This is to certify that the

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Alleviation of imbibitional chilling injury in snap bean (Phaseolus vulgaris) seeds

presented by

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ALLEVIATION OF INSECTICIDAL CHILLING INJURY IN

SNAP BEAN (*Phaseolus vulgaris*) SEEDS

By

EDWARD A. OAKLEY

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

ALLEVIATION OF IMBIBITIONAL CHILLING INJURY IN SNAP BEAN (Phaseolus vulgaris) SEEDS

By

Rufaro M. Makoni

Coating snap bean seeds with antitranspirants, Vapor Gard, Wiltpurf, and a postharvest fruit wax, Pacrite 383, reduced imbibition at 5, 10, and 20C. These waxes also reduced damage to cotyledons and axes and improved germination of imbibitionally chilled snap bean seeds in pots. Raising the initial moisture content to 15-20% and 25-30% from 6-9% reduced damage to axes and cotyledons of snap beans chilled during imbibition. Germination of snap beans chilled 24 hours during imbibition was improved by initial moisture contents greater than 15%. Polyethylene glycol solutions of -5 and -15 bars reduced water uptake in the first four hours of imbibition at 5, 10 and 20C. Priming solutions of -5 and -15 bars also reduced damage to cotyledons and axes and improved germination of seeds chilled during imbibition. Assessment of damage by both staining with tetrazolium chloride and growth assays of the axis and cotyledon proved to be reliable methods.

To my husband, Casper, whose love and encouragement facilitated this accomplishment and to my daughter, Rutendo, for being so patient.

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I. LITERATURE REVIEW

A. INTRODUCTION

Chilling injury is a physiological disorder occurring in some crops at temperatures between 0 and 10-15C. Chilling sensitive crops originate from tropical and subtropical regions and chilling injury affects all stages of growth and development in plants, from germination to senescence. Chilling injury during germination results in inhibition of germination or abnormal seedling development (Christiansen, 1967; Pollock and Toole, 1966; Wolk and Herner, 1982). Two types of chilling injury prevent seeds of chilling sensitive crops from germinating (Herner, 1986). The first occurs when the seed has already germinated and affects the radicle of the plants. In this type of chilling injury, the seed does not germinate at the low temperatures but only germinates when the temperatures rise. Exposure of the germinating seeds to chilling temperatures causes necrosis of the tissue just behind the radicle tip and injures the root cortex. The second type is where the chilling injury occurs in the initial stages of germination when the seed is taking in water. This is called imbibitional chilling injury.

Legume seed, especially snap beans (Phaseolus vulgaris L.) and lima beans (Phaseolus lunatus L.), are susceptible to imbibitional chilling injury.

This greatly reduces germination of these two legume crops if they are planted in cool spring soils. Pollock and Toole (1966) reported that lima bean seeds and excised embryonic axes can be injured during imbibition at temperatures below 20C. Christiansen (1968) and Bramlage et al. (1978) reported the same thing for cotton (Gossypium hirsutum) and soybean (Glycine max), respectively. If seeds of cotton or lima bean are first imbibed at 31C and then transferred to 5C, they are less severely damaged than if they had started imbibition at 5C. Four hours at 5C is enough to cause imbibitional chilling injury in cotton (Christiansen, 1968). The early imbibition stage is the critical stage in imbibitional chilling injury (Pollock and Toole, 1966). The symptoms of imbibitional chilling injury in snap beans and lima beans are transverse cotyledon cracking, abnormal germination and failure to germinate due to increased decay. Poor and slow germination result if these crops are planted in cold soils (Kooistra, 1971). Snap beans and lima beans originate from tropical and subtropical regions High temperatures are required both for germination and growth of these crops.

Many factors affect imbibitional chilling injury including temperature, relation between timing of temperature exposure and stage of germination, initial seed moisture content, speed of imbibition, seed coat characteristics and integrity, seed vigor and cultivar or species. The lower the temperature the greater the imbibitional chilling injury. If exposure to low temperature occurs after the seed has already taken in some water, then the seed will not be affected. Seed with a high initial moisture content (12-20%) is less susceptible to imbibitional chilling injury than

that with a low initial moisture content (6-7%) (Herner, 1986). A rapid imbibitional rate enhances imbibitional chilling injury. Firmly attached and undamaged seed coats reduce imbibitional chilling injury. The causes, theories and methods of alleviating imbibitional chilling injury are hereby reviewed.

B. CAUSES OF IMBIBITIONAL CHILLING

Rapid rate of water uptake, solute leakage and seed coat characteristics may cause imbibitional chilling injury.

1. Rapid rate of water uptake

Dickson (1971) and Powell and Matthews (1978) showed that the rapid rate of cold water uptake during the early stages of imbibition results in imbibitional chilling injury. Tully, et al. (1981) also reported that the sensitivity to imbibitional chilling injury is a consequence of the imbibition rate of pea (Pisum sativum) (chilling resistant crop) and soybean (a chilling sensitive crop). They reported that the imbibition of pea seeds proceeds slowly in cold water, whereas soybean seeds imbibe cold water rapidly and suffer significant vigor loss. When the imbibition rate of peas was increased by nicking the seed coats, the peas became susceptible to imbibitional chilling injury. This was probably due to the fast rate of imbibition in the nicked seeds. When the rate of soybean imbibition was slowed with a solution of polyethylene glycol, its susceptibility to imbibitional chilling was lessened. Cold imbibition of intact soybeans reduced vigor by about 83%. In nicked peas, the vigor as

a result of cold imbibition was also reduced by about 80% (Tully et al, 1981). As a result of this observation, Tully and coworkers attributed the resistance of chilling injury in peas to the rate of imbibition.

Duke et al. (1986) also reported that the rapid imbibition of soybean seeds greatly increases the leakage of intracellular substances and decreases seedling survival. They also reported that the testa epidermis of the soybean seed decreases the leakage of intracellular substances both during rapid and slow imbibition and increases survival. They found testa tissues other than the epidermis to have little effect on the intracellular components during slow imbibition but these tissues greatly decreased leakage of seeds during rapid imbibition and increased subsequent seedling survival.

Wolk (1988) studied effects of imbibition rates on germination of low moisture (7.5-9%) P. vulgaris seeds (cv. "Tendercrop" and "Kinghorn Wax") using four different ways to control water uptake rate. These were positioning the seed relative to the hilum, incremental addition of water, varying number of layers of filter paper on which seeds were imbibed and applying wax to the seed's hilum region. He found that each imbibition method affected the rate of water entry into the seeds; those methods that reduced imbibition rate were significantly correlated with increased germination. He then proposed that a hydrophobic compound, which when applied to a seed retards imbibition rates, would be beneficial and might be important on a commercial scale. Wolk (1988) also reported that cotyledon tissue is more readily injured by rapid imbibition than the

axes. Injury to the cotyledon is deleterious to axes germination. He also suggested that an uninjured, fully imbibed axes might confer a degree of protection to the cotyledon and that the sequence in which embryo tissues imbibe might be a factor in the injury process.

Powell and Matthews (1978) did not observe any staining with tetrazolium chloride on the outer surfaces of the cotyledons of imbibed pea seeds. These researchers concluded that the unstained cells were killed possibly by the rush of water into the seed. However, later work showed that the unstained cells are not necessarily killed because they stain if imbibed in succinate solution or if imbibed in water and then treated with succinate and tetrazolium salt (Powell and Matthews, 1981). Simon and Mills (1983) suggested that the incomplete staining of imbibed embryos indicate that succinate and other dehydrogenase substrates are lost from the cells. This was supported by work by Duke et al. (1983) which showed that imbibing soybean seeds lose dehydrogenase enzymes.

The tetrazolium test distinguishes between viable and dead or injured tissues of the embryo on the basis of their relative respiration rates in the hydrated state. The test utilizes the activity of dehydrogenase enzymes as an index to the respiration rate and seed viability (Copeland, 1976). The dehydrogenase enzymes reduce tetrazolium chloride to formazan, a red pigment which causes the red stain on live tissue. This further supports the fact that the unstained outer pea cells in Powell and Matthews' (1978) work was possibly due to loss of dehydrogenase enzymes and not necessarily due to a breakdown of cell organization.

The absolute rate of imbibition is not the only factor correlated with chilling injury. When soybean seeds of different initial moisture content are imbibed, those with 44% initial moisture content imbibe more rapidly than those with 6% initial moisture content (Bramlage et al., 1978). Pollock (1969) showed the same response in lima beans. Even though seeds with high initial water content imbibe moisture more rapidly, these seeds are protected from chilling injury. Also, the rate of imbibition of seeds at low temperature is slower than at high temperature yet damage to the seeds is greater when seeds are imbibed at low temperature (Leopold, 1980). The most critical stage of imbibition is the first few minutes or hours (Herner, 1986).

2. Solute leakage

Solute leakage is also one of the possible causes of imbibitional chilling injury. It is also possible that solute leakage is just a symptom of chilling injury.

The legume seed testa protects the embryo from massive cellular rupture and leakage of intracellular substances during imbibition (Duke and Kakefuda, 1981; Duke et al., 1983). Various intracellular substances leak from imbibing legume seeds which is negatively associated with seed vigor (Larson, 1968; Matthews and Bradnock, 1968; Perry and Harrison, 1970; Bramlage et al., 1978; Yaklich et al., 1979). Leachates may reflect a general deterioration of seed tissues which results in loss of seed vigor (Parrish and Leopold, 1978). Seed leachates may also serve as substrates for pathogen growth (Simon, 1974). Cells ruptured by imbibition may serve

as infection sites for seed pathogens (Duke et al., 1983).

Substances and ions leaking out of seeds during imbibition include amino acids, sugars, organic acids, gibberellic acids, phenolics, phosphates (Simon, 1974), succinate (Powell and Matthews, 1981) and enzymes (Duke et al., 1983). Duke et al. (1983) reported that embryos with intact testa from soybean, navy bean, pea and peanut leak detectable activities of either intracellular enzymes of the cytosol (glucose-6-phosphate dehydrogenase) or enzymes found in both the cytosol and organelles (malate dehydrogenase, glutamate dehydrogenase, glutamate oxaloacetate, transaminase, and NADP-isocitrate dehydrogenase) after six hours imbibition at 2.5C. They unequivocally stated that the testa of legume seeds inhibit the leakage of high molecular weight intracellular substances.

Leakage of intracellular substances from legume seeds during imbibition can involve two processes:

(i) passive diffusion of low molecular weight substances through cell membranes during a period of membrane reorganization (Chabot and Leopold, 1982; Duke et al., 1983; Simon, 1978) and

(ii) release of the entire cellular contents of ruptured testa and/or embryo cells (Duke and Kakefuda, 1981; Duke et al., 1983).

Testa integrity has a large effect on both phenomena in legume seeds (Larson, 1968; Simon, 1974; Powell and Matthews, 1978; Duke and Kakefunda, 1981; Tully et al., 1981; Duke et al., 1983). Other factors which affect the leakage of intracellular substances from legume seeds during imbibi-

tion include seed moisture content (Hobbs and Obendorf, 1972; Parrish and Leopold, 1977; Simon and Wiebe, 1975), temperature (Pollock et al., 1969; Bramlage et al., 1978; Leopold, 1980; Duke et al., 1983; Marbach and Meyer, 1985), water potential (Knypl et al., 1980; Woodstock and Taylorson, 1981a; Duke et al., 1983) and seed aging (Parrish and Leopold, 1978). Duke et al. (1986) found that anoxia has little or no effect on soybean seed leakage during imbibition and on subsequent seedling survival.

Duke and Kakefuda (1981) suggested that leakage of electrolytes and ultra violet (u.v.) absorbing compounds, the two most commonly used methods of measuring leakage, do not accurately reflect the state of membrane integrity because many smaller molecules diffuse freely through the membrane. Instead they assert that determination of enzymatic activity in seed leachate should be used as an assay for membrane damage.

3. Seed coat characteristics

Seed coat characteristics are not really causes of imbibitional chilling but compound both the rate of solute leakage out of the seed and the rate of water entrance into the seed. Imbibition may also be rapid, resulting in damage and poor seed germination if the seed coat is damaged during seed development or during harvesting and handling. The peanut (Arachis hypogea) seed coat is very thin and its presence or absence has little effect on embryo imbibition. Leakage from the embryo increases greatly when the seed coat is removed, decreasing the vitality and vigor of the embryo (Abdel Samad and Pearce, 1978). Powell and Matthews (1978) showed that if pea seeds are nicked with a razor blade water uptake is more rapid

and germination at 2C is much poorer than in controls imbibed at 25C. They concluded that the seed coat protects pea embryos from rapid imbibition, prevents extensive leakage and stops chilling damage. Tully et al. (1981) reported that cold tolerance during imbibition relates to the seed coat condition or to its pigmentation, both of which affect the rate of imbibition.

Powell et al. (1986) tested differences in the field emergence of 30 commercial seed lots of dwarf French bean associated with the color of the testa. They reported that eleven lots with a white testa had a lower mean field emergence of 67% compared with 91% for lots with black or brown testa. The white seeded lots also had higher leakage conductivities and imbibed more rapidly than black or brown seed lots. Dickson (1971) also showed that cultivars with white seeds produce weaker and less vigorous seedlings than those with colored seeds, especially under unfavorable field conditions. This susceptibility of white seeded cultivars to imbibitional chilling injury has been associated with the degree of seed coat adherence to the cotyledons (Powell and Matthews, 1981).

However, Wolk's (1988) work with "Kinghorn Wax" (with white seed coat) and "Tendercrop" (with pigmented seed coat) shows that the opposite can be true as far as pigmentation is concerned. "Kinghorn Wax" had more resistance to imbibitional chilling injury than "Tendercrop." The relationship to injury and adherence of the seed coat to the cotyledons, however, still holds. "Kinghorn Wax" has a semi-hard seed compared with variety "Tendercrop" which has a loosely bound and pigmented seed coat.

