calculated ratios.")

Cultivar	Moisture Level	Fatty Acid* (% by wt)					Calculated Ratios		
		16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
TC	High	27.1	4.3	13.6	34.2	20.8	2.3	1.4	1.7
	Low	34.8	4.2	12.0	30.9	18.1	1.5	1.3	1.9
KHW	High	32.1	4.0	6.6	26.3	30.0	1.9	1.5	1.9
	Low	33.1	3.2	6.3	27.2	28.2	1.8	1.5	1.0
	LSD (0.05)	N.S.	N.S.	2.2	5.5	9.5	N.S.	N.S.	N.S.

See Key, page 49.

Table 2.8--Effect of cultivar on the fatty acid content of phosphatidylethanol-amine extracted from seeds of Phaseolus vulgaris L., cv.'s 'Tendercrop' (TC) and 'Kinghorn Wax' (KHW).

Cultivar	Fatty Acid* (% by wt)					Calculated Ratios		
	16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
TC	31.0	4.2	12.8	32.5	19.5	1.9	1.4	1.8
KHW	32.5	3.6	6.4	26.7	29.8	1.8	1.5	1.0
LSD (0.05)	N.S.	N.S.	1.5	3.2	2.2	N.S.	N.S.	0.3

See Key, page 49.

Table 2.9--Effect of initial seed moisture on the fatty acid content of phosphatidylethanolamine extracted from seeds of Phaseolus vulgarlis L., cv.'s 'Tendercrop' and 'Kinghorn Wax.' High = 23% moisture; Low = 8% moisture.

Moisture Level	Fatty Acid* (% by wt)					Calculated Ratios		
	16:0	18:0	18:1	18:2	18:3	UNSAT	DBI	LINO
High	29.6	4.2	10.1	30.2	25.9	2.1	1.5	1.3
Low	33.9	3.7	9.2	29.1	23.1	1.7	1.4	1.5
LSD (0.05)	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

See Key, page 49.

In every case the single greatest difference between the cultivars is found in the linolenic acid content. In general, both cultivars contain equal relative quantities of palmitic acid (16:0). 'Tendercrop' contains 2% to 6% more stearic, oleic, and linoleic acids than 'Kinghorn Wax.' 'Kinghorn Wax' makes up for these cumulative differences by having 10% to 12% more linolenic acid than 'Tendercrop.'

In recent years a wealth of information has been accumulated by a large number of researchers implicating a role of primary importance in chilling resistance, low temperature acclimation and frost hardening to increased levels of linolenic acid. St. John et al. (1975) have shown that inhibition of linolenic acid biosynthesis lowers the chilling tolerance of cotton seedlings. In their study, levels of stearic and oleic acids remained constant in both treated and control plants. However, in seedlings treated with the inhibitor, levels of linoleic acid are significantly higher than control plants. The inhibiting compound effectively blocks the final desaturation creating a large precursor pool of linoleic acid in the treated plants. This alters the LINO ratio to higher values for treated plants.

Other studies have shown that during the acclimatization of wheat seedlings, linolenic acid levels increase at the expense of oleic and linoleic acids (DeLaRoche, et al., 1972). Extremely cold-tolerant rye seedlings increase their linolenic acid content by 63% to 77% during hardening while less cold-tolerant varieties

increase by only 10% to 56% (Farkas, et al., 1975). Dogras, et al. (1977) reported that the linolenate content of phosphatidylcholine increased three to four-fold in chilling-resistant broadbeans and peas during germination at 10 C while no change was observed in linolenic acid content in the chilling-sensitive lima bean during the same period. Leaves of Passiflora species with more chilling tolerance consistently contained more linolenic acid than those less chilling-tolerant Passiflora species (Patterson, et al., 1978). Chilling-resistant alfalfa cultivars contain almost twice as much linolenate than do chilling-sensitive varieties (Peoples, et al. 1978).

In the present studies, the total percent unsaturated fatty acid within each phospholipid type never varied between 'Kinghorn Wax' and 'Tendercrop.' As a result, the UNSAT ratio showed no differences between the two cultivars. However, the distribution of the unsaturated fatty acids varied significantly between 'Kinghorn Wax' and 'Tendercrop.' These differences in distribution led to differences in both the DBI and LINO ratio calculations. Using these two values, differences between the two cultivars were detected in each category of phospholipid. The DBI and LINO calculations add emphasis to the degree of unsaturation. The DBI was first suggested by Richardson and Tappel (1961) and has been used extensively since. It was determined that the LINO ratio might be useful after reviewing various reports of fatty acid analyses involving chilling and non-chilling plant species in which there seemed to be a consistent

trend toward greater relative amounts of linolenic acid in chilling-resistant plants than in chilling-sensitive plants.

While the exact nature of fatty acid desaturation in plant systems is not fully understood and in some quarters remains an area of controversy, there is a general agreement that the major pathway leading to unsaturated 18-carbon fatty acids has a stearic acid intermediate. Oleic, linoleic and linolenic acids represent progressive desaturations from stearate. Thus, a close relationship exists between linoleic and linolenic acid.

It has been shown that seeds tend to produce more highly unsaturated fatty acids when grown under cool climatic conditions (Harris and James, 1969). These polyunsaturates are synthesized at the expense of less highly unsaturated fatty acids (Hilditch, 1956). As the temperature of growth decreases, seeds forming on flax and rape increase their levels of linolenic acid dramatically (Canvin, 1965). Wheat and rye seedlings increase levels of linolenic acid at the expense of oleic and linoleic acids during low temperature hardening (Farkas, et al., 1975; DeLaRoche, et al., 1972, 1973). Calculating a LINO ratio from the data reported by Dogras, et al. (1978) shows that the value dropped in both chilling-sensitive and chilling-resistant species after six days at 10°C. The chilling-resistant species; LINO value decreases much more than do the chilling-sensitive species.

While these examples are derived from the dynamic process of low temperature hardening during which time the plants' metabolic

activities are functioning at a high rate as compared to the minimal metabolic rate of the dormant seeds used in our studies, they all point to a connection between cold tolerance and increased levels of linolenic acid. These reports also bear out the intimate relationships between the relative levels of the 18-carbon fatty acids. Peoples, et al. (1978) have shown that chloroplast membranes isolated from alfalfa cultivars differ greatly in their respective levels of linolenic acid. Those varieties with higher quantities of linolenic acid are invariably more chilling-tolerant than those with lesser amounts. In all of the reports reviewed, LINO ratios calculated from published data showed chilling-resistant species to have lower LINO values than chilling-sensitive species. Therefore, we have determined that the LINO ratio may prove to be a valuable insight into the relationship between fatty acid composition and chilling tolerance.

The question naturally arises as to the cause of the differences in LINO values found between 'Kinghorn Wax' and 'Tendercrop.' Because seeds forming at lower temperatures have greater amounts of unsaturated fatty acids, might not the differences observed here be due to differences in field temperatures during the period of seed development rather than to inherent differences in chilling tolerance? In order to avoid such a possibility, seeds used in our analyses were purchased from two different sources. Seeds from at least two different seed lots representing different years' production were obtained from each source for each cultivar.

The results of the fatty acid analyses of the seeds from each source and seed lot were always consistent with the findings from seeds of different sources and lots. The differences between cultivars were constant, regardless of source or lot.

The inability to detect differences in the UNSAT ratio is interesting. Lyons and Asmundson (1965) have reported a strong correlation between the UNSAT value and chilling sensitivity. Generally, chilling species have UNSAT ratios below 2.5 while chilling-resistant plants have a value of 2.5 or above. 'Tendercrop's' UNSAT ratio is 1.93 and 'Kinghorn Wax's' is 1.87. Lyons' findings apply to interspecies comparisons. One of the underlying purposes of the experiments reported here was to make an intraspecies comparison in order to determine if the differences found between chilling and non-chilling species might exist between cultivars of the same species that display different levels of tolerance to chilling. Phaseolus vulgaris is a chilling-sensitive species and the UNSAT ratio for each of the two cultivars falls well within the bounds of chilling-sensitive plants as defined by Lyons. It may well be that the UNSAT ratio is too gross a measurement for such fine differences existing on the intraspecies level. The DBI and LINO values, based as they are on the relative degree of saturation, have a tendency to magnify and define these small differences. From this perspective, then, it should not seem unusual that no differences exist between 'Tendercrop' and 'Kinghorn Wax' in their UNSAT ratios while the same data give cultivar differences in the DBI and LINO