Taylor and Dickson (1987) determined that the semi-hard seed coat characteristic in snap beans delayed the onset of imbibition at low initial moisture levels and reduced imbibitional chilling injury. Water entry into the semi-hard seeds takes place primarily through the chalaza and raphe rather than the hilum and micropyle resulting in a slower rate of water uptake (Holubowicz et al., 1988).

C. EXPLANATIONS (THEORIES) FOR IMBIBITIONAL CHILLING INJURY

Many theories have been proposed to explain imbibitional chilling injury. These ideas include membrane compositional differences, seed coat characteristics, membrane disruption, membrane reorganization and the water replacement hypothesis.

1. Membrane compositional differences

Chilling resistance is correlated with a greater degree of unsaturation in the lipid components of membranes. Lyons et al. (1964) showed that membrane lipids of chilling sensitive plants contain less unsaturated fatty acids than those of chilling resistant plants. Dogras et al. (1977) reported differences in percent ^{14}C glycerol incorporated into phosphotidyl choline (PC), phosphotidyl ethanolamine (PE) and phosphitidyl glycerol (PG) in imbibing seeds between chilling resistant species (broad beans and peas) and the chilling sensitive species (lima beans). The type of phospholipid synthesized may be related to sensitivity to chilling injury at 10C in the imbibition stage. This could be taken as evidence for the role of membrane compositional differences in affecting chilling injury.

In order to eliminate the species differences Wolk (1980) studied the fatty acid composition of two cultivars of P. vulgaris which differ significantly in their resistance/sensitivity to imbibitional chilling injury. The differences in membrane fatty acid saturation between the two cultivars were very slight and did not support the membrane phase transition hypothesis as the mechanism of low temperature injury in imbibing seeds. Priestley and Leopold (1980) also reported the same observation and suggested that the difference in chilling sensitivity between pea and soybean is not related to compositional differences in the major lipid components of the seed membranes. Stewart and Bewley (1981) also did not find any correlation between membrane compositional differences and imbibitional chilling injury. These analyses were all performed on bulk membrane lipids extracted from seed and may not reflect the true membrane lipid composition of individual membranes such as the plasma membrane. In addition, the synthesis of new membrane lipids may be quite different between chilling sensitive and resistant species or cultivars.

The differences in membrane composition (if there are any) may result in a change in the physical state of the chilling sensitive membranes at low temperatures which adversely affects energy supply and metabolism and results in the build-up of such toxins as acetaldehyde and ethanol. Membranes also increase in leakiness as a result of this phase change.

2. Membrane disruption

Larson (1968) proposed that cell membranes are ruptured by the rapid in-

rush of water as a seed imbibes, increasing leakage from the tissue. Powell and Matthews (1978) showed damage to a layer of cells on the abaxial surface through failure to stain with triphenyl tetrazolium chloride (TTC). Seeds with high rates of water uptake had cracks in the seed coats. A high proportion of seeds with cells not stained by TTC were low in vigor, had high amounts of solute leakage and exhibited poor field emergence (Powell and Matthews, 1979). Powell et al. (1986) noted that the pattern of leakage over time is the same for both living and dead seeds and concluded that it was a purely physical phenomenon and reflected disruption of cell membranes caused by imbibition.

3. Membrane reorganization

The theory of membrane reorganization is supported by the time course of initial water entry into dry cotyledons. It shows a period of rapid non-linear entry lasting 30 minutes or more (Leopold, 1980). The initial time course of solute leakage from the imbibing cotyledon also shows a period of rapid non-linear leakage followed by a linear phase beginning at the same time that water entry becomes linear. Leopold (1980) suggested that the initial rapid phases of these two events (imbibition rate and solute leakage) represent a period during which the membranes were relatively disorganized. The linear phases represented steady state processes through the reorganized membranes. Simon and Mills (1983) also proposed that membranes are disorganized in dry seeds no longer forming an intact barrier around the cytoplasm of each cell but regain their normal semi-permeable condition during imbibition. In a short period at the start of imbibition the membrane constituents in each cell may be going through a

phase of reorganization, and solutes could leak out. During imbibition, the water front penetrates slowly into the body of a seed or embryo, wetting each layer of cells, in turn, and allowing leakage from all the wet cells.

Simon and Wiebe (1974) have suggested that the membrane of developing seed corresponds to the bilayer structure seen in bulk phospholipid/water mixtures. As the seed matures, the plasma membrane dries out. The molecular architecture of the membrane constituents is thought to change when its water content falls below 20% (Simon and Wiebe, 1974). The membrane forms a hexagonal phase, holding the remaining water more tightly and at the same time leaving gaps or leaks in the membrane. Solutes leak from these gaps.

Recently several reports have been published refuting the theory that the membrane changes structure in dry seeds (McKersie and Stinson, 1980; Seewaldt et al., 1981). Investigations of membrane structure in dry seeds by X-Ray diffraction of both extracted (McKersie and Stinson, 1980) and in-situ (Seewaldt et al., 1981) seed membrane lipids refuted the hexagonal array hypothesis. ^{31}P-NMR studies of membrane structure in dehydrated pollen grains also indicated that the bilayer configuration is maintained even under conditions of extreme desiccation. With the doubts discussed above of the events taking place during imbibitional chilling a new hypothesis has been suggested to explain the phenomenon.

4. Water replacement hypothesis

Vertucci and Leopold (1983) studied the wetting reaction of soybean embryo tissue. They found that PEG acts not only as an osmotic agent retarding imbibition rate, but also as a surfactant and, in dilute solutions (<2%), actually increased the rate of imbibition in embryos with initial moisture contents less than 24%. They suggested that the wetting reaction, a purely physical process, was the critical step in the injury process. This led them into the area of anhydrobiosis. These studies are focussed on the maintenance of cell structure during dehydration. The central hypothesis is the "water replacement hypothesis" as reviewed by Clegg (1986), one of its principal proponents.

The water replacement hypothesis states that certain polyhydroxyl compounds such as the sugar trehalose are structured in a way that allows their hydroxyl groups to form hydrogen bonds to macromolecular sites in dehydrating tissues that would normally be occupied by water. In the presence of trehalose the membrane bilayer structure is maintained at low water activities, the fusion of vesicles is prevented and the activity of membrane bound enzyme systems is retained upon rehydration (Rudolp and Crowe, 1985; Crowe, Crowe and Jackson, 1983). Seeds do not contain trehalose (Kandler and Hope, 1966), but Caffrey et al. (1988) have recently demonstrated in-vitro the ability of raffinose/sucrose mixtures, sugars common in seeds, to preserve the bilayer configuration of phospholipid mixtures at low water activities.

Wolk (1988) indicated that the injury incurred by low moisture seeds during imbibition is the same at non-chilling temperatures. He also

reported that imbibition rate maxima of embryo tissue reflect transitions in the states of seed water. The experiments he did with NMR T_1 support the application of the water replacement hypothesis to seeds.

D. ALLEVIATION OF IMBIBITIONAL CHILLING INJURY

The causes of imbibitional chilling injury and the theories explaining it are not fully understood. This makes the alleviation of imbibitional chilling injury difficult. Genetic improvements, including good pod and plant types that can germinate at 8 to 10C (Dickson, 1971) and seed coat characteristics allowing slow imbibition (Taylor and Dickson, 1987), are viable methods of preventing imbibitional chilling injury in seeds. The other methods tried include seed priming, raising initial moisture content and coating of seeds.

1. Seed priming

Osmotic conditioning or priming is a technique based upon controlled hydration of seeds to a level that permits pre-germinative metabolic activity to proceed, but prevents actual emergence of the radicle (Bradford, 1986). Early attempts to achieve this relied on alternately imbibing the seeds then redrying prior to the completion of germination (May et al., 1962; Henckel, 1964). This process was termed hardening. A consistent result of such treatments was more rapid germination (Heydecker and Coolbear, 1977). Henckel (1964) proposed that it was the dehydration that was responsible for the hardening effect but Hanson (1973) showed that the effective invigoration of seed occurs in the

imbibition period and is subsequently "fixed" by drying. Heydecker et al. (1973, 1975) achieved the same end by using an osmotic solution to inhibit radicle emergence, but allowing the maintenance of sufficient hydration for metabolism to proceed. Salt solutions had previously been used for this purpose (Ells, 1963; Koehler, 1967) but Heydecker et al. (1973) used PEG of high molecular weight (average 6000) as an inert osmoticum.

The basic process, which Heydecker et al. (1975) called priming, consisted of imbibing seeds in an aerated PEG 6000 solution of sufficient osmotic strength to prevent visible germination. The seeds were held under these conditions for periods of up to several weeks, then rinsed and redried to the original water content. When planted, such seeds often germinated much more rapidly and uniformly, particularly under adverse temperature or moisture conditions.

A considerable amount of success has been achieved by the use of PEG to improve germination of several crop species under adverse conditions. Table 1 shows some of the crop species in which PEG has had an effect on germination, the water potentials and the sources of information.

TABLE 1: Seed priming conditions for crops (modified from Bradford, 1986)

CROPS	SOLUTION	TEMP. C	DURATION DAYS	RESULTS	SOURCE
Barley (*Hordeum vulgare*)	PEG (-0.5 to -1.5 MPa)	10	1-8	accelerated germination and improved uniformity	Bodsworth and Bewley(1981)
Corn (*Zea mays*)	PEG (-0.5 to -1.5 MPa)	10	1-8	accelerated germination and improved uniformity	Bodsworth and Bewley(1981)
	PEG(250g/kg +0.2% thiram)	15	8	improved germination; increased seedling growth rate	Khan *et al.* (1978)
Pea (*Pisum sativum*)	PEG(250-500g per kg)	15	4;8	accelerated germination;increased root and shoot growth	Khan *et al.* (1978)
Wheat (*Triticum aestivum*)	PEG(-0.5 to -1.5MPa)	10	1-8	improved germination	Bodsworth and Bewley(1981)
Soybean (*Glycine max*)	PEG(-0.5 to -1.5MPa)	10	1-8	accelerated germination;improved uniformity	Bodsworth and Bewley(1981)
	PEG(250-350g per kg+0.2% thiram	15	4-10	accelerated emergence; increased seedling growth rate	Khan *et al.* (1978)
	PEG(250g/kg)	15	11	accelerated emergence	Khan *et al.* (1980-81)
	PEG(250g/kg + 0.2% thiram + 1200 U penicillin	10	4	accelerated germination	Knypl *et al.* (1980)

The major obstacle to commercial application of seed priming is the variability of results among species, varieties, and even seed lots (Heydecker, 1977). The specific treatment conditions must be optimized essentially by trial and error for each species, cultivar or seed lot. Priming results in increasing seed initial moisture content before planting and thus has a beneficial effect. Drying the seed reduces seed initial moisture content and therefore loss of the beneficial effect of priming (Heydecker and Wainwright, 1976). Primed seeds can be stored at low temperatures to maintain the priming effect (Irwin and Price, 1981). Storing the primed seed at low temperatures and then planting it at subtropical temperatures has no benefit. Heydecker (1977) reported one major disadvantage of PEG 6000. PEG 6000 reduces oxygen availablity within the solution compared with that in water which in some cases may already be limiting. In PEG 6000, oxygen solubility is 50% that of water and oxygen mobility is only 10%, depressing relative oxygen availability to the order of 5% (Mexal et al., 1975).

Despite the success of seed priming only a few studies have been carried out on the biochemistry and physiology of seed priming. Osmoconditioning of lettuce seeds reduces the time of imbibition required for the onset of ribonucleic acid (RNA) and protein synthesis, polyribosome formation and increases the total amount of RNA and protein synthesized (Khan et al., 1978; Khan et al., 1980-81). They also reported increased activity of enzymes like acid phosphatase and esterase following osmoconditioning. Osmoconditioning also caused qualitative changes in soluble proteins, acid phosphatases, esterases and 3-phoshoglyceraldehyde dehydrogenses as

indicated by electrophoresis on polycrylamide gels. Complete disappearance of abscisic acid (ABA) was also reported after osmoconditioning (Khan et al. (1978). From their results Khan et al. (1978; 1980-81) suggested that the increased synthesis of RNA, protein and enzymes in treated seeds may be due either to the removal of certain inhibiting factors such as ABA or to the production of promotive factors. They also suggested that mobilization of storage materials such as sugars, fats and proteins by activation or de-novo synthesis of key enzymes may underlie the mechanism of osmoconditioning.

Hegarty (1978) concluded that while some work has been done on the physiology and biochemistry of seed priming "the physiology of incompletely hydrated seeds is a field in which very little information is available." The information that is available does not provide easily measurable parameters that can be correlated with successful priming treatments.

While seed priming depends on the control and manipulation of seed hydration, little attention has been paid to the water relationships of the seed during and after treatment. Bradford (1986) put together some basic information on water relations of seed priming.

In seeds the water content equilibrium attained at any given water potential (Ψ) depends upon solute potential (Ψ_π) and pressure potential (Ψ_p) of the embryo cells. Solutes present in the cells lower Ψ_π and provide the driving force for water uptake in a relatively high Ψ range.

Resistance of the cell walls to expansion as water is taken up results in turgor pressure, which increases the Ψ of the cell and reduces the driving force for water uptake. At the water content plateau, there is no net movement of water and the external water potential (Ψ_o) is equal to the water potential of the cell (Ψ_c) which is the sum of Ψ_π and Ψ_p.

In seed priming Ψ_o is either set sufficiently low that radicle expansion cannot occur, or the duration of priming at higher Ψ_o is shortened to be within the plateau period. Damage often results from dehydration as radicle growth has begun (that is, water content has begun to increase from the plateau level). Effective priming treatments are concerned with achieving and maintaining a near equilibrium between Ψ_o and Ψ_c.

2. Raising initial moisture content

Experiments with seeds at different water contents have shown that there is no injury in cotton and soybean seeds at moisture contents above 13% (Christiansen, 1968; Hobbs and Obendorf, 1972). The corresponding figure for lima bean is 20% (Pollock, 1969; Cal and Obendorf, 1972). Simon and Raja Harun (1972) reported that pea embryos that have first been allowed to imbibe some water through a small part of their surface leak relatively slowly when subsequently immersed in water. The greater the initial imbibition the slower the subsequent leakage. Embryos taken from peas that were harvested when succulent and tender only showed slow leakage. Simon and Wiebe (1975) showed that leakage from embryos with an initial water content of 17% is reduced and there is little sign of rapid leakage from embryos already containing 48% water. The rate declines steadily as

water content rises from air-dry value to levels of between 17-25% and then declines further as the embryos become more hydrated. At water contents above 30-35% leakage is reduced to a relatively low rate.

Cohn and Obendorf (1976) measured enzyme metabolism in corn with 5% and 13% initial moisture content measured at 0, 12, 24 and 48 hours at 25C subsequent to imbibition at 5C. The interaction of low kernel moisture with imbibition at 5C resulted in reduced radicle growth in seedlings. Oxygen uptake of whole kernels after imbibitional chilling was independent of initial kernel moisture. Differences in initial moisture did not alter ATP levels of embryos and embryonic axes after chilling. Mitochondria isolated from embryos of low moisture kernels exhibited slightly higher respiratory rates 24 hours after cold imbibition but not at other sampling times. This showed that a disruption of energy metabolism was not the primary cause of kernel moisture mediated imbibitional chilling injury. Respiration after imbibitional chilling was the same for both low and high initial moisture kernels.

3. Waxing or coating of seeds

Imbibitional chilling of seeds is generally associated with a rapid entry of water at low temperatures. A way of alleviating chilling injury would be to retard the entry of cold water into the seed. Attempts to slow the rate of imbibition of soybean, corn and cotton seeds through the application of a thin coat of lanolin (20-30g per kg seed) provided alleviation of chilling injury in the susceptible soybean and cotton (Priestly and Leopold, 1986). Lanolin coated soybean seeds imbibed water

at a greatly reduced rate compared to untreated seeds. When subjected to an imbibitional chilling stress (18 hours at 2C) coated seeds had higher percentage emergence and individual weight of the emerged seedlings than the controls. Very little work has been done elsewhere on the use of waxes to control imbibitional chilling injury. The hypothesis in this study was that imbibitional chilling injury is caused by a rapid rate of cold water uptake.

The objectives of this study were:

1. To try to alleviate imbibitional chilling injury in snap beans and lima beans by:
   (a) seed coating with waxes to reduce rate of cold water uptake by the seeds
   (b) allowing slow imbibition in aqueous solutions of PEG of different osmotic potential
   (c) gradually increasing the initial seed moisture content at room temperature before imbibition.

2. To determine the effectiveness of the above methods by assessing
   (a) damage to axes and cotyledons, and
   (b) germination of treated seed in pots

II. MATERIALS AND METHODS

Three methods to control imbibitional chilling injury in snap bean (Phaseolus vulgaris L.) and lima bean (Phaseolus lunatus L.) were carried out. These were coating seeds with hydrophobic waxes to reduce rate of water uptake by the seed during imbibition, raising initial seed moisture content before imbibition and finally, imbibing the seed at different temperatures in polyethylene glycol 6000 to reduce the rate of water uptake by the seed.

Seeds of snap bean, cv. Tendercrop, and lima bean cv. Fordhook 242, were obtained from Harris Moran Seed Company, New York. The stated seed quality characteristics were: germination percentage 84, purity 99% and 1328 seeds per pound for Tendercrop (lot number 83-3745); 86% germination and 388 seeds per pound for Fordhook 242 (lot number 26-3622). Both seed lots were prepared for commercial use and therefore pretreated with the fungicide Captan. In the laboratory the seed was stored in paper bags at 5C and 35% relative humidity until needed. Seeds in all experiments were presorted by hand and excessively small, large and/or damaged seeds were discarded. The three methods used in this study are described separately below.

A. SEEDCOATING WITH WAXES AND OILS

1. Selection and preparation of waxes

Twenty different waxes and oils were screened for their ability to effectively coat snap and lima bean seeds, and to reduce the rate of water uptake by the seeds. These waxes and oils were also selected for their ability to dry on the seed without leaving a sticky residue that impedes easy handling.

The waxes and oils were applied 24 hours before the imbibition. Twenty different seed lots (corresponding to the twenty waxes/oils), each of about 30 seeds, were hand sorted for both lima and snap bean. During application the wax/oil was added to a seed lot in a Petri dish and gently stirred to allow complete coverage of the seed by the wax/oil. The seeds were then spread out on paper towels to dry. Two experimental units of ten seeds each were counted from each waxed/oiled seed lot. Each unit was weighed to the nearest hundredth of a gram on a Mettler PJ4000 balance.

For imbibition treatments, each experimental unit (10 seeds per replication) was immersed in 20ml distilled water in a 57mm aluminum foil dish at 20C. The amount of water uptake was determined every 20 minutes for the first 2 hours of imbibition. At the end of each 20 minute period the seeds were removed from the water, thoroughly blotted on paper towels and weighed on the Mettler PJ4000 balance. The seeds were then reimmersed in water for the next 20 minute period. Amount of water uptake was based on the seed fresh weight increments.

The following four waxes were chosen for further study:

(a) Vapor Gard from Miller Chemical and Fertiliser Corporation, Hanover, Pennsylvania,

(b) Wiltpruf from Wiltpruf Products Inc., Greenwich, Connecticut,

(c) Sta-fresh 320 manufactured by FMC corporation Florida and California, and

(d) Pacrite 383 manufactured by American Machinery Corporation, Orlando, Florida.

Preliminary work indicated that most wax formulations contained enough water that allowed some imbibition of water to occur during wax application. To prevent this from occurring all wax formulations were placed in a rotovap to remove water for 1-2.5 hours at 45-50C. Since the four waxes selected were of different formulations and therefore different water contents, the amount of excess water removed (percentage of original wax volume placed in the rotovap) varied as shown in Table 2. The waxes/oils were applied by mixing them with the seed 24 hours before the imbibition studies. After thoroughly mixing the seed and wax the seeds were dried at air temperature. A completely randomized design was used to analyze the differences in treatments.

TABLE 2: The amount of water evaporated from different waxes of different formulations

Wax	Water Removed (% original wax volume)
Wiltpruf	80
Sta-fresh 320	84
Pacrite 383	60
Vapor Gard	60

2. Amount of water uptake

Amounts of water uptake (water imbibition) were determined at three different temperatures, 5, 10 and 20C, for the first 4 hours of imbibition for both snap and lima beans. The determinations were done 15, 30, 60, 120 and 240 minutes (4 hours) after the beginning of imbibition.

Ten seeds were used as one experimental unit and each unit was weighed before the start of imbibition. There were three replications for each treatment. For imbibition the seed was placed between two wet Whatman No.1 filter papers in 15 x 100ml plastic Petri dishes. The filter papers were wetted by water equilibrated to the respective temperature regime. Water was added to wet the filter papers as needed. At the end of each observational period (15, 30, 60, 120 and 240 minutes) the seed was removed from the filter papers, dried on paper towels and weighed.

3. Testing for damage with 2% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC)

Waxed seeds imbibed at 5, 10 and 20C were assessed for damage 24 hours after the beginning of imbibition using triphenyl tetrazolium chloride (Aldrich Chemical Company, Milwaukee, Wisconsin). Ten seeds were placed in 20ml of 2% TTC solution for four hours followed by a thorough rinse in distilled water. The seed was then checked for uniform stain, cotyledon cracks, and damage to axes under a lighted magnifying glass (x3 magnification). Seed with greater than three cotyledon cracks and large transverse cotyledon cracks on both cotyledons, damaged axes and seed that did not take up any stain was considered nonviable and unable to germinate.

4. Growth assay of axes and cotyledon tissue and chlorophyll determinations in cotyledons

Waxed seed (and non-waxed control seed) imbibed at 5, 10 and 20C for 24 hours was assayed for growth. The assay used required growth of separate axes and cotyledons at 20C for ten days. After imbibition for 24 hours the axes and cotyledons were separated using a scalpel blade. The seed coat was also removed from the cotyledons. In snap bean five axes were used as one experimental unit in the axes growth assay while twenty cotyledons comprised one experimental unit in the cotyledon growth assay. The corresponding figures in lima beans were five axes and ten cotyledons. The large size of the lima bean cotyledons restricted the number of cotyledons per experimental unit to ten. Each experimental unit was weighed before the beginning of the assay. Three replications were used for each treatment combination in both the axes and cotyledon growth assays.

The axes were placed on one Whatman No. 1 filter paper in 15 x 100ml plastic Petri plates and the cotyledons were carefully placed flat side down between two filter papers also in 15 x 100ml Petri plates. Two percent sucrose solution was added to wet the filter papers in both the Petri plates with the axes and cotyledons. These were then kept in a growth chamber under continuous flourescent light at 20C for ten days. The nutrient solution (2% sucrose solution) was replenished on a daily basis by keeping the filter papers wet. After ten days the axes were checked for growth using fresh weight increases and length of the radicle and plumule. The cotyledons were also checked for growth using fresh

weight increases. Damage to the cotyledons was determined by measuring chlorophyll content in the cotyledons. This was based on the presumption that only living cotyledon tissue produces chlorophyll and that the amount of chlorophyll extracted from each cotyledon experimental unit was related to the extent of damage inflicted by the respective temperature regime.

The amount of chlorophyll in the cotyledons was extracted using N,N-dimethyl formamide (DMF) as described by Moran and Porath (1980). For both snap and lima bean the cotyledons were cut into small pieces and weighed before being placed in medium sized test-tubes containing 20ml of DMF. The test-tubes were then wrapped in aluminum foil and placed at 5C in the dark for 48 hours. The aluminum foil was to ensure darkness at all times. After 48 hours, absorbance was read for the extracted chlorophyll solutions at 664, 647 and 625nm on a Beckman recording quartz spectrophotometer. A blank of 98% DMF was used in all cases and before each absorbance reading. Chlorophyll "a", "b" and "p" chlorophyll determinations were computed in ug/ml/g of cotyledon material according to the following equations by Moran (1982):

Chlorophyll "a" = $12.65A_{664} - 2.99A_{647} - 0.04A_{625}$

Chlorophyll "b" = $-5.48A_{664} + 23.44A_{647} - 0.97A_{625}$

"p" Chlorophyll = $-3.49A_{664} - 5.25A_{647} + 28.3A_{625}$

where A_{664}, A_{647} and A_{625} are absorbance readings at 664, 647 and 625nm, respectively.

5. Germination of treated seeds in pots

Treated snap and lima bean seed was planted in 15cm diameter plastic pots with Bacto (from Michigan Peat Company, Sandusky, Michigan) as the planting mix. The pots were prepared, watered and transferred to the respective constant temperature rooms (5, 10 and 20C) 24 hours before planting to equilibrate the soil with the required temperature. Five replications for each treatment combination were completed. Five seeds were planted per pot. Immediately after planting, the pots were watered with water equilibrated to the required temperature and left in the constant temperature rooms for 24 hours after which they were transferred to the greenhouse. In the greenhouse the pots were watered every day. Germination percentage and heights of the germinated plants were recorded two weeks after planting for snap beans and three weeks after planting for lima beans. Germination was considered as emergence from the soil.

B. MOISTURE CONTENT EXPERIMENTS

Three different seed moisture contents were used for both snap and lima beans. The moisture contents used were 6-9%, 15-20% and 25-30%. For all experiments moisture content was determined by drying seeds for 48 hours at 95C in a forced draft drying oven. All moisture contents are expressed on a fresh weight basis.

1. Moisture adjustments

Seed moisture contents were adjusted to the desired levels in humidity chambers made by placing some distilled water in air tight glass desiccators. The seeds were placed on netted wires above the distilled

water. Six to nine percent was the original moisture content of the seed from storage. For snap bean 300 seeds were kept in the humidity chamber for 48 hours to raise the moisture content to 15-20% and for five days to obtain 25-30% moisture content. For lima beans 300 seeds were kept in the humidity chamber for three and a half days to obtain 15-20% moisture content and for eight days to obtain 25-30% moisture content.

2. Testing for damage with 2,3,5-triphenyl-2H-tetrazolium chloride (TTC)

Seeds of three different moisture contents (6-9%, 15-20% and 25-30%) were imbibed for 24 hours at 5, 10 and 20C between two wet Whatman No.1 filter papers in 15 x 100ml plastic Petri plates. Ten seeds per Petri plate were used as one experimental unit. Five replications were completed for each treatment combination. The low moisture and 20C combination was the control treatment. After 24 hours of imbibition the seed was then assessed for damage using 20ml of 2% TTC solution as described in section A(3).

3. Growth assays and chlorophyll determination in cotyledons

Assays for the amount of damage to the axes and cotyledons after imbibition at the three temperatures were also carried out for the three moisture content levels. For both snap bean and lima bean five axes made up one experimental unit in the axes growth assay while ten cotyledons were one experimental unit in the cotyledon growth assay. Five replications were completed for each treatment combination.

The method described in section A(4) was used in growing the axes and

cotyledons. Fresh weight increases and the length of radicle and plumule were used to assess growth. Chlorophyll content per gram of cotyledon was used to reflect imbibitional chilling damage of the cotyledon tissue.