values. If anything, these findings emphasize the importance of the relative distribution of unsaturated fatty acids in a given membrane and not simply on a measurement like total unsaturates.

Phosphatidylcholine was the most highly unsaturated phospholipid in both 'Kinghorn Wax' and 'Tendercrop.' Roughan (1975) has shown that once stearic acid is desaturated to oleate, the oleic acid chain is esterified to a molecule of phosphatidylcholine. Further desaturations to linoleic and linolenic acids occur while the chain is a part of the phosphatidylcholine molecule. When the desaturation is completed, the diene or triene chain can either remain on the molecule or be transesterified to another glycerolipid. It has been demonstrated that the ratio of phosphatidylcholine to phosphatidylethanolamine rises dramatically in chilling-resistant plants undergoing low temperature hardening (Dogras, et al., 1977). This may be due to the increased requirement for polyunsaturated fatty acids.

The analysis of phosphatidylcholine shows 'Tendercrop' to have significantly more stearic and oleic acids than 'Kinghorn Wax' while the latter contains much higher relative amounts of linolenic acid. Thus, 'Kinghorn Wax' not only contains highly unsaturated phosphatidylinositol and phosphatidylethanolamine fractions but also has a large pool of linolenic acid previously synthesized and ready to be transferred to other membrane lipid molecules.

In only one instance was a difference in fatty acid composition found to change with moisture level. High moisture seeds

contained slightly more oleic acid in the phosphatidylinositol fraction. This difference was not great enough to cause any significant difference in either the UNSAT, DBI or LINO values.

The results of this study support the Lyons-Raison hypothesis. The phospholipids and thus, membranes, of the chilling-resistant cultivar 'Kinghorn Wax' are more highly unsaturated than the phospholipids of the chilling-sensitive 'Tendercrop' cultivar. It follows that 'Tendercrop's' membranes will have a higher transition temperature than the membrane of 'Kinghorn Wax.' The present study did not undertake to determine the transition temperatures of either cultivars' membranes. Therefore, it cannot be stated that 'Kinghorn Wax' membranes remain in a liquid-crystalline form at 5°C while 'Tendercrop' membranes have become a solid-gel.

The combined results of both Sections I and II can be plausibly interpreted using the Lyons-Raison hypothesis concerning the relationship between membrane fatty acid saturation and chilling sensitivity in concert with the knowledge that the membranes of dry seeds must go through a period of membrane reorganization immediately upon imbibition. Each species and, as in this case, intraspecies cultivar exhibits a unique combination of saturated and unsaturated membrane fatty acids and will thus exhibit a unique temperature at which the membranes undergo a phase change from the liquid-crystalline to solid-gel state. Because 'Tendercrop' suffered considerably more chilling injury than 'Kinghorn Wax,' regardless of moisture level, when chilled at 5°C, the more highly saturated

membrane lipids of 'Tendercrop' might solidify at some temperature above 5°C. 'Kinghorn Wax's' greater degree of phospholipid unsaturation may result in a transition temperature somewhat lower than 5°C. If this was so, 'Tendercrop's' membranes would have solidified during the chilling treatments while 'Kinghorn Wax's' would have remained fluid and physiologically functional.

When the concept of membrane disorganization in low moisture seeds is imposed upon the Lyons-Raison hypothesis, a speculative yet reasonable model for the mechanism of chilling injury in seeds might be suggested as follows:

'Kinghorn Wax' seeds may be protected from chilling injury by having highly unsaturated membranes that exhibit a low transition temperature. High moisture seeds of this cultivar have intact, fully organized membranes at the outset of imbibition and, as a result, do not suffer the loss of intracellular solutes. Low moisture 'Kinghorn Wax' seeds must go through a period of membrane reorganization during which time solutes may be lost resulting in some injury. If the degree of unsaturation is sufficient, 'Kinghorn Wax' seed would suffer no injury due to membrane phase transition. 'Tendercrop' membranes may be highly enough saturated to undergo a phase transition at 5°C or above. If so, membrane lipids in both high and low moisture seed would be in a solid-gel state during imbibition and germination at 5°C. The deleterious metabolic consequences of low temperature membrane phase transitions have been discussed earlier in this thesis. High moisture 'Tendercrop' seed would have structurally functional membranes and be protected from injury due to

solute leakage. Low moisture 'Tendercrop' seed membranes would start the imbibition process in a disorganized, highly permeable state. If 5°C is sufficiently low enough a temperature to induce a phase transition in this cultivar's membrane lipids, the membrane components of chilled seeds would lose their liquid mobility and could not properly rearrange during the influx of water. The membranes would be arrested in a disorganized state. Thus, the metabolic activities associated with membrane structure would be absent and the leakage of intracellular solutes would never be halted. The combination of these two adverse situations appear to act synergistically and result in almost total injury in the case of 'Tendercrop' seeds.

This model, then, proposes the possibility of two different types of injury occurring during the imbibition period. One might be called "low moisture" injury. Such injury is not dependent upon temperature but is strictly a function of membrane structure as determined by seed moisture content and the time required for membrane reorganization during imbibition. The other type of injury is true chilling injury that occurs as a result of a membrane phase transition and the range of metabolic consequences attendant to such a transition. Low moisture 'Tendercrop' seed represents an example of interaction between these two types of injury.

If the hypothesized model is correct, two results of a fatty acid analysis of high and low moisture seeds could be predicted: (1) 'Kinghorn Wax' seeds contain greater relative amounts of

polyunsaturated fatty acids than 'Tendercrop;' and (2) the initial seed moisture level should have no influence on the membrane's fatty acid composition. The results of this study tend to bear these two predictions outs. 'Kinghorn Wax' and 'Tendercrop' membranes both contain approximately equal amounts of unsaturated fatty acids. The distribution of the unsaturates, however, is such that 'Tendercrop' contains higher levels of oleic and linoleic acids while 'Kinghorn Wax' has more linolenic acid. The result is that 'Kinghorn Wax' membranes are, in fact, more heavily unsaturated than 'Tendercrop's.' The DBI and LINO ratio values are indicators of this basic difference in membrane composition.

While the differences in saturation were consistently found between cultivars throughout the various phospholipid classes analyzed, in only two instances were fatty acid differences attributed to differences in moisture level. Though one cannot completely disregard these differences, they were quite minor and did not contribute enough weight to cause any differences in the UNSAT, DBI or LINO ratios in the cases where they occurred. One possible explanation for such differences may be due to the increased metabolic rate of the high moisture seed which is better able to comply with the requirements for increased desaturase activity when initially confronted with chilling temperatures. Regardless of the exact nature of such differences, however, changes in seed moisture levels resulted in only slight differences in membrane fatty acid composition at best. The overwhelming bulk of data from this study point

to fundamental differences in membrane composition between the two cultivars, not between seed moisture levels.

These results do not confirm the proposed model but they do support it. The predictions of membrane composition based on the model were generally borne out by the membrane analysis. These findings are also congruent with the vast majority of work in this area which correlates chilling sensitivity/tolerance with the degree of membrane saturation/unsaturation. 'Tendercrop,' the chilling-sensitive cultivar, is more highly saturated than 'Kinghorn Wax,' the chilling-resistant cultivar.