4. Germination of seeds of different moisture content in pots

Both snap bean and lima bean seeds at three different moisture contents were planted in pots at 5, 10 and 20C. The pots were prepared, watered and equilibrated to the respective temperature as outlined in section A(5). Five seeds were planted per pot and there were five replications (with one pot as the experimental unit) for all treatment combinations. The pots were left in the constant temperature rooms for 24 hours after planting followed by transfer to the greenhouse. In the greenhouse the pots were watered every day. Germination percent and heights were measured two weeks after planting for snap beans and three weeks after planting for lima beans.

C. PRIMING EXPERIMENTS

Polyethylene glycol (PEG) 6000 obtained from Sigma Chemical Company (St. Louis, Missouri) was used for all the priming experiments. PEG at two osmotic potentials, -5 MPa and -15 MPa, was used to control rate of water uptake in the seeds. Distilled water at 0 MPa was used as the control. The osmotic potentials of PEG were calculated according to the two equations below adopted from Michel (1983);

(a) Relationship between PEG 6000 osmotic potential and concentration within a temperature range of 5-40C is expressed as follows:

$$= 1.29\ [PEG]^2T - 140\ [PEG]^2 - 4.0\ [PEG]$$

(b) The quadratic solution for PEG concentration of equation (a) is as follows:

$$[PEG] = \frac{(4 - (5.16\ T - 560 + 16)^{0.5})}{2.58T - 280}$$

where T is the temperature,

is the osmotic potential desired in bars and,

[PEG] is the concentration of polyethylene glycol.

1. Water uptake

Amounts of water uptake were determined at 5, 10 and 20C for the first four hours of imbibition using the three solutions of different osmotic potential (-1.5, -0.5 and 0 MPa). The experimental unit was ten seeds and each unit was weighed before the beginning of imbibition. Amount of water uptake was determined 15, 30, 60, 120 and 240 minutes after the start of imbibition. There were three replications in the experiment. For imbibition, seed from each experimental unit was placed between two wet filter papers in plastic Petri plates. The filter papers were then wetted with the respective solutions. At the end of each observation period (15, 30, 60, 120 and 240 minutes) the seed was removed from the Petri dish, quickly dried on paper towels, then weighed.

2. Testing for damage with TTC

Snap bean and lima bean seeds imbibed with solutions of different osmotic potential at 5, 10 and 20C were assessed for damage with TTC as described in sections A(3) and B(2). Ten seeds were used as one experimental unit

and three replications were made for all treatment combinations. After 24 hours of imbibition all the seed were thoroughly rinsed with distilled water before being placed in the TTC solution.

3. Growth assays and chlorophyll determinations in cotyledons

Seed imbibed in the three different solutions (of different osmotic potential) and at 5, 10 and 20C was also assayed for growth. The axes and cotyledons were separated as outlined in section A(4). In both snap bean and lima bean, five axes and ten cotyledons were used as experimental units in the axes and cotyledon growth assay, respectively. The assay was replicated three times for all treatment combinations. Fresh weight increases and radicle and plumule length were used in the assessment of growth. In the cotyledon assay chlorophyll content showed the extent of imbibitional chilling injury.

4. Pot germination of seed imbibed in three PEG solutions of different osmotic potential

Snap bean and lima bean seeds imbibed in the three osmotic potential solutions at 5, 10 and 20C in Petri dishes for 24 hours were also planted in pots with Bacto planting mix at the three temperatures (5, 10 and 20C). The pots were prepared as in sections A(5) and B(4). Five seeds were planted in each pot and three replications were used for each treatment combination. The pots were kept in the constant temperature rooms for 24 hours and then transferred to the greenhouse where they were watered everyday. Germination percent and heights were taken two weeks after planting for snap beans and three weeks after planting for lima beans.

A completely randomized design was used to analyze the results. Except for the water uptake experiments, in which the treatments were arranged in a three factor factorial, all the experiments were arranged in a two factor factorial. Analysis of variance was carried out and 5% level of significance was considered as significant in all cases. All mean separations were carried out by least significance differences.

III. RESULTS

The lima bean seed used in these experiments showed a lot of inconsistency and poor germination even under ideal conditions. The results reported in this study pertain mostly to snap bean. Summaries of the lima bean results are included in the appendix.

A. EFFECT OF SEEDCOATING WITH WAXES

1. Water uptake

Wax-coated snap bean seeds imbibed water at reduced rates compared to untreated seeds. In snap bean Vapor Gard and Pacrite 383 reduced water uptake by as much as 45% and were better ($p>0.01$) than Wiltpruf and Sta-fresh 320 whose corresponding figure was 35%. Figure 1 shows the effect of different waxes in reducing water uptake with time. There was clear separation between the waxes and control as well as among the waxes themselves as imbibitional time increased, giving rise to wax x time interaction ($p>0.001$).

For all the treatments, water uptake increased with temperature. The highly significant ($p>0.001$) temperature x time interaction is represented in Figure 2. The high water uptake at 20C compared to the chilling injury inducing temperatures (5 and 10C) suggests that the rate of water uptake *per se* might not be the only cause of imbibitional chilling injury.

FIGURE 1. The effect of coating snap bean seeds with waxes on water uptake in the first few hours of imbibition.

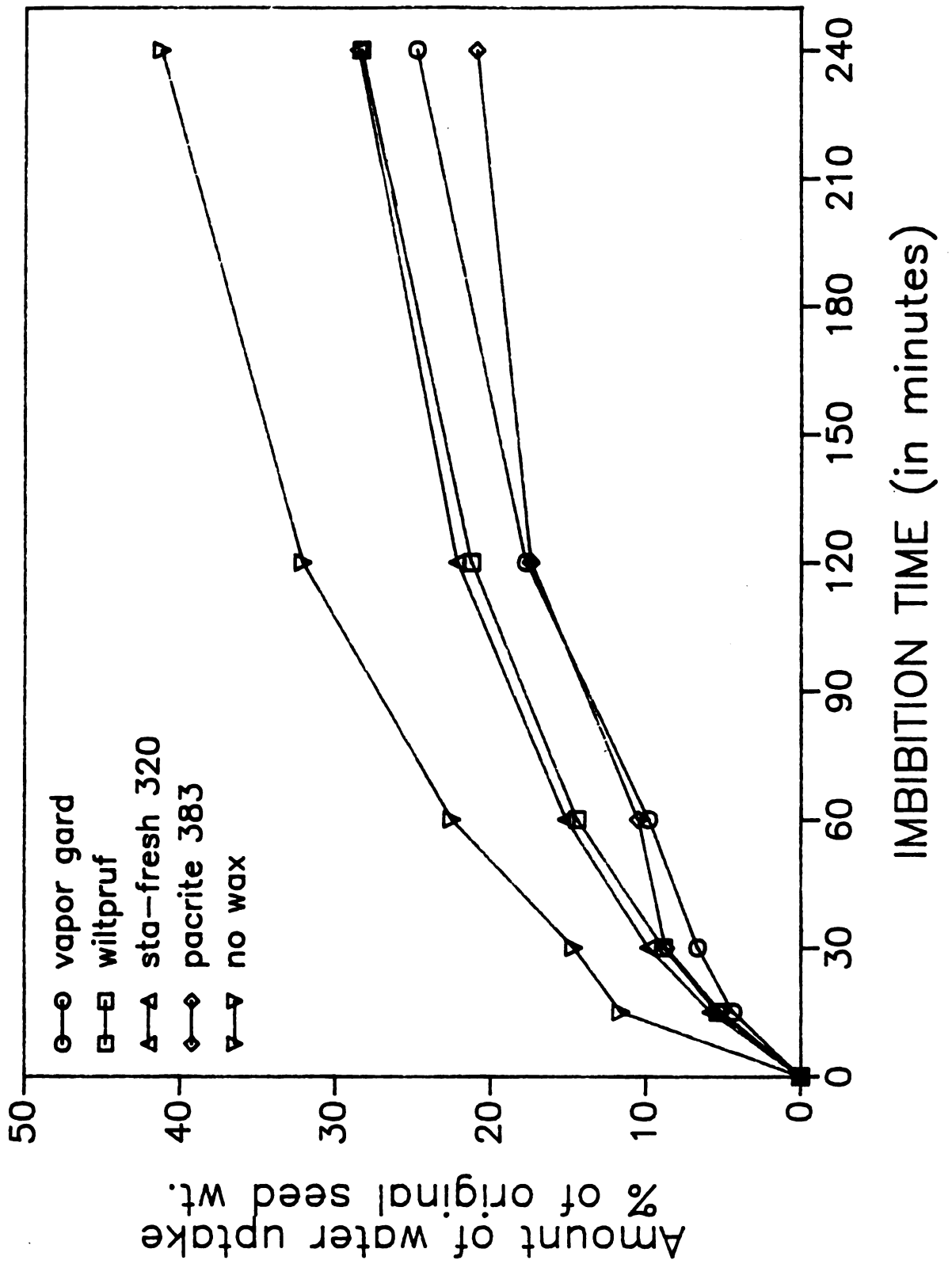
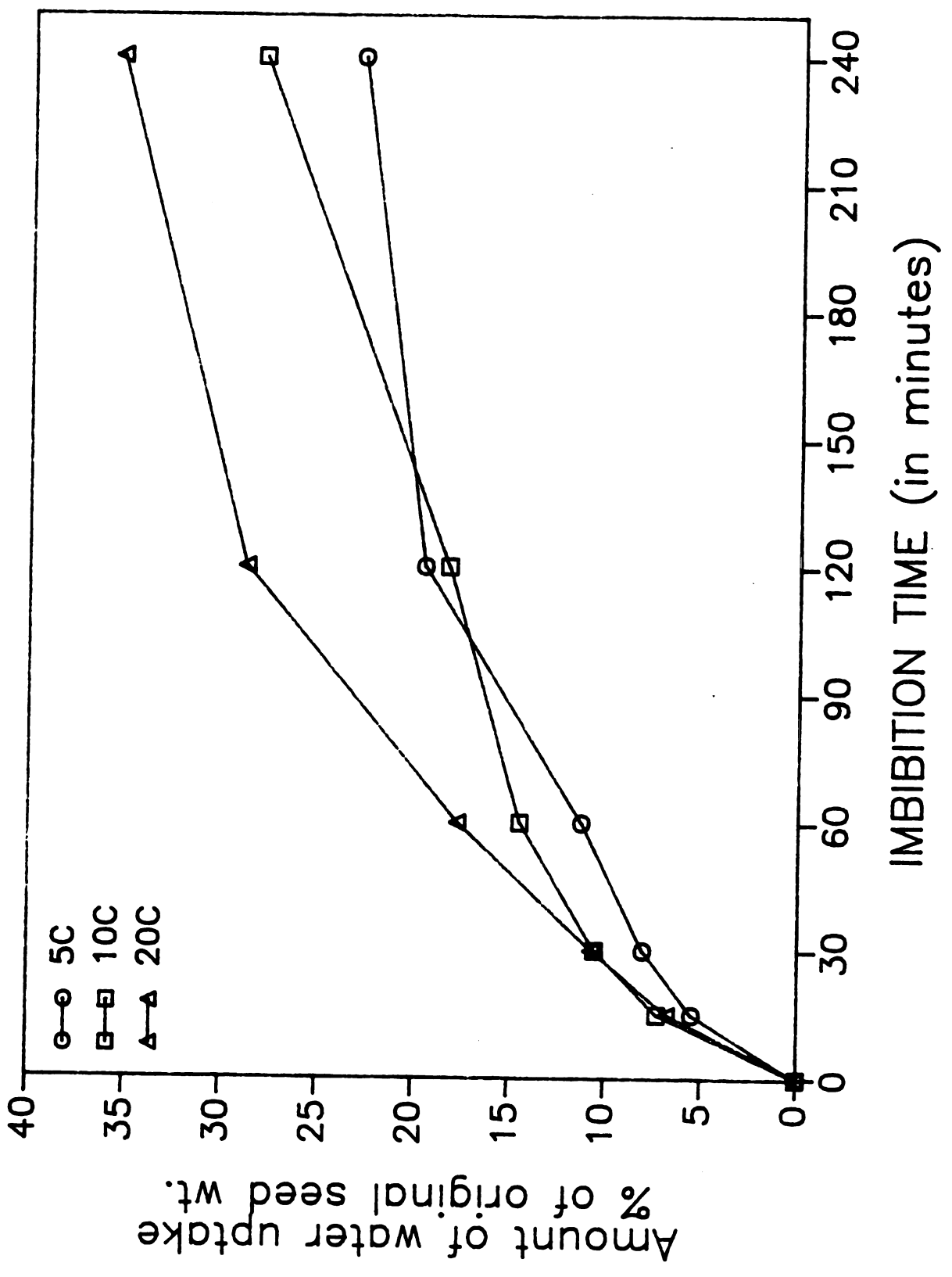

Amount of water uptake
% of original seed wt.
50
40
30
20
10
0
0
30
60
90
120
150
180
210
240
IMBIBITION TIME (in minutes)
vapor gard
wiltpruf
sta-fresh 320
pacrite 383
no wax

FIGURE 2. The effect of temperature and time on the rate of water uptake in wax-coated snap bean seeds.


Amount of water uptake
% of original seed wt.
40
35
30
25
20
15
10
5
0
0
30
60
90
120
150
180
210
240
IMBIBITION TIME (in minutes)
5C
10C
20C

2. Assessment of seed damage

In a preliminary study there was a high positive correlation (r=0.87) between germination percent of bean seeds imbibed at 5 and 10C with the sum of seeds with uniform TTC stain, normal axes and cotyledons with at most three small cracks. This indicated that these three measurements were reliable estimates of normal (viable) embryos after imbibition at low temperatures. At 5 and 10C all waxes increased the number of normal embryos compared to nonwaxed controls (Figure 3). Wiltpruf, Vapor Gard, Pacrite 383, and the nonwaxed controls were similar at 20C while Sta-fresh 320 reduced the number of normal embryos.

There was no effect of temperature and waxes on the number of damaged cotyledons. Wiltpruf, Pacrite 383 and Vapor Gard reduced the number of damaged axes while Sta-fresh 320 had no effect (Table 3). More axes were damaged at 5 and 10C than at 20C. This is expected since there is imbibitional chilling injury at these temperatures.

Assessment of seed damage by uniform TTC stain, normal axes and cotyledon cracks is very subjective and can be inaccurate. Growth perfomances of axes and cotyledons were also used to assess seed damage after imbibition at low temperatures. Cotyledon and axes weight increases were improved by all waxes (Table 3). Only Vapor Gard, Wiltpruf and Pacrite 383 increased total chlorophyll content. The temperature effect was only significant for axes germination ($p>0.01$) and radicle length ($p>0.001$). The wax temperature interaction for these two parameters was also significant (Table 4). Wiltpruf and Pacrite 383 increased axes germination

FIGURE 3. The effect of wax coating and temperature on the number of normal embryos (measured as the sum of seeds with uniform TTC stain, normal axes and cotyledons after 24 hours of imbibition) in snap bean. Small letters on bar graphs indicate levels of significance at 5%, comparing wax treatments at each temperature.

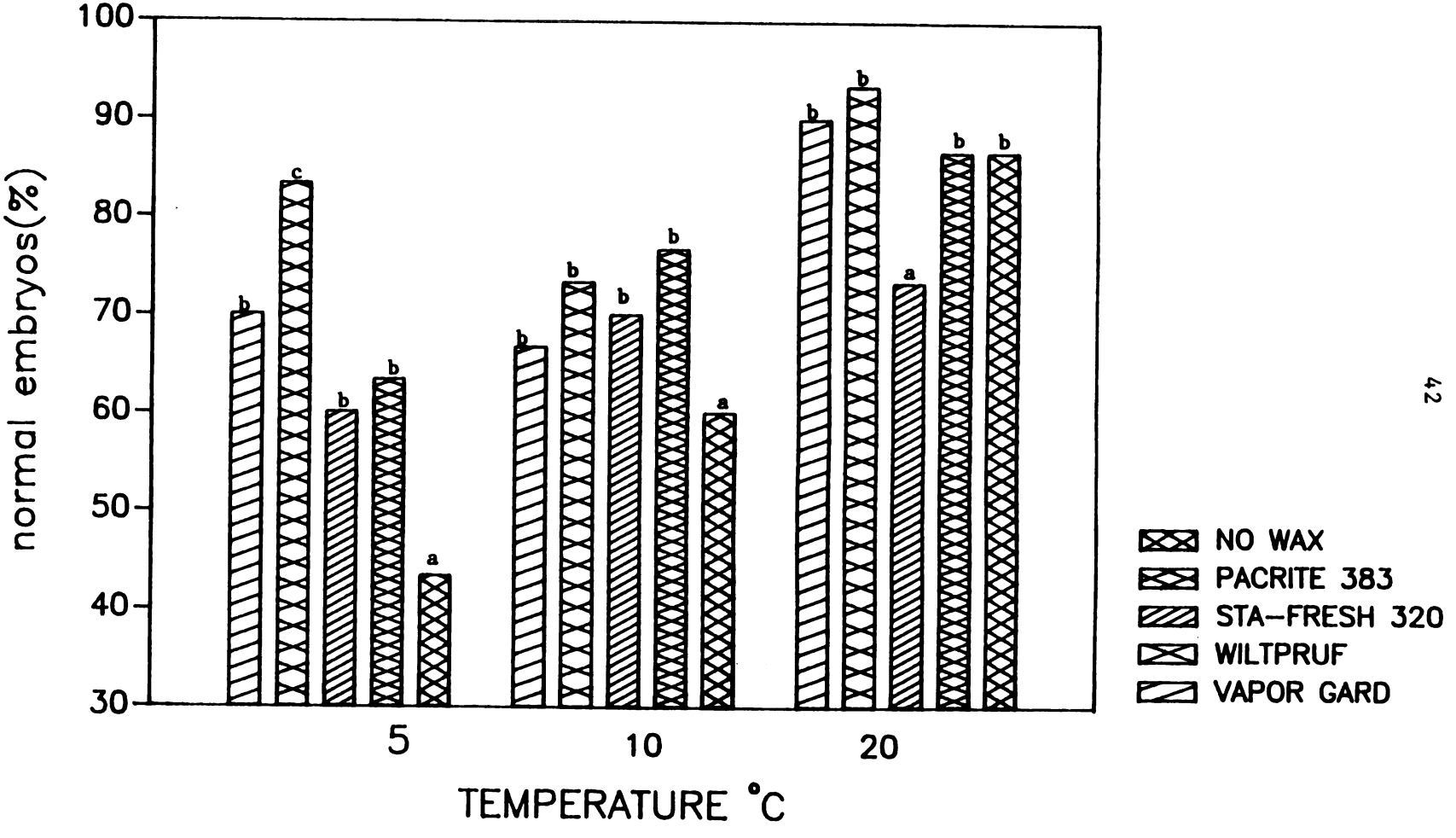

100
90
80
70
60
50
40
30
normal embryos(%)
5
10
20
TEMPERATURE °C
NO WAX
PACRITE 383
STA-FRESH 320
WILTPRUF
VAPOR GARD

TABLE 3: The effect of seedcoating on damaged axes, axes and cotyledon weight increases and total cotyledon chlorophyll content in snap bean

Waxes	Damaged axes (%)	Axes weight increase(g)	Cotyledon weight increase(g)	Total* chlorophyll	Germination in pots (%)
Vapor Gard	6.7[a]	0.050[b]	0.104[b]	0.629[b]	86.7[c]
Wiltpruf	4.4[a]	0.056[b]	0.133[b]	0.525[b]	84.0[c]
Sta-fresh 320	15.6[b]	0.054b	0.134[b]	0.257[a]	37.3[a]
Pacrite 383	7.8[a]	0.049[b]	0.168[b]	0.518[b]	81.3[c]
Control**	13.3[b]	0.031[a]	0.092[a]	0.246[a]	66.7[b]

* expressed as ug/ml/g of cotyledon tissue
** unwaxed seeds

TABLE 4: The effect of wax coating and temperature on axes germination and radicle length

Waxes	Axes Germination (%) (Temperature °C) 5	10	20	Radicle length (cm) 5	10	20
Vapor Gard	73.3	66.7	93.3	2.63	3.41	3.86
Wiltpruf	86.7	93.3	93.3	2.82	3.46	4.25
Sta-fresh 320	60.0	60.0	53.3	2.78	3.18	2.95
Pacrite 383	93.3	93.3	93.3	2.81	3.52	4.05
No wax	46.7	46.7	100.0	2.17	3.02	4.53

at 5, 10 and 20C. All the waxes, except Sta-fresh 320, increased axes germination. For all the waxes except Sta-fresh 320, radicle length increased with increases in temperature.

3. Germination of treated seeds in pots

In snap bean, Pacrite 383, Wiltpruf and Vapor Gard increased germination at 5 and 10C and Sta-fresh 320 reduced germination compared to the nonwaxed controls (Figure 4). Plant height in snap bean was also increased by Wiltpruf, Vapor Gard and Pacrite 383. At 20C, germination of Sta-fresh treated seeds was significantly decreased compared to the nonwaxed control and other waxes used in the experiment.

B. EFFECT OF INITIAL SEED MOISTURE CONTENT

1. Assessment of seed damage

In snap bean the number of normal embryos was affected by both initial seed moisture content and temperature. The number of normal embryos was increased by high initial seed moisture content and reduced by low (5 and 10C) imbibitional temperatures (Figure 5).

At high initial seed moisture contents (25-30%) temperature had little or no effect on the number of damaged cotyledons (Figure 6). At the low initial seed moisture contents increasing temperature decreased the number of damaged cotyledons. Neither initial seed moisture content nor temperature had an effect on the number of damaged axes. (Data not presented.)

Initial seed moisture content (Table 5) had significant ($p>0.01$) effect on all the growth assay measurements. Initial seed moisture content above 15% showed a consistent increase in axes germination, radicle length, axes and cotyledon weight increases. Plumule length and total cotyledon

FIGURE 4. Effect of wax treatments and temperature on germination of snap bean seeds after 24 hours of imbibition at the indicated temperatures. Small letters on bar graph indicate significance at the 5% level comparing wax treatments at each temperature.

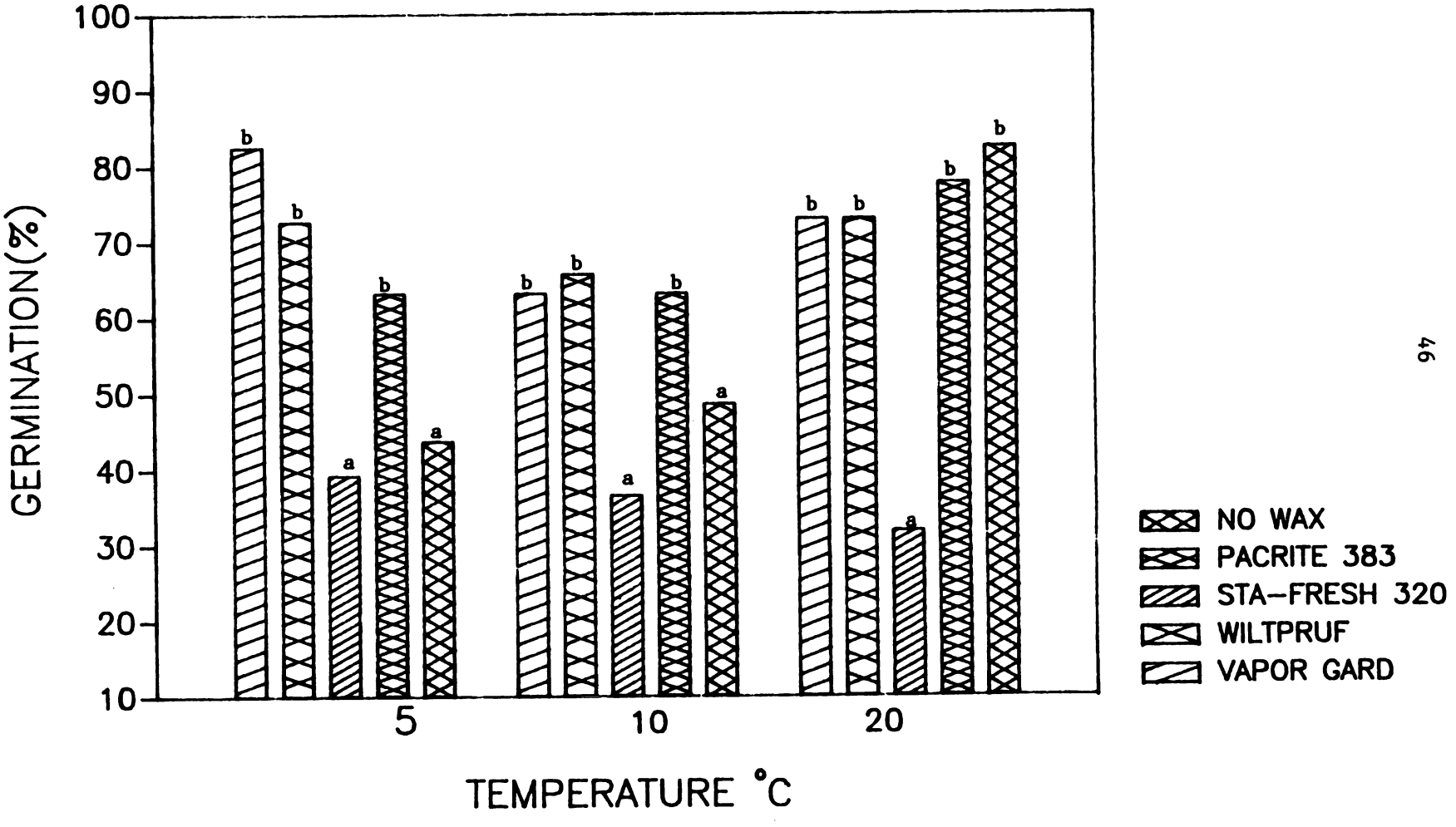

100
90
80
70
60
50
40
30
20
10
GERMINATION(%)
b
b
a
b
a
b
b
a
b
a
b
b
b
a
b
b
5
10
20
TEMPERATURE °C
NO WAX
PACRITE 383
STA-FRESH 320
WILTPRUF
VAPOR GARD

FIGURE 5. Effect of varying initial seed moisure content at different temperatures on the number of normal embryos in snap bean (as shown by number of seeds with uniform TTC stain, normal axes and cotyledons) after 24 hours of imbibition. Small letters on bar graph indicate the 5% level of significance comparing moisture content at the same temperature.