To date there are no data that contradict or refute the Lyons-Raison hypothesis. Yet, it must be noted that after 20 years of concerted effort on the behalf of numerous workers representing research institutions from around the world, no one has been able to definitively demonstrate that a decrease in the degree of membrane saturation imparts a greater resistance to chilling temperatures by itself. For this reason, there have been suggestions that the chilling phenomenon might be determined by factors other than membrane saturation or by some combination thereof. Yamahi and Uritani (1973) have turned their attention to the hydrophobic interactions between membrane lipids and membrane-bound proteins. Plant sterols may depress the membrane's Tc (Chapman, 1968) and act to increase a plant's chilling resistance.

Several researchers have shown that abscisic acid can ameliorate the effects of chilling (Rikin, et al., 1975; Rikin, et

al., 1976). On the basis of such findings, some have proposed a role of ABA in chilling. It would seem, however, based on the majority of information available at this time, that ABA exerts its influence on secondary and tertiary effects of chilling, that is, on the symptoms of the injury and not on its primary cause. The point remains though, that other avenues of approach are being investigated.

All work on the primary cause of chilling injury is not centered around the degree of membrane saturation. The shift from this focal point stems not so much from the Lyons-Raison hypothesis breaking down as it does from the frustration of not being able to provide more conclusive evidence today than was available 15 years ago. McNair gave a general body to the hypothesis in 1929 and Lyons formalized it in 1965. The idea is fairly simple and from a naive standpoint would seem easy enough to test. This, however, has not proven to be the case. Comparative lipid analyses, respiratory and membrane permeability studies and metabolite evaluations abound in the literature. All tend to support the basic hypothesis and bolster the confidence of those individuals working in this area. But still, we remain fixed in the same spot in terms of understanding the true nature of chilling injury.

Does a change in the degree of membrane phospholipid saturation alter a plant's chilling-sensitivity? St. John and Christiansen (1975) were able to show that inhibiting linolenic acid synthesis decreased the chilling-tolerance of cotton. Can we demonstrate that an increase in polyunsaturates will increase the chilling-tolerance

in and of itself? To date, the only methods used to increase the levels of unsaturate phospholipids have been by exposing the plant to low temperatures. Plants respond to low temperature with a host of adaptive measures thus making it difficult, if not impossible, to single out one factor for study. A method that would allow one to increase membrane polyunsaturates without exposure to low temperatures might provide a valuable insight into the problem.

Lipid analyses evaluate the end products of lipid metabolism without regard for the various factors that might contribute to such production. Another approach to the chilling injury problem might be to take a closer look at the desaturase systems in chilling and non-chilling species. Do chilling-tolerant plants have greater amounts of the enzymes responsible for desaturating fatty acids than chilling-sensitive plants? What are the kinetics of various plants' desaturase systems in response to changes in temperature? Answers to such questions need to be provided as the next logical step toward understanding the mechanism of chilling injury.

Concerning the Use of BHT

The results (not included) of the experiment in which BHT was employed as an antioxidant were essentially identical to those reported here where no antioxidant was used. Statistically significant differences found in experiments without BHT were the same as those determined with BHT. This included differences between cultivars within a given fatty acid type and in the calculated UNSAT, DBI and LINO values. However, overall UNSAT and DBI values were

slightly higher and the LINO value slightly lower in the BHT experiment than overall calculated values without BHT. This is indicative of a greater relative degree of unsaturation measured with BHT. Because of the painstaking care in keeping all samples away from oxidizing atmospheres and the favorable comparison between the results from experiments with and without BHT, confidence can be placed in the data presented in this report. A great deal of information concerning fatty acid unsaturation has been generated in the past without the use of antioxidants and such work will most probably continue on into the future. The methods described in this report are valid and reliable and have been used in lipid research for many years. However, use of antioxidants may greatly facilitate this type of experimentation. In the recent past a relatively large body of information has become available concerning the use of antioxidants such as BHT and BHA in almost all phases of lipid research. The ease and simplicity with which these antioxidants can be employed and the degree of protection they afford against sample degradation will seem a godsend to the experimenter who has often been frustrated by the autooxidation of lipid samples.

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