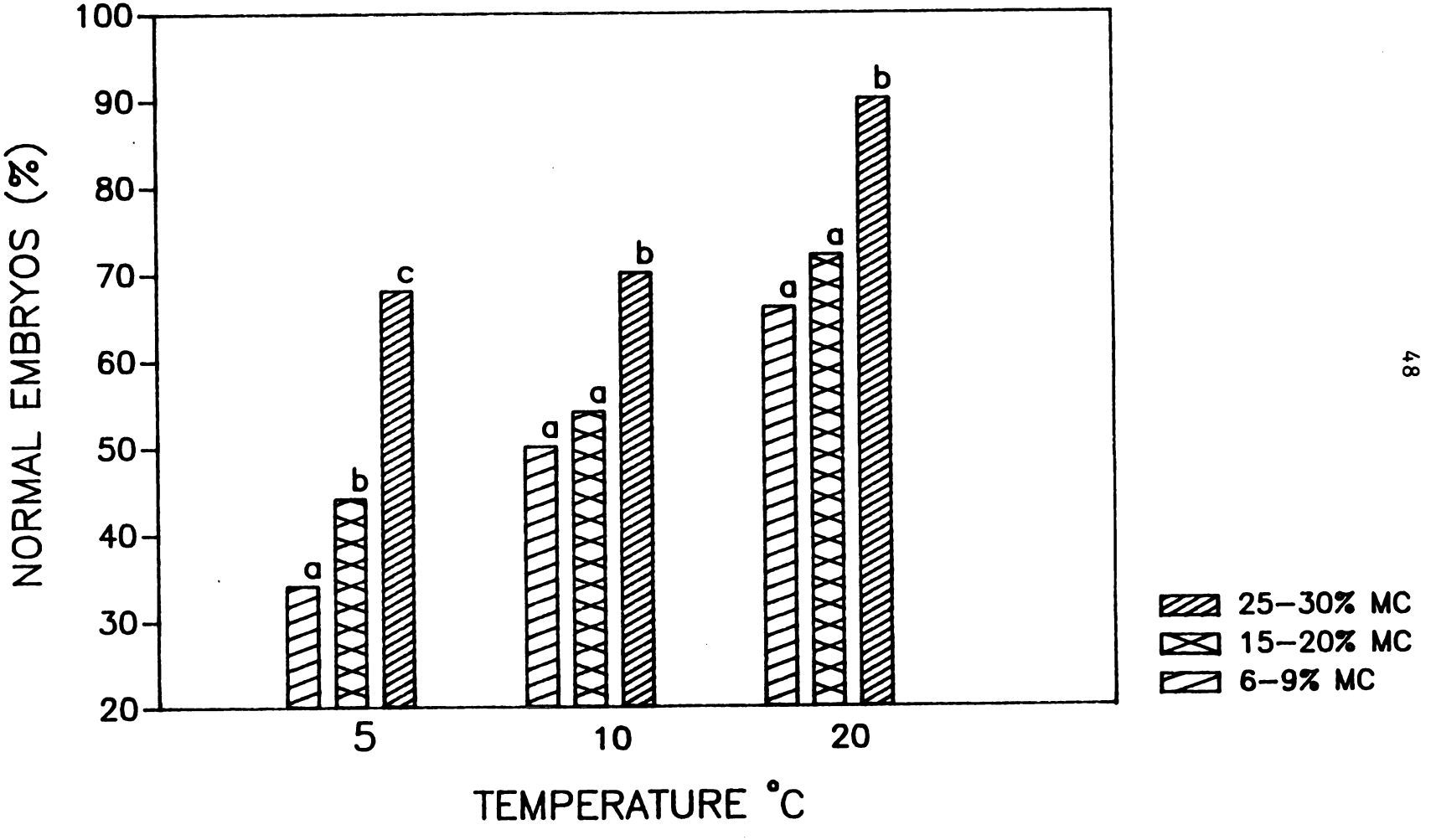
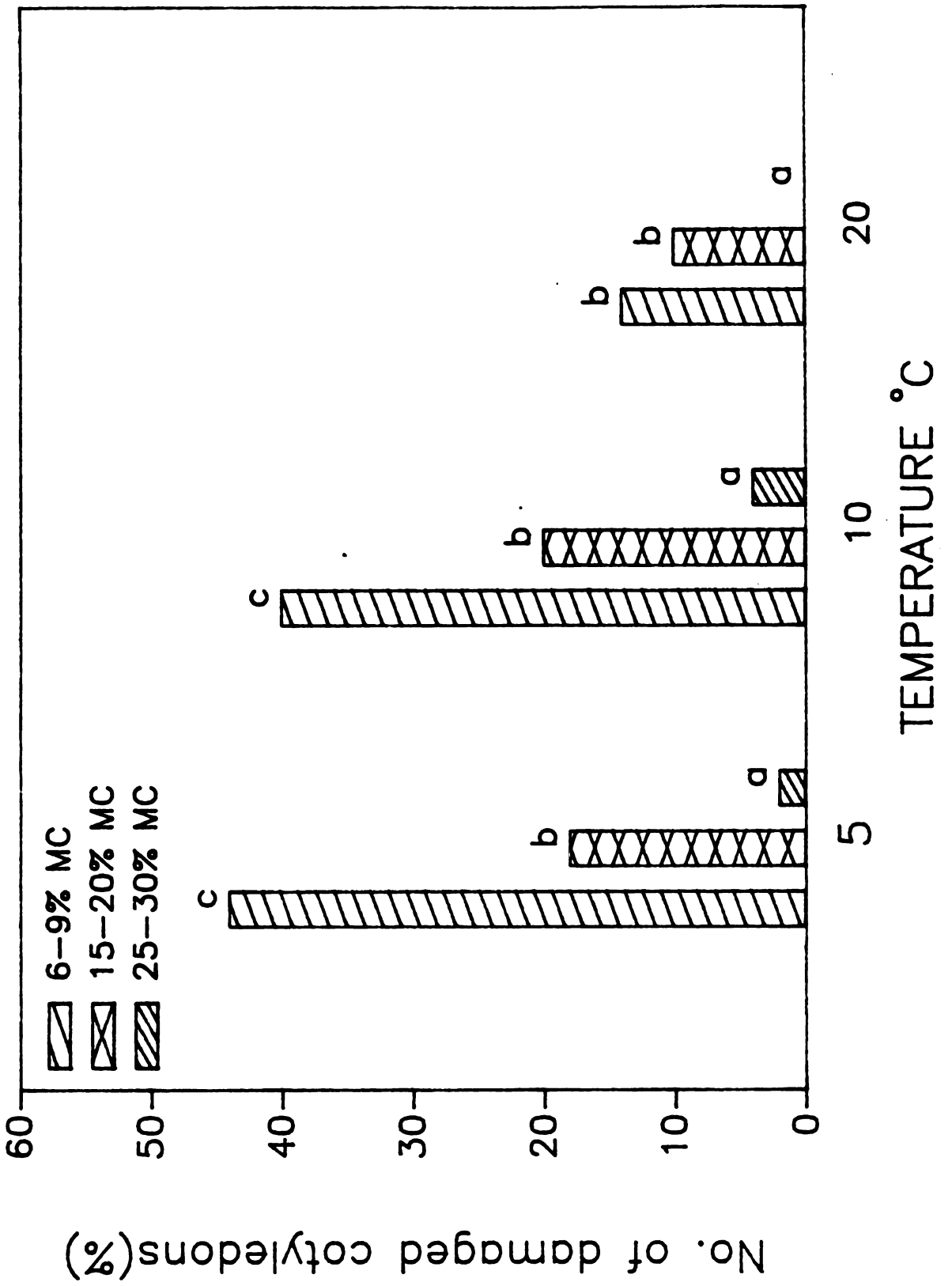

NORMAL EMBRYOS (%)
100
90
80
70
60
50
40
30
20
a
b
c
a
a
b
a
a
b
5
10
20
TEMPERATURE °C
25–30% MC
15–20% MC
6–9% MC

FIGURE 6. Effect of varying initial seed moisture content and temperature on the number of damaged cotyledons in snap beans after 24 hours of imbibition. Small letters on bar graph indicate the 5% level of significance comparing moisture content at the same temperature.


No. of damaged cotyledons(%)
60
50
40
30
20
10
0
6-9% MC
15-20% MC
25-30% MC
a
b
c
a
b
c
a
b
b
a
b
5
10
20
TEMPERATURE °C

chlorophyll content were only increased by the 25-30% initial seed moisture content. Axes germination, axes weight increase, cotyledon weight increase and plumule length were all improved at 20C as compared to 5 and 10C. Radicle length increased with increase in temperature and both 10 and 20C increased total chlorophyll content in snap beans.

TABLE 5: Effect of initial seed moisture content on axes germination, radicle and plumule length, axes and cotyledon weight increase, and total cotyledon chlorophyll content in snap bean

Moisture content (%)	Axes germ. (%)	Axes wt. increase (g)	Radicle length (cm)	Plumule length (cm)	Cotyledon wt. increase (g)	Total* chlor.
6-9	63.3[a]	0.025[a]	1.83[a]	0.471[a]	0.083[a]	0.202[a]
15-20	88.0[b]	0.035[b]	2.38[b]	0.479[a]	0.152[b]	0.237[a]
25-30	85.3[b]	0.039[b]	2.61[b]	0.535[b]	0.150[b]	0.334[b]

* expressed as ug/ml/g of cotyledon tissue

2. Germination in pots

Initial seed moisture content greater than 15-20% increased snap bean germination in pots and plant height after two weeks from planting. Temperature did not have a significant effect on either seed germination or plant height. Raising the initial seed moisture content increased germination of seeds in pots (Table 6).

TABLE 6: Effect of initial seed moisture content on germination of snap bean in pots

Moisture content %	Germination % in pots
6-9	57.8[a]
15-20	81.1[b]
25-30	81.1[b]

C. EFFECT OF OSMOTIC POTENTIAL SOLUTIONS

(a) Water uptake

In snap bean, water uptake was affected by osmotic potential, temperature and time. Generally water uptake increased with increase in temperature and imbibition time. At both -0.5 and -1.5 MPa water uptake was reduced at all three temperatures (Figure 7). There were no significant ($p<0.05$) differences between the -0.5 and -1.5 MPa treatments. There was also greater reduction of water uptake with increase in imbibition time and osmotic potential. The significant ($p>0.001$) osmotic potential x temperature x time interaction suggests that the greatest reductions in water uptake are achieved by low osmotic potential at low temperatures after long periods of imbibition.

2. Assessing seed damage

Both PEG priming solutions and temperature significantly ($p>0.001$) affected the number of normal embryos. The number of normal embryos was increased by osmotic solutions of -0.5 and -1.5 MPa and were reduced at

5 and 10C (Figure 8). More axes and cotyledons were damaged at low temperatures (5 and 10C) and priming solutions of -0.5 and -1.5 MPa significantly alleviated this damage (Figure 9).

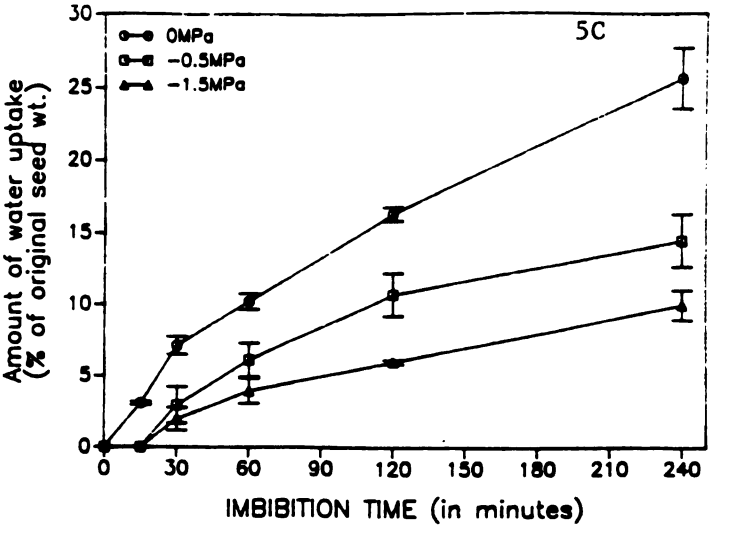
Osmotic solutions of -0.5 and -1.5 MPa increased axes germination, axes weight increase, and cotyledon weight increase in snap beans (Table 7).

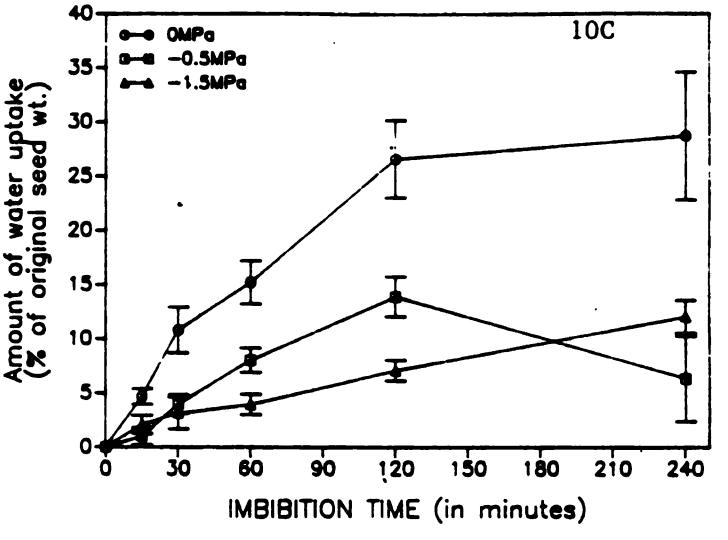
TABLE 7: Effect of osmotic solutions on axes germination and axes and cotyledon weight increase

Osmotic Potential(MPa)	Axes germ %	Axes wt. increase(g)	Cotyledon wt. increase(g)
0	66.7[a]	0.05[0a]	0.084[a]
-0.5	93.3[b]	0.066[b]	0.158[b]
-1.5	88.9[b]	0.068[b]	0.197[c]

Axes germination increased with increases in temperature. Low temperatures reduced cotyledon weight increase and total cotyledon chlorophyll content. There was no osmotic solution by temperature interaction for axes germination, axes weight increase, and cotyledon weight increase. For all the priming solutions, total chlorophyll content increased with increases in temperature (Table 8). At 5C, -0.5 MPa osmotic solution reduced chlorophyll content but for 10 and 20C, chlorophyll content increased with increase in osmotic potential of the priming solution.

FIGURE 7. Effect of osmotic potentials and temperatures on water uptake in the first four hours of imbibition in snap beans.


5C
0MPa
−0.5MPa
−1.5MPa
Amount of water uptake
(% of original seed wt.)
IMBIBITION TIME (in minutes)


10C
0MPa
−0.5MPa
−1.5MPa
Amount of water uptake
(% of original seed wt.)
IMBIBITION TIME (in minutes)

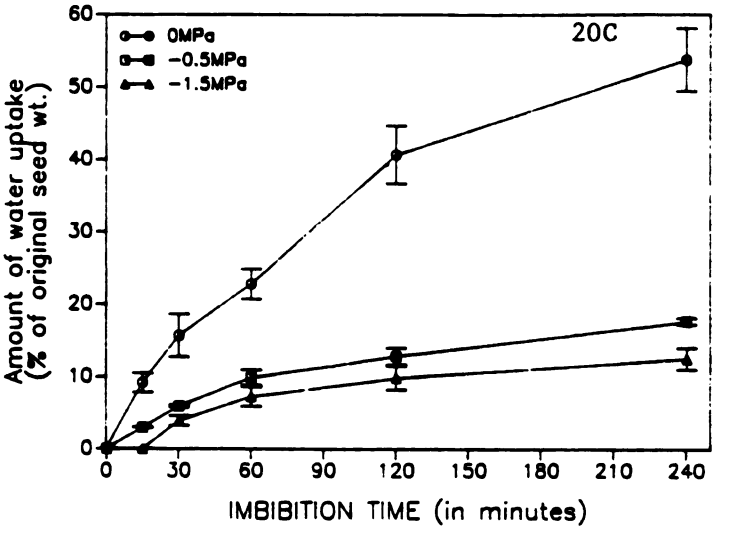

20C
0MPa
−0.5MPa
−1.5MPa
Amount of water uptake
(% of original seed wt.)
IMBIBITION TIME (in minutes)

FIGURE 8. Effect of osmotic potential and temperature on the number of normal embryos in snap beans after 24 hours of imbibition. Small letters on bar graph indicate 5% level of significance comparing osmotic solutions at each temperature.

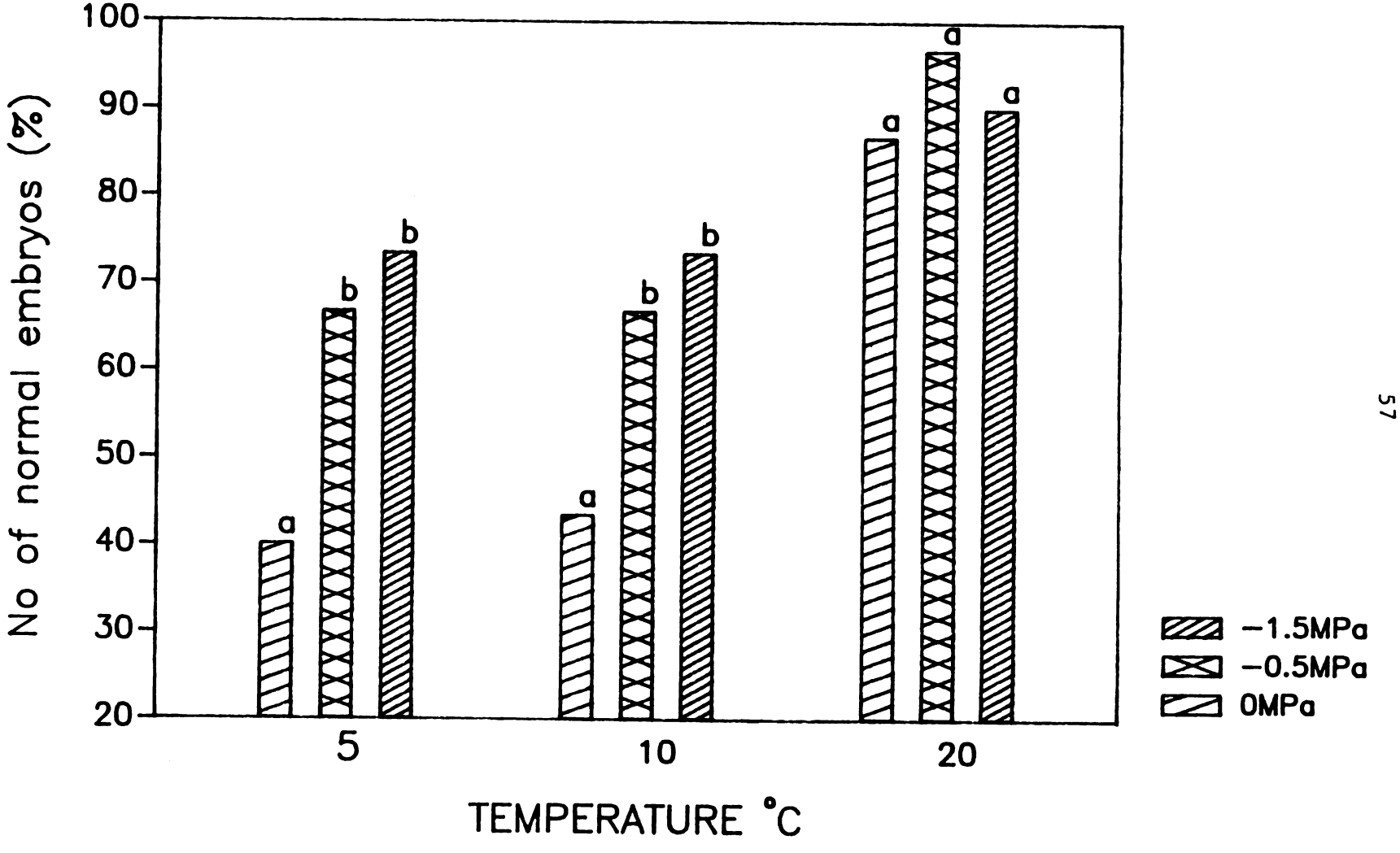
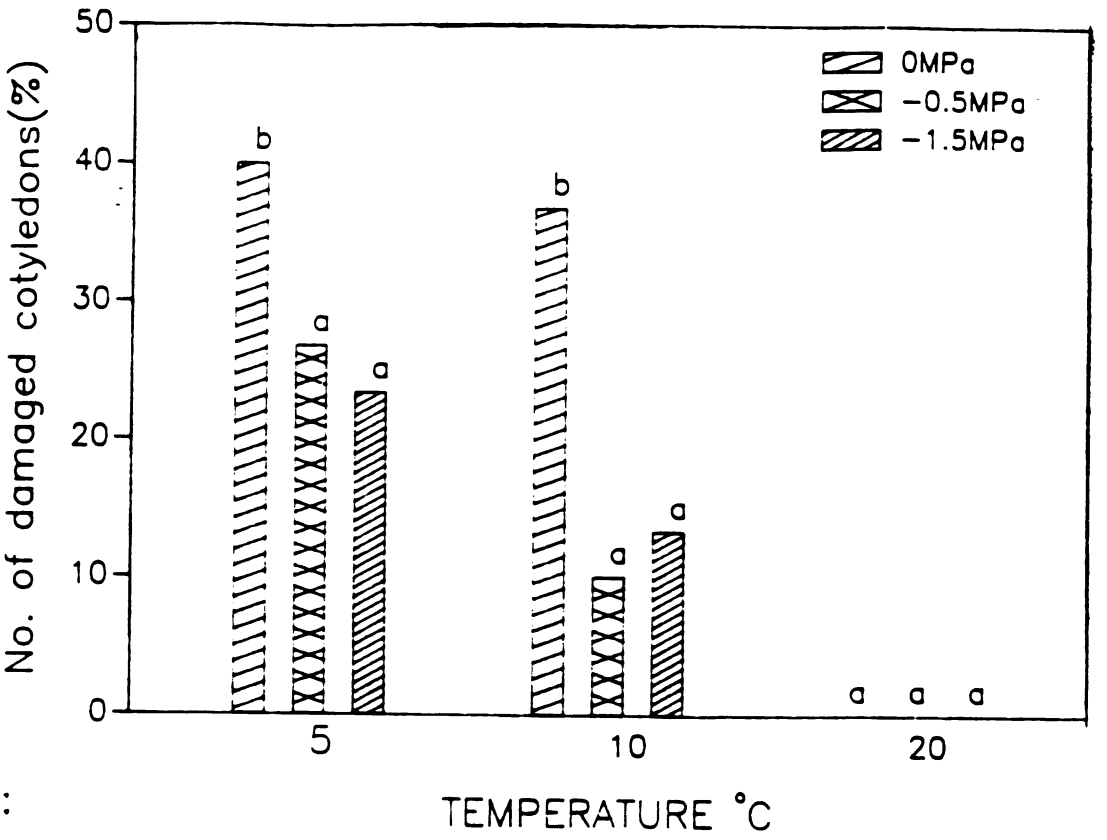

No of normal embryos (%)
100
90
80
70
60
50
40
30
20
a
b
b
a
b
b
a
a
a
5
10
20
TEMPERATURE °C
−1.5MPa
−0.5MPa
0MPa

FIGURE 9. Effect of osmotic potential and temperature on the number of damaged cotyledons and axes in snap beans after 24 hours of imbibition. Small letters on bar graph indicate 5% level of significance comparing osmotic solutions at each temperature.


No. of damaged cotyledons(%)
50
40
30
20
10
0
0MPa
−0.5MPa
−1.5MPa
b
a
a
b
a
a
a a a
5
10
20
TEMPERATURE °C

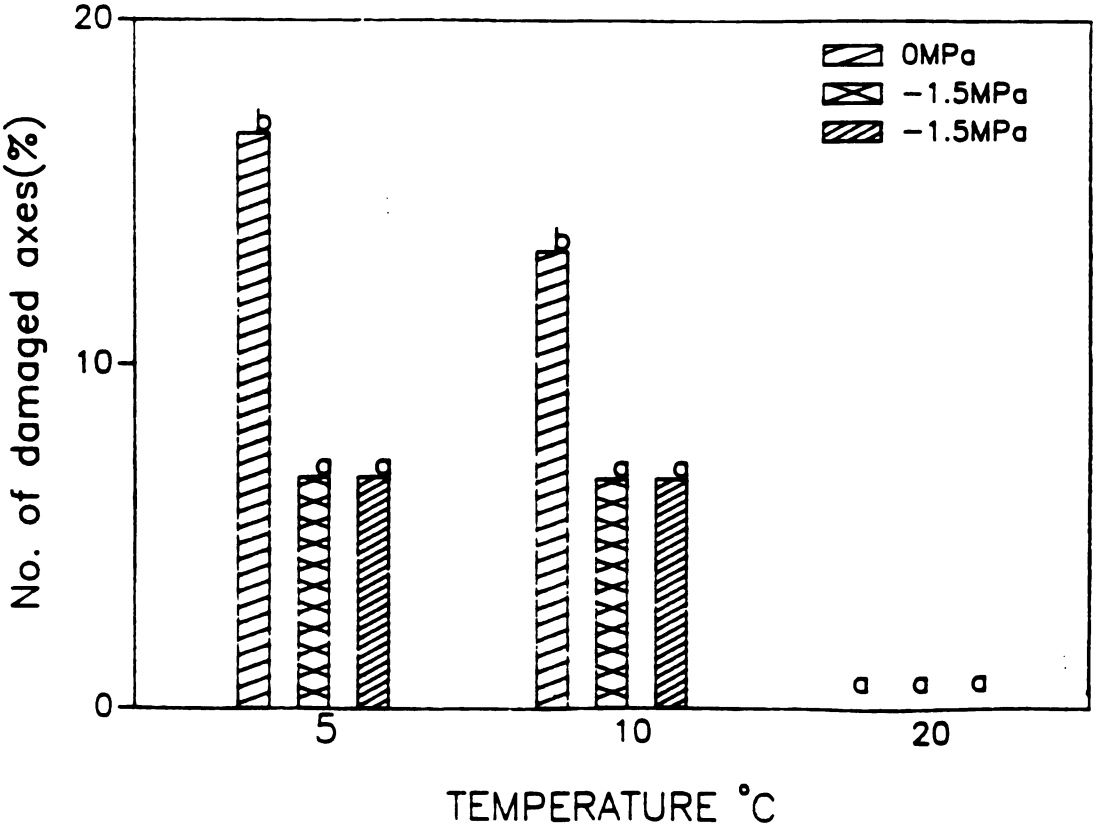

No. of damaged axes(%)
20
10
0
0MPa
−1.5MPa
−1.5MPa
b
a
a
b
a
a
a a a
5
10
20
TEMPERATURE °C

TABLE 8: Effect of osmotic solutions and temperature on total chlorophyll content in snap beans

Osmotic solution(MPa)	Temperature (°C) 5	10	20
0	0.186	0.408	0.535
-0.5	0.123	0.490	0.786
-1.5	0.339	0.614	0.978

3. Germination of primed seeds in pots

In snap beans, both the priming solutions and temperature affected seed germination but not plant height. Priming solutions of -0.5 and -1.5 MPa increased germination percent compared to the untreated controls (Table 9). There was a general increase in germination with increase in temperature. There was no PEG priming solution by temperature interaction on germination of snap beans in pots.

TABLE 9: Effect of osmotic solutions on germination of snap bean in pots

PEG priming solution(MPa)	Germination % in pots
0	42.2[a]
-0.5	66.7[b]
-1.5	71.1[b]

CHAPTER IV. DISCUSSION

A. SEEDCOATING WITH WAXES

The results obtained indicate that imbibitional chilling injury in snap beans can be alleviated by using hydrophobic materials (waxes) that reduce the rate of water uptake during the first few hours of imbibition. The waxes act as physical barriers to water uptake. The reduced water uptake in seeds coated with Vapor Gard, Wiltpruf and Pacrite 383 consistently increased the number of normal embryos and germination in pots. Priestley and Leopold (1986) reported similar results with the use of lanolin coating in soybean; however, lanolin is very difficult to handle because it is thick and sticky. It, therefore, had to be melted and diluted with acetone for ease of application to the seed. Waxes used in this experiment were all thin, easy to handle and apply, and all dried on the seed without stickiness; properties that encourage their widespread use. Although Sta-Fresh 320 reduced water uptake by the seed, it decreased germination in pots and the number of normal embryos especially at 20C. It is quite possible that this wax had some toxic effects. No further comment can be given because the wax compositions were not known.

The beneficial effects of these waxes were shown at 5 and 10C, the chilling injury inducing temperatures. Apart from Sta-Fresh 320, these

waxes showed no deleterious effects at 20C which is an advantage since they can still be applied when one is not sure imbibitional chilling inducing temperatures will prevail. The relationship between the wax mediated reduction in water uptake and the reduced number of damaged axes, increased cotyledon and axes weight and increased total cotyledon chlorophyll content suggested that both axes and cotyledons are injured during imbibition at low temperatures. The reduction in water uptake rate by waxed seeds may result in less injury because there is less free water causing damage between cotyledons and between the testa and cotyledons (Wolk, 1988).

Several authors have tried to explain the mechanism of imbibitional chilling injury (Dickson, 1971; Powell and Mathews, 1978 and 1981; Leopold, 1980; Tully et al., 1981; and Wolk, 1988). The apparent consensus is that the rate of cold water uptake is a major cause of imbibitional chilling injury. The current study supports this general understanding. However it remains unresolved as to whether imbibitional chilling injury is the physical damage from water entrance or is a result of some biological phenomenon triggered by cold water uptake.

B. RAISING INITIAL SEED MOISTURE CONTENT

Raising the initial seed moisture content before imbibition at low temperatures has the desired effects of reducing seed damage and increasing germination. The absence of any statistical differences between 15-20% and 25-30% initial moisture content treatments supports the existence of a critical moisture content above which seed is protected

from imbibitional chilling injury. Thirteen percent initial seed moisture has been suggested as the critical value for snap bean (Wolk, 1988) and soybean (Christiansen, 1968; Hobbs and Obendorf, 1972). A higher figure of 20% has been suggested for lima bean (Pollock, 1969; Cal and Obendorf, 1972).

How the raising of initial seed moisture content alleviates imbibitional chilling injury is not clear. It cannot be explained by the reduction of rate of cold water uptake because seeds of high initial moisture content imbibe water more rapidly than those of low initial moisture contents (Bramlage et al., 1978).

C. EFFECT OF OSMOTIC POTENTIAL SOLUTIONS

Osmotic solutions reduced seed water uptake. The consistent relationship between the reduction in water uptake and the reduced number of damaged seeds together with enhanced germination indicates that osmotic solutions alleviate imbibitional chilling injury by reducing cold water uptake. The use of priming solutions reduced the number of damaged axes and cotyledons, increased axes and cotyledon weights as well as cotyledon chlorophyll content. This supports an earlier inference that both axes and cotyledons are damaged when seeds are imbibed at low temperatures.

The reduced rate of cold water uptake explains the alleviation of imbibitional chilling injury by osmotic solutions. PEG solutions reduced water uptake because they are of higher osmotic potential (hold water more tenaciously) and therefore make less water available for uptake by the

seed. The fact that osmotic priming with PEG increases germination under cold temperatures has already received attention from other researchers (Khan et al.,1978; 1980-81; Bodsworth and Bewley, 1981). In a series of experiments involving priming seeds for several days these researchers concluded that the tolerance to imbibitional chilling injury imparted through PEG priming is a result of seed physiological and biochemical changes. Although physiological and biochemical changes were not monitored in this study, it is quite reasonable to accept that these changes contributed to the alleviation of imbibitional chilling injury.

The data from this study suggests that there are several causes of imbibitional chilling injury. Results from the use of waxes and PEG solutions support the idea that rapid cold water uptake is one of the major causes. The fact that high initial moisture content in the seed imparts protection against imbibitional chilling injury suggests phenomena other than just rapid rate of cold water uptake. Vertucci and Leopold (1983) suggested that the wetting reaction was the critical step in the injury process, an observation supported by Christiansen (1968) who elucidated that the effects of imbibitional chilling injury are set within the first few minutes of imbibition. The water replacement hypothesis propounded by Clegg (1986) and supported by Wolk (1988) can explain how high initial seed moisture contents impart tolerance to imbibitional chilling injury in seeds. Using NMR techniques Wolk (1988) showed that imbibition rate maxima of embryo tissue reflects transitions in the states of seed water. It is probable that the state of seed water in high initial seed moisture contents imparts resistance to imbibitional chilling

injury. The high leakage of solutes at low temperatures (Bramlage et al., 1978; Duke et al., 1983; Marbach and Meyer, 1985) and initial seed moisture content (Hobbs and Obendorf, 1972; Simon and Wiebe, 1975; Parrish and Leopold, 1977) is a phenomenon which can also be used in explaining imbibitional chilling injury.

The three methods used to control imbibitional chilling injury in snap bean in this study were all successful. The use of wax coating of seeds seems to be the most practical and easiest method to use. The successful application of the waxes on Captan treated seeds suggests that some pesticides could be incorporated into the waxes before application to the seed. The success of this method, however, depends on the waxes chosen. Although raising the initial moisture content reduced imbibitional chilling injury in both snap and lima beans, there are several problems with this method. Raising initial moisture content of the seed enhances faster deterioration in seed quality and therefore high moisture seeds cannot be stored for long periods. Seeds of high initial moisture content also lose moisture if stored under dry conditions and therefore lose the ability to protect seeds against imbibitional chilling injury. Raising the initial moisture content and coating seeds with waxes could be combined to reduce the moisture loss problem and enhance seed germination.

Priming with PEG was also effective in reducing imbibitional chilling injury in snap beans, but this method has several disadvantages. The first is that after priming, the seed has to be dried for easy handling. This, however, has shown to result in some retardation of germination or

loss of some of the beneficial effects of priming (Heydecker and Wainwright, 1976). Storage of primed seed also results in loss of the beneficial effects of priming (Irwin and Price, 1981). For large seeded crops like soybean there is a limit to the number of days the seed can be primed, since there is disintegration of the testa and cotyledon separation with handling (Bodsworth and Bewley, 1981).

CONCLUSIONS

1) Three of the waxes used (Wiltpruf, Vapor Gard and Pacrite 383) reduced the rate of water uptake and damage to cotyledons and axes and improved germination of imbibitionally chilled snap beans. Stafresh 320 also reduces rate of water uptake, but it was phytotoxic to the seed.
2) Raising the initial seed moisture content reduced damage to cotyledons and axes and improved germination of imbibitionally chilled snap bean seeds.
3) Polyethylene glycol of -5 and -15 bars reduced water uptake and damage to cotyledons and axes and also improved germination of imbibitionally chilled snap beans.
4) Assessment of damage with TTC and growth assay of separate axes and cotyledons showed good consistency with germination of imbibitionally chilled snap beans and can therefore be used to assess damage in seed.

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APPENDIX A

Screening of Waxes

Results

TABLE 1: **Effect of waxes on reduction of water uptake.**

Wax/oil	Mean water uptake (% of original seed weight
Sunflower oil	39.44[b]
Vegetable oil	37.05[b]
Corn oil	39.77[b]
Peanut oil	38.53[b]
Safflower oil	39.37[b]
Vapor Gard	29.17[a]
Wiltpruf	29.67[a]
Apple wax	35.94[b]
Olive oil	44.82[c]
Sta-fresh 560	33.71[a]
Sta-fresh 360	37.72[b]
Sta-fresh 320	27.52[a]
Pacrite Sunshine wax	38.65[b]
Lustre Dry	38.74[b]
Prime Shine	43.91[c]
Sealbrite 74	52.82[d]
Citrashine 801	36.14[b]
Pacrite 383	25.72[a]
Sealbrite 65	39.14[b]
Control (no wax)	44.79[c]

Vapor Gard, Wiltpruf, Sta-fresh 320, Pacrite 383 and Sta-fresh 560 reduced the rate of water uptake by the seed in the first two hours of imbibition. Sta-fresh 560 was not chosen because it does not dry on the seed.

APPENDIX B

Lima Bean Result Summaries

A. Effect of waxy seedcoating on imbibitional chilling injury in lima beans

TABLE 2: Effect of waxes on water uptake, number of damaged cotyledons, number of normal embryos, and germination in pots

Waxes	Water Uptake (% orig wt)	Number of Damaged cotyledons (out of 10)	Number of normal embryos (%)	Germination in pots (%)
Vapor Gard	5.53[a]	1.22[a]	61.1[c]	13.33
Wiltpruf	6.27[a]	1.33[a]	60.0[c]	5.33
Sta-fresh 320	5.72[a]	1.79[a]	45.6[b]	8.00
Pacrite 383	5.05[a]	1.67[a]	57.8[c]	14.67
No wax	7.87[b]	2.44[b]	32.2[a]	18.67

All the waxes reduced water uptake and number of damaged cotyledons to the same extent and increased the number of normal embryos. The waxes had no effect on germination of lima beans in pots.

TABLE 3: Effect of temperature on water uptake, number of damaged cotyledons, number of normal embryos, and germination in pots

Temperature °C	Water Uptake (% orig wt)	Number of Damaged cotyledons (out of 10)	Germination in pots (%)
5	5.53[a]	2.53[b]	6.67[a]
10	5.75[a]	1.80[b]	8.55[a]
20	6.87[b]	0.73[a]	28.76[b]

5 and 10C reduced water uptake, increased the number of damaged cotyledons, and reduced germination percent in pots, but generally there was very poor germination of lima beans.

The waxes had no effect on axes germination, axes weight increase, cotyledon weight increase, total chlorophyll content and plumule and radicle length.

B. Effect of moisture contents on imbitional chilling injury in lima beans

TABLE 4: Effect of initial seed moisture content on the number of damaged cotyledons, normal embryos, and germination of lima beans in pots

Moisture Content (%)	Number of Damaged Cotyledons (out of 10)	Normal Embryos (%)	Germination in Pots (%)
6-9		40.67[a]	7.61[a]
15-20		43.33[a]	64.69[b]
25-30		59.33[b]	71.23[b]

Initial moisture content of greater than 15% increased germination percent in pots but only 25-30% initial moisture content increased normal embryos. Raising the initial moisture content had no effect on the number of damaged axes and cotyledons.

TABLE 5. Effect of initial seed moisture content on axes germination, radicle and plumule length, axes and cotyledon weight increase and total cotyledon chlorophyll content in lima bean

Moisture content (%)	Axes germ. (%)	Radicle length (cm)	Plumule length (cm)	Axes weight (g)	Total Cotyledon chlorophyll content (ug/ml/g)
6-9	47.0[a]	2.16[a]	0.47[a]	0.072[a]	0.105[a]
15-20	72.8[b]	2.99[b]	0.56[b]	0.105[b]	0.179[b]
25-30	62.2[b]	3.24[b]	0.55[b]	0.098[b]	0.194[b]

Initial moisture content greater than 15% consistently increased axes germination, radicle length, plumule length, axes weight increase and total chlorophyll content. Raising the initial moisture content had no effect on cotyledon weight increase.

In all the moisture content experiments, 5 and 10C decreased germination percent and the number of normal embryos and all the growth assay measurements made.

C. Effect of osmotic solutions on imbibitional chilling injury in lima beans

TABLE 6. Effect of osmotic solutions on water uptake, damages axes and cotyledons, number of normal embryos and germination of lima beans in pots

Osmotic solutions (MPa)	Water Uptake (% of original seed weight)	Normal embryos (%)	Germination in pots (%)
0	6.11[b]	34.4[a]	4.4[a]
-0.5	2.80[a]	46.7[b]	20.0[b]
-1.5	2.00[a]	47.8[b]	17.8[b]

PEG osmotic solutions of -0.5 and -1.5 MPa reduced water uptake and increased both the number of normal embryos and germination percent in pots. Generally germination percent of the lima beans in pots was very poor. The primary solutions did not, however, have any effect on the number of damaged axes and cotyledons.

TABLE 7. Effect of temperature on water uptake, the number of damaged axes and cotyledons, number of normal embryos and germination of lima beans in pots

Temperature °C	Water uptake (% original seed weight)	Number of damaged (of 10)	Number of damaged (of 10)	Normal embryos (%)	Germination in pots (%)
5	3.51[a]	3.22[b]	3.0[c]	34.4[a]	0.00[a]
10	3.01[a]	3.22[b]	2.0[b]	25.6[a]	0.00[a]
20	4.40[b]	1.22[a]	1.1[c]	68.9[b]	42.20[b]

5 and 10C reduced water uptake, the number of normal embryos and resulted in no germination in pots; these temperatures also increased the number of damaged cotyledons and axes.

Priming solutions of -0.5 and -1.5 MPa only increased axes germination and cotyledon weight increase in the growth assay.

DISCUSSION

The poor and erratic germination obtained from work with lima beans probably indicates that the batch of seeds used in this experiment was very poor quality and, therefore, these results cannot be interpreted properly.